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Atypical transcriptional activation by TCF via a Zic transcription factor in *C. elegans* neuronal precursors

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Summary

Transcription factors of the TCF family are key mediators of the Wnt/ β -catenin pathway. TCF usually activates transcription on *cis*-regulatory elements containing TCF binding sites when the pathway is active and represses transcription when the pathway is inactive. However, some direct targets display an opposite regulation (activated by TCF in the absence of Wnt) but the mechanism behind this atypical regulation remains poorly characterized. Here we use the *cis*-regulatory region of an opposite target gene, *ttx-3*, to dissect the mechanism of this atypical regulation. Using a combination of genetic, molecular and biochemical experiments we establish that, in the absence of Wnt pathway activation, TCF activates *ttx-3* expression via a Zic binding site by forming a complex with a Zic transcription factor. This mechanism is later reinforced by specific bHLH factors. This study reveals an atypical mode of action for TCF that may apply to other binary decisions mediated by Wnt signaling.

Graphical abstract

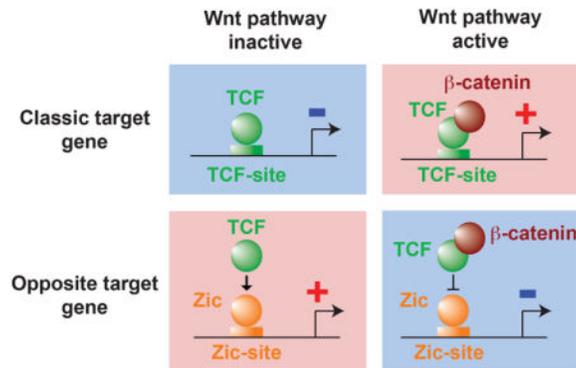
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Introduction

The Wnt/ β -catenin pathway is a key signaling pathway that plays important roles at several steps during animal development, cell proliferation and differentiation. Studies in model organisms have established that the key transcriptional mediators of this pathway are transcription factors of the TCF family (MacDonald et al., 2009). Following activation of the Wnt pathway the coactivator β -catenin is stabilized, enters the nucleus and associates with TCF. The TCF/ β -catenin complex then directly activates the expression of target genes via TCF binding sites present in their *cis*-regulatory regions. On the contrary, when the Wnt pathway is inactive, β -catenin is degraded, TCF is not associated with β -catenin and it represses the expression of Wnt target genes via TCF binding sites (Figure 1A).

Most of the direct target genes of the Wnt/ β -catenin pathway display this classic type of regulation (“classic target genes”, Figure 1A). However in *Drosophila* and vertebrates a few direct target genes have been observed to display the opposite regulation. These “opposite target genes” are repressed by TCF in presence of Wnt and/or activated by TCF in absence of Wnt (Blauwkamp et al., 2008; Cadigan, 2012; Jamora et al., 2003) (Figure 1A). The mechanism by which TCF mediates this intriguing opposite regulation remains poorly characterized (Cadigan, 2012). In this study we used the asymmetric divisions of *C. elegans* neuronal precursors as an experimental model to analyze this mechanism.

In both vertebrates and invertebrates, postmitotic neurons are often generated by asymmetric divisions of neuronal progenitors (Sawa, 2010). In *C. elegans* most neurons are generated during neurulation (called epidermal enclosure) by asymmetric divisions oriented along the antero-posterior axis (Sulston et al., 1983). These terminal divisions are regulated by a particular Wnt pathway called the Wnt/ β -catenin asymmetry pathway (Bertrand and Hobert, 2009a, b). This pathway also regulates multiple other asymmetric divisions during *C. elegans* embryonic and larval development (Bertrand and Hobert, 2010; Mizumoto and Sawa, 2007; Phillips and Kimble, 2009). Following asymmetric division this pathway is active in the posterior daughter where the TCF transcription factor POP-1 associates with its coactivator, a β -catenin called SYS-1, to directly activate the transcription of genes specific of the posterior daughter via TCF binding sites. In the anterior daughter SYS-1 is low or absent and POP-1 represses the target genes specific of the posterior daughter following a “classic target gene” logic. On the contrary the target genes expressed in the anterior

daughter display an “opposite target gene” logic: they are activated in the absence of SYS-1 (in the anterior daughter) and repressed in the presence of SYS-1 (in the posterior daughter). While the mechanism by which classic target genes are activated in posterior daughters has been well characterized (Phillips and Kimble, 2009), how opposite target genes are activated in anterior daughters remains poorly understood (Bertrand and Hobert, 2010).

In this study we used the opposite target gene *ttx-3*, which encodes a LIM homeodomain transcription factor, as an entry point to analyze how gene expression is activated in anterior daughter cells. *ttx-3* expression starts at the end of gastrulation in a neuroblast (ABpl/rpapaaa) that generates the SMDD and AIY neurons (Bertrand and Hobert, 2009a). We refer to this neuroblast cell from here on as NB^{SMDD/AIY}. The NB^{SMDD/AIY} neuroblast is produced by an asymmetric division which generates the NB^{SMDD/AIY} neuroblast (anterior daughter) and the NB^{SIAD/SIBV} neuroblast (posterior daughter) (Figure 1B). The NB^{SMDD/AIY} neuroblast divides afterwards to generate the SMDD motoneuron and the AIY interneuron, while the NB^{SIAD/SIBV} neuroblast divides to generate the cholinergic motoneurons SIAD and SIBV. In wild type embryos *ttx-3* is only expressed in the NB^{SMDD/AIY} neuroblast but not in its sister NB^{SIAD/SIBV} and this expression is regulated by the Wnt/ β -catenin asymmetry pathway (Bertrand and Hobert, 2009a). We have previously shown that when the Wnt/ β -catenin asymmetry pathway is inactivated just before this division using temperature-sensitive alleles of the upstream kinases *lit-1* and *mom-4*, the *ttx-3* gene becomes ectopically expressed in the NB^{SIAD/SIBV} neuroblast (Bertrand and Hobert, 2009a). Vice versa, when the pathway is ectopically activated in the NB^{SMDD/AIY} neuroblast by overexpression of the Wnt receptor *mom-5*, expression of *ttx-3* is lost (Bertrand and Hobert, 2009a). Therefore *ttx-3* expression is activated in the anterior daughter where the Wnt/ β -catenin asymmetry pathway is inactive and is repressed in the posterior daughter where the Wnt/ β -catenin asymmetry pathway is active. Following division of the NB^{SMDD/AIY} *ttx-3* is expressed in both the SMDD and AIY postmitotic neurons in the embryo but then gradually disappears from the SMDD neuron to be restricted to the AIY neuron at larval and adult stages where it regulates AIY terminal differentiation. There are two AIY lineages, one on either side of the embryo.

Here we use a combination of genetic, molecular and biochemical analysis to dissect how the TCF transcription factor POP-1 activates *ttx-3* expression in the NB^{SMDD/AIY} neuroblast. We show that *ttx-3* activation by POP-1 in this anterior daughter is primarily mediated by the Zic transcription factor REF-2, which forms a complex with POP-1 and activates transcription via a Zic binding site present in the *ttx-3* cis-regulatory region. The asymmetric activation of *ttx-3* expression is later reinforced via bHLH binding sites by the bHLH factor HLH-3 whose expression becomes gradually asymmetric and is regulated by the Wnt pathway. This study reveals the mechanism of transcriptional activation in an anterior daughter and suggests an atypical mode of action for a TCF factor, which may apply to opposite target genes in other cellular and organismal contexts.

Results

POP-1/TCF activates *ttx-3* expression in the absence of SYS-1/ β -catenin

To establish how *ttx-3* expression is activated in this anterior daughter we first tested whether the classic transcriptional effectors of the Wnt/ β -catenin asymmetry pathway, the TCF transcription factor POP-1 and its coactivator the β -catenin SYS-1, are involved in the initiation of *ttx-3* expression. The Wnt/ β -catenin asymmetry pathway usually regulates the nuclear level of both SYS-1 and POP-1 by modulating the degradation of SYS-1 and the nuclear export of POP-1 (Phillips and Kimble, 2009). Following asymmetric division of their mother cell, the anterior NB^{SMDD/AIY} neuroblast displays a high nuclear concentration of POP-1 and low concentration of SYS-1 while the posterior NB^{SIAD/SIBV} neuroblast has a low nuclear concentration of POP-1 and high concentration of SYS-1 (Figure 1B). This suggests that nuclear POP-1 is mostly free of SYS-1 in the NB^{SMDD/AIY} neuroblast (anterior) and mostly bound to SYS-1 in the NB^{SIAD/SIBV} neuroblast (posterior), as observed in other asymmetric divisions regulated by this pathway (Phillips and Kimble, 2009). In *sys-1* loss of function mutants *ttx-3* becomes ectopically expressed in the NB^{SIAD/SIBV} neuroblast similar to what we have previously observed when upstream components of the pathway are inactivated (Figure 1C). Interestingly, in *pop-1* loss of function mutants *ttx-3* expression is lost (Figure 1C, an effect is observed in a bit less than the 25% *pop-1(q772)* homozygote embryos expected from mendelian segregation; this is possibly due to remains of the very strong maternal contribution of *pop-1* observed at earlier stages). Loss of *ttx-3* expression in *pop-1* mutants is not due to a loss of the NB^{SMDD/AIY} neuroblast itself since the NB^{SMDD/AIY} neuroblast still expresses *hlh-16*, a very restricted marker that is turned on in the NB^{SMDD/AIY} and NB^{SIAD/SIBV} neuroblasts just before *ttx-3* (*hlh-16p::his::mCherry, stIs10546*, (Bertrand et al., 2011)) (Supplementary Figure 1A). The NB^{SMDD/AIY} neuroblast also still undergoes its terminal division at the right time during neurulation (Supplementary Figure 1B). Taken together, our data suggest that POP-1 is required for the activation of *ttx-3* expression in the anterior daughter and that this activation is blocked in the posterior daughter by SYS-1.

Zic and bHLH binding sites contribute to the asymmetry of *ttx-3* expression

In order to determine how POP-1 activates *ttx-3* in the absence of SYS-1 we examined the *cis*-regulatory regions of *ttx-3*. We have previously identified a 113 bp *cis*-regulatory element responsible for the initiation of *ttx-3* expression in the NB^{SMDD/AIY} neuroblast (Bertrand and Hobert, 2009a). This element does not contain any POP-1/TCF binding sites but contains binding sites for bHLH and Zic transcription factors that are required for the initiation of *ttx-3* expression (Bertrand and Hobert, 2009a) (Figure 2). To determine whether these binding sites could mediate the asymmetric activation in the anterior daughter (NB^{SMDD/AIY} neuroblast) versus the posterior daughter (NB^{SIAD/SIBV} neuroblast) we generated multimers of bHLH or Zic binding sites, placed them upstream of *GFP* and tested their activity in transgenic embryos. Multimers of 6 or 12 bHLH binding sites drive expression in both the anterior and posterior daughters (Figure 2). Interestingly, while the activity is initially mostly symmetrical, it becomes stronger in the anterior daughter than in the posterior as the neuroblasts approach their terminal division (Figure 2). A multimer of 6 Zic binding sites does not drive any expression on its own but greatly increases the antero-

posterior asymmetry of bHLH binding sites both early and late (Figure 2). Zic binding sites increase the expression in the anterior daughter and decrease the expression in the posterior daughter. As expected, the activity of this multimer of Zic and bHLH binding sites is regulated by *pop-1* (Supplementary Figure 2). In *pop-1* mutants the expression of the multimer is either lost or its asymmetry is reduced. Taken together this suggests that both bHLH and Zic binding sites contribute to the asymmetric activation of *ttx-3* expression. Zic binding sites contribute to the asymmetry both early and late, while bHLH binding sites contribute mostly to the late asymmetry.

REF-2/Zic forms an activator complex with POP-1/TCF

REF-2 is the only transcription factor of the Zic family in *C. elegans*. In *ref-2* mutants the initiation of *ttx-3* expression in the NB^{SMDD/AIY} neuroblast is lost (Figure 3A, (Bertrand and Hobert, 2009a)). By Electrophoretic Mobility Shift Assay (EMSA) we observed that *in vitro* produced REF-2 is able to bind directly to the Zic binding site present in the *ttx-3* promoter while *in vitro* produced POP-1 is unable to directly bind to this Zic binding site (Figure 3B, Supplementary Figure 4A). REF-2 may therefore mediate the asymmetric activity of POP-1 on Zic binding sites. The REF-2 protein is expressed at a similar level in the NB^{SMDD/AIY} and NB^{SIAD/SIBV} neuroblasts (Figure 3D, assessed with a REF-2::VENUS translational fusion that rescues the *ref-2* mutant phenotype (Bertrand and Hobert, 2009a)) suggesting that POP-1 does not regulate REF-2 expression but rather regulates its activity at a post-translational level. Consistent with this idea, REF-2 expression is not affected in *pop-1* mutants (Supplementary Figure 1C). Interestingly a genome wide yeast two-hybrid screen has revealed that POP-1 and REF-2 can directly interact (Simonis et al., 2009). Using co-immunoprecipitation we confirmed that POP-1 and REF-2 form a complex *in vivo* in the *C. elegans* embryo (Figure 3C). This suggests that POP-1 directly regulates the activity of the REF-2 protein by binding to it.

We then analyzed whether POP-1 and REF-2 are sufficient to activate transcription via Zic binding sites by using a heterologous mammalian cell culture system. Mammalian COS-7 cells were transfected with a multimer of Zic binding sites placed upstream of a luciferase reporter and cotransfected with various combinations of REF-2, POP-1 and SYS-1. We observed that POP-1 increases the ability of REF-2 to activate the multimer (Figure 4A) confirming that the REF-2:POP-1 complex acts as a transcriptional activator on Zic binding sites. In addition we also observed that REF-2 is able to activate a reporter of POP-1 activity (multimer of TCF binding sites placed upstream of *mCherry*) in transgenic *C. elegans* (Supplementary Figure 4B) suggesting that the REF-2:POP-1 complex may also act as a transcriptional activator on TCF binding sites.

In *C. elegans* embryos SYS-1 represses the activation of *ttx-3* expression in the posterior NB^{SIAD/SIBV} neuroblasts (Figure 1C). In addition, in our heterologous mammalian cell culture system addition of SYS-1 blocks the ability of REF-2 and POP-1 to activate transcription on Zic binding sites (Figure 4A). This suggests that SYS-1 blocks the activity of the REF-2:POP-1 complex. Interestingly, a recent study in vertebrates (Pourebahim et al., 2011) has shown that Zic2, TCF4 and β -catenin can form a Zic:TCF: β -catenin trimolecular complex (β -catenin binding to the N-terminal domain of TCF and Zic binding

to the HMG domain of TCF), and that this trimolecular complex is not able to activate transcription on TCF binding sites. This suggests that in *C. elegans* SYS-1/ β -catenin could block the activity of the REF-2:POP-1 complex in the posterior daughter by binding to the POP-1 N-terminal domain. To test this model, we induced, at the time when the NB^{SMDD/AIY} and NB^{SIAD/SIBV} neuroblasts are generated from their mother cell, the expression of a version of POP-1 that lacks its N-terminal SYS-1 interaction domain (Kidd et al., 2005) (NPOP-1, deletion of the amino acids 1 to 43, this deletion does not affect POP-1 nuclear asymmetry (Lo et al., 2004; Maduro et al., 2002)). This construct may form an activator complex with REF-2 that would be resistant to blockage by SYS-1. Indeed, when driven by the weak *hlh-16* promoter, NPOP-1, but not wild type (full) POP-1, ectopically activates *ttx-3* expression in the NB^{SIAD/SIBV} neuroblast where SYS-1 is present (Figure 1D). When driven by a strong heat shock promoter both NPOP-1 and full POP-1 are able to ectopically activate *ttx-3* expression in the NB^{SIAD/SIBV} neuroblast probably because the level of full POP-1 driven by the heat shock promoter is sufficiently high for large fractions of full POP-1 to escape SYS-1 binding (Supplementary Figure 1D).

Taken together, these data suggest that *ttx-3* expression is activated in the NB^{SMDD/AIY} by a REF-2:POP-1 complex via a Zic binding site and that this activation is blocked in the NB^{SIAD/SIBV} by SYS-1.

HLH-3/Achaete-scute protein level becomes gradually asymmetric

Contrary to the effects of Zic binding sites, bHLH binding sites do not show an asymmetric activity early but the activity becomes gradually asymmetric as the neuroblasts progress in their cell cycle. We have previously identified two bHLH factors involved in the regulation of *ttx-3* expression: the E/Daughterless ortholog HLH-2 and the Beta3/Olig ortholog HLH-16 (Bertrand et al., 2011; Bertrand and Hobert, 2009a). In *hlh-2(RNAi)* treated embryos *ttx-3* expression is lost in nearly all the NB^{SMDD/AIY} lineages while in *hlh-16* mutants *ttx-3* expression is lost in around 20% of the NB^{SMDD/AIY} lineages (Figure 3A). As general E/Daughterless proteins, like HLH-2, often act as heterodimers with other more tissue specific bHLH factors like Beta3/Olig or Achaete-Scute factors (Bertrand et al., 2002) it is likely that another tissue specific bHLH factor acts in parallel to HLH-16 in the initiation of *ttx-3* expression. During a genetic screen for regulators of *ttx-3* expression (Bertrand and Hobert, 2009a; Sarin et al., 2010) we have identified a mutant, *ot354*, that displays a loss of *ttx-3* expression in around 20% of the AIY neurons at larval and adult stage (Supplementary Figure 3A, B, C). In *ot354* mutant larvae we still observe a neuron at the position normally occupied by AIY and this neuron still expresses the pan-neuronal marker *rgef-1* (Supplementary Figure 3D) suggesting that the neuron is correctly generated but its subtype-specific identity (marked by *ttx-3*) is not correctly specified.

We cloned *ot354* using whole genome sequencing of the mutant strain and confirmed its identity with another allele and by transformation rescue experiments (Supplementary Figure 3A, B, C). *ot354* animals contain an early stop in the bHLH factor HLH-3 (Supplementary Figure 3A). HLH-3 belongs to the Achaete-Scute family and can heterodimerize with HLH-2 (Krause et al., 1997). As in *hlh-16* mutants, *ttx-3* expression is lost in around 20 % of the AIY lineages in *hlh-3* mutant embryos and larvae (Figure 3A,

Supplementary Figure 3C). In *hll-3; hll-16* double mutants *ttx-3* expression is lost in nearly all AIY lineages similarly to what is observed in *hll-2* RNAi treated animals (Figure 3A) suggesting that *ttx-3* expression may be regulated by two partly redundant bHLH complexes HLH-2:HLH-3 and HLH-2:HLH-16.

We analyzed the expression of HLH-2, HLH-3 and HLH-16 by using rescuing translational fusions (HLH-2: :GFP (Nakano et al., 2010); HLH-3: :YFP generated by fosmid recombineering, see Experimental Procedures; and HLH-16: :GFP (Bertrand et al., 2011)). While the level of HLH-2 or HLH-16 protein is similar between the NB^{SMDD/AIY} neuroblast and its posterior sister the NB^{SIAD/SIBV} neuroblast (Figure 3D), the HLH-3 protein displays some asymmetry. The level of HLH-3 protein is initially symmetric just after the generation of the NB^{SMDD/AIY} and NB^{SIAD/SIBV} neuroblasts but becomes gradually asymmetric as the cell cycle progresses (Figure 3D) which mirrors the asymmetry in the activity of the bHLH binding sites (Figure 2). This late asymmetry of HLH-3 protein levels is also dependent on the Wnt pathway as it is reduced by a RNAi knockdown of *sys-1* (Figure 3D). This suggests that the early asymmetry generated by the REF-2:POP-1 complex is later reinforced by an asymmetry in the level of HLH-3 protein.

Discussion

Mechanism of transcriptional activation by POP-1/TCF in the absence of SYS-1/ β -catenin

In this paper we have analyzed how the Wnt/ β -catenin asymmetry pathway regulates the expression of a target gene *ttx-3* in an anterior daughter following asymmetric division of neuronal precursors. The genetic, *cis*-regulatory and biochemical analyses we have performed suggest an interesting atypical mechanism (Figure 4B). Following asymmetric division the level of POP-1/TCF is high and the level of SYS-1/ β -catenin is low in the anterior daughter nucleus. POP-1 associates with the Zic transcription factor REF-2 and the REF-2:POP-1 complex activates *ttx-3* expression via a Zic binding site present in its *cis*-regulatory region. On the contrary, in the posterior daughter nucleus the level of POP-1/TCF is low, the level of SYS-1/ β -catenin is high and SYS-1 blocks the activation of *ttx-3* expression by the REF-2:POP-1 complex. The precise biochemical mechanism by which POP-1 activates REF-2 transcriptional activity remains to be determined. There is clear evidence that the POP-1 and REF-2 proteins physically interact (co-immunoprecipitation we have performed in *C. elegans* embryos and yeast two-hybrid). One could imagine that POP-1 and REF-2 form a long term complex on Zic sites and that this complex is able to interact with the basal transcription machinery in a manner different from REF-2 alone. Another possibility is that the direct interaction between the POP-1 and REF-2 proteins is transient and induces a long lasting conformational change in the REF-2 protein modifying its activity. The activation of REF-2 by POP-1 is blocked in the posterior daughter by SYS-1. While we have not analyzed in detail the mechanism of this blockage by SYS-1, a study in vertebrates has shown that Zic2 can directly bind a TCF4: β -catenin complex on TCF binding sites blocking transcriptional activation by TCF4: β -catenin (Pourebahim et al., 2011). One possible mechanism is therefore that in the NB^{SIAD/SIBV} neuroblast SYS-1 blocks the activation of *ttx-3* expression by binding to the REF-2:POP-1 complex blocking its ability to activate transcription.

The REF-2:POP-1 mechanism seems to be the main driver of the asymmetric transcriptional activation. However this process is later reinforced by a partial asymmetry in bHLH activity. While initially symmetric, HLH-3 protein levels and transcriptional activity mediated by bHLH binding sites become gradually asymmetric, decreasing in the posterior daughter. The gradual disappearance of the HLH-3 protein in the posterior daughter is regulated by the Wnt pathway and could be due to an increased degradation or a decreased production in the posterior daughter. Interestingly it has been recently reported that another bHLH factor, the Atonal ortholog LIN-32, displays a similar pattern (gradual disappearance from the posterior daughter) during asymmetric divisions in male specific neuronal lineages of the *C. elegans* larva and this pattern is also regulated by the Wnt/ β -catenin asymmetry pathway (Miller and Portman, 2011). The regulation of asymmetries in the level of proneural bHLH between daughter cells by the Wnt/ β -catenin asymmetry pathway may therefore be a common theme of the specification of neuronal lineages in *C. elegans*.

Intriguingly, the activation of target genes in the anterior daughter of another embryonic blastomere, the endomesoderm precursor EMS, seems to follow a different logic (Broitman-Maduro et al., 2009; Owraghi et al., 2010). In this context the POP-1:SYS-1 complex seems to activate the expression of the GATA transcription factors END-1 and END-3 in the posterior daughter, which subsequently restrict the expression of the anterior transcription factor *ceh-51* to the anterior daughter by repressing it in the posterior daughter. Therefore transcriptional activation in anterior daughters can involve either a direct activation by POP-1 in the absence of SYS-1 in the anterior daughter (*ttx-3* in the NB^{SMDD/AIY} neuroblast) or an indirect repression by the POP-1:SYS-1 complex in the posterior daughter (*ceh-51* in the EMS lineage). We currently do not know whether both mechanisms (direct activation of an anterior target by POP-1 in the absence of SYS-1 and direct activation of a posterior target by the POP-1:SYS-1 complex) can operate together in the same asymmetric cell division.

Regulation of the expression of opposite target genes by TCF

Can we place the atypical mechanism of transcriptional activation by POP-1 described here in a more general framework of transcriptional activation by TCF transcription factors? In both vertebrates and invertebrates it has been observed that a few direct target genes can display an “opposite” type of regulation: repressed by the TCF: β -catenin complex when the Wnt pathway is active and/or activated by TCF when the Wnt pathway is inactive. The mechanism behind this intriguing opposite regulation remains poorly characterized (Cadigan, 2012). In the case of the *E-cadherin* and *p16* genes in mice or of the *stripe* gene in *Drosophila* it has been observed that direct repression by the TCF: β -catenin complex is mediated by classic TCF binding sites. However, it is unclear why TCF: β -catenin bound to these sites represses rather than activates transcription (Delmas et al., 2007; Jamora et al., 2003; Piepenburg et al., 2000). The analysis of the *Ugt36Bc* gene in *Drosophila* suggests a second mechanism (Blauwkamp et al., 2008; Zhang et al., 2014). In this case TCF binds to a non canonical binding site and binding to this site is suggested to induce an allosteric change in TCF that reverts the transcriptional output. In the case of the *ttx-3* gene of *C. elegans* we propose a third mechanism (Figure 4C). Here, TCF forms a complex with another transcription factor X, regulating transcription via an X binding site (for *ttx-3*, X = the Zic

transcription factor REF-2). When Wnt signaling is inactive the X:TCF complex activates transcription, while when Wnt signaling is active β -catenin blocks this activation. It will be interesting to see whether this atypical mechanism also applies to other opposite target genes in *C. elegans* or other organisms.

Experimental Procedures

Genetics

ot354 was isolated from an automated genetic screen using EMS as a mutagen and a worm sorter to identify mutants in which *ttx-3p::gfp (otIs173)* expression is defective (Bertrand and Hobert, 2009a; Sarin et al., 2010). Identification of variants in the *ot354* mutant strain by whole genome sequencing was performed as described (Sarin et al., 2010) using MAQGene (Bigelow et al., 2009) as data analysis tool. The strain was backcrossed four times before phenotypic characterization. RNAi treatment was performed as described (Bertrand and Hobert, 2009a).

Expression constructs and transgenic strains

The making of constructs and transgenic strains is described in detail in the Supplemental Data.

Cell identifications

In the embryo the NB^{SMDD/AIY} and NB^{SIAD/SIBV} neuroblasts and their daughter cells (the SMDD, AIY, SIAD and SIBV neurons) were identified by using the *hlh-16p::his::mCherry* transgene (*otIs10546*) which labels only a small subset of neuroblasts and neurons at epidermal enclosure (Bertrand et al., 2011). At larval stages the AIY neuron was identified using DIC optics based on its stereotypical location (Sulston et al., 1983).

Luciferase assay

COS-7 cells (10^5) were transfected using Lipofectamine 2000 (Invitrogen) with the Zic site firefly luciferase reporter construct (200 ng) with or without expression vectors for REF-2 (500 ng), POP-1 (500 ng) and SYS-1 (2 μ g). After an incubation of 48h, Luciferase assay was performed according to manufacturer's guideline (Dual-Luciferase Reporter Assay, Promega). Measurements were normalized to a *Renilla* luciferase co-transfected control and performed on 12 biological replicates per conditions.

Biochemistry

The conditions used for co-immunoprecipitation and Electrophoretic Mobility Shift Assay are described in detail in the Supplemental Data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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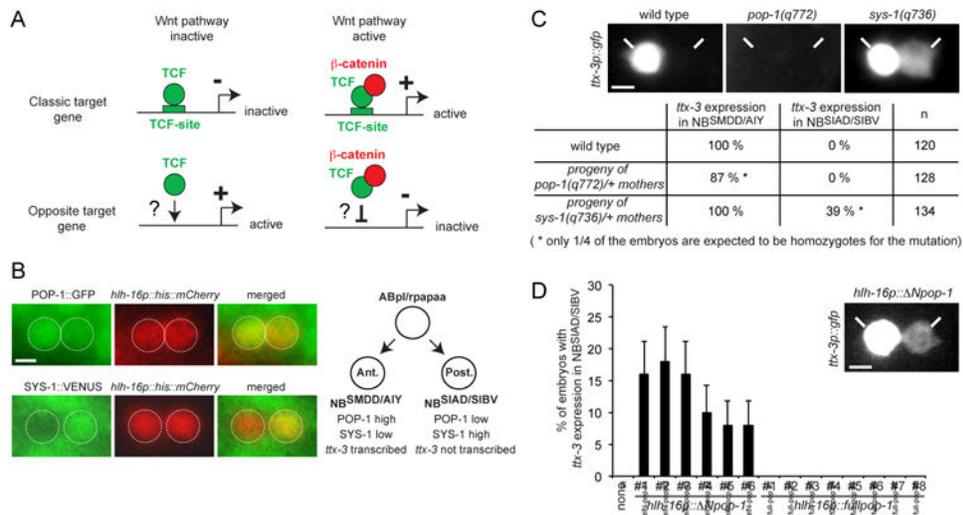


Figure 1. Activation of *ttx-3* expression by POP-1 and repression by SYS-1

(A) Classic target genes are activated by TCF associated with β -catenin when the Wnt pathway is active and repressed by TCF when the Wnt pathway is inactive. Opposite target genes are activated by TCF when the Wnt pathway is inactive and repressed by the TCF: β -catenin complex when the Wnt pathway is active.

(B) Expression of the POP-1::GFP (*qIs74*) and SYS-1::VENUS (*qIs95*) fusion proteins in the NB^{SMDD/AIY} (Ant.) and NB^{SIAD/SIBV} (Post.) neuroblasts identified with the *hllh-16p::his::mCherry* transgene (promoter of the *hllh-16* gene driving an histone fused to mCherry, *stIs10546*) at epidermal enclosure.

(C) Effect of *pop-1* and *sys-1* loss of function mutants on the initiation of *ttx-3* expression (*ttx-3p::gfp*, *otIs173*) at epidermal enclosure. As *pop-1(q772)* and *sys-1(q736)* homozygote mutants are lethal (at later embryonic stages), we scored the progeny of heterozygote mothers. Around 1/4 of the progeny displays a phenotype as expected from mendelian segregation (n = number of lineages analyzed). Note that in *sys-1* mutants the ectopic expression in NB^{SIAD/SIBV} is always weaker than the endogenous expression in NB^{SMDD/AIY} (see picture). This may be due to the fact that *sys-1* loss of function is only partial (due to maternal contribution) and/or to the fact that NB^{SIAD/SIBV} has a lower nuclear concentration of POP-1 than NB^{SMDD/AIY}. See also Figure S1.

(D) Expression of a POP-1 version lacking the SYS-1 interaction domain (*hllh-16p::Npop-1*) but not a full length version (*hllh-16p::fullpop-1*) ectopically activates *ttx-3* expression (*ttx-3p::gfp*, *mgIs18*) in the NB^{SIAD/SIBV} neuroblast. The *hllh-16* promoter which drives expression in NB^{SMDD/AIY} and NB^{SIAD/SIBV} is used as a driver. 6 independent lines were analyzed for *hllh-16p::Npop-1* and 8 for *hllh-16p::fullpop-1* (none: control without any *pop-1* transgene). The percentage of embryos showing ectopic expression in each of the *hllh-16p::Npop-1* lines is low but statistically significant ($p < 0.01$, Fisher's exact test; n=50; error bars show standard error of proportion). Note that the ectopic expression in NB^{SIAD/SIBV} is always weaker than the endogenous expression in NB^{SMDD/AIY} (see picture). Scale bar = 2 μ m.

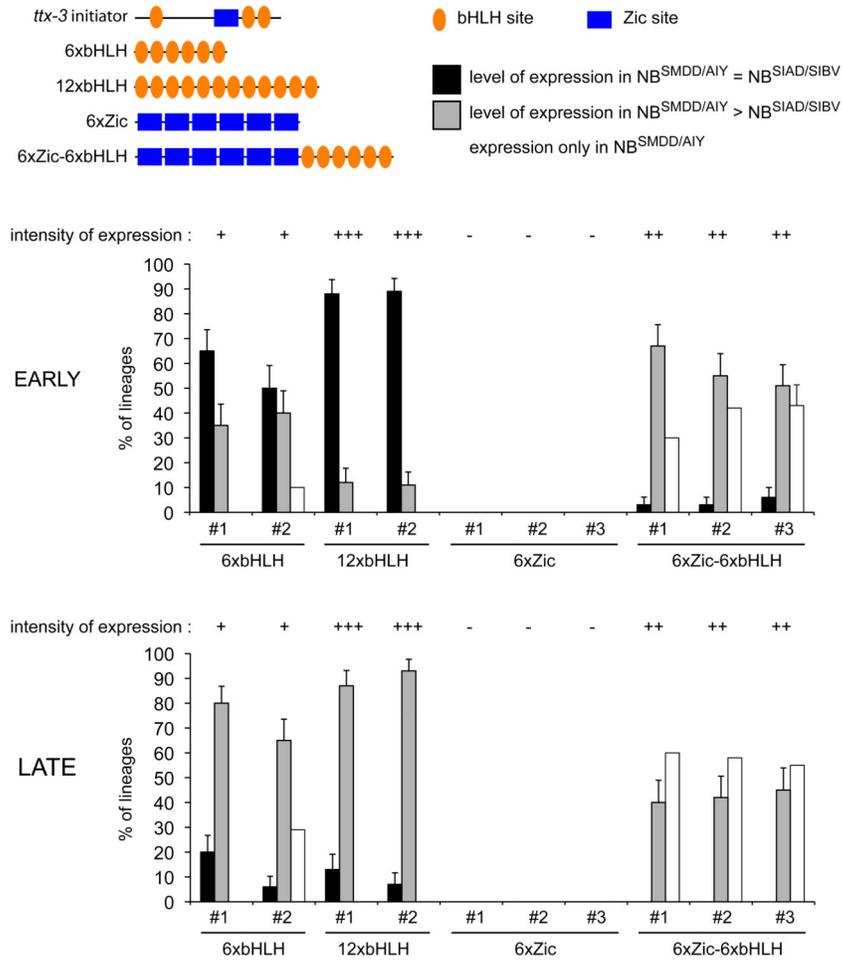


Figure 2. Role of the Zic and bHLH binding sites in the generation of the asymmetry
Activity of multimers of Zic or bHLH binding sites placed in front of *gfp*. On top the structure of the *cis*-regulatory element responsible for the initiation of *ttx-3* expression is shown (“*ttx-3* initiator”). The graphs present 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), or expression only in NB^{SMDD/AIY} (white). Expression in the NB^{SMDD/AIY} and NB^{SIAD/SIBV} neuroblasts was scored early (during interphase) or late (when entering mitosis as monitored by chromosome condensation using *hll-16p::his::mCherry* and by the appearance of the cleavage furrow) (n>30, error bars show standard error of proportion). The intensity of the GFP signal in the NB^{SMDD/AIY} neuroblast in each line is indicated above the graph (+++: strong, ++: medium, +: low). Numbers below graph indicate independent lines. See also Figure S2.

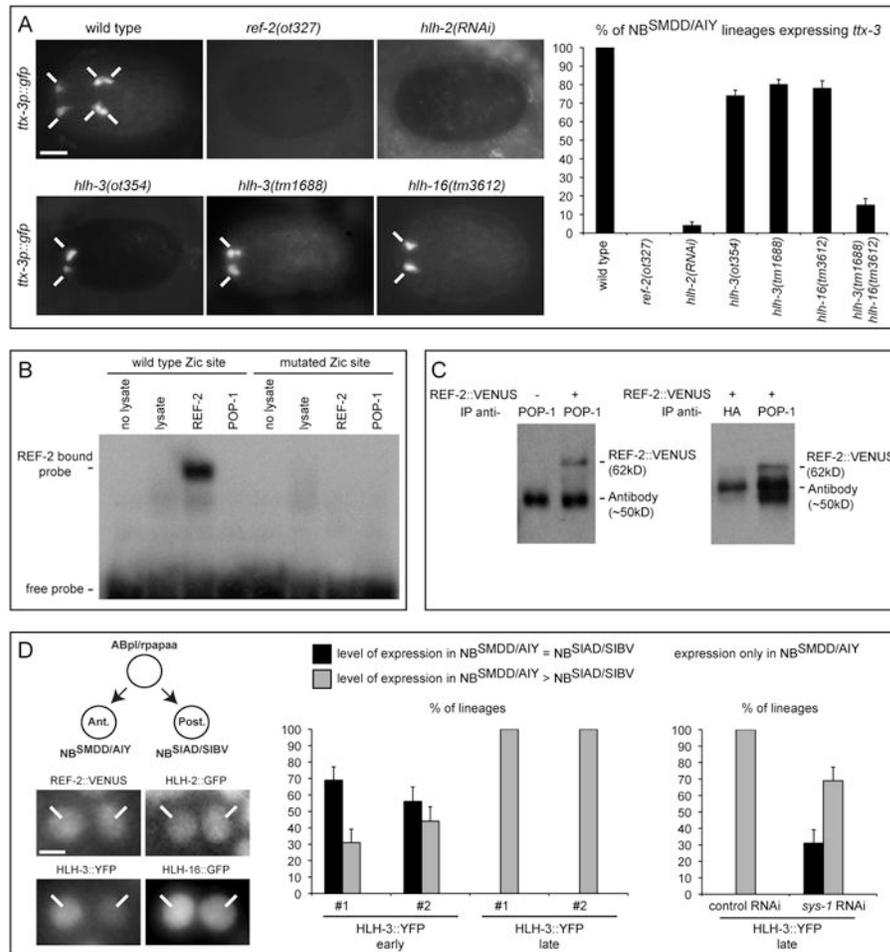


Figure 3. Regulation of the initiation of *ttx-3* expression by REF-2, HLH-2, HLH-3 and HLH-16
(A) Left: expression of *ttx-3* (*ttx-3p::gfp*) in embryos at epidermal enclosure just after division of the NB^{SMDD/AIY} neuroblast (ventral view, anterior is left, scale bar = 10 μ m, note that the expression of *ttx-3* in the AIN lineage (AINm, an unrelated neuronal lineage that also expresses *ttx-3*) is affected by loss of *ref-2* and *hlh-2* function but not by loss of *hlh-3* and *hlh-16* function). Right: percentage of NB^{SMDD/AIY} lineages that display expression of *ttx-3* (*ttx-3p::gfp*) at epidermal enclosure ($n > 100$, error bars show standard error of proportion). See also Figure S3.

(B) EMSA using *in vitro* (reticulocyte lysate) produced REF-2 or POP-1 proteins on probes containing the wild type or mutated Zic site from the *ttx-3* promoter. See also Figure S4.

(C) Coimmunoprecipitation from *C. elegans* embryos expressing the REF-2::VENUS (*otEx3091*) translational fusion (+) or not (-), using an anti-POP-1 antibody or control anti-HA antibody for immunoprecipitation and an anti-VENUS antibody for western blot. (Note that in addition to the REF-2::VENUS band (62kD) an additional band corresponding to the antibody used for immunoprecipitation (~50kD) is also detected. The same quantity of anti-POP-1 and anti-HA antibodies were used in the immunoprecipitations, differences of intensity of the antibody band between anti-POP-1 and anti-HA reflects difference of affinity of these antibodies with the western blot antibodies).

(D) Left: expression of the translational fusions REF-2: :VENUS (*otEx3091*), HLH-2: :GFP (*nIs47*), HLH-3: :YFP (*otEx4140*), HLH-16: :GFP (*otEx4503*) in the NB^{SMDD/AIY} (Ant.) and NB^{SIAD/SIBV} (Post.) neuroblasts at epidermal enclosure (scale bar = 2 μ m). Right: percentage of lineages expressing the translational fusion HLH-3: :YFP in both NB^{SMDD/AIY} and NB^{SIAD/SIBV} at similar level (black), in both NB^{SMDD/AIY} and NB^{SIAD/SIBV} with higher level in NB^{SMDD/AIY} (grey), or only in NB^{SMDD/AIY} (white). Two independent HLH-3: :YFP lines (#1 *otEx4140*, #2 *otEx4142*) were analyzed. Expression in the NB^{SMDD/AIY} and NB^{SIAD/SIBV} neuroblasts was scored early (during interphase) or late (when entering mitosis as monitored by chromosome condensation using *hll-16p::his::mCherry* and by the appearance of the cleavage furrow). The effects of control and *sys-1* RNAi were tested on *otEx4140*. (n>30, error bars show standard error of proportion).

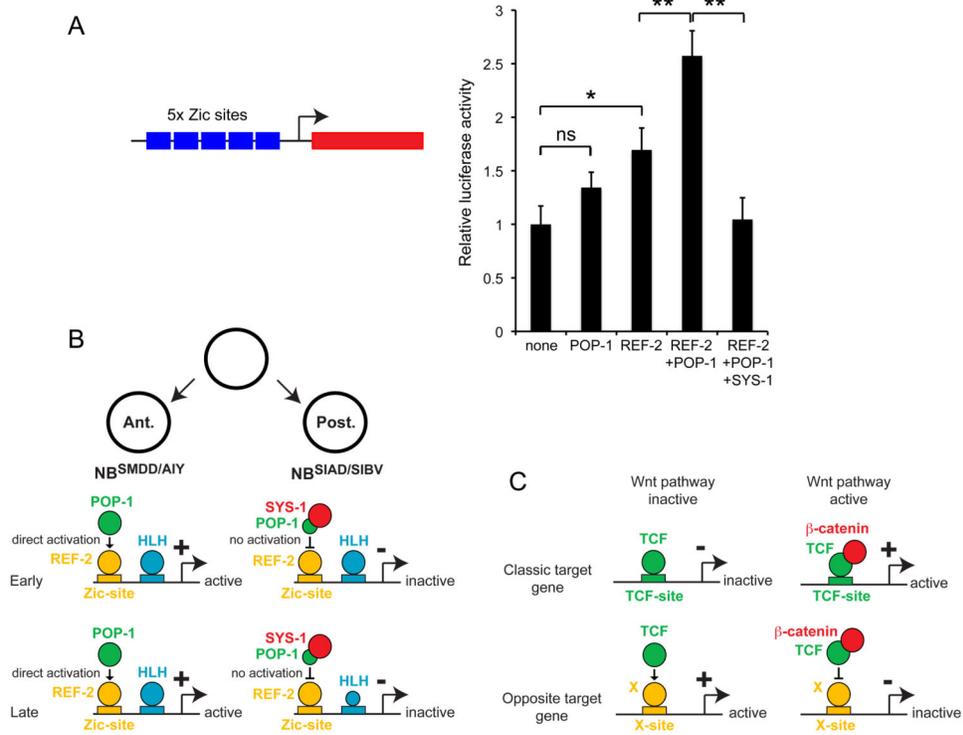


Figure 4. Action of REF-2, POP-1, SYS-1 on Zic binding sites and model

(A) Analysis of the activity in COS-7 cells of a multimer of Zic binding sites placed in front of a luciferase reporter. The multimer is cotransfected with vectors expressing REF-2, POP-1, SYS-1 or an empty vector (none). (*: $p < 0.05$, **: $p < 0.01$, ns: not significant, Student's t-test; $n = 12$, error bars show standard error of the mean).

(B) Activation of the opposite target gene *ttx-3* by the TCF transcription factor POP-1. “HLH” represents the 3 bHLH factors HLH-2, HLH-3 and HLH-16. The smaller circles in the NB^{SIAD/SIBV} neuroblast represent lower levels of POP-1 and HLH-3.

(C) General model for the activation of opposite target genes by TCF. X = REF-2 in the case of *ttx-3*.