Continuous polo-like kinase 1 activity regulates diffusion to maintain centrosome self-organization during mitosis

Robert Mahen*, Anand D. Jeyasekharan*, Nicholas P. Barry*, and Ashok R. Venkitaramana,1

*Medical Research Council Cancer Cell Unit, Hutchison/Medical Research Council Research Centre and 1Medical Research Council Laboratory of Molecular Biology, Cambridge, CB2 0XZ, United Kingdom

Whether mitotic structures like the centrosome can self-organize from the regulated mobility of their dynamic protein components remains unclear. Here, we combine fluorescence spectroscopy and chemical genetics to study in living cells the diffusion of polo-like kinase 1 (PLK1), an enzyme critical for centrosome maturation at the onset of mitosis. The cytoplasmic diffusion of a functional EGFP-PLK1 fusion correlates inversely with known changes in its enzymatic activity during the cell cycle. Specific EGFP-PLK1 inhibition using chemical genetics enhances mobility, as do point mutations inactivating the polo-box or kinase domains responsible for substrate recognition and catalysis. Spatial mapping of EGFP-PLK1 diffusion across living cells, using raster image correlation spectroscopy and line scanning, detects regions of low mobility in centrosomes. These regions exhibit characteristics of increased transient recursive EGFP-PLK1 binding, distinct from the diffusion of stable EGFP-PLK1-containing complexes in the cytoplasm. Chemical genetic suppression of mitotic EGFP-PLK1 activity, even after centrosome maturation, causes defects in centrosome structure, which recover when activity is restored. Our findings imply that continuous PLK1 activity during mitosis maintains centrosome self-organization by a mechanism dependent on its reaction and diffusion, suggesting a model for the formation of stable mitotic structures using dynamic protein kinases.

Results

Cell Cycle-Dependent Changes in EGFP-PLK1 Mobility in the Centrosome and Cytoplasm. We used a previously described (15) retinal pigment epithelium cell line immortalized using human telomerase (hTERT-RPE1), which expresses an EGFP-PLK1 fusion protein that functionally replaces endogenous PLK1 (EGFP-PLK1/PLK1<sup>−/−</sup>). We first measured the exchange between cytoplasmic and centrosomal EGFP-PLK1 during different cell-cycle stages using FRAP (16). Centrosomal EGFP-PLK1 exchanged almost completely within seconds after photobleaching during all cell-cycle stages, as shown by a FRAP recovery curve plateau at ~85–95% after ~40 s (Fig. L4). Our findings imply that, on this timescale, centrosomal populations of EGFP-PLK1 freely exchange with molecules in the cytoplasm and that little (~5–15%) of the protein is fixed within the structure, after image correction for photobleaching. Interestingly, the dynamic exchange between centrosomal and cytoplasmic EGFP-PLK1 is cell-cycle dependent. Fluorescence recovery was significantly slower during prophase and metaphase (time taken for half-maximal recovery, t<sub>1/2</sub> = 5.6 ± 0.3 s and 6.4 ± 0.7 s) than during either interphase or cytokinesis (t<sub>1/2</sub> = 4.2 ± 0.5 s and 2.4 ± 0.7 s, t test, P < 0.01) (Fig. L4). Notably, the slowed exchange rate during prophase and metaphase coincides with the known peak in PLK1 kinase activity (17, 18) marked by phosphorylation (19, 19) of PLK1 on threonine 210 (Fig. S1).

The relatively low expression of EGFP-PLK1 in our experimental system, and rapid fluorescence recovery in the cytoplasm, precluded analysis of cytoplasmic diffusion using FRAP. We therefore measured cytoplasmic EGFP-PLK1 mobility using fluorescence correlation spectroscopy (FCS), which is well suited to the analysis of fast-diffusing species present in low concentrations (20). FCS records fluorescence intensity fluctuations in a small confocal volume (~0.25 fL in our instrument) as a function of time, enabling inferences concerning physical properties including the rate of effective diffusion within the crowded and heterogeneous cellular environment (21) (Fig. 1B).

Using a previously described experimental setup (22), we find that EGFP-PLK1 fluorescence fluctuations exhibit a clear FCS autocorrelation function (ACF), whereas autofluorescence in wild-type RPE cells does not. As expected, the ACF for EGFP-PLK1 was significantly shifted to longer times compared with that for free EGFP alone, indicative of slower diffusion (Fig. 1C). The ACF for free EGFP could be fitted to a one-component model of 3D diffusion (23, 24) to give an effective diffusion coefficient, D<sub>e</sub>, of 31.1 μm<sup>2</sup>/s, consistent with values previously reported in vitro.

The authors declare no conflict of interest.

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<sup>1</sup>To whom correspondence should be addressed. E-mail: arv22@cam.ac.uk.

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Inhibition of EGFP-PLK1 Catalytic Activity Increases Mobility. The inverse correlation between EGFP-PLK1 diffusion and variations in PLK1 activity at different cell-cycle stages prompted us to predict that a reduction in PLK1 enzymatic activity should increase its mobility. To test this hypothesis, we exploited a previously established chemical genetic strategy (15), wherein endogenous PLK1 has been replaced with EGFP-PLK1(as), a form of the enzyme in which the catalytic pocket has been engineered to accommodate a nonnatural ATP analog, 3-MB-PP1, which serves as a rapid and specific inhibitor without detectable effects on normal protein function (27). Indeed, we confirmed that the introduction of EGFP-PLK1(as) itself had no effect on dynamics in the absence of inhibitor, nor did the addition of 3-MB-PP1 have any effect on EGFP-PLK1 WT in PLK1 cells (Fig. S3 A and B).

We treated cells expressing EGFP-PLK1(as) with 20 μM 3-MB-PP1 for 2.5 h. As expected from previous studies using this system (15), this treatment caused a modest increase in the percentage of cells with 4N DNA content in the G2/M phases of the cell cycle compared with DMSO mock-treated cells, as well as the appearance of cells arrested in prometaphase (Fig. S3 C and D). Notably, the rapid and specific inhibition of EGFP-PLK1(as) activity by this chemical genetic strategy was accompanied by an increase in its cytoplasmic diffusion measured by FCS (Fig. 2C). Thus, during prophase, the diffusion coefficient of EGFP-PLK1(as) increases to 9.9 ± 1 μm²/s after 3-MB-PP1 treatment compared with 5.4 ± 0.5 μm²/s in DMSO mock-treated cells (t test, P < 0.01).

For further confirmation that PLK1 kinase activity can affect its mobility, we studied the diffusion of a catalytically inactive mutant form of EGFP-PLK1, K82M, in which a lysine residue involved in ATP binding has been substituted with methionine (28). In interphase cells transiently expressing the mutant enzyme, analysis of cytoplasmic diffusion using FCS reveals that the D value for the cytoplasmic ATP-binding mutant is significantly increased in comparison with the wild-type enzyme (D = 8.6 ± 0.5, 5.7 ± 0.3 μm²/s, t test, P < 0.01). Therefore, results from two independent systems support the notion that cytoplasmic EGFP-PLK1 diffusion is inversely correlated with catalytic activity (Fig. 2B).

In addition to its kinase catalytic domain, PLK1 contains a phosphopeptide-binding domain [the Polo box domain (PBD)] that mediates substrate recognition (29). We used a well-characterized mutant form of PLK1 PBD mutant in which two amino acids (histidine/lysine 558/540) in the PBD are mutated to alanine and methionine, respectively, reducing the strength and duration of phosphopeptide–substrate binding (29). It has previously been reported using FRAP that an EGFP-PLK1 HK538/540 AM mutant exhibits increased dynamic exchange in the centrosome of transiently transfected interphase cells (30). Therefore, we first extended these studies to compare the FRAP curves of EGFP-PLK1 and the EGFP-PLK1 HK538/540 AM mutant at the centrosomes of stably expressing EGFP-PLK1 RPE cells at different cell-cycle stages. The difference in the dynamic exchange of the wild-type and mutant proteins peaked during prophase, when t1/2 values were 3.27 ± 0.3 and 0.9 ± 0.1 s, respectively (Fig. S4). Notably, the cytoplasmic diffusion of mutant EGFP-PLK1 HK538/540 AM measured by FCS was also increased during late G2/prophase compared with the wild-type enzyme (Fig. 2C), with effective diffusion enhanced to 7.44 ± 0.44 μm²/s. Taken together, our results demonstrate that disruption of the substrate-binding PBD domain of EGFP-PLK1 increases diffusional mobility both at the centrosome and in the cytoplasm, as does the specific inhibition of EGFP-PLK1 catalytic activity.

Raster Image Correlation Spectroscopy (RICS) Reveals a Region of Low EGFP-PLK1 Mobility at the Centrosome with Characteristics of Transient Binding. We explored the role of PLK1 mobility in the maintenance of centrosomal structure by simultaneously mapping EGFP-PLK1 diffusion in the cytoplasm and centrosomes of living cells, using RICS. RICS is a confocal laser scanning image analysis technique (31) that detects fluctuations between many focal points in the sample to determine the diffusion coefficient in different cellular locales (Fig. 3 A and B). We validated this technique in our experimental system using numerous control measurements in vitro and in vivo to minimize possible artifacts before taking EGFP-PLK1 measurements (summarized in SI Discussion and Fig. S5).

We found that the shapes of the RICS ACF curves (rACFs) were markedly different in the centrosome versus the cytoplasm. A representative analysis in a single cell (Fig. 3C) shows that the.
A continuous random walk (Fig. 3) stabilizes assembly into massive multiprotein complexes that exhibit cytoplasmic mobility. Protein diffusion within cells (32, 33), two modes of intermolecular interaction, and the interaction with other molecules in this environment. As reported in previous studies of protein diffusion within cells (32, 33), two modes of intermolecular interaction can be envisioned. Centrosomal EGFP-PLK1 could be slowed either through transient binding interactions that induce a “stop–start” motion or, instead, through stable assembly into multiprotein complexes that exhibit a continuous random walk (Fig. 3E, i). We used a previously described analysis of the shape of the rACFs to distinguish between these two possibilities (32). Whereas the slow diffusion of massive stable complexes broadens the rACF at long pixel shifts because particle movements between pixels are detected, rapid particle binding and unbinding in the microsecond–millisecond timescale conversely causes the rACF to narrow at long pixel shifts. We quantified the frequency of these characteristics in the RICS readings taken in the centrosome and cytoplasm (SI Methods and Fig. S7). Centrosomal rACFs frequently showed characteristics of transient binding (in 74% of 31 cells from two independent experiments) compared with cytoplasmic measurements (only 26%) (Fig. 3E, i). Our data are therefore consistent with the interpretation that transient recursive binding contributes to the slowing of EGFP-PLK1 mobility in the centrosome.

Continuous PLK1 Activity Is Required During Mitosis to Maintain PCM Structure Even After Centrosome Maturation. The data in Figs. 3 and 4 collectively identify two unrecognized features of EGFP-PLK1 mobility in living cells. First, EGFP-PLK1 exhibits considerable heterogeneity of effective diffusional motion within the cellular environment, evident in the marked slowing of its mobility in the centrosome compared with the cytosol. Second, the slowing of EGFP-PLK1 diffusion in the centrosome is accompanied by characteristics of continuous, but transient, recursive binding within this organelle. Together with our data demonstrating an inverse correlation between EGFP-PLK1 activity and mobility (Figs. 1 and 2), these features suggest that EGFP-PLK1 is organized into centrosomes by a mechanism that depends on its activity and diffusion. We therefore reasoned that continuous EGFP-PLK1 activity during mitosis would be required to

Fig. 2. Inhibition of EGFP-PLK1 catalytic activity increases mobility. (A) Chemical genetic inhibition of EGFP-PLK1 (as) kinase activity increases cytoplasmic mobility. FCS analysis was performed as described in Fig. 1 in EGFP-PLK1 (as) PLK1+ cells treated for 2.5 h with 20 μM 3MB-PP1 (blue) or with DMSO (black). (B) Genetic inactivation of EGFP-PLK1 kinase activity increases cytoplasmic mobility. RPE cells transiently expressing either EGFP-PLK1 wild type (black) or the kinase-dead mutant, KB2M (red) were analyzed by FCS in the cytoplasm during interphase as described in Fig. 1. (C) Genetic inactivation of PBD-substrate interactions increases the cytoplasmic mobility of EGFP-PLK1. Cells stably expressing EGFP-PLK1 wild type (black) or the PBD mutant, HK538/540 AM (red), were analyzed by FCS. In A–C, the horizontal bars show the mean ± SEM; each dot represents the measurement in a single cell, produced from 10 × 5 s readings. Data represent two independent experiments.

rACF at the centrosome was more extended in the vertical scanning direction at short pixel shifts, engendering a rounder overall shape, suggestive of slower diffusion. Indeed, fitting of the rACFs generated from multiple cells with a one-component model of anomalous diffusion (Eq. S3 in SI Methods) showed that the centrosomal EGFP-PLK1 pool consistently had a slower diffusion coefficient compared with the cytoplasmic pool (0.9 ± 0.3 μm^2/s versus 5.9 ± 0.4 μm^2/s, mean ± SEM, five independent experiments). Mapping back the diffusion coefficients onto a confocal slice area of the cell strikingly highlights how EGFP-PLK1 mobility is corralled at the centrosome, but relatively unimpeded in the cytoplasm (Fig. 3D). This difference does not simply reflect differences in the physical properties of the two environments or a general property of other proteins, because an inert tracer, free mCherry, expressed in the same cells exhibited no centrosomal coralling (Fig. S6).

We therefore surmised that the diffusion of EGFP-PLK1 might be slowed in the centrosome through its interaction with other molecules in this environment. As reported in previous studies of protein diffusion within cells (32, 33), two modes of intermolecular interaction can be envisioned. Centrosomal EGFP-PLK1 could be slowed either through transient binding interactions that induce a “stop–start” motion or, instead, through stable assembly into multiprotein complexes that exhibit a continuous random walk (Fig. 3E, i). We used a previously described analysis of the shape of the rACFs to distinguish between these two possibilities (32). Whereas the slow diffusion of massive stable complexes broadens the rACF at long pixel shifts because particle movements between pixels are detected, rapid particle binding and unbinding in the microsecond–millisecond timescale conversely causes the rACF to narrow at long pixel shifts. We quantified the frequency of these characteristics in the RICS readings taken in the centrosome and cytoplasm (SI Methods and Fig. S7). Centrosomal rACFs frequently showed characteristics of transient binding (in 74% of 31 cells from two independent experiments) compared with cytoplasmic measurements (only 26%) (Fig. 3E, i). Our data are therefore consistent with the interpretation that transient recursive binding contributes to the slowing of EGFP-PLK1 mobility in the centrosome.

Line Scanning Reveals a Region of Restricted EGFP-PLK1 Mobility at the Centrosome. RICS has limited spatial resolution (31, 34), which in our system we found to be an area of size ∼3.5 × 3.5 μm in control measurements (these areas are depicted in Fig. 3C, i). Therefore, to investigate PLK1 centrosomal coralling further at higher spatial resolution, we used line-scanning FCS as described previously (31, 35, 36). A single line of 64 pixels encompassing the centrosome and surrounding cytoplasm was scanned rapidly (5-μs dwell time on each pixel) and repeatedly (100,000 lines) to sample many locations simultaneously (Fig. 4A). The scanned line is represented as a “carpet diagram” of fluorescence intensity, with time plotted on the y axis and pixel position along the scan line on the x axis (Fig. 4 B and C). The temporal ACF calculated down each column as in FCS (Eq. S1 in SI Methods) represents the fluorescence fluctuations in a diffraction-limited spot that has a volume of ∼0.8 fL in our experimental system.

Line-scanning FCS analysis of EGFP-PLK1 diffusion supported the conclusions drawn from RICS. The timescale of ACF decay at the centrosome was much longer than in the adjacent cytoplasm (Fig. 4E, compare approximately column 30 with approximately column 50), indicative of the slower mobility of EGFP-PLK1 in this location, consistent with RICS analysis (Fig. 3). Notably, a plot of the diffusion coefficient in square micrometers per second versus pixel position along the scanning line shows a relatively sharp decay in diffusion coefficient at the position corresponding to the centrosome (Fig. 4F, compare the line graph with the intensity carpet). Thus, collectively, our observations using line-scanning FCS also suggest EGFP-PLK1 is corralled in the centrosome through transient recursive binding interactions.

Continuous PLK1 Activity Is Required During Mitosis to Maintain PCM Structure Even After Centrosome Maturation. The data in Figs. 3 and 4 collectively identify two unrecognized features of EGFP-PLK1 mobility in living cells. First, EGFP-PLK1 exhibits considerable heterogeneity of effective diffusional motion within the cellular environment, evident in the marked slowing of its mobility in the centrosome compared with the cytosol. Second, the slowing of EGFP-PLK1 diffusion in the centrosome is accompanied by characteristics of continuous, but transient, recursive binding within this organelle. Together with our data demonstrating an inverse correlation between EGFP-PLK1 activity and mobility (Figs. 1 and 2), these features suggest that EGFP-PLK1 is organized into centrosomes by a mechanism that depends on its activity and diffusion. We therefore reasoned that continuous EGFP-PLK1 activity during mitosis would be required to
material centrosome structure by such a mechanism. To test this idea, we exploited chemical genetics to rapidly and specifically inactivate or restore PLK1 activity, using 3MB-PP1 in EGFP-PLK1-(as) cells synchronized in prometaphase with nocodazole (Nz). Nz-induced mitotic arrest occurs after centrosome maturation in early mitosis, and therefore, as expected, Nz-arrested cells contained two centrosomes that were enriched in PCM relative to interphase. Strikingly, exposure to 10 μM 3MB-PP1 induced a marked dissolution in centrosome structure compared with the control, with a significant decrease in multiple PCM components, including γ-tubulin, NEDD1, and PCNT (Fig. 5 A and B; ? test, P < 0.01). PCM dissolution occurred rapidly after 3MB-PP1 treatment; changes were visible in 30 min, which became significant at 2 h (Fig. S8). This effect could be reversed

Fig. 3. Raster image correlation spectroscopy (RICS) reveals a region of low EGFP-PLK1 mobility at the centrosome with characteristics of transient binding. (A) Schematic representation of RICS methodology. (A, i) Particle movement (blue arrow) can be detected in raster scanning image time series from a confocal microscope (black lines). (A, ii) Fluctuations between pixels in different locations are detected and represented with the RICS autocorrelation function (rACF), GRICS, the shape of which reveals diffusion time. GRICS(0, 0) is plotted at the center of the grid. (B) RICS data collection area, denoted by the white box, at the centrosome and surrounding cytoplasm of a single EGFP-PLK1 PLK1→−→−− cell. (Scale bar, 5 μm.) (C) RICS analysis in the subcellular area shown in b, using a pixel dwell time of 12.61 μs and 125 frames. (C, i) A single frame of the time series, pseudocolored relative to fluorescence intensity. (Scale bar, 1.4 μm.) (C, ii) rACF calculated inside the subregions indicated in C, i, using a moving average of eight frames, pseudocolored relative to GRICS(x, y). (C, iii) rACF fits in 3D, using Eq. 57 in SI Methods to obtain the diffusion coefficients shown. Fit residuals are shown above the data. (D) EGFP-PLK1 diffusion is correlated at the centrosome. A diffusion map of the subcellular area indicated in B is shown, created by iteratively applying the RICS analysis to 64 × 64 pixel subregions. D, i shows a single frame of the time series for reference and D, ii shows the same area pseudocolored according to diffusion coefficient. (Scale bar, 1.4 μm.) (E) An increased proportion of centrosome rACFs show characteristics of transient binding compared with the cytoplasm. (E, i) Schematic representation of slowing through either “stop-start” binding or diffusion in massive complexes. (E, ii) The bar graph illustrates the average percentage of measurements classified as either binding or diffusion on the basis of analysis of the rACF shape relative to the point spread function (PSF) and after moving average length titration (described further in ref. 32 and SI Discussion). Data in B–D show a single cell representative of five independent experiments, whereas E, ii is representative of 30 individual cells analyzed in two independent experiments. Experimental averages are given in the main text.
Fig. 5. Continuous PLK1 activity is required during mitosis to maintain PCM structure even after centrosome maturation. (A) Nocodazole (Nz)-arrested EGFP-PLK1(as) cells were treated with the EGFP-PLK1(as) inhibitor 3MB-PP1 or mock treated with DMSO, before either fixation or washout of 3MB-PP1 and recovery after either fixation or washout. (B) Dot plot quantification of average fluorescence intensity of the PCM staining performed in A, whereby each dot represents a single cell (≥27 cells per sample), and horizontal lines show mean ± SEM. Data are representative of three independent experiments. (C) Model for the regulation of centrosome self-organization by PLK1 mobility. Black dots represent PLK, and red dots show putative substrates. (C, 1) PLK1 mobility is controlled by activity (indicated by ATP) in both the cytoplasmic and centrosomal pools. (C, 2) Mobility is spatially heterogeneous due to transient, recursive interactions. (C, 3) Continuous PLK1 activity enhances transient, recursive interactions required for the maintenance of centrosome structure even after centrosome maturation.

Discussion

We surmise that the behavior of PLK1 is an example of stigmergy, the principle whereby system components influence each other through dynamic interactions that leave traces or marks in their environment (1, 37). Thus, substrate modification by PLK1 may, in turn, stimulate the transient recursive binding interactions that slow its diffusion, and this stigmergic feedback loop may enable self-organization in structures like the centrosome. Interestingly and in support of our proposal, the rate of exchange of a centrosomally localized PLK1 substrate, Cmn, between the PCM and cytosol has recently been shown to affect centrosome structure (7). Thus, our proposal may more generally help to explain why PLK1 phosphorylates several centrosomal proteins whose modification is necessary for centrosomal structure (38, 39) and why artificial tethering of a PLK1 fusion protein within centrosomes triggers abnormalities in structure (6). From this perspective, two fundamental determinants of PLK1 behavior—diffusion and reaction—may be considered interdependent rather than separable. These considerations provide insight into how dynamic enzymes can self-organize in a manner dependent on their catalytic activity into stable cytoplasmic organelles (1–3).

Consistent with this model, a simple in silico simulation incorporating the diffusion and binding parameters we have measured for EGFP-PLK1 suggests that recursive binding interactions may allow the steady-state localization of PLK1 into a region of high substrate concentration (Fig. S9 and SI Discussion). Which of the 300 predicted PLK1 substrates is responsible for this behavior remains to be determined, although several are reported to concentrate in the centrosome (e.g., ref. 7), raising the possibility that multiple enzyme–substrate reaction sequences may contribute.

In conclusion, we present in this work three key observations that together suggest a model wherein centrosome PCM self-organization is sustained by the continuous activity of the PLK1 kinase through a reaction–diffusion mechanism. We first show using FRAP and FCS that EGFP-PLK1 substrate binding and catalytic activity regulate its cytoplasmic mobility, with decreased activity increasing the mobility of the enzyme. Second, RICS and line-scanning FCS reveal that a region of restricted EGFP-PLK1 diffusion in the centrosome correlates with characteristics of transient recursive interactions of the enzyme in this structure. Finally, a specific chemical genetic strategy confirms that ablation of EGFP-PLK1 activity even after centrosome maturation triggers defects in centrosome structure that can be reversed by the restoration of enzyme activity. Thus, our observations exemplify a reaction–diffusion mechanism (1) whereby the activity of a mitotic kinase contributes to the self-organization of diffusing mitotic proteomes into a key mitotic organelle.

Methods

FCS. One focus FCS was performed with the Zeiss Confocor2 system and a C-Apochromat 40× numerical aperture 1.2 water immersion objective as previously described (22). Samples were excited using a 488-nm laser operating at 0.8% and imaging over the 505- to 550-nm spectral band. The size of the lateral radius (w₀) and the structural parameter (S) were determined using a 10-nM rhodamine 6G (Rh6G) solution in water at 25 °C 100 μm above the coverslip, assuming a diffusion coefficient of 400 μm²/s. We thus obtained values of 0.2 μm and 5 for w₀ and S, respectively, which were kept constant during fitting. The normalized ACF G(c) of fluorescence fluctuations was calculated using Zeiss Confocor2 software.
where $I(t)$ is the fluorescence intensity, $\tau$ is the lag time, and $\langle \cdot \rangle$ represents a temporal average. Models used to fit the autocorrelation function are given in SI Methods. Diffusion coefficients (D) were calculated with

$$D = \frac{a^2}{4\tau}$$

For cellular readings each measurement consisted of repeating 10 readings, each 5 s in length. The first reading was always discarded to exclude any contributions from immobile molecules being bleached at the start of the reading (22). Readings showing obvious large deviations in count rate characteristic of cell movement were discarded.

RICS Data Collection and Analysis. RICS has been described previously (31), and the theoretical basis is given in SI Methods. A Zeiss LSM 710 laser scanning microscope with an incubation chamber and a 40x 1.2 NA water immersion lens was used. Data acquisition parameters were optimized using control measurements in vitro of Rhodamine R110 and EGFP and in vivo of EGFP (Fig. 55 and SI Discussion). The 488-nm laser was used with 1% of transmission power and the signal was detected using a long-pass emission filter (505 nm). The pinhole was set to 1 airy unit, detector gain was set to 800 V, and the smooth surface button was used twice in simFCS software. Readings were analyzed to ensure that only particle fluctuations rather than gross movements were recorded; occasional gross movements of the centrosome visible as intensity changes across >20 pixels along the scan line were discarded. Intensity drifts in Z due to movement or bleaching were corrected for using a previously described correction (35), which divides the time trace from one column in segments of 20 s each and adds random uncorrelated counts in each segment to match the intensity of the segment with the most counts.

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Supporting Information

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SI Discussion

Fitting of FCS Curves. We have discussed in detail elsewhere (1) the considerations that affect the choice of model used to fit FCS data. Here, we find that a simple one-component model of 3D diffusion (2) for fitting the ACF of EGFP-PLK1 allowed a systematic correlation in the fit residuals, with high residual values, suggesting that this simple model is inadequate to account for our data. This result is consistent with previous studies, including our own, on the diffusion of cellular proteins (1, 3).

An acceptable fit to our experimental data could be obtained either by the inclusion of an anomaly parameter or by using a two-component model of diffusion; both approaches reduced the fit residuals and the systematic correlation between them (Fig. S2). Both the two-component and the anomalous diffusion models have previously been used to fit in vivo FCS data (2, 3). Biologically, the two-component model represents the presence of additional diffusing species with different mobilities, whereas the anomalous diffusion model accounts for subdiffusive processes caused by, for example, molecular crowding of proteins within the nucleus.

Because there is no significant difference between the standardized residuals of the fits of these two models to our data (Fig. S2), we chose an anomalous diffusion model to fit our data, recognizing that the addition of a second diffusing component may be an equally valid method (4, 5). However, it should be emphasized that the conclusions drawn in our study are independent of the model chosen to fit the raw FCS data. Instead, we rely on the observed relative changes between internally controlled experimental comparisons, using the same model.

RICS Control Measurements. Suitable RICS data collection and analysis parameters for EGFP-PLK1 were determined from a range of in vitro and in vivo control measurements (Fig. S5). We sampled a range of different known mobilities from ~1–300 μm²/s using rhodamine R110, 175-nm diameter fluorescent latex beads (PS-Speck Microscope Point Source kit; Molecular Probes), and EGFP in water. Multiple acquisition settings such as pixel dwell time and frame number were optimized using these controls (see ref. 6 for a discussion of RICS methodology). EGFP expressed in multiple cell types served as an in vivo model of diffusion and binding were performed using simFCS software (www.lfd.uci.edu), followed by RICS data collection and analysis. Parameters were taken from the cellular FCS and RICS measurements: A cube of size 6.4 × 6.4 × 6.4 μm was used as the environment for the simulation, within which PLK1 particle concentration started at 50 nM, with Brownian diffusion at 5 μm²/s. Binding in an area of high substrate concentration ~1 μm width resulted in the steady-state accumulation of transiently bound PLK1 (Fig. S9 A, i and B, i). Local diffusional slowing was also apparent in this region by RICS analysis (Fig. S9B, iii). Both these effects were dependent on the time spent in the bound state (i.e., the time “on” of binding) (Fig. S9C); simulations incorporating transient recursive binding events each lasting ~1 ms caused diffusional slowing consistent with our in vivo observations. Therefore, in this simple hypothetical situation, our measured cellular parameters are sufficient to sustain the steady-state accumulation of PLK1 into a region of high substrate concentration. We emphasize, however, that our simulation represents a simple model that may have limited predictive value. Thus, the multiplicity of potential centrosomal substrates for PLK1 (8) and the lack of information concerning the characteristics of binding together complicate and constrain the power of the simulation.

SI Methods

Cell Culture and Transfection. Cells were grown at 37 °C with 5% CO₂ in F12:DMEM (1:1) media supplemented with 10% FCS, sodium bicarbonate, glutamate, and penicillin. They were imaged in MatTek poly-l-lysine–coated no.1 glass-bottomed 35-mm dishes, in media without phenol red at 37 °C. Transfection was with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Constructs. EGFP-PLK1 and EGFP-PLK1 K82M in the vector EGFP-C2 (Clontech) were kind gifts from Matthew Daniels (Oxford University, Oxford, United Kingdom). HK538/540 AM was made by PCR from EGFP-PLK1 using the following complementary primers:

F: CAACTTCTTCCAGGATGCCACCATGCTCATCTTGTCGCCACTG
R: CAGTTGGCCACAAGATGAGCATGGTGCCATCTTGGAAGAAGTTG

Fluorescence Recovery After Photobleaching (FRAP). FRAP was as described previously (9), using a Zeiss LSM 510 META confocal microscope with a heated stage, using a 100x NA 1.4 oil objective lens. Bleaching was performed in a rectangular spot encompassing the whole centrosome (~1 × 1 μm) using a 40-mW argon laser operating at 100% and took 1.25 s. Recovery was monitored by imaging at 3% laser power at 0.5-s intervals. Fluorescence values were corrected for both bleaching due to imaging and depletion of the cellular pool of protein. Curves were normalized between 0 and 100% in GraphPad Prism software and fitted to a single-order exponential equation Y = Ymax · (1-e⁻ᵏᵗ), where k = ln(2) / t₁/₂ to yield t₁/₂ and plateau (Ymax) values.

Fluorescence Correlation Spectroscopy (FCS). FCS theory has been described (10). Calculation and fitting of the autocorrelation function was performed in Zeiss Confocor 2 software. The normalized autocorrelation function (ACF) G(τ) of fluorescence fluctuations is

\[ G(\tau) = \frac{\langle I(t)I(t+\tau) \rangle}{\langle I(t) \rangle^2}. \]

where I(t) is the fluorescence intensity, τ is the lag time, and ⟨ ⟩ represents a temporal average. Assuming a 3D Gaussian profile in the focal volume, the following formula is used to describe three-dimensional diffusion (2).
where $\tau D$ is the dwell time in the observation volume, $N = CV_{eff}$ is the total number of molecules in the detection volume, $w_0$ is the $1/e^2$ radius of the Gaussian detection volume, and the structure parameter $S = z_0/w_0$, measures its aspect ratio. $T$ is the triplet fraction and $\tau_t$ the triplet time. These two parameters were determined from control EGFP readings and then kept fixed during the fitting routine, or the curve was fitted 100 µs onward. The model for anomalous diffusion is (11)

$$
G(\tau) = \frac{1}{N} \frac{1 - T + T e^{(-\tau/\tau_D)}}{1 - T} \left(1 + \frac{\tau}{\tau_D}\right)^{-1} \left(1 + \frac{\tau^2}{w_0^2 \tau_D^2}\right)^{-(1/2)},
$$

[S2]

where $\alpha$ is an anomaly parameter that describes the nonlinearity of mean square deviation, i.e., $\text{MSD} = 6D\alpha$, and $\tau D$ is replaced by $\tau D^\alpha$, the time for anomalous diffusion. Fitting control measurements on EGFP with a one-component model of anomalous diffusion gave an anomaly parameter (\(\alpha\) in Eq. S3) of 0.73, similar to previously published cytoplasmic values (12). We fixed it to this value when fitting EGFP-PLK1 FCS data. The model for two-component analysis is (2)

$$
G(\tau) = \frac{1}{N} \frac{1 - T + T e^{(-\tau/\tau_D)}}{1 - T} \left[\frac{F_1}{1 + \frac{\tau}{\tau_{D1}}} \left(1 + \frac{\tau}{\tau_{D2}}\right) \left(1 + \frac{\tau}{\tau_{D3}}\right)\right]
$$

[S4]

where $F_1$ is the fraction of the fast-diffusing component with a diffusion time $\tau_{D1} = w_0^2/4D_{fast}$, whereas $\tau_{D2} = w_0^2/4D_{slow}$ is the diffusion time of the slow component. $\tau D$ is related to the diffusion coefficient by

$$
\tau D = \frac{w_0^2}{4D}
$$

[S5]

**Immunofluorescence.** Cells grown on coverslips were fixed for 5 min in ice-cold methanol at -20 °C, with the exception of T210-P staining, for which cells were fixed in 4% paraformaldehyde at room temperature for 15 min. They were blocked in 5% BSA for 1 h at room temperature before incubation with primary antibodies in blocking solution at room temperature for 3 h. Antibodies and dilutions used were mouse anti-γ-tubulin (GTU-88, 1:1,000), rabbit anti-pericentrin (1:1,000), anti-tubulin (GTU-88, 1:4448) rabbit anti-NEDD1 (1:75336) and mouse anti-PLK1 pT210 (BD Pharmingen, 558400). Following washes in PBS, cells were incubated with 1:400 dilution of secondary antibodies for 30 min (donkey anti-mouse/rabbit conjugated to Alexa 568; Molecular Probes). Coverslips were mounted in 4,6-diamidino-2-phenylindole (DAPI) containing mounting medium (Vector shield) to visualize chromatin.

**Quantification of Centrosomal Fluorescence Intensity from Immunofluorescent Staining.** Quantification of the average centrosomal fluorescence intensity was performed using the freehand selection tool in Zeiss LSM ConfoCor 3 software. Maximum intensity z-projections were formed from z-stacks of confocal slices taken at 0.8-µm intervals. Cell-cycle stage was determined on the basis of nuclear morphology and EGFP-PLK1 localization. Centrosomes were identified by using the EGFP-PLK1 channel before measurement of T210 intensity in a different channel. Pixel intensities were never saturated or clipped off using offset and exposure settings that were identical between samples. Background fluorescence intensity was subtracted from all images.

**Fluorescence-Activated Cell Sorting (FACS).** FACS analysis was as described previously (13). Cells were harvested by trypsinization, washed in PBS, fixed in ice-cold 70% ethanol for 2 h, and stained with propidium iodide.

**Raster Image Correlation Spectroscopy (RICS) Theoretical Basis.** RICS has been described previously (6). The temporal–spatial RICS ACF (\(r\text{ACF}\)) is calculated in each frame with

$$
\text{GRICS}(\xi, \psi) = \frac{(I(x,y)I(x + \xi, y + \psi))}{\langle I(x,y) \rangle^2}; \quad \langle I(x,y) \rangle = \langle I(x,y) \rangle_{\text{eff}} + \langle \alpha \rangle \langle I(x,y) \rangle_{\text{cor}}.
$$

[S6]

where $I(x, y)$ is a matrix representing one image of the stack, $\xi$ and $\psi$ are shifts in the x and y directions, respectively, and the angled brackets represent averaging over all of the spatial locations in both the x and the y directions. As molecular diffusion and the scanning motion are independent, the spatial correlation can be split into two terms that describe it, adding in a term accounting for the detector movement:

$$
G_s(\tau) = S(\tau).G(\tau).
$$

[S7]

The following equation describes the scanning part of the correlation function,

$$
S(\xi, \psi) = \exp\left(-\frac{1}{2}\left(\frac{256\psi}{w_0^2} + \frac{256\xi}{w_0^2}\right)^2\right) (1 + 4D(\tau D + \tau \psi) w_0^2)
$$

[S8]

where $\xi$ and $\psi$ are the horizontal and vertical coordinates, respectively, $\alpha$ is the pixel size, and $\tau$ is the residence time at a pixel or along a line.

**RICS Diffusion and Binding Analysis.** The principles of the RICS diffusion and binding analysis have been described previously (15). The shapes of the rACFs were analyzed at long pixel shifts in both the horizontal and vertical scanning directions in two different ways. First, the rACF was plotted relative to the Gaussian correlation of the PSF at a fixed moving average length of 10. Second, the moving average length was varied to 4, 10, and 20. Criteria determined through simulated comparisons of transient binding and a continuous random walk using simFCS software were then used to classify each measurement as either diffusion or binding. According to these criteria binding was defined as being restricted to the PSF at long pixel shifts, and it changed shape across different areas of the curve (depending on the horizontal or vertical direction) in response to variation of the moving average length (see Fig. S6 for examples).
**Diffusion and Binding Simulations.** PLK1 diffusion and binding simulations were performed using simFCS software. simFCS is described in detail at www.lfd.uci.edu. The “binding 2D” function was used to simulate a localized area of high substrate concentration, varying the “time on” between 0 and 1,000. Two hundred cycles were run to completion. For the RICS data collection simulation, particle brightness was 100,000 counts per second per molecule and raster scanning was simulated using a 3D Gaussian point spread function of radial waist 0.3 μm. RICS analysis was performed as described in the raster image correlation spectroscopy data collection and analysis methods sections.


![Fig. S1.](image-url) Immunofluorescent staining of PLK1 T210 phosphorylation at the centrosome peaks in metaphase and prophase. EGFP-PLK1 WT PLK1−/− cells were stained with anti-PLK1 T210-p antibody and imaged by confocal microscopy. Cell-cycle stage was determined from nuclear morphology and EGFP-PLK1 fluorescence. Each circle represents the quantification of centrosomal anti-PLK1 T210-p signal from a single cell; horizontal lines show mean ± SEM.
Fig. S2. Fitting of EGFP-PLK1 FCS data. EGFP-PLK1 FCS curves were fitted in Zeiss ConfoCor2 software using three different models of diffusion as described in SI Discussion. (A, i) Average EGFP-PLK1 amplitude normalized ACF curve from interphase cells as described in Fig. 1 (black dots) with three different fits (colored solid lines). (A, ii) Fit residuals. (B) $\chi^2$ residuals of the fits in A.
Fig. S3. Dynamic and phenotypic characterization of EGFP-PLK1(as) cells. (A) EGFP-PLK1 shows the same mobility in EGFP-PLK1(as) and EGFP-PLK1(wt) cells. The graph shows FCS curves taken in the cytoplasm of interphase cells; values are mean ± SEM from ≥20 readings taken in 20 cells. Data are representative of two independent experiments. (B) EGFP-PLK1(wt) cells show no change in FCS dynamics after treatment for 2.5 h with 20 μM 3MB-PP1. Measurements were made and values depicted as in A. (C) FACs analysis shows a modest increase in the 4N DNA content of EGFP-PLK1(as) cells after 3MB-PP1 treatment (C, i) compared with mock DMSO treatment (C, ii). A total of 10,000 cells were analyzed per sample. (D) A representative example of a prometaphase-arrested EGFP-PLK1(as) cell visible 2.5 h after 3MB-PP1 treatment. The image shows a rounded-up cell with condensed DNA and EGFP-PLK1 localized to centrosomes and kinetochores. (Scale bar, 3 μm.) Thirty-five percent of cells (n = 1,000) exhibited this phenotype after overnight incubation with 3MB-PP1.
Fig. S4. Genetic inactivation of PBD substrate interactions increases the rate of exchange between the centrosome and the cytoplasm. FRAP analysis was performed as described in Fig. 1, by bleaching the centrosome in RPE cells stably expressing either EGFP-PLK1 (black curve) or EGFP-PLK1 HK538/540 AM (red curve). Dots and $t_{1/2}$ values are mean ± SEM from 25 measurements and are representative of two independent experiments.
Fig. S5. Determining the dynamic range of RICS in vitro and in vivo. (A) RICS negative control readings on a Zeiss LSM 710 microscope show little false-positive correlation. RICS time series were taken without laser illumination and the rACF was calculated. (Left and Center) rACF in the horizontal and vertical scanning directions, respectively. (Right) 2D rACF color coded according to \(G(x,y)\) amplitude, with blue representing zero. (B–D) Control readings used to find the dynamic range of the assay. The images show a single frame of raw data (Left) and the rACF (Center and Right) fitted with a one-component model to obtain an expected diffusion coefficient \(D\). (B) A total of 40 nM Rhodamine R110 in water, measured at 21 °C using a pixel dwell time of 5.09 μs using 100 frames. (C) A total of 50 nM EGFP in water, measured at 21 °C with a pixel dwell time of 5.09 μs using 100 frames. (D) EGFP expressed in RPE cells, measured at 37 °C using a pixel dwell time of 12.61 μs and 100 frames. (E) Slowly moving objects (in the seconds timescale) were prevented from affecting the rACF using the previously described (6) moving average (Methods). Two 175-nm diameter fluorescent latex beads stuck to the coverslip are shown (white arrow), surrounded by diffusing beads in water (red arrow). The two relatively immobile beads dominate the rACF (Center), unless a moving average of 10 frames is applied (Right). Data in B–D are from \(\geq 15\) measurements taken in 15 different cells, in two independent experiments.
**Fig. S6.** mCherry mobility is not restricted at the centrosome. (A) EGFP-PLK1(as) cells were transfected with mCherry. The images show an example subcellular region containing the centrosome in which RICS was performed. (B) RICS analysis of mCherry in the subcellular area shown in A. (B, i) A merge of the two channels depicted in A. (B, ii) Fits of the rACFs of mCherry calculated in the subregions indicated in B, i, pseudocolored relative to GRICS(x, y), using a pixel dwell time of 12.61 μs, 150 frames, and a moving average of 6. Fit residuals are shown above the data. (Scale bar, 1.4 μm throughout.)

**Fig. S7.** Representative rACFs taken at the centrosome and in the cytoplasm. (A) Horizontal scanning direction of rACF plotted relative to the correlation from the point spread function (PSF). Note the decreased overlap between the data and the PSF at longer pixel shifts in the centrosome compared with the cytoplasm (arrow). (B) Vertical scanning direction of rACF analyzed with different lengths of moving average. Note the narrowing with decreasing moving average length in the centrosome, but not in the cytoplasm (arrow). These readings show examples from a total dataset of 30 cells taken in two independent experiments.
Fig. S8. Specific inactivation of PLK1 with 3MB-PP1 rapidly disrupts PCM structure during mitosis. EGFP-PLK1 cells were arrested in prometaphase with Nm and treated with 10 μM 3MB-PP1 for the indicated times before fixation and staining with anti-NEDD1 antibody. Circles show the average centrosomal immunofluorescence of NEDD1 staining in single cells (n ≥ 15 cells), and horizontal bars show mean ± SEM. The results are representative of two experiments.

Fig. S9. In silico modeling of PLK1 diffusion and binding. (A and B) Simulations were performed in simFCS software using the parameters obtained from cellular EGFP-PLK1 measurements. Particle diffusion was set at 5 μm²/s and concentration started at ~50 nM, in a cube of size 6.4 x 6.4 x 6.4 μm. Binding within a region of high substrate concentration ~1 μm in diameter was simulated with varying binding times. RICS data acquisition was simulated using similar parameters to those used in cellular measurements; raster scanning was with a pixel dwell time of 15.6 μs and a point-spread function radial waist size of 0.3 μm. (A, i and B, i) A single frame of the simulation upon completion; red dots represent PLK1, and the arrow denotes the position of the region of binding. (A, ii and B, ii) Average of the RICS image stack of the simulation in A, i and B, i, false colored relative to fluorescence intensity. (Scale bar, 0.64 μm.) (A, iii and B, iii) RICS diffusion map of the simulation in A, i and B, i, created as described in Fig. 3, and example rACFs in the indicated regions. (C) The graph depicts the measured diffusion coefficients inside and outside the binding region plotted against the simulated binding time, calculated from RICS analyses of the simulations described in A and B.