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Skeletal muscle myogenesis is regulated by G protein-coupled receptor kinase 2

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Abstract

G protein-coupled receptor kinase 2 (GRK2) is an important serine/threonine-kinase regulating different membrane receptors and intracellular proteins. Attenuation of *Drosophila Gprk2* in embryos or adult flies induced a defective differentiation of somatic muscles, loss of fibers, and a flightless phenotype. In vertebrates, GRK2 hemizygous mice contained less but more hypertrophied skeletal muscle fibers than wild-type littermates. In C2C12 myoblasts overexpression of a GRK2 kinase-deficient mutant (K220R) caused precocious differentiation of cells into immature myotubes, which were wider in size and contained more fused nuclei, while GRK2 overexpression blunted differentiation. Moreover, p38MAPK and Akt pathways were activated at an earlier stage and to a greater extent in K220R-expressing cells or upon kinase downregulation, while the activation of both kinases was impaired in GRK2-overexpressing cells. The impaired differentiation and fewer fusion events promoted by enhanced GRK2 levels were recapitulated by a p38MAPK mutant, which was able to mimic the inhibitory phosphorylation of p38MAPK by GRK2, whereas the blunted differentiation observed in GRK2-expressing clones was rescued in the presence of a constitutively active upstream stimulator of the p38MAPK pathway. These results suggest that balanced GRK2 function is necessary for a timely and complete myogenic process.

Introduction

Skeletal muscle mass is maintained by signaling pathways that control cell proliferation, differentiation, protein synthesis and degradation. Loss of muscle mass occurs in multiple settings including cancer, AIDS, cachexia, and neuromuscular disorders, as well as during aging, and is an important factor contributing to morbidity (Glass, 2010b). On the other hand, skeletal muscle hypertrophy, the increase in muscle mass that occurs as a result of an increase in load, is characterized by increased fiber size and protein synthesis (Serrano et al., 2008). Understanding the molecular pathways that regulate gain or loss of muscle mass is therefore crucial for treating muscle wasting-associated disorders. In *Drosophila melanogaster*, fusion between two different types of cells leads to the formation of a muscle precursor cell/myotube, which further fusion events transform into a multinucleated myotube. In vertebrates, skeletal myogenesis is initiated by the generation of myoblasts followed by their differentiation to myocytes and the formation of myofibers (Buckingham et al., 2003). In the mouse (*Mus musculus*), differentiated myoblasts elongate and migrate towards other myoblasts thus giving rise to a nascent and, later, to a mature myotube through fusion events among equivalent cells (Simionescu and Pavlath, 2011). In all cases, the determination of myoblasts and their differentiation are controlled by muscle regulatory factors that are activated at specific stages during myogenesis (Abmayr and Pavlath, 2012).

In vertebrates, at least two important signalling pathways are strictly required for the success of this multistep complex program of myogenesis: those of phosphatidylinositol 3-kinase (PI3-K) and p38 mitogen activated protein kinase (p38MAPK), which regulate the activation of key muscle transcription factors as MyoD. PI3-Ks are important mediators of tyrosine kinase receptor signal transduction. Their inhibition blocks myogenesis (Glass, 2010a) as so happens with Akt, an important downstream target of PI3-K (Fujio et al., 1999; Jiang et al., 1999; Conejo and Lorenzo, 2001). p38MAPK is also a central player in this process (Conejo et al., 2002; Perdiguero et al., 2007a) acting through several well described mechanisms (Lluis et al., 2006). p38MAPK are a family of Ser/Thr kinases, including p38 α MAPK, p38 β MAPK, p38 γ MAPK and p38 δ MAPK, that regulate important cellular processes such as stress responses,

differentiation and cell cycle control (Nebreda and Porras, 2000; Zarubin and Han, 2005). p38 α MAPK is the isoform involved in the induction of myogenesis (De Alvaro et al., 2008) and, consistently, treatment with p38 α/β MAPK inhibitors or experimental depletion of the p38MAPK α isoform prevent the fusion of immortalized myoblasts into myotubes, and also the induction of specific muscle differentiation genes (Cuenda and Cohen, 1999; Zetser et al., 1999; Li et al., 2000; Perdiguero et al., 2007a, b; Alonso-Chamorro et al., 2011), thus demonstrating the requirement of p38MAPK α/β during myogenesis (de Angelis et al., 2005; Keren et al., 2005). The p38MAPK pathway seems also to be involved in the modulation of skeletal muscle differentiation by cell-to-cell contact and adhesion (Krauss, 2010) or mechanical stimulation (Niu et al., 2013) and in the epigenetic control of muscle regeneration (Palacios et al., 2010). In marked contrast to the PI3-K/Akt pathway, little is known about the upstream modulators of the p38MAPK pathway in myogenesis other than MAPK kinase (MAPKKs) (Nebreda and Porras, 2000). Accordingly, it has been reported that forced activation of p38MAPK by ectopic expression of a constitutively active mutant of MKK6 is sufficient to override the inhibitory factors present in proliferating cells and to induce both the expression of differentiation markers and the appearance of multinucleated myotubes, reinforcing the idea that p38MAPK activity plays an essential role in muscle differentiation (Li et al., 2000; Wu et al., 2000; Perdiguero et al., 2007a). However, the mechanisms that regulate p38MAPK activity during muscle cell differentiation remain unidentified, although they appear to be different from those involved in the response to stress or cytokines.

G protein-coupled receptor kinase 2 (GRK2) is a ubiquitous, essential protein kinase that is emerging as an integrative node in many signalling networks. Moreover, changes in GRK2 abundance and activity have been identified in several inflammatory, cardiovascular disease, and tumor contexts, suggesting that those alterations may contribute to the initiation or development of certain pathologies (Penela et al., 2003; Salcedo et al., 2006; Garcia-Guerra et al., 2010). GRKs were initially identified as key players in the desensitization and internalization of multiple GPCRs, but GRK2 also phosphorylates several non-GPCR substrates

and dynamically associates with a variety of proteins related to signal transduction (Peregrin et al., 2006; Ribas et al., 2007; Penela et al., 2010) including, among others, certain p38MAPK isoforms (Peregrin et al., 2006). However the implication of GRK2 in the mechanisms controlling the regulation of skeletal muscle development and function as well as how this regulation could affect the functionality of this tissue remain to be investigated. In this study, we report the importance of GRK2 in the regulation of skeletal muscle myogenesis and elucidate possible downstream effectors.

Results

Grpk2 is required for myogenesis in Drosophila melanogaster

Drosophila is a model system that had been successfully used to analyze the effects of *Grpk2*, a GRK ortholog in the fly, on signaling of Smoothed (Molnar et al., 2007) and recapitulates the complex myogenesis process of vertebrates in a simpler manner. We reduced *Grpk2* activity by injecting *Grpk2* dsRNA into *Dmef2Gal4>UAS-GPF* embryos, and directly observed the muscle pattern under a fluorescence microscope. Injection of *Grpk2* dsRNA caused aberrant morphology and late detachment of many muscles, what worsened with time (Figure 1A-D). This phenotype indicates a defective differentiation of somatic muscles under attenuation conditions for *Grpk2*, suggesting a role for this protein in myogenesis.

Due to strong maternal contribution for *Grpk2* and its early requirement during oogenesis, the study of its function during embryonic myogenesis is not possible, so an in depth analysis was performed during *Drosophila* adult myogenesis. We focused on the development of a subset of the indirect flight muscles (IFMs), the dorsal longitudinal muscles (DLMs) that consist in six muscles per hemithorax (Fernandes et al., 1991). We first attenuated *Grpk2* function using the *1151Gal4* line to drive the expression of an RNAi silencing construct, the *UAS-Ri-Grpk2*, at 29°C in all adult muscle precursors and found a significant increase in the number of flies with a flightless phenotype (31%) compared with controls (9%) (Figure 1E). However, the over-expression with the same driver of the full-length protein had low effect in the flying ability of the adults (Figure 1E). When we made use of a combination of the Gal4/UAS and the Flp-mediated recombination systems for generating stronger loss-of-function conditions (see Materials and Methods, and (Duffy, 2002)) the penetrance of the flightless phenotype drastically increased to 100%. This phenotype was associated with a reduction in the number of DLMs already visible at 48h after puparium formation (APF) (Figure 1F-I), suggestive of a failure in the splitting of the DLM larval templates (Roy and VijayRaghavan, 1998). We also observed motor defects in a test for climbing ability in these flies: 3 day-old flies deprived of *Grpk2* function in adult muscles, showed a reduction in climbing speed compared to their control siblings (Supplementary Movies S1-2), demonstrating that in the absence of *Grpk2* the

development of DLM muscles is compromised. However, due to the late expression of the *1151Gal4* driver in adult muscle tendons (Soler et al., 2004), we could not discard a contribution of *Gprk2* tendon-requirement to the late phenotype. To investigate whether there is a late requirement of *Gprk2* function in DLM muscles we attenuated *Gprk2* in differentiating DLM muscles using the IFM-specific *UH3Gal4* driver (Katzemich et al., 2012). Besides an absence of any apparent defect in DLM splitting *UH3Gal4>UAS-Ri-Gprk2* flies were flightless (100%, Figure 1E), indicating a late requirement of *Gprk2* for muscle differentiation.

GRK2 hemizygous mice present less skeletal muscle fibers and increased fiber size

To investigate whether GRK2 could play similar functions in vertebrates, we analyzed hindlimb muscles (gastrocnemius) from wildtype (Wt) and GRK2^{+/-} mice. These haploinsufficient mice constitute a good model to recapitulate differences in GRK2 expression of the same range to those observed during pathological conditions (Ribas et al., 2007; Penela et al., 2010) as well as to evaluate the potential of therapeutic strategies aimed at globally decreasing GRK2 functionality *in vivo*. Downregulation of GRK2 expression levels in skeletal muscle of hemizygous mice were confirmed by immunoblot (Figure 2A) and also by qPCR (Figure 2B) and correlates with a decrease in kinase activity that can be detected in muscle lysates (data not shown).

We observed no differences in muscle weight (Figure 2C) between Wt and GRK2^{+/-} mice at 3 months of age in the absence of significant differences in either food or water intake (Supplementary Figure S1). Morphological analysis of muscle sections revealed a decreased number of fibers with wider cross sections in GRK2^{+/-} mice at both 3 and 9 months of age (Figure 2D-F). This decrease in fiber number in GRK2^{+/-} animals was not apparent in newborn mice (Figure 2G-I) although they already showed a larger fiber size (Figure 2H), suggesting that fiber loss was taking place after birth and before 3 months of age while fiber growth is an earlier phenotype. These results suggest that GRK2 plays an important role in regulating myofiber growth and integrity also in vertebrates.

GRK2 overexpression impairs muscle differentiation in C2C12 myoblasts while a kinase-deficient mutant accelerates this process, giving rise to immature myotubes

We analyzed the expression of GRK2 protein during C2C12 myoblast differentiation, a well-established *in vitro* model for this process (Liu et al., 2002; de Alvaro et al., 2005; De Alvaro et al., 2008). A moderate but significant decrease in GRK2 can be observed along C2C12 differentiation (which correlates with a decrease in kinase activity as quantified in cell lysates, data not shown) suggesting that GRK2 expression is regulated during this process (Figure 3A). To investigate the contribution of GRK2 to myogenic differentiation, we generated several stable C2C12 cell lines that moderately overexpress (circa 2-fold increase) two different GRK2 constructs: pcDNA3-GRK2-Wt (pc-GRK2) and pcDNA3- GRK2-K220R (pc-K220R) with a point mutation that impairs the kinase activity of GRK2 and inhibits events dependent on GRK2 kinase activity but not those due to scaffolding abilities (Figure 3B and Supplementary Figure S2).

C2C12 cells proliferate in culture as undifferentiated cells in high serum; upon confluence and serum withdrawal (differentiation medium), myoblasts differentiate into myocytes, which subsequently fuse into multinucleated myotubes (Buckingham, 2001; Jansen and Pavlath, 2008). As expected, differentiated C2C12 myoblasts display a multinucleated tubular morphology, which was similarly observed in control cells after 4 days in culture (Figure 3C and Supplementary Figure S2). Interestingly, pc-K220R myoblasts fully differentiated to an extent that appeared similar to that of pcDNA3 cells. We observed multinucleated myotubes in pc-K220R myoblasts whereas pc-GRK2 myocytes were primarily mononuclear, exhibiting a severe defect in their ability to form multinucleated cells, even after four or more days in low serum (Figure 3C and Supplementary Figure S2).

Differentiation of skeletal muscle cells involves the sequential expression of specific transcription factors such as MyoD, and also early and terminal differentiation markers such as myogenin and myosin heavy chain (MHC), respectively (Weintraub, 1993). As expected, pcDNA3 myotubes showed a temporal expression pattern of differentiation markers similar to that reported for C2C12 cells (De Alvaro et al., 2008). Interestingly, the elevation of MyoD and

myogenin levels started one day earlier in pc-K220R-expressing clones than in pcDNA3 myotubes (Figure 3D and Supplementary Figure S2) and remained significantly higher than in control cells all along the process even when terminal differentiation was achieved. On the contrary, late differentiation markers such as Caveolin-3 and MHC, indicative of mature myotubes, were expressed at lower levels in pc-K220R myotubes along the process (Figure 3D and data not shown) and Caveolin-3 remained below control levels even at day four. On the other hand, pc-GRK2 cells did not express any of these skeletal muscle differentiation markers after four or more days in low serum (Figure 3D and Supplementary Figure S2). This cannot be ascribed to increased cell death occurring in GRK-overexpressing clones (Supplementary Figure S3 and data not shown). These results clearly demonstrate that enhanced GRK2 expression abrogates differentiation, whereas interfering with GRK2 function triggers an untimely differentiation process with increased early differentiation markers and a decreased expression of proteins characteristic of fully mature myotubes.

GRK2 regulates the formation of membrane protrusions in myocytes

Formation of membrane protrusions caused by polymerization of cortical actin at the plasma membrane is a first step towards myoblast fusion into multinucleated myotubes (Stoker and Gherardi, 1991). At the beginning of the differentiation process, low serum induced the formation of membrane ruffles in pc-K220R and pcDNA3 myoblasts (Figure 4A and B), which resulted in an enhanced percentage of cells with fused nuclei (Figure 4A and C). pc-K220R myotubes presented higher number of nuclei and a wider diameter than pcDNA3 myotubes (Figure 4D and E). In contrast, pc-GRK2 myoblasts failed to form membrane protrusions and fusion was impaired (Figure 4A-C). These data suggest that GRK2 kinase activity negatively regulates the fusion process of myoblasts into myotubes and that this could be due at least in part to the inability to form membrane protrusions. On the other hand, interfering with GRK2 kinase activity generates membrane protrusions that are able to promote more fusion events per myotube.

Myoblast differentiation requires growth arrest and cycle cell exit, and thus we analyzed the expression of several cell cycle regulators such as p21 (Shen et al., 2003); retinoblastoma (Rb), (Poznic, 2009); and proliferating cell nuclear antigen (PCNA) (Shen et al., 2003; Essers et al., 2005). We detected Rb in the hyperphosphorylated state and PCNA protein in proliferating myoblasts of the transfectant cells. Four days of differentiation in low serum produced sequential growth arrest with up-regulation of the expression of p21, reduction of Rb phosphorylation and down-regulation of PCNA (Supplementary Figure S4). The percentage of G0/G1 cells after four days in low serum was significantly increased to the same extent in all three transfectants, thus indicating that all cells exited the cell cycle in a similar manner (Supplementary Figure S4). Thus, GRK2 does not play a role in the growth arrest associated to skeletal muscle differentiation.

GRK2 kinase activity regulates the Akt, p38MAPK and ERK routes during myogenic differentiation

Differentiation of pcDNA3 myotubes occurs with enhanced phosphorylation of Akt and decreased phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 in C2C12 cells (Figure 5A), in agreement with our previous observations (Conejo and Lorenzo, 2001; de Alvaro et al., 2005; De Alvaro et al., 2008). Interestingly, we detected a moderately increased Akt phosphorylation on Ser473 in pc-K220R myoblasts at the earliest steps of the process (already visible at days 1 and 2), indicating that Akt is prematurely activated in these cells (Figure 5A). Furthermore, ERK1/2 remained phosphorylated after 2 days in low serum, but this phosphorylation declined more abruptly from day 3 in pc-K220R cells (Figure 5A). Phosphorylation of Akt was hardly detectable in undifferentiated pc-GRK2- myoblasts, whereas a decreased phosphorylation of ERK1/2 after day two was also observed in these cells (Figure 5A).

We also observed a clear phosphorylation on the activation loop residues Thr180/Tyr182 of p38MAPK, an important pathway required for muscle differentiation (Lluis et al., 2006; Perdiguero et al., 2007a), after day two in pc-DNA3 myotubes, whereas this phosphorylation

was blunted in pc-GRK2 cells (Figure 5B). This activation of p38MAPK during the differentiation process was accelerated in pc-K220R cells (Figure 5B). Thus, upon interference with GRK2 kinase activity, there is a prompt and sustained activation of p38MAPK that probably helps establish the precocious but immature differentiation program detected in pc-K220R cells (see Discussion).

In line with these results, silencing of GRK2 by adenovirus-driven expression of an sh-construct specific for this kinase (Lafarga et al., 2012) led to a differentiation dynamics most reminiscent to that observed in K220R-expressing cells (Supplementary Figure S5), including the upregulated phosphorylation profile of both p38MAPK and Akt proteins (Figure 5C). These data indicate that the phenotypic changes detected when GRK2 levels vary depend mostly on its kinase activity and are related to an altered p38MAPK activation status. Also, Western blot analysis of GRK2-expressing cells (Figure 5B and Supplementary Figure S6A) showed a decreased amount of phospho-p38MAPK (Thr180/Tyr182) in pc-GRK2, whereas phospho-p38MAPK (Thr123), an inhibitory residue target for GRK2 located at the docking groove of the protein (Peregrin et al., 2006), was enhanced. In contrast, in pc-K220R cells we observed that the increase in phospho-p38MAPK (Thr180/Tyr182) paralleled a decrease in the phospho-p38MAPK (Thr123) signal of endogenous p38MAPK. These results may explain how lowering GRK2 activity enhances p38MAPK activation and myogenesis, were consistent with what has been already described during the adipocyte differentiation process (Peregrin et al., 2006), and suggest a possible role for Thr123-p38 phosphorylation in downregulating p38MAPK activation in myogenesis.

Modulation of the p38MAPK pathway correlates with the inhibitory effect of GRK2 on myogenesis

To understand whether phosphorylation of p38MAPK by GRK2 is involved in the effect of the latter on myogenesis, C2C12 myoblasts were transiently transfected with Flag-p38 α T123D, a p38MAPK mutant that mimics phosphorylation by GRK2, and with Flag-p38 α WT as a control (Supplementary Figure S6B). Then cells were induced to differentiate in low serum and fusion

parameters were studied. There were no significant phenotypic differences between Flag-p38 α WT and C2C12 cultures in any of the parameters analyzed. In contrast, myotube formation process in Flag-p38 α T123D cells was disrupted (Figure 6A and B). Accordingly, we observed that these cells failed to form membrane protrusions at the beginning of the differentiation process (Figure 6B and C) and this observation was accompanied by a low number of cells with fused nuclei (25% and 10% respectively) (Figure 6D). Consequently, the few myoblasts that fused into myotubes contained lower number of nuclei (Figure 6E) and displayed a shorter diameter (Figure 6F) than control cells.

Interestingly, when differentiation-incompetent GRK2-overexpressing cells were infected with a constitutively active mutant of the MKK6 protein (termed MKK6-E), a potent and direct activator of p38MAPK, a reversion of the GRK2-induced phenotype was detected, and cells started to differentiate in a manner more reminiscent of controls (Figure 6G), and the expression of key myogenic markers such as MHC (almost undetectable in control GRK2-cells 4 days after differentiation) was rescued along with stimulatory p38-phosphorylation in MKK6-E-infected GRK2-expressing cells (Figure 6H and Supplementary Figure S6C). These results demonstrate that overexpression of GRK2 negatively regulates the activation of signalling cascades implicated in skeletal muscle differentiation and that this inhibition can be overcome by activation of the p38MAPK pathway.

Discussion

Skeletal myogenesis is a highly orchestrated multistep process that involves the determination of multipotential mesoderm cells to give rise to myoblasts. Exit of myoblasts from the cell cycle is followed by an increase in the expression of myogenic transcription factors and formation of multinucleated myotubes and by the expression of myofibrillar proteins (Buckingham et al., 2003; Jansen and Pavlath, 2008). A timely regulation of skeletal muscle formation is essential for normal development. Its dysregulation is the basis for certain pathological conditions such as muscular dystrophies and inflammatory myopathies in which prominent muscle loss and degeneration take place (Glass, 2010b). In the present study, we characterize the mechanisms underlying the effects of GRK2 on muscle differentiation, and reveal the important role of this kinase in the regulation of the kinetics of the myogenic process.

Drosophila melanogaster constitutes one of the key model systems to study myogenesis since it recapitulates the process observed in vertebrates but simplified to one precursor cell giving rise to one myotube and one adult fiber. Using this model to target *Gprk2*, a *Drosophila* ortholog of vertebrate GRKs (Molnar et al., 2007), we have detected both in *Drosophila* embryos and in newborn flies severe muscular dysfunction accompanied by fiber loss upon *Gprk2* silencing or depletion. *Gprk2* silencing in adult flies impaired motor activities such as climbing and flight behavior, and this phenotype further deteriorated with age. A reduction in the number of DLMs present in *Gprk2*-silenced or depleted flies, which was more evident with age, demonstrated an essential role for *Gprk2* both on the differentiation and on the stability of muscles in this system. Importantly, GRK2 hemizygous mice show a significantly reduced fiber number in muscles both at 3 and at 9 months of age accompanied by an increased fiber size. This skeletal muscle hypertrophy can be regarded as a compensatory response of the tissue to try to counteract the decreased fiber number, or, instead, as a phenomenon triggered by GRK2 downregulation in parallel to fiber loss. This second hypothesis seems to better fit the results observed in vertebrates, where the same amount of fibers that are, however, already larger in size are detected in newborn mice. The contribution to the observed muscle phenotype of other cell

types that also present lower levels of GRK2 in hemizygous mice cannot be ruled out. However, the hypertrophic phenotype seems not to be specific for skeletal muscle, since we also detect a hypertrophy of cardiomyocytes of GRK2^{+/-} mice with aging (unpublished data) with no differences in basal blood pressure in both genotypes (Avendaño et al., 2014).

The observed phenotypes in *Drosophila* and *Mus musculus* are reminiscent of those observed for the attenuation of the inhibitor of muscle differentiation gene *him* (Soler and Taylor, 2009) and of the reduction of p38MAPK activity both in *Drosophila* and in vertebrates (Perdiguerro et al., 2007b; Vrillas-Mortimer et al., 2011) (discussed below). However, it cannot be ruled out that, as so happens with the Notch ligand delta 1 (Schuster-Gossler et al., 2007) (a known inhibitor of the differentiation of myoblasts to myocytes), GRK2 loss can provoke a premature and excessive differentiation that would eventually lead to a loss of muscle fibers.

In cell lines overexpressing GRK2, the expression of early and late key myogenic markers, such as MyoD and myogenin, or Caveolin-3 and MHC respectively, was strongly reduced in GRK2-overexpressing cells. Accordingly, GRK2-expressing myoblasts did not differentiate properly. This is consistent with the phenotype observed for mice deficient for these marker proteins that show severe defects during myogenesis (Sabourin et al., 1999; Galbiati et al., 2001). In K220R-expressing cells, a kinase-deficient mutant of GRK2 able to recapitulate events dependent on GRK2 scaffolding properties, but not those dependent on its kinase activity, the expression of early myogenic markers was not blunted, but rather accelerated what was indicative of a premature differentiation. This early maturation process was apparently successful in giving rise to multinucleated myotubes since, at day 4, K220R cells were morphologically indistinguishable from control cells. However, K220R myotubes did not reach the same degree of maturity as control cells, as indicated by the expression pattern of Caveolin-3 or MHC.

In the absence of apparent differences in cell death or cell cycle exit between control and GRK2-expressing cells, other important early events are also decompensated. The activation of key protein kinases such as Akt and p38MAPK is impaired in GRK2 myoblasts and accelerated in both K220R-expressing C2C12 cells and upon GRK2 silencing. This effect was observed in

the phosphorylation of the Ser473 of Akt, which has been described to correlate with Akt kinase activity along myoblast differentiation (Gonzalez et al., 2004). More importantly, p38MAPK activation was more prominent all along the differentiation process in K220R-expressing or GRK2-silenced myoblasts, and blunted when GRK2 was elevated.

This event can have important consequences in the development of myogenesis (Gonzalez et al., 2004). Treatment with p38MAPK α/β inhibitor SB203580 prevented the fusion of myoblasts into myotubes, and the induction of muscle-specific genes (Cuenda and Cohen, 1999; Li et al., 2000; Wu et al., 2000). Myoblasts obtained from mice lacking p38MAPK α showed impaired myoblast differentiation and fusion, with reduced expression of myogenin and MHC and blunted myotube formation (Perdiguero et al., 2007b). So, the kinase activity of p38MAPK is essential along the differentiation process, but it also serves to ensure the timely activation of different subsets of differentiation factors that guarantee a successful terminal differentiation (Penn et al., 2004). For instance, inhibition of p38MAPK activity during late stages of C2C12 cell differentiation resulted in increased expression of certain skeletal muscle genes (Suelves et al., 2004). In particular, p38MAPK phosphorylated *in vitro* and *in vivo* certain residues in Nt-MRF4, leading to a repressive but selective effect during terminal differentiation. Therefore, activation of p38MAPK at early time points triggers premature differentiation, but when that activation is maintained (as so happens with K220R overexpression or GRK2 silencing), it activates a repressive role of MRF4 over late myogenic markers and impairs terminal differentiation (Suelves et al., 2004). This effect might explain why in K220R-overexpressing cells differentiation is detected earlier but does not reach the degree of maturation observed in control cells. These cells, even when they differentiate well into long multinuclear myotubes containing more fused nuclei than control cells (data not shown), conserve the biochemical characteristics of immature myocytes, since they express significantly larger amounts of early differentiation markers such as MyoD and myogenin relative to control cells even when terminal differentiation is reached.

Overall, our data suggest that the phenotypic changes on the kinetics and degree of the myogenic process detected when GRK2 levels vary depend mostly on its kinase activity and are related to an altered p38MAPK activation status. This is in full accordance with the described effect that GRK2 protein has on the process of adipocyte differentiation in 3T3-L1 preadipocytes. GRK2-mediated phosphorylation of the p38MAPK docking domain at the Thr123 residue prevents its interaction with substrates and activators and impedes progress of the differentiation process in a kinase-dependent manner (Peregrin et al., 2006). GRK2 might regulate skeletal muscle differentiation through a similar mechanism, since a phospho-mimetic mutant of p38MAPK (T123D) is able to recapitulate the effects of GRK2 overexpression on the myogenic process and to prevent formation of membrane protrusions, a process that should precede the formation of fused myotubes. On the other hand, the blunted differentiation observed in GRK2-expressing clones can be rescued by the activation of the p38MAPK route upon MKK6-E expression, strongly supporting the notion that modulation of the p38MAPK pathway is key to the effects of GRK2 in muscle differentiation. The fact that K220R-expressing cells do not reach full differentiation maturity could be ascribed to an untimely p38MAPK activation pattern as discussed above, although we cannot rule out the possibility that at later stages of the normal differentiation process GRK2 activity towards alternative targets would contribute to some aspects of the differentiation process.

The very early event of nuclei fusion is also altered by GRK2 expression. Myoblast fusion is initiated by finger-like membrane protrusions that search and engage neighboring fusion-competent myoblasts to initiate the fusion process. Thus, the formation of filopodia/lamelipodia-like structures represents one of the earliest detectable events in the myocyte fusion process. Such actin-labeled protrusions are almost absent in GRK2-overexpressing cells while K220R cells are fully able to generate membrane protrusions as control cells do. At day zero, before differentiation is initiated, K220R cells appear at least as competent as control cells to fuse to neighboring myoblasts, while GRK2 cells would be less able to extend and fuse their membranes. At day four, even when control cells and K220R cells

had the same amount of membrane protrusions, K220R cells had wider myotubes indicating more fusion events per myotube had occurred earlier on along the process. These results indicate that K220R cells are more competent or efficient in terms of promoting successful fusion events. So, it could be inferred that K220R cells possess a small but very precocious advantage during the first stages of fusion what eventually translates into larger myotubes with more fused nuclei. These myotubes would be, however immature, since early differentiation markers are still upregulated (and therefore would be causing differentiation to prolong), and late markers do not reach the levels achieved in control cells. Thus, these larger and multinucleated myotubes would remain immature in nature and, therefore, more prone to deterioration and detachment. GRK2 downregulation would cause fiber retraction due to hypercontraction of immature or dysfunctional myotubes, or by mechanical loss upon fiber detachment when the first contractile movements take place, what would explain the fiber loss that we detect in flies soon after birth. This phenomenon would also explain the fiber hypertrophy detected in adult mice, since the remaining fibers would grow larger in size and competency in an effort to compensate for fiber loss. Finally, it would also explain the lack of differences in muscle mass detected in GRK2-deficient animals, since deteriorated immature fibers can be replaced by hypertrophied larger ones, thus compensating for muscle mass loss.

Given the connection between GRK2 activity and p38 pathways described in this work, investigating the extracellular stimuli regulating muscle GRK2 expression/activity during myogenesis or in muscle pathologies constitutes a very interesting avenue for future research. These extracellular signals may act through GPCR such as muscarinic or adrenergic receptors, leading to subsequent release of activating G $\beta\gamma$ subunits. A variety of GPCR and growth factors can also promote activation of kinases known to modulate GRK2 levels and/or activity, such as PKC, c-Src or MAPK (Ribas et al., 2007; Penela et al., 2010). β -adrenoceptors (β ARs) play an important role in myogenesis and skeletal muscle regeneration after myotoxic injury cells (reviewed in Lynch and Ryall, 2008), and are also upregulated during differentiation of C2C12. On the other hand, the IGF1 pathway, which plays an important role in muscle growth,

maintenance and repair, has been shown to upregulate GRK2 expression in other cell types (Penela et al., 2010). Interestingly, GRK2 levels increase in muscle during aging and upon development of insulin resistance (Garcia-Guerra et al., 2010). Whether this increase contributes to age-related muscle wasting is however not known. Interestingly, β AR agonists have therapeutic potential for attenuating muscle wasting associated with age-, cancer cachexia, and neuromuscular diseases such as the muscular dystrophies (reviewed in Koopman et al., 2009). Also, a very recent report demonstrates that the loss in self-renewal detected in satellite cells from aged mice can be overcome by p38 α / β inhibition (Bernet and Rudnicki, 2014). Whether GRK2 is found mutated or dysregulated in muscle wasting contexts or in human patients of muscular diseases are interesting questions for future studies.

In sum, we can conclude that GRK2 provides important signals required to prevent uncontrolled early differentiation and to ensure sustained muscle differentiation during development. Modulation of GRK2 activity is of essential importance for skeletal muscle formation during development, and also determines fiber size and characteristics in adult mammalian tissue, probably by regulating the timely differentiation program and the development of fusion events at very early time points during the myogenic process.

Materials and methods

Drosophila stocks

The following stocks were used: *Dmef2Gal4>UAS-GPF*, *1151Gal4* (Dutta et al., 2004), *UH3Gal4* (from Upendra Nongthomba, IISc, Bangalore, India) *kettin-GFP* (Kelso et al., 2004), *Df(3R)Gprk2* and *UAS-Gprk2i* (Molnar et al., 2007), *UAS-Gprk2*, (a gift from C. Molnar and J.F. de Celis, CBMSO, Madrid).

Cell line generation

Mouse C2C12 myoblasts (American Type Culture Collection) (Blau et al., 1985) were maintained in DMEM supplemented with 10% fetal serum (FS) and antibiotics, at 37°C and 5% CO₂. C2C12 cells were stably transfected according to the LipofectAMINE™ protocol (Invitrogen), with 4 µg of pcDNA3-GRK2-WT-neo (pc-GRK2) or pcDNA3-GRK2-K220R-hygro (pc-K220R) or with the empty vector pcDNA3-hygro (pcDNA3) as a control. Afterwards, transfected cell lines were selected by neomycin (0.5 mg/ml) or hygromycin (0.5 mg/ml) for 3 weeks. Four clones were obtained from each construct using this procedure, which were analyzed for GRK2 overexpression and differentiation pattern. Cells showed stable GRK2 expression after several passages and no significant phenotypical differences among the four clones were observed in any group of cell lines. Accordingly, a representative clone from each group, pcDNA3, pc-GRK2 and pc-K220R, was used in this study. All cell lines were grown to confluence in 10% FS-DMEM before differentiation in low serum medium, DMEM supplemented with 2% horse serum (HS) for up to 4 days. Furthermore, C2C12 myoblasts were also subjected to transient transfection Flag-p38αWT or Flag-p38αT123D. These cells were also differentiated in 2% HS-DMEM for 4 days.

Animal protocols

Experiments were performed on Wt and hemizygous-GRK2 (GRK2^{+/-}) mice maintained on the hybrid 129/SvJ C57BL/6 background. The animals were bred and housed on a 12-hour light/dark cycle with free access to food and water. Muscle samples were collected at the

corresponding ages and immediately weighted and frozen in liquid nitrogen and stored at -80°C.

All animal experimentation described in this study was conducted in accordance with accepted standards of human animal care and approved by the University Committee.

Please, refer to Supplementary Material for further information on this section.

Acknowledgments

This work is dedicated to the memory of Prof. Margarita Lorenzo, who passed away April 7, 2010, at the age of 51.

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Figure legends

Figure 1 *Gprk2* is required for *Drosophila* myogenesis. (A-C) lateral views of a stage 16 *Dmef2Gal4>UAS-GPF* control embryo (A) showing the normal pattern of lateral and ventral somatic muscles (schematically represented in D), and the same *Dmef2Gal4>UAS-GPF* embryo visualized at 20h (B) and 24h (C) after injection of *Gprk2* dsRNA. Note the aberrant morphology of the LT muscles in the anterior region of injected embryo (corresponding to the site of injection, arrow in B) and their detachment 4 h later (arrow in C) and compare to LT muscles in the posterior region of the embryos (arrowheads in A-C and arrow in the scheme shown in D). (E) Flightless phenotypes obtained after manipulation of *Gprk2* expression using the *UAS/Gal4* system at 29°C. *UAS-Ri-Gprk2*, RNAi silencing construct for *Gprk2*. (F-I) Transverse sections of pupal (F, G) and adult thoraces (H, I). (F, H) *w/+; UAS-Flp/+; FRT82 Df(3R)Gprk2 / FRT82 M* control thoraces, showing the wild-type arrangement of the six pairs of DLM muscles. (G, I) *1151Gal4/+; UAS-Flp/+; FRT82 Df(3R)Gprk2 / FRT82 M* P8 pupa (G) and three days old fly (I) displaying a reduction in the number of DLM muscles (45%, *n*=11 in pupae, 47%, *n*=15 in 3 days old adults; arrow in panel G points at the left hemithorax containing 5 DLM muscles).

Figure 2 GRK2 hemizygous mice show decreased fiber number and increased skeletal muscle fiber size. Determination of GRK2 protein and gene expression in Wt and GRK2^{+/-} mice by immunoblotting (A) and qPCR (B). Wt and GRK2^{+/-} muscles were isolated and weighted (C), embedded in paraffin, cut in sections (5 µM) and stained with hematoxylin and eosin (D). Representative images are shown in 20× magnification. Scale bar 100 µm. The cross-sectional area of myofibers of the muscle from each individual mouse was measured to determine relative fiber size (Wt 3m=18.08±0.7 a.u.; Wt 9m=11.81±0.05 a.u.) (E) and the number of fibers (Wt 3m=48.33±4.86; Wt 9m=50.91±2.36) (F) per field. Values are expressed as percentage over wildtype animals. Muscle sections from neonates were stained with MHC (G), and relative fiber size (Wt=3.01±0.08 a.u.) (H) and number of fibers (Wt=187.8±9.0) (I) per field were determined. Scale bar: 50 µm (20×) and 20 µm (60×). Values are expressed as percentage over wildtype animals. * *P*<0.01.

Figure 3 GRK2 impairs and GRK2-K220R accelerates myoblast differentiation. **(A)** C2C12 myoblast were differentiated and lysed in successive days along the culture to analyze proteins by SDS-PAGE. Cell lines with increased expression of wild type GRK2 (pc-GRK2), catalytically inactive GRK2 (pc-K22R) or empty vector (pcDNA3) were used. **(B)** GRK2 protein expression was immunodetected by western blot in control cells. **(C)** Phase-contrast images were taken along the differentiation process. **(D)** Representative western blot results using antibodies against MyoD, Myogenin, Caveolin-3, MHC and β -actin, and densitometric analysis after standarization using β -actin content. Both are results from 3 independent experiments. Values are expressed as percentage over zero days of differentiation. Different symbols show $P<0.05$; Asterisks depict differences between pcDNA3 and K220R, triangles between pcDNA3 and GRK2.

Figure 4 Overexpression of GRK2 abrogates myoblasts fusion and membrane protrusions in low serum. Myoblasts from the cell lines pcDNA3, pc-GRK2 and pc-K220R were grown in 10% FS-DMEM until confluence and then further cultured in 2% HS-DMEM for four days to induce differentiation. **(A)** Cells were fixed and actin cytoskeleton was visualized by fluorescence microscopy of cells stained with rhodamine-phalloidin. Nuclei were stained with DAPI. **(B)** Quantification of phalloidin–stained cells was conducted by scoring individual cells from 10 different fields for the presence or absence of membrane ruffles. Membrane protrusions are indicated by white arrows. The percentage of cells with fused nuclei **(C)**, the number of nuclei per myotube **(D)** and the diameter of myotubes (a.u.) **(E)** were determined. * $P<0.05$.

Figure 5 GRK2 kinase activity regulates the Akt, p38MAPK, and ERK1/2 pathways during myogenic differentiation. Cells were lysed at different days of culture and total protein (30 μ g) was subjected to SDS-PAGE and immunodetected with total and phosphorylated forms of Akt, ERK 1/2 **(A)** and p38MAPK antibodies **(B)**. Graphs in **A** and **B** show the densitometric analysis of P-Akt (Ser473), P-p38MAPK (Thr180/Tyr182) and P-ERK1/2 after standarization using total Akt, p38MAPK and ERK1/2, respectively. Values are expressed as percentage over zero days of differentiation. Results show representative blots of four independent experiments.

Asterisks represent differences between pcDNA3 and K220R, triangles between pcDNA3 and GRK2. * $P < 0.01$. (C) C2C12 cells were infected with either a control adenovirus carrying GFP (Ad-CTRL) or an sh-GRK2 adenovirus (Ad-sh-GRK2), differentiated and lysed at different days along the differentiation process. Representative western blot results using antibodies against phosphorylated and total p38MAPK (Thr180/Tyr182) and AKT (Ser473) are shown. Graphs show densitometric analysis after standarization. * $P < 0.05$, $n = 3$.

Figure 6 Effects of a p38MAPK mutant that mimics its phosphorylation by GRK2 and of constitutive p38MAPK activation on a GRK2-overexpressing background. (A) Phase-contrast images of the terminally differentiated cells for detection of multinucleated myotubes. Magnification, 10 \times . (B) C2C12 cells transfected with Flag-p38 α WT or Flag-p38 α T123D were differentiated, fixed and immunostained with rhodamine-phalloidin (red) and DAPI to visualize actin cytoskeleton or nuclei, respectively. The percentage of membrane protrusions (C), percentage of cells with fused nuclei (D), number of nuclei per myotube (E), and myotube diameter (F) were determined. Four independent experiments were performed in triplicates. The images shown are representative from at least 10 different fields from independent coverslips. * $P < 0.05$. (G) Both C2C12 and pcGRK2 stable cell lines were infected with a retrovirus carrying a constitutively active MKK6 mutant (MKK6-E) using an empty vector as a control. Cells were differentiated to take phase-contrast images and then lysed at the zero and the fourth day of the process. (H) Lysates were subjected to SDS-PAGE and immunodetected with antibodies against MHC, GRK2 and actin.

Figure 1

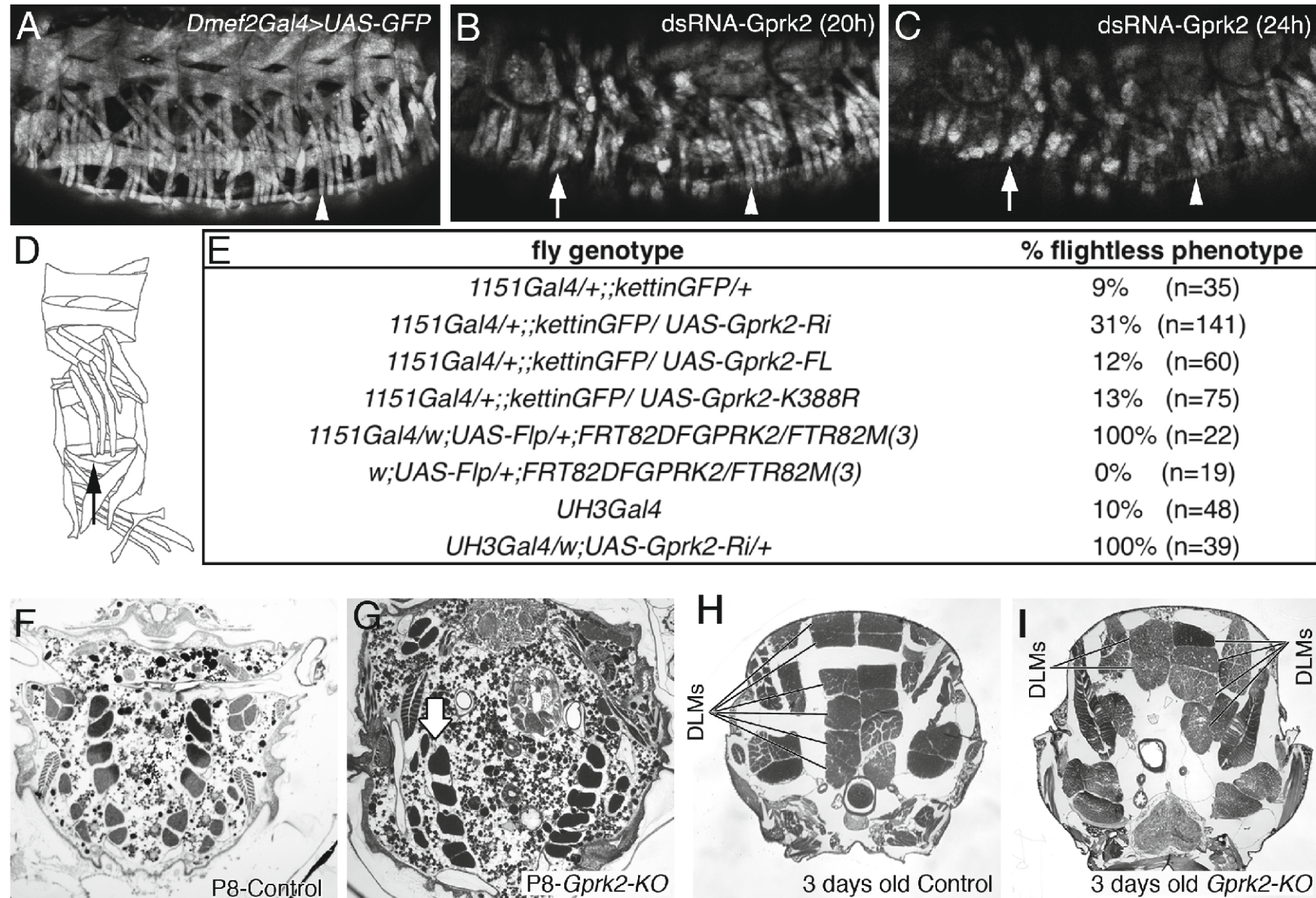


Figure 2

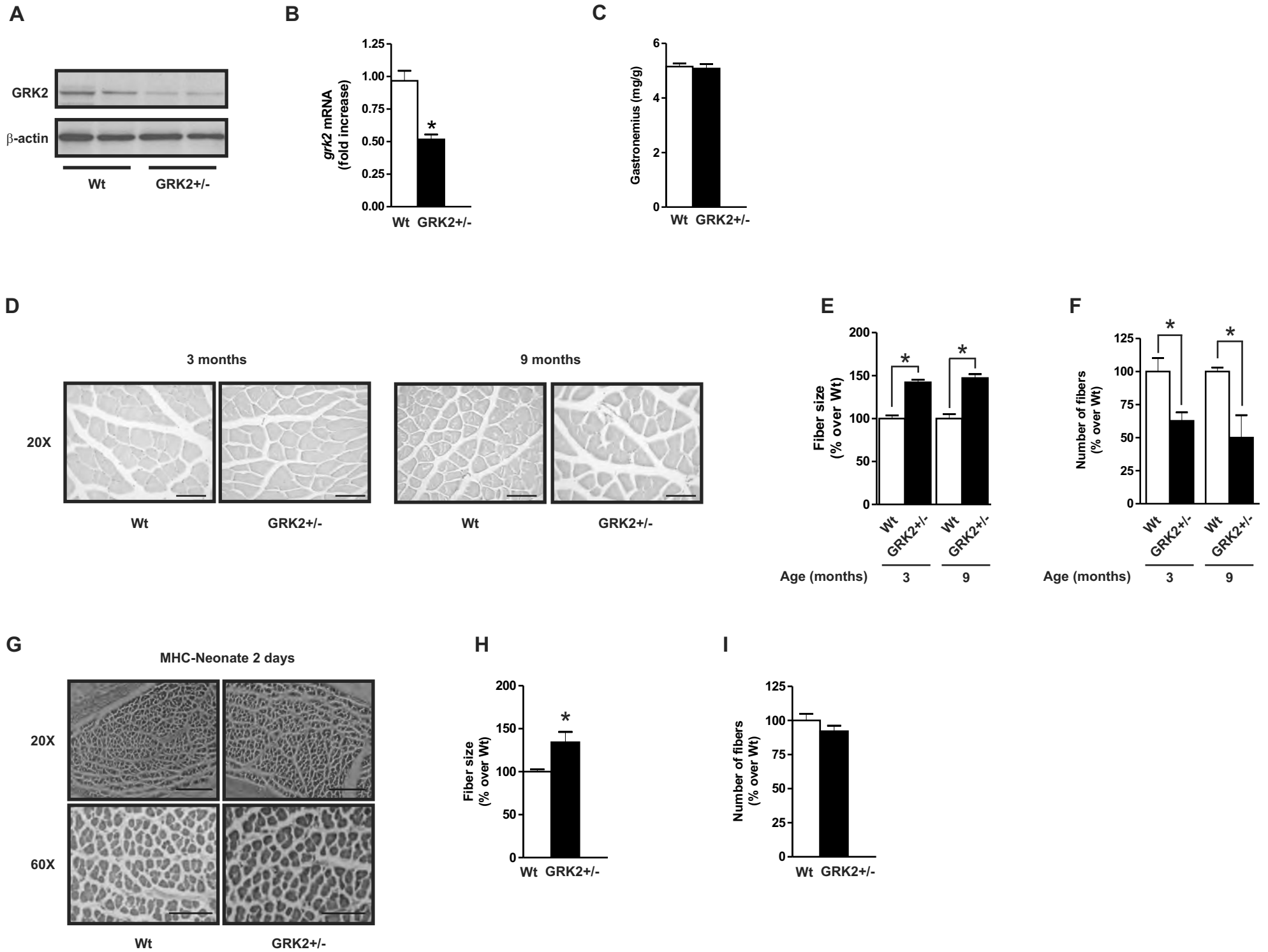


Figure 3

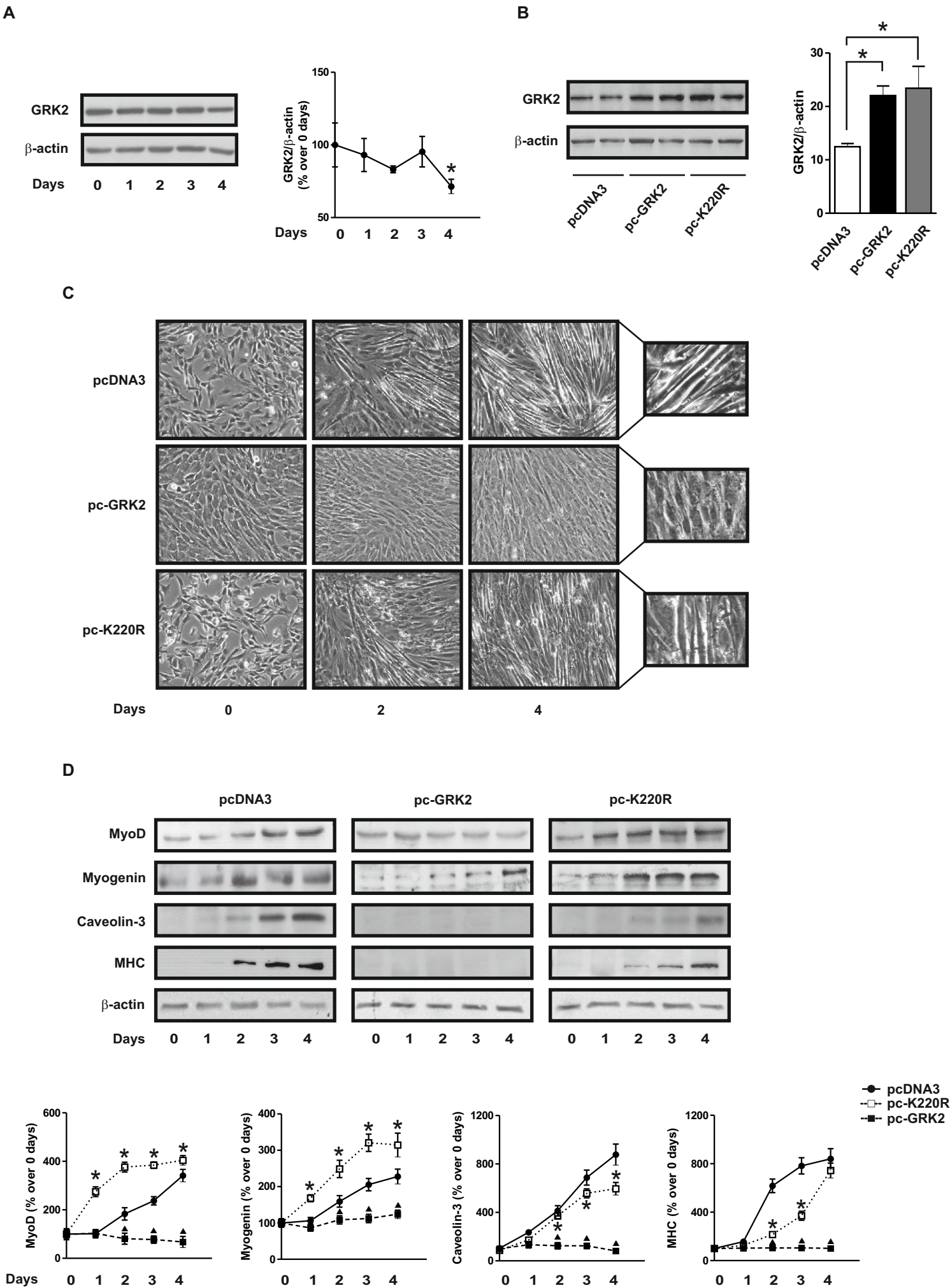


Figure 4

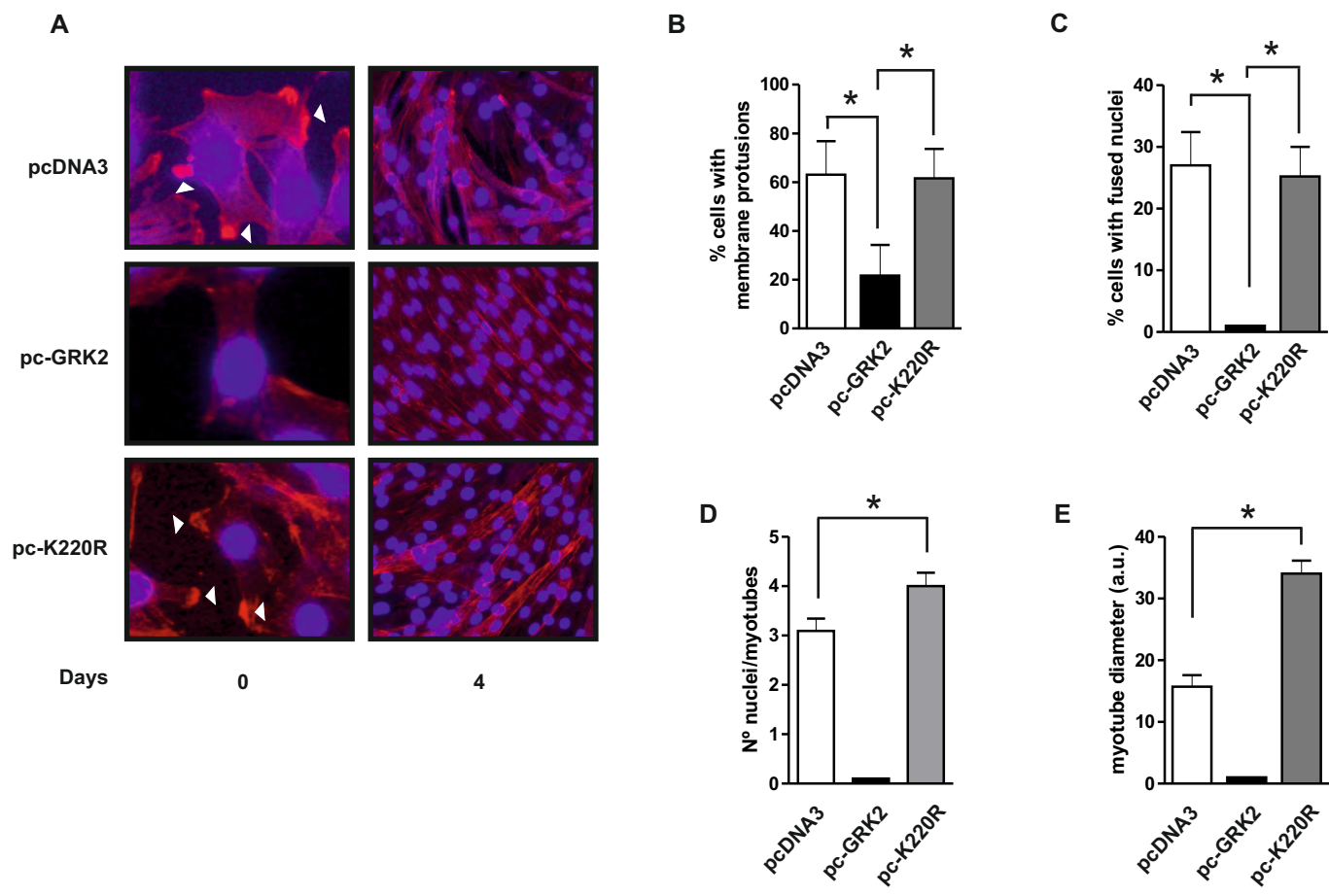
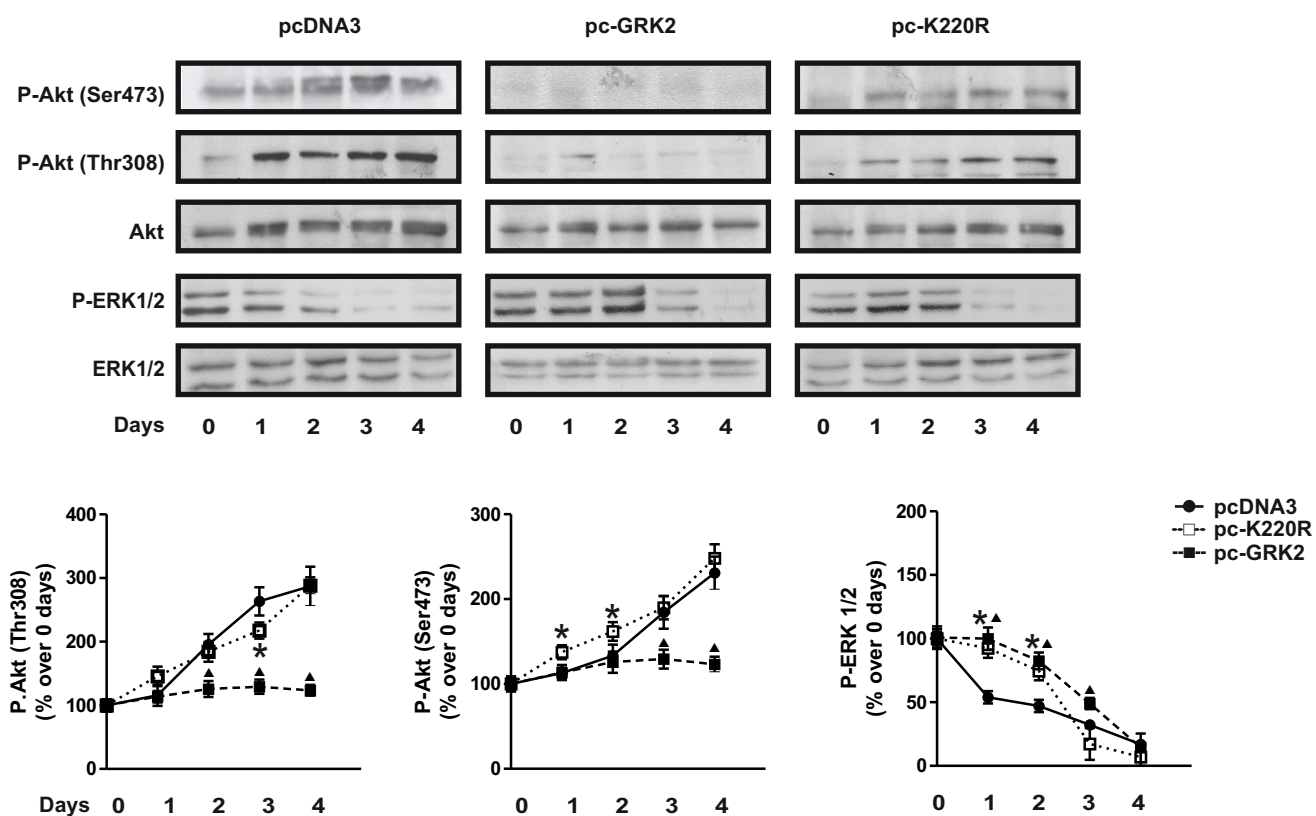
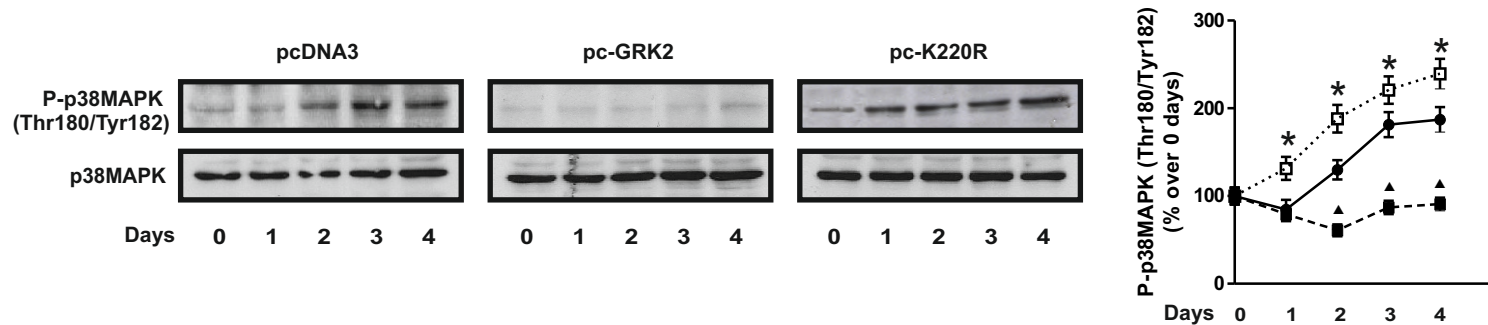


Figure 5

A



B



C

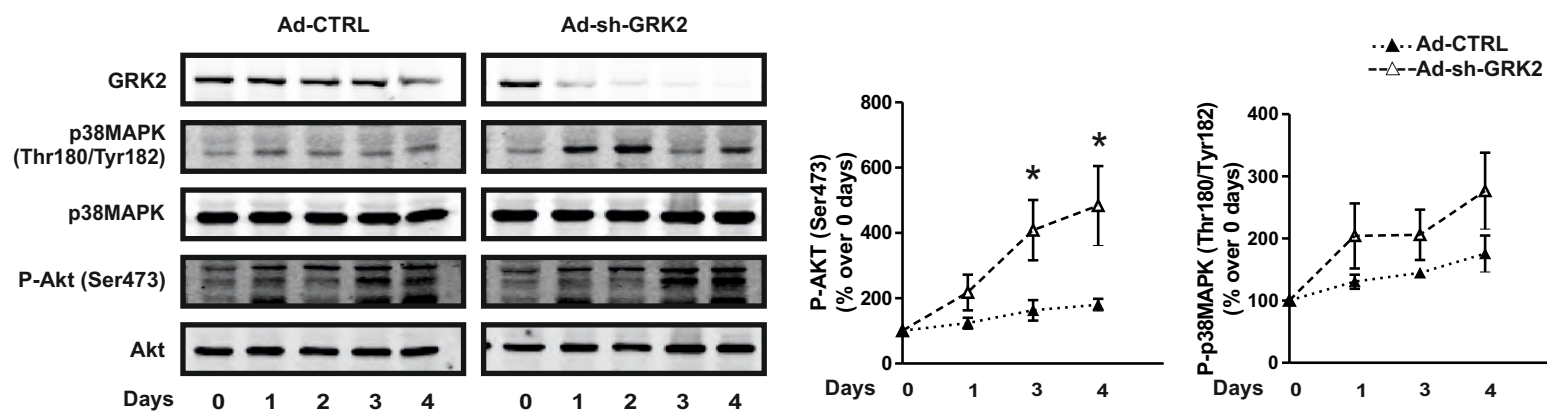
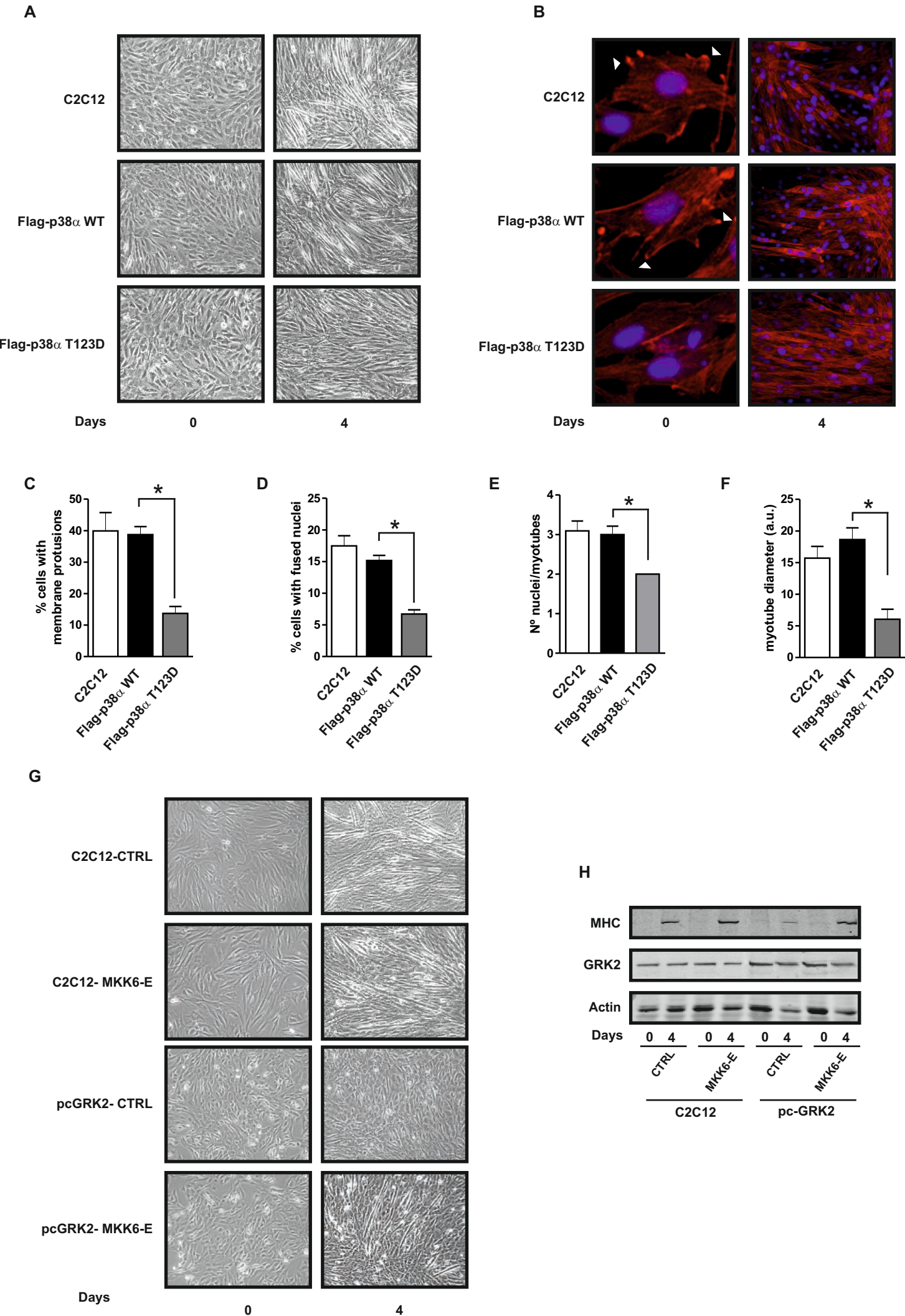


Figure 6



SUPPLEMENTAL MATERIAL

SUPPLEMENTAL DESIGN AND METHODS

Reagents

LipofectamineTM, culture media and sera were from Invitrogen (Paisley, UK). Neomycin and Hygromycin were purchased from Invitrogen (Carlsbad, CA). Antibodies were used against MyoD (sc-760), p38MAPK α (sc-535), myogenin (sc-576), MHC and GRK2 (sc-562) from Santa Cruz (Palo Alto, CA); β -actin from Sigma-Aldrich (St. Louis, MO); caveolin-3 from Transduction Laboratories (Lexington, KY); total and phosphorylated Akt (Ser473 and Thr308), p38MAPK (Thr180/Tyr182) and ERK1/2(Thr202/Tyr204) from Cell Signalling (Beverly, MA). The polyclonal anti-phospho-Thr123p38 serum was generated as described (Peregrin et al., 2006). Autoradiography films were supplied by GE Healthcare (Rainham, UK). The plasmids pcDNA3-hygro, pcDNA3-GRK2-WT-neo, pcDNA3-GRK2-K220R-hygro, Flag-p38 α WT or Flag-p38 α T123D were previously used in our laboratory (Penela et al., 2008; Peregrin et al., 2006). All other reagents used were of the purest grade available.

dsRNA injection into embryos

dsRNA was synthesized using the MEGAscript kit (Ambion) as in (Kennerdell and Carthew, 1998) with modifications. 1h-old *Dmef2Gal4>UAS-GPF* embryos were injected with 2-3 μ M of *gprk2*-dsRNA in the ventral region at a central position using standard procedures, Voltalef oil covered embryos were let to develop at 17°C in a humidified chamber and were observed at different times using a fluorescence microscope.

Generation of *Drosophila gprk2* mutant adult muscles and thorax sections

To obtain adult muscles mutant for *Gprk2* w; *UAS-Flp*; *FRT82Df(3R)FRT82/TM6B* females were crossed to 1151*Gal4*; *FRT82M/TM2* males at 25°C. Females *1151Gal4/+; UAS-Flp/+; FRT82 Df(3R)Gprk2 / FRT82 M* were used to score for LOF phenotypes whereas their *w/Y; 3 UAS-Flp/+; FRT82 Df(3R)Gprk2 / FRT82 M* siblings served as experimental controls. Pupae

(P8) and adult thoraces were fixed with 4% paraformaldehyde (PFA) overnight, washed twice in water, dehydrated in acetone series and embedded in Epon. Sections (10 μ m) were cut using a microtome and stained with Toluidine Blue.

Analysis of cortical actin protrusions and fused nuclei.

Overnight serum-starved cells plated in 35-mm tissue culture dishes and cultured as described above were fixed with 3.7% formaldehyde in PBS (pH 7.4) for 10 min at room temperature, washed in PBS, permeabilized in 0.2% Triton X-100 for 5 min, washed in PBS and incubated for 45 min with rhodamine-phalloidin (0.125 mg/ml) and DAPI to visualize cortical polymerized actin in protrusions of the cell membrane or the cells with fused nuclei respectively (Conejo and Lorenzo, 2001). Quantification of cells with actin-filled membrane protrusions was conducted by scoring individual cells from ten different fields for the presence of phalloidin-stained membrane protrusions. The percentage of cells with fused nuclei was determined by dividing the number of DAPI-stained nuclei within myotubes (two or more nuclei) by the total number of nuclei analyzed. Number of nuclei in cells was counted and expressed as a percentage of the total number of nuclei analyzed. Myotube diameter was determined as the average from three measurements per myotube.

Western blot.

Cellular proteins (30 μ g) were subjected to SDS-PAGE, transferred to Immobilon membranes and blocked using 5% nonfat dried milk in 10 mM Tris-HCl and 150 mM NaCl (pH 7.5), and incubated overnight with several antibodies as indicated in each case in 0.05% Tween-20, 1% non-fat dried milk in the same buffer. Immunoreactive bands were visualized using the enhanced chemiluminescence (ECL-Plus) Western blot protocol from GE Healthcare. In experiments using X-ray films, different exposure times were used to ensure that bands were not saturated.

Real-time quantitative RT-PCR (QPCR) assays.

Total RNA was extracted from the gastronemius muscle of 3-month old GRK2^{+/-} and wildtype mice (n=12, 6 mice per group) with Trizol reagent (Invitrogen). 1 µg of DNaseI-treated RNA was converted to first-strand cDNA with “High Capacity cDNA reverse transcription kit” (Applied Biosystems). The cDNA was subjected to real-time PCR assay for *grk2* gene expression using the Taqman Gene Expression Assays from Applied Biosystems. Data obtained was analysed using the comparative method and by normalization of expression values of 18S rRNA expression. The results are expressed as fold increased over *grk2* expression in wildtype animals.

Analysis of myofiber size, total number of fibers and MHC staining.

Samples were fixed in 4% paraformaldehyde and embedded in paraffin for histological analysis. Transverse paraffinized muscle sections (5 µm) were mounted on D-polyisinated glass slides and stained with hematoxylin and eosin. MHC staining was performed in freshly isolated muscles from 2-day old newborn mice fixed in 4% formalin and embedded in paraffin. Transverse sections (7 µm) were deparaffinized and rehydrated, and 3% H₂O₂ was used for blocking endogenous peroxidase activity. Sections were incubated with MHC antibody (Millipore). Immunohistochemical detection of MHC was performed following the staining procedure according to the vectastatin Elite ABC system (Vector laboratories) and counterstaining with hematoxylin. For each experimental group, five sections per animal were stained and a negative control without the primary antibody was performed. Digital images of skeletal muscle sections were captured using a light microscope (Olympus, Germany) at 20 and 60X magnification. Relative fiber size and number of fibers were calculated in arbitrary fields; by quantitation of 50-100 adjacent muscle fibers in at least three different randomly-chosen fields per mouse, using image analysis software (ImageJ).

Propidium iodide incorporation assay.

C2C12 myoblasts, pc-GRK2 and pc-K220R cell lines were cultured and differentiated as previously mentioned. Fresh differentiation medium was added every two days. At day four cells were washed twice with phosphate-buffered saline (PBS) and resuspended in Staining Buffer (PBS 1X, 1% BSA, 0.01% NaN₃, 1% FBS) with a final concentration of propidium iodide (PI) of 1µg/ml. Analysis was carried out in a BD FACS Calibur flow cytometer (BD-Bioscience) and propidium iodide-positive cells were quantified using CellQuest Software (BD-Bioscience) and analyzed with the FlowJo Software.

Adenoviral infection.

Cells were cultured in 10% FS-DMEM without antibiotics 24 hours before infection, then infected at a 150 M.O.I. with a rat adenovirus carrying an sh-GRK2 construct (Lafarga et al., 2012) using a GFP-containing adenovirus as a control. After 8 hours of infection, the medium was replaced with 2% HS-DMEM up to 4 days.

Retroviral infection.

293T cells were transiently transfected using standard LipofectAMINE™ protocol with 5 µg of the packaging construct pCL-Eco and 5 µg of pMSCVp38α or pCLNCX MKK6E plasmids (kindly provided by Drs. Eusebio Perdiguero and Pura Muñoz; Perdiguero et al., 2011). The next day the medium was replaced with fresh medium. After 24 hours, the virus-containing medium was collected and centrifuged. 100 µl of HEPES buffer solution (GIBCO, 1M) and 40 µl of polybrene solution (1 mg/ml) were added to the virus-containing supernatant, which was then added to C2C12 myoblasts and pc-GRK2 cell line. After 12 hours of infection, the medium was replaced with 2% HS-DMEM.

Data analysis

Results are means ± S.E. from 4 to 10 independent experiments. Statistical significance was tested with the unpaired Student's t test or with the one-way ANOVA followed by the protected

least-significant different test. Differences were considered statistically significant when P values were <0.05.

LEGENDS TO SUPPLEMENTAL MATERIAL

Supplemental Movie 1. Climbing assay in 3 day-old control flies. Climbing control assay in three days old *w/+; UAS-Flp/+; FRT82 Df(3R)Gprk2 / FRT82 M* flies.

Supplemental Movie 2. Climbing assay in 3 day-old *Gprk2* mutant flies. Climbing assay of three days old *1151Gal4/+; UAS-Flp/+; FRT82 Df(3R)Gprk2 / FRT82 M* flies showing that in the absence of the *Gprk2* adult muscle function the adults present a reduction in climbing speed compared with the control.

REFERENCES TO SUPPLEMENTAL MATERIAL

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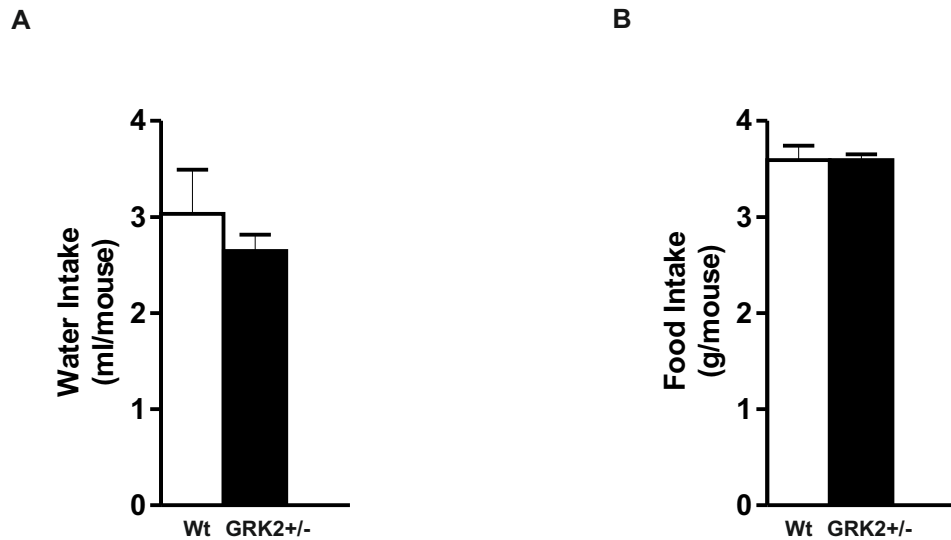
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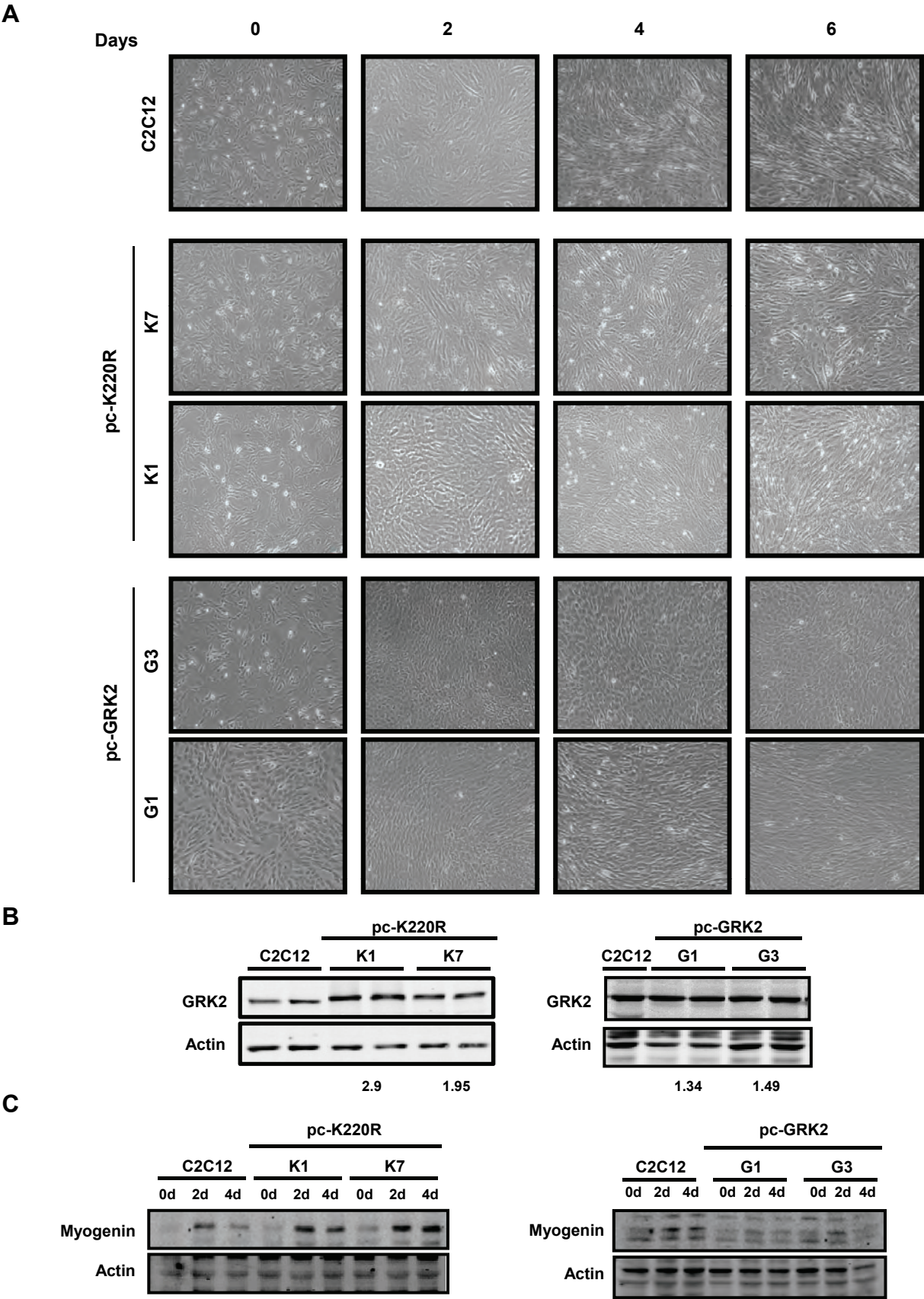
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Supplemental Figure S1



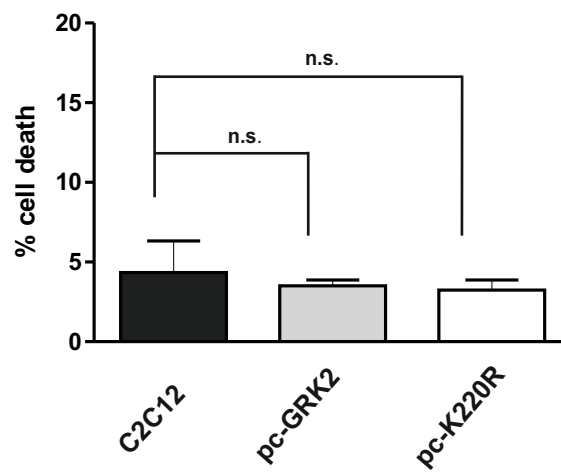
Supplemental Figure S1: GRK2^{+/-} mice display no differences in food or water intake compared to control littermates. Water **(A)** and food **(B)** intake were measured periodically in LabMaster metabolic cages, from TSE Systems (Bad Homburg, Germany) along 72 hours. Before the measure, mice were allowed to adapt to the new environment for 72 h. Measurements were taken every 10 min during 24 h. Results are expressed as ml of water or g of chow respectively, per mouse and day. Data were analysed with Phenomaster software (TSE Systems) and are means \pm SEM of measurements obtained for from 5 animals per group.

Supplemental Figure S2



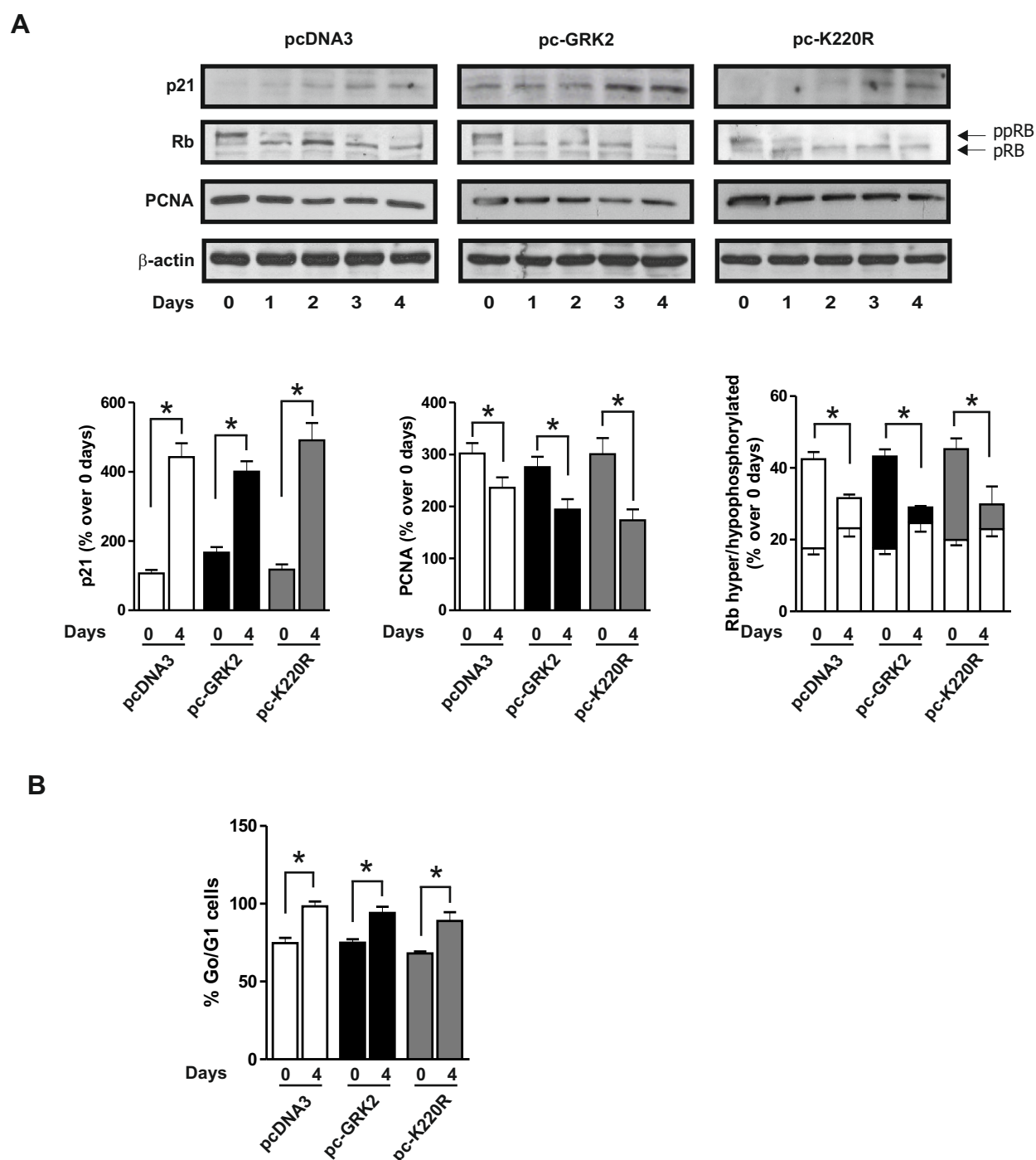
Supplemental Figure S2: Different clones were generated among transfected C2C12 myoblasts and different clones overexpressing either wild type GRK2 (pc-GRK2) or a catalytically inactive GRK2 mutant (pc-K220R) were selected. **(A)** Phase-contrast images were taken during the differentiation process. **(B)** GRK2 levels in undifferentiated C2C12, pc-GRK2 and pc-K220R myoblast lysates were immunodetected by Western Blot. The fold overexpression was quantified by densitometric analysis and shown below the blots. **(C)** Representative Western blot results using antibodies against Myogenin and Actin.

Supplemental Figure S3



Supplemental Figure S3: C2C12, pc-GRK2 and pc-K220R cell lines were differentiated and cell viability was determined at day four using a propidium iodide incorporation assay by FACS analysis. The percentage of propidium iodide-positive cells was calculated as a measure of cell death in each population. Data are mean±SEM of three independent experiments.

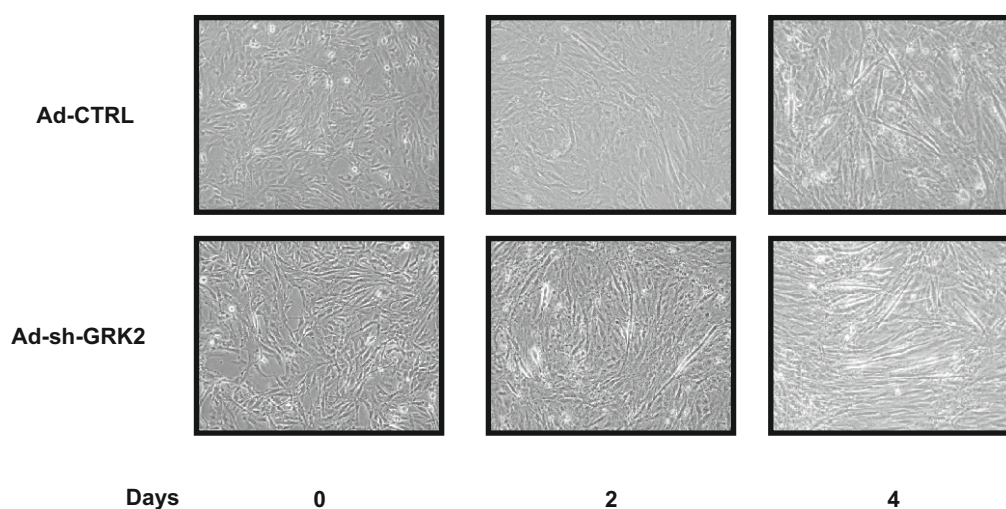
Supplemental Figure S4



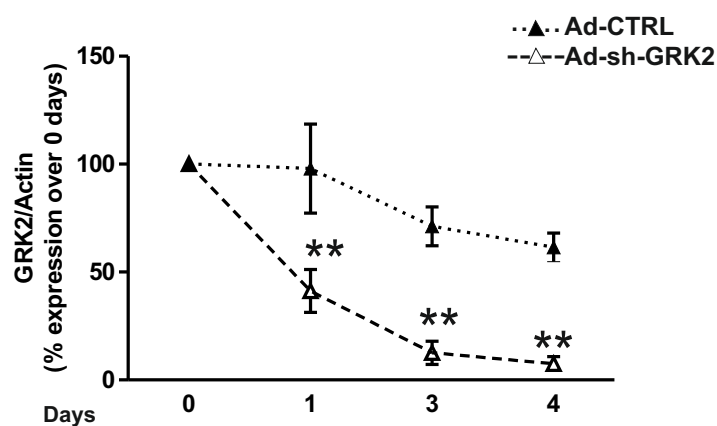
Supplemental Figure S4: Overexpression of GRK2 or GRK2-K220R does not alter cell cycle exit. C2C12 myoblast were differentiated and lysed in successive days along the culture to analyze proteins by SDS-PAGE. Cell lines with increased expression of GRK2 (pc-GRK2), catalytically inactive GRK2 (pc-K220R) or empty vector (pcDNA3) were used. **(A)** Representative Western blot results using antibodies against PCNA (Roche Molecular Biochemicals); Rb (Pharmingen); p21 (sc-397, Santa Cruz) and β-actin are shown. Graphs show densitometric analysis after standardization using β-actin content. Values are expressed as percentage over 0 days of differentiation. **(B)** Analysis of cellular DNA content was performed by flow cytometry. Cells were trypsinized, counted, washed with PBS, and fixed with cold ethanol (70%). Then they were resuspended in PBS (10⁶ cells/ml), treated with ribonuclease (Roche Diagnostics, Indianapolis, IN) for 30 min at 37°C, and analyzed by flow cytometry. Results are expressed as percentage of cells in the phases G0/G1 of the cell cycle. * P<0.01

Supplemental Figure S5

A

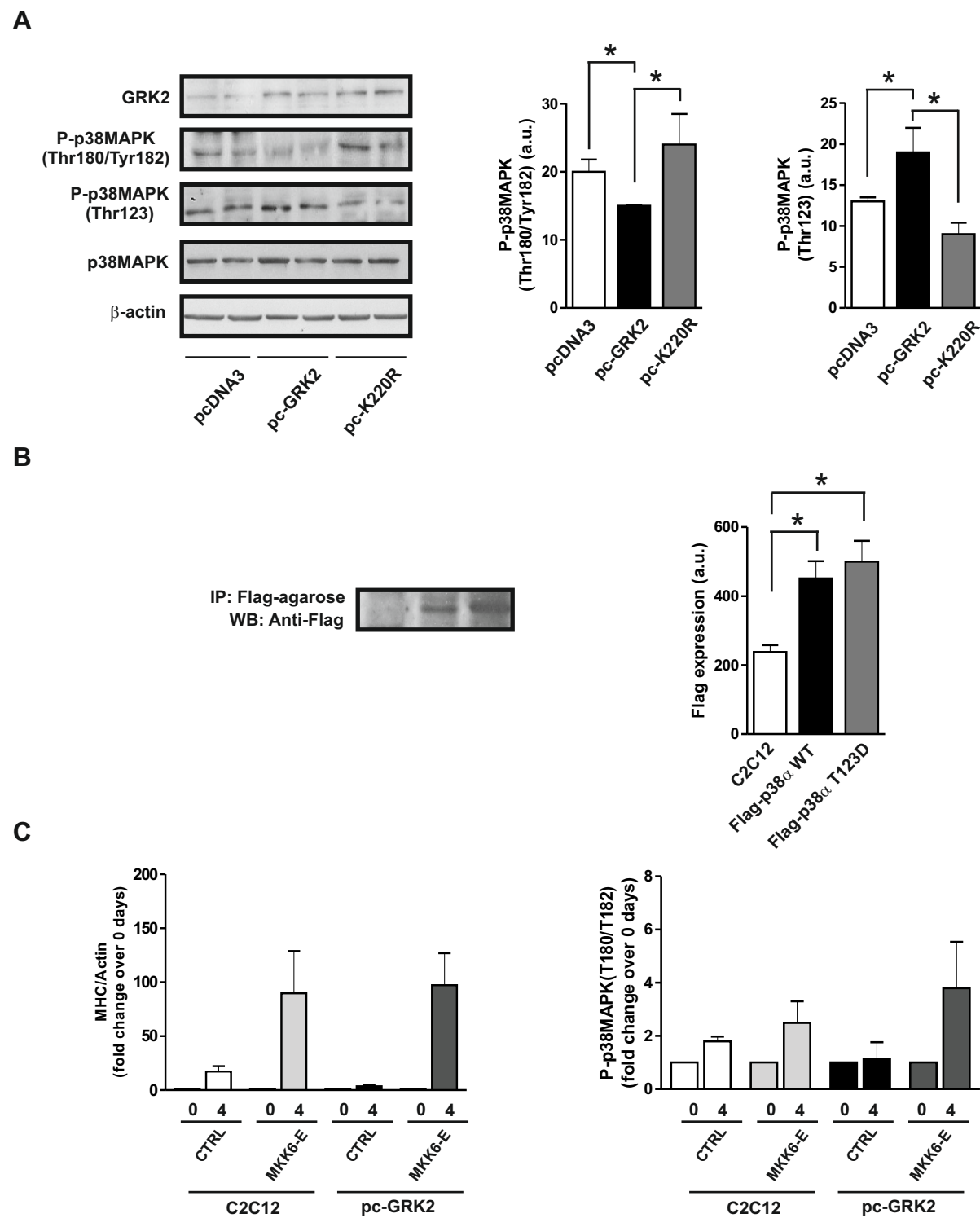


B



Supplemental Figure S5: C2C12 cells were infected with either a control adenovirus carrying GFP (Ad-CTRL) or an sh-GRK2 adenovirus (Ad-sh-GRK2) before differentiation. **(A)** Phase-contrast images were taken during the differentiation process. **(B)** The cells were lysed in successive days from day 0 to 4 and 30 μ g of lysates were subjected to SDS-PAGE and immunodetected with an antibody against GRK2. Graphs show densitometric analysis after standardization. Data correspond to representative sets out of at least three independent experiments ** $P < 0.01$.

Supplemental Figure S6



Supplemental Figure S6: (A) Myoblasts from the cell lines pcDNA3, pc-GRK2 and pc-K220R were grown in 10% FS-DMEM and lysed. Lysates were immunodetected with antibodies against GRK2, P-p38MAPK (Thr180/Tyr182), P-p38MAPK (Thr123), total p38MAPK and β-actin. Graphs show densitometric analysis after standarization using p38MAPK. Values are expressed as arbitrary units (a.u.). Results show representative blots of four independent experiments. * P<0.01. **(B)** C2C12 cells transfected with Flag-p38α WT or Flag-p38α T123D were analyzed for Flag expression by immunoprecipitation and western blot using Anti-Flag antibodies. A densitometric quantitative analysis is shown. * P<0.05. **(C)** C2C12 and pc-GRK2 cells were infected with a retroviral construct carrying MKK6-E, diferenciaded and then lysed at days 0 and 4. Lysates were subjected to SDS-PAGE and immunodetected with antibodies against MHC,GRK2, P-p38MAPK (Thr180/Tyr182), p38MAPK and actin. Graphs show densitometric analysis after standarization. Values are expressed as fold increase over cero days of differentiation. Data are mean±SEM of three independent experiments.