Supporting Information

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SI Materials and Methods

Cell-Cycle Analysis, Cell Synchronization, and Proliferation. HeLa cells and MEFs were synchronized at G1/S transition using a thymidine-aphidicolin double block. Subconfluent cells were cultured in the presence of 2-mM thymidine for 12 h and then in culture medium for 12 h. They were then incubated in the presence of 5-μg/mL aphidicolin for 12 h before release into fresh medium. Primary human endothelial cells from umbilical vein (HUVEC) were arrested in G0 by plating subconfluent cells into culture medium supplemented with 0.1% FBS for 48 to 72 h and released in 10% serum for different periods of time. The DNA content of cells growing asynchronously or upon release from a cell-cycle arrest was determined by flow cytometry analysis. Cells were rinsed in cell cycle-staining buffer (0.1% sodium citrate, 0.3% Nonidet P-40, 50-μg/mL propidium iodide and 20-ng/mL RNase in PBS) and incubated for 30 min at room temperature. The fluorescent cells were detected with FACS Calibur (Becton-Dickinson) and data analyzed using the CellQuest Software. DNA synthesis rates were determined by [^{3}H]thymidine uptake analysis. Cells were seeded at 70% confluence in 24-well culture plates, serum-starved for 48 h, and stimulated with 10% serum for different times. Cells were then pulse-labeled with 1 μCi/mL [^{3}H]thymidine for the last 4 h of stimulation, washed twice with PBS, and incubated in 10% trichloroacetic acid at 4 °C for 10 min. DNA was solubilized in 0.2 M NaOH, 0.1% SDS for 2 h at 37 °C. Radioactivity incorporated into DNA was determined in a scintillation β-counter.

Cell Culture and Cellular Transfections. HEK-293, MCF7, MDA-MB-468, HeLa cells (American Type Culture Collection) and immortalized mouse embryonic fibroblasts (MEFs), either wild-type, double knock-out for both β-arrestin 1 and 2 (βarr1/2-KO) (1), Pin1-deficient, or cyclin-dependent kinase 2 (CDK2)-deficient were maintained in DMEM (DMEM) supplemented with 10% (vol/vol) FBS (Sigma-Aldrich), at 37 °C in a humidified 5% CO_2 atmosphere. Next, 184B5 and MCF-10Acells were cultured in DMEM/F12-HAMS medium containing 5% horse serum, 10-μg/mL insulin, 20-ng/mL EGF, 100-ng/mL cholera toxin, and 0.5-μg/mL hydrocortisone. HUVECs were isolated and cultured up to the third passage. Cells were seeded on tissue culture dishes coated with 0.5% gelatin and grown in 199 medium from Bio Whittaker supplemented with 10% FBS, 50 IU/mL penicillin, 50-μg/mL streptomycin, and 2.5-μg/mL fungizone. After the first passage, cells were supplemented with 50-μg/mL growth supplement prepared from bovine brain and 100-μg/mL heparin. HeLa or HEK-293 cells (70–80% confluent monolayers in 60- or 100-mm dishes) were transiently transfected with the chosen combinations of cDNA constructs using the Lipofectamine/Plus method (Life Technologies Inc.), following manufacturer's instructions. Stably-transfected HeLa cells overexpressing either GRK2-wt, $GRK2_{436–689}$,, empty vector or the mutant constructs GRK2-S670A or GRK2-S670D were generated as described previously (2). Cells were characterized and collected as distinct pooled positive transfectant cells with different expression levels of the GRK2 constructs.

GRK2 Knock-Down. GRK2 knock-down was achieved by RNA interference using and adenovirus vector bearing the target GRK2 shRNA sequence (5′- GCAAGAAAGCCAAGAACAAGC-3′) using an unrelated vector as control as previously described (2). Recombinant adenoviruses expressing the GRK2 shRNA were produced using BLOCK-iT Adenoviral RNAi Expression System (Invitrogen) according to the manufacturer's instruction.

cDNA Constructs. The cDNAs encoding GRK2-wt, GRK2-K220R and the phosphorylation mutants GRK2-S670D and GRK2-S670A have been previously described (3–5). The constructs GST-$GRK2_{1–185}$, GST-$GRK2_{185–467}$ and GST-$GRK2_{436–689}$ were previously described (6). The $GRK2_{1–147}$ construct was kindly provided by J.L. Benovic (Thomas Jefferson Cancer Institute, Philadelphia, Pennsylvania). The $GRK2_{436–689}$ insert was subcloned into the pREP4 vector. Expression vectors for Pin1-wt, Pin1-Y23A, Pin1-C109A and the GST-Pin1-wt construct were generously provided by G. del Sal (Laboratorio Nazionale C.I.B., Trieste, Italy). Plasmids for the expression of the GST fusion proteins GST-ESS1 and GST-7PL were from M. Sudol (Mount Sinai School of Medicine, New York). The CDK2 plasmid was from C. Murga (Centro de Biología Molecular Severo Ochoa, Madrid, Spain).

Reagents and Antibodies. CDK2/cyclin A and CDK1/cyclin B recombinant complexes were from Upstate Biotechnology. Purified activated MAPK was obtained from Stratagene Laboratories. Affinity-purified rabbit polyclonal antibodies raised against GRK2 (C-15), cyclin A (H-432), the affinity-purified goat polyclonal antibody raised against actin (I-19), lamin B (M-20), the affinity-purified anti-GST (B-14), anti-HA (F-7), anti-cyclin D (HD11), and anti-p53 (DO-1) mouse monoclonal antibodies, the anti-phosphotyrosine monoclonal antibody conjugated to horseradish peroxidase (PY99-HRP), the polyclonal C-16 and C-14 antibodies that recognize ERK1 and ERK2 were purchased from Santa Cruz Biotechnology Inc.. The anti-PUMA rabbit polyclonal and the anti-Noxa mouse monoclonal antibodies were from Abcam. The anti-GRK2/3 mouse monoclonal and the anti-Pin1 rabbit polyclonal antibodies were purchased from Upstate Biotechnology. The polyclonal antibodies Ab-FP1 and Ab-FP2 were raised against GST fusion proteins, including the N-terminal RH domain and the C-terminal region of GRK2, respectively (1). Different antibodies were used for analyzing GRK2 levels during cell cycle progression. Because some of these antibodies could also detect GRK3, the observed down-regulation during the cell cycle could in principle be ascribed to both GRK isoforms, although we do not observe the slightly faster migrating band that would correspond to GRK3. In addition, GRK3 does not display the consensus phosphorylation site at the S670 position, so this kinase could not undergo the phosphorylation-dependent regulation that we observe for GRK2. Anti-$pSer_{670}$-GRK2 polyclonal antibody was from Biosource International. The anti-pTyr15-CDK1, anti-CDK1, anti-$pSer_{15}$-p53, and the anti-phospho-ERK1/2 polyclonal antibody were purchased from Cell Signaling Technologies. The anti-poly(ADP-ribose) polymerase p85-fragment polyclonal antibody was from Promega. The anti-cyclin B1 monoclonal antibody was from BD Biosciences Pharmingen. Thymidine, aphidicolin, nocodazol, heparin, GW8510, juglone, and etoposide were obtained from Sigma. Cycloheximide, was supplied by Calbiochem. MG132 was from BIOMOL Research Laboratories. Glutathione-Sepharose 4B beads were obtained from Pharmacia Amersham Biotech and G protein Sepharose from Invitrogen. [γ^{32}P]-ATP (3000Ci/mmol) was purchased from Amersham. Pin-1 and CDK2-deficient MEFs were generously provided by G. del Sal (Laboratorio Nazionale C.I.B., Trieste, Italy) and M. Barbacid (Centro Nacional de Investigaciones Oncológicas, Madrid, Spain), respectively. All other reagents were of the highest commercially-available grades.

Immunoprecipitation and Immunoblotting. For immunoprecipitation of GRK2, cells were lysed in 500 μl per 100-mm dish of lysis buffer A [50-mM hepes, pH 7.5, 150-mM NaCl, 1% Brij-96 V (FLU.K.A), 2-mM EDTA, 1-mM NaF, 1-mM sodium orthovanadate]. Upon centrifugation (15,000 × *g*, 10 min), supernatants were incubated with a specific anti-GRK2 monoclonal antibody (clone C5/1.1, Upstate Biotechnology). Immune complexes were resolved in 7.5% SDS/PAGE and transferred to nitrocellulose membranes. After incubation with the indicated antibodies, blots were stripped and reprobed with a polyclonal antibody directed against the immunoprecipitated GRK2 protein (C15, Santa Cruz Biotechnology Inc). Blots were developed using a chemiluminiscent method (ECL, Amersham). Band density was quantitated by laser densitometric analysis and the amount of coprecipitated protein normalized to the amount of the immunoprecipitated protein, as assessed by the specific antibodies.

Protein Degradation Assays. Metabolic labeling and pulse-chase experiments were performed as described (4). Protein extracts were immunoprecipitated with the specific GRK2 polyclonal antibody AbFP1 or with the GRK2 monoclonal antibody clone C5/1.1 (Upstate Biotechnology) as reported (4). Immunoprecipitates were resolved by SDS/PAGE and transferred to PVDF membranes to be treated with the Enhancer Autoradiography Starter Kit (EABiotech Ltd.) according to the manufacturer's protocol. Band density of ^{35}S-labeled GRK2 was quantitated by laser densitometry analysis and data were corrected according to total GRK2 protein detected by immunoblotting. Protein decay was also determined using translation shut-off assays. Cycloheximide (25 μg/mL) was added for different times to asynchronously growing MEFs or to HeLa cells that were synchronized at G1/S and released into the S or G2 phase (≈3 h or 7 h after the exit from the arrest, respectively).

Protein Purification. Bovine GRK2 was overexpressed and purified from baculovirus-infected Sf9 cells as described (6). GST-fusion proteins were purified from BL21(DE3) *Escherichia coli* (Stratagene), as previously described (6). GST-ESS1, GST-7PL, and GST-Pin1wt expression were induced with 0.4mM IPTG for 3 h at 37 °C (GST-Pin1wt) or 30 °C (GST-ESS1, GST-7PL). Bacteria were lysed in PBS plus 1%Triton-X100, 10-mM EDTA, and 5-mM DTT. For GST fusion protein purification, the soluble fraction of the bacterial lysates was incubated with Glutathione-Sepharose 4B (Amersham) and bound proteins were eluted with 10-mM Glutathione (Sigma) in 50mM Tris-HCl pH 8.0.

GST Pull-Down Assays. For GST pull-down assays, 20×10^6 wild-type or CDK2-deficient MEFs and 10×10^6 control or HeLa cells stably overexpressing GRK2-wt (HeLa wt5), the mutant protein GRK2-S670A (HeLa A1), the C-terminal construct $GRK2_{436–689}$, or an empty vector (HeLa-Ctrl), either arrested in the G2 phase by etoposide treatment or growing asynchronously, were rinsed in binding buffer A (50-mM Tris-HCl pH 7.5, 150-mM NaCl, 0.1% Triton-X100, 1-mM EDTA, 5-mM EDTA, 1-mM sodium orthovanadate, plus protease inhibitors). Cell lysates were incubated with GST or GST-Pin1wt (2 μg) immobilized to glutathione-Sepharose 4B beads. Precipitated proteins were subjected to SDS/PAGE and analyzed by immnublotting with the anti-GRK2 polyclonal antibody C-15. For in vitro association of GRK2 with Pin1, phosphorylation assays were performed with 125-ng recombinant GRK2 in the presence or absence of purified activated MAPK accordingly to the manufacturer's protocol (Stratagene). Then, GRK2 was incubated in binding buffer B (50-mM Tris-HCl pH 7.5, 100-mM NaCl, 0.1% Tween 20, 1-mM EDTA, and 1-mM NaF) with the indicated GST fusion proteins (300 ng) immobilized to glutathione-Sepharose 4B for 2 h at room temperature.

Kinase Phosphorylation Assays. Recombinant GRK2 protein at the concentrations indicated or GST-GRK2 proteins (100 ng) were incubated with 100 ng or 50 ng of the purified CDK2-cyclin A complex (combined purity 46%, Upstate Biotechnology), respectively, in 50-μl kinase buffer (20-mM hepes pH 7.2, 2-mM EGTA, 1-mM sodium orthovanadate, 1-mM DTT, 1-mM NaF, 20-mM MgCl2, 10-mM glycerol-phosphate, 100-μM ATP, 4,000 cpm/pmol [γ^{32}P]-ATP) in the presence or absence of 2.5 μg/mL heparin to inhibit GRK2 autophosphorylation. After the indicated time at 30 °C, the reaction was stopped by the addition of SDS sample buffer. Phosphorylated proteins were resolved by SDS/PAGE and visualized by autoradiography.

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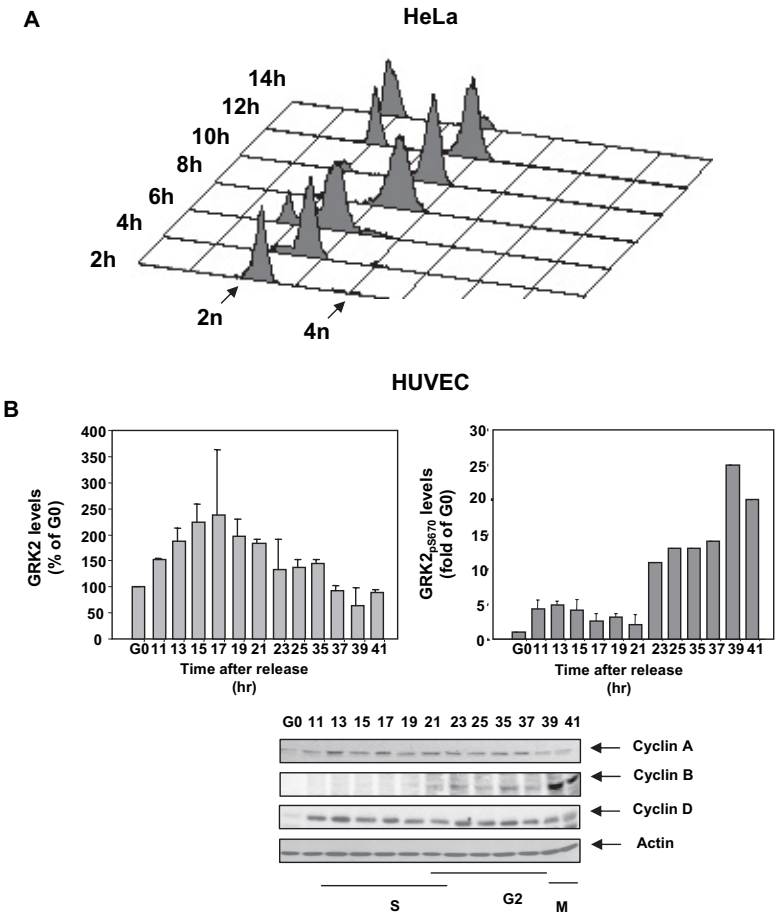

Fig. S1. (*A*) HeLa cells synchronization. Cells synchronized at the G1/S transition, were harvested at the indicated times after release of the block and subjected to flow cytometry analysis of the DNA content to determine the cell cycle status as detailed in *Methods*. The *x* axis represents DNA content and the *y* axis represents cell number. (*B*) GRK2 protein levels are down-regulated in primary cells progressing into the G2 phase. Primary human endothelial cells (HUVEC) were synchronized in G0 by incubation in 0.1% serum for 72 h before the treatment with 10% serum for the indicated time points. Levels of total and phospho-S670 GRK2 were determined in whole-cell lysates by immunoblotting with specific antibodies. Expression of cyclin B, cyclin A, and cyclin D were monitored in the same cellular samples to confirm that cells were synchronously entering the cell cycle. After correction of the GRK2 densitometry values for actin expression, phospho-S670 band densities were normalized to cognate total GRK2 densities. Data are mean ± SEM from two independent experiments. Representative blots are shown.

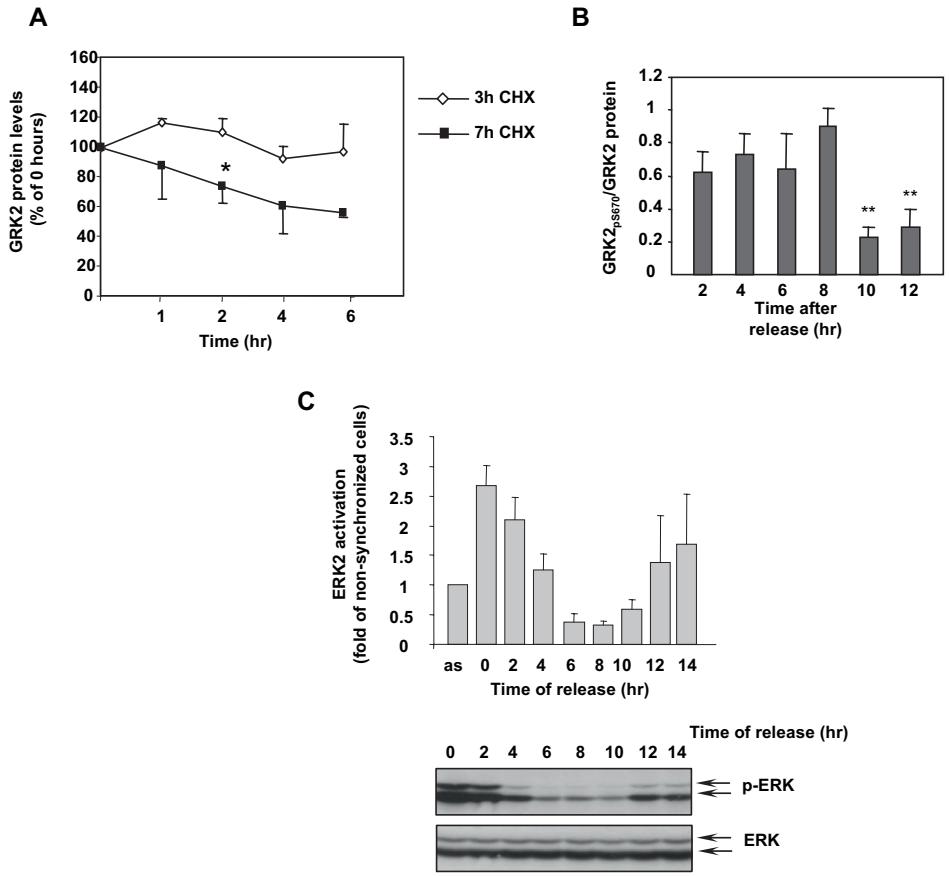

Fig. S2. (*A*) Stability of the GRK2 protein is reduced at G2 phase. To determine whether GRK2 down-regulation was reflecting changes in its degradation rate during cell cycle progression, we treated HeLa cells synchronized at the G1/S boundary with cycloheximide (CHX) for different times at either 3 h or 7 h after release from the block, and GRK2 protein levels were assessed. Upon translational shut-off, a GRK2 decay was clearly detected in G2 phase cells (7-h CHX treatment) but not in cells progressing into S phase (3-h point), thus pointing to the occurrence of a higher GRK2 turnover in G2. Cells were collected at the indicated times, lysed, and subjected to immunoblot analysis. The amount of GRK2 at either 3 h or 7 h was taken as 100%. Data are the mean ± SEM of three independent experiments. *, $P < 0.05$ comparison between 7-h and 3-h CHX-treated cells. (*B*) Analysis of the phosphorylation ratio of GRK2 at S670 during cell cycle progression. GRK2 immunoprecipitates from HeLa cells progressing through the cell cycle as in Fig. 1*A* were analyzed using a phospho-specific anti-pS670 GRK2 antibody followed by a polyclonal anti-GRK2 antibody after membrane stripping. Maximum pS670 levels normalized to cognate total GRK2 protein were arbitrarily defined as 1. **, $P < 0.01$, compared with values at 8 h. (*C*) Analysis of ERK1/2 activation during G2 progression. HeLa cells were arrested in G1/S and released back synchronously into the cell cycle as detailed in *SI Materials and Methods*. Cells harvested at the indicated times were lysed and ERK1/2 activation was determined by using an anti-phospho-ERK1/2 antibody (p-ERK). The immunoblot was then stripped and the total cellular ERK1/2 was detected with specific antibodies. Phospho-ERK2 band densities were normalized to cognate total ERK2 values. Data are mean ± SEM from three to four independent experiments. A representative blot is shown.

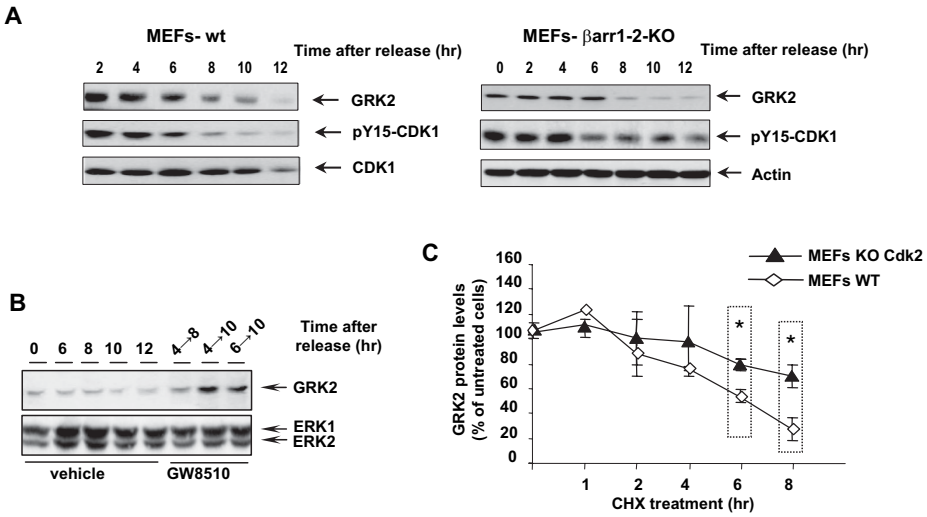

Fig. S3. (*A*) GRK2 protein levels are down-regulated in a β-arrestin-independent manner during G2 progression. MEFs deficient in both β-arrestin-1 and -2 isoforms (βarr1-2-KO) and wild-type cells were arrested in G1/S and released back synchronously into the cell cycle, as detailed in *SI Materials and Methods*. Cells harvested at the indicated times were lysed and protein levels of GRK2, total and phospho-CDK1 and actin were determined by immunoblotting with specific antibodies. Representative blots from two independent experiments are shown. (*B*) CDK2 mediates GRK2 down-regulation. HeLa cells synchronized at the G1/S transition and released from the block were treated with GW8510, a CDK2 inhibitor, for the indicated periods of time. GRK2 levels were assessed as above and ERK1/2 levels determined as a loading control. Blots are representative of three independent experiments. The fact that the addition of GW8510 in the early G2 phase (6-h point) was sufficient to prevent GRK2 protein decay at 10 h suggested that the critical phosphorylation events controlling GRK2 stability were confined to the G2 phase, further pointing to a role for CDK2 in such process. (*C*) Normal decay of GRK2 protein is impaired upon CDK2 knockdown. GRK2 protein levels were examined by immunoblot analysis in both wild-type and CDK2-deficient MEFs upon treatment with cycloheximide (CHX) for the indicated times. The amount of GRK2 at 0 h was defined as 100%, and data normalized by actin protein levels. Data are the mean ± SEM of four independent experiments (*, $P < 0.05$).

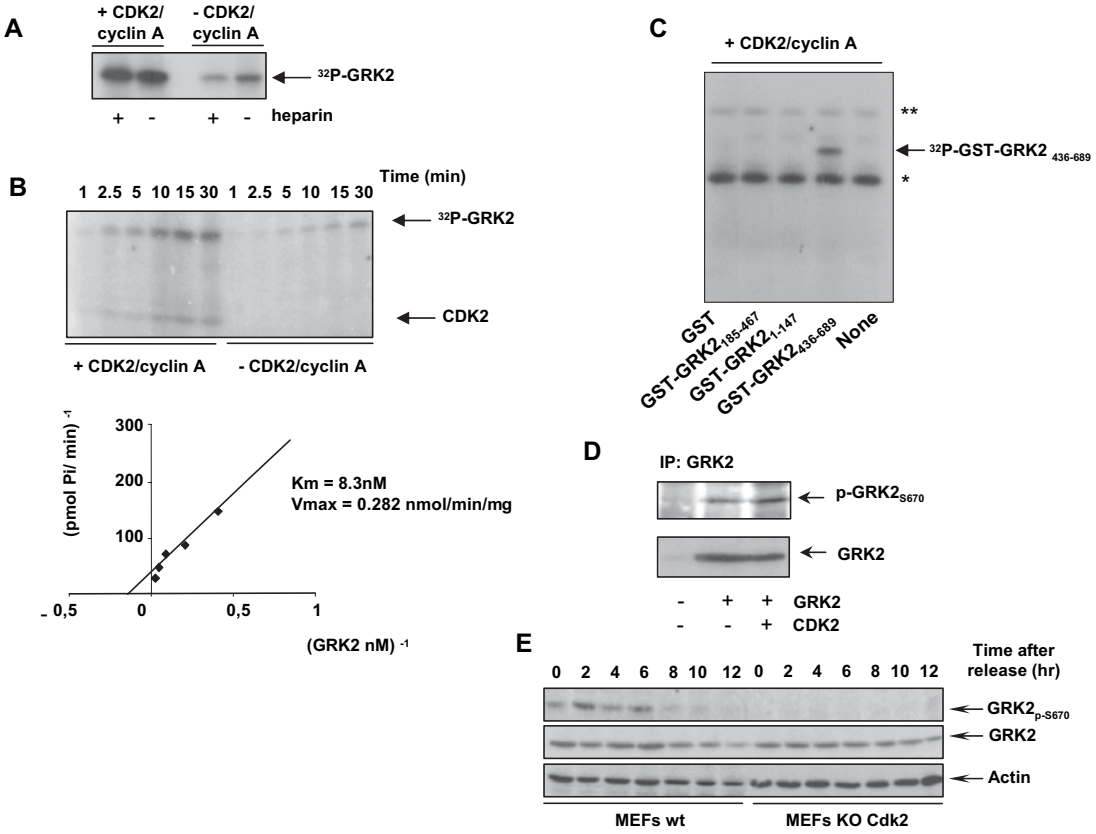

Fig. S4. GRK2 is phosphorylated by CDK2 at S670. (*A*) Recombinant GRK2 was incubated for 15 min in the absence (autophosphorylation control) or presence of purified CDK2-cyclin A with or without heparin under phosphorylation conditions, as described in *SI Materials and Methods*, followed by SDS/PAGE and autoradiography. (*B*) (*Upper*) Time course of GRK2 phosphorylation by CDK2-cyclinA. Samples containing recombinant GRK2 (50 nM) and CDK2-cyclinA (100 nM) or vehicle were incubated for the indicated times. (*Lower*) The kinetic parameters of GRK2 phosphorylation (V_{max} and K_m) were estimated by double-reciprocal plot analysis as detailed in *SI Materials and Methods*. Data are the mean from four independent experiments. (C) Identification of CDK2 phosphorylation site in GRK2. Despite the broad consensus motif X-S/T-P-XX-K/R has been defined for CDK/cyclin complexes, the phosphorylation sites of many well-established CDK2 substrates do not conform to this sequence. Such suboptimal phosphorylation sites are in conjugation with a cyclin-docking motif (RLX) that improves kinase efficiency, affinity, and specificity. Interestingly, GRK2 harbors three potential suboptimal phosphorylation sites for CDK2 at residues S388, S449, and S670, and several RLX-like sequences in the C-terminal half of the protein. When purified GST-fusion proteins spanning different regions of GRK2 were incubated with recombinant CDK2/cyclinA, only GST-GRK2436-689, but not the GST-GRK21-185 or GST-GRK2185-467 constructs, was phosphorylated, thus pointing to S670 as the main phospho-acceptor residue. *GST* fusion proteins were incubated with CDK2-cyclinA for 15 min and processed as in Fig. 1*E*. Asterisks denote unrelated proteins present in the purified sample of CDK2-cyclinA that are phosphorylated by CDK2. Gels representative of three or four experiments are shown. (*D*) Expression of CDK2 increases the extent of GRK2-S670 phosphorylation. Control cells and HeLa cells transiently cotransfected with GRK2-wt and CDK2 or empty vector were processed for GRK2 immunoprecipitation as detailed in *SI Materials and Methods*. In the immune complexes, GRK2 phosphorylation was assessed by with a phospho-specific anti-pS670 GRK2 antibody. After stripping, the presence of total GRK2 was immunodetected in the same gels. Similar results were obtained in two independent experiments. (*E*) GRK2 phosphorylation at S670 during cell cycle is inhibited in CDK2-deficient MEFs. Cellular lysates from wild-type and CDK2-deficient MEFs synchronously cycling after G1/S arrest were subjected to immunoblot analysis with a phospho-specific anti-pS670 GRK2 antibody. Blots were then stripped and probed with a polyclonal anti-GRK2 antibody. Gels are representative of two independent experiments.

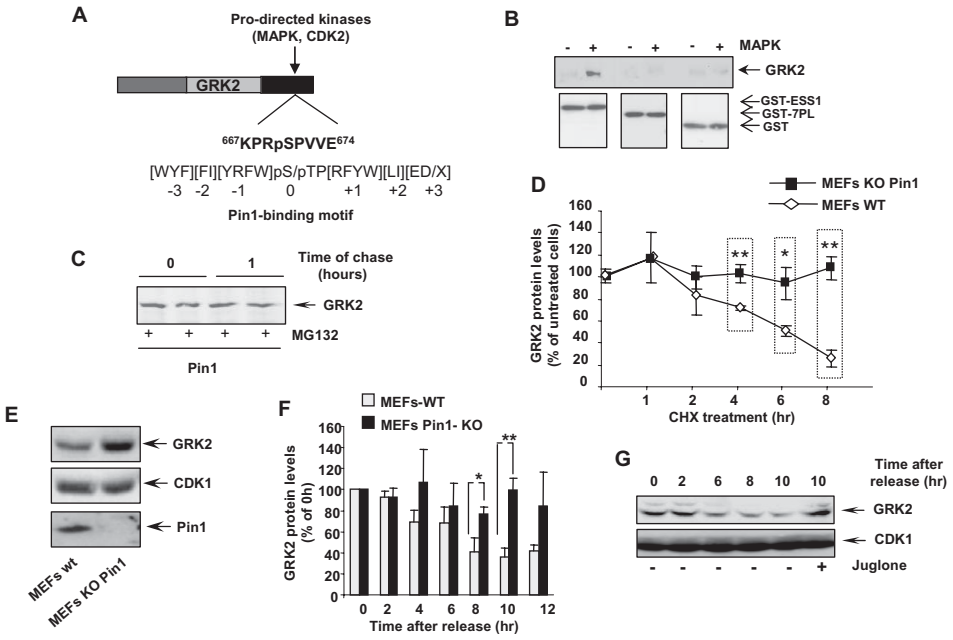

Fig. S5. (*A* and *B*) The WW domain of ESS1, a functional ortholog of Pin1, binds directly to GRK2 in a phosphorylation-dependent manner. The schematic diagram shows the sequence of GRK2 flanking S670, the residue targeted by MAPK and CDK2 phosphorylation. Such phosphorylated sequence resembles the optimal consensus motif for Pin1 binding depicted below. (*B*) GRK2 previously phosphorylated by MAPK specifically pulled down GST-ESS1, whereas no detectable GRK2 protein was precipitated by GST or an unrelated GST fusion protein (GST-7P). Recombinant GRK2 was incubated in phosphorylation conditions with or without purified MAPK as described in *SI Materials and Methods*. Kinase reactions were stopped and GST alone, GST-ESS1, or GST-7PL, an unrelated fusion protein, bound to glutathione-Sepharose beads were added as detailed in *SI Materials and Methods*. Precipitated proteins were washed and subjected to immunoblot analysis to detect both associated GRK2 and GST protein levels in the same blots with specific antibodies. Similar results were obtained in three independent experiments. (*C*) Pin1 promotes the degradation of GRK2 by the proteasome pathway. HEK-293 cells were cotransfected with Pin1 and GRK2 turnover was analyzed by pulse-chase assays in the presence or absence of the proteasome inhibitor MG132 as described in *SI Materials and Methods*. (*D*) GRK2 stability is enhanced in the absence of Pin1. GRK2 protein levels were examined by immunoblot analysis in both wild-type and Pin1-deficient MEFs upon treatment with cycloheximide (CHX) as in Fig. S3*C*. *, $P < 0.05$; **, $P < 0.01$. (*E*) The knock-down of Pin1 increases the steady-state levels of GRK2. Lysates from wild-type and Pin1-null MEFs were analyzed by immunoblotting. CDK1 expression was used as loading control. (*F*) GRK2 down-regulation in the G2 phase is prevented in cells lacking Pin1 expression. GRK2 levels were quantified by immunoblotting in cell lysates from synchronized wild-type and Pin1-deficient MEFs and normalized to the actin expression. Data are mean ± SEM from three independent experiments. *, $P < 0.05$; **, $P < 0.01$ compared to wild-type cells. (*G*) GRK2 down-regulation in the G2 phase is precluded on inhibition of Pin1 activity. Synchronized HeLa cells were treated 6h postrelease with juglone (50 μM). Whole-cell lysates were analyzed by immunoblotting with anti-GRK2 and anti-CDK1 antibodies.

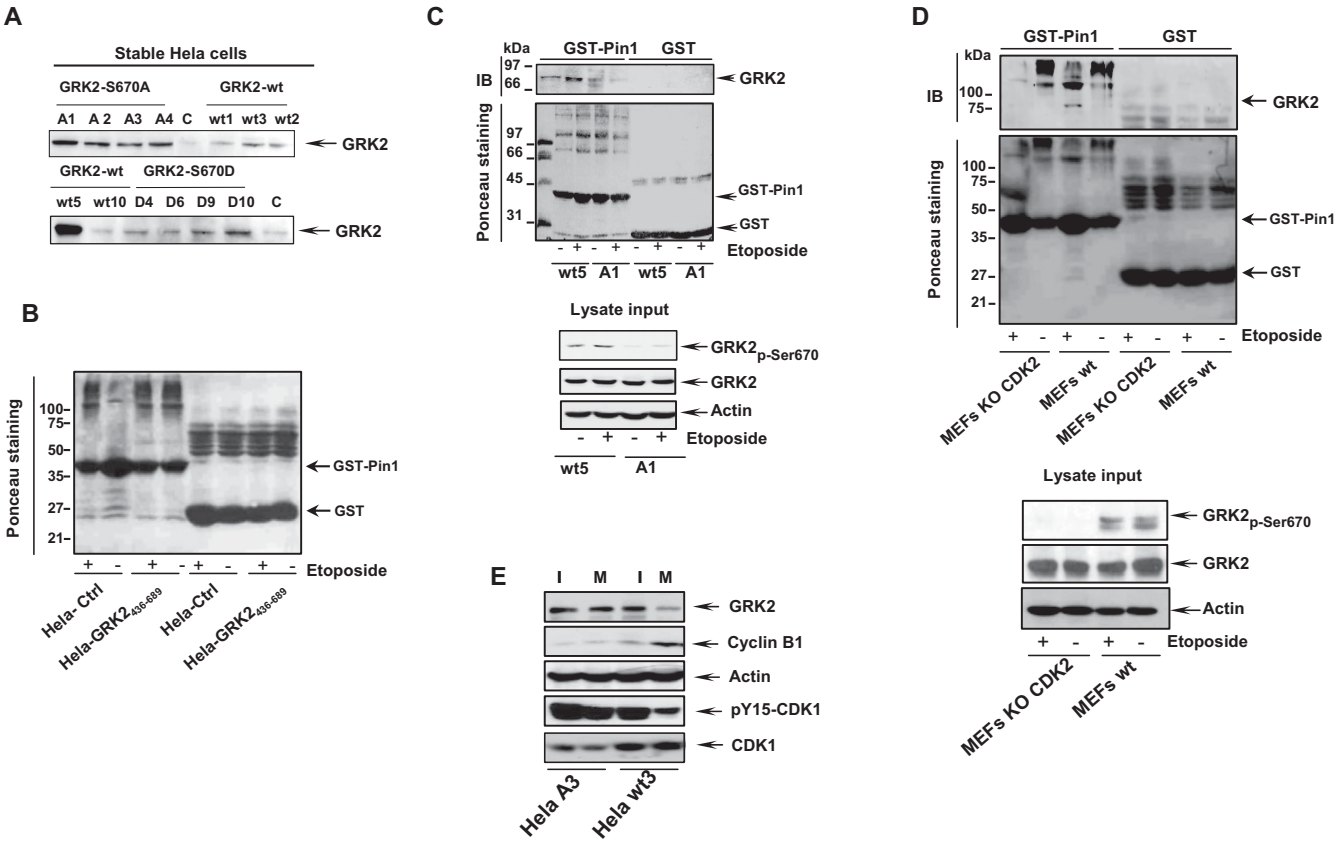

Fig. S6. (*A*) Generation of HeLa cells transfectants stably expressing different GRK2 mutants. Cells stably transfected with GRK2-wt or the phosphorylation mutants GRK2-S670A or GRK2-S670D were generated as described in *SI Materials and Methods*. GRK2 levels were analyzed by immnunoblotting in whole-cell lysates and compared to HeLa parental cells (control cells, C). (*B*) Control Ponceau staining showing equal loading of GST fusion proteins of the experiment shown in Fig. 3*C*. (*C*) GRK2-wt but not GRK2-S670A interacts with Pin1 in situ. Stably transfected cells with similar levels of GRK2wt (HeLa-wt5) or GRK2-S670A (HeLa-A1) expression were arrested in G2 phase with etoposide as described in *SI Materials and Methods* or maintained in exponential growth. In wt-GRK2-expressing cells, an increase in the cellular phospho-S670 content and in the interaction of GST-Pin1 with GRK2 was noted, whereas such changes were completely prevented upon expression of equivalent levels of the GRK2-S670A mutant. (*D*) The absence of CDK2 abrogates Pin1 binding to endogenous GRK2. Wild-type or CDK2-deficient MEFs were arrested in G2 phase with etoposide or maintained in exponential growth as above. In *C* and *D*, lysates were incubated with either GST or GST-Pin1. Precipitated proteins were subjected to SDS/PAGE and analyzed by immnublotting with the anti-GRK2 polyclonal antibody C-15. Equal loading of GST fusion proteins was confirmed by Ponceau staining of the blots. pS670-GRK2, GRK2, and actin protein levels were assessed in a 2% volume of input lysates. Gels are representative of two independent experiments. (*E*) Impairment of S670 phosphorylation prevents both cyclin B up-regulation and CDK1 activation. Cell lines expressing GRK2-wt (HeLa-wt3) or GRK2-S670A (HeLa-A3) were arrested at mitosis with nocodazol and cell lysates were analyzed by immunoblotting with anti-GRK2, anti-cyclin B and anti-pY15-CDK1 antibodies. Activation of CDK1 was monitored as a decrease in the extent of the inhibitory Y15 phosphorylation. The gel is representative of three independent experiments.

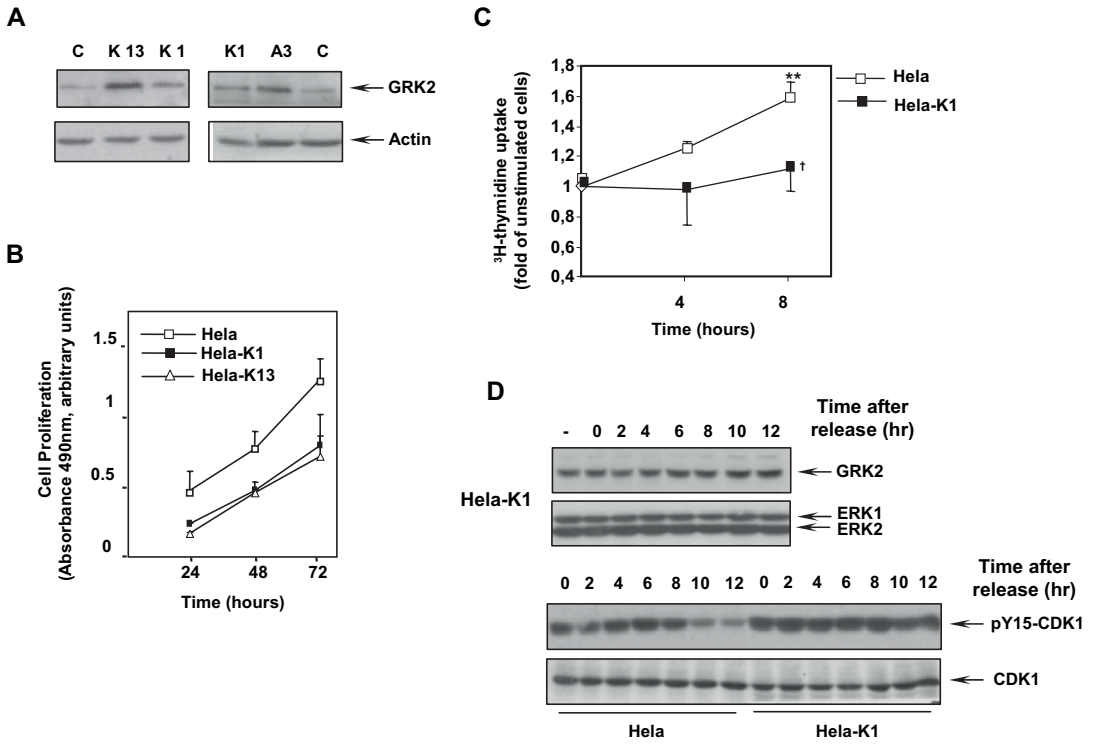

Fig. S7. Effect of GRK2 accumulation on cell cycle progression and cell proliferation. Expression of extra GRK2-K220R was analyzed in total lysates from cells stably transfected with this mutant (K13, K1) and compared to parental HeLa cells (*C*) and HeLa-A3 cells stably expressing the S670A mutant (*A*). Expression of the kinase-inactive mutant GRK2-K220R reduces cellular proliferation as monitored by using either a nonradioactive proliferation assay (*B*) or a H^3-thymidine uptake assay (*C*). Data are mean ± SEM of three to four independent experiments. **, $P < 0.01$, when compared to unstimulated serum-starved cells; †, $P < 0.05$ comparison between HeLa control cells and cells expressing GRK2-K220R (HeLa-K1). (*D*) The GRK2-K220R protein displays an impaired down-regulation in G2/M, which is paralleled by a deficient CDK1 activation. HeLa cells stably expressing GRK2-K220R (HeLa K1) were synchronized at G1/S as described in *SI Materials and Methods* and whole-cell lysates analyzed by immunoblotting with anti-GRK2 and anti-pY15-CDK1 antibodies. After stripping, blots were probed with anti-ERK1/2 and anti-CDK1 to check equal loading. Gels are representative of at least three independent experiments. The fact that impairment of normal GRK2 down-regulation in G2 by expression of two different mutants (S670A and K220R) promotes similar functional consequences strongly argues against an artifactual nature of such effects.

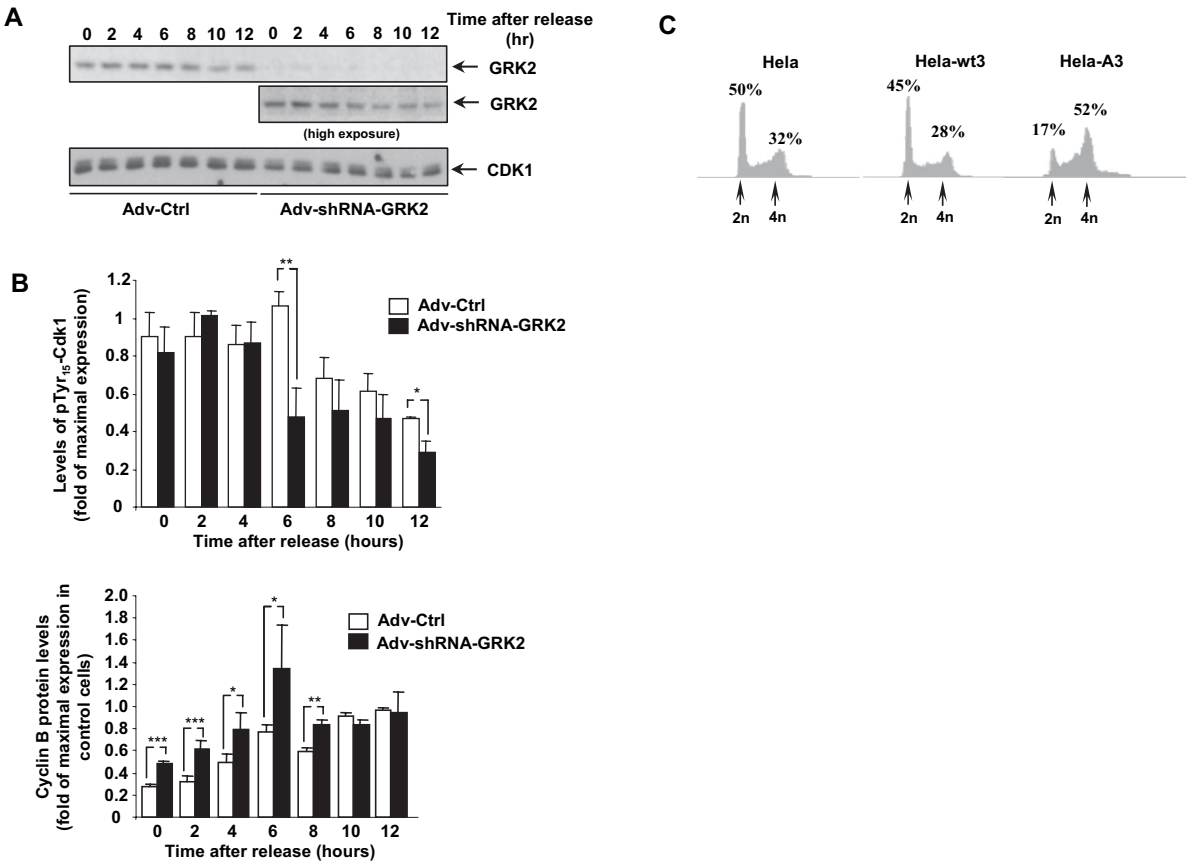

Fig. S8. Effect of GRK2 modulation on cell cycle progression. (*A* and *B*) Silencing of GRK2 expression induces an early activation of the mitotic CDK1-cyclinB complex. HeLa cells infected with an adenoviral-GRK2 shRNA or a control construct as described in *SI Materials and Methods* were synchronized at the G1/S transition and released into the cell cycle for the periods indicated. Cellular lysates were immunoblotted with anti-GRK2, anti-cyclin B, and anti-Y15-CDK1 antibodies. After stripping, blots were probed with anti-actin and anti-CDK1 antibodies for normalization and control loading. Data are mean ± SEM of three independent experiments performed in duplicate. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, when compared to control-infected cells. (*C*) Prevention of GRK2 phosphorylation delays G2/M transition. Cell cycle profiles of control HeLa cells or with extra GRK2-wt (HeLa-wt3) or GRK2-S670A (HeLa-A3) growing asynchronously were determined by flow cytometry. Percentage of cells in G1 (2n) and G2/M (4n) is shown.

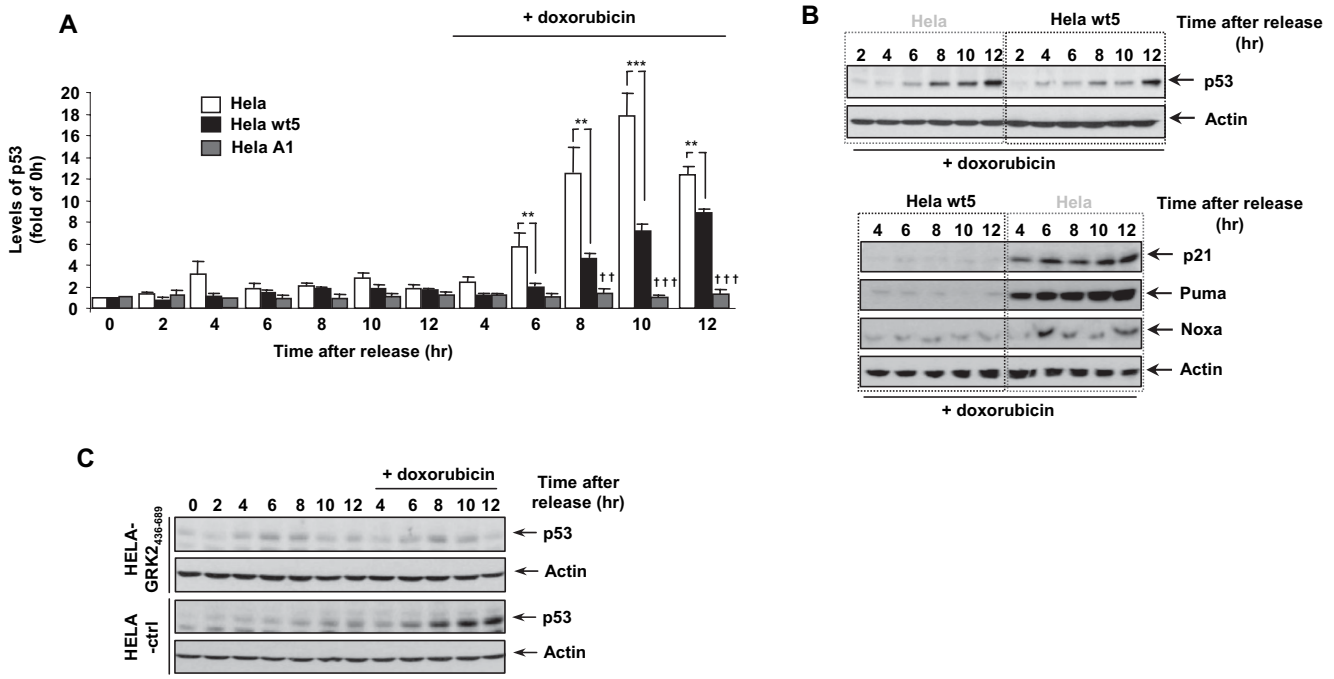

Fig. S9. Doxorubicin-triggered GRK2 stabilization in the G2-checkpoint response correlates with attenuated p53 up-regulation and induction of p53-induced genes. *(A)* Synchronized cells with or without extra wild-type GRK2 (HeLa wt5) or mutant GRK2-S670A (HeLa-A1) were exposed to doxorubicin or vehicle and levels of total p53 were immunodetected with specific antibodies and quantitated. p53 densitometry values were corrected for actin expression. **, $P < 0.01$; *** $P < 0.001$; ††, $P < 0.01$; and †††, $P < 0.001$ when compared to HeLa cells or to HeLa-wt5 cells, respectively. (*B*) The restrained p53 response observed is paralleled by a reduction in its transcriptional targets p21 and the proapoptotic PUMA and Noxa proteins. Cells as in *A* treated with doxorubicin were subjected to immunoblot analysis to determine expression levels of p53, p21, PUMA and Noxa with specific antibodies. (*C*) Inhibition of the cell cycle down-regulation of GRK2 by expression of the competitor construct $GRK2_{436–689}$ abrogates p53 induction upon DNA damage. HeLa cells stably expressing GRK2 436–689 or an empty vector (HeLa-Ctrl) were synchronyzed and treated with doxorubicin as in *A*. Levels of p53 and actin were determined in cellular lysates. Gels are representative of two independent experiments.