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Spenito and Split ends act redundantly to promote Wingless signaling

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Abstract

Wingless (Wg)/Wnt signaling directs a variety of cellular processes during animal development by promoting the association of Armadillo/β-catenin with TCFs on Wg-regulated enhancers (WREs). Split ends (Spen), a nuclear protein containing RNA recognition motifs (RRMs) and a SPOC domain, is required for optimal Wg signaling in several fly tissues. In this report, we demonstrate that Spenito (Nito), the only other fly protein containing RRMs and a SPOC domain, acts together with Spen to positively regulate Wg signaling. The partial defect in Wg signaling observed with *spen* RNAi was enhanced by simultaneous knockdown of *nito* while it was rescued by expression of *nito* in wing imaginal discs. In cell culture, depletion of both factors causes a greater defect in the activation of several Wg targets than RNAi of either *spen* or *nito* alone. These nuclear proteins are not required for Armadillo stabilization or the recruitment of TCF and Armadillo to a WRE. Loss of *Wg* target gene activation in cells depleted for *spen* and *nito* was not dependent on the transcriptional repressor Yan or Suppressor of Hairless, two previously identified targets of Spen. We propose that Spen and Nito act redundantly downstream of TCF/Armadillo to activate many Wg transcriptional targets.

Keywords: Drosophila; Wingless; Wnt; Split ends; Spenito; SHARP; Mint; OTT; RBM-15

Introduction

The *Drosophila* gene *wingless* (*wg*) encodes a member of the Wnt family of secreted and palmitoylated glycoproteins that are conserved throughout the animal kingdom (Cadigan and Nusse, 1997; Clevers, 2006; Mikels and Nusse, 2006). Wg influences cell behavior through stabilization and nuclear translocation of Armadillo (Arm), known as β-catenin in vertebrates (Kikuchi et al., 2006). This Wnt/β-catenin pathway controls numerous cell fate decisions in development and adult tissues (Logan and Nusse, 2004). Misregulation of this signaling cascade leads to a variety of diseases including several forms of human cancer (Logan and Nusse, 2004; Clevers, 2006; Johnson and Rajamannan, 2006).

In the absence of Wg/Wnt stimulation, cytosolic Arm/β-catenin is constitutively phosphorylated by the coordinated action of a group of proteins including the scaffolds Axin and adenomatous polyposis coli protein (APC), as well as casein kinase I and glycogen synthase kinase-3 (GSK-3). Phosphorylated Arm/β-catenin is then ubiquitinated and degraded by the proteosome (Kikuchi et al., 2006). Wg/Wnt signaling inhibits this process (Cadigan and Liu, 2006), leading to the accumulation and nuclear translocation of Arm/β-catenin. Once in the nucleus, Arm/β-catenin associates with members of the TCF/Lef1 (TCF) family of DNA-binding proteins. Arm/β-catenin converts TCFs from transcriptional repressors to activators in part by displacing the transcriptional corepressor Groucho (Gro; Städeli et al., 2006; Parker et al., 2007).

Several positive regulators of TCF/Arm/β-catenin-mediated transcription have been identified (Willert and Jones, 2006; Städeli et al., 2006; Parker et al., 2007). One of the potential positive factors is Split ends (Spen), a nuclear protein that is required for Wg signaling (Lin et al., 2003). The *spen* gene encodes several large isoforms (>5500 amino acids) that contain three RNA recognition motifs (RRMs) in their N-terminal portions and a Spen paralog and ortholog C-terminal (SPOC) domain at their C-termini (Wiellette et al., 1999; Rebay et al., 2000; Kuang et al., 2000). *spen* is required for optimal Wg signaling in several imaginal discs in fly larvae, but Wg

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signaling occurs normally in embryos lacking maternal and zygotic *spen* activity (Lin et al., 2003). Spen appears to be a tissue-specific positive regulator of the Wg pathway.

SHARP, the human ortholog of *Spen*, was recently shown to be required for Wnt/β-catenin signaling in several human cell lines (Feng et al., 2007). SHARP could potentiate the ability of Lef1 (a vertebrate TCF) to activate reporter genes independently of β-catenin (Feng et al., 2007). This suggests that SHARP promotes Lef1 DNA binding or acts through a transcription activation mechanism that does not require β-catenin.

In addition to Wg signaling, Spen has been implicated in several other processes in flies. In the developing eye, *spen* is required to inhibit Notch signaling and promote epidermal growth factor (EGFR)/Ras/MAP kinase (MAPK) signaling (Doroquez et al., 2007). In addition, loss of *spen* results in increased levels of the ETS-domain transcriptional repressor Yan (Doroquez et al., 2007), an antagonist of EGFR signaling (Rebay and Rubin, 1995). *spen* is also required for Hox-mediated repression of head identity in the embryonic trunk (Wiellette et al., 1999), specification, survival and axonal guidance of specific neurons (Chen and Rebay, 2000; Kuang et al., 2000), cell cycle regulation (Lane et al., 2000), and epidermal wound repair (Mace et al., 2005). The molecular mechanisms underlying the pleiotropic phenotype of *spen* mutants have not been elucidated.

In mammals, SHARP (known as Mint in the mouse) has been shown to act as a corepressor for nuclear hormone receptors (Shi et al., 2001) and RBP-Jκ, a DNA-binding protein critical for Notch signaling (Oswald et al., 2002, 2005; Kuroda et al., 2003; Tsuji et al., 2007; Yabe et al., 2007). The SPOC domain appears to be essential for the ability of Spen proteins to repress transcription and can physically interact with various proteins including the universal corepressor SMRT/NCoR, histone deacetylases, the CtIP/CtBP corepressors and the E2 ubiquitin-conjugating enzyme UbcH8 (Shi et al., 2001; Ariyoshi and Schwabe, 2003; Oswald et al., 2005; Li et al., 2006). In contrast to these repression roles, Mint has been shown to act with Runx2 to activate *osteocalcin* (*OC*) transcription, possibly through specific DNA binding to a G/T-rich element in the *OC* control region mediated by its RRMs (Newberry et al., 1999; Sierra et al., 2004).

The Spen family is defined by the presence of N-terminal RRMs and a C-terminal SPOC domain (Wiellette et al., 1999; Kuang et al., 2000). In addition to Spen and its homologs, there are several smaller (<1000 aa) family members that include Spenito (Nito) in flies (Rebay et al., 2000), as well as one-twenty-two/RNA-binding motif protein 15 (OTT1/RBM-15) in mammals. OTT1/RBM-15 (referred to as OTT1 hereafter) was originally identified as a fusion protein at a recurring chromosomal translocation in megakaryocytic acute leukemia (Ma et al., 2001; Mercher et al., 2001).

Although they share similar domains, several reports indicate that the smaller Spens are functionally distinct from their larger relatives. Like, SHARP/Mint, OTT1 can bind to RBP-Jκ and repress Notch signaling, but in some cell types, it activates the pathway (Ma et al., 2007). Even more dramatic differences were noted in the fly, where *nito* was shown to act antagonistically with *spen* in the developing eye (Jemc and Rebay, 2006).

In this report, we demonstrate that Nito is a physiological regulator of Wg signaling. In contrast to other processes where they act antagonistically, Spen and Nito act redundantly to promote Wg signaling in flies and cultured cells. Simultaneous depletion of both genes results in a more severe loss of Wg activation of target genes than either gene alone and expression of *nito* can rescue the defect in cells with reduced *spen*. Consistent with their nuclear localization, Spen and Nito act downstream of Arm stabilization/nuclear translocation but WRE recruitment of TCF and Arm are unaffected in *spen*, *nito* depleted cells. Spen and Nito do not appear to act on Wg targets through regulation of the transcriptional repressor Yan or suppressor of Hairless (Su(H)), the fly homolog of RBP-Jκ. However, loss of the Wg target *naked cuticle* (*nkd*) in *spen* and *nito* RNAi-treated cells was reversed by depletion of *groucho* (*gro*). This interaction between *gro* and *spen–nito* was not seen at another Wg target, and some targets of the pathway are independent of *spen* and *nito*. Thus, these factors act redundantly downstream of TCF-Arm complex formation to regulate Wg targets in a gene-specific manner.

Materials and methods

Fly strains

The P[*da-Gal4*], P[*arm-Gal4*], P[*GMR-Gal4*], P[*UAS-wg*], P[*GMR-Arm**], and P[*UAS-EGFR^{DN}*] stocks are previously described (Lin et al., 2003). The P[*GMR-hid^{l0}*], P[*GMR-rpr^{2}*] stocks and P[*ptc-Gal4*], P[*dpp-lacZ*] and P[*UAS-lacZ*] strains are previously described (Guan et al., 2007; Li et al., 2007). P[*UAS-yan*] (Rebay and Rubin, 1995) was obtained from the Bloomington Stock Center.

P[*UAS-spen^{RNAi}*] and P[*UAS-nito^{RNAi}*] (*UAS-nito^{RNAi}-2*) strains were constructed as follows. *spen* was amplified by PCR with primers 5′ CATGTCTAGACTTACCAGGCCATCAACT CATC3′ and 5′CATGTCTAGAGAATAACCTGCGAGGCGTTTCC3′ and *nito* with 5′CATGTCTAGACAATGTCCCCACCGGACTATGA3′ and 5′CATGTCTAGACTCCGTATT TGCCAAAGATG CGA3′ (*Xba*I sites used for cloning are underlined). The regions targeted in each gene are indicated in Fig. 1A. The amplified PCR products were cut with *Xba*I and ligated into the *Avr*II and *Nhe*I ends of the pWIZ vector in opposite directions (Lee and Carthew, 2003). The P[*UAS-nito*] strains were generated by amplifying the *nito* ORF from cDNA RE36227 (obtained from *Drosophila* Genomics Resource Center at Indiana University) with primers 5′CAGGAATTCATGAGTAGTCATCGAGACGGAG CCGGA3′ (*Eco*RI site is underlined) and 5′TGTGGTACCTCAGGCCGTTCC-GCC-GCGCACCACC-A3′ (*Kpn*I site is underlined) and was ligated into the *Eco*RI and *Kpn*I sites in *pUAST* (Brand and Perrimon, 1993). Transgenic flies were generated following the standard P element methodology using *w^{1118}* embryos. An additional P[*UAS-nito^{RNAi}*] line (*UAS-nito^{RNAi}-1*) targeting a non-overlapping region of *nito* transcripts than *UAS-nito^{RNAi}-2* (see Fig. 1A) was obtained from Ilaria Rebay (Jemc and Rebay, 2006).

Whole-mount staining and microscopy

In situ hybridization and immunostaining were performed as previously described (Lin et al., 2004). The probe against *nito* was generated using a PCR product from genomic DNA with the following oligos: 5′GGTGG-CTATTCTCCGTATCCACCTA3′ and 5′GTAATACGACTCACTATAGGGC-GACCACAATCACCAGGTGATCCTCCTT3′. Guinea pig anti-Sens antibody (1:1000) was described previously (Fang et al., 2006). Mouse monoclonal Wg antibody (4D4; 1:100) was from the Developmental Studies Hybridoma Bank (University of Iowa). Cy3-conjugated antibodies and Alexa Flour 488-conjugated antibodies were purchased from Jackson Immunochemicals (West