The E3 SUMO ligase Nse2 regulates sumoylation and nuclear-to-cytoplasmic translocation of skNAC-Smyd1 in myogenesis

Running title: Smyd1 sumoylation in myogenesis

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Summary

Skeletal and heart muscle-specific variant of the alpha subunit of nascent polypeptide associated complex (skNAC) is exclusively found in striated muscle cells. Its function, however, is largely unknown. Previous reports could demonstrate that skNAC binds to Smyd1/m-Bop, a multi-functional protein regulating myogenesis both via the control of transcription and the modulation of sarcomerogenesis, and that both proteins undergo nuclear-to-cytoplasmic translocation at later stages of myogenic differentiation. Here, we show that skNAC binds to the E3 SUMO ligase mammalian Mms21/Nse2 and that knockdown of *Nse2* expression inhibits specific aspects of myogenic differentiation, accompanied by a partial blockade of the nuclear-to-cytoplasmic translocation of the skNAC/Smyd1 complex, retention of the complex in PML-like nuclear bodies, and disturbed sarcomerogenesis. In addition, we show that the skNAC interaction partner Smyd1 contains a putative sumoylation motif and is sumoylated in muscle cells, with depletion of Mms21/Nse2 leading to reduced concentrations of sumoylated Smyd1. Taken together, our data suggest that the function, specifically the balance between nuclear and cytosolic roles of the skNAC/Smyd1 complex, might be regulated by sumoylation.

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Introduction

Skeletal and heart muscle-specific variant of the alpha subunit of nascent polypeptide associated complex (skNAC) is encoded by a splice variant of the alpha subunit of nascent polypeptide associated complex, a ubiquitous protein which plays a role in the targeting of newly synthesized polypeptide chains within the cell (Wiedmann *et al.*, 1994).

In contrast to αNAC, skNAC is exclusively found in skeletal and heart muscle cells. Its function, so far, is still enigmatic.

In 1996, Yotov and St.-Arnaud could show that skNAC enhances transcription of the *Myoglobin* gene, suggesting that the protein might act as a transcriptional (co)activator for this gene and that it might be involved in the regulation of oxidative versus glycolytic metabolism in muscle fibers, namely, fiber type specification.

In addition, we and others could previously show that the *skNAC* gene is not expressed in proliferating myoblasts, however, expression is induced after the induction of myogenic differentiation or during muscle regeneration (Yotov and St.-Arnaud, 1996, Munz *et al.*, 1999). Furthermore, *skNAC*-deficient mice are characterized by cardiac defects and also by skeletal muscle hypotrophy, aberrant fiber type specification/metabolism, and regeneration defects (Park *et al.*, 2010), suggesting that the skNAC protein might be involved in the regulation of skeletal and heart muscle development, homeostasis, and regeneration.

Finally, a function for skNAC in transcriptional regulation is also suggested by the 2002 paper of Sims *et al.*: The authors showed for the first time that skNAC binds to the mBop/Smyd1 protein, a SET-domain-containing histone methyltransferase which also contains a MYND domain known to be involved in the recruitment of HDACs. Interestingly, the skNAC/Smyd1 complex localizes to the nucleus in early-differentiating myoblasts and then translocates to the cytosol at later stages of differentiation, where at least Smyd1 is physically associated with sarcomeric structures (Just *et al.*, 2011, Li *et al.*, 2011), suggesting that besides regulating transcription, the proteins might also exert a cytoplasmic function at later stages of myogenesis.

In fact, in 2009, Li and colleagues could demonstrate that zebrafish embryos in which *skNAC* expression had been knocked down by a morpholino antisense oligonucleotide-mediated approach were paralyzed and showed no heartbeat. This was most likely due to defects in myofibrillogenesis and/or sarcomerogenesis: Embryos in which *skNAC* expression had been knocked down showed irregular distribution of actin and myosin filaments, and no formation of well-structured sarcomeres. Correspondingly, we could recently demonstrate defective sarcomerogenesis in murine C2C12 myoblasts after skNAC depletion using an siRNA-mediated approach (Berger *et al.*, 2012, Berkholz *et al.*, 2013). These data implicate skNAC in the regulation of sarcomere formation.

In addition, an essential role in the regulation of sarcomere assembly has recently also been demonstrated for the skNAC binding partner Smyd1 (Tan *et al.*, 2006, Just *et al.*, 2011, Li *et al.*, 2011, Li *et al.*, 2013), indicating that the two proteins operate together in the control of myofibrillogenesis and sarcomerogenesis.

Thus, given potential functions of the skNAC/Smyd1 complex in both the nuclear and the cytoplasmic compartments, we wondered how the translocation of the two proteins to the cytoplasm in later myogenesis might be regulated. One possibility is sumoylation.

Sumoylation is a process in which the small proteins SUMO-1, -2, and -3 become covalently attached to their target proteins by the sequential action of three enzymes or enzyme families, the E1, the E2, and the E3 SUMO ligases. In contrast to the mechanistically similar process of protein ubiquitination, however, sumoylation does not target the respective proteins for proteasomal decay, but plays an important role in the regulation of various cellular functions, such as the modulation of gene expression or the regulation of nucleocytoplasmatic shuttling (for review, see Garcia-Dominguez *et al.*, 2009, Hannoun *et al.*, 2010).

Little is known about possible functions of protein sumoylation in skeletal muscle cells. However, Riquelme *et al.* (2006b) could demonstrate that blocking expression of the *Ubc9* gene, which encodes the only E2 SUMO ligase known to date, strongly inhibits the terminal differentiation of C2C12 myoblasts. These data suggested for the first time that sumoylation might play an important regulatory role in myogenesis.

Indeed, specific sumoylation targets in mammalian skeletal muscle cells have been identified, such as the GLUT1 and GLUT4 glucose transporters (Giorgino *et al.*, 2000), suggesting a role in insulin resistance (Kampmann *et al.*, 2011), the myocyte enhancer factor-2 (MEF2) family of transcription factors (Grégoire and Yang, 2005, Kang *et al.*, 2006, Riquelme *et al.*, 2006a), the myogenesis-inducing protein SnoN (Hsu *et al.*, 2006, Wrighton *et al.*, 2007), the PPARγ nuclear receptor (Chung *et al.*, 2011a), or – most recently – the myogenic determination factor PAX7 (Luan *et al.*, 2012), and the basic helix-loop-helix transcription factor Sharp-1 (Wang *et al.*, 2013). In addition, it has been known for quite a while that sumoylation increases the stability of histone deacetylases (HDACs) (for review, see Garcia-Dominquez *et al.*, 2009), which might result in the repression of the genes encoding the MEF2 class of myogenic transcription factors (for review, see Potthoff and Olson, 2007, Glass, 2007). Finally, specific interaction with certain E3 SUMO ligases, without subsequent sumoylation, appears to regulate binding affinity for particular targets, such as in the case of the homeobox transcription factor Msx1, which needs binding to the E3 SUMO ligase PIAS1 in order to suppress the expression of myogenic regulatory genes such as MyoD (Lee *et al.*, 2006).

Here, we demonstrate that skNAC binds to the E3 SUMO ligase Nse2 and that inhibition of *Nse2* expression represses specific aspects of skeletal muscle differentiation, such as expression of the *MyHC* myogenic marker and the formation of well-structured sarcomeres, and reduces nucleocytoplasmic translocation of the skNAC/Smyd1 complex. In addition, we show that the skNAC binding partner Smyd1/mBop can get sumoylated in skeletal muscle cells and might thus represent a potential Nse2 target. Taken together, our data suggest that sumoylation plays an important role in balancing the nuclear and the cytosolic functions of the skNAC/Smyd1 complex.

Results

skNAC binds to the SUMO ligase mammalian Mms21/Nse2. When searching for novel skNAC binding partners, we inadvertently observed that in C2C12 murine myoblasts, this protein binds to the E3 SUMO ligase Nse2/mammalian homolog of yeast Mms21. The interaction was confirmed via co-immunoprecipitation of recombinant, FLAG-tagged skNAC truncation mutants and endogenous Nse2 protein. Particularly, we found that a fragment located at the carboxyterminal end of the skNAC-specific middle domain (aa 1712-1995) specifically bound to the endogenous Nse2 protein. By contrast, an skNAC fragment located more aminoterminally only weakly bound to Nse2 when overexpressed at very high levels (data not shown). Two unrelated, FLAG-tagged proteins (pFLAG-CMV-NADK and pFLAG-CMV-LRP6$_{\text{cytoplasmic domain}}$) did not bind to Nse2 (Fig.1). *Nse2* was broadly expressed in mammalian tissues and organs, with particularly high expression levels in skeletal and heart muscle, liver, skin, and brain (data not shown).

Expression of the *Nse2* gene declines during myogenic differentiation. To analyze the expression kinetics of the *Nse2* gene in myogenesis, C2C12 cells were differentiated *in vitro* and *Nse2* expression was determined via Northern blot (Fig.2A), semi-quantitative RT-PCR analysis (Fig.2B), and Western blot (Fig.2C). In addition, we studied the question whether depletion of skNAC or its other known binding partner Smyd1 has a detectable effect on *Nse2* expression. Overall, we found that *Nse2* expression was first slightly induced, peaking at t=48h after the induction of differentiation, and then gradually declined both at the mRNA and at the protein level. Inhibition of *skNAC* and *Smyd1* expression using specific siRNAs had no effect on *Nse2* expression levels at all time points analyzed (24-120 h after the induction of differentiation), when compared to the respective controls.

Nse2 translocates to the cytoplasm during skeletal muscle cell differentiation. When analyzing the intracellular localization of the Nse2 protein in skeletal muscle cells using immunofluorescence analysis, we observed staining of both the nuclear and the cytoplasmic region in proliferating and early-differentiating myoblasts and myotubes. Interestingly, in most cells, the nuclear staining was characterized by small dots and staining of the perinuclear region was particularly strong. Soon after the induction of myogenic differentiation, however, overall nuclear

staining became weaker. In differentiated myotubes, finally, nuclear staining was almost absent. By contrast, strong cytoplasmic staining could be detected, indicating a nuclear-to-cytosolic translocation of the Nse2 protein in myogenesis, reminiscent of the skNAC/Smyd1 intracellular translocation in myogenic differentiation (Fig.2D). To prove the nuclear-to-cytosolic translocation of the Nse2 protein, C2C12 cells were again induced to differentiate and nuclear proteins were separated from the cytosolic fraction. As shown in Fig.2E, using Western blot analysis, we could indeed demonstrate a strong decline of nuclear Nse2 protein within the first five days after the induction of differentiation.

Inhibition of *Nse2* expression influences expression of genes encoding myogenic differentiation markers, and of skNAC target genes. To functionally study a potential role of the Nse2 protein in myogenesis, we blocked expression of the respective gene in C2C12 cells using specific siRNAs. As shown in Fig.3A, using this approach, we could inhibit *Nse2* expression in these cells at the RNA and at the protein level by about 60%. To exclude off-target effects, two different Nse2 siRNA species were employed and all major results were reproduced with both species. Morphologically, cells in which *Nse2* expression had been knocked down appeared normal throughout the differentiation process (Fig.3B). Nevertheless, expression of certain myogenic differentiation markers, such as *MyoD* or *MyHC*, as well as *Smyd1*, was reduced at the mRNA level, whereas expression of others was unchanged or induced (Fig.3C). Since we could recently demonstrate a role for skNAC in the control of sarcomerogenesis in mammalian cells (Berger *et al.*, 2012, Berkholz *et al.*, 2013), we hypothesized that, given an interaction between skNAC and Nse2, the latter might also be involved in the regulation of sarcomerogenesis. Indeed, when we analyzed the intracellular staining pattern of α-sarcomeric actin and MyHC, the two major sarcomere proteins, in our siRNA-transfected cells, we could detect a diffuse and weak cytoplasmic staining, accompanied by strong staining immediately beneath the plasma membrane. This staining pattern was clearly different from that observed in untransfected or scr-siRNA-transfected controls, in which the typical, homogeneously distributed spotty staining of newly forming sarcomeres could be detected (Fig.3D), and was reminiscent of the MyHC and α-sarcomeric actin staining patterns we previously observed in myoblasts in which *skNAC* expression had been knocked down (Berger *et al.*, 2012, Berkholz *et al.*, 2013). The perturbed

sarcomerogenesis we had previously seen in skNAC siRNA-transfected cells was dependent on an induction of *calpain* gene expression and calpain enzymatic activity (Berger *et al.*, 2012). Calpains are a family of calcium-dependent proteases with a proven role in the regulation of skeletal and heart muscle homeostasis (Sorimachi and Ono, 2012). Thus, we hypothesized that induction of *calpain* expression might also be responsible for the inhibition of proper sarcomere formation in our Nse2 siRNA-transfected cells. Indeed, in these cells, we found a profound upregulation of *calpain 1* gene expression (Fig.3E), similarly as previously demonstrated in the skNAC siRNA-transfected cells, indicating that inhibition of sarcomerogenesis might actually be caused by enhanced calpain proteolytic activity in these cells. To functionally prove this hyopothesis, cells that had been transfected with the Nse2 siRNA were treated with the calpain inhibitor ALLN **(**N-[N-(N-Acetyl-L-leucyl)-L-leucyl]-L-norleucine). As shown in Fig.3F, this treatment indeed rescued the phenotype of defective sarcomerogenesis: The respective α sarcomeric actin staining resembled that seen in untransfected controls.

Inhibition of global protein sumoylation in myoblasts blocks morphological signs of myogenic differentiation. Next, we wondered whether a complete blockade of protein sumoylation to some extent mimicks the effects seen after Nse2 depletion. In fact, when we blocked global protein sumoylation by treating the cells with ginkgolic acid or by transfecting them with siRNA directed against the general E2 SUMO ligase Ubc9 (Fig.4A), we found that the appearance of morphological signs of myogenic differentiation, such as the formation of spindle cell-shaped myocytes and multinucleate myotubes, was reduced and delayed (Fig.4B). Consistently, expression of specific myogenic differentiation markers was repressed (Fig.4C), an effect that has also previously been described by Riquelme *et al.*, 2006b, for cells in which Ubc9 had been knocked down. Interestingly, similarly as in cells that had been transfected with an skNAC-specific siRNA (Berkholz *et al.*, 2013), *calpain* gene expression was strongly induced in the ginkgolic acid-treated or Ubc9-siRNA-transfected cells (Fig.4D). Consistently, sarcomerogenesis was disturbed similarly as in the skNAC- and the Nse2-depleted cells (Fig.4E), indicating that blocking sumoylation influences the expression of skNAC target genes. Taken together, these data suggest that sumoylation indeed plays a central role in the regulation of myogenesis.

Inhibition of *Nse2* expression or global protein sumoylation blocks the nuclear-to-cytoplasmic translocation of the skNAC/Smyd1 complex in myogenic differentiation. To test our initial hypothesis that nuclear-to-cytoplasmic translocation of the skNAC/Smyd1 complex might be regulated by Nse2, C2C12 cells that had been transfected with the Nse2-specific siRNA were analyzed for subcellular localization of the skNAC and the Smyd1 proteins by immunofluorescence staining and by cell fractionation/Western blot. Using this approach, we could detect partial inhibition of the nuclear-to-cytosolic translocation, which is normally seen for both proteins at later stages of myogenesis (Fig.5, A-C). In addition, nuclear skNAC was concentrated in small speckles, whereas cytosolic skNAC was more evenly distributed (Fig.5A). Consistently, when we inhibited global protein sumoylation by treating the cells with ginkgolic acid or transfecting them with Ubc9 siRNA, we observed a similar effect: Cytoplasmic translocation of skNAC was partially inhibited, accompanied by a spotty skNAC staining in the nucleus (Fig.5D,E). Transfection with the Ubc9 siRNA also inhibited the nuclear-to-cytosolic translocation of the Smyd1 protein (Fig.5D). These data suggest that Nse2-dependent sumoylation is important for the nuclear-to-cytosolic translocation of the skNAC/Smyd1 complex.

Nse2 and skNAC colocalize with PML nuclear bodies. The spotty nuclear staining of Nse2, skNAC, and Smyd1 is reminiscent of the pattern seen when staining for PML nuclear bodies. Thus, we tested whether Nse2 and skNAC colocalize with the PML protein using double immunofluorescence staining, which was indeed the case (Fig.6A,B). In addition, when transfecting a vector encoding a GFP-SUMO-1 fusion protein, we could also co-localize skNAC with SUMO-1 protein.

mBop/Smyd1 contains a putative sumoylation motif and is sumoylated in muscle cells. Given the known SUMO ligase activity of Nse2 as well as its binding to the skNAC-Smyd1 complex, we aimed at identifying a potential specific Nse2 sumoylation target within this system. Whereas skNAC itself does not contain a classical sumoylation site, a sumoylation consensus motif (LKDD) can be found in the Smyd1 sequence and is well-conserved in mammals (Fig.7A), indicating that this protein might be a potential sumoylation target. And indeed, we could pull out sumoylated Smyd1 from C2C12 lysates using affinity chromatography with an immobilized anti-SUMO-1 antibody (Fig.7B). To prove the hypothesis that Smyd1 is sumoylated in skeletal muscle cells, we immunoprecipitated the protein from C2C12

lysates and analyzed protein sumoylation by Western blot. Interestingly, when we co-transfected SUMO-1 and Smyd1, we observed a strong signal for sumoylated Smyd1, which was almost completely repressed when we co-transfected an expression plasmid for the SUMO protease SuPr1 in addition. Furthermore, when we blocked *Nse2* expression by around 60% in the *SUMO-1/Smyd1*-overexpressing cells using specific siRNAs, concentrations of sumoylated Smyd1 were much lower than in cells without Nse2 depletion 48h after the induction of differentiation (Fig.7C). Taken together, these data suggest that Smyd1 is sumoylated and that this sumoylation is dependent on the presence of Nse2, indicating that Smyd1 might indeed be an Nse2 target.

Discussion

skNAC and its known binding partner Smyd1 have been demonstrated to regulate myogenesis, specifically skeletal myoblast migration and sarcomerogenesis (Tan *et al.*, 2006, Li *et al.*, 2009, Li *et al.*, 2011, Just *et al.*, 2011, Berger *et al.*, 2012, Berkholz *et al.*, 2013, Li *et al.*, 2013). Since both proteins undergo nuclear-to-cytoplasmic translocation during myogenesis (Sims *et al.*, 2002), it is likely that they fulfill specific functions in both cellular compartments: In early myogenesis, they might act as transcriptional modulators, presumably via two specific domains within the Smyd1 protein – the so-called SET domain, a histone methyltransferase moiety, and the MYND domain, a potential recruiter of histone deacetylases (HDACs). Later in myogenesis, the skNAC-Smyd1 complex might have specific functions in the cytoplasm, namely the control of sarcomerogenesis.

Our finding that skNAC binds to the E3 SUMO ligase Nse2 indicates that sumoylation might directly or indirectly be involved in the regulation of the biological activities of skNAC and/or Smyd1, or that skNAC modulates Nse2 functions. Specifically, it is possible that skNAC's transcriptional coactivator and/or its sarcomere-organizing function might be regulated by sumoylation or that skNAC binding to Nse2 might stimulate or repress Nse2 sumoylation activity or its binding to other factors. Alternatively, since sumoylation plays an important role in the regulation of nucleocytoplasmic shuttling (for review, see Zhao, 2007), and since the skNAC-Smyd1 complex is known to translocate from the nucleus to the cytoplasm at later stages of myogenic differentiation (Sims *et al.*, 2002), it is possible that Nse2 might be involved in the regulation of this process.

The fact that a considerable amount of Nse2 protein localizes to the nucleus (and also to the perinuclear region) in proliferating and early-differentiating myoblasts and translocates to the cytosol at later stages is intriguing, since this pattern is reminiscent of the nuclear-to-cytoplasmic translocation described by Sims *et al.*, 2002, for the skNAC/Smyd1 complex, suggesting that these three proteins might indeed operate as a complex. Upon inhibition of *Nse2* expression in myoblasts, we found reduced and delayed expression of myogenic differentiation markers, such as *MyoD* or *Myosin heavy chain*, despite the fact that myotube formation proceeded more or less normally. Interestingly, expression of the *Smyd1* gene was also

reduced, indicating that regulation of this gene was directly or indirectly dependent on Nse2. Since both skNAC and Smyd1 have been implicated in the control of sarcomerogenesis, we also analyzed the staining patterns of α sarcomeric actin and myosin heavy chain in the Nse2 siRNA-transfected cells. We observed a staining pattern reminiscent of that seen in skNAC or Smyd1 siRNA-transfected cells: Weak and diffuse staining of the myotube center, accompanied by a stronger staining of the region immediately beneath the plasma membrane, whereas in control cells, the typical punctuate actin and myosin staining, characteristic for newly forming sarcomeres, was observed. These data indicate that Nse2 is important for sarcomere formation, or, in other words, that sarcomerogenesis cannot proceed normally in the absence of Nse2. Similarly as in skNAC and Smyd1-depleted myoblasts (Berkholz *et al.*, 2013), this appears to be at least in part the result of elevated calpain activities in these cells, since we could also detect enhanced *calpain 1* expression in the Nse2 siRNA-transfected cells. These data suggest that skNAC, Smyd1, and Nse2 operate together within a specific pathway to control myogenesis and, particularly, sarcomerogenesis. So far, however, it is unknown if this is only achieved via the control of *calpain* gene expression, i.e. via skNAC/Smyd1/Nse2 functions in the nucleus, or if cytosolic skNAC/Smyd1/Nse2 at later stages of differentiation also possess a specific role in the control of sarcomerogenesis.

Our finding that inhibition of *Nse2* expression and also repression of total cellular sumoylation blocks the nuclear-to-cytosolic translocation of the skNAC-Smyd1 complex suggests that sumoylation might control the balance between nuclear and cytosolic functions of this protein complex.

Since total skNAC levels remained unchanged after both Nse2 siRNA transfection (Fig.3C), or Ubc9 siRNA transfection / ginkgolic acid treatment (Fig.4C), it is unlikely that the different skNAC staining patterns are the result of different expression levels of the *skNAC* gene. For Smyd1, we cannot totally exclude this, since Smyd1 expression was repressed by around 50% under all three conditions.

Interestingly, the nuclear staining pattern of Nse2 in early myogenesis showed a speckled pattern, with strong staining of the perinuclear region in some cells. This pattern resembles that seen for PML nuclear bodies: These are nuclear matrix-associated domains that recruit a variety of different proteins, are organizers of key

nuclear functions such as replication, transcription, DNA repair, and epigenetic silencing, and, most remarkably, are centers of protein sumoylation (for review, see Lallemand-Breitenbach and de Thé, 2010). Indeed, we could colocalize Nse2 and skNAC with PMLs. Since the yeast Nse2 homologue, Mms21, as well as mammalian Nse2 have been implicated in DNA repair, recombination, and chromosome cohesion and telomere integrity (for review, see Potts, 2009, Stephan *et al.*, 2011), and since Mms21/Nse2 is a key component of PMLs (Potts and Yu, 2007, Brouwer *et al.*, 2009, Chung *et al.*, 2011b), it seems likely that Nse2, skNAC, and Smyd1 function as a PML-associated complex in early myogenesis.

Most remarkably, skNAC and Smyd1, which – upon Nse2 depletion or inhibition of sumoylation - were retained in the nucleus even at later stages of myogenesis, and persisted in PML body-like structures, suggesting that their release from these structures was somehow inhibited by the absence of Nse2 or a global blockade of sumoylation. Interestingly, we could demonstrate that at earlier stages of myogenesis, skNAC, Smyd1, and Nse2 indeed colocalize with SUMO-1 within these speckles. Thus, in the future, it will be crucial to determine the mechanism by which sumoylation might be involved in the regulation of the nuclear-to-cytosolic translocation of the skNAC / Smyd1 complex and thereby in the control of its potential subcellular-compartment-specific. Interestingly, a specific role for Mms21, the yeast Nse2 homolog, in the regulation of nucleocytoplasmic shuttling via the import receptor Kap114, has recently been demonstrated (Rothenbusch *et al.*, 2012, Werner and Melchior, 2012).

A search for putative sumoylation sites in both the skNAC and the Smyd1 sequences revealed the presence of such a site within the Smyd1, but not within the skNAC sequence and prompted us to test whether Smyd1 might be sumoylated. We found that Smyd1 is indeed sumoylated in skeletal muscle cells, an effect that was dependent on the presence of Nse2. Thus, in the future, it will be interesting to eliminate this sumoylation motif using site-directed mutagenesis and thereby analyze the specific functions of Smyd1 sumoylation in myogenesis in more detail.

Finally, most recently and remarkably, Simpson-Lavy and Johnston, 2013, could demonstrate that the yeast Nse2 homolog, Mms21, can sumoylate the SNF1 protein, which is the yeast homolog of the mammalian AMP-activated protein kinase

(AMPK), thereby inhibiting its catalytic activity. Since in mammalian skeletal muscle cells, AMPK is a central player in the regulation of cell metabolism and fiber type specification, and since skNAC has been implicated in the control of fiber type specification (Yotov and St.-Arnaud, 1996, Park *et al.*, 2010, J. Berkholz and B. Munz, unpublished data), this finding suggests an interesting link between Nse2/Mms21 and skeletal muscle plasticity.

Taken together, we provide evidence for the existence of an skNAC-Smyd1-Nse2 complex, in which Smyd1 is sumoylated in an Nse2-dependent manner. We show data suggesting that this sumoylation is important for the nuclear-to-cytosolic translocation of the skNAC/Smyd1 complex at later stages of myogenesis and might thus be crucial with respect to balancing the functions of this protein complex in both cellular compartments (Fig.8).

Materials and Methods

Tissue culture. Murine C2C12 cells were cultured in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum (growth medium) at 37°C and 5% CO_2. To induce differentiation, cells were grown to 80 to 90% confluence and then switched to differentiation medium (Dulbecco's modified Eagle's medium containing 2% horse serum).

Treatment of cells with ginkgolic acid. Ginkgolic Acid (50 µM) was added to cells in parallel to switching them to differentiation medium. The optimal ginkgolic acid concentration was determined in a series of dose-response analyses prior to the actual experiments.

ALLN treatment of cells. The calpain inhibitor ALLN (N-[N-(N-Acetyl-L-leucyl)-L-leucyl]-L-norleucine) (50 µM), was added 48h after the induction of differentiation and cells were then fixed for immunofluorescence analysis after a total of 96h.

Transfection with expression vectors. For transient transfection of expression vectors into C2C12 cells, the "TurboFect" reagent (Fermentas) was employed according to the manufacturer's instructions. Transfection efficiency was controlled with a GFP expression vector and was 40-50% in all experiments. pCI-skNAC and pBK-CMV-Smyd1 were gifts from René St.-Arnaud, Montreal, and Haley Tucker, Austin, Texas, respectively; pcDNA-HA-SUMO-1, pcDNA-HA-Ubc9, pGFP-SUMO-1, and pEGFP-SuPr1 were gifts from Hans Will, Hamburg, Germany.

Co-immunoprecipitation assay. Whole cell extracts were prepared as previously described (Munz et al., 2002). Co-IP analyses were performed with equal amounts of total cellular protein (~500µg), which was incubated in a rotatory shaker with anti-Flag magnetic beads (Sigma Aldrich) at 4°C overnight. Beads were then washed twice with TBS (pH=7.4), followed by addition of 25µl 1x Laemmli buffer (without SDS). Subsequently, the beads were resuspended and boiled for 10min. Samples were separated by SDS-PAGE and analysed by Western blotting. All Co-IP analyses were performed at least three times with samples from at least three independent transfections.

Transfection with specific siRNAs. siRNA transfection was carried out using predesigned, specific siRNAs purchased from Sigma. For inhibition of *Nse2* expression, two different, unrelated siRNA species were employed in parallel to rule out off-target effects: Nse2 siRNA-1: sense: 5'-CAUGGUUGAGUUUGCUAAA-3', antisense: 5'-UUUAGCAAACUCAACCAUG-3'; Nse2 siRNA-2: sense: 5'-GUCUACAAUCAAUCAUGUA-3', antisense: 5'-UACAUGAUUGAUUGUAGAC-3'. For inhibition of *Smyd1*, *skNAC*, and *Ubc9* expression, the following siRNAs were used: Smyd1 siRNA: sense: 5'-CACAUCUUUGGUGUGAUCA-3', antisense: 5'-UGAUCACACCAAAGAUGUG-3'; skNAC siRNA: sense: 5'-GACAGUUCCUGUUGAGAAAUU-3', antisense: 5'-UUUCUCAACAGGAACUGUCUU-3'; Ubc9 siRNA: sense: 5'-CCAUCAAACAGAUCUUAUU-3', antisense: 5'-AAUAAGAUCUGUUUGAUGG-3'. As a negative control, a non-gene-specific, "scrambled" siRNA was employed: scrambled siRNA: sense: 5'-CGUACGCGGAAUACUUCGAUU-3', antisense: 5'-UCGAAGUAUUCCGCGUACGUU-3'. C2C12 cells were transfected using the transfection reagent Interferin (Polyplus Transfection) according to the manufacturer's instructions.

RNA isolation, Northern blot analysis, and qPCR. RNA isolation and Northern blot analysis were carried out as previously described (Munz *et al.*, 2002). Semi-quantitative real time PCR analysis was carried out using the Rotor-Gene 2000 system (Corbett Research, Mortlake, Australia). Gene expression was analyzed using the GoTaq qPCR Master Mix (Promega). For detection of calpain 1 and 3, skNAC, Smyd1, and Nse2, self- and pre-designed primers (Qiagen QuantiTect Primer Assays) were used. For detection of MyHCI, only pre-designed primers were employed. Primer sequences are listed in Table 1. In each experiment, melting curve analysis was performed to verify that a single transcript was produced. RT-qPCR relative gene expression was calculated using the comparative CT ($2^{-\Delta\Delta C}{}_{\mathrm{T}}$) method, where expression was normalized to GAPDH. Non-RT- and non-template controls were run for all reactions. Unless otherwise specified, data from at least three independent experiments were expressed as means ± SEM, n=3–5. Significance was accepted at P<0.05.

Western blot analysis and immunofluorescence. Western blot and immunofluorescence analyses were carried out as previously described (Munz et al.,

2002), specifically, 20mM N-ethylmaleimide was added to the cell lysis buffer to inbibit de-sumoylation (Riquelme et al., 2006a,b). Cytoplasmic and nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo scientific) according to manufacturer's instructions. Confocal laser scanning microscopy was performed with a Zeiss LSM 700 confocal microscope (Carl Zeiss Microscopy GmbH, Germany), equipped with a 63× glycerin immersion lens. Digital images were processed using the Zeiss LSM ZEN software 2010 (Carl Zeiss). All antibodies employed in the study are listed in Table 2. For confocal microscopy, secondary antibodies coupled with Alexa Fluor dyes were used. All immunoblots were performed at least three times with samples from at least three independent transfections.

Purification of sumoylated proteins. To isolate sumoylated proteins from crude cell lysates, the SUMO-Qapture-T Kit (Enzo Life Sciences) was used according to the manufacturer's instructions.

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Conflict of interest

The authors declare no conflict of interest.

References

Berger, F., Berkholz, J., Breustedt, T., Ploen, D. and Munz, B. (2012). Skeletal muscle-specific variant of nascent polypeptide associated complex alpha (skNAC): Implications for a specific role in mammalian myoblast differentiation. *Eur. J. Cell Biol.* **91**, 150-155.

Berkholz, J., Zakrzewicz, A. and Munz, B. (2013). skNAC depletion stimulates myoblast migration and perturbs sarcomerogenesis by enhancing calpain 1 and 3 activity. *Biochem. J.* **453**, 303-310.

Brouwer, A.K., Schimmel, J., Wiegant, J.C., Vertegaal, A.C., Tanke, H.J. and Dirks, R.W. (2009). Telomeric DNA mediates de novo PML body formation. *Mol. Biol. Cell* **20**, 4804-4815.

Chung, S.S., Ahn, B.Y., Kim, M., Kho, J.H., Jung, H.S. and Park, K.S. (2011a). SUMO modification selectively regulates transcriptional activity of peroxisome-proliferator-activated receptor γ in C2C12 myotubes. *Biochem. J.* **433**, 155-161.

Chung, I., Leonhardt, H. and Rippe, K. (2011b). De novo assembly of a PML nuclear subcompartment occurs through multiple pathways and induces telomere elongation. *J. Cell Sci.* **124**, 3603-3618.

Garcia-Dominguez, M. and Reyes, J.C. (2009). SUMO association with repressor complexes, emerging routes for transcriptional control. *Biochim. Biophys. Acta* **1789**, 451-459.

Giorgino, F., de Robertis, O., Laviola, L., Montrone, C., Perrini, S., McCowen, K.C. and Smith, R.J. (2000). The sentrin-conjugating enzyme mUbc9 interacts with GLUT4 and GLUT1 glucose transporters and regulates transporter levels in skeletal muscle cells. *Proc. Natl. Acad. Sci. USA* **97**, 1125-1130.

Glass, D.J. (2007). Two tales concerning skeletal muscle. *J. Clin. Invest.* **117**, 2388-2391.

Grégoire, S. and Yang, X.J. (2005). Association with class IIa histone deacetylases upregulates the sumoylation of MEF2 transcription factors. *Mol. Cell. Biol.* **25**, 2273-2287.

Hannoun, Z., Greenhough, S., Jaffray, E., Hay, R.T. and Hay, D.C. (2010). Post-translational modification by SUMO. *Toxicology* **278**, 288-293.

Hsu, Y.H., Sarker, K.P., Pot, I., Chan, A., Netherton, S.J. and Bonni, S. (2006). Sumoylated SnoN represses transcription in a promoter-specific manner. *J. Biol. Chem.* **281**, 33008-33018.

Just, S., Meder, B., Berger, I.M., Etard, C., Trano, N., Patzel, E., Hassel, D., Marquart, S., Dahme, T., Vogel, B., Fishman, M.C., Katus, H.A., Strähle, U. and Rottbauer, W. (2011). The myosin-interacting protein SMYD1 is essential for sarcomere organization. *J. Cell Sci.* 124, 3127-3136.

Kampmann, U., Christensen, B., Nielsen, T.S., Pedersen, S.B., Ørskov, L., Lund, S., Møller, N. and Jessen, N. (2011). GLUT4 and UBC9 protein expression is reduced in muscle from type 2 diabetic patients with severe insulin resistance. PLoS *One* **6**(11):e27854.

Kang, J., Gocke, C.B. and Yu, H. (2006). Phosphorylation-facilitated sumoylation of MEF2C negatively regulates its transcriptional activity. *BMC Biochem.* Feb. 14, **7**:5.

Lallemand-Breitenbach, V. and de Thé, H. (2010). PML nuclear bodies. *Cold Spring Harb. Perpect. Biol.* **2**, a000661.

Lee, H., Quinn, J.C., Prasanth, K.V., Swiss, V.A., Economides, K.D., Camacho, M.M., Spector, D.L. and Abate-Shen, C. (2006). PIAS1 confers DNA-binding specificity on the Msx1 homeoprotein. *Genes Dev.* **20**, 784-794.

Li, H., Randall, W.R. and Du, S.J. (2009). skNAC (skeletal Naca), a muscle-specific isoform of Naca (nascent polypeptide-associated complex alpha), is required for myofibril organization. *FASEB J.* **23**, 1988-2000.

Li, H., Xu, J., Bian, Y.H., Rotilant, P., Shen, T., Chu, W., Zhang, J., Schneider, M. and Du, S.J. (2011). Smyd1b_tv1, a key regulator of sarcomere assembly, is localized on the M-line of skeletal muscle fibers. *PLoS One* **6**(12) e28524

Li, H., Zhong, Y., Wang, Z., Gao, J., Xu, J., Chu, W., Zhang, J., Fang, S. and Du, S. (2013). Smyd1b is required for skeletal and cardiac muscle function in zebrafish. *Mol. Biol. Cell* **24**, 3511-3521.

Luan, Z., Liu, Y., Stuhlmiller, T.J., Marquez, J. and Garcia-Castro, M.I. (2012). SUMOylation of Pax7 is essential for neural crest and muscle development. *Cell. Mol. Life Sci.*, 2012 Dec 18.

Munz, B., Wiedmann, M., Lochmüller, H. and Werner, S. (1999). Cloning of novel injury-regulated genes. Implications for an important role of the muscle-specific protein skNAC in muscle repair. *J. Biol. Chem.* **274**, 13305-13310.

Munz, B., Hildt, E., Springer, M.L. and Blau, H.M. (2002). RIP2, a checkpoint in myogenic differentiation. *Mol. Cell. Biol.* **22**, 5879-5886.

Park, C.Y., Pierce, S.A., von Drehle, M., Ivey, K.N., Morgan, J.A., Blau, H.M. and Srivastava, D. (2010). skNAC, a Smyd1-interacting transcription factor, is involved in cardiac development and skeletal muscle growth and regeneration. *Proc. Natl. Acad. Sci. USA* **107**, 20750-20155.

Potthoff, M.J. and Olson, E.N. (2007). MEF2: a central regulator of diverse developmental programs. *Development* **134**, 4131-4140.

Potts, P.R. (2009). The Yin and Yang of the MMS21-SMC5/6 SUMO ligase complex in homologous recombination. *DNA Repair* **8**, 499-506.

Potts, P.R. and Yu, H. (2007). The SMC5/6 complex maintains telomere length in ALT cancer cells through SUMOylation of telomere-binding proteins. *Nat. Struct. Mol. Biol.* **14**, 581-590.

Riquelme, C., Barthel, K.K. and Liu, X. (2006a). SUMO-1 modification of MEF2A regulates its transcriptional activity. *J. Cell. Mol. Med.* **10**, 132-144.

Riquelme, C., Barthel, K.K.B., Qin, X.-F. and Liu, X. (2006b). Ubc9 expression is essential for myotube formation in C2C12 cells. *Exp. Cell Res.* **312**, 2132-2141.

Rothenbusch, U., Sawatzki, M., Chang, Y., Caesar, S. and Schlenstedt, G. (2012). Sumoylation regulates Kap114-mediated nuclear transport. *EMBO J.* **31**, 2461-2472.

Sims, R.J. 3rd, Weihe, E.K., Zhu, L., O'Malley, S., Harriss, J.V. and Gottlieb, P.D. (2002). m-Bop, a repressor protein essential for cardiogenesis, interacts with skNAC, a heart- and muscle-specific transcription factor. *J. Biol. Chem.* **277**, 26524-26529.

Simpson-Lavy, K.J. and Johnston, M. (2013). SUMOylation regulates the SNF protein kinase. *Proc. Natl. Acad. Sci. USA* **110**, 17432-17437.

Sorimachi, H. and Ono, Y. (2012). Regulation and physiological roles of the calpain system in muscular disorders. *Cardiovasc. Res.* **96**, 11-22.

Stephan, A.K., Kliszczak, M. and Morrison, C.G. (2011). The Nse2/Mms21 SUMO ligase of the Smc5/6 complex in the maintenance of genome stability. *FEBS Lett.* **585**, 2907-2913.

Tan, X., Rotllant, J., Li, H., De Deyne, P. and Du, S.J. (2006). Smyd1, a histone methyltransferase, is required for myofibril organization and muscle contraction in zebrafish embryos. *Proc. Natl. Acad. Sci. USA* **103**, 2713-2718.

Wang, Y., Shankar, S.R., Kher, D., Ling, B.M.T. and Taneja, R. (2013). Sumoylation of the basic helix-loop-helix transcription factor Sharp-1 regulates recruitment of the histone methyltransferase and function in myogenesis. *J. Biol. Chem.* **288**, 17654-17662.

Werner, A. and Melchior, F. (2012). SUMO unloads the Kap114 cab. *EMBO J.* **31**, 2439-2440.

Wiedmann, B., Sakai, H., Davis, T.A. and Wiedmann, M. (1994). A protein complex required for signal-sequence-specific sorting and translocation. *Nature* **370**, 434-440.

Wrighton, K.H., Liang, M., Bryan, B., Luo, K., Liu, M., Feng, X.H. and Lin, X. (2007). Transforming growth factor-beta-independent regulation of myogenesis by SnoN sumoylation. *J. Biol. Chem.* **282**, 6517-6524.

Yotov, W.V. and St.-Arnaud, R. (1996). Differential splicing-in of a proline-rich exon converts alphaNAC into a muscle-specific transcription factor. *Genes Dev.* **10**, 1763-1772.

Zhao, J. (2007). Sumoylation regulates diverse biological processes. *Cell. Mol. Life Sci.* **64**, 3017-3033.

Figure legends

Fig.1. skNAC binds to the E3 SUMO ligase mammalian Nse2. Co-immunoprecipitation of a FLAG-tagged skNAC fragment (*, aa 1712-1995, refer to schematic drawing) and endogenous Nse2 from lysates of C2C12 murine myoblasts. FLAG-tagged NAD^+ kinase (NADK) was used as a negative control.

Fig.2. *Nse2* expression in myogenesis, with and without inhibition of *skNAC* and *Smyd1* expression.

(A) C2C12 myoblasts were transfected with skNAC- or Smyd1-specific siRNAs, an unspecific ("scrambled") control, or left untreated. Cells were harvested at different time points after the induction of differentiation as indicated, and analyzed for *Nse2* and *skNAC* or *Smyd1* expression via Northern blot. Concentration of the 18S rRNA transcript was analyzed as a control for equal loading (bottom panels).

(B) In parallel, *Nse2* expression was quantified 24 and 96h after the induction of differentiation via qPCR analysis.

(C) 0h, 48h, and 96h after the induction of differentiation, protein lysates were generated from the cells and analyzed for Nse2 protein levels by Western blot. The blot was reprobed with an antibody directed against α-tubulin as a control for equal loading (bottom panel).

(D) Subcellular Nse2 localization was also analyzed in untransfected cells by immunofluorescence and confocal laser sacanning 0h, 48h and 96h after the induction of differentiation using an Nse2-specific antibody. Note the spotty, nuclear Nse2 localization in some cells (arrowheads, 0h and 48h) and the prominent perinuclear staining in others (arrows) (0h), as well as the almost complete nuclear-to-cytosolic translocation of the protein 120h after the induction of differentiation. Representative pictures of at least n=3 independent experiments are shown.

(E) Analysis of subcellular localization of the Nse2 protein via cell fractionation and Western blot. Nuclear/cytoplasmic fractionation was performed during C2C12 differentiation, and Western blotting was performed on 30μg of cell extract for each time point. Membranes were probed with antibodies against Nse2, emerin (a nuclear

marker), and α-tubulin (a cytoplasmic marker). Note that emerin was not detectable in the cytoplasmic and α-tubulin was hardly detectable in the nuclear fraction, indicating effectivity of the fractionation.

Fig.3. Inhibition of *Nse2* expression and effects on myogenic differentiation.

C2C12 cells were transfected with an Nse2-specific siRNA or an unrelated "scrambled") control.

(A) At different time points after the induction of differentiation, total cellular RNA and total protein were isolated from the cells and *Nse2* expression was determined at the mRNA level by qPCR and at the protein level by Western blot as indicated. *: $p<0.05$.

(B) Cell morphology was analyzed in siRNA-transfected cells and in controls by light microscopy 96h after the induction of differentiation. Representative pictures of at least n=3 independent experiments are shown. The cells' fusion index (percent nuclei in cells with at least three nuclei) was also counted (bottom panel; mean with SEM; n=3).

(C) Expression of myogenic differentiation markers was analyzed in siRNA-transfected cells and controls at the RNA and at the protein level 72h after the induction of differentiation as indicated. *: $p<0.05$.

(D) Sarcomerogenesis was analyzed in Nse2 siRNA- and scr-transfected cells by immunofluorescence staining of α sarcomeric actin (top panels), and MyHC (bottom panels) 96h after the induction of differentiation. Representative pictures of at least n=3 independent experiments are shown. DAPI was used to stain and visualize nuclei.

(E) *Calpain 1* expression in Nse2 siRNA-transfected cells and controls was analyzed by qPCR and Western blot analysis 72h after the induction of differentiation as indicated. *: $p<0,05$.

(F) Nse2 siRNA-transfected cells were treated with the calpain inhibitor ALLN **(**N-[N-(N-Acetyl-L-leucyl)-L-leucyl]-L-norleucine) before the induction of differentiation (right panel). Nse2 siRNA-transfected C2C12 cells and scr-transfected controls were

differentiated for 96h. Subsequently, cells were analyzed for α sarcomeric actin intracellular distribution by confocal microscopy. Nuclei were stained with DAPI.

Fig.4. Global inhibition of sumoylation using ginkgolic acid or transfection with a specific siRNA directed against Ubc9 inhibits some aspects of myogenic differentiation.

C2C12 myoblasts were transfected with a specific siRNA directed against the sole E2 SUMO ligase Ubc9 or treated with ginkgolic acid. Cells transfected with an unspecific ("scrambled") control siRNA or treated with an equal amount of the ginkgolic acid solvent DMSO served as controls as indicated.

(A) Sumoylated proteins were detected in total cellular lysates by Western blot 48h after the induction of differentiation as indicated. A band most likely corresponding to sumoylated RanGAP1 is indicated. In the cells that had been transfected with the Ubc9-specific siRNA, *Ubc9* expression was also quantified by qPCR to confirm efficiency of repression. *: $p<0.05$.

(B) 96h after the induction of differentiation, cell morphology was analyzed by light microscopy. Note the reduced formation of multinucleate myotubes in the cells that had been treated with ginkgolic acid or transfected with the Ubc9-specific siRNA. In addition, the cells' fusion index (percent nuclei in cells with at least three nuclei) was counted; mean with SEM; n=3). Representative pictures of at least n=3 independent experiments are shown.

(C) 72h after the induction of differentiation, expression of specific myogenic differentiation markers was analyzed in the cells by qPCR and Western blot analysis as indicated. The two bands representing the Smyd1 signal on the Western blot are both specific and might represent differentially sumoylated species.

(D) *Calpain 1* and *3* gene expression was analyzed in ginkgolic acid-treated or Ubc9 siRNA-transfected cells by qPCR 72h after the induction of differentiation. In the ginkgolic acid-treated cells, *calpain 1* expression was also analyzed by Western blot as indicated. *: $p<0.05$.

(E) Sarcomerogenesis was analyzed in Ubc9 siRNA-transfected cells via immunofluorescence and confocal laser scanning analysis of α sarcomeric actin and MyHC 96h after the induction of differentiation. Note the strong staining immediately beneath the plasma membrane in contrast to the weak and diffuse staining of the cell center in the myotubes that had been transfected with the Ubc9 siRNA, indicative of disturbed sarcomerogenesis. Nuclei were stained with DAPI. Representative pictures of at least n=3 independent experiments are shown.

Fig.5. Depletion of Nse2 or inhibition of global protein sumoylation partially blocks the nuclear-to-cytosolic translocation of skNAC and Smyd1.

C2C12 myoblasts were transfected with an Nse2-specific siRNA and differentiated for 96h. Subsequently, subcellular localization of the skNAC and Smyd1 proteins was analyzed via immunofluorescence/confocal laser scanning using specific antibodies (A,B), or via cell fractionation and Western blot with emerin and α-tubulin as loading controls (C). Note the prominent, spotty nuclear skNAC staining in the cells that had been transfected with the Nse2-specific siRNA (A). A similar pattern was observed when staining with a Smyd1-specific antibody (B). (D) and (E) skNAC and Smyd1 were also partially retained in the nucleus when blocking global sumoylation via Ubc9 siRNA transfection (D), or ginkgolic acid treatment (E, only analyzed for skNAC). The bottom panels in (A), (B), and (E) show higher magnifications. Representative pictures of at least n=3 independent experiments are shown.

Fig.6. Colocalization of Nse2 (A) and skNAC (B) with PML nuclear bodies and SUMO-1 (C).

C2C12 cells were co-stained with PML (green) and Nse2/skNAC (red) 0h (A, Nse2) or 48h (B, skNAC) after the induction of differentiation. Cells co-transfected with pGFP-SUMO-1 and pCI-skNAC were switched to DM for 48h and then co-stained (C). Superimposing the two colors (merge) results in a yellow signal, where both proteins co-localize.

Fig.7. Smyd1 is sumoylated and might be a Nse2 target.

(A) Alignment of the Smyd1 proteins of different species; potential sumoylation motifs are indicated. Note the well-conserved LKDD motif and the additional alternative sumoylation motif found in the *m. musculus* sequence.

(B) SUMO assay. Affinity chromatography with immobilized SUMO-1 antibody. C2C12 cells were transfected with expression vectors as indicated and differentiated for 48h. Subsequently, lysates were purified via affinity chromatography for SUMO-1. Total cellular lysates ("input") and sumoylated proteins ("eluate") were analyzed by Western blot as indicated.

(C) pBK-CMV-Smyd1 and pcDNA-HA-SUMO-1HA expression plasmids were co-transfected into C2C12 cells. pcDNA-HA-Ubc9 was cotransfected in parallel as a negative control. 48h after the induction of differentiation, immunoprecipitation was carried out using an HA-specific antibody and sumoylated Smyd1 was detected by Western blot using a Smyd1-specific antibody. HA-SUMO-1 and HA-Ubc9 both run at 17-18kD. Note the strong decline in the signal specific for sumoylated Smyd1 when cells were co-transfected with Nse2-specific siRNA or a SuPr1 expression vector (pEGFP-SuPr1).

Fig.8. Partially hypothetic Model of skNAC, Smyd1, and Nse2 functions in muscle cells. In early myogenesis, skNAC and Smyd1 are found in the nucleus, where they might regulate transcription, presumably by influencing histone methylation via Smyd1's SET domain, and potentially histone deacetylation via Smyd1's MYND domain. Particularly, the skNAC/Smyd1 complex directly or indirectly inhibits transcription of the genes encoding calpains 1 and 3, the latter being potent inhibitors of sarcomere formation (Berkholz *et al.*, 2013, and references therein). At later stages of myogenesis, Nse2 binds to the skNAC component of the complex and sumoylates Smyd1, which allows translocation of the complex into the cytoplasm, where at least Smyd1 physically associates with sarcomeric structures (Just *et al.*, 2011, Li *et al.*, 2011) and might thus also directly be involved in the regulation of sarcomerogenesis.

Fig.1

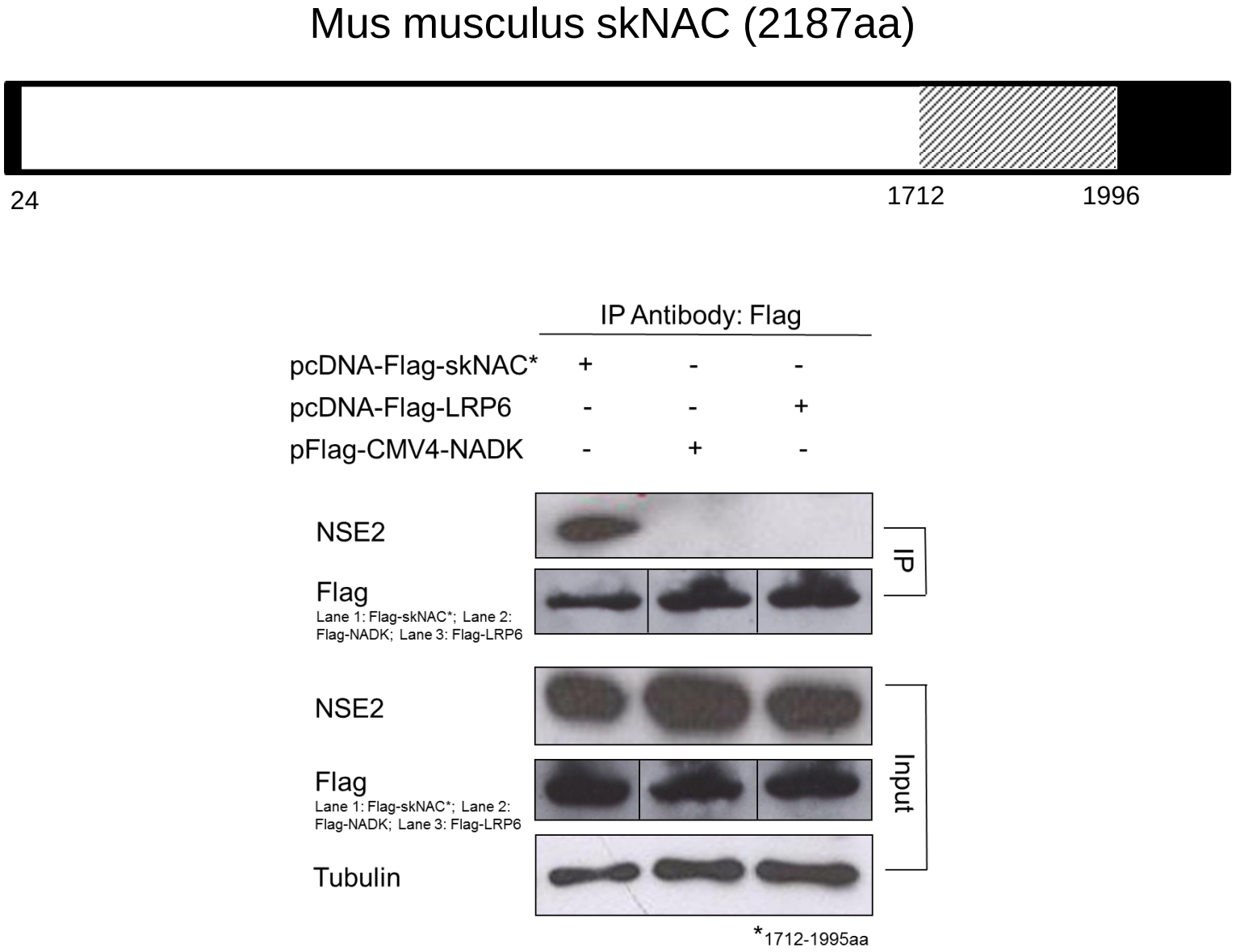


Fig.2

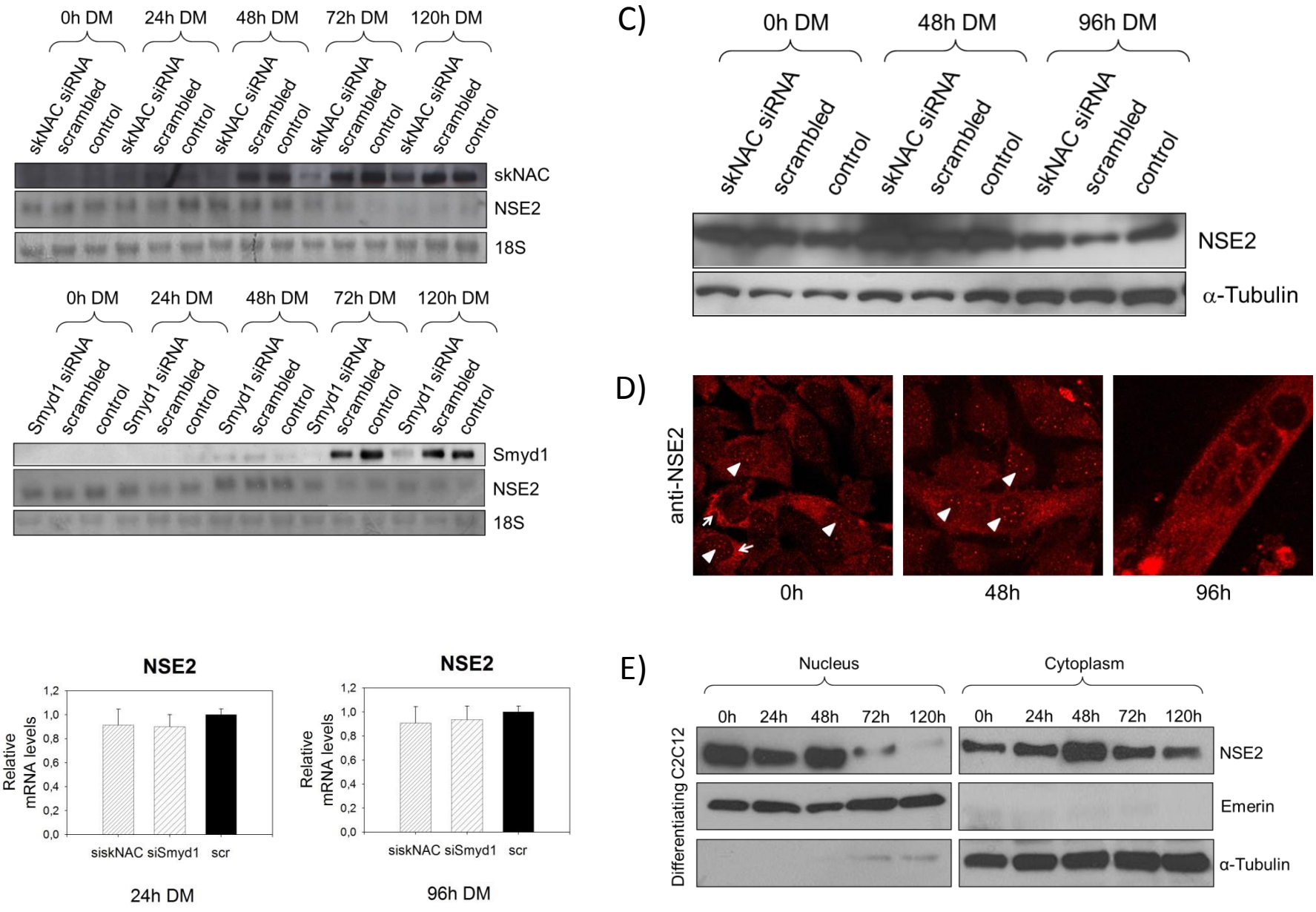


Fig.3

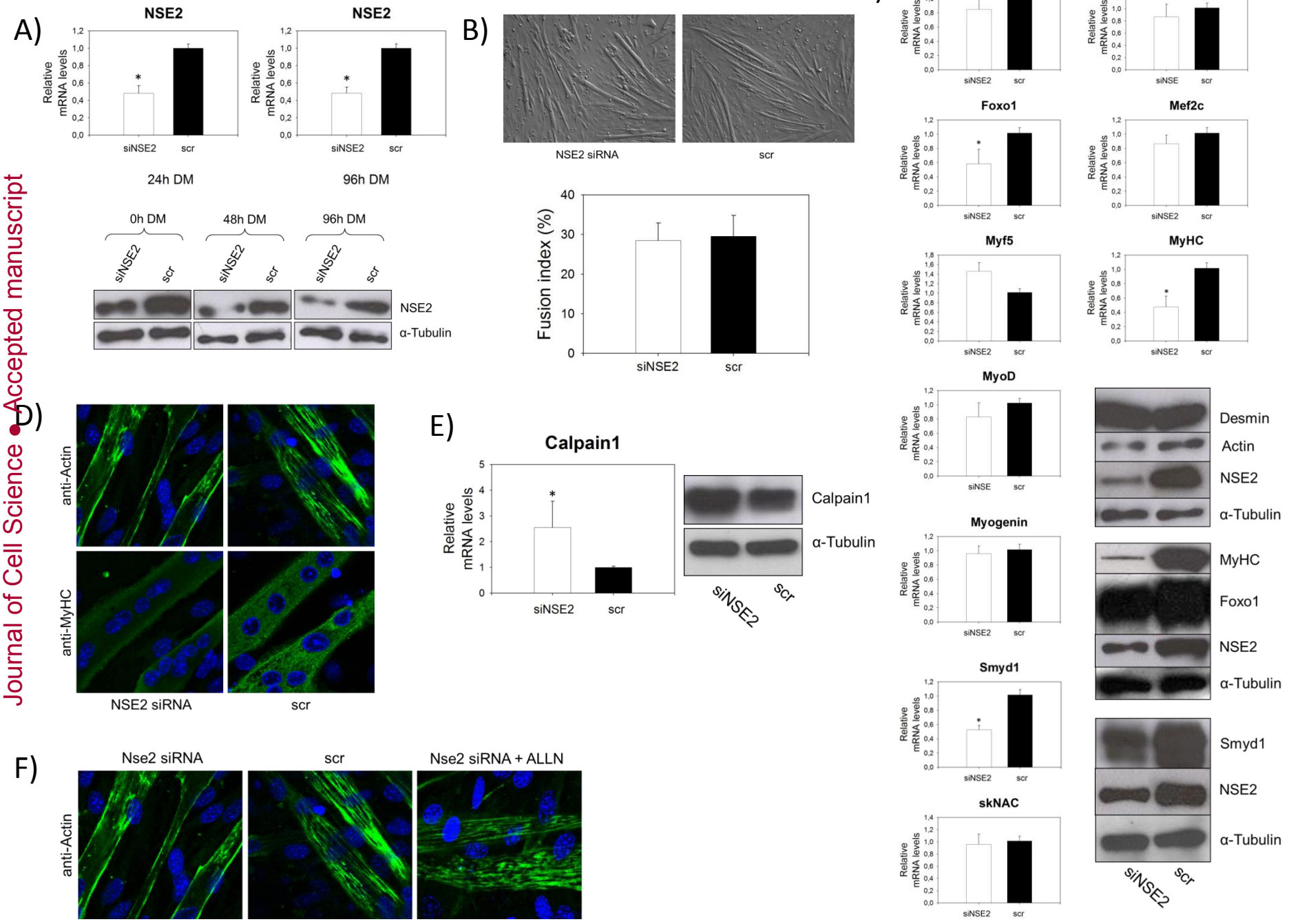


Fig.4

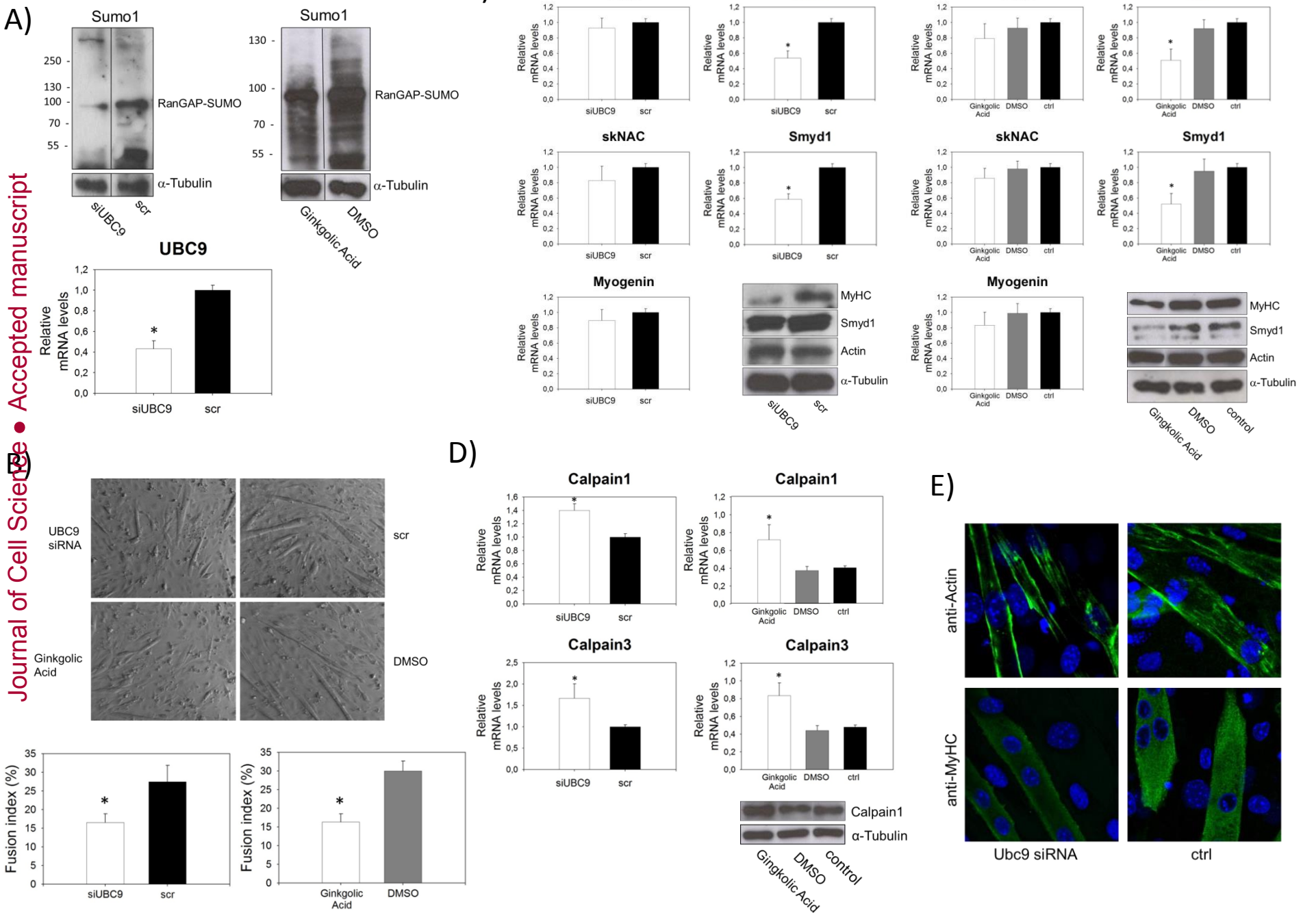


Fig.5

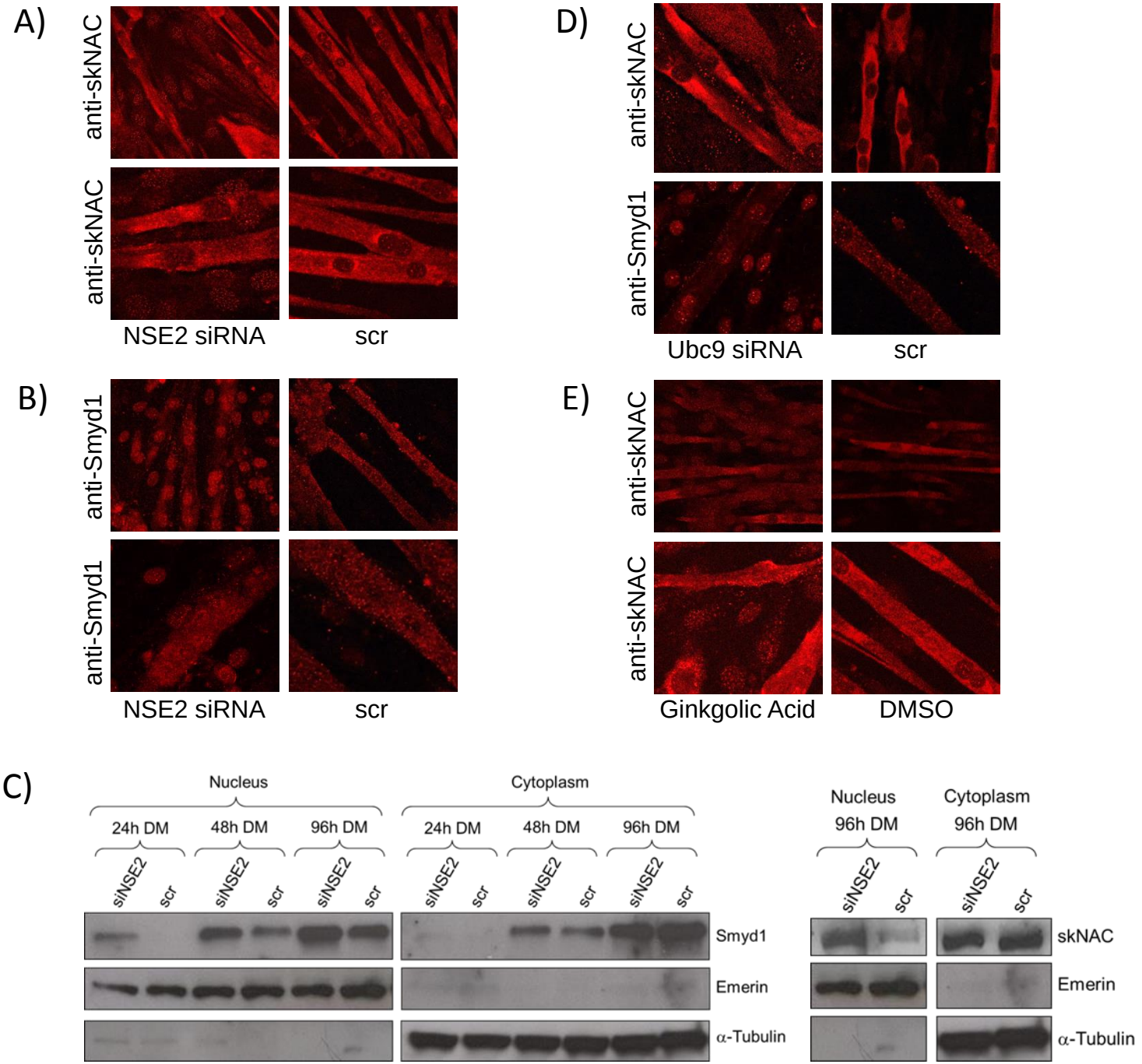
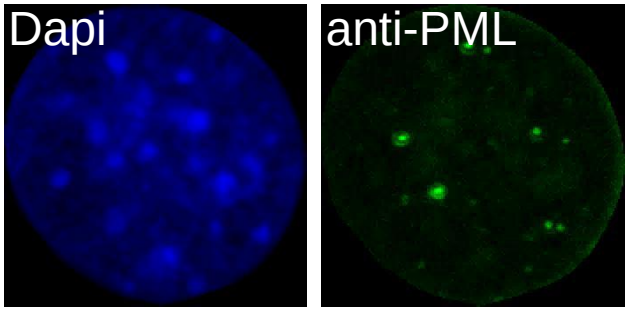
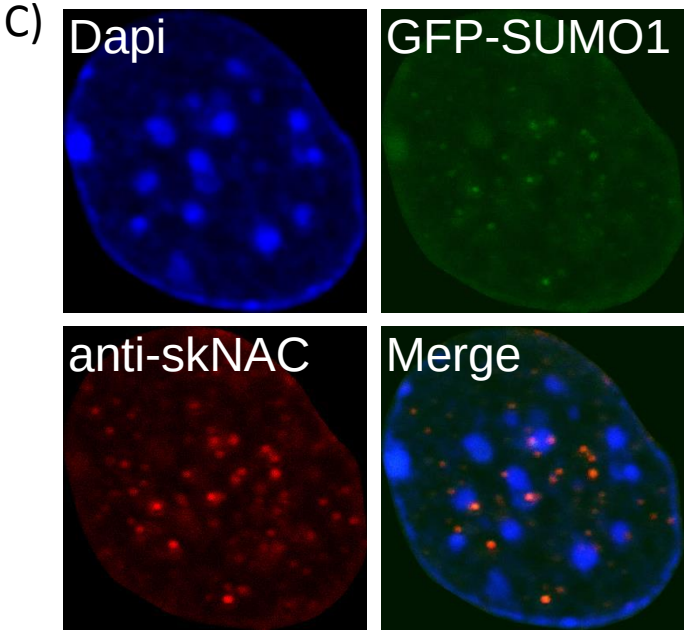


Fig.6





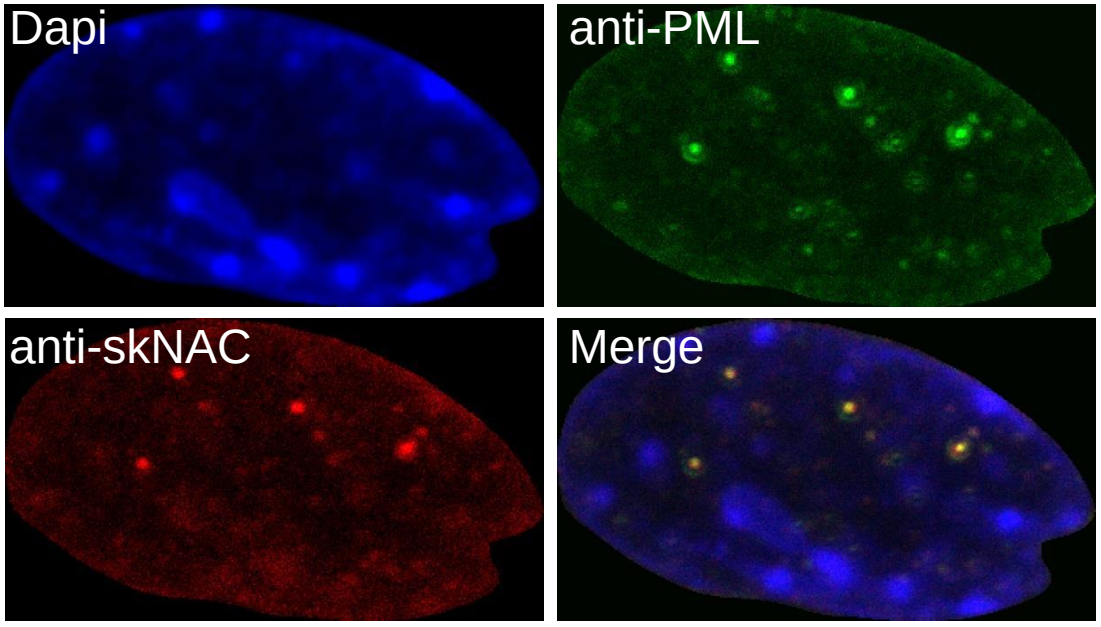
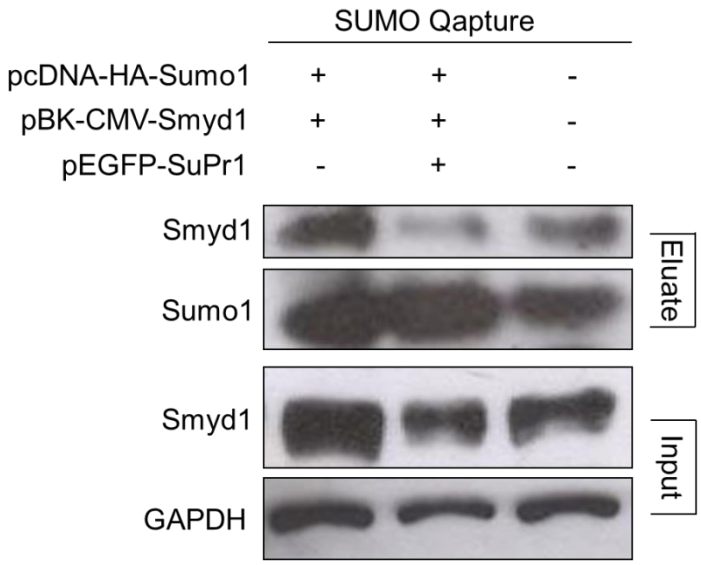


Fig.7

A)

H.sapiens	275	-	CEHCQKKLKDDLFLGVKDNPKPSQE	-	301
P.troglodytes	275	-	CEHCQKKLKDDLFLGVKDNPKPSQE	-	301
M.mulatta	262	-	CEHCQKKLKDDLFLGVKDNPKVHAT	-	288
C.lupus	275	-	CEHCQKKLKDDLFLGVKDDPKPSQE	-	301
B.taurus	275	-	CEHCQKGLKDDLFLGVKDNPKPSQE	-	301
M.musculus	262	-	CEHCQKGLKDDLFLAAKEDPKPSQE	-	288
R.norvegicus	275	-	CEHCQKGLKDDLFLAVKEDPKPSQE	-	301

B)



C)

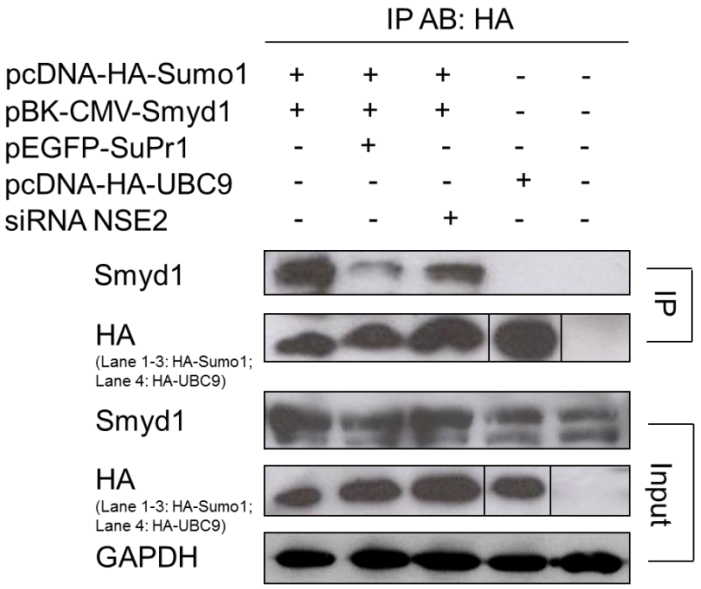
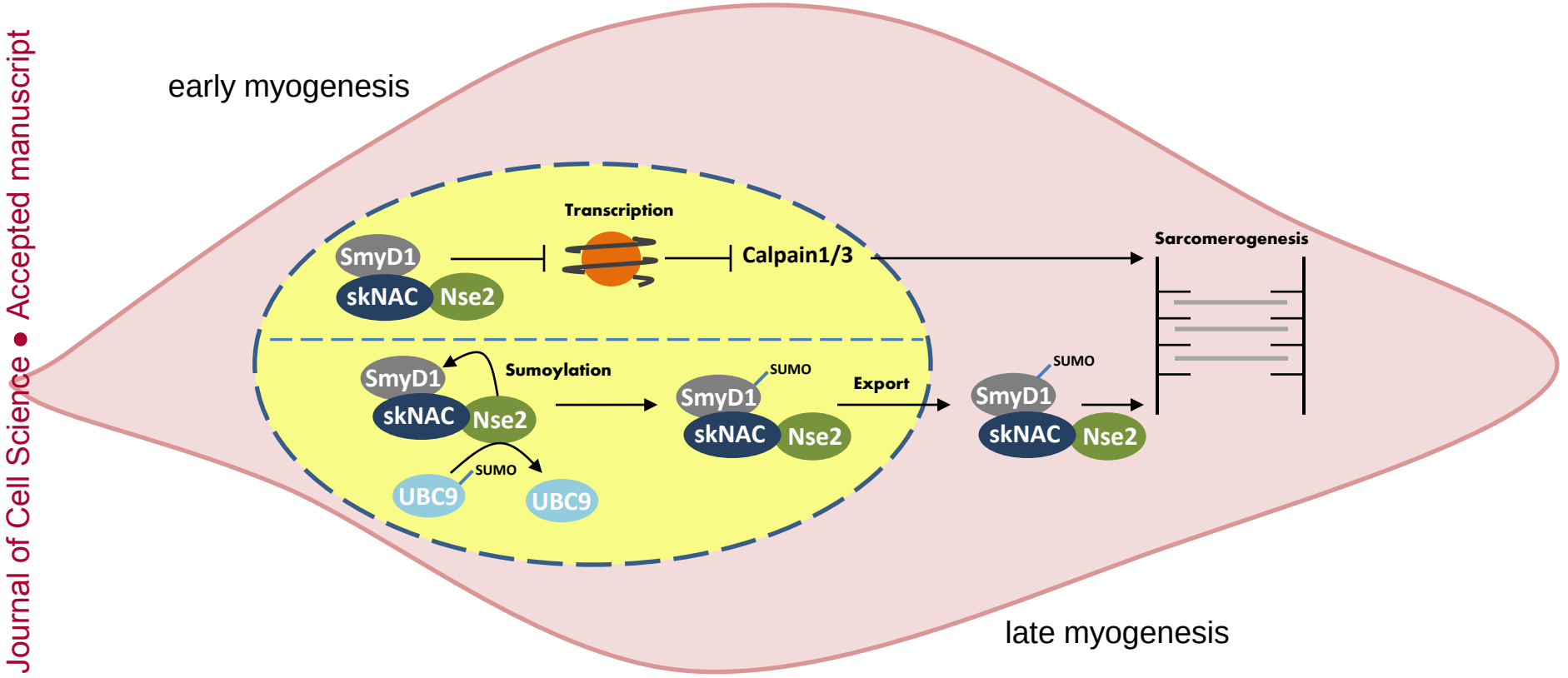


Fig.8



Gene name	Forward primer (5'→3')	Reverse primer (5'→3')
Calpain 1	CACCAAGGAAGCCAGCCCCAG	GTTTTCATGGCGGCCCAAGCC
Calpain 3	TCAGAGACTCAGACCTGGACCCCA	TCCGCAGCCGCACCAACTTC
skNAC	AGGGTGCCCCGGCTATGACC	TGGGGAAGTAGGAGGCACATGTTGT
Mef2c	GAGCTGAGCGTGCTGTGCGA	GCTCTCGTGCGGCTCGTTGT
Myogenin	TGGGTGTGCATGTGAGCCCC	CGCTGGGCTGGGTGTTAGCC
Smyd1	GCATCTTCCCCAACCTGGGCCT	GGGCCCGGAGCTCAATCCTCAT
Nse2	CCACCCTGTATCTCAGTGTGGGC	TGGGGACAAAGGACATGGAGGCA
Actin	AGGGCCAGAGTCAGAGCAGCA	GGGGCATCATCCCCGGCAAA
Desmin	TCCTCCTACCGCCGCACCTT	ACCGAAGCCTGCTCGAGGGA
Foxo1	CCTGTCGTACGCCGACCTCATCAC	GTCCATGGACGCAGCTCTTCTCCG
Myf5	TGAGGGAACAGGTGGAGAAC	AGCTGGACACGGAGCTTTTA
MyoD	AGCATAGTGGAGCGCATCTC	GGTCTGGGTTCCCTGTTCTG

Antibody	Company	Cat.no
α sarcomeric actin	Abcam (clone 5C5)	ab49672
α-Tubulin	Cell Signaling (11H10)	2125
Calpain 1	Cell Signaling	2556
Desmin	Abcam	ab15200
Emerin	Abcam	ab
Flag	Sigma-Aldrich	F7425
FoxO1	Cell Signaling (C29H4)	2880
GAPDH	Abcam	ab9484
HA	Santa Cruz (F-7)	sc-7392
Nse2	Sigma-Aldrich (Santa Cruz (N-12))	SAB3500490 (sc-87338)
MyHC (recognizes α and β chains of skeletal and cardiac myosins)	Novus (clone 3-48)	NB300-284
PML	Santa Cruz (N-19)	sc-9862
skNAC	polyclonal rabbit antiserum	H. Tucker, Austin, Texas
Smyd1	Santa Cruz (H-21) Abcam	sc-134016 (ab34472)
SUMO-1	Cell Signaling Santa Cruz (D11)	4930 sc-5308