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Report

Wnt Signaling and CEH-22/tinman/Nkx2.5 Specify a Stem Cell Niche in *C. elegans*

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Summary

Wnt signaling regulates many aspects of metazoan development, including stem cells [1–3]. In *C. elegans*, Wnt/MAPK signaling controls asymmetric divisions [4, 5]. A recent model proposed that the POP-1/TCF DNA binding protein works together with SYS-1/β-catenin to activate transcription of target genes in response to Wnt/MAPK signaling [6]. The somatic gonadal precursor (SGP) divides asymmetrically to generate distal and proximal daughters of distinct fates: only its distal daughter generates a distal tip cell (DTC), which is required for stem cell maintenance [7]. No DTCs are produced in the absence of POP-1/TCF or SYS-1/β-catenin, and extra DTCs are made upon overexpression of SYS-1/β-catenin [6, 8, 9]. Here we report that POP-1/TCF and SYS-1/β-catenin directly activate transcription of *ceh-22/nkx2.5* isoforms in SGP distal daughters, a finding that confirms the proposed model of Wnt/MAPK signaling. In addition, we demonstrate that the CEH-22/Nkx2.5 homeodomain transcription factor is a key regulator of DTC specification. We speculate that these conserved molecular regulators of the DTC niche in nematodes may provide insight into specification of stem cell niches more broadly.

Results and Discussion

The *C. elegans* DTC is specified by Wnt/MAPK signaling, which controls an asymmetric cell division during early gonadal development (Figures 1A and 1B) [6]. When Wnt/MAPK signaling is compromised, both SGP daughters adopt proximal cell fates, the Sys (for symmetric sister) phenotype [9, 10]. *sys-3(q632)* mutants display typical Sys defects [10]. Whereas wild-type hermaphrodites possess two DTCs and two gonadal arms, most *sys-3(q632)* mutants lack one or both DTCs and also lack one or both gonadal arms (Figure 1D, top line) [10]. In hermaphrodites, the presence of a gonadal arm serves as a simple morphological readout for the presence of a DTC, because DTCs control formation of the elongate arm ("leader function") as well as germline stem cell maintenance ("niche function") [7]. Wild-type males have two DTCs that function solely to maintain germline stem cells [7], but most *sys-3(q632)* males have no DTCs and consequently possess little or no germline. Genetic analyses indicated that *sys-3* acts in parallel to or downstream of POP-1/TCF [10].

We cloned the *sys-3* gene to further understand the molecular regulation of the SGP asymmetric division. To this end, we first mapped *sys-3(q632)* to a site near the *ceh-22* locus (see Supplemental Experimental Procedures available with this article online). Three lines of evidence suggest that *sys-3(q632)* is allelic to *ceh-22*. First, *sys-3(q632)* and the *ceh-22(cc8266)* internal deletion [11] (Figure 1C) failed to complement each other for the Sys phenotype. Second, reduction of *ceh-22* by RNA interference (RNAi) resulted in loss of gonadal arms (20% 0 arm, 31% 1 arm, 49% 2 arms, n = 64), a typical Sys defect. Finally, *ceh-22* genomic DNA rescued the *q632* Sys phenotype: most *q632* mutants carrying genomic *ceh-22* made two DTCs and two gonadal arms and were fertile (Figure 1D, *ceh-22(genomic)*). To identify the *q632* molecular lesion, we sequenced *ceh-22* genomic DNA from *sys-3(q632)* homozygotes. We found one lesion, a deletion of 400 bp within the first intron (Figure 1C). To explore the importance of the DNA deleted by *sys-3(q632),* we generated a transgene in which the first intron was deleted, but the *ceh-22* coding region was intact (Figure 1D, *ceh-22(Δ1stIntron)*). This transgene failed to rescue *q632* (Figure 1D), a result consistent with a previous study showing that *ceh-22* cDNA rescued pharyngeal, but not gonadal, defects of *cc8266* mutants [12]. We conclude that the first intron of *ceh-22* is critical for gonadal development.

The *ceh-22* gene generates transcripts of at least two sizes [13]. The exon/intron composition of the longer mRNA, which we dub *ceh-22a,* is well established [13] (Figure 1C). Our finding that the first intron is critical for gonadal development suggested to us that the first intron might act as a promoter to drive transcription of a shorter *ceh-22* mRNA; by this model, the shorter mRNA is predicted to lack the first exon. To test this idea, we performed RT-PCR with primers designed to identify *ceh-22* cDNAs lacking the first exon and found two *ceh-22* isoforms, which we call *ceh-22b* and *ceh-22c* (Figure 1C; see Experimental Procedures). The *ceh-22b* transcript contains exons 2 to 7 and carries SL1 *trans*-spliced directly to exon 2; its first methionine codon in-frame with the homeodomain occurs in exon 4 (Figure 1C). The *ceh-22c* transcript includes a fragment of the first intron plus exons 2 to 7; this isoform harbors within the first intron a methionine codon and potential initiation codon that occurs in-frame with the *ceh-22* coding region of the second exon (Figure 1C, blue M). We did not detect *trans*-splicing of SL1 to the *ceh-22c* isoform. To ask whether *ceh-22b* and *ceh-22c* mRNAs are functional, we generated two transgenes. The first, called *ceh-22b(genomic)* (Figure 1D), contained the first intron plus exons 2 through 7 of the *ceh-22* coding region, but lacked exon 1 and other upstream sequences. The second, called *ceh-22b(cDNA)*, was similar, but also lacked all introns except the first one (Figure 1D). Both *ceh-22b(genomic)* and *ceh-22b(cDNA)* transgenes

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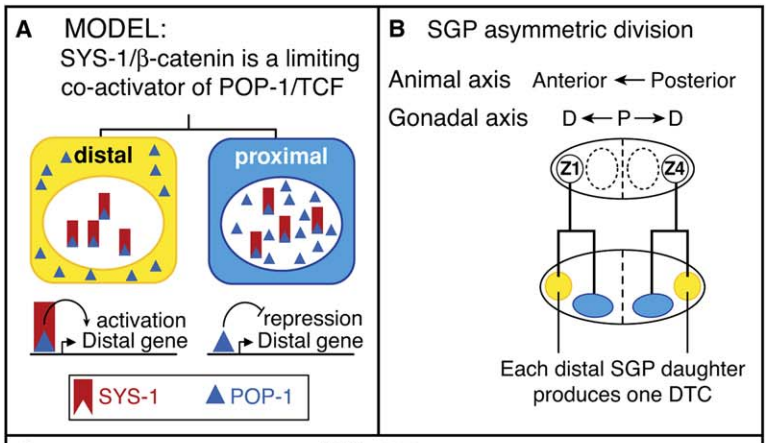

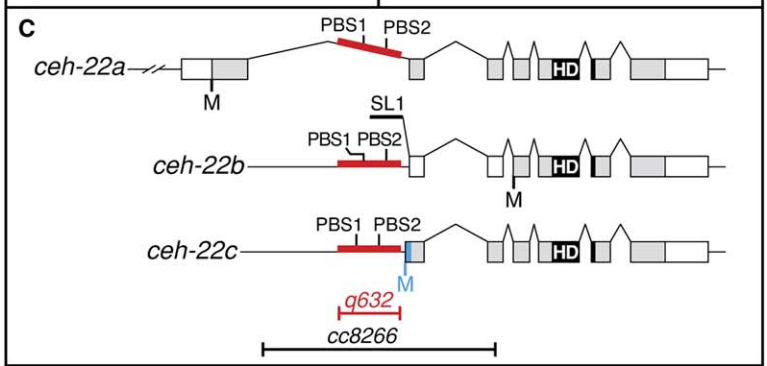

D Transgene	Number of DTC in *ceh-22(q632)* mutants (%) Two	One	None	n	Rescue?
none	24	38	38	150	NA
ceh-22(genomic)	97	3	0	127	+
ceh-22(Δ1stIntron)	25	35	40	92	–
ceh-22b(genomic)	97	3	0	160	+
ceh-22b(cDNA)	95	5	0	114	+
ceh-22b(ΔC)	23	48	28	89	–
ceh-22c(mutMet)	71	21	8	117	+/–
ceh-22c(mutPBS)	26	42	32	149	–

Figure 1. Control of SGP Asymmetric Division and Molecular Identification of *q632* as an Unusual *ceh-22* Allele

(A) Model for control of target genes by POP-1/TCF and SYS-1/β-catenin. In this model, the amount of available SYS-1 determines whether POP-1 functions as a transcriptional activator or repressor. In the distal daughter (yellow), the abundance of nuclear POP-1 is reduced so that most POP-1 is associated with SYS-1 and activates transcription. In the proximal daughter (blue), abundance of nuclear POP-1 is high, and therefore most POP-1 cannot associate with SYS-1 and cannot activate transcription.

(B) SGP asymmetric division. Top diagram, gonadal primordium containing SGPs (Z1 and Z4) at poles and primordial germ cells centrally. Bottom diagram, SGP daughters: yellow, distal daughters; blue, proximal daughters. D, distal; P, proximal. The distal daughter either differentiates as a distal tip cell (males) or generates a distal tip cell in its next division (hermaphrodites). The male DTC (mDTC) functions solely as a stem cell niche [7]; the hermaphrodite DTC has "leader" function in addition to niche function; therefore, the hermaphrodite DTC not only controls germline stem cells, but also controls formation of the elongate U-shaped gonadal arm [7].

(C) *ceh-22* gene structure. Boxes, exons; white, noncoding sequence; gray, coding sequence; black, coding for homeodomain (HD); M, predicted initiation codon for each isoform; bent lines, introns. The regions deleted by *cc8266* and *q632* are shown below. The extent of the *q632* deletion is represented in red in each isoform. PBS, POP-1 binding site; SL1, *trans*-spliced leader.

(D) Both the first intron and the *ceh-22* coding region and POP-1 binding sites are required for rescue of *q632* mutants. Transgenes (see Supplemental Experimental Procedures for details), conventions as in Figure 2A; numbers of DTCs were scored by counting numbers of elongated gonadal arms. The *ceh-22(genomic)* fragment contained ~2 kb of 5′ flanking sequence, all exons and introns plus 90 bp of 3′ flanking sequence.

rescued *q632* Sys defects as efficiently as the full genomic region (Figure 1D). Importantly, the *ceh-22* coding sequence was required for rescue: a *ceh-22b(ΔC)* transgene lacking exons 6 and 7 lost rescuing activity (Figure 1D). To ask if the *ceh-22c*-specific methionine in the first intron might be employed, we mutated the methionine codon to a stop codon in the *ceh-22c(mutMet)* transgene; this mutant diminished, but did not abolish, rescue (Figure 1D, *ceh-22c(mutMet)*). Therefore, it seems likely that both *ceh-22b* and *ceh-22c*

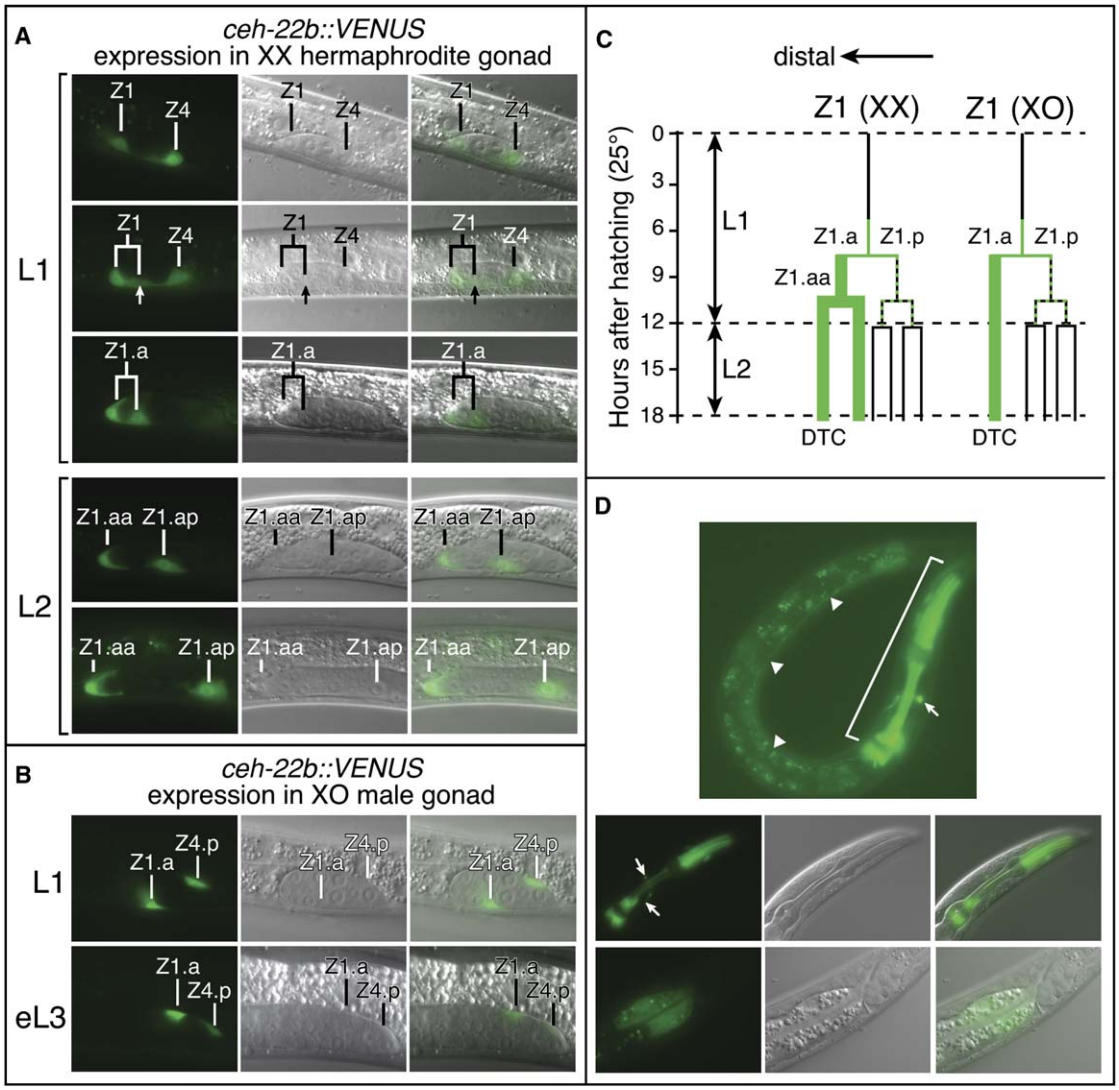

Figure 2. *ceh-22b::VENUS* Is Expressed Asymmetrically in the SGP Lineage

(A and B) Each row shows immunofluorescent (left), Nomarski (middle), and merged (right) images. Z1, anterior SGP; Z4, posterior SGP; Z1.a, distal Z1 daughter; Z1.p, proximal Z1 daughter. Images were taken at different exposures, so levels of expression are not comparable from stage to stage.

(A) *ceh-22b::VENUS* expression in hermaphrodites (XX) gonads. During L1, expression is detected in both SGPs, remains high in the distal SGP daughter (Z1.a), and is reduced in the proximal SGP daughter (Z1.p) (arrow). Expression is increased and maintained in the distal SGP daughter and its progeny, Z1.aa and Z1.ap, throughout L2, but disappears from the proximal SGP daughter and its descendants.

(B) *ceh-22b::VENUS* expression in male (XO) gonads. Expression was detected in the distal SGP daughters but is not visible in proximal SGP daughters at this exposure.

(C) Summary of *ceh-22b::VENUS* expression in L1 and L2 SGP lineage. Only Z1 lineage shown, although Z4 lineage was similar. Each vertical line represents a cell and each horizontal line represents a division. Black line, no detectable reporter expression; green line, reporter is expressed at level represented by line thickness (thin lines indicate low expression and thick lines indicate high expression); dotted line, diminished expression.

(D) *ceh-22b::VENUS* expression in nongonadal tissues. Top, expression in pharynx (bracket), head neuron (arrow), and ventral nerve cord (arrowheads). Middle, lower exposure to show distinct areas of pharyngeal expression. Bottom, expression in posterior intestinal cells.

isoforms are used. For simplicity, we refer to both isoforms collectively as *ceh-22b.*

To test whether the first *ceh-22* intron has promoter activity and to learn whether it drives expression in the somatic gonad, we created *ceh-22b::VENUS,* an integrated reporter transgene that links the *ceh-22* first intron to the Venus coding sequence (see Supplemental Experimental Procedures); Venus is a bright variant of YFP [14]. Two independent lines displayed the same expression pattern (Figure 2). In first stage larvae (L1) of both sexes, *ceh-22b::VENUS* expression was not detected in SGPs at hatching, but became visible midway through the first larval stage (L1) (Figures 2A and 2B; data not shown). We note that Z1 and Z4 refer to the anterior and posterior SGPs, respectively (Figure 1A). After the SGP divided, the intensity of the reporter began to increase in distal SGP daughters (Z1.a and Z4.p) and began to diminish from proximal SGP daughters (Z1.p and

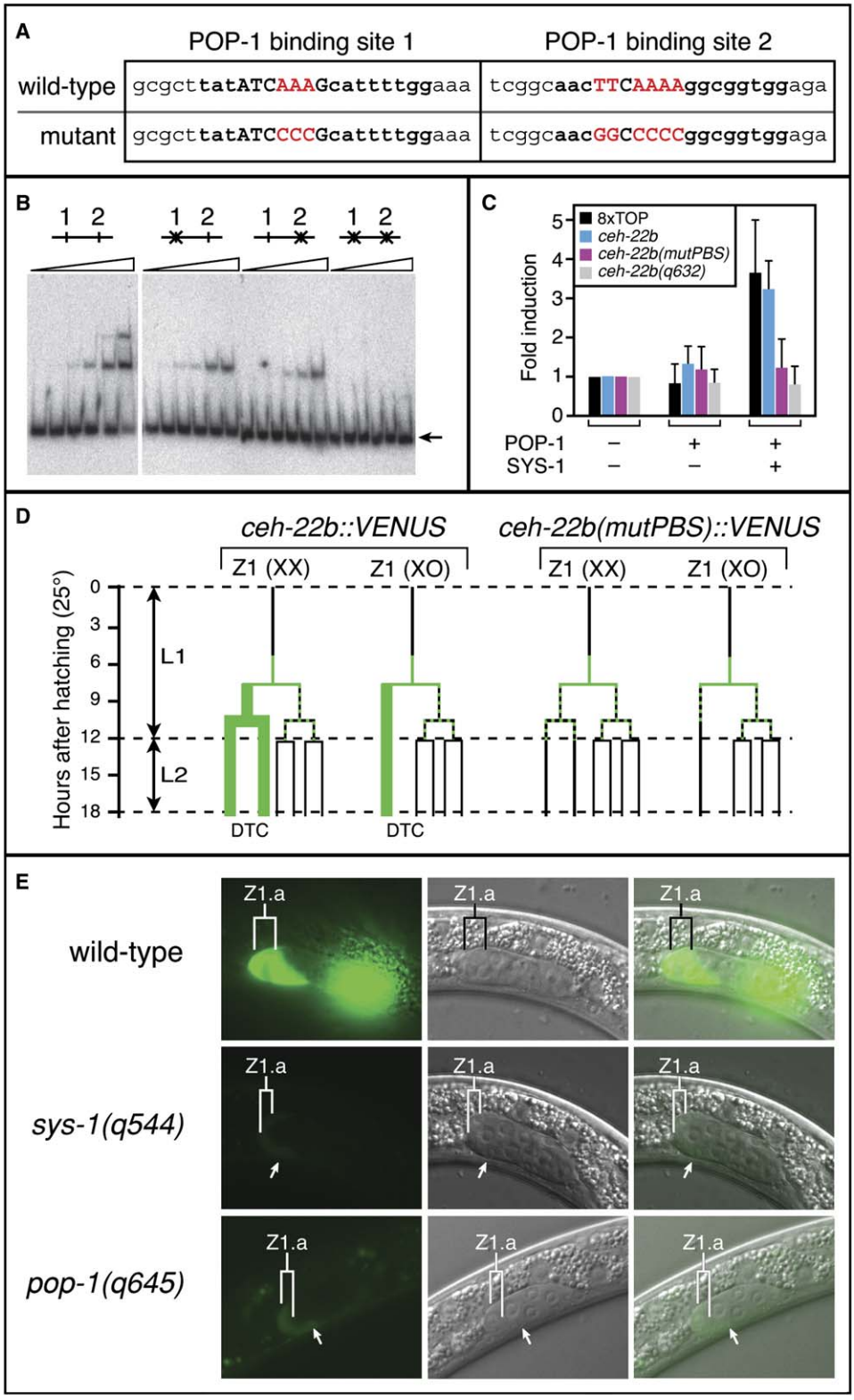

Figure 3. *ceh-22b* Is a Direct Target of POP-1 and SYS-1 Transcriptional Activation

(A) Sequence of POP-1 binding sites (PBS) in *ceh-22b* promoter. Capital letters indicate core binding region with sequence similarity to canonical TCF binding site of TTCAAAG. Red nucleotides show sequences mutated to abolish POP-1 binding and used as controls in the following experiments.

(B) Electrophoretic mobility assay. Above, diagram of probe; each is a 254 bp DNA fragment containing both PBS1 and PBS2 (1 and 2). Mutant sites indicated by asterisks. For each probe, 0, 0.3, 1, 3, 10, or 30 ng of recombinant POP-1 was added to the reaction. Arrow, unbound probes.

(C) Transcriptional activation in NCI-H28 cells. In each experiment, the luciferase coding region was driven by one of several promoters (see key in figure and text). Cells were transfected with reporters either alone (left), with a plasmid expressing POP-1 (middle), or with plasmids expressing

Z4.a) (Figures 2A–2C). In hermaphrodites, both progeny of the distal SGP daughter retained robust *ceh-22b::VENUS* expression through L2 or early L3 (Figures 2A and 2C). In males, the distal SGP daughter, which does not divide further, retained strong expression until L3 (Figure 2B); the expression decreased during L4. We conclude that the first intron can function as a promoter to drive expression asymmetrically in the SGP lineage. We term this intronic region the *ceh-22b* promoter.

The *ceh-22b::VENUS* reporter was also expressed in the pharynx, intestine, and ventral nerve cord as well as in unidentified neurons in the head and tail (Figure 2D, not shown). Expression in the pharynx and intestine was sustained throughout larval development into adulthood; expression in the ventral nerve cord was visible until L3. The significance of the nongonadal expression remains unknown, because no obvious nongonadal defect was seen in *q632* mutants.

The finding that *ceh-22b::VENUS* is expressed more strongly in distal than proximal SGP daughters suggested that *ceh-22* transcription might be controlled by Wnt/MAPK signaling. To ask if *ceh-22b* might be a direct target of transcriptional activation by POP-1/TCF and SYS-1/β-catenin, we first tested POP-1 binding to the *ceh-22b* promoter and identified two POP-1/TCF binding sites (PBS1 and PBS2) by a combination of sequence analysis and DNA footprinting (Figure 3A; see Experimental Procedures). Both sites had a similar sequence and a comparable POP-1 binding affinity to that of the consensus TCF binding site (TTCAAAG) (Figure 3A and not shown) [15, 16]. Remarkably, both sites were located within the *q632* deletion (Figure 1C). According to a gel electrophoretic mobility assay, recombinant POP-1/TCF bound specifically to both sites, but not to a mutated probe in which the sequence of the core TCF consensus element had been altered (Figures 3A and 3B).

To assay the function of the POP-1/TCF binding sites in the *ceh-22b* promoter, we first used a reporter assay in tissue-culture cells. A previous study showed that POP-1/TCF and SYS-1/β-catenin activate transcription from a promoter harboring eight copies of the consensus TCF binding site upstream of the luciferase coding region (8xTOPFlash) [6]. Here we replaced the 8xTOPFlash promoter with either the wild-type *ceh-22b* promoter or one of two control promoters. The *ceh-22b(q632)::luciferase* reporter harbors the *q632* deletion and *ceh-22b(mutPBS)::luciferase* carries mutated versions of PBS1 and PBS2 (see Supplemental Experimental Procedures). POP-1/TCF alone did not activate transcription from any of the reporters (Figure 3C, middle), but POP-1/TCF and SYS-1/β-catenin together enhanced *ceh-22b::luciferase* expression by 3- to 5-fold, a level comparable to that of the 8xTOPFlash reporter transgene (Figure 3C, right). Furthermore, POP-1/TCF and SYS-1/β-catenin did not enhance expression of either *ceh-22b(mutPBS)::luciferase* or *ceh-22b(q632)::luciferase* (Figure 3C). We conclude that POP-1/TCF and SYS-1/β-catenin transcriptionally activate the *ceh-22b* promoter via the PBS1 and PBS2 sites.

We next asked whether POP-1/TCF and SYS-1/β-catenin control expression of the *ceh-22b* promoter in nematodes. To this end, we compared expression of a *ceh-22b::VENUS* reporter transgene to that of a mutated transgene, *ceh-22b(mutPBS)::VENUS*, which carries mutations in PBS1 and PBS2, but otherwise is identical to *ceh-22b::VENUS*. Our results are summarized in Figure 3D. In nongonadal tissues, both transgenes expressed similarly in pharynx and neurons, but *ceh-22b(mutPBS)::VENUS* was not expressed in the intestine (not shown). In the SGPs, both transgenes were initially expressed midway through L1 as normal. Therefore, PBS1 and PBS2 have no apparent effect on initiation of *ceh-22b* expression in SGPs. By contrast, expression of the two transgenes was dramatically different in SGP daughters. Whereas *ceh-22b::VENUS* expression intensified in distal SGP daughters and was maintained at a high level in the distal SGP lineage through L2, *ceh-22b(mutPBS)::VENUS* was expressed similarly only at a low level in both distal and proximal SGP daughters, and usually disappeared by the time the SGP daughters began their division (65%, n = 14). No *ceh-22b(mutPBS)::VENUS* expression was seen in the late L1 gonads (n = 45). Therefore, PBS1 and PBS2 appear to be required specifically for the robust and sustained *ceh-22b* expression in the distal SGP daughters and their progeny as well as for intestinal expression.

We next compared expression of *ceh-22b::VENUS* in wild-type animals to that in *pop-1* and *sys-1* mutants. Specifically, we employed *pop-1(q645)* and *sys-1(q544)* mutants, which have fully penetrant Sys gonadal defects [8, 9]. In both mutants, *ceh-22b::VENUS* was initially expressed in the SGPs at a low level as normal; however, that expression did not intensify in distal SGP daughters and was not maintained in the distal SGP lineage (*pop-1(q645)*, n = 30; *sys-1(q544)*, n = 14) (Figure 3E). A similar effect was seen after *sys-1(RNAi)* (data not shown). We conclude that POP-1/TCF and SYS-1/β-catenin are both required for the robust and sustained *ceh-22b::VENUS* expression in the distal SGP lineage.

Given the fact that the *ceh-22b* promoter lacking POP-1 binding sites was able to drive low-level expression in the SGPs, we wondered if the sites were critical

POP-1 and SYS-1 (right) (see Experimental Procedures). Luciferase activity in the absence of POP-1 and SYS-1 was set at 1, and levels of induction were measured in other experiments. Results are the average of three independent experiments, each done in duplicate.

(D) Comparison of *ceh-22b::VENUS* and *ceh-22b(mutPBS)::VENUS* expression in hermaphrodite and male SGPs and their descendants. Conventions as in Figure 2C.

(E) Comparison of *ceh-22b::VENUS* in wild-type and mutant SGP daughters. The same transgene was used in all three genotypes, and all animals were at the same stage, just after the division of the SGP distal daughter, Z1.a. All images were obtained with the same exposure and were treated identically. In *sys-1* and *pop-1* mutants, VENUS levels were similar in distal and proximal SGP descendents. The two daughters of Z1.a are indicated by cell division diagram, and one daughter of Z1.p is marked by white arrow. Wild-type expression is shown in the top row for comparison; in wild-type gonads, expression in the distal SGP lineage is so bright that any faint expression in the proximal SGP lineage could not be determined at this exposure.

for *q632* rescue. We therefore compared the rescuing activities of *ceh-22b(genomic)* transgenes with wild-type or mutated POP-1 binding sites. Whereas *ceh-22b(genomic)* efficiently rescued *q632* mutants, the *ceh-22b(mutPBS,genomic)* transgene failed to rescue *q632* mutants (Figure 1C, *ceh-22b(mutPBS,genomic)*). Therefore, the POP-1 binding sites in the *ceh-22b* promoter are indeed crucial and the low level of expression that is POP-1 independent does not appear to be sufficient for rescue.

Our experiments demonstrate that POP-1/TCF and SYS-1/β-catenin control *ceh-22b* expression via POP-1 binding elements, and that POP-1/TCF and SYS-1/β-catenin achieve a high level of *ceh-22b* expression specifically in one daughter of an asymmetric division. These results provide the first example of a direct downstream target controlled by both POP-1/TCF and SYS-1/β-catenin and confirm the hypothesis that POP-1/TCF and SYS-1/β-catenin can transcriptionally activate target genes in nuclei with lowered POP-1/TCF abundance (Figure 1A) [6]. The idea that POP-1/TCF can transcriptionally activate target genes, rather than simply derepressing them in cells with lowered nuclear levels of POP-1, has also received support from experiments in the early embryo [17, 18].

The model depicted in Figure 1A also predicts that POP-1/TCF represses *ceh-22b* expression in proximal SGP daughters. We have not been able to see that POP-1/TCF repression. The VENUS reporter remains detectable at a low level in the proximal SGP daughters (Figures 3A and 3C), but that expression could either reflect perdurance of the reporter protein or a low level of transcription. VENUS disappears from the proximal SGP descendants at about the same stage in animals carrying either *ceh-22b::VENUS* (2 independent lines) or *ceh-22b(mutPBS)::VENUS* (3 independent lines), but a subtle difference might have been missed.

Although repression of *ceh-22b* may occur in the proximal daughters of SGP, one should note that POP-1 loss-of-function mutations have no effect on SGP proximal daughter fate [9], suggesting that POP-1 repression of target genes is not critical for proximal fate determination. This contrasts with EMS asymmetric divisions in which the repression of E-specific genes by high nuclear levels of POP-1/TCF is critical for the MS fate [17, 19, 20]. Therefore, both activation and repression of target genes by POP-1 are critical for determination of the E as well as MS fates [17, 19, 20]. We suggest that this difference between SGP and EMS divisions might be determined by strength of promoter activity of POP-1 target genes.

The control of *ceh-22* by POP-1/TCF and SYS-1/β-catenin by Wnt/MAPK signaling has intriguing similarities with the control of its vertebrate homolog, *nkx2.5*, by Wnt signaling. Thus, Wnt signaling is required for specification of cardiac progenitors, and the effect of Wnt signaling is commonly assayed by Nkx2.5 expression [21–23]. Therefore, a regulatory link between Wnt signaling and Nkx2.5 transcription factors has been conserved. In *C. elegans,* that link is direct, but in vertebrates, a direct link has not been demonstrated to date.

The identification of *ceh-22/nkx2.5* as a target of Wnt signaling suggested that this homeodomain transcription factor might be an essential regulator in the specification of the distal tip cell fate. Indeed, *ceh-22(q632)* loss-of-function mutants fail to make DTCs [10]. To ask whether *ceh-22b* is sufficient to specify DTCs, we used the heat shock promoter to drive the ectopic expression of CEH-22B (Figure 4A). As a marker of DTCs, we employed *lag-2::GFP* [24]. Without heat shock, all animals carrying the *hs::CEH-22b* transgene survived to adulthood, and all hermaphrodites and males contained two distal tip cells, the number typical of wild-type animals (Figures 4B and 4D). When *hs::CEH-22b* transgenic animals were heat-shocked soon after the SGP divided (see Experimental Procedures), more than half of the surviving adults possessed extra DTCs (XX, 14/19; XO, 6/8) (Figures 4C and 4E). In hermaphrodites, extra DTCs led to formation of extra gonadal arms (Figure 4C). A vulva was missing in half of the hermaphrodites that had four total DTCs (n = 8), indicating loss of the anchor cell that is normally produced by a proximal SGP daughter [25, 26]. In males, extra DTCs were always found in a disorganized gonad and a linker cell was usually not observed (Figure 4E), indicating defects in proximal SGP daughters. We conclude that CEH-22B is sufficient, when overexpressed, to specify the proximal daughter of SGP to the DTC fate in both hermaphrodites and males.

We have found that Wnt signaling and *ceh-22/nkx2.5* work together to specify the DTC fate. The common function of DTCs in hermaphrodites and males is that of a stem cell niche [7]. Wnt signaling has emerged as a key regulator of stem cells in many tissues and in many organisms, and that role relies on transcriptional activation by TCF/LEF and β-catenin transcription factors [27]. Our work suggests that one role of Wnt signaling may be to control the stem cell niche. A similar suggestion was recently put forward with respect to osteoblasts, which provide a niche for hematopoietic stem cells [28–30]. CEH-22/Nkx2.5 and its homologs have not previously been implicated in the control of stem cells. Indeed, the fly and vertebrate homologs, which are called tinman and Nkx2.5, respectively, are best known for their roles in heart specification and differentiation [31]. Nematodes have no heart, but CEH-22 controls development of the rhythmically contracting musculature of the pharynx [11], and zebrafish Nkx2.5 can functionally replace CEH-22 [12]. Therefore, the CEH-22/Nkx2.5 class of homeodomain transcription factors has broadly conserved functions in animal development.

A remaining question is whether CEH-22 control of the DTC fate reflects a conserved role for this class of homeodomain transcription factors in regulating stem cell niches. Mouse mutants deleted for Nkx2.5 die with a broad spectrum of defects, including severe defects in vasculogenesis and angiogenesis as well as hematopoiesis in the yolk sac [32]. Intriguingly, endothelial cells appear to function as stem cell niches [33–35]. It is tempting to speculate that the severe vasculature defects in Nkx2.5 mutants may reflect some role of this conserved regulator in control of a vertebrate niche, much as CEH-22 controls the DTC. Two important challenges for the future are to learn how CEH-22 specifies the DTC niche in *C. elegans* and to learn whether its homologs specify an analogous stem cell niche in flies and vertebrates.

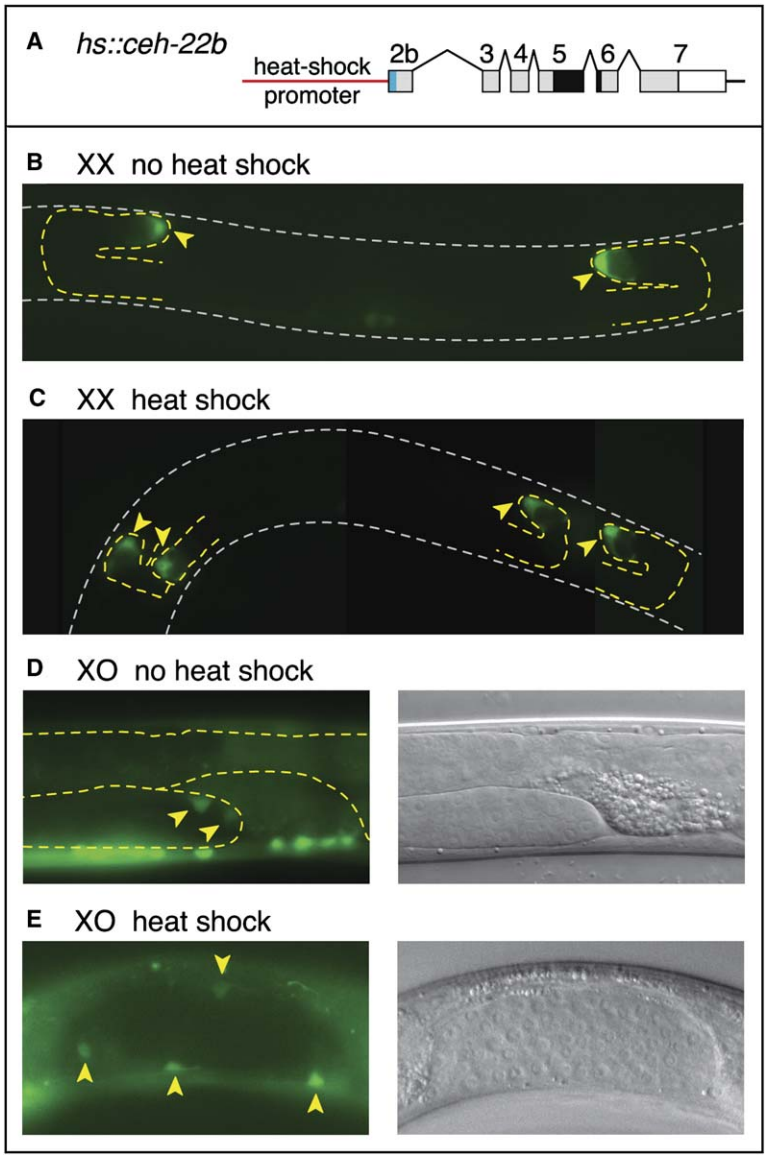

Figure 4. CEH-22B Is Sufficient to Specify Distal Cell Fate

Animals carrying *hs::ceh-22b* were heat-shocked or not. The number of distal tip cells was then scored by morphology and expression of the distal tip cell marker *lag-2::GFP*.

(A) Structure of the *hs::ceh-22b* transgene.

(B) Adult hermaphrodite without heat shock. Two distal tip cells are made as normal.

(C) Adult hermaphrodite after heat shock. In the animal shown, four distal tip cells are made.

(D) Male without heat shock; two distal tip cells and a gonad arm are made.

(E) Male after heat shock. In the animal shown, four distal tip cells are made. The gonad is disorganized; no gonad arm is formed.

Images showing distal tip cells are composed from multiple pictures taken from different focal planes. Nomarski pictures in (D) and (E) show one focal plane of the gonad. Gonad arms are delineated by dotted yellow lines. DTCs are marked with yellow arrows.

Experimental Procedures

Strains and Genetics

Standard protocols were used for culturing *C. elegans* strains. Strains were derived from the Bristol strain N2 and maintained at 20ºC unless otherwise noted. The following mutations were used for this work: *LG I*: *sys-1(q544)* [8], *pop-1(q645)* [9]; *LG V*: *ceh-22(cc8266)* [11], *sys-3(q632)* [10], *him-5(e1490)*. The integrated transgene *qIs56* [*lag-2::GFP*] was used.

Rescue Experiments

To rescue *q632*, different *ceh-22* DNAs (20 ng/μl) (Figure 2 and Supplemental Data) along with the coelomocyte marker *unc-122::GFP* (20 ng/μl) were injected into *q632* homozygote animals. Numbers of gonad arms were scored in animals carrying the transgenes. For each *ceh-22* DNA, at least three independent transgenic lines were analyzed.

RT-PCR

RNA (1 μg) from mixed staged worms was reverse transcribed with Oligo-dT primer and Superscript II reverse transcriptase (Invitrogen). *ceh-22b* was amplified by primers GGTTTAATTACCCAA GTTTGAG and GAGAAACGAGATGTATTCTGGGA. The 5′ primer anneals to the SL-1 splice leader. The 3′ primer anneals to the *ceh-22* 3′ UTR corresponding to nucleotides 20275 to 20297 of cosmid F29F11 (GenBank accession no. Z73974). *ceh-22c* was amplified by primers TGTCCGACTCCTTCACATTTCACC and GAGAAACGAGAT GTATTCTGGGA. The 5′ primer anneals to the first intron of *ceh-22* corresponding to nucleotides 18698 to 18721 of cosmid F29F11. The 3′ primer is the same used to amplify *ceh-22b*.

Transgenic Animals

To make *ceh-22b::VENUS* and *ceh-22b(mutPBS)::VENUS* transgenic animals, N2 (wild-type) worms were injected with *ceh-22::VENUS* (40 ng/μl) or *ceh-22(mutPBS)::VENUS* (50 ng/μl) together with influenza viral DNA (70 ng/μl). The transgenes were integrated by γ-irradiation. Animals having integrated transgenes were outcrossed five times. Male animals were obtained by crossing. To make *hs::ceh-22b* transgenic animals, JK3131 (*qIs56 him-5(e1490)*) worms were injected with *hs::ceh-22b* (30 ng/μl) and a coinjection marker (*ttx-3::DsRED*, a gift from Josh Kaplan) (30 ng/μl). The transgene was maintained as extrachromosomal arrays.

Identifying POP-1 Binding Sites in the *ceh-22b* Promoter

We found several sites similar to the consensus TCF binding sites (TTCAAAG) in the *ceh-22b* promoter by scanning the sequence. To test whether POP-1 binds the *ceh-22b* promoter specifically, we performed gel electrophoretic mobility assays. The histidine-tagged

HMG-box DNA binding domain of POP-1 was purified from *E. coli* [15]. Four overlapping fragments encompassing the first intron were used as probes. To identify specific binding, a DNA fragment containing six copies of consensus TCF binding site (6xTOP) was used as a positive control; a DNA fragment containing eight copies of a mutated TCF binding site (8xFOP) was used as a negative control. A typical 20 μl binding reaction contained 100 fmol DNA probe, 0.1 to 30 ng purified POP-1, 10 ng/μl poly dI-dC, and 100 ng BSA in 1× buffer (20 mM HEPES [pH 7.6], 40 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 10% glycerol). The binding reactions were performed at 4ºC for 20 min and separated by 4% native polyacrylamide gel electrophoresis.

Two potential POP-1 binding sites were identified in a 254 bp fragment (nucleotides 18468 to 18721 of F29F11). To fine map the POP-1 binding site, we performed DNaseI footprinting by using Core Footprint System (Promega). POP-1 protected two stretches of ~20 nt, each including a predicted POP-1 binding site in the assay (not shown).

Luciferase Reporter Assay

NCI-H28 cells (1×10^5) were transfected with 500 ng of luciferase reporters, 40 ng of TK-Renilla luciferase plasmid, 0 or 1 μg of POP-1 expression plasmid, and 0 or 1 μg of SYS-1 expression plasmid with Lipofectamine 2000 reagent (Invitrogen). Luciferase activities were measured with Dual luciferase system (Promega). Transfection efficiencies were normalized by Renilla luciferase activities.

Heat Shock

To test for phenotypes caused by overexpression of *ceh-22b*, L1 larvae (*hs::ceh-22b qIs56 him-5(e1490)*) at about 8.5 hr after hatching (25ºC) were subjected to a 60 min heat shock (33ºC). At the time of heat shock, Z1 and Z4 had just divided in most of the animals. This treatment showed toxicity to the animals. 70% of the animals carrying the transgene arrested at L1 (n = 106); the rest continued to develop to adulthood.

Supplemental Data

Supplemental Experimental Procedures can be found with this article online at http://www.current-biology.com/cgi/content/full/16/3/287/DC1/.

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