Supporting Information

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SI Materials and Methods

RNA-Sequencing Library Construction and Deep Sequencing. Upon ligation of both 5′ and 3′ of Solexa oligo adapters (DSFP5, 5′A-ATGATACGGCGACCACCGACTATGGATACTTAGTCAGGG-AGGACGATGCGG-3′; and DSFP3, 5′-CAAGCAGAAGA-CGGCATACGACCGCTGGAAGTGACTGACAC-3′, respectively), the cDNA library was enriched by 20 cycles of PCR, gel-purified (1), and used for cluster generation on Illumina's Cluster Station and then sequenced on Illumina HiSeq 2000 following vendor's instruction. Typically, a paired-end sequencing run with 101 nt read length for each read was performed for RNA Sequencing. Raw sequencing intensities were extracted and the bases were called by using Illumina's RTA software, followed by sequence quality filtering. The extracted sequencing reads were saved as a pair of FASTQ files for the first and second read, respectively.

Bioinformatic Analysis. Illumina reads (FASTQ, 36 bp single read, Phred33) were filtered according to Illumina's Chastity flag. Reads were eventually pruned with FASTQ_quality_filter (FASTX Toolkit 0.0.13.2) to include sequences with a quality score greater than 20 in more than 90% of read length. Adapters sequences were trimmed by using Flexible Adapter Remover, version 2.3 (2) with options "-end any," which cuts the adapter and leaves the longer part of the read. Sequences with more than one undetermined nucleotide were discarded. Processed reads were aligned to reference mouse mm10 (National Center for Biotechnology Information GRCm38) with BWA ALN (3) (bwa, 0.6.2-r126). Aligned tags were annotated with Ensembl GRCm38.69 *Mus musculus* gene notation. Multiple alignment of mammalian H19 sequences was conducted by using the ClustalW2 package (ebi.ac.uk).

RT-qPCR. Total RNA was isolated by using the miRNeasy mini kit (Qiagen) and retro-transcribed (50 ng) by using an miScript reverse transcription kit (Qiagen) according to the manufacturer's instructions or Transcriptor Reverse Transcriptase (Roche). Quantitative PCR was performed by using the miScript SYBR green PCR kit (Qiagen) or the RealMasterMix (5 PRIME) and the Realplex II Mastercycler (Eppendorf). The sequence-specific primers used for PCR reactions are listed in Table S2.

pri-miRNA in Vitro Processing Assays. Total cell extracts from C2C12 cells were prepared in 50 mM Tris·HCl, pH 8.0, 150 mM NaCl, 0.5% Triton X-100, 1× Complete Protease Inhibitors, 1× PhosSTOP Phosphatase Inhibitor Mixture (both from Roche), 10% (wt/vol) glycerol, and incubated (typically 40 μg per 25-μL reaction at 37 °C for the indicated times) with in vitro synthesized and uniformly labeled pri-miRNA (5 fmol) in processing buffer containing 100 mM KCH_3COOH, 2 mM $Mg(CH_3COOH)_2$, 10 mM Tris·Cl (pH 7.6), 2 mM DTT, 10 mM creatine phosphate, 1 μg creatine phosphokinase, 1 mM ATP, 0.4 mM GTP, 0.1 mM spermine, and 2 U Stop RNase Inhibitor (5 PRIME).

RNA in Vitro Degradation. Phosphorus 32-labeled RNAs were synthesized and used as substrates for in vitro degradation assays as reported (4). In some experiments immunocomplexes precipitated using anti-Flag antibody were preincubated with cell extracts at 4 °C for 90 min under continuous agitation (Thermomixer; Eppendorf).

RIP Assays. RIP assays were performed as previously described (5) with some modification. Briefly, cell lysates were immunoprecipitated with Dynabeads (Invitrogen) 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 Protease Inhibitors (Roche). Total RNA was prepared from immunocomplexes using the miRNeasy mini kit (Qiagen), retro-transcribed, and amplified by qPCR as described earlier. The primer sequences are detailed in Table S2.

Total, Nuclear, and Cytoplasmic Extract Preparation. Total cell extracts were prepared as detailed in a previous report (6), and nuclear and cytoplasmic (S100) extracts were prepared as previously described (4) in the presence of protease and phosphatase inhibitors.

MS2 Precipitation Assays. Glutathione–Sepharose beads (GE Healthcare) were washed four times with RNA binding buffer (RBB) (4), saturated with BSA, and washed four more times with RBB including a mixture of protein inhibitors (Complete; Roche) and RNase inhibitor (5 PRIME). Total lysates (200 μg) from HEK-293 cells transfected with GST-MS2BP plasmid (gift from Myriam Gorospe, National Institute on Aging, Bethesda, MD) together with pTAG2B-myog 3′UTR and pTAG2B-MS2-12X or pTAG2B-MS2-12XH19 were incubated with glutathione–Sepharose beads for 3 h at 4 °C under rotation. Beads were collected by centrifugation (30 s at 5,000 × *g*) and washed four times with complete RBB. RNA was extracted from beads by using Qiazol reagent (Qiagen).

EMSA. EMSAs were performed as detailed in ref. 6.

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3. Li H, Durbin R (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* 25(14):1754–1760.
4. Chen CY, et al. (2000) Nucleolin and YB-1 are required for JNK-mediated interleukin-2 mRNA stabilization during T-cell activation. *Genes Dev* 14(10):1236–1248.
5. Lin YY, et al. (2014) KSRP and MicroRNA 145 are negative regulators of lipolysis in white adipose tissue. *Mol Cell Biol* 34(12):2339–2349.
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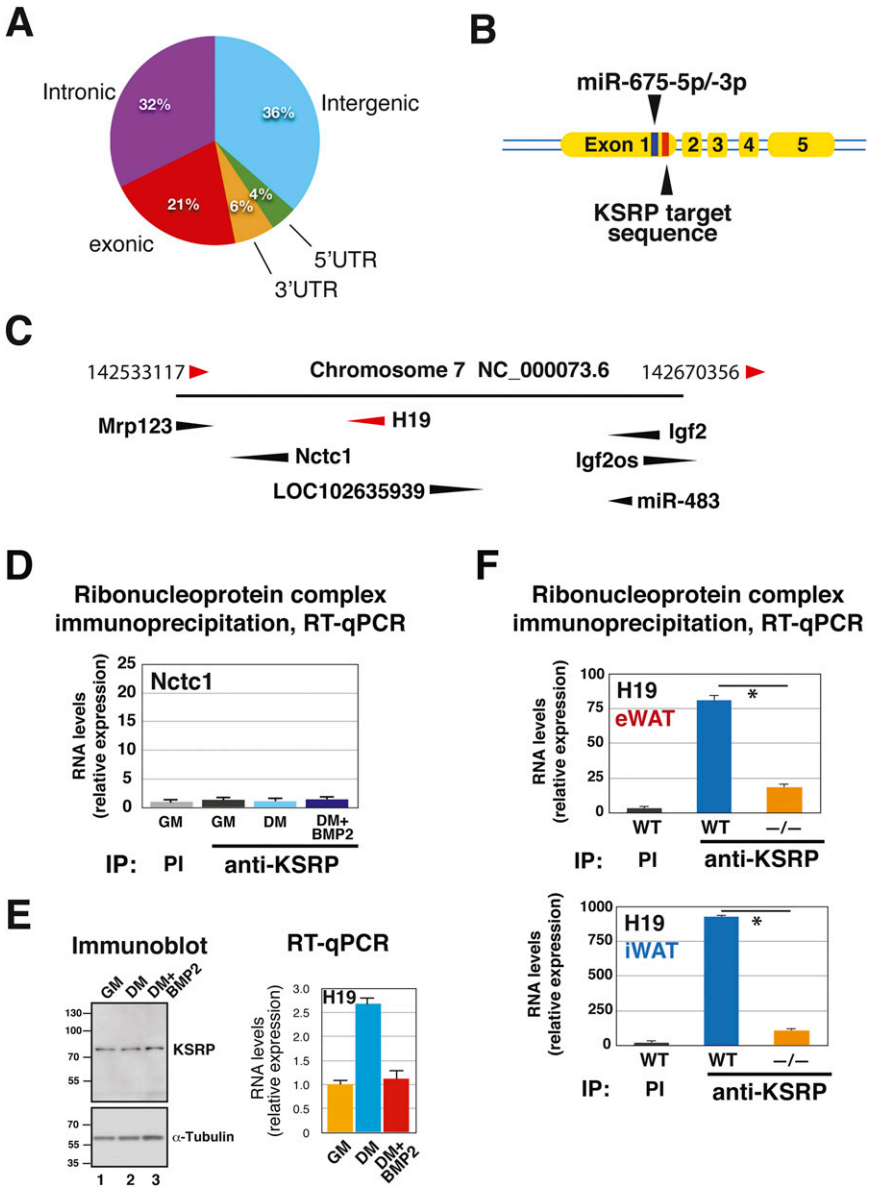

Fig. S1. H19 lncRNA interacts with KSRP. (*A*) Pie chart representing the percentage of KSRP HITS-CLIP read count mapped to distinct regions of mouse transcriptome. (*B*) Exon–intron arrangement of murine H19 lncRNA. The KSRP-bound region is indicated in red, and the miR-675 precursor region is indicated in blue, as indicated by arrows. (*C*) The gene encoding H19 maps to chromosome 7 in mouse. Neighboring transcriptional units are indicated by arrowheads. (*D*) C2C12 cells were cultured in GM, DM, or DM plus 300 ng/mL BMP2 for 24 h. Total cell extracts were immunoprecipitated as indicated. RNA was purified from immunocomplexes and analyzed by RT-qPCR to detect Nctc1 lncRNA. Analysis of KSRP protein (*E*, *Left*) and H19 (*E*, *Right*) expression levels in C2C12 cells cultured in GM, DM, or DM supplemented with 300 ng/mL BMP2 for 24 h. KSRP (as well as control α-tubulin) was quantified by immunoblot, and H19 was quantified by RT-qPCR analysis. (*F*) Total extracts were prepared from epididymal white adipose tissue (eWAT) or inguinal adipose tissue (iWAT) of 1-mo-old WT or *Ksrp*$^{-/-}$ mice and immunoprecipitated as indicated. RNA was purified from immunocomplexes and analyzed by RT-qPCR to detect H19 lncRNA. The values of RT-qPCR experiments shown are averages (±SEM) of three independent experiments performed in triplicate (*$P < 0.001$).

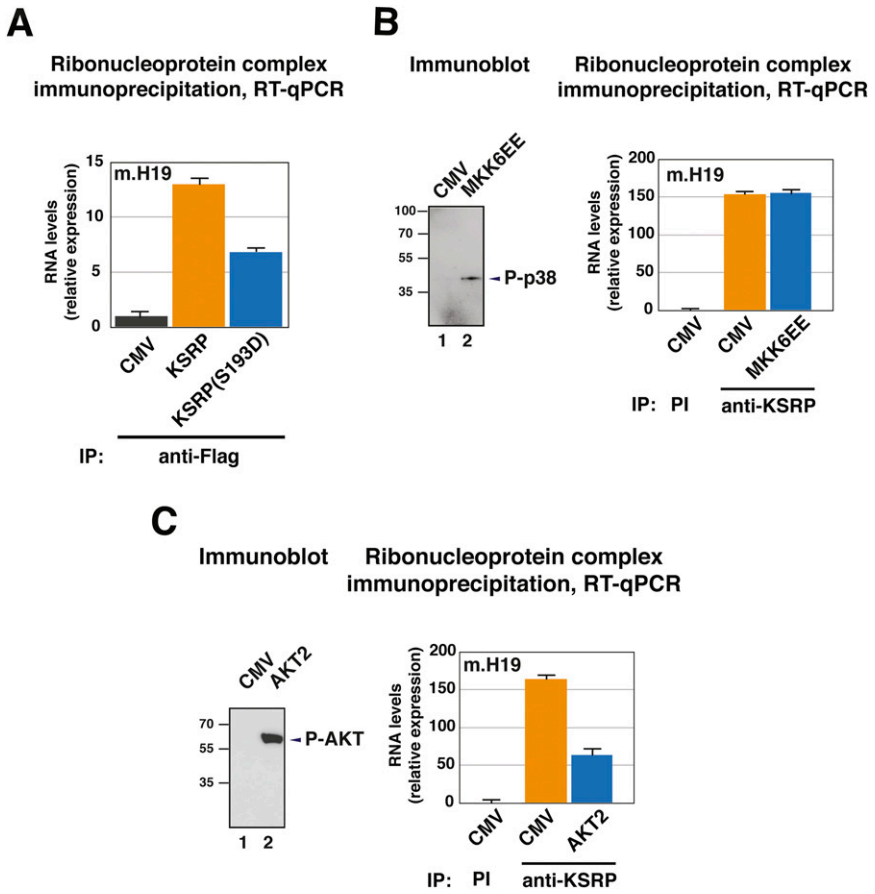

Fig. S2. Phosphorylation by AKT impairs KSRP's ability to interact with H19 lncRNA. (*A*) WT KSRP or an aspartic acid mutant [KSRP(S193D)] were expressed in HEK-293 cells together with a vector expressing murine H19. Total cell extracts were prepared 48 h after transfection and immunoprecipitated with anti-Flag antibody. RNA was purified from immunocomplexes and analyzed by RT-qPCR to detect transfected murine H19 (m.H19). (*B*) HEK-293 cells were transfected with control empty pCDNA vector (CMV) or with pCDNA3 vector expressing a constitutively active form of murine MKK6 (MKK6EE) together with a vector expressing murine H19. Total cell extracts were prepared 48 h after transfection and subjected to immunoblot analysis to detect phospho-p38 (P-p38; *Left*) or immunoprecipitated with anti-KSRP antibody (*Right*). RNA was purified from immunocomplexes and analyzed by RT-qPCR to detect transfected murine H19 (m.H19). (*C*) HEK-293 cells were transfected with control empty pCDNA vector (CMV) or with pCDNA3 vector expressing a constitutively active myristoylated form of murine AKT2 (AKT2) together with a vector expressing murine H19. Total cell extracts were prepared 48 h after transfection and subjected to immunoblot analysis to detect phospho-AKT (P-AKT; *Left*) or immunoprecipitated with anti-KSRP antibody (*Right*). RNA was purified from immunocomplexes and analyzed by RT-qPCR to detect transfected murine H19 (m.H19). The values of RT-qPCR experiments shown are averages (±SEM) of three independent experiments performed in triplicate (*$P < 0.01$).

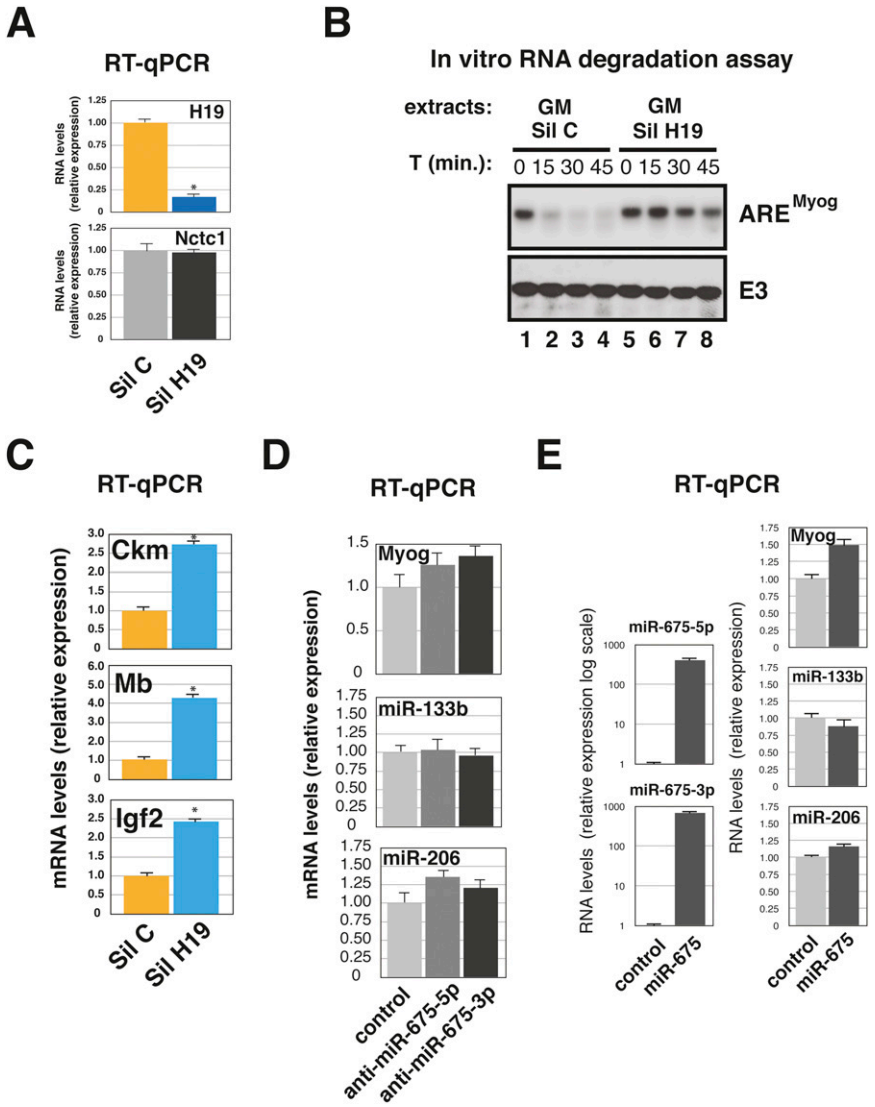

Fig. S3. Silencing of the H19 lncRNA leads to myogenin mRNA stabilization and to KSRP-dependent induction of myogenic miRNAs. (*A*–*C*) H19 was transiently silenced in C2C12 cells cultured in GM by using a combination of a sequence-specific siRNA and GAPmeR (collectively indicated as Sil H19). Parallel cultures were mock-silenced by using a combination of control siRNA and control GAPmeR (collectively indicated as Sil C). Cells were used for experiments 48 h after transfection. (*A*) RNA was prepared from transfected C2C12 cells and H19 or Nctc1 lncRNA levels were quantified by RT-qPCR. (*B*) In vitro RNA degradation assays performed by using the indicated cell extracts. Internally ^{32}P-labeled and capped RNA substrates were incubated with the extracts and their decay monitored for the indicated times. RNA was analyzed by denaturing polyacrylamide gel electrophoresis followed by autoradiography. E3 is a stable unrelated transcript. Representative autoradiograms are displayed. (*C*) RNA was prepared from transfected C2C12 cells cultured in GM as described in *A* and the levels of the indicated mRNAs were quantified by RT-qPCR. (*D*) C2C12 cells cultured in GM were transfected with control antagomiR or miRNA-specific antagonists (anti-miR-675–5p, anti-miR-675–3p). RNA was prepared 48 h after transfection and analyzed to detect the expression of myogenin, miR133b, and miR-206. (*E*) C2C12 cells cultured in GM were transfected with control RNA or a mixture of miRNA mimics corresponding to mature miR-675–5p and miR-675–3p. RNA was prepared 48 h after transfection and analyzed to detect the expression of miR-675–5p or miR-675–3p (*Left*), myogenin, miR133b, and miR-206 (*Right*). The values of RT-qPCR experiments shown are averages (±SEM) of three independent experiments performed in triplicate (*$P < 0.001$).

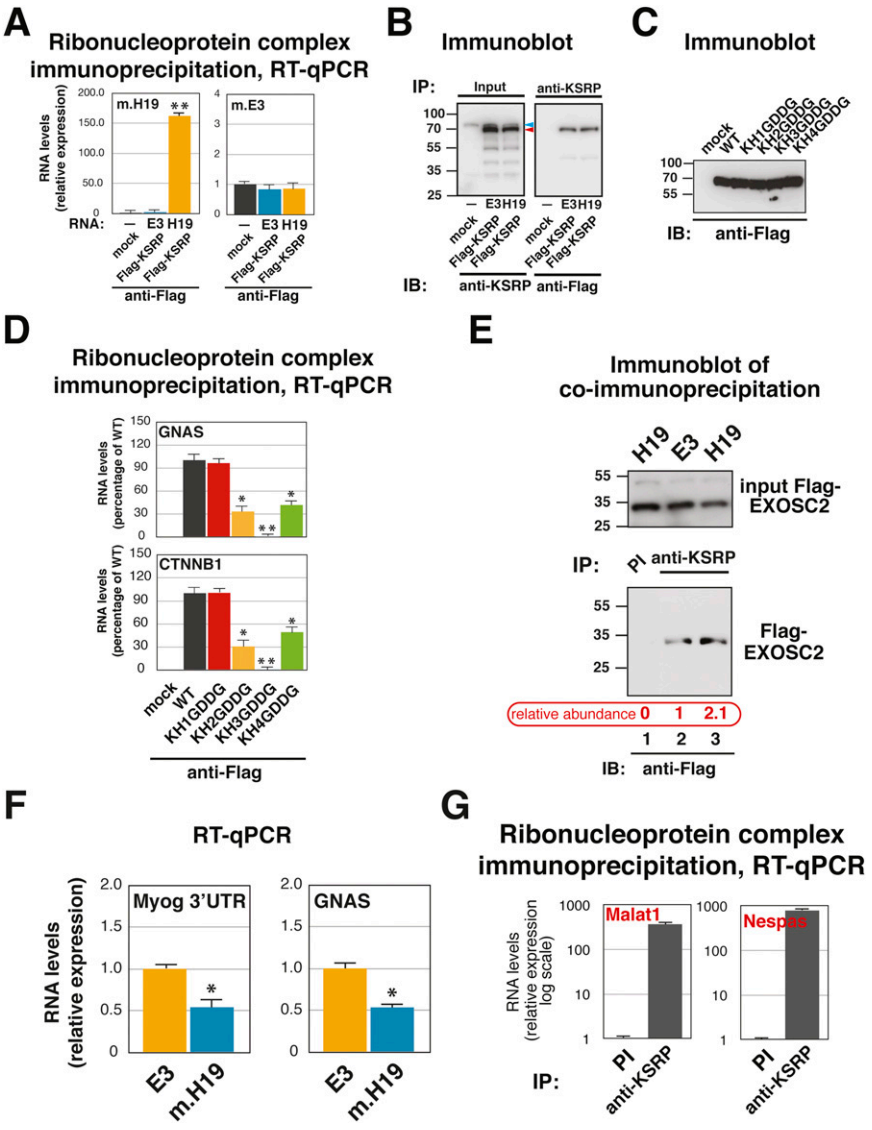

Fig. S4. The interaction with H19 favors the decay-promoting function of KSRP. (*A* and *B*) HEK-293 cells were transiently cotransfected with pTAG2B-myog 3′UTR together with (*i*) empty vector (mock control cells), (*ii*) pTAG2B-E3 (E3) plus pCDNA3-Flag-KSRP (Flag-KSRP), or (*iii*) pTAG2B-H19 (H19) 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 transfected murine H19 (m.H19) or transfected murine E3 sequence (m.E3; *A*). Total extracts were also immunoprecipitated with anti-KSRP antibody and analyzed by immunoblot to detect Flag-tagged KSRP expression levels (*B*, *Left*). Input cell extracts were analyzed by immunoblot using anti-KSRP antibody to detect endogenous (blue arrowhead) and transfected (red arrowhead) KSRP (*B*, *Right*). (*C*) HEK-293 cells were transiently cotransfected with pCMV-TAG2B-KSRP (expressing Flag-tagged 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 analyzed by immunoblotting using anti-Flag antibody. (*D*) HEK-293 cells were transiently cotransfected as described in *C*. 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 GNAS or CTNNB1 (also known as β-catenin) endogenous transcripts. (*E*) HEK-293 cells were transiently cotransfected with pCDNA3-flag-EXOSC2 together with pTAG2B-H19 (H19; lanes 1 and 3) or pTAG2B-E3 (E3; lane 2). Total cell extracts were prepared 48 h after transfection and immunoprecipitated with anti-KSRP antibody. The presence of transfected EXOSC2 in the immunocomplexes was detected by anti-Flag immunoblot (*Lower*). An aliquot of extracts was directly analyzed by SDS/PAGE and anti-Flag immunoblot to visualize inputs (*Upper*). The relative abundance of Flag-EXOSC2 in the anti-KSRP immunoprecipitates is presented as the average of three experiments. (*F*) HEK-293 cells were transiently cotransfected with pTAG2B-myog 3′UTR together with pTAG2B-E3 (E3) or pTAG2B-H19 (H19). RNA was prepared 48 h after transfection and analyzed to detect the expression of transfected murine myogenin 3′UTR (Myog 3′UTR) or endogenous GNAS. (*G*) Total cell extracts were prepared from HEK-293 cells and immunoprecipitated with anti-KSRP antibody. RNA was purified from immunocomplexes and analyzed by RT-qPCR to detect Malat1 or Nespas lncRNAs. The values of RT-qPCR experiments shown are averages (±SEM) of three independent experiments performed in triplicate (*$P < 0.01$ and **$P < 0.001$, Student *t* test).

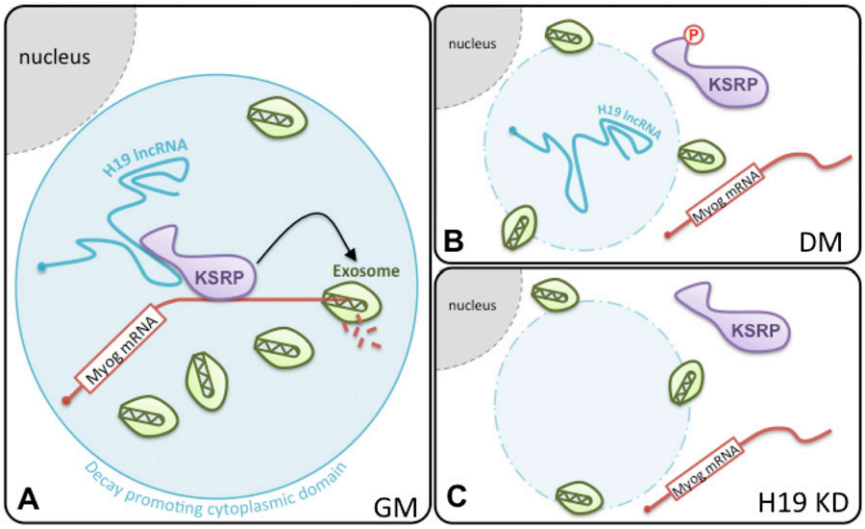

Fig. S5. Schematic model summarizing the role of H19 in modulating KSRP-dependent myogenin mRNA decay. (*A*) In C2C12 cells cultured in GM, KSRP associates with H19 and myogenin mRNA and, consequently, myogenin and KSRP are recruited to a cytoplasmic domain (represented by the light blue circle) that favors RNA exosome-mediated decay of myogenin. Upon switching to DM (*B*), KSRP is phosphorylated by AKT in Ser-193, and this results in its dissociation from H19 and release of KSRP and myogenin mRNA from the putative cytoplasmic decay domain. We suggest that H19 knock-down (KD; *C*) may similarly result in dissolution of the decay-promoting cytoplasmic domain, thus impairing the ability of KSRP to recruit the RNA exosome to myogenin mRNA.

Table S1. Primers used for gene silencing

Name	Sequence
Neg GapmeR Control	5′–TCATACTATATGACAG–3′
GapmeR mmu.H19	5′–CTAAGTGAATTACGGT–3′
siRNA mmu.H19	5′–CAUUCAUCCCGGUUACUUUTT–3′

Table S2. Primers used for qPCR reactions

Transcript	Forward primer	Reverse primer
mmu.miR-206	5′–TGGAATGTAAGGAAGTGTGTGG–3′	–
mmu.miR-133b	5′–TTTGGTCCCCTTCAACCAGCTA–3′	–
mmu.let-7	5′–TGAGGTAGTAGGTTGTATAGTT–3′	–
mmu.sno234	5′–TTCGTCACTACCACTGAGA–3′	–
mmu.pri-miR-206	5′–ACCCAGTGCCCTGTGTTCCCA–3′	5′–AGCGCCTCTTCTCGGTTTCCCT–3′
mmu.pri-miR-133b	5′–AGCAAGCCATATTTTCGGGGAGCC–3′	5′–AGGGGTATCCTCTGCTTTCGGCTT–3′
mmu.Myog	5′–ACTCCCCATTCACATAAGGCTA–3′	5′–TGGACTCCATCTTTCTCTCCTC–3′
mmu.Ckm	5′–AGAAGAAGCTGGAAAAGGGC–3′	5′–CAGAACCCAGAGGACAGAGC–3′
mmu.beta2-microglobulin	5′–AGTTAAGCATGCCAGTATGGCC–3′	5′–TTCTTTCTGCGTGCATAAATTGTAT–3′
mmu.Hprt	5′–CCTCACTGCTTTCCGGAGCGG–3′	5′–GGACTGCGGGTCGGCATGA–3′
Mmu.Myog.3′UTR	5′–GCCCCCTTGTTAATGTCCCT–3′	5′–GAGGGTGGAATTCGAGGCAT–3′
mmu.Nctc1	5′–CAGCAGGTAAAGCCACCGTA–3′	5′–TTACGTGTGAAGGGGTGTGG–3′
mmu.H19	5′–GCAGGTAGAGCGAGTAGCTG–3′	5′–TTCGAGACACCGATCACTGC–3′
mmu.H19ex1-2	5′–AAGAGCTCGGACTGGAGACT–3′	5′–GACCACACCTGTCATCCTCG–3′
mmu.E3	5′–CTGTCGGAGTGGGCAACTCTG–3′	5′–CTCTTTGAATTCAGTGGTTTC–3′
hsa.GNAS	5′–TCAGCGCATGCACCTTCGTCA–3′	5′–TCGGGGGAAGGGAAAGGTTGCT–3′
hsa.CTNNB1	5′–TTGGAACCTTGTTTTGGACA–3′	5′–ACCGCATTTTCTCTTGAAGC–3′
mmu.Mb	5′–GGGAAGGTGGAGGCCGAC–3′	5′–CCAGGGCTGTGAGCACGGTG–3′
Mmu.Igf2	5′–CCCGCCAAGTCCGAGAGGGA–3′	5′–TCCCGCGGACTGTCTCCAGG–3′
Hsa.Malat1	5′–TTTTAGCAACGCAGAAGCCC–3′	5′–GATAGCAGCACAACTCGTCG–3′
Has.Nespas	5′–ACCCTTGGGTGTGTTGATGG–3′	5′–GCAGTAAGCCCTCTCCTTGG–3′