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Research Paper

# G Protein-coupled Receptor Kinase 2 (GRK2) Promotes Breast Tumorigenesis Through a HDAC6-Pin1 Axis

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ABSTRACT

In addition to oncogenic drivers, signaling nodes can critically modulate cancer-related cellular networks to strength tumor hallmarks. We identify G-protein-coupled receptor kinase 2 (GRK2) as a relevant player in breast cancer. GRK2 is up-regulated in breast cancer cell lines, in spontaneous tumors in mice, and in a proportion of invasive ductal carcinoma patients. Increased GRK2 functionality promotes the phosphorylation and activation of the Histone Deacetylase 6 (HDAC6) leading to de-acetylation of the Prolyl Isomerase Pin1, a central modulator of tumor progression, thereby enhancing its stability and functional interaction with key mitotic regulators. Interestingly, a correlation between GRK2 expression and Pin1 levels and de-acetylation status is detected in breast cancer patients. Activation of the HDAC6-Pin1 axis underlies the positive effects of GRK2 on promoting growth factor signaling, cellular proliferation and anchorage-independent growth in both luminal and basal breast cancer cells. Enhanced GRK2 levels promote tumor growth in mice, whereas GRK2 down-modulation sensitizes cells to therapeutic drugs and abrogates tumor formation. Our data suggest that GRK2 acts as an important onco-modulator by strengthening the functionality of key players in breast tumorigenesis such as HDAC6 and Pin1.



## 1. Introduction

Malignant features such as sustained proliferative signaling, refractoriness to growth suppressors, resistance to cell death or aberrant motility and metastasis, can be triggered by a variety of distinctive mutations and signaling adaptations (Hanahan and Weinberg, 2011). This complexity translates into phenotypic variability among tumors. A prototypic example is breast cancer, a heterogeneous disease encompassing different histopathological entities with distinct molecular signatures, genetic and genomic variations (Bertos and Park, 2011). Ductal carcinomas (circa 80% of all breast cancers) are grouped in several types based on the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2 or ERBB2). The most common is the luminal type characterized by $ER^+$ and (or) $PR^+$ with a HER− (A subtype) or HER+ (B subtype) status. Along with the luminal B subtype, the HER2ERBB2-positive (ER and PR negative) and basal-like tumors lacking ER, PR and ERBB2 expression (triple negative) exhibit the worst prognosis (Bertos and Park, 2011; Hanahan and Weinberg, 2011). Enhanced activation of the RAS-mitogen activated protein kinase (MAPK) pathway triggered by amplification or up-regulation of growth factor receptors (ERBB2, IGF1R, EGFR), ER activation and alterations in the PI3K-AKT pathway are major contributors to the uncontrolled cellular proliferation and increased survival of breast tumor cells (Saini et al., 2013).

In addition to these oncogenic drivers, signaling nodes can act as cancer-associated factors by cooperating with oncogene-governed pathways or participating in compensatory transduction networks to strengthen tumor properties. G-protein-coupled receptor kinase 2 (GRK2) is emerging as one of such key nodes. Besides its canonical role in the desensitization of G protein-coupled receptors (GPCR) (Penela et al., 2010a; Premont and Gainetdinov, 2007), GRK2 is a positive effector of certain GPCR and receptor-tyrosine kinases (RTK)

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transduction cascades. Excessive signaling from diverse GPCRs can lead to aberrant survival, cellular growth and invasive motility in a variety of cancer cells (Lappano and Maggiolini, 2011). GRK2 has been identified as a rate-limiting mediator of MAPK activation and growth signaling triggered by some tumor-related GPCRs (Lipfert et al., 2013; Penela et al., 2008; Philipp et al., 2008). GPCRs can also trans-activate the EFGR by means of the GRK-β-arrestin-axis (Rozengurt, 2007). These effects may be explained by the ability of GRK2 to phosphorylate or dynamically interact with important modulators engaged along the MAPK pathway (Deiss et al., 2012; Penela et al., 2010b, 2008). Moreover, other relevant GRK2-interacting proteins or substrates are key players in the cellular stress response and survival (Lafarga et al., 2012b; Robinson and Pitcher, 2013) and references therein).

Despite such potential connectivity to signaling pathways related to cell transformation, and available data showing altered GRK2 levels in some tumoral contexts (reviewed in Evron et al. (2012), Penela et al. (2010a)) a comprehensive study of the role of GRK2 in tumor formation and progression has not been addressed. We report here that GRK2 levels are altered in breast luminal tumors and that increased GRK2 functionality in diverse breast cancer cell types represents a driving signaling event in the acquisition and maintenance of tumoral proliferation and survival, by mechanisms involving the potentiation of Histone Deacetylase 6 (HDAC6) function and the modulation of Pin1 (Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1) acetylation status and functionality.

## 2. Materials and Methods

### 2.1. Cell Culture, Cellular Treatments and Antibodies

All tumoral and non-tumoral breast cells were obtained from the American Type Culture Collection. Details on medium conditions, transfection methods, reagents for cellular treatments and antibodies utilized can be found in Supplemental Methods.

### 2.2. Cell Proliferation and Viability Assays

Cell proliferation was measured using the CellTiter96®AQueaous Non-Radioactive Cell Proliferation Assay according to the manufacturer's protocol (Promega). In addition, cell viability was also monitored in real-time assays using the xCELLigence System (Roche Applied Science) following the manufacturer's guidelines.

### 2.3. Mammary Tissue from Animal Models and Breast Cancer Patients

Mammary glands from 9-weeks old Myr-AKT transgenic mice and wild type littermates and paired non-tumoral and tumoral glands from 6 to 9 weeks old transgenic FVB-N-TgMMTV-HER2 mice were removed and snap-frozen. Human normal breast tissue and infiltrating ductal carcinoma samples were obtained from patients recruited at the Hospital Universitario La Paz (Madrid, Spain). Mice and human samples were processed as detailed in Supplemental Methods.

### 2.4. Colony Formation Assay

Tetracycline- or vehicle induced MCF7 Tet-on wt-GRK2 and GRK2-K220R cells were seeded in a soft agar medium, and cells were then fixed with methanol and stained with 0.005% Crystal Violet for subsequent analysis as detailed in Supplemental Methods.

### 2.5. Statistics

Data in all figures are expressed as mean ± SEM or SD as indicated. All results were confirmed in at least 2 separate experiments. Data were analyzed using Student's *t*-test. Two-tailed $p < 0.05$ was considered statistically significant. Statistical correlations obtained from samples of patients were analyzed using Pearson Test or a quadratic regression linear model with $p < 0.05$ considered as significant.

Protein and DNA arrays, western blot analysis and protein data normalizations, GST pulldown assays, protease-coupled peptidyl-prolyl isomerase assays, in vivo tumor implantation, immunohistochemical assays and study approval are described in the Supplemental Methods section.

## 3. Results

### 3.1. GRK2 protein expression is upregulated in breast cancer cell lines and cancer-prone animal models

We examined GRK2 expression in a panel of breast tumor cell lines representative of molecular features found in many of the primary tumor subtypes (Vargo-Gogola and Rosen, 2007). Luminal MDA-MB361, T47D and MCF7 cells expressed 3- to 10-fold higher GRK2 levels than non-transformed MCF10A and 184B5 lines (Fig. 1A). Conversely, total GRK2 levels were not increased in basal breast cancer-derived MDA-MB-231, MDA-MB157 and Hs578T cells. Enhanced GRK2 was also detected in MDA-MB468 cells, usually classified in the basal A subtype (Kao et al., 2009), but that share features with the luminal molecular phenotype (see below).

Interestingly, while GRK2 is upregulated in luminal cells irrespective of their p53 status (Fig. S1A), enhanced GRK2 concurs with either mutational activation of *PIK3CA* or inactivation of *PTEN* (Fig. 1B), and correlates with increased activation of the AKT pathway (Fig. 1C), a frequent feature of human luminal breast tumors (Eroles et al., 2012). These mutations are not displayed by the basal cells in our panel, except for MDA-MB468, which shows increased GRK2, consistent with previous data (Salcedo et al., 2006).

Elevated GRK2 was present in cells ER-PR+ (MDA-MB361, T47D and MCF7) and (or) displaying amplification of the EGFR (MDA-MB468) or HER2 (MDA-MB361) receptors (Fig. 1B), all of them contexts able to trigger PI3K-AKT activation (Renoir et al., 2013; Roskoski, 2014). Estrogen withdrawal promoted a decrease of GRK2 in both MCF7 and T47D ER+ cells (Fig. 1D), whereas estrogen exposure caused circa 2-fold increase (Fig. 1E). Moreover, GRK2 decayed in ER+ non-transformed 184B5 cells challenged with the estrogen antagonist tamoxifen, but not in tamoxifen-refractory MCF7 and T47D lines (Fig. S1B, C). Co-transfection of HER2 and Ras-V12, known to cooperatively induce mammary cell transformation (Wulf et al., 2004) increased both GRK2 protein and AKT stimulation in the non-malignant MCF10A and 184B5 cells (Fig. S1D) whereas epidermal growth factor receptor (EGFR) inhibition markedly reduced GRK2 levels and AKT activation in EGFR-overexpressing MDA-MB468 cells (Fig. 1F).

These results suggested that different signaling pathways altered in luminal breast cancer cell lines converge in promoting an AKT-mediated increased in GRK2 levels. Consistently, GRK2 expression was increased specifically in those mammary glands of transgenic MMTV-HER2 mice that spontaneously develop tumors (Fig. 1G), in parallel with higher activation of AKT, and in mammary glands of transgenic mice expressing myr-AKT (Fig. 1H), a constitutively active membrane-bound construct (Blanco-Aparicio et al., 2007).

### 3.2. GRK2-dependent regulation of HDAC6 strengthens growth factor-triggered signaling pathways in breast cells

Cell lines with enhanced GRK2 levels also displayed increased proliferation rates and expression of key proliferation markers compared to non-transformed and basal cells (Fig. S2a, b). Consistent with the notion that GRK2 up-regulation was not a mere bystander but was playing a role in the acquisition or strengthening of oncogenic properties, GRK2 overexpression in either non-transformed MCF10A (Fig. S3a, b) or 184B5 (Fig. S3c–f) cells promoted a significant increase in the levels of the mitotic entry marker pHis3 as well as of Pin1, a pivotal regulator

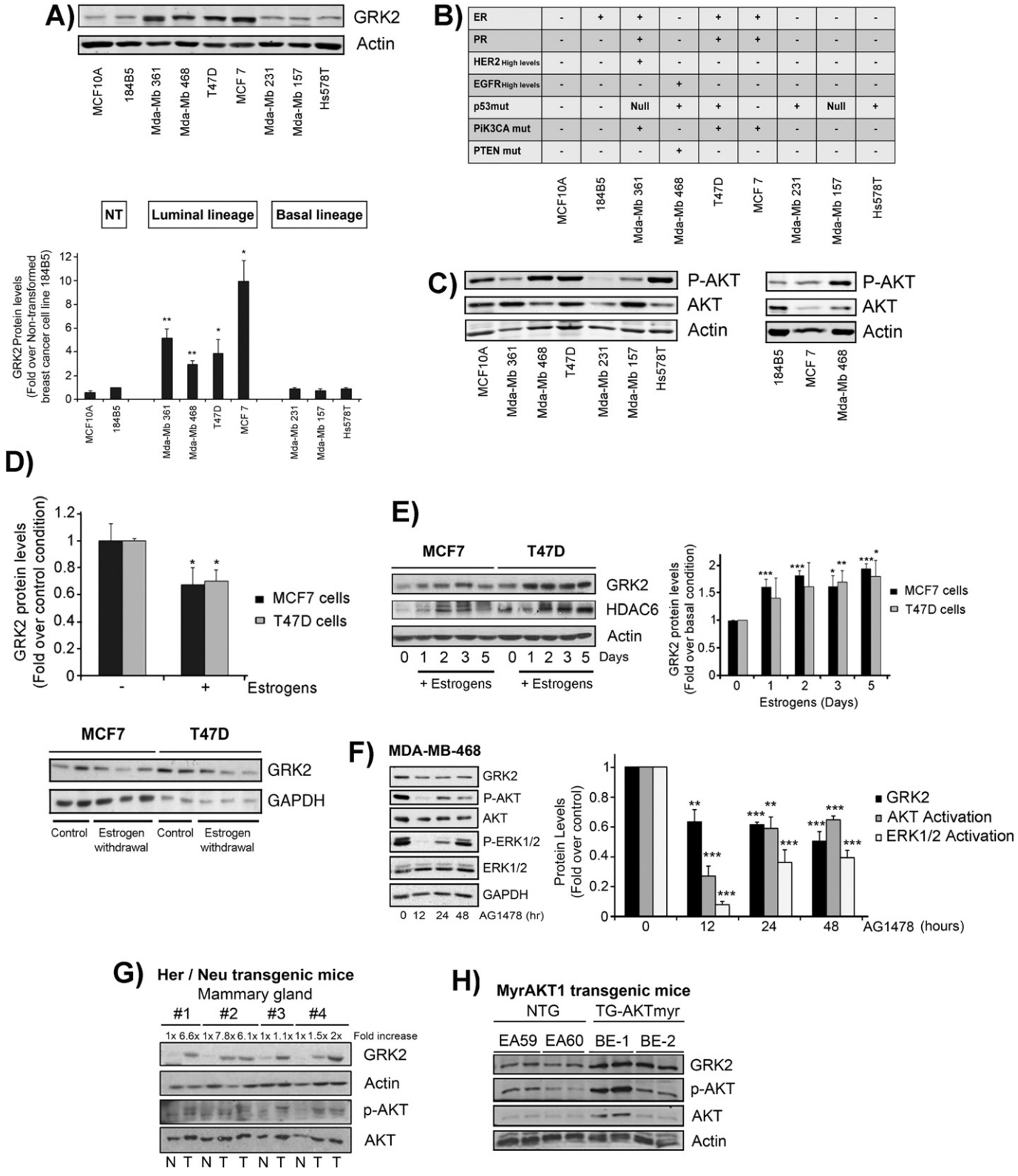



**Fig. 1.** Luminal breast tumors oncogenic pathways promote increased GRK2 protein levels in cellular and animal models. (A) Western blot analysis of GRK2 expression levels relative to 184B5 cells in non-transformed (NT), "luminal-like" and "basal-like" transformed breast cancer cells. (B) Molecular features of cell lines used in (A): +, presence; −, absence; null, homozygous deletion. (C) Immunoblot analysis of pSer473- AKT and pan-AKT levels in panel A cell lysates. (d, e) Analysis of GRK2 and HDAC6 expression in luminal breast tumor cells in control or estrogen-depleted conditions (D), or upon estrogen re-stimulation (E). (f) Levels of GRK2 and AKT activation in MDA-MB-468 cells treated with the EGFR-inhibitor AG1478. (G, H). Mammary expression of GRK2 and AKT activation in tumor-bearing HER2-transgenic mice (n = 4, g) or MyrAKT1-transgenic animals (n = 2 per group, h) as compared to normal paired glands or non-transgenic littermates. All data are mean ± SEM, n = 3–4. Actin or GAPDH expression was used as loading control were indicated. *p < 0.05; **p < 0.01; ***p < 0.001.

of HER2 and ER-mediated signaling in breast cancer (Frasor et al., 2004), to an extent similar to that of the oncogenic drivers Ras-Her2. Notably, adenovirus-mediated transduction of wild-type GRK2 in either 184B5 (Fig. S4a) or MCF10A cells (Fig. S4b) potentiated EGF-triggered Ras activation, whereas a catalytically inactive GRK2-K220R construct did not, suggesting that GRK2-mediated phosphorylation processes were required for enhancing mitogenic signaling. Moreover, the already altered levels of Pin1 and Ras proteins in transformed MCF7 cells were further increased by extra GRK2 but markedly reduced upon its shRNA-mediated knockdown (Fig. S4c). Further stressing a potential causal effect of GRK2 activity on Pin1 protein levels, adenovirus-mediated expression of wild-type GRK2 (but not of the kinase-dead K220R) enhanced Pin1 expression in a different luminal cell line (T47D) or in several basal breast cancer cells (MDA-MB-231, MDA-MB468 and Hs578T) (Fig. S4d), whereas an interfering shRNA-GRK2 construct significantly diminished Pin1 levels in most of these cells.

Remarkably, stable overexpression of GRK2 in 184B5 cells also significantly facilitated both mitogenic (ERK1/2) and pro-survival signaling (AKT) in response to heregulin (Fig. S5a) or EGF (Fig. S5b). Similar consequences were also noted in the time-course and dose-response effects of EGF in GRK2-overexpressing MCF7 cells in a catalytic-dependent manner (Fig. S6a, b), whereas kinase down-modulation markedly attenuated EGF signaling (Fig. 2a).

We hypothesized that the cytoplasmic type II histone deacetylase 6 (HDAC6) could play a relevant role underlying these effects. HDAC6 has been associated with malignant transformation in breast cancer (Li et al., 2013) and its tubulin-deacetylase activity contributes to maintain the EGFR at the plasma membrane, thus promoting sustained activation of downstream cascades (Deribe et al., 2009; Gao et al., 2010). We have reported that EGF-induced phosphorylation of GRK2 at S670 by ERK1/2 was required for GRK2-mediated phosphorylation of HDAC6, in turn promoting full tubulin deacetylase activity (Lafarga et al., 2012a). Notably, enhanced levels of HDAC6 were detected in the luminal-like breast cancer cells displaying GRK2 up-regulation (Fig. S7a), while most of both luminal and basal breast cancer cells tested displayed increased levels of phospho-S670 GRK2 (Fig. S7b), suggesting that a GRK2-HDAC6 axis could be operative in these cells. Remarkably, an HDAC6 mutant unable to be phosphorylated by GRK2 (Lafarga et al., 2012a) blocked the GRK2-triggered increase in EGF-mediated signaling to ERK1/2 in 184B5 cells (Fig. S7c) and led to enhanced levels

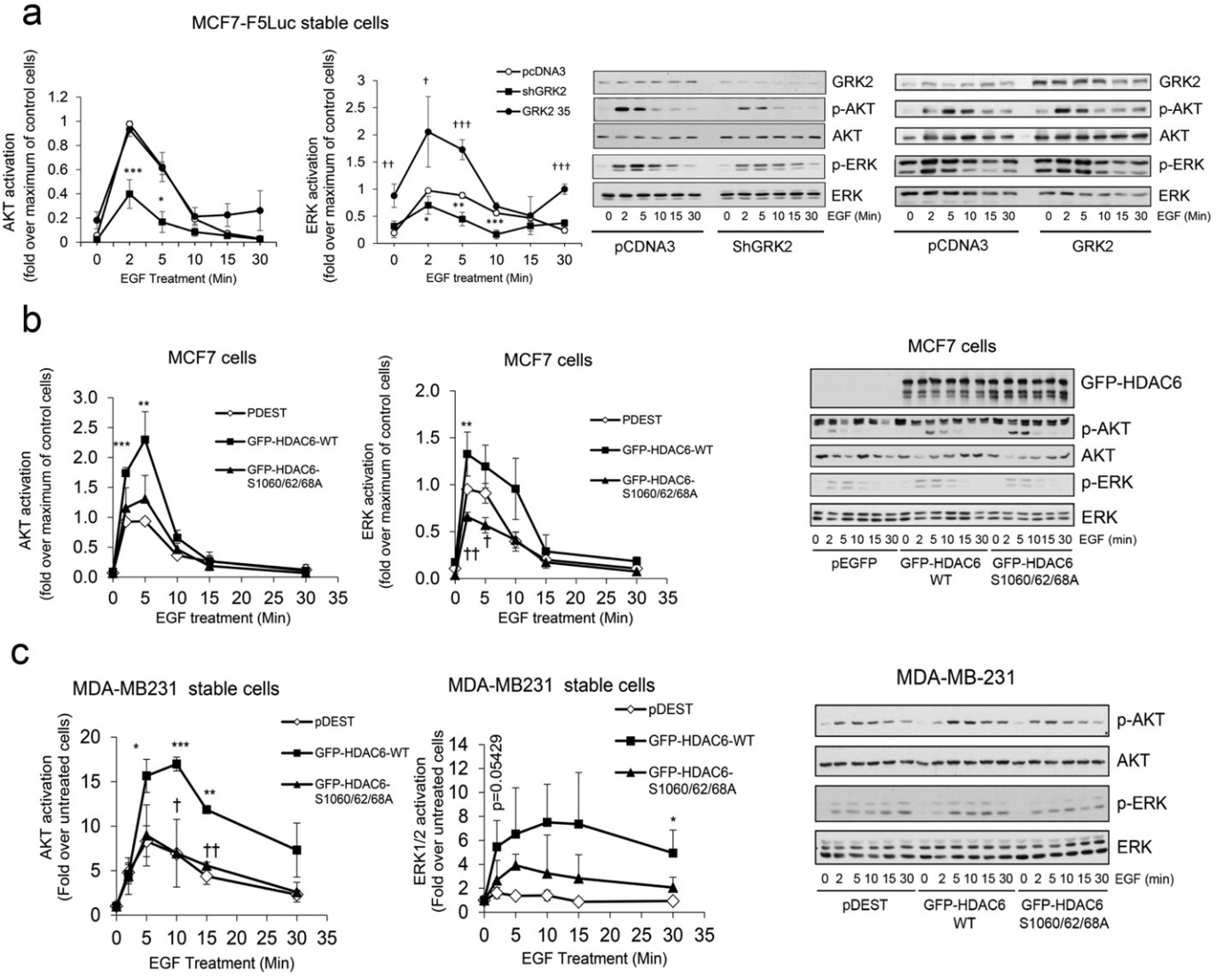



**Fig. 2.** GRK2 over-expression potentiates mitogenic signaling pathways in breast cancer cells via HDAC6. (a) Analysis of both AKT and ERK1/2 responses to 100 ng/ml EGF in MCF7-F5luc cells with extra (GRK2 35 clone) or silenced (shGRK2) expression of GRK2. *p < 0.05, **p < 0.01 and ***p < 0.001, and T p < 0.05, TT p < 0.01 comparing pcDNA3 to shGRK2 and GRK2 conditions, respectively. (b–c) Analysis of EGF-triggered ERK1/2 and AKT stimulation upon transfection of GFP-HDAC6-wt or GRK2-phosphodefective HDAC6 constructs in MCF7 cells (b), or stable transfection in MDA-MB-231 cells (c). *p < 0.05, **p < 0.01 and ***p < 0.001 compared to empty vector-bearing cells and T p < 0.05, TT p < 0.01 for comparison of HDAC6-wt to HDAC6 mutant-expressing cells. In all panels data are mean ± SEM (n = 3–4). Representative blots are shown.

of tubulin acetylation (Fig. S7d). Akin to non-transformed cells, expression of this phospho-defective HDAC6 mutant in either MCF7 luminal (Fig. 2b) or MDA-MB-231 basal cells (Fig. 2c) failed to mimic the enhanced EGF signaling promoted by wild-type HDAC6, strongly suggesting that a reinforced GRK2-HDAC6 functional interaction was playing a role in increasing EGF-modulated cascades in different breast cell types.

### 3.3. The GRK2-HDAC6 module stimulates Pin1 functionality by triggering de-acetylation of its lysine 46 residue

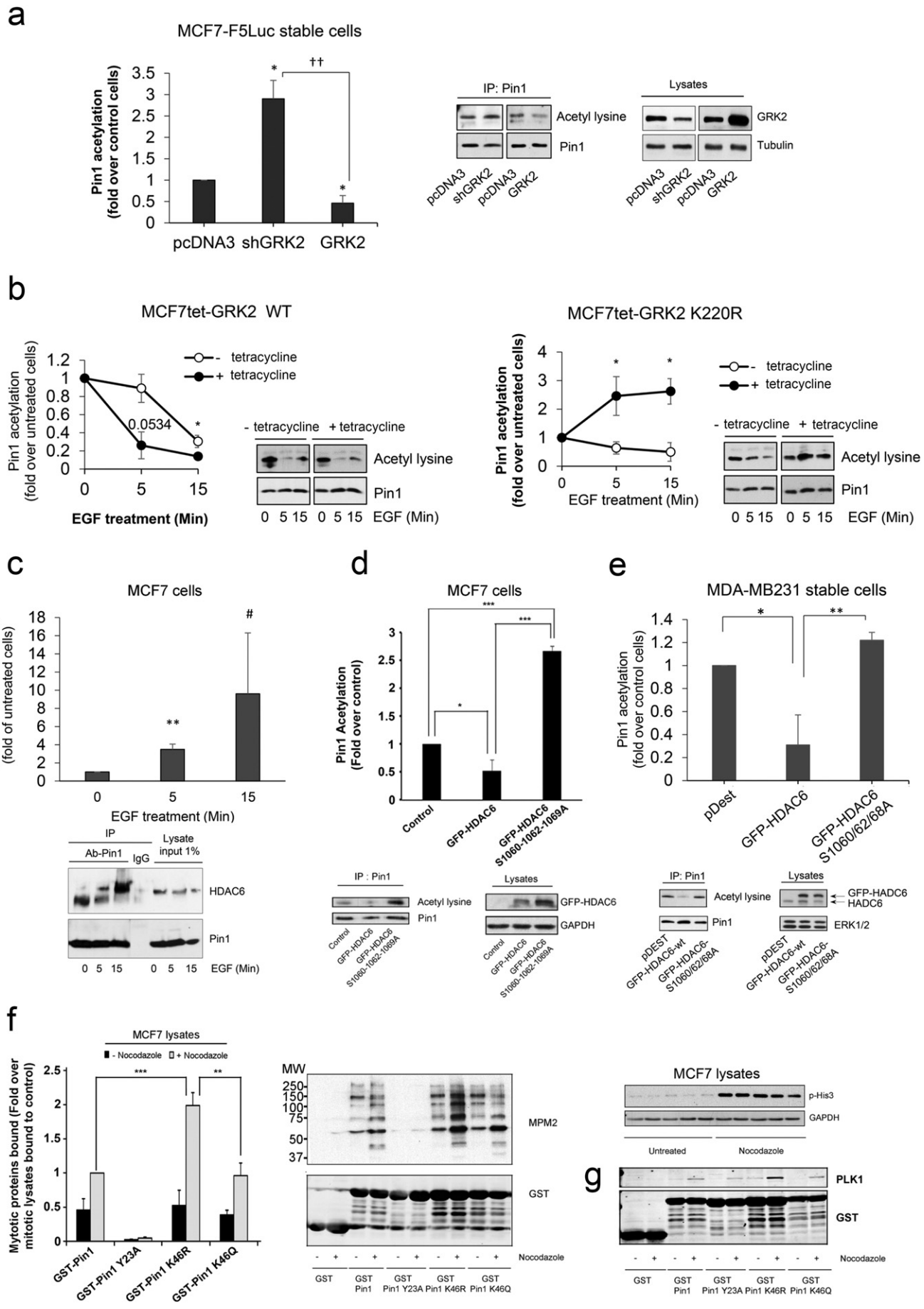
The notion that enhanced EGF signaling could be explained by an increased GRK2-dependent HDAC6-triggered deacetylation of α-tubulin would be consistent with the marked deacetylation of non-histone substrates reported in breast cancer (Suzuki et al., 2009). However, the extent of total tubulin deacetylation in our panel of cancer cell lines (Fig. S8a) did not straightforward correlate with the extent of GRK2 expression or phosphorylation status. We thus hypothesized that GRK2 might affect the HDAC6-dependent deacetylation of other key factors in the EGF pathway. As indicated above, GRK2 expression upregulated in different luminal and basal breast cancer cell lines the protein levels of Pin1 (Fig. S4c, d), a key mitotic modulator and distal EGF-signaling component which positively regulates other downstream factors such as Cyclin D1 (Lu, 2003). Interestingly, Pin1 was previously identified as a target of acetylation in a large-scale proteomics screen (Choudhary et al., 2009). Therefore, we addressed whether Pin1 acetylation could be modulated by HDAC6 in a GRK2-mediated manner. The presence of HDAC6 inhibitors caused hyperacetylation of endogenous Pin1 (Fig. S8b) to an extent similar to that of tubulin in MCF7 cells (Fig. S8c), putting forward Pin1 as a target of HDAC6 activity. Stable expression of extra GRK2 in MCF7 cells markedly reduced the steady-state levels of Pin1 acetylation, whereas kinase downmodulation promoted the opposite effect (Fig. 3a). Moreover, acetyl-Pin1 decreased progressively in MCF7 cells correlating with tetracycline-mediated induction of active GRK2, whereas a kinase-dead mutant promoted increasing accumulation of acetylated Pin (Fig. S8d). Further confirming that catalytic activity of GRK2 regulates de-acetylation of Pin1, inhibition of GRK2 with the small-molecule Cpmd101 promoted hyper-acetylation of Pin1 and abrogated Pin1 de-acetylation triggered by GRK2 overexpression (Fig. S8e). Moreover, EGF stimulation caused a rapid decrease of acetyl-Pin1 levels in MCF7 cells in a GRK2-mediated manner, since extra GRK2 potentiated deacetylation while an inactive kinase showed the opposite effect (Fig. 3b). Interestingly, EGF promoted the endogenous association of Pin1 and HDAC6 in MCF7 cells in parallel to increased Pin1 de-acetylation (Fig. 3c). Substantiating that GRK2 regulates Pin1 deacetylation through HDAC6 phosphorylation, acetylation of endogenous Pin1 was efficiently counteracted by the expression of HDCA6-wt in both MCF7 (Fig. 3d) and MDA-MB-231 cells (Fig. 3e), while extra levels of a HDAC6 mutant unable to be phosphorylated by GRK2 had the opposite effect, leading to a marked increase in the extent of Pin1 acetylation in both cell lines. Overall, these data indicated that Pin1 was specifically and dynamically deacetylated in breast tumor cells through a GRK2-dependent activation of HDAC6.

To evaluate the impact of the acetylation status on Pin1 functionality, we engineered mutations at lysine 46, a residue within the flexible linker domain of Pin1 previously identified as the acetyl-acceptor site by mass-spec analysis (Choudhary et al., 2009), that either mimicked the change in charge linked to acetylation (K-to-Q mutants) or preserved the charge by blocking acetylation (K-to-R mutants). Expression of such constructs confirmed that lysine 46 was the main site for Pin1 acetylation (Fig. S9a). Prevention of acetylation increased the protein stability of a tagged Pin1 construct (Fig. S9b, c), consistent with the observed effects of GRK2 dosage on Pin1 protein levels (Fig. S3a, c and S4c, d) and with the GRK2-induced deacetylation of Pin1 (Fig. 3a, b and Fig. S8d, e). Furthermore, catalytic activity assays indicated that the non-acetylated GST-Pin1-K46R mutant displayed a 7-fold lower Km and increased Kcat to Km ratio compared to GST-Pin1-K46Q or GST-Pin1-wt and suggested that deacetylation promotes a higher substrate affinity and isomerase activity (Fig. S9d, e). Moreover, using a GST-Pin1 pulldown assay in nocodazole-treated MCF7 cells (Fig. 3f), we found that, compared to GST-Pin1-wt, the acetylation-impaired mutant GST-Pin1-K46R displayed a two-fold increased ability to bind mitotic protein monoclonal 2 (MPM-2) antigens, known Pin1 substrates present in mitotic cells, particularly within the high molecular weight range. On the other hand, in contrast to GST-Pin1-Y23A, a WW-domain mutant unable to interact with substrates, the acetylation-mimicking K46Q mutation did not abolish protein binding, suggesting that the status of this residue acts as a regulatory factor in Pin1 substrate binding. A similar trend was noted for the mitotic target Polo-like kinase 1 (PLK1) (Fig. 3g), an early trigger of G2/M transition and a key regulator of mitotic events. Together, these results showed that Pin1 undergoes dynamic and HDAC6 regulated acetylation-deacetylation and that GRK2-HDAC6-induced Pin1 deacetylation would enhance protein stability and functionality.

### 3.4. GRK2 enhances proliferation and transforming growth properties of breast cancer cell lines by modulating HDAC6 and Pin1 functionality

We next investigated whether increased GRK2 dosage could endow breast cells with proliferative advantages in tumor-related contexts. Non-transformed 184B5 cells overexpressing GRK2 displayed a markedly increased proliferation rate in low-serum conditions compared to parental cells in a way dependent on GRK2 kinase activity (Fig. S10a, b). Similarly, increasing GRK2 dosage fostered the already high real-time growth rate of both MCF7 and MDA-MB-231 transformed cells in normal serum conditions, whereas the catalytically inactive GRK2 mutant had no effect (Fig. 4a, b). Conversely, GRK2 down-regulation significantly inhibited proliferation of either these cells or MDA-MB-468 tumoral cells (Fig. 4b and Fig. S10c, d). Such inhibitory effects could not be rescued by the expression of the inactive GRK2-K220R mutant (Fig. S10e, f). Interestingly, siRNA-mediated knockdown of HDAC6 expression suppressed the GRK2-dependent promotion of cell proliferation in MCF7 cells (Fig. 4c). Further confirming that GRK2-dependent phosphorylation of HDAC6 is relevant in breast cancer cell proliferation, expression of wild-type HDAC6 but not the HDAC6-S1060-1062-1068A mutant strongly potentiated MDA-MB-231 cell growth (Fig. 4d). Consistent with a role of the GRK2-HDAC6 axis in controlling cell proliferation via regulation of Pin1 acetylation and functionality, transient expression of either Pin1 wild-type or Pin1-K64R (permanent deacetylation-mimicking, higher functionality mutant) in MCF7 cells previously devoid of endogenous Pin1 using a mixture of siRNAs, significantly fostered

**Fig. 3.** GRK2 fosters Pin1 functionality by regulating its acetylation status in a HDAC6-dependent manner. (a) Effect of GRK2 dosage on Pin1 acetylation status. Levels of acetyl-Pin1 in MCF7-F5luc cells stably over-expressing GRK2 or a silencing shRNA-GRK2 construct were determined as detailed in Materials and Methods. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to empty vector, TT $p < 0.01$ for the indicated comparison ($n = 3–4$). (b) GRK2 promotes Pin1 de-acetylation in a catalytic-dependent manner. Pin1 acetylation levels were assessed in MCF7tet-GRK2 and MCF7tet-GRK2-K220R cells treated or not with tetracycline for 24 h and stimulated with EGF (100 ng/ml) for the indicated times. $^{*}p < 0.05$, when comparing EGF-stimulated tetracycline-induced vs non-induced conditions. (c) EGF-induced co-immunoprecipitation of endogenous HDAC6 and Pin1 in tetracycline-induced MCF7tet-GRK2wt cells. $^{**}p < 0.01$ (or $^{\#}p < 0.05$) when compared to unstimulated cells in a two-tail (or one-tail) *t*-test analysis ($n = 3$). (d, e). Effect of GRK2 on Pin1 de-acetylation by HDAC6. Pin1 acetylation decays upon transient (d) or stable (e) over-expression of HDAC6-wt, but not of an HDAC6 mutant defective in GRK2 phosphorylation, in MCF7 (d) or MDA-MB-231 (e) cells. (f, g) Prevention of K46-acetylation enhances the binding of Pin1 to mitotic phospho-proteins. Lysates of MCF7 cells in exponential growth or in mitotic arrest by Nocodazol were pulled-down with GST or GST-Pin1 constructs and analyzed by immunoblotting with anti-MPM2 (f) or anti-PLK1 (g) antibodies. Equal loading of GST fusion proteins and mitotic arrest were confirmed by western blot with anti-GST and anti-phospho Histone3 antibodies. In these panels data are mean ± SEM, $n = 2–5$. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.Representative blots are shown.


a
MCF7-F5Luc stable cells
Pin1 acetylation (fold over control cells)
pcDNA3 shGRK2 GRK2
IP: Pin1
Acetyl lysine
Pin1
Lysates
GRK2
Tubulin
b
MCF7tet-GRK2 WT
MCF7tet-GRK2 K220R
Pin1 acetylation (fold over untreated cells)
- tetracycline
+ tetracycline
0.0534
EGF treatment (Min)
Acetyl lysine
Pin1
c
MCF7 cells
HDAC6/Pin1 association (fold of untreated cells)
IP
Ab-Pin1
IgG
Lysate input 1%
HDAC6
d
MCF7 cells
Pin1 Acetylation (Fold over control)
Control
GFP-HDAC6
GFP-HDAC6 S1060-1062-1069A
IP : Pin1
Lysates
GFP-HDAC6
GAPDH
e
MDA-MB231 stable cells
pDest
GFP-HDAC6
GFP-HDAC6 S1060/62/68A
GFP-HADC6
HADC6
ERK1/2
f
MCF7 lysates
- Nocodazole + Nocodazole
Mytotic proteins bound (Fold over mitotic lysates bound to control)
GST-Pin1
GST-Pin1 Y23A
GST-Pin1 K46R
GST-Pin1 K46Q
MW
MPM2
GST
Nocodazole
g
MCF7 lysates
p-His3
GAPDH
Untreated
Nocodazole
PLK1
GST

cellular growth (Fig. 4e), whereas the Pin-K46Q mutant (acetylation-mimicking construct) was incompetent to support cellular proliferation.

Interestingly, analysis of changes in the activity of transcription factors in response to chronic EGF stimulation in parental and GRK2-overexpressing 184B5 cells suggested a relationship with enhanced Pin1 functionality mediated by extra GRK2 (Fig. S11a–c), whereas p53 was clearly attenuated in GRK2-overexpressing 184B5 cells in agreement with our previous findings (Penela et al., 2010b). Both p53 attenuation and increased Pin1-Ras functionality favored by enhanced GRK2 could bolster up cell transformation (Liou et al., 2011; Thomassen et al., 2008). Consistent with this notion and with the role of these factors in anchorage-independent cell growth (Hanahan and Weinberg, 2011; Wulf et al., 2004), induction of wtGRK2 expression strongly increased (>10-fold after 3-weeks in culture) the ability of MCF7 cells to form colonies in non-adherent conditions, whereas the GRK2-K220R mutant did not mimic such effect (Fig. 5a–d). Proportion and size of larger colonies (Fig. 5c, d) also increased at both 1 week and 3 weeks of culture in wild-type vs GRK2-K220R-expressing cells. Likewise, adenovirus-mediated expression of wild-type GRK2 in MDA-MB-231 cells potentiated net colony formation in soft-agar as well as the proportion of larger colonies, while downregulation of GRK2 weakened their transforming phenotype (Fig. 5e). Notably, Pin1-knockdown-MCF7 cells transiently expressing wild-type Pin1 or Pin1-K46R formed a higher number of total colonies and of larger-size in soft-agar than Pin1-K46Q-expressing cells (Fig. 5f and Fig. S11d). Furthermore, extra wild-type HDCA6 strengthened the transforming phenotype of MDA-MB-231 cells in a GRK2-mediated phosphorylation-dependent manner (Fig. 5g). Overall, these results indicated that GRK2 kinase activity is a strong facilitator of the processes triggered to promote proliferation in non-anchorage conditions and to bypass anoikis irrespective of the p53 status and related to HDAC6 and Pin1 functionality.

### 3.5. GRK2 favors survival of breast cancer cells in a kinase activity-dependent manner

Intrinsic and acquired resistance to cell death is another important feature of cancer progression. The weak apoptotic response of MCF7 cells toward commonly used cytotoxic compounds, as paclitaxel or etoposide, was potentiated upon GRK2 down-modulation (Fig. S12a–d). On the other hand, p53 levels and the pro-apoptotic phosphorylation of p53 at Ser15 (Maclaine and Hupp, 2011), were reduced in response to cytotoxic compounds in cells stably over-expressing GRK2 as compared to parental cells (Fig. S12e, f).

HDAC6 inhibitors can be used as anti-cancer agents, and pan or selective HDAC6 inhibitors such as suberoylanilide hydroxamic acid (SAHA) or tubacin, respectively, are able to induce growth inhibition and apoptosis in different cancer cell types (West and Johnstone, 2014). Consistent with the notion that GRK2 would potentiate HDAC6 activity and cellular survival, the anti-proliferative and death-promoting effects of these inhibitors were attenuated upon overexpression of functional wt-GRK2 in either 184B5 or MDA-MB-231 cells (Fig. S13a, b).

### 3.6. GRK2 is upregulated in breast tumor patients and correlates with Pin1 functionality and with prognostic markers

We determined GRK2 expression by western blot analysis in a randomly-collected group of 27 patients with infiltrating breast ductal carcinoma. An important proportion (41%) of tumor samples (Fig. 6a and Fig. S14a–c) displayed increased GRK2 protein levels compared to normal specimens, consistent with recurrent amplification or mutation of the GRK2-upregulating signaling pathways discussed above in the major luminal A-B and HER2 clinical subtypes of breast primary tumors (Eroles et al., 2012). Interestingly, AKT activity was up-regulated in a similar proportion (48%) (Fig. 6b), and a positive correlation was noted in the GRK2-positive tumors with AKT stimulation status (Fig. S14d, e). This trend was confirmed by immunohistochemistry analysis in 100% of AKT-positive samples of an independent cohort of 49 metastatic infiltrating ductal carcinomas, in which up-regulated GRK2 was also detected in 80% of primary tumors from patients that underwent metastasis (Fig. 6c, d). Interestingly, in the former cohort GRK2 expression positively correlated with increased tumor size (Fig. 6e) and with increased signals of the Ki67 proliferation marker (Fig. 6f), but inversely with p53-positive samples (Fig. 6h), in agreement with a p53-downmodulating effect of GRK2. Consistent with a relevant role of GRK2 in the regulation of Pin1 functionality in breast tumors, enhanced GRK2 protein levels positively associates with Pin1 expression (Fig. 6h and S14F), while an inverse non-linear correlation between GRK2 expression levels and the extent of acetylated-Pin1 was noted in breast cancer patients, but not in non-tumoral samples (Fig. 6i). Further substantiating the relevance of the GRK2-HDAC6 axis in Pin1 expression and functionality in human breast tumors, the occurrence of increased Pin1 (Pin1+) is higher in GRK2-positive samples, either HDAC6-positive or -negative, comparing to GRK2 n.c or negative samples (Fig. S15) in which Pin1-positive and –negative samples are evenly represented irrespective of HDAC6 levels. These results may suggest that GRK2 overexpression could efficiently mediate deacetylation and protein stabilization of Pin1 even at low HDAC6 expression levels.

### 3.7. GRK2 is a relevant modulator of tumor growth in vivo

To test the role of GRK2 in driving or maintaining tumor development in vivo, we investigated whether xenograft tumor growth in mice was influenced by GRK2 levels in human breast cancer cells. Using a doxycycline-dependent inducible expression strategy, we observed that tumors formed by MCF7 cells over-expressing wtGRK2 (but not those harboring the inactive K220R mutant) developed earlier and reached significantly higher sizes (Fig. 7a). Staining of sections from MCF7-wtGRK2-induced tumors demonstrated an increase in the proliferation marker Ki67 along with a decrease in p53 and cleaved caspase3 immunostaining, indicative of resistance to apoptosis, whereas no significant changes were observed in MCF7-K220R-derived tumors (Fig. 7b).

These findings were confirmed using an alternative experimental setting using adenoviral vectors. More important, down-regulation of GRK2 expression completely abrogated the growth of tumors (Fig. 7c), which display at early time points a substantial reduction in the proliferation marker Ki67 and a marked increase in p53 levels, suggestive of enhanced apoptosis (Fig. 7d). Simultaneous expression in the silenced MCF7 cells of the GRK2-K220R mutant did not rescue the blockade of tumor induction, indicating that kinase activity was required for the tumor-promoting effect of GRK2 in vivo (Fig. S16a).

Further suggesting a clinical relevance for GRK2 in breast cancer, tumors formed by MCF7-F5Luc cells stably over-expressing wild-type GRK2 developed earlier and reached higher volume than those promoted by parental cells in murine mammary glands, an orthotopic model in which specific fat-pad stromal interactions with transformed epithelial cells are recapitulated (Fig. 7e). Conversely, growing of GRK2-knockdown cells was halted compared to parental cells despite similar initial engraftment (otherwise hampered in the less permissive xenograft model), suggesting that expression of GRK2 is required for maintenance of aberrant growth in the breast tissue.

Interestingly, the ability of GRK2 to modulate tumor growth in vivo was also detected in mutant p53 contexts. As with MCF7, tumors induced by wtGRK2-overexpressing MDA-MB-468 cells (ER-, HER2-, mutated p53 and PTEN) and MDA-MB-231 cells (also triple-negative but non-mutated PTEN) were slightly higher in size (Fig. S16b, c), whereas kinase down-modulation markedly inhibited tumor formation (Fig. S16b), suggesting a more general role for GRK2 in breast cancer development.

## 4. Discussion

We report that increased GRK2 levels and functionality in either luminal or basal cancer cells can drive the acquisition and maintenance of

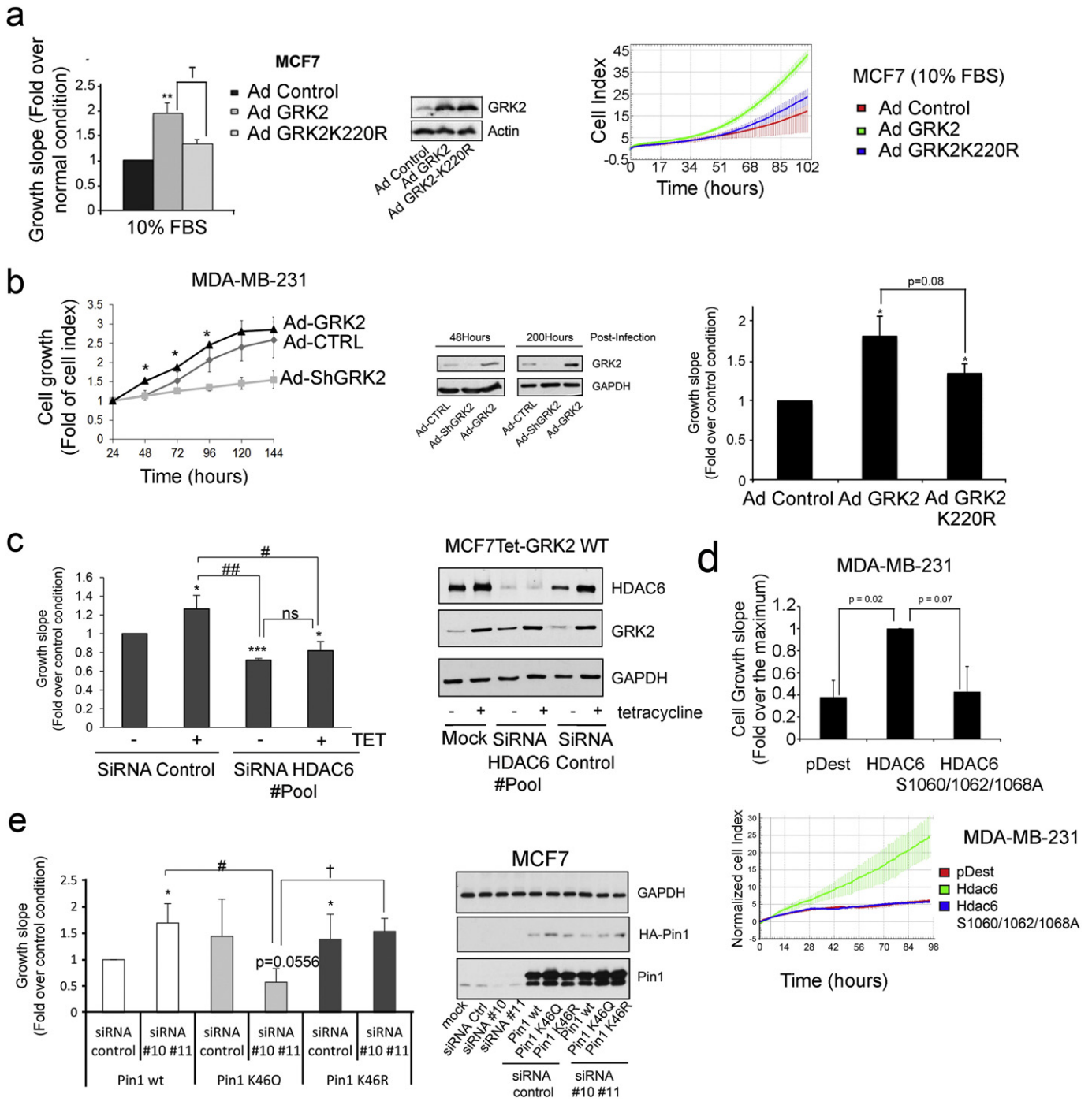



**Fig. 4.** GRK2 endows transformed breast cells with proliferative advantages. (a, b) Growth of parental, shRNA-GRK2, wt or K220R mutant GRK2-infected MCF7 (wt p53) (a), or MDA-MB-231 (mutant p53) (b) cells in normal culture medium was monitored using the xCELLigence technology as described in Materials and Methods. * or T, $p < 0.05$ and **$p < 0.01$ when compared to control infected cells. (c–d) HDAC6 underlies the growth-promoting effects of GRK2. (c) xCELLigence-based analysis of growth of non-induced or tetracycline-induced (24 h) GRK2wt-MCF7-Tet cells upon HDAC6 silencing with a pool of siRNAs as detailed in Materials and Methods. *$p < 0.05$ and ***$p < 0.001$ (two-tail), when compared to siRNA control-transfected and untreated cells, #$p < 0.05$ and ##$p < 0.01$ (one-tail). Proper knockdown of HDAC6 and induction of GRK2 is shown in a representative blot. (d) Stable expression of HDAC6-wt but not of the HDAC6-S1060-1062-1068A mutant fosters growth in MDA-MB-231 cells. (e) Acetylation of Pin1 impairs proliferation of tumor cells. Growth of MCF7 cells co-transfected either with HA-Pin1-wt, HA-Pin1-K64R or HA-Pin1-K46Q and a combination of two specific Pin1 siRNAs or a control siRNA was determined as above. *$p < 0.05$, #$p < 0.05$, T $p < 0.05$ in one-tail *t*-test analysis. Similar expression of Pin1 constructs and knockdown of endogenous Pin1 was confirmed by immunoblot with anti-HA and anti-Pin1 antibodies. A fragment that co-migrates with endogenous Pin1 is detected with a specific Pin1 antibody upon HA-Pin1 transfection. In all panels data are mean ± SEM ($n = 3$).

tumoral proliferation and survival. Elevated GRK2 protein levels are detected in different breast cancer cell lines, in tumors developed in MMTV-HER2 animals, in the mammary gland of Myr-AKT transgenic mice and in a significant proportion of two independent groups of patients diagnosed with invasive ductal carcinoma, in which luminal subtypes predominate (Bertos and Park, 2011). Interestingly, the stimulation of different transduction pathways (estrogen or EGFR receptors, the Ras-HER2 and PI3K-AKT cascades) known to be hyper-activated in luminal and in certain non-luminal types of breast cancer (Eroles et al., 2012) not only correlates with increased GRK2 levels but appears to converge in promoting enhanced GRK2 expression in transformed and non-transformed breast epithelial cells.

Our results are consistent with a relevant role for the AKT pathway in promoting GRK2 up-regulation. Cancer cells where GRK2 is enhanced

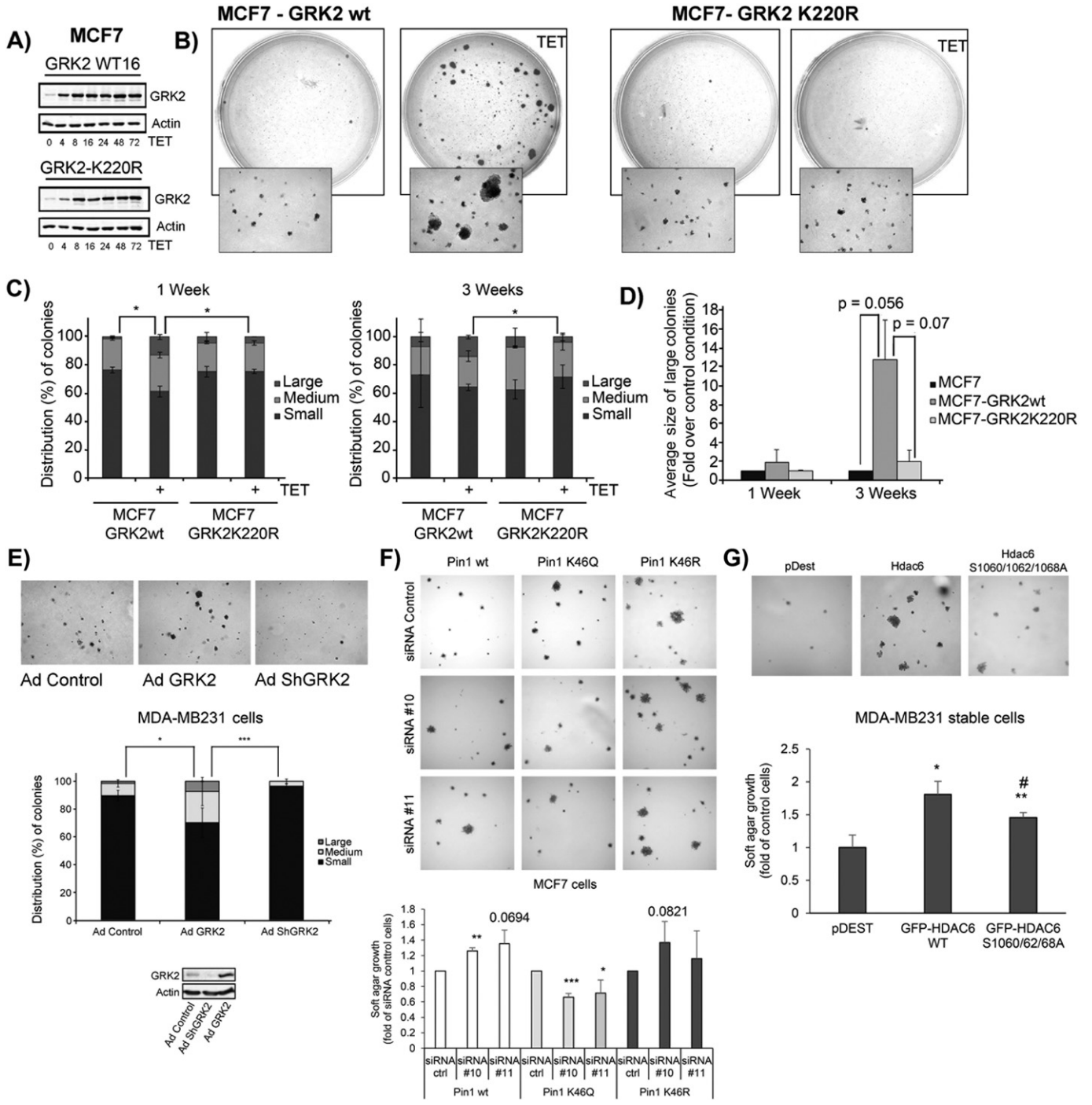



**Fig. 5.** GRK2 favors the anchorage-independent growth of breast tumor cells in a kinase-dependent manner via regulation of HDAC6 and Pin1. (A) Time-course of GRK2 levels in tetracycline (TET)-induced MCF7tet-GRK2wt or -GRK2-K220R cells. Colony formation by either un-induced or tetracycline-induced MCF7tet-GRK2wt and -GRK2-K220R cells (B–D) or by GRK2- or shRNA-GRK2-infected MDA-MB-231 cells (E) in soft-agar medium was analyzed as described in Materials and Methods. The area of colonies was measured using Image J and the size of colonies was scored as Large, Medium and Small as described in Materials and Methods. Bar-graphs show the distribution (%) of the colonies according to their size (C, E) and the median size (D) of colonies. Data are mean ± SD of 2 duplicate independent experiments. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). (F, G) The GRK2-HDAC6-Pin1 axis fosters malignant growth of luminal and basal breast tumor cells. Soft agar colony formation of MCF7 cells (F) transiently co-transfected with either HA-Pin1-wt, HA-Pin1-K64R or HA-Pin1-K46Q and specific Pin1 siRNAs or a control siRNA, as well as MDA-MB-231 cells (G) stably expressing either HDAC6-wt or the GRK2-deficient phosphorylation mutant HDAC6-S1060-1062-1068A in 96-multiwell plates was monitored by Alamar Blue staining as described in Materials and Methods. Data are mean ± SEM of 3 independent experiments performed by triplicate. *$p < 0.05$, **$p < 0.01$, when compared to empty-vector or siRNA-control transfected cells in each condition and #$p < 0.05$, comparing HDAC6-wt with HDAC6 mutant. Representative images of cellular foci are shown.

displays genetic alterations in this pathway (*PI3KCA, PTEN*) or hyper-stimulation of receptors (EGFR, HER2, ER) able to trigger AKT stimulation (Renoir et al., 2013; Roskoski, 2014), which in turn can stabilize GRK2 protein by means of hampering Mdm2-mediated GRK2 proteasome degradation (Salcedo et al., 2006). However, the occurrence of post-transcriptional mechanisms cannot be ruled out, since canonical transcriptional modulators as estrogens promoted GRK2 up-regulation in ER+ MCF7 and T47D cells.

Importantly, GRK2 levels play a driving role in the acquisition of oncogenic features. GRK2 up-regulation in either untransformed or cancer

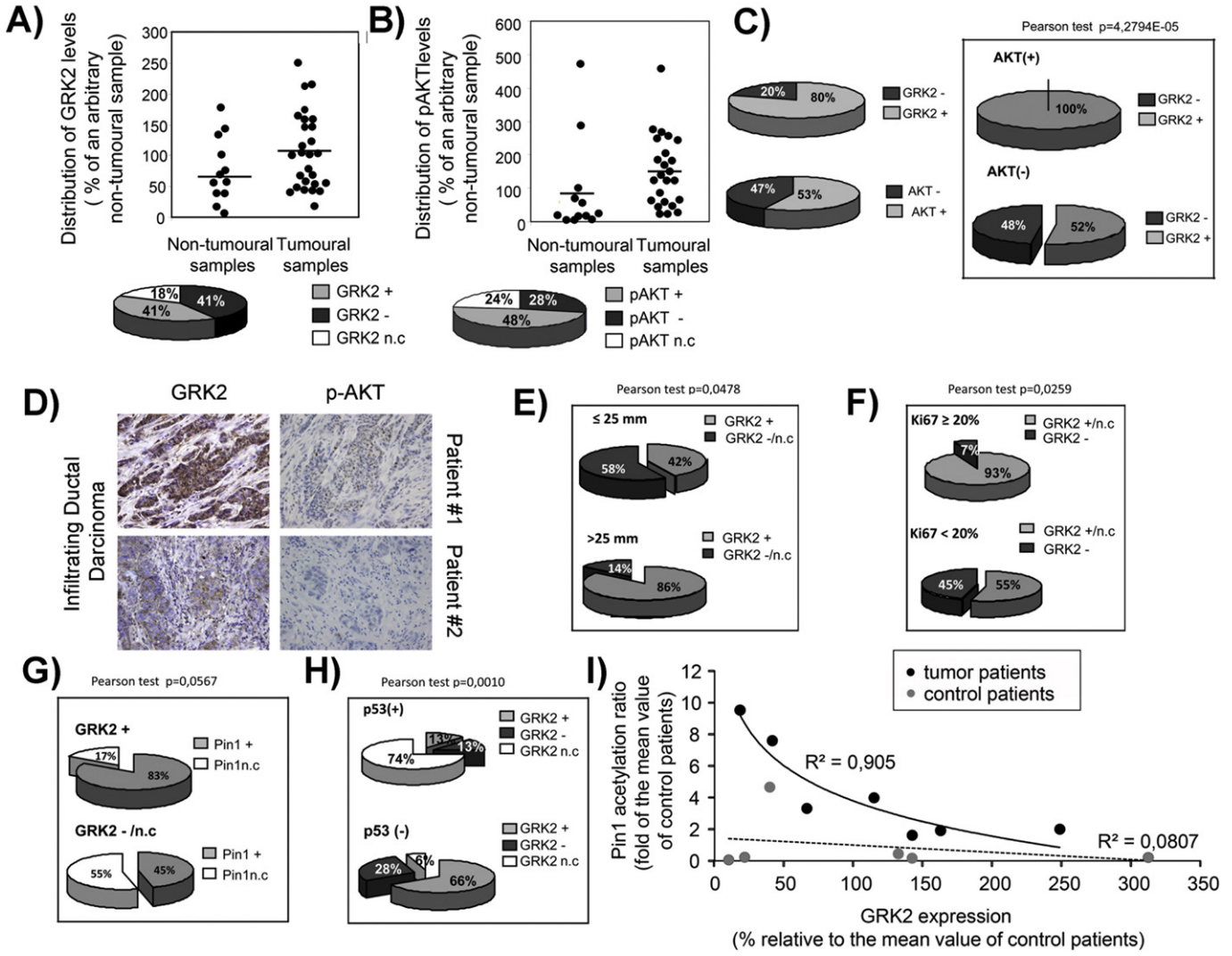



**Fig. 6.** GRK2 protein levels are up-regulated in a significant proportion of breast cancer patients and positively correlate with relevant prognostic markers of tumor aggressiveness. GRK2 levels (A) and p-S473 AKT (B) were analyzed by western blot in 27 samples from breast cancer patients with infiltrating ductal carcinoma and 12 controls. Bars indicate median values. Pie charts show group tumor patients based on the degree of change in GRK2 and p-AKT expression levels compared to an arbitrary control [(+), higher than control; (−), lower than control; nc, within the range of normal variation (see Materials and Methods)]. (C, D) Samples of 49 patients were analyzed by immunohistochemistry to detect GRK2 and p-AKT. Samples were scored as positive (moderate or strong staining, +) or negative (none or weak staining, −) for GRK2 and p-AKT levels (C). Samples were stratified by p-AKT levels and the distribution of GRK2 groups plotted in a pie chart. Representative sections of GRK2 and p-AKT staining of two patients are shown in panel d. The distribution of tumor size (E), Ki67 (F) and p53 (G) in the cohort of 27 patients with IDC was plotted according to GRK2 expression in a pie chart. p53 levels were determined by immunostaining and the remaining markers were obtained from clinical data (+, presence; −, absence). (H, I) GRK2 correlation with Pin1 expression and its de-acetylation status. Levels of Pin1 and GRK2 were determined by western blot (+, higher than control; −, lower than control; n.c, within the range of normal variation). Pie chart shows GRK2 distribution according to Pin1 expression (H). Pearson test was performed for statistical analysis (C–H). The acetylation status of Pin1 (I) was determined by immunoprecipitation of Pin1 and subsequent immunoblotting with acetyl-lysine and Pin1 antibodies. Pin1 acetylation ratio was plotted against GRK2 levels and the curve was fitted into a quadratic regression linear model using the BestCurFit program.

breast cells leads to a reinforcement of mitogenic (ERK1/2) and survival (AKT) pathways and to increased expression of the proliferation mediator Pin1, thereby enhancing growth potential under low-serum or normal conditions and resistance to the induction of cell death by different therapeutic agents. Moreover, GRK2 up-regulation markedly favors anchorage-independent growth of MCF7 or MDA-MB-231 cells and increases their competence to trigger tumor growth in vivo. Conversely, decreasing GRK2 levels have the opposite effect in either MCF7, MDA-MB-231 or MDA-MB-468 cells and sensitize cells to apoptotic stimuli, indicative of a critical role for GRK2 in this process.

The pro-survival and proliferative effects of GRK2 reported herein are clearly dependent on its kinase activity, since expression of GRK2-K220R, a mutant that lacks catalytic activity, is not able to mimic the effects of the wild-type protein in vitro or in vivo. In this context, we put forward the HDAC6-Pin1 axis and p53 as relevant direct and indirect targets, respectively, underlying the effects of GRK2 on breast cancer cells. We have reported that GRK2-mediated HDAC6 phosphorylation driven by phosphorylation of GRK2 itself at S670 in response to EGF specifically enhances HDAC6-mediated de-acetylation activity and migration of epithelial cells (Lafarga et al., 2012a). We find that HDAC6 levels and (or) pS670-GRK2 are increased in the tested breast cancer cells, wherein both proteins could interact with the EGFR (Deribe et al., 2009; Evron et al., 2012; Gao et al., 2010; Penela et al., 2010a) and modulate its signaling. Since HDAC6 potentiates EGFR signaling by decreasing EGFR internalization (Deribe et al., 2009; Gao et al., 2010), we hypothesize that the higher GRK2 and HDAC6 levels in luminal breast cancer cells would favor an enhanced local deacetylase turnover and more sustained signaling in response to activation of EGFR and other ErbB family members (Deribe et al., 2009), or ER alpha estrogen receptors, which interact also with HDAC6 (Azuma et al., 2009) and promote GRK2-S670 phosphorylation (Dominguez et al., 2009).

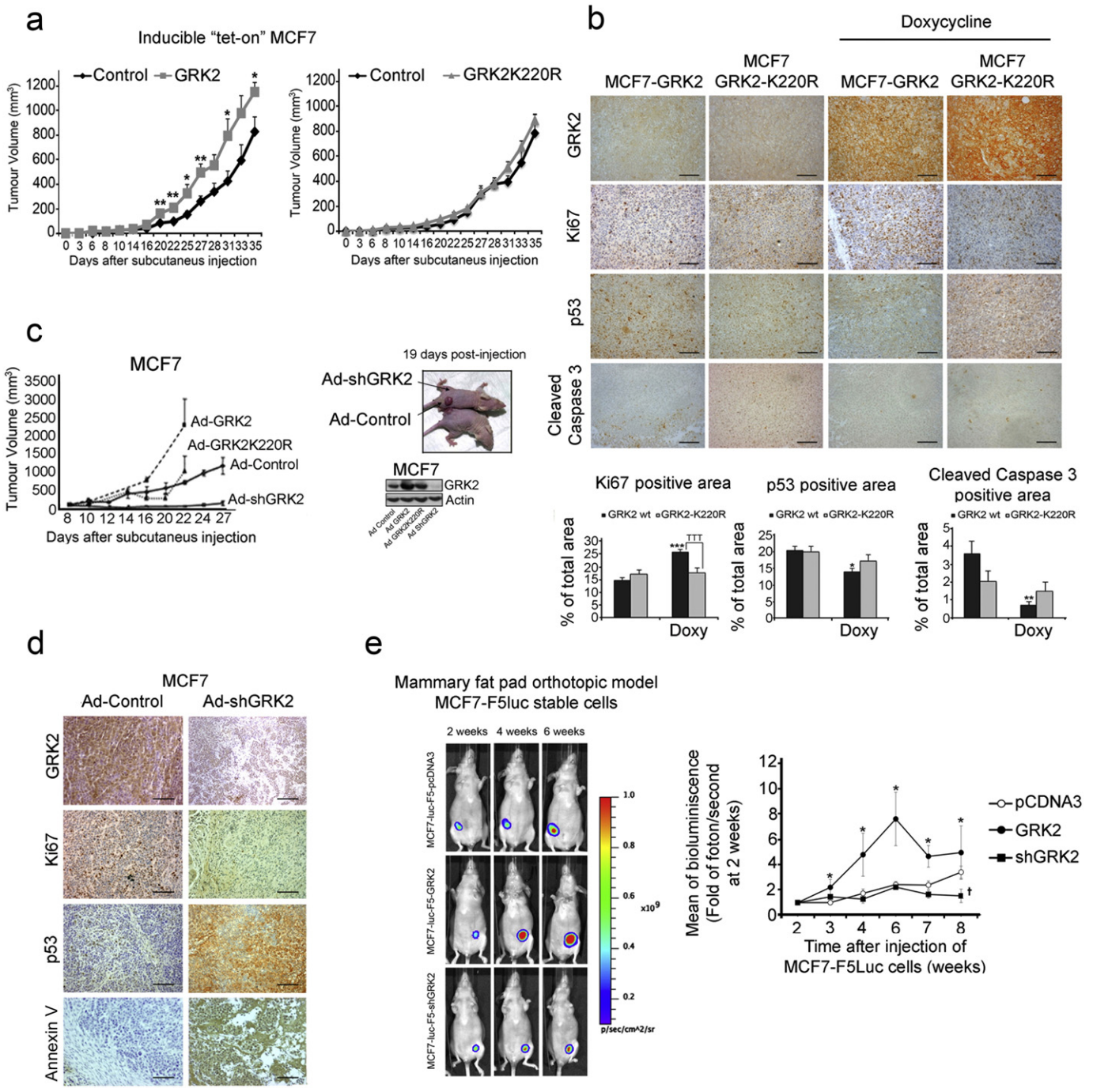



**Fig. 7.** GRK2 modulates in vivo tumor growth in a kinase-dependent manner by regulating proliferative and apoptotic pathways. (a) MCF7tet-GRK2wt or -GRK2-K220R cells pre-treated with tetracycline or vehicle (control) were subcutaneously implanted in doxycycline-treated or un-treated (control) nude mice. Tumor volume was measured and endpoint tumors were immuno-stained for GRK2, Ki67, p53 and cleaved caspase-3 (b) as described in Materials and Methods. (Scale bar: 100 μm). Data are the mean ± SEM from tumor masses of 6–8 mice per group. (c) Tumor growth was monitored (6–10 mice per condition) in nude mice subcutaneously injected with MCF7 cells infected with the indicated adenoviral constructs and verified for proper GRK2 levels by western blot. (d) Immunohistochemical analysis of GRK2, Ki67, p53 and annexin V expression in sections of tumors 8-days post-injection. (Scale bar: 100 μm). (e) Analysis of the orthotopic growth of MCF7-F5luc cells stably expressing GRK2-wt or a silencing shRNA-GRK2 construct injected in the mammary fat pad of nude mice with Xenogen imaging, using luciferin substrate once per week after implantation of cancer cells. Signals from primary breast tumors at 2, 4 and 6 weeks after implantation are shown. Data are mean ± SEM from 3 to 5 mice per condition. In all panels $^{*}p < 0.05$ or T $p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to control condition.

In addition, we have identified a regulatory mechanism by which HDAC6 de-acetylates Pin1 at the residue K46 in a stimulus-dependent manner. EGF triggers a rapid interaction of these proteins and the concurrent Pin1 de-acetylation is fostered by GRK2 expression through HDAC6 modulation in different breast cancer cell types, whereas a correlation between GRK2 expression and Pin1 levels and de-acetylation status is detected in breast cancer patients. Importantly, the extent of K46 acetylation has functional consequences, since a GRK2-mediated de-acetylated status enhances Pin1 stability and also bolsters its functionality, by increasing its affinity toward a canonical peptide substrate, its catalytic efficiency compared to acetylated Pin, and binding to mitotic cellular substrates. There is compelling evidence for Pin1 substrate binding involving the N-terminal WW domain (residues 1–39) and the C-terminal PPIase region (residues 50–163) (Innes et al., 2013), and for complex functional inter-domain interactions that impact both on Pin1 catalytic activity and on binding affinity and selectivity. Some

targets such as PLK1 require simultaneous interactions with both the WW and the PPIase domains, while the interaction of others relies exclusively on the WW domain (Innes et al., 2013). The weaker binding in the former situation is subsequently counterbalanced by the contribution of the PPIase domain, which ligand affinity is enhanced upon WW domain occupancy. Both Pin1 functional domains are tethered through a structurally disordered glycine-rich linker (residues 45–53) located at the N-terminal portion of the PPIase region. Since the PPIase domain is sensitive to inter-domain contacts that trigger allosteric communication, the inter-domain linker might play an active role in such process, by helping to position both domains in close contact and by increasing the local concentration of substrate available to the PPIase active site (Wilson et al., 2013). It is tempting to suggest that dynamic acetylation-deacetylation-mediated variations in charge at the residue K46 (conserved from humans to yeast) might alter the inter-domain linker folding (perhaps via electrostatic interactions with the proximal E52 residue), thus modulating inter-domain allosteric contacts and thus Pin1 ligand affinity and catalytic activity.

Potentiation of the HDAC6-Pin1 signaling module by GRK2 in specific breast cancer cells could be envisaged as an adaptation to enhanced activity of different tumor-promoting cascades that would trigger a self-perpetuating positive feedback cycle promoting cell proliferation and increased survival. Notably, HDAC6 is required for oncogenic Ras- and ErbB2-dependent fibroblast transformation, for sustained ERK1/2 and AKT signaling, for anchorage-independent growth of MCF7 cells and cancer cell survival (Lee et al., 2008; Li et al., 2013). Consistent with a regulatory role of GRK2 on HDAC6 tumoral competences, we find that extra GRK2 levels lessen the cytotoxic effectiveness of HDAC6 inhibitors (West and Johnstone, 2014). Therefore, it is tempting to suggest that GRK2-mediated phosphorylation and activation of HDAC6 makes this molecule less sensitive to its inhibitors, thereby debilitating their therapeutic effects. Such notion would be consistent with ineffective or partial responses shown by pan-HDAC inhibitors in GRK2-overexpressing (MCF7, MDA-MB361) (Tate et al., 2012) and with different efficacies among breast tumor cells with p53 mutant status (Li et al., 2011) and different pS670-GRK2 ratios. Therefore, treatment of certain types of breast cancer may benefit from the combined inhibition of GRK2 and HDAC6.

It is worth noting that the HDAC6's substrate Pin1, which levels are over-expressed in human breast cancers (Ryo et al., 2003) and transcriptionally enhanced by oncogenic HER2 or Ras signaling, is also critical for the HER2-Ras-triggered transformation of mammary epithelial cells (Ryo et al., 2002), for EGF signaling and HER-2 expression in breast cancer cells (Khanal et al., 2010), for regulation of the activity of estrogen receptors (Lucchetti et al., 2013), and for coordination of cell cycle progression (Liou et al., 2011). By using silencing approaches and (or) HDAC6 or Pin1 mutants unable to undergo GRK2-mediated phosphorylation or de-acetylation, our data support a relevant role of the GRK2-HDAC6-Pin1 signaling axis in reinforcing growth factor signaling, proliferation and anchorage-independent growth. Since the higher growth rates of breast tumor cells rely on Pin1 binding to cell-cycle associated proteins (Khanal et al., 2010; Liou et al., 2011; Lucchetti et al., 2013; Ryo et al., 2002), our data suggest that GRK2 would foster proliferation by triggering HDAC6-mediated Pin1 deacetylation, leading to enhanced interaction with mitotic regulators, particularly which those displaying an allosteric binding mode such as PLK-1 or Cdc25 phosphatase (Innes et al., 2013). Therefore, concurrent Pin1 and GRK2 up-regulation also emerges as a relevant component of the tumorigenesis pathway.

On the other hand, the simultaneous GRK2-dependent down-modulation of wild-type p53 protein and extra activation of the pro-survival AKT route might also help to counteract the effects of relevant cytotoxic compounds. We observe that down-regulating GRK2 levels increases sensitivity to apoptotic agents in cultured cells and triggers a strong p53 response in xenograft-derived tumors in vivo. Conversely, increasing GRK2 expression in non-transformed breast cells reduces genotoxic-dependent cell death and the p53 response, as previously noted in genotoxic cell cycle-arrested cells (Penela et al., 2010b). These results are consistent with the occurrence of an inverse GRK2-p53 correlation in the xenograft tumor model and in tumors of patients with breast cancer scored as p53-negative (either by gene-deletion or by protein down-modulation), suggesting that GRK2 might be an important modulator of the intact p53 pathway either through direct modulation of HDAC6 (Tang et al., 2008) or of other upstream regulators, with potential implications in therapy. An important proportion of breast cancer ERα-+ patients treated with estradiol antagonists or aromatase inhibitors become resistant to these compounds, what urges to identify new therapeutic targets (Renoir et al., 2013). We found that tamoxifen-resistant MCF7 cells can build stronger apoptotic responses in the absence of GRK2, suggesting that pharmacological inhibition of this kinase might be effective to inactivate tamoxifen-resistance pathways and to sensitize cells to general chemotherapeutic agents.

Interestingly, GRK2 down-modulation similarly inhibited tumor growth of breast tumor cell lines-derived xenografts irrespective of their p53 status, suggesting that the ability of GRK2 to modulate tumor development in vivo is independent of the p53 status or, alternatively, that it has a double-edge sword role, attenuating p53's tumor-suppressor functions in cells retaining the wild-type factor, but otherwise promoting its oncogenic roles in contexts of mutant p53 expression. In this regard, mutant p53 stability and oncogenic gain-of-function of p53 mutants relies on the presence of Pin1 (Girardini et al., 2011) and HDAC6 (Li et al., 2011), which levels and activity are both positively modulated by GRK2.

In sum, our data identify the GRK2 signaling node as a relevant player in the development of breast cancer. GRK2 up-regulation emerges as a convergent feature of the stimulation of diverse pathways altered in luminal breast cancer, in parallel to that of other key proteins such as HDAC6 or Pin1. In such conditions as well as in cellular settings of increased phosphorylation of GRK2 on S670 by means of hyper-activation of ERK1/2 or other kinases (often found in both luminal and basal tumoral contexts), the switch-on of the GRK2-HDAC6-Pin1 signaling module would help to trigger a self-perpetuating positive feedback loop of growth factor transduction cascades driving cell survival and proliferation. GRK2 could also inhibit wild-type p53 in a AKT and Mdm2-dependent manner in luminal cells, while in tumor cells with mutant p53 GRK2 could act as an amplifier of oncogenic p53 functions by means of the increased functional competence of Ras-Pin1 and HDAC6.

However, the pro-tumoral role of GRK2 might be more complex due to the concurrent and opposite changes of GRK2 in the epithelial (up-regulation) and stromal (down-modulation) components of breast tumors. The down-modulation of GRK2 specifically in the surrounding tumor endothelium results in vasculature dysfunction and hypoxia, thereby impelling a more aggressive tumor progression (Rivas et al., 2013). Therefore, a better knowledge of the mechanisms underlying such cell type–specific modulation and roles of GRK2 may help to understand its integrated role in cancer development and to design future therapeutic strategies.

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## Conflict of Interests

XZZ and KPL are inventors of PIN1 technology, which was licensed by Beth Israel Deaconess Medical Center (BIDMC) to Pinteon Therapeutics. Both authors own equity in, and consult for, Pinteon. Their interests were reviewed and are managed by BIDMC in accordance with its conflict of interest policy. The remaining authors declare no conflicts of interest.

## Author Contributions

LN, CR, VR, AS, VL, PR, and MN designed and carried out experiments; KS, XZZ + and KPL provided tools and key advice; MM, AB and DH provided and analyzed clinical samples; FM and PP designed and supervised the research and wrote the manuscript.

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## Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2016.09.030.

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