Supporting Text

Supporting Methods

Reagents. Antibodies used were against β-tubulin (Sigma), tankyrase 1 (gift from N.-W. Chi, Massachusetts Institute of Technology, Cambridge), cullin 2 (BD Biosciences), Sak1 (gift from J. Dennis, Mt. Sinai Hospital, Canada), MyoIIA (gift from D. Cohen, Cornell University, Ithaca, NY), various Ankyrin and spectrin isoforms (gift from K. Beck, University of California, Davis), GalT (gift from E. Berger, University of Zurich, Zurich), manII (gift from K. Moremen, University of Georgia, Athens), and GM130 (Translab). H89 (Calbiochem) was made up as a 10 mM stock solution in DMSO and stored at 4°C. All transfections were done with FuGENE (Roche Molecular Biochemicals).

Cell Synchronization. NRK cells were G_1/S arrested after 16 h in 10 μg/ml aphidicolin (Sigma). Between 8 and 10 h after release from aphidicolin, virtually all cells had entered or were in mitosis. Before 7.5 h, there were no cells in mitosis based on assessing chromatin condensation with Hoechst 33342 (Roche Molecular Biochemicals) and staining with antibodies to histone H3 serine 10 phosphorylation (gift from M. Dasso, National Institutes of Health, Bethesda).

Imaging. All images were obtained with a Zeiss LSM 510 confocal microscope. Live cells were held at 37°C during imaging. For quantification purposes, a ×40/1.3 N.A. objective was used with the pinhole fully open to collect fluorescence from the entire cell depth. Images were collected on 12-bit photomultiplier tubes. All fluorescence correlation spectroscopy (FCS) measurements were done with the Zeiss Confocor system with a water-immersion objective (×40/1.2 N.A) and a 70-μm collection pinhole. Metaphase cells were chosen under epifluorescence, and a single image was acquired under confocal illumination, from which collection spots for FCS were selected. The typical collection time was 10 sec for each spot. Photobleaching experiments were performed as described (1). Briefly, a region of interest (e.g., Golgi) was “drawn” by

using Zeiss LSM510 FRAP software. After initial scans of the whole cell with low-intensity laser light, the region of interest was photobleached by scanning with high-intensity laser light for a short period (typically 1–3 sec). Imaging in the post-photobleach recovery period was again performed with low-intensity laser light. KALEIDAGRAPH graphing software (Synergy Software, Reading, PA) was used for plotting and analysis of photobleaching recoveries.

Supporting Results

Analysis of Spindle Formation and Chromosome Morphology in H89-Treated Cells. Cytoplasmic microtubules and the spindle body were visualized by confocal microscopy in NRK cells treated with and without 50 μM H89. Fixed cells were processed for immunofluorescence by staining with an antibody against β-tubulin to visualize microtubules and with the dye Hoechst 33342 to observe DNA (Fig. 7*a*). Microtubules showed normal morphology at all stages of the cell cycle in H89-treated cells. This included a radial distribution in interphase, a mitotic spindle organization in metaphase, and a bipolar spindle arrangement in telophase. Chromosome behavior in H89-treated cells was normal in early stages of mitosis, including alignment at the metaphase plate (Fig. 7*a*, blue).

To further analyze chromosome dynamics in H89-treated cells we expressed histone 2B-CFP in NRK cells (Fig. 7*b*). Cells were treated with H89 shortly before entry into mitosis, and individual cells were imaged as they progressed through mitosis. In the majority of dividing cells examined, sister chromosome pairs remained attached at their ends (so-called bridged). Two examples illustrating this phenotype are shown in Fig. 8*b*. Note that despite the presence of chromosome bridges, H89-treated cells were able to decondense their chromosomes after segregation.

H89 Treatment Does Not Affect Dynamics of COPII Machinery in Mitosis. Previous work has suggested that H89 treatment in interphase affects the activity of ER exit sites (2). At the concentrations of H89 (50 μM) used in our study, the drug had no noticeable

effect on the behavior of ER exit sites in interphase or in mitosis as determined by live cell imaging of the COPII component, Sec13-YFP (3). As shown in Fig. 8, Sec13-YFP changed from a punctate pattern (representing ER exit sites) in interphase and prophase, to a completely diffuse pattern in metaphase, and back to a punctate pattern in telophase in the presence or absence of H89.

Peripheral Golgi Proteins Are Dispersed Before Golgi Structures Disassemble. Peripheral Golgi proteins are dispersed during acute BFA treatment (Fig. 9). Cells were treated with BFA (5 μg/ml) for either 3 or 30 min, fixed, and then costained with antibodies to peripheral Golgi proteins and to Golgi membrane markers (mannosidase II, GM130). After 3 min of BFA treatment, all peripheral proteins had dispersed into the cytoplasm, even though Golgi structures containing Golgi membrane markers were still present. After 30 min of BFA treatment, both peripheral and membrane markers of the Golgi were found widely dispersed within cells. Further analysis of the effects of short and extended BFA treatments are in Fig. 6*C*.

Use of FCS to Assess Whether Arf1-GFP in Mitotic Cells Exists Free in the Cytoplasm or Is Attached to Small Vesicles or Membranes. ***Method and results.*** We assessed the state of activation of Arf1-GFP in metaphase cells by monitoring its potential association with small vesicles or membrane structures. Arf1 is homogeneously distributed within metaphase cells (Fig. 3*A*); however, the spatial resolution of confocal microscopy does not allow us to conclude that all Arf1 is soluble (i.e., inactive) as some Arf1 could be active and associated with membrane structures that are not resolvable by confocal microscopy. Thus, we used fluorescence correlation spectroscopy (FCS) to assess the state of activation of Arf1 with great sensitivity. FCS monitors the diffusion of fluorescence particles, moving in and out of a confocal volume (Fig. 10*a*). The emitted fluorescence of particles dwelling in the excitation volume is collected with a single-photon counter to achieve time-resolved detection of individual molecules. Small molecules (e.g., cytoplasmic Arf1) diffuse fast and have a short dwell time within the confocal volume, whereas bigger fluorescent objects (e.g., vesicle-bound Arf1) diffuse slower and thus have a longer dwell time. In FCS, the dynamics of these fluctuations are

analyzed by using an autocorrelation function to yield the characteristic residency time of each molecule within the confocal volume, its diffusion coefficient (*D*), its hydrodynamic diameter, and the number of molecules comprised of that particular fluorescent species.

In the specific case of Arf1 in metaphase cells there can be only two fluorescent species in the confocal volume: Arf1GDP-GFP, which is cytoplasmic, and Arf1GTP-GFP, which is vesicle bound. If Arf1GTP-GFP dissociates from membranes during mitosis and accumulates in its inactive GDP-bound form, then Arf1's diffusional mobility in mitotic cells should be comparable to the diffusional mobility of free GFP expressed in mitotic cells. To test this, FCS measurements were carried out on (*i*) Arf1-GFP-expressing cells in metaphase, (*ii*) GFP-expressing cells in metaphase, (*iii*) GFP in PBS, and (*iv*) 40-nm fluorescent beads in PBS (this bead size was chosen because it is the typical size of the smallest known vesicle in a cell). Each FCS measurement was fitted with the following three-dimensional diffusion fit:

$$G(\tau) = \frac{1}{N}\frac{1}{1+\frac{\tau}{\tau_D}}\sqrt{\frac{1}{1+\frac{\tau}{\alpha\tau_D}}},$$

where $G(\tau)$ is the autocorrelation function of the collected fluorescence, N is the average number of fluorescent object in the confocal volume V, α is a geometric parameter, characterizing the longitudinal to transversal dimensions of V (we fixed this parameter by calibration with a solution of fluorescein), and τ_D is the typical diffusion time scale of the fluorescent object across V.

For all fluorescent objects under study here the diffusion time scale τ_D is

$$\tau_D = \frac{\omega^2}{4D} = \frac{3\pi}{4}\frac{\omega^2}{k_B T}\eta d_h,$$

where ω is the waist of the illumination beam, d_h is the hydrodynamic diameter of the diffusing fluorescent object, D is its diffusion coefficient, k_B is the Boltzmann constant, and T is the temperature. For our setup,

$$\frac{d_h}{\text{nm}} = 0.02 \times \frac{\eta_{water}}{\eta_{medium}} \times \left(\frac{\tau_D}{\mu\text{s}} \right).$$

Fig. 10*b* shows the autocorrelation curves that we obtained for GFP in PBS (blue), GFP in metaphase (red), Arf1-GFP in metaphase (green), and 40-nm fluorescent beads in PBS (black). The typical diffusion time scales extracted from the autocorrelation curves are given in the figure caption. These time scales were converted to D and reported in Fig. 3*B*. Because no free GFP was observed when we fractionated Arf1-GFP-expressing cells and blotted with anti-GFP antibodies (data not shown), the measured D represented only Arf1-GFP. Note that Arf1-GFP and GFP have closely similar autocorrelation curves suggesting that Arf1GDP-GFP is the predominant fluorescent species at metaphase. The similarity of the diffusion coefficient of GFP (≈30 kDa) and Arf1-GFP (≈47 kDa) might be surprising at first glance, but the hydrodynamic radius of proteins are directly related not to their molecular weight but rather to their molecular dimensions, which are comparable.

What Fraction, if Any, of Arf1-GFP Is Transiently Associated with Vesicles in Metaphase Cells? To address the possibility that a small fraction of Arf1-GFP may be associated with vesicles in metaphase cells, we simulated autocorrelation functions for conditions in which different percentages of Arf1-GFP were either free in the cytoplasm or bound to small vesicles (diameter 40 nm), and then we compared these simulations with our experimental FCS data on Arf1-GFP. Described below is our methodology and the results from this analysis.

If Arf1-GFP is in the cytoplasmic fraction with a probability p and a diffusion time scale of $\tau_1 = 215$ μs, and it is associated with a 40-nm-diameter vesicle with a probability ($1 - p$) and a diffusion time scale of $\tau_2 = 6.5$ ms (this was extrapolated from our measure of

the change in diffusion time scales of GFP in PBS vs. metaphase cytoplasm), then the autocorrelation function, $G(t)$, for its diffusion is the sum of the two components (4) is

$$G(t) \propto p(1-p) \times \left(\frac{1}{p} \times \frac{1}{1+\frac{t}{\tau_1}} + \frac{1}{1-p} \times \frac{1}{1+\frac{t}{\tau_2}} \right).$$

Two limit conditions can be considered:

1) All Arf1-GFP are cytoplasmic ($p = 1$), then

$$G(t) \propto \frac{1}{1+\frac{t}{\tau_1}} \quad or \quad \ln\left(\frac{1}{G(t)} - 1\right) \cong \ln(t) - \ln(\tau_1).$$

2) All Arf1-GFP are membrane-bound ($p = 0$), then

$$G(t) \propto \frac{1}{1+\frac{t}{\tau_2}} \quad or \quad \ln\left(\frac{1}{G(t)} - 1\right) \cong \ln(t) - \ln(\tau_2).$$

For any intermediate condition ($p \neq 0$ *or* 1), we introduce a processed correlation function $u(t)$ and use time limits to have a graphic estimate of p:

$$\begin{aligned} u(t) = \ln\left(\frac{1}{G(t)} - 1\right) &\underset{t \to 0}{\cong} \ln\left(\frac{t}{T_0}\right) \quad \text{with} \quad T_0 = \left(\frac{1-p}{\tau_1} + \frac{p}{\tau_2}\right)^{-1}, \\ u(t) = \ln\left(\frac{1}{G(t)} - 1\right) &\underset{t \to +\infty}{\cong} \ln\left(\frac{t}{T_\infty}\right) \quad \text{with} \quad T_\infty = (1-p)\tau_1 + p\tau_2. \end{aligned} \quad]$$

In other words, the graph of $u(t) = 1n(1/G(t) - 1)$ as a function of $\ln(t)$ gives a graphic representation of the value of p (Fig. 11 *Right*). For small p, the graph looks like the

graph for all cytoplasmic Arf1-GFP $T_0 = T_\infty = \tau_1$, whereas for p close to 1, the graph looks like the one for all vesicle-bound Arf1-GFP $T_0 = T_\infty = \tau_2$. For any intermediate p, $T_0 \neq T_\infty$, and the graph of $u(t)$ as a function of $\ln(t)$ displays a kink [see theoretical graphs of $u(t)$ for different values of P in Fig. 11 *Left*]. In Fig. 11 *Right*, Arf1-GFP (red) showed no kink and remained similar to GFP (blue) throughout the autocorrelation lag time rather than shifting toward the fluorescent bead profile (green); consequently, we can estimate that at least 98% of Arf1-GFP during metaphase is cytoplasmic and hence inactive GDP bound.

Analysis of Arf1 Binding and Release Kinetics in Interphase and Prophase Cells. To analyze Arf1-GFP's distribution changes in mitosis we constructed a simple kinetic model consisting of two compartments: Arf1-GFP in cytoplasm and Arf1-GFP on Golgi membranes. Arf1-GFP recruitment from the cytoplasm to the Golgi membranes was represented by the rate constant k_{on}, and dissociation of Arf1-GFP from Golgi membranes was represented by the rate constant k_{off}. Our previous live cell imaging work in interphase cells analyzing Arf1-GFP dynamics showed that exchange of Arf1-GFP between Golgi and cytoplasmic pools is well fitted with single association and dissociation rate constants that encompass the multiple intermediate steps in these processes (1).

The differential equation characterizing the amount of Arf1-GFP on the Golgi can be represented as

$$\frac{d}{dt}(\text{ArfGolgi}) = k_{on} * \text{ArfCytoplasm} - k_{off} * \text{ArfGolgi}.$$

Analytical solution of the differential equation describing this model reveals that recovery of Golgi fluorescence $f(t)$ after photobleaching proceeds with exponential kinetics and can be fitted to an equation of the form

$$f(t) \propto \left(1 - e^{-t/\tau}\right) \quad with \quad \frac{1}{\tau} = k_{on} + k_{off}$$

Note that τ is *not* the half-life for recovery, but rather the intrinsic characteristic time scale of Arf1 dynamics (i.e., association and dissociation of Arf1 with the Golgi during the recovery period) in our system. By definition, τ is the time when 37% (= 1/*e*) of the fluorescence has recovered. Fitting this equation to the FRAP data only determines the sum of the association and dissociation rate constants (i.e., $k_{on} + k_{off}$), one additional measurement is required to determine individual values of the two rate constants. Because the steady state distribution of fluorescent Arf1 between Golgi and cytoplasm is determined by the ratio $\boldsymbol{K} = k_{on}/k_{off}$, we measured total Arf1-GFP fluorescence in the Golgi and the cytoplasm to calculate the ratio $\boldsymbol{K}$. Thus, we combined these two measurements to obtain the rate constants

$$k_{on} = \frac{K}{1+K}\frac{1}{\tau} \quad and \quad k_{off} = \frac{1}{1+K}\frac{1}{\tau}.$$

and these rates were calculated for interphase cells and prophase cells. All of the calculations and least-squares fits were done simultaneously by using the numerical module of the SAAM II software (Version 1.1.2, SAAM Institute, Seattle). As described in the manuscript, as cells progressed from interphase to prophase we found that the mean association rate was ≈3-fold decreased (0.0051% sec^{-1} ± 0.0012 in interphase to 0.0018% sec^{-1} ± 0.0011 in prophase) while the mean dissociation rate was practically unchanged (0.0203% sec^{-1} ± 0.0046 in interphase to 0.0227% sec^{-1} ± 0.0064 in prophase).

We compared k_{on} and k_{off} for Arf1 in interphase and prophase cells at different expression levels of Arf1-GFP. We found that there could be up to a decade difference in the Arf1-GFP expression level within the Arf1-GFP-expressing cells that we studied. However, this had little impact on the calculated association and dissociation rates. To illustrate this, we present eight prophase cells where we have plotted their total Arf1-GFP levels against their association and dissociation rates (sec^{-1}), which were calculated from their respective FRAP curves and steady-state distribution ratios (Fig. 12).

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