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# Stau1 regulates Dvl2 expression during myoblast differentiation

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ABSTRACT

Post-transcriptional regulation of gene expression by RNA-binding proteins has pivotal roles in many biological processes. We have shown that Stau1, a conserved RNA-binding protein, negatively regulates myogenesis in C2C12 myoblasts. However, its target mRNAs in regulation of myogenesis remain unknown. Here we describe that Stau1 positively regulates expression of Dvl2 gene encoding a central mediator of Wnt pathway in undifferentiated C2C12 myoblasts. Stau1 binds to 3′ untranslated region (UTR) of Dvl2 mRNA and Stau1 knockdown shortened a half-life of the mRNA containing Dvl2 3′ UTR. After induction of myogenic differentiation, association of Stau1 with 3′ UTR of Dvl2 mRNA was decreased. Correlated with the decrease in the association, the Dvl2 mRNA level was reduced during myogenesis. A forced expression of Dvl2 markedly inhibited progression of myogenic differentiation. Our results suggest that Dvl2 has an inhibitory role in myogenesis and Stau1 coordinates myogenesis through the regulation of Dvl2 mRNA.



## 1. Introduction

Post-transcriptional regulation of gene expression has a significant role in various cellular processes such as cell survival, cell differentiation, adaptation to stress, and cell death. Post-transcriptional regulation that includes the processing, degradation, transport, localization, and translation of messenger RNA (mRNA) is coordinated by association of specific trans-acting factors to specific mRNA sequences usually found in the 5′ or 3′ untranslated region (UTR) [1]. Cellular signals modify post-transcriptional regulation by altering associations of RNA-binding proteins with target mRNAs [2].

AU-rich element (ARE) is a *cis*-acting element that leads to decreased mRNA stability [3]. The ARE-mediated mRNA regulation is involved in myogenesis. Myogenesis is regulated by set of myogenic transcription factors such as MyoD and myogenin [4]. Both MyoD and myogenin possess ARE in their 3′ UTRs of the mRNAs [5]. While MyoD and myogenin mRNAs are rapidly degraded in undifferentiated myoblasts in an ARE-dependent manner, the ARE-mediated mRNA decay is canceled following induction of myogenesis [5]. RNA-binding protein KSRP has been shown to associate with the 3′ UTRs of MyoD and myogenin mRNAs and to promote a rapid decay of these mRNAs in undifferentiated myoblasts [6]. In response to induction of myogenesis, p38 phosphorylates KSRP and this phosphorylation relieves destabilizing function of KSRP by decreasing its RNA-binding activity [6]. RNA-binding protein HuR has a role in stabilization of these myogenic transcripts. HuR resides in nucleus prior to differentiation and cytoplasmic accumulation of HuR is observed in differentiated cells, suggesting that stabilization of myogenic mRNAs by HuR is regulated in a nuclear export-dependent manner [7].

*Drosophila* Staufen is an RNA-binding protein that has five double-stranded RNA-binding domains (dsRBDs). Staufen is involved in cell fate determination during development by regulating mRNA localization [8]. Stau1, a mammalian ortholog of Staufen, has conserved dsRBDs without dsRBD1. Stau1 associates with the transported ribonucleo-protein complexes (RNPs) in neurons [9]. Deletion of dsRBD3 in Stau1 results in a decrease in RNA-binding activity and an impaired RNPs localization, suggesting that Stau1 has a role in mRNA localization [10]. The role of Stau1 in mRNA metabolism has been revealed by analyses of Stau1-binding protein. Stau1 associates with Upf1, a component of nonsense-mediated mRNA decay (NMD) machinery [11]. Aberrant mRNAs that have premature termination codon are rapidly degraded by the NMD machinery containing Upf1, Upf2, and Upf3. In contrast to NMD, Stau1 binds to target mRNAs and promotes mRNA decay in cooperation with Upf1, but not Upf2 and Upf3, which is termed Stau1-mediated mRNA decay (SMD) [11]. We have previously reported a role of Stau1 in myogenesis [12]. Knockdown of Stau1 in C2C12 myoblasts results in increased expression of myogenin, whereas knockdown of Upf1 does not affect myogenin expression, suggesting that Stau1 negatively regulates myogenesis in a SMD-independent mechanism. However, molecular basis of Stau1 in the regulation of myogenesis remains obscure.

Several lines of investigations suggest that Wnt signaling pathways coordinate proliferation and differentiation of myogenic cells during development and regeneration [13–15]. β-Catenin, a

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component of Wnt pathway, has been reported to associate with MyoD and positively regulates myogenesis in C2C12 myoblasts [13]. Besides canonical Wnt/β-catenin pathway, planer cell polarity (PCP) pathway that is a non-canonical Wnt pathway also has a role in regulation of myogenic stem cells during regeneration [15]. Dishevelled (Dvl) is a central mediator of both canonical and non-canonical Wnt pathways [16]. In mammalian genome, three Dvl family genes, Dvl1, Dvl2, and Dvl3, have been identified, and Dvl1 and Dvl3 mRNAs are listed in the Stau1-associated mRNAs by the genome-wide analysis [17]. In this manuscript, we describe that Stau1 associated with Dvl1, Dvl2, and Dvl3 mRNAs and that only the Dvl2 expression was positively regulated by Stau1 in undifferentiated C2C12 cells. Stau1 binds to the 3′ UTR of Dvl2 mRNA and Stau1 knockdown shortened a half-life of the mRNA containing the Dvl2 3′ UTR. While association of Stau1 with Dvl2 mRNA and the Dvl2 mRNA level were decreased after induction of myogenic differentiation, a forced expression of Dvl2 markedly inhibited progression of myogenesis. Our results suggest that Stau1 coordinates myogenesis through the regulation of Dvl2 expression.

## 2. Materials and methods

### 2.1. Cell culture, differentiation and knockdown

C2C12 myoblasts were cultured and differentiated as previously described [12]. Stealth siRNA for Stau1 was purchased from Invitrogen and transfected with RNAi MAX (Invitrogen).

### 2.2. Plasmids and retroviral experiments

Mouse Stau1 is a generous gift from Dr. Michael Kiebler (Medical University of Vienna, Austria). A cDNA of mouse Dvl2 was kindly supplied by Dr. Akira Kikuchi (Osaka University, Japan). Expression vectors pCMV-FLAG and pCMV-HA were used to express tagged proteins [18]. Retroviral-mediated gene expression was performed as previously described [12].

### 2.3. Analyses of mRNAs

Total RNAs were prepared from cells and then analyzed by Northern blot analysis as previously described [12]. To examine half-lives of transcripts, cells were cultured and then Actinomycin D (2.5 μg/mL) was added. Total RNAs were prepared at 0, 2, 4, and 6 h after addition of Actinomycin D. The level of luciferase mRNA was normalized with that of ribosomal RNA. Percentage of remaining mRNAs was plotted and the half-lives of mRNAs were calculated. Data represent the means standard deviations from three independent experiments.

### 2.4. Analyses of proteins

Total cell extracts were prepared and then analyzed by Western blot analysis as previously described [12]. Fixed cells were permeabilized and then immunofluorescence was performed with anti-myoglobin (Dako) and rhodamine-conjugated anti-rabbit IgG antibodies.

### 2.5. Immunoprecipitation and reverse transcription-PCR (RT-PCR) analysis

Stau1 protein was immunoprecipitated from the extract of C2C12 myoblasts by using anti-Stau1 antibody [12]. FLAG-tagged Stau1 protein was immunoprecipitated with anti-FLAG antibody (M2 Sigma). Co-immunoprecipitated RNAs were purified by RNeasy Minelute cleanup kit (QIAGEN). RT-PCR was performed using Superscript One-Step RT-PCR system (Invitrogen) with gene specific primers. Products of RT-PCR were resolved by 2% agarose gel and visualized by staining with SYBR Safe (Invitrogen).

### 2.6. In vitro transcription

Coding region (positions 635–1108 relative to the transcription start site) and 3′ UTR (positions 2421–2888) of Dvl2 were amplified by PCR with 5′ primer containing T7 RNA polymerase promoter sequence (TAATACGACTCACTATAGGG) and 3′ primer. PCR products were purified by Minelute PCR purification kit (QIAGEN). Biotinylated RNAs were synthesized by *in vitro* run-off transcription with MEGAscript T7 transcription kit (Ambion) and biotin-11-CTP (PerkinElmer).

### 2.7. RNA pull-down assay

Cell extracts (20 μg) were incubated with 5 μg of biotinylated RNA in 400 μL of binding buffer (10 mM Hepes–KOH at pH 7.5, 5% glycerol, 5 mM $MgCl_2$, 40 mM KCl, 1 mM dithiothreitol, 1.5 μg/μL BSA, 67 ng/μL yeast tRNA, 1.5 μg/μL heparin, 0.2 U/μL RNasin) for 30 min at room temperature. RNA–protein complexes were isolated with streptavidin-coupled Dynabeads (Dynal). Proteins were subjected to Western blot analysis with anti-HA or anti-Stau1 antibody.

## 3. Results

### 3.1. Stau1 is required for expression of Dvl2 in C2C12 myoblasts

Dvl1 and Dvl3 mRNAs have been listed in Stau1-associated mRNAs [17]. To investigate a possible involvement of Stau1 in Dvls expression in myogenic cells, we first examined association of Stau1 with Dvl1, Dvl2, and Dvl3 mRNAs in C2C12 myoblasts. As

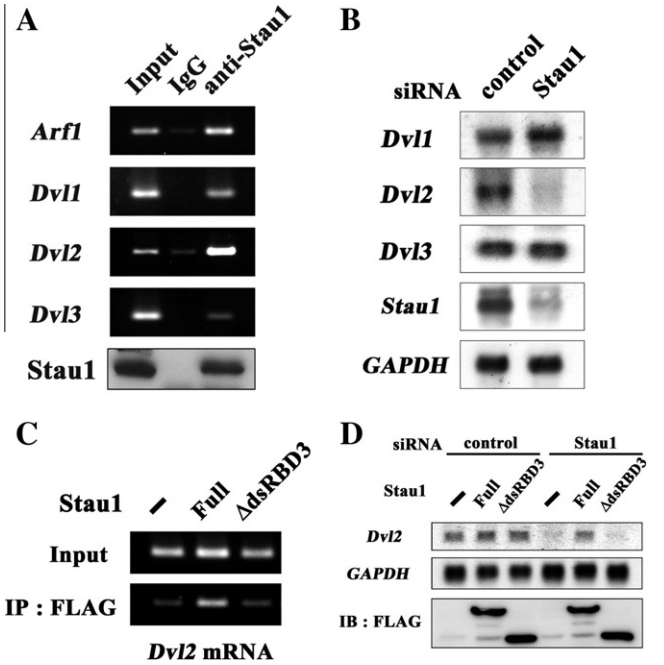


**Fig. 1.** Stau1 positively regulates Dvl2 expression. (A) Association of Stau1 with Dvl mRNAs. (B) The levels of Dvl family mRNAs in Stau1 knockdown cells. (C) The association of FLAG-Stau1 or Stau1ΔdsRBD3 with Dvl2 mRNA. (D) The level of Dvl2 mRNA. siRNA-resistant FLAG-Stau1 or Stau1ΔdsRBD3 was exogenously expressed and then endogenous Stau1 was silenced by siRNA.

shown in Fig. 1A, mRNAs of Dvls were detected in the cell extracts prepared from undifferentiated C2C12 myoblasts (Fig. 1A, input). We collected Stau1-associated mRNAs by immunoprecipitation with the anti-Stau1 antibody and confirmed that the Stau1-associated mRNAs contained Dvl1, Dvl2, and Dvl3 mRNAs as well as Arf1 mRNA, a known Stau1 target mRNA (Fig. 1A, anti-Stau1). To examine whether Stau1 is involved in regulation of Dvls expression, we analyzed Dvls expression in Stau1-knockdown C2C12 myoblasts. When Stau1 was silenced by siRNA, the Dvl2 mRNA level was significantly decreased as compared with control cells (Fig. 1B). In contrast, the Dvl1 and Dvl3 mRNA levels were similar in control and Stau1-knockdown cells. The reduction of Dvl2 mRNA in Stau1-knockdown cells was rescued by expression of siRNA-resistant Stau1 (Fig. 1D), indicating that knockdown of Stau1 is responsible for reduction of Dvl2 mRNA.

It has been shown that dsRBD3 of Stau1 is necessary and sufficient for binding to mRNA [19]. To examine whether Stau1 regulates Dvl2 expression by binding to Dvl2 mRNA through the dsRBD3, we constructed a mutant Stau1 that lacked its dsRBD3 (Stau1ΔdsRBD3). Consistent with a previous observation, the binding of Stau1ΔdsRBD3 to Dvl2 mRNA was decreased compared with that of full-length Stau1 (Fig. 1C). We then examined whether dsRBD3 was also required for Dvl2 expression using complementation analysis in Stau1 knockdown cells. The Dvl2 mRNA level was rescued by expression of full-length Stau1, but not by that of Stau1ΔdsRBD3 (Fig. 1D). These results suggest that the dsRBD3-mediated RNA-binding of Stau1 is required for Dvl2 expression.

### 3.2. Stau1 knockdown shortened a half-life of transcript containing Dvl2 3′ UTR

It has been shown that RNA-binding proteins affect mRNA stability through binding to 3′ UTRs of their target mRNAs [2]. We then examined whether Stau1 associated with 3′ UTR of Dvl2 mRNA. Biotinylated RNA corresponding to Dvl2 coding region (positions 635–1108) or 3′ UTR (positions 2421–2888) were incubated with C2C12 extracts and subsequently analyzed by pull-down assay with streptavidin beads (Fig. 2A). As shown in Fig. 2B, Stau1 was co-precipitated with the Dvl2 3′ UTR but not the coding region. To examine whether the Dvl2 3′ UTR determinates its stability, we measured the half-life of a chimeric transcript that is conjugated luciferase to the Dvl2 3′ UTR (Fig. 2C, D). While the luciferase reporter mRNA lacking the sequence of the Dvl2 3′ UTR was degraded at a similar rate in control and Stau1 knockdown cells (Fig. 2C), the luciferase reporter mRNA harboring the Dvl2 3′ UTR was more rapidly degraded in Stau1 knockdown cells as compared with control cells (Fig. 2D). These results suggest that Stau1 associates with the Dvl2 3′ UTR and this association regulates the mRNA stability.

We further mapped Stau1-binding region in the Dvl2 3′ UTR by pull-down assay. Stau1 was co-precipitated with U1 region (2421–2653), but not with U2 region (2654–2887) within the Dvl2 3′ UTR (Fig. 2E, F). To investigate effect of U1 region on mRNA stability, we compared the half-lives of chimeric luciferase mRNAs containing U1 and U2, U1 alone, or U2 alone (Fig. 2D, G, H). As shown in Fig. 2G, H, the half-life of mRNA containing U1 alone or U2 alone was similar in control and Stau1-knockdown cells, indicating that both U1 and U2 regions are required for the Stau1-mediated mRNA stability control. It should be noted that the half-life of the mRNA containing U2 alone was much shorter than when compared with those of the reporter mRNA containing U1 and U2 and the reporter mRNA containing U1 alone (Fig. 2D, G, H). These results suggested that U2 region contains a destabilizing sequence and association of Stau1 to U1 region protects the mRNA from the U2 region-dependent destabilization.

### 3.3. Stau1 dissociates from the Dvl2 3′ UTR during myogenesis

We have previously shown that Stau1 negatively regulates myogenic differentiation [12]. We analyzed whether the association of Stau1 with Dvl2 mRNA is affected by differentiation stimuli. Biothinylated RNAs containing the Dvl2 3′ UTR were incubated with the extracts prepared from undifferentiated cells or differentiating cells following induction of myogenesis and subsequently precipitated with streptavidin beads. As shown in Fig. 3A, although the Stau1 protein level was not altered during myogenesis, the association of Stau1 with Dvl2 3′ UTR was significantly reduced following induction of myogenesis and disappeared after three days.

We next examined the correlation between the association of Stau1 with Dvl2 3′ UTR and the Dvl2 mRNA level during myogenesis. Although Dvl2 mRNA was expressed prior to differentiation, the Dvl2 mRNA level was dramatically reduced following induction of myogenesis (Fig. 3B). These results suggest that Stau1 protects Dvl2 mRNA from decay in undifferentiated cells and the dissociation of Stau1 from the Dvl2 3′ UTR after the induction of myogenesis confers to decrease Dvl2 mRNA.

### 3.4. Dvl2 has an inhibitory role in myogenesis

Expression of Dvl2 was dramatically reduced when myogenin, an early inducible myogenic marker, was expressed (Fig. 3B). We hypothesized that Dvl2 has an inhibitory role in myogenesis and its expression is down-regulated during myogenesis. To examine this possibility, we generated the cells constitutively expressing Dvl2 and induced to differentiate into myotube. Three days after induction of myogenesis, 15% of control cells expressed myoglobin, a muscle specific marker, whereas only 3% cells were myoglobin positive in the cells constitutively expressing Dvl2 (Fig. 4A, B). While myogenin expression was induced by differentiation stimuli in control cells (Fig. 4C, vector), this induction was also severely inhibited by the constitutive expression of Dvl2 (Fig. 4C, Dvl2). These results suggest that Dvl2 has an inhibitory role in myogenesis and that the down-regulation of Dvl2 expression during differentiation is important for progression of myogenesis.

As we reported previously [12], Stau1-knockdown cells results in aberrant expression of myogenin and spontaneous progression of myogenesis in the absence of differentiation stimuli (Fig. 4D, siRNA Stau1, vector). If this aberrant expression of myogenin caused by Stau1-knockdown was mediated by the down-regulation of Dvl2 expression, a forced expression of Dvl2 would reduce the myogenin expression in Stau1-knockdown cells. To test this possibility, C2C12 cells were infected with virus expressing Dvl2 with or without its 3′ UTR. In cells expressing Dvl2 without the 3′ UTR, the myogenin expression in the Stau1 knockdown cells was significantly decreased (Fig. 4D, siRNA Stau1, Dvl2). Myogenin expression was also decreased by the expression of Dvl2 bearing 3′ UTR, but the effect was weaker than that of Dvl2 without 3′ UTR (Fig. 4D, siRNA Stau1, Dvl2-3′ UTR). We also examined the Dvl2 protein levels derived from Dvl2 with or without 3′ UTR constructs. While the level of Dvl2 protein generated from exogenous Dvl2 gene without 3′ UTR was not affected by Stau1 knockdown, the Dvl2 protein level from exogenous Dvl2 gene with 3′ UTR was reduced by Stau1 knockdown (Fig. 4E). These results support the model that Stau1 negatively regulates myogenesis through the regulation of the Dvl2 mRNA level.

## 4. Discussion

RNA-binding proteins modify expressions of target genes at post-transcriptional level. In this study, we described the regulation of Dvl2 expression by Stau1-mediated mRNA stabilization

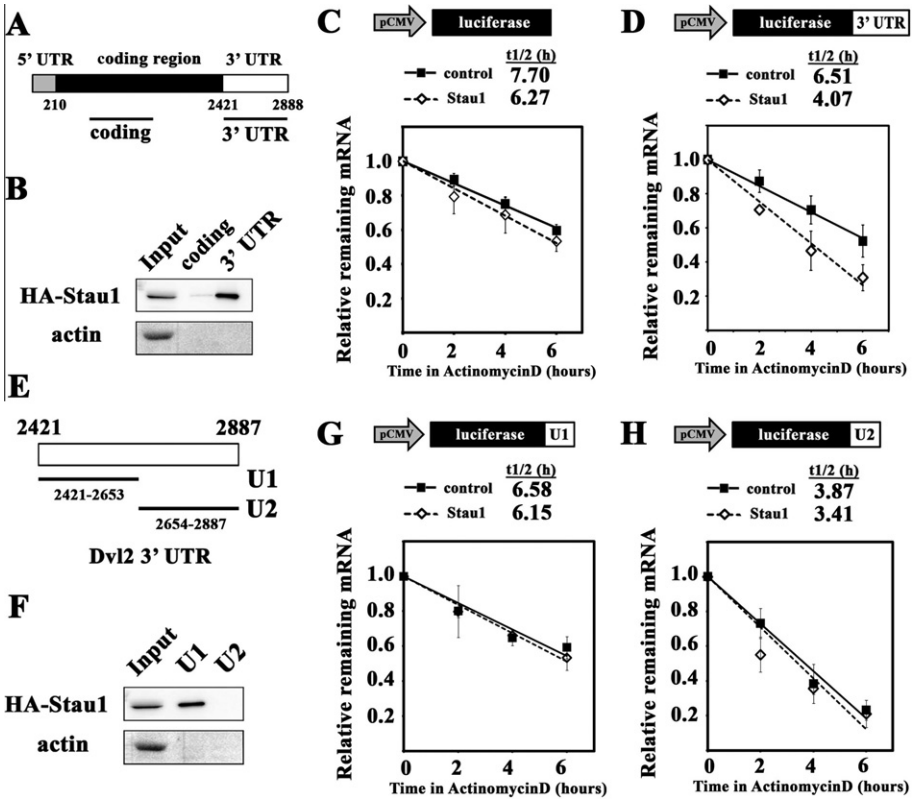


**Fig. 2.** Stau1 regulates the mRNA stability by binding to 3′ UTR of Dvl2 mRNA. (A) Schematic representation of the transcripts used for binding analyses. Positions are relative to the transcription start site. (B) The association of HA-Stau1 with biotinylated Dvl2 RNAs. (C, D) mRNA decay rates of luciferase-Dvl2 3′ UTR in control or Stau1 knockdown cells. (E) Schematic representation of the Dvl2 3′ UTR fragments. (F) The association of HA-Stau1 with Dvl2 3′ UTR fragments. (G) and (H) Half-lives of luciferase-Dvl2 3′ UTR in control and Stau1 knockdown cells.

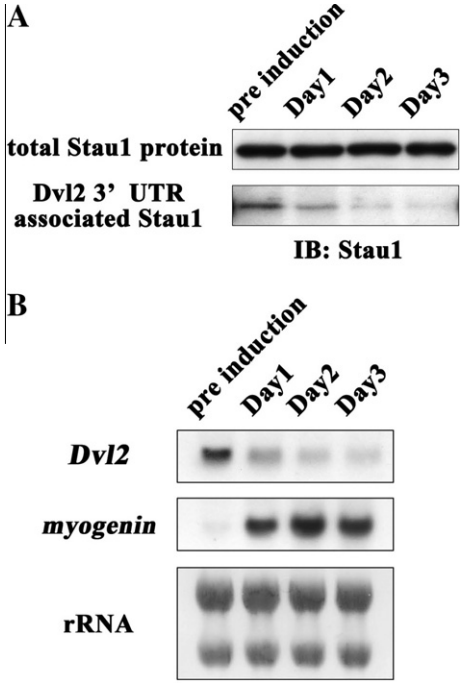


**Fig. 3.** Stau1 dissociates from Dvl2 mRNA in response to differentiation stimuli. (A) The association of Stau1 with biotinylated Dvl2 3′ UTR during myogenic differentiation. (B) Expression of *Dvl2* and *myogenin* mRNA during myogenesis.

during myogenesis. Stau1 associates with Dvl2 mRNA through the 3′ UTR and protects it from decay in undifferentiated cells (Figs. 1 and 2). Association of Stau1 with the 3′ UTR of Dvl2 mRNA is abolished by stimulation of myogenic differentiation, and simultaneously the Dvl2 mRNA level was reduced (Fig. 3). A forced expression of Dvl2 inhibited myogenic progression in Stau1-knockdown cells (Fig. 4). Taken together, our observations suggest that Stau1 contributes inhibition of myogenesis through the stabilization of Dvl2 mRNA in undifferentiated myoblasts.

It has been shown that ARE containing AUUUA (class I and II) or U-rich region (class III) leads to rapid degradation of mRNA [20]. The Dvl2 mRNA studied in this work does not have typical RNA sequences characteristic in class I and II, however, two U stretches characteristic in class III are located at middle region of the Dvl2 3′ UTR. Stau1 binds to the U1 region containing first U stretch. While the reporter RNA containing both the U1 and U2 regions was more rapidly degraded in the absence of Stau1 (Fig. 2D), the reporter RNA containing only U1 was not rapidly degraded (Fig. 2G). These results suggest that the first U stretch in the U1 region is not a target for mRNA decay machinery. On the other hand, the reporter RNA containing the U2 region containing the second U stretch was rapidly degraded even in the presence of Stau1. These observations suggest that ARE-mediated decay targets the second U stretch in the U2 region of the Dvl2 3′ UTR. The association of Stau1 with the U1 region may prevent the U2 region-dependent mRNA decay. We demonstrated that Stau1 associates with not only Dvl2 mRNA but also Dvl1 and Dvl3 mRNAs, however, the expression of Dvl2, but not Dvl1 or Dvl3, was affected by Stau1 knockdown. Dvl1 and Dvl3 mRNAs might have only Stau1-binding region, but not the decay element like the U2 region of the Dvl2 mRNA.

It has been reported that degradation of ARE-containing mRNAs is blocked by secondary structure at 5′ position to the ARE [21]. In

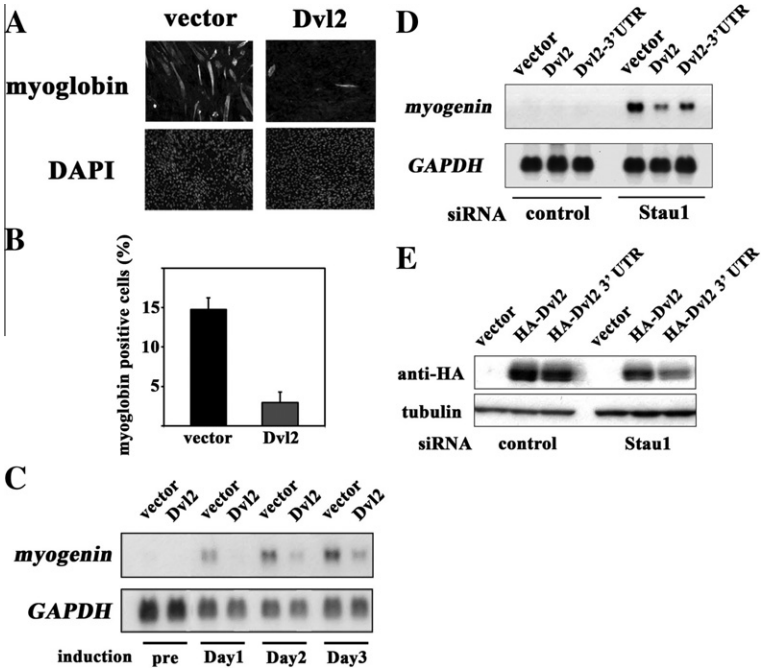


**Fig. 4.** Forced expression of Dvl2 inhibits the expression of muscle markers. (A, B) Expression of myoglobin in exogenous Dvl2 expressing cells. Percentage of myoglobin-positive cells was counted in exogenous Dvl2 expressing cells. (C) Myogenin expression level in exogenous Dvl2 expressed cells. (D) The level of myogenin mRNA in control or Stau1 knockdown cells. Effect of exogenous DVL2 expression on myogenin level in cells expressing DVL2 or DVL2-3′ UTR. (E) The level of HA-DVL2 protein in cells transfected with HA-Dvl2 or HA-Dvl2-3′ UTR vector.

our further binding analyses, U rich sequence in the U1 region was required for association of Stau1 with Dvl2 3′ UTR (Fig. S1). Since Stau1 did not associate with the U2 region containing another U rich sequence, Stau1 may recognizes secondary structure of the mRNA rather than the U rich sequence itself. Since Stau1 is reported to bind to stem structures [22], Stau1 may protect Dvl2 mRNA from degradation by associating with the secondary structure in the U1 region which is located at 5′ position of second U stretch in the U2 region.

It has been reported that several RNA-binding proteins are involved in stabilization or destabilization of myogenic transcripts [4–7]. Their association with target mRNAs determinates their stability, which is regulated by several mechanisms, e.g. modulation of RNA-binding activity, translocation [6]. Although the protein level of Stau1 or localization did not alter during myogenesis, Stau1 dissociated from Dvl2 mRNA following induction of myogenesis (Fig. 3 and our unpublished observation). Thus, it is possible that the RNA-binding activity of Stau1 is regulated by protein modification during myogenesis. We examined the effect of Stau1 overexpression on myogenesis, but overexpression of Stau1 did not affect the Dvl2 mRNA level during myogenesis (data not shown). This result also supports the idea that the protein modification affecting the RNA binding activity is more important than the amount of Stau1 protein. The dissociation of Stau1 from Dvl2 mRNA may allow decay factors to associate with Dvl2 mRNA.

Previous observations suggest that canonical and non-canonical Wnt signaling pathways coordinate proliferation and differentiation of myogenic cells [15,23]. Although several Wnts affect myogenesis, involvement of Dvls in myogenesis remains obscure. We found that the expression of Dvl2 is decreased during myogenesis and forced expression of Dvl2 inhibits myogenesis (Figs. 3 and 4). Dvl1 and Dvl3 were also expressed in undifferentiated C2C12 myoblasts but their expression levels were not altered during myogenesis (data not shown). Previous report showed that canonical Wnt/$\beta$-catenin pathway positively regulates myogenesis [13]. It is possible that Dvl1 and Dvl3 are responsible for the function of canonical Wnt/$\beta$-catenin pathway in myogenesis. Dvl2 has been reported to have a modest effect on canonical Wnt/$\beta$-catenin pathway as compared with Dvl1 or Dvl3 [24]. PCP pathway, a non-canonical Wnt signal, is a candidate for Dvl2 mediated inhibition of myogenesis. Silencing of Vangl2, a component of PCP pathway, in satellite cell- derived myoblasts results in the increase of myogenin expression [15], suggesting that the PCP pathway contributes expansion of myogenic progenitor cells. Dvl2 may be involved in expansion of myoblasts through inhibition of myogenesis.

Stau1 has been reported to be involved in mRNA decay in cooperation with Upf1, which is termed SMD [11]. The activity of SMD is increased during myogenesis, which leads to the degradation of Pax3 mRNA encoding a transcription factor that negatively regulates myogenesis [25]. We have previously shown that inhibitory role of Stau1 in myogenesis is independent of SMD. Furthermore, the Dvl2 mRNA level is not altered in Upf1 knockdown cells (data not shown). It remains unclear how Stau1 affects the mRNA stability both positively and negatively. One possibility is that Stau1 affects mRNA stability depending on mRNA sequence and/or binding site. Alternatively, the effect of Stau1 may be modulated by its partner protein.

In summary, our findings indicate that Stau1 has the ability to stabilize Dvl2 mRNA and the Stau1-dependent stabilization of the Dvl2 mRNA is modified by differentiation stimuli. A genome-wide approach showed that Stau1 associates with 7% of transcripts expressed in HEK293T cells [17]. Although we do not neglect the possibility that Stau1 represses myogenesis by regulating not only Dvl2 but also unknown targets, Dvl2 mRNA is one of the critical target mRNA for Stau1 during myogenesis.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.11.133.

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