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RASSF4 is required for skeletal muscle differentiation

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Abstract

RASSF4, a member of the classical RASSF family of scaffold proteins, is associated with alveolar rhabdomyosarcoma, an aggressive pediatric cancer of muscle histogenesis. However, the role of RASSF4 in normal myogenesis is unknown. We demonstrate here that RASSF4 is necessary for early *in vitro* myogenesis. Using primary human myoblasts, we show that RASSF4 expression is dramatically increased during *in vitro* myogenic differentiation, and conversely that RASSF4-deficient myoblasts cannot differentiate, potentially because of a lack of upregulation of myogenin. In microscopy studies, we show that RASSF4 protein co-localizes with proteins of the myogenic microtubule organizing center (MTOC) both before and after myogenic differentiation. RASSF4-deficient cells subject to differentiation conditions demonstrate a lack of shape change, suggesting that RASSF4 plays a role in promoting microtubule reorganization and myoblast elongation. In biochemical studies of myotubes, RASSF4 associates with MST1, suggesting that RASSF4 signals to MST1 in the myogenic differentiation process. Expression of MST1 in myoblasts partially reversed the effect of RASSF4 knockdown on differentiation, suggesting that RASSF4 and MST1 coordinately support myogenic differentiation. These data show that RASSF4 is critical for the early steps of myogenic differentiation.

Keywords

RASSF4; myogenesis; myogenic differentiation; microtubule

1. Introduction

Ras association domain family member 4 (*RASSF4*) is one of 10 *RASSF* genes in the human genome. This gene family encodes non-catalytic proteins that act as scaffold regulators of the Hippo developmental pathway. For the most part, *RASSF* genes are epigenetically downregulated in human cancer, and therefore characterized as tumor

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Conflicts of interest

The authors declare no potential conflicts of interest.

suppressors (Volodko et al. , 2014a). However, *RASSF4* was recently found to play a divergent role in the pediatric skeletal muscle cancer, alveolar rhabdomyosarcoma (aRMS). In aRMS, *RASSF4* is upregulated in human cell lines and tumors and is oncogenic, portending a worse clinical prognosis (Crose et al. , 2014). Mechanistically, the signature aRMS mutant fusion oncoprotein PAX3-FOXO1 transcriptionally upregulates *RASSF4* expression to promote cell proliferation and senescence evasion. While that prior work demonstrated a critical role for *RASSF4* in the aRMS disease model, the role of *RASSF4* in normal skeletal muscle biology is not clear.

Myogenesis, the developmental process whereby myogenic precursors including myoblasts differentiate to become mature skeletal muscle, is a highly conserved but complex series of events with a distinct genetic program that causes distinct cellular changes. One of the most dramatic changes in myogenesis is reorganization of the cytoskeleton, including the architecture of the microtubules. For example, while in single cell myoblasts microtubules are arrayed in a radial fashion, in multinucleated myotubes microtubules run parallel (Tassin et al. , 1985a). This change in architecture is due in part to alterations in the microtubule organizing center (MTOC), the cell structure that nucleates and organizes microtubules. During myogenic differentiation, the MTOC relocalizes from the centrosome of myoblasts to the nuclear membrane of myotubes (Bugnard et al. , 2005, Srsen et al. , 2009, Tassin, Maro, 1985a). More recently, Golgi elements have been shown to act as MTOC sites in fully mature myofibers (Oddoux et al. , 2013). Early expression of myogenin is crucial for this redistribution of centrosomal proteins and Golgi complex reorganization (Zaal et al. , 2011).

While the initial findings of microtubule reorganization during myogenic differentiation were made nearly 30 years ago, little is known about the protein regulators and signaling pathways associated with this critical process. Herein, we show that RASSF4 is upregulated during early differentiation, is critical for myogenic differentiation, and lies upstream of myogenin. In myoblasts, RASSF4 protein is centrosomal but then co-localizes with MTOC proteins to the nuclear surface of myotubes, suggesting a role in myogenic microtubule reorganization. Indeed, RASSF4-deficient myoblasts do not express myogenin and are not able to elongate during the early stages of differentiation. Thus, RASSF4 is a critical centrosomal protein upstream of myogenin and microtubule reorganization during early myogenic differentiation.

2. Materials and Methods

2.1. Reagents

The following antibodies were used for immunoblots and immunofluorescence: RASSF4 (Novus, NBP-1–89249), Erk1 (Santa Cruz Biotechnology, c-16, sc-93), alpha-tubulin (Sigma, T6199), actin (Sigma, A5441), M2-Flag (Sigma, F3165), PCM-1 (Cell Signaling, G2000, 5213), HA (Cell Signaling, 6E2, 2367) MST1 (Cell Signaling, 3682), MOB1 (Cell Signaling, 3863), and phospho-MOB1 (Cell Signaling, T35, 8699). To clarify nomenclature, note that the gene name for MST1 is *STK4*.

2.2. Cell culture and tissue

Human skeletal muscle myoblasts (HSMMs, Lonza) were grown and differentiated according to the Lonza myotube differentiation protocol. Human muscle tissue was obtained from the Cooperative Human Tissue Network, which is funded by the National Cancer Institute. Other investigators may have specimens from the same subjects.

2.3. qPCR

RNA isolation, cDNA generation, and qPCR was performed as described (Crose, Galindo, 2014, Naini et al. , 2008). All qPCR expression was normalized to qPCR for *GAPDH*. Primer sequences are as follows (all listed 5' to 3'): *RASSF4* FW: TTC TCT ATC AAC GGC CAC TTC, REV: CTT CCA CCC TAA ATT TGT TCA GC. *MYOD* FW: GGT CCC TCG CGC CCA AAA GAT, REV: CAG TTC TCC CGC CTC TCC TAC. *MYF5* FW: ATG GAC GTG ATG GAT GGC TG, REV: GCG GCA CAA ACT CGT CCC CAA. *MRF4* FW: CCC CTT CAG CTA CAG ACC CAA, REV: CCC CCT GGA ATG ATC GGA AAC. *MYOG* FW: CAG TGC ACT GGA GTT CAG CG, REV: TTC ATC TGG GAA GGC CAC AGA. *STK4* FW: AGA GGA TGA GGA AGA GGA AGG, REV: CCA GGT ACA CTC TTG CCA AAG. *GAPDH* FW: ATG GGG AAG GTG AAG GTC G, REV: GGG GTC ATT GAT GGC AAC AAT A.

2.4. Differentiation assays and MF20 staining

Differentiation assays for HSMMs and C2C12s and MF20 (DSHB Hybridoma Product MF20) staining to detect myosin heavy chain were performed as described (Linardic et al. , 2007). The hybridoma, developed by D.A. Fischman, was obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242. Unless specified, differentiated HSMMs were analyzed after 5 days in differentiation-inducing conditions.

2.5. RNAi

RASSF4 RNAi using pLKO.1-mediated stable shRNA expression was performed as described (Crose, Galindo, 2014).

2.6. Immunofluorescence

Immunofluorescence was performed as described (Crose, Galindo, 2014). Cells were fixed in 4% paraformaldehyde for 1h at room temperature, washed 5min x 3 with TBS-Triton X-100 (0.1%), then blocked in 10% goat serum. Cells were incubated with primary antibodies for 1h at room temperature, then washed 5min x 3 with TBS. Secondary antibodies (1:500) used included Alexa 647- and 488-conjugated secondary antibodies (Molecular Probes), and Cy3-conjugated secondary antibodies (Jackson ImmunoResearch). Nuclei were labeled by Hoechst 33342 (Invitrogen). Samples were mounted with SlowFade™ Gold Antifade Mountant (ThermoFisher) and images were acquired using a Leica SP5 inverted confocal microscope or Zeiss 710 inverted confocal microscope and analyzed using the Leica Application Suite 2.6.0 and FIJI software.

2.7. Immunoprecipitation

Immunoprecipitation of FLAG-RASSF4 was performed using M2-agarose (Sigma) according to the manufacturer's specifications. Briefly, cells were collected and lysed with lysis buffer (50mM Tris HCl, pH7.4, with 150mM NaCl, 1mM EDTA, and 1% Triton X-100). Lysate was centrifuged for 10min at 12,000xg and supernatant was collected into a chilled tube. Anti-FLAG M2 affinity gel was packed into a small column, with the column first rinsed with TBS, 0.1M glycine HCl, pH3.5, and then TBS. Then the protein lysate was loaded onto the column. TBS was used to wash out non-specific binding. Finally, bound protein was eluted by 0.1M glycine HCl, pH3.5, into tubes containing 1M Tris, pH 8.0.

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism Software. Data represents mean ± standard deviation, unless otherwise noted. P values were considered significant at *, $P< 0.05$; **, $P<0.01$; ***, $P<0.001$; and ****, $P<0.0001$.

3. Results

3.1. RASSF4 is transiently upregulated during myogenic differentiation

While we found in prior studies that *RASSF4* plays a critical role in the childhood skeletal muscle cancer aRMS, whether *RASSF4* also has regulatory roles during normal myogenic differentiation had not been examined. To address this, we first performed *in vitro* differentiation assays using non-transformed HSMMs and monitored myogenic gene expression changes during this process. We compared gene expression between proliferating HSMMs and differentiated myotubes (Figure 1A). As expected, *MYF5* (encoding the protein myogenic factor 5, a marker of early myogenesis) showed a significant decrease after five days of differentiation, while *MYOG* (encoding the protein myogenic factor 4, also known as myogenin, a marker of late myogenesis), showed a significant increase. Interestingly, we found that after five days of differentiation, *RASSF4* also increased >10-fold. The upregulation of *RASSF4* at the protein level was verified by immunoblot (Figure 1B).

We next examined the time course of RASSF4 induction during differentiation. *RASSF4* mRNA upregulation began at day one of differentiation, with a dramatic increase days two through four, but with RASSF4 protein levels increasing as early as day one (Figure 1C,D). *RASSF4* mRNA levels at day five were generally 20% less than the peak expression between days two through four. To investigate whether RASSF4 levels continued to change, we extended *in vitro* differentiation to 14 days (Figure 1E). As we had observed previously, there was a decrease in *RASSF4* mRNA between day three and day five of differentiation. RASSF4 expression gradually down-regulated throughout the 14 day differentiation time course. To confirm these findings in independent myoblast preparations, we performed time course analyses in HSMMs derived from a different donor, and in murine C2C12 myoblast cells, and observed the same trend of RASSF4 increase during differentiation (Supplemental Figure 1A,B). Since altogether these data could indicate that RASSF4 levels transiently increase during myogenic differentiation, we further compared *RASSF4* mRNA levels in early differentiating myoblasts to those in human mature skeletal muscle tissue (Figure 1F).

RASSF4 mRNA levels in human muscle (human SkM samples #1750 and #1751) were higher than undifferentiated HSMMs (day zero in differentiation). Significantly, early differentiation HSMMs (day three in differentiation) had the highest *RASSF4* mRNA expression among all the samples. Altogether, these data suggest that *RASSF4* is upregulated during early myogenic differentiation, with a peak at 3–4 days followed by a slow decline to baseline.

3.2. RASSF4 is necessary for myoblast differentiation

Since we observed transient upregulation of RASSF4 during early myogenesis, we next examined whether it is essential for myogenic differentiation. We suppressed RASSF4 expression by introducing lentiviral *RASSF4* shRNAs at either early (before differentiation was initiated) or late (after five days of differentiation) stages (Figure 2A,D). Knockdown efficiency was examined by qPCR (Figure 2B,E). After 5 days of *in vitro* differentiation, HSMMs were stained for the differentiation marker myosin heavy chain (MHC). While there was minimal effect on MHC staining when RASSF4 levels were depleted late (Figure 2F), we observed dramatic abrogated MHC staining when RASSF4 was suppressed prior to the differentiation stimulus (Figure 2C). In addition to loss of MHC staining, HSMM cells with early RASSF4 depletion did not induce *MYOG* expression (Figure 2G). These data suggest that early RASSF4 upregulation is required during early steps myogenic differentiation.

3.3. RASSF4 co-localizes with the centrosomal protein PCM-1 in pre- and post-differentiated HSMMs

While we had found RASSF4 transiently upregulated during and essential for myogenesis, we did not have insight into its role in the myogenic process. Since RASSFs are known to bind to microtubules, we investigated the relationship of RASSF4 to the MTOC, whose reorganization is a crucial event during early myogenesis (Tassin, Maro, 1985a, Tassin et al. , 1985b, Zaal, Reid, 2011). To test the possibility that RASSF4 might be involved in MTOC reorganization, we analyzed RASSF4 subcellular localization in undifferentiated myoblasts versus differentiated myotubes. We compared RASSF4 localization to that of pericentriolar material 1 (PCM-1), a known MTOC centrosomal protein. In undifferentiated myoblasts, transiently transfected HA-tagged RASSF4 protein was a perinuclear dot, and co-localized with PCM-1 (Figure 3A). As a control, we verified that transiently transfected HA-tagged RASSF4 co-localized with endogenous RASSF4 (Figure 3B). After differentiation, we first examined the expression pattern of PCM-1, and found that it accumulated in a ring-like pattern at the periphery of myotube nuclei (Figure 3C), consistent with previously published studies (Bugnard, Zaal, 2005, Zaal, Reid, 2011). Remarkably, transiently expressed Flag-tagged RASSF4 displayed a similar ring-like pattern and co-localized with PCM-1 (Figure 3D). Last, endogenous RASSF4 protein also accumulated in a ring-like fashion around myotube nuclei (Figure 3E). Transiently expressed Flag-tagged control vector appropriately had neither a ring-like pattern nor co-localization with PCM-1 (refer back to Figure 3C). To our surprise, the co-localization of RASSF4 and PCM-1 did not required the SARAH domain of the RASSF4 protein (Supplemental Figure 2), which is required for binding to and modulation of MST1. Altogether, these data suggest that

RASSF4 is a centrosomal protein and may be involved in MTOC reorganization during myogenesis.

3.4. RASSF4 regulates myoblast elongation and microtubule architecture

Since RASSF4 co-localized with other proteins of the myogenic MTOC, we used RASSF4 suppression by shRNA to examine the role of RASSF4 in HSMM microtubule reorganization. Importantly, since we were using RNAi to suppress RASSF4 at three days of differentiation, we first verified that expression of the shRNAs were still resulting in RASSF4 suppression at day three of differentiation (Figure 4A). Then, we examined microtubule architecture. In undifferentiated myoblasts, whether expressing a control NT construct or *RASSF4* shRNAs, the microtubule architecture was radial (Figure 4B, left column). Following three days of culturing in differentiation media, control cells expressing a NT shRNA exhibited microtubule restructuring associated with myoblast elongation, as expected. However, RASSF4-deficient cells failed to elongate, instead maintaining the radial configuration (Figure 4B, right column). These data suggest that RASSF4 may be induced during early stages of myoblast differentiation to promote microtubule reorganization and myoblast elongation.

3.5. RASSF4 regulates myogenesis in part via MST1

To gain insight into the RASSF4-mediated signaling associated with myogenic differentiation, we examined the proteins that associate with RASSF4 in differentiated HSMMs. One of the best characterized RASSF binding proteins is MST1 (gene name *STK4*), a Ste20-like kinase associated with tumor suppression, apoptosis, and the Hippo signaling cascade (Avruch et al. , 2012). MST1 associates with all of the classic RASSFs (RASSF1–6) via their respective SARAH domains (Chan et al. , 2013). Since we and others have shown that RASSF4 associates with MST1 in aRMS cancer cells, we examined whether RASSF4 associates with MST1 in myotubes. We differentiated our stably-expressing FLAG-RASSF4 HSMMs *in vitro* for five days and examined association with MST1 by co-immunopurification. We observed association between FLAG-RASSF4 and endogenous MST1 (Supplementary Figure 3A). This interaction was dependent on an intact RASSF4 SARAH domain, as FLAG-RASSF4ΔSARAH immunoprecipitates did not co-purify MST1.

Since RASSF4 protein associated with MST1 protein in myotubes, we next examined MST1 (*STK4*) mRNA levels in differentiating HSMMs. A small but significant increase in *STK4* mRNA was observed at three days of differentiation (Figure 5A). *STK4* mRNA levels then returned to baseline. These data suggest that in contrast to *RASSF4*, which displays robust mRNA upregulation starting at day 2, *STK4* mRNA levels remain mostly constant during *in vitro* myogenic differentiation. We also examined *STK4* mRNA expression during early differentiation in the setting of RASSF4 suppression, but did not see a consistent change (Supplementary Figure 3B), suggesting that the major mode of MST1 regulation during differentiation is post-translational. Based on the RASSF4-MST1 protein-protein interaction, we next examined whether altering MST1 activity could reverse the RASSF4 suppressed phenotype in HSMMs. We established HSMM cells expressing control vector, MST1 WT, or a kinase inactive MST1 (MST1 K59R). Cell lines were validated by immunoblot for MST1

expression and activation of downstream signaling to MOB1 (Figure 5B). Overexposure of the film verified that MST1 is expressed at baseline in HSMMs. Each cell population was transduced with *RASSF4* shRNA, differentiated *in vitro* for five days, then analyzed for MHC staining. Cells expressing a vector control and NT shRNA displayed robust MHC induction (Figure 5C, panel a). Similar results were observed in cells expressing MST1 and NT shRNA (Figure 5C, panel b). However, cells expressing MST1 K59R and the NT shRNA were deficient in myotube formation (Figure 5C, panel c). Similar to previous experiments, we observed defects in myotube formation in RASSF4-deficent cells (Figure 5C, panel d,g). However, MST1 expression but not MST1 K59R expression partially reversed the differentiation defects in RASSF4-deficient cells (Figure 5C, panels e,f,h,i), suggesting that MST1 activity is critical for myoblast differentiation. Interestingly, MST1 K59R expression combined with RASSF4 shRNA induced more severe myogenic defects than RASSF4 loss or MST1 K59R expression alone. These data suggest that RASSF4 and MST1 regulate differentiation of HSMMs.

4. Discussion

Since its discovery in 2000, study of the *RASSF* gene family has uncovered common and distinct roles for RASSF proteins 1–10 (Volodko, Gordon, 2014a). RASSF proteins regulate many cellular processes including microtubule reorganization, cell cycle progression, and cell survival. Classical RASSF proteins (RASSF1–6) regulate the RAS and Hippo/MST signaling pathways (Avruch et al. , 2009, Rodriguez-Viciana et al. , 2004). RASSFs have also been shown to regulate other cellular pathways including those containing cyclins, p53, NFkB, and MAPK (Volodko, Gordon, 2014a), suggesting a complex role for this gene family.

While RASSFs have a broad tissue distribution, the tissue-specific roles for most RASSFs are largely unknown. RASSF4 was shown to be upregulated in differentiating myoblasts derived from human muscle biopsies (Sterrenburg et al. , 2004), however its function remained unknown. We show here that in HSMMs *RASSF4* is critical for early myogenic differentiation. Early expression of RASSF4 coincides with the later upregulation of *MYOG* in differentiated myotubes. We further show that early suppression of *RASSF4* in myoblasts impairs elongation and myotube formation. We do not know whether this elongation and myotube formation can be rescued by later RASSF4 expression – ie whether the stimulus to promote microtubule reorganization is stalled, or irreversibly halted, but this would be important to understand for biologic situations in which one would want to drive myogenic differentiation forward. Furthermore, although essential for early myoblast differentiation, RASSF4 might be dispensable for later phases of myogenesis. After the dramatic upregulation of RASSF4 in the first two days of differentiation, RASSF4 expression gradually declined over 14 days. This transient increase in RASSF4 expression is similar to other critical proteins in the myogenic differentiation cascade. In addition, late knockdown of RASSF4 after myoblasts had been cultured for five days in fusion media had minimal impact on differentiation. These results suggests that RASSF4, directly or indirectly acting upstream of *MYOG*, is critical during early differentiation.

Microtubule restructuring is a signature process in muscle differentiation, which involves the re-localization of the MTOC to the nuclear membrane and Golgi (Bugnard, Zaal, 2005, Oddoux, Zaal, 2013). We observed that *RASSF4* suppression by RNAi prevents myoblast elongation during HSMM differentiation. We also found that RASSF4 is co-localized with the centrosomal protein PCM-1 in both undifferentiated and differentiation HSMMs. While this is the first examination of RASSF4 function in myogenic differentiation, a role for RASSFs in microtubule restructuring has been demonstrated in other studies (Arnette et al. , 2014, Oh et al. , 2006, van der Weyden et al. , 2005). It is very likely that during myogenesis, RASSF4 is involved in regulating microtubule dynamics through its function on MTOC reorganization. This will need further investigation.

The effects of RASSF4 on myogenic differentiation are due in part to regulation of MST1. RASSF4 associates with MST1 in myotubes, and the RASSF4 knockdown phenotype is partially rescued by MST1 expression, but not kinase inactive MST1. This is in line with previous studies of MST1 in skeletal muscle biology, which show that MST1 is upregulated in skeletal muscle in response to denervation (Wei et al. , 2013) and promotes myogenic differentiation downstream of caspase 3 (Fernando et al. , 2002). However, the regulation of MST1 activation in these contexts is incompletely understood. At early differentiation time points, we observed an increase in MST1 expression that was small relative to the robust upregulation of RASSF4. This may suggest that MST1 is regulated during differentiation by expression of accessory proteins or post-translational modifications. The fact that the RASSF4ΔSARAH mutant (which abrogated binding to MST1) could still localize with the MTOC suggests that the localization and function of RASSF4 may be separable. Indeed, MST1-independent roles of RASSF4 in myogenic differentiation are likely. For example, recent studies in non-transformed human cells show that RASSF4 controls store-operated calcium entry and the formation of endoplasmic reticulum-plasma membrane junctions (Chen et al. , 2017). Future studies of the RASSF4-MST1 (and other RASSF4-protein) complexes, and their regulation of downstream pathways, will provide critical information about their roles in skeletal myogenesis.

While we have begun to understand a role for RASSF4 in myogenic differentiation, much remains to be investigated. This is especially true of the regulation of RASSF4 expression. What are the mechanisms that control *RASSF4* transcription and RASSF4 protein stability? It is clear from the qPCR and immunoblot studies that RASSF4 protein increases rapidly with the onset of myogenic differentiation, but the factors that control this are unknown. We speculate that master myogenic regulators such as MYOD1 occupy *RASSF4* gene promoters and/or enhancers to increase its expression. Other RASSFs are controlled by post-translation modifications and these may influence protein stability, protein-protein interactions, or subcellular localization (Volodko et al. , 2014b). It will also be important to understand the role of RASSF4 beyond skeletal muscle development, including its roles in muscle regeneration and diseases of skeletal muscle origin including muscular dystrophies and other cancers of skeletal muscle histogenesis. Development of additional methodology including RASSF4 immunohistochemistry, and comprehensive identification of RASSF4 post-translational modifications will be required.

5. Conclusions

In conclusion, we have identified RASSF4 as a critical component of early myogenic differentiation. RASSF4 is upregulated in response to myogenic differentiation stimulus, and early suppression of RASSF4 in differentiating HSMMs leads to failed upregulation of myogenin expression and myotube elongation defects. Although the underlying cellular mechanisms that coordinate the re-localization of MTOC proteins and control microtubule reorganization remain unclear, our studies identify RASSF4 as a novel centrosomal protein that is upstream of myogenin and critical for early myogenic differentiation. Furthermore, a RASSF4-MST1 complex may regulate differentiation, as exogenous MST1 was able to partially rescue RASSF4 loss of function. Additional studies are necessary to further elucidate the roles of RASSF4 during skeletal myogenesis and repair, and its dysregulation in disease of skeletal muscle origin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

6. Acknowledgements

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Abbreviations

aRMS	alveolar rhabdomyosarcoma
HSMM	human skeletal muscle myoblast
MTOC	microtubule organizing center
MHC	myosin heavy chain
RASSF4	RAS association domain family member 4

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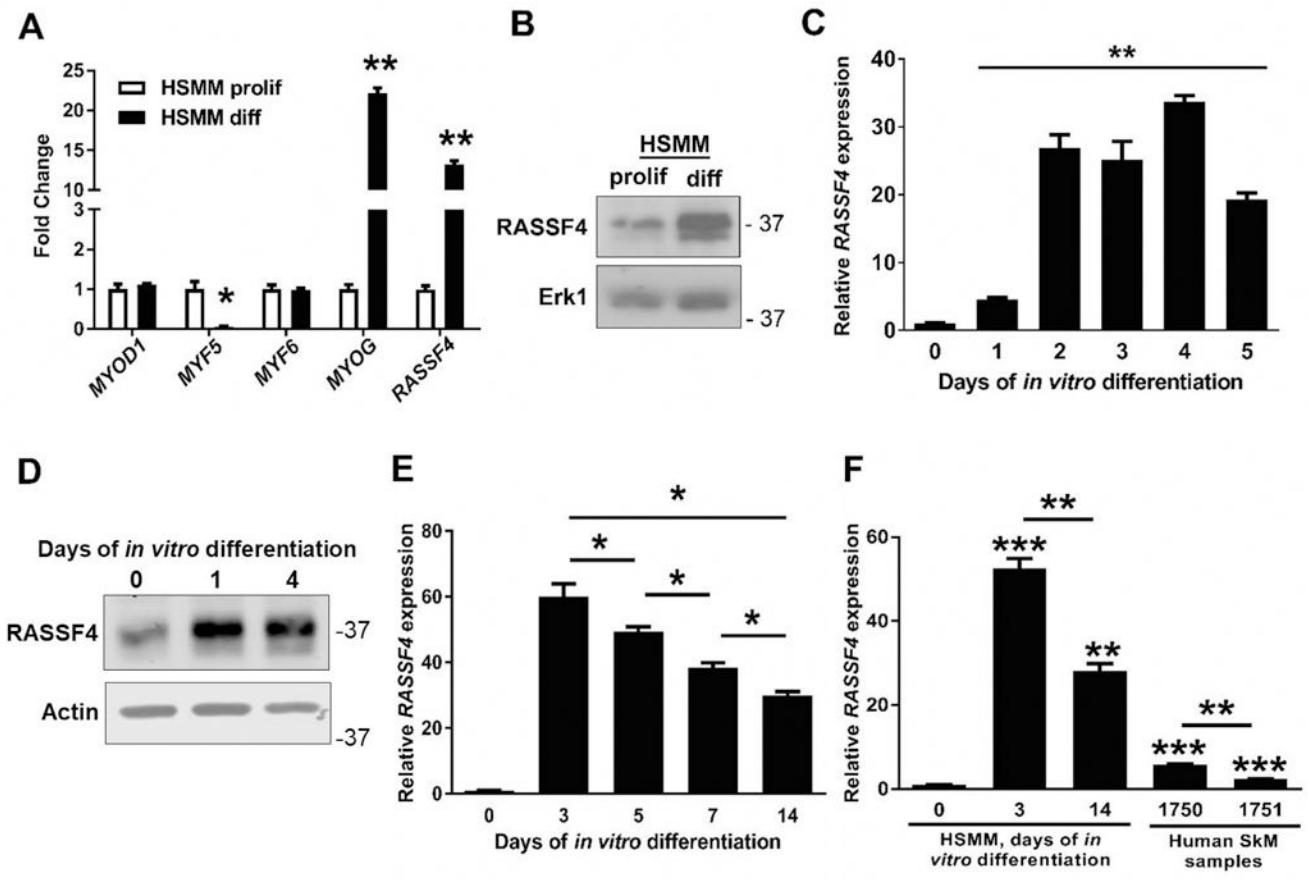

Figure 1. RASSF4 expression increases transiently during early myogenic differentiation.

(A) Expression of myogenic markers in proliferating versus differentiated HSMMs as assessed by qPCR. ***(B)*** Immunoblot validation of RASSF4 protein upregulation in response to differentiation. ERK1 used as a loading control. ***(C-E)*** Time courses of *RASSF4* mRNA or RASSF4 protein expression during *in vitro* differentiation. ***(F)*** *RASSF4* mRNA expression in *in vitro* differentiated HSMMs versus human skeletal muscle tissue (samples 1750 and 1751). *, P< 0.05; **, P<0.01 and ***, P<0.001.

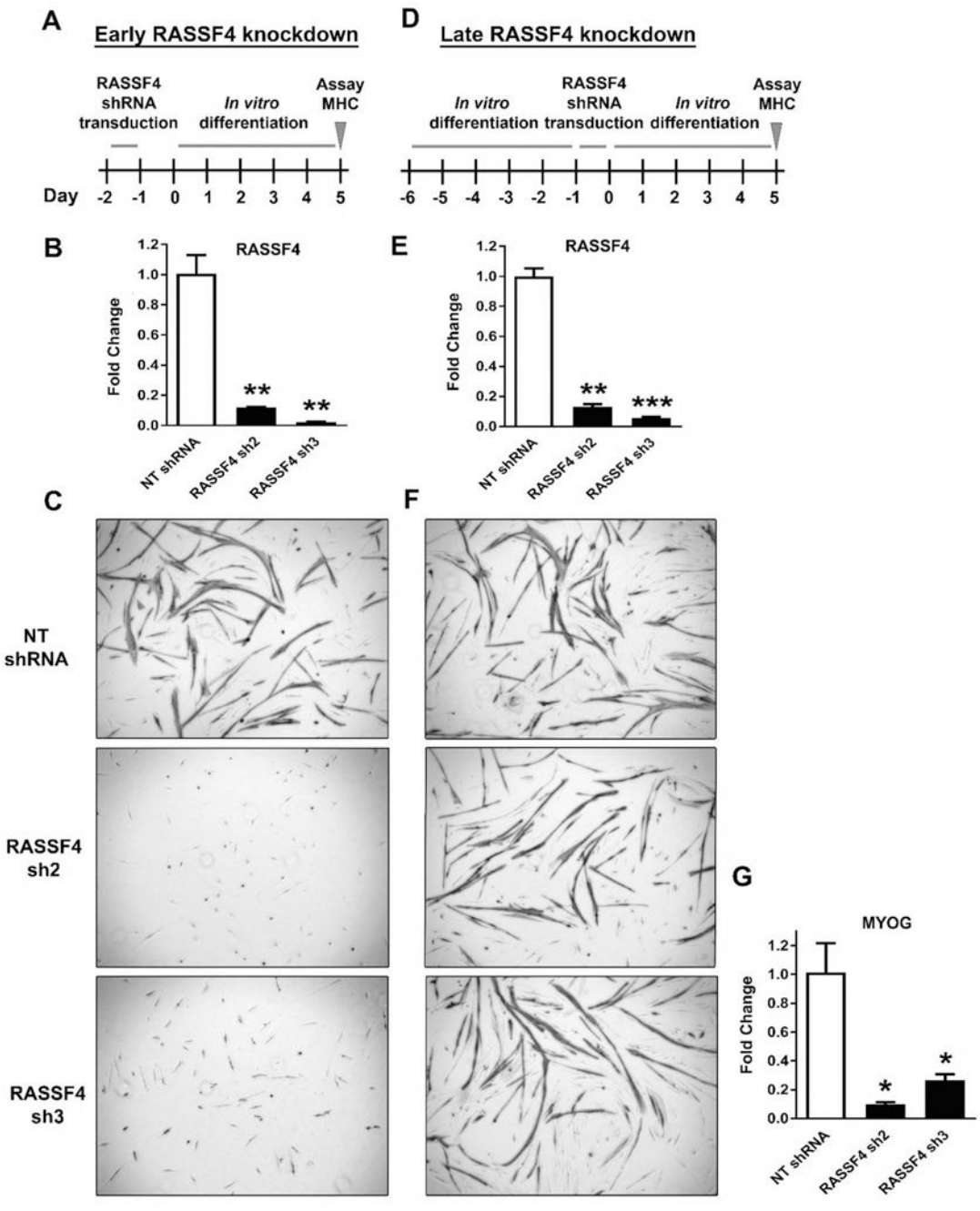

Figure 2. RASSF4 is necessary for early steps of *in vitro* myogenic differentiation and is upstream of myogenin

(A, D) Schematic of experimental design to examine the effects of *RASSF4* suppression early or late during the differentiation process. ***(B, E)*** Validation of *RASSF4* suppression by shRNAs as assessed by qPCR. ***(C, F)*** MF20 staining of HSMMs to detect MHC after 5 days of early or late *RASSF4* suppression. ***(G)*** Early *RASSF4* suppression early prevents induction of myogenin expression as measured by qPCR. *, P< 0.05; **, P<0.01 and ***, P<0.001.

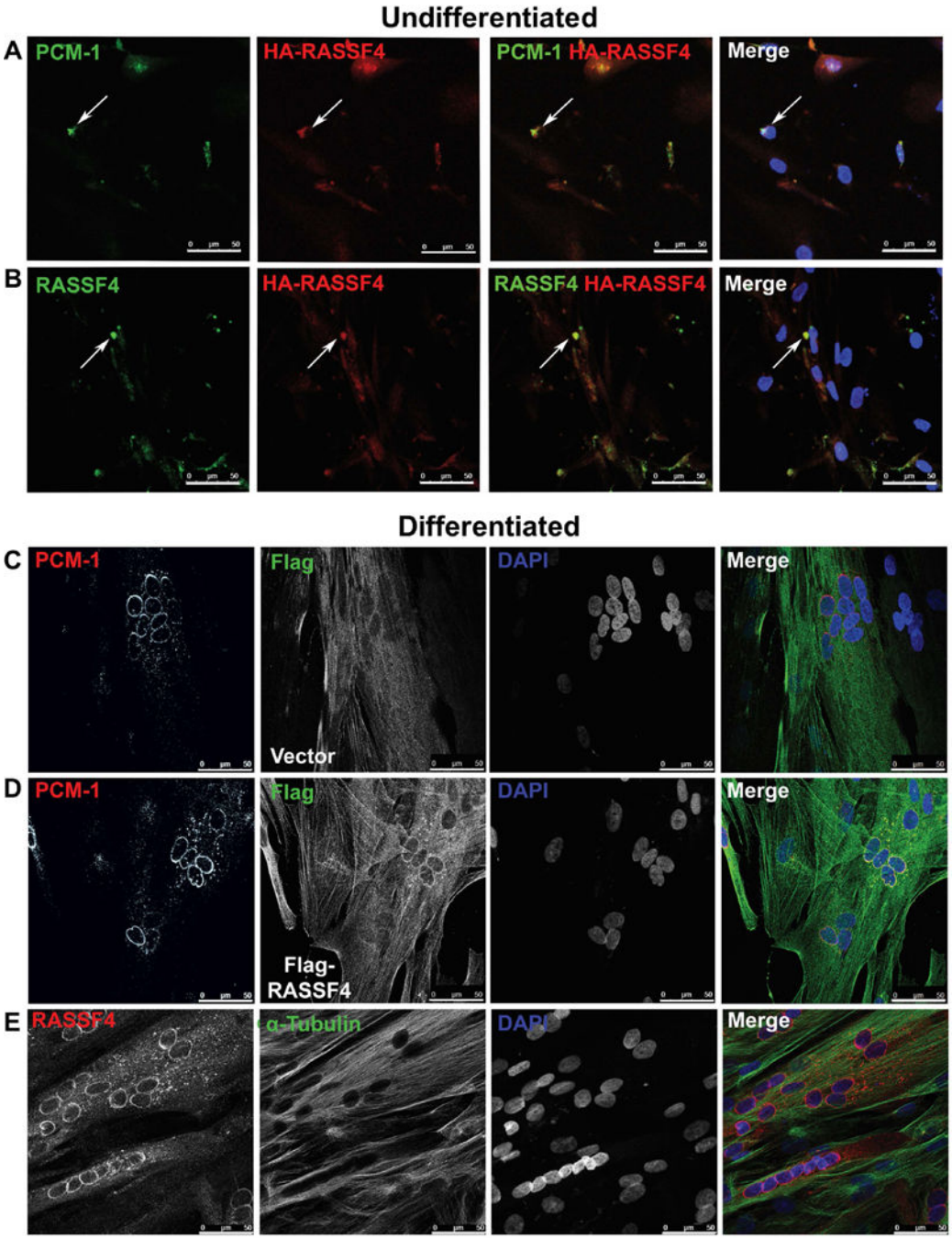

Figure 3. RASSF4 co-localizes with the centrosomal protein PCM-1 in pre and post-differentiated myoblasts.

(A, B) In undifferentiated myoblasts, HA-epitope-tagged RASSF4 and endogenous RASSF4 co-localize in a punctate distribution with PCM-1. The merge column includes DAPI staining to show nuclei. ***(C)*** In myoblasts subject to differentiation conditions for five days (forming multinuclei myotubes), PCM-1 is found ring-like around nuclei. ***(D-E)*** FLAG-epitope-tagged-RASSF4 and endogenous RASSF4 are similarly found ring-like around nuclei and co-localize with PCM-1. α-Tubulin used to label microtubules. The DAPI

column shows nuclei, and the Merge column provides color signal overlay. Scale bars represent 50μm.

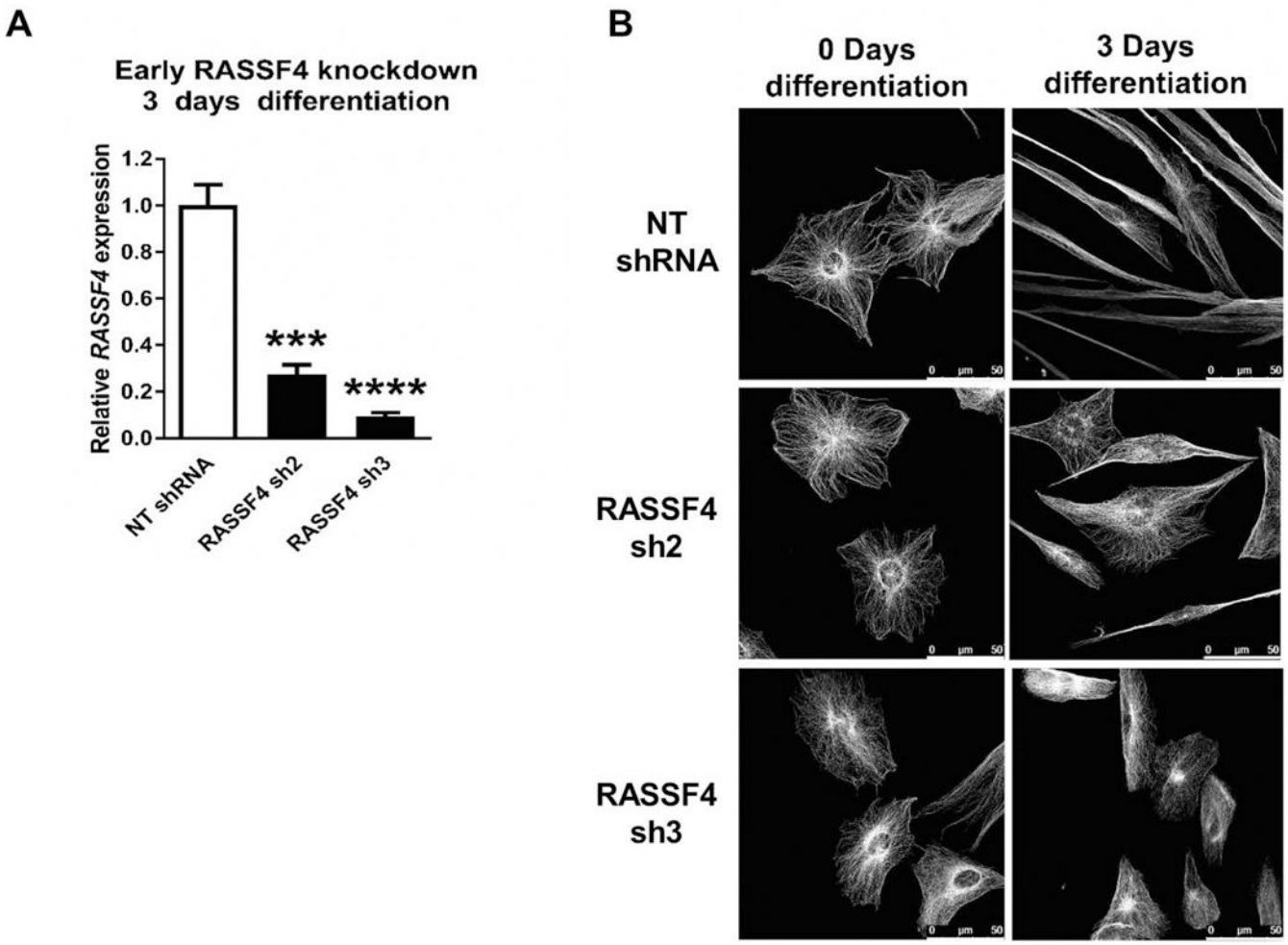

Figure 4. RASSF4 suppression prevents myoblast elongation.

(A) Validation of *RASSF4* suppression in differentiated HSMMs as assessed by qPCR. ***(B)*** Evaluation of microtubule distribution and cell shape in HSMMs transduced with NT or *RASSF4* shRNAs as assessed by measured by immunofluorescence microscopy. Cells with *RASSF4* shRNAs showed impaired elongation after three days of culturing in differentiation conditions. ***, P<0.001 and ****, P<0.0001. Scale bars represent 50μm.

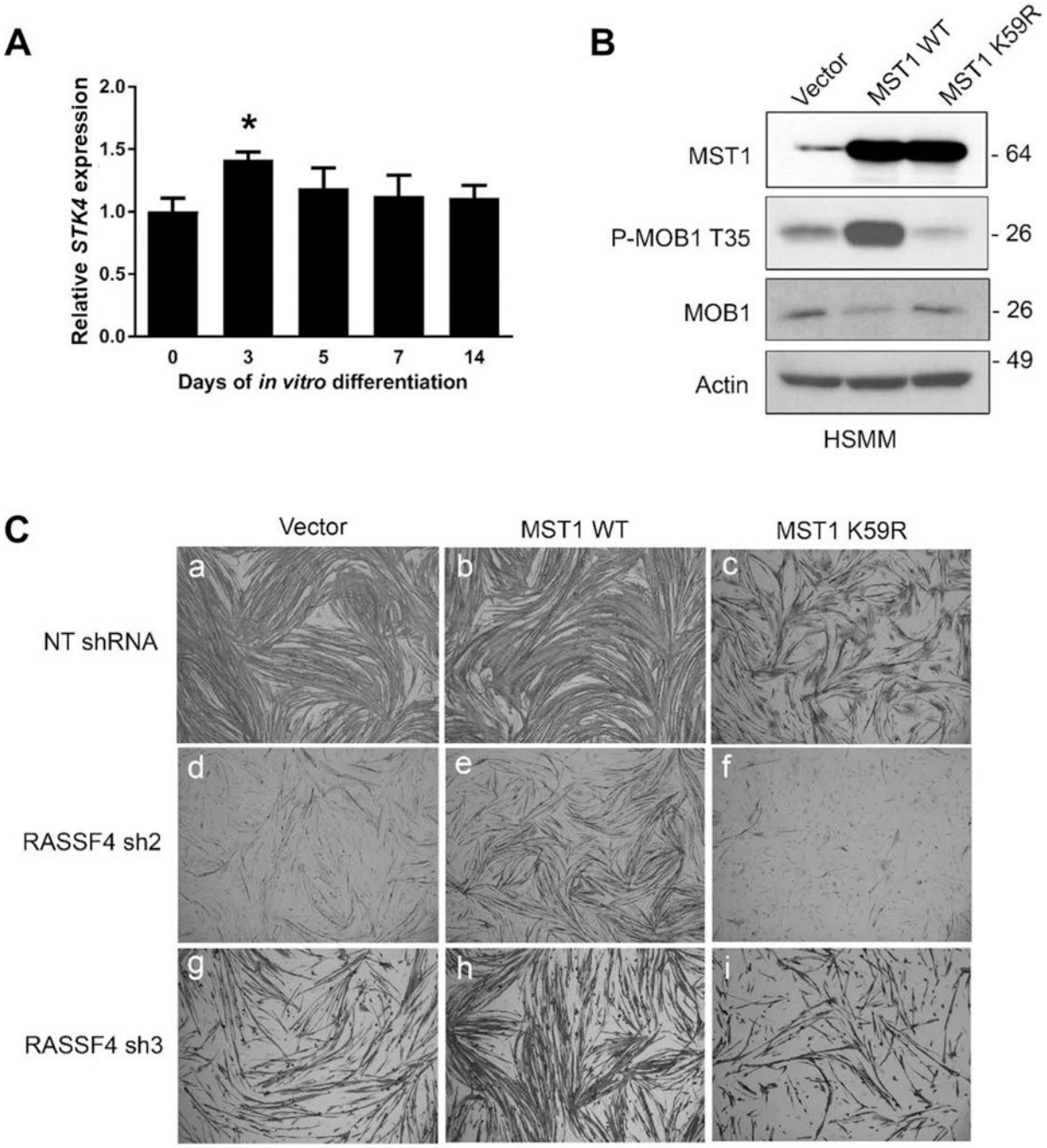

Figure 5. RASSF4-MST1 signaling promotes myoblast differentiation.

(A) As measured by qPCR, *STK4* mRNA (encoding MST1) is transiently induced during early myogenic differentiation but returns to baseline after day three. ***(B)*** HSMM cell lines were transduced to establish stable cell lines expressing empty vector, MST1 WT, or MST1 K59R. Cell lines were validated by immunoblot. ***(C)*** HSMMs expressing empty vector, MST1 WT, or MST1 K59R were transduced with shRNAs for RASSF4, differentiated *in*

vitro for five days, then stained for MF20, demonstrating that exogenous MST1 is able to partially rescue RASSF4 loss of function. *, P< 0.05.