# Nestin as a regulator of Cdk5 in differentiating myoblasts

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**ABSTRACT** Many types of progenitor cells are distinguished by the expression of the intermediate filament protein nestin, a frequently used stem cell marker, the physiological roles of which are still unknown. Whereas myogenesis is characterized by dynamically regulated nestin levels, we studied how altering nestin levels affects myoblast differentiation. Nestin determined both the onset and pace of differentiation. Whereas depletion of nestin by RNAi strikingly accelerated the process, overexpression of nestin completely inhibited differentiation. Nestin down-regulation augmented the early stages of differentiation, at the level of cell-cycle withdrawal and expression of myogenic markers, but did not affect proliferation of undifferentiated dividing myoblasts. Nestin regulated the cleavage of the Cdk5 activator protein p35 to its degradation-resistant form, p25. In this way, nestin has the capacity to halt myoblast differentiation by inhibiting sustained activation of Cdk5 by p25, which is critical for the progress of differentiation. Our results imply that nestin regulates the early stages of myogenesis rather than maintains the undifferentiated state of progenitor cells. In the bidirectional interrelationship between nestin and Cdk5, Cdk5 regulates the organization and stability of its own nestin scaffold, which in turn controls the effects of Cdk5. This nestin–Cdk5 cross-talk sets the pace of muscle differentiation.

## INTRODUCTION

Stem and progenitor cells of various tissues are characterized by the specific expression of the intermediate filament (IF) protein nestin. Therefore nestin has been broadly used as a marker for stem and precursor cells. During development, progenitor cells respond to various intrinsic and extrinsic signals that guide their differentiation.

Simultaneously, nestin is down-regulated, often along with its assembly partner vimentin, and is then replaced by other developmental stage- and tissue-specific IF proteins, such as neurofilaments in neurons, glial fibrillary acidic protein (GFAP) in astrocytes, and desmin in muscle. Despite nestin's highly specific expression profile, only a few studies have addressed its physiological functions. We have previously shown that nestin protects neuronal progenitor cells from oxidative stress (Sahlgren et al., 2006). Furthermore, the asymmetric distribution of nestin during the division of neuronal progenitors has been reported to regulate cell fate, leaving the nestin-negative daughter cells committed to apoptosis (Bieberich et al., 2003). More recently, nestin was shown to serve as a cytoplasmic anchor to unliganded glucocorticoid receptor (GR; Reimer et al., 2009). Such an anchoring function was proposed to serve as an important regulatory mechanism in embryonic development, as nuclear accumulation of GR, consequently enforcing cell-cycle arrest, was observed in cells depleted of nestin. Intriguingly, nestin expression defines a diverse range of cancers, including CNS, colon, prostate, breast, and pancreatic cancer (Kleeberger et al., 2007; Yang et al., 2008; Kawamoto et al., 2009; Liu et al., 2010). The motility of prostate

cancer cells is known to be regulated by nestin, the down-regulation of which severely retards migration (Kleeberger *et al.*, 2007).

During myogenesis and muscle regeneration, muscle cell precursors, myoblasts, proliferate, migrate, and subsequently withdraw from the cell cycle, align with each other, and fuse to form multinucleated myotubes (reviewed by Buckingham *et al.*, 2003; Shi and Garry, 2006). Differentiation is orchestrated by tightly controlled transcription factors, triggering the expression of muscle-specific genes. The muscle regulatory factors—Myf5, MyoD, MRF4, and myogenin—have been identified as master regulators of skeletal muscle differentiation. Their function is potentiated by the myocyte enhancer-binding factor 2 family (Buckingham *et al.*, 2003; Shi and Garry, 2006). Moreover, differentiation requires the expression and activity of cyclin-dependent kinase (Cdk) inhibitors, such as p21 and p27, critical for the withdrawal of myoblasts from the cell cycle (Kitzmann and Fernandez, 2001). During myoblast differentiation, nestin is down-regulated and replaced by the IF protein desmin in fully differentiated myotubes with the exception of specific nestin expression at the myotendinous and neuromuscular junctions (NMJs; Vaittinen *et al.*, 1999).

Cdk5 is a signaling factor serving critical functions during embryogenesis, as indicated by Cdk5 knockout (KO) mice that exhibit perinatal mortality associated with abnormal corticogenesis (Ohshima *et al.*, 1996). In addition to the impairments observed in migrating neurons, and thus in cortical development (Ohshima *et al.*, 1999), deficiencies in Cdk5 activity affect muscle tissue by inhibiting myogenesis (Lazaro *et al.*, 1997; Philpott *et al.*, 1997) and compromising signal transduction at the NMJs (Fu *et al.*, 2001, 2005). In a previous study, we demonstrated that, in differentiating myoblasts, Cdk5 activity is critically regulated by the atypical protein kinase C ζ (PKCζ). PKCζ phosphorylates the Cdk5 activator protein p35 and induces calpain activity, two events that collectively regulate the generation of a degradation-resistant activator, p25 (de Thonel *et al.*, 2010). The occurrence of the Cdk5/p25 signaling complex seems essential for maintaining adequate Cdk5 activity throughout the differentiation process. In addition, we have reported that Cdk5 regulates the reorganization of nestin filaments during myoblast fusion (Sahlgren *et al.*, 2003). Inhibition of the PKCζ–Cdk5 pathway disturbs the organization of nestin, simultaneously hampering differentiation (de Thonel *et al.*, 2010). Furthermore, nestin IFs have turned out to constitute a dynamic scaffold for the Cdk5 signaling complex, as nestin mediates cell survival during oxidative stress by regulating the activity and subcellular localization of the Cdk5/p35 complex through interaction (Sahlgren *et al.*, 2006).

In light of the studies pointing to the importance of nestin for cellular features often connected to stem and progenitor cells, we decided to investigate whether nestin functions in maintaining the undifferentiated state of progenitor cells or in the regulation of the progress of differentiation. Furthermore, we wanted to delineate whether these functions could be linked to the PKCζ-Cdk5–mediated signaling pathway that we already have demonstrated to determine myogenic differentiation. We report that nestin has the capacity to determine the progress of myoblast differentiation. Overexpression of nestin effectively inhibits the differentiation of myoblasts to multinucleated myotubes, whereas the ablation of nestin accelerates the differentiation process. Nestin regulates the initial phases of differentiation by an interplay with Cdk5, where nestin regulates Cdk5 activity by controlling p35 processing to the stable activator protein, p25. At the same time, Cdk5 activity is critical for nestin stability during myoblast differentiation, indicating that Cdk5 regulates its own scaffold and inhibitor.

## RESULTS

### Nestin controls the induction of differentiation in C2C12 and mouse primary myoblasts

To assess the functional role of nestin in myogenesis, we down-regulated nestin using the RNAi technique in C2C12 myoblasts, skeletal muscle–derived cells commonly used to study the mechanisms of muscle differentiation. C2C12 myoblasts were transfected with either RNAi control oligos (scrambled) or oligos targeting nestin mRNA and were induced to differentiate by removal of serum mitogens. Down-regulation of nestin significantly accelerated differentiation when comparing C2C12 cells targeted with nestin RNAi to cells targeted with scrambled RNAi oligos (Figure 1A). The relative amount of myosin heavy chain (MHC)-positive myoblasts undergoing fusion at 36 and 48 h of differentiation was almost twofold upon nestin down-regulation (Figure 1A). The difference was even more pronounced when assessing the characteristic morphological features of differentiated myoblasts, as most of the nestin-depleted cells showed prominent alignment and elongation compared with the scrambled oligo-targeted cells, among which only a fraction of cells had started to align and elongate (Figure 1A). In agreement with accelerated differentiation, the nestin-depleted cells exhibited a more efficient withdrawal from the cell cycle, as demonstrated by the increased expression of the Cdk2 inhibitors p21 and p27 (Figure 1B), both of which act as key regulators of the cell cycle. Moreover, the ablation of nestin dramatically enhanced the expression of the muscle regulatory factor myogenin as well as the downstream myogenic markers troponin and MHC (Figure 1C). The presence of the nestin polymerization partner, the IF protein vimentin, is presented as a control to demonstrate the efficient and highly specific down-regulation of nestin (Figure 1C; vimentin levels are down-regulated only at the late stages of myoblast differentiation). To corroborate our findings, we monitored the effect of nestin down-regulation on myoblast differentiation using two separate RNAi oligos instead of an oligo mixture (Supplemental Figure 1) Similar effects were observed with both approaches, confirming the ability of nestin to critically regulate differentiation.

Increased differentiation coincides with decreased proliferation, and many proliferation-promoting mechanisms inhibit differentiation. For example, signaling launched by transforming growth factor β has been proven to significantly increase proliferation, thereby incurring a delay in differentiation (Schabort *et al.*, 2009). The deliberate inhibition of the leukemia inhibitory factor–stimulated JAK1–STAT1–STAT3 signaling pathway inhibits the proliferation of myoblasts, simultaneously accelerating differentiation (Sun *et al.*, 2007). We wanted to investigate if nestin depletion affects the proliferation of actively dividing myoblasts in growth medium. No difference was detected, however, in either the metabolic MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphe-nyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt) assay (a measure of metabolic activity, which in turn can be used as a cell number indicator; Figure 1D) or the protein amounts of Ki-67 and proliferating cell nuclear antigen (PCNA), both of which are characteristic cellular proliferation markers (Figure 1E). Furthermore, nestin-depleted myoblasts showed no signs of cell-cycle withdrawal if maintained in growth medium (i.e., before being subjected to differentiation), as indicated by the equal levels of p21, p27, and Cyclin D1 in both nestin down-regulated and control cells (Figure 1E). In addition to the arrest of proliferation, the ability of myoblasts to migrate is another important aspect of muscle development and regeneration (Rochlin *et al.*, 2010). Cell migration bringing myoblasts to close proximity occurs before cell fusion. Stromal derived factor 1α (SDF-1α) and its receptor CXC chemokine receptor 4 are associated
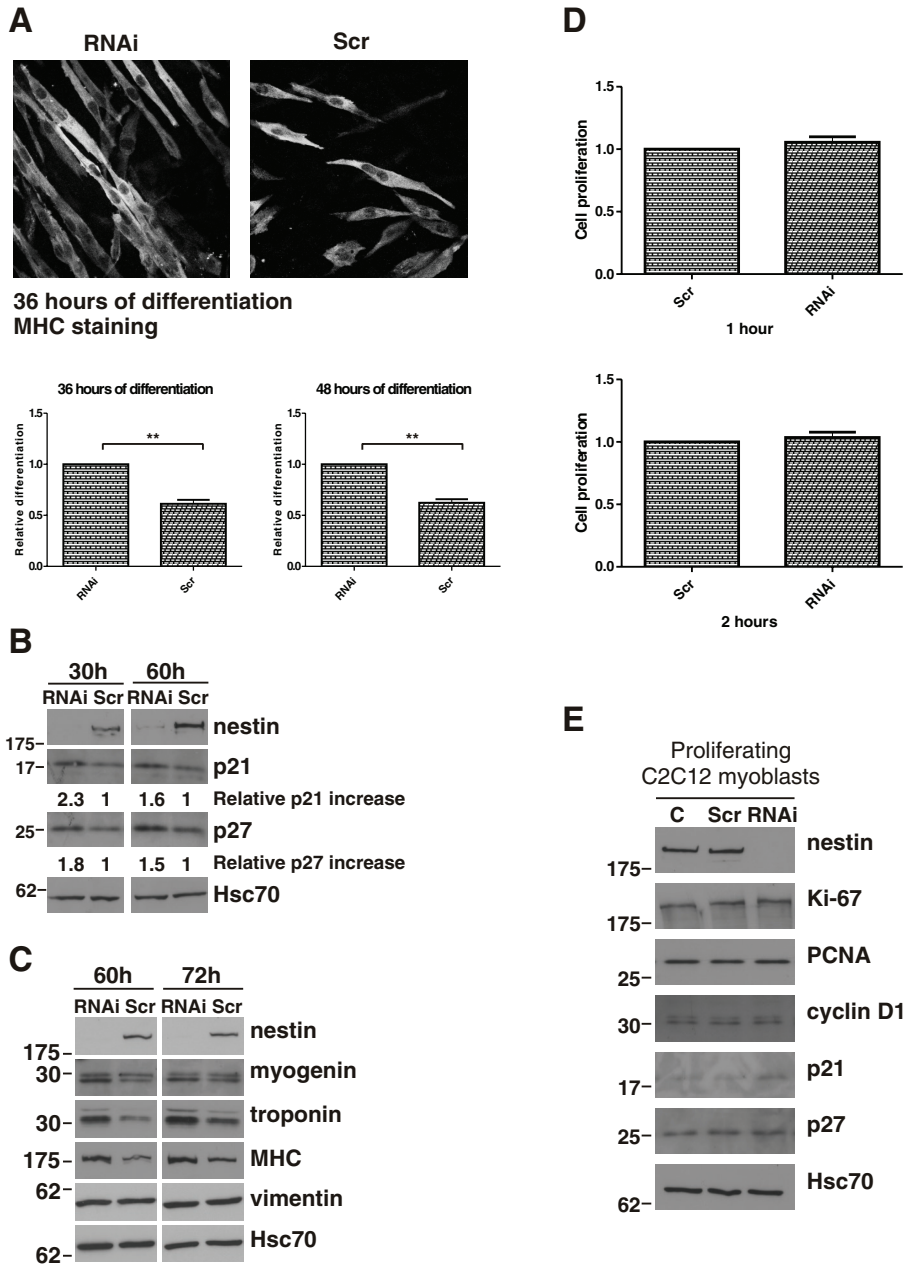
**A** RNAi    Scr

**36 hours of differentiation
MHC staining**

36 hours of differentiation

48 hours of differentiation

**B**

|   | 30h | 60h | |
|---|-----|-----|---|
| | RNAi Scr | RNAi Scr | |

175—
17—    nestin
         p21
**2.3  1    1.6  1    Relative p21 increase**
25—    p27
**1.8  1    1.5  1    Relative p27 increase**
62—    Hsc70

**C**

|   | 60h | 72h | |
|---|-----|-----|---|
| | RNAi Scr | RNAi Scr | |

175—   nestin
30—    myogenin
30—    troponin
175—   MHC
62—    vimentin
62—    Hsc70

**D**

Cell proliferation

Scr    RNAi
**1 hour**

Scr    RNAi
**2 hours**

**E**

Proliferating
C2C12 myoblasts

C   Scr  RNAi

175—   nestin
175—   Ki-67
25—    PCNA
30—    cyclin D1
17—    p21
25—    p27
62—    Hsc70

**FIGURE 1:** Nestin down-regulation accelerates the differentiation of myoblasts. (A) C2C12 myoblasts were transfected with nestin or scrambled RNAi oligos and induced to differentiate. Samples were fixed and labeled for MHC at the indicated time point. The amount of MHC-positive cells was counted from three independent experiments, and results are presented as relative cell differentiation. Statistical difference was determined with paired *t* test (**p < 0.01). Graphs show SEMs. (B and C) Cell lysates of RNAi or scrambled/ transfected myoblasts were prepared at indicated time points and subjected to Western blotting to detect markers of cell-cycle progression (B) and myogenic differentiation (C). Nestin immunoblot indicates successful down-regulation. p21 and p27 Western blots were quantified by densitometric analysis (ScionImage GelPlot 2), and the obtained values were normalized to loading controls. Numbers indicate the relative increase in p21/p27 in nestin down-regulated cells compared with cells targeted with scrambled oligos at each time point. Vimentin was used as a control to determine the specificity of nestin RNAi oligos. Hsc70 demonstrates equal loading. (D) To asses the effect of nestin on proliferation, the proliferative capacity of nestin-depleted myoblasts was determined by MTS assay. Results representing the relative proliferation are obtained from five independent experiments. Statistical difference was determined with paired *t* test (ns, p > 0.05), and the results are illustrated by GraphPad Prism. Graphs show SEMs. (E) The expression of different cell-cycle markers in nestin down-regulated myoblasts compared with myoblasts targeted with scrambled oligos was analyzed by Western blotting.

with chemotaxis of many different cell types, including myoblasts (Odemis *et al.*, 2007). We did not observe changes in the migratory response of nestin-deficient cells to SDF-1α compared with cells targeted with scrambled oligos (unpublished data). This observation illustrates that the regulatory function of nestin specifically relates to the early stages of differentiation processes. More specifically, whereas nestin depletion efficiently accelerates processes related to already initiated differentiation, it has no effect on cell proliferation by itself and is not sufficient to induce differentiation but requires additional differentiation-mediating cues.

Prompted by the striking effect of nestin depletion on C2C12 differentiation, we examined the reverse situation (i.e., the consequences of nestin overexpression. To obtain stable levels of overexpressed nestin, we used the N-terminal nestin fragment containing the first 640 amino acids (Sahlgren *et al.*, 2006). It has a longer half-life compared with full-length nestin as reflected by apparent stabilization and more prominent protein levels (Sahlgren *et al.*, 2006). Intriguingly, the overexpression of nestin efficiently repressed differentiation (Figure 2A). In nest-640–overexpressing cells, we observed a strikingly low number of nest-640/MHC double-positive cells among all differentiated, MHC-positive cells at 36 and 48 h of differentiation (Figure 2A). Similar results were obtained with another differentiation marker, troponin (Figure 2B). The nest-640–expressing cells showed no signs of differentiation, without any effects on viability and without signs of abnormal morphology. Furthermore, the amounts of p21 and p27 at 12 and 24 h of differentiation remained low in nestin-overexpressing cells compared with cells overexpressing empty green fluorescent protein (GFP)-vector pointing to a less efficient withdrawal from the cell cycle (Figure 2C). Similarly, the levels of myogenin, troponin, and MHC were reduced upon nestin overexpression (Figure 2D).

To validate the results obtained from C2C12 myoblasts, we analyzed mouse primary myoblast cultures derived from satellite cells isolated from neonatal limb muscles. Using nestin-specific RNAi, we managed to obtain a good down-regulation of nestin, as confirmed by Western blotting (Figure 3). Similarly to C2C12 myoblasts, the ablation of nestin in primary myoblasts triggered the cell-cycle withdrawal under differentiation-promoting conditions, as indicated by the increased amounts of p21 and p27 (Figure 3A). Consistently, the levels of myogenin and troponin were reproducibly
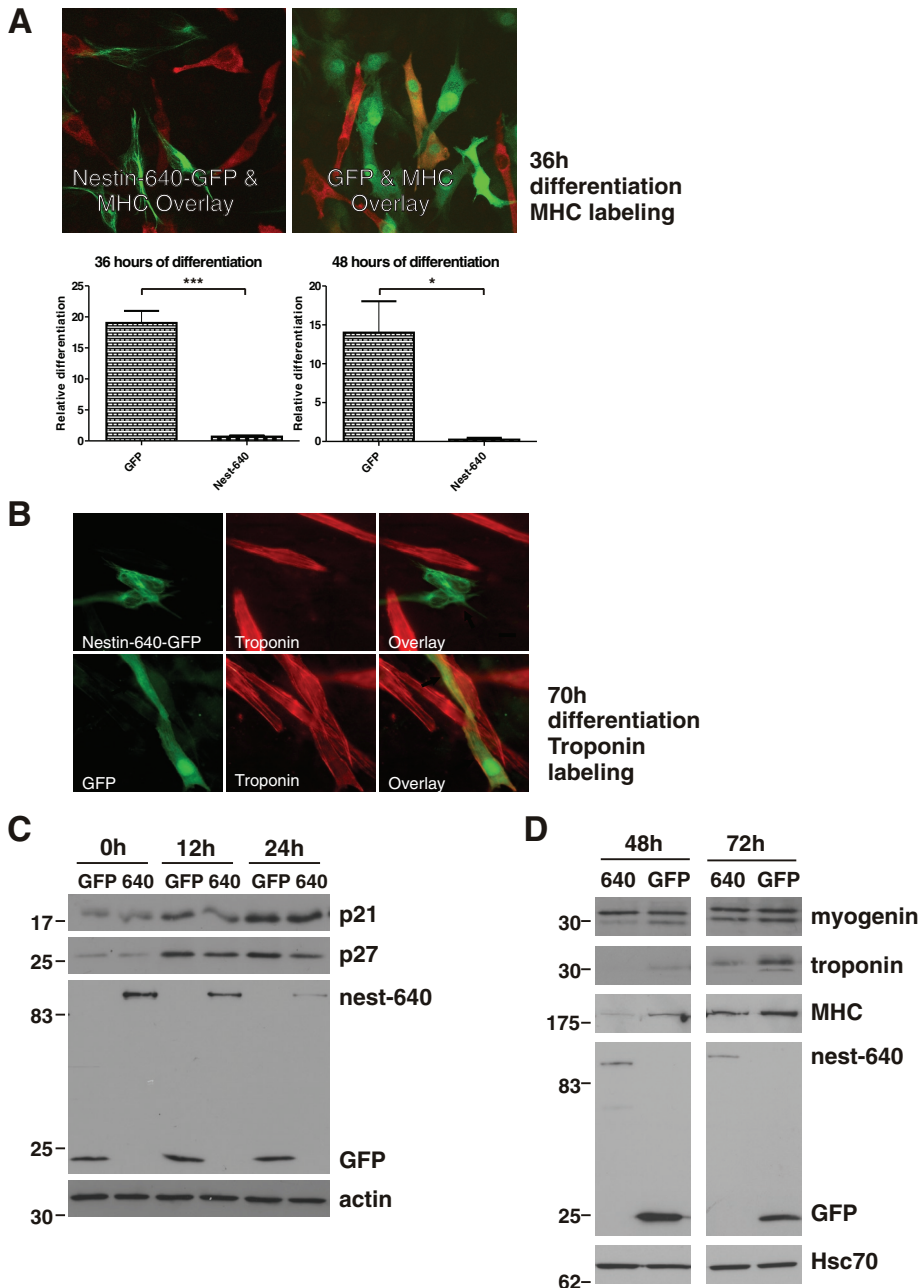
**FIGURE 2:** Overexpression of nestin represses the differentiation of myoblasts. (A and B) C2C12 myoblasts were transfected with GFP-tagged nestin fragment, nest-640, or empty GFP vector and induced to differentiate. Myoblasts were fixed and stained with MHC (A) and troponin (B) to visualize differentiating cells. (A) The amount of GFP/MHC double-positive cells compared with all MHC-positive cells at 36 and 48 h of differentiation was calculated from six and three independent experiments, respectively, and the relative values were illustrated by GraphPad Prism. Statistical difference was determined with unpaired $t$ test (***$p < 0.001$, *$p < 0.05$). Graphs show SEM. (C and D) Cell lysates prepared at indicated time points were resolved by SDS–PAGE and analyzed by Western blotting for the presence of Cdk inhibitors (C) and myogenic markers (D). GFP antibody was used to detect nest-640 and GFP to confirm successful transfection. Actin and Hsc70 were used as loading controls.

Previous studies have shown that the absence of nestin polymerization partner affects both nestin protein levels and assembly state (Eliasson et al., 1999). To analyze differentiation as a consequence of the assembly state of nestin, we isolated primary myoblasts from vimentin KO mice and investigated their differentiation profile. Although the increase in the total amount of nestin observed during differentiation (Sahlgren et al., 2003) was equal in both wild type (WT) and KO myoblasts (Figure 3A), the mechanical and nonmechanical properties of nestin polymers were postulated to be altered in the absence of vimentin in light of previous studies (Eliasson et al., 1999). Interestingly, the cell-cycle withdrawal seemed more prominent in vimentin KO cells after the induction of differentiation, as indicated by the increased amounts of p21 and p27 (Figure 4A). Similarly, the elevated amounts of differentiation markers desmin and troponin in vimentin KO myoblasts (Figure 4B) suggest that the modulation of nestin organization by vimentin depletion affects the progress of myogenic differentiation.

## Nestin-mediated regulation of the Cdk5/p35 signaling complex influences myoblast differentiation

Cdk5 signaling has been implicated as a critical regulator of myogenesis (Lazaro et al., 1997; Philpott et al., 1997). We have previously demonstrated a complex interrelationship between nestin and Cdk5 both in muscle and in neuronal cells (Sahlgren et al., 2003, 2006; de Thonel et al., 2010). To gain insight into the mechanistic role of nestin as a regulator of myoblast differentiation and to elucidate whether the nestin-induced effect on differentiation would be mediated by Cdk5, we examined Cdk5 signaling during differentiation in the absence and presence of nestin. Overexpression of dominant-negative dnCdk5 (dnCdk5) (Figure 5A) or inhibition of Cdk5 activity with roscovitine (Figure 5B) reversed the accelerating effect of nestin down-regulation, implicating that nestin and Cdk5 function in the same differentiation-promoting pathway.

Our previous results imply that nestin might serve as a scaffold regulating the activity of the Cdk5/p35 signaling complex (Sahlgren et al., 2003, 2006). Hence we wanted to test whether such a scaffolding function of nestin would be operational during myoblast differentiation also. The stabilized form of nestin, nest-640, sequestered Cdk5 in C2C12 myoblasts while inhibiting differentiation (Figure 5C). More importantly, as our recent study implicates that the accumulation of p25 is critical for Cdk5 activity and myoblast differentiation (de Thonel et al., 2010), we investigated whether the modifications in the IF scaffold affect the p35

elevated in nestin-depleted cells, pointing to accelerated differentiation (Figure 3B). These results clearly demonstrate that the concept of nestin-mediated regulation of myogenesis is generally applicable.

Vimentin is implicated as a specific polymerization partner of nestin, which alone is unable to form filaments (Steinert et al., 1999).

## A



| | 24h | | 48h | | |
|---|---|---|---|---|---|
| | Scr | RNAi | Scr | RNAi | |
| 175– | | | | | nestin |
| 17– | | | | | p21 |
| | 1 | 1.2 | 1 | 1.2 | Relative p21 increase |
| 25– | | | | | p27 |
| | 1 | 1.9 | 1 | 1.4 | Relative p27 increase |
| 62– | | | | | Hsc70 |

## B

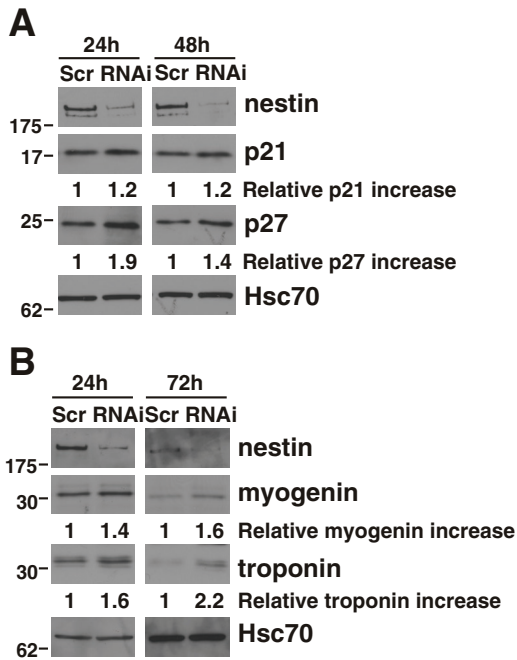| | 24h | | 72h | | |
|---|---|---|---|---|---|
| | Scr | RNAi | Scr | RNAi | |
| 175– | | | | | nestin |
| 30– | | | | | myogenin |
| | 1 | 1.4 | 1 | 1.6 | Relative myogenin increase |
| 30– | | | | | troponin |
| | 1 | 1.6 | 1 | 2.2 | Relative troponin increase |
| 62– | | | | | Hsc70 |

FIGURE 3: Nestin determines the commencement of differentiation in mouse primary myoblast. (A and B) Cultures of primary myoblasts established from the limb skeletal muscles of 2-d-old FVB-n mice were transfected with RNAi oligos and induced to differentiate. Samples were harvested at indicated time points and subjected to Western blotting with antibodies indicating cell-cycle withdrawal (A) and differentiation (B). Western blots were quantified by densitometric analysis, and the obtained values were normalized to loading controls. Numbers indicate the relative increases in p21, p27, myogenin, and troponin in nestin down-regulated cells compared with cells targeted with scrambled oligos.

## A



| | 0h | | 3h | | 24h | | 36h | | |
|---|---|---|---|---|---|---|---|---|---|
| | WT | KO | WT | KO | WT | KO | WT | KO | |
| 62– | | | | | | | | | vimentin |
| 175– | | | | | | | | | nestin |
| 17– | | | | | | | | | p21 |
| 25– | | | | | | | | | p27 |
| 30– | | | | | | | | | actin |

## B

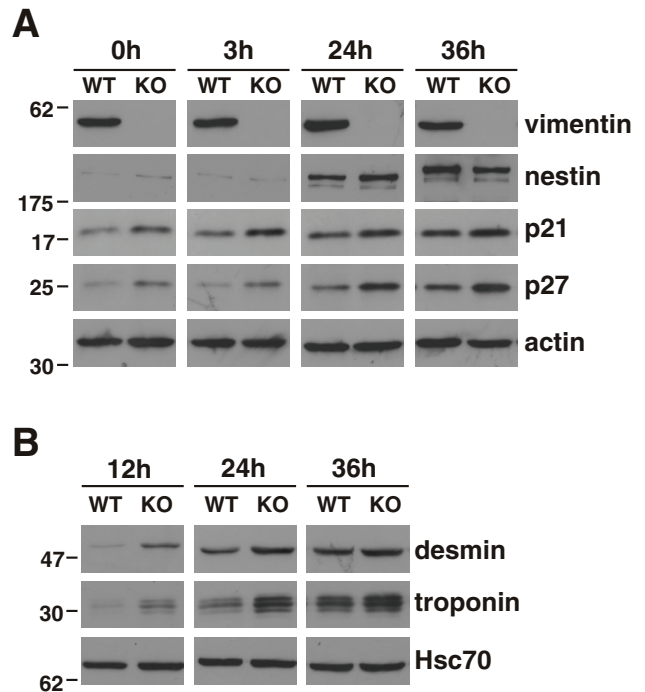| | 12h | | 24h | | 36h | | |
|---|---|---|---|---|---|---|---|
| | WT | KO | WT | KO | WT | KO | |
| 47– | | | | | | | desmin |
| 30– | | | | | | | troponin |
| 62– | | | | | | | Hsc70 |

FIGURE 4: Depletion of the nestin polymerization partner vimentin modulates the differentiation similarly to nestin down-regulation. (A and B) Primary myoblasts were isolated from the limb muscles of 2-d-old WT and vimentin KO mice. Equal amounts of WT and KO myoblasts were plated in 12-well plates, and the cultures were induced to differentiate after having reached 80% confluence. Western blot samples were harvested at indicated time points, and the presence of IF proteins, Cdk inhibitors (A), and myogenic markers (B) was assessed.

processing in this system. Indeed, p25 formation was clearly reduced when nestin was overexpressed (Figure 6A), whereas the amount of p25 generated during differentiation in nestin-depleted cells was considerably increased compared with control cells as evaluated by Western blotting (Figure 6B).

To further analyze the influence of the nestin scaffold on the calpain-mediated processing of p35, which we have recently mapped to be a key component of myoblast differentiation (de Thonel et al., 2010), we transfected HeLa cells either with GFP-tagged nest-640 or an empty GFP vector together with Cdk5 and p35. Nest-640 was only partially incorporated into the filament network composed of vimentin in HeLa cells. A small fraction of overexpressed nestin remained soluble, indicating disturbed polymerization into filaments (Supplemental Figure 2A). Similarly, nest-640 remained partially soluble in differentiating C2C12 myoblasts (Supplemental Figure 2B). We observed that the loss of the p35 form on A23187 treatment (de Thonel et al., 2010) was reduced in the presence of nest-640 (Figure 6C). This observation implies that nestin has a significant impact on $Ca^{2+}$/calpain-mediated processing of p35. Moreover this result prompted us to evaluate whether the fate of p35 is determined by the nestin incorporated into filaments or the fraction of nestin remaining insoluble. As a model system we used vimentin-positive (v+) and vimentin-negative (v−) clonal variants of SW13 adenocarcinoma cells. Cells were transfected with nest-640 together with p35, and the formation of the Cdk5/p35 complex was analyzed. Indeed, more Cdk5 associated with p35 was observed in v− SW13-clone where nest-640 remained soluble (Figure 6D). Vimentin

appeared not to have any effect on Cdk5/p35 complexing (Figure 6E), hence corroborating the specific regulatory effect of nestin over the Cdk5/p35 signaling complex. Furthermore $Ca^{2+}$-dependent p35 processing was more prominent in nest-640–transfected v+ SW13 cells (Figure 6F). Therefore it can be concluded that soluble nestin promotes the interaction with Cdk5/p35 and that the increased sequestration of Cdk5/p35 attenuates p35 processing.

### Dynamic interplay between nestin and Cdk5 during myogenic differentiation

In addition to the efficient, interaction-based regulation of Cdk5, nestin itself is a target for Cdk5. We have previously shown that Cdk5 phosphorylates nestin on Thr-316 during myogenic differentiation, thereby regulating nestin reorganization and disassembly into soluble fragments (Sahlgren et al., 2003). Here we induced C2C12 cells to differentiate and analyzed the levels of both nestin and nestin phosphorylated on Thr-316. The results showed that the amount of phosphorylated nestin increases along with the overall protein amount of nestin starts to decline at the later stages of differentiation, suggesting that Cdk5-mediated phosphorylation protects nestin from degradation (Figure 7A). Based on the ratio between the total nestin signal and that of p-Thr-316 nestin, the phosphorylation seems to occur at relatively low stoichiometry, indicating that phosphorylation of a subpopulation may be sufficient for both stabilization and inhibitory effect. To establish the hierarchy of the signaling complex, with Cdk5 indeed regulating the stability of nestin, we showed that inhibition of the PKCζ–Cdk5 signaling axis (de Thonel et al., 2010), by both PKCζ inhibition (upstream of Cdk5)
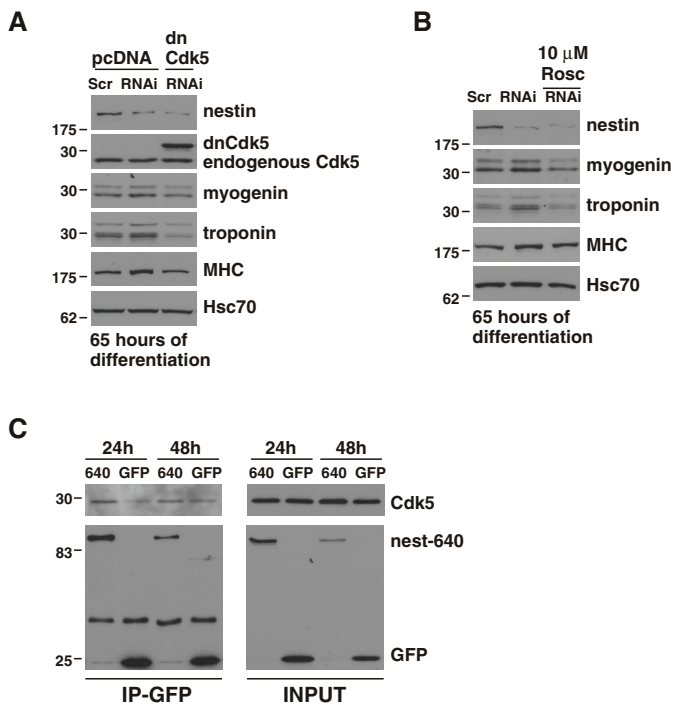
**FIGURE 5:** Increased differentiation of nestin-depleted cells can be reversed by Cdk5 inhibition. (A) C2C12 myoblasts were transfected with nestin or scrambled RNAi oligos followed by another transfection with dnCdk5 or an empty vector (pcDNA). Cells were induced to differentiate and harvested 65 h later. Western blotting was performed with indicated antibodies. (B) C2C12 myoblasts were transfected with nestin or scrambled RNAi oligos. Six hours after transfection, cells were induced to differentiate. Roscovitine (10 μM) was added 12 h after the induction of differentiation. Cell lysates were prepared after 65 h in differentiation medium and subjected to Western blotting with indicated antibodies. (C) C2C12 myoblasts were transfected with either nest-640 or empty GFP vector and induced to differentiate. The ability of nestin truncation to sequester Cdk5 in differentiating C2C12 myoblasts was assessed by coimmunoprecipitation of GFP-tagged nest-640 and endogenous Cdk5. The interaction between nestin and Cdk5 appears specific, as Cdk5 does not form a complex with GFP only. The presence of Cdk5 and GFP in the cell lysates prior to immunoprecipitation is shown in the right panel (Input).

and inhibition of Cdk5 by dominant-negative transfection and roscovitine, efficiently reduced nestin protein levels (Figure 7, B and C), without negative effects on nestin transcription (Figure 7D). Hence, while nestin regulates p35 processing and Cdk5 activity, Cdk5 in turn has the ability to modulate its own activity by regulating the levels and organization of its own scaffold and inhibitor, nestin.

## DISCUSSION

Nestin is characterized by its specific expression in the developing CNS and muscle tissue (Lendahl *et al.*, 1990; Sejersen and Lendahl, 1993). Whereas its expression has been studied extensively, especially in various contexts related to stem cell differentiation, the roles of nestin for the development of these tissues have not been addressed. This study shows that nestin contributes to the execution of myogenesis. The regulation of cell-cycle exit on mitogen withdrawal and the induction of myogenic genetic program, as indicated by the expression of differentiation markers, are critically affected by nestin. In contrast, baseline proliferation and the motility of dividing,

undifferentiated myoblasts are not modulated by nestin, further supporting a role for nestin at the regulatory steps at which differentiation has already been commenced. Therefore nestin functions are related to regulating the progress of differentiation rather than maintaining the undifferentiated state of progenitor cells.

Since the discovery of IFs as major phosphorylation targets (Inagaki *et al.*, 1987), the role of numerous reported IF–kinase interactions (Pallari and Eriksson, 2006; Hyder *et al.*, 2008; Eriksson *et al.*, 2009) has become increasingly evident, as they have been related to the regulation of specific signaling pathways. In fact, the ability of IFs to regulate multiple cellular processes by directly interacting with different signaling determinants is a rapidly expanding area of IF research. IFs have been demonstrated to sequester or act as a scaffold for, besides kinases, receptors and 14-3-3 proteins, thereby linking IFs to the integration of signals related to stress responses, cell survival, and cell growth (Pallari and Eriksson, 2006; Hyder *et al.*, 2008; Eriksson *et al.*, 2009). The highly specific spatiotemporal expression patterns of IF proteins make them versatile regulators of tissue- and cell-specific signaling processes. Given the precisely controlled expression of nestin during myoblast differentiation, nestin is potentially an attractive regulator of signaling events in myogenesis.

We have previously shown that nestin interacts with the Cdk5/p35 signaling complex during myogenic differentiation (Sahlgren *et al.*, 2003) and that nestin efficiently regulates the activity and subcellular localization of this complex during oxidative stress in neuronal progenitor cells (Sahlgren *et al.*, 2006). Here we show that nestin influences the differentiation of myoblasts in a Cdk5-dependent manner, by affecting the cleavage of p35 to more stable p25. Although the formation of the Cdk5/p25 complex is known to be responsible for the detrimental Cdk5 activity in neurodegenerative disorders (Ikiz and Przedborski, 2008; Kim *et al.*, 2008), we have recently proposed a developmentally important role for Cdk5/p25 and have shown that the calpain-mediated generation of p25 is required to maintain adequate Cdk5 activity during myoblast differentiation (de Thonel *et al.*, 2010). In further support of such nondetrimental, physiological effects of p25, the Cdk5/p25 complex has been demonstrated to have an important role in synaptic plasticity, synaptogenesis, learning, and memory (Fischer *et al.*, 2005). Transient expression of p25 in hippocampus induced an increase in N-*methyl-d-aspartate* signaling, spine density, and the number of synapses and facilitated learning and memory, whereas prolonged p25 activity led to a neuronal loss and severe cognitive defects. The authors proposed that, in neuronal systems, p25 activity is not harmful but can easily lead to neurodegeneration when additional factors increase p25 levels over the threshold. Here we provide evidence that nestin plays a key role in the regulation of p35 processing. Overexpression of nest-640, a stabilized form of nestin that allows for elevated expression of this otherwise tightly regulated protein, efficiently inhibited the generation of p25 in myoblasts, as well as calpain-mediated p35 reduction.

Nestin was shown to affect p35 cleavage and Cdk5 activity in an assembly-specific manner. We observed a stronger interaction between Cdk5 and p35 when nestin was expressed without a copolymerization partner vimentin, previously demonstrated to affect the assembly state of nestin (Eliasson *et al.*, 1999). p35 has been shown to be protected from calpain-mediated cleavage through Cdk5-dependent regulation (Kamei *et al.*, 2007). Our data suggest that the soluble pool of nest-640 sequesters Cdk5 more efficiently and facilitates Cdk5/p35 complexing, thus impeding the targeting of calpains to p35. In nestin-depleted myoblasts, the physiological regulation of Cdk5/p35 by nestin is absent, leading to uncontrolled p35
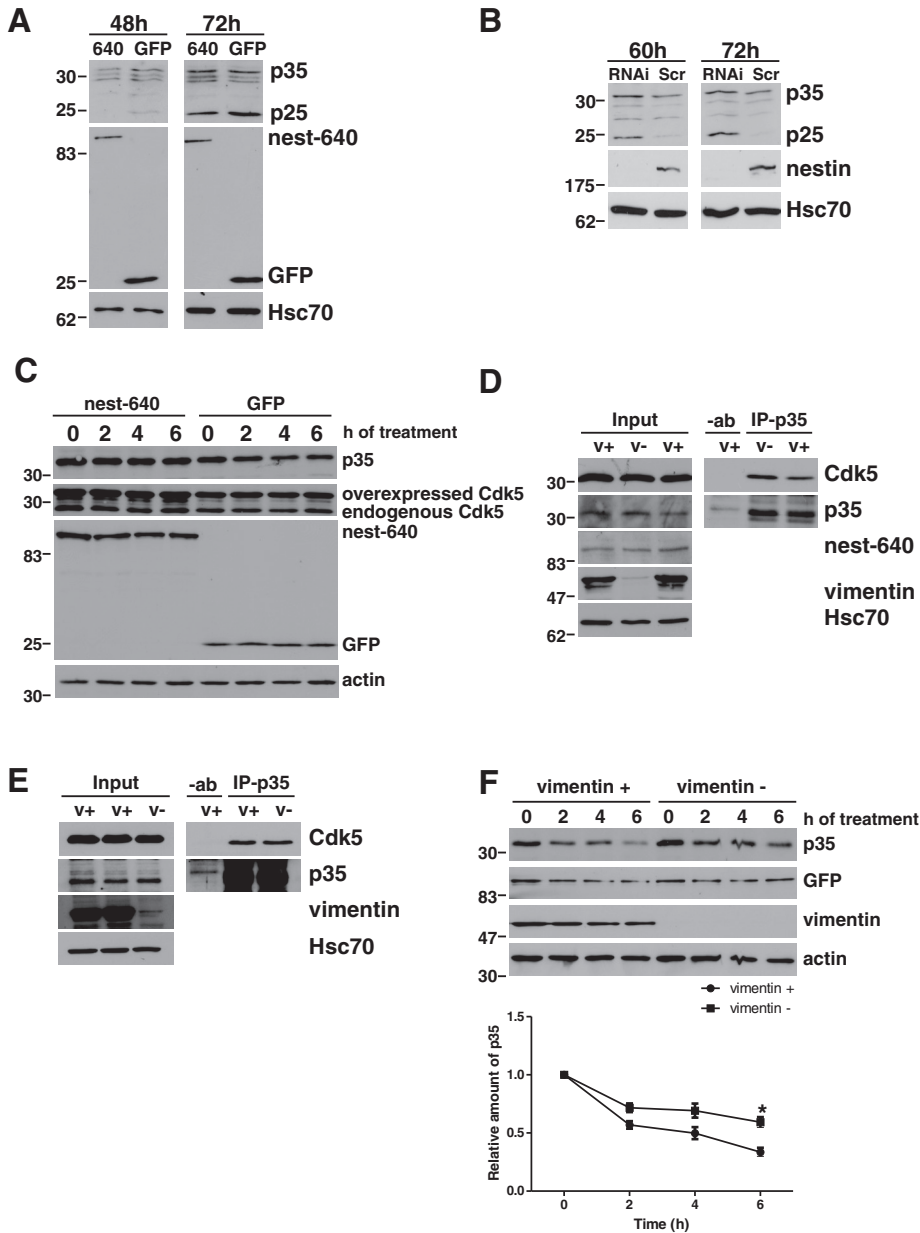
FIGURE 6: Nestin scaffold regulates the processing of p35. (A) Nestin overexpressing or (B) nestin-depleted C2C12 myoblasts were induced to differentiate. Samples were collected at indicated times, and the generation of p25 was assessed by Western blotting. (C) HeLa cells were transfected with either nest-640 or empty GFP vector together with Cdk5 and p35, and the effect of calcium ionophore A23187-induced increase in intracellular Ca²⁺ levels on p35 cleavage was investigated. Cells were treated with 10 μM A23187 and harvested at different times of treatment, and the cell lysates were subjected to Western blotting. Transfection efficiency was confirmed by p35, Cdk5, and GFP antibodies. (D and E) V– and v+ SW13 cells were transfected either with GFP-tagged nest-640 and p35 (D) or with p35 and an empty vector (E) to maintain an equal plasmid amount. Coimmunoprecipitation of p35 and Cdk5 was performed. The interaction between p35 and Cdk5 was demonstrated by Western blotting. Input samples collected before immunoprecipitation indicate the equal amount of proteins and the presence/absence of vimentin. (F) V+ and v– SW13 cell clones were transfected with plasmids encoding nest-640 and p35. To trigger calpain activity, 10 μM A23187 was added to cells for indicated times. Cell lysates were resolved by SDS–PAGE, and p35 processing was assessed by Western blotting. Western blots from three independent experiments were quantified with densitometric analysis, and the samples were normalized to untreated control samples (value 1). Statistical significance at the 6-h time point was determined with paired *t* test (*p < 0.05), and the results are illustrated by GraphPad Prism.

processing and Cdk5 activity. These results reflect the potent ability of nestin in controlling the Cdk5/p35/p25 signaling complex and strengthen the results of our previous report, presenting nestin as a key determinant in p25 generation (Sahlgren et al., 2006). Most importantly, this model implies that strict control of nestin stability and polymerization is essential during myogenic differentiation, as nestin can have a pronounced effect on cell fate.

Although relatively little is known about the functions of nestin, it has been implicated in critical cell fate decisions. Apart from its specific expression during development, it is characteristically elevated on various types of neurotrauma (Pekny and Lane, 2007) and muscle damage (Vaittinen et al., 1999; Sahlgren et al., 2003), in some cases along with a seemingly similar IF protein synemin (Jing et al., 2007). Asymmetric distribution of nestin occurs in mitotic neuronal progenitor cells affecting cell survival (Bieberich et al., 2003), and in avian neuroepithelium the ortholog of nestin, transitin, determines the mitotic segregation of numb, important regulator of neurogenesis, to one of the daughter cells (Wakamatsu et al., 2007). Moreover, nestin has been hypothesized as an important regulator in embryogenesis as nestin-mediated scaffolding of GR prevents the nuclear accumulation of GR enforcing cell-cycle arrest (Reimer et al., 2009). We have previously provided evidence of the signaling functions of nestin describing the influence of nestin-dependent Cdk5/p35 regulation on the execution of oxidant-induced cell death (Sahlgren et al., 2006). In light of these examples, it is not surprising that nestin could participate in the decision making also during myoblast differentiation.

The interrelationship between nestin and Cdk5 is bidirectional: Whereas Cdk5 activity is required to elevate the expression of and to stabilize nestin, conversely, nestin has the capacity to efficiently regulate the Cdk5/p35 signaling complex. We have revealed that the Cdk5-mediated phosphorylation of nestin is important both for nestin stability and reorganization on myoblast fusion (Sahlgren et al., 2003; de Thonel et al., 2010). These results point out a feedback mechanism wherein Cdk5 regulates its own scaffold and its negative inhibitory loop during myoblast differentiation. Analogously, Cdk5 has been shown to inhibit talin head domain ubiquitylation by E3 ubiquitin ligase Smurf1 and, thereby, talin degradation (Huang et al., 2009). Moreover, the Cdk5-mediated phosphorylation of tumor suppressor protein p53
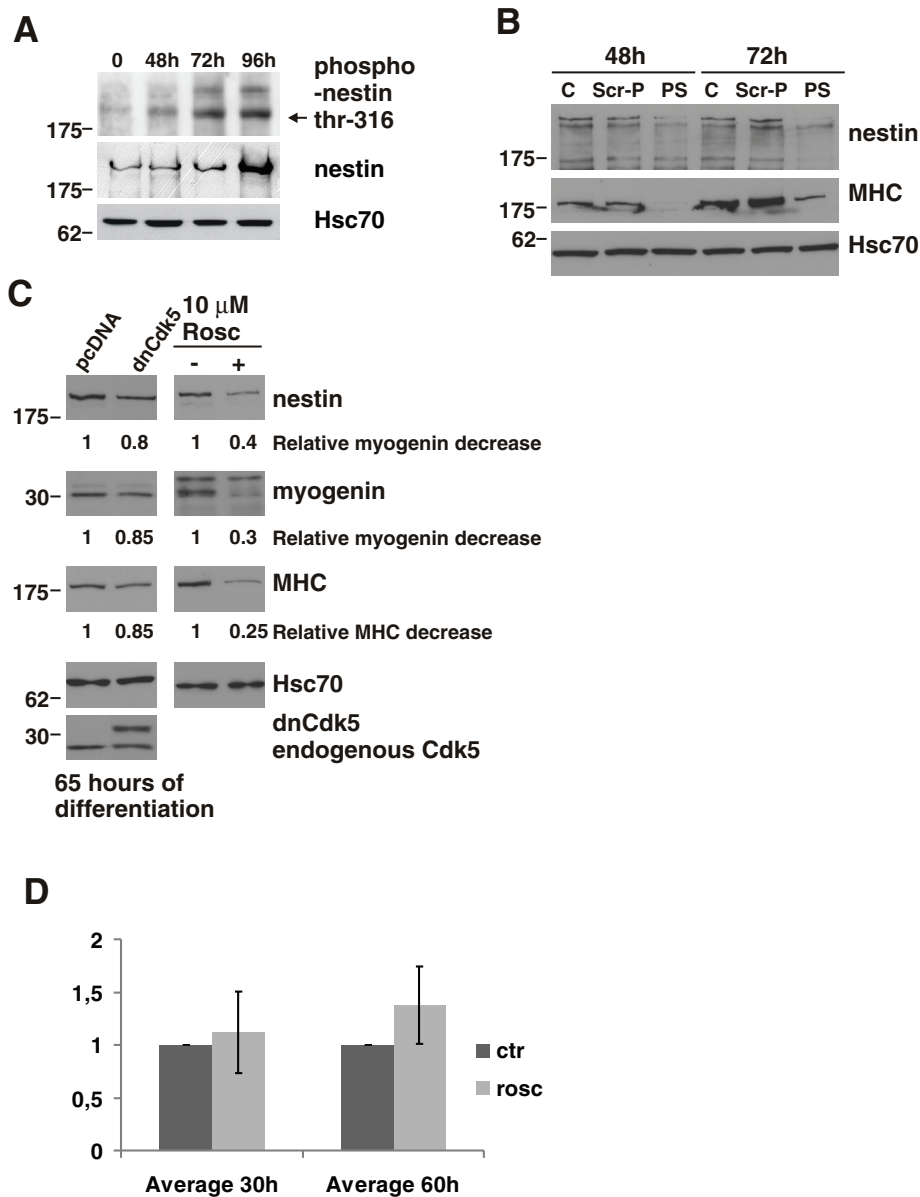
**FIGURE 7:** Differentiation-associated Cdk5 signaling is an important regulator of nestin protein amount. (A) C2C12 myoblasts were induced to differentiate, and the levels of nestin phosphorylated on Thr-316 as well as the overall nestin protein levels were determined by immunoblotting. (B) The role of PKCζ–Cdk5 signaling pathway as a regulator of nestin stability was analyzed by differentiating the C2C12 myoblasts in the presence of the PKCζ pseudosubstrate inhibitor (PS). Scrambled peptide (Scr-P) was used as a negative control. MHC indicates the PKCζ-dependent inhibition of myoblast differentiation. (C) The effect of Cdk5 inhibition on nestin during myoblast differentiation was investigated by transfecting C2C12 myoblasts with dnCdk5 before the induction of differentiation or treating the differentiating myoblasts with 10 μM Cdk5 inhibitor Roscovitine. The protein amounts of nestin and myogenic markers were studied by Western blotting. Western blots were quantified by densitometric analysis, and the obtained values were normalized to loading controls. Numbers indicate the relative decreases in nestin, myogenin, and MHC in Cdk5-inhibited myoblasts compared with control myoblasts. (D) RT-PCR analysis of differentiating myoblasts treated with 10 μM Roscovitine was performed to rule out the negative effect of Cdk5 inhibition on nestin expression.

disperses the interaction between p53 and Mdm2, thereby arresting p53 ubiquitylation and down-regulation (Lee *et al.*, 2007). These results imply that one modus operandi of Cdk5 is to regulate protein stability of critical components of the cellular decision-making machinery. Related to this scheme, it is attractive to speculate that

the observed effects of nestin on cell-cycle inhibition at the early phases of myogenesis (24–30 h), would be due to the previously observed p53 stabilization (Lee *et al.*, 2007) leading to a subsequent elevation of the Cdk inhibitors p21 and p27.

Cdk5-mediated phosphorylation seems to be critical to retain nestin protein levels during differentiation before nestin is replaced by desmin. In light of the modus operandi described in this study, one could envision that there would be a similar bidirectional interaction between Cdk5 and nestin at the NMJs, where Cdk5 is operational (Fu *et al.*, 2001, 2005) and where nestin, along with Cdk5 and p35, specifically accumulates in fully differentiated myotubes (Sahlgren *et al.*, 2003). Nestin could form a scaffold for the Cdk5/p35 complex at the NMJs. Cdk5, by its effects on nestin, would have the capacity to modulate its own activity and targeting by specifically determining nestin stability. Cdk5 has also been depicted as essential for the dispersion of the acetylcholine receptor clusters on myofibers that have not been innervated (Lin *et al.*, 2005). Thus, the dynamic interaction between Cdk5 and nestin could be a way to modulate receptor organization through nestin-mediated scaffolding at the NMJs.

An interesting paradigm has been presented for the IF proteins GFAP and vimentin in astrocytes, where they have been described to inhibit regenerative processes (Kinouchi *et al.*, 2003; Potokar *et al.*, 2010). According to the presented model, the IFs in astrocytes are beneficial in situations of acute stress and severe trauma but may actually constitute a barrier to favorable regeneration on milder injury (for a review, see Pekny and Lane, 2007). The neuronal development in vimentin- and GFAP-deficient mice, however, has been described as relatively normal (Pekny and Lane, 2007). Similarly, when nestin mouse models become publicly available, the prediction from the studies with GFAP/vimentin-depleted mice would be that myogenesis in a nestin-deficient mouse is more or less normal. Important myogenic Cdk5-dependent processes involving Cdk5–nestin interactions and nestin-mediated fine-tuning of Cdk5 activity could be disturbed, however. For example, the organization of NMJs and regenerative processes following injury could be affected as accelerated differentiation could lead to an unfavorable outcome. The prediction is also that myogenesis would be severely disturbed in nestin overexpression models.

In summary, we propose that nestin adjusts the optimal rate for myoblast differentiation. During the differentiation process, nestin regulates the Cdk5/p35 signaling complex by affecting the
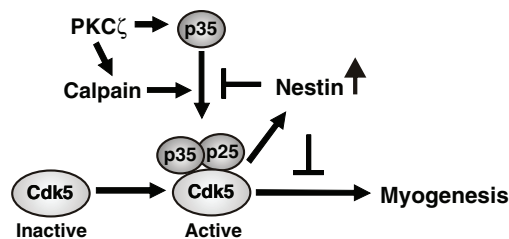
**FIGURE 8:** A model of nestin as a regulator of Cdk5 signaling pathway in differentiating myoblasts. Nestin regulates the Cdk5/p35 signaling complex through sequestration. In differentiating myoblasts, nestin scaffold participates in the regulation of p35 processing and p25 generation, thus promoting the differentiation process. PKCζ is an important upstream regulator of p35, as PKCζ-mediated phosphorylation facilitates the calpain-dependent p35 cleavage (de Thonel et al., 2010). Cdk5 critically regulates the stability and reorganization of its own nestin scaffold.

susceptibility of p35 to calpain-dependent processing. Conversely, the stability and organization of nestin are critically regulated by Cdk5, indicating a dynamic cross-talk between the two key proteins in development, differentiation, and regeneration. The operational model for the molecular mechanisms underlying this cross-talk is depicted in Figure 8. In the model, Cdk5 regulates nestin stability and thereby creates a negative feedback loop on both generation of p25 and on myogenesis. Cdk5-mediated phosphorylation of Thr-138 on p35 has been reported to suppress calpain-dependent cleavage of p35 to p25 (Kamei et al., 2007). For simplicity, this inhibitory loop has been omitted from the model. These two inhibitory loops, however, could obviously act in concert in the spatiotemporal regulation of p25 generation and Cdk5 activity. This model of nestin as a scaffold and self-induced inhibitor of Cdk5, and variations of the same theme, could be applicable in different situations in which nestin and Cdk5 are interacting during development, normal homeostasis, and regeneration.

## MATERIALS AND METHODS

### Cell culture
C2C12 and HeLa cells obtained from the American Type Culture Collection (ATCC) were maintained in DMEM (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and antibiotics (penicillin and streptomycin; growth medium) in an atmosphere of 5% $CO_2$ at 37°C. Subconfluent C2C12 cell cultures were switched to DMEM supplemented with 1% FBS, 2 mM L-glutamine, and antibiotics (differentiation medium) to initiate the differentiation.

Human adenocarcinoma SW13 cells, purchased from ATCC, were cultured in a humidified 5% $CO_2$ atmosphere at 37°C in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics (penicillin and streptomycin). SW13 cells expressing vimentin in a mosaic pattern were subcloned by dilution cloning. Cell clones were upscaled and examined for vimentin expression by Western blotting. V– and v+ subclones were selected for experimentation.

### Preparation of primary myoblasts
Cultures of primary myoblasts were established from the limb skeletal muscles of 2-d-old WT and vimentin KO 129/SV mice (Colucci-Guyon et al., 1994). Cultures of primary myoblasts to be transfected with nestin RNAi oligos were established from the limb skeletal muscles of 2-d-old FVB-n mice. Muscle tissue was minced and enzymatically digested by incubation in 0.2% (wt/vol) type XI collagenase (Roche Diagnostics, Mannheim, Germany) and 0.1% (wt/vol)

trypsin at 37°C for 30 min, after which the enzymatic activity was inhibited by the addition of FBS. The resulting slurry was filtered to remove large pieces of tissue, centrifuged at 1000 rpm for 5 min, and rinsed with phosphate-buffered saline (PBS). Cells were centrifuged at 1000 rpm for 5 min, resuspended in growth medium (Hams F-10 [Sigma-Aldrich] supplemented with 15% FBS, 2 mM L-glutamine, 100 U/ml penicillin G, 100 μg/ml streptomycin, 2.5 ng/ml fibroblast growth factor-β [Promega, Madison, WI], 20 μg/ml insulin [Sigma-Aldrich] and 4 μl/ml amphotericin B [Sigma-Aldrich]), and seeded into tissue culture dishes. After attainment of 80% confluence, the differentiation was induced by replacing growth medium with differentiation medium (DMEM supplemented with 2% FBS, 2 mM L-glutamine, and antibiotics).

### Transient transfections and cell treatments
C2C12 myoblasts, HeLa cells, and SW13 subclones were transfected with the following plasmids: empty GFP-vector (Clontech, Mountain View, CA), nest-640–GFP (Sahlgren et al., 2006), dnCdk5 (provided by Harish Pant, National Institutes of Health, Bethesda, MD) or myc-tagged p35 (Addgene, Cambridge, MA). C2C12 cells were transfected with jetPEI reagent (Polyplus Transfection, New York, NY) according to the manufacturer's protocol. Differentiation was induced 6–8 h following transfection. HeLa and SW13 cell clones were transfected using electroporation. Cells were collected, resuspended in OptiMEM (Life Technologies/Invitrogen, Carlsbad, CA), and up to 30 or 25 μg of plasmid was added, respectively. Cells were subjected to a single electric pulse (220 V, 975 μF) in 0.4 cm electroporation cuvettes (BTX, Holliston, MA) using a Bio-Rad Gene Pulser electroporator followed by dilution in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics. Cells were incubated for 24–48 h before experimental procedures.

To down-regulate nestin, C2C12 and mouse primary myoblasts were transfected with RNAi oligos purchased from Qiagen (Gaithersburg, MD) after attainment of 80% confluence. To deliver oligos, C2C12 and primary myoblasts plated on 12-well plates were transfected with 50 nM siRNA using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. After 8–9 h, the medium was replaced with differentiation medium or growth medium when aiming for MTS assay. For double transfections, C2C12 cells were transfected first with RNAi oligos for 6 h followed by a jetPEI-based transfection with dnCdk5 for an additional 6 h.

To inhibit Cdk5 activity, the Cdk inhibitor roscovitine (Calbiochem, EMD Chemicals, Darmstadt, Germany) was used at a final concentration of 10 μM for the indicated time periods. PKCξ was inhibited in differentiating myoblasts with 20 μM pseudosubstrate peptide inhibitor (PS; Myr-SIYRRGARRWRKL; MilleGen Prologue Biotech, Labege Cedex, France). Scrambled peptide (Scr-P; Myr-RLYRKRIWRSAGR) was used similarly at a final concentration of 20 μM to rule out the unspecific effect of peptide treatment. Both of the peptides were linked to a myristoyl group to facilitate the transport through the cell membrane. To trigger the calpain activity, a 10 μM concentration of the calcium ionophore A23187 (Calbiochem, Darmstadt, Germany) was added to cells.

### Immunocytochemistry
C2C12 myoblasts were grown on coverslips and fixed in 3% paraformaldehyde for 10 min at indicated points of differentiation. Cells were permeabilized in 0.5% Triton X-100 in PBS for 10 min. Blocking was performed in 1% enhanced bovine serum albumin (BSA)/PBS followed by an incubation with primary antibody against MHC (H-300; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at

room temperature (RT) in 1% BSA/PBS/0.5% Triton X-100. After three washes in PBS/0.5% Triton X-100, coverslips were treated with secondary antibody (Alexa 568 goat anti–rabbit; Invitrogen-Molecular Probes, Eugene, OR) for 1 h at RT, washed, and mounted on VECTASHIELD (Vector Laboratories, Burlingame, CA). Samples were visualized using a Zeiss 510 LSM Meta laser scanning microscope.

## MTS assay
Equal amounts of C2C12 myoblasts transfected with either nestin RNAi or scrambled RNAi were seeded on 12-well plates 24 h after the transfection and were cultured in growth medium for an additional 24 h. MTS (Promega) supplemented with phenazine ethosulfate (Sigma-Aldrich) was added to each well, and incubation was continued for 1 or 2 h. Optical density was measured at 490 nm with a plate reader (Wallac 1420 VICTOR2 multilabel counter; Perkin Elmer-Cetus).

## Immunoprecipitation
To immunoprecipitate the GFP-tagged nest-640, cell pellets were lysed on ice in immunoprecipitation buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.05% SDS, 5 mM EDTA, 5 mM EGTA, Complete Protease Inhibitor Cocktail [Roche Diagnostics]) for 30 min followed by centrifugation at 15,000 × g for 10 min at 4°C. Samples were taken for input controls, and each lysate was precleared using a 50% slurry of protein A-Sepharose beads (Sigma-Aldrich) for 1 h at 4°C. The cleared cell lysates were immunoprecipitated with 3 μl of polyclonal anti-GFP antibody (Living Colors; Clontech, Mountain View, CA) for 1 h at 4°C followed by the capture of immunocomplexes by protein A-Sepharose beads for 4 h at 4°C. Immunoprecipitates were washed three times with immunoprecipitation buffer, resuspended in Laemmli buffer, and analyzed by SDS–PAGE.

For immunoprecipitation of p35, cell pellets were lysed on ice in p35-immunoprecipitaion buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, Complete Protease Inhibitor Cocktail) for 30 min. The insoluble material was centrifuged at 15,000 × g for 10 min at 4°C after which samples were taken for input control. The lysates were precleared with protein A-Sepharose for 1 h at 4°C and then incubated first with 7.5 μl of anti-p35 antibody (C-19; Santa Cruz Biotechnology) for 1 h and second with protein A-Sepharose beads for 4 h under rotation at 4°C. After incubation, the Sepharose beads were washed three times with p35-immunoprecipitation buffer, then resuspended in Laemmli sample buffer. The immunoprecipitated proteins were analyzed by Western blotting.

## Western blotting
C2C12 and mouse primary myoblasts as well as A23187-treated HeLa cells were harvested at indicated time points by directly lysing in Laemmli sample buffer. Separation of IF fractions was performed as previously described (Sahlgren et al., 2006). Proteins were resolved by SDS–PAGE and transferred to a nitrocellulose membrane (Protran nitrocellulose; Schleicher & Schuell, Dassel, Germany) by using a wet transfer apparatus (GE Healthcare, Piscataway, NJ). Membranes were blocked with 5% nonfat milk in PBS containing 0.3% Tween-20 for 1 h at RT and incubated with the following primary antibodies overnight: nestin (BD PharMingen, Franklin Lakes, NJ), p35 (C-19; Santa Cruz Biotechnology), Cdk5 (DC34; Biosource Invitrogen, Camarillo, CA), p21 (C-19; Santa Cruz Biotechnology), p27 (C-19; Santa Cruz Biotechnology), myogenin (M-225, Santa Cruz Biotechnology), troponin T (JLT-12;

Sigma-Aldrich), MHC (H-300; Santa Cruz Biotechnology), desmin (RD301; Santa Cruz Biotechnology), vimentin (BD PharMingen), V9 (Sigma-Aldrich), Ki-67 (Abcam, Cambridge, UK), PCNA (Sigma-Aldrich), pThr-316 nestin (Sahlgren et al., 2003), GFP (JL-8; Living Colors, BD Biosciences Clontech), Hsc70 (SPA-815; Stressgen, Ann Arbor, MI), and actin (AC-40; Sigma-Aldrich). After washes with PBS/0.3% Tween-20, membranes were probed with horseradish peroxidase–conjugated secondary antibodies purchased from Promega, Southern Biotechnology, and GE Healthcare. Proteins were visualized using the ECL detection kit (GE Healthcare, Amersham, Buckinghamshire, UK).

## Quantitative real-time RT-PCR
Total RNA was extracted from cells by using an RNeasy kit (Qiagen, Hilden, Germany). From each sample, 1 μg of RNA was treated with RQ1 RNase-Free DNase (Promega) and reverse transcribed by using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The cDNA was diluted and used for real-time quantitative PCR. Primers and probes for nestin were designed by Universal ProbeLibrary Assay Design Center (Roche Applied Science, Basel, Switzerland) and used at a final concentration of 100 nM. Sequences were as follows: nestin forward 5′-tgcaggc-cactgaaaagtt-3′, nestin reverse 5′-Tctgactctgtagaccctgcttc-3′ and nestin probe (Universal ProbeLibrary probe no. 1) 5′-FAM cctggagc BHQ-3′ (Roche). The primers and probe for β-actin designed by Marjo Linja (Turku Center for Biotechnology, Turku, Finland) were purchased from Oligomer (Helsinki, Finland) and used at final concentrations of 300 nM (primers) and 50 nM (probe). The sequences were as follows: β-actin forward 5′-TGGCTCCTAGCACCAT-GAAGA-3′, β-actin reverse 5′-GTGGACAGTGAGGCCAGGAT-3′ and β-actin probe 5′-FAM CAAGATCATTGCTCCTCCTGAGCGCA BHQ-3′. KAPA probe fast ABI Prism qPCR master mix (KAPA Biosystems, Boston MA) was used to prepare the reaction mixes, and PCR was performed with the 7900HT Fast Sequence Detection System (Applied Biosystems) at the following conditions: 15 min for 1 cycle (95°C 15 s, 60°C 1 min) for 40 cycles. All reactions were done in triplicate. Relative nestin mRNA quantities were normalized to β-actin. The results were analyzed with SDS 2.4 and RQmanager 1.2.1 software (Applied Biosystems).

## Statistics
The comparisons of differentiation and p35 degradation were made using GraphPad Prism software (San Diego, CA). Unpaired Student's t tests were performed for data from nestin-overexpressing myoblasts, whereas paired Student's t tests were performed for nestin down-regulation and p35 degradation data. Statistical significance is marked by asterisks (***$p < 0.001$; **$p < 0.01$; *$p < 0.05$), and "ns." stands for "not statistically significant." The graph bars represent mean and SEM values.

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