Raf Kinase Inhibitor Protein1 is a myogenic inhibitor with conserved function in avians and mammals.

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Abstract

Raf Kinase Inhibitor Protein1 (RKIP) is a tumour suppressor that is present in a number of adult tissues. It functions as an inhibitor of both Raf/Mek/Erk and NFκB signalling when unphosphorylated, but following phosphorylation the ability to inhibit Raf/Mek/Erk signalling is lost and RKIP becomes an activator of G-protein coupled receptor signalling. In neonates and adults RKIP is known to be expressed in muscle, however, its physiological function is currently unknown. In this study we show by in situ hybridisation and immunofluorescence that RKIP is also expressed in developing chick embryonic muscle, and mouse C2C12 myoblasts. Furthermore, we demonstrate that in these systems it functions as an inhibitor of myogenesis: increased levels of RKIP suppress myotube differentiation whilst decreasing RKIP promotes differentiation. Additionally we show that the ability of RKIP to inhibit myogenesis is dependent upon its phosphorylation state as only the non-phosphorylated form of RKIP suppresses myogenesis. This study therefore clearly demonstrates that RKIP has conserved functions as a myogenic inhibitor in both mammalian and avian muscle.

Introduction

Raf Kinase Inhibitor Protein1 (RKIP) is a member of the phosphatidylethanolamine binding (PEBP) protein family. Although originally characterised by its ability to bind phosphatidylethanolamine, RKIP has also been shown to bind to a number of other molecules and consequently to regulate their function within a cell. Many of the target molecules for RKIP's actions are components of intracellular signalling pathways (Al-Mulla et al., 2011; Al-Mulla et al., 2012; Eves et al., 2006; Lorenz et al., 2003; Yeung et al., 1999; Yeung et al., 2001). The first pathway shown to be regulated by RKIP was the MAP kinase pathway (Yeung et al., 1999). In this context RKIP binds to Raf, or its target MEK, and inhibits their interaction thus blocking the ability of Raf to phosphorylate and activate MEK and consequently inhibiting downstream ERK signalling.

This discovery was closely followed by the finding that RKIP also binds and inhibits kinases that regulate other signalling pathways, including NFκB inducing kinase, Tak1 (Yeung et al., 2001) and GRK2 (Lorenz et al., 2003). NFκB inducing kinase and Tak1 both phosphorylate IκB which induces its dissociation from the IκB/NFκB complex. Once released NFκB is free to bind and activate transcriptional targets. The inhibition of Raf, NFκB inducing

kinase and Tak1 by RKIP therefore all result in inactivation of these intracellular signalling pathways. However, the actions of RKIP can activate other target pathways. For example RKIP inhibition of GRK2 blocks phosphorylation of activated G protein coupled receptors and consequently maintains them in an activated state thus enhancing signalling (Lorenz et al., 2003).

RKIP is a substrate for phosphorylation by Protein Kinase C (Deiss et al., 2012; Lorenz et al., 2003), however, phosphorylation at Serine 153 does not inhibit the function of RKIP but promotes dimerization (Deiss et al., 2012) which consequently changes its binding specificity. Of the binding partners tested it is known that Raf, NFκB inducing kinase and Tak1 bind to monomeric non-phosphorylated RKIP whilst GRK2 binds the phosphorylated dimeric form (Deiss et al., 2012; Lorenz et al., 2003; Yeung et al., 1999; Yeung et al., 2001). Thus phosphorylation of RKIP changes the balance of signalling within a cell from repression of MAP kinase and NFκB, to enhanced G-protein coupled receptor signalling.

Recently RKIP has also been found to modulate other cell signalling pathways that are important during embryonic development. For example it has been shown to bind and stabilise active GSK3β (Al-Mulla et al., 2011) a key negative regulator in the canonical Wnt signalling pathway. This would therefore lead to inhibition of canonical Wnt signalling as phosphorylation of β-catenin, a key component of the transcriptional complex of the canonical Wnt pathway, by GSK3β results in its degradation (MacDonald et al., 2009; Rao and Kuhl, 2010). There is also evidence that RKIP can inhibit signalling via the transcription factors STAT3 (Chatterjee et al., 2005) and Nrf2 (Al-Mulla et al., 2012) although the mechanisms by which these occur are not fully elucidated.

As RKIP can modulate a number of developmentally important intracellular signalling pathways, this raises the possibility that it functions during embryological development. However, mice null for RKIP are viable and exhibit no obvious physical defects. Postnatally, however, these mice develop an olfactory deficit over time and also exhibit behavioural problems (Theroux et al., 2007). Since RKIP is expressed widely in adult mouse tissues this lack of overt phenotype suggests that either RKIP is not expressed in embryonic tissues, which appears unlikely, or that there may be functional redundancy with another RKIP. However, in adult mice RKIP2 is reported to be only expressed in testes, and RKIPs 3, 4 and 5 are thought to be pseudogenes (Theroux et al., 2007), therefore redundancy appears unlikely unless there

are significant differences between embryonic and adult expression. It is therefore possible that in the RKIP null mice any changes are subtle and are not overtly apparent.

In rats, RKIP levels in the Tibialis Anterior muscle are at their highest immediately after birth and thereafter decline sharply(Sun et al., 2009) suggesting that RKIP functions in developing muscle. During embryogenesis muscle development follows a defined cellular program. Mononuclear Pax3/Pax7 expressing precursor cells express myogenic regulatory factors (MRFs), including Myf5 and MyoD, to become committed to the myogenic fate. These cells then express other MRFs, including Myogenin, and progress to a more differentiated state whereupon they fuse to one another to produce Myosin Heavy Chain (MHC) positive myotubes. A number of signalling pathways, including Wnt, BMP and FGF, are known to regulate these key steps in myogenesis (For recent review of myogenesis see (Bentzinger et al., 2012). As RKIP can regulate signalling downstream of these growth factors this therefore led us to hypothesise that RKIP regulates myogenic differentiation in embryonic muscle.

To investigate this we used three model systems of muscle development. Initially we used retroviral over-expression of RKIP in both embryonic chick limb in vivo, and cultured primary chick limb cells in vitro. These studies were then extended to the myogenic cell line C2C12, a model for mammalian satellite cell differentiation, where we determined the effect of both gain and loss of RKIP function. Additionally, we assessed the requirement for RKIP phosphorylation in muscle development. We show that RKIP is expressed in developing embryonic muscles of the chick and also both myoblasts and myotubes in C2C12 cultures. Furthermore we demonstrate that in both chick and mouse RKIP regulates the rate at which myoblasts differentiate into myotubes, and that in mouse this function is dependent on its phosphorylation state.

Results

RKIP is expressed in developing chick wing muscles.

Wholemount in situ hybridisation showed that in limb buds of HH stage 23 embryos expression levels of RKIP are low within the central mesenchymal core of the bud but much higher in peripheral mesenchyme (Fig 1A, 1A1). High levels of expression are maintained in the peripheral mesenchyme up to stage 26 (Fig 1B,C) at which stage weak expression can be seen in regions of myogenesis (white arrows in Fig 1C,C1) whilst expression in the condensing

mesenchyme of the skeletal elements decreases (asterisk in Fig 1C1). At Stages 27-29 expression of RKIP in muscle tissues increases further. This is clearly illustrated by comparison to myogenin expression (arrows in Fig 1D1, white asterisks in 1D2, E1). Additionally, expression appears higher in more mature muscle, i.e stage 29 and more proximal muscles of stage 27 (Fig 1 D1, E1) compared with the less differentiated, stage 27 distal muscle (Fig 1D1). At these stages of limb development expression of RKIP in some, but not all, peripheral mesenchyme is decreased (Fig 1D1, D2, E1) whilst expression in the now chondrogenic skeletal elements is lost (black asterisks in 1D2, E1).

Overexpression of RKIP in chick wings in vivo inhibits myogenic differentiation.

To test the effect of RKIP on muscle development the right presomitic mesoderm was infected with RKIP or GFP expressing retrovirus by injection with infected chick fibroblasts. In all cases the fibroblasts used in experiments were verified to be 100% positive for viral GAG protein. In some cases an RKIP GFP fusion protein was used and these cells were verified to be 100% positive for both GFP tagged RKIP and viral GAG prior to use (data not shown).

Embryos were fixed 3 days following cell injection at approximately stage 27 and assessed for both viral spread and muscle differentiation (myogenin expression) by in situ hybridisation. This showed that despite RKIP infected fibroblasts being injected continuously along a length of PSM expression of RKIP was limited to patches largely within the somitic derivatives. This was in contrast to control embryos where widespread unilateral expression was seen along a significant length of the embryo (Fig 2A, B). Sectional analysis of these embryos followed by immunolocalisation of viral GAG to further demonstrate viral spread (Fig 2A1, A2, B1, B2) showed myogenic tissues were positive for virus in control embryos (white arrows in Fig 2A2) but largely negative in RKIP infected embryos (Fig 2B2) although in these embryos some non-myogenic tissues were positive.

Furthermore at sites where expression of RKIP was evident these were often associated with changes in somite structure. Given the limited viral spread and altered somite size and shape at sites of infection disruptions in myogenesis seen in these embryos (Fig 2C,D) could be secondary to effects on somitogenesis. We therefore attempted to address this question in the developing limb. However similar to the injections into presomitic mesoderm RKIP infected limb buds showed significant degrees of skeletal anomaly (chondrodysplasia) at HH stage 32 (Fig 3I,J) compared with GFP infected controls which were phenotypically normal. As assessment of muscle differentiation was confounded by the abnormalities seen at these later

stages of development we went on to examine myogenesis at early stages of development (HH 24-26) before signs of chondrodysplasia appear. In situ hybridisation for myogenin, showed a loss of some muscle in three RKIP infected wings compared with their contra-lateral control limb (Asterisks in Fig 3A,B; arrows in 3C,D). These changes were predominantly in the dorsal muscle masses, however, this is to be expected as in these experiments cells are injected into the dorsal side of the limb bud so that cell entry and retention can be visualised at the time of injection. This decrease was never seen in control RCASA grafted embryos (data not shown). Similarly, MHC expression was decreased with RKIP compared to contralateral controls (Arrows in Fig 3E,F). Viral presence in affected tissue was verified by staining for vGAG protein (Fig 3G,H). These data therefore suggest that RKIP acts as a negative regulator of muscle differentiation.

Overexpression of RKIP in chick limb bud micromass culture inhibits myogenic differentiation.

Given the difficulties in assessing myogenic differentiation in vivo due to the effects of RKIP on non-muscle tissues we went on to investigate this in two in vitro models. Firstly we used micromass cultures of primary mesenchymal cells from embryonic chick limb buds. These were infected with either control (RCASA) or RKIP (RCAS-RKIP) expressing retrovirus and cultured to allow differentiation of myotubes. Analysis of total numbers of Myosin Heavy Chain positive myotubes showed that RKIP reduced the number of myotubes compared with RCASA controls (green myotubes in Fig 4A,B,C). There were similar numbers of MHC/MyoD +ve myotubes (Green myotubes with yellow nuclei in Fig 4A,B) but significantly fewer single MHC positive myotubes (green myotubes in Fig 4A,B) in RKIP treated cultures (Fig 4C). In contrast, the number of single positive MyoD cells (red nuclei) in RKIP treated cultures was increased compared with RCASA controls (Fig 4A-C).

RKIP is expressed in C2C12 myoblasts and differentiating myotubes.

As the chick micromass cultures are a mixed population of cells it is possible that changes in myogenesis may be secondary to effects on non-myogenic cell populations within the culture. In order to clarify this we determined the effects of RKIP in a pure myogenic culture; the C2C12 myoblast cell line. As RKIP function is regulated by phosphorylation we

also determined whether phosphorylation status of RKIP is important for inhibition of myogenesis.

Firstly in order to demonstrate that RKIP is expressed in this cell line, and to ascertain when RKIP may be acting during myogenesis, a series of timepoints throughout the course of myogenic differentiation were analysed for RKIP expression by RT-PCR. This showed (Fig 5A) that RKIP mRNA is expressed at similar levels in C2C12 cultures at different stages of differentiation. As RT-PCR does not give any information regarding which cells in a population express a particular gene we performed immunostaining for RKIP and MHC on cultures containing both myoblasts and differentiated myotubes (Fig5B). This demonstrated that RKIP protein is present in the majority of MHC positive myotubes (Fig 5B merge yellow vs green myotubes), and also myoblasts (Fig 5B merge red cells). Immunolocalisation of pERK in these cultures showed that it is absent or very low in myoblasts, but present in a subset of myotubes (Fig 5C). This is supported by western blots which show that in growth medium, in which C2C12 cells maintain their myoblastic state, levels of pERK are low in myoblasts that have normal (GFP) or enhanced levels of RKIP (RKIP WT (Fig 5D)). Following culture in differentiation medium, which promotes formation of myotubes in C2C12 myoblasts, differences in pERK/ERK levels were seen between control and RKIP expressing cultures (Fig 5E). In controls the absolute levels of pERK-42 and 44 were increased with differentiation but increased activation was only seen for pERK-42 suggesting that it is this isoform of ERK that is important during myogenesis in C2C12 cells. However, in RKIP overexpressing cells levels of both absolute ERK and active ERK were unchanged, compared with cultures in growth medium, suggesting that RKIP overexpression may be inhibiting the Raf/Mek/ERK pathway in these cells.

Stable RKIP transfected C2C12 cells produce ectopic protein.

As there are differential functions between phosphorylated and non-phosphorylated RKIP we produced mutant forms of RKIP that can either not be phosphorylated (RKIP SA) or are phosphomimetic (RKIP EE) as we hypothesized that only one of these would suppress muscle differentiation and this would therefore give an indication of which downstream signaling pathways may be important.

These vectors, and also a GFP vector, were used to derive stable overexpressing C2C12 polyclonal cell populations. The RKIP proteins were GFP tagged to enable visualisation of

ectopic RKIP protein (Fig 6A-D). Furthermore immunolocalisation analysis using an anti RKIP antibody confirmed the presence of elevated levels of RKIP compared with GFP transfected cells (data not shown). We also undertook duplex RT-PCR analysis for RKIP-GFP transgene expression in these cells which showed that the levels of ectopic RKIP expression were comparable for the different RKIP expressing populations (Fig 6E).

RKIP transfected C2C12 cells show decreased levels of myogenesis.

To assess myogenesis cultures of RKIP and GFP expressing C2C12 cells were differentiated prior to analysis of myotube formation. This showed that control and RKIP EE expressing cultures contained more differentiated myotubes (Fig 6F,I) than cultures over-expressing either wild-type or S153A RKIP (Fig 6G,H), which contained more myoblasts. Quantification by measuring numbers of nuclei incorporated into myotubes or area of myotubes showed that the levels of myogenesis were significantly reduced for RKIP WT and S153A compared with control (GFP) or RKIP EE expressing myoblasts (Fig 6J,K). As the rate of proliferation of C2C12 cells can influence differentiation, with higher levels resulting in increased myogenesis, we determined proliferation rates in our cell lines to determine whether this could be a contributing factor. This showed that in both of the cell lines that did not differentiate well the rates of proliferation were comparable or significantly greater than those in cell lines that underwent differentiation (Fig 6L).

Decreased RKIP expression results in enhanced myogenic differentiation.

Analysis of differentiation in 4 clonal populations of RKIP RNAi showed significantly increased myogenesis in two of these compared with control (GFP RNAi) cultures (Fig 7A-C) The overall average levels of myogenic differentiation in RKIP RNAi compared with control GFP RNAi myoblasts was also significantly increased (Fig 7C). Loss of RKIP transcripts was confirmed by duplex RT-PCR, with GAPDH as an internal standard. In both myoblastic and differentiated cultures a significant ($P<0.05$) average decrease in expression of approximately 50% was found (Fig 7D).

Discussion

RKIP is a known modulator of a number of signalling pathways that control cell differentiation and behaviour. It is expressed in a number of adult tissues, however, its expression and function during embryonic development are currently unknown. In mice null for RKIP there are no obvious anatomical defects at birth (Theroux et al., 2007), therefore any changes in tissue differentiation or phenotype are subtle. As RKIP is expressed in both neonatal and adult skeletal muscle (Sun et al., 2009) we investigated its expression and function during embryonic muscle development.

Initial analysis showed that RKIP is expressed in developing appendicular (Fig 1) and axial muscles (data not shown) in chick embryos and also in cultures of C2C12 mouse myoblast cells (Figs 5A, 7D). This therefore suggested that RKIP may indeed function in developing muscle tissue. Viral overexpression of RKIP in developing chick embryos was problematic as its failure to spread efficiently coupled with fundamental changes in behaviour of non-muscle tissues that would not normally express RKIP hindered analysis. However, in limb overexpression was seen to inhibit some muscle differentiation (Fig 3), prior to the onset of RKIP induced chondrodysplasia, suggesting it may regulate myogenesis.

Therefore to further confirm this we used two in vitro models of myogenesis; chick limb bud micromass cultures and the murine myoblastic cell line C2C12. In both of these models it was evident that overexpression of RKIP resulted in significant decreases in myoblast differentiation. In vivo, and in the chick micromass culture, the developing myoblasts are in an environment that contains other cell types. There is therefore the potential that RKIP could be regulating myogenesis via effects on cells other than muscle, however, as decreased myogenesis is also seen in C2C12 cultures, a pure myogenic cell line, this demonstrates that RKIP is able to regulate myoblast differentiation directly.

As phosphorylated and non-phosphorylated RKIP regulate different signalling pathways in order to gain insight into the mechanism of action we tested both a non phosphorylatable form (S153A) and a phosphomimetic form (SL153/7EE) of RKIP for inhibition of myogenesis in C2C12 cultures. This analysis confirmed RKIPs ability to suppress myogenesis in mouse myoblasts and showed that both the WT and S153A form of RKIP were able to do this but that the SL153/7EE form was not (Fig 6). Furthermore we demonstrated that endogenous RKIP was negatively regulating myogenesis in C2C12 cultures as decreasing its expression resulted in increased myogenesis (Fig 7). Taken together these results demonstrate

that RKIP regulates myogenesis and that this function is conserved between avians and mammals.

Currently, however, the mechanism by which RKIP functions to inhibit myogenesis remains unknown. A related, but not functionally equivalent, protein PEBP4 has previously been shown to regulate myogenesis in humans however this protein was shown to be a promoter of myogenesis as loss of PEBP4 inhibited myogenesis whilst increasing levels promoted myogenesis. In this study MEK/ERK signalling was proposed to be the key regulator of differentiation with increased levels of MEK/ERK activation following loss of PEBP4 inhibiting myogenesis (Garcia et al., 2009).

In our study changes in MEK/ERK signalling could potentially regulate myogenesis. There is some evidence that blockade of ERK signalling can inhibit myogenesis (Cho et al., 2007; Gredinger et al., 1998; Li and Johnson, 2006) and in our study the levels of pERK increased in control cultures undergoing differentiation but not in RKIP expressing cultures that failed to differentiate. This data could support pERK being a positive regulator of differentiation, however, as C2C12 cultures overexpressing WT and S153A RKIP produce few myotubes the failure to activate ERK, which occurs in myotubes (Fig 5C,E), could merely reflect an inability to differentiate rather than being its cause. There is a large body of work demonstrating that ERK signalling can inhibit myogenesis (Bennett and Tonks, 1997; Coolican et al., 1997; Yang et al., 2006; Yokoyama et al., 2007) therefore the low levels seen in myoblasts may reflect a permissive level for myogenesis to occur and activation in myotubes post differentiation may perform other functions. Given this and that RKIP regulates a number of other intracellular signalling pathways there are other potentially other mechanisms for the regulation of myogenesis.

In terms of phosphorylation status the non-phosphorylated form of RKIP is known to inhibit the NFκB (Yeung et al., 2001) signalling pathway. However, as classical NFκB signalling is known to reduce muscle differentiation (Bakkar et al., 2008; Guttridge et al., 1999; Guttridge et al., 2000; Langen et al., 2001) inhibition of NFκB by RKIP would be predicted to increase differentiation an outcome opposite to that seen in our study.

There are however several signalling pathways that are known to be modulated by RKIP, but in which the requirement for its phosphorylation are not documented. These include GSK3β (Al-Mulla et al., 2011), STAT3 (Chatterjee et al., 2005) and KEAP/Nrf2 (Al-Mulla et

al., 2012), and of these the interactions with STAT and GSK3β could both potentially inhibit myogenesis.

STAT3 is known to be expressed during myogenesis (Kami and Senba, 2002; Sun et al., 2007; Trenerry et al., 2011; Wang et al., 2008; Yang et al., 2009) and is a component of pathways that both promote myoblast proliferation whilst inhibiting differentiation (JAK1/STAT1/STAT3)(Sun et al., 2007) and also drive myotube differentiation (JAK2/STAT2/STAT3)(Wang et al., 2008; Yang et al., 2009). Potentially inhibition of STAT3 would affect the activity of both these JAK/STAT pathways, however, experimentally loss of STAT3 reflects the expected outcome for the loss of JAK2/STAT2/STAT3 driven differentiation, i.e. loss of differentiation, rather than the enhanced differentiation that would be expected following loss of JAK1/STAT1/STAT3 (Sun et al., 2007; Wang et al., 2008). The ability of RKIP to inhibit STAT3 could therefore potentially result in the inhibition of differentiation seen in this study.

Similarly GSK3β is a key component in the canonical Wnt signalling pathway (MacDonald et al., 2009; Rao and Kuhl, 2010), which is one of the key inducers of myogenesis in the embryo (Abu-Elmagd et al., 2010; Anakwe et al., 2003; Cossu and Borello, 1999) and myoblasts in vitro (Bernardi et al., 2011; Tanaka et al., 2011). GSK3β acts as a negative regulator of signalling as it phosphorylates β-catenin, a key component of the transcriptional complex, and promotes its degradation resulting in a loss of signalling (MacDonald et al., 2009). In cultured cells RKIP has been shown to bind and stabilise GSK3β (Al-Mulla et al., 2011), thus increasing its activity and decreasing canonical Wnt signalling, which could decrease myogenic differentiation.

Given that the effects of RKIP on both STAT3 and GSK3β would be predicted to give the decrease in myogenesis seen in this study these pathways represent attractive targets for further investigation.

In conclusion this study demonstrates for the first time that RKIP, a multifunctional signalling regulator, is an important regulator of myogenesis in both avians and mammals. However, further study is required to determine the exact mechanism by which RKIP inhibits muscle differentiation.

Experimental Procedures

Wholemount in situ hybridisation.

For generation of RKIP riboprobe the open reading frame (ORF) and a portion of the 3' untranslated region were amplified by RT-PCR from total RNA generated from Hamburger and Hamilton stage 20 chick embryos using the following primers 5' CCATGGCGGTGGAGCTG and 3' CTTTGAGTGGCAACAGAGATCAG. The resultant PCR product was ligated into pGEM Teasy and this plasmid linearised and used for the generation of riboprobes. Digoxygenin-labelled antisense riboprobes for chick RKIP and Myogenin were produced by transcription with the appropriate RNA polymerase and Dig RNA labelling mixture (Roche). Wholemount in situ hybridisation was then performed on either whole chick embryos, or for older embryos (stage 27-29) sections (50 µm) of agar embedded wing, essentially as described previously (Nieto et al., 1996).

Immunofluorescence

For immunofluorescent staining cells and tissues were first permeabilised with a 15 minute incubation in cold (4°C) permeabilisation buffer (20 mM hepes, 300 mM sucrose, 50 mM NaCl, 0.5% triton X100, 3 mM MgCl, 0.05% Na azide). Cells/tissues were then washed in PBS (3x5minutes) and incubated for 1hour in wash buffer (5% FCS, 0.05% w/v Na azide, in PBS). Samples were then incubated in the appropriate primary antibody before washing (3x10 minutes) in wash buffer and addition of appropriate fluorescently labelled secondary antibody (Alexa 488 or Alexa 555). Primary antibodies used in this study were RKIP (1:200, Santa Cruz), Pax7 (1:4, Developmental Studies Hybridoma Bank), MyoD (1:200, Santa Cruz), MHC (1:10 Developmental Studies Hybridoma Bank), pERK (1:200), vGAG (3C2, 1:4, Developmental Studies Hybridoma Bank).

Production of retrovirally infected chick fibroblasts and viral supernatants

To generate the RCAS RKIP retroviral vector for use in chick studies the ORF of chick RKIP was amplified by PCR using the 5' primer described above plus the 3' primer CTACTTCCCAGACAGCTGCTC. The resultant PCR product was then digested with NcoI and ligated into the NcoI/SmaI sites of the shuttle vector pSlax12Nco. The inserted sequence was then removed from the shuttle vector by digestion with ClaI and inserted into the ClaI site

of RCAS(BP)A. RCAS RKIP and control RCASA infected cells for grafting were then produced and grafted as described below.

DF1 chick fibroblasts were routinely cultured in DMEM containing 10% FCS and antibiotics (Penicillin/Streptomycin). For transfection with RCASA and RCAS-RKIP plasmids cells were plated in 24-well plates and incubated for 6 hours with 2 µg of DNA and 2 µl of Transfectin transfection reagent (Bio-Rad) according to the manufacturer's instructions. Cells were then passaged, until all cells were positive for viral GAG, prior to grafting or viral harvest and concentration. In some experiments a virus expressing GFP tagged RKIP was used in which case cells were verified as producing GFP tagged protein and also being viral GAG positive. Similar results were produced with tagged or untagged RKIP protein. For viral harvest cells were amplified into T75 tissue culture flasks and grown in a minimum amount (3 ml) of low serum (2%) DMEM which was harvested twice daily and stored at -80°C. Viral supernatants were concentrated by pelleting the virus by centrifugation for 3 hours at 48,000g followed by re-suspension in a small volume of medium.

Micromass cultures

Limb buds were harvested from stage 20-22 chick embryos, washed briefly in PBS, and transferred into 0.25% Trypsin/EDTA at room temperature for ectoderm removal. Following ectoderm removal the mesenchymal cells of the limb bud were dispersed by incubating in fresh 0.25% trypsin EDTA at 37°C with agitation. Once dispersed into a single cell suspension trypsinisation was stopped by addition of DMEM/FCS and cell number counted. Cells were then pelleted by centrifugation and re-suspended in DMEM/FCS/antibiotics at 10^7 cells/ml and an MOI of 250-500 of the appropriate RCAS virus. Cells were then plated as 10 µl spots in 24-well plates and allowed to attach for 1-2 hours before flooding with medium. After 4 days the cultures were fixed in 4% PFA for analysis of muscle differentiation by immunostaining for MHC and MyoD.

Grafting of infected fibroblasts in vivo.

High density cells suspensions of retrovirally infected DF1 chick fibroblasts were produced as follows. Infected cells, produced as described above, were detached by

trypsinisation before addition of DMEM/FCS/Antibiotics and pelleting by centrifugation (1000RPM, 5minutes) in a microcentrifuge. The supernatant was then removed leaving just enough medium to resuspend the cell pellet to give the final high density suspension. For injection into presomitic mesoderm and embryonic limb fertilised eggs from White Leghorn chickens (Henry Stewart, UK) were incubated, until the 12-16 somite stage or embryonic stage 20-22 respectively, and a small region of shell removed to allow access to the embryo. The cell suspension was then injected into the presomitic mesoderm (3-4 somites length) or the mesenchymal core of the wing bud using a pulled glass capillary needle before resealing the shells with tape and reincubating. At the end of each experiment embryos were removed, washed in PBS, and fixed in 4% PFA for analysis by in situ hybridisation for gene expression.

Generation of stable RKIP overexpressing/RNAi C2C12 populations.

To produce vectors expressing human RKIP the ORF of human RKIP was amplified by RT-PCR from osteoblast total RNA using the following primers, 5'1 tcgccatggcggtggac and 3'1 gatc**ctcgag**cttcccagacagctgctcg. The resulting amplified ORF lacks a stop codon but contains a XhoI site (shown in bold text) that was used to fuse the ORF to an eGFP sequence previously inserted into the multicloning site of pCDNA3.1+.

To generate an RKIP mutant that could not be phosphorylated Serine 153 was mutated to Alanine, which cannot be phosphorylated, and to create a phosphomimetic RKIP Serine 153 and Lysine 157 were changed to Glutamic acid. Overlapping fragments of the ORF containing each mutation were generated by PCR using the 5'1 and 3'1 primers above in combination with one of the following two pairs of primers, 5' caaggtggcg**gcg**ttccgtaaaaag and 3' ctttttacggaa**cgc**cgccaccttg for S153A and 5' caaggtggcg**gag**ttccgtaaa**gag**tatgagctcag and 3' ctgagctcata**ctc**tttacggaa**ctc**cgccaccttg for SK153EE (mutated codons are shown in bold text). The resulting pairs of PCR products were then stitched together by PCR using the two overlapping PCR products as template with 5'1 and 3'1 primers described above. The full length mutated ORFs were then inserted into eGFP tagged pCDNA3.1+ as above.

RNAi vectors targeting mouse RKIP were generated by annealing the following DNA oligomers (RKIP target sequences shown in bold text) and ligating into the EcoRI and BamHI sites of the vector pLVXshRNAi2 (Clontech).

1. gatccCCGG**CAGCAACAAGTCTGGAGACAA**CTCGAG**TTGTCTCCAGACTTGTTGCTG**TTTTTg

2. aattcAAAAA**CAGCAACAAGTCTGGAGACAA**CTCGAG**TTGTCTCCAGACTTGTTGCTG**CCGGg

This vector, and a control GFP RNAi vector, were then used to derive multiple clonal cell populations.

C2C12 cells were routinely grown in DMEM/10%FCS/antibiotics and all analyses were undertaken on cells at less than passage 25. For generation of stable cell populations cells were plated in 24-well plates and transfected when 70-75% confluent.

For GFP-tagged RKIP and GFP overexpressing lines C2C12 cells were transfected with 2 µg DNA and 2 µl transfection reagent (Transfectin (Bio-Rad) or Turbofect (fermentas)) and stable overexpressing cells, evident by their production of GFP, selected with G418 (800 µg/ml). Cells were then maintained as mixed clonal populations in medium containing 200µg/ml G418 throughout culture. For each construct (GFP, RKIP WT, RKIP SA and RKIP EE) four separate populations of mixed clonal cells were generated and assayed.

For RKIP and GFP RNAi lines C2C12 cells were transfected with 2 µg RNAi plasmid DNA plus 200 ng pCDNA 3.1+ DNA and 2 µl transfection reagent (Transfectin (Bio-Rad) or Turbofect (fermentas)). The RNAi plasmid vector has no antibiotic resistance but contains a GFP, however addition of pCDNA to the transfection mixture allows the short term (<1week) selection with G418 to remove non-transfected cells. This enhanced cell population was then plated at low density into 10 cm dishes, in normal medium, and the cells cultured until they formed distinct clonal colonies. Clones that expressed GFP and therefore contained the RNAi vector were chosen and ring-cloned to produce multiple clonal GFP-RNAi and RKIP-RNAi populations for assay.

C2C12 Differentiation Assays.

To assess myogenic differentiation cells were plated (40,000 cells/well) into 24-well plates and cultured for 2 days until almost confluent. The medium was then changed to a differentiation medium (DMEM/2% Horse serum/antibiotics) to promote the formation of myotubes. After 4 days in differentiation medium cultures were washed with PBS and fixed for 10 minutes in methanol.

Myogenic differentiation was assayed by either staining for Myosin Heavy Chains and counting the percentage of nuclei present in MHC positive myotubes (5-10,000 nuclei counted per treatment) or measuring the percentage area of differentiated myotubes per culture.

Four independently derived populations for each cell line were tested for differentiation on three separate occasions each producing similar results.

For the RNAi experiments cultures were stained and assayed as described previously (Velica and Bunce, 2011). Briefly, Jenner and Giemsa staining was undertaken, to colour myotubes purple and myoblasts pink, followed by analysis using Image J software (NIH) to calculate the area of myoblasts per culture. Each Culture was assessed on two to three separate occasions.

Proliferation assays

Populations of cells to be assayed were plated in duplicate in 24 well plates (6 wells per cell line) at a density of 10,000 cells/well. One of the duplicates was fixed with 4% PFA after 1 hour (cell attachment) and the second after 24 hours before both were stained with DAPI. The numbers of cells at the start and end points were then determined by counting 4 fields per well to determine an average value for each well. The fold increase was then determined by dividing the final numbers of cells for each well by the average starting number per well for each cell line.

Duplex RT-PCR analysis.

To determine mRNA expression in cultures total RNA was extracted using Trizol reagent for RT-PCR analysis. To ensure the procedure was quantitative the reactions were set up using primer ratios of 4:1 assayed gene:GAPDH internal standard as a duplex reaction. For each sample the whole procedure was carried out as a single tube RT-PCR reaction with a 1 hour reverse transcription and 25-35 cycles of PCR. The ratio of primers in the reaction and numbers of cycles of PCR were determined to ensure the reactions were stopped during their exponential phase of amplification. Primers used were Myosin Heavy Chain 5' ctgtgatgcattatgggaac, 3' gttcttgaaggaggtgtctg; Myogenin 5' ctgatggagctgtatgagac, 3' aggcaacagacatatcctc; RKIP 5' gatcctgggaaactctacac, 3' ctaagaactcccaatgagag; GAPDH 5' cagtggcaaagtggagattg, 3' ggttcacacccatcacaaac. In all cases primer pairs span more than one

exon therefore any product produced from genomic DNA would be significantly larger than that from mRNA. Each RT-PCR was undertaken at least twice and produced similar results on both occasions.

SDS-PAGE and Western blot analysis

Total protein was isolated from cultures of C2C12 myoblasts, immediately before and following 4 days growth in differentiation medium, quantified and normalised. Proteins were separated by SDS-PAGE and transferred onto a PVDF membrane (Sigma) for Western blot analysis. Membranes were blocked in 5% BSA (Bovine Serum Albumin, Sigma) in TBST (Tris-buffered saline, plus Tween-20) for 1 hour at room temperature, followed by an overnight incubation at 4^{o}C with P-p44/42 MAPK (T202/Y204) primary antibody (Cell Signalling Cat#9101S, 1:1000 dilution) diluted in 5% BSA/TBST. Following three 5 minute washes in TBST, membranes were exposed to goat anti-rabbit IgG-horseradish peroxidase (Dako) secondary antibody diluted in 5% non-fat powdered milk (Marvel)/TBST (1:10,000 dilution) for 1 hour at room temperature. Membranes were subjected to five 5 minute washes in TBST at room temperature and horseradish-peroxidase-conjugates were detected using enhanced chemiluminescence (200 mM Tris pH 8.5, 250 mM Luminol and p-Coumaric plus 30% H_2O_2 and H_2O) and Super RX film (Fuji). Films were developed using an automated developer (Konica SRX-101A). For the detection of p44/42 MAPK, complexes were removed by incubating the membrane in a 50^{o}C water bath for 30 minutes in stripping solution (62.5mM Tris-HCl pH 6.7,100 mM β-mercaptoethanol, 2% SDS), followed by three 15 minute washes in TBST, to remove any residual stripping buffer. The membrane was re-blocked with 5% BSA/TBST at room temperature for 1 hour, and p44/42 MAPK was detected as previously described using p44/42 MAPK primary antibody (cell signalling Cat#9102, 1:1000 dilution), and goat anti-rabbit IgG-horseradish peroxidase (Dako) secondary antibody (1:10,000 dilution).

Figure Legends.

Figure 1. RKIP is expressed in the developing musculature of the embryonic chick limb. RKIP expression was determined by whole mount in situ hybridisation on embryos (A, B and C) followed by cryosectioning (A1, C1) or on agar embedded sections (D1, D2, E1). Chick

embryo stages (Hamburger and Hamilton) are shown in the lower left corner of each set of images. Gene expression appears purple and dashed lines in A, C, D and E show the plane and position of section of the corresponding section images. In all images black asterisks show the position of either cartilaginous skeletal elements (D2,E2) or condensing pre-cartilaginous mesenchyme (C1). Arrows in C, C1, D1 and white asterisks in D2 and E1 indicate the developing musculature (myogenin positive in D1, D2 and E1).

Figure 2. RKIP expressing virus does not spread well in chick embryos and induces somite malformations.

A,B In situ hybridisation for GFP showed that injection of retrovirally infected cells into the PSM of 12-16 somite stage chick embryos results in widespread expression of GFP virus (A) but only patches of infection following injection of RKIP-GFP fusion infected cells (B). Panels A1 and B1 show sections through the trunk of embryos A and B respectively. Panels A2 and B2 show immunofluorescent localisation of viral GAG in sections A1 and B1 verifying the limited viral infection in myogenic tissues (white arrows) in RKIP infected embryos compared with controls which have extensive expression in muscle. C,D Analysis of muscle differentiation (myogenin expression; shown in purple) showed regions in RKIP injected embryos where differentiation appeared decreased (black asterisks in D) but these regions also corresponded to areas where the somites appeared smaller and more irregular (white asterisks in D).

Figure 3. Overexpression of RKIP in developing chick limb inhibits myogenesis.

RKIP, or control RCASA, infected chick fibroblasts were injected into embryonic chick wing buds at Stage 20-22 and myogenesis was assessed 48 hours later. A-D show in situ hybridisation for myogenin showing loss of myogenin in several developing muscles in wholemount (A, B) and cryosection (C, D) of the RKIP-infected wing (asterisks in B, arrows in D) compared with the contralateral control wing (asterisks in A, arrows in C). E and F show immunofluorescent staining for MHC-positive myotubes indicating the loss of myotubes in RKIP-infected (arrows in F) compared with the contralateral control (arrows in E) wings. G and H show immunofluorescence for viral GAG showing presence and spread of virus in the

infected (H) but not the contralateral control (G) wing. In control RCASA grafted embryos no differences were seen in myogenin expression between grafted and contralateral control wings.

I,J Long term incubation following injection of RKIP infected cells into wing bud mesenchyme often results in severe chondrodysplasia (J compared with the contralateral non injected wing I) thus limiting the time when muscle development can be assessed.

Figure 4. Overexpression of RKIP in micromass culture inhibits myogenesis. Micromass cultures of HH22 chick limb buds were infected with RCAS-RKIP, or control RCASA, and myogenesis (numbers of myotubes/myoblasts) assessed after 4 days in culture. A and B show immunofluorescent staining of MHC (green) and MyoD (red) in control (A) and RKIP-overexpressing (B) cultures. There are fewer green MHC expressing myotubes in RKIP overexpressing cultures compared with controls. There are also increased numbers of single MyoD-positive (red nuclei indicated by arrows) myoblasts in RKIP infected cultures compared with control cultures. Numbers of newly differentiated myotubes, MHC- and MyoD-positive (arrowheads in C, D) are similar. C shows graphical representation of the data where each bar represents the mean±SD. Statistical analysis between Control and RKIP expressing cultures was by the student T-test with $P<0.05$ considered significant. Scale bars in A and B are 100µm.

Figure 5. RKIP expression and ERK activation in C2C12 myoblasts and differentiating myotubes. Cultures of C2C12 myoblasts differentiated for up to 5 days were assessed for RKIP expression and ERK activation by RT-PCR (A), Immunofluorescence (B,C,D) and Western blot (E) analysis. A shows mRNA expression of RKIP in C2C12 cultures form proliferating myoblasts (1) up to 5 day differentiated cultures (5). This shows that RKIP mRNA levels are relatively constant throughout differentiation compared with myogenin and Myosin Heavy Chain which increase with differentiation. B shows expression of RKIP protein (red) and MHC (green) positive myotubes. RKIP protein is clearly visible in both myoblasts (red in merged image) and a large number of differentiated myotubes (yellow in merged image). C shows pERK (red) and MHC (green) positive myotubes. Activated phosphorylated ERK is clearly visible in a subset of myotubes (yellow in merged) but absent (or at very low levels) in myoblasts. D shows negative (no primary antibody control) stained for MHC showing lack of non-specific staining. E. Western blot of pERK and ERK in control (GFP) and RKIP

overexpressing (RKIP WT) C2C12 cells maintained in growth or differentiation medium for 5 days. Levels of pERK are comparable in GFP and RKIP expressing cells cultured in growth medium but when cultured in differentiation medium absolute ERK and phosphoERK-42 levels are increased in control compared with RKIP expressing cultures. Scale bars in B-D are 200µm.

Figure 6. Overexpression of RKIP in C2C12 myoblasts inhibits myogenesis. Transgenic populations of C2C12 myoblasts expressing WT and mutant (S153A and S153/7EE) RKIP proteins c-terminally tagged with GFP, plus a GFP control, were established and assessed for RKIP production. A-D show expression of GFP in these cultures indicating that these cells produce either GFP (control) or GFP tagged RKIP. Levels of RKIP transgene expression were also assessed by duplex RT-PCR (E) and these were largely comparable for the different RKIP populations but absent from the GFP control as expected. These populations were subsequently assessed for their ability to differentiate in vitro in response to serum starvation. F-I show representative images from control GFP (F) and RKIP-overexpressing (WT, G; S153A, H; S153/7EE, I) cultures stained for Myosin Heavy Chains. Compared with controls (F) there are fewer myotubes in WT and non-phosphorylated RKIP overexpressing cultures (G,H) but no difference with phosphomimetic RKIP (I). J and K shows a graphical representations of the percentage of nuclei incorporated into MHC positive myotubes and percentage area of myotubes per culture respectively. Results are shown as mean±SD. L shows proliferation rates in populations of myoblasts expressing either GFP (average for 4 different populations) or RKIP proteins (2 different populations for each). Statistical analyses were by Anova followed by a multiple comparison test with $p<0.05$ considered significant. a, $P<0.0001$ compared with GFP control; b $P<0.0001$ comparted with RKIP EE; c, $P<0.0002$ compared with RKIP EE; d, $P<0.0002$ compared with GFP; e, $P<0.004$ compared with GFP; f, $P<0.002$ compared with RKIP EE. Scale bars in A-D are 100µm and F-I are 200µm.

Figure 7. Knockdown of RKIP expression enhances myogenesis in C2C12 cells. Stable clonal populations of C2C12 cells containing RNAi vectors for RKIP or GFP were assessed for their ability to differentiate following serum starvation. Analysis shown is for two of four GFP RNAi populations, which all showed similar levels of differentiation, and four RKIP RNAi populations. Analysis showed that in the RKIP RNAi clones differentiation was

enhanced compared with the GFP RNAi clones. A and B show representative cultures for GFP RNAi (A) the most differentiated control clone and RKIP RNAi 7a (B). C shows a graphical representation of percentage area of myotubes per culture for each clone plus the overall averages for Control and RKIP RNAi. Results shown are mean±SD for each. Statistical analysis for clonal comparisons was by anova followed by multiple comparison and for average control vs RKIP data student T-test. a, $P<0.0001$ vs GFP RNAi 2; b, $P<0.0001$ vs GFP RNAi 4; c $P<0.0005$ vs GFP RNAi 4; e $P<0.005$ vs average GFP control value. D shows results for duplex RT-PCR analysis of RKIP expression in clones before (myoblasts) or after differentiation (myotubes) demonstrating that RKIP RNAi clones show significantly ($P<0.05$), on average approximately 50%, lower expression of RKIP. Scale bars in A,B are 200µm.

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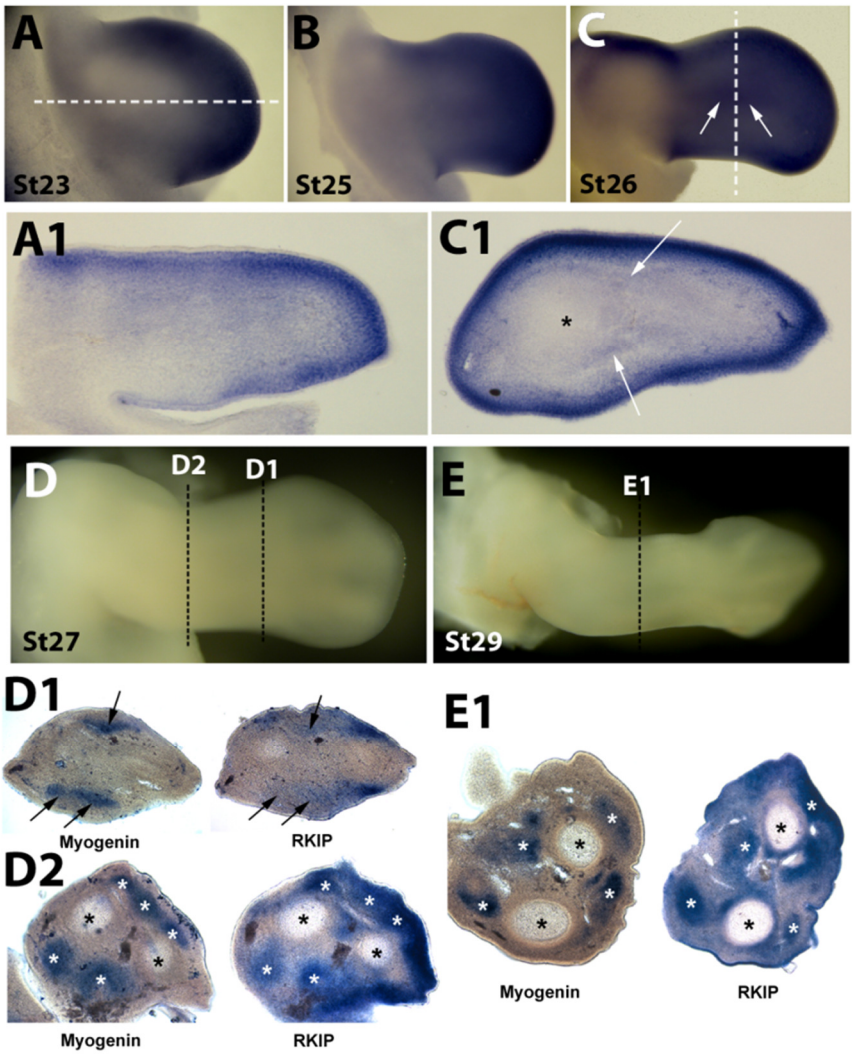
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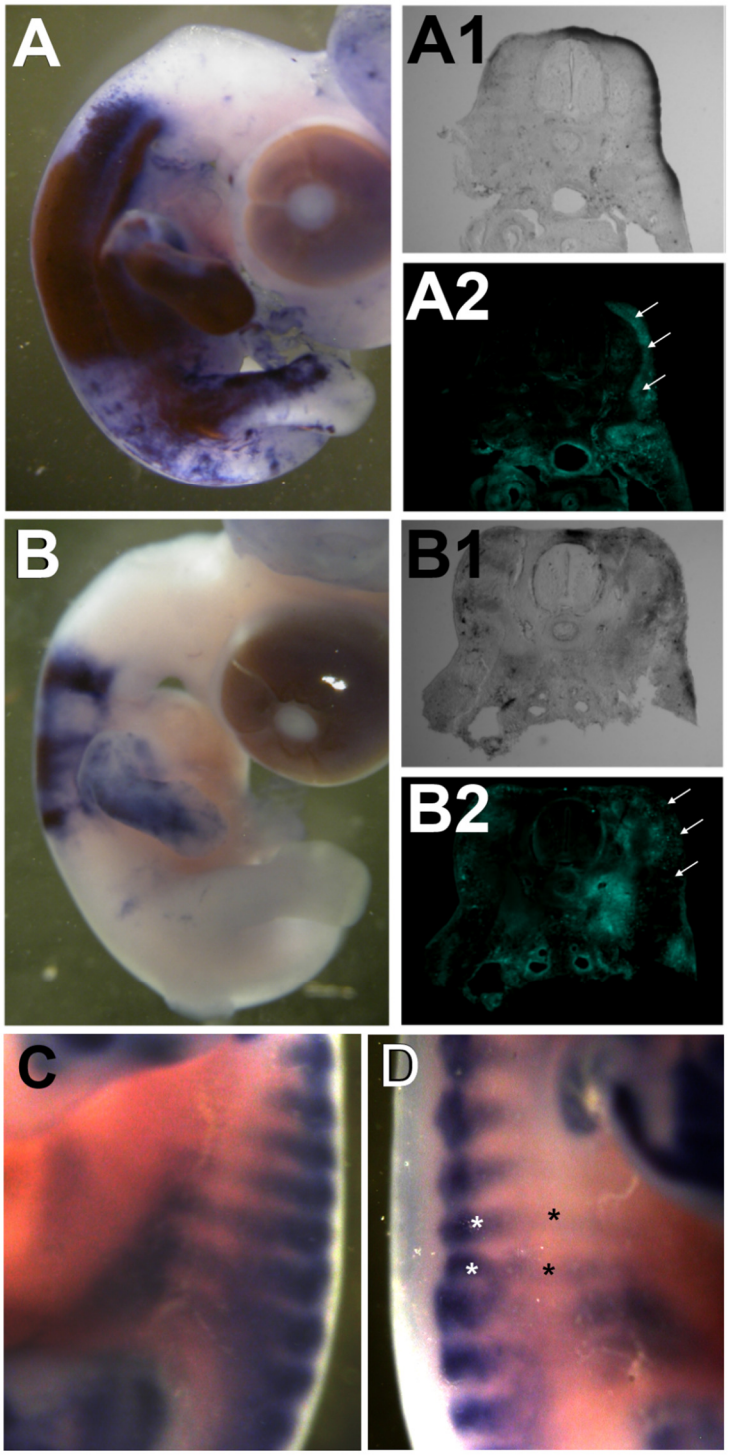
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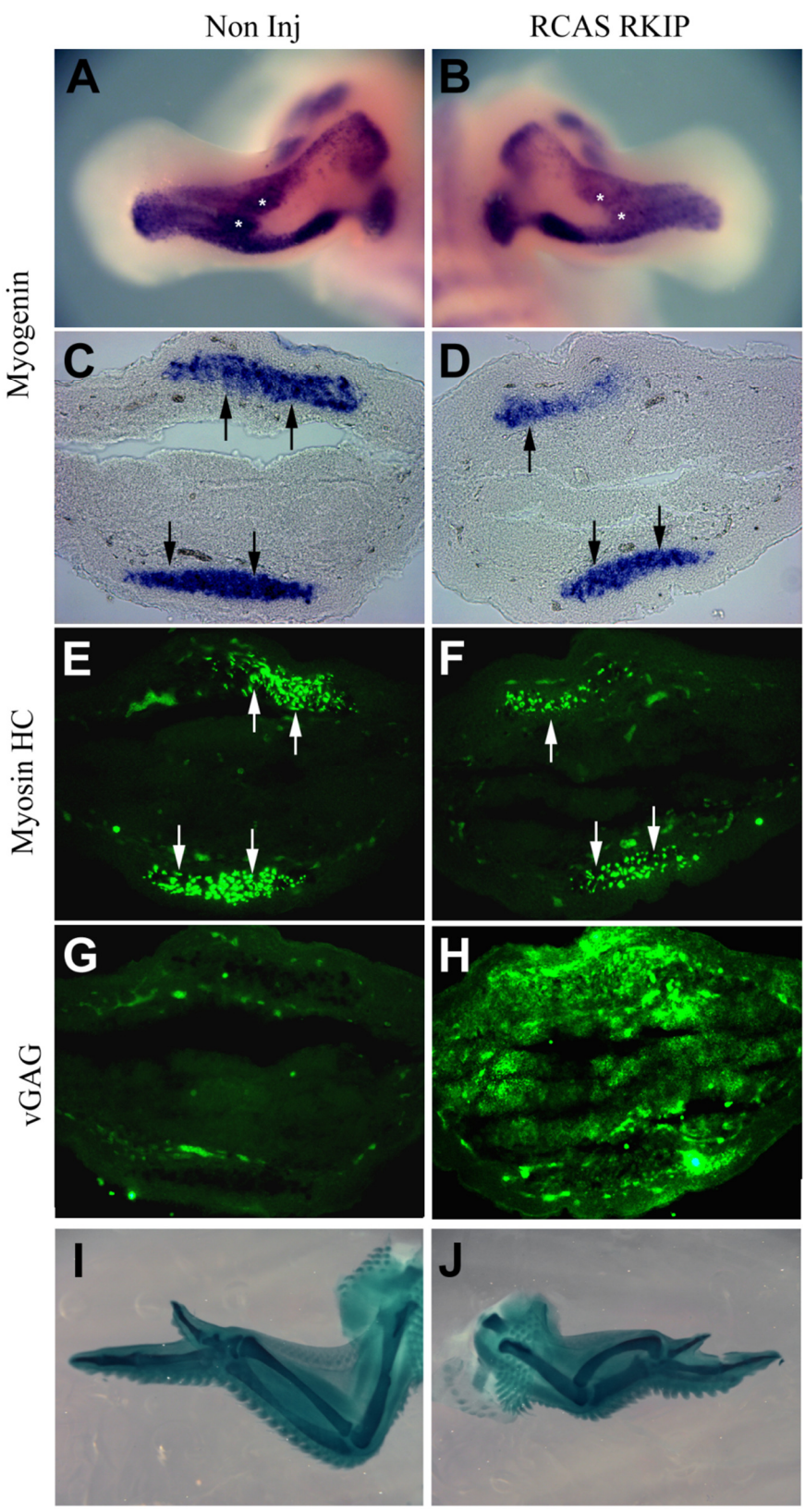
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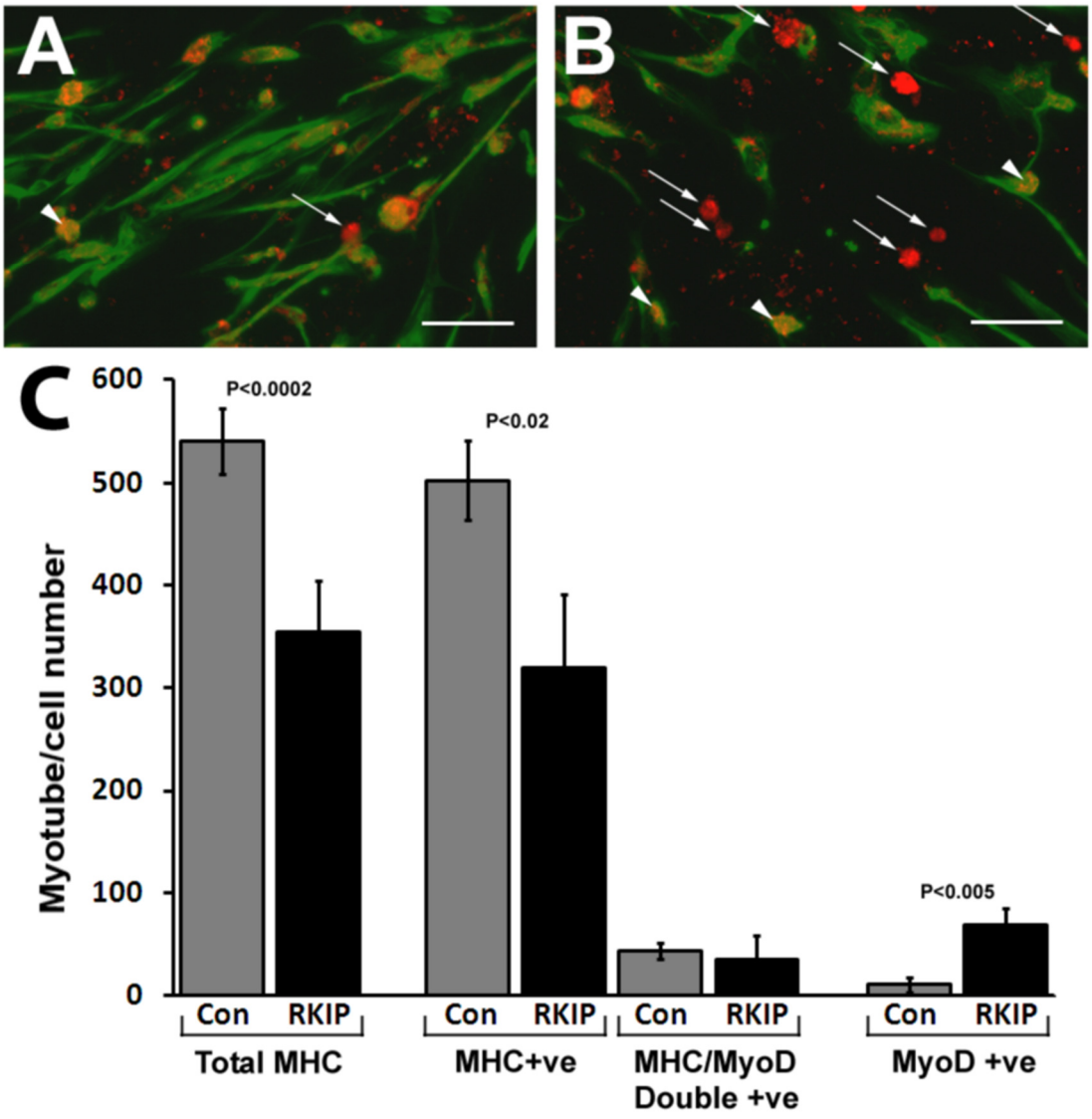
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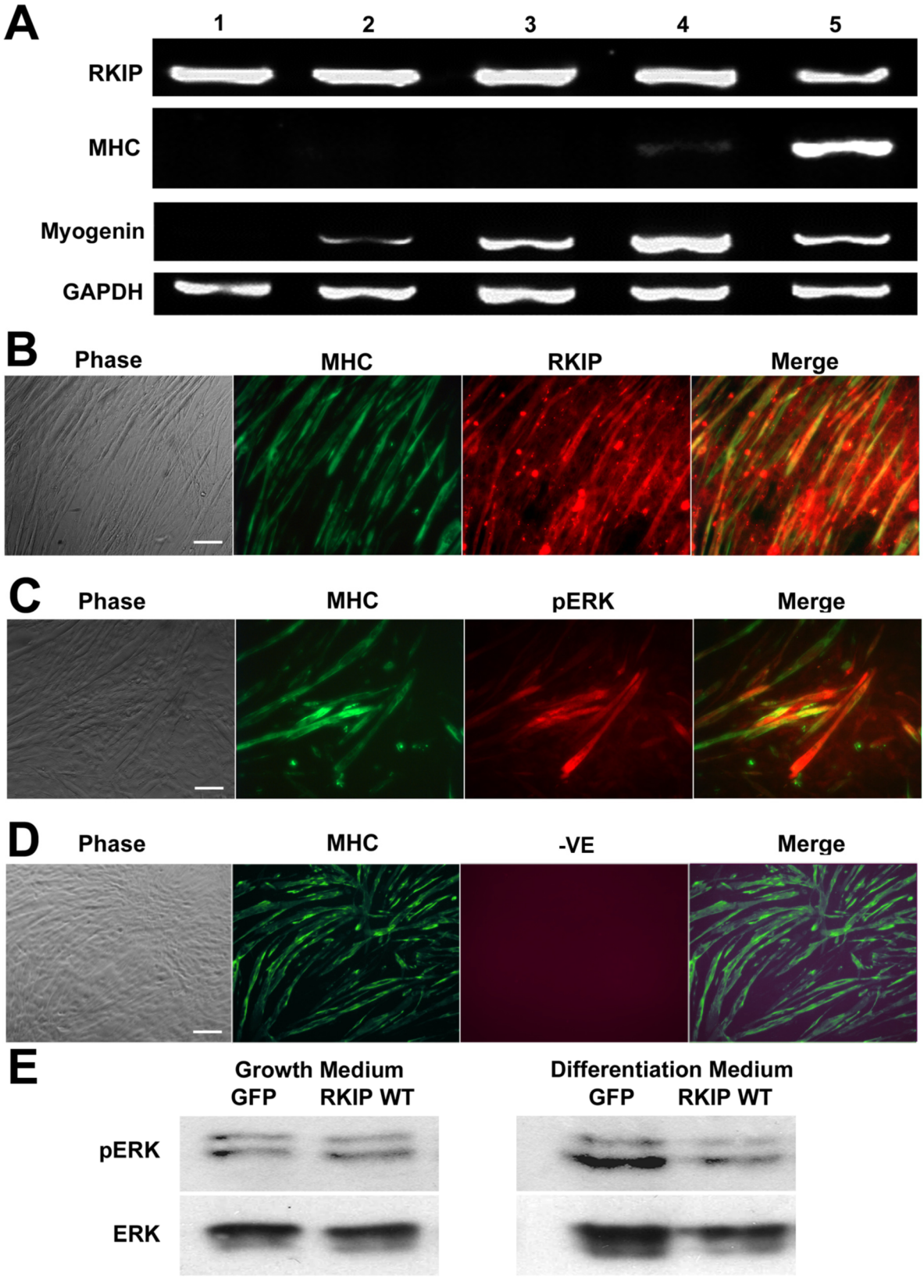
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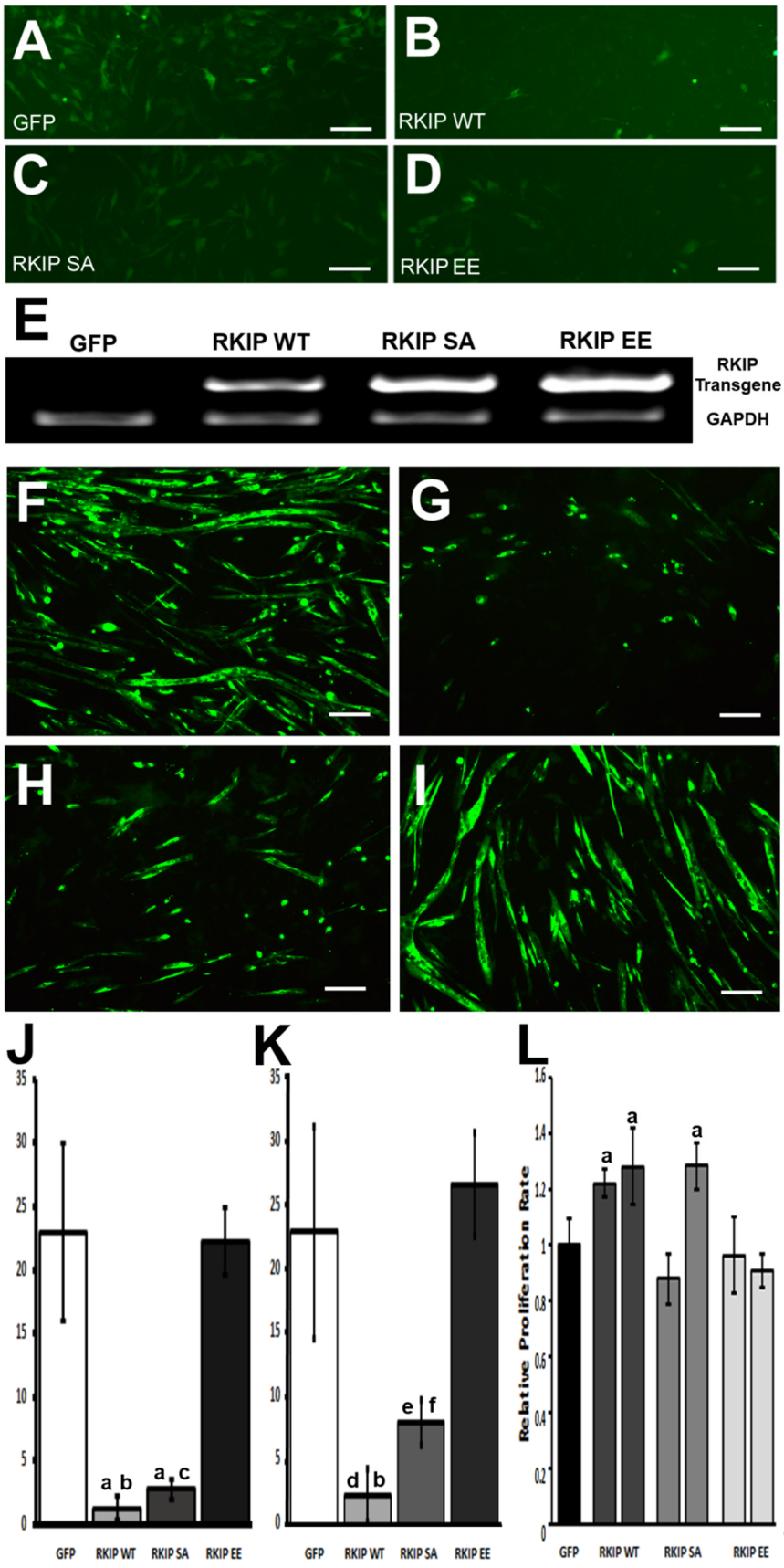
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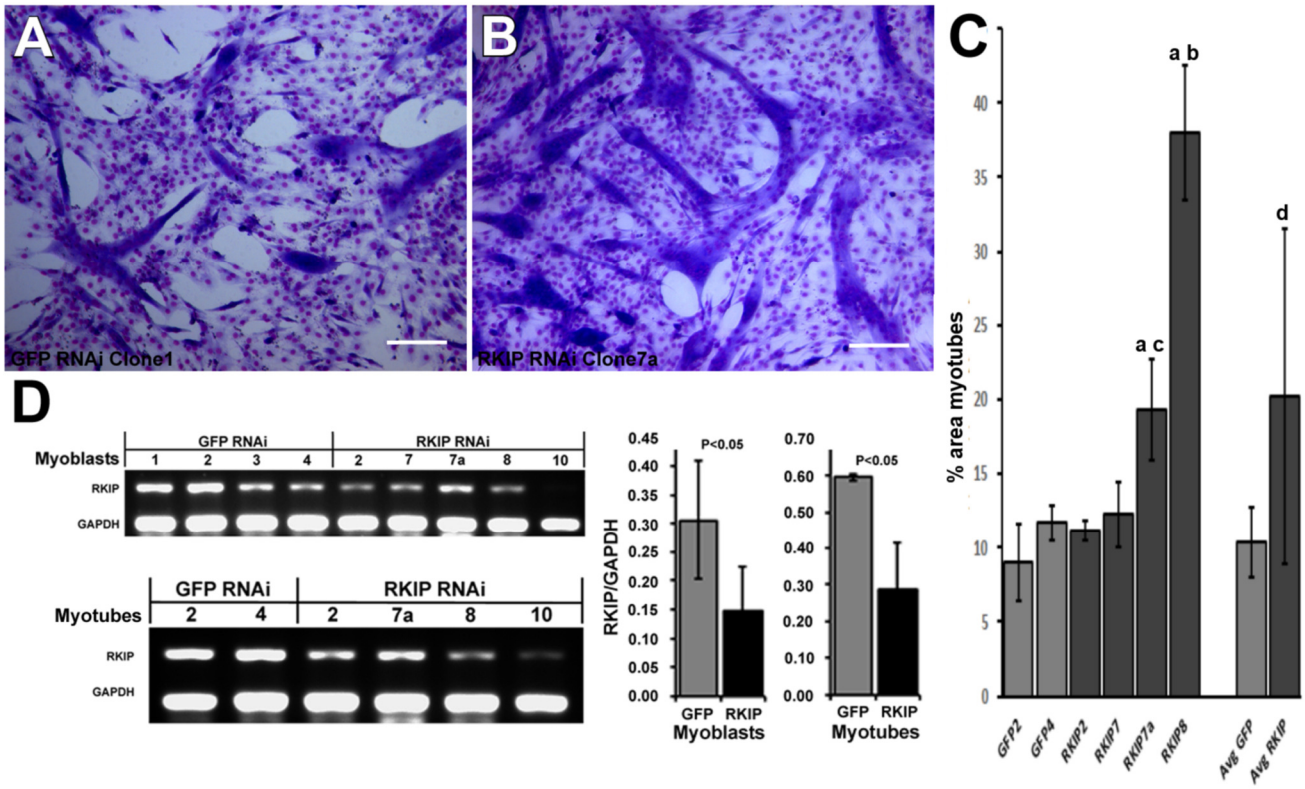
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