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Bone Morphogenetic Protein/SMAD Signaling Orients Cell Fate Decision by Impairing KSRP-Dependent MicroRNA Maturation

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SUMMARY

MicroRNAs (miRNAs) are essential regulators of development, physiology, and evolution, and their biogenesis is strictly controlled at multiple levels. Regulatory proteins, such as KSRP, modulate rates and timing of enzymatic reactions responsible for maturation of select miRNAs from their primary transcripts in response to specific stimuli. Here, we show that KSRP silencing in mesenchymal C2C12 cells produces a change in the transcriptome largely overlapping that induced by bone morphogenetic protein 2 (BMP2) signaling activation. This induces osteoblastic differentiation while preventing myogenic differentiation. KSRP silencing- and BMP2-dependent myogenic miRNA (myomiR) maturation blockade is required for osteoblastic differentiation of C2C12 cells. Our results demonstrate that phosphorylated R-SMAD proteins, the transducers of BMP2 signal, associate with phosphorylated KSRP and block its interaction with primary myomiRs. This abrogates KSRP-dependent myomiR maturation, with SMAD4, SMAD5, and SMAD9 silencing being able to rescue KSRP function. Thus, SMAD-induced blockade of KSRP-dependent myomiR maturation is critical for orienting C2C12 cell differentiation toward osteoblastic lineage.

INTRODUCTION

Posttranscriptional regulation of gene expression is a multilayered process used by cells to modulate many fundamental functions, and in this context, microRNAs (miRNAs) have emerged as critical regulators (Krol et al., 2010). Reflecting the broad impact of miRNAs on gene expression, their deregulation has been demonstrated in a variety of cancers as well as in some inflammatory, neurodegenerative, and cardiovascular diseases (Mendell and Olson, 2012).

It is now clear that miRNA maturation from precursors is dynamically regulated in response to a variety of stimuli (Trabucchi et al., 2009a). Once transcribed, primary miRNAs (pri-miRNAs) are cleaved into stem-loop-structured precursor miRNAs (pre-miRNAs) by Drosha-containing complex. Each pre-miRNA is exported into the cytoplasm and cleaved by Dicer-containing complex into a short duplex with one strand being selected and incorporated into the effector machinery, which determines gene silencing (Krol et al., 2010).

We and others have reported that the single-strand RNA-binding protein KH-type splicing regulatory protein (KSRP) interacts with select miRNA precursors and promotes their processing (Gherzi et al., 2010). This, in turn, contributes to convey cell signaling into changes of miRNA maturation (Zhang et al., 2011; Briata et al., 2012). KSRP displays functional plasticity and can regulate several additional aspects of RNA life with the decay-promoting function of labile mRNAs being extensively studied (Gherzi et al., 2010). We recently reported that a dynamic switch between distinct KSRP functions, following its phosphorylation by AKT, is essential for maturation of muscle-specific miRNAs (myogenic miRNAs [myomiRs]) and for myogenic differentiation of C2C12 mesenchymal cells (Briata et al., 2012). KSRP-deficient mice do not display overt muscular abnormalities, but KSRP absence impairs myomiR maturation in the course of injury-induced muscle regeneration even though the effect of KSRP knockout is transient (Briata et al., 2012). Interestingly, a similar phenotype of delayed skeletal muscle regeneration in response to injury has been recently described in miR-206 knockout mice (Liu et al., 2012).

Bone morphogenetic proteins (BMPs), members of the transforming growth factor beta (TGF-β)/BMP superfamily, generate a wide range of biological effects in various cell types and are potent inducers of osteoblastic differentiation from mesenchymal progenitors (Rosen, 2009). BMPs bind to specific transmembrane receptors and, by sequentially inducing receptor serine kinase activity, cause SMAD protein (SMAD)

phosphorylation (Miyazono et al., 2010). The receptor-regulated SMADs (R-SMADs: SMAD1, SMAD5, and SMAD9) once phosphorylated and complexed with SMAD4, translocate into the nucleus where they induce transcription of osteoblastic determination and differentiation factors (Miyazono et al., 2010). Recently, Hata and coworkers reported that SMADs interact with select pri-miRNAs in a sequence-specific manner and favor their maturation in a smooth muscle cell line (Davis et al., 2008, 2010).

Mesenchymal cell differentiation is regulated by integrated signaling networks that orchestrate changes in gene expression leading to commitment to specific cell lineages (Asakura et al., 2001). C2C12 cells possess the ability to differentiate into myofibers upon serum withdrawal or into osteoblasts upon addition of BMP to the culture medium, thus representing a powerful tool to dissect molecular events underlying cell differentiation (Katagiri et al., 1994).

In this study, we identified an unanticipated role for the phosphorylated-R-SMADs/SMAD4 complex that, interacting with KSRP, impairs its ability to bind to primary myomiRs and to promote their processing. These events are required to orient fate decisions of mesenchymal C2C12 cells determining osteoblast differentiation.

RESULTS

KSRP Knockdown in C2C12 Cells Reshapes the Transcriptome Favoring Osteoblast Differentiation

We investigated the consequences of KSRP knockdown on gene expression in C2C12 cells that are able to differentiate either toward myotubes upon serum withdrawal (differentiation medium, DM) or toward osteoblasts upon addition of BMP2 to DM (Katagiri et al., 1994). KSRP was stably knocked down (shKSRP) in undifferentiated C2C12 cells (GM, growth medium; Figure S1A), and gene expression was analyzed by transcriptome sequencing (RNA-seq). Analysis of data obtained comparing mock-transfected and shKSRP cells revealed gene expression changes reminiscent of those previously described in cells treated with BMP2 to induce osteoblastic differentiation (Balint et al., 2003) (Figure 1A, examples of upregulated osteogenic markers [top] and downregulated myogenic markers [bottom]; Table S1). Results have been validated by quantitative RT-PCR (qRT-PCR) analysis, immunoblotting, and cell staining. Increased expression of *Runx2*, a critical regulator of the osteogenic lineage, as well as of osteomodulin (*Omd*), fibromodulin (*Fmod*), and collagen 6a2 (*Col6a2*), was detected in shKSRP C2C12 cells (Balint et al., 2003) (Figures 1B, 1C, and S1B; data not shown) and was reproduced by transient KSRP silencing (siKSRP; Figure S1C). Importantly, alkaline phosphatase (AP) activity was present in shKSRP C2C12 cells cultured in GM (Figure 1D; Rattner et al., 2000). Strikingly, systematic bioinformatic analysis of the global transcriptome changes consequent to KSRP silencing in C2C12 cells cultured in GM (compared to mock-transfected cells) showed that 93% of the significantly upregulated and 87% of the significantly downregulated transcripts were concordantly regulated by BMP2 treatment (Figure 1E). Furthermore, KSRP silencing enhanced the magnitude of the BMP2-dependent osteoblastic marker induction (Figures S1D and S1E, right panels) and abrogated the induction of myogenic markers upon a myogenic stimulus (DM), inducing the expression of osteoblastic transcripts and of AP (Figures 1F and S1E, left panels).

Considering that KSRP is known to promote rapid decay of unstable mRNAs, we assumed that transcripts whose levels were downregulated upon KSRP silencing reflected an indirect modulation through KSRP-regulated factors able to impair gene expression, and we initially focused on labile transcripts whose expression was upregulated in KSRP knockdown cells. Interestingly, we found that the levels of transcripts encoding members of the BMP/SMAD signaling pathway were upregulated in shKSRP C2C12 cells (Figure 1A; Table S1; data not shown). *BMP-receptor 2* (*Bmpr2*), *Smad5*, and *Smad9* expression was significantly increased in KSRP-silenced cells when compared to mock-silenced cells (Figures 2A, 2B, and S2A). We found that KSRP interacts with the 3′ UTRs of Bmpr2, Smad5, and Smad9 mRNAs, and its knockdown enhances the stability of these labile mRNAs (Figures S2B and S2C). Importantly, as revealed by the induction of phosphorylated SMAD5 and SMAD9, the BMP/SMAD signaling pathway was activated in KSRP knockdown cells (Figures 2B and S2D). Comparable results were obtained by silencing KSRP in another mesenchymal cell line (C3H/10T1/2, Figure S2E). The relevance of BMP/SMAD signaling activation occurring in shKSRP C2C12 cells in determining the osteoblastic phenotype in the absence of any stimuli was underscored by the reduction of *Runx2* and *Fmod* expression consequent to SMAD5 and SMAD9 knockdown (Figure 2C). Altogether, our data suggest the implication of KSRP in the BMP/SMAD signaling pathway, allowing us to hypothesize that its function is required at the crossroad between myogenic and osteoblastic differentiation to orient C2C12 cell fate.

BMP/SMAD Signaling Impairs KSRP Ability to Promote Primary myomiR Maturation

We observed that KSRP silencing in undifferentiated C2C12 cells induced the expression of proteins that, apart from being involved in osteoblastic differentiation, are validated targets of myomiRs (i.e., CONNEXIN 43, also known as GJ1A, and HDAC4, Figures 1A and S3A; Chen et al., 2006; Inose et al., 2009). Considering this finding and the essential role of KSRP in pri-miRNA processing (Ruggiero et al., 2009; Trabucchi et al., 2009b; Zhang et al., 2011; Briata et al., 2012), we wanted to explore KSRP-dependent modulation of miRNA maturation in osteoblast differentiation. We first investigated the expression of miRNAs that have been previously described to be modulated during osteoblast differentiation (Kapinas and Delany, 2011) and observed that most of them are not regulated in C2C12 cells under our experimental conditions except for miR-26a, miR-125b-5p, and miR-19b (Figure S3B; data not shown). However, the expression of miR-26a, miR-125b-5p, and miR-19b was not affected by KSRP silencing (Figure S3B), and their functional role was not further explored.

Among miRNAs whose function has been implicated in both myogenic and osteoblastic differentiation, although with opposite outcome (Inose et al., 2009; Kim et al., 2006; Chen et al., 2006; Li et al., 2008), we focused on miR-206 and miR-133b

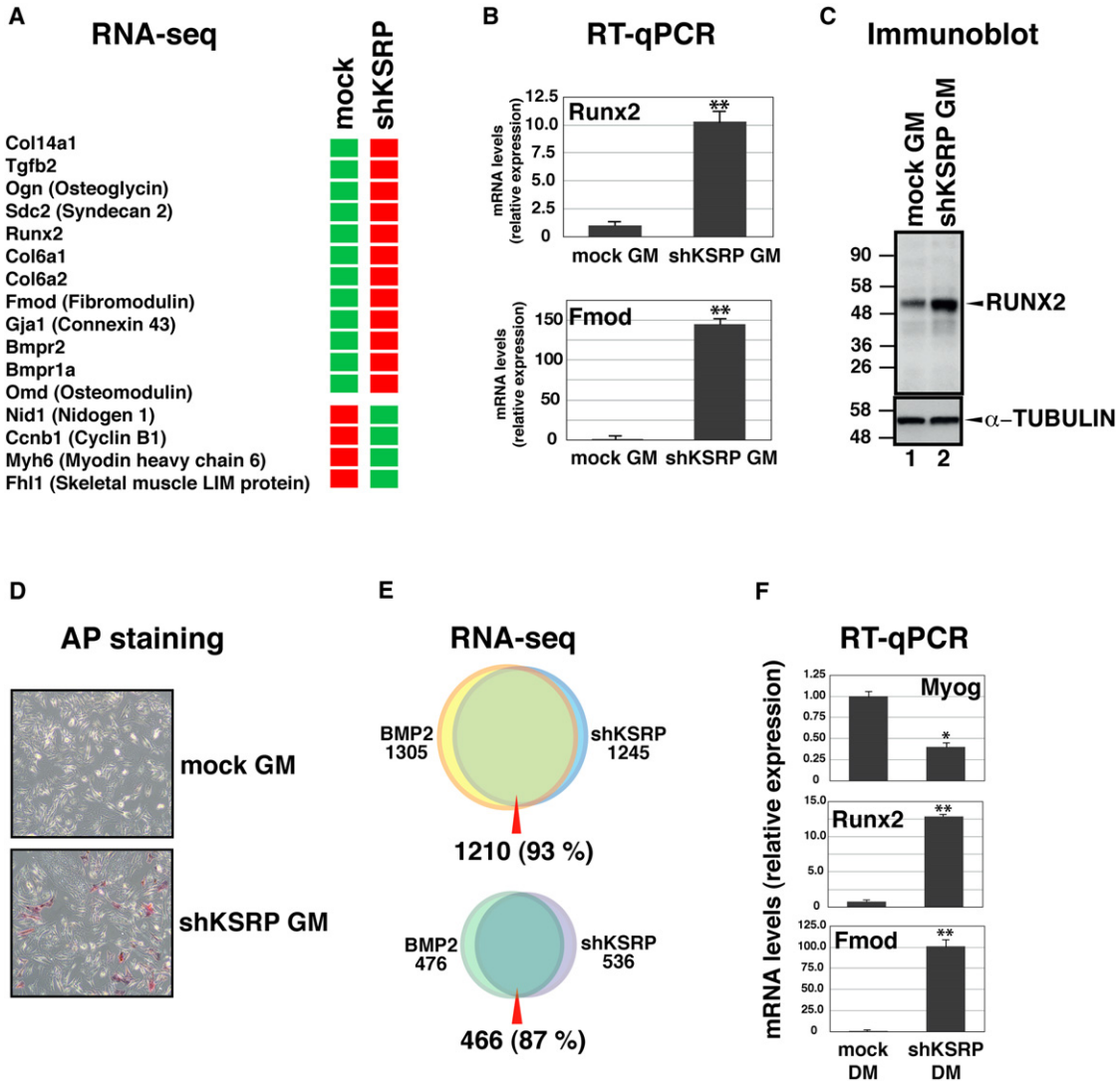

Figure 1. KSRP Knockdown in C2C12 Cells Favors Osteoblast Differentiation

(A) Selection of transcripts either upregulated (top) or downregulated (bottom) in stably KSRP-silenced (shKSRP) C2C12 cells (when compared to mock-transfected [mock] C2C12 cells) derived from a comparison of the results obtained by transcriptome analysis (RNA-seq) with data published in Balint et al. (2003). Green and red colors in the heatmap indicate a decrease and increase of mRNA levels, respectively.

(B) qRT-PCR analysis of the indicated transcripts in either mock or shKSRP C2C12 cells cultured in GM.

(C) Immunoblot analysis of total-cell extracts from either mock or shKSRP C2C12 cells cultured in GM using the indicated antibodies. The position of molecular mass markers is indicated on the left. Representative gels are shown.

(D) AP staining of either mock or shKSRP C2C12 cells cultured in GM. A representative staining is displayed.

(E) Bioinformatic analysis of the RNA-seq data. Schematic representation of the overlap between transcripts either upregulated (top) or downregulated (bottom) in BMP2-treated mock-C2C12 cells and transcripts concordantly regulated in shKSRP C2C12 cells cultured in GM. Transcripts derived from mock-transfected C2C12 cells cultured in GM were first compared either with transcripts derived from mock-transfected cells cultured in DM plus BMP2 or with transcripts derived from shKSRP C2C12 cells cultured in GM. Only mRNAs whose expression significantly ($p < 0.01$) differed were included in further comparisons. Arrowheads point to the percentage of overlapping transcripts (whose regulation is in common between BMP2-treated mock-transfected C2C12 cells and shKSRP C2C12 cells cultured in GM).

(F) qRT-PCR analysis of the indicated transcripts in either mock or shKSRP C2C12 cells cultured in DM.

The values of qRT-PCR experiments shown are averages (±SEM) of three independent experiments performed in triplicate. Statistical significance: *$p < 0.005$, **$p < 0.001$ (Student's t test).

See also Figure S1.

(hereafter indicated as myomiRs) that are expressed at relevant levels also in C2C12 cells cultured in GM (data not shown). Indeed, KSRP knockdown abrogated the basal maturation of miR-206 and miR-133b observed in GM and, similarly to BMP2 treatment, blocked their DM-induced maturation from primary transcripts (Figure 3A; Sato et al., 2009). Finally, KSRP

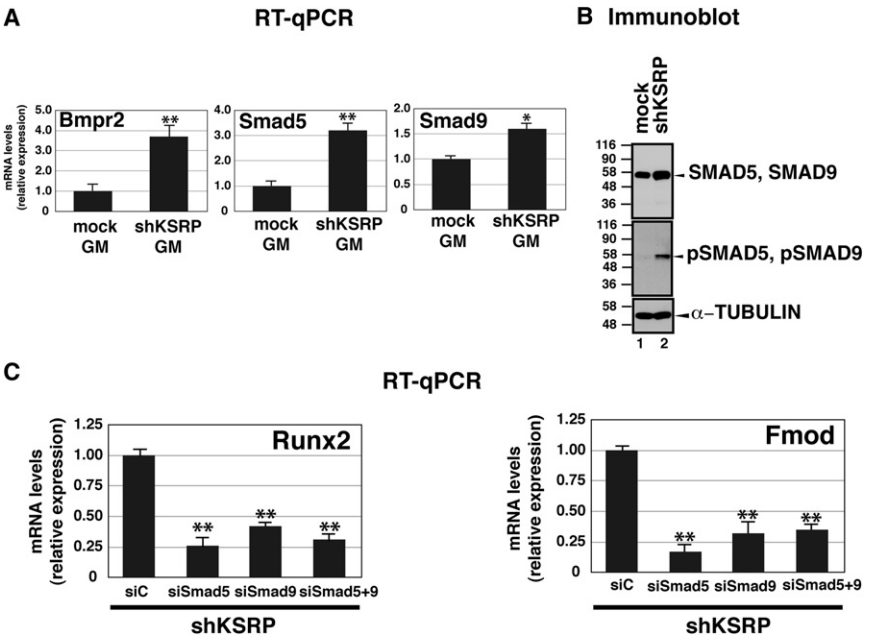

Figure 2. BMP-SMAD Signaling Activation Is Implicated in KSRP Knockdown-Dependent Phenotype Changes in C2C12 Cells

(A) qRT-PCR analysis of the indicated transcripts in either mock or shKSRP C2C12 cells cultured in GM.

(B) Immunoblot analysis of total-cell extracts from either mock or shKSRP C2C12 cells cultured in GM using the indicated antibodies. The two antibodies recognize either total or phosphorylated SMAD1, SMAD5, and SMAD9, but the expression of *Smad1* mRNA was barely detectable in C2C12 cells (data not shown). The position of molecular mass markers is indicated on the left. Representative gels are shown.

(C) qRT-PCR analysis of the indicated transcripts in KSRP-silenced C2C12 cells transfected with either control siRNA or siRNAs to SMAD5 and SMAD9 (as indicated).

The values of qRT-PCR experiments shown are averages (±SEM) of three independent experiments performed in triplicate. Statistical significance: *$p < 0.01$, **$p < 0.001$ (Student's t test). See also Figure S2.

knockdown and BMP2 treatment showed additive effects completely abrogating myomiR maturation (Figure 3A). The levels of pre-myomiRs mirrored those of the mature forms indicating that the arrest of maturation induced by BMP2 treatment and KSRP silencing occurred at the Drosha-processing level (data not shown). Forced re-expression of myomiRs in KSRP-silenced cells (Figure S3C) was able to counteract the increased expression of the components of the BMP/SMAD signaling pathway (Figure S3D) and caused a reduction of osteoblastic marker levels, demonstrating the essential role played by the blockade of myomiR maturation in the phenotype of shKSRP C2C12 cells, with transfected miR-206 also inducing a significant increase in *myogenin* expression (Figure S3E).

We hypothesized that the mechanism by which BMP signaling activation blocks myomiR maturation depends on the inhibition of KSRP function. UV-crosslinking/immunoprecipitation and ribonucleoprotein immunoprecipitation (RIP) experiments revealed that myogenic induction strongly enhanced the binding activity of KSRP to pri-miR-206 and pri-miR-133b, whereas the interaction was severely impaired by BMP2 treatment (Figures 3B, 3C, and S3F). As expected based on our previous data, KSRP was unable to interact with pri-miR-23b in C2C12 cells under all tested experimental conditions (Figure S3F, lower panel; Trabucchi et al., 2009b). RIP experiments showed that a transfected KSRP mutant unable to undergo AKT phosphorylation (S193A; Gherzi et al., 2006; Díaz-Moreno et al., 2009; Briata et al., 2012) was unable to interact with pri-miR-206 upon AKT signaling activation induced by expression of a constitutively active form of AKT1 (myrAKT1; Gherzi et al., 2006; Figures S3G and S3H). Accordingly, a transfected phosphomimetic KSRP mutant (S193D; Díaz-Moreno et al., 2009) was able to interact with pri-miR-206 in the absence of AKT activation (Figures S3G and S3H). Consistently, RNA/GST pull-down experiments performed using total-cell extracts from C2C12 cells cultured in DM revealed that a mutation in the AKT phosphorylation site (S193A) abrogated KSRP ability to interact with endogenous pri-miR-206 (Figure 3D).

In vitro pri-miRNA-processing assays confirmed that BMP2 treatment compromises the ability of cell extracts to process pri-miR-206. The processing activity was restored by the addition of an excess of purified recombinant KSRP to the reaction mixtures (Figure 3E).

Notably, BMP2 treatment did not affect either KSRP expression levels or its cellular localization or its phosphorylation (Figures S3I and 3F). Altogether, these results suggest that the functional interaction between AKT-phosphorylated KSRP and other regulatory proteins belonging to the BMP/SMAD signaling pathway might account for the blockade of myomiR maturation required for osteoblastic differentiation of C2C12 cells.

KSRP/SMAD Interaction Prevents myomiR Maturation and Orients C2C12 Cell Differentiation

We observed that phosphorylation of R-SMADs occurs early upon BMP2 treatment of C2C12 cells and is rapidly followed by inhibition of primary myomiR maturation (Figures S4A and S4B; data not shown). R-SMADs have been reported to bind to the R-SMAD-binding element (R-SBE, either CAGAC or CAGGG) in a group of pri-miRNAs in response to TGF-β/BMP signaling activation in pulmonary smooth muscle cells (Davis et al., 2008, 2010), and we confirmed that this was indeed the case also in C2C12 cells cultured in DM plus BMP2 (examples are provided in Figure S4C, left panels). On the contrary, we could not find any R-SMAD/primary myomiR interactions in C2C12 cells as expected on the basis of the absence of the R-SBE in the 300 nucleotides flanking the mature myomiRs (Figure S4C, right panels).

Strikingly, we found that phosphorylated R-SMADs interact with KSRP in C2C12 cells cultured in DM plus BMP2 as revealed

by both coimmunoprecipitation and GST pull-down experiments (Figures 4A and 4B). This interaction was RNase A insensitive and was abrogated by the S193A mutation (Figures 4B and S4D). Furthermore, a transfected SMAD5 lacking the C-terminal phosphorylation sites (phmSMAD5), differently from its wild-type counterpart, was unable to interact with KSRP (Figure 4C). It is known that, in response to BMP2, phosphorylated R-SMADs associate with SMAD4 and translocate into the nucleus (Miyazono et al., 2010). Indeed, data presented in Figure 4D indicate that KSRP was part of a complex including also SMAD4 in BMP2-treated cells. Among the four KH domains that enable KSRP to interact with RNA targets as well as other proteins, the third and fourth, which account for the majority of pri-miR-206-binding activity (R.G. and P.B., unpublished data), are predominant in the KSRP/R-SMAD interaction (Figure 4E).

Altogether, these findings allowed us to hypothesize that the interaction with SMADs could hinder the ability of KSRP to interact with primary myomiRs. RIP experiments performed in BMP2-treated C2C12 cells revealed that silencing of SMAD4, SMAD5, and SMAD9 (Figures S5A and S5B) was able to restore KSRP binding to primary myomiRs (Figure 5A), whereas it failed to affect KSRP interaction with unstable mRNAs (i.e., *Bmpr2* transcript in Figure S5C; data not shown). To rule out possible indirect consequences of SMAD silencing and to prove the direct effect of a protein complex including SMADs on KSRP function, we performed in-vitro-binding experiments using proteins purified by immunoprecipitation from C2C12 cell extracts. As shown in Figure 5B, preincubation with SMADs immunopurified from C2C12 cells cultured in DM plus BMP2, but not with SMADs immunopurified from C2C12 cultured in DM, impaired the ability of immunopurified phosphorylated KSRP to associate with in-vitro-synthesized pri-miR-206. This indicated that a complex including phosphorylated R-SMADs was able to directly inhibit phosphorylated KSRP binding to pri-miR-206. In keeping with results described in this paragraph, SMAD silencing in C2C12 cells cultured in DM plus BMP2 relieved the blockade of myomiR maturation from their primary transcripts (Figure 5C), significantly enhanced the expression of myogenic markers, and reduced the expression of osteoblastic markers (Figures 5D, S5D, and S5E).

In conclusion, upon DM plus BMP2 treatment, phosphorylated KSRP associates to a complex including phosphorylated R-SMADs and SMAD4, and this impairs its ability to interact with primary myomiRs and to promote their processing. This mechanism contributes to the osteoblast differentiation of C2C12 cells.

DISCUSSION

We have identified an unanticipated BMP/SMAD-dependent inhibition of KSRP's ability to promote maturation of myomiRs from their primary transcripts. This event is required to orient mesenchymal C2C12 cells toward osteoblast differentiation (Figure S6).

KSRP silencing in C2C12 cells causes a reshaping of the transcriptome, largely overlapping that produced by BMP/SMAD signaling activation. myomiRs have been implicated, with opposite outcomes, in both myogenic and osteoblastic differentiation, and we made the striking observation that miR-206 re-expression in KSRP-silenced cells is sufficient to enhance myogenin expression and to abrogate the osteoblastic phenotype. Thus, we propose that the most critical consequence of KSRP silencing is the impairment of myomiR maturation and that this is sufficient to determine C2C12 cell fate.

Our observations indicate that miR-206 and miR-133b have a functional role also in undifferentiated C2C12 cells because KSRP silencing-mediated inhibition of their expression induces osteoblast differentiation markers in cells cultured in GM. This underscores the relevance of maintaining a basal KSRP-mediated myomiR-processing activity to keep C2C12 cells poised to respond to myogenic stimuli. Instead, upon BMP2 treatment, KSRP-mediated myomiR maturation needs to be completely abrogated.

We have previously shown that phosphorylation by AKT at S193 enables KSRP to promote myomiR maturation upon a myogenic stimulus (Briata et al., 2012). However, in C2C12 cells cultured in DM plus BMP2, KSRP, although phosphorylated in S193, is unable to process primary myomiRs. Our data indicate that the functional repression of phosphorylated KSRP is a consequence of its interaction with a nuclear complex including phosphorylated R-SMADs as well as SMAD4.

The previously described R-SBE (Davis et al., 2010) is absent in primary myomiRs, and this is in keeping with the fact that we could not find any SMAD/primary myomiR interactions. Interestingly, although R-SBE-containing pri-miRNAs interacted with R-SMADs also in C2C12 cells, their expression was not modulated by culture in DM plus BMP2 (M.P., P.B., and R.G., unpublished data), and this observation underscores the importance of the cellular context in determining the final outcome of TGF-β/BMP signaling activation. Here, we report a distinct and unanticipated function for the SMAD complex that hinders the interaction of KH domains 3 and 4 with primary myomiRs. Our observations allow us to hypothesize the existence of at least two different classes of miRNAs regulated by SMADs. The maturation of R-SBE-containing pri-miRNAs is positively controlled by R-SMADs. Conversely, distinct pri-miRNAs, whose maturation is favored by KSRP in C2C12 cells, do not directly interact with SMADs, and their expression is negatively regulated through protein-protein interactions.

The inhibition of KSRP function that we describe here is different from the antagonism operated by either Lin28 or hnRNPA1. When Lin28 is expressed in undifferentiated embryonic stem cells, it occludes the accessibility of pri-let-7 family members to KSRP, whereas when Lin28 expression is abrogated, KSRP is allowed to promote maturation of let-7 family precursors (Trabucchi et al., 2009b). In different cells, hnRNPA1 directly competes with KSRP for binding to pri-let-7a (Michlewski and Cáceres, 2010). Notably, both Lin28 and hnRNPA1 directly interact with pri-miRNAs, whereas the inhibition operated by SMADs is RNA-binding independent. Neither hnRNPA1 nor Lin28A/B silencing affects C2C12 cell fate (P.B. and R.G., unpublished data), and these observations reinforce the notion that distinct cellular milieux play an essential role in governing the dynamic assembly of ribonucleoprotein complexes. In a sense, KSRP appears to exert a "default"-positive maturation function on select pri-miRNAs with cell- and pathway-specific negative modulators restricting its role.

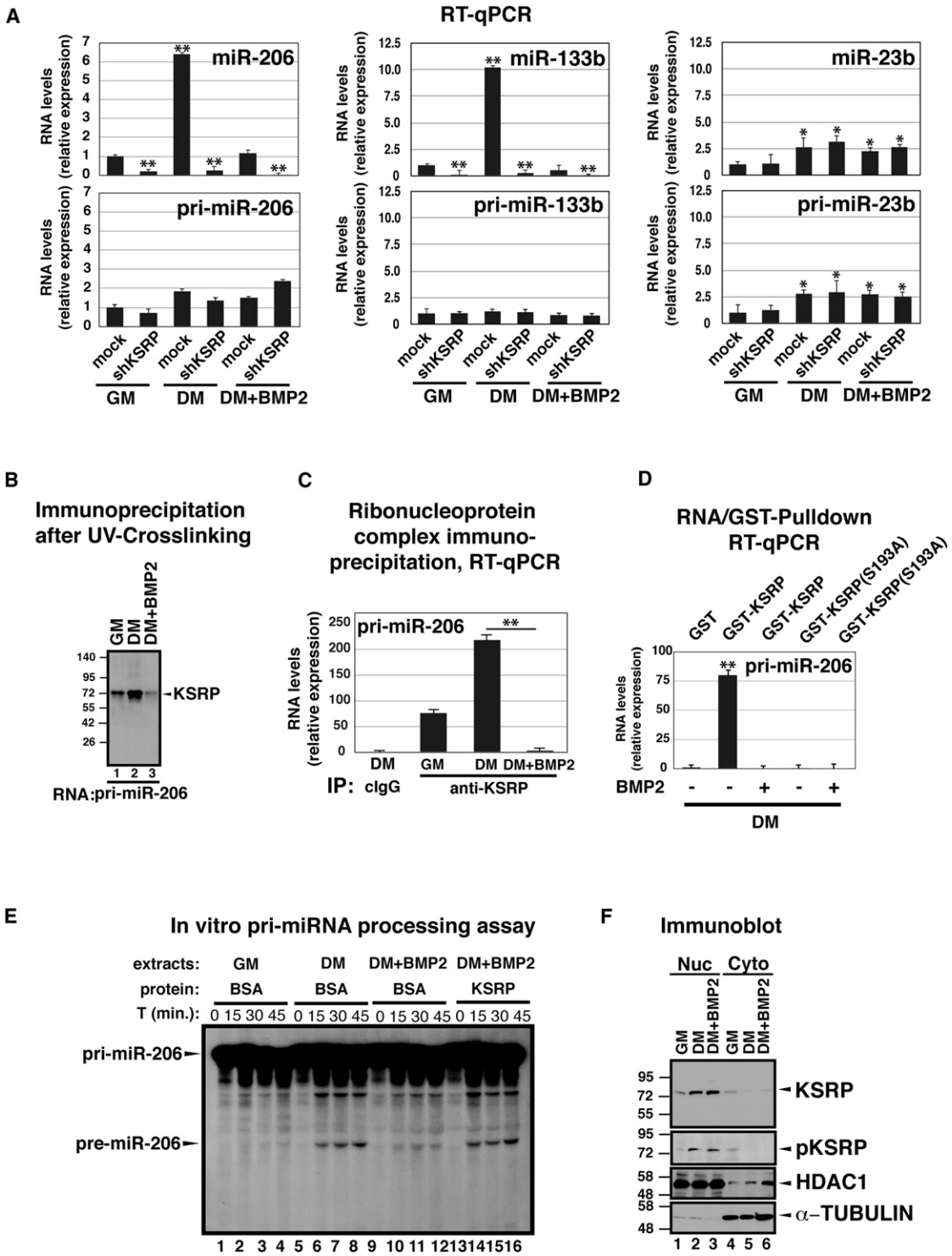

Figure 3. BMP/SMAD Signaling Impairs KSRP Ability to Promote Primary myomiR Maturation

(A) qRT-PCR analysis of miR-206, miR-133b, and miR-23b (upper panels) and their respective primary transcripts (lower panels) in either mock or shKSRP C2C12 cells cultured in either GM, DM, or DM plus BMP2.

(B) Total extracts from C2C12 cells cultured in GM, DM, or DM plus BMP2 were UV crosslinked to in-vitro-synthesized ^{32}P-labeled pri-miR-206 and subject to immunoprecipitation using KSRP antibody. Immunocomplexes were analyzed by SDS-PAGE and autoradiographed. A representative autoradiogram is shown.

(C) C2C12 cells were cultured in GM, DM, or DM plus BMP2. Total-cell extracts were immunoprecipitated (IP) as indicated. RNA was purified from immunocomplexes and analyzed by qRT-PCR to detect pri-miR-206.

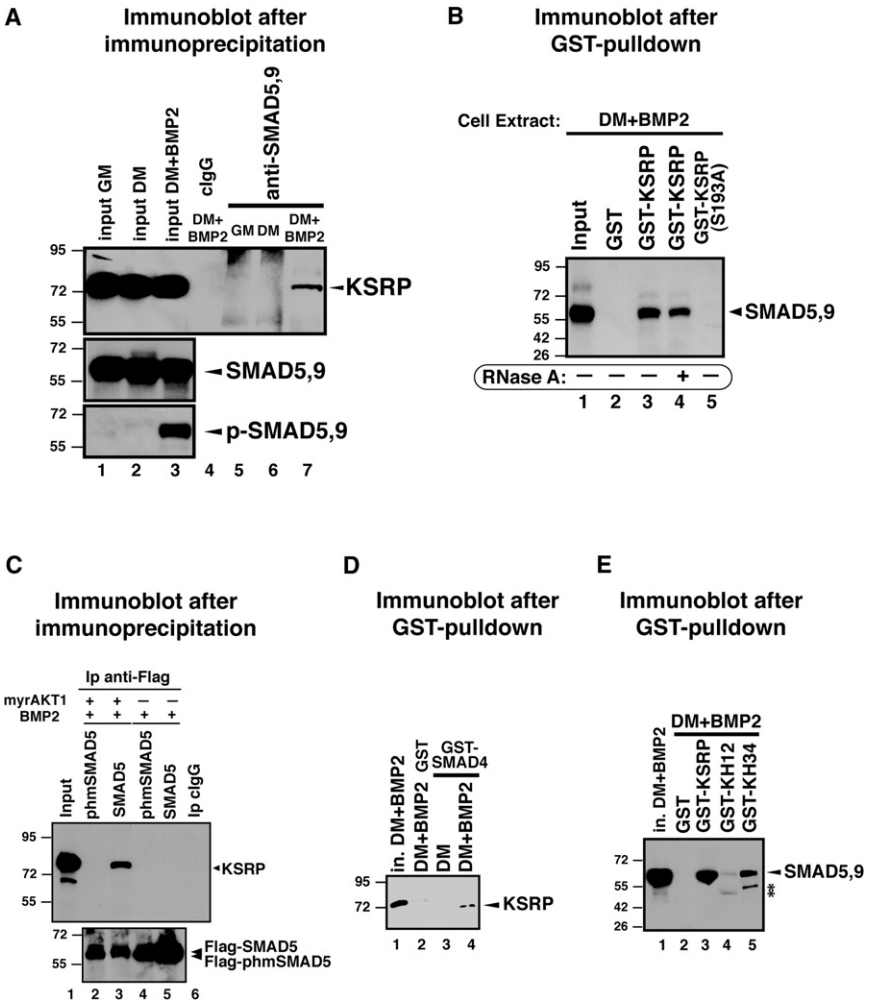

Figure 4. KSRP Interacts with SMAD Proteins upon BMP Signaling Activation

(A) Coimmunoprecipitation of SMAD5 and SMAD9 and KSRP in total extracts from C2C12 cells cultured in DM plus BMP2. Cell lysates were immunoprecipitated as indicated and analyzed by immunoblotting using monoclonal KSRP antibody (top panel). Inputs were probed also with either anti-SMAD5, anti-SMAD9 or anti-phospho-SMAD5, anti-phospho-SMAD9 (p-SMAD5, p-SMAD9).

(B) GST pull-down of endogenous SMAD5 and SMAD9 from total extracts of C2C12 cells cultured in DM plus 300 ng/ml BMP for 24 hr using either control GST, GST-KSRP or GST-KSRP(S193A) as indicated. Cell extracts were preincubated with either RNase A dissolved in water (15 μg/ml, +) or with water (–).

(C) Coimmunoprecipitation of either Flag-tagged SMAD5 or Flag-tagged four amino acid C-terminal deletion mutant of SMAD5 (phmSMAD5) with endogenous KSRP in total-cell extracts from HEK293 transiently transfected with either a plasmid expressing a constitutively active form of AKT1 (myrAKT1) or the empty vector and treated with BMP2.

(D) GST pull-down of endogenous KSRP from total extracts of C2C12 cells cultured in GM, DM, or DM plus BMP2 using either control GST or GST-SMAD4. Proteins were analyzed by immunoblotting.

(E) GST pull-down of endogenous SMAD5 and SMAD9 from total extracts of C2C12 cells cultured in DM plus 300 ng/ml BMP for 24 hr using either control GST, GST-KSRP or two KSRP deletion mutants fused with GST (GST-KH12 comprises the first two KH domains, whereas GST-KH34 comprises the third and fourth KH domains). Proteins were analyzed by immunoblotting. Asterisks mark nonspecific immunoreactivity.

The position of molecular mass markers is indicated on the left of each gel. Representative gels are shown for each experiment.

See also Figure S4.

TGF-β/BMP signaling is involved in the vast majority of cellular processes and is fundamental during the entire life of metazoans with its deregulation leading to developmental defects and/or diseases, including cancer (Massagué, 2008; Ikushima and Miyazono, 2010). Taking into account that members of the TGF-β superfamily are known to exert a myriad of influences on oncogenesis, implications of SMADs in the pleiotropic aspects of posttranscriptional control of gene expression pave the way toward the discovery of novel targets of intervention.

EXPERIMENTAL PROCEDURES

Cell cultures and transfections, antibodies, qRT-PCR, and pri-miRNA in-vitro-processing assays are described in Extended Experimental Procedures.

(D) RNA/GST pull-down performed using the indicated GST fusion proteins. Proteins were incubated for 16 hr at 4°C with total extracts (supplemented with protease and phosphatase inhibitors) prepared from C2C12 cells cultured for 24 hr in either DM or DM plus 300 ng/ml BMP2. Glutathione-sepharose-bound GST-protein pellets were extensively washed; protein-bound RNA was extracted and analyzed by qRT-PCR.

(E) In vitro pri-miR-206-processing assays performed using total extracts from C2C12 cells cultured in GM, DM, or DM plus BMP2 and preincubated (1 hr at 4°C) with either BSA (lanes 1–12) or purified recombinant KSRP (300 nM, lanes 13–16). Internally ^{32}P-labeled pri-miR-206 RNA substrate was added and its processing monitored as described in Experimental Procedures. A representative autoradiogram is shown.

(F) Immunoblot analysis of either nuclear (Nuc) or cytoplasmic (Cyto) extracts (20 μg) from C2C12 cells cultured in GM, DM, or DM plus BMP2 using antibodies directed toward the indicated protein (pKSRP is S193-phosphorylated KSRP). The position of molecular mass markers is indicated on the left. Representative gels are displayed.

The values of qRT-PCR experiments shown are averages (±SEM) of three independent experiments performed in triplicate. Statistical significance: *$p < 0.01$, **$p < 0.001$ (Student's t test).

See also Figure S3.

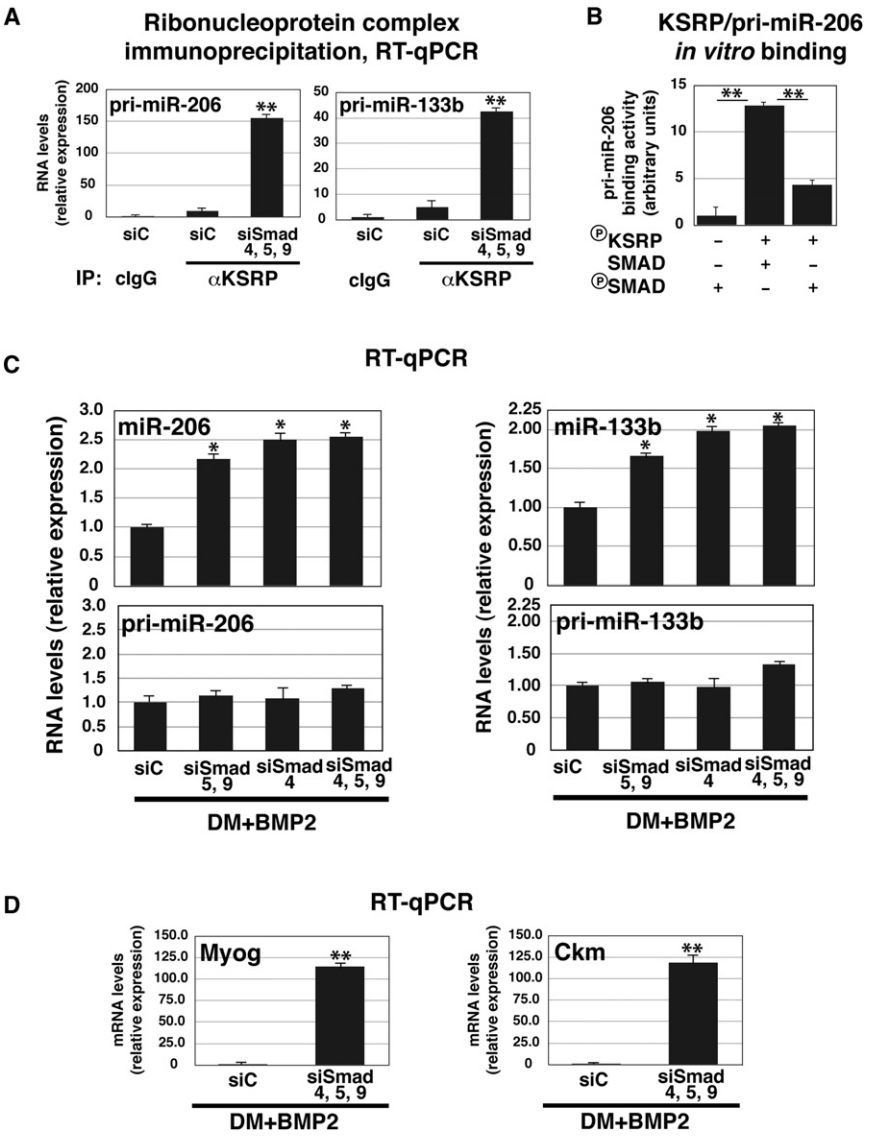

Figure 5. SMAD Protein Silencing Restores KSRP Ability to Promote myomiR Maturation Favoring Myogenic Differentiation of C2C12 Cells

(A) C2C12 cells were transfected with either control siRNA or siRNAs to SMAD4, SMAD5, and SMAD9 and then cultured in DM plus BMP2. Total-cell extracts were immunoprecipitated as indicated. RNA was purified from immunocomplexes and analyzed by qRT-PCR to detect either pri-miR-206 or pri-miR-133b.

(B) Phosphorylated KSRP ($^{(P)}$KSRP) immunopurified from C2C12 cultured in DM was pre-incubated (for 4 hr at 4°C) with SMAD proteins immunopurified from C2C12 cells cultured either in DM (SMAD) or in DM plus BMP2 ($^{(P)}$SMAD). Immunopurified proteins were incubated (1 hr at 4°C) with in-vitro-synthesized pri-miR-206 in the presence of heparin and yeast tRNA to inhibit nonspecific binding. Upon extensive washes of the pellets, RNA was extracted, and protein-bound pri-miR-206 was quantitated by qRT-PCR. The interaction observed in the absence of KSRP corresponds to background binding activity.

(C) C2C12 cells were transfected with either control siRNA or siRNAs to SMAD4, SMAD5, and SMAD9 (as indicated) and then cultured in DM plus 300 ng/ml BMP for 24 hr. RNA was prepared and analyzed by qRT-PCR to quantitate the expression of either mature miR-206 and miR-133 (upper panels) or the corresponding primary transcripts (lower panels).

(D) C2C12 cells were transfected with either control siRNA or siRNAs to SMAD4, SMAD5, and SMAD9 and then cultured in DM plus BMP2. RNA was prepared and analyzed by qRT-PCR in order to quantitate the expression of either myogenin or muscle creatine kinase (*Ckm*).

The values of qRT-PCR experiments shown are averages (±SEM) of three independent experiments performed in triplicate for each panel. Statistical significance: *$p < 0.005$, **$p < 0.001$ (Student's t test).

See also Figure S5.

RNA-Seq Library Construction and Deep Sequencing

The RNA-seq library was generated from total RNA prepared using miRNeasy (QIAGEN) and purified twice with Sera-mag Magnetic Oligo(dT) Beads (Thermo Fisher) according to Illumina's sample preparation instruction at LC-Sciences. Further details are provided in Extended Experimental Procedures.

Data Analysis and Bioinformatic Procedures

Procedures to analyze RNA-seq data sets are detailed in Extended Experimental Procedures. In order to detect the presence of the sequences CAGAC or CAGGG (binding sites for R-SMAD proteins described in Davis et al., 2010), we utilized the "Fuzznuc" tool in the EMBOSS package (Rice et al., 2000).

Plasmids, Recombinant Proteins, and Antibodies

The construction of both pCDNA-based and pGEX-based plasmids containing wild-type KSRP, KSRP(S193A), KH12, and KH14 mutants has been described elsewhere (Gherzi et al., 2006). To generate pCDNA-based and pGEX-based KSRP(S193D) mutant, we utilized the QuikChange Site-Directed Mutagenesis Kit (Agilent Technologies). To generate pCDNA-based and pGEX-based SMAD4 and SMAD5, specific cDNAs were obtained by PCR. To generate pCDNA-based and pGEX-based phmSMAD5 mutant, we cloned a PCR fragment encompassing a human SMAD5 cDNA fragment lacking the codons encoding the four C-terminal amino acids (SSVS). All plasmids were sequenced prior to their utilization. Baculovirus-based production and purification of His-tagged human KSRP were previously described (Briata et al., 2005).

siRNA and shRNA-Mediated Knockdown

siRNAs utilized to knock down KSRP expression were described previously by Trabucchi et al. (2009b). In order to knock down the expression of SMAD4, SMAD5, or SMAD9, we utilized siRNAs purchased from Santa Cruz Biotechnology.

RNA In Vitro Degradation

^{32}P-labeled RNAs were synthesized and used as substrates for in-vitro-degradation assays as reported (Briata et al., 2005).

RIP Complex Assays

RIP assays were performed as previously described (Briata et al., 2012) with some modification. Briefly, cell lysates were immunoprecipitated with

Dynabeads (Invitrogen, Carlsbad, CA, USA) coated with protein A/protein G and precoupled to specific antibodies at 4°C overnight. Pellets were washed four times with a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% Triton X-100, and 1× cOmplete (Roche). Total RNA was prepared from immunocomplexes using the miRNeasy Mini Kit (QIAGEN), retro-transcribed, and amplified by qPCR as described above. The primer sequences are detailed in Extended Experimental Procedures.

Nuclear and Cytoplasmic Extract Preparation

Nuclear and cytoplasmic (S100) extracts were prepared as previously described (Briata et al., 2005) in the presence of protease and phosphatase inhibitors.

RNA/GST Pull-Down Assays

Beads were incubated (16 hr, 4°C, rotating) with total-cell extracts supplemented with protease, phosphatase, and RNase inhibitors. After extensive washes in PDB supplemented with protease, phosphatase, and RNase inhibitors, RNA was extracted from beads and analyzed by qRT-PCR for pri-miRNA quantitation.

UV Crosslinking followed by Immunoprecipitation

Total-cell extracts were prepared in the presence of protein phosphatase inhibitors (PhosSTOP Phosphatase Inhibitor Cocktail; Roche, Mannheim, DE, USA) (200 μg protein), and ^{32}P-labeled RNA (5.0 ng = 2 × 10^6 cpm) was incubated at 20°C for 20 min in a RNA-binding buffer (200 μl) containing 10 mM HEPES (pH 7.6), 3 mM $MgCl_2$, 100 mM KCl, 2 mM DTT, 5% glycerol, 0.5% NP-40, yeast RNA (10 μg), and heparin (10 μg). Reaction mixtures were transferred to a 24-well plate and irradiated at 4°C for 10 min with a UV Stratalinker (Stratagene) at a distance of 5 cm. After subsequent digestion with RNase A (2 μg per reaction) for 10 min at 37°C, samples were subjected to immunoprecipitation overnight at 4°C, separated by SDS-PAGE, and ^{32}P-labeled proteins were visualized by autoradiography.

AP Staining

AP staining was performed using a commercial kit from Sigma-Aldrich (St. Louis) according to manufacturer's instructions.

ACCESSION NUMBERS

The Gene Expression Omnibus accession number for the data reported in this paper is GSE38907.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, two tables, and Extended Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.10.020.

LICENSING INFORMATION

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