p27^{Kip1}-stathmin interaction influences sarcoma cell migration and invasion

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Summary

Emerging evidences suggest that cyclin-dependent kinase inhibitors (CKIs) can regulate cellular functions other than cell cycle progression, such as differentiation and migration. Here, we report that cytoplasmic expression of p27^{kip1} affects microtubule (MT) stability following cell adhesion on extracellular matrix (ECM) constituents. This p27^{kip1} activity is due to its ability to bind and impair the function of the MT-destabilizing protein stathmin. Accordingly, upregulation of p27^{kip1} or downregulation of stathmin expression results in the inhibition of mesenchymal cell motility. Moreover, high stathmin and low cytoplasmic p27^{kip1} expression correlate with the metastatic phenotype of human sarcomas in vivo. This study provides a functional link between proliferation and invasion of tumor cells based on diverse activities of p27^{kip1} in different subcellular compartments.

Introduction

The tight crosstalk that exists between cells and surrounding extracellular matrix (ECM) components influences both cell growth and survival. ECM also provides background information and architectural scaffold for cellular adhesion and migration, eventually affecting the behavior of both normal and neoplastic cells (Bissell and Radisky, 2001). Signals from the ECM are transduced mainly from a family of glycoproteins called integrins (reviewed in Hood and Cheresh, 2002) that activate several intracytoplasmic pathways, resulting in the formation of focal adhesion contacts and cytoskeleton reorganization, finally leading to cell cycle progression (reviewed in Giancotti and Ruoslahti, 1999). It is well established that integrin stimulation is necessary for the correct proliferation of adhesion-dependent growing cells. The observation that adhesion to ECM directly influences the expression levels of cyclin D1 or of the CDK inhibitor (CKI) p27^{kip1} proteins (reviewed in Schwartz and Assoian, 2001) provides a molecular explanation for this phenomenon. More recently, a role for cell cycle-regulating proteins in cell adhesion or motility on ECM substrates has been proposed (Juliano, 2003). Similarly, data from different laboratories suggest that CKIs can exert additional physiological functions, not exclusively dependent on their ability to bind and inhibit the cyclin-CDK complexes (Ohnuma et al., 1999; Baldassarre et al., 1999a; Goukassian et al., 2001; Sun et al., 2001). It is now accepted that CKI nuclear localization is of primary importance for the control of cell cycle progression (reviewed in Blain et al., 2003), yet in both normal and tumor cells CKIs can shuttle from the nucleus to the cytoplasm, suggesting that the same protein exerts additional activities in different subcellular compartments. Among the cell cycle regulators an important role has been recently proposed for the CKI p27^{kip1} (hereafter p27) in the regulation of cell motility. p27 seems to act as an inhibitor of cell motility directed by chemotactic or aptotactic stimuli in smooth muscle (Sun et al., 2001), mesangial (Daniel et al., 2004), and endothelial cells (Goukassian et al., 2001), although the precise molecular mechanism is still unclear.

In transformed cells, the low expression levels of p27 protein are strictly linked to patients' survival in many types of examined

SIGNIFICANCE

This study explores the relationship between cell cycle regulation and cell adhesion and motility through or within the ECM, both of which represent important issues in several pathological alterations such as tumor invasion and metastatization. Here, we describe a pathway that links p27^{kip1} nucleocytoplasmic shuttling and the regulation of MT stability by stathmin, pointing to MT dynamics as an important determinant of cell motility and, more generally, of cancer cells' invasion and metastatization potential. Moreover, the finding that in mammalian cells a CKI contributes to regulate MT stability during interphase strictly connects the regulation of proliferation to the control of cell migration in both normal and tumor cells.

tumors (reviewed in Slingerland and Pagano, 2000). Moreover, in several types of cancer, such as colon (Thomas et al., 1998) and bladder carcinomas (Lacoste-Collin et al., 2002) or liposarcoma (Oliveira et al., 2000), the absence of p27 has been linked to invasion or metastasis. By contrast, the only functional study that is available reports an increased scatter motility in HepG2 hepatoma cells following transduction with a TAT-p27 fusion protein (McAllister et al., 2003).

Taking into account that the role of p27 in cell migration is still controversial and the mechanisms of action in normal as well as in cancer cells remain poorly elucidated, we investigated the influence of p27 on cell migration induced by cell-ECM contact. The present results demonstrate that p27 expression inhibits cell motility and that this activity depends on the ability to bind to and limit the function of the microtubule (MT)-destabilizing protein stathmin in the cell cytoplasm.

Results

p27 inhibits ECM-driven cell migration

The fibrosarcoma HT-1080 cells display a high rate of migration on ECM substrates in vitro through fibronectin (FN)- or vitronectin (VN)-coated membranes (Figure 1A and data not shown), thus representing a good in vitro model for motility studies. HT-1080 were stably transfected with an expression vector encoding for the human $p27^{wt}$ cDNA. Four cell clones expressing different amounts of p27 protein (Figure 1B) were tested in a standard assay to evaluate migration as a function of time (Spessotto et al., 2002). Overexpression of p27 reduced the migration through FN-coated membranes by more than 50% after 3 and 4 hr and almost completely during the first hour of incubation. The migration inhibition exerted by p27 correlated with p27 expression levels, suggesting that p27 acts in a dose-dependent manner (Figures 1A and 1B). Similarly, when VN was used as a substrate, 60% $\pm$ 7% of parental HT-1080 and only 35% $\pm$ 3% and 25% $\pm$ 5% of HT-1080 p27 clones B9 and D11 migrated within 3 hr, respectively (data not shown). These observations were confirmed using the green fluorescent fused proteins (EGFP) to track transiently transfected cells during migration. EGFP-$p27^{wt}$, but not EGFP alone, strongly inhibited migration of HT-1080 cells through either FN or VN (Figure 1C and data not shown). Similarly, both EGFP- or Flag-tagged $p27^{wt}$ proteins inhibited the motility of two other cell lines (Flow-6000 and SKUT-1; data not shown). In HT-1080 cells, exogenous p27 was expressed both in the cytoplasm and in the nucleus (Figure 1A), raising the question of which fraction was responsible for the inhibition of cell motility. Thus, EGFP-p27-transfected HT-1080 cells were treated for 3 hr with leptomycin B (LMB), an inhibitor of nuclear export that determines p27 nuclear accumulation (Ishida et al., 2002). LMB treatment strongly reduced cytoplasmic localization of p27 (data not shown) and severely impaired p27-mediated migration inhibition, whereas it did not affect the motility of EGFP-transfected cells (Figure 1C). In addition, an EGFP-p27 fusion protein that, carrying the nuclear localization signal (NLS) of the SV40 large antigen (EGFP-$p27^{NLS}$), remained mainly located in the nucleus (Figure 1D) almost completely failed to block cell motility (Figure 1E). We next examined p27 localization on both sides of Fluoroblok membranes through which HT-1080 cells transfected with EGFP-p27 vectors migrated for 3 hr. EGFP-$p27^{wt}$ was mainly cytoplasmic in nonmigrated cells (upper side of the membrane) and almost always nuclear in migrated cells (downside of the membrane), whereas EGFP-$p27^{NLS}$ was predominantly nuclear on both sides of the membrane (Supplemental Figures S1A and S1B at http://www.cancercell.org/cgi/content/full/7/1/51/DC1/). Overall, these data strongly suggest that cytoplasmic localization of p27 is a prerequisite for its inhibition of cell migration.

Immortalized mouse fibroblasts derived from wild-type (wt) or *p27* null embryos were serum starved and then allowed to migrate through FN-coated membranes for up to 6 hr. Under these experimental conditions, *p27* null cells migrated through FN at a 2-fold faster rate with respect to controls (Figure 1F). Similar results were obtained using wt or null *p27* primary MEFs (Supplemental Figure S1C at http://www.cancercell.org/cgi/content/full/7/1/51/DC1/). Moreover, reduction of p27 expression with p27 antisense oligodeoxynucleotide (AS ODN) in $p27^{+/+}$ fibroblasts (Figure 1F, inset) resulted in a 2-fold increase of cell migration through FN (Figure 1F). All these results strongly point to a physiological role of p27 in cell migration inhibition. Importantly, adhesion of serum-starved fibroblasts to FN induced a prompt nucleocytoplasmic shuttling, as revealed by Western blot and IF analysis (Supplemental Figures S1D and S1E). On the contrary, p27 was mainly nuclear when cells were either serum starved or in suspension (Supplemental Figures S1D and S1E), demonstrating that detachment per se did not alter p27 localization and further confirming the requirement of p27 cytoplasmic localization for cell motility inhibition.

The C-terminal portion of p27 is required for migration inhibition

Although the p27-dependent migration inhibition seemed to be associated to its cytoplasmic localization, a p27 mutant (EGFP-$p27^{1-150}$) that lacks its NLS and thus is mainly retained in the cytoplasm (Baldassarre et al., 1999b; Figure 2A) failed to inhibit cell migration (Figures 2B and 2C). The characterization of several p27 deletion mutants (Figure 2A) led to the identification of the last 28 amino acids ($p27^{1-170}$) as the necessary sequence for the migration inhibition activity (Figures 2B–2D). $p27^{wt}$ and $p27^{1-170}$ mutant displayed similar growth inhibition activity either in growing cells (Supplemental Figures S2A–S2C at http://www.cancercell.org/cgi/content/full/7/1/51/DC1/) or following adhesion on FN (Supplemental Figures S2D–S2E). Moreover, they displayed similar ability to bind cyclin A and cdk2 (Supplemental Figure S2F) or cyclin B1 and CDK1 (data not shown) and were similarly displaced in the cytoplasm following cell adhesion to FN (Supplemental Figure S2G). Stable transduction of *p27* null fibroblasts with $p27^{wt}$ or $p27^{1-170}$ retroviral vectors led to the expression of exogenous proteins at levels that were comparable to the expression of p27 observed in wt fibroblasts (Supplemental Figures S3A and S3B). The proliferation of the different cell lines was similar in the different conditions tested (Supplemental Figures S3C and S3D), whereas only $p27^{wt}$ protein expression was able to inhibit cell motility through FN (Supplemental Figure S3E). Altogether, these results indicated that p27 can inhibit cell migration when localized in the cytoplasm and when the cyclin-CDK binding sites and the last 28 amino acids are conserved. Interestingly, the p27-induced cell cycle arrest is not sufficient to explain its function on cell migration.

p27 expression interferes with cell motility but not with cell adhesion on ECM substrates

To test the possibility that inhibition of migration was the consequence of a defect in the number of cells adhered to the ECM,

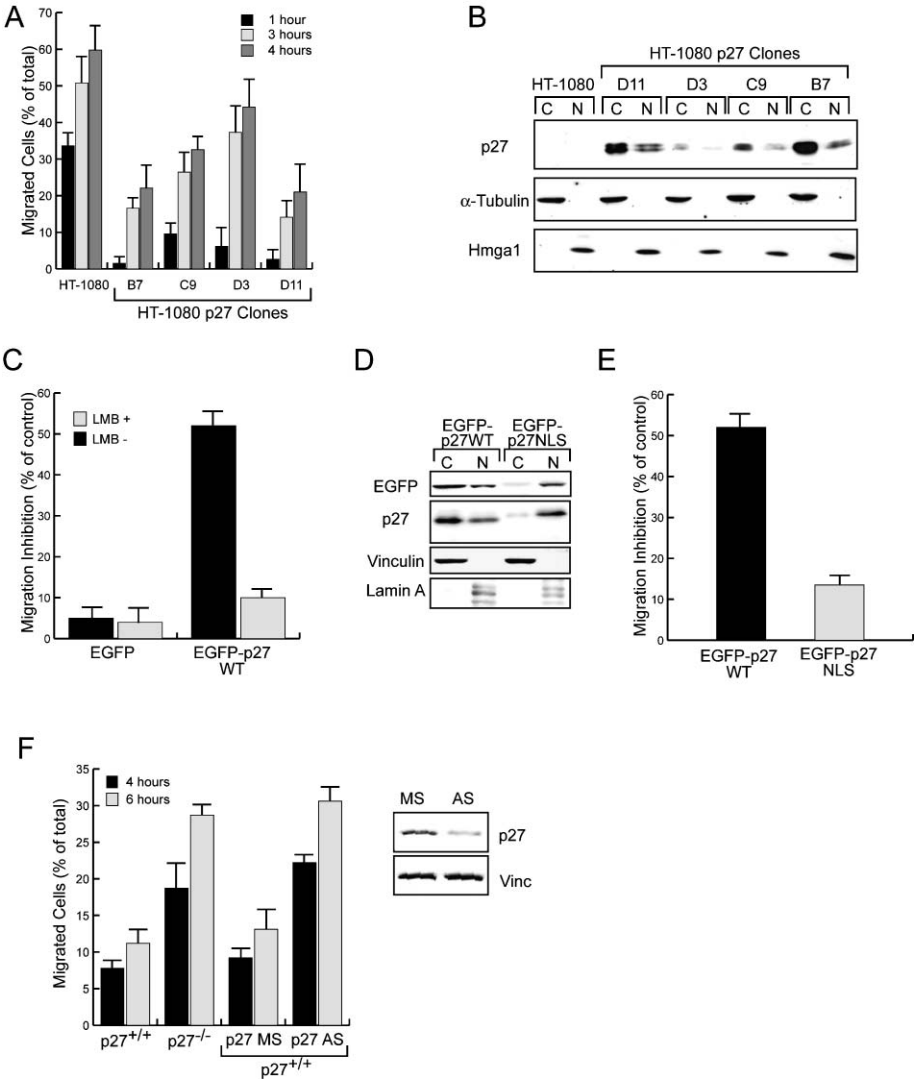

Figure 1. Cytoplasmic p27 inhibits cell migration

A and B: Percentage of migrated parental or p27-transfected HT-1080 cells through FN (**A**) and their p27 expression (**B**). α-tubulin and Hmga1 were used as control for the cytoplasmic (C) and nuclear (N) fractions, respectively.

C: Percentage inhibition of EFGP and EGFP-p27^{wt} in migrated cells through FN-coated membranes, treated with LMB or not.

D: Expression and localization of EGFP-p27^{wt} or EGFP-p27^{NLS} proteins evaluated using anti-EGFP or anti-p27 abs. Vinculin (cytoplasm) and Lamin A (nucleus) were used as controls.

E: Percentage inhibition of EGFP-p27^{wt} or EGFP-p27^{NLS} migrated cells through FN.

F: Percentage of *p27* wt, *p27* null, or *p27* wt fibroblasts treated with p27 missense (MS) or antisense (AS) ODN migrated through FN. (Inset) Expression of p27 and vinculin (loading control) in MS- or AS ODN-treated cells. The data (±SD) represent the mean of at least three independent experiments.

we compared the adhesion levels of HT-1080 cells, of two p27-derived cell clones, and of *p27* wt and null cells. Expression of p27 had no effect on the adhesion of HT-1080 cells (Figure 2E) or of mouse fibroblasts (Figure 2F) on several ECM substrates. Moreover, transient transfection experiments demonstrated that EGFP-p27^{wt} and EGFP-p27$^{1\text{-}170}$ did not influence adhesion of HT-1080 cells assayed over the time (from 5 to 90 min of adhesion) (Figure 2G), or by FN titration from 0.5 to 10 μg/ml (Figure 2H), thus demonstrating that adhesion on ECM substrates was not quantitatively compromised by p27 expression.

p27 interacts with the MT-destabilizing protein stathmin

Based on the above results, we speculated that the C terminus of p27 could be the binding site for proteins involved in the regulation of cell motility. Hence, using a p27 fragment containing the last 100 amino acids (aa 98–198) as bait in a yeast two-hybrid screen, we identified the MT-destabilizing protein stathmin (Cassimeris, 2002) as a p27 binding partner (data not shown). To assess the importance of this interaction in vivo, stathmin and p27 were cotransfected in HT-1080 cells. Coimmunoprecipitation (co-IP) analysis showed that p27^{wt} interacted in vivo with stathmin and that this interaction was severely impaired by the deletion of the last 27 amino acids of p27 (Figure 3A). Stathmin is a ubiquitous protein expressed at highest levels in brain, which peaks in the neonatal period and decreases in adult life (Koppel et al., 1990). Consequently, stathmin was readily co-IP with p27 from mouse fetal brain (day 18 p.c.) (Figure 3B). Stathmin and p27 associated in adult pork brain, in mouse fibroblasts adherent to FN but not in serum-starved cells (Supplemental Figures S4A and S4B at http://www.cancercell.org/cgi/content/full/7/1/51/DC1/), suggesting a physiological role for p27-stathmin interaction.

p27 counteracts the tubulin sequestering activity of stathmin in vitro

Since stathmin is a MT-destabilizing protein (Cassimeris, 2002), we tested the effects of p27-stathmin interaction in an in vitro tubulin polymerization assay. Purified bovine brain tubulin was incubated for 15 min in MSB buffer at 37°C to allow MT formation (Figure 3C, first lane). The presence of 2.2 μM stathmin decreased the polymerization rate of tubulin to 40%. This effect

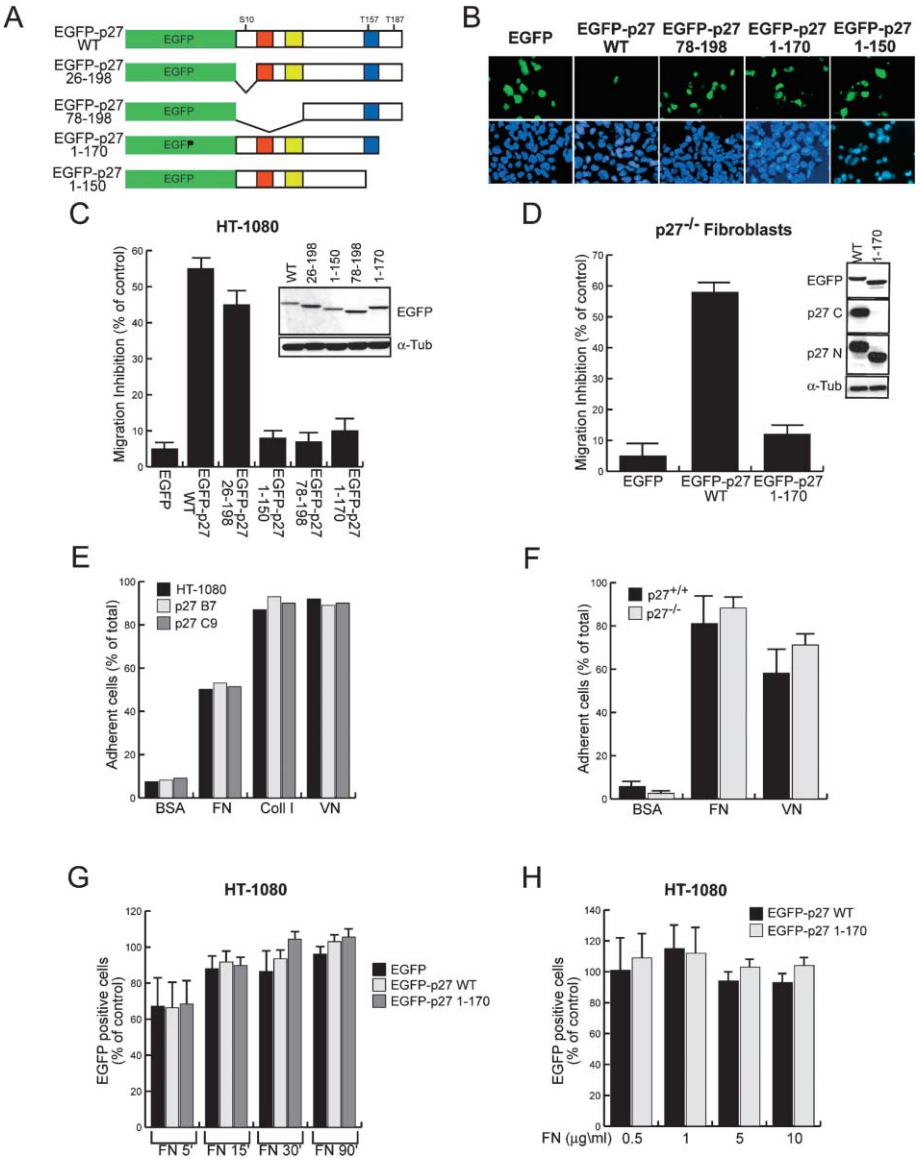

Figure 2. Inhibition of cell migration requires the C terminus of p27 protein

A: Schematic representation of p27 deletion mutants used. The binding domains for Cyclins and CDKs and the NLS are shown in red, yellow, and blue, respectively.

B: (Upper panels) Migrated EGFP-transfected cells; (bottom panels) Hoechst staining identifying all the migrated cells.

C and D: Percentage inhibition of HT-1080 (**C**) or *p27* null fibroblasts (**D**) transfected with EGFP-derived vectors and allowed to migrate on FN-coated Fluoroblok. (Insets) Expression of EGFP-p27-fused proteins. In **D**, the p27-C ab raised against p27 C terminus recognizes only $p27^{wt}$.

E and F: Percentage of HT-1080 and HT-1080 p27 B7 and C9 (**E**) or $p27^{+/+}$ and $p27^{-/-}$ fibroblasts (**F**) adherent to BSA (negative control), FN, VN, and Collagen I.

G and H: Percentage of EGFP-positive HT-1080 cells adherent to 10 μg/ml of FN for the indicated times (**G**), or to different concentration of FN for 30 min (**H**). Bars are SD.

was counteracted by $p27^{wt}$ in a dose-dependent manner (Figure 3C and Supplemental Figures S4D and S4E at http://www.cancercell.org/cgi/content/full/7/1/51/DC1/), whereas the $p27^{1-170}$ mutant had only little effect (Figure 3C). Similarly, in a time-dependent assay 2.2 μM stathmin severely impaired the polymerization of tubulin (Figure 3D). $p27^{wt}$ alone did not significantly interfere with this process, but when added together with stathmin it completely restored the MT formation rate (Figure 3D). As expected, $p27^{1-170}$ had no effect on tubulin polymerization—neither when incubated alone (data not shown) nor in the presence of stathmin (Figure 3D). Altogether, these results clearly indicate that p27 is able to interfere with stathmin activity in vitro and that this effect depends on the last 28 amino acids of the protein.

It has been reported that stathmin displays two different MT-destabilizing activities that can be discerned using deletion mutants. The N-terminal portion retains the catastrophe-promoting activity and stimulates the transition from MT elongation to shortening, whereas a mutant maintaining the central and C-terminal region is able to sequester the free tubulin heterodimers, thus decreasing the MT-polymerization rate (Howell et al., 1999). To evaluate whether p27 interfered with the catastrophe-promoting activity of stathmin or with its tubulin-sequestering ability, we generated two deletion mutants, one containing the N-terminal portion ($Stathmin^{1-99}$) and the other starting at amino acid 25 ($Stathmin^{25-149}$). An in vitro binding assay using recombinant proteins revealed that $p27^{wt}$ (but not $p27^{1-170}$) was able to pull down the $stathmin^{25-149}$ deletion mutant but not the $stathmin^{1-99}$ protein (Supplemental Figure S4C at http://www.cancercell.org/cgi/content/full/7/1/51/DC1/). Moreover, $p27^{wt}$ counteracted the effects of $stathmin^{25-149}$ on tubulin polymerization in a dose-dependent manner in an in vitro assay, whereas it was ineffective on the activity of $stathmin^{1-99}$ (Supplemental Figures S4D and S4E), suggesting that p27 affected the stathmin tubulin sequestering activity. To test this hypothesis, HT-1080 cells transfected with flag-stathmin alone or with increasing concentration of p27

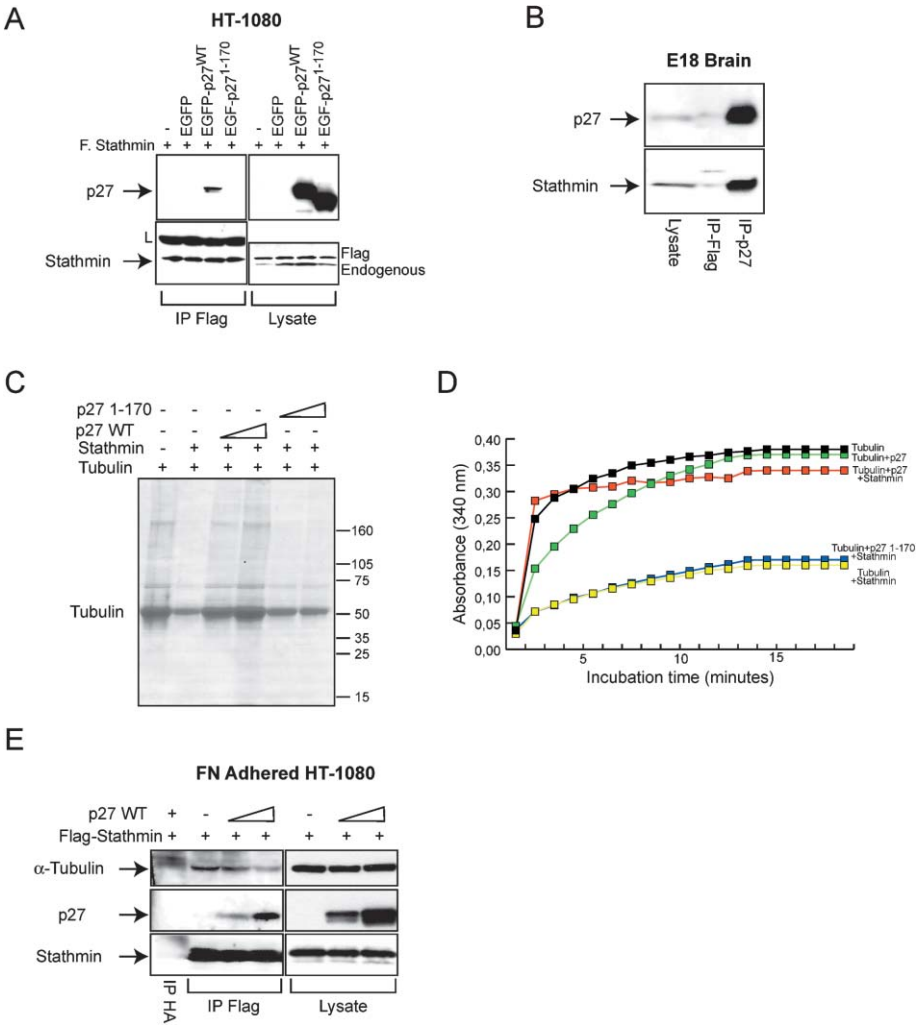

Figure 3. p27 binds and inhibits the MT-destabilizing protein stathmin

A: Flag IP proteins (left panels) and whole-cell lysate (right panels) of HT-1080 cells, transfected with the indicated vectors and analyzed for p27 and stathmin expression.

B: p27 IP of mouse embryo brain lysate probed with anti-stathmin and anti-p27 abs.

C: In vitro tubulin polymerization assay (30 min at 37°C) in the presence of the indicated recombinant proteins. Polymerized MTs were separated on SDS-PAGE gel and stained with Coomassie blue.

D: In vitro tubulin polymerization assay performed as a function of time.

E: Flag IP proteins (left panels) and whole-cell lysate (right panels) of HT-1080 cells transfected with the indicated vectors, adherent to FN for 60 min, and probed for α-tubulin, p27, and flag expression.

were allowed to adhere to FN for 1 hr. Cells were then lysed, and whole-cell lysates were used in co-IP experiments. p27 expression strongly reduced in a dose-dependent manner the interaction of α-tubulin with flag-stathmin as visualized by Western blot (Figure 3E) supporting the possibility that p27 interferes with the stathmin tubulin-sequestering activity also in vivo.

p27 contributes to FN-dependent MT stabilization

In mouse fibroblasts adherent to FN for 30 min, p27 and stathmin colocalized in the perinuclear region of the cells, as assessed by confocal microscopy (Figure 4A). This finding suggested that p27 could affect MT stability by locally counteracting stathmin activity. To test this hypothesis, we took advantage of antibodies able to specifically recognize modified tubulins that represent widely accepted markers of MT stability (Schulze et al., 1987; Palazzo et al., 2004). In most cells, in fact, MTs can be subdivided into dynamic MTs, which have a half-life of 5–10 min and contain unmodified tyrosinated tubulin, and stable MTs which persist for hours and can be identified by the content of acetylated or glutamylated (detyrosinated) tubulin (Schulze et al., 1987; Palazzo et al., 2004). In HT-1080 cells transfected with EGFP-p27^{wt} or EGFP-p27$^{1-170}$ vectors and allowed to adhere to FN for 1 hr, acetylated MTs increased in the perinuclear region of cells expressing p27^{wt} in the cytoplasm (80% ± 11% of these cells showed increasing levels of acetylated tubulins) but not in the nucleus (15% ± 8%), whereas they were almost unaffected by transfection with p27$^{1-170}$ irrespective of its localization (26% ± 10%) (Figure 4B). These data were confirmed by the nocodazole resistance assay (Khawaja et al., 1988) in which HT-1080 transfected with the EGFP-derived vectors were treated with 2 μM nocodazole for 30 min, thus specifically depolymerizing dynamic MTs (Khawaja et al., 1988). Expression of p27^{wt} in the cytoplasm increased the number of cells containing acetylated MTs, whereas the p27$^{1-170}$ mutant slightly affected the levels of acetylated MTs (Figures 4C and 4E). Moreover, the tubulin dilution assay, which distinguishes between dynamic and stable MTs (Khawaja et al., 1988), demonstrated that HT-1080 cells contained only few stable MTs, with respect to p27-overexpressing cells, in which the MT network was readily observable, as revealed by α-tubulin staining (Figures 4D and 4F).

Similarly, wt fibroblasts contain more stable MTs than *p27* null cells as evaluated by several experimental procedures. Cell

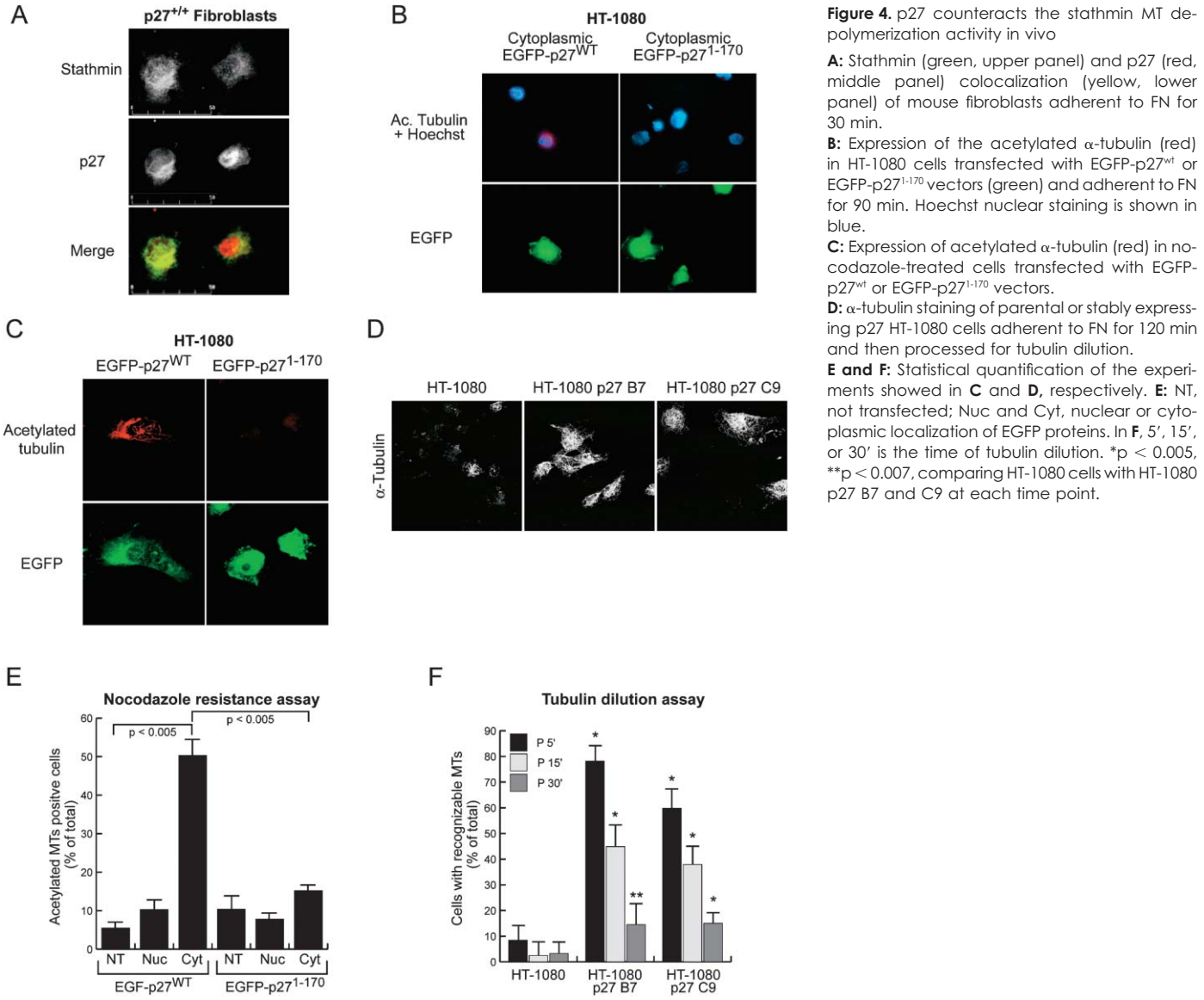

Figure 4. p27 counteracts the stathmin MT depolymerization activity in vivo

A: Stathmin (green, upper panel) and p27 (red, middle panel) colocalization (yellow, lower panel) of mouse fibroblasts adherent to FN for 30 min.

B: Expression of the acetylated α-tubulin (red) in HT-1080 cells transfected with EGFP-$p27^{wt}$ or EGFP-$p27^{1-170}$ vectors (green) and adherent to FN for 90 min. Hoechst nuclear staining is shown in blue.

C: Expression of acetylated α-tubulin (red) in nocodazole-treated cells transfected with EGFP-$p27^{wt}$ or EGFP-$p27^{1-170}$ vectors.

D: α-tubulin staining of parental or stably expressing p27 HT-1080 cells adherent to FN for 120 min and then processed for tubulin dilution.

E and F: Statistical quantification of the experiments showed in **C** and **D**, respectively. **E:** NT, not transfected; Nuc and Cyt, nuclear or cytoplasmic localization of EGFP proteins. In **F**, 5′, 15′, or 30′ is the time of tubulin dilution. *$p < 0.005$, **$p < 0.007$, comparing HT-1080 cells with HT-1080 p27 B7 and C9 at each time point.

lysate fractionation, which allows the separation of soluble from cytoskeletal tubulin, demonstrated that in $p27^{+/+}$ cells the amount of polymerized tubulin and in particular of its glutamylated form in FN-adherent cells was double that observed in *p27* null fibroblasts (Figure 5A, first and second panels). The tubulin dilution assay performed on cells adherent to FN for 2 hr confirmed that *p27* wt fibroblasts contained more stable MTs with respect to *p27* null cells, as revealed by IF (Figure 5B). Taken together, these data strongly suggest that p27 contributes to MT stability, probably by interfering with the destabilizing activity of stathmin.

Since cell adhesion to FN contributes to the formation of stable MTs in mouse fibroblasts (Palazzo et al., 2004), we investigated the adhesion-dependent MT modifications in a *p27* wt or null background. $p27^{+/+}$ and $p27^{-/-}$ cells were serum starved for 24 hr, then allowed to adhere to FN for 120 min and analyzed for the content of glutamylated, acetylated, or tyrosinated MTs. In *p27* wt fibroblasts, the glutamylated and acetylated modified MT network was prominent and extended from the perinuclear region to the periphery of the cell, with local MT accumulation in the perinuclear region (Figure 5C, first and second columns). By contrast, *p27* null cells showed a marked reduction of fluorescence intensity for both glutamylated and acetylated MTs and displayed perinuclear accumulation of modified MTs in less than 20% (19% ± 6%) of the cells (Figure 5C, first and second column). No significant difference was observed for the more dynamic MTs, identified using an anti-tyrosinated tubulin ab (Figure 5C, third column). Reintroduction of $p27^{wt}$ in $p27^{-/-}$ cells in the cytoplasm resulted in increased levels and perinuclear accumulation of modified MTs in 82% ± 10% of transfected cells. When $p27^{wt}$ was exclusively nuclear, it had no effect on the MT array, and the expression of cytoplasmatic $p27^{1-170}$ did not significantly alter MT organization (34% ± 15% of positive cells showed increased expression and perinuclear accumulation of modified MTs) as evaluated by IF using anti-acetylated (Figure 5D) and anti-glutamylated tubulin abs (data not shown).

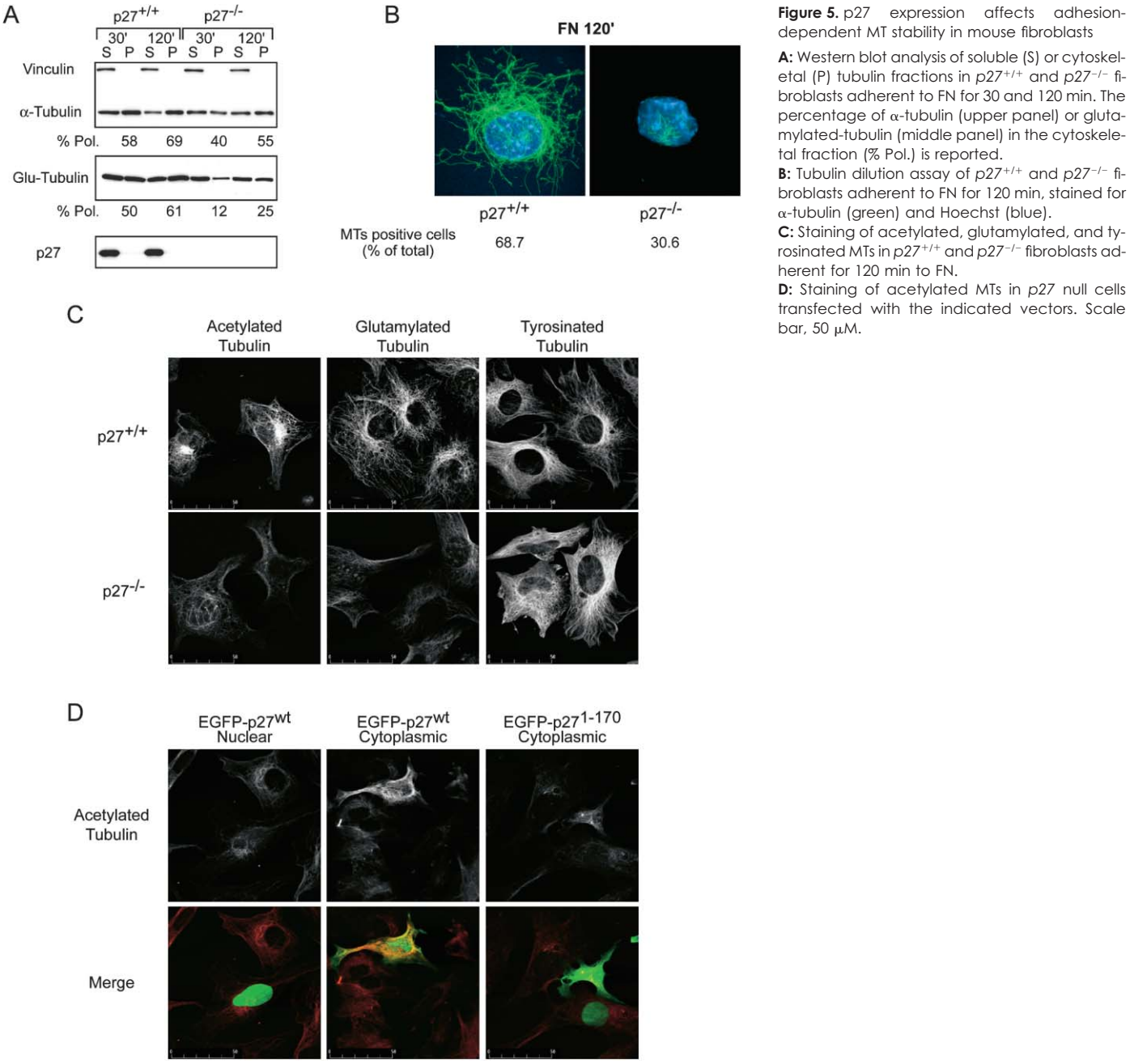

Figure 5. p27 expression affects adhesion-dependent MT stability in mouse fibroblasts

A: Western blot analysis of soluble (S) or cytoskeletal (P) tubulin fractions in $p27^{+/+}$ and $p27^{-/-}$ fibroblasts adherent to FN for 30 and 120 min. The percentage of α-tubulin (upper panel) or glutamylated-tubulin (middle panel) in the cytoskeletal fraction (% Pol.) is reported.

B: Tubulin dilution assay of $p27^{+/+}$ and $p27^{-/-}$ fibroblasts adherent to FN for 120 min, stained for α-tubulin (green) and Hoechst (blue).

C: Staining of acetylated, glutamylated, and tyrosinated MTs in $p27^{+/+}$ and $p27^{-/-}$ fibroblasts adherent for 120 min to FN.

D: Staining of acetylated MTs in *p27* null cells transfected with the indicated vectors. Scale bar, 50 μM.

Stathmin counteracts the p27-imposed migration inhibition

MT dynamics has been proposed as one of the mechanisms involved in the regulation of cell migration acting either on lamellipodia formation (Rodriguez et al., 2003) or on the control of contraction and focal adhesion formation (Small et al., 2002). To verify whether the effects of p27-stathmin interaction on MT stability were accompanied by changes in cell motility, EGFP, EGFP-$p27^{wt}$, or EGFP-$p27^{1-170}$ vectors were transfected in HT-1080 cells in the absence or in the presence of a 3-fold higher concentration of Flag-tagged stathmin vector. Only EGFP-$p27^{wt}$ was able to inhibit cell migration toward FN, but this effect was almost completely counteracted by coexpression of stathmin (Figure 6A). Transfection with stathmin alone slightly enhanced HT-1080 migration (Figure 6A), whereas EGFP or EGFP-$p27^{1-170}$ expression did not significantly interfere with cell migration either in the presence or in the absence of stathmin (Figure 6A). Stathmin overexpression stimulated migration of $p27^{+/+}$ fibroblasts, whereas it was ineffective in a *p27* null background (Figure 6B), suggesting the existence of a tight relationship between p27, stathmin, and the regulation of cell motility. The stathmin$^{25-149}$ construct displayed similar activity to the wt protein in stimulating cell migration (data not shown), thus implying that tubulin sequestration is sufficient to stimulate cell migration and that the effects of stathmin on both tubulin sequestering and ECM-induced cell motility are efficiently counteracted by

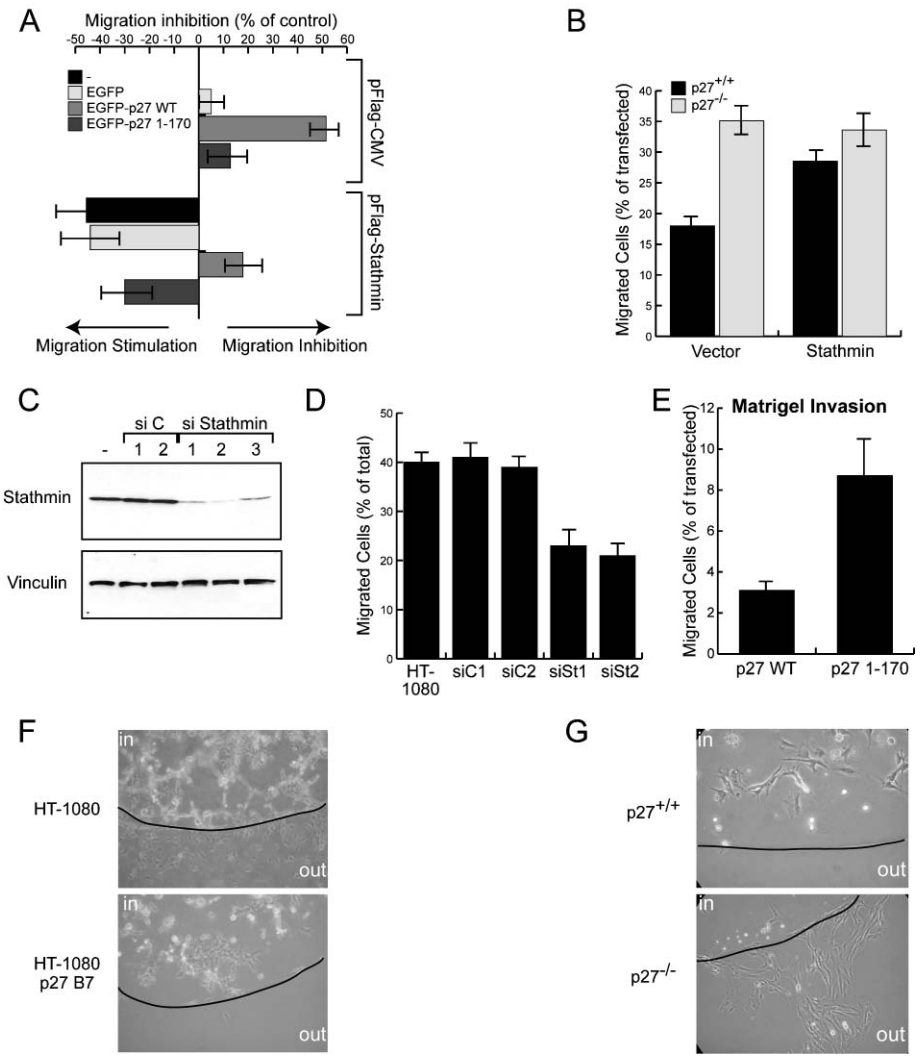

Figure 6. Stathmin counteracts the p27 migration inhibition activity

A: Migration inhibition through FN exerted by EGFP fusion proteins transfected alone or with Flag-stathmin in HT-1080 cells.

B: Effect of stathmin expression on the migration of *p27* wt and null fibroblasts.

C: Expression of stathmin and vinculin in parental (−), control (siC 1 and 2), and stathmin (siStathmin 1, 2, and 3) siRNA-transfected HT-1080 cells.

D: Migration assay through FN of HT-1080 cells treated as in **C**.

E: Invasion assay of HT-1080 cells transfected with EGFP-$p27^{wt}$ or EGFP-$p27^{1-170}$ in Matrigel at 48 hr.

F and G: Matrigel evasion assay of parental or HT-1080 p27 B7 cells cultured for 3 days (**F**) and of wt and *p27* null fibroblasts cultured for 5 days (**G**) in complete medium. Each experiment has been performed at least two times in quintuplicate.

p27. The reduction of endogenous stathmin expression by a siRNA targeting stathmin mRNA (Figure 6C) resulted in a 50% decrease of HT-1080 migration through FN (Figure 6D). Similar results were obtained with *p27* null fibroblasts and SK-LMS1 cells treated with AS ODN against stathmin (data not shown), demonstrating that the stathmin effects on cell migration through FN are not restricted to a single cell type.

p27 inhibits ECM-driven cell migration: A three-dimensional view

The motile behavior of several cell types may vary when cultured in a three-dimensional matrix compared to the two dimensions (Sahai and Marshall, 2003), and it is likely that the 3D context better represents what happens in living organisms. To verify the role of p27 in 3D migration, HT-1080 cells were transiently transfected with $p27^{wt}$ or $p27^{1-170}$ mutant and then allowed to migrate in Matrigel-coated Fluorobloks for 2 days. Transfection of HT-1080 cells with $p27^{wt}$ reduced to less than 50% their invasion with respect to the $p27^{1-170}$ mutant (Figure 6E). Since $p27^{wt}$ and $p27^{1-170}$ deletion mutants displayed similar effects on cell cycle progression (Figure 2 and Supplemental Figures S2 and S3 at http://www.cancercell.org/cgi/content/full/7/1/51/DC1/), the difference in cell motility in 3D could not be ascribed to different proliferation rates. In another 3D assay, HT-1080 cells were included in Matrigel and allowed to move and exit from the matrix. In this Matrigel evasion assay, parental cells were attracted outside Matrigel drops by serum and were consistently outside after 72 hr (Figure 6F, upper panel). In the same time frame, p27-stably expressing cells remained entrapped in the matrix (Figure 6F, lower panel). No significant difference was noticed between the various cell lines in terms of MMP production (data not shown), suggesting that p27 expression affected cell ability to move out of the Matrigel rather than to degrade the ECM. Similarly, normal fibroblasts cultured in Matrigel drops for 5 days in the presence of serum failed to exit from the matrix, (Figure 6G, upper panel), whereas *p27* null fibroblasts egressed quite effectively (Figure 6G, lower panel), indicating that the absence of p27 conferred a motility advantage also in a three-dimensional context.

Stathmin expression is necessary for p27-induced migration inhibition

To further confirm the importance of stathmin-p27 interaction in the regulation of cell motility, we generated primary MEFs

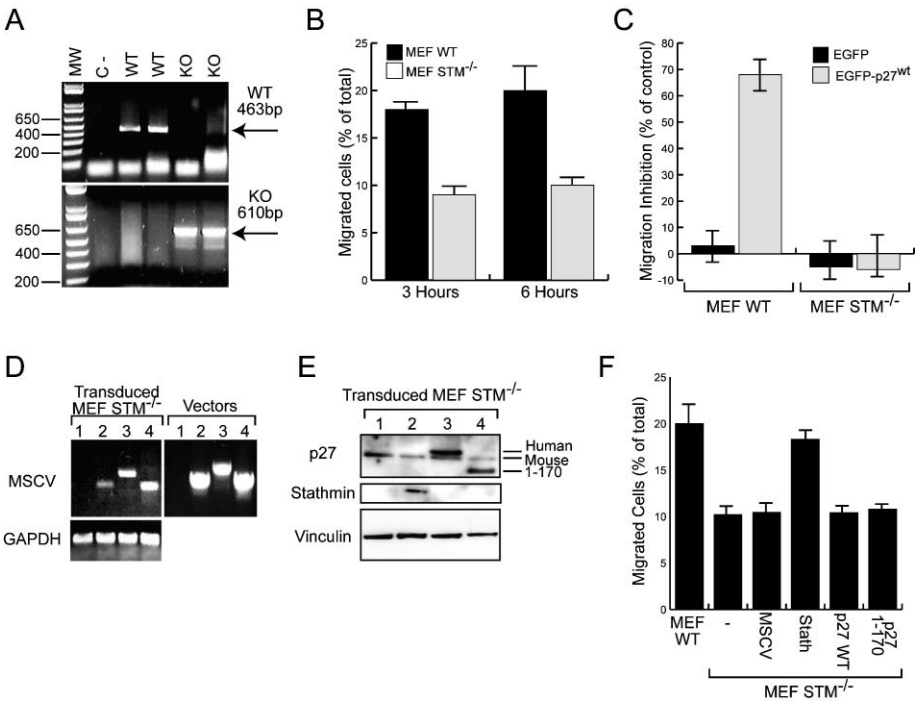

Figure 7. p27 fails to inhibit cell migration of *stathmin* null MEFs

A: Genotype of *stathmin* wt and null MEFs.

B: Percentage of MEFs migrated through FN-coated Fluorobloks.

C: Percentage of inhibition by EGFP-p27^{wt} of *stathmin* wt and null MEFs migrated through FN for 6 hr.

D and E: RT-PCR (**D**) and Western blot (**E**) analysis of transduced vectors in *stathmin* null cells. Lane 1, pMSCV; Lane 2, pMSCV-Stathmin; Lane 3, pMSCV-p27^{wt}; Lane 4, pMSCV-p27$^{1-170}$.

F: Migration assay through FN of *stathmin* null MEFs treated as in **E**. The data (± SD) represent the mean of two independent experiments performed in duplicate.

from *stathmin* knockout mice (Schubart et al., 1996). The correct genotype of *stathmin* wt and null MEFs was established by PCR (Figure 7A), and cells were tested in a transwell migration assay through FN. *Stathmin* null cells migrated more slowly than wt both at 3 and at 6 hr (Figure 7B), and EGFP-p27^{wt} was ineffective in the inhibition of their motility, whereas it inhibited wt MEF (Figure 7C). These data were further confirmed using retroviral transduction. *Stathmin* null MEF, transduced with stathmin, p27^{wt}, or p27$^{1-170}$, expressed the expected mRNAs (Figure 7D) and proteins (Figure 7E), but both p27^{wt} and p27$^{1-170}$ were ineffective in inhibiting their migration (Figure 7F). Importantly, the motile behavior of *stathmin* null MEFs was rescued by stathmin expression (Figure 7F), demonstrating that this defect was due to the loss of stathmin functions.

The p27/stathmin expression ratio correlates with sarcoma cell migration and tumor metastasis formation

Emerging evidences indicate that in cells of mesenchymal origin like fibroblasts or fibrosarcomas (this work), smooth muscle (Sun et al., 2001), endothelial (Goukassian et al., 2001), and mesangial cells (Daniel et al., 2004) p27 acts as inhibitor of cell migration and/or invasion. Thus, we extended our analysis to three other leiomyosarcoma (SKUT-1, SKLMS-1, and MES-SA)- and one fibrosarcoma (HS-913T)-derived cell lines. The expression levels of stathmin and p27 were evaluated by Western blot (Figure 8A), and the ratio between p27 and stathmin correlated with the ability of tumor cells to migrate through FN-coated Fluorobloks (Figure 8B). SKUT-1 cells that expressed the lowest p27/stathmin ratio migrated at the fastest rate, whereas MES-SA, which displayed the highest ratio, migrated more slowly than the other cell lines. Moreover, SKUT-1 cells stably transfected with p27 showed more than 50% decrease in cell migration rate through FN (Figure 8C), whereas decreasing p27 levels by AS ODN treatment in SKLMS-1 cells increased their motility by about 5-fold (Figure 8D), pointing to the p27/stathmin ratio as an important determinant in sarcoma migration.

We next analyzed the expression of p27 and stathmin in the cytoplasm and in the nucleus of ten primary and ten metastatic tumors diagnosed as fibrosarcoma or leiomyosarcoma. In primary tumors, p27 expression was low or absent in three out of ten tumors and high or moderate in seven out of ten. Tumors with high p27 demonstrated an expression level similar to the surrounding normal tissue (Supplemental Figure S5A at http://www.cancercell.org/cgi/content/full/7/1/51/DC1/). Importantly, in these tumors p27 expression was mainly localized in the cytoplasm (Figure 8E and Supplemental Figure S5B). In metastatic diseases, p27 was expressed at moderate/high levels only in four out of ten cases, and in two of them its expression was prevalently nuclear (Figure 8E and Supplemental Figure S5B). Stathmin expression was present at moderate/high levels in four primary tumors and at low levels in six cases, whereas it was high in eight and low in two cases of metastatic diseases (Figure 8E). When the cytoplasmic p27/stathmin ratio was calculated it became clear that primary sarcomas showed a high ratio ($\geq$1) in nine out of ten cases, whereas metastatic diseases always displayed a ratio $<$1 (Figure 8F), supporting the possibility that changes in the levels of cytoplasmic p27 and stathmin contribute to metastases development in human sarcomas.

Discussion

ECM-dependent interaction between stathmin and p27

Here, we provide experimental evidences unveiling a previously unknown function of p27 and stathmin in the regulation of cell motility and supporting the idea that MT dynamics plays a crucial role in the control of cell motility through or within the ECM. Our data demonstrate that cell-ECM contact, inducing a prompt

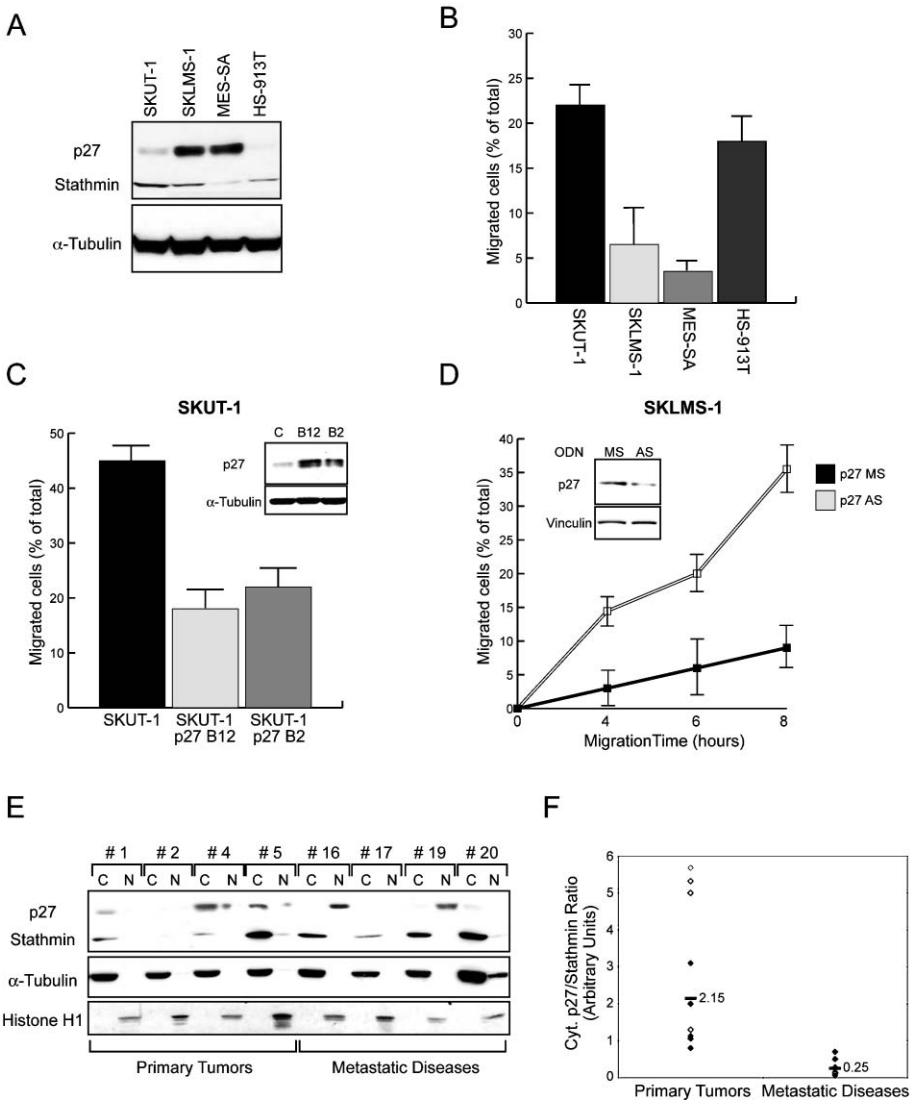

Figure 8. The p27/stathmin ratio correlates with cell migration capacity and tumor metastasis formation in human sarcomas

A: Stathmin and p27 proteins expression in four sarcoma-derived cell lines. α-tubulin was used as loading control.

B: Percentage of sarcoma cells migrated for 6 hr through FN.

C: Percentage of parental SKUT-1 and two p27-expressing clones migrated for 18 hr through FN. (Inset) p27 and α-tubulin expression.

D: Percentage of migrated SKLMS-1 cells treated for 48 hr with ODN missense (MS) or antisense (AS) against p27 for 8 hr through FN. (Inset) p27 and vinculin expression.

E: Expression of p27 and stathmin in nuclear (N) and cytoplasmic (C) extracts derived from primary or metastatic sarcomas. α-tubulin and histone H1 were used as control for the cytoplasmic and nuclear fractions, respectively.

F: Cytoplasmic p27/stathmin ratio in ten primary and ten metastatic diseases. The median value is shown. p = 0.001 (Student's t test). Error bars are SD.

p27 nucleocytoplasmic shuttling, favors p27-stathmin interaction. This interaction, in turn, influences cell motility, thus determining a tight link between regulation of cell proliferation and cell movement. Interestingly, a recent report (Boehm et al., 2002) describes the interaction between p27 and Kinase Interacting with Stathmin (KIS; Maucuer et al., 1997), supporting the existence of a macromolecular complex governing p27-stathmin functions. KIS activation, due to growth factor stimulation, induces p27 phosphorylation on serine 10 and contributes to its shuttling from the nucleus to the cytoplasm (Boehm et al., 2002), where p27 loses its growth inhibitory activity but can still interact with stathmin. Whether p27 cytoplasmic delocalization due to cell adhesion to ECM is dependent, at least in part, on KIS activity is still matter of speculation. The analysis of p27 deletion mutants revealed that the cyclin-cdk binding domain as well as the last 28 amino acids of p27 are necessary for its motility inhibition activity. It is of note that the C-terminal portion of p27 is highly conserved from *Xenopus* to humans, suggesting that the mechanism that we describe here could be retained along the evolution. Moreover, stathmin is a substrate of CDK1 and CDK2, and its phosphorylation seems to affect cell motility (Niethammer et al., 2004). It is also possible, and this is a matter for future studies, that p27 binding to CDK1 and 2 could contribute to the modulation of stathmin phosphorylation, eventually leading to an altered cell motility.

Role of stathmin in cell motility

Our data suggest that modulation of MT stability in cells in contact with and moving through or within ECM substrates by p27/stathmin interaction could represent an important pathway linking proliferation and motility. In line with these observations, we propose stathmin as a pivotal regulator of cell migration. This is based on several observations: first, *stathmin* null MEFs migrated more slowly than the wt cells through FN, and this phenotype was reversed by stathmin reintroduction. Second, stathmin downregulation in sarcoma-derived cell lines determined a strong migration inhibition, whereas its overexpression increased FN-directed motility. These observations could be of particular interest for cancer cells where stathmin is often upregulated and in which its role has not yet been fully eluci-

dated (for a review, see Curmi et al., 1999). Third, stathmin is also expressed at high levels in brain and testis, and it is interesting to note that in *Drosophila* (in which, differently from mammals, only one stathmin-related gene is present) stathmin downregulation is accompanied by defective migration of germ cells and altered neurite elongation (Ozon et al., 2002). Finally, inhibition of stathmin expression impaired neurons' migration in rats (Jin et al., 2004), demonstrating that this protein plays a pivotal role in cell motility also in vivo.

p27-stathmin interaction influences the MT stability

We demonstrated that stathmin MT depolymerization activity is well counteracted in vitro and in vivo by p27. Our data support the possibility that p27 interferes with the ability of stathmin to sequester the free tubulin heterodimers, and this function seems to affect interphase MTs rather than the mitotic spindle catastrophe (Holmfeldt et al., 2001). Although we cannot exclude the possibility that other proteins interact with p27 to modulate its activity on cell motility, the finding that p27 does not inhibit migration of stathmin null cells suggests that stathmin is the principal p27 target for this activity. The effects of p27/stathmin interaction on FN- or VN-driven cell motility seem to be directly linked to the modulation of FN-dependent MT stability, since p27 expression restores the content of stable MTs in *p27* null fibroblasts or in HT-1080 cells. Thus, our data provide evidence for the existence of an intracellular pathway inducing MT stability following adhesion to ECM substrates, an observation recently suggested by Gundersen and colleagues (Palazzo et al., 2004).

The different distribution of stable modified MTs in *p27* wt versus null fibroblasts suggests that p27/stathmin perinuclear interaction (Figure 4A) contributes to locally determine the stability of the MT cytoskeleton. In accord with this hypothesis, it has been demonstrated that migrating cells display a fine regional regulation of MT dynamics (Wadsworth, 1999) that, at least in part, could be assured by the regulation of stathmin functions.

The contrasted role of p27 in cell motility

p27 has been alternatively reported as inhibitor (Sun et al., 2001; Goukassian et al., 2001; Daniel et al., 2004) or stimulator (McAllister et al., 2003) of cell migration in primary or stabilized cells of different origins, leaving the role of p27 in cell motility still open (Assoian, 2004). We propose that the present study could reconcile the apparent controversial role depicted for p27, based on its activity on MT dynamics. The major difference between the reports suggesting an inhibitory role for p27 in cell motility and the works that support a promigratory role of p27 is, in our opinion, the type of cell motility assayed. Cells migrating through the pores of FN-coated transwell or in 3D and in vivo can use amoeboid movements that respond to attractant stimuli lacking an obvious polarization (Webb and Horwitz, 2003), assume a roundish/elliptoid morphology, and are largely dependent on propulsive forces and on cytoplasmic streaming for their motility (Friedl and Wolf, 2003). In this case, p27 could act as an inhibitor of cell migration by altering MT stability, thus impairing the generation of propulsive forces and/or the cytoskeletal modifications necessary for the cell to move. Accordingly, *p27* null smooth muscle cells' migration in transwell is fully inhibited by the MT-stabilizing drug taxol (Sun et al., 2001), which acts downstream of p27-stathmin functions on MT dynamics. Conversely, MT stability has been proposed as one of the mechanisms contributing to a proper directional motility in in vitro assays, like wound healing. Altered MT dynamics impairs the direction of cell motility in this assay, resulting either in reduced cell migration (Liao et al., 1995) or in altered trajectories in the wound (Kodama et al., 2003). Thus, it is possible that p27, contributing to stabilize the perinuclear network of MTs, enforces cell polarity and favors the movements of highly polarized cells. Our preliminary results indicated that this is the case in *p27* null fibroblasts during wound healing assay, since they migrated toward the wounded area with altered cell trajectories and failed to reorient glutamylated MTs to the scratched area (unpublished data). Interestingly, while this manuscript was in preparation, Roberts and colleagues reported that p27 expression stimulates wound healing cell motility by decreasing the RhoA-Rock1 activity (Besson et al., 2004), a pathway known to be necessary for amoeboid cell migration (Sahai and Marshall, 2003). The inhibitory role played by p27 in the regulation of cell motility may be of particular relevance in tumor cells that frequently use amoeboid movements to invade and metastasize (Condeelis and Segall, 2003) and in which stathmin is often upregulated.

Role of p27 and stathmin in tumor progression

In a wide variety of human cancers, p27 downregulation has been linked to a worse prognosis, increased recurrence of local disease, and a higher incidence of local invasion or distant metastasis (reviewed in Slingerland and Pagano, 2000). More recently, it has been proposed that the subcellular localization of p27 is also important in tumor formation and/or progression, since its cytoplasmic displacement results in a loss of function related to cell cycle progression inhibition (Blain et al., 2003). On the other hand, cytoplasmic localization of p27 in thyroid (Baldassarre et al., 1999b) and in mammary carcinomas (Liang et al., 2002) has been associated with lower malignancy in comparison with p27 absence. The present finding that p27 cytoplasmic expression inhibits migration and invasion of tumor cells can at least in part explain this latter observation and is in accord with the finding that in sarcoma samples low cytoplasmic expression of p27 and high stathmin levels correlate with distant metastasis formation. Importantly, it has been demonstrated that in a mouse model of sarcomas the absence of p27 but not p21 was able to increase the number of metastatic sarcomas and to shorten their time of appearance (Martin-Caballero et al., 2004).

Stathmin is expressed and could influence time to progression in metastatic non-small cell lung cancer (Rosell et al., 2003) and is associated with the more aggressive phenotype in Wilms tumors (Takahashi et al., 2002). Similarly, we found that in sarcomas stathmin expression is higher in metastatic than in primary tumors. Moreover, the fact that p27 cytoplasmic expression contributes to MT stabilization should be taken into account when using tubulin-acting agents in cancer therapy. p27 influences the response to taxol in 3D cultured cancer cells (St. Croix et al., 1996) and probably in patients (Van Poznak et al., 2002). Similarly, stathmin expression has been correlated with the resistance to MT-acting drugs in cultured cells (Alli et al., 2002) as well as in patients with non-small cell lung cancer metastasis (Rosell et al., 2003). In summary, this study opens a window on the molecular mechanisms contributing to the regulation of cancer cell invasion and represents another step toward the comprehension of the relationship existing between proliferation and motility in mammalian cells.

Experimental procedures

Cell culture, transfection, and treatments

All cell lines used were grown in DMEM supplemented with 10% FBS (Sigma) and transfected using FuGene 6 reagent (Roche). Primary wt and *stathmin* null MEFs were prepared from day 13.5 embryos according to standard procedures. Dr. U.K. Schubart and Dr. M. Kuhn kindly provided *Stathmin* null mice, maintained in a CI57Bl/6 background. Primary *p27* null MEFs were provided by Dr. J. Roberts, and *p27* wt and null fibroblasts were provided by Dr. M. Fero.

Proliferation, adhesion, and migration assays

Cell proliferation was evaluated using the BrdU incorporation assay or FACS analysis as described (Baldassarre et al., 1999a). Adhesion and migration assays were performed essentially as reported (Spessotto et al., 2002). Percent of inhibition was calculated as described (Baldassarre et al., 1999b). For 3D motility assay, cells (3×10^6/ml) were included in Matrigel (Becton Dickinson) drops and incubated for the indicated times in complete medium.

Immunoblotting, immunoprecipitation, and immunofluorescence

Total, nuclear, and cytoplasmic protein fraction preparation, IP, Western blot analysis, and IF assays were performed essentially as described (Baldassarre et al., 1999b). IF-labeled cells were studied using a confocal laser-scanning microscope (Diaphot 200 Nikon; MRC-1024 BioRad Laboratories) or a Leica epifluorescent microscope.

Two-hybrid yeast screening

The bait plasmid pAS2-1/p2797-198 was generated by PCR, and the two-hybrid screening was performed as described (Morrione et al., 1999). Briefly, Y190 yeast strain was first transformed with the bait plasmid and then with a mouse embryo cDNA library, cloned in pVP16 vector (kind gift of Dr. Stanley Hollenberg and Dr. Ann Vojtek). Cotransformants were plated onto −trp-leu-his selective medium, supplemented with 25 mM 3-aminotriazole. His+ colonies were then assayed for β-galactosidase activity by a filter assay (Morrione et al., 1999). cDNA library clones were isolated from yeast, transformed into bacteria, and analyzed by automated sequencing.

Production and purification of recombinant proteins

For production of recombinant proteins, $p27^{wt}$ or $p27^{1-170}$ and $stathmin^{wt}$, $stathmin^{1-99}$, or $stathmin^{25-149}$ cDNAs were cloned in the pQE-30 (Qiagen) modified with a 5′ Flag tag, or in the pET-2.1c (Novagen) vectors.

In vitro and in vivo MT studies

In vitro tubulin polymerization assay was performed essentially as reported (Holmfeldt et al., 2001). Recombinant stathmin was either from Calbiochem or produced as described above. Recombinant Flag-tagged or his-tagged proteins were used with no significant variation. Tubulin dilution assay was performed essentially as described (Khawaja et al., 1988). Briefly, cells adherent to FN-coated coverslips were incubated in PEM buffer containing 0.1% of Triton X-100 for 1 min at 37°C, then washed and incubated in PEM buffer for the indicated times at 37°C. Cells were then fixed and processed for IF.

Supplemental data

For a more detailed explanation of the Experimental Procedures, see the Supplemental Data at http://www.cancercell.org/cgi/content/full/7/1/51/DC1/.

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