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Polo-like kinase 1-mediated phosphorylation of the GTP-binding protein Ran is important for bipolar spindle formation ☆

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Abstract

Polo-like kinase functions are essential for the establishment of a normal bipolar mitotic spindle, although precisely how Plk1 regulates the spindle is uncertain. In this study, we report that the small GTP/GDP-binding protein Ran is associated with Plk1. Plk1 is capable of phosphorylating co-immunoprecipitated Ran *in vitro* on serine-135 and Ran is phosphorylated *in vivo* at the same site during mitosis when Plk1 is normally activated. Cell cultures over-expressing a Ran S135D mutant have significantly higher numbers of abnormal mitotic cells than those over-expressing either wild-type or S135A Ran. The abnormalities in S135D mutant cells are similar to cells over-expressing Plk1. Our data suggests that Ran is a physiological substrate of Plk1 and that Plk1 regulates the spindle organization partially through its phosphorylation on Ran.
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The major functions of the GTP/GDP-binding protein Ran include nucleo-cytoplasmic transport, cell cycle progression, microtubule nucleation and spindle assembly in Xenopus oocytes [1]. Recent reports have shown that in Xenopus extracts, GTP-bound Ran stimulates formation of aster microtubules [2]. Ran modulates microtubule dynamics through increasing rescue frequency and stimulations of a plus-end-directed microtubule motor [3]. Recent studies have indicated that Ran's role in spindle assembly could be extended to mammalian cells [4].

Polo-like kinase (Plk1) is a mammalian member of the polo family of serine/threonine kinases, a family which is conserved among organisms from yeast to mammals [5]. Polo-like kinases (Plks) are involved in multiple stages of the cell cycle, including initiation of M phase, bipolar spindle formation, mitotic exit, cytokinesis [5], and DNA damage checkpoint regulation. Plk1 interacts with a number of proteins such as Cdc25C phosphatase [6], α, β, γ-tubulins [7], proteasomes [8], anaphase-promoting complex [9], and NudC [10]. Both the wide variety of Plk1 interacting proteins and the striking changes in localization of Plk1 during mitosis and cytokinesis suggest that Plk1 may be

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a partner in several distinct multi-protein complexes. In accord with this idea, we previously found that the fraction of cellular Plk1 associated with proteasomes does not interact with tubulins [8]. Therefore, it seems likely that Plk1 interacts with distinct proteins at different stages of the cell cycle and at different subcellular locations. Although Plk1 interactions with identified proteins have clarified some of the molecular details of Plk1 functions, it seems likely that more interacting proteins and potential substrates remain unknown. We have therefore focused on identifying such new Plk1 partners that may reveal additional clues about the roles of Plk1 in the cell cycle and possibly how aberrant Plk1 functions are involved in disease. In this study, we show that Ran is phosphorylated during mitosis and that the kinase responsible is likely to be Plk1. We further demonstrate that during some stages of mitosis, Plk1 and Ran are concentrated at spindle poles. Finally, we show that over-expression of S135D mutant Ran induces significant mitotic abnormalities.

Materials and methods

Materials. Ran monoclonal antibody was purchased from Pharmingen BD Bioscience (San Diego, CA). Ran goat polyclonal antibody was purchased from Santa cruz Biotechnology (Santa Cruz, CA). MPM2 monoclonal antibody was purchased from DAKO (Carpinteria, CA). α and β-tubulin monoclonal antibodies were purchased from Sigma (St. Louis, MO). HA tag mAb was purchased from BabCo (Richmond, CA). Sequencing grade trypsin and Asp-N were purchased from Roche (Indianapolis, IN).

Cell culture and transfection. CA46 (human B lymphocyte lymphoma cell line) cells were maintained in RPMI1640 medium with 10% FBS. Cells were synchronized to G1 with mimosine (300 μM), to G1/S boundary with aphidicolin (0.75 μM), to metaphase with nocodazole (100 ng/ml) for 16 h.

HEK293 cells were cultured in DMEM with 10% FBS. Cells were transfected with plasmid DNA with Fugene 6 (Roche) reagent according to the manufacturer's instructions. Twenty-four hours post-transfection, cells were synchronized to mitosis with nocodazole.

The canine osteosarcoma cell line (D-17) was obtained from ATCC (#CCL-183).

D17 cells were transfected with a plasmid encoding the avian subgroup A receptor (tva) [11] and puromycin-*N*-acetyl-transferase. The transfected cells were selected after 24 h with medium containing 1 μg/ml puromycin. Selected clones were susceptible to infection by viruses expressing the ALU(A) envelope such as RCASBP(A).

Avian viral vectors. Construction of the avian vector is described in supporting text (online access).

Immunoprecipitations. Cell lysates were made in NP40 lysis buffer (1% NP40, 50 mM Tris, pH 8.0, 250 mM NaCl, 10 mM EGTA) supplemented with 1 mM DTT and protease inhibitors. The lysates were clarified by centrifugation at 100,000*g* for 1 h at 4 °C. The supernatant was used for immunoprecipitation. Each immunoprecipitation reaction included 1 mg lysate protein, 5 μg antibodies, and 20 μl of protein G-sepharose. The mixture was rotated at 4 °C overnight, and washed extensively with the lysis buffer.

Immunofluorescence. Cells grown on coverslips were fixed in freshly made 4% paraformaldehyde at RT for 30 min, followed by permeabilization in 0.5% Triton X-100. Cells were blocked in 4% normal goat serum for 1 h, and incubated with primary antibodies overnight at 4 °C at dilution indicated: anti-Ran (1:500), anti-Plk1 (1:500), anti-myc (1:1000) and anti-tubulin (1:500). Incubation with secondary antibodies was for 1 h at RT as indicated: FITC-conjugated goat anti-mouse IgG or rhodamine-conjugated goat anti-rabbit at 1:300. DNA was stained with DAPI at 1:12,000 dilution for 10 min at RT. Coverslips were mounted and cells were examined under an Axio fluorescence microscope.

Kinase reactions. Immunoprecipitates were washed with the kinase buffer, and the kinase reaction was performed as described in [7] and a second round of immunoprecipitation performed with specific antibodies.

Metabolic labeling. CA46 cells were first synchronized, conditioned in phosphate-free RPMI1640 with phosphate-free FBS, and labeled with [^{32}P]orthophosphate (1 mCu/ml) for 2 h in phosphate-free medium. Cell-synchronizing drugs were kept in the medium during labeling.

Phosphoamino acid analysis. Phosphoproteins were hydrolyzed in 6 N HCl at 110 °C for 1 h. The samples were dried using a speed-vac, and separated on thin layer chromatography plates according to Hunter et al. [12].

Mapping of phosphorylation sites. Mapping was performed as previously described [13]. Briefly, the phosphoprotein was digested with sequencing grade trypsin or Asp-N. The phosphopeptides were purified by HPLC. ^{32}P-labeled peptide was covalently bound to Sequalon disks and subjected to solid phase Edman degradation with an Applied Biosystems model 420 sequencer. Cycle fractions were spotted onto Water #1 paper and radioactivity was quantitated with an Amersham phosphoimager.

MALDI-TOF. Coommassie Blue stained protein gel bands were digested as described [14]. Samples were desalted with C18 Zip Tips (Millipore, Bedford, MA) as per manufacturer's protocols and stored at −20 °C until analysis. Mass spectrometric analysis: 1 μL of sample was co-crystallized with 1 μL of a 20% solution of α-cyano-4-hydroxycinnamic acid in 50% ACN, 1% trifluoroacetic acid, and spotted directly onto a stainless steel matrix-assisted laser desorption ionization (MALDI) target plate. Mass spectra were acquired using an Applied Biosystems 4700 MALDI-TOF/TOF mass spectrometer (Applied Biosystems Inc., Foster City, CA) using a laser frequency of 200 Hz. MALDI spectra were internally calibrated (<20 ppm) using known trypsin autolysis peptides. Post-acquisition baseline correction and smoothing was carried out using software provided with the TOF/TOF instrument. The peptides were identified via peptide mass fingerprinting using Mascot (http://matrixscience.com).

Results

The small GTP-binding protein Ran interacts with Plk1

Mammalian Plk1 is a cell cycle-dependent serine/threonine kinase. Mitotic Plk1 displays strong activities toward α-casein in an *in vitro* kinase assay. Some cellular proteins co-precipitated with Plk1 are also phosphorylated in this assay. Among the proteins phosphorylated by Plk1 is a low molecular weight protein of ~29 kDa (Fig. 3A, lane 2). This phosphoprotein was isolated, digested with trypsin, and analyzed by mass spectrometry. Sequences of three peptides derived from human Ran were identified, KYVAT LGVEV HPLVF HTNR, SNYNF EKR, and FLWLA R. These peptides match 33 of 216 amino acids (15%) of the human GTP-binding protein Ran.

To confirm the mass spectrometric results, Plk1 immunoprecipitations from human CA46 cells were analyzed by Ran immunoblotting (Fig. 1A). Ran was co-immunoprecipitated with Plk1 from both interphase and mitotic cell lysates. While Ran is an abundant protein in cells, we found that only a fraction of Ran protein was associated with Plk1, with a larger amount remaining in supernatants after Plk1 immunoprecipitation. This reflects the multi-role functions of Ran in cells. Plk1 could also be detected in Ran immune complexes, regardless of which Ran antibodies were used.

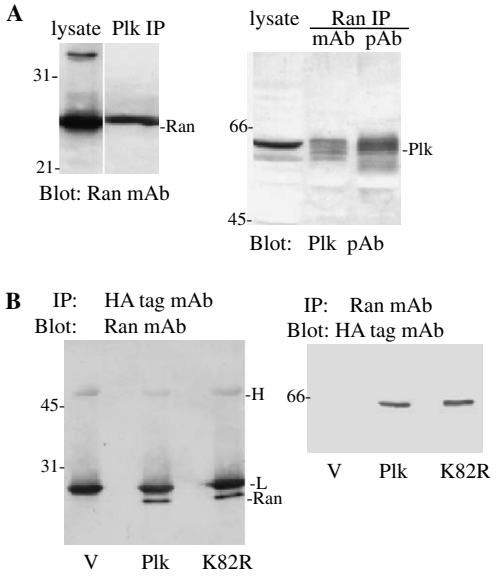

Fig. 1. Reversible co-immunoprecipitation of Ran GTP-binding protein and Plk1 in CA 46 cells and transfected 293 cells. (A) Left panel, total cell lysate or Plk1 immunoprecipitates were probed with Ran mAb. Right panel, Ran was immunoprecipitated from lysates with mAb or goat pAb, and immunoprecipitates were probed with Plk1 pAb. (B) HEK293 cells were transfected with pcDNA3 control vector (V), wild-type HA tagged-Plk1 (Plk1), or kinase inactive HA-K82R (K82R). Left panel, HA tagged Plk1 proteins were immunoprecipitated with HA tag mAb, and the presence of Ran was detected with Ran mAb. Right panel, Ran immunoprecipitates were probed with HA tag mAb.

Similar co-immunoprecipitations were performed in HEK293 cells transfected with HA tagged wild-type Plk1 or kinase dead Plk1 (K82R). As shown in Fig. 1B, Ran was present in HA immunoprecipitates from cells transfected with wild-type or kinase dead Plk1, and not present in HA immune complexes isolated from non-transfected cells. Both HA-Plk1 and HA-K82R were detected in reciprocal Ran immunoprecipitations and HA immunoblots. These results confirm that Plk1 interacts with Ran in cells and further indicates that the kinase activity of Plk1 is not required for the interaction.

Interaction of Plk1 and Ran was further supported by their co-localization in native cells (Fig. 2). D17 canine osteosarcoma cells were used because these cells display epithelial flat morphology even at mitosis, allowing better visualization. Endogenous Ran and Plk1 were shown by fluorescent stainings with their specific antibodies. In mitotic cells, Ran protein localization generally followed the staining pattern of Plk1 on spindle poles and fibers at various stage of mitosis, with the only exception being in the midbodies. During interphase, Ran protein is predominantly nuclear.

Ran is phosphorylated by Plk1 in vitro and possibly in vivo

The next critical question is whether Ran might be a substrate of Plk1. Phosphorylation of Ran has not been reported, although several members of the Ras superfamily are phosphorylated and their distribution or activities are affected by their phosphorylation status. These include Rab1, Rab4, Rab6, Rab8, RhoA, REM, RAD, and Gem [15–22]. We have decided to approach this question at multiple levels. First, HEK293 cells were transfected with a control vector, HA-Plk1 or HA-K82R. HA immunoprecipitates from transfected cells were subjected to *in vitro* kinase reactions, SDS–PAGE, and autoradiography. A protein at about 29 kDa was phosphorylated in HA-Plk1 but not in HA-K82R immunoprecipitates, indicating this phosphorylation is most likely mediated by Plk1 (Fig. 3A). To confirm that the 29 kDa protein was Ran, kinase reaction mixtures were treated with hot SDS to dissociate protein interactions, diluted in buffer, and a second immunoprecipitation performed with a monoclonal Ab specific to Ran (lanes 4–6). Ran protein was indeed the 29 kDa phosphoprotein seen in Lane 2, and it is specifically phosphorylated by Plk1.

Since Ran and Plk1 interact, we reasoned that there should be serine/threonine kinase activity associated with Ran immunocomplexes. We added the artificial substrate α-casein, a preferred substrate for Plk1, to Ran immunoprecipitates from mitotic cells and performed kinase reactions with the immune complexes (Fig. 3B). As expected, *in vitro* reactions demonstrated a strong kinase activity associated with Ran that could phosphorylate casein in addition to endogenous Ran. Therefore, Ran is constitutively associated with a kinase activity that is able to phosphorylate Ran and exogenously added casein, and Plk1 is capable of phosphorylating Ran *in vitro*.

Ran undergoes cell cycle-dependent phosphorylation in vivo

Having determined that Plk1 is capable of phosphorylating Ran *in vitro*, we then asked if similar phosphorylation occurs *in vivo*. To investigate this possibility, we arrested CA46 cells at different stages of the cell cycle, radio-labeled them with [^{32}P]orthophosphate, then isolated Ran protein, and analyzed it by autoradiography. Indeed, Ran protein was phosphorylated, and the phosphorylation was more pronounced in mitotic cells (Fig. 4A). Ran is an abundant protein in cells, so this relatively weak metabolic labeling may indicate that only a subset of Ran protein undergoes phosphorylation *in vivo* at M phase. This phenomenon is reminiscent of the result in Fig. 1 showing that a small fraction of total Ran protein is associated with Plk1. It is also possible that Ran is more heavily phosphorylated than is indicated by this experiment since Plk1 activity is down-regulated by DNA damage and [^{32}P]orthophosphate will certainly damage DNA [23].

In addition to metabolic labeling studies, the possible *in vivo* phosphorylation was also tested with a non-labeling approach by utilizing a monoclonal antibody MPM2. The MPM2 antibody specifically recognizes a subset of mitotic phosphoproteins that are phosphorylated on serine/threonine residues. Mitotic cells were lysed and immunoprecipitations performed using the MPM2 antibody followed by

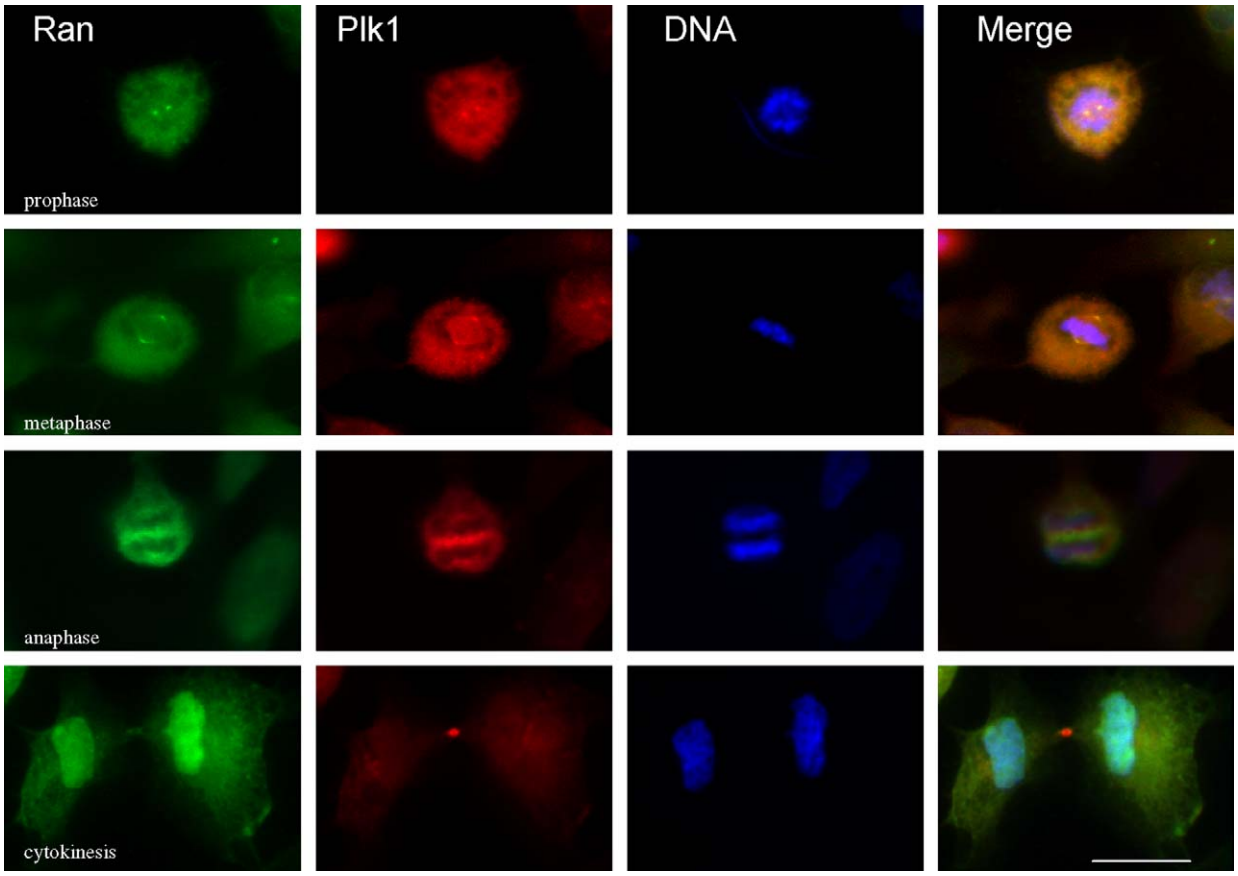

Fig. 2. Co-localization of Ran and Plk1 at different stages of mitosis. D17 cells were fixed and stained as described in Materials and methods. Representatives of cells at prophase, metaphase, anaphase, and cytokinesis are shown here with Ran (green), Plk1 (red), and DNA stainings. At the end of each row, all three stainings were merged. Scale bar 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

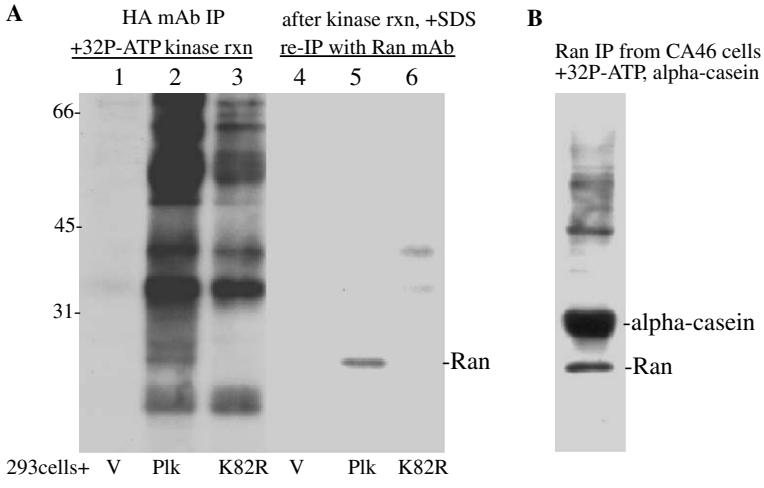

Fig. 3. Ran is specifically phosphorylated by co-precipitated Plk1 *in vitro*. (A) HEK293 cells were transfected with the control vector, HA-Plk1 or HA-K82R. Cells were synchronized to M phase 36 h after transfection. HA tagged-Plk1 proteins were immunoprecipitated from lysates with HA tag mAb. *In vitro* kinase assay was performed with the immune complexes in the presence of [γ-^{32}P]ATP (Lanes 1, 2, and 3). Samples were resolved on SDS–PAGE and transferred, autoradiography is shown. Lanes 4, 5, and 6: duplicate samples were boiled in 1% SDS after the kinase reaction, and diluted for second round of immunoprecipitation with mAb specific to Ran. The autoradiography of the second immunoprecipitation is shown. (B) Ran was immunoprecipitated from mitotic CA46 cells with Ran mAb. After extensive wash the immune complex was subjected to kinase reaction with α-casein and [γ-^{32}P]ATP added. Autoradiography shows phosphorylated Ran and casein.

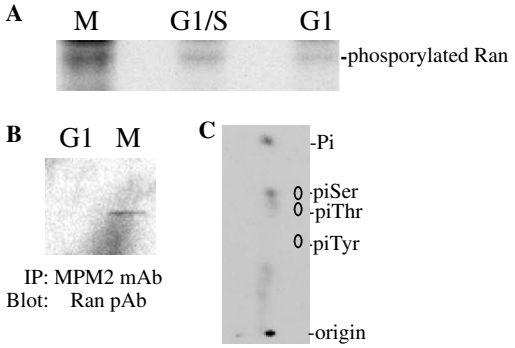

Fig. 4. Ran GTP-binding protein undergoes serine phosphorylation at M phase *in vivo* and *in vitro*. (A) CA46 cells were synchronized to M, G1/S or G1 phase, and labeled with [^{32}P]orthophosphate. Lysates made in NP 40 lysis buffer were clarified and used for Ran immunoprecipitation. Figure shows autoradiography of Ran immunocomplexes. (B) CA46 cells were synchronized to G1 or M phase, lysate supernatants were subjected to MPM2 mAb (specific to mitotic proteins phosphorylated on serine/threonine) immunoprecipitation. Samples were then probed with goat pAb specific to Ran. (C) Ran protein co-immunoprecipitated with Plk1 from mitotic CA46 cells was labeled in an *in vitro* reaction. The immunoprecipitates were boiled in 1% SDS, diluted for a second immunoprecipitation with Ran mAb. The sample was resolved by SDS–PAGE and transferred to an immobilon membrane. After autoradiography Ran protein was isolated from the membrane, and phosphoamino acid analysis was performed by standard methods. Pi, position of free phosphates. PiSer, PiThr, and PiTyr, positions of phosphoamino acid serine, threonine, and tyrosine.

immunoblot analysis using a goat polyclonal Ab to Ran (to distinguish Ran protein from the closely migrating mouse IgG light chain). Ran was only detected in mitotic MPM2 immunoprecipitates, but not in interphase samples (Fig. 4B). These results confirm that Ran is phosphorylated during mitosis, and agree with the idea that Plk1 mediates its phosphorylation.

Plk1 phosphorylates Ran on serine-135

The phosphorylated Ran protein isolated from radio-labeled cells did not contain enough radioactivity for further analysis. Therefore, we labeled Ran protein through an *in vitro* phosphorylation reaction of Ran immunoprecipitates, and separated Ran from other proteins with a second round of immunoprecipitation. Phosphoamino acid analysis was performed on *in vitro* phosphorylated Ran protein by standard methods. Our results showed that Plk1 phosphorylated Ran on serine residues only (Fig. 4C).

Further analysis was performed to map the potential phosphorylation sites by proteolytic digestion with trypsin and Asp-N. After proteolytic digestions the resulting phosphopeptides were purified by HPLC and subjected to automated Edman degradation. We found that serine-135 on Ran VKAKSIVFHR (basic-S-hydrophobic-uncharged, sequence based on NCBI P17080) was the only site modified by Plk1 *in vitro*. The MPM2 consensus epitope has been reported to be hydrophobic-T/S-pro-hydrophobic-uncharged/basic.The site we have identified is fairly consistent with the MPM2 consensus and suggests that it is the site on mitotic Ran that is recognized by the MPM2 antibody.

Further mapping analysis was performed on Ran protein isolated directly from cells. For reasons explained in the previous section (small fraction of Ran associated with Plk1 and reduced Plk1 kinase activity in radio-labeled cells), analysis of phosphorylation site could not be done with Ran protein from radio-labeled cells. Instead, Ran was isolated from non-labeled mitotic cells. This "cold" Ran sample was subjected to tryptic digestion and MALDI-TOF. This analytical tool allows mapping of phosphorylation site on non-radio-labeled proteins by comparing experimentally observed peptide masses with a database of theoretical Ran tryptic peptide masses that had been modified to include all possible phosphoseryl (pS)-containing peptides. A mass of 79.966 Da was added to each seryl residue. In Supplemental Fig. 1a, peptide masses with m/z 966.4849 and 1037.5545 agree with the theoretical masses of the seryl phosphorylated version of the peptides pSIVFHRK (theoretical $m/z = 966.4926$) and AKpSIVFHRK (theoretical $m/z = 1037.5297$), respectively. This indicates that serine-135 in AKSIVFHPK of Ran protein indeed bears phosphate *in vivo* during mitosis. Therefore, Ran undergoes phosphorylation *in vivo* only at serine-135, which is clearly the only site phosphorylated by Plk1 *in vitro*. It is highly likely that Ran is physiological substrate of Plk1.

We have compared the sequences surrounding S135 of human Ran with homologues from various species, and found that S135 is conserved among mouse, rat, dog, cow, chicken, Xenopus, drosophila, and fish, although it's threonine in budding yeast and nematode.

Mutation of S135D increases frequency of abnormal spindles in mitotic cells

Both Plk1 and Ran proteins have multiple cellular functions, and both have regulatory roles in spindle assembly/formation. The next question is whether the Plk1-mediated phosphorylation of Ran affects spindle assembly during mitosis. To this end, a mammalian vector containing Ran sequence was initially created to transfect HEK293 cells, however, the expression of exogenous Ran was poor. We then seeked another approach to introduce Ran to mammalian cells. As described in Materials and methods, myc-Ran and its mutant forms (S135A and S135D) were expressed through RCAS retroviral vector into D17 canine osteosarcoma cells. With this vector, all three forms of Ran were expressed well. More importantly, both the endogenous and transfected Rans were co-immunoprecipitated with Plk1 (Fig. 5). Both wild-type and mutant Ran were associated with Plk, indicating phosphorylation status has little effect on binding affinity between Ran and Plk1. Therefore, we decided to use these cells for functional studies of Ran mutations.

Localization of wild-type Ran and mutant proteins, spindle morphology and cell cycle profiles were first exam-

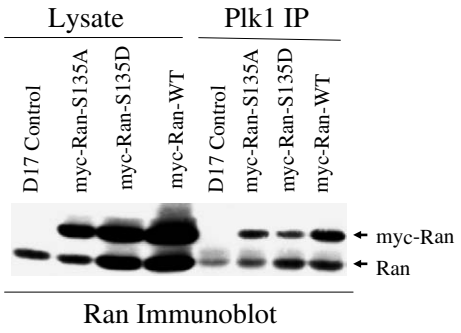

Fig. 5. Co-immunoprecipitation of Ran with Plk1 in D17 canine cells. Ran wild-type, S135A or S135D mutants were expressed through a retroviral RCAS vector. Expression level of each protein was examined by Western blot of lysate with Ran mAb. Equal amount of lysate was used for Plk1 immunoprecipitation with a Plk1 pAb. The presence of endogenous and myc-tagged Ran in Plk1 immunocomplexes was detected with Ran mAb.

Table 1
Mitotic index and percentage of abnormal mitotic cells in D17 cells over-expressing wild-type Ran, S135A, or S135D

	Mitotic index	Abnormal mitosis index
Wild-type Ran	2.21% ± 0.005	6.26% ± 0.011
S135A mutant	2.14% ± 0.006	6.36% ± 0.011
S135D mutant	2.32% ± 0.006	11.83% ± 0.007

ined (Supplemental Fig. 2a and b). In mitotic D17 cells, both the wild-type and mutants follow staining pattern of endogenous Ran (Fig. 2). In interphase cells, wild-type Ran is predominantly (>90%) in nuclei. While this is also true with S135A mutant, S135D mutant is approximately 60% in nuclei.

Although mutations on Ran protein did not change overall mitotic index, the frequency of abnormal mitotic cells did increase significantly in cells over-expressing S135D mutant (Table 1). These cells may have 4–8 spindle poles and multipolar spindles (Fig. 6). Chromosomes were retained in giant cells with incomplete mitosis, or were separated unevenly into daughter cells. These abnormalities are very similar to abnormal spindles caused by over-expression of Plk1 [24]. The consequence of such abnormalities is cancerous cells with aneuploidy chromosomes. Similar results were seen when GFP-Ran fusion proteins, instead of myc-tagged Ran, were expressed in D17 cells (data not shown). These results indicate that constitutive phosphorylation of Ran on S135 deregulates signals for proper mitotic spindles. Therefore, Plk1-mediated phosphorylation of Ran is important for normal bipolar spindles.

Discussion

In this study, we have presented evidence that Plk1 is associated with the small GTPase protein Ran during mitosis. Previously, we have observed similar association between Plk1 and other proteins complexes such as tubulins and proteasomes [7,8]. Because Plk1 kinase activity is tightly regulated, stable associations of Plk1 with its substrates might allow rapid phosphorylations when it is required. We have tested for other Plk1-interacting proteins in Ran immunoprecipitates, and did not detect either tubulins or proteasomes, therefore Plk1/Ran represents yet another distinct complex. It seems that Plk1 is associated with multiple distinct complexes, likely at discrete subcellular locations at different stages of the cell cycle, allowing Plk1 to achieve its versatile functions. Similarly, Ran-binding proteins, RanBP1, RanGAP or RCC1, were not detected in this Plk1/Ran complex, indicating this is different from other Ran-containing protein interactions.

We have shown for the first time that Ran undergoes phosphorylation *in vivo*, and that Plk1 is capable of phosphorylating Ran *in vitro* on the same site that is phosphorylated *in vivo*. Although the function of Ran is known to regulate by its GTP/GDP-binding status, reversible phosphorylation provides an additional regulatory mechanism such as has been observed for several other small GTP-binding proteins [15–22]. Ran is generally found in its GTP-bound form in the nucleus and in GDP-bound form in the cytoplasm. This gradient is favored by the nuclear/cytoplasmic transport functions of Ran. However, Ran regulates spindle assembly in a transport-independent fashion [1]. For this function of Ran, reversible phosphorylation could provide an adequate control. Our observations suggest that proper phosphorylation of Ran is required for establishment of bipolar spindles. Deregulation of Ran phosphorylation disrupts normal spindle structure and segregation of chromosomes. At this point, it is not known how signal from Ran phosphorylation is relayed to microtubule functions during mitosis. It is known that active Ran-GTP stimulates the phosphorylation and activity of the Xenopus Aurora A kinase, Eg2 [25], which in turn regulates spindle assembly and microtubule dynamics. It will be interesting to find out whether phosphorylation of Ran regulates this pathway. The staining pattern of Ran is not directly on microtubules, but rather diffusively around microtubules. This agrees with our findings that Ran is not directly associated with α- and β-tubulins.

Numerous studies have shown that Plk1 function is essential for bipolar spindle formation. The mechanism of this regulation is still unknown, but our studies suggest that Ran may be one of Plk1's targets in spindle poles. First, GTP-bound Ran is capable of stimulating spindle assembly in Xenopus mitotic extract and mammalian cells [4] independent of its function of nuclear-cytoplasmic transportation. The interaction of Plk1 and Ran connects the kinase to its role in normal spindle establishment. Ran may further modulate downstream proteins, such as NuMA and Aurora A kinase. This notion is supported by our observations that Ran-S135D mutant cells and Plk1-over-expressing cells display similar aberrant multipolar spindles [24]. Furthermore, in both budding yeast and fission yeast, there exists a multi-protein complex contain-

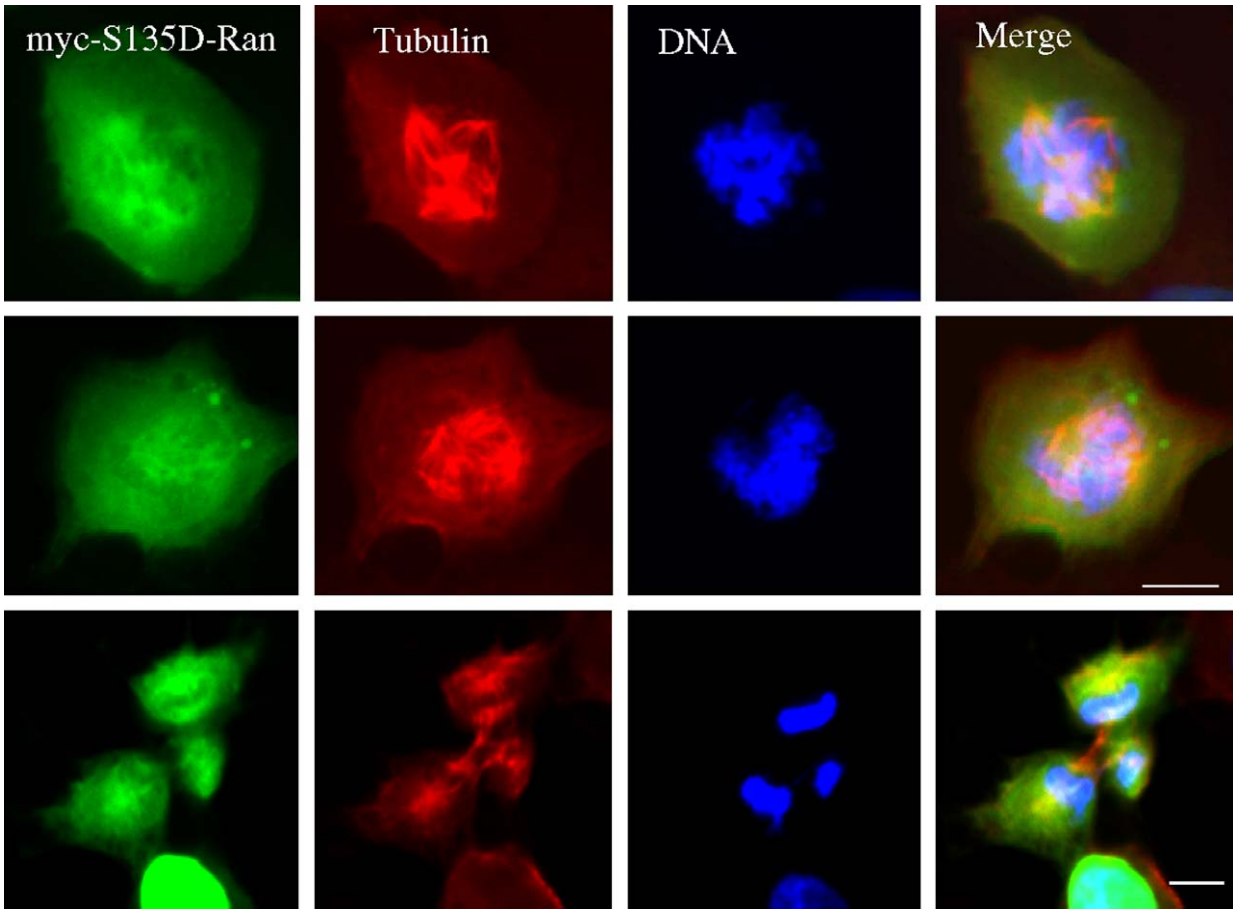

Fig. 6. Examples of abnormal mitotic cells in Ran-S135D mutant transfected cells. D17 cells selected for S135D mutant expression were stained with myc-tag Ab (green), α, β-tubulin mAb (red) and DNA stainings (blue). Scale bar 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

ing GTP-binding protein and Plks, and the complex is involved in cytokinesis and completion of mitosis. In *Saccharomyces cerevisiae*, the multi-protein complex, termed the mitotic exit network (MEN), is composed of: Tem1, a small GTP-binding protein; Cdc15; the cerevisiae Plk1 homolog cdc5; Dbf2 kinases; Cdc14 phosphatase; and Mob1, a protein of unknown function. The mitotic exit network is required for the actin ring formation, and is, therefore, crucial for the regulation of cytokinesis and mitotic exit [26,27]. In *Saccharomyces pombi*, the complex is named septum-initiation network (SIN), and it contains the GTPase-activating protein (GAP), Cdc16 and Byr4, and the GTP-binding protein, Spg1. The pombe Plk1 homolog Plo1 regulates this pathway and the pathway feeds back and inhibits Plo1 [28]. Mammalian Ran is a close relative of the cerevisiae GTP-binding protein Tem1. In both yeasts, the Plk1 homolog activities are required to initiate cytokinesis for the formation of bud neck and septum [28,29]. In the absence of yeast polo function, multi-nucleated cells are accumulated due to the malfunction of both spindles and cytokinesis, and this is similar to what has been observed in mammalian cells after Plk1 function is knocked out by Plk1 antibody microinjection [30]. Now we have observed similar abnormalities, multipolar spindles, in Ran S135D mutant. It is possible that the Plk1-Ran interaction in mammalian cells takes part in similar functions during cytokinesis and actin ring formation. Lastly, since the main function of Ran is transporting proteins to nuclei, Plk1-Ran interactions (or Plk1-mediated phosphorylation of Ran) may change cellular localization of centrosomal proteins.

The *in vitro* Plk1 phosphorylation site on Ran has been identified as S135 (AKSIVF). Previously, a consensus phosphorylation site, D/EXS/T, autophosphorylated by Plx1 [31] and phosphorylated by Plk1 on NudC [10], has been reported. However, Plx1 has also been reported to phosphorylate Claspin on SSS^{934}FLT [32], which lacks the negative charged residue at −2 position. As Plx1 may represent an activity unique to oocytes, it is likely that mammalian polo kinases may have somewhat different substrate specificities and phosphorylation sites. In fact, in HeLa cells Plk1 regulate nuclear entry of cyclin B1 at prophase through its phosphorylation on AFS^{147}DVI [33], which is similar to the phosphorylation site on Ran.

We have discussed briefly in the results section why Plk1 substrates have consistently shown low levels of ^{32}P-label-

ing in classical metabolic labeling experiment: Plk1 activity is greatly reduced by DNA damage induced by radio-isotope ^{32}P in the metabolic labeling experiment [23]. Alternative ways to avoid this reduction include using low-energy isotope ^{33}P for labeling or a non-labeling system. We have employed the MPM2 monoclonal antibody to detect *in vivo* phosphorylation of Ran in a native cell system. The S135 site in Ran (AKSIVF) fits the basic requirement for an MPM2 epitope. It has been reported that in Drosophila, Polo kinase is required for the phosphorylation of MPM2 epitopes, and that Polo is co-localized with MPM2 labeling at centrosomes, centromeres, and midbodies [34]. Based on our data it is likely that Plk1 is the MPM2 kinase for Ran.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.08.028.

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