CEH-20/Pbx and UNC-62/Meis function upstream of rnt-1/Runx to regulate asymmetric divisions of the C. elegans stem-like seam cells

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Summary
Caenorhabditis elegans seam cells divide in the stem-like mode throughout larval development, with the ability to both self-renew and produce daughters that differentiate. Seam cells typically divide asymmetrically, giving rise to an anterior daughter that fuses with the hypodermis and a posterior daughter that proliferates further. Previously we have identified rnt-1 (a homologue of the mammalian cancer-associated stem cell regulator Runx) as being an important regulator of seam development, acting to promote proliferation; rnt-1 mutants have fewer seam cells whereas overexpressing rnt-1 causes seam cell hyperplasia. We isolated the interacting CEH-20/Pbx and UNC-62/Meis TALE-class transcription factors during a genome-wide RNAi screen for novel regulators of seam cell number. Animals lacking wild type CEH-20 or UNC-62 display seam cell hyperplasia, largely restricted to the anterior of the worm, whereas double mutants have many additional seam cells along the length of the animal. The cellular basis of the hyperplasia involves the symmetrisation of normally asymmetric seam cell divisions towards the proliferative stem-like fate. The hyperplasia is completely suppressed in rnt-1 mutants, and rnt-1 is upregulated in ceh-20 and unc-62 mutants, suggesting that CEH-20 and UNC-62 function upstream of rnt-1 to limit proliferative potential to the appropriate daughter cell. In further support of this we find that CEH-20 is asymmetrically localised in seam daughters following an asymmetric division, being predominantly restricted to anterior nuclei whose fate is to differentiate. Thus, ceh-20 and unc-62 encode crucial regulators of seam cell division asymmetry, acting via rnt-1 to regulate the balance between proliferation and differentiation.

Key words: C. elegans, Seam cell, Asymmetric cell division, ceh-20/Pbx, unc-62/Meis, rnt-1/Runx

Introduction
Asymmetric cell divisions provide an important mechanism for the generation of cellular diversity during development and tissue regeneration. Furthermore, misregulation of asymmetric divisions has been associated with carcinogenesis, underscoring the biomedical importance of understanding this process (Knoblich, 2010; Neumüller and Knoblich, 2009). There are many examples of asymmetric divisions in biology, but one of the most notable is that of a stem cell, which characteristically produces one daughter that adopts a differentiated fate and another that remains a stem cell and proceeds to proliferate further (Knoblich, 2008). Since the discovery that multiple cell types may be derived from single self-renewing stem cells, the potential of these cells to generate all tissue types has led to intensive research into their unique properties.

In C. elegans the neuroectodermal seam cells provide a useful model for stem cell regulation. During larval development, they undergo reiterated asymmetric divisions in order to both self-renew and differentiate into epidermal cells, neurons, glia and ray precursor cells of the male tail. Worms hatch with 10 seam cells on each side (H0, H1, H2, V1–6 and T). The general pattern of division involves an asymmetric division at each larval stage, producing a posterior daughter that retains the ability to divide further and an anterior daughter that adopts a differentiated fate, most commonly contributing to epidermal tissue by fusing with the hyp7 syncytium (Sulston and Horvitz, 1977). In addition, there is a single symmetrical division at L2 in the V lineage, where both daughter cells retain proliferative ability and consequently expand the pool of seam cells so that adult hermaphrodites have 16 seam nuclei per side.

We, and others, have previously shown that the Runx transcription factor rnt-1 and its DNA binding partner bro-1 (a homologue of the Runx binding factor CBFβ) are crucial to regulate the balance between seam cell proliferation and differentiation, acting to promote the proliferative fate in posterior seam daughters (Kagoshima et al., 2007; Nimmo et al., 2005; Xia et al., 2007). Thus, mutations in rnt-1 or bro-1 reduce the number of seam cells due to failures in particular seam cell divisions, whereas overexpressing these genes leads to seam cell hyperplasia at the expense of other differentiated cell types.
(Kagoshima et al., 2007; Nimmo et al., 2005). Strikingly, Runx and CBFβ proteins in other organisms are also important for balancing proliferation and differentiation, particularly in stem cell lineages, for example during haematopoiesis (Blyth et al., 2009; Okuda et al., 1996; Okumura et al., 2007). This underscores the applicability of the seam cell system for understanding stem cell biology and the usefulness of using seam cell number as an assay for identifying genes that are required to control the balance between cell proliferation and differentiation. In particular, rnt-1 and bro-1 are the solo homologues of Runx and CBFβ, therefore studying the molecular pathways involving these genes in C. elegans is not confounded by the redundancy issues experienced in other model systems.

RNT-1 and BRO-1 are required for divisions throughout larval development, and are likely to interact directly with cell cycle regulators (Kagoshima et al., 2007; Nimmo et al., 2005; Xia et al., 2007). One recently identified direct regulator of bro-1 is the GATA factor ELT-1, which has dual roles in both promoting proliferation (via bro-1) and inhibiting differentiation (via the fusogen eff-1) (Brabin et al., 2011). Other regulators (and targets) of rnt-1 and bro-1 are yet to be identified.

Wnt signalling has been shown to be essential for establishing seam cell division asymmetry, in common with its role in a variety of organisms (Clevers, 2006; Grigoryan et al., 2008; Hayden et al., 2007; Yamamoto et al., 2011). In C. elegans, a variant of Wnt signalling termed the Wnt/β-catenin asymmetry pathway regulates asymmetric divisions throughout seam cell development (reviewed by Mizumoto and Sawa, 2007b) and is thought to act in parallel to the rnt-1/bro-1 pathway (Gleason and Eisenmann, 2010). Asymmetry in the dividing mother cell is established by the β-catenin WRM-1, which is enriched at the anterior cortex during telophase via a microtubule-dependent mechanism (Sugioka et al., 2011), thereby excluding WRM-1 from the anterior daughter nucleus (Mizumoto and Sawa, 2007a; Nakamura et al., 2005; Takeshita and Sawa, 2005). WRM-1 in the posterior daughter nucleus causes the export of the TCF/LEF homologue POP-1, thus setting up nuclear reciprocal asymmetry between these two factors (Lo et al., 2004; Nakamura et al., 2005; Rocheleau et al., 1999). The further reciprocal asymmetry between POP-1 and the β-catenin SYS-1 leads to the subsequent transcriptional activation of target genes in the posterior (signalled) daughter and repression of target genes in the anterior daughter, thus establishing the developmental fate appropriate to each daughter (Calvo et al., 2001; Huang et al., 2007; Lin et al., 1998; Lo et al., 2004; Phillips et al., 2007; Rocheleau et al., 1997; Shetty et al., 2005). The engrailed homologue ceh-16 is also required to control seam cell number, and is thought to act in the Wnt pathway. Perturbation of Wnt components suppresses the seam defects associated with ceh-16 mutants, suggesting that ceh-16 may act as an upstream regulator of the Wnt pathway (Huang et al., 2009).

Here, we describe a genome wide RNAi screen to identify novel regulators of seam cell divisions. Two genes, ceh-20 and unc-62, encoding interacting Pbx and Meis transcription factors, were isolated as a result of their knockdown phenotype causing significant seam cell hyperplasia, resulting from the symmetrisation of divisions that are normally asymmetric. Epistasis analysis suggests that ceh-20 and unc-62 act upstream of rnt-1 to repress inappropriate seam cell proliferation in daughters destined to differentiate. Thus, our studies show that CEH-20 and UNC-62 are two novel components of the molecular circuitry controlling seam cell divisions. It is striking that all the factors we have identified thus far as being crucial for the correct coordination of proliferation and differentiation in C. elegans seam cells have human homologues that are implicated in carcinogenesis, particularly acute myeloid leukaemia (Blyth et al., 2005; Cameron and Neil, 2004; Geerts et al., 2005; Licht, 2001; Mikesch et al., 2007; Osato, 2004; Wildonger and Mann, 2005; Wong et al., 2007).

**Materials and Methods**

**Strains and maintenance of worms**

Strains were derived from the wild type N2 Bristol strain and maintained at 20°C as described previously (Brenner, 1974). Strains used are detailed in supplementary material Table S1.

**Microscopy**

DIC (Nomarski) and fluorescent imaging was carried out using a Zeiss Axioskop2 microscope with a Zeiss AxioCamMR digital camera. Photomicrographs were taken using an ×63 oil immersion objective (Zeiss) and Axiovision software (Release 4.5). Images of whole worms were compiled using Adobe Photoshop 7.0 and the backgrounds merged. Confocal images were taken on a Leica TCS SP5II using Leica Application Suite Advanced Fluorescence Lite software (Release 2.2.1). In both cases, animals were mounted on agarose pads (2% agarose, 0.5% 1-phenoxy-2-propanol in M9) in 0.2% 1-phenoxy-2-propanol.

**Lineage analysis**

Lineage analysis was performed as described previously (Sulston and Horvitz, 1977). Seam nuclei were distinguished from hypodermal nuclei based on their morphology in addition to the expression of the seam specific GFP reporter, scm::gfp. Microscopy was performed with Nomarski (DIC) optics and a ×100 oil immersion objective (Zeiss) using a Zeiss AxioPlan microscope.

**RNAi**

RNAi was performed using the Ahringer RNAi library and protocol (Kamath and Ahringer, 2003). dsRNA was delivered by feeding to L4 stage JR667 animals which carry the integrated seam specific scm::gfp marker (Koh and Rothman, 2001). For experiments involving the wrm-1(ice1982ts) strain EB93, L4 animals were placed onto RNAi plates at 15°C. The parents were removed once eggs were laid (approximately 32 hours) and the plates transferred to the restrictive temperature (26.5°C). In all experiments, seam cell number was counted in L4 offspring. Control RNAi was performed using HT115 bacteria transformed with an empty L4440 vector.

The unc-62 feeding clone was not available in the library and was constructed separately by PCR amplification from cDNA using primer pair 1 (supplementary material Table S2). A 1.4 kb fragment was sub-cloned into the Fire Lab RNAi vector L4440 (Timmons and Fire, 1998). The unc-62 RNAi clone was verified by sequencing prior to transformation into HT115 bacteria.

**ceh-20 cDNA::GFP translational reporter construction**

ceh-20::gfp translational reporter constructs were obtained by fusion PCR (Hobert, 2002). ceh-20 with a GFP tag was amplified from cDNA using primer pair 2 (supplementary material Table S2). In parallel, the GFP ORF was ampliﬁed from the Fire Lab vector pPD95.73 with primer pair 3. These PCR fragments were used as a template for fusion PCR with primer pair 4. The resulting PCR product was cloned into pCR2.1-TOPO® (Invitrogen). pAWS24 consisted of ceh-20::gfp from wild type animals while the ceh-20(ay9):gfp (pAWS47) and ceh-20(m290):gfp (pAWS531) constructs were generated using cDNA from the respective mutant animals.

**ceh-20 promoter driven cDNA::GFP translational reporters**

The 2180 bp endogenous ceh-20 promoter was ampliﬁed from genomic DNA (primer pair 5, supplementary material Table S2). The fragment was sub-cloned into the Fire Lab vector pPD49.26 (pAWS30). The ceh-20::gfp plasmids (pAWS24, pAWS31 and pAWS47) were digested with SpeI and EcoRV ligated into pAWS30, previously digested with Nhel and EcoRV. Thus ceh-20::gfp (pAWS24, ceh-20::gfp (pAWS47) and ceh-20(m290):gfp (pAWS536) and ceh-20(ay9):gfp (pAWS50) were generated.

**Seam speciﬁc GFP translational reporters**

To drive speciﬁc expression in the seam, the bro-1 ICNE (pAWS62) was used, which comprises the bro-1 conserved non-coding element (CNE) and pes-10 minimal promoter (Brabin et al., 2011). Plasmids pAWS24, pAWS31 and pAWS47 were digested using SpeI and EcoRV and cloned into pAWS62, previously digested with...
NH$_e$l and EcoRV, generating pAW538 (bro-1CNE::ceh-20(WT)::gfp), pAW537 (bro-1CNE::ceh-20(mu290)::gfp) and pAW551 (bro-1CNE::ceh-20(ay9)::gfp).

Site directed mutagenesis
Amino acid 191 within NLS I of ceh-20 was converted from a lysine (AAA) to a proline (CCG). In this instance, DNA was PCR amplified from pAW538 using primer pair 6 generating pAW538 (bro-1CNE::ceh-20(mutated NLS I)::gfp). The same primers were used to amplify DNA from pAW537 to generate pAW539 (bro-1CNE::ceh-20(mutated NLS I+mu290)::gfp).

Construction of transgenic worms
Plasmids were injected into the syncytial gonad of L4 hermaphrodite animals at a concentration of 10–20 ng/µl as described previously (Mello and Fire, 1995).

Electrophoretic Mobility Shift Assay plasmids
PCR was used to amplify the full length ceh-20 and unc-62 cDNA using primer pairs 7 and 8 respectively (supplementary material Table S2) and cloned into pCR®-XL-TOPO® (Invitrogen) resulting in plasmids pAW521 (ceh-20) and pAW579 (unc-62). The TNT® Quick Coupled Transcription/Translation kit (Promega) was used for in vitro transcription and translation of cDNA constructs. The probe of a consensus Pbx/Meis binding site 5'CGGAGGACCCCGTAT-TGAACGGTCCTCC-3' (Shanmugam et al., 1999) was labelled with [γ-32P]ATP using polynucleotide kinase (Promega). The oligos were annealed by heating at 95°C for 5 minutes followed by gradual cooling to room temperature. The DNA binding reaction was carried out on ice for 30 minutes in Ficoll 400 (20% w/v) with PolyD/Ic (1 mg/ml). Reactions were run on a 7% non-denaturing polyacrylamide gel at 4°C in 0.5xTBE.

Real-time quantitative PCR analysis
RNA was extracted from L4 synchronised larvae using the hot phenol method (Furger et al., 2001). mRNA levels of rnt-1 and bro-1 (primer pairs 9 and 10, supplementary material Table S2) and a housekeeping gene mu2-0 (primer pair 11) were measured using GoTaq® qPCR Master Mix (Promega) with a StepOnePlus™ Real-Time PCR System (Applied Biosystems). The Ct values of rnt-1 and bro-1 were measured in all strains and normalised to mu2-0, an NADH ubiquinone oxidoreductase expressed in all seam cells, to correct for seam cell number. Expression levels were assayed using the ∆∆Ct method (Schmittgen and Livak, 2008).

Results
A genome-wide RNAi screen identified ceh-20 as a regulator of seam cell proliferation
A genome-wide RNAi by feeding screen was undertaken to identify novel regulators of seam cell proliferation, using animals carrying the integrated scm::gfp seam-specific marker (strain JR667) (Koh and Rothman, 2001). Seam cell number was counted in late L4, after the final asymmetric division but before terminal differentiation. We identified 307 genes that when silenced by RNAi, altered seam cell number in L4 animals (Table 1; supplementary material Table S3). Of these, 137 genes increased the number of seam cells while the remainder reduced the number of seam cells to below 16. The most striking phenotype observed was the seam cell hyperplasia caused by ceh-20 knockdown. Seam cell number is approximately doubled in ceh-20(RNAi) animals (Fig. 1A,C). Two viable ceh-20 missense alleles (Jiang et al., 2009; Takács-Vellai et al., 2007; Yang...
et al., 2005) were next tested for seam cell hyperplasia and mu290, but not the weaker allele ay9, displayed an increase in seam cell number, comparable to that observed with RNAi (Fig. 1A,C). In both cases (mu290 and ceh-20(RNAi)), seam hyperplasia showed a strong anterior bias in all animals observed (Fig. 1A, Fig. 2).

The CEH-20 binding partner UNC-62 is also essential for limiting seam cell proliferation. CEH-20 is a member of the conserved Pbx family of transcriptional regulators, initially identified as human leukaemic proto-oncogenes (Laurent et al., 2008), that are known to bind cooperatively to Meis proteins via interaction of their conserved PBC domains with the Meis Homothorax-Meis (HM) domain (Abu-Shaar et al., 1999; Rieckhof et al., 1997; Ryoo et al., 1999; Stevens and Mann, 2007). Pbx proteins are members of the Three Amino acid Loop Extension (TALE) class of homeodomain proteins, which have been shown to be important transcription factors or transcriptional co-factors in development throughout the animal kingdom (Moens and Selleri, 2006). C. elegans has two members of the Meis class of TALE proteins, psa-3 and unc-62 (Arata et al., 2006; Van Auken et al., 2002). We found no change in overall seam cell number following psa-3 RNAi (data not shown).

unc-62 was not isolated in our screen because it is not present in the RNAi feeding library. We therefore constructed an unc-62 RNAi feeding clone that caused a modest elevation in seam cell number when fed to worms (Fig. 1B,C). The mutant allele ku234 gave a much stronger phenotype while a second mutant allele, e644, did not have any effect on seam cell number (Fig. 1B,C). The hyperplasia in ku234 animals was again characterized by an obvious anterior bias, similar to that of ceh-20(mu290) and ceh-20(RNAi) animals (Fig. 2).

unc-62 is subject to alternative splicing (Van Auken et al., 2002). The ku234 allele results in a point mutation in the start codon of the 1b transcript. This has been suggested to shift the start codon downstream, likely resulting in a truncated protein lacking the important Pbx-interacting HM domain (Van Auken et al., 2002). The e644 allele introduces a stop codon in exon 7b, which may disrupt the homeodomain (Van Auken et al., 2002). It is thus not clear at present why seam hyperplasia is observed in ku234 but not e644 animals. Given the relative ineffectiveness of unc-62 RNAi,
The ceh-20; unc-62 double mutant, strain AW679, displayed much more extensive seam hyperplasia, up to 70 seam cells per side, extending throughout the length of the animal (Fig. 2), suggesting that CEH-20 and UNC-62 interact in a complex to control the correct development of the seam lineage. This mirrors the function of CEH-20/UNC-62 in other tissues, for example the M lineage and the vulva (Jiang et al., 2009; Yang et al., 2005).

Regulatory interactions between CEH-20 and UNC-62

UNC-62 has been shown to bind to CEH-20 in a yeast 2 hybrid assay (Jiang et al., 2009), consistent with previously described Meis/Pbx interactions in other systems (Abu-Shaar et al., 1999; Arata et al., 2006; Potts et al., 2009; Rieckhof et al., 1997; Ryoo et al., 1999; Van Auken et al., 2002). To confirm this interaction we performed an Electrophoretic Mobility Shift Assay (EMSA), which demonstrated binding to the consensus Pbx/Meis DNA binding site by a complex of CEH-20 and UNC-62 (supplementary material Fig. S1). In addition, Meis proteins have previously been shown to be required for the nuclear localisation of Pbx (Abu-Shaar et al., 1999; Arata et al., 2006; Potts et al., 2009; Rieckhof et al., 1997; Ryoo et al., 1999; Stevens and Mann, 2007). We therefore examined the subcellular localisation of CEH-20 in seam cells using ceh-20::gfp constructs driven by the strong seam enhancer bro-1CNE (Brabin et al., 2011). Overall, we observed that wild type ceh-20 constructs were expressed predominantly in seam nuclei (Fig. 3B). However, a construct containing the mu290 mutation (associated with seam hyperplasia) was strongly enriched in the cytoplasm (Fig. 3B). The mu290 mutation affects one of the two nuclear localisation signals (NLSs) in ceh-20, and our analysis demonstrated that this NLS (NLS II, but not NLS I) is essential for correct nuclear localisation in seam cells (Fig. 3B). Enriched cytoplasmic localisation was also observed in unc-62(ku234) mutants, indicating that UNC-62 is indeed required for nuclear enrichment of CEH-20 in seam cells (Fig. 3B).

The seam cell hyperplasia in ceh-20 and unc-62 mutants is a consequence of symmetrisation of seam cell divisions

To investigate the cellular basis of the ceh-20 and unc-62 hyperplasia phenotypes, lineage analysis was performed on wild type and mutant strains (Fig. 4). In both single mutants, additional seam cells first appeared at the L1 division (Fig. 4B), with the normally asymmetric division of H1 being symmetrised towards the proliferative fate (the H2 L1 division is symmetric in wild type animals). The seam identity of these daughters was established by the fact that they continued to express scm::gfp whereas non seam daughters usually lose their GFP within one or two hours of the L1 division (data not shown). Both daughters of H1 and H2 were seen to divide again, in the symmetric pattern, at the L2 stage. Significantly, the H2.a daughter did not divide several hours before the L2 division, as is normal, but instead delayed the timing of its division to coincide with that of other seam daughters at L2, suggesting a complete conversion of developmental fate to that of the posterior daughter. These defects occurred in all animals observed. Lineages were not followed beyond L2 because there were simply too many seam cells to observe.
While the hyperplasia in the single ceh-20 and unc-62 mutants is most striking in the H1 and H2 lineages, the V lineages were also affected, but with much more variable outcomes (Fig. 4B). Here, early Vn divisions (during L1) were sometimes symmetrised towards the proliferative fate, but Vn.a divisions were also observed to be symmetrised towards the hypodermal (differentiative) fate. We also often observed the Vn.p daughter undergoing an asymmetric division at the beginning of L2, instead of the normal symmetric division. Thus, in the posterior of the worm, one lineage might produce more cells with the seam fate than normal, resulting in clusters of extra seam cells, whereas others could produce fewer, producing gaps in the seam.

In the double mutant, however, extensive symmetrisation events were observed throughout H and V lineages (Fig. 4C), especially after L1, leading to much more severe hyperplasia along the length of the worm (Fig. 2).

The most common outcome of an asymmetric division in the seam lineage is a single seam daughter plus a differentiated hypodermal daughter that expresses dpy-7 and fuses with hyp7. Therefore, the abnormal expansion of seam cell number would be expected to occur at the cost of the hypodermal fate. To confirm this, we used an integrated dpy-7p::yfp reporter and found significantly fewer DPY-7::YFP positive nuclei in ceh-20 and unc-62 mutants compared to wild type (Fig. 4D).

ceh-20 and unc-62 function downstream of or in parallel to the Wnt pathway to regulate seam divisions

Given the well-characterised role of Wnt signalling in regulating asymmetric cell division, we tested possible interactions between ceh-20, unc-62 and the Wnt pathway. In wild type animals, the β-catenin WRM-1 has been shown to be asymmetrised during asymmetric V5 and T seam cell divisions, being enriched in posterior daughter nuclei and at the anterior cortex (Mizumoto and Sawa, 2007a; Takeshita and Sawa, 2005) This is thought to be essential for correct cell fate patterning, as WRM-1 in the nucleus causes the export of POP-1/TCF and thus subsequent adoption of the signalled fate (which is proliferative in the case of the seam cells) (Calvo et al., 2001; Gleason and Eisenmann, 2010; Herman, 2001; Huang et al., 2001; Lin et al., 1998; Lo et al., 2004; Phillips et al., 2007; Shetty et al., 2005). Using a wrm-1::gfp reporter (strain HS1417), we confirmed that WRM-1 asymmetric localisation also occurs in anterior seam cell lineages (H1 and H2) during the L3 asymmetric division (Fig. 5A).

In contrast, ceh-20(RNAi) animals displayed distinct nuclear WRM-1::GFP localisation in all daughters (Fig. 5B,C). An important question, however, is whether abnormal WRM-1 localisation in ceh-20(RNAi) animals causes the symmetrisation of seam divisions (implying that ceh-20 works upstream of WRM-

Table 2. Seam hyperplasia is not dependent upon the presence of WRM-1.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Temperature (˚C)</th>
<th>n</th>
<th>Average seam cells per side±s.e.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type; control(RNAi)</td>
<td>25</td>
<td>62</td>
<td>16±0.08</td>
</tr>
<tr>
<td>Wild type; control(RNAi)</td>
<td>26.5</td>
<td>113</td>
<td>17±0.12</td>
</tr>
<tr>
<td>Wild type; ceh-20(RNAi)</td>
<td>26.5</td>
<td>107</td>
<td>33±0.96</td>
</tr>
<tr>
<td>wrm-1(ne1982ts); control(RNAi)</td>
<td>25</td>
<td>67</td>
<td>16±0.25</td>
</tr>
<tr>
<td>wrm-1(ne1982ts); control(RNAi)</td>
<td>26.5</td>
<td>103</td>
<td>5±0.15</td>
</tr>
<tr>
<td>wrm-1(ne1982ts); ceh-20(RNAi)</td>
<td>26.5</td>
<td>107</td>
<td>33±0.96</td>
</tr>
</tbody>
</table>

At both the permissive (25˚C) and restrictive temperature (26.5˚C) wild type animals (strain JR667) have 16 seam cells per side, whereas wrm-1 (ne1982ts); scm::gfp (strain EW95) animals have just 5 seam cells on average at the restrictive temperature. However, seam cell hyperplasia is evident to the same extent in WT and wrm-1 ts mutants following exposure to ceh-20 RNAi at the restrictive temperature.
regulate if not the Wnt pathway? rnt-1 and bro-1 are known regulators of seam cell proliferation and self-renewal (Kagoshima et al., 2007; Nimmo et al., 2005; Nimmo and Woollard, 2008; Xia et al., 2007), therefore we tested for regulatory interactions using rnt-1 and bro-1 single and double mutants. The seam cell hyperplasia (and symmetrisation of divisions) observed in ceh-20 and unc-62 mutants is completely suppressed in rnt-1 and/or bro-1 mutant backgrounds (Fig. 7A,B), suggesting that rnt-1 and/or bro-1 may be normally repressed by CEH-20 and UNC-62 in cells that are not destined to proliferate further. In order to test this, we measured mRNA levels using q-PCR. We found upregulation of rnt-1 mRNA in ceh-20 and unc-62 single mutants, with a larger increase in double mutants (Fig. 7C). bro-1 mRNA expression remained unchanged in these strains (Fig. 7C). Thus, the symmetrisation of seam cell divisions towards the proliferative fate in ceh-20 and unc-62 mutants can be explained, at least in part, by the de-repression of proliferative targets such as rnt-1.

ceh-20 and unc-62 act redundantly with rnt-1 and bro-1 during early development

Intriguingly, our analysis of the bro-1 rnt-1: unc-62 triple mutant revealed that seam cell number was slightly lower in this strain than in bro-1 rnt-1 double mutants (Fig. 7A). This suggests a possible role for ceh-20 and/or unc-62 in seam cell proliferation that is redundant with rnt-1/bro-1. Indeed, we observed that bro-1 rnt-1: unc-62 triple mutants and bro-1 rnt-1: ceh-20(RNAi) animals frequently hatched with fewer than 10 seam cells per side indicative of defects in embryonic development, a phenotype never observed in bro-1 rnt-1 double mutants or ceh-20/unc-62 mutants (supplementary material Fig. S2A). In addition triple mutants displayed high levels of embryonic and larval lethality, with synthetic lethality being even more prominent in bro-1 rnt-1: unc-62; ceh-20(RNAi) animals (supplementary material Fig. S2B). Reduced seam cell number is not usually correlated with lethality, suggesting that bro-1, rnt-1, unc-62 and ceh-20 have hitherto undescribed overlapping roles during embryogenesis and larval development, distinct from their post-embryonic roles in seam development.

Asymmetric distribution of CEH-20 following seam cell divisions

During post-embryonic seam cell divisions, rnt-1 appears to be a downstream target of CEH-20 and UNC-62, as seam cell hyperplasia is repressed in rnt-1 mutants and rnt-1 expression is upregulated in ceh-20/unc-62 mutants. Thus, we were interested to test whether ceh-20 and/or unc-62 are differentially expressed in anterior vs posterior daughters. We used full-length ceh-20 and unc-62 translational reporters that were clearly observed in seam cell nuclei (Fig. 8A,B). ceh-20::gfp expression was observed to be higher in anterior daughters of asymmetric seam cell divisions compared with posterior daughters while no obvious differences in distribution of unc-62::CFP were observed (Fig. 8C).

Taken together, our data suggest that CEH-20 and UNC-62 work together to repress inappropriate rnt-1 expression during post-embryonic seam cell divisions in order to regulate the balance between proliferation and differentiation. In ceh-20 and unc-62 mutants this balance is lost and both daughters adopt the proliferative fate, resulting in a symmetrical division pattern.

Discussion

The phenotypes associated with ceh-20(mu290) and unc-62(ku234) alleles are very similar, with a high level of seam
cell hyperplasia, predominantly at the anterior of the worm. Lineage analysis revealed a clear cellular basis for the hyperplasia phenotype; divisions in the anterior seam cell linages H1 and H2 are completely symmetrised towards the seam fate of further cell proliferation, giving rise to the expansion in seam cell number. In no cases were extra rounds of division observed; ceh-20 and unc-62 mutations only affected the symmetry of scheduled divisions, suggesting that mechanisms controlling the timing of seam cell divisions (such as the heterochronic pathway) are intact in ceh-20 and unc-62 mutants.

In ceh-20; unc-62 double mutants, extensive hyperplasia was observed throughout the length of the animal. Thus, CEH-20 and UNC-62 act redundantly in V lineages, but non-redundantly in H lineages, to prevent inappropriate proliferation. This regional specialization in CEH-20/UNC-62 function may be suggestive of interaction with an additional factor. An obvious candidate for such a factor would be a Hox gene, as these are well-characterised regulators of positional identity. Indeed, Meis and Pbx proteins have been shown to act as Hox co-factors in several different systems (Shanmugam et al., 1999; Shen et al., 1999). In C. elegans, CEH-20 and UNC-62 form a tripartite complex with LIN-39 during vulval development (Yang et al., 2005), and with MAB-5 or LIN-39 during mesoderm development (Jiang et al., 2009). Furthermore, there is already an established link between the alternative Meis psa-3, Pbx (ceh-20) and the posterior Hox (nob-1) in regulating cell fate determination during the first division of the T cell. Here, ceh-20, psa-3 and nob-1 mutants have similar phenotypes, involving the failure of posterior T daughters to acquire neural fate and it has been suggested that...
PSA-3 and NOB-1 form a tripartite complex with CEH-20 to direct the fate choice of the posterior daughter cell (Arata et al., 2006). However, when we knocked down all six Hox genes individually by RNAi either in wild type, ceh-20(mu290) or unc-62(ku254) backgrounds we found no obvious differences in seam cell number or division pattern (S.H., unpublished), so possible interactions of CEH-20 and UNC-62 with Hox factors in H and V seam lineages remain unclear.

Although we observed that WRM-1 localisation is perturbed in ceh-20(RNAi) animals, wrm-1 was clearly not required for the hyperplasia in ceh-20(RNAi) animals, suggesting that ceh-20 and presumably unc-62 act downstream of WRM-1, or in a parallel pathway. Furthermore, we observed that WRM-1 distribution in wild type seam cell divisions does not always follow the expected pattern; we always found WRM-1 to be asymmetrically localised between seam daughters with enrichment at the posterior nucleus, even during the L1 division of H1 (which is reversed) and the L2 symmetrical division of Vn cells. This fits with previous observations of the distribution of POP-1, which has also been reported to be asymmetric during the L2 symmetrical division (Wildwater et al., 2011). Therefore, seam cells must have some mechanism of overriding WRM-1/POP-1 asymmetry when polarity needs to be reversed, or when a symmetrical division is required.

The absolute dependence of hyperplasia in ceh-20/unc-62 mutants on rnt-1 but not wrm-1 allows us to hypothesise that ceh-20/unc-62 function in the rnt-1 pathway, upstream of rnt-1, and either downstream of or in parallel to Wnt signalling. Given that rnt-1 is thought to act in parallel to Wnt signalling (Gleason and Eisenmann, 2010), the most likely interpretation is that ceh-20/unc-62 encode upstream regulators of rnt-1 working in parallel to Wnt signalling to regulate seam cell divisions. This mechanism is in contrast to that described for the action of CEH-20/PSA-3/NOB-1 in the T cell, where POP-1 directly activates psa-3 transcription in the posterior daughter of T (and no other seam cell) in conjunction with CEH-20 and NOB-1 (Arata et al., 2006). UNC-62, in contrast to PSA-3, appears to have uniform localisation throughout the seam, with no apparent bias to either daughter nucleus at division, and no obvious binding sites for POP-1 (C.B., unpublished). Overall, therefore, it is clearly not possible to directly relate this model to the more anterior lineages simply by substituting unc-62 for psa-3 and invoking the involvement of an anterior Hox gene.

We found that rnt-1 expression is upregulated in ceh-20 and unc-62 mutants, suggesting that CEH-20/UNC-62 repress rnt-1 expression. At present we do not know whether this repression is direct or indirect. The asymmetric expression of ceh-20 in seam daughters suggests a model in which CEH-20 and UNC-62 repress rnt-1 in anterior daughters destined not to proliferate further. In support of this, rnt-1 is known not to be normally expressed in anterior daughters such as hyp7 and can be observed to disappear at or soon after division (Kagoshima et al., 2007). Forced overexpression of rnt-1, on the other hand, is known to cause seam hyperplasia due to inappropriate symmetrisation of seam divisions (Kagoshima et al., 2007), thus it is crucial to tightly regulate rnt-1 expression in order to maintain the correct pattern of seam cell divisions. ceh-20 and unc-62 define an important mechanism to achieve this.

Interactions between rnt-1, bro-1, ceh-20 and unc-62 appear to be rather different during embryonic development, however, with high levels of embryonic and larval lethality in triple and quadruple mutants. This suggests redundant roles for these factors during embryogenesis, likely outside of the seam cells. Possible functions of rnt-1 and bro-1 during embryogenesis are unknown at present, although it is intriguing that synthetic lethality between rnt-1 or bro-1 and a diverse collection of developmental genes have been reported, including dpy-22 (Xia et al., 2007), lon-1 (Ji et al., 2004), pha-2 and eat-3 (Mörck and Pilon, 2006). This suggests that bro-1 and rnt-1 may function in several different tissues in combination with other factors.

Overall, it is striking that many of the genes isolated in unbiased screens for regulators of seam cell development have human homologues implicated in various cancers. In particular, Pbx and Meis proteins and Runx and CBFβ, are all known to regulate the proliferative potential of haematopoietic stem cells (Chen et al., 2009; Okuda et al., 1996; Thorsteinsdottir et al., 2001; Wang et al., 2006). The strong connections between perturbations in asymmetric cell divisions and tumourigenesis, underscored here in the context of hyperplasia of the stem-like seam cells, highlight the usefulness of the C. elegans seam cell model for defining novel elements and interactions of these pathways in a system that is not hampered by genetic redundancy experienced in systems with multiple paralogues of Runx, Pbx and Meis.

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Competing Interests

The authors have no competing interests to declare.

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