The H19 long noncoding RNA controls the mRNA decay promoting function of KSRP.

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ABSTRACT

Long noncoding RNAs interact with protein factors to regulate different layers of gene expression either transcriptionally or post-transcriptionally. Here we report on the functional consequences of the unanticipated interaction of the RNA binding protein KSRP with the H19 long noncoding RNA (H19). KSRP directly binds to H19 in the cytoplasm of undifferentiated multipotent mesenchymal C2C12 cells and this interaction favors KSRP-mediated destabilization of labile transcripts such as myogenin. AKT activation induces KSRP dismissal from H19 and, as a consequence, myogenin mRNA is stabilized while KSRP is repurposed to promote maturation of myogenic micro-RNAs thus favoring myogenic differentiation. Our data indicate that H19 operates as a molecular scaffold that facilitates effective association of KSRP with myogenin and other labile transcripts and we propose that H19 works with KSRP to optimize an AKT-regulated post-transcriptional switch that controls myogenic differentiation.

Significance Statement

Long non-coding RNAs (lncRNAs) provide new layers of complexity to gene expression control. We report on the functional consequences of the interaction between the single-stranded RNA-binding protein KSRP with H19 lncRNA (H19) in multipotent C2C12 cells able to differentiate in culture towards myotubes in response to activation of cell signaling pathways including AKT. KSRP and H19 interact exclusively in undifferentiated C2C12 cells and this favors KSRP ability to interact with the pro-myogenic transcript myogenin and to favor its degradation. AKT activation induces KSRP dissociation from H19 and, as a consequence, from myogenin mRNA that is stabilized. H19 likely acts as a scaffold that favors KSRP-mediated degradation of myogenin to contribute to the maintenance of the undifferentiated state of C2C12 cells.

Short Title: H19 long noncoding RNA controls the mRNA decay

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INTRODUCTION

Regulatory non-coding RNAs and RNA-binding proteins (RBPs) act side-by-side to convert extracellular signals into changes of gene expression regulated at post-transcriptional level.

Among small non-coding RNAs, microRNAs (miRNAs) repress gene expression by inhibiting translation and/or promoting decay of target mRNAs (1) with RBPs being able to either control miRNA maturation from precursors or influence miRNA function (1, 2). A new class of transcripts referred to as long-noncoding RNAs (lncRNAs, arbitrarily defined as longer than 200 nucleotides (nt)) has recently moved to the forefront of regulatory RNA research (3). lncRNAs had been originally considered epigenetic regulators of gene expression but the emphasis placed on the ways they regulate chromatin state likely obscures the full repertoire of their functions (4-6). Other roles of lncRNAs include post-transcriptional regulation, post-translational control of protein activity, organization of protein complexes, as well as cell-cell signaling (4, 6). lncRNAs have been implicated in cellular events as different as cell cycle regulation, pluripotency, apoptosis and DNA damage response, to name just a few (5, 6). Not surprisingly, lncRNA expression is altered in cancer and it is becoming clear that some lncRNAs can control cell transformation by regulating vital cellular functions (7). Nevertheless, the composition and function of ribonucleoprotein complexes including lncRNAs is generally uncharacterized.

Studies performed in primary and cultured cells as well as in mice proved that the KH-type splicing regulatory protein (KSRP), a single-stranded RNA-binding protein that interacts with nucleic acids through four hnRNPK-homology (KH) domains, is able to integrate different levels of gene expression and is required for proper immune response, lipid metabolism, cell fate decisions and tissue regeneration (8 for a recent review). We and others have found that KSRP negatively regulates gene expression via at least two distinct and integrated post-transcriptional mechanisms: *i)* by promoting decay of unstable mRNAs (mainly targeting AU-rich elements [ARE] in their 3'UTRs) (8, 9) and *ii)* by favoring maturation of select miRNAs from precursors (8, 10). Briefly, KSRP recruits the RNA exosome and other enzymes to labile transcripts and promotes decay of inherently unstable mRNAs that encode factors critical for disparate cell functions (8,

9). On the other hand, KSRP associates with Drosha and Dicer complexes and promotes maturation of precursors to generate miRNAs that exert important functions in cell proliferation, differentiation, and metabolism in response to extracellular stimuli (8, 10-12). The critical role exerted by KSRP in the myogenic differentiation of multipotent mesenchymal C2C12 cells and the profound transcriptome reshaping that we have recently described in these cells upon KSRP silencing (13, 14) emphasizes the possibility that KSRP modulates a wide range of RNA targets.

In order to systematically detect regulatory RNA species interacting with KSRP, we have performed a transcriptome-wide analysis of KSRP-interacting RNAs in C2C12 cells and identified, among other RNAs, the H19 lncRNA (hereafter indicated as H19). We demonstrate that KSRP directly interacts with H19 in the cytoplasm of proliferating undifferentiated C2C12 cells and that this interaction favors the decay-promoting function of KSRP on labile transcripts such as myogenin. AKT activation induces KSRP dissociation from H19 and, as a consequence, myogenin mRNA is stabilized while KSRP is able to promote maturation of myogenic miRNAs thus favoring myogenic differentiation.

RESULTS

KSRP directly interacts with the H19 lncRNA.

An unbiased search for potential KSRP interactions with target RNAs was performed by high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP), a method that has been used to map the *in vivo* interaction of proteins with their RNA targets (15, 16). Experiments were performed in undifferentiated multipotent mesenchymal C2C12 cells (GM, growth medium) as well as in C2C12 cells induced to differentiate either towards myotubes upon serum withdrawal (differentiation medium, DM) or towards osteoblasts upon addition of BMP2 to DM (14). Extracts from UV-crosslinked cells were immunoprecipitated using either a polyclonal anti-KSRP antibody or the corresponding pre-immune serum (9). As shown in Fig. S1*A*, bioinformatic analysis of HITS-CLIP results revealed KSRP interaction with exonic sequences (including 3' and 5' untranslated regions (UTRs)), intronic sequences, as well as intergenic regions of the transcriptome. In this study, we focused on the unanticipated interaction of KSRP with H19, an intergenic lncRNA (Fig. 1*A*, Fig. S1*B* and *C*).

H19 is the developmentally regulated product of a paternally imprinted gene located on chromosome 7 in mouse and its first exon encodes the precursor of two conserved microRNAs: miR-675-5p and miR-675-3p (17, 18) (Fig. S1*B* and *C*). Both HITS-CLIP and Ribonucleoprotein Complex Immunoprecipitation (RIP) analyses revealed that KSRP/H19 interaction takes place in proliferating undifferentiated cells but is abrogated by either myogenic or osteoblastic differentiation (Fig. 1*A* and *B*, a negative control is shown in Fig. S1*D*). Notably, KSRP levels were unaffected by the differentiation state of C2C12 cells while H19 expression increased in C2C12 cells cultured in DM and remained unchanged in cells cultured in DM plus BMP2 (Fig. S1*E*). The KSRP target sequence is located at the 3' end of the first exon of H19 (Fig. S1*B*) and is well conserved among mammalian species (Fig. 1*C*). This sequence does not display any AU-rich motif while it presents short G-rich stretches that we previously demonstrated to efficiently interact with KSRP (8, 10). The specificity of the anti-KSRP immunoprecipitations was validated by RIP analysis in mouse embryonic fibroblasts (MEFs) derived from either wild-type or *Ksrp*-/- mice (Fig. 1*D*). Further, we ruled out the possibility that KSRP interacts with H19 only in cells where the lncRNA is very abundant (such as C2C12 cells and MEFs) by showing KSRP-H19 association also in tissues that

express limited amounts of H19 such as adipose tissue (Fig. S1*F*). H19 is present in both nuclear and cytoplasmic fractions of C2C12 cells cultured in GM although it is enriched in the cytoplasm (Fig. 1*E*, left panel) where its interaction with KSRP takes place (Fig. 1*E*, right panel).

Altogether these data revealed that KSRP interacts with the H19 lncRNA in the cytoplasm of proliferating C2C12 cells.

KSRP-H19 interaction is abrogated by AKT signaling activation.

AKT signaling is rapidly activated in C2C12 cells cultured in DM and we have previously shown that AKT phosphorylates KSRP on Ser 193 affecting its function during myogenic differentiation (13, 19). Based on the evidence that KSRP dissociates from H19 upon the induction of differentiation (Fig. 1*A* and *B*), we investigated whether AKT activation impairs KSRP-H19 interaction. Expression of constitutively active AKT2 in C2C12 cells cultured in GM strongly reduced the interaction of KSRP with H19 (Fig. 2*A*). In order to explore whether this effect was due to direct KSRP phosphorylation by AKT, purified recombinant KSRP was pre-incubated with either active recombinant AKT2 or kinase buffer alone. As shown in Fig. 2*B* (left panel), *in vitro* phosphorylation impaired KSRP-H19 interaction. Conversely, phosphorylation by AKT2 did not affect the interaction of recombinant purified KSRP mutated in the AKT phosphorylation site (S193A, ref. 19) with H19 (Fig. 2*B*, right panel). Accordingly, an aspartic acid mutant (S193D) that we have reported to destabilize the structure of the first KH domain (KH1), where Ser 193 is located (20), impaired the interaction of KSRP with murine H19 transiently expressed in HEK-293 cells (Fig. S2*A*).

We have previously shown that KSRP also undergoes phosphorylation by MAPK p38 (p38) during myogenic differentiation (21). However, as shown in Fig. S2*B*, p38 activation by a constitutively active form of the upstream kinase MKK6 (MKK6EE) failed to affect KSRP-H19 interaction in transfected HEK-293 cells while, as predicted, constitutively active AKT2 impaired the interaction in the same cellular context (Fig. S2*C*).

Altogether, these data suggest that AKT activation impairs the interaction of KSRP with H19 in C2C12 cells.

H19 silencing in undifferentiated C2C12 cells promotes myogenin mRNA stabilization and maturation of myomiRs from precursors.

In order to gain functional insights into KSRP-H19 interaction, we efficiently silenced H19 in proliferating C2C12 cells (Fig. S3*A*). The expression of myogenin is both transcriptionally and post-transcriptionally regulated during myogenic differentiation of C2C12 cells (22-24) and we have previously reported that KSRP silencing impairs its rapid decay (21). As shown in Fig. 3*A*, H19 silencing induced stabilization of myogenin mRNA and significantly enhanced its expression (Fig. 3*B*) in proliferating C2C12 cells. *In vitro* decay assays supported that this stabilization is mediated through the ARE present in the myogenin 3' UTR (Fig. S3*B*). Further, H19-depleted C2C12 cells, although cultured in GM, showed increased expression levels of pro-myogenic factors including muscle creatine kinase (Ckm), myoglobin (Mb) and Igf2 (Fig. S3*C*).

Based on our previous observations that AKT activation impairs KSRP association with myogenin mRNA while enhances its association with myogenic miRNA primary transcripts (13), we investigated the consequences of H19 silencing on the formation of ribonucleoprotein complexes including KSRP. Our RIP experiments showed that H19 silencing impairs KSRP association with myogenin mRNA (Fig. 3*C*) and favors KSRP association with pri-miR-206 and pri-miR-133b (Fig. 3*C*) with accumulation of mature miR-206 and miR-133b (hereafter indicated as myomiRs) without affecting the levels of their primary transcripts (Fig. 3*D*). Further, *in vitro* processing assays confirmed that extracts from H19-depleted C2C12 cells cultured in GM were able to promote the formation of pre-miR-206 from the primary transcript, similarly to extracts from cells induced to myogenic differentiation (DM) (Fig. 3*E*).

The first exon of H19 encodes two miRNAs, miR-675-5p and miR-675-3p (18, and schematic in Fig. S1*B*) that are expressed, although at low levels, in C2C12 cells cultured in GM and increase over the course of myogenic differentiation (25). In order to investigate whether the observed effects of H19 silencing can be ascribed, at least in part, to the abrogation of miR-675-5p and miR-675-3p function, we silenced each miRNA in proliferating C2C12 cells. As presented in Fig S3*D*, we did not observe any difference in either myogenin or myomiR expression upon transfection of either miR-675-5p or miR-675-3p antagomirs. Similarly, overexpression of both miR-675-5p and miR-675-3p in proliferating C2C12 cells did not affect either myogenin or myomiR expression (Fig. S3*E*).

Altogether, our silencing experiments suggest that H19 plays a critical role in favoring KSRP-mediated myogenin mRNA decay and in preventing KSRP-dependent maturation of myomiRs.

The interaction with H19 favors the decay promoting function of KSRP.

In order to gain molecular insight into the way H19 regulates the decay-promoting function of KSRP, we transiently transfected HEK-293 cells with a vector expressing either murine H19 or the control sequence E3 (a region derived from the mouse Pitx2 3'UTR that does not interact with KSRP, ref. 26) together with Flag-tagged KSRP. Murine H19 ectopically expressed in HEK-293 cells associated with KSRP (Fig. S4*A*) and favored its interaction with either co-expressed myogenin 3'UTR or endogenous GNAS, a labile mRNA that we previously characterized as a target of KSRP (27) (Fig. 4*A*). Notably, Flag-tagged KSRP was similarly immunoprecipitated in the presence of either E3 or H19 (Fig. S4*B*). The above observation, together with the evidence that KSRP binds to both myogenin mRNA and H19 in undifferentiated C2C12 cells while it fails to interact with both transcripts in C2C12 cells differentiating towards myoblasts (Fig. 1*B* and ref. 13), prompted us to investigate whether H19 is present in a complex including myogenin mRNA and, possibly, other KSRP target mRNAs. We expressed murine H19 tagged with MS2 RNA hairpins (MS2-H19) in HEK-293 cells together with myogenin 3'UTR as well as GST-fused MS2 binding protein (GST-MS2BP). Total cell extracts were precipitated using Glutathione-Sepharose beads and GST-MS2BP-associated RNA was extracted and analyzed by RT-qPCR. Results presented in Fig. 4*B* indicate that MS2-H19 expression enhanced the association of GST-MS2BP with transfected myogenin 3'UTR. Importantly, also the association of the endogenous labile mRNA GNAS was similarly regulated.

Based on these observations, we wanted to explore the mode of KSRP interaction with either H19 or labile mRNAs. We have recently generated a series of KSRP mutants in which the hallmark GxxG RNA binding loop present in each KH domain is substituted by the GDDG sequence (28). Thus, we obtained four KSRP mutants in which the RNA binding ability of each single KH domain is abrogated while the structure and stability of the protein is unaffected (KH1GDDG, KH2GDDG, KH3GDDG, KH4GDDG; ref. 28). We transiently expressed each KSRP mutant (or wild-type KSRP) in HEK-293 cells (Fig. S4*C*) together with both murine H19 and myogenin 3'UTR. As shown in Fig. 4*C* and Fig. S4*D*, the interaction of KSRP mutants with either transfected myogenin 3'UTR or

endogenous KSRP mRNA targets (GNAS and CTNNB1, also known as beta-catenin) occurred according to the previously described mode with KH2, KH3, and KH4 playing the major role in the RNA recognition and KH1 resulting dispensable (28, 29). The interaction of KSRP with H19 was unaffected by mutations in KH4 while resulted impaired not only by mutations in KH2 and 3 but also in KH1 (Fig. 4*C*). The fact that KH1 is required for optimal KSRP binding to H19 but not to ARE-containing mRNAs (Fig. 4*C*, Fig. S4*D* and ref. 29), together with results shown in Fig. 3*C* and Fig. 4*B* made reasonable to hypothesize that H19 operates as a molecular scaffold favoring both KSRP binding to mRNA targets and its decay promoting activity.

In order to verify this hypothesis, Flag-tagged KSRP was immunoprecipitated from extracts of HEK-293 cells transiently transfected with either H19 or E3 negative control sequence. Immunocomplexes were pre-incubated with S100 extracts prepared from KSRP-silenced C2C12 cells cultured in GM that are unable to promote decay of labile mRNAs (14). As shown in Fig. 4*D*, KSRP immunopurified from H19 transfected cells promoted myogenin 3'UTR decay more efficiently than KSRP immunopurified from cells transfected with the negative control E3 sequence. On the contrary, KH1GDDG mutant immunoprecipitated from H19 co-transfected HEK-293 cells was not able to induce myogenin 3'UTR rapid decay (Fig. 4*D*).

Next, we explored whether H19 expression was able to favor the interaction of the RNA exosome with ARE-containing RNAs. RIP analysis was performed using extracts from HEK-293 transiently transfected with two distinct Flag-tagged exosome components (EXOSC2 and EXOSC5) together with either H19 or the E3 sequence. As shown in Fig. 4*E*, the co-expression of H19 significantly enhanced the interaction of the RNA exosome with either co-transfected Myog 3'UTR or endogenous GNAS mRNA. As predictable, H19 was able to favor the interaction of the RNA exosome with KSRP as revealed by co-immunoprecipitation experiments performed in transiently transfected HEK-293 cells (Fig. S4*E*). Notably, the expression of myogenin 3'UTR and GNAS mRNA was reduced in H19-transfected cells emphasizing H19 relevance in the control of the steady-state levels of KSRP-regulated unstable mRNAs. (Fig. S4*F*).

On the whole our data indicate that H19, interacting with KSRP, favors its decay-promoting function and recruitment of the RNA exosome to labile mRNAs.

DISCUSSION

We have identified H19 as a lncRNA that directly interacts with the multifunctional RNA binding-protein KSRP and defined its role as a regulator of rapid KSRP-dependent mRNA decay in undifferentiated multipotent mesenchymal C2C12 cells.

H19 is expressed in all embryonic and neonatal tissues but after birth is generally down-regulated with the exception of skeletal muscle where it remains abundant (reviewed in ref. 17). Although the role of H19 in tumorigenesis is still debated, it is considered as an oncogenic lncRNA with pro-tumorigenic properties in a variety of cell types and has also been reported to play an active role in promoting tumor metastasis (30-32). However, the molecular mechanisms underlying its function(s) are poorly understood.

lncRNAs, like other regulatory RNAs, are endowed with the ability to interact with both protein factors and nucleic acids thus displaying the potential to direct ribonucleoprotein complexes to specific RNA or DNA target sites (4, 6, 33). Thus, it is not surprising that different roles have been described for lncRNAs in regulating various stages of gene expression (4, 6, 33). Besides the originally reported ability to interact with transcriptional regulators modulating chromatin accessibility, a few lncRNAs recently proved capable of associating with RNA-binding proteins implicated in various RNA metabolism checkpoints (5, 6). Interestingly, recent reports indicated that some lncRNAs can function as competing endogenous RNAs (ceRNAs) by base-paring to and sequestering specific miRNAs (34) while others can modulate mRNA stability by interacting with RBPs (35-37).

In this report, we have identified an unanticipated mechanism by which cytoplasmic H19 post-transcriptionally modulates gene expression in proliferating C2C12 cells. We propose that H19 acts as a scaffold to favor the interaction of KSRP and the RNA exosome with target mRNAs enhancing the mRNA decay-promoting function of KSRP on myogenin mRNA (and, possibly, other labile transcripts). The modulation of KSRP function operated by H19 contributes to the maintenance of the undifferentiated state in these cells.

We previously showed that AKT signaling activation determines a series of changes in KSRP functions (including interaction with the adaptor protein 14-3-3 and nuclear translocation) that enable the protein to switch between the cytoplasmic mRNA decay-promoting activity to the nuclear primary-myomiR processing activity (13, 19, 20).

We propose that H19 silencing, similarly to AKT activation, impairs the function of a cytoplasmic "mRNA decay-promoting domain" in which KSRP accumulates to exert its role in undifferentiated C2C12 cells. A model summarizing our idea on the mechanism by which H19 can affect myogenin mRNA decay through KSRP regulation is presented in Fig. S5.

Induction of myogenic differentiation involves the activation of at least two distinct cell signaling pathways – AKT and MAPK p38 (22) – and we previously reported that KSRP phosphorylation by these kinases (in distinct protein domains) is instrumental to achieve some of the gene expression changes crucial to myogenic differentiation (13, 21). The AKT phosphorylation site is located in KH1, a domain that does not actively participate in KSRP interaction with labile mRNAs (19, 20). Our experiments have now revealed that KH1 is required for optimal KSRP-H19 binding. Thus, it is plausible that KH1 phosphorylation by AKT, abrogating the interaction with H19, limits the decay-promoting function of KSRP. Conversely, although we have demonstrated in the past that phosphorylation by p38 is able to limit KSRP interaction with myogenin and other labile myogenic transcripts (21), it does not impair KSRP interaction with H19. Based on our present and previous experimental evidences, we can speculate that dual phosphorylation of KSRP, is required to achieve the complete inhibition of KSRP-dependent mRNA decay promoting function.

Interestingly, HuR, an RBP that controls decay of labile mRNAs and also interacts with lncRNA similarly to KSRP (36, 38, 39), associates with KSRP to destabilize the mRNA encoding the cell cycle regulator Nucleophosmin in C2C12 cells (40). Considering that HuR also has been reported to associate with H19 (41), it will be interesting to investigate in the future whether H19, KSRP and HuR collaborate during C2C12 differentiation..

While our studies were in progress, Huang and coworkers reported that H19 functions as a ceRNA for let-7 miRNA to control muscle differentiation and that H19 silencing induces myogenic differentiation in a let-7-dependent way (42). Conflicting results were published by Dutta and coworkers that showed a pro-myogenic function of H19 attributable to miR-675-5p and -3p (processed from H19 exon 1) whose expression is induced by culturing C2C12 cells in DM (25). Our data are in agreement with the anti-myogenic function of H19 described by Huang and coworkers (42) but point to a different molecular mechanism independent of let-7 modulation and dependent on H19 ability to

favor the mRNA decay-promoting function of KSRP in undifferentiated C2C12 cells. Further, our experiments cannot exclude that, in the course of myogenic differentiation, miR-675-5p and miR-675-3p might exert a pro-myogenic function as proposed in Ref. 25.

The analysis of the noncoding transcriptome has suggested that the expression of lncRNAs is more cell type restricted than the expression of protein-coding genes (43). This, together with our evidence that KSRP associates with distinct lncRNAs in other cell types (Fig. S4*G*), suggest the possibility that the role played by H19 in C2C12 cells to modulate KSRP function might be operated by different lncRNAs in other cell types.

Our work suggests a cell-specific role for select lncRNAs in amplifying and consolidating the function of RBPs ultimately controlling cell fate and differentiation programs.

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MATERIALS AND METHODS

HITS-CLIP experiments.

To perform HITS-CLIP experiments we adopted the procedure utilized by Gene Yeo and coworkers (44) with the following modifications. Sub-confluent C2C12 cells (16 X 100 mm dishes per experimental point were UV irradiated (120 mJ/cm^2 254 nm) at 4ºC. Crosslinked lysates were treated with RNase I (Ambion). Immunoprecipitations were carried out using either the anti-KSRP polyclonal antibody (9) or the correspondent pre-immune serum. A detailed protocol and a list of linkers and primers used will be provided upon request.

Cell cultures and transfections.

Murine mesenchymal C2C12 cells (obtained from ATCC, no. CRL-1772, Lot no. 58236521) were cultured in DMEM plus 20% FBS (Growth Medium, GM). Myogenic differentiation of C2C12 cells was induced by incubation in DMEM plus 2% horse serum (Differentiation Medium, DM). Osteoblastic differentiation of C2C12 cells was induced by the addition of 300 ng/ml of recombinant BMP2 (R&D Systems). HEK-293 cells (from ATCC) were cultured in DMEM plus 10% FBS.

C2C12 cells were transfected using the Nucleofector II (Amaxa), according to manufacturer's instructions and, to generate stable transfectants, Puromycin (Invivogen) was used at 1.5 µg/ml for selection. Pools of transfected cells were used for experiments. HEK-293 cells were transfected using Attractene transfection Reagent (Qiagen). MEFs were prepared from either WT and *Ksrp*-/- mice as described previously (45).

Plasmids, Recombinant proteins, and Antibodies.

To generate pTAG2B-H19 expression vector, a fragment of 2166 nucleotides (nt) from murine H19 obtained by RT-qPCR was cloned in the EcoRI/SalI sites of pCMV-TAG2B plasmid (Agilent). To generate pTAG2B-myog 3'UTR expression vector, a fragment of 595 nt from murine Myogenin 3' UTR obtained by RT-qPCR was cloned in the SalI/XhoI sites of pCMV-TAG2B plasmid. To generate pTAG2B-E3 expression vector, a fragment of 122 nt of the murine E3 region from Pitx2 locus (22) obtained by RT-qPCR was cloned in the SalI/XhoI sites of pCMV-TAG2B plasmid. To generate the pTAG2B-MS2-12X vector, a fragment encompassing 12 MS2 binding sites was excised from the

pSLMS2-12X (Addgene) and cloned in the EcoRI/EcoRV sites of pCMV-TAG2B. To generate the pTAG2B-MS2-12XH19 expression plasmid, a fragment of 1695 nt from murine H19 was obtained by RT-qPCR and cloned in the EcoRV/XhoI sites of the pTAG2B-MS2-12X plasmid. All plasmids were sequenced prior to their utilization. pcDNA3-Flag-KSRP, pCMV-TAG2B-KSRP, pCMV-TAG2B-KSRP(S193D), pCMV-TAG2B-KH1GDDG, pCMV-TAG2B-KH2GDDG, pCMV-TAG2B-KH3GDDG, pCMV-TAG2B-KH4GDDG, pcDNA-3-myrAKT2, pcDNA3-MKK6EE, pcDNA3-EXOSC2, pcDNA3-EXOSC5 plasmids were described elsewhere (13, 14, 21, 28, 46). Adenoviral vector expressing myristoylated AKT2 and the respective negative control were from Vector Biolabs.

GST-KSRP and GST-KSRP(S193A) were previously described (14). Affinity-purified rabbit polyclonal anti-KSRP antibody and the corresponding pre-immune serum were previously described (9); anti-phospho-AKT and anti-phospho-p38 were from Cell Signaling Technology, M2 anti-Flag antibody was from Sigma.

Gene silencing.

siRNAs utilized to knock-down murine H19 were from Ambion (38). GAPmeRs utilized to to knock-down murine H19 expression were from Exiqon (see Table S2 for sequences). KSRP silencing was obtained as previously described (13). Anti-miR-675-5p, anti-miR-675-3p, mature miR-675-5p, and mature miR-675-3p were from Qiagen.

Bioinformatic Analysis, Quantitative RT-PCR, Pri-miRNA in vitro processing assays, RNA in vitro degradation, Ribonucleoprotein complexes immunoprecipitation (RIP) assays, nuclear and cytoplasmic extracts preparation, MS2 precipitation, Electrophoretic Mobility Shift Assays are described in detail under Supporting Information online.

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FIGURE LEGENDS

Figure 1. KSRP directly interacts with H19 lncRNA in the cytoplasm of proliferating C2C12 cells.

(A) C2C12 cells were cultured in GM, DM, or DM supplemented with 300 ng/ml BMP2 for 24 hours, UV-crosslinked, lysated and subjected to HITS-CLIP analysis. Immunoprecipitations were performed using either anti-KSRP polyclonal antibody or the respective preimmune serum (PI). The results of a representative experiment (of three distinct performed) are expressed as read count mapped to mouse H19 lncRNA (on a logarithmic scale). (B) C2C12 cells were cultured in GM, DM, or DM plus 300 ng/ml BMP2 for 24 hours. Total cell extracts were immunoprecipitated as indicated. RNA was purified from immunocomplexes and analyzed by RT-qPCR to detect H19. (C) Alignment of the murine H19 sequence corresponding to the KSRP binding site (as revealed by HITS-CLIP analysis) with sequences belonging to different mammals. (D) Murine embryonic fibroblasts (MEFs) were prepared from E14.5 either WT or *Ksrp* -/- embryos, total extracts were prepared and immunoprecipitated as indicated. RNA was purified from immunocomplexes and analyzed by RT-qPCR to detect H19. (E) RNA was prepared from either nuclear or cytoplasmic fractions of C2C12 cells cultured in GM and H19 levels were quantitated by RT-qPCR (left panel). Either cytoplasmic or nuclear extracts from C2C12 cells cultured in GM were immunoprecipitated as indicated. RNA was purified from immunocomplexes and analyzed by RT-qPCR to detect lncRNA H19 using primers spanning the exon 1-exon 2 junction (right panel).

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

Figure 2. KSRP-H19 interaction is abrogated by AKT signaling activation.

(A) C2C12 cells cultured in GM were treated with either a negative control adenoviral vector (AdNull) or with a constitutively active myristoylated AKT2-expressing adenoviral vector (AdAKT2) for 48 hours. Total cell extracts were immunoprecipitated as indicated and RNA was purified from immunocomplexes and analyzed by RT-qPCR to detect H19. The values of RT-qPCR experiments shown are averages (±SEM) of three independent experiments performed in triplicate. Statistical significance: (*) $p<0.001$ (Student's t-test).

(B) Either wild-type or S193A mutant recombinant purified KSRP (increasing amounts from 30 to 300 nM) was preincubated in either AKT2 kinase assay buffer (KB) or in KB supplemented with active recombinant AKT2 (30 min at 30º C, +AKT2). The interaction between ^{32}P-labeled RNAs (as indicated) and KSRP was evaluated by EMSA. Representative autoradiograms are shown.

Figure 3. H19 silencing in undifferentiated C2C12 cells promotes myogenin mRNA stabilization and maturation of myomiRs from precursors.

H19 was transiently silenced in C2C12 cells cultured in GM using a combination of a sequence-specific siRNA and a GAPmeR (collectively indicated as Sil H19). Parallel cultures were mock-silenced using a combination of a control siRNA and a control GAPmeR (collectively indicated as Sil C). Cells were used for experiments 48 h after transfection. (A) Cells were treated with 100 μM DRB, total RNA was isolated at different times (as indicated) after the addition of DRB, and analyzed by RT-qPCR to detect myogenin mRNA expression. (B) RNA was prepared from transfected C2C12 cells and myogenin levels were quantitated by RT-qPCR. (C) Total cell extracts were immunoprecipitated as indicated. RNA was purified from immunocomplexes and analyzed by RT-qPCR to detect either myogenin or primary miRNAs. (D) RNA was prepared from transfected C2C12 cells and the levels of the indicated miRNAs and primary miRNAs were quantitated by RT-qPCR. (E) *In vitro* pri-miR-206 processing assays performed using total extracts from C2C12 cells either mock- (Sil C) or H19- (Sil

H19) silenced and cultured in either GM or DM (for 36 hours). Internally ^{32}P-labeled pri-miR-206 RNA substrate was added and its processing monitored as described under Experimental Procedures. A representative autoradiogram is shown. The intensity of background bands is ascribable to the long exposure of gels due to the low processing efficiency of pri-miR-206. The arrow points to pre-miR-206 band that is visible only in experiments performed using extracts from cells treated with either DM/Sil C or GM/Sil H19. The bar graph below the autoradiogram is a quantitation of the pre-miR-206 levels measured in two distinct processing assays. The intensity of the bands corresponding to pri–miR-206 and pre-miR-206, quantified with ImageJ software (http://rsb.info.nih.gov/ij/index.html), was expressed as percentage (±SEM calculated on two experiments) of pre-miR-206 generated from pri-miR-206 at each time point. To avoid signal saturation, the quantitation was performed on underexposed autoradiograms.

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

Figure 4. The interaction with H19 favors the decay promoting function of KSRP.

(A) HEK-293 cells were transiently co-transfected with pTAG2B-myog 3'UTR together with either empty vector (mock control cells), or pTAG2B-E3 (E3) plus pCDNA3-Flag-KSRP (flag-KSRP), or pTAG2B-H19 (H19) plus pCDNA3-Flag-KSRP (flag-KSRP). Total cell extracts were prepared 48 h after transfection and immunoprecipitated with anti-Flag antibody. RNA was purified from immunocomplexes and analyzed by RT-qPCR to detect either transfected murine myogenin 3'UTR (m.Myog 3'UTR) or endogenous GNAS. (B) HEK-293 cells were transiently transfected with GST-fused MS2 binding protein (GST-MS2BP) together with pTAG2B-myog 3'UTR and either pTAG2B-MS2-12XH19 (in which murine H19 was tagged with MS2 RNA hairpins, MS2-H19) or pTAG2B-MS2-12X (empty vector, MS2). Total cell extracts were prepared 48 h after transfection and precipitated by Glutathione-Sepharose beads. RNA was purified and analyzed by RT-qPCR to detect either transfected murine myogenin 3'UTR (m.Myog 3'UTR) or endogenous GNAS. (C) HEK-293 cells were transiently co-transfected with either pCMV-TAG2B-KSRP (expressing Flag-tagged wild-type KSRP, WT KSRP) or pCMV-TAG2B expressing the indicated Flag-tagged KSRP mutants (KH1GDDG, KH2GDDG,

KH3GDDG, KH4GDDG) together with pTAG2B-myog 3'UTR and pTAG2B-H19. Total cell extracts were prepared 48 h after transfection and immunoprecipitated by anti-Flag antibody. RNA was purified from immunocomplexes and analyzed by RT-qPCR to detect either myogenin 3'UTR (m.Myog 3'UTR) or transfected murine H19 (m.H19). (D) *In vitro* RNA degradation assays using S100 extracts from shKSRP C2C12 cells preincubated (for 90 min at 4ºC) with anti-Flag immunoprecipitates from HEK-293 cells transiently transfected with either Flag-tagged wild-type KSRP (KSRP) or Flag-tagged KH1GDDG mutant (KSRP[KH1GDDG]) together with either the E3 sequence or murine H19 cloned in expression vectors. Internally ^{32}P-labeled and capped RNA substrates were incubated with the above reaction mixtures and their decay monitored for the indicated times. RNA was analyzed by denaturing polyacrylamide gel electrophoresis followed by autoradiography. E3 is a stable transcript used to detect background decay. Representative autoradiograms are displayed. (E) HEK-293 cells were transiently co-transfected with pTAG2B-myog 3'UTR together with either empty vector (mock control cells), or pTAG2B-E3 (E3) plus pCDNA3-Flag-EXOSC2 and pCDNA3-Flag-EXOSC5, or pTAG2B-H19 (H19) plus pCDNA3-Flag-EXOSC2 and pCDNA3-Flag-EXOSC5. Total cell extracts were prepared 48 h after transfection and immunoprecipitated with anti-Flag antibody. RNA was purified from immunocomplexes and analyzed by RT-qPCR to detect either transfected murine myogenin 3'UTR (m.Myog 3'UTR) or endogenous GNAS.

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