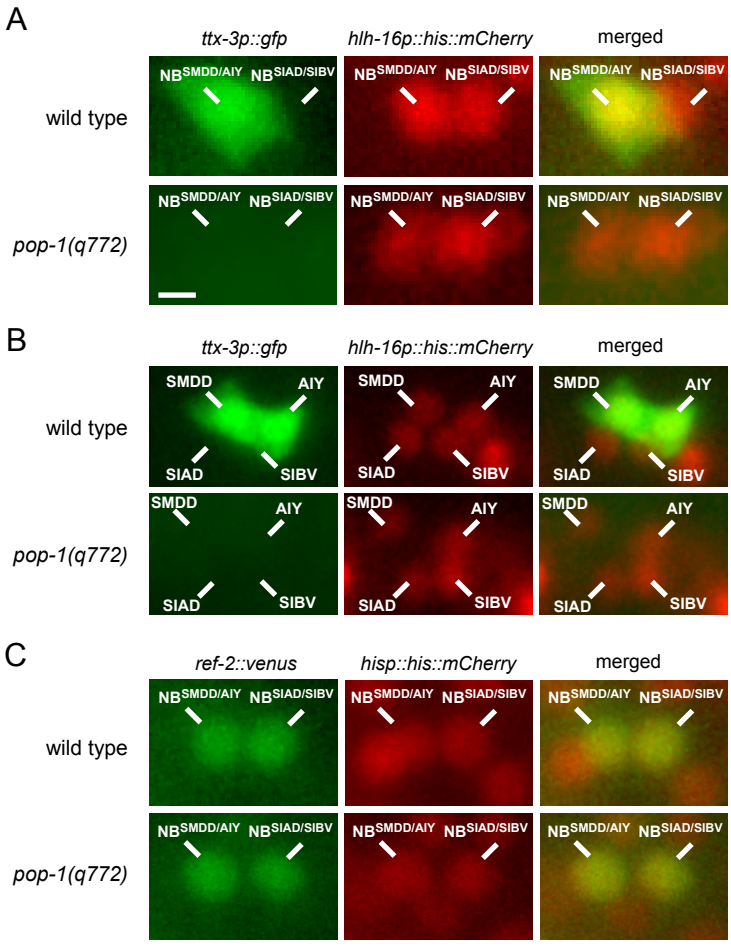
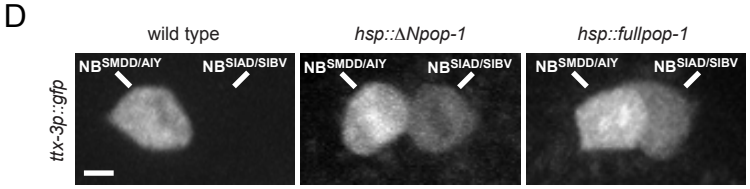
SUPPLEMENTAL INVENTORY





	ttx-3 expression in NB$^{SMDD/AIY}$	*ttx-3* expression in NB$^{SIAD/SIBV}$	n
wild type no heat shock	100 %	0 %	40
wild type heat shocked	100 %	0 %	48
hsp::ΔNpop-1 no heat shock	100 %	0 %	45
hsp::ΔNpop-1 heat shocked	100 %	29 % ***	45
hsp::fullpop-1 no heat shock	100 %	0 %	50
hsp::fullpop-1 heat shocked	100 %	29 % ***	38

Figure S1

Figure S1. Effect of *pop-1* on the expression of *ttx-3*, *hlh-16* and *ref-2*. (Related to Figure 1)

(A, B) Expression of *ttx-3* (*ttx-3p::gfp*, *mgIs32*) and *hlh-16* (*hlh-16p::his::mCherry, stIs10546*) in *pop-1(q772)* mutant embryos before (A) or after (B) the terminal division of the $NB^{SMDD/AIY}$ and $NB^{SIAD/SIBV}$ neuroblasts. Note that while *pop-1(q772)* affects the initiation of *ttx-3* expression in the SMDD/AIY lineage it has no effect on *hlh-16* expression in this lineage or on the terminal division of the $NB^{SMDD/AIY}$ neuroblast.

(C) Expression of *ref-2* (*ref-2::venus* fosmid translational fusion, *vbaEx55,* marked with *hisp::his::mCherry*) in *pop-1(q772)* mutant embryos before the terminal division of the $NB^{SMDD/AIY}$ and $NB^{SIAD/SIBV}$ neuroblasts. *ref-2* expression is not affected by the *pop-1(q772)* mutation : in the F1 progeny of either wild type or *pop-1(q772)/+* mothers, 100% of the transgenic embryos are positive for *ref-2::venus* expression (n=100 for each).

(D) Strong overexpression of a POP-1 version lacking the SYS-1 interaction domain (*hsp::ΔNpop-1*, *huIs4*) or of a full length version (*hsp::fullpop-1*, *teEx1*) using a heat shock promoter ectopically activates *ttx-3* expression (*ttx-3p::gfp, mgIs18*) in the $NB^{SIAD/SIBV}$ lineage. Embryos were heat shocked before the division of the mother of the $NB^{SMDD/AIY}$ and $NB^{SIAD/SIBV}$ neuroblasts and analyzed at the end of epidermal enclosure (n = number of embryos analyzed, *** : $p<0.001$ Fisher's exact test comparison with wild type heat shocked). Pictures : wild type, *hsp::ΔNpop-1* and *hsp::fullpop-1* heat shocked embryos ; note that the ectopic expression in $NB^{SIAD/SIBV}$ is only slightly weaker than the endogenous expression in $NB^{SMDD/AIY}$ (but was much weaker with *hlh-16p::ΔNpop-1*, Figure 1D). (scale bar = 2 µm).

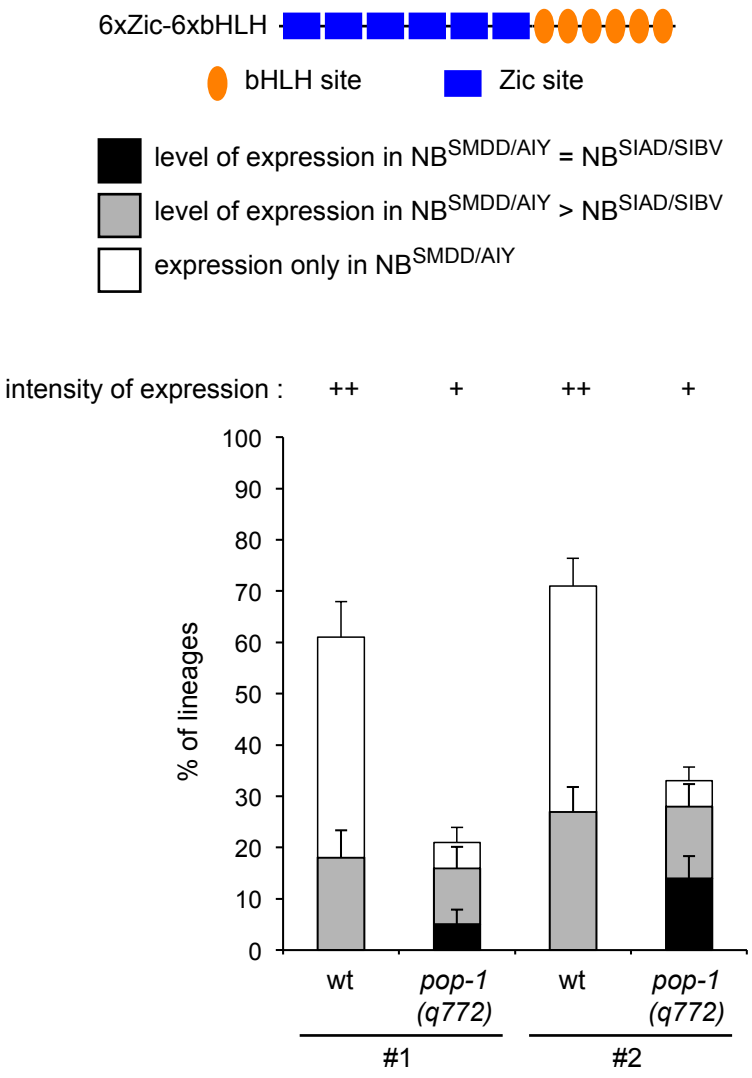

6xZic-6xbHLH
bHLH site
Zic site
level of expression in NB^SMDD/AIY = NB^SIAD/SIBV
level of expression in NB^SMDD/AIY > NB^SIAD/SIBV
expression only in NB^SMDD/AIY
intensity of expression : ++ + ++ +
% of lineages
100
90
80
70
60
50
40
30
20
10
0
wt
pop-1 (q772)
wt
pop-1 (q772)
#1
#2

Figure S2

Figure S2. POP-1 regulates the activity of a multimer of Zic and bHLH binding sites. (Related to Figure 2)

Activity of a multimer of 6 Zic and 6 bHLH binding sites placed in front of *gfp* in wild type (wt) or *pop-1(q772)* embryos. The graph presents the percentage of lineages with expression in both $NB^{SMDD/AIY}$ and $NB^{SIAD/SIBV}$ at similar level (black), expression in both $NB^{SMDD/AIY}$ and $NB^{SIAD/SIBV}$ with higher level in $NB^{SMDD/AIY}$ (grey), expression only in $NB^{SMDD/AIY}$ (white) or no expression at all (remaining fraction). The *pop-1(q772)* mutation reduces both the percentage of lineages showing expression as well as the degree of asymmetry when expression is still present. Expression in the $NB^{SMDD/AIY}$ and $NB^{SIAD/SIBV}$ neuroblasts was scored late (when entering mitosis) (n>50, error bars show standard error of proportion). Two independent transgenic lines for the multimer (#1 and #2) were analyzed. *pop-1(q772)* homozygote embryos were recognized by the absence of a marked balancer. The intensity of the GFP signal in the $NB^{SMDD/AIY}$ neuroblast in each line and condition is indicated above the graph (++: medium, +: low).

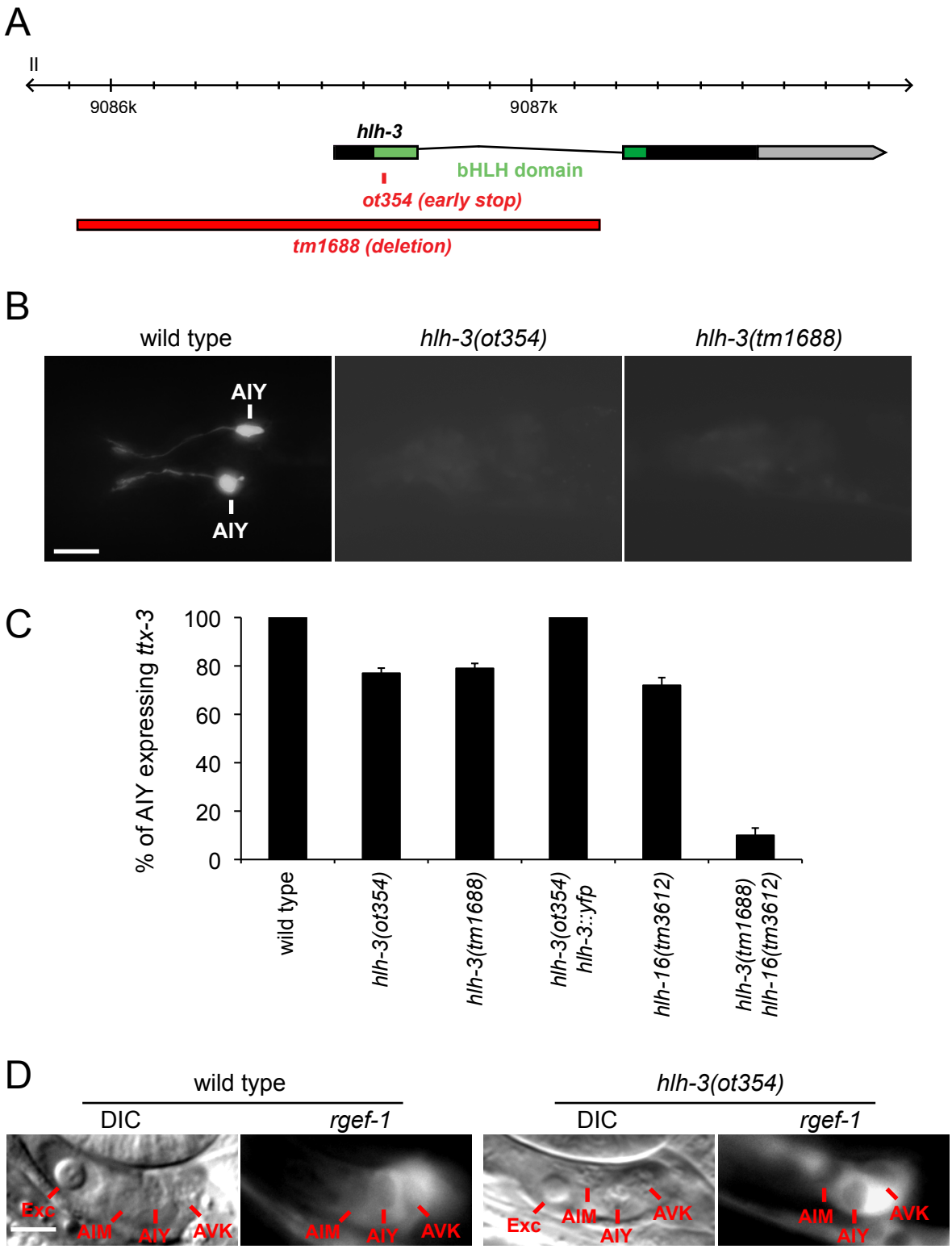

A
II
9086k
9087k
hlh-3
bHLH domain
ot354 (early stop)
tm1688 (deletion)
B
wild type
hlh-3(ot354)
hlh-3(tm1688)
AIY
AIY
C
% of AIY expressing ttx-3
100
80
60
40
20
0
wild type
hlh-3(ot354)
hlh-3(tm1688)
hlh-3(ot354) hlh-3::yfp
hlh-16(tm3612)
hlh-3(tm1688) hlh-16(tm3612)
D
wild type
hlh-3(ot354)
DIC
rgef-1
DIC
rgef-1
Exc
AIM
AIY
AVK
AIM
AIY
AVK
Exc
AIM
AIY
AVK
AIM
AIY
AVK

Figure S3

Figure S3. Effect of *hlh-3* on the specification of the AIY neuron at larval stage. (Related to Figure 3)

(A) *ot354* is a stop mutation (Q41(CAA) to stop(TAA)) at the beginning of the bHLH domain and *tm1688* is a deletion that removes a large portion of the bHLH domain.

(B) Loss of *ttx-3p::gfp* expression (*otIs173*) in *hlh-3* mutant larvae (head of L4 larvae, ventral view, anterior is left, scale bar = 10 µm).

(C) Percentage of AIY neurons expressing *ttx-3p::gfp* (*otIs173*) at L4 larval stage (n>100, error bars show standard error of proportion).

(D) In wild type L4 larvae AIY is located in a group of three neurons just posterior to the excretory cell (Exc) and expresses the pan-neuronal marker *rgef-1* (*rgef-1p::dsRed2*, *otIs173*, n=12). In *hlh-3(ot354)* mutants when *ttx-3p::gfp* expression is lost a neuron is still present at the position normally occupied by AIY and still expresses *rgef-1p::dsRed2* (n=11) (scale bar = 5 µm). Note that the *rgef-1p::dsRed2* signal is mostly cytoplasmic (lower in the nucleus).

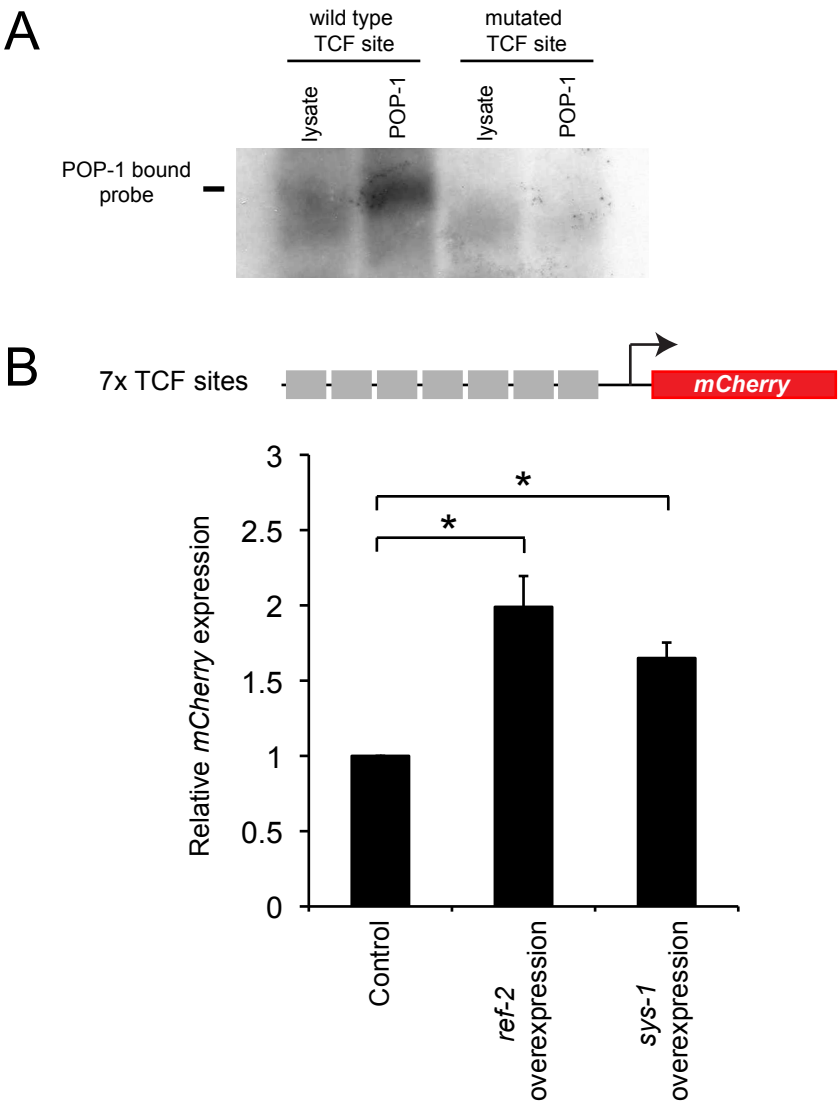

A
wild type TCF site
mutated TCF site
lysate
POP-1
lysate
POP-1
POP-1 bound probe
B
7x TCF sites
mCherry
Relative mCherry expression
3
2.5
2
1.5
1
0.5
0
*
*
Control
ref-2 overexpression
sys-1 overexpression

Figure S4

Figure S4. EMSA with POP-1 on TCF binding sites and activation of a multimer of TCF binding sites by REF-2. (Related to Figure 3)

(A) EMSA using *in vitro* (reticulocyte lysate) produced POP-1 proteins on probes containing a wild type or mutated TCF site. POP-1 proteins shift wild type but not mutated TCF sites showing that *in vitro* produced POP-1 is functional for DNA binding.

(B) Activation of a multimer of TCF binding sites by REF-2. *C. elegans* strains containing a multimer of TCF binding sites placed in front of the *mCherry* reporter (*syIs187*) and a heat shock promoter driving the expression of *ref-2* (*ref-2* overexpression, *muIs97*) or *sys-1* (*sys-1* overexpression, *vbaIs2*) or without heat shock promoter transgene (control), were subjected to heat shock (30 min at 37°C followed by 60 min at 20°C, 3 times) followed by a quantification of their *mCherry* mRNA level using quantitative RT-PCR. The relative expression level of the *mCherry* reporter in different strains was then calculated with the ΔΔCt method using the housekeeping gene *act-1* as a normalizer. n=3, error bars show the standard error of the mean, * : $p<0.05$ Student's t-test.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Expression constructs and transgenic strains

The 6xbHLH and 6xZic constructs were made by cloning between the HindIII and XbaI sites of pPD95.75 the following inserts: for 6xbHLH two-times (TAACACATGTTTAAGCAGGTGCACTCCCTCTTGAAT) and for 6xZic six-times (ATATGCACCCCGCTGACAAT). The 12xbHLH and 6xZic-6xbHLH constructs were then made by cloning the 6xbHLH insert between the XbaI and XmaI sites of the previously obtained 6xbHLH-pPD95.75 and 6xZic-pPD95.75 constructs respectively. These plasmids were injected at 100 ng/µL with 80 ng/µL of pRF4 coinjection marker.

As *ref-2(ot327)* homozygotes are larval lethal the penetrance of the phenotype was measured using a strain bearing the labeled rescuing extrachromosomal array *vbaEx2*. *vbaEx2* was generated by injecting the *ref-2* rescuing fosmid WRM0627cB02 at 50 ng/µL with 100 ng/µL of pRF4 and 50 ng/µL of *elt-2p::gfp* coinjection markers.

The HLH-3::YFP rescuing translational fusion was generated by inserting YFP before the stop of the *hlh-3* gene in the WRM0625aA01 fosmid using recombineering in bacteria (Tursun et al., 2009). The linearized fosmid was then injected at 15 ng/µL with 4 ng/µL of linearized pRF4 and 150 ng/µL of bacterial DNA to generate the *otEx4140* and *otEx4142* extrachromosomal arrays.

The REF-2::VENUS reporter array (*vbaEx55*) was generated by injecting the *ref-2::venus* fosmid translational fusion (Bertrand and Hobert, 2009) at 80 ng/µL with a ubiquitous *hisp::his::mCherry* coinjection marker at 50 ng/µL and the pRF4 coinjection marker at 100 ng/µL.

The hlh-16p::fullpop-1 and hlh-16p::ΔNpop-1 constructs were generated by replacing in the hlh-16prom(-514)::gfp vector (Bertrand et al., 2011) the CDS of *gfp* by the full *pop-1* cDNA or the *pop-1* cDNA lacking the first 43 amino acids. The constructs were injected at 100 ng/µL with 80 ng/µL of pRF4 coinjection marker.

For the mammalian cell culture system, the Zic binding site reporter vector was generated by cloning five Zic binding sites 5x(ATATGCACCCCGCTGACAAT) between the SacI and NheI sites of the pTA-Luc vector. For expression in cell culture the *ref-2* cDNA (with a V5 tag) or *sys-1* cDNA (with an HA tag) were cloned between the XbaI and HindIII sites of the pCDNA3.1(-) vector. For POP-1 expression a previously generated FLAG-POP-1 vector (Korswagen et al., 2000) was used.

Heat shock experiments

Two-cell stage embryos from an *hsp::ΔNpop-1* strain (*huIs4*), a *hsp::fullpop-1* strain (*teEx1*) or a wild type strain were mounted on slides, incubated 200 min at 20°C (until the mother of the $NB^{SMDD/AIY}$ and $NB^{SIAD/SIBV}$ neuroblasts is about to divide) then shifted to 37°C for 20 min and put back to 20°C until analysis at epidermal enclosure.

Co-immunoprecipitation

Embryos were collected from gravid adults and sonicated in co-IP buffer (Calvo et al., 2001) with protease inhibitors (Sigma tablet, 5µg/ml Leupeptin, 5mM Benzamidin, 1mM polymethyl sulferyl fluoride) and phosphatase inhibitors (10mM NaF, 1mM $Na2PO_4$, 1mM Na_3VO_4, 10mM β-glycerophosphate). For each co-IP, 50µl slurry of sepharose protein A was pre-coated with 87 ng of affinity-purified rabbit anti-POP-1 antibody (Lin et al., 1995) or an equal amount of rabbit anti-HA antibody (Cell Signaling Technology) in co-IP buffer at a final volume of 500µl for 4 hours. 400µg of embryo protein extract were pre-cleared for 1 hour on 50µl slurry of sepharose protein A and then incubated with the pre-coated beads over night. The beads were washed three times in co-IP buffer with 385mM KCl and then twice in co-IP buffer. The immunoprecipitates were analysed by Western blotting using mouse anti-GFP antibody (Roche) as primary antibody and goat anti-mouse-HRP antibody (Jackson) as secondary antibody. Revelation was done with the ECL prime system (Amersham).

Electrophoretic Mobility Shift Assay (EMSA)

FLAG-POP-1 (Korswagen et al., 2000) and HIS-REF-2 (Alper and Kenyon, 2002) were produced in vitro using a reticulocyte lysate system (TnT Quick Coupled Transcription/Translation Systems, Promega). Probes with the wild type or mutated Zic binding site from the *ttx-3* initiator element or with a wild type or mutated TCF binding site were generated by annealing complementary oligonucleotides:

wild type Zic (TTCATATATGCACCCCGCTGACAATAAGAA)
mutated Zic (TTCATATATGCACAAAGCTGACAATAAGAA)
wild type TCF (TTCATATATCTTTGATCAATAAGAA)
mutated TCF (TTCATATATAGGGACCCAATAAGAA)

The resultant double-stranded DNA fragments were labelled with [α-^{32}P]dATP using Klenow DNA polymerase (Promega) and purified on Microspin G50 columns (GE Healthcare). For Zic binding sites (Figure 3B) the EMSA binding reaction consisted of 10µl of synthesized protein, 2µl of α-^{32}P-labelled probe (at 25000CPM) and 50ng/µl poly[dI-dC] in 1x binding buffer (Yagi et al., 2004) with a final volume of 20 µl. For TCF binding sites (Supplementary Figure 4A) the EMSA binding reaction consisted of 8µl of synthesized protein, 2µl of α-^{32}P-labelled probe (at 25000CPM) and 10ng/µl poly[dI-dC] in 1x binding buffer (Gorrepati et al., 2013) with a final volume of 20 µl. The reaction was

incubated at room temperature first 15 minutes without the probe, then 30 minutes with the probe. The binding mixture was then subjected to electrophoresis in 0.5X TBE-buffer on a 4% native polyacrylamide gel at room temperature. After electrophoresis the gel was dried and analysed by autoradiography.

Quantitative RT-PCR

Total RNA was extracted from nematodes using Trizol (Ambion) according to standard procedures and treated with DNase I (Invitrogen). 0.5-1 µg of total RNA was used for reverse transcription using qScript cDNA SuperMix (Quanta, BioSciences). 10 ng of cDNA were then analysed by QPCR on a Bio-Rad CFX using PerfeCTa SYBR Green SuperMix ROX (Quanta, BioSciences) and oligos against the *mCherry* reporter and the housekeeping gene *act-1* as a normalizer. The relative expression level of the *mCherry* reporter in different strains was then calculated using the ΔΔCt method with the CFX Manager Software.

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