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# ARI-1, an RBR family ubiquitin-ligase, functions with UBC-18 to regulate pharyngeal development in *C. elegans*

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**Abstract**

The LIN-35 retinoblastoma protein homolog and the ubiquitin-conjugating enzyme UBC-18 function redundantly to control an early step of pharyngeal morphogenesis in *C. elegans*. In order to identify ubiquitin-ligases acting downstream of UBC-18, we carried out a two-hybrid screen using UBC-18 as the bait molecule. Our screen identified three putative ubiquitin-ligases, one of which, ARI-1, showed genetic interactions leading to defective pharyngeal development that were identical to that previously observed for UBC-18. ARI-1 is a member of the RBR family of ubiquitin-ligases and contains a C-terminal motif that places it within the highly conserved Ariadne subfamily of RBR ligases. Our analyses indicate that ARI-1 is the principal Ariadne family member in *C. elegans* that is involved in the control of pharyngeal development with UBC-18. Using GFP reporters, we find that ARI-1 is expressed dynamically in a wide range of tissues including muscles and neurons during embryonic and postembryonic development. We also provide evidence that dsRNA species containing 14 or fewer base pairs of contiguous identity with closely related mRNAs are sufficient to mediate off-target silencing in *C. elegans*.
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## Introduction

The regulation of protein stability through ubiquitin-mediated proteolysis has been shown to play a critical role in a wide range of cellular and developmental processes. For example, in *C. elegans*, the ubiquitin pathway controls cell polarity and asymmetric cell division in the early embryo, a process that is essential for the precise segregation of maternal determinants and establishment of the anterior–posterior axis (Cowan and Hyman, 2004; Levitan et al., 1994; Rappleye et al., 2002). Correspondingly in mammals, the ubiquitin-ligase Smurf1 targets degradation of the small GTPase RhoA, thereby controlling cytoskeletal dynamics and consequent changes in cell polarity and shape, and in epithelial cell differentiation (Bryan et al., 2005; Ozdamar et al., 2005; Wang et al., 2003). Other roles for ubiquitin and ubiquitin-like molecules have been described in *C. elegans* including the regulation of cell cycle progression (Fay et al., 2002; Feng et al., 1999; Kipreos et al., 1996, 2000; Kitagawa et al., 2002; Kurz et al., 2002; Shakes et al., 2003; Zhong et al., 2003), germline establishment and meiosis (DeRenzo et al., 2003; Furuta et al., 2000; Golden et al., 2000; Pintard et al., 2003; Yeong, 2004), cell signaling (Hubbard et al., 1997; Liao et al., 2004; Yoon et al., 1995), and morphogenesis (Fay et al., 2003; Nayak et al., 2002), in addition to other functions.

The process by which cellular proteins become marked for destruction is mediated by a series of hierarchical enzymes (termed E1 to E4) that lead to the covalent attachment of a ubiquitin moiety to the target substrate (Glickman and Ciechanover, 2002; Pickart, 2001). Initially, a broad-spectrum ubiquitin-activating enzyme (E1) transfers the 76-amino-acid ubiquitin peptide via a thiol ester intermediate to a ubiquitin-conjugating enzyme (E2/UBC). The E2 then associates with a ubiquitin-ligase enzyme (E3), which is required for the transfer of ubiquitin to a specific lysine residue within the target protein. The E3 enzymes are generally credited with providing specificity to the process. Additional ubiquitin monomers may then be consecutively added by a distinct ubiquitin-ligase enzyme (E4) to produce the poly-ubiquitinated product that

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serves as a substrate for degradation by the 26S proteasome (Hatakeyama and Nakayama, 2003). Alternatively, substrates marked with one or several ubiquitins may be targeted for endocytosis and transport to lysosomes, or may remain in the cell, albeit with altered activities (Hicke, 2001; Schnell and Hicke, 2003).

In most organisms, there is a single E1, a sizeable but limited number of E2s, and a large number of E3s. For example, in *C. elegans*, there is one E1, ~23 E2s, and >130 putative E3s, the majority of which contain either a RING or HECT domain. A general model to have emerged is that most E2s probably act in conjunction with multiple E3s, and that individual E3s are themselves likely to recognize multiple target substrates (Castro et al., 2005; Glickman and Ciechanover, 2002; Pickart, 2001). Further adding to the complexity of the system is the existence of E3s that are composed of multiple subunits such as the Skp1–cullin–F-box-protein complex (SCF) and the anaphase-promoting complex/cyclosome (APC; Castro et al., 2005; Petroski and Deshaies, 2005). In particular, given the large number of Skp1 and F-box proteins encoded by most genomes (the *C. elegans* genome encodes 21 Skp-1-related proteins and >300 F-box proteins), there exists the potential for an impressively large number of subunit combinations, each of which may have distinct functions and enzymatic properties (Kipreos and Pagano, 2000; Nayak et al., 2002).

The RING finger class of E3 ligases encompasses a large number of distinct family members, some of which function within large multisubunit complexes and many others that act as single subunits or homodimers (Glickman and Ciechanover, 2002; Pickart, 2001). One such family member, the RBRs (for RING-Between rings-RING), includes most notably the human disease gene Parkin; mutations in Parkin are the leading cause of hereditary parkinsonism, although the underlying etiological mechanism is not well understood (Kahle and Haass, 2004; Kitada et al., 1998; Marin et al., 2004). RBR family ligases contain two $C_3HC_4$ RING fingers that are separated by a unique $C_6HC$ domain that has been called the IBR (in-between-RINGS) or DRIL (double RING finger-linked) domain (Morett and Bork, 1999; van der Reijden et al., 1999). The precise function of the IBR/DRIL domain, as well as the C-terminal RING finger of RBR ligases, is currently unclear (Aguilera et al., 2000; Ardley et al., 2001; Moynihan et al., 1999). RBR ligases are generally thought to function as single-subunit E3s, although a noteworthy gene fusion between one family member and a cullin gene has led to the suggestion that RBRs may in some cases function within larger complexes (Marin and Ferrus, 2002; Marin et al., 2004). RBR family members include the Parkin, Dorfin, Plant I/II, ARA54, XAP3, and Ariadne subfamilies; the last group seems to be the most ancient and predates the origin of plants, animals, and fungi (Marin and Ferrus, 2002).

We have previously shown that the ubiquitin-conjugating enzyme UBC-18 functions in a parallel pathway to LIN-35, the *C. elegans* Retinoblastoma/pocket protein homolog, to regulate an early step in pharyngeal morphogenesis (Fay et al., 2003). Whereas single mutants in *ubc-18* and *lin-35* show no obvious defects, *lin-35; ubc-18* double mutants are nonviable because (at least in part) of the inability to consume nutrients. The defect in pharyngeal development affects the ability of the anterior-most pharyngeal cell precursors to undergo a stereotypical reorientation in their apical-basal polarities (Fay et al., 2003; Portereiko and Mango, 2001). This in turn leads to the failure of the primordial pharynx to attach to the mouth (or buccal cavity), resulting in a misshapen nonfunctional organ. We have also demonstrated a role for a third protein, PHA-1, in the processes of pharyngeal morphogenesis (Fay et al., 2004). Based on genetic evidence, PHA-1 appears to constitute an additional redundant pathway with LIN-35 and UBC-18. Whereas a partial reduction in *pha-1* activity has little or no effect on viability, double mutants with *pha-1* and either *lin-35* or *ubc-18* are lethal and display the identical phenotype to *lin-35; ubc-18* double mutants.

We report here the identification of the downstream E3 partner of UBC-18 in pharyngeal development, ARI-1. ARI-1 is a member of the highly conserved Ariadne subfamily of RBR ligases. Interestingly, the phenotypes and expression patterns of the *ariadne* orthologs in worms and flies suggest that Ariadne family functions may be partially conserved across species (Aguilera et al., 2000).

## Materials and methods

### *Strains*

Maintenance, culturing, and genetic manipulation of *C. elegans* were carried out following standard procedures at 16°C and 20°C (Steirnagle, 2005; www.wormbook.org). Strains used in the phenotypic analysis include N2, DP38 [*unc-119(ed3)*], PD4792 [mIs11 IV *myo-2*: :GFP], MH1384 [kuIs46 X *ajm-1*: :GFP], WY83 [*lin-35; ubc-18; kuEx119*], WY162 [*pha-1(e2123)*; *sup36*], WY164 [*pha-1(e2123)*; *sup37*], WY180 [*pha-1(e2123)*; *myo-2*: :GFP], WY131 [*vab-7 (e1562), pha-1(e2123)*; *ajm-1*: :GFP], NL2099 [*rrf-3(pk1426)*], and GE24 [*pha-1(e2123)*]. The extrachromosomal array *kuEx119* expresses both wild-type *lin-35* as well as a ubiquitously expressed GFP reporter (*sur-5*: :GFP; Fay et al., 2002).

### *Two-hybrid screen and assays*

PCR amplification for bait and prey constructs was carried out using Pfu DNA polymerase (Stratagene), and all resulting constructs were confirmed by sequence analysis. The bait construct containing the full-length UBC-18 cDNA was fused in frame to the GAL4 DNA-binding domain (DB) using Gateway cloning methods (pDEST32; Invitrogen) and suitable primers 5′-ggggacaagtttg-tacaaaaaagcaggcttgatgtcagcgacacg-3′/5′-ggggaccactttgtacaagaaagctgggtactatt-caggccgctt-3′. The bait construct was introduced into yeast strain Mav203 and failed to activate the three reporter genes (*HIS3*, *URA3*, and *lacZ*) together with the empty prey vector pEXP-AD502. Sequential transformation was carried out to introduce the cDNA prey library (ProQuest pre-made cDNA library, Invitrogen). From ~$5 \times 10^5$ yeast colonies, 110 were identified that grew on the synthetic medium lacking Leu, Trp, and His and containing 75 mM 3-amino-1,2,4-triazole (3AT). Thirty-three of the 110 isolates were also positive for two other reporter genes, *URA3* and *lacZ*. The prey plasmids were rescued from positive yeast strains into *E. coli*. They were then retransformed into Mav203 together with the DB–UBC-18 construct or the empty bait vector (pDBLeu) and examined for induction of the reporter genes. Positive prey vectors that induced all three reporter genes in the presence of the DB–UBC-18 construct but not in the presence of pDBLeu (no-insert control), were sequenced to identify the inserts.

For the DB–ARI-1 construct, full-length *ari-1* cDNA was released from the prey construct by *Not*I and *Sac*I and subcloned in vector pDBLeu in frame with the GAL4 DNA-binding domain. N-terminally truncated *ari-1* was amplified using suitable primers: *ari-1*Δ19 (lacking the first 19 N-terminal amino acids),

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