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# The role of the Hippo effector YAP in Duchenne Muscular Dystrophy

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*Für*

*Max Koski*

*Und*

*Erwin & Gisela Reitmeier*

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For a more concise language, I use active voice in this dissertation according to *Nature*'s writing guidelines.

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*“Wenn du eine weise Antwort verlangst, musst du vernünftig fragen.“*

Johann Wolfgang von Goethe

# Abstract

Duchenne Muscular Dystrophy (DMD) is a severe, genetic muscle disease that affects ~1 in 5000 newborn boys. The loss of the dystrophin protein causes progressive pathological features, including inflammation, fibrosis and muscle tissue degeneration, and leads to an early death due to cardiac or respiratory failure. Several lines of mainly animal research suggest that overactivity of the Hippo pathway member YES-associated protein (YAP) might contribute to the pathophysiology of DMD, but it is unclear whether this is also the case in DMD patients. This assumption is mainly based on the findings that YAP binds to members of the dystrophin glycoprotein complex (DGC) and is constitutively active in the muscles of dystrophic (mdx) mice. Additionally, YAP acts as a transcriptional coactivator and drives the expression of *Connective Tissue Growth Factor* (*CTGF*), which is upregulated in the muscles of DMD patients and whose overexpression alone causes a DMD-like phenotype in the muscles of mice. Several independent groups demonstrated that simvastatin, previously identified as a YAP inhibitor, improves many pathological features in dystrophic mice, including fibrosis, oxidative stress and muscle strength, but none of these studies attributed these improvements to changes in YAP activity or CTGF expression. Therefore, I aimed to answer the following questions in this dissertation:

1. How is YAP regulated in healthy and dystrophic, immortalised human skeletal muscle cells?
2. What are the effects of simvastatin on YAP signalling in healthy and dystrophic, immortalised human skeletal muscle cells?
3. Does YAP bind to DGC members or other myopathy-associated proteins in the muscles of wild-type or mdx mice?

To answer the first two questions, I performed immunofluorescence microscopy, reverse transcriptase quantitative polymerase chain reaction and Western Blot analysis to assess YAP activity *in vitro* using immortalised myoblasts and myotubes from two DMD donors and one healthy donor. While I found no difference in YAP localisation, the abundance of total or phosphorylated YAP (Serine 127), gene expression analysis revealed a higher differentiation-associated increase in *CTGF* mRNA levels in one of the dystrophic cell lines versus controls. Secondly, I provide experimental data that shows that simvastatin (10 µM) treatment over 24 hours decreased YAP signalling, as indicated by cytoplasmic accumulation and reduced mRNA levels of *CTGF*, *ANKRD1* and *CYR61* by ~60-80%. However, simvastatin did not significantly affect the abundance of total or phosphorylated YAP (Serine 127). Lastly, to characterise the YAP interactome in the muscles of wild-type and mdx mice, I immunoprecipitated YAP from gastrocnemius muscles and identified potential binding partners using a mass spectrometry-based proteomics approach. While there was no evidence that

YAP binds to DGC members or other myopathy-associated proteins, I detected RPS14, HMHA1 and PGAM5 exclusively in the precipitates of mdx muscles.

In summary, the findings of this dissertation provide a novel insight into the mechanisms by which simvastatin might alleviate the skeletal muscle pathology in DMD. Since DMD is still considered an untreatable disease, future studies should further investigate the therapeutic potential of simvastatin or other known YAP inhibitors to reduce CTGF. In addition, I detected previously unknown binding partners of YAP in dystrophic muscles, but this finding should be regarded with caution due to technical issues.

# Zusammenfassung

Die Duchenne Muskeldystrophie (DMD) ist eine schwere genetische Muskelerkrankung, die etwa 1 von 5000 neugeborenen Jungen betrifft. Der Verlust des Dystrophin Proteins verursacht progressive pathologischen Merkmalen wie Entzündungen, Fibrose und Degeneration des Muskelgewebes und führt zu einem frühen Tod durch Herz- oder Atemversagen. Mehrere Forschungsarbeiten, hauptsächlich an Versuchstieren, legen nahe, dass Überaktivität eines Effektors des Hippo Signaltransduktionsweg, YES-associated protein (YAP), zur Pathophysiologie von DMD beitragen könnte, aber es ist unklar, ob dies auch bei DMD-Patienten der Fall ist. Diese Annahme basiert hauptsächlich auf den Befunden, dass YAP an Mitglieder des Dystrophin-Glykoprotein-Komplexes (DGC) bindet und in den Muskeln von dystrophen (mdx) Mäusen konstitutiv aktiv ist. Darüber hinaus wirkt YAP als transkriptioneller Koaktivator und stimuliert die Expression des Connective Tissue Growth Factor (CTGF), der in den Muskeln von DMD-Patienten hochreguliert ist und dessen Überexpression allein eine DMD-ähnlichen Phänotypen in den Muskeln von Mäusen verursacht. Mehrere unabhängige Gruppen zeigten, dass Simvastatin, das zuvor als YAP-Inhibitor identifiziert wurde, viele pathologische Merkmale in dystrophen Mäusen verbessert, einschließlich Fibrose, oxidativem Stress und Muskelkraft, aber keine dieser Studien führte diese Verbesserungen auf Veränderungen in der YAP-Aktivität oder CTGF-Expression zurück. Daher zielte ich in dieser Dissertation darauf ab, die folgenden Fragen zu beantworten:

1. Wie wird YAP in gesunden und dystrophen, immortalisierten menschlichen Skelettmuskelzellen reguliert?
2. Welche Auswirkungen hat Simvastatin auf die YAP-Signalaktivität in gesunden und dystrophen, immortalisierten menschlichen Skelettmuskelzellen?
3. Bindet YAP an DGC-Mitglieder oder andere myopathieassoziierte Proteine in den Muskeln von Wildtyp- oder mdx-Mäusen?

Um die ersten beiden Fragen zu beantworten, führte ich Immunfluoreszenzmikroskopie, reverse Transkriptase quantitative Polymerase-Kettenreaktion und Western Blot-Analysen durch, um die YAP-Aktivität *in vitro* unter Verwendung immortalisierter Myoblasten und Myotuben von zwei DMD-Spendern und einem gesunden Spender zu ermitteln. Während ich keinen Unterschied in der YAP-Lokalisierung, der Abundanz an totalem oder phosphoryliertem YAP (Serin 127) fand, zeigte die Genexpressionsanalyse einen höheren differenzierungsassoziierten Anstieg der *CTGF* mRNA-Level in einer der dystrophen Zelllinien im Vergleich zu den Kontrollen. Außerdem präsentiere ich experimentelle Daten, die zeigen, dass die Behandlung mit Simvastatin (10 µM) über 24 Stunden die YAP-Signalaktivität verringerte, was durch die zytoplasmatische Akkumulation und die reduzierten mRNA-Level

von *CTGF*, *ANKRD1* und *CYR61* um etwa 60-80% angezeigt wurde. Simvastatin hatte jedoch keinen Effekt auf die Menge an totalem oder phosphoryliertem YAP (Serin 127). Um das YAP-Interaktom in den Muskeln von Wildtyp- und mdx-Mäusen zu charaktierisieren, immunpräzipitierte ich YAP aus Gastrocnemius-Muskeln und identifizierte potenzielle Bindungspartner mittels eines massenspektrometriebasierten Proteomics-Ansatzes. Während es keine Hinweise darauf gab, dass YAP an DGC-Komponenten oder andere myopathieassoziierte Proteine bindet, detektierte ich RPS14, HMHA1 und PGAM5 ausschließlich in den Präzipitaten von mdx-Muskeln.

Zusammenfassend bieten die Ergebnisse dieser Dissertation neue Einblicke in die Mechanismen, durch die Simvastatin die Skelettmuskelpathologie bei DMD lindern könnte. Da DMD immer noch als unheilbare Krankheit gilt, sollten zukünftige Studien das therapeutische Potenzial von Simvastatin oder anderen bekannten YAP-Inhibitoren zur Reduktion von CTGF weiter untersuchen. Darüber hinaus habe ich bisher unbekannte Bindungspartner von YAP in dystrophen Muskeln detektiert, aber dieses Ergebnis sollte aufgrund technischer Probleme mit Vorsicht betrachtet werden.

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# List of Figures and Tables

# Abbreviations

| | |
|---|---|
| 3-hydroxy-3-methylglutaryl coenzyme A | HMG-CoA |
| 3-MH | 3-Methylhistidine |
| 6MWT | 6-Minute-Walk-Test |
| AAV | Adeno-associated virus |
| Acc. No. | Accession Number |
| AGC | Automatic gain control |
| AMOTL1/2 | Angiomotin Like 1/2 |
| AMPK | AMP-activated protein kinase |
| ANKRD1 | Ankyrin repeat domain 1 |
| aPKC | Atypical protein kinase C |
| ARHGAP45 | Rho GTPase-activating protein 45 |
| BAG3 | Bcl2-associated athanogene 3 |
| BMD | Becker Muscular Dystrophy |
| Bp | Base pairs |
| Cdx2 | Caudal-type homeobox 2 |
| CH1/2 | Calponin homology domains 1 and 2 |
| CK | Creatine kinase |
| CTGF | Connective tissue growth factor |
| CYR61 | Cysteine-rich angiogenic inducer 61 |
| DAG1 | Dystroglycan 1 |
| DAPC | Dystrophin-Associated Protein Complex |
| Dg | Dystroglycan |
| DGC | Dystrophin Glycoprotein Complex |
| DMD | Duchenne Muscular Dystrophy |
| DNA | Deoxyribonucleic acid |
| DMSO | Dimethyl sulfoxide |
| ECM | Extracellular matrix |
| EDTB | Endotubin |
| FDA | Federal Drug Administration |
| FBS | Fetal bovine serum |
| F-actin | Filamentous actin |
| GPCR | G Protein-coupled receptor |

| | |
|---|---|
| HBS | Hepes-buffered saline |
| HCD | Higher energy collision induced dissociation |
| Hpo | Hippo |
| HMHA1 | Minor histocompatibility protein HA-1 |
| hTERT | Human telomerase reverse transcriptase |
| IgG | Immunoglobulin G |
| Kbr | Kibra |
| LATS1/2 | Large tumour suppressor kinase 1/2 |
| LC-MS/MS | Liquid chromatography tandem mass spectrometry |
| LFQ | Label-Free Quantification |
| LPA | Lysophosphatidic acid |
| LDH | Lactate dehydrogenase |
| Mats | Mob as tumour suppressor |
| maxIT | Maximum injection time |
| MLCK | Myosin light chain kinase |
| MS | Mass spectrometry |
| MST1/2 | Mammalian Ste20-like kinase 1/2 |
| mTORC1 | Mammalian target of rapamycin complex 1 |
| NCE | Normalised collision energy |
| NF-κB | Nuclear factor kappa B |
| nNOS | Neuronal nitric oxide synthase |
| NOX2 | NADPH oxidase 2 |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| PDE5 | Phosphodiesterase 5 |
| PGAM5 | Phosphoglycerate mutase family member 5 |
| PSM | Peptide spectrum match |
| PVDF | Polyvinylidene difluoride |
| RhoGAP | Rho GTPase-activating protein |
| ROCK | Rho-associated protein kinase |
| ROS | Reactive oxygen species |
| RPS14 | Ribosomal protein S14 |

| | |
|---|---|
| RT-qPCR | Reverse transcriptase-quantitative polymerase chain reaction |
| SAV1 | Salvador family WW domain-containing protein 1 |
| SCFBeta-TRCP | Skp, Cullin, F-box (SCF) complex containing β transducin repeat containing protein |
| Sd | Scalloped |
| SGCδ | Sarcoglycan δ |
| SMAD | Sma gene mothers against decapentaplegic peptide |
| TAD | Transcriptional activation domain |
| TAZ | WW domain-containing transcription regulator 1 |
| TBST | Tris-buffered saline – Triton X100 |
| TEAD | TEA domain family member |
| TEF | TEA domain family transcription factor |
| TGF-β | Transforming growth factor beta |
| VGLL4 | Vestigial-like family member 4 |
| YAP | YES-associated protein |
| Yki | Yorkie |
| Wts | Warts |
| ZO | Zona occludens |

# 1 Introduction

Skeletal muscle comprises ~31% of women's and ~38% of men's total body mass (Janssen et al., 2000) and plays a central role in whole-body metabolism (Wolfe, 2006). It is composed of the largest mammalian cells of tubular shape, called muscle fibres, which consist to 75-80% of smaller myofibrils. Myofibrils mainly contain the force-generating thick and thin myofilaments, myosin and actin, respectively, and are surrounded by more contractile proteins (e.g. tropomyosin), several cytoskeletal (e.g. tubulin, desmin, actin) and membrane-associated proteins (e.g. dystrophin, spectrin, talin, vinculin, ankyrin) (Henderson et al., 2017). The myofibrils cover ~70-85% of the intracellular area and are enclosed by the sarcolemma, the cell membrane (MacDougall et al., 1982). Each muscle fibre has multiple nuclei and is protected by a thin sheathe of connective tissue, the basal lamina, that is rich in fibronectin, laminin, α-dystroglycan and other proteins and hosts the muscle's stem cells, the satellite cells (Sanes, 2003). This layer contributes to the thick collagenous extracellular matrix (ECM), which maintains a pool of non-muscle cells like immune cells, fibro-adipogenic progenitor cells, fibroblasts, adipocytes, endothelial cells and pericytes (Englund et al., 2021). Muscle fibres are bundled in so-called fascicles, confined by a connective tissue layer termed perimysium. The whole muscle contains hundreds to thousands of fascicles and is encapsulated by another layer of connective tissue, the epimysium (Friederich & Brand, 1990).

Besides its role in locomotion, skeletal muscle is a major contributor to glucose control (Merz & Thurmond, 2020), a determinant of survival in cancer patients (Zhang et al., 2024) and closely associated with all-cause mortality (de Santana et al., 2021; Y. Wang et al., 2023). Therefore, maintaining skeletal muscle mass is critical to health and quality of life. Several diseases indirectly impair the quantity and quality of skeletal muscle, but there is also a broad spectrum of genetic muscle diseases directly causing structural or metabolic alterations. Duchenne Muscular Dystrophy (DMD) is the most common muscular dystrophy caused by mutations in the dystrophin gene (*DMD*) and features a plethora of structural and metabolic alterations progressively leading to loss of mass and function (Duan et al., 2021). This results in the loss of ambulation around the age of 12, the need for assisted ventilation and treatment of cardiac failure. Early diagnosis and implementation of medical care have increased the life expectancy of DMD patients to ~28 years (Weber et al., 2022), but the development of drugs for approval presents a major challenge.

Several studies suggest that aberrant activity of the evolutionary conserved Hippo pathway member YES-associated protein (YAP) might contribute to the pathophysiology of DMD. This includes the findings that YAP interacts with dystrophin-associated proteins (Morikawa et al., 2017) and is constitutively active in the muscles of dystrophic mice (Iyer et al., 2019). In this dissertation, I provide experimental evidence that partially contradicts the hypothesis that YAP is dysregulated in the muscles of DMD patients and that YAP binds to dystrophin-associated

proteins in the muscles of dystrophic mice. Moreover, I show for the first time that simvastatin reduces YAP signalling in skeletal muscle cells of DMD patients. This translates to a marked reduction in the expression of a therapeutic target, Connective Tissue Growth Factor (CTGF), which further supports simvastatin as a considerable choice for treating DMD.

## 1.1 Duchenne Muscular Dystrophy

### 1.1.1 Duchenne Muscular Dystrophy is a severe, progressive muscle disease

DMD is a severe, progressive muscle disease caused by mutations in the dystrophin gene (*DMD*) (Duan et al., 2021; Hoffman, Brown, et al., 1987; Hoffman, Knudson, et al., 1987). This disease affects 1 in ~5000 newborn boys (Mah et al., 2014) who would appear healthy when held, but the first signs of weakness show when they try to stand on their legs. These signs manifest in a delayed ability to stand and limited walking skills at 2-3 years of age, coinciding with progressive swelling of skeletal muscles (**Figure 1a**) (Emery, 1965). The patients might maintain this status throughout early childhood, but progressive weakening and atrophy cause wheelchair dependency around 10-12 years of age (Duchenne, 1867; Leon et al., 2023). Severe fibrosis and progressive degeneration of muscle fibres contribute to kyphoscoliosis (**Figure 1b**), resulting in the need for assisted ventilation, but also to inevitable cardiac failure and death at 20-40 years of age with improved care (Duan et al., 2021).

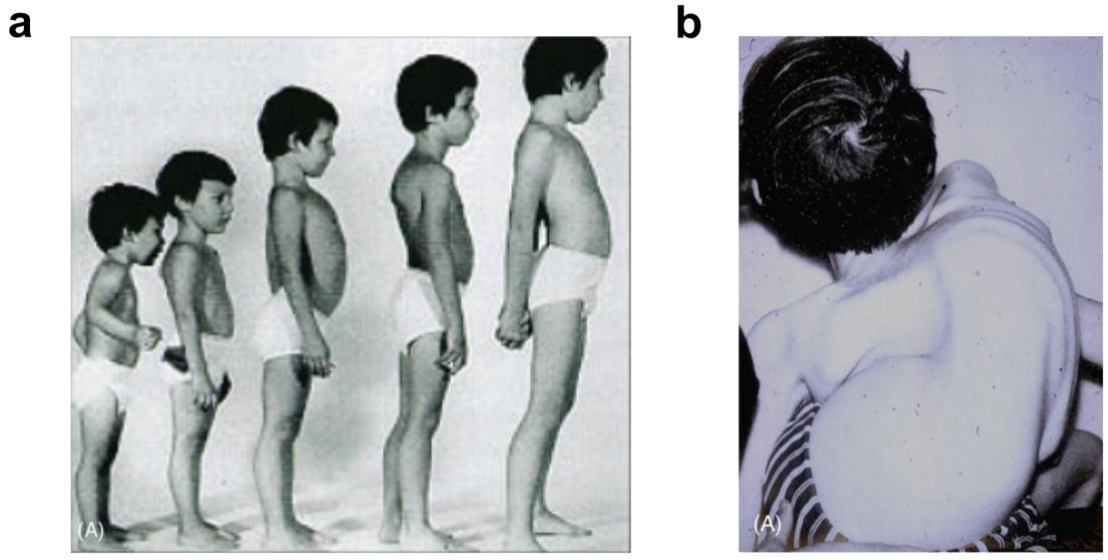


**Figure 1** Representative images of characteristic posture in DMD patients. **a** Boys of different ages showing progressive curvature of the spine and compensatory posture for weakened paraspinal muscles. **b** Severe kyphoscoliosis probably attributable to fibrosis in paraspinal muscles in a DMD patient of unknown age. Adapted from Tidball et al. (Tidball et al., 2018)

There was no consensus on what DMD means before pinning down a common region on the X chromosome as a putative cause for the disease, given the variety of muscular dystrophies of unknown cause (Dubowitz, 1960). Clinicians argued that DMD is inherited by a sex-linked recessive gene (Walton, 1956) and that female carriers are primarily asymptomatic but can be identified by increased serum creatine kinase (CK) levels (Pearce et al., 1964; Richterich et al., 1963). Over 100 years after Duchenne's early observations were published (Duchenne,

1867), Hoffman et al. identified the protein in 1987 as the product of the gene whose mutation they reported earlier and named it dystrophin (Hoffman, Brown, et al., 1987; Hoffman, Knudson, et al., 1987). Only a few years after the breakthrough discovery that dystrophin is absent in DMD, the Polymerase Chain Reaction (PCR) technique superseded the muscle biopsy to confirm the diagnosis by detecting common deletions in the *DMD* gene (Mah et al., 2014).

Despite technological advances in genetic treatments, pharmaceutical interventions are mostly limited to managing secondary pathologies (Verhaart & Aartsma-Rus, 2019). Several compounds in development were discontinued due to lack of efficacy, leaving glucocorticoids as the primary but unfavourable choice (Weber et al., 2022). Therefore, identifying targets and developing novel therapeutic approaches is as important as ever.

### 1.1.2 Duchenne Muscular Dystrophy is the most common muscular dystrophy

About 5 in 100.000 adult males worldwide are registered DMD patients, which is considerably less than the ~12.5 in 100.000 boys affected. Considering the even higher incidence of ~11 to 28 in 100.000 births, one can already estimate the effect that DMD has on the life expectancy of these individuals. In contrast, less than 1 in 1.000.000 females, mostly in combination with Turner syndrome, are reported to be notably affected. This is because the *DMD* gene is located on the X chromosome, of which males only have one, i.e. they have only one copy of the gene, while females have two where the intact allele can substitute for the mutated allele (Aartsma-Rus et al., 2006; Dubowitz, 1960; Walton, 1956). Besides these rare cases, carriers are usually asymptomatic but might show moderately elevated serum CK. This was established in an attempt to identify carriers and inform them of the risk of bearing an affected child (Emery et al., 1972; Richterich et al., 1963).

But even if this strategy were successful, it would not be able to eradicate the disease. About a third of all cases are caused by *de novo* germline mutations. Although this number seems high, it is less surprising considering the size of the gene of interest. The *DMD* gene is the largest human gene at 2.3 Mb of genomic DNA, of which only 11 kb cover the 79 exons making up the coding sequence (**Figure 2**). Over 4700 mutations are registered in the Leiden DMD mutation database (**Figure 2**). About 72% of these are intragenic deletions, mainly affecting exon 45 or exons 45-47 but can extend to exons 45-53. Single or multiexon duplications are less common (~7%) and primarily affect exon 2 but can extend to exon 2-20. The remaining ~20% are small deletions and insertions or point mutations resulting in frameshifts or nonsense codons (Mah et al., 2014). This heterogeneity increases the complexity of developing therapies to repair the primary defect of DMD.

Not all of these mutations result in total loss of the dystrophin protein due to premature truncation. DMD's closest relative, Becker muscular dystrophy (BMD), is also caused by mutations in the *DMD* gene. In contrast to DMD, BMD patients have low levels of full-length dystrophin due to large in-frame deletions affecting up to 35 exons in the central rod domain or exons 2-9 (**Figure 2**) (Aartsma-Rus et al., 2006). The prevalence is notably lower, with ~1.5 in 100.000 males and ~1.4 in 100.000 boys (Mah et al., 2014). Compared to DMD, the onset of the disease occurs later in life, and the symptoms are generally less severe despite the loss of ambulation at a later stage. This results in a near-normal life expectancy with optimised care and early treatment. Lastly, mutations in the *DMD* gene can also cause x-linked dilated cardiomyopathy without the involvement of skeletal muscles. The onset can be delayed until the 20s, but the disease is most often lethal within a few years after the first symptoms (Aartsma-Rus et al., 2006). Apart from dystrinopathies, there are over 40 other genes whose gain or loss of function is linked to a great variety of muscular dystrophies (Mercuri et al., 2019).

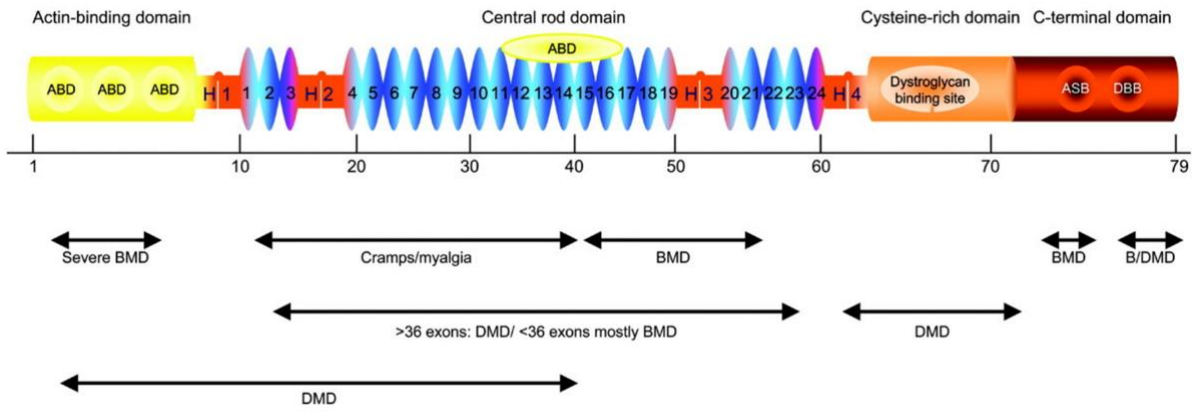


**Figure 2** Schematic showing the domains of the dystrophin protein and the regional mutations with the respective disease association. Adapted from Aartsma-Rus et al. (2006)

### 1.1.3 Pathophysiology of Duchenne Muscular Dystrophy

Dystrophin is a 427 kDa cytoskeletal protein located on the inner surface of the muscle cell membrane (**Figure 3**). It acts as a mechanical link between the actin cytoskeleton within the cell and the ECM through a transmembrane complex called the dystrophin glycoprotein complex (DGC; **Figure 3b**). The DGC comprises an α-dystroglycan at the outside of the membrane, a group of transmembrane sarcoglycans, a β-dystroglycan, sarcospan, and on the cytoplasmic side dystrophin, syntrophin, neuronal nitric oxide synthase (nNOS) and dystrobrevin. Dystrophin binds to members of the DGC but also to proteins initially not attributed to DGC, which is why the literature often refers to the dystrophin-associated protein complex (DAPC). The N-terminal domain of dystrophin contains two calponin homology domains (CH1/2) facilitating the direct interaction with F-actin. On the other end, the cysteine-rich domain contains a WW domain (Sudol et al., 1995) that binds to a proline-rich motif (PPxY motif) in the cytoplasmic tail of β-dystroglycan (Huang et al., 2000) and the C-terminal domain associates with dystrobrevin and syntrophins. Dystrophin gets its mechanical properties from the spectrin-like repeats in the central rod domain. These probably give dystrophin the ability to act as a shock absorber, protecting the cell from contraction-induced damage (Petrof et al., 1993).

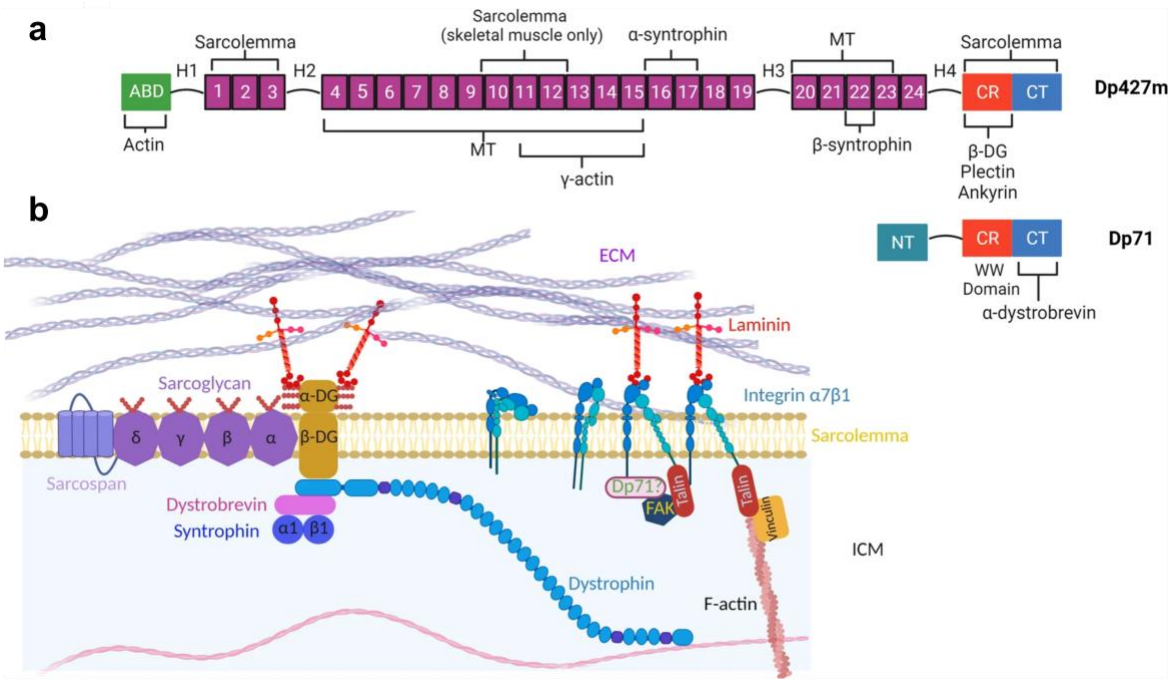


**Figure 3** The dystrophin glycoprotein complex. **a** Schematic depicting the full-length and truncated dystrophin isoforms with their key binding partners. **b** The dystrophin glycoprotein complex as a physical link between the ECM and actin cytoskeleton (F-actin). Adapted from Wilson et al. (2022)

In DMD, dystrophin is truncated, losing the cysteine-rich and C-terminal domains and disrupting sarcolemmal integrity, resulting in total loss of dystrophin (**Figure 4c**, **g**). This leaves the cell susceptible to mechanical stress-induced damage. Tears in the sarcolemma ("delta lesions"), observed by electron microscopy (Mokri & Engel, 1975; Petrof et al., 1993), promote intracellular calcium accumulation and release of cytoplasmic proteins. Calcium accumulation and oxidative stress trigger an inflammatory cascade associated with disease severity. The release of cytoplasmic proteins into circulation sets off an innate immune response characterised by early T-cell and macrophage infiltration in skeletal muscle tissue (**Figure 4a**, **e**) (Tidball et al., 2018). Together, this results in a mismatch of pro- and anti-inflammatory pathways, driving the degeneration of muscle fibres, excessive fibrosis and accumulation of adipose tissue in skeletal muscles (Bez Batti Angulski et al., 2023). This contributes to regeneration failure, although some studies suggest that this failure is an immediate consequence of DAPC disassembly, as it might affect the commitment of satellite cells (Duan et al., 2021).

These primary defects lead to progressive swelling, known as pseudo-hypertrophy, following the months after the onset of weakness, accompanied by interstitial connective tissue accumulation (Duchenne, 1867) (**Figure 4a-b**, **e**-**f**). Bell and Conen (1967) report larger fibre diameters until 5 years of age, followed by increased fibre size variation, mainly due to abnormally small fibres (**Figure 4d**, **h**). Emery (1965) also observed these variations in size in women identified as carriers, including structural changes and few necrotic fibres, a common feature found in the muscles of DMD patients (Gilbert & Hawk, 1963). This might explain the increased urinary 3-methylhistidine (3-MH) excretion, a catabolism marker (Mussini et al., 1984). Further, impaired autophagy promotes the accumulation of defective organelles and dysfunctional proteins, contributing to the loss of proteostasis and potentially cellular degeneration (Duan et al., 2021).

Fibrosis, as a hallmark of DMD, results from repetitive cycles of injury and regeneration (**Figure 4b**, **f**). It involves TGF-β signalling, usually high in injured or dystrophic muscles and induces the synthesis of ECM proteins like collagen and fibronectin from fibroblasts. On the other hand, TGF-β decreases the breakdown of ECM proteins. Additionally, CTGF is highly abundant in dystrophic muscles (Sun et al., 2008a) and can potentiate the effects of TGF-β by increasing collagen typ1, α5 integrin and fibronectin (Kharraz et al., 2014). Considering that overexpression of CTGF alone induces fibrosis (Morales et al., 2011a), this growth factor presents a clinically relevant target.

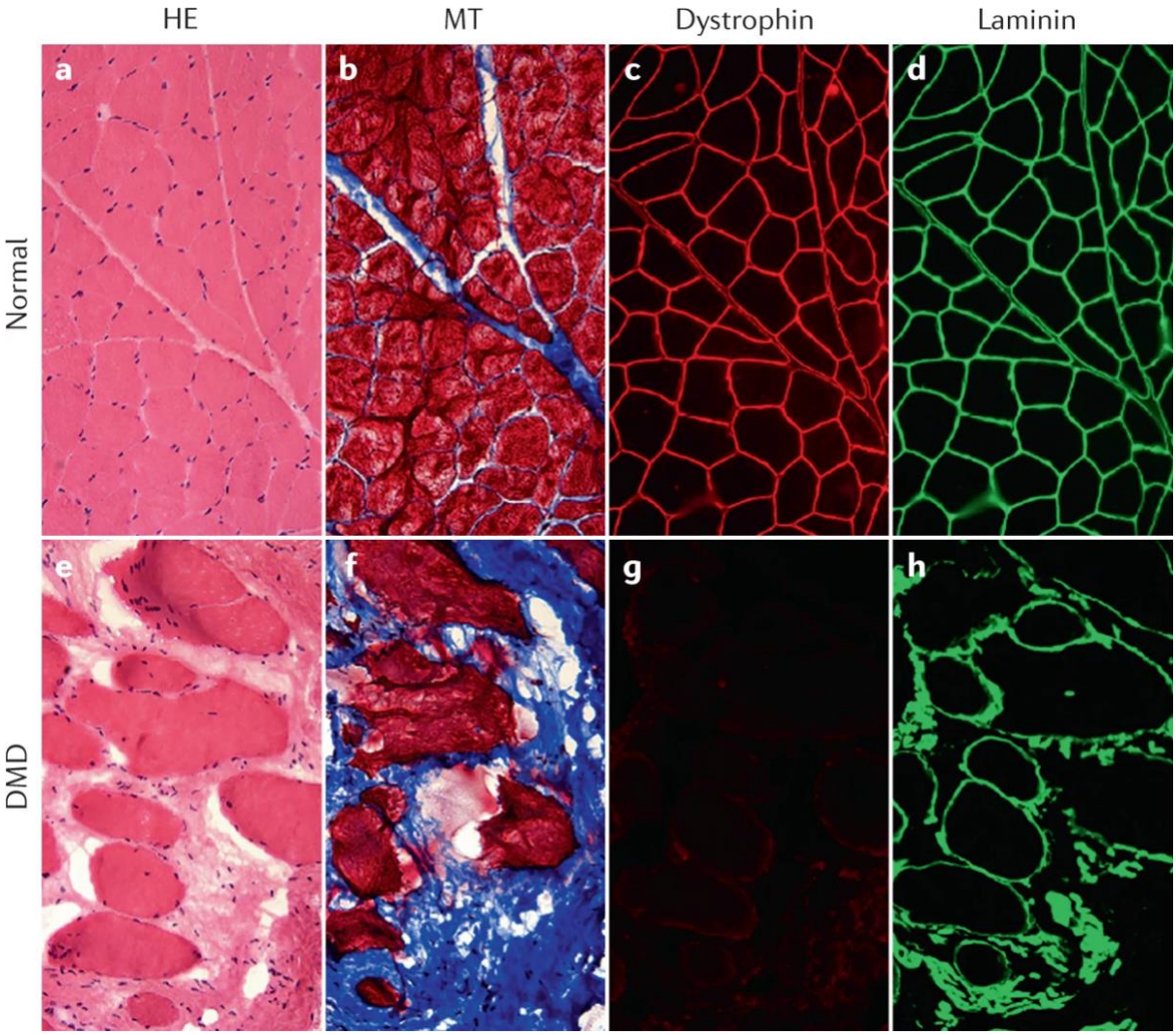


**Figure 4** Histological analyses of cross-sections of muscle biopsies from a healthy subject (**a-d**) and a patient with Duchenne muscular dystrophy (DMD; **e-h**). Haematoxylin and eosin (HE) staining indicates central nucleation, (immune) cell infiltration, variable muscle fibre size and accumulation of non-muscle tissue in the DMD section (**a**, **e**). Masson trichrome (MT) staining suggests severe fibrosis (blue colour = connective tissue) in the DMD section (**b**, **f**). Immunolabelling of dystrophin reveals a lack of dystrophin in the DMD section (**c**, **g**). Immunolabelling of laminin reveals irregular outline and variable muscle fibre size in the DMD section (**d**, **h**). No scale bar available. Adapted from Duan et al. (2021)

DMD is also associated with several metabolic irregularities, such as higher serum aldolase, lactate dehydrogenase (LDH) (Soltan & Blanchaer, 1959) and CK (Pearce et al., 1964; Richterich et al., 1963). This is consistent with reduced intramuscular ATP levels and maximal ATP synthesis (Percival et al., 2013). In addition, oxidative stress is a significant contributor to the pathogenesis of DMD. Whitehead et al. (2010) identified NADPH oxidase 2 (NOX2) as a critical source of reactive oxygen species (ROS) in the muscles of mdx mice. This might be attributed to the loss of dystrophin disorganising the microtubule lattice, which is associated with the NOX2-activating Rac1 (Duan et al., 2021). Similarly, nNOS dissociates from the DAPC and is a source of reactive nitrogen species (Kim et al., 2013). In healthy muscles, nNOS

localises to the sarcolemma, releasing nitric oxide to the peripheral vascular system for vasodilation. Since dystrophin no longer links nNOS to the sarcolemma, malperfusion might cause ischaemic damage to muscle cells (Duan et al., 2021).

These factors contribute to a progressive decline in motor function during childhood. Consistent with a reduced contraction velocity and relaxation time (McComas & Thomas, 1968), this is most notable in the knee and elbow extensors between 4 and 10 years of age. In comparison, the 6-minute walking distance usually declines from 9 years of age, resulting in loss of ambulation 2-3 years later (Leon et al., 2023). With some delay but just as severe, upper body motor function also declines to the point of life-threatening respiratory failure. However, the most frequent cause of death is congestive heart failure (Weber et al., 2022). Electrocardiographic abnormalities appear early in life (Manning & Cropp, 1958; Perloff et al., 1967) and reflect the beginning of progressive dilated cardiomyopathy, resulting in sudden death at ~30 years of age (Weber et al., 2022). Collectively, the loss of dystrophin causes a plethora of abnormalities present in skeletal and cardiac muscle, some of which are an immediate consequence of truncated dystrophin, and some can be ascribed to the progressive degeneration of muscle fibres and fibrosis.

### 1.1.4 Duchenne Muscular Dystrophy is currently an untreatable disease

Despite remarkable efforts to develop therapies, DMD is still an untreatable disease. At best, early diagnosis and improved care standards can slow disease progression. This includes ventilation and cough assistance, nutrition management, and cardiomyopathy treatment.

As outlined previously, the effects of dystrophin loss are wide-ranging, and treatments that do not target the primary defect are limited to ameliorating single aspects at a time. Glucocorticoids, like prednisolone or deflazacort, are immunosuppressants that reduce inflammation (Reitter, 1995). The effects of most glucocorticoids are mediated by transrepression, which means inhibition of the NF-κB pathway in this case, but also transactivation, to which many adverse events such as weight gain, stunted growth and insulin resistance are attributed. The goal is to develop agents that perform transrepression but less transactivation. Vamorolone could be a promising candidate as an alternative to prednisolone, but long-term data is still warranted (Heier et al., 2013).

Oxidative stress presents a seemingly approachable target, given the availability of antioxidants. However, a recent meta-analysis reported no clinical benefits of pentoxifylline, superoxide dismutase or vitamin E treatment. Supportive evidence exists for flavonoids- and omega-3-based compounds to increase strength and for Coenzyme Q10 to improve some clinically relevant markers. Lastly, idebenone is currently the most promising antioxidant in terms of its safety and improvement of pulmonary function, which is a critical outcome in DMD patients (Ren et al., 2022).

Several trials investigated phosphodiesterase 5 (PDE5) inhibitors like sildenafil and tadalafil in improving ischaemia-induced muscle damage due to compromised nitric oxide-cyclic GMP signalling is compromised in DMD. Unfortunately, the positive effects in mdx mice on cardiomyopathy, respiratory function, or muscle damage did not translate to effects in DMD patients. Instead, sildenafil was deemed unsafe after trials in BMD patients and tadalafil, which has a better safety profile, did not significantly improve the 6-Minute Walk Test (6MWT) or other outcomes in a large phase III clinical trial (Nio et al., 2017).

Myostatin signalling is a negative regulator of skeletal muscle mass, as demonstrated by two-fold increases in muscle weight in myostatin knock-out mice (Slack, 1997), potentially revealing a target to counter the loss of muscle tissue in DMD. This is supported by the finding that crossing myostatin-knockout mice with mdx mice increases muscle mass and reduces fibrosis. In contrast to studies in mdx mice, anti-myostatin antibodies had limited success in clinical trials (Muntoni et al., 2024). This is at least partially explained by the notion that myostatin levels are 50 times lower in DMD patients compared to mdx mice, indicating that myostatin signalling plays a different role in regulating skeletal muscle mass in humans than in mice (Verhaart & Aartsma-Rus, 2019).

Unlike the above therapeutic approaches, genetic treatments often face the overwhelming heterogeneity of genetic mutations causing DMD (Aartsma-Rus et al., 2006). To overcome this challenge, viral vectors can deliver a transgene encoding for a protein of interest instead of correcting the mutation. This is limited to adeno-associated viruses (AAV) for skeletal muscle tissue, for which the sequence for full-length dystrophin would be too long. The pathophysiology of BMD patients suggests that smaller dystrophins, termed microdystrophins, containing the actin-binding and dystroglycan domains but lacking most of the central rod domain, are sufficient for a milder progression than in DMD. Microdystrophin therapy has shown promising results in skeletal muscle but poses a risk of accelerating cardiac disease progression (Hart et al., 2024). In addition, the turnover of muscle tissue requires re-treatment with AAV, but since the patients develop antibodies against the previously used AAV, other AAV serotypes would have to be used. It is unclear if microdystrophin therapy would alter long-term disease progression because BMD is also progressive, yet patients have a near-normal life expectancy (Duan, 2018).

Microdystrophins are also the result of exon skipping therapy, which relies on the synthesis of an antisense oligonucleotide correcting the reading frame by skipping the exon in the dystrophin pre-mRNA transcript. Given that there are hotspots of mutations, this treatment could be developed for subgroups of patients instead of tailoring the antisense strand specifically to each patient. Exon skipping had limited success in clinical trials, but eteplirsen treatment is associated with prolonged survival of DMD patients (Iff et al., 2024). Casimersen has already been approved by the Federal Drug Administration (FDA), but a phase III clinical trial is ongoing (Assefa et al., 2024).

The development of the CRISPR-Cas9 gene editing technology opened up the possibility of correcting mutations on a DNA level, correcting the reading frame for every RNA transcript. Skeletal muscle fibres are post-mitotic, i.e., terminally differentiated, meaning CRISPR-Cas9 can only be used for non-homologous end joining repair. Technically, CRISPR-Cas9 can target multiple exons, but this is less efficient. *In vitro* and animal studies suggest that this technology effectively restores dystrophin levels. The disadvantage is that the AAV carrying the transgenes cannot target satellite cells. This means that when muscle damage occurs, and satellite cells donate new myonuclei to regenerate the muscle fibre, these new myonuclei still carry the defect. Additionally, genome editing techniques always inherit the risk of producing off-target effects.

Lastly, ~13% of DMD patients carry nonsense mutations and could benefit from stop codon readthrough therapy with compounds like ataluren. These compounds bind to premature stop codons and induce the incorporation of an amino acid instead of terminating translation.

Specifically, ataluren decelerated the decline in 6MWT but is expensive (McDonald et al., 2022). In summary, there are many approaches targeting primary and secondary pathology with meagre success rates. Still, the most convincing data exists for glucocorticoids delaying the loss of ambulation, decline in pulmonary function, heart failure, and overall reduced mortality (Weber et al., 2022). Despite few effective treatments being available, the improvements in overall care have increased the life expectancy of DMD patients over time. While DMD patients born before 1970 had a life expectancy of ~18 years, it increased to ~24 years for patients born between 1970 and 1990. According to more recent estimates, patients born after 1990 have a life expectancy of ~28 years (**Figure 5**) (Broomfield et al., 2021).

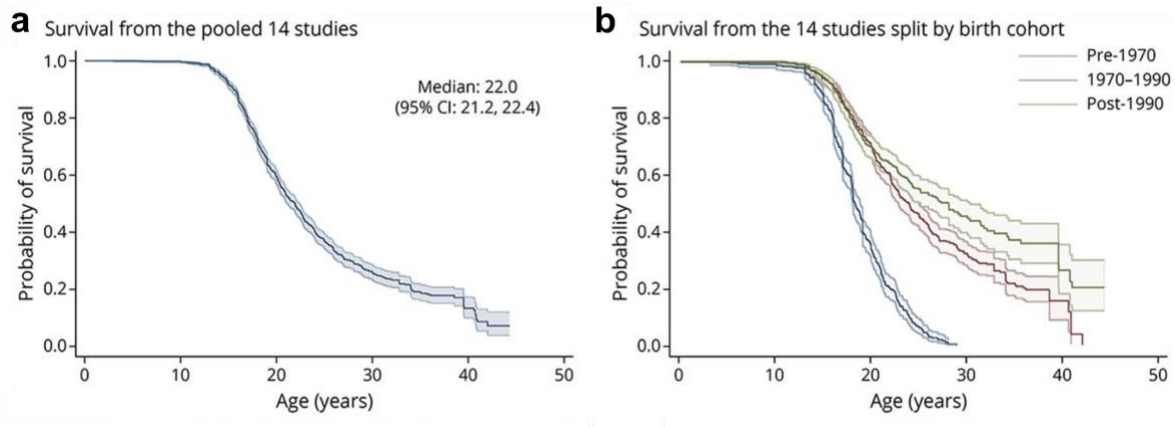


**Figure 5** Kaplan-Meier estimates of survival, pooled from 14 studies (**a**) and split by birth cohort (**b**). Adapted from Broomfield et al. (2021)

## 1.2 The Hippo Signalling Pathway

### 1.2.1 The Hippo pathway is an evolutionary conserved signalling network

The Hippo pathway is an evolutionarily conserved signalling network involved in controlling growth, proliferation and cell fate in response to various intra- and extracellular cues (Phillips et al., 2024). The first core components were identified when independent groups performed genetic mosaic screens in *Drosophila melanogaster* and found remarkably similar overgrowth phenotypes caused by mutations in *salvador* (*sav*), *warts* (*Wts*) and *hippo* (*hpo*). The conceptualisation of a network emerged when co-immunoprecipitation experiments confirmed that Sav and Wts interact and that Hpo phosphorylates Sav (Gokhale & Pfleger, 2019). Shortly after, *Mob as tumour suppressor* (*Mats*) was added to this list, but the discovery of Yorkie (Yki) as the downstream transcriptional effector presented a major step forward. Consequently, the upstream regulators Merlin, Expanded, and Fat were identified. The missing piece that allowed Yorkie to act as a transcriptional co-activator was a DNA-binding partner, which turned out to be the TEAD/TEF family homolog scalloped (Sd).

In mammalian cells, the homologues of the core components are the upstream kinases Ste20-like kinases 1 and 2 (MST1/2) for Hpo, their binding partner Salvador family WW-domain-containing protein 1 (SAV1) for Sav, the central kinases large tumour suppressor kinases 1 and 2 (LATS1/2, Wts) of the NDR family, their scaffold protein MOB kinase activator 1A and 1B (MOB1A/B), the downstream targets Yes-associated protein 1 (YAP1 or YAP, Yki) and its paralogue WW-domain-containing transcription regulator 1 (TAZ), and their transcription factors TEAD1-4 (Sd). When the Hippo pathway is active (**Figure 6a**), MST1/2 and Sav1 heterodimerise so that MST1/2 can phosphorylate LATS1/2 and MOB1. Activated LATS1/2, phosphorylate YAP/TAZ on serine residues 127 and 89, respectively. Serine phosphorylation at these sites creates binding sites for 14-3-3 proteins and YAP/TAZ, thereby promoting cytoplasmic retention and sequestration of YAP/TAZ. YAP/TAZ can also be subject to proteasomal degradation when casein kinase 1 phosphorylates them at serine residues 381 and 311, respectively. This creates a phosphodegron motif recognised by the $SCF^{Beta\text{-}TRCP}$ E3 ligase, leading to ubiquitination and, subsequently, proteasomal degradation. In the absence of nuclear YAP/TAZ, TEAD binds to the Vg domain-containing protein VGLL4 and exerts active repression of YAP/TAZ transcriptional targets. When the Hippo pathway is inactive (**Figure 6b**), YAP/TAZ are dephosphorylated and enter the nucleus, where the YAP/TAZ-TEAD1-4 heterodimer binds primarily to distal enhancers but also proximal promoters. This leads to active transcription of genes in a tissue- or cell-specific manner, including upstream regulators of YAP/TAZ to ensure tissue homeostasis via a negative feedback mechanism. YAP/TAZ also bind to other transcription factors, such as sma gene mothers against decapentaplegic peptide (SMAD) or TBX5.

YAP and TAZ are paralogues that share important features, including the WW domain, TEAD-binding, PDZ-binding, and transcriptional activation (TAD) domain. LATS1/2 phosphorylate both, but YAP has five, and TAZ has four known serine phosphorylation sites. Many studies demonstrate that YAP/TAZ are redundant, but as the amino acid sequence identity of ~41% suggests, they have overlapping and distinctive functions (Callus et al., 2019). These are best described in the context of development and cancer (Reggiani et al., 2021).

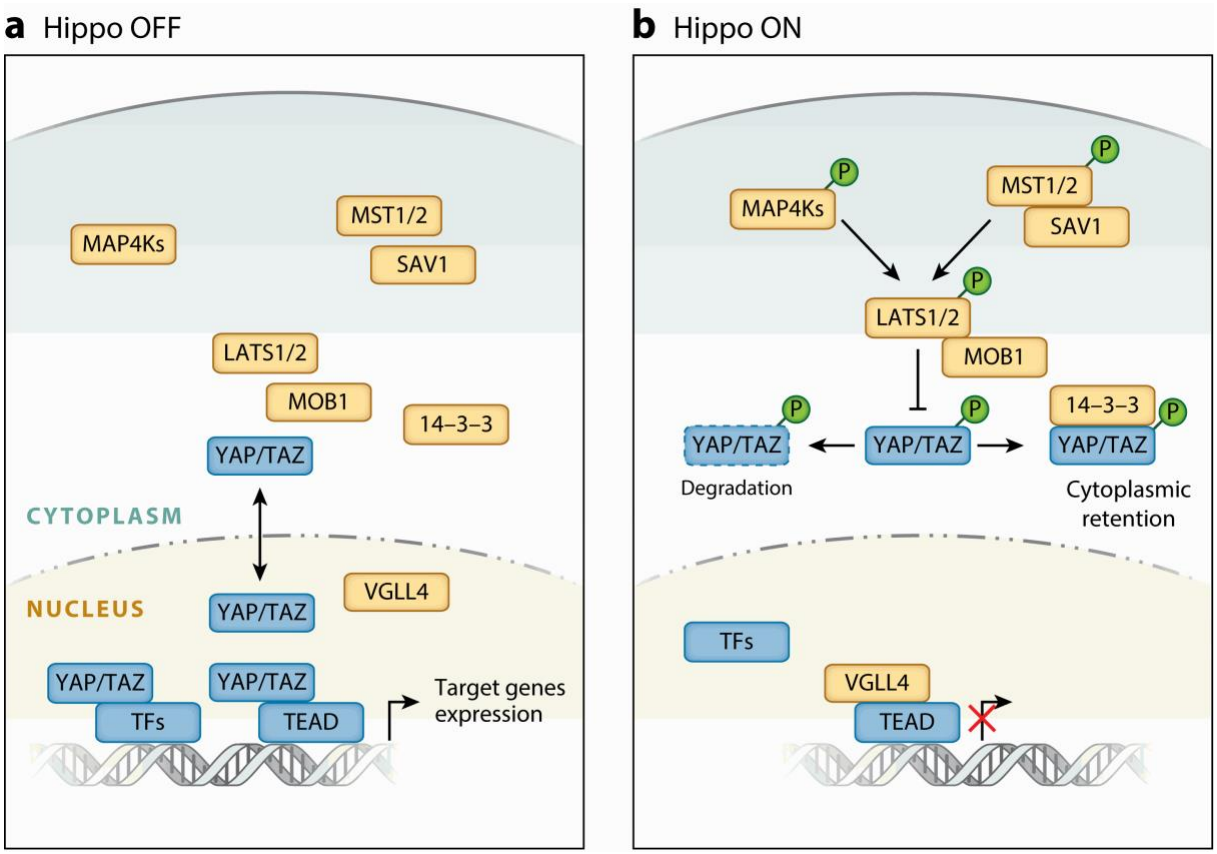


**Fig. 6** Core components of the mammalian Hippo pathway. **a** When the Hippo pathway is inactive, YAP/TAZ are dephosphorylated, accumulate in the nucleus to bind to TEAD and other transcription factors and regulate the expression of cell-specific target genes. **b** When the Hippo pathway is active, LATS-mediated phosphorylation of YAP/TAZ promotes degradation and association with 14-3-3 proteins, leading to cytoplasmic retention. VGLL4 competitively binds to TEAD and actively represses the target gene expression. Adapted from Ma et al. (2019)

Since the discovery of the core components of the Hippo pathway, the regulation of organ size quickly became the most prominent feature owed to the dramatic overgrowth phenotypes of Hippo mutants. The role of YAP/TAZ in growth control has been demonstrated in a series of studies in various tissues overexpressing or knocking down members of the Hippo pathway, resulting in similar over- and undergrowth phenotypes (Watt et al., 2017). As one of the primary examples, liver-specific induction of a constitutively active phospho-mutant YAP (S127A)

increased livers of adult mice ~4-fold compared to controls. This phospho-mutant describes the mutation of the critical serine residue in 127 to an alanine. This results in the failure of LATS1/2 to phosphorylate and inhibit YAP at S127, which is the regulation associated with cytoplasmic accumulation and 14-3-3-mediated sequestration. The overgrown livers returned to those of normal size upon inactivation of the expression of phospho-mutant YAP (Camargo et al., 2007). More recently, Kowalczyk et al. (2022) questioned the central hypotheses predicting that the Hippo pathway components are indispensable for regular tissue growth, that Hippo pathway activity changes in a cell proliferation-dependent manner and that the genetic overgrowth programme of Hippo mutants is similar to that over regular growth. After rigorously testing the validity of this hypothesis, the authors concluded that Hippo signalling is not a master regulator of physiological growth. Further, they discuss that the overgrowth phenotypes caused by Hippo mutations can be explained by ectopic activation of YAP/TAZ or Yki and might require the expression of several target genes unrelated to physiological growth (Kowalczyk et al., 2022). However, as has been extensively reviewed, the Hippo pathway plays a dominant role during the development of many vertebrate and *Drosophila* tissues (Bornhorst & Abdelilah-Seyfried, 2021; Ma et al., 2019; Misra & Irvine, 2018; Phillips et al., 2024; Zhong et al., 2024). As such, the increase in cell number during the development of tissue like lung, brain, epidermis, liver, bone, vasculature, adipose tissue and heart is associated with YAP/TAZ activity (Davis & Tapon, 2019). This is supported by the notion that YAP is high in stem/progenitor compartments of many tissues, and this is associated with metabolic programmes that support proliferation (Meinhold et al., 2024). On a cellular level, Hippo signalling impacts development by affecting cell fate decisions. During embryogenesis in mice, the first significant cell fate decision is the differentiation of blastocysts into the trophectoderm or inner cell mass. These form the outer layer and give rise to the embryo proper, respectively. YAP was found active and nuclear in outer blastocysts and induced the TEAD4-mediated expression of the trophectoderm-specific transcription factor Cdx2. These cells will differentiate into the trophectoderm, while in the inner cells, Hippo signalling is active and inhibits YAP, promoting the differentiation of the inner cell mass (Misra & Irvine, 2018).

In general, active YAP/TAZ induce a genetic programme in stem cells promoting proliferation, resistance to apoptosis and organisation of the ECM and cytoskeleton. More specifically, some evidence supports context-dependent roles of YAP/TAZ in stem cells, e.g. the regenerative response to tissue damage (Zhong et al., 2024). This includes the maintenance of the pool of undifferentiated stem/progenitor cells of the nervous system and intestine in *Drosophila* and the notion that regulation of YAP/TAZ is required for stem cell fate in the skin, nervous system, muscle, liver, lung, teeth, mammary gland and pancreas in mammals (Davis & Tapon, 2019; Misra & Irvine, 2018). While YAP/TAZ can induce self-renewal and differentiation in stem cells during development, they mostly induce self-renewal in stem cells and even de-differentiation in terminally differentiated cells of mature tissues (Driskill & Pan, 2023). These functions might

be relevant for treating various diseases, but it will be challenging to understand the context-specific signals that cue the Hippo pathway to control stemness and differentiation *in vivo* (Cao et al., 2020).

Beyond cell-autonomous regulation of cell fate, a recent review discussed several mechanisms of non-cell-autonomous functions of Hippo signalling. These mechanisms include yet-to-be-fully-elucidated direct cell-cell communication and indirect cell-cell communication. The latter describes the release of soluble factors such as the YAP/TAZ targets cysteine-rich angiogenesis-inducing factor 61 (CYR61) and CTGF. The release of CYR61, for example, has been associated with increased survival of neighbouring cells, induction of pluripotency and inflammation. Additionally, Hippo signalling is implicated in the biogenesis and release of extracellular vesicles, which are small particles that deliver nucleic acids, proteins, metabolites and lipids to other cells and tissues. While it is well-recognised that ECM stiffness regulates Hippo activity, Hippo signalling can also modulate ECM composition and affect surrounding cells. Together, the Hippo pathway covers a wide array of functions in an autonomous and non-autonomous manner (Nita & Moroishi, 2024).

In several recent reviews, additional functions of the Hippo pathway have been described, including regeneration (Nita & Moroishi, 2024; Riley et al., 2022; J. Wang et al., 2023; Zhong et al., 2024), chromatin remodelling (Hillmer & Link, 2019), glucose metabolism (Kashihara & Sadoshima, 2024), vascular homeostasis (Lv & Ai, 2022), bone homeostasis (Li et al., 2024) and immune homeostasis (Tang et al., 2023). Given this broad spectrum of functions, especially the effects on proliferation and cell fate decision, it is no surprise that Hippo signalling has attracted considerable attention in disease research (Fu, Hu, et al., 2022). This has led to a flood of reviews discussing the Hippo pathway's implication in various diseases, which is beyond the scope of this introduction. As one might expect, Hippo signalling has been studied and reviewed exhaustively in relation to cancer (Baroja et al., 2024; Cobbaut et al., 2020; Franklin et al., 2023; Kumar & Hong, 2024; Zhu et al., 2023). Fibrosis is one of the key intersections of cancer and other diseases associated with Hippo signalling (Mia & Singh, 2022). As outlined previously, TGF-β signalling and the YAP/TAZ target CTGF are major drivers of fibrosis. CTGF is produced by fibroblasts, tumour cells, muscle cells and stellate cells and is well-recognised as a prime target in several fibrotic diseases like hepatic, pulmonary, renal and cardiac fibrosis, systemic sclerosis, some types of cancer and DMD. Specifically in muscles, CTGF-mediated fibrosis was shown to be independent of TGF-β signalling, suggesting that other mechanisms might regulate YAP/TAZ activity and CTGF expression (Fu, Peng, et al., 2022). Studies on osteoarthritis, another disease characterised by loss of ECM homeostasis, revealed contrasting results regarding YAP/TAZ signalling. While some evidence shows that YAP is more nuclear in sections of osteoarthritic sections,

consistent with higher CTGF, other studies found that increasing YAP activity improved osteoarthritis in animal and *in vitro* studies (Sun et al., 2023).

Other recently reviewed disease contexts include respiratory diseases (Tang et al., 2022), ischemia-associated central nervous system diseases (Wei et al., 2023), retinal diseases and neurodegenerative diseases (Zhao et al., 2024), mitochondrial metabolism (Biswal et al., 2024), cardiac ageing (Leng et al., 2024), rheumatoid arthritis (T. Wang et al., 2023) and atherosclerosis (Liu et al., 2022). In summary, the Hippo pathway is now a well-established signalling network that affects proliferation, growth, fate decision and regeneration in a cell-, tissue- and context-dependent manner. This raises the question of what signals precisely regulate the Hippo pathway in relation to a specific function.

### 1.2.2 Regulation of the Hippo signalling pathway

A growing number of extra- and intracellular signals regulate the Hippo pathway and its downstream targets YAP/TAZ. The most compelling evidence exists for mechanical cues, cell density and polarity, soluble factors, and stress signals (**Figure 7**) (Ahmad et al., 2022; Dasgupta & McCollum, 2019; Dupont, 2016; Dupont et al., 2011; Fu, Hu, et al., 2022; Rausch & Hansen, 2020; Schroeder & Halder, 2012; Zou et al., 2020). This is unsurprising considering the Hippo pathway's implication in various functions and diseases.

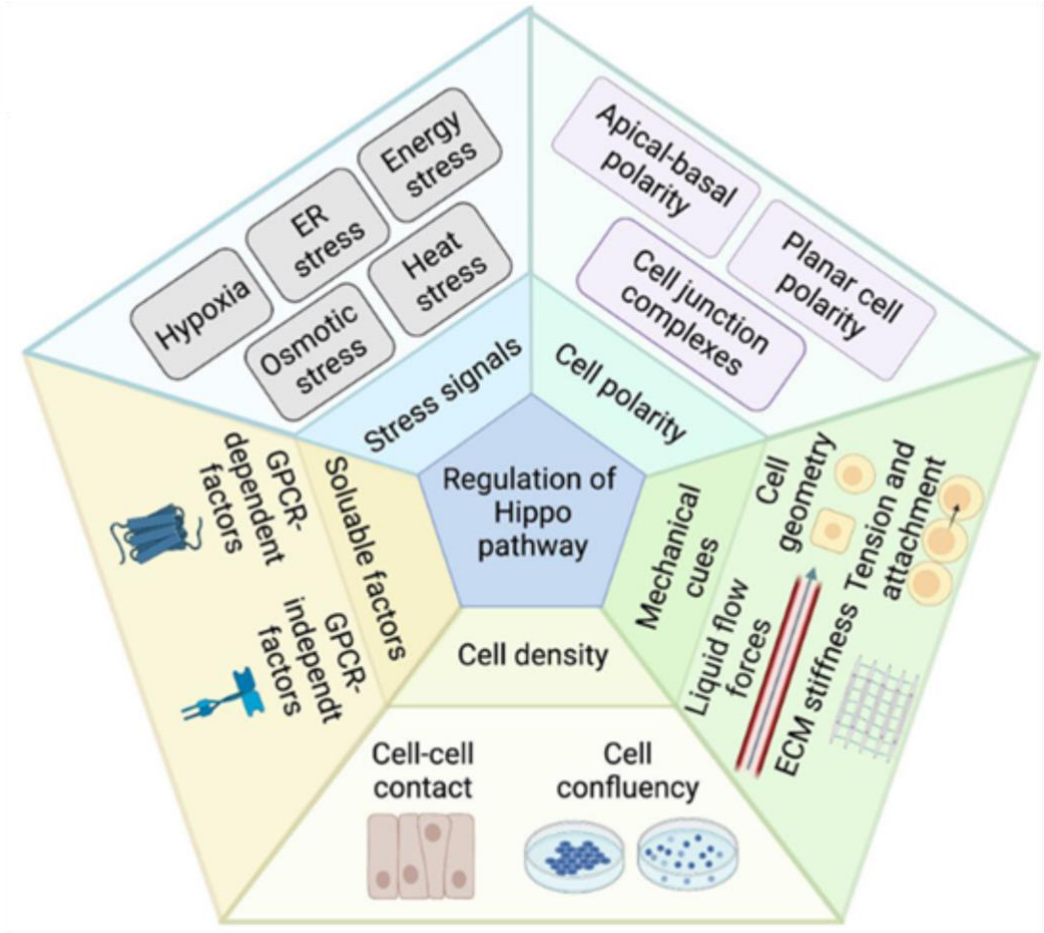


**Figure 7** The five major upstream signals regulating the Hippo pathway are mechanical cues, cell density, soluble factors, stress signals and cell polarity. Adapted from Fu et al. (2022)

Mechanotransduction refers to the cell's ability to convey mechanical signals such as tension, pressure or shear stress into biochemical signals. These external forces can depend on the stiffness of the ECM. The stiffness of the ECM is affected by the ECM composition, the concentration of certain macromolecules and posttranslational modifications. A cell's ability to sense its mechanical environment, i.e. the ECM stiffness, is based on its property to generate

internal forces through the polymerisation of contractile F-actin, myosins and the upstream Rho-associated protein kinase (ROCK) and myosin light chain kinase (MLCK). Integrins are focal adhesion proteins required for mechanosensing and form a reciprocal feed-forward loop with F-actin bundles: the establishment of focal points promotes the growth of actin bundles, which in turn drives the stabilisation and maturation of focal adhesions (Dupont, 2016). As reviewed by Dupont (2016), F-actin contractility controls YAP/TAZ activity in mammalian cell culture and Drosophila and requires Rho activity. As such, growing cells on stiff ECM substratum induces the spreading of cells associated with nuclear localisation of YAP/TAZ, whereas soft ECM substrates inhibit YAP/TAZ. Similar results are reported when cells grow on large or small surface areas, indicating that cell geometry affects YAP/TAZ activity (Dupont, 2016). Consistent with these results, Pereira et al. (2020) cultured C2C12 murine muscle progenitor cells, called myoblasts, onto rectangular and square fibronectin micropatterns and observed that the rectangular shape of the cell increased the cytoplasmic to nuclear ratio of YAP/TAZ. In a recent review, Fu et al. (2022) discuss that the ECM stiffness-related control of YAP/TAZ is likely mediated by the Ras-related GTPase RAP2 and Agrin. At lower ECM stiffness, RAP2 is active, leading to activation of LATS1/2 and subsequent inhibition of YAP/TAZ (Fu, Hu, et al., 2022). In contrast, Agrin is an ECM proteoglycan whose expression increases with higher stiffness and promotes YAP activity by decreasing Merlin and LATS1/2 phosphorylation (Chakraborty et al., 2017). In addition to ECM stiffness, liquid flow-induced shear stress can also increase YAP/TAZ target gene expression (Fu, Hu, et al., 2022). The regulation through these mechanisms is implicated in stem cell differentiation, cardiovascular biology, fibrosis and breast cancer. Thus, mechanical signals might be essential in controlling YAP/TAZ's key functions related to health and disease (Cai et al., 2021; Dupont, 2016). However, as Dupont (2019) pointed out, it is often unclear whether a stimulus's mechanical or biochemical nature initiates the cellular response. This is not only true for the varying composition of the ECM but especially for stimuli related to cell-cell contact.

Apart from the ECM, the Hippo pathway is also a crucial response element to other cells in a cell's surroundings. For instance, several studies in various cell types demonstrate that with increasing cell density in monolayer cell culture, phosphorylation of YAP increases (Ma et al., 2019). Increasing cell contact also stimulates cell polarity, which describes the asymmetric spatial distribution of cell contents. Hippo signalling is primarily associated with apicobasal polarity, the division of the plasma membrane into an apical domain and a basal domain by cell junction complexes. The Hippo pathway components MST1/2, CRB3, MER, KIBRA and AMOT localise to the apical membrane to engage with apical polarity components (Schroeder & Halder, 2012).

Particularly in epithelial and endothelial cells, adherens and tight junctions are crucial protein complexes that provide mechanical stability and biochemical exchange between cells. The

adherens junction protein α-Catenin is a member of the E-cadherin-catenin complex and promotes cytoplasmic accumulation, probably by interacting with 14-3-3 proteins. This is supported by several *in vivo* and *in vitro* experiments showing that nuclear YAP drives hyperproliferation after loss of function of a-cat or other components of the E-cadherin-catenin complex (Karaman & Halder, 2018). Other adherens junction proteins activating the Hippo pathway or inhibiting YAP directly include NF2/Merlin, the Pez family phosphatases FERM-domain containing nonreceptor protein tyrosine phosphatase type 14 PTPN14 and Ajuba (Ahmad et al., 2022).

At tight junctions, the Crb complex is indispensable for apical-basal cell polarity in epithelial cells. It is a negative regulator of YAP, as loss of CRB3 is associated with nuclear accumulation of YAP/TAZ. Inactivation of YAP/TAZ is indirectly and directly mediated by the Angiomotin family adaptor proteins (Karaman & Halder, 2018). In confluent cells, the Angiomotin family of proteins (AMOTL, AMOTL1/2) are tight junction and scaffolding proteins for LATS1/2, promoting LATS1/2 phosphorylation by MST1/2, which in turn inactivates YAP/TAZ., AMOT proteins can also directly inhibit YAP/TAZ through WW-PPxY interactions. Additionally, they can recruit the ubiquitin ligase AIP4, targeting YAP for ubiquitination (Karaman & Halder, 2018). In subconfluent cells, Endotubin (EDTB) keeps AMOT from binding YAP by facilitating the sequestration of AMOT on endosomal membranes. In contrast, AMOT in HEK293 cells is nuclear, binds to nuclear YAP and prohibits phosphorylation bei LATS1/2. Hence, Amot proteins exert context-specific, direct and direct control of YAP localisation and activity (Karaman & Halder, 2018). Tight junctions also host the atypical protein kinase C (aPKC) complex and Zonula occludens (ZO, ZO-1/2/3) proteins. The aPKC complex comprises a serine-threonine kinase and the Par-3 and -6 adaptor proteins and acts as an upstream regulator of several Hippo pathway components. In comparison, Zonula occludens proteins bind YAP/TAZ directly via a PDZ domain. This probably inactivates YAP as silencing of ZO-2 induces YAP-mediated renal hypertrophy, but other studies found opposing results (Fu, Hu, et al., 2022; Karaman & Halder, 2018). Lastly, Claudin-2 and -6 are also tight junction proteins that can activate the Hippo pathway or inhibit YAP directly (Ahmad et al., 2022).

Soluble factors are major regulators of many physiological processes, largely through binding to G protein-coupled receptors (GPCRs). GPCRs are the largest group of transmembrane receptors that stimulate a receptor-dependent signalling cascade inside the cell upon binding a soluble factor. Thus, GPCR signalling can activate or inactivate YAP/TAZ, depending on the ligand and the receptor. For example, lysophosphatidic acid (LPA) and sphingosine 1-phosphate, two regular fetal bovine serum components in most cell culture media, bind to G12/13 receptors, inhibit LATS1/2 leading to activation YAP/TAZ. Conversely, hormones like glucagon or epinephrine bind to Gs-coupled receptors and activate LATS1/2, leading to the inhibition of YAP/TAZ (Fu, Hu, et al., 2022; Yu et al., 2012). In addition, glucocorticoids might

indirectly promote YAP activation due to increased fibronectin expression. Several other factors shown to modulate Hippo pathway activity include TGF-b, IL17A, IL-6, insulin or insulin-like growth factors, epidermal growth factors, vascular, endothelial growth factors and bone morphogenic proteins (Fu, Hu, et al., 2022).

During the restriction of amino acids and growth factors, cells are programmed to initiate autophagy, a degradation system mediated by ATG1/ULC-51. YAP is subject to autophagy-dependent degradation, but in the presence of amino acids and growth factors, the mammalian target of rapamycin complex 1 (mTORC1) cascade inhibits autophagy and YAP degradation. Additionally, multiple mechanisms are proposed through which mTORC2 activates YAP directly and indirectly. Conversely, active YAP/TAZ promote mTORC1 activation indirectly through enhancement of insulin/IGF-1 signalling. In addition to insulin and IGF-1, several amino acids, especially leucine, activate the mTORC1 signalling cascade. Active YAP/TAZ may stimulate the uptake of some amino acids, including leucine, by increasing the expression of several amino acid transporter genes (Honda et al., 2023).

Restriction of nutrients can also induce energy stress, primarily affecting glucose metabolism. The AMP-activated protein kinase (AMPK) is the crucial energy sensor of the cell, often regarded as the opposing regulator of mTOR (González et al., 2020). Given the multiple interactions of YAP/TAZ and mTORC1/2, it is unsurprising that glucose metabolism and AMPK contribute to the control of YAP/TAZ. Several studies demonstrate that active AMPK can indirectly decrease YAP signalling through LATS1/2 or directly through phosphorylating the serine residue required for YAP-TEAD binding, thereby reducing YAP target gene expression. The abundance of glucose and the rate of glycolysis is also directly associated with YAP/TAZ activity. For instance, YAP/TAZ become inactive in response to inhibition of glucose uptake or glycolysis, but the abundance of glucose enhances YAP-TEAD interaction and, thereby, YAP target gene expression (Santinon et al., 2016). This mechanism is particularly relevant for proliferation since YAP is a driver of proliferation, which can only be sustained in the presence of sufficient glucose (Enzo et al., 2015). In summary, the notion that a plethora of signals regulate the Hippo pathway and YAP/TAZ activity creates an image of overwhelming complexity. Considering that most of the evidence is limited to gain or loss of function or *in vitro* experiments, it will be challenging to understand how these signals and YAP/TAZ activity contribute to physiological regulation *in vivo*.

### 1.2.3 The Hippo signalling pathway in skeletal muscle

After discovering that the core Hippo pathway components are ubiquitously expressed in hindlimb muscles of mice (Watt et al., 2010), a series of studies confirmed the previously established roles of Hippo signalling in proliferation (Jeong et al., 2010; Watt et al., 2010), cell fate decisions (Judson et al., 2012; Mohamed et al., 2016; Tremblay et al., 2014) and size control (Goodman et al., 2015; Judson et al., 2013; Watt et al., 2015; Wei et al., 2013). In the first study on the Hippo pathway in skeletal muscle, Watt et al. (2010) demonstrated that YAP accumulated in the nuclei of C2C12 myoblasts cultured in serum-rich growth medium but accumulated increasingly in the cytoplasm of confluent cells cultured in serum-low differentiation medium. Along with decreasing total YAP abundance, this translocation corresponded to a 20-fold and 28-fold increase in phospho-YAP abundance after 24 and 48 hours in differentiation medium compared to growth medium. A similar course was observed during post-natal maturation, but the phospho- to total YAP ratio remained consistent (Watt et al., 2015).

Watt et al. (2010) also demonstrated that C2C12 myoblasts transfected with phospho-defective human YAP (S127A) failed to differentiate into myotubes as indicated by reduced markers of terminal differentiation such as dysferlin, muscle CK and dystrophin. Expression analyses of myogenic factors and cell cycle regulators supported the hypothesis that S127 phosphorylation is necessary for cell cycle withdrawal (Watt et al., 2010). However, the knockdown of YAP prevented differentiation, suggesting that cytoplasmic accumulation of YAP is a necessary event during the differentiation of C2C12 myoblasts (Chen et al., 2017). In contrast to YAP, overexpression of TAZ increases myoblast differentiation into myotubes in association with the myogenic regulatory transcription factor MyoD (Jeong et al., 2010). Together, these data suggest that YAP/TAZ are sensitive to cell-cell contact inhibition and are critical regulators of terminal differentiation in C2C12 myoblast. These results largely align with previous studies in several other cell types (Ma et al., 2019). In addition to confluence, the withdrawal of serum-rich growth medium, high in soluble factors that act through GPCR signalling, optimises the conditions for myoblast differentiation into myotubes. Since YAP is sensitive to various factors acting through GPCRs (Yu et al., 2012), it is reasonable to assume that the change from growth medium to differentiation contributes to cytoplasmic accumulation of YAP, thereby promoting terminal differentiation (Wackerhage et al., 2014).

In concert with the results from other cell types (Cao et al., 2020; Driskill & Pan, 2023), YAP/TAZ also play a role in the fate decisions of satellite cells (Judson et al., 2012; Mohamed et al., 2016; Tremblay et al., 2014). Satellite cells reside between the basal lamina and the sarcolemma of muscle fibres, are usually quiescent and become activated in response to muscle injury or certain growth factors. Once active, satellite cells proliferate and commit a fate decision between self-renewal or differentiation into myoblasts. They can be studied *ex vivo*

by isolating single fibres and culturing them with their satellite cells in their niche (Engquist & Zammit, 2021). Coherent with increased *Yap* expression, YAP became readily detectable upon activation of satellite cells and co-localised with PAX7- and MYOD-positive cells (Judson et al., 2012). To find out whether YAP is a driver or a passenger of active satellite cells, Judson et al. (2012) transfected cultured satellite cells and satellite cell-derived myoblasts with phospho-defective human YAP (S127A) and found that proliferation increased by ~75% and ~79%, respectively. Conversely, shRNA-mediated knockdown reduced the proliferation of satellite cell-derived myoblasts by ~40% (Judson et al., 2012). Moreover, overexpression of phospho-defective human YAP (S127A) and human wild-type YAP drove the proliferation of PAX7-positive satellite cells but decreased the number of cells positive for the myogenic transcription factor myogenin. Additionally, overexpression of human YAP S127A increased the transcription of genes related to cell cycle, ribosome biogenesis and angiotensin signalling (Judson et al., 2012). To characterise common and distinct functions of YAP and TAZ, Sun et al. (2017) knocked out TAZ globally in muscles and YAP specifically in satellite cells. They found that TAZ knockout mice had a reduced body weight and reduced weight of hindlimb muscles but no impaired regeneration. Instead, mice with a satellite cell-specific knockout of YAP showed signs of impaired regeneration in response to cardiotoxin-induced injury. This can be partially explained by the different target genes whose expression YAP and TAZ regulate (Sun et al., 2017). After determining that YAP expression is high in embryonal rhabdomyosarcoma, a form of childhood soft tissue cancer, Tremblay et al. (2014) found that satellite cell-specific expression of constitutively active YAP is sufficient to develop ERMS-like tumours in mice. The development of ERMS required the cardiotoxin-induced activation of satellite cells, as YAP does not independently activate satellite cells. Similarly, TAZ was also identified as an oncogene in rhabdomyosarcoma (Mohamed et al., 2016). In summary, these results suggest that YAP/TAZ are potential regulators of satellite cell fate decisions and might be key drivers in the formation of satellite cell-borne rhabdomyosarcoma.

Several *in vivo* studies in mice addressed the role of YAP as a size regulator in skeletal muscles. In the first study, Judson et al. (2013) induced the skeletal muscle-specific expression of constitutively active, human YAP (S127A) in adult mice. Surprisingly, they found that 5-7 weeks of human YAP S127A expression reduced the body weight by 20-25% and the skeletal muscle weight by ~35% compared to controls. Histological analyses revealed obvious signs of severe degeneration and regeneration, an abundance of necrotic fibres and central nucleation. These alterations were also evident in transcriptomic changes related to regenerative myogenesis and protein degradation. Intriguingly, the silencing of constitutively active YAP reversed nearly all of the variables (Judson et al., 2013). These results are in contrast to the previous finding that the expression of constitutively active YAP led to a reversible 4-fold increase in liver size (Camargo et al., 2007). Nevertheless, these data indicate that YAP might

be a negative regulator of skeletal muscle size and that constitutively active YAP might induce a myopathy-like phenotype in skeletal muscles.

In comparison, shRNA-mediated knockdown of YAP by 72% in skeletal muscles of adult mice resulted in decreased target gene expression, significantly decreased muscle mass and cross-sectional area per fibre (Watt et al., 2015). These changes were in line with lower protein synthesis rates, which was not reflected in the phosphorylation status of mTORC1 signalling components. Furthermore, the authors observed no changes in the activity of the ubiquitin-proteasome system, indicating that YAP knockdown caused muscle atrophy by altering protein synthesis in a mTORC1-independent manner. Conversely, overexpression of wild-type YAP (mouse isoform) increased skeletal muscle mass and mean fibre CSA by 13% and 10%, respectively. In addition, further experiments provided evidence that the hypertrophic effect of YAP overexpression depended on YAP-TEAD interactions (Watt et al., 2015). These results align with a study published shortly after, confirming that YAP overexpression induced hypertrophy in a mTORC1-independent manner (Goodman et al., 2015). Furthermore, this study was the first to show that YAP is sensitive to mechanical overload by synergist ablation. However, the increase in YAP phosphorylation indicated that mechanical overload inhibited YAP, while the ratio to total YAP remained constant (Goodman et al., 2015). Another study reported no differences in muscle fibre size after muscle-specific deletion of YAP (Zhao et al., 2017). However, the same study demonstrated that YAP deletion prevented the formation of neuromuscular junctions, resulting in various pre- and postsynaptic defects. Additionally, this study showed that YAP was necessary to reinstate denervated muscles (Zhao et al., 2017). In summary, these results implicate YAP as a critical regulator of skeletal muscle size, although it is unclear whether active YAP increases or decreases muscle size. Similar to the results in other organs and settings (Kowalczyk et al., 2022), the time point and duration of YAP manipulation might have contributed to the controversy surrounding these results (Watt et al., 2018).

## 1.3 The Hippo signalling pathway as a potential mediator of the Pathophysiology in Duchenne Muscular Dystrophy

Several lines of indirect and direct evidence indicate YAP as a potential mediator of the pathophysiology in DMD. Considering that the Hippo pathway integrates mechanical cues such as ECM stiffness through the actin cytoskeleton and that the DGC acts as mechanical between the ECM and the actin cytoskeleton, it seems reasonable to assume that Hippo components interact with DGC components. As mentioned before, dystrophin has a WW domain, characterised by two tryptophane residues about 20-23 amino acids apart within a 35-40 amino acids protein module that binds to a PPxY motif in β-dystroglycan (Huang et al., 2000; Sudol et al., 1995). WW domains occur rarely in the human proteome (Huang et al., 2020) but are overrepresented in the Hippo signal transduction network (Sudol & Harvey, 2010). Sun et al. (2017) immunoprecipitated YAP from C2C12 myoblasts and myotubes and identified potential binding partners using mass spectrometry-based proteomics. They detected utrophin, which also has a WW domain, but not dystrophin. and shares substantial homology with dystrophin. Utrophin shares substantial homology with dystrophin and binds to the same proteins but is locally confined to the myotendinous and neuromuscular junction in adult muscles (Ervasti, 2007). They also found that YAP bound the molecular cochaperone Bcl2-associated athanogene 3 (BAG3). BAG3 is also an intriguing finding because the loss of BAG3 induces a severe myopathy in mice and leads to early death after 4 weeks (Homma et al., 2006). Additionally, BAG3 also has a WW domain and was shown to be a positive regulator of YAP in response to mechanical stress (Ulbricht et al., 2013).

In comparison, Morikawa et al. (2017) immunoprecipitated YAP from the cardiac muscles of wild-type mice and found that the DGC members sarcoglycan δ (SGCδ) and dystroglycan 1 (DAG1) bound to YAP. However, neither DGC member was detectable in YAP precipitates of the cardiac muscles of mdx mice. Furthermore, their data indicated that the Hippo pathway and the DGC cooperatively promoted cytoplasmic accumulation of YAP. Although there was no difference in nuclear accumulation of YAP in the cardiac muscles of control and mdx mice, knockout of Salvador in mdx mice further increased nuclear accumulation and target gene expression than Salvador knockout in control mice (Morikawa et al., 2017). Similarly, a study on the dystroglycan (Dg) interactome in *Drosophila* muscles showed that dystroglycan binds the Hippo component Kibra (Kbr) and might also bind Yorkie (Yatsenko et al., 2020). Moreover, the authors demonstrated that *kbr* mutants copied the degenerative phenotype of *Dg* mutants. This and other experiments led the authors to conclude that the physical and functional interaction of Dg and the Hippo components is necessary for muscle tissue integrity (Yatsenko et al., 2020). Together, these data suggest that YAP binds members of the DGC and other myopathy-associated proteins, but it is unclear why the dissociation from the DGC did not

affect the localisation of YAP in the cardiac muscles of mdx mice (Morikawa et al., 2017; Morikawa et al., 2015).

As outlined earlier, Hippo signalling has several implications in developing fibrosis (Fu, Peng, et al., 2022; Mia & Singh, 2022), a hallmark of DMD (Kharraz et al., 2014), through regulating the transcription of the YAP target CTGF (Zhao et al., 2008). Muscles of DMD patients show high levels of CTGF (Sun et al., 2008b), which is considered a relevant target in treating fibrosis in DMD (Kharraz et al., 2014) and many other diseases (Fu, Peng, et al., 2022). Morales et al. (2011a) demonstrated that overexpression of CTGF using adenovirus-mediated delivery resulted in the rapid development of severe pathological features in the tibialis anterior muscles of wild-type mice. These features include necrotic damage and fibrosis followed by an increase in markers of regeneration and a decrease in specific force ($mN/mm^2$) (Morales et al., 2011b), closely resembling some features of DMD (Kharraz et al., 2014). Moreover, this study reported that the normalisation of CTGF levels coincided with the disappearance of all pathological features, suggesting that attenuating CTGF expression could be a valid strategy for treating DMD (Morales et al., 2011b). To test the validity of this hypothesis, Morales et al. (2013) crossbred mdx mice with mice heterozygous for CTGF deletion and found that a 50% reduction in CTGF abundance compared to mdx mice corresponded to significant improvements in several pathological features, including fibrosis (**Figure 8a-b**). Similar results were obtained when mdx mice were treated with a neutralising anti-CTGF antibody (FG-3019; (Aikawa et al., 2006)) for 8 weeks (Morales et al., 2013). FG-3019, also known as pamrevlumab, was recently tested in a small Phase II trial and decelerated the deterioration of the primary and secondary endpoints (Connolly et al., 2023). None of these studies considered YAP/TAZ signalling, but a more recent study demonstrated that LPA injection in muscles also increases the expression of CTGF and the ECM components collagen type 3, fibronectin and periostin, reflecting a robust fibrotic response (Córdova-Casanova et al., 2022). LPA is a well-recognised YAP activator in other cell types (Yu et al., 2012), but LPA injection did not affect phospho-YAP levels (Córdova-Casanova et al., 2022). However, the authors did not assess total YAP levels. Therefore, it is possible that LPA increased total YAP abundance, and a larger fraction was active and nuclear. In summary, these studies support the importance of developing treatments targeting CTGF in DMD, but it is unclear whether YAP plays a role in regulating CTGF expression in muscles.

A number of studies investigated YAP activity directly in *in vivo* and *in vitro* models of DMD, like mdx mice and dystrophin-mutated cells, and reported conflicting results (Hulmi et al., 2013; Iyer et al., 2019; Morikawa et al., 2017; Ramirez et al., 2022; Vita et al., 2018). In the first study, Hulmi et al. (2013) compared total and phospho-YAP levels in gastrocnemius muscles of 14-week-old mdx mice to age-matched wild-type controls. They found that mdx mice displayed higher phospho-YAP but a disproportional increase in total YAP, suggesting a larger active

fraction of YAP in mdx mice (Hulmi et al., 2013). Secondly, Morikawa et al. (2017) demonstrated that siRNA-mediated knockdown of *Dmd* increased YAP abundance in the nuclear fraction and decreased YAP abundance in the cytoplasmic fraction of C2C12 myotubes. Although the knockdown was highly efficient compared to the control, it should be noted that dystrophin expression in C2C12 cells seems to be low and sometimes not even detectable. As mentioned above, the same study showed no difference in YAP localisation in the cardiac muscle of mdx mice (Morikawa et al., 2017). Shortly after, Vita et al. (2018) reported decreased YAP abundance and increased phospho-YAP abundance in biopsies of DMD patients and muscles of mdx mice. This was the first and, so far, the only study investigating YAP activity in human DMD samples, but the results contrast previous and subsequent studies. However, these results should be regarded with great caution because the described methods to assess phospho-YAP abundance appear questionable and deviate from standard procedures in the field (Luo et al., 2022).

Furthermore, Iyer et al. (2019) showed compelling evidence of aberrant YAP signalling in tibialis anterior muscles of ~3-month-old mdx mice. This includes three main findings: i) total YAP abundance was higher, and phospho-YAP abundance was lower in whole-tissue lysates of mdx mice compared to age-matched, wild-type controls; ii) YAP was readily detectable in nuclear fractions of mdx mice but barely detectable in that of wild-type muscles; iii) mechanical loading increased cytoplasmic and nuclear YAP and decreased the phospho- to total YAP ratio in wild-type muscles but had no effect in mdx muscles. The differences in the unloaded state were consistent with increased co-localisation with nuclei in mdx muscles, as determined by fluorescence microscopy, and increased YAP target gene expression in mdx muscles compared to wild-type muscles, as determined by RT-qPCR (Iyer et al., 2019). Lastly, Ramirez et al. (2022) transfected C2C12 myoblasts with the *DMD* gene carrying mutations resemblant of those found in DMD patients, leading to reduced nuclear accumulation of YAP compared to wild-type C2C12 myoblasts. In summary, these studies convincingly show that the loss of dystrophin directly affects YAP activity. Despite some of these data suggesting reduced YAP activity (Ramirez et al., 2022; Vita et al., 2018), most data, especially in skeletal muscles of mdx mice, indicate higher YAP activity in DMD models (Hulmi et al., 2013; Iyer et al., 2019; Morikawa et al., 2017).

While the above-presented studies suggest an association between DMD and the Hippo pathway, including the notion that YAP is more active in some DMD models, limited evidence explains how active YAP actively contributes to the pathophysiology in DMD. Considering that overexpression of constitutively active YAP (S127A) in muscle fibres promotes a myopathy-like phenotype similar to DMD (Judson et al., 2013) (**Figure 8c-d**), it is conceivable that aberrant YAP activity contributes to the pathophysiology in DMD, particularly by driving the CTGF-mediated fibrosis. Intriguingly, simvastatin, a potent YAP inhibitor (Sorrentino et al.,

2014), improved several outcomes in mdx mice (Amor et al., 2021; Bourg et al., 2022; Kim et al., 2019; Whitehead et al., 2015; Xu et al., 2020). In a series of experiments, Whitehead et al. (2015) were the first to demonstrate that long-term treatment (2-8 months) in young (3-week-old) and old (12-month-old) significantly reduced inflammation, oxidative stress and fibrosis (**Figure 8e-f**). This resulted in vastly reduced muscle damage, an increase in the specific force of the diaphragm and the tibialis anterior muscle and muscle endurance (Whitehead et al., 2015). These results have been partially reproduced and extended to cardiac muscle (Amor et al., 2021; Bourg et al., 2022; Kim et al., 2019; Xu et al., 2020), but two other studies reported no benefits from simvastatin treatment (Mucha et al., 2021; Verhaart et al., 2021). However, one of those studies failed to demonstrate meaningful increases in plasma simvastatin levels (1-3 ng/ml versus ~170 ng/ml in (Whitehead et al., 2015)) (Verhaart et al., 2021; Whitehead et al., 2021) while the other study also reported low plasma levels of simvastatin and the treatment duration was shorter than in the other studies (1 versus 3-8 months) (Mucha et al., 2021). Statins are commonly prescribed drugs to treat hypercholesterolemia by inhibiting the rate-limiting enzyme of cholesterol synthesis, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (Demierre et al., 2005). Sorrentino et al. (2014) first identified statins as YAP/TAZ inhibitors and demonstrated that the mevalonate pathway is necessary for YAP/TAZ function. Further, they showed that this regulation is mediated by geranylgeranylation of Rho GTPases and independent of LATS1/2 activity (Sorrentino et al., 2014). To sum up, considerable evidence supports simvastatin as a promising treatment for various pathological features of DMD. Despite the observation that these improvements partially resemble those by CTGF-targeted treatment (Morales et al., 2013), none of these studies addressed the YAP-CTGF signalling axis. Therefore, how simvastatin improves such a wide range of pathological features is largely unknown.

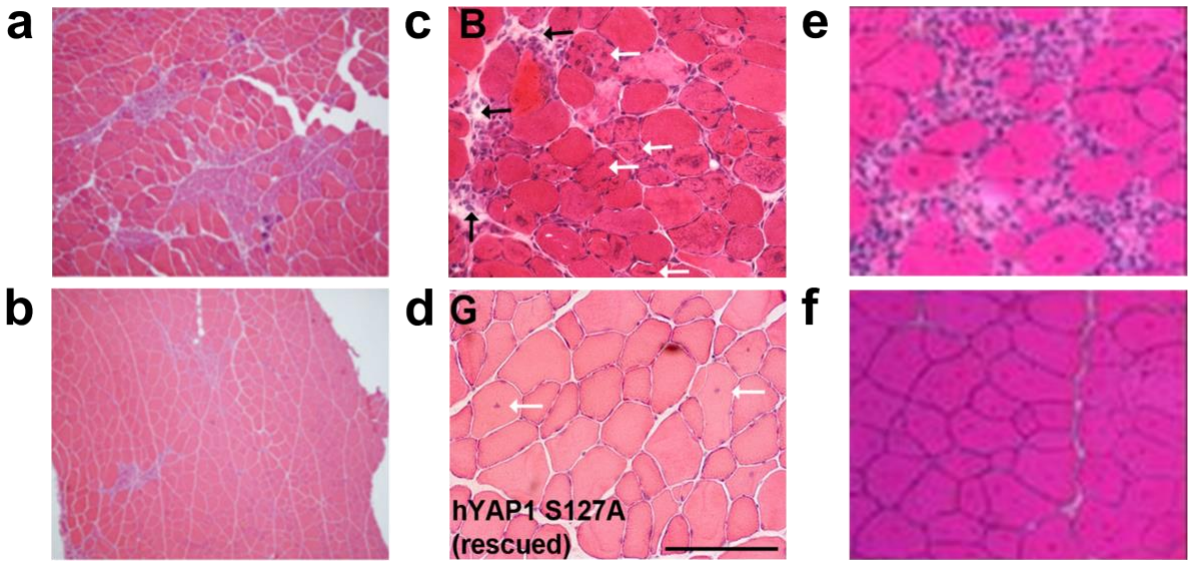


**Figure 8** Histological analyses of mdx muscles (**a**) after CTGF deletion (**b**; adapted from Morales et al. (2013)), overexpression of constitutively active YAP S127A (**c**) and rescue of wild-type YAP (**d**; adapted from Judson et al. (2013)) and of mdx muscles (**e**) treated with simvastatin for 8 months (**f**; adapted from Whitehead et al. (2015)).

## 1.4 Aims and Objectives

Several studies in mice or C2C12 cells suggested that YAP is predominantly active and nuclear in models of DMD. This might be relevant to the pathogenesis of this disease, as YAP regulates the expression of *CTGF*, a prime target for fibrosis in DMD. However, except for one questionable study, no evidence exists so far on YAP activity in human models of DMD. Therefore, Aim 1 of Study 1 was to find out whether YAP is constitutively active in dystrophic, immortalised human skeletal muscle cells. Specifically, I performed *in vitro* experiments using immortalised myoblasts from two donors carrying two different *DMD* mutations and from one healthy donor to determine

1.1) YAP localisation in myoblast and myotubes using immunofluorescence microscopy,
1.2) the mRNA levels of three well-established YAP target genes (*CTGF*, *ANKRD1*, *CYR61*) in myoblasts and myotubes using Reverse Transcriptase-quantitative Polymerase Chain Reaction (RT-qPCR) and
1.3) the total and phospho-YAP (S127) abundance in myoblasts and myotubes using Western Blot analysis.

Simvastatin improved cardiac and skeletal muscle pathology in DMD models, which is interesting because simvastatin was identified as a potent inhibitor of YAP. However, none of the previous studies considered the effects of simvastatin on YAP activity or the notion that YAP might be dysregulated in DMD. It is entirely unknown if simvastatin also decreases YAP signalling in skeletal muscle cells. Thus, Aim 2 of Study 1 was to investigate whether simvastatin inhibits YAP signalling in dystrophic, immortalised human skeletal muscle cells. Specifically, I performed *in vitro* experiments using immortalised myoblasts from two donors carrying two different *DMD* mutations and from one healthy donor to determine the effects of simvastatin or vehicle treatment on

2.1) YAP localisation in myoblast and myotubes using immunofluorescence microscopy,
2.2) the mRNA levels of three well-established YAP target genes (*CTGF*, *ANKRD1*, *CYR61*) in myoblasts and myotubes using RT-qPCR and
2.3) the total and phospho-YAP (S127) abundance in myoblasts and myotubes using Western Blot analysis.

Two previous studies indicated that YAP is more active and nuclear in mdx mice (Hulmi et al., 2013; Iyer et al., 2019), although only one of them reported decreased phospho-YAP abundance in mdx compared to wild-type mice (Iyer et al., 2019). Hence, Aim 1 of Study 2 was to investigate whether YAP is constitutively active in dystrophic mouse skeletal muscles of mice. Specifically, I performed *in vivo* experiments using extracted gastrocnemius muscles of wild-type and mdx mice to determine

1.1) YAP localisation in cryosections using immunofluorescence microscopy,
1.2) the mRNA levels of three well-established YAP target genes (*Ctgf*, *Ankrd1*, *Cyr61*) using RT-qPCR and
1.3) the total and phospho-YAP (S127) abundance using Western Blot analysis.

Studies in the cardiac muscle of mice revealed that YAP bound to the DGC members DAG1 and SGC and utrophin in C2C12 cells, but no evidence exists for these interactions in the skeletal muscles of healthy or dystrophic mice. Therefore, Aim 2 of Study 2 was to determine whether YAP binds to members of the DGC or other myopathy-associated proteins in healthy and dystrophic mouse skeletal muscles of mice. Specifically, I performed *in vivo* experiments using extracted gastrocnemius muscles of wild-type and mdx mice to

1.1) co-immunoprecipitate YAP and potential binding partners using an anti-YAP antibody or normal IgG and magnetic beads and
1.2) identify potential binding partners in the precipitates using a mass spectrometry-based proteomics approach.

# 2 Methods

## 2.1 Study 1

Cell Culture

To study the regulation of the Hippo pathway member YAP in a human *in vitro* model of DMD, Dr. Vincent Mouly (Center for Research in Myology, Sorbonne University, Paris) provided immortalised, dystrophic and healthy human myoblasts (Mamchaoui et al., 2011). As described in **Table 1**, I relabeled these in this manuscript for better readability. I cultured myoblasts in Cellovations® Skeletal Muscle Growth Medium (PELOBiotech, PB-MH-272-0090), including the Growth Supplements kit (PELOBiotech, PB-SH-272-0000) including 5% FBS, GlutaMAX™ (Gibco™, 35050061) and Gentamycin (Gibco™, 15750045). The culture medium was changed every two days. For myotube formation experiments, I switched media at a state of complete cell confluence Cellovations® Skeletal Muscle Growth Medium (PELOBiotech, PB-MH-272-0090) supplemented with 5% Horse Serum (Gibco, 16050130), GlutaMAX™ (Gibco™, 35050061) and Gentamycin (Gibco™, 15750045), and differentiated myotubes for seven days before treatments. For each replicate, I thawed a new vial of cells from the same stock and passage number on different days. To study the effects of simvastatin on YAP signalling, I prepared a 10 mM stock solution of simvastatin (Sigma-Aldrich®, S6196) in dimethyl sulfoxide (DMSO; Roth®, A994.2). I incubated cells at a final concentration of 10 µM simvastatin or 0,01% DMSO (Roth®, A994.2; v/v) as a control for 24 hours. To avoid interference with regular sera replenishment (Yu et al., 2012), I added simvastatin or DMSO (Roth®, A994.2) to the medium 24 hours after the last medium change.

**Table 1** Characteristics of the human, immortalised skeletal muscle cell lines.

| **Name** | **Relabeled as** | **Patho-logy** | **Mutation** | **Muscle** | **Age (years)** | **Sex** |
|---|---|---|---|---|---|---|
| AB1190 | Control | - | - | Paravertebral | 16 | Male |
| KM1316 | DMD1 | DMD | Duplication exon 10-11 | Paravertebral | 14 | Male |
| AB1071 | DMD2 | DMD | Deletion exon 45-52 | Paravertebral | 13 | Male |

Immunocytochemistry

For immunolabelling of myoblasts, I seeded myoblasts on chamber slides (ibidi, 80841) and treated them with 10 µM simvastatin or 0,01% DMSO (Roth®, A994.2; v/v) for 24 hours. For the immunolabelling of myotubes, I seeded cells on round glass coverslips within the same

100 mm dishes I used for Western Blots and RNA sequencing. After the treatment, I washed cells twice with phosphate-buffered saline (PBS), followed by fixation in 4% paraformaldehyde (Roth®, 0335.2) in PBS for 10 minutes, washing twice with PBS and permeabilisation with Tris-buffered saline (TBS)-Tween20 (0.3%) for 10 minutes. After blocking with 10% goat serum (Dako, X0907) in TBS-Tween20 (0.1%) for 1 hour, cells were incubated using a monoclonal mouse anti-YAP (Santa Cruz Biotechnology, sc-101199) primary antibody diluted 1:200 in TBS-Tween20 (0.1%) containing 2% goat serum (Dako, X0907) overnight. After four washes with TBS-Tween20 (0.1%), cells were incubated with a goat anti-mouse secondary antibody (Jackson ImmunoResearch, 115-545-003) diluted 1:700 in TBS-Tween20 (0.1%) containing 2% goat serum (Dako, X0907) for 1.5 hours, followed by another four washes with TBS-Tween20 (0.1%). For labelling of nuclei, I incubated cells with Hoechst 33342 (Sigma-Aldrich, B2261; 1:2000 of a 2 mg/ml stock solution in PBS) for 10 minutes with two final washes with PBS before mounting them using ibidi Mounting Medium (ibidi®, 50001) or ProLong Gold (Invitrogen, P36930) antifade solutions. I imaged the samples using a Zeiss Axiocam 503 mono digital camera attached to a Zeiss Imager.Z1m epifluorescence microscope.

RNA isolation and RT-qPCR

For gene expression analysis of myoblasts, I cultured cells in 60 mm dishes and extracted RNA using the NucleoSpin Plus kit (Macherey-Nagel, 740984.50) per the manufacturer's protocol for adherent cells. After aspirating the medium, I pipetted 350 µL of LBP lysis buffer into each well, scraped off the cells and collected the lysate in 0,5 mL DNA LoBind® tubes (Eppendorf, 022431021). I loaded the cell lysates onto NucleoSpin gDNA removal columns and centrifuged them at 11.000 g for 30 seconds to remove genomic DNA. After mixing the flowthrough with 100 µL BS Binding Solution, the lysate was loaded onto NucleoSpin RNA Plus columns and centrifuged at 11.000 g for 15 seconds. I washed the membrane with 200 µL WB1 buffer by centrifugation at 11.000 g for 15 seconds, followed by one wash with 600 µL of buffer WB2 by centrifugation at 11.000 g for 15 seconds and one last wash with 250 µL of buffer WB2 by centrifugation at 11.000 g for 2 minutes. Finally, RNA was eluted by pipetting 15 µL RNase-free water onto the membrane, centrifuging it at 11.000 g for 1 minute, followed by a second elution in 10 µL RNase-free water in 1,5 mL DNA LoBind® tubes (Eppendorf, 022431021). Finally, I stored the samples at -80° C. For gene expression analysis of myotubes, the treated cells were incubated with a minimal trypsin solution to detach myotubes, followed by centrifugation at 300 g for 3 minutes to separate myotubes from mono-nucleated cells. The myotube-enriched pellet was then resuspended in 350 µL of LBP lysis buffer and handled the same way as the myoblast lysate, as mentioned before. I quantified RNA concentrations using the Qubit™ RNA Broad Range kit (ThermoFisher Scientific, 10210) on a Qubit™ Fluorometer 4 (ThermoFisher Scientific, Q33226) and checked the eluates for contamination using a NanoDrop® ND-1000 (NanoDrop Technologies) at 230, 260, and 280 nm.

I reverse-transcribed 350-1000 ng of total RNA into cDNA using the qScript™ Ultra SuperMix® (Quantabio, 95217-100) kit as per manufacturer's protocol and performed real-time quantitative PCR using a Rotorgene RG-6000 (Corbett Research) with the PerfeCTa® SYBR® Green SuperMix (Quantabio, 95054-500) in duplicates comprising 5 µL Master Mix, 3 µL RNase-free water, 0.5 µL of each forward and reverse primer and 1 µL of cDNA template (equals to 17.5-50 ng CDNA). All primers were designed using the NCBI Primer-BLAST tool (Ye et al., 2012) and delivered by OLIGO (Sigma-Aldrich). The primer sequences are shown in **Table 2**. Data were normalised to the reference gene *RPLP0* (Ai et al., 2024; Wu et al., 2024), and gene expression was quantified using the delta-delta-Ct method (Pfaffl, 2001).

**Table 2** Primer sequences for qPCR.

| **Target gene** | **Primer sequences (5´-3´)** | **NCBI Acc. No.** | **Length of PCR product (bp)** |
|---|---|---|---|
| *CTGF* | CCAATGACAACGCCTCCTG<br>TGGTGCAGCCAGAAAGCTC | NM_001901.4 | 159 |
| *ANKRD1* | TAGCGCCCGAGATAAGTTGC<br>GGTTCAGTCTCACCGCATCA | NM_014391.3 | 155 |
| *CYR61* | GAAGCGGCTCCCTGTTTTTG<br>CGGGTTTCTTTCACAAGGCG | NM_001554.5 | 174 |
| *RPLP0* | TGGAGAAACTGCTGCCTCATA<br>CAGCAGCTGGCACCTTATTGG | NM_053275.4 | 108 |

Western Blotting

For Western Blots of myoblasts, I cultured cells in 60 mm dishes and extracted protein using a modified RIPA buffer containing Hepes-buffered saline instead of PBS, 20 mM sodium fluoride, 10 mM sodium orthovanadate, 1 mM EDTA, 1X Halt™ 100X protease (Thermo Scientific, 1862209) and 1X Halt™ 100X phosphatase inhibitor cocktails (Thermo Scientific, 1862495).

For Western Blots of myotubes, I cultured cells in 100 mm dishes and detached myotubes using a 0.25X trypsin solution, and the supernatant was split into two tubes before gentle centrifugation. One pellet was used for protein extraction as described for myoblasts and one was used for RNA extraction for transcriptomic analyses as described before.

I obtained protein concentrations by measuring optical density using a Bradford assay (Bio-Rad Laboratories 500-0006) on a Tecan (Sunrise) plate reader. Cell lysates were mixed with Laemmli buffer (Bio-Rad Laboratories 161-0747) including 10% β-mercaptoethanol (Roth®, 4227.1) and boiled at 95° C for 5 minutes. For detection of total and phospho-YAP (Ser127), I separated proteins using SDS gel electrophoresis by loading 30 µg of protein onto Mini-

PROTEAN® TGX Stain-Free™ precast gels (4-15%; Bio-Rad Laboratories, 45-8087) and running them at 80V for 30 minutes followed by 120 Volts until the blue dye was about 0.5 cm above the bottom edge of the gel (~50 minutes). Proteins were then transferred to Trans-Blot® Turbo™ PVDF membranes using the Trans-Blot® Turbo™ transfer system (Bio-Rad Laboratories). Membranes were washed in TBS-Triton X100 (1%; TBST) for 5 minutes, blocked in 5% milk powder in TBST for 1 hour, followed by overnight incubation in primary antibodies diluted 1:1000 in 5% BSA in TBST. Primary antibodies were mouse anti-YAP (Santa Cruz Biotechnology, sc-101199) and rabbit anti-phosphoYAP (Ser127; Cell Signaling Technology®, 4911S). The next day, I washed the membranes three times in TBST and incubated them with appropriate HRP-conjugated antibodies (Anti-mouse Cell Signaling Technology® 7076S; Anti-rabbit 7074S) diluted 1:5000 in blocking buffer for 1 hour. After four washes in TBST-T, I developed the membranes in Clarity Max™ ECL substrate (Bio-Rad Laboratories, 1705062). Mean density was obtained in FIJI and normalised to the total protein level of each lane using Ponceau S staining.

### Statistics

I determined statistically significant differences between groups using Student's *t*-test (two-tailed, unpaired) in Microsoft Excel. The significance level was set at $P < 0.05$. RT-qPCR results are shown as geometric means and geometric standard deviation, and Western Blot results are shown as means and standard error of the mean.

## 2.2 Study 2

Muscle specimen

I received three snap-frozen gastrocnemius muscles of 4-week-old mdx mice and age-matched wild-type controls, shipped on dry ice, as a gift from Dr. Bert Blaauw (University Padova). The muscles were stored at -80°C until further processed. These samples were used for all subsequent analyses.

Immunohistochemistry

To find out whether I can reproduce the finding that YAP is nuclear in muscles of mdx mice, I cut 10 µM cryosections at ~-22°C of mdx and wild-type gastrocnemius muscles using a Leica CM350S cryostat. Sections were stored at -20°C and immunolabelled YAP as described for immunocytochemistry in Study 1.

RNA isolation and RT-qPCR

To analyse gene expression of dystrophic and wild-type gastrocnemius muscles, I homogenised 20-30 mg of tissue in 500 µL NucleoZol (Macherey-Nagel, 740404.200) using Precellys CK14 (Bertin Technologies, P000912-LYSK0) tissue lysis tubes. Total RNA was isolated using the NucleoSpin RNA kit (Macherey-Nagel, 740406.50). Per the manufacturer's protocol, I added 200 µL RNase-free water to the lysate, vortexed and incubated for 10 minutes, followed by centrifugation for 15 minutes at 12.000 g at 4° C. Next, I mixed 500 µL of the supernatant with 500 µL buffer MX, loaded the lysate to a NucleoSpin RNA Column and centrifuged at 8000 g for 30 seconds. I washed the membranes once with 700 µL buffer RA3 by centrifugation at 8000 g for 30 seconds and a second time with 350 µL buffer RA3 by centrifugation at 8000 g for 2 minutes. After leaving the membrane to dry for 3 minutes, I eluated the RNA in 25 µL RNase-free water by 1 minute centrifugation at 11000 g. All subsequent steps were performed identically to the procedures described in Study 1. Primer sequences are listed in **Table 3**.

**Table 3** Primer sequences for qPCR.

| Target gene | Primer sequences (5´-3´) | NCBI Acc. No. | PCR product length (bp) |
|---|---|---|---|
| *Ctgf* | GAGTGTGCACTGCCAAAGAT<br>AAATGTGTCTTCCAGTCGGTAGG | NM_010217.2 | 287 |
| *Ankrd1* | AGTTGTGAAGGAGCCAGAAC<br>CGTCCGTTTATACTCATCGCA | NM_013468.3 | 151 |
| *Cyr61* | ACTAAGAGGCTTCCTGTCTTTGG<br>ACTGGAGCATCCTGCATAAGT | NM_010516.2 | 304 |
| *Rplp0* | GGACCCGAGAAGACCTCCTT<br>GCACATCACTCAGAATTTCAATGG | NM_007475.5 | 154 |

Co-immunoprecipitation

To identify differences in YAP interactomes between dystrophic and healthy muscles, I immunoprecipitated YAP and potential binding partners from the gastrocnemius muscles of mdx and age-matched wild-type mice using the Universal Magnetic Co-IP kit (Active Motif®, 54002). Per the manufacturer's protocol, frozen gastrocnemius muscles were sliced into pieces and mechanically homogenised in 100 µL/10 mg lysis buffer on ice using an Ultra-Turrax® homogeniser. After determining protein concentrations using a Bradford assay, I equilibrated 1,5 mg of total protein in a total of 500 µL of Co-IP buffer. After incubating the lysate with 25 µL of protein G magnetic beads slurry to reduce unspecific binding, I added 6 µL of polyclonal anti-YAP antibody (Novus Biologicals, NB110-58358) or rabbit IgG control (Sigma-Aldrich, 12-370) to the pre-cleared lysate for 10 hours, followed by another 2 hours with 25 µL of protein G magnetic beads slurry. During all incubation steps, I kept the samples on a rotator wheel at 5 rpm at 4° C and on ice during pipetting steps and subsequent washes. I pulled the magnetic beads aside using a magnetic tube rack, aspirated the lysates and stored an aliquot in 4X Laemmli buffer (Bio-Rad Laboratories 161-0747) with 10% β-Mercaptoethanol at -20° C. I washed the beads attached to the precipitates four times with 500 µL wash buffer containing protease inhibitors, followed by another two washes with 500 µL PBS containing protease inhibitors. To wash the beads, I cut the pipette tips to widen the orifice and gently pipetted the solution 20 times up and down. I eluted the precipitates in 20 µL 2X Laemmli buffer (Bio-Rad Laboratories 161-0747) containing 10% β-Mercaptoethanol (Roth®, 4227.1) by boiling them at 95° C for 5 minutes. I isolated the precipitates by pulling the beads aside in the magnetic tube rack and stored the aspirated precipitates at -20° C.

Western Blotting

To validate that I immunoprecipitated YAP from the soluble fraction of muscle tissue lysates, I subjected the eluates to SDS-PAGE followed by immunoblotting. I pooled triplicates of the lysates incubated with normal Rabbit IgG, later termed PreIP, and of the lysates incubated with the anti-YAP antibody, later termed PostIP and loaded 60 µg of protein and 8 µL of each eluate onto 4-15% TGX precast gels (Bio-Rad Laboratories, 45-8087). I performed SDS-PAGE and immunoblotting as described above in Study 1. Using the lysates used for immunoprecipitation of YAP, I determined total and phospho-YAP (Serine 127/112) abundance as described in Study 1.

Mass spectrometry-based proteomics

*Sample preparation.* For each Co-IP eluate described above, in-gel trypsin digestion was performed according to standard procedure (Shevchenko et al., 2006). Briefly, samples were run on a Nu-PAGE™ 4%–12% Bis-Tris protein gel (ThermoFisher Scientific) for about 1 cm. Subsequently, the still not size-separated single protein band per sample was cut out, reduced (50mM dithiothreitol), alkylated (55mM chloroacetamide) and digested overnight with trypsin (Trypsin Gold, mass spectrometry grade, Promega). The peptides obtained were dried to completeness and resuspended in 25 µL of 2% acetonitrile and 0.1% formic acid in HPLC-grade water. Finally, 5 µL of each sample was injected per mass spectrometric (MS) measurement.

*LC-MS/MS data acquisition.* Liquid chromatography tandem mass spectrometry (LC-MS/MS) data acquisition was carried out on a Dionex Ultimate 3000 RSLCnano system coupled to a Q-Exactive HF-X mass spectrometer (ThermoFisher Scientific, Bremen). Injected peptides were delivered to a trap column (ReproSil-pur C18-AQ, 5 µm, Dr. Maisch, 20 mm × 75 µm, self-packed) at a flow rate of 5 µL/min in 0.1% formic acid in HPLC grade water. After 10 minutes of loading, peptides were transferred to an analytical column (ReproSil Gold C18-AQ, 3 µm, Dr Maisch, 450 mm × 75 µm, self-packed) and separated using a 50 min gradient from 4% to 32% of solvent B (0.1% FA, 5% DMSO in acetonitrile) in solvent A (0.1% FA, 5% DMSO in HPLC grade water) at 300 nL/min flow rate. The Q-Exactive HF-X mass spectrometer was operated in data dependent acquisition (DDA) and positive ionisation mode. MS1 spectra (360–1300 m/z) were recorded at a resolution of 60k using an automatic gain control (AGC) target value of 3e6 and maximum injection time (maxIT) of 45 msec. Up to 18 peptide precursors were selected for fragmentation. Only precursors with charge states 2 to 6 were selected, and dynamic exclusion of 30 sec was enabled. Peptide fragmentation was performed using higher energy collision induced dissociation (HCD) and a normalised collision energy

(NCE) of 26%. The precursor isolation window width was set to 1.3 m/z. MS2 Resolution was 15.000 with an automatic gain control (AGC) target value of 1e5 and maximum injection time (maxIT) of 25 msec.

*LC-MS/MS data analysis.* Peptide identification and quantification were performed using the software MaxQuant (Tyanova et al., 2016) (version 1.6.3.4) with its built-in search engine Andromeda (Cox et al., 2011). MS2 spectra were searched against the UniProt mouse reference protein database (UP000000589, 17038 protein entries, downloaded July 2020), supplemented with common contaminants (built-in option in MaxQuant). Trypsin/P was specified as a proteolytic enzyme. Carbamidomethylated cysteine was set as a fixed modification. Methionine oxidation and acetylation at the protein N-terminus were specified as variable modifications. Results were adjusted to a 1% false discovery rate on peptide spectrum match (PSM) level and protein level employing a target-decoy approach using reversed protein sequences. Label-Free Quantification (LFQ) (Cox et al., 2014) intensities were used for protein quantification with at least 2 peptides per protein identified. The minimal peptide length was defined as 7 amino acids, and the “match-between-runs” functionality was disabled. For differential abundance analysis, raw LFQ intensity values were transformed into their log10 values. Further, the dataset was filtered for proteins that were detected in at least two out of three biological replicates in at least one condition (mdx, mdx_mock, WT or WT_mock). Missing values were imputed by a protein-specific constant value, which was defined as the lowest detected protein-specific LFQ-value over all samples divided by two. Additionally, a maximal imputed LFQ value was defined as a 15% quantile of the protein distribution from the complete dataset. Differential abundance was determined by a Student’s t-test, applying a fold-change cutoff > 2 and a p-value cutoff < 0.05 (not corrected for multiple hypothesis testing).

# 3 Results

## 3.1 Study 1

YAP/TAZ can shuttle between the nucleus and the cytoplasm. This shuttling determines the activity of YAP/TAZ, as they can only be active as transcriptional co-factors if they are in the nucleus. This shuttling is dynamic rather than a fixed steady state (Kofler & Kapus, 2023; Manning et al., 2020). To determine YAP localisation in dystrophic, human myotubes versus healthy controls, I immunolabelled YAP in myotubes on day 8 of differentiation and found that YAP accumulated in some myotubes predominantly in the cytoplasm with little detection in the nucleus (**Figure 9a-i**). However, YAP accumulated mainly in the nucleus and little in the cytoplasm in other myotubes of the same sample (**Figure 9a-i**). This was evident in all three cell lines (**Figure 9a-i**). This is surprising because previous studies in C2C12 cells established that YAP phosphorylation increased during differentiation of myoblasts, which is associated with cytoplasmic accumulation of YAP (Watt et al., 2010). Based on these results, YAP does not appear to be constitutively nuclear in dystrophic compared to healthy skeletal muscle cells (Iyer et al., 2019).

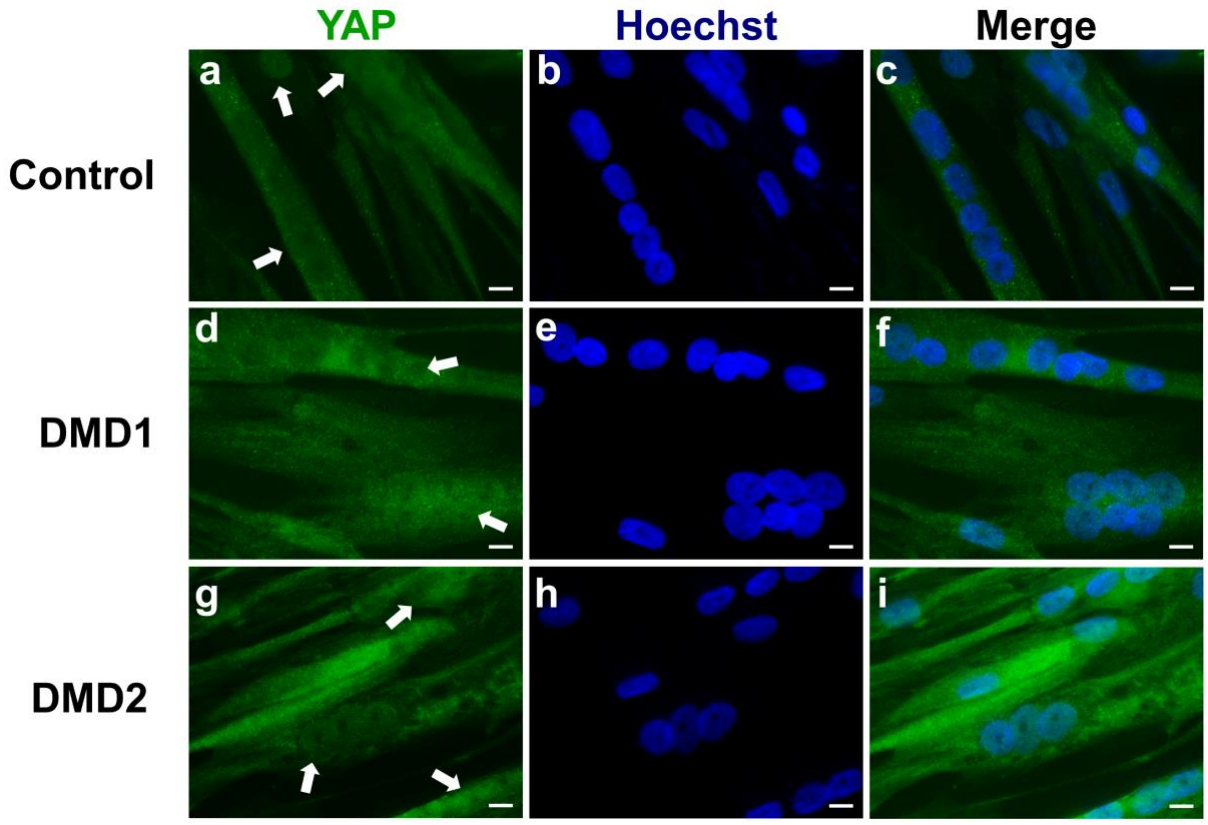


**Figure 9** YAP localisation in healthy and dystrophic myotubes. Control (**a-c**), DMD1 (**d-f**) and DMD2 (**g-i**) myotubes were differentiated for 8 days and immunolabelled for YAP (**a, c, d, f, g, i**) or nuclei (Hoechst**; b, c, e, f, h, i**). Scale bar represents 10 µm

When active and nuclear, YAP/TAZ bind to TEAD1-4 transcription factors, which regulate the expression of target genes such as *CTGF*, *ANKRD1* or *CYR61* (Ma et al., 2019). Terminal differentiation of myoblasts depends on LATS1/2-mediated phosphorylation of YAP at S127 (Chen et al., 2017; Watt et al., 2010), and cytoplasmic accumulation is consistent with decreasing *CTGF* mRNA levels (Knyazeva et al., 2020). To find out whether YAP is more active in dystrophic compared to healthy cells, I quantified the expression of *CTGF*, *ANKRD1* and *CYR61* in myoblasts at ~60-70% confluence (**Figure 10a**) and myotubes at day 8 of differentiation (**b**). In contrast to the literature (Iyer et al., 2019), I did not find that dystrophy-associated *CTGF* was higher in either DMD1 or DMD2. However, when I compared *CTGF* expression in myotubes to myoblasts of each respective cell line, I found that the differentiation-associated increase was significantly higher in DMD1 compared to Control (6,#-fold versus 2,#-fold, respectively; p = 0.02); **c**). This increase was less pronounced in DMD2 (3,#-fold; p = 0.35), suggesting a donor-specific *CTGF* regulation. *ANKRD1* was downregulated in DMD1 in myoblasts (**a**) but not significantly in myotubes (**b**), while I found a trend that it might have been upregulated in DMD2. *CYR61* expression did not significantly differ between dystrophic and healthy cells (**a**,**b**). Consistent with the literature (Knyazeva et al., 2020), *ANKRD1* and *CYR61* decreased in myotubes compared to myoblasts (**c**). Together, these results provide limited support for the hypothesis that YAP is constitutively active in dystrophic muscle cells.

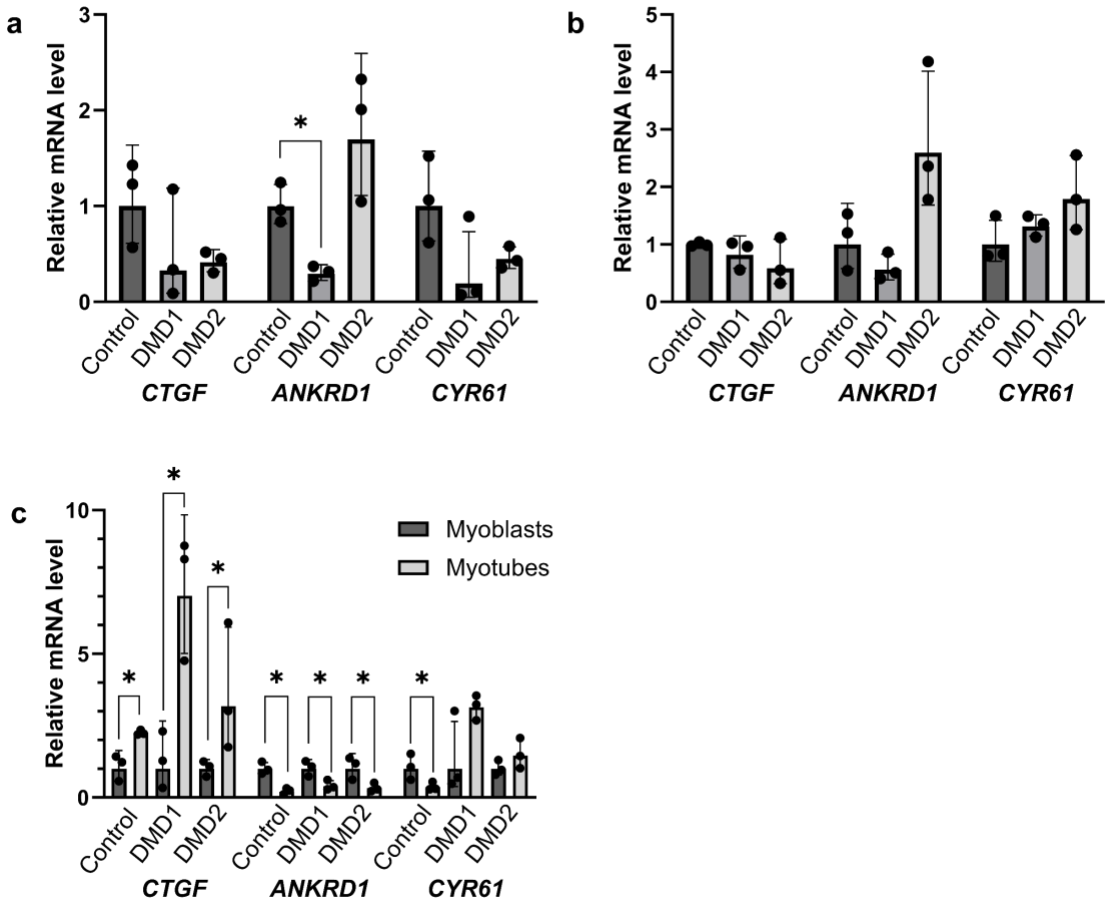


**Figure 10** YAP target gene expression analysis in healthy and dystrophic myoblasts and myotubes collected at 60-70% confluence or after 8 days of differentiation, respectively. **a** In myoblasts, *ANKRD1* is lower in DMD1 compared to Control. **b** In myotubes, target gene expression is similar between cell lines. **c** *CTGF* expression is higher in myotubes compared to myoblasts. This is more pronounced in DMD1 and DMD2 than in Control. *ANKRD1* decreases in myotubes of all cell lines, but *CYR61* only in Control. n = 3; * $p < 0.05$, two-tailed t-test

Next, I investigated whether YAP regulation differs on protein level (**Figure 11**). When comparing dystrophic myoblasts and myotubes to healthy cells, I found no significant differences in total YAP (**b**), phospho-YAP (S127, **c**), or phospho- to total YAP ratio (**d**). However, in line with previous results (Watt et al., 2010), total YAP abundance decreased in myotubes by ~68%, ~75% and ~60% in Control, DMD1 and DMD2 myotubes (**b**), respectively. This corresponded to a ~2.9-fold, ~4.2-fold and ~4.1-fold increase in phospho- to total YAP ratio (**d**). These results are consistent with the finding that YAP target gene expression was similar between healthy and dystrophic cells (**Figure 10**). Notably, the differentiation-associated increase in *CTGF* expression in DMD1 (**Figure 10c**) was not reflected in the protein data. This suggests that differences in YAP phosphorylation on Serine 127 (or lack thereof) is

not entirely indicative of YAP target gene expression or that other (co-) factors regulate *CTGF* expression in skeletal muscle cells.

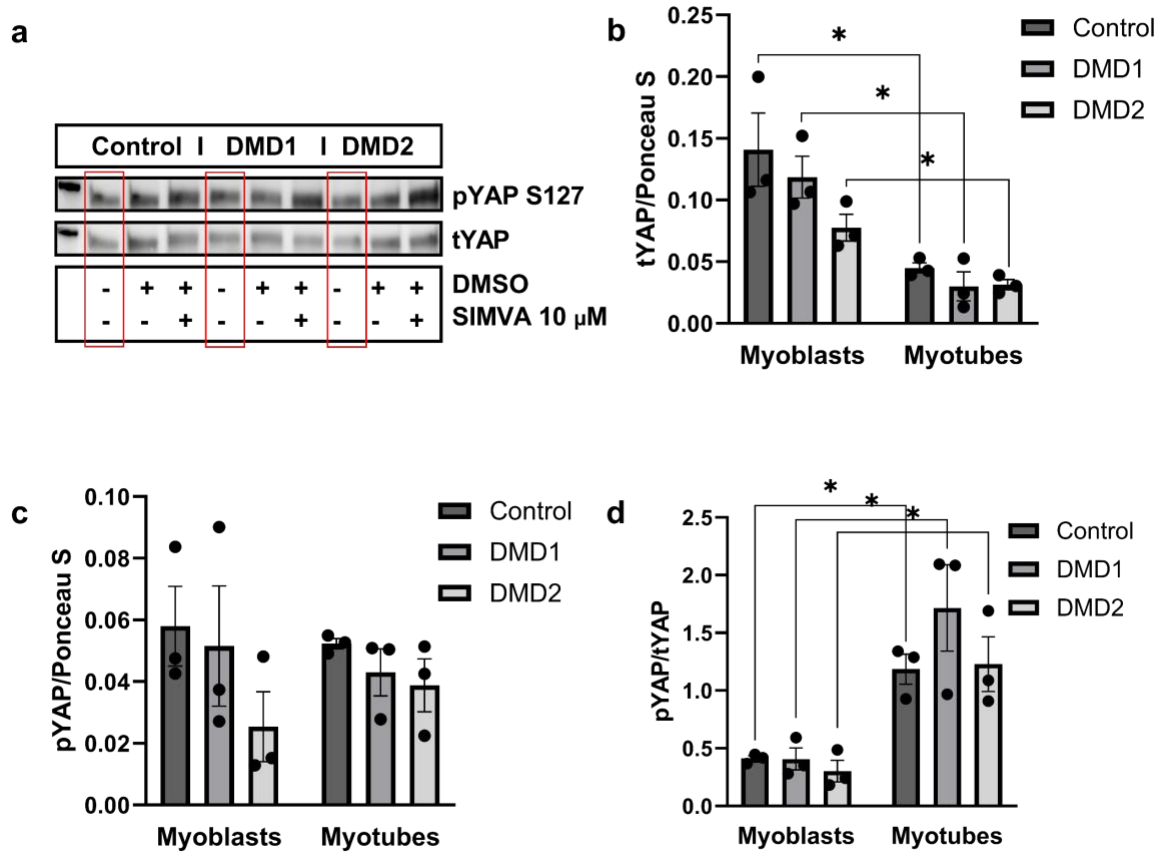


**Figure 11** Total (tYAP) and phospho-YAP (pYAP S127) abundance in healthy and dystrophic myoblasts and myotubes collected at 60-70% confluence or after 8 days of differentiation, respectively. **a** Representative immunoblot showing total and phospho-YAP abundance in 30 µg of protein from whole-cell lysates of myotubes; **b** Quantification of mean grey values suggests little difference in total YAP abundance between cell lines but significantly lower abundance in myotubes compared to myoblasts when normalised to total protein; **c** Quantification of mean grey values suggests little difference in phospho-YAP (S127) abundance between cell lines and between myoblasts and myotubes when normalised to total protein; **d** Quantification of mean grey values suggests little difference in phospho- to total YAP ratio (normalised to total protein) between cell lines but increases in myotubes. n = 3; * $p < 0.05$, two-tailed t-test

Despite little evidence that YAP is constitutively active in human, dystrophic skeletal muscle cells, I sought to determine if simvastatin reduces YAP signalling in these cells. Since cell-cell contact inhibits YAP signalling, I seeded myoblasts at low density, in which YAP is typically active and accumulates in the nucleus. Immunofluorescence microscopy revealed that simvastatin reduced YAP nuclear accumulation in Control (**Figure 12a-f**) and DMD1/2 (**g-r**) myoblasts after 24 hours.

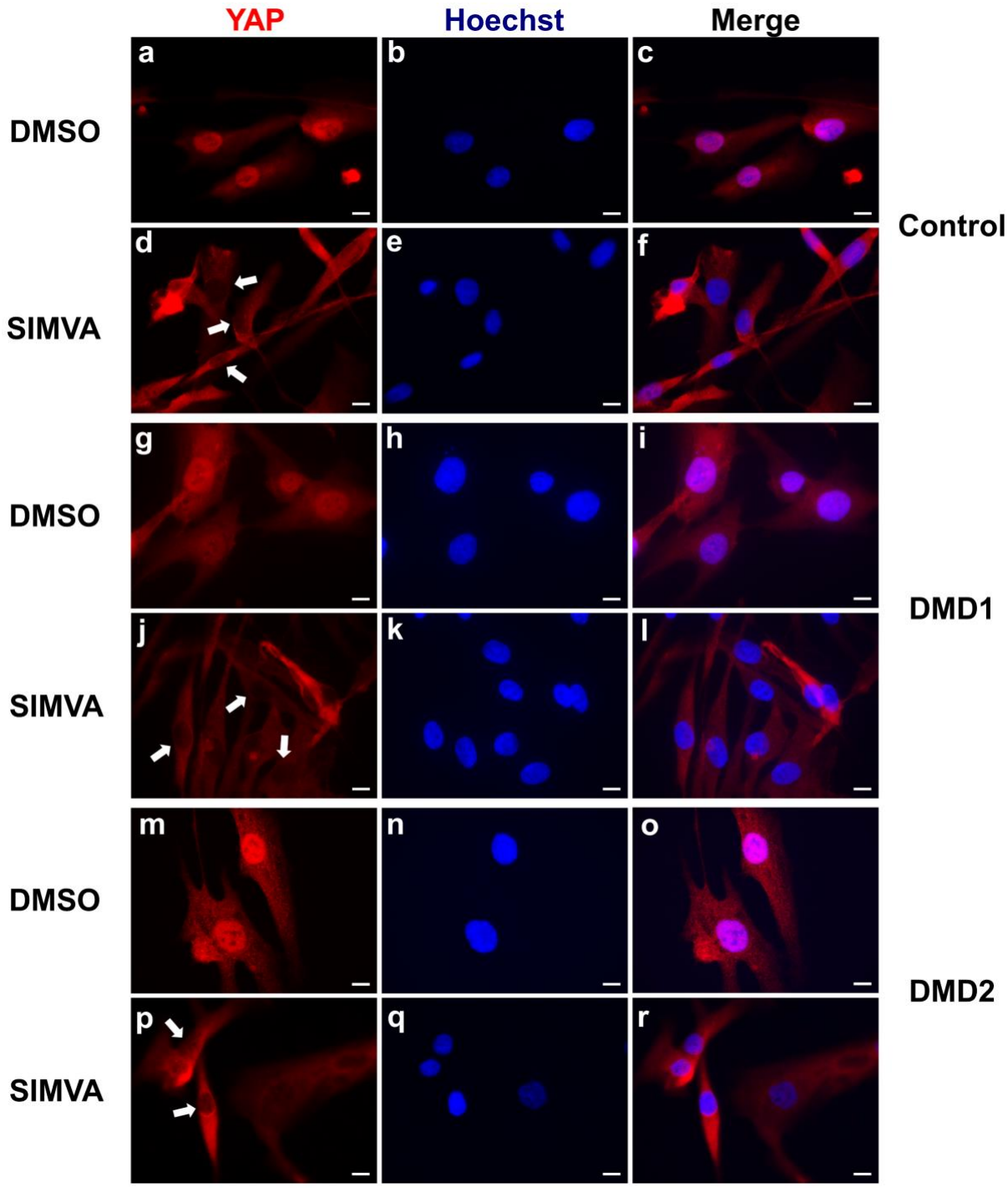


**Figure 12** YAP localisation in healthy and dystrophic myoblasts. Cells were treated with DMSO or 10 µM simvastatin (SIMVA) for 24 hours before fixation. Control (**a-g**), DMD1 (**g-l**) and DMD2 (**m-r**) cells were immunolabelled for YAP (**a**, **c**, **d**, **f**, **j**, **l**, **m**, **o**, **p**, **r**) and nuclei (Hoechst; **b**, **c**, **e**, **f**, **h**, **i**, k**, l**, **n**, **o**, **q**, **r**). Scale bar represents 10 µm

To see whether simvastatin also translocates YAP in myotubes, where I found YAP accumulating in the nucleus and the cytoplasm (**Figure 9**), I differentiated Control, DMD1 and DMD2 myotubes for 7 days and treated them for 24 hours before fixation. As expected, I detected YAP mostly cytoplasmic in the treated condition but nuclear in some myotubes when

treated with DMSO. Together, these results suggest that simvastatin markedly reduced YAP detection in the nuclei of healthy and dystrophic myoblasts and myotubes.

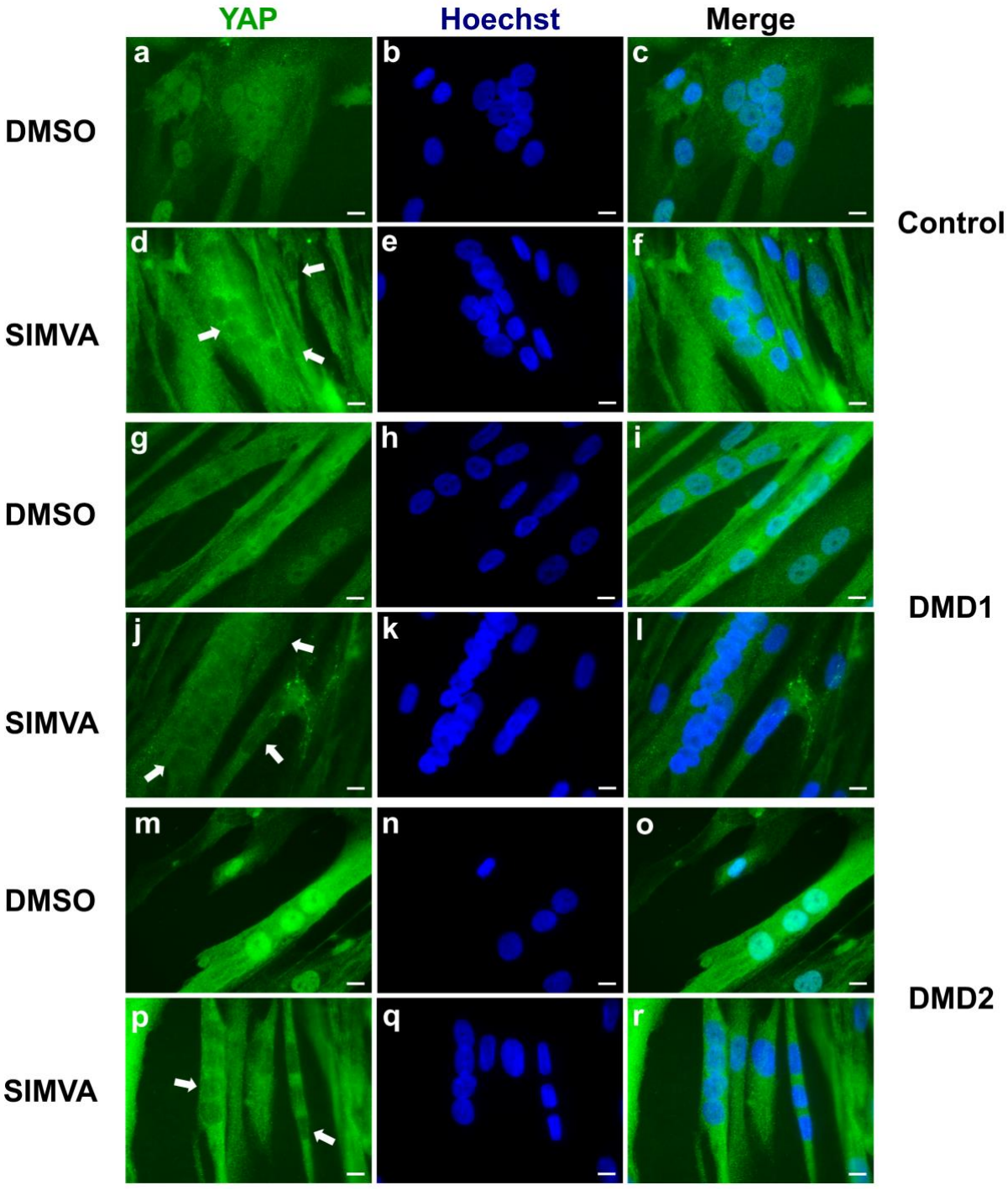


**Figure 13** YAP localisation in healthy and dystrophic myotubes. Cells were treated with DMSO or 10 µM simvastatin (SIMVA) for 24 hours before fixation. Control (**a-g**), DMD1 (**g-l**) and DMD2 (**m-r**) cells were immunolabelled for YAP (**a**, **c**, **d**, **f**, **j**, **l**, **m**, **o**, **p**, **r**) and nuclei (Hoechst; **b**, **c**, **e**, **f**, **h**, **i**, **k**, **l**, **n**, **o**, **q**, **r**). Scale bar represents 10 µm

To examine if these effects correspond to changes in gene expression, I treated myoblasts and myotubes with 10 µM simvastatin or DMSO for 24 hours and measured YAP target gene expression using RT-qPCR (**Figure 14**). Consistent with the literature (Sorrentino et al., 2014), I found that simvastatin decreases *CTGF* by ~79±5% and ~76±6%, *ANKRD1* by ~75±17% and ~59±13% and *CYR61* by ~68±9% and ~76±4% in myoblasts (**a**) and myotubes (**b**), respectively. Moreover, simvastatin reduces the differentiation-associated increase in *CTGF* expression in DMD1 to levels I found for Control in untreated conditions (**c**). This raises the question of whether simvastatin could potentially normalise *CTGF* expression in dystrophic patients.

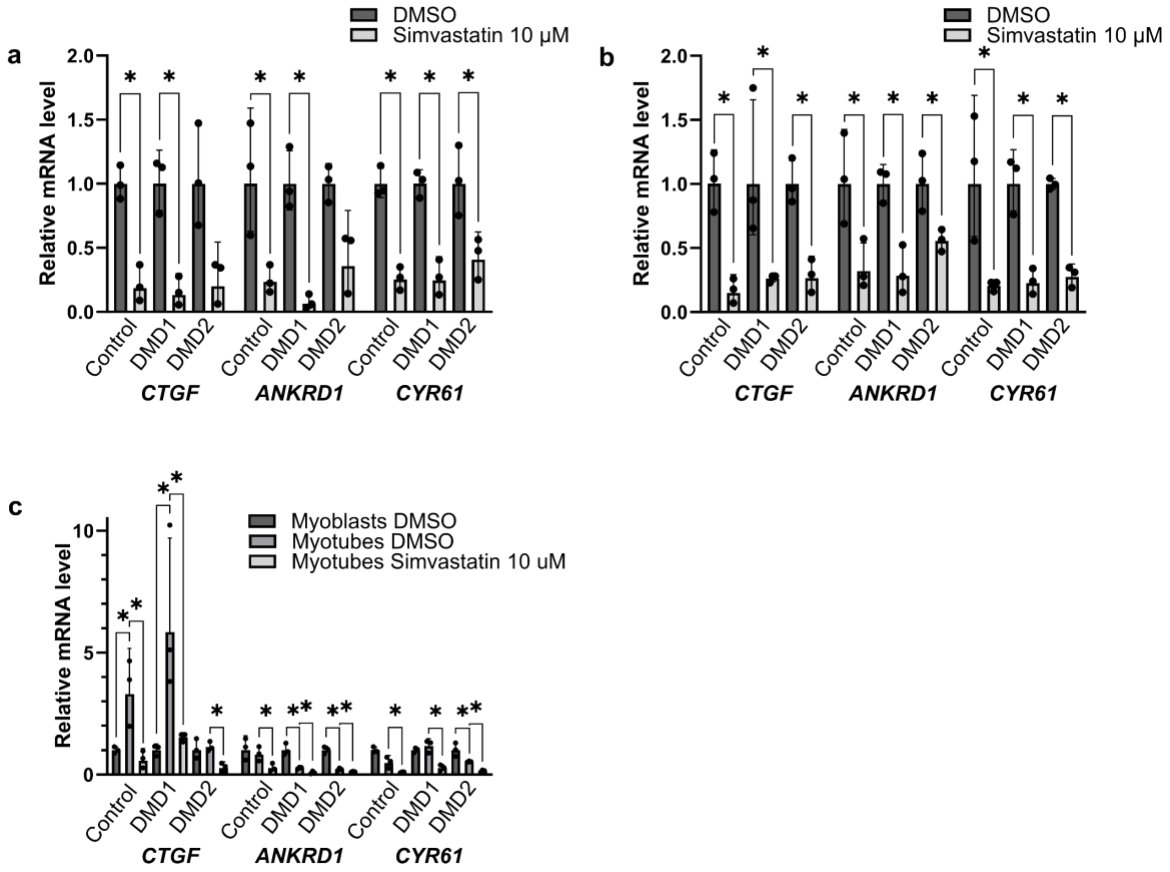


**Figure 14** YAP target gene expression in healthy and dystrophic myoblasts and myotubes. **a** Simvastatin decreases YAP target gene expression by ~ 73% after 24 hours in myoblasts. **b** Simvastatin decreases YAP target gene expression by ~70% after 24 hours in myotubes. **c** Simvastatin decreases the differentiation-associated increase in *CTGF* expression in dystrophic myotubes. * $p < 0.05$, two-tailed t-test

Lastly, I investigated whether simvastatin affects total and phospho-YAP (S127) levels in myoblasts and myotubes of all three cell lines. Again, I treated myoblasts and myotubes with 10 µM simvastatin or DMSO for 24 hours. Incoherent with the finding that simvastatin causes cytoplasmic accumulation of YAP and significantly reduced YAP target expression, simvastatin

had no systematic effect on total or phospho-YAP abundance as determined by Western Blot analysis. This might be explained by the already detectable levels of phospho-YAP.

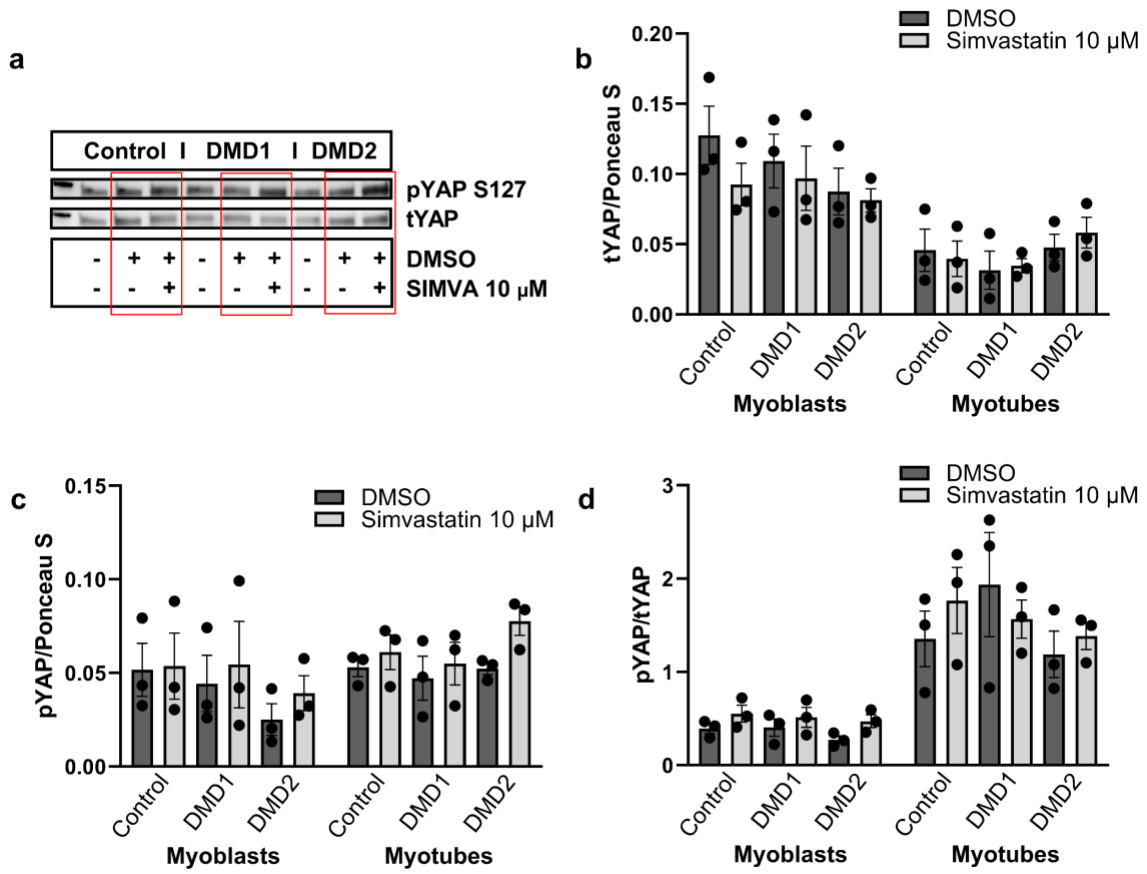


**Figure 15** Total (tYAP) and phospho-YAP (pYAP S127) abundance in healthy and dystrophic myoblasts and myotubes. Cells were treated with DMSO or 10 µM simvastatin (SIMVA) for 24 hours before collection. **a** Representative immunoblot showing total and phospho-YAP (S127) abundance in 30 µg protein from whole-cell lysates of myotubes; **b** Quantification of mean grey values suggests small but non-significant decreases in total YAP abundance in myoblasts but not in myotubes when normalised to total protein; **c** Quantification of mean grey values suggests small but non-significant increases in phospho-YAP (S127) abundance when normalised to total protein; **d** Quantification of mean grey values suggests small but non-significant increases in phospho- to total YAP ratio (normalised to total protein). n = 3; * $p < 0.05$, two-tailed t-test

In summary, I found little evidence that YAP is constitutively active in human, dystrophic skeletal muscle cells *in vitro*. This is limited to the finding that the differentiation-associated increase in *CTGF* expression was considerably higher in DMD1 compared to Control. However, our data suggest that simvastatin potently reduces YAP signalling in human myoblasts and myotubes, including the differentiation-associated increase in *CTGF* expression.

## 3.2 Study 2

In a previous study, Iyer et al. (Iyer et al., 2019) demonstrated that YAP accumulated in the nuclei of mdx muscles and in the cytoplasm of wild-type muscles. To verify that YAP accumulates in the nuclei of mdx muscles, I immunolabelled YAP in cryosections of the same wild-type and mdx muscles that I used for subsequent analyses (**Figure 16**). In gastrocnemius muscles of wild-type mice, I found that YAP did not co-localise with nuclei (**a-d**). However, the gastrocnemius muscles of mdx mice showed substantial overlap of the YAP signal and that of nuclei (**e-h**). Additionally, mdx muscles displayed central nucleation, typical in dystrophic muscles (**g-h**) (Iyer et al., 2019).

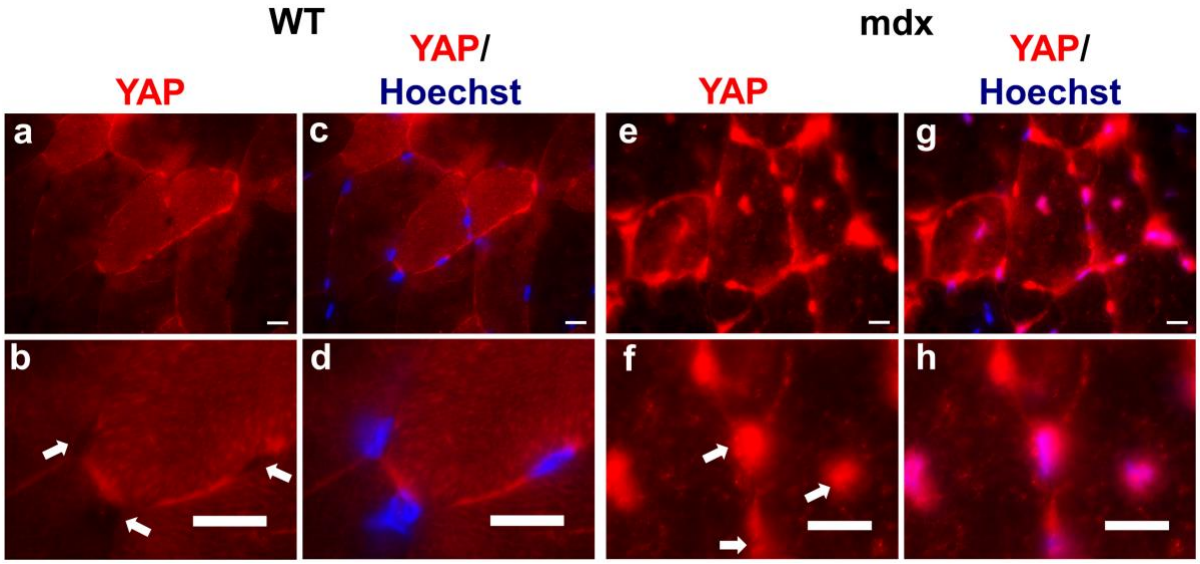


**Figure 16** YAP localisation in wild-type (WT) and mdx gastrocnemius muscles. Gastrocnemius muscles of wild-type (**a-d**) and of age-matched mdx (**e-h**) were snap-frozen, and 10 µm cryosections were labelled for YAP (**a-h**) and nuclei (Hoechst; **c-d**, **g-h**). Scale bar represents 10 µm

Next, I sought to find out whether this translated to YAP target gene expression and found that *Ankrd1* expression is ~5-fold higher in gastrocnemius of mdx mice compared to wild-type mice and a trend that *Cyr61* is ~2-fold higher ($p = 0.065$), as determined by RT-qPCR (**Figure 17a**). Accordingly, Western Blot analysis revealed that total YAP appeared more abundant in mdx than wild-type mice, while there was little difference in phospho-YAP abundance (**b**). Since YAP is typically phosphorylated when cytoplasmic, these results suggest that a larger fraction of YAP is active and nuclear. This is consistent with the finding that YAP target gene expression was partially higher in dystrophic muscles (**a**), but in contrast to a previous study that reported lower levels of phospho-YAP in muscles of mdx than in control mice (Iyer et al., 2019).

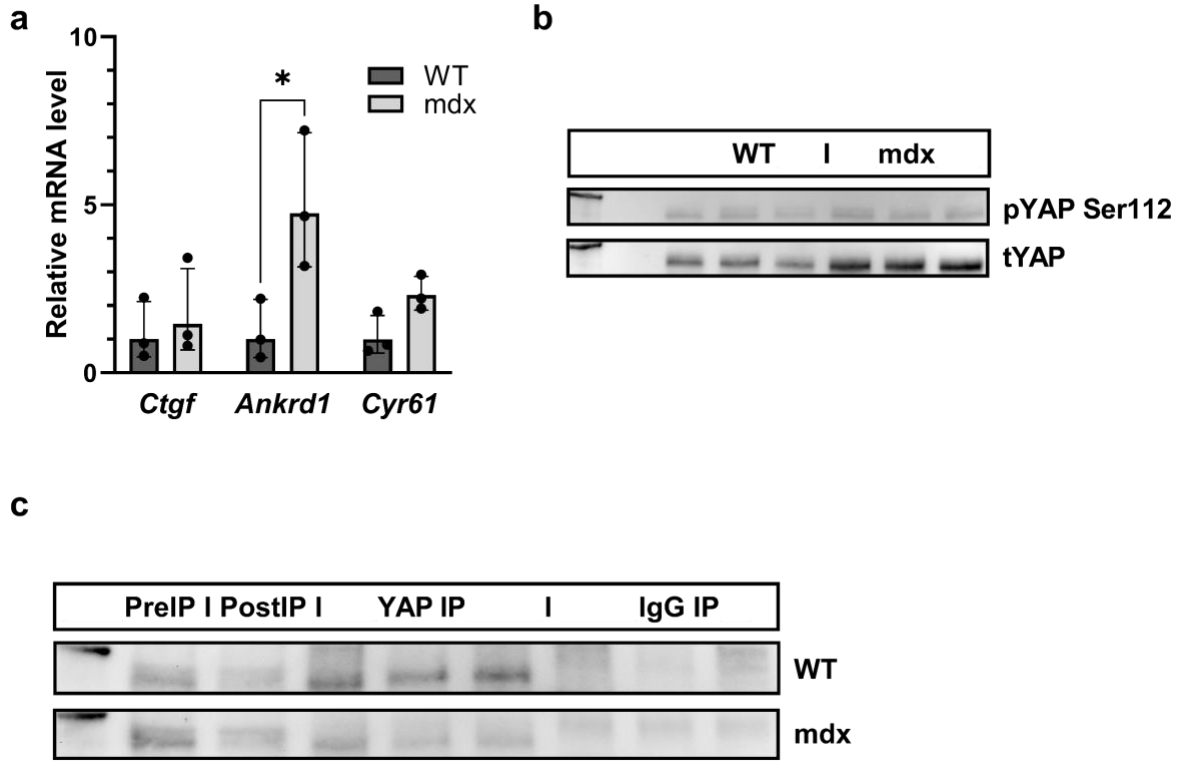


**Figure 17** YAP signalling in wild-type (WT) and mdx gastrocnemius muscles. **a** YAP target gene expression in wild-type and dystrophic muscles determined by RT-qPCR; **b** Immunoblot showing total and phospho-YAP (S112) abundance in 30 µg protein from tissue lysates of wild-type and mdx gastrocnemius muscles; **c** Immunoblot showing YAP abundance in lysates before (PreIP) and after immunoprecipitation (PostIP), and in precipitates using an anti-YAP antibody or normal rabbit IgG of wild-type and mdx muscles. n = 3; * $p < 0.05$, two-tailed t-test

Together, these results indicate that YAP might be dysregulated in the muscles of mdx mice. Finally, I aimed to determine if YAP preferentially binds to members of the DGC in wild-type muscles compared to mdx muscles. I performed immunoprecipitation (**c**) coupled with mass spectrometry and found 38 proteins significantly enriched in the precipitates of mdx muscles. It should be noted that YAP was detectable in the precipitates but was not among the most enriched proteins as determined by mass spectrometry. This might suggest suboptimal immunoprecipitation conditions and increases the likelihood of false positives. In line with the Western Blot analysis of the precipitates (**Figure 17c**), YAP was not detectable by mass spectrometry in IgG control precipitates. After removing proteins that were abundant in IgG control precipitates, 40S ribosomal protein S14 (RPS14), minor histocompatibility protein HA-1 (HMHA1) and serine/threonine-protein phosphatase PGAM5 remained as significantly enriched proteins in YAP precipitates of mdx muscles. RPS14 belongs to the universal ribosomal protein uS11 family, ubiquitously expressed in most tissues and localised to the endoplasmic reticulum. However, single-cell sequencing data of skeletal muscle tissue suggests that *Rps14* expression is higher in T-cells and macrophages than in skeletal muscle

cells. Given that mdx muscles display T-cell and macrophage infiltration (Spencer et al., 1997), this finding should be interpreted cautiously. HMHA1, also known as Rho GTPase-activating protein 45 (ARHGAP45), has been identified as a RhoGAP regulating GTPase activity, cytoskeletal remodelling and cell spreading in cancerous cells (de Kreuk et al., 2013). Mechanical signals, such as ECM stiffness, regulate YAP in a Rho-GTPase-dependent manner. However, little is known about the involvement of Arhgap proteins in this, potentially making binding to HMHA1/ARHGAP45 a novel finding. PGAM5 localises to the outer mitochondrial membrane and is involved in stress response signalling, but no links to YAP signalling exist so far.

Nevertheless, neither did I find that YAP bound to members of the DGC, nor was I able to detect potential binding partners that are exclusively enriched in YAP precipitates of wild-type muscles. This is in contrast to my expectations because the results of the other experiments suggested that YAP might be predominantly cytoplasmic in wild-type but a considerable fraction nuclear in mdx muscles. Collectively, these data provide limited support for the hypothesis that YAP signalling is remarkably different in muscles of mdx compared to wild-type mice.

# 4 Discussion

To my knowledge, these were the first studies to investigate YAP signalling in immortalised, human skeletal muscles from DMD donors and the YAP interactome in skeletal muscles of mdx mice. Here, I demonstrate that there is little support for the hypothesis that YAP is differentially regulated between healthy and dystrophic human myoblasts and myotubes. This is limited to the differentiation-associated increase in *CTGF* mRNA levels in one of the dystrophic skeletal muscle cell lines compared to the healthy control. More importantly, my results suggest that simvastatin is a potent inhibitor of YAP, as reflected in the cytoplasmic accumulation and considerable decreases in the expression of the YAP target genes *CTGF*, *ANKRD1*, and *CYR61*. In the second study, I immunoprecipitated YAP from skeletal muscles of wild-type and mdx mice and identified potential binding partners using a mass spectrometry-based proteomics approach. While I did not detect DGC members or other myopathy-associated proteins as YAP binding partners, I found RPS14, HMHA1 and PGAM5 enriched in the precipitates from mdx but not from wild-type muscles.

In contrast to some of the previous studies (Hulmi et al., 2013; Iyer et al., 2019; Morikawa et al., 2017), biochemical assays of YAP activity in Study 1 revealed no systematic differences between healthy and dystrophic skeletal muscle cells. This might be explained by the heterogeneity in the studied conditions. Two previous studies investigated YAP activity in *in vitro* DMD models using C2C12 cells (Morikawa et al., 2017; Ramirez et al., 2022). Morikawa et al. (2017) mimicked the loss of dystrophin in myotubes by knocking down *Dmd* with siRNA, which led to the enrichment of YAP in the nuclear fraction, reflecting higher YAP activity. Although this study did not address changes in phospho-YAP levels, the decrease in cytoplasmic YAP, where YAP is mostly phosphorylated and inactive, might indicate a converse increase in phospho-YAP levels. I found no significant differences in total or phospho-YAP abundance in myoblasts or myotubes from DMD patients compared to those of a healthy, 16-year-old male subject. The comparison to one healthy subject might be a limitation as the heterogeneity in YAP signalling might differ between healthy individuals. The second *in vitro* study also used C2C12 cells, which lacked dystrophin or expressed dystrophin with mutations associated with DMD or BMD (Ramirez et al., 2022). While the lack of dystrophin or the expression of mutated dystrophin decreased the nuclear/cytoplasmic ratio of YAP, it should be noted that this experiment was conducted in myoblasts only (Ramirez et al., 2022). YAP is expected to be active and nuclear in myoblasts (Watt et al., 2010), but the presumably representative images in the publication clearly show cytoplasmic accumulation of YAP in all conditions (Ramirez et al., 2022). Therefore, comparing these results with the previous study by Morikawa et al. (2017) might be unreasonable. Nevertheless, it remains to be clarified why the loss of dystrophin affected YAP localisation in C2C12 but not in human myotubes. Interestingly, when Iyer et al. (2019) cultured muscle fibres of wild-type and mdx mice *ex vivo*

on culture plates with hard (50 kPa) and soft (1 kPa) substrate stiffness, YAP became only detectable in the nuclei of fibres plated on the hard substrate. In contrast to their *in vivo* results, there was no difference in YAP localisation between wild-type and mdx fibres. This mechanosensitive response is consistent with a more recent study that showed that YAP in C2C12 myoblasts became increasingly detectable in the nuclei with the stiffening of the substrate up to 32 kPa (Silver et al., 2021). Together, these results suggest that the loss of dystrophin might only affect YAP localisation *in vivo*.

The general school of thought is that YAP is active and nuclear in proliferating myoblasts, becomes inactive and cytoplasmic in a confluent monolayer and subsequent differentiation into myotubes (Watt et al., 2010). The cytoplasmic accumulation of YAP is associated with increased YAP phosphorylation (Watt et al., 2010) and declining mRNA levels of target genes like *CTGF* and *CYR61* (Knyazeva et al., 2020). While I found that *ANKRD1* decreased significantly in differentiated myotubes compared to myoblasts in all cell lines, *CYR61* decreased significantly only in Control myotubes. In contrast to the literature, *CTGF* expression increased in myotubes compared to myoblasts of all three cell lines. This increase was more pronounced in DMD1 (~6-fold) compared to DMD2 (~3-fold) and especially to Control (~2-fold). These cell line-specific differences did not correspond to differences in total or phospho-YAP levels. CTGF is also regulated by other pathways, but the finding that simvastatin decreased CTGF expression in myoblasts and myotubes of all three cell lines suggests that this was largely driven by YAP activity.

Considering the putative role of CTGF in the pathogenesis of DMD (Morales et al., 2011a; Morales et al., 2013; Sun et al., 2008a), this finding might be relevant for repurposing already approved drugs like simvastatin (Pushpakom et al., 2019). The use of simvastatin for treating DMD is supported by previous studies (Amor et al., 2021; Bourg et al., 2022; Kim et al., 2019; Whitehead et al., 2015; Xu et al., 2020), but this is the first study to show that simvastatin reduces YAP signalling, including *CTGF* expression, in immortalised human myoblasts and myotubes. The decreases in target gene expression by ~60-80% are consistent with previous findings (Sorrentino et al., 2014). Two other mechanisms have been proposed by which simvastatin might alleviate skeletal muscle pathology in DMD. Firstly, Whitehead (2016) suggested that simvastatin reduces oxidative stress by inhibiting NOX2, thereby promoting autophagy, which is impaired in DMD. As further discussed in this publication, simvastatin might also prevent the prenylation of RAC1, an activator of NOX2, and the autophagy inhibitor mTOR (Whitehead, 2016). Secondly, Amor et al. identified associations with altered cholesterol metabolism in DMD, the primary target of statins. Simvastatin treatment improved muscle pathology and decreased cholesterol abundance in skeletal muscle tissues but not serum cholesterol. Incoherent with the hypothesis that simvastatin improves autophagy by reducing oxidative stress, simvastatin had no effect on NOX2 or LC3b, which the authors

attributed to the young age of the mdx mice. Together, it is conceivable that simvastatin improves muscle pathology through various pathways, but it remains unclear if YAP signalling is also affected *in vivo*. If this were the case, it would open up the possibility of testing other known YAP inhibitors like dobutamine (Bao et al., 2011), forskolin (Yu et al., 2013) and several other GPCR agonists/antagonists (Yu et al., 2012). The use of statins to alleviate muscle pathology might be counterintuitive as statins are suspected to cause myopathy-like symptoms in a small proportion of users (Abd & Jacobson, 2011). However, one might argue that the infrequent symptoms (muscle aches, increased CK levels) are small, considering the severe pathology of DMD. Additionally, a recent systematic review of randomised controlled trials on statin use in 336 children with neurofibromatosis type 1 concluded that statins were well tolerated by children with a mean age of ~11 years (Agouridis et al., 2023). While both young and old mdx mice benefitted from long-term simvastatin treatment (Whitehead et al., 2015), treatment at an early age might be reasonable to partially prevent muscle wasting in DMD.

In contrast to the *in vitro* results in human skeletal muscle cells, I found that YAP colocalised with nuclei in mdx but not wild-type muscle sections, which corresponded to higher total YAP abundance and significantly higher *Ankrd1* expression in mdx muscles compared to wild-type. These results partially align with previous reports on YAP activity in mdx muscles. Consistent with my results, Hulmi et al. (2013) found increased phospho-YAP and an overproportionate increase in total YAP in ~14-week-old mdx mice compared to age-matched wild-type controls but did not assess YAP localisation or target gene expression. YAP hyperactivity was more pronounced in the study by Iyer et al. (2019), where mdx mice of similar age showed increased total YAP and decreased phospho-YAP levels, corresponding to a ~20-fold increase in *Ankrd1* expression versus a ~5-fold increase in my results. It is unlikely that using the tibialis anterior instead of the gastrocnemius caused this, as total and phosphor-YAP levels are similar between these muscles (Watt et al., 2010).

In this first attempt to characterise the YAP interactome in skeletal muscles of wild-type and mdx mice, I could not detect DGC members or other myopathy-associated binding partners. The association with DGC members has previously only been demonstrated in the cardiac muscles of wild-type mice (Morikawa et al., 2017) and in Drosophila muscles (Yatsenko et al., 2020), which was lost in the cardiac muscles of mdx mice (Morikawa et al., 2017). It is not unreasonable to assume that the YAP interactome differs between cardiac and skeletal muscles, given the tissue- and cell-specific functions of YAP, but it is also possible that technical issues contributed to this result. Nevertheless, the mass spectrometry-based proteomics suggest that YAP interacted with RPS14, HMHA1, and ARHGAP45 in mdx but not in wild-type muscles. None of these proteins have previously been associated with the Hippo pathway or DMD.

Several limitations confound the interpretation of these results. While it can be considered a strength that this is the first study to investigate YAP activity in human patient-derived skeletal muscle cell lines, it bears reminding that the Leiden DMD mutation database registers over 4700 different mutations (Aartsma-Rus et al., 2006). It is currently unclear how different mutations affect pathological features like the severity of fibrosis or oxidative stress. Secondly, immortalised cell lines can undergo phenotypic drifts that limit reproducibility (Mamchaoui et al., 2011). It is also unknown whether the immortalisation, including the transfection with *human telomerase reverse transcriptase* (*hTERT*) and *cyclin-dependent kinase 4* (*CDK-4)*, affects Hippo activity.

Co-immunoprecipitation coupled with mass spectrometry-based proteomics is a valid approach to identifying previously unknown protein interactions but is subject to overinterpretation due to false positives (ten Have et al., 2011). For instance, homogenisation decompartmentalises the tissue, which increases the likelihood of proteins interacting with each other that would normally be separated by membranes in intact tissues. Immunoprecipitation from whole-tissue lysates is also biased by the different cell types resident in or infiltrating the given tissue (Englund et al., 2021). This is particularly the case in the mdx muscles used in this study, which are usually infiltrated with different types of immune cells (Bez Batti Angulski et al., 2023). Therefore, the findings of this study should be interpreted cautiously and warrant further investigation.

# 5 Conclusion and Outlook

DMD is the most common and among the most severe genetic muscle diseases caused by various mutations in the *DMD* gene (Hoffman, Brown, et al., 1987). Several studies revealed a wide spectrum of pathological features, including muscle damage, inflammation and fibrosis, leading to loss of ambulation, cardiac failure and early death (Duan et al., 2021). In these studies, I addressed the role of the Hippo pathway member YAP as a potential contributor to some of these pathological features and investigated the effects of simvastatin on YAP activity.

In summary, this is the first study to demonstrate that there is no systematic difference in YAP activity in immortalised skeletal muscle cells from DMD patients versus cells from a healthy donor. However, my results indicate that the fibrosis-driver *CTGF* disproportionally increases in the myotubes of one of the dystrophic cell lines. More importantly, I show for the first time that simvastatin decreases *CTGF* expression and other markers of YAP activity in human skeletal muscle cell lines, suggesting a previously unexplored mechanism by which simvastatin could improve many pathological features. This finding is relevant as simvastatin is a well-characterised, approved drug, which is also tolerated by children (Agouridis et al., 2023), and should now be verified in cells from donors with other mutations.

In the second study, I found supporting evidence that YAP is overactive in the muscles of mdx mice, which warrants further investigation in human DMD patients. However, it is unclear how the finding that YAP associates with RPS14, HMHA1 and PGAM5 exclusively in mdx muscles contributes to understanding the pathogenesis in DMD.

Future studies should now address the efficacy of simvastatin to lower CTGF levels. Technically, CTGF levels can be measured minimally-invasive in serum, but serum CTGF levels are not elevated in DMD patients with a mean age of ~10 years (Alonso-Jiménez et al., 2021). This is surprising because tissue CTGF levels are higher at an earlier age (Sun et al., 2008b). Serum CTGF levels are higher in adult BMD patients (Alonso-Jiménez et al., 2021), which could mean that the dystrophy must have progressed further until CTGF is higher in serum, but no data on serum CTGF in adult DMD patients is available. Therefore, it should first be established that serum CTGF levels reliably indicate the disease state in adult DMD patients to avoid the necessity to take muscle biopsies. Secondly, if simvastatin lowered CTGF levels acutely, it will be important to investigate whether long-term simvastatin treatment reduces fibrosis and other pathological features, as demonstrated in mdx mice (Amor et al., 2021; Bourg et al., 2022; Kim et al., 2019; Whitehead et al., 2015; Xu et al., 2020). Lastly, the notion that the decrease in *CTGF* coincided with the decrease of other markers of YAP activity supports the hypothesis that YAP might be a target to lower CTGF levels in DMD. This extends the therapeutic options beyond statins to several other pharmaceuticals like dobutamine (Bao

et al., 2011), forskolin (Yu et al., 2013) and many other GPCR agonists/antagonists (Yu et al., 2012).

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