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PI3K/AKT signaling determines a dynamic switch between distinct KSRP functions favoring skeletal myogenesis.

Running title: AKT-dependent and KSRP-regulated maturation of myomiRs.

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ABSTRACT

Skeletal myogenesis is orchestrated by distinct regulatory signaling pathways, including PI3K/AKT, that ultimately control muscle gene expression. Recently discovered myogenic micro-RNAs (miRNAs) are deeply implicated in muscle biology. Processing of miRNAs from their primary transcripts is emerging as a major step in the control of miRNA levels and might be well suited to be regulated by extracellular signals. Here we report that the RNA binding protein KSRP is required for the correct processing of primary myogenic miRNAs upon PI3K/AKT activation in myoblasts C2C12 and in the course of injury-induced muscle regeneration, as revealed by *Ksrp* knock-out mice analysis. PI3K/AKT activation regulates in opposite ways two distinct KSRP functions inhibiting its ability to promote decay of Myogenin mRNA and activating its ability to favor maturation of myogenic miRNAs. This dynamic regulatory switch eventually contributes to the activation of the myogenic program.

Keywords: myogenesis, mRNA degradation, microRNA maturation, RNA-binding protein.

INTRODUCTION

Myogenic differentiation is the end point of a cascade of events that remove myoblasts from the cell cycle and ultimately determine the characteristic pattern of skeletal muscle gene expression and the formation of multinucleated myotubes¹. Once differentiated, adult muscles retain the capacity to regenerate in response to exercise or injury by activation and proliferation of satellite cells^{2,3}. Skeletal myogenesis is orchestrated by distinct regulatory signaling pathways, including phosphatidylinositol-3-kinase (PI3K)/AKT, that ultimately change tissue-specific gene expression programs during muscle cell differentiation and tissue reconstruction after trauma⁴. In the recent few years extensive analysis of the composition of the transcriptome assembled on the chromatin of myogenic loci has been performed (reviewed in ref. 5) while knowledge on the mechanisms that post-transcriptionally regulate muscle differentiation and regeneration is more limited⁶⁻⁹.

Recently, genetic and biological evidence of an essential role of microRNAs (miRNAs) in muscle development and function has been accumulating and a group of miRNAs, highly enriched in skeletal muscle (referred to as myomiRs), has been identified (reviewed in refs. 10, 11). MyomiRs influence multiple aspects of muscle development and function through their regulation of key genes controlling myogenesis¹²⁻¹⁴. Deregulated expression of some myomiRs results in pathological conditions¹⁵⁻¹⁷.

miRNA encoding genes are transcribed as primary miRNA transcripts (pri-miRNAs) by RNA polymerase II. The mechanism by which pri-miRNAs are processed first to stem-loop structured miRNA precursors (pre-miRNAs) and then to mature miRNAs involves two sequential endonucleolytic cleavages operated by multiprotein complexes including the RNase III enzymes Drosha, in the nucleus, and Dicer, in the cytoplasm (reviewed in ref. 18). Mature miRNAs, in the context of the RNA Induced Silencing Complex (RISC), are able to silence gene expression by binding to partially complementary target sites predominantly located in the 3' untranslated region (3'UTR) of target messenger RNAs (mRNAs)¹⁸. This results either in the repression of translation or in the deadenylation and destabilization of the target mRNA (reviewed in ref. 19).

In the last few years, the relevance of post-transcriptional control of miRNA biogenesis has been discovered (ref. 20 for a recent review) and, notably, miRNA processing defects result in pathological conditions including cancer^{21,22}. Inactivation of Dicer in muscles results in perinatal lethality due to skeletal muscle hypoplasia thus highlighting the essential role of the miRNA processing machinery in muscle homeostasis²³. Recently, Paroo et al.²⁴, reported that MAPK Erk phosphorylates TRBP, a protein factor belonging to the core Dicer complex, and affects miRNA maturation. This observation points to the relevance of post-translational modifications of factors involved in miRNA biogenesis in the modulation of miRNA expression and function. However, regulation of a general molecular machine, such as the Dicer complex, does not explain how different signaling pathways can regulate maturation of different subsets of miRNAs in specialized cellular environments.

It has been recently shown that different regulatory proteins, acting as either activators or repressors of Drosha and Dicer machineries, influence the processing of select miRNAs (reviewed in ref. 25). Among them, the single-strand RNA binding protein KSRP (reviewed in ref. 26) interacts with the terminal loop of select miRNA precursors and promotes their maturation in cultured cells²⁷⁻²⁹. Importantly, KSRP has been shown to regulate gene expression also promoting the decay of inherently labile mRNAs in several cellular contexts including C2C12 myoblasts²⁶. KSRP distinct functions are regulated by different protein kinases, including MAPK p38, PI3K/AKT and ATM^{8,29,30}. An unresolved issue is whether a specific signaling pathway regulates only a single KSRP function (i.e. affecting either mRNA decay or miRNA maturation) or is able to induce a dynamic change between KSRP functions in order to produce relevant phenotypes in a specific cellular context.

Here we report that activation of the PI3K/AKT signaling is responsible for myomiR maturation during C2C12 myoblasts differentiation with KSRP being required for this function. Importantly, myomiR maturation in the course of injury-induced muscle regeneration is impaired in *Ksrp* knock-out mice. PI3K/AKT signaling activation which occurs during myogenesis determines a switch between two distinct KSRP functions, inhibiting its ability to promote the decay of Myogenin mRNA while activating its ability to favor maturation of myogenic miRNAs.

RESULTS

PI3K/AKT signaling induces myomiR maturation in C2C12 myoblasts.

In order to assess the contribution of the PI3K/AKT signaling pathway to myomiR induction during differentiation of C2C12 myoblasts, we continuously exposed cells to PI3K inhibitor LY294002 (LY) throughout the transition from myoblasts to myotubes induced by differentiation medium (DM). LY was able to impair DM-induced expression of myogenic differentiation markers (Supplementary Figure S1a, and ref. 31) and to strongly inhibit the expression of miR-1a, miR-133b, and miR-206 (members of the myomiR family^{10,11}) induced by DM (Figure 1a). Importantly, the comparative analysis of primary and mature miRNAs revealed that LY specifically impairs myomiR maturation (Figure 1a, and b). AKT kinases are the major downstream effectors of PI3K³². Since the relative contribution of the two AKT isoforms expressed in C2C12 cells (Akt1 and Akt2) to differentiation is still debated³³, we down-regulated either isoform prior to exposure to DM (Supplementary Figure S1b) and obtained inhibition of the expression of myogenic differentiation markers (Supplementary Figure S1c, and ref. 31). Silencing experiments demonstrated that both Akt1 and Akt2 are required for DM-induced maturation of miR-1a, miR-133b, and miR-206 (compare the expression of mature and primary miRNA in Figure 1c and Figure 1d). In agreement with a role of AKT in myomiR maturation, expression of a membrane-bound myristoylated and constitutively active form of Akt2 (myr-AKT2³⁰) strongly enhanced the expression of myomiRs in C2C12 cells cultured in DM without affecting the expression of the corresponding pri-miRNAs (Figure 1e, 1f).

PI3K/AKT could crosstalk with other signaling pathways activated during DM-induced myoblast differentiation and this could influence myomiR maturation. In order to minimize this potential interference, we activated PI3K/AKT signaling in exponentially growing cells (cultured in growth medium, GM). Indeed, the expression of myr-AKT2 in C2C12 cells cultured in GM (myr-AKT2/GM) induced the expression of differentiation markers (Supplementary Figure S2a). To obtain a comprehensive picture of the miRNome changes induced by myr-AKT2/GM in C2C12 myoblasts, we performed miRNA microarray analysis and found 37 miRNAs significantly regulated (Figure 2a). Since our aim was to investigate the contribution of PI3K/AKT activation in the context

of physiological C2C12 myoblast differentiation, we focused exclusively on miRNAs whose expression was similarly affected by myr-AKT2/GM and by DM (Figure 2a and Supplementary Figure S2b). miRNA array results were validated (by both quantitative RT-PCR [qPCR] analysis and RNase protection assays) with the exception of miR-21 whose expression was not affected by myr-AKT2/GM (Figure 2b, c). Importantly, AKT activation enhanced miRNA expression inducing their maturation without significantly affecting the corresponding primary miRNA levels with the only exception of miR-351 whose primary transcript levels were significantly up-regulated (Figure 2b).

Insulin and IGF-I are known as physiological activators of PI3K/AKT signaling in different cell types including C2C12 myoblasts³⁴. Treatment of serum-starved C2C12 cells with insulin (10^{-6} M) was able to induce muscle differentiation markers (Supplementary Figure 2c) and to enhance the expression of mature miR-1a, 133b, 206, 378, and 532-5p without increasing the levels of their primary transcripts (Figure 2d and Supplementary Figure 2d).

Connexin 43 is one of the few validated targets of miR-1a and miR-206^{13,35} and, consistent with the increased expression of these myomiRs, we observed a similar down-regulation of Connexin 43 protein levels in C2C12 myoblasts upon either PI3K/AKT signaling activation or culture in DM (Figure 2e).

Altogether these data demonstrate that either physiological or forced activation of PI3K/AKT signaling in C2C12 myoblasts induces the expression of a group of myogenic miRNAs activating their maturation.

KSRP is required for AKT-induced myomiR maturation.

We reported that KSRP is a target of PI3K/AKT signaling in different cell lines and that phosphorylation by AKT1 and 2 causes an impairment of its decay promoting function on a variety of inherently labile mRNAs²⁶. We investigated whether KSRP function is required for PI3K/AKT induced maturation of myomiRs in C2C12 myoblasts and whether KSRP phosphorylation by AKT affects its ability to promote miRNA precursor maturation.

We transiently down-regulated KSRP in myr-AKT2/GM C2C12 myoblasts (Supplementary Figure S3a). KSRP knock-down impaired the myr-AKT2-dependent

induction of differentiation markers (Supplementary Figure S3b) and the maturation of myogenic miRNAs (Figure 3a). We obtained similar results analyzing C2C12 transfectants in which the expression of KSRP was stably down-regulated by shRNA (Supplementary Figure S3c).

Using a primary miRNA in vitro processing assay²⁷ we showed that extracts from both myr-AKT2/GM- and DM-cultured C2C12 myoblasts were able to process pri-miR-206 into pre-miR-206 in vitro while extracts from C2C12 myoblasts cultured in GM were not (left panels in Supplementary Figure S3d, e). In order to investigate the possibility that phosphorylation by AKT directly affects the ability of KSRP to process pri-myomiRs to pre-myomiRs in vitro, either unphosphorylated or AKT2-phosphorylated recombinant KSRP³⁰ was added to extracts from C2C12 myoblasts cultured in GM. Unphosphorylated KSRP was unable to promote pri-miR-206 and pri-miR-1a-2 processing while in vitro phosphorylation by AKT2 activated the processing function of KSRP (Figure 3b and data not shown). S193A mutation, that abrogates AKT phosphorylation, impairs the ability of AKT2 to activate the pri-miR-206 processing activity of GST-KH1-4 (a protein that includes the 4 KH domains of KSRP, comprises the AKT phosphorylation site³⁰, and recapitulates KSRP activity to favor pri-miRNA processing) (Supplementary Figure S4).

These results suggest the existence of a PI3K/AKT > KSRP pathway that activates maturation of myomiRs in C2C12 myoblasts.

KSRP knock-out impairs myomiR maturation in the course of muscle regeneration.

We have recently generated *Ksrp* knock-out (*Ksrp*^{-/-}, Supplementary Figure S5a, b) mice that have been described elsewhere³⁶. Adult skeletal muscle regenerates in response to injuries with a process in which PI3K/AKT signaling activation plays an essential role (reviewed in ref. 37 and Supplementary Figure S1a). Since the regenerative process has been recently linked to induction of myomiR expression³⁸ we asked whether *Ksrp* knock-out affects miomyR processing in injury-dependent muscle regeneration.

In order to evaluate the expression of myomiRs during muscle regeneration, both *Ksrp*^{-/-} and their wild-type (WT) littermates were injected with cardiotoxin in the *tibialis anterioris* muscle and sacrificed at different times (Supplementary Figures S5c). The analysis of proliferation/differentiation markers in regenerating muscles revealed a stronger and more sustained induction of *Ccna2* (also known as Cyclin A2) in *Ksrp*^{-/-} in comparison to WT mice (Supplementary Figures S5d). Conversely, Myogenin induction at day 4 after injury was significantly impaired in *Ksrp*^{-/-} in comparison to WT mice (Supplementary Figure S5e). Hematoxylin-Eosin staining of cross-sections of non-injured (day 0) and injured muscles showed dishomogeneous and hypernucleated regenerating myofibers at day 7 after cardiotoxin injection in *Ksrp*^{-/-} mice (Supplementary Figure S5f).

As shown in Supplementary Figure S5g and in the left panel of Figure 4a, miR-206 expression transiently declined at day 2 post-injection in both WT and *Ksrp*^{-/-} mice and strongly increased starting from day 4 to day 14 in WT animals. miR-206 increase was significantly reduced in *Ksrp*^{-/-} mice when compared to WT animals (Figure 4a, left panel). miR-133b and miR-1a expression levels sharply decreased between days 2 and 7 while almost reached pre-injection levels at day 14 (Figure 4a, middle and right panels). A significant reduction of the expression of miR-133b and miR-1a was detectable at day 14 in *Ksrp*^{-/-} mice when compared to WT (Figure 4a, middle and right panels). Importantly, pri-miR-206 and pri-miR-133b induction, occurring at day 4 post-injury, was significantly higher in *Ksrp*^{-/-} than in WT mice suggesting an accumulation of unprocessed primary myomiRs (Figure 4b, left and middle panels). Also pri-miR-1a-2 levels, although with a different kinetic compared to pri-miR-206 and pri-miR-133b, were significantly higher at day 4 in *Ksrp*^{-/-} mice when compared to WT animals (Figure 4b, right panel).

Altogether, these data indicate that the induction of myomiRs occurring during muscle regeneration is impaired in *Ksrp*^{-/-} mice due to maturation blockade.

PI3K/AKT inhibits KSRP ability to promote decay of Myogenin mRNA while promotes its recruitment to myogenic miRNA processing complexes.

We and others have shown that stabilization of inherently labile muscle specific transcripts takes place during C2C12 cell differentiation⁶⁻⁸. Myogenin and p21 mRNAs

are unstable in C2C12 myoblasts cultured in GM and KSRP is required for their decay while MAPK p38-induced inactivation of KSRP (which occurs in differentiating myoblasts) contributes to Myogenin and p21 induction⁸. Figure 5a and Supplementary Figure S6a show that AKT increased the half life of Myogenin and p21 mRNAs when activated in C2C12 myoblasts cultured in GM. On the basis of this observation and the above data, we hypothesized that PI3K/AKT signaling activation reduces KSRP ability to promote mRNA decay while induces its ability to promote miRNA precursor maturation in the course of C2C12 myoblasts differentiation.

To address this hypothesis we first performed in vitro mRNA degradation assays supplementing extracts from C2C12 myoblasts cultured in DM with purified recombinant KSRP⁸. In vitro phosphorylation by AKT2 impairs the decay-promoting activity of KSRP on a Myogenin mRNA substrate (Figure 5b) thus indicating that, besides inducing a subcellular re-distribution of KSRP (Supplementary Figure S6b, and ref. 26), phosphorylation by AKT2 directly affects KSRP function on Myogenin mRNA decay.

This finding, together with the observation that phosphorylation by AKT2 enhances the ability of KSRP to promote pri-miR-206 processing (Figure 3b) prompted us to address the possibility that PI3K/AKT activation in C2C12 myoblasts displaces KSRP from myogenic mRNA destabilizing complexes and relocates it to primary myomiR processing complexes. Indeed, ribonucleoprotein immunoprecipitation (RIP) assays show that PI3K/AKT activation in C2C12 cells, as well as culture in DM, caused dissociation of KSRP from Myogenin mRNA while increased KSRP interaction with pri-miR-133b, pri-miR-206, and pri-miR-532-5p, (Figures 5c, d and data not shown). Finally, we investigated whether PI3K/AKT activation affects KSRP ability to interact with the enzymatic complexes responsible for either labile mRNA decay or pri-miRNA processing. Indeed, once phosphorylated by AKT2, KSRP was unable to interact with the Exosome complex (represented by EXOSC10 component, Figure 5e) while became capable to interact with the Drosha complex (Figure 5f). As a control for AKT2-dependent inhibition of KSRP interaction with the Exosome, we showed that the Exosome binding ability of a recombinant KSRP mutated in the critical Ser193³⁰ was unaffected by AKT2 phosphorylation (Figure 5e).

Altogether these data suggest that phosphorylation by AKT displaces KSRP from myogenic mRNA destabilizing complexes relocating it to primary myomiR processing complexes.

DISCUSSION

In this manuscript we report that PI3K/AKT signaling activation occurring at early phases of C2C12 myoblast differentiation induces the expression of myomiRs activating their processing from primary transcripts with KSRP playing a required role in this pathway. AKT regulates in opposite ways two distinct KSRP functions inhibiting its ability to promote decay of Myogenin mRNA and activating its ability to favor maturation of myomiRs from precursors. This dynamic regulatory switch eventually contributes to the activation of the myogenic program.

PI3K/AKT signaling has been reported to regulate the expression of some miRNAs in fibroblast and epithelial cells³⁹ while a role for the pathway in controlling miRNA maturation has been only postulated⁴⁰. Our data indicate that either physiological or forced activation of PI3K/AKT favors the processing of primary myomiRs leading to accumulation of their mature forms. We and Others have shown that KSRP promotes processing of a select group of miRNAs, including the let-7 family, in HeLa and other cell lines^{27,29,41}. KSRP silencing in C2C12 myoblasts down-regulates the expression of the above miRNAs (Supplementary Figure S6c) thus indicating that an overlap between miRNAs whose expression is affected by KSRP knock-down in HeLa and C2C12 cells does exist. However, the overlap is partial since some KSRP-regulated miRNAs expressed in C2C12 myoblasts are not expressed in HeLa cells (miR-1a, miR-133a, miR-133b, miR-206, miR-378) and, conversely, other KSRP-regulated miRNAs are expressed in HeLa cells but not in C2C12 myoblasts (miR-335, miR-595) (according to miRNA microarray analyses performed in this study and in ref. 27). KSRP interacts in C2C12 myoblasts with myogenic (Figure 5d) as well as with non-myogenic pri-miRNAs (Supplementary Figure S6d). However, PI3K/AKT activation in C2C12 cells strongly enhances KSRP interaction with myogenic pri-miRNAs (Figure 5d), as well as their processing into miRNAs (Figure 2b) while it does not increase either KSRP interaction with non-myogenic pri-miRNA or the expression of the corresponding miRNAs (Supplementary Figure S6d, e, and (in the case of miR-21) Figure 2b). It is also noteworthy that maturation of primary let-7a-1 is constitutive in C2C12 myoblasts and is not affected by either DM or myr-AKT2/GM (right panels in Supplementary Figure S3d, e). Based on these data, we are tempted to hypothesize that phosphorylation by AKT

might increase the ability of KSRP to interact with ribonucleoprotein complexes specifically comprising myomiR precursors. This could be achieved through the interaction of AKT-phosphorylated KSRP with a specific co-activator of myogenic pri-miRNA processing.

Studies performed in cultured myoblasts and in animals have demonstrated that myomiRs regulate fundamental aspects of myoblast biology such as differentiation and proliferation^{10,11}. However, the molecular mechanisms by which the expression of myomiRs is dynamically regulated by activation of distinct signaling pathways during skeletal myogenesis have not been elucidated. Our data highlight the relevance of PI3K/AKT signaling as an exquisite regulator of myomiR maturation. Noticeably, culture in DM, differently from activation of AKT signaling in GM, strongly induces pri-miR-133b and pri-miR-206 expression and moderately enhances pri-miR1a-2 levels (Figure 1b). PI3K/AKT pathway inhibition in C2C12 myoblasts cultured in DM does not reduce the expression of primary myomiRs (Figure 1b, d) while strongly impairs their maturation (Figure 1a, c). This is in agreement with previous data from different laboratories demonstrating the transcriptional regulation of primary myomiR levels upon myoblast exposure to DM (reviewed in ref. 10). Altogether, these observations and our findings suggest that a stringent control of myomiR expression occurs during myogenesis at different regulatory levels, with PI3K/AKT signaling being central in the control of maturation.

As other single-strand RNA binding proteins, KSRP proved to be involved at different levels in RNA metabolism with its ability to promote labile mRNA decay as well as miRNA precursor maturation as the most extensively studied functions²⁶. However, despite the evidence that KSRP is targeted by distinct signaling pathways^{8,29,30}, the possibility that a specific signal induces a biological effect by regulating both KSRP main functions in an integrated and dynamic way has never been addressed. C2C12 myoblasts, an established culture model of muscle satellite cells, that we have previously utilized to demonstrate KSRP function on labile myogenic mRNA decay⁸, were ideal to investigate this possibility. Indeed, phosphorylation by AKT inhibits KSRP ability to interact with the Exosome complex and to promote decay of Myogenin mRNA while activates KSRP ability to interact with Drosha complex and to favor the processing of myogenic miRNAs.

Although studies on cultured muscle cells allowed us to identify the PI3K/AKT>KSRP>myomiR processing pathway, we obtained definitive evidence of the physiological relevance of KSRP in primary myomiR processing using the *Ksrp* knock-out mice that display an impaired myomiR maturation during injury-induced muscle regeneration. However, the effect of *Ksrp* knock-out on myoblast proliferation and differentiation markers as well as on myofibers morphology is transient. One possible explanation is that, besides PI3K/AKT signaling, other pathways are activated during the course of muscle regeneration⁵. These pathways, targeting effector proteins different from KSRP, can modulate the molecular events implicated in muscle regeneration in a KSRP-independent way and, in principle, compensate for the molecular defects due to KSRP ablation. Another possibility is that *Ksrp* gene deletion leads to the overexpression of RNA binding proteins (some have been previously described as post-transcriptional targets of KSRP [reviewed in ref. 26]) that could be able to compensate for some of the KSRP functions in mice lacking *Ksrp*. Further studies will be required to investigate the consequences of KSRP deletion and the reduced myomiR expression on the function of regenerated muscles.

PI3K/AKT signaling affects myogenesis at different levels, regulating chromatin remodeling at myogenic loci and controlling Cyclin D1 expression at post-transcriptional level^{5,9}. Our present data reveal an unanticipated additional effect elicited by PI3K/AKT signaling which dynamically integrates two KSRP functions contributing to the post-transcriptional control of skeletal myogenesis.

MATERIALS AND METHODS

Cell cultures, transfections, plasmids and adenoviral infections.

Murine C2C12 myoblasts (obtained from ATCC, #CRL-1772, Lot # 58236521) were cultured in DMEM plus 20% FBS (Growth Medium, GM). Myogenic differentiation was induced by incubation in DMEM plus 2% horse serum (Differentiation Medium, DM). For transient transfections, C2C12 cells were electroporated using the Nucleofector II, according to manufacturer's instructions (Amaxa, Walkersville, MD, USA). C2C12 cell transfections aimed to obtain stable shKSRP transfectants were performed using Lipofectamine Plus (Invitrogen, Carlsbad, CA, USA) and Puromycin (Invivogen, San Diego, CA, USA) was used at 2.5 $\mu\text{g/ml}$ for selection. Pools of transfected cells were used for experiments. The adenoviral vectors pAdCMVnull (Ad-Control) and pAdmyrAKT2 (myr-AKT2) were purchased from Vector Biolabs (Eagleview, PA, USA) and used as previously described⁸.

Recombinant proteins and antibodies.

Production and purification of recombinant GST-KH1-4 (comprising the 4 KH domain of human KSRP) were performed as previously described⁸. Baculovirus-based production and purification of His-tagged human KSRP were previously described⁸. Affinity-purified rabbit polyclonal anti-KSRP antibody was previously described⁸. Affinity-purified rabbit polyclonal anti-Drosha was from Abcam (Cambridge, UK). Affinity-purified rabbit polyclonal anti-AKT and anti-Phospho-AKT (Ser 473) were from Cell Signaling (Danvers, MA, USA). Affinity-purified rabbit polyclonal anti-EXOSC10, mouse monoclonal antibody anti-alpha-Tubulin, and rabbit polyclonal antibodies anti-connexin 43 were from Sigma (St. Louis, MO, USA).

siRNA and shRNA-mediated knockdown.

siRNA utilized to knock-down Ksrp expression were described previously⁸. In order to knock-down the expression of either Akt1 or Akt2, we utilized siRNAs purchased from Santa Cruz (Santa Cruz, CA, USA).

In vitro kinase assays.

AKT2 kinase assays were performed using the preactivated enzyme purchased from Millipore (Billerica, MA, USA) as recommended by manufacturer. We have previously demonstrated that both AKT1 and AKT2 phosphorylate to a similar extent recombinant KSRP³⁰.

miRNA profiling.

MicroRNA microarray assays were provided by LC Sciences (Houston, TX, USA). Total RNA was prepared from either control-infected (cultured in GM or in DM) or myr-AKT2-infected (cultured in GM) C2C12 myoblasts using Trizol (Invitrogen, Carlsbad, CA, USA), enriched with RNA smaller than 40 nt using PureLink miRNA Isolation Kit (Invitrogen, Carlsbad, CA, USA) and used to interrogate a HumanMouseRat miRNA Array (LC Sciences, Houston, TX, USA) comprising a total of 17,341 mature miRNA sequences, based on Sanger miRBase release 16.0. Data were analyzed by first subtracting the background and then normalizing the signals using a LOWESS filter (Locally-weighted Regression). The ratio of the two sets of detected signals (log₂ transformed, balanced) and p-values of the t-test were calculated; differentially detected signals were those with less than 0.01 p-values.

Quantitative RT-PCR.

Total RNA was isolated using the miRNeasy mini kit (Qiagen, Milano, Italy), treated with DNaseI (Promega, Madison, WI, USA), and retro-transcribed using miScript reverse transcription kit (Qiagen, Milano, Italy) according to manufacturers' instructions. For quantitative RT-PCR (qPCR), 100 ng of DNase I-treated total RNA were retro-transcribed and PCR reactions were performed using the miScript SYBR green PCR kit (Qiagen, Milano, Italy) and the Realplex II Mastercycler (Eppendorf, Milano, Italy). The sequence-specific primers used for PCR reactions are listed in Supplementary Table S1.

Northern Blot and RNase-protection assays.

Total RNA (10 µg/lane) was resolved on 15% polyacrylamide-urea gels, and electroblotted onto HyBond N+ membranes (GE Healthcare, Buckinghamshire, England). Membranes were hybridized overnight with radiolabeled antisense miRNAs in

ExpressHyb solution (Clontech, Mountain View, CA, USA). After hybridization, membranes were washed three times with 2X SSC and 0.05% SDS, twice with 0.1X SSC and 0.1% SDS, exposed overnight to imaging screens, and analyzed using a Storm 860 PhosphorImager (GE Healthcare, Buckinghamshire, England). The same blot was hybridized (upon stripping in boiling 0.1% SDS) with three distinct probes, including control U6 RNA.

RNase protection assays were performed using the *mirVana* miRNA Probe Construction and *mirVana* miRNA Detection kits from Ambion (Austin, TX, USA) according to manufacturers' instructions.

Pri-miRNA *in vitro* processing assays.

For pri-miRNA processing assays, total cell extracts from C2C12 myoblasts were prepared in 50mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.5% Triton X-100, 1X Complete [Roche, Mannheim, De], 10% glycerol) and incubated (typically 40 μ g per 25 μ l reaction at 37° C for the indicated times) with in-vitro synthesised and uniformly labeled pri-miRNA (5 fmol) in processing buffer containing 100 mM KCH₃COOH, 2 mM Mg (CH₃COOH)₂, 10 mM Tris-Cl (pH 7.6), 2 mM DTT, 10 mM creatine phosphate, 1 μ g of creatine phosphokinase, 1 mM ATP, 0.4 mM GTP, 0.1 mM spermine, 2 units of Stop RNase Inhibitor (5 PRIME, Hamburg, Germany).

RNA in Vitro degradation.

³²P-labeled RNAs were synthesized and used as substrates for in vitro degradation assays as reported⁸.

RNA *in vitro* degradation assay and measure of mRNA half-life in intact cells.

³²P-labeled RNAs were synthesized and used as substrates for *in vitro* degradation assays as reported⁸. ARE^{Myog} and the E3 sequence from Pitx2 3'UTR were described previously^{8,9}.

To measure the mRNA half-life, intact C2C12 myoblasts were treated with 100 μ M DRB (5,6-Dichloro-1- β -D-ribofuranosylbenzimidazole, 5,6-Dichlorobenzimidazole

riboside), harvested at the indicated times, and total RNA was isolated and analyzed as above.

Ribonucleoprotein complexes immunoprecipitation (RIP) assays.

RIP assays were performed as previously described in ref. 27. Briefly, cell lysates were immunoprecipitated with Protein A-coupled antibodies at 4° C overnight. Pellets were sequentially washed with the following buffers: Buffer I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl); Buffer II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl); and Buffer III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1). Total RNA was prepared from immunocomplexes using Trizol (Invitrogen, Carlsbad, CA, USA) retro-transcribed and amplified by qPCR as described above. The primer sequences are detailed in Supplementary Table S1.

Nuclear and S100 extracts preparation.

Nuclear and S100 extracts were prepared as previously described⁸.

GST pull-down assays.

Escherichia coli, transformed with pGEX-based recombinant plasmids (GE Healthcare) were grown until the A_{600} reached 0.6, induced with 1 mM of isopropyl- β -D-thiogalactoside (IPTG) for 3 h, harvested, washed with PBS, and lysed by sonication. After centrifugation (14,000 \times g for 15 min at 4 °C) the supernatants were incubated with glutathione-Sepharose beads (GE Healthcare) for 1 h at 4 °C under rotation. Beads were washed four times with Pull-Down Buffer (PDB, 150 mM NaCl, 20 mM Tris-HCl pH 8.0, 0.2% Nonidet P-40, 10% glycerol, and a mixture of protein inhibitors (Complete, Roche)), saturated with bovine serum albumin, washed again with PDB, and incubated with 300 μ g of cell extracts (2h, 4° C, rotating). Beads were collected by centrifugation (30 sec 5000 \times g) and washed 4 times with PDB. Bound proteins were eluted in SDS sample buffer, resolved by SDS-PAGE, and analyzed by Immunoblot. Plasmids GST-KH1-4 and GST-KH1-4(S193) were described previously³⁰.

Generation of Ksrp-null mice.

Generation of Ksrp-null mice (Ksrp^{-/-}) is described elsewhere (ref.). Briefly, a hygromycin cassette flanked by loxP sites was designed to replace exon 1 to exon 13 of Ksrp gene and electroporated into V6.5 mouse embryonic stem (ES) cells. The cells were subsequently selected by hygromycin (250 µg/ml) and ganciclovir (2 µM). ES colonies resistant to hygromycin and ganciclovir were genotyped by Southern blot analysis and the Ksrp-targeted ES clones, after Cre recombinase-mediated removal of the hygromycin cassette, were injected into C56BL/6 blastocysts to generate chimera mice. Male chimeras were further backcrossed with C56BL/6 females for 8 generations and were then used for the experiments. All animal studies were conducted in accordance with guidelines for animal use and care established by the University of Alabama at Birmingham Animal Resource Program and the Institutional Animal Care and Use Committee.

Injury-induced muscle regeneration.

Animals were anesthetized by inhalation of Isoflurane and the site of injection was shaved and sterilized according to the animal care requirements. 25 µl of 10 µM Cardiotoxin (Latoxan, Fr) (in sterile PBS) was slowly injected into the tibialis anterioris muscle. Animals were euthanized at different time points after injection (0-14 days) using CO₂ asphyxiation, and the musculature of both lower limbs was removed and processed for Hematoxylin-Eosin staining and RNA preparation.

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TITLES AND LEGENDS TO FIGURES

Figure 1. PI3K/AKT signaling controls maturation of myogenic miRNAs in differentiating C2C12 myoblasts.

(a, b) Quantitative RT-PCR (qPCR) analysis of miR-1a, miR-133b, miR-206 (panel a) and their respective primary transcripts (panel b) in C2C12 cells cultured either in growth medium (GM) or differentiation medium (DM) for 2 days in the presence of either DMSO (the solvent of LY294002 (LY), labeled as DM), or LY (25 μ M, labeled as DM+LY). The values shown are averages (\pm SEM) of three independent experiments performed in triplicate. (c, d) qPCR analysis of miR-1a, miR-133b, miR-206 (panel c) and their respective primary transcripts (panel d) in C2C12 cells transfected with either siControl, siAkt1, or siAkt2 (40 nM) and cultured either in GM or DM for 2 days. The values shown are averages (\pm SEM) of three independent experiments performed in triplicate. (e) Northern blot analysis of total RNA purified from C2C12 myoblasts cultured either in GM or DM (1 day) and then infected with either a control empty adenoviral vector (1d DM) or an adenoviral vector expressing constitutively active myristoylated AKT2 (1d DM+myr-AKT2) for further 2 days. Representative blots sequentially hybridized with miR-133, miR-1a, and U6 probes (left panels) or with miR-206, let-7a, and U6 probes (right panels) are displayed. (f) qPCR analysis of pri-miR-1a-2, pri-miR-133b, pri-miR-206 in the RNA samples from C2C12 cells treated as in panel e. Statistical significance: three asterisks $p < 0.001$ (Student's t-test).

Figure 2. Identification and validation of miRNAs whose expression is induced by PI3K/AKT signaling in C2C12 myoblasts cultured in GM.

(a) C2C12 myoblasts cultured in GM were infected with either a control empty adenoviral vector (GM#1.0, 2.0, and 3.0 represent three distinct experiments) or an adenoviral vector expressing myr-AKT2 (A2#1.0, 2.0, and 3.0 represent three distinct experiments) and harvested after 2 days. Total RNA was extracted and analyzed by miRNA microarrays. Green or red color on the heat map indicates a decrease or increase of miRNA levels, respectively, and color intensities correspond to relative signal levels on a logarithmic scale. The green arrow points to miR-21 whose expression was significantly reduced also by a 2-day treatment of C2C12 myoblasts

with DM. The red arrow and the red brackets indicate miRNAs whose expression was significantly enhanced also by a 2-day treatment of C2C12 myoblasts with DM. Statistical analysis was performed using Student's t-test. (b) qPCR analysis of miRNAs and their respective primary transcripts (as indicated) in C2C12 myoblasts cultured in GM and infected with either a control or a myr-AKT2-expressing adenoviral vector. The values shown are averages (\pm SEM) of three independent experiments performed in triplicate. (c) RNase protection assay performed on the same RNA samples displayed in paned b. Samples were analyzed for the expression of either miR-206 or miR-16 (as control). Lanes corresponding to assays performed in the absence of RNA (no RNA) or in the presence of only RNA probes without digestion (probe) are also displayed. (d) qPCR analysis of miRNAs and their respective primary transcripts (as indicated) in C2C12 myoblasts serum-starved for 16 hours and cultured for 2 additional hours in the same medium in the absence or in the presence of 10^{-6} M insulin. The values shown are averages (\pm SEM) of three independent experiments performed in triplicate. (e) Immunoblot analysis of total cell extracts from C2C12 cells cultured either in GM (and infected with control or myr-AKT2 vectors) or in DM (for 2 or 3 days). Blots were sequentially hybridized with anti-Connexin 43 and anti-alpha-tubulin antibodies and films subjected to densitometric scanning. Densitometric values (%) of Connexin 43 expression after normalization for alpha-tubulin expression are presented. Representative blots are displayed.

Statistical significance: two asterisks $p < 0.01$, three asterisks $p < 0.001$ (Student's t-test).

Figure 3. KSRP is required for AKT-induced myomiR maturation and phosphorylation by AKT activated KSRP ability to process pri-miR-206.

(a) qPCR analysis of miRNAs (upper) and their respective primary transcripts (lower) in C2C12 myoblasts cultured in GM, transfected with either siControl or siKsrp, and infected with either a control (Ad-Control) or a myr-AKT2-expressing adenoviral vector. The values shown are averages (\pm SEM) of three independent experiments performed in triplicate. (b) *In vitro* pri-miR-206 processing assays performed using total extracts from either C2C12 myoblasts cultured in GM pre-incubated with BSA (lanes 1-4), KSRP (30 nM, lanes 5-8), AKT2-phosphorylated KSRP (30 nM, lanes 9-12), respectively.

Internally ^{32}P -labeled, pri-miR-206 RNA substrate was added and its processing monitored as described under ref. 8.

Statistical significance: two asterisks $p < 0.01$, (Student's t-test).

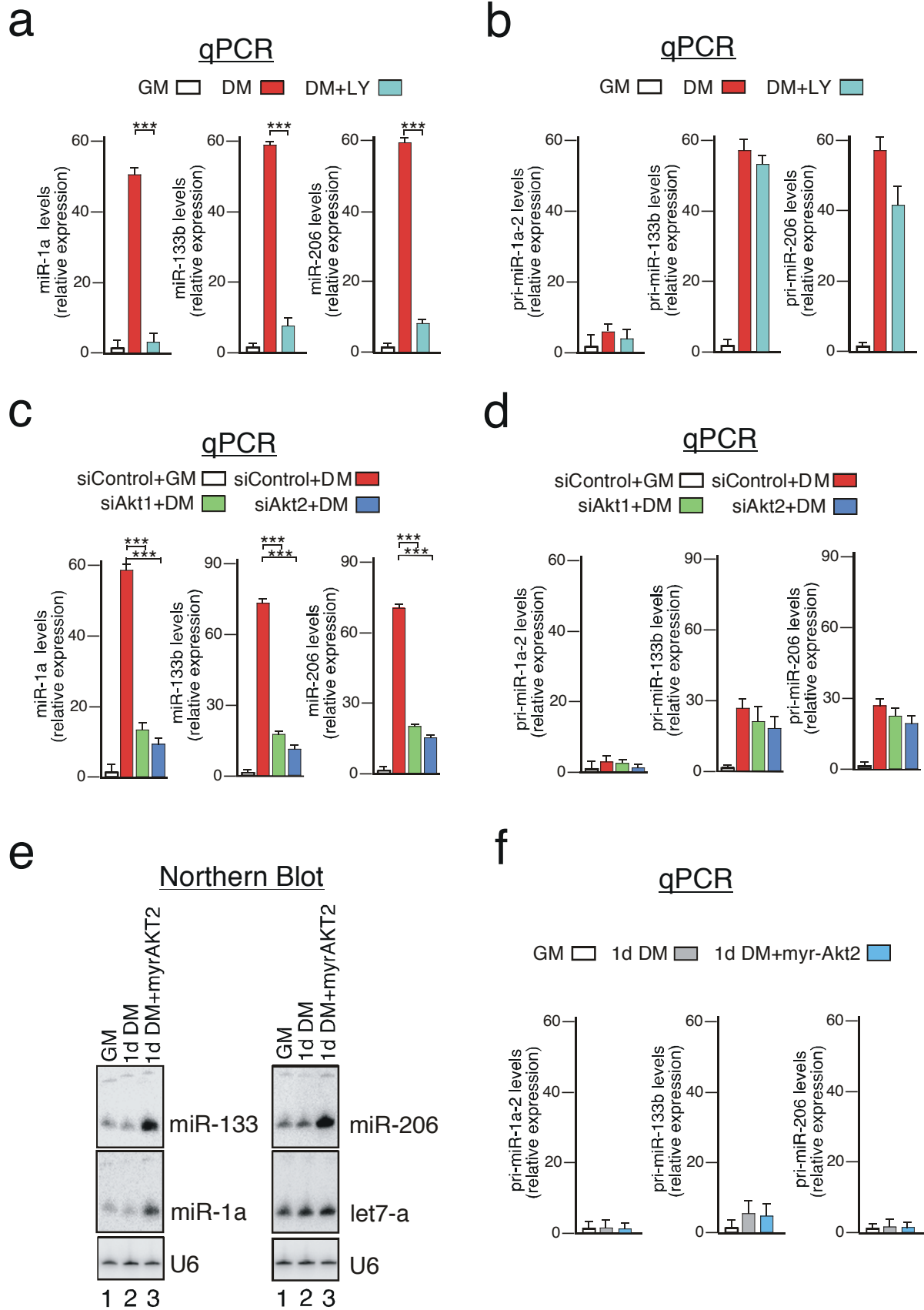
Figure 4. Impaired maturation of myomiRs during muscle regeneration in Ksrp knock-out mice.

(a, b) qPCR analysis of miRNAs (panel a) and their respective primary transcripts (panel b) in total RNA samples extracted from tibialis anterioris muscles of either wild-type (Ksrp^{+/+}) or Ksrp^{-/-} mice before and at different intervals of time after injection of cardiotoxin as indicated. The values shown are averages (\pm SD) of five independent animals for each experimental group. Assays were performed in triplicate. Statistical significance: one asterisk $p < 0.05$, two asterisks $p < 0.01$, three asterisks $p < 0.001$ (Student's t-test).

Figure 5. AKT signaling activation inhibits KSRP ability to participate in a Myogenin mRNA decay complex while favors its association with a primary myomiR processing complex.

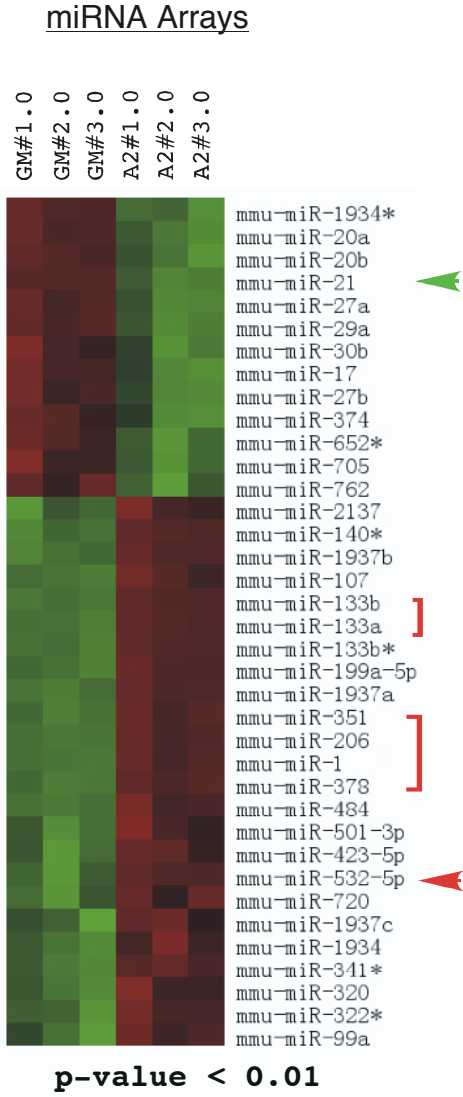
(a) qPCR analysis of Myogenin and p21 (also known as Cdkn1a) expression in C2C12 myoblasts cultured in GM and infected with either a control (GM) or a myr-AKT2-expressing adenoviral vector. Total RNA was isolated at the indicated times after the addition of DRB. The values shown are averages (\pm SEM) of three independent experiments performed in triplicate. (b) *In vitro* RNA degradation assays using S100 extracts from C2C12 myoblasts cultured in DM pre-incubated with BSA (lanes 1-4), KSRP (30 nM, lanes 5-8), AKT2-phosphorylated KSRP (30 nM, lanes 9-12), respectively. Internally ^{32}P -labeled and capped RNA substrates were added and their decay monitored as described under Material and Methods. E3 is a stable unrelated transcript⁹. Representative gels are displayed. (c) C2C12 myoblasts were cultured in GM and infected with either control (GM) or myr-AKT2-expressing adenoviral vector. After two days cells were lysed, and total cell extracts were immunoprecipitated as indicated. RNA was purified from immunocomplexes and analyzed by qPCR to detect Myogenin mRNA. The values shown are averages (\pm SEM) of three independent experiments performed in triplicate. (d) C2C12 myoblasts were cultured in GM and

infected with either control (GM) or myr-AKT2-expressing adenoviral vector. After two days cells were lysed, and total cell extracts were immunoprecipitated as indicated. RNA was purified from immunocomplexes and analyzed by qPCR to detect primary myomiRs as indicated. The values shown are averages (\pm SEM) of three independent experiments performed in triplicate. (e, f) GST pull-down of endogenous Exosc10 (panel e) and Drosha (panel f) present in total extracts from C2C12 cells cultured in GM using the following proteins: GST, GST-KH1-4, GST-KH1-4 *in vitro* phosphorylated by AKT2, GST-KH1-4(S193A), or GST-KH1-4(S193A) *in vitro* phosphorylated by AKT2 as indicated. GST-KH1-4(S193A) is mutated in Ser 193 in order to abolish the AKT phosphorylation site as described under ref. 30. Proteins were analyzed by immunoblotting using the indicated antibodies. Representative blots are displayed. Statistical significance: three asterisks $p < 0.001$ (Student's t-test).

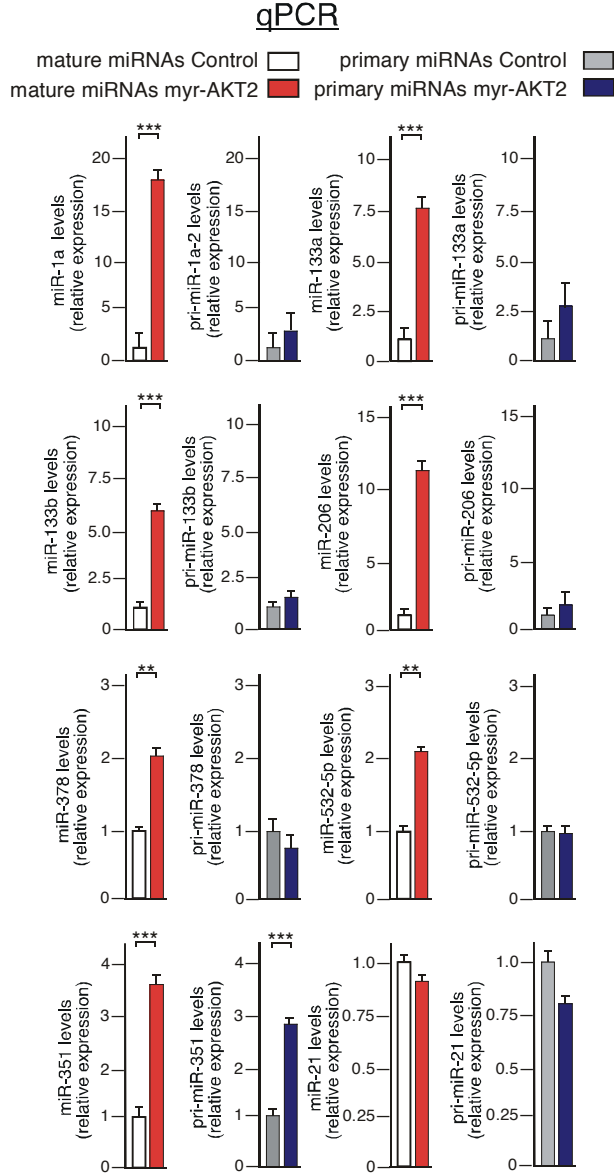


Briata et al. Figure 1

a

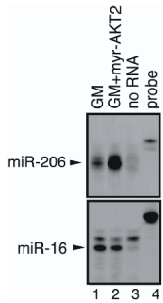


b



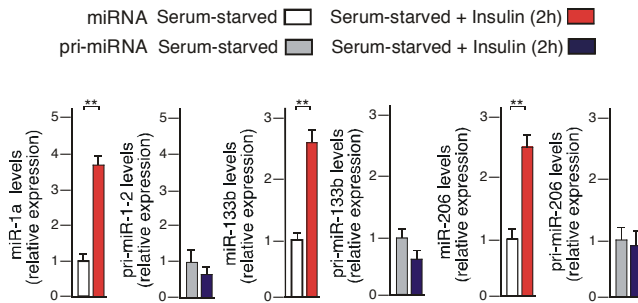
c

RNase-protection Assay



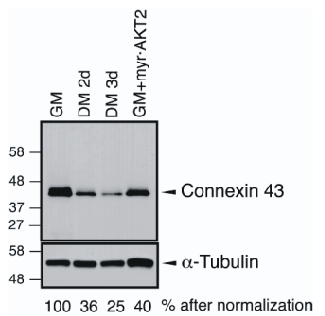
d

qPCR



e

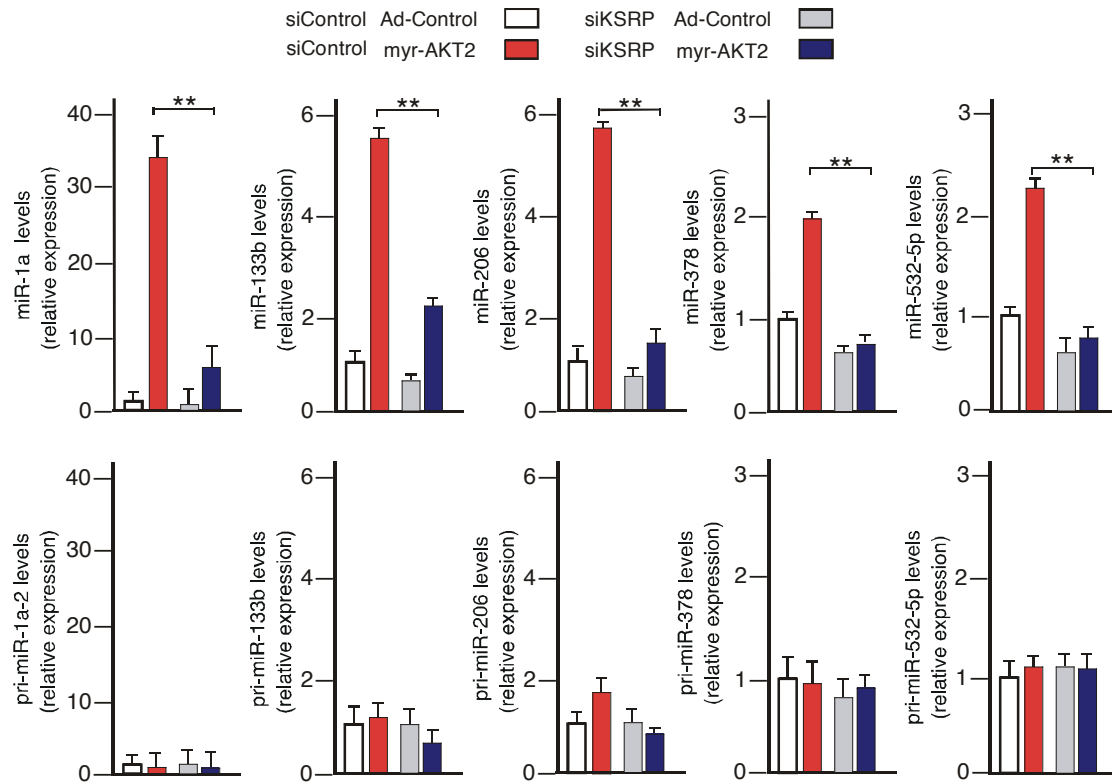
Immunoblot



Briata et al. Figure 2

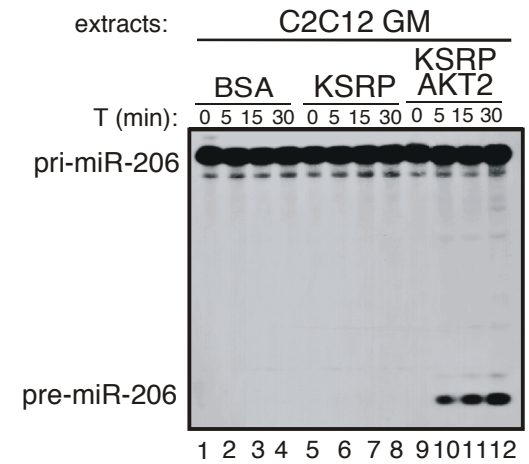
a

qPCR



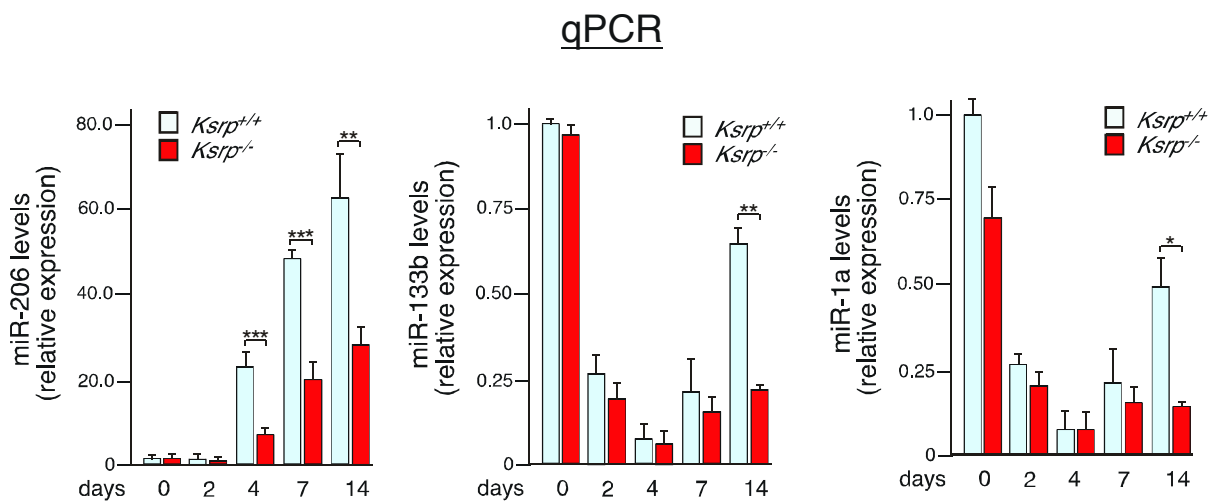
b

In vitro pri-miRNA Processing Assay

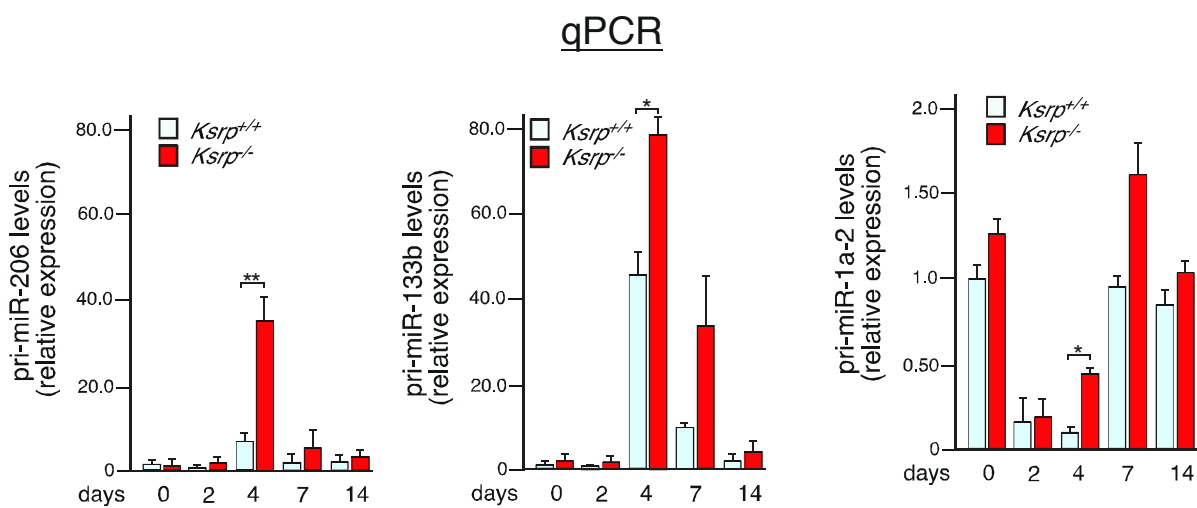


Briata et al. Figure 3

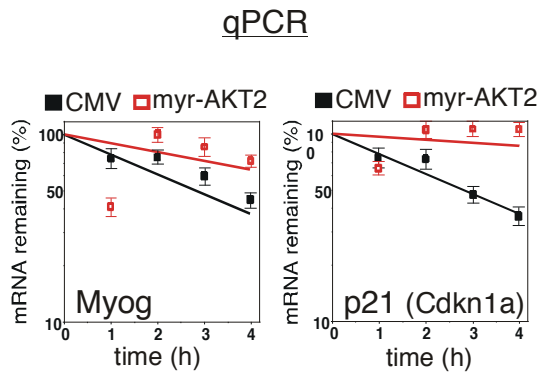
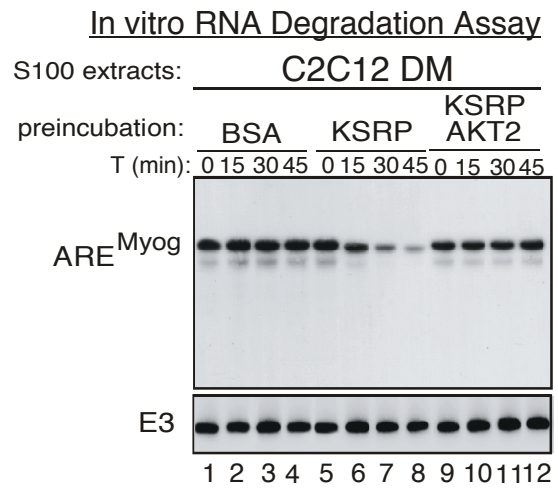
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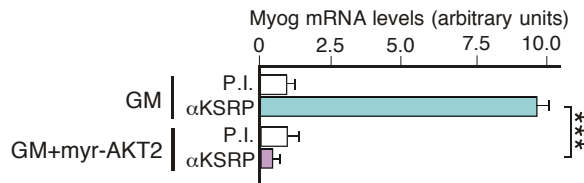
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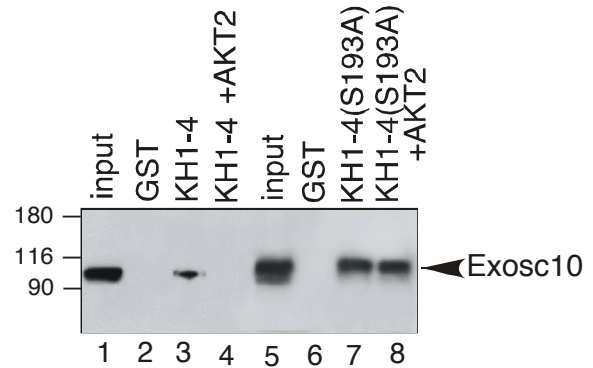
Briata et al. Figure 4

a**b****c**

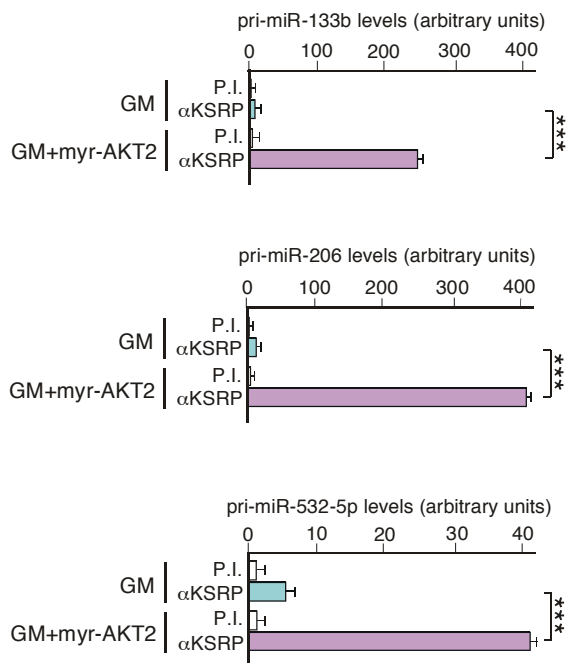
Ribonucleoprotein complex immunoprecipitation, qPCR

**e**

GST Pulldown, Immunoblot

**d**

Ribonucleoprotein complex immunoprecipitation, qPCR

**f**

GST Pulldown, Immunoblot

