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

Anna T. Chao, Whitney M. Jones and Amy Bejsovec*

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; GSK3β 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 GSK3β, 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).
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 chordogenesis (reviewed in Kamachi et al., 2000; Wilson and Koopman, 2002). In addition, Xenopus X Sox3, as well as X Sox17α and X Sox17β (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 X Sox3 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 N C14 are from the Bloomington Stock Center. w^g^{E22} is a missense mutation that disrupts cell-to-cell movement of Wg ligand without abolishing its secretion or signaling capacity (Dierick and Bejsovec, 1998). w^g^{CX4} is an RNA-null allele (Baker, 1987). Existing SoxN mutations (sox^NC14, sox^NC40/47 and sox^NC21/89) 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 Watering et al. (van de Watering 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. 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^g^{A4} allele produces a ‘lawm of denticles’ phenotype (C), which is partially suppressed by the SoxN^NC14 mutation (D). (E) wg^g^{A4} mutant embryos lose epidermal expression of the Wg target gene en before stage 10 (compare with wild-type pattern in Fig. 2G). (F) wg^g^{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.

**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^G11192 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^G11192 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^582 mutant homozygotes. Embryos are oriented with anterior to the left and dorsal side up.

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transgene expression in a stripe along the anteroposterior boundary of each imaginal disc (Johnston and Schubiger, 1996). Epistasis experiments were performed with the strong arm7 allele (arm7D53) and were verified with the weaker arm8 (armH8.1) allele (Peifer and Wieschaus, 1990) and the strong Tc2 allele (van de Wetering et al., 1997). The gro deficiency gro^{Rx22} (Cavallo et al., 1998) is also known as Df(3R)Exel22.

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 D2-3 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 glutamate 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-catΔphans were gifts from P. Casey (Duke University Medical Center, Durham, NC). TOP-ΔL. and TOP-ΔL. were generated by deleting the 1.681 kb Xbal 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 μg), 0.25 ng pBHR-G (Promega) internal control and a total of 0.5 μg expression plasmid was used for each transfection with lipofectamine2000 (Invitrogen). TOPFlash expression was induced by co-transfection with 0.25 μg of pcDNA-beta-catΔphans 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 μl ProtG beads (Zymed) with 1 μl 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^{NC14} 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 mutation with respect to wg and also to generate recombinant chromosomes that bear the NC14 mutation without the wg^{NC14} 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 recombinated the NC14 mutation onto a chromosome bearing a null allele of wg, wg^{NC14}, 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^{NC14}, 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^{NC14}, gro and wg^{NC14}, 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 SoxNNC14 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 SoxNNC14 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 SoxNNC14/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 groBGG2, a deficiency removing the locus, produces milder pattern disruptions both dorsally and ventrally (compare with Fig. 2F). (E, F) Double homozygotes for arm2 and either SoxN allele show the arm ‘lawn of denticles’ phenotype (E), but embryos are smaller and have stronger dorsal pattern disruptions than do arm2 single mutants (F) (data shown in Table 2). (G) Mutants homozygous for SoxN that were derived from groBGG2 heterozygous mothers show increased naked cuticle (n=140). (H, I) SoxN; Tcf 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 TcfPN-expressing embryos are partially rescued when SoxN dosage is reduced. The SoxNNC14 mutation is linked to the E22C-Gal4 insertion in this experiment, so that all embryos ubiquitously expressing UAS-TcfPN 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 SoxNNC14. 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-LaGraff 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 SoxNNC14 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 SoxNNC14 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
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 harvested 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 SoxNNGA1192 and SoxNGA1192; Tcf2/+; arm4/FM7, a previously isolated protein-null allele (Buescher et al., 2002). SoxNNGA1192 in trans with the deficiency produces the same extent of excess naked cuticle as does SoxNNC14 in trans with the deficiency, indicating similar allele strength (see Fig. S1 in the supplementary material). We routinely show SoxNNC14 because this mutant chromosome was extensively recombined to remove extraneous mutations. During random EMS mutagenesis, such as that in which the NCI4 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 SoxNNC14 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 SoxNNC14 and SoxNNGA1192 (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

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

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<th>Transgene</th>
<th>UAS-SoxN</th>
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<td>Embryonic lethal (%)</td>
<td>Pupal lethal (%)</td>
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<td>E22C-Gal4</td>
<td>dpp-Gal4</td>
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<td>n</td>
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<td>99.6</td>
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<td>UAS-Tcf DN</td>
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**Table 2. Epistasis analysis of SoxN**

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<th>Wild type (%)</th>
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<th>Wild type (%)</th>
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</table>

For each SoxN allele, non-balancer F₁ female progeny from arm¹/FM7 × SoxN/CyO were crossed to the deficiency stock +/Y; Df(2L)Exel7040/CyO; non-balancer F₁ progeny from Tcf²/Y × SoxN/CyO were crossed to each other. All cuticle 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 TcfDN 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 TcfDN 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-expression of 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).

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 POPFlash (far from optimal), which has mutated Tcf-binding sites (Korinek et al., 1998;
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
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 indicated that the 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. Fewer are known to exert negative regulatory effects downstream of Arm. The vertebrate Sox proteins – Sox9 (Akiyama et al., 2004), XSox3, XSox17α and XSox17β (Zorn et al., 1999) – as well as Chibby, a conserved nuclear effector (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 Clevets and van de Wetering, 1997; Kamachi et al., 2000; Wilson and Koopman, 2002), although XSox3 and XSox17β 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-terminal, 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 (Bejszovec 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.


