

The HMG-box transcription factor SoxNeuro acts with Tcf to control Wg/Wnt signaling activity

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Wnt signaling specifies cell fates in many tissues during vertebrate and invertebrate embryogenesis. To understand better how Wnt signaling is regulated during development, we have performed genetic screens to isolate mutations that suppress or enhance mutations in the fly Wnt homolog, *wingless* (*wg*). We find that loss-of-function mutations in the neural determinant *SoxNeuro* (also known as *Sox-neuro*, *SoxN*) partially suppress *wg* mutant pattern defects. *SoxN* encodes a HMG-box-containing protein related to the vertebrate Sox1, Sox2 and Sox3 proteins, which have been implicated in patterning events in the early mouse embryo. In *Drosophila*, *SoxN* has previously been shown to specify neural progenitors in the embryonic central nervous system. Here, we show that *SoxN* negatively regulates Wg pathway activity in the embryonic epidermis. Loss of *SoxN* function hyperactivates the Wg pathway, whereas its overexpression represses pathway activity. Epistasis analysis with other components of the Wg pathway places *SoxN* at the level of the transcription factor Pan (also known as Lef, Tcf) in regulating target gene expression. In human cell culture assays, *SoxN* represses Tcf-responsive reporter expression, indicating that the fly gene product can interact with mammalian Wnt pathway components. In both flies and in human cells, *SoxN* repression is potentiated by adding ectopic Tcf, suggesting that *SoxN* interacts with the repressor form of Tcf to influence Wg/Wnt target gene transcription.

KEY WORDS: SoxN, Wg, Wnt, *Drosophila*, Embryo, Signal transduction

INTRODUCTION

The Wnt gene family encodes cysteine-rich secreted growth factors that are highly conserved throughout the animal kingdom (reviewed in Dierick and Bejsovec, 1999; Logan and Nusse, 2004). In vertebrate embryos, different Wnt proteins promote a variety of cell-fate decisions, including the patterning of the limbs, brain and other organs. In *Drosophila*, a single Wnt molecule, encoded by the *wingless* (*wg*) gene, directs a remarkably similar set of patterning events. Excess Wnt pathway activity in vertebrate tissues is associated with a variety of cancers, particularly colorectal tumors (reviewed in Bienz and Clevers, 2000; Polakis, 2000). Although excess Wg activity in flies does not create tumors, it does produce profound tissue alterations that are easily detected. All of the cell surface and intracellular Wnt pathway components are highly conserved between vertebrates and *Drosophila*, and many were first identified in *Drosophila* through mutational disruptions of patterning (reviewed in Bejsovec, 2006).

At the end of *Drosophila* embryogenesis, epidermal cells secrete a highly patterned cuticle layer. The ventral surface displays segmental denticle belts, which are 'tractor tread' arrays of hooked elements interspersed with 'naked' cuticle. *wg* loss-of-function mutant embryos secrete a uniform lawn of denticles, lacking the naked cuticle that should separate the belts (Fig. 1C, Fig. 2A). High levels of Wg signaling convert all of the ventral epidermis to the naked cuticle cell fate (Noordermeer et al., 1992; Hays et al., 1997). Mutations in downstream components that positively activate the pathway show a *wg*-like phenotype, whereas mutations in negative regulators show an excess-naked-cuticle phenotype (reviewed in Dierick and Bejsovec, 1999).

Epistasis experiments with these mutations have shown that the pathway hinges on the regulation of Armadillo (Arm), which is the fly beta-catenin homolog (reviewed in Bejsovec, 2000; Peifer and Polakis, 2000; Jones and Bejsovec, 2003). In the absence of Wg signaling, Arm levels are kept low by a set of proteins known collectively as the destruction complex. These proteins, which include the Axin and Apc scaffolding molecules and the serine-threonine kinase Shaggy (also known as Zeste-white3, Zw3; GSK3 β in vertebrates), target Arm for destruction via ubiquitylation. When Wg binds to its receptor complex, which consists of Arrow (LRP5/6 in vertebrates) and Frizzled, this inactivates the destruction complex and allows Arm to accumulate. In the simplest view, Arm accumulation drives its interaction with Tcf, an HMG-box transcription factor, in the nucleus. Tcf can bind DNA in the absence of Arm and represses Wg target gene expression in conjunction with Groucho (Gro), a transcriptional co-repressor (Cavallo et al., 1998; Roose et al., 1998). When Arm binds to Tcf, it displaces Gro and recruits other proteins to form a transcriptional activation complex that promotes Wg target gene expression (Brunner et al., 1997; van de Wetering et al., 1997; Kramps et al., 2002; Daniels and Weis, 2005).

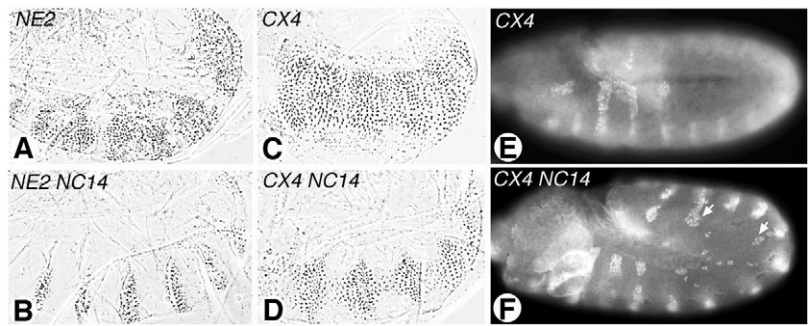
The balance between repression and activation properties of the Tcf complex is crucial for Wnt target gene regulation. Recent work suggests that some negative regulators, such as Apc and GSK3 β , may act in the nucleus in regulatory complexes at the promoters of Wnt target genes (Sierra et al., 2006), and that these complexes act at least in part through chromatin remodeling. However, the mechanism that switches Tcf from repressor to activator is still unclear. To identify new molecules that regulate Wg/Wnt pathway activity, we have performed genetic screens in *Drosophila* for mutations that suppress weak *wg* loss-of-function phenotypes. Here, we describe a strong suppressor mutation that partially rescues both hypomorphic and null mutant alleles of *wg*. This mutation is allelic with previously isolated mutations in *SoxNeuro* (*SoxN*), a gene that is required for the specification of neural progenitors in the embryonic central nervous system (Buescher et al., 2002; Overton et al., 2002).

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Fig. 1. *SoxN* loss of function suppresses *wg* mutant phenotypes.

(A) Hypomorphic *wg^{NE2}* mutation reduces the zones of naked cuticle, which separate denticle belts, on the ventral side and disrupts dorsal patterning, resulting in strong curvature of the embryonic cuticle. (B) *SoxN^{NC14}* mutation rescues *wg^{NE2}* ventral patterning to almost wild type (compare with Fig. 2A), without rescuing dorsal patterning. (C,D) The RNA-null *wg^{CX4}* allele produces a 'lawn of denticles' phenotype (C), which is partially suppressed by the *SoxN^{NC14}* mutation (D). (E) *wg^{CX4}* mutant embryos lose epidermal expression of the Wg target gene *en* before stage 10 (compare with wild-type pattern in Fig. 2G). (F) *wg^{CX4}, SoxN^{NC14}* double-mutant homozygotes retain some epidermal *en* expression (arrows) even at late stages. *SoxN^{NC14}* is linked to *wg* on the second chromosome; single and double homozygotes are recognized by the absence of GFP from a marked balancer chromosome. Embryos are oriented with anterior to the left and dorsal side up.



Sox proteins, like Tcf proteins, contain HMG domains, which bind DNA. SoxN is most closely related to vertebrate Class B Sox family members – Sox1, Sox2 and Sox3 (Cremazy et al., 2000; Cremazy et al., 2001) – which control cell-fate decisions in developmental processes ranging from sex determination to chondrogenesis (reviewed in Kamachi et al., 2000; Wilson and Koopman, 2002). In addition, *Xenopus* XSox3, as well as XSox17 α and XSox17 β (Zorn et al., 1999; Sinner et al., 2004), and the mouse Sox9 protein (Akiyama et al., 2004) interfere with Wnt signaling by physically interacting with beta-catenin through their C-termini. However, other work suggests that the interaction with beta-catenin is not sufficient to explain the *in vivo* Wnt-modulating function. Zhang et al. (Zhang et al., 2003) found that the DNA-binding domain, rather than the beta-catenin-binding region, is crucial for the influence of XSox3 proteins on Wnt target expression. Our data support the idea that Sox proteins act as true repressors *in vivo*. We show that SoxN strongly represses Wg/Wnt-mediated target gene

transcription, and we find no evidence for an interaction of SoxN with Arm. Instead, we detect a strong genetic interaction with Tcf, suggesting that SoxN is involved in the delicate balance between the repressor and activator functions of Tcf.

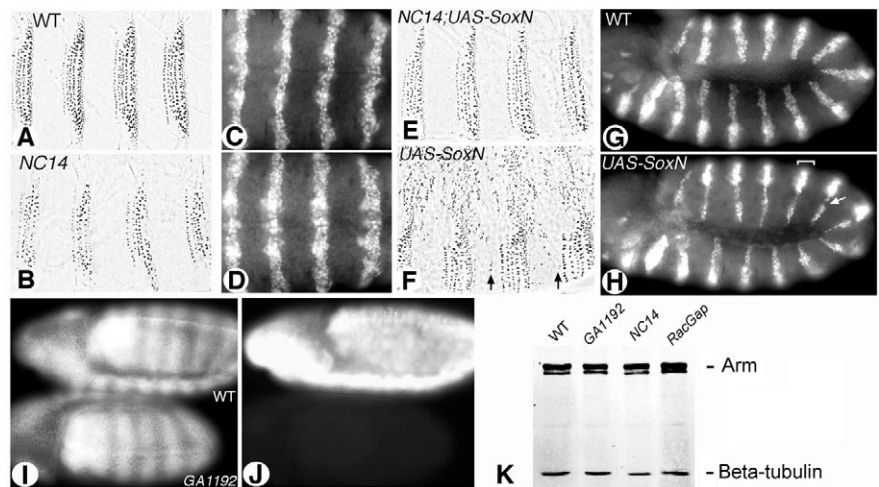
MATERIALS AND METHODS

Drosophila stocks and culture conditions

All deficiencies and P-element insertions used in mapping *NC14* are from the Bloomington Stock Center. *wg^{NE2}* is a missense mutation that disrupts cell-to-cell movement of Wg ligand without abolishing its secretion or signaling capacity (Dierick and Bejsovec, 1998). *wg^{CX4}* is an RNA-null allele (Baker, 1987). Existing *SoxN* mutations (*SoxN^{GA1192}*, *SoxN^{C463}* and *SoxN^{C2139}*) and *UAS-SoxN* flies were kindly provided by G. Tear and S. Russell, respectively (Seeger et al., 1993; Overton et al., 2002). *UAS-Tcf* and *UAS-Tcf^{DN}* (also known as ΔN) are as described by van de Wetering et al. (van de Wetering et al., 1997). Ubiquitous embryonic expression of *UAS* transgenes was achieved with either the *E22C-Gal4* or the *arm-Gal4* driver lines (both from the Bloomington Stock Center). *dpp-blink-Gal4* drives *UAS*

Fig. 2. *SoxN* regulates Wg pathway activity.

(A) Wild-type cuticle pattern shows a normal expanse of Wg-specified naked cuticle separating denticle belts. (B) *SoxN^{NC14}* single mutants produce excess naked cuticle. (C) Wild-type stripes of *en* expression span 2-3 cells in each segment. (D) *en*-expressing stripes in *SoxN^{NC14}* are broadened, similar to known phenotypes produced by ectopic Wg signaling. (E) Ubiquitous expression of wild-type *SoxN* with the *arm-Gal4* driver rescues the excess-naked-cuticle phenotype in *SoxN^{NC14}* homozygotes. This treatment does not rescue embryonic lethality. (F) Overexpressing *SoxN* at higher levels, using the *E22C-Gal4* driver in an otherwise wild-type embryo, affects Wg-mediated cuticle patterning. Ectopic denticles replace some of the ventral and ventrolateral naked cuticle (arrows), and dorsal patterning is disrupted, leading to curvature of the embryo. We observe an average of 12 ectopic denticles within the naked cuticle zone of a typical abdominal segment ($n=100$). (G) Stripes of *en* expression extend evenly from the ventral midline to the edge of the dorsal epidermis in wild-type stage-10 embryos. (H) These stripes are narrowed, particularly in the dorsolateral regions (arrow), when *UAS-SoxN* is driven with *E22C-Gal4*. Ventrally, expansion of *en* expression in the underlying central nervous system (bracket) can be seen; this *en* expression is not under the control of Wg (Bejsovec and Martinez Arias, 1991; Heemskerk et al., 1991) and presumably reflects the role of SoxN in specifying neuronal fates. (I) Side-by-side comparison showing no difference in anti-Arm staining between a *SoxN^{GA1192}* mutant (bottom) and a wild-type sibling (top). (J) Anti-GFP staining reveals the presence of the *twist-GFP* balancer chromosome in a wild-type sibling and its absence in the homozygous mutant embryo. (K) Quantitative immunoblot of lysates from hand-selected embryos shows equivalent Arm levels in homozygous mutants for *SoxN^{GA1192}* and *SoxN^{NC14}* compared with their wild-type *CyO-GFP*-bearing siblings. When normalized to the tubulin loading control, there is no detectable difference among the first three lanes. By contrast, Arm levels are 25% higher in *RacGap50C^{AR2}* mutant homozygotes. Embryos are oriented with anterior to the left and dorsal side up.



transgene expression in a stripe along the anteroposterior boundary of each imaginal disc (Johnston and Schubiger, 1996). Epistasis experiments were performed with the strong *arm⁴* allele (*arm^{YD35}*) and were verified with the weaker *arm⁸* (*arm^{H8.6}*) allele (Peifer and Wieschaus, 1990) and the strong *Tcf²* allele (van de Wetering et al., 1997). The *gro* deficiency *gro^{BX22}* (Cavallo et al., 1998) is also known as *Df(3R)Espl22*.

Most *Gal4-UAS* experiments were conducted at 28°C to allow maximal activity of the Gal4 protein. Experiments involving the *dpp-Gal4* driver were performed at 25°C because of increased lethality at the higher temperature. Hatching efficiencies and cuticle preparation were performed as described by Jones and Bejsovec (Jones and Bejsovec, 2005), except that all hatched larvae were also mounted for scoring. Because balancer chromosomes can introduce non-specific cuticle pattern defects, epistasis crosses were performed without marked balancers.

Isolation and characterization of the *NC14* mutation

Details of the EMS mutagenesis are described by Jones and Bejsovec (Jones and Bejsovec, 2005). The *NC14* mutation was mapped by standard meiotic recombination with adult-visible mutations. The Bloomington Deficiency Kit stocks *Df(2L)N22-14* and *Df(2L)N22-2* uncover the genetic interval containing the mutation, which was refined further by its complementation of *Df(2L)Exel7039* and failure to complement *Df(2L)Exel7040*.

Male site-specific recombination (Chen et al., 1998) was used to pinpoint the mutation with respect to molecularly characterized P-element insertions. A marked *NC14* mutant chromosome crossed into a $\Delta 2-3$ background was placed in trans to P-bearing chromosomes. Males were crossed back to flies with the same marked *NC14* chromosome to score viable recombinant progeny. For candidate genes within the refined interval, the complete sequence of all exons was analyzed to locate the EMS-induced *NC14* mutation. A single-nucleotide change in *SoxN* alters glutamine 351, according to the FlyBase annotated sequence AAF52712.1.

Antibody staining and western blotting

Antibody staining was as described by Dierick and Bejsovec (Dierick and Bejsovec, 1998). Mutant chromosomes were maintained over balancer chromosomes marked with *twist-Gal4 UAS-green fluorescent protein (GFP)*, to identify homozygotes by their failure to express GFP. Anti-En antibody was used at 1:50, and anti-Arm and anti-GFP at 1:500 [anti-En and anti-Arm from the Developmental Studies Hybridoma Bank (DSHB), anti-GFP from Chemicon]. Immunoblots were performed as previously described (Chao et al., 2003) with embryos that were hand-selected for the appropriate GFP genotype. Filters were stained with anti-Arm protein (DSHB) at 1:100 and anti-Tubulin at 1:5000 (Lab Vision). Cross-reacting proteins were detected and quantified using the Odyssey infrared imaging system and reagents (Li-Cor Technologies).

Cell-culture conditions and luciferase assays

The intronless *SoxN* sequence was cloned by PCR from genomic fly DNA to create pcDNA-SoxNflag, pcDNA-Tcf4myc and pcDNA-beta-cat Δ phos were gifts from P. Casey (Duke University Medical Center, Durham, NC). TOP- Δ L and FOP- Δ L were generated by deleting the 1.681 kb *XbaI* fragment containing most of the luciferase gene from the TOPflash- and FOPflash-reporter plasmids (Upstate). HEK293T cells were grown in DMEM medium supplemented with 10% FBS. TOPflash-reporter plasmid (0.1 μ g), 0.25 ng phRG-B (Promega) internal control and a total of 0.5 μ g expression plasmid was used for each transfection with lipofectamine2000 (Invitrogen). TOPflash expression was induced by co-transfection with 0.25 μ g of pcDNA-beta-cat Δ phos or by culturing cells in conditioned media from a Wnt3A stably transfected L-cell line (gift from P. Casey). Un-induced cultures were similarly treated using conditioned media from untransfected L-cells. Luciferase activities were determined 24–48 hours post-transfection using the Dual-luciferase reporter assay system (Promega). Duplicate transfections were made for each experiment and at least two independent experiments were performed.

For immunoprecipitation, antibody-conjugated beads were prepared by mixing 20 μ l ProtG beads (Zymed) with 1 μ l antibody [either anti-Myc (Cell Signaling) or anti-Flag M2 (Sigma)] in 1 ml PBS with protease inhibitors (Roche mini tablets) at 4°C overnight. Cell lysates were added to

the prepared beads and treated following standard protocols (Sambrook et al., 1989). Immunoblots were stained with anti-Myc (1:10,000) or anti-Flag M2 (1:10,000) and anti-beta-catenin (1:1000).

RESULTS

SoxN is a *wg* suppressor

In an EMS mutagenesis designed to recover mutations that suppress the hypomorphic *wg^{NE2}* phenotype (Fig. 1A), we isolated *NC14*, a linked second-chromosome mutation that showed significant rescue of patterning in homozygous mutant embryos (Fig. 1B). Standard meiotic recombination was used to map this mutation with respect to *wg* and also to generate recombinant chromosomes that bear the *NC14* mutation without the *wg^{NE2}* mutation. The *NC14* mutation on its own caused embryonic lethality, with a pattern defect showing slightly greater expanses of naked cuticle than is observed in wild-type embryos (Fig. 2A,B). Excess naked cuticle specification is diagnostic of ectopic Wg signaling. For example, maternal loss of the destruction-complex components *Axin*, *Apc2* or *zw3* artificially stabilizes Arm in the resulting embryos, which show an excess-naked-cuticle phenotype (Peifer et al., 1994; Hamada et al., 1999; McCartney et al., 1999). Only two known fly genes produce an excess-naked-cuticle phenotype in zygotically mutant embryos: the original segment polarity gene, *naked cuticle* (Jürgens et al., 1984), and the recently characterized *RacGap1* ortholog, *RacGap50C* (Jones and Bejsovec, 2005). Although the exact mechanisms of action for these two cytosolic gene products are still not clear, in both cases the phenotype reflects inappropriate activation of the Wg pathway.

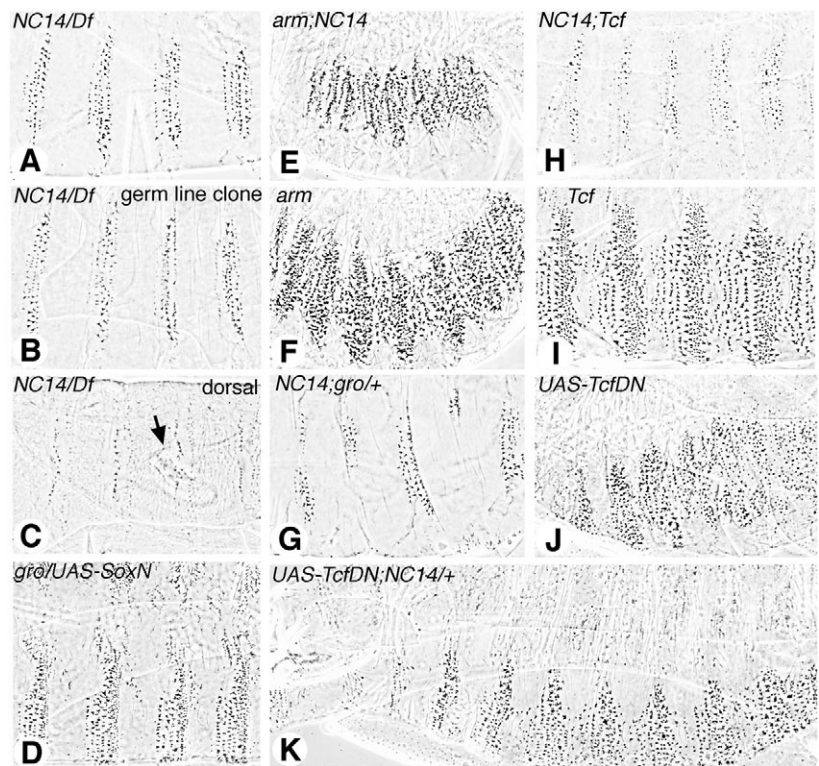
The *NC14* zygotic phenotype also indicates ectopic Wg pathway activity. We recombined the *NC14* mutation onto a chromosome bearing a null allele of *wg*, *wg^{CX4}*, and found that it was also suppressed, although to a lesser degree than the hypomorphic allele (Fig. 1C,D). Thus, *NC14* rescues segmental patterning in a ligand-independent fashion. Rescue could also be detected at the molecular level. The epidermal expression of *engrailed (en)*, in stripes of cells immediately posterior to the *wg*-expressing stripes of cells, is dependent on Wg signal transduction (DiNardo et al., 1988; Martinez Arias et al., 1988). In the absence of Wg activity, epidermal *en* expression was initiated normally by pair-rule-gene transcription factors but decays by developmental stage 9. In *wg^{CX4}*, *NC14* double-mutant embryos, however, some epidermal *en* expression continued to be detected throughout later stages of development (Fig. 1E,F). This derepression of Wg target gene expression is reminiscent of the derepression observed in *wg^{CX4}*; *gro* and *wg^{CX4}*; *Tcf* double-mutant embryos (Cavallo et al., 1998). On its own, the *NC14* mutation produced an expansion of the *en* expression domain (Fig. 2C,D). Wild-type stage-10 embryos expressed *en* in stripes that ranged from 2 to 3 cells wide, whereas *NC14* homozygotes consistently showed stripes ranging from between 3 and 5 cells wide. This degree of expansion is similar to that produced by hyperactivating the Wg pathway within each segment, either by overexpressing wild-type Wg or by removing a known negative regulator, such as *naked cuticle* (Noordermeer et al., 1992). The *NC14* mutation did not affect *wg* gene expression (data not shown) and partially restored target gene expression in a *wg*-null mutant (Fig. 1F); therefore, it is likely to disrupt a negative regulator downstream of Wg receptor activation.

To determine the molecular identity of the mutated gene, we subjected the *NC14* mutant chromosome to higher-resolution mapping techniques, including deficiency analysis and male site-specific recombination. In testing deficiencies that span the meiotic-

Fig. 3. Epistatic relationships of *SoxN* with the *Wg* pathway.

(A) The *SoxN^{NC14}* mutation placed in trans with a small deficiency for the region, *Df(2L)Exel7040*, shows no change from the homozygous mutant phenotype (compare with Fig. 2B), indicating that *SoxN^{NC14}* behaves as a null allele. (B) Removing maternal *SoxN* does not increase the severity of the mutant phenotype; therefore, *SoxN* acts zygotically. (C) Dorsal patterning elements show mild disruptions in some segments of *SoxN^{NC14}/Df*-mutant embryos (arrow; compare with more anterior segments, which have normal dorsal pattern elements). (D) Overexpressing *SoxN* in embryos derived from mothers that were heterozygous for *gro^{BX22}*, a deficiency removing the locus, produces milder pattern disruptions both dorsally and ventrally (compare with Fig. 2F). (E,F) Double homozygotes for *arm^d* and either *SoxN* allele show the *arm* 'lawn of denticles' phenotype (E), but embryos are smaller and have stronger dorsal pattern disruptions than do *arm^d* single mutants (F) (data shown in Table 2). (G) Mutants homozygous for *SoxN* that were derived from *gro^{BX22}* heterozygous mothers show increased naked cuticle ($n=140$). (H,I) *SoxN*; *Tcf²* double-mutant embryos also show increased naked cuticle (H). Thus, the *SoxN* mutant phenotype is epistatic to the *Tcf* 'lawn of denticles' phenotype (I) (data shown in Table 2). (J) *E22C-Gal4*-driven ubiquitous expression of dominant-negative *Tcf* produces a 'lawn of denticles' phenotype and severely reduces the size of the embryonic cuticle.

(K) Segmental patterning and body size of *Tcf^{DN}*-expressing embryos are partially rescued when *SoxN* dosage is reduced. The *SoxN^{NC14}* mutation is linked to the *E22C-Gal4* insertion in this experiment, so that all embryos ubiquitously expressing *UAS-Tcf^{DN}* are also heterozygous for *SoxN*. All show a milder phenotype regardless of whether the *SoxN* mutation was introduced from the mother or the father ($n=205$). Embryos are oriented with anterior to the left and dorsal side up.



map position of *NC14*, we identified several deficiencies that fail to complement the embryonic lethality of the mutation. Embryos trans-heterozygous for *NC14* and these deficiencies show an epidermal pattern with excess naked cuticle, identical to that of *NC14*-homozygous embryos (compare Fig. 3A with Fig. 2B). Thus, the *NC14* allele appears to be amorphic according to the classical definition for null alleles (Muller, 1932).

Male site-specific recombination (Chen et al., 1998) was used to force recombination at the position of molecularly defined P-element insertions. Candidate genes within the refined interval were then sequenced and a nonsense change at glutamine 351 in the *SoxN* gene was found in the *NC14* line. We performed complementation tests with existing *SoxN* mutations, which were isolated in a screen for nervous system disruption (Seeger et al., 1993; Buescher et al., 2002), and found that *NC14* was allelic with these mutations. We therefore refer to the *NC14* mutation as *SoxN^{NC14}*. A cuticle pattern defect has been noted previously for *SoxN* mutants (Buescher et al., 2002), but it has not been characterized in any detail. Our finding suggests that *SoxN* plays a role in regulating *Wg* pathway activity in addition to its role in neuroblast formation. *Wg* is known to control cell-fate choice in the developing nervous system (Chu-LaGriff and Doe, 1993), and, therefore, some of the neuronal defects of the *SoxN* mutants may be a secondary consequence of ectopic *Wg* pathway activation. However, the effects of *SoxN* loss of function on neuroblast formation and specification (Buescher et al., 2002; Overton et al., 2002) occur at earlier times and are more severe than those observed for ectopic *Wg* expression (Bhat, 1996), indicating that *SoxN* is likely to have separate roles in the two processes.

***SoxN* influences epidermal patterning**

The patterning defect of *SoxN^{NC14}* mutant embryos can be rescued by the expression of a *UAS-SoxN* transgene (Fig. 2E), confirming that *SoxN* is the gene responsible for the mutant phenotype. Although *SoxN* expression shows a pattern of ectodermal stripes during the late stages of embryogenesis (Cremazy et al., 2000; Buescher et al., 2002), rescue of *SoxN^{NC14}* pattern defects can be achieved by uniform expression with either the *arm-Gal4* or *E22C-Gal4* driver lines. Therefore, segmentally striped expression of *SoxN* is not required for its role in regulating *Wg* pathway activity. There did not appear to be a significant maternal contribution of *SoxN* (Fig. 3B). Embryos derived from homozygous mutant germline clones showed cuticle pattern defects indistinguishable from the zygotic mutant embryos (Fig. 3B). Likewise, homozygous mutant clones of adult tissue did not show any evidence of disrupted pattern, indicating that *SoxN* does not play a significant role in regulating *Wg* signal transduction in the imaginal disc (data not shown). We also tested for possible redundancy of *SoxN* with *Dichaete* (*D*), a second closely related SoxB-class gene in the fly genome (Nambu and Nambu, 1996; Russell et al., 1996). This gene has been found to function redundantly with *SoxN* in patterning the embryonic nervous system (Overton et al., 2002), but did not appear to influence the role of *SoxN* in *Wg* signaling (see Fig. S1 in the supplementary material).

Driving high levels of wild-type *SoxN* can produce profound disruptions in embryonic patterning, without affecting *wg* expression or *Arm* stability (see Fig. S2 in the supplementary material). In otherwise wild-type fly embryos, ectopic *SoxN* interfered with the normal specification of naked cuticle, resulting

Table 1. Ectopic expression of *SoxN* and *Tcf* in embryos or imaginal discs affects viability

Transgene	Driver line					
	× <i>arm-Gal4</i>		× <i>E22C-Gal4</i>		× <i>dpp-Gal4</i>	
	Embryonic lethal (%)	<i>n</i>	Embryonic lethal (%)	<i>n</i>	Pupal lethal (%)	<i>n</i>
<i>UAS-SoxN</i>	32.9	301	77.7	381	29.4	321
<i>UAS-Tcf</i>	1.0	394	1.8	496	36.7	291
<i>UAS-Tcf;UAS-SoxN</i>	87.6	372	99.6	460	100.0	297
<i>UAS-Tcf DN</i>	100.0	345	100.0	300	100.0	218

arm-Gal4 and *E22C-Gal4* drive transgene expression in the embryo: *arm-Gal4* is expressed throughout the embryo and *E22C-Gal4* is expressed at high levels in the epidermis. *dpp-blink-Gal4* drives expression along the anteroposterior boundary in imaginal discs.

in denticles within the naked zone (Fig. 2F). The cuticle defects produced by *SoxN* overexpression correlated with an inappropriate repression of *en* expression (Fig. 2G,H), again supporting a role for SoxN in the negative regulation of target gene expression. Segmental patterning on the dorsal surface was often more severely disrupted than on the ventral (Fig. 2F,H), leading to curvature of the cuticle. Loss of *SoxN* function also had variable and mild effects on dorsal cuticle patterning (Fig. 3C).

SoxN overexpression phenotypes are variable and dose sensitive. Driving *UAS-SoxN* expression with the strong epidermis-specific *E22C-Gal4* produced stronger pattern disruptions and greater embryonic lethality than did driving *UAS-SoxN* with the more widely expressed but less potent *arm-Gal4* driver (Table 1). In both cases, those embryos that hatched often survived to become normal adults, suggesting that ectopic SoxN plays no further role beyond embryogenesis. Consistent with this idea, expressing ectopic *UAS-SoxN* in the imaginal disc with a *dpp-Gal4* driver did not create any apparent pattern disruption in the adult, although it did diminish viability somewhat (Table 1). By contrast, *dpp-Gal4*-driven expression of the dominant-negative form of *Tcf*, a known repressor of Wg target gene expression, disrupts adult body pattern and results in complete pupal lethality (Table 1).

Position of *SoxN* in the Wg pathway

To determine where in the Wg cascade *SoxN* acts, we performed epistasis analysis between *SoxN* and known mutations in the Wg pathway. All epistasis experiments were performed with both *SoxN^{NC14}* and *SoxN^{GA1192}*, a previously isolated protein-null allele (Buescher et al., 2002). *SoxN^{GA1192}* in trans with the deficiency produces the same extent of excess naked cuticle as does *SoxN^{NC14}* in trans with the deficiency, indicating similar allele strength (see Fig. S1 in the supplementary material). We routinely show *SoxN^{NC14}* because this mutant chromosome was extensively recombined to remove extraneous mutations. During random EMS mutagenesis, such as that in which the *NC14* allele was isolated,

multiple mutations may be induced on a chromosome and these will homozygose along with the mutation of interest. Therefore, accurate phenotypic analysis requires development of a stock where only the gene of interest is mutationally disrupted. We know that the *SoxN^{NC14}* lesion is the only lethal mutation on the chromosome, because homozygous viable recombinants to the left and right of the lesion were recovered during male site-specific recombination mapping.

We tested *SoxN* mutations with strong (Fig. 3E,F; Table 2) and weak alleles of *arm*, and found that, in both cases, the *arm; SoxN* double mutants showed the ‘lawn of denticles’ phenotype typical of *arm* mutants. Double mutants could be distinguished from *arm* single mutants among the progeny of this cross because the *SoxN* mutation disrupted dorsal pattern and reduced the size of *arm* mutant embryos. Thus, *SoxN* slightly enhances the severity of the effects of *arm* on overall body patterning. Because *arm* gene activity is required for the specification of excess naked cuticle observed in *SoxN* mutants, SoxN must act upstream or in parallel with Arm. However, Arm protein levels are not artificially stabilized in *SoxN* mutant embryos (Fig. 2I-K) like they are in other zygotic-mutant conditions that produce excess naked cuticle, such as *RacGap50C* mutants (Jones and Bejsovec, 2005). *SoxN* mutant embryos showed stripes of Arm accumulation similar to those of wild-type siblings (Fig. 2I,J), indicating that the signal transduction machinery upstream of Arm functions properly and that epidermal cells respond normally to the striped production of Wg signal. This is true for both the *SoxN^{NC14}* and *SoxN^{GA1192}* (shown) alleles. Thus, *SoxN* is a unique zygotically acting mutation that hyperactivates the Wg pathway without affecting Arm stability.

By contrast, *SoxN; Tcf* double-mutant embryos showed the excess-naked-cuticle phenotype of *SoxN* (Fig. 3H,I; Table 2), suggesting that *SoxN* acts downstream of *Tcf* in the pathway. However, it must be kept in mind that interpretations of *Tcf* phenotypes are complicated by the dual role of Tcf in directing

Table 2. Epistasis analysis of *SoxN*

Mutant	Phenotype				<i>n</i>	χ^2
	<i>arm</i> (%)	<i>SoxN</i> (%)	Severe <i>arm</i> (%)	Wild type (%)		
<i>arm⁴/+</i> ; <i>SoxN^{GA1192}/+</i>	17.4	17.6	6.8	58.2	888	2.21
<i>arm⁴/+</i> ; <i>SoxN^{NC14}/+</i>	19.9	17.5	7.4	55.2	922	3.60

Mutant	Phenotype				<i>n</i>	χ^2
	<i>Tcf</i> (%)	<i>SoxN</i> (%)	Severe <i>SoxN</i> (%)	Wild type (%)		
<i>SoxN^{GA1192}/+</i> ; <i>Tcf²/+</i>	19.2	17.7	7.2	55.9	928	2.08
<i>SoxN^{NC14}/+</i> ; <i>Tcf²/+</i>	18.6	18.9	5.8	56.7	1019	0.43

For each *SoxN* allele: non-balancer F₁ female progeny from *arm⁴/FM7* × *SoxN/CyO* were crossed to the deficiency stock +Y; *Df(2L)Exel7040/CyO*; non-balancer F₁ progeny from *Tcf²/ey^P* × *SoxN/CyO* were crossed to each other. All cuticles from F₂-progeny, hatched and unhatched, were mounted for examination. F₂ phenotypes do not deviate significantly from numbers expected for the hypothesized double-mutant phenotypic class (18.75% for single mutant, 6.25% for double mutant). Significant deviation for 3 degrees of freedom is >7.815.

either repression or activation of Wg target genes, depending on its binding partners (Cavallo et al., 1998). Surprisingly, not only was the *SoxN* mutant phenotype epistatic to *Tcf* loss of function, but loss of *Tcf* enhanced the naked cuticle specification in *SoxN* mutant embryos. *SoxN*; *Tcf* double mutants showed more extensive naked cuticle than did the *SoxN* single mutant (Fig. 3H, Fig. 2B, Table 2). These data suggest that the repressive form of Tcf may act synergistically with *SoxN* to downregulate Wg pathway activity. Consistent with this idea, we found that reducing the dose of *gro* also enhanced the naked cuticle specification in *SoxN* mutant embryos (Fig. 2B, Fig. 3G). This effect was only observed when the *gro* allele was introduced from the mother. Previous work has shown that *gro* suppression of *wg* loss of function is also strictly a maternal phenotype (Cavallo et al., 1998). Conversely, reducing maternal *gro* reduced the severity of pattern disruptions caused by ectopic *UAS-SoxN* expression (Fig. 2F, Fig. 3D). Thus, maternally provided Gro⁺ functions as a Wg target co-repressor whether or not the *SoxN* gene product is present, and appears to increase the repressor activity of *SoxN* in a dose-dependent fashion.

Genetic interaction of *SoxN* and *Tcf*

We explored the interaction of *SoxN* and *Tcf* further by testing the effects of *SoxN* on the dominant-negative *Tcf* molecule. The *Tcf^{DN}* transgene expresses the DNA-binding portion of Tcf, but lacks the Arm-binding domain that would allow it to switch from repressor to activator (van de Wetering et al., 1997; Cavallo et al., 1998). Therefore, it strongly and constitutively represses Wg target gene expression. Lowering the dose of *SoxN* partially rescues the segmental pattern disruptions caused by *Tcf^{DN}*-transgene

expression, and also substantially rescues head cuticle defects and increases the overall size of the body (Fig. 3J,K). This indicates that wild-type SoxN activity contributes to the repressor activity of Tcf in a dose-dependent fashion.

The idea that SoxN interacts with Tcf is further supported by overexpression experiments involving the wild-type forms of both molecules. Overexpressing wild-type *Tcf* enhanced the repressive capacity of overexpressed wild-type *SoxN*. This was particularly obvious when the *UAS* transgenes were driven ubiquitously at lower levels with the *arm-Gal4* driver. Under these conditions, *UAS-Tcf* had no effect on either *en* expression or on cuticle pattern (Fig. 4A,B) and embryos typically hatched and grew to adulthood. *arm-Gal4*-driven *UAS-SoxN* showed an only modest narrowing of *en* expression domains (Fig. 4C) and few ectopic denticles (Fig. 4D). When both transgenes were driven simultaneously, *en* expression was more dramatically narrowed (Fig. 4E) and the cuticle pattern was more disrupted, both ventrally and dorsally (Fig. 4F). By calculating the rates of embryonic lethality in the transgenic crosses, we found that the synergy between *SoxN* and *Tcf* cannot be explained as simple additivity (Table 1). With either the *arm-Gal4* or *E22C-Gal4* embryonic drivers, fewer embryos co-expressing *SoxN* and *Tcf* survived than those where *SoxN* alone is overexpressed. With the potent *E22C-Gal4* epidermal driver, the embryonic lethality rate of double-transgenic embryos approached the rate of embryos expressing dominant-negative *Tcf*. Co-expression of *Tcf* with *SoxN* also affected adult patterning. The expression of either transgene individually in the imaginal disc with *dpp-Gal4* had modest effects on adult eclosion rates. Combining the transgenes produced pupal lethality as profound as that of the dominant-negative *Tcf* (Table 1).

These co-expression results argue against a simple model in which SoxN downregulates target expression by competing with Tcf for Tcf-binding sites. In this simple model, overexpressing wild-type Tcf should reduce the severity of ectopic wild-type SoxN; however, instead we observed an increase in severity. Furthermore, SoxN did not appear to act by sequestering Arm away from Tcf. Co-expressing *arm* with *UAS-SoxN* did not affect the *SoxN* overexpression phenotype ($n=467$), nor did reducing maternal *arm* dose ($n=553$). Likewise, we observed no evidence of *arm* dosage effects on the *SoxN* mutant phenotype (Table 2).

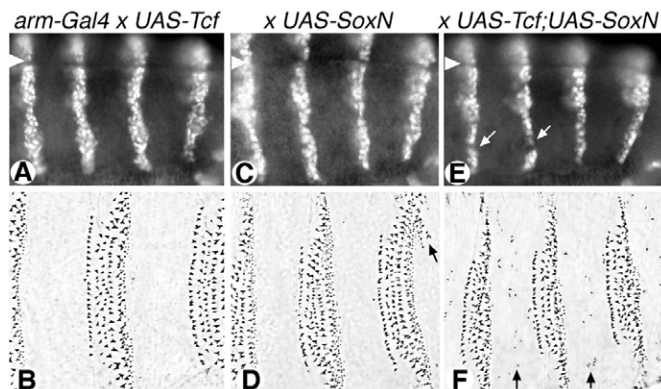


Fig. 4. *Tcf* acts synergistically with *SoxN*. (A,B) Overexpressing the wild-type form of *Tcf*, using the *arm-Gal4* driver, produces no effect on *en* expression (A) or on cuticle pattern (B), and most such embryos hatch into larvae (data shown in Table 1). (C,D) *arm-Gal4* drives a lower level of expression than *E22C-Gal4*, producing milder effects of overexpressed *SoxN*. Under these conditions, *en* expression is only slightly narrowed (C) and cuticle pattern is mostly normal (D), with only occasional ectopic denticle formation (arrow) and little dorsal curvature. (E,F) When *Tcf* and *SoxN* are expressed together, using *arm-Gal4* to drive a double-homozygous *UAS* stock, *en* expression is more severely narrowed (arrows, E), and ectopic denticles (arrows, F) appear with greater frequency and broader distribution across the ventral naked cuticle domain; cuticles are strongly curved due to defective dorsal patterning. The severity of the phenotype is comparable to expressing higher levels of *UAS-SoxN* alone with the stronger *E22C-Gal4* driver (see Fig. 2F,H). Arrowheads in A, C and E indicate the posterior end of the ventral midline. Embryos are oriented with anterior to the left and dorsal side up.

SoxN represses mammalian Wnt signal transduction

To determine whether the relationship between *SoxN* and *Tcf* is conserved in vertebrates, we made use of the TOPflash (Tcf optimal binding sites) reporter system expressed in human embryonic kidney 293T (HEK293T) cells (Korinek et al., 1998; Ishitani et al., 1999). We found that *SoxN* expression reproducibly diminished Tcf-mediated transcription in a dose-dependent fashion (Fig. 5A), comparable with other known negative regulators of Wnt gene expression, such as *gro* (Cavallo et al., 1998). This demonstrates that the fly SoxN protein interacts with vertebrate pathway components to antagonize Wnt-stimulated gene expression in mammalian cells. Similar repression is observed whether TOPflash is activated with Wnt-conditioned medium or by co-transfection with a constitutively active beta-catenin. Thus, artificially elevated beta-catenin levels do not affect Sox-mediated repression.

We tested whether the addition of extra Tcf-binding sites interferes with the SoxN repression of TOPflash-reporter activity. The TOPflash plasmid was altered to delete the luciferase structural gene (Fig. 5B). We made the same change in FOPflash (far from optimal), which has mutated Tcf-binding sites (Korinek et al., 1998;

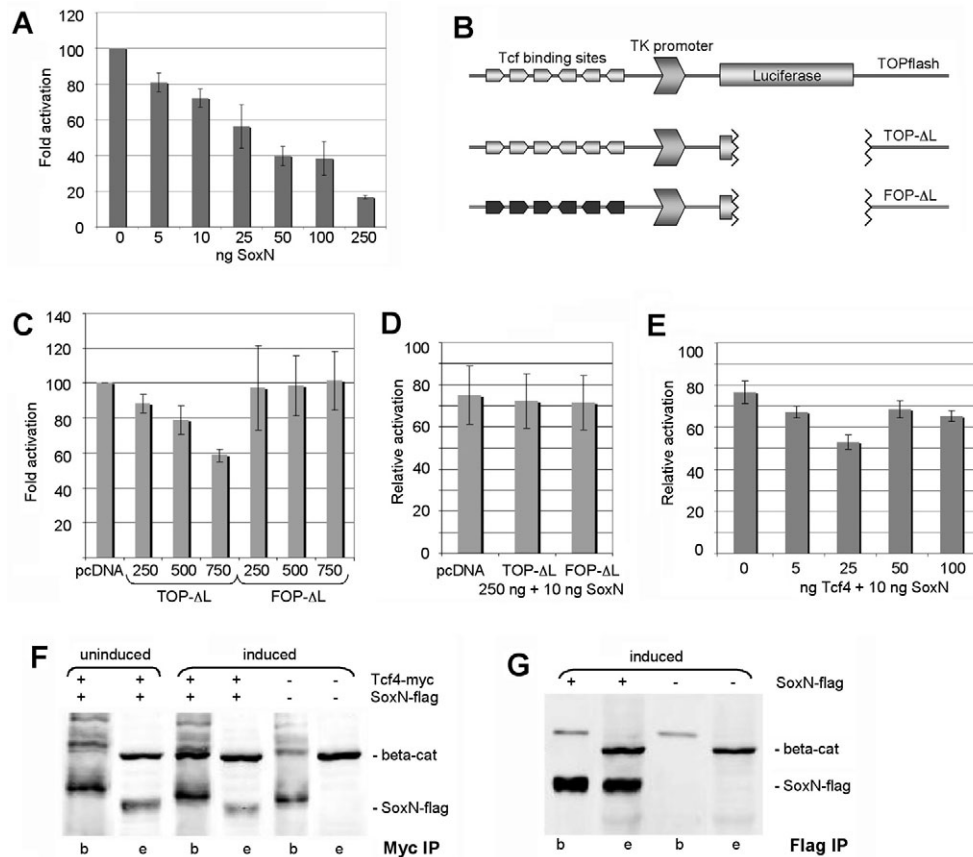


Fig. 5. SoxN represses Wnt signaling in HEK293T cells. (A) Increasing the amount of SoxN decreases the amount of TOPflash activity that is detected. Fold activation is defined as the ratio between relative luciferase units under induced versus uninduced conditions. For comparison between independent experiments, values were normalized to the 0 ng SoxN data point. (B) Schematic diagram of constructs used for Tcf-binding-site competition assay. FOP-ΔL is identical to TOP-ΔL in structure but carries mutated Tcf-binding sites. (C) Increasing amounts of TOP-ΔL, but not of FOP-ΔL, decrease the amount of TOPflash activity that is detected. Fold activation is defined as in A. (D) Extra Tcf-binding sites do not affect SoxN repression of TOPflash activity, expressed as the fold activation with 10 ng pcDNA-SoxNflag/fold activation with 0 ng pcDNA-SoxNflag. Each competitor DNA was present at 2.5× the amount of TOPflash reporter. No statistically significant difference in the degree of repression can be detected. (E) SoxN and Tcf4 at low doses synergistically repress TOPflash activity. Cell cultures containing 10 ng pcDNA-SoxNflag and 0, 5, 25, 50 or 100 ng of pcDNA-Tcf4myc were assayed. Empty pcDNA3.1 vector was used to hold constant the total amount of DNA added. Each bar represents relative activation, defined as fold activation with 10 ng pcDNA-SoxNflag/fold activation with 0 ng pcDNA-SoxNflag. (F) Beta-catenin, but not SoxN, is co-immunoprecipitated with Tcf in TOPflash cell extracts. pcDNA-TCF4myc (0.5 μg) and pcDNA-SoxNflag (0.05 μg) were co-transfected into HEK293T cells and grown under induced and uninduced conditions. Control cultures contained only pcDNA3.1 to reveal non-specific cross-reacting proteins. Cell extracts (e) were subjected to immunoprecipitation using Myc-antibody-conjugated ProtG beads (b). Immunoblot was stained with anti-Flag and anti-beta-catenin. SoxNflag is not detected in the Tcf4-myc-bound fraction under conditions where beta-catenin is found in the induced Tcf4-myc bound fraction (third lane). (G) SoxN and beta-catenin are not co-immunoprecipitated in TOPflash cell extracts. pcDNA-SoxNflag (0.5 μg) was transfected into HEK293T cells and grown under induced conditions. Cell extracts (e) were subjected to immunoprecipitation using flag-antibody-conjugated ProtG beads (b). Immunoblot was stained with anti-flag and anti-beta-catenin. Beta-catenin is not detected in the SoxN-flag-bound fraction (first lane).

Ishitani et al., 1999), in order for it to serve as a negative control. TOP-ΔL competitor DNA, added at levels in excess of 2.5 times the amount of TOPflash, reduced reporter activity, whereas FOP-ΔL competitor did not (Fig. 5C). This indicates that the intact Tcf-binding sites in the TOP-ΔL competitor can titrate endogenous Tcf in HEK293T cells. If SoxN preferentially binds to Tcf consensus sites, we would expect to see a reversal of SoxN-mediated repression with TOP-ΔL but not with FOP-ΔL. To sensitize the assay, we used 10 ng of SoxN, which provides an intermediate level of repression, and added competitor DNA at 2.5 times the amount of TOPflash reporter. Under these conditions, SoxN is limiting, but endogenous Tcf is not substantially compromised. We found that co-transfecting with either plasmid did not affect SoxN repression

in the TOPflash assay (Fig. 5D). This suggests that SoxN does not act by directly competing with Tcf for its consensus binding sequences.

We next asked whether the synergy between Tcf and SoxN observed in fly embryos can be detected in the TOPflash system. Tcf levels were increased by co-transfecting with a wild-type *Tcf4* transgene. We found that small amounts of ectopic Tcf initially potentiated the repression of SoxN, reducing reporter transcription, but, as levels of Tcf increased, the effect diminished (Fig. 5E). This suggests that a balance between Tcf and SoxN, and perhaps other transcriptional components, controls the output of Tcf-responsive promoters. What is not clear is the mechanism by which the potentiation occurs. SoxN and Tcf do not appear to interact with

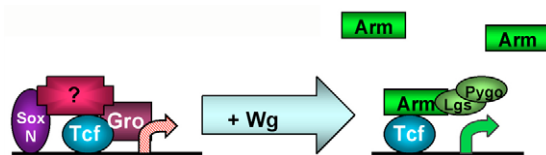


Fig. 6. Model for SoxN interaction with the Tcf-responsive promoter. SoxN may be able to bind DNA sequences adjacent to, or overlapping with, Tcf-binding sites and may contribute to the repressive capacity of Tcf, for example, by helping to recruit an unidentified scaffolding molecule that stabilizes the Tcf-Gro interaction.

each other directly, because they were not co-precipitated in TOPflash cell extracts (Fig. 5F). SoxN also does not appear to bind to beta-catenin, because these proteins did not co-precipitate from TOPflash cells under conditions where beta-catenin is robustly co-precipitated with Tcf (Fig. 5F,G). Co-precipitation experiments also failed to detect physical interaction between SoxN and Gro (data not shown). We propose a model in which SoxN and Tcf may bind to adjacent DNA sequences, and the presence of SoxN increases the efficiency with which the Tcf-Gro repressor complex forms or functions at the promoters of target genes (Fig. 6).

DISCUSSION

Our findings demonstrate that SoxN downregulates the Wg/Wnt pathway to reduce target gene expression. Downregulation is a crucial process because it sensitizes the signal response to allow rapid on/off switching and also keeps the system off in cells that are not actively responding to signal. Many genes have been shown to negatively regulate Wg/Wnt pathway activity through the destabilization of Arm/beta-catenin. Far fewer are known to exert negative regulatory effects downstream of Arm. The vertebrate Sox proteins – Sox9 (Akiyama et al., 2004), XSox3, XSox17 α and XSox17 β (Zorn et al., 1999) – as well as Chibby, a conserved nuclear factor (Takemaru et al., 2003), antagonize Wg/Wnt signaling by binding to Arm/beta-catenin and preventing it from partnering with Tcf to activate target gene expression. SoxN, however, did not bind beta-catenin in cell-culture assays, and does not share strong homology with the C-terminal sequences through which vertebrate Sox proteins bind this protein. Furthermore, we find that SoxN function is not influenced by Arm levels. No difference was observed in SoxN-mediated TOPflash repression when cells were induced by co-transfection with a constitutively stabilized beta-catenin versus with Wnt-induced medium. Instead, both our TOPflash and our genetic experiments indicate that SoxN function depends on Tcf and Gro, its co-repressor.

One way to explain our observations is that SoxN contributes to the assembly or stability of the Tcf repressor complex on DNA (Fig. 6). The consensus-sequence recognition for HMG domains in the Sox and Tcf families is reported to be similar (reviewed in Clevers and van de Wetering, 1997; Kamachi et al., 2000; Wilson and Koopman, 2002), although XSox3 and XSox17 β fail to bind a consensus Tcf DNA sequence (Zorn et al., 1999; Zhang et al., 2003). We show that SoxN does not compete for Tcf-binding sites as a means of repressing target gene transcription, but our data support a model in which SoxN might bind DNA elsewhere or might bind Tcf sites transiently to initiate or stabilize the assembly of a repressor complex.

A similar model may explain the results from *Xenopus* that showed that XSox3-mediated repression does not require interaction between XSox3 and beta-catenin (Zhang et al., 2003). XSox3 strongly interferes with dorsal fate specification in

Xenopus embryos and represses TOPflash-reporter activity in vitro. HMG-domain mutations render XSox3 inactive in embryos without affecting its interaction with beta-catenin or its repression in TOPflash assays. Thus, it is the DNA-binding domain, not the beta-catenin-interacting C-terminus, that is relevant to its in vivo function in dorsal determination in *Xenopus*. XSox3 represses the expression of the dorsal-specific Nodal-related gene *Xnr5* through optimal core binding sequences adjacent to and partially overlapping with Tcf sites in the *Xnr5* promoter (Zhang et al., 2003). By contrast, the fly SoxN shows no discrepancy between its behavior in TOPflash assays and its in vivo effects. This suggests that the synthetic Tcf-binding sites arranged in the TOPflash-reporter plasmid are sufficient to support SoxN repressor function.

Because adding Tcf-site competitor DNA does not diminish the repressive capacity of limiting amounts of SoxN (Fig. 5A-D), the role of SoxN in repression does not appear to be stoichiometric. Therefore, we favor the idea that Sox proteins may act in a catalytic fashion during repressor-complex assembly at Wnt target gene promoters, rather than forming a structural part of the repressor complex itself. We have been unable to detect direct binding of SoxN with either Tcf, Gro or Arm, raising the possibility that SoxN interacts with some as yet unidentified protein that chaperones assembly of the repressor complex. A SoxN-binding cofactor, SNCF, was previously identified in *Drosophila* (Bonneaud et al., 2003), but this gene is expressed only in pre-gastrulation embryos. Because Wg signaling occurs exclusively post-gastrulation, and specification of naked cuticle begins more than 4 hours after gastrulation (Bejsovec and Martinez Arias, 1991), we do not believe that SNCF is a likely candidate for mediating this aspect of SoxN function. Rather, it is likely to play a role in the neuronal specification events promoted by SoxN at earlier stages of embryogenesis.

We find it curious that uniformly overexpressed *SoxN* represses Wg signal transduction in dorsal epidermal cells more severely than in ventral cells. This effect is evident in both cuticle pattern elements and in *en* expression, and is reminiscent of defects observed in the ‘transport-defective’ class of *wg* mutant alleles, which includes *wg^{NE2}*. These mutations restrict Wg-ligand movement ventrally to promote only local signaling response while simultaneously abolishing all dorsal signaling (Dierick and Bejsovec, 1998), suggesting a fundamental difference in ventral and dorsal cell response. Perhaps it is not a coincidence that the *NC14* mutation was isolated in the *wg^{NE2}* mutant background. Further analysis of *SoxN* function may help us to determine the molecular basis for dorsoventral differences in Wg signal transduction.

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Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/134/5/989/DC1>

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