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RESEARCH ARTICLE

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

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ABSTRACT

Skeletal and heart muscle-specific variant of the α subunit of nascent polypeptide associated complex (skNAC; encoded by *NACA*) is exclusively found in striated muscle cells. Its function, however, is largely unknown. Previous reports have demonstrated that skNAC binds to m-Bop/Smyd1, a multi-functional protein that regulates myogenesis both through the control of transcription and the modulation of sarcomerogenesis, and that both proteins undergo nuclear-to-cytoplasmic translocation at the 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 promyelocytic leukemia (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 the nuclear and cytosolic roles, of the skNAC–Smyd1 complex might be regulated by sumoylation.

KEY WORDS: skNAC, Smyd1, m-Bop, Mms21, Nse2, Sumoylation, Myogenic differentiation

INTRODUCTION

Skeletal and heart muscle-specific variant of the α subunit of nascent polypeptide associated complex (skNAC) is encoded by a splice variant of the α subunit of nascent polypeptide associated complex (αNAC; encoded by *skNAC*, also known as *Naca*), 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; however, its function, so far, is still enigmatic.

In 1996, Yotov and St-Arnaud showed that skNAC enhances transcription of the myoglobin gene (Yotov and St-Arnaud, 1996), suggesting that skNAC 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 have previously shown 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 and 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 transcription regulation has also been suggested by Sims and colleagues (Sims et al., 2002); the authors showed for the first time that skNAC binds to the m-Bop/Smyd1 protein [note, muscle (m)-Bop is the isoform of the the *Smyd1* gene that is expressed in muscles], a SET-domain-containing histone methyltransferase that also contains a MYND domain known to be involved in the recruitment of histone deacetylases (HDACs). Interestingly, the skNAC–Smyd1 complex localizes to the nucleus in myoblasts at an early differentiation stage and then translocates to the cytosol at later stages of differentiation, where at least Smyd1 is associated with sarcomeric structures (Just et al., 2011; Li et al., 2011), suggesting that, besides regulating transcription, the proteins might also exert a function in the cytoplasm at later stages of myogenesis.

Furthermore, Li and colleagues (Li et al., 2009) have demonstrated that zebrafish embryos in which *skNAC* expression had been knocked down through use of a morpholino antisense oligonucleotide-mediated approach were paralyzed and showed no heartbeat. This was most probably 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 have recently demonstrated defective sarcomerogenesis in murine C2C12 myoblasts after skNAC depletion using a small interfering (si)RNA-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 also recently 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 the potential functions of the skNAC–Smyd1 complex in both the nuclear and the cytoplasmic compartments, we questioned how the translocation of the two proteins to the cytoplasm in later myogenesis might be regulated. One possibility is sumoylation.

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Sumoylation is a process in which the small proteins SUMO-1, SUMO-2 and SUMO-3 become covalently attached to their target proteins through the sequential action of three enzymes or enzyme families, the E1 activating and E2 conjugating enzymes, as well as the E3 SUMO ligases. In contrast to the mechanistically similar process of protein ubiquitylation, however, sumoylation does not target the substrate 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 a review, see Garcia-Dominguez and Reyes, 2009; Hannoun et al., 2010).

Little is known about possible functions of protein sumoylation in skeletal muscle cells. However, Riquelme et al. (Riquelme et al., 2006b) have demonstrated that blocking expression of the *Ubc9* gene (also known as *UBE2I*), which encodes the only E2 SUMO-conjugating enzyme 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, a specific subset of sumoylation targets in mammalian skeletal muscle cells have been identified, such as the GLUT1 and GLUT4 glucose transporters (Giorgino et al., 2000), indicating 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) and – most recently – the myogenic determination factor PAX7 (Luan et al., 2013) and the basic helix-loop-helix transcription factor Sharp-1 (Wang et al., 2013). In addition, it has been known for some time that sumoylation increases the stability of HDACs (for a 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 a 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* (also known as *Myod1*) (Lee et al., 2006).

Here, we demonstrate that skNAC binds to the E3 SUMO ligase Nse2 (also known as NSMCE2) and that inhibition of *Nse2* expression represses specific aspects of skeletal muscle differentiation, such as expression of the myosin heavy chain 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 m-Bop/Smyd1 can be 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 bound to the E3 SUMO ligase Nse2/mammalian homolog of yeast Mms21. The interaction was confirmed by using co-immunoprecipitation of recombinant FLAG-tagged skNAC truncation mutants and endogenous Nse2 protein. Particularly, we found that a fragment located at the C-terminal end of the skNAC-specific middle domain (amino acids 1712–1995) specifically bound to the endogenous Nse2 protein (Fig. 1). By contrast, an skNAC fragment that was located closer to the N-terminus only weakly bound to Nse2 when overexpressed at very high levels (data not shown). Two unrelated FLAG-tagged proteins, NADK and the cytoplasmic domain of LRP6 (encoded by pFlag-CMV4-NADK and pcDNA-Flag-LRP6, respectively), did not bind to Nse2 (Fig. 1). *Nse2* was broadly expressed in mammalian tissues and organs, with particularly high expression

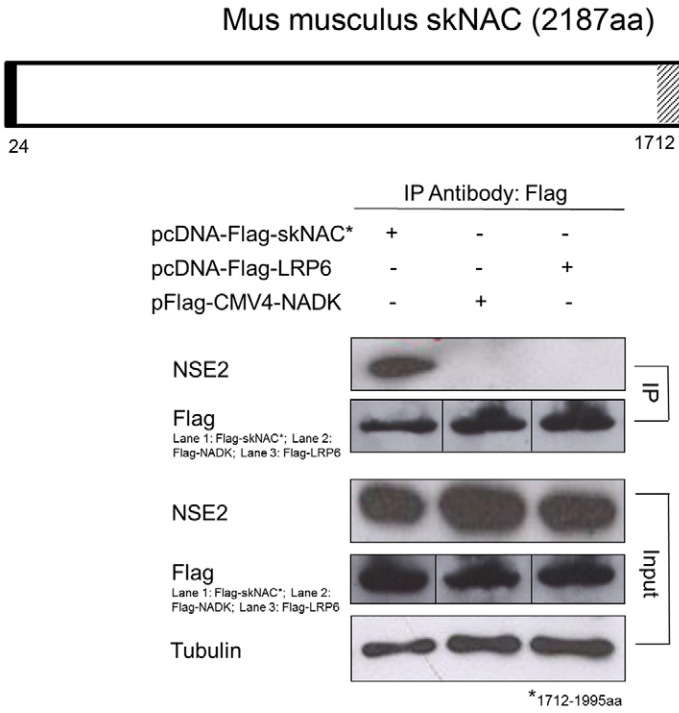

Fig. 1. skNAC binds to the E3 SUMO ligase mammalian Nse2. Co-immunoprecipitation of a FLAG-tagged skNAC fragment (*, amino acid residues 1712–1995, refer to schematic drawing) and endogenous Nse2 from lysates of C2C12 murine myoblasts. FLAG-tagged NAD^+ kinase and the cytoplasmic domain of LRP6 (pFlag-CMV4-NADK and pcDNA-Flag-LRP6, respectively) were used as negative controls. IP, immunoprecipitation.

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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 by northern blotting (Fig. 2A), semi-quantitative real-time PCR (qPCR) analysis (Fig. 2B) and western blotting (Fig. 2C). In addition, we investigated 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=48 h 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 with 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 (Fig. 2D), 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. Finally, in differentiated myotubes, 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. To prove the nuclear-to-cytosolic translocation of the Nse2 protein, C2C12 cells were again induced to differentiate and nuclear proteins were separated from

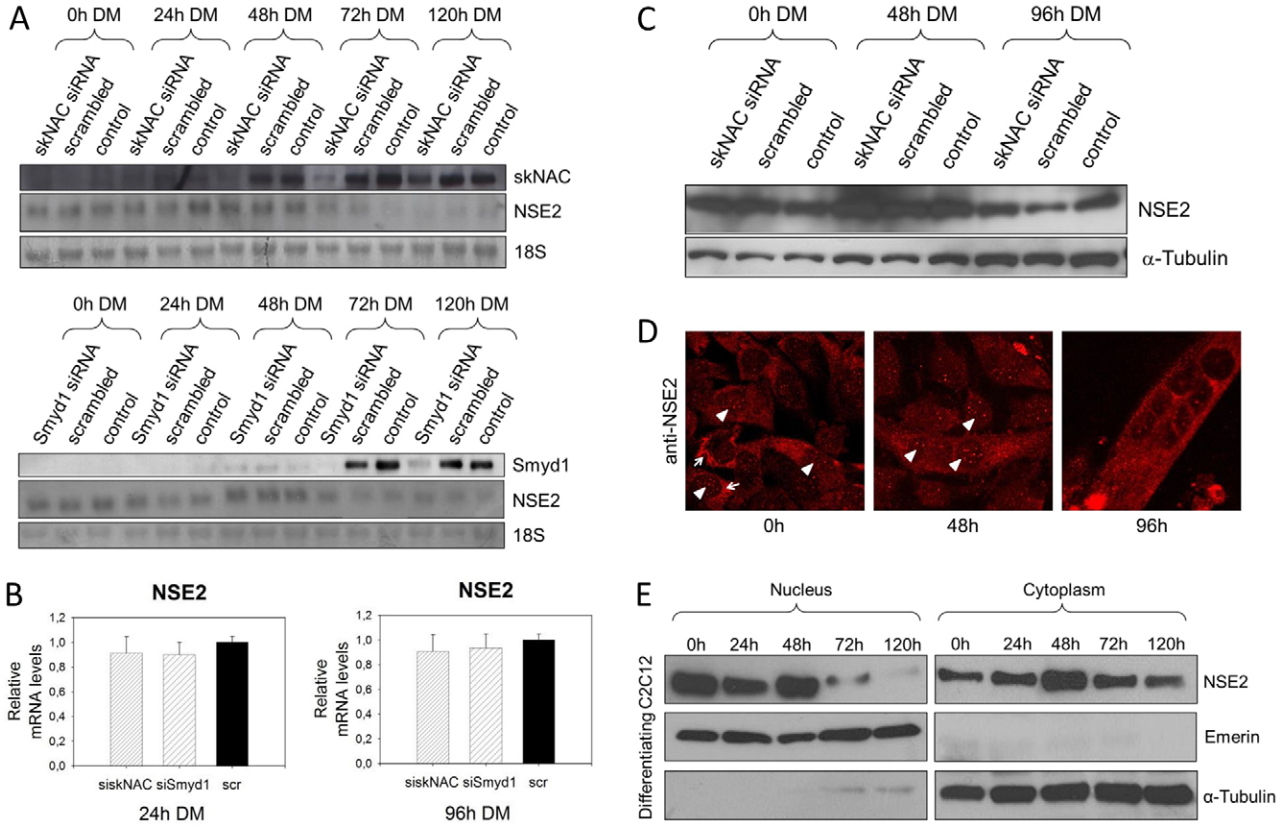

Fig. 2. *Nse2* expression in myogenesis, with and without inhibition of *skNAC* and *Smyd1* expression. (A) C2C12 myoblasts were transfected with skNAC- or Smyd1-specific siRNAs (skNAC siRNA and Smyd1 siRNA, respectively) a non-specific (scrambled) control, or left untreated. Cells were harvested at different time points after the induction of differentiation as indicated and then analyzed for *Nse2* and *skNAC* or *Smyd1* expression by northern blotting. The concentration of the 18S rRNA transcript was analyzed as a control for equal loading (bottom panels). (B) In parallel, *Nse2* expression was quantified by using qPCR analysis 24 and 96 h after the induction of differentiation. Means±s.e.m. are shown. scr, scrambled; siskNAC, siRNA against skNAC; siSmyd1, siRNA against Smyd1. (C) At 0 h, 48 h and 96 h after the induction of differentiation, protein lysates were generated from the cells and analyzed for Nse2 protein levels by western blotting. The blot was re-probed 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 using immunofluorescence and confocal laser scanning 0 h, 48 h and 96 h after the induction of differentiation using an Nse2-specific antibody. Note the spotty, nuclear Nse2 localization in some cells (arrowheads, 0 h and 48 h) and the prominent perinuclear staining in others (arrows, 0 h), as well as the almost complete nuclear-to-cytosolic translocation of the protein 120 h after the induction of differentiation. Representative pictures of at least three independent experiments are shown. (E) Analysis of subcellular localization of the Nse2 protein by cell fractionation and western blotting. Nuclear and cytoplasmic fractionation was performed during C2C12 differentiation, and western blotting was performed on 30 µg 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 fraction and α-tubulin was hardly detectable in the nuclear fraction, indicating effective fractionation. DM, differentiation medium.

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the cytosolic fraction. As shown in Fig. 2E, using western blotting, we could indeed demonstrate a strong decline of nuclear Nse2 protein within the first 5 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 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 protein level by approximately 60%. To exclude off-target effects, two different siRNAs against Nse2 were employed and all major results were reproduced by using both siRNA 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 myosin heavy chain (*MyHC*; also known as *Myh7*), as well as *Smyd1*, was reduced at the mRNA level, whereas expression of others was unchanged or induced (Fig. 3C). Because we had recently demonstrated a role for skNAC in the control of sarcomerogenesis in mammalian cells (Berger et al., 2012; Berkholz et al., 2013), we hypothesized

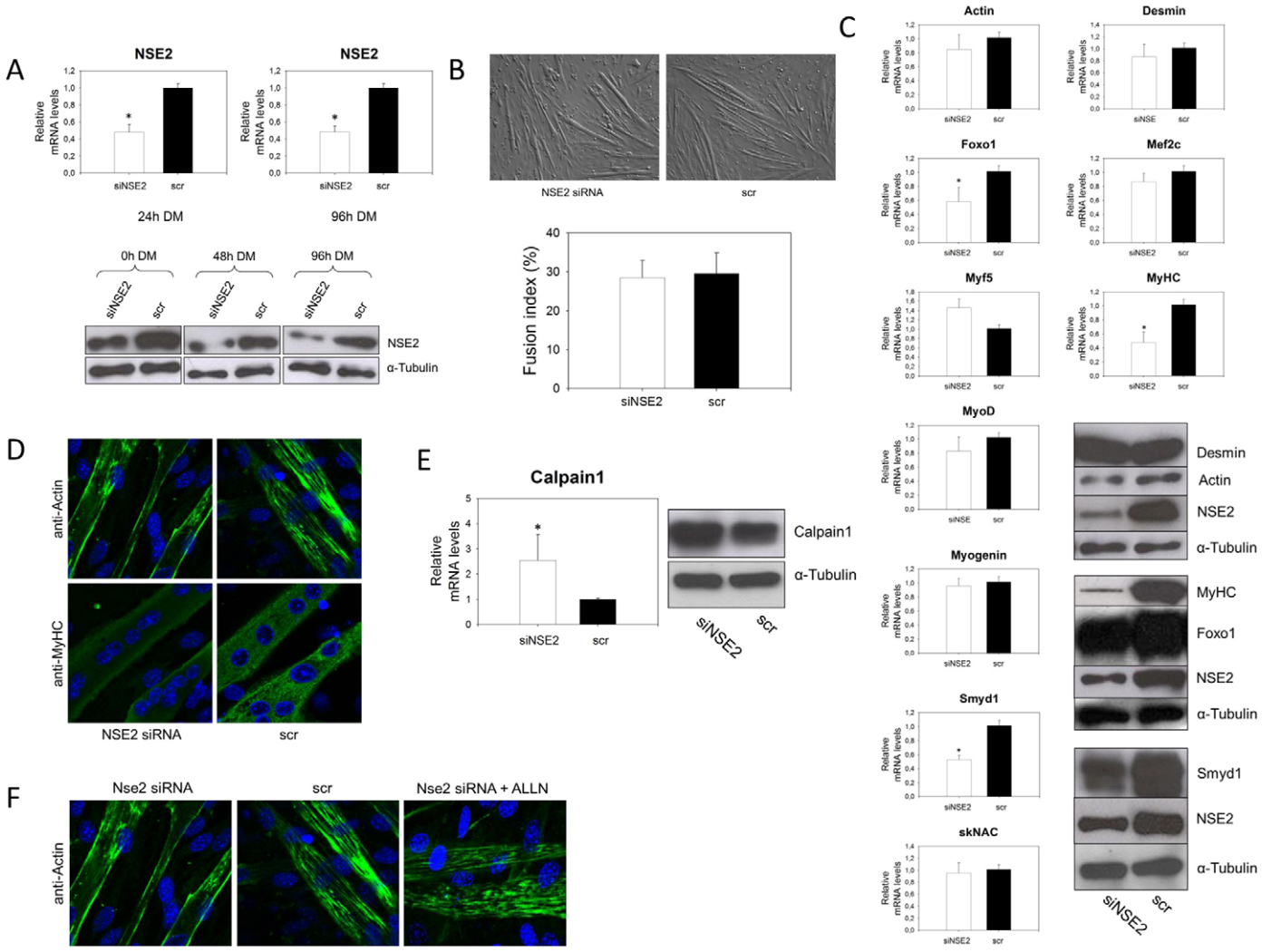

Fig. 3. Inhibition of *Nse2* expression and effects on myogenic differentiation. C2C12 cells were transfected with an Nse2-specific siRNA (siNse2) or an unrelated scrambled (scr) control. (A) At different time points after the induction of differentiation, total cellular RNA and total protein were isolated from the cells and *Nse2* expression was determined at the mRNA level by using qPCR and at the protein level by western blotting as indicated. (B) Cell morphology was analyzed in siRNA-transfected cells and in controls by using light microscopy 96 h after the induction of differentiation. Representative pictures of at least three independent experiments are shown. The fusion index of cells (percent nuclei in cells with at least three nuclei) was also calculated (bottom panel; mean±s.e.m.; $n=3$). (C) The expression of myogenic differentiation markers was analyzed in siRNA-transfected cells and controls at the RNA (qPCR charts) and protein levels (western blots) 72 h after the induction of differentiation as indicated. (D) Sarcomerogenesis was analyzed in cells that had been transfected with scrambled siRNA or an siRNA against Nse2 by using immunofluorescence staining of α-sarcomeric actin (top panels) and MyHC (bottom panels) 96 h after the induction of differentiation. Representative pictures of at least three independent experiments are shown. DAPI was used to stain and visualize nuclei (blue). (E) Calpain 1 gene expression in siNse2-transfected cells and controls was analyzed by using qPCR and western blot analysis 72 h after the induction of differentiation as indicated. (F) siNse2-transfected cells were treated with the calpain inhibitor ALLN before the induction of differentiation (right panel). siNse2-transfected C2C12 cells and scr-transfected controls were differentiated for 96 h. Subsequently, cells were analyzed for α-sarcomeric actin intracellular distribution by using confocal microscopy. Nuclei were stained with DAPI (blue). *$P<0.05$. DM, differentiation medium.

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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 predominant sarcomere proteins, in our siRNA-transfected cells, we detected 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 cells or those transfected with a scrambled siRNA, in which the typical, homogeneously distributed spotty staining of newly forming sarcomeres was detected (Fig. 3D), and was reminiscent of the MyHC and α-sarcomeric actin staining patterns we had observed previously in myoblasts in which *skNAC* expression had been knocked down (Berger et al., 2012; Berkholz et al., 2013). The perturbed sarcomerogenesis we had seen previously in cells that had been transfected with siRNA against skNAC was dependent on an induction of calpain gene expression and calpain enzymatic activity (Berger et al., 2012). Calpains are a family of Ca^{2+}-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 gene expression might also be responsible for the inhibition of proper sarcomere formation in cells that had been transfected with an siRNA against Nse2. Indeed, in these cells, we found a profound upregulation of calpain 1 gene expression (Fig. 3E) similar to that previously seen in the cells that had been transfected with siRNA against skNAC, indicating that inhibition of sarcomerogenesis might actually be caused by enhanced calpain proteolytic activity in these cells. To functionally prove this hypothesis, cells that had been transfected with siRNA against Nse2 were treated with the calpain inhibitor N-[N-(N-Acetyl-L-leucyl)-L-leucyl]-L-norleucine (ALLN). As shown in Fig. 3F, this treatment indeed rescued the phenotype of defective sarcomerogenesis – the 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 investigated whether a complete blockade of protein sumoylation to some extent mimics 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-conjugating enzyme 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 a specific subset of myogenic differentiation markers was repressed in cells in which Ubc9 had been knocked down (Fig. 4C), an effect that has also previously been described by Riquelme and colleagues (Riquelme et al., 2006b). Interestingly, 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 cells and in cells that had been transfected with siRNA against Ubc9 (Fig. 4D). Consistently, sarcomerogenesis was disturbed in a manner similar to that seen 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 during myogenic differentiation

To test our initial hypothesis that the 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 using immunofluorescence staining and cell fractionation followed by western blotting. Using these approaches, we could detect partial inhibition of the nuclear-to-cytosolic translocation, which was normally seen for both proteins at later stages of myogenesis (Fig. 5A–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 siRNA against Ubc9, we observed similar effects – cytoplasmic translocation of skNAC was partially inhibited, accompanied by spotty skNAC staining in the nucleus (Fig. 5D,E). Transfection with the siRNA against Ubc9 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 was reminiscent of the pattern seen when staining for PML nuclear bodies. Thus, we tested whether Nse2 and skNAC colocalized with the PML protein using double immunofluorescence staining, which was indeed the case (Fig. 6A,B). In addition, when co-transfecting a vector that encoded a GFP–SUMO-1 fusion protein, we could also colocalize skNAC with SUMO-1 protein (Fig. 6C).

m-Bop/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 to identify a potential specific Nse2 sumoylation target within this system. skNAC itself does not contain a classical sumoylation site, whereas 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 antibody against SUMO-1 (Fig. 7B). To investigate the hypothesis that Smyd1 is sumoylated in skeletal muscle cells, we immunoprecipitated the protein from C2C12 lysates and analyzed protein sumoylation by western blotting. 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. Furthermore, when we blocked *Nse2* expression by approximately 60% in the *Sumo-1-Smyd1*-overexpressing cells using specific siRNAs, concentrations of sumoylated Smyd1 were much lower than in cells without Nse2 depletion 48 h 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.

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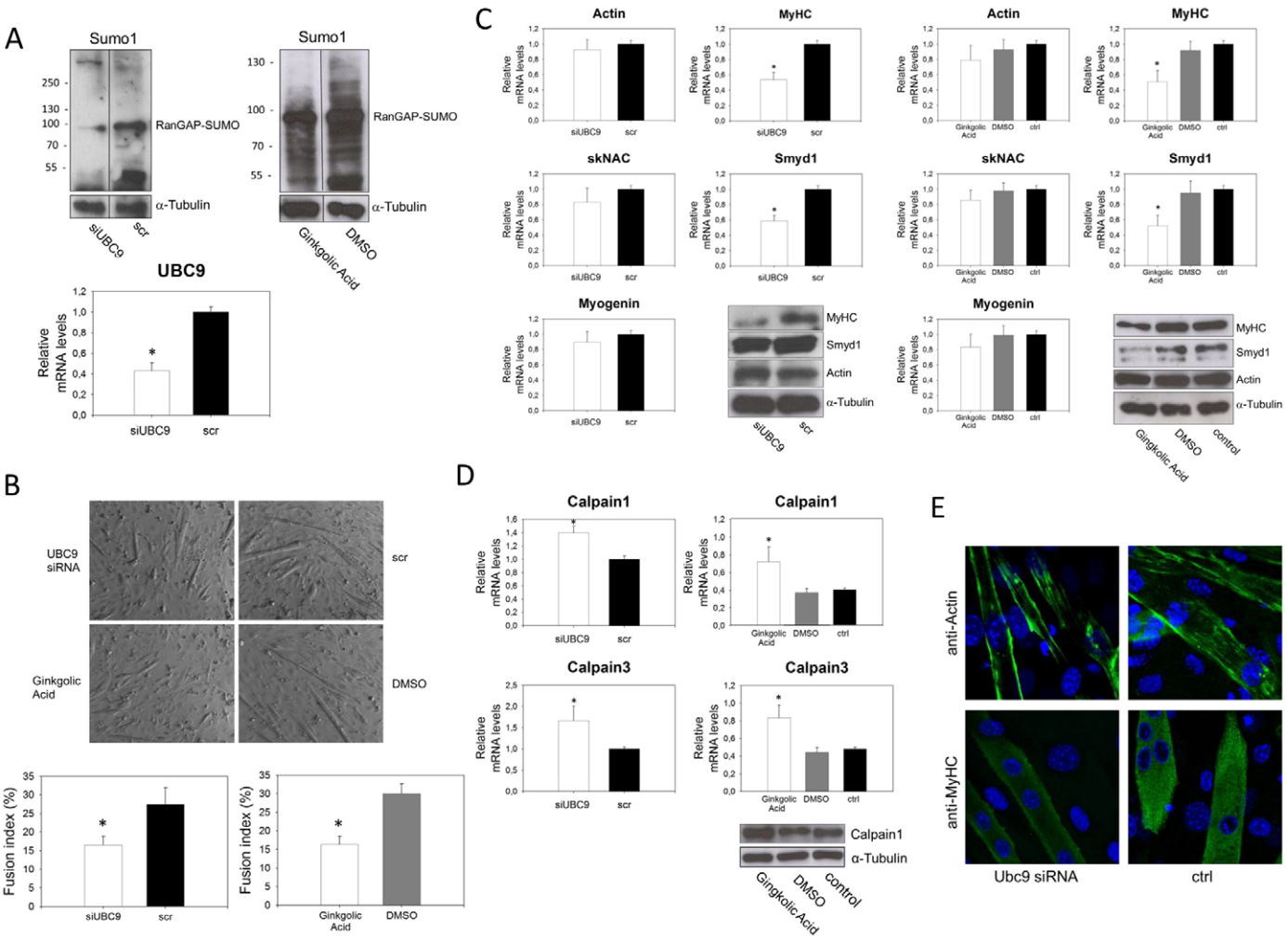

Fig. 4. Global inhibition of sumoylation using ginkgolic acid or transfection with a specific siRNA directed against Ubc9 inhibits some aspects of myogenic differentiation. C2C12 myoblasts were transfected with a specific siRNA directed against the sole E2 SUMO-conjugating enzyme Ubc9 (siUBC9) or were treated with ginkgolic acid. Cells that had been transfected with a scrambled (scr) 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 blotting 48 h after the induction of differentiation as indicated. A band that is most likely to correspond to sumoylated RanGAP1 is indicated. In the cells that had been transfected with the Ubc9-specific siRNA, *Ubc9* expression was also quantified by using qPCR to confirm efficiency of repression. (B) At 96 h after the induction of differentiation, cell morphology was analyzed by using 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 fusion index of the cells (percent nuclei in cells with at least three nuclei) was counted; mean±s.e.m.; $n=3$. Representative pictures of at least three independent experiments are shown. (C) At 72 h after the induction of differentiation, the expression of specific myogenic differentiation markers was analyzed in the cells by using qPCR and western blotting 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 calpain 3 gene expression was analyzed in ginkgolic-acid-treated or siUBC9-transfected cells by using qPCR 72 h after the induction of differentiation. In the ginkgolic-acid-treated cells, calpain 1 expression was also analyzed by western blotting, as indicated. (E) Sarcomerogenesis was analyzed in siUBC9-transfected cells through immunofluorescence and confocal laser scanning analysis of α-sarcomeric actin and MyHC 96 h 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 siRNA targeting Ubc9, indicative of disturbed sarcomerogenesis. Nuclei were stained with DAPI (blue). Representative pictures of at least three independent experiments are shown. *$P<0.05$. ctrl, control.

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). Because both proteins undergo nuclear-to-cytoplasmic translocation during myogenesis (Sims et al., 2002), it is probable that they fulfill specific functions in both cellular compartments; in early myogenesis, they might act as transcriptional modulators, presumably through two specific domains within the Smyd1 protein – the so-called SET domain, which is a histone methyltransferase moiety, and the MYND domain, which is a potential recruiter of 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.

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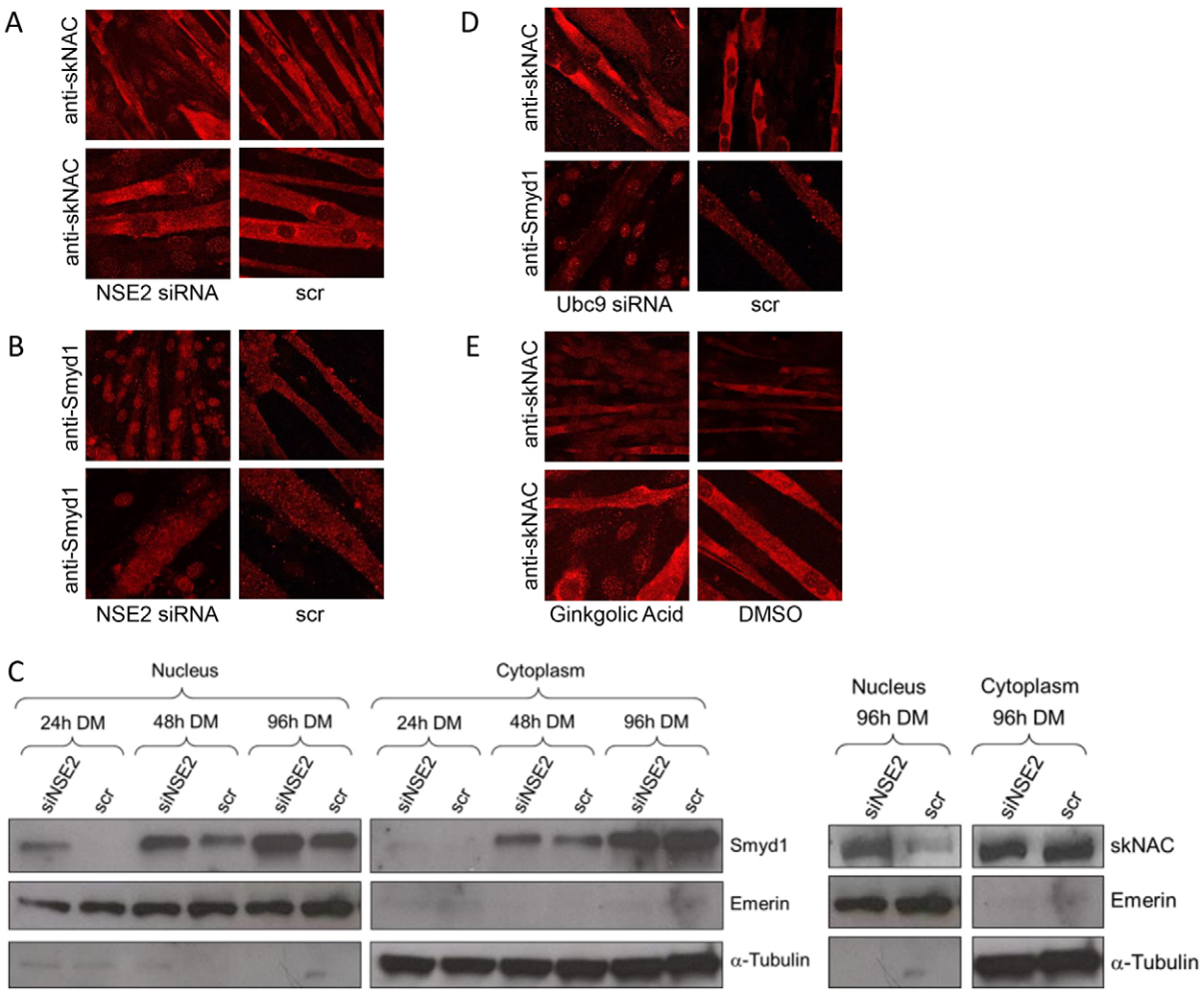

Fig. 5. Depletion of Nse2 or inhibition of global protein sumoylation partially blocks the nuclear-to-cytosolic translocation of skNAC and Smyd1. C2C12 myoblasts were transfected with an Nse2-specific siRNA (NSE2 siRNA, siNSE2) and differentiated for 96 h. Subsequently, subcellular localization of the skNAC and Smyd1 proteins was analyzed through immunofluorescence and confocal laser scanning using specific antibodies (A,B), or by using cell fractionation and western blotting 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,E) skNAC and Smyd1 were also partially retained in the nucleus when blocking global sumoylation through transfection with an siRNA against Ubc9 (D) or treatment with ginkgolic acid (E, only analyzed for skNAC). The bottom panels in A,B and E show higher magnification images. Representative pictures of at least three independent experiments are shown. DM, differentiation medium; scr, scrambled siRNA.

Specifically, it is possible that the transcriptional coactivator function and/or the sarcomere-organizing function of skNAC 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, because sumoylation plays an important role in the regulation of nucleocytoplasmic shuttling (for a review, see Zhao, 2007), and because 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 is 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 then translocates to the cytosol at later stages is intriguing because this pattern is reminiscent of the nuclear-to-cytoplasmic translocation described previously by Sims and colleagues (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 *MyHC*, 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. Because both skNAC and Smyd1 have been implicated in the control of sarcomerogenesis, we also analyzed the staining patterns of α-sarcomeric actin and MyHC in cells that had been transfected with an Nse2-specific siRNA. We observed a staining pattern reminiscent of that seen in cells that had been transfected with siRNA against skNAC or Smyd1 – 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 of newly forming sarcomeres, was observed. These data indicate that Nse2 is important for sarcomere formation, or,

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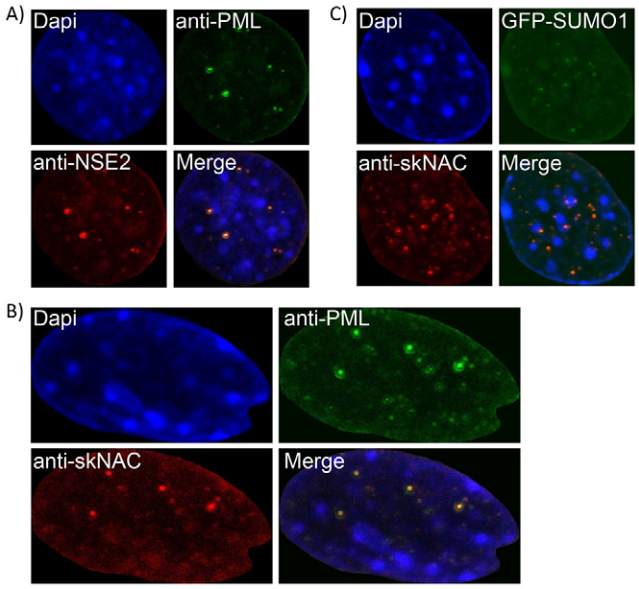

Fig. 6. Colocalization of Nse2 (A) and skNAC (B) with PML nuclear bodies and SUMO-1 (C). C2C12 cells were co-stained for PML (green) and Nse2 or skNAC (red) 0 h (A, Nse2) or 48 h (B, skNAC) after the induction of differentiation. Cells that had been co-transfected with pGFP-SUMO-1 and pCI-skNAC were switched to DM for 48 h and then co-stained (C). Superimposing the two colors (merge) results in a yellow signal, where both proteins colocalize.

in other words, that sarcomerogenesis cannot proceed normally in the absence of Nse2. Similar to skNAC- and Smyd1-depleted myoblasts (Berkholz et al., 2013), this appears to be, at least in part, the result of elevated calpain activity in these cells because we could also detect enhanced calpain 1 gene expression in cells that had been transfected with siRNA against Nse2. 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 whether this is only achieved through the control of calpain gene expression – i.e. through skNAC, Smyd1 and Nse2 functions in the nucleus – or whether cytosolic skNAC, Smyd1 and Nse2, at later stages of differentiation, also have a specific role in the control of sarcomerogenesis.

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

Because total skNAC levels remained unchanged after transfection with siRNA against Nse2 (Fig. 3C), or siRNA specific to Ubc9 and treatment with ginkgolic acid (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, because Smyd1 expression was repressed by approximately 50% under all three conditions.

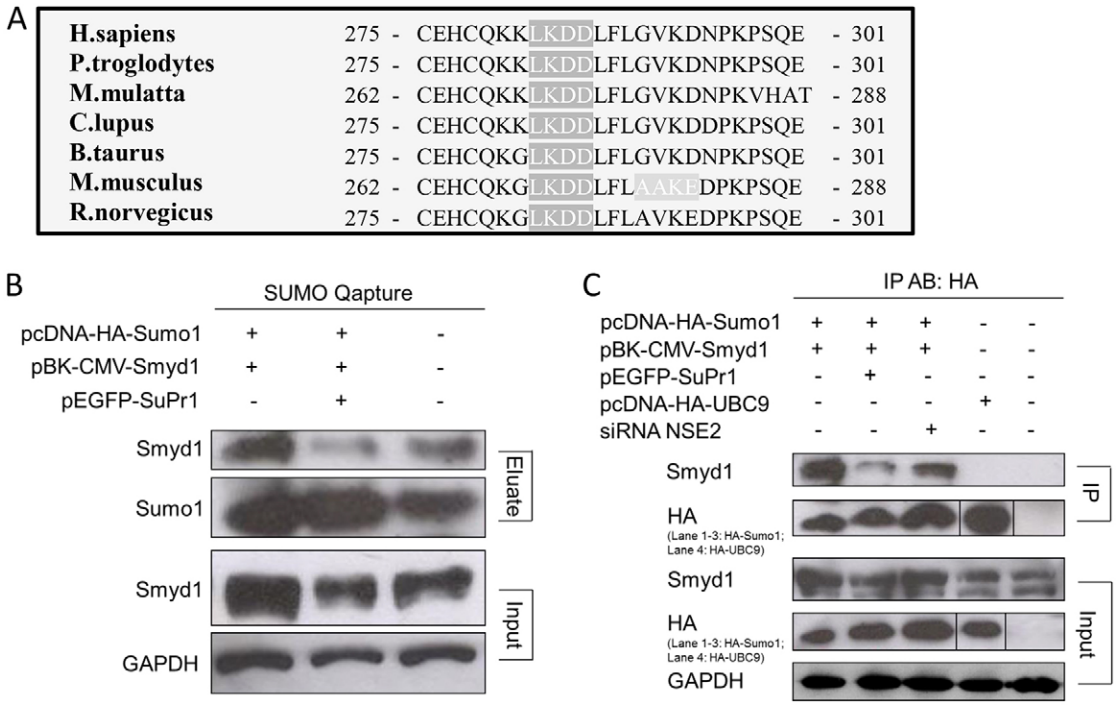

Fig. 7. Smyd1 is sumoylated and might be an Nse2 target. (A) Alignment of the Smyd1 amino acid sequences of different species; potential sumoylation motifs are indicated in grey. Note the well-conserved LKDD motif and the additional alternative sumoylation motif found in the *Mus musculus* sequence. (B) SUMO assay. Affinity chromatography using an immobilized SUMO-1 antibody. C2C12 cells were transfected with expression vectors as indicated and then differentiated for 48 h. Subsequently, lysates were purified using affinity chromatography for SUMO-1. Total cellular lysates (input) and sumoylated proteins (eluate) were analyzed by western blotting 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. At 48 h after the induction of differentiation, immunoprecipitation was performed using a hemagglutinin (HA)-specific antibody, and sumoylated Smyd1 was detected by western blotting using a Smyd1-specific antibody. HA-SUMO-1 and HA-Ubc9 both run at 17–18 kDa. 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). AB, antibody; IP, immunoprecipitation.

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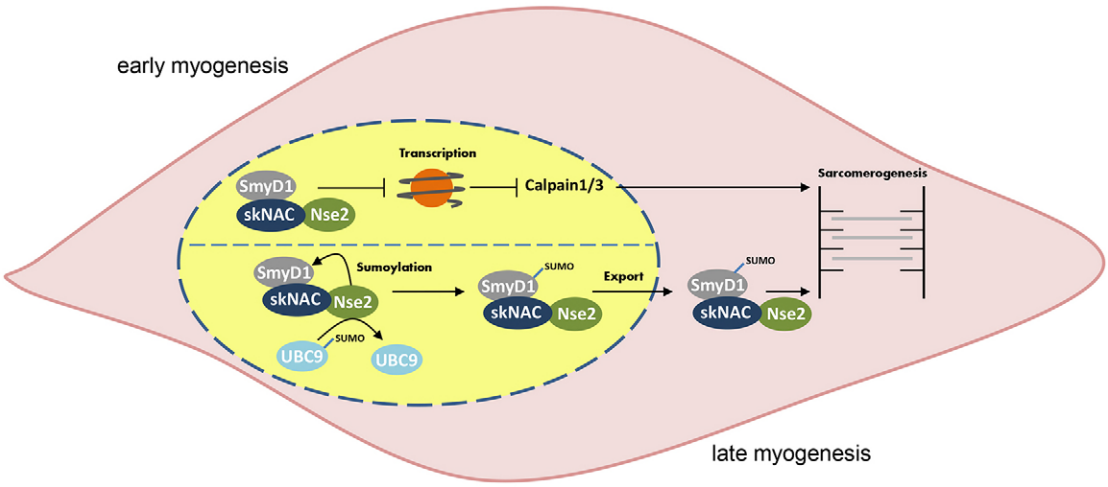

Fig. 8. Partially hypothetical model of skNAC, Smyd1 and Nse2 functions in muscle cells. In early myogenesis, skNAC and Smyd1 are found in the nucleus, where they might regulate transcription, presumably, by influencing histone methylation through the SET domain of Smyd1 and potentially through histone deacetylation through the MYND domain of Smyd1. Particularly, the skNAC–Smyd1 complex directly or indirectly inhibits transcription of the genes encoding calpain 1 and calpain 3, with at least the latter being a potent inhibitor 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 Smyd1, at the least, 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.

Interestingly, the nuclear staining of Nse2 in early myogenesis showed a speckled pattern, with strong staining of the perinuclear region in some cells. This pattern resembled 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 a review, see Lallemand-Breitenbach and de Thé, 2010). Indeed, we could colocalize Nse2 and skNAC with PML bodies. Because the yeast Nse2 homologue, Mms21, as well as mammalian Nse2 have been implicated in DNA repair, recombination, chromosome cohesion and telomere integrity (for a review, see Potts, 2009; Stephan et al., 2011), and because Mms21/Nse2 is a key component of PML bodies (Potts and Yu, 2007; Brouwer et al., 2009; Chung et al., 2011b), it seems probable that Nse2, skNAC and Smyd1 function as a PML-associated complex during 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, 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 colocalized 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 roles. Interestingly, a specific role for Mms21, the yeast Nse2 homolog, in the regulation of nucleocytoplasmic shuttling through 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 amino acid sequences revealed the presence of such a site within the Smyd1 sequence, but not within that of skNAC, 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 (Simpson-Lavy and Johnston, 2013) have demonstrated that the yeast Nse2 homolog, Mms21, can sumoylate the SNF1 protein, which is the yeast homolog of mammalian AMP-activated protein kinase (AMPK), thereby inhibiting its catalytic activity. In mammalian skeletal muscle cells, AMPK is a central player in the regulation of cell metabolism and fiber type specification, and skNAC has been implicated in the control of fiber type specification (Yotov and St-Arnaud, 1996; Park et al., 2010) (J.B. and B.M, unpublished data), suggesting that there is 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 provide data that suggest that this sumoylation is important for the nuclear-to-cytosolic translocation of the skNAC–Smyd1 complex at later stages of myogenesis and that this translocation 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℃ and under 5% CO_2. To induce differentiation, cells were grown to 80–90% 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 before 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 48 h after the induction of differentiation, and cells were then fixed for immunofluorescence analysis after a total of 96 h.

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 by using a GFP expression vector and was 40–50% in all experiments. pCI-skNAC and pBK-CMV-Smyd1 were gifts from René St-Arnaud (McGill University, Montreal, Canada) and Haley Tucker (University of Texas at Austin, Austin, TX), respectively; pcDNA-HA-SUMO-1, pcDNA-HA-Ubc9, pGFP-SUMO-1 and pEGFP-SuPr1 were gifts from Hans Will [Center of Experimental Medicine at the University Hospital Eppendorf (UKE), Hamburg, Germany].

Co-immunoprecipitation assay

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

Transfection with specific siRNAs

siRNA transfection was performed using predesigned specific siRNAs (Sigma Aldrich). For inhibition of *Nse2* expression, two different, unrelated siRNA oligonucleotides were employed in parallel to rule out off-target effects: Nse2 siRNA-1, sense, 5′-CAUGGUUGAGUU-UGCUAAA-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′-UGAUCAC-ACCAAAGAUGUG-3′; skNAC siRNA, sense, 5′-GACAGUUCCU-GUUGAGAAAUU-3′, antisense, 5′-UUUCUCAACAGGAACUGUC-UU-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′-UCGAA-GUAUUCCGCGUACGUU-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 performed as previously described (Munz et al., 2002). qPCR analysis was performed 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 calpain 3, skNAC, Smyd1 and Nse2, self- and pre-designed primers (Qiagen QuantiTect Primer Assays) were used. For detection of MyHC, only pre-designed primers were employed. Primer sequences are listed in supplementary material Table S1. In each experiment, melting curve analysis was performed to verify that a single transcript was produced. qPCR relative gene expression was calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method where expression was normalized to that of GAPDH. Controls lacking reverse transcriptase or template were run for all reactions. Unless otherwise specified, data from at least three independent experiments were expressed as means±s.e.m., $n=3$–5. Significance was accepted at $P<0.05$.

Western blot analysis and immunofluorescence

Western blot and immunofluorescence analyses were performed as previously described (Munz et al., 2002), specifically, 20 mM N-ethylmaleimide was added to the cell lysis buffer to inhibit de-sumoylation (Riquelme et al., 2006a; Riquelme et al., 2006b). Cytoplasmic and nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo scientific) according to the manufacturer's instructions. Confocal laser scanning microscopy was performed using a Zeiss LSM 700 confocal microscope (Carl Zeiss Microscopy GmbH, Germany) that was 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 supplementary material Table S2. 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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Competing interests

The authors declare no competing interests.

Author contributions

B.M. and J.B. designed the study; J.B. and L.M. carried out experiments; B.M., J.B., and L.M. wrote the paper.

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Supplementary material

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.150334/-/DC1

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Table S1. Sequences of all primers used in this study..

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

Table S2. Summary of all antibodies employed in this study.

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

Antibody	Company	Cat.no
FoxO1	Cell Signaling (C29H4)	2880
GAPDH	Abcam	ab9484
HA	Santa Cruz (F-7)	sc-7392
Nse2	Sigma-Aldrich	SAB3500490
	(Santa Cruz (N-12))	(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)	sc-134016
	Abcam	(ab34472)
SUMO-1	Cell Signaling	4930
	Santa Cruz (D11)	sc-5308