Molecular dissection of the APC/C inhibitor Rca1 shows a novel F-box-dependent function

Norman Zielke¹, Silvia Querings¹, Ruth Grosskortenhaus^{1,†}, Tânia Reis^{2,‡} & Frank Sprenger^{1,+}

¹Institute for Genetics, University of Cologne, Köln, Germany, and ²Fred Hutchinson Cancer Research Center, Seattle, Washington, USA

Rca1 (regulator of Cyclin A)/Emi (early mitotic inhibitor) proteins are essential inhibitors of the anaphase-promoting complex/cyclosome (APC/C). In *Drosophila*, Rca1 is required during G2 to prevent premature cyclin degradation by the Fizzy-related (Fzr)-dependent APC/C activity. Here, we present a structure and function analysis of Rca1 showing that a carboxy-terminal fragment is sufficient for APC/C inhibition. Rca1/Emi proteins contain a conserved F-box and interact with components of the Skp–Cullin–F-box (SCF) complex. So far, no function has been ascribed to this domain. We find that the F-box of Rca1 is dispensable for APC/C–Fzr inhibition during G2. Nevertheless, we show that Rca1 has an additional function at the G1–S transition, which requires the F-box. Overexpression of Rca1 accelerates the G1–S transition in an F-box-dependent manner. Conversely, S-phase entry is delayed in cells in which endogenous Rca1 is replaced by a transgene lacking the F-box. We propose that Rca1 acts as an F-box protein in an as yet uncharacterized SCF complex, which promotes S-phase entry.

Keywords: anaphase-promoting complex/cyclosome; *Drosophila*; F-box protein; G1–S transition; SCF complex

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INTRODUCTION

Tight regulation of anaphase-promoting complex/cyclosome (APC/C) activity is critical for progression through mitosis and establishment of G1. Activation of the APC/C depends on its phosphorylation state and the presence of the activator proteins Fizzy/cell division cycle 20 (Fzy/Cdc20) and Fizzy-related/CDC20 homologue 1 (Fzr/Cdh1; Vodermaier, 2004). Several other

molecules that regulate APC/C activity by using other mechanisms have been identified. Prominent members include the vertebrate Emi proteins, which restrict APC/C activity at different stages of the cell cycle (Schmidt *et al*, 2006). Emi1 was initially identified as an APC/C inhibitor specific for early mitotic stages, but further research has shown that Emi1 is also implicated in the G1–S transition (Reimann *et al*, 2001; Hsu *et al*, 2002). We have recently characterized the *rca1* (regulator of cyclin A) gene in *Drosophila*—an inhibitor of APC/C activity during G2 (Grosskortenhaus & Sprenger, 2002). Rca1 and the Emi proteins share several functional domains, including an F-box in their central region. F-box proteins are part of SCF (Skp–Cullin–F-box) ubiquitin ligases that mediate the degradation of numerous substrates. In such an SCF complex, F-box proteins are attached to the Cullin scaffold by an Skp protein and act as substrate recognition subunits (Vodermaier, 2004). Emi1 was identified in a screen for suppressor of kinetochore protein 1 (Skp1) interaction partners, in which deletion of the F-box prevents Skp1 binding *in vitro* (Reimann *et al*, 2001). Furthermore, a genome-wide yeast two-hybrid screen showed that Rca1 interacts with *Drosophila* SkpA and SkpB (Giot *et al*, 2003). These observations indicate that the Rca1/Emi proteins contain a functional F-box. However, several studies have shown that the F-box of the Emi proteins is dispensable for their inhibitory effect on the APC/C (Reimann *et al*, 2001; Schmidt *et al*, 2005). Thus, the *in vivo* function of the F-box in Emi/Rca1 remains unclear and it has to be explained whether they act in an SCF complex, targeting proteins for proteasomal degradation. Here, we show that the F-box of Rca1 is dispensable for APC/C inhibition in G2. However, we also identify a second F-box-dependent function of *rca1* in the transition from G1 to S phase.

RESULTS AND DISCUSSION

Rca1 shares several motifs with the Emi proteins (Fig 1A) including the F-box and the carboxy-terminal zinc-binding region (ZBR), which is important for APC/C inhibition (Reimann *et al*, 2001; Miller *et al*, 2006). To analyse the function of these motifs *in vivo*, we generated transgenic flies expressing different haemagglutinin (HA)-tagged deletion constructs with the upstream activation sequence (UAS)/Gal4 system (Rorth, 1998). Previously, we have shown that Rca1 is unstable during G1 (Grosskortenhaus

¹Institute for Genetics, University of Cologne, Zùlpicherstrasse 47, 50674 Köln, Germany

²Fred Hutchinson Cancer Research Center, 1100 Fairview Ave. N, PO Box 19024, Seattle, Washington 98109, USA

[†]Present address: Biotechnologisches Zentrum, Technische Universität Dresden, Tatzberg 47-51, 01307 Dresden, Germany

[‡]Present address: Department of Molecular and Cell Biology, UC Berkeley, 142 Life Science Addition, Berkeley, California 94720-3200, USA

⁺Corresponding author. Tel: +49 221 470 5259; Fax: +49 221 470 5264; E-mail: sprenger@uni-koeln.de

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Fig 1 | Rca1 restricts APC/C-Fzr activity during embryogenesis in an F-box-independent manner. **(A)** Schematic representation of the analysed Rca1 constructs. **(B)** To examine Rca1 stability, all constructs were expressed in alternating segments in otherwise wild-type (WT) embryos using *prd-Gal4* and stained for HA, Cyclin A (CycA) and DNA. In stage 11 embryos, most cells are in G2, indicated by high levels of CycA, and accumulate HA-Rca1. During stage 13, most epidermal cells are in G1 and lack CycA. Here, HA-Rca1 is unstable and fails to accumulate. HA-Rca1 $\Delta 203$ is degraded, similar to full-length Rca1, indicating that the amino terminus is not essential for Rca1 degradation. HA-Rca1 $\Delta 255$ was refractory to degradation in G1, suggesting that the region between 203 and 255 is involved in Rca1 turnover. **(C)** The F-box is not required for Rca1 function during embryogenesis. To monitor progression through mitosis, the number of epithelial cells in a given region (marked by the white box) of stage 13 embryos was determined. For quantification, cells were visualized with P-Tyr antibodies and DNA staining. The numbers of cells in segments A1 and A2 were counted after expression of the indicated Rca1 constructs in the A1 segment and compared with WT and *rca1*² mutants ($n \geq 10$). Both constructs lacking the F-box ($\Delta 203$ and Δ F-box) restore WT cell numbers in *paired* expressing segments. **(D)** The F-box of Rca1 is not essential for stabilization of CycA in *rca1*² embryos. HA-Rca1 $\Delta 203$ and HA-Rca1 Δ F-box were expressed in *rca1*² mutants using *paired-Gal4*. In both cases, premature degradation of CycA was prevented in segments expressing *paired*. APC/C, anaphase-promoting complex/cyclosome; Fzr, Fizzy-related protein; HA, haemagglutinin tag (YPYDVPDYA); NLS, nuclear localization signal; Rca1, regulator of Cyclin A; ZBR, zinc-binding region.

& Sprenger, 2002). We tested several constructs for their stability by expressing them in otherwise wild-type embryos. At stage 13 of embryogenesis, most epidermal cells are in G1 phase and fail to accumulate HA-Rca1 and Cyclin A (CycA; Fig 1B). HA-Rca1 $\Delta 203$ —which lacks most of the amino-terminal region—is also degraded in G1, showing that the N terminus is dispensable for Rca1 degradation. By contrast, further deletion up to amino acid 255 results in a stable protein, which is detectable even in epidermal cells that are in G1 phase (Fig 1B). This suggests that the region between amino acids 203 and 255 is involved in Rca1 destruction; however, so far, we have not identified a degron that is responsible for the instability during G1.

APC/C is F-box independent

The ability of several constructs to inhibit APC/C-Fzr activity was tested by expressing them in *rca1* mutant embryos. The *rca1* phenotype is characterized by premature degradation of mitotic cyclins in G2 of the 16th cell cycle and a subsequent failure to execute mitosis. Therefore, *rca1* mutant embryos have fewer cells than wild-type embryos. Expression of HA-Rca1 in *rca1* mutants can restore cell numbers to wild-type levels. Similarly, HA-Rca1 $\Delta 203$, which lacks the whole N terminus including the F-box and HA-Rca1 Δ F-box, was able to restore mitosis of cell cycle 16 in *rca1* mutant segments (Fig 1C). Further truncation up to residue 255 abolished Rca1 activity. In addition, mutation of a conserved

(Fig 2D,E). Finally, we found that the interaction with Cul1 is dependent on the F-box (supplementary Fig S1 online). These experiments show that Rca1 forms a complex with the core SCF components SkpA and Cul1.

S-phase induction by Rca1 is F-box dependent

The F-box in Rca1 is dispensable for the inhibitory effect on APC/C-Fzr, at least during embryogenesis. To identify instances that might require the F-box, we extended our analysis of Rca1 function to later developmental stages that undergo different cell-cycle modes. In eye imaginal discs, different cell-cycle stages are induced by the movement of the morphogenetic furrow (MF). Cells anterior to the MF are undifferentiated and proliferate asynchronously, whereas cells in the MF are synchronized in G1. Cells posterior to the MF fall into one subpopulation that stays in G1 and differentiates, whereas the remaining cells enter a terminal cell cycle called second mitotic wave (SMW; Thomas *et al*, 1994). A previous study showed that expression of Rca1 during eye development causes ectopic S phases and a rough eye phenotype (Dong *et al*, 1997). By contrast, a more recent study failed to show ectopic 5-bromodeoxyuridine (BrdU) incorporation after expression of Rca1 (Araki *et al*, 2003). In agreement with the first study, we observed ectopic BrdU labelling posterior to the SMW and a rough eye phenotype after overexpression of HA-Rca1 (Fig 3A–D). In addition, flow-cytometric analysis showed an increase of S-phase cells after Rca1 overexpression (supplementary Fig S2 online), indicating that Rca1 is able to induce ectopic

Fig 3 | Overexpression of Rca1 accelerates G1-S transition in an F-box-dependent manner. (A,C) Eye morphology and bromodeoxyuridine (BrdU) pattern in wild type (WT). (B,D) Overexpression of HA-Rca1 using GMR-Gal4 results in a rough eye phenotype and ectopic S phases in the posterior part of the eye disc visualized by BrdU incorporation. The brackets indicate the second mitotic wave and the arrow the morphogenetic furrow (MF). (E,F) Overexpression of HA-Rca1 $\Delta 203$ or HA-Rca1 $\Delta F\text{-box}$ using GMR-Gal4 does not affect eye morphology. (G) Expression of Cyclin A (CycA) in WT eye discs. (H) Flow-cytometric analysis of wing imaginal disc cells indicates that Rca1 overexpression accelerates the G1-S transition. Cells overexpressing HA-Rca1 (green) show a reduced number of cells in G1 and a concomitant increase in the S/G2 population compared with WT cells (black). Cell size measured by forward scattering (FSC) is not affected. Overexpression of HA-Rca1 $\Delta 203$ or HA-Rca1 $\Delta F\text{-box}$ (green) does not affect the cell-cycle profile. (I) Overexpression of HA-Rca1 (clones are marked by green fluorescent protein (GFP)) results in ectopic CycA staining in the MF. (J) Overexpression of HA-Rca1 $\Delta 203$ does not stabilize CycA in the MF. HA staining shows that HA-Rca1 $\Delta 203$ is unstable in the MF. (K,L) HA-Fizzy-related (Fzr) is readily detectable in clones coexpressing Rca1 and HA-Fzr, suggesting that Fzr is not a target of the putative Skp-Cullin-Rca1 complex. (M-P) Rca1 overexpression can induce S phase independent of Cyclin E/Cyclin-dependent kinase 2. (N) HA-Rca1 overexpression induces ectopic S phases in the MF. (O) Overexpression of *dacapo* (dap) blocks S-phase entry in the second mitotic wave, indicated by the absence of BrdU incorporation in the clone area. (P) Coexpression of Dap and HA-Rca1 does not affect the ability of Rca1 to induce ectopic S phases. (Q) Levels of Dap are not affected by HA-Rca1 coexpression. GMR, promoter element, named after Gerry M. Rubin, UC-Berkeley, CA, USA; HA, haemagglutinin tag (YPYDVPDYA); Skp, suppressor of kinetochore protein.

G1–S transitions in eye imaginal discs. To determine whether Rca1 accelerates G1–S transitions in other imaginal discs as well, we generated clones overexpressing HA–Rca1 in wing imaginal discs. Flow-cytometric analysis of these cells showed a significant decrease of the G1 population, indicating that cells overexpressing Rca1 progress faster through G1. Interestingly, we observed a simultaneous increase in the fraction of S/G2 cells (Fig 3H). This might be due to a recently discovered compensatory mechanism that keeps the overall cell-cycle duration constant (Reis & Edgar, 2004). Forward scattering (FSC) showed that Rca1-overexpressing cells do not differ significantly in cell size, supporting the idea that they undergo cell-cycle length compensation (Fig 3H). These results show that excess Rca1 protein can change the cell-cycle profile by accelerating the transition from G1 to S phase.

Cells in the MF reside in G1, and mitotic cyclins are down-regulated by the APC/C–Fzr complex (Pimentel & Venkatesh, 2005; Fig 3G). Overexpression of Rca1 in the MF results in high levels of CycA (Fig 3I) and Cyclin B (CycB; supplementary Fig S3 online), indicating downregulation of APC/C activity. This could be the direct cause of the inhibitory activity of Rca1. However, both constructs lacking the F-box ($\Delta 203$ and Δ F-box) failed to induce the rough eye phenotype and clones in the MF do not accumulate CycA (Fig 3J; data not shown). Overexpression of these proteins had no effect on the cell-cycle profile in imaginal discs (Fig 3H; supplementary Fig S2 online). Thus, S-phase induction by excess Rca1 relies on an F-box-dependent mechanism.

Rca1 is unstable in G1 cells and, similar to our analysis of Rca1 turnover in the embryo, we found that HA–Rca1 $\Delta 203$ was degraded in the MF and reaccumulated once cells entered S phase (Fig 3J). Interestingly, full-length HA–Rca1 that is subjected to degradation in G1 can accumulate in cell clones in the MF (Fig 3I). However, these cells have been shifted from G1 to S phase, in which Rca1 degradation is switched off. Constructs that fail to induce S phase remain in G1 and become degraded. In conclusion, Rca1 is able to induce S phase in imaginal discs, a feature that requires the F-box even though this motif is dispensable for APC/C inhibition.

F-box proteins are part of SCF complexes that initiate the degradation of target proteins. The F-box-dependent S-phase induction by Rca1 indicates that SCF/Rca1 might mediate degradation of a negative regulator of S-phase entry. The APC/C–Fzr complex is important for the establishment of G1 phase; therefore, an explanation for S-phase induction could be that Rca1 mediates the degradation of Fzr. However, we did not observe any change in HA–Fzr levels on coexpression of Rca1 (Fig 3K,L), which suggests that Fzr is not a target of the Rca1-containing SCF complex. Interestingly, coexpression of Rca1 and HA–Fzr prevents ectopic S-phase induction by Rca1, possibly by a titrating effect of Fzr on Rca1 (supplementary Fig S4 online).

The G1–S transition in *Drosophila* eye discs is normally mediated by Cyclin E (CycE)/Cdk2 (Richardson *et al*, 1995). To examine whether Rca1 relies on CycE to drive S phase, we coexpressed Rca1 with the CycE inhibitor *dacapo* (dap), which can block CycE-dependent S-phase induction (de Nooij *et al*, 1996; Fig 3O). Interestingly, Dap overexpression could not prevent induction of ectopic S phase by Rca1 (Fig 3P). Furthermore, dap levels were not influenced by Rca1 overexpression, indicating that Dap is not a substrate of the SCF–Rca1

complex (Fig 3Q). In summary, these experiments show that Rca1 is able to induce ectopic S phases in an F-box-dependent manner and does not rely on CycE/Cdk2 activity.

Rca1 lacking the F-box fails to maintain proliferation

We were interested in whether the F-box-dependent function of Rca1 is required for proliferation in general. Therefore, we generated clones that were mutant for *rca1* and tested whether Rca1 lacking the F-box could substitute for endogenous Rca1. Cells in *rca1* clones have a severe proliferation disadvantage compared with wild-type cells (Grosskortenhaus & Sprenger, 2002; Fig 4F; supplementary Fig S5 online). This proliferation disadvantage can be rescued by HA–Rca1, evidenced by clone sizes similar to wild type (Fig 4F; supplementary Fig S5 online). Remarkably, both constructs lacking the F-box ($\Delta 203$ and Δ F-box) did not completely restore the proliferation potential of *rca1* mutant cells. The average clone size was only one-third of wild-type levels (Fig 4F; supplementary Fig S5 online), indicating that these cells undergo limited divisions. Thus, constructs lacking the F-box can only partially compensate for the loss of Rca1.

In *rca1* clones, we observed reduced CycA levels, in agreement with the function of Rca1 as an APC/C–Fzr inhibitor. Additionally, these cells showed increased DNA levels due to endoreplication (Grosskortenhaus & Sprenger, 2002; Fig 4G). In *rca1* mutant clones that express HA–Rca1, we found normal DNA content and wild-type levels of CycA (Fig 4H). By contrast, *rca1* mutant clones that express HA–Rca1 $\Delta 203$ contained significantly reduced levels of CycA and normal DNA content (Fig 4I). In rare cases, we also observed cells with higher DNA content in these clones (Fig 4J). In addition, HA–Rca1 $\Delta 203$ was absent in large regions of the clone. Considering that HA–Rca1 $\Delta 203$ and CycA are unstable in G1, this suggests that these cells persist longer in G1. These findings show that Rca1 lacking the F-box cannot completely replace endogenous Rca1 in proliferating cells.

In conclusion, our results show that Rca1 can inhibit APC/C–Fzr activity during G2 even in the absence of the F-box. However, the F-box is essential for the G1–S transition in proliferating cells and induction of ectopic S phases by Rca1. As Rca1 can interact with core SCF components, we propose that Rca1 is part of an SCF complex involved in G1–S transition.

METHODS

Fly stocks. The stocks *rca1*²/CyO(wg-lacZ); MKRS/TM6B, *rca1*²/CyO(wg-lacZ); *prd*-Gal4/TM6B, Frt40A, *rca1*²/CyO(wg-lacZ); and UAS-dap(II.3) were described previously (Lane *et al*, 1996; Grosskortenhaus & Sprenger, 2002). Stocks used for clonal analysis were kindly provided by Thomas Klein (University of Cologne, Germany): w,hs-Flp(1.22); AyGal4(25),UAS-GFP/SM6-TM6 and w,hs-Flp(1.22),tub-Gal4,UAS-GFP/FM7; Frt40A,Gal80/CyO. Clones were induced 40–48 h after egg deposition by heat shock at 37 °C: 10 min for Flp-out experiments and 1 h for MARCM (mosaic analysis with a repressible cell marker) experiments. Imaginal discs were analysed 72 h after clone induction.

Antibody staining. Embryos were collected overnight at 18 °C, fixed for 20 min at 37 °C in 4% formaldehyde/PBS and stored in methanol at –20 °C. Imaginal discs from wandering larvae were dissected in PBS and fixed for 30 min at 24 °C in 4% paraformaldehyde/PBS. Primary antibodies were used in the following dilutions: rat-HA (1:100–1,000; Roche, Basel,

Fig 4 | Rca1 lacking the F-box fails to restore the proliferation disadvantage of *rca1*² mutant cells. *rca1*² mutant clones (positively marked by green fluorescent protein (GFP)) were generated by the MARCM technique, which allows the simultaneous overexpression of upstream activation sequence constructs with *tubulin-Gal4* (A–F). The proliferation rate was determined by counting cells per clone in the ventral part of wing imaginal discs ($n=25$). (A) Control (GFP-only) clones. (B) *rca1*² mutant clones have a severe proliferation disadvantage. (C) Expression of HA-Rca1 in *rca1*² mutant clones suppresses the proliferation disadvantage indicated by clone sizes similar to wild type (WT). (D,E) Expression of HA-Rca1Δ203 or HA-Rca1ΔF-box rescues the proliferation defect of *rca1*² mutant cells only partially. (G–J) Areas of wing imaginal discs containing MARCM clones of the indicated genotypes. Discs were stained for Cyclin A (CycA), DNA and HA. (G) *rca1*² mutant clones fail to maintain CycA levels and undergo endocycles indicated by increased nuclear size. (H) HA-Rca1 expression can suppress the premature degradation of CycA and allows normal proliferation. (I) In most *rca1*² mutant clones that express HA-Rca1Δ203, reduced CycA levels are visible and HA-Rca1Δ203 itself is unstable. This suggests that these cells persist in a G1 state. Occasionally, these clones contain cells with increased DNA level (J). HA, haemagglutinin tag (YPYDVPDYA); MARCM, mosaic analysis with a repressible cell marker.

Switzerland), rabbit-CycA (1:200), rabbit-CycB (1:1,000; J. Raff, University of Cambridge, UK), mouse-Dap (1:4; I. Hariharan, UC-Berkeley, CA, USA), mouse-pTyr (1:10; D. Morrison, National Cancer Institute, Frederick, MD, USA), rabbit-βGal (1:500; Cappel, Illkirch, France), rabbit-green fluorescent protein (1:500; Torrey Pines Biolabs, Houston, TX, USA) and mouse-BrdU (1:20; Becton Dickinson, Heidelberg, Germany). Secondary antibodies, purchased from Invitrogen (Karlsruhe, Germany), were used at a dilution of 1:500. DNA was visualized with propidium iodide or Hoechst. For BrdU labelling, third instar larvae were fed for 2.5 h with yeast supplemented with BrdU (1 mg/ml; Sigma, Munich, Germany) and processed according to Baker & Yu (2001). Images were acquired on a Zeiss (Göttingen, Germany) Axioplan Imaging2e fluorescent microscope or on a Leica (Bensheim, Germany) TCS-SP2. Adobe Photoshop and ACD-Canvas X software were used for image assembly.

Flow cytometry. Flow cytometry was carried out according to Reis & Edgar (2004). Clones were induced 40–48 h after egg

deposition by heat shock (30 min at 37 °C). Discs were analysed 72 h after clone induction.

Cell culture, immunoprecipitation and western blot analysis. *Drosophila* S2 cells were grown at 27 °C in Schneider's medium (Gibco BRL) supplemented with 5% FCS (Sigma). Transfections were carried out according to Battersby *et al* (2003). Immunoprecipitations were performed as described by Wilson *et al* (2004), by using mouse-HA antibody (Roche). Samples were separated by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Blotted membranes were blocked in blocking buffer for fluorescent western blotting (Rockland, Gilbertsville, PA, USA). Primary antibodies used were as follows: rat-HA (1:2,500; Roche), mouse-FlagM2 (1:1,500; Sigma), rabbit-cMyc (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit-Cul1 (1:250; Zymed, Karlsruhe, Germany). Proteins were detected with secondary antibodies (1:3,000; Rockland) for the Odyssey Infrared Imaging system (Li-Cor, Hamburg, Germany).

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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