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Negative regulation of cell cycle progression by RINGO/Speedy E

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Running title: Cell cycle regulation by RINGO/Speedy E

ABSTRACT

Cell cycle transitions are controlled by cyclin-dependent kinases (CDKs), whose activation is usually associated with the binding of cyclins. RINGO/Speedy proteins can also bind to and activate CDKs, although they do not have amino acid sequence homology to cyclins. The RINGO/Speedy family members studied so far positively regulate cell cycle progression. Here we report the biochemical and functional characterization of RINGO/Speedy E. We show that RINGO/Speedy E is a functionally distant member of this protein family that negatively affects cell cycle progression. RINGO/Speedy E overexpression inhibits the meiotic progression in *Xenopus* oocytes as well as the proliferation of mammalian cells. RINGO/Speedy E can bind to endogenous CDK1 and CDK2 in both cellular systems. However, the RINGO/Speedy E-activated CDKs have different substrate specificity than the CDKs activated by other RINGO/Speedy proteins, which may account for their different effects on the cell cycle. Our results indicate that although all RINGO/Speedy family members can activate CDKs, they may differently regulate cell cycle progression.

Keywords: cell cycle, CDK, cyclin, RINGO/Speedy, *Xenopus* oocyte

Abbreviations used: CDK, cyclin-dependent kinase; XRINGO, *Xenopus* RINGO; GST, glutathione-S-transferase; H1K, histone H1 kinase; IP, immunoprecipitation; MAPK, mitogen-activated protein kinase; MPF, M-phase or maturation promoting factor.

INTRODUCTION

Cell cycle progression is controlled by a family of serine/threonine protein kinases named cyclin-dependent kinases (CDKs). CDKs are essentially inactive as monomers and their activation requires the binding of activating subunits named cyclins as well as the phosphorylation of a conserved residue in the T-loop of the kinase domain [1-4]. In addition to CDK activation, cyclins contribute to substrate specificity determination as well as to the subcellular localization of CDKs [5-7]. CDKs can be negatively regulated by the phosphorylation of residues Thr-14 and Tyr-15 in the ATP binding pocket [2, 8].

Different CDKs have been implicated in the regulation of different cell cycle stages [9]. In particular, CDK1 bound to B-type cyclins, an activity that is usually referred to as M-phase or maturation-promoting factor (MPF), plays a key role in M phase entry and progression. CDK1-cyclin B complexes are formed prior to mitosis but are maintained inactive due to the phosphorylation of Thr-14 and Tyr-15 by the kinases Wee1 and Myt1. These inactive complexes are referred to as pre-MPF. Dephosphorylation of Thr-14 and Tyr-15 is carried out by phosphatases of the CDC25 family. MPF activation at the onset of mitosis results from both the inhibition of Wee1/Myt1 and the stimulation of CDC25, with phosphorylation playing an important role in both processes [8, 10-12]. MPF itself is thought to phosphorylate Wee1, Myt1 and Cdc25 forming a positive feedback loop mechanism, which contributes to MPF activation at mitotic entry. The regulation of MPF activity has been intensively studied in *Xenopus* oocytes [13-15]. Fully grown *Xenopus* oocytes are induced to enter meiosis by the hormone progesterone in a process called meiotic maturation. Progesterone stimulates several signal transduction pathways leading to Myt1 inhibition, CDC25 stimulation and pre-MPF activation [16]. In G2 arrested *Xenopus* oocytes, only 10-20% of CDK1 is associated with cyclin B forming the pre-MPF [17, 18]. The remaining CDK1 is present as a monomer and this subpopulation is thought to contribute to the activation of pre-MPF during the G2/M phase progression of oocytes [13, 19].

RINGO/Speedy proteins can activate some CDKs although they do not share significant sequence homology with cyclins [20]. The first member of this family was identified in *Xenopus* as a potent inducer of oocyte maturation [21, 22]. Endogenous XRINGO/Speedy accumulates in oocytes upon progesterone stimulation and contributes to meiotic progression, probably by CDK1 or CDK2 activation [21, 23, 24]. An orthologue named Spy1 or RINGO/Speedy A, has been proposed to participate in the G1/S transition [25, 26] and in the DNA damage response pathway [27, 28] in cultured mammalian cells. More recently, four additional mammalian RINGO/Speedy proteins have been identified [29, 30].

These proteins can all bind to and activate CDK1 and CDK2 while some of them can also activate CDK5 [29].

RINGO/Speedy E is a more distant and less characterized family member. We previously reported that RINGO/Speedy E (formerly Ringo1) was able to bind to and activate CDK1, CDK2 and CDK5 [29]. Here we provide more detailed biochemical and functional characterisation of this protein. We show that RINGO/Speedy E impairs cell cycle progression in *Xenopus* oocytes and in mammalian cells. Moreover, RINGO/Speedy E activates endogenous CDKs but provides different substrate specificity than the same CDKs activated by other RINGO/Speedy proteins. Thus, RINGO/Speedy family members may differently regulate cell cycle progression by providing CDKs with different substrate specificities.

EXPERIMENTAL

DNA cloning

Constructs for expression in *Xenopus* oocytes, FTX4–HA-CDK2 (*Xenopus*) wild type and different mutants, FTX5-XRINGO/Speedy, FTX5-RINGO/Speedy E, FTX5-RINGO/Speedy A and FTX5-Myt1 (*Xenopus*) C-terminus, as well as constructs to express recombinant proteins in *Escherichia coli*, glutathione-S-transferase (GST)-RINGO/Speedy E, GST-CDK1 (*Xenopus*) and GST-Myt1 (*Xenopus*) C-terminus, have been previously described [21, 29, 31]. A construct to express in *E. coli* GST-RINGO/Speedy A1 was prepared by cloning human RINGO/Speedy A1 into the pGEX-KG vector digested with EcoRI. For expression in mammalian cells with an N-terminal GFP-tag, RINGO/Speedy E was subcloned into the pEGFP-C2 vector as an EcoRI fragment.

Xenopus oocyte manipulation and injection

Xenopus oocytes were sorted, injected with *in vitro*-transcribed mRNAs and lysed as described previously [32].

Cell culture and transfection

U2OS cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Cells were transiently transfected in suspension using FuGENE 6 reagent (Roche). In a typical experiment, transfection of U2OS cells in suspension with FuGENE6 reagent resulted in more than 70% of transfection efficiency.

Cellular lysates were prepared in buffer containing 150 mM Tris-HCl pH 7.5, 1 mM EDTA, 1

mM EGTA, 10 mM NaF, 1% NP-40, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin, 0.2 mM PMSF, 0.1 mM sodium orthovanadate, 1 mM DTT and 1 mM benzamidine.

Immunoblotting and immunoprecipitation

Primary antibodies against *Xenopus* CDK1 (3E1 monoclonal) and cyclin B2 (affinity purified rabbit antisera) were provided by J. Gannon and T. Hunt (Cancer Research UK, Clare Hall Laboratories, South Mimms, UK). The rabbit antiserum 3297.1 was used to detect the mitogen-activated protein kinase (MAPK) Xp42/XMpk1 [21]. Antibodies for p90Rsk and Myc were purchased from Santa Cruz Biotechnology and the HA antibody was from Roche. A RINGO/Speedy E monoclonal antibody was generated by the CNIO Monoclonal Antibody Unit. The blots were developed using Alexa Fluor 680 (Molecular Probes) or Li-Cor IRDye 800 (Rockland) labelled antibodies with the Odyssey Infrared Imaging System (Li-Cor).

For immunoprecipitation (IP), 5 μ l of anti-Myc (Santa Cruz; sc-40 AC), 5 μ l of anti-HA (Santa Cruz; sc-7392 AC) or 10 μ l of Suc1/Cks (Upstate biotechnology) agarose conjugates were incubated with the oocyte lysates for 2 h at 4°C. IP of cellular lysates with a RINGO/Speedy E monoclonal antibody was done overnight at 4°C followed by incubation with IgG-agarose at 4°C for 1 h. The beads were washed three times in lysis buffer and analyzed by immunoblotting, or further washed in histone H1 kinase (H1K) buffer and used for kinase assays [32].

***In vitro* kinase assays**

H1K activity was assayed using 4 μ l of total oocyte lysates as described [21, 32].

For CDK1-RINGO/Speedy kinase assays, bacterially produced GST-CDK1 (2 μ g) was pre-incubated in concentrated *Xenopus* egg extracts and recovered on glutathione-Sepharose beads, as described [21]. The beads were washed and incubated at 30°C for 10 min with GST-RINGO/Speedy proteins (2 μ g) and then further incubated for 15 min in kinase buffer containing histone H1 (3 μ g) or GST-Myt1 C-terminus (2 μ g), as substrates. Phosphorylated proteins were separated in 15% SDS-PAGE and detected by autoradiography.

Cell cycle analysis, proliferation and apoptosis assays

Cell cycle distribution was analyzed by flow cytometry. Cells were trypsinized and fixed in chilled 70% ethanol for at least 1 h. Cells were then incubated in PBS containing 30 mg/ml of

RNase and stained with propidium iodide (25 $\mu\text{g/ml}$) for 30 minutes. Flow cytometry analysis was carried out using FACScan system and CellQuest program.

For proliferation assays, U2OS cells were transfected with GFP or GFP-RINGO/Speedy E constructs and seeded in a 96 well-plate at a density of 2,000 cells/well. Proliferation was followed for three days using the Cell Proliferation Kit, MTT (Roche Diagnostics). For colony formation assays, cells were trypsinized 48 h after transfection, counted and seeded (200 cells) in 10 cm dishes. Colonies were visualized by staining with crystal violet and counted 14 days after plating. Apoptotic cell death was evaluated by ELISA using the Cell Death Detection ELISA^{PLUS} kit (Roche) according to the manufacturer's instructions.

Detection of GFP-RINGO/Speedy E

U2OS cells were transfected with the expression construct for GFP-RINGO/Speedy E and cultured on glass coverslips for 24 h. Cells were then fixed with 4% formaldehyde and subcellular localization of RINGO/Speedy E was analyzed by fluorescence microscopy (Zeiss Jena).

Statistical analysis

All results are expressed as means \pm S.E. for at least 3 independent experiments. Statistical analysis was performed using the *t* test for paired and unpaired data versus control values using the statistic Prisma software (GraphPad, San Diego, CA). $p < 0.05$ was considered statistically significant.

RESULTS

Biochemical properties of RINGO/Speedy E differ from other RINGO/Speedy proteins

We examined the effect of RINGO/Speedy E expression on *Xenopus* oocyte maturation, the assay used to identify and characterize other RINGO/Speedy proteins. Expression of either XRINGO/Speedy or RINGO/Speedy A suffices to induce maturation of *Xenopus* oocytes in the absence of progesterone, while RINGO/Speedy B expression accelerates progesterone-induced maturation [21, 22, 25, 30]. In contrast with those results, we found that expression of RINGO/Speedy E not only was unable to induce oocyte maturation on its own, but also strongly inhibited oocyte maturation induced by progesterone (Fig. 1A). The lack of maturation was scored morphologically, by the absence of a white spot on the animal pole of the oocyte and the presence of the germinal vesicle inside the oocyte (Fig. 1A, left panel).

This was also confirmed biochemically, as MPF, MAPK and p90Rsk were all inactive in the RINGO/Speedy E-expressing oocytes treated with progesterone, contrary to progesterone-treated oocytes injected with water (Fig. 1A, right panel).

RINGO/Speedy E can bind to and activate several CDKs [29]. Previous studies indicated that binding of cyclins and XRINGO/Speedy to the CDKs involves residues in the PSTAIRE helix and the T-loop [29, 33]. To study the interaction between RINGO/Speedy E and CDKs, we co-expressed in *Xenopus* oocytes RINGO/Speedy E with a collection of CDK2 mutants [29] and then evaluated the binding by co-IP experiments. Surprisingly, all the CDK2 mutants tested, including K33A, R50A and R150A that are impaired in binding to XRINGO/Speedy and cyclin A1 [29], were able to bind to RINGO/Speedy E as efficiently as the wild type CDK2 (Fig. 1B, IP Myc). For a comparison, the same experiment was done using RINGO/Speedy A. As expected, the CDK2 mutants that were impaired in binding to XRINGO/Speedy and cyclin A1 did not bind to RINGO/Speedy A (Fig. 1C). We also tested the activation of the different CDK2 mutants by co-IP experiments. Consistent with the binding data, RINGO/Speedy E increased the activity of all the CDK2 mutants, except for the catalytically inactive K33A (Fig. 1B, IP HA). We could not perform the same experiment with RINGO/Speedy A, because this protein induces oocyte maturation and the concomitant upregulation of endogenous cyclins, making it difficult to elucidate the relative contribution of RINGO/Speedy A and cyclins to the H1K activity measured in CDK2 IPs. Altogether, our results suggested that RINGO/Speedy E binds CDKs differently from other RINGO/Speedy proteins or cyclin A1.

RINGO/Speedy E activates endogenous CDK1 in *Xenopus* oocytes

Although progesterone treated or untreated RINGO/Speedy E-expressing oocytes did not mature, based on morphological and biochemical criteria (Fig. 1A), the total lysates from these oocytes contained significant levels of H1 kinase activity (Fig. 2A). The H1 kinase activity detected in total lysates of mature oocytes originates mostly from active MPF. Interestingly, pre-MPF was not activated in the oocytes expressing RINGO/Speedy E, as indicated by the existence of a slow migrating and Thr-14/Tyr-15-phosphorylated CDK1 band (Fig. 1A, right panel). The H1 kinase activity in RINGO/Speedy E-expressing oocytes co-IP with RINGO/Speedy E (Fig. 2A), suggesting that it originated from the direct activation of endogenous kinases other than MPF by RINGO/Speedy E.

Considering the ability of RINGO/Speedy E to bind to different CDKs, we predicted that CDKs could account for the observed H1 kinase activity. Indeed, addition of the CDK

inhibitor roscovitine inhibited the H1 kinase activity in total lysates of RINGO/Speedy E-expressing oocytes as it did in oocytes treated with progesterone (Fig. 2B). Moreover, the H1K activity of oocytes expressing RINGO/Speedy E was IP with CDK1 antibodies and was also detected in pull-downs with the CDK1 binding protein Suc1/Cks (Fig. 2C). Interestingly, we detected binding of RINGO/Speedy E to endogenous CDK1 in the co-IP experiments, but the binding was restricted only to the faster-migrating CDK1 band, which is thought to correspond to cyclin-free, monomeric CDK1 non-phosphorylated on Thr-14 and Tyr-15 (Fig. 2D). Immunoblotting confirmed that CDK1 bound to RINGO/Speedy E was not in a complex with cyclin B2 (Fig. 2D). Altogether our results strongly suggest that RINGO/Speedy E binds to and activates endogenous monomeric CDK1 in *Xenopus* oocytes.

Differential substrate specificity of CDK1-RINGO/Speedy E

The ability of RINGO/Speedy E to stimulate the kinase activity of CDK1 on histone H1 without inducing oocyte maturation was intriguing. There is evidence that RINGO/Speedy proteins might contribute to the substrate specificity of CDKs [34]. Since different cyclins can modulate the substrate preferences of a given CDK [35-37], it is possible that RINGO/Speedy family members might also determine distinct specificities in the phosphorylation of CDK substrates. Therefore, we hypothesized that the reason why RINGO/Speedy E-activated CDK1 was not able to trigger *Xenopus* oocyte maturation could be because RINGO/Speedy E was impaired in targeting CDK1 to oocyte substrates relevant for meiotic maturation. To test this possibility, we followed the phosphorylation of Myt1 in oocytes expressing either XRINGO/Speedy or RINGO/Speedy E. Myt1 is an important regulator of the G2 arrest in *Xenopus* oocytes, which is known to be phosphorylated upon M-phase entry [31, 38-40]. As expected, XRINGO/Speedy induced the phosphorylation of Myt1, which resulted in reduced electrophoretic mobility of the protein. The expression of RINGO/Speedy E also induced Myt1 phosphorylation, but much less efficiently than the phosphorylation induced by XRINGO/Speedy. Moreover, when H1K activity levels were compared to the degree of Myt1 phosphorylation at the 3 h time point, it seemed clear that RINGO/Speedy E induced the phosphorylation of Myt1 less efficiently than XRINGO/Speedy (Fig. 3A).

Next, we performed *in vitro* kinase assays using recombinant CDK1 activated by either RINGO/Speedy E or RINGO/Speedy A, which is the mammalian family member most similar to XRINGO and is able to induce *Xenopus* oocyte maturation [30]. In agreement with the results in oocytes, we found that the two RINGO/Speedy proteins provided CDK1 with different abilities to phosphorylate either histone H1 or Myt1 *in vitro*. We needed to use about

four times more activity of CDK1-RINGO/Speedy E than CDK1-RINGO/Speedy A, based on histone H1 as a reference substrate, in order to obtain the same level of Myt1 phosphorylation (Fig. 3B). Thus, it is likely that the reduced efficiency of RINGO/Speedy E to target CDK1 to appropriate substrates accounts for its inability to induce oocyte maturation.

RINGO/Speedy E blocks oocyte maturation by sequestering CDK1

The activation of monomeric CDK1 has been shown to contribute to progesterone-induced MPF activation and oocyte maturation [19]. As RINGO/Speedy E can bind to monomeric CDK1 in oocytes (Fig. 2D), we postulated that the inhibition of oocyte maturation by RINGO/Speedy E could be due to the sequestration of endogenous monomeric CDK1. To test this possibility, we overexpressed CDK1 in the oocytes expressing RINGO/Speedy E. Interestingly, CDK1 overexpression was able to rescue the inhibitory effect of RINGO/Speedy E on progesterone-induced oocyte maturation. The same result was observed upon overexpression of the CDK1 mutant T161A, which cannot be phosphorylated in the T-loop. Moreover, the oocytes expressing RINGO/Speedy E together with either CDK1 wild type or T161A matured significantly faster in response to progesterone stimulation than water-injected oocytes (Fig 4A). On the contrary, the kinase-dead mutant CDK1 K33R was unable to rescue the inhibition by RINGO/Speedy E (Fig. 4A). We confirmed that CDK1 wild type and the two mutants were all able to bind to RINGO/Speedy E with similar efficiencies in co-IP experiments (Fig. 4B). We also found that the RINGO/Speedy E-triggered H1 kinase activity in oocyte lysates was increased upon co-expression with either CDK1 wild type or the T161A mutant, but not in the case of CDK1 K33R expression (Fig. 4B). These results indicate that RINGO/Speedy E can activate CDK1 in the absence of the T-loop phosphorylation, as it was previously reported for XRINGO/Speedy and RINGO/Speedy A [24, 34]. More importantly, the results strongly suggest that RINGO/Speedy E inhibits cell cycle progression in *Xenopus* oocytes by sequestering CDK1.

RINGO/Speedy E overexpression impairs cell cycle progression of U2OS cells

Next we investigated the effect of RINGO/Speedy E overexpression on the cell cycle of mammalian cells. GFP-RINGO/Speedy E overexpressed in U2OS cells was found to localize to the nucleus (Fig. 5A) and was able to bind to endogenous CDK1 and CDK2 in co-IP experiments (Fig. 5B). Interestingly, using flow cytometry analysis we found that the overexpression of RINGO/Speedy E severely disturbed cell cycle distribution of U2OS cells. Most notably, the G1 population was significantly reduced while the G2/M population, and to

a lesser extent the S phase population, proportionally increased, suggesting that RINGO/Speedy E overexpression might impair G2 and/or M-phase progression (Fig. 5C). We also noticed that the percentage of subG0 cells was higher in GFP-RINGO/Speedy E-expressing cells suggesting that inhibition of cell cycle progression by RINGO/Speedy E might eventually lead to apoptosis. This was confirmed by directly measuring apoptosis in RINGO/Speedy E-expressing cells (Fig. 5D). Consistent with these observations, cells expressing RINGO/Speedy E had reduced proliferation ability (Fig. 5E) and also produced significantly less colonies when compared with GFP-transfected cells (Fig. 5F). Taken together, these results indicate that RINGO/Speedy E overexpression interferes with cell cycle progression in mammalian cells.

DISCUSSION

CDK activity can be regulated by both cyclins and RINGO/Speedy proteins. Although the function of different members of the RINGO/Speedy family in cell cycle regulation is just starting to be addressed, the RINGO/Speedy proteins characterised so far positively modulate progression through the meiotic and mitotic cell cycles. We have now found that one member of this family, RINGO/Speedy E, negatively affects cell cycle progression although it can activate both CDK1 and CDK2.

XRINGO/Speedy was first identified as a potent inducer of the meiotic maturation in *Xenopus* oocytes and some of the mammalian RINGO/Speedy proteins were also shown to positively regulate *Xenopus* oocyte maturation [25, 30]. In contrast, RINGO/Speedy E overexpression inhibits progesterone-induced maturation. Intriguingly, although CDK1 activation is usually associated with oocyte maturation and RINGO/Speedy E can apparently activate the endogenous CDK1 in oocytes, the RINGO/Speedy E-expressing oocytes did not mature. We found that RINGO/Speedy E-activated CDK1 phosphorylates Myt1, an important regulator of pre-MPF activation in oocytes, less efficiently than other RINGO/Speedy proteins. This suggested that the reduced ability of RINGO/Speedy E to target CDK1 to certain substrates in oocytes might account for its inability to induce oocyte maturation. These results strongly argue that RINGO/Speedy proteins contribute to the substrate specificity of CDKs and that different RINGO/Speedy proteins could have diverse biological functions by providing CDKs with different substrate preferences, as cyclins do [35-37].

Overexpression of CDK1 was able to rescue the RINGO/Speedy E inhibition of progesterone induced maturation, suggesting that sequestration of endogenous CDK1 is a likely mechanism by which overexpression of this protein interferes with oocyte maturation.

This prediction is consistent with the observed interaction between RINGO/Speedy E and endogenous monomeric CDK1, and with the ability of kinase-dead CDK1 or CDK1 antibodies to inhibit progesterone-induced maturation [19]. One of the proteins that can activate monomeric CDK1 in oocytes is XRINGO/Speedy itself, which is upregulated during the meiotic maturation [23]. Therefore, it is attractive to speculate that RINGO/Speedy E might inhibit oocyte maturation by competing with the endogenous XRINGO/Speedy for binding to the monomeric CDK1. This possibility would be consistent with the observation that RINGO/Speedy E binds to CDK1 more efficiently than XRINGO/Speedy [29]. Therefore, if the two proteins are available for binding to endogenous CDK1, RINGO/Speedy E will probably out-compete XRINGO/Speedy.

The negative effect of RINGO/Speedy E on the meiotic cell cycle of *Xenopus* oocytes suggested that RINGO/Speedy E could also negatively regulate the mammalian cell cycle. Indeed, we have shown that RINGO/Speedy E overexpression impairs the proliferation of mammalian cells, which is just the opposite effect reported for RINGO/Speedy A overexpression [25]. We also found that the overexpressed RINGO/Speedy E interacts with endogenous CDK1 and CDK2 in mammalian cells and induces the accumulation of cells in the G2 and M phases of the cell cycle. Binding of RINGO/Speedy E to CDK1 may interfere with its role in the regulation of the G2-M transition and account for the negative effect of RINGO/Speedy E in mammalian cells, as in *Xenopus* oocytes. Alternatively, the phosphorylation of particular proteins by CDK1-RINGO/Speedy E might lead to slower progression through the G2 and M phases of the cell cycle.

RINGO/Speedy proteins are strong candidates to regulate cell cycle transitions through CDK1 and CDK2 activation. Current evidence indicates that XRINGO/Speedy and RINGO/Speedy A can positively regulate cell cycle progression. In contrast, RINGO/Speedy E negatively affects cell cycle progression, although it can potentially activate CDKs. Future work should investigate whether RINGO/Speedy E provides a general mechanism of negative cell cycle control and how different RINGO/Speedy proteins cooperate with cyclins to regulate cell cycle progression through CDK activation.

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FIGURE LEGENDS

Figure 1. Characterization of RINGO/Speedy E expressed in *Xenopus* oocytes

(A) Oocytes were injected with water or mRNA encoding RINGO/Speedy E and 14 h later were treated with progesterone. Meiotic maturation was monitored by the appearance of a white spot on the animal pole of the oocyte. Oocyte lysates were analyzed by immunoblotting with the indicated antibodies. Phosphorylated, active p42MAPK and p90Rsk showed reduced electrophoretic mobilities (closed arrowheads). MPF activation correlates with the disappearance of the Thr14 and Tyr15 phosphorylated CDK1 band (open arrowhead). (B) Oocytes were injected with Myc-RINGO/Speedy E mRNA, incubated 10 h, re-injected with mRNAs encoding different HA-CDK2 mutants and incubated for 12 h more. Total lysates were immunoblotted with HA (CDK2) antibody. Lysates from 35 oocytes were IP with Myc antibody and then immunoblotted with Myc (RINGO/Speedy E) and HA (CDK2) antibodies. For H1K assays, lysates from 8 oocytes were IP with HA antibody; half of the IP was assayed for H1K activity and the other half was immunoblotted with HA (CDK2) antibody. (C) Oocytes were injected with mRNAs encoding HA-CDK2 mutants, incubated 12 h and then re-injected with Myc-RINGO/Speedy A mRNA. After 10 h, lysates from 35 oocytes were IP with Myc antibody and then immunoblotted with Myc (RINGO/Speedy A) and HA (CDK2) antibodies. Molecular weight markers are indicated in kDa.

Figure 2. RINGO/Speedy E activates monomeric CDK1 in *Xenopus* oocytes

Oocytes were injected with Myc-tagged RINGO/Speedy E mRNA, incubated overnight and treated with progesterone for 12 h. (A) H1K activity was determined in oocyte lysates (4 μ l) or Myc IPs obtained from 5 oocytes. (B) Oocyte lysates were treated with DMSO (-) or 20 μ M Roscovitine (+) for 20 min before H1K activity was assayed. (C) Lysates from 5 oocytes were used for IP with CDK1 antibody or were incubated with Suc1/Cks agarose beads and then used for H1K assays, together with aliquots of the total lysates. (D) Lysates from 15 oocytes were IP with Myc antibody and then immunoblotted, together with an aliquot of the total lysates, with Myc (RINGO/Speedy E), CDK1 and cyclin B2 antibodies. CDK1 bound to cyclin B and phosphorylated on Thr14 and Tyr15 is indicated with an open arrowhead and the unphosphorylated, cyclin B-free CDK1 with a closed arrowhead. Molecular weight markers are indicated in kDa.

Figure 3. Myt1 is a poor substrate for CDK1-RINGO/Speedy E

(A) Oocytes were injected with mRNA encoding the Myc-tagged C-terminus of Myt1, incubated overnight and re-injected with XRINGO/Speedy or RINGO/Speedy E mRNAs. Lysates were prepared from oocytes collected at the indicated times after the second injection and were used for both immunoblotting and H1K assays. Molecular weight markers are indicated in kDa. (B) *In vitro* phosphorylation assays using CDK1 activated with RINGO/Speedy A or RINGO/Speedy E and either histone H1 or the C-terminus of Myt1 fused to GST, as substrates. The histograms show the quantification of 3 independent experiments (mean±s.d.).

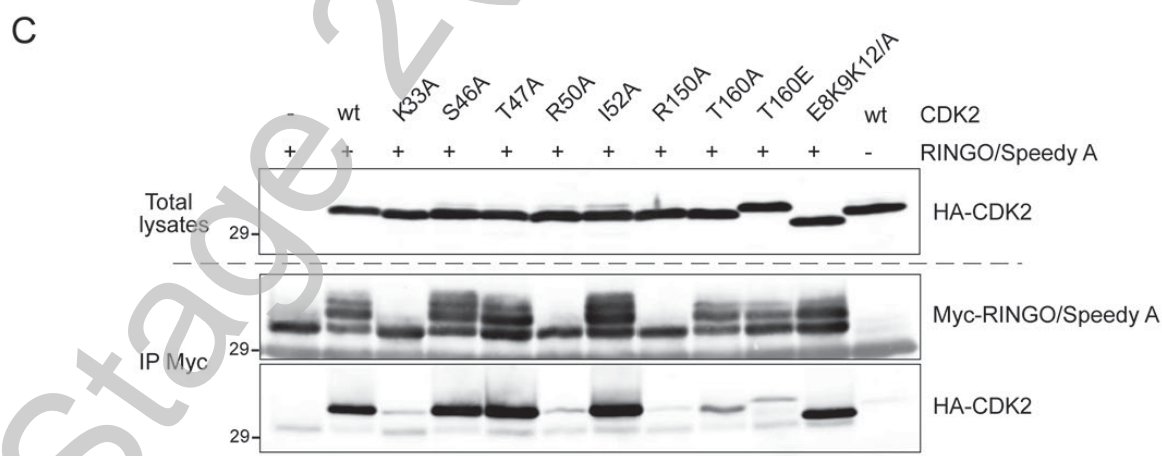
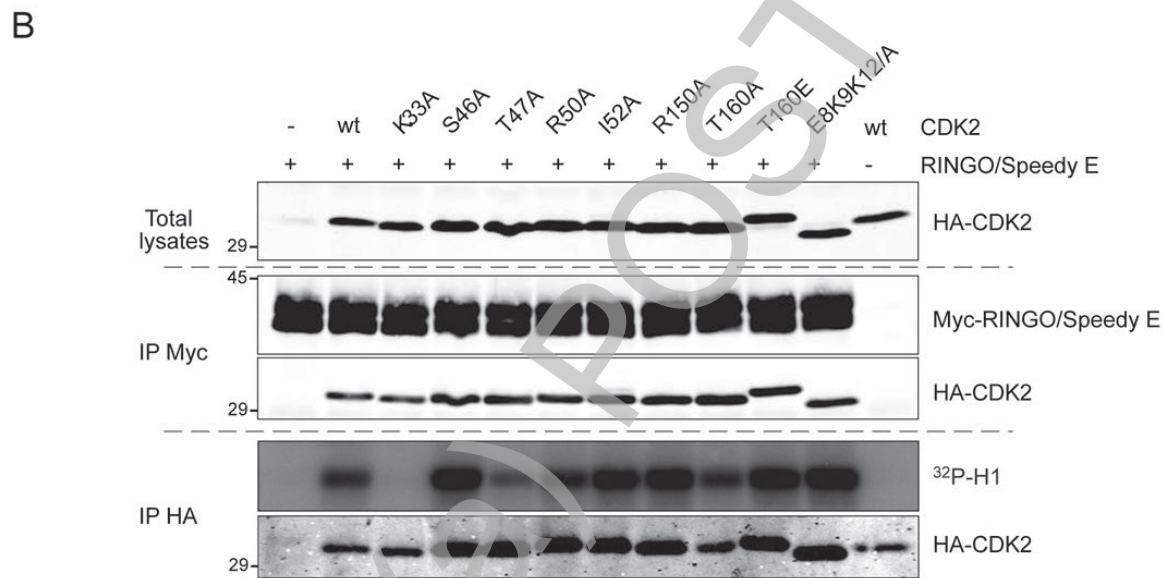
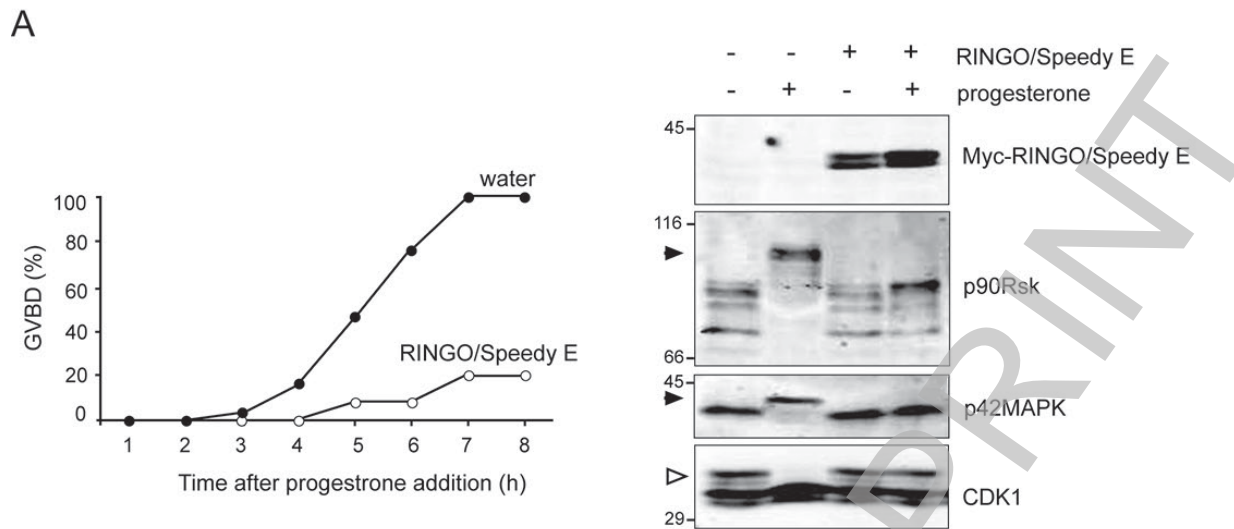
Figure 4. CDK1 overexpression rescues the inhibition of oocyte maturation by RINGO/Speedy E

(A) Oocytes were injected with water or Myc-tagged RINGO/Speedy E mRNA, incubated for 10 h and then re-injected with water or with mRNAs encoding CDK1 wild type, K33R or T161A proteins, as indicated. After 12 h, oocytes were treated with progesterone and meiotic maturation was monitored by the appearance of a white spot on the animal pole of the oocytes. (B) Oocytes were injected with Myc-tagged RINGO/Speedy E mRNA, incubated for 10 h and then re-injected with mRNAs encoding CDK1 wild type, K33R or T161A proteins, as indicated. After 12 h, lysates from 14 oocytes were IP with Myc antibody and then immunoblotted together with aliquots of the total lysates using either CDK1 or Myc (RINGO/Speedy E) antibodies. H1K activity was assayed in oocyte lysates and was quantified in a PhosphorImager. Molecular weight markers are indicated in kDa.

Figure 5. Effect of RINGO/Speedy E overexpression on cell cycle progression of mammalian cells

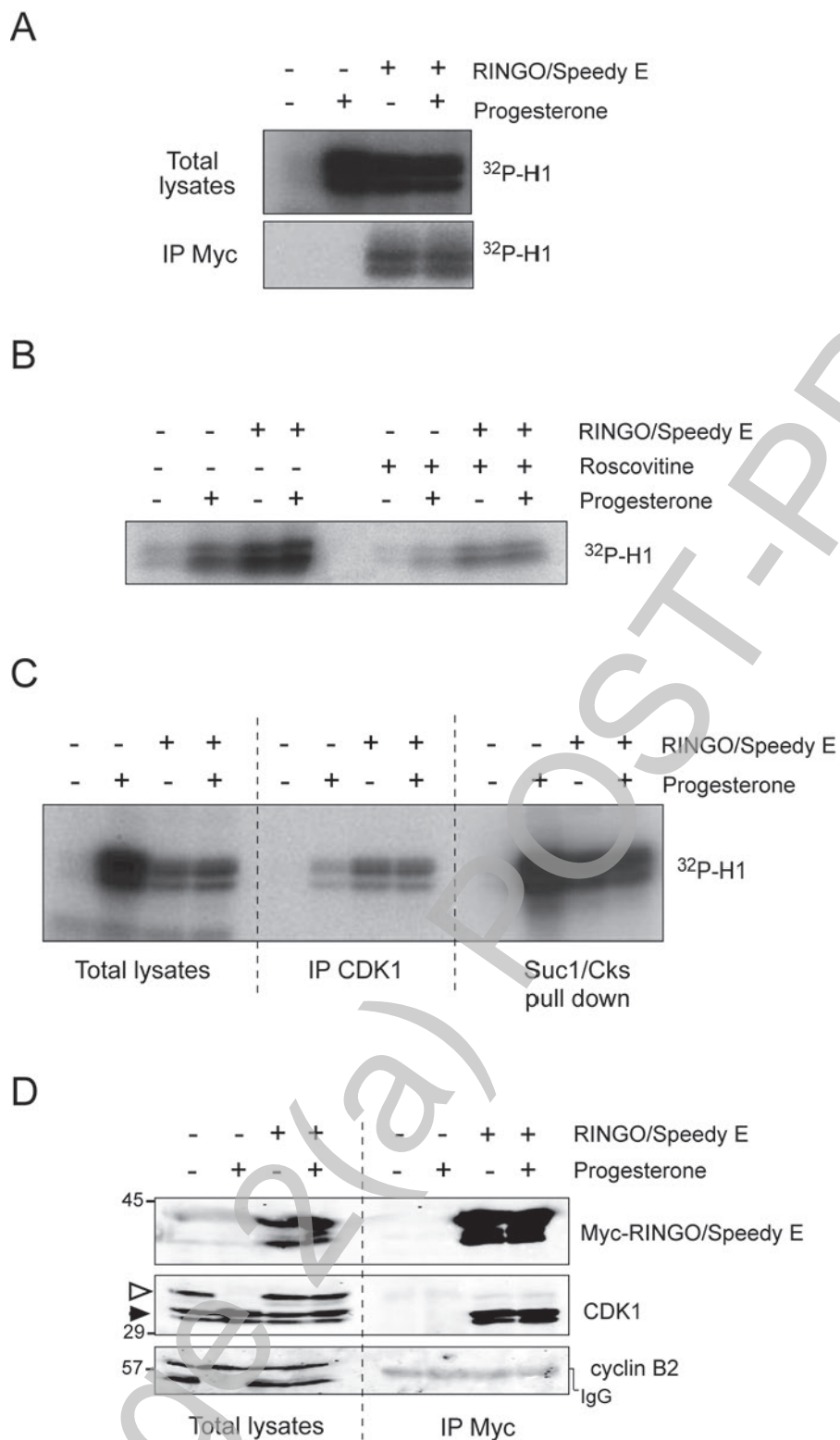
(A) Direct immunofluorescence of U2OS cells transiently transfected with GFP-RINGO/Speedy E. The middle panel shows a DAPI staining and the left panel phase contrast. (B) U2OS cells were transiently transfected with GFP or GFP-RINGO/Speedy E constructs. 48 h later, cells were lysed and immunoprecipitated with RINGO/Speedy E antibody. Total lysates and the IPs were analyzed by immunoblotting with the indicated antibodies. Molecular weight markers are indicated in kDa. (C) U2OS cells were transfected with GFP or GFP-RINGO/Speedy E (5 µg), pCDNA3.1 (3.5 µg) and pBabe-Puro (1.5 µg) constructs. 24 h after

transfection, cells were selected with puromycin (1 $\mu\text{g/ml}$) for 24 h. Cells were then allowed to proliferate 12 h more before the cell cycle profile was analyzed by flow cytometry. **(D)** U2OS cells were transfected with GFP or GFP-RINGO/Speedy E constructs. 48 h later apoptosis was quantified by measuring DNA fragmentation in a colorimetric assay. **(E)** U2OS cells were transfected with GFP or GFP-RINGO/Speedy E and cell proliferation was followed for 3 days using an MTT assay. **(F)** Colony assay of U2OS cells transiently transfected with GFP or GFP-RINGO/Speedy E. Two independent experiments were performed.

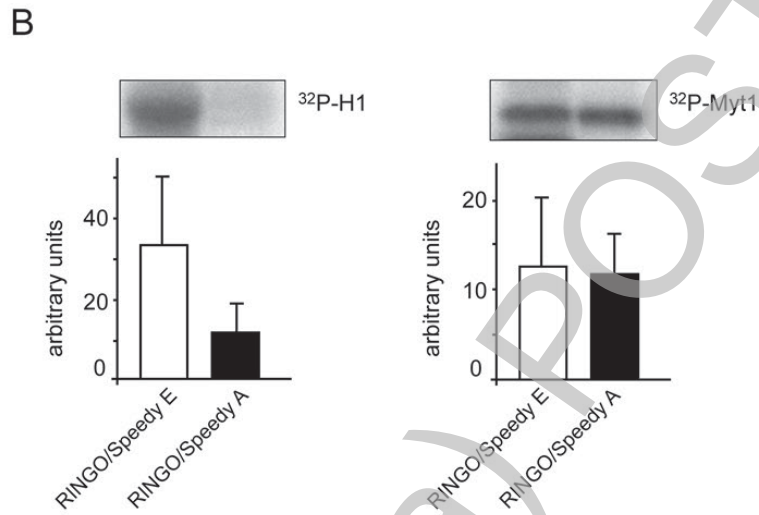
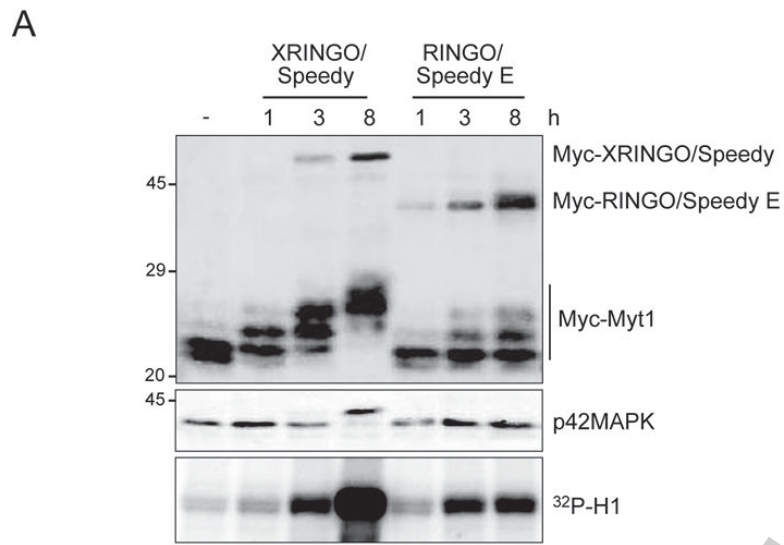


Dinarina et al., Figure 1

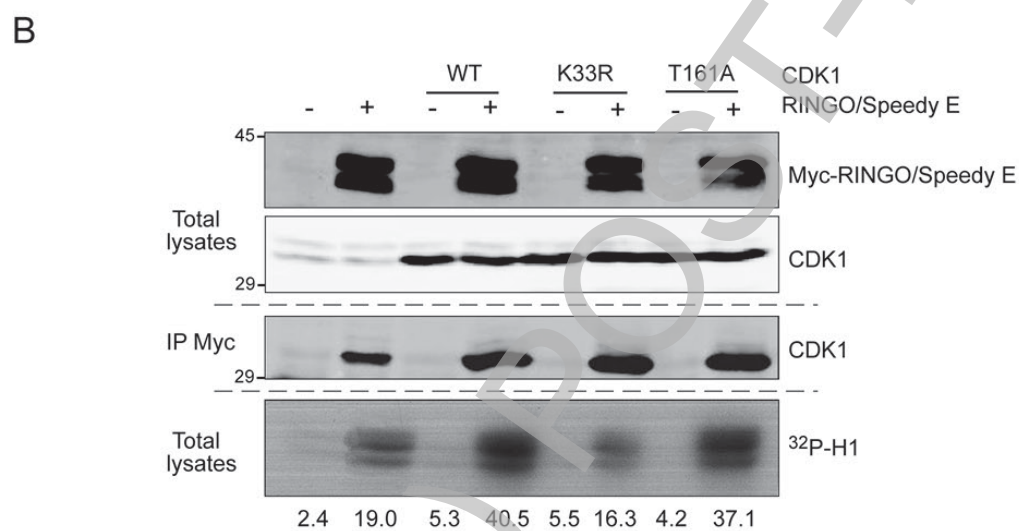
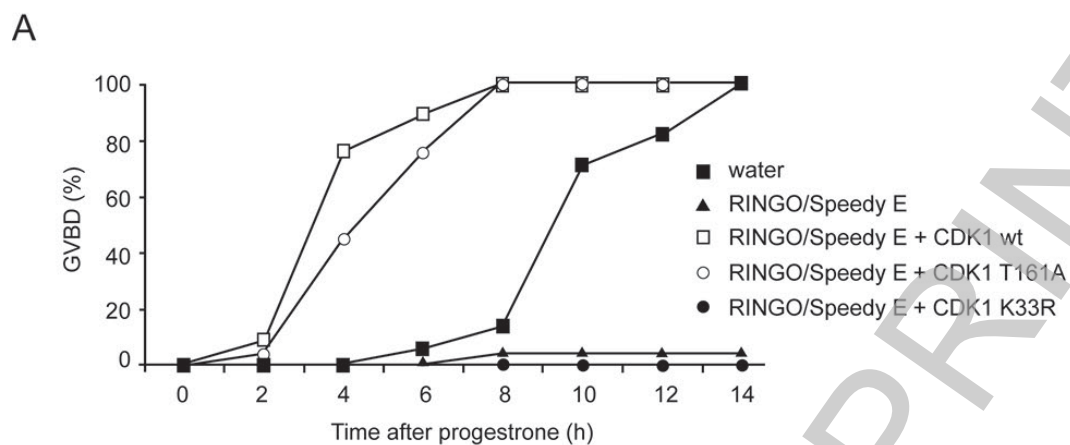
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Dinarina et al., Figure 2

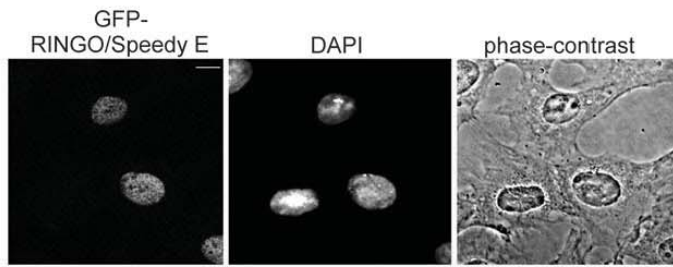


Dinarina et al., Figure 3

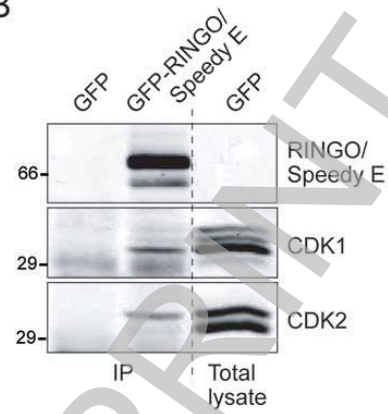


Dinarina et al., Figure 4

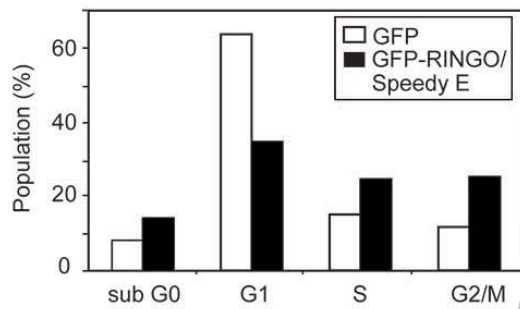
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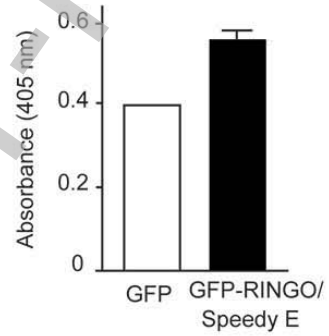
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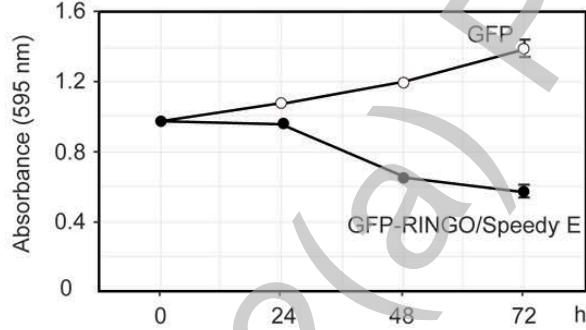
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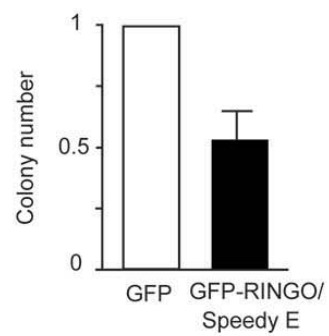
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Stage 2 POST-PRINT