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p57^{KIP2} control of actin cytoskeleton dynamics is responsible for its mitochondrial pro-apoptotic effect

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p57 (Kip2, cyclin-dependent kinase inhibitor 1C), often found downregulated in cancer, is reported to hold tumor suppressor properties. Originally described as a cyclin-dependent kinase (cdk) inhibitor, p57^{KIP2} has since been shown to influence other cellular processes, beyond cell cycle regulation, including cell death and cell migration. Inhibition of cell migration by p57^{KIP2} is attributed to the stabilization of the actin cytoskeleton through the activation of LIM domain kinase-1 (LIMK-1). Furthermore, p57^{KIP2} is able to enhance mitochondrial-mediated apoptosis. Here, we report that the cell death promoting effect of p57^{KIP2} is linked to its effect on the actin cytoskeleton. Indeed, whereas Jasplakinolide, an actin cytoskeleton-stabilizing agent, mimicked p57^{KIP2}'s pro-apoptotic effect, destabilizing the actin cytoskeleton with cytochalsin D reversed p57^{KIP2}'s pro-apoptotic function. Conversely, LIMK-1, the enzyme mediating p57^{KIP2}'s effect on the actin cytoskeleton, was required for p57^{KIP2}'s death promoting effect. Finally, p57^{KIP2}-mediated stabilization of the actin cytoskeleton was associated with the displacement of hexokinase-1, an inhibitor of the mitochondrial voltage-dependent anion channel, from the mitochondria, providing a possible mechanism for the promotion of the mitochondrial apoptotic cell death pathway. Altogether, our findings link together two tumor suppressor properties of p57^{KIP2}, by showing that the promotion of cell death by p57^{KIP2} requires its actin cytoskeleton stabilization function.
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p57 (Kip2, cyclin-dependent kinase inhibitor 1C) has been described as a potential tumor suppressor gene due to its ability to inhibit several of the hallmarks of cancer.[1] The importance of p57^{KIP2} in the suppression of cancer is highlighted by its mutation/inactivation in Beckwith-Wiedemann Syndrome, a cancer pre-disposing syndrome,[2] and its reported decrease in protein expression in cancers of the breast, lung, liver, and prostate, among others.[3–8] Inhibition of p57^{KIP2} expression in cancer cells is mediated by promoter DNA methylation at CpG islands and/or by histone modifications, such as methylation and deacetylation.[1] Worth a notice, the lack of p57^{KIP2} expression in tumors is associated with poor survival, and has been identified as prognostic marker for many of the aforementioned cancer types.[1]

Initially identified as a member of the Cip/Kip family of Cdk (cyclin-dependent kinase) inhibitors,[9,10] p57^{KIP2} has since been shown to have diverse cellular roles aside from cell cycle inhibition, for example, promotion of cell death,[11–13] inhibition of cell migration,[14] and regulation of cell differentiation.[15–17] Indeed, considering that p57^{KIP2} null mice have increased embryonic lethality and gross developmental defects as compared with p21$^{CIP1/CDKN1A}$ and p27$^{KIP2/CDKN1B}$ null mice, it appears that the cellular roles of p57^{KIP2} surpass that of the other Cdk inhibitors.[18–20]

The ease at which cancer cells migrate and invade a neighboring tissue demonstrates the importance of regulators of cell cytoskeleton structure in the cancer progression. The cytoskeleton remodeling factor cofilin disassembles actin filaments and thereby facilitates cell migration through increasing the actin mobile fraction.[21] Active unphosphorylated cofilin is associated with increased tumor metastasis.[22] Cofilin can be inactivated by LIM domain kinase-1/2 (LIMK-1/2),[23,24] which are themselves activated by phosphorylation on Thr508[25] through the Rho/Rho kinase (ROCK) signaling pathway.[26] Interestingly, p57^{KIP2} interacts with LIM domain kinase-1 (LIMK-1) and increases its kinase activity independently of Rho/ROCK signaling and thereby contributes to the stabilization of actin filaments and the inhibition of cell migration.[14]

p57^{KIP2} expression can enhance cell death induced by staurosporine (STS) and DNA damaging agents such as etoposide and cisplatin, all of which induce apoptotic mitochondrial events.[11,12] In contrast, p57^{KIP2} has no potentiating effect on apoptosis induced by death receptor ligand,[12] indicating that p57^{KIP2} enhances cell death specifically at the level of the mitochondria. Indeed, overexpression of Bcl-2 or

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Abbreviations: Cdk, cyclin-dependent kinase; Cyto. D, cytochalsin D; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; Dox, doxycycline; F-actin, filamentous actin; FRAP, flourescence recovery after photobleaching; G3PDH, glycerol 3 phosphate dehydrogenase; GFP, green fluorescent protein; HK-1, hexokinase-1; Jasp, jasplakinolide; LIMK-1, LIM domain kinase-1; p21/Cip1/CDKN1A, cyclin-dependent kinase inhibitor 1A; p27/Kip1/CDKN1B, cyclin-dependent kinase inhibitor 1B; p57/KIP2/CDKN1C, cyclin-dependent kinase inhibitor 1C; PARP, poly ADP-ribose polymerase; PBS, phosphate-buffered saline; ROCK, rho kinase; ROI, region of interest; STS, staurosporine; TMRE, tetramethylrhodamine ethyl ester; Tom40, translocase of outer membrane 40; VDAC, voltage-dependent anion channel; $\Delta\Psi$m, loss of mitochondrial transmembrane potential
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inhibition of the mitochondrial voltage-dependent anion channel (VDAC) inhibited p57^{KIP2}'s cell death promoting effect.[12] Interestingly, there are reports that actin can influence the conductance of VDAC channels. In *Neurospora crassa*, an abundance of monomeric actin facilitated the closure of VDAC channels, whereas stable filamentous actin (F-actin) was unable to do the same.[27] In fact, the presence of F-actin led to an increased ion conductance across the outer mitochondrial membrane. This indicated a possible link between the stability of actin cytoskeleton and the integrity of the mitochondrial transmembrane potential. In this study, we investigated the possibility that the cell death promoting effect of p57^{KIP2} requires its actin cytoskeleton-stabilizing effect.

Results

p57^{KIP2} stabilizes the actin cytoskeleton and promotes apoptosis. Two of the tumor suppressor characteristics of p57^{KIP2} include inhibition of cell migration and promotion of apoptosis.[11,12,14] In this study, we took advantage of HeLa Tet-On derived cell line stably transfected with tetracycline-inducible p57^{KIP2}, HeLa-p57^{KIP2} cells. These cells can be induced to express p57^{KIP2} selectively, upon administration of the tetracycline analog doxycycline (Dox) (Figure 1a). Induction of p57^{KIP2} expression in HeLa-p57^{KIP2} cells led to an increased formation of actin stress fibers as visualized by staining with phalloidin, which binds F-actin (Figure 1b). Additionally, fluorescence recovery after photobleaching (FRAP) analysis of green fluorescence protein (GFP)-labeled-actin revealed a reduced actin-GFP mobile fraction in HeLa-p57^{KIP2} cells treated with Dox as compared with untreated cells, providing additional evidence for the actin cytoskeleton-stabilizing function of p57^{KIP2} (Figure 1c). Although, induction of p57^{KIP2} expression did not provoke cell death *per se*, it sensitized cells to apoptosis triggered by STS (Figures 1d and e, Supplementary Figure S1).

It has already been established that stabilization of the actin cytoskeleton can induce apoptotic cell death.[28,29] Jasplakinolide (Jasp), an actin cytoskeleton-stabilizing agent, was used to investigate whether actin cytoskeleton stabilization increases cell death induced by STS, thereby mimicking the effect of p57^{KIP2}.[30] At the concentration used in this study, Jasp stabilized the actin cytoskeleton, as shown by FRAP measurement of the mobility of actin-GFP (Figure 1c), but did not increase cell death (Figures 1d and e, Supplementary Figure S1). Pre-treatment of HeLa-p57^{KIP2} cells with Jasp 30 min before STS treatment resulted in an increase in cells exhibiting apoptotic nuclear morphology (Figure 1d, Supplementary Figure S1) and poly ADP-ribose polymerase (PARP) cleavage (Figure 1e), similar to levels observed in cells co-treated with Dox and STS. Importantly, induction of p57^{KIP2} expression by Dox treatment did not significantly increase the cell death caused by Jasp and STS co-treatment indicating that indeed Jasp mimics the pro-apoptotic effect of p57^{KIP2}.

Destabilization of actin filaments inhibits p57^{KIP2} enhancement of STS-induced cell death. As actin cytoskeleton stabilization mimicked the pro-apoptotic effects of p57^{KIP2}, an actin cytoskeleton destabilizing agent cytochalasin D (Cyto. D),[31] was used to investigate whether depolymerization of actin filaments could prevent the promotion of cell death by p57^{KIP2}. Cyto. D was used at a concentration that does not induce cell death, but yet retains the ability to destabilize the actin cytoskeleton (Figures 2a–c). FRAP analysis of actin-GFP mobility in HeLa-p57 cells treated with Dox showed that treatment with Cyto. D for 3 h successfully reversed the actin cytoskeleton-stabilizing effect of p57^{KIP2} (Figure 2a). To assess the effect of actin depolymerization on the p57^{KIP2}-death promoting effect, HeLa-p57^{KIP2} cells were treated with Dox and STS with or without Cyto. D pre-treatment. Cells co-treated with Dox and STS presented reduced apoptotic nuclear morphology in the presence of Cyto. D (Figure 2b: 7.2% with Cyto. D *versus* 22.2% without Cyto. D). Furthermore, the increase in cleaved PARP in HeLa-p57^{KIP2} cells treated with STS was reduced in the presence of Cyto. D (Figure 2c). Therefore, one could conclude that actin cytoskeleton destabilization inhibits the ability of p57^{KIP2} to enhance STS-induced apoptotic cell death.

LIMK-1 is required for p57^{KIP2}-induced cell death. Activation of LIMK-1 kinase results in decreased cofilin activity through phosphorylation and as such, increased actin cytoskeleton stabilization.[26] It has previously been shown that p57^{KIP2} directly interacts with LIMK-1 resulting in an increase in LIMK-1 kinase activity, which is required for p57^{KIP2}-mediated actin cytoskeleton stabilization.[14] To investigate whether LIMK-1 is required for p57^{KIP2}-mediated apoptosis, small interfering RNA (siRNA) directed against LIMK-1 were used, which resulted in the specific knockdown of LIMK-1 protein levels (Figure 3a).

In HeLa-p57^{KIP2} cells co-treated with Dox and STS, there was a reduction in the apoptotic nuclei to 6.7% in LIMK-1 knockdown cells as compared with 15.2% in LIMK-1 expressing cells (Figure 3b). In fact, the number of apoptotic nuclei in LIMK-1 knockdown cells co-treated with Dox and STS were similar to those observed in cells treated only with STS (Figure 3b). Similarly, measurement of DEVDase activity also showed a decrease caspase-3 like activity in cells co-treated with Dox and STS when LIMK-1 expression was suppressed as compared with the control (Figure 3c). Furthermore, analysis of PARP cleavage by immunoblot confirmed that in LIMK-1 deficient cells, p57^{KIP2} was unable to enhance STS-mediated cleavage of this caspase-3 substrate (Figure 3d). Together, these results demonstrated that silencing of LIMK-1 prevented p57^{KIP2} enhancement of STS-induced apoptosis. Thus, this additionally established that loss of the actin-stabilizing effect of p57^{KIP2} is sufficient to prevent its pro-apoptotic effect.

Actin cytoskeleton stabilization by p57^{KIP2} positively modulates apoptosis at the mitochondrial level. So far, it has been established that the pro-apoptotic effect of p57^{KIP2} requires its ability to stabilize the actin cytoskeleton. This death promoting effect of p57^{KIP2} was previously shown to be mediated through the mitochondria.[12] Indeed treatment of HeLa-p57^{KIP2} cells with Dox and STS increased the loss of mitochondrial transmembrane potential ($\Delta\Psi$m) compared

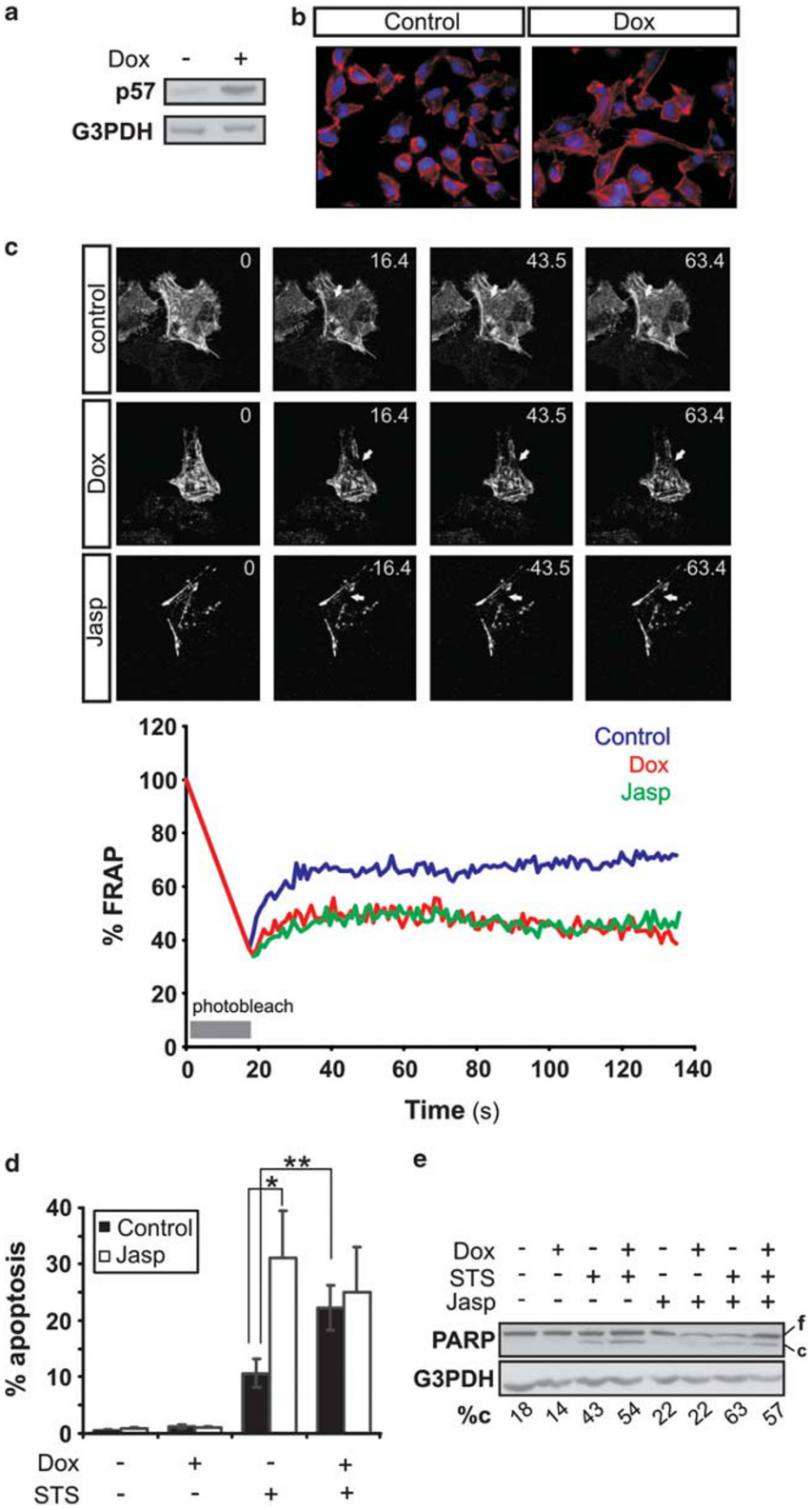

Figure 1 Cytoskeleton stabilization enhances STS-induced apoptosis. (**a**) HeLa-p57 TET-on cells were treated with Dox for 24 h. p57^{KIP2} expression was analyzed by immunoblotting, and (**b**) actin filaments were stained with phalloiden. (**c**) The mobile actin fraction following exposure to Dox or Jasp (3 h) was measured by FRAP analysis of cells transfected with actin-GFP. Values represent the % fluorescence recovery over time of actin-GFP after bleaching. Arrows indicate the photobleached area. (**d**) HeLa-p57^{KIP2} cells were treated with Jasp for 30 min, followed by treatment with STS for 3 h with or without exposure to Dox. Apoptotic nuclear morphology was quantified after Hoechst staining and expressed as the percentage of the total cells counted. (**e**) PARP cleavage was assessed by immunoblotting. G3PDH was used as a loading control. Full length (f) and cleaved (c) PARP was analyzed by densitometry and cleaved PARP was expressed as a % of total PARP (%c)

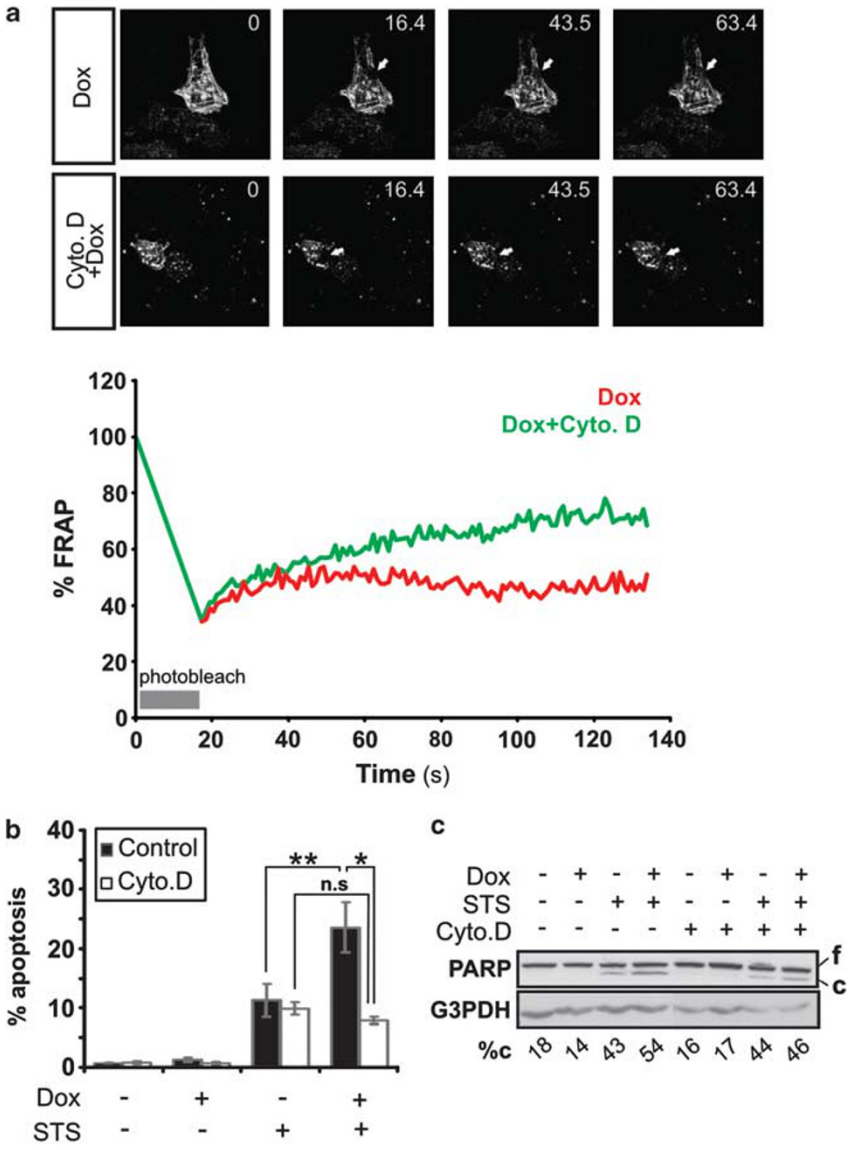

Figure 2 Cytoskeleton destabilization prevents the enhancement of apoptosis by p57^{KIP2}. (**a**) FRAP analysis of HeLa-p57^{KIP2} cells treated with or without cytochalsin D for 3 h. Values represent the % fluorescence recovery over time of actin-GFP after bleaching. Arrows indicate the photobleached area. (**b** and **c**) HeLa-p57^{KIP2} cells were treated with cytochalsin D for 1 h, followed by treatment with STS for 3 h. (**b**) Apoptotic nuclear morphology was quantified after Hoechst staining and expressed as a percentage of the total cells counted. (**c**) PARP cleavage was assessed by immunoblotting. G3PDH was used as a loading control. Full length (f) and cleaved (c) PARP was analyzed by densitometry and cleaved PARP was expressed as a % of total PARP (%c)

with STS treatment alone (Figure 4a). The importance of actin dynamic for maintenance of $\Delta\Psi$m is demonstrated by the ability of Jasp to enhance STS-induced loss in $\Delta\Psi$m (Figure 4b), and of Cyto. D to reduce the loss of $\Delta\Psi$m induced by Dox and STS co-treatment (Figure 4c). Furthermore, in the presence of a kinase-inactive mutant of LIMK-1 (LIMK-1-D460A), p57^{KIP2} expression did not enhance STS-induced loss of $\Delta\Psi$m (Figure 4d). In all, these results show that a stable actin cytoskeleton, induced by p57^{KIP2}, positively modulates apoptosis at the mitochondrial level.

Actin stabilization by p57^{KIP2} displaces hexokinase-1 (HK-1) from mitochondria. Previous work has shown that inhibition of VDAC channel opening using 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) counteracts the p57^{KIP2}-death promoting effect.[14] Therefore, the effect of p57^{KIP2} on the mitochondrial protein levels of HK-1, an important regulator of VDAC channel conductance, was examined. A decrease in mitochondrial localization of HK-1 was observed upon Dox treatment of HeLa-p57^{KIP2} cells (Figure 5a), Actin cytoskeleton stabilization by Jasp also resulted in a decrease in mitochondrial HK-1 (Figure 5a). Finally, the ability of HK-1 to rescue cells from apoptosis induced by p57^{KIP2} was investigated. Accordingly, overexpression of HK-1 in p57^{KIP2} expressing cells resulted in a decrease in cleaved caspase-3 staining compared with the pcDNA-transfected control (Figures 4b and c).

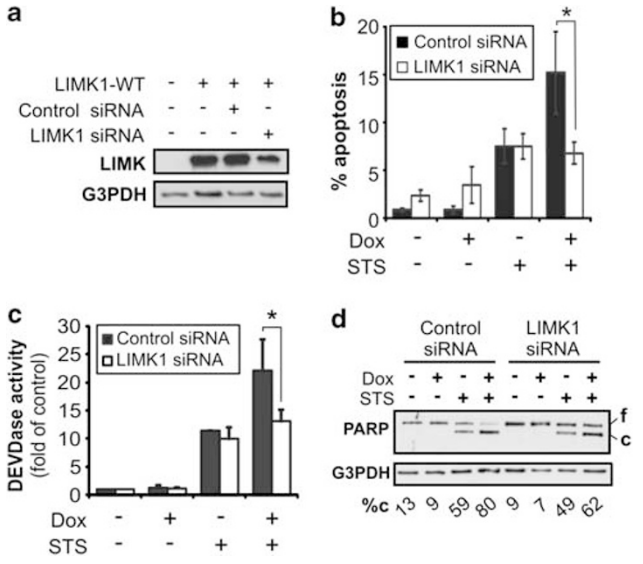

Figure 3 LIMK-1 is required for p57^{KIP2}-induced apoptosis. HeLa-p57^{KIP2} cells were transfected with scrambled sequence siRNA or LIMK-1 siRNA in the presence or absence of Dox (24 h) and STS (3 h). (**a**) LIMK-1 knockdown was confirmed by immunoblotting against LIMK-1, using G3PDH as a loading control. (**b**) Apoptotic nuclear morphology was quantified by Hoechst staining and expressed as a percentage of the total cells counted. (**c**) Activation of effector caspases was measured by DEVDase assay, expressed as fold increase of control. Values represent the mean +/− S.D. of three separate experiments. (**d**) PARP cleavage was assessed by immunoblotting, using G3PDH as a protein loading control. Full length (f) and cleaved (c) PARP was analyzed by densitometry and cleaved PARP was expressed as a % of total PARP (%c)

Discussion

p57^{KIP2} inhibits many of the hallmarks of cancer and as such has been hailed as a potential tumor suppressor gene.[1] The results in this study corroborate previous studies showing firstly that p57^{KIP2} inhibits cancer cell migration through stabilization of the actin cytoskeleton, and secondly that p57^{KIP2} enhances apoptotic cancer cell death through mitochondrial depolarization. This study examines the interplay between these two seemingly disparate functions of p57^{KIP2}, and shows that the pro-apoptotic effect of p57^{KIP2} depends on its ability to stabilize the actin cytoskeleton. Disturbing actin cytoskeleton dynamics affects the ability of p57^{KIP2} to promote cell death. We here show that stabilizing the actin cytoskeleton mimics the death promoting effect of p57^{KIP2}, and destabilizing actin filaments reverses this effect. Significantly, inhibition of the actin cytoskeleton-stabilizing effect of p57^{KIP2} (through LIMK-1 knockdown) also abrogates the death promoting effect of p57^{KIP2}, providing a direct link between the actin cytoskeleton-stabilizing function of p57^{KIP2} and the death promoting function of p57^{KIP2}. Stabilization of actin cytoskeleton has been shown to induce cell death.[28,32] Although to date, the mechanism by which F-actin sensitizes to cell death is not fully described, evidence suggests that it is through mitochondrial depolarization.[27,32] Further investigation into how p57^{KIP2} sensitizes cells to death revealed a link between actin cytoskeleton stabilization and mitochondrial hexokinase levels. Hexokinase is often found overexpressed in cancer cells, where its binding to mitochondria enhances the cell's rate of glycolysis through easy access to mitochondrial-generated ATP.[33] The closure of

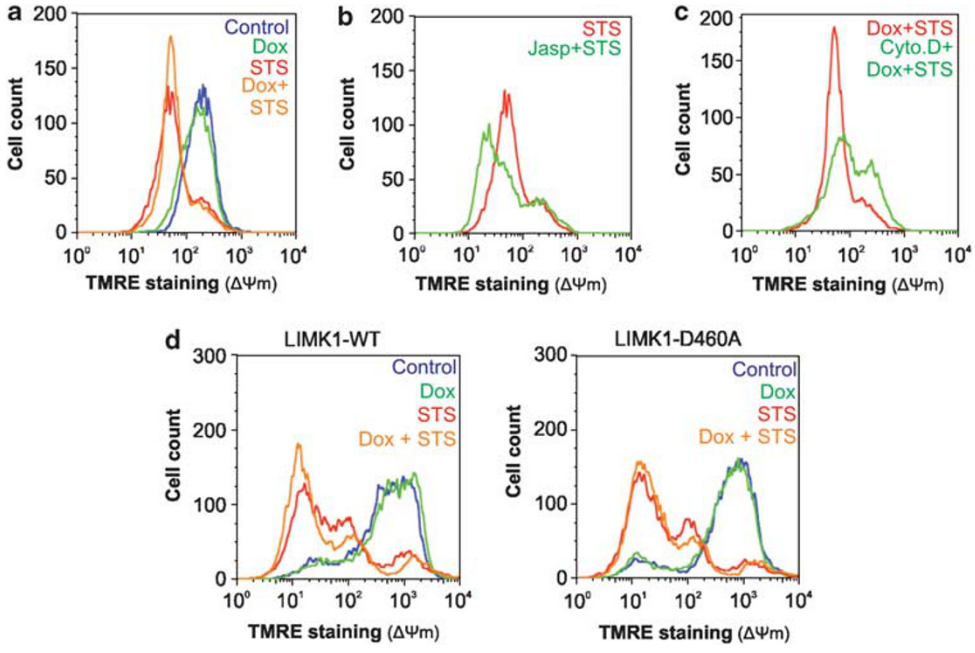

Figure 4 Actin cytoskeleton stabilization by p57^{KIP2} promotes ΔΨm. HeLa-p57^{KIP2} cells were treated STS in the presence or absence of Dox. (**a**) The ΔΨm was measured by flow cytometry after staining with TMRE. (**b**) HeLa-p57^{KIP2} cells were treated with Jasp for 30 min, followed by treatment with STS for 5 h. The ΔΨm was measured by flow cytometry after staining with TMRE. (**c**) HeLa-p57^{KIP2} cells were treated with Cytochalsin D for 30 min, followed by treatment with STS for 3 h in the presence of Dox. The ΔΨm was measured by flow cytometry after staining with TMRE. (**d**) HeLa-p57^{KIP2} cells were transfected with wild-type LIMK (LIMK-WT) or a kinase-inactive mutant of LIMK (LIMK-D460A) before treatment with Dox and STS. The ΔΨm was measured by flow cytometry after staining with TMRE

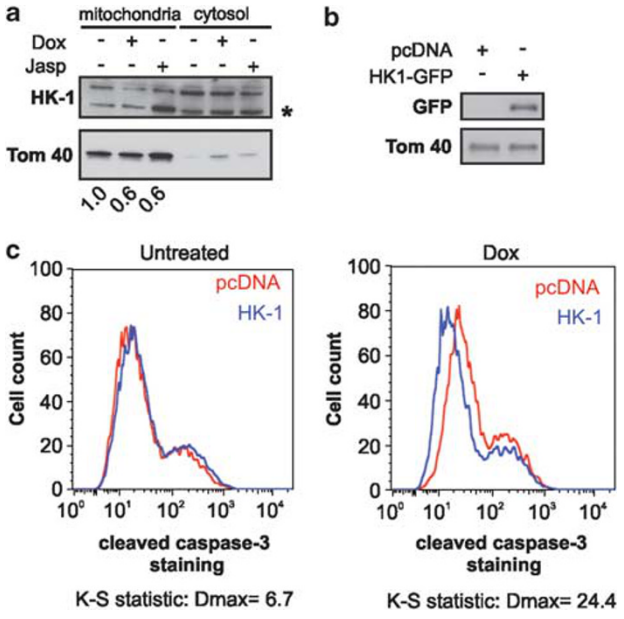

Figure 5 Actin stabilization by p57KIP2 promotes displacement of HK-1 from mitochondria. (**a**) HeLa-p57^{KIP2} cells were treated with either Dox or Jasp. Mitochondrial and cytosolic protein fractions were analyzed by immunoblotting using antibodies against HK-1. Tom40 was used as a mitochondrial loading control. Asterisk (*) indicates a cleavage product of HK-1. Values represent the fold decrease of full-length HK-1 compared with control. (**b**) HeLa-p57^{KIP2} cells were transfected with HK-1-GFP or pcDNA and grown in the presence or absence of Dox for 24 h. Overexpression of HK-1-GFP was analyzed by immunoblotting with GFP, using Tom40 as a loading control, and (**c**) cleaved caspase-3 staining was assessed by flow cytometry

VDAC channels upon hexokinase binding is one mechanism used by cancer cells to evade death.[34] This study shows that actin cytoskeleton stabilization either by Jasp or by p57^{KIP2} expression displaces HK-1 from the mitochondrial membrane. In fact, overexpression of HK-1 inhibits the promotion of cell death by p57^{KIP2}. This describes a scenario whereby stabilization of actin by p57^{KIP2} promotes cell death through the displacement of HK-1 from the mitochondria. Many cancer cells exhibit decreased p57^{KIP2} expression either through promoter DNA methylation, histone modifications, genetic mutation, silencing by microRNA's, or proteasomal degradation.[1] Interestingly, induced p57^{KIP2} expression in tumors forces regression of the cancer.[35] Therefore, as with many tumor suppressors, it is important for the cancer to keep expression of p57^{KIP2} at a reduced level. Consequently, p57^{KIP2} protein expression is being tested as a prognostic indicator in cancer. In this study, we have shown that the negative regulation of at least two cancer hallmarks, increased cell migration-metastasis and evasion of cell death, can be traced back to the ability of p57^{KIP2} to stabilize actin cytoskeleton. Potentially, other hallmarks of cancer could also be regulated by p57^{KIP2} regulation of the actin cytoskeleton dynamic. Already, increased actin mobility is known to enhance tumor angiogenesis.[36] These findings should increase interest in therapies that reactivate p57^{KIP2} expression in cancer cells.

Materials and Methods

Plasmids and siRNA. Myc-LIMK-1 and Myc-LIMK-1 D640A mutant in the FPC1 vector were gifts from K Mizuno (Tohoku University, Japan). Actin-GFP plasmid was a gift from Pirta Hotulainen (University of Helsinki, Finland). GFP plasmid was from Clontech (Invitrogen, Carlsbad, CA, USA). SMARTPool siRNA against LIMK-1 and non-targeted sequence were purchased from Dharmacon (Lafayette, CO, USA).

Cell culture, transfections, and treatments. Human cervical carcinoma HeLa cells expressing p57^{KIP2} under the control of the tetracycline promoter (HeLa-p57^{KIP2}) were a gift from S Okret, Karolinska Institutet and were cultured in MEM medium (Gibco, Carlsbad, CA, USA) supplemented with 10% Fetal bovine serum, penicillin/streptomycin, non-essential amino acids, sodium pyruvate, and L-glutamine. 2 μg/ml Dox was added to the culture medium for 24 h to induce expression of p57^{KIP2}. Transfections were performed with Lipofectamine and Plus reagent (Invitrogen) according to the manufacturer's protocol. Cells were treated with 0.1 μM Jasp, 1 μM Cytochalsin D, or 0.1 μM STS for the indicated times.

Protein extracts and immunoblotting. Cells were harvested and lysed by sonication in NP40 lysis buffer containing protease inhibitors.[17] Protein lysates were heated to 95 °C for 5 min in Laemmli buffer. In all, 20–40 μg proteins were electrophoresed on 12% SDS-PAGE acrylamide gels, and then immunoblotted onto nitrocellulose membrane. Membranes were blocked in 5% milk and incubated with anti-PARP (BD Pharmingen, Franklin Lakes, NJ, USA), anti-p57^{KIP2} (C-20, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-HK-1 (N-19, Santa Cruz Biotechnology), anti-GFP (Santa Cruz Biotechnology), anti-translocase of outer membrane 40 (tom40; Santa Cruz Biotechnology), or anti-glycerol 3 phosphate dehydrogenase (G3PDH; Trevigen, Gaithersburg, MD, USA) overnight at 4 °C, followed by incubation with the appropriate horseradish peroxidase secondary antibody (1 : 10 000) for 1 h at room temperature. Bands were visualized by enhanced chemiluminescence (Pierce, Rockford, IL, USA) following the manufacturers protocol.

Fluorescence recovery after photobleaching (FRAP). FRAP was used to assess the amount of mobile actin-GFP monomers. HeLa- p57^{KIP2} cells, grown on glass chamber slides, were transfected with actin-GFP and treated as indicated in the figure legends. Chamber slides were then placed in the POC-chamber/CTI Controller/heating insert P system for live cell imaging. After bleaching the region of interest (ROI) with 100% intensity of Argon/2 laser 488 nm (current 4.7 Amp), the time course of fluorescence recovery in the bleached ROI was monitored with 20 ms intervals. Samples were analyzed with Axiovert 200 m microscope (Zeiss, Oberkochen, Germany).

Measurement of effector caspase-3/7 (DEVDase) activity. DEVDase activity in cell lysates was calculated by measuring the rate of cleavage of the fluorogenic DEVD-MCA substrate (Peptide Institute Inc., Osaka, Japan). Free AMC was monitored in a Fluoroscan II plate reader (Labsystems, Waltham, MA, USA) using 355 nm excitation and 460 nm emission wavelengths.

Measurement of mitochondrial transmembrane potential. Cells were incubated with 25 nM tetramethylrhodamine ethyl ester (TMRE) (Molecular Probes, Carlsbad, CA, USA) at 37 °C for 30 min before harvesting. Cells were washed twice in phosphate-buffered saline (PBS), trypsinised, resuspended in PBS and analyzed by flow cytometry on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA) flow cytometer. Results were analyzed using CellQuest (Becton Dickinson) software.

Subcellular fractionation. Harvested cells were washed twice in PBS and resuspended for 10 min on ice in buffer A (10 mM Hepes, pH 7.9 at 4 °C, 1.5 mM $MgCl_2$, 10 mM KCl and 0.5 mM DTT). Cells were centrifuged at 2000 r.p.m. for 5 min at 4 °C. Cell pellet was resuspended in buffer A containing 0.2% NP40 and protease inhibitors for 2 min on ice. Cells were centrifuged at 500 r.p.m. for 2 min. The supernatant was collected and further centrifuged at 14 000 r.p.m. for 30 min. The supernatant and pellet contained the cytosolic and mitochondrial fractions, respectively.

Quantification of apoptotic nuclear morphology. Cells were grown on glass coverslips, treated, and fixed with 4% paraformaldehyde for 15 min at 4 °C. Fixed cells were then incubated in Hoechst (1 μg/ml) for 10 min at room temperature to stain nuclei. At least 200 cells were counted per sample.

Detection of F-actin. To visualize F-actin, cells grown on glass coverslips were fixed and incubated with rhodamine-conjugated phalloidin (1 : 8000) for 30 min at room temperature. Nuclei were stained by incubating coverslips in 1 μg/ml Hoechst (Molecular Probes) for 10 min at room temperature. Cells were visualized using a 510 Meta confocal microscope (Zeiss).

Measurement of cleaved caspase-3. Cells were fixed with 4% paraformaldehyde for 10 min at 37 °C and permeabilized with 90% methanol for 30 min at − 20 °C. Cells were incubated in blocking solution (0.5% BSA in PBS) for 10 min at room temperature, followed by anti-cleaved caspase-3 (Cell signaling, Danvers, MA, USA; 1 : 3200 in blocking solution) for 1 h at room temperature, and then with goat anti-rabbit Alexa 488 (Invitrogen; 1 : 200 in blocking solution) for 30 min. Cells were washed once in blocking solution between all antibody incubations, and were finally resuspended in PBS for analysis on a FACSCalibur (Becton Dickinson) flow cytometer. Results were analyzed using CellQuest software.

Statistical analysis. Statistical analyses were performed using two tailed, paired student's *t*-test where $*P<0.05$ and $**P<0.01$ were considered to be significant. Bars and error bars represent mean with S.E.M.

Conflict of Interest

The authors declare no conflict of interest.

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