

Identification and characterization of restricting host factors for viral replication

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Abstract

Viruses rely on host organisms for their replication. Over time, these hosts have developed a diverse array of mechanisms to actively inhibit the spread of viruses, with notable examples found within the adaptive and innate immune systems. However, several other factors also come into play to inhibit virus replication. These factors encompass all host proteins within the cell that limit the virus in its replication, e.g., by affecting the stability of viral DNA, RNA, or proteins. Identifying these proteins can be challenging, as they typically serve vital functions in maintaining cellular homeostasis and host physiology, unlike components of the innate immune system, which are primarily dedicated to antiviral defense.

Our hypothesis-driven approach has unveiled three key restricting factors in viral replication in three independent projects. Firstly, we have discovered the ubiquitin E3 ligase MDM2, which plays a role in regulating the stability of the Severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) entry receptor Angiotensin-converting enzyme 2 (ACE2). Our research demonstrates that depleting MDM2 leads to increased ACE2 levels, subsequently enhancing SARS-CoV-2 uptake. Additionally, we have identified the nudix hydrolase NUDT2 as a restriction factor for viruses dependent on triphosphorylated RNA (PPP-RNA). NUDT2 facilitates the conversion of viral PPP-RNA to monophosphorylated RNA (P-RNA), enabling its subsequent degradation through the canonical mRNA degradation pathway, facilitated by 5'-3'-Exoribonuclease 1 (XRN1). Finally, our work has shed light on the importance of the sigma70 factor within the RNA polymerase II complex in the assembly of various phages.

To conclude, the novel findings presented in my dissertation represent a significant leap in our understanding of virus-host interactions. These discoveries not only expand our current knowledge but also provide compelling evidence for conserved mechanisms of viral restriction that can serve as an inception point for the development of novel antiviral treatments.

Zusammenfassung

Viren sind für ihre Replikation auf Wirtsorganismen angewiesen. Im Laufe der Evolution haben diese Wirte eine Vielzahl von Mechanismen entwickelt, um die Ausbreitung von Viren aktiv zu verhindern, mit bemerkenswerten Beispielen innerhalb des adaptiven und angeborenen Immunsystems. Zusätzlich existieren jedoch auch eine Vielzahl anderer Faktoren, die die Virusreplikation hemmen. Diese Faktoren umfassen alle Wirtsproteine innerhalb der Zelle, die die Virusreplikation limitieren, d.h. die Stabilität der viralen DNA, RNA oder Proteine beeinflussen. Die Identifizierung dieser Proteine gestaltet sich aber oft schwierig, da sie typischerweise lebenswichtige Funktionen bei der Aufrechterhaltung der zellulären Homöostase und der Physiologie des Wirts erfüllen, im Gegensatz zu Komponenten des angeborenen Immunsystems, die hauptsächlich der antiviralen Verteidigung dienen.

Unser hypothesenbasierter Ansatz hat drei neue Schlüsselfaktoren für die virale Replikation in drei verschiedenen Projekten identifiziert. Der erste Faktor ist die Ubiquitin E3-Ligase MDM2, die die Stabilität des Severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) Eintrittsrezeptors ACE2 (Angiotensin-konvertierendes Enzym 2) regulieren kann. Unsere Ergebnisse zeigen, dass das Depletieren von MDM2 die Menge an ACE2 erhöhen kann, was anschließend die Aufnahme von SARS-CoV-2 verstärkt. Zweitens haben wir die Nudix-Hydrolase NUDT2 als einen Restriktionsfaktor für Viren identifiziert, die triphosphorylierte RNA (PPP-RNA) nutzen. NUDT2 wandelt virale PPP-RNA in monophosphorylierte RNA (P-RNA) um, was deren anschließenden Abbau über den kanonischen mRNA-Abbaupfad ermöglicht, der von der 5'-3'-Exoribonuclease 1 (XRN1) durchgeführt wird. Schließlich hat unsere Arbeit die Wichtigkeit des Sigma70-Faktors innerhalb des RNA-Polymerase-II-Komplexes bei der Assemblierung verschiedener Phagen herausgearbeitet.

Zusammenfassend stellen die in meiner Dissertation herausgearbeiteten neuen Erkenntnisse einen bedeutenden Sprung im Verständnis der Virus-Wirt-Interaktionen dar. Diese Entdeckungen erweitern nicht nur unser aktuelles Wissen, sondern liefern auch überzeugende Beweise für konservierte Mechanismen der Restriktionsfaktoren, die als Ausgangspunkt für die Entwicklung von neuen antiviralen Behandlungen dienen können.

Abstract short

We conducted several hypothesis-driven studies to identify and characterize restriction factors for viral replication. These include the ubiquitin E3 ligase MDM2, which affects the stability of the SARS-CoV-2 receptor ACE2, and NUDT2, which promotes the degradation of viral RNA. Additionally, the role of the sigma70 factor in the RNA polymerase II complex in the assembly of phages was highlighted. These findings provide new insights into virus-host interactions and could contribute to the development of novel antiviral therapies.

Kurzzusammenfassung

Unser hypothesenbasierter Ansatz hat drei neue Schlüsselfaktoren in der viralen Replikation identifiziert. Die E3 Ligase MDM2, die die Stabilität des SARS-CoV-2-Rezeptors ACE2 beeinflusst, sowie NUDT2, das die Degradation von viraler RNA initiieren kann. Zudem wurde die Rolle des sigma70-Faktors für die Assemblierung von Bakteriophagen herausgearbeitet. Diese Erkenntnisse bieten neue Einblicke in die Virus-Wirt-Interaktionen und könnten zur Entwicklung neuer antiviraler Therapien beitragen.

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Disclosure statements

Sections of this thesis have already been published in peer-reviewed journals as required by the graduate school. The introduction, material and methods, results and discussion of this thesis have been adapted from following publications:

Publication P1: MDM2 Influences ACE2 Stability and SARS-CoV-2 Uptake:

Emslander, Q., Krey, K., Hamad, S., Maidl, S., Oubraham, L., Hesse, J., Henrici, A., Austen, K., Mergner, J., Grass, V. and Pichlmair, A., 2023. MDM2 Influences ACE2 Stability and SARS-CoV-2 Uptake. *Viruses*, 15(8), p.1763.

Publication P2: NUDT2 initiates viral RNA degradation by removal of 5'-phosphates

Laudenbach, B.T.*, Krey, K.*, **Emslander, Q.***, Andersen, L.L., Reim, A., Scaturro, P., Mundigl, S., Dächert, C., Manske, K., Moser, M. and Ludwig, J., 2021. NUDT2 initiates viral RNA degradation by removal of 5'-phosphates. *Nature Communications*, 12(1), p.6918.

Publication P3: Cell-free production of personalized therapeutic phages targeting multidrug-resistant bacteria

Emslander, Q.*, Vogele, K*, Braun, P., Stender, J., Willy, C., Joppich, M., Hammerl, J.A., Abele, M., Meng, C., Pichlmair, A. and Ludwig, C., 2022. Cell-free production of personalized therapeutic phages targeting multidrug-resistant bacteria. *Cell chemical biology*, 29(9), pp.1434-1445.

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Competing interests

The author Quirin Emslander has filed two patents (see list patents) related to the findings in this thesis.

Otherwise the author does not declare any conflict of interest.

List of first-author publications

Asterisk indicates equal contribution:

- 1) **Emslander, Q.**, Krey, K., Hamad, S., Maidl, S., Oubraham, L., Hesse, J., Henrici, A., Austen, K., Mergner, J., Grass, V. and Pichlmair, A., 2023. MDM2 Influences ACE2 Stability and SARS-CoV-2 Uptake. *Viruses*, 15(8), p.1763.
- 2) Laudenbach, B.T.*, Krey, K.*, **Emslander, Q.***, Andersen, L.L., Reim, A., Scaturro, P., Mundigl, S., Dächert, C., Manske, K., Moser, M. and Ludwig, J., 2021. NUDT2 initiates viral RNA degradation by removal of 5'-phosphates. *Nature Communications*, 12(1), p.6918.
- 3) **Emslander, Q.***, Vogele, K.*, Braun, P., Stender, J., Willy, C., Joppich, M., Hammerl, J.A., Abele, M., Meng, C., Pichlmair, A. and Ludwig, C., 2022. Cell-free production of personalized therapeutic phages targeting multidrug-resistant bacteria. *Cell chemical biology*, 29(9), pp.1434-1445.

List of patents

- 1) Method for producing a modified bacteriophage without genome modification (17627861) (2022/8/18)
- 2) Host-independent expression of bacteriophages (17627854) (2022/8/18)

List of abbreviations

ACE2 - Angiotensin-Converting Enzyme 2

ACN - Acetonitrile

AGC - Automatic Gain Control

AIDS- Acquired Immunodeficiency Syndrome

A549 - Human Lung Carcinoma Cell Line A549

ATP - Adenosine Triphosphate

ATCC - American Type Culture Collection

BCA - Bicinchoninic Acid

BHI - Brain Heart Infusion

CAA - Chloroacetamide

CARD - Caspase Activation and Recruitment Domain

CBRND-EB - Chemical, Biological, Radiological, Nuclear Defense-Enabling Biotechnologies

CCR4/POP2/NOT1 - Carbon Catabolite Repression 4/Positive of Proliferation 2/Negative on TATA less 1 (components of the CCR4-NOT complex)

CD4 - Cluster of Differentiation 4

CIP - Calf Intestinal Phosphatase

CLP-P3 - A type of bacteriophage

COVID-19 - Coronavirus Disease 2019

CPD1/DCP2 - Decapping Protein 1/Decapping Protein 2

CRISPR/Cas9 - Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated protein 9

CTD - C-Terminal Domain

CTP - Cytidine Triphosphate

DMEM - Dulbecco's Modified Eagle Medium

DNA - Deoxyribonucleic Acid

DTT - Dithiothreitol

dsDNA - Double-stranded DNA

dsRNA - Double-stranded RNA

EcoRI - The restriction enzyme EcoRI used for cutting DNA at specific sequences

EDTA - Ethylenediaminetetraacetic acid

EMCV - Encephalomyocarditis Virus

FA - Formic Acid

FCS - Fetal Calf Serum

GFP - Green Fluorescent Protein

GTP - Guanosine-5'-triphosphate
HCD - Higher-energy Collisional Dissociation
HEK293R1 - Human Embryonic Kidney 293 Cells with R1 modification
HEK293T - Human Embryonic Kidney 293 cells with SV40 large T-antigen
HEPES - 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic Acid
HPLC - High-performance liquid chromatography
HSV-1 - Herpes Simplex Virus type 1
IAV-ren - Influenza A Virus with renilla luciferase
ICE Analysis - Inference of CRISPR Edits Analysis
IVT - In vitro transcription
K. pneumoniae - Klebsiella pneumoniae
LC-MS/MS - Liquid Chromatography with Tandem Mass Spectrometry
LFQ - Label-Free Quantification
LY6E - Lymphocyte Antigen 6 Complex, Locus E
MDM2 - Mouse Double Minute 2 homolog
MAVS - Mitochondrial Antiviral-Signaling Protein
MEFs - Mouse Embryonic Fibroblasts
MERS - Middle East Respiratory Syndrome
MOI - Multiplicity of Infection
mRNA - Messenger RNA
MS1 - First Mass Spectrometry
MS2 - Second Mass Spectrometry
MX1 - Myxovirus Resistance Protein 1
NTC - Non-Targeting Control
NUDT2 - Nudix Hydrolase 2
NEB - New England Biolabs
OD - Optical density
PBS - Phosphate-buffered saline
PEI - Polyethyleneimine
PRRs - Pattern Recognition Receptors
PTMs - Post-Translational Modifications
PCR - Polymerase Chain Reaction
RBD - Receptor Binding Domain
RIG-I - Retinoic Acid-Inducible Gene I
RNA - Ribonucleic Acid RNase L - Ribonuclease L
RT-qPCR - Reverse Transcription Quantitative Polymerase Chain Reaction
RVFV - Rift Valley Fever Virus

SARS-CoV - Severe Acute Respiratory Syndrome Coronavirus

SARS-CoV-2 - Severe Acute Respiratory Syndrome Coronavirus 2

SDS - Sodium Dodecyl Sulfate

SDS-PAGE - SDS-Polyacrylamide Gel Electrophoresis

SEAP - Secreted Alkaline Phosphatase

SFV - Semliki-Forest-Virus

VSV - Vesicular Stomatitis Virus

WT - Wild Type

XRN1 - 5'-3' Exoribonuclease 1

Introduction

Preface

Host organisms have evolved a fascinating repertoire of defense mechanisms against viral infection, leading to the emergence of a diverse adaptive and innate immune system. One prominent facet of the innate immune response involves pattern recognition receptors (PRRs), which are specialized proteins identifying viral components. Activation of PRRs, in turn, initiates the expression of genes which play a crucial role in suppressing viral replication (D. Li and Wu 2021; Chaplin 2010). Proteins that interfere with viral replication are commonly referred to as "restricting factors." In particular, when such restricting factors inhibit viral replication, are encoded in the germline, interact directly with viral elements, and are typically induced by interferon, they are precisely termed "restriction factors" (Duggal and Emerman 2012). All steps of the viral life cycle can be targeted by restriction factors with different modes of actions (Figure 1). This includes the viral uptake process, e.g., depletion of LY6E inhibits coronavirus replication by interfering with its clathrin-mediated endocytosis (Mar et al. 2023) and viral RNA integrity, e.g., RNase L can degrade viral RNA (Gusho, Baskar, and Banerjee 2020). As well as the viral transcriptional process, e.g., MXA inhibits influenza A virus RNA amplification by interfering with the viral ribonuclear protein (Villalón-Letelier et al. 2017) and many other aspects of the viral life cycle (Figure 1). Understanding which host proteins restrict the virus enables fundamental insights of virus-host relationship and potential therapeutic application.

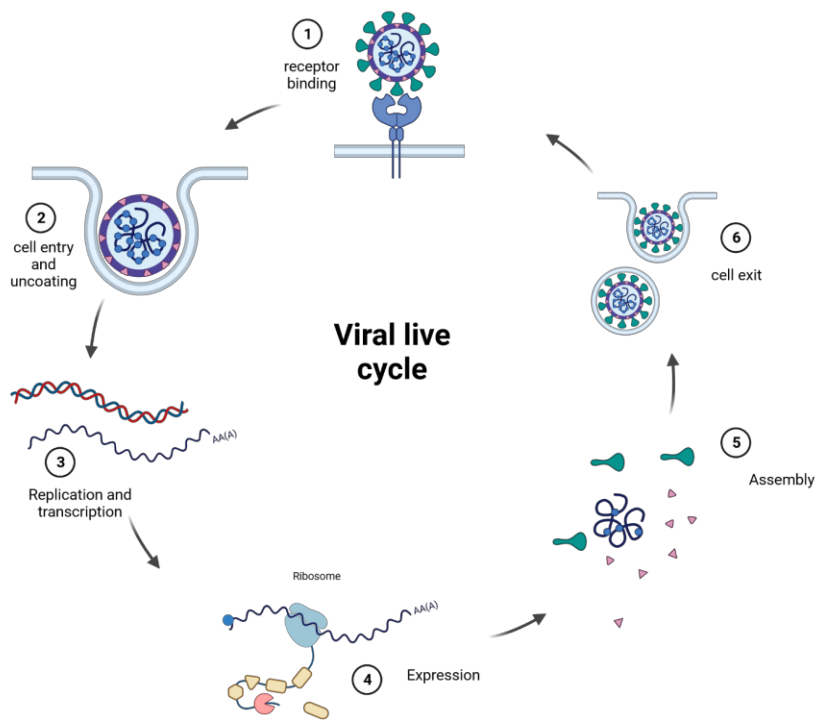


Figure 1: Schematic illustrating the viral replication cycle: The cycle begins with the virus binding to a specific cell receptor, initiating cellular entry. Upon entry, the viral genome is released into the cytoplasm, enabling replication and viral protein synthesis. Following these events, viral assembly occurs within the host cell, ultimately resulting in the release of mature virions.

Introduction P1: MDM2 influences ACE2 stability and SARS-CoV-2 uptake

Viral entry receptors are crucial in the initiation of viral infections, shaping host cell recognition, host range determination, and tissue tropism. These receptors primarily face the extracellular space, are embedded within the plasma membrane and are broadly classified into four categories, which are relevant for virus infection: cell adhesion molecules (Dalglish et al. 1984), integrins (Gavrilovskaya Irina N. et al. 1999; Gavrilovskaya et al. 1998), sialylated glycans (Suzuki et al. 2000), and phosphatidylserine family receptors (Hunt et al. 2011). Notably, the cell type-specific expression of these receptors influences the site of infection and the potential for interspecies transmission. A prominent example is provided by coronaviruses, with the Middle East respiratory Syndrome virus (MERS) originating from dromedary camels and also infecting humans through preferential binding to α 2,3-linked sialylated glycans, prevalent in the primary site of MERS infection, the lower respiratory tract (Hemida et al. 2017; Wentao Li et al. 2017).

The ACE2 entry receptor defines SARS-CoV-2 replication

The Severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2), responsible for the COVID-19 pandemic in 2019, likely originated in bats and adapted to utilize angiotensin 2 (ACE2) as its entry receptor (H. Wang et al. 2022; Lau et al. 2020; Wacharapluesadee et al. 2021). SARS-CoV-2 recognizes ACE2 via its spike protein (Q. Zhang et al. 2021), and mutations in the spike domain have expanded host range (Q. Wang et al. 2023; Naderi et al. 2023) and also infectivity within the human population (Ren et al. 2022; Karim and Karim 2021). The viral entry process commences with the binding of SARS-CoV-2 to ACE2 through its spike protein, comprising a subunit-1 (bearing the receptor-binding domain) and subunit-2 (Q. Zhang et al. 2021). This critical step requires cleavage at the furin site between the two spike subunits, a process facilitated by the cell surface protein TMPRSS2 or cathepsin in endosomes. Following membrane fusion, the viral genome is released into the cytoplasm, initiating viral replication (Koch et al. 2021).

Interestingly, ACE2 deviates from the typical categories of viral entry receptors, functioning as a peptidase involved in angiotensin I processing in various human cell types (Turner 2015). Moreover, ACE2 is not abundantly expressed in the lung, the primary site of SARS-CoV-2 infection (M.-Y. Li et al. 2020; Hikmet et al. 2020). Several co-receptors, such as AXL, sialic acid, and lectins, have been identified to support SARS-CoV-2 uptake, contributing to its extraordinary tropism and infectivity (Eslami et al. 2022). The expression levels of ACE2 are

central to SARS-CoV-2 infection (Hoffmann et al. 2020), and as previously mentioned, ACE2 levels are quite low in the respiratory system, with some lung cells lacking notable levels of ACE2 completely (Puray-Chavez et al. 2021). Nevertheless, several patients with severe COVID-19 outcomes display increased ACE2 expression. This gives reason to speculate about molecular mechanisms governing ACE2 levels (Gheware et al. 2022).

Protein abundance regulated by ubiquitination and proteasomal degradation

Protein abundance regulation, achieved through post-translational modifications (PTMs), significantly influences protein stability and turnover (Lee et al. 2023). Common PTMs include ubiquitination, acetylation, and phosphorylation (Schrader, Harstad, and Matouschek 2009; Zecha et al. 2022). Ubiquitination, involving isopeptide bond formation with K48 lysine residues initiates proteasomal degradation (Mallette and Richard 2012; Thrower et al. 2000). The transfer of ubiquitin to the substrate typically involves E1, E2, and E3 ubiquitin ligases. E1 activates the ubiquitin using adenosine triphosphate and transfers the activated ubiquitin to the mediator E2. Finally the E3 ligase executes the delivery of the ubiquitin to the substrate by simultaneously binding E2 and the substrate via protein-protein interaction (Potjewyd and Axtman 2021; Thrower et al. 2000). Acetylation and also phosphorylation have been reported to interfere with ubiquitination at the lysine side chain, thus preventing proteasomal degradation and stabilizing the protein. Acetylation of the amine of the lysine side chain has been shown to prolong protein stability, probably by disabling the nucleophilic attack necessary for ubiquitination. Unlike acetylation, phosphorylation does not happen on the amine group of the lysine side chain but in close proximity to it. It is not well characterized if this phosphorylation changes the conformation of the substrate proteins thus rendering it sterically impossible to interact with E3 ligase due to the negative charge so close to the ubiquitination site (Lee et al. 2023; Zecha et al. 2022; Gong et al. 2020; Saha et al. 2011). The enzymes ubiquitin ligases, acetyl-transferases and kinases, executing the transfer of these PTMs can strongly affect receptor stability and therefore restrict virus replication. For ACE2 all these three PTMs have been reported at the beginning of the COVID-19 pandemic at phosphositeplus (<https://www.phosphosite.org/>) but their functionality as well as the corresponding enzymes had not been characterized in detail.

Therapeutic relevance of the ACE2 receptor

Because ACE2 regulates SARS-CoV-2 infection, it poses great therapeutic potential (Bartolomeo et al. 2021; Winkler et al. 2020). Strategies aimed at modulating ACE2 expression or accessibility for SARS-CoV-2, such as microRNA targeting of ACE2 transcripts leading to decreased ACE2 expression (D. Lu et al. 2020), and the ACE2/ receptor binding domain interface disruption to directly prevent SARS-CoV-2 entry (Huang et al. 2021; Shin et al. 2022), hold promise in combating the virus, particularly in immunocompromised individuals ("Vaccines and Therapeutics for Immunocompromised Patients with COVID-19" 2023). As vaccines are commonly based on the SARS-CoV-2 spike protein, which displays a high mutation rate, it appears necessary to adapt the vaccine (J. Yang et al. 2022; Malik et al. 2022). Here the relatively slow mutation rate of host proteins like the ACE2 entry receptor underscores their potential as stable therapeutic targets (Suvarnapathaki et al. 2022). In summary, these reasons make ACE2 and host modulator of ACE2 a promising therapeutic target.

Introduction P2: NUDT2 initiates viral RNA degradation by removal of 5'-phosphates

In the context of gene expression in eukaryotic cells, ensuring the integrity of messenger RNA (mRNA) is essential, given that mRNA acts as a bridge between the genetic code and functional protein synthesis. To safeguard mRNA from rapid degradation by exonucleases, it is imperative to protect both its 5' and 3' ends. The 5' end is shielded by a cap structure, which comprises a 7-methylguanosine molecule linked to the first transcribed nucleotide through a 5' to 5' triphosphate bridge. This first nucleotide also undergoes methylation at the ribose O-2 position (Galloway and Cowling 2019). Meanwhile, the 3' end is safeguarded by a polyadenine (poly-A) tail (Adjibade and Mazroui 2014). In order to initiate the typical mRNA decay process, these protective modifications must be enzymatically removed. The 5' cap structure is mainly cleaved by DCP1/DCP2, making the monophosphorylated mRNA (P-RNA) vulnerable to degradation by the exonuclease XRN1 (Mugridge, Coller, and Gross 2018). Removal of the poly-A tail is orchestrated by CCR2/POP2/NOT1, allowing access to various degradation pathways (Adjibade and Mazroui 2014).

The Role of triphosphorylated RNA in Innate Immunity

In response to viral infection, eukaryotic hosts have developed diverse mechanisms to sense viral RNA, especially through the recognition of viral RNA patterns using pattern recognition receptors (PRR). These PRRs play a crucial role in detecting pathogen-associated molecular patterns (D. Li and Wu 2021). The PRRs include Toll-like receptors (TLRs), which recognize short dsRNA (TLR3) or ssRNA (TLR7 and TLR8) in endosomes (R. Yang et al. 2022). Additionally, MDA5 recognizes longer dsRNA strands, while RIG-I (Retinoic Acid-Inducible Gene I) detects triphosphorylated RNA (PPP-RNA) in the cytosol (Chan and Gack 2016). RIG-I is comprised of a C-terminal (CTD), two CARD (CARD) domains, and one helical domain. It remains in an auto-repressed state until the CTD binds to the 5' triphosphorylated or short viral RNA. Upon activation, RIG-I structures around the viral RNA and connects with the MAVS protein through the CARD domain (Thoresen et al. 2021). This protein complex initiates various downstream signaling events, including type I and III interferon responses, proinflammatory cytokine production, and apoptosis (Rehwinkel and Gack 2020; Maelfait, Liverpool, and Rehwinkel 2020). Induced interferons can be secreted to prime neighboring cells for viral defense or stimulate an antiviral response within the same cell through autocrine signaling (Michael Lavigne et al. 2021). Consistent with this, in vivo experiments have demonstrated that depleting RIG-I leads to increased infectivity by Influenza A virus, reduced type I interferon production, and an overall diminished adaptive immune response,

underscoring the crucial role of sensing viral PPP-RNA in host immunity (Kandasamy et al. 2016).

Degradation of triphosphorylated mRNA: A barrier for viral replication

Viruses face a complex environment within host cells, where diverse RNA degradation processes are employed to protect cellular integrity. To successfully navigate this environment, viruses have developed a range of strategies. General strategies involve the suppression of antiviral responses (Goodbourn, Didcock, and Randall 2000), retroviral conversion of RNA to DNA for integration into the host genome (Craigie and Bushman 2012), and the use of microRNAs to impede viral RNA degradation (Y. Li et al. 2013). At the level of viral RNA, protective measures may include modifying the 3' end with a poly-A tail (Kim et al. 2020). For shielding the 5' end from XRN1-mediated degradation (Mugridge, Collier, and Gross 2018), viruses may acquire the cap structure from host mRNA (De Vlugt, Sikora, and Pelchat 2018) or, intriguingly, employ triphosphorylation. The latter mechanism is employed by several viruses, including the Vesicular Stomatitis virus (Weber et al. 2013; Takeuchi and Akira 2008). Notably, the triphosphorylation structure is a particularly interesting protection mechanism, as bacteria use the triphosphorylation structure to stabilize and regulate their mRNA levels (Kaberdin, Singh, and Lin-Chao 2011). Also the corresponding viruses, the bacteriophages, possess enzymes modulating phosphorylation of RNA, which can affect their turnover rate, and the T7 RNA polymerase can generate triphosphorylated RNA (PPP-RNA) (Durand et al. 2012; Sy, Lan, and Tree 2020; Milligan et al. 1987). Importantly, in bacteria the process for dephosphorylation and subsequent degradation of PPP-RNA has been characterized: The bacterial nudix hydrolase RppH initiates bacterial mRNA degradation by removing the pyrophosphate group from the bacterial mRNA enabling 5'-3' degradation of the mono phosphorylated mRNA via RNases (Hsieh et al. 2013; Bischler et al. 2017). Because this triphosphorylation structure is shared between prokaryotes and eukaryote-infecting viruses, it is possible that a conserved mechanism for regulation of viral PPP-RNA turnover exists. In line with this hypothesis, it has been shown that depletion of XRN1 in mammalian cells increases viral load of human viruses relying on triphosphorylated RNA (Y. Li et al. 2013). This further suggests that another hydrolase, perhaps a nudix hydrolase, similar to RppH exists in eukaryotes facilitating the dephosphorylation of PPP-RNA, providing XRN1 with P-RNA for further degradation (Nagarajan et al. 2013).

Introduction P3: Cell-free production of personalized therapeutic phages targeting multidrug-resistant bacteria

The study of virus-host interactions presents a substantial challenge *in vivo*, given the intricate physiology of organisms, the diversity of tissue structures, and the array of distinct cell types involved. To simplify the complexity, research often commences with cancer cell lines, where cellular constituents can be more readily manipulated and studied. This includes the depletion or overexpression of single host proteins to test the effect on the virus, which revealed the different roles of the interferon stimulated genes (Hubel et al. 2019) or restricting factors for the viral cell-entry (Pfaender et al. 2020). Nevertheless, for many research questions a cell based system is still too complex. The reliance of certain viruses on host machinery, particularly for processes involved in gene expression means that perturbation of these host factors can have deleterious cellular effects. Their depletion can be lethal for the cell, which specifies these genes as essential (Du 2020; Nikolaitchik et al. 2023). Common essential genes include the components of the DNA polymerase complex (Bellelli et al. 2020), RNA polymerase II factors (Lynch et al. 2018) and transcription factors (D. I. Choi et al. 2021). The dependence on these host processes is one of the many characteristics that mammalian viruses and bacterial viruses, the bacteriophages, share (D. Walsh and Mohr 2011; Weigel and Seitz 2006). Due to the less complex nature of the bacterium-bacteriophage relationship, findings from this species can be used to infer information about the mammalian-host interactionship (Mathew and Chatterji 2006; Wein et al. 2023; Morehouse et al. 2020) .

Minimal systems to study essential genes

To dissect the molecular roles of these essential proteins within the viral life cycle, different approaches can be used, e.g., computational approaches (Yuan et al. 2012), engineered cells (Mnaimneh et al. 2004) and minimal systems such as cell-free systems. These cell-free systems recreate the living cell environment comprising functional DNA replication and protein expression machinery, offering a valuable platform to study these critical biological processes. Mammalian cell-free systems have facilitated the production of viruses such as human Encephalomyocarditis virus (EMCV) and Polyomavirus JC (Mikami, Kobayashi, and Imataka 2010; Kobayashi, Machida, and Imataka 2014; Nesper et al. 1997), while bacterial cell-free systems have successfully generated bacteriophages like T7, T4, and MS2 (Rustad et al. 2018, 2017). These systems, whether mammalian or bacterial, consist of a cell extract (HeLa or E.coli), an energy solution, metabolites and crowding agents. The cell extract is usually

generated by lysing actively dividing cells, which are processed to a lysate that can be stored for long-term use. The energy solution consists of physiologically relevant energy sources (ATP etc.), the metabolite solution includes all the relevant nucleotides for protein biosynthesis (nucleotides, amino acids, etc.) and crowding reagents contain the relevant buffers and ions (HEPES, magnesium etc.). The supplementation of these systems with the DNA or RNA of the corresponding virus/bacteriophage leads to the self-assembly of infectious virions (Kobayashi, Machida, and Imataka 2014; Rustad et al. 2017).

The RNA II polymerase complex

The RNA II complex consists of several essential gene products and the progress made in unraveling the structure of bacterial and eukaryotic transcription machinery has significantly enhanced our comprehension of transcription mechanisms. The bacterial RNA polymerase and eukaryotic RNA polymerase II share notable structural resemblances. These similarities encompass their overall structure and the positions of functional components including the subunits. These structural parallels are mirrored by similarities in how they interact with DNA (Ebright 2000). The bacterial RNA II complex includes five subunits: β , β' , ω , two α and σ subunit(s). The α part is relevant for transcription initiation and interaction with other regulatory proteins, β and β' for DNA binding and execution of RNA synthesis, ω promoting RNA polymerase assembly and σ for the promoter recognition. The assembly of all these components into the RNA II polymerase facilitates the transcription of the host genes. (Eick and Geyer 2013; Mathew and Chatterji 2006; Sutherland and Murakami 2018). Bacteriophages employ a wide range of mechanisms to disrupt or reconfigure bacterial functions, particularly by targeting the RNA polymerase. Many bacteriophages express proteins designed to selectively modulate the RNA polymerase activity of the bacterial host to serve their own developmental requirements (Nechaev and Severinov 2008, 2003; Liu et al. 2014). However, the intricacies of the subunits are not completely understood.

Aims and objectives of this thesis

This cumulative thesis aims to identify novel restricting factors for viral replication to better understand the underlying biological processes. With our data- and hypothesis-driven approach combined with state-of-the-art in vitro models, we were able to discover and in-depth characterize three previously uncharacterized host proteins that restrict different viruses in the early steps of their life cycle.

The emergence of SARS-CoV-2 and the following pandemic necessitated an in-depth understanding of the biological processes involved in SARS-CoV-2 replication. Leveraging previously published proteomics, interactome, and transcriptome data, a knockout screen and proteomics profiling was performed to identify host proteins restricting viral replication.

The degradation of viral PPP-RNA is not well understood, even though many viruses harboring the potential for an epidemic or even pandemic outbreak rely on this modification. It was hypothesized that nudix hydrolases (NUDT) could facilitate the initiation of viral PPP-RNA degradation in humans, because the anticipated mechanism has been discovered in bacterial nudix hydrolases.

RNA polymerase II is integral to transcription, a function conserved across the domains of life. Despite the recognition that bacteriophages exhibit stringent host specificity, typically governed by cell surface receptor patterns, the role of intracellular restrictive factors remains obscure. We reasoned that the sigma 70 subunit of RNA polymerase II is a major restriction factor for bacteriophage replication because it recognizes the promoter region, and the other parts of the complex could be redundant due to high sequence similarity.

Material and methods

Material and methods P1: MDM2 influences ACE2 stability and SARS-CoV-2 uptake

Cell line cultivation

The cell lines in this study comprised A549-H2B-mRFP and A549-H2B-mRFP-ACE2, along with HEK293R1 for the production of lentiviral vectors. The cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS) and 1% Penicillin/Streptomycin, as elaborated in a prior study (Stukalov et al. 2021). Mycoplasma screening was conducted to prevent of mycoplasma contamination. To generate the A549-H2B-mRFP-ACE2 knockout cell line multiplexed single-guide RNA (sgRNA) templates were cloned into the pLentiCRISPRv2 plasmid (Addgene plasmid #52961, Addgene, Watertown, MA, USA). The sgRNA sequence templates, except for the non-targeting control (NTC) sequences (Sanjana, Shalem, and Zhang 2014), were designed with Synthego (Redwood City, CA, USA). Subsequent steps included lentivirus production, cell transduction, and selection, following established protocols (Bergant et al. 2022). Briefly, lentiviruses encoding puromycin resistance, Cas9, and sgRNAs were introduced into A549-H2B-mRFP-ACE2 cells, which were subsequently subjected to a seven-day puromycin selection (2 µg/mL). The knockout efficiency of all resultant cell populations was validated through sanger sequencing, employing Synthego Performance Analysis (ICE Analysis, 2019, v3.0, Synthego, Redwood City, CA, USA, 06/2021).

For the generation of A549-H2B-mRFP-ACE2 and A549-H2B-RFP-ACE2 (K788R) cells, lentiviruses were produced as previously described, utilizing the pWpi-puro expression vector (Stukalov et al. 2021), which was sequenced before lentivirus generation to validate ACE2 integrity and K788R insertion. To ensure uniform lentiviral transduction efficiency, a low multiplicity of infection (MOI) of 0.1 was applied for transduction into A549-H2B-mRFP cells, followed by antibiotic selection to establish a stable cell population.

Preparation of virus stocks

The virus stocks employed in this study, including clinical SARS-CoV-2 parental strain and SARS-CoV-2-GFP strains, were prepared according to previously described protocols. SARS-CoV-2 strains were generated by infecting Vero E6 cells for a duration of two days at a multiplicity of infection (MOI) of 0.01 (Stukalov et al. 2021). Additionally, VSV-GFP, VSV-S and RVFV-GFP stocks, a kind gift from Friedemann Weber, were generated in accordance with established methodologies (Jocher et al. 2022; Pichlmair et al. 2011; Kainulainen et al. 2014).

Virus infection and live-cell imaging experiment

Infection experiments were conducted by seeding A549-H2B-mRFP-ACE2, A549-H2B-mRFP-ACE2 (K788R), and the knockout cell line one day prior to the experiment, employing 96-well plates. The cells were subjected to infections with the following reporter viruses, including SARS-CoV-2-GFP (MOI: 3), YFV-GFP (MOI: 1), VSV-GFP (MOI: 0.01), RVFV-

Katuschka (MOI: 0.1), and HSV-1-GFP (MOI: 0.1). The progression of infection was monitored utilizing the IncuCyte S3 Live-Cell Analysis System (Essen Bioscience), capturing green and red fluorescence signal (GFP and RFP), and phase contrast signals at intervals ranging from 3 to 4 hours, for a total of 72 hrs. Data was analyzed utilizing the internal IncuCyte S3 Software (Essen Bioscience, version 2020C rev1), entailing the normalization of the integrated intensity of the GFP (virus) signal either to the integrated intensity of the RFP signal (cell) or to the confluency measured via the phase contrast channel (cell). Statistical significance was determined with a two-sample t-test, with p-values indicated as $p < 0.05$ (*), < 0.01 (**), < 0.001 (***). Subsequently, for each infection, the maximum signal value for each knockout was selected, and the percentage change relative to the NTC was calculated.

For the screening of the drugs live-cell imaging experiments followed the same protocol as described previously, with the exception that cells underwent a 4-hour pre-treatment with either MG132 (medchem, HY-13259) or PR-619 (medchem, HY-11814). These inhibitors were dissolved in dimethyl sulfoxide (DMSO) and diluted in the cell culture medium before application. To assess cytotoxicity, some cells were left uninfected after inhibitor treatment, while others were pre-treated with the inhibitors for 4 hours before being infected with the reporter virus SARS-CoV-2-GFP (MOI: 3) to investigate the effect of the drugs on the virus.

Full proteome mass spectrometry sample preparation

For each replicate of A549-H2B-mRFP-ACE2 cells (including MDM2 knockouts and non-targeting controls), SARS-CoV-2 infection was performed at an MOI of 3. Subsequently, the samples were harvested (buffer: 4% SDC, 100 mM Tris-HCl, pH 8.5), heat-inactivated at 95 °C for 10 minutes, and sonicated at 4 °C for 15 minutes using a 30-second on/30-second off cycle at high settings. The protein concentrations were determined using the BCA assay (Pierce, Appleton, WI, USA) following the manufacturer's instructions. For each sample, 50 µg of protein material was subjected to reduction and alkylation for 5 minutes at 45 °C with TCEP (10 mM) and CAA (40 mM). Protein digestion was carried out overnight at 37 °C using trypsin (1:100 w/w, enzyme/protein; Promega, Madison, WI, USA) and LysC (1:100 w/w, enzyme/protein; Wako, Hiroshima-shi, Japan).

For proteome analysis, 20 µg of peptide material underwent desalting using SDB-RPS StageTips (Empore, Stellarton, NS, Canada). 1% trifluoroacetic acid (TFA) in isopropanol was added to the samples achieving a final volume of 200 µL and loaded onto StageTips. Subsequently, they were washed with 1% TFA in isopropanol and 0.2% TFA/5% acetonitrile (ACN, Concord, CA, USA). Peptides were eluted with 1.25% ammonium hydroxide (NH₄OH) in 60% ACN and then dried using a SpeedVac centrifuge (Eppendorf, Concentrator Plus, Hamburg, Germany). The dried peptides were resuspended in 0.1% formic acid (FA) before LC-MS/MS analysis. Peptide concentrations were assessed optically at 280 nm (Nanodrop 2000, Thermo Scientific, Waltham, MA, USA) and equalized using 0.1% FA.

LC-MS/MS data acquisition

The proteome samples were analyzed on an Eclipse mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled on-line to a Dionex Ultimate 3000 RSLCnano system (Thermo Fisher Scientific). The Peptides were reconstituted in 0.1% FA, firstly loaded to a trap column (ReproSil-pur C18-AQ, 5 μ m; Dr. Maisch, 20 mm $\times$ 75 μ m, self-packed) at a flow rate of 5 μ L/min in 100% solvent A (0.1% FA in HPLC-grade water). After 10 min, peptides were transferred to an analytical column (ReproSil Gold C18-AQ, 3 μ m; Dr. Maisch, 400 mm $\times$ 75 μ m, self-packed) at 50 °C and separated using an 80-min linear gradient from 4% to 32% of solvent B (0.1% FA, 5% DMSO in ACN) at a 300 nL/min flow rate.

The mass spectrometer was utilized in positive ionization and data-dependent acquisition modes. Full MS1 scans (360–1300 m/z) were measured in the orbitrap with a resolution of 60,000, a normalized automatic gain control (AGC) value of 100% with a maximum injection time of 50 ms. Peptide precursor selection for fragmentation was executed in a fixed cycle time of 2 s. precursors with charge states from 2 to 6 were selected, and dynamic exclusion of 35 s was enabled. Peptide fragmentation was performed using higher-energy collision-induced dissociation (HCD) and a normalized collision energy of 30%. The precursor isolation window width of the quadrupole was set to 1.3 m/z. MS2 spectra were acquired in the orbitrap with a resolution of 15,000, a fixed first mass of 100 m/z, a normalized AGC target value of 200%, and a maximum injection time of 22 ms.

MS data processing and analysis

Raw MS data files from the data-dependent acquisition experiments were processed using MaxQuant (version 2.0.3.1.) with default settings. The analysis included label-free quantification (LFQ) with parameters such as LFQ min ratio count 2 and classic normalization type, and intensity-Based Absolute Quantification (iBAQ) was enabled. Spectra were searched against the forward and reverse sequences of the reviewed human proteome, including isoforms (Uniprot, UP000005640), using the built-in Andromeda search engine.

The resulting protein groups were further subjected to analysis using Perseus (Version: 1.6.15.0). The proteinGroups.txt file was imported to Perseus and the analysis was conducted using the LFQ values (4941 proteins IDs), with columns filtered for default settings including reverse sequences, those only identified by site, and contaminants. Subsequently, the LFQ values were log2-transformed, and protein groups with at least 2 valid values per grouping were retained. Missing values were imputed using the normal distribution method (1.8 downshift, 0.3 width, separately for each column). A two-sample t-test was applied to the two conditions, leading to 267 significantly changing proteins (two-sided, permutation-based FDR, 0.05 FDR, 0.1 S0, 250 randomizations).

Upstream regulator analysis

To identify global regulators, the significant hits from the full proteome analysis were further analyzed using the Ingenuity Pathway Analysis software (version 84978992, Qiagen, Hilden, Germany). The Core analysis was performed using default settings, for p-value calculations the Ingenuity Knowledge Base served as the reference set. "Direct and indirect relationships" were considered for upstream regulator analysis. Following analysis, only upstream regulators with a p-value < 0.05 were deemed significant.

VSV-S and VSV infection

To investigate whether the observed effects on SARS-CoV-2 were dependent on viral uptake, a VSV pseudovirus bearing the SARS-CoV-2 spike (S) protein on its surface (VSV-S) was utilized, with parental VSV serving as a reference. Cells, including A549-H2B-mRFP-ACE2 MDM2 knockout and A549-H2B-mRFP-ACE2 non-target control cells, or A549-H2B-mRFP, A549-H2B-mRFP-ACE2, and A549-H2B-mRFP-ACE2 (K788R) cells, were seeded in a 96-well plate the day prior to infection. Subsequently, cells were infected with VSV-S (MOI: 3) and VSV-GFP (MOI: 0.01). Infection levels were monitored using the IncuCyte S3 Live-Cell Analysis System (Essen Bioscience) as described earlier. The obtained raw data underwent processing with the internal IncuCyte S3 Software (Essen Bioscience; version 2020C rev1). Normalization of the integrated intensity of the GFP signal (virus) was performed using the integrated intensity of the RFP signal (cell) or phase (cell). The normalized signal was further processed by median filtering and a Savitzky–Golay filter. The significance was calculated by comparing each of the infections for VSV and VSV-S to the control using a two-sample t-test for each time point (with p-value ns > 0.05, * < 0.05, ** < 0.01, *** < 0.001).

Virus-like particle uptake assay and immunofluorescence staining

The SARS-CoV-2 virus-like particles (VLPs) were generated as previously published, (Roessler et al. 2022). One day prior to the experiment A549-H2B-mRFP-ACE2 and A549-H2B-mRFP-ACE2 (K788R) cells were seeded. Cells were rinsed with PBS (4 °C), and a mixture of 100 µL of DMEM (10% FCS and 1% Penicillin/Streptomycin) and 100 µL of VLPs was added per replicate. To stain the cytosol, CellTracker™ Orange CMRA Dye (Thermo Fisher Scientific, Waltham, MA, USA, C34551) was included in the medium/VLP mix according to the manufacturer's instructions. The cells were incubated on ice to enable simultaneous uptake (30 min) and then the uptake continued in the incubator (1 h, 37 °C, 5% CO₂). The cells were washed three times with PBS, fixed with 4% PFA (15 min, RT) and again washed three times with PBS. Cell permeabilization was executed with 0.1% Triton-X100 at RT, washing with PBS, blocking with 5% FCS (1 h, RT), and washing with PBS. An in-house antibody dilution (1:1000) in 5% FCS (1 h, RT) was used to stain the VLPs, followed by PBS washing. The HA-tagged ACE2 samples were processed as described before and the HA-tag was stained in the dark (1:500) (Cell Signaling, Danvers, MA, USA, 3724S) in 5% FCS (1 h, RT). The Zeiss LSM 900 (Zeiss, Oberkochen, Germany) with an Airyscan 2 detector system and a Plan-APOCHROMAT 63×/1.4 Oil DIC Objective was used to generate super-resolution images. ImageJ software (version 1.53) was used for post-acquisition linear adjustments of image brightness. Additionally, confocal images were obtained using an Olympus FV10i microscope (Olympus, Tokyo, Japan) with a 60×/1.2 water immersion objective.

qRT-PCR analysis

To conduct qRT-PCR analysis, the corresponding cell lines were seeded one day prior to the experiment. Cells were infected with SARS-CoV-2 (MOI: 3) and samples were harvested at 1 hour, 12 hours, or 24 hours post-infection to quantify SARS-CoV-2 transcript levels. ACE2 levels were quantified in uninfected cells. In both cases the RNA was isolated with the NucleoSpin RNA Plus kit (Macherey-Nagel, Duren, Germany) following the manufacturer's instructions. Total RNA underwent reverse transcription with PrimeScript RT with a gDNA eraser (Takara, Kusatsu, Japan). Relative transcript quantification was performed using PowerUp SYBR Green (Applied Biosystems, Waltham, MA, USA). Primers for the SARS-CoV-2 nucleocapsid gene quantification (X. Lu et al. 2020) and ACE2 quantification (Wakahara et al. 2007) were utilized, with normalization to GAPDH gene levels (Jiang et al. 2018) or RPLP0 (Jocher et al. 2022). Data analysis was carried out in GraphPad Prism (V.9), with RNA level changes assessed by one-way ANOVA, comparing the levels of the knockouts to the control $p > 0.12$ (ns), <0.033 (*), <0.002 (**), <0.001 (***)

Western blotting

To assess ACE levels on protein level, A549-H2B-mRFP-ACE2 and A549-H2B-mRFP-ACE2 (K788R) cells were seeded one day prior to harvesting. After washing with PBS, cells were lysed in RSB buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% Glycerol, 50 mM DTT, 0.01% Bromophenol blue) and boiled at 95 °C for 5 minutes. Protein concentration was determined using Pierce 660 nm Protein Assay Reagent. 5 µg of each sample material was resolved on a NuPAGE 1 mm 4–12% Bis-Tris gel (Invitrogen) in 1× NuPAGE MES SDS Running Buffer (Invitrogen). PageRuler Prestained Protein Ladder (Thermo Fisher Scientific) served as a marker. A 0.45 µm nitrocellulose membrane (BioRad) was used to capture the separated proteins with transfer settings of 100 V for 1 hour in pre-cooled wet-blotting transfer buffer (25 mM Trizma base, 0.192 M Glycine, pH 8.3, 20% methanol). Following transfer, the membrane was blocked in 5% skim milk in PBS-T buffer (1 hour, RT) under constant agitation and then washed twice with PBS-T before being incubated with primary antibodies. The used antibodies were diluted with 5% milk in PBS-T (phosphate buffered saline with the addition of 0.25% Tween-20). ACE2, was detected using the primary antibody (abcam, Waltham, MA, USA, ab272690, 1:1000 dilution) and the secondary antibody rb-HRP (Agilent Dako, Glostrup, Denmark, P0448, 1:2500 dilution). The membrane was rinsed three times for 5 minutes with PBS-T between and after incubation with each antibody. Following the detection of the protein of interest, the membrane was stripped using Restore Western Blot Stripping Buffer (Thermo Fisher Scientific) and washed three times for 5 minutes with PBS-T before reprobing with the ACTB-HRP antibody (Santa Cruz, Dallas, TX, USA, sc-47778, 1:2500 dilution). Finally, the membrane was incubated with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) and imaged using the ChemiDoc XRS+ Imager. To assess the protein abundance the optical density was acquired using ImageJ (1.52 k, 1.8.0_172). Results were further analyzed in GraphPad Prism (V.9) using a student's t-test to evaluate statistical significance.

Material and methods P2: NUDT2 initiates viral RNA degradation by removal of 5'-phosphates

Cell lines and viruses

The HEK293T (ATCC CRL-3216) and HeLa (ATCC CCL-2) cell lines were acquired from ATCC and cultured as described before (Holze et al. 2018). Hep3B cells were supplied by Prof. R. Bartenschlager at the University Hospital Heidelberg, Germany. Mouse embryonic fibroblasts (MEFs) came from embryos at the 13.5-day developmental stage. Murine bone marrow was extracted from the femur and tibia of mice and cultured in DMEM with 10% fetal calf serum (FCS, GE Healthcare), 1 mM sodium pyruvate, 10 mM HEPES at pH 7.4, and 2 mM L-glutamine. Cell lines were cultured in DMEM (PAA Laboratories) supplemented with 10% FCS and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin). All utilized viruses were classified as BSL2 pathogens in Germany, and all experiments were conducted in compliance with official regulations. The following viruses were employed: Influenza A/SC35M-NS1-2A-renilla-2A-NEP (IAV-ren)40 (Reuther et al. 2015), VSV, VSV-M2 (Habjan et al. 2013), VSV-GFP (Dalton and Rose 2001), VSV-AV3-GFP (Stojdl et al. 2003), HSV-1 (F-strain) acquired from Soren Riis Paludan (Uni Aarhus, Denmark), VSV-Luc, obtained from Gert Zimmer (University Bern, Switzerland), and SFV, obtained from Andres Merits (University of Tartu, Estonia). The proteins XRN1, CIP, and RppH, as well as restriction enzymes for *in vitro* transcription, were acquired from NEB. The phosphate release assays and single nucleotides for *in vitro* transcriptions were ordered from Jena Bioscience. RNasin was supplied by Promega, TURBO DNase by Thermo Fisher Scientific, and T7 RNA polymerase was expressed and purified in-house by the Core Facility of the MPI of Biochemistry (Munich). The CellTiter-Glo assay kit as well as the luciferase substrate d-Luciferin (E1602) were procured from Promega. The used siRNAs were either obtained from Qiagen or synthesized by the Core Facility of the MPI of Biochemistry.

Preparation of triphosphorylated or capped RNA

As the RNA substrate, IVT4, a precisely defined double-stranded triphosphorylated RIG-I ligand, was utilized as described (Marion Goldeck et al. 2014). To investigate the sequence specificity of NUDT2, RNA substrates with varying initial nucleotides, encompassing single-stranded and double-stranded 5'-ends, were employed as previously outlined (Hsieh et al. 2013). The RNA substrates were generated by *in vitro* transcription synthesized from linear, double-stranded DNA templates, formed by hybridizing complementary oligodeoxynucleotides. Notably, the template for renilla RNA (Promega, E2231) was PCR-amplified in accordance with the manufacturer's instructions (2x HF Mastermix, NEB M0541S) using the corresponding oligodeoxynucleotides (Supplementary Table 3). The resulting linear dsDNA product was purified (Monarch PCR Cleanup, T1030S) and immediately employed for *in vitro* transcription. All templates contained a T7 ϕ 6.5 promoter, except RNA sequences commencing with 5'-AG featured a T7 ϕ 2.5 promoter. The antisense strand of the DNA template was annealed with sense-oriented oligos encompassing the respective T7 promoter region (Arnaud-Barbe et al. 1998). These oligonucleotides were mixed in a 1:1 ratio to achieve a final concentration of 10 µM each, in a buffer containing 10 mM Tris (pH 7.5–8.0), 50 mM NaCl, and 1 mM EDTA. The mixture was heated to 95 °C for 5 minutes and gradually cooled to 20 °C at a rate of 0.1 °C/s using a PCR cycler. For the production of renilla luciferase RNA,

the pRL-SV40 plasmid was linearized using BamHI cleavage site. To generate firefly luciferase RNA, the RiboMAX Large Scale RNA Production Systems Kit control plasmid (Promega) was employed. Transcription was conducted overnight at 37 °C, following the manufacturer's protocol of the T7 RiboMAX Large Scale RNA Production Systems kit (Promega). After a 30-minute DNase treatment at 37 °C using 1 U of RNase-free DNase per µg of DNA template, ivtRNA was extracted with the MinElute Cleanup kit (Qiagen) or the Monarch RNA Cleanup Kit (NEB).

The template for HCV PPP-RNA generation was the pFK-JFH plasmid DNA (Schaller et al. 2007) linearized with MluI digestion. The transcription was performed for 6–8 h at 37 °C in 5× transcription buffer (200 mM Tris pH 8, 30 mM MgCl₂, 10 mM Spermidine, and 5 mM DTT), 2.5 mM ATP, 2.5 mM UTP, 2.5 mM CTP, 2.5 mM GTP, RNasin (Promega), and 1000 U T7 RNA polymerase (Core Facility of the MPI of Biochemistry), in a total final volume of 100 µL. Additionally, 2 µL of TURBO DNase (Thermo Fisher Scientific) was added and incubated for 30 min at 37 °C. The products were purified using mini Quick Spin RNA Columns (Roche) following the manufacturer's protocol. To produce the 44-mer single-stranded RNA, the pGEX-6P-T7-44-mer-RNA plasmid (Abbas et al. 2013), linearized by EcoRI digestion, was the template for in vitro transcription. RNA was synthesized via in vitro transcription utilizing MEGAscript T7 Kit (Thermo Fisher Scientific). The reaction was carried out for 16 h at 37 °C followed by TURBO DNase (Thermo Fisher Scientific) treatment for 30 min at 37 °C. The resulting RNA purification was carried out using the Direct-zol RNA extraction kit (Zymo Research). To generate double-stranded RNA, a 20-nt antisense RNA (Metabion) (5'-acucucucucucucucuccc-3') was annealed to the in vitro-transcribed RNA. The ScriptCap Cap 1 Capping System (CellScript, C-SCCS1710) was used to cap the resulting RNA.

siRNA-mediated knockdown experiments

Cells were transfected with duplex siRNAs using siPrime transfection reagent (GE Healthcare) for the Nudix hydrolase knockdown screen or Metafectine Pro transfection reagent combined with the SI+Buffer (Biontex) for targeted gene knockdowns. Transfection was carried out following the manufacturer's guidelines for HeLa cells. Duplex siRNAs were either obtained from Qiagen or from the Core Facility at the MPI of Biochemistry. Cell viability following siRNA-mediated knockdown was assessed using the CellTiter-Glo (Promega) luminescent cell viability assay, following the manufacturer's protocol.

Virus infections and determination of virus titers and growth

To evaluate the effect of NUDT2 depletion on virus growth, MEFs and bone marrow were plated one day prior to infection. Cells were infected with various viruses at the specified multiplicities of infection (MOI): VSV wt (MOI 0.001), SFV (MOI 0.001), HSV-1 (MOI 2), VSV-Luc (MOI 0.001), IAV-renilla (MOI 0.1), and HSV-Luc (MOI 2). Bone marrow was infected with VSV wt and VSV M2 (MOI 1). At 24 h post-infection or detailed otherwise in the figure legend, cells were harvested for quantitative RT-qPCR and Western Blot. Also at 24 h post-infection or detailed otherwise in the figure legend the supernatant was collected for virus quantification using 50% tissue culture-infective dose (TCID₅₀) assays on Vero E6 cells. For assessing the effects of siRNA-mediated Nudix hydrolase knockdown, the following viruses were used for infection 48 h after the knockdown: VSV-Luc (MOI 0.001) for 24 h, IAV-renilla (MOI 1) for 48 h, VSV-GFP (MOI 0.001) for 24 h, and VSV-M2-GFP (MOI 0.0001) for 24 h. Levels of GFP were

quantified fluorometrically. To compare the knockdown effect on the viruses the z-scores were computed for each gene-virus combination from three independent experiments, as described before (J. H. Zhang, Chung, and Oldenburg 1999).

For transcript quantification by RT-qPCR, RNA was isolated using the NucleoSpin RNA Plus kit (Macherey Nagel) following the manufacturer's instructions. Cell pellets designated for proteomic analysis were snap-frozen in liquid nitrogen before further processing. For western blotting, cells lysis was performed in Laemmli buffer, boiled for 10 min at 95 °C, and subjected to SDS-polyacrylamide gel electrophoresis. For GFP-tagged viruses, GFP levels were measured using a microplate reader (Tecan Infinite 200 Pro) with excitation at 485 nm and emission at 535 nm to further analyze virus growth. For the quantification of luciferase-tagged viruses, cells were lysed in Passive Lysis Buffer (Promega), and firefly or renilla-luciferase activity was measured to quantify virus load. Growth kinetics of VSV-GFP were measured in the IncuCyte S3 Live-cell imaging system and analyzed with the corresponding internal software (Essen Bioscience). To test the effect of the depletion of NUDT2, XRN1, or NUDT2 and XRN1 in Heb3b cells, the cells were transduced with either a non-targeting control vector or CRISPR/Cas9-mediated depletion of the genes of interest. The cells were infected with VSV-GFP at an MOI of 0.01 in five technical replicates. Each technical replicate included two green fluorescence and phase-contrast images using a 10× objective. The background signal was reduced using a top-hat filter. The integrated intensity of GFP+ cells per image was normalized to the total cell area for each time point.

Protein expression and purification

Proteins were expressed, purified and quality was controlled by the Core Facility of the MPI of Biochemistry (Munich). DNA encoding the protein sequence of NUDT2, NUDT2 E58A, NUDT12, NUDT12 E369A, NUDT14, NUDT14 E121A, NUDT17, and NUDT17 E124A were purchased as gBlocks from IDT.

To evaluate phosphate release from mammalian-expressed NUDT2 cells, N-terminally 6-myc-tagged NUDT2, NUDT2 E58A, and NS1 of Influenza A virus (bearing mutated RNA binding domain (R38A, K41A) of strain A/PR8/34) were transiently expressed from pCS2-N-6xMyc plasmids. HEK293T cells were transfected with 1.3 µg of plasmid per 1e6 cells using a PEI to plasmid DNA ratio of 3:1. Cells were collected after two days, washed with PBS, pelleted, and frozen. Subsequently, cell lysis was performed using TAP lysis buffer (50 mM Tris-HCl at pH 7.5, 5% glycerol, 0.2% NP-40, 1.5 mM MgCl₂, 100 mM NaCl), supplemented with complete protease and phosphatase inhibitor cocktails (Roche), on ice for 30 min. The lysate was further processed by sonication (10 min, 4 °C) and cleared by centrifugation (4000 g, 30 min, 4 °C). The expressed, Myc-tagged proteins were then precipitated using an 80 µL slurry of equilibrated α-myc agarose beads (Chromotek, yta-20). Prior to performing the phosphate release assay the beads were washed three times with NEB 2 reaction buffer.

SDS-PAGE and western blotting

Verification of precipitation of Myc-tagged proteins: The immunoprecipitated proteins were eluted with 1x SDS sample buffer (comprising 62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 50 mM DTT, 0.01% bromophenol blue, pH 6.8), denatured at 95 °C for a duration of 5 minutes. The eluted proteins were thereafter separated utilizing a 4–12% Bis-Tris acrylamide gel (NuPAGE, Invitrogen) under an electric field of 100 V for a total period of 90 minutes. The

transfer of proteins onto a 0.2 μ m nitrocellulose membrane was performed through a wet-blotting procedure, applying a constant current of 300 mA for 1 hour. The subsequent step involved blocking the membrane with 5% milk, followed by three washes with PBS containing 0.25% Tween-20 (PBS-T) for a duration of 5 minutes each. Finally, primary antibodies were applied in 5% milk, and after that, the membrane was subjected to incubation with an appropriate secondary antibody.

Antibodies: the primary antibodies employed comprised the following: monoclonal mouse α -NUDT2 (Santa Cruz: sc-271410, diluted at 1:1000), monoclonal mouse α - β -Actin-HRP (Santa Cruz: sc-47778, diluted at 1:1000), monoclonal mouse α -myc-HRP (Roche, 11814150001, diluted at 1:2000), and a secondary antibody: horseradish peroxidase (HRP)-coupled antibody, rabbit α -mouse IgG (Cell Signaling, 7076, diluted at 1:2000).

Phosphate release assay

Time-resolved analysis: To conduct time-resolved assessments of phosphatase activities of the Nudix hydrolases, CIP, or RppH, the EnzChek Phosphate Assay (E6646, Sigma-Aldrich) was employed according to the manufacturer's instructions. For optimizing the assay, specific adjustments were introduced: a single replicate, with a reaction volume of 200 μ L, encompassed 600 nM NUDT protein or the corresponding mutant, 4.5 μ g of RNA substrate, 0.5 U of PNP, 0.2 mM MESG, and NEB 2.0 buffer. This experimental configuration was validated to yield optimal assay performance. The absorbance at 360 nm was recorded at three-minute intervals, and the phosphate release was calculated based on a linear standard curve tailored to each time point.

Single time-point analysis: The assessment of Nudix hydrolases' ability to liberate phosphates from RNA was conducted using in vitro transcribed RNA (ivtRNA) and ribonucleotide triphosphate (rNTPs). The malachite green assay (MAK307, Sigma-Aldrich) was used in accordance with the manufacturer's instructions following a 3-hour incubation of 900 ng IVT4 with NUDT2 or NUDT2 E58A in NEB2 buffer at 37 $^{\circ}$ C. This configuration was validated for its ability to deliver optimal assay performance. For probing NUDT2 activity against a range of potential substrates, NUDT2 (600 nM) and NUDT2 E58A (600 nM) were incubated for 3 hours with rATP, rGTP, rUTP, and rCTP (2.5 μ M) at 37 $^{\circ}$ C in NEB2 reaction buffer. The positive control CIP (5 U) (NEB) was used to measure inorganic phosphate release. In the case of immunoprecipitated NUDT2, NUDT2 E58A, or NS1 (IAV) derived from HEK cell lysate, 20 μ L of beads were mixed with 40 μ L NEB 2 reaction buffer and 900 ng IVT4. The mixture was incubated for 3 hours at 37 $^{\circ}$ C. Beads were subsequently separated (500 g, 1 minute, RT), and the phosphate release assay was conducted employing the malachite green assay, as described above.

Phosphate release from γ -32P-labeled RNA

γ -32P Labeled RNA Synthesis: The MEGAscript T7 Kit (Thermo Fisher Scientific) was followed to generate γ -32P-labeled RNA, with the addition of 0.17 μ M [γ -32P] GTP (Perkin Elmer). For the 44-mer single-stranded RNA, the pGEX-6P-T7-44mer-RNA plasmid (Abbas et al. 2013), linearized by EcoRI digestion, was employed as a template. For the generation of dsRNA IVT4, two annealed primer pairs (Marion Goldeck et al. 2014) served as templates. Following a 16-hour incubation at 37 $^{\circ}$ C and DNase digestion using TURBO DNase (Thermo Fisher Scientific) for 30 minutes at 37 $^{\circ}$ C, RNA purification was carried out as per the Direct-

zol RNA extraction kit (Zymo Research). To generate a double-stranded RNA from the 44-mer ssRNA, a 20-nt antisense RNA (Metabion) (5'-acucucucucucucucuccc-3') was annealed to the in vitro transcribed RNA.

Phosphate release assay: The labeled RNAs mixed with NUDT2 (600 nM), NUDT12 (600 nM), NUDT14 (600 nM), NUDT17 (600 nM), and RppH (5 U) from NEB, in a final volume of 20 μ L with NEB2 buffer and incubated at 37 °C. Samples (10 μ L) were withdrawn at 0 and 90 minutes and quenched with 2.5 μ L of EDTA (100 mM, pH 8.0). Analysis was performed by TLC on PEI-cellulose (Merck Millipore), developed with potassium phosphate buffer (0.3 M, pH 7.5), and spot intensities were captured using a GE Typhoon FLA 9000 Imager. For time-course experiments, the labeled RNA was mixed with NUDT2 or NUDT2 E58A (600 nM) in a final volume of 50 μ L solution containing NEB2 Buffer for durations ranging from 0 to 90 minutes at 37 °C. Samples (10 μ L) were collected at specific time intervals and quenched with 2.5 μ L of EDTA (100 mM, pH 8.0). The analysis was performed as described above.

Synthesis of pppApG and pppGpA

Dinucleotide Synthesis: Dinucleotides were generated with DMT-2'-O-TBDMS-rA(bz), DMT-2'-O-TBDMS-rG(ib) amidites, and rA(Bz) and rG(ibu) CPG (Chemgenes) on a 10 μ M scale, employing standard solid-phase oligoribonucleotide coupling techniques. Triphosphorylation of the CPG-bound dimers was done utilizing the cyclotriphosphate protocol of triphosphate synthesis (M. Goldeck et al. 2014). After deprotection of the dinucleotides, the reaction mixture was converted into the triethylammonium salt employing Dowex Et3NH⁺. Subsequently, reverse-phase chromatography on a Source RP column was conducted. DecNHpppApG and DecNHpppGpA RP-HPLC product peaks were transformed into pppApG and pppGpA, as described. Isolation of pppGpA and pppApG was performed using triethylammonium salts by ion-exchange chromatography on a Hi Screen DEAE FF column with a gradient of 0.0–0.6 M Triethylammonium bicarbonate. LC-MS analysis was used to verify the integrity of the pppApG and pppGpA dimers.

HPLC analysis

For HPLC analysis, a short RNA (pppGpA, 0.1 OD260) was mixed with recombinant NUDT2 (600 nM), NUDT2 E58A (600 nM), RppH (5 U), or was left untreated and was incubated for 2 hours at 37 °C in a 15 μ L solution with NEB2 buffer (1 $\times$). The reaction was stopped with 3.75 μ L of EDTA (100 mM, pH 8.0) and diluted 1:20 with water. In the case of time-course measurements, two short RNAs (pppGpA or pppApG, 0.1 OD260 each) were mixed with NUDT2 (600 nM), NUDT14 (600 nM), and NUDT2 E58A (600 nM), respectively, in a 75 μ L reaction mix with NEB2 buffer (1 $\times$) at 37 °C. Aliquots were harvested (15 μ L) at 0, 1, 3, 6, and 19 hours, the reaction was stopped with 3.75 μ L of EDTA (100 mM, pH 8.0), and diluted 1:20 with water. Subsequently, aliquots (80 μ L) were analyzed by reversed-phase (RP)-HPLC using an AGILENT 1100 System equipped with an XBridge C18 column (3.5 μ m, 150 $\times$ 2.1 mm, WATERS). Chromatography was conducted using two-eluent aqueous buffer systems: Buffer A consisted of an aqueous solution of 100 mM Et3NHOAc, pH 7.8, and Buffer B comprised an aqueous solution of 100 mM Et3NHOAc, pH 7.8, with 40% (v/v) MeCN. Chromatograms were analyzed at a wavelength of 260 nm. HPLC was executed using the following gradient conditions: 0% B over 4 minutes, increasing to 15% B over 20 minutes, further increasing to 18% B over 16 minutes, reaching 100% B over 10 minutes, followed by a

15 minute hold at 100% B, then returning to 0% B over 2 minutes, and a 13-minute hold at 0% B, all at a flow rate of 0.3 mL/min. The corresponding peak areas were integrated to quantify the eluted compounds.

Analysis of RNA integrity and transcript level analysis

To evaluate the integrity of RNA following treatment with recombinant Nudix hydrolases, small reaction samples underwent analysis using the Bioanalyzer (Agilent) and the Agilent Small RNA kit, following the manufacturer's protocol. For quantitative RT-qPCR analysis, total RNA was extracted from cells using the NucleoSpin RNA Plus kit (Machery-Nagel) following the manufacturer's instructions. In the case of RNA extraction from mouse cerebellum, heart, liver, lung, or spleen, the tissues were lysed directly in LBP buffer, and RNA purification was performed according to the manufacturer's instructions (NucleoSpin RNA Plus kit, Machery-Nagel). Subsequently, 200–500 ng of RNA was reverse-transcribed with PrimeScript RT Master Mix (TAKARA), followed by quantification via RT-qPCR using the QuantiFast SYBR Green RT-PCR Kit (Qiagen) and a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Each cycle included a 10 s denaturation step at 95 °C and a 30 s annealing/extension step at 60 °C, followed by a melting curve analysis.

Generation of CRISPR/Cas9 knockout cells

Guide sequences consisting of double-stranded 30-nt sequences targeting the human NUDT2 gene and a non-targeting control were designed and cloned into pLentiCRISPRv2 (Addgene #52961), following previously established protocols (Shalem et al. 2014; Sanjana, Shalem, and Zhang 2014). HEK293T cells were co-transfected with the resulting plentiCRISPR v2 vector (6 µg), pxPAX2 (3 µg), and pMD2G (1.5 µg) in a 10 cm dish using PEI as the transfection reagent. At 24 hours post-transfection, the medium was aspirated and changed with 10 mL of fresh DMEM. Seventy-two hours post-transfection, the medium was collected, filtered through a 0.45 µm pore size membrane (Millipore). Resulting lentiviruses were used for transduction of HeLa cells. Selection was started 24 h transduction with DMEM containing puromycin (1 µg/mL) for four passages. Westernblotting was used to verify the knockout.

RNA degradation assays

To evaluate the degradation of 1 µg of in vitro transcribed renilla or HCV PPP-RNA including luciferase reporter, the RNAs were incubated with various enzymatic treatments at 37 °C for 4 hours: 600 nM recombinant NUDT2, 1 U recombinant XRN1, 10 U RNase A, 5 U recombinant RppH, or combinations of 600 nM NUDT2 with 1 U XRN1, or 5 U RppH with 1 U XRN1. To assess RNA levels following incubation, renilla RNA was quantified via RT-qPCR, while the presence of HCV PPP-RNA was detected using 1% agarose gel electrophoresis. To determine the stability of in vitro transcribed HCV PPP-RNA encoding luciferase the RNA products were electroporated into HeLa NUDT2 knockout cells or corresponding control cells. HCV PPP-RNA abundance was determined at 4 and 6 hours post-electroporation by analyzing the levels of firefly luciferase RNA via RT-qPCR. To assess the degradation of capped or PPP-RNA, equimolar amounts of capped firefly luciferase and PPP-renilla luciferase were co-electroporated into Hep3B knockout cells lacking NUDT2, XRN1, or both NUDT2 and XRN1, or Hep3B control cells. Cells were subsequently cultured for 1 and 4 at 37 °C and the RNA levels were measured by quantifying renilla or firefly luciferase RNA using RT-qPCR, normalized to the housekeeping gene RPLP0 RNA.

Ap4A treatment

HeLa cells were transfected with either 1 µg poly(I:C) using 1 µL METAFECTENE Pro or 250 nmol Ap4A using 2 µL METAFECTENE Pro. Alternatively, 250 nmol of Ap4A was directly added to the cell culture media (Guerra et al. 2020; Jin et al. 2011). To test if the stimulus came from the nucleotides HeLa cells were transfected with PBS with and without METAFECTENE Pro, as controls. The cells were infected with SFV6-2SG Nano-Luc (MOI of 0.0035) or left uninfected 24 h post-treatment. Subsequently, cell lysis was performed 24 h after infection with Passive Lysis Buffer, and virus growth was evaluated by measuring renilla luciferase activity.

Generation of Nudt2 knockout mice

To disrupt the functionality of the Nudt2 allele, a poly(A) signal was introduced after exon 1 using a construct obtained from the European Conditional Mouse Mutagenesis Consortium (EUCOMM). A genetically modified ES cell clone (Clone ID: EPD0146_2_H06, Cell type: JM8.N4) carrying this construct was injected into C57BL/6 blastocyst donors. Male chimeras resulting from this injection were then bred with C57BL/6 females to produce heterozygous Nudt2^{+/tm1a} mice (Nudt2^{+/-}). These mice harbored a tm1a cassette, which encoded the lacZ gene, a neomycin resistance marker, a splice acceptor, and a polyA site flanked by FRT sites. Subsequently, heterozygous animals were interbred to generate homozygous Nudt2^{tm1a/tm1a} mice (Nudt2^{-/-}).

Ear punches were collected from mice aged 21 to 23 days and subjected to PCR for genotyping using the following primers: Nudt2_fwd: 5'-ccagcttctgtacaaagtgg-3', Nudt2 wt_fwd: 5'-gaatttctgctgctgcaggc-3', Nudt2_rev: 5'-cctagtgaagggacaaagcagc-3', and the following parameters: an initial denaturation step at 94 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 20 s, and extension at 72 °C for 1 min. The insertion of the tm1a cassette generated bands at 417 and 912 bp, while the wild-type allele was clearly characterized by its 492 bp band.

Ethics statement and mouse infections

All animal experiments were conducted in accordance with the German Animal Welfare Law (TierSchG BGBl. S. 1105; 25.05.1998). The mice were housed and managed in compliance with good animal practice guidelines established by FELASA. The responsible state office (Landesverwaltungsamt Sachsen-Anhalt) and the University of Magdeburg granted approval for all animal experiments under permit number AZ 42502-2-1344. During intranasal infections (i.n), mice aged 8–12 weeks were initially anesthetized through intraperitoneal (i.p.) injection with a ketamine (100 mg/g body weight) and xylazine (5 mg/g body weight) mixture. Subsequently, they were infected with 5e6 plaque-forming units (PFU) of VSV in 20 µL PBS, unless otherwise specified. Mice that exhibited a loss of body weight greater than 20% were humanely euthanized.

Proteomic analysis of Nudt2 knockout in mouse bone marrow cells

Cell lysis of bone marrow cells from wild-type and Nudt2 knockout mice (5e6/cell line/replicate) harvested in 300 μ L of lysis buffer (4% SDS, 10 mM DTT in 50 mM Tris pH 7.6) and mixed with protease inhibitor (Roche). The samples were denatured for 10 minutes at 95 °C and the protein concentration of the samples was determined (Pierce 660 nm Protein Assay, Thermo Fisher Scientific) following the manufacturer's instructions. 35 μ g of protein content from each of the four replicates was alkylated with 55 mM IAA for 20 minutes in the dark. After precipitation of the proteins with acetone and the samples were resuspended in 150 μ L of denaturation buffer (6 M Urea, 2 M Thiourea in 10 mM HEPES pH 8.0). Next trypsin and LysC enzymes at a ratio of 1:100 (protein:enzyme) was added. After incubating the samples overnight at room temperature the peptides were subsequently desalted using stage tips and analyzed by LC-MS/MS using the EASY-nLC 1200 system coupled to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific). The experimental setup included a 50 cm C18-reversed-phase column (Reprosil-Pur 120 C18-AQ, 1.9 μ M, 200 $\times$ 0.075 mm; Dr. Maisch) using a 180-minute linear gradient of 5–30% buffer B (0.1% formic acid and 80% ACN) at a flow rate of 250 nL/min to separate the peptides. The mass spectrometer operated in a Top10 method, involving full scans (300–1,600 m/z, R = 60,000 at 200 m/z) with a target of 3e6 ions, followed by the isolation of the ten most abundant ions, HCD fragmentation (target 1e5 ions, maximum injection time 120 ms, isolation window 1.4 m/z, NCE 27%, and an underfill ratio of 20%).

Raw data processing and statistical analysis

The raw data was processed in MaxQuant (version 1.6.17.0) with the default parameters including the following settings: fixed modification for carbamidomethylation of cysteine, variable modifications: oxidation of methionine and N-terminal protein acetylation, proteolytic enzyme: Trypsin/P. The minimal peptide length: 7 amino acids. Spectra were searched against forward and reverse sequences of the reviewed mouse proteome, including isoforms (Uniprot, UP000000589). The match-between-run option was disabled for LFQ determination.

The LFQ values from the proteingroups.txt file were analyzed in Perseus (version 1.6.14.0). The matrix was filtered according to the default settings, considering potential contaminants identified only by site and reverse hits. After log₂ transformation, the protein groups were filtered for at least three valid values in total. For statistical analysis a two-sided student's t-test (FDR 0.05) was conducted, after missing values were imputed (width 0.3 and downshift 1.8).

Phylogenetic analysis

To elucidate the phylogenetic relationships among various human Nudix hydrolases and bacterial RppH, protein sequences of E. coli RppH and all human Nudix hydrolases were subjected to analysis using MAFFT (Kato et al. 2002) with default parameters. The resulting phylogenetic tree was visualized using Dendroscope (Huson et al. 2007) and further adjusted for presentation using Adobe Illustrator CS6 (16.0.3).

Multiple sequence alignment

The Clustal Omega tool was used with default settings to align the protein sequences of NUDT2 from various species, including Homo sapiens, Danio rerio, Caenorhabditis elegans,

Mus musculus, *Xenopus laevis*, *Gallus gallus*, and *Drosophila melanogaster*. The multiple sequence alignment was presented using ESPript 3.0 (Robert and Gouet 2014).

Statistical analysis

Statistical analyses of all data were performed using either GraphPad Prism (8.4.3) or R (4.0.2) in conjunction with R Studio (1.3.1056). Data obtained from the Incucyte live-cell imaging platform were analyzed utilizing built-in IncuCyte Analysis Software (2019B Rev2).

Material and Methods P3: Cell-free production of personalized therapeutic phages targeting multidrug-resistant bacteria

Details of the microorganisms and phages

All microorganisms and bacteriophages used in this study were sourced from “Deutsche Sammlung von Mikroorganismen und Zellkultur” (DSMZ) or obtained as detailed below. Specifically, the Enteroaggregative *E. coli* (EAEC) strain was obtained from Jean-Paul Pirnay and Maya Merabishvili at the Laboratory for Molecular and Cellular Technology (LabMCT). The PhiA1122 phage was acquired from Shanmuga Sozhamannan at the Defense Biological Product Assurance Office, CBRND-EB, JPEO, Frederick, USA, and the CLB-P3 phage was provided by Laurent Debarbieux at the Department of Microbiology, Institut Pasteur, Paris, France. The *K. pneumoniae* strain (Bw1) and its corresponding MUC-5 phage were isolated as detailed below. MS2 bacteriophages (DSM 13767) were propagated using *E. coli* (DSM 613) in NZCYM medium (Sigma Aldrich) at 37 °C. T7 bacteriophages (DSM 4623) were amplified with *E. coli* (DSM 613) under the same conditions. CLP-P3 bacteriophage was amplified with the enteroaggregative *E. coli* following the protocol (Maura et al. 2012). The MUC-5 bacteriophage, isolated from wastewater, was amplified in the *K. pneumoniae* strain (Bw1) with wzj capsule type K17 in LB broth (Thermo Scientific) at 37°C. PhiA1122 bacteriophages (Garcia Emilio et al. 2003) were propagated in *Y. pestis* EV 76 (CCUG32133) in BHI broth (Merck) at 28°C. Phi29 bacteriophages (DSM 5546) were amplified in *B. subtilis* (DSM 5547) using NZCYM medium (Sigma Aldrich) at 37°C, while Goe1 bacteriophages (DMS 101030) were amplified in *B. subtilis* (DSM 402) under the same conditions. *E. coli* - DH5a (18258-012) was cultivated at 37°C in LB medium (Carl Roth).

Phage stock production

For the natural propagation of MS2, T7, CLP-P3, Phi29, and Goe1 phages, the appropriate bacterial strains were cultured in 1 L of NZCYM medium (Sigma Aldrich, USA) until an OD of 0.6–0.8 was reached (37°C, 230 rpm, Innova4 Incubator, Eppendorf, Germany) and infected with the corresponding phage. The *Yersinia pestis* EV 76 bacteria (CCUG32133) was propagated on Brain Heart Infusion (BHI, Merck) agar plates or in 5 mL BHI broth (Merck) utilizing 50 ml sterile tubes with shaking until an OD of 0.6–0.8 was reached (28°C, 130 rpm). The bacteria infected with 100 µL of PhiA1122 phage stock (10^6 – 10^{10} pfu/mL) and cultured until lysis. The sample was centrifuged to remove residual bacterial fragments (10 min, 7000 x g), and 10% [w/v] PEG8000 and 1M [w/v] NaCl (final concentrations) was added. The supernatant was chilled for 12 h at 4°C, and the phages were pelleted in Falcon tubes (VWR, USA) (4°C, 10 min, 7000 x g). The phages were then solubilized in 5 mL of phage buffer (1x PBS, 1 mM MgCl₂, 1 mM MgSO₄) per sample and sterile filtered (0.45 mm Rotilabo syringe filter, Roth, Germany).

Phenol-chloroform extraction

Equal volumes of phage stock (10^6 – 10^{10} pfu/mL) was mixed with Roti-Phenol/Chloroform/Isoamyl alcohol (pH 7.5–8.0) in a 5PRIME Phase Lock Gel™ tube (Quantabio, USA). After careful inverting and centrifugation for 5 min at 16,000 g at room temperature, additional two volumes of pure chloroform (Roth, Germany) were supplemented

to the top phase of the tube, which was then inverted and centrifuged as before. The supernatant, containing the DNA, was transferred to a separate Eppendorf tube. For DNA precipitation 20 μ L of 3M sodium acetate and 1 mL of -80°C pure ethanol (Roth, Germany) was added, the sample was then stored for 1 h at -80°C. The sample was centrifuged (16,000 x g, 15 min, room temperature), and the liquid was discarded. After adding 1 mL of -20°C 70% (v/v) ethanol (Roth, Germany), the DNA was pelleted (16,000 x g, 5 min, 4°C), and the ethanol was aspirated. The remaining ethanol was removed by evaporation (15 min, RT). The phage genome was solubilized in 30 μ L of nuclease-free water (Thermo Fisher Scientific, USA).

Cell extract preparation

For the generation of crude cell extract, a previously published protocol was followed (Falgenhauer et al. 2021).

Phage assembly

Phages were assembled as previously described (Rustad et al. 2017), with some modifications. This study used TX-TL system based on Sun and colleagues (Sun et al. 2013) with an energy solution, and an amino acid solution, with changes in Buffer BB (6 mM Mg-glutamate, 100 mM K-glutamate, 3 mM DTT, 1.5 mM each amino acid except leucine, 1.25 mM leucine, 50 mM HEPES, 1.5 mM ATP, and GTP, 0.9 mM CTP and UTP, 0.2 mg/mL tRNA, 0.26 mM CoA, 0.33 mM NAD, 0.75 mM cAMP, 0.068 mM folinic acid, 1 mM spermidine, 30 mM 3-PGA, 4% PEG-8000) (Garamella, Marshall, and Rustad 2016). For the assembly of six reactions a 13 μ L, 2.5 μ L PEG 8000 (36% w/v), 4 μ L dNTPs (25 mM), 0.8 μ L ATP (500 mM), 37.5 mL Buffer B, 2 μ L GamS (150 μ M), 28.5 μ L TX-TL and 1.6 μ L DNA (10 nM) were mixed and 2 μ L of nuclease free water was added to achieve 80 μ L total volume. All parts were mixed (except for the phage genome), chilled for 5 min on ice, then the phage DNA was supplemented. For phage assembly the 13 μ L assembly mix was incubated for 4 h at 29°C..

Plaque assay

For plaque assays, 0.5% agarose NZCYM medium was split into 4 mL aliquots, and maintained in a water bath at 48°C. Separately, the assembled phages were diluted in a phage buffer (1x PBS, 1 mM MgCl₂, 1 mM MgSO₄). Next, 100 μ L of each phage dilution was mixed with the same volume of an overnight culture of the corresponding host bacterium. This solution was then mixed with the 0.5% agarose NZCYM medium aliquots and added onto 1% NZCYM (or 1% BHI for *Y. pestis* EV76) agar plates. Once the suspension solidified at room temperature, the plates were incubated at 37°C (or 28°C for *Y. pestis* EV76) until plaques became apparent. Plaque counts (P) were calculated to titers (T) using the formula $T = P/D$, where D represents the dilution factor.

Isolation of *K pneumoniae* (Bw1)

The Bw1 is a *K. pneumoniae* strain that was a gift from the Bundeswehr military hospital (BwK) in Berlin. The sample was collected from a colonized patient's skin in accordance with relevant ethical guidelines and regulations. This strain is classified as a 4MRGN (carbapenem-resistant) pathogen, with a wzi capsule type of K17.

Isolation of vB_KpS_Muc5 from wastewater

The vB_KpS_Muc5 phage was isolated from wastewater samples obtained from the Gut Großlappen wastewater treatment plant in Munich (Münchner Stadtentwässerung). The isolation process involved a blind enrichment of water samples. These samples were centrifuged at 7000 g for 10 minutes at 4°C and sterile-filtered through a 0.2 µm filter. Subsequently, 350 µL of the host bacterium (OD 0.4-0.6) were incubated at room temperature with 100 µL of the filtered water sample for 10 minutes. Next, 5 mL of LB broth were added, and the culture was incubated at 37°C with shaking at 120 rpm for 3 hours. Following incubation, the culture was centrifuged (7000 g, 10 minutes, 4°C) and filtrated (0.2 µm). A plaque assay was performed to test for plaque-forming phage. Specifically, 100 µL of the filtrate and 350 µL of *K. pneumoniae* (OD 0.4–0.6) were mixed with 2.5 mL of warm (40°C) LB-Agar (0.6%) containing 1 mM CaCl₂ and immediately poured onto a 2% LB Agar plate. These plates were then incubated inverted at 37°C for 24 hours.

Transient engineering

Phages were assembled as previously described, with the addition of 0.2 nM of a plasmid (ordered from Twist Bioscience) based on the expression plasmid psb1c3-t7 (Addgene: 135173, (Calles et al. 2019)). This plasmid encoded the fusion of the minor capsid protein of the T7 phage Gp10B to HiBiT along with a 6xHis-tag. For plasmid amplification *E. coli* - DH5a (Invitrogen) were used and plasmids were purified according to the manufacturer's instructions (QIAprep Spin Miniprep kit, 27104, Qiagen).

His-Tag purification

After cell-free production the assembled phages were diluted to 10⁶ PFU/mL in 1x PBS and 20 mM imidazole immediately (800 µL total volume). This solution was then mixed with Ni-NTA agarose beads (250 µL of Ni-NTA Agarose from Qiagen ID: 30210) that had been pre-equilibrated with a washing buffer (1x PBS and 20 mM imidazole). Next, the beads were washed with 6 column volumes (800 µL) of 1x PBS and 20 mM imidazole. For phage elution one column volume of 1x PBS and 250 mM imidazole was used and the phage titer was determined as previously explained.

HiBiT assay

The cell-free reaction was conducted as previously explained, with or without the addition of 0.2 nM of a plasmid encoding either Gp10B-HiBiT-His or just HiBiT-His without G10B. T7 RNA polymerase at a concentration of 200 nM was added for transcription. Following incubation of the cell-free reaction, 20 µL of the reaction mixture was added to 1 mL of the corresponding *E. coli* cells (DSMZ 613) with an OD adjusted to 0.8 and incubated for 5 min at room temperature. The bacteria were pelleted by centrifugation at 7,000 x g at room temperature, and the supernatant was discarded. The cells were then resuspended in 1x PBS. HiBiT signal was detected after mixing with the Nano-Glo HiBiT Extracellular Detection System according to the

manufacturer's instructions (Promega N2420). The phage progeny were isolated from a plaque assay and adjusted to the same titer as reached in the cell-free system to confirm that the modification was only transient. This experiment was repeated, and luminescence was measured again with the Nano-Glo HiBiT Extracellular Detection System.

Polymerase chain reaction

For the polymerase chain reaction (PCR) the Phusion Flash High Fidelity Mastermix kit (Thermo Fisher Scientific, F548S) was used according to the manufacturer's instructions. The primer design was done using Benchling (2019). To complete the CLB-P3 genome, primers were used to amplify specific genome positions: 10.5 kb (10523–11287 bp), 16.5 kb (15852–16625 bp), 27 kb (26860–27736 bp), and 40.8 kb (40644–41504 bp). The primers were purchased from Eurofins Scientific (GER).

Nanopore sequencing

The phage genome sequencing was executed with the SQK-RAD004 kit (Oxford Nanopore Technologies, UK) on the FLO-MIN106 flow cell (Oxford Nanopore, UK) according to the manufacturer's protocol.

Illumina sequencing

The phage genome library was prepared using the Ultra II DNA library prep for Illumina (NEB) and sequenced using the MiSeq v3 600 cycle kit on the Illumina MiSeq platform according to the manufacturer's protocol. DNA was sheared using an E220 supersonicator (Covaris).

Genome assembly and annotation of phage genomes

For base-calling of the long reads from MinION sequencing (Oxford Nanopore, UK) guppy (ONT) version Version 2.3.7 + e041753 was used. Additionally the short-reads from Illumina MiSeq-derived FASTQ files (Miseq reporter version 2.6.2.3) were used. Reads that mapped to *E. coli* K-12 MG1655 (U00096.3) were removed from both MinION and MiSeq reads. To generate a genome a hybrid assembly approach combining both MinION and Illumina reads was executed using MaSURCA 3.3.2 in auto mode for the assembly graph k-mer size. The assembled sequence was controlled by re-aligning the sequence itself (using BLAST) with the Illumina or MinION reads to the sequence to create a coverage plot. The BLAST results were checked in a dot plot to control that the new sequence was a duplication of the actual genome. Likewise, in the first/second copy of the actual genome the aligned Illumina reads depicted a secondary alignment position. After deduplicating the assembled sequence, the same alignment check was performed to confirm the correct deduplication. These positions were also confirmed by Sanger Sequencing. PHANOTATE (McNair et al. 2019) was used to identify Open-reading frames (ORFs). To assign specific functions to each ORF representative members for each pVOG class were identified (Grazziotin, Koonin, and Kristensen 2017). Those ORFs that could be correlated to pVOGs are likely to be conserved because other bacteriophages also have similar sequences. The ORFs and the pVOG sequences were compared utilizing optimal pairwise global alignments with the PAM250 substitution matrix and affine gap costs (Gotoh 1982). Alignments were accepted if they showed sequence identity of

more than 40%. The remaining ORFs were blasted against all bacteriophage protein sequences downloaded from Genbank (file gbphg1.seq.gz). Here an alignment was accepted if more than 70% of the ORF length aligned and 50% of the pVOG sequence aligned, respectively. Overall, for 72 of the 87 ORF a pVOG assignment was found.

Sample preparation for mass spectrometry

3 μ L cell-free phage- or 5 μ L native sample (stock concentration $>10^6$ pfu/mL) was mixed with NuPAGE LDS sample buffer (ThermoFisher) to achieve 1x LDS sample buffer dilution. After reduction with 50m dithiothreitol (DTT) (70°C, 10 min) and alkylation with 55mM chloroacetamide (CAA) (RT, 30 min) in the dark, the samples were run on a NuPAGE 4–12% Bis-Tris protein gel (ThermoFisher) for 5 min (200V, 500 mA, 1x MOPS buffer). The samples were fixed (30min, 40%MeOH, 2% AA), colored (1x RotiBlue, Carl Roth, Germany) and destained (25% EtOH, 1% AA). After in-gel digest (Shevchenko et al. 2006), the collected supernatant (130 mL) was dried in a centrifugal evaporator (Centrivap Cold Trap -50, Labconco, USA). For MS measurement the sample was freshly suspended in 20 μ L washing solution (2% ACN, 0.1 FA). per measurement 5 μ L of the freshly dissolved sample was injected into the mass spectrometer per measurement.

Proteomic data acquisition

For the measurement LC-MS/MS the Ultimate 3000 RSLCnano system was connected to an Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Fisher Scientific Bremen, Germany). Each analysis involved injecting approximately 0.1 mg of peptides into a trap column (ReproSil-pur C18-AQ, 5 mm, Dr. Maisch, 20 mm $\times$ 75 mm, self-packed) at a flow rate of 5 mL/min in 100% solvent A (0.1% formic acid in HPLC grade water). Following a 10 min loading time, peptides were transferred to an analytical column (ReproSil Gold C18-AQ, 3 mm, Dr. Maisch, 400 mm $\times$ 75 mm, self-packed) and separation was achieved with a 50-minute gradient from 4% to 32% of solvent B (0.1% formic acid in acetonitrile and 5% (v/v) DMSO) at 300 nL/min flow rate. Notably, both nanoLC solvents contained 5% (v/v) DMSO. The Fusion Lumos Tribrid MS/MS was run in the data-dependent acquisition and positive ionization mode. MS spectra (360–1300 m/z) were recorded at a resolution of 60,000 using an automatic gain control (AGC) target value of $4e5$ and maximum injection time (maxIT) of 50 ms. Higher-energy collision-induced dissociation (HCD) was used for peptide fragmentation and MS2 spectra of up to 20 precursor peptides were acquired at a resolution of 15,000 with automatic gain control (AGC) target value of $5e4$ and maximum injection time (maxIT) of 22 ms. 1.3 m/z was chosen for the precursor isolation window width and normalized collision energy to 30%. Dynamic exclusion was used with 20 s exclusion time (mass tolerance ± 10 ppm). MS/MS spectra were singly-charged, unassigned, or with charge states $>6+$ were excluded.

Raw data processing and proteomic statistical analysis

Raw data was processed utilizing MaxQuant version 1.6.3.4 (Cox and Mann 2008) with the default settings including the proteomes E. coli BL21(DE3) (NCBI: 469008), phage CLB-P3 (de-novo assembled: P3.prot.fa) and phage T7 (Uniprot: UP000000840). A contaminant list (a built-in option in MaxQuant) was also added. Trypsin/P was specified as the proteolytic

enzyme. The precursor tolerance was set to 4.5 ppm, and fragment ion tolerance to 20 ppm. The FDR was set to 1% on the peptide spectrum match (PSM) level employing a target-decoy approach using reversed protein sequences. 7 amino acids was defined as the minimal peptide length. Intensity-based absolute quantification (iBAQ) (Schwanhäusser et al. 2011) was enabled. The iBAQ intensities were used throughout this study for protein quantification.

Heatmaps

Phage proteins from the phage assembly were further analyzed to investigate their expression pattern. The phage protein (RPOL_BPT7) cannot be distinguished from E. coli protein (A0A140NCE7_ECOBD) and was included in the analysis as well. The log10 transformed iBAQ intensity was used for the analysis, missing values were replaced with the lowest measured iBAQ intensity. For the generation of the heatmap the Euclidean distance together with the Ward linkage method (no scaling).

For the time resolved protein expression three replicates were analyzed over 12 time points. The log10 transformed iBAQ intensity was used for the analysis, missing values were substituted with the constant value of 5.5, which is slightly lower than the lowest measured values. The iBAQ intensities were z-score transformed within each replicate to remove any batch effect. The z-scored iBAQ intensities were controlled using beeswarm plots (R package Beeswarms version 0.2.3). For the categorization of the phage proteins on their kinetics, the z-scored iBAQ values of the three replicates condensed to their means, which were further scaled to the range of 0 to 1. Manhattan distance and Ward's method were used to cluster the final transformed intensities. Three phage proteins did not follow a clear expression trend and were therefore removed (P03751|GP73_BPT7, P03778|Y06_BPT7, P03797|Y38_BPT7). The analysis was conducted in R (version 3.6.1).

Statistical analysis

For statistical analysis the data was analyzed using either Graphpad Prism (V. 9.2.1 or 7) or R-studios with three replicates if not specified otherwise. All values are depicted as the mean $\pm$ standard deviation or SEM of the replicates.

Summary and discussion

Summary and discussion publication P1: MDM2 influences ACE2 stability and SARS-CoV-2 uptake

The coronavirus family has been a re-emerging threat to global health since the early beginning of the 21st century (Morens and Fauci 2020), making the understanding of virus-host interactions of emerging viruses of great scientific and therapeutic interest. The cell surface receptor ACE2 is the entry receptor for two coronaviruses, namely SARS-CoV-1 and SARS-CoV-2 (Wenhui Li et al. 2003)(Tai et al. 2020), with the latter being the causative agent of the COVID-19 pandemic (Zhu et al. 2020; Bchetnia et al. 2020). The receptor is ubiquitously expressed in almost all cell types in the human body (M.-Y. Li et al. 2020; Hikmet et al. 2020), and even though alternate entry receptors have been identified, such as NRP1 for SARS-CoV-2 (Cantuti-Castelvetri et al. 2020), ACE2 maintains its status as the primary entry receptor and thus is a therapeutic target. Surprisingly little is known about the host's post-translational regulation of ACE2 levels, even though post-translational modifications significantly impact protein functionality and abundance (C. T. Walsh, Garneau-Tsodikova, and Gatto 2005). Ubiquitination is the post translational modification mediating the protein abundance via the ubiquitination-proteasomal degradation pathway (Kornitzer and Ciechanover 2000; Erales and Coffino 2014). Here, we identified the E3 ubiquitin ligase MDM2 as a regulator of ACE2 levels, which directly influences SARS-CoV-2 uptake and subsequent replication.

MDM2 was identified by combining a genetic depletion screen with a live-cell imaging approach. Proteins were chosen as candidates for our screen if they changed significantly in at least one of the omics layers (transcriptomics, proteomics, and interactomics) in our previous SARS-CoV-2 study (Stukalov et al. 2021). We genetically depleted 23 proteins by CRISPR/Cas9-mediated knockouts separately in A549-H2B-mRFP-ACE2 cells and tested for kinetic changes in virus replication with five GFP reporter viruses. Interestingly, MDM2 depletion increased SARS-CoV-2 replication only but was antiviral or had no effect on the other tested viruses. Proteomics profiling of the MDM2 knockout cells revealed increased protein levels of ACE2 compared to the control. This was also validated by immunofluorescence imaging, indicating increased ACE2 levels on the cell surface. This prompted us to perform a SARS-CoV-2-specific uptake assay, as increased ACE2 levels would explain the SARS-CoV-2-specific effect. Therefore, we compared the replication of VSV-S-GFP, an engineered VSV strain bearing the SARS-CoV-2 spike protein, with VSV-GFP and could prove increased uptake of VSV-S compared to the control. The combined

results validated that MDM2 knockout could indeed increase ACE2 levels and subsequently SARS-CoV-2 uptake and replication. In line with our results, Shen and colleagues (Shen et al. 2020) discovered that MDM2 modulates ACE2 levels in patients with pulmonary hypertension and showed that the lysine K788 within ACE2 is the regulation site for ubiquitination by MDM2, leading to proteasomal degradation. Therefore, we investigated the relevance of this ubiquitination site for SARS-CoV-2 cell entry and replication. We could show that substituting lysine K788 with arginine (K788R) stabilizes ACE2 levels, as proteasomal degradation of ACE2 is hindered. Additionally, we show that SARS-CoV-2 uptake is increased, as generally more cells get infected, and more virus particles adhere to the cells, leading again to a highly increased SARS-CoV-2 replication. In conclusion, MDM2 can restrict SARS-CoV-2 by regulating the entry receptor ACE2 through proteasomal degradation in vitro.

The characterization of the role of MDM2 might be crucial in understanding why certain risk groups suffer from increased hospitalization rates and a more serious COVID-19 progression. In this regard, it is noteworthy that MDM2 exhibits significant downregulation in individuals with common diabetic nephropathy (Saito et al. 2016). One could speculate that the observed downregulation of MDM2 may contribute to ACE2 upregulation, as described in our study. This, in turn, could potentially facilitate increased SARS-CoV-2 infection rates, exacerbate renal tissue damage, and ultimately portend severe COVID-19 cases. This could be an explanation for the heightened susceptibility of diabetes patients to severe COVID-19 outcomes (He et al. 2022; Williamson et al. 2020).

For future perspectives, the in-depth understanding of the ACE2 expression and degradation process could be key for developing novel antiviral strategies. This could be particularly relevant for immunocompromised patients, e.g. people suffering from autoimmune disease or people with organ transplants, because of the reduced efficacy of the vaccination (Barnes et al. 2023; “Vaccines and Therapeutics for Immunocompromised Patients with COVID-19” 2023).

Summary and discussion P2: NUDT2 initiates viral RNA degradation by removal of 5'-phosphates

This publication was a collaborative effort between different research groups. I contributed to conceptualization, experiments, analysis and writing of the manuscript. I designed, executed and interpreted the experiments of the different phosphate release assays in Figure 3a,b,c,e as well as Supplementary Figure 3a,b,c and Supplementary Figure 4c. I analyzed and interpreted the LC-MS/MS experiments in Figure 6a supplementary Figure 5d and curated the relevant data that is available online. Moreover, I consulted on the design of all figures, contributed to the writing process of all sections of the manuscript and correspondence with the reviewers.

The mRNA serves as the intermediate step to translate their DNA into proteins for all living organisms and viruses. The regulation of mRNA abundance is tightly controlled (Newbury, Mühlemann, and Stoecklin 2006), as the copy number of mRNA can affect protein abundance (Koussounadis et al. 2015; Guimaraes, Rocha, and Arkin 2014). To decrease eukaryotic mRNA, the m7G cap structure is removed from the 5' mRNA end by DCP1/2, which generates monophosphorylated RNA (P-RNA). This p-RNA is further degraded by the exonuclease XRN1 (Adjibade and Mazroui 2014). Viruses have adapted to the mammalian host with two main strategies for stabilizing their 5' mRNA end. The first is identical to eukaryotes, stabilizing their mRNA with m7G cap structures, which they can steal from the host's mRNA, a process called cap snatching (De Vlugt, Sikora, and Pelchat 2018). Interestingly, the second strategy involves stabilizing their RNA with triphosphorylation at the 5' end (PPP-RNA), which is a mechanism found in bacterial mRNA (Weber et al. 2013; Kaberdin, Singh, and Lin-Chao 2011). Importantly, it has been shown that XRN1 depletion stabilizes viruses relying on PPP-RNA (Y. Li et al. 2013). This suggests a mechanism of dephosphorylation of PPP-RNA to P-RNA, because XRN1 only degrades p-RNA. Here, we identified and mechanistically characterized the human phosphatase NUDT2, of which the depletion increases virus infection. Furthermore, our study shows that NUDT2 can facilitate dephosphorylation on a broad spectrum of nucleotides and RNA structures by dephosphorylating PPP-RNA to P-RNA, which then serves as a substrate for XRN1.

The pyrophosphatase RppH, which belongs to the nudix family, was known to facilitate the conversion of PPP-RNA to p-RNA in bacteria (Hsieh et al. 2013; Bischler et al. 2017). Therefore, we searched for mammalian nudix enzymes known to play a role in nucleotide metabolism. We performed a knock-down screen on 16 endogenous nudix enzymes in HeLa

cells and analyzed their effect on reporter viruses that rely on ppp-RNA. The most significant pro-viral effect was observed for NUDT2, indicating that NUDT2 can restrict viral replication. Using thin-layer chromatography, we determined that NUDT2 could, as expected, facilitate dephosphorylation of ppp-RNA because we observed the release of radioactively labeled gamma phosphate from ssPPP-RNA, dsPPP-RNA, and IVT4, a synthetic RNA RIG-I trigger. Additionally, we found that NUDT2 is active on a broad range of nucleotides, highlighting its broad applicability to various RNA substrates. Our reverse-phase experiments showed that NUDT2 releases phosphates in a sequential manner, i.e. the release of two individual phosphates, and further in vitro experiments proved that the resulting p-RNA from NUDT2 can indeed be used as a substrate for XRN1. Finally, the study showed an increased virus load in different cell types (bone marrow, mouse embryonic fibroblasts) derived from NUDT2 knockout mice. Interestingly, the survival rate between NUDT2 knockout mice and wild-type mice was similar when infected with virus VSV. This concludes that NUDT2 is restricting viral RNA stability because it can sequentially convert ppp-RNA to p-RNA, which can be processed by XRN1 and potentially serve as a gatekeeper for viral mRNA sensing and stability.

Interestingly, even though NUDT2 knockout appears to be pro-viral in vitro, the accumulation of viral ppp-RNA, e.g., by NUDT2 depletion, could activate RIG-I and stimulate an antiviral interferon response *in vivo* (Kandasamy *et al.* 2016; Weber *et al.* 2013). Also, DUSP11 has been postulated to dephosphorylate ppp-RNA and mediating RIG-I response, suggesting potential redundancy in ppp-RNA processing or cell type-specific activity (J. H. Choi *et al.* 2020; J. H. Choi and Sullivan 2021). The possibility of several dephosphorylation mechanisms for ppp-RNA and the highly probable RIG-I response might partly explain why the virus infection in NUDT2 knockout mice was not more severe or lethal but led to an increased viral load in the selected cell types.

It remains to be investigated how the mammalian system balances the low immune sensitivity induced by p-RNA with a highly pro-inflammatory state induced by ppp-RNA/RIG-I. A deeper understanding of these viral ppp-RNA sensing, dephosphorylation, and degradation processes will be required to gain an understanding of how the innate immune system responds to these viruses, particularly as Influenza A and the bunyaviruses pose pandemic potential. Findings from this research could be used to develop novel antiviral strategies.

Summary and discussion P3: Cell-free production of personalized therapeutic phages targeting multidrug-resistant bacteria

This publication was a collaborative effort between different research groups. I contributed to conceptualization, experiments, analysis and writing of the manuscript. I conceptualized and designed the cell-free, transient engineering, genomics, and proteomics experiments, performed the nanopore sequencing, performed the MS sample preparation with the support of collaboration partners (Figure 1-4). Furthermore, I have consulted in the genome assembly of the phage (Figure 1, Supplementary Figure S1). I generated all the figures and spearheaded the writing of the manuscript.

It has been demonstrated that it is possible to generate functioning viruses in cell-free systems. Those systems are mostly reconstituted cell lysates (containing the replication, transcription and translation machinery) complemented with energy/metabolite solution to fuel the biosynthesis. The addition of purified viral DNA to this system starts the assembly process of the virus (Garamella, Marshall, and Rustad 2016; Nesper et al. 1997). One advantage of cell-free systems lies in their ability to study essential genes and proteins; they allow for modulation and monitoring of effects without the cell lethality commonly observed in living cells. In our research, we have shown that the RNA polymerase subunit sigma70 is the restricting factor for the functional assembly of *B. subtilis* phages. Also we demonstrate that the protein expression kinetics of the viruses within the system is identical to the behavior in living host cells.

We leveraged our own cell-free system for the production of a variety of bacteriophages, targeting *E. coli*, *Y. pestis* and *K. pneumoniae*, showcasing the broad applicability of one standardized system for different bacteriophages. We hypothesized that such a system would enable in-depth kinetic proteomics analysis of bacteriophage proteins, as cells infected with bacteriophages have the issue of varying infection rates and non-synchronized states of the replication cycle. Such a problem does not exist in the cell-free system, because the addition of DNA initiates the start of protein expression. So we focused on analyzing the kinetics of the T7 phage in the cell-free system to see whether we can recapitulate published findings from living cells. We could indeed clearly differentiate different states of protein expression patterns that are in line with our expectations, i.e., two distinct patterns between proteins expressed by the host DNA polymerase and the T7-polymerase (Boeckman et al. 2022). This proves the conclusion drawn from this synthetic system can be biologically correct and relevant.

Importantly, our cell-free approach revealed viral proteins which have not been found and/or experimentally validated before, suggesting a deeper proteomics coverage as compared to previous approaches. We could show that these proteins exist in several hundred other bacteriophages, highlighting the relevance of these understudied proteins.

Upon attempting to assemble various bacteriophages in the cell-free system, we noticed that some bacteriophages, particularly *B. subtilis* phages, failed to assemble. We hypothesized that this was due to the specificity of RNA polymerase subunits in restricting bacteriophage RNA transcription and phage production. The host factor crucial for transcribing these promoters is the sigma70 RNA subunit. Due to the high sequence similarity between the *E. coli* and *B. subtilis*, we expected that they could be exchangeable and facilitate transcription of *B. subtilis* phage. Therefore, we modified the RNA polymerase complex within the cell-free system by co-expressing the sigma70 factor in the cell-free system and could indeed generate a functioning phage. This suggests that the structural similarity of the interfaces between RNA polymerase subunits is highly conserved. This finding implies that, aside from host entry and exit mechanisms, transcription may be the primary barrier to bacteriophage assembly in various bacteria, and other host proteins exploited by phages, such as ribosomes, may be interchangeable.

In conclusion we demonstrated the possibility of producing a variety of bacteriophages in a unified cell-free system. This approach enables proteomics studies on the phage but also reveals a variety of uncharacterized phage proteins. Moreover, we could identify the restriction factor sigma70 for *B. subtilis* phage assembly. Particularly, the identification of the sigma70 factor shows the relevance of such a system, because such a finding could have not been achieved in a living cell, as perturbing the RNA polymerase subunits is mostly lethal.

Also the identified bacteriophage proteins need to be further characterized. Deeper understanding of their function could potentially help to also better understand human viruses and the mammalian immune system. Several publications show a conserved mechanism of bacteriophage- and human viral -proteins, which show ancient immune evasion strategies in human and bacterial cells (Mathew and Chatterji 2006; Wein et al. 2023; Morehouse et al. 2020).

Additionally, bacteriophages could be used as an extension to antibiotics (Lin, Koskella, and Lin 2017). Several multidrug-resistant bacteria can be lysed by bacteriophages, but there is no common production pipeline available. Also phage engineering would become easier because one could add an engineered genome of the phage, e.g., a synthesized DNA brick, to the cell free system and harvest the assembled phages. Generally, the cell-free system poses the potential for a standardized production. Our results show that it is possible to generate therapeutically relevant phages and extend the production repertoire to other phage types.

List of figures used in this thesis

Illustrations used in this thesis have been generated with BioRender:

Figure 1: Schematic illustrating the viral replication cycle

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Appendix

The Appendix includes the three first author publications used in this dissertation. Parts of the publications, i.e. supplementary tables, were compressed to fit in this dissertation.

- Full Manuscript: P1 - MDM2 Influences ACE2 Stability and SARS-CoV-2 Uptake
- Full Manuscript: P2 - NUDT2 initiates viral RNA degradation by removal of 5'-phosphates
- Full Manuscript: P3 - Cell-free production of personalized therapeutic phages targeting multidrug-resistant bacteria

Full Manuscript: P1 - MDM2 Influences ACE2 Stability and SARS-CoV-2 Uptake

Article

MDM2 Influences ACE2 Stability and SARS-CoV-2 Uptake

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Abstract: Angiotensin-converting enzyme 2 (ACE2) is the central entry receptor for SARS-CoV-2. However, surprisingly little is known about the effects of host regulators on ACE2 localization, expression, and the associated influence on SARS-CoV-2 infection. Here we identify that ACE2 expression levels are regulated by the E3 ligase MDM2 and that MDM2 levels indirectly influence infection with SARS-CoV-2. Genetic depletion of MDM2 elevated ACE2 expression levels, which strongly promoted infection with all SARS-CoV-2 isolates tested. SARS-CoV-2 spike-pseudotyped viruses and the uptake of non-replication-competent virus-like particles showed that MDM2 affects the viral uptake process. MDM2 ubiquitinates Lysine 788 of ACE2 to induce proteasomal degradation, and degradation of this residue led to higher ACE2 expression levels and superior virus particle uptake. Our study illustrates that cellular regulators of ACE2 stability, such as MDM2, play an important role in defining the infection capabilities of SARS-CoV-2.

Keywords: severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2); SARS-CoV-2 uptake; MDM2; angiotensin-converting enzyme 2 (ACE2); ubiquitination



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1. Introduction

Angiotensin-converting enzyme 2 (ACE2) is a transmembrane protein regulating the renin-angiotensin-aldosterone system and thereby contributing to blood pressure management [1]. Interestingly, it is also the central entry receptor for several coronaviruses, among them Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) [2], the causative pathogen for the COVID-19 pandemic. Genetic depletion of ACE2 or its blockage with antibodies inhibits SARS-CoV-2 infection in vivo [3], further supporting the central importance of this protein for virus entry. SARS-CoV-2 initiates its uptake by adhering to the receptor binding domain of the glycoprotein spike (S) to ACE2 [2]. To deliver the genome to the cytoplasm, the SARS-CoV-2 particle is either fused at the plasma membrane or in the endosome by employing TMPRSS2 [4] or a cathepsin L-dependent process [5], respectively. These proteases cleave the S protein and enable the fusion of the viral particle with cellular membranes [6]. Several SARS-CoV-2 variants of concern evolved adaptive mutations in the S protein, further underlining the central importance of ACE2. These adaptive mutations led to enhanced binding efficacy of the S/ACE2 interaction site, e.g., the D614G mutation, which stabilized the S-conformation towards an ACE2 binding-competent state [6], as well as T478I and N501Y, which modulated the host range of SARS-CoV-2 [7]. Overall, these amino acid substitutions and others increased the infectivity of the emerging SARS-CoV-2 variants and promoted the global spread of the pathogen [8,9].

SARS-CoV-2 predominantly infects the respiratory system. Surprisingly, compared to other organs, ACE2 is not highly expressed in lung cells (<https://www.proteinatlas.org/>,

accessed on 1 May 2023) [10], which gives us a reason to speculate on the relevance of additional proteins promoting entry or alternative receptors that contribute to defining the tropism of the virus. Indeed, it has been reported that the Tyrosine-protein kinase receptor UFO (AXL) can serve as an entry receptor independently of ACE2 [11], and neuropilin-1, which is highly expressed in the bronchus and susceptible brain areas, supports SARS-CoV-2 uptake by bringing virus particles in close proximity to ACE2 [12,13]. Also, matrix metalloproteinases (MMPs) have been shown to promote SARS-CoV-2 entry; e.g., ADAM17 can facilitate the cleavage of the spike, and their inhibition led to the downregulation of SARS-CoV-2 infection [14].

Even though understanding the SARS-CoV-2/ACE2 interface is essential for COVID-19 pathology and therapeutic approaches, surprisingly little is known about the regulation of ACE2 by the host. Particularly, the effect of post-translational modifications on ACE2 and the consequences for SARS-CoV-2 infection are only marginally understood. In a CRISPR/Cas9-mediated screening approach to identify pro-/antiviral host factors, we found that the depletion of MDM2 substantially increased infection with SARS-CoV-2. This E3 ubiquitin ligase is best described for its ability to ubiquitinate p53 and thereby regulate its abundance [15]. Here we show that MDM2 depletion leads to elevated levels of ACE2 on the cell surface, which correlates with superior uptake of SARS-CoV-2 particles. We further show that the mutation of the ubiquitinated Lysine at position 788 into Arginine (K788R) increased the stability of ACE2 and thus its overall abundance, leading to significantly better uptake of SARS-CoV-2 and subsequently higher infection rates.

2. Material and Methods

2.1. Cell Lines

The following cells were used in the experiments: A549-H2B-mRFP and A549-H2B-mRFP-ACE2 for infection experiments and HEK293R1 for lentivirus production. All cells were cultured as previously described in DMEM (Invitrogen, Carlsbad, CA, USA) with 10% FCS and 1% Penicillin/Streptomycin [16]. All cells were tested to be mycoplasma-free.

Generation of knockout A549-H2B-mRFP-ACE2 cells was performed by cloning multiplexed sgRNA template sequences into the pLentiCRISPRv2 plasmid (Addgene plasmid #52961, Addgene, Watertown, MA, USA). All target sequences were designed with Synthego (Redwood City, CA, USA), except the NTC sequences, which were used as previously published [17] (Supplementary Table S1). Lentivirus production, transduction of cells, and antibiotic selection were performed as described previously [18]. In short, lentiviruses encoding puromycin resistance, Cas9, and sgRNAs were added to A549-H2B-mRFP-ACE2, followed by a 7-day puromycin selection (2 µg/mL). All cells were validated for their respective knockouts by sequencing using Synthego Performance Analysis (ICE Analysis, 2019. v3.0. Synthego, Redwood City, CA, USA, 06/2021).

For the generation of A549-H2B-mRFP-ACE2 and A549-H2B-RFP-ACE2 (K788R) cells, the lentiviruses were produced as described before with the pWpi-puro expression vector [16]. To ensure similar lentiviral transduction efficacy, we used a low MOI (0.1) of lentivirus for transduction of A549-H2B-mRFP, followed by antibiotic selection for a stable cell population. Plasmids were sequenced to validate the successful incorporation of the construct and the mutation K788R, respectively.

2.2. Virus Stock Preparation

The following viruses were used in this study and were produced as previously described: clinical SARS-CoV-2 WT and SARS-CoV-2-GFP strains were produced by infecting Vero E6 cells (2 days, MOI 0.01) [16] and VSV-S as in [14]. VSV-GFP was produced as in [19], RVFV-GFP was a kind gift from Friedemann Weber, and virus stocks were prepared as previously published [20].

2.3. Virus Infection and Live-Cell Imaging Experiment

A549-H2B-mRFP-ACE2, A549-H2B-mRFP-ACE2 (K788R), and the knockout cell line were seeded one day prior to the experiment in 96-well plates. Cells were infected with the reporter viruses SARS-CoV-2-GFP (MOI: 3), YFV-GFP (MOI: 1), VSV-GFP (MOI: 0.01), RVFV-Katuschka (MOI: 0.1) and HSV-1-GFP (MOI: 0.1). The infection was measured with the IncuCyte S3 Live-Cell Analysis System (Essen Bioscience) by acquiring the GFP, RFP, and phase contrast signal over 24–72 h post-infection, with 3–4 h intervals depending on the kinetics of the virus using a 4x objective. The raw data were processed with the internal IncuCyte S3 Software (Essen Bioscience; version 2020C rev1) by normalizing the integrated intensity of the GFP (virus) signal to either the integrated intensity of the RFP signal (cell) or the confluency determined by the phase contrast channel (cell). For the calculation of significance, a two-sample *t*-test $p < 0.05$ (*), < 0.01 (**), < 0.001 (***) was used.

We then chose, for each infection, the maximum signal value of each knockout and calculated its percentage change compared to the NTC:

$$\% \text{ change} = \frac{\text{mean(knockout)}}{\text{mean(NTC)}} - 1 \quad (1)$$

Life-cell imaging experiments were conducted as explained above, but cells were pre-treated for 4 h with MG132 (medchem, HY-13259) or PR-619 (medchem, HY-11814). The inhibitors were solubilized in DMSO and, for the experiments, diluted in cell culture medium. Cells were either not infected to test the cytotoxicity or pre-treated with the drugs for 4 h and then infected with the reporter virus SARS-CoV-2-GFP (MOI: 3) to monitor the effect of the drug on the virus.

2.4. Full Proteome MS Sample Preparation

Each of the replicates of A549-H2B-mRFP-ACE2 cells (MDM2 knockouts and NTCs) was infected with SARS-CoV-2 (MOI 3). The samples were harvested in SDC buffer (4% SDC, 100 mM Tris-HCl, pH 8.5), heat-inactivated (95 °C, 10 min), and sonicated (4 °C, 15 min, 30 s on/30 s off, high settings). In short, protein concentrations were measured by the BCA assay (Pierce, Appleton, WI, USA) according to the manufacturer's instructions. 50 µg of protein material were reduced and alkylated for 5 min at 45 °C with TCEP (10 mM) and CAA (40 mM). For each sample, 50 µg of protein material was digested overnight at 37 °C using trypsin (1:100 *w/w*, enzyme/protein, Promega, Madison, WI, USA) and LysC (1:100 *w/w*, enzyme/protein, Wako, Hiroshima-shi, Japan).

For proteome analysis, 20 µg of peptide material was desalted using SDB-RPS StageTips (Empore, Stellarton, NS, Canada). Samples were diluted with 1% trifluoroacetic acid (TFA) in isopropanol to a final volume of 200 µL and loaded onto StageTips, subsequently washed with 1% TFA in isopropanol and 0.2% TFA/5% acetonitrile (ACN, Concord, CA, USA). Peptides were eluted with 1.25% ammonium hydroxide (NH₄OH) in 60% ACN and dried using a SpeedVac centrifuge (Eppendorf, Concentrator Plus, Hamburg, Germany). They were resuspended in 0.1% FA prior to LC-MS/MS analysis. Peptide concentrations were measured optically at 280 nm (Nanodrop 2000, Thermo Scientific, Waltham, MA, USA) and subsequently equalized using 0.1% FA.

2.5. LC-MS/MS Data Acquisition

Full proteome samples were measured on an Eclipse mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled on-line to a Dionex Ultimate 3000 RSLCnano system (Thermo Fisher Scientific). Peptides were reconstituted in 0.1% FA and delivered to a trap column (ReproSil-pur C18-AQ, 5 µm; Dr. Maisch, 20 mm × 75 µm, self-packed) at a flow rate of 5 µL/min in 100% solvent A (0.1% FA in HPLC-grade water). After 10 min of loading, peptides were transferred to an analytical column (ReproSil Gold C18-AQ, 3 µm; Dr. Maisch, 400 mm × 75 µm, self-packed) at 50 °C and separated using an 80-min linear gradient from 4% to 32% of solvent B (0.1% FA, 5% DMSO in ACN) at a 300 nL/min flow rate.

The mass spectrometer was operated in data-dependent acquisition and positive ionization modes. MS1 full scans (360–1300 m/z) were acquired in the orbitrap with a resolution of 60,000, a normalized automatic gain control (AGC) value of 100%, and a maximum injection time of 50 ms. Peptide precursor selection for fragmentation was carried out using a fixed cycle time of 2 s. Only precursors with charge states from 2 to 6 were selected, and dynamic exclusion of 35 s was enabled. Peptide fragmentation was performed using higher-energy collision-induced dissociation (HCD) and a normalized collision energy of 30%. The precursor isolation window width of the quadrupole was set to 1.3 m/z . MS2 spectra were acquired in the orbitrap with a resolution of 15,000, a fixed first mass of 100 m/z , a normalized AGC target value of 200%, and a maximum injection time of 22 ms.

2.6. MS Data Processing and Analysis

Raw MS data files of the experiments conducted in DDA mode were processed with MaxQuant (version 2.0.3.1.) using the default settings and label-free quantification (LFQ) (LFQ min ratio count 2, normalization type classic) and intensity-Based Absolute Quantification (iBAQ) enabled. Spectra were searched against forward and reverse sequences of the reviewed human proteome, including isoforms (Uniprot, UP000005640), by the built-in Andromeda search engine.

The protein groups were further analyzed using Perseus (Version: 1.6.15.0). The LFQ values of 4941 proteins were imported from the proteinGroups.txt file, and the columns were filtered for the default settings of reverse, only identified by site and contaminants. The LFQ values were log2 transformed, and the protein groups were filtered for at least 2 valid values per grouping. The missing values were replaced by the normal distribution (1.8 downshift, 0.3 width, separately for each column). The two conditions, now including 3767 protein IDs, were analyzed with a two-sample *t*-test, leading to 267 significant changing proteins (two-sided, permutation-based FDR, 0.05 FDR, 0.1 S0, 250 randomizations) (Supplementary Table S2).

2.7. Upstream Regulator Analysis

For the identification of global upstream regulators, significant hits from the full proteome analysis were processed in the Ingenuity pathway analysis software (version 84978992, Qiagen, Hilden, GER). The core analysis was performed using the default settings, including the Ingenuity Knowledge Base as the reference set for *p*-value calculations as well as “direct and indirect relationships” for upstream regulator analysis. After analysis, only upstream regulators were considered significant, exceeding a *p*-value < 0.05.

2.8. VSV-S and VSV Infection

To assess whether the observed effect on SARS-CoV-2 was uptake-dependent, we used a VSV pseudovirus that displayed the SARS-CoV-2 spike (S) protein on its surface (VSV-S) and a parental VSV as a control. The day prior to infection, 8e3 A549-H2B-mRFP-ACE2 MDM2 knockout and A549-H2B-mRFP-ACE2 NTC or A549-H2B-mRFP, A549-H2B-mRFP-ACE2, and A549-H2B-mRFP-ACE2 (K788R) cells were seeded in a 96-well plate. The cells were infected with VSV-S (MOI: 3) and VSV-GFP (MOI: 0.01). The infection was measured with the IncuCyte S3 Live-Cell Analysis System (Essen Bioscience) as above. The raw data were processed with the internal IncuCyte S3 Software (Essen Bioscience; version 2020C rev1) by normalizing the integrated intensity of the GFP (virus) signal to either the integrated intensity of the RFP signal (cell) or phase (cell). The resulting normalized signal was then processed by median filtering and then by a Savitzky–Golay filter. Significance was determined by comparison of each of the two conditions for VSV and VSV-S to the control using a two-sample *t*-test for each time point (with a *p*-value ns > 0.05, * < 0.05, ** < 0.01, *** < 0.01).

2.9. Virus-like Particle Uptake Assay and Immunofluorescence Staining

For the generation of SARS-CoV-2 VLPs, HEK293T cells were transiently transfected with plasmids encoding S, M, N, E, and CD63~BlaM and harvested from conditioned medium 72 h post-transfection as previously described [21]. Then, 1.6×10^4 A549-H2B-mRFP-ACE2 and A549-H2B-mRFP-ACE2 (K788R) cells were seeded in a 96-well plate one day prior. Cells were washed with PBS (4 °C), and 100 µL of DMEM (10% FCS and 1% Penicillin/Streptomycin) plus 100 µL of VLPs were added per well. Additionally, CellTracker™ Orange CMRA Dye (Thermo Fisher Scientific, Waltham, MA, USA, C34551) was added to the medium/VLP mix according to the manufacturer's instructions. The plate was incubated for 30 min on ice to enable simultaneous uptake and then moved to the incubator (37 °C, 5% CO₂) for 1 h. The medium was aspirated, and the cells were washed three times with PBS, fixed with 4% PFA for 15 min at RT, and washed three times with PBS. The cells were permeabilized with 0.1% Triton-X100 at RT, washed with PBS, blocked with 5% FCS (1 h, RT), and washed with PBS. The VLPs were stained with a previously published in-house antibody dilution (1:1000) in 5% FCS for 1 h at RT in the dark, followed by PBS washing.

For staining of HA-tagged ACE2 levels, cells were processed as above. The HA-tag was stained with (1:500) an antibody dilution (Cell Signaling, Danvers, MA, USA, 3724S) in 5% FCS for 1 h at RT in the dark.

Super-resolution Images were acquired using a Zeiss LSM 900 (Zeiss, Oberkochen, Germany) with an Airyscan 2 detector system using a Plan-APOCHROMAT 63×/1.4 Oil DIC Objective. Post-acquisition linear adjustments of image brightness were made using the ImageJ software version 1.53. Confocal images were acquired using an Olympus FV10i microscope (Olympus, Tokyo, Japan) with a 60×/1.2 water immersion objective.

2.10. qRT-PCR Analysis

The knockout, A549-H2B-mRFP-ACE2 and A549-H2B-mRFP-ACE2 (K788R) cells were seeded (130,000 cells) one day prior to the experiment. For SARS-CoV-2 transcript quantification, cells were infected with SARS-CoV-2 (MOI: 3) and incubated for 1 h, 12 h or 24 h post-infection. To quantify ACE2 levels, cells were not infected. The RNA was isolated using the NucleoSpin RNA Plus kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's protocol. Total RNA was used for reverse transcription with PrimeScript RT with a gDNA eraser (Takara, Kusatsu, Japan). For relative transcript quantification, PowerUp SYBR Green (Applied Biosystems, Waltham, MA, USA) was used. The previously published primers 5'-TTACAAACATTGGCCGCAAA-3' (sense) and 5'-GCCGACATTCGGAAGAA-3' (antisense) were used for the SARS-CoV-2 nucleocapsid gene quantification [22] and 5'-CATTGGAGCAAGTGTGGATCTT-3' (sense) and 5'-GAGCTAATGCATGCCATTCTCA-3' (antisense) for ACE2 quantification [23]. For normalization, the previously published primers 5'-GATTCCACCCATGGCAAATTC-3' (sense) and 5'-GGATCTGCTGCATCTGCTTG-3' (antisense) for the GAPDH gene [24] or housekeeping gene for the RPLP0 gene were used as previously described [14]. Data were analyzed in GraphPad Prism (V.9). RNA level changes were analyzed by one-way ANOVA, comparing the 24 h levels of the knockouts to the 24 h values of the NTC, with $p > 0.12$ (ns), <0.033 (*), <0.002 (**), <0.001 (***)

2.11. Western Blotting

For western blot analysis, A549-H2B-mRFP-ACE2 and A549-H2B-mRFP-ACE2 (K788R) cells were seeded for one day in a 6-well format. Cells were washed with PBS and lysed in 200 µL of SSB buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% Glycerol, 50 mM DTT, 0.01% Bromophenol blue), followed by boiling at 95 °C for 5 min. The protein concentration of the samples was measured using Pierce 660 nm Protein Assay Reagent with the addition of an ionic detergent compatibility reagent (Thermo Fisher Scientific). 5 µg cell lysate was resolved on a NuPAGE 1 mm 4–12% Bis-Tris gel (Invitrogen) in 1× NuPAGE MES SDS Running Buffer (Invitrogen). For the PageRuler Prestained Protein Ladder (Thermo

Fisher Scientific), 5 μ L was used as a marker. The separated proteins were subsequently transferred to a 0.45 μ m nitrocellulose membrane (BioRad, Hercules, CA, USA) at 100 V for 1 h in pre-cooled wet-blotting transfer buffer (25 mM Trizma base, 0.192 M Glycine, pH 8.3, 20% methanol). The membrane was blocked in 5% skim milk in PBS-T buffer (phosphate buffered saline with the addition of 0.25% Tween-20) for 1 h at room temperature under constant agitation and rinsed twice with PBS-T before being incubated with antibodies. All antibodies were diluted with 5% milk in PBS-T. The protein of interest was detected using the primary antibody against ACE2 (abcam, Waltham, MA, USA, ab272690, 1:1000 dilution) and the secondary antibody coupled to horseradish peroxidase rb-HRP (Agilent Dako, Glostrup, Denmark, P0448, 1:2500 dilution). The membrane was washed 3 $\times$ for 5 min with PBS-T in between and after incubation with primary and secondary antibodies. Before reprobing the membrane with the ACTB-HRP (Santa Cruz, Dallas, TX, USA, sc-47778, 1:2500 dilution) antibody, bound antibodies were stripped using Restore Western Blot Stripping Buffer (Thermo Fisher Scientific), and the membrane was washed 3 $\times$ for 5 min with PBS-T. The membrane was incubated with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) prior to developing the membrane using the ChemiDoc XRS+ Imager. Optical density was determined with ImageJ (1.52 k, 1.8.0_172), and statistical analysis was performed with GraphPad Prism (V.9), performing a *t*-test.

3. Results

3.1. Selection of Knockout Candidates Based on Multi-Omics Profiling Data

We selected 21 host proteins for a CRISPR/Cas9-based knockout screen targeting different pathways and contributing to various cellular functions that may be important for SARS-CoV-2 replication or spread. These proteins were selected based on multi-omics profiling of SARS-CoV-2 infection [16], in which they were significantly affected in their mRNA or protein expression or were identified as interaction partners of SARS-CoV-2 proteins. We particularly focused on interactions between ORF7B and ORF3, two multi-functional SARS-CoV-2 proteins necessary for SARS-CoV-2 spread. ORF7B localizes in the Golgi and the virion [25]. It is also reported to induce several cytokines and growth factors and mediate apoptosis regulation [26]. ORF3a is an accessory protein that blocks autolysosome fusion [27]. It is frequently mutating and induces different innate immune responses [28]. ORF7B and ORF3a both interact with the syntaxins STX6, STX10, STX12, and STX16, necessary for various roles in vesicle trafficking [29], the receptor-type tyrosine phosphatase PTPRM, the transmembrane protein DCBLD2, and the solute carrier SLC30A1. The endosomal transport protein VTI1A [30], the SHC transforming protein, and the G-protein coupled receptor 39 (GPR39) interacted with ORF7b, while the cell adhesion proteins PCDHAC2, PCDHGC3, NECTIN3, and CDH16 mainly interacted with ORF3. Matrix metalloproteinase MMP15 interacted with ORF7b and ORF3 and was significantly upregulated in the transcriptome after infection. The TGFB1 regulator, LTBP1 [31], interacted with ORF8. The cell surface protein TNS4 was highly upregulated in the full proteome after the SARS-CoV-2 infection. The transcript levels of OSMR were highly upregulated by SARS-CoV-2. Also, the transcripts of the E3-ubiquitin ligase MDM2 were significantly upregulated by the SARS-CoV-2 infection. The latter was particularly interesting because several publications suggested drugging this E3 ligase to increase p53 levels to counteract SARS-CoV-2 infection [32,33].

3.2. MDM2 Knockout Specifically Enhances SARS-CoV-2 Infection

We used a CRISPR/Cas9-based knockout strategy to deplete the described candidates in human lung carcinoma epithelial A549 cells expressing the SARS-CoV-2 entry receptor ACE2 and H2B-mRFP (A549-H2B-mRFP-ACE2) (Supplementary Table S1). The knockout efficiency was confirmed by sequencing. As a positive control for our screen, we knocked out lymphocyte antigen 6e (LY6E), a protein that impairs efficient entry for human Coronaviruses [34]. We used a recombinant SARS-CoV-2 that expressed green fluorescent protein (GFP) upon infection (SARS-CoV-2-GFP) and performed continuous live-cell imaging to

monitor the effect of the selected candidate on viral spread. We normalized the integrated GFP expression values to the integrated mRFP signal (representative of the cell count) and plotted the maximum value of each infection normalized to the non-targeting control (NTC) (Figure 1A). Our screening approach revealed several effects on SARS-CoV-2 replication. As expected, LY6E depletion led to a significantly increased accumulation of GFP, confirming the antiviral activity of this protein and the validity of the assay. Notably, three tested candidates, MDM2, SLC30A1, and GPR39, displayed a substantial increase in GFP signal (Figure 1B). Interestingly, the strongest decrease in infection was detected for the knockouts of three vesicular trafficking proteins (VTI1A, STX12 and STX16), highlighting the important role of vesicular trafficking for SARS-CoV-2 infection and replication. To test whether the identified proteins were specifically important for SARS-CoV-2, we complemented the analysis of SARS-CoV-2 with growth analyses of unrelated viruses, namely Vesicular Stomatitis Virus (VSV) and Yellow Fever Virus (YFV). As expected, the knockout of the restriction factor LY6E did not affect VSV and YFV but showed exclusive antiviral activity for SARS-CoV-2 (Figure 1B–D). Interestingly, the antiviral effect of syntaxin knockouts appeared to be reversed for VSV, particularly for STX16. We focused on the top four pro-viral and top three antiviral knockouts in the SARS-CoV-2 screen (including all the syntaxins) and the top pro- and antiviral hits of YFV and VSV. These candidates were further tested for their effect against the Rift Valley Fever Virus (RVFV) and Herpes Simplex Virus (HSV-1). Depletion of GPR39, a regulator of heat shock proteins and the hedgehog pathway [35], showed highly virus-specific effects: While GPR39 depletion increased SARS-CoV-2 replication, it decreased the propagation of all other viruses tested (Figure 1B–F). Better growth of SARS-CoV-2 and VSV was seen upon knockout of SLC30A1 (Figure 1B,C), which has been reported to elevate intracellular zinc levels and hence inhibit caspase activation and apoptosis [36,37]. Depletion of SHC1 increased the growth of VSV, YFV HSV-1, and RVFV but slightly inhibited the growth of SARS-CoV-2 (Figure 1B–F). The knockout of NECTIN3 was strongly antiviral for YFV, slightly repressive for VSV, and moderately pro-viral for SARS-CoV-2. No significant changes were observed for RVFV and HSV-1 (Figure 1B–F). However, among all candidates tested, depletion of MDM2 showed the strongest pro-viral effect and was exclusively important for SARS-CoV-2 (Figures 1A–F and S1A). This phenotype was very reminiscent of LY6E knockout, which resulted in an infection kinetic of SARS-CoV-2 that was comparable to MDM2 deletion, i.e., an early onset of the infection signal and a rapid increase of the fluorescent signal (Supplementary Figures S1B,C and S2).

3.3. MDM2 Knockout Stabilizes ACE2 and Increases SARS-CoV-2 Replication

We next confirmed the obtained results by quantifying SARS-CoV-2 nucleocapsid RNA between 12 and 24 h post-infection in our knockout cells. Considering the accumulation of viral RNA in NTC cells over time, only three knockout cell lines led to a significantly higher increase in SARS-CoV-2 levels. Notably, in line with our screening approach, the highest increase could be seen in MDM2 knockout cells (Figures 2A and S3). In contrast, among all tested knockouts, depletion of STX16 most strongly reduced viral accumulation. Thus, we could validate the screening results and confirm that depletion of MDM2 led to a significant increase in SARS-CoV-2 replication, while STX16 had the opposite effect.

Given the notable effect of MDM2 knockout, we further focused on the antiviral activity of this protein in the context of the SARS-CoV-2 infection. The comparable infection kinetics and specificity in MDM2 and LY6E-depleted cells led us to hypothesize that MDM2 may impair SARS-CoV-2 uptake, as reported for LY6E [34]. To test this, we infected the MDM2-depleted and control cells with a pseudotyped VSV-GFP reporter virus bearing the SARS-CoV-2 spike protein (VSV-S). We observed a significantly higher GFP signal in MDM2 knockout cells as compared to NTC cells (Figure 2B). As an additional control, we infected MDM2 knockout and NTC cells with the parental VSV-GFP but did not observe any significant difference in the GFP signal (Figure 2C). Collectively, these data showed that depletion of MDM2 specifically increased SARS-CoV-2 uptake but did not affect the

uptake of other tested viruses. Moreover, it showed that MDM2 serves as a prominent restricting factor for SARS-CoV-2 entry.

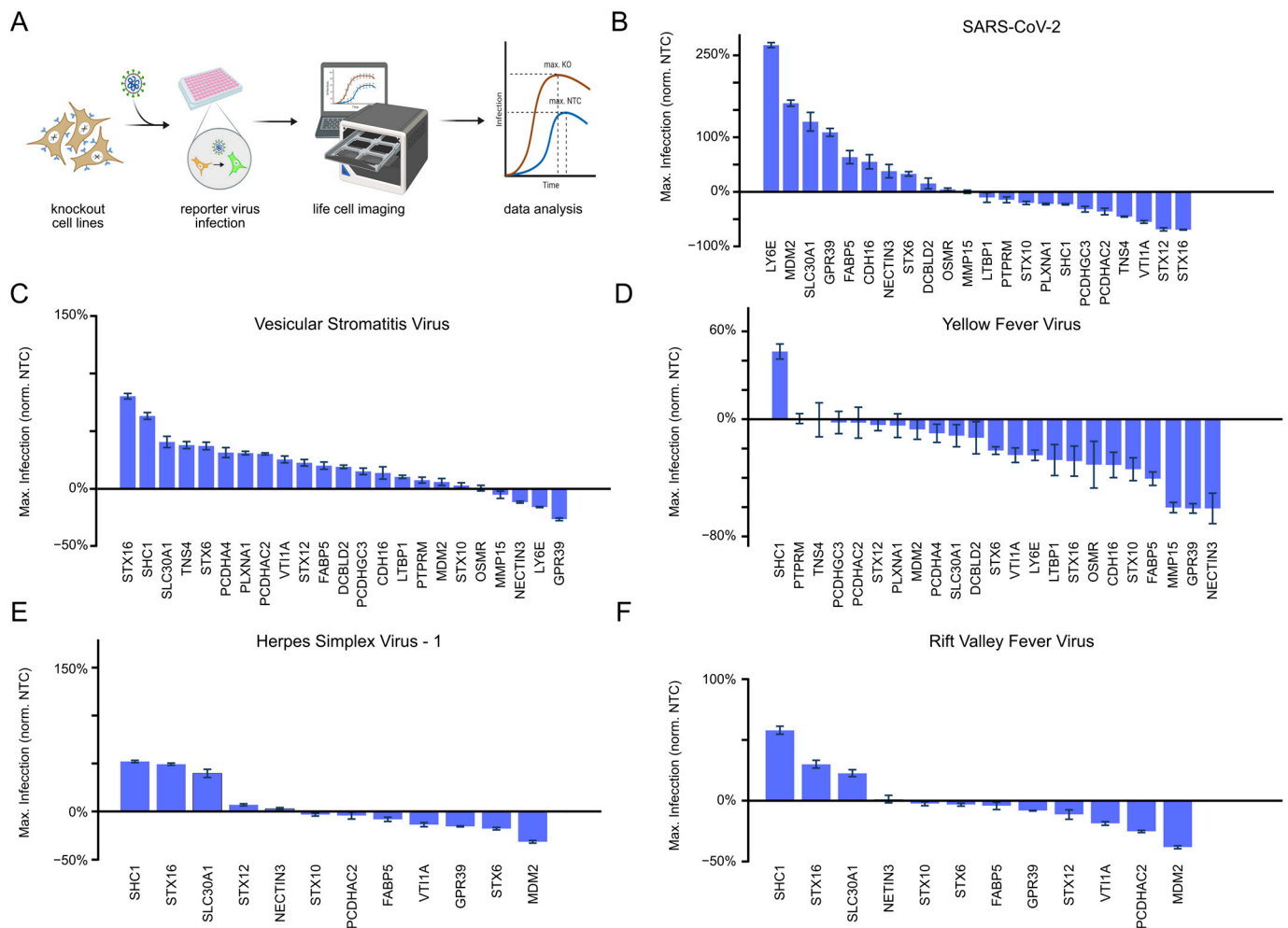


Figure 1. Loss of function screen identifies MDM2 as a SARS-CoV-2 restriction factor. (A) Experimental design and analysis of a loss-of-function screen. A549-ACE2-H2B-mRFP knockout and control cells were infected with 5 different GFP-expressing reporter viruses, and virus growth was measured using a live-cell imaging platform. The virus infection was quantified over time by normalizing the virus signal (integrated intensity of GFP) by A549-ACE2-H2B-mRFP (integrated intensity of mRFP). The maximal value during infection of infected knockout cells and NTC was used for further analysis and displayed as a bar plot in. The illustration was designed with BioRender (<https://www.biorender.com/>, accessed on 1 May 2023) (B–F). (B–F) Growth of SARS-CoV-2 (MOI 3), Vesicular Stomatitis Virus (MOI 0.01), Yellow Fever Virus (MOI 0.1), Herpes Simplex Virus 1 (MOI 0.1), and Rift Valley Fever Virus (MOI 0.1). Analysis was performed as described in (A). Each bar represents the mean ($n = 3$ biological repeats, $+/-$ SEM) normalized to the NTC to show the effect of the individual gene deletion on the virus (%) compared to the infection behavior in NTC cells.

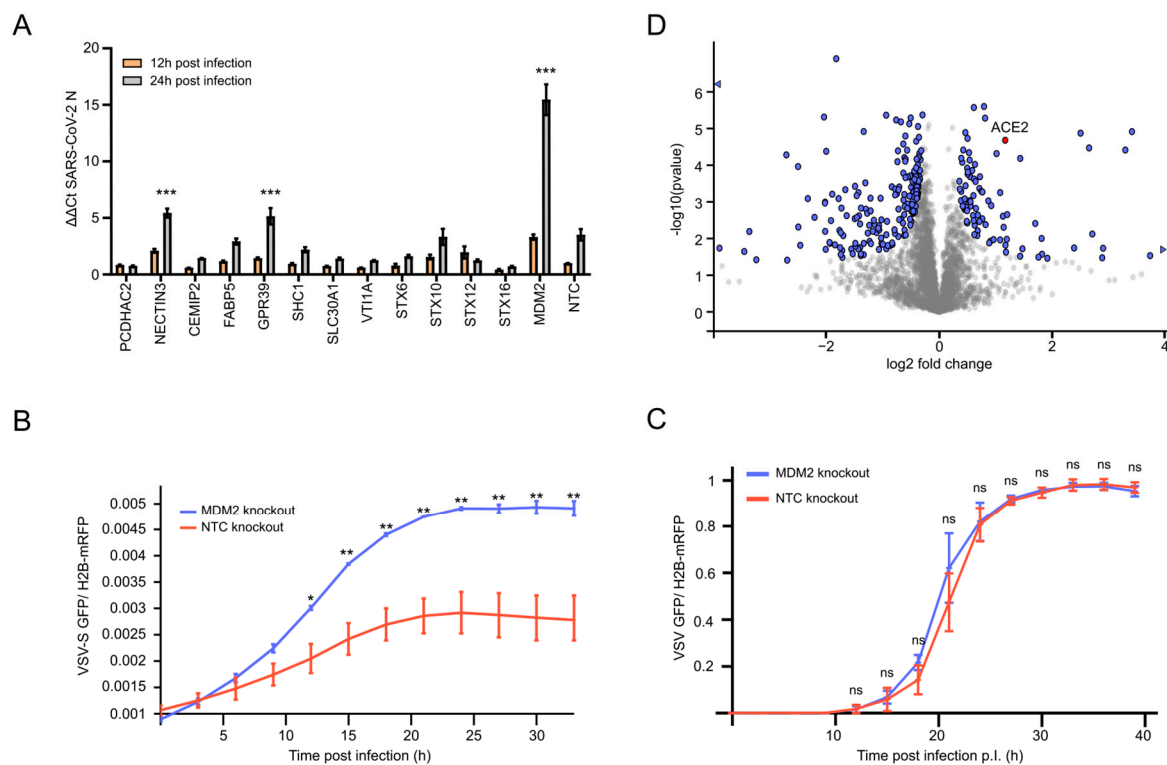


Figure 2. MDM2 knockout increases SARS-CoV-2 RNA abundance and supports virus uptake. (A) SARS-CoV-2 nucleocapsid (N) RNA levels in A549-ACE2-H2B-mRFP knockout and NTC cells infected with SARS-CoV-2 (wt) were analyzed 12 h and 24 h post-infection. SARS-CoV-2 transcripts were normalized to GAPDH and to the NTC sample. The graph shows a fold change in N abundance vs. 12 h NTC infected cells. Significance of the RNA level changes was analyzed by one-way ANOVA, comparing the 24 h levels of the knockouts to the 24 h values of the NTC, with $p < 0.001$ (***). Bars show mean signal $\pm$ SD of 3 independent experiments. Significance was displayed when $p < 0.001$ and the difference in $\Delta\Delta Ct$ (12 to 24 h) of the knockout was greater than the difference in $\Delta\Delta Ct$ of the NTC. (B) A549-H2B-mRFP-ACE2 MDM2 knockout and NTC cells were infected with VSV-S-GFP reporter virus (MOI 3), and the GFP expression was measured over time (mean $\pm$ SD, $n = 3$). Integrated GFP intensity was normalized to the H2B-mRFP intensity. Significance was calculated by a two-tailed Student's t -test using a two-sample t -test with $p < 0.05$ (*), < 0.01 (**) and non-significant $p > 0.5$ (ns). (C) As in (B) but with VSV-GFP (MOI 0.01). (D) Full proteome analysis of A549-H2B-mRFP-ACE2 MDM2 knockout cells and NTC. Volcano plot shows 3767 analyzed proteins (dots), of which 267 proteins showed significantly different expression in MDM2 knockout vs. NTC cells (blue symbols, red for ACE2). Triangles represent proteins outside the displayed x-axis scaling. Significance was calculated by a two-tailed Student's t -test (permutation-based FDR < 0.05 , $n = 4$ biological replicates).

Since MDM2 is an E3 ubiquitin ligase mediating proteasomal degradation of substrates, we proceeded to use proteomics analysis employing liquid chromatography coupled to mass spectrometry (LC-MS/MS) to analyze the global changes in the proteome after depletion of MDM2. In total, we could detect expression values for 4941 proteins in all tested conditions, of which 3767 were used for analysis. Among them, 267 proteins showed significant differences in their expression levels in MDM2 knockout cells as compared to NTC cells (Figure 2C, Supplementary Table S2). We performed an upstream regulator analysis on these hits to identify functional clusters and their responsible inducers that could explain the SARS-CoV-2-specific effect (Supplementary Table S3). As expected, this analysis identified p53 as an activated regulator (57 of the 267 proteins were regulated by p53) that was specifically enriched in the absence of MDM2 (p -value $< 2.14 \times 10^{-14}$). Surprisingly, a direct comparison between MDM2 knockout and control cells revealed a significantly

increased protein abundance of ACE2 in the absence of MDM2 (Figure 2D). Therefore, the knockout of MDM2 could increase the expression levels of ACE2, plausibly explaining enhanced infection and selective uptake of SARS-CoV-2. Indeed, ACE2 immunostaining showed that MDM2 knockout cells displayed higher levels of ACE2, including at the cell surface, as compared to NTC cells (Supplementary Figure S3B). Collectively, these data suggested that MDM2 controls ACE2 expression levels and that MDM2 depletion led to increased SARS-CoV-2 uptake, likely due to increased levels of ACE2.

3.4. Lysine 788 within ACE2 Mediates ACE2 Stability and SARS-CoV-2 Replication

In search of a potential molecular mechanism for the enhanced infectivity of MDM2 knockout cells, we mined a database for post-translational modifications [38] and identified ACE2 Lysine K788 as the only ubiquitination site accessible for cytosolic MDM2 (Supplementary Figure S4A). Importantly, this ACE2-K788 site has already been identified as a target of the E3-ligase MDM2 in patients suffering from idiopathic pulmonary arterial hypertension. It has been shown that MDM2 is upregulated in these patients and that MDM2 ubiquitinates ACE2 at position K788, which leads to a subsequent downregulation of ACE2 through proteasomal degradation. Also, mutation of this site or inhibiting ACE2-expressing cells with a proteasome inhibitor can protect ACE2 from proteasomal degradation, commonly induced by K48-ubiquitination [39]. However, while the consequences of this site for SARS-CoV-2 infection have not been investigated so far, the regulation of ACE2 levels by MDM2-dependent ubiquitination is in line with our infection experiments.

To confirm that MDM2 may target K788 for ubiquitination and subsequent degradation of ACE2, we mutated Lysine 788 into Arginine (K788R), following the hypothesis that preventing MDM2-dependent ubiquitination would stabilize ACE2 and lead to overall higher infection rates. We used lentiviral gene transfer to trans-complement ACE2-negative A549-H2B-mRFP cells with wild-type ACE2 (A549-H2B-mRFP-ACE2) or with a K788R mutant (A549-H2B-RFP-ACE2 (K788R)). Notably, the expression level of the ACE2 (K788R) mutant protein was clearly increased as compared to the expression levels of cells expressing wt ACE2, despite comparable levels of the β -actin (β -ACT) control by 60% (Figures 3A and S4B,C). Importantly, evaluation of the Ace2 transcript levels by quantitative PCR indicated identical Ace2 mRNA expression in both tested cell lines (Figure 3B), supporting that the increase of ACE2-K788R on the protein level was due to increased stability of the mutant rather than higher mRNA expression. To test if the increase in ACE2 level leads to better infection, we infected these cell lines with similar amounts of SARS-CoV-2-GFP reporter virus and monitored the accumulation of the fluorescent signal over time. Indeed, we observed significantly increased infection in the ACE2 (K788R) cells as compared to ACE2-wt controls (Figure 3C). Moreover, further analysis of the obtained pictures showed that more cells got infected with SARS-CoV-2-GFP in ACE2 (K788R) cells as compared to wt ACE2-expressing cells (Supplementary Figure S4D). To test if degradation via the proteasome influences ACE2 levels and thereby SARS-CoV-2 uptake, we treated A549-ACE2 cells with the proteasome inhibitor (MG132) and monitored SARS-CoV2 replication. However, proteasomal inhibition impaired SARS-CoV-2 replication, indicating that proteasomal activity is in general required for virus replication (Supplementary Figure S4E), which is in line with previous findings [16,40]. We speculated that inhibition of deubiquitinating enzymes (DUBs) would increase ACE2 degradation and thus reduce the infectivity of SARS-CoV-2. However, the broadly active deubiquitinase inhibitor (PR-619) did not considerably affect SARS-CoV-2 replication at non-cytotoxic concentrations (0.5 μ M) (Supplementary Figure S4F,G). More specific inhibitors and further functional experiments would be required to identify a chemical compound that targets the described pathway to reduce cellular ACE2 levels.

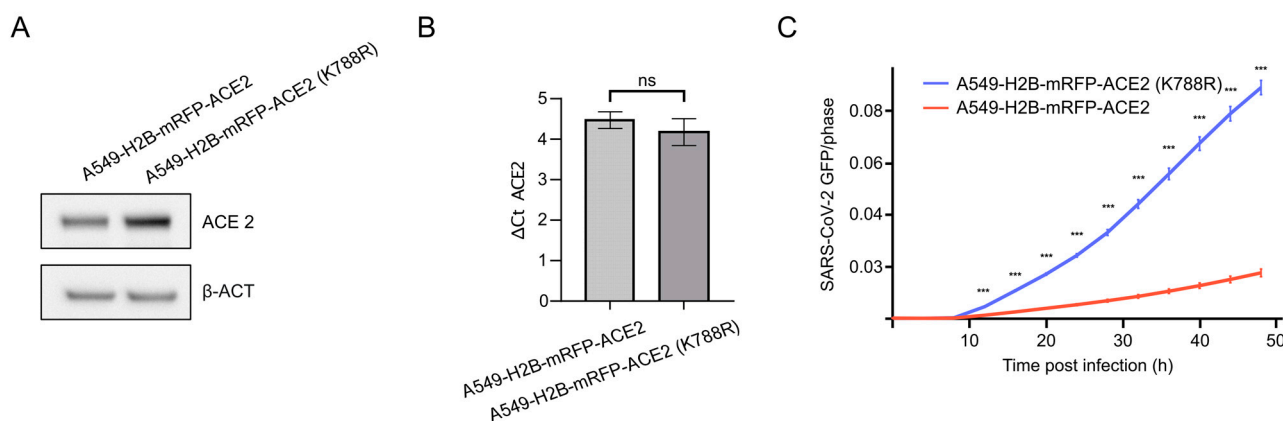


Figure 3. Mutation of ACE2 Lysine 788 increases protein levels of ACE2 and enhances SARS-CoV-2 infection. (A) Western blot showing ACE2 and β -actin (β -ACT) levels of A549-H2B-mRFP-ACE2 and A549-H2B-mRFP-ACE2 (K788R) cells. (B) Comparison of ACE2 mRNA levels (normalized to RPLP0) between A549-H2B-mRFP-ACE2 and A549-H2B-mRFP-ACE2 (K788R) cells. To compare mRNA levels, an unpaired two-tailed *t*-test was applied with ns: $p > 0.05$ (mean $\pm$ SD, $n = 4$). (C) Infection of A549-H2B-mRFP-ACE2-wt and A549-H2B-mRFP-ACE2-K788R with SARS-CoV-2-GFP reporter virus. The integrated GFP area (virus signal) was normalized to the area of cells. Significance was calculated using a two-sample *t*-test, $p < 0.001$ (***).

3.5. ACE2-K788R Increases SARS-CoV-2 Particle Uptake

These experiments prompted us to test whether ACE2 (K788R) may allow for better viral uptake. To test this, we infected A549-H2B-mRFP, A549-H2B-mRFP-ACE2, and A549-H2B-mRFP-ACE2 (K788R) cells with VSV-S and VSV GFP reporter viruses, respectively, and monitored GFP expression over time (Figure 4B,C). As expected, A549-H2B-RFP cells that were not trans-complemented with ACE2 could not be infected with VSV-S, while expression of ACE2 led to GFP accumulation over time. Notably, the number of cells infected with VSV-S was significantly higher in A549-H2B-mRFP-ACE2 (K788R) cells as compared to A549-H2B-mRFP-ACE2, further indicating that the uptake of VSV-S was superior in the ACE2 (K788R) cell line (Supplementary Figure S5A,B). Importantly, the infection with VSV was identical in all three tested cell lines (Figure 4C). Moreover, we infected A549-H2B-RFP-ACE2 and A549-H2B-mRFP-ACE2 (K788R) with SARS-CoV-2 and detected significantly higher SARS-CoV-2 nucleocapsid RNA levels at 1 h post-infection in A549-H2B-RFP-ACE2-K788R cells as compared to wt ACE2-expressing cells (Figure 4A).

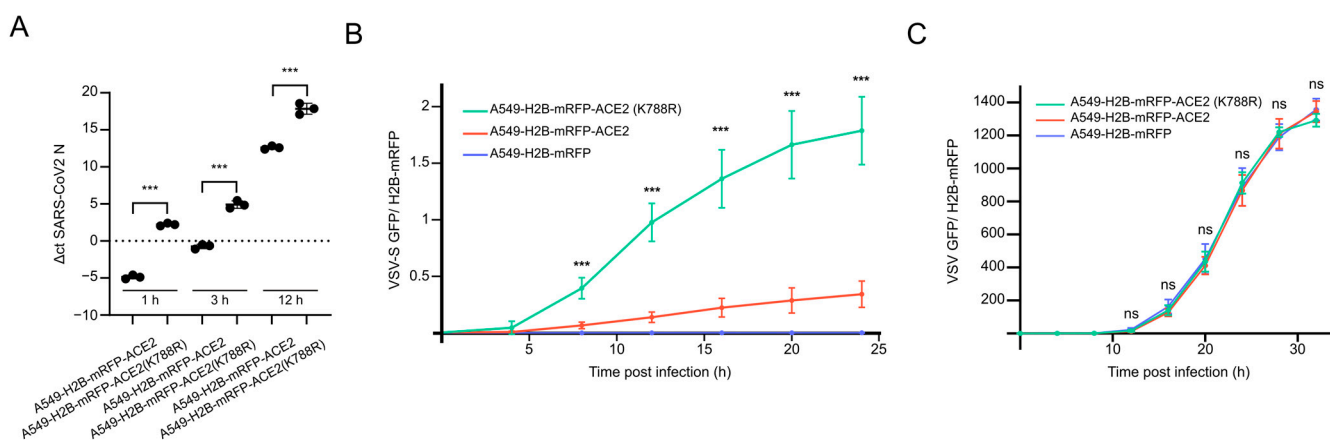


Figure 4. Cont.

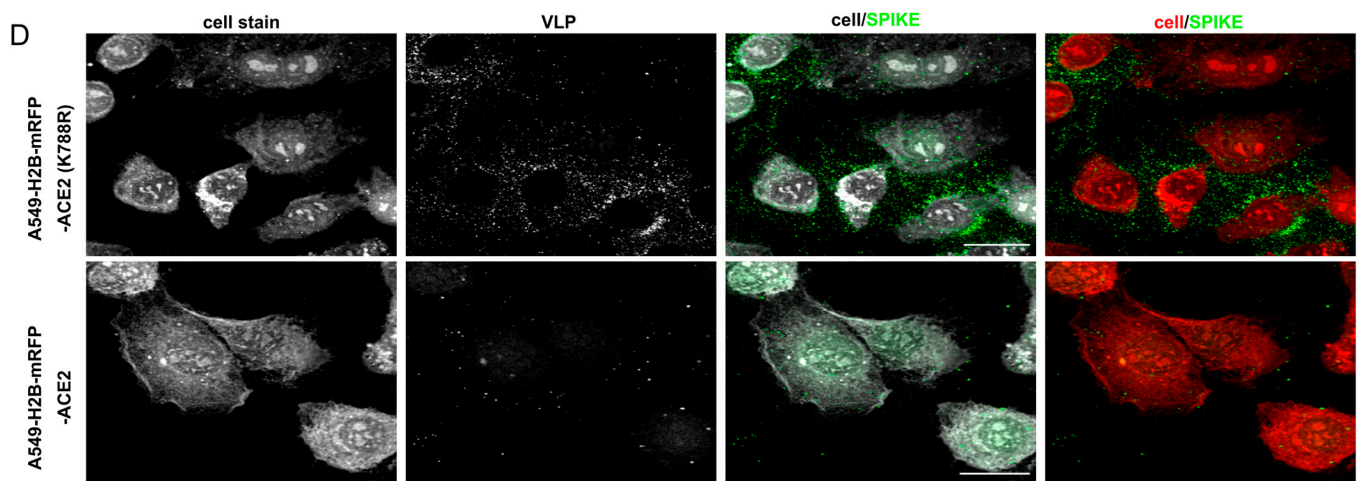


Figure 4. ACE2-K788R increases SARS-CoV-2 particle uptake. (A) Comparison of SARS-CoV-2 nucleocapsid (N) RNA levels between SARS-CoV-2 (wt) infected (MOI 3) A549-H2B-mRFP-ACE2 and A549-H2B-mRFP-ACE2 (K788R) cells at 1, 3 and 12 h post-infection. To compare the RNA levels (normalized to RPLP0 mRNA levels), an unpaired *t*-test was applied with $p < 0.0001$ (***) (mean $\pm$ SD, $n = 3$). (B) A549-H2B-mRFP, A549-H2B-mRFP-ACE2, A549-H2B-mRFP-ACE2 (K788R), and A549-H2B-mRFP were infected with VSV-S-GFP (MOI 3), and GFP expression was measured over time (mean $\pm$ SD, $n = 3$). The graph shows the ratio of the integrated intensity of the GFP signal over the RFP signal. Significance was calculated using a two-sample *t*-test with $p < 0.001$ (***). (C) As (A), but cells were infected with VSV-GFP (MOI 0.01) (mean $\pm$ SD, $n = 3$). Significance was calculated using a two-sample *t*-test with $p > 0.05$ (ns). (D) A549-H2B-mRFP-ACE2 and A549-H2B-mRFP-ACE2 (K788R) cells were exposed to SARS-CoV-2 Spike-coated virus-like particles (VLPs). Representative immunofluorescence staining of S after VLP incubation (1 h, 37 °C). The staining was performed with the cell stain CMRA orange and a polyclonal antibody against SARS-CoV-2 Spike (scale bar, 20 μ m).

To underline the better binding of SARS-CoV-2 to ACE2 (K788R) over ACE2-wt expressing cells, we compared the adhesion of SARS-CoV-2 virus-like particles (VLP) [21] on the two different cell lines using super-resolution microscopy. We expected to observe a higher amount of VLPs on the surface of A549-H2B-mRFP-ACE2 (K788R) as compared to A549-H2B-mRFP-ACE2 cells. To test this, we exposed both cell lines to identical amounts of VLPs on ice, followed by a temperature shift to 37 °C for 1 h. In line with the notion that ACE2-K788R increased uptake efficiency, we could observe an increased association of VLPs to the cell surface, as evaluated by staining for Spike (Figure 4D). Taken together, these experiments highlight the specific effect of MDM2-driven ACE2 regulation on SARS-CoV-2 entry.

4. Discussion

Here we selected host factors identified from SARS-CoV-2 multi-omics analysis [16] to further investigate their effect on SARS-CoV-2, YFV, VSV, HSV-1, and RVFV replication. Our analysis highlighted proteins that had pan-antiviral as well as virus-specific effects. The knockouts of syntaxin proteins showed virus-specific effects (Figures 1, 2 and S2). This was particularly evident for STX16, which displayed a strong antiviral effect for SARS-CoV-2 and was proviral for VSV. The effect on SARS-CoV-2 could be partly explained by the different roles of STX16, as this protein is necessary for late events of endocytosis, more specifically in lysosome biogenesis [41]. One additional role that could be impeded by STX16 knockout is the uptake of viral particles, as STX16 also plays an important role in vesicle formation for endocytosis [42]. SARS-CoV-2 has two main entry pathways: membrane fusion, which requires TMPRSS2 expression, or the endocytic pathway, which requires ADAM17/Cathepsin L [4] and thus the endolysosomal compartment. Lung cells, including the A549 cell line, display a negligible amount of TMPRSS2 (<https://www.>

proteomicsatlas.org/, accessed on the 1 May 2023). As SARS-CoV-2 depends on endocytic uptake in this cellular model, the observed antiviral effect of STX16 might be due to the reduced entry of SARS-CoV-2 particles. In conclusion, the role of STX16 in SARS-CoV-2 biology could be more relevant for the virus uptake than for the secretion of newly generated virions, which is underlined by the very slow infection rate in STX16-depleted cells (Figures 2A and S1B). Conversely, VSV infection appeared to be increasing, potentially because STX16 depletion reduces the degradation of the virus through the role of STX16 in autophagosome/lysosome formation.

GPR39, NECTIN3, and MDM2 knockout cells showed an exclusive and strong pro-viral effect for SARS-CoV-2 (Figures 1 and 2). Among them, MDM2 knockout was particularly interesting as it displayed a similar phenotype to LY6E knockout cells [34]. This observation is unexpected, as MDM2 is mainly described as an E3 ubiquitin ligase involved in p53 regulation, the latter being an antiviral protein for most viruses [43,44]. Interestingly, an antiviral effect of MDM2 could be confirmed for YFV, HSV-1, and RVFV but not for SARS-CoV-2 (Supplementary Figure S1A), suggesting a potentially different role of MDM2 in the case of SARS-CoV-2 infection. LY6E has been shown to specifically impair SARS-CoV-2 entry, and—given the similarity of the infection phenotype in our live-cell imaging screen (Supplementary Figure S1B,C)—we speculated that MDM2 might be involved in virus uptake. This theory was substantiated by viral particle uptake assays, as SARS-CoV-2 spike-pseudotyped VSV (VSV-S) replication was significantly higher in the MDM2 knockout cells as compared to the NTC cells (Figure 2B,C).

Our combined results suggested that the increase in SARS-CoV-2 replication was an uptake-dependent effect. Knowing the enzymatic activity of MDM2 and the observed modulation of ACE2 levels in our LC-MS dataset (Figure 2D), we further investigated the contribution of the ACE2 ubiquitination site used by MDM2 derived from patient data [39]. We generated stable A549-H2B-mRFP-ACE2 and A549-H2B-mRFP-ACE2 (K788R) cell lines, which were used to elucidate the effect on SARS-CoV-2 infection and uptake. Indeed, compared to the wild-type, the K788R substitution increased the amount of ACE2, even though the transcriptional levels of ACE2 remained similar (Figure 3A,B), highlighting the role of the MDM2 ubiquitination site for ACE2 turnover. Accordingly, the SARS-CoV-2 infection was significantly increased in the ACE2 mutated cells, likely due to the accumulation of ACE2 on the cell surface and consequently increased uptake of SARS-CoV-2 (Figure 3). To further substantiate the uptake-dependent effect, we used a SARS-CoV-2 VLP system. We could clearly observe more attached particles on A549-H2B-mRFP-ACE2 (K788R) cells after 1 h of incubation as compared to A549-H2B-mRFP-ACE2 (Figure 4D). Similar results were obtained by RT-qPCR on SARS-CoV-2-infected cells (Figure 4A). Additionally, our VSV-S assay underlined the importance of the K788R substitution for SARS-CoV-2 entry, as the effect was only observed for VSV-S but not with the parental VSV strain (Figure 4B,C). The observed pro-viral effect of MDM2 knockout seems to be dependent on ACE2-mediated virus uptake (Figure 2). This is further supported as the pro-viral effect of the MDM2 knockout was SARS-CoV-2-specific (Figures 1 and 2, Supplementary Figure S1), which we could recapitulate by mutating K788 within ACE2 (Figures 3 and 4).

Aside from MDM2, another proto-oncogene ubiquitin ligase (SKP2) has also been identified to modulate ACE2 levels. SKP2 is activated by CSE (cigarette smoke extract) and has been reported to downregulate ACE2 [45]. MDM2 appears to be more expressed in lung cells as compared to Skp2 (https://www.proteomicsatlas.org, accessed on 10 July 2023), even though the effect of SKP2 on ACE2 levels in lung cells was significant [45]. The coexistence of multiple ubiquitin ligases, such as MDM2 and SKP2, that can exert regulatory control over ACE2, highlights the intricate nature of ACE2 modulation. It is plausible that different ubiquitin ligases operate on ACE2 in a cell type-specific manner, depending on their expression level or specific triggers.

Our in vitro results on the function of MDM2 for SARS-CoV-2 infection could help to understand risk factors that affect disease severity. The lethality of COVID-19 can be correlated with risk factors such as pulmonary arterial hypertension and diabetes [46,47].

Recently, it has been shown that MDM2 can mediate the ubiquitination of ACE2 at position K788 and that MDM2 regulation is disrupted in patients suffering from pulmonary arterial hypertension [39]. Collectively, these data highlight the possibility that dysregulated MDM2 activity could be a risk factor for SARS-CoV-2 infection and COVID-19 pathogenicity.

MDM2 is also significantly downregulated in patients with common diabetic nephropathy [47]. It is known that SARS-CoV-2 infects the kidney, which can lead to severe kidney damage with a high mortality rate [48], and that patients suffering from diabetes are more likely to develop severe forms of COVID-19 [48,49]. Combining the latter reports with our results, one could speculate that the downregulation of MDM2 could lead to an upregulation of ACE2, subsequently leading to a higher SARS-CoV infection rate, higher tissue damage in the kidney, and an overall poor prognosis in cases of severe COVID-19.

As ACE2 is the central receptor for SARS-CoV-2 entry, insights gained on its regulation could be a promising drug development target for treating COVID-19. Multiple small molecules have been developed to target proteasomal degradation mediated by MDM2 [50]. Interestingly, inhibition of the proteasome has been shown to stabilize ACE2, implying a potential increase in SARS-CoV-2 uptake [39]. However, despite this effect, the overall impact of proteasome inhibition on SARS-CoV-2 replication has been demonstrated to be antiviral [16,40]. This paradox may arise from the necessity of proteasome activities to promote virus replication or from the induction of endoplasmic reticulum (ER) stress, which subsequently suppresses the biosynthesis of SARS-CoV-2 proteins [40,51]. Interestingly, a proteomics study has identified over 98 ubiquitin ligases and 29 deubiquitinating enzymes that interact with SARS-CoV-2 proteins [52], underlining the relevance of this post-translational modification not just for the host but also viral proteins. Understanding this interface might lead to the development of novel therapeutic approaches, such as targeting deubiquitinating enzymes to suppress SARS-CoV-2 replication [53]. These combined findings underline the need for further research to comprehend the intricate network of the ubiquitination and deubiquitination processes. This is particularly relevant for ACE2 and its multifaceted implications in diverse physiological and pathological contexts, as it is the central receptor for SARS-CoV-2 infection.

5. Conclusions

We observed a SARS-CoV-2-specific pro-viral effect by MDM2 depletion, which is not evident for other viruses tested, including VSV, YFV, RVFV, and HSV-1. Proteomics analysis highlighted that the SARS-CoV-2 entry receptor ACE2 was significantly upregulated in MDM2 knockout cells, which explains the enhanced SARS-CoV-2 infectivity. The effect of the MDM2 ubiquitination site on ACE2 was further investigated by mutating the ubiquitination site K788 of the ACE2 receptor. Our results highlighted the increased protein levels of ACE2-K788R, leading to more SARS-CoV-2 infections due to a higher viral uptake. Moreover, our data suggest that the expression levels of ACE2 affect SARS-CoV-2 infectivity and that the regulation of ACE2 by cellular proteins or by potential drug treatment could be a valuable means to regulate SARS-CoV-2 infectivity.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/v15081763/s1>, Table S1: Designed gRNAs for knockout screens; Table S2: Proteomic analysis of MDM2 knockout and NTC; Table S3: Upstream regulator analysis of the MDM2 knockout and NTC; Figure S1: Investigating the effect of MDM2 knockout phenotype on reporter Viruses; Figure S2: Comparison of virus growth in knockout cell lines; Figure S3: MDM2 knockout increases SARS-CoV2-RNA abundance and ACE2 protein levels in knockout cell lines; Figure S4: Comparison of SARS-CoV-2 infection of cells expressing ACE2 or ACE2-K788R; Figure S5: ACE2-K788R increases VSV-S but not VSV-infection.

Author Contributions: Experiments were designed by Q.E., K.K., V.G. and A.P. The experiments were conducted by Q.E., V.G., J.M., L.O., K.A., S.M., A.H. and J.H. The data was analyzed by Q.E., K.A. and S.H. The manuscript was written by Q.E. and A.P. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The files of the proteomic datasets and Maxquant output have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>, accessed on 1 May 2023) via the PRIDE partner repository under the project ID: PXD040891.

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Conflicts of Interest: The authors declare no conflict of interest.

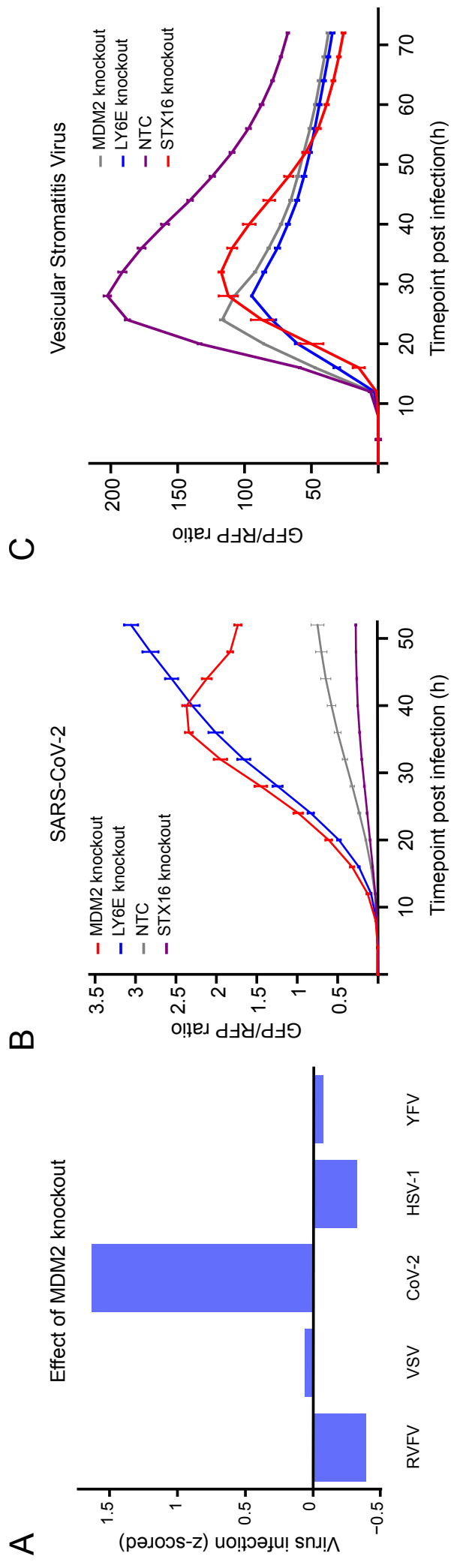
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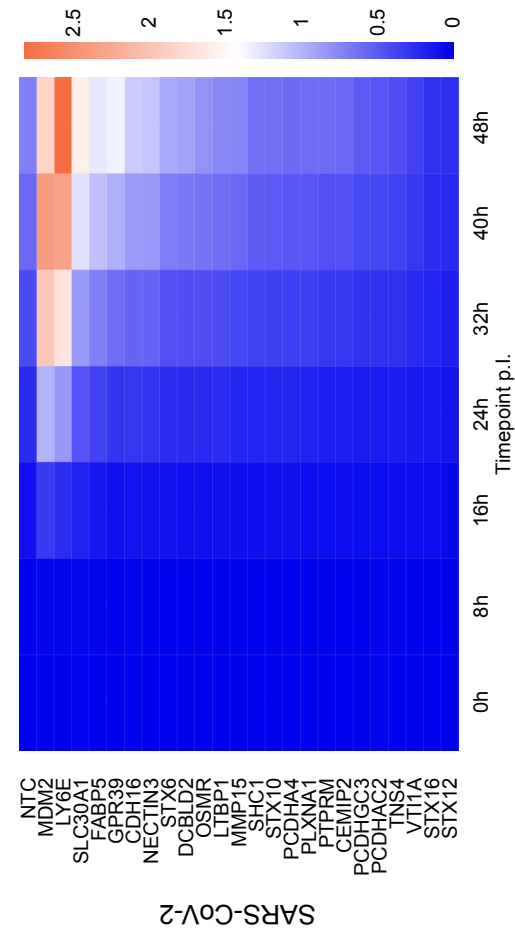
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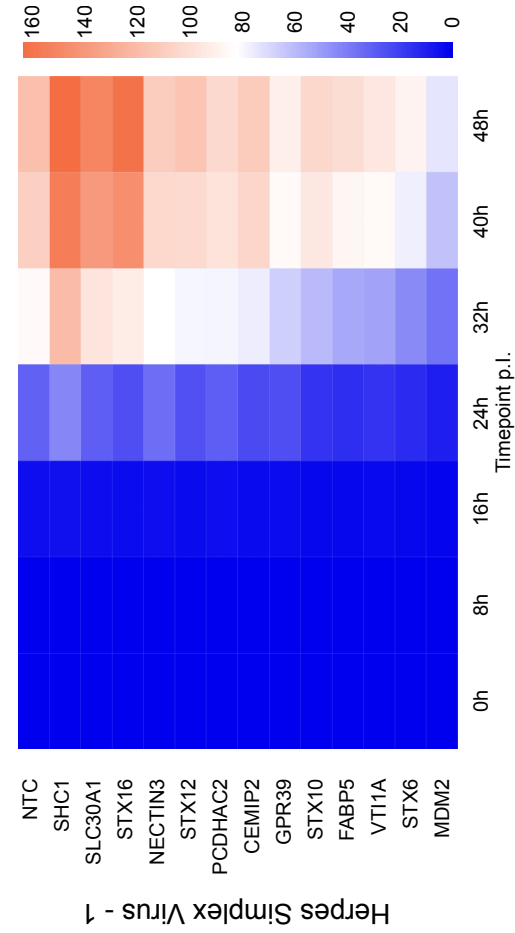


Supplementary Figure S1. Comparison of MDM2 knockout phenotype. (A) Related to Figure 1 - graph shows the z-score of the mean of the maximal infection value (integrated intensity GFP/mRFP ratio) for SARS-CoV-2, VSV, YFV, HSV-1, and RVFV of MDM2 knockout cells. (B-C) The indicated knockout cells or controls (NTC) were infected with SARS-CoV-2-GFP or VSV-GFP reporter viruses, and infection kinetics were measured. The virus signal (GFP integrated intensity) was normalized to the cell signal (mRFP integrated intensity) (mean $\pm$ SD, $n = 3$).

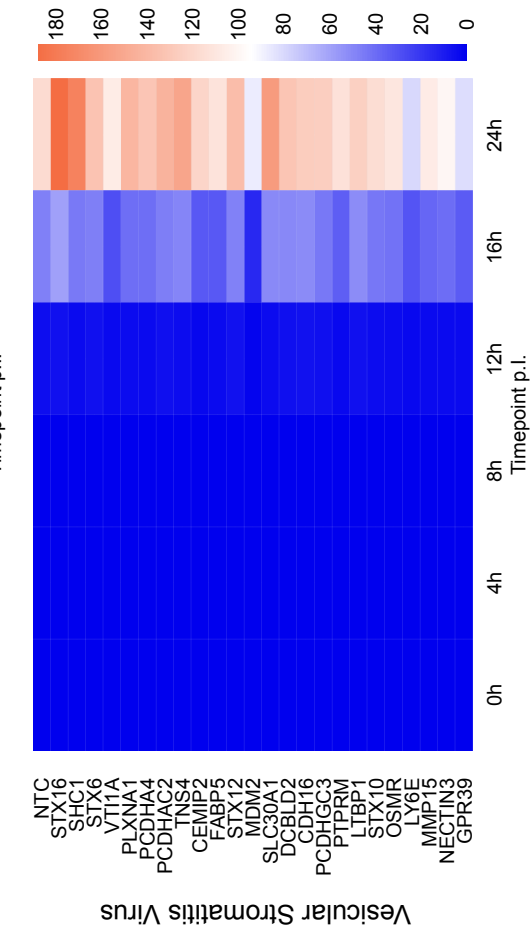
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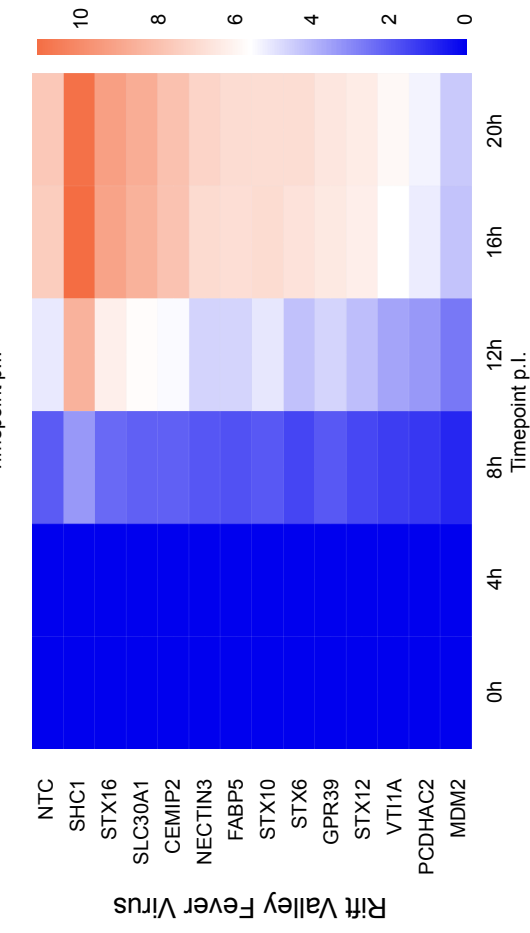
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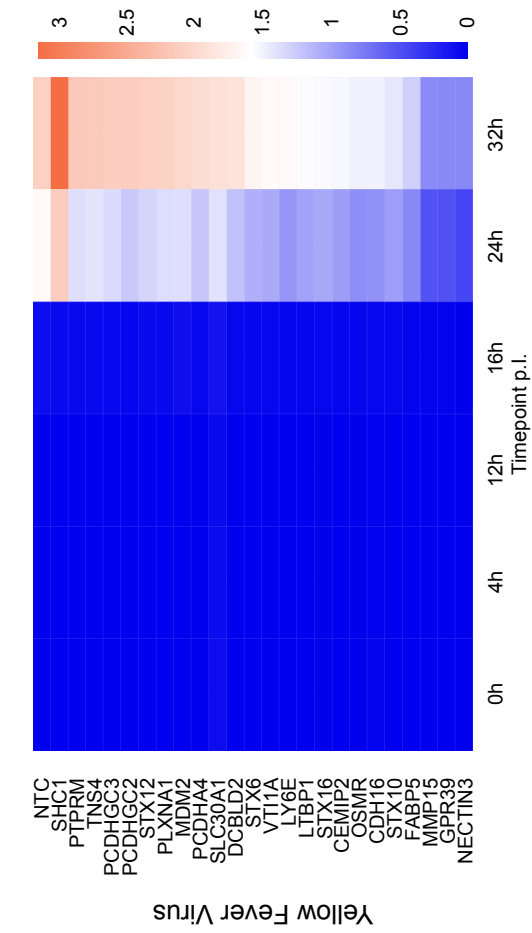
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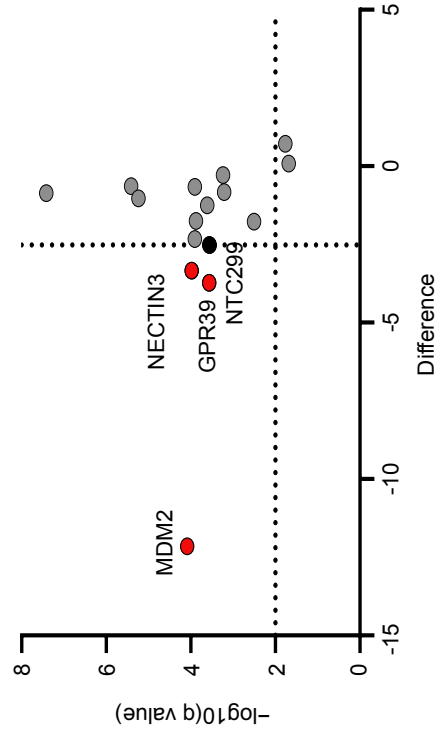


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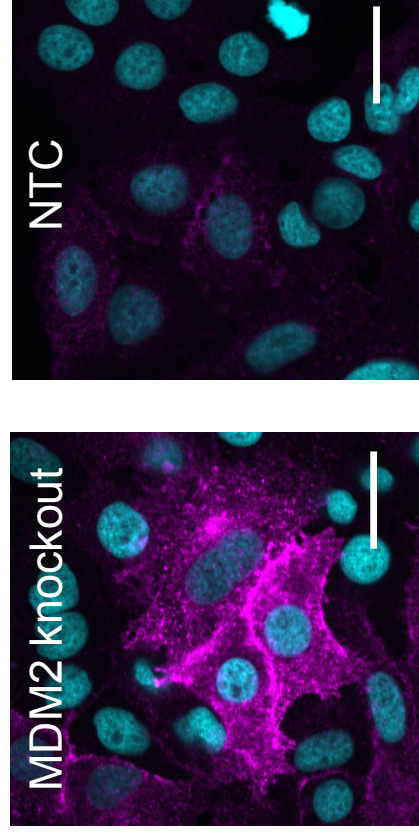


Supplementary Figure S2. Comparative virus growth in candidate knockout cell lines. Related to Figure 1 - The indicated A549-H2B-mRFP-ACE2 knockout cells were infected with (A) SARS-CoV-2-GFP (MOI 3), (B) Vesicular Stomatitis Virus (MOI 0.01), (C) Yellow Fever Virus (MOI 0.1), (D) Herpes Simplex Virus 1 (MOI 0.1), (E) Rift Valley Fever Virus (MOI 0.1). Heatmaps show the average signal (GFP/RFP) in relation to time post-infection.

A

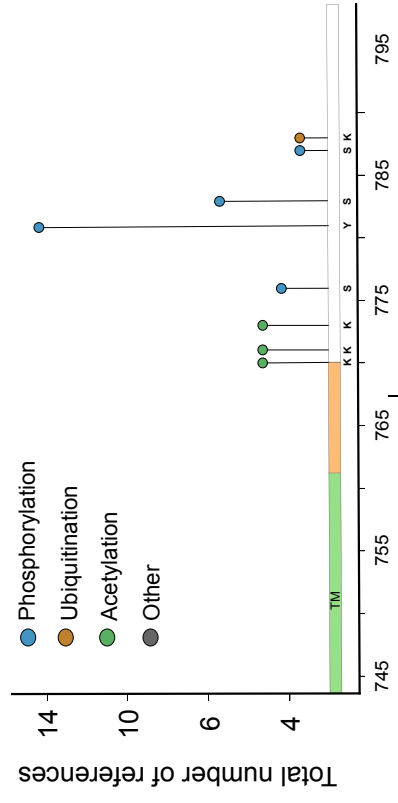


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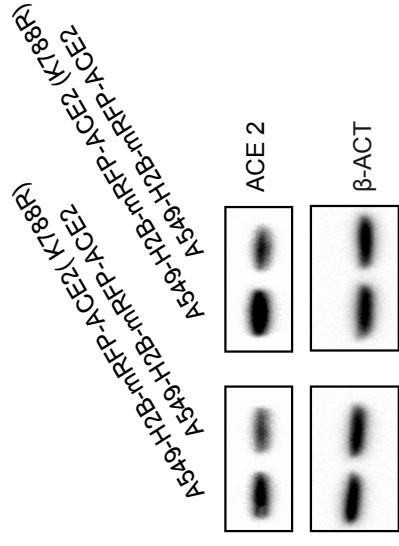


Supplementary Figure S3. MDM2 knockout increases SARS-CoV-2 RNA abundance in selected knockout cells and ACE2 protein levels (A) Difference in $-\Delta\Delta\text{Ct SARS-CoV-2}$ N RNA levels between 12 h to 24 h and corresponding q-value. The knockout cells that allowed a higher increase in transcript level as compared to the NTC are depicted in red (mean $\pm$ SD, $n = 3$). (B) Representative confocal images of A549-H2B-mRFP-ACE2 MDM2 knockout (MDM2 KO) or control cells (NTC). ACE2 (magenta), nuclei (cyan). Scale bar equals 25 μm .

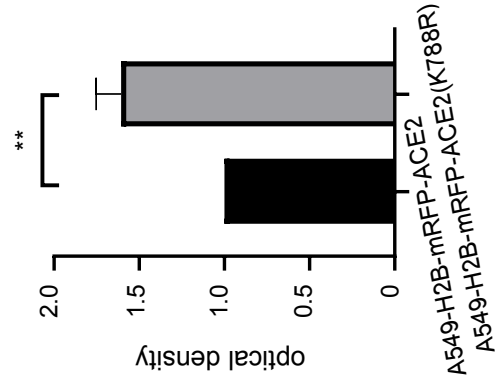
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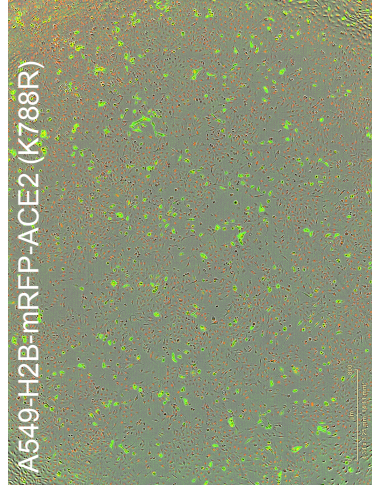
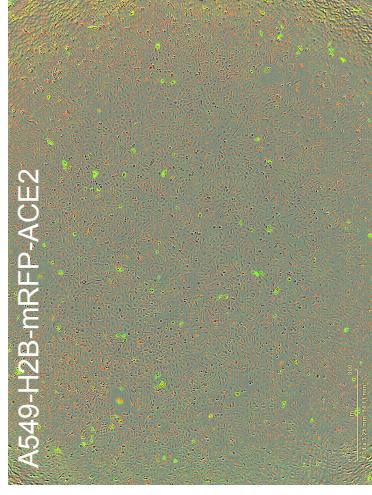
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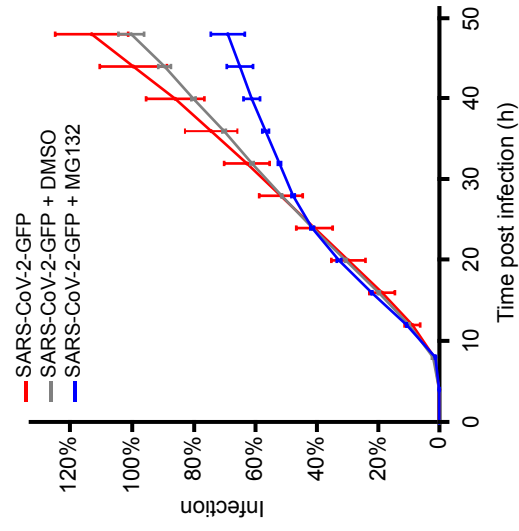
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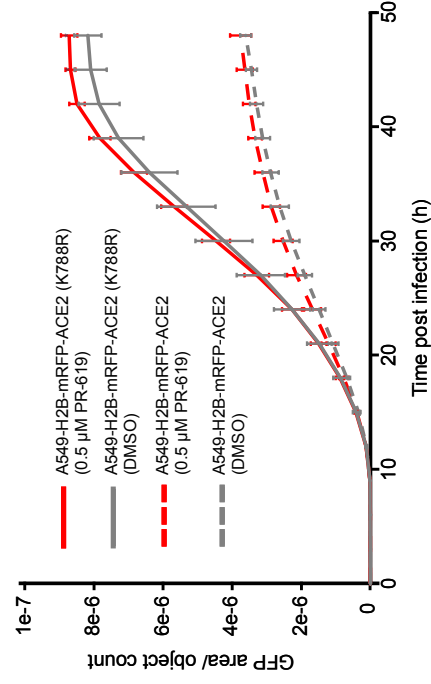
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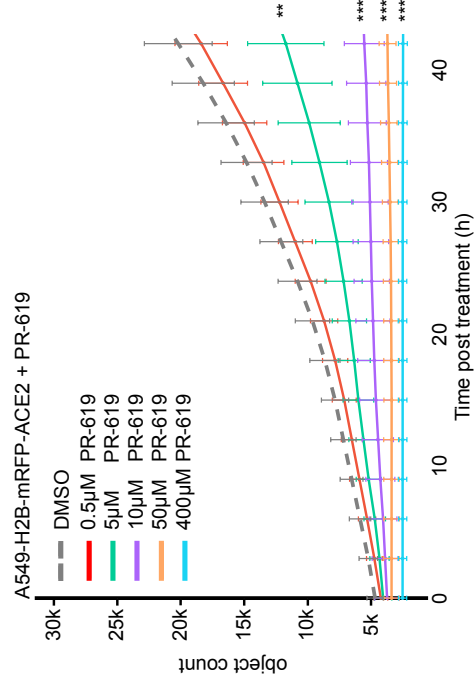
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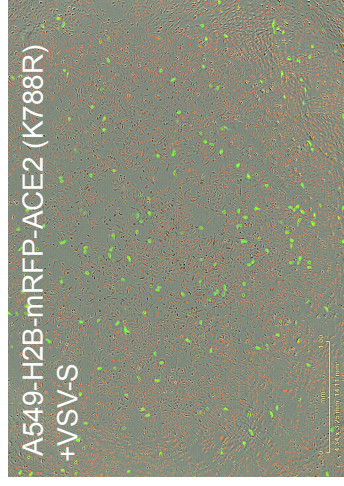
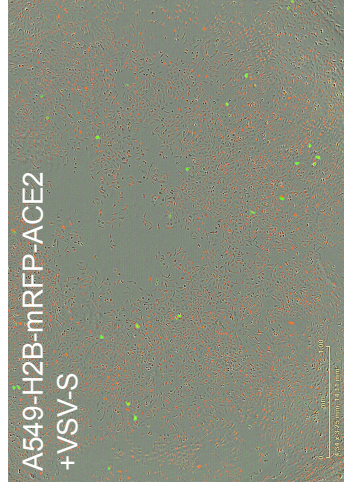


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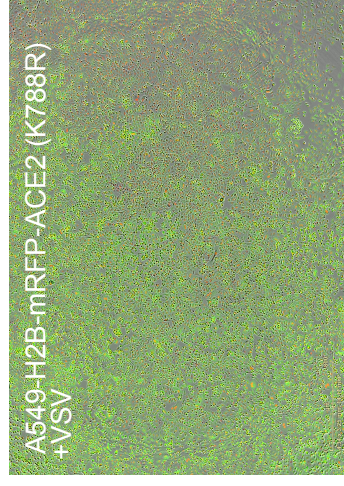
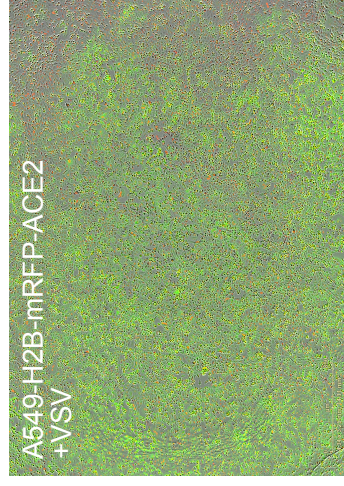


Supplementary Figure 4. SARS-CoV-2 infection of cells expressing ACE2 or ACE2-K788R. (A) ACE2 structure and designated post-translational modifications of the cytosolic domain includes only one ubiquitination site (adapted from PhosphositePlus, 2022). Lysine (K), Serine (S), Tyrosine (Y), Transmembrane (TM). (B) Western Blot showing ACE2 and β -actin (β -ACT) levels of A549-H2B-mRFP-ACE2 and A549-H2B-mRFP-ACE2(K788R) cells. (C) Western Blot quantification of ACE2 and ACE2(K788R) expressed in A549-H2B-mRFP-ACE2 and A549-H2B-mRFP-ACE2(K788R) cells shown in B and Figure 4A. ACE2 levels were normalized to the corresponding β -actin (β -ACT). The bar graph depicts the ACE2(K788R) levels (relative optical density) normalized to the ACE2. The relative optical density was determined with ImageJ, and the calculation of significance was performed with a two-sample t-test with $p < 0.01$ (**) ($n = 3$, $\pm$ SD). (D) Epifluorescence images of A549-H2B-mRFP-ACE2 and A549-H2B-mRFP-ACE2(K788R) (24 h post-infection) cells infected with SARS-CoV-2-GFP (MOI 3). The images were acquired by a live-cell imaging platform. (E) MG132 inhibits SARS-CoV-2 infection. A549-H2B-mRFP-ACE2 cells were pre-treated with MG132 ($1\mu\text{M}$, 4h), DMSO control or no treatment and infected with SARS-CoV-2-GFP reporter virus (MOI:3). Virus replication was measured for 48 h post-infection in a live-cell imaging platform (Incucyte S3) and analyzed by normalizing the virus signal (GFP area) to confluency (phase area). The signal of DMSO control was used to show the effect of the drug on infection (%). (F) A549-H2B-mRFP-ACE2 and A549-H2B-mRFP-ACE2(K788R) cells were pre-treated for 6 h with $0.5\mu\text{M}$ PR-619. The cells were infected with SARS-CoV-2-GFP (MOI:3) and virus replication was measured over 48h. Virus signal (GFP) was normalized to cell number (RFP). (G) Cell viability screening of PR-619. A549-H2B-mRFP-ACE2 cells were pre-treated 6h with PR-619 at the indicated concentrations. The cell number (RFP counts) was measured in a live-cell imaging platform (Incucyte S3). For statistical analysis a t-test between DMSO control (dashed line) and compound of interest (solid line) was performed at 24h and 48h post-infection with $p < 0.01$ (**), 0.001 (***).

A



B



Supplementary Figure S5. ACE2-K788R increases VSV-S but not VSV infection. A549-H2B-mRFP-ACE2 and A549-H2B-mRFP-ACE2(K788R) cells were infected with either (A) VSV-S (MOI 3) or (B) VSV (MOI 0.01). Representative epifluorescence images at 12 h post-infection are shown. The images were acquired by a live-cell imaging platform.

Supplementary Table 1

Gene Symbol	Sample_ID	crRNA
MMP15	MMP15_1	CUUCUUUGCAGAACUGGCUG
	MMP15_2	AUGGUGGACAU AUGGCGGCU
	MMP15_3	CAUGGUGGACAU AUGGCGGC
SLC30A1	SLC30A1_1	GCCGCAGCCAUGGGGUGUUG
	SLC30A1_2	GGGUGUUGGGGUCGGAACCG
	SLC30A1_3	GUUGGGGUCGGAACCGGGGC
OSMR	OSMR_1	UUAAGUGAUACAGGAGUCAA
	OSMR_2	CCUGAUGAU AAGGAAGGUUG
	OSMR_3	UCAAUUCCUGAUGAU AAGGA
SHC1	SHC1_1	GAGCUGAGCGGGCGGCUACA
	SHC1_2	CCAGGAUAGAGCUGAGCGGG
	SHC1_3	CGCCCGCUCAGCUCUAUCCU
GPR39	GPR39_1	GGGAGCAGUCACUGCCCGGG
	GPR39_2	AUGACUGUGAUCAAUGAUUU
	GPR39_3	AGGUGGCCACCUCAAACUCG
PTPRM	PTPRM_1	UCAUGCUGGUGAAUGCCUCU
	PTPRM_2	AUGCCUCUGGGAGACCUGAG
	PTPRM_3	AAGUUGGGGUAAGAGCAGGU
LTBP1	LTBP1_1	CACCAUUCAUACAUGGAAGA
	LTBP1_2	CAUCUCCAUGUAUGAAUGG
	LTBP1_3	AUGGUGGCCAGUGCAGUUCA
DCBLD2	DCBLD2_1	GGAUGUGGACACACUGUACU
	DCBLD2_2	AUGUAAGGGUUCCACUCUCA
	DCBLD2_3	GAUGUAAGGGUUCCACUCUC
MDM2	MDM2_1	UUGUUUUAGGAUCUUGAUGC
	MDM2_2	GCUGGUGUAAGUGAACAUUC
	MDM2_3	GAUUAUAGCCUUAUGUAAGA
CDH16	CDH16_1	CAGUUGCCGCUGCCCCGUGA
	CDH16_2	AGUUGCCGCUGCCCCGUGAG
	CDH16_3	AGUCCCCUGACAGCACGAUC
NECTIN3	NECTIN3_1	CUUAGCUGGACCAAUUAUUG
	NECTIN3_2	CCCAUACUGCUGUGACAUG
	NECTIN3_3	AGCCACAUGUCACAGCAGUA
PCDHGC3	PCDHGC3_1	CAUCGCCAUUUUGGGAGCUG
	PCDHGC3_2	UCAUCGCCAUUUUGGGAGCU
	PCDHGC3_3	UCCCAAAUGGCGAUGACAC
PCDHA4	PCDHA4_1	ACUAAAGAUGGAGUUUUCU
	PCDHA4_2	GUUUUCCUGGGGAAGCGGCC
	PCDHA4_3	AAGUAAGAGCAGCAGACGCC
PCDHAC2	PCDHAC2_1	GCGGCCAGUGGCUAUGGAGC
	PCDHAC2_2	GCAGGCGGGCACCAGACCUG
	PCDHAC2_3	CGUGGAUGCUCUGUCGCCGC
PLXNA1	PLXNA1_1	UUCACACAGUUCAGGGUCUG
	PLXNA1_2	CUCAGGGUUCACACAGUUCA
	PLXNA1_3	UGCACCUGAGGUGCCGGUGA
STX10	STX10_1	CCGCAGCGAGGUGCAGAAGG
	STX10_2	AAGGCGGUGAACACGGCCCCG
	STX10_3	AGGCGGUGAACACGGCCCCG

STX12	STX12_1	UGAAGUCUCUGCUGGGCGCUA
	STX12_2	ACGAGAUCCAGCUCUUGCUC
	STX12_3	AGUAUUGCCAGAGCAAGAGC
STX16	STX16_1	UGCCUGACACCAGUGCCAUA
	STX16_2	GACCGUAUGGCACUGGUGUC
	STX16_3	UUAGAUCCAGAAGCAGCGAU
STX6	STX6_1	GGCAGCCAGAACUGGAGCAC
	STX6_2	AACAGAUAAAUAUGGGCGUC
	STX6_3	AGCCAAUUCUCAUUUCAUUG
VTI1A	VTI1A_1	CCUCUUCUAGCUUGAACAGA
	VTI1A_2	UACAUCCCUCGACUUUGGGG
	VTI1A_3	CUGUACAUCCCUCGACUUUG
FABP5	FABP5_1	GACUUAACAUUCUACAGGAG
	FABP5_2	ACUUAACAUUCUACAGGAGU
	FABP5_3	AGCUGAUGGCAGAAAAACUC
CEMIP2 (TMEM2)	CEMIP2_1	CAUGUAUGCCACUGAUUCCA
	CEMIP2_2	AUGUAUGCCACUGAUUCCAG
	CEMIP2_3	GAAAGCAGGGGAGUGUCCCC
TNS4	TNS4_1	CGUUCUCCUGAGGCUCUGUC
	TNS4_2	AGGUGGCAUACGUUCUCCUG
	TNS4_3	GAACGUAUGCCACCUCUUUG
LY6E	LY6E_1	GCACAUCAAGCAGCUGGCUG
	LY6E_2	GGAGAAGCACAUCAAGCAGC
	LY6E_3	AUUGCUCUUCUGGUUCAAGC
NTC	NTC_1	ACGGAGGCUAAGCGUCGCAA
	NTC_2	CGCUUCCGCGGCCCGUUCAA
	NTC_3	AUCGUUCCGCUUACGGCG

Supplementary Table 2 (compressed)

Significant	P-value	Difference	Gene names
+	0,728362859	-0,169908524	SERPINB6
	0,814789722	-0,864319801	CEACAM5
	1,873723305	-1,018244267	hCG_1994888;DNAJC25;GNG10
	0,031689581	-0,019917965	hCG_39893;GRAMD1B
	1,362404437	0,092834949	HDLBP
	0,085279874	-0,016577244	EHD1
	0,81080322	-0,761882782	ELOF1
+	3,682598196	-0,346720695	PSMA2
	1,687059375	-0,470137119	TCEB3
	1,429048397	-0,216189861	BAG6;BAT3
	0,930420847	0,644726753	NDUFAF6
+	2,410504651	1,700295925	ANXA8;ANXA8L1;ANXA8L2
	0,1672107	-0,174683094	USP36
	0,299989506	0,179672718	KIAA1468
	0,35473545	-0,027374744	TXNRD1
	0,833276177	0,340192318	PDE4D
	0,377727721	0,130441189	HMOX2
	2,585264906	0,38597393	C11orf54
	0,264834262	0,096916676	UCHL3
	0,709161027	-0,972674847	NDUFS7
	0,072671077	-0,076232433	PTPRJ
	0,431078562	-0,026863098	KHSRP
	0,366047286	0,591464043	ALG13
	1,004692172	-0,17706728	PYCR2
	0,79947847	-0,16983223	UHRF1
	0,665045123	0,310656071	ERGIC2
	0,117801726	-0,464400291	CD9
	0,187398755	-0,097091198	VPS45
	0,861740059	-0,211658478	CTNBL1
	2,713288021	0,387627602	SPCS2
	0,520533978	-0,108691692	HNRNPDL
	1,712245163	-1,551377296	PEG10
	0,455820234	0,044096947	SLIRP
	2,318160787	0,188584805	EIF5B
	0,926753989	-0,435873508	IKBK
	0,183213125	0,058128834	MYCBP
	0,579649917	-0,224949837	MTHFD1L
	1,152989515	-0,137874603	CLTC
	0,529782563	0,08399868	EEF1A1;EEF1A1P5
	0,908339072	-0,608506203	NME4
	2,298373258	-0,362377167	POLR2E
	0,025997361	-0,004858017	SH3GLB1
	0,208018608	-0,509563446	EPT1
	0,09629897	-0,285163879	ABCB7
	1,234553966	-0,107539654	POLD2
	0,306740219	0,14711237	RBM15
	0,230348437	0,089078426	MAZ

	0,369937799	-0,330269814 THOC1
	0,641520097	-0,195741177 TPM3
	0,014333362	0,019113064 BCL10
	0,506623541	-0,103181362 TARDBP;TDP43
	0,039655345	-0,010179996 NDUFA10
	1,040522215	-0,072654724 RPL17;RPL17-C18orf32
	0,741741868	0,635159016 BCAM
	0,09728429	-0,01513052 ESYT2
	0,481250546	-0,192158699 HEATR6
	0,454482424	-0,150397778 VTA1
	0,961098456	0,172148228 AP2M1
	0,23707107	-0,048924446 UBE2D3;UBE2D2
	0,936005944	-1,300439835 SLC6A6
	1,292774073	-0,415577412 TAP2
+	2,079313864	-1,694324493 SEZ6L2
	0,775732166	-0,295161247 DHX29
	0,986696201	-2,15927887 SRP19
	1,691087748	0,231618404 PTGES3
	1,080788994	1,034701824 C16orf13
	0,172819844	-0,186377525 TSPAN6
	0,556299231	0,130285263 RAVR1
	0,361574238	-0,686845303 DHPS
	0,908786569	-0,172419071 EIF3H
	0,903015895	-0,169429779 IDH3B
	0,276948644	0,080099106 SAMD4B
	0,915782543	-0,155572414 BOLA2
	0,28650677	-0,574935913 KIF3A
	0,43982095	-0,127564907 SBDS
	0,503534053	0,49578619 ZFAND2B
	0,619099328	-0,218758106 RBM26
	1,01770088	-0,109050274 TJP1
	1,06205854	-0,114127159 OXA1L
	1,180270857	-0,229439259 SLC4A1AP
	0,779840811	0,17679739 YTHDF3
	0,03342819	0,007119179 SNX12
	0,326040211	0,41561985 SENP3-EIF4A1;SENP3
	0,101662918	-0,02485466 ACOT2;ACOT1
	0,351723934	0,11387682 OTUD6B
	0,605540372	-0,072073936 NOMO1
	0,317340956	0,078505516 SEP08
	0,112449095	0,049902916 ARFIP2
	0,145096443	0,353016853 CADM1
	1,825463147	0,233174324 PSME2
	0,120812156	0,105229855 KIAA1524
	0,000135772	-5,63E-05 KIAA1033
+	1,646748257	-3,444484711 RBFOX1;RBFOX2;RBFOX3
	0,087599258	0,166816235 MRPL45
+	2,530012594	-0,668288231 HECTD1
	1,904489941	-0,19197607 PSMC6
+	2,541946775	-1,491636276 DOCK1

	0,651064748	-1,60357666 RNF114
	0,607289585	-0,142037868 ADSL
	0,089639226	0,041647911 FAM21C;FAM21A
	0,004712964	0,000807762 C21orf33
	1,208654188	0,790381432 TXNRD2
	0,323333575	0,086538315 RTFDC1
	1,13115413	0,242204666 PYCRL
	0,622086226	0,16182375 WDR18
	0,264650074	0,304463863 SCLY
	0,281732709	-0,069200039 MYEF2
	0,586790797	0,151256084 MBNL1
+	2,229540449	-0,61059761 RAP1GAP
	0,105935377	0,292191982 NSA2
	3,044301524	-0,283095837 VDAC2
	0,270628418	0,863058567 CDK5RAP2
	0,091878951	-0,060711861 CHD1L
	0,308136758	-0,06009388 SNX6
	0,898108925	0,105578423 PCMT1
	0,158337105	0,044305325 ABLIM1
	0,976892441	-0,12801981 NOLC1
	1,105073176	-0,229252815 SNRPC
	0,148003588	-0,300843239 ABI1
+	3,252433503	-1,425474167 MARK2
	0,213067257	0,113321781 APTX
	2,253949122	-0,294890881 DDRGK1
+	4,315906698	1,01567173 GSN
	0,870959737	-0,527552605 EIF4G3
	0,754394476	0,300204754 PVR
	0,005534146	-0,001937866 EXOC7
	0,079597531	0,098640919 LDAH;C2orf43
	1,326403971	0,151841164 EXOC5
+	3,448755529	-0,367344379 TOR1AIP1
	0,285901344	0,372888565 ARHGAP5
	0,521691311	-0,062170982 AKR1C3
	0,279918029	-0,098852634 SLC4A7
	1,260860033	0,245349884 TAPBP
	0,097041052	-0,055044174 PITPNB
	1,272755964	-0,263389111 SCAF8
	2,297554108	-0,4478755 WDR36
	2,906287083	-0,257877827 RNF213
	0,190239599	-0,041009903 ILK
	0,701213178	-0,15132618 DBI
	0,656340091	0,13761282 NCEH1
	0,820519089	-1,698999405 CUL2
	0,117105838	-0,640005112 TTN
	0,793387357	-0,236335754 TMED7;TMED7-TICAM2
	1,444796139	-1,085977077 ZFP91-CNTF;ZFP91
	0,362628527	-0,152104378 CBWD6;CBWD3;CBWD5;CBWD2;CBWD1;CBWD7
	0,839340699	-0,930449963 PPAN;PPAN-P2RY11
	1,594707486	-0,174179554 LUC7L2

+	0,162743254	0,233884811 ST20-MTHFS;MTHFS
	3,685267511	0,548673153 SULT1A4;SULT1A3
	0,661888689	0,138836861 CORO7-PAM16;CORO7
	1,080530194	-0,160184383 NAA15
	0,190193759	-0,042623043 SRSF7
	0,543248315	-1,149913311 POLR2J3;POLR2J;POLR2J2
	1,248490905	-0,335744858 COMMD4
	0,237793045	-0,254471779 TRPV1;SHPK
	0,290666937	-0,132434368 RPS15
	0,37872994	-0,942509651 DLGAP4
	0,51782443	0,137296677 TPT1
	0,566590672	-0,898066998 PWP2
	0,025463867	0,016946316 GSTZ1
	1,558936263	-1,332937717 TM9SF4
	0,98957176	0,414097786 ZNF574
	3,026693324	0,871008396 MGLL
+	0,712537207	0,136023521 DLG1
	1,373895437	-0,658809662 NELFA
	0,13745045	0,026389599 NBN
	1,145625186	-0,102095127 RPSA
+	0,369740814	0,299670696 PEX1
	2,388486736	-1,139410973 HTRA2
	0,204342231	-0,428807735 ARL6IP4
	2,992722746	-0,271304607 DDX46
	1,414852322	0,308568478 ECI2
	0,288310608	-0,110361099 HLTF
	1,636491032	-0,825902462 SCOC
	0,296168715	0,249443531 EHMT1
	1,614411894	-0,180742264 CHD4
	2,000716931	0,124812603 CAPNS1
	0,765846723	-0,207889557 DDOST
	0,528134101	-0,134316921 POLR3E
	0,242432024	0,042902946 EPB41L1
	0,111215953	-0,053189754 CDK11A;CDK11B
	0,436167598	0,333742619 MEF2A;MEF2C;MEF2D
	0,296251875	-0,163513184 MAP4K4
	2,150688671	-0,173699856 DDX3X;DDX3Y
	5,001626497	-0,198185444 SPTAN1
	3,417033042	-0,296735764 TBL1XR1
	0,762092317	0,290791035 ARFGAP2
	0,67350278	0,094337463 DNM1
	1,789137294	-0,224144936 MYO18A
	0,952719196	0,264133453 HADH
	2,379991898	0,821681023 MECP2
	1,477159264	-0,991645336 FHL1
	0,347800778	0,691022873 UBE2A
	0,28529667	-0,325988293 STXBP1
	1,025516713	0,581327438 UBAP1
	0,009542336	-0,007425785 PHF6
	0,640432664	0,113881588 DIAPH1

+	2,775627695	-0,193385601 HSPA1B;HSPA1A
	1,243288995	1,54858923 C4B;C4A
	2,873403073	-0,478730202 NLRP2
	0,234292271	0,031170368 SCRIB
	1,13914052	0,231153011 HLA-A
	0,513013344	-0,132934093 ABR
	1,482247585	-0,146212101 PUF60
	1,252253475	-0,44915247 CIRH1A
	1,292134253	0,107256889 PON2
	0,350321459	-0,076809406
	0,161549064	-0,018565178 MATR3
	0,195114348	-0,296028614
	0,046068406	-0,068441868 SCARB1
	1,161854707	-0,762223721 CLASP2
	0,457772125	0,147920132 hCG_2043597
	0,204989256	-0,237968445 SCYL2
	1,244280544	-0,267426014 CD99L2
	0,678702533	-1,641050339 ROCK1
	0,0722867	-0,014292717 HIST1H2AJ;HIST1H2AH;
	0,655438505	-0,128014088 PLEKHG3
+	0,113055102	-0,127981186 PHF14
	0,307011959	0,080162048 MICU1
	3,208253375	-1,889185905 GATAD2B
	1,497367617	-0,265418053 PTBP1
	1,371715626	2,229851246 HLA-C
	0,097502061	0,085114002 DHX16
	1,189332691	-1,024199963 HLA-C;HLA-Cw
	0,768585171	1,36817503 PSMB8;PSM8
	0,432516352	-0,512089252 TAP1
	0,608455924	-0,169490337 LSM14A
+	1,050062285	-1,258779049 QIL1;C19orf70
	1,05054565	-0,38587141 ATP6AP2
	1,672970403	-1,713226318 FTO
	1,309662698	-0,173144341 ALDH7A1
	0,555642969	-0,049854755 RAB11FIP5
+	0,255277206	-0,256504059 ASAH1
	0,125106473	-0,026048183 PXN
	2,209244819	0,682600975 TJP2
	0,238418726	-0,158457279 DNMBP
	0,024123884	0,034423351 PCCA
+	0,934928756	0,096984386 KIAA1279
	1,737428946	-3,882367134 KIF21A
	0,901636237	-0,77736187 CLN3
	1,210780524	0,148189068 CTSD
	0,623799005	-0,199953556 UBE3A
	1,046546004	0,243782043 DPY19L1
	0,65075278	0,613327503 ACY1;ABHD14A-ACY1
	3,197361507	-0,335854053 DPYSL2
	0,458355219	-0,691742897 SFXN3
	1,226747892	-0,266545773 PIGT

	0,646791457	1,611577511
	0,631374073	0,555970669 STAT6
	0,564357552	0,808238029 ELP4
	0,972921262	-0,735767365 GOSR2
	0,062430921	-0,011232376 ME2
	0,21577148	-0,041196346 TCEA1
	1,123206276	0,527472019 GAMT
	0,229406831	0,135504246 SCARB2
	0,672743092	0,404600143 SMN1;SMN2
	0,094368166	0,085111141 HLA-A
	0,126283857	0,017486572 DDX17
+	3,736719711	-0,43251276 PHGDH
	1,645778134	0,49202013 RRM2
	2,077658718	-0,335278988 PPT1
	0,061565479	0,043353081 MON2
	0,549329478	-0,184351921 CAMK2G
	0,117643862	-0,204101563 HARS2
	0,305955136	-0,257339954 COMMD7
+	1,751472604	-1,397092819 BCAR1
	0,700751326	0,699309826 SPAST
	0,201257421	-0,20205307 EPS8
	1,155406326	0,797538757 SPG7
	0,093300897	0,082259655 CDK13
	0,273691285	-0,088255405 CTNNB1
	1,096061006	-0,926640987 ACBD6
	2,839769375	-0,368555069 CTCF
	0,084377909	-0,034286022 ATXN2
	0,654222165	-0,905222416 TSC2
+	3,403636041	-0,370729446 TBCE
	0,002795154	0,000862598 RDX
	0,868927655	0,188101292 COL4A3BP
	0,30420738	-0,491045952
+	1,813264145	-0,861447811
	0,249376998	-0,178666115 UCK2
	0,134044611	-0,042315483 RPL5
	0,255232572	0,254910946 PMS2
	0,267143496	-0,069053173 SUCLA2
	1,760159034	0,563446999 PPFIA1
	0,939260242	-0,194796085 RPS14
	0,451905685	-0,228932858 RPS24
	1,391888726	-0,284741879 RPS29
	0,606213595	-0,21337986 CUX1
	1,715595109	-0,251847267 SMARCE1
	1,261277365	-0,411441326 PFKM
	0,748153956	1,324109077 TPP1
	1,086308731	-0,095749855 HSD17B4
	1,912563566	-0,315261364 CSNK2A1;CSNK2A3
	0,358553737	-0,118914604 CASK
	0,22302115	-0,362576008 VPS33A
	0,06765054	-0,023247242 SEC23B

	0,396769844	-0,828278542 ARHGEF7
	0,78110679	-0,081682205 EEF1A2
	2,55709905	0,2221632 CD59
+	3,24582314	-0,37176466 KIF23
	0,484667691	-0,072369576 GPI
	1,236695939	-0,475982189 CLPB
	0,578465644	-0,614208698 NDUFA6
	0,811888881	-0,218902111 NCOR2
	1,34184709	0,994803905 PNPLA6
	1,275564742	0,256585598 FAM45A;FAM45B
	0,215821959	-0,034893036 MOGS
	0,069867896	0,134828091 CREG1
	1,455643826	-0,402237892 HMBS
	0,426792403	-0,76335907 C8orf33
	0,981730777	-0,236960411 SSR1
	0,707391442	-0,145592213 ECE1
	0,762949205	0,074572086 IDE
+	1,750610899	-1,749639034 POLA2
	1,650461596	-0,427359104 RPL29
	0,007395442	0,018862247 GSK3B
	2,055268781	-0,140314102 ABCD3
	0,955495512	-1,203084469 ATP5L
	2,080560699	0,306993008 THRAP3
	2,507254796	-0,226441383 MCM4
	0,095752639	-0,025681973 NHP2L1
	1,400040481	-0,474380016 HM13
	0,314189755	-0,445690632 GLA
	0,702411699	-0,139562607 PDCCD11
	0,053508472	-0,047473907 ENOSF1
	0,080464339	0,033769131 TACC3
+	1,870499019	-1,928652763 PARN
	1,297213959	0,223755836 LRBA
	0,527591639	-1,119845867 MYADM
	0,816788929	-0,184594631 DEK
	0,506221206	-0,668385506 SP3
	0,701161305	-0,85405159 RAD23A
	0,678432707	0,552836418 TYMP
	1,028339123	0,434545994 UROD
	1,790561218	-0,102396011 CLUH
	0,376906583	-0,529425144 TPP2
	0,454742103	-0,598351002 NFKB1
	0,03448483	-0,008070946 PEPD
	2,239979287	-0,206697941 NUP214
	0,438410668	0,111746788 GTF2I
	1,610097355	-0,141629219 FKBP2
	0,391483777	-0,098811626 FAM83H
	2,606063838	-0,376143456 SART3
	0,219092827	-0,184492588 WDR26
	0,728120745	-0,217412949 CUL1
	1,808300363	-0,267034531 CSNK1A1

	1,467064966	0,198958397 TSEN34
	0,95053789	-0,721313477 KCNAB2;KCNAB1
	0,473786979	-0,229084015 MSH3
	0,729728873	1,225908279 NIPBL
	0,938515538	0,112040997 SRSF6
	1,169169708	-0,806791782 NNT
	0,485482462	0,658258438 AMPD2
	0,751335132	-0,159946918 SDF4
	0,95412163	-0,106060028 TGOLN2
	0,826210339	-1,381551266 SPCS1
	0,202136629	-0,269656181
	2,14970286	-0,433907509 PRIM1
	0,929503358	-0,065615177 LDHB
	0,083883146	-0,062286377 SACM1L
	0,197923643	0,036836147 ALDH3A2
	0,69820319	-0,37964344 ACP2
	0,57600747	-0,367671013 PRIM2
	0,601858777	-0,249720097 ARSE
	0,063258263	-0,185395718 BRI3BP
+	3,400227521	-0,368741512 JAK1
	0,623374602	-0,681977272 EED
	0,086124761	-0,01325655 NELFB
	1,468481189	-0,351278305 PCM1
	1,624390475	-0,233304501 MDH1
	0,691235822	-0,461133957 MLH1
+	3,252748957	1,080066681 WFS1
	0,057234357	-0,005176067 CUL4B
	0,41013836	-0,110462189 BCAP29
+	3,616631647	-0,338318825 CTNND1
	0,691330837	-0,144882202 GBF1
	0,545301343	-1,05356741 CXXC1
+	3,736729309	-0,409246922 STAT1
	1,069040467	-0,960824966 TMEM30A
	0,058254695	-0,011480331 ADNP
	1,150774364	-0,156647205 USP7
	2,933767445	-0,31516552 LARS
	1,19061371	0,537624836 SVIL
+	3,089101686	-1,777090073 GNL3L
	1,137599489	-0,163698673 DNAJC7
	1,588190546	-0,205900192 GOLGA2
	0,17612891	-0,206816673 CEP41
	0,564818048	-1,002360344 IKBKAP
	0,544714476	0,405582428 CLCC1
	4,433828434	-0,244280338 AARS
	0,61049347	-0,94701767 CHMP1A
	0,259535045	0,037336826 MID1
	0,14158022	0,064642429 KIF2A
	0,227590111	-0,040563583 AIFM1
	0,088016762	0,027994633 GDAP1
+	2,220345545	-1,760680676 NEDD4L

	2,857475695	0,1095438 LMNA
	0,418316072	0,079741955 LRSAM1
	3,547254593	-0,18196106 PAFAH1B1
	0,250588639	0,100923538 TFG
	1,769941277	-0,170037746 GARS
	2,522048407	-0,124410152 POLR2A
	1,647534116	-0,474255562 TRIP12
	1,000301403	0,121375561 CAST
	0,152049707	-0,040589333 SPATA5
	0,741672106	-0,20131588 FANCI
	0,675493258	-0,102737904 INF2
+	1,462550378	1,914478302 MACROD1
	1,092493515	-0,188779831 NT5C2
	1,544775267	-0,236305714 GNE
	0,075827504	-0,02090168 HGS
	1,109468974	0,416215897 MARK3
+	4,679627857	1,168476582 ACE2
	1,928514843	-0,113292217 ITGB1
	1,15355597	-0,19246769 EIF3E
	1,762796544	-0,387464046 NACA
+	2,082177179	0,674748421 CTSC
	1,746125048	-0,214957714 HNRNPA3
	2,990371568	-0,238699913 IMPDH2
	1,853727739	-0,286218643 MTHFD2
	0,689996035	0,117161751 KPNA2
	0,178933292	-0,041579247 PABPN1
	0,35306819	0,045739174 SCFD1
	1,302827146	-0,212141991 NCL
	2,451683621	-0,103485107 HNRNPA1;HNRNPA1L2
+	1,924160511	-1,228615284 ATP6AP1
	0,759612996	-0,06636858 STAT3
	0,287198104	-0,083508015 ADAM9
	1,698174326	0,242724895 SPECC1
	0,758963015	-0,153012276 H2AFY2
	0,335358013	-0,033571243 DDX1
+	3,025296956	-0,44081068 LAS1L
	0,052406275	0,045151711 ZC3H13
	0,696782137	0,400927544 CTSA
	0,817963933	-0,218400002 YKT6
	0,463512343	-0,071554661 PABPC4
	2,491914877	-0,243359089 DCTN2
	0,538175417	0,089338779 NONO
	0,286705022	-0,04506731 NMT1
	1,052846822	0,115601063 PITRM1
	0,03438313	0,004817486 DNMT1
	0,067413803	0,026958942 ACBD5
	0,89502291	-0,267899513 RPL13A;RPL13a;RPL13AP3
	0,569139599	0,097151756 CTSB
	0,331587745	-0,331355572 PPIG
	0,041264599	-0,006518364 NEK9

	2,241003365	-0,250686169 GNAS
	0,358602283	0,057127476 NPM1
+	3,68728942	-0,411000252 PTGES2
	1,254431624	0,098656178 SGTA
	1,393352282	-0,309239864 ADCK4
	2,583484316	0,368982315 CTSB
	0,732661669	-0,108805656 KPNA4
	2,469387466	-0,159156799 EIF3I
	0,371795851	-0,085731983 RPL19
	2,20433494	-0,183359146 G3BP1
	0,693221521	-0,129811764 PRPF40A
	0,316825624	-0,091886044 MRPS22
	2,013089113	-0,336009026 RPS20
	0,214917406	-0,081088066 ARFGEF2
	0,145078878	0,060737133 VRK1
	0,642125688	0,206449986 AKAP8L
	0,490062989	-0,147351265 AATF
	0,071502255	-0,072423458 DST
	0,110533562	0,139400005 VLDLR
	0,346855372	0,37335062 SNX9
	0,918181096	-0,088743687 ACADM
+	3,23598632	-0,465562344 CTSZ
	0,07645271	-0,051609516 AAR2
	0,119442273	0,02169466 TCERG1
	0,484249119	-0,111551285 ANO6
	0,490966355	-0,76644659 SIRT5
	0,363581471	0,483232975 WAC
	0,259194438	0,091609001 CRAT
+	4,412551985	0,426944256 NAMPT
	1,206247679	-0,93223381 SMPD4
	0,566764163	-0,298011303 DDX20
	2,721410171	-0,191001892 SF1
	1,223584648	-0,168845654 OAS3
	3,099134686	0,173532486 P4HB
	0,2157179	0,040001869 ACSL3
	1,486081021	-0,243385315 BSG
	1,535314269	-0,105974674 HSP90B1
	0,659376329	-0,080189228 CANX
	0,685989929	-0,124922276 DLAT
	3,333656873	-0,225076675 EIF2AK2
	4,025419625	-0,246520042 SLC3A2
	1,454131087	-0,211437702 DNAJC10
	2,684774182	-0,302199841 VCP
	2,181446727	0,219437599 PPIB
	0,721955995	-0,100159645 DDB1
	2,201150985	-0,261662483 DCTN1;DKFZp686E0752
	0,931166167	-0,126811981 ADAR
	1,114323705	0,377319336
	0,130594573	-0,051434994 STAMBP
	2,386386728	-0,353191376 TBCD

	0,200209992	-0,246046543 GLE1
	5,102412337	-0,181296825 LRPPRC
	1,989886747	-0,428652287 TUBGCP2
	2,017678805	0,58031702 MLLT4
	1,19266187	0,155622482 MCCC2
	0,530049923	-0,610268593 MAN1B1
+	2,489415853	-1,910960197 UBA5
	1,73938518	-0,236660004 KIAA0368;ECM29
	0,686623099	-0,897145271
	0,213561316	-0,027544498 MAP4
	1,129181166	-0,252280712 AIP
	1,564403671	0,281826973 ITGA6
	0,001720655	-0,000279903 UBAP2
	0,087160202	-0,024734497 PDHX
	0,014600466	-0,018353462 MGA
	1,088713563	-0,234849453 RBM47
	0,41346237	-0,048337936 UBA6
+	3,584030124	-0,471444607 KIAA1598
	1,555175	-0,41066885 ILVBL
	0,370274	0,494355202 SH3PXD2B
	2,421660929	0,359832287 ACO2
	1,886691805	0,559928894 PRNP
	0,06858066	-0,156535625 RBM34
	0,974434659	-1,586150646 SLC39A7
	0,449708631	-0,545461178 MPG
	0,323146979	0,143489361 MRPL28
	0,30232862	-0,330667019 AURKA
	1,467851849	-0,693323612 VWA8
	0,693182317	-0,891070843 GTPBP10
	0,434798566	0,162642479 CNOT1
	0,155703901	-0,219007015 MAP1LC3B;MAP1LC3B2;MAP1LC3A
	1,462298138	-0,160878658 PGP
	0,541846802	-0,84332943 C5orf51
	0,480780167	0,14680481 RCCD1
	2,057923024	-0,215180397 HCFC1
	0,1004961	0,144906521 TSPAN14
	0,596996789	0,732271671 ZNF316
	1,186845974	-0,139750481 NUDT5
	0,803996808	1,377083302 WBP2
	0,557077234	1,403335094 DYNLT3
	0,825173578	-0,080542088 HN1L
	0,523524579	0,8990345 YIF1A
	0,868935554	3,103514671 TUBAL3
	0,867976164	-0,778095245 FBLL1
	0,556333968	-2,330364227 SMCHD1
	1,743302015	-0,411302567 METTL15
	0,225935382	0,62116909 CNIH4
	0,542409169	0,312271595 CRYZL1
	1,875104646	-0,323311806 CD81
	1,063658236	-1,872909546 POLA1

	0,665394286	-0,139703274 ENSA	
	1,005342704	-0,24972105 TP53BP1	
+	5,289399261	-0,501664639 CPNE1	
+	1,924306993	0,905072689 CRELD2	
	0,087187055	0,080426693 BTBD11	
	0,382475605	-0,157076836 POM121;POM121C	
	1,335929907	-0,31055975 FMR1	
	0,310016412	-0,060410023 RPL23A	
	1,650048142	-0,52020359 SNRPG;SNRPGP15	
	0,462986665	0,201251507 PPM1F	
+	4,918962307	-1,332740307 NAA20	
+	2,799853532	-1,293637276 DRG2	
	0,989222059	-0,314268589 SRRM1	
	0,613857368	-0,627840042 CSNK1E;CSNK1D	
	0,442180362	-0,082079887 EIF3L	
	0,200196308	0,094834328 EWSR1	
	0,939909858	0,087912083 ARCN1	
	1,162518921	-0,421777725 PIGK	
	0,49232968	-0,077850342 CAPZB	
+	1,699857521	4,632505417 CROCC	
	0,76937245	-0,138938904 DYNLRB1	
	0,275250184	-0,057535172 MRPL43	
	2,763150518	-0,328778267 PRPS1	
+	2,689146104	-1,270686626	Sep 06
	0,706631536	0,261687279 EXOSC1	
	0,6095445	-0,587409973 GUK1	
	1,205972525	-0,916720867 CD55	
	0,086114796	-0,015033722 RPS6KA3	
	0,276803492	-0,047083855 JMJD6	
	0,157523879	0,163084984 NDUFAF5	
	0,215250766	0,043515205 PTGES3L-AARSD1;AARSD1	
	0,264345496	-0,141183853 MORF4L1	
	0,813272137	-3,719054222 CENPH	
	0,979507115	0,538383961 CDIPT	
	1,572765343	-0,333661556 CNN2	
	0,880527354	-1,296373844 COX11	
	0,178527476	0,135731697 VWA9	
	1,885771882	-0,152205467 CS	
	0,687098549	-0,177160263 PWP1	
	1,072163676	-0,100843906 CARS	
	2,39072451	-0,245092392	
	0,009804587	-0,005229473 GOSR1	
	0,985441887	0,302306652 MGRN1	
	0,762085751	-0,38091898 SEC61A1	
	3,043866722	-0,244868279 STK24	
	0,027906743	-0,058334827 IXL;MED29	
	1,583969702	-0,182155609 FXR1	
	0,620306178	0,210732937 MAPK14	
	0,058886502	-0,132030487 STK26	
	0,831897149	-1,214161873 DERL1	

	0,719440687	0,069279194 RBPMS	
	1,334016566	2,069796562 LBH	
	0,442288089	-0,330500126 CFAP36	
+	3,398856016	-0,493841648 PES1	
	2,138340254	-0,222548962 RAN	
	1,748023615	-0,391517639	Sep 10
	0,908437958	0,20268631 HNRNPLL	
	0,807066445	-0,167591572 UQCRB	
	0,57310343	-0,078896523 RPL31	
	0,553529776	0,502157688 ST7	
	0,459759064	0,518147469 RTN3	
+	3,223974052	-0,371551037 DDX27	
	0,830622555	-0,942430973 CNOT3	
	0,208664484	-0,312703133 UBE4A	
	1,045691363	-0,221775532 ETF1	
	2,27267469	-0,41531086 KIFC3	
	0,048895807	0,012350559 SH3GLB2	
	0,812767237	-0,147422791 SMARCA1	
	0,337579491	0,206261158 INO80B;INO80B-WBP1	
	0,875528692	0,292453289 PTMA	
	1,961824474	0,518629074 GLS	
	0,149745675	-0,088646412 TAX1BP1	
	0,294215607	-0,059635639 SUMO1	
	0,867745958	-0,163665295 TCEB2	
	0,504204133	0,162559509 MRPL30	
	1,27579951	-0,11559248 SPATS2L	
	0,286477179	-0,147907734 IMP4	
	1,366175906	-0,380336761 SMARCD2	
	1,49851837	-0,196316242 WASH3P;WASH2P;WASH1;WASH4P	
	0,022325786	-0,008338451 ZC3HC1	
	0,413582371	-0,121644974 PFN2	
	0,667081138	-0,186853409 SRI	
	1,659496613	-0,876055241 RAPH1	
	1,058919204	-1,124765396 RNF181	
	0,103389081	-0,020139217 POLR2B	
+	2,162143916	-1,105959415 CMSS1	
	0,792418113	-0,541781425 B4GALT4	
	0,246881091	0,478962898 IMMT	
	0,547594084	-0,164908409 RPL37A	
	0,59898559	-0,36489296 IGF1R	
	0,43911029	0,346017838 NISCH	
+	1,879980381	0,885808945 NT5C3B	
	0,939804093	0,228692532 ALDH5A1	
	0,56476552	0,137913704 COX17	
	0,879334781	-0,179616451 RHEB	
	0,487366475	-0,092293739 NMD3	
	0,130672396	0,330261707 FXN	
+	2,626960121	0,600077152 PSPH	
	0,261062368	0,552043915 MRPS33	
	0,526529921	0,287036896 FAIM	

	0,915926247	-0,923169613 AAMP
	1,140965207	0,430987358 PCYT1A;PCYT1B
	1,818607044	-0,300918579 GPS1
	0,242274309	-0,06633234 CYCS
	1,159515888	-0,167196274 PELP1
	0,069580061	-0,008491039 APEH
	1,114726909	2,686302662 ATG4B
+	4,080970102	0,409926891 PSAP
	0,068274272	-0,012212276 MRPS34
	2,093423319	0,254612446 CAV1;CAV3
	0,184137122	-0,053814888 POLR2H
	1,740130093	0,342010975 OARD1
	1,395266865	-0,267741203 MTX2
+	3,784222522	-0,631594658 RPL24
	0,65072155	-1,601764679 CCDC58
	0,114210961	0,033353329 PPIH
	0,471684589	-0,179594517 C7orf50
	0,137772502	0,033662319 CHCHD3
	1,149887798	0,505205154 CGGBP1
	0,371888844	-0,124607563 DCUN1D1
	0,068705916	-0,033012867 L2HGDH
	3,977078367	-0,243285179 PSMC2
	0,227429268	0,051949024 SYPL1
	0,347811609	0,186826706 RPL22L1
	0,123090637	0,011034966 NRCAM
	0,656356184	-0,880946636 UBE2H
	2,464018715	-0,197467804 EIF4G2
	1,265697024	-0,994216442 PPP3R1
	0,096631128	-0,026947975 RPL32
	0,297542328	0,379991531 IRAK1
	0,873834744	0,122232437 CAMK2D
	1,981856839	-0,269021988 HNRNPAB
+	2,474744757	-0,491839886 TMA16
	0,850313184	-0,197563648 TACC2
	0,754525549	-0,209710598 TMEM33
+	3,285744397	0,391657829 MRPL27
	2,181641898	-0,300532818 GNPDA1
	0,013721461	-0,038700104 FAM169A
+	2,359869725	-1,234471321 PSMG4
	0,09941406	0,112491608 RELL1
	0,202823732	0,331182957 ELMOD2
	1,50047312	-0,186939716 EIF4E
	1,128638717	-0,188823223 NHP2
	1,01131747	-0,284093857 HSD17B11
	1,128551336	-0,073982716 HSPA9
	0,313070568	-0,091571808 LIMCH1
	0,687178808	-1,259829044 CWC27
	0,010182011	-0,00265646
	1,612457121	0,0710783 SEC31A
	0,8582439	-0,25463438 STX18

	0,405311956	-0,588130951 DCK	
	1,996091985	-0,226879597 RPS3A	
	0,43185553	0,125637054 UFSP2	
	0,042434041	0,090894699 COMMD10	
+	2,936076033	0,555298328 INPP4B	
	1,353840598	0,120244503 OPA1	
	0,393619618	0,864136696 GINS4	
	1,002500156	0,498000622 EPHX2	
	0,782249474	-0,232484818 NUDCD2	
	0,461621657	0,213434696 TATDN1	
	0,921743736	-0,751486301 VPS37A	
	0,764311029	0,106938362 ATOX1	
	0,264072018	0,349589825 RWDD1	
	0,123890378	0,326584339 REEP4	
	2,057644673	-0,399311543 PFDN1	
	1,478250146	-0,44547987 TCEB1	
	1,870326719	-0,302843571 ERLIN2	
	0,499860201	-0,228791237 RPL30	
	0,427812743	-0,205195427 DNPEP	
	1,420898196	-1,60594368 SLC39A14	
	1,538377951	-0,186870098 TBCA	
	0,422003274	-0,107984543 SKP1	
	1,38129625	-1,924246788 RDH10	
	0,528713623	0,176396847 COPS6	
	1,24309335	-0,388238907 SWAP70	
	0,207152307	0,037216187 CALM2;CALM1	
	0,062686638	-0,04857111 PPP1R2;PPP1R2P3	
	0,594869987	0,229466915 NPC2	
	0,539369957	-0,460915565 UBR5	
+	1,420656108	-3,23182106 ZNF143	
	2,263478718	0,270531178 ADD1	
+	3,521097648	-0,462253094 RPL14	
	2,076095521	0,496959209 IBTK	
	1,845921	-0,258543015	Sep 07
	0,289282494	-0,065550804 PRRC2C	
	0,543455839	1,169521809 CHEK1	
	0,656403039	-0,218238831 NDUFV2	
	2,3869713	-0,235816479 NAA50	
	0,041300611	-0,03947258 TMED2	
	0,13518056	0,018639088 EZR	
	0,50317754	-0,105520725 ACIN1	
	2,876086268	-0,258027554 HMGB3	
	0,093311068	-0,069599152 FAM91A1	
	0,271498261	-0,0817523 EIF2B4	
	0,762839119	-0,146038532 ZC3H18	
+	1,891774903	-1,93788147 RAB34	
	0,220958657	0,040691376 PTK2	
	0,1877017	-0,496133804 FAM114A2	
	0,103006412	0,054054737 MTA1	
	1,802249288	-0,196201801 DTX2	

	0,133993959	-0,017440796 HARS
	0,053936495	0,124876499 RBMS1;RBMS3
	0,197002803	-0,058920383 MRPL3
	2,770569825	0,134586811 PLOD2
	1,054605782	0,233113289 GPX8
	0,766509338	0,137973309 BZW2
	0,407991002	-0,867980957 G6PD
	0,365331195	0,766374111 DUS2
	1,505670893	0,073458195 EIF4G1
	2,137668941	-0,22306633 KLC1
	0,348916457	-0,049908161 PRPF31
	1,13827811	0,440927029 EXOC6
	0,036920652	-0,017227173 DHX36
	0,076360734	-0,015440464 EIF4B
	2,654096497	0,241373062 CALD1
	2,547142303	0,187319279 FKBP11
	0,137236234	-0,040813923 ALYREF
+	2,399315044	-1,397259712 CCNB1
	0,931472242	-1,4526577 CAV2
	2,875080792	-0,325380802 SMC4
+	5,055542227	-0,372099876 MYO1B
	0,17784484	-0,152680397 ANKHD1
	0,187075891	-0,411400795 MRPS17;hCG_1984214
	2,463973647	-0,120962143 FUBP1
	0,364859524	-0,104988575 NRP1
	0,206900951	-0,150145531 ZFX;ZFY
	1,240105266	-0,247047424 NUP155
	0,711213319	0,943540573 VCAN
	0,675408114	-0,11499548 TBL2
	2,185781826	-0,363517284 PPP2R5D
	0,728978268	0,102224827 APP
	0,655062696	-1,93399334 RASA1
	1,549099042	0,333045483 GBE1
	1,833245083	-0,229016781 TSN
	0,399827677	-0,418300152 RPS6KA1
+	4,577811628	-0,632550716 COPS8
	0,071442522	-0,038577557 GSDMD
	0,381256758	-0,144783974 CSTF2
	0,960580771	0,551712036 TCP11L1
	0,020897888	-0,042479038 COMMD5
	0,438142975	-0,381201744 MAPK3
	1,390582161	-1,222441673 SLC25A22;SLC25A18
	0,180277278	0,185094833 SNX15
	0,556554914	-0,520136833 SCYL1
	0,88465232	-0,086842537 PPP6R3
	0,165372571	0,087841988 PRMT1
	0,748504435	0,242314816 NDUFS8
	0,096710062	-0,094974995 PCF11
	0,806504789	-0,208519936 UBTF
	0,556566912	-1,13062048 BTF3L4

	0,728822458	-1,441014767	EEF1D
	0,240315019	-0,339936256	EIF1AD
	0,248588975	-0,025707722	NPEPPS
	2,03945598	-0,32238102	RPL27A
	0,010827813	-0,004467487	CSTF3
	0,113639643	0,090475559	SIRT3
	1,471756075	-0,341561317	C11orf58;SMAP
	0,070494157	-0,033156395	C11orf30;EMSY
	0,07057486	-0,162730694	CD151
	1,753045941	0,723561287	ARHGEF12
	0,100206039	0,026682377	LMO7
	0,547782333	-0,215368748	RNF214
+	2,619675135	-1,416282654	RNASEH2C
	0,220414656	0,464623451	TPD52L1
	0,49888138	-0,372223854	RIN1
	1,364026065	-1,042503357	FAR1
	0,346435675	-0,264387131	ZC3H11A
	0,942690292	1,45357132	PFDN4
	0,273132237	-0,039520264	UBXN1
+	1,819918487	1,173458576	CHMP4A
	0,177360741	0,104616642	TRRAP
	0,561997941	-0,167051315	LSM8
	1,189945473	-0,279040337	CCDC53
	1,320285771	-0,199174881	WNK1
	1,489097931	0,357730389	ATE1
	1,156240423	-0,511915207	RBM7
+	1,48269484	-1,709245205	COPS7A
	0,319418283	-0,628116608	REXO2
	0,252797091	0,062069893	STRN4
	1,793428108	-0,212963104	OTUB1
	0,440208023	0,234591961	PARD3
	0,715690937	0,994328499	PPFIBP1
	0,082263706	-0,028776646	GABARAPL1;GABARAP
	0,431107294	0,24281311	GPHN
	0,064040512	-0,09029007	ANAPC5
	0,512748344	-0,473995686	RAB35
	0,283028283	-0,070101261	PUS1
	2,936901662	-0,20476532	MTHFD1
	0,108762508	-0,035682678	SEC23A
	0,530393788	0,584895611	DHX37
	1,017749041	-0,194989204	TSG101
	1,444633942	-0,235756397	NAP1L1
	0,006080639	-0,002636433	GRSF1
	0,640707887	0,374011993	BCKDHA
	0,048537337	-0,05316782	NAT1
	0,680887813	-0,367375851	DDX55
	0,49597865	-0,582555771	HEATR5A
+	3,122660866	-0,379285336	MYO1C
	0,658242104	0,088066578	ATL3
	0,55745084	-0,130191326	MRPL48

+	2,608237157	1,128171921 IQGAP2
	0,696540017	0,275760174 ASH2L
	0,562825365	0,701505661 MVK
	0,698702645	-1,032558441 PDE12
	0,244973648	-0,381532669 LGALS8
	0,027818917	0,037013054 POLDIP3
	0,541235323	0,103337288 CD63
	2,363926939	-0,235626698 CAD
	1,568209232	1,182640553 C12orf75;OCC1
	0,863649496	-0,977071762 YEATS4
	0,897814745	-0,645735741 POC1B-GALNT4;GALNT4
	0,271406193	0,615210533 SLC38A2
	0,65120819	-0,187716961 COPZ1
	0,637460002	1,481048584 DENR
	0,072970752	0,016631603 SLC25A3
	0,13174364	-0,224903584 COQ5
	0,648103612	-0,448400974 CSRP2
	1,365253405	-0,158860683 SMARCC2
	0,102967901	0,044401169 SCAF11
+	2,752065615	-1,210884571 ZCRB1
	1,163233438	-0,324192047 PPP1CC
+	3,023470747	0,450311184 ARL1
	0,225770598	0,880902767 PCBP2
+	1,584328274	-1,527448177 C17orf49;BAP18;RNASEK-C17orf49
	0,064980951	-0,104504108 NUBPL
	0,406487028	0,141858578 PPHLN1
	0,124847565	-0,295318127 ARID2
	1,317408873	-0,249775887 AK2
	1,97612627	-0,162824154 UBAP2L
+	2,347871582	-1,063029289 BRE
	1,620612665	0,13728714 FAM162A
	0,850046767	0,230881691 TIA1
	0,637275663	0,078287125 RTN4
	0,40388978	-0,438310146 GAPVD1
	0,184164238	0,040035725 CCDC93
	0,118346443	-0,057235718 CLASP1
	1,520879142	-0,461601257 PNO1
	0,106157005	-0,078732491 ARPC4-TTLL3;ARPC4
	0,937028736	0,190593719 MYO5A
	1,614724822	-0,373519897 SEC62
	2,474915943	-0,216407776 CPSF6
	0,22144813	0,036142349 NDUFV1
	0,90144751	-1,107624531 ATP6V1H
	0,209930121	-0,253949165 B3GAT3
	1,029747727	0,182516098 DPP3
	0,712124271	-0,140311718 NUP160
	0,785187928	-0,161404133 API5
	0,30019887	-0,339933872 HNRNPC
	0,502501389	-0,397321224 MAX
	0,496889082	0,218964577 ATP6V1D

+	2,013209008	-1,025420666 ACYP1
	0,032700164	0,014682293 ATP5J2-PTCD1;ATP5J2;PTCD1
	1,274766941	-0,226976395 HNRNPC
	2,117947519	0,421764374 LGMN
	0,408195486	-0,291919231 VIPAS39
	1,557572182	-0,099265575 DDX24
	0,247622107	-0,055933952 C14orf2;MP68
	0,04540123	0,021233082 MIA2
	0,906201607	-1,064217091 CCNK
+	2,878054564	-0,568463802 CDK2
	0,204268076	0,053016663 SMEK1
	0,19754547	0,224938393 ZNF512
	0,658171987	-0,616017818 PAPOLA
	2,463940763	0,324350357 LAMB1
	0,081689634	0,148516178 SMARCB1
	0,279759847	0,269583225 USP11
	0,605614484	0,346458912 NFKBIB
	0,048586019	0,013614178 ELAC2
	1,83553788	-0,350190639 CPSF3
	1,220159216	-0,194579124 CLPTM1L
+	2,653295161	-0,615970612 CELF1
	0,55088208	-0,492893219 SRGAP1
	1,24395691	-0,187187195 MYL6
	0,151554462	0,015984535 HNRNPH1
	0,135775693	0,044335365 ERC1
	0,554641354	-0,901076794 USE1
	0,177737474	-0,30782795 TH1L;NELFCD
	0,065361862	0,037882805 CEP170
	0,876856646	-1,624931335 DPM1
	1,328190533	0,622509003 NF1
	0,421674926	0,844560623 ERI3
	0,371173606	-0,287417889 TMEM201
	0,930879377	-0,290291786 ERGIC3
	0,981408121	-0,211441994 SMPD2
	0,244385728	-0,255533218 TRIM33
	0,060261258	0,01687479 UFM1
	1,798724441	-0,14014101 TRIM16
	0,251822167	-0,220441341 PRCC
	0,530875512	1,178350449 GOLGA4
	0,578151494	-0,539157867 PTPRF
	0,221833444	-0,047122955 HNRNPD
	0,534951235	0,566851616 CENPC
+	4,873847213	2,501249313 MRPL1
	0,844166083	-0,239535809 UTP15
	0,495471409	-0,887296677 DNPH1
	0,178594952	-0,041344643 COPB2
	0,635685872	-0,074803829 PGM3
+	1,70403383	-1,062282085 PCBD2
	0,380287954	0,65666914 ARHGEF10
	0,106431235	0,208469391 IMPA1

	0,14759735	-0,064346313 TPD52
	0,172146225	0,208125114 ATPAF1
	0,086914657	0,014201641 STIM1
+	1,628317168	1,182821751 PLEC
	0,218015061	-0,213784218 MRPL49
	0,805921703	-0,159739017 TTC9C
	0,353548404	-0,111554146 REPS1
	0,063478662	-0,016687393 EIF3F
	1,186426831	-1,710175037 SSSCA1
	0,458206365	0,130972385 PUM1
	0,724846481	-0,562304974 C8orf82
	0,156394002	-0,034904957 HADHA
	0,540459956	-0,557979107 NGDN
	0,76727638	-0,210294247 RPL28
	1,649661014	-0,535906315 KNSTRN
+	2,001674269	1,81269455 MFGE8
	0,012410456	-0,008545876 USP3
	0,075952594	0,532693386 RPS27L
	0,663224754	-0,184792042 ANP32A
	1,427703785	0,226687908 WDR61
	0,455081104	0,245417595 SPPL2A
	2,779231747	0,303481579 SEC11A
	0,535335762	-1,355717182 DPH6
	0,666989125	1,050598145 CERS2
	1,719752777	-0,375455379 AKR7A2
	0,214480093	-0,376848221 NUB1
	0,407576526	-0,315459728 AKTIP
	0,162868939	-0,108824253 ZFAND6
	1,491695492	-0,168386459 ARPP19
	0,328383362	0,16483593 RNPS1
	0,439723612	-0,996209145 RPS15A
	2,676789458	-0,417955399 RPS17
	0,511688623	0,125314236 PDXDC1
	0,217128245	0,097616196 CSNK2A2
	0,125028389	-0,034538269 MPHOSPH6
	0,908431378	0,145922661 HEXA
	0,070472244	0,191413879 COQ7
	0,322848557	-0,10605669 RNF40
	0,497034451	-0,111768723 MACF1
	0,809839516	-0,263512611 ZNF598
	0,607800337	-0,162509918 NDUFB10
	0,074057093	0,018752575 HAGH
+	3,168902574	-1,456905842 NME3
	0,317515786	-0,391347885 ZFYVE19
	1,134710054	-1,089458466 UBF1
	0,204150928	0,06499052 EARS2
	0,256341342	-0,446379662 ARL2BP
	0,828176775	-0,489882946 GCA
	0,047330173	0,013820171 DHX30
	0,722130941	0,571256638 RHOT1

+

0,984235312	-1,347034931 NUPL1
0,934807883	-0,325078487 SMARCAL1
0,844496934	-0,230797291 CIT
0,144360336	-0,031366348 TPM1
0,616680625	-0,196851254 SAP18
0,546414001	1,049273968 UHRF1BP1
0,323200715	-0,067444324 APOO
0,023716441	0,085647583 EPB41L1
0,846528347	-0,203147411 EBNA1BP2
1,251242175	-1,72918272 THUMPD3
0,05893792	0,012862206 IAH1
1,077242716	0,28659296 GCOM1;POLR2M
2,331163	-0,325508595 TMEM189-UBE2V1;UBE2V1
0,018624475	-0,004151344 DDX19A
0,980706747	-0,266709805 RNF167
1,001005281	0,118403435 NSF
1,320015916	-0,555142403 CCDC137
0,013541602	0,00428009 SLC25A11
1,419450538	0,383594513 FLYWCH2
4,094782224	-0,736399174 MPDU1
0,439808979	-0,55977726 RHOT2
0,269740271	-0,412850857 UBE2G1
0,502095631	-0,46075964 DVL2
0,494204892	0,487465858 LCMT1
0,339174923	0,039974213 EIF5A;EIF5AL1
0,341206563	-0,524804592 CHMP6
0,178079414	-0,230011463 TOM1L1
1,599320095	0,085106373 ABCC1
0,357114128	-0,077780724 KDM2A
0,543188625	-0,207399845 DPF2
0,105419444	0,061150551 CCS
0,58632136	-0,699816227 TBL3
0,153982455	-0,034482479 SRSF2
0,987150572	-0,140171051 LUC7L3
0,603740371	0,187101841 DNAJB12
0,250562918	-0,030933857 OLA1
0,666090105	-2,435258865 COPS7B
0,544410814	-0,097314358 PTRH2
1,253588722	0,186126709 SUMF2
0,556009166	0,265613556 APOL2
0,342502189	-0,452867031 BABAM1
0,554330552	-1,069108009 CCDC12
0,256037223	-0,03712368 ARHGDI1
0,170105594	-0,124201298 NFAT5
0,016789468	-0,036869526 ICT1
1,65367784	-0,661201477 DCXR
0,434751772	0,387434959 MBP
1,772256408	-0,138356209 DDX5
0,586108989	0,073286533 SRSF1
0,42610101	0,169847488 SCO1

	0,393101794	-0,55997467	SCRN2
	1,332335997	-0,430846214	SAP30BP
	0,001208443	0,000388145	MRPS7
	0,506409468	-0,114995956	RPL18
	0,6640869	1,790374756	RPL26
	1,570977327	-0,549991608	SUZ12
+	3,032417828	0,388594627	PNPO
	0,008473476	-0,003083229	YTHDC1
	0,56365644	0,09223175	MYL12A;MYL12B;MYL9
	0,231027588	-0,06610775	ZNF207
	0,398038352	-0,510374069	LLGL2
	1,825682146	-0,180222511	BUB3
	0,566132277	-0,775503159	CHCHD6
	0,288710216	-0,261458874	C17orf75
	0,141798408	-0,049195766	MBD3
+	2,623162886	-1,633878708	SMAD4
	1,429299742	0,083806038	LONP1
	1,016529101	-0,119158745	AP1M1
	2,478407787	-0,209653378	DAZAP1
	0,593303371	0,238049984	C19orf25
	1,016300812	-0,555308342	PPP1R12C
	1,564873081	-0,273303509	LSM12
	2,392204517	-0,193386078	PRKCSH
	0,029683496	0,008362293	TMEM205
	0,589817572	-0,14978075	EIF1
	0,302663481	-0,244722843	PSMG2
	0,067356825	0,03689909	RBM42
	0,072377923	-0,145683289	POP4
	0,947686054	-0,133261681	FARSA
	0,523865977	-1,079406261	TMEM256
	0,959374898	-0,103373051	TK1
	0,208421917	-0,354587555	
	0,394005669	0,263499737	DCAKD
	0,001840141	0,001103401	SLC35A4
+	3,839063884	-0,439992905	SARS2
	0,350770639	-0,140974998	GTF2F1
	1,400095069	0,280212879	ZC3H4
	0,71831999	-1,165410995	PIH1D1
	0,144763914	-0,183983326	PNKP
	2,414632682	-0,423342228	
	1,282530888	-0,503326416	IRF3
	0,293972536	-0,498417377	GRAMD1A
	0,210778187	-0,050428391	ARHGEF1
	1,750172388	-0,212698936	RPS5
	0,825019554	0,448928833	ZNF414
	0,376981092	0,055452347	RPL18A
	0,190464458	-0,068852425	EPS15L1
	0,943119757	-0,942765236	KDELRL1
	0,28897663	-0,054530144	POLD1
	0,307652996	0,04031086	SUGP2

	1,021185863	0,211458206 PRPF18
	0,127291865	-0,016001225 AGPS
	0,078265551	0,02242136 DDX39A
	0,339300082	0,111024857 PDLIM1
	1,89551184	-0,576489449 ACOT7
	1,49705411	-0,252768517 SNAP23
	0,703600879	-0,495010376 HAX1
	0,393679764	0,466900826 STXBP3
	1,128791269	0,184811592 RAB27B
	0,273122174	0,045960903 AP3B1
	1,561416812	-0,106788635 PSMD11
	0,030614415	0,00604248 PSMD12
	1,378691053	-0,157783508 PSMD9
+	3,920046578	0,496889114 PGRMC1
	0,602657393	-0,060741901 SUPT5H
	0,95674325	-0,194858551 DFFA
	0,095511865	0,044602394 HIP1
	1,142149252	-0,149366856 CLIC1
+	3,365506173	0,712311745 DCTN6
	0,823524425	-0,132541656 WASL
	2,4593248	-0,217758179 IPO5
	1,54059509	-0,537933826 POLRMT
	0,61531973	0,166240215 IGF2BP3
	0,712921894	-0,100728035 DNMT1L
	0,194763041	0,168775082 RTCA
	0,690526071	0,336256027 PIK3C2A
	0,119515404	-0,051120758 GOLIM4
	0,024453313	-0,013207912 AGRN
	1,382675422	-0,211988449 NDUFA4
	0,664244102	0,095881462 PSMD14
+	2,514885493	-0,704225063 BIN1
	0,574384584	0,226737976 KPNA3
	0,182371354	-0,028940201 SDCBP
	0,838654429	-0,123455048 MPHOSPH10
	0,760269153	-0,073519707 NOP56
	1,214069462	-0,815656662 CYR61
	3,025145572	-0,291208267 PIR
	0,216830768	-0,360287189 PPP6C
	0,267613312	-0,118107319 UBE2C
	0,81958736	0,093918324 PDXK
	1,219769784	0,310095787 SCD
	0,646888775	-0,200965405 ARID1A
	0,442558327	0,113217354 CDK2AP1;CDK2AP2
	0,692993913	-0,13517952 TXNDC9
	1,028691306	-1,644535065 COX7A2L
	0,497892823	0,183887959 NDUFAB1
	2,577176466	0,17992878 COPE
	1,431608415	0,283771038 AP3D1
+	2,829842922	-1,726307392 TOR1A
	0,274689208	0,0406394 TOR1B

	1,750808062	-0,244000435 ADAM10
	0,636141247	-1,094667912 PTGES
	1,81490682	-0,384084225 PDCD5
	1,197953027	-0,149326324 PRMT5
	0,245728629	-0,049434185 SLC9A3R1
	0,486471732	0,507878304 TNFRSF10B
	1,632223207	-0,230357647 PSMA7
	0,1320061	0,036036015 SCAMP3
	0,816173114	0,454601765 MGST3
	0,964512297	-0,243057251 GIPC1
	2,268228769	-0,227782249 HAT1
	1,066098158	-1,543670654 UBE2L6
	0,287091721	-0,121958733 UQCRQ
	0,845230159	-1,552728653 CLGN
	1,305350574	-0,198179245 PPP1R12A
	0,516617935	0,244840145 SLC27A2
	1,218133899	-0,22314024 XPO1
+	1,97078139	0,963270187 ZNF609
	0,177274772	0,032192707 SEC16A
	0,053548238	-0,022538185 PLXNB2
	2,35094786	-0,277027607 U2SURP
	0,030832151	0,028625965 KIF3B
	3,510739865	-0,205219746 PFAS
+	1,716475258	-1,429294109 ANKRD28
	0,269887866	-0,147919655 KIAA0391
+	3,519238475	-1,303966999 CHUK
	0,285740792	-0,417821884 LSM1
	1,168637432	-0,388517857 NPC1
	0,530026387	-0,500970364 DEGS1
	0,061358001	0,218514442 SCAMP1
	0,781459984	0,206324577 SCAMP2
	2,381321351	0,37260437 ARPC1B
	0,68405282	-0,121967793 ARPC2
	0,441274533	0,064920425 ARPC3
	0,108043304	-0,019817352 POLR1C
	0,649651705	0,589520454 TRIM24
	1,847656135	0,332971573 PGRMC2
	1,58567475	-0,330661774 PFDN6
	0,267134995	-0,240533829 NKRF
	0,087102346	-0,037421227 LAMA5
	0,537845757	0,097723484 ZNF185
	0,559301276	0,290979862 CASC3
	0,03402289	0,007329941 MRPS12
	0,174881563	-0,527494431 ACOX3
	0,670701968	0,902008533 RER1
	0,869054638	-0,369951248 SURF4
	0,81839804	0,899827003 SPTLC1
	0,943913832	0,500662804 SPTLC2
+	3,418962598	-0,390678406 OGT
	0,480971816	-0,089886665 PMM2

+
+

2,728743068	-0,210035324 PPM1G
2,410702391	-0,397807121 EIF3D
1,190142102	0,922492981 NVL
0,06194429	0,020634174 BCAT2
0,071142777	0,225540638 STX7
0,20856125	0,03142643 ABCC3
0,044237063	0,021484852 ABCC4
0,460625927	0,523351669 CD3EAP
0,049611607	0,131146908 P4HA2
1,450275272	-0,153716087 ARPC5
0,020370149	-0,036890507 POLR2D
2,945654779	-0,331569195 DHX15
3,099890325	-1,212633133 ZZEF1
3,410376637	-0,836200714 TTI1
0,384707158	-0,115302563 RRP8
0,248132473	-0,214422703 PJA2
0,070733083	0,01555109 CYB5B
0,890794406	-0,151862144 PRPF4
0,059833716	0,028521538 NDUFS4
0,200802999	0,027492523 DYNC1LI2
0,191543079	0,025794029 PSMD3
0,870804154	0,157282829 PAPSS1
0,089925459	0,039776802 ZW10
2,376560318	-0,282292843 SART1
0,854522163	0,316726208 SPINT2
0,449743834	-0,574500084 GPAA1
1,436003682	-0,123708725 EEF1E1;EEF1E1-BLOC1S5
0,712331799	0,052393436 HNRNPR
0,405455779	-0,715180397 PRPF3
0,209424543	-0,043106079 TXNL1
2,696231259	-0,177985668 TPD52L2
0,341757408	0,06213665 EPB41L2
0,124753548	0,128516197 LAMTOR5
1,611217958	-0,183919907 RNF13
1,563932405	-0,202914715 CA12
0,589148448	-0,792534351 XPOT
0,845072129	-0,089082718 TIMM44
0,926471098	-1,217328548 TRAPPC3
0,761971253	0,194048405 CHMP2A
0,585549459	-0,212238789 PLRG1
1,05006146	0,114295959 PRC1
1,038730528	-0,219591618 RGS10
0,715682348	0,731568813 NDUFB3
0,201116802	-0,03097105 NDUFA2
0,8218939	-0,163263321 ASNA1
0,723920636	-0,046471596 ACTN4
0,780228752	-0,787306786 WBSCR22
0,088480971	0,038321495 HTATSF1
1,386677568	-0,413132191 AP1G1
0,735357337	0,271446228 SYNGR2

	1,409757612	-0,133121967 NARS
	0,860331006	-0,127909184 NUDT21
	0,130470959	0,069337368 LANCL1
	0,961948405	0,219181061 STRN
+	2,992265034	-2,015674114 RRP9
	1,178074283	-0,231882572 NRD1
	1,278245761	-0,097500324 CALU
	0,685829993	-0,218902588 AHCYL1;AHCYL2
	1,86614464	0,354152203 NDUFS5
	1,517009929	-0,740807056 ORC4
	1,09407659	-0,138819695 RAD21
	0,910081949	-0,054085732 AKR1B10
	0,653238242	-0,188446045 PRPSAP2
+	5,128576119	-0,526760101 SMARCA5
	2,32606058	-0,263392925 SPAG9
	0,014292263	0,010898113 URB1
	0,715064275	-0,51777935 SIPA1L3
	0,17141008	0,065793991 AQR
	0,249711825	-0,113090038 KDM1A
	1,49060922	-0,323969841 TBC1D4
	1,172948858	-0,670578003 FADS1
+	3,346599122	-0,774498463 DFNA5
	0,766060025	0,212170124 ACSL4
	0,951426593	-0,173707008 SNX3
	0,892505459	-0,147093773 MGEA5
	1,136776355	0,224315166 SORBS3
	0,797425145	-0,092944622 SYNCRIP
	0,355037537	0,125984192 NEMF
	0,909276253	-0,85132885 GMDS
	0,692729562	-0,364383221 CCNT1
	0,754445396	0,175221443 PLOD3
	0,142296737	-0,065728664 EIF4E2
	1,218388576	-0,116196632 PLIN3
	0,114896799	0,022382736 PRMT3
	1,502072667	-0,370406628 KPNA6
	0,392872837	0,031035423 UGDH
	1,426370883	-0,187691212 SNX2
	0,417802581	-0,035725117 USO1
	0,539371136	0,768973827 MRPS14
	0,261675465	-0,064562798 HIST1H2BN;HIST1H2BM;
	0,155935496	-0,051852703 CCDC22
	0,869829818	-1,557552814 PQBP1
	1,285740918	-1,387953281 TIMM17B
	0,114340342	0,23672533 PRAF2
	0,242904805	-0,038124561 DKC1
	0,985440359	-0,145627975 EDF1
	0,138109773	-0,028497219 DNAJA2
	0,638162755	-1,313603878 BRD4
	1,93030899	-0,470557213 CUTA
	0,60374159	-0,31695652 RNASEH1

	1,89016244	-0,206084728 WDR1
	0,655412553	-0,11089468 ROCK2
	0,513766521	-0,059199333 CPNE3
	0,135444278	0,107511044 HIP1R
	0,23374179	0,597142696 TSC22D2
	0,798451824	-0,556251049 DNAJC13
	0,591721919	-0,152754307 COQ9
	1,746040827	0,478929043 GGCT
	0,674312819	0,168212891 NDUFS2
+	1,565946098	-1,738662243 ZPR1
	0,098629186	-0,031685829 GBAS
	0,179514488	0,043345928 PDCD6
	0,606528685	0,169268131 ATP6V1G1
	0,124456193	0,030773163 VPS4B
	2,060366509	-0,196282387 H2AFY
+	2,430760034	0,493242741 SH3BGRL
	0,526049452	-0,037791729 FLNB
	0,812368781	-0,199701786 NDUFS6
	1,852226062	-0,303205013 PEX14
	1,440037959	-0,390081882 SPAG7
	0,054805756	-0,091962814 MRPL33
	1,165137765	0,154045105 SEC22B
	0,411561986	0,106096268 TACC1
	0,323158612	0,100773811 VPS26A
	0,469672244	-0,230348587 NDUFB1
	1,806399703	0,296869755 PMPCB
	2,481050795	-0,226748943 PSIP1
	0,957086348	-0,192072392 ERLIN1
	1,089485772	0,16952467 NDUFS3
	0,639080961	0,206415176 SRSF10
	0,632782774	-0,601428509 HSBP1
	1,226692426	-0,160902977 BANF1
	1,069647689	-0,115549088 SF3B1
	0,082717677	0,017271519 CSDE1
	0,236901761	-0,045374393 PRKRA
	1,078012208	-0,251589298 NPM3
	0,03580958	0,007894993 LYPLA1
	0,164424771	-0,051958084 ERAL1
	1,905124106	-0,195277214 SNRNP200
	1,964374817	0,368515491 TIPRL
	1,612742405	-0,648854256 SURF6
	0,840889657	-0,224402905 PPM1B
	0,011738621	-0,008206844 UTP20
+	3,097966135	-1,034279346 WDHD1
	0,183874186	-0,239377499 CRTAP
	1,587519793	-0,328186989 CDC123
+	2,653559914	1,203798294 RPP40
	1,633386726	-0,208870411 EIF3G
	0,077409059	-0,018157482 EIF3J
	0,106687312	-0,029225826 CBR3

	0,580430717	0,154073715 PSMD10
	0,893841332	-0,137209415 ZMPSTE24
	0,120069195	0,019769192 IDH1
	0,060396206	-0,017442226 RBBP9
	0,73652028	0,339962482 STAM2
	1,314667514	0,261686802 DHRS3
	1,108993742	0,249576092 ARL6IP5
	1,241355803	-0,248078823 BCAS2
	0,810460884	-0,232183456 DCTN3
	0,731670075	-0,139104843 DNAJC8
	0,13426992	-0,428081036 SMNDC1
	0,885084757	-0,179720879 ATP5H
	1,302155118	-0,853176117 FLOT1
	0,91377369	-0,146368504 CPD
	0,178375549	-0,015535355 GLRX3
	0,006726577	0,00163126 RSL1D1
	1,715668105	-0,254183292 CLPX
	0,354744413	-0,070124149 CIAO1
+	2,74166712	-0,488175392 SRP72
	0,879739889	0,097679138 DDAH1
	0,374290899	0,60430479 URI1
	0,120542347	-0,061434746 MTA2
	3,386397603	-0,223680973 TOMM70A
	0,156565292	0,475310326 SEC24D
	0,781502407	-0,120325565 UFL1
	1,987843321	0,500758171 PROSC
	0,75626985	-0,125203133 PRPF6
	0,089765012	-0,038208008 DKK1
+	2,424192182	0,500535488 GLS
	1,03501844	-0,221002579 AP2A2
+	3,708147859	-0,511597633 HEXIM1
	0,160598372	-0,131233215 TJP3
	0,435627646	0,105388165 AGFG2
	0,753702441	-0,754918575 PRSS23
	0,797235401	-0,325772285 SNUPN
	0,285928188	-0,069621086 ZFPL1
	0,397921882	-0,198139191 NDUFB4
	0,439264597	0,491037846 NDUFB8
	1,241864838	0,210764408 NDUFA7
	0,621388601	0,208669662 RTN3
	1,124535444	0,150327682 LETM1
	0,074279662	0,036642075 ZRANB2
	0,163540122	-0,291924953 SNX4
	0,074576832	-0,079565525 KIF20A
+	4,79607052	-0,595663548 KIF4A
	0,71428298	-0,138567448 VAPB
	0,477844305	-0,74064827 MPZL1
	0,879432454	0,29676342 NDUFC2
	0,873629069	-0,292186737 FKBP9
	2,055768544	0,179533005 PGLS

+	4,730861889	0,503343105 PAPSS2	
	2,551652347	-0,38720274 SMC2	
	0,057324648	-0,15549469 ATG7	
	0,113782916	-0,043462276 LYPLA2	
	3,469166104	-0,28922224 IPO7	
	0,015475119	0,036327839 CD2BP2	
	0,206250386	0,041503429 AHSA1	
	1,142264965	0,366286278 PSMG1	
	+	2,086682244	-0,616518021 FMNL1
		1,543990794	-0,22297287 SGPL1
0,245838349		0,122254372 SEC24A	
1,130554961		-0,277241707 SEC24B	
0,817119431		1,444609642 VNN1	
1,895804373		0,187617779 ETHE1	
0,080197848		-0,037091255 POLR1A	
+		4,470792591	2,651939392 ZBTB11
		1,520199741	-0,356673241 CDS2
		0,249339972	0,222359657 HERC2
	0,423292758	0,121132374 SNAP29	
	0,148017877	-0,025148869 OXSR1	
	1,269489296	0,281416893 HSPA4L	
	0,73182749	0,212785244 PTBP3	
	2,86559949	0,14509201 AP2A1	
	0,142092127	-0,017339706 STAU1	
	1,039156248	-0,656330585 TTC4	
+	0,222191338	0,058944702 SDPR	
	1,439992131	-0,358318806 BAG2	
	0,06856447	-0,016709328 BAG3	
	0,591826221	-0,075124741 BPNT1	
	0,624829655	-0,119516373 FADS2	
	0,77060553	1,143009663 DDAH2	
	0,494668086	-0,103225708 TXNDC12	
	1,774154724	-0,393523216 ECD	
	1,313415558	0,449113369 SYF2	
	+	3,293965101	-0,44371748 AGR2
1,359539064		-0,206743717 CLPTM1	
2,858528368		-0,283058643 TOMM40	
1,034240217		-0,33973074 PAK4	
1,702493003		-0,157593727 ACTL6A	
1,062391385		1,610132694 MT-CYB	
+		3,170202575	0,607333183 CYB5A
		1,290862836	0,109187126 LDHA
		1,567329725	0,173477173 ALDH1A1
		1,647776143	0,15068388 GLUD1;GLUD2
	0,580493658	0,751193523 DHFR	
	+	4,138851635	0,540028572 CYB5R3
		2,141900556	-0,163256168 GSR
		0,511674634	-0,273229599 MT-CO1
		0,354090923	0,045659542 MT-CO2
		0,10659217	0,08542347 MT-CO3

	2,215776534	0,261237144 SOD1
	0,444971714	0,054051399 PNP
	0,624264647	0,077103615 HPRT1
	1,194066949	-0,191658497 GOT2
	2,269255879	-0,193200111 EGFR
	2,277092833	0,160155773 PGK1
	0,167140538	0,044673443 AK1
+	3,684952292	0,721615791 ADA
	1,30136912	-1,102601528 CST3
	0,015264252	0,03152132 NRAS
	0,178081097	0,061932087 HRAS
	0,142251159	0,43585825 KRAS
	0,323258715	-0,724194527 HLA-H
+	1,721011554	1,473229408 FN1
+	2,122942308	2,709003448 RBP4
	2,914232083	-0,367262363 TFRC
	2,194707837	0,331073284 FTL
	0,511294526	0,081912994 FTH1
	0,224478294	0,404315472 MT-ND1
	0,063330448	0,014719009 MT-ND5
	2,713326333	0,323335171 CAT
	1,366784463	-0,258431911 GBA
	0,732683602	0,092717171 ALDOA
	0,14615327	0,03329277 CSTB
	2,117412327	0,166935921 ANXA1
	0,523587518	0,849162579 APOB
	0,061087316	-0,01728487 NR3C1
+	2,883575077	0,555392265 SOD2
	2,84916711	-0,258791447 OAT
	0,896307996	-0,117631435 TUBB4A
	0,434615555	-0,042637825 GAPDH
	2,260471232	0,354132652 HSPB1
	1,492676831	0,357827663 TYMS
	2,063347349	-0,203861237 RPN1
	1,49236574	-0,223529816 RPN2
	0,0821225	0,164307594 GNAI2
	1,382408753	0,284733772 SLC4A2
	0,291970365	-0,036584377 ATP1A1
	2,815161083	-0,351552486 ATP1B1
+	3,757700021	0,504998207 ALDH2
	1,685165279	0,783399105 SERPINE1
	0,484246372	-0,051941872 SLC25A5
	1,915078821	-0,140668869 EIF2S1
	1,102624363	-0,141708374 RPLP1
	0,226484671	0,028682232 RPLP2
	1,302033178	-0,175091743 RPLP0;RPLPOP6
	0,259999929	0,031652927 SSB
	5,01986819	0,23499012 KRT18
	1,1189427	-0,921012402 RB1
	2,718242503	-0,294803619 CDK1;CDC2

	0,517390616	0,04156065 ATP5B
	0,592792802	0,117901802 S100A6
	1,384036556	-0,106162071 ENO1
	1,792222757	0,247002602 PYGL
+	2,683864354	-0,458141327 ITGAV
+	3,561625157	0,358533859 EPHX1
	1,43756984	-0,262107372 H1FO
	0,411115902	0,030652046 ANXA2;ANXA2P2
	1,765052426	0,207569599 CAPN1
	1,538537205	-0,226290703 TUBB
	0,057488714	0,009304047 HEXB
	0,937724695	-0,113769531 PFN1
	2,537342536	0,26432991 APRT
	2,267984575	-0,128633499 EPRS
	1,779607146	-0,178480148 HSP90AA1
+	3,286751432	0,748666286 UQCRH
	0,552926671	-0,037728786 FH
	0,198495983	0,10717392 ERCC1
	1,108631892	-0,590745449 SP1
	1,770498511	-0,198866844 ANXA6
	3,289986902	-0,162461281 HSP90AB1
	1,353050743	-0,320940495 ASNS
	1,313665845	-0,095347881 PDHA1
	1,396614667	0,17090416 CYC1
	0,689858016	-1,201047897 SNRNP2
+	3,032872036	0,543220043 MFI2
	3,343751661	-0,241240978 SNRNP70
	1,948292898	0,615180969 ITGA5
	0,215437554	0,011278152 VIM
	0,903687254	-0,169520855 GNAI3
	0,963992271	0,156962872 ANXA5
	0,561018433	-0,076889992 SNRPA
	0,294443784	-0,049127579 ENO2
	0,557437634	-0,187942028 ACAA1
	2,420801809	-0,301172733 GSTP1
	1,501355854	-0,248477936 LGALS1
	0,161382521	0,044229031 QDPR
	0,089998225	0,020967484 HMGB1;HMGB1P1
+	2,793780374	0,453884602 CLTA
	0,672052447	0,186814308 CLTB
+	4,190368798	0,392619133 ANXA4
	0,296321936	-0,05803442 CNP
+	3,003187967	-0,469970226 HMOX1
	0,907752585	-0,103513241 DLD
	1,590760362	-0,255390167 SNRPA1
	0,759833172	0,161381245 COX6C
	3,697757123	-0,319998264 PARP1
	0,658421652	-0,077950001 UCHL1
	0,29440869	0,015231133 LTA4H
	0,604817648	-0,230924606 H2AFV;H2AFZ

+

0,147892697	0,207908154 CISD3
0,153002053	0,441569328 FDX1
1,243529967	0,175807953 TROVE2
0,926488791	-0,699445248 COX8A
2,820908914	0,650337696 GAA
1,069519371	-0,147405624 RRAS
0,150361801	0,035916805 TXN
0,968106698	0,174993992 COX5B
0,093185358	-0,072629929 MGST1
0,077066876	-0,03722477 PRKAR1A
0,368698298	-0,034662724 ESD
1,437127242	-0,156777859 HSPD1
0,428533466	0,123809338 CLU
1,790830804	-0,089307308 HSPA5
0,526684924	0,233917713 LAMC1
0,01513318	-0,001636982 HSPA8
1,435063778	0,187552452 SLC2A1
2,218824948	-0,310943604 UMPS
2,955611669	-0,305540085 PDHB
2,806372394	-0,290171146 PYGB
0,168850655	0,30284071 RALA
0,486927844	0,226212978 RALB
0,802518438	-0,131833076 LAMP1
1,866789755	-0,195539951 TOP1
2,022792985	-0,241924763 TOP2A
2,398080201	-0,139167786 G6PD
2,631372482	0,236397743 PC
1,286784982	-0,285401344 IGF2R
0,030629537	-0,00808382 ADH5
1,078543353	0,294582367 CDK4
1,221936053	-0,438556671 PRPS2
1,862209732	-0,128673553 PABPC1
3,284419781	-0,24805975 PCNA
0,385040108	0,171878338 COX6A1
1,231260298	1,533736229 SLC25A4
1,233240028	-0,377893448 SLC25A6
1,120172681	-0,150233746 TPR
0,209810302	-0,016795158 ANXA3
3,424952423	-1,486383438 CKMT1A;CKMT2
4,154455816	-0,327854156 ACTN1
1,084735159	-0,214005947 CDH1
0,164593058	0,0484972 SRC
1,744998437	-0,164815426 XRCC6
0,659228004	-0,080570698 XRCC5
0,066086283	0,011962414 COX4I1
0,957547494	-0,205241203 LAMP2
1,77635071	-0,241389275 RNH1
0,31456369	0,710279465 CYBA
2,044691641	-0,153614998 EEF2
0,605759963	0,036367893 PDIA4

+

+

	0,87484755	0,227710247 P4HA1
	3,384332618	-0,240848064 PLS3
	1,694850184	0,259913445 ETFA
	0,10611416	-0,03209877 PRKAR2A
+	1,742011165	2,386847019 ENO3
	0,225256211	-0,07500267 GTF2F2
	0,634870393	0,136666775 MIF
	0,355693025	-0,122034073 CD99
	1,497915336	-0,271612644 FDPS
	2,181465762	0,216950417 AKR1A1
	0,089309148	-0,01083374 PKM
	1,059586238	-0,351802826 SNRPB;SNRPN
	1,732192872	0,290354252 COX6B1
	2,745578939	-0,094181061 HNRNPL
	0,293008055	-0,06883812 DARS
	1,561892534	-0,201829433 JUP
	1,355757183	0,182283878 AKR1B1
	0,037683295	-0,007661343 GSPT1
	1,69631405	-0,644130707 B4GALT1
+	3,854794121	0,585429668 HPGD
	0,350469788	-0,06776762 CD46
	0,479835859	2,002365112 NME1
	0,077451834	0,013530254 NQO1
	1,481258169	0,266021729 GNS
	3,396584254	-0,304449081 RPS2
	0,249441261	-0,040109158 DSP
	1,157237006	-0,326271534 RPA2
	0,75742607	-0,320732117 COX7C
	1,150052261	0,081985474 CD44
	0,579609777	0,162026882 NQO2
	1,242680862	0,163042068 ITGB4
	0,09729981	-0,015226841 CBR1
	0,341782708	-0,171210766 GLB1
+	2,756130291	-1,211042881 HIST1H1B
	1,193768193	-0,316245079 HIST1H1C
	0,545342904	-0,05439043 POR
	1,039445442	-0,115684032 ATP2A2
	0,334629077	-0,158533573 FAH
+	2,51021646	-1,020596027 STMN1
	1,695729174	0,298719883 YBX3
	0,885360852	-0,127848148 HMGA1
	1,707074401	0,247116089 TMEM11
	2,151087067	-0,190964222 GOT1
	1,153212617	0,339476109 PRKCA
	1,701140027	0,464068413 ITGA2
	1,767671232	0,422551155 NDUFB7
	0,15013778	0,060318947 PRKACA
	2,437093209	0,252596378 CAPN2
	0,318824752	-0,455561638 CEBPB
	1,176956765	-0,227212429 PTPN2

+	3,855429327	-0,338078976 CTPS1
	0,427519237	-0,109153271 PFKL
+	3,810621283	0,509854794 LGALS3
+	3,267083895	-0,385894299 PSMC3
	0,429106118	-0,060388088 TCP1
	0,328106639	0,054629803 RPL35A
	0,190111127	-0,066515923 ITGB5
	0,740916117	-0,134341717 ARF4
	0,216684629	-0,048197746 RPL7
	2,451729385	0,155935287 VCL
	0,185373224	0,0387187 SON
	0,082348336	-0,098116398 NELFE
	0,009154382	0,002055645 PGAM1
	1,07464012	-0,10253334 RCC1
	0,667961117	-0,15731287 SDC1
	0,974472684	-0,26205349 LIG1
	0,706689421	-0,150131226 ATP5J
	1,378517912	0,377133846 XRCC1
	0,07965546	0,089103699 PLCG1
	1,729479805	0,81717968 UGT1A6
	0,19489955	0,278921604 HK1
	1,310521827	-0,246679306 POLR2C
	0,099591629	-0,048146248 GGT1;GGT3P;GGT2
	0,953096551	-0,751265526 TRIM21
	0,40945286	0,117011547 SRM
	0,546317012	-0,084210396 ATP2B1
	3,214273227	-0,217236519 EIF2S2
	0,036873836	0,013149738 ANXA7
	0,598875906	-0,262957096 BTF3
	0,157666827	0,080797195 RAB5A
	0,980194681	-0,128249168 RAB6A
	2,399482639	-0,116827965 PSMB1
	0,158743776	0,131432056 M6PR
	1,223936899	0,133113861 COX5A
	2,082350727	-0,161041737 LMNB1
	0,487948453	-0,721680641 IMPDH1
	1,264821848	-0,181407928 ATP6V1B2
	2,018727571	-0,235427856 ATP6V1C1
	2,108974316	-0,227734089 CSRP1
	2,716995722	-0,1366992 FLNA
	0,57809371	-0,095918179 ACO1
	1,322728422	-0,108139515 VDAC1
	0,579130676	0,071139336 SDHB
	0,412111044	0,172756195 COMT
	3,562455365	0,257327557 TGM2
	1,650150281	-0,259867191 OSBP
	0,046659721	0,007742882 FBL
	2,390667951	-0,228747368 GART
+	3,557289791	-0,382279873 PAICS
	0,66621026	0,101975918 SCP2

	2,597244455	-0,282665253 UBA1
+	5,283605419	0,813436508 FDXR
	3,784713379	-0,274968147 HNRNPA2B1;HNRPA2B1
	1,520107592	0,245369911 IGFBP4
	0,605023386	0,061499119 UQCRC2
	1,892587654	0,409918308 FECH
	0,474993288	0,021066666 SFPQ
	0,133869928	-0,024343491 TUBG1;TUBG2
+	1,534333896	-1,384776592 GLDC
	0,330707643	-0,040219307 WARS
	1,205042921	-0,235263348 RPS3
	3,291284149	-0,33992672 AHCY
	0,98414423	0,09644413 CFL1
	0,052020437	-0,020617008 CPT2
	2,243647337	-0,333458424 DTYMK
	1,828861568	-0,221077442 RRM1
	0,138776787	-0,088254929 NR2F2;NR2F1
	0,66235592	-0,095948219 EEF1B2
	0,640630242	-0,110467911 ATP5F1
	1,444903999	0,208094597 ACP1
	0,304231344	0,050652504 ACAT1
	2,240905385	-0,363777637 MCM3
	1,506841924	0,295937538 MPST
	2,332363124	-0,241339684 RPS12
	2,177072896	-0,395222187 YY1;YY2
	0,998303317	-0,149294853 DNAJB1
	0,400141496	-0,046486378 ATP5A1
	1,200222971	-0,154876232 PSMA1
	0,640579088	0,09645462 PSMA3
	2,927044403	-0,315246105 PSMA4
	0,216043926	-0,734931946 S100P
	0,177433231	0,040048122 ITGA3
	1,27213111	0,075281143 MSN
	0,167496608	-0,016273022 DDX6
	0,010728105	0,001554966 U2AF2
	0,611615484	0,094172001 RPL13
	2,104460846	0,332172871 S100A4
	2,131811296	0,402673244 HMGB2
	3,305117309	-0,32080555 TARS
	0,573576351	-0,047047138 VARS
	0,394160816	-0,039141178 EEF1G
	0,622304687	0,180566311 STOM
	0,689068251	0,245791435 AK4
+	2,364379309	0,628632545 ANXA13
	1,093031882	-0,146724701 YWHAQ
	0,142961959	-0,071509838 ATP6VOC
	1,265634654	-0,268792629 RPL10
	1,078784156	-0,140661716 RPA1
	0,884276683	-0,08647728 APEX1
	0,284438936	0,038136959 CALR

	0,990434041	-0,156706333 PSMA5
	1,007975285	-0,252040386 PSMB4
	1,187646299	-0,243691444 PSMB6
	0,455730513	-0,079785824 PSMB5
	0,766022418	0,068742275 NDUF51
	0,004015401	-0,000930786 MAPK1
	0,547675566	-1,622712612 RBL1
	0,482845183	0,214733124 GRN
	0,318717168	0,025765896 LAP3
+	3,623513809	0,616539955 CD38
	1,058867534	-0,242074966 GTF2E1
	0,291823247	-0,129817009 GTF2E2
	0,294539996	0,077567101 EPHA2
	1,017514178	-0,853471756 SHC1
	1,332299821	-0,152480125 TKT
	2,71673781	-0,212305546 EEF1D
+	2,706395398	-0,441936493 MARCKS
	1,023033397	-0,817189693 GNA11
	2,02893753	-0,44158411 ERP29
	0,388058708	0,041948795 PRDX6
	1,672079456	-0,146521568 BLVRB
	1,241359621	0,101793766 PRDX5
	0,616174789	0,053546906 DDT;DDTL
	0,216728592	-0,032062054 PRDX3
	0,392477183	-0,126359463 ATP5D
	1,792615146	0,155715466 RPL12
	0,534444741	0,052490711 ECHS1
	2,062747749	0,302422047 CMPK1
	3,383885353	0,147278309 PEBP1
	2,446520015	-0,12487936 PDIA3
	1,083248546	-0,108387947 PPP2R1A
	1,307450313	-0,669754982 PPP2R1B
	0,541259027	-0,611945152 CDC27
	0,214751839	-0,566101074 PPIF
+	3,57012101	-0,362252712 ADSS
	0,080362268	0,014189243 LRPAP1
	2,318041002	-0,190042496 CLIP1
+	3,083163448	0,453858376 SERPINB1
	0,265317026	-0,28663063 SLC7A1
	3,358805334	0,27046299 ALDH1B1
+	5,600353997	0,793962955 ALDH3A1
	0,570518001	-0,05749464 SDHA
	0,389260415	-0,088827133 GDI1
	2,003008463	-0,178979874 MAT2A
+	6,903977476	-1,81822443 CPS1
	0,325512728	-0,029150963 DNAJA1
	0,029032186	-0,017006397 AKT1
+	2,149176188	-1,260266304 AKT2
	1,669664868	0,129414558 UQCRC1
	1,581571024	0,276723862 HIBADH

	1,40294145	-0,098940849 ATIC
	0,10808446	-0,013366222 HNRNPH3
	1,313547188	-0,256044388 YWHAB
	0,356990139	0,098879814 SFN
	1,893419327	-0,163888931 STIP1
	0,7488935	-0,148060799 S100A11
	0,563359399	0,320323944 DCTD
	2,015242106	-0,335342884 PYCR1
	1,201580752	-0,21328783 RPL9
	3,385661377	-0,290922165 KIF5B
	2,066747278	0,485369205 DUT
	0,108649429	0,070839405 CKS2
	1,217426136	0,527441978 S100A3
	1,785850595	-0,295162201 MCM5
	3,469102783	-0,241856575 MCM7
	1,390194769	-0,269776344 SHMT1
	0,13526998	-0,019821644 SHMT2
	2,663344874	-0,265286446 HSPA4
	0,773825784	0,111854553 MPI
	0,585397775	-0,1672616 GPC1
	0,258627692	0,112944603 CA8
	0,925066606	0,084127426 CTNNA1
	2,290061283	-0,141872883 PHB
	0,561735801	-0,079274654 RPA3
	0,997799681	-0,198974133 RFC4
	1,600088438	-0,296031952 RFC2
+	3,405543247	-0,705304146 RFC1
	0,848627859	-0,212830544 RPL22
	1,653455862	-0,187880993 SPR
	0,824935268	-0,779362679 PTGS2;COX-2
	1,33818859	-0,101844788 MYH9
	0,734933391	-0,104584694 MYH10
	0,279399207	-0,126969337 SOAT1
	1,369818301	-0,321614265 FUS
+	2,096397824	-0,590360165 GLRX
	1,246715869	1,002795219 HMGCL
	1,308632487	-0,217902184 ARL2;ARL2-SNX15
	1,391088322	-0,179463863 ARL3
	0,691906808	-0,643915653 TRIM23
	0,801462783	0,134118557 MAP2K2
+	2,880684835	-0,415664673 ATP5C1
	0,022751139	-0,003296375 ATP6V1E1
	0,162458866	-0,027413845 CPOX
	2,377360052	-0,092642784 RPL4
	1,362723331	-0,366125107 NUDT1
	0,321965543	0,071969509 PGM1
+	1,533453746	3,73037529 GNL1
	0,961755238	-0,751591206 LTBR
	0,119309773	0,032987595 POLR2I
	0,977673447	0,066158772 DLST

	1,386461473	-0,212928295 SRP14
	0,322306005	-0,106704712 NUP62
	1,1867927	-0,113773823 HPCAL1;HPCA
	0,139703175	-0,120194435 FDFT1
	1,581316393	-0,144757748 TAGLN2
	1,045491021	-0,081604958 TALDO1
	2,028210083	0,239331245 ETFB
	2,262028361	0,260363102 RBMX
	1,722555979	0,160681248 ATP6V1A
	1,621625149	-0,385159492 EIF4A3
	4,470296686	-0,288256168 RPS19
	0,854661148	-0,09239006 RPL3
	2,190708225	-0,22435236 FEN1
	1,5041339	0,401806831 CAPG
	0,988125768	0,340079784 CAP2
+	2,538026103	0,764044762 CEACAM6
	0,920597791	-0,315747261 TXLNA
	2,827459336	-0,173973083 CCT6A
+	4,341981143	0,529414654 NNMT
	0,379546762	0,464057922 PSMB10
	0,0910483	-0,021809578 USP8
	0,669894237	0,121923447 PEX19
	0,643614137	0,072832108 MDH2
	1,704267712	-0,306993008 RFC5
	1,128100183	-0,193723679 RFC3
	0,876624525	-0,130327225 EIF2S3;EIF2S3L
	0,199050237	0,074737549 CETN2
	0,016763923	-0,005123615 EIF2D
	0,603259373	0,064016819 BUD31
	0,100690988	-0,027783394 NAA10
	0,544495952	-0,120849133 CSK
	1,562233651	-0,148514748 IARS
	0,738708097	-0,523688316 PRKCI
	0,059085422	0,051168919 ACTR1B
	0,26358846	0,035608768 ECI1;DCI
	3,921845321	-0,267029762 TMPO
	0,451024718	-0,066699505 TMPO
	1,952629947	-0,170780659 SKIV2L2
	0,094601848	0,024716377 MTOR
	0,43481743	-0,380719185 CASP3
	1,169407429	-0,150166512 RPS27
	0,710621312	0,070819378 ACAA2
	1,360443054	-0,298095703 RPL35
	0,911432778	-0,693394661 SLC1A4
	1,492853158	-0,283755779 MSH2
	0,078895704	-0,023605347 GPD2
	0,140865875	0,033691883 ALDH3B1
	0,559679209	-0,132085323 RANBP1
	1,391716515	-0,085152626 PSMC4
	0,176666864	-0,050088406 TSFM

	2,153423971	0,400212288 ACADSB
	0,08127219	-0,022188663 CBX5
	2,14131759	-0,171017647 USP5
	1,302057607	-1,403150082 MAP2K4
	0,055249854	0,007894993 MKI67
	0,933383232	-0,154231071 RANGAP1
	0,773758206	-0,125668049 RECQL
	1,11807027	-0,112866879 NOP2
	1,521206708	-0,293025494 ATRX
	0,95205892	-0,08560133 CRK
	0,037991809	0,019829273 CRKL
	1,072379717	0,157629967 MAP2K3
	2,9714255	-0,266840935 BRCC3
	0,063940558	0,027230263 RPL21
	0,525468966	-0,093986034 RPS9
	0,118699262	0,019686222 RPS10;RPS10-NUDT3
	1,312621129	-0,070930004 MAP1B
	0,281695818	-0,034750938 NEDD4
	0,416544728	0,342456818 UTRN
	2,099313392	-0,124590874 IQGAP1
	1,531221302	-0,278634548 GYG1
	0,192852419	-0,037427902 STT3A
	2,577250408	0,407137871 PLA2G4A
	1,033134088	-0,156982422 CAPZA2
	1,775861853	0,397990704 EIF1AX;EIF1AY
	2,01429033	0,504234791 ALDH1A3
	0,173928581	-0,03250885 QARS
	0,101848609	-0,035725117 UQCRFS1;UQCRFS1P1
	2,710477471	-0,241652489 ATP5O
	2,160896464	-0,36242485 LIMS1
	0,985564245	-0,228883266 PREP
	0,88859238	-0,113108158 ME1
	0,982616629	-0,278708935 IREB2
+	6,216463842	-4,9029212 LSS
	0,714944846	-0,093987465 GCLC
	1,837991136	-0,155944824 GCLM
	1,016737753	-0,114271164 PSMD8
	0,202854947	-0,203086853 PRRC2A
	1,716249281	-0,228892803 GSS
+	5,370206995	-0,293417931 CCT5
	1,104020955	0,142566681 IDH2
	0,628674601	-0,543459892 CD97
	1,350717193	-0,184752464 ALDH9A1
	0,181526168	0,08643055 RPL34
	0,757201379	-0,687325954 RPIA
	0,264419368	-0,088617325 LMAN1
	0,777860529	-0,103657246 NASP
	3,103875518	-0,264735699 FASN
	0,144738747	0,051848888 FNTA
	0,912572752	-0,27200222 FNTB

	2,985507103	-0,197272301	CCT3
	0,96029108	-0,222613335	MRPL19
	1,535601497	-0,330897331	ARRB1
	0,177835326	0,024092197	TUFM
	1,017088969	-0,302033901	SRP9;DKFZp564M2223
	1,872805531	-0,353499889	SARS
	1,698243466	-0,304348946	PSMB3
	0,098678788	0,014823437	PSMB2
+	3,564554342	-0,470483303	MCM2
	3,442239741	0,281098366	ACADVL
	0,102096752	0,016602993	YLPM1
	0,028816746	-0,004956245	TMED10
	2,206833096	-0,170048714	RBM25
	0,240822772	-0,048187733	NUMB
	0,774859564	-0,461894989	EIF2B2
	0,322338145	-0,084658623	HINT1
	1,641858401	-0,239845753	NUP153
	3,125115046	-0,238510609	RANBP2
	0,193573149	-0,058711529	GSK3A
	2,93355383	-0,333752155	SEPHS1
	2,507702315	-0,214643478	GMPS
	2,445493679	-0,451046944	LIG3
	0,041589773	-0,009928226	MRE11A
	0,799247538	-0,530235291	GNAQ
	0,973155035	-0,078252792	IDH3A
	1,883827095	0,397569656	CRIP1
	0,632278034	0,052461147	GDI2
	0,910212842	0,180616856	EMD
+	2,940856543	-0,557826996	CPT1A
	0,427985314	0,100811958	SERPINB9
	1,230656379	0,100673199	SERPINH1
	1,357604012	0,144816875	ST13;ST13P5;ST13P4
	1,269151257	-0,179278851	VASP
	0,685627935	-0,091258049	DNM2
	1,245626944	-0,129436493	METAP2
	0,5488859	-0,588842869	NUDT2
+	2,197307897	-1,172504902	KNTC1
	0,875475762	-0,664868355	RASSF2
	1,49151719	-1,179663181	CDK9
	3,438626309	-0,338238716	CCT8
	0,446317533	-0,078668118	CCT4
	1,12307233	-0,111288548	ANXA11
	1,323567929	-0,219183922	FXR2
	0,002026167	0,000462532	RAB5C
	0,463677238	-0,029121399	RAB7A
	1,009526211	-0,227199078	RAB9A
	0,791261587	-1,412258625	RAB13
	0,716682093	0,189250946	DAP3
	0,131497207	-0,042774677	DUSP3
	1,140457637	-0,125396252	IDH3G

	0,050253911	0,025623322 GALK1
	1,601380826	-0,1551404 SSR4
	0,463471664	-0,17157793 BCAP31
	0,794218884	-1,730660915 TPMT
	0,153441045	-0,017495155 PSMD7
+	1,723485409	-0,93444252 STAT5B
	0,138950544	0,177760124 VAMP7
	1,080159889	-0,440681934 NR0B1
	0,307103861	0,044904709 HDGF
	0,386046527	0,053031445 NDUFA8
	1,558745759	-0,124729633 PGD
	1,532177308	-0,136499882 HNRNPM
+	4,379093507	-1,995652199 KPNA1
	0,121580448	-0,060549259 NCBP2
	2,199055368	0,441039085 RAP1GDS1
	1,72146929	-0,159695148 AGFG1
	0,206464934	0,043011189 HNRNPF
	0,632118119	-0,074244499 MSH6
	0,886446145	-0,890118122 KIF11
	0,449053762	-0,926179886 HRSP12
	0,751179337	-0,162123203 SMS
	0,4951308	-0,110150337 THOP1
	0,084350185	-0,011685371 AKR1C2
	1,200203606	-0,197084427 CAPZA1
+	2,489466033	0,611361027 CRIP2
	2,572370323	-0,258791924 NUP98
	1,081691421	-0,071378708 BLVRA
	1,442135626	0,227287292 SLC25A1
	3,026756353	-0,296133041 PPP5C
	0,213748731	-0,071585655 ARFIP1
	0,078270367	-0,043744564 NUBP1
	3,475416314	-0,140681744 ACLY
	0,175467105	0,07265377 METAP1
	0,079292083	0,020744324 SUCLG1
	0,112544057	0,020849228 COPB1
	0,053081404	0,010625362 COPA
	0,939056399	-0,066000938 HCCS
	0,016062698	-0,064829826 SLC16A1
	0,274303232	0,040199757 IST1
	1,190052689	-0,347285271 SEC24C
	0,427996752	-0,288314342 SUB1
	2,693377908	-0,237571239 RARS
	1,893733137	-0,144715309 YARS
	0,38683474	-0,033182144 USP14
	1,462016772	-0,144323826 HSPA2
	0,677508203	-0,113206387 ATP1B3
	0,893019079	-0,16813612 RAD23B
	0,562346	0,075976372 ALDH18A1
	1,91112605	0,136109829 NAPA
	0,542970415	-0,101282597 EIF5

	2,657650375	-0,391980648 SLC12A2
	0,601853174	-0,130727768 PSMD4
+	4,006034681	-0,376437187 CSE1L
	1,121781212	-0,164633751 MFAP1
	0,93874792	0,109544277 HADHB
	1,028972827	-0,097057343 MANF
	0,22241517	-0,0917449 CASP7
	0,333843925	0,111991405 ADK
	0,003590653	0,001102448 SEC13
	0,496454278	-0,116528988 HNRNPH2
	2,397725861	-0,304664135 EIF3B
	0,252049493	-0,06494379 BID
+	5,313620907	-2,034236431 NDUFV3
	0,112565345	-0,036716461 RRP1
	0,129021748	-0,035385132 MARS
	0,44157252	-0,78972578 ATP5I
	1,417701136	-0,320879936 EIF6
	1,548390229	-0,351057053 CTBP2
	0,945399929	0,262303829 SYNJ2BP;SYNJ2BP-COX16
	0,668492821	-0,129694939 NUP107
	0,844913128	-0,197089672 MTPN
	0,854907971	0,118001461 TPI1
	0,8407494	0,102824688 SEC61B
	0,866444157	-0,73032093 PPP4C
	0,102786206	-0,165719986 UBE2G2
+	1,808831603	-0,906865597 ACTB
	1,36645412	-0,240981102 EIF4A1
	0,369490842	-0,041227818 PSMA6
	0,24814486	0,145563602 S100A10
	0,306926231	-0,060462952 CDC42
	1,237397954	-0,212934017 DSTN
	0,344417397	-0,514881134 GMFB
	0,297221532	-0,083972931 RAB8A
	0,781476346	-0,129567623 SRP54
	0,005357813	-0,000833511 RAB2A;RAB2B
	0,255050517	-0,396330357 RAB5B
	0,592810627	-1,406074524 CKS1B
	1,337410017	0,252563953 RAB10
+	3,121172629	-0,393379688 UBE2M
	0,373551882	0,061122894 UBE2K
	2,286851811	-0,231118202 UBE2N;UBE2NL
	0,693117981	-0,121256828 RAB14
	2,197926999	-0,193729877 ACTR3
	1,240772983	-0,223190784 ACTR2
	0,096727152	-0,018731594 ACTR1A
	0,660338733	-0,069654942 COPS2
	0,195197917	-0,351716042 ARF3
	2,425555211	-0,282473564 ABCE1
	1,568924443	-0,252563477 RAP1B;RAP1A
	0,372244893	-0,042561054 PSME3

+	4,320888006	-0,348796368 RPL15
+	3,219492002	-0,747404575 MAGOH;MAGOHB
	1,031410203	-0,199431896 RPL27
	0,516747892	-0,147558212 ATP6VOD1
	1,056234387	-0,160464764 PCBD1
	0,116747155	0,022175789 RHOA
	2,873469841	0,20366478 HSPE1
	1,86753127	-0,462586403 VBP1
	2,587114094	0,310776234 B2M
	0,861178432	3,005496502 SUMO2;SUMO3;SUMO4
	0,152167319	-0,363472462 DCAF7
	0,573302533	-0,709893227 WDR5
	0,911137125	0,169989586 NUTF2
	0,055484858	0,007277012 HNRNPK
	0,747314045	0,115038395 YWHAG
	0,364136465	-0,712655544 RRAS2
	0,907382845	0,257689953 TIMM10
	1,97544118	-0,306707382 RPS7
	0,696520166	-0,06079483 PPP1CA
	0,053037865	-0,017459393 PPP1CB
	1,588470762	-0,154866219 PSMC1
	2,921204778	-0,224601746 PSMC5
	1,077243621	-0,171898842 RPS8
+	3,262720792	-0,450351715 RPS15A
	1,441481225	-0,3405509 RPS16
	1,595276277	-0,112051487 YWHAE
	0,212634291	0,149258137 RPS23
	0,917242015	-0,11573267 RPS18
	1,782511754	-0,253871918 RPS13
	1,201940422	-0,2368536 RPS11
	1,379598427	-0,25144577 SNRPE
	1,04031425	-0,217504978 SNRPF
	0,166810269	-0,330582142 LSM6
	1,204406669	0,306404114 SNRPD1
	2,106731814	-0,30409193 SNRPD2
	2,038564276	-0,401155472 SNRPD3
	0,227951313	0,162658215 TMSB4X
	0,76850165	-0,316924095 ARF6
	1,285607729	-0,179729462 RPL7A
	0,523532943	-0,685890198 POLR2G
	1,481931916	-0,282940865 CNBP
	1,588797753	-0,191308022 RPS4X;RPS4Y2
	0,135304005	0,193252563 RHOB
	0,58427239	-0,125074863 RPS6
	0,428619801	-0,128537178 HIST1H4A
	1,313258364	0,315258026 RAB1A
	1,912551777	-0,314135075 RPL23
	0,893796303	-0,125572681 RPS25
+	2,734146587	-0,427005768 RPS26;RPS26P11
	0,243520607	-0,055972576 RPS28

0,509487461	-0,125575066 GNB1
0,414074228	-0,594883442 POLR2L
1,358820764	-0,20263052 GNB2
0,446065768	0,062898159 RPL10A
0,446620584	-0,069955826 RPL11
0,235477078	-0,036052227 RPL8
0,333868111	-0,055925846 PPIA
2,294455157	-0,228496075 FKBP1A
2,494456615	-0,316302299 RPS27A;UBC;UBB;UBA52
0,914604318	0,158889294 GRB2
1,499715645	-0,220832825 TRA2B
0,611260135	0,098286629 RAC1;RAC3;RAC2
2,637645545	-0,208308697 AP2B1
0,442978716	-0,097554207 GNAI1
1,804752827	0,204172134 YWHAZ
1,060256949	-0,126558304 PPP2R2A
0,608143793	-0,226067543 DYNLL1;DYNLL2
0,99940905	-1,336907387 DYNLT1
2,538176079	-0,288896084 RPL38
0,427276531	-0,793848991 GNG5
1,371604821	-0,185033321 RPS21
3,566890982	-0,326172352 GNB2L1
0,405213786	-0,043572426 ACTG1
0,428457713	-0,693758011 SUPT4H1
1,81619361	0,398023605 UBE2I
1,006724462	-0,216883183 TMSB10
2,947820077	-0,348060131 PPP2CA
1,314382422	-0,156431675 YBX1
0,427564141	-0,159766674 CSNK2B;CSNK2B-LY6G5B-1181;
1,978148288	-0,534655094 TPM4
0,78317366	0,353081703 ACTA1;ACTC1
2,081039162	0,152178288 UBE2L3
1,271085019	-0,146959305 TUBA1B
1,472724904	-0,293507099 TUBA4A
1,863300111	-0,206072807 TUBB4B
0,437320038	0,060793877 PAFAH1B2
0,129645688	-0,064510345 HIST1H3A
1,023563874	3,579037189 HBA1;HBA2
1,469005337	1,261335373 NOP14
0,963731617	0,183151245 IGBP1
0,811664532	-0,399802208 SIRPA
0,290941706	0,072602749 RPP30
0,378610318	-0,246910095 PIP4K2B
0,016576677	0,015887737 SRPK2
2,212860205	-0,151509762 CCT2
1,516803224	-0,256961346 RAE1
0,575102549	0,084453106 GSTO1
2,584029368	-0,130945206 PRKDC
0,184027238	-0,261573315 ADAM17
0,649094364	-0,343735218 CRADD

	0,005818491	-0,003534317 IFI35
	0,919874174	-0,066914558 NUCB2;Nucb2
	1,525176326	-0,332060337 BASP1
	1,96990947	0,464151382 MRPS25
	0,239620844	-0,62031889 MRPS10
	0,884498739	0,145974159 MRPS35
	0,457905041	0,098521709 MRPS5
	1,957536351	-0,265716553 MRPS36
	1,167112041	-1,130595207 MRPS11
	0,04439583	0,029506207 MRPS6
	0,090292297	0,019526482 MRPS9
	1,339950455	0,797163486 HMGN5
	2,41884964	-0,256491661 SARNP
	2,098514927	0,307932377 LACTB
	0,437203771	-0,543375492 CBX1
	2,098632922	-0,264323235 ARF1
	0,229250911	-0,133293152 ARF5
	0,383940408	-0,091790676 ERH
	0,738546729	-0,17530632 RHOG
	0,473974268	-0,112169743 SRSF3
	0,853808922	-0,102899075 FOXK1
	0,886532808	1,275826454 CCZ1B;CCZ1
+	2,898083588	0,922234058 MUC5AC
+	1,735642313	2,899540424 HSPG2
	1,311866034	-0,552807808 XIAP
	1,063607147	-0,16258812 RBM10
	0,949423405	-0,188248634 RBM3
	0,433076188	-0,069504738 TFAM
	0,956835507	-0,143904209 PITPNA
	0,538358317	0,312416553 CDK6
	0,297336002	0,116569042 CDK5
	1,497221534	-0,324164867 PURA
	2,223822745	-0,511320114 NFKB2
	1,897925151	-0,182424545 FKBP3
	0,245783424	0,158723831 REEP5
	0,536882729	-0,156556606 SORD
	1,31647162	-0,108740807 HNRNPU
	0,557633035	-0,133643627 U2AF1
	2,621098163	-0,159244061 SPTBN1
	0,57368146	0,111800671 TIAL1
	1,595250752	-0,152616024 SET
	1,422557344	0,186908245 FABP5
	0,641679253	-0,104646683 CAP1
+	3,269641047	-0,402356148 HMGCs1
+	3,329791788	-0,716586113 SLC7A5
	1,23441831	-0,169810772 EXOSC10
+	4,184493091	1,4344697 OTUD4
+	4,209133803	-0,365093708 PFKP
	0,38054343	0,628417492 XPC
	0,285443297	0,126269817 OCRL

	0,163327305	-0,243015766	PLCB3
	1,302417147	-0,125593185	OGDH
	0,052401177	-0,14330864	ALDH6A1
	0,259072824	-0,056755066	MAP2K1
	1,571989631	-0,138926029	FKBP4
	0,189404166	0,035552979	PLOD1
	0,307452488	-0,039796829	NUCB1
	0,542126103	-0,068143845	RPL6
	0,572154351	0,127530098	TOP2B
+	4,20722277	-0,614117146	AKAP12
	1,663773658	-0,26610899	TNFAIP2
	0,048140997	-0,00718832	LMNB2
	0,593737467	0,086976528	CEBPZ
	1,358401049	-0,122262955	GLO1
	0,640104306	-0,0698843	AKR1C1
	1,548271131	-0,251528263	SSBP1
	0,392245957	-0,100108147	YWHAH
	0,611152359	-2,092146397	PLP2
	0,116311127	0,026983738	CSTF1
	0,771792515	-0,03944397	PTPN12
	0,101860149	-0,05069828	SRSF11
	0,558997722	0,116333961	PTPN11
	0,424404701	-0,633409977	PPAT
	0,623047528	-0,05776453	GFPT1
	0,428913667	-0,882288933	EXOSC9
	0,277343847	0,061798096	PSME1
	0,264531696	0,323509693	APLP2
	0,391877163	-0,537308216	GABPA
	0,393992512	-0,153559685	RING1
	0,187661072	-0,015667915	PRDX1
	0,949568761	-0,227712631	C1QBP
	2,53115621	-0,144436359	CKAP4
	0,298185944	-0,230397224	SPAG1
	0,754241482	0,117706299	KHDRBS1
+	2,839372041	0,455819607	BAX
	0,268205051	0,035235405	ARHGAP1
	1,019742818	-0,242370605	CYP24A1
	0,208213835	0,070721626	AES
+	2,107256041	0,624128819	PPP3CA;PPP3CB
	1,219535551	-0,133563042	DHX9
	0,807071902	0,090513229	CRYZ
	1,41785239	0,335057735	GOLGA3
	0,01570356	0,005036354	LGALS3BP
	0,482272634	-0,071296692	PPID
	2,56136635	-0,232010365	SSRP1
	0,176538186	-0,083918571	CAMSAP2
	0,305796501	-0,304131031	VAC14
	0,70267408	-0,071900368	NSUN2
	0,482516238	-0,10614109	RBBP4
	1,631992217	-0,299361706	NCBP1

	3,803727926	-0,237941265	AHNAK
	1,185646226	-0,264411926	HSPA14
	0,023833168	-0,04420948	MGAT2
	0,175321638	-0,030940056	GALNT2
	0,99868305	0,41666317	GALNT1
	0,030578397	-0,014043331	AP1B1
	1,277024425	0,300292015	CPSF1
	1,468248986	0,319543362	PMPCA
	1,240016685	-0,165504932	SCRN1
	0,047711674	0,021232605	KIAA0196
	1,518087306	-0,713027	GTF3C1
	0,603359362	0,065294266	TWF1
	1,530267909	0,222632408	ASPH
	0,553144828	-1,029201031	TFCP2
	0,678397849	0,194682121	AKAP13
+	3,269807164	0,766069889	STX4
	3,481051851	0,249636173	CNTN1
+	1,963383007	1,080222607	CDH17
	0,16948837	-0,039478779	SF3A3
	1,686208609	0,271988869	TRIM26
	0,774387093	-0,058448315	AIMP1
	0,047233622	-0,008649349	ILF2
	0,730029099	-0,038377285	ILF3
	0,914430643	-0,068368912	LMAN2
	2,103912867	-0,194244385	TRAP1
	0,396260527	-0,37421751	TRAF2
	0,742845414	-0,090132236	MYO1E
	0,236409318	-0,071983814	PPP1R8
	1,249785569	0,804201603	NFX1
	1,402585398	0,18452692	ECH1
	0,425990301	-0,154999256	MLLT11
	0,990895022	0,329377174	STRN3
	0,431625562	0,410718918	CDC16
	0,832614206	-1,245201111	STK4
	0,579802385	-0,119005203	FLII
	0,42472154	-0,339127541	TRIM32
	0,096897664	0,016484261	COASY
	0,380348351	0,060003757	ACACA
	0,102267263	0,064379215	CHAF1A
	0,84491765	-0,830289841	CHAF1B
	0,057316918	0,023923874	IK
	0,498537786	-0,573746681	PRKAA1
	0,752777968	-1,252891064	CALCOCO2
	0,072782684	0,014659405	HNRNPA0
	1,132947582	-0,165831089	AIMP2
	0,698751367	-0,21263361	FADD
	2,082191282	-0,218906879	PRDX4
	0,883710797	-0,067492485	PAK2
	0,77029435	-0,137073994	CBX3
	0,341916296	-0,431916237	STX5

	1,857482892	-0,196302891 PSMD2
	0,081756937	0,032683849 DNAJC3
	0,57601206	0,458504677 PAPPB
	2,634942546	-0,383777142 SRSF9
	1,358799471	-0,288875558 SRSF5
	1,192113148	-1,29956007 MAD2L1
	0,645142431	-0,068341732 TRIM28
	0,686322304	-0,733417511 NMI
	0,032079143	0,067419529 IFIT5
	0,679498418	0,16338253 CTBP1
	0,568748514	-0,100979328 DYNC1I2
	0,758345692	-0,253723145 XRCC4
	1,34750181	-0,151665211 TCOF1
	1,20328202	-0,118190289 SF3B2
	0,033441723	-0,081683159 OS9
	0,459020975	0,101376057 PDAP1
	0,121466344	-0,204300404 TMED1
	0,153990471	-0,071181774 FKBP5
	0,8855636	-0,116950512 PICALM
+	4,166910647	-0,39841938 SQSTM1
	0,072030652	0,026569366 MTX1
	0,024428568	0,006366253 TUBB3
+	2,499044612	-1,153269768 PRPF4B
	1,956651369	-0,298610687 PIN1
	0,662296995	-0,289321899 EIF4EBP1
	1,182758096	0,547173023 RIPK1
	1,205191965	-0,430925846 HDAC1
	1,204358883	1,318874836 PKD2
	0,155374849	-0,063184261 NAE1
	1,036218572	-0,141214848 SNW1
	0,399199458	0,118360043 TRA2A
	0,268953578	0,063281536 SNX1
	2,278173875	-0,313963413 CUL3
	0,594764632	-0,250261784 CUL4A
	0,094573994	0,038640976 TSTA3
	1,637151151	-0,672353268 RAB31
	1,239987384	-0,279932022 RAB32
	2,14788217	0,415928364 TPBG
	1,363567186	-0,122116566 ALCAM
	0,257165249	-0,04117918 THOC5
	0,530520449	-0,106911659 GNL2
	0,474524791	-0,074016094 DDX39B
	0,739244266	-0,228510857 BLMH
	0,887818967	-0,426997185 EXOSC2
	1,458738129	-0,605295181 TUBB2A;TUBB2B
	0,292937853	0,034999371 BYSL
	0,047352788	-0,00846386 IDI1
	0,925480841	-0,734955311 CBFB
	0,97353175	-0,104038239 CKAP5
	1,016683987	-0,172510624 CIRBP

	0,051132532	0,007103443 COTL1
	1,481669675	1,370909214 BAAT
	0,147787511	0,029811859 IL18
	0,515669594	-0,397354603 DAG1
	0,603494156	-0,060809135 DSG2
	0,822781355	-0,343048573 BOP1
	0,71421224	0,227974415 SAFB2
	1,045075092	-0,062270164 EIF3A
	0,381697431	0,083359718 MLEC
	0,778465819	-0,117978096 TTLL12
	0,656157557	0,15849638 FHL2
+	1,790611181	-1,480943203 DPYSL3
	2,934415254	-0,152731419 DYNC1H1
	0,014694454	-0,035871506 EIF2B1
	0,946926213	0,266077995 EIF4A2
	2,402367778	0,247880936 MAP7
	0,24856791	-0,01738596 CTTN
	0,187397508	0,182585239 ENDOG
	0,627056957	-0,109853745 FLOT2
	0,659011587	-0,175275803 RCN2
	0,881642069	-0,092017174 TRIM25
	1,176192694	-1,033002853 FKBP8
	1,260049036	0,297497272 FAM50A
	0,6229267	-0,283316612 GNA13
	1,402647693	0,170821667 GALE
	0,12035211	-0,161261559 PDE3A
	1,889446111	-0,236901283 CAPRIN1
	1,263410231	-0,168510437 RBM39
	0,655336864	-0,482824326 PDIA5
	1,428813998	-0,29613924 PRPSAP1
	3,490825405	-0,224954128 MCM6
+	2,850477515	0,478495598 ITPR3
	0,721293773	-0,082574844 PLS1
+	3,089427938	-2,321201324 LAGE3
	0,276514251	-0,053350449 CLINT1
	3,252657663	-0,23711586 SMC1A
	0,288997636	-0,069124699 RRP1B
	1,22439451	1,856111526 GINS1
	0,309588991	0,038426399 BMS1
	0,631848874	-0,058970451 USP10
	0,534876655	0,129495621 MESDC2
	3,278617374	-0,151751041 GANAB
	0,109758322	-0,026982784 LBR
	0,489436841	0,060460567 MVP
	0,895423471	-0,111508846 GOLGB1
	0,780842592	0,192052841 NAA30
	0,800996728	-0,171939373 LASP1
	0,682417	0,123090267 PTGR1
	0,769532614	0,111227512 ZNF638
	2,550075675	-0,244194031 KPNB1

2,720299271	-0,255056858 NUMA1
1,50227472	-0,872588158 PSME4
0,130863882	-0,039902687 NAA25
2,297092368	-0,393657684 NCAPH
0,012290688	-0,004639626 KIAA0101
0,257212074	0,087770462 EMC2
1,157902571	-1,17561245 WTAP
0,622102939	0,066675186 PSMD6
0,061404372	-0,10819149 MORF4L2
0,985779993	-0,321464539 FAM175B
2,198243945	-0,18405056
1,461587689	-0,193110943 NCAPD2
0,569817259	0,631123543 EXOSC7
2,441213657	-0,179254532 EFTUD2
0,484639594	-0,545423031 SNX17
0,729074263	-0,219558716 RAB3GAP1
3,299807101	-0,291862488 KARS
0,684460276	-0,219184875 RRS1
1,518682073	-0,146636486 EIF4H
0,479173963	-0,50980711 KIF14
0,349693562	0,049876213 WDR43
0,371083352	-0,066936016 ACOX1
2,399507903	0,432849884 EEA1
2,520210566	-0,195524693 PDIA6
1,529836759	0,06192255 PAFAH1B3
1,059902379	0,123081684 PEA15
0,728701763	-1,320213795 EBP
0,425615084	-0,106269836 PMVK
2,57179782	-0,181019783 PLEC
1,430180623	-0,687613487 TCEAL1
0,747065901	-0,058064938 PPA1
0,631843577	-0,711255074 STK38
0,458237	-0,095106125 PPP2R4;DKFZp781M17165
0,946577041	0,126072407 RCN1
4,027914214	-0,307087898 PCBP1
2,733583793	-0,218331337 PCBP2
0,428301523	-0,617888451 UBE3C
1,228828211	-0,148134708 SF3B3
2,829658074	-0,301076889 KIAA0020
0,337461421	-0,164415836 RSU1
0,928206409	-0,154262066 CNN3
0,404284841	-0,072962761 SAFB
0,238640557	-0,068638802 SF3B4
0,63122666	-0,134251595 SF3A2
0,661240688	0,127990246 PPP1R7
1,325741925	-0,121544361 SF3A1
0,086913781	0,158303261 SKIV2L
0,409091265	-0,119971275 SURF1
0,452952023	0,395656109 SURF2
0,88315199	-0,218469143 MAPRE2

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	0,49994201	0,152959347	TGFBI
+	1,844977241	-0,994673252	SLC9A3R2
	0,057006873	0,024692535	TRADD
	0,169892084	-0,037972927	TRAM1
	1,99087347	-0,289013863	TRIP10
	0,305947819	0,175001621	TRIP11
	2,321606951	-0,389546394	TRIP13
	0,052735085	-0,083771229	MED1
	0,663911733	0,086508274	TRIP6
	2,670844075	-0,335312843	MAPRE1
	0,84708152	-0,12964344	ELAVL1
	1,922023034	-0,213352203	NSDHL
	0,16172362	-0,173407078	TAB1
	0,95954427	0,33793211	HERC1
	2,237179938	-0,507723808	SLC1A5
	0,163873636	-0,312096596	MLF2
	1,123759525	-0,229024887	TOMM34
	1,970967313	-0,332456589	SMAD2
	0,897269619	-0,290641308	STXBP2
	0,417389795	-0,092820644	VAMP3;VAMP2
	1,548117744	-0,440775871	NEDD8
	0,968794285	0,094582081	RAB11B;RAB11A
+	1,838711885	-0,824762821	EZH2
	1,272302151	-0,150569916	ZYX
+	1,474813411	2,883953094	ADRM1
	0,546154239	-0,227679729	CCDC6
	1,603369688	0,237061501	INA
	2,875888753	-0,312884331	PSMD5
	0,418042163	-0,226122379	PKN1
+	1,86812253	-1,480671406	PPP2R5E
	0,696316465	-0,155241966	MRPL23
	0,502642233	-0,141205311	CDC37
	0,278999094	-0,568781853	SYPL1
	0,364981546	-0,052321434	RBBP7
	0,941860461	-0,178507328	DBN1
	1,739859975	0,22790575	FSCN1
	0,152185193	0,023675919	DECR1
	0,077968059	-0,096036911	MAN2A1
	0,088989737	-0,020517826	NDUFA5
	0,324707248	-0,064222336	KYNU
	0,048667932	0,013362408	CLPP
	0,628519628	0,152927876	TST
	0,284114362	0,098389149	UBE2S
	0,585961096	1,781630516	HIST2H2AC;HIST2H2AA3
	0,130306866	-0,03948307	NDUFA9
	0,044442041	0,016527176	PCK2
	0,828256524	-0,661804199	UPP1
	0,625055371	-0,332399368	CYP51A1
	0,172484351	-0,027701378	UGP2
	0,035964789	0,01014185	ATP6V1F

	0,570526436	-0,046826363 IMMT
	1,11121201	-0,431728363 KNOP1
	3,276362078	-0,245164394 HNRNPUL2;HNRNPUL2-BSCL2
	0,547291663	-0,093138695 ARHGAP12
	1,334529116	0,279967785 PDS5A
	1,087751841	-0,73200798 ERCC6L
	2,009146201	-0,362812519 TSR1
+	1,880093177	-0,830016613 QRICH1
+	3,348376432	0,365879059 RELA
	0,199167724	-0,061387062 SMU1
+	4,392019479	-0,726612568 HKDC1
	0,109556734	0,022167683 LRRFIP1
	0,00067666	-0,001263142 LEPRE1
	0,016649306	-0,006548882 TRMT5
	0,263628823	-0,039524078 NME1-NME2;NME2;NME1;NME2P1
+	2,639533935	0,685329437 LMNA
	0,002695489	0,003544807 UAP1L1
	1,072324095	0,116385937 DAK;TKFC
	0,434522686	-0,173947334 RABL6
	0,692555636	-1,168767452 VMA21
	2,394764052	-0,264137745 TIMM50
	0,213437799	-0,331953526 GLYR1
	0,125660723	0,075150013 VPS26B
	0,23438055	-0,385961056 LARP7
	0,270853694	-0,282517433 ACSF3
	0,059895473	-0,028209209 TBC1D10B
	0,246132301	-0,263453484 GRIPAP1
	1,748596192	-0,335558891 FAM98B
	0,448606106	-0,801292896 PDCD4
	0,950486202	0,826273441 FNDC3B
	0,572248201	-0,379253387 AGPAT9
	1,462769775	-0,202939034 CEP55
	0,213157145	-0,365224838 C17orf85
+	4,889671354	0,464577675 TP53I3
	0,78762623	-0,844206333 C11orf73
	0,684647931	-0,119217873 HSD17B12
+	3,614899719	-0,435444832 USP39
	0,619202995	-0,119843006 AGK
	2,206481561	0,221721172 LACTB2
	0,994786821	-2,196103573 ANKRD39
	0,696223663	-0,148315907 BOLA3
	0,293999881	-0,046428204 HS1BP3
	0,282950978	0,111224651 OCIAD2
	1,974991722	-0,30524683 HSP90AB2P
	0,749549589	0,354795933 TMEM41B
	1,395540511	0,305610657 ODF2
	2,283383381	-0,218779087 ZNF326
	0,09142172	0,037608624 HERC4
	0,780629726	-0,226857185 MAGED2
	0,237127981	0,071586132 SH3KBP1

0,745906824	0,221674919 MIA3
0,553295441	-0,686168194 WDR44
0,170474607	0,041558266 RRP12
0,294484293	0,051161766 COA6
1,202095557	-0,328870296 CRNKL1
0,518032643	-0,201111794 DNTTIP2
0,233847935	0,086344242 MRPS18A
1,433745381	-0,314568043 RALY
0,452580716	0,115960121 PSMF1
0,229508124	0,414924622 COX20
0,630502575	-0,714743137 EXOSC6
0,638116593	0,17868185 NUP188
0,954801934	-0,159579277 HP1BP3
0,173063075	-0,256735802 NOL9
0,011315414	-0,033905506 ABRACL
+	1,51747904 1,43343401 GSN
	0,087152505 -0,100729942 FNBP1L
	1,959068979 0,347281456 SH3BGRL3
	0,268921217 0,108972073 FLAD1
	1,193440996 -0,386270523 FKBP15
	0,295699403 -0,612542152 C9orf114
	1,233343577 -0,104976177 HEATR1
	0,299597427 0,31408596 HECTD3
	2,945578161 -0,261328697 UBR4
	0,287543055 -0,635655403 HNRNPK
	0,998562122 0,220911026 C9orf41
+	3,9629877 -2,492995739 MED4
	0,037756425 0,031656265 DCAF8
	0,009756809 -0,001788616 TPM2
	0,854278626 -0,739033222 SMOX
	1,575286137 -0,476420879 HIST2H3PS2
	0,24084526 -0,03200531 NT5DC1
	2,063506713 -0,390512943 NDRG3
	0,197911191 -0,209759712 DNAJC11
	0,272890383 -0,7707901 LYRM7
	1,112062686 -0,424377918 RIF1
	0,803715235 -0,731776237 RPRD2
	0,828998556 -0,808110237 RNF20
	0,449607922 -1,316464901 ARHGEF16
	1,068159175 0,200666428 BROX
	0,242345542 0,123034 FOCAD
	0,241780585 -0,104540348 LYPLAL1
	0,90144672 -0,860321045 HAUS6
	0,099765699 0,047121048 DENND4C
	0,049489069 -0,067053795 NAA35
	0,323520005 0,339905739 TOR3A
	0,353038638 0,279492378 KANK2
	0,455600854 0,201980114 TBC1D9B
	0,022738408 -0,004322052 MAP1S
	0,094477201 0,103171825 GLT8D1

+

2,693776143	-0,208643436 TNS3
0,093912894	-0,118942738 INTS3
0,134535009	0,211250782 CWF19L1
0,639736503	-0,260319233 ZNF787
1,39604738	-0,307076931 RPL7L1
0,521401998	-0,268371582 NXN
0,113432381	-0,216719627 TTC19
1,816212044	-2,454294682 POLR2M
0,689305683	-0,086135864 CIAPIN1
0,222831961	-0,078337193 SMYD5
0,505604707	-0,152312756 PTRHD1
1,402815553	-0,857495308 THOC7
0,857389039	-0,884575844 ELP2
1,325408106	0,142281055 LAMTOR1
0,392683576	0,09632349 TWF2
1,306465139	-0,382714272 MRM1
0,556856965	-0,122006416 SLC25A24
1,312948268	0,40396595 RETSAT
0,744370069	0,144693851 TMEM214
0,631840192	0,099792004 HIBCH
1,531855062	0,100311279 PTRF
2,866745583	0,310019016 ZCCHC8
1,578493863	-0,341897011 CDC73
0,77998415	0,237271786 MRPL14
0,155657832	0,352109432 SLC27A4
0,082053279	-0,135396004 CC2D1A
0,663637599	-0,938213825 METTL2B;METTL2A
0,234786767	-0,088432789 EDC4
3,329497234	-0,293735981 PRPF8
0,138175498	0,15592432 TTC27
1,213961879	0,486569405 MTFR2
0,371884543	-0,08728838 FAHD1
1,298451463	0,289794922 ALKBH5
0,35629253	-0,286612988 TLDC1
1,806293666	0,648972988 PGM2L1
1,318539747	-0,697255135 CTR9
0,934320453	-1,26585865 AAGAB
0,45086294	-0,155838013 TTC37
1,449140248	-0,191316128 DARS2
1,082549852	-0,193811417 BRAT1
1,591283565	-0,419537544 ZC3H14
0,101385138	-0,013751507 LARP1
0,308754896	0,214678288 ATAD2
3,67896492	-0,554641247 CPLX2
0,936127149	-2,164755344 WDR74
0,780220418	-1,565181255 AGTRAP
0,732263867	-0,494020462 FIP1L1
0,760552191	-0,947332382 LCLAT1
1,508219923	-0,302567005 WDR82
0,243863799	-0,042372227 MPRIP

+

	0,379420857	0,681287289 RAB11FIP1
	1,661660965	-0,290422916 GIGYF2
	1,33448779	-0,322203636 CD109
	0,419972109	0,09286499 HSDL2
	0,016824913	0,037777901 GDPGP1
+	2,366435908	-0,508508682 QSOX2
	1,189191811	-1,281790257 TMTC3
	0,097765131	0,054342747 VPS13C
	1,541572563	-0,678147316 UNC13D
	0,102468346	-0,017495155 IKBIP
	1,084840211	-1,708661079 UBE2R2;CDC34
	2,227183675	-0,325819016 LARP4
	0,546917744	0,103700638 SUPT6H
	0,569666486	-0,053770542 SND1
	0,117711423	0,022163391 TRMT10C
	2,490923002	0,18710947 BZW1
	0,381507424	0,264621258 ASRGL1
	0,526206173	0,481453419 KCTD9
	1,939804946	-0,220064163 EIF3M
	0,011176082	-0,025436401 MEPCE
	1,366382527	-0,114247799 CYFIP1;CYFIP2
	0,948392312	-0,858931065 NDUFAF7
	0,70818874	-0,30603838 FA2H
	1,230541828	-0,949168682 TAOK1
	0,734059163	-0,665132523 FASTKD5
	1,580397556	-0,155797958 MOB1B;MOB1A
	0,20009186	-0,071476936 KDM3B
	0,930189089	0,321214676 HS2ST1
	1,003821919	-0,22932291 PHF5A
	0,142980377	-0,040841579 ERMP1
	0,032282315	0,013356209 TRMT1L
	0,158445743	-0,026547432 ZC3HAV1
	0,85300767	-0,159814835 EFTUD1
+	2,801606069	-0,718114853 NUP54
	1,481153377	-0,489643097 POGZ
	0,183457002	-0,055876255 ZFYVE16
	1,074155271	-1,156857491 MYH14
+	3,016171335	-0,466455936 NUFIP2
	0,038797808	0,009559631 MAVS
	1,264468416	-0,863983154 TRMT11
	0,719440954	0,116913795 HDDC2
	0,049817782	0,010848522 HDGFRP2
	1,367772735	-0,311908245 IRF2BP2
	0,048467061	-0,116158962 RBBP6
	0,021236418	0,003910542 HUWE1
	0,56895012	-0,223807335 CTU1
	0,431443843	-0,552485466 UBE2Q1
	1,738819932	-0,376978874 TMED4
	2,119329931	-0,51325655 MRPL10
	0,002200152	0,006057262 CENPV

+

2,374712323	0,326201916 GALNT7
1,25875214	0,7215662 GALNT10
0,211383136	-0,52840519 GLRX5
1,025202513	-0,37859869 PATL1
0,719276367	-0,811126709 DPP9
1,225754215	-0,266392231 SETD3
0,790968134	-0,642210007 METTL3
0,113692134	0,631757736 PBRM1
1,253809368	0,200070858 MTDH
5,233066717	-0,758464336 RTN4RL2
0,395290721	-0,02789402 KTN1
0,274369875	-0,134026527 LUZP1
0,907893105	-0,188805103 IQGAP3
1,6171294	-0,146901131 CAND1
0,144927261	-0,197281837 FAM134C
0,022183862	0,013088226 HOOK3
1,289378854	-1,705842972 THOC6
0,960810596	-1,472508907 METTL16
0,109132556	0,025408268 NR2C2AP
0,606440621	0,845446587 CCDC25
0,085958898	-0,10017252 LSR
0,407264887	-0,129881859 CARM1
0,431796207	0,156958103 NIT1
0,547487011	-0,111216545 COMMD2
0,10261303	0,146127224 NCAPG2
0,653549185	-0,158887386 ANKLE2
0,0283373	-0,006410599 DDX42
0,557575238	-0,262560844 SPATS2
0,17697838	-0,021895409 DNAAF5
0,309644956	-0,324549675 PTRH1
0,605272433	-0,395679951 STX12
0,241317671	-0,416497231 GATAD2A
1,417798825	-1,054696083 HPS6
0,427096751	-0,553828716 IRF2BP1
0,780288978	-0,765169621 RHPN2
0,155692955	-0,272070408 MINA
0,438721929	0,250526905 CRLF3
0,561920009	-0,57659626 PLD3
0,081403367	-0,136748791 ERI1
0,680252023	-0,035055637 AHNAK2
0,183295952	0,250743866 LEPREL2
0,111379026	-0,042194366 MCAT
1,314912105	-0,163756847 MISP
0,134910162	-0,298769951 WDR75
0,229728225	-0,330354214 ITPRIP
0,510215245	0,105539322 FAM114A1
0,371086789	-0,383839607 GCC2
0,0843421	-0,018121243 CHERP
0,101437134	0,036679268 CCAR1
0,412286183	0,124298573 MRPL41

	1,299733397	0,194599628 RBM12B
	0,230535549	-0,376439571 SMC5
	0,275625691	-0,411299229 CMIP
	0,876677569	-0,554476738 YAF2
	1,12395852	-0,289750099 FTSJ3
	0,046635903	-0,089487553 SMAP1
	0,258601158	0,317734718 SUPV3L1
	0,428671779	-0,121979237 GSPT2
	0,440565486	0,371611118 EXOC8
	0,362770996	0,487689018 THNSL1
	0,029935681	0,011012554 PM20D2
	0,0099083	-0,003420353 MICU2
	1,231281911	0,120912552 ALDH16A1
	0,547477702	0,555594444 TNS4
	0,83615414	0,132980347 SPG20
	0,498722044	-0,211900711 TTC5
	3,075628545	-0,232077599 CCAR2
	0,522214058	-1,399042606 NDUFAF2
+	3,3000701	-0,399015903 NUP93
	1,915794851	0,214764118 LRRC47
	1,026704867	-0,534957409 ARHGEF28
	2,535948101	0,239989281 ARMC10
	1,485411054	0,22619772 ABHD12
	0,238989853	-0,067243099 SLC35F6
	1,282989588	-0,356705666 ARHGAP18
	0,197317708	0,058851242 ASCC3
	1,094800205	-0,309647083 EHBP1L1
+	2,883394102	0,416883945 MPP5
	1,35752936	-0,895886898 STAG2
	1,000439974	0,366921425 SYNPO
	0,470877803	0,105933666 HDDC3
	1,387470503	-0,523467064 CHCHD4
	0,157256234	-0,062485218 MMGT1
	0,975221919	-1,215476036 OGFO1
	0,209633906	-0,055994987 CISD2
	0,704028868	0,541540623 JAGN1
	2,174170922	0,292311668 MRPL50
	0,577138851	-0,66689539 COMMD1
	0,242417844	0,083300114 CPSF7
	0,395540376	0,089921474 METTL13
	0,780119793	-0,141096115 ARFGAP1
	0,044297392	-0,008390427 EMC1
	1,546265426	-0,403441429 PAF1
	0,324780211	-0,066036701 UBR7
	0,158177764	-0,165039539 DDX51
	0,834365259	-0,113072872 ENAH
	0,013496554	0,005841732 ZNF579
	0,203285899	-0,252915382 MLKL
	0,128341653	0,276379585 PDDC1
	0,589998983	-0,781637669 ANKRD52

	0,323994928	-0,060662746 NHLRC2
	2,839148881	-0,268045902 GOLM1
	2,911138291	-0,370085716 COLGALT1
	0,21553478	0,287709236 POGLUT1
	0,324820859	-0,2264781 RDH13
	0,183548904	0,036262512 TXNDC5
	0,382294403	-0,76747036 SPC24
	0,632686884	-0,124016762 ATAD1
	1,937270717	0,432074547 SCCPDH
	0,166507563	-0,034699917 SERBP1
	0,83925185	-0,076700687 LEMD2
	0,6687646	-0,223240852 NOA1
	0,620156858	-0,118423939 FAM98A
	0,056831191	0,015347958 APOA1BP
	0,072577176	0,031501293 NPEPL1
	0,029297794	0,012693882 EHBP1
	2,444944416	-0,207793713 ABCF1
	1,639768236	0,296478748 MCU
	0,78761735	0,366596222 SRFBP1
	0,349014735	-0,162766933 PDZD8
	1,503425794	0,385771275 FBXO22
	0,110842548	0,178956985 LPCAT1
	1,343482066	0,312502861 SYNE1
	1,507900559	-0,287277222 BOD1L1
	1,270205845	-0,299222946 NUP43
	0,136716178	0,045983315 NUP37
	0,499491675	0,55623436 NUP35
	0,315625843	0,12189579 GPRC5A
	0,313220103	-0,049375534 TOR1AIP2
	1,224761973	0,472242355 CMAS
	1,808023107	-0,462844849 ATL2
	0,469871762	0,110890388 MCFD2
	0,464648957	0,123108864 THOC2
+	1,572757202	1,81987524 GADD45GIP1
	0,584515887	-0,074922085 NPLOC4
	0,731878493	-0,650802135 GOLGA5
+	2,161065659	-1,739556313 KLHDC4;DKFZp434G0522
	1,007521624	-0,177759171 UBA3
	0,031642988	0,012223244 PIP4K2C
	0,240544119	-0,067556381 TBC1D15
+	4,160921973	-0,590817451 RDH11
	0,108280186	0,024561882 NT5C
	0,752904882	0,279019356 STT3B
	0,451671357	-0,09254694 PNPT1
	0,127721206	-0,222308159 BICD2
	1,273756233	-0,204107761 DDX54
	1,446452131	-0,228197575 BRIX1
	0,095162619	-0,071298122 DTD1
	0,864951287	-0,175213814 NUP210
	0,241031413	0,044986248 GEMIN5

	0,806274755	-0,104730606 IPO4
	0,064170816	-0,082435131 PPP4R1
	1,27227661	-0,643507004 UBASH3B
	0,236271274	-0,593069077 GIPC2
	1,746643445	0,532517433 WIPF2
	0,109148278	0,037385464 NOC3L
	1,474066949	-0,25492382 ZC3H15
	0,635077443	0,291044712 GTF3C2
	1,83785303	0,17137146 TSKU
	0,507558262	0,878433704 TMEM263
	2,241220403	-0,294644356 NUP133
	0,222172382	0,013277054 PDCC6IP
	0,75942618	-0,246522903 BRK1
	0,989020748	-0,172540188 CHMP7
	0,196860049	0,082015991 THEM6
	0,046864076	0,101872444 NSMCE1
	0,734864225	-0,166147232 LEO1
	0,318640568	-0,173518181 TFB1M
	1,621025427	0,346655369 POF1B
	2,101362068	0,435122013 UBLCP1
+	2,465455355	0,714165688 PCNP
	1,316900173	-0,92864275 C2orf47
	0,430055969	0,37974453 TRUB1
	0,810918926	-0,127825737 ATXN2L
	0,190530136	-0,146130085 PHIP
	0,073313399	0,021101475 PALLD
	0,052276034	-0,077215195 SREK1
	0,943973204	1,011601925 GEMIN6
	2,182199787	0,20152235 PSPC1
	1,631502049	0,216763973 SYNE2
	1,50734678	-0,466989517 DNAJC9
+	4,413582554	3,292779446 AHCTF1
	0,231659692	-0,034772873 OVCA2
	0,170683576	-0,052968502 IRGQ
+	3,005435098	0,55636549 DDB2
	0,306367517	0,064541817 FAM3C
+	4,40903614	-0,565301895 H1FX
	0,619506818	1,074675083 RTF1
	0,538607719	-0,096387863 NCSTN
	0,158463527	-0,030767918 MRPS27
	0,324980403	0,434389591 WASF1
	0,262100239	0,0822258 AP3S1
	0,001203836	0,000720978 UBXN4
	0,518757834	0,470062733 PHF3
+	3,555915204	0,64449358 NDRG1
	2,245176373	-0,148839951 HSPH1
	1,557111426	-1,038430691 RQCD1
+	2,529446633	-0,513130188 LPGAT1
+	3,189261893	-0,776418686 TBC1D5
+	4,654507369	-0,403973103 LARP4B

	3,962323454	-0,188268185 GCN1L1
+	3,439459091	0,467178822 DHX38
	1,679727479	-0,171189308 NUP205
	0,710914325	-0,961900234 MRPS31
	0,505632655	-0,182782173 AKAP1
	0,728872536	-0,137493134 ANP32B
+	1,409630787	-2,685304165 PVRL2
	1,24830241	0,376950741 HTRA1
	0,413091571	0,105219841 HDAC2
	0,218425257	-0,570240974 STAM
	1,463690047	-0,148066044 CREBBP
	0,022875554	0,008190632 SYMPK
	0,162959411	-0,055418968 TAF15
	0,347344188	-0,279492855 GOLGA1
	0,963047059	-0,223722458 GGH
	0,080546975	0,049537659 NEO1
	2,289055413	-0,218225956 RAD50
	0,031974682	0,015818119 OSTF1
	0,022807859	0,006146431 ABCC2
	0,368193139	-0,080051899 UFD1L
	0,296187485	-0,021819592 GLG1
	1,684714843	-0,163456917 UPF1
	0,385585103	0,118014336 COPS5
	0,492266847	-0,120398998 GPKOW
+	3,146194333	-0,443586349 SMARCC1
	1,382061339	0,519600391 RAB8B
	2,355608546	-0,240266323 TNPO1
	0,55518132	-0,350193501 EMG1
	0,911061908	-0,097157001 USP9X
	0,647303473	0,12676096 ATP6V0A1
	0,268780554	0,032922745 LPP
	0,375703846	0,617702007 PTP4A1
	0,346849717	-0,090768814 HGD
	0,126063345	0,064008713 TSR2
	0,409011943	1,300062656 RFK
+	4,280372822	-2,700436115 MYDGF
	0,432047689	0,102926731 YIPF5
	0,251720011	-0,604541779 RPL36AL;RPL36A
	0,298037999	-0,115862846 ZNF622
	0,226199554	-0,081635475 NCLN
	0,220472531	-0,204896927 MUL1
	1,009465686	0,083463192 ERGIC1
+	3,201859526	-0,401497364 TBRG4
	0,49457218	-0,177403927 PPP1R14A
	0,235319664	-0,067783833 CCDC47
	1,376561293	0,25829649 MRPL24
	1,113845851	-0,20208931 SYAP1
	0,640712857	-1,112969398 TMEM230
	1,707267886	0,267930984 EXOC4
	0,876652774	0,22371769 ISOC2

	0,589011889	-0,517892361 FERMT2
+	1,979336343	1,1977458 PNPLA2
	3,560672679	-0,18282795 LRRC59
	0,780326762	-1,766212463 PBXIP1
	1,817949106	-0,428495884 KIAA1143
	1,732169135	-0,471045971 RPE
	0,217947618	-0,074860096 FKBP10
	0,197892783	0,066440582 CLDN23
	0,332532463	0,099854469 AKT1S1
	0,178468505	0,214755058 RNF25
	0,290066108	-0,097168446 AIDA
	1,427411295	-0,56959486 COA7
	1,510899218	-0,492930889 TESC
	0,626507469	0,611678123 PTER
	1,377046064	-0,179232597 FAM136A
	0,051617823	-0,01350069 EFHD2
	0,237667576	-0,102982998 GALM
	0,876815149	-0,191571712 DCPS
	0,038784293	0,136965275 PPP1R14B
	0,492513703	-0,40161562 ISOC1
	2,678999724	-0,372558117 FAF2
	0,010082621	-0,002634525 CCDC124
	1,069436625	-0,258839607 OPTN
	1,397041636	0,401755333 CMBL
	0,110202332	0,04618597 MSI2
	1,681329466	-0,194860458 SNRNP40
	0,406590753	0,125832558 MRPL38
	0,380779376	0,91793251 ASB9
	0,217318344	-0,239857197 MRRF
	0,792849696	-0,342840195 TCEAL4
	0,04763987	-0,0211339 GNP NAT1
	0,166312515	-0,097513676 KTI12
	0,639623951	-1,227645397 MRPS24
+	2,055454148	-0,720200539 MRPL53
+	2,468371169	-0,920308113 SAAL1
	0,616715993	0,196098328 CCDC51
	0,587509131	-0,118300438 DNAJA3
	0,936405387	-0,14467907 PTC D3
	1,581415501	0,137151718 MMAB
	0,087932662	0,034805775 CCDC97
	1,601631797	-0,561728001 EDC3
	0,556725404	-0,257689476 CPNE2
	0,791948687	0,223082066 DTD2
	0,496485844	-0,131993771 S100A16
	1,097103247	-0,311662197 TRMT61A
	0,239898602	-0,104914665 PGM2
	0,147539528	-0,076717377 SDSL
	0,363762868	-0,481618404 VMP1
+	1,580754059	-1,383579254 AURKB
	0,258498688	-0,384530544 TMEM261

	0,43421973	0,102783203 FAHD2A;FAHD2B
	0,126553229	-0,528460979 SMARCD1
+	1,490826562	1,75675869 APIP
	0,167046068	-0,175372124 MYO19
	0,82486044	-3,530167103 ZC3HAV1L
	0,775450525	0,095740795 PDLIM5
	0,788512229	0,0649786 ERO1L
	1,229759291	0,237660408 PGAM5
	0,563117187	-0,480051041 TMEM41A
	0,00046065	0,000565052 DHTKD1
	0,53612614	0,100859165 FUBP3
	0,685214234	-0,8080163 RBM17
	0,057961009	0,102974892 NARS2
	0,534117143	0,076925755 SUCLG2
	0,421248271	-0,115476608 GMPPA
	0,914067274	0,502152443 BOD1
	2,159731042	0,3404212 ABHD14B
	0,677143372	-2,284226894 USMG5
	3,25863051	-0,25089407 PAWR
	0,352871938	-0,07961607 THOC3
	0,072980456	0,031945229 CDK5RAP3
	0,169115717	-0,070240021 VCPIP1
	0,117157805	0,038645267 TMX3
	0,425181732	-0,110387802 CHAMP1
	0,845341694	0,247338772 CLMN
	0,446556119	0,179175854 ZNF668
	0,829111631	-0,377164364 USP47
	0,029564203	-0,005435944 PBK
	0,640649869	-0,305438519 DNAJC1
	2,171308124	0,144620419 CNDP2
	0,675657808	-0,190208912 ZFR
	0,0667817	-0,018103123 EP400
	0,152208565	-0,255047321 SNX27
	0,726541523	-0,877100468 IFT74
	1,666153996	-0,288491726 TRIM47
	1,180627257	-1,184316158 UBE2E2;UBE2E3
	1,474192932	0,341582775 PRRC1
	0,182891598	0,097980499 NDNL2
	2,338827418	0,368162155 MBOAT7
	0,040562619	-0,063868523 NSUN5;NSUN5P2
	0,019432452	0,005947113 IPO9
+	2,468639642	0,488807201 DCBLD2
	0,223920036	0,020308018 RBM14
	0,292995349	-0,439250469 QKI
	0,00134441	-0,000424862 PUS7
	0,453953108	-0,07267046 TRNT1
	0,341157703	0,118482113 PPP1R10
	1,972822132	-0,169513702 VPS35
	0,317702935	0,209748745 PURB
	0,06617367	-0,021271706 SPAG5

	1,494629226	-0,679433823 UIMC1
	0,063052725	0,013799191 GFM1
	0,031690014	0,012337685 NUDCD1
	0,604467815	0,170911789 ERBB2IP
	1,323818983	-0,163571835 PIGS
	2,328539624	-0,387176991 PPP1R9B
	0,352179254	0,096107483 SRPK1
+	1,827651703	-1,84078598 SMC6
	0,263301876	-0,554719925 STRBP
	2,236319121	-0,395784855 CYP2S1
	0,634878999	-0,173207283 IWS1
	0,397691798	-0,177928925 SIN3A
	0,024513538	-0,012003422 OSBPL9
	1,230636291	-0,261860847 RUFY1
	2,249670853	-0,480961323 MMS19
	0,159754038	0,045199871 FAM129B
	0,794007764	0,124526501 YME1L1
	0,948148448	-0,166493416 TBCB
	0,511769399	0,093275547 PSMB7
	0,009758333	0,002480984 CDC5L
	0,234440815	-0,021856785 PSMD1
	1,120665037	-0,235180855 PFDN5
	0,396870686	0,113380909 RNF2
	1,502620364	-0,326326847 PARK7
	0,048487986	-0,01315546 VAT1
	1,118296994	0,215500832 PLIN2
	0,982622979	-1,411132336 DNAJC2
	0,761618019	0,301448822 MAP3K14
	1,291824969	-0,208683014 NUP88
	0,425884913	-0,141945362 POP1
	0,378776842	0,078279972 S100A13
	0,287144297	-0,468446255 TIMM17A
+	3,050786676	-0,627937317 TSNAX;DISC1
	1,801715641	-0,211183548 EIF3C;EIF3CL
	0,425659767	-0,138563156 TTC1
+	3,201191794	-0,614264488 C12orf57
	2,776429932	-0,398589134 PHB2
	0,096710643	-0,048134804 SDHC
	0,345874023	0,082111359 CHP1
	1,676850055	-0,191182137 HSD17B10
	0,642679308	-1,022241116 SIGMAR1
	0,00205536	-0,000212193 NAP1L4
	0,758153949	0,312540531 NAPG
	0,537085964	0,261516571 TXN2
	0,201025578	-0,138809204 MIPEP
	0,352596134	0,05548954 TM9SF2
	0,946543165	3,427260399 SLC29A1
+	3,094037203	-0,395282745 CCT7
	0,406388293	-0,460960865 MYD88
	1,486028655	0,327336788 PKP2

	1,033755544	0,219064713 NIPSNAP1
	2,289721155	-0,395448685 NCAPG
	0,415682057	-0,771924019 ARPC5L
	1,105475069	-0,62349844 PAIP2
+	2,869859812	-0,609536171 DDX50
+	2,421639443	-0,637793064 C19orf43
	0,260280275	-0,041163921 GRWD1
	0,993076476	-0,173829556 WDR77
+	3,157296607	-0,5240798 TUBA1C
	1,572542382	0,092591763 MYBBP1A
	0,904369917	0,174182892 MGME1
	0,621117325	0,131031513 CORO1B
	2,021028559	0,403503895 TXNDC17
	1,155502197	0,3114748 CPPED1
	0,034922937	0,015739441 VPS25
	0,419178522	-0,452047348 PAAF1
	0,952552524	-0,09673357 WIBG
	0,055107678	-0,027647495 AIFM2
	0,204468491	-0,05129528 ADPGK
+	4,915791321	3,410456657 GPATCH1
	1,30428077	-1,443283558 LLPH
	0,848801591	-0,172753811 PELO
	0,618506865	-0,195045471 GINS3
	0,197746358	0,195001125 TRIM56
	0,484036392	-0,054594517 ERP44
	0,25342781	0,068152428 LXN
	0,573978188	-0,533414364 NOL10
	0,254970904	-0,077585697 NTPCR
	0,705032803	1,350096226 AGMAT
	1,058527877	-0,257496834 TACO1
	1,351586342	-0,769533634 HDHD3
	0,227118024	0,039990425 ESYT1
	0,002374935	-0,000930786 CNPY3
	0,177476697	-0,190681458 MTG1
	0,175698144	0,412849903 ALG1
	0,620172601	0,839285374 HAUS8
	0,266685877	-0,076104641 COPS4
	0,584962912	-0,100334167 DIDO1
	0,88005489	-0,367396355 MCMBP
	0,009322305	-0,013021946 DCUN1D5
	0,538262608	0,140972614 PAGR1
	1,564522523	0,382332802 URM1
	0,161294185	0,019503117 ANP32E
	1,480596855	-1,499150753 PI4K2A
	0,836005706	-0,207369328 TMEM43
	0,478120713	0,717009544 HGH1
	0,738464519	-0,42973423 LMF2
+	2,238937294	-1,057930946 MMTAG2
	1,346558481	2,352155685 DOHH
	2,880957892	-0,29166317 TUBB6

+

2,200138982	-0,177615166 HNRNPUL1
0,698912982	-0,178135872 MSTO1
0,889486831	-0,098083019 PDCD10
1,581077075	0,163211346 HTATIP2
0,286850972	-0,081375599 DDX23
0,638082092	0,630517483 MRI1
1,408203372	0,263120651 MLPH
0,191774532	0,035795689 ADI1
0,091590567	-0,043688297 MECR
1,039271307	-0,248036385 NTMT1
1,150637258	-0,394551754 TMEM109
0,128425299	0,051762104 PBDC1
0,51880646	-0,681495667 PTDSS2
0,207828106	0,061840057 NOC4L
0,390760639	-0,083599567 UTP14A
1,926187571	0,418519974 DUSP23
0,816925057	-0,280068874 TMED9
1,418394299	-0,216219902 GNL3
0,006816164	0,010318279 RIOK2
0,742189142	-0,526997089 TIMM21
0,205453194	0,214939117 SARG
0,429610542	-0,502288818 KIFC1
1,49566442	-0,263996124 NUP85
0,038990441	-0,087566853 HIGD2A
2,295221136	-0,298585892 IFT27
0,23831313	0,061313629 NUDT9
0,102705144	-0,053442478 TARS2
5,181758112	-0,67444706 ACAT2
0,535556707	-0,455354214 REPIN1
3,486300231	-0,235823631 RBM4
0,644601887	-0,145019531 SF3B5
1,931947759	0,132491112 CHID1
0,676807628	0,106073856 HINT2
0,353716701	0,073835373 BCL2L13
1,485868432	-0,156299591 SRRT
0,946900524	0,516303539 NUSAP1
0,025342456	0,011701107 C14orf142
0,291378477	0,038519382 CECR5
0,221266003	-0,511940479 MAK16
0,041499158	-0,008800507 ITPA
1,289542016	0,34830904 CHMP4A
2,450454408	-0,369256973 EIF2A
0,747902239	-0,949640274 PECR
0,740850433	0,209967136 MRPL13
0,150696535	-0,364289761 MRPL9
0,08408699	-0,035942554 MRPL4
1,533775197	-0,381819725 NIFK
0,132531935	-0,218939781 PARD6B
0,037999103	0,007686138 SRXN1
0,982547513	-0,766512394 MRPS26

	1,365653494	-0,126983643 NLN
	1,190718826	2,471914768 TBC1D2
	0,397677671	-0,062686443 MRPL37
	0,630490895	-0,136394024 GTPBP4
	0,197513893	0,072793484 ASPSCR1
	0,055489885	-0,018368721 UACA
	2,362673401	0,32148838 WDR11
	0,178949173	0,087073803 UBL5
	0,376896997	1,664764881 DPY30
	0,109952387	-0,165792942 ZDHHC5
	0,070155626	-0,008688927 TNKS1BP1
	0,17657787	-0,032633781 UBE2O
	0,181117193	0,032795906 TANC1
	0,090304646	0,139322758 TTYH3
	0,125030842	0,218346596 MTMR12
	1,385760486	0,408502579 WDR33
	1,772696115	-0,308246136 WDR12
	0,057627889	-0,029856205 C20orf27
	1,995396428	-0,276128292 POLR1E
	1,58955889	0,599538326 SRR
	0,141543309	0,066923618 NIF3L1
	2,100713361	-0,317415714 EGLN1
	0,096723857	-0,128130913 FAM192A
	0,755714444	0,215277195 MFF
	0,818262997	-0,269431114 TMEM126A
	1,980380778	-0,268758774 PAIP1
	0,31477053	0,122574329 LSG1
	1,04870864	0,174512863 FAM107B
	1,517841642	-0,329973221 NAT10
	0,034096489	0,018205166 KLC2
	1,809981269	-0,589198112 ILKAP;ILKAP3
	1,247397775	-0,129179955 XRN2
	0,027544076	-0,007430553 TOLLIP
	0,888989941	-0,250882626 NSRP1;CCDC55
+	4,39560904	-0,317606449 RACGAP1
	0,04532779	0,024607182 CSTF2T
	1,635319576	-0,455596447 NT5C3A
	0,741796877	-0,302735806 QRSL1
	2,376461245	-0,289717674 DDX47
	1,289413374	-0,15349865 RAB1B;RAB1C
	0,444892282	-0,174254894 MRPL18
+	2,280949659	-1,074947834 LMAN2L
	0,095604921	0,041726112 SMG9
	0,038606789	-0,097033024 ANAPC1
	1,371404059	-0,323831081 UNC93B1
+	1,868560503	-1,331184864 CYSTM1
	2,715698973	0,313883781 NUCKS1
	0,49415589	-0,462412357 TMX4
	0,771530061	0,212043762 ISCU
	0,207939813	-0,15491581 EHD4

	0,247129515	0,088320732 VPS33B
	0,882520187	1,575277805 VPS16
	0,958781655	0,246869087 TRIOBP
	0,21622697	-0,033634186 SLK
	0,168444774	-0,379430294 PPIL3
	1,072551113	-0,156918526 PDCL3
	1,368627647	0,658471584 TAOX3
	1,79009828	-0,31467104 RAB3GAP2
	4,432399632	0,167561531 PPA2
	0,823228904	-0,184770107 MRPL46
	1,057737926	-0,149869919 PNN
	0,34020319	-0,642299652 TMEM245
+	2,573479417	-1,455098152 C11orf68
	0,437864513	0,525476456 GHITM
	1,945529464	-0,089779854 TMX1
	0,262070197	-0,08346796 ACBD3
	0,131998896	-0,095114231 PTPN23
	2,351046331	-0,220463753 UNC45A
	0,568586311	0,457649708 PDPF
	0,056243011	-0,126863003 DNAJC5
	1,668921658	-0,312255383 CHMP4B
	0,153944649	-0,321361065 FN3K
	0,776490629	-0,311788559 POFUT1
	0,371122082	-0,04240799 RNPEP
+	2,968072367	-2,018241882 GOLPH3
	0,048252448	-0,020002365 OSBPL3
	0,781223552	-0,220132828 ESF1
	0,683372923	-0,228895187 TFB2M
	0,513266666	-0,695633411 FAM96A
+	1,862052106	-1,206107616 BICC1
	0,784498824	-0,222991943 CCDC86
	0,370381299	-0,473026276 NARFL
	2,034255399	-0,1533494 NOL6
	0,098321665	-0,01397562 EPS8L2
	0,676452772	-0,10523653 RPAP3
+	2,30748255	1,141347408 ANTXR1
	0,005781437	-0,002206802 WDR55
	0,292923065	0,085396767 RANBP3
	0,619251386	0,103248596 DCTPP1
	1,520380857	0,25010252 SH2D4A
	0,03506831	0,008511543 RPF2
	0,461022135	-0,499633789 DOCK5
	0,026246306	-0,009691238 SCAF1
	0,165200088	0,462731838 PHAX
	0,497448015	-0,146178722 UBE2Z
	0,253477379	0,682907104 ACAD9
	0,932776248	-0,611682415 NOL11
	0,261197727	-0,326994419 ECT2
	0,378973926	0,111165524 GORASP2
	0,245536072	0,800823212 QTRTD1

	0,134566324	-0,180247784 ARMT1
	0,310836287	-0,181107998 CNOT10
	0,696520229	-0,28570652 LRRC40
	0,834859408	0,082431316 SFXN1
	0,00890309	0,003166199 MRPL44
	1,011355164	-1,408623219 NHEJ1
	1,15822905	-0,332743645 ELP3
	0,059869786	0,117675304 RPF1
	0,607431273	0,361440182 POLR1B
	0,722710985	-1,058278561 FN3KRP
	0,713827444	-0,758896351 CARS2
	0,022444527	0,038541794 PPCS
	0,510393172	-0,156587601 XPO5
	0,436914681	0,121359348 GRPEL1
	0,0452651	-0,008529186 C12orf10
	0,20694502	-0,29970932 SCPEP1
	0,092275687	-0,024083138 CACYBP
	0,07315031	0,196819305 RHOF
	1,033538213	-0,206297874 RDH14
+	2,746967706	-0,457231522 CYP4F11
+	2,577834313	-2,202299118 TMEM165
	0,91710269	0,093036652 EML4
	0,302658584	0,097409725 RNMTL1
	1,8790984	0,149578571 GLOD4
	0,116079404	0,442443371 CBX8
	0,37744494	-0,133691311 MUC5B
	0,354186372	0,137144089 NCOA5
	0,480133897	-0,406645298 METTL14
	0,58972782	0,230632782 GPN1
	0,140070173	-0,199989319 SDF2L1
+	4,021865177	0,634159565 XAB2
	0,197566707	-0,096343994 PREB
	0,661795348	0,5522542 SRA1
	0,434713286	-0,106637001 ATP13A1
	0,711770034	0,255183697 GOPC
	2,026811267	0,282126904 MRPL47
	0,40681644	0,079471111 TM9SF3
	0,36263641	0,04008913 APMAP
	1,064224449	0,288949966 ARFGAP3
	0,141464244	-0,13644886 HMG20A
+	3,928370754	-0,405744553 RAB18
	2,485256411	-0,25744009 SSU72
	0,093163995	0,19277668 MRPS30
	0,989251452	-0,134770393 EMC7
	0,117847185	0,267860889 MID1IP1
	0,216410646	-0,326654434 ENY2
	0,176772748	0,058498383 EXOSC4
	0,141577179	-0,059206009 UBE2T
	0,131193005	-0,300105095 NOP10
	0,918624041	-0,305006981 ISYNA1

	1,095484128	-0,150475502 ACOT13
	0,204160455	-0,304736137 NXT1;NXT2
	0,237402968	0,115641594 TIMMDC1
	0,357684564	-0,112086773 RIC8A
	2,637999039	-0,348025322 LUC7L
	0,881914177	-0,676733971 MRPL40
	0,840189121	0,243618965 TIGAR
	0,672244704	0,486902714 MANBAL
	1,464720826	-0,300049305 RPRD1B
	2,231065282	-0,234435558 NIT2
	0,768664578	0,925043583 EXOSC5
	0,178076791	0,013664246 EXOSC3
	0,251812732	0,142103195 KIF13B
+	2,758416396	0,495228767 ANLN
	0,510064092	-0,157075405 XPNPEP1
	1,776073369	0,133966446 UTP3
	0,761165396	-0,116924763 BIRC6
	0,820196072	-0,715802193 PDLIM7
	0,369523857	0,082792759 DIABLO
	3,422251025	-0,34465313 DDX21
	1,640781777	-0,264210701 SAR1A
	0,157004862	0,050813675 NANS
	1,620344626	-0,473605156 EIF2B3
	0,909524856	-1,73823595 POLE3
	0,104036828	-0,263759613 AAAS
+	3,331617112	-1,657990932 BAZ1A
	0,898735916	-0,168649673 AASDHPPT
	0,43082037	-0,741639137 UBQLN4
	0,947284621	0,111244202 HEBP1
	1,63468755	0,25060606 APOBEC3C
	0,941375128	-0,185146332 MRPL17
	1,807542731	0,441614151 PHPT1
	0,144351484	-0,059407711 ARHGAP35
	0,489348018	-0,09158659 HELLS
+	3,661786135	-0,593887329 TOMM22
	0,656247625	-0,614926815 LANCL2
	1,718206117	-0,1738801 FARSB
	0,56721957	-0,040648937 IARS2
	0,196011782	-0,375727177 FAM207A
	0,580813379	-0,369251251 ATG3
	0,404731142	-0,182332039 PDS5B
	0,568770542	-0,11862278 RBM12
	0,494653909	-1,151055336 MDN1
	1,5277727	0,180140495 ABHD10
	0,769439577	-0,296995163 DDX28
	0,945194428	-0,776272774 SLC39A9
	0,853790652	0,357638836 TMEM106B
	0,574988026	-0,4237957 LIN7C
	0,732886827	-0,640445232 AGPAT5
	0,304110442	-0,590633392 TXLNG

1,914890544	-0,625304222 ABCF3
2,259899674	-0,343276024 FAM49B
0,827950371	-0,279864311 IMP3
1,131785831	0,254460335 EXOC1
1,279365832	0,430705547 PARVA
1,248556455	-3,836298943 INTS7
0,383428047	0,096961498 ATAD3A;ATAD3B
0,205310389	-0,042856693 ARL8B;ARL8A
3,321711124	-0,316654205 DDX18
0,924461699	-0,262343407 ARMC1
0,146257536	-0,171708107 MTPAP
0,899109094	-1,826732635 NLE1
0,652348421	-0,175223351 RBM28
1,249703629	-0,644834995 ANO10
0,50264299	-0,857722759 RBM22
0,252597101	-0,071513176 WDR70
0,565764286	0,069099426 SLTM
0,48613285	0,535607815 OXSM
0,968162382	0,815200806 GID8
1,410766991	0,32286787 C1orf123
1,190617556	0,357190132 CLN6
0,333832383	0,66320467 C4orf27
0,021969407	0,048222065 COMMD8
0,172010884	-0,689754963 NDUFB11
0,25996944	-0,307420731 MRPL16
0,333001356	0,064134121 OCIAD1
0,931401347	-0,213140488 MARCH5
0,270701359	-0,175572395 HYPK
0,318639386	-0,127761841 LYAR
0,732057154	-0,930436611 IMPAD1
1,026718683	0,241749763 TEX10
1,005169225	0,144256592 THUMPD1
0,738475073	-0,763241768 TOR4A
0,911966028	-0,590500355 CDKN2AIP
0,502394196	-0,175240517 GAR1
0,119953595	-0,032506943 PPP4R2
0,619113613	-0,101696491 DDX56
0,334113599	0,071250439 TERF2IP
0,455864687	-0,048456192 BCLAF1
0,459059445	0,880041122 UTP6
1,208962846	-1,371525288 COA4
0,625789914	0,128461838 MRPL39
0,474217784	-0,64544487 ZAK
2,119617838	-0,226143837 TMOD3
1,344754515	-0,156373024 UGGT1
1,128952664	-0,746843815 CDK12
1,30325556	-0,221064091 FASTKD2
2,823118318	-0,559735775 TECR
0,041828834	-0,035391808 ERAP1
0,687174464	-0,553159714 ACTR10

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	0,657222293	0,329753399 CISD1
	0,913571897	-0,085380077 FAM120A
+	2,140434924	-0,779764175 UBP1
	1,667187591	-0,126810551 IGF2BP1
+	2,926613313	-0,47810936 HSPBP1
	0,683038063	-0,136044979 MAT2B
	1,966717946	-0,158147812 MYOF
	1,181725181	0,460141182 GLTSCR2
	0,305900612	0,037858009 EHD2
	0,657499997	-0,139745235 OGFR
	0,032458435	-0,009482861 LMCD1
	0,001299019	0,000307083 MPP6
	1,948982887	-0,284852982 CHMP5
	0,954948886	-0,286596775 COMMD9
	0,868326286	-0,881037712 CWC15
	0,111321797	-0,036496162 MRPL15
	0,476892636	-0,585343838 CRIPT
	1,553415749	-0,417390347 NDUFAF4
	0,817962728	-0,160112381 HACD3
	0,055723125	0,086257935 EMC3
	0,775674248	-0,156362534 NDUFA13
	0,218755594	0,112924099 KCMF1
	0,263189666	0,064072132 RAI14
	1,739828013	0,156905174 VAPA
	0,438131547	0,095710754 GSKIP
	0,003069484	-0,000586987 RCC2
	2,801726178	-0,380255699 BCCIP
	0,343222233	0,80194521 CTTNBP2NL
	2,521066059	-0,219622612 RRBP1
	0,561992528	0,267096996 CPSF2
	0,632874622	-0,813153267 RBM27
	0,196093923	-0,042746067 ANKFY1
	0,15219216	0,195562363 DPM3
	0,872207629	-0,1396451 ATXN10
	1,648544139	0,590096951 NCDN
	0,680890002	-0,500159264 ORC3
	1,089274015	-0,088796139 SAE1
	1,545422352	-0,175701618 COPG2
	0,031859896	-0,016317368 MRC2
	1,293697213	0,280565262 COMMD3;COMMD3-BMI1
	0,178914258	0,040325642 GNG12
	1,230777959	0,379827976 METTL1
	1,467750258	1,04340744 GULP1
	1,94658538	-0,202474117 VPS29
	0,873598774	-0,173689365 EIF3K
	1,348251377	0,17952776 GRHPR
+	4,762007503	-0,355842113 DNAJB11
	0,416088492	-0,457794189 RNF14
	1,031585853	-0,158389568 UBA2
	0,362344157	-0,428991795 NXF1

	0,023018472	0,006003857 SEL1L	
	0,406266268	-0,874351978 PEF1	
	0,15365052	0,098448277 CLIP2	
	1,305561521	-0,248847961 UQCR10	
	0,948913062	0,795758724 DNAJB4	
	0,118273426	0,260607243 CFDP1	
	0,635835612	0,515081882 ZNF629	
	0,351278943	-0,724017143 STK39	
	1,161708032	-0,730147839 LRWD1	
	0,271540225	-0,086330414 ABCF2	
	2,241670808	0,243156433 TES	
	0,375368255	-0,12955761 LIMD1	
	0,494924352	-0,071401596 SEC63	
+	2,829981849	-1,37978363 SUSD2	
	0,041308937	0,012033939 SUN2	
	0,142319902	0,052053928 LAMTOR3	
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	1,428387036	-0,165490627 SRP68	
	0,585030011	-0,07072258 CHORDC1	
	0,342223377	0,044029236	Sep 09
	0,127960133	-0,055898666 UBQLN2	
	0,319120131	0,232018948 DCDC2	
	0,348771645	0,067650795 PCYOX1	
	0,390435857	0,704500675 DPP7	
	0,684595922	-0,1239748 TMEM2	
	0,423586827	-0,098709583 CYB5R1	
	0,783727091	0,184867859 BAIAP2L1	
	0,864325075	-0,48601532 ZNHIT2	
	0,025090152	-0,010430813 PFDN2	
	0,48591873	-0,136476994 NRBP1	
	0,035472304	0,0195961 ENOPH1	
	0,067987839	0,021053791 NDUFA12	
	0,284059634	-0,070487499 IPO11	
	1,867253926	-0,397053719 TRMT112	
	0,122794188	-0,023824215 XPO7	
	0,70219661	-0,078942299 BAZ1B	
+	2,313446663	-2,489084721 ATP1F1	
	0,964788663	0,129043579 AK3	
+	2,103703919	-0,933286667 LNPEP	
	0,360552217	0,05021286 NAGK	
	2,162972955	-0,434703827 TRMT6	
	0,732632001	-0,488028049 TSR3	
	0,175228496	-0,044744015 SLC25A13	
	0,136300849	0,021765709 DBNL	
	0,217394156	-0,3545928 DDX41	
+	2,195479608	-2,0136199 DCTN4	
	2,076698317	-0,414978027 ANAPC7	
+	4,107924842	-0,598379135 STOML2	
	0,516581115	-0,158003807 VPS28	
	0,245959754	-0,207435131 FAM208A	

	2,016117271	-0,167570114 HN1
+	5,363608701	-0,935703278 MRT04
	0,704982929	-0,382388592 APPL1
	0,09356994	-0,122184753 GPATCH8
	0,295879224	0,102859497 PARP4
	0,117454707	0,05329752 RCOR1
	0,001010221	-0,002409458 GTF3C4
	0,385934755	0,417996407 AGO2
	0,224943852	-0,030751705 NUP50
	0,497359691	0,075853348 CDV3
	0,727222587	0,328432083 CNOT11
	0,806060458	0,167072773 RAB21
	0,493422944	-0,129364491 MCTS1
	1,675859658	-0,057499886 MTUS1
	0,198416902	-0,199474812 MKL2
	1,278241356	-0,128550053 CORO1C
	0,67476202	-0,184711456 TPX2
	0,617356186	0,329425335 NOB1
	2,42059496	-0,436993122 TMCO1
	0,278529374	-0,368038177 EPDR1
	0,974298937	-0,237381935 NFU1
	2,408572645	-0,424253941 PRPF19
	3,108883671	-0,289458752 UBQLN1
	0,651967099	-0,130870819 NENF
	0,699072542	-0,668284893 NOL7
	0,572526814	-0,091098309 VPS4A
	0,33249586	-0,050891876 G3BP2
	0,484849347	-0,196503162 STUB1
	1,033951495	-0,078204632 PACSIN2
	0,409415791	-0,115224361 DUSP12
	0,260674521	-0,081226349 STX8
	2,773950467	-0,214067936 PSMD13
+	2,19139947	-3,354775429 PROCR
	0,540767551	-0,127749443 PPIE
+	2,238254242	-0,788363934 ABCG2
	0,363529109	0,27384758 DIMT1
	0,685127505	-0,311266422 TIMELESS
	0,77305887	-0,092563629 COPS3
	0,546753923	-0,153726578 MINPP1
	0,536464629	-0,140363693 RPL26L1
	3,785708808	-0,302958012 NSFL1C
	1,309876894	-1,067677498 COG5
	1,388562474	0,350954533 PPP6R1
	0,56951401	-0,565159798 USP22
	0,072866449	-0,074699402 USP24
	2,207811985	-0,206134796 SRRM2
	1,915012251	-0,219518185 PA2G4
	3,007196747	-0,30728817 SMC3
	2,938476689	-0,337177277 NIP7
	2,506529229	-0,215237617 C14orf166

+

1,607261261	0,104722977 RUVBL2
0,648269156	-0,212216854 PIN4
1,220530172	-0,368409634 POMP
1,373607536	-0,790974617 GINS2
2,121281877	0,228747845 PLAA
2,936586027	-0,352578163 RUVBL1
3,186692049	-0,279081345 NUDC
0,235772806	-0,125732422 VDAC3
2,043132555	-0,312336922 CFL2
0,336117368	-0,713806152 ITM2B
1,615123578	-0,25961113 DRG1
1,304772495	-0,255356789 NCKAP1
0,596816999	0,088623047 CNPY2
0,344730486	-0,083262444 DIS3
0,243344933	-0,324839115 RCL1
5,573678918	0,615096092 GSTK1
0,631030796	-0,175908089 COA3
0,174395833	-0,055264473 DDX52
1,039570757	-0,161065578 POLDIP2
1,425551164	-0,271074772 AP3M1
0,050862447	0,077448845 LEMD3
2,76327852	-0,292694092 CARHSP1
0,066827885	-0,025130749 WBP11
0,505948961	0,092099667 NOP58
1,393053258	0,385126591 GIT1
1,298257947	-1,26816082 ZNF281
0,978537882	-0,254368305 SUGT1
1,662671181	-0,234265327 YARS2
0,618723964	-0,300316811 COQ6
0,651283961	-0,13381958 ACOT9
3,147516421	0,226699829 NOSIP
0,709158957	-0,099601746 DERA
1,312810895	0,219529629 MEMO1
0,034290431	-0,015562534 TMX2
0,753313374	-0,232042789 LSM2
0,02144667	0,005560398 CAB39
1,876760123	-0,332220078 DHRS7
0,580802931	-0,15585804 MRPS2
0,290701642	-0,218790531 UTP11L
1,014122278	-0,118228436 RRP7A
1,195465295	0,684426785 TMED5
1,064317142	-0,310354233 SF3B6
0,618097721	-0,132026196 MRPL11
0,824593097	0,263337612 RRP15
1,461987035	-0,157631874 NOP16
1,288884578	-0,129847527 TPRKB
1,662736308	-0,316180229 PPIL1
0,011873604	0,003940105 FAM96B
0,042092693	-0,018104076 MRPS16
0,698343774	-0,994628906 FIS1

0,265922311	-0,144947052 AK6;TAF9
1,218360087	0,32051754 MRPS23
1,126843904	-0,227919102 HDGFRP3
0,859627031	-0,82294035 BOLA1
1,196655682	-1,323470116 CHMP3
2,415070642	-0,180064678 STRAP
1,131645182	-0,076858521 RTCB
0,306136016	-0,362582684 TMED3
0,474863928	-0,121652603 NOC2L
0,611506323	-0,075507164 RPL36
0,06477062	-0,019237041 CCDC9
0,992915079	-0,252466202 CHTOP
0,46438543	-0,374047279 SAMHD1
0,085014724	-0,019341469 PKP3
0,326419298	-0,09414053 HBS1L
0,685423206	-0,279176712 PRKAB1
2,921990365	-0,122487068 TLN1
0,814688754	-0,190573215 MTCL1
1,371102504	0,563826561 VPRBP
0,107720024	-0,246621609 TCAF1
1,320150706	-0,258929729 USP15
1,064475957	-0,135284424 HYOU1
0,335432273	0,128256798 AFG3L2
0,312522686	0,10215044 ARIH1
0,202476282	0,173744202 LSM4
0,130621388	-0,057625294 SAMM50
0,798424339	-0,068447113 PPME1
0,626567168	-0,195121765 YTHDF2
0,053717229	-0,054388523 CTDP1
1,301645639	-0,197780609 SUPT16H
0,48116886	-0,155345917 UTP18
0,226021007	-0,046006203 TIMM9
0,255781116	-0,060695648 UCHL5
0,608999524	0,081885338 CD2AP
0,88184958	-0,217645168 TNPO3
0,601561731	-1,029376507 TIMM13
1,619217073	0,362432957 SRPRB
0,706152985	-0,114186764 GMPPB
0,229385201	0,063084126 GTF3C5
1,895175011	-0,195592403 GTF3C3
0,269571024	0,102070808 CDC42BPB
0,049676091	0,01257515 RBM8A
1,377944543	-0,990385532 SNX8
1,664891369	-0,218151569 SNX5
0,259250171	0,79466486 NUBP2
1,706094666	-2,004419327 HEBP2
0,292514687	-0,055314541 LRRFIP2
1,437133379	0,837605953 FHOD1
3,429015685	-0,168010712 PSAT1
0,676089996	-0,209550381 F11R

+

0,849980427	-0,885581017 GPC6
0,262873783	0,060712814 NPTN;DKFZp566H1924
0,491280669	-0,811628819 SPIN1;SPIN3
1,784045908	0,436677933 MRPS18B
0,641353506	-0,106533527 COPG1
1,087333796	0,196629047 AUP1
0,169722868	-0,0404706 CLIC4
0,479740979	-0,759532928 NFS1
0,394819782	0,221867085 SAR1B;DKFZp434B2017
0,061072158	0,01074028 MTCH2
1,535590925	-0,211251259 DYNC1LI1
0,426883724	1,146391392 CHCHD2;CHCHD2P9
1,211490353	-0,262298107 EPN1
0,281273033	-0,099945068 TEX264
0,003108337	-0,008012295 SLC30A1
0,382784989	0,075255394 NDUFB9
0,156572308	-0,0344944 SQRDL
1,129204671	-1,734200954 WASF2
0,114305787	0,047197819 IVNS1ABP
1,995925736	-0,240486145 SEC23IP

Supplementary Table 3 (compressed)

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Upstream Regulator	p-value of overlap	Mechanistic Network
TGFB1	1.98E-12	134 (20)
TP53	2.14E-12	125 (21)
beta-estradiol	1.19E-11	133 (19)
lipopolysaccharide	2.18E-11	127 (17)
HNF4A	3.54E-11	
COPS5	6.65E-10	84 (7)
cisplatin	1.13E-09	109 (16)
CDKN1A	1.84E-09	98 (15)
YAP1	2.30E-09	113 (16)
FOXO1	2.77E-09	106 (19)
MYC	5.00E-09	126 (19)
medroxyprogesterone acetate	5.31E-09	130 (19)
ESR2	1.12E-08	117 (18)
8-bromo-cAMP	2.20E-08	131 (18)
tamoxifen	2.68E-08	130 (19)
E2F1	1.27E-07	98 (15)
trichostatin A	1.63E-07	112 (17)
5-fluorouracil	2.88E-07	112 (14)
CYLD-AS1	3.36E-07	
resiquimod	5.27E-07	93 (7)
LUCAT1	5.96E-07	83 (7)
CTNNA1	7.91E-07	126 (19)
NFE2L2	8.03E-07	98 (11)
NPPB	8.82E-07	59 (3)
calcitriol	8.82E-07	117 (20)
CST5	9.79E-07	
PML	1.00E-06	121 (21)
metribolone	1.12E-06	100 (7)
RB1	1.49E-06	106 (17)
CEBPB	1.56E-06	103 (16)
decitabine	1.76E-06	122 (15)
ATF3	2.78E-06	81 (7)
HRAS	3.31E-06	111 (18)
pirinixic acid	3.97E-06	112 (16)
LCN2	4.34E-06	105 (20)
CLDN7	4.59E-06	
RABL6	4.63E-06	91 (11)
UNC1999	4.72E-06	
NRG1	4.99E-06	102 (17)
dexamethasone	5.10E-06	136 (21)
PPARG	6.48E-06	117 (19)
E2F8	6.76E-06	
Z-LLL-CHO	7.38E-06	106 (15)
mir-8	7.49E-06	91 (13)
ATF4	9.43E-06	79 (8)

antioxidant	1.00E-05	45 (7)
ERBB2	1.02E-05	107 (16)
aflatoxin B1	1.06E-05	
D-glucose	1.40E-05	111 (18)
CCNE1	1.63E-05	94 (13)
NPC1	1.94E-05	
STX18	2.12E-05	
ERBB3	2.26E-05	109 (16)
ESR1	2.68E-05	128 (18)
TWIST1	2.74E-05	
E2F7	2.78E-05	
BHLHE40	3.03E-05	
EGR1	3.13E-05	113 (17)
OGA	3.18E-05	106 (12)
sirolimus	3.26E-05	122 (18)
PPARA	3.28E-05	81 (13)
CDK19	3.46E-05	
arsenic trioxide	4.39E-05	107 (16)
NSUN6	4.39E-05	
AGT	4.66E-05	128 (20)
gemcitabine	5.12E-05	49 (7)
MDL 73811	5.31E-05	
TNF	5.45E-05	129 (18)
ampelopsin	5.49E-05	103 (16)
methylselenic acid	5.54E-05	119 (12)
CD38	5.56E-05	89 (10)
1,2-dithiol-3-thione	5.62E-05	21 (2)
Lh	6.26E-05	96 (10)
SP1	6.33E-05	108 (17)
AURKB	6.44E-05	83 (11)
FLCN	6.76E-05	88 (10)
Immunoglobulin	7.47E-05	110 (17)
CpG ODN 2006	7.84E-05	114 (15)
mir-142	8.06E-05	
streptozocin	8.12E-05	118 (19)
MAP2K3	8.28E-05	105 (12)
MAPT	9.09E-05	80 (8)
CREB1	9.38E-05	
CUX1	9.67E-05	
CDKN2A	9.81E-05	95 (14)
eflornithine	9.88E-05	111 (17)
PLPP3	1.02E-04	
AKAP1	1.02E-04	67 (7)
camptothecin	1.04E-04	109 (15)
AGN194204	1.08E-04	45 (3)
KLF6	1.20E-04	91 (7)
silibinin	1.21E-04	110 (19)
BRCA1	1.21E-04	105 (15)
PD98059	1.25E-04	120 (17)
resveratrol	1.27E-04	101 (17)

IL4	1.36E-04	114 (17)
ethanol	1.42E-04	103 (16)
FOXO1	1.45E-04	121 (19)
CHI3L1	1.49E-04	
HTT	1.49E-04	106 (17)
dihydrotestosterone	1.52E-04	128 (20)
MFAP4	1.58E-04	
TXN	1.62E-04	121 (17)
pifithrin alpha	1.72E-04	101 (15)
N-Ac-Leu-Leu-norleucinal	1.72E-04	108 (18)
TP63	1.78E-04	94 (14)
UBQLN2	1.78E-04	
SMARCA4	1.83E-04	
MAPK1	1.84E-04	118 (17)
ZBTB17	1.84E-04	91 (13)
let-7	1.88E-04	86 (10)
imatinib	1.98E-04	104 (15)
mesalamine	2.09E-04	115 (19)
trehalose	2.09E-04	75 (7)
2,3-bis(4-hydroxyphenyl)-propionitrile	2.13E-04	19 (2)
lactacystin	2.17E-04	92 (11)
TP73	2.19E-04	101 (15)
LAMP2	2.27E-04	118 (19)
MAFB	2.34E-04	
AICAR	2.34E-04	121 (21)
apigenin	2.40E-04	115 (20)
APP	2.45E-04	112 (17)
forskolin	2.54E-04	123 (20)
enalapril	2.55E-04	71 (6)
cinnamaldehyde	2.55E-04	109 (11)
carbon tetrachloride	2.60E-04	105 (17)
nitric oxide	2.62E-04	114 (18)
PDGF BB	2.64E-04	94 (15)
NFKBIA	2.72E-04	118 (21)
INSR	2.75E-04	51 (7)
Eldr	2.76E-04	
IL1	2.78E-04	107 (18)
etoposide	2.86E-04	108 (18)
Akt	2.91E-04	107 (15)
nitrofurantoin	2.97E-04	
mir-21	3.00E-04	
E2f	3.00E-04	77 (8)
EGFR	3.03E-04	126 (16)
dibenzo[a,l]pyrene diol-epoxide	3.03E-04	
KLHL42	3.03E-04	
proTAME	3.03E-04	
EPAS1	3.14E-04	90 (13)
ALDH2	3.30E-04	
imipramine blue	3.30E-04	
D-galactose	3.41E-04	

SMOC2	3.41E-04	
COL9A1	3.41E-04	
TSC2	3.48E-04	90 (15)
H89	3.48E-04	109 (16)
HSF1	3.54E-04	52 (7)
IKBKB	3.57E-04	108 (19)
MITF	3.65E-04	85 (8)
SCAP	3.66E-04	28 (4)
IL10	3.75E-04	114 (17)
LAS1L	3.79E-04	
NRAS	3.82E-04	109 (14)
CSF2	4.01E-04	111 (17)
3-methyladenine	4.09E-04	108 (17)
ZBTB7A	4.16E-04	105 (11)
ANGPT2	4.22E-04	88 (10)
monorden	4.24E-04	89 (9)
berberine	4.36E-04	119 (19)
SIX2	4.41E-04	
PLX5622	4.49E-04	
COL5A1	4.64E-04	
SFTPC	4.64E-04	
GLI1	4.68E-04	
PGR	4.70E-04	133 (18)
doxorubicin	4.77E-04	106 (16)
VDR	4.89E-04	99 (15)
palmitic acid	5.03E-04	102 (14)
mir-3622	5.17E-04	
10-nitrooleate	5.17E-04	
costunolide	5.17E-04	67 (7)
torkinib	5.48E-04	
ETV4	5.73E-04	
E2F4	6.00E-04	
PH755	6.02E-04	
Bvr	6.02E-04	
SNHG1	6.02E-04	
SAPCD2	6.02E-04	22 (4)
TEX11	6.02E-04	
MAF1	6.02E-04	
glucuronyl glucosamine glycan sulfate	6.02E-04	
CTNNAL1	6.02E-04	
NOC2L	6.02E-04	
DEETGE-CAL-Tat	6.02E-04	
karenitecin	6.02E-04	
ibrutinib	6.17E-04	
doxifluridine	6.24E-04	
PURPL	6.24E-04	
IL19	6.24E-04	44 (7)
KRAS	6.25E-04	106 (17)
GnRH analog	6.30E-04	
IGFBP3	6.33E-04	104 (12)

ADRB	6.43E-04	89 (7)
troglitazone	6.58E-04	124 (18)
ONECUT1	6.64E-04	
tyrphostin AG490	6.65E-04	113 (18)
ALOX15	6.98E-04	110 (16)
ETS1	6.99E-04	95 (13)
NAMPT	6.99E-04	112 (17)
hydrogen peroxide	7.28E-04	110 (18)
LMO2	7.39E-04	
tunicamycin	7.39E-04	106 (15)
NR1H3	7.42E-04	
SYVN1	7.58E-04	
miR-124-3p (and other miRNAs w/seed	7.64E-04	
thapsigargin	7.64E-04	104 (15)
miR-9-5p (and other miRNAs w/seed CL	8.15E-04	
cuprizone	8.20E-04	32 (4)
JUN	8.26E-04	123 (18)
acetaminophen	8.35E-04	58 (8)
chloropromazine	8.41E-04	
IFNG	8.42E-04	120 (19)
vorinostat	8.43E-04	104 (17)
TFAZZIN	8.65E-04	51 (8)
KDM3B	8.68E-04	
MIR101	8.75E-04	
SLC29A1	8.75E-04	
superoxide	8.75E-04	96 (12)
LDB1	9.10E-04	
LDLR	9.11E-04	
methylprednisolone	9.14E-04	52 (7)
PTEN	9.34E-04	100 (17)
IL6	9.54E-04	121 (18)
TLR9	9.64E-04	118 (18)
SPDEF	9.81E-04	
ATM	9.81E-04	109 (18)
cadmium	9.81E-04	103 (15)
NLRP3	9.95E-04	112 (18)
ETHE1	9.96E-04	
C1GALT1	9.96E-04	
GLS	9.96E-04	
PPP1R13B	9.96E-04	
geraniol	9.96E-04	
ozone	1.00E-03	99 (14)
topotecan	1.04E-03	81 (7)
TBX2	1.04E-03	91 (10)
ERK	1.05E-03	117 (18)
LGALS3	1.08E-03	126 (20)
FSH	1.08E-03	106 (7)
IRF4	1.08E-03	
pioglitazone	1.08E-03	101 (17)
thymoquinone	1.09E-03	101 (15)

ZNF106	1.09E-03	
TRIB3	1.09E-03	
2-amino-1-methyl-6-phenylimidazo-4-5	1.09E-03	
JAK2	1.11E-03	112 (17)
4-phenylbutyric acid	1.12E-03	
JUNB	1.16E-03	104 (10)
FOXA2	1.16E-03	14 (2)
diphenyleneiodonium	1.17E-03	99 (17)
ciprofloxacin	1.18E-03	
GSK3A	1.18E-03	
l-asparaginase	1.23E-03	
CSF1	1.24E-03	98 (14)
BCR (complex)	1.29E-03	96 (14)
TCF3	1.32E-03	
MTDH	1.33E-03	
ARID1A	1.34E-03	
BTK	1.34E-03	106 (16)
SP2509	1.35E-03	
VEGFA	1.38E-03	109 (16)
RNF187	1.39E-03	91 (9)
ADRA1D	1.40E-03	
hydroxyurea	1.46E-03	116 (21)
NQO1-DT	1.48E-03	
conduritol epoxide	1.48E-03	
hesperidin	1.48E-03	93 (12)
Atg5	1.48E-03	
Snhg20	1.48E-03	
PMVK	1.48E-03	
CYP26B1	1.48E-03	
CSE1L	1.48E-03	
APOBEC3B	1.48E-03	
venetoclax	1.48E-03	
C1QTNF2	1.48E-03	
CBLC	1.48E-03	
PAXIP1	1.48E-03	
MIA-459	1.48E-03	
MIA-313	1.48E-03	
dec-RRLL-cmk	1.48E-03	9 (2)
liproxstatin-1	1.48E-03	
propyl-2-(8-(3,4-difluorobenzyl)-2',5'-di	1.48E-03	
WT1	1.49E-03	
GABA	1.55E-03	
oxymatrine	1.55E-03	73 (8)
ACTB	1.55E-03	
Rb	1.63E-03	93 (14)
TRAF2	1.63E-03	87 (8)
tazemetostat	1.67E-03	
fenofibrate	1.70E-03	94 (14)
rosiglitazone	1.71E-03	117 (19)
TIMP3	1.75E-03	44 (7)

SNAI1	1.76E-03	97 (10)
BRD7	1.76E-03	
LY294002	1.81E-03	114 (17)
MAP2K5	1.88E-03	
ADRA1A	1.88E-03	
PP2/AG1879 tyrosine kinase inhibitor	1.90E-03	120 (17)
APOE	1.97E-03	103 (16)
CDH2	1.99E-03	96 (12)
aphidicolin	1.99E-03	85 (6)
pidnarulex	2.00E-03	
melatonin	2.01E-03	104 (18)
miR-328-3p (and other miRNAs w/seed	2.06E-03	
CDC20	2.06E-03	85 (9)
3-hydroxykynurenamine	2.06E-03	
adiporon	2.06E-03	
Histone h3	2.07E-03	
COL18A1	2.10E-03	91 (13)
dextran sulfate	2.13E-03	76 (6)
BAX	2.16E-03	
EIF2AK4	2.16E-03	
ATF6	2.21E-03	
ZNF768	2.23E-03	
alpha-tocopherol	2.23E-03	118 (21)
iron	2.31E-03	103 (19)
SREBF1	2.34E-03	91 (14)
KDM5A	2.36E-03	
lithium chloride	2.45E-03	102 (15)
SIRT1	2.46E-03	112 (18)
BIRC5	2.47E-03	102 (18)
CBL	2.47E-03	105 (17)
D-galactosamine	2.47E-03	102 (16)
XIAP	2.49E-03	95 (13)
nilvadipine	2.49E-03	
ellagic acid	2.49E-03	104 (16)
kaempferol	2.49E-03	90 (11)
chrysin	2.49E-03	
trans-hydroxytamoxifen	2.51E-03	
PPARGC1A	2.51E-03	108 (15)
GCN2iB	2.55E-03	
Insulin	2.57E-03	112 (15)
tetrachlorodibenzodioxin	2.58E-03	129 (19)
obeticholic acid	2.64E-03	67 (7)
PRKAA	2.64E-03	123 (18)
ITGAV	2.64E-03	97 (16)
PSEN1	2.64E-03	93 (12)
diclofenac	2.67E-03	97 (9)
SLC25A4	2.73E-03	
chlorpyrifos	2.73E-03	
protocatechuic acid	2.73E-03	81 (10)
Collagen type VI	2.73E-03	

CASR	2.74E-03	69 (8)
E. coli serotype O127B8 lipopolysacchar	2.75E-03	101 (16)
sodium selenite	2.77E-03	67 (7)
SLC39A8	2.77E-03	
PLA2R1	2.77E-03	
ZNF507	2.77E-03	
CLUH	2.81E-03	
CAT	2.81E-03	98 (17)
docetaxel	2.84E-03	94 (10)
L-glutamic acid	2.94E-03	109 (15)
CEBPA	2.99E-03	115 (13)
molybdenum disulfide	3.05E-03	
mibolerone	3.05E-03	
PG3-Oc	3.06E-03	
ACTL6A	3.06E-03	81 (7)
mir-26	3.06E-03	67 (7)
CDKN2B	3.06E-03	
PELP1	3.15E-03	89 (6)
TO-901317	3.17E-03	111 (17)
actinomycin D	3.17E-03	106 (16)
nickel chloride	3.18E-03	
maslinic acid	3.20E-03	
mir-210	3.20E-03	
15-deoxy-delta-12,14 -PGJ 2	3.33E-03	111 (18)
clofibrate	3.34E-03	118 (19)
LLGL2	3.38E-03	
FAM117B	3.38E-03	20 (2)
torin1	3.47E-03	
cephaloridine	3.49E-03	
bendamustine	3.49E-03	
AAMDC	3.49E-03	
miR-142-5p (and other miRNAs w/seed	3.49E-03	
genipin	3.49E-03	
di-spiropyrrolizidino oxindole androgra	3.49E-03	
racemic flurbiprofen	3.49E-03	
tangeretin	3.49E-03	67 (7)
trigonelline	3.49E-03	20 (2)
D-alpha-hydroxyglutarate	3.49E-03	
STAT6	3.54E-03	122 (18)
CD44	3.59E-03	128 (19)
3,5-dihydroxyphenylglycine	3.62E-03	
butyric acid	3.66E-03	126 (19)
MDM4	3.71E-03	81 (7)
dithiothreitol	3.71E-03	
epigallocatechin-gallate	3.77E-03	120 (18)
cholesterol	3.77E-03	102 (13)
EIF2AK2	3.80E-03	89 (7)
N-nitro-L-arginine methyl ester	3.80E-03	104 (18)
POR	3.89E-03	
PDX1	3.89E-03	

THPO	3.96E-03	111 (16)
SHH	4.00E-03	70 (10)
SEMA7A	4.07E-03	67 (7)
mir-31	4.07E-03	
ACVR1C	4.07E-03	104 (16)
letrozole	4.07E-03	
valproic acid	4.10E-03	109 (7)
gefitinib	4.11E-03	106 (16)
mir-34	4.13E-03	15 (3)
TSC1	4.13E-03	94 (13)
methotrexate	4.15E-03	80 (7)
FOXO3	4.18E-03	102 (15)
RORC	4.23E-03	
SOD2	4.30E-03	103 (20)
GW9662	4.30E-03	98 (15)
BML-111	4.33E-03	
WDR4	4.33E-03	
alpha-santalol	4.33E-03	
2-hydroxy-1-naphthylaldehyde isonicot	4.33E-03	
perilla alcohol	4.33E-03	
AI-1	4.33E-03	
NCF1	4.44E-03	109 (19)
mir-124	4.44E-03	87 (7)
miR-199a-3p (and other miRNAs w/seed	4.44E-03	
DHX9	4.44E-03	68 (7)
ST6GAL1	4.44E-03	81 (10)
vitamin K3	4.44E-03	115 (18)
sodium tungstate	4.44E-03	
TERT	4.46E-03	
26s Proteasome	4.56E-03	111 (15)
PIK3CA	4.58E-03	85 (11)
bucladesine	4.68E-03	109 (20)
MLXIPL	4.70E-03	
deoxycholate	4.73E-03	85 (12)
progesterone	4.82E-03	117 (17)
miR-24-3p (and other miRNAs w/seed C	4.84E-03	
asciminib	4.84E-03	
4-(2-aminoethyl)benzenesulfonylfluoric	4.84E-03	103 (14)
bardoxolone	4.84E-03	43 (5)
CXCL8	4.84E-03	98 (12)
DNMT1	4.84E-03	93 (15)
1-methyl-4-phenyl-1,2,3,6-tetrahydropy	4.84E-03	114 (19)
MMP12	4.99E-03	
Mapk	5.04E-03	117 (16)
uranyl nitrate	5.04E-03	
L-triiodothyronine	5.08E-03	106 (14)
puromycin aminonucleoside	5.23E-03	
PAK1	5.25E-03	45 (7)
green tea polyphenol	5.25E-03	100 (17)
ursodeoxycholic acid	5.26E-03	89 (10)

L-serine	5.26E-03	
CYP46A1	5.26E-03	
CD53	5.26E-03	
mir-874	5.26E-03	
salinomycin	5.26E-03	84 (7)
WNT5B	5.26E-03	
UBE2E2	5.26E-03	20 (2)
USP11	5.26E-03	
TP53BP2	5.26E-03	
LRPPRC	5.26E-03	
CHRNA3	5.26E-03	
CYBA	5.26E-03	
manumycin A	5.26E-03	91 (13)
tyrphostin AG 1296	5.26E-03	
arachidonyltrifluoromethane	5.26E-03	
nandrolone	5.26E-03	
NR3C1	5.30E-03	120 (17)
tretinoin	5.42E-03	132 (20)
FAS	5.42E-03	102 (12)
RBM20	5.44E-03	
CpG ODN 2216	5.69E-03	95 (10)
mir-214	5.69E-03	
PARK7	5.69E-03	112 (18)
andrographolide	5.69E-03	100 (16)
bortezomib	5.72E-03	89 (9)
ELOVL3	5.82E-03	
IKZF3	5.82E-03	
glutathione	5.82E-03	114 (15)
NGF	5.84E-03	123 (17)
diethylnitrosamine	6.01E-03	114 (15)
CD40	6.03E-03	100 (11)
P38 MAPK	6.03E-03	132 (21)
sulforafan	6.10E-03	105 (15)
L-arginine	6.14E-03	67 (7)
PRKCZ	6.14E-03	108 (19)
amiodarone	6.14E-03	18 (3)
ST1926	6.19E-03	
embelin	6.27E-03	
OICR-9429	6.27E-03	
naringin	6.27E-03	97 (13)
liquiritigenin	6.27E-03	
DDB2	6.27E-03	101 (10)
FOXD3	6.27E-03	
AJUBA	6.27E-03	
MAP1LC3B	6.27E-03	
CXCL16	6.27E-03	
roflumilast N-oxide	6.27E-03	
trientine	6.27E-03	
dehydrocostus lactone	6.27E-03	
zinostatin	6.27E-03	

androstenediol	6.27E-03	92 (7)
miR-16-5p (and other miRNAs w/seed #	6.40E-03	
curcumin	6.58E-03	113 (18)
UBE2I	6.62E-03	83 (7)
HIF1A	6.74E-03	126 (19)
plicamycin	6.77E-03	
NUPR1	6.82E-03	
cyclic AMP	6.94E-03	127 (23)
ETV5	7.01E-03	
SIRT3	7.05E-03	67 (7)
IFT88	7.12E-03	
discodermolide	7.12E-03	
IKZF1	7.24E-03	8 (2)
SAR1A	7.37E-03	
NACC1	7.37E-03	
CH25H	7.37E-03	
APOC3	7.37E-03	
meloxicam	7.37E-03	
N,N-dimethylsphingosine	7.37E-03	71 (10)
MEF2D	7.39E-03	67 (7)
ERG	7.39E-03	71 (7)
MYCN	7.43E-03	103 (15)
1,4-bis[2-(3,5-dichloropyridyloxy)]benz	7.52E-03	
paclitaxel	7.56E-03	121 (19)
ITCH	7.64E-03	
DDX3X	7.64E-03	
pimagedine	7.64E-03	80 (10)
F2	7.70E-03	103 (16)
MAP2K6	7.73E-03	115 (21)
SP3	7.75E-03	
AMPK	7.78E-03	107 (19)
testosterone	7.93E-03	120 (18)
mifepristone	7.99E-03	114 (16)
AZ-1	8.09E-03	
ursolic acid	8.09E-03	114 (18)
OSM	8.14E-03	53 (9)
H-7	8.18E-03	93 (12)
SN-38	8.18E-03	94 (10)
CD40LG	8.20E-03	115 (17)
AHR	8.22E-03	124 (19)
cigarette smoke	8.31E-03	113 (18)
benzo(a)pyrene	8.31E-03	101 (11)
fluticasone propionate	8.43E-03	
FGF21	8.45E-03	67 (7)
folic acid	8.45E-03	75 (7)
SOD1	8.52E-03	111 (16)
UCA1	8.54E-03	67 (7)
allyl isothiocyanate	8.54E-03	
trans-stilbene oxide	8.54E-03	
delta-aminolevulinic acid	8.54E-03	

polycyclic aromatic hydrocarbons	8.54E-03	50 (6)
pyrimidin-2-one beta-ribofuranoside	8.54E-03	
ML385	8.54E-03	
SALL4	8.66E-03	80 (7)
isoproterenol	8.66E-03	118 (19)
carbamylcholine	8.74E-03	
propofol	8.74E-03	109 (19)
oxaliplatin	8.83E-03	90 (11)
fulvestrant	8.87E-03	98 (16)
IL3	8.90E-03	81 (7)
BRD4	9.13E-03	84 (10)
uric acid	9.18E-03	96 (13)
celecoxib	9.18E-03	125 (18)
rottlerin	9.21E-03	103 (13)
pituitary adenylate cyclase-activating peptide	9.33E-03	
FOXA1	9.37E-03	14 (2)
fatty acid	9.48E-03	79 (16)
IL13	9.58E-03	109 (19)
IL5	9.60E-03	
particulate matter	9.78E-03	
mir-373	9.78E-03	
GLUL	9.78E-03	
CAPN3	9.78E-03	
SDHB	9.78E-03	
POLDIP2	9.78E-03	
ginkgolide B	9.78E-03	
ethylene dimethanesulfonate	9.78E-03	
zeranol	9.78E-03	
zinc protoporphyrin IX	9.78E-03	101 (13)
(-)-norephedrine	9.78E-03	
N-carbobenzyloxy-leucine-leucine-norvaline	9.78E-03	
asiatic acid	9.78E-03	81 (7)
IRAK1	9.94E-03	104 (16)
FUS-DDIT3	9.94E-03	
lysophosphatidylcholine	1.01E-02	
trilinolein	1.01E-02	
12(R)-hydroxyeicosatetraenoic acid	1.01E-02	
aucubin	1.01E-02	
SMAD decoy ODN	1.01E-02	
APIO-EE-07	1.01E-02	
cortodoxone	1.01E-02	
beta,beta-dimethylacrylalkannin	1.01E-02	
lactotrehalose	1.01E-02	
ND-13	1.01E-02	
eicosa-11Z, 14Z-dienoic acid	1.01E-02	
ZYG11B	1.01E-02	
dendrobium officinale polysaccharide	1.01E-02	
Ggt	1.01E-02	
thymidine phosphorylase	1.01E-02	
ASC-JM17	1.01E-02	

Aspp	1.01E-02
SERPINE3	1.01E-02
phosphorodiamidate morpholino oligor	1.01E-02
3-tert-butyl-4-hydroxyanisole	1.01E-02
AEOL 10113	1.01E-02
HSD17B11	1.01E-02
FBXL17	1.01E-02
BRIP1	1.01E-02
MACC1	1.01E-02
Lamin b	1.01E-02
ELSPBP1	1.01E-02
ZNF667	1.01E-02
LRRC4	1.01E-02
ZYG11A	1.01E-02
cis-vaccenic acid	1.01E-02
PTPRM	1.01E-02
1-deoxysphinganine	1.01E-02
RGS5	1.01E-02
miR-3180-5p (miRNAs w/seed UUCCAG	1.01E-02
mir-3180	1.01E-02
miR-638 (miRNAs w/seed GGGAUCG)	1.01E-02
mir-638	1.01E-02
PPP2R5E	1.01E-02
EIF2B2	1.01E-02
AMPH	1.01E-02
NUAK2	1.01E-02
PSMD1	1.01E-02
CYB5A	1.01E-02
KRT19P3	1.01E-02
HGSNAT	1.01E-02
TUB	1.01E-02
MPT0B214	1.01E-02
PSMD3	1.01E-02
LINC01564	1.01E-02
dulanermin	1.01E-02
HMGN5	1.01E-02
GMEB2	1.01E-02
ASP5878	1.01E-02
RO0281675	1.01E-02
pantoprazole	1.01E-02
benzo(a)pyrene-3,6-quinone	1.01E-02
HA-1004	1.01E-02
phorone	1.01E-02
GNA002	1.01E-02
CA-074	1.01E-02
ferricyanide	1.01E-02
E2F3	1.01E-02
SP600125	1.02E-02
bleomycin	1.04E-02
aspirin	1.04E-02

DDIT3	1.04E-02
reactive oxygen species	1.04E-02
LARP1	1.04E-02
ROR1	1.06E-02
RBM5	1.06E-02
GPX1	1.06E-02
cilostazol	1.06E-02
baicalein	1.06E-02
honokiol	1.06E-02
imiquimod	1.06E-02
docosahexaenoic acid	1.07E-02
CpG oligonucleotide	1.07E-02
misoprostol	1.11E-02
prostaglandin A2	1.11E-02
(Z,E)-5-(4-ethylbenzylidene)-2-thioxothiazolidine-4-carboxamide	1.11E-02
SAR1B	1.11E-02
DPH5	1.11E-02
TRIM31	1.11E-02
SLC2A3	1.11E-02
ATG16L1	1.11E-02
TGFBR3	1.11E-02
DGAT1	1.11E-02
mir-290	1.11E-02
1,1-bis(3'-indolyl)-1-(4-hydroxyphenyl)pyrene	1.11E-02
di-2-pyridylketone 4-cyclohexyl-4-methyl-2-pyridylmethanol	1.11E-02
rhodioloside	1.11E-02
rebamipide	1.11E-02
sildenafil	1.12E-02
NR4A1	1.14E-02
RAC1	1.14E-02
GCG	1.14E-02
tanespimycin	1.14E-02
PNPLA2	1.18E-02
MAPK7	1.18E-02
ATN1	1.18E-02
tanshinone II	1.19E-02
AGTR1	1.19E-02
XBP1	1.21E-02
MYB	1.21E-02
SLC15A4	1.21E-02
NFkB (family)	1.22E-02
IRF5	1.22E-02
romidepsin	1.22E-02
PPARD	1.23E-02
AKT1	1.23E-02
IL33	1.23E-02
(+)-catechin	1.25E-02
triclosan	1.25E-02
NOP2	1.25E-02
CBLB	1.25E-02

HDAC8	1.25E-02
bosutinib	1.25E-02
ethyl pyruvate	1.25E-02
decursin	1.25E-02
phenyl-N-tert-butylNitrone	1.25E-02
allyl sulfide	1.25E-02
DIM-C-pPhOH-3-Cl-5-OCH3	1.25E-02
REL	1.26E-02
lysophosphatidylinositol	1.26E-02
MASTL	1.26E-02
mir-19	1.26E-02
acetovanillone	1.26E-02
GRN	1.27E-02
TFEB	1.29E-02
NOS2	1.30E-02
ATG5	1.32E-02
DRD2	1.32E-02
TWIST2	1.33E-02
4-methylnitrosoamino-1-(3-pyridinyl)-1	1.33E-02
parthenolide	1.33E-02
IL2	1.34E-02
5-azacytidine	1.36E-02
prednisolone	1.36E-02
budesonide	1.37E-02
BCR-ABL1	1.37E-02
mannitol	1.40E-02
C1QTNF1	1.40E-02
PHLPP2	1.40E-02
necrostatin-1	1.40E-02
p85 (pik3r)	1.40E-02
AKR1B1	1.40E-02
protectin D1	1.40E-02
PARP1	1.40E-02
JAK3	1.41E-02
IKBKE	1.42E-02
KDM1A	1.47E-02
chloroquine	1.47E-02
elaidic acid	1.47E-02
ritonavir	1.48E-02
dasatinib	1.48E-02
ALB	1.48E-02
filgrastim	1.49E-02
MYOD1	1.51E-02
Pka	1.52E-02
TFAP2A	1.52E-02
ATF2	1.53E-02
desmopressin	1.53E-02
3-deoxy-2-octulosonic acid(2)-lipid A	1.55E-02
Rp-cAMPS	1.55E-02
C2CD5	1.55E-02

oridonin	1.55E-02
CYTOR	1.55E-02
RHOJ	1.55E-02
PROM1	1.55E-02
volasertib	1.55E-02
NEDD4	1.55E-02
acetyl-L-carnitine	1.55E-02
helenalin	1.55E-02
taxifolin	1.55E-02
melphalan	1.55E-02
PAX3	1.56E-02
SRF	1.56E-02
roscovitine	1.56E-02
SRC (family)	1.56E-02
temozolomide	1.56E-02
PF543	1.56E-02
UQCC3	1.56E-02
HGF	1.59E-02
KAT5	1.59E-02
NFKB1	1.60E-02
RPTOR	1.61E-02
TLR2	1.63E-02
DYSF	1.64E-02
SREBF2	1.64E-02
CKAP2L	1.65E-02
NLRX1	1.65E-02
YTHDF1	1.65E-02
MMP1	1.65E-02
N-formyl-Met-Leu-Phe	1.65E-02
FOS	1.68E-02
LEP	1.68E-02
EGF	1.69E-02
doxycycline	1.69E-02
Sos	1.70E-02
PRL	1.71E-02
kawain	1.71E-02
TMBIM6	1.71E-02
NAB2	1.71E-02
TP53COR1	1.71E-02
HNRNPAB	1.71E-02
antimycin A	1.71E-02
FDI6	1.71E-02
SQSTM1	1.73E-02
PLK1	1.73E-02
MYBL2	1.73E-02
carbamazepine	1.73E-02
Am 580	1.73E-02
IL1B	1.74E-02
triptolide	1.74E-02
bee venom	1.75E-02

N-acetyl-L-cysteine	1.75E-02
TNFSF13B	1.79E-02
SFRP1	1.82E-02
CD 437	1.82E-02
Interferon alpha	1.84E-02
MDM2	1.87E-02
Irgm1	1.87E-02
MUC1	1.87E-02
IMD-0354	1.88E-02
PPOX	1.88E-02
PTP4A3	1.88E-02
MGLL	1.88E-02
RND3	1.88E-02
CREB3L1	1.88E-02
NR6A1	1.88E-02
mangiferin	1.88E-02
halofuginone	1.88E-02
SPP1	1.90E-02
SIRT1 inhibitor III	1.91E-02
mir-30	1.91E-02
Vegf	1.93E-02
EIF2AK3	1.93E-02
lovastatin	1.93E-02
TCOF1	1.94E-02
bezafibrate	1.94E-02
oleic acid	1.98E-02
CLU	2.00E-02
luteolin	2.00E-02
SIRT6	2.00E-02
mir-29	2.00E-02
NCOA3	2.00E-02
GS-444217	2.01E-02
guanosine	2.01E-02
cerebroside 3-sulfate	2.01E-02
eleutherococcus senticosus extract	2.01E-02
Clock-Bmal1	2.01E-02
Mellitin	2.01E-02
VX-944	2.01E-02
atiprimod	2.01E-02
BayCysLT2	2.01E-02
PYDC2	2.01E-02
CP-724,714	2.01E-02
prunetin	2.01E-02
2-methoxycinnamaldehyde	2.01E-02
Rbx1	2.01E-02
PLEKHM1	2.01E-02
ZBTB2	2.01E-02
TCHP	2.01E-02
ZFAT	2.01E-02
FAM168A	2.01E-02

RBM17	2.01E-02
GFRAL	2.01E-02
API5	2.01E-02
AVL9	2.01E-02
MAGEA2/MAGEA2B	2.01E-02
PCDH9	2.01E-02
CENPL	2.01E-02
BHLH	2.01E-02
ADH5	2.01E-02
PHOSPHO1	2.01E-02
arecaidine propargyl ester	2.01E-02
bucillamine	2.01E-02
hyaluronidase	2.01E-02
MYO1C	2.01E-02
CPI-613	2.01E-02
safflor yellow B	2.01E-02
DGKD	2.01E-02
ASNS	2.01E-02
BCL2L2-PABPN1	2.01E-02
16:0/20:4 phosphatidylcholine	2.01E-02
mir-663	2.01E-02
SALL2	2.01E-02
ALMS1-IT1	2.01E-02
NOXO1	2.01E-02
CST7	2.01E-02
HPS1	2.01E-02
ULK2	2.01E-02
SHMT2	2.01E-02
PLIN3	2.01E-02
NKIRAS1	2.01E-02
MUC16	2.01E-02
IBTK	2.01E-02
ADAMTS13	2.01E-02
COMP	2.01E-02
SVIL	2.01E-02
ING5	2.01E-02
CDC23	2.01E-02
KHDRBS2	2.01E-02
GPBP1	2.01E-02
CYP24A1	2.01E-02
PRIMA1	2.01E-02
2-methoxy-N-(3-methyl-2-oxo-1,4-dihy	2.01E-02
PRSS3	2.01E-02
LOC100359583/Ptma	2.01E-02
RANBP2	2.01E-02
Anp32e	2.01E-02
nexrutine	2.01E-02
NRIR	2.01E-02
BMS-863233	2.01E-02
PFK-15	2.01E-02

tyrosine kinase inhibitor	2.01E-02
S7	2.01E-02
SI163	2.01E-02
S29	2.01E-02
reactive nitrogen species	2.01E-02
daphnoretin	2.01E-02
XK469	2.01E-02
BIBX1522	2.01E-02
CGP77675	2.01E-02
AN-207	2.01E-02
glyoxal	2.01E-02
SSR180575	2.01E-02
E 3330	2.01E-02
4-nitrophenol	2.01E-02
heme arginate	2.01E-02
fascaplysin	2.01E-02
chitosan oligosaccharide	2.01E-02
all-trans-4-Oxo-retinoic acid	2.01E-02
isoalantolactone	2.01E-02
luteolin 7-O-beta-D-glucoside	2.01E-02
swainsonine	2.01E-02
N(6)-carboxymethyl-L-lysine	2.01E-02
ceapin-A7	2.06E-02
glycine	2.06E-02
sitagliptin	2.06E-02
IgG1	2.06E-02
HSP90AA1	2.06E-02
ZNF148	2.06E-02
mir-515	2.06E-02
TNFRSF11A	2.06E-02
ITGA6	2.06E-02
MAP3K5	2.06E-02
IND S1	2.06E-02
PTPN3	2.06E-02
NSC23766	2.06E-02
coenzyme Q10	2.06E-02
V-PYRRO/NO	2.06E-02
probucol	2.06E-02
idarubicin	2.06E-02
farnesol	2.06E-02
PSEN2	2.06E-02
hemin	2.06E-02
1,1-diarylethylene-diammonium deriva	2.09E-02
RBL2	2.13E-02
CGA	2.13E-02
CAV1	2.18E-02
miR-27a-3p (and other miRNAs w/seed	2.19E-02
PIM1	2.19E-02
telmisartan	2.19E-02
EP300	2.23E-02

zidovudine	2.24E-02
A-769662	2.24E-02
NADPH oxidase	2.24E-02
C1QBP	2.24E-02
KIF3A	2.24E-02
epirubicin	2.24E-02
CHUK	2.24E-02
RANBP3L	2.28E-02
SGPL1	2.28E-02
thioctic acid	2.28E-02
acyline	2.34E-02
miR-146a-5p (and other miRNAs w/seed)	2.34E-02
phenethyl isothiocyanate	2.34E-02
FN1	2.38E-02
TNFRSF8	2.38E-02
IFNAR1	2.40E-02
HAVCR1	2.41E-02
wortmannin	2.42E-02
UHRF2	2.42E-02
SH3PXD2A-AS1	2.42E-02
mir-101	2.42E-02
CCAT2	2.42E-02
CYP27A1	2.42E-02
DYRK1A	2.42E-02
RAPGEF3	2.42E-02
FABP1	2.42E-02
diallyl trisulfide	2.42E-02
7-ketocholesterol	2.42E-02
diosgenin	2.42E-02
Ni2+	2.42E-02
isobutylmethylxanthine	2.47E-02
RAF1	2.47E-02
RHOA	2.48E-02
genistein	2.48E-02
NPM1	2.58E-02
PAX3-FOXO1	2.58E-02
L-methionine	2.59E-02
carbon monoxide	2.59E-02
FAM83A	2.62E-02
E2F5	2.62E-02
COMMD1	2.62E-02
TNFSF11	2.63E-02
metformin	2.63E-02
peptidoglycan	2.63E-02
phosphate	2.63E-02
simvastatin	2.67E-02
miR-30c-5p (and other miRNAs w/seed)	2.70E-02
TNFAIP3	2.70E-02
ATP5IF1	2.70E-02
emodin	2.70E-02

ARNT	2.76E-02
EGR2	2.76E-02
HMG20A	2.78E-02
GNA15	2.78E-02
RELA	2.79E-02
SNCA	2.80E-02
gentamicin	2.81E-02
tributyrin	2.81E-02
secondhand smoke	2.81E-02
E2F6	2.81E-02
CDX1	2.81E-02
sulindac	2.81E-02
PHF1	2.82E-02
IL31	2.82E-02
CDC73	2.82E-02
SLC2A4	2.82E-02
HEXIM1	2.82E-02
FOXF2	2.82E-02
H-[1,2,4]oxadiazolo[4,3-alpha]quinoxali	2.82E-02
TAL1	2.82E-02
NOTCH1	2.85E-02
tetradecanoylphorbol acetate	2.88E-02
methylmercury	2.92E-02
KIT	2.94E-02
bisindolylmaleimide I	2.94E-02
RICTOR	2.94E-02
alitretinoin	2.95E-02
phosphatidylethanolamine	3.00E-02
PDGF receptor tyrosine kinase inhibitor	3.00E-02
L-tyrosine	3.00E-02
3-aminoisobutanoate	3.00E-02
L-ornithine	3.00E-02
7alpha-hydroxycholesterol	3.00E-02
extremely high molecular weight hyalur	3.00E-02
Ewsr1	3.00E-02
methysticin	3.00E-02
MIR100HG	3.00E-02
CpG 10101	3.00E-02
LINC01134	3.00E-02
ZNF346	3.00E-02
NOMO1 (includes others)	3.00E-02
TTC5	3.00E-02
BIRC6	3.00E-02
IL3r	3.00E-02
AKIP1	3.00E-02
LINC00924	3.00E-02
MSRA	3.00E-02
MCTS1	3.00E-02
USP50	3.00E-02
RPS6KA	3.00E-02

METTL7B	3.00E-02
OXGR1	3.00E-02
2-(3-chlorophenylamino)phenylacetic a	3.00E-02
CKLF	3.00E-02
MINDY3	3.00E-02
SDF4	3.00E-02
TMEM106B	3.00E-02
UBE2D3	3.00E-02
Fetal Hemoglobin	3.00E-02
GEM231	3.00E-02
cerebrolysin	3.00E-02
ZMAT3	3.00E-02
RNF144B	3.00E-02
vistusertib	3.00E-02
GSK461364	3.00E-02
IVNS1ABP	3.00E-02
MAT2B	3.00E-02
COL3A1	3.00E-02
GSTA4	3.00E-02
FBXL20	3.00E-02
miR-520g-3p (and other miRNAs w/seed AAAACGU	3.00E-02
miR-424-3p (miRNAs w/seed AAAACGU	3.00E-02
mir-368	3.00E-02
apitolisib	3.00E-02
SEC23B	3.00E-02
ACS84	3.00E-02
AP2A2	3.00E-02
RBFOX1	3.00E-02
PES1	3.00E-02
ERCC6	3.00E-02
Lycium barbarum polysaccharides	3.00E-02
CLCA1	3.00E-02
MT3	3.00E-02
GBP2	3.00E-02
GK	3.00E-02
Naip1 (includes others)	3.00E-02
GBP3	3.00E-02
UIMC1	3.00E-02
HILPDA	3.00E-02
COPS6	3.00E-02
AIM-100	3.00E-02
3K3A-APC protein	3.00E-02
UBE2D2	3.00E-02
(E)-1-(2-nitrovinyl)naphthalene	3.00E-02
(E)-9-(2-nitrovinyl)anthracene	3.00E-02
norcantharidin	3.00E-02
temuterkib	3.00E-02
OPC-13213	3.00E-02
methoxyacetic acid	3.00E-02
mivebresib	3.00E-02

tempo	3.00E-02
fomepizole	3.00E-02
methacholine	3.00E-02
GSK-3beta inhibitor II	3.00E-02
LFM-A13	3.00E-02
fosbretabulin	3.00E-02
(S)-duloxetine	3.00E-02
ofloxacin	3.00E-02
N-methylsulfonyl-12,12-dibromododec	3.00E-02
NSC63002	3.00E-02
NSC43067	3.00E-02
NSC269621	3.00E-02
silver nitrate	3.00E-02
lanthanum chloride	3.00E-02
MitoBloCK-6	3.00E-02
lead nitrate	3.00E-02
nickel subsulfide	3.00E-02
imisopasem manganese	3.00E-02
oleuropein aglycone	3.00E-02
Schiff base zinc (II) derivative complex	3.00E-02
L-JNK inhibitor I	3.00E-02
GENE-317	3.00E-02
quinine	3.00E-02
alpha-ergocryptine	3.00E-02
CDP ethanolamine	3.00E-02
stavudine	3.00E-02
NCGC00378430	3.00E-02
epiallopregnanolone	3.00E-02
EBF1	3.01E-02
TNFRSF1A	3.01E-02
peoniflorin	3.02E-02
PADI2	3.02E-02
HSPB1	3.02E-02
FABP3	3.02E-02
ECSIT	3.02E-02
FGF9	3.02E-02
GW7647	3.02E-02
4-nitroquinoline-1-oxide	3.02E-02
K-252	3.02E-02
WR 1065	3.02E-02
MCC950	3.02E-02
rifaximin	3.02E-02
tauroursodeoxycholic acid	3.02E-02
glutamine	3.02E-02
FOXO4	3.02E-02
EHMT1	3.02E-02
PRKN	3.02E-02
arsenite	3.02E-02
PDK1	3.04E-02
Go 6976	3.04E-02

HNF1B	3.08E-02
raloxifene	3.08E-02
HDAC1	3.14E-02
KL	3.16E-02
Gcg	3.16E-02
trovafloxacin	3.16E-02
3,3'-diindolylmethane	3.16E-02
cytarabine	3.16E-02
TCF4	3.19E-02
DDX5	3.19E-02
CDX2	3.21E-02
SB203580	3.22E-02
lipofermata	3.23E-02
MC4R	3.23E-02
BTRC	3.23E-02
HDAC9	3.23E-02
IAPP	3.23E-02
SLC2A1	3.23E-02
IND S7	3.23E-02
TRIM21	3.23E-02
SMARCC1	3.23E-02
PRKCB	3.28E-02
EFNA1	3.28E-02
PTP4A1	3.28E-02
PTGS2	3.32E-02
Collagen type I (complex)	3.40E-02
TSLP	3.40E-02
rotenone	3.40E-02
NFkB (complex)	3.43E-02
trans-cinnamaldehyde	3.45E-02
RAD21L1	3.45E-02
SH3TC2	3.45E-02
MAP3K12	3.45E-02
miR-223-3p (miRNAs w/seed GUCAGUL	3.45E-02
SUB1	3.45E-02
STAG3	3.45E-02
MEL S3	3.45E-02
NPC2	3.45E-02
7(R)-maresin 1	3.45E-02
acteoside	3.45E-02
methylnitronitrosoguanidine	3.45E-02
isoflurane	3.45E-02
deoxynivalenol	3.45E-02
paraquat	3.45E-02
mir-133	3.52E-02
FASN	3.52E-02
ITGB3	3.52E-02
TGFB2	3.54E-02
nicotine	3.56E-02
SMAD4	3.60E-02

MTOR	3.60E-02
TARDBP	3.63E-02
infliximab	3.65E-02
PLAU	3.65E-02
STK11	3.65E-02
POLG	3.67E-02
olaparib	3.67E-02
CASP3	3.67E-02
GCK	3.67E-02
GDF15	3.67E-02
BGN	3.67E-02
SMC1B	3.67E-02
VAV3	3.67E-02
pirfenidone	3.67E-02
ramipril	3.67E-02
R-WIN 55,212	3.67E-02
bardoxolone methyl	3.67E-02
cannabidiol	3.67E-02
phytohemagglutinin	3.71E-02
PTH	3.71E-02
KCNJ2	3.73E-02
KDM8	3.78E-02
SHC1	3.78E-02
caspase	3.90E-02
MAPK11	3.90E-02
VAV1	3.90E-02
CXCR3	3.90E-02
LACTB	3.90E-02
TUG1	3.90E-02
oltipraz	3.90E-02
proteasome inhibitor PSI	3.90E-02
epoxomicin	3.90E-02
carbenoxolone	3.90E-02
arsenic	3.90E-02
PTN	3.91E-02
5-hydroxytryptamine	3.92E-02
MAPK8	3.93E-02
sofalcone	3.98E-02
miR-98 inhibitor	3.98E-02
choline plasmalogens	3.98E-02
flavokawain A	3.98E-02
5-deoxy-rutaecarpine	3.98E-02
heat-not-burn cigarette smoke extract	3.98E-02
BC-11	3.98E-02
ML264	3.98E-02
tyrosine kinase	3.98E-02
PLD	3.98E-02
15-LOX	3.98E-02
yangonin	3.98E-02
FEZF1	3.98E-02

Smad3-Smad4	3.98E-02
Metalloprotease	3.98E-02
tozasertib	3.98E-02
tanshinone I	3.98E-02
CC8490	3.98E-02
ticrynafen	3.98E-02
DIRAS2	3.98E-02
ZFP42	3.98E-02
GNL3L	3.98E-02
DNAJB4	3.98E-02
MYOT	3.98E-02
WAPL	3.98E-02
SLC13A5	3.98E-02
DUOX1	3.98E-02
LAPTM4B	3.98E-02
USP24	3.98E-02
ORAI3	3.98E-02
cathepsin L inhibitor	3.98E-02
NDUFA4L2	3.98E-02
SIRT5	3.98E-02
XRN2	3.98E-02
LINC00173	3.98E-02
AZD7762	3.98E-02
nintedanib	3.98E-02
danusertib	3.98E-02
NCF2	3.98E-02
kahweol	3.98E-02
MOAP1	3.98E-02
IBSP	3.98E-02
CELA2A	3.98E-02
PKIA	3.98E-02
miR-200a-5p (and other miRNAs w/see)	3.98E-02
mir-431	3.98E-02
mir-340	3.98E-02
NNMT	3.98E-02
BOK	3.98E-02
TOPORS	3.98E-02
PDE4A	3.98E-02
DUXAP8	3.98E-02
TGFBI	3.98E-02
KPNA1	3.98E-02
GCN1	3.98E-02
ARSA	3.98E-02
CHRM4	3.98E-02
CASP14	3.98E-02
CELF2	3.98E-02
IRAK1BP1	3.98E-02
PTP4A2	3.98E-02
BICD2	3.98E-02
USP13	3.98E-02

GP1BA	3.98E-02
LDB2	3.98E-02
LGALS2	3.98E-02
VSNL1	3.98E-02
PLCD4	3.98E-02
SCIN	3.98E-02
PPP1CC	3.98E-02
ANP32E	3.98E-02
CYP4V2	3.98E-02
MK-8776	3.98E-02
RRM2B	3.98E-02
DPYSL2	3.98E-02
unsaturated guluronate oligosaccharide	3.98E-02
Apela	3.98E-02
LOC105374325	3.98E-02
AZD-1208	3.98E-02
azurin 50-77	3.98E-02
GR-MD-02	3.98E-02
4-(4-(3-(4-trifluoromethoxy-phenyl)urei	3.98E-02
PDGFRbeta-recognizing cyclic peptide	3.98E-02
MRS2211	3.98E-02
griseofulvin	3.98E-02
bafilomycin	3.98E-02
BO-653	3.98E-02
iopanoic acid	3.98E-02
tipifarnib	3.98E-02
bilobalide	3.98E-02
glabridin	3.98E-02
centrinone	3.98E-02
moxifloxacin	3.98E-02
lomefloxacin	3.98E-02
methyl cellosolve	3.98E-02
protoporphyrin IX	3.98E-02
salsalate	3.98E-02
salicylamide	3.98E-02
4-methylcatechol	3.98E-02
mycophenolate mofetil	3.98E-02
cadmium sulfate	3.98E-02
chlorophyllin	3.98E-02
SF2523	3.98E-02
sertindole	3.98E-02
Phe-Pro-Arg-chloromethyl ketone	3.98E-02
tiotropium	3.98E-02
isoacteoside	3.98E-02
thymeatoxin	3.98E-02
Co2+	3.98E-02
Pb2+	3.98E-02
thyroid hormone	4.01E-02
E2F2	4.04E-02
IL10RA	4.10E-02

CDKN1B	4.11E-02
SCH 58261	4.13E-02
grape seed extract	4.13E-02
PCLAF	4.13E-02
mir-193	4.13E-02
HSPB8	4.13E-02
pitavastatin	4.13E-02
carvedilol	4.13E-02
dimethyl fumarate	4.13E-02
glycyrrhizic acid	4.13E-02
LMNA	4.16E-02
CLPP	4.18E-02
THZ1	4.21E-02
levodopa	4.24E-02
Ap1	4.25E-02
RUNX2	4.31E-02
RBL1	4.32E-02
miR-199a-5p (and other miRNAs w/seed)	4.32E-02
PLG	4.32E-02
Hif1	4.32E-02
25-hydroxycholesterol	4.32E-02
losartan potassium	4.33E-02
prostaglandin J2	4.37E-02
fluoromethyl 2,2-difluoro-1-(trifluoromethyl)-1H-imidazole-5-carboxylate	4.37E-02
PHF12	4.37E-02
HAND1	4.37E-02
CNOT7	4.37E-02
TRPV4	4.37E-02
HSPD1	4.37E-02
rosuvastatin	4.37E-02
diallyl disulfide	4.37E-02
TAS4464	4.37E-02
IGF1R	4.40E-02
SRSF1	4.46E-02
TRAF6	4.46E-02
FGF7	4.46E-02
cerivastatin	4.46E-02
IL1A	4.48E-02
GSK3B	4.51E-02
KLF11	4.51E-02
indomethacin	4.55E-02
miR-141-3p (and other miRNAs w/seed)	4.60E-02
CCL5	4.60E-02
TFAP2C	4.60E-02
fenretinide	4.60E-02
THEM6	4.61E-02
MALT1	4.61E-02
ANXA2	4.61E-02
SOST	4.61E-02
THBS4	4.61E-02

RAS	4.62E-02
estrogen receptor	4.69E-02
mir-181	4.74E-02
MAP3K7	4.74E-02
IGF1	4.75E-02
MRTFB	4.84E-02
indican	4.86E-02
PRKDC	4.86E-02
miR-26a-5p (and other miRNAs w/seed	4.86E-02
CA9	4.86E-02
ING1	4.86E-02
PSMD10	4.86E-02
PHB1	4.86E-02
betulinic acid	4.86E-02
choline	4.86E-02
eplerenone	4.86E-02
TIA1	4.89E-02
nelfinavir	4.89E-02
FIIN-2	4.95E-02
taumuricholic acid	4.95E-02
taurochenodeoxycholate	4.95E-02
chondroitin sulfate	4.95E-02
lunasin	4.95E-02
AOPEP	4.95E-02
W146	4.95E-02
aurapten	4.95E-02
CYP	4.95E-02
cefditoren	4.95E-02
SLC22A2	4.95E-02
DESI2	4.95E-02
CYP26A1	4.95E-02
FENDRR	4.95E-02
NSRP1	4.95E-02
SPATA2	4.95E-02
ICMT-DT	4.95E-02
YIPF6	4.95E-02
NDST2	4.95E-02
SNHG3	4.95E-02
CDH16	4.95E-02
SIRT4	4.95E-02
PLXND1	4.95E-02
PCA3	4.95E-02
proanthocyanidin derivative	4.95E-02
WAY 200070	4.95E-02
VTRNA2-1	4.95E-02
ALDH3A2	4.95E-02
azilsartan	4.95E-02
KPNA4	4.95E-02
SH3GLB1	4.95E-02
AZD1480	4.95E-02

MIR3936HG	4.95E-02
omipalisib	4.95E-02
FOXK1	4.95E-02
AGAP2	4.95E-02
TAOK2	4.95E-02
AKT1S1	4.95E-02
miR-331-3p (miRNAs w/seed CCCCUGG	4.95E-02
miR-1285-3p (and other miRNAs w/seed	4.95E-02
mir-612	4.95E-02
MIR3781	4.95E-02
KCND2	4.95E-02
UTS2	4.95E-02
FBP1	4.95E-02
PFN1	4.95E-02
FUT4	4.95E-02
DCX	4.95E-02
CANX	4.95E-02
CUL5	4.95E-02
CEL	4.95E-02
BBC3	4.95E-02
SH3GL1	4.95E-02
VCAM1	4.95E-02
SRSF6	4.95E-02
CDC27	4.95E-02
copanlisib	4.95E-02
DDT	4.95E-02
PLAA	4.95E-02
HOXD12	4.95E-02
Muc1	4.95E-02
RP11_394O46	4.95E-02
ciliobrevin A	4.95E-02
methylamine	4.95E-02
4-vinyl-1-cyclohexene dioxide	4.95E-02
N-hydroxy-N'-(4-butyl-2-methylphenyl)hydrazide	4.95E-02
L-796449	4.95E-02
pyrazole	4.95E-02
silicon phthalocyanine	4.95E-02
SQ 29548	4.95E-02
plevitrexed	4.95E-02
ethacrynic acid	4.95E-02
phenol derivative	4.95E-02
Collagen type V	4.95E-02
aurothiomalate	4.95E-02
opioid	4.95E-02
flavonoid	4.95E-02
H-8	4.95E-02
phloretin	4.95E-02
sodium azide	4.95E-02
mini-GAGR	4.95E-02
TH1834	4.95E-02

angustibalin	4.95E-02
SAR407899	4.95E-02
AL162253.2	4.95E-02
1-Naphthalenesulfonyl-IW-CHO	4.95E-02
colcemid	4.95E-02
pergolide	4.95E-02
azelaic acid	4.95E-02
floxuridine	4.95E-02
L-buthionine (SR)-sulfoximine	4.95E-02
N-acetylglucosaminono-1,5-lactone O-(	4.95E-02
Hg2+	4.95E-02
AR	4.95E-02
vancomycin	6.98E-02
deferoxamine	7.20E-02
WNT5A	8.57E-02
ELAVL1	1.05E-01
HOXA10	1.15E-01
SCD	1.25E-01
ID3	1.43E-01
STAT5B	1.67E-01
FMR1	2.08E-01
quercetin	2.17E-01
lenalidomide	2.58E-01
cyclosporin A	3.11E-01

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NUDT2 initiates viral RNA degradation by removal of 5'-phosphates

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While viral replication processes are largely understood, comparably little is known on cellular mechanisms degrading viral RNA. Some viral RNAs bear a 5'-triphosphate (PPP-) group that impairs degradation by the canonical 5'-3' degradation pathway. Here we show that the Nudix hydrolase 2 (NUDT2) trims viral PPP-RNA into monophosphorylated (P)-RNA, which serves as a substrate for the 5'-3' exonuclease XRN1. NUDT2 removes 5'-phosphates from PPP-RNA in an RNA sequence- and overhang-independent manner and its ablation in cells increases growth of PPP-RNA viruses, suggesting an involvement in antiviral immunity. NUDT2 is highly homologous to bacterial RNA pyrophosphatase H (RppH), a protein involved in the metabolism of bacterial mRNA, which is 5'-tri- or diphosphorylated. Our results show a conserved function between bacterial RppH and mammalian NUDT2, indicating that the function may have adapted from a protein responsible for RNA turnover in bacteria into a protein involved in the immune defense in mammals.

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All living organisms use RNA as the messenger molecule to translate their DNA encoded genetic information into proteins. Besides trans-activating sequences encoded on messenger (m)RNA and the subcellular localization of the RNA molecule, the abundance of mRNA is the primary determinant to modulate gene expression. mRNA abundance is regulated based on transcription and the stability of the molecule. In eukaryotes, the most prominent and evolutionary conserved processes that modulate the stability of mRNA are 5'-to-3' RNA degradation by the cellular exonuclease 1 (XRN1) and 3'-to-5' degradation by the RNA exosome complex. Eukaryotic mRNAs are protected from 5'-to-3' degradation by a 5'-m⁷G cap structure, co-transcriptionally added to the mRNA molecule. To proceed with the 5'-to-3' degradation process, the mRNA cap needs to be removed by the decapping protein 2 (DCP2) to generate a monophosphorylated (P-)RNA substrate, which can then be degraded by XRN1 (Fig. 1a)^{1,2}. Genomic RNAs and RNA transcripts of many viruses, including Orthomyxo-, Arena-, Paramyxo- and Bunyaviruses, do not bear a 5'-cap but present a 5'-

triphosphate (PPP-) group. This terminal PPP- moiety linked to double-stranded RNA serves as a pathogen-associated molecular pattern (PAMP) sensed by the cellular helicase RIG-I, which activates a MAVS-dependent innate immune signaling cascade. Eventually, this culminates in the accumulation of type-I interferon (IFN) and IFN-stimulated genes and is critical to prevent virus spread³. It is currently unclear why some viruses bear terminal triphosphates despite the selective pressure of the innate immune system. However, a possible explanation may be the increased stability of PPP-RNA that inhibits 5'-to-3' degradation by XRN1. Interestingly, despite the stabilizing PPP-group on viral RNA, specific highly secondary structured viral RNA sequences expressed by Flavi-, Bunya-, and Arenaviruses impair the activity of XRN1^{4,5}. Moreover, the depletion of XRN1 increases the stability of viral RNA⁶. Collectively, this suggests that 5'-to-3' RNA degradation processes are operative in the case of viral RNA. In analogy to the eukaryotic mRNA degradation system, however, the degradation of viral PPP-RNA would require a processing step that removes the PPP-group. Indeed, recently the

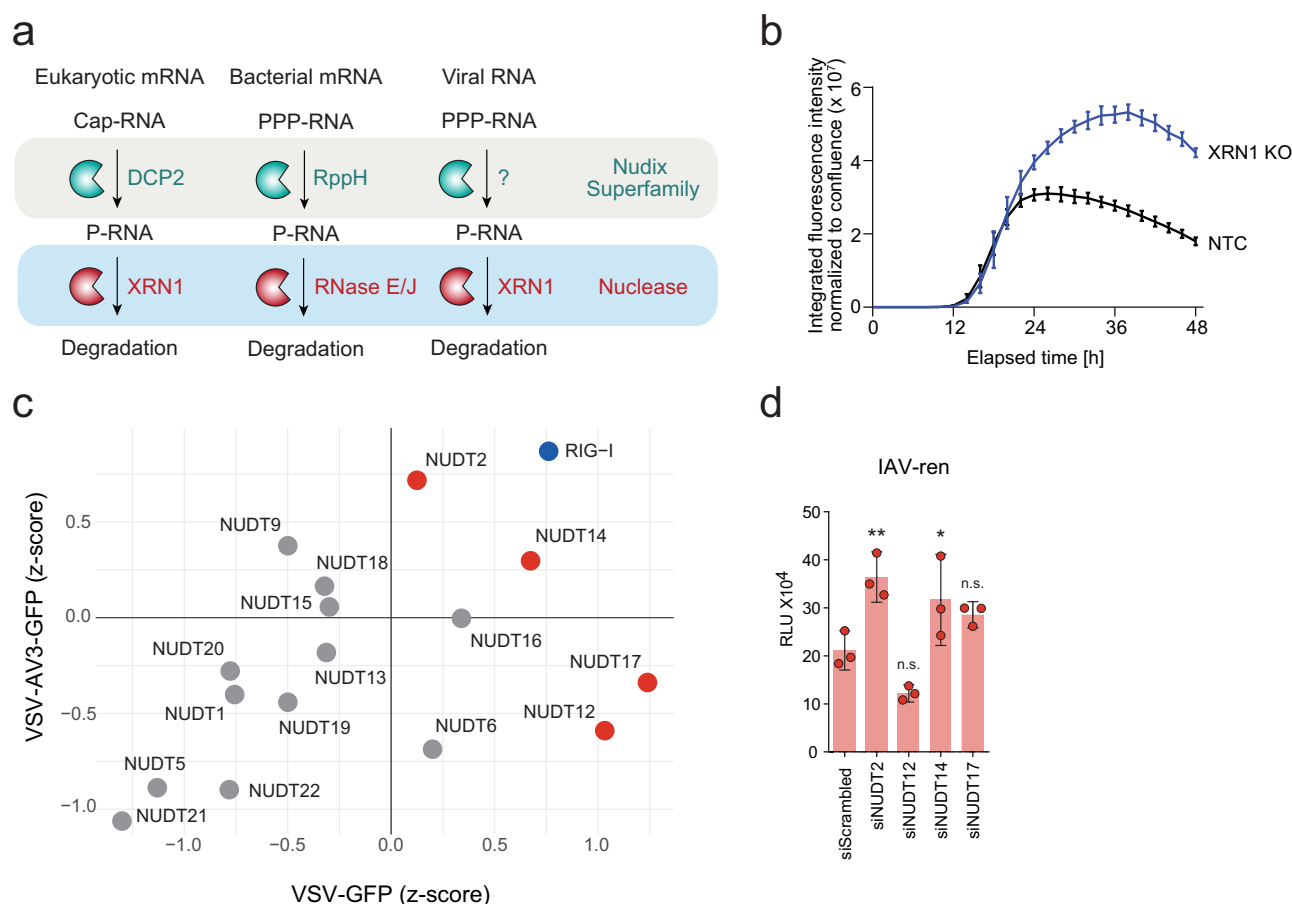


Fig. 1 Influence of Nudix depletion on virus growth. **a** Schematic representation of processes involved in 5'-to-3' RNA degradation. Eukaryotic and bacterial triphosphorylated messenger (m)RNA are processed by proteins DCP2 and RppH, respectively, to generate monophosphorylated substrates for the 5'-3' exonuclease XRN1, RNase E or RNase J. We hypothesize that a mammalian Nudix hydrolase could prepare viral RNA for degradation by removing pyrophosphate groups. **b** Hep3B cells with a CRISPR/Cas9-mediated KO of XRN1 or Hep3B cells transduced with a non-targeting-control vector (NTC) were infected with VSV-GFP at MOI 0.01 for 48 h. GFP expression was continuously quantified by live-cell microscopy. The data points are displayed as the mean of five technical replicates $\pm$ SD. One representative experiment of three is shown. **c** HeLa cells transfected with siRNA pools targeting the indicated Nudix gene for 48 h were infected with VSV-GFP (x-axis) or VSV-AV3-GFP (y-axis). Twenty-four hours after infection, GFP expression was analyzed by fluorometric analysis. The displayed z-scores were calculated from three independent reactions. **d** HeLa cells were treated with siRNA pools for 48 h targeting the transcript of *NUDT2*, *NUDT12*, *NUDT14*, *NUDT17*, or non-targeting siRNA control (siScrambled) and infected with Influenza A reporter virus expressing the renilla luciferase (IAV-ren) at MOI 1. Forty-eight hours post-infection, renilla luciferase activity was analyzed. The means of three biological replicates $\pm$ SD are shown; ** $p = 0.004$, * $p = 0.03$ as analyzed by two-way analysis of variance (ANOVA) statistics. RLU relative light units. n.s. not significant.

triphosphatases RNA/RNP complex-1-interacting phosphatase (DUSP11) and Decapping and exoribonuclease protein (DXO, also known as DOM3Z) were shown to dephosphorylate PPP-RNA to P-RNA that can be further degraded by XRN1^{7–9}. However, the mechanism of PPP-RNA dephosphorylation and the substrate specificity of DUSP11 is not yet well understood.

Similar to viral RNA, prokaryotic mRNA also bears a 5'-PPP or 5'-PP group^{10,11}. In bacteria, the 5' phosphates can be removed by the RNA pyrophosphohydrolase (RppH) to prepare RNA for degradation by the bacterial 5'-to-3' endoribonuclease RNase E or 5' exonucleases (e.g., RNase J^{11–13} (Fig. 1a)). Interestingly, both mammalian DCP2 and bacterial RppH belong to the superfamily of Nudix hydrolases¹⁴, a protein family that is highly diverse in terms of sequence, domain organization, and substrate specificity¹⁵. Nudix proteins are characterized by a Nudix hydrolase domain, which contains characteristic catalytic and metal-binding amino acids¹⁶. They mostly function as pyrophosphohydrolases active towards substrates with the structure NDP-X (nucleoside diphosphate linked to another moiety, X), usually resulting in NMP (nucleoside monophosphate) and P-X as products^{16–18}. Since Nudix hydrolases process mammalian and bacterial mRNAs, we tested whether mammalian Nudix hydrolases may have dephosphorylation activity on PPP-RNA to initiate further exonucleolytic processing. Understanding such viral RNA degradation mechanisms is essential to further study cellular antiviral principles that are in place to limit the spread and pathogenicity of infectious pathogens and their involvement in antiviral immunity.

Results

Nudix hydrolases have an impact on virus growth. To establish a screening system that allows testing for 5'-to-3' dependent RNA degradation in virus-infected cells, we first evaluated whether CRISPR/Cas9-mediated depletion of XRN1 affects the replication of PPP-RNA-generating vesicular stomatitis virus expressing green fluorescent protein (VSV-GFP). Automated live-cell microscopy of VSV-GFP infected cells showed a distinct increase of GFP signal in XRN1 targeted cells as compared to controls (Fig. 1b), indicating that VSV-GFP is restricted by a 5'-to-3' exonuclease dependent pathway. We hypothesized that any Nudix hydrolase that may be involved in PPP-RNA processing should similarly negatively regulate virus growth. We, therefore, used siRNAs to deplete mammalian Nudix proteins, which are known to play a role in nucleotide metabolism¹⁷ (Supplementary Table 1) and quantified the viral growth of VSV-GFP and the IFN-stimulating variant VSV-AV3-GFP¹⁹. Compared to the control treatment, depletion of the PPP-RNA-sensing pattern recognition receptor RIG-I led to increased GFP expression after infection with either of the two viruses, confirming the validity of this approach (Fig. 1c). Notably, the depletion of four Nudix hydrolases (NUDT2, NUDT12, NUDT14, and NUDT17) led to an increase in VSV-GFP or VSV-AV3-GFP growth. As tested by cell titer glow assay, cell viability was not affected by the depletion of these Nudix proteins (Supplementary Fig. 1a). Depletion of NUDT5, NUDT19, NUDT21, and NUDT22 showed a negative impact on VSV growth (Fig. 1c). For NUDT19 and NUDT22, this phenotype could potentially be explained by reduced cell viability after depletion of these Nudix hydrolases. In total, we selected four Nudix hydrolases (NUDT2, -12, -14, and -17), which showed the highest suppressive effect on either of the two viruses for further experiments. We confirmed the knockdown efficiency of NUDT2, -12, -14, and -17 siRNA treatment in HeLa cells by RT-qPCR and western blotting (NUDT2) (Supplementary Fig. 1b, c). We used renilla-luciferase-expressing Influenza A virus (IAV-ren) as an alternative PPP-RNA generating virus to confirm the

antiviral activity of the four NUDTs (Fig. 1d). While the depletion of NUDT12 and NUDT17 did not significantly increase the renilla signal in this cell type, the depletion of NUDT2 and -14 led to a significant increase (Fig. 1d).

NUDT2 releases phosphates from a triphosphorylated RNA substrate. To characterize the four selected Nudix hydrolases for their potential ability to dephosphorylate PPP-RNA, we generated recombinant NUDT2, -12, -14, and -17 in *E. coli*. As controls, we mutated the metal-coordinating glutamic acid residue (E) in their conserved Nudix hydrolase domain motif (NUDT2 E58A, NUDT12 E369A, NUDT14 E121A, and NUDT17 E124A)²⁰. Successful purification of the recombinant proteins was confirmed by SDS-PAGE and mass spectrometry (Supplementary Fig. 2a, b). As expected, co-incubation with in vitro transcribed RNAs showed that the purified proteins and recombinant calf intestinal phosphatase (CIP) did not have RNase activity (Fig. 2a). RNase A-treated RNA, however, was clearly degraded. We next tested whether the recombinant Nudix proteins can dephosphorylate PPP-RNA. For this, we used thin-layer chromatography (TLC), which allows visualization of released γ -phosphates from γ -³²P-labeled single-stranded 44-mer PPP-RNA substrate. Notably, NUDT2 released substantial amounts of γ -phosphate (Fig. 2b, γ -³²P) and led to almost complete dephosphorylation of the substrate RNA (Fig. 2b, RNA). In contrast, the other tested Nudix proteins (NUDT12, NUDT14, and NUDT17) only showed a minute release of γ -³²P. The NUDT2-dependent dephosphorylation was time-dependent (Fig. 2c) and required Nudix hydrolase activity since the NUDT2 E58A mutant did not release radioactively labeled phosphates or change RNA substrate labeling (Fig. 2d). Dephosphorylation was also apparent when using double-stranded RNA substrate, which was generated by annealing antisense RNA to the radioactively labeled 44-mer substrate (dsRNA) and by using radioactively labeled IVT4 (hairpin RNA) as substrate (Fig. 2e). Bacterial RppH, known for its activity to release γ -phosphates from unpaired RNA 5'-overhangs¹¹, showed a similar, although less pronounced release of phosphates from single-stranded PPP-RNA substrates (Fig. 2e). These experiments indicated that human NUDT2, like bacterial RppH, has dephosphorylation activity, pointing towards NUDT2 as a candidate protein involved in PPP-RNA degradation and antiviral immunity.

Interestingly, sequence alignment analysis suggests that NUDT2 is highly conserved in evolutionary distant eukaryotic species (Supplementary Fig. 2c). Notably, after sequence alignment of all human Nudix hydrolases, NUDT2 shows the highest sequence similarity to bacterial RppH, further pointing towards a conserved function of both proteins (Fig. 2f). The crystal structures of RppH and NUDT2 have been solved^{20,21}, and alignment of these structures revealed striking similarities in which the active site of NUDT2 resembles the RNA binding pocket of RppH (Fig. 2g). It exhibits a negatively charged interface required for positioning the positively charged magnesium ions needed to coordinate the RNA's PPP-group. Furthermore, the RNA binding pocket contains a large, positively charged channel bearing space for the negatively charged RNA phosphate backbone (Fig. 2h).

NUDT2 is active on a broad range of PPP-RNA substrates. We focused on NUDT2 as a candidate protein involved in viral RNA degradation and therefore assessed the ability of NUDT2 to release phosphates from different substrates. To this aim, we used a time-resolved enzymatic assay to compare phosphate release from PPP-RNA. Titration of NUDT2 suggested an optimal signal-to-noise ratio when 600 nM of NUDT2 were used

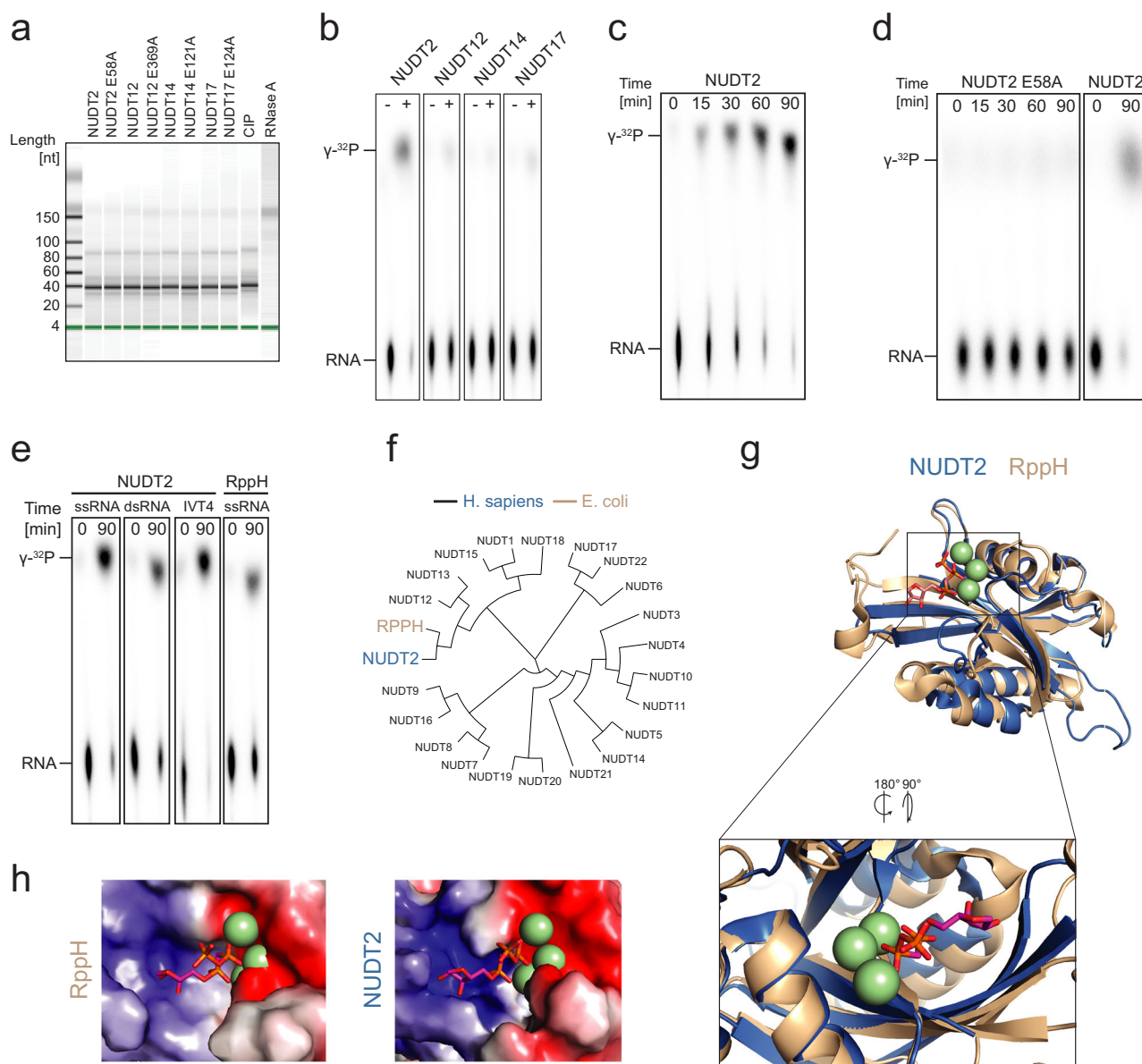


Fig. 2 Identification of NUDT2 as PPP-RNA phosphatase. **a** RNA was incubated for 3 h with the indicated Nudix hydrolase, and the integrity of the RNA was determined using a Bioanalyzer. One representative experiment of three is shown. **b** γ - 32 P-radiolabeled in vitro transcribed 44-mer ssRNA substrate was left untreated or incubated for 90 min with 600 nM NUDT2, NUDT12, NUDT14 or NUDT17 (+) or was left untreated (–). Reaction mixtures were analyzed by TLC and autoradiography. γ - 32 P: radiolabeled γ -phosphate; RNA: remaining input RNA after incubation. **c** As (**b**) but incubation for the time indicated at the top of the panel. 600 nM NUDT2 were used. **d** As (**c**) but 600 nM NUDT2 E58A or NUDT2 were used. **e** As (**b**) but γ - 32 P-radiolabeled in vitro transcribed single-stranded (ss-) or double-stranded (ds-) RNA generated by annealing an antisense oligo, or a RIG-I activating hairpin RNA (IVT4) were incubated with 600 nM NUDT2 and 5 U RppH, respectively. **f** Phylogenetic analysis of all human Nudix hydrolases and bacterial RppH was performed using MAFFT. **g** Structural alignment of the crystal structures of RppH (brown) with an RNA substrate (red sticks) (PDB: 4S2Y) and NUDT2 (blue) (PDB: 3U53) using PYMOL⁵⁷. The green-colored magnesium ions spheres are essential for hydrolase activity. **h** Visualization of the electrostatic surface charges of RppH and NUDT2 displayed with the aligned RNA of the RppH structure using PYMOL. Acidic amino acids are depicted in red, and basic residues are shown in blue.

(Supplementary Fig. 3a). Phosphatase activity of CIP was higher, probably due to more efficient or more complete dephosphorylation of the RNA substrate. As expected, NUDT2 mediated phosphate release is dependent on a glutamic acid at the catalytic site since the NUDT2 E58A mutant or control proteins (NUDT14, NUDT14 E121A) failed to yield a considerable signal (Fig. 3a).

To investigate the reactivity of NUDT2 on different substrates, we designed RNAs with or without different 5' single-stranded overhangs and variable nucleotides at the first (A, G) and the

second position (A, G, U, and C) of the RNA (Fig. 3b). NUDT2 processed all constructs, irrespective of the length of the 5' single-stranded overhang (Fig. 3c). Moreover, NUDT2 was also able to dephosphorylate RNA substrates with adenines at the 5' end and 5' single-stranded RNA overhang starting with adenines (Fig. 3c). As expected, the control NUDT2 E58A did not mediate significant phosphate release (Supplementary Fig. 3b). NUDT2 did not degrade the RNA substrates (Fig. 3d), confirming the lack of nuclease activity of NUDT2. Importantly, NUDT2 did not release any phosphates from single nucleotides (rATP, rGTP,

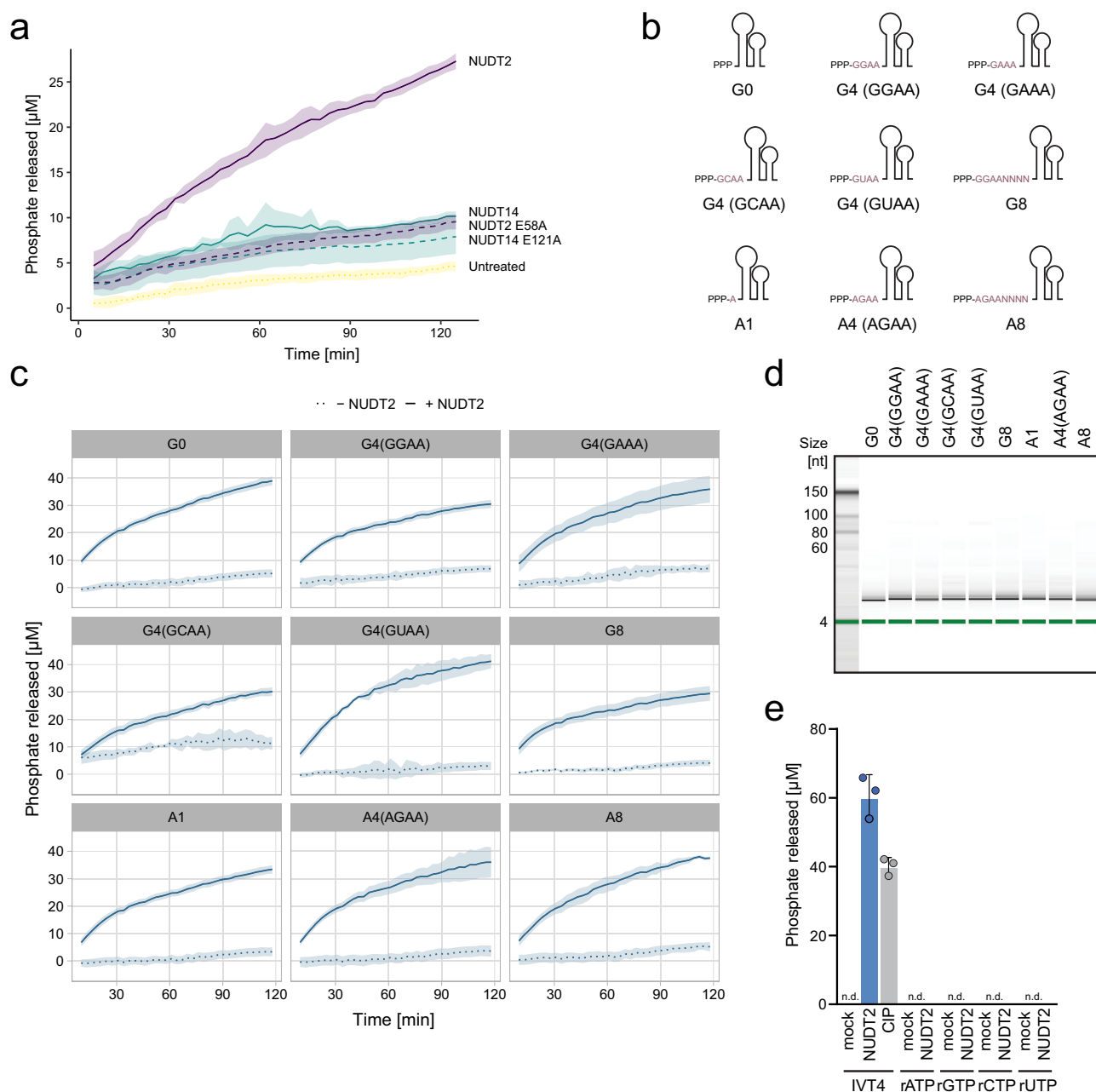


Fig. 3 NUDT2 is active on a broad range of substrates. **a** EnzChek assay to quantify released phosphate from in vitro transcribed double-stranded triphosphorylated hairpin RNA (IVT4) left untreated (untreated) or incubated with recombinant NUDT2, NUDT E58A, NUDT14, or NUDT14 E121A (600 nM), as indicated. Colorimetric measurements of the samples were performed every 3 min. The line graph shows the mean of three independent experiments with the ribbon indicating $\pm$ SD. **b** Illustration of in vitro transcribed RNA substrates with varying starting nucleotides with or without paired 5'-overhangs. **c** RNA substrates depicted in **(b)** were incubated without (dotted line) or with (solid line) NUDT2 at a concentration of 600 nM over a time course of 2 h, and phosphate release was evaluated using the EnzChek assay. Spectrophotometric measurements to quantify phosphate release were performed every 3 min. The line graph shows the mean of three independent experiments with the ribbon indicating $\pm$ SD. **d** The indicated RNA was incubated with NUDT2 (600 nM) for 3 h, and the integrity was analyzed using an Agilent small RNA chip and a Bioanalyzer. **e** The indicated ribonucleoside triphosphates (2.5 μM) and the positive control IVT4 were incubated for 3 h with NUDT2 (600 nM), and phosphate release was determined by malachite green assay. The mean of three independent reactions $\pm$ SD is shown.

rUTP, and rCTP) at comparable molarity (Fig. 3e), indicating that NUDT2 prefers polynucleotides as substrates. We concluded from these results, that NUDT2 is dephosphorylating PPP-RNAs irrespective of the first nucleotide (A or G), the 5'-terminus sequence, and the base-pairing of the 5'-end (single/double-stranded) of the substrate. Regarding substrate preference, NUDT2 appears to be different from RppH, which requires an unpaired 5' overhang for its activity¹¹ (Supplementary Fig. 3c).

NUDT2 releases monophosphates in a sequential manner. To assess the mode of phosphate removal by NUDT2, we synthesized PPP-dinucleotides (PPP-GpA and PPP-ApG) and tested how NUDT2 processes these substrates. Notably, NUDT2 sequentially released single phosphates resulting in a di-phosphorylated dinucleotide intermediate that is further converted into a mono-phosphorylated product (Fig. 4a–c). This contrasts with RppH, which predominantly releases

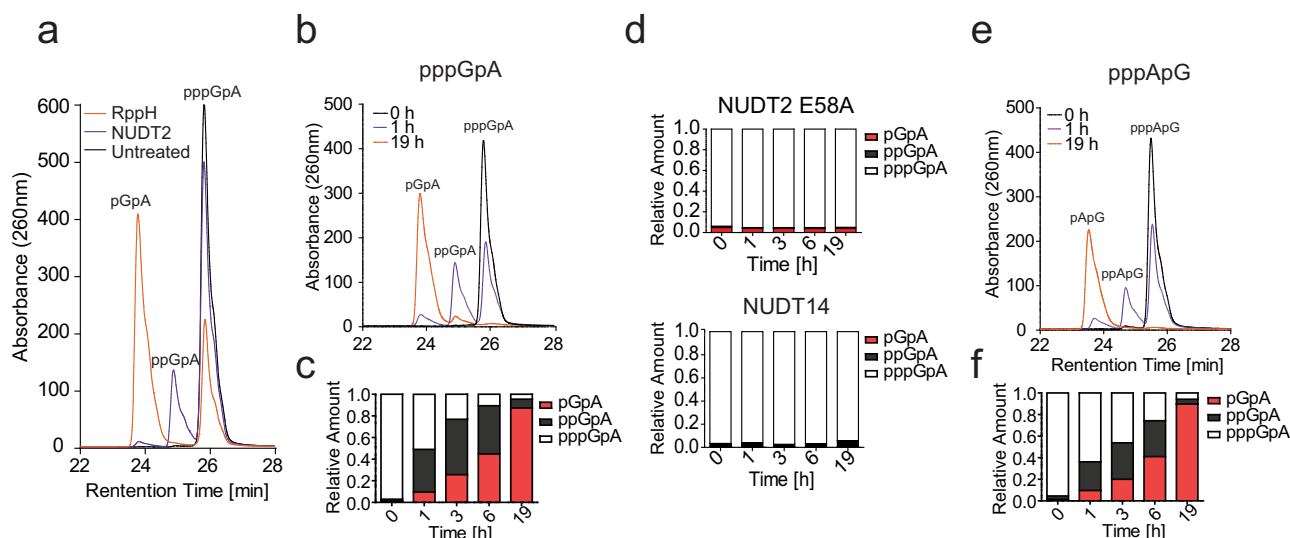


Fig. 4 NUDT2 sequentially releases phosphates from PPP-RNA. **a** A triphosphorylated RNA dinucleotide (pppGpA) was treated with NUDT2 (600 nM) or RppH (5 U) for 2 h at 37 °C. Additionally, the reference pppGpA was incubated in the same reaction buffer (untreated). Products were analyzed by reverse-phase HPLC. The absorbance at 260 nm at the indicated retention time is shown. **b** As **(a)** incubation with 600 nM NUDT2 for the indicated time. **c** As **(b)** but quantification of the generated RNA after incubation of pppGpA with NUDT2 for the indicated time. Quantification was performed by the integration of the corresponding peak area. **d** As **(b)** but incubation with NUDT2 E58A or NUDT14. **e** As **(b)**, but the triphosphorylated dinucleotide pppApG was used as substrate. **f** As **(e)** but quantification of the generated RNA after incubation of pppApA with NUDT2 for the indicated time. Quantification was performed by the integration of the corresponding peak area.

pyrophosphates from PPP-GpA in a one-step reaction without detectable PP-RNA intermediate²² (Fig. 4a). Neither NUDT2 E58A mutant nor NUDT14 changed the phosphorylation status of PPP-GpA, confirming the activity of NUDT2 in this assay (Fig. 4d). Removal of monophosphates by NUDT2 was also observed when using a PPP-ApG nucleotide as substrate, further corroborating that RNAs containing guanosines or adenosines at the 5' end are similarly dephosphorylated by NUDT2 (Figs. 3c, 4e, f).

NUDT2 and XRN1 cooperate for PPP-RNA degradation. We next tested whether NUDT2 can prepare PPP-RNA for degradation by the canonical XRN1 dependent 5'-to-3' RNA degradation machinery. As a substrate, we used Hepatitis C virus (HCV) encoding PPP-RNA. Incubation with NUDT2 or XRN1 did not affect the stability of the PPP-HCV RNA (Fig. 5a, b), indicating that the PPP-group protects RNA from degradation by XRN1 and that NUDT2 does not have exonuclease activity. Notably, co-incubation of HCV PPP-RNA with NUDT2 and XRN1 led to RNA degradation, confirming that the two proteins can act in concert to degrade PPP-RNA (Fig. 5a, b). Control experiments using bacterial RppH, as well as RppH/XRN1 co-treatment gave similar results. These data demonstrate the functional synergy between NUDT2 and XRN1 in a 5'-to-3' degradation pathway of PPP-RNA. As NUDT2 consecutively dephosphorylates PPP-RNA (Fig. 4a, b, e), this suggests that mammalian NUDT2 can, in principle, process PPP-RNA to prepare it for degradation by XRN1.

To extend this *in vitro* model to physiological conditions, we evaluated the activity of NUDT2 mammalian cells. In human cells, NUDT2 localizes to both the nucleus and the cytoplasm (Supplementary Fig. 4a), which suggests that NUDT2 can be biologically active in both compartments. NUDT2 precipitated from HEK293T cells could release significantly more phosphates from a PPP-RNA compared to NUDT2 E58A mutant or control proteins (Supplementary Fig. 4b, c), indicating that mammalian cell-expressed NUDT2 also has phosphatase activity.

Targeting endogenous NUDT2 in HeLa cells using CRISPR/Cas9 did not lead to any phenotypic alteration of cells compared to control treatments (Supplementary Fig. 6a). We transfected *in vitro* transcribed HCV-luciferase PPP-RNA into these cells and analyzed its abundance over time using RT-qPCR. As expected, the abundance of HCV-Luc PPP-RNA declined over time (Fig. 5c). Remarkably, in the absence of NUDT2, HCV-Luc PPP-RNA was considerably more stable, suggesting that NUDT2 might be involved in the degradation of viral RNA. To assess whether the observed effect is transferable to another cell type, we co-transfected PPP-RNA and capped-RNA into Hep3B cells or Hep3B cells lacking functional NUDT2, XRN1, or both. At 1 and 4 h post-transfection, NUDT2 depletion led to an increase in PPP-RNA stability as compared to the control (NTC) (Fig. 5d). Capped-RNA was not affected by NUDT2 depletion indicating specificity for degradation of PPP-RNA substrates. Importantly, depletion of XRN1 stabilized capped-RNA and PPP-RNA to a similar extent, underlining that XRN1 is in principle required to degrade both types of RNA. Similarly, cells co-depleted for NUDT2 and XRN1 showed increased PPP-RNA as well as capped-RNA levels. Notably, co-depletion did not result in synergistic effects compared to deleting single genes, further indicating that NUDT2 and XRN1 are operative in the same pathway (Fig. 5d). To further validate these findings, we infected Hep3B cells, depleted for NUDT2, XRN1, or NUDT2 and XRN1 with VSV-GFP (Fig. 5e). Both the lack of NUDT2 and XRN1 and the co-depletion led to a similar increase in GFP expression compared to the control, again pointing towards co-operative activities between NUDT2 and XRN1.

NUDT2 depletion in mice accelerates the growth of PPP-RNA generating viruses *in vitro*. We generated NUDT2 deficient mice from targeted embryonic stem cells (Supplementary Fig. 5a). Correct targeting of the *Nudt2* locus was confirmed by PCR (Supplementary Fig. 5b). Homozygous knockout mice were viable, bred with expected Mendelian ratios (Supplementary Fig. 5c), and did not show an obvious phenotype under

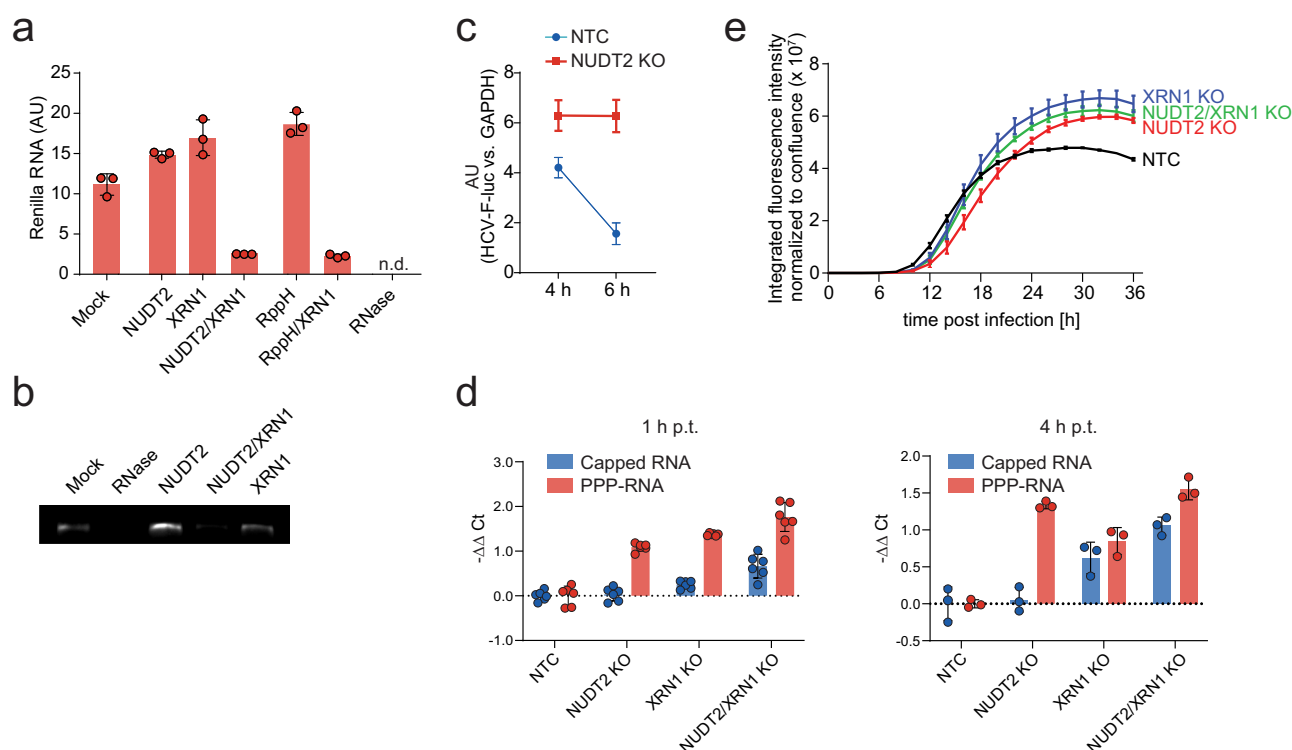


Fig. 5 NUDT2 is preparing RNA to serve as XRN1 substrate. **a** In vitro transcribed PPP-renilla RNA was left untreated (mock) or incubated with 600 nM NUDT2, 1 U XRN1, 600 nM NUDT2 together with 1 U XRN1, 5 U RppH, or 5 U RppH together with 1 U XRN1 and 10 U RNase for 4 h. The remaining RNA was quantified by RT-qPCR. The bar plot shows the fold change compared to input RNA as the mean $\pm$ SD of three technical replicates. **b** In vitro synthesized HCV RNA was treated with 5 U RNase, 600 nM NUDT2, 1 U XRN1, or co-treated with 600 nM NUDT2 and 1 U XRN1 and then analyzed on an agarose gel. **c** In vitro transcribed HCV RNA encoding luciferase was electroporated into HeLa CRISPR KO cells lacking NUDT2 or HeLa cells treated with a non-targeting-control vector (NTC). Viral RNA load was quantified 4 and 6 h post electroporation by the abundance of firefly luciferase RNA. **d** In vitro transcribed triphosphorylated renilla RNA and capped firefly luciferase RNA were co-electroporated into Hep3B CRISPR/Cas9 KO cells lacking NUDT2, XRN1, or both NUDT2 and XRN1, or Hep3B cells treated with a non-targeting-control vector (NTC). RNA load was quantified 1 and 4 h post electroporation by quantifying the abundance of firefly luciferase or renilla RNA. **e** Hep3B treated with CRISPR/Cas9 lentivectors targeting NUDT2, XRN1, NUDT2, and XRN1 or with a non-targeting-control vector (NTC) were infected with VSV-GFP at MOI 0.01. GFP expression was monitored using automated live-cell microscopy. The data points are displayed as the mean of five technical replicates $\pm$ SD. Shown is one representative experiment of three independent experiments.

non-infected conditions. To test whether NUDT2 deficiency may generally affect vulnerability to virus infections, we performed an unbiased proteome analysis of *Nudt2*^{-/-} and *Nudt2*^{+/+} bone marrow cells. This analysis allowed a parallel identification of 6288 proteins in total (Supplementary Table 2). As expected, NUDT2 could not be identified in the NUDT2 deficient mice (Fig. 6a). However, NUDT2 deficiency did not lead to significant differences in basal expression levels of the mouse proteome (considering 2866 proteins identified in 3 of 6 samples, FDR = 0.05, S0 = 0.1) (Supplementary Fig. 5d and Supplementary Table 2). Deficiency in the degradation of aberrant nucleic acids can lead to the accumulation of antiviral responses. However, compared to wild-type or heterozygous mice, *Nudt2*^{-/-} mice did not show spontaneous upregulation of mRNA encoding for classic antiviral proteins (e.g., Ifit3), inflammatory cytokines (e.g., IL-6), or U1 snRNA in heart, liver, lung, or spleen (Supplementary Fig. 5f) showing that NUDT2 depletion does not affect the basal immune status of mice. NUDT2 is known to cleave Ap₄A enzymatically, a dinucleotide generated under teratogenic conditions and during translational stress. To exclude that virus infection would elicit Ap₄A synthesis and that accumulation of this substance could be responsible for suppressing antiviral responses in cells, we tested the influence of Ap₄A on the growth of Semliki forest virus (SFV), a virus that is highly sensitive to antiviral activities of IFNs. Extracellular application of Ap₄A or its

delivery into HeLa cells through lipofection impaired virus replication in a comparable manner as the double-stranded RNA analog poly(I:C) (Supplementary Fig. 5e), indicating that accumulation of Ap₄A is not responsible for increased virus growth. Collectively, NUDT2 depletion does not result in a general perturbation of the cellular innate immune response, and increased Ap₄A levels caused by NUDT2-deficiency do not promote virus growth. These observations are in line with a more direct effect of NUDT2 on viral RNA, i.e., the dephosphorylation of triphosphorylated RNA and its subsequent degradation.

We isolated total bone marrow (BM) from *Nudt2*^{-/-} and littermate control mice and tested for replication of VSV wt and the IFN-stimulating variant VSV(M51A) (VSV-M2). Notably, accumulation of infectious virus particles was 10- to 30-fold increased in supernatants of bone marrow-derived from *Nudt2*^{-/-} mice compared to corresponding *Nudt2*^{+/+} littermate controls (Fig. 6b). Similarly, compared to controls, MEFs from *Nudt2*^{-/-} animals showed up to a 100-fold increase in VSV release into the supernatant and higher accumulation of VSV-N RNA (Fig. 6c, d). The increased virus growth also correlated with an increased virus-induced cytopathic effect in *Nudt2*^{-/-} compared to *Nudt2*^{+/+} MEFs (Fig. 6e). Importantly, infectious virus particles and viral RNA accumulation of HSV-1 (DNA virus), which does not generate PPP-RNA, were comparable in *Nudt2*^{-/-} and *Nudt2*^{+/+} MEFs (Fig. 6f, g). Similarly, SFV (a virus predominantly sensed by

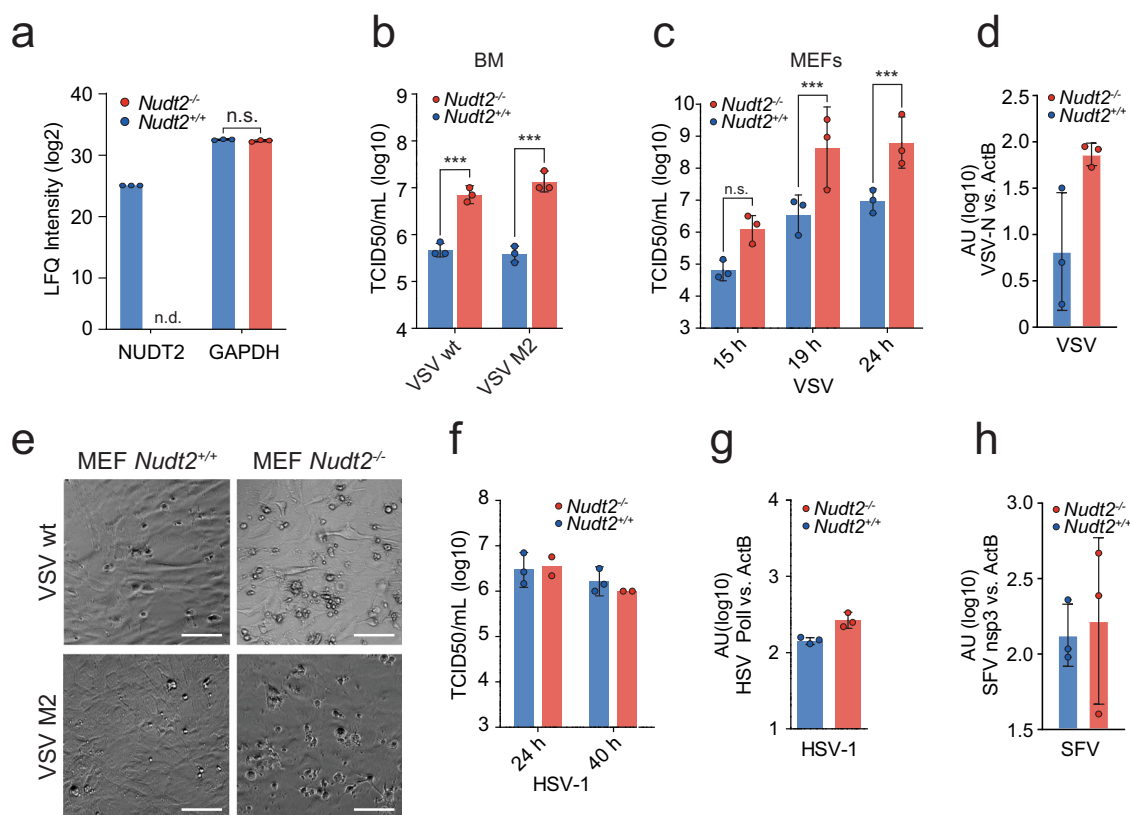


Fig. 6 Impact of NUDT2 on virus growth. **a** Bone marrow cells were isolated from *Nudt2* knockout (*Nudt2*^{-/-}), and control mice were tested for the abundance of NUDT2 by mass spectrometry. The mean LFQ intensities ± SD for NUDT2 and GAPDH of six biological replicates are shown. A two-sided students t-test (FDR 0.05) was performed. **b** Accumulation of infectious virus particles in supernatants of *Nudt2*^{+/+} or *Nudt2*^{-/-} bone marrow infected with either wild-type VSV, VSV-M2 at an MOI of 1 for 16 h. The average TCID50/mL of three biological replicates ± SD are shown. *** *p* < 0.001 two-way ANOVA with Bonferroni's post-test. **c** Accumulation of VSV in supernatants of *Nudt2*^{+/+} and *Nudt2*^{-/-} MEFs infected with VSV at an MOI of 0.001 for 16 h. Average TCID50/mL of three biological replicates ± SD. *** *p* < 0.001, two-way ANOVA with Bonferroni's post-test. **d** Viral RNA load in *Nudt2*^{+/+} or *Nudt2*^{-/-} MEFs infected with VSV. RNA levels were quantified by RT-qPCR analysis using specific primers for the VSV nucleoprotein transcript. Data were normalized to murine *ActB* RNA. The mean ± SD of three biological repetitions are shown in arbitrary units. **e** Phase-contrast micrographs of *Nudt2*^{+/+} or *Nudt2*^{-/-} MEFs infected with VSV for 16 h. The scale bars represent 100 μm. MEF cells of the indicated genotype were infected with HSV-1 (**f**, **g**) or SFV (**h**) at an MOI of 2 for 16 h. **f** Accumulation of infectious virus particles was analyzed. The graph shows the average TCID50/mL of three biological replicates ± SD. **g** Accumulation of viral RNA quantified by RT-qPCR analysis using specific primers for the DNA polymerase I of HSV or nsp3-transcript of SFV. Data were normalized to murine *ActB* RNA. Bar plots show average arbitrary units of three biological repeats ± SD.

MDA5)²³ replicated equally well in *Nudt2*^{-/-} compared to *Nudt2*^{+/+} MEFs (Fig. 6h). We concluded from these studies that mouse NUDT2 impairs the growth of PPP-RNA generating viruses in vitro, but it does not affect the accumulation of other viruses.

We proceeded to infect *Nudt2*^{+/+} and *Nudt2*^{-/-} mice with VSV using an intranasal infection regime, causing encephalitis. Surprisingly, despite significant growth differences in cell culture experiments, the infection in vivo did not lead to significant differences in survival rates when comparing *Nudt2*^{+/+} and *Nudt2*^{-/-} mice (Supplementary Fig. 6a). Moreover, the viral RNA load in the cerebellum of these mice was not significantly different. (Supplementary Fig. 6b) as well as transcripts for ISGs like *Ifi3* or cytokines like *IL-6* (Supplementary Fig. 5f). These experiments suggest that additional proteins with redundant functions are operative on an organismal level.

Discussion

RNA turnover is a highly conserved process and tightly controlled to regulate RNA abundance and to target erroneous RNAs for degradation. PPP-RNA, as present in viral genomes and to some extent in bacteria¹⁰, should, in principle, be protected from canonical cellular 5'-3' mRNA decay pathways present in higher eukaryotes. However, the half-life of PPP-RNA delivered into

cells is only ~0.5–1.0 h, which is relatively short compared to the 9 h average half-life of cellular mRNA^{6,24}. Although the PPP-group protects PPP-RNA from XRN1-dependent degradation, depletion of this exonuclease increases viral RNA half-life²⁵. This suggested the presence of a triphosphatase that converts PPP-RNA into an XRN1 substrate^{4,26,27}. Indeed, recently the phosphatases DUSP11 and DOM3Z (DXO) were identified as proteins that convert PPP-RNA into P-RNA, which serves as a substrate for XRN1^{8,9,28}. Since RNA degradation is an evolutionary, highly conserved process, we asked whether proteins known to be involved in PPP-RNA degradation in bacteria are also functionally active in mammalian cells. Using a siRNA screen, phosphate release assays, automated live-cell microscopy, and RT-qPCR, we identified that the protein NUDT2 could release phosphates from 5'-PPP RNA substrates and contributes to control the degradation of viral PPP-RNA. We show that NUDT2 is active on a broad range of PPP-RNA substrates but has a preference for processing longer substrates. NUDT2 sequentially releases two phosphates, and a monophosphorylated RNA is generated that can then serve as a substrate for the cellular exonuclease XRN1. This suggests that NUDT2 could be active in the degradation process of RNAs derived from diverse viruses.

The Nudix hydrolase RppH has been shown to play a role in the dephosphorylation of 5'-PPP and mRNA degradation RNA in bacteria¹¹. Although PPP-mRNA is present in bacteria, recent reports of Luciano et al. suggest that *E. coli* predominantly contain diphosphorylated mRNA, which appears to be the preferred substrate of RppH in *E. coli*¹⁰. The high structural similarity of RppH and mammalian NUDT2 indicates similar activities of both proteins, which is supported by the ability of NUDT2 and RppH to process phosphorylated RNA. Likely, NUDT2 has a preference for longer RNA constructs (compare Figs. 1b–e, 3a with Figs. 3c, 4a–c) and thus can also act on viral diphosphorylated RNA, characteristic for some viruses, including reoviruses²⁹, supported by the consecutive PPP-RNA dephosphorylation mechanism employed by NUDT2. Notably, NUDT2 is conserved throughout vertebrates, flies, worms, and bacteria, suggesting a broad functionality of this protein: Depending on the organism NUDT2 can be involved in physiological RNA turnover as well as antiviral defense mechanisms. NUDT2 deficiency in haploid KBM7 cells has been proposed to elicit an increased ISG expression pattern, which should result in reduced virus growth³⁰. However, this reported phenotype does not match our observations in various diploid human and murine cells, the phenotype of Nudt2 knockout mouse, or the viral growth phenotype in Nudt2 depleted systems.

The importance of RNA degradation is highlighted by the fact that many viruses evolved mechanisms to escape RNA decay pathways and potentially hide from NUDT2 activity. Potential evasion strategies include capping of RNA by either employing the virus-derived capping machinery or seizing it from cellular mRNAs in a process called cap snatching^{31,32}. Other RNA viruses, like viruses of the family Caliciviridae, protect their RNA by attaching a viral genome-linked protein (VPg). RNA of some viruses like Picornaviruses carry a structural element named internal ribosome entry site (IRES) that could also present a barrier for exonucleases like XRN1³³. HCV employs an unconventional mechanism to protect its RNA from degradation as it recruits the liver-specific microRNA miR-122 to its 5'-end. Through complementary binding to the HCV RNA, miR-122 protects the RNA from degradation by XRN1^{6,34} by sterically hindering XRN1 binding to the 5'-end. Although NUDT2 dephosphorylated all tested RNA substrates, it may be that the activity of NUDT2 is similarly inhibited by steric hindrance under very special conditions, as seen for XRN-I. Flaviviruses form membranous replication factories that cover viral RNA from cellular proteins³⁵, which may also be an evasion strategy to escape NUDT2 activity. It is not yet clear if NUDT2 can access such intracellular sub-compartments or if the activity of NUDT2 is limited to free 5'-PPP RNAs in the cytoplasm. Screening the impact of NUDT2 on more viruses would greatly enhance our understanding of the impact of NUDT2 dependent viral restriction and RNA decay. Since we could not observe a significant difference in survival rates between VSV-infected *Nudt2*^{+/+} and *Nudt2*^{-/-} mice, we cannot exclude that other cellular pyrophosphatases have redundant functions. Such redundancies have also been reported for the activity of pattern recognition receptors (PRRs) in vivo. Despite the requirement of certain PRRs to induce cytokines and reduce virus growth in vitro, in vivo phenotypes were often less convincing. The RNA sensor Toll-like receptor (TLR) 7, for instance, only reveals its in vivo phenotype in the absence of the RIG-I sensing pathway³⁶. Similarly, the in vivo activity of TLR3 strongly depends on the infection route of the pathogen³⁷. Moreover, in an infection model for the DNA virus mouse Cytomegalovirus, the DNA sensor TLR9 only shows an effect in livers but not in spleens, clearly indicating an organ-specific effect³⁸. Similarly shown for PRRs, the in vivo phenotype of NUDT2 may only be visible when using sophisticated in vivo

models or when specific infection models are used. On an organismal level, organ-specific expression differences or viral counter mechanisms could affect the impact of NUDT2.

Antiviral responses are often governed by proteins that are regulated by type-I IFNs. The expression of NUDT2 protein, however, does not change after cytokine treatment or virus infection. This may suggest that NUDT2, in addition to its ability to dephosphorylate viral RNA, may also have additional house-keeping functions. Interestingly, RNA Polymerase III products, including 5S ribosomal RNA, non-coding, and in some cases coding RNAs, should contain 5'-PPP termini. It is conceivable that proteins, such as NUDT2, DOM3Z (DXO), and DUSP11, are processing such aberrant RNAs or other RNA molecules to avoid stimulation of the innate immune system in the absence of pathogen encounter. Since self-RNA recognition may have dramatic effects on the cell, NUDT2, DUSP11, and DOM3Z (DXO) may fulfill redundant functions under steady-state conditions. Indeed, we could not obtain cells that lack both NUDT2 and DUSP11, despite the successful depletion of either of the two proteins. The full activity of these proteins may be required in case of viral infections since the abundance of viral PPP-RNA may exceed the catalytic capacity of either of these proteins.

Methods

Cells, reagents, and viruses. HeLa (ATCC CCL-2) and HEK293T (ATCC CRL-3216) cells were described previously and were authenticated by ATCC³⁹. Hep3B cells were obtained from Prof. R. Bartenschlager, University Hospital Heidelberg (Germany). Mouse embryonic fibroblasts (MEFs) were isolated from 13.5-day old embryos from heterozygous breeding pairs. Murine bone marrow was isolated from mouse femur, and tibia and cultured in DMEM supplemented with 10% fetal calf serum (FCS, GE Healthcare), 10 mM HEPES pH 7.4, 1 mM sodium pyruvate, and 2 mM L-glutamine. Cell lines were maintained in DMEM (PAA Laboratories) containing 10% FCS and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin). All viruses used are classified as BSL2 pathogens in Germany, and all experiments were carried out according to official regulations. Influenza A/SC35M-NS1-2A-renal-2A-NEP (IAV-ren)⁴⁰, VSV, VSV-M2⁴¹, VSV-GFP⁴², VSV-AV3-GFP¹⁹ have been previously described. HSV-1 (F-strain) was from Søren Riis Paludan (Uni Aarhus, Denmark), VSV-Luc was a gift from Gert Zimmer (University Bern, Switzerland), and SFV was a gift from Andres Merits (University of Tartu, Estonia). Recombinant XRN1, CIP, and RppH and restriction enzymes for in vitro transcriptions were obtained from NEB, single nucleotides for in vitro transcriptions and phosphate release assays were from Jena Bioscience, RNasin was from Promega, TURBO DNase was obtained from Thermo Fisher Scientific, and T7 RNA polymerase was produced by the Core Facility of the MPI of Biochemistry. The CellTiter-Glo assay kit and the luciferase substrate d-Luciferin (E1602) were purchased from Promega. siRNAs were purchased from Qiagen or were synthesized by the Core Facility of the MPI of Biochemistry (see Supplementary Table 1).

Preparation of triphosphorylated or capped RNA. As RNA substrate, IVT4, a defined double-stranded triphosphorylated RIG-I ligand, was used as described⁴³. To test the sequence specificity of NUDT2, RNA substrates with varying starting nucleotides, including single-stranded and double-stranded 5'-ends, were used as described¹³. In vitro transcribed RNA was generated from linear, double-stranded DNA templates, hybridized of complementary oligodeoxynucleotides (Supplementary Table 3). Except, the template for the generation of renilla RNA (Promega, E2231), which PCR amplified according to the manufacturer's instructions (2x HF Mastermix, NEB M0541S) with the corresponding oligodeoxynucleotides (Supplementary Table 3). The linear dsDNA product was purified (Monarch PCR Cleanup, T1030S) and directly used for in vitro transcription. RNA Sequences beginning with 5'-AG contained a T7 ϕ 2.5 promoter; all other templates contained a T7 ϕ 6.5 promoter. The antisense strand of the DNA template was annealed with sense-oriented oligos covering the respective T7 promoter region⁴⁴. These oligonucleotides were mixed 1:1 to a final concentration of 10 µM each in 10 mM Tris (pH 7.5–8.0), 50 mM NaCl, and 1 mM EDTA, heated to 95 °C for 5 min and cooled down to 20 °C at a rate of 0.1 °C/s on a PCR cyclor. For the preparation of renilla luciferase RNA, the pRL-SV40 plasmid was linearized by BamHI cleavage. To prepare firefly luciferase RNA, the RiboMAX Large Scale RNA Production Systems Kit (Promega) control plasmid was used. Transcription was carried out overnight at 37 °C according to the manufacturer's protocol of the T7 RiboMAX Large Scale RNA Production Systems kit (Promega). After DNase treatment for 30 min at 37 °C using 1 U of RNase-free DNase per µg of DNA template, ivtRNA was purified using either the MinElute Cleanup kit (Qiagen) or the Monarch RNA Cleanup Kit (NEB).

For the preparation of HCV PPP-RNA, the pFK-JFH plasmid DNA⁴⁵ was linearized by MluI cleavage. Transcription was carried out for 6–8 h at 37 °C in

5× transcription buffer (200 mM Tris pH 8, 30 mM MgCl₂, 10 mM Spermidine, and 5 mM DTT), 2.5 mM ATP, 2.5 mM UTP, 2.5 mM CTP, 2.5 mM GTP, RNasin (Promega), and 1000 U T7 RNA polymerase (Core Facility of the MPI of Biochemistry), in 100 µL final volume. 2 µL TURBO DNase (Thermo Fisher Scientific) was added for 30 min at 37 °C. Reactions were purified with mini Quick Spin RNA Columns (Roche) according to the manufacturer's protocol. For the 44-mer single-stranded RNA, the pGEX-6P-T7-44-mer-RNA⁴⁶ plasmid linearized by EcoRI cleavage served as a template. RNA was synthesized by *in vitro* transcription according to the MEGAscript T7 Kit (Thermo Fisher Scientific). After incubation for 16 h at 37 °C and TURBO DNase (Thermo Fisher Scientific) treatment for 30 min at 37 °C, the RNA was purified according to the Direct-zol RNA extraction kit (Zymo Research). For the generation of double-stranded RNA, a 20-nt antisense RNA (Metabion) (5'-acucucucucucucucuccc-3') was annealed to the *in vitro*-transcribed RNA. *In vitro* transcribed RNA was capped using the ScriptCap Cap 1 Capping System (CellScript, C-SCCS1710).

siRNA-mediated knockdown experiments. Duplex siRNAs were transfected using either siPrime transfection reagent (GE Healthcare) for the Nudix hydrolase siRNA screen or Metafectine Pro transfection reagent with the SI⁺ Buffer (Biont) for targeted gene knockdowns. Transfection was performed according to the manufacturer's instructions for HeLa cells. Briefly, we transfected 15 pmol of pooled siRNAs per 1e5 cells in a 24 well format. Cells were infected 48 h after siRNA knockdown. Duplex siRNAs were either purchased from Qiagen or synthesized by the Core Facility at the MPI of Biochemistry (Supplementary Table 1). Cell viability after siRNA-mediated knockdown was assessed with the CellTiter-Glo (Promega) luminescent cell viability assay, according to the manufacturer's protocol.

Virus infections and determination of virus titers and growth. To determine the impact of *NUDT2* knockout on virus growth, MEFs and bone marrow were seeded the day before infection. Cells were infected with VSV wt at a multiplicity of infection (MOI) of 0.001, SFV at an MOI of 0.001, HSV-1 at an MOI of 2, VSV-Luc at an MOI of 0.001, IAV-*renilla* at an MOI of 0.1, and HSV-Luc at an MOI of 2. Bone marrow was infected with VSV wt and VSV M2 at an MOI of 1. Twenty-four hours post-infection or as indicated specifically in the figure legend, cells were harvested for quantitative RT-qPCR, Western Blot or and the supernatant was harvested to test for virus accumulation by 50% tissue culture-infective dose (TCID₅₀) assays on Vero E6 cells. To quantify the effects of siRNA-mediated knockdown of Nudix hydrolases, cells were infected 48 h after the knockdown was performed with VSV-Luc at an MOI of 0.001 for 24 h, IAV-*renilla* at an MOI of 1 for 48 h, VSV-GFP at an MOI of 0.001 for 24 h, and VSV-M2-GFP at an MOI of 0.0001 for 24 h. GFP levels were determined fluorometrically. Z-scores were calculated from three independent experiments individually for every gene and virus, as previously described⁴⁷.

For RT-qPCR analysis, RNA was isolated using the NucleoSpin RNA Plus kit (Macherey Nagel) according to the manufacturer's protocol. For proteomic analysis, cell pellets were snap-frozen in liquid nitrogen before further processing. For western blot analysis, cells were lysed in Laemmli buffer, boiled for 10 min at 95 °C, and subjected to SDS-polyacrylamide gel electrophoresis and western blot analysis. For GFP-tagged viruses, virus growth was measured by determining GFP levels in a microplate reader (Tecan Infinite 200 Pro) with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. For luciferase-tagged viruses, cells were lysed in Passive Lysis Buffer (Promega), and virus load was determined by measuring firefly or *renilla*-luciferase. VSV-GFP growth kinetics were monitored and analyzed using the IncuCyte S3 Live-cell imaging system and software (Essen Bioscience). Heb3b cells, transduced with a non-targeting control vector or with a CRISPR/Cas9-mediated depletion of either *NUDT2*, *XRNI*, or *NUDT2* and *XRNI*, were infected with VSV-GFP at an MOI of 0.01 in five technical replicates. For each technical replicate, two phase-contrast and green fluorescence images were acquired by a 10× objective. Fluorescence images were further processed to remove the background signal using a top-hat filter. The integrated intensity of GFP+ cells per image was then normalized to the total area of cells by division for each time point.

Protein expression and purification. DNA sequences of *NUDT2*, *NUDT2* E58A, *NUDT12*, *NUDT12* E369A, *NUDT14*, *NUDT14* E121A, *NUDT17*, *NUDT17* E124A were obtained as gBlocks from IDT containing overhangs suitable for SLIC cloning. All constructs were cloned into pCoofy4, providing an N-terminal His6-MBP tag. Protein expression and purification were performed by the Core Facility of the MPI of Biochemistry. Briefly, plasmids were transformed in BL21-AI pRARE bacteria, and protein expression was carried out in an autoinduction medium containing 0.2% arabinose. Cells were lysed using an AVESTIN high-pressure homogenizer, and cleared lysate was used for protein purification using a HisTrap HP column (GE Healthcare: 17-5247-01) and further purified by gel filtration using a Superose 6 PC 3.2/30 (GE Healthcare: 17-0673-01) (mobile phase: 20 mM Tris-HCl pH 7.5, 250 mM NaCl, 10 mM MgCl₂, 1 mM DTT). Proteins were dialyzed with D-Tube Dialyser Midi (cutoff 6–8 kDa) in a buffer containing 20 mM Tris-HCl pH 7.5, 250 mM NaCl, 10 mM MgCl₂, and 1 mM DTT. The identity of recombinant proteins was confirmed by mass spectrometry.

To test phosphate release from mammalian expressed *NUDT2* cells, N-terminally 6-myc-tagged *NUDT2*, *NUDT2* E58A, and NS1 of Influenza A virus (with mutated RNA binding domain (R38A, K41A) of strain A/PR8/34) were transiently expressed from pCS2-N-6xMyc plasmids. For this, HEK293T cells were transfected using 1.3 µg of plasmid per 1e6 cells and a ratio of the transfection reagent PEI to plasmid DNA of 3:1. The cells were collected after two days, washed 3× with PBS, pelleted, and frozen. Subsequently, the cells were lysed with TAP lysis buffer (50 mM Tris-HCl at pH 7.5, 5% glycerol, 0.2% NP-40, 1.5 mM MgCl₂, 100 mM NaCl, supplemented with complete protease and phosphatase inhibitor cocktails (Roche)) on ice for 30 min, followed by sonication (10 min, 4 °C) and clearing of the lysate (4000 g, 30 min, 4 °C). The expressed, Myc-tagged proteins were then precipitated using 80 µL slurry of equilibrated α-myc agarose beads (Chromotek, yta-20). Beads were washed three times with NEB 2 reaction buffer to equilibrate for the phosphate release assay.

SDS-PAGE and western blotting. Validation of precipitation of myc-tagged proteins: Immunoprecipitated proteins were eluted in the presence of 1× SDS sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 50 mM DTT, 0.01% bromophenol blue, pH 6.8) and by boiling the samples at 95 °C for 5 min. Eluted proteins were then separated on a 4–12% Bis-Tris acrylamide gel (NuPAGE, Invitrogen) at 100 V for 90 min and transferred onto a 0.2 µm nitrocellulose membrane by wet-blotting at 300 mA for 1 h. The membrane was then blocked with 5% milk, 3× washed with PBS with 0.25% Tween-20 (PBS-T) for 5 min each, and stained with primary antibodies in 5% milk followed by incubation with an appropriate secondary antibody.

Primary antibodies used in this study were as follows: monoclonal mouse α-*NUDT2* (Santa Cruz: sc-271410, 1:1000 dilution), monoclonal mouse α-β-Actin-HRP (Santa Cruz: sc-47778, 1:1000 dilution), monoclonal mouse α-myc-HRP (Roche, 11814150001, 1:2000 dilution), and secondary horseradish peroxidase (HRP)-coupled antibody rabbit α-mouse IgG (Cell Signaling, 7076, 1:2000 dilution).

Phosphate release assay. For time-resolved analysis, the phosphatase activities of Nudix hydrolases, CIP, or RppH were analyzed with the EnzChek Phosphate Assay (E6646, Sigma-Aldrich) according to the manufacturer's instructions. For optimal performance, the following adjustments were made: one replicate (200 µL reaction volume) included 600 nM *NUDT* protein or the corresponding mutant, 4.5 µg of RNA substrate, 0.5 U of PNP, 0.2 mM MESG, and NEB 2.0 buffer. This setting was validated to yield optimal assay performance (Supplementary Fig. 3a). The absorbance was measured at 360 nm every three min. The phosphate release was calculated from a linear standard curve specific for each time point.

For single time-points, the ability of Nudix hydrolases to release phosphates from RNA was tested with *in vitro* transcribed RNA (ivtRNA), and ribonucleotide triphosphate (rNTPs). The malachite green assay was performed according to the manufacturer's instructions (MAK307, Sigma-Aldrich) after incubating 900 ng IVT4 with *NUDT2* or *NUDT2* E58A in NEB2 buffer for 3 h at 37 °C. This setting was validated to yield optimal assay performance. In order to test *NUDT2* activity on a panel of possible substrates, *NUDT2* (600 nM) and *NUDT2* E58A (600 nM) were incubated for 3 h with rATP, rGTP, rUTP, and rCTP (2.5 µM) for 3 h at 37 °C in NEB2 reaction buffer. CIP (5 U) (NEB) was used as a positive control for released inorganic phosphate in all experiments. For immunoprecipitated *NUDT2*, *NUDT2* E58A or NS1 (IAV) derived from HEK cell lysate, 20 µL of beads were incubated in 40 µL NEB 2 reaction buffer, including 900 ng IVT4, for 3 h at 37 °C. Beads were separated (500 g, 1 min, RT), and the phosphate release assay was performed using the malachite green assay as described above.

Phosphate release from γ-³²P-labeled RNA. γ-³²P labeled RNA was synthesized by *in vitro* transcription according to the MEGAscript T7 Kit (Thermo Fisher Scientific) supplemented with 0.17 µM [γ-³²P] GTP (Perkin Elmer). For the 44-mer single-stranded RNA, the pGEX-6P-T7-44mer-RNA⁴⁶ plasmid linearized by EcoRI cleavage served as a template. For the dsRNA IVT4, two annealed primer pairs (Supplementary Table 3)⁴³ were used as a template. After incubation for 16 h at 37 °C and DNase digestion with TURBO DNase (Thermo Fisher Scientific) for 30 min at 37 °C, the RNA was purified according to the Direct-zol RNA extraction kit (Zymo Research). For the generation of a double-stranded RNA of the 44-mer ssRNA, a 20-nt antisense RNA (Metabion) (5'-acucucucucucucuccc-3') was annealed to the *in vitro* transcribed RNA.

The labeled RNAs were incubated with purified *NUDT2* (600 nM), *NUDT12* (600 nM), *NUDT14* (600 nM), *NUDT17* (600 nM), and RppH (5 U) purchased from NEB in a solution (20 µL) containing NEB2 buffer at 37 °C. Reaction samples (10 µL) were taken at 0 and 90 min and quenched with 2.5 µL of EDTA (100 mM, pH 8.0), analyzed by TLC on PEI-cellulose (Merck Millipore), and developed with potassium phosphate buffer (0.3 M, pH 7.5). Spot intensities were imaged by using a GE Typhoon FLA 9000 Imager. For time-course experiments, the labeled RNA was incubated with purified *NUDT2* or *NUDT2* E58A (600 nM) in a solution (50 µL) containing NEB2 Buffer for 0–90 min at 37 °C. Reaction samples (10 µL) were quenched at time intervals with 2.5 µL of EDTA (100 mM, pH 8.0) and analyzed by TLC on PEI-cellulose (Merck Millipore) developed with potassium

phosphate buffer (0.3 M, pH 7.5). Spot intensities were imaged by using a GE Typhoon FLA 9000 Imager.

Synthesis of pppApG and pppGpA. Dinucleotides were synthesized using DMT-2'-O-TBDMS-rA(bz), DMT-2'-O-TBDMS-rG(ib) amidites, and rA(Bz) and rG(ib) CPG (Chemgenes) on a 10 μ M scale by using standard solid-phase oligoribonucleotide coupling techniques. The CPG-bound dimers were triphosphorylated using the cyclotriphosphate protocol of triphosphate synthesis⁴⁸, except that after deprotection of the dinucleotides, the reaction mixture was converted into the triethylammonium salt using Dowex Et3NH+ before reverse-phase chromatography on a Source RP column. DecNHpppApG and DecNHpppGpA RP-HPLC product peaks were converted into pppApG and pppGpA as described. The pure pppGpA and pppApG were isolated as triethylammonium salts by ion-exchange chromatography on a Hi Screen DEAE FF column using a gradient of 0.0–0.6 M Triethylammonium bicarbonate. The integrity of the pppApG and pppGpA dimers was verified by LC-MS analysis.

HPLC analysis. A short RNA (pppGpA, 0.1 OD260) was incubated for 2 h at 37 °C in a solution (15 μ L) containing NEB2 buffer (1 $\times$) with recombinant NUDT2 (600 nM), NUDT2 E58A (600 nM), RppH (5 U) or left untreated. Reactions mixtures were quenched with 3.75 μ L of EDTA (100 mM, pH 8.0) and diluted 1:20 with water. For time-course measurements, the two short RNAs (pppGpA or pppApG, 0.1 OD260 each) were incubated with purified recombinant NUDT2 (600 nM), NUDT14 (600 nM), and NUDT2 E58A (600 nM), respectively, in a solution (75 μ L) containing NEB2 buffer (1 $\times$) at 37 °C. Reaction samples (15 μ L) were taken after 0, 1, 3, 6, and 19 h, quenched with 3.75 μ L of EDTA (100 mM, pH 8.0), and diluted 1:20 with water. Aliquots (80 μ L) were analyzed by reversed-phase (RP)-HPLC on an AGILENT 1100 System using an XBridge C18 column (3.5 μ m, 150 $\times$ 2.1 mm, WATERS). Chromatography was performed using a two-eluent buffer system. Buffer A consists of an aqueous solution of 100 mM Et3NH₄OAc, pH 7.8, and buffer B consists of an aqueous solution of 100 mM Et3NH₄OAc, pH 7.8, and 40% (v/v) MeCN. Chromatograms were recorded at a wavelength of 260 nm. HPLC was performed using the following gradient condition: 0% B over 4 min, to 15% B over 20 min, to 18% B over 16 min to 100% B over 10 min, held at 100% B for 15 min, to 0% B over 2 min, held at 0% B for 13 min with a flow rate of 0.3 mL/min. Quantification of eluted compounds was performed by integration of the corresponding peak areas.

Analysis of RNA integrity and transcript level analysis. To assess RNA integrity after treatment with recombinant Nudix hydrolases, small reaction samples were analyzed by the Bioanalyzer (Agilent) using the Agilent Small RNA kit according to the manufacturer's protocol. For quantitative RT-qPCR analysis, total RNA from cells was isolated using the NucleoSpin RNA Plus kit (Machery-Nagel). RNA from mouse cerebellum, heart, liver, lung, or spleen was extracted using Lysing Matrix M and the FastPrep-24 (MP Biomedicals) instrument. Organs were directly lysed in LBP buffer, and RNA isolation was continued using the NucleoSpin RNA Plus kit (Machery-Nagel). Next, 200–500 ng of RNA was reverse-transcribed with PrimeScript RT Master Mix (TAKARA) and after that quantified by RT-qPCR using the QuantiFast SYBR Green RT-PCR Kit (Qiagen) and a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Each cycle included 10 s at 95 °C and 30 s at 60 °C, followed by a melting curve analysis. Primer sequences are depicted in Supplementary Table 4.

Generation of CRISPR/Cas9 knockout cells. Double-stranded 30-nt guide sequences (Supplementary Table 5) targeting the human *NUDT2* gene and a non-targeting control were designed and cloned into pLentiCRISPRv2 (Addgene #52961) as previously described^{49,50}. HEK 293T cells were co-transfected with the obtained plentiCRISPR v2 vector (6 μ g), pXPA2 (3 μ g), and pMD2G (1.5 μ g) in a 10 cm dish using PEI. 24 h post-transfection, the medium was replaced with 10 mL of fresh DMEM. Seventy-two hours post-transfection, the medium was collected, filtered through a 0.45 μ m pore size membrane (Millipore), and HeLa cells were infected with the lentivirus. Twenty-four hours post-infection, the medium was replaced with DMEM containing puromycin (1 μ g/mL) for four passages. Knockout efficiency was analyzed by western blotting.

RNA degradation assays. Degradation of 1000 ng of an in vitro transcribed renilla or HCV PPP-RNA carrying a firefly luciferase was assessed by incubating it either with 10 U RNase A, 600 nM recombinant NUDT2, 1 U recombinant XRN1, 5 U recombinant RppH, or both 600 nM NUDT2 and 1 U XRN1 or 5 U RppH and 1 U XRN1 for 4 h at 37 °C. To determine RNA levels after incubation, renilla RNA was quantified using RT-qPCR, and the presence of HCV PPP-RNA was assessed using 1% agarose gels. The stability of in vitro transcribed HCV PPP-RNA encoding luciferase was determined by electroporation into HeLa CRISPR/Cas9 KO cells lacking *NUDT2* or HeLa cells treated with a non-targeting control vector. HCV PPP-RNA load was quantified 4 and 6 h post electroporation by quantifying the abundance of firefly luciferase RNA by RT-qPCR. To determine degradation of capped or PPP-RNA, capped firefly luciferase and PPP-renilla luciferase were mixed in equimolar amounts and were co-electroporated into Hep3B CRISPR/Cas9 KO cells lacking *NUDT2*, *XRN1*, or both *NUDT2* and *XRN1*, or Hep3B cells

treated with a non-targeting-control vector. Cells were incubated for 1 and 4 h at 37 °C. To determine RNA levels after incubation, renilla or firefly luciferase RNA was quantified using RT-qPCR and normalized to RPLP0 RNA.

Ap4A treatment. HeLa cells were transfected with 1 μ g poly(I:C) using 1 μ L METAFACTENE Pro or 250 nmol Ap₄A using 2 μ L METAFACTENE Pro or 250 nmol of Ap₄A was added directly to the media^{51,52}. As controls, the cells were stimulated with PBS in combination with and without METAFACTENE Pro. Twenty-four hours post-treatment, the cells were infected with SFV6-2SG Nano-Luc at an MOI of 0.0035 or left uninfected for 24 h. The cells were lysed in Passive Lysis Buffer, and virus growth was assessed by measurement of renilla luciferase.

Generation of *Nudt2* knockout mice. The *Nudt2* allele was rendered unfunctional by introducing a poly(A) signal after exon 1 in a construct purchased from the European Conditional Mouse Mutagenesis consortium (EUCOMM). This genetically modified ES cell clone (Clone ID: EPD0146_2_H06, Cell type: JM8.N4) was injected into C57BL/6 blastocyst donors. Male chimeras were bred with C57BL/6 females to produce heterozygous *Nudt2*^{+/tm1a} mice (*Nudt2*^{+/−}) containing a tm1a cassette encoding for the lacZ gene, a neomycin resistance, a splice acceptor, and a polyA site flanked by FRT sites. Heterozygous animals were then bred to generate homozygous *Nudt2*^{tm1a/tm1a} mice (*Nudt2*^{−/−}).

For genotyping *Nudt2* knockout mice, ear punches were collected from 21 to 23-days old mice and genotyped by PCR using the following primers: *Nudt2*_{fw}: 5'-ccagcttctgtgtacaaagtgg-3', *Nudt2* wt_{fw}: 5'-gaattctgtctgctgcaggc-3', *Nudt2*_{rev}: 5'-cctagtggaaggagacaaagcagc-3' and using the following parameters: 94 °C for 1 min, followed by 35 cycles of 94 °C for 30 s, 65 °C for 20 s, and 72 °C for 1 min. The wild-type allele was detected as a band at 492 bp, whereas the inserted tm1a cassette was detected as bands of 417 and 912 bp.

Ethics statement and mouse infections. All animal experiments were performed in compliance with the German Animal Welfare Law (TierSchG BGBI. S. 1105; 25.05.1998). The mice were housed and handled in accordance with good animal practice as defined by FELASA. All animal experiments were approved by the responsible state office (Landesverwaltungsamt Sachsen-Anhalt), the University of Magdeburg, under permit number AZ 42502-2-1344. During intranasal infections (i.n), 8–12-week-old mice were first anesthetized by i.p. injection with a mixture of ketamine (100 mg/kg body weight) and xylazine (5 mg/kg body weight). They then were infected with 5e6 plaque-forming units (PFU) of VSV in 20 μ L PBS unless otherwise indicated. Mice that lost more than 20% of their body weight were sacrificed.

Proteomic analysis of *Nudt2* knockout in mouse bone marrow cells. For proteomic analysis, wild-type and *Nudt2* knockout bone marrow cells (5e6/cell line/replicate; 4 replicates) were lysed in 300 μ L of lysis buffer (4% SDS, 10 mM DTT in 50 mM Tris pH 7.6) supplemented with complete protease inhibitor (Roche) and boiled for 10 min at 95 °C. The protein concentration of cleared cell lysates was assessed using Pierce 660 nm Protein Assay (Thermo Fisher Scientific) according to the manufacturer's protocol. Protein concentrations for all cell lines and replicates were equalized to 35 μ g protein content and alkylated with 55 mM IAA for 20 min in the dark. Proteins were precipitated with acetone and resuspended in 150 μ L of denaturation buffer (6 M Urea, 2 M Thiourea in 10 mM HEPES pH 8.0). Protein digestion was performed by adding 1:100 (protein:enzyme) trypsin and LysC overnight at room temperature. Peptides were purified on stage tips and analyzed by LC-MS/MS using the EASY-nLC 1200 system coupled to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific). Peptide mixtures were separated on a 50 cm C18-reversed-phase column (Reprosil-Pur 120 C18-AQ, 1.9 μ m, 200 $\times$ 0.075 mm; Dr. Maisch) using a 180-minute linear gradient of 5–30% buffer B (0.1% formic acid and 80% ACN) with a flow rate of 250 nL/min. The mass spectrometer was set up to run a Top10 method acquiring full scans (300–1,600 m/z, *R* = 60,000 at 200 m/z) at a target of 3e6 ions, followed by isolation of the ten most abundant ions, HCD fragmentation (target 1e5 ions, maximum injection time 120 ms, isolation window 1.4 m/z, NCE 27%, and underfill ratio of 20%) and detection in the Orbitrap analyzer.

Raw data processing and statistical analysis. Peptide identification and quantification were performed using MaxQuant (version 1.6.17.0). For all MaxQuant searches, typical default parameters were employed. Spectra were searched against forward and reverse sequences of the reviewed mouse proteome, including isoforms (Uniprot, UP000000589). Settings included carbamidomethylation of cysteine as fixed modification and oxidation of methionine and N-terminal protein acetylation as variable modifications. Trypsin/P was specified as the proteolytic enzyme. The minimal peptide length was defined as 7 amino acids, and match-between-run was disabled for LFQ determination.

The protein groups were further analyzed with Perseus (1.6.14.0). The matrix included 6288 proteins and was filtered with the default settings: potential contaminant only identified by site and reverse. After log2 transformation, a two-sided students *t*-test (FDR 0.05) was performed, including proteins with at least three valid values (in total) and imputed (width 0.3 and downshift 1.8).

Phylogenetic analysis. To establish the phylogenetic relationship between different human Nudix hydrolases and bacterial RppH, protein sequences from all human Nudix hydrolases and *E. coli* RppH were analyzed using MAFFT⁵³ with standard settings. The resulting tree was displayed using Dendroscope⁵⁴ and visually adapted using Adobe Illustrator CS6 (16.0.3).

Multiple sequence alignment. Protein sequences of NUDT2 from *Homo sapiens*, *Danio rerio*, *Caenorhabditis elegans*, *Mus musculus*, *Xenopus laevis*, *Gallus gallus*, and *Drosophila melanogaster* were collected and aligned using the Clustal Omega tool with standard settings. The multiple sequence alignment was visualized using ESPript 3.0⁵⁵.

Statistical analysis. All data were analyzed either using GraphPad Prism (8.4.3) or R (4.0.2) using R Studio (1.3.1056). Data acquired with the Incucyte live-cell imaging platform were analyzed using IncuCyte Analysis Software (2019B Rev2).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The data supporting the findings of this study are available from the corresponding authors upon reasonable request. Source data for the figures and supplementary figures are provided as a Source Data file. The LC-MS/MS data and MaxQuant output generated in this study have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository⁵⁶ under accession code P9. Source data are provided with this paper.

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Author contributions

B.T.L., K.K., Q.E., L.L.A., A.R., C.D., K.M., A.K., S.M., and P.S. conducted the experiments. B.T.L., K.K., Q.E., L.L.A., and A.R. analyzed the data. J.L., D.W., M.M., and M.B.

contributed critical reagents. B.T.L., K.K., Q.E., and A.P. designed the experiments and wrote the paper.

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Competing interests

The authors declare no competing interests.

Additional information

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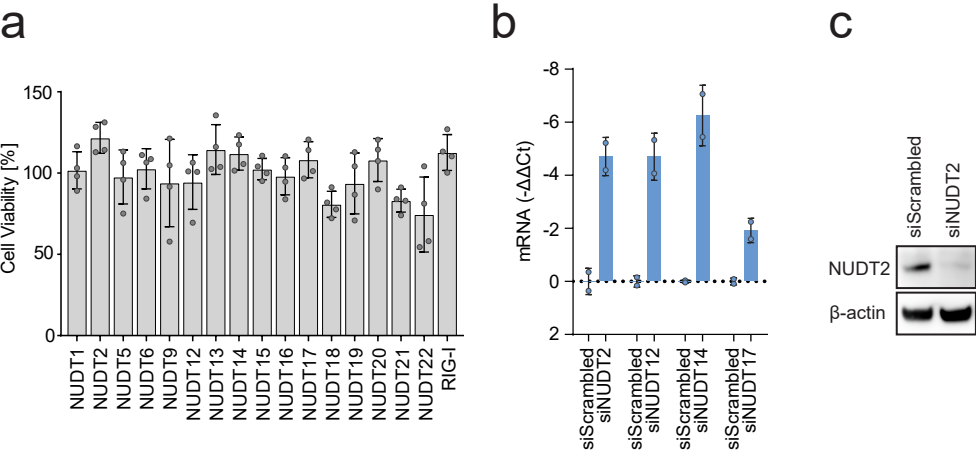
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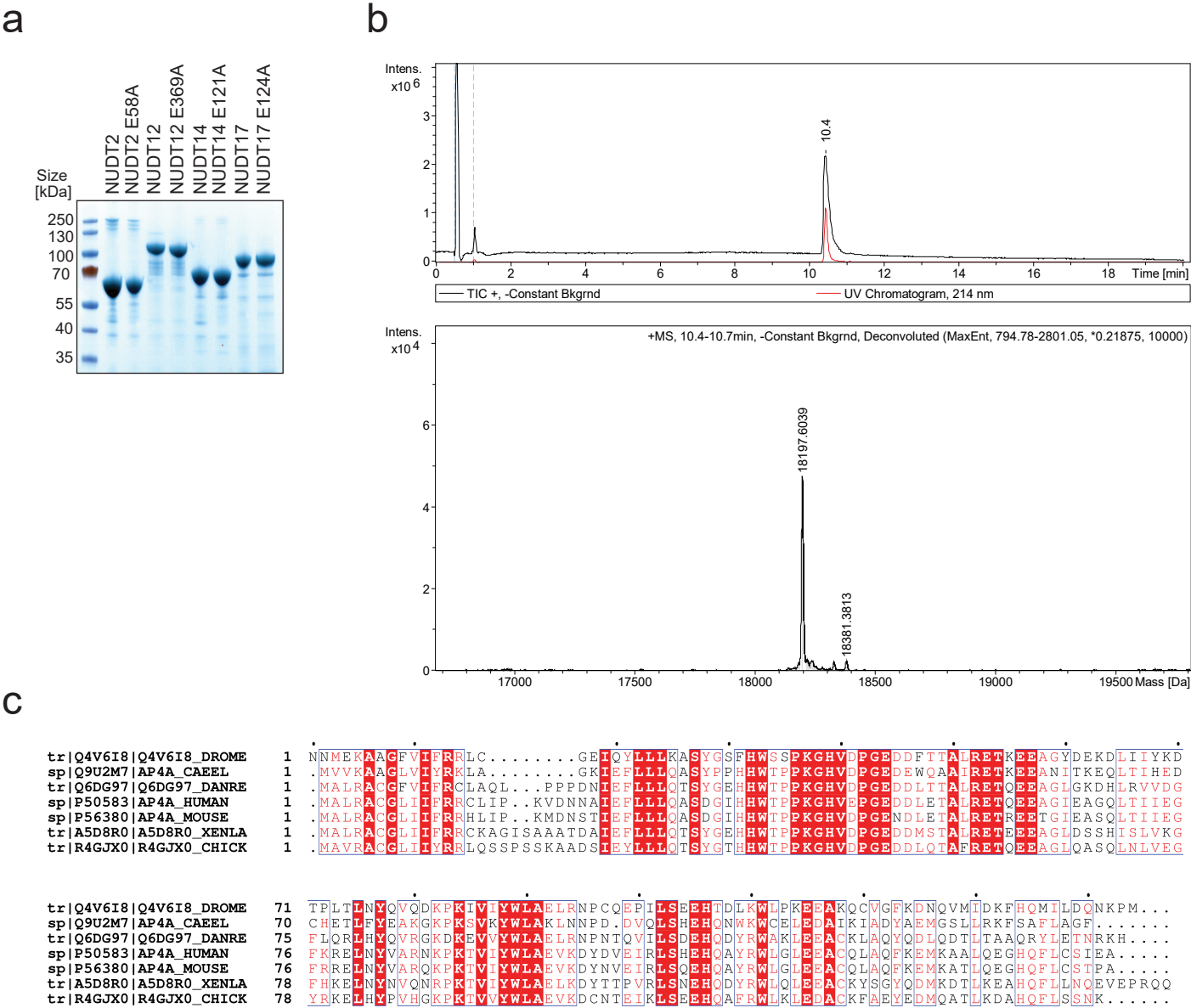
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Supplementary Figure 1



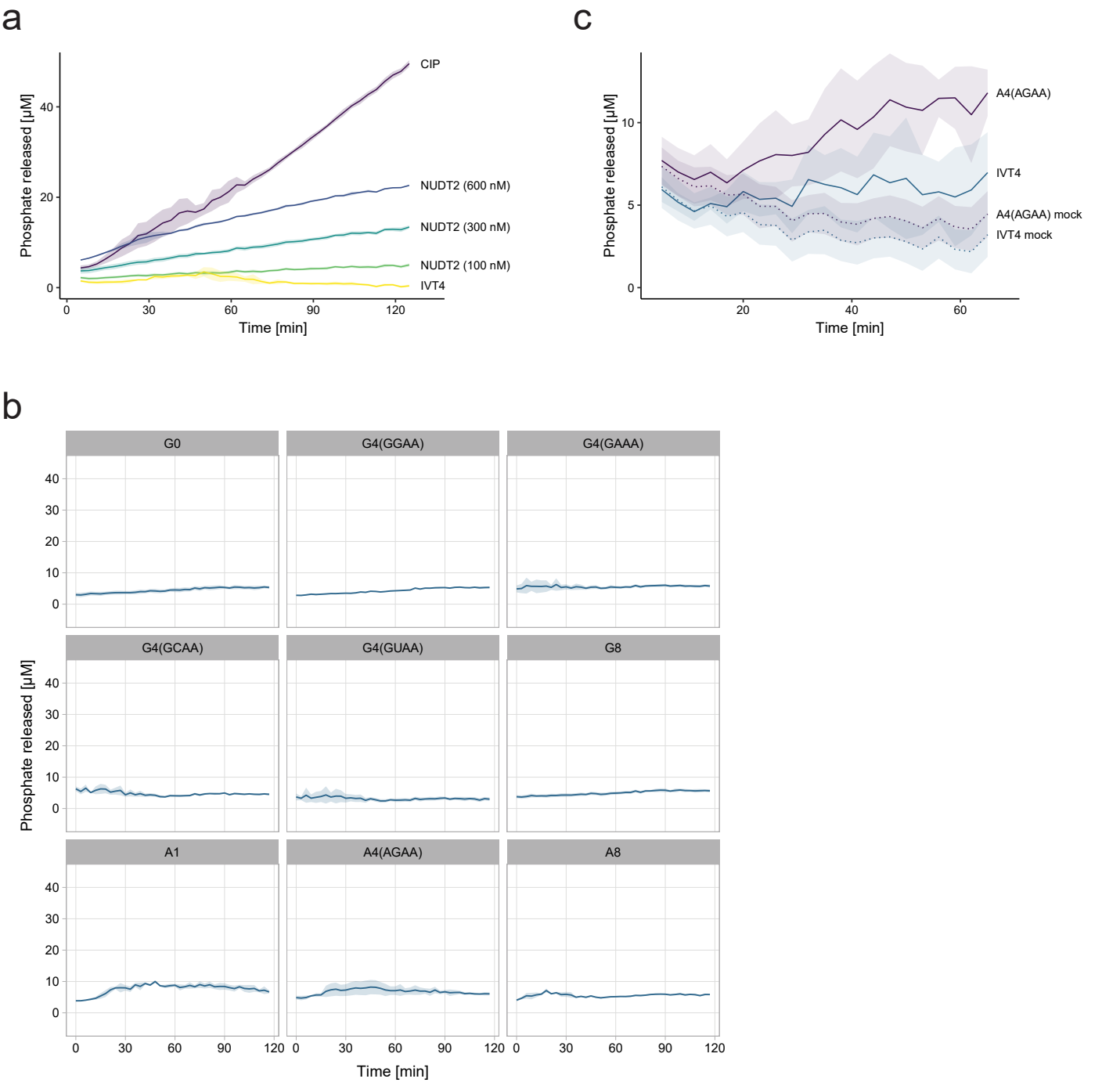
Supplementary Figure 1 | Cell viability after NUDT depletion and confirmation of knockdown. **(a)** Cell viability after siRNA knockdown. 72 h after siRNA transfection into HeLa cells, cell viability was tested using a cell titer glow assay. Relative cell viability was assessed by assuming the average cell viability of the whole siRNA screen set as 100% viable. The graph shows an average of four independent experiments done in technical duplicates $\pm$ SD. **(b)** Cells were treated with siRNA targeting the transcript of *NUDT2*, *NUDT12*, *NUDT14*, *NUDT17*, or control (siScrambled), and mRNA abundance of the respective cellular targets was quantified using RT-qPCR normalized to GAPDH. Bar graphs show the mean fold change of the targeted sequence vs. control $\pm$ SD of two independent replicates. **(c)** Expression of NUDT2 in cells transfected with siNUDT2 or control (siScrambled) assessed by western blotting.

Supplementary Figure 2



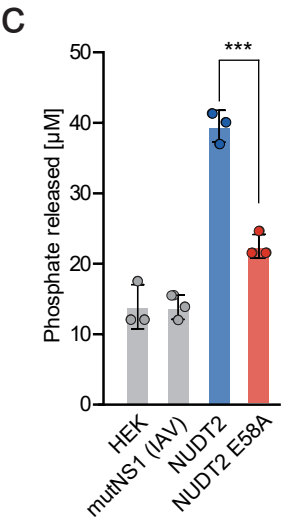
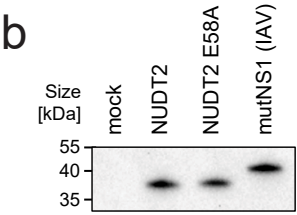
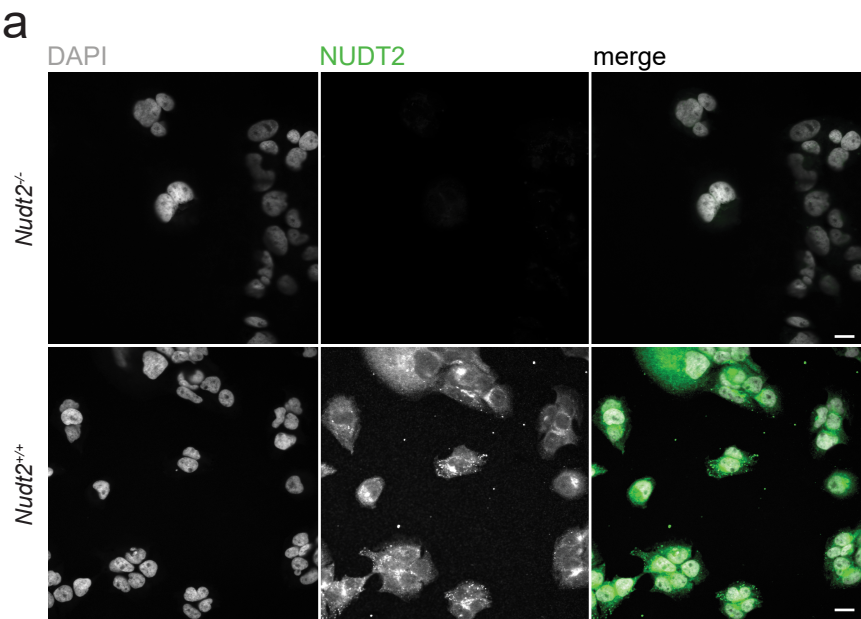
Supplementary Figure 2 | NUDT2 localization and cross-species sequence alignment. **(a)** Coomassie-stained SDS-PAGE of His6-MBP-tagged recombinant Nudix hydrolases, including mutants bearing point mutations in the catalytic Nudix box (n = 1). **(b)** Total mass determination of NUDT2 by top-down LC-MS analysis, including the total ion current chromatogram (TIC, top) and the extracted ion chromatogram (bottom). **(c)** Sequence alignment of NUDT2 proteins from *Homo sapiens*, *Danio rerio*, *Caenorhabditis elegans*, *Mus musculus*, *Xenopus laevis*, *Gallus gallus*, and *Drosophila melanogaster*. Colors are depicted as following: red box, white character shows a strict identity of residues; red character shows a similarity in a group; a blue frame shows similarities across groups.

Supplementary Figure 3



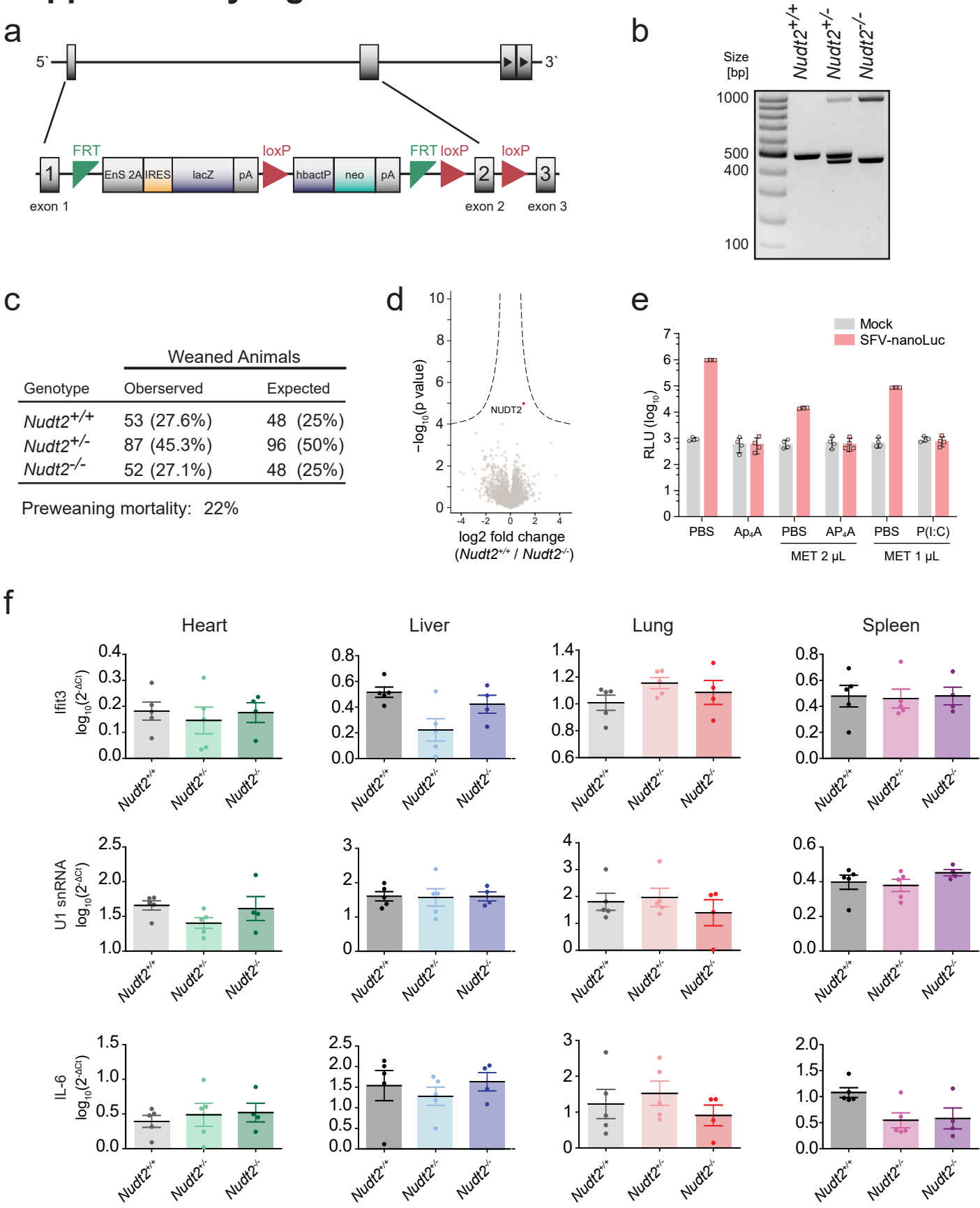
Supplementary Figure 3 | Time-resolved phosphate release assay. **(a)** Time-resolved EnzChek assay to quantify released phosphate from incubated IVT4 RNA with different concentrations of NUDT2. Spectrophotometric measurements of the samples were performed every 3 min. The line graph shows the mean of three independent experiments with the ribbon indicating $\pm$ SD. **(b)** Complementary to Fig. 3c. RNA substrates depicted in Fig. 3b were incubated with NUDT2 E58A mutant at a concentration of 600 nM over a time-course of 2 h utilizing the EnzChek assay to quantify phosphate release. Spectrophotometric measurements were performed every 3 min. The line graph shows the mean of three independent experiments with the ribbon indicating $\pm$ SD. **(c)** As (a) but using RppH to dephosphorylate an RNA substrate which is base-paired on its 5'-end (IVT4) and one that has an unpaired 5'-overhang (A4(AGAA)) in comparison to untreated RNA substrates. The line graph shows the mean of three independent experiments with the ribbon indicating $\pm$ SD.

Supplementary Figure 4



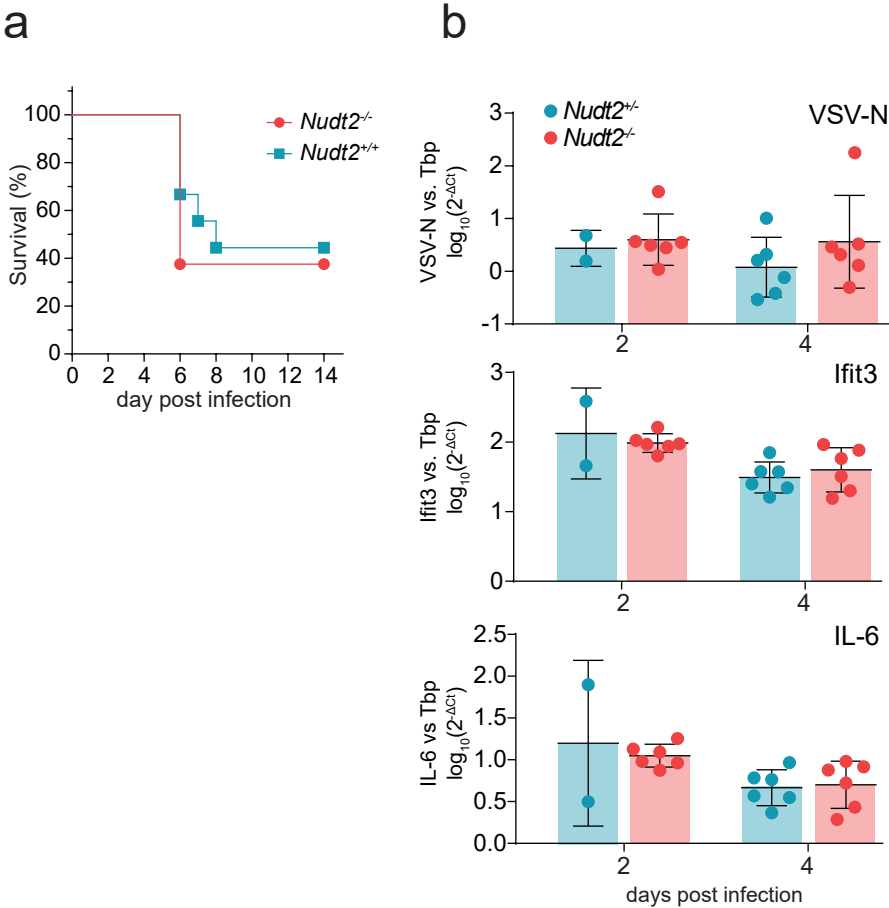
Supplementary Figure 4 | Human NUDT2 subcellular localization and function. **(a)** Huh7.5 and Huh7.5 deficient for NUDT2 cells were stained for NUDT2 and analyzed by confocal microscopy. Representative images of three biological repeats are shown. The scale bars represent 10 μm . **(b)** HEK293T cells were transfected with plasmids encoding Myc-tagged NUDT2, NUDT2 E58A, and the control mutNS1 (IAV) or left untransfected (mock). Myc-tagged proteins were precipitated, and precipitation efficiency was evaluated by western blotting against Myc. **(c)** Precipitates from (b) were incubated with PPP-RNA (IVT4) for 3 h and phosphate release evaluated using a malachite green assay. Bar graphs show the mean of three independent replicates $\pm$ SD, *** $p < 0.001$ as analyzed by nonparametric two-tailed t-test.

Supplementary Figure 5



Supplementary Figure 5 | Characterization of NUDT2 deficient mice. **(a)** Schematic overview of *Nudt2* targeting in mice. Depicted is the *Nudt2* gene locus with a tm1a cassette inserted after the first exon and encoding for the lacZ gene, with a splice acceptor, a polyA site, and a neomycin resistance gene. **(b)** Genotyping PCR of *Nudt2^{tm1a/tm1a}* (*Nudt2^{-/-}*) mice. PCR amplification results in a wild-type allele detectable at 492 bp, whereas the inserted tm1a cassette is detectable as bands of 417 bp and 912 bp. **(c)** Mendelian ratios were observed from 192 weaned animals originating from *Nudt2^{+/-}* breeding pairs compared to the expected Mendelian distribution. **(d)** Proteome analysis of bone marrow cells isolated from NUDT2 knockout (*Nudt2^{-/-}*) and control mice. Volcano plots display proteins that were significantly up-or down-regulated in cells lacking NUDT2 (right) or wild-type (left) bone marrows (student's *t*-test, FDR ≤ 0.05, 6 biological replicates). **(e)** Influence of extracellular applied or intracellular delivered Ap₄A or poly(I:C) on the growth of SFV6-2SG Nano-Luc (SFV) at an MOI of 0.0035. Virus growth was assessed by the measurement of renilla luciferase. The bar plot shows the mean ± SD of four independent replicates. **(f)** Levels of Ifit3, Il6, or U1 snRNA in the heart, liver, lung, or spleen of wild-type mice or *Nudt2^{-/-}* mice were quantified by RT-qPCR analysis. Data were normalized to murine Tbp RNA. Shown is the mean ± SEM. Every dot represents an individual mouse (n = 5 for *Nudt2^{+/+}* and *Nudt2^{+/-}* and n = 4 for *Nudt2^{-/-}*).

Supplementary Figure 6



Supplementary Figure 6 | in vivo infection experiments. (a) Survival rates of 8–12 week old wild-type (*Nudt2*^{+/+}) (9 animals) or NUDT2 knockout (*Nudt2*^{-/-}) mice (8 animals) infected intranasally with 5e6 PFU of VSV in 20 µL PBS. (b) Viral RNA load and levels of Ifit3 and Il-6 in the cerebrum of wild-type and knockout mice infected with VSV. RNA levels were quantified by RT-qPCR analysis using specific primers for the N-transcript of VSV. Data were normalized to murine Tbp RNA. Shown is the mean ± SD. Every dot represents an individual infected mouse (n = 2 for NUDT2^{-/-} at 2 days post infection and n = 6 for the other conditions).

Supplementary Table 1

siRNA target sequences used in the siRNA screen

Sequence Name	siRNA target sequence 5'->3'
Hs_NUDT1_3	CTCCTGCTTCAGAAGAAGAAA
Hs_NUDT1_4	CCGGGTTTCATCTGGAATTAA
Hs_NUDT1_5	CCGCGAGGTGGACACGGTCTA
Hs_NUDT2_10	CAGGCATCAGATGGCATTCA
Hs_NUDT2_7	TGCCAGGGTCCTGCAGTTATA
Hs_NUDT2_8	AGGATCCTTGTGGGCCTTCTA
Hs_NUDT5_2	CAGGCTTGTCAAAGTGTACTA
Hs_NUDT5_3	TACATGGATCCTACTGGTAAA
Hs_NUDT5_6	TTCCTACGCTCTAGCACTGAA
Hs_NUDT6_5	CACGCAGAATCGGATTCATCA
Hs_NUDT6_6	CTGGTTGTACAAGATCGAAAT
Hs_NUDT6_7	GAGCTGTATTTGATGAAAGTA
Hs_NUDT9_5	ATGGATAATCTTATGCTAGAA
Hs_NUDT9_6	CACGCTGCAGATCCCATTATA
Hs_NUDT9_3	AAGATTAGTGCCACACTGAAA
Hs_NUDT12_6	AGCCGAGCTATTGCACATCAA
Hs_NUDT12_7	ATGATTGGTTGCTTAGCTCTA
Hs_NUDT12_5	AAGGTCGGATATTAATAAGT
Hs_NUDT13_6	CAGGGAACGGAAGCCGTTGAA
Hs_NUDT13_5	CCACAACGTGTTGATTAACAA
Hs_NUDT13_7	TAGCCCGGATCAAGTCACTTA
Hs_NUDT14_1	CAGCGTGACCGTTCTCTTATT
Hs_NUDT14_2	AAAGGCTTGCATAGCTCCAAA
Hs_NUDT14_4	CTCCAGACAGACCATGTTCTA
Hs_NUDT15_4	CAGCAGTACTCTTCTCACTAA
Hs_NUDT15_6	CTCAAGAGCCTTTCAAGGGTA
Hs_NUDT15_7	ATCTGGTGTGATATTGTAATA
Hs_NUDT16_1	CTCCCTGTTTATATGCGTACA
Hs_NUDT16_2	CAGGTCAACACTAATACCACT
Hs_NUDT16_3	ATGTATGAAGGTGGTTCTCAA
Hs_NUDT17_7	CCCAACCATGGCAGAGGACAA
Hs_NUDT17_6	CCCGGATCCAACCAAACCCAA
Hs_NUDT17_5	TACCATCACATTGTTCTGTAT
Hs_NUDT18_1	CTGGGCCGAGATCACAGTGAT
Hs_NUDT18_2	AAGCGAGGAGTCCAAAGCTCA
Hs_NUDT18_3	ATCCTGCACCTGGTTGAACTA
Hs_NUDT19_4	TACGAAGTGAGAAGACTTGCA
Hs_NUDT19_3	CACGTTTATCCTAAGAACTCT
Hs_NUDT19_2	CACCGGATAGTGACATACCAT
Hs_DCP2_5	CCGGTGATTCATGTTTGTGAA
Hs_DCP2_6	CTGCTTATAAATGTTATTGTA
Hs_DCP2_1	CTGGGTTATCAGAGTAATGAA
Hs_NUDT21_5	CTGCACATATTACAAAGCCTA
Hs_NUDT21_1	ATCGTGATGAGAAACCTAATA
Hs_NUDT21_3	CCAAGTGTAGCTGAGCAATTA
Hs_NUDT22_4	AGGCGCCATCATCCTCTACAA
Hs_NUDT22_1	CTGGGCCTTACTTCCTACCGA
Hs_NUDT22_3	CCGAGACTTCCTGGGCACCAA
Hs_DDX58_6 (RIG-I)	AACGTTTACAACCAGAATTTA
Hs_DDX58_10 (RIG-I)	TTCTACAGATTTGCTCTACTA
Hs_DDX58_11 (RIG-I)	CTCCTCCTACCCGGCTTTAAA
Scrambled (control)	AAGGTAATTGCGCGTGCAACT

Supplementary Table 2

Proteomic analysis of bone-marrow cells derived from Nudt2^{-/-} animals and Nudt2^{+/+} littermate controls (WT_1-3 and KO_1-3). Three individual mice per genotype were analyzed. Table shows Protein IDs and label free quantification levels (LFQ) of individual measurements. (two-tailed Student's t-test, FDR 0.05, S0 = 0.1)

Supplementary Table 3

Nucleotide sequences used as templates in in vitro transcriptions

Sequence Name	Sequence 5' -> 3'
dsRNA IVT4	TTGTAATACGACTCACTATAGGGACGCTGACCCAGAAGATCTACTAGAAATAGTAGATCTTCTGGGTCAGCGTCCC
dsRNA IVT4_as	GGGACGCTGACCCAGAAGATCTACTATTTCTAGTAGATCTTCTGGGTCAAGCTCCCTATAGTGAGTCGTATTACAA
G0	AATTCCTGCAGTAATACGACTCACTATAGGCGCCGCGGTAACGCGGCGCCACGCGGAAACGCGCC
G0_as	GGCGCGTTTCCGCGTGCGCGCCGCTTACCGCGGCGCCTATAGTGAGTCGTATTACTGCAGGAATT
G8	AATTCCTGCAGTAATACGACTCACTATAGGAACAACGGCGCCGCGGTAACGCGGCGCCACGCGGAAACGCGCC
G8_as	GGCGCGTTTCCGCGTGCGCGCCGCTTACCGCGGCGCCGTTGTTCTATAGTGAGTCGTATTACTGCAGGAATT
G4[GGAA]	AATTCCTGCAGTAATACGACTCACTATAGGAAGGCGCCGCGGTAACGCGGCGCCACGCGGAAACGCGCC
G4[GGAA]_as	GGCGCGTTTCCGCGTGCGCGCCGCTTACCGCGGCGCCTTCTATAGTGAGTCGTATTACTGCAGGAATT
G4[GAAA]	AATTCCTGCAGTAATACGACTCACTATAGAAAGGCGCCGCGGTAACGCGGCGCCACGCGGAAACGCGCC
G4[GAAA]_as	GGCGCGTTTCCGCGTGCGCGCCGCTTACCGCGGCGCCTTCTATAGTGAGTCGTATTACTGCAGGAATT
G4[GCAA]	AATTCCTGCAGTAATACGACTCACTATAGCAAGGCGCCGCGGTAACGCGGCGCCACGCGGAAACGCGCC
G4[GCAA]_as	GGCGCGTTTCCGCGTGCGCGCCGCTTACCGCGGCGCCTTGCTATAGTGAGTCGTATTACTGCAGGAATT
G4[GUAA]	AATTCCTGCAGTAATACGACTCACTATAGTAAGGCGCCGCGGTAACGCGGCGCCACGCGGAAACGCGCC
G4[GUAA]_as	GGCGCGTTTCCGCGTGCGCGCCGCTTACCGCGGCGCCTTACTATAGTGAGTCGTATTACTGCAGGAATT
A8	AATTCCTGCAGTAATACGACTCACTATTAGAACAACGGCGCCGCGGTAACGCGGCGCCACGCGGAAACGCGCC
A8_as	GGCGCGTTTCCGCGTGCGCGCCGCTTACCGCGGCGCCGTTGTTCTAATAGTGAGTCGTATTACTGCAGGAATT
A4[AGAA]	AATTCCTGCAGTAATACGACTCACTATTAGAAGGCGCCGCGGTAACGCGGCGCCACGCGGAAACGCGCC
A4[AGAA]_as	GGCGCGTTTCCGCGTGCGCGCCGCTTACCGCGGCGCCTTCTAATAGTGAGTCGTATTACTGCAGGAATT
A1	AATTCCTGCAGTAATACGACTCACTATTAGGCGCCGCGGTAACGCGGCGCCACGCGGAAACGCGCC
A1_as	GGCGCGTTTCCGCGTGCGCGCCGCTTACCGCGGCGCCTAATAGTGAGTCGTATTACTGCAGGAATT
T7(φ2.5)_fwd	AATTCCTGCAGTAATACGACTCACTATAG
T7(φ6.5)_fwd	AATTCCTGCAGTAATACGACTCACTATTA
Ren_IVT_PCR	TTAAGGACGTCATTATGCTGAGTGAT

Supplementary Table 4

qRT-PCR primers used in this study

Sequence Name	Sequence 5'→ 3'
hu_NUDT2_fw	CACTGGACTCCTCCCAAAGG
hu_NUDT2_rv	TCCTCTTGGGTCTCCCTCAG
hu_NUDT12_fw	TTGGGCAAACTTGTATCGAC
hu_NUDT12_rv	CTTCATCCTCCTATGCCAGC
hu_NUDT14_fw	AAGAGAACGGTCACGCTGTC
hu_NUDT14_rv	CCGCCTCACCTACCTG
hu_NUDT17_fw	GACTGTCTTGCTAACCCGAAG
hu_NUDT17_rv	GGCAGGTGTAGTCCACTCTC
VSV-N_fw	ATCGGAATATTTGACCTTGTA
VSV-N_rv	ACTGTTCAATTGATCATTTTGC
ms_ActB_fw	CTCTGGCTCCTAGCACCATGAAGA
ms_ActB_rv	GTA AACGCAGCTCAGTAACAGTCCG
SFVnsp3_fw	GCAAGAGGCAAACGAACAGA
SFVnsp3_rev	GGGAAAAGATGAGCAAACCA
HSV-DNA Polymerase I_fw	AGCCTGTACCCAGCATCAT
HSV-DNA Polymerase I_rv	TGGGCCTTCACGAAGAACA
Firefly Luc_fw	CTCACTGAGACTACATCAGC
Firefly Luc_rv	TCCAGATCCACAACCTTCGC
hu_GAPDH_fw	GATTCCACCCATGGCAAATTC
hu_GAPDH_rv	AGCATCGCCCCACTTGATT
Renilla Luc_fw	GAATTTGCAGCATATCTTGAACCAT
Renilla Luc_rv	GGATTTACAGAGGCCATGATAA
hu_RPLP0_fw	GGATCTGCTGCATCTGCTTG
hu_RPLP0_rv	GCGACCTGGAAGTCCAACCTA
mu_TBP_fw	CCTTCACCAATGACTCCTATGAC
mu_TBP_rv	CAAGTTTACAGCCAAGATTCA
mu_Ifit3_fw	TGGTCATGTGCCGTTACAGG
mu_Ifit3_rv	GCTGCGAGGTCTTCAGACTT
mu_Ii6_fw	TAGTCCTTCCTACCCCAATTTC
mu_Ii6_rv	TTGGTCCTTAGCCACTCCTTC
U1 RNA_fw	ACTTACCTGGCAGGGGAGATAC
U1 RNA_rv	ACATCCGGAGTGCAATGGATAA

Supplementary Table 5

Nucleotide guide sequences cloned in pLentiCRISPRv2

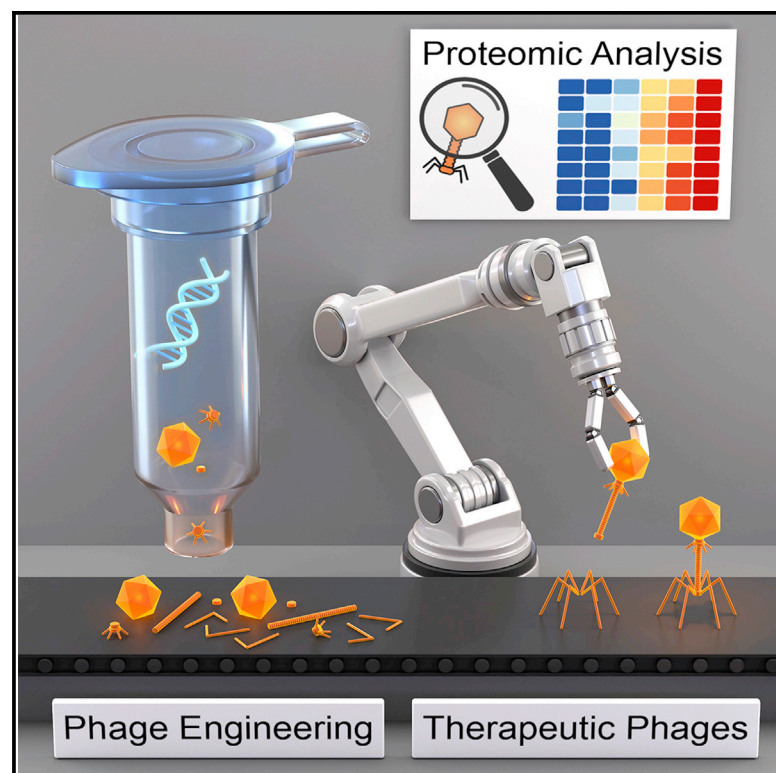
Sequence Name	Sequence 5' -> 3'
NUDT2_gRNA1	ATGAGCACCAAGCCTACCGC
NUDT2_gRNA2	AATTGCATTGTTGTCCACTT
NUDT2_gRNA3	TAACTAGGCCATGTGGAACC
NUDT2_gRNA4	CATCAGATGGCATTTCATCAC
NUDT2_gRNA5	TCTCCTTGAAGTGAAGCAAC
NUDT2_gRNA6	CCATTATTGAGGGGTTCAAA
XRN1_gRNA1	TTAAGAGAAGAAGTTCGATT
XRN1_gRNA2	TAAAACGCCTCCCACGCTGC
XRN1_gRNA3	GCTTGGATTAACAAGTCATG
XRN1_gRNA4	TAATGCGAAACAACACCTCC
XRN1_gRNA5	GTATCCCTGTCTCAGCGAAG
XRN1_gRNA6	TTTCAACCACTTCGCTGAGAC

Full Manuscript: P3 - Cell-free production of personalized therapeutic phages targeting multidrug-resistant bacteria

Cell Chemical Biology

Cell-free production of personalized therapeutic phages targeting multidrug-resistant bacteria

Graphical abstract



Authors

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In brief

Emslander, Vogele et al. demonstrate the personalized production of therapeutic bacteriophages in a cell-free system. This approach is faster and safer than the conventional method based on cultivating host bacteria. Cell-free production also allows for time-resolved proteome analysis during phage assembly and biosafe, non-genomic engineering of single-use phages targeting multidrug-resistant bacteria.

Highlights

- Personalized and cell-free production of phages against multidrug-resistant bacteria
- Extending cell-free production to phages targeting gram-positive bacteria
- Transient, non-genomic engineering of bacteriophages
- Time-resolved proteomics during phage assembly and validation of phage proteins



Resource

Cell-free production of personalized therapeutic phages targeting multidrug-resistant bacteria

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SUMMARY

Bacteriophages are potent therapeutics against biohazardous bacteria, which rapidly develop multidrug resistance. However, routine administration of phage therapy is hampered by a lack of rapid production, safe bioengineering, and detailed characterization of phages. Thus, we demonstrate a comprehensive cell-free platform for personalized production, transient engineering, and proteomic characterization of a broad spectrum of phages. Using mass spectrometry, we validated hypothetical and non-structural proteins and could also monitor the protein expression during phage assembly. Notably, a few microliters of a one-pot reaction produced effective doses of phages against enteroaggregative *Escherichia coli* (EAEC), *Yersinia pestis*, and *Klebsiella pneumoniae*. By co-expressing suitable host factors, we could extend the range of cell-free production to phages targeting gram-positive bacteria. We further introduce a non-genomic phage engineering method, which adds functionalities for only one replication cycle. In summary, we expect this cell-free methodology to foster reverse and forward phage engineering and customized production of clinical-grade bacteriophages.

INTRODUCTION

The growing number of multidrug-resistant (MDR) bacteria has been classified as one of the most critical global health threats by the World Health Organization (Leung et al., 2011; Tacconelli et al., 2018). It is estimated that costs of \$2.2 billion in the United States in 2014 alone (Thorpe et al., 2018) and 33,000 deaths per year in Europe are caused by MDR bacteria (Cassini et al., 2019). MDR is particularly severe in gram-negative bacteria, with most cases reported for *Escherichia coli*. However, classic hospital pathogens are also increasingly resistant and frequently found in bacterial superinfections in COVID-19 patients, especially the ESKAPE pathogen *Klebsiella pneumoniae* (He et al., 2020; Lai et al., 2020; Zhu et al., 2020). In addition, pathogens such as *Yersinia pestis*, a potential biological warfare agent, still contribute to naturally occurring outbreaks of epidemics in Africa with high morbidity rates (Chatterjee et al., 2016).

Because of the increasing need for such alternatives to antibiotics, Belgium and France have recently allowed the use of naturally occurring, well-characterized bacteriophages for personalized bacteriophage (short “phage”) therapy under the legal framework of magistral preparation (Pirnay et al., 2019). Unfortunately, the current production processes for natural and engineered phages pose considerable biosafety concerns and are often relatively inefficient and unreliable. In both cases, a pure culture of the pathogenic host is required for phage amplification and engineering (Dedrick et al., 2019; Jault et al., 2019). This process poses a high risk of prophage contamination (Rohde et al., 2018), which can induce virulence factors in bacteria (Colavecchio et al., 2017; Rohde et al., 2018). Moreover, the standard cultivation of phages with bacteria is time-consuming, generates relatively low titers, and requires the appropriate lab security level for the respective pathogen. These factors make it challenging to parallelize the production of personalized phage



cocktails that typically contain up to 16 phages (Azeredo and Silankorva, 2017). Recent double-blind patient trials have also revealed the instability and correspondingly low shelf half-life of prepared phage cocktails, emphasizing the importance of a fast and reliable production process (Jault et al., 2019).

In addition, the production of engineered phages is limited by the necessity of modifying phage genomes within living bacteria, e.g., by CRISPR-Cas or by using yeast artificial chromosomes, which reduces efficiency (Ando et al., 2015; Hatoum-Aslan, 2018).

Apart from the elaborate production process, the limited understanding of the molecular phage-host interaction can lead to erratic treatment outcomes (Brüssow, 2012; Lu and Koeris, 2011). This is partly due to the insufficient information on phage protein expression and function. Even though phages have been described for over a century (Twort, 1915), the characterization of phage proteomes is still largely obscure. Based on the analysis of several hundred phage genomes, recent studies determined over 60% of the annotated gene products as “hypothetical proteins” (Ando et al., 2015; Hatoum-Aslan, 2018) without any experimental validation.

We thus reasoned that cell-free production systems could address all of the challenges mentioned above, as shown in Figure 1: they could (1) produce phages efficiently and safely at high titers, (2) facilitate novel methodologies for phage engineering, and (3) enable a better characterization of phage proteomes and function, which is essential to improve phage therapies further. Cell-free expression systems have recently been employed for the biosynthesis of glycoproteins (Jaroentomeechai et al., 2018), the incorporation of non-canonical amino acids (Jaroentomeechai et al., 2018), and as components of minimal cells (Jia et al., 2017; Vogeley et al., 2018). However, for phage production, cell-free systems made from *E. coli* extracts were limited to the generation of model phages such as the *E. coli* phages T7, phiX174, and MS2 (Garenne et al., 2019; Rustad et al., 2017; Shin et al., 2012).

So far, neither the cell-free generation of clinically relevant bacteriophages nor a comprehensive characterization of phage proteomes has been achieved.

Here, we introduce the phage production pipeline “phactory,” which enables on-demand and safe generation of a broad spectrum of phages in a host-independent manner, including transiently engineered and therapeutic phages against antibiotic-resistant bacteria. This approach has also enabled us to validate the existence of dozens of highly conserved hypothetical proteins, including non-structural proteins. It also allowed us to systematically monitor phage assembly in correlation with its expression profile using time-resolved mass spectrometry. These innovations can significantly foster our understanding of molecular phage biology, accelerate future phage engineering, and improve therapeutic phage production.

RESULTS

Bottom-up phage proteome characterization

To date, phage proteomes, particularly proteins involved in phage assembly, are not yet characterized in detail. To assess if the incomplete phage proteome could be analyzed in-depth, we first compared the protein composition of the model T7

phage (NCBI ID: NC_001604.1) derived from classic bacteria-dependent amplification (phage stock) with phages synthesized in the cell-free reaction.

To this end, the cell-free system, based on *E. coli* cell extract, buffers, and metabolites, was mixed with purified phage DNA, which resulted in the assembly of functional phages. The phages from classic bacterial cultures and cell-free produced phages were subjected to in-gel digestion and analyzed by high-resolution liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Remarkably, the proteome coverage of phages generated by the cell-free system was 72% (41 of 57), with 28% (16 proteins) of the proteome represented by hypothetical proteins (Figure 2A). Thus, the cell-free system enabled the detection of almost all previously characterized proteins and validated the existence of at least half of the hypothetical proteins.

The unbiased clustering of proteins (rows in the heatmap) suggests overall two groups of proteins according to their abundance with a general trend toward the more abundant proteins corresponding to structural proteins, whereas the less abundant proteins were non-structural (Figure 2A). Non-structural proteins (based on the protein uniprot ID and their correlated function) exhibited a much lower abundance in the phage stock samples than in the cell-free products. We also found that several of the newly validated proteins from phage T7 (Table S1) are conserved in other clinically relevant phages targeting *K. pneumoniae* and *Y. pestis*, e.g., *Yersinia* phage PhiA1122 (NCBI ID: NP_848312.1) and *Klebsiella* phage (vB_KpnP_KpV767 GenBank ID: AOZ65500), suggesting that these proteins play a universal role in phage biology.

Motivated by the success in validating hypothetical proteins based on the well-characterized genome of T7, we next tested whether the cell-free approach could be applied to yet uncharacterized phages. We chose phage CLB-P3 (Maura et al., 2012), which is directed against a strain of pathogenic enteroaggregative *E. coli* (EAEC). To assemble a protein FASTA file for LC-MS/MS analysis, we *de novo* sequenced CLB-P3 phages using next-generation sequencing (using both Nanopore and Illumina Sequencing) and performed a hybrid genome assembly from MinION and MiSeq reads. We then analyzed the proteins from cell-free expression by MS/MS.

As shown in Figure 2B, we could identify most of the CLB-P3 phage proteins in both samples, but the cell-free system produced more stable results. In total, 68 of 87 predicted proteins were identified by LC-MS/MS. Only 38 of these 68 proteins could be directly assigned functional names based on genome annotation. The remaining 30 are labeled “hypothetical.” Importantly, the corresponding heatmap (Figure 2B) exhibits a similar distribution of the precursor signal as the heatmap obtained by analyzing the T7 phage. This comparison enables data-driven functionalization based on protein intensity and coverage. Similar to the heatmaps of the T7 phage, the intensities of the soluble factors of the CLB-P3 are lower in the phage stock sample than in the cell-free system. In contrast, structural proteins were present with high abundance in both sample types, as phage stocks (e.g., by polyethylene glycol [PEG] precipitation or ultracentrifugation) enrich completely assembled virion particles. Importantly, it was possible to track these proteins in sequencing data of several hundred phages targeting gram-negative and

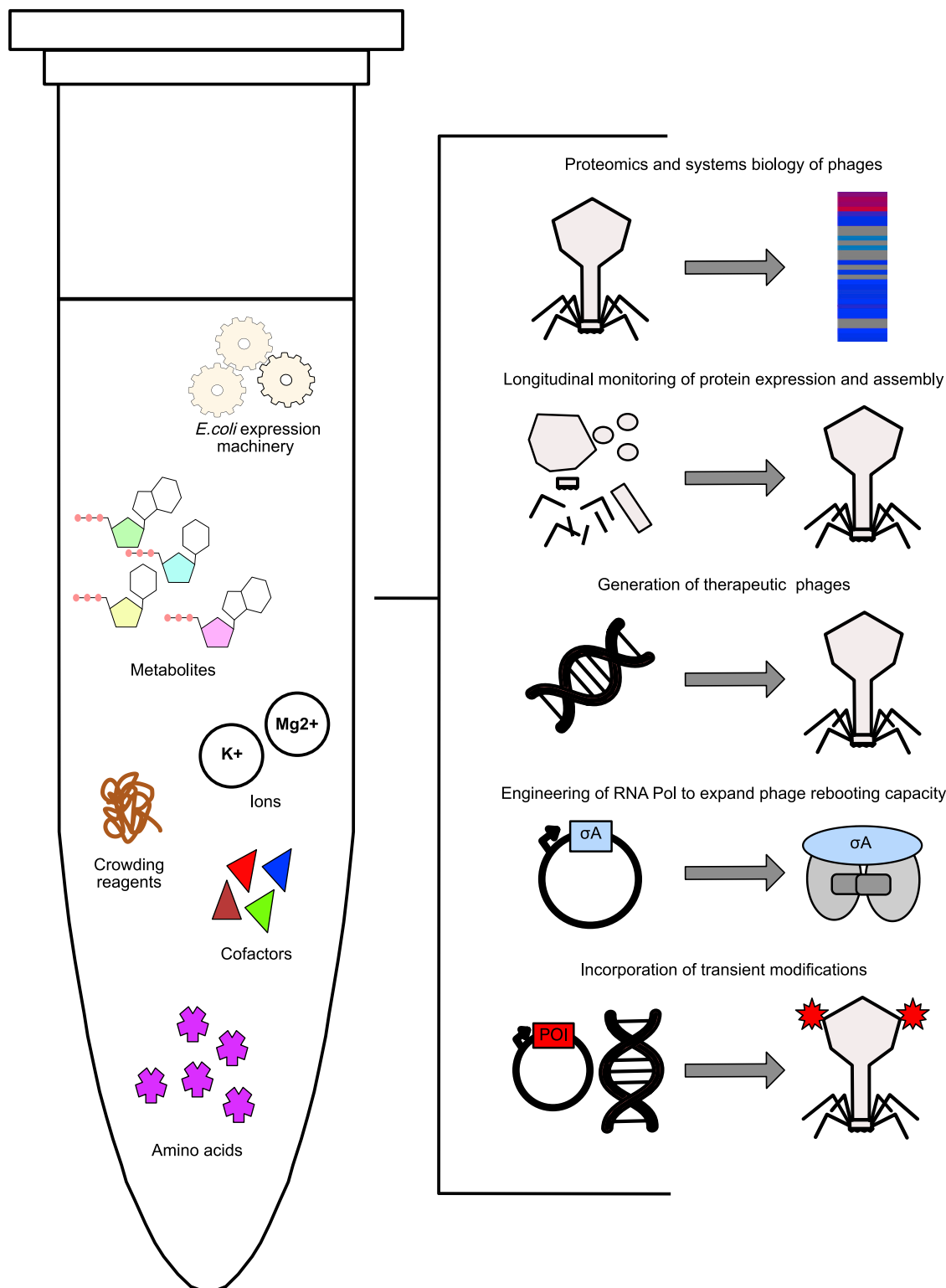


Figure 1. Schematic of 'phactory', a cell-free platform for reverse and forward engineering of therapeutic phages

An optimized transcription-translation system with additional host factors and crowding reagents can produce phages that target a broad range of Gram-positive and Gram-negative bacteria. Non-genomic phage engineering can safely add transient functionalities to phages. Cell-free produced phages can be analyzed by mass spectrometry without purification, facilitating the identification of non-structural phage proteins. Time-resolved proteomic analyses can further be correlated with the phage assembly process. At the microliter scale, a one-pot reaction enables the production of therapeutic phages against multidrug-resistant bacteria in a matter of hours at clinically relevant titers.

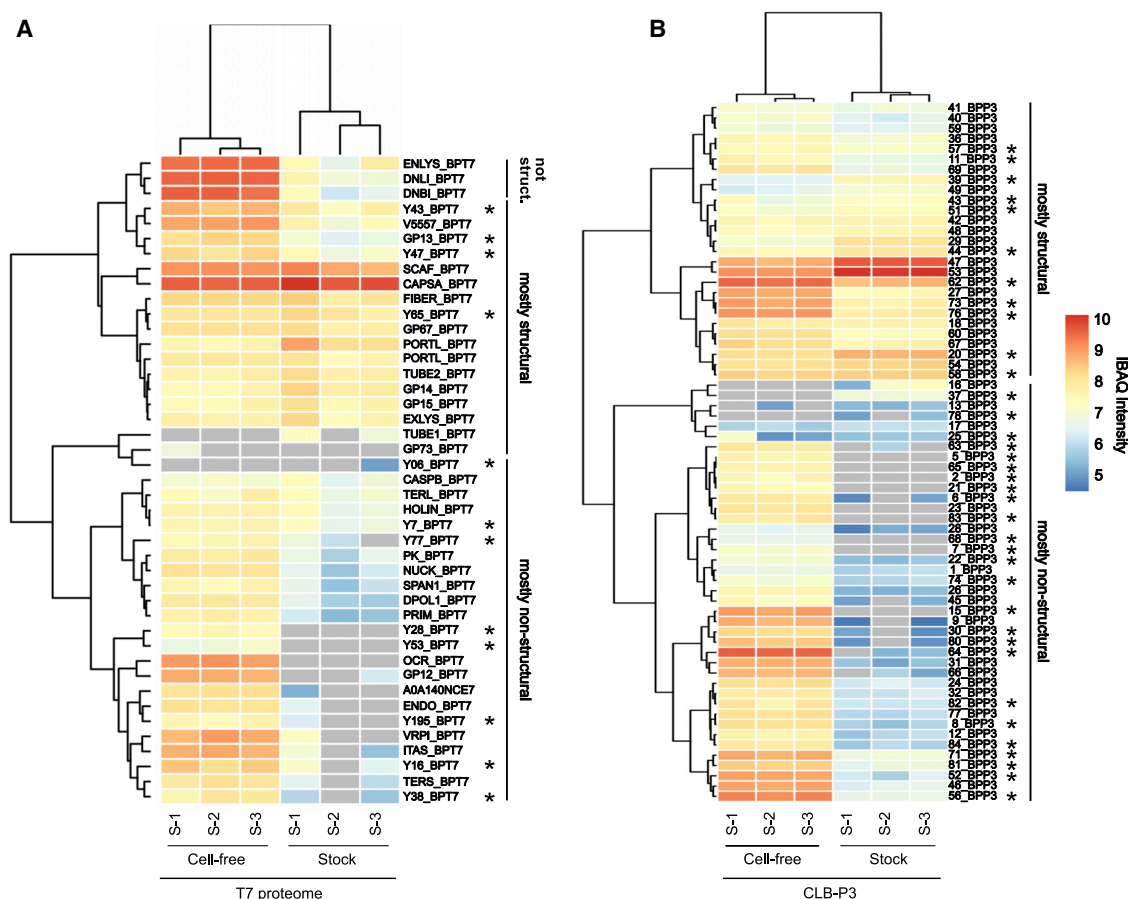


Figure 2. Highly resolved phage proteomes obtained from cell-free systems reveal hypothetical proteins

(A and B). Phage proteomes expressed in a cell-free system were analyzed by LC-MS/MS. The heatmaps from (A) T7 and (B) CLB-P3 proteomes were obtained from three independent replicates (S-1, S-2, S-3) for phage stock (stock) and cell-free assembled phages (cell-free). The logarithm transformed (base 10) iBAQ intensity was used to represent the protein abundance. Missing values were replaced with the lowest measured iBAQ intensity. The clustering was based on the Ward linkage method using the euclidean distance. Dendrograms display similarity between rows (or columns). Less abundant proteins in the phage stock proteome clustered mostly as non-structural proteins, e.g., not contained in the phage capsid, base-plate, and tail fiber. Stars indicate all hypothetical proteins validated in this study.

gram-positive bacteria. These include potentially therapeutic phages such as the *Shigella* phage psf-2 (NC_026,010.1), the *Salmonella* phage Jersey (NC_021,777.1), the *Klebsiella* phage Sushi (NC_028,774.1), and the *Cronobacter* phage phiES15 (NC_018,454.1). Moreover, the identified proteins are also encoded in phages potentially relevant for the food industry, such as *Lactococcus* phage TP901-1 (NC_002,747.1) and *Pseudomonas* phage phiPSA1 (NC_024,365.1) (Figure S1).

Time-resolved analysis of protein expression and assembly

Encouraged by the high proteome coverage, we reasoned that it should be possible to correlate protein expression to phage assembly by time-resolved mass spectrometry and plaque assay to analyze the tightly orchestrated protein expression with molecular precision. We thus focused on the T7 phage, for which a clustering into early-, middle-, and late-expressing genes has been known. Here, all proteins are produced in under 20 min, making it challenging to track the expression of every single protein in a time-resolved manner in bacteria (McAllister and Wu,

1978). Leveraging the cell-free system, we monitored the protein expression and T7 phage titer from a 70 μ L cell-free reaction over 10 time points (0–240 min). Specifically, half of the removed volume at each time point (3 μ L) was prepared for LC-MS/MS analysis by immediately adding lithium dodecyl sulfate (LDS) to stop the expression and assembly process (Figure 3A). From the remaining volume, the phage titer was determined via plaque assay (Figure 3B). The mass spectrometric results show that most phage proteins are present after 40 min (Figure 3A), but fully assembled and functional phages first appear after 60 min (Figure 3B).

Interestingly, only four proteins were expressed particularly late after 40 min. These four proteins entail the two subunits of the terminase (TerL and TerS, necessary for the loading of DNA into the phage capsid [Daudén et al., 2013]), the cell lysis protein holin (necessary for the timed release of the phage from the bacteria [Nguyen and Kang, 2014; Wang et al., 2000]), and the yet uncharacterized Y195.

Combined with the plaque assay results (Figure 3B), which show the first assembled phages between 60 and 90 min,

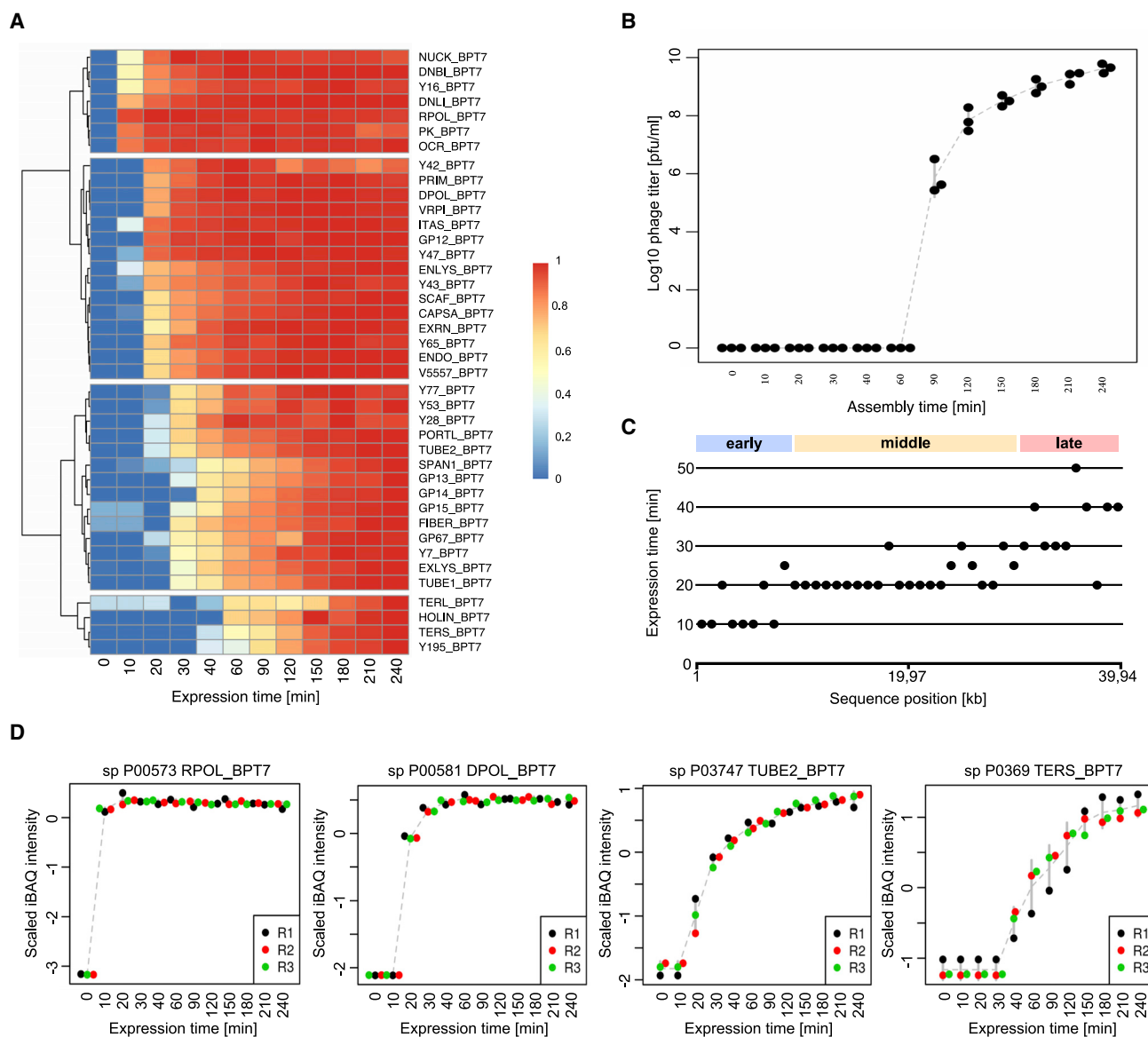


Figure 3. Time-resolved analysis of the cell-free protein expression of T7 phage elucidates the self-assembly process

Protein expression and phage assembly from a cell-free reaction were monitored with respect to proteome composition and titer over different time points. (A) The protein expression of the T7 proteome was analyzed by LC-MS/MS from 0 to 240 min within the cell-free system. The heatmap shows the relative abundance of proteins at different time points, with the dendrogram indicating the protein similarity derived from the measured peptides.

(B) Phage titers during the 240 min phage assembly in a cell-free reaction were determined with a plaque assay.

(C) Mapping of protein expression onto the phage genome accurately recapitulates early, middle, and late gene products in the synthetic system. Time points for which a protein was detected with a 50% max. MS intensities were aligned to the position in the T7 genome.

(D) Protein expression patterns can be differentiated with molecular precision. Early genes, e.g., DNA/RNA polymerase (P00581 and P00573), are already expressed after 10 min, whereas structurally relevant proteins, e.g., TUBE2 and late proteins such as TerS are expressed after 20 and 30 min. Data were obtained from three biological replicates. Error bars in b and d represent the SD of the mean (n = 3).

these proteins appear to be limiting factors for the assembly of fully functional phages or the release of the phages from the bacteria. On this basis, we aligned the protein expression patterns to the position of their coding sequence in the T7 genome. The trajectory obtained by time-resolved proteomics of the cell-free system is consistent with the current consecutive expression model proposed for T7 phage (Figure 3C). It is even possible to analyze the kinetics of cell-free expression

with single protein precision (Figure 3D), exemplarily shown for T7 RNA polymerase (RPOL), T7 DNA polymerase (DPOL), as well as the late genes, Tail tubular gp12 (TUBE2) and Termination S (TER S).

Personalized phage therapy

We next tested whether we could also produce biomedically relevant phages targeting bacteria from a different genus

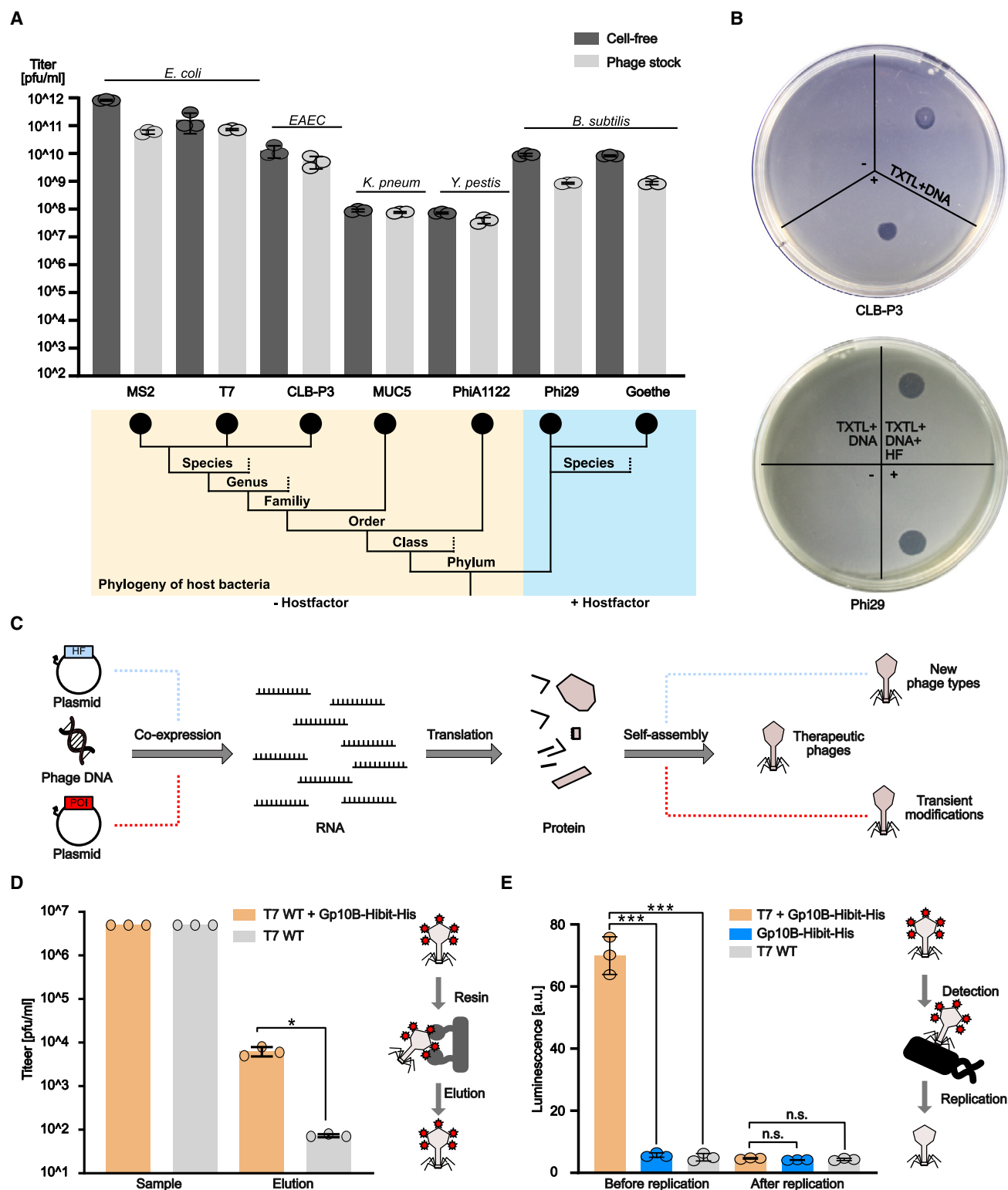


Figure 4. Cell-free production of phages against Gram-negative and Gram-positive bacteria, including therapeutic phages

(A and B) Quantification of the titers of the phages produced by the cell-free system. Titers sufficient for therapy ($>10^6$ pfu/mL) were obtained for CLB-P3 targeting EAEC, PhiA1122 targeting *Y. pestis*, and vB_KpS_Muc5 (MUC-5) phages targeting *K. pneumoniae* (*K. pneum.*). The mean phage titer is shown here $\pm$ SEM ($n = 3$). (B) Spot assay results from some of the phages shown in (a). The assay plates include the phage stock as a positive control (+) as well as the cell-free system with (+) phage DNA and without (–).

(legend continued on next page)

(Table S2). We thus chose PhiA1122 (NCBI ID: NC_004777.1) phage targeting *Y. pestis*, a possible biological warfare agent.

As shown in Figure 4A, it was indeed possible to produce clinically relevant titers (on the order of 10^8 PFU/mL) of specific phages within just 4 h.

Encouraged by this result, we assessed whether we could produce therapeutic phages on-demand that could target an MDR ESKAPE pathogen in a realistic patient-dependent scenario. We thus isolated a carbapenem-resistant *K. pneumoniae* strain (Bw1) with wzi capsule type K17 from colonized patient skin, which is untreatable with common antibiotics. We then isolated a bacteriophage, which we designated vB_KpS_Muc5 (MUC-5), from a wastewater plant via amplification in *K. pneumoniae* strain Bw1 followed by isolation of phage DNA. When we subsequently added MUC-5 phages produced in the cell-free system to the pathogenic bacteria, we observed bacterial lysis within approximately 30 min with sufficient phage titers produced in just a few microliters of the one-pot reaction (Figure 4A).

Collectively our results demonstrate a complete pipeline for personalized phage therapy (phactory) that includes the isolation of a multidrug-resistant pathogen from a patient, the selection of a specific therapeutic phage, its cell-free production, and successful application.

Rebooting of phages targeting gram-positive bacteria

To demonstrate the generalizability of phactory beyond phages targeting gram-negative bacteria, we sought to produce the phages Phi29 (NCBI ID: NC_011048.1) and Goe1 (vB_BsuP_Goe1, NCBI ID NC_049975.1) with the cell-free system, targeting the gram-positive bacterium *Bacillus subtilis*. (Figure 3C).

The early genes of a phage genome are usually transcribed by the host's RNA polymerase. Therefore, we reasoned that modifying the *E. coli* RNA polymerase by incorporating the *B. subtilis* housekeeping sigma factor SigA should enable the production of these phages (Figures 4A and 4C). The RNA polymerase holoenzymes of *E. coli* and *B. subtilis* show a 96.5% sequence similarity (Table S3), and the sigma factors were shown to be interchangeable (Bervoets et al., 2018).

Capitalizing on the direct genetic access in the cell-free system, we thus co-expressed the corresponding SigA host factor. This modification indeed enabled the assembly of the *B. subtilis*-targeting phages, shown by spot assay (Figure 4B). Only the combination of SigA, phage DNA, and the cell-free system led to the production of functional phages (Figure S2A), with a plaque size identical to phages amplified in their bacterial host (Figures S2B and S2C). To exclude lytic proteins generated in the cell-free system rather than by complete infectious phages as a possible cause for plaque formation, the plaques were

picked, the contained phages amplified with the appropriate host, and their identity validated by PCR with phage-specific primers (Figure S2C). This analysis confirmed that the adaptation of the RNA polymerase in the cell-free system was sufficient for generating fully functional phages. These data show cell-free phage production can be extended to the important class of phages targeting gram-positive bacteria.

Nongenomic engineering of single-use phages

Given the ease with which proteins can be co-expressed in the cell-free system, we reasoned that it should be possible to engineer phages at the protein level without altering the phage genome. To demonstrate this approach, we generated variants of the T7 phage with the modified minor capsid protein Gp10B, including a polyhistidine-tag (His-tag) and the split luciferase Hi-Bit part (Oh-Hashi et al., 2017). The HiBit luciferase consists of two parts. The minor C-terminal domain was genetically fused to the capsid protein structure, while the major N-terminal domain was part of the assay buffer. The complementation of both parts generates a bioluminescent signal. This approach thus enables the affinity purification and detection of the modified phage with a relatively small protein modification as compared with fluorescent proteins or other luciferase systems.

Upon co-expression of the His-tagged capsid protein, phages could be purified by affinity chromatography at nearly two log-fold higher titers, indicating that the G10B-His was integrated into the outer phage structure and modified phages were still effective against the host bacteria (Figure 4D). We also co-expressed HiBit with a His-tag fused to G10B, purified the resulting phages, and incubated them with *E. coli*. After washing the bacteria, a substantial luminescence signal was detected compared with phages produced by co-expression of his-tagged HiBit without the capsid protein G10B or wild-type T7 (Figure 4E). These data show that the engineered phages could attach to the host bacteria and potentially serve phage biotyping.

Notably, the bioluminescence signal disappeared after one phage replication cycle, showing that the phage modification at the protein level was only transient (Figure 4E).

DISCUSSION

We have introduced phactory, a cell-free phage production and analysis pipeline, which facilitates the host-independent one-pot synthesis of potentially clinically relevant phages enabling the systematic identification of phage proteins and phage engineering.

However, the success of cell-free production of other phages is still limited by the knowledge of required interaction with other

(C) Schematic of phage rebooting via co-expression of an appropriate host factor (HF, blue arrows) to produce phages targeting bacteria distant from *E. coli*. Nongenomic phage engineering is achieved by the co-expression of proteins of interest (POI, red) that co-assemble into the phage structure to produce phages with transiently modified properties.

(D) Titers of T7 phages with co-expression of a modified capsid protein (Gp10B) fused to split luciferase (HiBit) and a polyhistidine-tag (Gp10B-HiBit-His) before and after affinity purification (elution) compared to wildtype T7 phages (WT). Titers were adjusted to 10^6 pfu/mL for both conditions. The mean phage titer is shown here $\pm$ SEM ($n = 3$). * $p < 0.05$ for an unpaired two-tailed t-test with Welch's correction.

(E) The modification with a luciferase is present for only one replication cycle of the phage. Bioluminescent signals before and after the replication cycle of the phage by infection of the host bacteria. Host bacteria were incubated with modified phages, pelleted, and washed (before replication). The same experiment was repeated with the resulting phages, which lost the transient modification after replication. Phages produced with co-expression of Gp10B-HiBit-His, or controls (HiBit-His without Gp10B or WT). The mean luminescence signal is shown here $\pm$ SEM ($n = 3$). *** $p < 0.001$ for an ordinary two-way ANOVA with Tukey correction.

host proteins, the presence of membrane components, and DNA packing mechanisms.

Phactory increases the diversity of bacteriophages produced by cell-free expression beyond *E. coli* phages. We were able to produce phages (Phi29 and Goe1) against gram-positive bacteria by co-expressing the primary *B. subtilis* RNA polymerase sigma factor SigA, which mediates the interaction of RNA polymerase with *B. subtilis*-specific promoter sequences (Haldenwang, 1995) (Figure 4B). These results are in line with the finding by Whiteley and colleagues (Davison et al., 1980; Whiteley and Ernest Hemphill, 1970), demonstrating that the *B. subtilis* SigA factor can direct the *E. coli* RNA-polymerase to transcribe foreign promoters, even if the natural sigma70 cofactor is still active in the system.

Our results suggest that the *E. coli* and *B. subtilis* RNA-Polymerase core enzymes are sufficiently similar to allow some promiscuity in integrating the host factor. In addition, our data also make it appear likely that additional phages for hosts distinct from *E. coli* could be generated by supplementing appropriate host factors (Figures 4A–4C). Interestingly, the *E. coli* ribosomes can non-specifically translate other bacterial mRNAs (e.g., from *B. subtilis*), while this does not seem to occur vice versa (Vellanoweth and Rabinowitz, 1992; Roberts and Rabinowitz, 1989). The promiscuity of *E. coli* translation factors is thus a powerful feature of the phactory production process.

At the present stage, phactory relies on isolating phage DNA from purified phage stocks. With such phage genomes, approximately 0.126 (MS2), 0.05 (CLP-P3), 0.00002 (PhiA1122), 0.0025 (Phi29), and 0.0014 (Goe1) infectious phage particles per genome were obtained. In comparison, the T7 phage can replicate within the cell-free system, resulting in an infectious particle/genome ratio of 2.7 (Vogele et al., 2021).

Given that it is already possible to generate synthetic genomes between 5 and 1,500 kb (Kodumal et al., 2004; Richardson et al., 2017), we anticipate that phages can soon be *de novo* designed and produced from synthetic genomes. With this approach, it should be possible to generate a variety of phages that tackle different MDR bacteria for which the development of antibiotic resistance has become a major global health problem (Cassini et al., 2019; Tacconelli et al., 2018).

Phactory also enables the identification of non-structural phage proteins, particularly by comparing cell-free produced phages to phage stocks. It is known that phage stock samples enrich functioning phages, which include the structural phage proteins during PEG purification, e.g., tail fiber proteins, stem proteins, and base plate proteins (Yamamoto et al., 1970). On the other hand, soluble factors necessary for intracellular phage replication, e.g., polymerases, nuclease inhibitors, are probably lost during buffer exchange, as they are not physically connected to the phage structure.

Indeed, the proteomics data generated from phactory were highly reproducible and enabled the identification of over 44 proteins with almost complete proteome coverage that had been previously categorized only as hypothetical (Figure 2). In contrast, samples obtained via bacteria-dependent production displayed fewer identified proteins with a high variability of the obtained intensities. In these samples, many of the proteins could not be identified reproducibly (Figure 2).

We found that the set of proteins in both the cell-free and bacteria-derived samples of phage T7 could be assigned mainly to structural proteins instead of soluble factors not attached to the phage structure. By contrast, proteins exhibiting lower MS intensities were mainly related to proteins not part of the phage structure (Figure 2A). As the majority of the hypothetical proteins clustered together with already characterized non-structural T7 proteins, we assumed that these proteins also belong to the class of non-structural proteins. A similar intensity distribution was found for the previously uncharacterized and *de-novo* sequenced CLB-P3 phage (Figure 2B). We thus expected the proteins found in both types of preparations to be structural, as observed for T7 proteins of the "core proteome." Accordingly, we assumed that proteins that are exclusively well-represented in samples from the cell-free system are mainly non-structural. It is likely that this identification method for phage proteins, exemplarily demonstrated for CLB-P3, will facilitate the further characterization of other phages. It can be seen that a range of CLB-P3 proteins is well conserved in many bacteriophages, while others appear to be host-specific (*Enterobacteria/Escherichia* phage).

It is also interesting to see that CLB-P3 shares a large portion of proteins with *Escherichia* phage ADB-2 (JX912252.1) and *Shigella* phage pSf-2 (NC_026,010.1), highlighting its clinical potential (Figure S1). In addition, we observed that the proteins from the cell-free expression system itself (i.e., from the *E. coli* proteome) are ten times less concentrated than the actual bacterial cytosol, allowing the detection of also less abundant phage proteins by MS analysis. Given that all of these proteins are conserved in other phages, these results may pave the way for consecutive functional and structural analysis enabling a deeper understanding of phage biology or even interaction with the host. Notably, during their life cycle, bacteriophages interact with several host proteins. Some of these known interaction partners have been identified in the cell-free system, e.g., GroEL/ES (Garren et al., 2019).

Phactory furthermore allows for longitudinal monitoring of phage protein expression over time. When examining the time course of phage protein expression, the natural protein expression patterns for phage assembly is closely recapitulated *in vitro*. The *E. coli* RNA polymerase-dependent proteins cluster separately from the T7 RNA polymerase-dependent proteins (Figure 4A), and the T7 typical gene expression sequence from early via middle to late genes is confirmed (Figure 4C) (McAllister and Wu, 1978). However, the overall expression trajectory appears to be decelerated by about a factor of two to three compared to the *in vivo* case, where T7 phage replicates typically within 20–30 min (Heineman and Bull, 2007). This may be partially explained by the dilution of the bacterial cell extract compared to the bacterial cytosol (by a factor of ~10), which may lead to a decrease in reaction rates in the extract. At the same time, this deceleration enables the expression analysis in detail while probably still maintaining the natural sequence.

The time-resolved analysis allowed us to analyze the expression sequence between class II and class III genes (Carter et al., 1981) at the protein level with total proteome coverage. Interestingly our data indicates a fourth cluster of late-expressing genes, including TerS, TerL, holin, and Y195 (Figure 3A).

Previously, it was suspected that TerS and TerL were responsible for incorporating DNA into the phage capsid (Daudén et al., 2013) and holin for the disruption of the bacterial cell wall (Wang et al., 2000). Consistently with the phage titer (Figure 3B), we determined that the first infectious phages were generated within a time frame of 60–90 min.

The position at the end of the linear genome and their late expression, coincident with the increase in phage titer (Figures 3A and 3C, may indicate a function of these proteins, including the newly validated Y195, in delaying cell escape until phage assembly is complete (Figures 3A and 3C).

It may thus be possible that these type IV genes depend not only on the T7 RNA polymerase but might exploit additional promoter-independent regulatory elements, e.g., RNAs amenable to conformational changes. Besides the identical sequence of protein expression in the cell-free system, the complete DNA replication was also shown, including cleavage of the concatemers at the specific *pac* sites for the T7 phage (Rustad et al., 2017; Vogele et al., 2021). With respect to producing therapeutic phages, the titers obtained by phactory for PhiA1122, CLB-P3, and MUC-5 targeting *Y. pestis*, EAEC, and the MDR *K. pneumoniae*, respectively, were substantially higher (5-fold) than those achieved with amplification in bacterial liquid culture (Figure 4A). This may be explained by the fact that phactory provides a stably buffered solution with high metabolite concentration and is unaffected by phage-induced bacterial defense mechanisms such as CRISPR, cell death (Garneau et al., 2010), or differences in the relative efficiency of plating of host bacteria (Kutter, 2009).

Furthermore, we demonstrated an approach for transient phage engineering by temporarily integrating a luciferase and a polyhistidine tag in the capsid structure of the T7 phage. This form of phage engineering is modular and convenient, requiring only the addition of plasmids without altering the phage genome. The method also offers a high degree of biosafety because the non-genomic modification cannot be passed on to the offspring. This transient phage bioengineering at the protein level can thus become a rapid and adaptive method to systematically modify, e.g., tail-fibers to respond to MDR bacteria without risking the spread of genetically modified phages to the environment.

In summary, we have developed and deployed phactory, a cell-free production and analysis pipeline for bacteriophages, which enables the rapid, reliable, and host-independent production of a wide range of phages, including therapeutic phages. This versatile platform allows for the systematic analysis of phage proteomes and bioengineering of phages at the protein level, which will accelerate the biological development of tailor-made phage therapies against the growing number of pathogenic MDR bacteria.

Limitations of the study

This study was mainly focused on phages from the order of *Caudovirales* with a genome size below 50 kbp. We expect that for phages with a larger genome, such as Myoviridae, more energy may have to be provided for protein expression and phage assembly. This could be achieved with a continuous exchange of metabolites, e.g., via a continuous flow or fed-batch system.

We have currently supplemented sigma factors to enable the expression of Phi29 and Goe1. For other phages more distant

from *E. coli*, it may be necessary to augment the cell-free system with additional components of exogenous expression machinery such as RNA polymerase or ribosome subunits.

The one-pot reaction mixture allows for time-resolved and in-depth proteomic analyses of structural and non-structural phage proteins. Moreover, expression kinetics may be manipulated conveniently in the membrane-free system to form hypotheses on the molecular function of the individual phage components. However, natural model systems will be necessary to elucidate their exact molecular functions.

Furthermore, GMP-conform production has to be established, and clinical trials have to be conducted to prepare for the translation of cell-free produced phages into alternative therapeutic options.

SIGNIFICANCE

The increasing number of multidrug-resistant bacteria poses a growing threat to human health, and alternatives to conventional antibiotics are increasingly needed. Although bacteriophage therapy has been known for over 100 years, it is not yet a standard therapeutic option due to the lack of a reliable bacteriophage production method, an in-depth characterization, and a safe bioengineering method to optimize phage properties.

Here, we demonstrate how an optimized cell-free system based on reconstituted and stable *E. coli* lysates enables on-demand production of bacteriophages, multi-time point characterization of their proteomes, and transient phage engineering. For the first time, we have used a cell-free system to produce phages against multidrug-resistant and bio-hazardous bacteria such as *K. pneumoniae*, *Y. pestis*, and EAEC in clinically applicable titers. Importantly, we extended cell-free production to phages against Gram-positive *B. subtilis* by replacing the promoter recognition factor (SigA) within the cell-free system. In addition, we demonstrate the transient non-genomic modification of phages via co-expression of a modified T7 capsid protein that can add functionalities such as an affinity tag or a reporter signal, but only for one replication cycle, thus increasing biological safety.

Finally, we characterized the proteome of two cell-free produced phages by mass spectrometry, detecting dozens of highly conserved hypothetical proteins. Monitoring the phage assembly process with mass spectrometry also allowed us to analyze the temporal sequence of protein expression. We anticipate that the cell-free methodology will facilitate reverse and forward engineering of phages and open new possibilities for phage therapy.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.chembiol.2022.06.003>.

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AUTHOR CONTRIBUTIONS

Q.E. has conceptualized and designed the cell-free, transient engineering, genomics, and proteomics experiments, performed the nanopore sequencing, performed the MS sample preparation with the support of M.A. and C.L., the proteomics data analysis with support from C.M.; K.V. has designed the sigma factor experiments, conducted the cell-free phage assembly and executed the transient engineering experiments; M.J. has performed the CLB-P3 genome assembly in cooperation with Q.E.; P.B. performed work on *Y. pestis* and PhiA1122; C.W. has isolated the *K. pneumoniae* strain; J.S., K.V. and J.B. performed work on the *K. pneumoniae* phage. J.A.H. performed work on the MUC-5 phage. Q.E. and A.P. have generated the figures. F.C.S. and G.G.W. have guided experimental designs and supervised the work. Q.E. and G.G.W. have written the manuscript, incorporating comments from all authors.

DECLARATION OF INTERESTS

A patent application has been filed by the authors' employer, the Technical University of Munich on behalf of Q.E., K.V., and F.C.S.. K.V. is currently lead-

ing the Invitris team funded by an EXIST Transfer for Research grant by the Federal Ministry of Economy and Climate Action to pursue work related to this article. Q.E. is a scientific advisor to the team and F.C.S., and G.G.W. formally serve as mentors at TUM.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
No antibodies were used	/	/
Bacterial and virus strains		
<i>Escherichia coli</i>	DSMZ	DSM No. 613
<i>E. coli</i> - DH5 α	Invitrogen	Cat. 18258-012
enteroaggregative <i>E. coli</i>	(Maura et al., 2012)	N/A
<i>B. subtilis</i>	DSMZ	DSM No. 402
<i>B. subtilis</i>	DSMZ	DSM No. 5547
<i>Y. pestis</i> EV76	Bundeswehr Institute of Microbiology, 80937 Munich	N/A
<i>K. pneumoniae</i> strain (Bw1)	Isolated from a patient at Bundeswehrkrankenhaus Berlin, 10115 Berlin, Germany	N/A
T7 phage	DSMZ	DSM No. 4623
MS2 phage	DSMZ	DSM No. 13767
CLB-P3 phage	(Maura et al., 2012) Queen Astrid military hospital, 1120 Brussels, Belgium	OL800706 (GeneBank)
MUC-5 phage	Isolated from a wastewater plant in Munich for this paper	OM687892 (GeneBank)
PhiA1122 phage	(Garcia et al., 2003) Bundeswehr Institute of Microbiology, 80937 Munich	N/A
Phi29 phage	DSMZ	DMS No. 5546
Goe1 phage	DSMZ	DMS No. 101030
Biological samples		
No biological samples were used	/	/
Chemicals, peptides, and recombinant proteins		
ACN	Merck	Cat. 75-07-05
Agarose NEEQ Ultra Quality	Carl Roth	Cat. 2267.4
RTS Amino Acid Sampler	Biotechrabbit	Cat. BR1401801
Ammonium Bicarbonate	Sigma Aldrich	Cat. 9830
ATP	Sigma Aldrich	Cat. A8937
CAA	Merck	Cat. 79-07-2
cAMP	Sigma Aldrich	Cat. A9501
Chloramphenicol	Sigma Aldrich	Cat. C0378
CoA	Sigma Aldrich	Cat. C4248
CTP	Carl Roth	Cat. K057.4
DTT	Sigma Aldrich	Cat. 3483-12-3
EDTA	Sigma Aldrich	Cat. E5134
Ethanol	Carl Roth	Cat. 5054
Formic Acid	Sigma Aldrich	Cat. F0507
Folic Acid	Sigma Aldrich	Cat. F7878
Gam-S	Arbor Bioscience	Cat. 501024
GTP	Carl Roth	Cat. K056.4
HEPES	Sigma Aldrich	Cat. H6147
Imidazole	Sigma Aldrich	Cat. I2399

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Potassium-Glutamate	Sigma Aldrich	Cat. G1149
Magnesium-Glutamate	Sigma Aldrich	Cat. 4965
LB-Agar	Carl Roth	Cat. X.969.2
LB-Media	Carl Roth	Cat. X.968.2
LDS buffer	Merck	Cat. CG3009
Mg-Glutamate	Sigma Aldrich	Cat. 496005
Magnesium Chloride	Sigma Aldrich	Cat. M1028
Magnesium Sulfate	Sigma Aldrich	Cat. 63138
NAD	Sigma Aldrich	Cat. N6522
MOPS-SDS Buffer (20x)	VWR-Life Science	Cat. K855
Nuclease Free Water	Carl Roth	Cat. T143.2
NZCYM Medium	Sigma Aldrich	Cat. N3643
PBS	VWR-Life Science	Cat. E404
PEG-8000	Sigma Aldrich	Cat. 1546605
Phosphoenolpyruvate	Sigma Aldrich	Cat. 860077
PMSF	Sigma Aldrich	Cat. P7626
Roti-Phenol/Chloroform/ Isoamyl alcohol (pH 7.5-8.0)	Carl Roth	Cat. A156.1
Sodium Acetate	Sigma Aldrich	Cat. W302406
Spermidine	Sigma Aldrich	Cat. 85558
TAE-buffer (50x)	Carl Roth	Cat. CL86.3
Tris	Carl Roth	Cat. AE15.1
tRNA	Sigma Aldrich	Cat. R4752
Trypsin	Roche	Cat. 03 708 985 001
UTP	Carl Roth	Cat. K055.3
3-PGA	Sigma Aldrich	Cat. F8877
Urea	Sigma Aldrich	Cat. U5378

Critical commercial assays

Nano-Glo HiBiT Extracellular Detection System	Promega	Cat. N2420
Rapid sequencing kit	Nanopore	Cat. SQK-RAD004
Nanopore flow cell FLO-MIN106	Nanopore	Cat. R.9.4.1
Ultra II DNA library prep for Illumina	NEB	Cat. E7103
MiSeq v3 600 cycle kit	Illumina	Cat. MS-102-3003
Phusion Flash High Fidelity Master mix	Thermo Fisher	Cat. F548S
QIAprep Spin Miniprep Kit	Qiagen	Cat. 27104
DNA Gel Extraction Kit	NEB	Cat. T1020S

Deposited data

Mass spectrometry	This paper, PRIDE	PXD019543
Sequencing data	This paper, Mendeley Data	https://doi.org/10.17632/fszkgvg56j.1 (OM687892, OL800706)

Experimental models: Cell lines

No cell lines were used	/	/
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Experimental models: Organisms/strains

No organisms/strains were used	/	/
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Oligonucleotides

Primers for PCR on the phage phi29, see Table S4: Gcccaatgttcacgtgaaacat (phi29_fwd), gtcttta acgcgaacgtaatcaggtatag (phi29_rwd)	This paper	N/A
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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Primers for PCR on the phage Goe1, see Table S4 : Gtgaattacactgtgacacg (vB_BsuP-Goe1_fwd), g cccaatgttcacgtgaaacat (vB_BsuP-Goe1_rwd)	This paper	N/A
Primers for sequencing of the CLB-P3 genome position 10.500, see Table S4 : P3_10.5_F1: CAGGTCGGCTATAAGGAGGAT TTAACC P3_10.5_R1: GGTGGATTGTGTATCCTGGCTGAAATAC	This paper	N/A
Primers for Sequencing of the P3 genome at 16.500, see Table S4 : P3_16.50_F3: CAAGTATTGGTGC GGCAATGGAG P3_16.50_R3: CTCGCTCCTGCTCATCATCC	This paper	N/A
Primers for sequencing of the P3 genome 27.000, see Table S4 : P3_27_F1: CATAGACAACTGAATACTCGCGTC P3_27_R1: CACCACTGATTACATCAACTCCG	This paper	N/A
Primers for sequencing of the P3 genome position 40.800 bp, see Table S4 . P3_40.8_F2: GAGATCCTTACCGATAAAGGAGGC P3_40.8_R2: GTTTTGCCAGTCGTGCAAG	This paper	N/A
Recombinant DNA		
Backbone of the plasmids used in this paper Psb1c3-T7-sfGFP	(Calles et al., 2019)	Addgene plasmid: #135173
Plasmid for expression of the modified capsid protein with His-tag and luciferase pSB1C3_T7_gp10B_His_Hibit_His	This paper	N/A
Plasmid for expression of the His-tag and luciferase without the capsid protein pSB1C3_T7_Hibit_His	This paper	N/A
Software and algorithms		
MaxQuant version 1.6.3.4	(Cox and Mann, 2008)	https://www.maxquant.org/
iBAQ quantification	(Schwanhäusser et al., 2011)	https://www.maxquant.org/
R-studios R3.6.3 and R3.6.1	rstudio server v1.1.383	https://www.rstudio.com/products/rstudio/download/
Benchling	Benchling	https://benchling.com
guppy [MJ5] (ONT) version Version 2.3.7 + e041753	Oxford Nanopore Technologies	https://nanoporetech.com/nanopore-sequencing-data-analysis
MaSURCA 3.3.2	(Zimin et al., 2017)	https://genome.cshlp.org/content/27/5/787.long
PHANOTATE	(McNair et al., 2019)	https://github.com/deprekate/PHANOTATE
GOTOH algorithm	(Gotoh, 1982)	N/A
pVOG	(Grazziotin et al., 2017)	https://vogdb.org/
Graphpad Prism 9.2.1	This paper	https://www.graphpad.com/
BLAST	(Camacho et al., 2009)	https://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastDocs&DOC_TYPE=Download
pVOG assignment	This paper, see Mendeley data "pvog_analysis"	https://doi.org/10.17632/fszkgvgg56j.1
Other		
Illumina MiSeq Plattform	Illumina	Cat. SY-410-1003

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Minion Sequencer	Oxford Nanopore	MinION Mk1B
E220 super sonicator	Covaris	Cat. 500239
LC-MS/MS the Ultimate 3000 RSLCnano system	Thermo Fisher Scientific	N/A
Orbitrap Fusion Lumos Tribrid Mass Spectrometer	Thermo Fisher Scientific	N/A

RESOURCE AVAILABILITY**Lead contact**

Requests for further information and requests for reagents and resources should be directed to the Lead Contact, Gil Gregor Westmeyer (e-mail: gil.westmeyer@tum.de).

Materials availability

Genetic constructs generated in this study will be made available on request, but we may require a payment and/or a completed Materials Transfer Agreement if there is potential for commercial application. Requests should be directed either to the corresponding author or to Quirin Emslander or Kilian Voegelé.

Data and code availability

For the proteomic datasets (Figures 2 and 3), all LC-MS/MS data files and MaxQuant output files have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository with the dataset identifier PXD019543. All sequencing data (Figure 2, Figure S1) are available via Mendeley Data (<https://doi.org/10.17632/fszvkvgg56j.1>). No specific code was generated in this project. The genomes have been deposited at GeneBank (CLP-P3: OL800706, and Muc-5: OM687892). Any additional information required to reanalyze the data reported in this work is available from the [Lead Contact](#) upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All bacteria and phages were obtained from DSMZ except for the following organism. The Enterotoxigenic *E. coli* (EPEC) was obtained from Jean-Paul Pirnay and Maya Merabishvili (Laboratory for Molecular and Cellular Technology (LabMCT). The PhiA1122 phage was obtained from Shanmuga Sozhamannan (Defense Biological Product Assurance Office, CBRND-EB, JPEO, Frederick, USA), and the CLP-P3 phage from Laurent Debarbieux (Department of Microbiology, Institut Pasteur, Paris, 75015, France). The *K. pneumoniae* strain (Bw1) and corresponding MUC-5 phage were isolated in this manuscript (see [Method details](#)).

MS2 bacteriophages (DSM 13767) were amplified with *E. coli* (DSM 613) in NZCYM medium (Sigma Aldrich) at 37°C.

T7 bacteriophages (DSM 4623) were amplified with *E. coli* (DSM 613) in NZCYM medium (Sigma Aldrich) at 37°C.

CLP-P3 bacteriophages were amplified in enterotoxigenic *E. coli* (Maura et al., 2012) in NZCYM medium (Sigma Aldrich) at 37°C.

The from wastewater isolated MUC-5 bacteriophage was amplified in the *K. pneumoniae* strain (Bw1) with *wzi* capsule type K17 in LB broth (Thermo scientific) at 37°C.

PhiA1122 bacteriophages (Garcia et al., 2003) were amplified in *Y. pestis* EV 76 (CCUG32133) in BHI broth (Merck) at 28°C.

Phi29 bacteriophages (DSM 5546) were amplified in *B. subtilis* (DSM 5547) in NZCYM medium (Sigma Aldrich) at 37°C.

Goe1 bacteriophages (DSM 101030) were amplified in *B. subtilis* (DSM 402) in NZCYM medium (Sigma Aldrich) at 37°C.

E. coli - DH5 α (18258-012) was cultivated at 37°C in LB medium (Carl Roth).

METHOD DETAILS

Phage stock production. To naturally amplify MS2, T7, CLP-P3, Phi29, and Goe1 phages, the corresponding bacteria were each cultivated (230 rpm, 37°C, Innova4 Incubator, Eppendorf, Germany) in 1 L NZCYM medium (Sigma Aldrich, USA) until an OD of 0.6–0.8 (Spectrophotometer Nanogram, Implen, Germany) was reached. *Yersinia pestis* EV 76 (CCUG32133) was cultivated on Brain Heart Infusion (BHI, Merck) agar plates or in 5 ml BHI broth (Merck) using 50 ml centrifuge tubes with shaking (130 rpm, 28°C, Innova4 Incubator, Eppendorf, Germany) until an OD of 0.6–0.8 (Spectrophotometer Nanogram, Implen, Germany) was reached. The culture was infected with 100 μ L of PhiA1122 phage stock (10^6 – 10^{10} pfu/mL) and further incubated up to the lysis point. The lysed culture was centrifuged to eliminate residual bacterial fragments (10 min, 7000 $\times$ g). The supernatant was then mixed with 10 % [w/v] PEG8000 and 1 M [w/v] NaCl (final concentrations). The sample was cooled for 12 h at 4°C, and the phages were pelleted in Falcon tubes (VWR, USA) for 10 min at 7000 $\times$ g at 4°C. The phages were then suspended in 5 mL phage buffer (1x PBS, 1mM MgCl₂, 1 mM MgSO₄) per tube and sterile filtered (0.45 μ m Rotilabo syringe filter, Roth, Germany).

Phenol-chloroform extraction. The phage stock (10^6 – 10^{10} pfu/mL) was mixed at a 1:1 ratio with Roti-Phenol/Chloroform/Isoamyl alcohol (pH 7.5–8.0) in a 5PRIME Phase Lock GelTM tube (Quantabio, USA). The tubes were gently inverted and centrifuged for 5 min

at 16,000 g at room temperature. Afterward, two volumes of pure chloroform (Roth, Germany) were added to the upper phase of the tube, inverted and centrifuged as before. The supernatant was transferred to a separate Eppendorf tube, and 20 μ L of 3M sodium acetate was added. The DNA was precipitated with 1 mL of -80°C pure ethanol (Roth, Germany) for 1 h at -80°C . The DNA was centrifuged (16,000 $\times$ g, 15 min, RT), and the supernatant was discarded. After adding 1 mL of -20°C cold 70% (v/v) ethanol (Roth, Germany) the DNA was pelleted (16,000 $\times$ g, 5 min, 4°C), and the supernatant was discarded again. To evaporate the remaining ethanol, the sample was stored at RT for approximately 15 min. The DNA pellet was dissolved with 30 μ L of nuclease-free water (Thermo Fisher Scientific, USA).

Cell extract preparation

For the generation of crude cell extract, a protocol described as in Falgenhauer et al. (Falgenhauer et al., 2021) was used. In short, the *E. coli* Rosetta 2 (DE3) cells were cultivated in a 2 l lab-scale bioreactor with pO_2 monitoring and pH control. The culture was harvested in the mid-log phase and harvested and washed. The pellet was flash-frozen in liquid nitrogen and stored overnight at -80°C . On the next day, the pellet was thawed on ice and resuspended. A mixture of lysozyme and sonication was used for lysis, followed by a centrifugation step, an incubation at 37°C , and a subsequent centrifugation step. This remaining cell lysate was dialyzed and centrifuged for the last time before flash-frozen in liquid nitrogen and stored at -80°C till further usage.

Phage assembly

Phages were assembled according to Rustad and colleagues (Rustad et al., 2017), with the following changes. Phage DNA was mixed with a TX-TL system based on cell extract, an energy solution, and an amino acid solution as described by Sun and colleagues (Sun et al., 2013) with one amendment in Buffer B (6 mM Mg-glutamate, 100 mM K-glutamate, 3 mM DTT, 1.5 mM each amino acid except leucine, 1.25 mM leucine, 50 mM HEPES, 1.5 mM ATP, and GTP, 0.9 mM CTP and UTP, 0.2 mg/mL tRNA, 0.26 mM CoA, 0.33 mM NAD, 0.75 mM cAMP, 0.068 mM folinic acid, 1 mM spermidine, 30 mM 3-PGA, 4% PEG-8000) (Garamella et al., 2016). For 6 reactions $\times$ 13 μ L, 2.5 μ L PEG 8000 (36% w/v), 4 μ L dNTPs (25 mM), 0.8 μ L ATP (500 mM), 37.5 μ L Buffer B, 2 μ L GamS (150 μ M), 28.5 μ L TX-TL and 1.6 μ L DNA (10 nM) were mixed with nuclease-free water to a final volume of 80 μ L. All constituents were mixed (except the DNA), chilled for 5 min on ice, then the DNA was added. This 13 μ L assembly mix was incubated for 4 h at 29°C to generate the phage.

Plaque assay. For plaque-assays, 0.5% agarose NZCYM medium was melted, split into 4 mL aliquots, and stored in a water bath at 48°C . Separately, the assembled phages were diluted 10^2 - 10^8 in phage buffer (1x PBS, 1mM MgCl_2 , 1mM MgSO_4). 100 μ L of each dilution was mixed with an equal volume of an overnight culture of the corresponding host bacterium. This mixture was added to the 0.5% agarose NZCYM medium aliquots and poured on a 1% NZCYM (1% BHI for *Y. pestis* EV76) agar plate. After the suspension solidified at RT, the plates were incubated at 37°C (28°C for *Y. pestis* EV76) until plaques became visible. The counted plaques (P) were converted to titer (T) via $T = P/D$. (where D is the dilution factor).

Isolation of *K pneumoniae*

Bw1 is a *K pneumoniae* strain, isolated in the Bundeswehr military hospital (BwK) in Berlin. The sample was taken from the colonized patient's skin in accordance with relevant ethical guidelines and regulations. It is a 4MRGN (carbapenem-resistant) pathogen. The wzi capsule type is K17.

Isolation of vB_KpS_Muc5 from wastewater

The phage was isolated from wastewater samples collected in the wastewater treatment plant Gut Großlappen in Munich (Münchner Stadtentwässerung). We isolated vB_KpS_Muc5 from a wastewater sample of the second level of clarification, during which gross pollution is eliminated. A blind enrichment of the water samples was performed. The water samples were centrifuged at 7000 g for 10 min at 4°C and sterile filtered through a 0.2 μ m filter to remove all bacteria. 350 μ L of the host bacterium (OD 0.4-0.6) were incubated at room temperature with 100 μ L of the filtered water sample for 10 min, 5 mL of LB broth were added, and the culture was incubated at 37°C with shaking (120 rpm) for 3 h. Afterward, the culture was centrifuged (7000 g, 10 min, 4°C) and filtrated (0.2 μ m). A plaque assay was performed to test for plaque-forming phage: 100 μ L filtrate and 350 μ L *K pneumoniae* OD 0.4-0.6 were added to 2.5 ml of warm (40°C) LB-Agar (0.6%) with 1 mM CaCl_2 and were poured immediately on a 2% LB Agar plate. The plates were incubated inverted at 37°C for 24 h.

Transient engineering

Phages were assembled as before, except for the addition of 0.2 nM of a plasmid (ordered from Twist Bioscience) on the basis of the expression plasmid psb1c3-t7 (Addgene: 135173, Calles et al., 2019) encoding to fuse the minor capsid protein of the T7 phage Gp10B via a 3xGS Linker to HiBiT and a 6xHis-tag. (MLGVASTVAASPEEASVTSTEETLTPAQEAARTRAANKARKEAELAAATAEQGS GSGSVSGWRLFKKISHHHHHH). The plasmids were amplified in *E. coli* - DH5 α (Invitrogen) and isolated according to the manufacturer's instructions (QIAprep Spin Miniprep kit, 27104, Qiagen).

His-tag purification

The assembled phages were diluted to 10^6 PFU/mL in 1x PBS and 20 mM imidazole directly after the assembly in the cell extract in a total volume of 800 μ L. The phage suspension was then applied onto Ni-NTA Agarose beads (250 μ L of Ni-NTA Agarose from Qiagen ID: 30210), which had been pre-equilibrated with a washing buffer containing 1x PBS and 20 mM imidazole. Subsequently, the

column was washed with 6 column volumes (800 μ L) of 1x PBS and 20 mM imidazole. The phages were eluted with one column volume of 1x PBS and 250 mM imidazole before the titer was measured.

HiBit-assay

The cell-free reaction was carried out as described before with or without adding 0.2 nM of a plasmid encoding either Gp10B-HiBit-His or just HiBit-His without G10B. 200 nM of T7 RNA polymerase was added to conduct transcription. After incubating the cell-free reaction, 20 μ L was added to 1 ml of the corresponding bacteria (*E. coli* DSMZ 613) with an OD adjusted to 0.8 and incubated for 5 min at room temperature. The bacteria were pelleted down by centrifugation at 7,000 $\times$ g at room temperature, and the supernatant was discarded. The cells were resuspended in 1x PBS. The HiBiT was detected by measuring the luminescence after mixing with the Nano-Glo® HiBiT Extracellular Detection System (Promega N2420). The progeny of the phages were isolated from a plaque assay and adjusted to the same titer as reached in the cell-free system to confirm that the modification was only transient. The same experiment was repeated and luminescence was measured again with the Nano-Glo® HiBiT Extracellular Detection System.

Polymerase chain reaction (PCR)

All PCRs were performed with the Phusion Flash High Fidelity Mastermix kit (Thermo Fisher Scientific, F548S). Primers (10 μ M) and 50 ng of DNA were diluted in nuclease-free water and the PCR master mix according to the manufacturer's instructions. Primers (Table S4) were designed with Benchling (2019) using the genomes from Table S2. For Goe1 and Phi29 primers were designed to generate a 1 kb fragment. To finalize the de-novo sequenced CLB-P3 genome primers were designed to amplify the genome position 10.5 kb (10523–11287 bp), 16.5 kb (15852–16625 bp), 27 kb (26860–27736 bp) and 40.8 kb (40644–41504 bp). The oligonucleotides were ordered from Eurofins Scientific (GER).

Nanopore sequencing

Phage DNA was prepared with the SQK-RAD004 kit (Oxford Nanopore Technologies, UK) on an FLO-MIN106 flow cell (Oxford Nanopore, UK) according to the manufacturer's protocol.

Illumina Sequencing

The phage genome library (Ultra II DNA library prep for Illumina, NEB) and sequencing (MiSeq v3 600 cycle kit, Illumina) were performed with the Illumina MiSeq platform according to the manufacturer's protocol. The DNA was sheared with an E220 supersonicator (Covaris).

Genome assembly and annotation of phage genomes

The reads derived from Oxford Nanopore MinION sequencing were basecalled using guppy (ONT) version Version 2.3.7 + e041753. For the short reads, the Illumina Miseq derived FASTQ-files (Miseq reporter version 2.6.2.3) were used. Reads mapping to *E. coli* K-12 MG1655 (U00096.3) were filtered from both MinION and Miseq reads. Following this, a hybrid assembly from both MinION and Illumina reads was performed using MaSURCA 3.3.2 in auto mode for the assembly graph k-mer size. After assembly, quality control of the assembled sequence was performed by re-aligning the sequence itself (using BLAST) while aligning the Illumina or MinION reads onto the sequence for a coverage plot. The BLAST result was visualized in a dot plot, from which it was verified that the assembled sequence was a duplication of the actual genome. Likewise, the aligned Illumina reads showed a secondary alignment position in the first/second copy of the actual genome. After deduplicating the assembled sequence, the same alignment check was performed to confirm the correct deduplication. These positions were also verified by (Table S4), and the generated PCR products were confirmed by Sanger Sequencing. Open-reading frames (ORFs) were identified using PHANOTATE (McNair et al., 2019). Found ORFs were then compared to representative members for each pVOG class to determine the most similar representative, which allows assigning a function to a specific ORF (Grazziotin et al., 2017). Particularly those ORFs which could be associated with a pVOG are likely to be conserved since similar sequences had already been found in other bacteriophages. To compare the found ORFs and the pVOG sequences, optimal pairwise global alignments, using the PAM250 substitution matrix and affine gap costs (gap open/extend costs –12 and –1), were computed (Gotoh, 1982). If for any alignment, sequence identity of the ORF with more than 40% was detected, the alignment was accepted, and the ORF was assigned the respective pVOG. The remaining ORFs (15) were blasted against all bacteriophage protein sequences downloaded from Genbank (file gbphg1.seq.gz). Again, an alignment was accepted if more than 70% of the ORF length aligned and 50% of the pVOG sequence aligned, respectively. Overall, for 72 of the 87 ORF a pVOG assignment was found. 8 ORFs were assigned a known bacteriophage protein and 7 ORFs remained unassigned.

Sample preparation for mass spectrometry

For sample preparation, 3 μ L cell-free phage- or 5 μ L native sample (stock concentration >106 pfu/mL) was diluted with NuPAGE LDS sample buffer (ThermoFisher). The samples were reduced with 50 mM dithiothreitol (DTT) at 70°C for 10 min and alkylated with 55 mM chloroacetamide (CAA) at room temperature for 30 min in the dark. The samples were run on a NuPAGE 4–12% Bis-Tris protein gel (ThermoFisher) for 5 min (200V, 500 mA, 1x MOPS buffer), 30 min fixated (40% MeOH, 2% AA), colored (1x RotiBlue, Carl Roth, Germany) and destained (25% EtOH, 1% AA). All samples were prepared via in-gel digest (Shevchenko et al., 2006). The collected

supernatant (130 μ L) was dried in a centrifugal evaporator (Centrivap Cold Trap -50, Labconco, USA). The sample was freshly suspended before MS measurement in 20 μ L washing solution (2% ACN, 0.1 FA), and 5 μ L was injected into the mass spectrometer per measurement.

Proteomic data acquisition

For LC-MS/MS the Ultimate 3000 RSLCnano system was coupled to an Orbitrap Fusion Lumos Tribrid Mass Spectrometer (Thermo Fisher Scientific Bremen, Germany). For each analysis, a final injection amount of around 0.1 μ g of peptides was delivered to a trap column (ReproSil-pur C18-AQ, 5 μ m, Dr. Maisch, 20 mm $\times$ 75 μ m, self-packed) at a flow rate of 5 μ L/min in 100% solvent A (0.1% formic acid in HPLC grade water). After 10 min of loading, peptides were transferred to an analytical column (ReproSil Gold C18-AQ, 3 μ m, Dr. Maisch, 400 mm $\times$ 75 μ m, self-packed) and separated using a 50 min gradient from 4% to 32% of solvent B (0.1% formic acid in acetonitrile and 5% (v/v) DMSO) at 300 nL/min flow rate. Both nanoLC solvents contained 5% (v/v) DMSO. The Fusion Lumos Tribrid Mass Spectrometer mass spectrometer was operated in the data-dependent acquisition and positive ionization mode. MS spectra (360–1300 m/z) were recorded at a resolution of 60,000 using an automatic gain control (AGC) target value of 4e5 and maximum injection time (maxIT) of 50 ms. After peptide fragmentation using higher-energy collision-induced dissociation (HCD), MS² spectra of up to 20 precursor peptides were acquired at a resolution of 15,000 with automatic gain control (AGC) target value of 5e4 and maximum injection time (maxIT) of 22 ms. The precursor isolation window width was set to 1.3 m/z and normalized collision energy to 30%. Dynamic exclusion was enabled with 20 s exclusion time (mass tolerance $\pm$ 10 ppm). MS/MS spectra were singly-charged, unassigned, or with charge states $>6+$ were excluded.

Raw data processing and proteomic statistical analysis

Peptide identification and quantification were performed using MaxQuant version 1.6.3.4 (Cox and Mann, 2008), and the reference proteomes *E. coli* BL21(DE3) (NCBI: 469008), phage CLB-P3 (de-novo assembled: P3.prot.fa) and phage T7 (Uniprot: UP000000840). A contaminant list (a built-in option in MaxQuant) was also added. For all MaxQuant searches, typical default parameters were employed. Those included carbamidomethylation of cysteine as fixed modification and oxidation of methionine and N-terminal protein acetylation as variable modifications. Trypsin/P was specified as the proteolytic enzyme. The precursor tolerance was set to 4.5 ppm, and fragment ion tolerance to 20 ppm. Results were adjusted to a 1% false discovery rate (FDR) on the peptide spectrum match (PSM) level employing a target-decoy approach using reversed protein sequences. The minimal peptide length was defined as 7 amino acids. Intensity-based absolute quantification (iBAQ) (Schwanhäusser et al., 2011) was enabled. The iBAQ intensities represent an estimate of the absolute concentration (rank) of a given protein in a sample and were the applied quantification unit throughout this study.

Heatmaps

To examine how the phage proteins are differentially expressed in the cell-free system and phage stock, triplicates of each sample were measured using tandem-MS. All phage proteins were selected and further analyzed. The phage protein (RPOL_BPT7) cannot be distinguished from *E. coli* protein (A0A140NCE7_ECOBD) and was included in the analysis as well. The logarithm transformed (base 10) iBAQ intensity was used to represent the protein abundance, missing values were replaced with the lowest measured iBAQ intensity. The Euclidean distance together with the Ward linkage method (no scaling) was used in the heatmap (Figure 3B).

Time-resolved heatmap and single protein analysis

Three replicates were measured over 12 time points. The logarithm transformed (base 10) iBAQ intensity was used to represent the protein abundance. Missing values were replaced with the constant value of 5.5, which is slightly lower than the lowest measured values. To remove the potential batch effect between the three replicates, the iBAQ intensities were z-score transformed within each replicate. The z-scored iBAQ values of all three replicates were then visually checked using beeswarm plots (Figure 4D, R package Beeswarms version 0.2.3). To cluster phage proteins according to their time-points of expression (Figure 4A), the z-scored iBAQ values of the three replicates were combined using their mean values, which were further scaled to the range of 0 to 1. Manhattan distance and Ward's method were used to cluster the final transformed intensities (Figure time_resolved.png). Three phage proteins (P03751|GP73_BPT7, P03778|Y06_BPT7, P03797|Y38_BPT7) were removed in this analysis because they did not show a clear trend over the time course. All analyses were performed in R (version 3.6.1).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

If not indicated otherwise data were acquired as at least 3 independent replicates and analyzed using either Graphpad Prism (V. 9.2.1 or 7) or R-studios. Values are expressed as mean $\pm$ standard deviation from the mean or SEM of the replicates, as indicated in the figure legends.

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Supplemental information

Cell-free production of personalized therapeutic phages targeting multidrug-resistant bacteria

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Supplemental Information

Personalized production, non-genomic engineering, and time-resolved proteomics of therapeutic phages for biohazardous and multidrug-resistant bacteria

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Table S1: List of validated and conserved hypothetical proteins; related to Figure 2

Name	Uniprot ID	Status
Protein 5.3	P03798	hypothetical
Protein 19.5	P03804	hypothetical
Protein 2.8	P03795	hypothetical
Protein 6.5	P03800	hypothetical
Protein 3.8	P03797	hypothetical
Protein 4.7	P03876	hypothetical
Protein 4.3	P03784	hypothetical
Protein 7.7	P03796	hypothetical
Protein 4.2	P03783	hypothetical
Protein 1.6	P03793	hypothetical
Protein 7	P03750	hypothetical
Protein 1.5	P03792	hypothetical

Table S2: List of cell-free produced phages; related to Figure 4

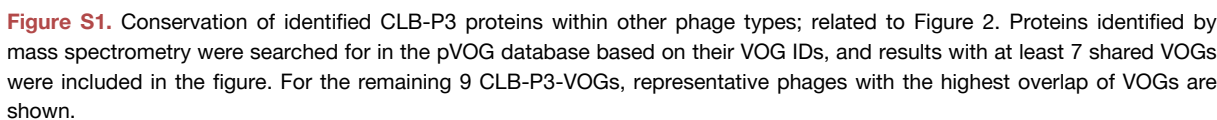
Phage	T7	MS2	CLB-P3	PhiA1122	MUC-5	Phi29	Goe1
NCBI Reference Sequence	NC_001604.1	NC_001417.2	Sequenced in this paper OL800706	NC_004777.1	isolated in this paper OM687892	NC_011048.1	NC_049975.1
Genome Type	linear dsDNA	linear ssRNA	linear dsDNA	linear dsDNA	linear dsDNA	linear dsDNA	linear dsDNA
genome size	39937 bp	3569 bp	49689 bp	37555 bp	48169 bp	19282 bp	18379 bp
viral type	lytic	lytic	lytic	lytic	lytic	lytic	lytic
taxonomic placement	Order: Caudovirales	Order: Levivirales	Order: Caudovirales	Order: Caudovirales	Order: Caudovirales	Order: Caudovirales	Order: Caudovirales
	Family: Autographiviridae	Family: Leviviridae	Family: Siphoviridae	Family: Autographiviridae	Family: Drexlerviridae	Family: Salasmaviridae	Family: Salasmaviridae
	Genus: Teseptimavirus	Genus: Levivirus		Genus: Teseptimavirus	Genus: Webervirus	Genus: Salasvirus	Genus: Beecentumtrevirus
Host bacterium	<i>E. coli</i> (ATCC 11303)	<i>E. coli</i> (ATCC 12435)	<i>E. coli</i> EAEC strain (55989Str)	<i>Y. pestis</i> EV76	<i>K. pneumoniae</i> strain (Bw1) with wzi capsule type K17	<i>B. subtilis</i> subsp. <i>subtilis</i> (DSM 5547)	<i>B. subtilis</i> (DSM 402)

Table S3: Comparison of the DNA-directed RNA polymerase subunits of *B. subtilis* and *E. coli*; related to Figure 4

Subunits of DNA-directed RNA polymerase <i>B. subtilis</i>	Subunits of DNA-directed RNA polymerase <i>E. coli</i>	Percentage Identity (Blast)
RpoA (sigA): alpha subunit (WP_174223679.1)	DNA-directed RNA polymerase subunit alpha (MXF67048.1)	96.50 %
RpoB: beta subunit (WP_128737407.1)	DNA-directed RNA polymerase subunit beta (EHP3501041.1)	89.91 %
RpoC: beta' subunit (WP_120027712.1)	DNA-directed RNA polymerase subunit beta' (HBB6316284.1)	77.50 %
YloH: omega subunit (WP_087992101.1)	DNA-directed RNA polymerase subunit omega (MXF68189.1)	85.07 %

Table S4: List of Primers; related to STAR methods.

Primer name	Nucleotide sequence (5'-3')
phi29_fwd:	AATCCAAGATGACTCACTCAAACTGC
phi29_rwd:	GTCTTTAACGGCAACGTAATCAGGTATAG
vB_BsuP-Goe1_fwd	GTGGAATTACACTGTGGCACG
vB_BsuP-Goe1_rwd	GCCCAATGTTCCACGTGAAACAT
P3_10.5_F1	CAGGTCGGCTATAAGGAGGATTAAACC
P3_10.5_R1	GGTGGATTGTGTATCCTGGCTGAAATAC
P3_16.50_F3	CAAGTATTGGTGCGGCAATGGAG
P3_16.50_R3	CTCGCTCCTGCTCATCATCC
P3_27_R1	CATAGACAACTGAATACTCGCGTC
P3_27_F2	CACCACTGATTACATCAACTCCG
P3_40.8_F2	GAGATCCTTACCGATAAAGGAGGC
P3_40.8_R2	GTTTTGCCAGTCGTGCAAG



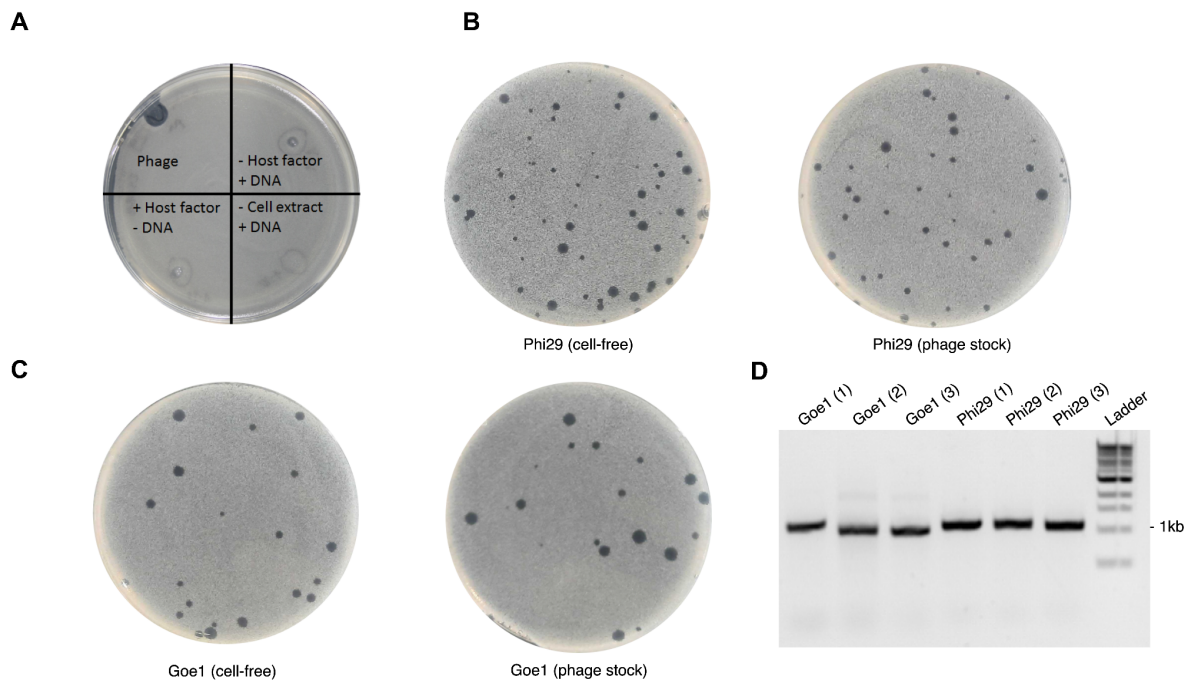


Figure S2. Quality control of the cell-free phages; related to Figure 4. (A) Spot assay of cell-free produced phage phi29 and controls. A functioning phi29 phage can only be produced by combining host factor (SigA), cell extract, and phage DNA. (B) Plaque assay of cell-free and bacterial amplified phi29 phage. Comparison of plaque size and shape between plaques generated by bacterial amplified (phage stock) and a cell-free produced phi29 phages. (C) Plaque assay of cell-free and bacterial amplified Goe1 phage. Comparison of plaque size and shape between plaques generated by bacterial amplified (phage stock) and a cell-free produced Goe1 phages. (D) Verification cell-free produced *B. subtilis* phages by PCR. Three independent plaques descended from cell-free produced phages were picked, and phages were replicated with *B. subtilis*. The resulting phages were analyzed by PCR with specific primers for Goe1 and phi29 phages. The 1.5% agarose gel shows the detection of the expected DNA fragments of 1kb (Goe1, (phi 29), respectively).

Appendix End