Chromosomal gain promotes formation of a steep RanGTP gradient that drives mitosis in aneuploid cells

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Many mitotic factors were shown to be activated by Ran guanosine triphosphatase. Previous studies in Xenopus laevis egg extracts and in highly proliferative cells showed that mitotic chromosomes were surrounded by steep Ran guanosine triphosphate (GTP) concentration gradients, indicating that RanGTP-activated factors promote spindle assembly around chromosomes. However, the mitotic role of Ran in normal differentiated cells is not known. In this paper, we show that although the steep mitotic RanGTP gradients were present in rapidly growing cell lines and were required for chromosome congression in mitotic HeLa cells, the gradients were strongly reduced in slow-growing primary cells, such as HFF-1 fibroblasts. The overexpression of RCC1, the guanine nucleotide exchange factor for Ran, induced steeper mitotic RanGTP gradients in HFF-1 cells, showing the critical role of RCC1 levels in the regulation of mitosis by Ran. Remarkably, in vitro fusion of HFF-1 cells produced cells with steep mitotic RanGTP gradients comparable to HeLa cells, indicating that chromosomal gain can promote mitosis in aneuploid cancer cells via Ran.

Introduction

Mitotic entry is marked by a strong increase in the dynamic instability of microtubules (MTs; Zhai et al., 1996), leading to increased MT dependence on local regulation. During prometaphase (PM), chromosome-, kinetochore-, and centrosome-centered mechanisms direct the self-assembly of MTs into the mitotic spindle and facilitate correct MT connections to kinetochores on each chromosome (Walczak and Heald, 2008; Wadsworth et al., 2011). In one model explaining the rapid MT–kinetochore attachments, the growth of centrosomal MTs toward kinetochores is promoted by a chromosomal gradient of MT stabilization activity (Wollman et al., 2005). In another model, such chromosomal signals promote MT growth within the clusters of PM chromosomes, accelerating the initially lateral MT–kinetochore attachments in PM (Magidson et al., 2011). In both models, chromosomes could contribute to their mitotic segregation by activating spindle assembly factors (SAFs) through Ran GTPase (Clarke and Zhang, 2008; Kaláb and Heald, 2008).

The chromatin binding of RCC1, the guanine nucleotide exchange factor for Ran, and the cytoplasmic localization of RanGAP1 drive the rise of a concentration gradient of RanGTP surrounding the mitotic chromosomes. The binding of RanGTP diffusing from chromosomes to its ligands induces downstream gradients, including a gradient of SAFs activated by their RanGTP-induced release from importins (Kaláb and Heald, 2008). Although the RanGTP or RanGTP-regulated gradients were detected in meiotic Xenopus laevis egg extracts, maturing mouse oocytes, and tissue-culture cell lines (Kaláb et al., 2002, 2006; Caudron et al., 2005; Dumont et al., 2007), the mitotic role of Ran in normal differentiated cells is not known.

Results and discussion

Cell type-specific diversity of the mitotic RanGTP and importin-β cargo gradients

To determine whether the RanGTP gradient supports mitosis in all human somatic cells or is an adaptation specific to certain

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kinds of cells, we measured RanGTP gradients in a panel of human cells, including primary cells, immortalized normal cells, cancer-derived cells, and tumorigenic cells (Fig. 1 and Table S1). These measurements were performed with fluorescence lifetime imaging microscopy (FLIM) using two previously developed Förster resonance energy transfer (FRET) sensors (Kaláb et al., 2002, 2006) with the donor–acceptor pairs replaced by mTFP-1 (Ai et al., 2008) and dsREACH (Materials and methods). For both sensors, we used live-cell FLIM measurements of their donor fluorescence lifetime ($\tau_{\text{donor}}$) to calculate FRET efficiency $E$ using $E = 1 - \tau_{\text{donor}}/\tau_{\text{donor \, REF}}$ (Sun et al., 2011), in which the $\tau_{\text{donor \, REF}} = 2.519$ ps is the mean $\tau_{\text{donor}}$ of mTFP-1 expressed in cells in the absence of the acceptor (Fig. S1, E and F).

To measure free RanGTP, we used RBP-4 (RanGTP-binding probe-4, modified YFP–RanGTP-binding domain (RBD)–CFP; Kaláb et al., 2002), which indicates RanGTP binding by decreased $E$ (Fig. 1, A and B). We quantified the mitotic RanGTP gradient by subtracting the mean chromatin $E$ from the cytoplasmic $E$ (RBP-4 $E - E$; Fig. 1 E), and we used the inverse of cytoplasmic $E$ ($RBP-4 E^{-1}$) as a measure of cytoplasmic free RanGTP levels (Fig. 1 G). Separately, we used FLIM of Rango-4 (modified Rango; Kaláb et al., 2006) to measure the RanGTP-regulated gradient of free importin-$\beta$ cargoes. Because Rango-4 signals its RanGTP-induced release from importin-$\beta$ by increased $E$ (Fig. 1, C and D), we quantified the free cargo gradient by subtracting mean cytoplasmic Rango-4 $E$ from its $E$ at the chromatin (Rango-4 $E - E$; Fig. 1 F).

Our screen revealed a striking, cell-specific diversity of mitotic RanGTP and cargo gradients (Fig. 1). Rango-4 FLIM showed that mitotic HeLa cell chromosomes were surrounded
by a free cargo gradient as previously observed (Kaláb et al., 2006; O’Connell et al., 2009; Soderholm et al., 2011), and RBP-4 FLIM detected the expected upstream RanGTP gradient (Kaláb et al., 2002). However, only in pancreatic adenocarcinoma-derived CFPAC-1 cells were both gradients as steep as in HeLa cells. The RanGTP gradient was at background level in HFF-1 fibroblasts and in 184-A1 breast epithelial cells and reduced in D551 (Detroit-551) fibroblasts (Fig. 1, A and E). A gradient steeper than that in D551 cells was present in tumorigenic DVH3 cells derived from a virally induced D551 cell fusion and in metastatic DVH3-t2 cells that were isolated from DVH3 cell–induced tumors (Duelli et al., 2007). A steep RanGTP gradient was detected in human telomerase reverse transcriptase (hTert)–RPE1 cells (normal retinal pigmented epithelial cells immortalized by telomerase), 293T cells (human embryonic kidney cells immortalized by adenoviral DNA), and immortalized breast epithelial cells MCF10A1 (Fig. 1, A and E). The amplitude of the RanGTP gradient correlated with increased cytoplasmic RanGTP levels (P = 0.0014; Fig. 1 G), showing that RanGTP could reach farther to the cytoplasm in cells with steep RanGTP gradients. Not surprisingly, given the absence of a RanGTP gradient, no mitotic cargo gradient was detected in HFF-1 and 184-A1 cells. However, the cargo gradient was also absent in D551, DVH3, and DVH3-t2 cells (Fig. 1 F), indicating their shared disconnect between the RanGTP and cargo gradients (Fig. 1, E, F, and H). The steep mitotic RanGTP gradient is therefore not universal in human somatic cells, and its coupling to the gradients of free importin-β cargoes is also cell type specific, raising questions about the causes and physiological role of such diversity.

Although FLIM provided sensitive and quantitative measurements, it required a long acquisition time (2 min) and complex data analysis. We therefore validated our results by using the more traditional emission intensity ratio method of FRET detection, after we replaced the nonfluorescent acceptor in our sensor with mCherry reporter carrying methyl-mimetic substitution showed strong chromosome binding in both cells (Fig. 2 A). However, HFF-1 cells contained less Ran (71 ± 2% HFF-1/HeLa) and markedly lower levels of RCC1 (17 ± 3% HFF-1/HeLa) and TPX2 (10 ± 3% HFF-1/HeLa; mean ± SD, n ≥ 4 for all comparisons; three or four repeats; Fig. 2 A).

Immunoblots from cells synchronized by nocodazole (NZ) shake-off showed that interphase and mitotic HeLa cells had similar RCC1 levels (the ratio of mitotic/interphase RCC1 was 92 ± 20%; mean ± SD, n = 2; one repeat; Fig. 2 B), but in HFF-1 cells, there was more RCC1 in mitotically synchronized than in interphase cells (171 ± 1% mitotic/interphase HFF-1 cells; Fig. 2 B). It is therefore unlikely that the much lower RCC1 levels in asynchronous HFF-1 cells versus asynchronous HeLa cells (Fig. 2 A) resulted from a lower fraction of mitotic cells in the HFF-1 cell population.

Ran localization was similar between the two cells (Fig. S2 A). However, the immunofluorescence (IF) showed low RanGAP1 levels at the kinetochores (Fig. S2 B) and strongly reduced RCC1 staining on the chromosomes (Fig. 2, C and D) in HFF-1 cells compared with HeLa cells. Because RCC1-chromatin binding drives RanGTP gradient formation, we examined this further.

Both the C-terminal catalytic domain of RCC1 and its short flexible N-terminal domain (NTD) directly bind to DNA and histones in the nucleosome (Makde et al., 2010), where the chromatin binding of the C terminus is modulated by the loading of Ran on RCC1 (Li et al., 2003), and the chromatin binding of the NTD provides crucial support (Hitakomate et al., 2010). The binding of the RCC1 N terminus to DNA requires α-N-methylation of Ser 2, which is mediated by the N-terminal RCC1 methylase (NRMT; Chen et al., 2007; Schaner Tooley et al., 2007; Hitakomate et al., 2010). In addition, three RCC1 splicing isoforms (RCC1α, β, and γ) expressed in human cells differ in their NTD composition and in how their binding to chromatin is affected by Ser 11 phosphorylation (Hood and Clarke, 2007).

Because quantitative PCR showed that the phosphorylation-sensitive RCC1γ isofrom (Hood and Clarke, 2007) was the least abundant and that RCC1α was the prevailing isofrom (Fig. S2 C), we focused on RCC1α and its methylation. Although immunoblotting with RCC1 α-N-trimethylation antibodies (Me3-SPK; Schaner Tooley et al., 2010) showed that, relative to RCC1 protein levels, the Me3-SPK on RCC1 was similar in HeLa and HFF-1 cells (41 ± 7% Me3-SPK HFF-1/HeLa), this comparison was limited by residual reactivity of the Me-3 antibody with nonmethylated RCC1 (Fig. 2 A and Fig. S2 E). We therefore analyzed the role of RCC1 N-terminal methylation using RCC1α-mCherry reporters expressed in HeLa and HFF-1 cells. As expected (Chen et al., 2007; Hitakomate et al., 2010; Schaner Tooley et al., 2010), the nonmethylatable ASPK-NTDα-mCherry (Schaner Tooley et al., 2010) did not bind to mitotic chromosomes (Fig. 2, E and F). However, the binding of wild-type (wt) NTDα-mCherry to mitotic chromosomes was weaker in HFF-1 cells, and the S2K NTDo-mCherry reporter carrying methyl-mimetic substitution showed stronger chromosome binding in both cells (Fig. 2, E and F).
cells coexpressing Rango-4 and RCC1α-mCherry. We found that the expression of the wt and particularly of the S2K RCC1 α-mCherry induced detectable mitotic cargo gradients in HFF-1 cells (Fig. 2 H), showing that increased RCC1 levels are sufficient to drive the formation of steeper mitotic RanGTP gradients. However, the amplitude of the RanGTP gradient is expected to be sensitive to the balance of the cellular concentrations of Ran and its regulators (Caudron et al., 2005; Kaláb et al., 2006). For example, the smaller amplitudes of mitotic RanGTP gradients in DVH3 and DVH3-t2 cells than in HeLa cells (Fig. 1, A and E) then potentially could be explained by the combined effect of lower RCC1 levels and higher RanGAP1 levels, together with slightly lower Ran levels (Fig. 2 A). Specifically, relative to HeLa cells, the Ran levels were 81 ± 17% in DVH3 cells and 73 ± 12% in DVH3-t2 cells. At the same time, relative to HeLa cells, DVH3 cells contained 141 ± 32% total

We verified the role of N-terminal methylation in full-length RCC1-chromatin binding using FRAP (Fig. 2 G and Fig. S2, F and G). In cells expressing wt or ASPK RCC1α-mCherry, we photobleached an area of the nucleus or mitotic chromosomes and measured the FRAP I_{1/2} (Fig. 2 G and Fig. S2, F and G), which is inversely proportional to the chromatin binding of the fluorescent reporter (Hitakomate et al., 2010). In mitotic and interphase cells, the binding of wt RCC1α-mCherry to chromatin was stronger in HeLa than in HFF-1 cells. The ASPK mutation strongly reduced the RCC1-chromatin binding in HeLa but had almost no effect on the already weak binding in HFF-1 cells (Fig. 2 G). The lower α-N-terminal methylation therefore significantly contributed to reduced RCC1 binding to chromatin in HFF-1 cells throughout the cell cycle.

To examine the role of RCC1 levels in RanGTP gradient formation, we measured the mitotic cargo gradients in HFF-1 cells coexpressing Rango-4 and RCC1α-mCherry. We found that the expression of the wt and particularly of the S2K RCC1α-mCherry induced detectable mitotic cargo gradients in HFF-1 cells (Fig. 2 H), showing that increased RCC1 levels are sufficient to drive the formation of steeper mitotic RanGTP gradients.

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As previously reported (Schaner Tooley et al., 2010), the NRMT RNAi reduced the RCC1 methylation (Fig. S3 B) and disrupted RCC1-chromatin binding (Fig. 3 C). Although NRMT RNAi induced a subtle increase of RCC1 levels (to 151 ± 27%; Fig. S3 B), the RanGTP gradient was reduced by the treatment, as shown by the decreased mitotic cargo gradient and decreased mean free Rango-4 in mitotic cells (Fig. 3, D and E). The NRMT knockdown induced a significant increase in the PM fraction of mitotic HeLa cells (31.2 ± 2.7% control vs. 45.9 ± 4.8% NRMT RNAi; P < 10^{-3}; Fig. 3 F and Fig. S3 C), indicating that the steep RanGTP gradient is required for the assembly of a fully functional bipolar spindle capable of driving the chromosome congression during PM. We confirmed these results by perturbing the RanGTP gradient via RanGAP1 RNAi in HeLa cells and via RCC1 inhibition in the temperature-sensitive tsBN2 cell line (Fig. S3, E–I; Li and Zheng, 2004). Consistent with previous studies (Joseph et al., 2002; Arnaoutov and Dasso, 2005), the depletion of RanGAP1 and RCC1 both induced strong defects in chromosome congression and segregation (Fig. S3, F–I). Both interventions also caused the accumulation of PM cells (Fig. S3, F–I), supporting the results of the NRMT knockdown.

Because of the low TPX2 levels in HFF-1 cells (Fig. 2, A and B), we expected that increasing the RanGTP gradient by RCC1 overexpression in HFF-1 cells (Fig. 2 H) might have only a subtle effect on spindle assembly and function. Indeed, S2K-RCC1α-mCherry overexpression caused only a statistically insignificant decrease in PM cells with astral MTs (19.9 ± 6.5 vs. 31.1 ± 9.6%; two-way analysis of variance [ANOVA], P > 0.05; RanGAP1 (sum of RanGAP1 + RanGAP1-SUMO) and 68 ± 19% RCC1. Similarly, DVH3-t2 cells contained 126 ± 12% total RanGAP1 and only 69 ± 14% RCC1 compared with HeLa (all aforementioned relative expression values were derived from data in Fig. 2 A and its biological repeats; n = 4). Future studies will be needed to verify this model and what causes the absence of the cargo gradient in certain cells.

Steep mitotic RanGTP gradient promotes bipolar spindle assembly in PM

To examine how differences in RanGTP gradient steepness affect mitosis, we first compared mitotic progression in unperturbed HeLa and HFF-1 cells by live-cell microscopy (Fig. 3, A and B; and Fig. S3 A). Although the mitosis was shorter in HFF-1 cells (54 ± 12 vs. 61 ± 7 min in HeLa; P = 0.003), the PM time in HFF-1 cells was longer and more variable (17 ± 7 vs. 9 ± 2 min in HeLa; P < 10^{-3}; Fig. 3 A and Fig. S3 A), indicating that the chromosome congression was slower and more stochastic in HFF-1 cells. Metaphase was longer in HeLa cells (18 ± 4 vs. 10 ± 5 min in HFF-1 cells; P < 10^{-9}; Fig. 3 A and Fig. S3 A), and unlike in HFF-1 cells, its duration in individual HeLa cells was inversely proportional to their PM time (Fig. 3 B), perhaps indicative of a longer time needed for sorting erroneous MT–kinetochore attachments formed during rapid spindle assembly.

Next, we examined whether the RanGTP gradient is responsible for such differences by inducing changes in the RanGTP gradient and following their effects on mitosis. We reduced the RanGTP gradient by decreasing RCC1-chromatin binding via NRMT RNAi in HeLa cells (Schaner Tooley et al., 2010). As previously reported (Schaner Tooley et al., 2010), the NRMT RNAi reduced the RCC1 methylation (Fig. S3 B) and disrupted RCC1-chromatin binding (Fig. 3 C). Although NRMT RNAi induced a subtle increase of RCC1 levels (to 151 ± 27%; Fig. S3 B), the RanGTP gradient was reduced by the treatment, as shown by the decreased mitotic cargo gradient and decreased mean free Rango-4 in mitotic cells (Fig. 3, D and E). The NRMT knockdown induced a significant increase in the PM fraction of mitotic HeLa cells (31.2 ± 2.7% control vs. 45.9 ± 4.8% NRMT RNAi; P < 10^{-3}; Fig. 3 F and Fig. S3 C), indicating that the steep RanGTP gradient is required for the assembly of a fully functional bipolar spindle capable of driving the chromosome congression during PM. We confirmed these results by perturbing the RanGTP gradient via RanGAP1 RNAi in HeLa cells and via RCC1 inhibition in the temperature-sensitive tsBN2 cell line (Fig. S3, E–I; Li and Zheng, 2004). Consistent with previous studies (Joseph et al., 2002; Arnaoutov and Dasso, 2005), the depletion of RanGAP1 and RCC1 both induced strong defects in chromosome congression and segregation (Fig. S3, F–I). Both interventions also caused the accumulation of PM cells (Fig. S3, F–I), supporting the results of the NRMT knockdown.

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Chromosomal gain drives steep mitotic RanGTP gradient

Because slower diffusion is expected to increase the steepness of the RanGTP gradient (Caudron et al., 2005), we wondered whether chromosomal crowding in cells with high chromosome numbers contributes to their steep mitotic RanGTP gradient. To test this, we measured mitotic RanGTP gradients in polyploid HFF-1 cells prepared in vitro (Jansen et al., 2007) by fusion between HFF-1 cells expressing RBP-4 and HFF-1 cells expressing MT plus end–binding protein EB3-mCherry. Remarkably, fused mitotic HFF-1 cells displayed steep RanGTP gradients comparable with mitotic HeLa cells (compare Fig. 4 B and Fig. 1 E), demonstrating that chromosomal gain is sufficient to drive the formation of a steep mitotic RanGTP gradient.

Virus-induced cell fusion (Duelli et al., 2007), mitotic slippage, or cytokinesis failure is thought to produce unstable tetraploid precursors of aneuploid cancer cells (Ganem et al., 2007; Vitale et al., 2011). The chromosomal gain-driven mitotic RanGTP gradient in such intermediates could selectively promote proliferation of intermediates expressing Ran-regulated mitotic factors. Intriguingly, the transcriptional analysis in human breast cancers and in mouse models identified a conserved TPX2-centered expression network that strongly predicted metastasis susceptibility in both species (Hu et al., 2012).

Our results indicate that the diversity of mitotic RanGTP gradients can be understood if Ran is considered as a component of a highly dynamic and spatially organized intracellular system. Although elevated RCC1 levels and increased chromosomal crowding in cells with high chromosome numbers contribute to their steep mitotic RanGTP gradient (Caudron et al., 2005), we wondered whether chromosomal crowding in cells with high chromosome numbers contributes to their steep mitotic RanGTP gradient. To test this, we measured mitotic RanGTP gradients in polyploid HFF-1 cells prepared in vitro (Jansen et al., 2007) by fusion between HFF-1 cells expressing RBP-4 and HFF-1 cells expressing MT plus end–binding protein EB3-mCherry. Remarkably, fused mitotic HFF-1 cells displayed steep RanGTP gradients comparable with mitotic HeLa cells (compare Fig. 4 B and Fig. 1 E), demonstrating that chromosomal gain is sufficient to drive the formation of a steep mitotic RanGTP gradient.

Materials and methods

Cell culture

tsBN2 cells were obtained from T. Nishimoto (Kyushu University, Fukuoka, Japan) and from M. Dasso (National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD) and were grown at 33°C in 5% CO2. All other cells were grown at 37°C in 5% CO2. Human adenocarcinoma epithelial cells (HeLa; CCL-2) were obtained from American Type Culture Collection and used to isolate the single cell–derived clone Hela 6 (Kalab et al., 2006; Kalab and Soderholm, 2010), which was used throughout this study. Human ductal adenocarcinoma–derived CFPAC-1 cells (CRL-1918), human embryonic kidney 293T cell line (CRL-11268), normal newborn human male foreskin fibroblasts (HFF-1; SCRC-104), normal human skin fibroblast D551 (CCL-110), and telomerase-immortalized normal retinal pigmented epithelial cells hTertRPE1 (CRL-4000) were purchased from American Type Culture Collection and cultured in 10% FBS/DMEM (Invitrogen). The MCF10A1 normal immortalized breast epithelial cells (Santer et al., 2001) were purchased from the Karmanos Cancer Institute and cultured in DMEM/F12 (1:1) with 5% horse serum, 0.029 mM Na bicarbonate, and 10 mM Hepes supplemented with 10 µg/ml insulin, 20 ng/ml EGF, 0.5 µg/ml hydrocortisone, and 100 ng/ml cholina.

For IF, cells were grown on cleaned glass coverslips or on dishes (IbiTreat 60 µDish; Ibi). After fixation with the antigenspecific method (see next paragraph), the cells were permeabilized for 2–5 min with 1% Triton X-100 and 2 µg/ml Hoechst 33342 (Sigma-Aldrich) in general tubulin buffer (GTB; 80 mM Pipes, pH 6.9, 1 mM MgCl2, and 1 mM EGTA) and blocked at 4°C for 1 h or overnight with IF buffer (IFB; 6% normal donkey serum [Jackson ImmunoResearch Laboratories, Inc.], 0.2% saponin [Sigma-Aldrich], and 0.02% sodium azide in GTB). Samples were incubated with primary antibodies diluted in IFB at room temperature for 1–2 h or overnight at 4°C followed by washes with 0.2% saponin/GTB and a 0.5–1-h incubation with secondary antibodies (dye-labeled purified donkey IgG; Jackson ImmunoResearch Laboratories, Inc.) diluted in IFB. After final washes in GTB containing 0.2% saponin and 0.1% Triton X-100, the samples were mounted using Prolong Gold Antifade (Invitrogen) and stored at −20°C or photographed immediately. EXP-Booster-Atto594 (ChromoTek) was used to detect mCherry-tagged RCC1 or mCherry in fixed cells for experiments in
Fig. 3 C. For RCC1 and tubulin detection (Fig. 2 C and Fig. 3 C), cells were fixed for 2–5 min at −30°C in 5% acetic acid, 50% methanol, and 45% ethanol. For tubulin and DNA detection (Fig. S3, C, D, and G), the cells were fixed with freshly prepared 4% paraformaldehyde in GTB for 20 min at 37°C. For the detection of tubulin, RanGAP1, and DNA (Fig. S2 B), cells were fixed in 2% paraformaldehyde in GTB for 15 min at room temperature. The fixation and IF of tubulin and DNA in synchronized tsBN2 and BHK cells (Fig. S3 I) are described in the paragraph Analysis of mitosis progression in tsBN2 cells treated with nonpermissive/permissive temperature.

Antibodies
The following commercial antibodies were used for immunoblotting and IF assays: rabbit monoclonal to RCC1 (used for IF and immunoblotting; #5134, Epitomics), goat polyclonal to RCC1 (used for immunoblotting; sc-1161, Santa Cruz Biotechnology, Inc.), mouse monoclonal to Rho (610340; BD), rabbit polyclonal to TPX2 (NB500-179; Novus Biologicals), mouse monoclonal E7 antibody to tubulin (Developmental Studies Hybridoma Bank), rabbit polyclonal to β-tubulin (#A01203; GenScript), human anticientromere antibody (HCT 0100; Immunovision), and rabbit polyclonal to phospho-Ser 10 in histone H3 (#E173; Epitomics). The rabbit polyclonal antibody to MeJ-3SPK (Chen et al., 2007; Schaner Tooley et al., 2010) was a gift from C. Tooley and I. Macara (University of Virginia, Charlottesville, VA), the affinity-purified rabbit polyclonal antibody to RanGAP1 was a gift from A. Arnaoutov and M. Dasso (National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD), and goat polyclonal antibody to RanGAP1 was a gift from F. Melchior (Heidelberg University, Heidelberg, Germany).

Cell transduction with lentiviruses and cell transfection
Lentiviral production was performed in house or contracted to the Protein Expression Laboratory, SAIC-Frederick, Inc., using protocols recommended by Invitrogen. Human embryonic kidney cells, 293FT (Invitrogen), were transfected with the transfection reagent (FuGENE 6; Roche) according to the instructions of the manufacturer.

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Antibodies described in this paragraph. 3 d after the start of the treatment, control and NRMT RNAi-treated cells were transfected with a plasmid for the expression of Rango-4 (pK314 [pSG8 Rango-4]); FLM analysis of both cell samples was performed 5 d after the start of treatment.

Scoring mitotic phenotypes in HFF-1 and Hela cells
Mitotic phenotypes were manually scored by inspecting the fixed and immunostained (tubulin or TPX2; DNA) cells under 100×, 1.4 NA oil immersion objective on an inverted microscope (IX81; Olympus) equipped with a system controller (IX2-UCB; Olympus) and a shutter controller (Lambda 10-5; Sutter Instrument) and operated via MetaMorph software (Molecular Devices).

Mitotic phenotypic analysis
FRET was scored in PM, metaphase, anaphase, and telophase. As the defining difference between PM and metaphase cells, we considered the state of chromosome congression rather than bipolar spindle assembly. Because the spindle axis was defined in HFF-1 cells tilted vertically, all cells were inspected by focusing along the z axis to discriminate between the spherical (PM) or disc-shaped (metaphase) arrangement of chromosomes. Cells with the most chromosomes dispersed and showing no sign of organization to the metaphase plate were scored as PM, and within that category, we scored the MT organization: MT ball, asters, and spindle. The MT ball corresponds to MTs showing no clear polar or radial structures, clearly radial MT arrays were scored as asters, and MT structures showing clear pole-to-pole connections were scored as spindles. Cells with spindles that were also showing clear evidence of chromosomal congression to the disc-shaped metaphase plate were scored as metaphase, and within that category, we scored the number of spindle poles and number of lagging chromosomes. Similarly, we scored anaphase and telophase cells to categories with and without lagging chromosomes.

HFF-1 cell fusion
The fusion of HFF-1 cells was induced using a modification of the previously described protocol (Jansen et al., 2007). HFF-1 cells were seeded on 6-well plates and separately transduced with lentiviruses for the expression of RBP-4 FRET sensor or with lentiviruses for EB3-mCherry microtubule plus-end marker. 2 d later, the cells were trypsinized, and the cells expressing RBP-4 were mixed 1:1 with cells expressing EB3-mCherry and seeded on fibronectin-coated 35-mm diameter wells with annealed and deprotected siRNA oligonucleotide (oligo) targeting a unique sequence in human RanGAP1 (5′-GAAAACCUGCUUGAGAUGAGdT6′3′; obtained from Thermo Fisher Scientific) using Lipofectamine 2000 and a protocol recommended by the manufacturer (Invitrogen). Control cells were treated with Lipofectamine 2000 only. After 5 d of treatment, cells were harvested for immunoblotting (using a protocol described for Fig. 2 A in Electrophoresis and quantitative immunoblotting) or fixed for IF with tubulin antibodies and DNA stain.
kept in culture for 2–3 d to achieve optimal adherence. The cultures were then supplemented with 10 μM N2 to add their growth media and incubated for 6 h at 37°C in 5% CO2, to synchronize in mitosis. The dishes were rinsed with fresh media containing 10 μM N2 and vortexed in 3–4 ml of 10 μM N2/media for 10 s at maximum speed (Vortex Genie 2; Diaguer).

The mitotic cells dissociated from the dishes were then washed and resuspended in warm DPBS and rapidly processed to prepare samples for SDS-PAGE and protein concentration measurements as described in Electrophoresis and quantitative immunoblotting. To collect interphase cells, the cells remaining on plates after shake-off were washed with warm DPBS and vortexed again to dissociate remaining mitotic cells, and the adhered cells were harvested by trypsinization, washed with warm PBS-containing media to neutralize trypsin, and processed as described for the mitotic cells. The HeLa cells were detached using trypsin-EDTA, and Petri dishes (Pyrex 3160) were incubated in 1 M HCl at 60–65°C overnight, washed extensively in distilled water, and autoclaved.

Confocal live-cell microscopy

Confocal images were sequentially acquired using ZEN 2009 software (Carl Zeiss) on a confocal system (LSM 710; Carl Zeiss) including a 34-channel spectral detector (Carl Zeiss) with an inverted microscope (Observer.Z1; Carl Zeiss) and an infrared laser (680–1,080 nm; Chameleon Ultra II; Coherent, Inc.). For most-live-cell imaging experiments, cells were grown in a 2-well or 4-well chambered coverslip (Lab-Tek II; Thermo Fisher Scientific). For FRAP measurements of different RCC1-mCherry constructs, HeLa cells were grown in 8-well chambers (μ-Slide ibiTreat; Ibidi). An incubator (Incubator XL S1; PeCo) mounted on the microscope stage and equipped with a 2°C, 35% CO2, CO Module S1, and a PC-Module heater. Unit XL S1 was used to maintain the cells at 37°C in 5% CO2 and constant humidity. During all live-cell imaging experiments, cells were kept in the same growth media as for maintaining the cell culture.

Time-lapse bright-field imaging microscopy of cell division

Bright-field images were collected using the same microscope system as the confocal-live cell microscopy described in the previous section, except the cells were illuminated by a 532-nm HeNe laser (LASG Lander Lasertechnik GmbH) at 0.2% maximum power using the Plan Apochromat 20×, 0.8 NA lens, and the transmitted light was detected using the internal transmitted light photomultiplier tube transmitted light detector of the microscope. The pixel dwell time of the laser was fixed at 1.27 μs, and the detector gain was adjusted at each imaging field at the beginning of the time lapse to avoid under- or overexposure. Images were acquired as z stacks of five slices with 4-μm intervals every 80 s for ≤23 h and were stitched together sequentially with ZEN 2009 software to create time-lapse videos. ZEN autofocus macros was used between each time frame to prevent focus drifting. We acquired two videos for HeLa and seven videos for HFF-1 cells. Using the time stamp feature of the ZEN software, in all cells that underwent complete mitosis, we recorded the time of the (1) nuclear envelope breakdown, (2) metaphase plate formation, (3) metaphase plate disassembly, and (4) nuclear envelope reformation. The PM time corresponds to the interval between 1 and 2, the metaphase time corresponds to the interval between 2 and 3, and the interval between 3 and 4 is the anaphase + telophase time.

FLIM

Spatially resolved, time-correlated single-photon counting (TCSPC) datasets were collected using a Plan Apochromat 63×, 1.40 NA oil immersion lens on an inverted nonlinear optics (NLO) microscope (LSM 710) equipped with a TCSPC controller (SPC-830; Becker & Hickl). Samples were excited by one-photon 440-nm pulses generated by a frequency-doubling 80-MHz, 880-nm infrared laser using a harmonic generator (Mira Femto System Harmonic Generator 9300; Coherent, Inc.). The emission was collected from a custom side port, filtered through a 510/84 band-pass filter (510/84; Chroma Tech), and detected by a module (HPM-100-40; Becker & Hickl) containing a hybrid photomultiplier (R10467-40 GaAsP; Hamamatsu Photonics). 100-ps infrared laser using a harmonic generator (Mira Femto System Harmonic Generator 9300; Coherent, Inc.) were used for the in silico assembly and for sequencing of all constructs.

Cloning general procedures

Gene construction kits (Textco BioSoftware) and Vector NTI software (Invitrogen) were used for the in silico assembly and for sequencing of all constructs. Site-directed mutagenesis was performed using the site-directed mutagenesis kit (QuikChange Lightning Multi; Agilent Technologies). PCRs were performed with high fidelity polymerases (Pfu Ultra or PfX; Invitrogen) assuming incomplete two-exponential decay and using trapezoid integration. The fluorescence lifetime measurements were analyzed using SPCI automatic calculation routine. At least eight mitotic cells were analyzed per cell type and FRET sensor (range 8–34, 261 total; 132 with Rango-4, and 129 with RBP-4). Excluded from further analyses were FRET sensor-expressing cells displaying mean cellular T

FRET emission intensity ratio imaging

FRET emission intensity ratio images were collected using a NLO microscope (LSM 710) with the same objective lens and light source used for the FLIM microscopy described in the FLM section. The master gain was fixed at 900 and 1,150 for MTP-1 and YPet channels, respectively, to directly compare the value of T

FRAP

FRAP measurements of mCherry-tagged RCC1 constructs expressed in live cells were performed on a confocal NLO microscope (LSM 710) using a Plan Apochromat 63×, 1.40 NA oil immersion lens (Carl Zeiss) and a diode-pumped solid-state 561-nm, 10-mW laser 561-nm excitation line. Five prebleach scans were followed by photobleaching with two passes of a 100-µW laser 561-nm laser and 30 (interphase cells) or 35 (mitotic cells) postbleach scans. In the interphase cells, the photobleached area was a 1-µm circle inside the nucleus. To suppress the noise caused by chromosomal motion during recovery, the photobleached area comprised approximately one half of the metaphase plate in the mitotic cells. Pre- and postbleach scans were acquired at 1 frame/s, and the excitation power was adjusted to assure no detectable bleaching in neighboring cells that were not subjected to FRAP (corresponding to 0.2–0.7% of maximum 561-nm laser power). ZEN 2009 software was used to calculate the fluorescence recovery r1/2.

Cloning of RCC1-mCherry constructs

PCR cloning was used to insert human fulllength RCC1α or RCC1α (1–27 aa) between XhoI and AgeI sites and mCherry between AgeI and NotI sites in pEGFPN1, resulting in pRCC1α-mCherry (pK260) and pRCC1α-NTD-mCherry (pK295) plasmids for transfection-mediated expression in tissue-culture cells. PKG (phosphoglycerate kinase) Histone H2B-mCherry (plasmid

FRET data analysis

Out-of-cell fluorescence was omitted from the analysis by choosing a threshold at 1% of the mean photon count inside of the cell. Fluorescence lifetime images were produced and analyzed using SPCI (single photon counting) software (Becker & Hickl). In all samples, the median donor fluorescence lifetime T

FRET emission intensity ratio imaging

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Cloning general procedures

Gene construction kits (Textco BioSoftware) and Vector NTI software (Invitrogen) were used for the in silico assembly and for sequencing of all constructs. Site-directed mutagenesis was performed using the site-directed mutagenesis kit (QuikChange Lightning Multi; Agilent Technologies). PCRs were performed with high fidelity polymerases (Pfu Ultra or PfX; Invitrogen) using the pENT63.V5-TOPO TA Cloning kit (Invitrogen), the PirViral HIFPlPerform TREX Gateway Expression System (Invitrogen), or the pENTR.D-TOPO Cloning kit (Invitrogen). All constructs were verified by restriction digests and sequencing across fragments generated by PCR.

Cloning of RCC1α-mCherry constructs

PCR cloning was used to insert human fulllength RCC1α or RCC1α (1–27 aa) between XhoI and AgeI sites and mCherry between AgeI and NotI sites in pEGFPN1, resulting in pRCC1α-mCherry (pK260) and pRCC1α-NTD-mCherry (pK295) plasmids for transfection-mediated expression in tissue-culture cells. PKG (phosphoglycerate kinase) Histone H2B-mCherry (plasmid
21217; Addgene; Kito-Matsuo et al., 2009) was used as the PCR template for mCherry. The pK260 and pK295 plasmids were then used as templates for the PCR-mediated assembly of lentiviral expression plasmids in linearized topoisomerase-conjugated plentid3/VS-TOPO. Full-length wt RCC1-mCherry was amplified from pK260 by PCR, using 5’ RCC1x and 3’ mCherry primers. After the addition of TA overhangs using Taq polymerase, the purified PCR product was inserted by TOPO cloning into plentid3/VS-TOPO, resulting in plentid3/RCC1-mCherry (pK267). Using the same strategy and PCR with mutated 5’ RCC1x oligos, we constructed plenti RCC1-mCherry with S2K (pK286) and ASPK (pK285) mutations. Similarly, using the same primers, but with p-RCC1x NTD-mCherry (pK295) as a template, we prepared constructs for the lentiviral expression of wt and mutated mCherry-tagged NTD: plenti wNTD-mCherry (pK296), plenti ASPK-NTD-mCherry (pK297), and plenti S2K-NTD-mCherry (pK306).

Cloning of FRET sensors

As in YFP-RBD-CFP (Kalab et al., 2002), the sensory domain in RBP-4 and RBP-4 Y FRET sensors is an RBD of Yrb1 (Saccharomyces cerevisiae homologue of RanBP1). In Rango-4 Y and Rango-4 Y, the sensory domain is identical to the importin-β-binding domain (Iβ) of human snurportin 1, which was used in previous versions of Rango (Kalab et al., 2006; Kalab and Saderholm, 2010). The FRET donor in RBP-4 Y and Rango-4 Y is the monomeric teal fluorescent protein mTFP-1 (purchased as pmTFP-1-ER plasmid from Allele Biotechnology; Ai et al., 2008), and the acceptor is YPet (Nguyen and Daugherty, 2005). The FRET donor in RBP-4 and Rango-4 is again mTFP-1, and the acceptor is a moderately dimerizing nonfluorescent acceptor, dREACh, that we developed by removing the monomerization mutation R223F from dREACh (gift from R. Yasuda, Duke University Medical Center, Durham, NC; Murakoshi et al., 2008) and introducing a dimerization-promoting mutation, S208F. Plasmid for transfection-mediated expression of Rango-4 in HeLa (pK215 [pSG Rango-4]) was prepared by stepwise assembly of mTFP-1, snurportin Iβ, and dsREACh sequences in a pSG8 vector using PCR and restriction-ligation cloning.

Using PCR and restriction-ligation cloning, the open reading frames for RBP-4 Y, Rango-4 Y, RBP-4, and Rango-4 were inserted into pENTR/D-TOPO/V5. In this manner, we prepared pKC224 (pENTR RBP-4 Y), pKC225 (pENTR Rango-4 Y), pKC226 (pENTR RBP-4), and pKC221 (pENTR Rango-4). LR Gateway recombination reactions (Invitrogen) of the aforementioned pENTR vectors with plentid3/TO/V5-DEST were then used to prepare pKC322 (plenti RBP-4 Y), pKC321 (plenti Rango-4 Y), pKC324 (plenti RBP-4), and pKC343 (plenti Rango-4) from data shown in Fig. 2 A and from at least three additional biological repeats (new cell culture, SDS-PAGE, and immunoblot) of this experiment. The relative expression values were calculated from such background-subtracted signals that were normalized to the levels of tubulin detected in the same lane on the same blot. The relative expression values for asynchronous cell cultures of HFF-1, HeLa, DHV3, and DHV3-42 cells were derived from data shown in Fig. 2 A and from at least three additional biological repeats (new cell culture, SDS-PAGE, and immunoblot) of this experiment. The comparisons of RCC1 levels in cell cycle–synchronized HeLa and HFF-1 cells were obtained from data shown in Fig. 2 B and from one additional biological repeat of this experiment. The comparison of RCC1 levels in HeLa cells treated with control or NRMT RNAi was obtained from data in Fig. S3 B and three additional repeats of the NRMT RNAi experiment. Recombinant N-terminally biotinylated RCC1x (Halpin et al., 2011) in Fig. S2 was provided by D. Halpin and R. Heald (University of California, Berkeley, Berkeley, CA).

Imaging

Phenotype CS3 (Adobe) was used to crop and arrange all images, and contrast was adjusted separately for each RGB channel using the Auto Color Correction Options with no clipping. Figures were assembled and annotated using Illustrator CS3 (Adobe).

Statistical analyses

Statistical analyses were performed with Prism version 6 (GraphPad Software) and/or Excel (Microsoft).

Online supplemental material

Fig. S1 shows validation of the RBP-4 and Rango-4 sensors and data on reference \( t_{\text{donor}} \) and on background \( t_{\text{donor}} \) gradient. Fig. S2 shows the localization of Ran and RanGAP1, quantitative RCC1 PCR, Me3-SPK antibody characterization, and RCC1-mCherry FRAP data. Fig. S3 shows data on live cell lifetimes, cell NIH cells, RanGAP RNAi in HeLa cells, and RCC1 inhibition in

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