A novel GRK2/HDAC6 interaction modulates cell spreading and motility

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ABSTRACT

Cell motility and adhesion involves dynamic microtubule acetylation/deacetylation, a process regulated by enzymes as HDAC6, a major cytoplasmic α-tubulin deacetylase. We identify G protein-coupled receptor kinase 2 (GRK2) as a key novel stimulator of HDAC6. GRK2, which levels inversely correlate with the extent of α-tubulin acetylation in epithelial cells and fibroblasts, directly associates with and phosphorylates HDAC6 to stimulate α-tubulin deacetylase activity. Remarkably, phosphorylation of GRK2 itself at S670 specifically potentiates its ability to regulate HDAC6. GRK2 and HDAC6 co-localize in the lamellipodia of migrating cells, leading to local tubulin deacetylation and enhanced motility. Consistently, cells expressing GRK2-K220R or GRK2-S670A mutants, unable to phosphorylate HDAC6, exhibit highly acetylated cortical microtubules and display impaired migration and protrusive activity. Finally, we find that a balanced, GRK2/HDAC6-mediated regulation of tubulin acetylation differentially modulates the early and late stages of cellular spreading. This novel GRK2/HDAC6 functional interaction may have important implications in pathological contexts.

Keywords: GRK2 / HDAC6 / Microtubules / Migration / Spreading

INTRODUCTION

Cell chemotaxis involves the projection of organelle-free extensions (termed pseudopodia or lamellipodia depending on the cell type) in the direction of the chemoattractant source. These extensions establish new adhesions to the substratum and create centripetal contractile tension, leading to the detachment and retraction of the cell tail, thus allowing the cell body to translocate forward (Berzat and Hall, 2010; Kay et al., 2008). In fibroblasts or epithelial cells, locomotion is initiated by chemoattractant binding to a variety of membrane sensors, as G protein-coupled receptors (GPCR) or tyrosine-kinase receptors (Cotton and Claing, 2009) which trigger downstream signals responsible for polarized and stable cell protrusion during migration (Berzat and Hall, 2010). Such protrusive activity is mainly driven by enhanced actin polymerization adjacent to the leading edge membrane (Insall and Machesky, 2009), mediated by factors as Arp2/3, m-Dia2 or cofilin (Kay et al., 2008). In addition, microtubules (MTs) may also play a significant role in cell protrusion formation depending on the cell type and physiological context (Watanabe et al., 2005). MTs may be directly involved in the generation of a protrusive activity, counteracting the contractile action of the actin-myosin cortex (Levina et al., 2001), or stimulate cortical F-actin nucleation via local delivery to the MT plus-ends in protruding regions of several small G proteins GEFs (Fukata et al., 2002; Krendel et al., 2002; Nalbant et al., 2009). The MT network can also provide polarized routes for kinesin-dependent trafficking of intracellular vesicles, thus supporting membrane extension (Kay et al., 2008; Reed et al., 2006). Finally, dynamically growing MTs would favor focal adhesion (FA) disassembly and directional motility, whereas depolymerization or the presence of less-dynamic MTs would favor the formation of stress fibers and large FA, thus compromising migration (Kaverina et al., 1999; Wagner et al., 2002). Interestingly, MT dynamics is different in protruding and retracting regions of polarized, motile cells (Salaycik et al., 2005), strengthening an essential role for MT modulation in this process.

MT acetylation at the amino-terminus of α-tubulin subunits (Zhang et al., 2003; Watanabe et al., 2005) has a prominent role in cell migration and adhesion by affecting MT stability and dynamics (Hubbert et al.,2002; Matsuyama et al., 2002). Tubulin acetylation levels are finely regulated by the opposite action of acetyltransferases (Creppe et al., 2009) and of the major deacetylases SIRT2 and HDAC6 (Hubbert et al.,

2002; North et al., 2003; Zhang et al., 2003). Increased chemotaxis is observed upon over-expression of HDAC6 in different cell types either in a deacetylase activity-dependent (fibroblast and epithelial tumour cells) or independent manner (lymphocytes) (Valenzuela-Fernandez et al., 2008). Conversely, inhibition of HDAC6 markedly enhances MT acetylation and decreases cell migration (Haggarty et al., 2003; Hubbert et al., 2002). Besides tubulin, HDAC6 triggers deacetylation of other substrates as diverse as cortactin, Hsp90 or β-catenin, and also interacts with a broad spectrum of signaling partners, what underlies its role in other cellular processes (Boyault et al., 2007; Valenzuela-Fernandez et al., 2008). Despite its functional relevance, very little is known about the mechanisms that regulate HDAC6 functionality in the cell migration context.

GPCR stimulation can modulate tubulin polymerization by altering the functionality of different proteins that regulate the overall dynamics of MTs as a result of its binding to soluble and polymerized tubulin, association to the plus-ends of microtubules (+TIPs proteins) or promotion of tubulin post-translational modifications (Etienne-Manneville, 2010; Westermann and Weber, 2003). Most GPCR are regulated by G protein-coupled receptor kinases (GRKs), which phosphorylate agonist-occupied receptors, allowing the subsequent binding of beta-arrestins, what in turn blocks G-protein dependent receptor signalling and promote receptor internalization (Moore et al., 2007). Besides such regulatory role, the ubiquitous GRK2 isoform has been shown to modulate a growing number of signaling sensors, switchers and effectors (some of them related to cell migration) in a phosphorylation-dependent or –independent way (Penela et al., 2010; Ribas et al., 2007). Consistently, changes in GRK2 expression and/or activity have been reported to alter chemotactic motility in a cell-type specific manner (Vroon et al., 2006; Penela et al., 2009). We have recently shown that GRK2 positively regulates integrin-dependent motility in epithelial cell types and fibroblasts (Penela et al., 2008). Such effect involves the GRK2-dependent modulation of the scaffold function of GIT-1 in the activation of the Rac/PAK/MEK/ERK1/2 pathway. The positive effects of GRK2 on cell motility seem to involve the promotion of F-actin remodelling at the cell periphery and FA turnover (Cant and Pitcher, 2005; Penela et al., 2008). Interestingly, GRK2 interacts with and phosphorylates β-tubulin subunits (Pitcher et al., 1998), but whether this kinase could also affect cell migration by means of the modulation of MT has not been investigated.

We report herein that GRK2 modulates MT acetylation in a HDAC6-dependent manner in order to regulate key cellular processes relying on cytoskeletal rearrangements such as migration, polarity and cell spreading.

RESULTS

Effect of GRK2 expression levels on α-tubulin acetylation

As directional locomotion requires both the dynamic reorganization of MTs and proper regulation of tubulin acetylation in different cell types (Azuma et al., 2009; Hubbert et al., 2002; Watanabe et al., 2005), and GRK2 levels are critically involved in the modulation of the chemotactic migration of murine embryonic fibroblasts (MEFs) and epithelial cells (Penela et al., 2008), we decided to analyze the extent of tubulin acetylation in cells with different GRK2 expression levels. Decreased expression of GRK2 in MEFs derived from hemizygous GRK2 (+/-) mice (which have 40-50% less kinase protein as compared to wt animals) clearly enhanced (1.8-fold) tubulin acetylation compared to MEFs from wt animals (Fig 1A). Such effect was not accompanied by global changes in the expression of either HDAC6 or SIRT2 deacetylases. Moreover, GRK2 downregulation lead to a higher accumulation of acetylated tubulin in both wt and GRK2 +/- MEFs (Fig.S1A-C) or in Hela cells (Fig.1B), in parallel with the reduced motility caused by a decrease in GRK2 levels (Penela et al., 2008 and Fig. S1B). Consistent with such inverse correlation between tubulin acetylation and cell migration, fibronectin-induced chemotaxis was reduced in +/- MEFs compared to wt (Fig 1C, control conditions) and treatment with the general HDAC inhibitor trichostatin A (TSA) (Hubbert et al., 2002; Matsuyama et al., 2002), but not with sodium butyrate (NaB, a compound that inhibits other HDAC family members but not HDAC6) inhibited migration of both +/- and wt MEFs (Fig. 1C).

Notably, acetylated tubulin markedly accumulated in HeLa cells that stably over-express either a catalytically inactive mutant of GRK2 (GRK2-K220R; HeLa-K1 cells) or a mutant at the S670 regulatory site (GRK2-S670A; Hela-A1 cells) (Fig. 1D). Such increased tubulin acetylation takes place in the absence of changes in HDAC6 protein expression (Fig. 1E) or in the extent of other tubulin posttranslational modifications (Fig. S1D) and is coincidental with the impaired ability of Hela-A1 to migrate towards chemotactic cues (reported in Penela et al., 2008) . A similar trend was

noted in wound healing experiments. Increased expression of wild-type GRK2 enhanced wound-healing closure, whereas this process was blocked in HeLa-K1 cells (Fig. S1E), as observed upon GRK2 silencing (Penela et al., 2008) Intriguingly, expression of extra wt GRK2 in HeLa-wt5 cells did not alter the steady-state levels of global tubulin acetylation, despite its chemotactic response and motility was clearly enhanced (Penela et al. 2008 and Fig. S1E). This might reflect that either endogenous kinase is sufficient to maximally modulate tubulin deacetylation or that localized GRK2-mediated increases in deacetylation are involved in such effects.

GRK2 associates with and phosphorylates HDAC6 to stimulate tubulin deactetylase activity

We next explored the potential functional interactions between GRK2 and HDAC6. A significant fraction of GRK2 co-immunoprecipitated with a HA-tagged construct of HDAC6 in HEK-293 cells transiently transfected with these proteins (Fig. 2A). Furthermore, co-immunoprecipitation of endogenous HDAC6 and GRK2 can be detected in cytoplasmatic extracts from Hela cells (Fig. 2B), indicating a specific association of these proteins at steady-state physiological conditions. (Fig. 2B)To identify the GRK2-binding region in HDAC6, a battery of HA-tagged HDAC6 truncated constructs were used (Fig. 2C). Only deletion mutants containing at least one *hdac* catalytic domain (DD1 or DD2) appear to be able to co-immunoprecipitate with GRK2, although the more critical determinants of the interaction reside in the N-terminal half of the protein encompassing the first *hdac* domain, since its removal strongly reduced HDAC6/GRK2 association (Fig. 2C).

In vitro kinase assays revealed that purified GST-HDAC6 was readily phosphorylated by recombinant GRK2 with an apparent K_m of ~45 nM (Fig. 2D). The ability of GRK2 to phosphorylate HDAC6 but not HDAC5 (Martini et al., 2008) strongly suggested that such modification was specific. Notably, we found that a construct encompassing the second *hdac* domain and the C-terminal portion of HDAC6 (C+DD2) was phosphorylated by GRK2 as efficiently as the full-length protein, whereas the truncated protein N+DD1 (able to efficiently interact with GRK2) was not (Fig. 2E). Overall, our data indicated that whereas several HDAC6 domains are involved in a multi-site interaction with GRK2, the main phospho-acceptor site(s) is located in or near to the second catalytic domain of HDAC6. Interestingly, this domain is the only one that possesses α-tubulin deacetylation activity and is the target of

HDAC6-inhibitors (Haggarty et al., 2003). Therefore, we performed in vitro deacetylation assays to test whether GRK2-mediated phosphorylation could alter HDAC6 activity. Pre-incubation with GRK2 clearly enhanced both the extent and kinetics of HDAC6-mediated α-tubulin deacetylation (Fig. 2F), indicating that GRK2 was a direct positive modulator of HDAC6 activity.

Since both *hdac* domains of HDAC6 interact with GRK2, and the first one has been proposed to serve as a α-tubulin-anchoring domain, the possibility that GRK2 was favoring HDAC6 activity by acting as a scaffold protein could not be ruled out. However, the kinase-deficient recombinant GRK2-K220R mutant did not stimulate the ability of GST-HDAC6 to deacetylate brain tubulin *in vitro* (Fig. 3A), consistent with increased tubulin acetylation levels in cells expressing this mutant (see Fig. 1D). However, tubulin acetylation was also augmented in cells expressing GRK2-S670A (Fig. 1D), a mutant described to keep unaltered its catalytic function towards GPCR (Pitcher et al., 1999). The inability of these mutants to modulate HDAC6 is not due to a binding defect, since the amount of GRK2-S670A or GRK2-K220R protein associated to HA-tagged HDAC6 was undistinguishable from that of wt-GRK2 (Fig. 3B). Likewise, association of tubulin to HDAC6 was not altered in the presence of GRK2-S670A or GRK2-K220R compared to wt protein (Fig. 3C) arguing against the possibility that such mutants could impair the potential bridging role of β-tubulin, with which GRK2 can interact (Pitcher et al., 1998), in such association (Zhang et al., 2003).

Taken together, these data supported that regulation of HDAC6 activity by GRK2 was strictly dependent on its kinase activity. Remarkably, we observed that recombinant GRK2-S670A showed a markedly reduced ability to phosphorylate HDAC6 compared to wt-GRK2, despite phosphorylation of other established GRK2 substrates was not significantly affected in this mutant (Fig. 3D). These data indicated that phosphorylation of GRK2 at the S670 regulatory site acts as a key switch that specifically modulates its ability to phosphorylate HDAC6 and thus to affect its activity.

GRK2 activity towards HDAC6 and tubulin deacetylation promote efficient pseudopodia extension in response to chemotactic cues

To determine the physiological implications of HDAC6 regulation by GRK2, we analyzed their subcellular localization in HeLa-wt5 cells directionally migrating to close an in vitro scratch. In such polarized cells, endogenous HDAC6 showed a broad

cytoplasmic distribution, although a clear accumulation was noted in the leading edge (Fig. 4A). Acetylated MTs displayed an asymmetric distribution, with an increased density towards the wound, but were excluded from the lamellipodium at the leading edge (zoomed images in Figs. 4A and 4B), in agreement with earlier reports (Gao et al., 2007; Hubbert et al., 2002; Salaycik et al., 2005). Such non-overlapping distribution at the cell border is consistent with a role for HDAC6-mediated deacetylation in motility and with an active tubulin deacetylation taking place at the dynamic leading edge. Remarkably, GRK2 was also enriched at the leading edge of migrating cells (Fig. 4B), displaying a marked co-localization with HDAC6 in the lamellipodial region (Fig. 4C) devoid of acetylated tubulin. These results strongly suggested that GRK2 would interact with HDAC6 at the cell periphery to positively regulate its activity to promote local tubulin deacetylation, what would help to maintain a gradient of MT instability that seems to be critical for migration (Salaycik et al., 2005; Siegrist and Doe, 2007; Zilberman et al., 2009). To further substantiate this point, we performed pseudopodia purification assays, which allow the isolation of both lamellipodia and adjacent lamellae structures (Cho and Klemke, 2002), in HeLa cells stably expressing either extra wt GRK2 or mutant GRK2-S670A and GRK2-K220R proteins. In response to serum, both parental and HeLa-wt5 cells extended pseudopodia through 3.0-μm porous membranes as determined by protein recovery on the underside of the membrane (Fig. 5A). Such parameter was clearly impaired in cells expressing GRK2 mutants kinase-defective towards HDAC6 (pseudopodial protein content 0.29-fold and 0.27-fold in HeLa-A1 and -K1 cells, respectively, compared to parental cells), consistent with inefficient protruding activity and defective locomotion in these cell lines (Fig. S1E and Penela et al., 2008).

We also observed that serum-induced pseudopodia extension was accompanied by local tubulin deacetylation in cells that expresses either endogenous GRK2 or extra wt protein, while relative acetylation levels remained unaltered in HeLa-A1 and –K1 cells lamellipodia (Fig. 5B). In addition, a local increase in HDAC6 and GRK2 protein levels was detected biochemically in the cortical edge of parental and HeLa-wt5 cells but not in HeLa-A1 or K1 cells (Fig. 5C). Interestingly, a marked up-regulation of GRK2 phosphorylation at S670 was specifically noted in parallel in the pseudopodia of the former cells (Fig. 5C), probably driven by the enhanced ERK activity in the protruding membrane (data not shown). As we have found that a GRK2 protein unable to be phosphorylated at S670 does not efficiently phosphorylate HDAC6, our data

strongly suggest that dynamic GRK2 posttranslational modification at this residue would take place at the leading edge and thus favor the kinase activity of GRK2 towards its co-localized substrate HDAC6.

GRK2 regulates cell adhesion and cellular spreading by promoting tubulin deacetylation

We next investigated the potential involvement of GRK2-mediated HDAC6 modulation in a distinct cellular process affected by changes in MT dynamics. Cell spreading is a multiphase process in which spherical cells in suspension initially contact with the extracellular matrix (phase P0), rapidly increase the contact area with a continuous membrane protrusion (phase P1) and finally undergo periodic contractions and focal adhesion stabilization (phase P2), leading to a maximal spread area before cells become polarized and adopt a final morphology (Dubin-Thaler et al., 2008). The implication of MTs and MT acetylation in cell spreading has been established in different cell types (Rhee et al., 2007; Tran et al., 2007).

We monitored by confocal microscopy the isotropic spreading of HeLa cells that express endogenous or extra wt GRK2 or mutant proteins S670A and K220R on fibronectin-coated surfaces. Spreading of parental and HeLa-wt5 cells progressed in a sigmoidal way with an initial period of low contact area (10min) that includes the lag time for spreading initiation, followed by a period of fast area growth (between 20 and 60 min after plating). Afterward, spreading slowed down and 2h after plating these cells attained an extension area of 548±66 and 513±55 m^2, respectively (Fig. 6A). Such spreading kinetics was similar to that reported for HeLa and other adherent cells (Cuvelier et al., 2007; Dubin-Thaler et al., 2008). Interestingly, expression of either GRK2-S670A or GRK2-K220R notably altered such normal spreading pattern. The initial phase of cell spreading proceeded in an exponential way, the spreading area 20 min after plating being 27±5% and 43±13% higher in Hela-A1 and Hela-K1 cells, respectively, compared to cells expressing similar amounts of the wt GRK2 protein (Fig. 6A and B). However, despite such initial enhanced spreading, A1 and K1 cells did not attain a larger final cellular area (2h after plating the surface of Hela-A1 and Hela-K1 was 337±75 and 380±31 m^2, respectively, versus 548±66 $m^2$of Hela). We reasoned that cells expressing such GRK2 mutants might fail to sustain the spreading at later stages, when cell expansion becomes mostly dependent on FA formation and substrate traction forces (Dubin-Thaler et al., 2008). In support of this notion, both Hela-A1 and –K1 cells spreading for 60min on FN display a low number of (aberrant)

FAs as compared to Hela or wt GRK2 cells (Fig. S2A), consistent with our previous report showing that expression of GRK2-S670A promotes the loss of FA due to abrogation of the scaffolding function of GIT-1 (Penela et al., 2008). This defect in adhesion could compromise the maintenance of a fully spread area.

We also investigated the qualitative features of cell spreading in the aforementioned cells by analyzing the organization of the actin and tubulin cytoskeleton. In parental and Hela cells expressing extra wt-GRK2 the MT network, condensed around the nucleus at early times (10min), rapidly expanded to the periphery after 40min of spreading, with a parallel increase in tubulin acetylation (Fig. 6B). Extension of such cells was accompanied by the formation of round membrane protrusions or blebs detected both at early and late stages of spreading (Fig. 6B, and inserts therein). In line with previous reports, actin stress fibers were not observed in the period of fast continuous spreading, but cortical actin cables, central fibers and actin transverse arcs were detected at later stages (60 and 120min of spreading (Fig. 6B and Fig. S2B), together with actin microspikes and filopodia. Interestingly, the organization of the MT and actin cytoskeleton was quite different in cells expressing extra GRK2-S670A or –K220R. Expansion of the MT network initiated earlier in these cells, with a higher proportion of acetylated MTs compared to controls. After 20min of plating onto FN, a well-organized net of MTs was formed around the nucleus, with longer filaments lining the edge of the cell and covering the more extended area that the K1 and A1 cells occupy at this time point (compare the surface of the different cells at 20min of spreading), while such level of organization is not noted until much more later in Hela cells, suggesting that modulation of HDAC6-mediated MT acetylation by GRK2 regulates the early phase of cell spreading. On the other hand, at later times there were markedly less central stress fibers and actin transverse arcs in A and K1 lines compared to control, while F-actin at the cortical border and filopodia were detected as in Hela cells (compare panels at 120min in Fig 6B and Suppl. Fig. S2B). Such failure to develop F-actin bundles correlates with the phase of impaired spreading in Hela-A1 and K1 cells, pointing again to defects in adhesion/contractility.

Interestingly, many MTs were found into the newly formed membrane protrusions at the cell periphery of the different stable Hela cells (Fig. 6B inserts), resembling the highly dynamic pioneer MTs that are extended towards the lamellipodium in motile cells (Salaycik et al., 2005). We observed that such leading MTs are less acetylated at their distal than at their proximal ends (see insert images in

Fig.6B) during both early and late spreading of parental and Hela-wt5 cells, but only at early spreading in Hela-A1 and K1 cells. Indeed, 2h after plating onto FN most MTs are acetylated in these cells, even those that reach the cellular edge (Fig. 6B, inserts). Since either wt, GRK2-S670A or K220R are co-localizing with cortical F-actin during spreading (Fig. S3), but tubulin deacetylation is only detected at later spreading times in cells expressing wt GRK2 (Fig. 6B), maintenance of de-acetylated MTs in the edge of spread cells at this stage seems to be dependent on the recruitment to this region of GRK2 catalytically competent towards HDAC6.

The modulatory role of GRK2 in cell spreading was further investigated by real-time resistance measurements in HeLa cells expressing endogenous or extra wt GRK2, a silencing GRK2 shRNA construct, or mutant proteins S670A and K220R, spread over a FN-coated gold electrode sensor plate, using the XCELLigence system (Roche Applied Science). Cellular impedance was continuously recorded and converted to a cell index (CI) that allows for the assessment of attached cells on the electrodes and sensing of different outputs such as cell proliferation, cell surface coverage or cellular adhesion strength. Cells were plated and allowed to attach and spread for several hours until reaching a stable base-line, when cells are maximally spread out and polarize to adopt their regular shapes (Fig. S4). Such process, which is followed by a lag period before cell growth initiates, takes 6-8 h in both HeLa and Hela-wt5 cells, while it is markedly reduced to 3h in Hela-A1 and –K1 cells, thus confirming that the later cells spread more rapidly (Fig. S4A). Interestingly, CI values at the point of plateau spreading were >2-fold higher in Hela cells compared to Hela-A1 or –K1 stable cell lines (Fig.S4A), indicating that the later cells are smaller than control cells, in line with data obtained using confocal microscopy. CI values were next normalized to the point at which all cells have reached a plateau in spreading (6h after plating) in order to circumvent cell type-specific differences in absolute CI values. Plotting of normalized CI confirmed that both Hela-A1 and –K1 cells spread more rapidly than Hela-wt5 and control cells, as the slope of the increase in CI was higher in the former cells (Fig. S4A). Consistently, the period of time required to achieve such steady-state CI values was significantly lower in Hela-A1 and –K1 cells (Fig. 7A). Downregulation of GRK2 levels also promoted enhanced cell spreading (Fig. S4B). Noteworthy, the spreading time of both Hela-wt5 and control cells can be similarly lowered upon treatment with an HDAC6 inhibitor (Fig. 7A). These results indicate that either pharmacological inhibition of HDAC6 or functional downregulation of the positive HDAC6-modulator

GRK2 might accelerate the rate of spreading by increasing the global acetylation of tubulin. Indeed, global levels of acetylated tubulin underwent a modest increase within the first 20-30 min of spreading in parental and Hela-wt5 cells and remained stable thereafter. As expected, Hela-A1 and –K1 cells accumulated much more acetylated tubulin before substrate attachment and during spreading in comparison with control cells (Fig. 7B). Remarkably, the burst in the extent of tubulin acetylation in Hela-A1 and –K1 cells was paralleled by a lack of GRK2 phosphorylation at S670, while in Hela-wt5 and control cells such modification increased up to 60min of spreading (Fig. 7C). This event could propitiate the stimulation of HDAC6 activity and help to keep a balanced acetylation of tubulin during early spreading of cells.

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DISCUSSION

We describe a novel functional interaction between GRK2 and HDAC6 that plays a key role in the dynamic modulation of MTs taking place during oriented migration of fibroblasts and epithelial cells and in cell spreading. HDAC6 is increasingly being characterized as a relevant molecular sensor and effector that modulates diverse cellular responses in ways either dependent- or independent of its catalytic activity (Boyault et al., 2007; Valenzuela-Fernandez et al., 2008). Highly dynamic MTs are present at specific sites of the cell cortex of motile cells, thereby reinforcing cell polarization and enabling directional migration. MT acetylation promotes the interaction of molecular motors with MTs (Reed et al., 2006) and inversely correlates with MT dynamics (Tran et al., 2007; Westermann and Weber, 2003; Zilberman et al., 2009). Consistently, HDAC6 upregulation and decreased tubulin acetylation enhance the motility of different cell types including fibroblasts (Hubbert et al., 2002) and breast cancer cells (Azuma et al., 2009; Saji et al., 2005).

Several lines of evidence support GRK2 as a new endogenous stimulator of HDAC6 tubulin deacetylase activity in motile cells. First, GRK2 and HDAC6 can be found endogenously in the same protein complex in cells, directly interact in vitro, are co-localized in the leading front of polarized, motile wound-edge cells, and both proteins are specifically co-recruited to chemoattractant-induced pseudopodia. Second,

recombinant GRK2 protein stimulates the tubulin deacetylase activity of HDAC6 in vitro, in a process involving direct phosphorylation of HDAC6, since neither a catalytically inactive protein (GRK2-K220R) nor a GRK2 mutant that specifically fails to phosphorylate HDAC6 (GRK2-S670A) can promote this effect. Third, down-regulation of GRK2 levels or functional silencing of endogenous GRK2 by means of over-expression of such kinase mutants with impaired activity towards HDAC6 markedly increase the extent of whole-cell acetylated tubulin. Moreover, we find that the extent of integrin- or serum-directed cell migration as well as pseudopodia extension positively correlates with enhanced GRK2 levels whereas these processes are strongly inhibited in the presence of the GRK2-K220R or GRK2-S670A mutants. Noteworthy, MTs present in the leading pseudopodia of motile cells that express endogenous or extra wt GRK2 show low levels of α-tubulin acetylation, whereas cortical MTs are highly acetylated in K220R or S670A-expressing cells. Such cells seem to be unable to establish or maintain polarized, stable protrusions as evidenced by lack of persistent lamellae toward the wound edge (Fig.S5).

Our results suggest that chemoattractant-induced translocation of GRK2 to the plasma membrane (Penela et al., 2008) might help to recruit HDAC6 to the lamellipodium and that phosphorylation of HDAC6 by GRK2 at such specific location would allow full stimulation of its activity thus enhancing local tubulin deacetylation. Importantly, we also uncover that the modulatory effect of GRK2 on HDAC6 can be dynamically regulated. The marked inability of GRK2-S670A to phosphorylate HDAC6 but not other substrates constitutes the first evidence that phosphorylation at S670 would confer a novel layer of GRK2 regulation by switching its substrate repertoire. As phosphorylation of GRK2 at S670 is rapidly up-regulated by chemotactic cues (Penela et al., 2008) and we find it specifically increased in pseudopodia of motile cells, such modification might be instrumental in enhancing localized phosphorylation of HDAC6 in situ.

We propose that GRK2-triggered, HDAC6-mediated dynamic deacetylation of tubulin at the plus-ends of MTs would be important for maintaining the cortical polarization underlying pseudopodia extension and directed migration (Fig.8A). Deacetylation might favor the dynamic anchoring of pioneering MTs to the cell cortex, propitiating actin polymerization through local recruitment of different Rac activators, such as IQGAP1 via the +TIP protein CLIP-170 (Fukata et al., 2002). It is also possible that GRK2-mediated modulation of HDAC6 activity may trigger the deacetylation of

substrates other than tubulin also critically involved in migration, such as cortactin (Zhang et al., 2007) or Hsp90 (Gao et al., 2007), a chaperone with which GRK2 directly interacts (revised in Ribas et al., 2007). Alternatively, scaffolding functions of HDAC6 could be altered by GRK2. In this regard, changes in both expression and deacetylase activity of HDAC6 have been shown to affect localization of +TIP proteins and MT regulatory functions (Zilberman et al., 2009), and HDAC6 interacts with EB1 and with $p150^{glued}$, which are key in MT nucleation and growth (Conde and Caceres, 2009; Valenzuela-Fernandez et al., 2008; Zilberman et al., 2009). Whether GRK2 can alter such potential alternative HDAC6 activities remains to be addressed in future studies.

It has been shown that extrinsic polarity cues derived from GPCR activation can translocate GRK2 to the plasma membrane (Penela et al., 2009), while pioneering MTs act as cell-intrinsic components of the polarization machinery of the cell (Siegrist and Doe, 2007). Therefore, it is tempting to speculate that GRK2, through regulation of tubulin-deacetylase activity and/or localization of HDAC6 to the leading edge, acts as a signalling node to engage and coordinate both extrinsic and intrinsic pathways relevant in controlling polarity.

GRK2 regulation of MT acetylation might also affect motility by modulating cellular adhesion. Targeting of dynamic, hypoacetylated MTs to FA promotes their disassembly, what lessens the strength of cellular adhesion and increases motility (Kaverina et al., 1999). Consistently, a higher turnover of FA has been found in cells upon GRK2 up-regulation (Penela et al., 2008), what would reduce acetylation. Strikingly, cells expressing GRK2-K220R or GRK2-S670A proteins, unable to stimulate HDAC6 and thus displaying MT hyper-acetylation, show a retracted morphology with reduced actin stress fibers and aberrant FA rather than the expected FA stabilization. Thus, these mutants must be interfering with the activity of other factors engaged in FA assembly/disassembly (Penela et al., 2008), resulting in impaired cell migration.

Our data uncover as well that GRK2 may modulate the rate of cell spreading by regulating tubulin acetylation. Furthermore, our results also suggest that the mechanisms by which MT acetylation impacts such process are more complex than previously anticipated (Fig. 8B). Our data support the notion that in the initial P1 phase, which progresses in the absence of stress fibers and mature FA (Dubin-Thaler et al., 2008) acetylated MTs would stimulate membrane protrusion and area growth in an

adhesion-independent way. During such continuous spreading protrusive forces are generated by the formation of F-actin and exocytosis at the plasma membrane, in a way resembling lamellipodium extension (Kay et al., 2008). Hyperacetylation of MTs underneath the cell periphery would improve the delivery of regulatory and structural surface components in a kinesin-dependent manner (Reed et al., 2006), thus enabling membrane extension. Consistent with this model, global tubulin acetylation levels increase at this early stage in Hela cells, and hyperacetylation in those expressing GRK2-K220R or –S670A results in enhanced rate of area growth during early spreading, in line with data showing that loss of HDAC6 activity increase the rate of the initial phase of cell spreading (Tran et al., 2007). After early spreading, actomyosin contraction is activated and cell extension starts to proceed with actin filaments pulling integrins and assembling new adhesions. At this adhesion-dependent stage, acetylated MTs would help to promote an increase in cellular area by inhibiting turnover of focal adhesions (Tran et al., 2007). Despite their higher levels of tubulin acetylation, this process is not efficient in K1 or A1 cells due to their intrinsic defects in FA dynamics (see above). Finally, at later spreading times, an active process of tubulin deacetylation seems to take place at the cell edge, in a process that requires the presence of GRK2 catalytically competent towards HDAC6 , since it is not detected in the A1 or K1 lines, and that may help to attain full surface growth. Therefore, balanced tubulin acetylation might serve cell extension and adhesion by different independent, overlapping mechanisms, which particular contribution would vary as cell transits through different phases of the spreading process (Dubin-Thaler et al., 2008; Rhee et al., 2007).

It is worth noting that HDAC6 activity has been associated with malignant transformation and invasive motility in breast cancer (Azuma et al., 2009). Moreover, both HDAC6 expression and tubulin deacetylation are required for a complete TGFβ-induced epithelial-mesenchymal transition in a lung adenocarcinoma cell line (Shan et al., 2008), and reduced tubulin acetylation was associated with malignant breast cancer progression (Suzuki et al., 2009). Conversely, inactivation of HDAC6 confers cell resistance to oncogenic transformation and tumor formation (Lee et al., 2008). Notably, GRK2 is up-regulated in different malignant mammary cell lines compared to normal cells (Salcedo et al., 2006), and enhanced GRK2 levels not only increases epithelial cell motility upon integrin and GPCR engagement (Penela et al., 2008) but also lead to reduced DNA damage-induced p53 responsiveness (Penela et al., 2010). In the light of the results reported herein, it is tempting to suggest that GRK2 up-regulation in human

tumour malignancies, by increasing the deacetylase activity of HDAC6 towards α-tubulin (and perhaps toward other substrates) would favor aberrant cell motility, adhesion and transformation. The potential role of this novel GRK2/HDAC6 interaction in cancer via the modulation of cellular processes related to cellular growth, motility or stress surveillance opens an interesting field for future research.

Materials and methods

Cell culture and treatments

Hela and HEK-293 cells were maintained in Dulbecco´s modified Eagle,s medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) at 37ºC in a humidified 5% CO_2 atmosphere. Hela cell lines stably over-expressing GRK2-wt (Hela-wt5) or mutant constructs GRK2-K220R (Hela-K1) and GRK2-S670A (Hela-A1) were previously described (Penela et al., 2008). Mouse embryonic wild-type (WT) and GRK2 (+/-) fibroblasts (MEFs) were obtained as described (Penela et al., 2008). Hela or HEK-293 cells (70-80% confluent monolayers in 60- or 100-mm dishes) were transiently transfected with the chosen combinations of cDNA or shRNAi constructs using the Lipofectamine/Plus method, following manufacturer's instructions. The cells were serum-starved for 12-16 hours and stimulated with either immobilized fibronectin (FN) in serum-free DMEM media (20 g/ml of FN for transwell migration assays and 10 gr/ml for motility scratch assays) or with 10% FBS (pseudopodia purification experiments). When indicated, cells were treated with the histone deacetylase inhibitors TSA (0.4 M) or NaB (0.4 mM).

Tubulin deacetylase (TDAC) assay

TDAC assays were developed as described (Hubbert et al., 2002). Briefly, recombinant HDAC6 protein (12.5 nM) was incubated in the presence or absence of GRK2 wt or GRK2 K220R (25 nM) for 30 min at 30ºC in kinase buffer (10µl). Then, deacetylase activity of both phosphorylated and un-phosphorylated HDAC6 proteins was assessed toward either 1µg of α/β tubulin dimers (Cytoskeleton, Inc.) or immunoprecipitated tubulin from TSA-treated HeLa cells in 100 µl of deacetylation buffer (10 mM Tris-HCl pH 8, 10 mM NaCl). Reactions were allowed to proceed at 37 °C for the indicated times and then stopped on ice for 10 min. Levels of whole and acetylated α-tubulin were analyzed by western blot.

Cellular adhesion and spreading assays

Cells were detached and kept in suspension on 150-mm Petri dishes pre-coated with 1% BSA (lipid-free) for 2 h in serum-free medium. Cells were then either immediately lysed (cells in suspension (S)) or allowed to adhere and spread for the indicated time periods to culture dishes coated with 10 g/ml FN, followed by lysis in RIPA buffer to quantitate by immunoblot analysis the expression levels of GRK2 and tubulin and their extent of phosphorylation and acetylation, respectively.

To monitor the process of spreading in live cells at real-time, we measured in parallel changes in cellular electrical impedance using the xCELLigence system (Roche Applied Science) as detailed in the Supplemental section.

Pseudopodia purification

Purification of pseudopodia was assessed as described (Matsuyama et al., 2002). Briefly, 1–1.5 × 10^6 cells were serum-starved for 16 hour and then seeded onto 1 g/ml of Fibronectin-coated 24 mm Transwell filters with 3.0-μm pores (Costar) in the presence of serum-free DMEM media in both upper and bottom chambers. After 2 hours, cells were induced or not to form pseudopodia for 1 hour by adding 10% FBS or vehicle (control), respectively, to the bottom chamber. Cells were rinsed in excess cold PBS and rapidly fixed in 100% ice-cold methanol. Cell bodies on the upper side of the membrane filter were scraped into PBS, and lysed with RIPA buffer. Remaining debris on the upper membrane were manually removed with a cotton swab and pseudopodia on the under surface scrapped into loading buffer for immunoblot analysis.

Other detailed methods are available in the Supplemental Information section.

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“Supplementary information is available at The EMBO Journal Online’

Author contributions: VL planned and performed most of the experimental work and participated in writing the manuscript. IA characterized the relationship between cellular GRK2 levels and tubulin acetylation. OT performed some of the immunofluorescence experiments. FM and PP coordinated the project, planned experiments and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Figure 1. GRK2 expression levels modulate the extent of tubulin acetylation in MEFs and Hela cells in a kinase-activity dependent manner. (A,B) Downregulation of GRK2 expression enhances tubulin acetylation. MEFs derived from wt or hemizygous GRK2 mice (A) or Hela cells transfected with either a control or a GRK2 silencing construct (B) were lysed and levels of HDAC6, SIRT2, GRK2, tubulin, acetylated tubulin (Ac-tubulin) and actin or GADPH (as loading controls) were determined by western blot analysis. Data of normalized Ac-tubulin levels are mean±SEM of 3 independent experiments. **(C)** Motility of MEFs depends on GRK2 expression levels and HDAC6 activity. Cells as in (A) were seeded on Transwell filters pre-coated with FN (20 μg/ml) in the presence of HDAC inhibitors (TSA, NaB) or vehicle (control). Cell migration was assessed as detailed in Materials and methods. Data are mean±SEM of 3 independent experiments performed in duplicate. **(D-E)** Ac-tubulin markedly accumulates in HeLa cells upon expression of catalytically inactive GRK2 or of the S670A mutant. Parental Hela cells or cells stably expressing GRK2 wt (Wt5), GRK2-S670A (A1) or GRK2-K220R (K1) mutants were analyzed for GRK2 levels and the extent of tubulin acetylation (**D**) and HDAC6 expression **(E)** as above. Data of normalized Ac-tubulin or HDAC6 levels are the mean±SEM of two independent experiments. Representative blots are shown in most panels.

Figure 2. GRK2 associates with and phosphorylates HDAC6 to stimulate tubulin deactetylase activity. (A) HDAC6 co-immunoprecipitates with GRK2**.** HEK293 cells were transfected with GRK2 alone or together with HA tagged-HDAC6. Protein association was analyzed by HA immunoprecipitation followed by immunobloting using anti-HA or anti-GRK2 antibodies**.** The same antibodies were used to check GRK2 and HDAC6 expression in cell lysates. (B) Association of endogenous HDAC6 and GRK2. Cytoplasmic extracts obtained from Hela cells were incubated with anti-HDAC6 or IgG antibodies as indicated. Immunoprecipitates (IP) or total cell extracts (Input) were analyzed by western blot using specific antibodies. **(C)** Identification of the GRK2-binding region in HDAC6. HEK293 cells were transfected with the indicated HA tagged-HDAC6 constructs (see scheme) and co-immunoprecipitation assays were performed as above. The expression of HDAC6 constructs or GRK2 in cell lysates was verified as above. Quantification of two independent co-immunoprecipitation experiments is shown. (D) HDAC6 is a GRK2 substrate. GRK2 (50 nM) and GST-HDAC6 (50-500nM) were incubated in the presence of [γ-^{32}P] -ATP as detailed in

Materials and methods. The kinetic parameters of the reaction (Vmax and Km) were estimated by double-reciprocal plot analysis. Data are the mean from 4 independent experiments. **(E)** A region bearing the second deacetylase catalytic domain of HDAC6 is the main target of GRK2 phosphorylation. HEK293 cells were transfected with different HA-tagged HDAC6 deletion mutants (N+DD1 or C+DD2). HA-immunoprecipitates were incubated under phosphorylation conditions with recombinant GRK2 (100 nM), followed by SDS-PAGE and autoradiography (upper panel). Overexpression of HA-tagged HDAC6 constructs was monitored by immunoblot with an anti-HA antibody. **(F)** Phosphorylation of HDAC6 by GRK2 enhances tubulin deacetylation. GST-HDAC6 was pre-incubated with GRK2 or vehicle under phosphorylation conditions, followed by addition of tubulin isolated from TSA-treated Hela cells. Deacetylase activity was monitored for the indicated times with an anti-acetylated and anti-α-tubulin antibodies. Acetylated band densities were normalized to total tubulin values. A blot representative of two independent experiments is shown.

Figure 3. Regulation of HDAC6 activity by GRK2 is strictly dependent on its kinase activity and is modulated by GRK2 phosphorylation status. (A) A catalytically inactive GRK2 mutant is unable to stimulate HDAC6-mediated deacetylation. GST-HDAC6 was pre-incubated with GRK2, GRK2-K220R or vehicle under phosphorylation conditions, followed by analysis of deacetylation activity as in Fig. 2F. GRK2 and HDAC6 levels were monitored to confirm equal loading. **(B)** GRK2-K220R and GRK2-S670A mutants interact normally with HDAC6. HEK293 cells were co-transfected with HA tagged-HDAC6 in the presence or absence of GRK2 wt or GRK2-S670A or K220R mutants. GRK2/HADC6 interaction was analyzed by co-immunoprecipitation as described in Fig. 2A. **(C)** HDAC6 association to tubulin is not affected by the presence of extra GRK2 wt or mutant proteins. HEK293 cells were transfected with HDAC6 and GRK2 constructs as above and tubulin immunoprecipitates analyzed for the presence of HDAC6 and GRK2. Expression levels of these proteins in cell lysates were monitored as above. **(D)** The GRK2 S670A mutant displays a markedly reduced ability to phosphorylate HDAC6, but not other GRK2 susbtrates. Phosphorylation of either GST-HDAC6 (100 nM), rhodopsin (25 nM) or Tubulin (100 nM) was performed in the presence of [γ-^{32}P] -ATP using either recombinant GRK2 wt, GRK2 S670A or GRK2 K220R proteins as described in Materials and methods and Fig.2D. Intensity of ^{32}P-bands was quantified by

densitometry and plotted as percentage of wt-GRK2-triggered ^{32}P incorporation. Data from 2-3 independent experiments and representative autoradiographies are shown.

Figure 4. HDAC6 and GRK2 co-localize in the leading edge of migrating cells. Hela cells stably expressing GRK2wt were plated in FN (10 g/ml)-coated dishes and scratched to promote wound healing as indicated in Materials and methods. After 16h of migration, cells were fixed and potential co-localization of acetylated α-Tubulin with HDAC6 (A) or GRK2 (B) and of HDAC6 with GRK2 (C) was determined by confocal microscopy upon staining with specific antibodies. Arrows indicate the leading edge of migrating cells and asterisks the wound direction.

Figure 5. GRK2-stimulated HDAC activity is relevant for pseudopodia formation in response to chemotactic cues. (A) Expression of GRK2 mutants unable to phosphorylate HDAC6 inhibits pseudopodia formation. Parental, wt5, A1 or K1 Hela cells were serum-starved for 16 h and subjected to transwell migration assays as detailed in Materials and methods in the absence or presence of serum in the bottom chamber. Levels of pseudopodia protein recovered on the underside of porous filters were analyzed using the Bradford method. Data are mean±SEM of 3 independent experiments. **(B-C)** Accumulation of both HDAC6 and GRK2 phosphorylared at S670 at pseudopodia correlates with local deacetylation of tubulin. Cells as in **(A)** were allowed to migrate in the absence or presence of a serum gradient, and 2 h later purified pseudopodia were collected and the levels of α-Tubulin acetylation **(B)** or of GRK2, its phosphorylation at S670 and HDAC6 **(C)** were determined using specific antibodies. Data in **(B)** are mean±SEM from 3-4 experiments Representative blots are shown.

Figure 6. Expression of GRK2 mutants defective in HDAC6 regulation results in an altered cell spreading pattern. (A-B) Parental, wt5, A1 or K1 Hela cells were plated on cover slips coated with FN (10 g/ml), fixed at the indicated times and analyzed by confocal microscopy. The spreading area was quantified by morphometric analysis **(A)** and cells were triple-stained **(B)** for acetylated α-Tubulin (blue), α-Tubulin (green) and F-actin (Phalloidin, red) as described in Materials and methods. Zoomed images are shown at 20, 40 and 120 min of spreading. Blue, green and white arrows and white arrow-heads indicate acetylated MTs, non-acetylated MTs, pioneer MTs, and blebs, respectively.

F**igure 7. GRK2-dependent regulation of HDAC6 activity modulates cell spreading kinetics and tubulin acetylation levels. (A)** Impairment of GRK2-mediated HDAC6

phopshorylation or pharmacological inhibition of HDAC6 accelerates spreading. The spreading kinetics of parental and Hela-wt5 pretreated or not with TSA (1mM) or Hela-A1 or –K1 cells was analyzed using the XCELLigence system as detailed in Materials and methods. Total time needed to achieve a maximum cell index during spreading and the extent of tubulin acetylation at this stage was determined for each cell line. Data are mean±SEM from 3 independent experiments. **(B)** Both the extent and time course of tubulin acetylation during cellular spreading are altered in the presence of GRK2 mutants defective in HDAC6 phosphorylation. Parental and HeLa-wt5, -A1 and -K1 cells were kept in suspension for 2 h and then allowed to adhere and spread into FN-coated plates for the indicated times. Acetylated α-tubulin and total α-tubulin levels were immunodetected with specific antibodies. A representative blot and quantification of tubulin acetylation is shown. **(C)** Levels of GRK2-pS670 are differentially regulated during spreading and inversely correlate with the spreading rate. Cells were serum-starved and collected after kept in suspension (S) or allowed to adhere and spread for 1 h (A) on FN-coated plates. The extent of GRK2 phosphorylation at S670 and total GRK2 levels were analyzed by western blot. A representative blot from two independent experiments is shown.

Figure 8.- Models depicting the intertwinement of GRK2 and HDAC6-mediated tubulindeacetylation in directed cell motility and cellular spreading.

(A) Chemotactic-movement of cells involves the projection of a dominant cell protrusion in the direction of the chemoattractant source, as a result of localized actin polymerization and the establishment of new adhesions to the substratum. The leading edge is dominated by interrelated structures such as lamella and the organelle-free lamellipodia, which are characterized by different actin and MT networks and distinct extent of focal adhesion (FA) maturation. Association of actin bundles to adhesion sites creates centripetal contractile tension that leads to detachment and retraction of the cell at the rear edge, allowing cell body translocation forward. An increased gradient of MT acetylation is present from the rear to the lamella. This would support cell polarity by facilitating the targeting and dissolution of FA at the rear edge and the delivery of regulatory and structural components to the leading edge. In the lamellipodium, GRK2 would be recruited in a Gβγ-dependent manner to sites of the plasma membrane wherein chemotactic activation is taking place. At such specific locations, chemokine receptor stimulation would promote the phosphorylation of GRK2 at S670 by MAPK,

what would in turn switch on the ability of GRK2 to phosphorylate co-localized HDAC6. Phosphorylated HDAC6 would display a higher de-acetylase activity toward tubulin at such location, contributing to keep down MT acetylation specifically at the lamellipodium. The presence of highly dynamic, hipo-acetylated MTs would stimulate cortical F-actin polymerization by helping to recruit at their plus-ends different small G proteins-GEF activities (that are directly recruited by tubulin or indirectly by the microtubule-interacting +TIP proteins).

(B) In the early phase of spreading, hyper-acetylation of MTs would increase the rate of spreading as a result of "pushing forces" generated by the sustained growth of stable MT that extend the membrane forward and facilitate the trafficking processes that drive protein cargo to the cell periphery and bring back membrane rafts that were endocytosed during the non-attached, rounded-state of cells before spreading. The extent of bulk MT acetylation would be counterbalanced by the action of HDAC6 in a GRK2-dependent manner. At this stage, local assembly of actin filaments occurs rapidly at the leading membrane edge as integrin contacts with substratum are taking place. MTs entering into this region seem to play a role akin to that of MTs in lamellipodium, displaying lower acetylation levels even in the absence of HDAC6 regulation by GRK2.

(C) At later spreading phases, the process turns to rely on FA assembly and actin stress fibers that connect FAs to generate myosin II-dependent traction forces on the substratum. At this phase, hyper-acetylation of MTs increases the spreading area by means of stabilization of FA and enhancement of actomyosin contractility, while deacetylated MTs in the lamellipodium contribute to membrane protrusion. In late spreading, the extent of tubulin acetylation of both cortical and non-cortical MTs is determined by the functional interaction of GRK2 with HDAC6.

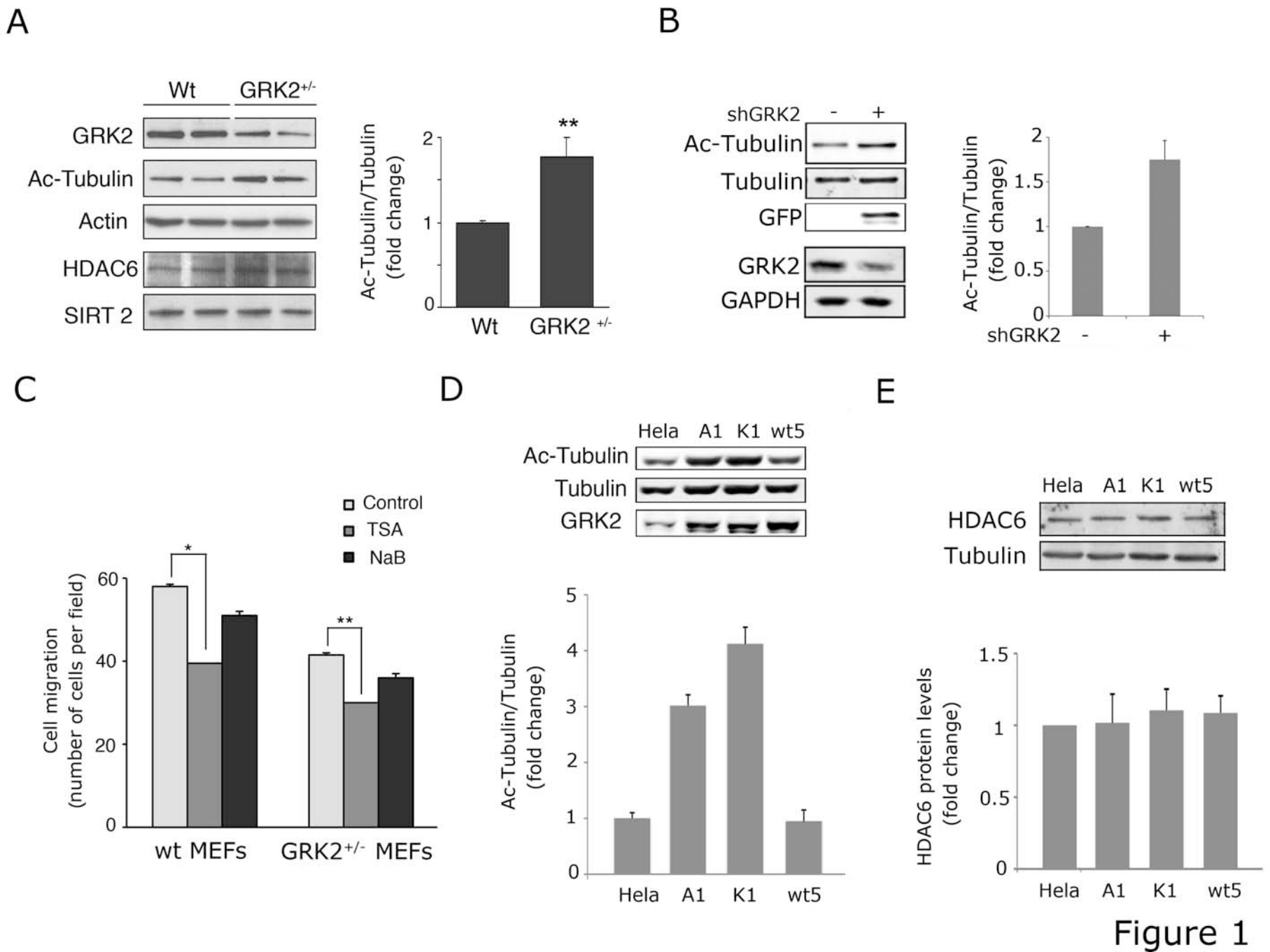

A
Wt
GRK2+/-
GRK2
Ac-Tubulin
Actin
HDAC6
SIRT 2
Ac-Tubulin/Tubulin (fold change)
**
Wt
GRK2 +/-
B
shGRK2 - +
Ac-Tubulin
Tubulin
GFP
GRK2
GAPDH
Ac-Tubulin/Tubulin (fold change)
shGRK2 - +
C
Control
TSA
NaB
Cell migration (number of cells per field)
*
**
wt MEFs
GRK2+/- MEFs
D
Hela A1 K1 wt5
Ac-Tubulin
Tubulin
GRK2
Ac-Tubulin/Tubulin (fold change)
Hela A1 K1 wt5
E
Hela A1 K1 wt5
HDAC6
Tubulin
HDAC6 protein levels (fold change)
Hela A1 K1 wt5

Figure 1

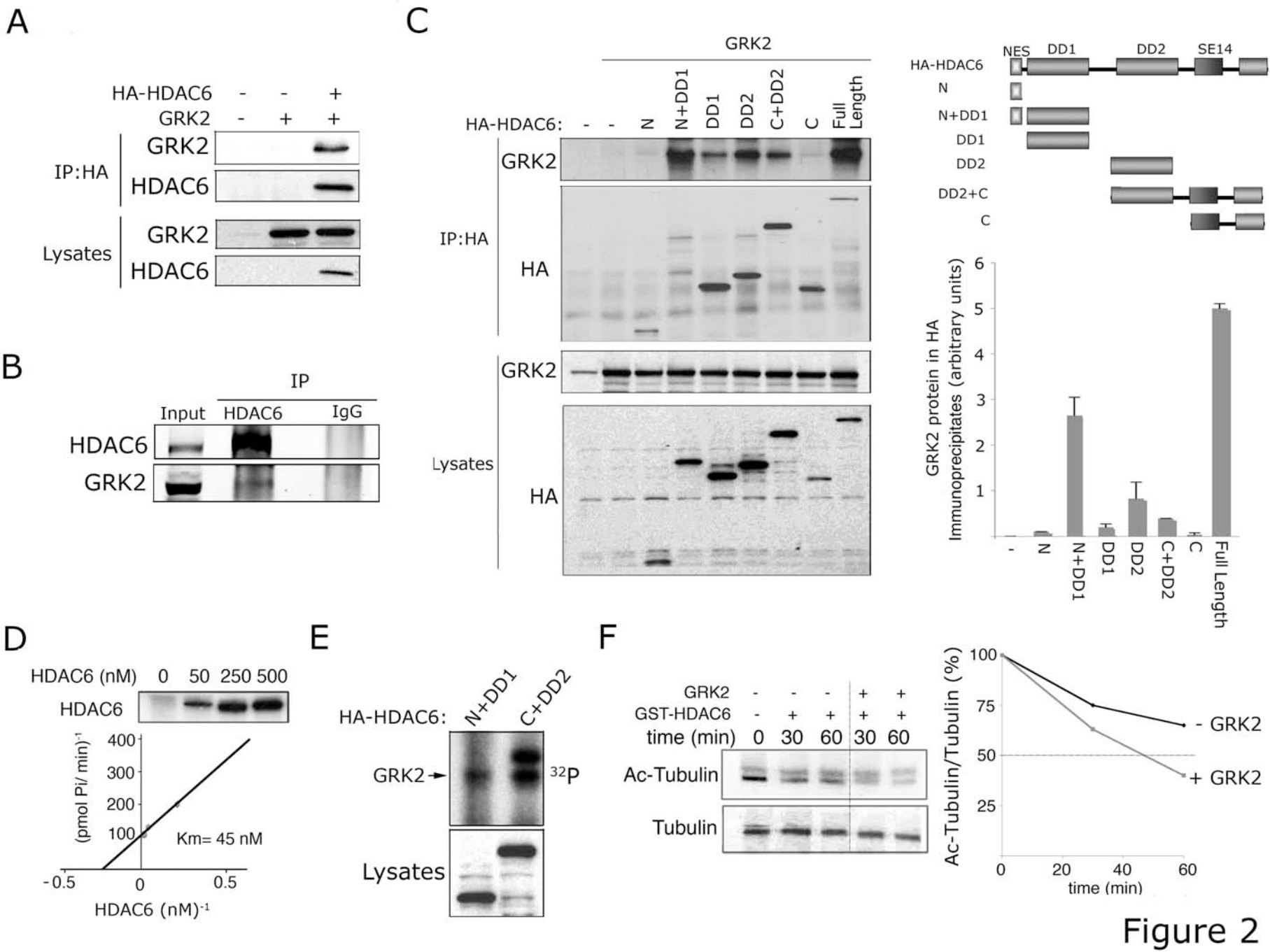

A
HA-HDAC6 - - +
GRK2 - + +
IP:HA
GRK2
HDAC6
Lysates
GRK2
HDAC6
B
IP
Input HDAC6 IgG
HDAC6
GRK2
C
GRK2
HA-HDAC6: - - N N+DD1 DD1 DD2 C+DD2 C Full Length
IP:HA
GRK2
HA
GRK2
Lysates
HA
HA-HDAC6
N
N+DD1
DD1
DD2
DD2+C
C
NES DD1 DD2 SE14
GRK2 protein in HA Immunoprecipitates (arbitrary units)
0 1 2 3 4 5 6
- N N+DD1 DD1 DD2 C+DD2 C Full Length
D
HDAC6 (nM) 0 50 250 500
HDAC6
(pmol Pi/ min)-1
100 200 300 400
Km= 45 nM
-0.5 0 0.5
HDAC6 (nM)-1
E
HA-HDAC6: N+DD1 C+DD2
GRK2
32P
Lysates
F
GRK2 - - - + +
GST-HDAC6 - + + + +
time (min) 0 30 60 30 60
Ac-Tubulin
Tubulin
Ac-Tubulin/Tubulin (%)
100 75 50 25
- GRK2
+ GRK2
0 20 40 60
time (min)

Figure 2

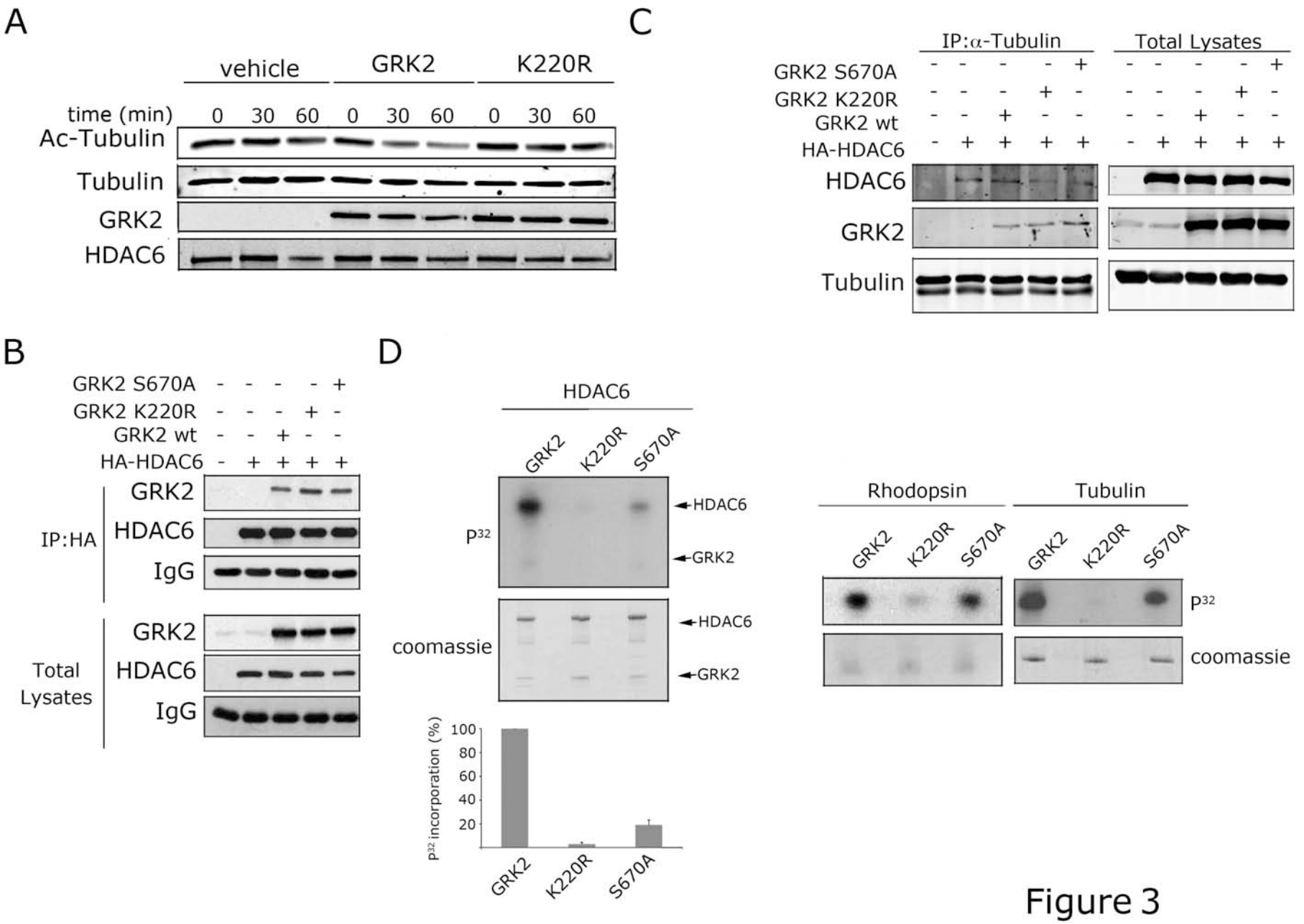

A
vehicle
GRK2
K220R
time (min)
0 30 60 0 30 60 0 30 60
Ac-Tubulin
Tubulin
GRK2
HDAC6
B
GRK2 S670A
GRK2 K220R
GRK2 wt
HA-HDAC6
IP:HA
GRK2
HDAC6
IgG
Total Lysates
GRK2
HDAC6
IgG
C
IP:α-Tubulin
Total Lysates
GRK2 S670A
GRK2 K220R
GRK2 wt
HA-HDAC6
HDAC6
GRK2
Tubulin
D
HDAC6
GRK2
K220R
S670A
P32
HDAC6
GRK2
coomassie
HDAC6
GRK2
P32 incorporation (%)
100
80
60
40
20
GRK2
K220R
S670A
Rhodopsin
Tubulin
GRK2
K220R
S670A
GRK2
K220R
S670A
P32
coomassie

Figure 3

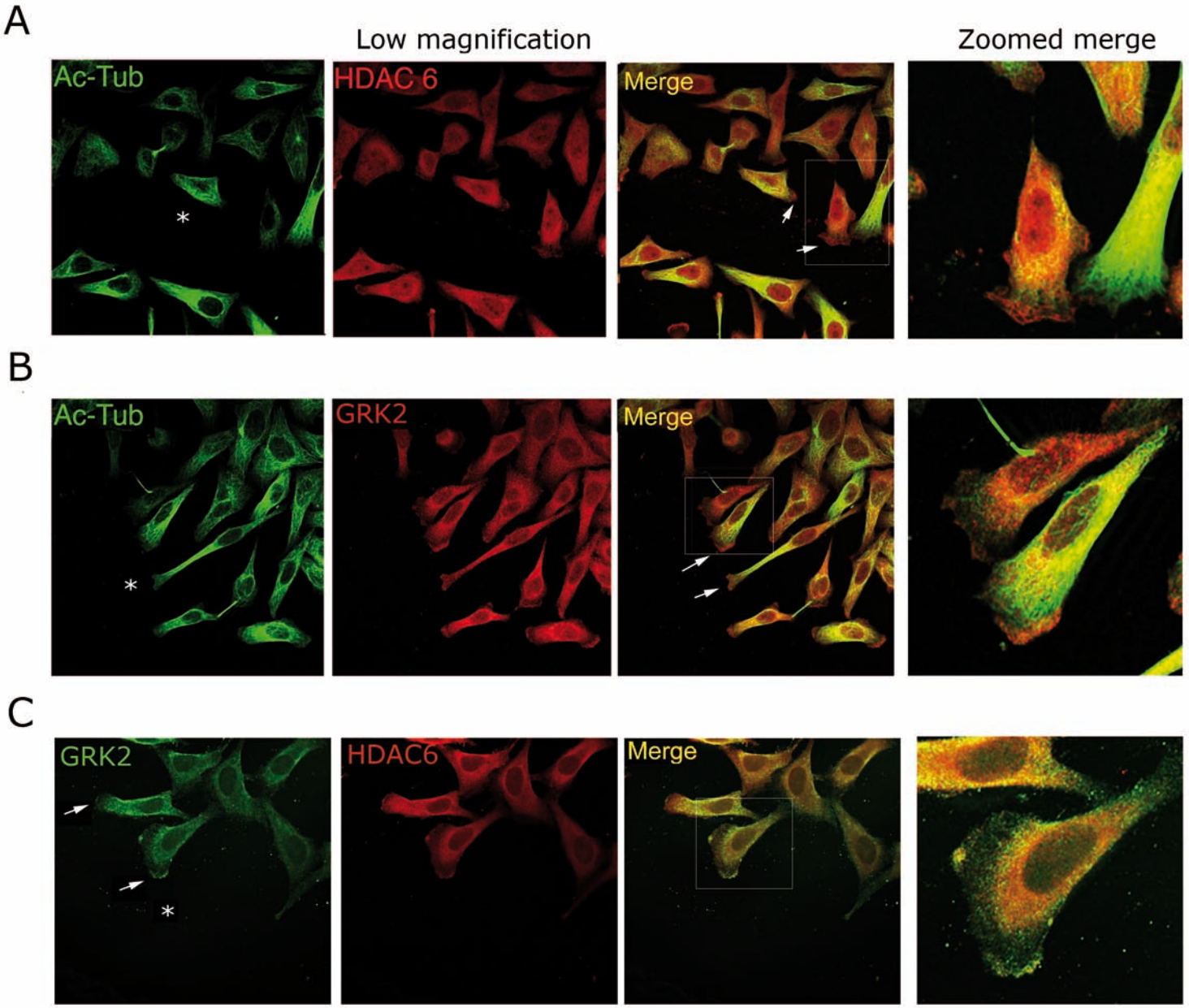

A
Low magnification
Zoomed merge
Ac-Tub
HDAC 6
Merge
B
Ac-Tub
GRK2
Merge
C
GRK2
HDAC6
Merge

Figure 4

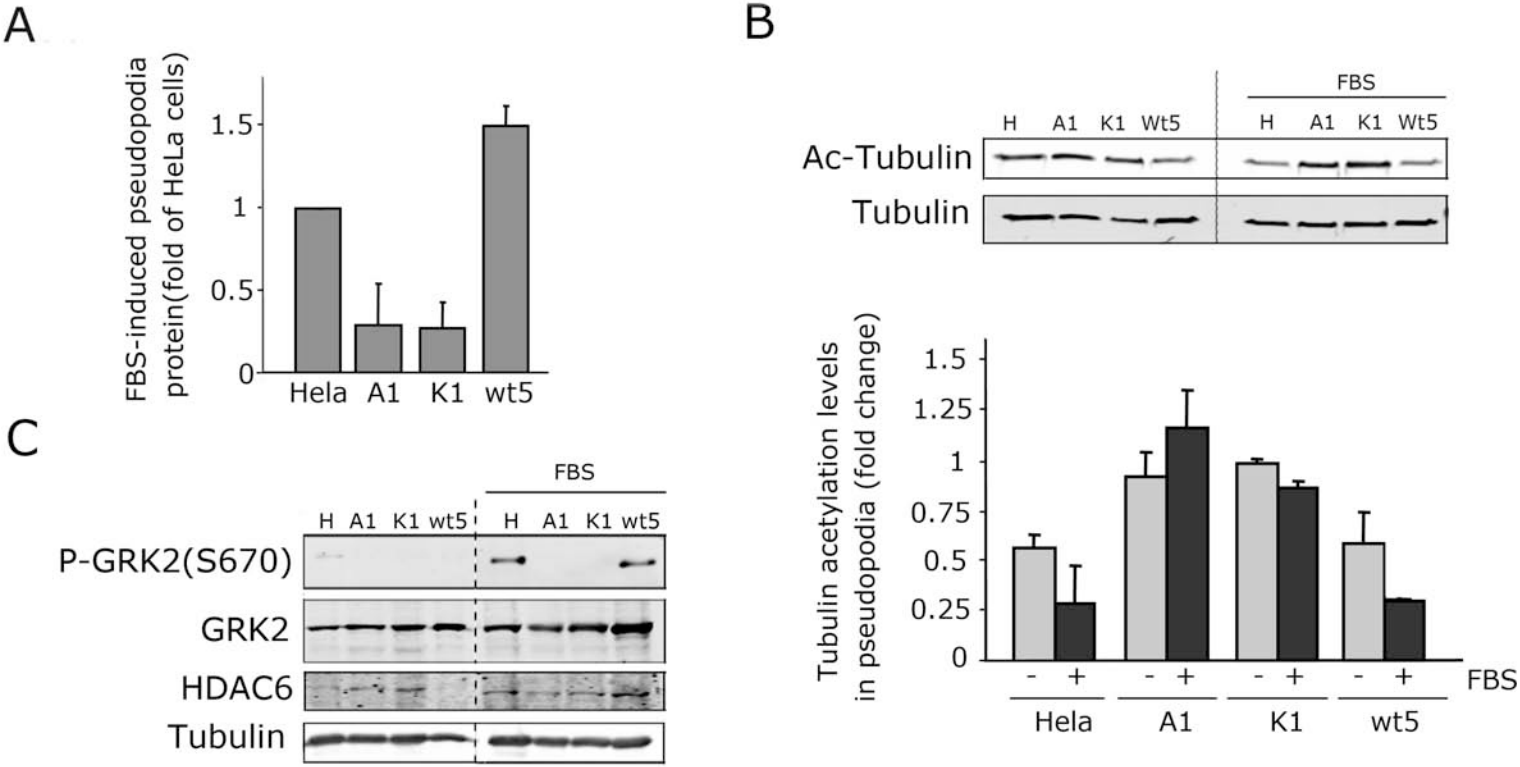

A
FBS-induced pseudopodia protein(fold of HeLa cells)
1.5
1
0.5
0
Hela A1 K1 wt5
B
FBS
H A1 K1 Wt5 H A1 K1 Wt5
Ac-Tubulin
Tubulin
Tubulin acetylation levels in pseudopodia (fold change)
1.5
1.25
1
0.75
0.5
0.25
0
- + - + - + - +
FBS
Hela A1 K1 wt5
C
FBS
H A1 K1 wt5 H A1 K1 wt5
P-GRK2(S670)
GRK2
HDAC6
Tubulin

Figure 5

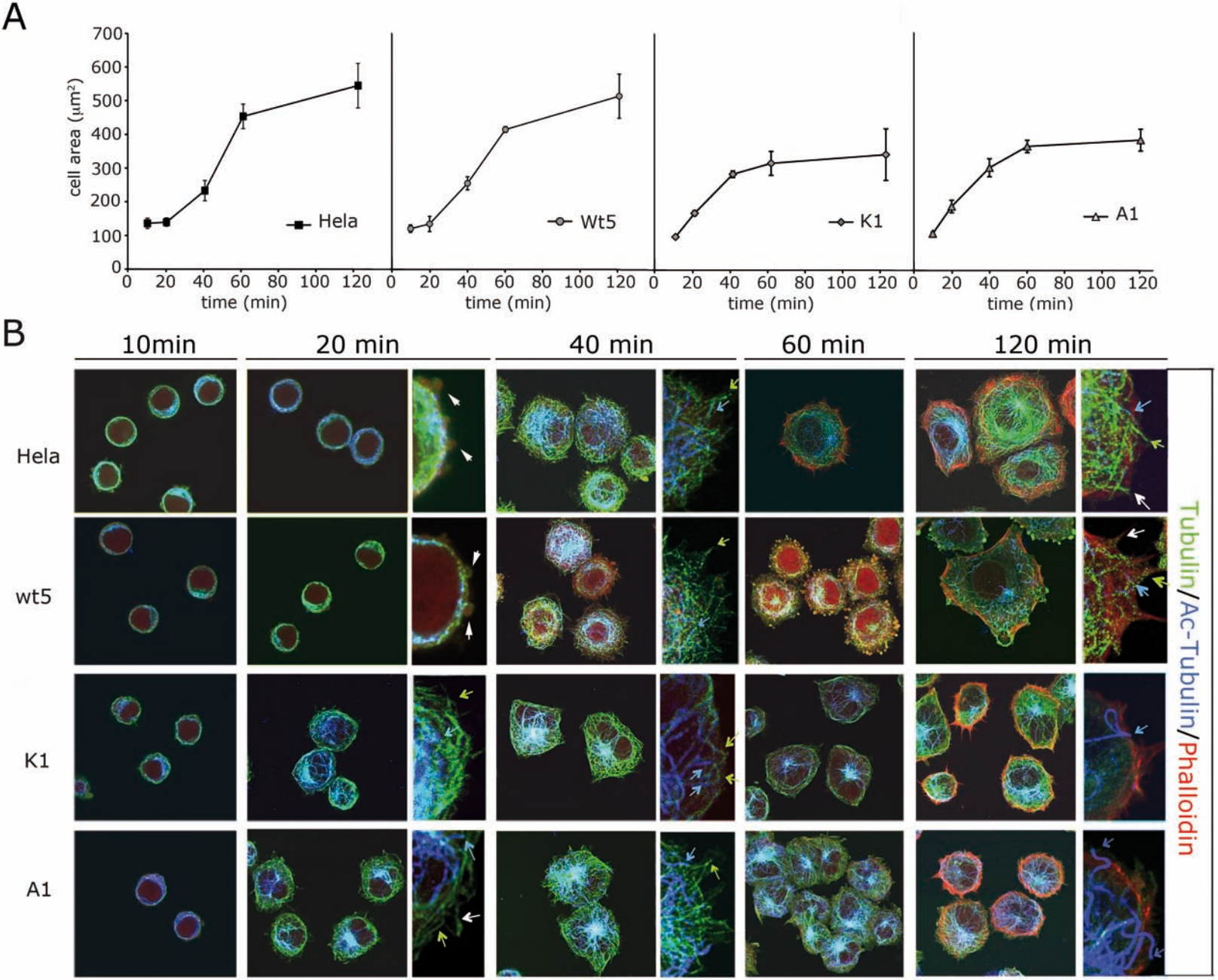

A
cell area (μm²)
700
600
500
400
300
200
100
0
0 20 40 60 80 100 120
time (min)
Hela
Wt5
K1
A1
B
10min
20 min
40 min
60 min
120 min
Hela
wt5
K1
A1
Tubulin/Ac-Tubulin/Phalloidin

Figure 6

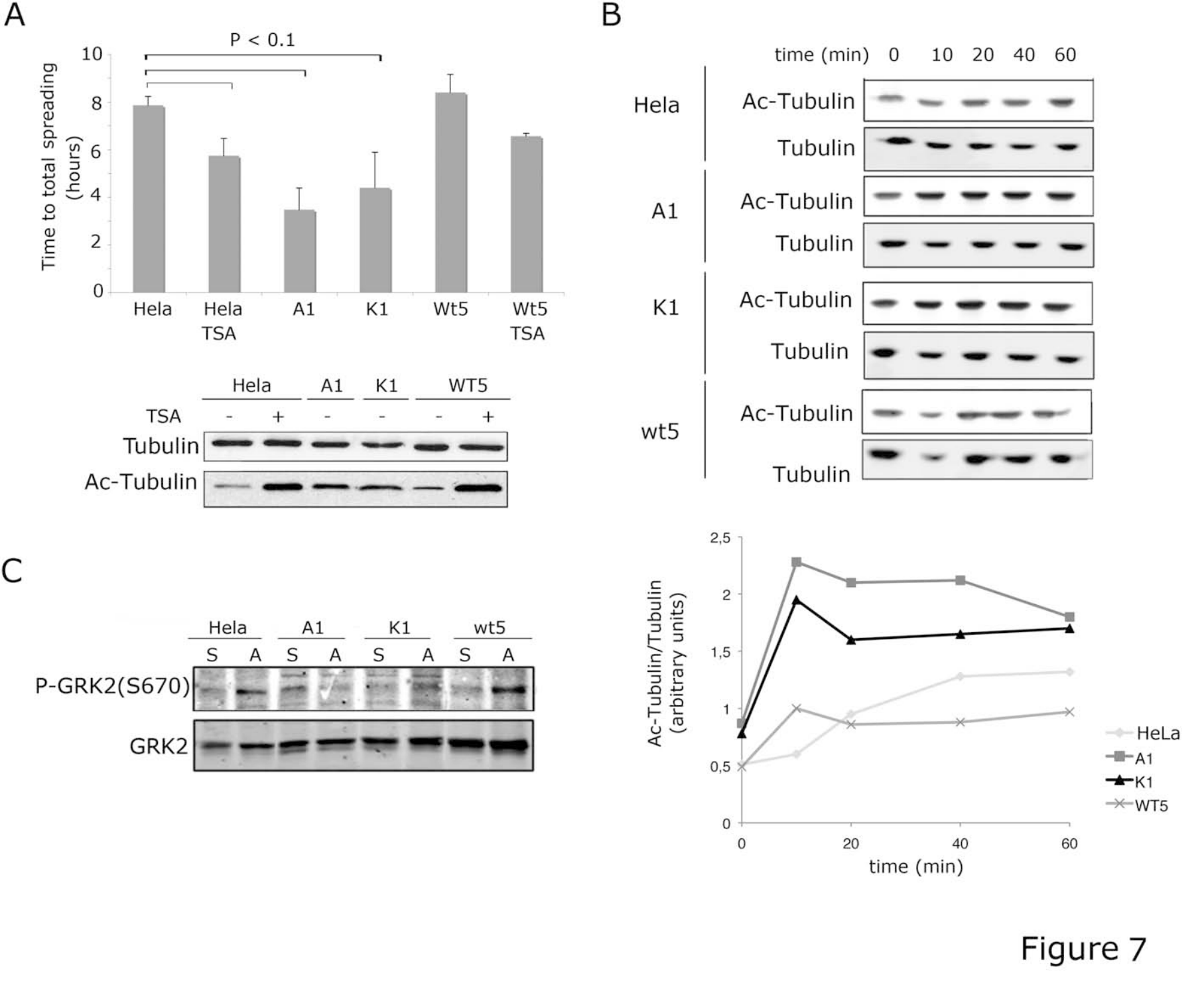
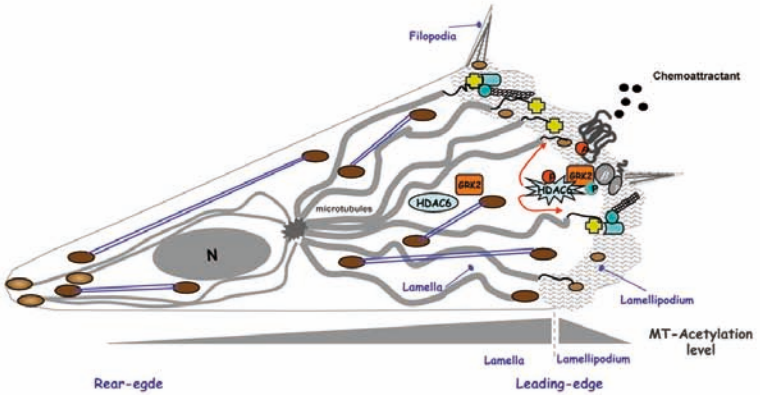
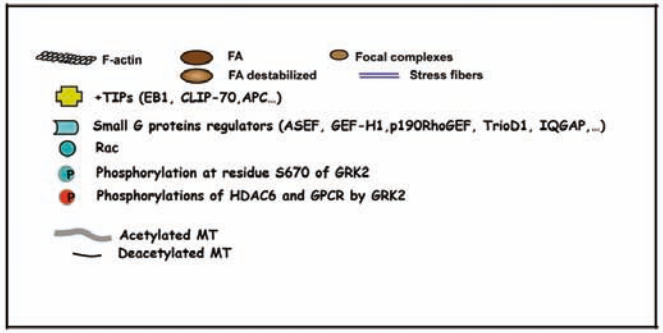

A
P < 0.1
Time to total spreading (hours)
10
8
6
4
2
0
Hela
Hela TSA
A1
K1
Wt5
Wt5 TSA
Hela
A1
K1
WT5
TSA
- + - - - +
Tubulin
Ac-Tubulin
B
time (min) 0 10 20 40 60
Hela
Ac-Tubulin
Tubulin
A1
Ac-Tubulin
Tubulin
K1
Ac-Tubulin
Tubulin
wt5
Ac-Tubulin
Tubulin
C
Hela
A1
K1
wt5
S A S A S A S A
P-GRK2(S670)
GRK2
Ac-Tubulin/Tubulin (arbitrary units)
2,5
2
1,5
1
0,5
0
0
20
40
60
time (min)
HeLa
A1
K1
WT5

Figure 7

A

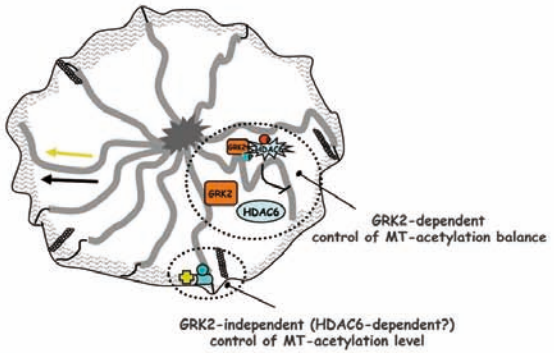




GRK2 and HDAC6-mediated MT deacetylation in cell migration

B

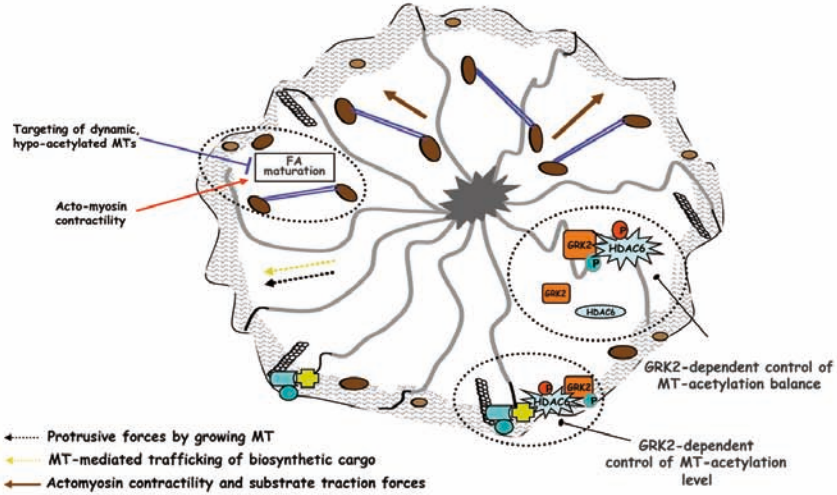
Adhesion-independent, early spreading phase



Protrusive forces by growing MT
MT-mediated trafficking of biosynthetic cargo and membrane rafts

GRK2 and HDAC6-mediated MT deacetylation in early cell spreading

Adhesion-dependent, late spreading phase



Protrusive forces by growing MT
MT-mediated trafficking of biosynthetic cargo
Actomyosin contractility and substrate traction forces

GRK2 and HDAC6-mediated MT deacetylation in late cell spreading

Figure 8