A novel motif governs APC-dependent degradation of *Drosophila* ORC1 in vivo

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Regulated degradation plays a key role in setting the level of many factors that govern cell cycle progression. In *Drosophila*, the largest subunit of the origin recognition complex protein 1 (ORC1) is degraded at the end of M phase and throughout much of G1 by anaphase-promoting complexes (APC) activated by Fzr/Cdh1. We show here that none of the previously identified APC motifs targets ORC1 for degradation. Instead, a novel sequence, the O-box, is necessary and sufficient to direct Fzr/Cdh1-dependent polyubiquitylation in vitro and degradation in vivo. The O-box is similar to but distinct from the well characterized D-box. Finally, we show that O-box motifs in two other proteins, *Drosophila* Abnormal Spindle and *Schizosaccharomyces pombe* Cut2, contribute to Cdh1-dependent polyubiquitylation in vitro, suggesting that the O-box may mediate degradation of a variety of cell cycle factors.

[**Keywords:** APC; cell cycle; ORC1; proteolysis; replication]

Received August 5, 2005; revised version accepted August 16, 2005.

Progression through the cell cycle relies critically on the scheduled degradation of an ensemble of proteins. Misregulation of this degradation can cause developmental defects, cell death, and cancer. It is thus of great interest to understand the molecular mechanisms governing the degradation of cell cycle regulators.

Cell cycle-dependent degradation of proteins is carried out by the proteasome. Substrate proteins are marked by E3 ubiquitin (Ub) ligases, which catalyze the covalent attachment of poly-(Ub) chains, the signal for degradation by proteasomes (for review, see Ciechanover 1994; Hochstrasser 1996). Precise control of E3 activity achieves the temporally and spatially regulated protein degradation.

One of the key E3s in cell cycle regulation is the anaphase-promoting complex/Cyclosome (APC). The APC regulates progression through and exit from mitosis by sequentially degrading first the A- and B-type cyclins and subsequently securin/Pds1 (as well as other proteins; for review, see Zachariae and Nasmyth 1999; Harper et al. 2002; Peters 2002). During M and G1, two different factors sequentially activate the APC: Fizzy(Fzy)/Cdc20 and Fizzy-related(Fzr)/Cdh1. Each activator confers a different substrate specificity to the APC; thus, the timing of substrate degradation is determined in part by whether Fzy/Cdc20 or Fzr/Cdh1 is involved. To date, four different APC targeting motifs in substrates have been identified: the D-box (Glutzer et al. 1991), KEN-box (Pfleger

and Kirschner 2000), A-box (Littlepage and Ruderman 2002), and GxEN-box (Castro et al. 2003). The mechanism by which each mediates interaction with the APC activator complex is not yet clear, although recent experiments show that the D-box binds directly to Cdh1, Cdc20, and also to the APC itself (Yamano et al. 2004; Burton et al. 2005; Kraft et al. 2005).

In addition to directing the degradation of M-phase proteins, the APC governs the degradation of DNA replication factors. To date, *Drosophila* origin recognition complex protein 1 (ORC1) (Araki et al. 2003), mammalian Cdc6 (Petersen et al. 2000), *Xenopus* Geminin (McGarry and Kirschner 1998), and *Saccharomyces cerevisiae* DBF4 (Ferreira et al. 2000) have been shown to be degraded by the APC. Each of these proteins is thought to play a key role in either formation of the prereplication complex (pre-RC) or subsequent events at the replication origin (for review, see Bell 2002; Bell and Dutta 2002; DePamphilis 2003; Kearsey and Cotterill 2003; Diffley 2004; Stillman 2005). APC-mediated degradation thus may play a significant role in the disassembly of origin-bound complexes and in the resetting of origins after initiation.

We previously showed that *Drosophila* ORC1 is degraded by APC that is activated by Fzr/Cdh1 (Araki et al. 2003). The *cis*-acting signal mediating degradation was mapped to the N-terminal 555 residues of ORC1. This portion of the protein contains several previously identified targeting motifs that we assumed were responsible for its degradation. In the present work, we show that none of these sequences mediates destruction of ORC1. Instead, the critical destabilizing sequences map to a novel motif, the O-box (ORC1-destruction box),

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Article published online ahead of print. Article and publication date are at <http://www.genesdev.org/cgi/doi/10.1101/gad.1361905>.

which is essential for ubiquitylation in vitro and degradation in vivo.

Results

No role for previously identified APC-targeting motifs in ORC1 degradation

The signal that mediates Fzr/Cdh1-dependent ubiquitylation and degradation of ORC1 resides between amino acids 1 and 555. Near the N terminus of this domain is a KEN-box, which is a Fzr/Cdh1-specific signal in other proteins and therefore an attractive candidate for the ORC1 destruction motif. To determine whether it mediates degradation in vivo, we mutated the KEN-box (mkb), prepared a transgene encoding a mutant fragment of ORC1 fused to GFP, and expressed the gene in eye-imaginal disc cells under the control of the *GMR* promoter, which is constitutively active in all cells behind the morphogenetic furrow.

As shown in Figure 1C, mutation of the KEN-box has essentially no effect on the stability of the ORC1-GFP fusion, which accumulates in a stripe of S-, G2-, and M-phase cells immediately posterior to the morphogenetic furrow, but is otherwise degraded in the majority of

the cells which are in G1/G0 (Fig. 1B). Mutation of the KEN-box also has almost no effect on ubiquitylation of an N-terminal ORC1 fragment in vitro (Fig. 1D), consistent with the idea that the KEN-box plays no role in its degradation.

The degradation signal was next localized to residues 242–556, which contain three canonical D-box motifs (Fig. 1A). A priori, it seemed unlikely these were responsible for targeting ORC1 in vivo, since D-boxes mediate both Fzr/Cdc20- and Fzr/Cdh1-dependent degradation whereas ORC1 is specifically targeted by Fzr/Cdh1. However, to test their potential role, we mutated the three D-box motifs and expressed the mutant protein in the eye disc; as shown in Figure 1C, the D-boxes play no apparent role in ORC1 degradation. Consistent with this observation, an ORC1 fragment bearing ablated D-boxes is polyubiquitylated normally in vitro (Fig. 1D). To rule out possibilities that the ORC1 fragments might present the KEN- or D-motifs in an inappropriate context or that these motifs act redundantly, we prepared a transgene encoding a derivative of full-length ORC1 (fused to GFP) in which the KEN-box and all three D-boxes are ablated. As shown in Figure 1C, the quadruply mutant ORC1 protein is still cell cycle-regulated in the eye imaginal disc. As the N-terminal 554 residues of ORC1 do not

Figure 1. None of the previously identified APC-targeting motifs is responsible for ORC1 degradation and ubiquitylation. (A) Drawing of the deletion derivatives analyzed in C and D, substitutions denoted by red asterisks. In the mutant KEN-box (mkb) protein KEN at residues 4–6 is replaced by AAA. In the triply mutant D-box (3× mdb) protein, the conserved amino acids of D-boxes at residues 313–321, 388–395, and 539–546 (RxxL-xxxxD, RxxLxxxxN, and RxxLxxxxD, respectively) are substituted with alanine. (B) As the morphogenetic furrow (MF, marked with an arrowhead) sweeps from posterior (P) to anterior (A), most eye disc cells first undergo a synchronous cell cycle transition and then enter a prolonged G1/G0 phase. (C) Expression of various proteins under control of the *GMR* promoter, which is active in all cells posterior to the MF (arrowhead) in the eye disc. In discs where the GFP fusion is degraded following M phase (i.e., ORC1), a minor population of cells scattered throughout the posterior retains GFP because they arrest in G2 where the APC is inactive (Araki et al. 2003). High-magnification views are inset. (D) In vitro APC^{Fzr/Cdh1}-dependent ubiquitylation assay with purified enzymes for D- and KEN-box mutant derivatives of ORC1 (unreacted input in the left lane of each pair). Arrowheads indicate ³⁵S-labeled substrates.

contain either of the other characterized destruction motifs (e.g., GxEN- or A-box), we infer the existence of another APC-targeting signal.

The O-box, a novel *Fzr/Cdh1*-targeting motif

To identify the ORC1 destruction motif, we prepared a series of deletion derivatives (Fig. 2A) and used these as substrates for ubiquitylation *in vitro* (Fig. 2B). The efficiency of substrate utilization was monitored by estimating the mean molecular weight of the (heterogeneous) high-molecular-weight reaction products and by measuring the residual unreacted substrate. These experiments suggest the degradation signal is between amino acids 245 and 307. The critical interval was further narrowed to residues 283–303 using a second series of deletion derivatives (Fig. 2C).

To better define the degradation signal, we next performed alanine-scanning mutagenesis, separately mutating each residue from P283 to E303. The ubiquitylation of each mutant was then tested *in vitro* (Fig. 2D). Substitution at two residues, L295 and N299, eliminated high-order polyubiquitylation. In addition, substitution at nearby residues P291, S293, P294, E297, and K301 significantly decreased the formation of high-molecular-weight products. Based on these results we determined that the targeting signal is PASPLTEKN^{AK} (essential residues underlined), and named this element the ORC1-destruction box (O-box).

The O-box governs cell cycle-dependent ORC1 degradation in a variety of cell types *in vivo*

We next wished to determine whether the O-box is responsible for the cell cycle-dependent degradation of ORC1 *in vivo*. Both wild-type ORC1 and a mutant version bearing alanine substitutions in the two critical O-box residues (L295 and N299) were expressed as GFP fusion in eye-imaginal discs under *GMR* promoter con-

trol. As shown previously (Araki et al. 2003), wild-type ORC1 is degraded and undetectable in most of the G1-phase cells (Fig. 3A). In contrast, the ORC1 derivative with a mutant O-box is stable, accumulating in essentially every cell to the same level. We confirmed this result by dissociating the eye-imaginal discs into single cells and subjecting these to analysis by fluorescence-activated cell sorting (FACS). As shown in Figure 3B, the ORC1 derivative bearing a mutant O-box accumulates in G1/G0 cells, unlike wild-type ORC1, which is stable only in S- and G2-phase cells when *Fzr* is inactive. Thus, the O-box mediates cell cycle-dependent degradation of full-length ORC1 in eye imaginal disc cells.

To test whether O-box-dependent degradation might be a peculiarity of eye disc cells, we drove expression of GFP fusions to both wild-type ORC1 and the O-box mutant derivative in the posterior compartment of wing discs using the *engrailed-GAL4* driver and in epidermal cells of the embryo using the *actin5C-GAL4* driver. In both cell types, wild-type ORC1–GFP accumulates in G2 cells and is destabilized in G1 cells; in contrast, the ORC1–GFP fusion with an ablated O-box is stable in both G1 and G2 cells (Fig. 3D). Thus, the O-box appears to govern cell cycle-dependent degradation of ORC1 in a variety of cell types.

The O-box is sufficient to confer *Fzr/Cdh1*-specific polyubiquitylation

Having demonstrated that the O-box is necessary for ubiquitylation of ORC1, we wished to determine whether it is sufficient to direct modification of a naive substrate. To address this question, we swapped the O-box with another APC-targeting motif—the D-box of human Cyclin B (CycB).

Degradation of mammalian CycB is mediated by a canonical D-box (RxxLxxxxN) (Brandeis and Hunt 1996). We first showed that polyubiquitylation of an N-terminal fragment of human CycB is dependent on the integ-

Figure 2. ORC1 residues 283–303 are essential for *Fzr/Cdh1*-dependent polyubiquitylation.

(A) Drawing of the deletion derivatives analyzed in B and C. (B–D) *In vitro* APC^{*Fzr/Cdh1*}-dependent ubiquitylation assays with purified enzymes for a series of ORC1 fragments. Each reaction was analyzed on a gel appropriate for the molecular weight of the input substrate (left lane in each pair). Note that ORC1^{1–245} is oligo-ubiquitylated *in vitro*, but this level of reactivity is not sufficient to destabilize the protein *in vivo* (Fig. 1C), consistent with a previous report that polyubiquitylation is necessary for efficient degradation (Thrower et al. 2000). (D) Analysis of a series of single-alanine-substituted derivatives of ORC1^{1–397}, as described for B and C above. The two critical residues are boxed, and other residues at which substitution more subtly affects ubiquitylation are in a larger font. The figure was assembled from the results of two experiments, as indicated.

Figure 3. Substitutions in the O-box abolish cell cycle-dependent ORC1-degradation in vivo. (A) Eye-imaginal discs expressing GFP, GFP-tagged full-length wild-type ORC1, and GFP-tagged ORC1 bearing a mutant O-box (*mob*) (alanine substitutions at L295 and N299). (B,C) Eye-imaginal and wing disc cells were dissociated from transgenic animals and analyzed by FACS. Distributions of GFP-positive cells are shown in green, and all cells are shown in black. Note that the proportion of G1 cells in eye disc samples is slightly higher than in wing disc samples, because the *GMR* promoter is active in a region of the eye disc where many cells have exited the cell cycle in G1/G0 and under terminal differentiation, whereas the *en* promoter used to drive expression in wing discs is active in proliferating cells. (D) Confocal images of epithelial cells of stage 12–13 embryos in which transcription of either wild-type or mutant ORC1–GFP fusion proteins is driven ubiquitously by *actin5C-GAL4*. Most of these cells are in G1; only a minority that is marked by high levels of Cyclin B is still in S or G2.

ity of this D-box, which resides at residues 42–50. As shown in Figure 4A, mutation of the highly conserved residues of the D-box essentially abolishes ubiquitylation by both Fzy/Cdc20- and Fzr/Cdh1-activated APC. We next prepared derivatives in which the D-box is replaced with either a wild-type O-box or, as a control, a mutant O-box. When tested in vitro, the O-box bearing protein is polyubiquitylated, but only when the APC is stimulated with Fzr/Cdh1; the derivative with a mutant O-box is inert toward both Fzy/Cdc20 and Fzr/Cdh1. ORC1 responds robustly to O-box-dependent ubiquitylation, whereas CycB is considerably less reactive. Although we have not systematically investigated the source of this apparent difference, similar context-dependent activity has been described for the D-box (Zur and Brandeis 2002). Nevertheless, the major conclusion from these experiments is that the O-box is a portable motif that directs modification specifically by the Fzr/Cdh1-activated APC.

We also performed a reciprocal swap, substituting the human CycB D-box for the O-box in *Drosophila* ORC1. The ORC1 derivative bearing a wild-type D-box is ubiquitylated in the presence of both Fzy/Cdc20 and Fzr/Cdh1 (Fig. 4B). Thus, the O- and D-boxes are functionally interchangeable—each determines APC activator specificity and acts as an autonomous signal to direct ubiquitylation.

Despite sequence similarities, O- and D-boxes are distinct

Both D- and O-box motifs are information-poor, with only three and two amino acids rigidly specified, respectively. Based on current definitions, the two motifs are

superficially similar, each bearing critical L and N residues separated by four and three residues: RtaLgdiGn (D-box) versus paspLtekNak (O-box). Given these similarities, we wished to determine whether the O-box is a D-box relative or a distinct APC-targeting signal. To this end, we constructed plasmids that encode substrates with chimeric D- and O-boxes, interchanging the R and A residues at –3 (with respect to the L common to both motifs) and the interstitial GDIG and TEK residues between the critical L and N residues.

As shown in Figure 5A, Cdh1-dependent ubiquitylation driven by an O-box is only slightly enhanced by swapping the –3 residue, and is unaffected by swapping the L–N spacer. In contrast, Cdh1-dependent ubiquitylation driven by a D-box is significantly impaired by either of the reciprocal substitutions: Swapping the –3 residue or the L–N spacer significantly reduces reactivity, and introduction of both changes reduces polyubiquitylation below the level achieved by the native O-box (Figs. 4A, 5B). Presumably other features of the native O-box contribute to its activity, consistent with the alanine-scan analysis of Figure 2D. By these criteria, the O- and D-boxes appear to be quite different.

We also examined the activities of all chimeras in reactions with Cdc20-activated APC. The main conclusion from these experiments is that the R at –3 is a critical determinant both for efficient reaction with Cdc20 and for specifying a D-like identity. Swapping the R for the A at –3 of the O-box significantly enhances the Cdc20-driven reaction. The resulting chimera has the same core RxxL motif as the D-box; substrates with either this chimera or the native D-box are similarly sensitive to the length of the L–N spacer (Fig. 5A,B). In the reciprocal experiment, substitution of the R at –3 of the D-box reduces reactivity to a very low level.

Figure 4. The O-box is a portable motif for APC^{Fzr/Cdh1}-directed ubiquitylation. (A) APC-dependent ubiquitylation in vitro of a fragment of human Cyclin B, with the sequence of its endogenous wild-type D-box (wtodb) highlighted. This sequence was substituted as shown *above* each set of reactions to install a mutant D-box (mdb), and either wild-type or mutant O-boxes (wtob and mob, respectively). (B) APC-dependent ubiquitylation in vitro of fragments of *Drosophila* ORC1 bearing either the endogenous O-box or the indicated substitutions.

In summary, both the presence of an R at -3 and the difference in the length of the L-N spacer distinguish the D- and O-boxes. Activity of the O-box is insensitive to the identity of the residue at -3 and to the length of the spacer, whereas activity of the D-box is acutely sensitive to both features. Most of these chimeric motifs were assayed in both the human CycB (residues 1–106) substrate and the *Drosophila* ORC1 (residues 1–554) substrate. The relative effect of each substitution is similar for both substrates (data not shown), which refutes the idea that the O-box is a degenerate D-box that functions efficiently only due to the influence of flanking ORC1 sequences. We conclude that the D- and O-boxes are distinct.

We next wished to determine whether the O-box competes with the D-box, either directly for binding to Cdh1 (or the APC) or perhaps at a downstream step in the polyubiquitylation reaction. To address this question, we performed peptide competition experiments, using a short D-box peptide that has been shown to compete effectively in APC-dependent ubiquitylation assays (Yamano et al. 1998; Kraft et al. 2005). We prepared three substrates in which a single motif directs polyubiquitylation: Fragments of human CycB and *Drosophila* ORC1 described above (bearing the native D- and O-boxes, respectively), and an otherwise identical fragment of ORC1 in which a KEN-box is substituted for the

O-box. Each substrate was incubated with various concentrations of either wild-type or mutant D-box peptide. The wild-type D-peptide inhibits Cdh1-dependent ubiquitylation of all three substrates to a similar extent at various concentrations, only one of which is shown in Figure 5C; at the same concentration, a mutant D-peptide does not detectably inhibit reaction with any of the substrates. We do not know whether the observed inhibition occurs at the level of binding to Cdh1, binding to the APC, or at a subsequent step in the reaction. However, the main conclusion is that the D- and O-box motifs direct utilization of common downstream components and thus are functionally homologous.

Figure 5. The O- and D-boxes are distinct motifs. In vitro ubiquitylation assays with *Drosophila* ORC1^{1–554} (A) and human CycB^{1–106}-6× myc (B) derivatives. Derivatives with the endogenous degradation signal flanking various substitutions are shown. The L and N residues that are critical features of both the O- and D-box are underlined, and altered amino acids are shown in a larger font. As in Figure 4, each trio of lanes is from unreacted input substrate, reaction with Cdc20-activated APC, and reaction with Cdh1-activated APC. (C) Competition for Cdh1-dependent ubiquitylation of various substrates with either 333 μM wild-type (LRPRTALGDIGNKVS) or mutant (LRPATAAGDIGAKVS) D-box peptide from human CycB. The wild-type human CycB^{1–106}-6× myc (D-box) and *Drosophila* ORC1^{1–554} (O-box) substrates are as above; the KEN-box substrate is a derivative of *Drosophila* ORC1^{1–554} with a substitution of the human Cdc20 KEN box (KENQPEN) for residues 293–301. We do not believe that the remaining O-box residues (291 and 292) significantly modify activity of the KEN-box, as substitution of KEN to AAA completely abolishes reactivity (data not shown).

O-boxes in other proteins

Since the O-box is a portable, autonomous motif, we asked whether other proteins have O-boxes that mediate reaction with Cdh1-activated APC. Although the information content of the short O-motif is rather low, we were able to identify O-box-like elements in a number of sequences using various pattern-finding algorithms, including Flybase Pattern Search and BLAST searches for short, nearly exact matches. Subsequent testing revealed that two of these candidates, each implicated in control of cell cycle progression, contain functional O-boxes. The *Drosophila* abnormal spindle protein (Asp), which is thought to play a role in spindle pole organization during mitosis (do Carmo Avides and Glover 1999), and the *Schizosaccharomyces pombe* anaphase inhibitor Cut2 (Pds1/securin) (Funabiki et al. 1996) each contain a 6/11 match to the ORC1 O-box (Fig. 6A). In addition to this sequence, Asp contains a KEN-box and Cut2 contains two D-boxes, deletion of which has been shown to stabilize the protein (Funabiki et al. 1997).

As shown in Figure 6B, ablation of the O-box in Asp has a significant effect on the extent of Cdh1-driven ubiquitylation, either in the presence or absence of the KEN motif. Similarly, deletion of the D-boxes from Cut2 reduces but does not eliminate ubiquitylation; ablation of the remaining O-box essentially eliminates reactivity. Thus, the O-motifs in two other proteins behave much as does the O-box in ORC1, directing Cdh1-dependent modification in vitro.

Discussion

The O-box is an addition to the family of motifs that mediate APC-dependent degradation. Like the D-box, the O-box is a short portable motif that is sufficient to

direct polyubiquitylation in vitro and cell cycle-dependent degradation in vivo. The O- and D-box signals compete at some currently unknown level in ubiquitylation reactions, demonstrating that, although they are structurally and functionally distinct, both motifs converge on at least one common component of the degradation machinery. We have identified functional O-boxes in two other proteins; given the low sequence complexity of the motif and the current definition of essential residues solely by alanine scanning (Fig. 2D), it seems likely that O-boxes exist in other proteins.

Two recent reports (Burton et al. 2005; Kraft et al. 2005) significantly extended earlier observations (Pfleger and Kirschner 2000; Burton and Solomon 2001; Hilioti et al. 2001), demonstrating a direct interaction between Cdh1 and both KEN- and D-box peptides. Given the functional homology among the various destruction motifs, we asked whether we could detect interactions between O-box-containing peptides and Fzr in GST-pull-down, coimmunoprecipitation (co-IP), and yeast two-hybrid experiments. We were unable to detect specific binding of the O-box to Fzr/Cdh1; perhaps more sensitive assays either using purified components (Burton et al. 2005) or cross-linkable peptide substrates (Kraft et al. 2005) will be necessary to detect an inherently weak and transient interaction.

The N-terminal 554 amino acids of ORC1 contain three degradation motifs: one O-box, one KEN-box, and three D-boxes. What roles do these play? The O-box is primarily responsible for regulating cell cycle-coupled degradation of full-length ORC1. Our results also clearly indicate that the KEN- and D-boxes, at best, play only minor roles in directing ubiquitylation in vitro and degradation in vivo. The most likely explanation for the inactivity of these motifs is that they are situated in an unfavorable context (as has been observed in other cases—Zur and Brandeis 2002). When substituted for the O-box at positions 291–301 of ORC1, both D- and KEN-motifs direct very efficient polyubiquitylation in vitro (Figs. 4B, 5C).

We assume that degradation of ORC1 is integral to the process of resetting origins of replication after the initiation of S phase. In human cells, ORC1 is degraded during S phase by the SCF (Mendez et al. 2002). Negative regulators of pre-RC formation, such as Geminin and Cyclin B, are degraded by the APC at the G2/M transition, and the pre-RC can then reform in G1 after ORC1 is resynthesized. In *Drosophila*, ORC1, Cyclin B (Sigrist et al. 1995), and presumably Geminin (Quinn et al. 2001) are all degraded by the APC; and it is thus unclear when the pre-RCs are re-established. One possibility is that ORC1 acts during the narrow window between activation of Fzr (leading to the destruction of Cyclin B and Geminin) and Fzr (leading to the destruction of ORC1) during the G2/M transition. Another possibility is that in *Drosophila* pre-RC formation does not take place until late in G1 when Fzr activity drops and ORC1 is resynthesized under the direction of E2F (Asano and Wharton 1999).

Expression of the stable O-box mutant derivative of

Figure 6. Functional O-boxes in other cell cycle regulators. (A) Sequences of the O-boxes from *D.m.* ORC1 and Asp, as well as *S.p.* Cut2. (B) Cdh1-dependent ubiquitylation of various fragments of Asp (wild-type residues 1–400) and Cut2 (wild-type residues 1–301). To generate mutant O-box (mob) derivatives, the boxed residues in A were substituted with alanine. To generate the mutant KEN-box (mkb) derivative of Asp, KEN residues 229–231 were substituted with AAA. Unreacted substrate is in the left lane of each pair.

ORC1 fused to GFP caused no significant cellular (Fig. 3) or organismal phenotypes using a variety of GAL4 drivers (data not shown). However, expression of either tagged or untagged wild-type ORC1 using the same constellation of GAL4 drivers fails to phenocopy the organismal and cellular phenotypes that are associated with low-level constitutive expression of ORC1 (Asano and Wharton 1999). We assume that, as is the case for many other cell cycle regulatory factors (e.g., APC) (Rudner and Murray 2000), ORC1 activity is likely regulated by redundant mechanisms. If so, a more sensitive test of the role of ORC1 degradation would be to ask whether the stable O-box mutant form of the protein can substitute for all of the known or suspected roles of the wild-type protein, including not only replication control but also heterochromatin-associated transcriptional silencing (Pak et al. 1997), chromosome condensation (Loupert et al. 2000), and the control of synaptic plasticity (Pinto et al. 1999; Rohrbough et al. 1999). Such experiments await the isolation and characterization of an *ORC1*[−] mutant.

Materials and methods

GMR-GAL4, *en-GAL4*, and *actin5C-GAL4* lines as well as the *UAS-GFP-NLS* line were obtained from the Bloomington stock center. Other transgenic flies were generated by microinjection of *w¹¹¹⁸* embryos by standard methods. Third-instar larval eye-imaginal discs were dissected, mounted in PBS (130 mM NaCl, 7 mM Na₂HPO₄, and 3 mM NaH₂PO₄) without fixation, and immediately observed under the fluorescence microscope. The dissociation of imaginal disc cells and FACS analysis was performed as described by Neufeld et al. (1998). Embryo staining and construction of *ORC1-GFP* fusion genes and transgenic flies were as described previously (Araki et al. 2003). Plasmids for in vitro APC assays were constructed by insertion of PCR fragments into pSP73 (Promega) at the BglII site downstream of SP6 promoter (details upon request). In vitro ubiquitylation assays were carried out essentially as described (Tang and Yu 2004). *D.m. asp* and *S.p. cut2* cDNAs were isolated by RT-PCR from poly(A)⁺ mRNA and PCR amplification from genomic DNA, respectively. For the competition experiments shown in Figure 5, peptides (synthesized by Sigma-Genosys) were preincubated at final concentrations from 33 μM to 3.3 mM with the Cdh1-activated APC for 1 min prior to the addition of substrates.

Acknowledgments

We thank Zhanyun Tang for help in preparing in vitro assay material; Mike J. Cook and Lynn M. Martinek for FACS analysis; Tammy Lee, Laura Mitchell, Cary D. Gardner, and Mami Kawaguchi for technical help; Glenda Johnson and Jian Chen for media preparation; and Sandy Curlee for administrative help. We are grateful to Robin P. Wharton and Joseph R. Nevins for their generous support and encouragement throughout this work. This work was supported by NIH grants to M. Asano (GM64348) and H.Y. (GM61534). M. Araki is supported by a post-doctoral fellowship from the Uehara Memorial Foundation.

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Genes Dev. 2005, **19**:

Access the most recent version at doi:[10.1101/gad.1361905](https://doi.org/10.1101/gad.1361905)

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