Proteasome regulation of the chromodomain protein MRG-1 controls the balance between proliferative fate and differentiation in the C. elegans germ line

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ABSTRACT

The level of stem cell proliferation must be tightly controlled for proper development and tissue homeostasis. Multiple levels of gene regulation are often employed to regulate stem cell proliferation to ensure that the amount of proliferation is aligned with the needs of the tissue. Here we focus on proteasome-mediated protein degradation as a means of regulating the activities of proteins involved in controlling the stem cell proliferative fate in the C. elegans germ line. We identify five potential E3 ubiquitin ligases, including the RFP-1 RING finger protein, as being involved in regulating proliferative fate. RFP-1 binds to MRG-1, a homologue of the mammalian chromodomain-containing protein MRG15 (MORF4L1), which has been implicated in promoting the proliferation of neural precursor cells. We find that C. elegans with reduced proteasome activity, or that lack RFP-1 expression, have increased levels of MRG-1 and a shift towards increased proliferation in sensitized genetic backgrounds. Likewise, reduction of MRG-1 partially suppresses stem cell overproliferation. MRG-1 levels are controlled independently of the spatially regulated GLP-1/Notch signalling pathway, which is the primary signal controlling the extent of stem cell proliferation in the C. elegans germ line. We propose a model in which MRG-1 levels are controlled, at least in part, by the proteasome, and that the levels of MRG-1 set a threshold upon which other spatially regulated factors act in order to control the balance between the proliferative fate and differentiation in the C. elegans germ line.

KEY WORDS: E3 ubiquitin ligase, MRG-1, RFP-1, Substrate recognition subunit, Proteasomal degradation, Stem cell proliferative fate, Caenorhabditis elegans

INTRODUCTION

Proper development and tissue homeostasis require precise control of the extent of stem cell proliferation. Excess proliferation can lead to tumor formation, while reduced proliferation can lead to stem cell depletion. Multiple levels of gene control are often employed to ensure that the amount of proliferation is aligned with the needs of the tissue. In addition, the mammalian embryonic stem cell (ESC) system is highly dependent upon transcriptional regulation to control self-renewal (proliferation) and differentiation, relying heavily on the Sox2, Nanog and Oct4 (Pou5f1) transcription factors (Avilion et al., 2003; Chambers et al., 2003; Nichols et al., 1998); however, other modes of gene regulation have also been identified as being involved in regulating ESC proliferation, including protein phosphorylation (Li et al., 2011; Phanstiel et al., 2011), microRNA-mediated gene regulation (Martinez and Gregory, 2010) and the epigenetic modification of DNA (Meissner, 2010). Recently, protein degradation has also been implicated in regulating mammalian stem cell pluripotency and self-renewal (Buckley et al., 2012). We previously found that a reduction in proteasome function enhances overproliferation of stem cells in the C. elegans germ line (MacDonald et al., 2008), suggesting that a role for the proteasome in regulating stem cell proliferation might be conserved.

Proliferating cells in the C. elegans germ line are confined to the proliferative zone (PZ) at the distal ends of both gonad arms (Fig. 1A). As cells move proximally, they enter into meiotic prophase. From the very distal end of the gonad to where cells first show signs of entering meiotic prophase is a distance of ∼20 cell diameters (Crittenden et al., 1994; Hansen and Schedl, 2013; Kershner et al., 2013). The primary signal regulating whether a cell adopts a proliferating (mitotic) or differentiating (meiotic) fate is the canonical GLP-1/Notch signalling pathway, with high levels of signalling promoting the proliferative fate and low or absent levels more proximally allowing cells to enter meiotic prophase (Hansen and Schedl, 2013; Kershner et al., 2013) (Fig. 1B,C). GLP-1 signalling indirectly inhibits the activities of factors in two genetic pathways, referred to as the GLD-1 and GLD-2 pathways, that inhibit the proliferative fate and/or promote entry into meiotic prophase (Eckmann et al., 2004; Francis et al., 1995; Hansen et al., 2004b; Kadyk and Kimble, 1998) (Fig. 1B,C). GLD-1 is a translational regulator that contains a K homology (KH) RNA-binding domain (Crittenden et al., 2002; Hansen et al., 2004b; Jones et al., 1996; Jones and Schedl, 1995). GLD-1 levels are low in the distal end of the gonad, but increase gradually until reaching their maximum level in the transition zone (Jones et al., 1996). GLD-2 is the catalytic portion of a poly(A) polymerase and functions with GLD-3, a BicC homologue (Eckmann et al., 2004; Kadyk and Kimble, 1998). Factors in the GLD-1 pathway (GLD-1 and the Nanos-related NOS-3 protein) function redundantly with factors in the GLD-2 pathway (GLD-2 and GLD-3), such that each pathway is sufficient for relatively normal proliferative levels. However, if the activities of both pathways are disrupted, overproliferation occurs resulting in a germline tumor (Eckmann et al., 2004; Hansen et al., 2004b; Kadyk and Kimble, 1998).

This core genetic network controls the balance between the proliferative fate and differentiation. Presumably, this network promotes the expression of factors needed for proliferation, such as cell cycle regulators, in the PZ, and factors needed for meiosis, such as synaptosomal complex proteins, in the transition zone and beyond. Additionally, there are other factors, some promoting the proliferative fate and others promoting meiotic entry, that appear to work in...
parallel to the GLP-1 and GLD-1 GLD-2 pathways, presumably to reinforce or fine-tune the regulation accomplished through these pathways (Hansen and Schedl, 2013). As cells move from the distal mitotic region to the more proximal meiotic region, not only do meiotic proteins need to be expressed, but the activities of proteins that are necessary for proliferation must also be reduced. The role of the proteasome in regulating the balance between proliferative fate and differentiation might be to degrade proteins that promote the proliferative fate, as well as proteins that are necessary for proliferation, thereby allowing cells to differentiate. We previously demonstrated that partially reducing proteasome activity in a sensitized genetic background results in overproliferation (MacDonald et al., 2008). Therefore, when proteasome function is reduced, proteins that promote the proliferative fate may persist, resulting in stem cell overproliferation.

Proteins to be degraded by the proteasome are targeted through ubiquitylation (Komander, 2009; Pickart, 2001). An E1 ubiquitin-activating enzyme first places ubiquitin on an E2 ubiquitin-conjugating enzyme. Then, an E3 ligase binds to both the E2 and to the substrate protein and facilitates the transfer of ubiquitin from the E2 enzyme to the substrate (Komander, 2009; Pickart, 2001). E3 ligases can either be monomeric, in which different domains within a single protein bind to the E2 enzyme and substrate, or multi-subunit, in which multiple proteins accomplish these different functions and form a complex (Metzger et al., 2012) (Fig. 1D,E). Binding of the E3 ligase to the substrate protein is a critical step in providing specificity for this targeting reaction. Either the monomeric protein, or the substrate recognition subunit (SRS) of a multi-subunit complex, binds directly to the target protein and provides specificity. There are estimated to be more than 600 mammalian E3 ligases (Li et al., 2008), and potentially even more in \textit{C. elegans} (Kipreos, 2005).

Here, we identify five putative E3 ubiquitin ligases or SRSs that enhance overproliferation when their activity is reduced. The RING finger protein RFP-1 shows the strongest overproliferation phenotype. We confirm that RFP-1 binds to MRG-1, which is homologous to the mammalian MRG15 (MORF4L1) chromodomain protein. MRG-1 has multiple functions in \textit{C. elegans}, including facilitating synaptonemal complex-independent pairing of homologous chromosomes (Dombecki et al., 2011), maintaining genomic integrity in germ cells (Xu et al., 2012) and repressing the expression of X-linked genes in the germ line (Takasaki et al., 2007). Additionally, MRG-1 is necessary for proliferation of \textit{C. elegans} primordial germ cells (Fujita et al., 2002; Takasaki et al., 2007), and loss of MRG15 reduces the proliferation capacity of neural precursor cells in mice (Chen et al., 2009). We demonstrate that MRG-1 also contributes to the proliferation capacity of stem cells in adult...
RESULTS
Identification of E3 ligases involved in regulating stem cell proliferative fate

We previously determined that reduction in proteasome activity results in increased proliferation in sensitized genetic backgrounds in the *C. elegans* germ line (MacDonald et al., 2008), suggesting that the proteasome normally functions to inhibit the proliferative fate in the germ line. Our current model is that, in order for cells to enter meiotic prophase, the proteasome degrades proteins that promote the proliferative fate (which we refer to as proliferative fate-promoting proteins). We sought to identify these proliferative fate-promoting proteins and understand how their degradation contributes to the control of stem cell proliferation. Since a partial reduction of proteasome function results in increased proliferation in sensitized genetic backgrounds (MacDonald et al., 2008), we reasoned that a reduction in the activity of the ubiquitin system that targets proteins for degradation would also result in increased proliferation in these same genetic backgrounds. We further reasoned that since the SRS or a domain of the monomeric E3 ubiquitin ligase physically interacts with the protein to be degraded, we could use this as a means to identify proliferative fate-promoting proteins.

To identify the SRSs/monomeric E3 ligases that are involved in controlling stem cell proliferation, we performed an RNAi screen of the majority of the predicted SRSs/monomeric E3 ligases in the *C. elegans* genome. We speculated that there could be many proliferative fate-promoting proteins and that different SRSs/monomeric E3 ligases could target many of these proteins; therefore, reducing the function of any single SRS/monomeric E3 ligase might not result in a significant stem cell proliferation phenotype. We devised a screen that allowed us to detect even subtle amounts of overproliferation using the *glp-1* (ozf264gf) gain-of-function allele at 20°C as a highly sensitive genetic background for overproliferation (Kerins et al., 2010). These animals also carried a transgene containing a GFP-tagged version of the GLD-1 protein, the levels of which are low or absent in proliferating cells and high as cells enter meiotic prophase (Jones et al., 1996; Schumacher et al., 2005) (Fig. 1F). An increase in the number of proliferative cells results in a larger PZ, which in turn causes the region where GLD-1 levels increase to be moved more proximally in the gonad (Hansen et al., 2004b). To identify animals containing modest amounts of overproliferation, we screened for animals in which the two regions of GLD-1 expression (from the two gonad arms) were further apart than in wild-type animals (Fig. 1G), as well as for animals with proliferative cells in the region where GLD-1 is normally expressed, or with proximal proliferation (see below). The animals screened also lacked *rfp-1* activity, which reduces the effectiveness of RNAi in many somatic tissues, but not in the germ line (Kumsta and Hansen, 2012; Sijen et al., 2001). This reduced the possibility of an SRS/monomeric E3 ligase also functioning in other somatic processes, the disruption of which might mask a germline overproliferation phenotype.

We generated a list of 854 potential SRSs/monomeric E3 ligases by identifying those with domains typical of such proteins, including F-box, HECT, U-box, BTB/POZ, RING finger, SOC-box and VHL-box domains (supplementary material Methods and Table S1). We obtained 714 bacterial feeding RNAi clones from commercial libraries (Kamath and Ahringer, 2003; Rual et al., 2004) and constructed 109 clones that were not contained in these libraries, for a total of 823 potential SRSs/monomeric E3 ligases that we screened by feeding RNAi (supplementary material Table S1). Initial screening identified five potential candidates that appeared to increase proliferation in both the primary screen and a subsequent repeat of the RNAi (Fig. 2). To verify that reduction of function in these genes results in overproliferation, we performed RNAi by dsRNA injection and dissected and stained the gonads with anti-REC-8 and anti-HIM-3 antibodies, which mark proliferative and differentiating cells, respectively (supplementary material Table S2). We found that RNAi against *fbxb-54*, *fbxa-171* and *rfp-1* resulted in overproliferation primarily in the proximal end of the gonad (proximal tumor (Pro) phenotype), which can be due to excess proliferation during larval development and contact with somatic cells that are normally in contact with proliferative cells (McGovern et al., 2009), although defects in processes other than stem cell proliferation can also cause this phenotype (Hansen and Schedl, 2013). RNAi against *vps-11*, when performed by dsRNA injection, resulted in embryonic lethality, precluding further analysis (supplementary material Table S2). RNAi against *rfp-1*, which encodes a RING finger protein (Crowe and Candido, 2004), gave the strongest phenotype, with proliferative cells present in patches throughout the gonad arm (as described below; Fig. 3D). Subsequent analyses focused primarily on this gene.

Characterization of *rfp-1* involvement in the proliferative fate versus differentiation decision

To help determine how *rfp-1* functions in the proliferative fate versus differentiation decision, we more closely analyzed the overproliferation phenotype and determined how *rfp-1* interacts with other genes in the genetic pathway regulating this decision. To determine if the enhancing activity of *rfp-1* is specific to the *glp-1* (oz264gf) allele, we examined whether it could also enhance the *glp-1* (ar202gf) gain-of-function allele, which has been used extensively to analyze enhancement of proliferation (Hansen et al., 2004a; Pepper et al., 2003). We found that reducing RFP-1 activity either through RNAi or using the *rfp-1* (ok572) presumed null allele (supplementary material Fig. S1) caused significant overproliferation in *glp-1* (ar202gf) animals, as compared with
either single mutant (Fig. 3, Table 1). The regions of overproliferation appear as large areas of proliferative cells, interspersed with regions of meiotic cells (Fig. 3D,F). Most gonads have proximal proliferation (Pro; 96%, n = 24), with the locations and sizes of the interspersed proliferative regions differing somewhat from gonad to gonad (data not shown). Using the loop region where the gonad arm reflexes back as a landmark, we found that 93% of *rfp-1(ok572)* glp-1(ar202) gonad arms have proliferative cells in this region, whereas this phenotype is not displayed by gonads in any of the single mutants (Table 1). Indeed, *rfp-1(ok572)* single mutants have a wild-type polarity, with proliferative cells only being found in the distal end, and the PZ being even smaller than that in wild-type animals (Table 2). The smaller PZ is likely to reflect a function for RFP-1 other than in regulating the proliferative fate versus differentiation decision, such as regulating progression through the mitotic cell cycle. Smaller PZs have been observed for *puf-8* and *gld-1* single mutants, even though both genes strongly enhance *glp-1(gf)* overproliferation, suggesting that they also have additional functions (Hansen et al., 2004a; Racher and Hansen, 2012).

To determine where *rfp-1* might function in the genetic pathway regulating the proliferative fate versus meiotic entry decision, we examined whether *rfp-1* functions in the GLD-1 or GLD-2 pathways that function downstream of GLP-1 signalling (Fig. 1B,C). The genes in these pathways inhibit the proliferative fate and/or promote meiotic entry, and their loss enhances overproliferation in sensitized genetic backgrounds, similar to that observed with the loss of *rfp-1*. If the activity of one pathway is removed, the balance between the proliferative fate and meiosis is essentially the same as in wild-type animals. However, if the activities of both pathways are removed simultaneously, most cells fail to switch from the proliferative fate to meiotic entry, resulting in a germline tumor (Hansen and Schedl, 2013; Kadyk and Kimble, 1998). We found that *rfp-1(ok572)* did not form a synthetic tumor with either *gld-1(q485)* or *gld-2(q497)*, suggesting that *rfp-1* does not function in either pathway (Table 2). These results are consistent with *rfp-1* functioning parallel or downstream to the GLP-1 and GLD-1 GLD-2 genetic pathways.

**Reduction in MRG-1 expression partially suppresses *rfp-1(ok572)* glp-1(ar202) overproliferation**

RFP-1 is likely to be a monomeric E3 ubiquitin ligase. It contains a conserved RING finger domain in its carboxy terminus (Crowe and Candido, 2004) and interacts with the E2 conjugating enzyme UBC-1 (Crowe and Candido, 2004). RFP-1 homologues in other systems, including BRE1 from yeast and rat Staring, have been shown to have E3 ligase activity (Chin et al., 2002; Ishino et al., 2014; Liu et al., 2009; Wood et al., 2003; Xuan et al., 2013).
Therefore, reducing RFP-1 activity would be expected to impair the degradation of target proteins by the proteasome. This model suggests that the enhancing activity of rfp-1(ok572) or rfp-1(RNAi) is likely to be due to perdurance of proliferative fate-promoting proteins that are normally targets of proteasomal degradation. Reducing the expression of these proliferative fate-promoting proteins in rfp-1(ok572) glp-1(1ar202gf) animals should reduce the extent of overproliferation. However, since E3 ligases may target multiple proteins for degradation, reducing the activity of a single proliferative fate-promoting protein might not completely suppress the overproliferation in rfp-1(ok572) glp-1(1ar202gf) animals. RFP-1 was previously proposed to bind to MRG-1 based on a yeast two-hybrid and bacterially expressed protein co-immunoprecipitation experiments, we have confirmed that MRG-1 and RFP-1 directly interact (Fig. 4). MRG-1 is necessary for proliferation of the primordial germ cells, with mutants lacking both maternal and zygotic activity completely lacking a germ line due to failure of the primordial germ cells to proliferate (Fujita et al., 2002). Additionally, the murine homologue of MRG-1, MRG15, promotes proliferation of neural precursor cells (Chen et al., 2009). Therefore, MRG-1 is a good candidate for a proliferative fate-promoting protein, the accumulation of which may be controlled through proteasomal degradation.

To test this we first performed RNAi of mrg-1 in rfp-1(ok572) glp-1(1ar202gf) animals and found that the extent of overproliferation was dramatically reduced as compared with control RNAi (Fig. 5-C-E). Indeed, whereas 100% of rfp-1(ok572) glp-1(1ar202gf) gonad arms have regions of proliferative cells outside of the PZ, 13% of rfp-1(ok572) glp-1(1ar202gf) mrg-1(RNAi) gonad arms have a wild-type polarity. Additionally, rfp-1(ok572) glp-1(1ar202gf) mrg-1(RNAi) gonads are reduced in the extent of ectopic proliferation, with only 43% of gonad arms having proliferative cells in the loop region as compared with 97% with the control RNAi (Table 1). We confirmed the RNAi results by analyzing rfp-1(ok572) glp-1(1ar202gf) mrg-1(tm1227) triple-mutant animals, where the extent of ectopic proliferation was reduced compared with the rfp-1(ok572) glp-1(1ar202gf) double mutant (Fig. 5-F and Fig. 3-F). However, the suppression was slightly weaker than with mrg-1 RNAi, which was likely to be due to a maternal contribution of MRG-1 in the mrg-1(tm1227) mutant. Since rfp-1(ok572) glp-1(1ar202gf) mrg-1(tm1227) MzMZ animals lacked a germ line, we could only analyze MZ animals, in which some maternal MRG-1 would still be present (supplementary material Fig. S2A). Consistent with MRG-1 promoting the proliferative fate, mrg-1(RNAi) results in a smaller than normal PZ in otherwise wild-type animals (Xu et al., 2012), partially suppresses glp-1(1ar202gf) overproliferation, and slightly enhances the glp-1(bn18ts) smaller PZ phenotype (supplementary material Fig. S3).

The role of MRG-1 in influencing the proliferative fate versus differentiation decision could be accomplished by working in parallel to the GLP-1 and GLD-1 GLD-2 pathways, and affecting the activity of downstream proliferative fate-promoting and/or differentiation-promoting proteins. Alternatively, MRG-1 could directly affect the activity of the GLP-1 and GLD-1 GLD-2 pathways, either promoting GLP-1 signalling or inhibiting the GLD-1 and/or GLD-2 pathways. Since our genetic analysis of rfp-1 suggested that it functions parallel to, or downstream of, these

Table 1. Loss of mrg-1 suppresses the rfp-1(ok572); glp-1(ar202) tumorous germline phenotype

<table>
<thead>
<tr>
<th>Genotype</th>
<th>WT (%)</th>
<th>REC-8 cells in loop (%)</th>
<th>Sperm (%)</th>
<th>Oocytes (%)</th>
<th>n²</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2 (wild type)</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>&gt;50</td>
</tr>
<tr>
<td>rfp-1(ok572)</td>
<td>100</td>
<td>0</td>
<td>100</td>
<td>100</td>
<td>&gt;50</td>
</tr>
<tr>
<td>glp-1(ar202)</td>
<td>89</td>
<td>93</td>
<td>60</td>
<td>4</td>
<td>84</td>
</tr>
<tr>
<td>rfp-1(ok572) glp-1(ar202)</td>
<td>0</td>
<td>93</td>
<td>43</td>
<td>0</td>
<td>222</td>
</tr>
<tr>
<td>rfp-1(ok572) glp-1(ar202) mrg-1(RNAi)</td>
<td>13</td>
<td>43</td>
<td>43</td>
<td>0</td>
<td>178</td>
</tr>
<tr>
<td>rfp-1(ok572) glp-1(ar202) mrg-1(tm1227)</td>
<td>2</td>
<td>18</td>
<td>67</td>
<td>37</td>
<td>207</td>
</tr>
</tbody>
</table>

¹Percentage gonad arms with a wild-type (WT) germline phenotype. WT phenotype is defined here as the absence of ectopic proliferative germ cells in the gonad arm outside of the PZ. In glp-1(1ar202) animals, gonad arms had a larger PZ compared with that in the wild type, but as long as no other proliferative cells were found outside the PZ, the phenotype was categorized as WT.

²Percentage gonad arms with a significant number of anti-REC-8 immunopositive cells in the loop region.

³Sample size refers to the number of gonad arms examined. Animals were randomly selected at the L4 stage and gonad phenotypes were analyzed at the young adult stage by immunostaining with anti-REC-8 and anti-HIM-3 antibodies. DAPI was used to stain germ cell nuclei and facilitate the identification of sperm and oocytes.

Table 2. rfp-1 interaction with components of the proliferative fate versus differentiation pathway

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
<th>Average germ cells in PZ (s.d.; range)</th>
<th>n²</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2 (wild type)</td>
<td>WT</td>
<td>207 (10; 195-221)</td>
<td>10</td>
</tr>
<tr>
<td>rfp-1(ok572)</td>
<td>WT</td>
<td>165 (11; 145-180)</td>
<td>10</td>
</tr>
<tr>
<td>gld-1(q485)</td>
<td>Gld-1</td>
<td>137 (12; 121-156)</td>
<td>10</td>
</tr>
<tr>
<td>gld-1(q485): male</td>
<td>WT</td>
<td>N.D.</td>
<td>&gt;30</td>
</tr>
<tr>
<td>rfp-1(ok572) glp-1(ar202)</td>
<td>Glp-1</td>
<td>96 (6; 88-107)</td>
<td>10</td>
</tr>
<tr>
<td>rfp-1(q572)</td>
<td>WT</td>
<td>N.D.</td>
<td>&gt;30</td>
</tr>
<tr>
<td>gld-2(q497); gld-3(q497)</td>
<td>Gld-2**</td>
<td>384 (34; 331-421)</td>
<td>10</td>
</tr>
<tr>
<td>rfp-1(q572)</td>
<td>Gld-2**</td>
<td>354 (22; 321-394)</td>
<td>10</td>
</tr>
</tbody>
</table>

¹Phenotype refers only to the main germine phenotype. Some mutants had a slightly larger or smaller PZ than wild-type animals. In all cases, n=30 gonad arms were analyzed and 100% displayed the indicated phenotype.

²PZ, proliferative zone; N.D., had proliferative cells in the germ line, although the number was not determined.

³The number of gonad arms counted to assess the average germ cells in PZ.

⁴Gonads with the Gld-1 phenotype develop tumorous germ cell lines due to defective meiotic progression, not a defect in the proliferative fate versus differentiation decision (Francis et al., 1995). To circumvent this masking phenotype, gld-1(q485) males, which do not have a defect in meiotic progression, were used to facilitate the analysis. Whole-mount DAPI staining was used to analyze germine phenotypes of the males.

⁵Gonads with the Gld-2 phenotype show an accumulation of germ cells at the proximal end of the germ line that are arrested at an early stage of oogenesis. In gld-2(q497); rfp-1(q572) animals these cells were degraded, which was likely to be through apoptosis, and no germ cell accumulation was found.
pathways, if MRG-1 is regulated by RFP-1 then we expect MRG-1 to also function parallel to, or downstream of, these pathways. To test this we determined whether loss of MRG-1 activity could suppress proliferation when the activities of the GLP-1 and GLD-1 GLD-2 pathways are eliminated. gld-2(q497) gld-1(q485); glp-1(q175) animals have completely tumorous gonads (Hansen et al., 2004a). When MRG-1 levels were reduced by RNAi in these animals, 12% (n=136; data not shown) of the gonads contained large regions of cells that entered meiotic prophase, whereas none of the control gonads contained these regions (Fig. 6). This partial suppression suggests that at least part of the influence of MRG-1 on the proliferative fate versus differentiation decision does not involve controlling the GLP-1 and GLD-1 GLD-2 pathways. However, we cannot rule out the possibility that MRG-1 has some influence on these pathways, in addition to its role that is independent of them. That mrg-1 and rfp-1 both genetically function parallel to, or downstream of, the GLP-1 and GLD-1 GLD-2 signalling pathways is consistent with RFP-1 regulating MRG-1 activity.

**MRG-1 is degraded by the proteasome**

To further test our model that proteasomal degradation contributes to the control of MRG-1 levels, we analyzed the levels of MRG-1 protein in animals with reduced proteasome activity. PAS-5 is a highly conserved alpha subunit of the proteasome, with pas-5(oz237) mutant animals having a partial reduction in proteasome activity (MacDonald et al., 2008). We found that MRG-1 levels increase ~1.8-fold in pas-5(oz237) animals (Fig. 7A), suggesting that the wild-type levels of MRG-1 are likely to be controlled, at least in part, by proteasomal degradation. To further verify that MRG-1 levels increase when proteasome activity is reduced, we treated wild-type animals with the proteasomal inhibitor lactacystin. We found that MRG-1 levels increase ~1.9-fold at 10 µM and ~2.4-fold at 20 µM (Fig. 7B). We also observed that MRG-1 becomes ubiquitylated (supplementary material Fig. S4). Therefore, MRG-1 levels are likely to be reduced in wild-type animals through proteasomal degradation.

To determine whether RFP-1 is likely to be a monomeric E3 ubiquitin ligase that targets MRG-1 for degradation, we analyzed MRG-1 levels in rfp-1(ok572) animals and found that they increase ~2.5-fold as compared with wild-type animals (Fig. 7A). To verify that this increase in MRG-1 accumulation occurs in the tissue relevant to germline proliferation, we used indirect immunofluorescence to analyze MRG-1 levels in dissected gonads and found an increase of ~2-fold in rfp-1(ok572) mutants as compared with wild-type animals (Fig. 7C,D).

**Spatial regulation of MRG-1**

We suggest that MRG-1 normally promotes the proliferative fate in the germ line, and proteasomal degradation of MRG-1 helps to control the level of proliferation. We envision two models for how this could occur. First, MRG-1 levels might be spatially regulated, with high levels in the distal end of the gonad where proliferative cells are located. Proteasomal degradation of MRG-1 would occur more proximally, allowing cells to cease proliferating and enter into meiotic prophase. In the second model, MRG-1 levels would be relatively uniform throughout the gonad, and the amount of MRG-1 sets a threshold upon which other spatially regulated molecules act. To distinguish between these possibilities, we analyzed the spatial distribution and overall level of MRG-1 in wild-type and rfp-1(ok572) gonads. We found that MRG-1 levels are relatively constant throughout the distal end of the gonad in wild-type gonads, with no observable decrease in levels as cells enter into meiotic prophase (Fig. 7C,E). Indeed, MRG-1 levels even appear slightly higher more proximally. Overall MRG-1 levels are higher in the rfp-1(ok572) mutant, and show the same general pattern of accumulation as in wild-type gonads (Fig. 7C-E). This accumulation pattern is more consistent with our second model, whereby MRG-1 could be active throughout the gonad, and other factors ensure that this activity promotes proliferation only in the PZ. However, we cannot rule out the possibility that MRG-1 activity is restricted to this region through some form of post-translational modification (see Discussion).

The GLP-1 and GLD-1 GLD-2 signalling network is the primary mechanism by which spatial control of the proliferative fate versus differentiation is achieved (Hansen and Schedl, 2013). If MRG-1 does set a threshold for this signalling network, it would be expected that this network would not control MRG-1 levels. To test this, we analyzed MRG-1 levels in gld-2(q497) gld-1(q485) double mutants. Since GLD-1 and GLD-2 inhibit the proliferative fate and/or promote meiotic entry, if they regulate MRG-1 levels then
such levels would be expected to increase in gld-2(q497) gld-1(q485) mutants. However, we observed the same level of MRG-1 accumulation in gld-2(q497) gld-1(q485) mutants as in wild-type animals, suggesting that this signalling pathway does not control MRG-1 levels (Fig. 7F). It is possible that GLP-1 signalling might promote MRG-1 levels through a pathway that is independent of GLD-1 and GLD-2, including through the hypothesized third pathway that functions redundantly with GLD-1 and GLD-2 (Hansen et al., 2004a). However, MRG-1 levels are not decreased when glp-1 activity is also removed, suggesting that this is not the case (Fig. 7F). Therefore, the level of MRG-1 accumulation is important in regulating the balance between the proliferative fate and differentiation; however, its accumulation is not controlled by the GLP-1 and GLD-1 GLD-2 signalling pathways. Rather, MRG-1 levels are independently controlled and might set a threshold upon which this pathway acts.

**DISCUSSION**

**Role of the proteasome in regulating stem cell proliferation**

Although many modes of gene regulation have been implicated in regulating the balance between the proliferative fate and differentiation in various stem cell systems, there has been relatively little exploration of proteasome-mediated protein turnover in controlling this balance. Here we describe the role of the proteasome in regulating the accumulation of MRG-1, a protein implicated in promoting the proliferative fate in various systems (Chen et al., 2009; Tominaga et al., 2005; Zhang et al., 2010). We found that reducing proteasome activity, or mutating the putative E3 ligase RFP-1, elevated the levels of MRG-1 and enhanced stem cell overproliferation. Given that RFP-1 and MRG-1 physically interact, RFP-1 is likely to function as the E3 ubiquitin ligase that targets MRG-1 for degradation, although we cannot rule out the possibility that RFP-1 could regulate the activity of another factor...
A lack of maternal and zygotic MRG-1 results in MRG-1 promoting the proliferative fate and differentiation.

Regulating the activity of factors that control the balance between the proliferative fate and differentiation is likely to be a conserved mechanism for organisms, including Drosophila, yeast, and humans, that MORF/MRG family proteins are associated with complexes that are involved in the activation or repression of target gene transcription. Some of these target genes, including B-myb, cdc-2 and p53, are known to control cell proliferation (Alekseyenko et al., 2006; Chen et al., 2011, 2010; Kind et al., 2008; Leung et al., 2001; Martrat et al., 2011; Peña and Pereira-Smith, 2007; Peña et al., 2011; Smith et al., 2005; Tominaga et al., 2003). Therefore, MRG-1 could both promote the expression of proteins necessary for proliferation and repress the expression of proteins necessary for differentiation. Its influence on stem cell proliferation might be conserved, as lack of MRG15, the MRG-1 homologue in mammals, results in smaller and fewer murine neurospheres (Chen et al., 2009).

**MRG-1 sets a threshold upon which other factors act**

The balance between the proliferative fate and differentiation in the C. elegans germ line is regulated by a number of factors with activities that are spatially regulated (Hansen and Schedl, 2013; Kershner et al., 2013). GLP-1 signalling activity is presumed to be high in the distal end of the gonad in the PZ, but decreases more proximally as GLD-1 and GLD-2 pathway levels increase, allowing cells to cease proliferation and enter into meiotic prophase (Hansen and Schedl, 2013; Kershner et al., 2013). This signalling pathway presumably promotes the accumulation and activity of proteins in the distal end of the gonad that are necessary for proliferation and of proteins that are necessary for meiosis more proximally.

Here, we demonstrate that MRG-1 promotes the proliferative fate; however, MRG-1 protein is not enriched in the PZ (Takasaki et al., 2007) (Fig. 7E). Additionally, the GLP-1 and GLD-1 GLD-2 pathways do not control the level and pattern of MRG-1 accumulation (Fig. 7F). It remains possible that MRG-1 activity is restricted to the PZ through some other mechanism, such as post-translational modification; however, we consider this unlikely because MRG-1 has been shown to have functions in more proximal regions of the gonad, including maintaining genomic integrity in meiotic cells, silencing X-linked genes and facilitating pairing of homologous chromosomes (Dombecki et al., 2011; Fujita et al., 2002; Olgun et al., 2005; Takasaki et al., 2007; Xu et al., 2012). We propose a model in which MRG-1 sets a
threshold for the proliferative fate upon which the spatially regulated molecules of the GLP-1 and GLD-2 pathways act (Fig. 8). As part of this model, MRG-1 levels are controlled, at least in part, by the proteasome. MRG-1 could be thought of as priming cells for the proliferative fate, and if MRG-1 levels are too high, as in the *rfp-1* mutant, cells are more primed for the proliferative fate.

All proteins within the GLD-1 and GLD-2 pathways appear to affect the level of translation and/or stability of target mRNAs, although targets specific to the proliferative fate versus differentiation decision are yet to be characterized (Hansen and Schedl, 2013; Kershner et al., 2013; Racher and Hansen, 2010). Since MRG-1 homologues associate with acetyltransferases, MRG-1 could control the level of transcription of many genes and could set the threshold by keeping the abundance of mRNAs at a level that allows their translation or stability to be properly controlled by GLD-1 and GLD-2 pathway proteins. If MRG-1 levels are too high, this could result in mRNAs – presumably encoding proteins that are necessary for proliferation – to be expressed at too high a level to be properly repressed by GLD-1 and GLD-2 pathway proteins in the proximal region of the gonad. For those genes that are repressed by MRG-1 – which may encode proteins that promote meiotic entry – transcription levels might be too low in the presence of excess MRG-1 for GLD-1 and GLD-2 pathway proteins to properly stabilize their mRNAs or promote translation. Although the higher levels of MRG-1 that are present in *rfp-1* mutants are not sufficient on their own to cause overproliferation, when they are combined with a *glp-1(gf)* mutation, which presumably lowers GLD-1 and GLD-2 pathway activity, overproliferation occurs (Fig. 8). Identification of genes whose transcription is influenced by MRG-1 activity, and whose translation is regulated by the GLD-1 and GLD-2 pathways, will be the next step in understanding how MRG-1 levels help to control the extent of proliferation in the *C. elegans* germ line.

**MATERIALS AND METHODS**

**General methods and nematode strains**

*C. elegans* handling, genetic manipulation and maintenance were performed using standard methods (Brenner, 1974). Experiments were performed at 20°C and animals were prepared by L1 synchronization as described by Wang et al. (2014) unless otherwise stated. Lactacystin treatment was performed as previously described (Ding et al., 2007). Lactacystin (Cayman Chemicals, #70980) in DMSO, or DMSO alone, was added to synchronized wild-type L1 animals in M9 minimal medium containing OP50 bacteria and grown at 20°C for 2–3 days. Alleles used: linkage group I (LGI): *glp-1(ar202gf)*, *rrf-1(pk1417)*, *glp-1(q485)*; LGII: *glp-1(q264)*, *unc-32(e189)*, *glp-1(q175)*, *glp-1(ar202)*, *glp-1(q264)*, *rfg-1(tm1227)*.

**Computational search for *C. elegans* SRSs and monomeric E3 ligases**

A list of potential *C. elegans* SRSs and monomeric E3 ligases was compiled through a computational search using WormMart (http://www.wormbase.org) as outlined in the supplementary material Methods.

**RNA interference**

RNAi was performed by feeding as previously described (unless otherwise stated) (Wang et al., 2014). RNAi vectors for genes not available from commercial RNAi libraries were constructed through TA cloning as previously described (Holton and Graham, 1991) using the L4440 vector (Timmons and Fire, 1998). For the SRS/monomeric E3 ligase RNAi screen, L4 and young adult (1 day past L4) stage *rfp-1(pk1417)*; *ctxs2 (GLD-1::GFP)*; *rrf-1(ok572)* animals were grown on RNAi plates at 20°C. Animals were prepared by L1 synchronization as described by Wang et al. (2014) unless otherwise stated. Lactacystin treatment was performed as previously described (Ding et al., 2007). Lactacystin (Cayman Chemicals, #70980) in DMSO, or DMSO alone, was added to synchronized wild-type L1 animals in M9 minimal medium containing OP50 bacteria and grown at 20°C for 2–3 days. Alleles used: linkage group I (LGI): *glp-1(ar202gf)*, *rrf-1(pk1417)*, *glp-1(q485)*; LGII: *glp-1(ok572)*, *unc-32(e189)*, *glp-1(q175)*, *glp-1(ar202)*, *glp-1(q264)*, *rfg-1(tm1227)*.

Gonad dissection, immunostaining and imaging
Gonad dissection and staining were performed as previously described (Jones et al., 1996). Dissected gonads were fixed with 3% paraformaldehyde for 5-8 min at room temperature. Post-fixation was performed with 100% methanol overnight at −20°C. Gonads were blocked with 30% goat serum at room temperature for 1 h and incubated in primary antibodies overnight at 4°C. Primary antibodies were: anti-REC-8 (Pasierbek et al., 2001) (1:200), anti-HIM-3 (Zetka et al., 1999) (1:400), anti-Nop1p (EnCor Biotech, MCA-38F3; 1:200) and anti-MRG-1 (Novus Biologicals, 4913.00.02; 1:1000) (Fujita et al., 2002). Secondary antibodies used were rabbit Alexa 594 (Molecular Probes, A21207; 1:500) and rat and mouse Alexa 488 (Molecular Probes, A21208 and A21202; 1:200). DNA was visualized by 4′,6-diamidino-2-phenylindole (DAPI) staining. Images were captured using a Zeiss Imager Z.1 microscope with an AxioCam MRm camera (Zeiss). Dissected gonads and other worm tissues were mounted on slides. To achieve consistent display, other tissues were cropped out of the immunofluorescence images in Fig. 1A,F and Figs 3, 5 and 6, so that only the gonad arms are shown. Fluorescence intensity was measured as described in the supplementary material Methods.

Western blotting
Western blotting was performed as previously described (Wang et al., 2012). For further details, see the supplementary material Methods.

Yeast two-hybrid analysis
mrg-1 and rfp-1 cDNAs were PCR amplified from wild-type (N2) cDNA using primers A61 and A62 (for mrg-1) and B21 and B22 (for rfp-1), and cloned into the pGEM-T Easy vector (Promega); for primers see supplementary material Methods. Ndel and EcoRI sites (for mrg-1) and Smal and BamHI sites (for rfp-1) were used to subclone mrg-1 and rfp-1 into pGBK7T (DNA-binding domain) and pGAD7T (activation domain) from the Matchmaker Two-Hybrid System 3 (Clontech). Combinations of pDH310 (mrg-1 in pGBK7T), pDH311 (mrg-1 in pGAD7T), pPD339 (rfp-1 in pGAD7T), pPD340 (rfp-1 in pGBK7T) and empty vector controls were co-transformed into the AH109 yeast strain (Matchmaker Two-Hybrid System 3).

Bacterial co-immunoprecipitation
Bacterial co-immunoprecipitation was performed as previously described (Wang et al., 2012). MRG-1 (1-337 aa) and RFP-1 (1-834 aa) cDNAs were PCR amplified (C1 and C2 primers for mrg-1, C3 and C4 for rfp-1; see supplementary material Methods) and cloned into pGEX-4T1 (GE Healthcare) and pET-29a (Novagen), respectively, to generate plasmids pDH354 and pDH355. GST-tag and His6- tag proteins were co-expressed in E. coli BL21(DE3) pLysS. His6- RFP-1 fusion proteins that bound to GST- MRG-1 were co-immunoprecipitated using glutathione agarose beads (GE Healthcare). GST-MRG-1 proteins that interact with His6-RFP-1 were purified on Ni-NTA beads (Qiagen).

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Competing interests
The authors declare no competing financial interests.

Author contributions
P.G. and X.W. performed experiments associated with mrg-1 and helped in preparation of the manuscript. L.L., B.B. and X.W. performed experiments associated with the SRS RNAi screen and downstream genetic analyses of rfp-1. C.W. performed experiments related to the co-immunoprecipitation. K.J. helped with mrg-1 RNAi experiments. D.H. developed the concepts, experimental approaches and prepared the manuscript.

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Supplementary material
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References

Fig. 8. MRG-1 sets up a threshold for proliferation and differentiation. White and grey bars represent hypothetical levels of GLD-1 GLD-2 pathway activity in the distal and proximal germ line, respectively. Dashed lines indicate a hypothetical threshold set by MRG-1 and the corresponding activity of factors controlled by MRG-1. Differentiation occurs if GLD-1 GLD-2 pathway levels are above the MRG-1 threshold and proliferation occurs if the pathway levels are below the threshold. In the wild type and in permissive gpl-1(ar202gf) conditions, GLD-1 GLD-2 pathway levels are sufficient to promote differentiation in the proximal germ line, but not in the distal germ line, even though GLD-1 GLD-2 pathway levels are likely to be lower in gpl-1(ar202gf) animals. In rfp-1(ok572) gpl-1(ar202gf) animals, MRG-1 levels increase (due to decreased degradation), raising the threshold, such that proliferation occurs in the proximal end. In mrg-1(1/fl) animals (M Z −), the threshold is lowered; therefore, even minimal levels of GLD-1 GLD-2 pathway activity promote differentiation.


