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# Cell cycle regulation of the mammalian CDK activator RINGO/Speedy A

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ABSTRACT

**Cell cycle progression is regulated by cyclin-dependent kinases (CDKs), whose activation requires the binding of regulatory subunits named cyclins. RINGO/Speedy A is a mammalian protein that has no amino acid sequence homology with cyclins but can activate CDKs. Here we show that RINGO/Speedy A is a highly unstable protein whose expression and phosphorylation are periodically regulated during the cell cycle. RINGO/Speedy A is degraded by the proteasome and the process involves the ubiquitin ligase SCF$^{Skp2}$. Overexpression of a stabilized RINGO/Speedy A form results in the accumulation of high levels of RINGO/Speedy A at late stages of mitosis, which interfere with cytokinesis and chromosome decondensation. Our data show that tight regulation of RINGO/Speedy A is important for the somatic cell cycle.**

*Structured summary:*
MINT-7226413:*RINGO A* (uniprotkb:Q5MJ70) *physically interacts* (MI:0914) with *Ubiquitin* (uniprotkb:P62988) by *anti bait coimmunoprecipitation* (MI:0006)MINT-7226431, MINT-7226448:*RINGO A* (uniprotkb:Q5MJ70) *physically interacts* (MI:0914) with *Skp2* (uniprotkb:Q13309) by *anti tag coimmunoprecipitation* (MI:0007)
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## 1. Introduction

The mammalian cell division cycle is governed by the periodical activation of cyclin-dependent kinases (CDKs), whose activity is modulated by the binding of regulatory subunits named cyclins. Two mechanisms that contribute to the periodical accumulation of cyclins during cell cycle progression are transcriptional activation and protein destruction. Cyclin degradation is mediated by the ubiquitin-proteasome system (UPS) and involves the covalent attachment of a chain of ubiquitin molecules to the cyclin by the sequential action of three enzymes, referred to as E1, E2 and the ubiquitin ligase E3 [1]. Two major classes of ubiquitin ligases mediate ubiquitination of cyclins: the SCF (Skp1-Cul1-F-box protein) and the APC/C (Anaphase Promoting Complex/Cyclosome) complexes [2–4]. The proteolysis of cyclins by the UPS is a key mechanism that ensures accurate levels of CDK activity during the cell cycle. Consistently, overexpression of non-degradable cyclin mutants impairs cell cycle progression [5,6]. Some cyclins have also been reported to be phosphorylated in a cell cycle-dependent manner [7].

RINGO/Speedy proteins can bind to and activate CDKs in spite of having no amino acid sequence similarity to cyclins [8,9]. The first member of this family was identified as a potent inducer of the meiotic maturation in *Xenopus* oocytes [10,11]. A mammalian homologue was subsequently identified and named Spy1; this protein was proposed to regulate cell cycle progression via CDK2 activation and p27$^{Kip1}$ phosphorylation [12]. More recently, five different mammalian members of this protein family have been described with Spy1 corresponding to RINGO/Speedy A [13]. Intriguingly, the distant family member RINGO/Speedy E has been proposed to negatively regulate cell cycle progression [14]. To understand the contribution of RINGO/Speedy proteins to the periodical activation of CDKs, it is important to know their regulation during the cell cycle. In this regard, *Xenopus* RINGO/Speedy accumulates transiently during the meiotic cell cycle of oocytes owing to a tight control by the UPS [15]. Here, we report the regulation of RINGO/Speedy A (RINGO A for simplicity) during the mammalian mitotic cell cycle.

## 2. Materials and methods

### 2.1. Expression constructs

Details on DNA cloning and mutagenesis are provided as Supplementary data.

### 2.2. Cell culture and manipulation

The protocols used for cell culture and transfection, time-lapse microscopy, immunofluorescence, flow cytometry analysis and qRT-PCR are indicated in Supplementary data.

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Cells were synchronized in mitosis by incubation with Nocodazole (100 ng/ml) for 16 h followed by mechanical shake-off. The G1 population was obtained releasing mitotic cells into fresh medium for 4 or 8 h, corresponding to G1a or G1b phases, respectively. In experiments with only one G1 time point, this corresponds to 8 h after release. For G1/S synchronization, cells were incubated in medium containing Thymidine (2.5 mM) for 16 h, released into fresh medium for 10 h and then incubated again with 2.5 mM Thymidine for another 12 h. These cells were released into fresh medium for 5 or 9 h to obtain S and G2 phase populations, respectively.

*2.3. Protein analysis*

Cell pellets were resuspended in cold IP buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 5 mM EDTA, 0.1 mM Na Vanadate, 1 mM PMSF, 2 μM microcystin, 2.5 mM benzamidine and 10 μg/ml each of aprotinin, leupeptin and pepstatin. Lysates were centrifuged at maximal speed in an Eppendorf centrifuge for 15 min at 4 °C and the supernatants were used for immunoprecipitation, immunoblotting, kinase assays, ubiquitination assays or alkaline phosphatase treatments as indicated in Supplementary data.

## 3. Results

*3.1. RINGO A regulation during the cell cycle*

Expression of cyclins is tightly regulated during the cell cycle by several mechanisms, including transcriptional activation. Previous work, using RT-PCR, has reported that RINGO A mRNA was exclusively expressed at the G1/S transition in synchronized 293T cells [16]. In contrast, we detected the RINGO A mRNA in all stages of the cell cycle in two human cell lines, U2OS and HeLa, using quantitative RT-PCR (qRT-PCR) (Supplementary Fig. S1). Differences in the techniques used for cellular synchronization and transcript

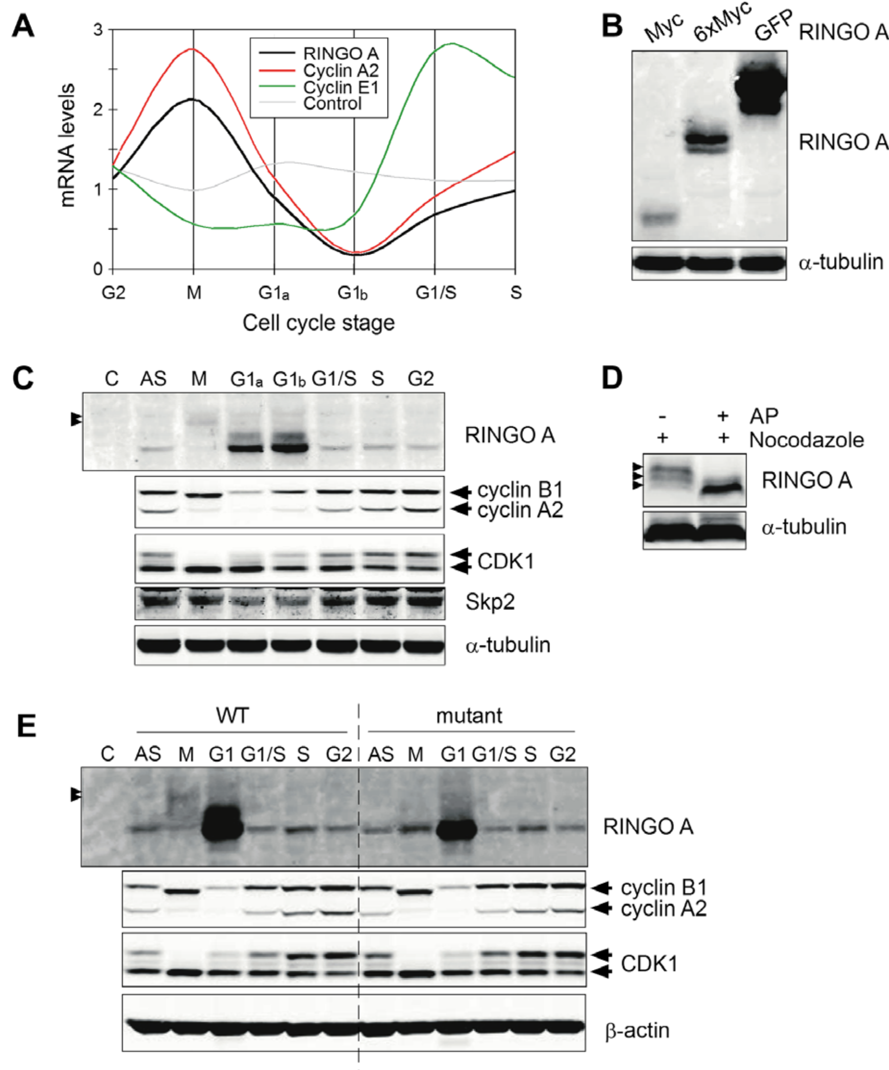


**Fig. 1.** RINGO A regulation during the cell cycle. (A) Relative amounts of RINGO A, cyclin A2, cyclin E1 and control mRNA levels in different stages of the cell cycle in U2OS cells. Transcript levels were determined by qRT-PCR and were normalized using asynchronous population (AS) levels as a reference. Cell cycle profiles are shown in Supplementary Fig. S1. (B) U2OS cells were transfected with the indicated RINGO A constructs and 24 h later total lysates were analysed by immunoblotting. (C) RINGO A protein accumulation in U2OS cells synchronized in different cell cycle stages. (D) Cells expressing inducible 6×Myc-RINGO A were treated with both tetracycline and nocodazole to induce RINGO A expression and mitotic arrest, respectively. Cell lysates were split in two aliquots that were processed equally but one received alkaline phosphatase and the other not, and were analysed by immunoblotting. (E) U2OS cells expressing RINGO A wild-type (WT) or the phosphorylation mutant (with Thr10 and Ser242-249 all changed to Ala) were synchronized in different cell cycle stages and total lysates were analysed by immunoblotting. Control lysates were prepared from non-transfected U2OS cells. Arrowheads indicate phosphorylated RINGO A forms.

analysis might account for the discrepancies with previous reports [16]. The relative RINGO A transcript levels oscillated during cell cycle progression so that they were higher in M phase and lower in G1 phase, resulting in an expression pattern nearly identical to that for cyclin A2 (Fig. 1A and Supplementary Fig. S1). Cyclin E mRNA levels were lower in the M and G1 phases and higher during S phase entry, in agreement with previous reports [17].

Cyclins accumulate periodically during cell cycle progression at both mRNA and protein levels [18]. To follow the expression pattern of the endogenous RINGO A protein during the cell cycle, we generated sensitive antibodies that could detect as little as 1 ng of recombinant RINGO A protein. Nevertheless, we have not been able to detect the endogenous protein in cell lines that express RINGO A mRNA (not shown), indicating that

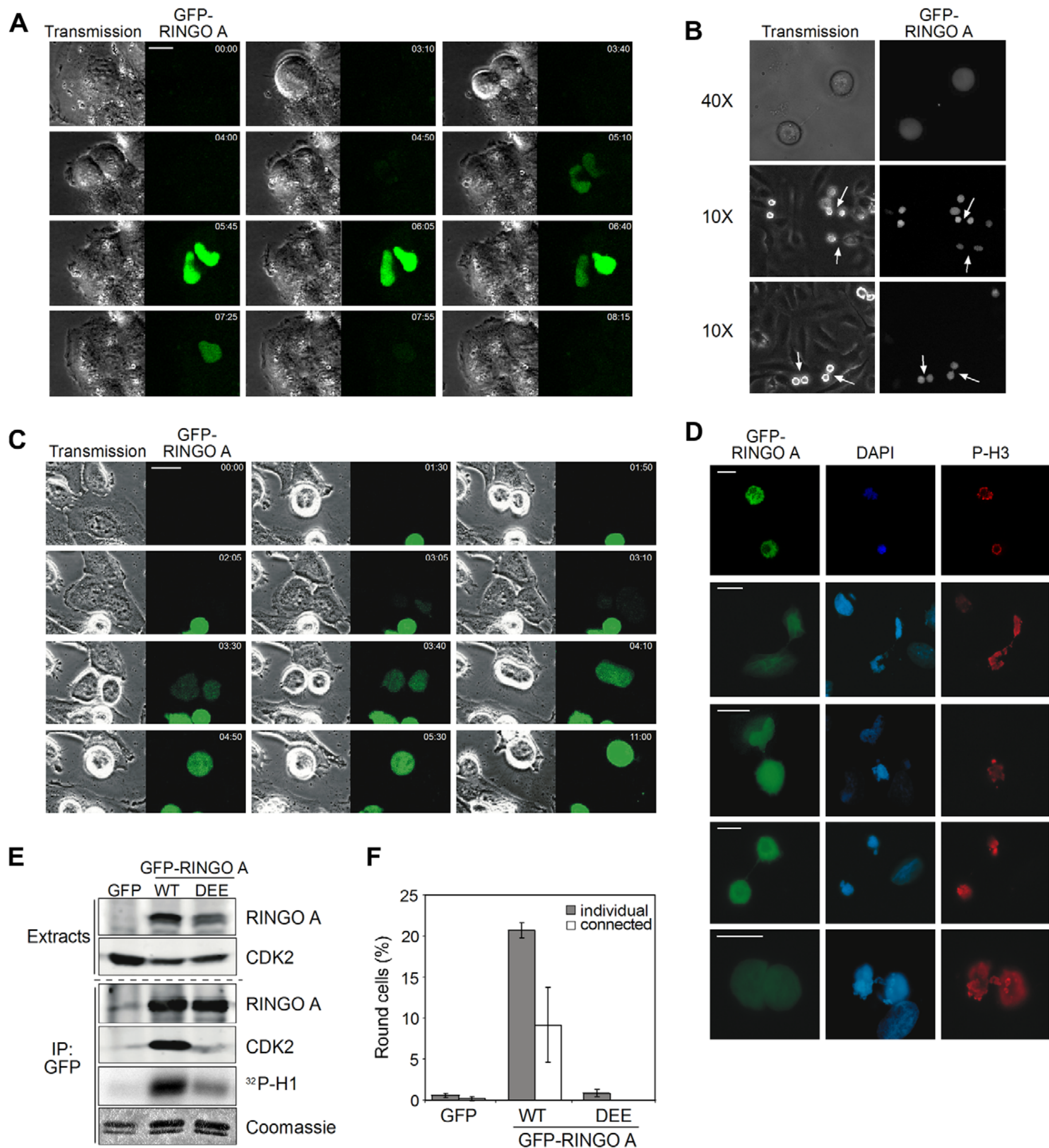


**Fig. 2.** GFP-RINGO A expression impairs cell cycle progression. (A and C) U2OS cells were transfected with GFP-RINGO A and were analysed by video microscopy. Time is indicated in the upper right corner. Scale bar, 10 mm. (B) Images of GFP-RINGO A-transfected cells were acquired in transmission and fluorescent channels using 10× or 40× magnification objectives, as indicated. GFP-RINGO A positive cells connected via a cytoplasmic bridge are indicated by white arrows. (D) U2OS cells were transfected with GFP-RINGO A. Phosphorylation of Ser10-histone H3 (P-H3), a mitotic marker, was examined by immunofluorescence 24 h after transfection. Scale bars, 10 mm. (E) 293T cells were co-transfected with GFP or GFP-RINGO A, either WT or with Asp135, Glu136 and Glu137 changed to Ala (DEE mutant), and FLAG-CDK2. Total lysates were immunoprecipitated with GFP antibody and analysed by immunoblotting with FLAG antibody to detect CDK2, as well as by kinase activity towards Histone H1. Coomassie staining of H1 is shown as a loading control. (F) U2OS cells were transfected with GFP or GFP-RINGO A-WT and -DEE and 24 h later individual rounded cells and cells connected with a cytoplasmic bridge were scored and represented as percentage of transfected cells.

RINGO A protein levels are likely to be very low. In fact, we noticed that even the overexpressed RINGO A protein was difficult to detect, which is consistent with previous reports showing poor expression of transfected RINGO A, unless the N-terminal 60 amino acids were deleted [9]. We found that N-terminal tagging improved the accumulation of transfected RINGO A, with a good correlation between the size of the N-terminal tag and RINGO A expression levels, so that a fusion to GFP was better expressed than to 6×Myc and this better than a fusion to 1×Myc (Fig. 1B). To follow the accumulation of RINGO A protein during the cell cycle, we generated a mammalian cell line that constitutively expressed Myc-RINGO A. These cells were synchronized at different stages of the cell cycle and the levels of RINGO A protein were analysed by immunoblotting (Fig. 1C). We found that RINGO A accumulated periodically, being higher in G1 phase, although small levels were detectable in all cell cycle stages. As expected, endogenous A and B-type cyclins also accumulated periodically in our experiments. Thus, RINGO A is both transcriptionally and post-transcriptionally regulated during the cell cycle. Indeed, the apparent discrepancy between the peaks of RINGO A mRNA and protein levels during cell cycle progression resembles human cyclin A regulation [19], whose mRNA levels peak in mitosis when the protein is already absent due to proteasomal degradation (see also Fig. 1A and C). This suggests a possible high turnover rate for RINGO A in mitosis.

### 3.2. RINGO A protein is phosphorylated in mitosis

RINGO A usually appeared as a smear in immunoblottings of mitotic cell extracts (Fig. 1C–E, arrowheads). Since these changes in electrophoretic mobility are usually associated with protein modifications, mostly phosphorylation, we tested the possibility that the shifted RINGO A bands represented phosphorylated forms. To be able to analyse the low amounts of RINGO A protein present in mitotic cell extracts, we generated a U2OS-derived cell line expressing Tetracycline-inducible 6×Myc-RINGO A (Supplementary Fig. S2). By immunoblotting, we detected several 6×Myc-RINGO A bands in extracts of mitotic cells (Fig. 1D, arrowheads). Incubation of the extracts with alkaline phosphatase converted the slower migrating RINGO A forms to a faster migrating form, indicating that this protein was phosphorylated in mitosis, probably at multiple sites. To identify phosphorylated residues in RINGO A, we generated a collection of mutants with potential phosphorylation residues changed to Ala (Supplementary Fig. S3). We found that mutation of an N-terminal Thr-Pro site together with mutation of a stretch of eight Ser located in the C-terminus abolished the mitotic shift (Fig. 1E and Supplementary Fig. S3, mutant C). Next, we generated a cell line that constitutively expressed the phosphorylation-deficient RINGO A mutant and analysed its accumulation during the cell cycle. We found that the cell cycle accumulation profiles of wild-type (WT) RINGO A and the non-phosphorylatable mutant were very similar, except in mitosis where the mutant did not display the characteristic band shift and appeared as a single fast-migrating band (Fig. 1E, arrowheads). These results indicate that RINGO A is hyper-phosphorylated in mitosis. However, this phosphorylation does not appear to have a major effect on RINGO A protein accumulation during the cell cycle.

### 3.3. Overexpression of a stabilized RINGO A form impairs cell cycle progression

A fusion with GFP was the best expressed form of RINGO A that we could obtain (Fig. 1B), indicating that this fusion protein was more resistant to the degradation mechanisms that conferred poor expression to other RINGO A constructs. Since degradation-resistant mutants of cyclins are known to impair cell cycle progression [5,6], we investigated the effect of GFP-RINGO A on the cell cycle.

By using live-cell video microscopy, we found that about half (54 ± 12%) of the cells expressing GFP-RINGO A progressed normally through the cell cycle and showed only transient RINGO A accumulation. Typically, the protein appeared shortly after mitosis and was then degraded (Fig. 2A; Supplementary movies 1 and 2), in agreement with the results from our synchronization experiments (Fig. 1C and E). However, the other half of the GFP-RINGO A-expressing cells (46 ± 12%) displayed cell cycle abnormalities. In particular, these cells had a rounded morphology and were often connected in pairs via what we referred to as the cytoplasmic bridge (Fig. 2B). The cytoplasmic bridge frequently contained a structure similar to the midbody, suggesting a cytokinesis defect

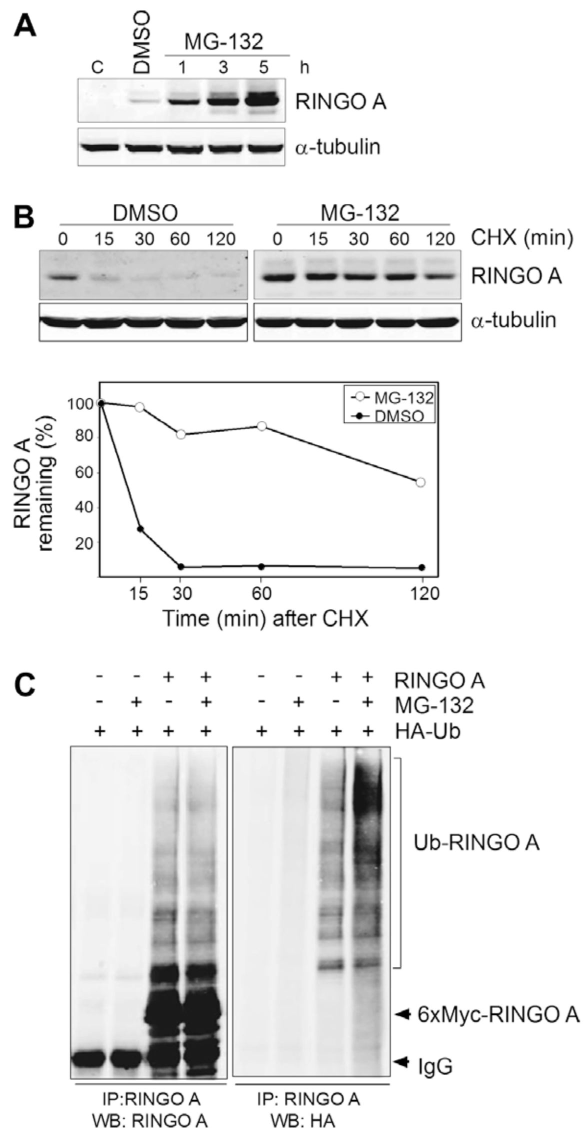


**Fig. 3.** Degradation of RINGO A by the UPS. (A) U2OS cells stably expressing Myc-RINGO A were incubated either with DMSO for 5 h or with MG-132 (25 μM) for the indicated times and the lysates were analysed by immunoblotting. Control lysates were prepared from non-transfected U2OS cells. (B) U2OS cells expressing Myc-RINGO A were pre-incubated with DMSO or MG-132 (25 μM) for 2 h prior to cycloheximide addition (CHX, 30 μg/ml). Cells were collected at the indicated times after CHX addition and the expression of RINGO A was determined by immunoblotting. Quantification of the blots is shown in the lower panel. (C) U2OS cells were transfected with HA-ubiquitin (HA-Ub) or 6×Myc-RINGO A and incubated with MG-132. Cell lysates were immunoprecipitated with RINGO A antibody and then blotted with HA or RINGO A antibodies, as indicated.

(Fig. 2B). By using live-cell imaging, we found that these cells displayed constitutive RINGO A expression after mitosis, which usually resulted in cell rounding (Fig. 2C; Supplementary movies 3 and 4). Frequently, two connected cells fused back in a single cell (Fig. 2C; Supplementary movie 4), resembling the "reversal of mitosis exit" process observed upon forced stabilization of cyclin B in mitosis [20]. In addition, these cells showed abnormal chromatin condensation and morphology (Fig. 2D). We have not observed a similar phenotype by overexpressing other RINGO A constructs that are expressed at lower levels, such as 6×Myc-tagged forms. We therefore believe that this phenotype is a direct consequence of the presence of high levels of GFP-RINGO A protein.

Given that RINGO A can activate CDKs, primarily CDK2, it is possible that unscheduled and/or increased CDK activity resulting from RINGO A accumulation at late stages of mitosis may interfere with cell cycle progression. To test this possibility we mutated residues 135–137 of RINGO A to Ala and confirmed that this mutant (RINGO A-DEE) was unable to bind to and activate CDK2 (Fig. 2E), as expected from the mutation of equivalent residues in *Xenopus* RINGO and mammalian RINGO E [8]. Interestingly, GFP-RINGO A-DEE induced neither the appearance of cells with rounded morphology nor cells connected via the cytoplasmic bridge (Fig. 2F), even when expressed at the same level as GFP-RINGO A wild-type (Supplementary Fig. S4). This data suggests that high levels of GFP-RINGO A impair cell cycle progression by a mechanism that involves binding to endogenous CDKs.

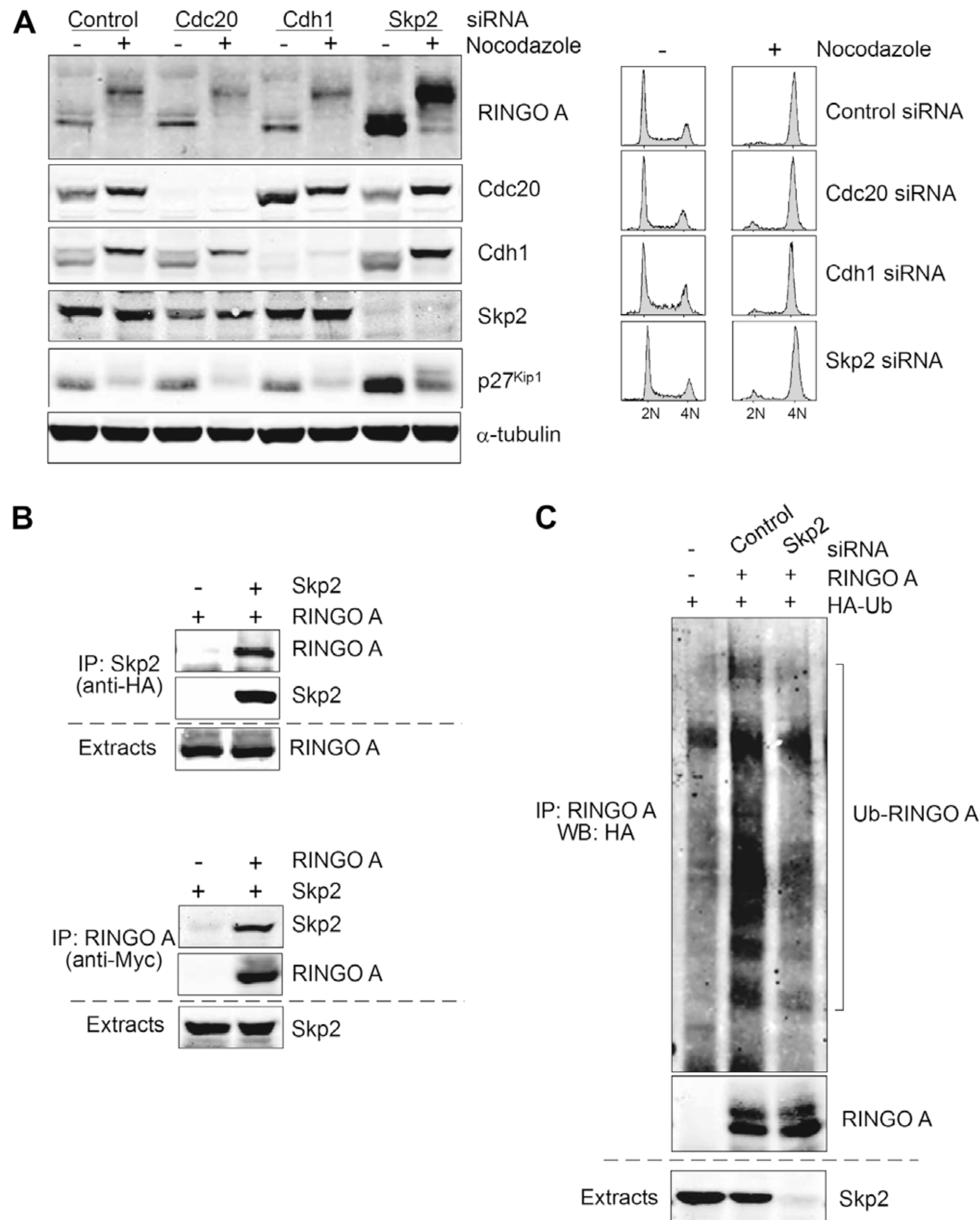


**Fig. 4.** RINGO A degradation is mediated by Skp2. (A) U2OS cells expressing Myc-RINGO A were transfected with control, Cdc20, Cdh1 or Skp2 siRNAs and 48 h after transfection were incubated with Nocodazole. Asynchronous and mitotic cell populations were analysed by immunoblotting. Flow cytometry analysis are shown in the right panels. (B) U2OS cells were transfected with HA-Skp2 and Myc-RINGO A, as indicated. Total lysates were immunoprecipitated with HA (upper panel) or Myc (lower panel) antibodies and then were analysed by immunoblotting. (C) U2OS cells were co-transfected with HA-ubiquitin or 6×Myc-RINGO A and 24 h later were transfected again with the indicated siRNAs. Cell lysates were immunoprecipitated with RINGO A antibody and then immunoblotted with HA antibody. Note that Skp2 depletion did not cause strong accumulation of 6×Myc-RINGO A in contrast with Myc-RINGO A.

### 3.4. RINGO A degradation by the UPS

Proteolysis mediated by the UPS is a key mechanism controlling cyclin levels during the cell cycle [18]. To examine whether RINGO A levels were regulated by the UPS, we treated RINGO A-expressing U2OS cells with the proteasome inhibitor MG-132. This resulted in rapid RINGO A accumulation (Fig. 3A), indicating a fast turn-over of RINGO A in proliferating cells. By treating cells with the protein synthesis inhibitor cycloheximide, we estimated that the half-life of RINGO A was less than 15 min (Fig. 3B). As expected, MG-132 significantly increased the half-life of RINGO A in the presence of cycloheximide.

Interestingly, GFP tagged RINGO A protein also showed an increased half-life (Supplementary Fig. S5). To examine whether RINGO A was ubiquitinated, U2OS cells were co-transfected with RINGO A and HA-tagged ubiquitin. We detected high molecular weight HA-immunoreactive forms in the RINGO A immunoprecipitates, which were confirmed to correspond to the ubiquitinated RINGO A by re-probing with a RINGO A antibody (Fig. 3C).

### 3.5. SCF-Skp2 mediates RINGO A degradation

Since SCF and APC/C complexes are two important ubiquitin ligases controlling the timely proteolysis of cell cycle regulators, we investigated their implication in UPS-mediated RINGO A degradation. We used siRNA against the APC/C activators Cdc20 and Cdh1 as well as against Skp2, the F-box protein of SCF. Downregulation of Skp2, but not of Cdc20 or Cdh1, resulted in RINGO A accumulation in both asynchronous and mitotic cells suggesting that SCF-Skp2 complex could be involved in RINGO A degradation (Fig. 4A, left). As a control, treatment with Skp2 siRNA also induced accumulation of $p27^{Kip1}$, a well-known Skp2 substrate [21]. We confirmed that RINGO A accumulation was not a consequence of changes in cell cycle distribution induced by Skp2 siRNA (Fig. 4A, right). Interestingly, overexpressed RINGO A co-immunoprecipitated with Skp2 (Fig. 4B) and Skp2 downregulation impaired RINGO A ubiquitination in cells (Fig. 4C). We also observed that the expression of Skp2 inversely correlated with the accumulation of RINGO A during the cell cycle (Fig. 1C), supporting the idea that Skp2 mediates RINGO A ubiquitination and degradation.

## 4. Discussion

Our data indicate that the similarity between RINGO/Speedy proteins and cyclins goes beyond CDK binding and activation. Like cyclins, RINGO A is a very unstable protein that is periodically expressed during the cell cycle and is degraded by the UPS. We found that the $SCF^{Skp2}$ ubiquitin ligase is a good candidate to regulate RINGO A ubiquitination and degradation. It has been recently proposed that NEDD4, a HECT-type ubiquitin ligase, might target RINGO A for degradation [22]. Although we have not tested this possibility, it is well documented that Skp2 substrates are usually targeted by additional ubiquitin ligases [21].

Overexpression of a stabilized form of RINGO A fused to GFP impairs cell cycle progression. In particular, a high percentage of the cells expressing GFP-RINGO A at late stages of mitosis round up and stop cycling. These cells often show a phenotype similar to what has been reported for cells that express high levels of non-degradable cyclin B [23] or cells that undergo the process called "reversal of mitotic exit" [20] in which cyclin B accumulation induces premature CDK1 activation and prevents mitotic exit. Similarly, mutation of the RINGO A core, which is essential for CDK binding, abolishes the negative effects of GFP-RINGO A expression on cell cycle progression suggesting that these are likely due to unscheduled activation of CDK/RINGO A complexes. The tight control of RINGO A protein levels by UPS-mediated degradation could be important to ensure the low levels of CDK activity required for some cell cycle transitions, such as mitotic exit. RINGO A binds to and activates preferentially CDK2, but it can also bind to CDK1 when both proteins are overexpressed [8]. Although CDK2 has been mainly implicated in S-phase, it also coordinates mitotic events [24]. Indeed, downregulation of RINGO A results in delayed progression both at G1/S and G2/M transitions (unpublished data). It remains to be elucidated whether binding to CDK2 accounts for the phenotype induced by RINGO A overexpression and, in this case, which substrates could be targeted by the CDK2/RINGO A complex. Given the central role of Skp2 in promoting tumorigenesis, which cannot always be explained by $p27^{Kip1}$ downregulation [21], it would be also interesting to elucidate the biological relevance of RINGO A regulation by Skp2, determining precisely the timing and the events that lead to its degradation.

Future studies should investigate how RINGO A interplays with cyclins to tightly regulate CDK activity during cell cycle progression and how timely expression and degradation of these proteins coordinate cell cycle events by selectively targeting CDKs to specific substrates.

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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.febslet.2009.07.028.

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