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Role of the CRL2^{LRR-1} E3 ubiquitin-ligase in the development of the germline in *C. elegans*

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The ubiquitin-proteolytic system (UPS) regulates a variety of cellular and biological processes by controlling the stability of regulatory proteins, in space and time. Not surprisingly, defects in this system have been associated with various syndromes and pathologies, including cancer, illustrating the importance of understanding the regulation and the multiple functions of this system. *C. elegans* is a powerful model system to identify components of the UPS and to study their function during development in multicellular organisms. In *C. elegans*, the evolutionarily conserved CRL2^{LRR-1} E3-ligase is critical for the development of the germline. Inactivation of the CUL-2 scaffold or the LRR-1 substrate-recognition subunit leads to a cell cycle arrest in germline stem cells resulting in sterility. Through a genetic screen, we have identified a *cul-2* temperature-sensitive allele and we have used this allele to show that CUL-2 plays multiple roles in the development of the germline. CUL-2 (1) promotes germ cell proliferation, (2) influences the balance between mitotic proliferation and meiotic differentiation, and (3) inhibits the first step of meiotic prophase. Here, we discuss how CUL-2 regulates and coordinates these different processes. We suggest that ubiquitin-mediated protein degradation constitutes an important additional layer of regulation that contributes to the spatial organization of the germline.

Keywords: CUL-2, LRR-1, E3 ubiquitin-ligase, *C. elegans*, DNA replication checkpoint, germline, proliferation, differentiation

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CRL2^{LRR-1} E3-ligase is Essential for Germline Development and Embryogenesis in *C. elegans*

The ubiquitin-proteolytic system (UPS) comprises a series of enzymatic reactions leading to the covalent addition of ubiquitin chains onto lysine residues of protein substrates, targeting their subsequent degradation by the 26S proteasome, a large macromolecular complex with protease activities.[1] Polyubiquitination of the substrate requires the coordinated action of three enzymes: E1 ubiquitin-activating enzymes, E2 ubiquitin conjugating-enzymes, and E3 ubiquitin-ligases.[2,3] Together, these enzymes activate and transfer ubiquitin to target proteins by promoting the formation of an isopeptide bond between a lysine residue of the substrate and the C terminus of ubiquitin. Reiteration of this catalytic cycle assembles ubiquitin chains on the substrate, targeting its recognition and degradation by the 26S proteasome.

The paramount regulatory step in the system is the selective recognition of the substrate, which is achieved by E3-enzymes. The most prominent family of E3-enzymes is composed of multisubunit Cullin-RING E3-Ligases (CRLs) comprising exchangeable substrate-recognition modules nucleated by a specific Cullin RING-based catalytic core.[4-8] Eukaryotic genomes encode several cullin subunits, which function as platform of specific E3-enzymes. Whereas most cullins are evolutionarily conserved from yeast to human, Cul2 is present only in multicellular organisms.[9,10] However, the

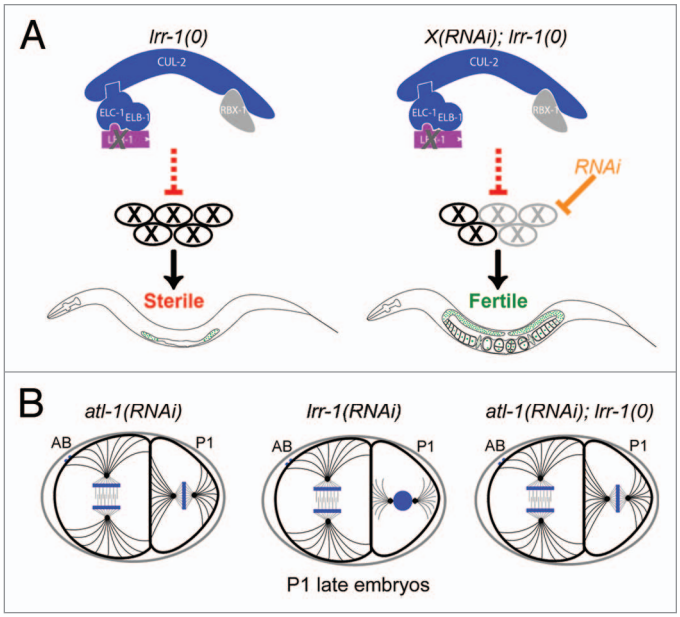

Figure 1. Hyperactivation of the DNA replication checkpoint pathway in *lrr-1(0)* mutant animals. (**A**) Flow-chart of the RNAi-based visual suppressor screen employed to search for *lrr-1(0)* suppressors. The CRL2^{LRR-1} complex is presented, the scaffold CUL-2, the adaptor ELC-1 and ELB-1 are in blue, RBX-1 in gray, and the LRR-1 substrate-recognition subunit in purple. X, substrates or pathways activated upon loss of *lrr-1* function. The screen was designed to search for genes whose inactivation by RNAi suppress *lrr-1(0)* mutant sterility. (**B**) Schematic of dividing embryos of the indicated genotypes. Note that inactivation of *lrr-1* delays division of the P1 blastomere and this delay is suppressed by inactivation of the DNA replication checkpoint.

role of Cul2 and the majority of its targets remain to be found.

In *C. elegans*, CUL-2 is highly expressed in the germline where it is essential for cell cycle progression.[11] *cul-2* loss-of-function animals are defective in germline stem cell (GSC) proliferation resulting in animal sterility. To further dissect *cul-2* function in the germline, we searched for the substrate-recognition subunit (SRS) acting together with CUL-2 to regulate GSC proliferation. Substrate-recognition modules of CRL2 complexes comprise the core subunits ELC-1 and ELB-1 that link the N-terminal part of CUL-2 to specific SRS termed BC-Cul2 box because these proteins share small regions, termed BC and Cul2 box, which are required for binding ELC-1 and CUL-2, respectively.[12,13] We and others identified the evolutionarily conserved Leucine Rich Repeat protein LRR-1 as the specific SRS acting together with CUL-2 to regulate germ cell proliferation.[14,15] LRR-1 is abundant in the germline and *lrr-1*-null [*lrr-1(0)*] mutant animals, similarly to *cul-2* mutants, are defective in germ cell proliferation resulting in animal sterility.

Hyperactivation of the DNA Replication Checkpoint Causes Germ Cell Cycle Arrest and Sterility in *lrr-1* and *cul-2* Mutants

In order to understand the cause of the cell cycle arrest in *lrr-1(0)* mutant germ cells, we performed a visual RNAi-based suppressor screen. We constructed an *lrr-1(0)* strain expressing the Histone H2B fused to GFP under the control of the germline-specific promoter *pie-1* and screened for genes whose inactivation by RNAi suppressed the germ cell cycle arrest of *lrr-1(0)* mutant, and restored their fertility (**Fig. 1A**). This visual screen led to the identification of CHK-1 (Chk1 in humans for checkpoint kinase 1) and ATL-1 (ATR, Ataxia telangiectasia and Rad3 related) kinases, which are core components of the DNA replication checkpoint pathway.[16-18] This checkpoint pathway is typically activated in response to defects in DNA replication, such as stalled replication forks, and blocks cell cycle progression in G2 phase.[19,20] These observations indicated that the DNA replication checkpoint is hyperactivated in *lrr-1(0)* mutants and prevents mitotic proliferation of GSC resulting in animal sterility.[14]

Hyperactivation of the DNA replication pathway in absence of *lrr-1* function is not only occurring in the germline but also in early embryos where RNAi-mediated knockdown of *lrr-1* causes activation of the DNA replication checkpoint resulting in a severe delay in the division of the P1 blastomere at the two-cell stage (aka P1 late phenotype, **Fig. 1B**).[14]

To further dissect the role of the CRL2^{LRR-1} E3-enzyme, we searched for temperature-sensitive (ts) mutants affecting the function of this enzyme. We took advantage of the unique phenotype of the *lrr-1(0)* mutant to screen for ts mutants presenting similar phenotypes. More specifically, we screened a collection of temperature-sensitive mutants presenting a P1 late phenotype and searched for mutants that are sterile at restrictive temperature (25°C) but fertile in absence of DNA replication checkpoint, like *lrr-1(0)* mutants. By this approach, we identified or209, the first *cul-2* temperature-sensitive allele. *cul-2(or209ts)* mutant animals are largely sterile at restrictive temperature but recover fertility in absence of DNA replication checkpoint pathway components. The finding of a *cul-2* allele in this simple genetic screen confirmed our previous observations, indicating that the DNA replication checkpoint blocks cell cycle progression in the germline upon inactivation of the CRL2^{LRR-1} E3-enzyme.[14]

Why is the ATL-1 checkpoint pathway hyperactivated when CRL2^{LRR-1} function is compromised? We believe that one function of this E3-ligase is to regulate DNA replication integrity in germ cells and in early embryos. The ATL-1 pathway is most likely primarily activated in response to DNA replication defects in the *lrr-1(0)*[14] and *cul-2(or209ts)* mutants.[21] Consistent with this hypothesis, ssDNA accumulates in *lrr-1* mutant germ cells, as revealed by the appearance of RPA-1 foci.[14] RPA-1 binds single-stranded (ss) DNA and ssDNA-RPA-1 complexes contribute to the recruitment and activation of the DNA replication checkpoint pathway leading to cell cycle arrest in germline stem cells.

What causes the accumulation of ssDNA? We have shown that a fraction of germ cells accumulate with a DNA content greater than 4N in *lrr-1(0)* mutants suggesting that some regions of the genome might undergo re-replication;[14] however, what causes DNA re-replication in these animals is currently unknown. LRR-1 may regulate the stability of factor(s) required for DNA replication. For instance, it has been shown in budding yeast that several key factors are limiting for DNA replication initiation[22] and LRR-1 might control the stability of one of these factors. Alternatively, LRR-1 might regulate the activity of cyclin-dependent kinase 1, which regulates DNA replication initiation. The Cdk inhibitor CKI-1 has been proposed to be one of the targets of the $CRL2^{LRR-1}$ complex in the germline.[15] However, in our hands, inactivation of *cki-1* by RNAi using different methods failed to suppress the germ cell proliferation defect observed in *lrr-1(0)* or *cul-2ts* mutant animals indicating that CKI-1 might not be the critical LRR-1 target in the germline. Consistent with this hypothesis, CKI-1 is apparently not expressed in the germline.[23] Therefore, the $CRL2^{LRR-1}$ substrate involved in DNA replication remains to be found. The role of the $CRL2^{LRR-1}$ enzyme in DNA replication integrity is likely identical in germ cells and in early embryos. In summary, the $CRL2^{LRR-1}$ enzyme promotes germ cell proliferation, most likely by regulating DNA replication integrity and, thereby, prevents activation of the DNA replication checkpoint.

Beyond Germ Cell Proliferation: Role of the $CRL2^{LRR-1}$ E3-ligase in the Spatial Organization of the Germline

lrr-1- and *cul*-2-null mutants are defective in germ cell proliferation, therefore analyzing a role of the $CRL2^{LRR-1}$ complex in later steps of germ cell development was not possible until the identification of the *cul-2ts* allele. Using this allele, we discovered additional phenotypes associated with the loss of *cul-2* function. In particular, we obtained evidences indicating that $CRL2^{LRR-1}$ influences the balance between germline stem cell proliferation and meiotic differentiation and we showed that this enzyme inhibits the assembly of the synaptonemal complex (SC).

In *C. elegans,* the balance between germ cell proliferation and meiotic differentiation is controlled by the GLP-1/Notch signaling pathway.[24] The distal tip cell (DTC), which caps the distal end of the germline, provides the notch ligand. Downstream of Notch signaling, a regulatory network of post-transcriptional nature coordinates the decision to either proliferate or differentiate by entry into meiosis. Central in this network are the almost identical FBF-1/2 (pumilio) RNA-binding proteins, which repress in GSCs the translation of meiotic-promoting factors, including GLD-1, GLD-2/3, and CKI-2.[23,25,26]

We started to investigate a potential role of *cul-2* in regulating the balance between germ cell proliferation and meiotic differentiation when we found that *cul-2ts* animals are hypersensitive to RNAi-mediated inactivation of FBF-1/2, an observation that we confirmed using genetic alleles. These results suggest that FBF-1/2 and CUL-2 may share common targets to inhibit meiotic differentiation by acting at the mRNA and protein level, respectively. Such complementary mechanisms would provide robustness to the meiotic entry decision. However, the CUL-2 target(s) involved in this process remains to be identified.

The decision to enter into meiosis must be coordinated with the timely production of meiotic chromosomal proteins, such as SC components, and their recruitment on meiotic chromosomes. FBF-1/2 plays a role in this coordination by repressing in GSC the expression of the SC components, including HIM-3, HTP-1/2, and SYP-1.[27] However, the recruitment of these proteins on chromosomes depends on the activity of the HORMA-domain protein HTP-3.[28,29] HTP-3 is expressed at low levels in GCSs but in contrast to the other structural components of meiotic chromosomes, HTP-3 expression is not regulated by FBF-1/2.[27] We obtained substantial evidences indicating that HTP-3 is a substrate of $CRL2^{LRR-1}$ E3-ligase. HTP-3 physically interacts with LRR-1 and accumulates upon inactivation of *cul-2* or proteasome subunits. Whereas, inappropriate HTP-3 accumulation in GSC promotes the recruitment of HIM-3 on chromosomes, it is however not sufficient to trigger meiotic entry. These observations suggest that several independent pathways act together to regulate and coordinate meiotic entry. Notably, the cyclinE/Cdk2 kinase (CYE-1/CDK-2 in *C. elegans*) recently emerged as a critical regulator of the mitosis to meiosis entry decision.[30,31] Indeed, *cye-1* inactivation force mitotic-proliferating germ cells to enter into meiosis,[21,30] suggesting that CYE-1/CDK-2 might inhibit the different pathways promoting meiotic entry.

Concluding Remarks

The identification of a *cul-2* temperature-sensitive revealed exciting new insights into the role of the $CRL2^{LRR-1}$ E3-ligase in the development of germline. This E3-enzyme promotes germ cell proliferation, most likely by controlling DNA replication integrity, but also influences the balance between mitotic proliferation and meiotic differentiation and prevents the premature assembly of the synaptonemal complex by regulating HTP-3 stability. It will be critical to identify the molecular mechanisms providing spatial regulation of HTP-3 degradation. Given that LRR-1 is expressed throughout the germline, we suspect that HTP-3 post-translational modifications in germline stem cells might promote its interaction with LRR-1. Equally important will be the identification of the other targets of the $CRL2^{LRR-1}$ enzyme. Finally, given that LRR-1 is evolutionarily conserved, it will be interesting to determine whether LRR-1 plays similar functions in other organisms.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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