**CRL2^{LRR-1} E3-Ligase Regulates Proliferation and Progression through Meiosis in the Caenorhabditis elegans Germline**

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**Abstract**

The ubiquitin-proteolytic system controls the stability of proteins in space and time. In this study, using a temperature-sensitive mutant allele of the *cul-2* gene, we show that CRL2^{LRR-1} (CUL-2 RING E3 ubiquitin-ligase and the Leucine Rich Repeat 1 substrate recognition subunit) acts at multiple levels to control germline development. CRL2^{LRR-1} promotes germ cell proliferation by counteracting the DNA replication ATL-1 checkpoint pathway. CRL2^{LRR-1} also participates in the mitotic proliferation/meiotic entry decision, presumably controlling the stability of meiotic promoting factors in the mitotic zone of the germline. Finally, CRL2^{LRR-1} inhibits the first steps of meiotic prophase by targeting in mitotic germ cells degradation of the HORMA domain-containing protein HTP-3, required for loading synaptonemal complex components onto meiotic chromosomes. Given its widespread evolutionary conservation, CUL-2 may similarly regulate germline development in other organisms as well.

**Introduction**

The ubiquitin-proteolytic system has emerged as a central mechanism to regulate protein turnover spatially and temporally [1,2]. In this system, ubiquitin, a small polypeptide of 76 amino acids, is covalently linked to a target protein through an enzymatic cascade, and the assembly of a poly-ubiquitin chain typically specifies that target protein for rapid degradation via the 26S proteasome [3]. The system can rapidly turn “off” regulatory proteins with high selectivity and is essential for numerous cellular processes such as transcription, signaling, DNA replication, DNA repair, cell cycle progression and differentiation. Not surprisingly, defects in the ubiquitin-proteolytic system have been implicated in a number of human diseases including cancers and neurodegenerative disorders [4–6].

An enzymatic cascade of three enzymes mediates the attachment of ubiquitin to substrate protein: the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-ligase (E3) [7]. Repeated cycles of ligation to the initial ubiquitin lead to poly-ubiquitination. The assembly of poly-ubiquitin chains can occur at different lysine residues within ubiquitin, with conjugation at lysines 11 and 48 typically leading to proteasomal degradation [8–11–12].

The paramount regulatory step in the cascade is the selective recognition of substrates, which is achieved by the E3-Ligase. Many E3 enzymes are nucleated around cullin scaffold subunits, with at least five cullin family members conserved in all metazoans [13,14]. Each cullin scaffold nucleates multiple E3-ligase complexes that all contain a similar catalytic core but use different substrate recognition modules to engage their cognate substrate(s) [15,16]. Over the past several years, Cullin RING E3-ligases nucleated around the cullins 1, 3 and 4 have been investigated extensively. By comparison, the functions of CRL2 complexes are relatively less well understood, with the noticeable exception of the CRL2^{Von Hippel Lindau (VHL)} complex that regulates the hypoxic response [for review see [17]]. A Cul2 knockout mouse has not been reported and the roles of Cul2 during vertebrate development remain largely unknown.

In *Caenorhabditis elegans*, CUL-2 is highly expressed in the germline and in early embryos, where it regulates numerous developmental processes including germ cell proliferation [18–20], sex determination [20], progression through oocyte meiosis [21,22], cell polarity [21], cell fate determination [23] and cell cycle progression [19]. CUL-2 accomplishes these various functions by recruiting, via the adaptor protein elongin C (ELC-1), distinct substrate-recognition subunits (SRS) that specifically engage their substrates (Figure 1A) [17]. Several SRS have been identified in *C. elegans*, though in most cases their relevant targets remain to be identified. For instance, the Leucine Rich Repeat...
Author Summary

Maintenance of the germline depends on the presence of a germline stem cell pool with self-renewal potential that produces gametes upon meiotic differentiation. Factors regulating the balance between germline stem cell self-renewal and meiotic differentiation ensure germline homeostasis, whereas disruption of these regulatory mechanisms can lead to sterility or cancer. In this study, we show that the ubiquitin-proteolytic system (UPS), which selectively targets regulatory proteins for proteasomal degradation, controls germline development by acting at three different levels. The UPS promotes germ cell proliferation, regulates the balance between self-renewal and meiotic differentiation, and limits progression through meiotic prophase. In particular, we show that an E3 ubiquitin-Ligase nucleated around the cullin 2 (CUL-2) protein and using the Leucine rich repeat protein LRR-1 as substrate recognition subunit regulates in germ stem cells the stability of HTP-3, which is required for progression through meiotic prophase. These findings identify a previously unknown role for proteolytic regulation in germline development and also explain how the critical balance between germ cell proliferation and meiotic differentiation can be tightly and robustly controlled with multiple, parallel regulatory inputs.

protein LRR-1 is an SRS expressed throughout the germline where it is essential for germ cell proliferation [19], but the precise function of the CRL2<sub>LRR-1</sub> E3-Ligase in the germline is still poorly understood.

We report here the isolation of a temperature-sensitive mutation in the C. elegans cul-2 genome. The cul-2(209) mutation recapitulates all the cul-2(RNAi) and cul-2(0) phenotypes and also reveals several novel functions in the C. elegans germline. More specifically, we show that besides promoting germ cell proliferation, CUL-2 has at least two other functions in the germline: it participates in the proliferation and meiotic differentiation, and limits progression through meiotic prophase. Furthermore, we show that the CRL2<sub>LRR-1</sub> E3-ligase inhibits meiotic prophase progression at least in part by promoting degradation of HTP-3, a HORMA domain containing protein. HTP-3 is required for progression through meiotic prophase and for the assembly of the synaptonemal complex, the zipper-like structure that tethers homologous chromosomes together.

Results

Identification of a temperature-sensitive allele in the cul-2 gene: cul-2(209)

The evolutionarily conserved CRL2<sub>LRR-1</sub> E3-ligase is essential for germline development in C. elegans: lrr-1(0) and cul-2(0) null mutants are sterile with a small germline [18,19]. However, the precise function of this enzyme is still poorly understood as the sterility in null mutants limits analysis of gene requirements. To circumvent this problem, we screened for temperature-sensitive (ts) mutants that resemble lrr-1(0) mutants. While lrr-1(0) animals are sterile, this phenotype is suppressed by inactivation of the DNA replication ATR/ATL-1 checkpoint pathway [19]. Therefore, we screened for ts mutants that, like lrr-1(0) mutants, are sterile at the restrictive temperature of 25°C but recover fertility upon atl-1 inactivation by RNAi (Figure 1B). Such mutants might be specifically defective in the function of the CRL2<sub>LRR-1</sub> complex and allow for a more careful analysis of gene requirements in the germline at different temperatures that only partially compromise E3-ligase function.

After screening a collection of ts mutants (Figure S1, Table S1) we found one that fulfilled the above criteria: cul-2(209). Like lrr-1(0) mutants, most cul-2(209) mutant animals are sterile at 25°C but recover fertility upon atl-1 inactivation by RNAi (Figure 1C). cul-2(209) has previously been linked to chromosome III [24], and we used single nucleotide polymorphisms to further map the cul-2(209) mutation to the end of this chromosome, which contains the cullin gene cul-2 (21.36 cM) (Figure S1). We next found that cul-2(209) fails to complement a known cul-2 null allele (material and methods), and we then sequenced the cul-2 gene from cul-2(209) genomic DNA and found a single nucleotide change relative to wild-type in the splicing donor site at the fifth exon-intron boundary (Figure 1D). This mutation substantially reduces CUL-2 protein levels even at the permissive temperature (15°C), but this effect is aggravated at the restrictive temperature (25°C) (Figure 1E). Consistent with the partially conditional effect on protein levels, cul-2(209) animals present high levels of embryonic lethality (30%) and a severe reduction in brood size even at the permissive temperature of 15°C, compared to wild-type (Figure S1). Further analysis revealed that cul-2(209) animals present typical cul-2 loss-of-function phenotypes, including the accumulation of abnormally high levels of the TRA-1 protein, presumably causing feminization of the germline (Figure 1F), and of the PIF-1 protein (Figure S2), both well-defined targets of CRL2<sub>FEM-1</sub> and CRL2<sub>ZIF-1</sub> E3-ligases, respectively [23,25]. We conclude that cul-2(209) is a conditional allele of cul-2. To our knowledge, cul-2(209) is the first temperature-sensitive allele yet identified that affects a metazoan cullin gene.

CRL2<sub>LRR-1</sub> E3-ligase promotes germ cell proliferation in adult germlines

Using the cul-2(209) allele, we re-examined CUL-2 function in the germline. The C. elegans germline is spatially organized and contains - from the distal to proximal end - mitotically proliferating stem cells, meiotic germ cells, and gametes (sperm or oocyte). The mitotic zone extends 18-20 germ cell diameters (gcd) along the gonadal axis from the distal end, with the total number of germ cell nuclei exceeding 200 [26] (Figure 2A). In the transition zone (TZ), just proximal to the mitotic zone, cells enter into meiotic prophase [27,28]. At this stage, structural components accumulate on meiotic chromosomes (e.g HIM-3 [29]) such that germ cell nuclei present a characteristic crescent shape that is readily evident by DAPI staining [30].

Corroborating our previous observations indicating that loss of the CRL2<sub>LRR-1</sub> E3-ligase results in a cell cycle arrest [19], the number of germ cells in the mitotic zone was severely reduced and nuclei were enlarged in cul-2(209) mutants raised from early larval stages (L1) at the restrictive temperature of 25°C. As expected, this phenotype was largely suppressed by inactivation of the ATL-1/DNA replication pathway (Figure 2). In addition, we noticed that the size of the mitotic zone was reduced in cul-2(209) animals, as determined by scoring the distance, in gcd, between the distal end of the germline and the appearance of both nuclear crescents and HIM-3 positive cells (Figure 2B, arrows and 2C-D). The mitotic zone was reduced to 15 gcd in cul-2(209) mutants raised to adulthood at permissive temperature (15°C), and even more reduced in mutants raised at the restrictive temperature (25°C). However, it was difficult to rigorously quantify the phenotype at 25°C because germ cell nuclei were both enlarged and reduced in number (Figure 2). We thus analyzed the size of this region in atl-1(RNAi); cul-2(209) mutants, who have smaller mitotic zones in these animals. The size of the mitotic zone was similarly reduced in atl-1(RNAi); lrr-1(0) animals but was unaffected in the control atl-1(RNAi) (Figure 2B and 2D). Finally, ZYG-11 is another substrate-recognition subunit of a CRL2 complex that appears dispensable in germline stem
Figure 1. or209 is a conditional cul-2 temperature-sensitive allele. A- Schematic representation of the CRL2LRR-1 E3-ligase. This complex is composed of two modules nucleated around the CUL-2 subunit (arc-shaped in blue): the substrate recognition module and the catalytic site. The substrate recognition module comprises the adaptor protein ELC-1, ELB-1 (blue) and the LRR-1 substrate recognition subunit (purple), whereas the catalytic module contains the RING finger protein RBX-1 (grey). B- Schematic drawing of the RNAi-based screen used to search for temperature-sensitive (ts) alleles affecting the function of the CRL2LRR-1 complex. Irr-1(tm3543) mutant animals (Irr-1(0)) are sterile but recover fertility upon inactivation of atl-1. The or209ts mutant fulfilled these criteria (lower panel). C- The or209ts mutation phenocopies inactivation of the CRL2LRR-1 complex. Graph showing the percentage of fertile or209 animals (25°C) after control (orange bars) and RNAi-mediated depletion of atl-1 (green bars). An average of eight different experiments is presented with 30 animals analysed in each experiment. D- Structure of the cul-2 gene (upper panel); red asterisk depicts the location of the or209 mutation. Chromatograms showing the T to A transversion found in the cul-2(or209ts) mutant (lower panels). E- Embryonic extracts of the indicated genotype were separated by SDS-PAGE and immunoblotted with CUL-2 (upper panel) and tubulin (lower panel) antibodies (loading control). The asterisk marks the position of truncated forms of CUL-2 that probably lack the C-terminal part of the protein. The value at the bottom is the ratio between CUL-2 and tubulin signal intensities. The wild-type value was arbitrary defined as 1. F- The sex determination factor TRA-1 accumulates in cul-2(or209ts) mutant animals, presumably causing the feminisation of the germline. Micrographs of adult worms of the indicated genotype were separated by SDS-PAGE and blotted with TRA-1 (upper panel) antibodies. The asterisk marks the position of a non-specific band. The value at the bottom is the ratio between TRA-1 and actin signal intensities. The wild-type value was arbitrary defined as 1.
Figure 2. CRL2LRR-1 E3-ligase promotes germ cell proliferation. A- Schematic drawing of an adult distal germline. The distal tip cell (DTC) niche is located at the distal end. The DTC expressed the Notch ligand LAG-2, which is shown in grey. The mitotic zone contains germ cells in the mitotic cell cycle (red), including a germline stem cell (GSC) pool distally and possibly transit-amplifying germ cells proximally; at its proximal edge,
some germ cells have entered pre-meiotic S-phase. The transition zone contains germ cells in meiotic S-phase and in meiotic prophase (nuclear crescents) that start to accumulate HIM-3 (green), a marker of meiotic prophase. The dashed line marks the boundary between the mitotic and transition zones (left panel). A simplified pathway with key regulators of the balance between GSC renewal and meiotic differentiation is shown in the right panel. Positive regulation (arrows) and negative regulation (bars). B- Representative images of dissected gonads of animals of the indicated genotypes taken 24 hours after the mid-L4 stage stained with HIM-3 antibodies (green) and DAPI (red). The distal end of the germline (arrowhead) is oriented toward the left, and the proximal end is oriented toward the right in this and other figures. Arrow, nuclei with crescent shape that is typical of nuclei in meiotic prophase. Germlines in each panel were treated identically and fluorescent images taken at the same settings. Scale bars: 50 μM.

C- Graphs showing the mean of the total number of germ cells in the mitotic zone of the germline and D- the quantification of the size of the mitotic zone in C. elegans. At least ten germlines of each genotype were scored.

Cul-2 participates in the proliferation versus meiotic entry decision

We thus investigated whether cul-2, in addition to regulating mitotic proliferation, might also have a role in preventing meiotic entry, by looking for genetic interactions between cul-2 and genes regulating the proliferation versus meiotic entry decision. GLP-1/Notch signaling controls the decision between self-renewal and entry into the meiotic cell cycle [28]. Downstream of Notch, the nearly identical Puf (Pamilo and FB)-domain RNA-binding proteins FBF-1/2 prevent meiotic entry by repressing the translation of meiotic promoting factors and structural components of meiotic chromosomes (Figure 2A) [32,33]. We therefore constructed double mutants between cul-2[or209ts] and glp-1[bn18], a temperature-sensitive gfp-1 mutant in which the Notch signaling pathway is partially defective [34], and between cul-2[or209ts] and fbf-1[0] or fbf-2[0], and scored meiotic entry in the double versus single mutants at the semi-permissive temperature of 20°C. At this temperature, germ cell proliferation is only modestly affected in cul-2[or209ts] mutants (Figure 3A).

To score meiotic entry in these different mutant backgrounds, we used a combination of cellular morphology (nuclear crescents) and molecular markers. More specifically, we monitored the appearance of HIM-3 and SUN-1 Ser6-Pi (P-SUN-1) on meiotic chromosomes [35]. P-SUN-1 is first detected in the mitotic zone, at nuclear periphery, in germ cells that are in mitosis from prometaphase onward (Figure 3A, yellow arrows), and then in meiosis, in the transition zone (TZ) at foci and patches, as well as over the nuclear envelope, as reported previously [35]. As shown in Figure 3A, the cul-2ts mutant enhanced the premature meiotic entry phenotype of the gfp-1[bn10] mutant, as evidenced by the premature meiotic entry in the gfp-1[bn10] cul-2[or209ts] double mutants, compared to the single mutants. Likewise, when combined with cul-2[or209ts], both fbf-1 and fbf-2 mutants showed a smaller mitotic zone than single mutants (Figure 3B) indicating that cul-2 influences the size of the mitotic zone. Collectively, these results indicate that Cul-2 acts with the Notch signaling pathway and FBF-1/2 to prevent meiotic entry.

CRL2LRR-1 regulates HTP-3 stability in the mitotic zone of the germline

CRL2LRR-1 acts through HTP-3 in the C. elegans germline. Entry into meiosis requires the coordination of a number of events: upon exit from the mitotic zone, germ cells initiate the chromosome dynamics required for meiotic pairing and synopsis. In preparation for this transition, proximal cells in the mitotic zone activate the expression of both regulators of meiotic entry and chromosomal proteins required for synopsis (e.g. HIM-3) that are then recruited on meiotic chromosomes. Cul-2 may thus inhibit meiotic entry by controlling the stability of regulators of meiotic entry in the mitotic zone of the germline.

Interestingly, an RNA interference (RNAi)-based screen [JM and LP, unpublished data] for suppressors of cul-2[or209ts] lethality at a semi-permissive temperature (25°C) identified, in addition to ATL-1 and its regulator MUS-101/TopBP1 [36], the axial element component HTP-3, which is required for the progression through meiotic prophase [37,38], and SYP-1, a core component of the synaptonemal complex (SC) [39] (Figure 4A and 4B).

Htp-3 belongs to the family of HORMA (Hop1-Rev1-Mad2) domain-containing proteins [40], and is required for preparing chromosomes for meiosis. More specifically, HTP-3 is required for loading on chromosomes the meiotic cohesin REC-8 and the axial and transverse components of the synaptonemal complex, including HIM-3 and SYP-1; for the formation of double-strand breaks that initiate meiotic recombination; and for implementation of the meiotic DNA damage ATM-1/checkpoint [38,41,42].

Given that HTP-3 promotes HIM-3 and SYP-1 loading on chromosomes, we investigated the potential role of CRL2LRR-1 in HTP-3 regulation. As expected, the accumulation of HIM-3 on chromosomes in cul-2[or209ts] germ cells depends on HTP-3 because HIM-3 fails to accumulate on chromosomes in htp-3[RNAi]; cul-2[or209ts] mutant germlines (Figure 4C). To determine whether LRR-1 acts through HTP-3 in the germline, we asked whether htp-3 reduction-of-function suppressed brl-1(0) mutant sterility. To this end, we took advantage of the htp-3[vu75] allele, which only modestly compromises HTP-3 function [42], and constructed brl-1(tm3543); htp-3[vu75] double mutant animals. Importantly, a significant fraction (34%, n = 115) of htp-3[vu75]; brl-1(tm3543) double mutant animals were fertile and produced embryos, in contrast to the highly penetrant sterility phenotype observed in brl-1(tm3543) single mutants (Figure 4D). The observed suppression of the brl-1(tm3543) sterility by the htp-3[vu75] allele is not merely resulting from an inactive ATL-1 checkpoint pathway in the htp-3[vu75] mutant because the DNA replication checkpoint is fully functional in this mutant (Figure 4E and Figure S3). Collectively, these results indicate that LRR-1 acts through HTP-3 in the germline (Figure 4F).

CRL2LRR-1 and the 26S proteasome control HTP-3 levels in the mitotic zone of the germline. To test whether CRL2LRR-1 regulates HTP-3 stability, we assessed HTP-3 protein levels by immunostaining cul-2[or209ts] and brl-1(0) mutant germlines. In control germlines, HTP-3 was expressed at low levels in the most distally located germ cells and at high levels on chromosomes in the most proximal mitotic germ cells (Figure 5A) [38,43]. In contrast to wild-type, HTP-3 was abundant in the most distally located germ cells in both cul-2[or209ts] and brl-1(tm3543) mutants (Figure 5A). HTP-3 similarly accumulates in atl-1(RNAi); cul-2[or209ts] and atl-1(RNAi); brl-1(tm3543) germlines, indicating...
that down-regulation of the DNA replication checkpoint does not affect HTP-3 levels. Importantly, HTP-3 also accumulates in the distal region after inactivation of PBS-5, a proteasome subunit, suggesting that CRL2LRR-1 may target HTP-3 for degradation (Figure 5A).

To ask whether the negative regulation of HTP-3 by CRL2LRR-1 E3 ligase involves physical interaction between the two proteins, we incubated total worm extracts with a recombinant LRR-1/ELC-1/ELB-1 trimeric complex or with a similar complex containing a truncated LRR-1 protein lacking the substrate binding interface. As shown in Figure 5B, endogenous HTP-3, but not MUS-101, was specifically retained on the trimeric complex containing full length LRR-1, but not on the complex containing the truncated form of LRR-1. Taken together, these results indicate that LRR-1 physically interacts with HTP-3 and regulates its levels in the mitotic zone of the germline, suggesting that HTP-3 is likely a direct target of the CRL2LRR-1 E3-ligase.

CRL2LRR-1 acts through HTP-3 to control HIM-3 loading on chromosomes. Our observations indicate that CRL2LRR-1 and the proteasome regulate HTP-3 stability in the mitotic zone of the germline, possibly to prevent entry into meiosis. To further investigate this possibility, we asked whether HTP-3 accumulation upon inactivation of cul-2, br-1 or the proteasome could force ectopic proliferative cells to enter meiosis.

GLP-1 promotes the proliferative fate, and the meiotic promoting factors GLD-1, NOS-3, GLD-2 and GLD-3 act downstream of Notch/GLP-1, in two parallel pathways (GLD-1/NOS-3 and GLD-2/GLD-3), to promote entry into meiosis. Simultaneous loss of both pathways causes a defect in meiotic entry that leads to germline overproliferation and prevention of gamete production [44–46]. Therefore, we asked whether loss of CUL-2 would cause ectopic proliferative germ cells to enter meiosis in gld-3 nos-3 double mutants. As a control for this experiment, we depleted CYE-1 (Cyclin E in C. elegans) because it has been shown recently that CYE-1 depletion forces ectopic proliferative germ cells to load HIM-3 and SUN-1 Ser8-Pi (P-SUN-1) and to enter meiosis [47].

As shown in the Figure 6C, HTP-3 accumulates in tumorous gld-3 nos-3 germlines upon inactivation of cul-2, as revealed by western blot analysis. We then used immunofluorescence to test whether this accumulation is accompanied by a change in germ cell fate by monitoring the accumulation of the HIM-3 and SUN-1 Ser8-Pi (P-SUN-1) and to enter meiosis [47].

As shown in the Figure 6C, HTP-3 accumulates in tumorous gld-3 nos-3 germlines upon inactivation of cul-2, as revealed by western blot analysis. We then used immunofluorescence to test whether this accumulation is accompanied by a change in germ cell fate by monitoring the accumulation of the HIM-3 and SUN-1 Ser8-Pi (P-SUN-1) and to enter meiosis [47].
Figure 4. CRL2/LRR-1 E3-ligase acts through HTP-3 in the C. elegans germline. A- Flow-chart of the cul-2(ts) RNAi-based suppressor screen. At 15°C, synchronised cul-2(ts) L1 larvae were fed with bacteria expressing dsRNA, then were shifted to a semi-permissive temperature (23°C) at the L4 stage. After 11 hours, young adults were removed, and embryonic viability was determined and plotted. B- Graphs showing the percent viability of cul-2(or209ts) animals after RNAi depletion of the indicated genes. C- Representative images of dissected gonads of the indicated genotypes stained with HIM-3 antibodies (red) and DAPI (green) are shown. Scale bars: 50 μm. Arrow marks the distal end of the germline. D- Reduction of htp-3 function suppresses lrr-1(0) mutant animal sterility. Micrographs of adult worms of the indicated genotypes were analysed by DIC microscopy. Thirty-four percent of lrr-1(0); htp-3(vc75) double mutants are fertile (n = 112). The germline is outlined, and the presence of oocytes and embryos is indicated. The asterisk marks the distal region of the germline. E- The DNA replication checkpoint is functional in htp-3(vc75) mutant animals. Animals of indicated genotypes were exposed to 10 mM Hydroxyurea (HU) and germlines were dissected 24 h post-L4 and stained with DAPI (upper panel). The number of germ nuclei in a given volume was determined and plotted (bottom panel). Note that HU treatment of wild-type (N2) animals leads to S-phase arrest that manifests as enlarged nuclei with an overall reduction in the number of nuclei in the mitotic zone of the germline. HU treatment similarly arrested htp-3(vc75) mutant germ cells, whereas checkpoint deficient atl-1(tm853) mutant germ cells fail to arrest cell cycle progression, indicating that the DNA replication checkpoint is functional in the htp-3(vc75) mutant. F- Pathway diagram summarizing the observed genetic interactions.
CUL-2 and LRR-1 counteracts the DNA replication checkpoint pathway, which is composed of MUS-101 and ATL-1, and inhibits HTP-3, which promotes the recruitment of HIM-3, SYP-1 and the assembly of the synaptonemal complex (SC). Positive regulation (arrows) and negative regulation (bars).

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to express HIM-3 to high levels. However, these germ cells remain mitotic because they do not express the other meiotic marker P-SUN-1 to significant levels. Similar results were obtained using the cul-2; gld-3 nos-3 triple mutant (Figure 6B and 6C). As reported previously, CYE-1 depletion causes gld-3 nos-3 germ cells to enter into meiosis as revealed by the accumulation of HIM-3 and P-SUN-1 meiotic markers.

Collectively, these results indicate that CUL-2 and LRR-1 inhibit only the first step of meiotic prophase by controlling HTP-3 stability and HIM-3 recruitment, whereas the CYE-1/CDK-2 kinase has a broader function in preventing meiotic entry.

Discussion

Through the isolation and characterization of a unique temperature-sensitive mutant allele of the cul-2 gene, we have shown that CUL-2 plays a critical role in germline development in C. elegans. In particular, CUL-2, in combination with LRR-1, i)
promotes germ cell proliferation, ii) participates in the proliferation versus meiotic entry decision and iii) inhibits progression through the first step of meiotic prophase by regulating the stability of the axial element HTP-3.

cul-2(or209ts) mutant: A sensitized genetic background to analyze CRL2 functions during C. elegans development

Temperature-sensitive (ts) alleles have been instrumental for discovering the function of essential genes in C. elegans, in

Figure 6. CRL2LRR-1 E3-Ligase controls HIM-3 loading on chromosomes. A: Schematic drawing showing that mitotic germ cells proliferate throughout the germline with no sign of meiotic entry in the gld-3 nos-3 double mutant germlines (upper panel). B: A simplified pathway with key regulators of the mitosis/meiosis decision is shown in the lower panel. C: HIM-3 accumulates in gld-3 nos-3 tumorous germlines upon cul-2(RNAi). Total gld-3 nos-3 worm extracts from control or cul-2(RNAi) were separated by SDS-PAGE and immunoblotted with HIM-3 (upper panel) and actin (lower panel) antibodies (loading control). The value at the bottom is the ratio between HIM-3 and actin signal intensities. The wild-type value was arbitrary defined as 1. D: Representative images of dissected gonads, of the indicated genotypes stained with HIM-3 (red) and SUN-1 Ser8-Pi (P-SUN-1, green) antibodies and with DAPI (blue). RNAi treatments were performed from the L1 (control, cul-2, lrr-1, cye-1) or L3 (pbs-5) stage at 20°C. Germlines in each panel were treated identically and fluorescent images taken at the same settings. Scale bars: 50 μm. The boxed regions, encompassing representative nuclei, are shown at higher magnification on the right panels. Scale bar: 5 μm. E: Graph showing the percentage of tumorous gld-3 nos-3 germlines HIM-3 and SUN-1 Ser8-Pi negative (white bars), HIM-3 positive and P-SUN-1 negative (light grey), and both, HIM-3 and P-SUN-1 positive (dark grey), as scored by counting the number of nuclei rows that contain at least 5 gcd with cells expressing HIM-3 and P-SUN-1 positive.

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particular for genes like CUL-2 with roles in multiple processes. Indeed, ts alleles present numerous advantages: they often only partially reduce gene function even at the fully restrictive temperature, and they allow for modulation of activity through analysis at multiple temperatures.

CUL-2 is an essential gene in *C. elegans* that is highly expressed in the germline and in the early embryo where it has been implicated in numerous processes. Our phenotypic analysis revealed that the *cul-2(ts209ts)* mutant recapitulates all the *cul-2* loss of function phenotypes including feminization of the germline (Figure 1E), defects in cell fate determination with PIE-1 accumulation in somatic blastomeres (Figure S2), and defects in cell cycle progression in the early embryo [24] (data not shown). The penetrance of each phenotype varies with the duration of the shift at restrictive temperature, and thus this *cul-2(ts209ts)* mutation provides a useful sensitized genetic background for identifying new *in vivo* functions of CRL2 complexes and, most importantly, for identifying its targets. Finally, other temperature-sensitive mutants affecting the ubiquitin-proteolytic system have been identified in *C. elegans* [48–50], but *cul-2(ts209ts)* is to our knowledge the first conditional mutation reported for a metazoan cullin.

**CRL2/LRR-1** regulates germ cell proliferation by counteracting the DNA replication checkpoint

Using the *cul-2(ts209ts)* allele, we confirmed our previous observations indicating that loss of the CRL2/LRR-1 E3-ligase causes hyperactivation of the ATL-1/DNA replication checkpoint in germ cells, resulting in a cell cycle arrest and adult sterility [19]. Germ cell nuclei were enlarged and reduced in number in the *cul-2(ts209ts)* mutant at 25°C, a phenotype that was partially suppressed by RNAi-mediated inactivation of *atl-1* (Figure 2). Furthermore, reducing *mas-101* and *atl-1* function by RNAi increased the hatching rate of the *cul-2(ts209ts)* mutant (Figure 5), further demonstrating that loss of CRL2/LRR-1 function triggers activation of the ATL-1/DNA replication checkpoint pathway.

Why is the ATL-1 checkpoint pathway hyperactivated in *cul-2(ts209ts)* and *br-1(0)* mutants? We believe that one function of the CRL2/LRR-1 complex is to regulate DNA replication integrity, both in germ cells and in early embryos [19]. The ATL-1 pathway is thus activated primarily in response to DNA replication defects in the *br-1(0)* [19] and *cul-2(ts209ts)* mutants (this study). In addition, our observations suggest that the inappropriate accumulation of structural components of meiotic chromosomes in *br-1(0)* mutants may also contribute to the activation of the ATL-1 checkpoint pathway (see below).

**CUL-2** participates in the proliferation versus meiotic entry decision

Besides promoting germ cell proliferation, our results suggest that CUL-2 influences the balance between stem cell self-renewal and meiotic differentiation possibly by regulating the stability of meiotic promoting factors. In the *C. elegans* germline, GLP-1/Notch signaling controls the decision between self-renewal and entry into the meiotic cell cycle [28]. Downstream of Notch, the RNA-binding proteins FBF-1/2 prevent meiotic entry by repressing the translation of meiotic promoting factors, and of structural components of meiotic chromosomes [32,33,51]. In addition to this regulatory network, which is largely translational in nature, there is increasing evidence that post-translational regulations play also a critical role in regulating the proliferation versus meiotic entry decision. For instance, the Cyclin E/Cdk2 kinase acts with the Notch pathway to promote the proliferative fate and to prevent meiotic entry [47,52], and our results indicate that CUL-2 plays also a role in this process. Indeed, the *cul-2* mutant enhanced the premature meiotic entry phenotype of the *glp-1(bn18)* mutant and when combined with the *cul-2* mutant, both *fbf-1* and *fbf-2* mutants showed a smaller mitotic zone than single mutants (Figure 3). This function of CUL-2 in influencing the balance between germ cell self-renewal and meiotic entry is likely independent of the DNA replication checkpoint pathway given that *atl-1* depletion does not affect the size of the mitotic zone (Figure 2).

These observations suggest the existence of a complex network of post-transcriptional and post-translational regulatory mechanisms to regulate the balance between germ cell self-renewal and meiotic entry.

What is the role of CUL-2 in this network? CUL-2 might act independently at multiple levels, for instance by regulating the activity of the CYE-1/CDK-2 kinase, and by regulating the stability both of meiotic promoting factors and of structural components of meiotic chromosomes in the mitotic zone of the germline. A gradient of high/low CYE-1/CDK-2 kinase is established along the distal to proximal end of the germline, and this gradient appears important for the self-renewal versus meiotic entry decision [47,52]. Three complementary mechanisms establish this gradient: in the meiotic zone i) GLD-1 inhibits CYE-1 translation [53], ii) CYE-1 subunit is targeted for degradation by an E3-ligase nucleated around CUL-1 [47] and iii) the cyclin-dependent kinase inhibitor CKI-2 accumulates and inhibits CYE-1/CDK-2 activity [53]. In the mitotic zone, CKI-2 translation is inhibited by Notch and FBF-1/2. CKI-2 is not essential for germ cell proliferation but plays a role in the maintenance of the germline by influencing the proliferation versus meiotic entry decision [54]. CUL-2 has been implicated in the regulation of CKIs stability [18] and thus CUL-2 might target CKI-2 for degradation in the mitotic zone of the germline to maintain high CYE-1/CDK-2 activity in this region.

Alternatively, CUL-2 might act with CYE-1/CDK-2 to control the stability of meiotic promoting factors in the mitotic zone of the germline. Consistent with this hypothesis, substrate phosphorylation often is a pre-requisite for recognition by an E3 ligase [15], and it has been shown recently that CYE-1/CDK-2 phosphorylates GLD-1 and thereby regulates its stability in the mitotic zone of the germline [52], suggesting that CUL-2 might regulate GLD-1 degradation in germline stem cells. However, we failed to detect significant GLD-1 accumulation in germ cells located in the most distal part of the germline upon inactivation of *cul-2* (data not shown), suggesting that CUL-2 might not be involved in GLD-1 degradation. Nevertheless, we cannot exclude the possibility that a small fraction of GLD-1 accumulates upon inactivation of *cul-2* and thereby contributes to the *cul-2* phenotype.

**CRL2/LRR-1** acts through HTP-3 to inhibit assembly of the synaptonemal complex

Although the mechanisms by which CUL-2 influences the proliferation versus meiotic entry decision remain to be identified, our results indicate that CRL2/LRR-1 negatively regulates progression through meiotic prophase by controlling the stability of HTP-3 (Figure 7). HTP-3 accumulates in germ cells upon inactivation of *cul-2*, *br-1* and the proteasome, and HTP-3 physically interacts with LRR-1 *in vitro*. Furthermore, reducing *htp-3* function, using the *htp-3*(or209) allele or with RNAi, suppressed *br-1(tm3543)* sterility and increased the hatching rate of the *cul-2(ts209ts)* mutant, respectively. In addition, reducing *syb-1* function also increased the hatching rate of the *cul-2(ts209ts)* mutant. These results suggest that premature accumulation of HTP-3 on
inactivation of Ser8-Pi on chromosomes of ectopic proliferating germ cells upon appearance to enter into meiosis because we failed to detect SUN-1 development in *C. elegans* meiotic entry and thus emerged as a critical regulator of germline levels in the germline to coordinate germ cell proliferation and mitotic zone of the germline (Figure 7).

In conclusion, our results indicate that CUL-2 acts at multiple conditions. Our study demonstrates the inhibitory role of CUL-2 in promoting meiotic entry and supports the hypothesis that CUL-2 acts independently of HTP-3 to prevent meiotic entry presumably by controlling the stability of unknown meiotic promoting factor(s).

**Materials and Methods**

**Nematode strains, strain construction, and culture conditions**

*C. elegans* strains, strain construction, and culture conditions were obtained from the MRC Geneservice (Cambridge, U.K.) or209 wild isolate, and recombinant F2 lines. In this study, *C. elegans* strain positions the *cul-2* mutation between 27.2 cM and 21.25 cM along the third chromosome. This region contains unknown meiotic promoting factor(s).

**Single nucleotide polymorphism (SNP) mapping and pyrosequencing**

For this study, *or209* worms were mated to the Hawaiian CB4856 *C. elegans* wild isolate, and recombinant F2 lines homozygous for the *or209* mutation were isolated based on embryonic lethality at the restrictive temperature. Several SNPs located between −27.2 cM and 21.25 cM along the third chromosome were amplified by PCR from these recombinants, essentially as described [59], and genotyped using pyrosequencing technology. Briefly, PCR amplifications were performed from single worms using Taq DNA polymerase (New England Biolabs) and specific primers for each SNP. The purification of single-stranded PCR amplicons and the pyrosequencing reactions were subsequently performed according to manufacturer’s instructions using a PyroMark Q96 ID instrument (Biotage). This analysis positioned the *or209* mutation between +18.52 cM and the right end of chromosome III. This region contains *cul-2*, and we showed that all embryos from *or209/cul-2(ok1)* trans-heterozygotes (*ek1* is a null allele of *cul-2* [18]) failed to hatch.

**RNA interference (RNAi)**

*Escherichia coli* clones expressing dsRNA to deplete *C. elegans* genes were obtained from the MRC Geneservice (Cambridge, U.K.). RNAi feeding was performed as described using 2 mM IPTG (RNAi plates) [62].

**Statistical analysis**

The results are presented as means ± S.E.M. In all graphs, data were compared by a Mann-Whitney test (two-tailed p) or Student's t-test (Figure 3 and Figure 5). All calculations were performed with InStat3 software (Graphpad). * p<0.05; ** p<0.01; *** p<0.001.

**Hydroxyurea treatment**

L4 animals were fed on NGM plates containing 10 mM final HU prior to analysis. Germlines were then dissected and stained with DAPI. Quantification of HU-induced cell cycle arrest was performed by counting the number of nuclei in a defined volume (20 000 µm²).

**Microscopy, immunochemistry, and fluorescence microscopy**

Germlines were dissected in PBS followed by freeze-crack, immersion in cold MeOH (−20°C) for 1 min, and fixation in 1× PBS, 0.08 M HEPES (pH 6.9), 1.6 mM MgSO₄, 0.8 mM EGTA and 3.7% paraformaldehyde for 30 min in a humidity chamber at 4°C. The samples were then processed for fluorescence microscopy.
room temperature. Slides were washed 3 × 5 min, blocked for 1 h in PBT (1 × PBS, 0.1% Triton X-100, and 5% BSA), and incubated overnight at 4°C with primary antibodies diluted in PBT. Working dilutions for the primary antibodies were 1:1000 for rabbit anti-HIM-3 (M. Zetka), 1:100 for rabbit anti-HTP-3 (M. Zetka) and 1:1000 anti-SUN-1 Ser8-Pi (V. Jantsch). Slides were later incubated for 30 min at room temperature with secondary antibodies coupled to the Alexa 488 and 568 fluorophore (1:600, Molecular Probes). Next, germlines were mounted in Vectorshield Mounting Medium with DAPI (Vector). Fixed germlines were imaged using either a TCS SP5 confocal microscope (Leica) or a LSM 710 confocal microscope (Zeiss) with 40× objectives. Confocal images correspond to the projection of confocal Z-stacks spanning maximum 5 μm. Captured images were processed using ImageJ and Adobe Photoshop.

For whole-worm images, worms were immobilized with 20 mM levamisole and mounted on 2% agarose pads. Images were then acquired using an Axiovert 200 inverted microscope equipped with DIC optics.

Protein extracts and antibodies

Standard procedures were used for SDS-PAGE and western blotting. The following antibodies were used in this study: primary antibodies were directed against CUL-2 [19], ELC-1 [19], HTP-3 (M. Zetka), HIM-3 (M. Zetka), SUN-1 Ser8-Pi [35], Tubulin (Sigma), Actin (Sigma), TRA-1 (Sigma), TRA-1 [63] and MUS-101 [36]; secondary antibodies conjugated to peroxidase against rabbit or mouse were purchased from Sigma.

LRR-1/ELC-1/ELB-1 trimeric complexes were expressed in E. coli and purified as described [19]. Total worm extracts were prepared by cryolysis, as previously described [64], and loaded onto trimeric complexes immobilized on T7-agarose beads (Novagen) for 2 hours at 4°C. After five washes, proteins were eluted with sample buffer and separated by SDS-PAGE.

Supporting Information

Figure S1 Identification of a temperature-sensitive allele in the cul-2 gene. A- Flow chart of the approach used to screen temperature-sensitive mutants affecting the function of the CRL2/LRR-1 complex. The basic idea is to screen for mutants that behave like br-1(0) mutant animals. br-1(0) animals are sterile with a small germline but this phenotype is fully suppressed by levamisole and mounted on 2% agarose pads. Images were then acquired using an Axiovert 200 inverted microscope equipped with DIC optics.

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