SETDB1 modulates the TGFβ response in Duchenne muscular dystrophy myotubes

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In memory of our collaborator and friend Armando Felsani

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Highlights

- TGFβ induces nuclear accumulation of SETDB1 in healthy myotubes
- SETDB1 is enriched in DMD myotube nuclei with intrinsic TGFβ pathway overactivation
- SETDB1 LOF in DMD myotubes attenuates TGFβ-induced pro-fibrotic response
- Secretome of TGFβ-treated DMD myotubes with SETDB1 LOF is less deleterious on myoblast differentiation

SUMMARY

The histone lysine methyltransferase SETDB1 is involved in muscle differentiation and has been shown as a regulator of the TGFβ pathway in cancer contexts. Here, we investigated the role of SETDB1 in Duchenne muscular dystrophy (DMD) myotubes harboring an overactivated TGFβ pathway. Our data show that challenging healthy myotubes with TGFβ induces nuclear accumulation of SETDB1, while in DMD myotubes SETDB1 is constantly accumulated in nuclei in TGFβ-dependent fashion. SETDB1 loss-of-function (LOF) leads to a decrease of the TGFβ downstream target genes in DMD myotubes, suggesting the involvement of a TGFβ/SETDB1 axis in DMD physiopathology. Transcriptomics show that many targets of SETDB1 code for secreted factors involved in fibrosis, extracellular matrix remodeling and inflammation and are downregulated when SETDB1 is silenced. Conditioned medium assays show that SETDB1 LOF in DMD myotubes abrogates the deleterious effect of the secretome on myoblast differentiation and impairs their pro-fibrotic response. Together, our findings indicate that SETDB1 potentiates the TGFβ response in DMD muscles and participates in fibrosis.

INTRODUCTION

Histone lysine methylation/demethylation is a major epigenetic mechanism that controls chromatin states (Mozzetta et al., 2015), with lysine 9 of histone 3 trimethylation (H3K9me3) being a hallmark of repressed chromatin (Padeken et al., 2022). SETDB1 (SET Domain, Bifurcated 1, also called KMT1E, or ESET in the mouse) (Markouli et al., 2021) is one of the H3K9 SUV39 KMT family. SETDB1 regulates many cellular states, such as stemness and terminal differentiation, including skeletal muscle terminal differentiation (Markouli et al., 2021; Mozzetta et al., 2015). Although SETDB1 is mainly nuclear in proliferating myoblasts, where it represses terminal differentiation genes through H3K9me3 deposition, we have uncovered an original mechanism whereby murine Setdb1 is exported to the cytoplasm of differentiating myoblasts in a Wnt-mediated manner, allowing the de-repression of late differentiation genes and the formation of mature multinucleated myotubes (Beyer et al., 2016). SETDB1 is associated with many human diseases including inflammatory bowel disease (IBD) (Juznic et al., 2021), many cancer types (Strepkos et al., 2021), neuropsychiatric, genetic and cardiovascular diseases (Markouli et al., 2021).

Dystrophinopathies are a spectrum of muscle genetic diseases caused by alterations in the *Dystrophin* gene, and Duchenne muscular dystrophy (DMD) is the most severe form, caused by total absence of the coded protein (Hoffman et al., 1987; Mendell et al., 2012). Dystrophin is essential for the maintenance of muscle membrane integrity by providing a strong mechanical link between the actin cytoskeleton and the extracellular matrix (ECM) (Hoffman, 2020; Petrof et al., 1993). Thus, the lack of functional Dystrophin is deleterious to membrane integrity, at the origin of the continuous degeneration of skeletal muscles (Lapidos et al., 2004), leading to exhaustion of muscle stem cells (MuSCs), progressive muscle wasting, chronic inflammation and severe fibrosis (i.e., accumulation of ECM components such as Collagens), which is the most prominent feature of DMD. Fibrosis is defined as pathological wound healing with an abnormal deposition of ECM resulting in the replacement of functional tissue by fibrotic connective tissue, which leads to an alteration of tissue and organ homeostasis (Walton et al., 2017) (Kharraz et al., 2014). Fibrosis involves intracellular and extracellular soluble effectors also called secretome, including pro- and anti-inflammatory cytokines, growth factors and ECM remodelers such as TGFβ, Matrix metalloproteinases

(MMP)/Tissue Inhibitors of MMPs (TIMPs) and Collagens) (Serrano et al., 2011). The main pathway that sustains this excessive pro-fibrotic response is the TGFβ pathway, which is overactivated in DMD (Caputo et al., 2020; Choi et al., 2016).

In DMD patients, TGFβ levels, monitored by the nuclear accumulation of phosphorylated SMAD2/3 (pSMAD2/3) transcriptional modulators, are elevated in both plasma and muscle, and were shown to contribute to pathological fibrosis (Bernasconi et al., 1995). However, the signaling pathways contributing to human DMD fibrosis, and the signals coordinating diverse cell types to maintain or restore muscle tissue homeostasis, remain largely unknown. Yet, TGFβ pathway overactivation is the common feature observed in Dystrophin-deficient myotubes *in vitro* independently of the type of mutation (Caputo et al. 2020; Choi et al. 2016) and exacerbated TGFβ/SMAD pathway has been shown to lead to fusion defects in *in vitro* DMD myotubes. Moreover, TGFβ pathway has been described as a molecular brake of myoblast fusion and, thus, muscle regeneration, while its inhibition leads to the formation of giant myofibers *in vivo* (Girardi et al. 2021).

SETDB1 has been shown to regulate the TGFβ response in cancer and in T-cells (Du et al., 2018; Ryu et al., 2019; Wakabayashi et al., 2011; Wu et al., 2014) and pulmonary fibrosis (Liu et al., 2022) contexts. Here we investigated the SETDB1/TGFβ interplay in a model of DMD myotubes derived from hiPSCs or immortalized myoblasts (Caputo et al, 2020).

We show that SETDB1 contributes to the deregulated TGFβ signaling in DMD, indicating that SETDB1 may participate in muscle dysfunction and increased fibrosis. Indeed, TGFβ induces nuclear accumulation of SETDB1 in healthy myotubes, while SETDB1 is constantly accumulated in DMD myotube nuclei with intrinsic overactivated TGFβ pathway. Moreover, transcriptomics showed that SETDB1 silencing attenuates the TGFβ-dependent pro-fibrotic and anti-differentiation response in DMD myotubes. Interestingly, many TGFβ/SETDB1-dependent genes code for secreted proteins involved in ECM remodeling and inflammation. Conditioned medium assays show that TGFβ-treated myotubes with SETDB1 LOF produce a secretome which reduces the negative impact of TGFβ response on myoblast differentiation and impairs their pro-fibrotic response. Our findings point to a role of SETDB1 in the fine-tuning of the TGFβ response in muscles, which is deregulated in DMD, participating in fibrosis.

RESULTS

SETDB1 relocalizes in the nuclei of healthy differentiated human myotubes in response to TGFβ pathway activation

All our experiments were conducted in human models generated from either human induced pluripotent stem cells (hiPSCs, which we have already described (Caputo et al., 2020), or from immortalized myoblasts derived from healthy or DMD patients (Mamchaoui et al. 2011). hiPSCs can be directly induced to differentiate into myotubes using a transgene based epigenetic reprogramming strategies (Caputo et al. 2020).

We have already showed that in murine myoblasts Setdb1 is both cytoplasmic and nuclear, and during terminal muscular differentiation Setdb1 is exported to cytoplasm in a Wnt-dependent manner, allowing de-repression of muscle genes (Beyer et al., 2016). Thus, we have first investigated, and quantified (as depicted in **Figure S1A**), the subcellular localization of SETDB1 in proliferating *versus* differentiating healthy human myoblasts, and in myotubes in response to TGFβ/SMAD pathway activation (**Figure 1A**). TGFβ/SMAD pathway is constitutively activated in human proliferating myoblasts while nuclear phospho-SMAD3 (p-SMAD3) levels decrease upon differentiation (**Figures 1B, S1B**), as already shown in murine muscle cells (Girardi et al., 2021). Differentiated myotubes are still responsive to TGFβ/SMAD pathway activation since p-SMAD3 nuclear levels are restored upon TGFβ1 treatment (**Figure 1F, S1B**). TGFβ treatment efficiency was evidenced by the activation of many TGFβ target genes concomitant to a decrease in many differentiation genes (**Figure S1D**).

We have confirmed in human muscle cells that SETDB1 is also highly enriched in the nuclei of proliferating myoblasts while it is depleted in nuclei of differentiated myotubes (**Figures 1B, 1C**), as in murine cells (Beyer et al., 2016). Interestingly, TGFβ/SMADs pathway activation in muscle cells is correlated with SETDB1 presence in nuclei (**Figures 1B, 1C**) and we were able to trigger SETDB1 nuclear enrichment in differentiated myotubes after TGFβ1 treatment (**Figures 1B, 1C**), in absence of significant changes in SETDB1 mRNA and protein levels (**Figures 1D, 1E, S1C**). This result was

confirmed in our healthy human hiPSCs-derived model (Caputo et al., 2020) of muscle cells (**Figure S2B** upper panels). We next used the ALK4/5/7 inhibitor SB-431542 in myotubes to block TGFβ pathway activation prior to TGFβ1 addition (**Figure 1G**). The SB-431542 efficiency was evidenced by its effect on phospho-SMAD3 nuclear levels and TGFβ target and *MYOD1* gene expression (**Figures 1H, 1J, S1E**). Autocrine TGFβ signal blocking does not change SETDB1 localization in myotubes in basal condition but blocks SETDB1 nuclear relocalization in response to TGFβ1 (**Figures 1H, 1I**).

Altogether, these data showed that SETDB1 is excluded to the cytoplasm upon terminal differentiation of human myoblasts, and TGFβ pathway activation is directly responsible for SETDB1 nuclear relocalization in myotube nuclei.

SETDB1 is accumulated in DMD myotube nuclei in TGFβ-dependent manner

We next investigated SETDB1 level and subcellular localization in the context of DMD in which the TGFβ pathway is overactivated both *in vivo* (Bernasconi et al., 1995; Yamazaki et al., 1994) and in *in vitro* cellular models (Caputo et al., 2020; Choi et al., 2016).

First, immunohistochemistry assays show that SETDB1 co-localizes with phospho-SMAD3 in the centrally localized nuclei in DMD patient muscle histological slices (**Figure 2A**). To further confirm this observation a more quantitative manner, we efficiently differentiated three healthy myoblasts or hiPSCs lines and three DMD lines carrying different *Dystrophin* mutations (myoblasts with exons 45-52 deletion (**Figure 1B**), myoblasts with point mutation in exon 59 (**Figure S2B**), and hiPSCs with exon 45 deletion (**Figure S2C**)). Compared to healthy differentiated myotubes, DMD myotubes display overactivated TGFβ/SMAD pathway, as depicted by *TGβ1* mRNA expression (**Figure S2A**) and nuclear phospho-SMAD3 protein level (**Figures 2D, 2F,** and (Caputo et al., 2020; Choi et al., 2016)), as expected.

The quantification of SETDB1 nuclear/cytoplasmic ratio in healthy myotubes shows that SETDB1 translocated in the nuclei after 6h of TGFβ1 treatment and this relocalization tends to

decrease after 24h (**Figure 2B-C, S2A**). Interestingly, in DMD myotubes, SETDB1 is accumulated in the nuclei at steady state and is persistent in response to TGFβ pathway activation (DMD exon 45 deletion in myoblasts or hiPSCs, **Figures 2B, 2C**; DMD point mutation, **Figure S2A**) and is associated with a higher activation of *TGFβ1* expression (**Figure S2A**). The effect of TGFβ pathway activation on the subcellular localization of SETDB1 is further confirmed with the blotting of nuclear and cytoplasmic fractions of healthy and DMD myotubes (**Figures 2D, 2E, 2F**) and also in our hiPSC-derived DMD myotubes (exon 45 deletion, **Figure S2C**). We also blocked the autocrine signal of TGFβ in DMD myotubes with SB-431542 and found that it leads to decrease SETDB1 levels in nuclei with and without TGFβ1 treatment (**Figure 2G**). Overall, these data showed an effect of TGFβ on SETDB1 nuclear relocalization in at least 3 different DMD patient myotubes with different *Dystrophin* gene mutations.

Taken together, these data showed that TGFβ induces SETDB1 nuclear enrichment in normal and DMD myotubes, regardless of the genetic background. This suggested that SETDB1 could modulate the cellular response to TGFβ.

SETDB1 loss-of-function attenuates the TGFβ response in human DMD myotubes

To investigate the role of SETDB1 in the TGFβ response, we performed SETDB1 loss-of-function (LOF) in both healthy and DMD myotubes treated or not with TGFβ (as depicted in **Figure 3A**). While the siRNA mediated SETDB1 knockdown (KD, more than 80%) was efficient (**Figures 3B, S3A**), it did not show any effect on the levels of SMAD2/3 proteins nor their phosphorylation levels (**Figure 3B**). However, SETDB1 LOF led to a decrease in the TGFβ response, as depicted by the expression of *ad hoc* TGFβ target genes involved in pro-fibrotic response and inflammation, such as *TGFβ1*, *TIMP1* (*Tissue Inhibitor of Metalloproteinases 1*), *IL6* (*Interleukin 6*), *FN1* (*Fibronectin 1*) and *CTGF* (*Connective Tissue Growth Factor*) **(Figure 3C** and **S3C**). Since these TGFβ target genes are downregulated in SETDB1 KD condition, we hypothesized that in normal condition SETDB1 could target and repress inhibitors of the TGFβ pathway. A gene candidate approach showed that, indeed, SETDB1 LOF led to an increase in *SKIL* and *SMAD7* genes only in DMD (**Figures 3D, 3E,**

S3B), two known inhibitors and fine tuners of the TGFβ pathway. Of note, concomitant to the attenuation of the TGFβ response, SETDB1 LOF in 3-days differentiated myotubes induced a significant increase in muscle early differentiation marker *Myogenin* and regeneration marker *MYH3 (embryonic Myosin Heavy Chain*), but not in the muscle late differentiation marker *MYH1* (**Figure 3F, S3B**).

Thus, SETDB1 LOF attenuates the TGFβ1-induced fibrotic response while promoting regeneration and could be beneficial in DMD patients.

SETDB1 alters the TGFβ regulated secretome in human DMD myotubes

To deepen the role of SETDB1 in the TGFβ response at the global level, we performed RNA-seq comparing DMD myotubes with healthy myotubes in response to TGFβ, with or without SETDB1 LOF. Principal component analysis (PCA) of gene expression levels showed that the triplicates and duplicates were distinctively separated and that the main source of variability between samples are the cell line source, healthy *versus* DMD. The second source seems to be the effect of the TGFβ treatment and SETDB1 silencing (**Figure S4A**).

To track changes in gene expression, first in response to TFGβ, we performed differential gene expression analysis (**Figure 4A**). We identified 1043 TFGβ-dependent differentially expressed genes (DEGs) in healthy myotubes (489 up- and 554 down-regulated genes) and only 480 in DMD myotubes (363 up- and 117 down-regulated genes) (Top genes in **Tables 1** and **2**). These data showed that while the TGFβ pathway is already intrinsically activated in DMD myotubes it is not saturated and can still be further activated *in vitro* (see also **Figure 2 & 3**, and (Caputo et al., 2020)). Interestingly, only TFGβ-dependent 184 TFGβ-dependent genes (corresponding to 18% of healthy and 39% of DMD DEGs) are commonly deregulated in healthy and DMD conditions (**Figure 4A**).

Gene ontology (GO) analysis of the TFGβ-responsive genes in heathy myotubes showed a significant enrichment in terms related to the regulation of MAPK cascade, negative regulation of locomotion, cellular component movement, cell adhesion, SMAD protein phosphorylation, migration,

motility, chemotaxis, vessel and nervous development (**Figure S4B**). While in DMD myotubes, GO showed that the main categories are related to cytokine-mediated signaling, extra cellular matrix (ECM) remodeling and organization, inflammation, SMAD protein phosphorylation and positive regulation of cell motility (**Figures 4B**, **S4C**), reminiscent of disease traits. Collectively, these data validated the responsiveness of both healthy and DMD myotubes to the TFGβ treatment.

Second, we have checked the DEGs in SETDB1 LOF condition, both in healthy and DMD myotubes treated with TGFβ. We found that SETDB1 LOF affected 320 genes in response to TFGβ in healthy myotubes (**Figure 4A,** top genes on **Table 3**). To determine the signature of the transcriptional changes, we applied gene set enrichment analysis (GSEA) and found that the SETDB1-dependent genes belong to ECM and cell surface categories (**Figure S4D**). Together, these data already suggest that SETDB1 LOF affects genes coding for secreted factors.

In DMD myotubes, SETDB1 LOF affected 212 genes in response to TFGβ (**Figure 4A** & **4C**, top genes on **Table 4**), 120 were less abundant and 92 more abundant. In particular, SETDB1 LOF induced less abundant mRNAs associated with ECM remodeling, inflammation and fibrosis, including *THBS1* (*Thrombospondin-1*)*, Myostatin, BMPR2 (Bone Morphogenetic Protein Receptor type 2), ADAMTS8 (ADAM Metallopeptidase with Thrombospondin type 1 motif 8), MMP14 (Matrix Metallopeptidase 14), SERPINE1 (Plasminogen Activator Inhibitor Type 1 or PAI1), and WNT5A* (**Figure 4C**, **4F, S4E**). All these genes, gene categories and pathways are notably involved in the DMD disease traits. Furthermore, enrichment analysis for biological processes and gene network of biological pathway analyses highlighted that the SETDB1-dependent genetic programs in DMD myotubes in response to TFGβ are mainly involved in inflammation, signaling and cell surface (**Figure 4D-E**).

Altogether, here we validated the efficacy of TGFβ to induce fibrotic and inflammation genetic programs and highlighted that SETDB1 silencing alters the transcription of many secreted factors (secretome) and ECM components, attenuating the deleterious effect of TGFβ overactivation in DMD myotubes.

SETDB1 LOF in myotubes has a beneficial impact on the TGFβ−induced secretome: it improves muscle differentiation and reduces fibrosis

So far, our data showed that SETDB1 LOF in myotubes could modulate the TGFβ effect on environing cells and affect muscle regeneration. To test this, we produced conditioned medium (CM) in healthy or DMD myotubes with or without SETDB1 LOF, treated or not with TGFβ (**Figure 5A**). Then, CM from healthy myotubes was applied on confluent healthy myoblasts, and CM from DMD myotubes on DMD myoblasts (**Figure 5A**).

As a control, we first checked whether CM could impact myoblast differentiation as compared to fresh differentiation medium and found no changes (**Figure S5A-C)**. Thus, receiving myoblasts treated with fresh medium or CM supplemented with TGFβ1 showed a less efficient differentiation (**Figure S5A-C)**. In absence of medium change, CM from TGFβ-treated myotubes completed impaired fusion, as expected, meaning that TGFβ1 is not consumed by the cells and remain high in the CM and though hide the myotube response (Girardi et al., 2021).

We then included medium change after acute TGFβ1 treatment to study the intrinsic myotube pro-fibrotic response (**Figure 5A**). Our results showed that DMD myoblasts, but not healthy ones, have a decreased fusion index when differentiated with CM from TGFβ-treated myotubes, but myoblasts incubated with CM from TGFβ-treated and SETDB1 LOF myotubes show a more normal fusion index (**Figures 5B, 5C**).

The discrepancy between the effects of the CM in healthy versus DMD myoblasts could be due to the higher differentiation potential of the healthy myoblasts (**Figure S5D**).

Interestingly, the receiving myoblasts incubated with CM from TGFβ-treated and SETDB1 LOF myotubes showed a significant decrease in fibrotic markers *SERPINE1* and *MSTN* (**Figure 5D**). Concomitantly, they displayed an increase in differentiation markers expression such as *MYOD1*, *Myogenin*, *MCK* (*Muscle Creatine Kinase*) and *MYH1* (**Figures 5E, 5F**).

Together, these data show that the response of myofibers to TGFβ pathway activation could be attenuated by targeting SETDB1 in DMD patients. Thus, SETDB1 LOF in myofibers could be

beneficial for the myofibers themselves but also to the differentiation of the surrounding myoblasts and potentially limit fibrosis.

DISCUSSION

Tissue repair, such as muscle regeneration after injury, involves many cell types which communicate together through secreted molecules, the so-called secretome, to orchestrate replacement of damaged cells with new functional ones and re-establish tissue homeostasis. Thus, in addition to their contractile properties, myofibers have also a central role in cell-cell communication since they are able to secrete the so-called myokines especially during regeneration and differentiation, including dedicated vesicles acting on muscle adaptation to damage or exercise (Bittel and Jaiswal, 2019). This myofibers secretome plays important roles in intercellular communication of the muscle resident cells, i.e., MuSCs, myofibers themselves, the fibro-adipogenic progenitors (FAPs) and macrophages (Le Bihan et al., 2012).

Although FAPs and macrophages have been described as major TGFβ sources, and as fibrosis and inflammation effectors, myofibers are also responsive to TFGβ1 and have been shown to participate in TGFβ/SMAD response during muscle regeneration *in vivo* (Accornero et al., 2014; Hillege et al., 2022). TGFβ-induced fibrosis is a key pathological feature in muscle disease, such as DMD, and TGFβ overactivation worsens muscle degeneration by preventing proper repair. Moreover, even though myoblasts display a TGFβ/SMAD autocrine signal required for proliferation, TGFβ pathway prevents myoblast fusion by controlling actin remodeling and its activation decreases upon muscle differentiation. Interestingly, DMD muscle cellular models have been shown to recapitulate TGFβ/SMAD overactivation pathological feature *in vitro* associated with muscle defects and fibrosis (Caputo et al., 2020; Choi et al., 2016). Hence, targeting TGFβ-induced pro-fibrotic response in DMD to slow down disease progression appears as a promising therapeutic approach.

TGFβ signaling nuclear endpoint mediated by the two major TGFβ downstream transcription factors SMAD2 and SMAD3, also involves many chromatin-modifying enzymes including SETDB1.

SETDB1 regulates different cell fates including stemness and terminal differentiation (Padeken et al., 2022). SETDB1 is also involved in fibrosis (Liu et al., 2022), inflammatory response and diseases (Hachiya et al., 2016; Johnson et al., 2023; Juznic et al., 2021; Wang et al., 2020) and has been linked to TGFβ response in many non-muscle contexts (Du et al., 2018; Ryu et al., 2019; Wakabayashi et al., 2011; Wu et al., 2014). SETDB1 has been previously described by us and others as a major negative regulator of muscle terminal differentiation through repression of muscle gene expression in myoblast nuclei (Beyer et al., 2016; Song et al., 2012; Song et al., 2015). SETDB1 is responsive to Wnt signaling that is known to have a pro-differentiation effect on myoblasts and causes SETDB1 export to the cytoplasm allowing muscle gene de-repression. Interestingly, SETDB1 has also been shown to be a regulator of TGFβ/SMAD pathway in cancer (Du et al., 2018; Ryu et al., 2019; Wakabayashi et al., 2011; Wu et al., 2014) and pulmonary fibrosis (Liu et al., 2022) contexts. In this study, we have shown that SETDB1 nuclear localization follows TGFβ/SMAD activation in human muscle cells. SETDB1 is exported to the cytoplasm during muscle terminal differentiation, while TGFβ/SMAD pathway activation decreases, but it can be transiently relocalizes in myotube nuclei in response to TGFβ pathway activation.

In the effort to identify new key players of TGFβ response whose dysregulation is a key driver of DMD progression, we hypothesized that SETDB1 could participate in TGFβ response in muscle and, though, could be involved in deregulation of TGFβ pathway in DMD. We found that SETDB1 is constitutively accumulated in DMD myotube nuclei correlating with phospho-SMAD3 high nuclear levels *in vivo* and *in vitro*. As expected, DMD myotubes are more responsive to TGFβ activation as compared to healthy ones. Interestingly, we were able to block SETDB1 nuclear localization by inhibiting intrinsic autocrine TGFβ signal in DMD myotubes. We also performed a loss-of-function of SETDB1 and studied TGFβ-induced pro-fibrotic response. We found that healthy but especially DMD myotubes lacking SETDB1 display an attenuated response to TGFβ/SMAD pathway activation without changing phospho-SMAD3 levels. These results point to unprecedented link between SETDB1 and TGFβ response in muscle, and more especially in DMD context. Nonetheless, the exact mechanisms through which SETDB1 localization is controlled by either TGFβ or Wnt pathways are not yet understood.

To better understand how SETDB1 impacts TGFβ response in myotubes, we performed global transcriptomic analysis on healthy and DMD myotubes. We found that SETDB1 loss-of-function leads to a global decrease of RNA levels of gene coding for factors involved in ECM remodeling, inflammation and TGFβ and Wnt pathway signaling that respond to TGFβ. Since SETDB1 is known for its repressive action on gene expression, we made the hypothesis that SETDB1 could repress TGFβ inhibitors as already described in cancer context (Ryu et al., 2019). Interestingly, we found that SETDB1 loss-of-function leads to an increased expression of the TGFβ inhibitor genes *SKIL* and *SMAD7*. The precise mechanism through which SETDB1 controls the expression of these genes remain unclear. Chromatin immunoprecipitation assays on SETDB1 and H3K9me3 marks would be required to confirm that SETDB1 represses TGFβ inhibitor expression but remain challenging in myotubes.

Finally, since most of SETDB1 direct or indirect targets are coding for secreted factors involved in fibrotic response and that TGFβ is known for its anti-differentiation role, we tested the effect of myotube secretome on myoblasts. Moreover, DMD muscle cells display fusion defects in some DMD models of hiPSC-derived myotubes and our model of immortalized myoblast-derived myotubes. Here, TGFβ-treated myotube secretome negatively impacts myoblast differentiation and more specifically fusion, but SETDB1 LOF in DMD myotubes has a beneficial cell non-autonomous effect on myoblast fusion. We show here the effect of SETDB1 on myotube secretome that affects environing cells. Targeting SETDB1 in post-mitotic myotubes does not seem to have deleterious effect on cell viability but its targeting in muscle tissue would be challenging since we do not know the impact on other cell types such as FAPs and macrophages. Nevertheless, we highlight interesting, secreted targets displaying aberrant expression in DMD myotubes that could play a role in deleterious environment of DMD. Moreover, some of deregulated targets have an unknown function such as *ANKRD33B* but could be involved in muscle regeneration process.

In summary, this study highlights the role of SETDB1 in post-mitotic muscle cells in adaptation to the environment cues. Even though the mechanism involved in SETDB1 regulation remains elusive, we unraveled a functional effect of its LOF on muscle cell differentiation and shed light on gene networks under SETDB1 control that would be worthy to study individually in the context

of DMD. At last, we did not investigate Wnt and TGFβ interaction in this study but it seems that SETDB1 acts as a mediator of these pathway communication in muscle. Even though Wnt and TGFβ are described as having opposite effects on muscle terminal differentiation, some studies also described their collaboration in muscle (Biressi et al., 2014; Rudolf et al., 2016). Here, we showed *in vitro* the cell non-autonomous effect of SETDB1 inhibition in myotubes on the surrounding myoblast differentiation through the control of secreted factor expression induced by TGFβ/SMAD pathway. Nevertheless, it is unclear if this effect could be beneficial *in vivo* during regeneration since some of the targets have been described in literature for their double-edged effect on muscle regeneration and this could depend on the cell type involved. It is likely that a fine-tuned balance has to be established in muscle tissue to allow a proper regeneration and SETDB1 could be involved in this mechanism by controlling muscle differentiation, but also by regulating myofiber response to environmental cues.

METHODS

Establishment of stable cell lines and cell culture

The human induced pluripotent stem cell lines, control and DMD, were obtained from Coriell. iPSC lines were generated by Sendai Virus reprogramming (SBP Lake Nona). IPSCs were maintained in culture in mTesR1 medium and dissociated using ReLeSRTM (StemcellTM). They were transfected with epB-Puro-TT-mMyoD and epB-Bsd-TT-FlagBaf60c2 by electroporation using the Neon Transfection System as described in Caputo et al., 2020. Selection was performed using 5 µg/ml of Puromycin and 10 µg/ml of Blasticidin at the same time.

Human control and DMD immortalized myoblasts were obtained from AFM-MyoBank. Immortalization was performed as described in: (Mamchaoui et al., 2011), using human telomerase-expressing and cyclin-dependent kinase 4-expressing vectors. They were cultured on Gelatin-coated plates and propagated in DMEM high glucose GlutaMAX™ (Invitrogen, 61965-026)/Medium 199 (Invitrogen, 41150020) 4:1 mixture, supplemented with 20% FBS (Sigma, F7524), 50 µg/mL Fetuin, 5 µg/mL insulin, 0.5 ng/mL bFGF, 5 ng/mL hEGF and 0.2 µg/mL Dexamethasone (Sigma D4902).

Cells	*Reference*	*Patient*
Induced pluripotent stem cells (IPSCs)	AG08C5 Coriell	Skin fibroblast, 1 year old healthy male
	GM25313	Skin fibroblast, 13-year-old DMD male patient, deletion ex45
Immortalized myoblasts (hTERT/CDK4) (hMB)	AB1190	Paravertebral, 16-year-old male
	AB1167	Fascia lata, 20-year-old male
	AB1023DMD11Q	Quadriceps, 11-year-old male
	AB1071DMD13PV	Paravertebral, 13-year-old male

Myogenic differentiation of iPSCs and human immortalized myoblasts

iPSCs stably expressing MyoD and Baf60C Tet-ON inducible transgenes were propagated in mTeSR1 on Matrigel-coated wells. To induce myogenic differentiation starting from iPSC colonies, doxycycline (200 ng/mL) was added in cells maintained in mTeSR1 (day 0). After 24 h of treatment with doxycycline (day1), cells were dissociated as single cells using TrypLE and plated 25.10^3/cm2 in growth media (GM) (knockout DMEM (Invitrogen) supplemented with 1 mM L-glutamine, 20% knockout serum replacement medium (KOSR, Invitrogen), Glutamax 1X, 0.1 mM nonessential aminoacids (NEAA, Invitrogen), 50 U/mL streptomycin (Invitrogen) plus hES cell recovery supplement (10 μM) (Stemgent) and doxycycline. On day 3, GM medium was switched to differentiation media (DM) (knockout serum-free DMEM containing 1X Insulin-Transferrin-Selenium (ITS) (SIGMA)) supplemented with doxycycline until day 7.

Human control and DMD immortalized myoblasts were grown at > 80% confluence and the medium was switched for DM media.

Immunofluorescence on cells and tissue

Muscle histological slides were provided by the Institute of Myology and arised from muscle biopsies of 15-years old DMD patient paravertebral muscle and and a healthy 17-years old individual. Frozen samples were fixed in ice-cold acetone for 1 min and then air-dried. They were blocked in 4%BSA PBS solution for 45 min at room temperature and then incubated with the primary antibodies at 4°C

overnight. Secondary antibodies were applied for 45 min at room temperature in the dark before mounting.

Cells were grown on Matrigel- and Gelatin-coated coverslips for iPSCs and human myoblasts respectively. They were fixed with 4% PFA for 20 min and they were saturated with 50 mM NH4Cl for 10 min at room temperature. Then, they were permeabilized with 0,5% Triton X-100 for 5 min. Cells were washed 3 times with PBS between each steps. Cells were next incubated with primary antibodies at the concentration indicated in the antibody table below. Alexa-488 or 555 or 647 were used as secondary antibodies (Invitrogen). Nuclei were counterstained with DAPI.

Images were acquired with a Leica DMI-6000B microscope and analysis was performed using Fiji software. In-house macro was used to performed nuclear and cytoplasmic signal quantification. Acquisition of iPSCs-derived myotubes was performed using ImageXpress Micro High Content Screening System. All the conditions were acquired with identical settings and were analyzed with an in-house macro on ImageJ/Fiji to quantify nuclear and cytoplasmic signal.

RNA and Quantitative Reverse Transcription-PCR (RT-qPCR)

Total RNA was extracted using RNeasy micro-kit (Qiagen) following manufacturer's procedures. DNase (Qiagen) treatment was performed to remove residual DNA. 1 μg of total RNA was reverse transcribed with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time quantitative PCR was performed to analyze relative gene expression levels using SYBR Green Master mix (Applied Biosystems) following manufacturer indications. Relative expression values were normalized to the housekeeping genes mRNA *PPIA* or *UBC*. Primers are listed in the Table below.

Nuclear and cytoplasmic fractionation

Cells were scraped directly in 3 volumes of buffer A (20 mM HEPES pH7.0, 0.15 mM EDTA, 0.15 mM EGTA, 10 mM KCl) supplemented with 0.15 mM Spermine, 0.5 mM Spermidine, protease inhibitor cocktail 1X (SIGMAFAST™) and phosphatase inhibitor cocktail 1X (Sigma). Cells were lysed with 0.5% NP-40 and mixed gently by inversion. 0.88 volume of buffer SR (50 mM HEPES

pH7, 0.25 mM EDTA, 10 mM KCl, 7% sucrose) supplemented with Spermine, Spermidine, protease and phosphatase inhibitor cocktails was added before centrifugating the cells for 5 min at 2000 x g at 4°C and the supernatant was collected as the cytoplasmic fraction. The core nuclei pelleted at 2000 x g was resuspended in 1 volume of Low Salt buffer (20 mM Tris pH7.65, 25% glycerol, 1.5 mM MgCl2, 0.2 mM EDTA, 20 mM NaCl) with 3X of protease and phosphatase inhibitor cocktails. One volume of High Salt buffer (same as Low Salt but with 900 mM NaCl) supplemented with 5 mM ATP while vortexing. The nuclei samples were kept on ice for 30 min and mix by inversion every 5 min. 1 volume of sucrose buffer (20 mM Tris pH7.65, 60 mM NaCl, 15 mM KCl and 0.34 M sucrose) was added before treating the cells with 0.0025 U/μL of MNase for 10 min at 37°C and 1 mM CaCl2. Reaction was stopped with 4 mM EDTA. Samples were next sonicated for 10 min and ultracentrifugated at 40000 rpm at 4°C for 30 min. Supernatant was collected as nuclear fraction.

Western blot

Cells were lysed in RIPA buffer (20 mM Tris pH 7.65, 150 mM NaCl, 0.1% SDS, 0.25% NaDoc and 1% NP-40) supplemented with protease inhibitor 1X (SIGMAFAST™) and phosphatase inhibitor 1X (Sigma) and kept on ice for 30 min. Cell lysates were sonicated at 4°C for 10 min (30 sec ON, 30 sec OFF) at medium frequency (Bioruptor Diagenode). Then, the lysates were centrifugated for 10 min at 4°C at maximum speed and the supernatants were kept as the samples. Extracts were resolved on pre-cast polyacrylamide gel cassettes (NuPAGE® 4-12% Bis-Tris) (Invitrogen) and 1X NuPAGE MOPS SDS Running Buffer and transferred into nitrocellulose membrane (Amersham) in 20 mM phosphate transfer buffer (pH 6.7). Membrane was blocked in 5% skim milk in PBST Buffer (1X PBS, 0.2% Tween 20) and incubated overnight at 4°C with the primary antibody (see Table of antibodies). Membranes were washed twice 5 minutes in PBST, incubated with appropriate secondary antibody IRDye® (Li-Cor) in PBST, washed twice 10 minutes in PBST, once 10 minutes in PBS and then imaged on Odyssey® Imaging System.

Transcriptome analysis and bioinformatics

RNA was isolated as described above. Two or three independent biological replicates were sequenced depending on the cell conditions. Libraries were generated using the Ion AmpliSeq™ Transcriptome Human Gene Expression Kit (A24325; Ion Torrent™). Sequencing was performed on an Ion S5 sequencer (Ion Torrent™). The reads were analyzed using the plugin AmpliseqRNA of Torrent Suite (release 5.10) and mapped on the panel of human AmpliseqRNA which reveals the expression of 20813 genes. Out of these 18185 genes were uniquely mapped on human reference gene names (retrieved from ENSEMBL_102 release).

Using the raw count table generated, genes having low counts were filtered out based on the log2 of Counts Per Million (logCPM). We kept genes that have at least a 1 logCPM mean expression level in at least one of the experimental conditions, leaving 12 060 expressed genes for all the downstream analyses. The differential expression analysis of the filtered data was performed using the edgeR package of R and the limma-trend method described in (Law et al. 2016 https://doi.org/10.12688%2Ff1000research.9005.3). For each experimental setting Differentially Expressed Genes (DEGs) were further analyzed for all the available enrichments. Enrichment analysis and visualization of the DEGs were performed using the clusterProfiler R package (Wu, et al. 2021 https://doi.org/10.1016/j.xinn.2021.100141) using Gene Ontologies, DOSE, the MSigDB database and GSEA (Liberzon, et al. 2015 https://doi.org/10.1016/j.cels.2015.12.004).

Statistical analyses

Statistical analyses were carried out using Excel and R. Data are represented as mean +/- SEM as described in the figure legend. Graphs are prepared using Excel and R. Double tail *t* test was used for statistical analysis, *$*p<0.05$, $**p<0.01$, $***p<0.001$*.

Antibodies

Antibody	**reference**	**Use & dilution**
SETDB1 (rabbit)	Santacruz sc-66884	Immunofluorescence (1:200)
SETDB1 (mouse)	Abcam ab107225	Western Blot (1:1000)

SETDB1 (mouse)	Thermofisher MA5-15722	Immunofluorescence (1:200)
Myosin heavy chain, MYH1E	DSHB	Immunofluorescence (1:10), Western blot (1:200)
Phospho-SMAD3 (rabbit)	Abcam ab52903	Immunofluorescence (1:200), Western Blot (1:1000)
SMAD3 (mouse)	Proteintech 66516-1-Ig	Western Blot (1:1000)
PAI1 (rabbit)	Proteintech 13801-1-AP	Western Blot (1:1000)
RNA polymerase II (rabbit)	Abcam ab5095	Western Blot (1:1000)
Lamin A/C (rabbit)	Proteintech 10298-1-AP	Western Blot (1:1000)
H3 (C-16) (goat)	Santacruz sc-8654	Western Blot (1:1000)

Primers

Targets	**Forward**	**Reverse**
SETDB1	CAATACCGGGACAGTAGCTC	TCTGGTCTTTTGGAGTTCTGC
TGFB1	GCCTGAGGCCGACTACTA	CTGTGTGTACTCTGCTTGAACT
TIMP1	TTCTGCAATTCCGACCTCG	TCATAACGCTGGTATAAGGTGG
IL6	TAGTGAGGAACAAGCCAGAGC	TGGGTCAGGGGTGGTTATTG
SKIL	GCCCCAAATGTGTCACTTAC	TCCCACTTTGTTTATGTCTCTGA
SMAD7	GTGTTGCTGTGAATCTTACGG	TCGGGTATCTGGAGTAAGGAG
MYOGENIN	AATGCAGCTCTCACAGCGCCTC	TCAGCCGTGAGCAGATGATCC
MYH1	CTGTTGCAGTTTCTCATTGGTG	CCAGGCAGTACTTCATTGGG
MYH3 (embryonic)	CAAGAGTTCTCAGGATGGGAAG	GCCATGTCTTCGATCCTGTC
FN1	AGCCGAGGTTTTAACTGCGA	CCCACTCGGTAAGTGTTCCC
CTGF	TGTGCACCGCCAAAGAT	GCACGTGCACTGGTACTT
SERPINE1	TCCACAAATCAGACGGCAGC	TCGTAGTAATGGCCATCGGG
THBS1	CTGGCCCAATGAGAACCTGG	GCCCTGAGTTGGGAAGGTTG
LIF	CCAACTGGCACAGCTCAATG	CTTGTCCAGGTTGTTGGGGA

MSTN	AGCGATGGCTCTTTGGAAGAT	TTTGGGTTTTCCATCCACTTGC
BMPR2	GCAGGTTCTCGTGTCTAGGG	CCTGGTCCCAACAGTCTTCG
WNT5A	GCAGCACTGTGGATAACACC	GCTCACCGCGTATGTGAAGG
ADAMTS8	GCAGCGCCATGTATCTCACA	TGTGGGGAGGGGCAGG
ANKRD33B	GGGGAACACAGCCCTAATCA	GCGTTCCTCCTTTCAAGGTCA
MMP14	GGCTGCCTACCGACAAGATT	GCCCTGAGCTCTTCGTTGAA
MYOD1	TGCTCCGACGGCATGATGGACTA	TTGTAGTAGGCGCCTTCGTAGCAGTT
MCK	TGGAGAAGCTCTCTGTGGAAGCTC	TCCGTCATGCTCTTCAGAGGGTAGTA

Data and Code Availability

Data corresponding to all the experiments described in this study are available upon request.

SUPPLEMENTAL INFORMATION

5 supplemental figures and 4 Tables are provided.

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AUTHOR CONTRIBUTION

Conceptualization, S.A.S and S.A; Methodology A.G, M.Z, L.D.M, C.B, V.J, S.A and S.A.S. Software, C.B.; Formal Analysis, C.B and A.G; Investigation, A.G, M.Z, L.D.M, C.B, V.J, S.A and S.A.S; Data Curation, C.B.; Writing – Original draft, A.G and S.A.S; Writing – Review & Editing, A.G, M.Z, L.D.M, C.B, V.J, S.A and S.A.S; Supervision, S.A and S.A.S; Funding Acquisition, S.A.S; Project Administration, S.A.S.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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FIGURE LEGENDS

Figure 1: SETDB1 is excluded from myotube nuclei upon differentiation and can be relocated in response to TGFβ pathway activation

A. Scheme of experimental design. Cells were collected in proliferating phase (myoblasts) or after 3 days of differentiation (myotubes) with or without TGFβ1 treatment at 20 ng/mL.

B. Immunostaining of SETDB1 (red) and phospho-SMAD3 (green). Nuclei were stained with DAPI (blue). Scale bar, 10 μM.

C. Quantification of SETDB1 nuclear/cytoplasmic signal ratio.

D. RT-qPCR of *SETDB1* in healthy proliferating myoblasts and differentiated myotubes +/- TGFβ1.

E-F. Quantification of SETDB1 and phospho-SMAD3 protein levels in proliferating myoblast and myotube treated or not with TGFβ1 in total extracts. Alpha tubulin was used as a loading control to normalize samples.

G. Scheme of experimental design with TGFβ inhibitor SB-413542.

H. Immunostaining of SETDB1 (red) and phospho-SMAD3 (green). Nuclei were stained with DAPI (blue). SB-413542 blocks SETDB1 relocalization in myotube nuclei upon TGFβ1 treatment. Scale bar, 10 μM.

I-J. Quantification of SETDB1 nuclear/cytoplasmic signal ratio (I), and phospho-SMAD3 nuclear signal (J).

For all panels: Statistics were performed on ≥3 biological replicates (>100 nuclei for immunostaining quantification) and data are represented as average +/- SEM *p<0.05; **p<0.01; ***p<0.001 (unpaired Student's t test).

Figure 2: DMD differentiated muscle cells display a constitutive relocalization of SETDB1 in the nuclei and a higher activation of TGFβ/SMAD pathway

A. Immunostaining of SETDB1 (green), pSMAD3 (magenta) and laminin (red) on histological slides from healthy individual or DMD patient paravertebral muscles. Nuclei were stained with DAPI (blue). White arrows identify centrally localized nuclei of damaged myofibers in DMD muscle section. Scale bar, 10 μM.

B. Immunostaining of SETDB1 (red) in healthy#1 and DMD del ex 45 myotubes. Nuclei were stained with DAPI (blue). Scale bar, 10 μM.

C. Quantification of SETDB1 nuclear/cytoplasmic signal ratio.

D. Western Blot of nuclear and cytoplasmic fractions of healthy and DMD myotubes in response to TGFβ1 showing protein levels of SETDB1 and phospho-SMAD3. RNA polymerase II and alpha tubulin were used as loading controls of nuclear and cytoplasmic fractions respectively.

E-F. Quantification of SETDB1 nuclear/cytoplasmic ratio (E), and phospho-SMAD3 nuclear signal (F).

G. Immunostaining of SETDB1 (red) and phospho-SMAD3 (green) in DMD del ex45 myotubes and quantification of SETDB1 nuclear/cytoplasmic signal ratio. Nuclei were stained with DAPI (blue). Scale bar, 10 μM.

For all panels: Statistics were performed on ≥3 biological replicates (>100 nuclei for immunostaining quantification) and data are represented as average +/- SEM *p<0.05; **p<0.01; ***p<0.001 (unpaired Student's t test).

Figure 3: SETDB1 silencing leads to a decreased response to TGFβ1 in DMD myotubes while keys myogenic markers increase

A. Scheme of experimental design. Cells were treated with siRNAs scrambled (CTL) or against SETDB1 for 2 days after 3 days of differentiation (myotubes) and then treated or not with TGFβ1 at 20 ng/mL.

B. Western blot showing SETDB1, phospho-SMAD3 and total SMAD3 protein levels. H3 was used as a loading control.

C. RT-qPCR of TGFβ/SMADs pathway known targets *TGFβ1, IL6* and *TIMP1* in healthy and DMD myotubes +/- siSETDB1 +/- TGFβ1.

D. RT-qPCR of TGFβ/SMADs pathway inhibitors *SKIL* and *SMAD7* in healthy and DMD myotubes +/- siSETDB1 +/- TGFβ1.

E. RT-qPCR of myogenic markers *Myogenin, MYH1* and *MYH3 (embryonic MHC).*

For all panels: Statistics were performed on ≥3 biological replicates and data are represented as average +/- SEM *p<0.05; **p<0.01; ***p<0.001 (unpaired Student's t test).

Figure 4: SETDB1 silencing in DMD myotubes leads to a decreased expression of TGFβ-dependent genes involved in ECM remodeling, receptor signaling transduction and inflammation

A. Venn diagram shows that a different set of TGFβ1-responsive genes were differentially expressed upon SETDB1 silencing in healthy and DMD myotubes.

B. Volcano plot of differentially expressed genes in DMD myotubes +/- TGFβ1.

C. Heatmap of expression level z-scores for DEGs in DMD myotubes for TGFβ *versus* TGFβ+siSETDB1 comparison. Some genes found to be increased upon TGFβ1 treatment decrease upon SETDB1 silencing (*IGFBP3, THBS1, IL6ST*).

D-E. Enriched categories tree plot (D) and enriched gene-concept network for biological pathways deregulated for TGFβ *versus* TGFβ+siSETDB1 in DMD myotubes.

F. mRNA levels of gene validated by RT-qPCR involved in ECM-remodeling (*THBS1, SERPINE1*) and inflammation (*LIF*).

For all panels: Statistics were performed on ≥3 biological replicates and data are represented as average +/- SEM *p<0.05; **p<0.01; ***p<0.001 (unpaired Student's t test).

Figure 5: Secretome of SETDB1 deficient myotubes in response to TGFβ1 reduces the negative impact of TGFβ treatment on myoblast differentiation

A. Diagram of the conditioned medium experiments.

B. Immunofluorescence of myosin heavy chain (red) and nuclei staining with DAPI (blue). Scale bar, 50 μM.

C. Quantification of fusion index after 6 days of differentiation in the conditioned medium.

D. RT-qPCR of fibrotic markers *SERPINE1* and *MSTN* after 3 days in the conditioned medium. **E.** RT-qPCR of early myogenic markers *MYOD1* and *MYOGENIN*.

F. RT-qPCR of late myogenic markers *MYH1* and *MCK.*

For all panels: Statistics were performed on ≥3 biological replicates (>100 nuclei for immunostaining quantification) and data are represented as average +/- SEM *p<0.05; **p<0.01; ***p<0.001 (unpaired Student's t test).

SUPPLEMENTAL FIGURE LEGENDS

Figure S1: SETDB1 localization depends on TGFβ/SMAD pathway activation during muscle terminal differentiation

A. Scheme of microscopy analysis and nuclear/cytoplasmic signal quantification. Nuclear masks were setup from DAPI pictures and applied on SETDB1 pictures and total cell masks were selected on SETDB1 pictures. Cytoplasmic quantification was performed by subtracting nuclei mask from total cell masks.

B. Quantification of phospho-SMAD3 nuclear fluorescence in proliferating myoblasts, myotubes treated or not with TGFβ1.

C. Protein levels of SETDB1 and phospho-SMAD3 in total extracts of myoblasts and myotubes treated or not with TGFβ1.

D. RT-qPCR of early myogenic (*MYOD1, Myogenin*) and TGFβ-related genes (*TGFB1, SERPINE1*) in myoblasts and myotubes treated or not with TGFb1.

E. RT-qPCR of early myogenic marker *MYOD1* and TGFβ-related genes (*SERPINE1, TGFB1*) in myotubes treated or not with TGFβ1 and/or its inhibitor SB-431542.

For all panels: Statistics were performed on ≥3 biological replicates (>100 nuclei for immunostaining quantification) and data are represented as average +/- SEM *p<0.05; **p<0.01; ***p<0.001 (unpaired Student's t test).

Figure S2: SETDB1 translocate into muscle cell nuclei in response to TGFβ/SMAD pathway activation and show more persistent nuclear signal in DMD myotubes irrespectively of the type of DMD mutation

A. RT-qPCR of *TGFB1* shows a higher response of DMD myotubes to TGFB1 treatment as compared to WT cells.

B. Immunostaining of SETDB1 (red) in healthy#2 and DMD point mutation myotubes. Nuclei were stained with DAPI (blue). Scale bar, 10 μM.

C. SETDB1 and MyHC immunostaining in proliferating muscle cells and in myotubes treated or not with TGFβ1 derived from iPSCs of healthy or DMD individuals. Nuclei were stained with DAPI (blue). Scale bar, 10 μM. Diagrams represent the percentage of SETDB1 signal intensity measure in nuclei and cytoplasm of the cells.

For all panels: Statistics were performed on ≥3 biological replicates (>100 nuclei for immunostaining quantification) and data are represented as average +/- SEM *p<0.05; **p<0.01; ***p<0.001 (unpaired Student's t test).

Figure S3: Efficient siRNA-mediated SETDB1 knockdown in TGFβ-treated DMD myotubes leads to a decrease in TGFβ target gene expression and an increase in pro-myogenic factors *SKIL* and *MYH3*

A. RT-qPCR of *SETDB1* shows an acute decrease (>80%) of *SETDB1* expression upon siRNAs-mediated silencing. *SETDB1* expression decreases upon TGFβ1 treatment in healthy myotubes but not in DMD myotubes.

B. Western Blot showing protein levels of SETDB1, SKIL, embryonic MYH and phospho-SMAD3 in healthy *versus* DMD myotubes upon SETDB1 silencing and TGFβ1 treatment.

C. RT-qPCR of TGFβ/SMADs pathway known targets *FN1* and *CTGF* in healthy and DMD myotubes +/- siSETDB1 +/- TGFβ1. TGFβ-related gene expression is decreased in SETDB1-silenced *versus* siCTL DMD myotubes in response to TGFβ1.

For all panels: Statistics were performed on ≥3 biological replicates and data are represented as average +/- SEM *p<0.05; **p<0.01; ***p<0.001 (unpaired Student's t test).

Figure S4: Healthy and DMD myotubes respond differently to TGFβ/SMAD pathway activation but display some SETDB1 target gene signatures in common

A. Principal component analysis of the filtered (look at Mat. And Methods) RNA-seq data, depicting a well grouping of all the samples for each of the 6 experimental conditions of the study. The variability captured by the first PC corresponds to the healthy vs. DMD conditions and the one of the second PC to the TGFβ treatment.

B. Gene Ontology enrichment of DEGs from healthy myotubes basal *versus* TGFβ comparison.

C. Gene Ontology enrichment of DEGs from DMD myotubes basal *versus* TGFβ comparison.

D. Gene Set Enrichment Analysis (GSEA) of DEGs from healthy myotubes TGFβ +/- siSETDB1.

E. Validation by RT-qPCR of genes coding for proteins involved in ECM-remodeling (*ADAMTS8, MMP14*), TGFβ/BMP and Wnt pathway (*MSTN, BMPR2, WNT5A*) and unknown function but predicted as regulator of muscle differentiation (*ANKRD33B*).

For all panels: Statistics were performed on ≥3 biological replicates and data are represented as average +/- SEM *p<0.05; **p<0.01; ***p<0.001 (unpaired Student's t test).

Figure S5: TGFβ/SMAD pathway activation leads to fusion defects in muscle cells

A. Diagram of the experimental design.

B. Immunofluorescence of pSMAD3 (green) and MHC (red) in myoblasts differentiated in fresh or conditioned medium +/- TGFβ1. Nuclei were stained with DAPI (blue). Scale bar, 10 μM.

C. Myogenic and fusion index of the myoblasts differentiated in fresh or conditioned medium +/- TGFβ1.

D. Raw myogenic and fusion index in basal conditioned medium show difference in differentiation rate between healthy and DMD myotubes.

For all panels: Statistics were performed on ≥3 biological replicates (>100 nuclei for immunostaining quantification) and data are represented as average +/- SEM *p<0.05; **p<0.01; ***p<0.001 (unpaired Student's t test).

Figure 1, Granados et al.

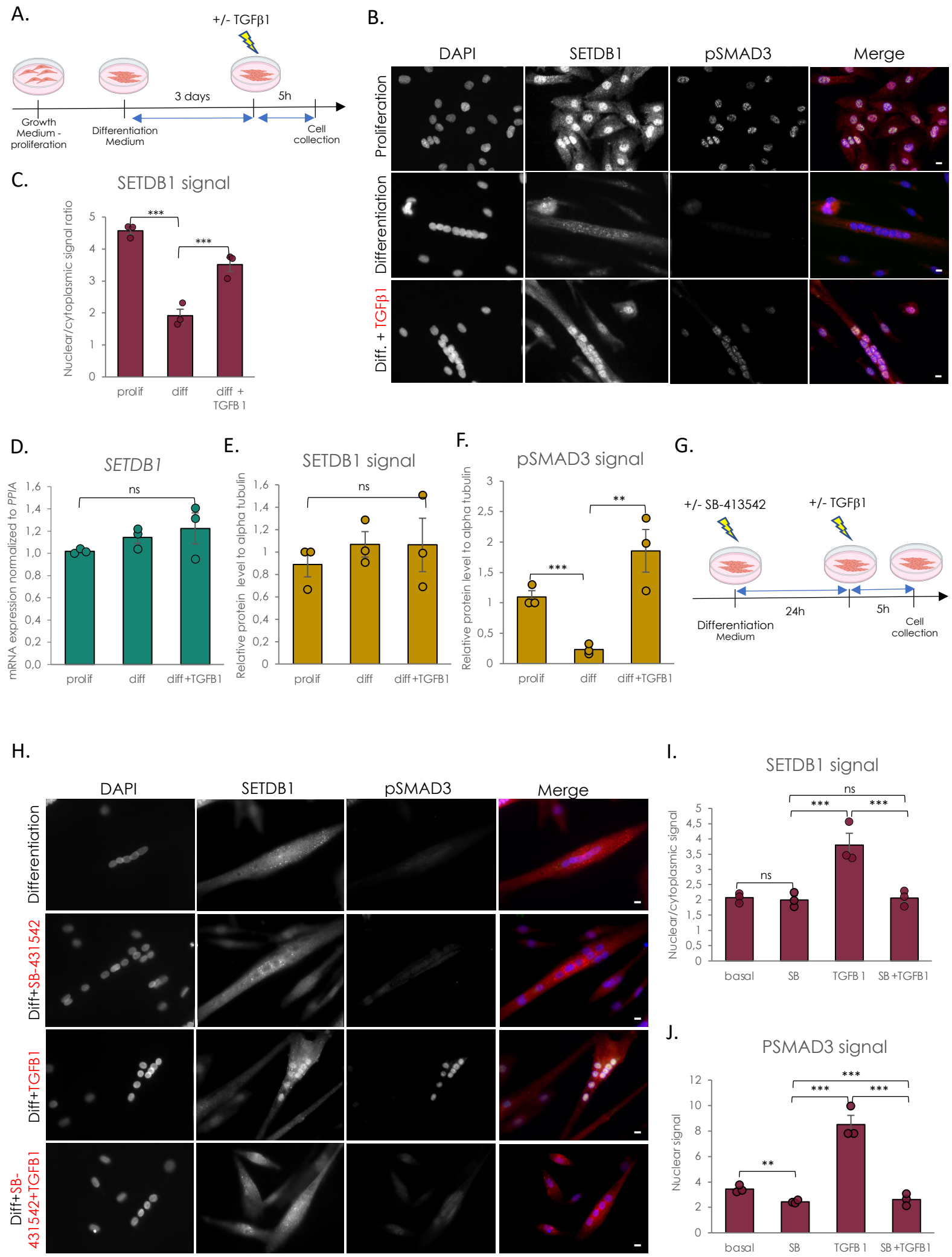


Figure 2, Granados et al.

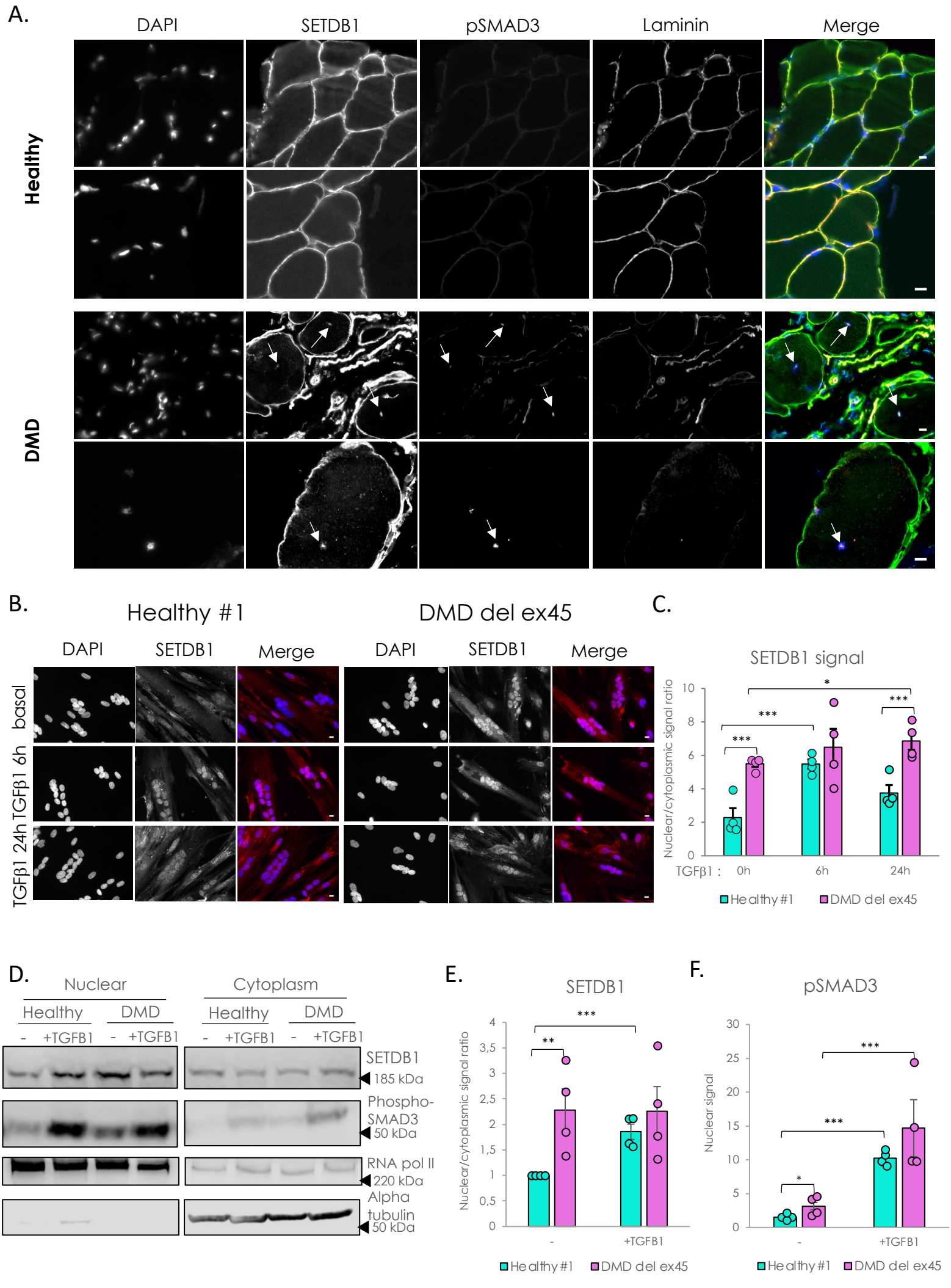
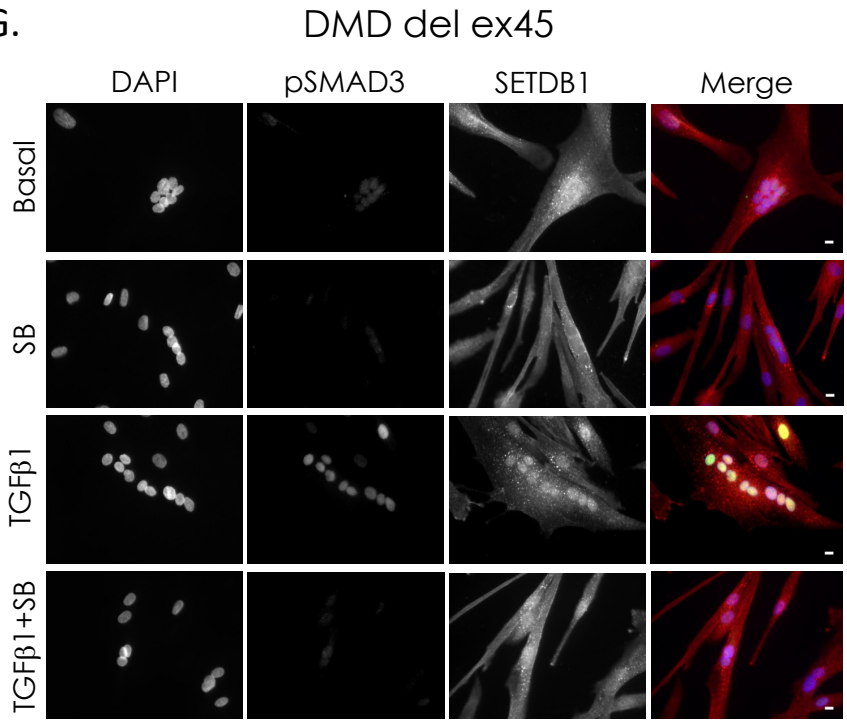


Figure 2, Granados et al.

G.



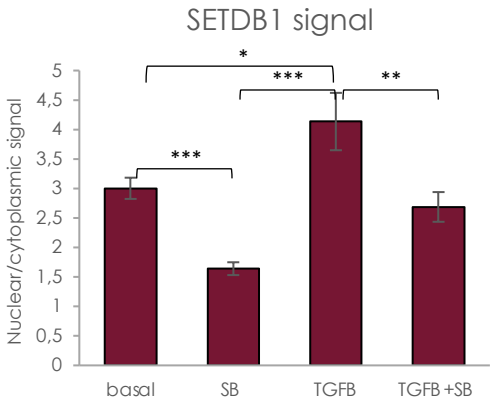
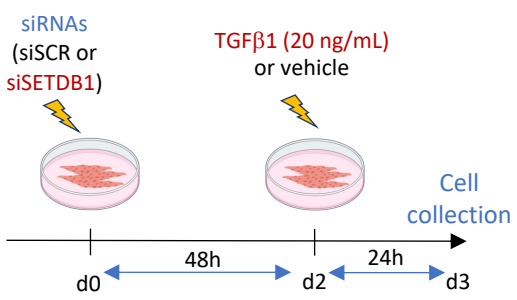


Figure 3, Granados et al.

A.



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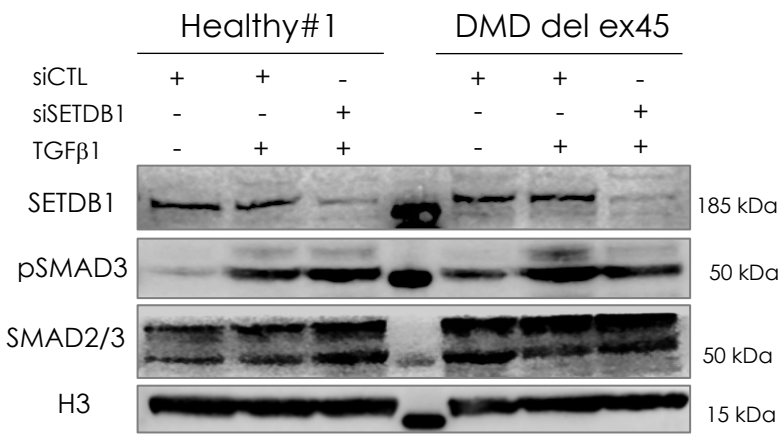

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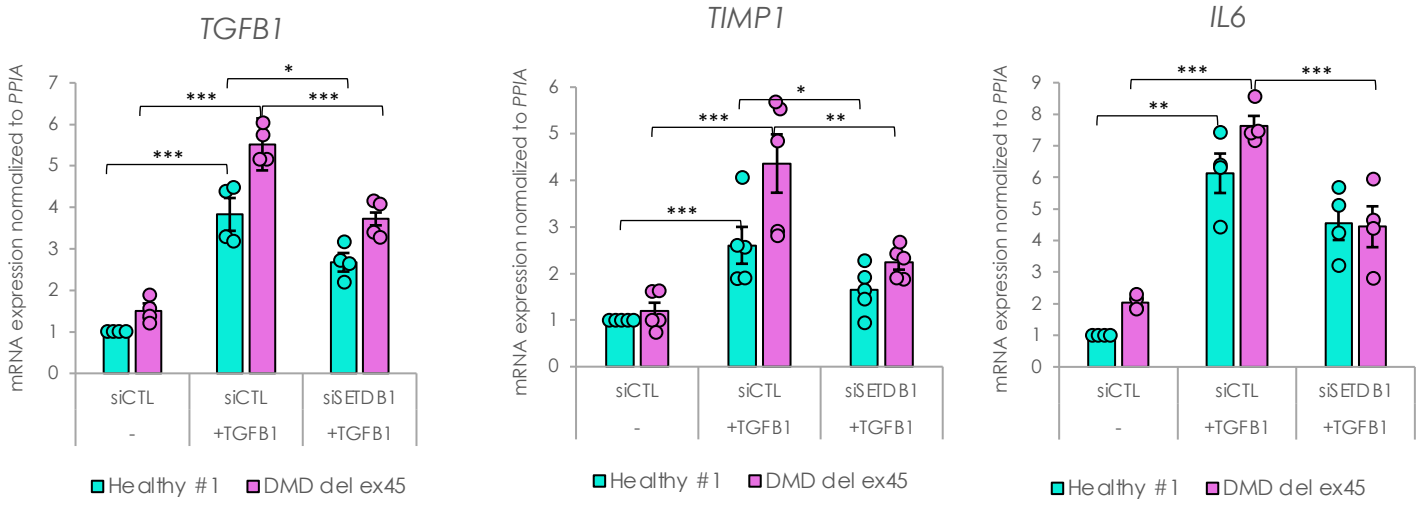

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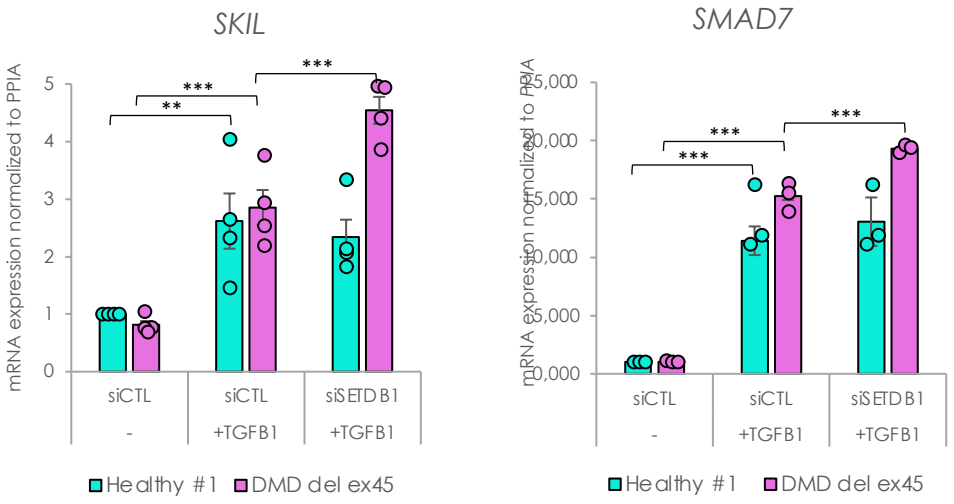


E.

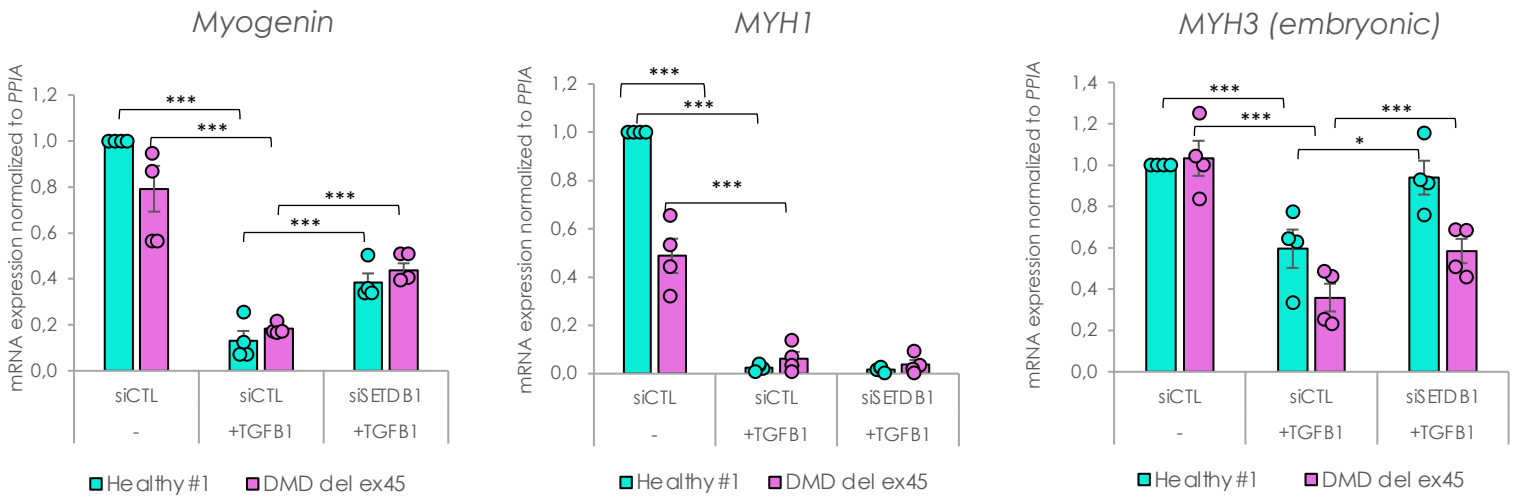


Figure 4, Granados et al.

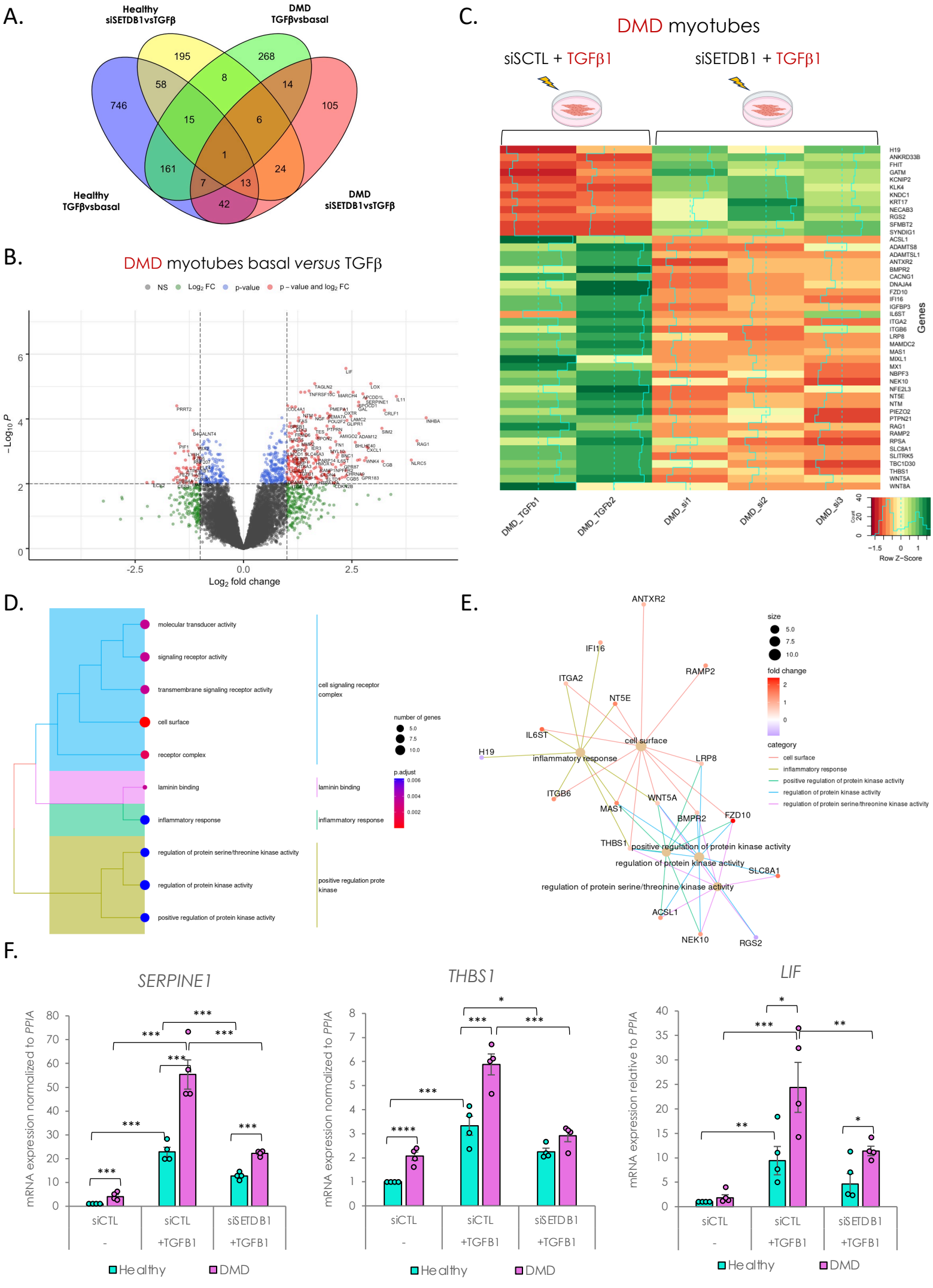


Figure 5, Granados et al.

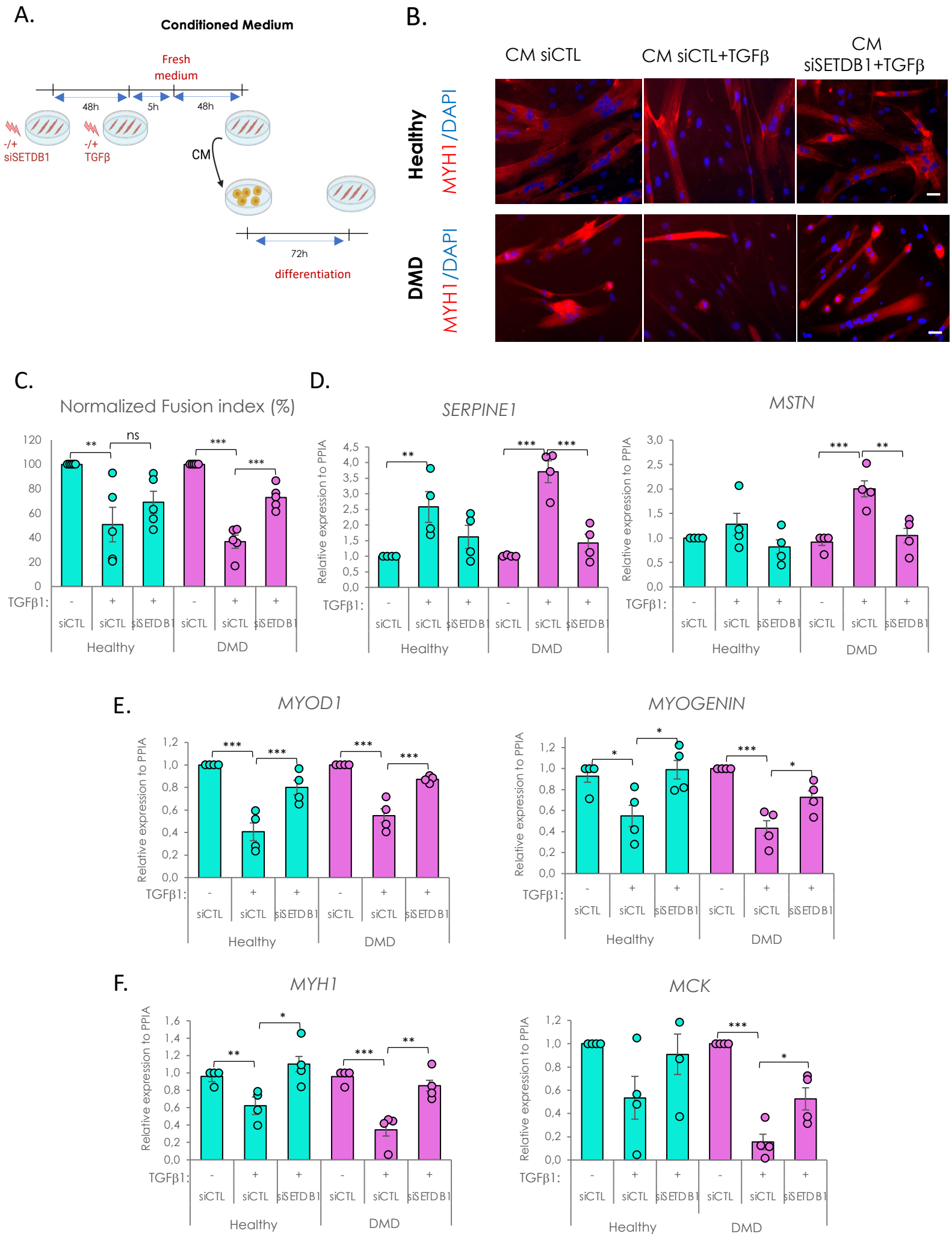
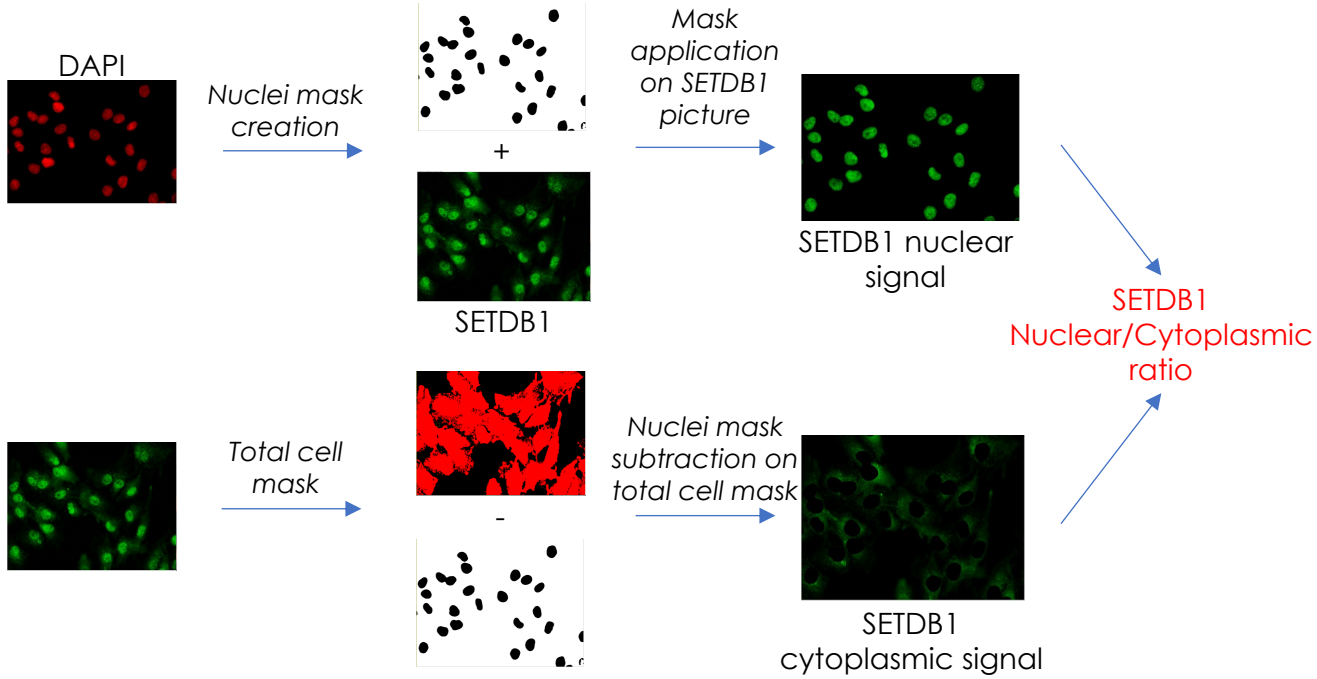


Figure S1, Granados et al.

A.



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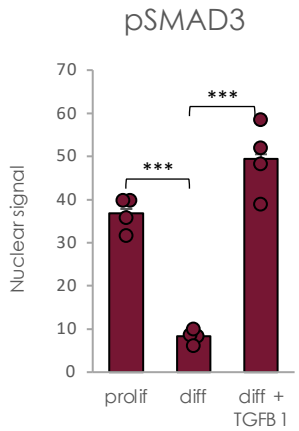

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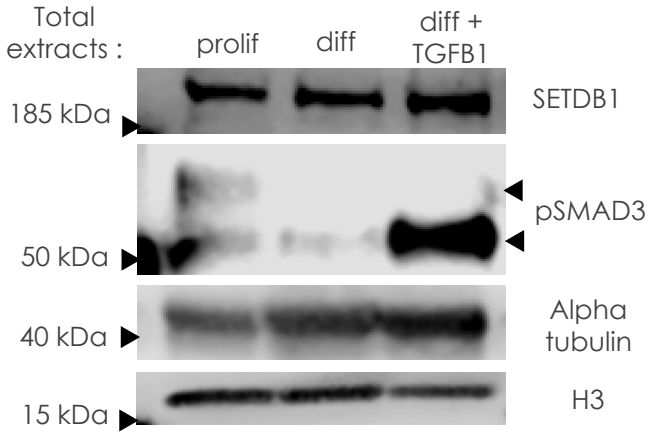

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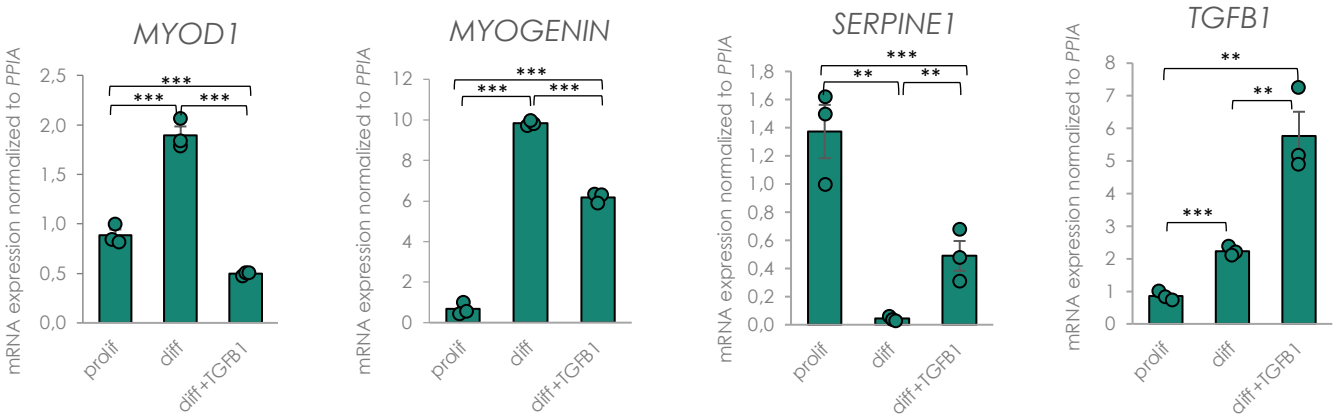

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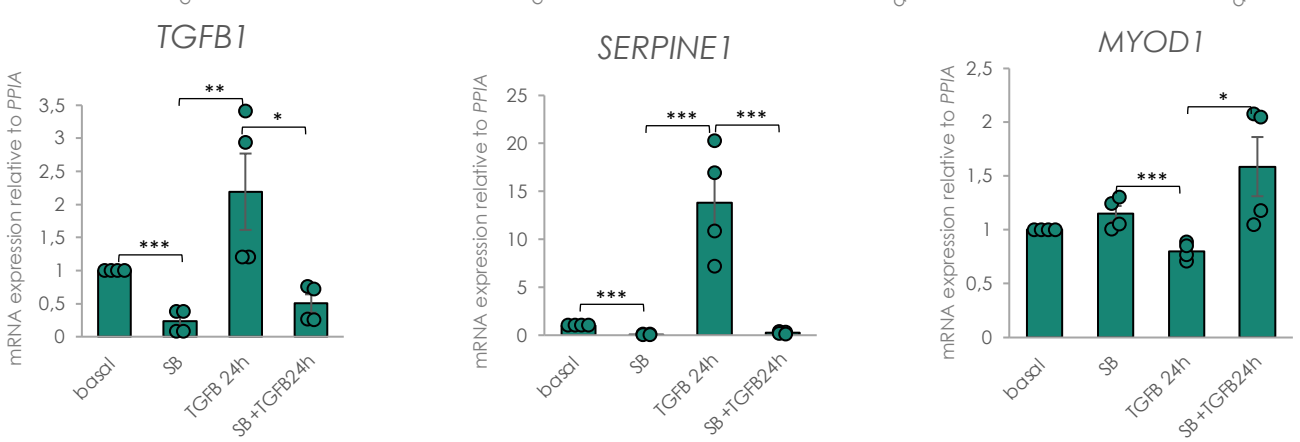
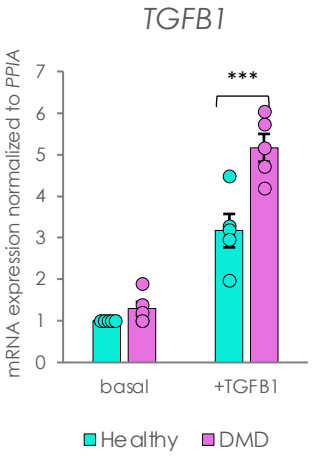


Figure S2, Granados et al.

A.



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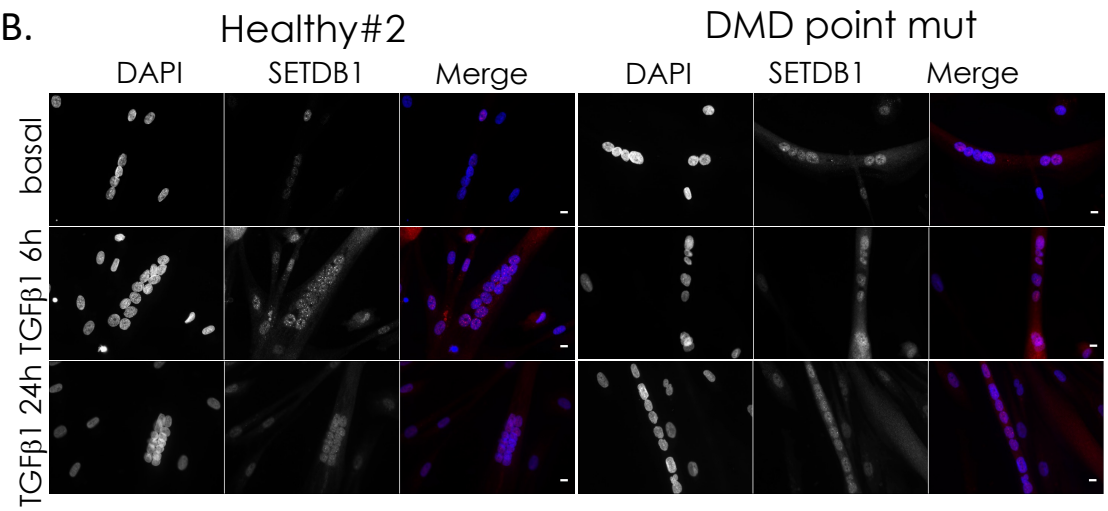

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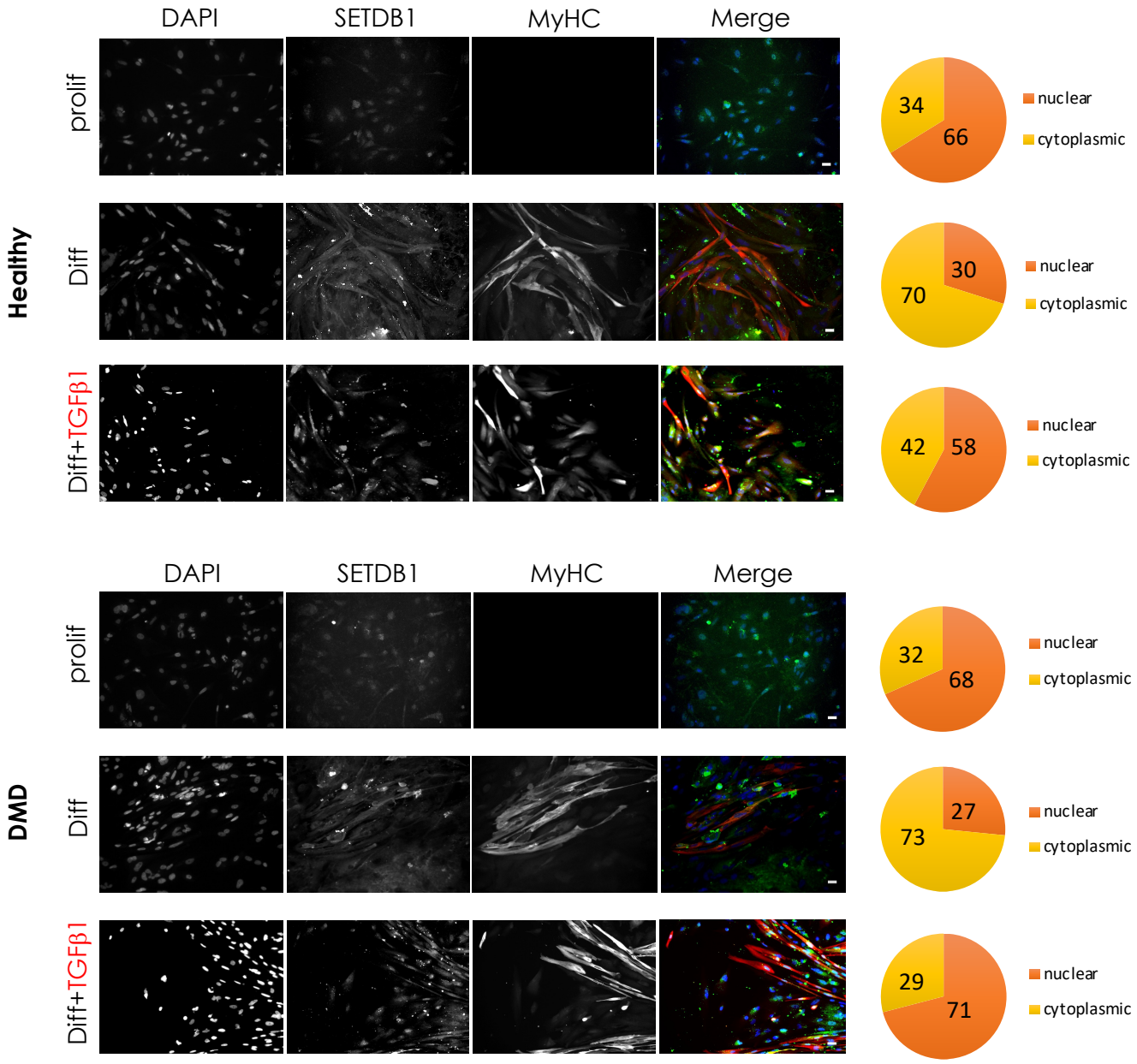
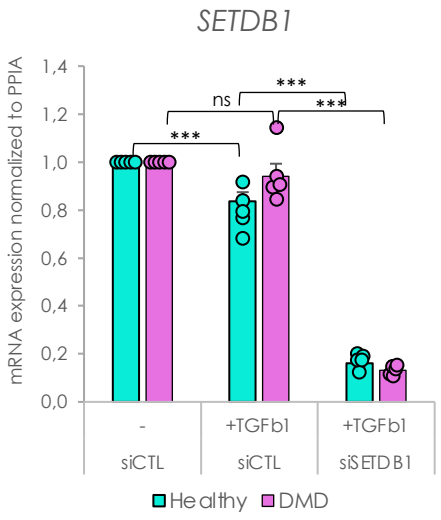


Figure S3, Granados et al.

A.



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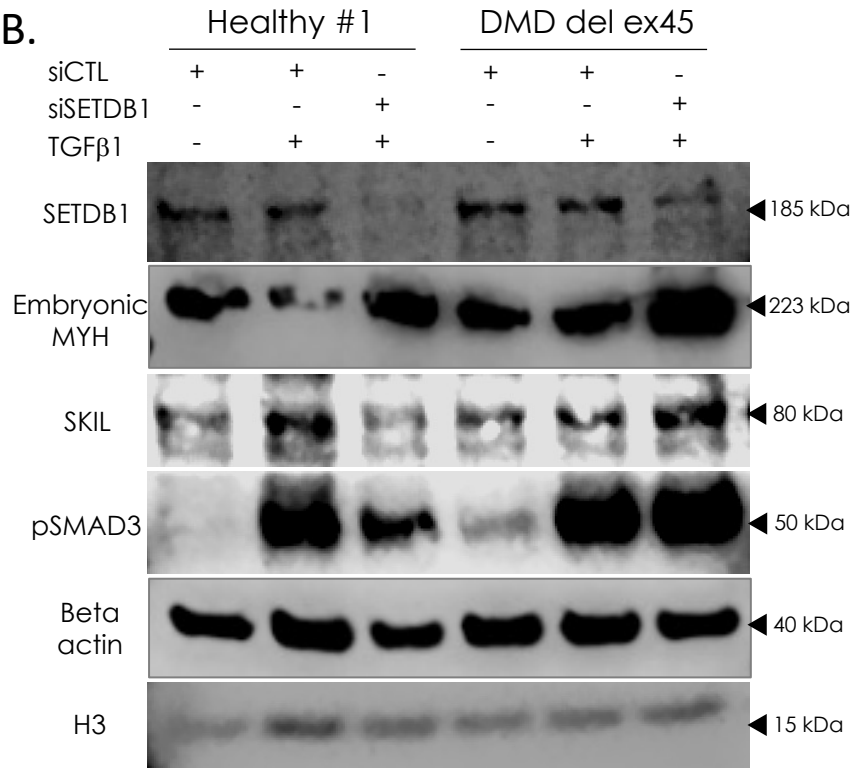

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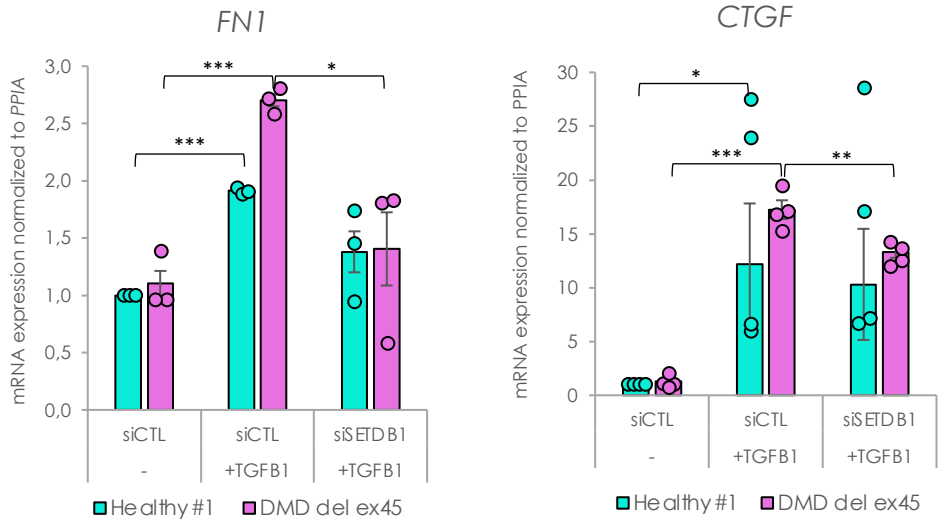


Figure S4, Granados et al.

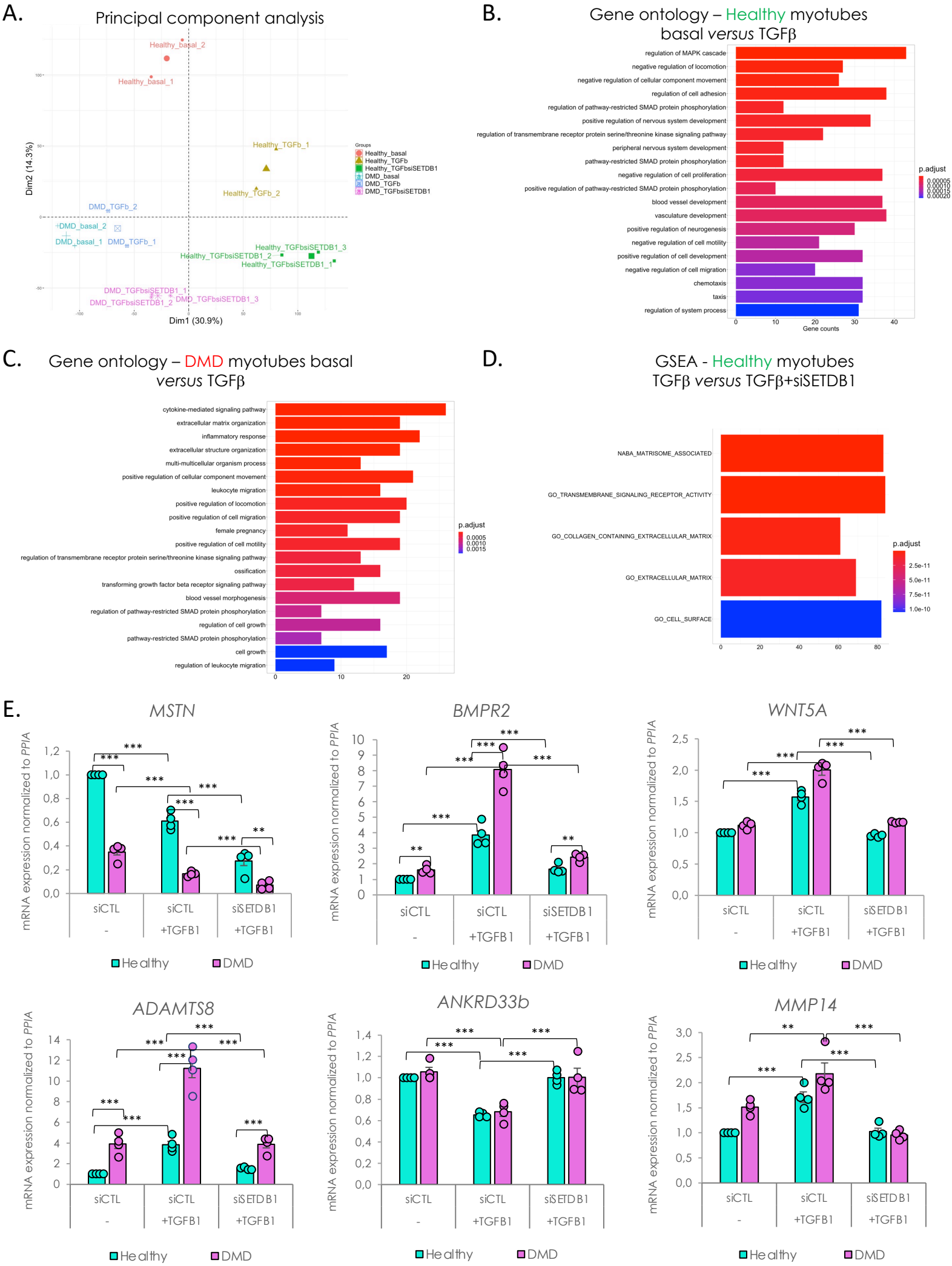
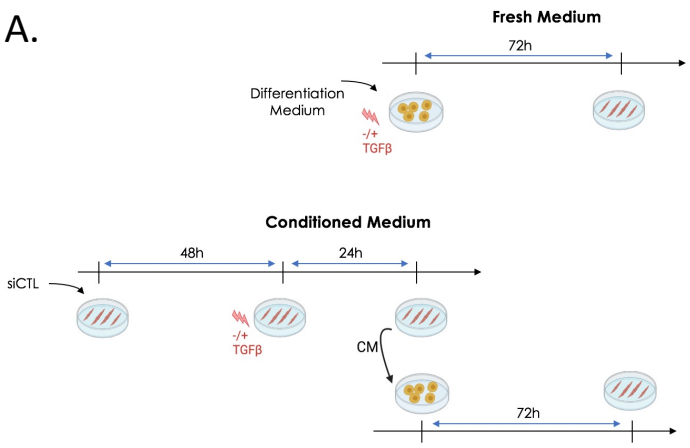


Figure S5, Granados et al.

A.



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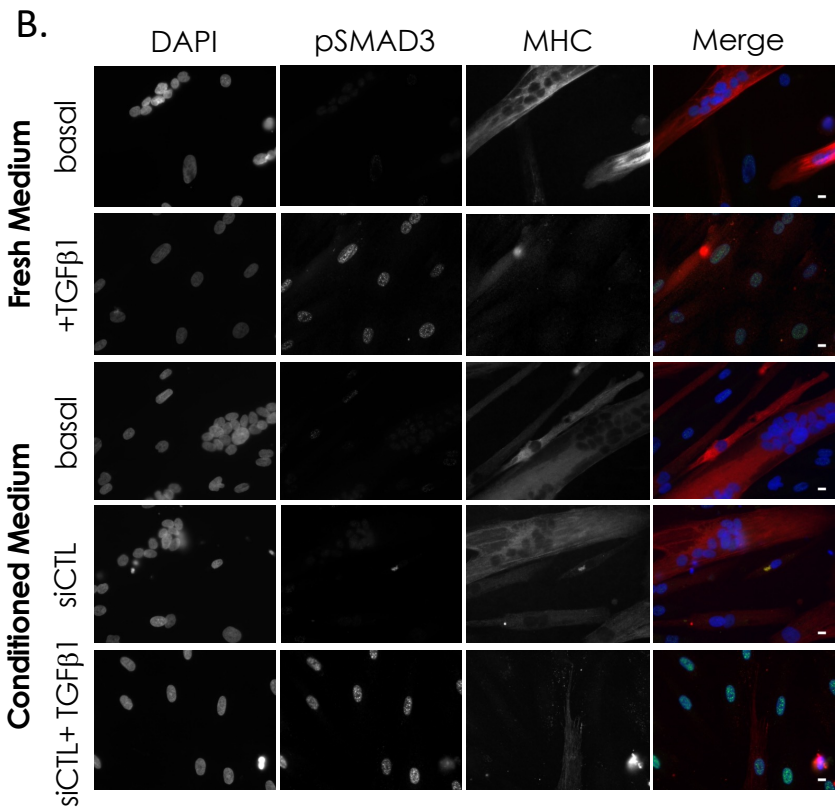

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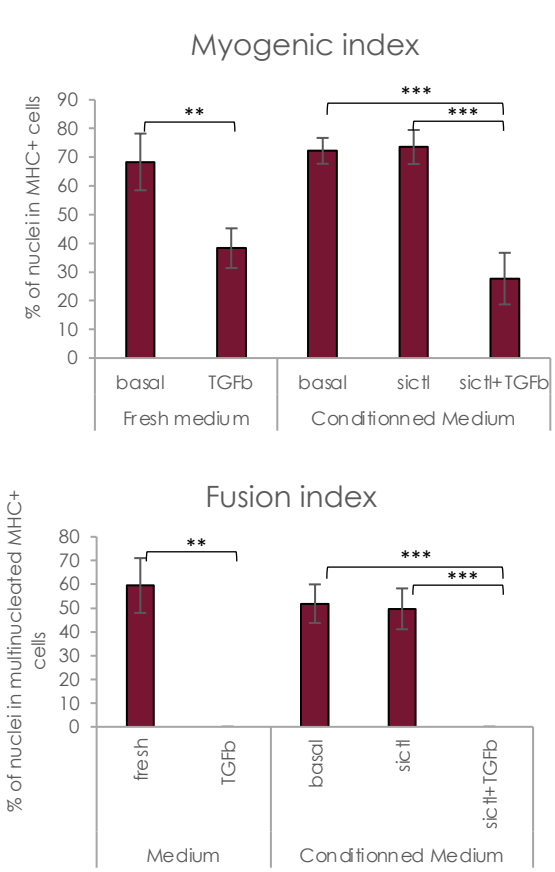

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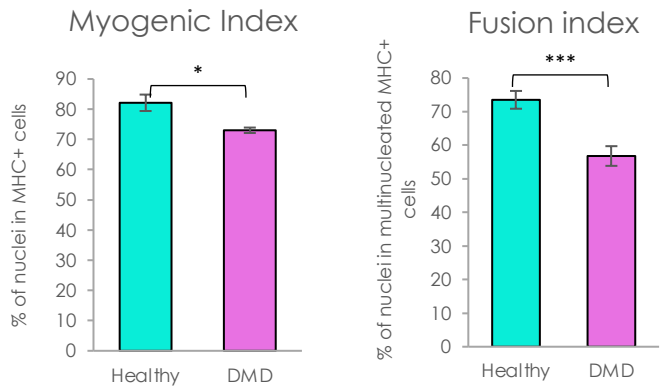

Table 1: Top DEGs + *versus* – TGFB1 in healthy myotubes

Genes	LogFC	Adj p-value	Genes	LogFC	Adj p-value	Genes	LogFC	Adj p-value
TAGLN2	2,117414122	0,0000029	FZD1	-1,709895607	0,000820576	CYGB	1,91232727	0,004636356
PMAIP1	1,822825158	0,0000295	CPT1A	1,892601801	0,000847677	CHRNA9	2,131251441	0,004655553
SPSB1	1,954444533	0,0000431	FLRT3	-1,666029718	0,000907678	HES6	1,511256537	0,004686457
LEFTY2	2,378015571	0,0000633	SPOCD1	2,072190972	0,000955143	BHLHE40	1,577516443	0,004688974
MYL10	2,775733677	0,000102766	KY	1,741592873	0,000987349	DUSP15	1,504729237	0,004688974
NGF	1,657626522	0,000102766	MMP24	1,785794877	0,001060517	PRTG	-1,939105695	0,004688974
HSD11B2	2,056381007	0,00014734	PTPRN	1,572910987	0,001060517	TAS2R50	-1,617874683	0,004707551
CPEB1	2,008260893	0,00014734	SPHK1	1,553357986	0,001060517	CAPG	1,617433777	0,004761555
GRIK3	1,897772958	0,00014734	SLC6A15	-1,604281321	0,001060517	IL15	1,632420492	0,004800381
CAMK2B	1,66141848	0,00014734	RGS10	1,775216817	0,001091293	RCSD1	1,50536248	0,005066626
LIF	1,609459933	0,00014734	ACAP1	-1,518147984	0,001091293	BCHE	-1,514123565	0,00508091
FAS	1,65594723	0,000176389	CDH22	1,952879439	0,001160065	HAPLN1	-1,939131798	0,005275934
COMP	3,079879046	0,000193172	VDR	2,38037933	0,001185628	TRAPPC11	-1,683727235	0,005288437
KRT17	2,249228221	0,000193172	MADCAM1	2,012788312	0,001218815	E2F2	-1,769263425	0,005304981
CYSLTR1	-1,686154285	0,000193172	IL11	1,720981756	0,00125013	ABHD1	-1,604574292	0,005424992
EFNA5	-1,539212907	0,000193172	ATF3	1,505914322	0,001406572	EDN3	-2,42948784	0,005452562
CBFA2T3	1,585761128	0,00021232	BNC1	1,727769999	0,001463008	KCNG3	-2,006427044	0,005469823
TINAGL1	2,040560934	0,000214261	UGT8	-2,091670121	0,001463008	MT2A	2,078060952	0,005630917
ST6GALNAC2	2,621131534	0,000278225	MDGA1	-1,57495199	0,001500196	MMRN1	-5,596523239	0,005630917
MYL7	1,928998074	0,000278225	LRRN3	-2,023789769	0,001564602	NPPB	2,22275891	0,005782479
RAB11FIP4	1,68799875	0,000278225	APCDD1L	1,573817321	0,001603986	SFRP1	-1,766514568	0,005851596
CADM2	-1,756947008	0,000278225	LEFTY1	2,948081363	0,001646694	NUPR1	2,251896144	0,006190195
KCNH3	1,526606981	0,000294724	INSC	1,984068026	0,001654329	MAPK10	-1,765559007	0,006190195
SH2D4A	1,542140036	0,000304292	FGFBP3	-1,960631592	0,001750743	TENM1	-1,823947329	0,006245981
BMP8A	1,66627264	0,000311971	LRP2	-2,613936968	0,001789815	ABCA7	-1,6412153	0,006245981
UBASH3B	1,556460437	0,000311971	KCNK3	2,416533797	0,001890051	ADAMTS3	-1,554488887	0,006363145
GAL	2,095209135	0,000339293	DSCAML1	2,603034024	0,001909552	SPATA22	1,86416685	0,006490852
FZD5	-2,217424853	0,000339293	LPAR4	-2,840036833	0,002027283	MAB21L2	-2,344727736	0,006490852
ERBB4	-1,608195924	0,000339293	BMP6	1,797677275	0,002067634	PREX2	-1,895214593	0,00654097
JUNB	1,651100338	0,000363745	DRD2	1,911310104	0,002119699	LRRIQ1	-2,230304268	0,006747866
SYTL1	2,38078558	0,000381256	INHBA	1,943531045	0,002198855	PLXDC1	1,720148106	0,006814196
APOBEC3C	1,532547029	0,000381256	HHIPL2	-3,371136543	0,002252125	CBR1	2,746093702	0,006991214
SNAI2	1,80924925	0,000391447	CRLF1	1,941126358	0,002294054	PTPRZ1	-2,289835098	0,006997138
RASL10A	1,877442599	0,000415202	ADAMTSL2	1,594546261	0,002388538	GATA3	1,58890351	0,007566377
PDK4	2,83974063	0,00043132	FZD3	-1,5642083	0,00246262	SLC6A3	-1,725541298	0,007724626
FBLN1	-1,588350862	0,000444297	MKI67	-1,620940441	0,002506658	EPHA4	-1,949119966	0,007737852
BMP3	-1,918899291	0,000449801	CXCR2	1,854163088	0,00256305	LTBP4	-1,510220625	0,008205986
LAMP5	1,686374304	0,000451403	FOSL1	1,871421395	0,002601514	RASD1	1,776910188	0,008344487
NDRG1	1,551820455	0,000451403	ZNF385B	-1,81912287	0,002601514	FAT4	-1,540323008	0,008352329
KCNT2	-1,6565026	0,00046383	SLC15A3	-1,776559618	0,002623801	TAS2R3	-2,803397109	0,008662833
PCDH18	-1,908015374	0,00048586	GPR26	2,554885167	0,002627384	FAM228B	1,920625724	0,008786462
C1QL3	2,750006723	0,000496238	PHLDA2	1,912747543	0,002627384	CGB5	1,997764466	0,008933737
SUMO4	-1,986991477	0,000506112	RRAD	2,053971838	0,002728733	DUSP2	1,65487497	0,008983737
ARHGDIB	1,850929182	0,000509686	FREM2	-1,565177808	0,002728733	ROBO1	-1,537260889	0,008983737
PCSK6	1,711984469	0,000509686	CAV1	1,914603656	0,002899024	GPR183	2,015068932	0,009086429
IRF1	1,590773748	0,000509686	WNK4	1,997056036	0,002931427	TTF1	-1,94369392	0,009313694
KIAA1211L	2,76892938	0,000517694	UGT3A1	-1,686489198	0,003345925	TAS2R31	-2,897454085	0,009353729
BBC3	1,535319531	0,000517694	MAPRE2	1,610130633	0,003380818	CD34	-3,228526136	0,009577181
ROBO2	-2,353637478	0,000534263	KDR	-1,538782171	0,003406179	ZNF493	-1,597943058	0,009582422
MSTN	-2,419217607	0,000572993	TFAP2C	1,719741735	0,003623613	OSGIN1	2,246915673	0,009631694
SFRP2	-2,547991562	0,00060178	PODN	1,685594321	0,003623613	ANK1	1,501084248	0,009652588
ZNF488	1,613702593	0,000668475	ASPH	-1,680643217	0,003696591	SCN3A	-1,978797967	0,009826333
CYP26C1	-2,60465348	0,000697753	NPR1	-1,93870055	0,00402619	SAC3D1	1,586634217	0,009949286
CDH6	-1,630541842	0,000707553	HES5	-5,038583814	0,004080276	TMEM168	-2,739006335	0,009993603
ADAMTS5	-1,875618352	0,000718764	SRGAP2	-1,614350913	0,004106754			
MX1	3,4674981	0,000725331	ISG15	1,862365951	0,004133911			
HMOX1	1,659405071	0,000725331	BIK	1,73069843	0,00451334			
HIST1H4D	-1,571617314	0,000814482	CACNG7	-1,885120997	0,004522138			
CDK5R2	1,65690867	0,000820304	IRS4	-2,193265101	0,004527926			
BMP5	-1,950914501	0,000820304	SLC7A8	-1,852506781	0,004527926			

Table 2: Top DEGs + *versus* – TGFB1 in DMD myotubes

Genes	LogFC	Adj p-value	Genes	LogFC	Adj p-value
TGFBI	2,75040849	5,16E-08	BNC1	2,22770933	0,001244942
IL32	2,163620694	2,67E-07	QPCT	1,556242386	0,001244942
LIF	2,335338653	4,04E-06	MYL10	1,972655907	0,00146367
TAGLN2	1,614876963	5,10E-06	C6orf15	1,678899961	0,001517905
LOX	2,910516076	1,49E-05	ACTN3	-1,617310124	0,00159821
IL11	3,500409887	1,56E-05	S100A11	1,542630473	0,001754586
ANGPTL4	2,505569257	1,56E-05	HMOX1	1,644698523	0,002038795
MARCH4	2,157892488	1,56E-05	PARP14	1,696207068	0,002076847
ITGA2	1,980632839	1,56E-05	FSTL3	2,029350269	0,002148251
APCDD1L	2,725494981	1,81E-05	NLRC5	3,843801654	0,00225234
CCL4	5,438924629	2,45E-05	WNK4	2,802240205	0,00225234
PRRT2	-1,567243856	3,16E-05	SLCO2B1	2,732760296	0,00225234
SPOCD1	2,629199572	4,54E-05	ARHGDIB	2,226199038	0,002446559
GAL	2,598934135	6,15E-05	PTHLH	4,711087434	0,002450317
PMEPA1	1,967218429	6,15E-05	IL6ST	2,123553616	0,002450317
SEMA7A	1,911459216	6,15E-05	F2RL1	1,701706227	0,002546264
SIM2	3,167334171	6,29E-05	CPA4	1,642588269	0,002547328
SERPINE1	2,798165062	6,57E-05	TRIML2	2,65612546	0,002803042
INHBA	4,186346592	7,54E-05	RRAD	2,356494017	0,002830075
GDF15	1,973998395	8,06E-05	CAV2	1,944528389	0,002907175
JUNB	1,952571749	8,06E-05	DUSP5	1,614895079	0,002978339
OXTR	2,296104211	8,32E-05	LTBP2	2,603423885	0,003245881
LAMC2	2,448525099	0,000121314	RAMP1	1,731342839	0,003662479
CRLF1	3,222009526	0,00013696	TAC1	1,590107658	0,003700528
AMIGO2	2,19537543	0,000188579	GPR87	2,290047196	0,003705592
POU2F2	1,920477198	0,000199134	EBI3	1,834331335	0,003890978
ANXA4	1,524080736	0,000203676	CLCA2	1,568271593	0,004203243
NGF	1,575477534	0,000214729	MX1	2,151667583	0,004673777
ADAM12	2,627168827	0,000241171	NPFFR2	2,071848324	0,004673777
PTPRN	1,898344697	0,000278524	FOSL1	2,033533552	0,004848003
TNFRSF11B	2,052407425	0,000306379	C1QL1	-1,518618864	0,00567038
FN1	2,097273737	0,000316043	CHRNA9	2,341930935	0,006317128
LDLRAD4	1,885583009	0,000366423	PROKR2	2,187361985	0,006317128
SPON2	1,664023037	0,000367141	CDKN2B	2,078064312	0,006317128
TES	1,638095057	0,000367141	C1QL3	2,251176908	0,006486544
LEFTY2	1,665034244	0,000405062	CLDN4	1,75335273	0,006486544
RAG1	3,975923848	0,000483132	MSTN	-1,615087924	0,006486544
MMP9	1,928522542	0,000499646	HSPA12A	1,709492127	0,006879139
PIF1	-1,50786107	0,000529838	CGB5	2,351065776	0,007003126
CXCL1	2,816696053	0,000539842	ANKRD1	1,667202737	0,00716927
NT5E	2,187868134	0,000665576	SRPX2	1,946225219	0,007347603
TAP1	1,706154724	0,000665576	GPR26	1,770766319	0,007671595
BHLHE40	2,552101744	0,000728008	COMP	1,563971118	0,007671595
IER3	1,535483982	0,00095399	GPR183	2,697987123	0,008448068
GLIPR1	2,359671334	0,001126099	CYGB	1,810473194	0,008551238

Table 3: Top DEGs siSETDB1 *versus* siCTL during TGFB response in WT myotubes

Genes	LogFC	Adj p-value	Genes	LogFC	Adj p-value	Genes	LogFC	Adj p-value
MAMDC2	-1,520498038	5,65E-05	ARSJ	-1,061549661	0,008451464	RPS6KL1	-1,267489634	0,023290191
SETDB1	-2,312260334	6,09E-05	FLT1	-1,332461615	0,008613958	CLCA2	-1,650785368	0,024689191
ACSL1	-1,455424424	0,000224437	HAS2	-1,247511382	0,008751755	KCNG3	-2,004431023	0,024951235
THBS1	-1,301394066	0,000224437	HMCN1	-1,044961141	0,008751755	APLNR	-2,306740676	0,027475948
BMPR2	-1,176640246	0,000224437	GPC6	-1,294102823	0,008794726	LAMA1	-1,105272513	0,028027906
ATP8B2	-1,05885524	0,000224437	ITGB6	-1,119913491	0,008794726	GSDMA	-1,111110953	0,028371421
PTGFRN	-1,53323687	0,000474681	LRP8	-1,042762389	0,008794726	GRK4	1,686838586	0,028644611
LIMA1	-1,175745808	0,000501383	ST8SIA2	-1,054476505	0,009159413	FREM1	-1,676226071	0,029134271
IGFBP3	-1,471141886	0,00057845	SCN11A	-1,758429933	0,009303076	MAN1A1	-1,378856426	0,029134271
TMED7	-1,443559104	0,00057845	DOK3	-1,010817943	0,009434878	NAALADL2	-1,525850019	0,029709578
TMEM109	-1,281138773	0,00057845	SUSD5	-1,206994107	0,009994918	LAMA2	-1,445964152	0,029886349
IGSF22	-1,179795818	0,00057845	MPZL2	-2,408914913	0,010148671	RPL13	1,177052671	0,030194496
NTM	-1,097552605	0,000596109	LRRN3	-2,423741639	0,010347444	LRRC2	-1,122755043	0,030247404
GRIK3	-1,382794973	0,000633272	DSCAML1	-1,583808697	0,010432176	PCDH18	-1,142379056	0,030682091
PRSS23	-1,21234796	0,000633272	HMOX1	1,01627009	0,010534753	CLDN22	-1,451975925	0,030829524
PARM1	-1,196752186	0,000723086	SLC39A11	-1,348927723	0,011182178	SCN2A	-1,301431103	0,031312572
RAMP2	-1,709990009	0,00072624	ENPP6	-1,329673444	0,011182178	DNAJC22	-1,866109729	0,031497413
PCDHA4	-1,179801452	0,000866001	SLC27A1	-1,039664553	0,011182178	PRKCQ	-1,748274946	0,031643005
SLC8A1	-2,168674612	0,001035925	KY	-1,136090745	0,011569641	TLL2	-1,382705171	0,031643005
IGSF3	-1,452586062	0,001260586	HOXA1	-1,869923761	0,011589371	HPDL	-1,0274529	0,031691539
UGGT2	-1,373806653	0,001260586	WNT8A	-1,723404066	0,011605214	CLGN	-1,00964704	0,031709166
CD109	-1,206613739	0,001260586	DCX	-1,172252082	0,011605214	SMARCAD1	-2,674991532	0,032096292
ZFYVE27	-1,146034744	0,001260586	CHRM3	-1,328414995	0,01184128	C1QL3	-1,110617004	0,032165845
AKR1C3	1,196726021	0,001288236	ARSB	-1,05339903	0,01184128	KIAA1549L	-1,301619635	0,032682881
ARHGAP26	-1,44235698	0,001370776	RAG1	-1,520711756	0,0120045	TMED8	-1,039140935	0,033560494
GPR137C	-1,350324674	0,001387303	LRFN2	-1,269349114	0,012103738	OAF	-1,149040279	0,033574106
FGF13	-1,003563077	0,001387303	NFIA	-1,56438338	0,01222235	ISM1	-1,131304626	0,033574106
NRP2	-1,303642909	0,001435207	SLC4A8	-1,423660225	0,012780684	LRRC37A	-1,179829418	0,033755698
OR2H2	-3,608774047	0,001483697	ITGA4	-1,093763889	0,012860251	PER3	-1,000955169	0,034677493
TMEM87B	-1,158116859	0,001779461	MMP25	-1,194320365	0,013314621	LRFN5	-1,607087431	0,035473366
SMTNL1	-1,069024474	0,001779461	OLFML1	-1,985558064	0,013852692	DPY19L2	-1,043176127	0,035841864
TAL2	-1,110677244	0,001884083	CNTNAP3	-1,246093716	0,014099073	MAST1	-1,282552641	0,036121551
CHDH	-1,49571412	0,002016351	CLDN1	-1,078499463	0,014200411	CCL4	1,885798209	0,037313963
PAK3	-1,059555572	0,002178155	CACNG4	-1,004577351	0,01422263	FAT3	-1,074630898	0,037313963
RIMS1	1,002106455	0,002207039	MKI67	-1,476461877	0,014255468	SPIB	-1,503325119	0,037761996
PLCD4	-1,709870369	0,002291852	MXRA8	-1,068346915	0,014255468	CDH4	-1,671716499	0,037870918
POLR3D	-1,05688535	0,002378671	GPLD1	-1,024911621	0,014268962	CBR1	-2,167825525	0,038060638
FRMPD1	-1,092657246	0,002688295	KCNK3	-1,637967552	0,014865901	ADAM33	-1,124290334	0,039108986
CACNA1E	-1,119064387	0,003649151	FAM71F1	1,003725779	0,015144667	UBE2Q2	-1,050446746	0,03956611
GRPR	-1,333005464	0,003905121	PAPPA	-1,235866455	0,015144667	DESI2	-1,177585114	0,040182971
SLC37A3	-1,035129517	0,003905121	GARNL3	-1,06505093	0,017145732	PODN	-1,010497684	0,040182971
PRIMA1	-2,433823631	0,004160811	RNF220	-1,073998499	0,017340312	GJC1	-1,132522857	0,041033645
DKK2	-1,476262445	0,004326207	ABCC9	-1,238756215	0,017696163	COL22A1	1,691834247	0,041291631
GAL3ST4	-1,296668125	0,004399025	CXCL1	-1,582775383	0,018516113	SCN4B	-1,181753524	0,042784027
ZNF107	-1,129045081	0,004399025	BRCA2	-1,44530328	0,018516113	HIST2H3D	-1,10116306	0,042784027
DPY19L3	-1,009527364	0,004399025	DOC2B	-1,133045331	0,018516113	YTHDC2	-1,074434231	0,044167886
KRT80	-1,272269317	0,005432878	THBD	-1,04413245	0,018516113	SLC6A3	1,32166898	0,044288292
TMEM182	-1,076767897	0,005750453	GAS2L1	-1,029502553	0,018516113	CNTN5	-1,150597818	0,044381061
CAMK4	-1,51980501	0,005834327	KDR	-1,412431822	0,019245021	SCN7A	-1,467305844	0,044542055
SPTLC3	-2,155845794	0,006288784	ROR1	-1,044621628	0,019603878	GRIN2A	-1,412058254	0,047051083
FSD1L	-1,190564652	0,006555192	PRDM8	-1,214084634	0,019779272	PCDH17	-1,143147116	0,049034992
KSR2	-1,802189364	0,00723519	VCAN	-1,192075825	0,019779272	TSPAN11	-1,52753589	0,049416366
SEZ6	-1,161203502	0,00723519	CDH6	-1,046088047	0,020401625	DNAJA4	-2,230979822	0,049955982
KIAA1211	-1,005076581	0,00723519	CNTN2	-1,109787194	0,020995772	FAXC	-1,046733564	0,049974705
APCDD1L	1,149403017	0,007527773	NEK10	-1,131980357	0,021155723			
ARNT2	-1,077264685	0,007527773	RRAD	1,152195301	0,021663764			
TENM3	-1,003246377	0,00807358	THEMIS2	-1,141961836	0,021663764			
TENM2	-1,01075277	0,008088306	SPTBN5	-1,200082097	0,022154881			
NT5E	-1,337136771	0,008215045	CARNS1	-1,189226427	0,022569992			

Table 4: Top DEGs siSETDB1 *versus* siCTL during TGFB response in DMD myotubes

Genes	LogFC	Adj p-value	Genes	LogFC	Adj p-value
MAMDC2	-1,637289048	1,59E-05	TBC1D30	-1,656334075	0,017074581
NTM	-1,190266731	0,000624532	TMEM109	-0,852260252	0,019233362
CACNG1	-1,183738436	0,001856085	MIXL1	-1,554596572	0,019233362
MAS1	-1,521127791	0,001856085	WNT8A	-2,267660056	0,020236735
IGFBP3	-1,376670225	0,00219827	RGS2	0,830392825	0,020236735
SLC8A1	-1,793845414	0,002530987	PRSS23	-0,872430004	0,021751043
ACSL1	-1,287676377	0,003389855	HEXB	-0,59923785	0,022743141
KLK4	1,787192625	0,003389855	OLIG3	-1,219844213	0,022795667
NT5E	-1,535868776	0,003691319	ANO2	-0,809761055	0,023330054
RAG1	-3,601343309	0,003691319	CXCL12	-1,052378458	0,023330054
KCNIP2	1,004157391	0,003691319	IL6ST	-1,816664708	0,023330054
ADAMTSL1	-1,233489406	0,003799356	DNAJA4	-1,462582406	0,0237548
ANTXR2	-0,898772223	0,004091009	C6orf118	-1,270419296	0,025251564
LRP8	-0,874823587	0,004452942	UCP2	0,591231944	0,025251564
FZD10	-2,477744095	0,010340651	FHIT	0,974006361	0,025251564
IFI16	-1,095725152	0,010354658	PCDHB5	-0,847006813	0,026603158
ADAMTS8	-0,749833348	0,010652676	MOXD1	-1,300307077	0,026603158
ITGA2	-0,833861933	0,010652676	NECAB3	0,57770924	0,026603158
ANKRD33B	1,309182508	0,010772045	MX1	-1,671442415	0,026673574
VEZT	-0,59658059	0,010948764	IGSF22	-0,898697771	0,027562876
LIMA1	-0,805583317	0,010948764	SETDB1	-1,0498379	0,028675644
ITGB6	-1,363673345	0,011201951	FBLN1	-0,829507714	0,02996258
POLN	-0,950024758	0,011211256	RPSA	-1,790736619	0,033731882
RAMP2	-1,299151192	0,011334712	MATN2	-0,849749358	0,035265666
NBPF3	-1,164405324	0,011446775	MLIP	-1,211291173	0,035922112
CANX	-0,679162696	0,012181954	PGPEP1	0,632953024	0,035922112
BMPR2	-0,753326146	0,012181954	SFMBT2	0,683128284	0,035922112
ZFYVE27	-0,809883445	0,012181954	RAP1GAP	0,71600913	0,035922112
NFE2L3	-0,939940084	0,012181954	NKAIN2	-1,137475403	0,039898636
KNDC1	0,708511646	0,012238511	MARCH2	-0,595011539	0,039948549
WNT5A	-0,703188943	0,013030024	KRT17	0,948663616	0,042060417
TMEM30A	-0,705417259	0,013030024	FRY	-0,58182675	0,042984885
THBS1	-0,73807264	0,013030024	PTGFRN	-0,743069331	0,042984885
NEK10	-1,269427146	0,013030024	RTL1	-0,890963897	0,042984885
PTPN21	-0,775583543	0,013160275	ARRDC2	-1,191091084	0,042984885
FAM20A	-0,869507338	0,013160275	AMHR2	-2,339438416	0,042984885
KIT	-0,917148702	0,013160275	GATM	0,521594077	0,042984885
PIEZO2	-1,18208656	0,013160275	FXYD4	3,180179625	0,042984885
SLITRK5	-1,053308863	0,015640425	ZFP28	-1,866547884	0,045979659
SYNDIG1	0,893507731	0,015866611	DDOST	-0,696025419	0,047060967
LIF	-0,657896325	0,017074581	PRDM8	-1,346055025	0,047060967
LRTM1	-0,907971475	0,017074581	SLA	1,287085305	0,048993455