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# Perturbation of the Chromosomal Binding of RCC1, Mad2 and Survivin Causes Spindle Assembly Defects and Mitotic Catastrophe

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## ABSTRACT

Mitotic catastrophe is a form of cell death that results from aberrant mitosis. Currently, the mechanisms involved in this form of cell death remain poorly understood. We found that actinomycin D induces mitotic catastrophe with severe spindle assembly defects. We have studied the nature of three groups of chromosome binding proteins in mitotic cells treated with actinomycin D. We found that actinomycin D reduced the binding affinity of RCC1 to the mitotic chromosome, which led to a reduction of RanGTP level. In addition, Mad2 was not concentrated at the kinetochores, indicating that the mitotic spindle checkpoint was affected. Furthermore, the localization of survivin was altered in cells. These data suggested that chromosomal binding of the mitotic regulators such as RCC1, Mad2 and survivin is essential for mitotic progression. Mitotic chromosomes not only carry the genetic material needed for the newly synthesized daughter cells, but also serve as docking sites for some of the mitotic regulators. Perturbation of their binding to the mitotic chromosome by actinomycin D could affect their functions in regulating mitotic progression thus leading to severe spindle defects and mitotic catastrophe. J. Cell. Biochem. 105: 835–846, 2008. 

**KEY WORDS:** SPINDLE ASSEMBLY; RCC1; SURVIVIN; MAD2; MITOTIC CATASTROPHE; ACTINOMYCIN D

Mitotic catastrophe is a form of cell death that results from aberrant mitosis. It has been described in the literature that mitotic catastrophe is death that occurs during mitosis, resulting from a combination of deficient cell cycle checkpoints and cellular damage [Castedo et al., 2004]. The definitions of mitotic catastrophe encompass a variety of phenotypes including those presenting with cellular demise in mitosis or following G1 exit after an aberrant mitosis. Generally, such cells may present with multi- or micro-nucleated phenotype after errors in the checkpoint sensing mechanism or die with hallmarks of apoptosis in cases that cell cycle checkpoints have been bypassed. Very little is known about the mechanism(s) that underlies these responses. Nevertheless, it is agreed upon that cell cycle checkpoints come into play upon sensing of genotoxic insult and hold the cell in arrest until eventual activation of cell death pathways. After mitotic arrest, a cell could exit mitosis to enter G1 followed by reentering the cell cycle, senescence, or apoptosis. Alternatively, a cell might die during a mitotic arrest [Weaver and Cleveland, 2005]. Recently, it has been demonstrated that Polo-like kinase 1 (Plk1) activity is inhibited through dephosphorylation of Plk1 when DNA is damaged in mitosis [Jang et al., 2007]. In addition, a chromosome passenger protein, survivin, is important in protecting the cells from entering mitotic catastrophe [Carvalho et al., 2003; Lens et al., 2003]. These studies suggested that mitotic regulators could play important roles in regulating mitotic catastrophe.

The mitotic chromosome is a highly condensed entity and many of the mitotic regulators are found on it. While cohesins hold sister chromatids together by encircling two DNA duplexes within its coiled-coil arms, condensin I and II regulate mitotic chromosome condensation through provision of a centrally located protein scaffold [Losada and Hirano, 2005; Belmont, 2006]. In addition, chromokinesins, HKIF4A and Kid, bind to chromosome arms. Depletion of HKIF4A has been shown to result in mitotic defects such as chromosome misalignment and spindle abnormalities [Mazumdar et al., 2004; Zhu et al., 2005].

Abbreviations used: GDP, guanosine diphosphate; GTP, guanosine triphosphate; GEF, guanine nucleotide exchange factor; DMEM, Dulbecco's Modified Eagles's Medium; DAPI, 4,6-diamidino-2-phenylindole.

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Chromatin-bound RCC1 generates a mitotic RanGTP gradient to regulate spindle formation. Ran is a small GTPase, which exists in GDP- and GTP-bound forms regulated by guanine nucleotide exchange factor for Ran, RCC1 and RanGTPase activating protein, RanGAP1, respectively. During mitosis, a number of spindle assembly factors are sequestered by importin β binding. RanGTP generated by chromatin-bound RCC1 releases the spindle assembly factors from the inhibitory binding of importin β near the chromosomes [Trieselmann and Wilde, 2002; Tsai et al., 2003; Gruss and Vernos, 2004]. Recently, it has been shown that dynamic interaction of RCC1 with the mitotic chromosome is essential to generate RanGTP gradient in mitosis. RCC1 interacts with histone H2A/H2B of the nucleosomes. This specific interaction enhances RCC1 GEF activity by 20% [Nemergut et al., 2001]. In addition, a model has been proposed in which the binary complex of RCC1-Ran has better affinity for the core histones on the chromatin than RCC1 or Ran alone. The stable binding of the binary complex could allow coupling of nucleotide exchange. Upon successful nucleotide exchange, the two proteins would have reduced binding affinity thus enabling them to dissociate from chromatin, driving RanGTP production on the chromatin surface [Li et al., 2003, 2007]. Post-translational modification of RCC1 could regulate its binding to the chromatin, which in turn affects the RanGTP production. For instance, phosphorylation of RCC1 at serine 11 enhances the binding affinity of RCC1 to the chromatin by preventing the inhibitory binding of importin β [Li and Zheng, 2004; Li et al., 2007]. N-terminal α-methylation of RCC1 is required for stable chromatin association in order to generate RanGTP to sustain the formation of spindle during mitosis [Chen et al., 2007].

Kinetochore constituents such as those involved in checkpoint signaling (Mad and Bub proteins) and those comprising the mammalian chromosomal passenger complex are dynamic entities that coordinate numerous events driving mitotic progression. Their dynamic behavior and unique localization on the mitotic apparatus are two criteria central to their respective functions [Maiato et al., 2004].

Checkpoints constitute a surveillance mechanism to ensure the fidelity of chromosome segregation [Shah and Cleveland, 2000; Musacchio and Hardwick, 2002; Maiato et al., 2004]. Checkpoint proteins localize to the outer plate of the kinetochore, a large assembly of >60 proteins, where they monitor microtubule attachments to kinetochores and mediate checkpoint signaling. The checkpoint signal is generated at the kinetochore and as few as one unattached kinetochore is sufficient to initiate checkpoint activation [Chan and Yen, 2003; Maiato et al., 2004]. The spindle checkpoint proteins Bub1, Bub3, Mad1, Mad2 and BubR1 only localize to kinetochores during mitosis [Shah and Cleveland, 2000; Musacchio and Hardwick, 2002]. The chromosomal passenger complex (CPC) in humans comprise of four members, that is, the aurora B serine/threonine kinase, and three non-enzymatic subunits survivin, the inner centromere protein (INCENP) and Borealin/Dasra-B. The CPC proteins show distinct localization to the mitotic apparatus: they move towards the inner centromeric chromatin from chromosome arms during prometaphase, relocate to the central spindle at the metaphase–anaphase transition, and finally concentrate at the midbody during telophase/cytokinesis [Fortugno et al., 2002]. This localization is correlated to their role in orchestrating various processes during mitosis, such as chromosome alignment, histone modification, and cytokinesis. Central to these functions is the enzymatic activity of aurora B kinase, which in turn relies on proper and timely localization of this complex throughout cell division [Carvalho et al., 2003].

In this report, we found that actinomycin D induces mitotic catastrophe with severe spindle defects. Actinomycin D is an antibiotic produced by *Streptomyces antibioticus*. This molecule consists of a phenoxazone ring system to which two cyclic pentapeptides are attached. The aromatic ring system is well apt to intercalate into DNA, preferably at GC steps [Hou et al., 2002]. This mode of action interferes with the action of the transcriptase and replicase complexes and also prevents chromosome-binding proteins from interacting with chromatin. Interestingly, we found that chromosomal binding of mitotic regulators such as RCC1, Mad2 and survivin is essential for mitotic progression. Perturbation of these interactions by actinomycin D would lead to failure of mitosis and thus induce mitotic catastrophe.

## MATERIALS AND METHODS

### CELL CULTURE, TRANSFECTION AND DRUG TREATMENTS

HeLa cells were maintained in DMEM GlutaMax medium (Gibco, Invitrogen) containing 10% fetal calf serum (Hyclone), and 1% Penicillin/Streptomycin at 37°C in a humidified atmosphere with 5% carbon dioxide. Transfection was done using Lipofectamine® Reagent (Invitrogen) according to manufacturer's protocol. 1 μg of plasmid DNA was used per transfection reaction. For drug treatment, HeLa cells were treated with 600 nM nocodazole (Sigma, MO) for 6 h and drug washout was done by incubating the cells in DMEM for 30 min with three changes of medium. For actinomycin D (Sigma) treatment, cells were supplemented with 400 nM actinomycin D 2 h into nocodazole treatment and following nocodazole washout, cells were allowed to recover in the presence of 400 nM actinomyin D.

### IMMUNOFLUORESENCE STAINING AND TIME LAPSE MICROSCOPY

For immunofluorescence staining, cells were collected and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and permeabilized with 0.5% Triton X-100 for 5 min at room temperature. Primary antibodies in 4% bovine serum albumin in Tris-buffered saline (TBS) with 0.05% Tween 20 were incubated for 1 h at room temperature or overnight at 4°C. Cells were washed three times and incubated with appropriate secondary antibodies for 1 h at room temperature. Slides were mounted in Prolong Gold Anti-Fade medium with DAPI (Invitrogen). Images were collected and analyzed on an Axiovert 200 M inverted fluorescence microscope (Carl Zeiss Inc., Germany). For live cell imaging, an Axiovert 200 M inverted fluorescence microscope (Carl Zeiss Inc.) equipped with a 10× phase contrast objective (Carl Zeiss Inc.) was used. Cells were maintained at 37°C with 5% $CO_2$ for the duration of the time-lapse acquisition. Images were recorded at 5 min intervals and analyzed using the Axiovision software (Carl Zeiss Inc.). For imaging of live cells expressing RCC1-GFP, images were obtained using same exposure

times for each channel, offsetting any effect the difference in expression levels might have on the outcome.

### FLUORESCENCE LOSS IN PHOTOBLEACHING

Cells were plated on a coverslip and mounted onto a glass slide with a depression containing culture medium. FLIP were performed on a Zeiss 510 LSM confocal microscope (Carl Zeiss Inc.) using the 488 nm laser line of an argon laser. For FLIP experiments, five single scans were acquired, followed by repeated photobleaching using a single bleach pulse at intervals of 1 s for 200 iterations in defined regions. Single section images were then collected at 1 s intervals. For imaging, the laser power was attenuated to 1% of the bleach intensity. The relative fluorescence intensity in a region of interest was determined by normalizing fluorescence intensity in the region to the total fluorescence in the same region during prebleach.

### FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET)

FRET detection was performed on a Zeiss 510 LSM confocal microscope (Carl Zeiss Inc.) via the Acceptor Photobleaching method of FRET detection. Fluorescence of donor cyan fluorescent protein (CFP) and acceptor yellow fluorescent protein (YFP) in the YIC probe both before and after photobleaching of the YFP fluorophore were recorded. CFP was excited by 458 nm light and the emission was collected through an HQ 470–500 nm bandpass filter. YFP was excited by 514 nm light and the emission was collect through an HQ 525–575 nm bandpass filter. Photobleaching of YFP was performed by repeatedly scanning a region of the specimen with the 514 nm laser line set at maximum intensity to photobleach the original acceptor fluorescence. The fluorescence emission from the donor and the acceptor are collected sequentially using the multitrack function of the Zeiss LSM 510 software. Average fluorescence intensities of the donor are measured before and after bleaching. FRET efficiency was calculated as

$$E_F\,(\%) = (I_6 - I_5) \times \frac{100}{I_6}$$

where $I_5$ and $I_6$ represent the CFP fluorescence intensity of the fifth and sixth images immediately before and after photobleaching of YFP (Karpova et al., 2003). The change in donor fluorescence was assessed in at least 10 cells for each experimental set. Mean FRET efficiencies were then reported ± standard error.

## RESULTS

### ACTINOMYCIN D INDUCES MITOTIC CATASTROPHE IN HeLa CELLS

We have tested a panel of cytotoxic drugs to induce mitotic catastrophe. Surprisingly, we found that actinomycin D was able to cause cell death in mitosis, as opposed to its effect on interphase cells that seemed predominantly apoptotic after prolonged treatment. To investigate the effect of the actinomycin D on mitotic cells, we arrested HeLa cells stably expressing histone H2B-GFP in mitosis by incubating them in 600 nM nocadozole for 2 h before actinomycin D was added. The cells were released from nocadozole arrest by thorough washing with normal medium and recovery was followed using time-lapse microscopy in the absence or presence of actinomycin D (Fig. 1A). We found that treatment of actinomycin D caused significant cell death during mitosis or cytokinesis (~70%). In contrast, there was only about 12% of cell death during mitosis or cytokinesis in control experiments. H2B-GFP serves as a marker to visualize chromatin. Several anomalies were observed more frequently in actinomycin D treated cells. Some cells reverted to interphase with multinucleated phenotype while others appear to die in G1 phase after cell division has occurred, however, the segregation of chromosomes was clearly asymmetric. We also observed cases in which actinomycin D treated cells undergo division before proper metaphase alignment had been achieved, resulting in mis-segregation and finally death (Fig. 1B,C).

### ACTINOMYCIN D AFFECTS SPINDLE ASSEMBLY DURING MITOSIS

It is prudent to speculate that disrupted spindle assembly might plausibly account for the cell death in mitotic progression. Cells were fixed and stained with histone H3 phosphorylated at Ser10 and tubulin after nocodazole washout at the indicated time points as shown in Figure 2A. Phosphorylation of histone H3 occurs exclusively as an event in mitosis. Hence, pH3 serves as a mitosis marker. The cells were co-stained with anti-α tubulin antibodies to visualize the spindle.

We found that most of the cells treated with actinomycin D remained at prometaphase whilst majority of the control cells went on to complete cell division within 2 h after nocadozole washout (Fig. 2B). This observation is consistent with time lapse experiments performed earlier. Approximately, actinomycin D treatment caused two folds more of cells exhibiting severe spindle assembly defects as compared with control one hour after nocadozole washout (Fig. 2C). Furthermore, most of the kinetochores were not attached to microtubules in cells treated with actinomycin D. This is probably due to a decrease in kinetochore fibers emanating from the spindle poles that were able to capture chromosomes successfully. These data suggested that actinomycin D inhibited spindle assembly during mitosis (Fig. 3).

It has been well documented that actinomycin D inhibits transcription by preventing transcription factors from binding to the chromosomes thus leads to apoptosis. Structural analysis also revealed that actinomycin D intercalates to the DNA through the GC steps [Hou et al., 2002]. We believed that actinomycin D could intercalate to the DNA of mitotic chromosomes and thereby knocking off some of the essential chromosomal binding proteins for spindle assembly. To test this hypothesis, the nature of several chromosomal binding proteins, which are essential for spindle assembly and mitotic progression, were analyzed.

### ACTINOMYCIN D DISRUPTS RCC1 MOBILITY AND RanGTP PRODUCTION

Recent studies have revealed that a signaling pathway mediated by RanGTPase plays an important role in mitosis [Li et al., 2003]. In *Xenopus* egg extracts, microtubule dynamics and spindle assembly are dependent upon the concentration of RanGTP [Dasso, 2002]. It is believed that chromatin-associated RCC1 protein creates an increased local concentration of RanGTP in the vicinity of chromosomes, helping to direct the formation of bipolar spindles.

RCC1 is associated with the chromosome throughout the cell cycle [Li et al., 2003]. The N-terminus of RCC1 binds to DNA and the

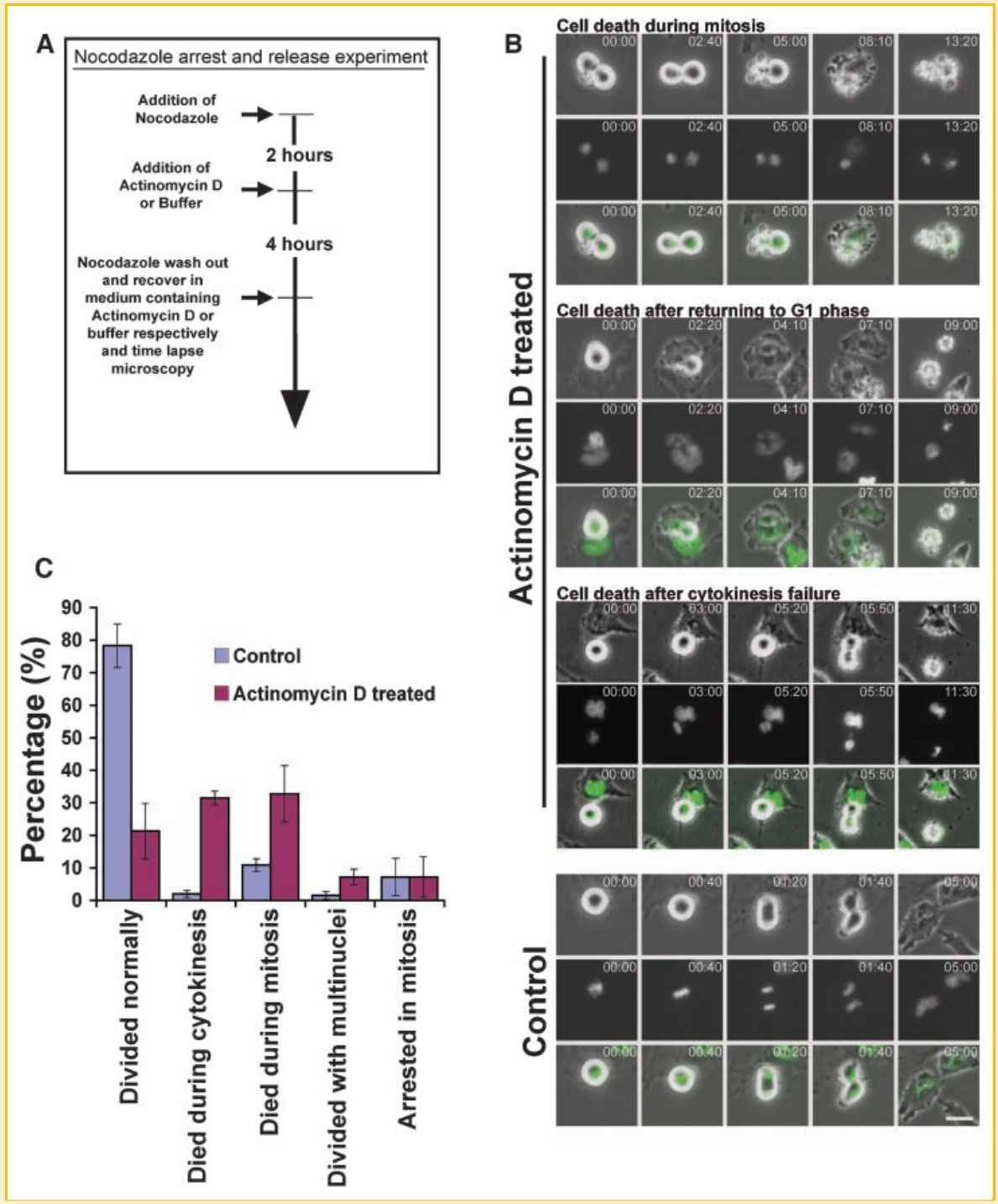


Fig. 1. Actinomycin D induces mitotic catastrophe in HeLa cells. A: Schematic diagram showing the nocodazole arrest and release experiment. Cells were incubated with 600 nM nocodazole for 2 h and 400 nM actinomycin D was added for an additional 4 h before nocodazole washout was done by washing with complete medium. Recovery was allowed in the presence or absence of 400 nM actinomycin D. B: Time lapse experiment was carried out using a H2B-GFP stable cell line. Representative cells displaying different phenotypes in control and actinomycin D treated samples are shown in phase contrast, GFP and merged views. Scale bar indicates 20 μm. C: Quantification of various phenotypes observed. An average of 100 mitotic cells were quantified in three independent time-lapse experiments.

rest of the protein associates with chromatin via an interaction with histones H2A and H2B. Binding of the histones stimulates a modest increase in RCC1's activity as Ran's GEF [Nemergut et al., 2001]. Recently, it has been suggested that the binary complex of RCC1-Ran has better affinity for the core histones on the chromatin than RCC1 or Ran alone [Li et al., 2003]. The stable binding of the binary complex could allow coupling of nucleotide exchange to the chromatin. Upon successful nucleotide exchange, the two proteins

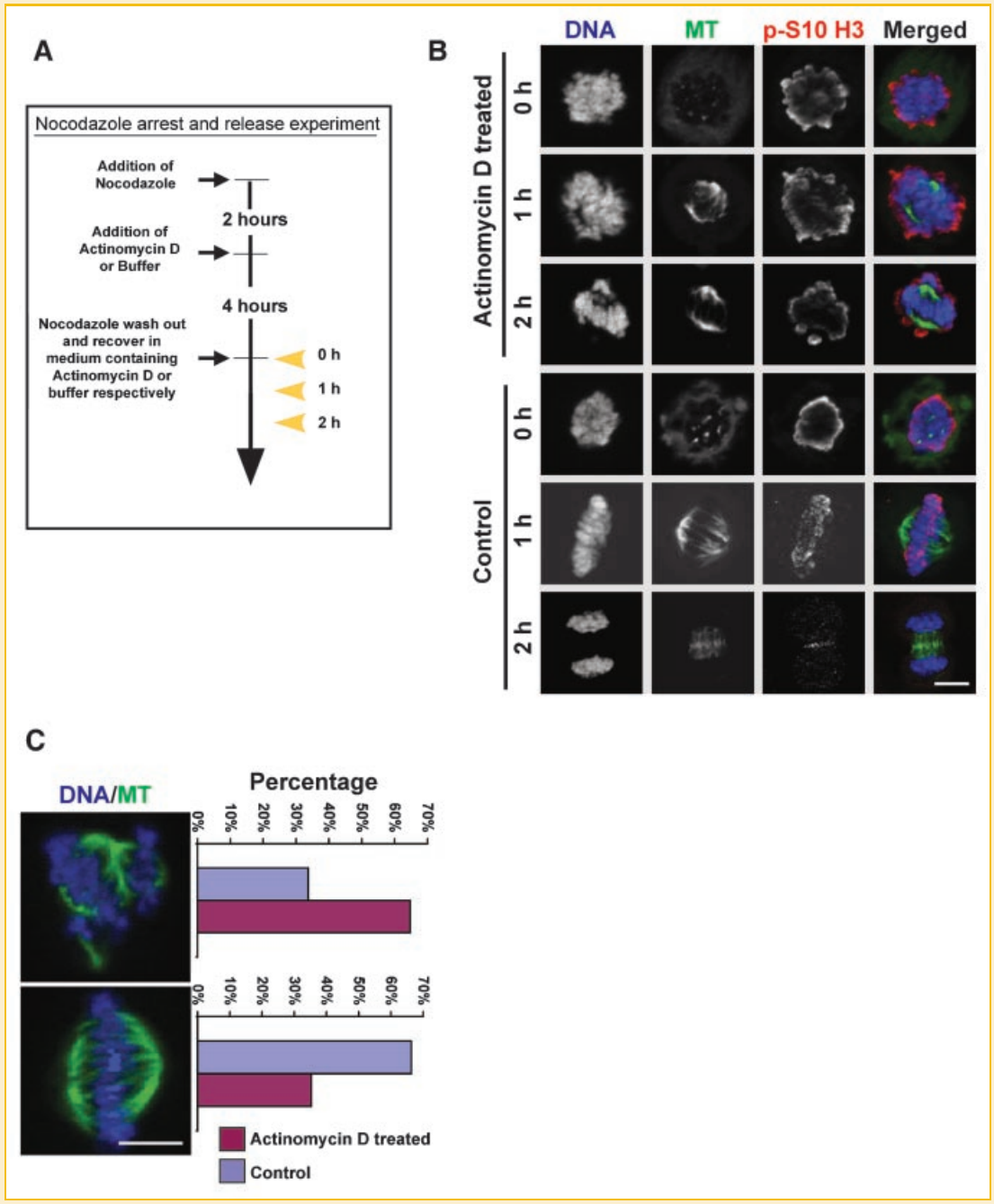


Fig. 2. Actinomycin D affects spindle assembly during mitosis. A: Schematic diagram showing the nocodazole arrest and release experiment. B: Immunofluorescence images of representative cells stained for microtubules (green), phosphorylated histone H3 (red) and DNA (blue) at different time points indicated after drug treatment. C: Spindle morphologies in control and actinomycin D treated cells. Spindles are shown in green and DNA is shown in blue. The percentages of normal bipolar spindles (upper row) and abnormal spindles (bottom row) were calculated. Scale bar indicates 10 μm.

would have reduced binding affinity thus enabling them to dissociate from chromatin, driving RanGTP production on the chromatin surface.

It is possible that actinomycin D interferes the binding of the N-terminus of RCC1 to the mitotic chromosome by intercalating to DNA. Consequently, RanGTP production could be suppressed and thus spindle assembly could be also affected. To study the effect of actinomycin D on the nature of RCC1, we examined the kinetic behavior of RCC1-green fluorescent protein (GFP) fusion protein in HeLa cells. The RCC1-GFP construct was transfected into HeLa cells. Statistically, the average total intensity are comparable, suggesting the expression levels of RCC1-GFP in cells treated with or without actinomycin D are similar (Fig. 4C). Interestingly, however, we found that RCC1-GFP was concentrated on the mitotic chromo-

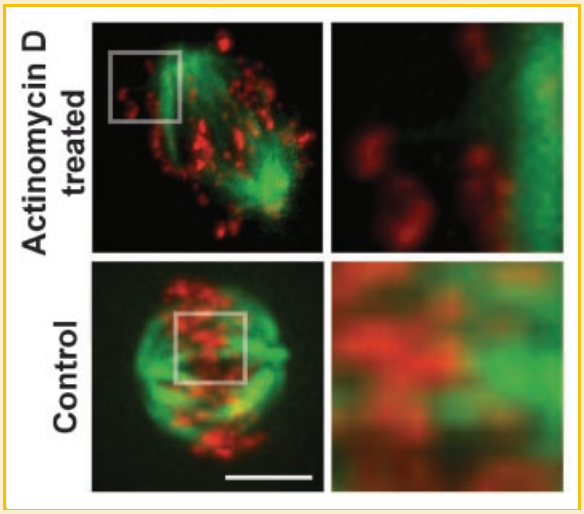


Fig. 3. Microtubule attachments to kinetochores appear to be disrupted in actinomycin D treated cells. Microtubules are shown in green and the centromere marker ACA in red. Insets show magnified regions boxed in white. DAPI staining is not shown. Scale bar indicates 10 μm.

somes in control cells whereas it was found in both mitotic cytosol and on the mitotic chromosomes in actinomycin D treated cells (Fig. 4A,B). This suggested that the binding affinity of RCC1-GFP to mitotic chromosomes was reduced in the presence of actinomycin D. To verify this idea, we examined dissociation kinetic of RCC1-GFP by Fluorescence Loss in Photobleacing (FLIP). In a FLIP experiment, a fluorescent cell is repeatedly photobleached within a small region while sequential images of the whole cell are continuously collected. Upon actinomycin D treatment, an increase in the rate of fluorescence loss was observed as compared with control, indicating that actinomycin D increased the dissociation kinetic of RCC1-GFP from the mitotic chromosomes (Fig. 4E,F). Additionally, immunoblots for Ran, RCC1 and importin β in mitotic cells showed that their protein levels remained constant for both control and actinomycin D treated cells (Fig. 4D).

We next investigated whether RanGTP production was affected in actinomycin D treated cells. We used YIC as biosensor to detect the presence of RanGTP in the mitotic cells. The YIC biosensor is held in an extended conformation by binding of importin β in the absence of RanGTP, inhibiting occurrence of FRET. In the presence of RanGTP, the importin is released. Following which, donor-acceptor pair can interact and give rise to FRET. Cells were transfected with YIC followed by fluorescence resonance energy transfer (FRET) experiments. FRET experiments were performed by the acceptor photobleaching technique. In acceptor photobleaching FRET, energy transfer between the donor and acceptor is reduced or eliminated when the acceptor is irreversibly bleached, resulting in an increase in donor fluorescence as an indicator of physical interaction between the test proteins [Kalab et al., 2002; Karpova et al., 2003]. Upon drug treatment, we found that there was a significant reduction in FRET efficiency as compared to control (Fig. 4G).

Collectively, these results indicated that RanGTP production is obliterated in HeLa cells treated with actinomycin D, presumably via its effect in the interruption of RCC1 binding to mitotic chromosomes. This, in turn, could result in continual sequestration of spindle assembly factors, which explains the deformed spindles and prolonged arrest.

## ACTINOMYCIN D DISPLACED Mad2 FROM THE KINETOCHORES

The spindle assembly checkpoint has been proposed to be bipartite, with one arm monitoring kinetochore occupancy with microtubules [Waters et al., 1998] and the other spindle tension exerted on kinetochores [Taylor et al., 2001]. Extended prometaphase in actinomycin D treated cells suggested that mitotic delay could be due to activation of the spindle assembly checkpoint. Activation of spindle assembly checkpoint signaling is initiated by recruitment of a stably bound Mad1/Mad2 complex to unattached kinetochore early in prometaphase [Chung and Chen, 2002; De Antoni et al., 2005]. Spindle checkpoint regulators accumulate on unattached kinetochores in close correlation with checkpoint activation. As few as one unattached kinetochore is sufficient for checkpoint activation [Rieder et al., 1995; Rieder et al., 1997].

It has been reported that premature spindle checkpoint inactivation causes cells to exit mitosis with multi-nuclei or mitotic cell death [Castedo et al., 2004]. We reasoned that actinomycin D could interfere with the spindle checkpoint by preventing checkpoint proteins from binding to the unattached kinetochores and thus initiate mitotic cell death as shown in Figure 1B,C. To test this idea, we performed double immunostaining against the centromere marker ACA and checkpoint protein Mad2 or BubR1. Kinetochores assembled on chromatin in nocodazole-arrested HeLa cells can be visualized as discrete foci by immunostaining with antibodies against centromeric proteins (ACA). Addition of actinomycin D did not alter ACA staining in any detectable manner, suggesting that the underlying structure of the kinetochore remains intact.

In accordance to its role in checkpoint signaling, Mad2 and BubR1 are depleted from kinetochores after microtubule capture and alignment. The overall Mad2 or BubR1 level at prometaphase kinetochores was determined by normalizing their immunofluorescence intensities against ACA immunofluorescence intensity. We found that Mad2 was predominantly localized to the kinetochores in control cells, indicating that Mad2 is retained at kinetochores due to the lack of microtubule attachment at the kinetochores. In contrast, there was a significant reduction of Mad2 levels in cells treated with actinomycin D. (Fig. 5A,B,E). To further dissect the mechanism which lies behind this phenomenon, we also examined levels of BubR1, a known regulator responsible for recruiting Mad2 in early prometaphase. BubR1 levels seemed to be unaffected after actinomycin D treatment (Fig. 5C–E). Expression levels for both proteins were confirmed by immunoblotting (Fig. 5F). We believe that kinetochores devoid of Mad2 could indicate that attachments can be established in the presence of actinomycin D. We have previously shown that microtubule attachments to kinetochores were more often found to be defective in the presence of actinomycin D (Fig. 3). This led us to the hypothesis that

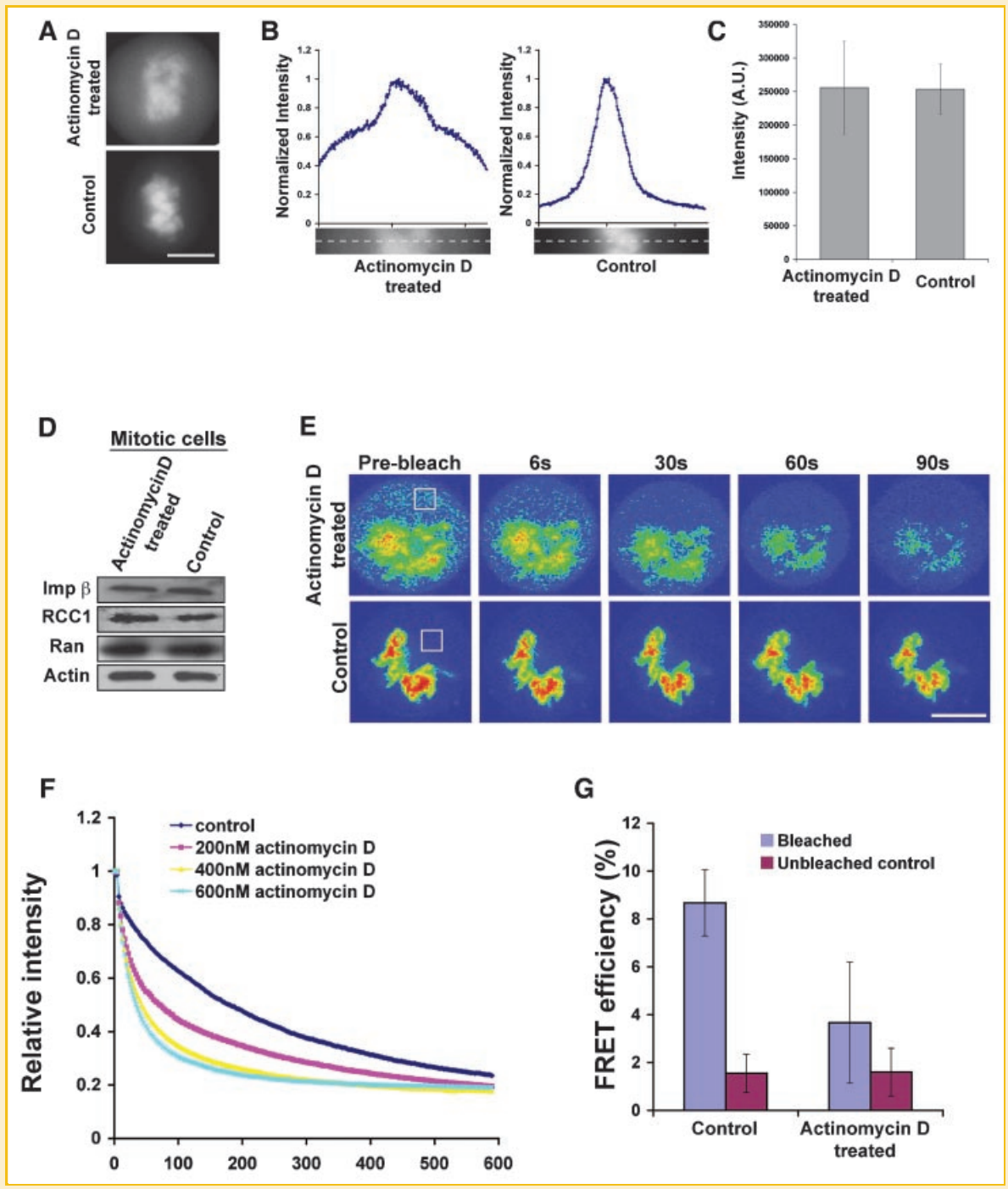


Fig. 4. Actinomycin D disrupts RCC1 mobility and RanGTP production. A: Representative images of HeLa cells transfected with RCC1-GFP for control and actinomycin D treated cells. B: Intensity profiles of live cells expressing RCC1-GFP for control and actinomycin D treated cells. C: Fluorescence intensity (AU) of whole cell expressing RCC1-GFP for both actinomycin D treated cells or untreated cells were measured. Intensities were comparable between actinomycin D treated cells and control cells. D: Western blotting shows expression levels of Ran, RCC1 and importin β in mitotic cells to remain constant both in control and in actinomyin D treated sets. E: A spot marked by a white box in the mitotic cytosol was repeatedly photobleached in HeLa cells expressing RCC1-GFP. Representative FLIP images are shown. F: Fluorescence intensity of the RCC1-GFP was quantified after each bleach pulse and plotted as relative intensity versus time. RanGTP production is affected under drug treatment. G: Histogram shows FRET efficiency calculated for mitotic cells in the absence and presence of actinomycin D. Error bars indicate standard deviation. Scale bar indicates 10 μm.

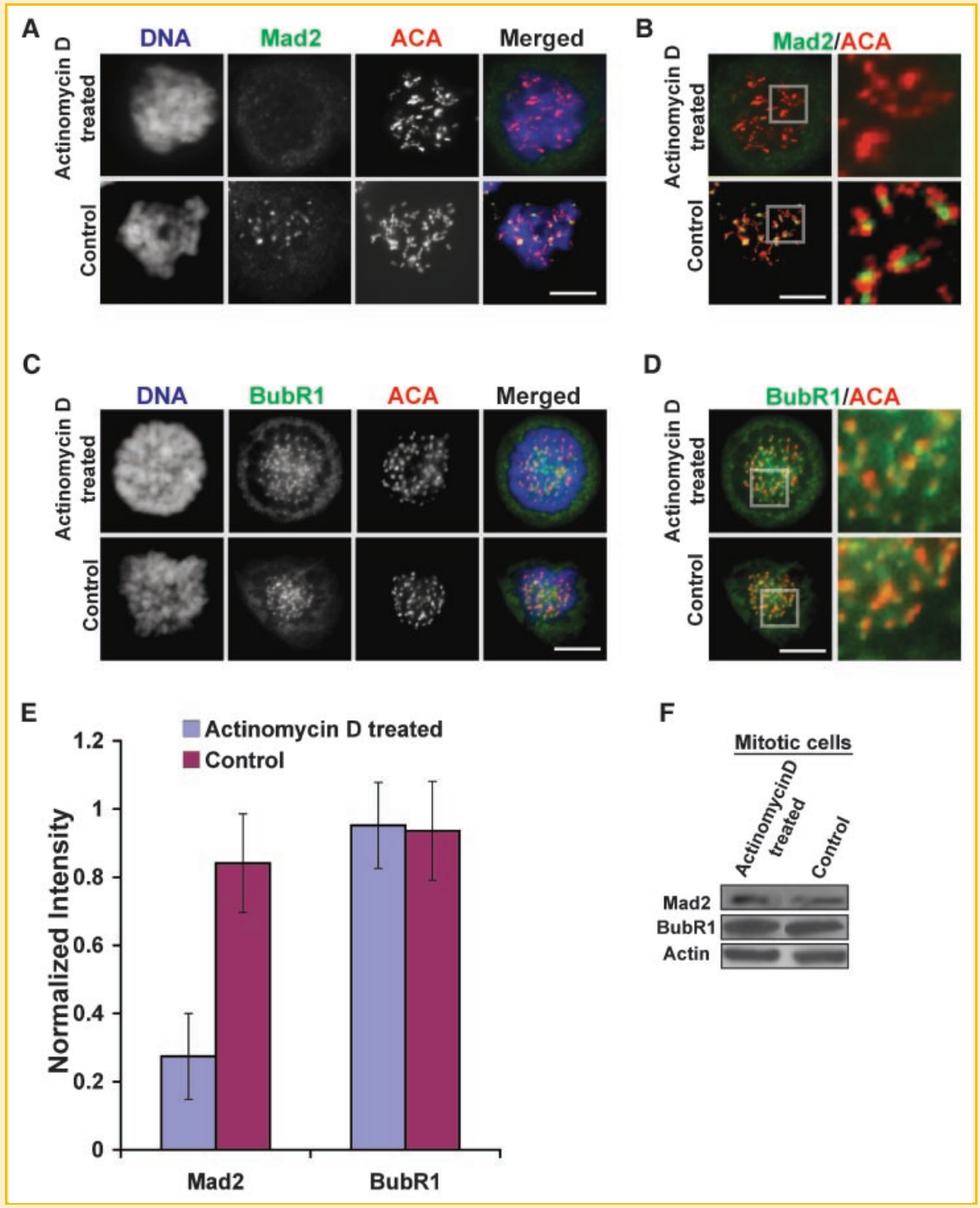


Fig. 5. The spindle checkpoint signal is sustained by the BubR1-mediated but not the Mad2-mediated pathway. Immunofluorescence images of representative cells stained for Mad2 (A) or BubR1 (C) (green), centromere marker ACA (red) and DNA (blue) in control and cells after drug treatment. B,D: Selected kinetochores were magnified to show Mad2/BubR1 levels on kinetochores. E: An average of 20 cells was selected for quantification purposes and fluorescence intensity of Mad2 and BubR1, respectively, was normalized against ACA intensity and presented as a ratio. Error bars indicate standard deviation. F: Western blotting shows expression levels of Mad2 and BubR1 in mitotic cells to remain constant both in control and in actinomyin D treated sets. Scale bar indicates 10 μm.

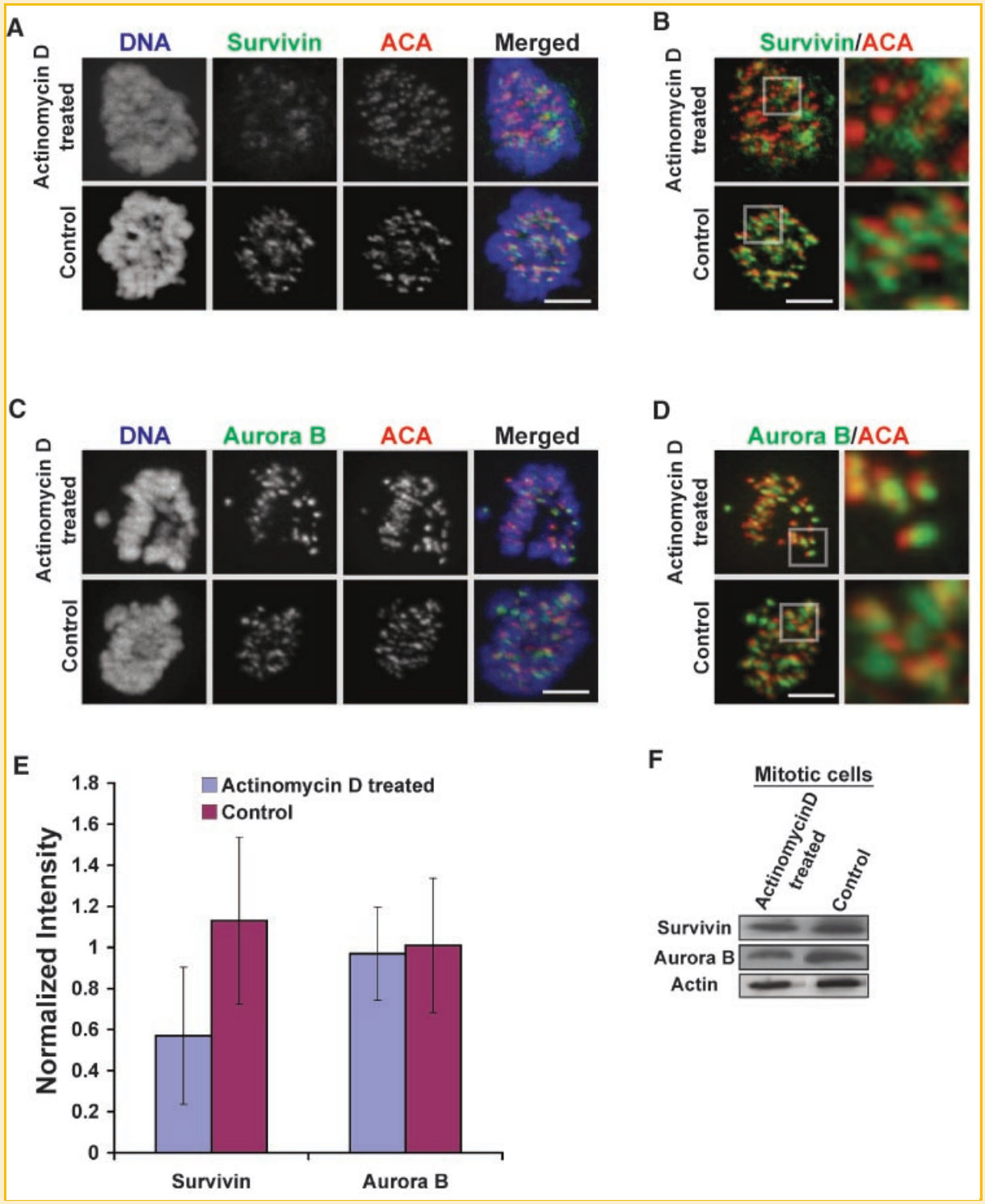


Fig. 6. Actinomycin D disrupts survivin localization in mitotic cells. Immunofluorescence images of representative cells stained for survivin (A) or aurora B (C) (green), centromere marker ACA (red) and DNA (blue) in control and cells after drug treatment. B,D: Selected kinetochores were magnified to show survivin/aurora B levels on kinetochores. E: An average of 30 cells was selected for quantification purposes and fluorescence intensity of survivin and aurora B, respectively, was normalized against ACA intensity and presented as a ratio. Error bars indicate standard deviation. F: Western blotting shows expression levels of survivin and aurora B in mitotic cells to remain constant both in control and in actinomyin D treated sets. Scale bar indicates 10 μm.

kinetochores are unable to recruit comparable levels of Mad2, which could signify a weak checkpoint signal that is easily bypassed, resulting in premature exit from mitosis or initiation of mitotic cell death (Fig. 1B,C).

### LOCALIZATION OF SURVIVIN WAS ALTERED UPON ACTINOMYCIN D TREATMENT

Previous reports have shown that a number of chromosomal passenger proteins play important roles in the spindle checkpoint, both in terms of recruitment of various checkpoint proteins such as Mad2 and BubR1, and also for correction of attachment errors [Vagnarelli and Earnshaw, 2004].

Together with INCENP and Borealis, survivin and aurora B form the chromosome passenger complex in human. We examined the localization of survivin and aurora B in cells treated with actinomycin D. We performed double immunostaining against the centromere marker ACA with either survivin or aurora B during mitosis. We observed an increased number of kinetochores positive for ACA with diffused survivin staining in actinomycin D treated cells. However, there was a strong focused survivin staining at the kinetochore in control cells (Fig. 6A,B,E). On the other hand, Aurora B staining did not show striking differences upon drug treatment (Fig. 6C–E) whereas both survivin and aurora B levels did not change after drug treatment (Fig. 6F). Thus, actinomycin D treatment disrupted survivin localization but not aurora B, which could lead to chromosome mis-segregation and aberrant mitosis.

## DISCUSSION

Mitotic catastrophe can result from a combination of deficient mitotic checkpoints in tumor cells, anti-microtubular drugs and premature mitosis. It has been proposed that the irreversibly damaged cell has a broad repertoir of ''final options'' that allow it to react to certain stressors, according to the multiposition switch model of cell death [Abend, 2003]. This idea is consistent with the concept that a decrease in apoptosis is compensated for by increase in the fraction of cells that undergo permanent growth arrest with phenotypic features such as that of mitotic catastrophe [Roninson et al., 2001].

In interphase cells, actinomycin D causes cell death by repressing gene transcription through its intercalating action into DNA. In mitotic cells, however, gene expression is minimal mainly due to the highly compressed state of chromatin in forming the mitotic chromosomes. Thus, the concerns of actinomycin D usage in interphase cells do not greatly limit our model in this respect.

In this report, we have studied contributions of various mitotic regulators in the event of prolonged arrest as a consequence of disrupted spindle assembly followed by mitotic catastrophe. RanGTP has been reported independently in many sources to play a role in providing a directional bias in guiding microtubules to kinetochore regions [Gruss et al., 2001; Gruss and Vernos, 2004]. A model for RCC1 function, the sole guanine nucleotide exchange factor for Ran, describes a coupled mechanism between interaction with chromosomes and production of RanGTP [Li et al., 2003]. RCC1 binds to chromatin via its N-terminal region. It is possible that administration of a general DNA intercalator might inhibit the binding of RCC1 to chromatin thus impeding its function.

Many proteins that function during mitosis relocate to the mitotic apparatus after nuclear envelope breakdown. The prolonged arrest could be due to activation of the spindle checkpoint regulated by the BubR1 arm [Skoufias et al., 2001; Shannon et al., 2002]. The spindle checkpoint kinase BubR1 was detected at kinetochores during prometaphase while Mad2 levels at kinetochores appeared to be significantly reduced in actinomycin D treated cells. Mad2 is a highly conserved protein from yeast to vertebrates. This is consistent with its pivotal role in mitotic checkpoint signaling. The HORMA domain (for Hop1p, Rev7p and MAD2) was found to be conserved and homology has been established in yeast proteins involved in DNA repair. HORMA-domain-containing proteins share a common feature, that is, they are able to associate directly with chromatin [Aravind and Koonin, 1998]. In sharp contrast, protein-protein interaction seems to be responsible for BubR1 recruitment to the checkpoint signaling complex [Johnson et al., 2004; Bolanos-Garcia et al., 2005]. Recruitment of checkpoint proteins has to occur in a timely and orderly fashion and through RNAi studies, the protein kinase Bub1 is localized to the kinetochores prior to other components, which follows in the sequence CENP-F, BubR1, CENP-E and Mad2 [Johnson et al., 2004]. However, the structural details of how the various components interact with one another have yet to be elucidated and more effort is called for in order to gain a more wholesome understanding of the detailed mechanism.

The chromosomal passenger proteins show unique localization patterns throughout different phases of mitosis [Adams et al., 2001]. They regulate multiple and diverse aspects of mitosis. Aurora B provides the enzymatic core whilst survivin dictates localization of the chromosomal passenger complex [Lens et al., 2006]. Deletion mutants of survivin lacking the Baculovirus IAP Repeat (BIR) domain failed to localize properly to the inner centromere [Vader et al., 2006]. Therefore, in a way, RCC1, Mad2 and survivin can be considered chromosome-binding proteins and it is logical that intercalation of actinomycin D into DNA disrupts their binding and in turn, affect their biological activity.

Proteins are dynamic entities. The cell assembles its paraphernalia prior to entering mitosis. Proteins move in an orchestrated fashion to fulfill their respective designated roles. This highlights the importance of the ability of certain proteins to relocate to specific locations in the cell or to act in a timely fashion for execution of vital processes needed for activation of downstream effectors. We studied the kinetic behavior of several proteins using photobleaching techniques. RCC1 catalyzes the production of RanGTP, which has multiple functions in the mitotic cell. Thus, our results demonstrate that protein mobility has far reaching consequences, especially in cases where catalysis is the outcome. Changes in protein mobility accompany changes in conformation during the trans-activation of enzymes, which in turn strongly correlate with the microscopic rates of substrate turnover [Eisenmesser et al., 2002; Hoofnagle et al., 2001].

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