Development 135, 1259-1269 (2008) doi:10.1242/dev.014969

CDC-25.1 stability is regulated by distinct domains to restrict cell division during embryogenesis in *C. elegans*

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Cdc25 phosphatases are key positive cell cycle regulators that coordinate cell divisions with growth and morphogenesis in many organisms. Intriguingly in *C. elegans*, two *cdc-25.1(gf)* mutations induce tissue-specific and temporally restricted hyperplasia in the embryonic intestinal lineage, despite stabilization of the mutant CDC-25.1 protein in every blastomere. We investigated the molecular basis underlying the CDC-25.1(*gf*) stabilization and its associated tissue-specific phenotype. We found that both mutations affect a canonical β-TrCP phosphodegron motif, while the F-box protein LIN-23, the β-TrCP orthologue, is required for the timely degradation of CDC-25.1. Accordingly, depletion of *lin-23* in wild-type embryos stabilizes CDC-25.1 and triggers intestinal hyperplasia, which is, at least in part, *cdc-25.1* dependent. *lin-23*(RNAi) causes embryonic lethality owing to cell fate transformations that convert blastomeres to an intestinal fate, sensitizing them to increased levels of CDC-25.1. Our characterization of a novel destabilizing *cdc-25.1(lf)* intragenic suppressor that acts independently of *lin-23* indicates that additional cues impinge on different motifs of the CDC-25.1 phosphatase during early embryogenesis to control its stability and turnover, in order to ensure the timely divisions of intestinal cells and coordinate them with the formation of the developing gut.

KEY WORDS: cdc-25.1, lin-23, F-box, β-TrCP, Intestine, Hyperplasia, Cell fate, Embryogenesis, *C. elegans*

INTRODUCTION

Precise spatiotemporal coordination between developmental pathways and the cell cycle machinery is essential to ensure the timely and accurate growth and differentiation of cells and organs. In many cells, this is controlled at the G1-S phase transition, while in others this coordination occurs at the G2-M boundary. These transitions are modulated by the action of key cell cycle regulators that ultimately fine-tune the activity of cyclin/cyclin-dependent kinase (cyclin/CDK) complexes (Nigg, 2001; Nurse et al., 1998; Pines, 1995). This regulation can occur at multiple levels, including cell cycle phase-dependent transcriptional modulation of CDKs, cyclins and cyclin-dependent kinase inhibitors (CKIs) coupled with activating or inhibitory phosphorylation events, subcellular relocalization and/or ubiquitin-mediated degradation of key cell cycle regulators. Together, these regulatory controls ensure the integrity and the faithful segregation of the genome throughout successive division cycles (Ang and Harper, 2005; DeSalle and Pagano, 2001; Morgan, 1995; Nilsson and Hoffmann, 2000; Schafer, 1998).

During unperturbed cycles, the timely degradation of cell cycle regulators is coordinately controlled by two major ubiquitin E3 ligases, the anaphase-promoting complex/cyclosome (APC/C) and the Skp1/Cullin/F-box (SCF) E3 ligase that trigger irreversible progression into the next phase (Castro et al., 2005; Nakayama and Nakayama, 2006; Vodermaier, 2004). Interestingly, after incomplete replication or DNA damage, the cells rely on checkpoint pathways that impinge on the same E3 ubiquitin ligases to delay the cell cycle phase transition and to allow for repair of the defect, thus preventing genomic instability (Bartek et al., 2004; Sancar et al., 2004).

The Cdc25 phosphatase is a key positive regulator of cell cycle progression through its ability to remove inhibitory phosphates from cyclin/CDK complexes (Moreno et al., 1990; Sebastian et al., 1993). Multicellular organisms often contain multiple Cdc25 paralogues with overlapping, yet distinct, phase- and tissue-specific functions. In mammals, Cdc25A activates cyclinE(A)/Cdk2 for progression into S phase, while cooperating with Cdc25B and Cdc25C to promote G2/M transition (Boutros et al., 2006). The ubiquitin-mediated degradation of the different Cdc25 paralogues is triggered by the APC/C^{Cdh1} (activator=Cdc20 homologue) at the end of mitosis through recognition of a KEN box, while during S and G2 phase the abundance of Cdc25A is regulated by the $SCF^{\beta TrCP}$ (F-box=β-transducin repeat-containing protein) E3 ubiquitin ligase that recognizes a phosphorylated β-TrCP-like destruction motif ($DSGX_4S$) (Busino et al., 2003; Donzelli et al., 2002; Jin et al., 2003). This $SCF^{\beta TrCP}$-mediated degradation is accelerated after DNA damage during S and G2 phase in an ATM/Chk2-ATR/Chk1-dependent manner (Falck et al., 2001; Sorensen et al., 2003).

Cdc25 has also been shown to integrate spatiotemporal information to coordinate timely cell cycle progression with developmental processes. In *Drosophila melanogaster*, the two Cdc25 paralogues *twine* and *string* have mutually complementing and overlapping functions in the female germ line and in early embryos, respectively (Edgar and Datar, 1996). During embryogenesis the zygotic expression of *string* becomes the limiting factor for successive cell divisions in the mitotic domains of the fly embryo (Edgar and O'Farrell, 1989; Edgar and O'Farrell, 1990; Foe, 1989). Transcription of *string* is highly regulated through elements in it large, complex promoter region (Edgar et al., 1994; Lehman et al., 1999), while degradation of *string* protein is also developmentally controlled by an ubiquitin-mediated pathway involving *tribbles* (Mata et al., 2000).

The *C. elegans* genome encodes four *cdc-25* paralogues (Ashcroft et al., 1998), among which *cdc-25.1* shows the greatest homology to the human Cdc25A gene. *cdc-25.1* is expressed in the hermaphrodite germ line where it sustains germ cell proliferation (Ashcroft et al., 1999). Maternal *cdc-25.1* gene product is contributed to oocytes and early embryos and is required for completion of meiosis and to ensure timely mitotic divisions until it is degraded after the 28-cell stage (Ashcroft and Golden, 2002). Consistent with its role in

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Accepted 29 January 2008

regulating proliferation, it has been considered a proto-oncogene in humans, and overexpression of *cdc-25.1* results in unscheduled S phase initiation (Kostic and Roy, 2002). We and others previously reported that gain-of-function (*gf*) mutations in *cdc-25.1* cause embryonic hyperplasia restricted to the intestinal (E) lineage (Clucas et al., 2002; Kostic and Roy, 2002). The *gf* mutant CDC-25.1(*rr31*) protein becomes stabilized and is still present in all embryonic cells past the 100-cell stage. Despite this abnormal perdurance, CDC-25.1(*rr31*) induces only a single supernumerary division in the intestinal precursor cells at a specific time after the embryonic E^8-stage (embryo containing eight intestinal cells).

To determine whether the increased stability of the mutant protein and the hyperplasia are linked, we developed a means to test candidate genes that could influence the stability of CDC-25.1, which in turn would provide further clues to explain the tissue specificity of this mutation. One candidate, the *C. elegans* orthologue of the mammalian F-box protein β-TrCP, LIN-23 is required for the timely degradation of CDC-25.1 during early embryogenesis and its differential embryonic expression may shed some light on the tissue specificity of *cdc-25.1(gf)*. In addition, we report the isolation and characterization of a viable loss-of-function intragenic suppressor of *cdc-25.1(gf)* that affects a novel site required for CDC-25.1 stability, suggesting that multiple controls contribute to the stabilization of this cell cycle regulator through independent domains to control its activity appropriately during embryogenesis.

MATERIALS AND METHODS

Strains and culture

All *C. elegans* strains were cultured at 20°C according to standard procedures (Brenner, 1974) unless stated otherwise. The following alleles and transgenes were used for this study: wild-type Bristol (N2); *cdc-25.1(rr31)I; cdc-25.1(ij48)I; cdc-25.1(rr31rr36)I; lin-23(ot1)II; lin-23(e1883)II*; *unc-119(ed3)III; rrIs01[elt-2::gfp; unc-119+]X*; *rrIs12[pie-1::gfp::lin-23; unc-119+]*; *wIs137[end-3::end-3(P202L)::gfp; rol-6D]* (gift from M. Maduro); *otEx731[lin-23::gfp; rol-6(su1006)]; otEx840[lin-23::lin-23::gfp; rol-6(su1006)]*; *rrEx128/129/130[end-3::gfp::cdc-25.1(wt); unc-119+]; rrEx131/132/133[end-3::gfp::cdc-25.1(rr31); unc-119+]; rrEx155/156/157[end-3::gfp::cdc-25.1(rr31rr36); unc-119+]*; *rrEx212/213[end-3::gfp::cdc-25.1(rr36); unc-119+]; rrEx134/135/136/137 [end-3::gfp::cdc-25.1(wt); elt-2::gfp]; rrEx138/139/140[end-3::gfp::cdc-25.1(rr31); elt-2::gfp]; rrEx161/162/163[end-3::gfp::cdc-25.1(rr31rr36); elt-2::gfp]; rrEx148/149/150/206/207/208/209 [end-3::lin-23; inx-6::gfp]* and *rrEx152/153/154[end-3::lin-23(ΔSalI); inx-6::gfp]*.

DNA constructs

pMR174 *end-3::gfp* was generated by cloning the 990 bp *Bam*HI/*Spe*I GFP fragment from pJH4.52 (Strome et al., 2001) between a 1455 bp *Hin*dIII/*Bam*HI *end-3* promoter fragment (no *end-3* coding sequence) and a 270 bp *Spe*I/*Apa*I *end-3* 3′UTR fragment amplified from pMM446 *end-3::end-3(P202L)::gfp* (gift from M. Maduro) into pPD49.26*ΔHin*dIII/*Apa*I. pMR513 *end-3::gfp::cdc-25.1(*wild-type*)*, pMR514 *end-3::gfp::cdc-25.1(rr31)* and pMR556 *end-3::gfp::cdc-25.1(rr31rr36)* were generated by inserting a 1818 bp *Spe*I fragment of *cdc-25.1* cDNA from wild type, *cdc-25.1(rr31)* and *cdc-25.1(rr31rr36)* mutants, respectively, into the *Spe*I site of pMR174. pMR579 *end-3::gfp::cdc-25.1(rr36)* was generated by PCR-mediated mutagenesis from pMR513. pMR562 *end-3::lin-23* was constructed by inserting a wild-type 2.6 kb *Bgl*II/*Spe*I fragment of genomic *lin-23* into pMR174*ΔBam*HI/*Spe*I. pMR572 *end-3::lin-23(ΔSalI)* was generated by removing the 1.9 kb *Sal*I fragment from pMR562. pMR576 *pie-1::gfp::lin-23* was constructed by inserting a wild-type 2.6 kb *Spe*I fragment of genomic *lin-23* into pJH4.52 *ΔSpe*I together with a rescuing *unc-119+* fragment into the *Sac*II site. pMR911 *inx-6::gfp* was created by introducing a 3.1 kb *Sph*I/*Xba*I fragment of the *inx-6* promoter into pPD95.77 and used as a transformation marker. Primer sequences are available upon request.

Microinjection and transformation

C. elegans strains were transformed as previously described (Mello et al., 1991). Plasmids were injected at a concentration of 25 ng/μl. At least three independent transgenic lines were analysed for each construct. pMR174, pMR513, pMR514, pMR556 and pMR579 were microinjected into *unc-119(ed3)* with the *unc-119+* rescuing plasmid pDP#MM051 (Maduro and Pilgrim, 1995) or into N2 with the intestinal-specific pJM67 *elt-2::gfp* construct (Fukushige et al., 1999). pMR562 was microinjected into *rrIs01*, (*cdc-25.1(rr31); rrIs01*) and *rrEx134[end-3::gfp::cdc-25.1(wt); elt-2::gfp]* animals with pMR911 as a marker. pMR572 was microinjected into *rrEx134[end-3::gfp::cdc-25.1(wt); elt-2::gfp]* animals with pMR911.

Germline expression of *pie-1::gfp::lin-23* was achieved by bombarding pMR576 into *unc-119(ed3)* worms as previously described (Praitis et al., 2001). A similar expression pattern was detected with a complex array (Kelly et al., 1997).

RNA interference

lin-23(RNAi) was performed by injecting 1 μg/μl *lin-23* dsRNA into young adults (Fire et al., 1998). Embryos were dissected from 36 and 48 hour post-injection hermaphrodites and mounted for microscopic observation. Feeding RNA interference experiments were carried out according to standard procedures (Kamath et al., 2003). Hypomorphic RNAi was achieved by shortening the time during which animals were fed on dsRNA producing bacteria.

Semi-clonal screen for maternal effect suppressors of *cdc-25.1(rr31)* and cloning of *rr36*

MR142 *cdc-25.1(rr31); rrIs01* animals were mutagenized at the L4 stage with 40 mM ethylmethanesulfonate (EMS) (Brenner, 1974). The F3 generation was screened under a fluorescent dissecting microscope for animals with reduced *cdc-25.1(rr31)*-dependent hyperplasia. A total of 5456 haploid genomes were analyzed and five suppressors isolated. Owing to its strong suppression and tight genetic linkage with *cdc-25.1(rr31)*, we hypothesized *rr36* represented an intragenic suppressor. Independent sequencing analyses of MR183 *cdc-25.1(rr31rr36)* identified the second mutation. *rr36* is a typical G to A EMS-induced transition that affects nucleotide 817 of *cdc-25.1* cDNA.

Microscopy and image processing

Light microscopy, image analysis and processing were performed essentially as previously described (Kostic and Roy, 2002).

RESULTS

cdc-25.1(gf) mutations that cause supernumerary cell divisions in the E lineage affect a highly conserved β-TrCP-like phosphodegron

We previously described a G47D amino acid change in CDC-25.1(*rr31*) that stabilizes the protein in all embryonic cells (Kostic and Roy, 2002). In an independent study, a S46F substitution in CDC-25.1(*ij48*) was identified and found to cause a similar intestinal-specific hyperplasia (Clucas et al., 2002), indicating that this domain plays an important regulatory function. We therefore sought to better understand when and how these *gf* mutations affect CDC-25.1 stability, in addition to providing insights concerning the molecular mechanisms responsible for the tissue-specific cell cycle defect associated with both *cdc-25.1(gf)* mutations.

Both S46F and G47D mutations affect adjacent residues in the N-terminal regulatory domain of the phosphatase, and although the kinetics of CDC-25.1(*ij48*) turnover have not been described, both proteins appear to behave similarly at higher temperatures (Table 1). Sequence analysis of the affected region predicts a conserved $DSGX_4S$ motif previously described as a phosphodegron recognized by the F-box protein β-TrCP when phosphorylated on both serines (Fig. 1) (Fuchs et al., 2004). It is therefore quite plausible that both mutations enable the corresponding CDC-25.1(*gf*) proteins to escape

Table 1. *cdc-25.1(rr31)* and *cdc-25.1(ij48)* are temperature-sensitive mutations that cause supernumerary cell divisions exclusively in the developing *C. elegans* intestine

	Number of intestinal cells in L1 hatchlings at the indicated temperature*		
Genotype	15°C	20°C	25°C
Wild type (N2)	20 (*n*=40)	20 (*n*=40)	20 (*n*=40)
cdc-25.1(rr31)	42.7±3.8 (*n*=30)	40.8±3.6 (*n*=40)	20.4±1.1 (*n*=40)
cdc-25.1(ij48)	38.3±3.9 (*n*=30)	37.4±3.4 (*n*=30)	20.6±1.3 (*n*=30)

*All strains carry an integrated intestinal-specific *elt-2::gfp* reporter to allow accurate quantification of intestinal cell numbers.

timely degradation during embryogenesis by independently affecting two highly conserved residues within a potential β-TrCP-like phosphodegron.

Enhanced stability of CDC-25.1 perturbs cell cycle timing in a specific developmental window

Although the identified CDC-25.1(*gf*) mutations affect the appropriate turnover of CDC-25.1, whether this enhanced stability is responsible for the supernumerary E divisions observed remains questionable. This is particularly intriguing as CDC-25.1(*rr31*) perdures in every embryonic cell beyond the 100-cell stage (corresponding to E^8 stage), whereas only the intestinal lineage is affected (Kostic and Roy, 2002). Therefore, to test whether an alteration of the putative β-TrCP-like phosphodegron stabilizes CDC-25.1 and whether this stabilization is the basis of the intestinal hyperplasia, we designed a degradation assay to assess the stability and the function of the wild-type and CDC-25.1(*rr31*) proteins during *C. elegans* embryogenesis. By taking advantage of the early intestinal-specific expression of *end-3* (Maduro et al., 2005), we monitored the kinetics of wild-type and gain-of-function GFP-tagged CDC-25.1 variants in the E lineage. In wild-type animals that express the wild-type variant GFP::CDC-25.1[WT], nuclear fluorescence was detected from the E^2 to the E^8 stage with a rapid loss of signal within the first 20-25 minutes of E^8 (*n*=30/30), consistent with the GFP::CDC-25.1[WT] fusion protein being degraded during this period (Fig. 2). The gain-of-function GFP::CDC-25.1[G47D] variant, however, was still detectable ~120 minutes after the E^8 stage before it completely disappeared (*n*=30/30). Furthermore, the GFP::CDC-25.1[G47D] variant consistently triggered an extra mitosis at the stage when *cdc-25.1(rr31)* mutant embryos display their supernumerary division, around 40-45 minutes into the E^8 stage (~200-cell stage) (Fig. 2).

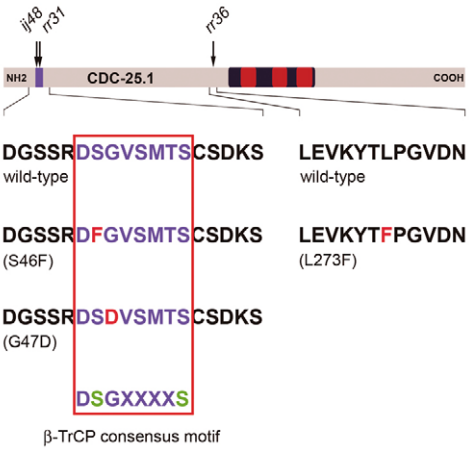

Fig. 1. Two *cdc-25.1(gf)* mutations affect a putative β-TrCP-like phosphodegron, while *rr36* corresponds to a new temperature-sensitive *lf* mutation. Schematic representation of the CDC-25.1 phosphatase highlighting the corresponding amino acid changes: the two gain-of-function mutations within the β-TrCP motif (blue) in the N-terminal regulatory domain and the intragenic *rr36* mutation adjacent (N-terminal) to the catalytic phosphatase domains (black and red) (see Materials and methods for description). The amino acid changes are in red. The consensus β-TrCP phosphodegron motif is boxed, with its phosphorylable serines in green.

By scoring intestinal cells in L1 hatchlings, we found that transgenic animals expressing GFP::CDC-25.1[WT] underwent normal intestinal development and had ~20 intestinal cells (9/10 independent transgenic lines), whereas fewer than 3% (*n*=200) exhibited slight intestinal hyperplasia (<25 intestinal cells). One exception was observed (wild-type#4) where 84% of the animals displayed modestly increased intestinal cell counts (~30 intestinal cells) owing to higher transgene copy numbers (Table 2 and data not shown). Conversely, 90% (*n*=200) of the animals expressing GFP::CDC-25.1[G47D] displayed severe intestinal hyperplasia with ~40 intestinal cells (10/10 independent transgenic lines), similar to what is detected in both *cdc-25.1(gf)* mutants at 20°C (compare Table 1 with Table 2). All the transgenic lines that displayed intestinal hyperplasia (wild-type#4 and the gain-of-function CDC-25.1 variants) were suppressed by *cdc-25.1*(RNAi), confirming that the cell cycle defects observed in these animals were due to the *cdc-25.1* gene product (data not shown). No intestinal defects were seen post-embryonically in any of the transgenic animals.

These results indicate that the degradation machinery that destabilizes CDC-25.1 is still functional at the beginning of the embryonic E^8 stage, while confirming that the G47D change caused by *rr31* perturbs the timely turnover of the phosphatase, causing it to perdure beyond this stage. Our findings also reveal a window of sensitivity in the E^8 stage during which the intestinal cells can

Table 2. Expression of an intestinal-specific *cdc-25.1(rr31)* transgene triggers hyperplasia, which is suppressed by the intragenic *rr36* mutation

	Number of intestinal cells in L1 hatchlings expressing *end-3::gfp::cdc-25.1**		
	Wild type	*rr31*	*rr31rr36*
Line 1	20.5±2.4 (*n*=30)	40.7±10.4 (*n*=40)	20.4±1.3 (*n*=40)
Line 2	21.2±2.4 (*n*=30)	40.2±7.0 (*n*=40)	20.6±1.7 (*n*=40)
Line 3	21.6±3.3 (*n*=40)	37.5±7.8 (*n*=40)	20.2±0.6 (*n*=40)
Line 4†	30±6.8 (*n*=40)		

*In all cases, wild-type N2 animals were injected with the indicated variant of the *cdc-25.1* transgene together with the intestinal marker *elt-2::gfp* for quantification of intestinal cell numbers. The results of three typical transgenic lines are shown for each construct at 20°C.
†The wild-type extrachromosomal line 4 contains a higher copy number transgenic array compared with the other three transgenic strains (lines 1-3).

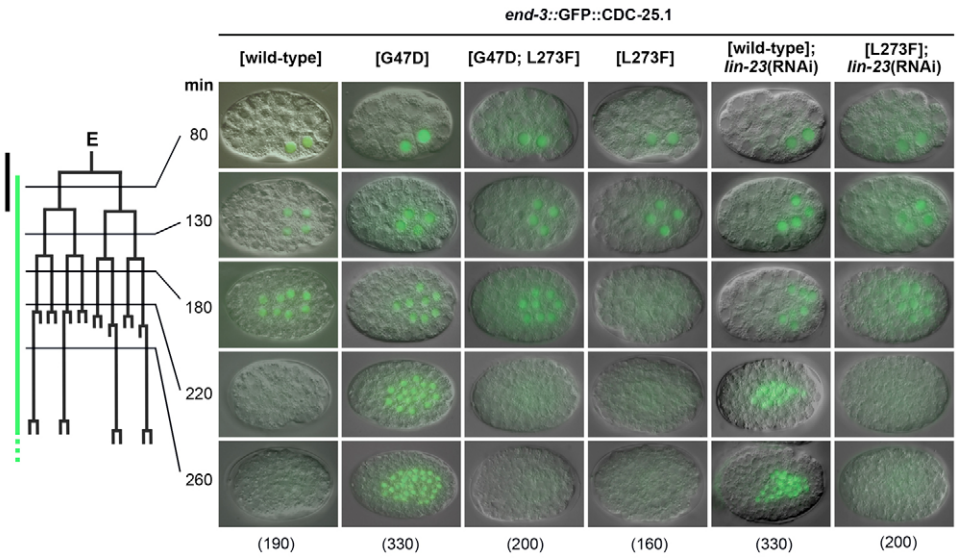

Fig. 2. Stabilization of CDC-25.1 triggers intestinal hyperplasia during a specific developmental window. The wild-type intestinal lineage is shown on the left with its typical division timing in minutes after pronuclear meeting at 20°C. The *end-3* gene is transcribed during the E^1 and E^2 stages (black bar) (Maduro et al., 2007), while GFP alone expressed from the *end-3* promoter is visible from E^2 to threefold stage (~550 minutes) (green bar). DIC/GFP overlays of wild-type transgenic embryos expressing the indicated GFP::CDC-25.1 fusion protein variants under the control of the *end*-3 promoter. The average time in minutes at which the degradation was complete is shown beneath each column. Degradation of the GFP::CDC-25.1[WT] fusion protein occurs ~20 minutes after the E^{4-8} cell division. GFP::CDC-25.1[G47D] resists degradation for more than 120 minutes and triggers a supernumerary division ~40-45 minutes into the E^8-cell stage, resulting in a precocious E^{16}-like stage and an abnormal hyperplasic E^{32}-cell stage. The intragenic mutation restores proper degradation of GFP::CDC-25.1[G47D;L273F] with kinetics similar to the wild type, while GFP::CDC-25.1[L273F] is destabilized prematurely. Stabilization and hyperplasia typical of GFP::CDC-25.1[G47D] protein are detected in *lin-23*(RNAi)-treated animals expressing GFP::CDC-25.1[WT], while the [L273F] substitution suppresses these phenotypes. These *lin-23*(RNAi) embryos do not undergo cell fate transformation (see text). Exposure time was increased to similar levels in all 220 and 260 minute embryos. All embryos were assayed at 20°C. In the last two columns, these embryos were classified based on their intestinal cell stage, owing to the effect of *lin-23*(RNAi) treatment on the E division timing (Z. Bao, personal communication).

respond to inappropriate levels of CDC-25.1 activity, thus providing a conclusive link between the stabilization of the phosphatase and the observed cell cycle defect.

Loss-of-function in the β-TrCP orthologue *lin-23* triggers intestinal hyperplasia by two independent mechanisms during embryogenesis

As the affected sequence in both *cdc-25.1(gf)* mutations matches a consensus β-TrCP destruction motif, we wondered whether the cell cycle defects observed in *cdc-25.1(rr31)* could be phenocopied by loss-of-function in the *C. elegans* β-TrCP orthologue *lin-23*. LIN-23 was first described as a general negative cell cycle regulator that restricts embryonic and post-embryonic cell divisions (Kipreos et al., 2000), while it is also implicated in proper axon growth and pathfinding (Mehta et al., 2004), and in postsynaptic glutamate receptor recycling through destabilization of β-catenin (Dreier et al., 2005). *lin-23*(RNAi) and *lin-23(0)* embryos arrest without undergoing morphogenesis and display severe embryonic hyperplasia (Kipreos et al., 2000). We quantified the intestinal cells in terminally arrested *lin-23*(RNAi) embryos and counted up to 100 cells instead of the typical 20 or 38-40 observed at similar stages in wild-type and *cdc-25.1(gf)* embryos, respectively (Fig. 3A), indicating that loss of *lin-23* causes more severe intestinal hyperplasia than stabilization of CDC-25.1 alone.

Owing to the involvement of the Wnt pathway in the E cell fate specification (Goldstein, 1992; Huang et al., 2007; Phillips et al., 2007; Rocheleau et al., 1997; Thorpe et al., 1997) and as LIN-23 is known to target at least one β-catenin orthologue (BAR-1) for degradation in *C. elegans* (Dreier et al., 2005), we asked whether the *lin-23*(RNAi)-associated hyperplasia originates from a cell cycle defect or a cell fate transformation in the early embryonic blastomeres that result in extra intestinal cells. Using a combination of intestinal-specific markers, we found that the specification of the E lineage occurs normally at the seven-cell stage in all *lin-23*(RNAi) embryos, but thereafter one or two ectopic E blastomeres were detected in the posterior cells of some embryos (Fig. 3B). Sixty percent of *lin-23*(RNAi) embryos ectopically express the E marker in the descendants of Cp, while around 5% of them also expressed GFP in MSp descendants (n=91). These cell fate transformations were *skn-1* dependent (Bowerman et al., 1992), as none of the *lin-23*(RNAi); *skn-1*(RNAi) double mutant embryos that lack endodermal specification produced any E or ectopic E-like cells (n=50). This is consistent with *lin-23* being at least partially required in Cp and MSp to inhibit the SKN-1-dependent endoderm specification pathway (Maduro et al., 2005; Maduro et al., 2001; Zhu et al., 1997). Thus, these multiple E lineages generate a variable source of intestinal hyperplasia in *lin-23* compromised embryos.

To determine whether the observed *lin-23(*RNAi) hyperplasia is due exclusively to these cell fate transformations or whether it may also depend on stabilization of its putative cell cycle target CDC-25.1 in the E lineages, we performed *lin-23*(RNAi) in wild-type animals that express GFP::CDC-25.1[WT] and monitored the kinetics of its turnover after the E^8-stage. In these *lin-23*(RNAi) embryos, the tagged phosphatase became stabilized compared with control embryos, causing it to perdure much like the GFP::CDC-25.1[G47D] variant. Furthermore, as in the gain-of-function mutants and their corresponding transgenic variants, these animals displayed supernumerary cell divisions in the E lineage (Fig. 2). To quantify the degree to which the E lineage contributes to the *lin-23*(RNAi)-associated hyperplasia, E blastomeres were isolated from *lin-23*(RNAi) embryos by laser ablation. We found that ~40 intestinal cells originated from the E blastomere under these conditions, indicating that these cells undergo a single supernumerary division when *lin-23* is downregulated (Fig. 3C). Thus, through these and reciprocal laser ablation experiments (E ablated) (data not shown),

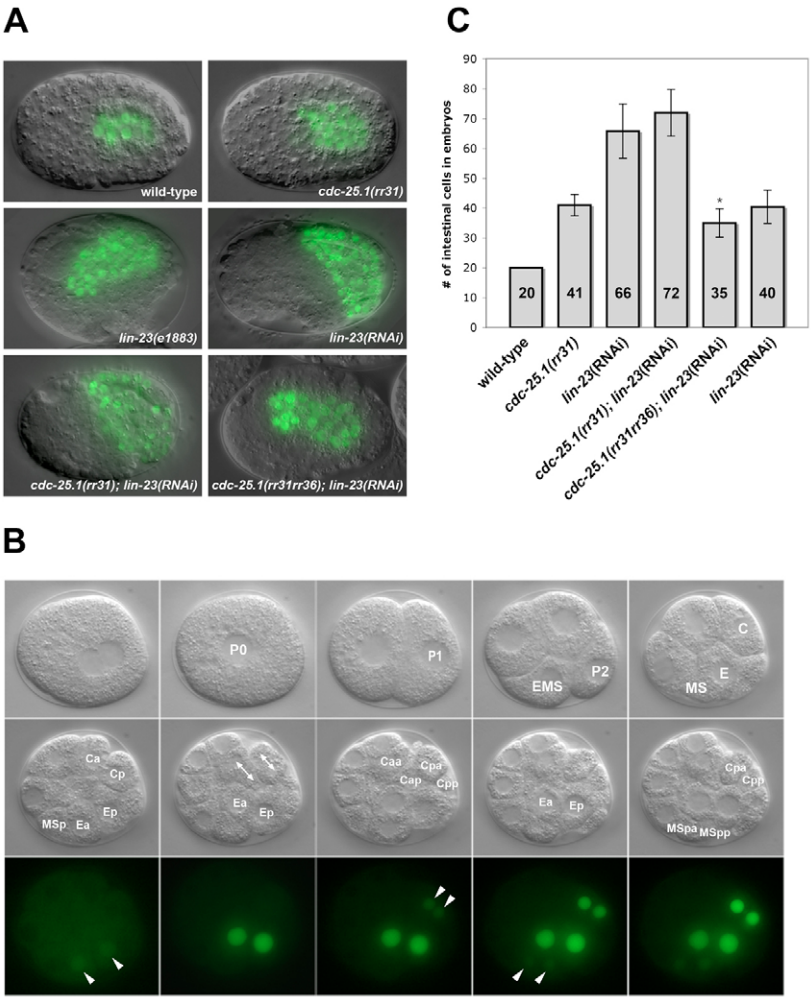

Fig. 3. Loss-of-function in *lin-23* triggers cell fate transformations and *cdc-25.1*-dependent hyperplasia. (**A**) DIC/GFP overlays of representative embryos expressing the intestinal-specific *elt-2::gfp* marker at 20°C. A wild-type embryo with 16 intestinal cells and a *cdc-25.1(rr31)* mutant with 30 intestinal cells are shown before morphogenesis. *lin-23(e1883)* null, *lin-23*(RNAi) and *cdc-25.1(rr31); lin-23*(RNAi) embryos at their terminal stages display obvious hyperplasia and lack morphogenetic movements. A *cdc-25.1(rr31rr36)* mutant treated with *lin-23*(RNAi) showing typical suppression of the *lin-23*-dependent intestinal hyperplasia. (**B**) Time lapse imaging of an embryo dissected from a *lin-23*(RNAi)-treated hermaphrodite undergoing Cp and MSp to E transformations. This embryo carries an integrated *end-3::end-3(P202L)::gfp* transgene that marks descendants of intestinal cells. Cells in the E, C- and MS-like lineages are highlighted. Double-headed arrows indicate the division axis of Ca- and Cp-like. Daughters of the normal E blastomere and those of two ectopic E-like cells are indicated by arrowheads as they begin to express the GFP marker. (**C**) Quantification of intestinal cell number in terminal stage *lin-23*(RNAi) and control embryos. The *lin-23*(RNAi)-dependent hyperplasia is not additive to *cdc-25.1(gf)*, while penetrant and significant suppression (*) of the cell cycle defects is seen when CDC-25.1 levels are reduced by *rr36*. E blastomeres (*n*=10) from *lin-23*(RNAi)-treated embryos (rightmost bar) were isolated by laser ablation as described by Zhu et al. (Zhu et al., 1997) and the number of intestinal cells, based on the expression of *elt-2::gfp* marker, was quantified 24 hours thereafter.

we found that ~40% of the total *lin-23*(RNAi)-mediated intestinal hyperplasia originates from the E blastomere, while the remaining ~60% arises from ectopic E-like cells.

To confirm whether indeed *lin-23* and *cdc-25.1* act cooperatively in a linear pathway to regulate the intestinal cell divisions, we quantified the severity of the intestinal hyperplasia of terminally arrested *lin-23*(RNAi); *cdc-25.1(rr31)* double mutants. In these animals, the intestinal defect was only slightly more pronounced than that of *lin-23*(RNAi) single mutants and non-additive with *cdc-25.1(rr31)* (Fig. 3C), suggesting that both *lin-23(lf)* and *cdc-25.1(rr31)* act in a linear pathway and consistent with *lin-23* negatively regulating *cdc-25.1*, probably through protein destabilization.

Homozygous viable *lin-23(ot1)* mutants also display mild *cdc-25.1*-dependent intestinal hyperplasia that arises during embryogenesis (~26 intestinal cells at hatching) (Fig. 4A). Partial depletion of the phosphatase by *cdc-25.1*(RNAi) entirely suppressed this *lin-23(ot1)*-dependent cell cycle defect, while *cdc-25.1(rr31); lin-23(ot1)* double mutants did not display stronger intestinal hyperplasia than *cdc-25.1(rr31)* (Fig. 4B). As in *cdc-25.1(rr31)* mutants, no other embryonic or post-embryonic cell cycle perturbation nor any evidence of cell fate changes was observed, suggesting that *lin-23(ot1)* represents a weak hypomorph.

In summary, we show that *lin-23* compromise causes mild to severe intestinal hyperplasia originating from two independent sources: through MSp and/or Cp to E cell fate transformations that generate extra E-like cells during early embryogenesis and through stabilization of CDC-25.1 that probably contributes to the supernumerary cell divisions in the E descendants, much like that observed in both *cdc-25.1(gf)* mutants. Furthermore, that the *lin-23(ot1)* mutation affects both intestinal cell cycle progression and appropriate neurotransmission would argue against the possibility of separable cell cycle and neuronal-specific target recognition domains in the LIN-23 protein, as previously hypothesized (Mehta et al., 2004).

Efficient LIN-23-mediated suppression of the CDC-25.1-associated intestinal hyperplasia requires an intact phosphodegron

Our findings suggest that *lin-23* mediates the timely degradation of CDC-25.1, but the importance of the putative phosphodegron sequence in this process remains unclear. We therefore questioned whether LIN-23 might be limiting and whether simply increasing LIN-23 levels could suppress the intestinal hyperplasia typical of *cdc-25.1(rr31)* mutants. To test this, we generated a transgene that expresses *lin-23* specifically in the E lineage prior to the stage when *cdc-25.1(rr31)*-dependent supernumerary divisions occur. The transgene completely rescues the *lin-23(ot1)*-dependent hyperplasia (5/5 independent transgenic lines), yet it had no effect on the intestine when expressed in wild-type animals. When expressed in *cdc-25.1(rr31)* mutants, we observed a slight, but reproducible suppression of the intestinal hyperplasia (Table 3),

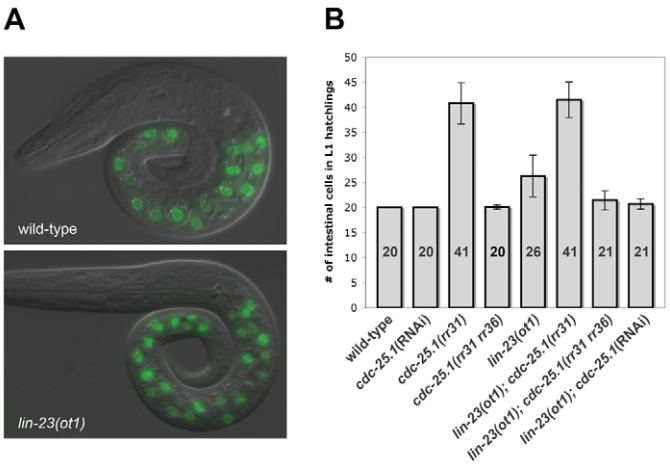

Fig. 4. *lin-23(ot1)* mutants display a mild *cdc-25.1*-dependent intestinal-specific hyperplasia during embryogenesis. (**A**) DIC/GFP overlays of wild-type and *lin-23(ot1)* L1 hatchlings expressing the intestinal-specific *elt-2::gfp* marker. The wild-type larvae hatch with 20 intestinal cells, whereas the *lin-23(ot1)* mutants typically display mild intestinal hyperplasia (here, 28 intestinal cells). (**B**) Quantification of intestinal cell number in L1 hatchlings at 20°C (*n*=40). The *cdc-25.1(gf)* and *lin-23(ot1)* hyperplasia are not additive, whereas the loss-of-function *cdc-25.1(rr36)* intragenic mutation significantly suppresses both the *cdc-25.1(rr31)* and the *lin-23(ot1)*-dependent cell cycle defects. Similar results were observed after hypomorphic *cdc-25.1*(RNAi) treatment of *cdc-25.1(rr31)* (data not shown) and *lin-23(ot1)*.

consistent with a reduced capacity of LIN-23 to trigger efficiently the degradation of the phosphatase with the mutant β-TrCP phosphodegron.

To test the importance of the phosphodegron sequence in the degradation process, we used the transgenic line (wild-type#4) that has an intact phosphodegron and causes modest intestinal hyperplasia. Following overexpression of LIN-23, we noted that the majority of animals containing both arrays displayed wild-type intestinal cell counts and morphology (Table 4). This suppression required an intact F-box protein, as a functionally compromised LIN-23 variant that lacked the typical F-box WD repeats and the F-box domain that are required for interaction between substrate and the E3 ligase components, respectively, did not suppress the extra divisions (Table 4).

Because the GFP::CDC-25.1[wild-type#4]-associated defect is suppressed by overexpression of LIN-23, while the CDC-25.1(*rr31*)-dependent hyperplasia is only marginally ameliorated,

Table 3. Overexpression of the F-box protein LIN-23 in the developing intestine reduces *cdc-25.1(rr31)*-dependent hyperplasia

	Number of intestinal cells in *cdc-25.1(rr31)* L1 hatchlings with (+) or without (–) the *end-3::lin-23* transgenic array*		
	(–)	(+)	*P*
Line 1	41.0±3.6 (*n*=30)	37.7±3.3 (*n*=30)	5.0×10^{-5}
Line 2	40.9±3.3 (*n*=30)	38.1±2.4 (*n*=50)	7.4×10^{-7}
Line 3†	34.7±5.8 (*n*=24)	31.6±4.5 (*n*=30)	1.9×10^{-2}

*The (–) control animals have lost the *end-3::lin-23* transgene, while the (+) animals express *lin-23* specifically in the early intestinal cells. All animals contain the intestinal marker *elt-2::gfp* for accurate quantification of intestinal cell numbers.
†This line was tested at 23°C and displays temperature-dependent suppression of the *cdc-25.1(rr31)* hyperplasia (see Table 1).

Table 4. Efficient LIN-23-mediated suppression of the *cdc-25.1*-associated hyperplasia requires an intact phosphodegron

Number of intestinal cells in L1 hatchlings* expressing the indicated transgene		
Control	*end-3::lin-23*	*end-3::lin-23(ΔSalI)*†
30±6.8 (*n*=40)	22.6±3.6 (*n*=44)	29.1±5.6 (*n*=40)
	21.8±2.9 (*n*=40)	32.2±5.1 (*n*=44)
	21.8±3.2 (*n*=40)	29.5±6.1 (*n*=40)

*All animals already contain *end-3::gfp::cdc-25.1(wild-type)* line 4 which induces mild intestinal hyperplasia (Table 2) together with the intestinal marker *elt-2::gfp* for quantification of the intestinal cell numbers.
†The *SalI* restriction sites encompass both the F-box and the WD repeat domains.

it is likely that an intact phosphodegron/destruction signal is required for the timely and efficient degradation of CDC-25.1 by LIN-23.

An intragenic suppressor mutation reveals a second domain that controls CDC-25.1 stability

Although CDC-25.1(*rr31*) perdures beyond the 100-cell stage in most cells in the embryo it eventually disappears (Kostic and Roy, 2002). We have shown that the GFP::CDC-25.1[G47D] fusion protein also gradually disappears in our transgenic assay compared with the control GFP (Fig. 2). This suggests that another pathway could control CDC-25.1 levels through a secondary mechanism that may be independent of β-TrCP/LIN-23 activity. Using genetic analysis to isolate suppressors of the *cdc-25.1(rr31)* intestinal hyperplasia, we identified *rr36*, a recessive, maternal-effect intragenic mutation in *cdc-25.1(rr31)* that restores intestinal cell counts to wild-type levels (Fig. 4B). *rr36* causes a L273F substitution adjacent to the rhodanese-like catalytic domain of the phosphatase (Fig. 1). *cdc-25.1*-null mutations and *cdc-25.1*(RNAi) are embryonic lethal (Ashcroft and Golden, 2002; Ashcroft et al., 1999), but incomplete depletion of CDC-25.1 by RNAi in *cdc-25.1(rr31)* animals suppresses their supernumerary intestinal divisions (Kostic and Roy, 2002). We noticed that *rr36* is temperature-sensitive embryonic lethal, wherein 10% of the embryos die at 15°C or 20°C, but this lethality increases to 100% when shifted to 25°C. Furthermore, when young larvae were upshifted at 25°C, they developed into sterile adults and displayed phenotypes very similar to *cdc-25.1(0)* mutants or *cdc-25.1*(RNAi)-treated animals, with very few germ cells (Ashcroft and Golden, 2002; Ashcroft et al., 1999) (and data not shown), suggesting that *rr36* represents a new, temperature-sensitive, hypomorphic *cdc-25.1* allele.

Because the *rr36* lesion is in close proximity to the catalytic domain, we wondered whether *rr36* restores the intestinal cell divisions by compromising the catalytic efficiency of the phosphatase or whether it re-establishes the proper turnover of CDC-25.1(*rr31*). Wild-type animals that express a transgene that mimics this suppressor variant (GFP::CDC-25.1[G47D;L273F]) in the early E lineage displayed timely degradation of the fusion protein after the E^8 stage, with only ~10 minutes delay compared with GFP::CDC-25.1[WT] (Fig. 2), while most of these transgenic animals displayed wild-type intestinal division patterns that resulted in 20 intestinal cells at hatching (Table 2). Interestingly, a GFP::CDC-25.1[L273F] fusion protein that mimics the *rr36* mutation alone, was less stable than the wild-type variant, and was degraded before the E^8 stage (Fig. 2). This would not be expected if the *rr36* mutation resulted in a catalytically compromised CDC-25.1. Thus, it is most likely that the domain affected by *rr36* is required for the normal stability of CDC-25.1, rather than for its catalytic activity, although the latter cannot be entirely ruled out.

Table 5. The terminal *lin-23*(RNAi) embryonic phenotype is suppressed by reduction of the *cdc-25.1* gene product

Genotype (*n*)	Disorganized embryos (%)*	Comma stage or later (%)	Hatched dead L1 (%)
lin-23(RNAi) (132)	98	2	0
cdc-25.1(rr31); lin-23(RNAi) (100)	100	0	0
cdc-25.1(rr31rr36); lin-23(RNAi) (104)	54	37	9

*These embryos die as a mass of cells without undergoing morphogenesis (see Kipreos et al., 2000).

rr36 suppresses the *lin-23(lf)*-associated intestinal hyperplasia in a *lin-23*-independent manner

To determine whether the *rr36* mutation destabilizes CDC-25.1 by enhancing the effects of LIN-23 or the SCF^{LIN-23} complex, we tested whether this mutation could suppress the *cdc-25.1*-dependent intestinal hyperplasia typical of *lin-23*(RNAi) embryos. By quantifying the intestinal cells in *cdc-25.1(rr31rr36)* mutants treated with *lin-23*(RNAi), we noticed that *rr36* reduced the *lin-23(*RNAi)-mediated intestinal hyperplasia by ~70% (Fig. 3C), while allowing a significant number of embryos to develop beyond the embryonic arrest typical of *lin-23(*RNAi) (Table 5). A positive effect of destabilizing CDC-25.1 was also observed in *cdc-25.1(rr31rr36); lin-23(ot1)* double mutants, wherein the intragenic mutation completely restored the intestinal cell counts to wild-type (Fig. 4B). However, the frequency of the *lin-23*(RNAi)-dependent cell fate change was unaffected and still occurred in *cdc-25.1(rr31rr36); lin-23*(RNAi) embryos (data not shown).

To confirm that effects observed with *rr36* were indeed LIN-23-independent, we performed *lin-23*(RNAi) in transgenic embryos expressing GFP::CDC-25.1[L273F] during the early intestinal divisions. The GFP fusion was detected until the middle of the E^8-stage, similar to GFP::CDC-25.1[G47D;L273F], and unlike *lin-23*(RNAi)-treated GFP::CDC-25.1[WT] embryos, neither abnormal perdurance nor extra intestinal cell divisions were detected (Fig. 2).

These results reveal the possibility that independent and antagonistic signals may impinge upon distinct domains in CDC-25.1 to fine-tune its timely turnover during embryogenesis. Furthermore, and perhaps most surprising, the general hyperplasia characteristic of terminal *lin-23*(RNAi) embryos appears to be, at least in part, *cdc-25.1* dependent, as some animals lacking *lin-23*, which normally arrest as overproliferated balls of cells, progressed to the hatch when CDC-25.1 was destabilized.

Differential expression of zygotic LIN-23 in the E lineage

Although the stability of CDC-25.1 may depend on a LIN-23-dependent interaction with its N-terminal regulatory domain, the tissue specificity of the *cdc-25.1(gf)*-associated defect remains enigmatic. One means of achieving this tissue specificity could include the differential expression, stability or activity of the putative SCF^{LIN-23} degradation complex in the developing embryo. By in situ hybridization, *lin-23* mRNA was shown to be maternally provided

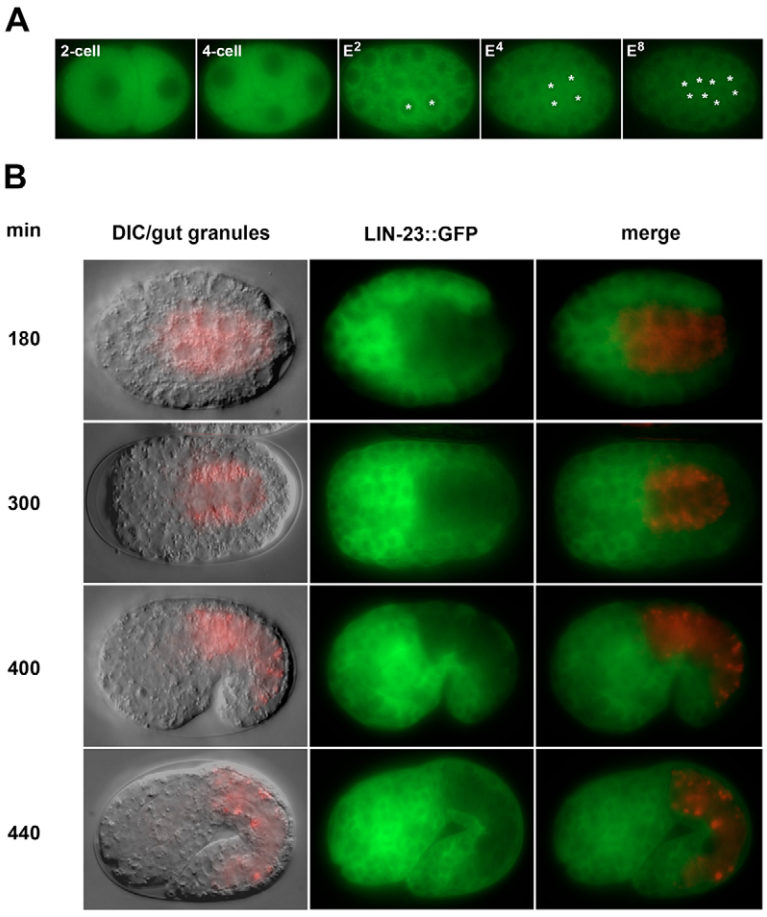

Fig. 5. The β-TrCP orthologue *lin-23* is differentially expressed during embryogenesis. (**A**) The progeny of transgenic hermaphrodites expressing GFP::LIN-23 exclusively in their germ line (*pie-1* promoter) display ubiquitous and mainly cytoplasmic localization of maternal GFP::LIN-23 until the beginning of the E^8-cell stage (~180 minutes). Asterisks indicate the intestinal cells. (**B**) Zygotic LIN-23::GFP is detected ~120 minutes after fertilization (E^4-stage), and shows differential expression levels between the E lineage and the rest of the embryo. The differential expression of *lin-23* continues throughout embryogenesis, with the highest levels in the head region and the lowest in the intestine. Intestinal cells were recognized by morphology, localization and the presence of birefringent gut granules (pseudo-red). The corresponding developmental time in minutes following pronuclear meeting is shown on the left.

to embryos, where it is ubiquitous (Kipreos et al., 2000). We confirmed the presence of maternal LIN-23 in all cells until the 100-cell stage (E^8) in transgenic worms expressing GFP::LIN-23 in their germ line (Fig. 5A). LIN-23 was reported to be zygotically expressed in most embryonic cells (Mehta et al., 2004). Consistent with this, our analysis of zygotic LIN-23::GFP indicates that it is expressed at relatively high levels in most cells, but surprisingly, very low, if any, GFP signal was detectable in the developing intestine. This differential expression was apparent at the onset of GFP detection at approximately the 51-cell stage (E^4-stage) and lasted until the end of embryogenesis (Fig. 5B). To discriminate whether the synthesis or the stability of LIN-23 may be differentially controlled in the E lineage, we analyzed the expression pattern of a *lin-23* promoter fusion. The transcriptional GFP pattern looked indistinguishable from the translational fusion (data not shown), confirming that the zygotic transcription of *lin-23* is differentially regulated such that LIN-23 levels are lower in the E lineage. Therefore, the downregulation of a putative SCF^{LIN-23} E3 ligase in the developing intestine may account for some of the tissue specificity associated with the *cdc-25.1(gf)* mutation and may provide insight into why other tissues are refractory to CDC-25.1(*gf*).

DISCUSSION

The early cell divisions of the *C. elegans* embryo are precisely controlled by gene products that are provided from the maternal germ line. The isolation of two gain-of-function alleles of CDC-25.1 that demonstrate a strict maternal effect indicates that this regulation can be perturbed, resulting in supernumerary cell divisions specifically within the E lineage. How these mutations give rise to the extra divisions is unclear, although CDC-25.1*(rr31)* was observed to be more stable than its wild-type counterpart. By developing a GFP-based transgenic assay to assess the dynamics of protein degradation during early embryogenesis, we show that the stabilization of CDC-25.1 is mediated by a point mutation within a conserved $DSGX_4S$ β-TrCP-like phosphodegron in both *cdc-25.1(gf)* mutants. This stabilizes the protein, resulting in the abnormal presence of CDC-25.1 during a short, yet crucial, window during early development, which is presumably the cause of the observed cell cycle defect.

The β-TrCP orthologue *lin-23* acts through the CDC-25.1 $DSGX_4S$ motif to trigger its degradation

The equivalent phosphodegron/destruction motif is recognized by β-TrCP F-box containing proteins in other systems (Fuchs et al., 2004). Both serines in the consensus site must be phosphorylated to constitute a phosphodegron trigger that is efficiently recognized by an $SCF^{β\text{-}TrCP}$ E3-ligase (Winston et al., 1999). In mammals, the checkpoint protein Chk1 facilitates the $SCF^{β\text{-}TrCP}$-mediated degradation of Cdc25A during unperturbed S phases by phosphorylating several N-terminal regulatory residues, but the kinase that modifies the actual β-TrCP motif remains unidentified (Busino et al., 2003; Jin et al., 2003). Our findings suggest that in *C. elegans*, the equivalent $DSGX_4S$ motif in CDC-25.1 is targeted by the β-TrCP F-box orthologue LIN-23, but in a developmentally controlled manner, so that cell divisions are coordinated with the ongoing cellular processes that normally occur during embryogenesis.

By using our transgenic assay to monitor the dynamics of CDC-25.1 degradation in the E lineage, we show that the wild-type protein is stabilized when *lin-23* activity is reduced, while the embryonic E lineage overproliferates, much like in *cdc-25.1(gf)* mutants. We also confirmed that the turnover of CDC-25.1 is regulated in a linear pathway that includes *lin-23*. Our data suggest that a putative SCF^{LIN-23} E3 ligase actively targets CDC-25.1 for destruction during early embryogenesis, while this targeted elimination requires an intact, functional β-TrCP destruction box for efficient degradation. In the *cdc-25.1(gf)* mutants, this degradation process is impaired because of the change in the β-TrCP motif, resulting in abnormal stabilization of this protein. The potential targeting kinase and its upstream regulators may also be crucial for the timely regulation of this process, as their activity would ultimately trigger the degradation cascade.

Additional domains contribute to CDC-25.1 stability

Interestingly, the intragenic suppressor *cdc-25.1(rr31rr36)* displays phenotypes indistinguishable from the *cdc-25.1(0)* mutants when grown at the restrictive temperature (Ashcroft and Golden, 2002; Ashcroft et al., 1999). This is consistent with either the compromise of *cdc-25.1* catalytic activity or changes in the levels of the protein. Our data indicate that *rr36* destabilizes the protein in a manner independent of LIN-23 and/or its recognition site.

At present, it is not clear how the domain affected by *rr36* confers this function, but it may provide a second regulatory interface to ensure the timely elimination of the phosphatase in response to spatial and/or temporal developmental cues. Using various bioinformatic approaches, we were unable to identify any known motifs associated with post-translational modification around the L273F lesion, with the exception of a strong phosphoacceptor value attributed to Y_{271}. It is, thus, conceivable that a phosphorylation event near L_{273} stabilizes the phosphatase, similar to what is seen after phosphorylation of key residues in p53, p21 or CDC25A (Buschmann et al., 2001; Chehab et al., 1999; Lavin and Gueven, 2006; Li et al., 2002; Mailand et al., 2002). Alternatively, the region affected by the *rr36* lesion could modify the association between CDC-25.1 and small regulatory proteins such as 14-3-3 or Pin1, both of which affect the localization and/or stability of mammalian Cdc25A orthologues (Chen et al., 2003; Conklin et al., 1995; Crenshaw et al., 1998; Stukenberg and Kirschner, 2001). The fact that the mutated leucine is conserved among the four *C. elegans cdc-25* paralogues, the two *Drosophila* Cdc25 orthologues and human CDC25A suggests that it could potentially be part of an important regulatory domain that would control protein stability.

lin-23(lf) embryos display pleiotropic phenotypes

The cell cycle phenotypes of *cdc-25.1(gf)* and *lin-23(lf)* are similar but not equivalent. Loss of *lin-23* results in a more severe embryonic hyperplasia that does not seem to be restricted to the E lineage (Kipreos et al., 2000), similar to the one observed in *cul-1(0)* (cullin) or *skr-1/2(0)* (Skp-related) mutants (Kipreos et al., 1996; Nayak et al., 2002). Our observations indicate that a majority of *lin-23(0)* embryos undergo one or two cell fate transformations, giving rise to separate populations of intestinal-like cells that divide throughout embryogenesis. Interestingly, transformation toward the E fate has also been detected in blastomeres of *skr-1/2*(RNAi) embryos (Z. Bao, personal communication). These extra-intestinal phenotypes cannot be solely attributed to stabilized CDC-25.1. $SCF^{β\text{-}TrCP}$ targets multiple proteins for degradation, including β-catenin, IκB, Emi1/2, Wee1A and CDC25A/B (Nakayama and Nakayama, 2005; Nakayama and Nakayama, 2006); thus, the pleiotropic effects seen in *lin-23*(RNAi) suggest that LIN-23 also controls distinct aspects of *C. elegans* embryogenesis through multiple targets.

Interestingly, most of the hyperplasia that occurs in *lin-23(lf)* embryos, even that outside the usual E lineage, also appears to be *cdc-25.1*-dependent. It is therefore tempting to associate the general *lin-23*(RNAi)-mediated embryonic hyperplasia to misregulated CDC-25.1 activity. Indeed, the hyperplasia and lethality typical of *lin-23*(RNAi) is partially suppressed by a *cdc-25.1(rr31rr36)* mutant variant that has no apparent effect on normal cell cycle progression per se. Alternatively, it is plausible that the loss of *lin-23* alone can induce hyperplasia independently of changes in CDC-25.1 stability. Thus, the absence/reduction of CDC-25.1(*rr31rr36*) protein would prevent excessive cell divisions in *lin-23*-depleted embryos indirectly, as would be the case by eliminating any other crucial cell cycle regulator.

Conversely, the *lin-23(lf)*-dependent early cell fate transformations are *cdc-25.1* independent and are more likely to be the result of inappropriate regulation of developmental signals downstream, or in parallel, to the *skn-1*-dependent endodermal specification pathway. Interestingly, embryos depleted of *gsk-3*, the GSK3β orthologue, display *skn-1*-dependent Cp to E cell fate transformations (Maduro et al., 2001; Schlesinger et al., 1999), similar to what we observe in *lin-23*(RNAi) animals. As the Wnt pathway plays multiple roles in early cell fate specification (Huang et al., 2007; Phillips et al., 2007; Rocheleau et al., 1997; Thorpe et al., 1997), the misregulation of *wrm-1* or *sys-1* (two embryonically expressed β-catenin-like molecules) could affect MS, C, D or even other blastomeres to give rise to the extra intestinal cell populations observed in *lin-23* mutant embryos. Interestingly, other mutants with such C-derived extra intestinal cells may indirectly affect the Wnt pathway (Shirayama et al., 2006).

Early developmental events and E lineage-specific sensitivity to CDC-25.1(*gf*)

The transgenic GFP::CDC-25.1[G47D] resists degradation and triggers supernumerary divisions of the E daughters ~40-45 minutes into the E^8-stage, very similar to what is observed in *cdc-25.1(rr31)* mutants, defining a crucial period of sensitivity midway into the E^8-stage (~200-cell stage embryo). This window of sensitivity may reflect a temporal transition where maternal *cdc-25.1*-dependent regulation of cell cycle progression is gradually replaced by zygotic control, possibly through expression of other *cdc-25* paralogues or even other positive or negative S-phase regulators. Our data suggest that abnormally high levels of CDC-25.1 that surpass a crucial threshold during this transition period may be sufficient to trigger extra intestinal divisions before zygotic cell cycle control would limit further overproliferation.

During embryogenesis, cell cycle timing is regulated through the control of S phase progression (Edgar and McGhee, 1988), wherein each lineage could potentially use distinct mechanisms of coordinating cell division timing with other concurrent developmental events. This maternal to zygotic transition may indeed be one such property that is particular to the E lineage, thus rendering it sensitive to changes in CDC-25.1 turnover.

Differential expression of *lin-23* in the developing endoderm

The differential expression of zygotic *lin-23* in embryos from the 51-cell stage until the end of embryogenesis may help to explain the intestinal specificity associated with the *lin-23(ot1)* and *cdc-25.1(gf)* mutations. The combination of maternal and zygotic LIN-23 in non-E tissues could indicate a higher catalytic efficiency of their SCF^{LIN-23} E3 ligase compared with that acting in the E lineage. Consistent with this idea, *lin-23(ot1)* animals display weak *cdc-25.1*-dependent hyperplasia solely in the intestinal tissue, despite wild-type levels and pattern of LIN-23(*ot1*) expression (Mehta et al., 2004). Thus, LIN-23*(ot1)* probably still recognizes its embryonic targets, although with reduced efficiency. Accordingly, lower levels or reduced activity of $SCF^{LIN-23(ot1)}$ in E would stabilize CDC-25.1 to varying degrees causing moderate intestinal hyperplasia, while the higher E3 ligase activity in other tissues would prevent any cell cycle perturbation. Albeit speculative, this could also explain the tissue specificity of both *cdc-25.1(gf)* alleles. Although CDC-25.1(*rr31)* perdures in all cells, it does not cause extra divisions in every tissue, suggesting that despite the impaired phosphodegron, its levels are still maintained below a crucial threshold outside the E lineage. However, overexpression of LIN-23 in the early E lineage can only reduce the *cdc-25.1(rr31)*-mediated hyperplasia by a small margin. Therefore, LIN-23 may not be the only limiting factor for the catalytic efficiency of SCF^{LIN-23}, inferring that other components of this complex may also be limiting in the developing intestine.

The reason for the zygotic LIN-23 expression pattern remains unclear and may reflect intrinsic differences in the requirement for *lin-23*-dependent pathways between lineages. For example, early expression of *lin-23* in Cp and MSp would be required to restrict the intrinsic potential of these lineages to adopt endodermal fates, while downregulation of SCF^{LIN-23} in the E lineage may be required for the Wnt-dependent migration of Ea and Ep (Lee et al., 2006), and possibly subsequent morphogenetic movements associated with gastrulation. From this perspective, the levels of CDC-25.1 in the developing gut may be held very close to a sensitive threshold and small increases resulting from defects in SCF^{LIN-23}-dependent degradation may result in intestinal hyperplasia.

We thank Dr Christian Rocheleau, Dr François Fagotto, Ivana Kostic and the members of the Roy laboratory for their help, comments and critical reading of the manuscript. We are also grateful to Dr Zhirong Bao for sharing results before publication. We thank Dr Morris Maduro and the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR), for *C. elegans* strains and reagents. This work was supported by a Research Award from the Canadian Cancer Society. R.R. is a CIHR New Investigator.

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