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The microtubule cytoskeleton is required for a G2 cell cycle delay in cancer cells lacking stathmin and p53

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Abstract

In several cancer cell lines, depleting the microtubule-destabilizing protein stathmin/ oncoprotein18 leads to a G2 cell cycle delay and apoptosis. These phenotypes are observed only in synergy with low levels of p53, but the pathway(s) activated by stathmin depletion to delay the cell cycle are unknown. We found that stathmin depletion caused greater microtubule stability in synergy with loss of p53, measured by the levels of acetylated α -tubulin and the rate of centrosomal microtubule nucleation. Nocodazole or vinblastine-induced microtubule depolymerization abrogated the stathmin-depletion induced G2 delay, measured by the percentage of cells staining positive for several markers (TPX2, CDK1 with inhibitory phosphorylation), indicating that microtubules are required to lengthen G2. Live cell imaging showed that stathmin depletion increased time in G2 without an impact on the duration of mitosis, indicating that the longer interphase duration is not simply a consequence of a previous slowed mitosis. In contrast, stabilization of microtubules with paclitaxel (8 nM) slowed mitosis without lengthening the duration of interphase, demonstrating that increased microtubule stability alone is not sufficient to delay cells in G2.

Keywords

microtubule; stathmin; G2; interphase duration; acetylated tubulin

Introduction

Stathmin/Oncoprotein 18 is a microtubule (MT) destabilizing protein that is highly over-expressed in many cancers (Belletti et al. 2008; Bieche et al. 1998; Brattsand 2000; Chen et al. 2003; Friedrich et al. 1995; Kouzu et al. 2006; Melhem et al. 1997; Nakashima et al. 2006; Ngo et al. 2007; Nishio et al. 2001; Nylander et al. 1995; Price et al. 2000; Yuan et al. 2006). We and others have shown that depleting stathmin in many cancer cell lines slows cell proliferation and ultimately leads to apoptosis (Alli et al. 2007; Carney and Cassimeris 2010; Mistry et al. 2005; Wang et al. 2009; Zhang et al. 2006). The slower proliferation observed in stathmin depleted cancer cells is likely a result not only of apoptosis, but also of a delay during G2 of the cell cycle (Carney and Cassimeris 2010). In contrast to cancer-derived cell lines, stathmin depletion is not deleterious to non-transformed cells (Carney and Cassimeris 2010; Zhang et al. 2006) and stathmin knockout mice are viable (Schubart et al.

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1996). However, stathmin depletion is deleterious in combination with loss of the tumor suppressor p53, in both cancer-derived cell lines and in normal human fibroblasts (Carney and Cassimeris 2010), as originally proposed by Alli et al (2007). Although these data support the idea that stathmin, or those pathways that its level regulates, may be targets for selectively inhibiting proliferation of many cancers, the pathway(s) activated by stathmin depletion has not been identified.

Stathmin's only characterized function is as a MT destabilizing protein, where reducing stathmin level has an overall stabilizing effect on the MT cytoskeleton. More specifically, stathmin depletion increases the concentration of MT polymer and decreases the concentration of free tubulin dimers (Holmfeldt et al. 2006; Howell et al. 1999a; Ringhoff and Cassimeris 2009b; Sellin et al. 2008), inhibits MT dynamic turnover (Howell et al. 1999a; Howell et al. 1999b; Ringhoff and Cassimeris 2009b), increases MT nucleation from centrosomes (Ringhoff and Cassimeris 2009b) and increases the amount of acetylated α -tubulin (Belletti et al. 2008), a marker of non-dynamic, long-lived MTs (Perdiz et al. 2011; Schulze et al. 1987). In in vitro MT assembly assays, stathmin has two MT destabilizing activities: sequestration of tubulin dimers (Belmont and Mitchison 1996; Curmi et al. 1997; Howell et al. 1999b), preventing their polymerization, and a more direct promotion of MT catastrophes (the switch from MT growth to shortening states) (Belmont and Mitchison 1996; Howell et al. 1999b). Stathmin is active as a MT destabilizer during interphase of the cell cycle; it is phosphorylated on all 4 serine residues and turned off during mitosis (Holmfeldt et al. 2001; Larsson et al. 1997).

While stathmin depletion stabilizes the MT cytoskeleton in many cell types, it is not clear whether overly stable MTs will slow cell cycle progression during G2, a phenotype observed in cancer-derived cell lines depleted of stathmin (Carney and Cassimeris 2010). Stabilization of MTs by paclitaxel does not slow progression through interphase, but rather blocks cells in mitosis (Uetake and Sluder 2007). Others have shown that MT depolymerization slows cell cycle progression, particularly during G2 (Balestra and Jimenez 2008; Blajeski et al. 2002; Rieder and Cole 2000), but Uetake and Sluder (2007) have argued that an interphase MT integrity checkpoint does not exist; rather it is the length of the previous mitosis that matters. Taken together, these data suggest that a drug-induced change in MT stability does not alter cell cycle progression outside of M phase, raising the possibility that stathmin depletion does not slow G2 via increased MT stability.

As a first step to understand why several cancer-derived cell lines require stathmin for proliferation, we examined whether interphase MTs are required for the G2 delay observed after stathmin depletion. Live cell imaging demonstrated that stathmin-depleted cells proceed through mitosis with normal kinetics, but remained in interphase for a longer time. The interphase duration showed no correlation with the length of the previous mitosis, eliminating a mitotic delay as the source of the slower interphase. To examine whether stathmin depletion could relay a signal via MTs, we examined changes to the MT cytoskeleton in cells with reduced levels of stathmin, p53, or both proteins. We found that stathmin depletion caused greatest MT stability in combination with negligible p53 levels, where stability was measured by both the presence of post-translational modifications to tubulin and increased MT nucleation from centrosomes. Additionally, MT

depolymerization, by incubation in either nocodazole or vinblastine, was sufficient to abrogate the G2 delay. In contrast, treatment of cells with paclitaxel to stabilize MTs extended mitotic duration without affecting interphase duration. Together, these results indicate that stathmin depletion, in combination with reduced levels of p53, delays cells in G2 by a signal that requires MTs, but that stabilization of MTs alone is not sufficient to delay the cell cycle in G2.

Results

Stathmin depletion delays G2 without affecting mitotic duration

Previously we demonstrated that stathmin depletion from HeLa cells (which also lack detectable p53) delayed cells in G2 of the cell cycle, as measured by the percent of cells staining positive for several markers (Carney and Cassimeris 2010). To understand the mechanism responsible for this cell cycle delay we first used long-term live cell imaging to follow individual HeLa cells through several cell cycles. Consistent with our previous results, siRNA transfection reduced stathmin protein level significantly in HeLa cells, as shown in Figure 1A for cell lysates prepared 48 h after transfection. We previously demonstrated that stathmin levels are reduced by at least 75% compared to control transfected cells within the first 24 h, and that this depletion is maintained for at least 3 days (Carney and Cassimeris 2010). Figure 1B shows a series of phase contrast images taken from a long-term time series for a cell entering and exiting mitosis. Interphase was considered to have ended, and mitosis to have begun, when the nuclear envelope was no longer clearly detectable (time marked 0 in Figure 1B; see also Methods). Mitosis was considered complete when the cell first showed constriction of the plasma membrane (45 min in Figure 1B). Phase contrast images were collected at 5 min intervals for 2 - 3 days and cell fates were then determined from the image series. As shown in Figure 1C, stathmin depletion significantly slowed interphase duration compared to untreated cells, increasing the average interphase duration by 4.6 hours, without affecting the time in mitosis. We asked whether the duration of mitosis is correlated with the duration of the next interphase for all stathmin-depleted cells where the pair of durations was available. As shown in Figure 1D, mitotic duration did not predict the duration of the next interphase ($R^2 = 0.002$ for the linear curve fit to the data).

We confirmed that the longer interphase measured in stathmin depleted cells was due to a delay in G2 by synchronizing cells using a double thymidine block and then examining the rate at which cells reached mitosis after release from the second S phase block. As shown in Figure 1E, 50% of control siRNA transfected cells had initiated mitosis by ~12 h after release from the block, and by 28 h nearly all cells had divided. Stathmin-depleted cells were slower to enter mitosis, it took ~16 h for 50% of the cells to enter mitosis and 30% of cells had still not entered mitosis by 42 h after release from the block. Taken together, these data link stathmin depletion and delayed cell cycle progression during G2 in HeLa cells.

Stathmin depletion stabilizes MTs to a greater extent in cells lacking p53

Our previous results demonstrated that stathmin depletion increased the population of cells staining positive for markers of G2 only in combination with reduced or no expression of p53 (Carney and Cassimeris 2010). If this cell cycle delay depends on stathmin regulation of

the MT cytoskeleton, we predicted that stathmin depletion would have a greater MT stabilizing effect in cells when combined with low levels of p53. We tested this prediction using HeLa cells because we previously demonstrated that we could lower stathmin by siRNA and restore p53 by depleting the HPV E6 protein, which normally targets p53 for destruction in these cells (Carney and Cassimeris 2010) (Figure 2A). To enhance detection of p53 on immunoblots, cells were first treated with doxorubicin, a DNA-damaging agent, for 24 h prior to cell lysate isolation. Doxorubicin was only used to test whether E6 depletion was sufficient to restore p53 and was not used in experiments to measure MT phenotypes.

Measurement of MT polymer density at the periphery of cells showed that stathmin depletion from HeLa cells increased MT polymer by ~1.5 fold compared to control-transfected cells (data not shown), consistent with results reported previously by us and others (Holmfeldt et al. 2006; Howell et al. 1999a; Ringhoff and Cassimeris 2009b; Sellin et al. 2008). But, to compare MT stability between 4 different conditions to manipulate both stathmin and p53 levels, we used two other measures of general MT stability: the levels of acetylated α -tubulin, which serves as a marker of stable, long-lived, non-dynamic MTs (Belletti et al. 2008; Cambray-Deakin and Burgoyne 1987; Perdiz et al. 2011; Schulze et al. 1987), and centrosomal emergence of new MTs bound by EB1, a marker of MT nucleation rate (Piehl and Cassimeris 2003). We chose nucleation rate as a marker of MT assembly because it is sensitive to the level of a number of proteins that regulate MT dynamics, centrosome function, or both processes (Srayko et al. 2005) and because we previously showed that up or down regulation of stathmin has a significant impact on MT nucleation rate from centrosomes (Ringhoff and Cassimeris 2009b). These two measures of general MT stability showed less cell-to-cell variation within a treatment group than did MT polymer density, facilitating comparison between multiple treatment groups.

To examine non-dynamic, stable MTs by the amount of acetylated α -tubulin, we first used HeLa cells depleted of either stathmin, HPV E6 (restoring p53) (Figures 1A, 2A), or both stathmin and HPV E6 (Carney and Cassimeris 2010). By immunoblot, cells depleted of stathmin showed a large increase in acetylated α -tubulin (Figure 2B-E). In contrast, depleting E6 (restoring p53) and stathmin together resulted in an acetylated α -tubulin level only slightly higher than that in controls, and approximately the same level as that seen in cells depleted of E6 alone (Figure 2B-D). We also examined individual cells after fixation and staining for acetylated α -tubulin and observed the same effect, where cells depleted of stathmin showed the greatest amount of acetylated α -tubulin in MTs, while restoring p53 reduced the amount of acetylated α -tubulin in MTs to the level in control siRNA transfected cells, regardless of stathmin level (Figure 2C-E). We used two methods to quantify the changes in level of acetylated tubulin in MTs: measurement of staining intensity within individual cells and counts of cells having >25 acetylated MTs at the cell periphery, similar to a method used previously by Nguyen et al (1997). Both measurements demonstrated that acetylated α -tubulin in MTs is increased significantly only when both stathmin and p53 levels were reduced. Note that a fraction of stathmin-depleted cells did not show a change in the amount of acetylated α -tubulin, but these cells were included in the quantitative measurements. The mechanism responsible for the observed variation is not known. We did not observe any detyrosinated MTs in HeLa cells under any experimental conditions,

confirming results of Bulinski et al. (1988) that HeLa cells lack this tubulin post-translational modification. Therefore, to confirm the increase in acetylated α -tubulin in HeLa cells, we examined α -tubulin acetylation in a second cell type, using matched colon cancer cell lines differing in p53 genotype. Acetylated α -tubulin increased in HCT116^{p53^{-/-}} cells after stathmin depletion, while the matched HCT116^{p53^{WT}} cells showed no change compared to control transfected cells (Figure S1).

As a second measure of overall MT stability and of MT assembly, we examined MT nucleation from centrosomes. Previously we showed that MEFs lacking both copies of the stathmin gene have a significantly greater rate of MT nucleation from centrosomes (Ringhoff and Cassimeris 2009b). To measure centrosomal MT nucleation rate we observed live cells expressing EB1-GFP (Piehl et al. 2004). HeLa cells were depleted of stathmin, HPV E6, or both proteins and then incubated for 24 hours. Cells were then transfected with the EB1-GFP plasmid and incubated for an additional 48 hours. Stathmin depletion increased nucleation rate significantly compared to siGlo transfected controls, while restoring p53 had no effect on the rate of MT formation compared to the siGlo controls (Figure 3). Cells depleted of both stathmin and HPV E6 (restoring p53) had a nucleation rate nearly identical to that in control-treated cells. Taken together with results of acetylated α -tubulin levels, these data demonstrate that overall MT stability was increased to a greater extent when stathmin was depleted in cells having reduced (HeLa) or no (HCT116^{p53^{-/-}}) p53. Thus, stathmin depletion from cells lacking appreciable p53 caused both a G2 cell cycle delay (Carney and Cassimeris 2010), and above) and increased MT stability. Next we tested whether MTs are required for the stathmin-depletion induced G2 cell cycle delay or merely correlated with the delay.

Drug-induced MT depolymerization allows cells to escape the G2 block induced by stathmin depletion

To test whether MTs are necessary for the G2 cell cycle delay in stathmin-depleted cells, we asked whether drug-induced MT depolymerization eliminated the delay. HeLa cells were allowed to grow for 43-45 h post transfection with siRNA directed against stathmin or a control siRNA and then incubated in 33 μ M nocodazole for 3 - 5 h prior to fixation. Rieder and Cole (2000) previously demonstrated that brief exposure to high concentrations of nocodazole, sufficient to depolymerize MTs, can drive non-tumor derived cell lines to revert from prophase to G2. This phenomenon is transient; cells recover from the stress quickly and then block in mitosis. Our experimental timing was chosen to be of sufficient duration to avoid possible contributions from prophase cells transiently reverting back into G2. After 3 - 5 h of exposure to 33 μ M nocodazole, the MT network was depolymerized to near completion (Figure 4A). Cells were also stained with two markers to identify cells in G2. Although MTs were no longer present, interphase and mitotic cells were still easily differentiated by cell and nuclear morphologies.

Consistent with our previous results (Carney and Cassimeris 2010), HeLa cells depleted of stathmin showed an approximate 1.5 fold increase in the percent of interphase cells staining positively for TPX2, a protein expressed in the nucleus in late S and G2 (Brito and Rieder 2006), when compared to control treated cells (Figure 3; examples of stained cells are shown

in Figure S2). The percent of TPX2 positive cells returned to control levels when cells were also treated with 33 μ M nocodazole (Figure 4B). Using phospho-CDK1(Y15P) (Borgne and Meijer 1996), as a second marker of G2 cells, we saw a similar pattern, where nocodazole treatment reduced the percent of stathmin-depleted interphase cells staining positive for this marker (Figure 4C). For both markers the percentage of nocodazole-treated, stathmin-depleted interphase HeLa cells staining positive for G2 markers was not significantly different from control-transfected cells. Using the same experimental protocol in HCT116^{p53}^{-/-} cells confirmed the results in HeLa cells (Figure S3). As demonstrated previously (Carney and Cassimeris 2010), stathmin depletion increased the percentage of TPX2-positive interphase cells (Figure S3). After MT depolymerization the percent of TPX2-positive interphase cells was similar to that in control-transfected cells, with or without nocodazole treatment (Figure S3). Taken together, these results indicate that MT depolymerization by nocodazole was sufficient to abrogate the G2 delay.

To verify the results observed with nocodazole-induced MT depolymerization, we treated cells with 10 nM vinblastine (Jordan et al. 1992), a concentration sufficient to completely depolymerize MTs in 3 h (Figure 5A). HeLa cells were depleted of stathmin, transferred to medium supplemented with 10 nM vinblastine 45 h after siRNA transfection, and fixed after 3 h in the drug. Cells were then stained for TPX2 expression to determine the percent of interphase cells in G2. Similar to results with 33 mM nocodazole, stathmin-depleted cells incubated in 10 nM vinblastine had a percent of TPX2 positive interphase cells similar to that in control transfected cells (Figure 5B).

The nocodazole and vinblastine concentrations tested above completely depolymerized the MT network. We also examined whether incubation in 100 nM nocodazole, a drug concentration that reduced MT polymer content but did not completely depolymerize MTs (Figure 6), was sufficient to abrogate the stathmin depletion-induced G2 delay. We found that incubation of HeLa cells for 3 h in 100 nM nocodazole reduced MT polymer (Figure 6A) and reduced the percentage of stathmin-depleted interphase cells staining positive for TPX2 (Figure 6B).

Microtubule stabilizing drugs do not affect interphase progression

While our results indicated that MT depolymerization abrogates the stathmin depletion-induced delay in G2, it is not clear whether increased MT stability alone is sufficient to delay cell cycle progression during G2. In a previous study, Uetake and Sluder (2007) demonstrated that 10 - 100 nM paclitaxel treatment had no effect on interphase duration in either RPE1 or primary fibroblast when the previous mitosis was of normal duration. It is possible that cancer-derived cell lines respond differently to increased MT stability during interphase, or that stathmin-depletion induces a G2 delay through a mechanism that is not simply caused by a general increase in MT stability. To address this question, we used live cell imaging to examine interphase and mitotic durations in HeLa cells treated with 8 nM paclitaxel, the half-maximal concentration needed to block these cells in mitosis (Jordan et al. 1993). Jordan et al. (1993) previously found that the total amount of MT polymer was not significantly increased at this drug concentration, although the interphase MT array showed increased density around the nucleus (Figure 7). Note that Jordan et al. (1993) demonstrated

that paclitaxel is concentrated within cells and at 8 nM added to the medium, the intracellular concentration of paclitaxel should reach ~ 2 - 5 μ M after 20 h incubation.

Hela cells were treated with 0.1% DMSO or 8 nM paclitaxel and imaged for 45 - 60 hr by phase contrast microscopy (see Methods). As shown in Figure 7, paclitaxel did not slow interphase duration compared to DMSO-treated cells. However, as expected, paclitaxel significantly increased mitotic duration (Figure 7). These longer mitotic durations did not show any correlation with the length of the subsequent interphase (Figure 7C; $R^2 = 0$ for the linear curve fit to the data). These data demonstrate that enhanced MT stability alone is not sufficient to delay interphase, either directly or by a previous delay in mitosis.

Discussion

Previously we demonstrated that stathmin depletion, from cells expressing negligible p53, delay during G2 of the cell cycle (Carney and Cassimeris 2010). Here we explored the mechanism responsible for the G2 delay. We found that the ~ 5 h delay during interphase was not the result of a previous delay during mitosis. Cells depleted of both stathmin and p53 showed greater MT stability, as measured by either post-translational α -tubulin acetylation or MT nucleation rate from centrosomes, than did cells having reduced levels of either protein alone. MT depolymerization was sufficient to abrogate the G2 cell cycle delay in stathmin-depleted cells, but MT stabilization by paclitaxel did not lengthen interphase, even after a long duration in mitosis. Taken together, our results demonstrate that stathmin depletion delays cells in G2 through a mechanism that requires the MT cytoskeleton, but that MT stability alone is not sufficient to delay interphase cell cycle progression.

Stathmin depletion causes more robust MT stabilization in combination with low levels of p53

Two methods were used to assay overall MT stability, the amount of acetylated α -tubulin (representing the amount of long-lived, non-dynamic MTs) and the rate of MT nucleation from centrosomes (representing the rate of new MT formation). By either method, stathmin depletion increased MT stability most significantly in cells lacking p53. The level of acetylated α -tubulin in MTs is a marker of non-dynamic MTs (Janke and Kneussel 2010), but could also reflect a shift in the balance between α -tubulin's acetylase and deacetylase enzymes to favor greater net acetylation. We do not have evidence to suggest that these enzymes are regulated by stathmin, and therefore think the amount of acetylated α -tubulin represents the pool of long-lived, non-dynamic MTs. The rate of EB1-GFP comet emergence from the centrosome provides an assay for MT nucleation rate (Piehl and Cassimeris 2003) and the increased rate of MT formation indicates that stathmin-depleted cells are producing new MTs at a faster rate than control transfected cells.

The greater overall MT stability observed after stathmin depletion is consistent with many previous studies in both cell lines (Belletti et al. 2010; Holmfeldt et al. 2007; Howell et al. 1999a; Ringhoff and Cassimeris 2009b) and *Xenopus* egg extracts (Budde et al. 2001). However, in the Hela cells used for most experiments reported here, stathmin depletion only significantly increased MT stability in cells lacking detectable levels of p53. These results were not confined to Hela cells because stathmin depletion only increased acetylated α -

tubulin in HCT116^{p53-/-} cells and not the matched HCT116^{p53WT} line. At present it is unclear why p53 expression influences the outcome of stathmin depletion, but several possible mechanisms could explain our observations. First, MT dynamic instability differs slightly in cells expressing p53 or a dominant negative p53 mutant (Galmarini et al. 2003), making it possible that stathmin and p53 normally function synergistically to regulate MT dynamics. P53 may also impact centrosome function since it has been shown to localize to centrosomes and affect centrosome amplification (Tarapore and Fukasawa 2002). Alternatively, cells differing in p53 status could be dissimilar in more general ways that subsequently impact the degree of MT stability observed after stathmin depletion. For example, a large number of gene expression changes have been noted after depletion or knockout of either stathmin or p53 (Ringhoff and Cassimeris 2009a; Sur et al. 2009).

Stathmin depletion, paclitaxel addition, and MAP4 over-expression yield different cell cycle phenotypes

While our data are consistent with a model where increased MT stability, downstream of stathmin depletion, delays cell cycle progression during G2, these results differ from that observed after treatment with the MT stabilizing drug, paclitaxel. Treating cells with paclitaxel suppresses MT dynamics (Yvon et al. 1999) and blocks cells in mitosis (Gascoigne and Taylor 2008; Shi et al. 2008) without slowing interphase progression (Figure 7) (Uetake and Sluder 2007). Therefore, MT stability alone cannot be the signal acting downstream of stathmin depletion to delay interphase cell cycle progression.

Cells over-expressing MAP4 (Nguyen et al. 1997), a MAP that functions in opposition to stathmin to regulate the amount of tubulin assembled into MTs (Holmfeldt et al. 2007), also disrupts cell cycle timing. Mouse L cells over-expressing MAP4 displayed slower proliferation, without an increase in mitotic index, compared to the parent cell line (Nguyen et al. 1997). In these cells, MAP4 over-expression increased the time required to enter S phase, implicating MAP4 level in control of the timing of G1. Thus, three treatments that increase MT polymer and generate more stable MTs, delay cells in different cell cycle stages. Why cells respond differently to these treatments is not known. It remains possible that stathmin depletion and/or MAP4 over-expression relay signals that require more than increased numbers of MTs. Alternatively, each may modify the MT cytoskeleton in unique ways, which have consequences at different cell cycle stages.

Why does stathmin depletion only result in a G2 delay in cells lacking p53?

Stathmin depletion only slows proliferation, cell cycle progression and induces apoptosis in the absence of p53 (Alli et al. 2007; Carney and Cassimeris 2010). Here we demonstrated that stathmin depletion requires an intact MT cytoskeleton to delay cells in G2, but these results do not provide insight into the synergy between stathmin, or MTs, and p53. It is possible that p53 and stathmin function as part of a single pathway, where loss of two components is sufficient to interrupt cell cycle progression and/or lead to apoptosis. In support of this model, the expression of both stathmin and MAP4 are repressed by activated p53 (Ahn et al. 1999; Johnsen et al. 2000), putting both these MT regulators downstream of active p53. Curiously though, stathmin and MAP4 act in opposition to regulate the extent of tubulin polymerization and experimental depletion of both proteins returns MT polymer

level to that in untreated cells (Holmfeldt et al. 2002). Alternatively, cells lacking p53 may differ in a more general way from cells expressing p53, making them sensitive to loss of stathmin (or increased MT stability). For example, p53 is one component regulating whether most cellular ATP is generated via glycolysis or oxidative phosphorylation (Maddocks and Vousden 2011). In this type of model, the absence of p53 could make cells more reliant on glycolysis, or some other general condition, that then renders them more susceptible to loss of stathmin.

Materials and Methods

Cell Culture

Cells were grown at 37°C in a humidified atmosphere of 5% CO₂. HeLa cells were grown in MEM (GIBCO) supplemented with 2.2g/L sodium bicarbonate, 1X antibiotic/antimycotic (Sigma) and 10% fetal bovine serum (FBS; GIBCO-Invitrogen), or DMEM (Sigma) supplemented with 1X antibiotic/antimycotic, 1% L-Glutamine (Sigma), and 10% FBS. HCT116^{p53WT} or HCT116^{p53-/-} cells were also maintained in DMEM. In some experiments, HeLa cells were synchronized through a double thymidine block by overnight incubation in 5mM thymidine (in DMEM), 8 h release in DMEM during which time they were transfected with either siRNA to stathmin or control siGlo RNA, and then 16 h incubation in 5mM thymidine (in DMEM). Cells were transferred to DMEM and followed via live cell imaging as described below. Paclitaxel (Molecular Probes) was added to culture medium at a final concentration of 8 nM from a 8 µM stock prepared in DMSO.

RNA Interference and Transient Transfection

RNA interference (RNAi) was achieved using GeneSilencer reagents following the manufacturer's protocol as described previously (Carney and Cassimeris 2010). Briefly, cells were grown on 35 mm dishes for 1-2 days before the addition of siRNA. Cells were serum starved 30 minutes pre-transfection and 4 hours post-transfection to improve transfection efficiency. RNAi oligonucleotides (Dharmacon; 2 µg per 35 mm dish) used included: SMTN1 (Op18-443), 5'-CGUUUGCGAGAGAAGGAUAdtdt-3', (Holmfeldt et al. 2007) and HPV E6 (18E6-385), 5'-CUAACACUGGGUUAUACAAdtdt-3' (restores p53 by depleting the HPV E6 protein) (Koivusalo et al. 2005). SiGlo Risc-Free siRNA (Dharmacon) was used as a control siRNA sequence for these experiments. We previously demonstrated that cells transfected with either siRNA or shRNA targeting stathmin showed an approximate 75% reduction in stathmin level (see also Figure 1A), that depleting HPV E6 restores p53 in HeLa cells (see also Figure 2), and that stathmin and E6 can be co-depleted in these cells (Carney and Cassimeris 2010).

For plasmid transfection, cells were grown similarly to those treated with siRNA except that Eugene 6 (Piehl and Cassimeris 2003; Warren et al. 2006) was used to transfect cells with 2 µg EB1-GFP plasmid per 35 mm dish as previously described (Piehl and Cassimeris 2003).

Indirect immunofluorescence and confocal microscopy

Cells were fixed, stained and imaged as described previously (Carney and Cassimeris 2010; Piehl and Cassimeris 2003; Ringhoff and Cassimeris 2009b). Primary antibodies used were

mouse anti- α -tubulin; mouse anti-acetylated- α -tubulin (1:1000; Clone 6-11B-1; Sigma-Aldrich), rabbit anti-TPX2 (1:1000; Garrett et al. 2002) (Gift from Duane Compton, Dartmouth Medical School), and rabbit anti-phospho-CDK1 (Y15P) (1:50; Cell Signaling Technology). Goat anti-mouse or rabbit Alexa Fluor 488 or 563 (1:200; Invitrogen) were used as the secondary antibodies in these experiments. Confocal microscopy was used to image stained cells as described previously (Carney and Cassimeris 2010; Warren et al. 2006). Images were acquired using a 40X/1.3NA objective. Image stacks were converted to maximum intensity projections, exported as TIFF files and assembled using Photoshop. For all cell cycle markers, we counted interphase cells as stained or unstained, and did not count mitotic cells.

Acetylated α -tubulin staining intensities were determined using MetaView imaging software (Molecular Devices, Sunnyvale, CA). For each individual cell, a region was drawn to encompass the entire cell and 100-125 Hela cells or ~ 200-250 matched HCT116 cells were measured for each treatment group. A background intensity region outside of the cell was subtracted from each measurement and the data reported as mean intensity $\pm$ SD for 3 independent experiments (Howell et al. 1999a; Ringhoff and Cassimeris 2009a). Counts of the number of acetylated MTs extending to the cell periphery included all acetylated MTs within 10 μ m of the cell edge.

Live Cell Imaging

Confocal microscopy was used to image live cells expressing EB1-GFP as described previously (Piehl and Cassimeris 2003; Warren et al. 2006). Briefly, images were collected every 2 s, for up to 2 minutes, from an image plane centered on the centrosome. EB1-GFP comets were counted as they emerged from the centrosome and used as a measure of nucleation rate.

To follow cell fates over several days, Hela cells were plated on Mattek dishes and were imaged using a Nikon Biostation IM. Throughout imaging cells were maintained in a humidified chamber and in 5% CO₂ atmosphere provided by an MIGM Gas Mixer (Tokken, Inc.). Cells were imaged with phase contrast optics using a 20X objective and images collected at 5 m intervals for 24-72 h. Cell fates were tracked from the image series. Mitotic entry was scored either by the first image showing loss of nuclear envelope integrity or by extensive cell rounding making it impossible to see the nuclear envelope (see Figure 1). Mitotic exit was scored as the first image showing indentation of the plasma membrane, indicative of the beginning of cytokinesis (see Figure 1). For experiments where cells were released from an S phase block, the time from the second thymidine washout to the disappearance of the nuclear envelope was recorded for each cell.

Protein Isolation and Western Blotting

Soluble cell extracts were prepared for SDS-polyacrylamide gel electrophoresis as described previously (Carney and Cassimeris 2010). To enhance detection of p53, the protein was stabilized by cell incubation in doxorubicin (0.5 μ M) for 24 h prior to lysate preparation (Carney and Cassimeris 2010). Protein concentrations were measured by Bradford assay (Bradford 1976). Membranes were probed and imaged as previously described (Carney and

Cassimeris 2010) using primary antibodies rabbit anti-stathmin (1:1000; Sigma-Aldrich), mouse anti- α -acetylated-tubulin (1:1000; Clone 6-11B-1; Sigma-Aldrich), mouse anti-p53 (1:1000; Vision Bio Systems), rabbit anti-actin (1:1000; Sigma-Aldrich), or goat anti-GAPDH (1:1000; Abcam) followed by goat anti-mouse (1:2000; Sigma-Aldrich), goat anti-rabbit (1:2000; Sigma-Aldrich) or rabbit anti-goat (1:2000; Abcam) horseradish peroxidase-linked IgG.

Data Analysis

Statistical analysis of cell counts and intensity measurements were performed using unpaired t-tests in Microsoft Excel or GraphPad Software (www.graphpad.com/quickcalcs/ttest1.cfm).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Stathmin depletion slows interphase but not mitosis in HeLa cells. (A) Immunoblot confirming stathmin depletion from HeLa cells. Blots were re-probed for actin to serve as a loading control. See also Carney and Cassimeris (2010). (B) Phase contrast image series for a cell entering and exiting mitosis. Mitotic entry (and the end of interphase) is shown at time 0. Mitotic exit is shown at 45 m. Scale bar = 10 μ m. (C) HeLa cells were transfected with stathmin siRNA and cell fates determined from phase contrast image series as described in Methods. Box plots are shown in C, summarizing data from > 100 cells and 3 independent experiments per condition. Stathmin depletion increased interphase duration by approximately 4.6 h (**, $p < 0.001$), but cells progressed through mitosis with normal kinetics. (D) The durations of mitosis and the corresponding time of the next interphase are plotted. No correlation is observed between the time in mitosis and the duration of interphase. Linear curve fit to the data is shown, slope = -0.9, $R^2 = 0.002$. (E) Stathmin depleted cells are slower to enter mitosis following release from a double thymidine block. Representative experiments are shown from a total of 2 siGLO and 3 stathmin siRNA experiments.

Figure 2.

Stathmin depletion from HeLa cells increases acetylated α -tubulin levels. HeLa cells were transfected with siGLO control siRNA or siRNA targeting stathmin, HPV E6 (which restores p53 in these cells), or both siRNAs. (A) Immunoblot demonstrating restoration of p53 expression in HeLa cells by depletion of the HPV E6 protein, confirming our previous results (Carney and Cassimeris 2010). For this blot, doxorubicin was added to cell cultures for 24 h to induce DNA damage and p53 stability. Doxorubicin was only used to enhance p53 detection in these samples and not in any other experiments. (B) Anti-acetylated α -tubulin immunoblot from HeLa cell lysates isolated 72 h after transfection. Tubulin acetylation is greatest in cells depleted of stathmin. Restoring p53 (E6 siRNA) alone, or in combination with stathmin depletion, resulted in a small increase in total acetylated α -tubulin relative to control-transfected cells. GAPDH is shown as a loading control. (C) HeLa cells were fixed 72 h after transfection and stained with antibodies against acetylated α -tubulin. Tubulin acetylation in MTs was greatest in stathmin depleted cells lacking p53. Restoring p53, independent of stathmin level, did not increase the amount of acetylated tubulin in MTs. Representative images of each treatment are shown. Scale bar = 20 μ m. (D) Quantitation of acetylated tubulin staining intensities from immunofluorescent images. Staining intensity increased significantly in HeLa cells depleted of stathmin (in the

absence of p53). Restoring p53 expression by HPV E6 depletion, with or without stathmin depletion, did not result in any significant change in acetylated α -tubulin staining intensity relative to control transfected cells. *** denotes $p < 0.0001$. (E) The percent of cells with greater than 25 acetylated MTs at the cell periphery is significantly increased in stathmin-depleted cells, and only in combination with low levels of p53. **denotes $p < 0.001$.

Figure 3.

Stathmin depletion increased MT nucleation rate in synergy with low p53 level. MT nucleation rates were determined by transfecting Hela cells with EB1-GFP 24 h after indicated siRNA treatments. Living cells were imaged 48 h after EB1-GFP transfection and the number of EB1-GFP comets emerging from the centrosome per minute were counted. A box plot summing 3 independent experiments and 20 - 25 cells per treatment is shown. ***denotes $p < 0.0001$.

Figure 4.

Depolymerizing MTs with nocodazole abrogates the G2 delay in HeLa cells. Cells were transfected with either siGLO control siRNA or siRNA targeting stathmin. Forty three - 45 h after transfection, cells were treated with DMSO, as a control, or 33 μ M nocodazole for 3-5 h prior to fixation and staining with antibodies to α -tubulin and TPX2 or phospho-CDK1(Y15) as noted. (A, B) Representative images of anti-tubulin stained cells fixed after transfection with siGLO (A) or stathmin siRNA (B), allowed to grow for ~45 h and incubated for 3 h in medium supplemented with 33 μ M nocodazole. MTs are completely depolymerized in this time frame. Scale bar = 20 μ m. (C, D) HeLa cells transfected with siRNAs as noted were fixed after 3 - 5 h incubation in medium supplemented with DMSO or 33 μ M nocodazole. Cells were stained with antibodies recognizing tubulin and either TPX2 or phospho-CDK1(Y15P). Representative images are shown in Figure S2. Interphase cells were differentiated from mitotic cells based on cell and nucleus morphologies. Plots are shown for the percent interphase cells staining positive for TPX2 (C) or phospho-CDK1(Y15) (D) as measures of cells in G2. The population of interphase cells scored as positive for a G2 markers increased when cells were depleted of stathmin, and this increase was reversed by incubation in 33 μ M nocodazole. Each plot represents the summary of 3 (C) or 4 (D) independent experiments $\pm$ SD. *denotes p < 0.01.

Figure 5.

MT depolymerization by 10 nM vinblastine abrogates the G2 delay in stathmin-depleted HeLa cells. HeLa cells were transfected with siGLO or siRNA targeting stathmin and incubated for ~45 h, and then incubated in medium supplemented with 10 nM vinblastine for 3 h. (A) Representative micrograph of anti-tubulin stained HeLa cells after a 3 h incubation in 10 nM vinblastine. MTs were completely depolymerized in either siGLO or stathmin siRNA transfected cells at this time point. (B) Fixed cells were stained with antibodies recognizing tubulin and TPX2. The percent of interphase cells staining positively for TPX2 was used as a marker for cells in G2. Plot of the percentage of interphase cells staining positively for TPX2 under the experimental conditions. Data shown are the mean of 3 independent experiments $\pm$ SD. *denotes $p < 0.01$, **denotes $p < 0.001$.

Figure 6.

Partial MT depolymerization in 100 nM nocodazole abrogates the stathmin-depletion induced G2 delay in HeLa cells. (A).

Representative images of MT organization in control siRNA transfected cells after 3 h incubation in DMSO or 100 nM nocodazole. Stathmin-depleted cells showed a similar reduction in MT polymer. (C). Cells treated with 100 nM nocodazole were stained with TPX2 and the percent of positively stained interphase cells was counted as a measure of cells in G2. Incubation of stathmin depleted cells in 100 nM nocodazole for 3 h was sufficient to reduce the percentage of TPX2 positive interphase cells to approximately the percentage measured in control siRNA transfected cells, with or without nocodazole treatment. The plot represents the mean of 3 independent experiments $\pm$ SD. ***denotes $p < 0.0001$, **denotes $p < 0.001$.

Figure 7.

MT stabilization by incubation in 8 nM paclitaxel does not lengthen interphase duration in HeLa cells. (A) MT organization in cells incubated for 24 h in medium supplemented with 0.1% DMSO (left) or 8 nM paclitaxel (right). MT density around the nucleus appears greater in paclitaxel treated cells. (B) Box plots of interphase and mitotic durations. Mitotic durations were significantly longer in paclitaxel (***) denotes $p < 0.0001$). Note the break in the Y-axis for mitotic durations for comparison of DMSO and paclitaxel-treated cells. Data shown are pooled from 3 independent experiments and > 100 cells. (C) Plot of mitotic duration vs. the subsequent interphase duration for individual cells. The duration of mitosis does not correlate with the duration of the next interphase (slope of linear curve fit = 0.02, R^2 value equal to zero).