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RINGO/Speedy proteins, a family of non-canonical activators of CDK1 and CDK2

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Abstract

Cyclin-dependent kinases (CDKs) require the binding to a regulatory subunit to acquire enzymatic activity, and cyclins are the canonical CDK activators. However, there are specific situations in which CDKs can be activated by non-cyclin proteins that are less characterized. This review focuses on the family of RINGO/Speedy proteins, which have no sequence amino acid homology to cyclins but can bind to and activate CDK1 and CDK2. Interestingly, RINGO/Speedy proteins can activate CDKs under conditions in which CDK-cyclin complexes would not be active, and there is evidence that RINGO/Speedy-activated CDKs can phosphorylate different sites than the cyclin-activated CDKs. RINGO/Speedy proteins were originally described in *Xenopus* oocytes, but their roles in mammalian cells have also been addressed. We will summarize the properties of RINGO/Speedy proteins and how they trigger CDK activation, and discuss recent studies that characterized their physiological functions. In particular, studies using genetically modified mice have shown that RingoA, also known as Spy1, plays a key role in meiosis regulation. Emerging evidence also suggests a potential role for RingoA/Spy1 in cancer.

1. Introduction

The periodic activation of so-called cyclin-dependent kinases (CDKs) plays a key role in cell cycle progression. The activation of these kinases is controlled by their association with regulatory subunits, and also by phosphorylation. The association with a cyclin subunit leads to partial CDK activation. Full kinase activation requires the phosphorylation of a Thr on the T-loop (Thr161 in CDK1 and Thr160 in CDK2) as well as the removal of the inhibitory phosphorylations Thr14 and Tyr15. CDK activity can be further regulated by the binding of CDK inhibitors of the INK4 and Cip/Kip families such as $p21^{Cip1}$ and $p27^{Kip1}$ [1]. The structures, regulatory mechanisms and functions of CDKs have been extensively reviewed [2, 3]. The roles and regulation of cyclins are the subjects of several reviews in this issue.

Although cyclins are universal CDK activators, there are cases in which CDKs can be activated by proteins that do not belong to the cyclin family. CDK5 is one of the best-known examples, as this kinase can be activated by the proteins p35, p39 or p25. CDK5 has important roles in post-mitotic neuronal cells and has been implicated in neurodegeneration [4]. Growing evidence also implicates CDK5 and its activators in cancer [5, 6].

This review focuses on the cyclin-like CDK activators of the RINGO/Speedy family. These proteins were discovered in *Xenopus* by two independent screenings. One was an expression-cloning screening aimed to find proteins that upon overexpression in G2-arrested *Xenopus* oocytes were able to trigger M phase entry, the initial stage of the meiotic maturation, and named them RINGO (Rapid INducer of G2/M progression in Oocytes) [7]. The other screening searched for *Xenopus* genes that were able to confer resistance to UV and γ-radiation when overexpressed in a Rad1-deficient mutant of the yeast *S. pombe*, and named them Speedy [8]. The role of *Xenopus* RINGO/Speedy (XRINGO) in oocyte maturation has been extensively studied, and is reviewed below.

Mammalian genes with similarity to XRINGO have been also identified. The first human XRINGO homolog was named Spy1 [9], which is also known as Ringo3, SpeedyA or RingoA, and here it is referred to as RingoA/Spy1. Other mammalian homologs of XRINGO were named RingoC (Ringo2) and RingoE (Ringo1) in human, and RingoB (Ringo4) and RingoD (Ringo5) in mouse [10]. A number of variants of this protein family in different species and their phylogenetic relationship have been reported [11]. Of note, RINGO/Speedy family proteins are notably different between human and mouse, with RingoA/Spy1 being the most conserved across species [10, 11].

2. Non-canonical CDK activation

The initial characterization showed that XRINGO was able to bind to and increase the kinase activity of both CDK1 and CDK2 [7]. Similar results were later reported for mammalian RingoA/Spy1, RingoB, RingoC and RingoE [9, 12, 13]. Intriguingly, overexpression experiments in *Xenopus* oocytes have shown that RingoE but not RingoA/Spy1 or RingoC, can also bind to CDK5 [13]. The RINGO/Speedy proteins share a conserved central region of approximately 100 residues referred to as the RINGO or S/R box, which is required for CDK binding [12, 13].

Removing the N-terminal and C-terminal regions of XRINGO does not affect the ability of the protein to induce *Xenopus* oocyte maturation and CDK2 activation *in vitro* [13], but the C-terminal region of RingoA/Spy1 might contribute to CDK activation [12]. In addition, the N-terminal domain of RingoA/Spy1 seems to destabilize the protein, as removal of this region enhances RingoA/Spy1 expression in transfected HEK293 cells [12]. The RINGO/Speedy proteins have no similarity in amino acid sequence with cyclins, however, recent structural studies on RingoA/Spy1 have shown that the RINGO box adopts a cyclin-box fold (CBF) that binds CDK2 in the same position as the N-terminal CBF of CyclinA. Moreover, the secondary structural elements of the RINGO box at the CDK PSTAIRE interphase align with CyclinA [14]. The largest interface between RingoA/Spy1 and CDK2 comprises the PSTAIRE region of CDK2 and the helixes α3 and α5 of RingoA/Spy1. These observations agree with an earlier mutagenic analysis that identified key residues in the PSTAIRE region of CDK2 and in the α3 helix of XRINGO and RingoA/Spy1, which were required for binding of the two proteins [13, 15].

In vitro studies using purified recombinant proteins and injections into *Xenopus* oocytes showed that XRINGO is able to activate CDK1 and CDK2 independently of T-loop phosphorylation [16] (Figure 1). This was a surprising observation, considering that the kinase activity of cyclin-bound CDK1 and CDK2 strongly increases upon phosphorylation on the T-loop. The biochemical results have been confirmed by recently determined crystal structures showing that the non-phosphorylated form of CDK2 bound to RingoA/Spy1 adopts a conformation similar to the T-loop phosphorylated CDK2 bound to CyclinA [17]. Thus, upon RingoA/Spy1 binding, the PSTAIRE region and the T-loop of CDK2 adopt a conformation associated with the active kinase, explaining why RingoA/Spy1 binding alone suffices to activate CDK2 regardless of the T loop phosphorylation. Mutational analysis showed that among the RingoA/Spy1 residues that interact with the CDK2 T-loop, Asp97 and Glu135 are critical for kinase activation [14]. Interestingly, it was also shown that the Wee1 family kinase Myt1 is less efficient at inhibiting CDK-XRINGO than CDK-cyclin complexes

[16], suggesting that CDK-RINGO complexes are less sensitive to the inhibition mediated by the phosphorylation on Thr14 and/or Tyr15 than CDK-cyclin complexes. It has been also reported that CDK2 activated by RINGO/Speedy proteins has reduced sensitivity to $p21^{Cip1}$ and $p27^{Kip1}$. These CDK inhibitors bind to the cyclin CBF through the MRAIL region, which RINGO/Speedy proteins lack, providing an explanation for the weaker binding and poorer inhibition observed in CDK2-RINGO compared with CDK2-CyclinA [14, 16]. Taken together, the ability of RINGO/Speedy proteins to activate CDK1 and CDK2 regardless of the activating T-loop phosphorylation and with less sensitivity to Myt1 or CDK inhibitors suggests that CDK-RINGO complexes might play important roles in specific situations when CDK-cyclin complexes are inhibited (Fig. 1).

In addition to enable the activation of CDKs under particular conditions, it is conceivable that RINGO/Speedy proteins might also affect CDK substrate specificity. This was first addressed in studies using peptide libraries, which concluded that RingoA/Spy1-activated CDK2 has a less strict preference for Lys at +3 position of the phospho-acceptor site compared with CDK2-CyclinA [18]. Further work using Myt1 as a substrate provided evidence that RINGO/Speedy proteins can lead CDKs to phosphorylate different sites than the cyclins [19]. *In vitro* experiments showed that XRINGO-bound CDK1 or CDK2 are able to phosphorylate three residues at the C-terminal regulatory domain of Myt1, which promote the inhibition of Myt1 kinase activity, but these residues are poorly phosphorylated by CDK1- or CDK2-cyclin complexes. Interestingly, evidence was presented that the RNL sequence of Myt1, which is required for binding to CyclinB, was not required for Myt1 phosphorylation by XRINGO-activated CDKs. This suggests that XRINGO and cyclin bind to different regions of Myt1, which may account for the different sites phosphorylated by the two CDK complexes [19]. The three sites identified are likely phosphorylated during the meiotic maturation of *Xenopus* oocytes and can prime Myt1 for the binding to the kinase p90Rsk1, which further phosphorylates Myt1 leading to its complete inhibition required for the full activation of CDK1-CyclinB complexes and the G2/M progression [20]. Therefore, CDK-RINGO complexes may have different substrate specificity, in addition to being potentially active under conditions in which CDK-cyclin complexes are inactive, such as in G2 arrested *Xenopus* oocytes.

3. Cell cycle regulation and the DNA damage response

RingoA/Spy1 is the most studied mammalian RINGO/Speedy protein. Human RingoA/Spy1 mRNA was shown to be expressed in G1/S phase of the cell cycle in HEK293 cells [9]. Further work in U20S cells detected RingoA/Spy1 mRNA expression in all stages of the cell cycle in an oscillating pattern, peaking in M phase and reaching the lowest levels in mid-late G1, very similar to the expression pattern of CyclinA2 [21]. Moreover, myc-RingoA/Spy1 overexpressed in U20S cells was found to accumulate periodically, reaching the highest levels in the G1 phase. The overexpressed myc-RingoA/Spy1 protein was also phosphorylated in mitosis [21].

RingoA/Spy1 protein overexpressed in U2OS cells was very unstable, and was found to bind to the E3 ubiquitin ligase Skp2, which triggered its degradation by the proteasome [21]. NEDD4 is another ubiquitin ligase that was found to bind to RingoA/Spy1 and to target it for degradation in HEK293 cells [22]. Interestingly, the expression of a stabilized form of RingoA/Spy1 fused to GFP resulted in impaired cell cycle progression, with cells having abnormal chromatin condensation and morphology. These cells have a similar phenotype as those expressing a stable form of CyclinB that prevents mitotic exit, which has been called the "reversal of mitosis exit" [23]. This RingoA/Spy1 phenotype is CDK dependent, as it was not observed upon expression of a GFP-RingoA/Spy1 mutant unable to bind to CDK [21]. The observation that RingoA/Spy1 overexpression interferes with mitotic progression in somatic cells suggests that degradation of this protein might be required for the completion of somatic mitosis. Previous work showing that XRINGO induces cleavage arrest upon injection into the blastomere of two-cell mouse embryos further supports that XRINGO/Speedy proteins expressed out of context may have cytostatic activity [24].

RingoA/Spy1 has been proposed to control the G1/S transition in human cancer cells, based on the observations that RingoA/Spy1-overexpressing HEK293 cells showed a reduced G1 phase length, whereas treatment with RingoA/Spy1 siRNA led to an increase in G1 phase and reduction in G2/M phases [9]. The ability of human RingoA/Spy1 to interact with $p27^{Kip1}$ might explain the observed changes in G1. Consistent with this idea, RingoA/Spy1 overexpression was shown to overcome the $p27^{Kip1}$-induced G1 arrest in HEK293 cells [25], which correlated with CDK2-mediated phosphorylation of $p27^{Kip1}$ on Thr187 [26]. More recently, the direct binding of RingoA/Spy1 has been reported to destabilize $p27^{Kip1}$, whereas RingoA/Spy1 binding to CDK2 does not seem to affect $p27^{Kip1}$ stability, suggesting that RingoA/Spy1 might regulate $p27^{Kip1}$ stability independently of Thr187 phosphorylation [27]. These observations are intriguing considering that cyclin-bound CDK2 is well-known to

phosphorylate $p27^{Kip1}$ on Thr187 and induce its degradation [28]. It is therefore unclear how RingoA/Spy1 can trigger $p27^{Kip1}$ degradation independently of Thr187 phosphorylation.

RINGO/Speedy proteins have been proposed to have a role in the DNA damage response. First, XRINGO overexpression in Rad1-deficient *S. pombe* was found to confer resistance to UV [8]. Subsequently, the same group published several reports supporting that RingoA/Spy1 may allow to bypass DNA damage checkpoint activation in mammalian cells. Thus, RingoA/Spy1 overexpression was reported to increase the survival of U2OS cells treated with genotoxic drugs [29], and the RingoA/Spy1-overexpressing U2OS cells showed reduced levels of phospho-Chk1 and phospho-RPA, as well as reduced sensitivity to UV-induced apoptosis [30]. The pro-survival role of RingoA/Spy1 in response to UV was proposed to depend on the expression of p53 and $p21^{Cip1}$, and siRNA-mediated knockdown of RingoA/Spy1 was described to increase γH2AX foci formation and Chk1 phosphorylation in U20S cells [31]. Another study has reported that RingoA/Spy1 expression improves the viability of neuronal NSC34 cells overexpressing the superoxide dismutase (SOD1) mutant G93A, which have an activated DNA damage response [32].

Besides RingoA/Spy1, there is evidence implicating other mammalian RINGO/Speedy family members in the regulation of various cell cycle steps. For example, siRNA-mediated downregulation of RingoC in HEK293 cells resulted in longer G2 phase, whereas the overexpression of RingoC led to faster progression through late S and G2 phases and override of the G2 DNA damage checkpoint [33]. Another study using several human cancer cell lines has shown that RingoC downregulation leads to premature mitosis exit and chromosome alignment problems, with decreased recruitment of spindle assembly checkpoint components [34]. RingoC was shown to associate with Aurora B, but did not seem to control its kinase activity based on CENPA and H3 phosphorylation, although Thr232 phosphorylated AuroraB was found to miss-localize in prometaphase upon RingoC downregulation [34]. RingoE has also been linked to cell cycle regulation. Surprisingly, unlike other RINGO/Speedy proteins, RingoE was found to inhibit progesterone-induced *Xenopus* oocyte maturation, probably by sequestering CDK1 [15]. Although RingoE can activate CDK1 *in vitro* and in *Xenopus* oocytes, CDK1-RingoE phosphorylates Myt1, a key negative regulator of oocyte maturation, with reduced efficiency compared with CDK1-RingoA/Spy1. These results suggest that different RINGO/Speedy proteins could determine the substrate preferences of CDKs and potentially regulate different biological functions. In agreement with the observations in *Xenopus* oocytes, overexpression of RingoE has been reported to inhibit the proliferation of U20S cells, reducing the amount of cells in G1 while

enhancing the cells in G2/M [15]. Of note, as for RingoA/Spy1, RingoC and RingoE overexpressed in *Xenopus* oocytes have been also reported to interact with $p27^{Kip1}$ [13], although it remains to be determined whether this has any physiological significance.

4. Meiotic functions

There is evidence supporting that XRINGO is both necessary and sufficient for triggering the G2/M transition in *Xenopus* oocytes. XRINGO was identified for its ability to induce G2 arrested *Xenopus* oocytes to enter meiotic maturation in the absence of progesterone stimulation [7], suggesting a role of this protein in meiosis regulation. Further work showed that endogenous XRINGO protein accumulates transiently during meiosis I in progesterone-treated oocytes and then is downregulated. Two proteasome-mediated mechanisms have been proposed to contribute to the regulation of XRINGO expression. One leads to partial processing generating a C-terminally truncated form, which may contribute to the oocyte G2 arrest. This involves the ubiquitin ligase $SCF^{\beta TrCP}$ and requires XRINGO phosphorylation by protein kinase A and glycogen synthase kinase-3β. On the other hand, XRINGO degradation after meiosis I is mediated by the ubiquitin ligase Siah-2 and requires phosphorylation on Ser243 [35]. As mentioned above, the ability of CDK-XRINGO to phosphorylate Myt1 may account for its ability to induce *Xenopus* oocyte maturation. Myt1 can phosphorylate CDK1 on Thr14 and Tyr15, resulting in kinase inhibition, and is believed to play an important role in keeping the preformed CDK1-CyclinB complexes inhibited in the G2 arrested *Xenopus* oocytes [36]. Dephosphorylation of these two residues by Cdc25 phosphatases leads to CDK1-CyclinB complex activation [37, 38]. In late G2 phase, there is a switch in the balance between Myt1 and Cdc25, which leads to the dephosphorylation of CDK1 bound to Cyclin B and activation of the complex, allowing the oocyte to enter into meiosis I. CDK-XRINGO can phosphorylate three Ser on the C-terminal non-catalytic domain of Myt1, inhibiting its kinase activity, and these sites are poorly phosphorylated by CDK-cyclin complexes. Taken together, these results support that XRINGO plays an important triggering role at the G2/M transition of *Xenopus* oocytes by inducing Myt1 inhibition [19, 20].

The role of RINGO/Speedy proteins in mammalian oocytes has also been investigated. The injection of a porcine RINGO/Speedy homolog has been shown to accelerate the maturation of porcine oocytes [39], and mammalian RINGO/Speedy proteins can induce *Xenopus* oocyte maturation, albeit with less efficacy than XRINGO [9, 12, 13]. Moreover, the injection of XRINGO mRNA suffices to induce germinal vesicle breakdown (GVBD) and first polar body extrusion in mouse oocytes [24]. These findings suggest that RINGO/Speedy

proteins may have a role in the maturation of mammalian oocytes. However, mice bearing RingoA/Spy1$^{lox/lox}$ alleles and Zp3-Cre, which is expressed in postnatal oocytes, seem to be affected neither in oocyte maturation nor in female fertility [40]. It is therefore possible that endogenous RingoA/Spy1 might not be required for oocyte maturation in mice. However, it remains to be confirmed that RingoA/Spy1 expression is indeed abolished in the oocytes from RingoA/Spy1$^{lox/lox}$ Zp3-Cre mice.

Independently of whether RingoA/Spy1 might be involved in mammalian oocyte maturation, recent studies using genetically modified mice have demonstrated a key role for this protein in meiosis regulation. Thus, RingoA/Spy1-deficient mice are sterile [40, 41], and phenocopy the defects of CDK2-deficient mice [42]. This observation, together with the fact that RingoA/Spy1 can interact with and activate CDK2, suggests that RingoA/Spy1 is an essential activator of this kinase during the meiotic process. Mice expressing a CDK2 mutant that is deficient in catalytic activity show similar phenotypes as the CDK2 knockout and RingoA/Spy1 knockout mice, suggesting that the kinase activity of CDK2 is essential in the process [43]. RingoA/Spy1 was found to localize to the telomeric regions of spermatocytes, overlapping with TRF1 from leptotene to pachytene. This telomeric location is very similar to the one described for CDK2. RingoA/Spy1 is detectable in telomeres located at the nuclear envelope in pre-leptotene, co-localizes with CDK2 in telomeres during leptotene and zygotene, and then the signal is reduced as cells enter diplotene (Fig. 2A). Moreover, RingoA/Spy1 and CDK2 co-localize along the asynapsed axial elements of the sex chromosomes in about 60% of their surface. The interaction between endogenous RingoA/Spy1 and CDK2 in testis was confirmed by immunoprecipitation [40, 41]. In testis, RingoA/Spy1 protein expression starts from postnatal day 12 (P12), and in female germ cells, mRNA expression starts around 14.5 days post coitum (dpc), when they enter meiosis, and decreases at 18.5-19.5 dpc, being dramatically reduced in adult ovaries [40] (Fig. 2A).

Studies in spermatocytes showed that RingoA/Spy1 is essential for the tethering of telomeres to the nuclear envelope during meiotic prophase. Spermatocytes from RingoA/Spy1 knockout mice are arrested in mid-pachytene of meiosis I, display non-homologous pairing, telomere fusions, and inefficient double-strand break repair, and also lacked sex-body formation (Fig. 2B). Interestingly, CDK2 is absent from telomeres of RingoA/Spy1 knockout spermatocytes at the pachytene-like stage, suggesting that RingoA/Spy1 is necessary for the recruitment of CDK2 to telomeres [40, 41]. This was further supported by overexpression experiments showing that a CDK2 mutant deficient in RingoA/Spy1 binding was unable to locate to telomeres in spermatocytes. Moreover, it has been reported that TRF1 directly

interacts with RingoA/Spy1 whereas CDK2 interacts with TRF1 through RingoA/Spy1 [44]. Therefore, RingoA/Spy1 has been proposed to function as a scaffold that mediates CDK2 recruitment to telomeres.

RingoA/Spy1 deficient spermatocytes also lack telomeric Sun1, a protein essential for telomere tethering to the nuclear envelope. CDK2-RingoA/Spy1 can directly phosphorylate Sun1 *in vitro*, and Sun1 phosphorylation could therefore account for most of the phenotypes observed in RingoA/Spy1 KO spermatocytes. However, it seems unlikely that Sun1 phosphorylation could explain the observed telomere fusion phenotype [41].

According to the serious meiotic defects observed, both male and female RingoA/Spy1 knockout mice are sterile. RingoA/Spy1 knockout mice show normal testes at post-natal day 7 (P7) but from two months of age their testes are atrophied, hypoplastic and lack round and elongated spermatids. The histological analysis showed abnormal epithelium, narrower seminiferous tubules and lack of post-meiotic cells. In females, there is an almost total loss of oocytes from P5 onwards, and ovaries are also atrophic and mainly composed of interstitial and stromal cells [40, 41].

In general, RINGO/Speedy family members tend to be highly expressed in testes but very little is known on whether other RINGO/Speedy proteins besides RingoA/Spy1 are implicated in meiosis. We have generated RingoB knockout mice and found that they are fertile (L Gonzalez and A Nebreda, unpublished observations) indicating that RingoB does not play an essential role in meiosis regulation.

5. A potential role in cancer

The ability of RINGO/Speedy proteins to activate CDKs under conditions that are not favorable for CDK-cyclin activation, suggest that these proteins may potentially control cycle progression in special situations, such as in cancer. A number of studies have reported correlations between RingoA/Spy1 overexpression and bad prognosis in human tumors, including hepatocellular carcinoma [45], glioma [46], non-Hodgkin's lymphoma [47], ovarian [48], and colorectal [49]. However, these results should be taken with caution, as they are based on immunohistochemistry analysis and no controls were provided to demonstrate that the staining really corresponds to RingoA/Spy1 expression.

The possible role of RingoA/Spy1 in breast cancer has been documented in several studies. It has been reported that RingoA/Spy1 expression is upregulated in breast cancer tissue microarrays (TMAs) and several breast cancer cell lines, and both MDA-MB-231 and MCF-7 cells shown decreased proliferation upon siRNA-induced knockdown of

RingoA/Spy1 [50]. RingoA/Spy1 expression has also been described to change during mammary gland development. Thus, RingoA/Spy1 mRNA and protein levels have been detected at higher levels in virgin mammary glands and during early pregnancy to be then dramatically reduced in lactation. The same study reported that HC11 mammary epithelial cells stably overexpressing RingoA/Spy1 were able to refill the cleared mammary fat pad of syngeneic mice at a faster rate, showed increased ductal branching and produced enhanced mammary tumorigenesis compared with parental HC11 cells [51]. Using the same fat pad transplantation model, HC11 cells expressing an N-terminally truncated (more stable) RingoA/Spy1 mutant were shown to form tumors faster [50], whereas cells expressing RingoA/Spy1 mutants deficient in either $p27^{Kip1}$ or CDK2 binding formed tumors more slowly [52]. The activation of ERα has been proposed to induce RingoA/Spy1 upregulation in MCF-7 cells, which might in turn control the ERK1/2 mediated phosphorylation of ERα on Ser118, and sensitivity to tamoxifen tested by injection of RingoA/Spy1 overexpressing MCF-7 cells in zebrafish [53]. RingoA/Spy1 levels have been also proposed to be controlled by p53 in HC11, MDA-MB-231 and HEK293 cells in which RingoA/Spy1 is overexpressed, but there is no evidence that the endogenous protein follows the same dynamics [54]. It should be noted that the quality of the loading controls in some of these experiments is not optimal, and further work would be needed to confirm the statements. Moreover, cMyc overexpression in mouse embryonic fibroblasts has been reported to induce the upregulation of RingoA/Spy1 protein and mRNA [51]. However, other studies have not identified RingoA/Spy1 among the cMyc-regulated genes [55].

A recent study has reported that transgenic mice expressing MMTV-RingoA/Spy1 show enhanced hepatic cell proliferation and increased incidence of liver tumors, which led the authors to propose that RingoA/Spy1 may promote susceptibility to non-cirrhotic hepatocellular carcinoma [56]. Moreover, studies based on the use of a single siRNA concluded that RingoA/Spy1 knockdown reduces cell proliferation and increases the number of cells in G0/G1 phases in the ovarian cancer cell line SKOV-3 [48] and the leukemia cell line Jurkat [47].

RingoA/Spy1 has been also proposed to play a role in glioma. It has been reported that higher levels of RingoA/Spy1 (*SPDYA* gene) amplification in glioma samples correlated with reduced patient survival based on the evaluation of data from the Rembrandt NCI database [57]. The same study concluded that RingoA/Spy1 protein is overexpressed in some human glioma tumors, based on the analysis of a small number of samples. However, we have not been able to find correlations between *SPDYA* mRNA expression levels and the prognosis of

glioma patients using other tumor databases, nor evidence that RingoA/Spy1 is overexpressed in glioma tumors by immunohistochemistry analysis (unpublished observations). Experiments using human cancer cell lines in culture support a potential role of RingoA/Spy1 in glioma [57]. Thus, shRNA-mediated downregulation of RingoA/Spy1 was reported to reduce the proliferation of glioma cells. Moreover, RingoA/Spy1 mRNA expression was found enriched in $CD133^+$ cells and was proposed to regulate the stemness properties. RingoA/Spy1 overexpression was also shown to enhance clonality and viability of glioma cells, while reducing their capacity to differentiate, based on the qRT-PCR analysis of markers. Finally, RingoA/Spy1 knockout human brain cancer cells were reported to divide in a more asymmetric manner, therefore suggesting a role for RingoA/Spy1 in the self-renewal potential of brain tumor cells with stemness properties [57]. Differentiated neuroblastoma SH-SY5Y cells have been also reported to have decreased RingoA/Spy1 protein levels, and RingoA/Spy1 overexpression resulted in increased self-renewal in neurosphere assays and delayed cell differentiation [58]. Another study has proposed that RingoA/Spy1 associates with CLIPR-59, when both proteins were overexpressed in HEK-293-T cells, which might in turn reduce the association of CLIPR-59 with the de-ubiquitinating enzyme CYLD and affect the de-ubiquitination of RIP1 [59]. It is unclear how in this study RingoA/Spy1 co-localizes with CLIPR-59 in the cytoplasm, since previous work has shown that overexpressed RingoA/Spy1 localizes in the nucleus [9, 21].

6. Conclusions and perspectives

There is good evidence that the binding of RINGO/Speedy proteins suffices to increase the kinase activity of CDK1 and CDK2, as demonstrated by biochemical experiments using purified proteins. Importantly, recent structural studies have provided the molecular basis for the activation of CDK2 by RingoA/Spy1 interaction. Moreover, it has been demonstrated that T-loop phosphorylation of the CDK is not necessary for its activation by RINGO/Speedy proteins, and that RINGO/Speedy-activated CDKs are less sensitive to CKIs. Altogether, these observations indicate that CDK-RINGO/Speedy complexes can be active under conditions where CDK-cyclin complexes are likely to have low activity, and therefore can potentially perform different functions. In addition, XRINGO-activated CDKs can phosphorylate different sites in the kinase Myt1 than the same CDKs activated by cyclins. This can be mechanistically explained because RINGO/Speedy binds to a different region of Myt1 than cyclins and therefore both regulatory subunits position the CDK differently. Whereas these results provide a clear example of how CDKs can phosphorylate different sites

when activated by RINGO/Speedy or cyclins, we are not aware of any evidence that CDK-RINGO/ Speedy complexes can phosphorylate new substrates not targeted by CDK-cyclins.

At the physiological level, there is evidence that XRINGO regulates the meiotic maturation of *Xenopus* oocytes. In mammals, experiments using genetically modified mice have demonstrated that RingoA/Spy1 is essential for the prophase of meiosis I. Accordingly, RingoA/Spy1 knockout mice both males and females are sterile. However, these mice appear to be otherwise normal, which agrees with RingoA/Spy1 being mainly expressed in testis and embryonic ovary, with very low or absent expression in other tissues. Altogether the available data suggest that RingoA/Spy1 is crucial for meiosis in mammals but seems to be dispensable for the mitotic cell cycle in somatic tissues. Nevertheless, it remains to be clarified whether RingoA/Spy1 could play a role in particular cell types or situations in which the cell cycle might be differently regulated.

There are also several reports suggesting a role for RingoA/Spy1 in cancer, which usually link RingoA/Spy1 overexpression with enhanced tumorigenesis. The majority of these studies were performed in established cancer cell lines and, although some observations are potentially interesting, they are often based on overexpression experiments. In many cases, there is a lack of support from loss-of function experiments or evidence that the endogenous RingoA/Spy1 protein behaves in a similar way, and some of the data published is of borderline quality. Therefore, conclusions should be considered with caution.

In our experience, detection of the endogenous RingoA/Spy1 protein is technically challenging, and many studies that report RingoA/Spy1 expression in different tumor types, lack controls to prove the specificity of the antibodies used. Moreover, although several studies point to the overexpression of RingoA/Spy1 mRNA in tumors, this correlation is not detected in publicly available databases of human tumor samples, and RingoA/Spy1 seems to be frequently expressed at very low levels in tumors (L Gonzalez and A Nebreda, unpublished observations). Finally, there are no studies analyzing the effect of the endogenous RingoA/Spy1 protein depletion using genetic models, except for the two recently published papers showing that mice lacking this protein are sterile.

In summary, RINGO/Speedy proteins are atypical activators of CDK1 and CDK2, which play key roles in meiosis. Further work will be needed to establish their role in particular situations of somatic cells, for example in response to stress, and during tumorigenesis.

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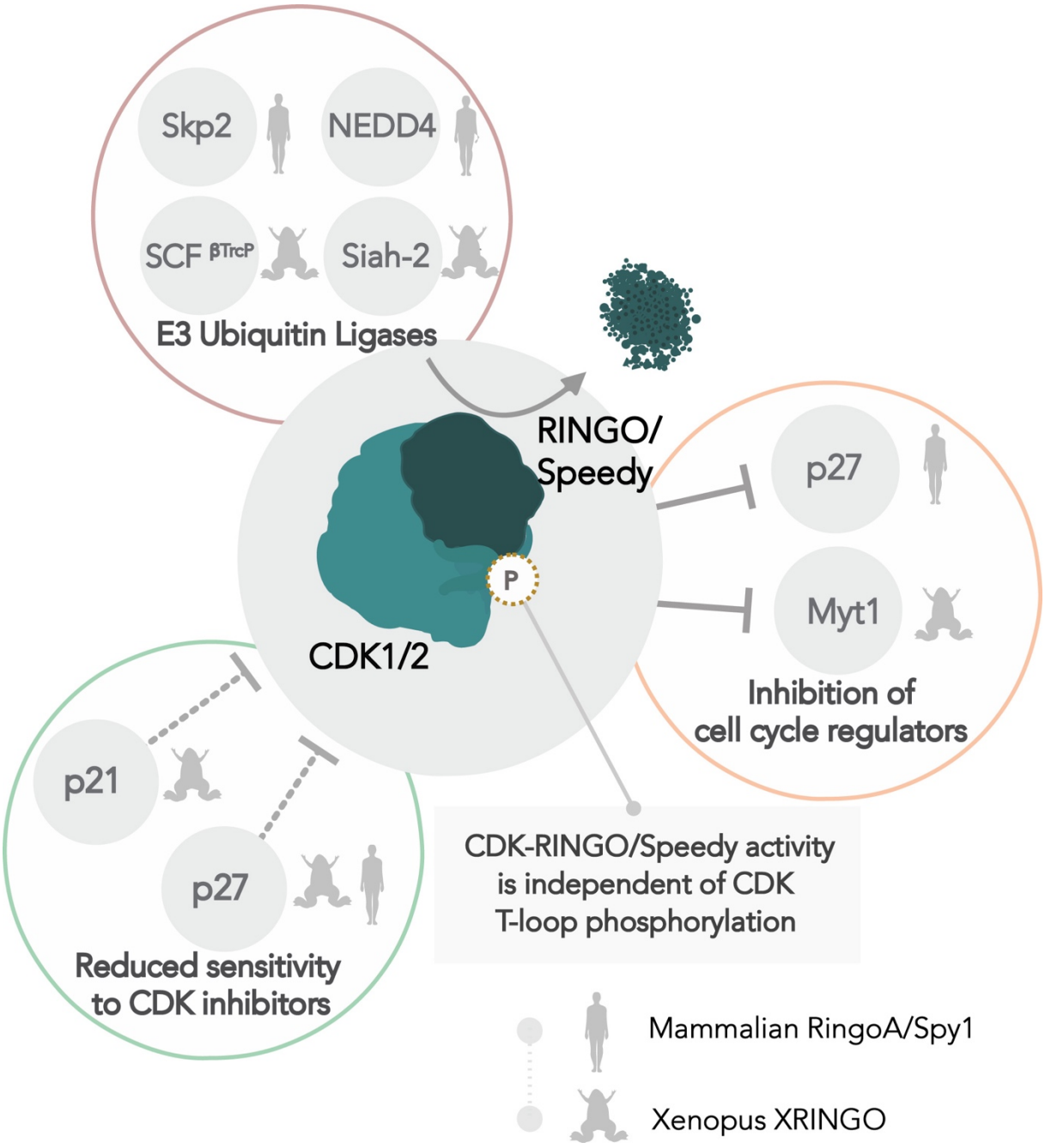

Fig. 1. Mechanisms that regulate the function of RINGO/Speedy proteins. Binding of RINGO/Speedy proteins to CDK1 and CDK2 suffices to induce activation of these kinases under conditions in which CDK-cyclin complexes would not be active. In particular, CDK-RINGO/Speedy complexes are equally active regardless of the CDK phosphorylation on the T-loop, are less affected by the $p21^{Cip1}$ and $p27^{Kip1}$ CDK inhibitors, and are able to phosphorylate and inhibit the CDK negative regulators $p27^{Kip1}$ and Myt1. The E3 ubiquitin ligases Skp2 and NEDD4 can interact with and induce the degradation of the overexpressed RingoA/Spy1, while $SCF^{\beta TrcP}$ and Siah-2 regulate XRINGO processing and degradation,

respectively. Gray symbols indicate whether the links have been made using mammalian RingoA/Spy1 or *Xenopus* XRINGO.

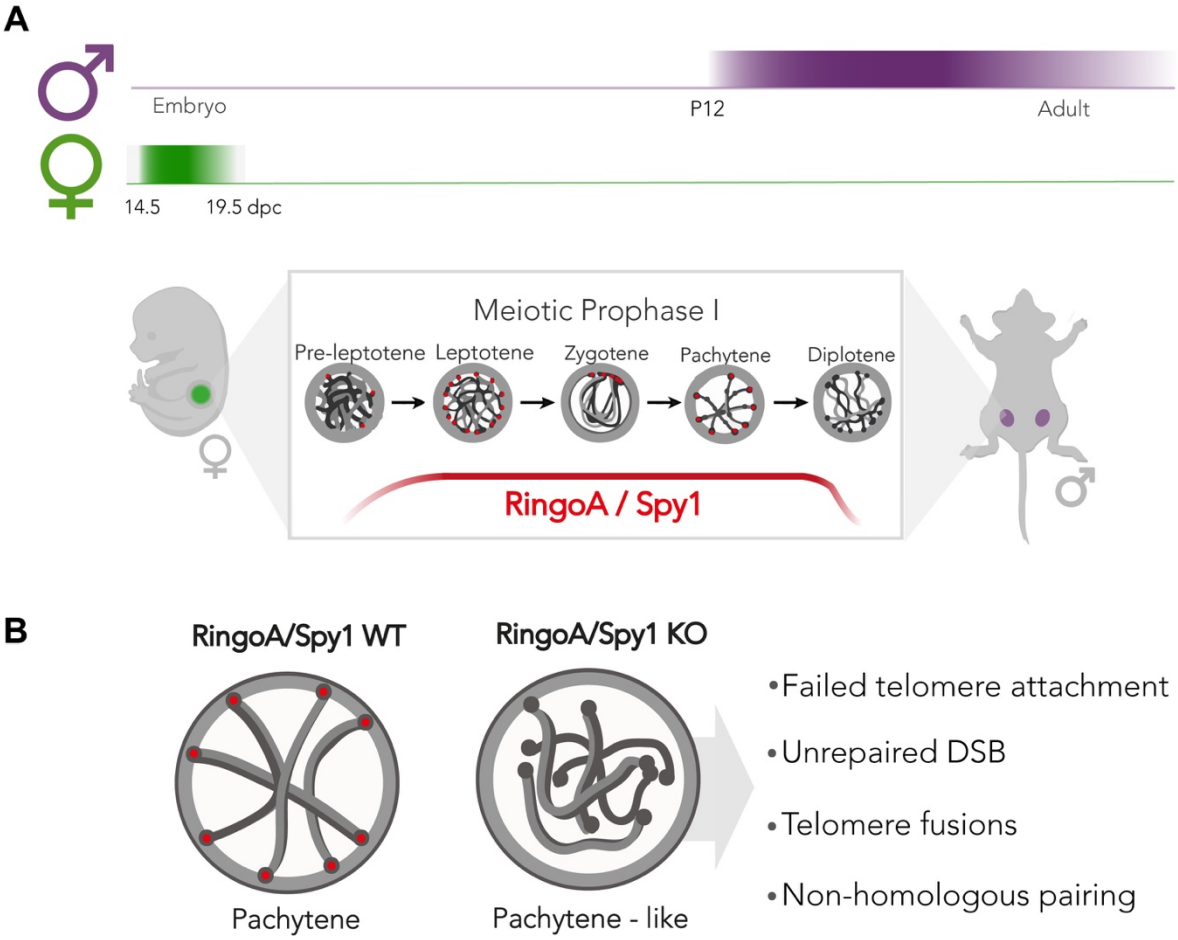

Fig. 2. RingoA/Spy1 is essential for meiotic prophase I. (A) RingoA/Spy1 is expressed in male gonads from postnatal day 12 (P12) onwards (purple) and in females mainly during embryonic stages, approximately from 14.5 to 19.5 days post-coitum (dpc) (green). Specifically, RingoA/Spy1 is highly expressed in prophase I of meiosis. During this stage, telomeres attach to the nuclear envelope at pre-leptotene, chromosome synapsis starts at zygotene and concludes at pachytene, when meiotic recombination occurs. RingoA/Spy1 expression starts at pre-leptotene and is not detectable as cells enter the diplotene stage (red line). (B) RingoA/Spy1 localizes at telomeres during prophase I of meiosis. RingoA/Spy1 knockout mice are sterile and their gametocytes arrest at a pachytene-like stage showing impaired tethering of telomeres to the nuclear envelope, unrepaired double-strand breaks (DSB), telomere fusions, and non-homologous chromosome pairing.