The CRL2\(^{LRR-1}\) ubiquitin ligase regulates cell cycle progression during \textit{C. elegans} development

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SUMMARY
The molecular mechanisms that regulate cell cycle progression in a developmental context are poorly understood. Here, we show that the leucine-rich repeat protein LRR-1 promotes cell cycle progression during \textit{C. elegans} development, both in the germ line and in the early embryo. Our results indicate that LRR-1 acts as a nuclear substrate-recognition subunit of a Cullin 2-RING E3 ligase complex (CRL2\(^{LRR-1}\)), which ensures DNA replication integrity. LRR-1 contains a typical BC/Cul-2 box and binds CRL2 components in vitro and in vivo in a BC/Cul-2 box-dependent manner. Loss of \(lrr-1\) function causes cell cycle arrest in the mitotic region of the germ line, resulting in sterility due to the depletion of germ cells. Inactivation of the DNA replication checkpoint signaling components ATL-1 and CHK-1 suppresses this cell cycle arrest and, remarkably, restores \(lrr-1\) mutant fertility. Likewise, in the early embryo, loss of \(lrr-1\) function induces CHK-1 phosphorylation and a severe cell cycle delay in P lineage division, causing embryonic lethality. Checkpoint activation is not constitutive in \(lrr-1\) mutants but is induced by DNA damage, which may arise due to re-replication of some regions of the genome as evidenced by the accumulation of single-stranded DNA-replication protein A (ssDNA-RPA-1) nuclear foci and the increase in germ cell ploidy in \(lrr-1\) and \(lrr-1\); \(atl-1\) double mutants, respectively. Collectively, these observations highlight a crucial function of the CRL2\(^{LRR-1}\) complex in genome stability via maintenance of DNA replication integrity during \textit{C. elegans} development.

KEY WORDS: E3 ubiquitin ligase, DNA replication, Checkpoint, Cell cycle, ATR/ATL-1, Chk1/CHK-1, \textit{Caenorhabditis elegans}

INTRODUCTION
DNA replication is a period of extreme vulnerability for the genome of eukaryotic cells. During this complex process, replication forks frequently encounter obstacles that impede their progression. Stalled forks are unstable structures that have to be stabilized and restarted in order to achieve faithful duplication of the genome. To cope with stalled replication forks and other forms of DNA damage, cells have evolved surveillance mechanisms, termed DNA damage and replication checkpoints, that scrutinize DNA damage and replication stress and orchestrate the appropriate cellular responses (Harper and Elledge, 2007; Paulsen and Cimprich, 2007). These signaling cascades are initiated by loading and activation of the PI3 kinase-related kinase ataxia telengectasia and Rad3 related (ATR) at sites of damage (Cimprich and Cortez, 2007). Once recruited to the stalled replication fork, ATR phosphorlates and thereby activates Chk1, which in turn blocks cell cycle progression, prevents origin firing, stabilizes stalled replication forks and facilitates the restart of collapsed forks.

The ATR/Chk1 signaling pathway is also mobilized during development to control cell cycle duration. For example, in the \textit{C. elegans} embryo, this pathway is specifically activated by developmental cues to control cell cycle timing (Brauchle et al., 2003). This checkpoint is preferentially activated in the P lineage, or future germ line of the animal, such that at the two-cell stage the P1 blastomere invariably enters mitosis 2 minutes after the anterior AB blastomere (Brauchle et al., 2003; Moser et al., 2009). This asynchrony of cell division is crucial for germ line and embryonic development, as shortening the delay through inactivation of the ATL-1/CHK-1 (the \textit{C. elegans} orthologs of ATR/Chk1) pathway leads to sterility, whereas lengthening the cell cycle through hyperactivation of this pathway causes patterning defects and embryonic lethality (Encalada et al., 2000; Brauchle et al., 2003; Kalogeropoulos et al., 2004; Holway et al., 2006). Therefore, activation of the ATL-1/CHK-1 pathway by DNA damage is actively suppressed in early embryos so that P lineage cell divisions occur on schedule (Holway et al., 2006). DNA damage resulting from severe DNA replication defects does eventually delay cell cycle progression in a checkpoint-dependent manner and the P1 blastomere is affected more strongly by this delay than the AB blastomere (Brauchle et al., 2003).

In sharp contrast to the early embryo, in which the fast pace of development is favored at the expense of genome stability, germ cells are highly sensitive to genotoxic insults and replication stress (Gartner et al., 2000). Under these conditions, the ATL-1/CHK-1 pathway is required to transiently arrest cell cycle progression (Garcia-Muse and Boulton, 2005), presumably to allow time for DNA repair. This cell cycle arrest causes a severe reduction in the number of germ cell nuclei, which become enlarged because nuclear and cellular growth continue during the arrest (Gartner et al., 2000).

In this study, we report that leucine-rich repeat protein 1 (LRR-1) is an essential determinant of genome stability in \textit{C. elegans} and acts as a substrate-recognition subunit of a Cullin 2-RING E3 ligase complex (CRL2\(^{LRR-1}\)). LRR-1 is a nuclear protein that contains a typical BC/Cul-2 box, which is the signature of CRL2 substrate-recognition subunits, and binds CRL2 components.
through this motif both in vitro and in vivo. Although lrr-1 is an essential gene, maternal rescue allows the analysis of lrr-1 loss of function in adult tissues. lrr-1 mutants are sterile owing to severe defects in germ cell proliferation. Inactivation of ATL-1/CHK-1 checkpoint components suppresses this proliferation defect and, remarkably, fully restores lrr-1 mutant fertility. Likewise, in the early embryo, lrr-1 inactivation leads to hyperactivation of the ATL-1/CHK-1 pathway, which delays mitotic entry and results in embryonic lethality. Checkpoint activation is not constitutive in lrr-1 mutants but is induced by DNA damage, which may arise due to DNA re-replication problems.

MATERIALS AND METHODS

Nematode strains, strain construction and culture conditions
C. elegans strains were cultured and maintained using standard procedures (Brenner, 1974). The lrr-1(tm3543) allele was generated and kindly provided by S. Mitani of the National BioResource Project for the nematode, Japan. Deletions were backcrossed four times with the wild-type N2 strain (Bristol) and then balanced with the balancer chromosome m1n1 (Edgley and Riddle, 2001). Strains of the following genotypes were used: lrr-1(tm3543); leads30[junc-119(+)] pie-1::GFP::lrr-1 [this study]; XA3501: unc-119(ed3); ruls32[unc-119(+)] pie-1::GFP::his-11 III; ojs1[junc-119(+)] pie-1::GFP::hhb-2; div-1(or148ts) (Encalada et al., 2000); and atl-1(tm853) IV/nT1[qIs50] (IV;V) (Garcia-Muse and Boulton, 2005).

RNAi-mediated interference (RNAi)
RNAi was performed by the feeding method (Kamath et al., 2001). RNAi-depleted embryos that were able to divide at least until the four-cell stage were analyzed. For strains expressing fluorescent proteins, RNAi was performed at 20°C or 25°C.

Hydroxyurea (HU) treatment
L1 larvae were grown until adulthood on NGM plates containing HU (6 and 8 mM) at 20°C. Animals were then paralyzed with levamisole (20 mM; Sigma) and examined under a Zeiss Axiosmager A1 microscope equipped with DIC optics.

DNA manipulation and worm injection
Molecular biology was performed using standard procedures (Sambrook et al., 1989). The polycistronic vector pST39 was obtained from S. Tan (Tan, 2001). The fosmid WRM0634DD04 containing the lrr-1 gene was obtained from Geneservice and prepared according to the manufacturer’s instructions. WRM0634DD04 (20 ng/µl) was injected into lrr-1(tm3543) heterozygous animals together with pRF4 (80 ng/µl) containing the rol-6(D) (su1006) mutant gene (Mello et al., 1991) and myo-2::RFP (50 ng/µl) as a co-injection marker, giving a total concentration of 150 µg/ml per injection.

Antibodies, protein extracts and immunopurification
LRR-1, CUL-2 and ELC-1 proteins were expressed as fusions with glutathione-S-transferase (GST) in E. coli BL21, purified from inclusion bodies and used to immunize rabbits (Charles River Laboratories). The generated antibodies were affinity purified on nickel cellulose strips as described previously (Pintard et al., 2003). The antibodies used in this study included FLAG (1/1000; Sigma), T7 (1/6000; Novagen), Mab144 (1/500; Covance), phospho-serine histone H3 (1/100; Cell Signaling), phospho-Y15 CDK-1 (Calbiochem), phospho-S345 CHK-1 (Abcam), CDC-25.1 (Segref et al., 2010), RPA-1 (Lee et al., 2010), GFP (1/1000; Roche), mouse TrueBlot horseradish peroxidase (1/1000; E Bioscience), ELC-1 (1/500; this study), CUL-2 (1/500; this study) and LRR-1 (1/50 (immunofluorescence) and 1/500 (western blot); this study).

Protein extraction and immunoprecipitation experiments were performed as previously described (Luke-Glaser et al., 2007). The expression of recombinant proteins was induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside to 1-liter cultures of E. coli BL21 before incubation overnight at 18°C. After pelleting, the bacteria were resuspended in 20 ml 0.15 M NaCl, 0.5 mM dithiothreitol, 40 mM imidazole in PBS, pH 7.3, before lysis using an Emulsiflex homogenizer (Avestin). The soluble portion of the lysate was loaded on a 1-ml Hi-Trap HP Column (GE Healthcare). The column was washed with ten volumes of lysis buffer, and bound proteins were eluted in lysis buffer containing 300 mM imidazole.

HEK293T cell culture and transfection
Human embryonic kidney HEK293T cells were cultured as described (Luke-Glaser et al., 2007). Six hours before immunopurification, the proteasome inhibitor MG132 (20 µM) was added to the culture.

Immunostaining, live imaging and fluorescence microscopy
The gonads were dissected by opening the worms in PBS buffer behind the pharyngeal bulb. Worms and embryos were freeze-cracked by flipping off the coverslip, immobilized on poly-lysine-coated slides and fixed for 20 minutes in methanol at −20°C. Affinity-purified LRR-1 antibody was used at a dilution of 1:50 and the secondary antibodies were coupled to the fluorophores Alexa 488 or Alexa 543 (Molecular Probes) and used at 1:400. Embryos were mounted in Vectashield Mounting Medium with DAPI (Vector). To determine cell cycle length, early embryo divisions were recorded at 20°C by taking images at 10-second intervals using a 63× objective on a DMI 6000 microscope (Leica) equipped with DIC optics and a high-resolution CoolSNAP HQ2 camera (Photometrics) driven by Metamorph software (Universal Imaging). Fixed germ lines and embryos were imaged on a DM IRB inverted microscope (Leica), a CSI-10 spinning Nikon confocal unit (Yokogawa Electric, Tokyo, Japan) equipped with a CoolSNAP HQ camera (Fig. 2A), a TCS SP5 confocal microscope (Leica) (Fig. 3C, Fig. 5 and Fig. 6C) or an Axiovert 200 inverted microscope (Zeiss) equipped with the ApoTome module (Zeiss) (Fig. 2B).

Propidium iodide staining and genomic DNA quantification
Wild-type and lrr-1(tm3543) mutant germ lines were dissected on the same slide 24 hours post L4 stage, permeabilized and fixed as described above. After RNase A treatment, germ cells were stained with propidium iodide (40 µg/ml). Slides were mounted and z-stacks were imaged at 0.3 µm with a 63× objective on an SP2 confocal microscope (Leica) at maximum resolution, using the same parameters for each slide. We then reconstructed each germ line in three dimensions using Imaris v.5.5 software (Bitplane). To determine the quantity of genomic DNA per germ cell nucleus, we quantified the fluorescence of each pixel contained within the volume of the nucleus. To define a 2N- and 4N-equivalent amount of genomic DNA, we used germ cell nuclei in anaphase as a reference, in which each mass of DNA was assumed to have a 2N DNA content. The nuclei contained in the mitotic region were classified according to their DNA content (2N, 2N<X<4N, 4N or >4N).

RESULTS

LRR-1 (F33G12.4) is a substrate-recognition subunit of a CRL2 complex in C. elegans
Cullin-RING E3 ligases (CRLs) are multisubunit enzymes that comprise cullin-based catalytic cores and exchangeable substrate-recognition modules, which selectively recruit substrates for ubiquitylation by an associated E2 enzyme (Petroski and Deshaies, 2005). In Cullin 2-based ubiquitin ligases (CRL2), the substrate-recognition module comprises the adaptor protein Elongin C (ELC-1), which, along with Elongin B (ELB-1), bridges the interaction between the N-terminal part of Cullin 2 (CUL-2) and the BC/Cul-2 box-containing protein that selectively recruits substrates (Fig. 1A). The BC/Cul-2 box includes two short sequences, termed BC and the Cul-2 box, that are required for ELC-1 and CUL-2 binding, respectively (Vasudevan et al., 2007; Mahour et al., 2008). Through proteomic screens in mammalian cells (HEK293T), we and others have identified LRR-1 as a potential substrate-recognition subunit of a CRL2 complex (Kamura et al., 2004; Olma et al., 2009). Although LRR-1 is evolutionarily conserved, its function remains elusive.
The *C. elegans* genome encodes one open reading frame with similarities to human LRR-1 (PPIL5 – Human Genome Nomenclature Committee) (Flicek et al., 2010). The uncharacterized F33G12.4 protein (henceforth called LRR-1) has a similar organization and, like its human counterpart, contains several leucine-rich repeats (LRRs) followed at the C-terminus by a putative BC/Cul-2 box (residues 336-375) (Fig. 1B). Multiple sequence alignment of LRR-1 with selected BC/Cul-2 box-containing proteins from *C. elegans* revealed that these two motifs are conserved in LRR-1. In particular, the key residues that mediate binding to ELC-1 and CUL-2 (Stebbins et al., 1999; Vasudevan et al., 2007; Mahrour et al., 2008) are invariant in LRR-1 (Fig. 1B), suggesting that it is a bona fide substrate-recognition subunit of a CRL2 complex in *C. elegans*.

To test this hypothesis, we expressed LRR-1 in *E. coli* and performed in vitro binding experiments with CRL2 components. The LRR-1 polypeptide, however, failed to fold properly in bacteria and was consistently recovered in the insoluble fraction of the cell extract (data not shown). To circumvent this problem, we co-expressed 6xHis-LRR-1 together with ELC-1 and ELB-1 from a polycistronic vector (see Materials and methods). Under these conditions, 6xHis-LRR-1 was readily recovered in the soluble fraction of the bacterial extract and was retained on nickel beads in a complex containing ELB-1 and ELC-1 (Fig. 1C, lane 2), whereas neither ELB-1 nor ELC-1 was retained on nickel beads in the absence of LRR-1 (Fig. 1C, lane 4). Importantly, the domain encompassing the LRR-1 BC/Cul-2 box (residues 278-399) was sufficient to co-purify ELC-1 and ELB-1 (Fig. 1C, lane 6). LRR-1 also bound CRL2 components in vivo, as shown by its specific co-precipitation from total worm extracts with a FLAG-tagged version of CUL-2 (Fig. 1D).

*C. elegans* LRR-1 therefore has the hallmarks of a substrate-recognition subunit of a CRL2 complex: (1) the presence of a BC/Cul-2 box and an additional protein-protein interaction domain (LRRs); (2) a physical interaction with ELC-1 and ELB-1 in a BC/Cul-2 box-dependent manner; and (3) an association with CUL-2 in vivo.

**LRR-1 is a nuclear protein and its localization depends on CUL-2 and ELB-1**

Next, we analyzed the subcellular localization of LRR-1 by immunofluorescence using specific antibodies (Fig. 2). Given that *lrr-1* mRNA is germ line enriched (Reinke et al., 2000), we examined LRR-1 protein levels and localization in the adult germ line and in early embryos. The *C. elegans* germ line consists of two ‘U-shaped’ tubes, each of which contains a syncitium of hundreds of nuclei subdivided into a mitotic zone and a meiotic zone (Fig. 2A) (Kimble and Crittenden, 2007). The mitotic zone is located at the distal end of each tube and contains the mitotic germ cells, which serve as transient amplifying cells (Kimble and White, 1981). As germ nuclei move proximally, they exit the mitotic cycle to enter meiosis. As shown in Fig. 2A, LRR-1 was present throughout the germ line in both the mitotic and meiotic zones, where it localized to the nucleus, but it appeared to be excluded from the chromosomes as no co-staining with DAPI could be detected. In early embryos, LRR-1 was also enriched in the nuclear compartment, and its localization was dependent on CUL-2 and ELB-1.
nucleus, but was excluded from chromatin during mitosis (Fig. 2B). We confirmed the nuclear localization of LRR-1 by analysis of a transgenic line expressing a functional GFP::LRR-1 fusion protein (Fig. 2D). Notably, LRR-1 was no longer nuclear in cul-2(RNAi) and elb-1(RNAi) embryos (7 of 7 and 27/27 embryos, respectively; Fig. 2B) and accumulated in the cytoplasm. However, LRR-1 protein levels remained unchanged under these conditions (Fig. 2C).

We conclude that LRR-1 is a nuclear protein in the germ line and in early embryos, in which its proper localization requires core subunits of the CRL2 complex.

**LRR-1 is essential for cell cycle progression in the C. elegans germ line**

To determine the function of LRR-1, we characterized *tm3543*, a null allele of *lrr-1* (Fig. 3A). Given that *lrr-1* is a maternal-effect gene, homozygous *lrr-1(tm3543)* embryos derived from heterozygous mothers are rescued by the maternal gene product and develop until adulthood. They systematically develop into sterile adults, however, with a protruding vulva. During the course of vulval development, 22 cells are produced in wild-type animals (Sternberg, 2005); by contrast, *lrr-1(tm3543)* mutants produced a reduced number of vulva cells, resulting in a small and asymmetric L4 invagination (*n*=20). Consequently, following eversion, vulvae from *lrr-1* animals protruded abnormally. These phenotypes were fully rescued by wild-type *lrr-1* transgenes, demonstrating that they were caused by the *lrr-1(tm3543)* mutation (Fig. 3B).

The observation that *lrr-1(tm3543)* animals are sterile and display a protruding vulva suggests that LRR-1 might regulate cell cycle progression in the vulva and in the germ line. Cytological examination of fixed germ lines from *lrr-1(tm3543)* mutant animals revealed a highly disorganized mitotic zone with aberrant nuclei (Fig. 3C, upper panel). The total number of germ cells was severely reduced [48±1 nuclei per gonad arm in *lrr-1* mutants (*n*=15) versus 740±17 nuclei in heterozygous animals (*n*=4)], and the nuclei were unevenly distributed. Moreover, the mutant nuclei were almost twice the diameter of control nuclei [7.68±1.34 μm (*n*=70) versus 4.25±0.66 μm (*n*=40)].

We next analyzed the cell cycle profile of *lrr-1* mutant germ cells by staining wild-type and *lrr-1(tm3543)* mutant germ lines with propidium iodide and determining the DNA content per germ cell by quantifying the fluorescence (see Materials and methods). Most (>40%) *lrr-1(tm3543)* mutant germ cell nuclei were arrested at the DNA content per germ cell by quantifying the fluorescence (see Materials and methods). Most (>40%) *lrr-1(tm3543)* mutant germ cell nuclei were arrested with a 4N DNA content (Fig. 3D). To corroborate this observation, we analyzed the presence of the G2 marker phospho-Y15 Cdk1, the inactive form of Cdk1 (Norbury et al., 1991; Norbury and...
cycle defects observed in the progression (Harper and Elledge, 2007). To test whether the cell Cdc25, the Cdk1 (Y15) phosphatase, thereby preventing cell cycle replication defects or damaged DNA. Stalled replication forks checkpoint to prevent mitotic entry in the presence of DNA phosphorylation of the cell cycle kinase Cdk1 on Y15. This

In eukaryotes, the onset of mitosis is controlled by inhibitory germ line cycle progression in the 

lrr-1(tm3543) than 90% and 60% of atl-1(RNAi); lrr-1(tm3543) and lrr-1(tm3543) of these two genes also restored lrr-1(tm3543) morphology of mutant germ line, as compared with the wild-type

C. elegans germ line.

Consistent with this hypothesis, lrr-1 was identified in a genomewide RNAi-based screen combined with video recording of the first embryonic divisions as a gene required for determining the timing of the P lineage division (Sonnichsen et al., 2005). We confirmed these observations and found that downregulation of lrr-1 by RNAi resulted in a penetrant embryonic lethal phenotype, as more than 95% of the embryos derived from RNAi-treated animals failed to hatch (n=200). Time-lapse GFP fluorescence and differential

The ATL-1/CHK-1 checkpoint pathway prevents cell cycle progression in the lrr-1(tm3543) mutant germ line

In eukaryotes, the onset of mitosis is controlled by inhibitory phosphorylation of the cell cycle kinase Cdk1 on Y15. This mechanism of mitotic control is also used in the DNA replication checkpoint to prevent mitotic entry in the presence of DNA replication defects or damaged DNA. Stalled replication forks activate the ATR/Chk1 checkpoint pathway, which inactivates Cdc25, the Cdk1 (Y15) phosphatase, thereby preventing cell cycle progression (Harper and Elledge, 2007). To test whether the cell cycle defects observed in the lrr-1(tm3543) mutant germ line were dependent on the ATR/Chk1 pathway, we inactivated atl-1 and chk-1 in lrr-1(tm3543) mutants. Interestingly, atl-1 and chk-1 inactivation restored the germ cell proliferation and germ line morphology of lrr-1(tm3543) mutants. More strikingly, inactivation of these two genes also restored lrr-1(tm3543) mutant fertility (Fig. 4A). The suppression phenotype was highly penetrant, as more than 90% and 60% of atl-1(RNAi); lrr-1(tm3543) and chk-1(RNAi); lrr-1(tm3543) animals, respectively, were fertile (n>100), whereas
interference contrast (DIC) microscopy confirmed that the timing of P1 cell division was aberrant in lrr-1(RNAi) embryos (Fig. 5A). Whereas in wild-type embryos, there was invariably a 2-minute delay between anaphase onset in the AB and P1 blastomeres (124±3 seconds, n=8), this time window reached more than 6 minutes in lrr-1(RNAi) embryos (390±59 seconds, n=9). Consistent with LRR-1 being part of a CRL2 complex, similar, although more severe, phenotypes were observed upon inactivation of the core CRL2 subunit ELB-1 (1059±59 seconds, n=10). The P1 blastomere was delayed prior to mitosis in these embryos as confirmed by the accumulation of phospho-Y15 CDK-1 (Fig. 5B). As anticipated, this delay was dependent on the ATL-1/CHK-1 pathway as it was entirely suppressed in atl-1(tm853) mutant embryos (Fig. 5C,D). We also confirmed these observations by evaluating atl-1(RNAi); lrr-1(tm3543) embryos expressing histone H2B fused to GFP and similarly observed that atl-1 was epistatic to lrr-1(tm3543) with respect to cell cycle timing: the elapsed time between AB and P1 anaphase was 86±1.02 seconds in atl-1(RNAi); lrr-1(+/–) embryos (n=11) versus 90±0.79 seconds in atl-1(RNAi); lrr-1(–/–) embryos (n=20). Taken together, these genetic data indicate that the ATL-1/CHK-1 pathway is activated in early embryos upon inactivation of the CRL2LRR-1 complex.

To demonstrate molecular activation of this pathway, we used an antibody that recognizes the activated form of CHK-1 (phospho-S345 CHK-1). Under our conditions, this antibody stained the P granules and centrosomes in wild-type embryos (n=40), but this staining was not significantly reduced in chk-1(RNAi) or atl-1(RNAi) embryos (n=59 and n=15, respectively). By contrast, nuclear staining was specifically detected in lrr-1(RNAi) and elb-1(RNAi) embryos (38 of 44 and 41 of 48 embryos, respectively) (Fig. 5E), demonstrating that CHK-1 was specifically activated upon inactivation of the CRL2LRR-1 complex. These observations indicate that LRR-1 affects the AB-P1 asynchrony of cell division in the early C. elegans embryo by negatively regulating the DNA replication checkpoint. LRR-1 has no impact, however, on the levels or localization of PLK-1 (data not shown) or CDC-25.1 (Fig. 5F), which localize asymmetrically between the AB and P1 blastomeres and regulate the AB-P1 asynchrony of cell division (Budirahardja and Gonczy, 2008; Rivers et al., 2008).

ssDNA-RPA-1 nuclear foci activate the DNA replication checkpoint in lrr-1 mutants

Why is the ATL-1/CHK-1 checkpoint pathway hyperactivated upon lrr-1 inactivation? Checkpoint activation may arise from defects in DNA replication and its integrity, or, alternatively, the checkpoint might be constitutively activated independently of DNA replication defects. The finding that ATL-1/CHK-1 pathway inactivation readily suppressed lrr-1(tm3543) mutant sterility suggests that this pathway is not activated as a consequence of severe DNA replication defects. Indeed, if the checkpoint were
activated as a consequence of hampered DNA replication, the lrr-1(tm3543); atl-1(tm853) double mutants would be sterile owing to the persistence of DNA replication defects. Consistent with this prediction, RNAi-mediated inactivation of atl-1 exacerbated the sterility phenotype of the DNA replication mutant div-1(or148), which exhibits inefficient priming of Okazaki fragments (Encalada et al., 2000) (Fig. 6A). Likewise, atl-1(tm853) larvae exposed to low concentrations of hydroxyurea (HU), which induces stalled replication forks by depleting the pool of dNTPs, readily developed into sterile animals, whereas wild-type N2 animals were eightfold more resistant to HU-induced sterility at 8 mM HU (Fig. 6B).

Although these observations suggest that the DNA replication checkpoint is not activated as a consequence of severe DNA replication defects, they do not exclude the possibility that some forms of DNA damage accumulate in the lrr-1 mutants that activate the DNA replication checkpoint. To further investigate this possibility, we analyzed the formation of replication protein A (RPA-1) nuclear foci in lrr-1 mutants using a specific antibody (Lee et al., 2010). RPA-1 binds single-stranded (ss) DNA, and ssDNA-RPA-1 complexes contribute to the recruitment and activation of ATL-1 at sites of DNA damage (Garcia-Muse and Boulton, 2005). Unlike wild-type worms, lrr-1 mutants exhibited stretches of ssDNA, as evidenced by the accumulation of RPA-1 nuclear foci, and this phenotype was even more pronounced in atl-1(RNAi); lrr-1(tm3543) animals (Fig. 6C). Taken together, these observations indicate that a form of DNA damage accumulates in lrr-1 mutants and activates the DNA replication checkpoint.

To determine the origin of this DNA damage, we analyzed the cell cycle profile of atl-1(RNAi); lrr-1(tm3543) germ cell nuclei after propidium iodide staining, as described previously in Fig. 3D.
Remarkably, this analysis revealed that a large proportion of germ cells (30%) showed a DNA content greater than 4N in atl-1(RNAi); lrr-1(tm3543) mutants (Fig. 6D), suggesting that some regions of the genome might have undergone DNA re-replication under these conditions. Notably, this analysis also showed that a small percentage of germ cell nuclei (<2%) showed a DNA content greater than 4N in the single lrr-1(tm3543) mutant, whereas this phenotype was never observed in wild-type or atl-1(RNAi) animals (Fig. 6D).

Taken together, these observations suggest that LRR-1 is required to suppress re-replication. The underlying molecular mechanisms, however, are currently unclear. Interestingly, CUL-2 has been previously implicated in the degradation of the CDK inhibitor CKI-1 (Feng et al., 1999), and it has been shown, at least in mitotic cells, that CKI-1 accumulation in S phase contributes to the re-replication phenotype (Kim et al., 2008). Therefore, we investigated whether LRR-1 might be involved in CKI-1 degradation. Although we could detect a specific physical interaction between LRR-1 and CKI-1 in a heterologous human HEK293T cell system (Fig. 6E), cki-1 depletion by RNAi (feeding, soaking or injection) failed to suppress cell cycle arrest in C. elegans lrr-1 germ cells, suggesting that CKI-1 might not be the only LRR-1 target. Nevertheless, CKI-1 accumulation might contribute to the observed re-replication phenotype.

**DISCUSSION**

We have identified the leucine-rich repeat protein LRR-1 in *C. elegans* and shown that it functions as a substrate-recruitment subunit of a Cullin 2-based E3 ligase with an essential role in cell cycle progression. lrr-1(tm3543) homozygous mutants develop into sterile adults with a small germ line. This severe reduction in germ cell number is due to a failure in germ cell proliferation. Consistent with a proliferation defect, we observed cell cycle arrest in G2 phase in these mutants due to hyperactivation of the DNA replication checkpoint, as this phenotype was entirely suppressed by inactivation of the DNA replication checkpoint genes *atl-1* and *chk-1*. Importantly, inactivation of these genes not only released the cell cycle block, but also rescued fertility of the lrr-1(tm3543) mutants, suggesting that LRR-1 is required for mitotic proliferation of the germ line but is dispensable for meiosis. Similar to the situation in germ cells, RNAi inactivation of *lrr-1* in early embryos led to hyperactivation of the DNA replication checkpoint, causing a cell cycle delay in the division of the P lineage and resulting in embryonic lethality. Hyperactivation of this checkpoint is not constitutive in *lrr-1* mutants, but is likely to be a consequence of the accumulation of stretches of ssDNA, which may arise due to DNA re-replication (Fig. 7). Collectively, our observations identify the CRL2LRR-1 complex as an important determinant of replication integrity and genome stability in *C. elegans*.

**Identification of a Cullin 2-based E3 ligase containing the LRR-1 protein in *C. elegans***

The LRR-1 protein is evolutionarily conserved, but its function remains elusive. Protein sequence analysis revealed that LRR-1 contains a typical BC/Cul-2 box at its C-terminus and binds other CRL2 subunits both in vitro and in vivo in a BC/Cul-2 box-

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**Fig. 6. LRR-1 controls DNA replication integrity.** (A) The percentage of fertile animals of the indicated genotypes after a control treatment or RNAi inactivation of *atl-1*. The results are representative of three independent experiments. (B) The percentage of fertile wild-type and *atl-1(tm853)* animals upon exposure to 6 and 8 mM hydroxyurea (HU). Error bars in A and B represent ± s.d. (C) Representative images of mitotic germ lines of the indicated genotypes stained with an RPA-1 antibody (green) and counterstained with DAPI (blue). Scale bars: 20 μm. (D) The percentage of germ cell nuclei of the indicated genotypes with a DNA content of 2N (white), between 2N and 4N (gray), 4N (dark gray) and greater than 4N (black). Wild type, *n* = 389; *atl-1(RNAi)*, *n* = 923; *lrr-1(tm3543)*, *n* = 251; and *atl-1(RNAi); lrr-1(tm3543)*, *n* = 691 nuclei. (E) FLAG::LRR-1 immunoprecipitates from HEK293T cells expressing GFP or GFP::CKI-1 were immunoblotted with specific GFP (top) and FLAG (bottom) antibodies.
Hyperactivation of the DNA replication checkpoint upon loss of the CRL2\textsuperscript{LRR-1} complex

Phenotypic analysis of \textit{lr-1(tm3543)} mutants and \textit{lr-1(RNAi)} embryos revealed that the DNA replication checkpoint was hyperactivated in the absence of LRR-1. Activation of this checkpoint culminates in the recruitment and activation of ATR kinase at sites of DNA damage or stalled replication forks (Cimprich and Cortez, 2008; Segurado and Tercero, 2009; Zegerman and Diffley, 2009). When DNA polymerases stall, the replicative minichromosome maintenance (MCM) helicases continue to unwind the DNA ahead of the fork, generating ssDNA. ssDNA-RPA-1 nuclear foci then recruit and activate ATL-1, which, together with the CHK-1 kinase, prevents CDK-1 activation (dephosphorylation via CDC-25) and cell cycle progression.

However, we did not detect massive DNA damage or replication failure in \textit{lr-1} mutants. For example, DNA segregation is often impaired when DNA replication is compromised (Mouysset et al., 2008) and this phenotype is exacerbated in absence of a functional DNA replication checkpoint (Brauchle et al., 2003). Massive DNA segregation defects were not apparent in \textit{lr-1(tm3543)} or \textit{atl-1(RNAi)}; \textit{lr-1(tm3543)} embryos, although some minor DNA bridges were detected by spinning-disk microscopy in the latter case (data not shown). Furthermore, whereas the ATL-1/CHK-1 checkpoint pathway is essential for animal fertility when DNA replication is challenged, it appears to be dispensable in \textit{lr-1} mutants given that \textit{lr-1}; \textit{atl-1} double-mutant animals were fully fertile.

These observations suggest that although DNA damage is relatively minor in \textit{lr-1} mutants, it is sufficient to trigger a checkpoint response, which is consistent with recent data indicating that a stretch of 200-1000 nucleotides is sufficient to trigger a robust checkpoint response, at least in vitro (Choi et al., 2010).

The CRL2\textsuperscript{LRR-1} complex and the integrity of DNA replication

The origin of the DNA damage that occurs in \textit{lr-1} mutants is currently unclear, as ssDNA-RPA-1 is a structural intermediate common to several DNA metabolic processes. Given that \textit{atl-1(RNAi)}; \textit{lr-1} mutant germ cells accumulated with a DNA content greater than 4N, it is tempting to speculate that some regions of the genome might have undergone re-replication. DNA replication is a complex task that involves the activation of a large number of replication origins distributed throughout the chromatin. Strict temporal regulation of DNA replication and its separation in two distinct steps ensure that DNA is replicated only once per cell cycle (Blow and Dutta, 2005; Arias and Walter, 2007). These two steps are regulated by the periodic oscillation of CDK activity during the cell cycle. In a first step, which takes place at the end of mitosis and during early G1, when CDK activity is low, pre-replicative complexes (pre-RCs) assemble on replication origins. In a second step, during S phase, CDKs and DDks (Dbf4-dependent kinases) activate the MCM replicative helicase (Labib, 2010).

During S phase, the assembly of new pre-RCs must be prevented and several mechanisms contribute to pre-RC inactivation, including Cdt1 degradation and Cdc6 nuclear export (Arias and Walter, 2007). In \textit{C. elegans}, the CRL4\textsuperscript{Cdc2} complex is instrumental for pre-RC disassembly by promoting the degradation of the licensing factor CDT-1 (Zhong et al., 2003) and facilitating CDC-6 nuclear export via degradation of the CDK inhibitor CKI-1 (Kim et al., 2008). Interestingly, CUL-2 has been implicated in the degradation of CKI-1 in the germ line (Feng et al., 1999) and LRR-1 physically interacts with CKI-1, suggesting that CKI-1 is an LR-1 target. \textit{cki-1} depletion by RNAi, however, failed to suppress cell cycle arrest in \textit{lr-1} germ cells, suggesting that CKI-1 might not be the only target of LR-1. Consistent with this prediction, CKI-1 levels are unchanged in \textit{cul-2(ek1)} mutant embryos (Feng et al., 1999). Nevertheless, CKI-1 might contribute to the observed phenotype. Further characterization of the CRL2\textsuperscript{LRR-1} complex and identification of its critical target(s) will certainly shed light on this important new mechanism in the maintenance of genome integrity during DNA replication.

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**Fig. 7. Model for CRL2\textsuperscript{LRR-1} function.** Loss of \textit{lr-1} function induces re-replication and causes the accumulation of stretches of ssDNA. ssDNA-RPA-1 nuclear foci then recruit and activate ATL-1, which, together with the CHK-1 kinase, prevents CDK-1 activation (dephosphorylation via CDC-25) and cell cycle progression.
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Competing interests statement
The authors declare no competing financial interests.

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