Direct conversion of somatic cells utilizing CRISPR/Cas9

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1 Abstract

Midbrain dopaminergic (DA) neurons, which are implicated in the control of voluntary movement, degenerate during Parkinson’s disease. Currently, there is no cure with treatments mostly focusing on alleviation of motor symptoms. Cell replacement therapy utilizing directly reprogrammed cells is therefore regarded as a promising alternative. Somatic cells such as fibroblasts can be directly converted to DA neurons e.g. by overexpression of the transcription factors Ascl1, Lmx1a and Nurr1 (A+L+N). However, the obtained neurons were found to be negative for the midbrain DA neuron specific marker Pitx3 and therefore do not resemble the DA subtype lost in Parkinson's disease patients. The aim of this thesis was to test new strategies in order to improve the reprogramming efficiency in vitro and to identify factors enabling the generation of Pitx3 expressing DA neurons.

PITX3+ DA neurons were successfully obtained by treating A+L+N transduced fibroblasts with forskolin, an activator of cAMP signaling, suggesting the promising generation of ‘true’ midbrain DA neurons. In these experiments, co-transduction of a single cell by three individual lentiviruses encoding Ascl1, Lmx1a and Nurr1 seemed to be a limiting factor. Therefore, a tri-cistronic vector carrying all three transcription factors was tested. However, a substantial proportion of Ascl1, Lmx1a and Nurr1 was expressed as fusion proteins resulting in a significant decrease of reprogrammed cells rendering the tri-cistronic approach unsuitable for increasing the yield of TH+ neurons.

In order to overcome these limitations, the potential of the CRISPR/Cas9 technology regarding the induction of endogenous genes and direct reprogramming of somatic cells was investigated. Nuclease deficient dCas9 proteins together with sequence specific gRNAs were used as shuttles to deliver transcriptional activators as fusion proteins to the promoter region of target genes thus inducing their expression. For the proof-of-principle experiments in this thesis, Ascl1 was chosen as target gene since overexpression of this transcription factor was shown to be sufficient to directly convert astrocytes or fibroblasts into neurons. Screenings for suitable gRNA combinations and dCas9 fusion proteins were performed and a novel synergistic effect of two transcriptional activator systems – VPR and SAM – was found to strongly activate murine Ascl1 expression. Furthermore, for the first time murine cortical astrocytes were directly converted into neurons by CRISPR/Cas9 mediated induction of Ascl1. These are promising findings highlighting the potential of the CRISPR/Cas9 technology for direct reprogramming and cell replacement therapies.
Zusammenfassung


muriner kortikaler Astrozyten in Neurone verwendet. Diese vielversprechenden Ergebnisse zeigen das Potential der CRISPR/Cas9 Technologie für die direkte Reprogrammierung und die Anwendung für Zellersatztherapien.
INTRODUCTION
3 Introduction

3.1 Midbrain dopaminergic neurons and cell replacement strategies

3.1.1 Midbrain dopaminergic neurons and Parkinson’s disease

The neurotransmitter dopamine is synthesized and released by dopaminergic (DA) neurons in different parts of the mammalian brain. The rate limiting enzyme and DA neuron marker Tyrosine hydroxylase (TH) converts L-Tyrosine into L-Dihydroxyphenylalanine (L-DOPA) followed by decarboxylation to dopamine which is catalyzed by Aromatic L-amino acid decarboxylase [1]. Subsequently, dopamine is packaged into synaptic vesicles and released into the synaptic cleft upon neuronal excitation. The Dopamine transporter (DAT) enables re-import into the presynaptic neuron thus stopping dopamine signaling followed by recycling of the neurotransmitter [1].

The largest and most intensively studied group of DA neurons are the meso-diencephalic dopaminergic (mdDA) neurons involved in the control of voluntary movement, working memory and reward [2]. mdDA neurons can be divided in three clusters known as retrorubral field (A8), substantia nigra pars compacta (SNc, A9) and the ventral tegmental area (VTA, A10) [3]. mdDA neurons of the VTA and the retrorubral field are mainly implicated in cognition and reward and project to the ventral striatum and limbic structures [4]. mdDA neurons of the SNc are predominantly involved in the control of voluntary movement via their axonal projections to the dorsal striatum [4]. Parkinson’s disease (PD) is characterized by the progressive degeneration and loss of mdDA neurons in the SNc [5]. This leads to a reduction of dopamine in the striatum resulting in motor symptoms including bradykinesia, rigidity and resting tremor [6-8]. However, also non-motor symptoms such as cognitive impairment and mood disorders are observed [9]. PD is a neurodegenerative disorder affecting about 1% of people at the age of 60 and increasing with age [7]. PD is regarded as a sporadic disease with unclear etiology and ageing as a major risk [10]. However, exposure to environmental toxins such as rotenone, paraquat or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), has also been linked to PD and approximately 5 - 10% of PD cases have a genetic component [11-13].

The DA neuron subpopulation susceptible to neurodegenerative stress is characterized by the expression of Paired-like homeodomain transcription factor 3 (Pitx3) [14]. Pitx3 is not only a marker gene for mdDA neurons but has also been shown to be critical for differentiation, long term survival and stress resistance by induction of the survival factor Brain derived neurotrophic factor (Bdnf) [14, 15]. Interestingly, Pitx3 deficient aphakia mice show a specific loss of dopaminergic neurons in the SNc and to a much lesser extend in the VTA [14, 16], thus resembling the cell loss in PD.
To date there is no cure for PD. Treatments focus on motor symptoms by replenishing and stabilizing dopamine levels in the striatum utilizing L-DOPA or by deep brain stimulation [17]. However, the effects are often only short term effective and neuronal degradation cannot be stopped or reverted [17]. New approaches such as direct reprogramming and cell replacement therapies are therefore a promising alternative.

### 3.1.2 Different routes for cell replacement strategies in PD

PD is one of the prime candidates for direct reprogramming and cell replacement therapies due to the localized degeneration of a specific cell type [18]. Since the 1980s efforts were made to replace degenerated mdDA neurons in PD patients including allografts of human fetal ventral mesencephalon tissues [19-21]. However, severe side-effects including graft-induced dyskinesias and adaptive immune responses against allografts were observed [22, 23]. A completely new field for cell replacement strategies was born when Yamanaka and colleagues reported the reprogramming of fibroblasts into induced pluripotent stem cells (iPSC) in 2006 [24].

![Figure 1: Different routes for the conversion of fibroblasts to neurons](image)

**Figure 1: Different routes for the conversion of fibroblasts to neurons**

Schematic illustration of different routes to convert one somatic cell type into another one. Reprogramming: Induced pluripotent stem cells (iPS cells) are generated by overexpression of Yamanaka factors (Oct4, Sox2, c-Myc and Klf4). Targeted differentiation by overexpression of lineage specific transcription factors (TFs) leads to the generation of neuronal stem cells (NSCs) and neuronal precursor cells (NPCs) which can be further differentiated to neuronal subtypes by addition of specific morphogens. Pluripotency mediated (PM) transdifferentiation: Overexpressing of Yamanaka factors for a limited time to generate neuronal precursor cells (NPCs) which can then be differentiated to a specific neuronal subtype by supplementing specific morphogens. Transdifferentiation or direct conversion / direct reprogramming: Overexpression of lineage specific transcription factors (TFs) is utilized to directly convert one somatic cell type into another one without passing through a pluripotent cell stage. **Abbreviations**: iPS cell: induced pluripotent stem cell, NPC: neuronal precursor cell, NSC: neuronal stem cell, PM: pluripotency mediated, TF: transcription factor.
This was achieved by overexpression of POU domain, class 5, transcription factor 1 (Pou5f1 or Oct4), Sex determining region Y-box 2 (Sox2), Myelocytomatosis oncogene (c-Myc) and Kruppel-like factor 4 (Klf4) in mouse and human fibroblasts reprogramming them into embryonic stem cell-like cells [24, 25]. iPSCs are undifferentiated cells which undergo asymmetric cell division generating two daughter cells with different properties. While one cell stays pluripotent allowing self-renewal the second daughter cells can differentiate into all types of cells in the body [24, 25]. One of the main advantages of iPSCs compared to embryonic stem cells is that pluripotent cells can be generated from easily accessible somatic cells of the patient such as skin fibroblasts. This overcomes immunologic reactions after transplantation and does not require destruction of embryos as it is the case for the isolation of embryonic stem cells. Targeted differentiation of iPSCs to a cell type of interest such as mdDA neurons therefore has great potential for cell replacement therapies enabling personalized regenerative medicine (see Figure 1).

A commonly used model for PD are 6-hydroxy dopamine (6-OHDA) treated animals were DA neurons are selectively degenerated by the neurotoxin 6-OHDA [26]. Dopaminergic neurons derived from iPSCs have been transplanted into the striatum of 6-OHDA treated rats and were found to integrate into the host tissue leading to behavioral improvement [27]. However, teratomas which are tumors containing cell types of more than one germ layer were observed indicating the presence of undifferentiated iPSCs [27]. This tumorigenic potential which has been observed in other animal models as well is a risk of stem cell based transplantation therapies in clinical applications [28, 29].

Recently, a new approach for cell reprogramming termed transdifferentiation has emerged which can be accomplished by two ways: Via generation of expandable neuronal precursor cells [30, 31] or by direct conversion to postmitotic DA neurons [32-37] (see Figure 1). Neuronal precursor cells can be obtained by temporal expression of the Yamanaka factors described earlier [30]. These precursor cells are then differentiated into DA neurons in a second step by addition of morphogens involved in the in vivo development of DA neurons [30]. This method is termed pluripotency mediated (PM) transdifferentiation. While showing comparable functionality to cells derived from iPSCs and their in vivo counterparts, neurons derived from transdifferentiation do not have the risk or tumorigenicity as they do not pass a pluripotent state [38, 39].

Finally, by overexpression of lineage-specific transcription factors (TFs) somatic cells such as fibroblasts can be directly transdifferentiated into another somatic cell type e.g. DA neurons without passing a proliferative state [32-37]. This is also referred to as direct conversion or direct reprogramming and opens a third route for patient-specific cell replacement therapies as indicated by the red arrow in Figure 1. This route of direct reprogramming was utilized in
the underlying thesis. Transplanted DA neurons obtained by direct conversion of fibroblasts were shown to alleviate symptoms in 6-OHDA treated mice underlining the potential of direct lineage reprogramming [32].

3.1.2.1 Transcription factor combinations utilized for the direct conversion of fibroblasts to DA neurons

Several groups are working on the direct conversion of somatic cells into DA neurons but there is still a need to increase the reprogramming efficiency and to steer the DA neuron subtype towards mdDA neurons. Table 1 gives an overview of published combinations of transcription factors for the direct conversion of fibroblasts to DA neurons. In these cases, three to six different TFs associated with mdDA neuron development were co-expressed in fibroblasts after retro- or lentiviral gene delivery. Reprogramming efficiencies ranged from 0.05% to 18% of TH+ DA neurons but a quantitative comparison is difficult due to differences in protocols, cell types used and species of origin.

<table>
<thead>
<tr>
<th>Table 1: Published combinations of transcription factors used for direct conversion of murine and human fibroblasts to DA neurons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell type used</td>
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<tr>
<td>----------------</td>
</tr>
<tr>
<td>Ascl1</td>
</tr>
<tr>
<td>Brn2</td>
</tr>
<tr>
<td>En1</td>
</tr>
<tr>
<td>Foxa2</td>
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<tr>
<td>Lmx1a</td>
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<td>Lmx1b</td>
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<tr>
<td>Myt1l</td>
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<tr>
<td>Ngn2</td>
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<td>Nurr1</td>
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<tr>
<td>Otx2</td>
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<tr>
<td>Pitx3</td>
</tr>
<tr>
<td>Sox2</td>
</tr>
<tr>
<td>TH+ cells/DAPI</td>
</tr>
<tr>
<td>PITX3+ cells</td>
</tr>
</tbody>
</table>
While TH⁺ DA neurons are found in several areas of the mammalian brain only midbrain (TH⁺/PITX3⁺) DA neurons are degenerating in PD [6-8]. It is therefore of interest to generate ‘true’ mdDA (TH⁺/PITX3⁺) neurons by direct reprogramming in order to replace the DA neuron subpopulation lost during PD. Of the different transcription factor combinations shown in Table 1 however, only Kim et al., and Sheng et al., report the generation of TH⁺/PITX3⁺ cells using five to six different transcription factors [32, 36]. This suggests that DA neurons generated by the remaining protocols may not be of midbrain identity. It is therefore of interest to both improve the reprogramming efficiency and to identify additional factors supporting the generation ‘true’ mdDA (PITX3⁺) neurons.

3.1.2.2 Roles of Ascl1, Lmx1a, Nurr1 in mdDA development and direct conversion of fibroblasts to DA neurons

While the combinations of transcription factors used for direct conversion of fibroblasts to DA neurons shown in Table 1 differ considerably, one factor is always included: Achaete-scute family bHLH transcription factor 1 (Ascl1, also known as Mash1). Ascl1 is a proneural gene of the basic helix-loop-helix family of transcription factors involved in the development of gamma-aminobutyric acid secreting neurons (GABAergic neurons) in the brain suppressing the alternative glia fate [40, 41]. Ascl1 is also expressed in mdDA progenitor cells during embryonic development and seems to be involved in activating mdDA neuronal maturation [42, 43]. Deletion of Ascl1 in mice leads to severe defects in neurogenesis and death at birth [44]. Ascl1 is a central factor for neuronal reprogramming purposes as it can bind closed chromatin which makes Ascl1 a so-called pioneer transcription factor [45]. Such a pioneer transcription factor is able to bind nucleosomal DNA by its own while regular transcription factors require cooperation with other factors [46]. This property is critical to overcome the epigenetic barriers e.g. of a fibroblast and convert it into a neuron. Exogenous Ascl1 expression in fibroblasts [45, 47] or astrocytes [48, 49] is sufficient to directly convert these cells to neurons. Interestingly, while astrocytes are converted into inhibitory GABAergic neurons resembling the in vivo role of Ascl1 [48, 49], fibroblasts become excitatory glutamatergic neurons upon overexpression of Ascl1 [47]. These data suggest that Ascl1 is sufficient for the induction of a neuronal fate but further factors are required to determine the neuronal subtype.

In order to generate DA neurons, up to five additional subtype specific transcription factors are commonly co-expressed [32-37]. Caiazzo et al., reported a minimal set of three transcription factors comprising Ascl1, LIM homeobox transcription factor 1 alpha (Lmx1α) and Nuclear receptor subfamily 4, group A, member 2 (Nr4a2 or Nurr1) [37]. Overexpression of Ascl1, Lmx1α and Nurr1 is not sufficient to directly convert fibroblasts into PITX3⁺ DA neurons (unpublished data, F. Meier, Helmholtz Zentrum München). This minimal
combination however, was used as a starting point for further screenings in order to improve reprogramming efficiencies and to identify new factors enabling TH⁺/PITX3⁺ mdDA neuron generation with less than the published five to six different TFs.

*Lmx1a* is a member of the LIM homeodomain transcription factors and is involved in the development of mdDA neurons mainly by regulating Msh homeobox 1 (*Msx1*) [50]. *Msx1* in turn induces neurogenesis by activation of the proneural gene Neurogenin 2 and inhibition of alternative cell fates via suppression of NK6 homeobox 1 [51]. Furthermore, *Lmx1a* was found to directly induce the expression of the mdDA neuron marker *Pitx3* in *vitro* [52]. *Lmx1a* is expressed from the dopaminergic progenitor cell stage (embryonic day nine (E9) in mice) onwards and stays active in mature mdDA neurons [50]. A loss of *Lmx1a* leads to a significant reduction but not complete loss of DA neurons in mouse models [53].

The orphan nuclear receptor/transcription factor *Nurr1* is expressed in postmitotic mdDA precursors of mouse embryos from E10.5 onwards but is also detected in non-dopaminergic areas such as hippocampus and cerebral cortex [54-56]. *Nurr1* is implicated in mdDA neuron specification, migration and target innervation [57]. Target genes include the survival factor *Bdnf* as well as DA neuron markers *Th, Dat* and the mdDA marker gene *Pitx3* [58-62]. While *Nurr1*-depleted mouse embryos develop PITX3⁺ DA precursors, maturation of these cells is arrested and they undergo apoptosis at a neonatal stage [61]. *Nurr1*⁻/⁻ mice completely lack *Th* expression and die shortly after birth [63]. This underlines *Nurr1* as an important component of post-mitotic DA neuron specification and maturation.
3.2 The CRISPR/Cas9 system and its implications for genome editing and transcriptional regulation

3.2.1 CRISPR/Cas9 - an adaptive immune system in bacteria and archaea

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR associated proteins (Cas) serve as adaptive immune system in approximately 40% of bacteria and 90% of archaea [64]. The CRISPR system acts via sequence specific recognition and subsequent cutting of foreign DNA by a protein-RNA complex [65]. Three types of CRISPR systems (I-III) have been described so far [64]. The type II CRISPR system is well characterized [66, 67] and was therefore used in the underlying project. Figure 2A shows the genomic organization of the type II CRISPR system comprising a Cas locus encoding the nuclease Cas9, a CRISPR array consisting of repeat and spacer sequences and a transactivating CRISPR RNA (tracrRNA) [68]. Upon infection of bacteria or archaea e.g. by bacteriophages short fragments of invader-derived DNA can be integrated as spacer sequences into the CRISPR-arrays of the host chromosome [65].

These new spacers subsequently serve as sequence-specific resistance to foreign DNA. Transcription of the CRISPR array results in a precursor transcript termed pre-CRISPR-RNA (pre-crRNA) consisting of repeats and newly integrated spacer sequences (see Figure 2A). The repeat region of pre-crRNAs is bound by co-expressed tracrRNA molecules via a 25-nucleotide sequence leading to the recruitment of RNase III and cleavage within the double-stranded repeat region (orange arrowheads in Figure 2A) [69]. Afterwards, the crRNA spacer is trimmed from the 5’ end by a yet unknown nuclease to a length of 20 nt [68] (yellow arrowhead in Figure 2A) resulting in a mature crRNA-trcrRNA hybrid.

This crRNA-trcrRNA hybrid is bound by a Cas9 nuclease and the resulting protein-RNA complex scans invader DNA for protospacer sequences (target sequences) complementary to the 20 nucleotides of the crRNA as shown in Figure 2B [66, 68, 70]. Upon binding Cas9 induces a double strand break in the foreign DNA (white arrowheads in Figure 2B). It is of importance for the CRISPR immune system to discriminate between ‘self’ (integrated spacer sequences) and ‘non-self’ (foreign DNA molecules) in order to only bind and cut invading DNA molecules. In this context protospacer adjacent motifs (PAMs) play an important role [71]. PAMs consist of three nucleotides immediately downstream of the 20 nt target sequence in the foreign DNA (see Figure 2B) marking a difference to the sequence of CRISPR repeats in the host genome [71, 72]. Cas9 proteins from different species require distinct PAM sequences. The type II-A Cas9 of Streptococcus pyogenes used in this project detects a 5’-NGG-3’ PAM sequence [73].
Figure 2: Illustration of crRNA maturation, Cas9 functionality and Cas9 protein domains

(A) Schematic illustration of crRNA maturation of type II CRISPR systems in bacteria and archaea serving as adaptive immune system. Cas genes are encoded in the Cas locus, the CRISPR locus contains repeat and spacer sequences. Short sequences of foreign DNA can be integrated as spacer sequences and serve as template for the recognition of invading DNA. A pre-crRNA is generated upon transcription of the CRISPR locus containing the newly integrated spacer sequences. tracrRNAs bind to repeat sequences of the pre-crRNA which is recognized and cleaved by RNase III (orange arrowheads). A yet unknown nuclease trims the spacer region to a length of 20 nucleotides as indicated by the yellow arrowhead. This results in a mature crRNA-tracrRNA hybrid. (B) Illustration of DNA cleavage by Cas9. crRNA-tracrRNA hybrids are bound by Cas9 nucleases and the protein-RNA complex recognizes and binds foreign DNA via the 20 nucleotides of the crRNA (red) leading to cleavage of the foreign DNA as indicated by white arrowheads. PAM sequences (green) are used to discriminate ‘self’ and ‘non-self’ DNA as they are only found in foreign DNA but not in the integrated spacer sequences of the CRISPR locus. (C) Illustration of S. pyogenes type II-A Cas9 protein domains. The nuclease activity is mediated by two domains: The discontinuous RuvC nuclease domain and the HNH nuclease domain. While the HNH nuclease domain cleaves the strand bound by the crRNA, RuvC cuts the non-complementary strand. The two nuclease domain lobes are separated by an α-helical lobe. Further domains are Arg (arginine rich domain, involved in DNA binding), Topo (Topo homology domain) and CTD (C-terminal domain). Abbreviations: bp: base pairs, Cas: CRISPR associated genes, CRISPR: clustered regularly interspaced short palindromic repeats, crRNA: CRISPR-RNA, PAM: protospacer adjacent motif, tracrRNA: transactivating crRNA.
S. pyogenes Cas9 comprises two nuclease domains and an α-helical domain illustrated in Figure 2C. The Cas9 protein is folded in a bilobed architecture with both nuclease domains in the first lobe and the α-helical domain in the second lobe [67]. Loading of Cas9 by a crRNA-tracrRNA hybrid induces a structural rearrangement with a central channel that binds to target DNA mediated by the arginine rich region (Arg) [67, 74]. The HNH nuclease domain cleaves the DNA strand complementary to the crRNA sequence (target strand) and the RuvC nuclease domain cleaves the non-complementary strand [66, 67]. This generates a blunt double-strand break three nucleotides upstream of the PAM sequence thus destructing foreign DNA sequences [66, 75, 76].

3.2.2 Repurposing CRISPR/Cas9 technology for genome editing

The adaption of the CRISPR/Cas9 technology for eukaryotic genome editing has revolutionized the field of molecular biology allowing simple and quick generation of genetically modified cells and animals or genome-scale screenings [77-82]. By exchange of the 20 targeting nucleotides of the crRNA basically any sequence preceding a 5’-NGG-3’ PAM can be targeted and cleaved [83, 84]. Furthermore, by using more than one gRNA multiple targets can be edited simultaneously [79, 83]. The simple and fast adaption of the CRISPR system to new target sequences is a big advantage over previously used genome editing tools such as zinc-finger nucleases and transcription activator-effector nucleases (TALENS) [85-87]. Zinc-finger nucleases and TALENS can also be modified to bind and cut specific target sequences. However, the DNA sequence specificity of these two systems is based on the protein sequence requiring individually designed proteins for each target which is cost and time intensive [88]. In contrast, targeting a new locus with the CRISPR/Cas9 system simply requires adjusting the 20 nt protospacer sequence.

For a faster and more efficient assembly of Cas9-RNA complexes so-called guide RNAs (gRNAs) were developed which are expressed as a synthetic fusion of crRNA and tracrRNA [66]. gRNAs therefore do not require the maturation process described in Figure 2A. These gRNAs are usually expressed from a RNA polymerase III promoter such as U6 or H1 and expression is terminated by a poly-T sequence [89-91].

The use of the CRISPR/Cas9 system for genome editing is based on the two major cell intrinsic DNA damage repair mechanisms depicted in Figure 3: Error-prone non-homologous end joining (NHEJ) and homology directed repair (HDR). Both pathways can be used to achieve distinctive goals. Re-ligation of the DNA by NHEJ leaves scars due to insertion/deletion mutations (yellow sequence in Figure 3) which can result in frame-shift mutations or premature stop-codons and is therefore often used for the generation of gene knockouts [92].
Figure 3: Genome editing by means of CRISPR/Cas9 technology
Illustration of genome editing utilizing the CRISPR/Cas9 system. Upon binding of the gRNA-Cas9 complex to a target sequence (red) e.g. in the eukaryotic genome Cas9 induces a double strand break three nucleotides upstream of the PAM sequence (green) as indicated by white arrowheads. The cell uses two major DNA repair mechanisms: Non-homologous end joining (NHEJ) and homology directed repair (HDR). NHEJ leads to insertion/deletion mutations (yellow) resulting in frame-shift mutations and can be used to generate gene knockouts. HDR can be used to insert a gene of interest (blue) in a scar-less fashion by offering a repair template with homology arms. Abbreviations: bp: base pairs, HDR: homology directed repair, NHEJ: non-homologous end joining, PAM: protospacer adjacent motif.

In order to insert or modify a gene of interest in a sequence specific and scar-less fashion the HDR mechanism can be utilized (blue sequence in Figure 3). For this purpose, a repair template with homology arms binding upstream and downstream of the cutting site is offered and integrated via homologous recombination [93].

3.2.3 Transcriptional modification by CRISPR/Cas9
The generation of a Cas9 version with depleted nuclease activity termed dead Cas9 (dCas9) opened a new field of applications for the CRISPR/Cas9 technology. For this purpose the RuvC (D10A) and HNH (H840A) nuclease domains of S. pyogenes Cas9 were mutated [66]. dCas9 can then be used as a shuttle to deliver epigenetic or transcriptional regulators to a sequence specific location e.g. to the promoter of a target gene without inducing a double strand break. This technology has been used to activate and suppress gene expression, to alter epigenetic marks and to change cell fate [89, 90, 94, 95]. For the induction of endogenous genes dCas9 is usually expressed as a fusion protein with different transactivation domains of transcriptional activators. Various systems have been published with varying gene induction potential depending on the transcriptional activators used. Figure 4 gives an overview on transcriptional activator systems analyzed in this thesis. The first published activation system comprises ten repeats of the transcriptional activation domain of Herpes simplex virus protein vmw65 (VP16) (residues 437 - 448) and is shown in Figure 4A [89]. These ten repeats of VP16 (termed VP160) are fused to the C-terminus of dCas9 and can directly interact e.g. with the General transcription factor IIB (TFIIB) [96]. TFIIB in turn binds the TATA-binding protein and is involved in the recruitment of RNA polymerase II thus contributing to the formation of the transcription initiation complex [97].
A slightly modified version termed Sso7d-dCas9-VP160 (Figure 4B) was developed at the institute by J. Truong. Here, the *S. solfataricus* DNA binding protein 7d (Sso7d) is fused to the N-terminus of dCas9 in order to putatively increase the time of dCas9 associated with the target promoter due to stronger binding and therefore potentially support transcriptional induction.

The SpyTag system shown in Figure 4C was also developed at the institute by J. Truong and allows recruitment of multiple transcriptional activators (VP160) to a single dCas9 protein. This system is based on a split version (SpyTag and SpyCatcher) of the second immunoglobulin-like collagen adhesion domain (CnaB2) of the *Streptococcus pyogenes* fibronectin-binding protein (FbaB) [98, 99]. Interaction of SpyTag and SpyCatcher leads to the formation of an isopeptide bond and thus covalent binding [98, 99]. For gene induction, multiple SpyTag repeats (four, eight or twelve) were fused to the C-terminus of the inactive nuclease (Figure 4C). These SpyTags can then be bound by SpyCatchers which in turn were fused to VP160 transcriptional activators. This allows recruitment of up to twelve copies of VP160 to a single dCas9 protein (Figure 4C). The underlying idea was that a significant increase in transcriptional activators might improve gene induction of target genes. Tanenbaum *et al.*, [100] developed a similar system termed SunTag shown in Figure 4D. Here, an array of 24 *S. cerevisiae* General control protein (GCN4) peptides termed SunTags was fused to dCas9. These tags can be targeted by single-chain variable fragment (scFv) antibodies which are in turn fused to four repeats of VP16 (VP64). This system therefore allows recruitment of up to 24 copies of VP64 to a single dCas9 molecule.

While the above-mentioned systems use varying amounts of VP16 repeats, the VPR system depicted in Figure 4E relies on a combination of three different transactivation domains [90]. Besides the commonly used VP16 repeats the VPR system also comprises transactivation domains of the P65 subunit of human NF-κB (residues 287 - 546). Furthermore, the transactivation domain of the Regulator of transcription activation (RTA, BRLF1: residues 416 – 605) of the *Human herpesvirus 8* is added resulting in a dCas9-VP64-P65-RTA fusion protein termed VPR system in the following. These two additional domains increase the variety of potential interaction partners which can be recruited to the dCas9 fusion protein at the promoter of a target gene. This includes e.g. the TATA-box-binding protein [101] and E1a binding protein p300 [102] by P65 or the cAMP-response-element-binding protein (CREB)-binding protein by RTA [103].
Figure 4: Schematic illustration of CRISPR/Cas9 systems used for gene induction in this study

Schematic illustrations of nuclease deficient *Streptococcus pyogenes* dCas9 (D10A, H840A version) fused to different transcriptional activator systems. Together with a sequence-specific gRNA dCas9 fusions are targeted to the promoter region of a gene of interest and thus induce the expression of the target gene. 

(A) dCas9-VP160 consisting of a C-terminal fusion of dCas9 with ten repeats of VP16, termed VP160. 

(B) Sso7d-dCas9-VP160 comprising dCas9 N-terminally fused to double-stranded DNA binding protein Sso7d and C-terminally to VP160. 

(C) SpyTag system consisting of dCas9 fused to multiple repeats of SpyTags (13 amino acids of the CnaB2 domain of the *Streptococcus pyogenes* fibronectin-binding protein FbaB) and SpyCatchers (complementary 116 amino acids of the CnaB2 domain). SpyCatches are expressed as fusion proteins with VP160 thus allowing recruitment of multiple copies of VP160 to a single dCas9. 

(D) SunTag system as described by Tanenbaum et al., [100]. dCas9 is fused to repeats of SunTags (GCN4 peptides) which can be bound by ScFv antibody fragments that are in turn fused to VP64 (4x repeats of VP16). 

(E) VPR system as described by Chavez et al., [90] where dCas9 is fused to VP64-P65-RTA. 

(F) SAM system as described by Konermann et al., [91]. A modified gRNA with two additional loops serving as MS2 aptamers is used. These aptamers are bound by MS2-P65-HSF1 fusion proteins which serve as additional transcriptional activators. This system is used in combination with a dCas9 fused to C-terminal VP64. 

**Abbreviations:** GCN4: general control protein 4, HSF1: heat shock transcription factor 1, MS2: bacteriophage MS2 coat protein, P65: P65 subunit of human NF-κB, RTA: Regulator of transcription activation, ScFv: single-chain variable fragment antibody, VP64/VP160: four/tent repeats of *Herpes simplex virus* protein VP16.
The synergistic activation mediator (SAM) system shown in Figure 4F uses an alternative strategy where transcriptional activators are recruited to hairpin aptamers added to tetraloop and stem loop 2 of the gRNA [91]. These aptamers are selectively bound by dimerized MS2 bacteriophage coat proteins indicated as MS2 in Figure 4F [104]. MS2 in turn is fused to murine P65 (residues 369 – 549) and the activation domain of the human Heat shock transcription factor 1 (HSF1) (residues 406 – 529). The HSF1 activation domain additionally allows the recruitment of e.g. chromatin remodeling complexes of the Switching defective/Sucrose non-fermenting (SWI/SNF) family [105]. The assembled complex comprising MS2-P65-HSF1 fusion proteins with a gRNA and dCas9-VP64 (Figure 4F) is termed SAM complex in the following.

In order to induce the expression of a target gene gRNAs are designed to target the promoter region of this gene. While some groups report best effects within the 250 nt upstream of the transcription start site [89, 91, 106] others have also used gRNAs binding up to 1 kb upstream of the transcription start site [90, 107]. The number of gRNAs required for a sufficient activation of the target gene seems to be gene and sequence dependent. Some reports show sufficient gene induction by a single gRNA [108, 109], whereas other reports suggest synergistic effects when a single gene is targeted by multiple gRNAs [89, 106, 110].

Early publications concentrated on improving the transcriptional levels of target genes but it soon became clear that the CRISPR/Cas9 technology could also be used to manipulate cell fates [90, 108]. The underlying idea is that targeting of the endogenous promoter rapidly remodels the epigenetic landscape and thus more closely resembles natural mechanisms which may be an advantage compared to forced overexpression of transcription factors [107]. Indeed, Black et al., reported an increase in histone 3 modifications (H3K4me3 and H3K27ac) at endogenous Ascl1 and POU domain class 3 transcription factor 2 (Brn2) loci induced by VP64-dCas9-VP64 three days post-transfection [107]. Tri-methylation of lysine 4 (K4me3) and acetylation of lysine 27 (K27ac) of histone 3 are both well-described markers of transcriptional activity [111, 112]. Interestingly, these modifications of endogenous promoters were not observed at this timepoint when Ascl1 and Brn2 were overexpressed from transfected vectors [107]. Furthermore, in contrast to neuronal transcription factors whose binding sites can be inaccessible in MEFs or astrocytes Cas9 binding was reported to be independent of the chromatin state [113]. This would give Cas9-based approaches a similar potential like pioneer factors such as Ascl1 which was described earlier.

Mouse embryonic stem cells have been differentiated into extraembryonic lineages by dCas9-VP64 induced expression of endogenous Caudal type homeobox 2 (Cdx2) and GATA binding protein 6 (Gata6) [106]. Others however, have reported that a simple dCas9-VP64
system was not sufficient to influence cell fate [90, 108]. By using the more sophisticated VPR system and a pool of 30 gRNAs, human induced pluripotent stem cells were differentiated to neurons by inducing either Neurogenin 2 (NGN2) or Neurogenic differentiation 1 (NEUROD1) expression. Direct reprogramming of one somatic cell type to another one has only been described in two cases so far [107, 108]. Both publications used a VP64-dCas9-VP64 system where the VP16 repeats were fused to both the N- and C-terminus of dCas9. Utilizing this system MEFs were converted to skeletal myocytes (induction of Myogenic differentiation 1 (Myod1)) [108] and just recently MEFs were also reprogrammed to neurons [107] (induction of Ascl1, Bm2 and Myelin transcription factor 1-like (Myt1l)). However, there is still a need to improve the CRISPR/Cas9-based direct conversion of cells, to identify new activator complexes and to apply these systems to additional cell types such as astrocytes.
4 Aim of the thesis

Parkinson’s disease is among the prime candidates for direct reprogramming and cell replacement therapies due to the loss of a specific and spatially restricted cell type – the midbrain dopaminergic neurons. The aim of this thesis was to develop new strategies in order to improve the reprogramming efficiencies of somatic cells to dopaminergic neurons \textit{in vitro} as this is a limiting factor of current protocols. Furthermore, new factors should be identified enabling the generation of PITX3$^+$ midbrain dopaminergic neurons. Only these PITX3$^+$ DA neurons resemble the subtype lost during Parkinson’s disease which is not achieved by most of the currently published transcription factor combinations used for direct reprogramming. The CRISPR/Cas9 system is a relatively new tool to modulate gene expression and could be a promising alternative to classical reprogramming by activating the expression of endogenous genes. In this thesis, a system should be established to induce the expression of endogenous \textit{Ascl1} utilizing transcriptional activators fused to Cas9. Furthermore, the reprogramming potential of this system should be investigated as proof-of-principle experiments to directly convert astrocytes to neurons which will then serve as a basis for cell replacement therapies.
5 Results

Due to the specific cell loss of midbrain dopaminergic neurons Parkinson’s disease is one of the prime targets for cell replacement therapy using reprogrammed cells and direct reprogramming in vivo. However, the reprogramming efficiency of current protocols is low and the generated DA neurons mostly do not resemble the population lost in PD as assessed by the missing expression of the mdDA marker gene Pitx3. It is therefore of interest to improve the reprogramming efficiency and to identify new factors enabling the generation of ‘true’ mdDA (PITX3+) mdDA neurons. The first part of the results concentrates on addressing these points utilizing different vector systems for the expression of exogenous transcription factors. In the second part the potential of the CRISPR/Cas9 technology regarding the induction of endogenous genes and the direct conversion of somatic cells is analyzed.

5.1 Exogenous gene expression strategies for direct conversion of MEFs to DA neurons

5.1.1 Limitations of direct reprogramming utilizing multiple viruses for gene delivery

Caiazzo et al., 2011 [37], published a minimal set of transcription factors (Ascl1, Lmx1a and Nurr1) for the direct conversion of mouse embryonic fibroblasts (MEFs) and adult human fibroblasts to DA neurons which were delivered by individual lentiviruses. This strategy however has the disadvantage, that a single cell needs co-transduction of all three lentiviruses which may pose a limiting effect on the reprogramming efficiency. To test this hypothesis MEFs were transduced with individual lentiviruses encoding Ascl1, Lmx1a and Nurr1 as shown in Figure 5A. Quantification of transduced cells revealed 28.5 ± 0.5% of cells to be ASCL1+ and 19.8 ± 0.5% were NURR1+ (Figure 5B). However, only 10.1 ± 0.9% of cells expressed both genes as shown in Figure 5C. To identify successful reprogramming cells were co-stained for the DA neuron marker Tyrosine hydroxylase (TH) 14 days after transduction. When looking at these TH+ cells it became clear that reprogrammed cells were always TH+/ASCL1+ (Figure 5D) or TH+/NURR1+ (Figure 5E). However, there was an excess of ASCL1+ and NURR1+ cells which did not express TH. This suggested that neither factor alone was sufficient to directly reprogram MEFs to TH+ neurons thus excluding cells that were not co-transduced by at least these two lentiviruses.
Figure 5: The need for co-transduction by multiple lentiviruses limits the reprogramming efficiency

Immunocytochemistry at 48 h (A) or 14 days (D and E) after transduction of MEFs with Ascl1, Lmx1a and Nurr1 encoding lentiviruses at a multiplicity of infection (MOI) of three. (A) Co-staining for ASCL1 and NURR1 revealed that only a fraction of cells expressed both transcription factors. (B) Quantification of all ASCL1+ and NURR1+ cells revealed a transduction efficiency of 28.5 ± 0.5% for Ascl1 and 19.8 ± 0.5% for Nurr1 encoding lentiviruses. (C) Quantification of double positive cells. 10.1 ± 0.9% of cells expressed both factors. (D) Co-staining for ASCL1 and TH. All successfully reprogrammed TH+ cells analyzed were also ASCL1+ suggesting the requirement of this factor for reprogramming. Many ASCL1+ cells however, were TH- indicating that Ascl1 alone was not sufficient for the generation of dopaminergic neurons. (E) Co-staining for NURR1 and TH. All TH+ cells observed were also NURR1+ showing the importance of this factor for successful reprogramming. Similar to Ascl1 however, Nurr1 alone also was not sufficient for a successful conversion to TH+ neurons as assessed by an excess of NURR+/TH- cells. Abbreviations: A+L+N: Ascl1, Lmx1a, Nurr1 expressed from individual lentiviruses, TH: Tyrosine hydroxylase. Scale bars: 50 µm. Data was derived from one experiment. Error bars represent mean ± SEM.

Caiazzo et al., have shown that efficient reprogramming requires co-expression of a third factor - Lmx1a [37]. Due to strong and unspecific background signal of the α-LMX1A antibody an immunocytochemical analysis for LMX1A+ cells could not be performed. Nevertheless, the proportion of cells co-transduced by all three lentiviruses would be expected to be even lower than what was found for NURR1 and ASCL1 in Figure 5A. Furthermore, since Tet-O constructs were used, a fourth virus carrying the activator rTTA2 was required to co-transduce cells in order to allow expression of the three factors from Tet-O promoters [114]. The idea therefore was to use a tri-cistronic vector encoding all three factors.
5.1.2 Limitations of multi-cistronic constructs for direct reprogramming

In order to omit the limitations of single viruses described above a tri-cistronic vector was generated at the institute by F. Meier carrying Ascl1, Lmx1a and Nurr1 termed ALN where individual transcription factors were separated by 2A peptides (see Figure 6A). 2A peptides consist of 18 – 22 amino acids and are cleaved at the c-terminal glycyl-prolyl peptide bond by ribosome skipping during protein synthesis [115, 116]. This leads to separation of the 2A peptide and the immediate downstream peptide which can be utilized for the expression of several genes from a single expression cassette. In comparison to internal ribosomal entry sites (IRES), 2A sequences have two advantages: 2A peptides are short (18 – 22 amino acids vs ≥ 500 nucleotides for IRES) and multiple proteins are produced at stoichiometric ratios [117]. In order to avoid repeated sequences which can be problematic for lentiviral packaging 2A sequences from Porcine teschovirus-1 (P2A) and Thoseaasigna virus (T2A) were chosen. Using lentiviruses carrying the newly generated Ascl1-T2A-Lmx1a-P2A-Nurr1 (ALN) construct depicted in Figure 6A MEFs were successfully reprogrammed to DA neurons at the institute (dissertation F. Meier).

However, reprogramming efficiencies did not reach the numbers originally published for Ascl1, Lmx1a and Nurr1 (< 1% TH+ cells in our hands vs approximately 18% TH+ by Caiazzo et al., [37]). Therefore, reprogramming efficiencies of single viruses (A+L+N) and the tri-cistronic (ALN) construct (see Figure 6A) were compared 14 days after transduction of MEFs (Figure 6B). In order to identify newly generated DA neurons cells were stained for the expression of the mature neuronal marker Microtubule-associated protein 2 (MAP2) and the DA neuron marker TH. Surprisingly, the percentages of MAP2 positive neurons (Figure 6C, A+L+N: 4.1 ± 0.8%, ALN: 1.0 ± 0.3% MAP2+ cells / DAPI) and TH positive DA neurons (Figure 6D, A+L+N: 2.2 ± 0.6%, ALN: 0.3 ± 0.1% TH+ cells / DAPI) were significantly decreased when using the ALN vector compared to single viruses.

In order to investigate a possible loss of cells after ALN transduction the number of cells per mm² was determined using Stereo Investigator software (MBF Bioscience) that randomly selected fields of 200 x 200 µm for counting. Indeed, wells transduced by the tri-cistronic ALN construct seemed to contain a slightly decreased number of DAPI+ cells per mm² when compared to the control or wells transduced with single viruses (Figure 6E, A+L+N: 932 ± 86 vs ALN: 709 ± 54 cells per mm²). Taken together, these results suggested possible adverse effects of the tri-cistronic ALN construct.
Figure 6: Utilizing the tri-cistronic ALN vector decreases the conversion efficiency to DA neurons
(A) Schematic illustration of lentiviral vectors used for reprogramming. (B) Cells stained for neuronal marker MAP2 and dopaminergic marker TH 14 days after transduction. Both MAP2+ and TH+ cells were decreased when using the tri-cistronic vector. White arrowheads indicate MAP2+/TH+ cells. (C) Quantification of MAP2+ cells/DAPI. Using the ALN virus resulted in a significant decrease in MAP2+ cells when compared to the single vectors. (D) Quantification of TH+ cells/DAPI: The amount of TH+ cells was significantly lower for ALN transduced wells when compared to single viruses. (E) Quantification of DAPI+ cells per mm² did not reveal a significant effect but hinted at lower cell numbers when using the tri-cistronic ALN construct. Abbreviations: ALN: Ascl1, Lmx1a, Nurr1 expressed from a single lentiviral vector, A+L+N: Ascl1, Lmx1a, Nurr1 expressed from individual lentiviral vectors, dox: doxycycline used for induction of Tet-O promoters. TH: Tyrosine hydroxylase, MAP2: Microtubule-associated protein 2. Scale bars: 50 µm. Data was derived from three independent experiments. Error bars represent mean ± SEM, Mann Whitney test, ns: not significant, *P < 0.05. This data was published by Theodorou and Rauser et al., 2015 [118].

5.1.3 Inefficient ribosome skipping at 2A sites results in fusion proteins
The reason for a reduced reprogramming efficiency of the tri-cistronic ALN construct could be the generation of fusion proteins due to inefficient ribosome skipping at 2A sites [119]. To investigate this, western blots were performed using the ALN construct from the above-mentioned reprogramming experiments (Ascl1-T2A-Lmx1a-P2A-Nurr1) and additionally a construct where P2A was replaced by IRES (Ascl1-T2A-Lmx1a-IRES-Nurr1) as well as a construct with additional eGFP (Ascl1-T2A-Lmx1a-P2A-Nurr1-IRES-eGFP) and the single Ascl1 and Nurr1 expressing viruses (see schematic illustrations in Figure 7A).
RESULTS

Figure 7: Western blot analysis reveals inefficient ribosome skipping at 2A sequences resulting in the generation of fusion proteins

HEK293 cells were transduced with individual viruses and western blot analysis was performed after 48 h. (A) Schematic illustration of vectors used. (B) Western blot detecting ASCL1. The expected ASCL1 band at 37 kDa was clearly detected in the positive control. The slight shift towards a higher mass for ASCL1 from A-T2A-L-P2A-N and A-T2A-L-IRES-N vectors was due to the additional residues from the T2A element. Possible fusions with LMX1A or LMX1A and NURR1 could not be detected due to strong unspecific binding of the ASCL1 antibody. (C) Western blot detecting LMX1A. Besides the WT LMX1A at 55 kDa, additional fusion proteins of LMX1A with ASCL1, NURR1 or with both proteins were observed as indicated by arrows. (D) Western blot detecting NURR1. WT NURR1 was expected at 66 kDa. Additional fusion proteins with LMX1A or LMX1A and ASCL1 were observed as indicated by arrows. Abbreviations: A: ASCL1, L: LMX1A, N: NURR1, P2A: 2A sequence form Porcine teschovirus-1, T2A: 2A sequence from Thosea asigna virus, IRES: internal ribosomal entry site. This data was published by Theodorou and Rauser et al., 2015 [118].

All ASCL1 western blots performed showed strong unspecific antibody binding (Figure 7 B). Therefore, only the correctly cleaved form of ASCL1 at 37 kDa could be detected (Figure 7B). For LMX1A (Figure 7C) and NURR1 (Figure 7D) however, a ladder of different fusion proteins was observed from the constructs Ascl1-T2A-Lmx1a-P2A-Nurr1 and Ascl1-T2A-Lmx1a-IRES-Nurr1. LMX1A (correct size 55 kDa) was partially fused to the N-terminal...
ASCL1 (92 kDa), the C-terminal NURR1 (121 kDa) or both proteins (158 kDa). NURR1 was detected at 66 kDa (correctly cleaved form), at 121 kDa (fusion with LMX1A) and at 158 kDa (ASCL1-LMX1A-NURR1 fusion). The replacement of P2A by IRES to separate LMX1A and NURR1 prevented the generation of LMX1A-NURR1 fusion proteins as expected. However, the use of IRES resulted in a reduced expression level of NURR1 when comparing the 66 kDa NURR1 band of Ascl1-T2A-Lmx1a-P2A-Nurr1 and Ascl1-T2A-Lmx1a-IRES-Nurr1 in Figure 7D. Taken together, these results confirmed incomplete ribosome skipping at 2A sites resulting in the generation of fusion proteins which are responsible for the low reprogramming efficiency.

The Tet-O promoter used in the ALN construct is known to induce gene expression at high levels [120]. In further experiments, it was therefore determined whether cleavage at 2A sites and reprogramming efficiency could be improved at lower expression levels by reducing the doxycycline concentration.

### 5.1.4 The reprogramming efficiency of ALN is independent of doxycycline concentrations

The Tet-O-promoter used in the previously described experiments is based on a Tet-ON system comprising tet operators upstream of a minimal promoter [114]. For the induction of the promoter a reverse Tet repressor (rTetR) fused to the transactivating domain (TA) of Herpes simplex virus protein vmw65 (VP16) is required [114]. This fusion protein termed rTTA only binds and induces the tet operator upon interacting with doxycycline [114]. This allows timing and fine tuning of the expression level by adjusting doxycycline concentrations.

In order to test whether ribosome skipping can be improved at lower expression levels, reprogramming experiments were repeated with reduced doxycycline concentrations shown in Figure 8A. Quantifications of reprogrammed cells did not reveal statistically significant differences in the percentage of MAP2+ cells (Figure 8B) or TH+ cells (Figure 8C) upon reducing doxycycline concentrations. However, results for the single viruses indicated a concentration dependent effect of doxycycline on the reprogramming efficiency favoring higher levels of gene expression (1.1 ± 0.3% TH+ cells at 0.1 µg/ml dox vs 2.2 ± 0.6% TH+ cells at 2.0 µg/ml dox). For ALN it seemed a concentration of 1.0 µg/ml doxycycline may be the most suitable (Figure 9C). At all conditions analyzed however, using ALN again seemed less suitable for direct reprogramming of MEFs to DA neurons than utilizing the single viruses. Next, it was analyzed on the protein level whether the decrease in doxycycline influences ribosome skipping at 2A sites.
Figure 8: Reducing the doxycycline concentration does not significantly affect the reprogramming efficiency

MEFs were transduced either with tri-cistronic ALN or single viruses encoding Ascl1, Lmx1a and Nurr1 and were cultured at different doxycycline concentrations for 14 days. (A) Co-staining for MAP2 and TH. Arrow heads indicate MAP2+/TH+ cells. (B) Quantification of MAP2+ cells showed no statistically significant effect on MAP2+ cells upon reducing doxycycline concentration within either ALN or A+L+N transduced cells. However, a trend was observed favoring a concentration of 1 µg/ml doxycycline for ALN and higher levels for A+L+N. (C) Quantification of TH+ cells. Reducing doxycycline concentration did not statistically significant affect the percentage of TH+ cells within the ALN or A+L+N transduced cells. Similar to the results for MAP2, again best results were obtained at 1 µg/ml doxycycline for ALN and higher levels for A+L+N. Abbreviations: ALN: Ascl1, Lmx1a, Nurr1 expressed from a tri-cistronic lentiviral vector, A+L+N: Ascl1, Lmx1a, Nurr1 expressed from individual lentiviral vectors, dox: doxycycline used for induction of Te-O promoters. Scale bars: 50 µm. Data was derived from three independent experiments. Error bars represent mean ± SEM, Kruskal-Wallis test, Dunn’s multiple comparison test. This data was published by Theodorou and Rauser et al., 2015 [118].
5.1.5 Ribosome skipping at 2A sites is not affected by expression levels

In order to test whether ribosome skipping at 2A sites may be impaired due to the high level of protein biosynthesis, western blot analysis for NURR1 was repeated at different doxycycline levels (Figure 9A). There seemed to be a concentration dependent effect of doxycycline on the overall amount of NURR1 expressed from the ALN construct (see Figure 9A and B, 23.4 ± 9.7% NURR1 protein at 0.1 µg/ml dox vs 45.2 ± 26.8% NURR1 protein at 2.0 µg/ml dox normalized to Tet-O-Nurr1).

**Figure 9: Lower expression levels do not affect ribosome skipping at P2A site**

HEK293 cells were transduced with Tet-O-Nurr1 encoding lentiviruses at different doxycycline concentrations. Western blot analysis was performed after 48 h. (A) Western blot detecting NURR1 shows WT NURR1 at 66 kDa and LMX1A-NURR1 fusion proteins at 121 kDa. (B) Quantification of total NURR1 protein. Changing the doxycycline concentration did not significantly affect the total amount of NURR1 but hinted at reduced levels upon decreasing the doxycycline concentration. (C) Quantification of unfused NURR1 protein at 66 kDa. Reducing doxycycline concentration did not significantly increase the amount of unfused NURR1 protein. Values were normalized to NURR1 overexpression from a single Nurr1 lentivirus. Abbreviations: ALN: Ascl1, Lmx1a, Nurr1 expressed from a single lentiviral vector, L: Lmx1a. N: Nurr1 expressed from individual lentiviral vectors, dox: doxycycline used for induction of Tet-O promoters. Data was derived from three independent experiments. Error bars represent mean ± SEM, Kruskal-Wallis test, Dunn’s multiple comparison test, ns: not significant. This data was published by Theodorou and Rauser et al., 2015 [118].
When analyzing the percentage of correctly cleaved NURR1 protein at 66 kDa it became clear that just 15.9 ± 6.3% of total NURR1 produced from the ALN construct was correctly cleaved at 2.0 µg/ml doxycycline (Figure 9C). Furthermore, the total amount of NURR1 produced by the ALN construct only reached about 50% of the level expressed by the single Nurr1 encoding lentivirus (Figure 9B). This could be an explanation for the low reprogramming efficiency of the tri-cistronic ALN construct. Reducing the doxycycline concentration from 2.0 µg/ml to 0.1 µg/ml did not significantly affect the proportion of correctly cleaved NURR1. However, at 1.0 µg/ml doxycycline the percentage of correctly cleaved NURR1 seemed to be slightly increased although not statistically significant. This corresponds to the slightly improved reprogramming efficiency of ALN observed at 1.0 µg/ml doxycycline in Figure 8. These results suggest that ribosome skipping at 2A sequences was independent of the expression level and could therefore not be rescued by choosing lower levels of expression. Taken together, the use of a tri-cistronic lentiviral vector did not help to overcome the limitations of multiple single viruses described earlier and was therefore not pursued any further.

5.1.6 Successful generation of PITX3+ DA neurons by forskolin treatment

Single viruses encoding A+L+N were found to be better suited for the direct conversion of MEFs to DA neurons than the tri-cistronic ALN construct. However, there was still a need to improve reprogramming efficiencies. Besides overexpression of transcription factors the addition of small molecules or morphogens during reprogramming experiments can also be beneficial [30, 121]. One promising factor is forskolin, a labdane diterpenoid isolated from the roots of Coleus Forskohlii. Forskolin directly activates adenylate cyclase which catalyzes the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) [122-124]. cAMP signaling is involved in a wide range of cellular processes including proliferation, differentiation and chromatin condensation [125]. Interestingly, forskolin was also found to induce anti-apoptotic responses, to improve neuronal regeneration and to increase the translation of TH mRNA [126-130].

It was therefore tested whether forskolin treatment of A+L+N transduced MEFs influences the direct reprogramming efficiency. Indeed, Figure 10A+B shows a trend toward higher reprogramming efficiency upon addition of forskolin to the cells (increase from 0.5 ± 0.2% to 0.8 ± 0.2% TH+ cells). In order to detect possible anti-apoptotic effects of forskolin, the number of DAPI+ cells per mm² was determined in Figure 10C. However, forskolin treatment did not increase the overall cell survival indicating that the trend in TH+ cells was not based on a generally higher survival of the cells.
Figure 10: Forskolin treatment of A+L+N transduced MEFs enables the generation of TH⁺/PITX3⁺ neurons

Analysis of reprogrammed MEFs 14 days after lentiviral transduction. 25 µM forskolin was added together with reprogramming medium three days after transduction. (A) Immunocytochemistry analysis of TH⁺ cells for A+L+N with or without forskolin (Fk) treatment. (B) Quantification of TH⁺ cells per DAPI revealed a positive trend upon addition of forskolin. (C) Quantification of DAPI⁺ cells per mm² that the slight increase in TH⁺ cells in (B) was not due to an overall increase of cells. (D) Immunocytochemistry analysis of reprogrammed cells from Pitx3⁺/GFP⁺ mice. GFP⁺ cells indicate the generation of 'true' TH⁺/PITX3⁺ mdDA neurons after addition of 25 µM forskolin to A+L+N transduced cells. (E) Quantification of PITX3⁺ cells per TH. While A+L+N transduced cells were never found to express GFP (PITX3), 46.7 ± 8.8% of all TH⁺ cells were PITX3⁺ upon addition of forskolin.

Abbreviations: Dox: Doxycycline, Fk: forskolin, A+L+N: Ascl1, Lmx1a, Nurr1 expressed from individual lentiviral vectors, PITX3: Paired-like homeodomain transcription factor 3. Scale bars: 50 µm. Data was derived from three independent experiments. Error bars represent mean ± SEM, Mann-Whitney test, ns: not significant.

The subtype of the reprogrammed DA neurons obtained by A+L+N based reprogramming was still an open question. While several types of DA neurons are found in the mammalian brain only midbrain dopaminergic neurons are affected in Parkinson’s disease [6-8]. This subpopulation is characterized by the expression of Pitx3 [14]. Various protocols and transcription factor combinations have been described for the direct conversion of fibroblasts to dopaminergic neurons [32-37]. However, the generation of TH⁺/PITX3⁺ neurons was only
reported for two protocols using five or six different transcription factors respectively (Brn2, Lmx1b, Ascl1, Nurr1, Otx2 [36] and En1, Foxa2, Lmx1a, Ascl1, Nurr1, Pitx3 [32]). Overexpression of Ascl1, Lmx1a and Nurr1 is not sufficient for the generation of TH+/PITX3+ cells (unpublished data F. Meier, Helmholtz Zentrum München). It is therefore of interest to identify factors enabling the generation of ‘true’ midbrain dopaminergic neurons (TH+/PITX3+) requiring less than five to six individual viruses.

It was thus tested whether forskolin treatment could influence the identity of the reprogrammed cells allowing the generation of TH+/PITX3+ mdDA neurons. For reprogramming experiments MEFs of Pitx3GFP/+ mice were used which allows visualization of PITX3+ cells due to GFP expression. Figure 10D shows successful generation of TH+/PITX3+ cells upon addition of forskolin to A+L+N transduced MEFs. 46.7 ± 8.8% of all TH+ cells were PITX3 positive (Figure 10E) which is a very promising finding for the generation of ‘true’ mdDA neurons.

However, the above described limitations of co-infection by multiple viruses and the inefficient ribosome skipping at 2A sequences called for a new reprogramming strategy. One promising new technology is the CRISPR/Cas9 system which can be used to induce endogenous gene expression. Due to epigenetic remodeling of targeted loci and the expression from endogenous promoters this technology more closely resembles physiological mechanisms of gene expression than forced overexpression of exogenous factors [107]. The potential of CRISPR/Cas9 for direct conversion of somatic cells is described in the following part.
5.2 Induction of endogenous genes and direct reprogramming of astrocytes to neurons utilizing the CRISPR/Cas9 technology

The CRISPR/Cas9 system allows targeting of virtually any locus in the genome by a protein-RNA complex consisting of a Cas9 nuclease and a specifically designed gRNA which binds complementary DNA sequences [83, 84]. Dead Cas9 (dCas9) variants which lack the DNA cutting properties are utilized as shuttles to transfer fused transcriptional regulators to a promoter of interest and modulate gene expression [89, 94, 95]. At the beginning of this project direct reprogramming of somatic cells to neurons using the CRISPR/Cas9 technology had not been described. Therefore, the aim was to set up a system which allows maximum induction of endogenous genes and to test the reprogramming properties as proof-of-principle experiments. The CRISPR/Cas9 technology enables the simultaneous induction of several genes by expressing an array of suitable gRNAs thus circumventing limitations in packaging capacity, co-transduction efficiency and fusion proteins.

For the proof-of-principle experiments of this thesis Ascl1 was chosen as target gene since ectopic expression of this transcription factor is sufficient to successfully reprogram MEFs or astrocytes to neurons [131, 132]. Some experiments also included Nurr1 as a second target gene since the long-term goal of this project was to directly reprogram somatic cells to DA neurons by induction of Ascl1, Lmx1a, and Nurr1. The following chapters describe screening experiments for suitable gRNAs, followed by screenings in which different dCas9-fusion proteins were analyzed regarding their gene induction properties.

5.2.1 Design of gRNAs and implementation of a screening assay to detect induction of endogenous genes

As a first step gRNAs targeting murine Ascl1 and Nurr1 promoters were designed to test the CRISPR/Cas9 technology for gene activation. The promoter region between -250 to -1 nucleotides (nt) upstream of the transcription start site (TSS) has been successfully targeted for gene induction using CRISPR/Cas9 by other groups [89, 91]. Therefore, eight gRNAs were designed covering these 250 nucleotides of the Ascl1 (ENSMUST00000020243.9) and Nurr1 (ENSMUST00000028166) promoters using an online tool at http://crispr.mit.edu/ [133]. For each gene, four gRNAs targeting the sense strand and four gRNAs targeting the antisense strand were chosen (gRNAs A1 – A8 for Ascl1, N1 – N8 for Nurr1, see Figure 11A and B) to check for possible orientation effects. In order to detect successful induction of endogenous genes, dual luciferase assays were performed 48 h after lipofection. This allowed a quantification of the activity of the induced target protein and not just an analysis of gene induction via mRNA levels.
**Figure 11**: gRNA A5 successfully induces Ascl1 expression in Neuro 2a cells

Position and orientation of gRNAs targeting the 250 nucleotides upstream of the transcription start sites (TSS) of murine (A) Ascl1 and (B) Nurr1. (C) Schematic illustration of the reporter system used for luciferase assays. Upon binding of dCas9 fused to transcriptional activators, the target gene i.e. Ascl1 was expressed and bound to specific Ascl1 binding sites on a luciferase reporter construct. Binding of ASCL1 then induced *firefly luciferase* expression and luciferase activity was determined by adding a suitable substrate and measurement of the generated luminescence in a luciferase assay. For normalization purposes *renilla luciferase* driven by a constitutively active promoter was co-transfected. (D) Luciferase assay screen for suitable gRNAs 48 h after transfection of Neuro 2a cells. The combination of gRNAs A1/A5 and A4/A6 showed the strongest luciferase activity. Data was derived from one experiment. (E) Luciferase assay screen for suitable gRNAs. gRNAs A5 and A6 reached similar levels of activation when tested alone or in combination with gRNAs A1 or A4, respectively. Data was derived from one experiment. (F) Luciferase assay comparing gRNA A5 and Ascl1 overexpression. gRNA A5 induced about 60% of luciferase activity (Ascl1 induction) when compared to Ascl1 overexpression. Data was derived from three independent experiments. **Abbreviations**: gRNAs A1-8: gRNAs targeting the murine Ascl1 promoter, gRNAs N1-N8: gRNAs targeting the murine Nurr1 promoter, Sso7d: *S. solfataricus* DNA binding protein 7d, TSS: transcription start site, VP160: ten repeats of the *Herpes simplex virus* protein vmw65 (VP16) transactivation domain. Error bars represent mean ± SEM, Kruskal-Wallis test, Dunn’s multiple comparison test, *P < 0.05.
Figure 11C depicts an illustration of the established luciferase assay system in which the induced proteins ASCL1 or NURR1 bound to specific binding sites on reporter constructs inducing firefly luciferase expression [134]. The activity of the firefly luciferase could then be determined in a luciferase assay after addition of a substrate and subsequent luminescence measurement. The *firefly* luciferase activity was normalized to the activity of a co-transfected constitutively expressed *renilla* luciferase. Figure 11D-F shows an exemplary screen for suitable Ascl1 gRNAs which was also performed for Nurr1 (appendix, Figure 25). For these initial experiments a dCas9 version termed *Sso7d-dCas9-VP160* was used which was developed by J. Truong at the institute. *Sso7d-dCas9-VP160* consists of dCas9 C-terminally fused to ten VP16 repeats (VP160) serving as transcriptional activators and N-terminally fused to the DNA binding protein Sso7d putatively improving DNA binding properties (see illustration in Figure 4B). In a first screen gRNA combinations with two gRNAs (A1/A5 or A4/A6) targeting the Ascl1 promoter showed the strongest activation (Figure 11D).

In a second screen gRNAs A5 and A6 were found to be responsible for the detected activating effect showing a comparable level of luciferase activity when used alone or in combination with gRNAs A1 or A4, respectively (Figure 11E). A general influence of the gRNA orientation was not detected. gRNA A5 was chosen for further experiments and reached about 60% of the luciferase activity when compared to direct Ascl1 overexpression as positive control (1.8 ± 0.2 vs 3.1 ± 0.8-fold gene induction).

5.2.2 Screening for dCas9 fusion proteins to induce endogenous Ascl1

The screenings for suitable gRNAs described in the above experiments were performed using *Sso7d-dCas9-VP160*. To identify a system with potentially even stronger gene induction properties, different dCas9 versions were tested in the following chapters where dCas9 was fused to diverse combinations of transcriptional inducers including the SpyTag and SunTag systems, SAM and VPR strategies.

5.2.2.1 The SpyTag system improves endogenous gene induction

First, it was determined whether the fusion of the additional DNA binding domain Sso7d to dCas9 variants benefits the gene induction potential. For these tests, HEK293 cells were used to also apply the system to human cells. A change in gRNAs was not required since both gRNAs (A5 for ASCL1 and N2 for NURR1) matched sequences in the corresponding human and murine promoter regions. Initial screens for ASCL1 (Figure 12A) and NURR1 (Figure 12B) induction suggested a positive effect of *Sso7d* on gene induction when compared to dCas9-VP160 (increased activity from 4.0 ± 0.5 to 6.4 ± 1.2-fold for ASCL1, and 0.9 ± 1.2 to 2.1 ± 1.6-fold for NURR1).
The fusion of the Sso7d DNA binding domain to dCas9 seemed to increase the activation of VP160 when compared to simple dCas9. SpyTag arrays consisting of four, eight or twelve SpyTags allowed recruitment of multiple VP160 copies to dCas9. Figure 12 shows that using the SpyTag system led to a significant increase in NURR1 expression when compared to simple dCas9-VP160.
(0.9 ± 0.2 vs 3.6 ± 0.7-fold activation). By rising the number of SpyTag repeats fused to dCas9 to twelve the activation level of NURR1 was further increased to 4.7 ± 0.5-fold activation (Figure 12C). Next, it was tested whether adding the DNA binding protein Sso7d to the SpyTag system would again benefit gene induction. However, in this case no additional effect was detected suggesting that the putatively stronger binding of the dCas9 system due to the Sso7d domain only supported gene induction when the overall level of induction is moderate (Figure 12D). Therefore, the dCas9-12x-SpyTag system was chosen for further comparative studies.

In the course of these studies Tanenbaum et. al., published a system similar to the SpyTag technology termed SunTag [100]. The SunTag system is based on scFv antibodies fused to four repeats of VP16 (VP64) that can bind an array of 24 SunTags fused to dCas9 (see illustration in Figure 4D). The SunTag system therefore allows recruitment of up to 24 copies of VP64 which may lead to an even higher gene induction than the 12x-SpyTag system described above.

Figure 13: The SpyTag system seems to be superior to the published SunTag system for the induction of ASCL1 and NURR1
Luciferase assays comparing SpyTag and SunTag systems in HEK293 cells 48 h after transfection. Preliminary screening data suggested the SpyTag system to be better suited for gene induction of (A) ASCL1 and (B) NURR1 than the published SunTag system. Abbreviations: gRNA A5: gRNA targeting murine and human Ascl1 promoters, gRNA N2: gRNA targeting murine and human Nurr1 promoters, scFv: single chain variable fragment antibody, Sso7d: S. solfataricus DNA binding protein 7d, VP64: four repeats of Herpes simplex virus protein VP16, VP160: ten repeats of Herpes simplex virus protein VP16. Data was derived from one experiment. Error bars represent mean ± SEM.

The activating effect of SpyTag vs SunTag system was therefore compared in a luciferase assay screen depicted in Figure 13. However, the SunTag system could not improve ASCL1 or NURR1 induction when compared to SpyTag and was therefore not used in further experiments (ASCL1 induction: SpyTag: 6.7 ± 0.9 vs SunTag: 4.4 ± 1.3-fold, NURR1 induction: SpyTag: 3.3 ± 0.1 vs SunTag: 3.1 ± 0.1-fold).
5.2.2.2 Requirement of a new screening platform for SAM and VPR systems

While the above screenings were performed two promising new systems termed VPR [90] and SAM [91] were published. VPR-based gene induction of NGN2 or NEUROD1 in human iPS cells was found to induce neuronal differentiation suggesting a sufficient level of gene activation to influence cell fate [90]. Here, dCas9 is fused to the transactivating domains of 4 x VP16 (VP64), P65 and RTA (see illustration in Figure 4E). The SAM system utilizes a new strategy with two additional loops added to the gRNA that are bound by the Phage protein MS2. MS2 is expressed as a fusion protein with the transcriptional activators P65 and HSF1 which allows recruitment of MS2-P65-HSF1 to MS2 aptamers at the gRNA (see Figure 4F for further details). This system is co-expressed with dCas9 fusion proteins such as dCas9-VP64 which was reported to significantly improve gene induction compared to dCas9-VP64 alone [91]. In order to test these new systems luciferase assays were performed using VPR and SAM for ASCL1 and NURR1 induction (Figure 14).

Surprisingly, SAM and especially VPR seemed to inhibit the expression of both genes showing each a 0.1 ± 0.0-fold induction in Figure 14A. In the dual luciferase system used for these screenings the firefly luciferase activity (induced by successful generation of ASCL1 or NURR1) was normalized to the activity of the constitutively expressed (SV40 promoter driven) renilla luciferase. This should compensate for variations in transfection efficiency or pipetting errors. Renilla luciferase activity however, seemed to be strongly influenced by VPR (17.3 ± 1.9-fold activation) and the Tet-O system (14.2 ± 1.0-fold activation) as depicted in Figure 14B. This suggested an interaction of the transcriptional activators with the SV40 promoter regulating renilla expression.

Figure 14C shows firefly activity without normalization to renilla and revealed the expected activating effects of the positive controls (Tet-O-Ascl1: 6.8 ± 0.6-fold, Tet-O-Nurr1: 9.6 ± 0.3-fold) but hardly an effect of SAM and VPR (both below two-fold activation). A closer look at the previously described screening experiments revealed that the so far used VP16 activation systems (including SpyTag and SunTag systems) did not influence renilla activity (data not shown). Nevertheless, the established luciferase assay platform was not suitable for further experiments using SAM or VPR systems. Most publications reporting CRISPR/Cas9 based gene induction show RT-qPCR data for quantification and comparative purposes [89-91, 110]. Further screenings were therefore performed by RT-qPCR to both omit the limitations of the luciferase assay system described above and to allow a better comparison with published data.
Figure 14: Using VPR and Tet-O-systems influences renilla activity rendering this system unsuitable for screening purposes

Luciferase assay measuring ASCL1 and NURR1 activity. (A) Firefly luciferase activity showing ASCL1 and NURR1 activation normalized to constitutively expressed renilla. Surprisingly, the use of the SAM and VPR systems seemed to have an inhibiting effect on the activity of both genes analyzed when firefly luciferase values were normalized to renilla luciferase activity. (B) Activity of the constitutively expressed renilla luciferase. Using the Tet-O-system and VPR had a strong activating effect on renilla luciferase activity rendering renilla luciferase activity unsuitable as control values for normalization. (C) Firefly luciferase activity showing ASCL1 and NURR1 activation without normalization to renilla. A clear activation upon overexpression of ASCL1 and NURR1 could be detected. Abbreviations: gRNA A5: gRNA targeting murine and human Ascl1 promoters, gRNA N2: gRNA targeting murine and human Nurr1 promoters, SAM: MS2-P65-HSF1 fusion protein, VP160: ten repeats of Herpes simplex virus protein VP16, VPR: VP64-P65-RTA fusion protein. Data was derived from one experiment. Error bars represent mean ± SEM.
Increasing the number of gRNAs has a synergistic effect on gene induction

VPR and SAM systems were both reported to strongly induce gene expression in the original articles [90, 91]. However, this could not be confirmed in the luciferase assay shown in Figure 14C even without normalization to renilla. A difference in the experimental setup between his thesis and the article introducing the VPR system was the number and position of gRNAs used. While the above described experiments included a single gRNA (gRNA A5), Chavez et al., 2015 used four gRNAs targeting the 900 bp upstream of the human ASCL1 transcription start site (Figure 15A) [90]. In order to test the reproducibility RT-qPCR analysis was performed using the four published gRNAs targeting the human ASCL1 promoter (hA1 – hA4).

Interestingly, while single gRNAs only had a limited activating effect on ASCL1 mRNA levels (each gRNA below 30-fold induction), combining three gRNAs (1.1 ± 0.2 x 10^2-fold induction) and especially four gRNAs (4.4 ± 0.1 x 10^3-fold induction) led to a synergistic activating effect as depicted in Figure 15B. The values for all four gRNAs closely resembled the findings of Chavez et al., 2015 [90] thus proving the reproducibility of the system.

Figure 15: Multiple gRNAs synergistically increase the activation of human ASCL1 in HEK293 cells

(A) Position and orientation of gRNAs used by Chavez et al., 2015 [90] for the induction of human ASCL1 in HEK293 cells. (B) RT-qPCR analysis of ASCL1 expression in HEK293 cells 48 h after transfection. While the activating effect of single gRNAs was low, combining three to four gRNAs had a synergistic activating effect on ASCL1 expression. Abbreviations: gRNAs hA1-hA4: gRNAs A-D targeting the human ASCL1 promoter, gRNA A5: gRNA targeting human and murine ASCL1 promoters, VPR: VP64-P65-RTA fusion protein. Data was derived from one experiment. Error bars represent mean ± SEM.
While single gRNAs have been successfully used for gene induction [91] and even direct reprogramming [108] other groups have reported similar findings of synergistic activating effects by increasing gRNA numbers [89, 106, 110, 113]. However, such a synergistic effect was not observed with the murine gRNAs tested earlier in this thesis (gRNAs A5 and N2, see Figure 11). The goal of this project was to implement a CRISPR/Cas9 based system for gene induction and direct reprogramming in vitro which could then as a next step be analyzed in mouse models in vivo. Due to sequence differences in the human and murine Ascl1 promoter region the human gRNAs (hA1 – hA4) do not bind to the murine promoter. It was therefore necessary to find new gRNAs targeting murine Ascl1 that also offer the synergistic activating effect observed for the human gRNAs.

The initial murine gRNAs such as gRNA A5 were designed based on high binding specificity meaning that the probability of binding off-targets was low (high off-target score). At this point a new online tool at https://benchling.com/ also offered a so-called on-target score for gRNAs indicating gRNAs with a putatively high binding affinity based on an algorithm by Doench et al., 2014 [135]. The three gRNAs with the highest on-target scores targeting the 250 bp upstream of the murine Ascl1 transcription start site termed mA1, mA2 and mA3 (Figure 16A) were tested in a first RT-qPCR screen.

Figure 16B shows a strong synergistic effect on murine Ascl1 mRNA levels when gRNAs mA1, mA2 and mA3 were combined reaching 1.0 ± 0.4 x 10^4-fold induction. Interestingly, when adding the previously used gRNA A5 the level of gene induction was reduced to 4.9 ± 0.4 x 10^3-fold showing that not all combinations of gRNAs are suitable for high levels of gene induction. Screening different combinations of two or three gRNAs suggested a maximal activation of Ascl1 when gRNAs mA1 and mA2 were used together as depicted in Figure 16C leading to 1.8 ± 0.2 x 10^3-fold activation. Adding gRNA mA3 did not further increase Ascl1 expression (1.7 ± 0.4 x 10^3-fold activation). Differences observed for the absolute numbers of gene induction between experiments are based on the fact that fold change values were calculated relative to the control condition with no or very low Ascl1 expression (Ct values between 33 and 36). Therefore, numbers can only be compared within an individual experiment.

Since an increase from three to four gRNAs had a positive effect in case of the human gRNAs in Figure 15B new murine gRNAs mA4 and mA5 were designed (see Figure 16A) to cover gaps between the previously tested gRNAs. However, Figure 16D shows that the additional gRNAs did not further increase Ascl1 mRNA levels leaving mA1 and mA2 as the most promising combination.
Figure 16: Combining two gRNAs synergistically increases Ascl1 induction in murine Neuro 2a cells. (A) Position and orientation of newly designed gRNAs targeting the murine Ascl1 promoter region at -1 to -250 nt upstream of the transcription start site. (B) RT-qPCR analysis of Ascl1 mRNA levels 48 h after transfection. While single gRNAs increased Ascl1 mRNA levels only moderately, combining gRNAs mA1, mA2 and mA3 had a synergistic activating effect. (C) RT-qPCR analysis of Ascl1 mRNA levels 48 h after transfection. Combining gRNAs mA1 and mA2 had the strongest activating effect which could not be further increased by the addition of gRNA mA3. (D) RT-qPCR analysis of Ascl1 mRNA levels 48 h after transfection. The addition of gRNAs mA4 and mA5 did not further increase Ascl1 expression when compared to gRNAs mA1 and mA2. Abbreviations: gRNAs mA1-mA5: gRNAs targeting the murine Ascl1 promoter, gRNA A5: gRNA targeting human and murine Ascl1 promoters, VPR: VP64-P65-RTA fusion protein. Data was derived from one experiment, error bars represent mean ± SEM.

5.2.2.4 SAM and VPR systems are superior to SpyTag for Ascl1 activation

Having established gRNAs mA1 and mA2 for murine Ascl1 activation, the SpyTag, VPR and SAM systems were compared next. Figure 17A shows a significant increase in Ascl1 mRNA levels when using VPR (423 ± 144-fold) or SAM (1.4 ± 0.1 x 10^3-fold) compared to the SpyTag system (3.1 ± 0.5-fold). These promising results raised the question whether a combination of SpyTag with SAM or VPR systems might improve the activating effect even further. The SAM system is based on recruitment of MS2-P65-HSF1 fusion proteins to MS2 aptamers at the gRNAs (see illustration in Figure 4) and co-expression of a dCas9 fusion protein. The idea therefore was to replace dCas9-VP160 by dCas9-12xSpyTag allowing recruitment of up to twelve VP160-SpyCatchers.
Surprisingly, the combination of SAM and dCas9-12x-SpyTag was less effective in Ascl1 activation than SAM and dCas9-VP160 as depicted in Figure 17B (0.10 ± 0.02 x 10³ vs 1.4 ± 0.1 x 10³-fold induction). In order to test whether recruitment of multiple VPR activating complexes to a single dCas9 protein would be beneficial, VPR-SpyCatchers were developed at the institute by J. Truong. Here, VP160 activators fused to SpyCatchers were replaced by VPR. However, this exchange in activators did not significantly affect Ascl1 induction as shown in Figure 17B (VP160-SpyCatchers: 3.1 ± 0.5-fold induction, VPR-SpyCatcher: 23.1 ± 5.3-fold induction). Furthermore, a combination of VPR-SpyCatchers and SAM was not beneficial for transcriptional activation as depicted in Figure 17B (SAM and VP160-SpyCatcher: 101 ± 25-fold, SAM and VPR-SpyCatcher: 106 ± 23-fold).

These data suggested an incompatibility of the SpyTag system with SAM and VPR possibly due to steric hindrances of the large activator complex being recruited to the SpyTag system.

**Figure 17:** VPR and especially SAM are superior to SpyTag for gene induction of Ascl1 in Neuro 2a cells

RT-qPCR analysis of Ascl1 mRNA levels 48 h after transfection. (A) Using SAM and VPR for Ascl1 gene induction significantly increased Ascl1 mRNA levels when compared to the SpyTag system. (B) Combining SpyTag with SAM slightly increased Ascl1 mRNA levels when compared to SpyTag alone but were lower than levels measured for SAM alone. Replacing VP160 of the SpyCatchers by VPR did not significantly increase Ascl1 induction neither alone nor in combination with SAM. The use of SAM or VPR without the SpyTag technology had the strongest effect on Ascl1 expression with best results obtained for SAM.

**Abbreviations:** gRNAs mA1, mA2: gRNAs targeting the murine Ascl1 promoter; SAM: MS2-P65-HSF1 fusion protein, VP160: ten repeats of *Herpes simplex virus* protein VP16, VPR: VP64-P65-RTA fusion protein. Data was derived from three independent experiments, error bars represent mean ± SEM, Kruskal-Wallis test, Dunn’s multiple comparison test, ns: not significant, *P < 0.05.
5.2.2.5 Combining SAM and VPR systems has a synergistic effect on Ascl1 expression

SAM and VPR were found to be the strongest activators tested in this project so far. It was therefore of interest whether it would be beneficial to combine these two systems without using the SpyTag system as illustrated in Figure 18A. Interestingly, the combination of SAM and VPR had a synergistic effect on Ascl1 induction reaching 7.3 ± 0.8 x 10^3-fold Ascl1 expression while an additive effect would have been expected at approximately 2.4 x 10^3-fold (0.20 ± 0.09 x 10^3-fold VPR alone, 2.2 ± 0.2 x 10^3-fold SAM alone). Although, this new combination of activators could not reach levels detected for direct Ascl1 overexpression (4.1 x 10^5 ± 1.0 x 10^5, Figure 18B) these results are very promising. Gene induction by the VPR system alone has already been shown to be sufficient to induce neuronal differentiation of iPS cells [90]. The significantly higher level of Ascl1 mRNA by combining SAM and VPR systems therefore might be a valuable tool for direct conversion of somatic cells to neurons. Interestingly, this synergistic effect of SAM and VPR systems for gene induction has not been reported by others so far.

Figure 18: Combining SAM and VPR synergistically increases Ascl1 mRNA levels in Neuro 2a cells
(A) Schematic illustration combining activators of the VPR system fused to dCas9 and the SAM complex which binds MS2 aptamers of modified gRNAs. (B) RT-qPCR analysis of Ascl1 mRNA levels 48 h after transfection. Combining SAM and VPR had a synergistic effect on Ascl1 expression which reached 7.3 ± 0.8 x 10^3-fold expression vs a calculated additive value of 2.4 x 10^3-fold (0.20 ± 0.09 x 10^3-fold VPR alone, 2.2 ± 0.2 x 10^3-fold SAM alone). Direct overexpression of Ascl1, led to an increase of 4.1 x 10^5 ± 1.0 x 10^5-fold Ascl1 mRNA levels. Abbreviations: gRNAs mA1, mA2; gRNAs targeting the murine Ascl1 promoter, SAM: MS2-P65-HSF1 fusion protein, VP64: four repeats of Herpes simplex virus protein VP16, VPR: VP64-P65-RTA fusion protein. Data derived from six independent experiments (three independent experiments for Ascl1 overexpression), error bars represent mean ± SEM. Kruskal-Wallis test, Dunn’s multiple comparison test, ns: not significant, *P < 0.05. Asterisk indicates significant changes to dCas9-VPR alone.
5 | RESULTS

Conversely, a recent article by Chavez et al., 2016 comparing SAM, VPR and the SAM-VPR combination shows no synergistic effect of the two systems [110]. Here, the induction of several genes including ASCL1 was analyzed in human HEK293 cells. These contradictory results could either be based on species differences (murine Neuro 2a cells vs human HEK293 cells) or differential effects of individual gRNAs used in the experiments.

In order to investigate this, gRNAs targeting the human ASCL1 promoter were designed with comparable properties (protospacer sequence and distance between gRNA binding sites/ to the TSS) as the murine gRNAs mA1 and mA2. Figure 19A shows an alignment of the 250 bp upstream of the transcription start sites of human and murine Ascl1. Large parts show sequence homologies including the binding site of gRNA mA1 (yellow) which could therefore also be used for human ASCL1 induction. gRNA mA2 (dark blue) also bound in a homology area, however with one mismatch at the 5' end of the target sequence. Therefore, a new gRNA termed seq. matched mA2 (grey) was designed targeting human ASCL1 at an almost identical sequence as mA2 with an adjusted 5' nucleotide. Due to additional nucleotides in the human ASCL1 promoter between the two gRNA binding sites the distance between mA1 and this new human seq. matched mA2 gRNA was increased when compared with the original mA1 and mA2 gRNAs. To account for possible distance effects a second gRNA termed dist. matched mA2 (green, Figure 19A) was designed targeting the human ASCL1 promoter at the same distance to mA1 as mA2 did in the murine Ascl1 promoter.

A comparative RT-qPCR analysis in Figure 19B revealed no significant differences of the sequence and distance matched gRNAs when used with either SAM or VPR systems alone in human HEK293 cells. Interestingly, when SAM and VPR were used together a trend towards higher activation was observed only with the sequence matched gRNA (3.9 ± 0.3 x 10³-fold for SAM, 4.9 ± 0.4 x 10³-fold activation for SAM with VPR) but not for the distance matched gRNA. However, this effect was not as prominent as in murine Neuro 2a cells earlier. In order to determine whether the synergistic effect in Neuro 2a cells required the combination of both gRNAs (mA1 and mA2), an additional RT-qPCR analysis was performed. Using gRNA mA2 alone with SAM and VPR systems did not lead to a significant activation of Ascl1 as shown Figure 19C. Interestingly, the combination of SAM and VPR had a synergistic effect when gRNA mA1 was used alone (VPR: 2.1 ± 03, SAM: 69.7 ± 17.1, SAM and VPR: 133.0 ± 34.6). However, the activating properties of gRNA mA1 alone were limited when compared to the combination of gRNAs mA1 and mA2 reaching 3.7 ± 0.9 x 10³-fold induction (Figure 19C).
Figure 19: The synergistic effect of SAM and VPR seems to be based on the gRNA sequence

(A) Alignment of human ASCL1 and murine Ascl1 promoter regions 250 nt upstream of the transcription start sites. (‘) indicates sequence homology, (−) indicates a mismatch, (-) indicates a gap in the sequence. gRNA mA1 (yellow) bound both sequences. For gRNA mA2 (blue) a human equivalent termed seq. matched mA2 (grey) was designed with an adjusted 5′ nucleotide. The human gRNA dist. Matched mA2 (green) was designed to compensate the additional nucleotides in the human ASCL1 promoter thus allowing binding at the same distance to gRNA mA1 as gRNA mA2 did in case of murine Ascl1. A Gli2 binding site (red rectangle) was identified adjacent to gRNA mA2 as gRNA mA2 bound both sequences. For gRNA dist. Matched mA2, a synergistic effect when SAM and VPR were combined, was observed for the induction of Ascl1, with a synergistic effect when SAM and VPR were combined. Using both gRNAs with SAM and VPR together however again synergistically increased the level of induction. (D) Illustration of GLI protein family consensus binding site derived from Genomatix software by D. Trüm bach. The larger a nucleotide symbol, the more conserved it is. Abbreviations: Gli: Glioma-associated oncogene family zinc finger, SAM: MS2-P65-HSF1 fusion protein, VP64: four repeats of Herpes simplex virus protein VP16, VPR: VP64-P65-RTA fusion protein. Data was derived from three independent experiments. Error bars represent mean ± SEM, Kruskal-Wallis test, Dunn’s multiple comparison test, ns: not significant, *P < 0.05.
The role of the transactivation domains comprising SAM and VPR fusion proteins is the recruitment of the transcription machinery to the targeted promoter. The exact binding site of the dCas9 complex in relation to binding sites of transcriptional activators or inhibitors likely influences the transcriptional induction. Binding of dCas9 without any fused transcriptional activators to promoter regions was previously shown to silence gene expression [136]. This may be based on blocked binding sites of endogenous transcription factors or the transcription initiation complex. Conversely, when using dCas9 variants for gene induction it may be favorable to block binding sites of repressor proteins or to recruit endogenous transcription factors to their natural binding sites near the dCas9 complex.

A potential candidate for such a gain-of-function effect is Glioma-associated oncogene family zinc finger 2 (GLI2) which has been reported to bind and induce the murine Ascl1 promoter [137]. A transcription factor binding site analysis by D. Trüm bach revealed a Gli2 binding site just downstream of gRNA mA2 indicated in red brackets in Figure 19A. Recruitment of transcriptional activators to this Gli2 binding site by SAM and VPR systems might therefore benefit Ascl1 expression. Interestingly, this binding site is not conserved in the human Ascl1 promoter due to single nucleotide exchanges (red nucleotides in Figure 19A, please note: GLI2 binds the reverse strand at the sequence indicated in Figure 19D). This may contribute to the differences in gene induction observed in human vs murine cells by SAM and VPR systems but has to be investigated in further detail.

Taken together, these data suggested that the choice of gRNAs and activator system are critical and that common rules for the prediction of a suitable system are difficult. Nevertheless, the SAM-VPR system described above seemed to be the most suitable for murine Ascl1 induction and was therefore chosen for further experiments.

5.2.3 Design and generation of lentiviral vectors for SAM and dCas9-VPR delivery

After having established a suitable system for Ascl1 induction, an efficient delivery system was required for reprogramming experiments. Lentiviruses were chosen due to the high packaging capacity and transduction efficiency as well as the independency of cell replication for the integration into the host genome. Figure 20A shows the basic components of a third-generation lentiviral vector which allows delivery of up to 7 kb additional insert reaching a total length of 10 kb.

An Eukaryotic translation initiation factor 1A (Efi1a) promoter was chosen for the expression of the genes of interest due to its stable and high activity in a wide range of tissues including fibroblasts and neurons [120, 138] and common use in lentiviruses [91, 139].
A

3rd generation lentiviral backbone  

\[ \text{LTR} \quad \Psi \quad \text{RRE} \quad \text{cPPT} \quad / / \quad \text{Up to 7 kb insert} \quad / / \quad \text{WPRE} \quad 3' \text{LTR} \]

B

\[ \text{hU6-mA2-miniEf1a-dCas9-VPR} \quad / / \quad \text{hU6} \quad \text{gRNA mA2} \quad \text{miniEf1a} \quad \text{dCas9-4xVP16-P65:RTA} \quad \text{miniWPRE} \quad / / \]

C

\[ \text{hU6-mA1-Ef1a-SAM-N-dCas9} \quad / / \quad \text{hU6} \quad \text{gRNA mA1} \quad \text{Ef1a} \quad \text{MS2:65:HSF1:Flag} \quad \text{P2A} \quad \text{Flag:dCas91-573:DNA-E-n} \quad / / \]

\[ \text{hU6-mA2-Ef1a-C-dCas9-VPR} \quad / / \quad \text{hU6} \quad \text{gRNA mA2} \quad \text{Ef1a} \quad \text{dnaE-c-dCas91-574:1364xVP16:65:RTA:MyTag} \quad / / \]

Figure 20: Schematic illustrations of lentiviral vectors carrying gRNAs, dCas9 and activator components

(A) Structure of a third generation lentiviral backbone flanked by long terminal repeats (LTRs). Further important components are the packaging signal \( \Psi \), the rev response element (RRE) that enables export of transcripts to the cytoplasm, the central polypurine tract (cPPT) which serves as recognition site for proviral DNA synthesis and the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) that supports mRNA export to the cytoplasm. Up to 7 kb of DNA including promoters can be added to the lentiviral vector for gene delivery. (B) Lentiviral vector carrying gRNA mA2 and dCas9-VPR driven by an intronless Ef1a promoter. Furthermore, a shorter version of WPRE was used in order to stay below the packaging limit. (C) Illustration of the SAM split dCas9-VPR system. The two vectors contained all necessary components for gene induction of murine Ascl1 by SAM and VPR. One vector carried gRNA mA1, SAM and N-dCas9 fused to N-intein (dnaE-n). The second vector contained gRNA mA2 and a C-intein-(dnaE-c)-C-dCas9-VPR fusion. Upon interaction N-intein and C-intein splice themselves out and generate a seamless dCas9-VPR protein. Abbreviations: C-dCas9: C-terminal dCas9 residues 574-1368, dnaE-c: C-terminal part of DNA polymerase III subunit alpha (C-intein), dnaE-n: N-terminal part of DNA polymerase III subunit alpha (N-intein), Ef1a: Eukaryotic translation initiation factor 1A promoter, VP16: Herpes simplex virus protein vmw65, P65: P65 subunit of human NF-κB, RTA: Human herpesvirus 8 Regulator of transcription activation, N-dCas9: N-terminal dCas9-residues 1-573, P2A: 2A sequence of Porcine teschovirus-1.

Furthermore, in contrast to the Tet-O system there would be no need to co-transduce cells with an additional lentivirus carrying the rTTA2 activator. In chapter 5.1.1 disadvantages of using multiple viruses were described with only a fraction of cells being transduced by all viruses thus decreasing reprogramming efficiencies. Therefore, the goal was to deliver all components required (gRNAs mA1 and mA2, SAM and dCas9-VPR) with the lowest possible number of viruses. However, the combination of one gRNA and Ef1a-dCas9-VPR already exceeds the lentiviral packaging capacity of 10 kb. This packaging limit is not clear-cut but titers decrease in a semi logarithmic fashion with increasing length [140]. Two strategies were therefore tested in order to decrease the length. First: reducing the size of the Ef1a promoter and the WPRE element in the lentiviral backbone and second: using an intein-mediated dCas9-VPR split version similar to the split-Cas9 system previously established at the institute [141].
Figure 20B shows a lentiviral vector containing gRNA mA2, an intronless Ef1a promoter (nucleotides 233-1179 deleted) and a shortened WPRE element (WPRE3) [142]. This allows an overall reduction in size by 1.3 kb to 9.2 kb and thus below the packaging limit. The second strategy with a split-dCas9-VPR system is depicted in Figure 20C. Here, the N-terminal residues 1-573 of dCas9 were fused to dnaE-n (N-intein) and the remaining C-terminal residues 574-1368 of dCas9 were fused to VPR and dnaE-c (C-intein). This resulted in the generation of two fusion proteins of which N-intein and C-intein spliced themselves out upon interaction leaving a seamless dCas9-VPR protein [141, 143]. In this case C-dCas9-VPR was combined with gRNA mA2 and N-dCas9 with gRNA mA1 and SAM. In order to simplify nomenclature this system is termed SAM split-dCas9-VPR in the following.

Figure 21: The SAM split-dCas9-VPR system proves to be suitable for gene induction
RT-qPCR analysis of Ascl1 induction 48 h after transfection of Neuro 2a cells. (A) The use of an intronless Ef1a and shorter WPRE version was found to result in an approximately threefold reduction of Ascl1 induction. Representative RT-qPCR run of two independent experiments. (B) The SAM split-dCas9-VPR system reached activation levels for Ascl1 which were not significantly decreased when compared to the expression of SAM and VPR from individual vectors. Abbreviations: C-dCas9: C-terminal dCas9 residues 574-1368, gRNAs mA1, mA2: gRNAs targeting the murine Ascl1 promoter, N-dCas9: N-terminal dCas9-residues 1-573, SAM: MS2-P65-HSF1 fusion protein, VPR: VP64-P65-RTA fusion protein. Data was derived from three independent experiments. Mann-Whitney test, ns: not significant. Error bars represent mean ± SEM.

Although 2A sequences are not cleaved completely as described in chapter 5.1.3 for the ALN vector, a P2A sequence was chosen to separate SAM and N-dCas9. As an alternative an IRES could have been used for the translation of N-Cas9 from a bi-cistronic mRNA. However, the downstream gene of IRES is known to be expressed at lower levels [144, 145] perturbing stoichiometric expression levels of N-dCas9 and C-dCas9. Furthermore, only one 2A sequence was used instead of two in the ALN construct (see Figure 6A) thus putatively reducing the overall effects of fusion proteins.
Last but not least, P2A has been shown to have the highest cleavage efficiency of commonly used 2A sequences in a variety of different cell types and was therefore chosen [119].

RT-qPCR analysis of the newly generated construct comprising an intronless Ef1a promoter and a short version of WPRE suggested a much lower level of Ascl1 induction compared to the full-length version depicted in Figure 21A and was therefore not followed up any further (6.1 ± 0.9 vs 1.8 ± 0.2 x 10³-fold induction). The new SAM split-dCas9-VPR system was not significantly different to SAM and dCas9-VPR expressed from individual vectors although a slight decrease in activity from 7.8 ± 1.0 x 10³ to 5.8 ± 0.9 x 10³-fold induction can be observed in Figure 21B. The SAM split-dCas9-VPR system was therefore used for further experiments and virus production since packaging limits were not exceeded and all components required for the induction of Ascl1 could be carried by just two lentiviruses.

5.2.4 Challenges in lentiviral Cas9 packaging

Having established the functionality of the newly generated SAM split-dCas9-VPR system by lipofection and RT-qPCR the next step was lentiviral production for reprogramming experiments. However, in several attempts lentiviral titers were low with only 1 x 10⁵ - 1 x 10⁶ total infectious particles per harvest whereas former harvests e.g. for ALN lentiviruses were in the range of 1 x 10¹⁰ - 1 x 10¹¹ viral particles. With such a low titer a complete harvest (1.5 x 10⁵ viral particles) would be required for a single well of a 24-well plate in order to transduce cells at a multiplicity of infection of three (MOI 3). Titers from colleagues also working with lentiviral Ef1a-Cas9 vectors were comparably low suggesting possible adverse effects of the dCas9 coding sequences or gRNA secondary structures for lentiviral packaging (personal communication). This was surprising, as similar designs have been published comprising e.g. an Ef1a promoter and dCas9-VP64 [91]. However, in this publication selection markers were used to obtain a stable cell line where low numbers of transduced cells are not the limiting factor. Unfortunately, such a selection process is not suitable for reprogramming of primary cells (unpublished experimental data of collaborating group).

One alternative to viral delivery of Cas9 would be lipofection which was utilized for the conversion of embryonic stem cells to extraembryonic lineages [106]. However, the efficiency of gene delivery in astrocytes was found to be << 1% by lipofection (approximately 70 ± 20 total cells 14 days after seeding 5 x 10⁴ cells, data not shown) compared to approximately 30 – 40% transduced cells by lentiviruses. This underlines the need for an efficient lentiviral delivery system.
Therefore, one idea was to invert the cassette including the gRNA sequence, dCas9 components and activator complexes in order to prevent unintentional translation from the viral RNA during packaging or possible inhibitory effects due to secondary structures of the gRNA. However, a first RT-qPCR analysis suggested a strong decrease in Ascl1 induction from $4.7 \pm 0.1 \times 10^3$ to $0.30 \pm 0.02 \times 10^3$-fold upon inversion of the cassette as depicted Figure 22A. This system was therefore not used for further tests. Another possible solution was the exchange of the Ef1a promoter as Black et al., [107] report in a current article for the first time reprogramming of fibroblasts to neurons using dCas9 expressed from lentiviruses by a human Ubiquitin C (hUBC) promoter. This was surprising as hUBC is a comparably weak promoter [120] and it was expected that high levels of expression would be beneficial for successful reprogramming (see Figure 8B and C). Nevertheless, sufficient lentiviral packaging must have been accomplished by Black et al., [107].

Therefore, lentiviral production was repeated with a hUBC promoter and additionally a Tet-O promoter as an alternative known for its high levels of expression [120]. Interestingly, the replacement of the Ef1a promoter increased lentiviral titers to $3 \times 10^8$ (hUBC-Cas9) and $1 \times 10^9$ (Tet-O-Cas9) total viral particles per harvest. As expected, Tet-O-Cas9 was expressed at much higher levels than hUBC-Cas9 which was close to the detection limit of the immunocytochemistry analysis depicted in Figure 22C.

The Tet-O construct was chosen for further experiments due to its high level of expression and the possibility to influence timing and activity by adjusting the doxycycline concentration (see chapter 5.1.5). A RT-qPCR screen shown in Figure 22B comparing the SAM split-dCas9-VPR system using Ef1a or Tet-O promoters confirmed the functionality of the new Tet-O constructs. Western blot analysis in Figure 22D using a Cas9 antibody revealed successful generation of N-dCas9 at 80 kDa from newly generated viruses with comparable band patterns and intensities to transfected cells. A fraction of total N-dCas9 was found as a fusion protein with SAM at 130 kDa due to inefficient ribosome skipping at the P2A site separating SAM and N-dCas9. Upon addition of C-dCas9-VPR viruses a shift in size was observed indicating successful assembly of N- and C-dCas9 parts at 218 kDa.

These findings were confirmed by visualization of C-dCas9-VPR via a MycTag in a second western blot in Figure 22E. The newly generated SAM split-dCas9-VPR viruses were therefore successfully packaged and dCas9-VPR was able to assemble itself after transduction.
Figure 22: Replacement of Ef1a by Tet-O or hUBC promoters enables lentiviral packaging of dCas9

(A) RT-qPCR analysis of Ascl1 induction in Neuro 2a cells 48 h after transfection. Inversion of the expression cassette of split-dCas9-VPR (hU6-mA1-Ef1a-SAM-N-dCas9 + hU6-mA2-Ef1a-C-dCas9-VPR) resulted in a strong decrease in Ascl1 expression. In this case, expression cassettes including promoters and gRNAs were inverted relative to the lentiviral backbone (see Figure 20C for comparison). (B) RT-qPCR of Ascl1 induction comparing split-dCas9-VPR system with Ef1a or Tet-O-promoters revealed similar levels of activation for the two promoters. (C) Immunocytochemistry analysis of Cas9 in HEK293 cells 48 h after transduction by 0.5 µl lentiviruses encoding hUBC-Cas9 or Tet-O-Cas9. While the percentage of Cas9+/DAPI+ cells was comparable the expression level in hUBC-Cas9 transduced cells was much lower as suggested by the different intensities of the Cas9 signal. (D) Western blot analysis using an α-Cas9 antibody detecting N-terminal Cas9 only. Viruses were functional as assessed by comparable bands of transfected and transduced cells. The P2A splicing between SAM and N-dCas9 was not complete as expected. However, with the majority of N-dCas9 correctly cleaved. Upon adding C-dCas9-VPR N- and C-parts assembled to dCas9-VPR at 218 kDa. (E) Western blot analysis using an α-MycTag antibody for the detection of C-dCas9-VPR. The C-dCas9-VPR virus was functional with protein bands similar to transfected cells showing successful assembly of dCas9-VPR upon addition of N-dCas9. Abbreviations: C-dCas9: C-terminal dCas9 residues 574-1368, Ef1a: Eukaryotic translation initiation factor 1A promoter, N-dCas9: N-terminal dCas9-residues 1-573, SAM: MS2-P65-HSF1 fusion protein, hUBC: human ubiquitin C promoter, VPR: VP64-P65-RTA fusion protein. Scale bars: 50 µm. Data was derived from one experiment. Error bars represent mean ± SEM.
5.2.5 Direct reprogramming of astrocytes to neurons utilizing SAM and VPR

As a final step the reprogramming potential of the established SAM split-dCas9-VPR system was tested in primary cortical astrocytes isolated from CD1 mice at the age of five to six days. This system was chosen over MEFs as astrocytes are closer to a future in vivo situation where viruses should be injected into the mouse brain. Forskolin was again added to the differentiation medium (see Table 6) since conversion efficiency of astrocytes to neurons and their survival are increased upon induction of the forskolin target gene B-cell leukemia/lymphoma 2 (Bcl2) [128, 146].

The disadvantage of primary cortical cells is the composition of the cell population which also includes neuronal precursors. In order to distinguish between newly reprogrammed neurons and those derived from precursors already present in the culture at the day of lentiviral transduction dsRed expressing lentivirus was added to control wells.

Figure 23A shows dsRed+ cells at day 16 post transduction of which a fraction was also MAP2+ indicating the basal level of neurons/neuronal precursors being transduced by lentiviruses. Immunocytochemistry analysis of cells transduced by SAM split-dCas9-VPR revealed FlagTag+ cells (marker for N-dCas9 and SAM) which were also MAP2+ suggesting putatively successful reprogramming of astrocytes to neurons in Figure 23A. Direct overexpression of Ascl1 clearly resulted in an increase of MAP2+ cells of which most seemed to be also ASCL1+ (Figure 23A).

Figure 23B shows a quantification of MAP2+ cells per DAPI with 9.3 ± 1.6% neurons per DAPI in the control condition (dsRed virus). This percentage was doubled to 18.5 ± 3.6% MAP2+ cells in the SAM split-dCas9-VPR condition demonstrating successful reprogramming of astrocytes to neurons. The increase in neurons was not based on a higher transduction efficiency of SAM split-dCas9-VPR encoding lentiviruses as Figure 23C shows comparable transduction efficiencies of the lentiviruses used. When only looking at transduced cells (dsRed+ or FlagTag+) the percentage of MAP2+ cells slightly increased from 13.4 ± 2.4% (dsRed) to 18.7 ± 2.2% (SAM split-dCas9-VPR) in Figure 23D.
Figure 23: Successful conversion of astrocytes to neurons utilizing the SAM split-dCas9-VPR system delivered by lentiviruses

(A) Immunocytochemistry analysis of reprogrammed cells 16 days after transduction revealed successful reprogramming of astrocytes to neurons using the SAM split-dCas9-VPR system. Tet-O-dsRed lentivirus was used at MOI 1. hU6-mA1-Ef1a-SAM-N-dCas9 at MOI 3. 0.5 µl of hU6-mA2-Ef1a-C-dCas9-VPR virus were used due to difficulties in the detection of the MycTag for titer determination and Tet-O-Ascl1 was used at MOI 1. (B) Quantification of MAP2+ neurons/DAPI revealed an increase from 9.3 ± 1.6% (dsRed) to 18.5 ± 3.6% MAP2+ cells for SAM split-dCas9-VPR and 35.0 ± 3.0% (Tet-O-Ascl1) MAP2+ cells suggesting successful reprogramming. (C) Quantification of transduced cells per DAPI. dsRed served as control for the transduction rate of neurons already present in the mixed culture at the day of transduction. The percentage of FlagTag+ (detection of N-dCas9 and SAM) and ASCL1+ (detection of Tet-O-Ascl1) cells was comparable to dsRed. (D) Quantification of MAP2+ neurons per transduced cells. Only dsRed+, FlagTag+ or ASCL1+ cells were checked for co-expression of MAP2. 13.4 ± 2.4% of dsRed transduced cells were MAP2+ indicating the basal fraction of neurons transduced by viruses. The use of SAM split-dCas9-VPR revealed a slight increase in MAP2+/FlagTag+ cells to 18.7 ± 2.2% indicating successful conversion of astrocytes to neurons. 64.4 ± 5.5% of all ASCL1+ cells were MAP2+. Abbreviations: MAP2: Microtubule-associated protein 2, SAM: MS2-P65-HSF1 fusion protein, TH: Tyrosine hydroxylase, VPR: VP64-P65-RTA fusion protein. Scale bars: 50 µm. Data was derived from two independent experiments. Error bars represent mean ± SEM.
These promising data indicated successful proof of concept experiments for the SAM split-dCas9-VPR system. Direct overexpression of Ascl1 as a positive control (64.4 ± 5.5% MAP2⁺ neurons / transduced cells) exceeded the effects of the SAM-VPR system putatively due to the higher expression level of Tet-O-Ascl1 already observed in RT-qPCRs and also based on the fact that the split-Cas9 system required co-transduction and assembly of N-dCas9 and C-dCas9. However, the CRISPR/Cas9 system is a promising new tool for direct conversion of somatic cells and might show its full potential when several genes have to be induced simultaneously e.g. for the generation DA neurons where common reprogramming strategies reach their limits.

RT-qPCR data revealed a synergistic effect when SAM and VPR systems were combined. In order to investigate a possibly similar effect for reprogramming different dCas9 versions were tested. At this point viruses carrying SAM or VPR alone were not available. DNA was therefore transferred using Lipofectamine LTX with Plus Reagent into astrocytes. Transfection efficiency of these primary cells was much lower than viral transduction (< 1%) and transiently expressed genes were hardly detectable by immunocytochemistry 16 days after transfection (data not shown). Therefore, dsRed which was still detectable at this time point was co-transfected with dCas9 components. All dsRed⁺ cells were analyzed for MAP2 expression and a neuronal morphology.

Figure 24A shows successfully reprogrammed cells for all dCas9 fusion proteins analyzed. Reprogramming efficiencies were quantified in Figure 24B showing successful reprogramming by SAM (15.2 ± 0.9% MAP2⁺ cells per dsRed), VPR (9.8 ± 2.9% MAP2⁺ cells per dsRed) or the combination of SAM and VPR (SAM split-dCas9-VPR, 9.8 ± 2.2% MAP2⁺ cells per dsRed). A synergistic effect of SAM and VPR however, could not be observed. This has to be analyzed in further experiments in detail.

Taken together, these reprogramming experiments for the first time show successful CRISPR/Cas9 based conversion of astrocytes to neurons. This is a very promising starting point for further optimizations of the system in order to increase reprogramming efficiencies and to investigate simultaneous activation of multiple genes.
Figure 24: The reprogramming potential of SAM or VPR alone is comparable to the SAM split-dCas9-VPR system in lipofection experiments.

(A) Immunocytochemistry analysis shows dsRed+/MAP2+ cells indicating successful reprogramming 16 days after lipofectamine LTX transfection of astrocytes for all dCas9 variants tested. dsRed was co-transfected with all conditions in order to identify transfected cells. (B) Quantification of reprogrammed cells 16 days after lipofection. All dsRed+ cells were checked for neuronal morphology and MAP2 expression. Due to different background levels of neurons in control wells, the percentage of dsRed+ neurons in control wells was subtracted from all wells of an individual experiment. SAM alone seemed most potent for reprogramming but was not significantly different from dCas9-VPR or the combination of SAM and dCas9-VPR. Scale bars: 50 µm. Data was derived from three independent experiments, error bars represent mean ± SEM, Kruskal-Wallis test, Dunn’s multiple comparison test, ns: not significant, *P < 0.05.
6 Discussion

6.1 Limitations of exogenous gene expression for direct reprogramming

6.1.1 The requirement of co-transductions limits the reprogramming efficiency

Direct conversion of somatic cells such as fibroblasts to DA neurons is seen as a promising source for cell replacement therapies in PD. For this purpose, combinations of three to six different transcription factors are usually delivered by individual lenti- or retroviruses [32-37]. However, in chapter 5.1.1 of this thesis it was shown that co-transduction is already a limiting factor when analyzing the co-expression of just two factors (Ascl1, Nurr1). Here, only a fraction of cells was ASCL1⁺/NURR1⁺ thus restricting the number of cells which could potentially be converted to DA neurons. In this case a multiplicity of infection of three (MOI 3) was used meaning that a threefold excess of viral particles was applied to cells.

One way to increase the transduction efficiency would be adding more viral particles. However, using Ascl1, Lmx1a and Nurr1 (A+L+N) encoding lentiviruses each at MOI 10 reduced the reprogramming efficiency by 90% indicating adverse effects of high lentiviral concentrations (see appendix Figure 26). Therefore, increasing the number of lentiviral particles was not found to be an adequate option to improve reprogramming efficiencies.

6.1.2 The high reprogramming efficiency of Caiazzo et al., seems to be influenced by the reporter system

Caiazzo et al., who originally described the A+L+N combination (unknown MOI) claimed a reprogramming efficiency of approximately 18% TH⁺ DA neurons [37]. These numbers could neither be reproduced in this thesis (approximately 2% TH⁺ cells, chapter 5.1.2) nor by others so far. A difference in the reprogramming protocols was the composition of the cell culture medium used during differentiation. While in the underlying thesis a 50:50 mixture of DMEM/F12 medium (addition of N2 supplement) and Neurobasal medium (addition of B27 supplement) was used (see Table 6 for further details), Caiazzo et al., conducted their differentiation in DMEM/F12 medium with N2 supplement comprising the same constituents as N2 but at partially altered concentrations [37, 147]. This included a five-fold insulin concentration, 0.5-fold transferrin and 0.1-fold putrescine (see appendix, Figure 27 for details) – three factors important for cell proliferation in serum free media [148]. However, since B27 supplement also comprises these factors - although at unknown concentrations due to confidentiality reasons - it is unclear how individual concentrations in the mixed medium of this thesis compare to the medium of Caiazzo et al.
Therefore, reprogramming experiments were repeated with the cell culture medium of Caiazzo et al. with no significant effect on the number of reprogrammed cells (appendix, Figure 27). Thus, the medium composition was excluded as possible cause for the low reprogramming efficiency.

A factor that might influence the reprogramming efficiency is the origin of the MEFs utilized. In the underlying thesis MEFs were derived from Pitx3\(^{GFP/+}\) mice [149] where TH expression has to be visualized by immunocytochemistry analysis. Caiazzo et al. utilized a transgene Th-Gfp reporter mouse line expressing Gfp under the control of the rat Th promoter [150] and GFP\(^+\) cells were counted as successfully reprogrammed TH neurons. The GFP\(^+\) domain in the respective mouse model however, was found to exceed the TH\(^+\) population with only 60% of GFP\(^+\) cells also expressing TH [150]. The numbers published by Caiazzo et al. may therefore overestimate the reprogramming efficiency. Taken together, co-transduction by multiple lentiviruses limits the reprogramming efficiency which could not be overcome by increasing the virus concentration or by changing the medium composition.

### 6.1.3 Forskolin treatment enables the generation of PITX3\(^+\) DA neurons

In order to improve reprogramming efficiencies small molecules can be beneficial as an alternative to the addition of lineage specific transcription factors [121]. For example, forskolin (activator of cAMP signaling) together with dorsomorphin (inhibitor of bone morphogenic protein (BMP) signaling) has previously been shown to enable direct conversion of Neurogenin2 transduced fibroblasts to cholinergic neurons, while Neurogenin2 alone was not sufficient for direct reprogramming [121]. The authors did not investigate the underlying mechanisms of forskolin and dorsomorphin regarding the reprogramming process but it became clear that activating general pathways such as cAMP signaling can benefit neuronal differentiation.

In chapter 5.1.6 of this thesis, forskolin treatment of A+L+N transduced MEFs was found to result in a slight increase in the reprogramming efficiency to TH\(^+\) DA neurons. Interestingly, forskolin does not affect TH expression but only the translation of TH mRNA via induction and binding of Poly(rC)-binding protein 2 (PCBP2) to the 3'-UTR of TH mRNA [126, 127]. The observed effect may therefore be based on higher levels of TH protein in low expressing cells which could now be detected by immunocytochemistry. Alternatively, the pro-survival effect of forskolin may play a role. Forskolin has previously been described to induce anti-apoptotic responses via upregulation of Bcl2 and inhibition of Caspase 3 and Caspase 9 [128]. This protective effect of forskolin seems to be based on reduced lipid peroxidation which is a hallmark of ferroptosis [146, 151]. Nevertheless, a general effect on the survival of cells by forskolin treatment was not observed in this thesis as assessed by the number of DAPI\(^+\) cells per mm\(^2\). This could be explained by the findings of Michel et al., who report a
specific effect of increased cAMP signaling on the survival of mdDA neurons in vitro while e.g. GABAergic and serotonergic neurons showed a much lower dependency [152]. With approximately 1 – 2% of all cells being reprogrammed to TH+ neurons these putatively cell type specific anti-apoptotic effects of forskolin therefore hardly affect the overall number of surviving cells but seem to be beneficial for the reprogrammed cells.

While several groups report direct conversion of fibroblasts to DA neurons, these cells often do not express the midbrain DA marker Pitx3 [33-35, 37] meaning that these cells do not correspond to the DA subtype affected in PD [14]. Previous experiments at the institute confirmed that A+L+N overexpression is not sufficient for the generation of PITX3+ DA neurons (dissertation F. Meier). This is somewhat surprising as both Lmx1a and Nurr1 were found to directly activate Pitx3 expression [52, 62]. A possible reason might be an inaccessibility of NURR1 and LMX1A binding sites in MEFs. In this thesis treatment of A+L+N transduced MEFs with the cAMP signaling activator forskolin was found to enable the generation of PITX3+ DA neurons. This is a very promising result, as previous reprogramming protocols required co-delivery of five to six different transcription factors to a single cell for the generation of PITX3+ DA neurons [32, 36].

Activation of cAMP signaling is known to support the differentiation of progenitor cells to mdDA neurons and is therefore commonly induced during terminal differentiation protocols of iPS cells, embryonic stem cells or ventral midbrain tissue [153-155]. A connection between forskolin and the direct reprogramming to DA neurons and Pitx3 expression however, had not been made yet. One possible link between cAMP signaling and Pitx3 expression could be Glial cell-line derived neurotrophic factor (GDNF). GDNF is a highly selective neurotrophic factor implicated in the survival of DA neurons [156] which has been shown to induce Pitx3 expression [15]. The pro-survival effect of GDNF on DA neurons was found to be potentiated by cAMP signaling in vitro suggesting a crosstalk between cAMP and GDNF signaling pathways [157]. Whether forskolin indeed influences Pitx3 expression via GDNF signaling however remains to be explored.

In conclusion, the addition of forskolin seems to support direct reprogramming of MEFs to DA neurons either via up-regulating TH translation or due to pro-survival effects. Furthermore, forskolin treatment of A+L+N transduced cells enabled the generation of ‘true’ mdDA neurons which is a promising starting point for further functional tests of these neurons in vivo.
6.1.4 Inefficient ribosome skipping at 2A sites results in fusion proteins and cell death

In order to overcome the limitations of co-transduction, a tri-cistronic lentiviral vector encoding Ascl1, Lmx1a and Nurr1 (ALN) was previously generated by F. Meier at the institute. Here, the three transcription factors were separated by 2A peptides (P2A, T2A) inducing ribosome skipping during protein biosynthesis and subsequent production of ‘cleaved’ ASCL1, LMX1A and NURR1 proteins. Surprisingly, reprogramming efficiencies with the tri-cistronic ALN vector were quite low (< 1% TH+ cells). In this thesis, the generation of fusion proteins in large quantities could be shown in chapter 5.1.3 which likely accounts for the low reprogramming efficiency of the ALN construct.

While some reports claim almost complete separation of proteins at 2A peptides [158, 159] others have also reported the generation of fusion proteins [119]. P2A and T2A differ not only in their sequence but also in the efficiency of ‘cleavage’ which is strongly influenced by the cell type ranging from 50 – 90% for T2A and 80 – 95% for P2A [119]. Across a number of cell types P2A was found to have the highest efficiency in peptide separation of all tested 2A sequences [119]. Using only P2A due to the higher performance rate could be an option for DNA based gene delivery, but in lentiviruses repeat sequences bear the risk of recombination and thus deletions [160]. Therefore, P2A and T2A were used in the tri-cistronic ALN vector. Inefficient cleavage of P2A and T2A peptides added up generating a high proportion of fusion proteins consisting of two or three transcription factors. The low reprogramming efficiency of ALN may therefore be caused by fused and thus inactive transcription factors resulting in lower expression of their target genes.

However, incorrect or completely unfolded transcription factors can also trigger a process termed unfolded protein response [161]. This was observed in a conditional Rosa26\textsuperscript{CAG:ALN+} mouse line generated at the Helmholtz Zentrum München which carries the tri-cistronic ALN cassette [118]. These mice were crossed to Tnap\textsuperscript{TgCreERt2/+} and Nestin-Cre mice for in vivo analysis of the reprogramming potential in pericytes and neural stem/precursor cells, respectively [118]. Surprisingly, while TH+ neurons were obtained in vitro, albeit at low numbers, in vivo reprogramming to DA neurons was completely absent. Further analysis in vivo revealed apoptosis in ALN expressing cells partially due to endoplasmatic reticulum stress and unfolded protein response [118]. This was not investigated in detail in vitro but likely contributes to the reduced reprogramming efficiency of the tri-cistronic vector observed in this thesis.

Alternatives to 2A peptides could be internal promoters, IRES sequences or proteolytic processing e.g. by furins. However, when using multiple promoters transcriptional interference and promoter suppression have been reported especially in RNA viruses [162,
Furthermore, the size of the vector would be significantly increased by additional promoters thus exceeding the lentiviral packaging limit. As mentioned earlier, IRES dependent expression usually reaches only 20 – 50% of the upstream gene thus preventing high levels of expression at equimolar ratios [144]. Finally, proteolytic processing by furins seems to be highly efficient [158] but can only be applied to secreted proteins as furins reside in the golgi apparatus [164]. Taken together, with all strategies having some drawbacks co-transduction by individual lentiviruses may be better suited than a tri-cistronic approach. However, several groups are working with up to six different transcription factors for the conversion of fibroblasts to DA neurons [32-34, 36]. In these publications reprogramming efficiencies were rather low in the range of 0.05 - 2.5% TH^+ cells certainly also due to the required co-transduction of up to six different viruses.

In conclusion, direct conversion of somatic cells by overexpression of exogenous factors has several downsides. When using single viruses to deliver the genes of interest, the efficiency of co-transductions is a limiting factor. By choosing a multi-cistronic vector the necessity of co-transduction can be overcome but the generation of fusion proteins leading to cell death and a decrease in the reprogramming efficiency was detected. It is therefore of interest to develop new tools circumventing these disadvantages. One promising system is the CRISPR/Cas9 technology which may show its full potential especially when a number of genes needs to be induced simultaneously by a pool of sequence specific gRNAs.

**6.2 Utilizing CRISPR/Cas9 technology for gene induction and direct cell conversion**

**6.2.1 The importance of gRNA screenings for transcriptional activation**

The design and selection of suitable gRNAs is crucial for any CRISPR/Cas9 based application in order to ensure specific and strong binding to target sites. Several factors should be considered including on- and off-target scores, position of the targeted sequence relative to the gene of interest and the combination of gRNAs. In this thesis, initially a single gRNA (gRNA A5) was found to efficiently induce the expression of Ascl1 in Neuro2a and HEK293 cells (see chapter 5.2.1). Co-expression with other gRNAs did not result in synergistic effects. While this finding is in line with some reports [108, 109], by now most groups work with multiple gRNAs showing synergistic effects on gene induction [90, 106, 113, 165].

It was therefore of interest to find gRNAs providing such a beneficial synergistic effect as direct conversion of cells seems to require high levels of expression [166]. While gRNA A5 was chosen due to a low off-target score, the second generation of gRNAs targeting the
murine Ascl1 promoter (mA1 - mA5) was chosen due to high on-target scores suggesting improved binding to DNA. gRNAs mA1 and mA2 were identified as top ranked gRNAs binding the Ascl1 promoter with on-target scores of 57 and 61 out of 100, respectively. Indeed, these new gRNAs showed a synergistic effect when applied together. Interestingly, the level of gene induction could not be further increased by adding additional gRNAs mA3, mA4 or mA5 (on-target scores of 61, 30, 41, respectively). The total number of gRNAs required for a maximum effect seems to be highly gene and locus dependent ranging mostly from one to four gRNAs [89, 108, 165]. Interestingly, replacement of mA2 by mA3 resulted in a much lower Ascl1 expression level although binding sites (shift by five nucleotides) and on-target scores (both 61) were almost identical. Chromatin accessibility was found not to influence CRISPR/Cas9 based gene induction [113]. It has therefore been speculated that differential effects of gRNAs with comparable properties could be due to competitive binding events with endogenous transcription factors [108, 113]. This suggests that the on-target score can be suitable to identify gRNAs with excellent binding properties but does not necessarily predict gene induction potential.

When thinking ahead to possible in vivo applications of CRISPR/Cas9-based gene induction the risk of off-target effects should be addressed. gRNAs were previously found to partially bind off-target sequences despite mismatches [167]. In case of wt Cas9 nuclease off-target effects can have severe consequences due to unwanted double-strands breaks and subsequent frame-shift mutations [168]. For gene induction however, off-target effects only play a minor role. The requirement to bind near a transcription start site (ideally within the first 250 nt upstream of TSS [89, 91, 106]) in order to induce gene expression already decreases the number of loci that could be affected. Furthermore, the observed synergistic effect of multiple gRNAs also plays a protective role as co-binding of several gRNAs to a single off-target promoter seems even less likely. This is supported by the findings of Perez-Pinera et al., who report no detectable off-target effects of CRISPR/Cas9-based gene induction in a RNA-seq analysis [113].

To conclude this part, although tools are available to predict binding properties of gRNAs, screenings are still required to identify an optimal set of gRNAs to induce the expression of target genes at high levels.

6.2.2 Screenings of dCas9 fusion proteins for an efficient gene induction

Besides identifying a set of gRNAs the choice of a suitable dCas9 system for gene induction is just as important. In this thesis, several dCas9-fusion proteins were analyzed regarding their potential for gene induction with the aim to identify the system with the highest activation effect. An increase in VP16 repeats was found to benefit transcriptional activation when comparing dCas9-VP160 with the SpyTag system (up to 12 x VP160 repeats) and the
SunTag system (up to 24 x VP64 repeats) [100] in chapter 5.2.2.1. The role of the fused VP16 transactivation domains is the recruitment of factors involved in the formation of the transcription initiation complex such as the general transcription factor TFIIB [96, 97]. Besides simply offering an increased number of VP16 repeats the success of SpyTag and SunTag systems may also be based on a wider coverage of putative DNA binding sites for transcriptional activators due to the extended geometry of the fusion proteins. Without knowing where exactly binding of the transcription complex is required at the promoter of a particular gene, an increased coverage of the genomic DNA by VP16 copies could be helpful. Although the total number of VP16 repeats is even higher in case of SunTag, the SpyTag system seemed at least as powerful. This may be based on the underlying molecular mechanisms of VP16 recruitment. Interaction of SpyTag and SpyCatcher results in the formation of a permanent isopeptide bond and thus a lasting fusion of VP160-SpyCatchers with the dCas9-SpyTag protein [98, 99]. In the SunTag system, VP64-repeats are fused to scFv antibodies which bind SunTag arrays at the C-terminus of dCas9 resulting in a binding equilibrium. For a maximum effect 24 scFv-VP64 fusion proteins have to bind to a single dCas9 at any given time which may be a limiting factor.

VPR and especially SAM systems [90, 91] were found in chapter 5.2.2.4 to induce Ascl1 expression at significantly higher levels than the SpyTag system with gRNAs mA1 and mA2. This seems to be based on the additional transactivation domains (P65 and HSF1 for SAM, P65 and RTA for VPR) allowing the recruitment of further cell-intrinsic components of transcription complexes. Amongst them are well known factors implicated in the regulation of gene expression such as the TATA-box binding protein, CREB which acts downstream of cAMP signaling and chromatin remodelers of the SWI/SNF family [101, 103, 105]. A recent publication [110] comparing SAM, VPR and SunTag systems in HEK293 cells reports similar findings confirming the results obtained in murine Neuro2a cells. Surprisingly, combining either SAM or VPR with the SpyTag system was not beneficial. A possible explanation could be steric hindrance. The SAM complex binds hairpin aptamers at the gRNA and was published in combination with dCas9-VP64 [91]. Upon binding of up to twelve VP160-Spycatchers to dCas9-SpyTag the interaction of SAM and gRNA aptamers might be impaired. This would explain why the level of activation by SAM plus SpyTag system dropped even below the values measured for SAM with d-Cas9-VP64. This could not be overcome by replacing the 12xSpyTag by a smaller 4xSpyTag version (see appendix, Figure 28). Similarly, substituting VP160 of the SpyTags by VPR did not seem to be an advantage possibly also due to steric hindrances by the increased size of VP64-P65-RTA vs VP160. To address this point, peptide linker sizes between dCas9 and the SpyTag array as well as in-between individual SpyTags (currently GSGGKGGSGG linker) could be increased.
allowing a putatively improved accessibility. An alternative would be the commonly used and highly flexible (GGGGS)$_n$-linker which can be used as tandem repeats [169].

Ascl1 mRNA levels obtained by direct overexpression of Ascl1 (Tet-O-Ascl1 vector) exceeded the levels observed for CRISPR/Cas9 based gene induction in RT-qPCR analysis. However, this was not surprising as a single cell receives several copies of each plasmid and Ascl1 can be expressed from all Tet-O-Ascl1 constructs simultaneously [170]. Furthermore, Tet-O is a rather strong promoter system [120]. In case of the CRISPR/Cas9 system Ascl1 can only be expressed from the endogenous Ascl1 promoter. A quantitative comparison is therefore difficult and Tet-O-Ascl1 values should only be treated as a qualitative positive control showing the functionality of the system. In any case, the aim was not to compete with transcriptional levels obtained by direct overexpression but to induce endogenous Ascl1 expression to more closely resemble physiological processes and to take advantage of epigenetic remodeling of targeted loci [107].

Taken together, offering a variety of different transactivators fused to dCas9 improves the gene induction potential compared to multiple repeats of VP16. However, not all systems seem to be compatible with each other.

6.2.3 Synergistic activating effect of SAM and VPR systems

While a combination of SpyTag with SAM or VPR systems was not successful, co-expression of SAM and dCas9-VPR was found to result in a synergistic activation of Ascl1 expression in Neuro2a cells using gRNAs mA1 and mA2 (see chapter 5.2.2.5). With SAM being described as one of the most powerful activating systems so far [110], a further increase in transcriptional induction by combining two systems is very promising for later reprogramming experiments where high levels of expression seem to be required [166]. However, in a recent article Chavez et al., 2016 [110] also compared SAM, VPR and the combination of both systems. Surprisingly, no beneficial effect was reported upon combining these two systems for ASCL1 and three other genes in HEK293 cells [110].

These contradictory results for ASCL1 induction may be based on experimental differences regarding gRNAs and cell types utilized. A general Neuro 2a specific effect was excluded as SAM and VPR did not show such a synergistic effect when other genes were induced in these cells (J. Schwab, data not shown). Chavez et al., 2016 used four gRNAs (hA1 – hA4) targeting 1 kb upstream of the human ASCL1 TSS in HEK293 cells. In this thesis two gRNAs (mA1, mA2) binding within the first 250 bp upstream of the murine Ascl1 TSS were tested in Neuro 2a cells. The question therefore was whether the synergistic effect of SAM and VPR systems observed in Neuro 2a cells could be reproduced in human HEK293 cells with gRNAs closely resembling mA1 and mA2. Therefore, two new gRNAs termed dist. matched
mA2 (identical distance between targeting sequences as in case of murine mA1 and mA2) and seq. matched mA2 (except for 5' nucleotide identical protospacer sequence as mA2 but increased distance to mA1 by 23 nt) were designed. Interestingly, when using the dist. matched mA2 gRNA there was no difference in activation when using the SAM system alone or in combination with VPR. This indicated that the synergistic effect observed in Neuro 2a cells was not based on the distance of the activation systems relative to each other or the TSS of Ascl1.

However, in case of the seq. matched mA2 gRNA a trend towards increased ASCL1 expression but not a synergistic effect as in murine cells was observed in HEK293 cells for SAM plus VPR when compared to SAM alone. This effect therefore seems to be sequence specific and possibly based on interactions of the fused transactivation domains with endogenous factors regulating Ascl1 expression. It has to be analyzed in further detail whether e.g. GLI2 plays a role here. GLI2 is a known activator of Ascl1 [137] and a GLI2 binding site was found just downstream of gRNA mA2 in a Genomatix promoter analysis. Recruitment of transcriptional activators to this GLI2 binding site by SAM and VPR systems might therefore benefit Ascl1 expression. Interestingly, during the gRNA screening process in chapter 5.2.2.3 the addition of gRNA mA4 to gRNAs mA1 and mA2 resulted in a reduced level of Ascl1 induction. mA4 binds just six nucleotides downstream of the GLI2 binding site thus possibly perturbing its binding. Furthermore, the GLI2 binding site is not conserved in the human ASCL1 promoter due to nucleotide mismatches and therefore not recognized by the Genomatix software. This may contribute to the differences in gene induction observed in human vs murine cells by SAM and VPR systems but has to be investigated in further detail.

Interestingly, Chavez et al., 2016 also report differences in the activation potential of individual dCas9 fusion proteins depending on target gene and cell type [110]. For example, SAM was found to be more potent than the VPR system for the induction of Hemoglobin subunit gamma 1 (HBG1) in HeLa cells whereas the opposite effect was observed in the MCF-7 breast cancer cell line [110]. For the induction of Titin (TTN) however, the SunTag system seemed to be more suitable than SAM or VPR [110]. These data suggest that for an efficient induction of a specific gene not only gRNAs have to be screened but also compatible dCas9 fusion proteins.

Taken together, several factors including the interaction of gRNAs and activator system as well as the respectively targeted locus influence the level of gene induction. Here, a seemingly unique synergistic effect of Ascl1 induction by SAM and VPR systems was found in Neuro 2a cells by using gRNAs mA1 and mA2. So far analysis of transcription factor binding sites is not included in the gRNA design but may be a valuable tool for future applications.
6.2.4 Challenges in lentiviral delivery of dCas9 systems

In this thesis, *SAM split-dCas9-VPR* vectors were generated to efficiently transduce astrocytes by lentviruses each carrying one gRNA and either SAM and N-dCas9 or C-dCas9-VPR. The intein-mediated assembly of N- and C-dCas9 was found not to be impaired by the fusion of VPR to C-dCas9 thus increasing the field of applications for the split-Cas9 system originally published for wt Cas9 [141]. Surprisingly, lentiviral production of the *SAM split-dCas9-VPR* system repeatedly resulted in very low titers (1 x 10^5-fold lower than for co-produced *Tet-O-Ascl1* virus). This suggested negative effects of the expression cassette on lentiviral packaging. Interestingly, in the original publications of VPR, SAM and SunTag systems stable cell lines expressing dCas9 fusion proteins were used thus omitting the need for efficient lentiviral dCas9 delivery [90, 91, 100]. However, this is not suitable for the direct conversion of primary cells such as MEFs or astrocytes which stop proliferation after few passages and can therefore not undergo lengthy selection processes for successful transgene expression [34, 171]. So far, direct conversion of somatic cells based on lentiviral delivery of dCas9 was achieved in only two reports where dCas9 was expressed under the control of either *Tet-O* or *hUBC* promoters [107, 108]. Indeed, lentiviral delivery of Cas9 was successful in this thesis when the *Ef1a* promoter was replaced by *Tet-O* or *hUBC* promoters in chapter 5.2.4. This was surprising as *Ef1a* is a commonly used promoter also in lentiviral vectors [91, 172].

The underlying mechanism was not further investigated but one explanation could be the loss of the large intronic sequence of the *Ef1a* promoter due to splicing events during packaging of the virus [173]. This results in an intron-less *Ef1a* promoter comparable to the *small Ef1a-dCas9-VPR* construct which was found in this thesis to induce only low levels of gene expression. Expression levels close to the detection limit of immunocytochemistry could be a limiting factor during titer analysis. However, such an intronic loss was not observed by Cooper *et al.*, where *Ef1a* was used to drive *Gfp* expression in a lentiviral vector [173]. This indicates a specific interaction between *Ef1a* promoter and gRNA or *Cas9* sequences adversely affecting lentiviral packaging or expression. Interestingly, for the *hUBC* promoter Cooper *et al.*, reported splicing events during lentiviral packaging and subsequently a fourfold reduction in expression [173]. Since *hUBC* is a weak promoter even without splicing [120] the *Tet-O* system was chosen to replace the *Ef1a* promoter as the aim of this thesis was to find a dCas9-based system allowing the highest possible induction of target genes.

In conclusion, the split-Cas9 strategy is also suitable for gene induction and the combination of promoter and coding sequence can significantly affect the efficiency of lentiviral delivery.
6.2.5 Direct conversion of astrocytes to neurons utilizing VPR and SAM

Using the SAM split-dCas9-VPR system astrocytes were successfully converted to neurons in chapter 5.2.5. These results are very promising as direct conversion of somatic cells had not been achieved with SAM or VPR systems before. Furthermore, astrocytes have not been directly converted with any CRISPR/Cas9 based system up to now. Therefore, these results act as proof-of-principle for the SAM split-dCas9-VPR system. It was not surprising that direct overexpression of Ascl1 resulted in a higher reprogramming efficiency than the CRISPR/Cas9 based approach. When inducing just a single gene the CRISPR/Cas9 based approach has some downsides including the limiting effect of the required co-transduction by two viruses (SAM-N-dCas9 and C-dCas9-VPR vs Tet-O-Ascl1). This means that only a fraction of FlagTag+ (N-dCas9+) cells were also co-transduced by a C-dCas9-VPR encoding lentivirus similar to the findings for Ascl1 and Nurr1 at the beginning of this thesis in chapter 5.1.1. Therefore, the percentage of MAP2+ cells per FlagTag+ cells underestimates the reprogramming potential of SAM split-dCas9-VPR.

Furthermore, N-dCas9 and C-dCas9-VPR have to assemble in order to become a functional dCas9-VPR protein. Finally, protein separation at P2A, which was used to separate SAM and N-dCas9 is also a limiting factor. However, these calculated downsides of the SAM split-dCas9-VPR system should only be prominent when a single gene is induced. The underlying idea to generate this new system was based on the simultaneous induction of several genes where this new technology may show its full potential. In this case, direct overexpression of multiple transcription factors would require co-transduction of several viruses whereas gRNA multiplexing can still be applied to express all required gRNAs from the two established vectors SAM-N-dCas9 and C-dCas9-VPR due to the small size of gRNA expression cassettes.

Indeed, in a recent article Black et al., report a twofold increase in reprogrammed neurons when inducing the expression of endogenous genes Brn2, Ascl1 andMyt1l in MEFs by VP64-dCas9-VP64 compared to direct overexpression of the three transcription factors [107]. So far, this is the only report of directly converted somatic cells to neurons by dCas9 mediated gene induction. Naturally, the performance of this system was compared to the SAM-VPR system. RT-qPCR analysis revealed no significant activation of Ascl1 expression when using VP64-dCas9-VP64 with gRNAs mA1 and mA2 (appendix, Figure 29). This reflects earlier findings of this thesis which suggested that a single kind of transcriptional activator (VP16) and the relatively weak hUBC promoter are not suited for a strong transcriptional induction (chapters 5.2.2.4 and 5.2.4). It could be argued that this is mainly based on the different performance of promoters used (weak hUBC for VP64-dCas9-VP64 versus strong Tet-O for SAM split-dCas9-VPR). However, Chavez et al., 2016 came to a
similar solution comparing Tet-O-VP64-dCas9-BFP-VP64 with Ef1a-SAM or CMV-VPR systems [110]. The latter three promoters reach comparable levels of expression [120] confirming actual differences in the activation potential of the tested systems. Here, gene induction by VPR and VP64-dCas9-BFP-VP64 were comparable for human ASCL1 and NEUROD1 but SAM performed approximately ten-fold stronger in both cases [110].

Along these lines, when testing the hUBC-VP64-dCas9-VP64 system with gRNAs mA1 and mA2 for reprogramming of astrocytes to neurons no significant increase in reprogrammed cells was observed (transfer by lipofection, appendix Figure 30). Besides the weak hUBC promoter the combination of specific gRNAs and the transcriptional activator system also seems to play a role. This was already suggested by the results regarding the synergistic effect of SAM and VPR systems for Ascl1 induction and the findings of Chavez et al. 2016, [110] who reported differential gene induction properties of dCas9 fusion proteins depending on gene and cell type. This might also explain the missing synergistic effect in the reprogramming potentials of SAM and VPR when used alone or in combination in lipofection based reprogramming experiments. However, the underlying mechanisms in astrocytes have to be analyzed in more detail before a final conclusion can be drawn.

Taken together, the successful conversion of astrocytes to neurons by CRISPR/Cas9 based gene induction of Ascl1 demonstrates the suitability of this system for the direct reprogramming of cells. It is therefore a promising starting point for further tests including simultaneous induction of multiple genes and in vivo experiments to analyze the functionality of reprogrammed cells. In vivo, CRISPR/Cas9-based gene induction may have further advantages by modifying the epigenetic landscape of targeted promoters thus resembling natural gene expression [107]. This was found to result in sustained levels of gene expression even when using transient expression systems [107]. This system may therefore be helpful to overcome the limitations of exogenous transcription factor expression as described in this thesis and by Theodorou and Rauser et al., [118].
CONCLUSION

AND FUTURE PERSPECTIVES
7 Conclusion and future perspectives

In this thesis limitations of exogenous gene expression for direct reprogramming were shown due to low efficiencies of co-transduction and the generation of fusion proteins. Promisingly, forskolin was found to support the direct conversion of Ascl1, Lmx1a and Nurr1 transduced MEFs to dopaminergic neurons and enabled the generation of TH⁺/PITX3⁺ midbrain dopaminergic neurons. These findings are highly promising as overexpression of Ascl1, Lmx1a and Nurr1 alone was not sufficient to generate PITX3⁺ mdDA neurons which therefore do not resemble the DA neuron subtype affected in PD. As next steps, the functionality of these neurons should be further characterized by electrophysiology and finally transplantation in lesioned mouse models to investigate the therapeutic benefits.

As an alternative to exogenous gene expression a combination of SAM and dCas9-VPR was found to strongly induce endogenous Ascl1 levels in murine Neuro 2a cells. Interestingly, the newly observed synergistic effect of these two systems seemed to depend on the chosen set of gRNAs and was specific for Ascl1 and the murine cell system used. For the first time SAM and VPR systems were utilized in this thesis to directly convert astrocytes to neurons. These promising data are a basis for further improvements in order to further increase the reprogramming efficiency and also guide reprogrammed cells towards a specific neuronal subtype such as mdDA neurons.

Currently, optimized versions are tested such as a SAM split-dCas9-VPR system driven by a GFAP promoter which restricts expression to astrocytes. This should help simplifying the quantitative analysis where newly converted neurons must be distinguished from neurons already present at the beginning of the primary cortical cell population. One central task for the future will be the simultaneous induction of multiple genes in order to generate specific neuronal subtypes. For this, multiplexing is required which allows expression of several gRNAs from a single vector.

Finally, the developed system will be tested in vivo by injecting the SAM dCas9-VPR system into the murine cortex to analyze the reprogramming and rescue potential in disease models such as 6-OHDA treated mice.

Taken together, in this thesis successful proof-of-principle experiments show the highly promising potential of the CRISPR/Cas9 technology for the direct conversion of somatic cells which will now be a basis for further developments.
MATERIAL AND METHODS
8 Material and methods

8.1 Material

Table 2: Equipment

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<th>Description</th>
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<td>Centrifuge Avanti J-30i; JA-30.50 Ti Rotor</td>
<td>Beckman Coulter, Germany</td>
</tr>
<tr>
<td>Centro LB 960 luminometer</td>
<td>Berthold Technologies, Germany</td>
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<tr>
<td>ChemiDoc MP imaging system</td>
<td>BioRad, Germany</td>
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<tr>
<td>Fusion SL imaging system</td>
<td>Vilber, Germany</td>
</tr>
<tr>
<td>Gel-Documentation system E.A.S.Y Win32</td>
<td>Herolab, Germany</td>
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<tr>
<td>LightCycler 480</td>
<td>Roche, Germany</td>
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<tr>
<td>NanoDrop® Spectrophotometer ND-1000</td>
<td>Peqlab, Germany</td>
</tr>
<tr>
<td>Olympus IX81</td>
<td>Olympus, Germany</td>
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<tr>
<td>PCR Gelelectrophoresis chambers</td>
<td>Peqlab, Germany</td>
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<tr>
<td>Thermo cycler Mastercycler pro</td>
<td>Eppendorf, Germany</td>
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<tr>
<td>Transilluminator</td>
<td>Herolab, Germany</td>
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<tr>
<td>Western Blot chambers Novex® Mini-Cell</td>
<td>ThermoFisher Scientific, Germany</td>
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Table 3: General consumables

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>50 ml syringe</td>
<td>300865</td>
<td>BD Plastipak, USA</td>
</tr>
<tr>
<td>AceGlow Luminol Enhancer Solution</td>
<td>37-3420</td>
<td>Peqlab, Germany</td>
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<tr>
<td>Adhesive Seal Sheets</td>
<td>AB-1170</td>
<td>ThermoFisher Scientific, Germany</td>
</tr>
<tr>
<td>AquaPolyMount</td>
<td>18606</td>
<td>Polysciences, USA</td>
</tr>
<tr>
<td>Blotting-grade Blocker, nonfat milk</td>
<td>170-6404</td>
<td>Biorad, Germany</td>
</tr>
<tr>
<td>Cell strainer, 70 µm</td>
<td>352350</td>
<td>BD Falcon, USA</td>
</tr>
<tr>
<td>Centrifugation tube for Avanti J-30i</td>
<td>357003</td>
<td>Beckman Coulter, Germany</td>
</tr>
<tr>
<td>Cover slips</td>
<td>1001/14</td>
<td>Karl Hecht, Germany</td>
</tr>
<tr>
<td>EasYFlask 225 cm²</td>
<td>159934</td>
<td>ThermoFisher Scientific, Germany</td>
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## MATERIAL AND METHODS

<table>
<thead>
<tr>
<th>Description</th>
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<tr>
<td>EasYFlask 75 cm²</td>
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<td>ThermoFisher Scientific, Germany</td>
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<tr>
<td>Framestar 384</td>
<td>4ti-0384/C</td>
<td>4ttitude, United Kingdom</td>
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<tr>
<td>GeneRuler 100 bp DNA Ladder</td>
<td>SM0243</td>
<td>ThermoFisher Scientific, Germany</td>
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<tr>
<td>GeneRuler 1kb DNA Ladder</td>
<td>SM0311</td>
<td>ThermoFisher Scientific, Germany</td>
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<tr>
<td>Immobilon P Transfer Membrane</td>
<td>IPVH00010</td>
<td>Merck Millipore, Germany</td>
</tr>
<tr>
<td>Nunc multidish 24</td>
<td>142475</td>
<td>ThermoFisher Scientific, Germany</td>
</tr>
<tr>
<td>Nunclon Delta Surface 96 well plate</td>
<td>13610</td>
<td>ThermoFisher Scientific, Germany</td>
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<tr>
<td>NuPAGE 4-12% Bis-Tris Gel</td>
<td>NP0321</td>
<td>ThermoFisher Scientific, Germany</td>
</tr>
<tr>
<td>NuPAGE® LDS Sample Buffer (4x)</td>
<td>NP0007</td>
<td>ThermoFisher Scientific, Germany</td>
</tr>
<tr>
<td>NuPAGE® MOPS SDS Running Buffer (20x)</td>
<td>NP0001</td>
<td>ThermoFisher Scientific, Germany</td>
</tr>
<tr>
<td>NuPAGE® Transfer Buffer (20x)</td>
<td>NP0006-1</td>
<td>ThermoFisher Scientific, Germany</td>
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<tr>
<td>Page Ruler Plus Prestained</td>
<td>26619</td>
<td>ThermoFisher Scientific, Germany</td>
</tr>
<tr>
<td>Phosphatase inhibitor cocktail</td>
<td>04906837001</td>
<td>Roche, Germany</td>
</tr>
<tr>
<td>Protease inhibitor cocktail</td>
<td>11836170001</td>
<td>Roche, Germany</td>
</tr>
<tr>
<td>SuperFrost™ Plus glas slides</td>
<td>4951PLUS4</td>
<td>ThermoFisher Scientific, Germany</td>
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<tr>
<td>Syringe 0.22 µm PES filter</td>
<td>SLGP033RS</td>
<td>Merck Millipore, Germany</td>
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<tr>
<td>Syringe 0.45 µm PES filter</td>
<td>SLHP033RS</td>
<td>Merck Millipore, Germany</td>
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<td>TaqMan Universal PCR MasterMix</td>
<td>5000991</td>
<td>ThermoFisher Scientific, Germany</td>
</tr>
<tr>
<td>Whatman paper</td>
<td>3030-931</td>
<td>Sigma-Aldrich, Germany</td>
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<table>
<thead>
<tr>
<th>Description</th>
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<th>Supplier</th>
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<tr>
<td>2-Mercaptoethanol</td>
<td>M7522</td>
<td>Sigma-Aldrich, Germany</td>
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<tr>
<td>Agarose</td>
<td>870055</td>
<td>Biozym, Germany</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>11593-027</td>
<td>ThermoFisher Scientific, Germany</td>
</tr>
<tr>
<td>Ampuwa</td>
<td>B230673</td>
<td>Fresenius Kabi, France</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>A3059</td>
<td>Sigma-Aldrich, Germany</td>
</tr>
<tr>
<td>D-(+)-glucose</td>
<td>G8270</td>
<td>Sigma-Aldrich, Germany</td>
</tr>
<tr>
<td>Description</td>
<td>Catalogue number</td>
<td>Supplier</td>
</tr>
<tr>
<td>--------------------------------------------------</td>
<td>------------------</td>
<td>-----------------------------------</td>
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<tr>
<td>Dimethyl sulfoxide</td>
<td>D5879</td>
<td>Sigma-Aldrich, Germany</td>
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<td>EDTA</td>
<td>E5134</td>
<td>Sigma-Aldrich, Germany</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1.00983.2500</td>
<td>Merck Millipore, Germany</td>
</tr>
<tr>
<td>Ethidiumbromide</td>
<td>2218.2</td>
<td>Carl Roth GmbH, Germany</td>
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<tr>
<td>Formalin, 10%</td>
<td>F5554</td>
<td>Sigma-Aldrich, Germany</td>
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<tr>
<td>Isopropanol</td>
<td>109634</td>
<td>Merck KGaA, Germany</td>
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<tr>
<td>KCl cell culture grade</td>
<td>P5405</td>
<td>Sigma-Aldrich, Germany</td>
</tr>
<tr>
<td>Methanol</td>
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<td>MgCl₂ cell culture grade</td>
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<td>Milk powder</td>
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<td>NaCl cell culture grade</td>
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<td>Sigma-Aldrich, Germany</td>
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<td>Sodium Chloride</td>
<td>106404</td>
<td>Merck KGaA, Germany</td>
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<tr>
<td>Triton X 100</td>
<td>T9284</td>
<td>Sigma-Aldrich, Germany</td>
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<tr>
<td>Trizma cell culture grade</td>
<td>T2319</td>
<td>Sigma-Aldrich, Germany</td>
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<tr>
<td>Tween 20</td>
<td>P1379</td>
<td>Sigma-Aldrich, Germany</td>
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Table 5: Cell culture media and supplements

<table>
<thead>
<tr>
<th>Description</th>
<th>Catalogue number</th>
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<tbody>
<tr>
<td>B27 Serum-Free-Supplement</td>
<td>17504044</td>
<td>ThermoFisher Scientific, Germany</td>
</tr>
<tr>
<td>B-27 Supplement Minus Vitamin A</td>
<td>12587010</td>
<td>ThermoFisher Scientific, Germany</td>
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<tr>
<td>BDNF</td>
<td>203702</td>
<td>Merck Millipore, Germany</td>
</tr>
<tr>
<td>bFGF</td>
<td>13256029</td>
<td>ThermoFisher Scientific, Germany</td>
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<tr>
<td>DAPI</td>
<td>D8417</td>
<td>Sigma-Aldrich, Germany</td>
</tr>
<tr>
<td>DMEM</td>
<td>21969</td>
<td>ThermoFisher Scientific, Germany</td>
</tr>
<tr>
<td>DMEM/F12 GlutaMAX™-I</td>
<td>31331</td>
<td>ThermoFisher Scientific, Germany</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>44577</td>
<td>Sigma-Aldrich, Germany</td>
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<tr>
<td>DPBS</td>
<td>14190</td>
<td>Sigma-Aldrich, Germany</td>
</tr>
<tr>
<td>EGF</td>
<td>E4127</td>
<td>Sigma-Aldrich, Germany</td>
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<tr>
<td>Fetal Bovine Serum</td>
<td>A2153</td>
<td>ThermoFisher Scientific, Germany</td>
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<tr>
<td>FGF-basic recombinant mouse</td>
<td>PMG0034</td>
<td>ThermoFisher Scientific, Germany</td>
</tr>
<tr>
<td>Forskolin</td>
<td>F3917</td>
<td>Sigma-Aldrich, Germany</td>
</tr>
<tr>
<td>Hanks balanced salt solution, Mg²⁺/Ca²⁺ free</td>
<td>14175-053</td>
<td>ThermoFisher Scientific, Germany</td>
</tr>
<tr>
<td>Horse serum</td>
<td>16050-122</td>
<td>ThermoFisher Scientific, Germany</td>
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### MATERIAL AND METHODS

<table>
<thead>
<tr>
<th>Component</th>
<th>Catalog Number</th>
<th>Manufacturer, Country</th>
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<tr>
<td>L-ascorbic acid</td>
<td>A4034100G</td>
<td>Sigma Genosys, Germany</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>16285</td>
<td>Roche, Germany</td>
</tr>
<tr>
<td>Lipofectamine 2000</td>
<td>11668-019</td>
<td>ThermoFisher Scientific, Germany</td>
</tr>
<tr>
<td>Lipofectamine® LTX with Plus™ Reagent</td>
<td>15338100</td>
<td>ThermoFisher Scientific, Germany</td>
</tr>
<tr>
<td>N2 Supplement-A</td>
<td>07152</td>
<td>Lab Life, Germany</td>
</tr>
<tr>
<td>Neurobasal medium</td>
<td>21103-049</td>
<td>ThermoFisher Scientific, Germany</td>
</tr>
<tr>
<td>Opti-MEM I</td>
<td>31985-047</td>
<td>ThermoFisher Scientific, Germany</td>
</tr>
<tr>
<td>Penicillin/Streptomycin</td>
<td>15070063</td>
<td>ThermoFisher Scientific, Germany</td>
</tr>
<tr>
<td>Poly-D-lysine</td>
<td>A-003-E</td>
<td>Merck Millipore, Germany</td>
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<tr>
<td>Trypsin-EDTA Solution (0.05%)</td>
<td>25300054</td>
<td>ThermoFisher Scientific, Germany</td>
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<tr>
<td>Trypsin-EDTA Solution (0.25%)</td>
<td>25200056</td>
<td>ThermoFisher Scientific, Germany</td>
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### Table 6: Composition of buffers and cell culture media

<table>
<thead>
<tr>
<th>Description</th>
<th>Constituents</th>
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</thead>
<tbody>
<tr>
<td>Agar plates</td>
<td>10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar, Add water to 1 L pH 7</td>
</tr>
<tr>
<td>Astrocyte dissection medium</td>
<td>HBSS buffer, 1% penicillin/streptomycin</td>
</tr>
<tr>
<td>Astrocyte plating medium</td>
<td>DMEM/F12 GlutaMAX™-I, 10% FCS, 5% horse serum, 45 mg/ml glucose additionally, 1x B27 supplement, 10 ng/ml EGF, 10 ng/ml bFGF, 1% penicillin/streptomycin</td>
</tr>
<tr>
<td>Astrocyte reprogramming medium</td>
<td>DMEM/F12 GlutaMAX™-I, 45 mg/ml glucose additionally, 1x B27 supplement, 1% penicillin/streptomycin, 25 µM forskolin, 20 ng/ml BDNF every fourth day</td>
</tr>
<tr>
<td>DMEM medium for HEK293, Lenti-X 293T, N2A cells</td>
<td>DMEM, 10% FCS, 1% L-glutamine</td>
</tr>
</tbody>
</table>
| **Immunocytochemistry blocking buffer** | PBS  
| | 1% BSA  
| | 0.5% Triton-X100  
| **LB medium** | 10 g tryptone  
| | 5 g yeast extract  
| | 10 g NaCl  
| | Add water to 1 L  
| | pH 7  
| **MEF medium (Fibroblast cultivation medium)** | DMEM  
| | 10% FCS  
| | 1% L-glutamine  
| | 1% penicillin/streptomycin  
| **N2-B27 medium (Fibroblast reprogramming medium)** | 50% DMEM/F12 GlutaMAX™,I  
| | 50% Neurobasal  
| | 0.5x B27 supplement minus vitamin A  
| | 0.5x N2 supplement  
| | 100 μM ascorbic acid  
| | 0.5% L-glutamine  
| | 20 ng/ml BDNF  
| | 1% penicillin/streptomycin  
| **RIPA buffer** | 50 mM Tris-HCl  
| | 150 mM NaCl  
| | 1% Triton-X100  
| | 0.5% Na-Deoxicholate  
| | 0.1% SDS  
| | 3 mM EDTA  
| | Addition of protease/phosphatase inhibitors  
| **TAE buffer** | 40 mM Tris  
| | 20 mM acetic acid  
| | 1 mM EDTA  
| **TBS-5 buffer** | 50 mM Tris-HCl, pH 7.8  
| | 0.13 mM NaCl  
| | 10 mM KCl  
| | 5 mM MgCl₂  
| **TBS-T buffer** | 20 mM Tris-HCl, pH 7.5  
| | 136 mM NaCl  
| | 0.1% Tween 20  
| **TE buffer** | 10 mM Tris-HCl  
| | 1 mM disodium EDTA  
| | pH 8.0  
| **Western blot blocking buffer** | TBS-T buffer  
| | 5% non-fat milk powder (5% BSA for ASCL1)  


### Table 7: Kits

<table>
<thead>
<tr>
<th>Description</th>
<th>Catalogue number</th>
<th>Supplier</th>
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</thead>
<tbody>
<tr>
<td>Dual-Luciferase® Reporter Assay System</td>
<td>E1910</td>
<td>Promega, USA</td>
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<tr>
<td>NEBuilder HiFi DNA Assembly Cloning Kit</td>
<td>E5520</td>
<td>New England Biolabs, Germany</td>
</tr>
<tr>
<td>Pierce BCA Protein Assay Kit</td>
<td>23225</td>
<td>ThermoFisher Scientific, Germany</td>
</tr>
<tr>
<td>PureLink® HiPure Plasmid Filter Maxiprep Kit</td>
<td>K210016</td>
<td>ThermoFisher Scientific, Germany</td>
</tr>
<tr>
<td>QIAprep Spin Maxiprep Kit</td>
<td>12162</td>
<td>Qiagen, Germany</td>
</tr>
<tr>
<td>QIAprep Spin Miniprep Kit</td>
<td>27104</td>
<td>Qiagen, Germany</td>
</tr>
<tr>
<td>QIAquick Gel Extraction Kit</td>
<td>28704</td>
<td>Qiagen, Germany</td>
</tr>
<tr>
<td>QIAquick PCR Purification Kit</td>
<td>28104</td>
<td>Qiagen, Germany</td>
</tr>
<tr>
<td>QuikChange Lightning Site-Directed Mutagenesis Kit</td>
<td>210518</td>
<td>Agilent, Germany</td>
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<tr>
<td>Rapid DNA Dephos &amp; Ligation Kit</td>
<td>04898117001</td>
<td>Roche, Germany</td>
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<tr>
<td>RNeasy plus Mini Kit</td>
<td>74134</td>
<td>Qiagen, Germany</td>
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<tr>
<td>StrataClone PCR Cloning Kit</td>
<td>240205</td>
<td>Agilent, Germany</td>
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<tr>
<td>SuperScript® VILO cDNA Synthesis Kit</td>
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<tr>
<td>TOPO TA Cloning Kit</td>
<td>45-0640</td>
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### Table 8: Enzymes

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<tr>
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<tbody>
<tr>
<td>BamHl</td>
<td>R3136</td>
<td>New England Biolabs, Germany</td>
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<tr>
<td>BbsI</td>
<td>R0539</td>
<td>New England Biolabs, Germany</td>
</tr>
<tr>
<td>BsiWI</td>
<td>R0553</td>
<td>New England Biolabs, Germany</td>
</tr>
<tr>
<td>BsmBI</td>
<td>R0580</td>
<td>New England Biolabs, Germany</td>
</tr>
<tr>
<td>EcoRI</td>
<td>R0101</td>
<td>New England Biolabs, Germany</td>
</tr>
<tr>
<td>EcoRI-HF</td>
<td>R3101</td>
<td>New England Biolabs, Germany</td>
</tr>
<tr>
<td>KAPA HiFi DNA polymerase</td>
<td>07-KK2100-01</td>
<td>VWR, Germany</td>
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<tr>
<td>NotI</td>
<td>R0189</td>
<td>New England Biolabs, Germany</td>
</tr>
<tr>
<td>NotI-HF</td>
<td>R3189</td>
<td>New England Biolabs, Germany</td>
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<tr>
<td>Q5 High-Fidelity 2X Master Mix</td>
<td>M0492</td>
<td>New England Biolabs, Germany</td>
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<tr>
<td>REDTaq ReadyMix PCR Reaction Mix</td>
<td>R2523</td>
<td>Sigma-Aldrich, Germany</td>
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<td>RsrlI</td>
<td>R0501</td>
<td>New England Biolabs, Germany</td>
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<td>SbfI-HF</td>
<td>R3642</td>
<td>New England Biolabs, Germany</td>
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### Table 9: Primary antibodies

<table>
<thead>
<tr>
<th>Target</th>
<th>Host</th>
<th>Dilution</th>
<th>Catalogue #</th>
<th>Supplier</th>
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</thead>
<tbody>
<tr>
<td>ASCL1</td>
<td>Mouse</td>
<td>ICC: 1:1000, WB: 1:250, 5%</td>
<td>556604</td>
<td>BD Biosciences, USA</td>
</tr>
<tr>
<td>Cas9</td>
<td>Mouse</td>
<td>ICC: 1:500</td>
<td>A-9000</td>
<td>EpiGentek, USA</td>
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<tr>
<td>FlagTag</td>
<td>Mouse</td>
<td>ICC: 1:1000, 10% NGS serum</td>
<td>F1804</td>
<td>Sigma-Aldrich, Germany</td>
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<tr>
<td>GFP</td>
<td>Chicken</td>
<td>ICC: 1:1000</td>
<td>PA1-9533</td>
<td>AVES, USA</td>
</tr>
<tr>
<td>GFP</td>
<td>Mouse</td>
<td>ICC: 1:1000</td>
<td>11814460001</td>
<td>Roche, USA</td>
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<tr>
<td>LMX1A</td>
<td>Rabbit</td>
<td>WB: 1:2000</td>
<td>ab10533</td>
<td>Merck-Millipore, Germany</td>
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<tr>
<td>MAP2</td>
<td>Rabbit</td>
<td>ICC: 1:1000</td>
<td>AB5622</td>
<td>Merck-Millipore, Germany</td>
</tr>
<tr>
<td>MycTag</td>
<td>Goat</td>
<td>ICC: 1:2000</td>
<td>ab9132</td>
<td>Abcam, Germany</td>
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<tr>
<td>MycTag</td>
<td>Mouse</td>
<td>ICC: 1:1000</td>
<td>M4439</td>
<td>Sigma-Aldrich, Germany</td>
</tr>
<tr>
<td>NURR1</td>
<td>Rabbit</td>
<td>ICC: 1:2000</td>
<td>sc-990</td>
<td>Santa Cruz, USA</td>
</tr>
<tr>
<td>PITX3</td>
<td>Rabbit</td>
<td>ICC: 1:300, WB: 1:100</td>
<td>38-2850</td>
<td>ThermoFisher Scientific, Germany</td>
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<tr>
<td>TH</td>
<td>Mouse</td>
<td>IHC: 1:600, ICC: 1:600</td>
<td>MAB318</td>
<td>Merck-Millipore, Germany</td>
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<td>TH</td>
<td>Rabbit</td>
<td>ICC: 1:1000</td>
<td>AB152</td>
<td>Merck-Millipore, Germany</td>
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<td>TUJ1</td>
<td>Rabbit</td>
<td>ICC: 1:500</td>
<td>ab18207</td>
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<tr>
<td>β-actin</td>
<td>Mouse</td>
<td>WB: 1:10000</td>
<td>ab6276</td>
<td>Abcam, Germany</td>
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### Table 10: Secondary antibodies

<table>
<thead>
<tr>
<th>Target</th>
<th>Conjugate</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Chicken IgG</td>
<td>Cy2</td>
<td>Rabbit</td>
<td>ICC: 1:250</td>
<td>703225155</td>
<td>Dianova, Germany</td>
</tr>
<tr>
<td>Goat IgG</td>
<td>Alexa 488</td>
<td>Donkey</td>
<td>ICC: 1:500</td>
<td>A-11055</td>
<td>ThermoFisher Scientific, Germany</td>
</tr>
<tr>
<td>Goat IgG</td>
<td>Alexa 594</td>
<td>Donkey</td>
<td>ICC: 1:500</td>
<td>A-11058</td>
<td>ThermoFisher Scientific, Germany</td>
</tr>
<tr>
<td>Mouse IgG</td>
<td>Alexa 350</td>
<td>Donkey</td>
<td>ICC: 1:250</td>
<td>A10035</td>
<td>ThermoFisher Scientific, Germany</td>
</tr>
<tr>
<td>Mouse IgG</td>
<td>HRP</td>
<td>Goat</td>
<td>WB: 1:5000</td>
<td>115-035-003</td>
<td>Dianova, Germany</td>
</tr>
</tbody>
</table>
Table 11: Primers for amplification and sequencing

<table>
<thead>
<tr>
<th>Description</th>
<th>Sequence 5’ – 3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>BsiWI-del-F</td>
<td>gcttagaaggacacgtagtgtagcagcagctcgacaat</td>
</tr>
<tr>
<td>C-Cas-BsiWI-F</td>
<td>ggactatcgctacgacacgataaagagtgccaccaagatctgg</td>
</tr>
<tr>
<td>C-Cas-SbfI-R</td>
<td>ggactatcctcagctagtcagctctctccctggaaatcacttc</td>
</tr>
<tr>
<td>dCasVP64-BsiWI-F</td>
<td>ggactatcgtacgccacatgcataaagatcttg</td>
</tr>
<tr>
<td>dCasVP64-SbfI-R</td>
<td>ggactatcgtacgctacaatcgtagcagctggcagacatc</td>
</tr>
<tr>
<td>Flag-Oligo-F</td>
<td>gtacagactacaagcagcagcagcagaatgtg</td>
</tr>
<tr>
<td>Flag-Oligo-R</td>
<td>aattctcacttattgtctgtctctgtgtct</td>
</tr>
<tr>
<td>gBlock-Flag-P2A</td>
<td>ctccccaaagcccaagcaccctctctctctctctctactagactacaagcagcagcagcataaagggaagccg</td>
</tr>
<tr>
<td>hUBC-ampl-F</td>
<td>ggactatgatcgcaccctgagggttacgtagctctctcaaggctcgctcttcaccctcaagagctgagctgccctc</td>
</tr>
<tr>
<td>hUBC-ampl-R</td>
<td>ggactatgatcgcaccctgagggttacgtagctctctcaaggctcgctcttcaccctcaagagctgagctgccctc</td>
</tr>
<tr>
<td>Int-C-Cas-Fw</td>
<td>tctccatcttcagtgctgatgtacacgtcataaccatgtcattgctccttc</td>
</tr>
<tr>
<td>Int-C-Cas-Rev</td>
<td>gagggtgattccagctgtcagctcttcagctcatttcagaatctctcaccctcaagagctgagctgagctgccctc</td>
</tr>
<tr>
<td>N-Cas-BsiWI-F</td>
<td>ggactatcgtacgccacatgcataaaccttcactctcagttcgg</td>
</tr>
<tr>
<td>N-Cas-Int F</td>
<td>gaagtagagcactgctgccg</td>
</tr>
<tr>
<td>N-Cas-Int R</td>
<td>agctgtgatcagctctctctagtctgtactgtagcagcagcagctcgctcttcaccctcaagagctgagctgccctc</td>
</tr>
<tr>
<td>N-Cas-SbfI-R</td>
<td>ggactatcgtacgctctctctactccagctctctctctactgtagcagctcgctcttcaccctcaagagctgagctgccctc</td>
</tr>
<tr>
<td>SAM-SbfI-R</td>
<td>ggactatcgtacgctctctctactccagctctctctctactgtagcagctcgctcttcaccctcaagagctgagctgccctc</td>
</tr>
<tr>
<td>TRE-ampl-F</td>
<td>ggactatgatcgcaccctgagggttacgtagctctctcaaggctcgctcttcaccctcaagagctgagctgccctc</td>
</tr>
<tr>
<td>TRE-ampl-R</td>
<td>ggactatgatcgcaccctgagggttacgtagctctctcaaggctcgctcttcaccctcaagagctgagctgccctc</td>
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</table>
Table 12: gRNA targeting sequences

<table>
<thead>
<tr>
<th>Description</th>
<th>Sequence 5' – 3' without PAM</th>
<th>Position to TSS</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>gRNA A1, murine Ascl1</td>
<td>GTTGTTGACGTGCGTCGCGC</td>
<td>-230 / anti-sense</td>
<td>On-target: 57</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Off-target: 92</td>
</tr>
<tr>
<td>gRNA A2, murine Ascl1</td>
<td>CGGTCGGCCCGCTTTTTCA</td>
<td>-25 / anti-sense</td>
<td>On-target: N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Off-target: 93</td>
</tr>
<tr>
<td>gRNA A3, murine Ascl1</td>
<td>GAGTTTGCAAAGAGCGGGCG</td>
<td>-179 / sense</td>
<td>On-target: N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Off-target: 42</td>
</tr>
<tr>
<td>gRNA A4, murine Ascl1</td>
<td>GCGGGCCAGGGCTGCGGTG</td>
<td>-47 / sense</td>
<td>On-target: N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Off-target: 77</td>
</tr>
<tr>
<td>gRNA A5, murine Ascl1</td>
<td>CTCCCCGCTGTGCAAGCGG</td>
<td>-92 / anti-sense</td>
<td>On-target: N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Off-target: 71</td>
</tr>
<tr>
<td>gRNA A6, murine Ascl1</td>
<td>ACGCAGTGCACAACAAACC</td>
<td>-211 / sense</td>
<td>On-target: N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Off-target: 70</td>
</tr>
<tr>
<td>gRNA A7, murine Ascl1</td>
<td>CCCAGCCCACGCCAGGGCC</td>
<td>-52 /anti-sense</td>
<td>On-target: N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Off-target: 57</td>
</tr>
<tr>
<td>gRNA A8, murine Ascl1</td>
<td>CGCAGGCAACTGGGAGGGGGG</td>
<td>-161 / sense</td>
<td>On-target: N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Off-target: 59</td>
</tr>
<tr>
<td>gRNA dist. matched ma2, human ASCL1</td>
<td>AGCCGAGAAAGGAAGGGA</td>
<td>-185 /sense</td>
<td>On-target: 55</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Off-target: 57</td>
</tr>
<tr>
<td>gRNA hA1, human Ascl1</td>
<td>CGGGGAGAAAGGAAGGGAAGGAG</td>
<td>-196 / sense</td>
<td>On-target: 30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Off-target: 51</td>
</tr>
<tr>
<td>gRNA hA2, human Ascl1</td>
<td>AAGAAGTAGCAAGCCAGGG</td>
<td>-451 /anti-sense</td>
<td>On-target: 50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Off-target: 74</td>
</tr>
<tr>
<td>gRNA hA3, human Ascl1</td>
<td>TCAATTTTCTAGGTCACC</td>
<td>-572 /sense</td>
<td>On-target: 68</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Off-target: 82</td>
</tr>
<tr>
<td>gRNA hA4, human Ascl1</td>
<td>GTTGTAGCCGCCTCTGAGG</td>
<td>-886 /anti-sense</td>
<td>On-target: 57</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Off-target: 83</td>
</tr>
<tr>
<td>gRNA mA1, murine Ascl1</td>
<td>AGCGCTCGCTGCAGCAGC</td>
<td>-83 / sense</td>
<td>On-target: 57</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Off-target: 43</td>
</tr>
<tr>
<td>gRNA mA2, murine Ascl1</td>
<td>GGCTGAATGGAGAGTTTGA</td>
<td>-190 / sense</td>
<td>On-target: 61</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Off-target: 36</td>
</tr>
<tr>
<td>gRNA mA3, murine Ascl1</td>
<td>AATGGAGAGTTTGAAGGAGG</td>
<td>-185 / sense</td>
<td>On-target: 61</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Off-target: 35</td>
</tr>
</tbody>
</table>
### gRNA mA4, murine Ascl1
- **Sequence**: GGAGGGAGCTGAGGAGGTGG
- **Target**: -130 / sense
  - On-target: 30
  - Off-target: 33

### gRNA mA5, murine Ascl1
- **Sequence**: ATTGAAAAGGCCGGCCGCACC
- **Target**: -26 / anti-sense
  - On-target: 41
  - Off-target: 48

### gRNA N1, murine Nurr1
- **Sequence**: TCCGACCTGACGTACGACT
- **Target**: -48 / anti-sense
  - On-target: N/A
  - Off-target: 95

### gRNA N2, murine Nurr1
- **Sequence**: TACCAAGCGAGCGGGCC
- **Target**: -10 / sense
  - On-target: N/A
  - Off-target: 94

### gRNA N3, murine Nurr1
- **Sequence**: AAGGTGGAACGCTACCT
- **Target**: -80 / anti-sense
  - On-target: N/A
  - Off-target: 84

### gRNA N4, murine Nurr1
- **Sequence**: CGTGTGAGGACGCAAGGTCT
- **Target**: -198 / sense
  - On-target: N/A
  - Off-target: 83

### gRNA N5, murine Nurr1
- **Sequence**: CTCCTGGCCCGCGCTCGCTT
- **Target**: -19 / anti-sense
  - On-target: N/A
  - Off-target: 80

### gRNA N6, murine Nurr1
- **Sequence**: TCCACCAAGTGGGTACCA
- **Target**: -86 / sense
  - On-target: N/A
  - Off-target: 77

### gRNA N7, murine Nurr1
- **Sequence**: TAGCATCACCACGGACTTCA
- **Target**: -152 / sense
  - On-target: N/A
  - Off-target: 74

### gRNA N8, murine Nurr1
- **Sequence**: AAGTGTGACTTCTGCAACCC
- **Target**: -139 / anti-sense
  - On-target: N/A
  - Off-target: 53

### gRNA seq. matched mA2, human ASCL1
- **Sequence**: AGCTGAATGGAGAGTTTCA
- **Target**: -208 / sense
  - On-target: N/A
  - Off-target: 35

---

**Table 13: Taqman probes**

<table>
<thead>
<tr>
<th>Description</th>
<th>Catalogue number</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB, human</td>
<td>Hs99999903_m1</td>
<td>ThermoFisher Scientific, Germany</td>
</tr>
<tr>
<td>Actb, mouse</td>
<td>Mm00607939_s1</td>
<td>ThermoFisher Scientific, Germany</td>
</tr>
<tr>
<td>ASCL1, human</td>
<td>Hs04187546_g1</td>
<td>ThermoFisher Scientific, Germany</td>
</tr>
<tr>
<td>Ascl1, mouse</td>
<td>Mm03058063_m1</td>
<td>ThermoFisher Scientific, Germany</td>
</tr>
<tr>
<td>Gapdh, mouse</td>
<td>Mm99999915_g1</td>
<td>ThermoFisher Scientific, Germany</td>
</tr>
</tbody>
</table>
### Table 14: DNA Vectors

<table>
<thead>
<tr>
<th>Description</th>
<th>Catalogue number</th>
<th>Supplier/reference/producer</th>
</tr>
</thead>
<tbody>
<tr>
<td>dSpCas9(D10A,H840A)_12xSpyTag</td>
<td>N/A</td>
<td>Available at the IDG; generated by J. Truong</td>
</tr>
<tr>
<td>dSpCas9(D10A,H840A)_4xSpyTag</td>
<td>N/A</td>
<td>Available at the IDG; generated by J. Truong</td>
</tr>
<tr>
<td>dSpCas9(D10A,H840A)_8xSpyTag</td>
<td>N/A</td>
<td>Available at the IDG; generated by J. Truong</td>
</tr>
<tr>
<td>dSpCas9 VP160 (SpCas9&lt;sup&gt;2-573&lt;/sup&gt; and C-Cas9 (SpCas9&lt;sup&gt;574-1368&lt;/sup&gt;))</td>
<td>N/A</td>
<td>Available at the IDG; generated by J. Truong</td>
</tr>
<tr>
<td>hU6-mA1-Ef1a-SAM</td>
<td>N/A</td>
<td>Generated in this thesis</td>
</tr>
<tr>
<td>hU6-mA1-Ef1a-SAM-N-dCas9 (D10A-SpCas9&lt;sup&gt;2-573&lt;/sup&gt;)</td>
<td>N/A</td>
<td>Generated in this thesis</td>
</tr>
<tr>
<td>hU6-mA1-Tet-O-SAM</td>
<td>N/A</td>
<td>Generated in this thesis</td>
</tr>
<tr>
<td>hU6-mA1-Tet-O-SAM-N-dCas9 (D10A-SpCas9&lt;sup&gt;2-573&lt;/sup&gt;)</td>
<td>N/A</td>
<td>Generated in this thesis</td>
</tr>
<tr>
<td>hU6-mA2-Ef1a-C-dCas9-VPR (H840A-SpCas9&lt;sup&gt;574-1368&lt;/sup&gt;)</td>
<td>N/A</td>
<td>Generated in this thesis</td>
</tr>
<tr>
<td>hU6-mA2-Tet-O-dCas9-VPR</td>
<td>N/A</td>
<td>Generated in this thesis</td>
</tr>
<tr>
<td>lenti dCAS-VP64_Blast</td>
<td>61425</td>
<td>Addgene, USA / [91]</td>
</tr>
<tr>
<td>lenti MS2-P65-HSF1_Hygro (SAM)</td>
<td>61426</td>
<td>Addgene, USA / [91]</td>
</tr>
<tr>
<td>lenti sgRNA(MS2)_zeo backbone (SAM gRNA)</td>
<td>61427</td>
<td>Addgene, USA / [91]</td>
</tr>
<tr>
<td>Luciferase reporter: Ascl1((E1)6-Luc)</td>
<td>N/A</td>
<td>Castro et al., 2006 [134]</td>
</tr>
<tr>
<td>Luciferase reporter: Lmx1a (Pitx3 promoter)</td>
<td>N/A</td>
<td>Available at the IDG; generated by C. Peng [15]</td>
</tr>
<tr>
<td>Luciferase reporter: Nurr1 (DeltaM-Luc)</td>
<td>N/A</td>
<td>Castro et al., 2006 [134]</td>
</tr>
<tr>
<td>Npu-DnaE-C-Intein_C-SpCas9(574-1368)</td>
<td>N/A</td>
<td>Available at the IDG, [141]</td>
</tr>
<tr>
<td>N-SpCas9(2-573)_Npu-DnaE-N-Intein</td>
<td>N/A</td>
<td>Available at the IDG, [141]</td>
</tr>
<tr>
<td>pBS-U6-chimaeric-F+E</td>
<td>N/A</td>
<td>Available at the IDG</td>
</tr>
<tr>
<td>pCAG-Cas9v2_Intein_C-Part_v1_new</td>
<td>N/A</td>
<td>Available at the IDG; generated by J. Truong</td>
</tr>
<tr>
<td>pCCLsin.PPT.hPGK.rtTAm2 (rTTA)</td>
<td>N/A</td>
<td>Caiazzo et al. 2011, [37]</td>
</tr>
<tr>
<td>pcDNA3.1 (+)</td>
<td>V79020</td>
<td>Thermo Fisher, Germany</td>
</tr>
<tr>
<td>pHRdSV40-dCas9-10xGCN4_v4-P2A-BFP (SunTag)</td>
<td>60904</td>
<td>Addgene, USA / [100]</td>
</tr>
<tr>
<td>pHRdSV40-NLS-dCas9-24xGCN4_v4-NLS-P2A-BFP-dWPRE (activators for SunTag)</td>
<td>60910</td>
<td>Addgene, USA / [100]</td>
</tr>
<tr>
<td>pKLV-U6gRNA(BbsI)-PGKpuro2ABFP</td>
<td>50946</td>
<td>Addgene, USA / [174]</td>
</tr>
<tr>
<td>pLV hUbC-dCas9 VP64-T2A-GFP</td>
<td>53192</td>
<td>Addgene, USA / [175]</td>
</tr>
<tr>
<td>pLV hUbC-VP64 dCas9 VP64-T2A-GFP</td>
<td>59791</td>
<td>Addgene, USA / [175]</td>
</tr>
</tbody>
</table>
### Material and Methods

- **pMD2.G**: Addgene, USA / gift from Didier Trono
- **pMDLg/pRRE**: Addgene, USA / [176]
- **pRL-SV40 Vector**: Promega, USA
- **pRSV-Rev**: Addgene, USA / [176]
- **pSC-B-amp/kan**: Agilent Technologies, USA
- **SP-dCas9-VP1R**: Addgene, USA / [90]
- **Sso7d_dSpCas9(D10A,H840A)_12xSpyTag**: Available at the IDG; generated by J. Truong
- **Sso7d_dSpCas9(D10A,H840A)_4xSpyTag**: Available at the IDG; generated by J. Truong
- **Sso7d_dSpCas9(D10A,H840A)_8xSpyTag**: Available at the IDG; generated by J. Truong
- **Sso7d_dSpCas9_VP160**: Available at the IDG; generated by J. Truong
- **Tet-O-ALN (Ascl1-T2A-Lmx1a-P2A-Nurr1)**: Available at the IDG; generated by J. Zhang
- **Tet-O-Ascl1**: Caiazzo et al. 2011, [37]
- **Tet-O-FUW**: Caiazzo et al. 2011, [37]
- **Tet-O-Lmx1a**: Caiazzo et al. 2011, [37]
- **Tet-O-Nurr1**: Caiazzo et al. 2011, [37]
- **Tet-O-Smarca1**: Available at the IDG; generated by B. Rauser
- **Tet-O-T2A-dsRed**: Available at the IDG
- **VP160_SpyCatcher**: Available at the IDG; generated by J. Truong

#### Table 15: Cell lines

<table>
<thead>
<tr>
<th>Description</th>
<th>Catalogue number</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK293</td>
<td>CRL-1573</td>
<td>ATCC, USA</td>
</tr>
<tr>
<td>Neuro-2a</td>
<td>CCL-131</td>
<td>ATCC, USA</td>
</tr>
<tr>
<td>Lenti-X™ 293T</td>
<td>632180</td>
<td>Takara Bio, USA</td>
</tr>
</tbody>
</table>

#### Table 16: Bacterial strains

<table>
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<tr>
<th>Description</th>
<th>Catalogue number</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5a</td>
<td>18265017</td>
<td>Thermo-Fischer Scientific, USA</td>
</tr>
<tr>
<td>XL10-Gold Ultracompetent Cells</td>
<td>200315</td>
<td>Agilent Technologies, USA</td>
</tr>
<tr>
<td>One Shot® TOP10</td>
<td>C404010</td>
<td>Thermo-Fischer Scientific, USA</td>
</tr>
</tbody>
</table>
### Table 17: Mouse strains

<table>
<thead>
<tr>
<th>Description</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>C67Bl6/N</td>
<td>Charles River; Germany</td>
</tr>
<tr>
<td>CD-1</td>
<td>Charles River; Germany</td>
</tr>
<tr>
<td>Pitx3(^{GFP/GFP})</td>
<td>Zhao <em>et al.</em> 2004, [149]</td>
</tr>
</tbody>
</table>

### Table 18: Software

<table>
<thead>
<tr>
<th>Description</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>CellInsight NXT</td>
<td>Thermo Scientific, Germany</td>
</tr>
<tr>
<td>GraphPad Prism 5.01</td>
<td>Graphpad Software, Inc., USA</td>
</tr>
<tr>
<td>Stereo Investigator 5.05.4</td>
<td>MBF Bioscience, USA</td>
</tr>
<tr>
<td>Fluoview 2.0b</td>
<td>Olympus, Germany</td>
</tr>
<tr>
<td>SDS 2.4.1</td>
<td>Applichem, Germany</td>
</tr>
<tr>
<td>MicroWin32 5.0.249</td>
<td>HeroLab, Germany</td>
</tr>
<tr>
<td>Vector NTI® Advance 11.5.2</td>
<td>Invitrogen, Germany</td>
</tr>
</tbody>
</table>

### 8.2 Methods

#### 8.2.1 Isolation and culture of primary cells and established cell lines

All experiments in cell culture were performed under sterile conditions. Primary cells were incubated at 37°C, 5% CO\(_2\). Established cell lines were cultured at 37°C, 7% CO\(_2\).

#### 8.2.1.1 Storage and culture of stable cell lines

HEK293, Lenti-X 293T and N2A cell lines were stored in liquid nitrogen in 10% DMSO/DMEM medium (Table 6). Cells were thawed at 37°C in a water bath and diluted in 10 ml DMEM medium before centrifugation at 200 x g for 3 min. The cell pellet was resuspended in fresh DMEM medium and cells were cultured in T75 flasks with 20 ml DMEM medium. Upon reaching a confluency of approximately 90% cells were passaged by washing the cells with sterile Dulbecco's Phosphate-Buffered Saline (DPBS) and subsequent trypsinization using 0.25% Trypsin/EDTA. After centrifugation for 2 min at 200 x g supernatant was removed and the cell pellet was resuspended in fresh DMEM medium. Cells were then seeded in a fresh flask in a 1:10 dilution.

#### 8.2.1.2 Isolation and culture of primary fibroblasts

Primary mouse embryonic fibroblasts (MEFs) were isolated from E14.5 CD1 or Pitx3\(^{GFP/+}\) mouse embryos. Head, viscera, extremities and spinal cord were removed in cold PBS containing 1% penicillin/streptomycin. The remaining tissue was chopped into pieces and incubated in 0.05% Trypsin/EDTA at 37°C for 10 min. Afterwards, trypsin was inactivated by the addition of MEF medium (Table 6) and the suspension was filtered using 70 μm cell strainers in order to obtain a single cell suspension. Cells were centrifuged at 200 x g for...
4 min and resuspended in fresh MEF medium. Centrifugation was repeated twice before 5 x 10^6 cells were seeded in a T75 flask containing 20 ml MEF medium. Upon reaching 80% confluency cells were trypsinized and stored in liquid nitrogen using 10% DMSO/MEF medium. These cells were considered passage one MEFs.

8.2.1.3 Isolation and culture of primary astrocytes
Primary cortical astrocytes were obtained from postnatal (p5 – p6) CD1 mice as described by Heinrich et al., 2011 [49]. Shortly, the brain was collected; cerebellum and optic chiasm were removed in cold astrocyte dissection buffer (Table 6). Afterwards the two hemispheres were separated by a longitudinal cut. Diencephalon and hippocampal formation were discarded leaving the cortex. Meninges were cleared away carefully and the remaining tissue was transferred to a tube containing cold astrocyte dissection buffer. The cortices of three animals were pooled. Dissection buffer was removed under sterile conditions and 5 ml astrocyte plating medium (Table 6) were added. A 200 µl pipette tip was stuck on the tip of a 10 ml pipette and the medium containing the tissue was carefully pipetted up and down three times in order to break down the cortices. Afterwards, the cell suspension was pipetted into a T75 flask and additional 15 ml of astrocyte plating medium were added. Cells were incubated for four days at 37°C. Flasks were tapped vigorously to loosen tissue chunks and loose cells such as oligodendrocytes or microglia. Then, medium was replaced by fresh astrocyte medium and cells were incubated for another six days. At this point astrocytes were trypsinized and directly used for experiments.

8.2.1.4 Coating of cover slips for cell attachment
In order to allow a qualitative and quantitative analysis of cell culture experiments after immunocytochemistry cells were seeded on glass coverslips in 24-well plates. These cover slips required coating by poly-D-lysine to enable cell attachment. Prior to coating the coverslips were autoclaved and placed in individual wells containing sterile water. The water was replaced by 500 µl 5% poly-D-lysine/PBS solution and the plates were incubated at 37°C overnight. The cover slips were washed three times with sterile water and dried at room temperature (RT) under sterile conditions. Plates were stored at 4°C for up to one week.

8.2.1.5 Lipofection
Lipofection of HEK293, Lenti-X™ 293T and N2A cells was performed using Lipofectamine2000 according to the manufacturer’s instructions. Shortly, Lipofectamine 2000 was diluted in serum free OPTI-MEM and incubated for 5 min. Afterwards, pre-diluted DNA in OPTI-MEM medium was added and the mix was incubated at RT for 20 min. The DNA/Lipofectamine 2000 mix was added dropwise to the cells. After 6 h medium was replaced by fresh medium. In order to enable a quantitative comparison of transcriptional activators by luciferase assays or RT-qPCR, plasmids with different sizes were adjusted on
the molar level. Within individual experiments the total amount of transfected DNA was equalized in all conditions by the addition of the empty plasmid pcDNA3. Table 19 states the amounts of individual plasmids used in lipofections conducted in this thesis.

| Table 19: Transfection of DNA plasmids using Lipofectamine 2000 |
|-----------------------------|-----------------------------|
| Plasmid                     | DNA per well in a 24-well plate |
| Firefly luciferase reporter constructs | 200 ng |
| Renilla luciferase          | 1 ng |
| CRISPR/Cas9 fusions         | 10 fmol |
| SAM activator complex       | 10 fmol |
| SpyCatcher                  | 40 fmol |
| scFv-VP64                   | 20 fmol |
| Tet-O-Ascl1, Tet-O-Lmx1a, Tet-O-Nurr1, Tet-O-Smarca1, rTTA | 10 fmol |
| gRNAs, combined amount of all gRNAs per well | 10 ng |
| pcDNA3                      | Adjustment to highest DNA amount of experiment |

Primary cortical astrocytes were transfected using Lipofectamine LTX with Plus Reagent according to the manufacturer’s instructions. However, the amounts of DNA, Lipofectamine LTX and Plus Reagent were doubled to approximately 1.1 µg DNA, 5 µl Lipofectamine LTX and 1 µl Plus reagent per well of a 24-well plate with 5 x 10⁴ primary astrocytes. The molar ratios of plasmids transfected are shown in Table 20. 100 µl Lipofectamine LTX/DNA mix were added to each well of a 24-well plate.

| Table 20: Transfection of DNA plasmids using Lipofectamine LTX |
|-----------------------------|-----------------------------|
| Plasmid                     | DNA per well in a 24-well plate |
| CRISPR/Cas9 fusions         | 40 fmol |
| SAM activator complex       | 40 fmol |
| Tet-O-Ascl1, Tet-O-T2A-dsRed, rTTA | 40 fmol |
| gRNAs, combined amount of all gRNAs per well | 20 ng |
| pcDNA3                      | Adjustment to highest DNA amount of experiment |

8.2.2 Preparation of lentiviruses and titer determination

Replication incompetent, self-inactivating lentiviruses were generated with a third-generation packaging system [176]. For each virus 1.6 x 10⁷ low passage Lenti-X 293T cells were seeded in two T225 flasks, respectively. On the next day, medium was replaced by 18 ml fresh DMEM medium in each flask 2 h before transfection. For the transfection of two T225 flasks DNA was diluted in 9 ml OPTI-MEM medium as indicated in Table 21.
Table 21: Transfection of DNA for lentivirus production

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>DNA for an individual virus [µg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transfer vector with gene of interest</td>
<td>59.5</td>
</tr>
<tr>
<td>pMDLg/pRRE</td>
<td>39.8</td>
</tr>
<tr>
<td>pRSV-Rev</td>
<td>15.4</td>
</tr>
<tr>
<td>pMD2.G</td>
<td>19.5</td>
</tr>
</tbody>
</table>

248 µl Lipofectamine2000 were diluted in 9 ml OPTI-MEM and incubated for 5 min. Lipofectamine and DNA mix were combined and incubated for further 20 min before 9 ml of the mix were added to the two T225 flasks. After 5 h medium was replaced by 21 ml fresh DMEM medium. Virus was harvested 72 h after transfection by combining the supernatant of the two flasks. In order to remove cell debris, the supernatant was centrifuged at 2,200 x g for 10 min. Afterwards, the supernatant was filtered using a 0.45 µm PES filter and centrifuged in an Avanti J-30I centrifuge at 50,000 x g, 4°C for 2 h. The pellet was washed with 30 ml cold TBS-5 buffer (Table 6) and centrifuged again at 50,000 x g, 4°C for 2 h. The supernatant was removed and the pellet dissolved in 100 µl TBS-5 buffer overnight at 4°C. Virus was then aliquoted and stored at -80°C. In order to determine the virus titer 5 x 10^4 HEK293 cells/well were seeded on a 24-well plate. After 4 h of cultivation 2 µl of lentivirus were diluted in 400 µl DMEM medium. 1 µl (containing 0.005 µl virus), 10 µl (containing 0.05 µl virus) or 100 µl (containing 0.5 µl virus) of the virus dilution were added to individual wells, respectively. For viruses containing a Tet-O promoter, 1 µl of rTTA2 virus and 2 µg/ml doxycycline were added to each well to enable expression from the Tet-O-promoter. 48 h after transduction cells were fixed and stained to detect the expression of the transduced gene (see 8.2.4). The percentage of transduced cells per DAPI+ cells was determined by scanning 50 fields of each well using the CellInsight NXT High Content Screening Platform. After calculating the corresponding number of transduced cells per 5 x 10^4 cells the titer was calculated as follows:

\[
\frac{\text{# transduced cells per } 5\times 10^4 \text{ seeded cells}}{\mu l \text{ of virus used (0.005, 0.05 or 0.5 } \mu l)} \times 10^3 = \text{ infectious particles per ml}
\]
8.2.3 Direct reprogramming of somatic cells

8.2.3.1 Reprogramming of MEFs

MEFs in passage one were thawed and cultured in a T75 flask in MEF medium (see Table 6) until reaching a confluency of approximately 80%. Cells were trypsinized and 5 x 10⁴ cells were seeded on poly-D-lysine coated cover slips (see 8.2.1.4) in 24-well plates. After 24 h cells were transduced with viruses at a multiplicity of infection of three for each individual virus. When using the Tet-O system, 1 µl rTTA2 lentivirus was co-transfected. 24 h after transduction the medium was replaced with 500 µl fresh MEF medium. From this point onwards 2 µg/ml doxycycline were added to the medium if Tet-O promoters were used. 72 h after transduction the medium was replaced by 500 µl differentiation medium (N2-B27 medium, see Table 6). Six days after transduction the medium was replaced by 500 µl fresh differentiation medium. Nine days after transduction 250 µl of the conditioned differentiation medium were replaced by fresh differentiation medium. At day 12 – 14 cells were fixed and stained for analysis (see 8.2.4). The percentage of neurons per DAPI was determined in 20 random fields (200 x 200 µm) on each coverslip using the Stereo Investigator system. Only TUBB3⁺ or MAP2⁺ cells with a mature neuronal morphology (compact soma, at least one neurite more than three times longer than the cell body) were considered successfully reprogrammed neurons. As an alternative to normalizing to DAPI, cells were stained for the expression of the delivered gene such as Cas9 and the percentage of mature neurons per Cas9⁺ cells was determined.

8.2.3.2 Reprogramming of astrocytes

Ten days after isolation of cortical astrocytes (see 8.2.1.3) cells were washed with PBS and trypsinized. 5 x 10⁴ cells were seeded in 100 µl astrocyte medium carefully in the middle of coated glass cover slips (see 8.2.1.4) in 24-well plates (Table 6). This prevented a loss of cells due to attachment to the plate outside the glass cover slip. 1 h after seeding, 400 µl additional astrocyte medium were added. At 3 h past seeding cells were transduced using lentiviruses at different multiplicities of infection (see individual experiments). On the next day, astrocyte plating medium was replaced by 1.5 ml astrocyte reprogramming medium (Table 6) containing 2 µg/ml doxycycline if Tet-O promoters were used. As an alternative to viral transduction cells were transfected 24h after seeding. In order to remove dead cells before the transfection, the medium was removed, sterile filtered with a 0.22 µm PES syringe filter and 400 µl of the medium were added to the wells again. The transfection was performed using Lipofectamine LTX as described in chapter 8.2.1.5. On the next day, medium was replaced by 1.5 ml astrocyte reprogramming medium (see Table 6) containing 2 µg/ml doxycycline if Tet-O promoters were used. Transduced and transfected cells were incubated for 14 days before fixation and staining (see 8.2.4). During these 14 days, BDNF was added at a concentration of 20 ng/ml every fourth day. The percentage of neurons per
DAPI was determined in 25 random fields (500 x 500 µm) on each coverslip using the Stereo Investigator system. As an alternative to normalizing to DAPI, cells were stained for the expression of the delivered gene such as Cas9 and the percentage of mature neurons per Cas9+ cells was determined. Due to the significantly lower DNA transfer efficiency of lipofection complete coverslips were analyzed in this case. In lipofection reprogramming experiments marker genes such as dsRed were co-transfected and all dsRed+ cells on the whole coverslip were checked for co-expression of neuronal markers. Only TUBB3+ or MAP2+ cells with a mature neuronal morphology (compact soma, at least one neurite more than three times longer than the cell body) were considered successfully reprogrammed neurons.

### 8.2.4 Immunocytochemistry and microscopy

Immunocytochemistry (ICC) was used to visualize gene expression of cells in *in vitro* experiments. For pre-fixation, an equal volume of 10% formalin was added to the cells in culture medium of a 24-well plate and incubated for 5 min at 37°C. The medium was then removed and replaced by 10% formalin for 10 min at 37°C. Afterwards cells were washed three times using DPBS. Primary antibodies were diluted according to Table 9 in blocking buffer (1% BSA, 0.5 % Triton-X-100 in PBS) and 200 µl antibody solution were added to each well in a 24-well plate. After incubation at 4°C overnight, cells were washed three times with PBS. Suitable secondary antibodies (see Table 10) were diluted in blocking buffer and 200 µl were added to each well and incubated in the dark for 1 h at RT. Afterwards the antibody solution was replaced by 200 µl DAPI (100 ng/ml DPBS) in order to visualize nuclei. DAPI was discarded after 1 min followed by three washing steps using PBS. Finally, cover slips were removed from the 24-well plate and mounted on glass slides using AquaPolyMount. Slides were dried and stored at 4°C in the dark.

Pictures of fluorescence stained cells were taken using an Olympus IX81 confocal microscope and Fluoview 2.0 software. For quantitative analysis, an Axioplan 2 microscope together with Stereoinvestigator software were used. Unless indicated otherwise, 20 – 25 random 200x200 µm fields evenly distributed over the whole coverslip were analyzed. Only fields with more than 10 cells were counted.

### 8.2.5 Luciferase assay analysis

Luciferase assays were performed to analyze transcription factor binding and intracellular signaling mechanisms. The assay was based on a dual-luciferase system. A *firefly luciferase* was expressed under the control of an assay dependent promoter (i.e. *Pitx3* promoter, binding sites for transcription factors or signaling molecules of interest). From a second plasmid *renilla luciferase* was expressed under the control of a constitutively active *SV40* promoter and served as a control for normalization. 5 x 10^4 HEK293 or N2A cells were
seeded in 24-well plates 24 h prior to transfection. **Firefly luciferase** plasmid and **renilla luciferase** plasmids were transfected together with further plasmids of interest using Lipofectamine 2000 (see 8.2.1.5). Cells were lysed using 100 µl passive lysis buffer provided in the promega dual luciferase assay kit 48 h after transfection by shaking at RT for 15 min. 10 µl of the lysate were used for the measurement of firefly and renilla luciferase activity in a white 96-well plate using a Centro LB 960 luminometer. The program settings for the MicroWin32 luminometer software are shown in Table 22.

**Table 22: Settings MicroWin32 luminometer software**

<table>
<thead>
<tr>
<th>Step</th>
<th>Operation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dispense</td>
<td>Volume</td>
<td>50 µl</td>
</tr>
<tr>
<td></td>
<td>Speed</td>
<td>Middle</td>
</tr>
<tr>
<td></td>
<td>Measured operation</td>
<td>By well</td>
</tr>
<tr>
<td></td>
<td>Repeated operation</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Duration</td>
<td>2 s</td>
</tr>
<tr>
<td>Delay</td>
<td>Measurement option</td>
<td>By well</td>
</tr>
<tr>
<td></td>
<td>Name</td>
<td>Firefly or renilla</td>
</tr>
<tr>
<td>Firefly/renilla</td>
<td>Counting time</td>
<td>5 s</td>
</tr>
<tr>
<td></td>
<td>Measurement option</td>
<td>By well</td>
</tr>
</tbody>
</table>

For data analysis firefly values were normalized to renilla activity and these were subsequently normalized to control values in order to receive fold change values. All experiments were performed with three technical replicates.

### 8.2.6 RT-qPCR

RT-qPCR was used to quantitatively assess mRNA levels of different genes of interest in cell culture experiments. 5 x 10^4 cells were seeded in 24-well plates 24 h before transfection. Experiments were performed in triplicates and Lipofectamine 2000 was used for DNA transfer (see 8.2.1.5). Six hours after lipofection medium was replaced and 2 µg/ml doxycycline was added to experiments where **Tet-O-promoters** were included. 48 h after transfection RNA was isolated using a RNeasy plus Kit according to the manufacturers protocol. 400 ng RNA were reversely transcribed from each well using the SuperScript VILO cDNA synthesis kit in a 20 µl reaction. Subsequently, 80 µl RNAse free water was added and this 1:10 dilution was used for RT-qPCR analysis. All measurements were carried out in technical triplicates. 9 µl cDNA were pipetted into a 384 well-plate. 1 µl TaqMan probe and 10 µl TaqMan universal PCR mastermix were added and the plate was sealed with adhesive seal sheets. RT-qPCR was carried out using an ABI Prism 7900 HT Real-Time PCR System and SDS 2.4.1 software with settings shown in Table 23.
In order to analyze the data the mean threshold cycle (ct) values of the gene of interest from three technical replicates were normalized to the mean ct of a control gene (b-actin, Gapdh). This value was termed Δct. Subsequently, fold changes were calculated as follows:

\[
\Delta ct = \frac{ct \text{ gene of interest}}{ct \text{ housekeeping gene}}
\]

\[
\Delta \Delta ct = \frac{\Delta ct \text{ treated cells}}{\Delta ct \text{ control}}
\]

*Fold change values* = \(2^{-\Delta\Delta ct}\)

### 8.2.7 Western blot

24 h after transfection cells were lysed by adding 100 µl RIPA buffer (Table 6) to each well of a 24-well plate. Protein concentration was determined using a Pierce BCA protein assay kit according to the manufacturer's instructions. Absorption was measured at 562 nm by a ChemiDoc imaging system. A linear equation was determined from the absorption values of the BSA standard which allowed calculation of protein sample concentration.

Afterwards, 5 µl NuPAGE LDS sample buffer containing 4% β-mercaptoethanol were added to 15 µl concentration-matched samples which were then incubated at 95°C for 5 min for denaturation. After cooling down on ice samples were pipetted into gel pockets of a 4 - 12% NuPAGE Novex Bis-Tris gel. For size determination 5 µl Page Ruler Plus Prestained were used. Gel electrophoresis was performed at 200 V in 1x NuPAGE MOPS SDS running buffer. PVDF membranes were activated prior to blotting by incubation in 100% methanol for 30 s followed by 2 min in water and 5 min in 1x NuPAGE transfer buffer/ 10% methanol. The SDS gel was equilibrated in 1x NuPAGE transfer buffer/ 10% methanol for 15 min. Blotting pads and whatman paper were also soaked in this buffer. Two blotting pads followed by a whatman paper, SDS gel and the PVDF membrane were placed into a blotting chamber. On top, another whatman paper was placed and the chamber was filled with several layers of blotting pads and 1x NuPAGE transfer buffer/ 10% methanol. Transfer of the proteins from gel to PVDF membrane was subsequently performed at 30 V for one hour or at 20 V, 4°C overnight for proteins > 100 kDa. Afterwards, the membrane was washed in TBS-T buffer (see Table 6) for 5 min and blocked in western blot blocking buffer (see Table 6) for 1 h.
Overnight, the membrane was incubated with a primary antibody (see Table 9) diluted in western blot blocking buffer at 4°C. On the next day, the membrane was washed with TBS-T buffer 3 x 5 min. A suitable secondary antibody coupled to horseradish-peroxidase (Table 10) was diluted 1:5000 in blocking buffer and incubated with the membrane for 1 h at room temperature. Subsequently, the membrane was washed 3 x 5 min in TBS-T buffer. For the detection of protein bands 0.5 ml Ace glow luminol enhancer per lane were pipetted onto the membrane. Pictures were taken using a Fusion SL imaging system. Quantitative analysis was performed with ImageJ software. Band intensities of the protein of interest and a housekeeping protein such as β-Actin were determined with ImageJ and the values of the protein of interest were divided by the values of the loading control. Afterwards, all values were normalized to the control condition in order to obtain fold change values.

8.2.8 Isolation of nucleic acids

8.2.8.1 Isolation of RNA
For the isolation of mRNA from cells a RNeasy plus mini kit was used according to manufacturer’s instructions.

8.2.8.2 Purification of DNA
In order to remove buffer components, primers or enzymes for subsequent enzymatic steps DNA was purified using a QIAquick PCR Purification Kit according to manufacturer’s instructions. For the isolation of DNA fragments from agarose gels the bands of interest were cut from gels with a scalpel and DNA was isolated using a QIAquick Gel Extraction Kit according to the manufacturer’s instructions.

8.2.8.3 Agarose gel electrophoresis
Agarose gel electrophoresis was performed for size estimation and separation of DNA fragments. Agarose powder was boiled in 1X TEA buffer (Table 6) and 1 µg/ml EtBr was added for the detection of DNA fragments before gels were cast. The agarose concentration was adjusted to the size of the analyzed DNA fragments (1 - 2% agarose for DNA <1 kb, 0.8 - 1% agarose for DNA >1 kb). DNA samples were mixed with loading buffer and applied to the gel. Gel electrophoresis was performed in 1x TAE buffer at 120 V and pictures were taken using a E.A.S.Y Win32 Gel-Documentation system.

8.2.9 DNA plasmid preparations
For mini plasmid preparations 5 ml LB-medium with a suitable selection marker were inoculated with a single bacterial colony from an agar plate. For a maxi plasmid preparation 200 ml of medium were used. Bacteria were cultured in a shaker at 37°C overnight. Plasmid preparations were performed using a QIAprep Spin Miniprep or QIAprep Spin Maxiprep Kit according to the manufacturer’s instructions. Plasmids for lentivirus production were isolated.
using the PureLink® HiPure Plasmid Filter Maxiprep Kit according to the manufacturer’s instructions in order to achieve superior plasmid purity.

8.2.10 Cloning of new constructs

8.2.10.1 Polymerase chain reaction
For PCRs requiring a proof-reading polymerase the Q5 High-Fidelity 2X Master Mix was used according to manufacturer’s instructions. For colony PCRs and other reactions not requiring proof reading REDTaq ReadyMix PCR Reaction Mix was used according to manufacturer’s instructions. Site-directed mutagenesis was carried out with a QuikChange Lightning Site-Directed Mutagenesis Kit. PCRs were performed in a Mastercycler pro. All primers used for amplification steps are shown in Table 11.

8.2.10.2 Digestion of DNA fragments
For subsequent cloning steps or as a control after cloning 1 – 5 µg of DNA fragments or plasmids were digested with restriction enzymes provided by New England Biolabs according to manufacturer’s instructions.

8.2.10.3 Ligation of DNA fragments
DNA fragments were ligated either by using a Rapid DNA Dephos & Ligation Kit or by gibson assembly (NEBuilder® HiFi DNA Assembly Master Mix) according to manufacturer’s instructions. Blunt end cloning was performed using a StrataClone PCR Cloning Kit according to manufacturer’s instructions.

8.2.10.4 Design and generation of gRNA constructs
The 20 nucleotide targeting sequence (crRNA) of a gRNA defines the sequence specific binding to DNA. Targeting sequences were designed within positions -250 to -1 upstream of the transcription start site of the respective gene. gRNAs N1 – N8 targeting the murine Nurr1 promoter and gRNAs A1 – A8 binding to the murine Ascl1 promoter were designed using the online tool http://crispr.mit.edu/. For each gene the eight targeting sequences with the highest off-target score (i.e. low probability of off-target binding) and an even distribution over the 250 nucleotides were chosen. Targeting sequences mA1 to mA5 binding the murine Ascl1 promoter were designed using the online platform https://benchling.com/. This tool offered an additional on-target score indicating gRNAs with higher binding properties. The five targeting sequences with the highest on-target scores were chosen (mA1 > mA5). Sense and antisense oligos (without PAM sequence) were ordered with the following additions: sense 5’-CACCGG-20nt-3’, anti-sense 5’-AAAC-20nt-CC-3’. These overhangs served for later sticky end cloning into the target vector and GG 5’ of the 20 nucleotides was required as a start signal for RNA polymerase III.
The lyophilized sense and antisense targeting sequence oligos were dissolved at 1 µg/µl in TE buffer (see Table 6). 1 µl of each oligo was pipetted to 100 µl TE buffer followed by incubation at 100°C for 5 min. The oligos were cooled down slowly to allow hybridization. These double stranded DNA fragments were then cloned into plasmids containing a RNA polymerase III promoter and the gRNA scaffold. As a non-viral gRNA expressing plasmid pBS-U6-chimaeric-F+E was used. pKL-U6gRNA(BbsI)-PGKpuro2ABFP served as gRNA vector suitable for lentiviral packaging and lenti-sgRNA(MS2)-zeo-backbone was used for gRNA expression in combination with the SAM system and for lentiviral packaging. 5 µg of these target vectors were digested using BbsI or BsmBI leaving suitable sticky end overhangs for ligation with the annealed oligos. After gel purification 50 ng digested gRNA backbone were ligated with 4 µl annealed oligos (see 8.2.10.3) and transformed into DH5α bacteria (see 8.2.11). Successful cloning was checked by sequencing at GATC Biotech.

8.2.10.5 Addition of a FlagTag to the SAM construct
To allow detection and titer determination of SAM the hygromycin resistance cassette of lenti-MS2-P65-HSF1_Hygro (SAM) was replaced by a FlagTag. The vector was digested by BsrGI and EcoRI. Flag-oligo-F and Flag-oligo-R (see Table 11) containing a FlagTag and stop codon were annealed similar to gRNA targeting sequences (see chapter 8.2.10.4) and ligated with the digested plasmid.

8.2.10.6 Design and generation of the split-Cas sytem with Ef1a promoters
The split-Cas system was based on an intein split system developed at the IDG previously [141] for adeno-associated virus (AAV) delivery. The system consisted of two vectors containing N-Cas (SpCas92-573) and C-Cas (SpCas9574-1368) fused to DnaE-N-Intein and DnaE-C-Intein respectively. Since the packaging limit of AAVs prevented the addition of SAM and VPR a lentiviral system was chosen. The aim was to generate a two-vector system containing all components necessary: N-Cas was combined with one gRNA and SAM, C-Cas was combined with a second gRNA and VPR.

First, a SAM-compatible gRNA scaffold together with a hU6 promoter from lenti sgRNA(MS2)zeo backbone was added to the SAM construct (lenti-MS2-P65-HSF1_Hygro) by AleI and AgeI digest and ligation. Next, N-Cas-N-intein was amplified by PCR from AAV vector N-SpCas9(2-573)_Npu-DnaE-N-Intein (N-Cas-Int F and N-Cas-Int R primers) and a gblock containing Flag-Tag and P2A sequences (gBlock-Flag-P2A) to connect SAM and N-Cas in a single expression cassette was ordered. The newly generated gRNA-SAM vector was digested by BsrGI/BstXI followed by gibson assembly of the gRNA-SAM backbone, the N-Cas-N-intein PCR product and the FlagTag-Stop gblock resulting in hU6-gRNA-Ef1a-SAM-P2A-N-Cas-N-intein (termed hU6-mA1-Ef1a-SAM-N-Cas).

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In order to generate a C-Cas-VPR construct pCAG-Cas9v2_Intein_C-Part_v1_new was digested using EcoRV and MluI resulting in pCAG-Cas9v2_Intein. The wildtype C-Cas was then replaced by C-Cas-VPR from the SP-dCas9-VPR construct by Gibson assembly thus generating C-intein-C-cas-VPR. Finally, the whole coding sequence was amplified (Int-C-Cas-Fw, Int-C-Cas-Rev primers) and transferred to the lentiviral lenti sgRNA(MS2)zeo backbone containing gRNA mA2. For this purpose the gRNA vector was digested by BsrGI/EcoRI thus replacing the zeomycin resistance cassette by C-intein-C-cas-VPR generating the hU6-mA2-Ef1a-C-intein-C-Cas-VPR construct.

8.2.10.7 Design and generation of CRISPR/Cas9 constructs using the Tet-O system

These constructs were designed to generate a split-Cas system with an inducible promoter system enabling regulation of the expression level and timing of gene induction. Furthermore, these constructs were generated with the same unique restriction sites allowing simple exchange of individual cassettes. gRNAs including SAM-compatible loops were flanked by Nhel/RsrII recognition sites, the Tet-O-promoter by RsrII and BsiWI cutting sites, and the open reading frame by BsiWI and SbfI.

The lenti-sgRNA(MS2)zeo backbone containing a gRNA scaffold suitable for the SAM system and the mA1 or mA2 gRNA was digested using BamHI and EcoRI. The backbone without promoter and zeomycin resistance was isolated by gel electrophoresis. The Tet-response element (TRE, Tet-O-promoter) was amplified from Tet-O-FUW using the primers TRE-ampl-F (containing BamHI, RsrII restriction sites) and TRE-ampl-R (containing EcoRI and SbfI restriction sites). The PCR product was blunt-cloned using the StrataClone kit (see 8.2.10.3) into pSC-B-amp/kan. The insert was then digested by BamHI/EcoRI and cloned into the digested lenti-sgRNA(MS2)zeo backbones. The new vectors were termed mA1-Tet-O and mA2-Tet-O.

The coding sequences of SAM-N-Cas and SAM were cloned into mA1-Tet-O, the coding sequences of C-Cas-VPR and dCas-VPR were cloned into mA2-Tet-O. The SAM-N-Intein-N-Cas coding sequence was amplified from mA1-Ef1a-SAM-N-Cas-N-intein using N-Cas-BsiWI-F and N-Cas-SbfI-R primers and cloned into the pSC-B-amp/kan vector using a Strataclone blunt cloning kit. By BsiWI and SbfI digest the coding sequence was then transferred into the mA1-Tet-O backbone generating mA1-Tet-O-SAM-N-Cas-N-intein. SAM-Flag (amplified with N-Cas-BsiWI-F and SAM-SbfI-R primers from Ef1a-SAM-Flag) was also cloned into the mA1-Tet-O construct. Similar, C-Cas-VPR (amplified using C-Cas-BsiWI-F, C-Cas-SbfI-R), dCas-VPR (amplified with VPR-BsiWI-F, VPR-SbfI-R), VP64-dCas-VP64 (amplified by VP64-Cas-VP64-Fw, VP64-Cas-VP64-Rv) were cloned into mA2-Tet-O.

dCas-VP64 was amplified from lenti dCAS-VP64_Blast (dCasVP64-BsiWI-F, dCasVP64-SbfI-R primers) and ligated into pSC-B-amp/kan using the Strataclone blunt ligation kit. An
internal BsiWI restriction site was removed by site directed mutagenesis (see 8.2.10.1) using BsiWI-del-F primer. Afterwards, dCas-VP64 was digested with BasiWI/SbfI and transferred into the mA2-Tet-O vector.

8.2.11 Transformation of competent bacteria
For the multiplication of plasmids chemically competent bacteria were transformed. Bacteria were carefully thawed on ice and 2 µl plasmid DNA were added to 50 µl of bacteria. After incubation on ice for 20 min cells were heat shocked for 30 s at 42°C in a water bath. After 2 min on ice, 250 µl of pre warmed (37°C) LB-medium was added and the tube was shaken at 37°C for 1 h. Afterwards bacteria were spread on agar plates containing suitable selection markers. Bacteria were incubated overnight at 37°C and single colonies were picked for further analysis.

8.2.12 Glycerol stock preparations
For long-term storage of bacteria containing plasmids of interest 700 µl overnight culture were mixed with 300 µl 80% glycerol and stored at -80°C immediately.

8.2.13 Statistical analysis
Statistical analysis of data was performed using GraphPad prism 5 software. Only experiments with at least three biological replicates were analyzed. In order to test for Gaussian distribution of the values a D'Agostino and Pearson omnibus normality test was performed. If data passed the normality test, either a t-test or one-way ANOVA test was conducted. Values with non-Gaussian distributions were analyzed by Mann-Whitney or Kruskal-Wallis tests respectively. Asterisks were assigned as follows: *P < 0.05. All data are shown with mean ± standard error of the mean (SEM).
9 References


9 | REFERENCES


9 | REFERENCES

10 Appendix

10.1 Supplementary data

This section includes supplementary information as indicated in the results or discussion sections.

Figure 25: gRNA N2 successfully induces Nurr1 in Neuro 2a cells
Luciferase assay screen for suitable gRNAs to induce murine Nurr1 48 h after transfection of Neuro 2a cells. (A) The combination of gRNAs N2/N6 showed the strongest luciferase activity. (B) gRNA N2 alone reached comparable levels as the combination of gRNAs N2/N6 together for Nurr1 induction in (A). gRNA N2 was therefore chosen for further screenings regarding the induction of murine Nurr1. Abbreviations: gRNAs N1-8: gRNAs targeting the murine Nurr1 promoter, Sso7d: S. solfataricus DNA binding protein 7d, VP160: ten repeats of the Herpes simplex virus protein vmw65 (VP16) transactivation domain. Data was derived from one experiment. Error bars represent mean ± SEM.

Figure 26: Increasing the MOI of Ascl1, Lmx1a, Nurr1 encoding lentiviruses to ten results in a decrease of TH⁺ cells
Quantification of reprogrammed TH⁺ neurons 14 days after transduction of MEFs with individual lentiviruses encoding Ascl1, Lmx1a and Nurr1. Here, the total number of TH⁺ cells / well was determined. An increase in the viral load from MOI3 to MOI10 (three and ten times more viral particles than cells, respectively) resulted in a reduction of reprogrammed TH⁺ cells indicating adverse effects of high lentiviral particle concentrations. Abbreviations: A: Ascl1, L: Lmx1a, MOI: multiplicity of infection, N: Nurr1. Data was derived from one experiment. Error bars represent mean ± SEM.
A

<table>
<thead>
<tr>
<th></th>
<th>N2 supplemented DMEM/F12</th>
<th>Caiazzo al., supplementation of DMEM/F12</th>
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<tr>
<td>Insulin</td>
<td>5 µg/ml</td>
<td>25 µg/ml</td>
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<tr>
<td>Transferrin</td>
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<td>50 µg/ml</td>
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<tr>
<td>Sodium selenite</td>
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<td>30 nM</td>
</tr>
<tr>
<td>Progesterone</td>
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</tr>
<tr>
<td>Putrescine</td>
<td>100 µM</td>
<td>0.1 µM</td>
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</table>

Figure 27: Switching from N2/B27 to the medium used by Caiazzo et al., does not influence the reprogramming efficiency

(A) Concentrations of N2 supplement components compared to concentrations used by Caiazzo et al., [37]. How final concentrations of these substances in a 50:50 mixture of N2/B27 medium used in this thesis (see Table 6 for details) compare to the medium by Caiazzo et al., is unclear as all substances shown in the table above are also included in B27 supplement at unknown concentrations. (B) Quantification of reprogrammed TH+ neurons 14 days after transduction of MEFs with individual lentiviruses encoding Ascl1, Lmx1a and Nurr1. Switching from the previously used N2/B27 medium to the differentiation medium used by Caiazzo et al., did not significantly affect the percentage of reprogrammed TH+ cells. Abbreviations: A: Ascl1, L: Lmx1a, N: Nurr1, N2/B27 medium: 50:50 mixture of DMEM/F12 medium (addition of N2 supplement) and Neurobasal medium (addition of B27 supplement). Data was derived from one experiment. Error bars represent mean ± SEM.

B

![Graph showing % TH+ cells / DAPI](image)

Figure 28: The combination of SAM and 4x-SpyTag does not reach the levels of Ascl1 activation observed for SAM and VPR

RT-qPCR analysis of Ascl1 mRNA levels 48h after transfection. Using a dCas9 version with four SpyTag repeats in combination with SAM did not induce Ascl1 expression at levels observed for the combination of SAM and VPR together. Abbreviations: gRNAs mA1, mA2: gRNAs targeting the murine Ascl1 promoter, SAM: MS2-P65-HSF1 fusion protein, VP160: ten repeats of Herpes simplex virus protein VP16, VPR: VP64-P65-RTA fusion protein. Data derived from one experiment, error bars represent mean ± SEM.
Figure 29: VP64-dCas9-VP64 fails to induce Ascl1 expression in Neuro 2a cells when used alone with gRNAs mA1 and mA2
RT-qPCR analysis of Ascl1 mRNA levels 48h after transfection of Neuro 2a cells. Using VP64-dCas9-VP64 in combination with gRNAs mA1 and mA2 did not induce Ascl1 at detectable levels. By combining VP64-dCas9-VP64 with SAM, Ascl1 expression was induced although not reaching the levels of SAM and dCas9-VP64. Abbreviations: gRNAs mA1, mA2: gRNAs targeting the murine Ascl1 promoter, SAM: MS2-P65-HSF1 fusion protein, VP64: four repeats of Herpes simplex virus protein VP16, VPR: VP64-P65-RTA fusion protein. Data was derived from three independent experiments, error bars represent mean ± SEM, Kruskal-Wallis test, Dunn’s multiple comparison test, *P < 0.05.

Figure 30: VP64-dCas9-VP64 is not sufficient to induce direct conversion of astrocytes to neurons
Quantification of reprogrammed cells 16 days after lipofection. In each condition dsRed was co-transfected allowing identification of cells which had received the DNA mix. All dsRed+ cells were checked for neuronal morphology and MAP2 expression and counted for the analysis. Due to different background levels of neurons in control wells, the percentage of dsRed+ neurons in control wells was subtracted from all wells of an individual experiment. The percentage of neurons obtained by VP64-dCas9-VP64 was not significantly different to dCas9-VP64 or the control. Abbreviations: gRNAs mA1, mA2: gRNAs targeting the murine Ascl1 promoter, VP64: four repeats of Herpes simplex virus protein VP16. Data derived from three independent experiments, error bars represent mean ± SEM, Kruskal-Wallis test, Dunn’s multiple comparison test, ns: not significant, *P < 0.05.
### 10.2 Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-OHDA</td>
<td>6-hydroxy dopamine</td>
</tr>
<tr>
<td>A</td>
<td>Ascl1 expressed from single vector</td>
</tr>
<tr>
<td>A+L+N</td>
<td>Ascl1, Lmx1a and Nurr1 expressed from single vectors</td>
</tr>
<tr>
<td>A1 – A8</td>
<td>gRNAs 1 – 29 targeting murine Ascl1 promoter</td>
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<tr>
<td>ALN</td>
<td>Ascl1, Lmx1a, Nurr1 expressed from tri-cistronic vector</td>
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<tr>
<td>Ascl1</td>
<td>Achaete-scute family bHLH transcription factor 1</td>
</tr>
<tr>
<td>Bdnf</td>
<td>Brain derived neurotrophic factor</td>
</tr>
<tr>
<td>Brn2</td>
<td>POU domain, class 3, transcription factor 2</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cMyc</td>
<td>Myelocytomatosis oncogene</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>CRISPR</td>
<td>Clustered regularly interspaced short palindromic repeats</td>
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<tr>
<td>crRNA</td>
<td>CRISPR-RNA</td>
</tr>
<tr>
<td>DA</td>
<td>Dopaminergic</td>
</tr>
<tr>
<td>DAT</td>
<td>Dopamine transporter</td>
</tr>
<tr>
<td>dCas9</td>
<td>Nuclease deficient CRISPR associated protein</td>
</tr>
<tr>
<td>Ef1a</td>
<td>Eukaryotic translation initiation factor 1A promoter</td>
</tr>
<tr>
<td>En1</td>
<td>Engrailed 1</td>
</tr>
<tr>
<td>FbaB</td>
<td>Fibronectin-binding protein</td>
</tr>
<tr>
<td>Foxa2</td>
<td>Forkhead box A2</td>
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<tr>
<td>GCN4</td>
<td>General control protein 4</td>
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<tr>
<td>Gdnf</td>
<td>Glial cell-line derived neurotrophic factor</td>
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<td>GFAP</td>
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<td>Gli2</td>
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<td>guide RNA</td>
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<td>hUBC</td>
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<td>IMR90</td>
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<tr>
<td>iPSC</td>
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<tr>
<td>IRES</td>
<td>Internal ribosomal entry site</td>
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<td>Klf4</td>
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<td>L</td>
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<tr>
<td>L-DOPA</td>
<td>L-Dihydroxyphenylalanine</td>
</tr>
<tr>
<td>Lmx1a</td>
<td>LIM homeobox transcription factor 1 alpha</td>
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Lmx1b  LIM homeobox transcription factor 1 beta
mA1 - mA5  gRNAs A – E targeting murine Ascl1 promoter
mdDA  Meso-diencephalic dopaminergic
MEF  Mouse embryonic fibroblast
MOI  Multiplicity of infection
MS2  MS2 coat protein
Msx1  Msh homeobox 1
Myt1I  Myelin transcription factor 1-like
N  Nurr1 expressed from single vector
N1 - N8  gRNAs 1 - 38 targeting murine Nurr1 promoter
Ngn2  Neurogenin 2
NHEJ  Non-homologous end joining
NPC  Neuronal precursor cells
NSC  Neuronal stem cell
nt  Nucleotide
Nurr1  Nuclear receptor subfamily 4, group A, member 2 (Nr4a2)
Oct4  POU domain, class 5, transcription factor 1 (Pou5f1)
Otx2  Orthodenticle homeobox 2
P2A  2A peptide of Porcine teschovirus-1
P65  P65 subunit of human NF-κB (residues 287-546)
PAM  Protospacer adjacent motifs
PD  Parkinson's disease
Pitx3  Paired-like homeodomain transcription factor 3
PM  Pluripotency mediated
RTA  Regulator of transcription activation (residues 416 – 605)
SAM  Synergistic activation mediator, consists of VP64-P65-HSF1
SNc  Substantia nigra pars compacta
Sox2  Sex determining region Y-box 2
Sso7d  S. solfataricus DNA binding protein 7d
SWI/SNF  Switching defective/ Sucrose non-fermenting
T2A  2A peptide of Thosea asigna virus
TALEN  Transcription activator-effector nuclease
TF  Transcription factor
TFIIB  General transcription factor IIB
TH  Tyrosine hydroxylase
TNAP  TRAFs and NIK-associated protein
tracrRNA  Transactivating CRISPR RNA
<table>
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<th>Description</th>
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<tr>
<td>VP16, -64, -160</td>
<td>Herpes simplex virus protein 16 (residues 437-448) repeats</td>
</tr>
<tr>
<td>VPR</td>
<td>Fusion protein of VP64-P65-RTA</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
</tbody>
</table>
10.3 Danksagung

Meinem Doktorvater Herrn Prof. Dr. Wolfgang Wurst bin ich sehr dankbar dafür, dass ich meine Doktorarbeit am Institut für Entwicklungsgenetik habe anfertigen dürfen. Insbesondere möchte ich mich für die regelmäßigen und offenen Diskussionen zu meinem Projekt und die hervorragende wissenschaftliche und persönliche Unterstützung bedanken. Ebenso gilt mein Dank meinen Betreuern Frau Prof. Dr. Nilima Prakash, Herrn Dr. Oskar Ortiz und Herrn Dr. Florian Giesert für die vielfältige Unterstützung und die zahlreichen Ratschläge. Ich möchte auch den Mitgliedern meines Thesis Komitees Frau Prof. Dr. Magdalena Götz und Frau Dr. Stefanie Hauck für ihre Unterstützung danken, die mich in vielerlei Hinsicht vorangebracht hat. Im Weiteren bin ich den Mitgliedern meines Prüfungskomitees Frau Prof. Angelika Schnieke, Ph.D. und Frau Prof. Dr. Aphrodit Kapurniotu dankbar für ihre Unterstützung bei meiner Verteidigung.

Besonderer Dank gilt auch meinem Kollegen Jeffery Truong für die Entwicklung und Bereitstellung der SpyTag und Sso7d Cas9 Varianten und für die zahlreichen konstruktiven Diskussionen. Zusätzlich danke ich meinen Kollegen Dr. Florian Meier, Dr. Sebastian Götz, Dr. Marina Theodorou, Dr. Jingzhong Zhang und Jessica Schwab für die wissenschaftliche und persönliche Unterstützung. Ich danke Susanne Badeke und Davina Fischer für die Hilfe im Labor und Anja Folchert für ihren Einsatz bei den Mäusen. Danke auch an Dietrich Trümbach für die Promoteranalysen und an Christoph Bach für die vergleichenden Experimente an humanen und murinen Promotoren. Zusätzlich gilt Annerose Kurz-Drexler ein besonderer Dank für ihre moralische Unterstützung, ihr stets offenes Ohr und das außergewöhnliches Fachwissen in sämtlichen Laborfragen. Außerdem danke ich meinen Kollegen Constantin, Artem, Petra, Clara, Anke, Michi und Luise für die gute Zeit im Labor und außerhalb der Arbeit. Vielen Dank auch an alle anderen Mitglieder des IDG für die gute Zusammenarbeit.


Vielen Dank!
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Alternativer Zivildienst im Ausland
10.5 Eidesstattliche Erklärung

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der TUM zur Promotionsprüfung vorgelegte Arbeit mit dem Titel:
Direct conversion of somatic cells utilizing CRISPR/Cas9

institut für Entwicklungsgenetik, HelmholtzZentrum München
(Fakultät, Institut, Lehrstuhl, Klinik, Krankenhaus, Abteilung)

unter der Anleitung und Betreuung durch Prof. Dr. Wolfgang Wurst

ohne sonstige Hilfe erstellt und bei der Abfassung nur die gemäß § 8 Abs. 6 und 7 Satz 2
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bei der Fakultät für ____________________________
der Hochschule ____________________________
unter Vorlage einer Dissertation mit dem Thema ____________________________

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