spenito is required for sex determination in *Drosophila melanogaster*

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***Sex-lethal* (*Sxl*) encodes the master regulator of the sex determination pathway in *Drosophila* and acts by controlling sex identity in both soma and germ line. In females Sxl maintains its own expression by controlling the alternative splicing of its own mRNA. Here, we identify a novel sex determination gene, *spenito* (*nito*) that encodes a SPEN family protein. Loss of *nito* activity results in stem cell tumors in the female germ line as well as female-to-male somatic transformations. We show that Nito is a ubiquitous nuclear protein that controls the alternative splicing of the *Sxl* mRNA by interacting with Sxl protein and pre-mRNA, suggesting that it is directly involved in Sxl auto-regulation. Given that SPEN family proteins are frequently mutated in cancers, our results suggest that these factors might be implicated in tumorigenesis through splicing regulation.**

germ-line stem cell | sex determination | alternative splicing

Sex determination in *Drosophila* is under the control of the master regulatory gene *Sex-lethal* (*Sxl*) (1). *Sxl* acts downstream of the X-chromosome counting mechanism and encodes a female-specific RNA binding protein. Once activated, Sxl maintains its own expression by regulating the alternative splicing of its pre-mRNA. Sxl controls female fate by controlling somatic and germ-line sex identity as well as dosage compensation (2). In female somatic cells, Sxl controls the alternative splicing of *transformer* (*tra*), which together with *transformer2* (*tra2*) controls the alternative splicing of *doublesex* (*dsx*) and *fruitless* (*fru*). *dsx* and *fru* in turn encode sex-specific transcription factors that control male versus female morphology, physiology, and behavior (3, 4). In addition, Sxl represses the male-specific dosage compensation system by regulating *male-specific lethal 2* (*msl-2*) both at the level of alternative splicing and translational control (5).

In the female germ-line Sxl regulates sex identity by a different mechanism, as *tra*, *tra2*, *msl-2* have no roles in the germ line (2). In the ovary, germ-line stem cells (GSCs) located at the anterior tip of the germarium divide to produce another GSC and a cystoblast (CB) that is committed to differentiate. Sxl protein accumulates to high levels in the GSCs/CBs and is required for the proper differentiation of the germ cells (6). Germ cells lacking Sxl cannot differentiate and instead produce stem cell tumors. The identity of Sxl target genes in the germ line is not well characterized; however, a recent study indicates that *nanos*, a gene required for GSC maintenance, is a Sxl target (7). Indeed, Sxl has been proposed to promote the differentiation of GSCs by downregulating Nanos levels in CBs by binding to the *nanos* 3′ UTR (7). In addition, Sxl is also important for repressing the expression of testis-specific genes, including *Phf7*, a male germ-line identity gene (8). In the absence of Sxl, *Phf7* is mis-expressed leading to germ-line tumors (9).

Sxl does not act alone to control splicing. Several genes, including *sans fille* (*snf*), *virilizer* (*vir*), *female-lethal-2-d* (*fl(2)d*), *SPF45*, *U1-70K*, *U2af38*, *U2af50*, and *protein partner of sans-fille* (*pps*) facilitate Sxl splicing autoregulation (10–18). Except for *pps*, these genes encode either general splicing factors or proteins associated with spliceosomes. They all act to maintain the Sxl autoregulatory splicing loop by interacting with Sxl itself. In addition, some of them are involved in the splicing of other Sxl splicing targets such as *tra* or *msl-2* (1). Interestingly, these genes have essential functions besides *Sxl* regulation and null mutations are associated with zygotic lethality in both sexes. Therefore, the roles of these factors in sex determination were revealed from genetic interactions (*snf*, *U1-70K, U2af38*) (11, 17), temperature-sensitive mutation (*vir*) (15), clonal analysis (*fl(2)d*) (10), or biochemical studies (*SPF45, U2af50* and *pps*) (12, 14, 17).

Here, we characterize *spenito* (*nito*), a novel regulator of Sxl, which is required to maintain sex identity and Sxl levels in both the female germ-line and somatic tissues. Nito is required for the proper alternative splicing of the *Sxl* pre-mRNA in both germ line and soma, and forms a complex with Sxl protein and its pre-mRNA, thus identifying an important component of the sex determination pathway.

Results

nito Is an Essential Gene Required for Ovarian GSC Differentiation.

nito was identified from our previous RNAi screen in *Drosophila* GSCs (19). Specifically, RNAi knockdown of *nito* driven by the germ-line-specific *MTD-Gal4* driver resulted in complete sterility in females. In wild-type ovarioles, two or three GSCs are located in the anterior tip of the germarium (Fig. 1 *A–B*′). Strikingly, *nito* shRNA ovarioles are filled with undifferentiated stem-cell-like cells, and nurse cells and oocytes are not formed (Fig. 1 *C* and *C*′, compared with WT in Fig. 1 *B* and *B*′). Further, stem-cell-like cells associated with *nito* shRNA ovaries retain their proliferative potential as shown by staining with the mitotic marker phosphorylated histone H3 (pH3) (Fig. 1*E*, compared with WT in Fig. 1*D*). Note that the same stem-cell tumor phenotype was observed with three independent *nito* shRNAs and two long dsRNA RNAi lines (*Methods*) (Fig. S1 *A–B*′), indicating that *nito* is an essential gene required for GSC differentiation.

nito Is Required for Sex Determination in the Soma.

Because the germ-line phenotype of *nito* could reflect perturbations in a number of developmental processes affecting either germ-line

Significance

Sex determination is a fundamental biological problem faced by all metazoans. To understand the sex determination pathway, it is important to identify all the genes involved in this process. In this study, we have identified a novel gene, *spenito* (*nito*), which is required for sex determination in *Drosophila melanogaster*. Loss of *nito* function in the soma transforms female tissues to male, and loss of *nito* function in female germ-line stem cells changes their sexual identity and prevents them from proper differentiation. We show that *nito* is a cofactor for *Sex-lethal* (*Sxl*) auto regulation, a process that remains an important textbook model for regulated alternative splicing.

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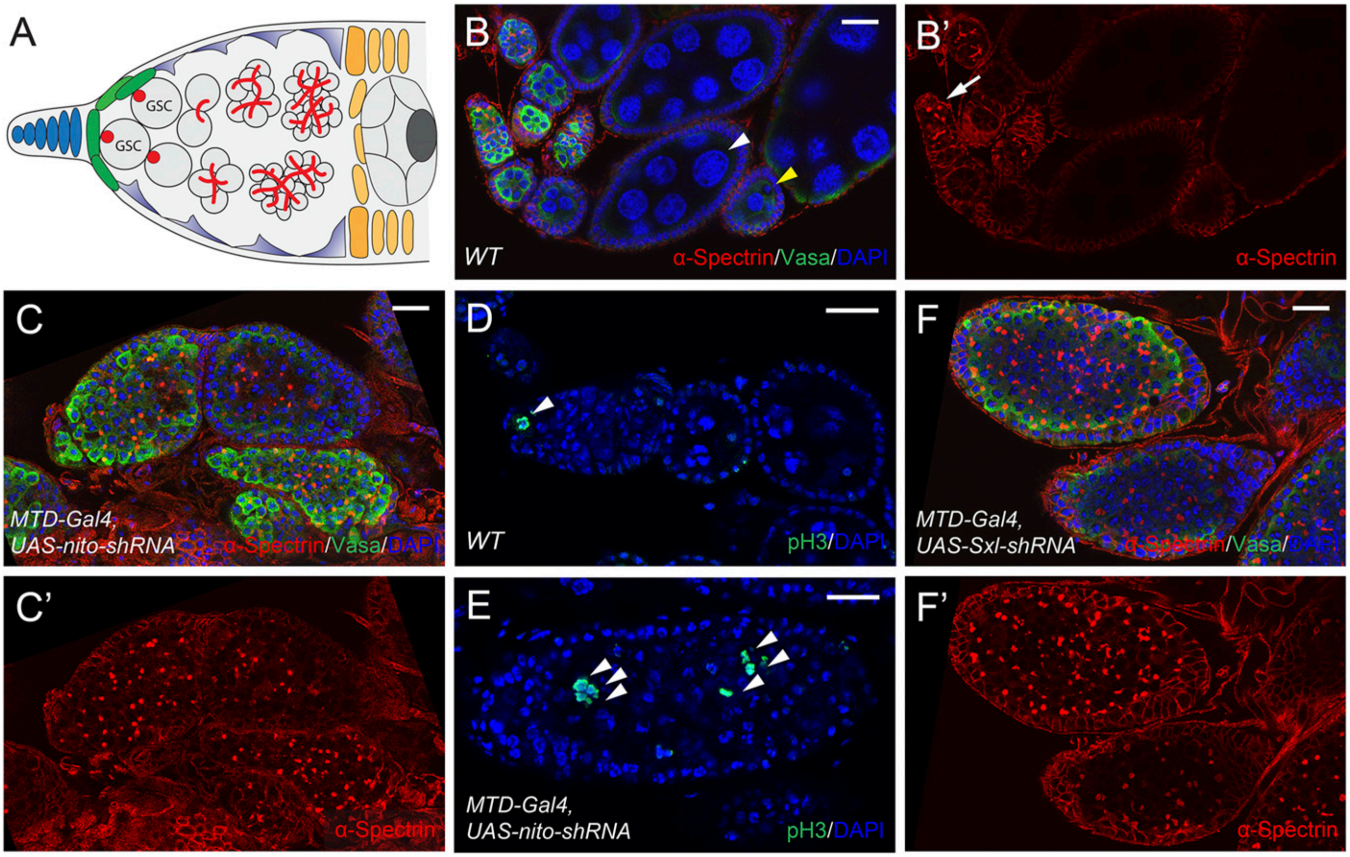

Fig. 1. Nito is essential for ovarian GSC differentiation. (*A*) Diagram showing the structure of a wild type germarium. (*B*–*B*′) WT ovarioles stained for α-Spectrin, Vasa and DAPI. The α-Spectrin antibody labels the round spectrosomes in GSCs (arrow); the Vasa antibody labels all germ cells and DAPI labels nuclei to monitor oocyte (yellow arrowhead) and nurse cell formation (white arrowhead). (*C*–*C*′) Egg chambers expressing *nito* shRNA by *MTD-Gal4* were stained for α-Spectrin, Vasa and DAPI. Note the numerous stem-cell-like cells labeled by α-Spectrin and the absence of differentiated nurse cells. (*D*–*E*) pH3 staining in WT egg chambers and egg chambers expressing *nito* shRNA. In WT egg chambers, pH3-positive cells were restricted to the anterior tip of the germarium but were detected throughout *nito* shRNA egg chambers (arrowheads). (*F*–*F*′) Egg chambers expressing *Sxl* shRNA stained for α-Spectrin, Vasa and DAPI. (Scale bars: 20 μm.)

proliferation or differentiation, we examined *nito* loss-of-function phenotypes in somatic tissues. Strikingly, expression of *nito* shRNA using *dome-Gal4*, that drives expression in both the leg and genital discs (Fig. 2 *A* and *B*), led to the transformation of female tissues into that of males. This is evidenced by the appearance of dark, thickened bristles, the male sex combs, in the forelegs of *nito* shRNA females (Fig. 2*E*, compare with WT in Fig. 2 *C* and *D*). This phenotype is almost fully penetrant and occurs in 97% ($n = 78$) of females examined. In addition, there are strong abnormalities in the genitalia of these female flies. First, a rotation defect has occurred in 71% ($n = 78$) of *dome-Gal4/nito-shRNA* females (Fig. 2*H*). Second, typical female external structures, such as vaginal bristles (Fig. 2*G*, white arrow), are absent in the genitalia (Fig. 2*H*). Third, structures resembling those of males, such as penis apparatus and claspers can be identified (Fig. 2 *F* and *H*). These transformations suggest that Nito is a component of the *Drosophila* sex determination pathway in the soma. Because *Sxl* shRNA generates a stem-cell-tumor phenotype in the germ line similar to that of *nito* (Fig. 1 *F* and *F*′), the *nito* germ-line phenotype therefore could be due to sex determination defects associated with Sxl (see below).

Nito Is a Ubiquitously Expressed Nuclear Protein That Is Crucial in Both Sexes. Nito, together with Split ends (Spen), are members of the SPEN protein family characterized by three N-terminal RNA recognition motifs (RRMs) and a C-terminal SPOC (Spen paralog and ortholog C-terminal) domain (Fig. 3*A*) (20, 21). To analyze Nito expression, we raised a polyclonal antibody against a 22 amino acid peptide (*Methods*). A Western blot showed that this antibody recognizes a protein of the expected ∼89 kDa size in *Drosophila* S2 cell lysates (Fig. 3*C*). Nito is ubiquitously expressed in all tissues examined, including imaginal discs and ovaries, and localizes to the nucleus (Fig. 3 *D* and *F*). Furthermore, expression of *nito* shRNA using *ap-Gal4* led to almost complete depletion of the Nito protein in the dorsal half of wing discs demonstrating the specificity of the antibody (Fig. 4*C* and Fig. S2 *A* and *B*).

Because *nito* affects sex determination, we tested whether its expression level is biased in females versus males. To exclude the maternal contribution from ovaries, we compared *nito* mRNA levels in wing discs. As shown in Fig. 3*B*, *nito* mRNA levels were similar in female and male wing discs. In addition, Nito antibody staining showed similar protein levels in female and male discs (Fig. 3 *D* and *E*). Further, Nito is not regulated by Sxl as its protein level is not affected in *Sxl* RNAi discs (Fig. 3 *G* and *G*′). Together, these data indicate that *nito* is not differentially expressed in males versus females.

We generated a null allele of *nito* by imprecise P-element excision (referred to as *nito*^{1}) to test whether *nito* is an essential gene. *nito*^{1} homozygous animals die during larval stages and homozygous mutant clones show the absence of Nito protein indicating that *nito*^{1} is a null mutation (Fig. 3 *H* and *H*′). Interestingly, *nito*^{1} causes lethality in both females and males, indicating that *nito* is an essential gene. Further, *nito*^{1} is lethal over

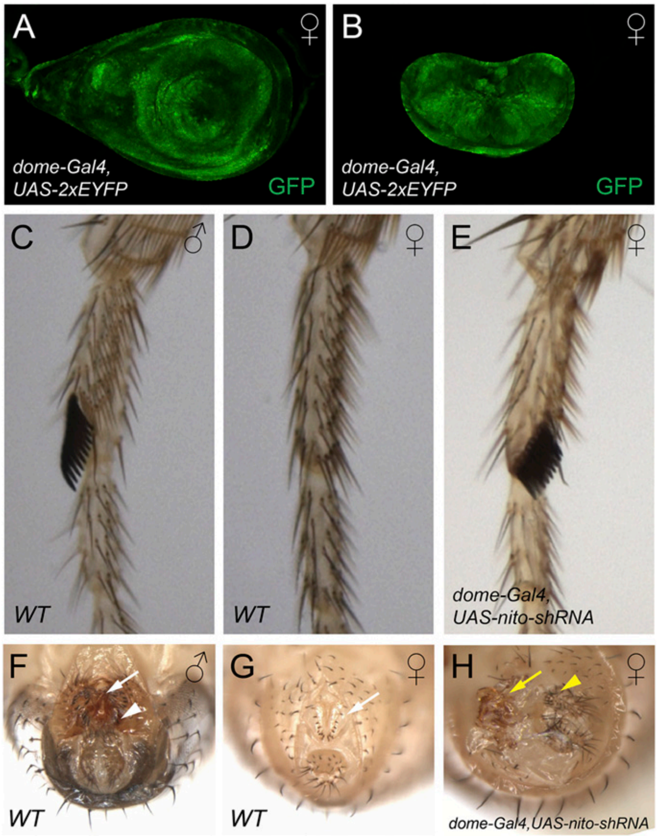

Fig. 2. Nito is required for sex determination in somatic tissues. (*A* and *B*) *dome-Gal4* drives expression in the first pair of leg discs (*A*) and genital discs (*B*), as shown by *UAS-2xEYFP*. (*C*) Foreleg of a WT male with the dark thickened sex comb bristles. (*D*) Foreleg of a WT female. Note the absence of sex combs. (*E*) Foreleg of a female fly expressing *nito* shRNA driven by *dome-Gal4*. Some bristles are transformed into male sex combs. (*F* and *G*) Genitalia of wild-type male (*F*) and female (*G*) flies showing distinct morphology, such as penis apparatus and claspers in male (F, arrow and arrowhead, respectively) and vaginal bristles in female (*G*, arrow). (*H*) *nito* shRNA driven by *dome-Gal4* transforms female genital morphology into male-like, as evidenced by the absence of vaginal bristles and appearance of structures resembling penis apparatus (arrow) and claspers (arrowhead).

a deficiency of the *nito* locus indicating that the lethality is likely due to the *nito* mutation. Consistent with this, *nito* RNAi driven by a ubiquitous Gal4 such as *actin-Gal4* or *tubulin-Gal4* is associated with larval lethality.

Nito Regulates Sxl Levels by Controlling *Sxl* Alternative Splicing. The phenotype associated with loss of Nito function in both soma and germ line suggests that Nito may regulate *Sxl* activity. In ovaries, Sxl is enriched in GSCs and their immediate daughter cells (Fig. 4*E*) (6), whereas in somatic tissues such as wing discs Sxl is expressed ubiquitously in females but absent in males (Fig. 4 *A* and *B*). We expressed *nito* shRNA in the germ line using *MTD-Gal4* and in the dorsal half of the wing disk using *ap-Gal4*. Strikingly, knockdown of *nito* in both tissues led to a significant reduction of Sxl levels (Fig. 4 *F*, *C*, and *C*′). Note that similar results were obtained using two additional *nito* shRNA lines as well as in homozygous *nito*^{1} mutant clones (Fig. 4 *D* and *D*′ and Fig. S2 *A*′ and *B*′). However, the level of Sxl is not affected when Split-ends (Spen), another member of the SPEN family, was knocked down by shRNA in the wing disk (Fig. S2*C*), indicating a specific role of Nito in the sex determination pathway. Altogether, we have identified a new component of the *Drosophila* sex determination pathway that acts in both the germ line and soma by affecting Sxl levels.

Sxl transcripts are alternatively spliced, with exon 3 containing a stop codon that is included in males but skipped in females, leading to truncated Sxl forms in males but functional proteins in females (Fig. 4*G*) (22). Because Nito has three RRMs, we asked whether Nito has a potential role in *Sxl* splicing and used a pair of primers that detects the small female and large male spliced *Sxl* products (Fig. 4*G*) (12) to analyze *Sxl* splicing by RT-PCR in the absence of Nito. In *nito* shRNA female wing discs or ovaries, a large band corresponding to the male-specific spliced form was clearly detected (Fig. 4*G*). Note that in wing discs and ovaries, female-specific transcripts are detected due to contributions from remaining WT disk cells or somatic follicle cells, respectively, as *nito* was knocked down in half of the discs or in the germ line only. Together, our results indicate that Nito regulates Sxl levels by controlling its alternative splicing.

Nito Interacts with Sxl Protein and its Pre-mRNA in S2 Cells. We then analyzed how Nito controls *Sxl* alternative splicing. Because the key protein that binds *Sxl* pre-mRNA and inhibits splicing of male-specific exon 3 is Sxl itself (2), we examined whether Nito interacts with Sxl using a coimmunoprecipitation (co-IP) assay in *Drosophila* S2 cells. GFP-Nito and HA-Sxl were expressed either individually or in combination in S2 cells, and GFP alone was used as a control (Fig. 5*A*). HA-Sxl was detected in the precipitate obtained using anti-GFP nanobody from GFP-Nito cells, but not from GFP-expressing cells. Similarly, GFP-Nito, but not GFP, was pulled down by HA-Sxl. Further, we tested whether the interaction between Nito and Sxl is dependent on the presence of RNA. Interestingly, the amount of Sxl-HA pulled down by GFP-Nito is strongly reduced in the presence of RNase, suggesting that the Nito/Sxl interaction is mediated or stabilized by RNA (Fig. 5*B*). Finally, we performed RNA immunoprecipitation (RIP) experiments in S2 cells to analyze whether Nito can interact with *Sxl* pre-mRNA, which was detected by RT-PCR using an intron 3-exon 4 primer pair (12). As shown in Fig. 5*C*, GFP-Sxl, GFP-Nito, but not GFP alone, can pull down *Sxl* pre-mRNA from cell lysates. Together, the specific interactions between Nito, Sxl, and Sxl pre-mRNA support the model that Nito forms a complex with Sxl and that they together regulate alternative splicing of *Sxl* mRNA (Fig. 5*D*).

Screen for Additional Splicing Genes Involved in Sex Determination. Because we identified *nito* as an important gene involved in sex determination by regulating *Sxl* splicing, we asked whether there are other unidentified genes in the *Drosophila* genome acting in this pathway. Because most known *Sxl*-autoregulatory proteins are associated with spliceosomes (2), we screened a collection of 316 RNAi lines representing 247 splicing-associated genes and RNA-binding proteins (Dataset S1), using *MTD-Gal4* for the germ line and *dome-Gal4* for somatic phenotypes. In addition, we also included in our screen a wing-specific driver, *nub-Gal4,* as many RNAi lines exhibit lethality with *dome-Gal4* and prevent the detection of potential sex determination phenotypes. Because *Sxl* is responsible for the larger wing size in females, examination of wing size using *nub-Gal4* allows detection of potential genes involved in sex determination, as shown in the case of *nito* (Fig. S3 *A*–*F*).

Our screen successfully identified known components of the sex determination pathway: *tra* and *tra2* RNAi showed strong female-to-male transformation in sex combs and genitalia when induced by *dome-Gal4*; and *fl(2)d* and *vir* were identified using the *nub-Gal4* driver as both have a stronger effect in female wings than in male wings. Strikingly, besides these genes we did not identify any other genes that showed a sex-related phenotype. However, we did characterize GSC differentiation phenotypes associated with *tsu*, *mago*, *RnpS1*, *Rbp9* RNAi lines, as well

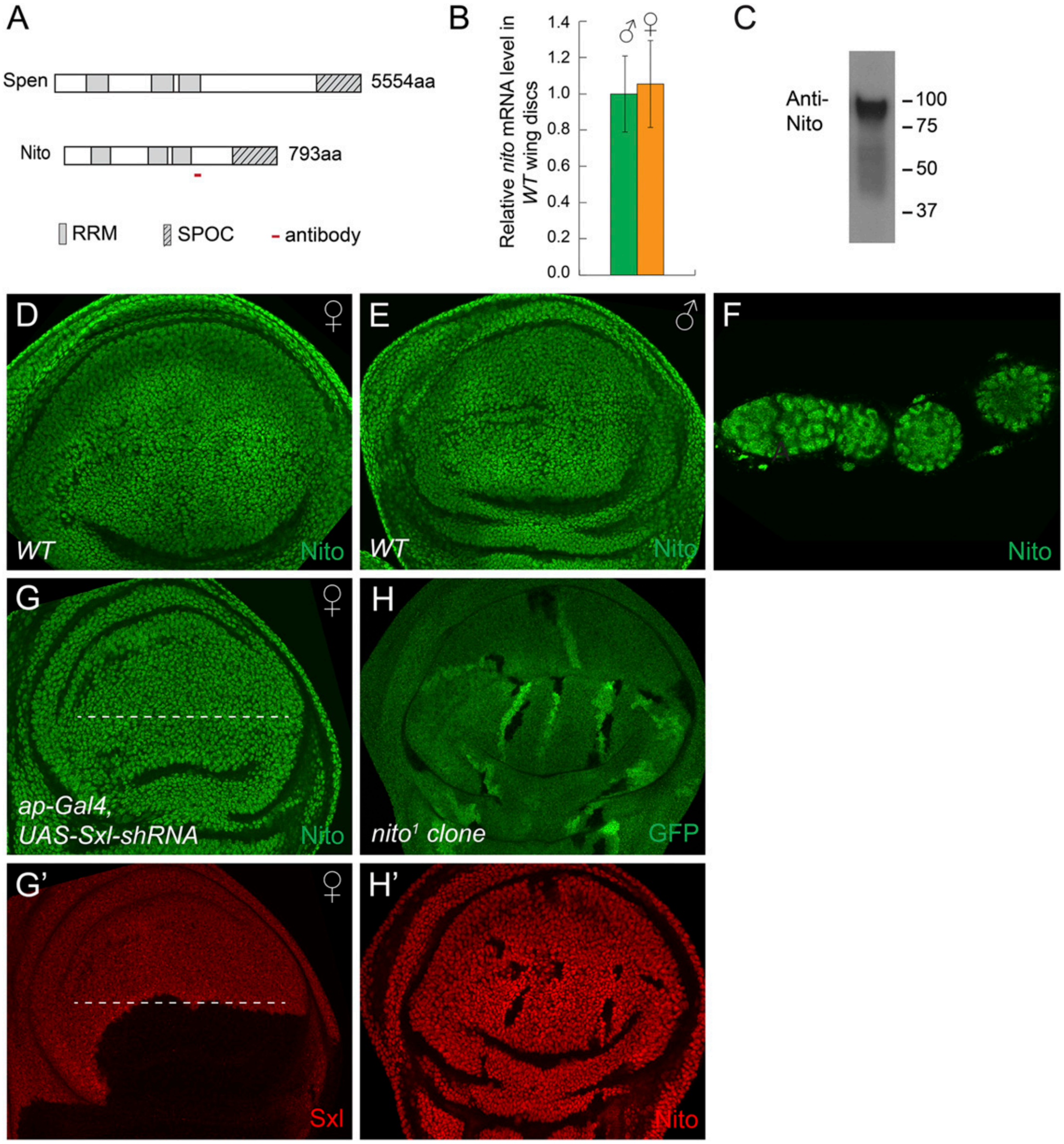

Fig. 3. Nito is a ubiquitous nuclear protein that shows no sex-biased expression. (*A*) Schematic diagram showing domain structures of Spen and Nito proteins and the peptide used to generate the Nito antibody. (*B*) *nito* mRNA levels in male and female WT wing discs were measured by qRT-PCR. Error bars represent SDs. (*C*) Nito antibody recognizes a single band of the predicted ~89 kDa size in S2 cell lysates. (*D*–*F*) Nito antibody stainings in WT female (*D*) or male (*E*) wing discs and ovarioles (*F*). (*G*–*G*′) Expression of *Sxl* shRNA in the dorsal half of the wing disk (below the dashed line) using *ap-Gal4* leads to depletion of Sxl protein, but has no effect on Nito protein levels. (*H*–*H*′) Nito antibody staining in wing discs containing $nito^1$ mutant clones, marked by the absence of GFP. Note the absence of Nito staining in $nito^1$ clones.

as wing growth and pattern defects with *kul*, *CG7879*, *ASPP*, *tst* and *Syp* RNAi lines. These data provide a valuable resource of phenotypes associated with splicing-related genes (data available at www.flyrnai.org/RSVP.html).

Discussion

We describe the characterization of Nito as a novel component of the *Drosophila* sex determination pathway. *Nito* loss-of-function results in stem-cell tumor phenotypes in the germ-line and sexual transformations in the soma. Interestingly, Nito affects Sxl protein levels in both GSCs and somatic tissues by regulating *Sxl* pre-mRNA alternative splicing, most likely directly as Nito interacts with the Sxl protein and pre-mRNA. The role of Nito is reminiscent of the previously reported roles of splicing factors in Sxl auto regulation, such as both subunits of U2AF (17), U1-70K (17, 18), Fl(2)d (10, 23), SPF45 (13, 14), Vir (15, 24), and Snf (11, 16). Our data support earlier reports that Sxl physically interacts with components of the spliceosome to simultaneously block utilization of the 3′ and 5′ splice sites of the male exon.

Nito and Spen are members of the SPEN protein family that are evolutionarily conserved from plants, worms, flies to mice and humans (25). Both proteins contain three N-terminal RRM domains and one C-terminal SPOC domain. The sequence similarity between these domains is low and there is no conservation outside these motifs, suggesting that they have evolved specific functions following a duplication event (20), as indicated by our observation that *spen* is not required for *Sxl* regulation (Fig. S2*C*). In *Drosophila*, *spen* was first identified in several genetic screens looking for components of the receptor tyrosine kinase (RTK) signaling pathway (26). Subsequent studies found

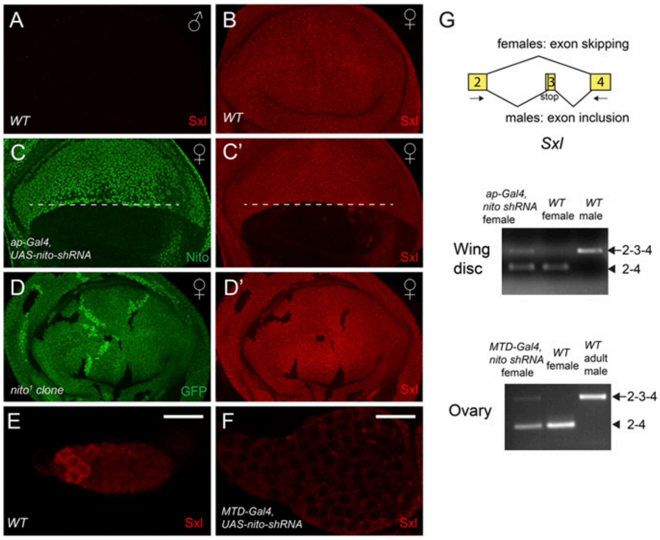

Fig. 4. Nito is required for Sxl levels and regulates *Sxl* mRNA splicing. (*A* and *B*) Sxl stainings in WT male (*A*) and female (*B*) wing discs. (*C–C′*) Expressing *nito* shRNA in the dorsal half of the disk (below the dashed line) using *ap-Gal4* leads to strong reduction of Nito (*C*) and Sxl (*C′*) stainings. (*D–D′*) Sxl antibody staining (*D′*) in wing discs containing *nito*^{1} mutant clones, which are marked by the absence of GFP (*D*). Note the absence of Nito and Sxl staining in *nito*^{1} clones. (*E* and *F*) Sxl stainings in WT egg chambers (*E*) or in egg chambers expressing *nito* shRNA by *MTD-Gal4* (*F*). Scale bars: 20 μm. (*G*) Diagram showing the alternative splicing event that produces the male- or female-specific S*xl* transcripts. The arrows indicate the primers used for RT-PCR. *Sxl* splicing was analyzed by RT-PCR using RNA extracted from wing discs or ovaries. Male-specific bands: 2–3-4. Female-specific bands: 2–4.

that *spen* is implicated in a variety of cellular and developmental processes including neuronal cell fate specification, axon guidance, cell cycle, Hox gene regulation, and cell death (27–30). These pleiotropic effects are likely due to the involvement of *spen* in multiple signaling pathways (31–33). However, the molecular mechanisms underlying the function of Spen in these pathways are not understood.

Genetic studies in *Drosophila* have shown that *nito* overexpression results in a rough eye phenotype (20) and that it plays a redundant role with *spen* in Wnt signaling (21), but how Nito is involved in these processes is not known. Biochemical studies indicate that Nito, like its human ortholog, copurify with the precatalytic spliceosome (complex B) (34). In addition, *nito*, as well as many other splicing factors, was identified in an RNAi screen for RAS/MAPK signaling components (35). Consistent with these findings, we find that *nito* is required for the alternative splicing of the master sex-determination gene *Sxl*. Previously, both Spen and Nito were thought to act mainly as transcription factors through their SPOC domains, our findings however clearly indicate that Nito is involved in mRNA splicing. It is intriguing to note that PPS, another important factor required for *Sxl* splicing, also has a SPOC domain (12). Similar to Nito, PPS also forms a complex with Sxl protein and its pre-mRNA (12). In the future it will be crucial to dissect how different protein domains contribute to the function of SPEN family proteins.

Then what is the "main" role of *nito*? On one hand, the phenotypes in the sex comb, genitalia and germ line appear specific to *Sxl* and such phenotypes do not depend on the genetic interaction with other genes in the sex determination pathway. On the other hand, *nito* clearly has other non-sex-specific functions, as revealed by the lethality, rough eye, and wing phenotype observed in both sexes (Fig. S3 *A–F*). Because a null allele of *nito* is associated with zygotic lethality, the RNAi knockdown approach is a powerful method to reveal sex-related phenotypes. Interestingly, our RNAi screen targeting splicing factors did not identify any new additional sex determination genes, indicating that there are a limited number of genes yet to be identified in this pathway. Finally, intriguingly, three recent studies have identified SPEN and Rbm15 (the mouse and human ortholog of Nito) as factors interacting with *Xist*, the long noncoding RNA that is essential for dosage compensation in mammals (36–38). Clearly, future experiments such as RNA-seq will be necessary to elucidate the mechanism and logic of Nito-mediated signaling events.

Rbm15, also known as OTT, was originally identified from infants with acute megakaryoblastic leukemia (AMKL) (39, 40). The *t*(1, 22) chromosomal translocation results in fusion of

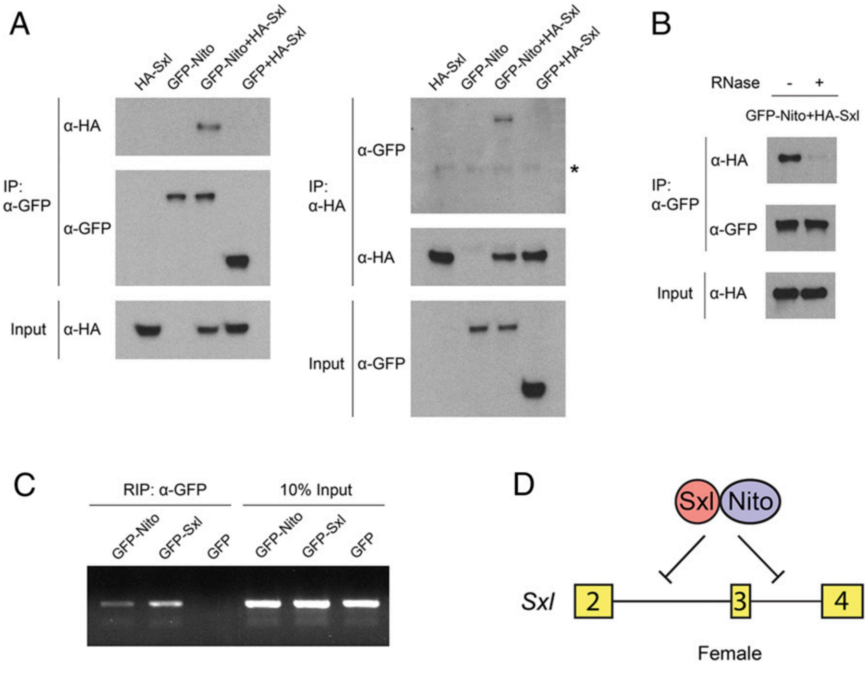

Fig. 5. Nito interacts with Sxl and Sxl pre-mRNA in S2 cells. (*A*) HA-Sxl, GFP-Nito or GFP expression vectors were transfected individually or together into *Drosophila* S2 cells. Cell lysates were immunoprecipitated using GFP nanobody or anti-HA antibody and analyzed by Western blot. GFP alone is used as a control. Asterisk indicates IgG heavy chain. (*B*) S2 cells were transfected with GFP-Nito and HA-Sxl, and Co-IP was performed using GFP nanobody in the absence or presence of RNase A and RNase T1. (*C*) S2 cells were transfected with GFP-Nito, GFP-Sxl or GFP and immunoprecipitated with GFP nanobody. The presence of Sxl pre-mRNA was detected by RT-PCR using an intron 3/exon 4 primer pair. GFP-Sxl was used as a positive control and GFP alone as a negative control. (*D*) Model: Nito forms a complex with Sxl and together they repress the splicing of *Sxl* exon 3 in female tissues.

RBM15 and MKL1, and the fusion protein is responsible for AMKL development as shown in a mouse model (41). In addition to this chromosome translocation, recent cancer genome sequencing projects have found that RBM15 and SPEN (also known as SHARP) are mutated in many different types of cancers, such as adenoid cystic carcinomas and bladder cancers (42). Given that SPEN family proteins are frequently mutated or deleted in cancers, they have been proposed to act as potential tumor suppressors (42). Studies of Spen and Nito in *Drosophila* will provide mechanistic insights to our understanding of this important family of proteins.

Methods

Details on the fly strains used in this study, as well as how the null *nito* mutation was isolated and how *nito* clones were generated can be found in *SI Methods*. Protocols used for antibody staining, reagents, how Nito antibodies were generated, coimmunoprecipitation protocols, RT-PCR, and information on primers and RNA immunoprecipitation (RIP), can be found in *SI Methods*.

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Supporting Information

Yan and Perrimon 10.1073/pnas.1515891112

SI Methods

Fly Strains. The following stocks were used in this study: *w^{1118}* (used as wild-type, WT), *MTD-Gal4*, *ap-Gal4*, *nub-Gal4*, *dome-Gal4, UAS-2xEYFP* (43), *Sxl* shRNA (HMS00609), *nito* shRNA (HMS00166), *nito* shRNA (HMS02013), *nito* shRNA (HMJ02081), *nito* dsRNA (VDRC 20942), *nito* dsRNA (VDRC 114704). Experiments presented in Figs. 1, 2, and 4 and Fig. S3 were done using *nito* shRNA (HMS00166).

To generate a null *nito* mutation, we used the homozygous viable P-element insertion *$nito^{HP25329}$* located in the 5′UTR of the *nito* gene. After mobilization of the HP25329 P-element, we screened for homozygous lethal lines and recovered a null allele, *$nito^1$*, that deletes 1,357 bp and is lethal over *Df(2R)Exel6055* (43F1 to 44A4) that uncovers the *nito* locus. The Nito antibody, raised against amino acids 479–500, cannot detect any Nito antigens in *$nito^1$* mutant clones (Fig. 3 *H* and *H′*).

To generate mutant clones, *$nito^1$* was recombined with *FRT^{G13}* and crossed to *y w hsflp; ubiGFP FRT^{G13}* flies. The progeny were heat-shocked at 37 °C for 1 h twice at first- and second-instar larval stage.

Antibody Stainings in Discs and Ovaries. Larval wing discs and female ovaries were stained as described (19). Briefly, tissues were dissected in PBS and fixed in 4% formaldehyde in PBST (PBS + 0.1% Triton X-100). After blocking in 1% normal donkey serum in PBST for 1 h, the samples were incubated with the primary antibody in the same solution at 4 °C overnight. After three washes in PBST, samples were incubated with the secondary antibody for 2 h at room temperature, washed in PBST three times, and subsequently mounted in Vectashield. All images were taken on a Zeiss LSM 780 microscope.

The following antibodies were used: mouse anti-α-Spectrin (1:10) (3A9, DSHB), rabbit anti-Vasa (1:250) (Santa Cruz Biotechnology), mouse anti-Sxl (1:10) (M18, DSHB), rabbit anti-Nito (1:500), rabbit anti-phospho-Histone H3 (1:1,000) (Millipore), rabbit anti-GFP (1:1,000) (Molecular Probes), mouse anti-GFP (1:200) (Molecular Probes), Alexa 488- or 555- conjugated secondary antibodies (1:1,000) (Molecular Probes) and DAPI (1:1,000) (Molecular Probes).

Nito antibodies were generated in rabbits against a peptide containing amino acids 479–500 (KSSKPPYDESALEYRRPEYDPY) and affinity-purified at YenZym Antibodies. Polyclonal antisera were raised in two rabbits, YZ3137 and YZ3138, and gave similar staining patterns. All of the experiments described in the paper were performed with antiserum from YZ3137.

Adult legs and wings were mounted in a 1:1 (vol/vol) mixture of Permount (Fisher Scientific) and xylene. The genitalia images were taken in stacks and rendered with HeliconFocus software.

Coimmunoprecipitation. To generate the GFP-Nito plasmid, a *nito* full-length cDNA (GH11110) was cloned into the *Drosophila* Gateway vector pAGW. HA-Sxl and GFP-Sxl were constructed following PCR of *Sxl* (the MS3 isoform) from *UAS-Sxl* flies (44) and cloned into pAHW and pAGW, respectively. GFP was cloned into pAWM as a control.

Drosophila S2 cells were maintained at 25 °C in Schneider's medium supplemented with 10% FBS. One microgram of total DNA was transfected into S2 cells in a single well of six-well plates with Effectene (QIAGEN). After 48 h, cells were lysed in IP lysis buffer (Pierce) with Halt Protease Inhibitor (Thermo Scientific). Lysates were incubated with anti-GFP nanobody agarose beads (Allele Biotechnology) or anti-HA agarose (Sigma) for 2 h at 4 °C. The beads were washed 3–4 times with 1 mL lysis buffer. Protein complexes were eluted and detected by Western blotting using anti-GFP antibody (A6455, Molecular Probes) or anti-HA antibody (3F10, Roche). For RNase treatment experiment, 100 μL of RNase A (10 mg/mL, Thermo Scientific) and 5 μL of RNase T1 (1,000 U/μL, Thermo Scientific) were added to 1 mL of lysate and incubated for 30 min at 30 °C, then overnight at 4 °C with beads (12, 17).

RT-PCR. Total RNA was extracted from dissected wing discs or ovaries using TRIzol (Invitrogen), digested with DNase I (Qiagen) and purified using the RNeasy Mini kit (Qiagen). cDNA was generated from 1 μg of purified RNA using the iScript cDNA Synthesis lit (Bio-Rad). For nonreal time PCR, TaKaRa Taq polymerase was used. For qPCR, iQ SYBR Green Supermix (Bio-Rad) was used and reactions were measured in a CFX96 Real-Time PCR detection system (Bio-Rad). qPCR results for *nito* expression in male and female wing discs (Fig. 3*B*) were normalized to the reference gene *αTubulin84B*. *Sxl* primers used in Fig. 4*G* are described in ref. 12. *nito* primers in Fig. 3*B* and *αTubulin84B* primers are listed below.

nito_PP17280_f	GGCTACAAGGTACTTTGCGTC
nito_PP17280_r	TACTCGCGGTACAGTGTCTCC
Sxl-f	GTGGTTATCCCCCATATGGC
Sxl-r	GATGGCAGAGAATGGGAC
αTubulin84B-f	CAACCAGATGGTCAAGTGCG
αTubulin84B-r	ACGTCCTTGGGCACAACATC

RNA Immunoprecipitation (RIP). RIP experiments were performed following previous protocols (45, 46). One microgram of DNA was transfected into S2 cells in 60-mm plates with Effectene (QIAGEN). After 48 h, cells were lysed in IP lysis buffer (Pierce) with Halt Protease Inhibitor (Thermo Scientific) and RNasin plus (40 U/mL, Promega). After centrifugation, 10% of the supernatant were saved as the input and the rest were incubated with anti-GFP nanobody agarose beads (Allele Biotechnology) for 2 h at 4 °C. The beads were washed 3–4 times with 1 mL of lysis buffer. To elute RNA from the RNA/protein complexes, the beads were treated with proteinase K solution for 10 min at 55 °C. Total RNA from the input and the beads were extracted by using RNeasy Micro kit (QIAGEN). cDNA was synthesized using SuperScript III First-Strand Synthesis System (Life Technologies) with four of the eluted RNA and random hexamers. TaKaRa Taq polymerase was used for two rounds of PCR amplification with primers and conditions described in ref. 12.

Sxl-intron	GAGGGTCAGTCTAAGTTATATTCG
Sxl-r	GATGGCAGAGAATGGGAC

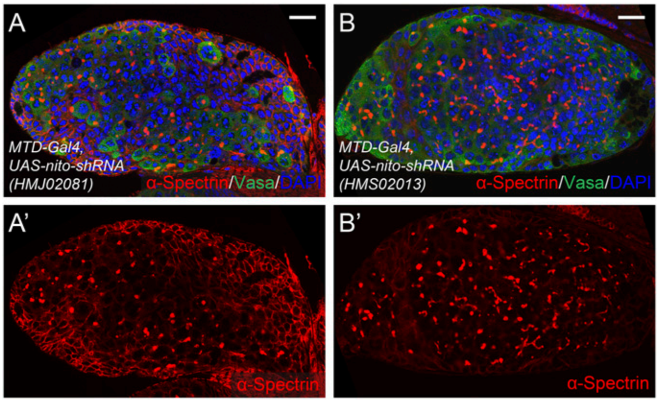

Fig. S1. Two independent *nito* shRNAs result in similar stem-cell-tumor in the germ-line. (*A*–*B*′) Egg chambers expressing shRNAs targeting *nito* (HMJ02081) or *nito* (HMS02013) using *MTD-Gal4* stained for α-Spectrin, Vasa and DAPI. (Scale bars: 20 μm.)

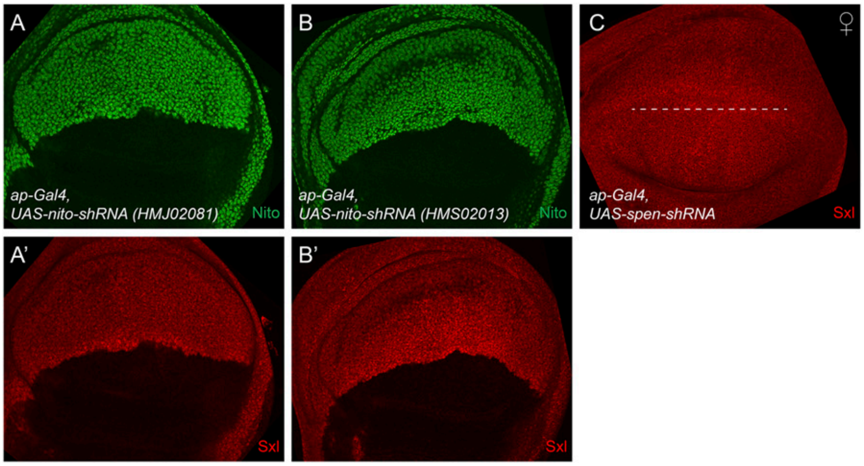

Fig. S2. *nito*, but not *spen*, regulates Sxl levels in wing discs. (*A*–*B*′) Expression of *nito* shRNA (HMJ02081 or HMS02013) in the dorsal half of the wing disk using *ap-Gal4* leads to a strong reduction of both Nito (*A* and *B*) and Sxl (*A*′ and *B*′) stainings. (*C*) Expression of *spen* shRNA in the dorsal half of the disk (below the dashed line) by *ap-Gal4* does not affect Sxl protein levels. *spen* shRNA generates embryonic lethality with cuticle and head defects when expressed using *MTD-Gal4* (47), which resembles the phenotype of the *spen* mutant, indicating that the shRNA is functional.

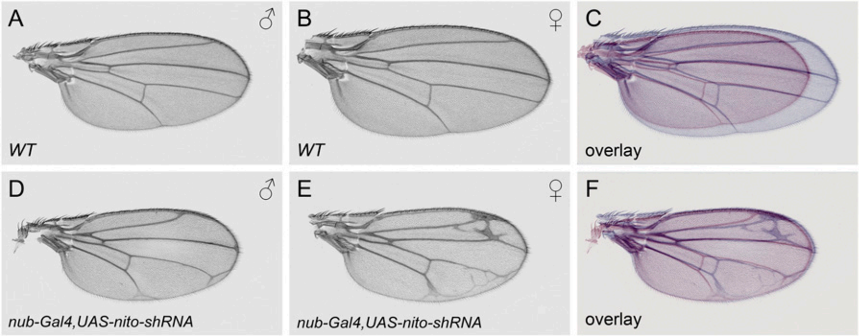

Fig. S3. Nito shRNA affects wing growth more strongly in females than in males. (*A*) WT male wing. (*B*) WT female wing. (*C*) Overlay of the images in *A* (red) and *B* (blue) shows that a WT female wing is about 30% larger than a WT male wing. Male (*D*) and female (*E*) wings in which *nito* shRNA was expressed using the *nub-Gal4* driver. (*F*) Overlay of *D* and *E* showing that both male and female wings reach about the same size upon *nito* knockdown.

Dataset S1. Screen results of 316 RNAi lines targeting 247 splicing genes using *MTD-Gal4*, *dome-Gal4* and *nub-Gal4*

Dataset S1