The DExH box helicase domain of Spindle-E is necessary for retrotransposon silencing and axial patterning during Drosophila oogenesis

Kristen M. Ott †,*, Tram Nguyen† and Caryn Navarro†,∗, ‡

† Department of Medicine
Biomedical Genetics
Boston University School of Medicine
Boston, MA 02118

∗ Graduate Program in Genetics and Genomics
Boston University School of Medicine
Boston, MA 02118
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‡ Corresponding author:

Caryn Navarro
72 E. Concord Street, E222
Boston University School of Medicine
Boston, MA 02118

Phone: (617) 638-4278
Email: cnavarro@bu.edu
ABSTRACT

Transposable selfish genetic elements have the potential to cause debilitating mutations as they replicate and reinsert within the genome. Therefore, it is critical to keep the cellular levels of these elements low. This is especially true in the germline where these mutations could affect the viability of the next generation. A class of small non-coding RNAs, the Piwi associated RNAs, are responsible for silencing transposable elements in the germline of most organisms. Several proteins have been identified as playing essential roles in piRNA generation and transposon silencing. However, for the most part their function in piRNA generation is currently unknown. One of these proteins is the *Drosophila melanogaster* DExH box/Tudor domain protein Spindle-E whose activity is necessary for the generation of most germline piRNAs. In this study we molecularly and phenotypically characterized 14 previously identified *spindle-E* alleles. Of the alleles that express detectable Spindle-E protein we found that five had mutations in the DExH box domain. Additionally, we found that processes that depend on piRNA function, including Aubergine localization, Dynein motor movement and retrotransposon silencing, were severely disrupted in alleles with DExH box domain mutations. The phenotype of many of these alleles is as severe as the strongest *spindle-E* phenotype, while alleles with mutations in other regions of Spindle-E did not affect these processes as much. From these data we conclude that the DExH box domain of Spindle-E is necessary for its function in the piRNA pathway and retrotransposon silencing.
INTRODUCTION

A large portion of both the human and Drosophila genomes are composed of transposable elements (TEs), which are capable of creating genome instability and a high mutation rate upon excision and re-integration within the genome (BELGNAOUI et al. 2006; GASIOR et al. 2006). In most organisms germ cells seem to be particularly sensitive to elevated levels of TEs, and TE deregulation ultimately leads to germ cell developmental defects and sterility (JULIANO et al. 2011). TE regulation in the germline is particularly important as germline DNA is inherited by offspring and mutations can hinder reproductive success or could be deleterious to the progeny.

The Drosophila ovary is composed of both somatic and germ cells and in both cell types a highly conserved class of small non-coding RNAs, piRNAs (Piwi-interacting RNAs), are responsible for silencing TE expression and transposition (GUZZARDO et al. 2013). Germline piRNAs are highly abundant and quite divergent in their sequences. Although the population of piRNAs is quite complex, most piRNAs can be mapped to a small number of genomic regions called “piRNA clusters” (BRENNECKE et al. 2007). Precursor piRNAs (Pre-piRNAs) are transcribed as long single-stranded RNAs from these clusters. Pre-piRNA transcripts are exported from the nucleus and processed into primary piRNAs. In germ cells transcription is controlled by several chromatin associated proteins including the HP1 paralog Rhino and its binding partner Cutoff (CUFF), the histone
methyltransferase, dSETDB1, as well as the Tudor domain proteins, Kumo/Qin and Vreteno (VRET) (Anand and Kai 2012; Handler et al. 2011; Klattenhoff et al. 2009; Pane et al. 2011; Rangan et al. 2011; Zamparini et al. 2011; Zhang et al. 2011). Primary transcripts are bound by the putative helicase, UAP56, and shuttled out of the nucleus where they are transferred to Vasa (VAS) within a specialized perinuclear cytoplasmic region known as the nuage (Lim and Kai 2007; Zhang et al. 2012). The nuage is believed to be the site of retrotransposon silencing (Lim and Kai 2007). These long transcripts are then processed further to mature primary piRNAs. The 5′ end of the mature primary piRNA is likely generated by the endonuclease Zucchini (Ipsaro et al. 2012; Nishimasu et al. 2012; Voigt et al. 2012). However, the complete mechanism by which the mature piRNAs are generated is currently unknown. Several other proteins have been identified as necessary to generate primary piRNAs, most localize to the nuage and several form complexes, however how many of these proteins function in piRNA biogenesis is not known (Czech et al. 2013; Guzzardo et al. 2013; Handler et al. 2013).

In germ cells cytoplasmic primary piRNAs also enter into an amplification cycle (Brennecke et al. 2007; Gunawardane et al. 2007). Here, proteins of the Argonaute family bind piRNAs. In Drosophila these include Piwi and Aubergine (Aub) (Brennecke et al. 2007; Gunawardane et al. 2007). It is unclear what role Piwi plays in germline piRNA generation. Deep sequencing of piRNAs bound by Aub has shown that it binds piRNAs that are mostly antisense to active TE
mRNAs. Active TE mRNAs are cleaved 10 nucleotides downstream of the piRNA terminal A most likely through AUB’s slicer activity, thereby generating secondary sense piRNAs (BRENNECKE et al. 2007; GUNAWARDANE et al. 2007). Sense piRNAs are loaded onto another Argonaute family protein Argonaute 3 (Ago3), which functions to cleave cluster-derived antisense transcripts to generate more antisense piRNAs. This mechanism of piRNA generation has been termed the “ping-pong” amplification cycle and provides an adaptive response to the presence of newly synthesized TE mRNA. This amplification cycle most likely takes place in the nuage (LIM and KAI 2007). Most proteins necessary for piRNA biogenesis localize to the nuage and a temporal hierarchical relationship governing nuage localization exists among these proteins. Vasa, a RNA helicase, localizes first, followed by the DExH box helicase/Tudor domain protein, Spindle-E (SPN-E) and the Tudor domain protein, Tejas (TEJ), both of which are dependent on Vas for their localization (FINDLEY et al. 2003; LIM and KAI 2007; MALONE et al. 2009; PATIL and KAI 2010). Other piRNA pathway proteins such as AUB, Ago3 and Krimper (KRIM) rely on VAS, SPN-E and TEJ for their localization. The cumulative data indicate that a large complex or several complexes form at the nuage or localize piRNA proteins to the nuage where the piRNA proteins along with their associated piRNAs act to silence retrotransposons.

How piRNAs function to silence TEs is currently not well established but evidence exists for both post-transcriptional, as described above, and transcriptional regulation. Recently it was shown that Piwi is responsible for regulating retrotransposon expression in ovarian somatic cells, as the occupancy of RNA
Polymerase II at retrotransposon promoters is increased along with steady state levels of transposon transcripts in piwi mutant cells (Huang et al. 2013; Le Thomas et al. 2013; Rozhkov et al. 2013; Sienski et al. 2012). It is currently unknown whether this mechanism also occurs in the germ cells. How piRNAs influence transcription is also currently unknown but it has been reported that heterochromatin protein binding and histone methylation at retrotransposon sequences changes in piRNA pathway mutants where piRNA levels are decreased (Klenov et al. 2007; Pal-Bhadra et al. 2004).

A critical protein involved in the generation of most germ cell piRNA species is Drosophila Spindle-E (Malone et al. 2009). SPN-E colocalizes to the nuage along with other piRNA pathway proteins and its function is required for either primary piRNA generation and/or the ping-pong cycle (Malone et al. 2009; Patil and Kai 2010). spn-E was originally identified as a gene necessary for microtubule network formation, RNA localization and embryonic pattern formation (Gillespie and Berg 1995; Klattenhoff et al. 2007; Martin et al. 2003). However it is not known whether SPN-E function in the piRNA pathway controls all of these processes. The SPN-E protein contains a DExH box helicase domain, a Tudor domain and a Zinc finger (Zn), which implicate its function in RNA processing, translational regulation, RNA decay, splicing, or protein-protein interactions (Fig 1A,B). However, the relative contribution of these domains to SPN-E function, particularly in the piRNA pathway, is currently unknown. Therefore, to begin to understand how SPN-E functions during oogenesis, in particular in TE silencing, we took advantage of several previously isolated spn-E mutant fly lines in an attempt to identify
mutations in the predicted functional domains. Our results provide evidence that
the DExH box helicase domain of SPN-E is necessary for TE silencing in the
germline.

MATERIALS and METHODS

Drosophila strains

The following Drosophila lines were used: \( w; \text{FRT[ry}^+\text{]}82B \text{ spn-E}^{4-4} \text{ e, w;} \text{FRT[ry}^+\text{]}82B \text{ spn-E}^{66-21} \text{ e, w;} \text{FRT[ry}^+\text{]}82B \text{ spn-E}^{100-37} \text{ e, w;} \text{FRT[ry}^+\text{]}82B \text{ spn-E}^{189-3} \text{ e, w;} \text{FRT[ry}^+\text{]}82B \text{ spn-E}^{114-33} \text{ e, w;} \text{FRT[ry}^+\text{]}82B \text{ spn-E}^{155-5} \text{ e, and w;} \text{FRT[ry}^+\text{]}82B \text{ spn-E}^{23-17} \text{ e were a kind gift from Ruth Lehmann and described in (STAeva-Vieira 2003).} \text{yw; FRT[ry}^+\text{]}82B \text{ spn-E}^{2A9-14}, \text{yw; FRT[ry}^+\text{]}82B \text{ spn-E}^{9A2-17}, \text{yw; FRT[ry}^+\text{]}82B \text{ spn-E}^{9A9-18}, \text{yw; FRT[ry}^+\text{]}82B \text{ spn-E}^{9D4-11}, \text{yw; FRT[ry}^+\text{]}82B \text{ spn-E}^{4E2-14}, \text{ and yw;} \text{FRT[ry}^+\text{]}82B \text{ spn-E}^{7G2-5} \text{ were a kind gift from Daniel St. Johnston and described in (Martin et al. 2003).} \text{spn-E}^{4125} \text{ was a kind gift from Celeste Berg and described in (Gillespie and Berg 1995).} \text{spn-E}^{653} \text{ and spn-E}^{616} \text{ were obtained from the Tubingen stock collection (Tearle and Nusslein-Volhard 1987a). The wild-type strain used was Oregon-R.}

Induction of germline clones

Germline clones were induced using the FLP/FRT system (Chou and Perrimon 1996). \text{FRT82B spn-E females were crossed to yw hsflp(ii); FRT82B UbiGFP male flies (Bloomington stock center). Second or third instar larvae were heat shocked}
at 37°C for two hours on two consecutive days to induce clones. Female flies of the correct genotype were dissected 10 days post heat shock.

**Sequencing**

Genomic DNA was isolated from 30 adult male \( spn-E^{\text{mutant}} / spn-E^{\Delta 125} \) flies as in (REHM). \( spn-E^{\Delta 125} \) is a deletion that completely removes the \( spn-E \) gene (GILLESPIE and BERG 1995). The \( spn-E \) gene was sequenced using a primer walking strategy. Sections of the \( spn-E \) gene were amplified using standard PCR conditions and Crimson Taq (New England Biolabs). Multiple sets of \( spn-E \) gene-specific primers that span the gene from the start codon to the stop codon, including introns were used (primer sequences available upon request). PCR products were purified using the Qiagen MinElute PCR purification kit. Sanger sequencing was done using the Big Dye termination kit.

**Aubergine and Spindle-E antibody production**

Rabbit polyclonal antisera directed against peptide MNLPPNPVIARGRGRG (amino acids 1-16) (BRENNECKE et al. 2007) of AUB and TNHRRKHSIGKFYRDQLG (amino acids 295-312) of SPN-E were generated by Pocono Rabbit Farm and Laboratory, Inc. using their Quick Draw 49 Day protocol.
The antiserum was affinity purified by Pocono Rabbit Farms using the appropriate peptide.

**Protein isolation and western blot analysis**

15-20 female flies that were two or three days old were placed on yeast overnight and ovaries were dissected in 1x Ephrussi Beadle Ringer’s buffer (EBR, 130mM NaCl, 4.7mM KCl, 1.9mM CaCl$_2$, and 10mM Hepes pH 6.9). Ovaries were homogenized 5-10 times in lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP40, 1x Halt protease inhibitor single-use cocktail [Thermo Scientific]). Samples were centrifuged twice for 5 minutes at 12,000g at 4°C and the supernatant was assayed for protein concentration. Protein was quantitated by a Bradford type assay using the Biorad Protein Assay kit. 40µg of protein was resolved on 7% SDS-polyacrylamide gels and transferred to PVDF membranes (Immobilon) using the Bio-Rad mini-PROTEAN tetra electrophoresis system. Western blots were done as in (NAVARRO et al. 2004). Primary antibodies were diluted at 1:1000 in 5% non-fat milk in Tris-buffered saline with 0.5% Tween. Antibodies used were mouse anti-ß-tubulin (Developmental Studies Hybridoma Bank) and affinity purified rabbit anti-Spindle-E. HRP-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories) were used at a dilution of 1:10,000. For detection, the blots were incubated in Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) according to the manufacturer’s instructions and exposed to X-ray film.
(Kodak Biomax light or Amersham). Bands were quantitated using NIH ImageJ software.

**D/V Patterning Assay**

8-10 female flies that were two or three days old of the specified genotypes (spn-\(E^{mutant}\)/Deficiency or spn-\(E^{mutant}\)/Balancer) were placed on yeast overnight, put into egg laying chambers, and allowed to lay eggs on apple juice agar plates with yeast paste overnight at 25°C. Eggs of each class were counted after 24 hours of egg laying for three consecutive days.

**Real Time Quantitative RT-PCR**

15-20 female flies (spn-\(E^{mutant}\)/Deficiency or spn-\(E^{mutant}\)/Balancer) that were two or three days old were placed on yeast overnight. Ovaries were dissected in EBR and placed in microcentrifuge tubes. The EBR was removed and the ovaries were flash frozen in liquid nitrogen or used fresh for RNA isolation. RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. RNA was treated twice with Turbo DNase (Ambion) to remove contaminating genomic DNA. cDNA was generated from 1µg of RNA using the Verso cDNA kit (Thermo Scientific) or the Maxima cDNA kit (Fermentas). A 10µl real time PCR reaction was performed with either 1x ABsolute Blue SYBR Green master mix
(Abgene), 0.075 mM of forward and reverse primers, and 1µl of cDNA reaction OR
1x Maxima SYBR master mix (Fermentas), 0.3 mM of forward and reverse
primers, and 1µl of cDNA reaction. Cycling parameters were: 50 °C, 2 min; 95 °C,
10 min; 95 °C, 15 sec; 60 °C, 1 min for 40 cycles using an ABI 7900HT. The
following previously published primers were used: HetA and TART as in (PANE et
al. 2007), I-Factor and roo as in (VAGIN et al. 2006), Blood as in (DONERTAS et al.
2013), gypsy as in (BRENNECKE et al. 2007) and Adh as in (KLENOV et al. 2007).
Data were analyzed using SDS software and relative RNA levels were calculated
by the 2-ΔΔCt method (LIVAK and SCHMITTGEN 2001). RNA was normalized to Adh
levels. Fold enrichments were calculated in comparison to respective RNA levels
obtained from heterozygous flies.

Immunohistochemistry and microscopy

Antibody staining was done as in (NAVARRO et al. 2004). Chicken anti-GFP
(Abcam) was used at a dilution of 1:5000, rabbit anti-Egalitarian was used at a
dilution of 1:5000 (NAVARRO et al. 2004), rabbit anti-Aubergine was used at a
dilution of 1:1000, mouse anti-Gurken clone 1D12 was obtained from the
Developmental Studies Hybridoma Bank and was used at a dilution of 1:20
(QUEENAN et al. 1999). Secondary antibodies (Cy3- Jackson Immunoresearch,
Alexa488- Molecular Probes) were used at a concentration of 1:500. Images were
RESULTS

Molecular analysis of \textit{spn-E} alleles

We obtained 12 \textit{spn-E} alleles from two independent EMS mutagenesis screens and determined if each line expressed ovarian SPN-E protein (\textsc{Martin \textit{et al.}} 2003; \textsc{Staeva-vieira} 2003) (\textbf{Fig 1C,D}). Eight alleles expressed protein of the correct size for full-length SPN-E. Four of these eight alleles (9A2-17, 2A9-14, 23-17, 4-48) expressed SPN-E protein at levels close to wildtype. Four other alleles produced protein below wildtype levels (8D4-11, 155-55, 66-21, 7G2). 66-21 mutant ovaries have approximately 75\% less SPN-E protein than wildtype ovaries. This allele displays only a mild phenotype (see below), therefore, it seems that the level of SPN-E produced by 66-21 is enough for mostly wildtype function. All of the other protein expressing \textit{spn-E} lines produce more protein than 66-21. Therefore, we conclude that the phenotypes that we describe below are probably not due to reduced SPN-E protein levels, but due to disruption of protein function by the point mutation. Four of the twelve alleles (114-33, 9A9-18, 4E2-14 and 100-37) as well as two additional \textit{spn-E} alleles that had been previously phenotypically characterized (616 and 653, (\textsc{Tearle} and \textsc{Nusslein-volhard} 1987b)) do not express detectable SPN-E protein (\textbf{Fig 1C}, \textbf{Fig S1}).
We next determined the genomic DNA sequence from the translational start codon to the stop codon, including introns, for each allele to determine if the spn-E gene contained mutations that lie in the known functional domains: the DExH box, the Tudor or the Zinc Finger (Fig 1A). Our sequencing of the protein expressing alleles identified 5 mutations in the DExH box domain, one mutation in the Zinc Finger domain and two mutations in highly conserved residues outside of the predicted functional domains (Fig 1A,B Table 1). Additionally, all alleles that did not express detectable protein had mutations that cause premature stop codons to be formed (Fig 1A,B Table 1).

DExH box helicase domains consist of several motifs, some of which have defined functions associated with helicase activity (LUKING et al. 1998). We identified mutations in motif I (2A9-14T145I - the Walker A box), a common motif found in proteins that bind and hydrolyze NTPs. Motif II (155-55E239K, 23-17H241Q - the DExH region), which is necessary for ATP binding/hydrolysis and interdomain contacts, and motif V (8D4-11S435F), which may be necessary for RNA binding. In addition, we identified a mutation that lies between motifs I and II in an amino acid that is conserved between species (7G2-5T233I) (Fig 1B). The identification of multiple mutations in the DExH box domain from two independent genetic screens indicates that the DExH box domain may be critical for SPN-E function.

Zn fingers are mostly known for their role in DNA binding and transcriptional regulation, however they have also been shown to be necessary for RNA, protein and lipid binding (GAMSJAEGER et al. 2007; HALL 2005; MATTHEWS and SUNDE 2002). The Zn finger found in SPN-E belongs to the Cys2His2-like fold group that is
identified by the sequence: $X_2$-Cys-$X_{2,4}$-Cys-$X_{12}$-His-$X_{3,4,5}$-His, where the two Cys and two His amino acids are important for coordinating Zn (PABO et al. 2001). The mutation that we identified changes the last His in the Zn finger region to a Leu (66-21, aa 1422). Because this amino acid is important for Zn coordination the mutation that we found most likely would disrupt the function of the domain.

Because we found mutations in two of the conserved functional domains of SPN-E we wanted to determine if these amino acid changes affect protein function. Therefore, we assessed the extent to which the mutations affect developmental processes known to require SPN-E function such as Dorsal/Ventral (D/V) eggshell patterning, AUB subcellular localization and dynein motor complex localization. Additionally we directly tested whether the piRNA pathway was affected in the mutant ovaries by measuring retrotransposon levels (Table 1).

**DExH box domain mutations in SPN-E perturb its function in Dorsal/Ventral patterning**

*spn-E* mutant females lay eggs with severe D/V patterning defects ranging from fused dorsal appendages to no dorsal appendages and collapsed eggs (GILLESPIE and BERG 1995). To evaluate the importance of the DExH box and Zn finger domains to the establishment of D/V eggshell polarity we examined eggs laid by the various mutant females. The eggs laid by the *spn-E* mutant flies that we describe here vary in the severity of D/V patterning defects (Table 2). In contrast to wildtype females that lay eggs with two distinct dorsal appendages, most of the eggs laid by females expressing SPN-E protein with DExH box domain mutations
showed a very severe phenotype similar to the strongest *spn-E* mutant egg phenotype, with a high percentage of collapsed eggs or eggs with no dorsal appendages. For example, female flies expressing SPN-E protein with a mutation in amino acid 145 (2A9-14^{T145I}), which lies in DExH box motif I, lay approximately 94% collapsed eggs and no wildtype eggs. This is in contrast to wildtype females that do not lay collapsed eggs (*spn-E^{Δ125}/Balancer*) but compares favorably with eggs laid by *spn-E* mutant females that do not express detectable SPN-E protein, which lay between 83-100% collapsed eggs (114-33^{R918*}, 9A9-18^{w632*}, 4E2-14^{R695*}). The same severe phenotype occurs in eggs laid by *spn-E* mutant females that have mutations located in DExH box motif V (8D4-11^{S435F}) and in between motifs I and II (7G2-5^{T233I}).

We found two mutations in amino acids in the DExH box motif, region II (155-55^{E239K}, 23-17^{H241Q}). Mutation of the Glu in this region leads to a less severe phenotype than the DExH box mutations discussed above where the majority of eggs laid are collapsed but a larger percentage of eggs with fused or no dorsal appendages are present. Additionally, mutation of the His in the DExH box (23-17^{H241Q}) sequence resulted in an even less severe phenotype with 55% of the eggs laid having wildtype patterning.

Females with mutations in SPN-E that lie outside of the predicted functional domains lay eggs with a milder phenotype than those with mutations within the DExH box domain. Mutation of amino acid 508 (9A2-17^{P508L}), which changes a conserved Pro to Leu in between the DExH box and the Tudor domains, produced
a mild D/V patterning defect with 60% of the eggs laid having a wildtype appearance (Fig 1A, Table 2).

Flies expressing SPN-E with a mutation in the Zn finger motif (66-21H1422L) lay eggs with an extremely mild D/V patterning phenotype resulting in 92% of the eggs having a wildtype appearance.

Interestingly eggs laid by females from lines 100-37splicesite, 616R1081* and 653Y636* show weaker D/V phenotypes than the other mutant spn-E alleles that also did not express detectable protein. Line 100-37 has a splice site mutation at base pair 488. If this intron was not spliced properly a truncated SPN-E protein should be made. This protein would truncate before the DExH box domain. Because this protein would stop before the peptide to which our antibody was made we were not able to determine if this shortened SPN-E protein was expressed in 100-37 ovaries. However, when RNA isolated from line 100-37 was analyzed by RT-PCR using primers surrounding the affected intron the amplified product ran at the same size as wildtype on an agarose gel, indicating that splicing was not altered in 100-37 mutant ovaries (data not shown). We also did not detect protein of the correct size for SPN-E by Western blotting. Nevertheless, the weaker mutant phenotype indicates that at least some functional protein may be made by this allele. Along these same lines, eggs laid by females expressing genetically characterized spn-E hypomorphic alleles, spn-E616, and spn-E653 (GILLESPIE and BERG 1995) show a similar phenotype to eggs laid by females from the spn-E100-37 allele. We have not been able to detect SPN-E protein from spn-E616 and spn-E653 ovaries even though our antibody should be capable of recognizing the truncated
form of SPN-E that could be made by this allele (Fig 1, Fig S1). It is possible that spn-E\textsuperscript{616}, spn-E\textsuperscript{653} and spn-E\textsuperscript{100-37} express protein that we are unable to detect by our assay.

To further analyze the D/V phenotype we examined the expression/localization of the Dorsal determinant Gurken (GRK) by immunohistochemistry. At oogenesis stage 8 GRK localizes to the dorsal-anterior corner of the oocyte where it is necessary to signal to the dorsal follicle cells to establish embryonic dorsal fate. In support of the above data, we find that GRK levels at the dorsal corner of the oocyte are reduced in the spn-E mutant ovaries to the same extent as the D/V phenotypes described above, with those alleles that show the most severe D/V patterning defects having the lowest GRK levels (Fig S3). Additionally, we did not detect any Grk expression in the stronger spn-E alleles, 2A9\textsuperscript{T145I}, 7G2\textsuperscript{T233I}, 8D4\textsuperscript{S435F}, 114-33\textsuperscript{R918*}, 9A9\textsuperscript{W632*}, or 4E2\textsuperscript{R695*}. For the 155-55\textsuperscript{E239K}, 100-37\textsuperscript{splicesite488}, 616\textsuperscript{R1081*}, and 653\textsuperscript{Y636*} alleles, which have moderate D/V patterning defects, we did not detect significant Grk levels at the dorsal-anterior corner of the oocyte at stage 9 and beyond, however, we did detect reduced GRK in the oocyte at the earlier stages (Supplementary Figure 3, arrows). Therefore, it is possible that some GRK is expressed and localized at the later oogenesis stages in these alleles but the level is not detectable by our immunofluorescent assay.

From the above data it seems that the DExH box domain of SPN-E is quite important for embryonic axis specification. However, the Zn finger seems to be dispensable.
The DExH box domain is important for SPN-E function in Aubergine nuage localization

AUB and SPN-E co-localize to the nuage in wild type ovaries and AUB localization depends on SPN-E function (FINDLEY et al. 2003; PATIL and KAI 2010). Therefore, we determined if AUB was localized properly in the different spn-E mutant ovaries using immunohistochemistry. In order to compare mutant and wildtype expression/localization in the same ovary we generated clones of homozygous mutant egg chambers in an otherwise heterozygous background using the FLP/FRT technique (CHOU and PERRIMON 1996). Similar to what we saw with the D/V patterning phenotype we found that AUB localization is variably affected in the different spn-E mutant ovaries. We found that AUB is completely delocalized from the nuage in ovaries isolated from spn-E mutant flies that show the strongest D/V patterning phenotypes, in particular those alleles with DExH box mutations and the spn-E alleles that did not express detectable protein. Additionally, when we compared spn-E mutant egg chambers side by side with wildtype egg chambers it appeared that AUB levels were strongly reduced in these mutant egg chambers (Fig 2D,E, data not shown). spn-E 23-17^{H241Q} and 9A2-17^{P508L} females laid eggs with mid-range D/V patterning phenotypes and in ovaries isolated from these alleles AUB partially localized to the nuage, giving a more punctate localization than wildtype (Fig 2C). In ovaries isolated from the weaker spn-E alleles, 66-21^{H1422L} and 4-48^{E717K}, AUB was localized to the nuage as in wild type (Fig 2B). The AUB localization studies shown above were done by analyzing homozygous spn-E mutant clones. In order to show that the phenotype that we see is due to the
mutation in the *spn-E* gene and not a secondary background mutation we also analyzed AUB localization in hemizygous *spn-E* mutant ovaries using the respective *spn-E* allele in trans to the *spn-E* deficiency, Δ125 ([Fig 5S2](#)). We find similar results to those shown above, with the exception of the 4-48<sup>E717K</sup> allele. 4-48 hemizygous mutant egg chambers showed a little more punctate AUB localization than the clones, however AUB was still localized to a certain extent. Therefore, similar to what we saw with D/V patterning, ovaries expressing SPN-E with mutations in the DExH box domain give a similar phenotype to the strongest *spn-E* mutant phenotype, with AUB delocalized from the nuage, demonstrating the importance of the DExH box domain to SPN-E function.

**The DExH box domain of SPN-E is necessary for ovarian dynein dependent molecular transport**

In wildtype egg chambers dynein motor complex proteins are transported from the nurse cells to the oocyte ultimately leading to a high concentration of protein in the oocyte and a more diffuse pattern in the supporting nurse cells ([Fig 3A](#)). This is in contrast to piRNA pathway mutant egg chambers where large aggregates containing components of the dynein motor machinery form in the nurse cells ([NAVARRO et al. 2009](#)). These aggregates contain the dynein core motor complex as well as the accessory proteins, Egalitarian (EGL) and Bicaudal-D (BIC-D) and may be sites of retrotransposon sequestration or degradation. We examined ovaries from the different *spn-E* mutants and determined whether dynein aggregates form by immunohistochemistry using EGL as a marker for the aggregates. Similar to what we found with the D/V patterning phenotype; those
spn-E mutant flies that lay eggs with the most severe D/V patterning defects formed dynein motor complex aggregates (Fig 3D,E); while the spn-E mutants that had milder D/V patterning defects did not form ovarian dynein aggregates (Fig 3B,C). Additionally, in the mutants with the most severe dynein aggregation phenotype the oocyte failed to grow properly whereas in the less severe mutants the oocyte appeared to grow normally (Fig 3). The failure of oocyte growth could indicate a failure of the nurse cells to transport their contents into the oocyte, which could result in collapsed eggs. Indeed, the lack of oocyte growth we find correlates well with the percentage of collapsed eggs laid by the most severe mutants. As above we confirmed the mutant phenotypes that we see in homozygous spn-E mutant clones in hemizygous ovaries and find the same phenotypes (Fig S3).

**Mutations in the DExH box of SPN-E cause elevated ovarian retrotransposon levels**

Mutations in piRNA pathway proteins result in increased retrotransposon RNA levels in the Drosophila ovary (Malone et al. 2009; Vagin et al. 2006). We measured the RNA level of several germline specific and one somatic cell specific retrotransposon in the spn-E mutant ovaries using quantitative real-time RT-PCR (Fig 4, Fig S4,5). The germline specific retrotransposons included the non-LTR retrotransposons: HetA, TART and I Factor and the LTR retrotransposons: Blood and roo, while the somatic retrotransposon used was gypsy. We found a similar trend in phenotypic severity for germline retrotransposon RNA expression in the spn-E mutant ovaries as we found for the D/V patterning defects, AUB localization
and dynein aggregate formation that we described above. Ovaries from flies expressing SPN-E protein with mutations in the DExH box region had the highest ovarian retrotransposon RNA levels. Whereas, mutations outside of the DExH box region, including the mutation in the Zn finger motif, produced a more moderate phenotype. Interestingly, all alleles showed increased levels of the Blood and HetA retrotransposons to some extent, however, those spn-E mutants that gave the strongest developmental phenotypes showed considerably higher levels of HetA and Blood compared to the other alleles. For example spn-E<sup>155-55(E239K)</sup> ovaries had ~500x the level of Blood and ~200x the level of HetA retrotransposon RNA as heterozygous controls, whereas spn-E<sup>66-21(H1422L)</sup> ovaries had only ~40x the level of Blood and ~50x the level of HetA as the heterozygotes. Additionally, only those alleles that gave the strongest developmental phenotypes also had high levels of I Factor, TART and roo. SPN-E has previously been reported to function only in the ovarian germline cells, therefore we did not expect to see an effect on the somatic retrotransposon gypsy (MALONE et al. 2009). However, we did detect a slight increase in gypsy expression in most of the spn-E alleles that we examined. The level of expression was significantly lower than that seen in the dSETDB1 mutant, egg. dSETDB1 function is required in both the somatic and germline cells of the ovary, however, its silencing of gypsy transcription has been attributed to its somatic function (RANGAN et al. 2011). Therefore, it is possible that SPN-E may have a function in silencing retrotransposons in the somatic ovary cells or gypsy may also be expressed in the germline cells of the ovary. In support of our data Malone et al., showed a slight decrease in gypsy piRNA ping-pong pairs in spn-E
mutant ovaries (MALONE et al. 2009). From the above data it seems likely that the resulting piRNA phenotypes are due to the upregulation of multiple retrotransposons.

As mutations in the DExH box region of SPN-E produce a similar phenotype to the strongest phenotypes ascribed to spn-E mutant ovaries in all of the assays we used, our cumulative data point to an important role for the DExH box region in SPN-E function during Drosophila oogenesis.

**DISCUSSION**

We characterized 14 spn-E mutant alleles using multiple phenotypic criteria and identified point mutations in the spn-E gene in these alleles. Our results strongly indicate that the DExH box helicase domain of SPN-E is necessary for function, whereas the Zn finger domain seems to be dispensable at least for the functions assessed here.

**The DExH box domain of SPN-E is necessary for function during oogenesis**

Our sequencing efforts identified five spn-E alleles with mutations in the DExH box domain. Three of the amino acids affected by these mutations have been shown to be important for DExH box helicase activity in other DExH box containing proteins. These include mutations in two motifs necessary for ATP hydrolysis and binding, motifs I and II. The mutation that we identified at amino acid 145 within motif I changes a Thr, a polar uncharged amino acid, to an Ile, a nonpolar hydrophobic amino acid. This amino acid seems to be critical for SPN-E function as mutant ovaries display a severe oogenesis phenotype. Previous mutational
studies of vaccinia virus Nucleoside Triphosphate Phosphohydrolase I (NPH-I) showed that an amino acid with a –OH group at the analogous position in the NPH-I DExH box is necessary for its ATPase activity (Martins et al. 1999). For example, when Thr62 of NPH-I is changed to an Ala or Val, both of which are nonpolar hydrophobic amino acids, the ATPase activity of NPH-I is decreased dramatically. However, when Thr62 is changed to Ser, an uncharged polar amino acid with a –OH group, ATPase activity was not affected. This finding is also supported by mutational analysis of the yeast splicing factor, Prp16, where expression of the protein with a change of Thr380 to either Ala or Val leads to lethality whereas a change of Thr380 to Ser does not affect yeast growth (Hotz and Schwer 1998).

Less severe phenotypes resulted from mutations in the DExH box motif itself. Mutation of Glu239 to Lys resulted in a moderate phenotype, while mutation of His241 to Gln resulted in an even milder phenotype. Mutational analysis of Glu140 within the NPH-I DExH box showed that an acidic side chain at this position is necessary for ATP hydrolysis (Martins et al. 1999). This is also true for the yeast splicing factor, Prp16 (Hotz and Schwer 1998). Our analysis agrees with this conclusion as the change we identified in SPN-E is a substitution of Glu, an acidic amino acid to Lys, a polar basic amino acid. The mutation at amino acid 241 results in a change from His to Gln. His and Gln are partially isosteric, which may account for the less severe phenotype. Mutational analysis of NPH-I also showed a similar result where a His to Gln change decreased ATPase activity to 42% whereas a more dramatic effect was seen when His was changed to Ala or Asp.
Again, this is also in agreement with changes in the comparable amino acid of Prp16 (HOTZ and SCHWER 1998; MARTINS et al. 1999).

We also have identified mutations in two uncharacterized amino acids within the DExH box domain. This includes a Ser to Phe change at amino acid 435 in motif V, which has been implicated in RNA binding (ROCAK and LINDE 2004). This mutation gave one of the strongest phenotypes, indicating that this amino acid is critical for SPN-E function and is consistent with the proposal that SPN-E interacts with RNA.

Our results indicate that the DExH box domain of SPN-E is necessary for retrotransposon silencing and oocyte patterning. Given that DExH box domains have helicase activity and work to change nucleic acid conformation how could the DExH box in SPN-E contribute to its function in piRNA biogenesis? Previous reports showed that in spn-E mutant ovaries the levels of all piRNAs are depleted and that piRNA ping-pong piRNAs are not generated (MALONE et al. 2009). Therefore, it is possible that SPN-E could function to change piRNA structure to make the RNA more accessible to other piRNA pathway proteins such as nucleases or chaperones at one or multiple points in the biogenesis pathway. This could include binding to and unwinding: 1) pre-piRNAs in order for primary piRNA processing to occur, 2) secondary piRNAs for incorporation into the silencing/cleavage complex or 3) retrotransposon RNAs for cleavage by Aub or Ago3. Alternatively, SPN-E could function indirectly in piRNA biogenesis by affecting the translation of a key piRNA pathway protein such as AUB. Given that AUB levels are reduced in spn-E mutant egg chambers it seems that SPN-E
function may be necessary to maintain AUB protein levels. Therefore, it is possible that SPN-E functions in AUB translation. However, it is just as likely that SPN-E function may be necessary for AUB stabilization. Given that AUB binds to piRNAs it is possible that without piRNAs AUB may become unstable. Therefore, because piRNA levels are low in spn-E mutant ovaries AUB may be degraded due to lack of piRNA binding. Alternatively, SPN-E may associate with AUB either in conjunction with or after piRNA processing. Without piRNA generation SPN-E and AUB may not form a complex leading to AUB destabilization.

Interestingly, mutation of amino acid 1422, which is located in the putative Zn finger produced a very mild phenotype with 92% of eggs laid being wildtype. The mutation that we identified is in an amino acid crucial for Zn coordination, indicating that this mutation would most likely disrupt Zn finger activity (PABO et al. 2001). The Zn finger seems to be unique to Drosophila as we did not find a Zn finger in SPN-E homologs such as TDRD9 from humans, mouse and zebrafish using the protein sequences deposited in the Ensemble Genome Browser release 73 (Fig 1A). These results indicate that Zn finger activity may not be important for SPN-E function in embryonic patterning.

**SPN-E may have functions independent of piRNA biogenesis**

We found that some spn-E alleles cause elevated levels of all of the retrotransposons that we examined, whereas others caused elevated levels of only HetA and/or Blood. Blood and HetA seem to be the most sensitive elements to piRNA pathway perturbation (LIM and KAI 2007; SIENSKI et al. 2012; VAGIN et al.
2006). This could be due to their placement within the piRNA clusters or perhaps, differential sensitivity of the clusters themselves to piRNA pathway perturbations. The alleles that caused only a mild elevation of a subset of retrotransposon RNA consistently produced weaker developmental phenotypes. It is possible that the higher levels of retrotransposon RNA that we found in the weaker alleles may be due to decreased SPN-E protein levels and that retrotransposon silencing is a more sensitive readout for changes in SPN-E function than the developmental phenotypes that we examined. For the most part, there is good correlation between elevated ovarian retrotransposon levels and the severity of the \textit{spn-E} phenotype, with those alleles that have high levels of all retrotransposons showing the strongest phenotypes.

The one exception to the above statement is \textit{spn-E}^{4-48}. \textit{Spn-E}^{4-48} ovaries only show a slight increase in retrotransposon levels yet the eggs laid by these females have a moderate D/V phenotype. Most piRNA pathway mutant ovaries have an active Chk-2 dependent DNA damage checkpoint (KLATTENHOFF \textit{et al.} 2007; PANE \textit{et al.} 2007). It is thought that checkpoint activation is due to the massive amounts of DNA double strand breaks that occur from elevated retrotransposition in these ovaries. However, this has not been directly shown. It is known that activation of the checkpoint leads to embryonic patterning defects for most piRNA pathway mutants (KLATTENHOFF \textit{et al.} 2007; PANE \textit{et al.} 2007). This does not seem to be the case for \textit{spn-E}, however, as the D/V patterning defects of \textit{spn-E} mutants are not suppressible by checkpoint inhibition (PANE \textit{et al.} 2007). Therefore, SPN-E probably has functions in addition to its function in piRNA biogenesis. One of these
may be to control cytoplasmic streaming, as premature cytoplasmic streaming has been reported for certain spn-E mutant alleles (MARTIN et al. 2003). orb (oo18 RNA binding protein), a Cytoplasmic Polyadenylation Element binding protein that functions in translational regulation, mutant ovaries also show premature cytoplasmic streaming similar to what is seen in spn-E mutant egg chambers (MARTIN et al. 2003). The streaming defects could cause grk RNA mislocalization that, in turn, would lead to embryonic patterning defects. Orb levels are reduced in spn-E mutant ovaries, therefore it is possible that Orb may be either a direct or indirect target of SPN-E function (MARTIN et al. 2003).

Additionally, it has been shown that in I-R hybrid dysgenic crosses where the only retrotransposon that is upregulated is I Factor, the DNA damage checkpoint is not activated yet moderate D/V patterning defects are seen in the eggs laid by the I-R dysgenic females (ORSI et al. 2010). The D/V patterning phenotype in IR dysgenic eggs is similar to what we saw for spn-E^{4-48} eggs. Additionally, the retrotransposon that is most affected in spn-E^{4-48} ovaries is I Factor. The D/V defects that arise in eggs laid by dysgenic females are thought to arise from a competition of I Factor and grk RNA for access to the microtubule motor machinery, which leads to the displacement of grk RNA and the resulting D/V patterning defects (VAN DE BOR et al. 2005). It will be interesting to determine whether the DNA double strand break checkpoint is activated in spn-E^{4-48} ovaries and why I Factor levels are the most impacted by the spn-E^{4-48} mutation.

In addition to D/V patterning and checkpoint activation it seems that dynein aggregate formation is also sensitive to retrotransposon levels. We find that in
spn-E mutants that have only a slight increase in retrotransposon levels dynein aggregates do not form. This is also true in I-R dysgenic ovaries (ORSI et al. 2010). Dynein aggregate formation is due to activation of the Chk-2 checkpoint in spn-E mutant ovaries (NAVARRO et al. 2009). Therefore, dynein aggregate formation, as well as checkpoint activation, is sensitive to ovarian retrotransposon levels.

Our experiments have identified several mutations in the DExH box helicase domain of SPN-E implicating helicase function as important for SPN-E function, especially in the piRNA pathway. Although these mutations could affect protein folding we think that the mutations we have identified affect protein function rather than protein structure since the ovarian protein levels for several of these new alleles were close to wildtype. It is interesting to note that we did not obtain mutations in the highly conserved Tudor domain. It is possible that mutations in the Tudor domain could render SPN-E unstable. However, it is curious that the 616R1081* allele has a mutation that causes a premature stop after the Tudor domain, whereas the rest of the alleles with premature stop codons would cause protein truncation before the Tudor domain. This allele has a weaker D/V patterning phenotype than these other alleles, indicating that if this allele does produce protein, the Tudor domain may be an important functional domain of SPN-E. A more directed mutagenesis approach may be necessary to determine the relevance of the Tudor domain for SPN-E function.

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LITERATURE CITED


Rehm, E., Inverse PCR & Cycle Sequencing of P Element Insertions for STS Generation, pp. in BDGP Resources.


FIGURE LEGENDS

Figure 1. Eight of the fourteen spn-E alleles express detectable protein and have point mutations in the SPN-E coding region. (A) Domain structure of Drosophila SPN-E and its human homolog, TDRD9. SPN-E contains a highly conserved DExH box and a Tudor domain as well as a Zinc Finger, whereas TDRD9 only has a DExH box and Tudor domain. The position of the two mutations outside of the conserved domains, the five mutations that do not produce detectable protein as well as in the Zinc Finger are shown. (B) The amino acid sequence of the SPN-E DExH box domain compared to its human homolog TDRD9, yeast splicing factor Prp16, and vaccinia virus protein NPH-I. The positions of the five mutations identified in the SPN-E DExH box domain are shown. Amino acid numbering is according to Ensemble Genome Browser release 73. (C) SPN-E protein expression in mutant ovary extracts as measured by western blotting. Protein was isolated from hemizygous ovaries of the genotype spn-E\textsuperscript{mutant}/spn-E\textsuperscript{A\textsubscript{125}}. Eight alleles express detectable protein of the correct size for SPN-E. Four alleles do not express detectable protein. Spn-E/Bal = spn-E\textsuperscript{A\textsubscript{125}}/Balancer chromosome. Line 7G2-5 is not shown. Several extraneous bands are found on the western blots shown above. We did not detect these bands when we used a second antibody developed in the lab of Dr. Toshie Kai (PATIL and KAI 2010, data not shown), therefore we think that the extra bands are most likely non-specific bands recognized by our SPN-E antibody. (D) SPN-E protein levels in the various mutant ovaries relative to spn-E\textsuperscript{A\textsubscript{125}}/Balancer. Error bars represent standard deviation of at least 2 separate protein isolates. SPN-E protein levels were
normalized to beta-tubulin. (E) A listing of each spn-E allele name along with its corresponding mutation.

**Figure 2.** AUB nuage localization is lost in some, but not all of the spn-E mutant egg chambers. spn-E mutant germline clones are marked by the absence of GFP. All egg chambers were stained with α-GFP (green), α-AUB (red), and DAPI to mark the DNA. In wild type egg chambers AUB localizes around the nurse cell nuclei to a structure known as the nuage (A-A”). spn-E4-48 (B-B”), and spn-E66-21 (not shown) show wild type localization of AUB to the nuage. spn-E23-17 (C-C”) and spn-E9A2-17 (not shown) show an intermediate phenotype where AUB expression is punctate and only partially localized to the nuage (C’, chamber outlined). In the spn-E155-55 DExH box mutant allele (D-D”) as well as most of the other DExH box alleles (not shown) AUB is not localized to the nuage and levels of AUB protein appear to be strongly decreased in mutant egg chambers (D’, outlined). This phenotype is also seen in spn-E9A9-18 mutant egg chambers (E-E”) as well as the remainder of the spn-E alleles that do not express detectable protein (not shown). Scale bars = 20µm.

**Figure 3.** Dynein motor complex aggregates form in some, but not all spn-E mutant ovaries. spn-E mutant germline clones are marked by the absence of GFP. All egg chambers were stained with α-GFP (green) to mark clones, α-Egalitarian (EGL) (red), and the DNA dye DAPI. In wildtype egg chambers, EGL is dispersed
throughout the nurse cells and localizes to the oocyte (A-A’’). *spn-E*1-49 (B-B’’), *spn-E*23-17 (C-C’’), as well as *spn-E*9A2-17 and *spn-E*66-21 (not shown) show wildtype EGL localization. In *spn-E*155-55 DExH box mutant egg chambers (D-D’’) EGL forms aggregates throughout the egg chamber. This phenotype is present in *spn-E*9A9-18 mutant egg chambers (E-E’’) as well as the DExH box alleles: *spn-E*2A9-14, *spn-E*7G2-5, *spn-E*8D4-11, and the remainder of the *spn-E* alleles that do not express detectable protein (not shown). Note the small size of the oocyte in *spn-E*155-55 and *spn-E*9A9-18 egg chambers (arrow in D’ and E’). Scale bars = 20µm.

**Figure 4.** Retrotransposon RNA levels are increased to varying degrees in the various *spn-E* mutant ovaries. (A) Quantitative real time RT-PCR for the retrotransposons Het-A and Blood using extracts from mutant ovaries (*spn-E*mutant/*spn-E*Δ125). (B) Quantitative real time RT-PCR for retrotransposons I Factor, TART, and Roo. Relative expression was calculated in comparison to respective RNA levels obtained from heterozygous siblings for each individual allele. All RNA was normalized to Adh. Error bars represent standard deviation of four experiments using two independent RNA isolates.
<table>
<thead>
<tr>
<th>Allele Name</th>
<th>SPN-E Protein</th>
<th>Mutation</th>
<th>Affected Domain</th>
<th>D/V Defects</th>
<th>Retrotransposon Expression</th>
<th>Dynein Aggregate Formation</th>
<th>Aub Nuage Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>2A9-14 +</td>
<td>+ Thr145lle</td>
<td>DExH Box (I)</td>
<td>severe</td>
<td>↑↑↑</td>
<td>yes</td>
<td>delocalized</td>
<td></td>
</tr>
<tr>
<td>7G2-5 +</td>
<td>+ Thr233lle</td>
<td>DExH Box (I/II)</td>
<td>severe</td>
<td>↑↑↑</td>
<td>yes</td>
<td>delocalized</td>
<td></td>
</tr>
<tr>
<td>155-55 +</td>
<td>+ Glu239Lys</td>
<td>DExH Box (II)</td>
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<td>↑↑↑</td>
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<tr>
<td>23-17 +</td>
<td>+ His241Gln</td>
<td>DExH Box (II)</td>
<td>mild</td>
<td>↑</td>
<td>no</td>
<td>partial</td>
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</tr>
<tr>
<td>8D4-11 +</td>
<td>+ Ser435Phe</td>
<td>DExH Box (V)</td>
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<td>↑↑↑</td>
<td>yes</td>
<td>delocalized</td>
<td></td>
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<tr>
<td>9A2-17 +</td>
<td>+ Pro508Leu</td>
<td>None</td>
<td>mild</td>
<td>↑</td>
<td>no</td>
<td>partial</td>
<td></td>
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<tr>
<td>4-48 +</td>
<td>+ Glu717Lys</td>
<td>None</td>
<td>mild</td>
<td>↑</td>
<td>no</td>
<td>Nuage</td>
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<tr>
<td>66-21 +</td>
<td>+ His1422Leu</td>
<td>Zn Finger</td>
<td>mild</td>
<td>↑</td>
<td>no</td>
<td>Nuage</td>
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<td>100-37 -</td>
<td>G→A bp 488</td>
<td>Truncated at aa 151</td>
<td>mid</td>
<td>↑↑↑</td>
<td>yes</td>
<td>Delocalized</td>
<td></td>
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<tr>
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<td>Arg918stop</td>
<td>Truncated at aa 918</td>
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<tr>
<td>9A9-18 -</td>
<td>Trp632stop</td>
<td>Truncated at aa 632</td>
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<td>↑↑↑</td>
<td>yes</td>
<td>Delocalized</td>
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<td>4E2-14 -</td>
<td>Arg695stop</td>
<td>Truncated at aa 695</td>
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<td>Arg1081stop</td>
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<tr>
<td>653 -</td>
<td>Tyr636stop</td>
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<td>↑↑↑</td>
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<td>Delocalized</td>
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+ or – for SPN-E protein indicates (+) SPN-E protein detected (-) SPN-E protein not detected on western blot analysis. Number in () next to DExH Box indicates the DExH box motif affected. Severe D/V defects indicate mostly collapsed eggs laid by mutant females, mid indicates the majority of eggs laid were collapsed however, a small percent have wildtype or fused dorsal appendages, mild indicates the majority of eggs laid were wildtype. ↑↑↑ indicates all retrotransposons were upregulated in the mutant ovaries, ↑↑ indicates that at least two retrotransposons tested were upregulated, ↑ indicates that one or no retrotransposons were upregulated in the mutant ovaries. bp numbering for 100-37 according to Ensemble Genome Browser. ND – not determined.
Table 2. Most *spn-E* mutant females lay eggs with Dorsal/Ventral patterning defects.

<table>
<thead>
<tr>
<th>Allele Name</th>
<th>% Wildtype</th>
<th>% Fused</th>
<th>% None</th>
<th>% Collapsed</th>
<th>Total Eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>spn-E</em>/<em>Bal</em></td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>600+</td>
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**Alleles with mutation in DExH box**

<table>
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<th>Allele</th>
<th>% Wildtype</th>
<th>% Fused</th>
<th>% None</th>
<th>% Collapsed</th>
<th>Total Eggs</th>
</tr>
</thead>
<tbody>
<tr>
<td>2A9-14</td>
<td>0</td>
<td>.48 +/- 0.078</td>
<td>5.9 +/- 3.5</td>
<td>93.7 +/- 3.6</td>
<td>428</td>
</tr>
<tr>
<td>7G2-5</td>
<td>4.3 +/- 3.3</td>
<td>3.9 +/- 1.9</td>
<td>3.4 +/- 2.6</td>
<td>88.5 +/- 7.8</td>
<td>759</td>
</tr>
<tr>
<td>155-55</td>
<td>5.7 +/- 2.4</td>
<td>12.1 +/- 2.5</td>
<td>18.5 +/- 9.3</td>
<td>63.7 +/- 9.5</td>
<td>742</td>
</tr>
<tr>
<td>23-17</td>
<td>54.7 +/- 9.1</td>
<td>31.5 +/- 2.1</td>
<td>10.7 +/- 2.5</td>
<td>4.2 +/- 3.3</td>
<td>970</td>
</tr>
<tr>
<td>8D4-11</td>
<td>0</td>
<td>0</td>
<td>0.95 +/- 0.13</td>
<td>99.1 +/- 1.3</td>
<td>181</td>
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**Alleles with mutation outside of predicted domains**

<table>
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<tr>
<th>Allele</th>
<th>% Wildtype</th>
<th>% Fused</th>
<th>% None</th>
<th>% Collapsed</th>
<th>Total Eggs</th>
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<tbody>
<tr>
<td>9A2-17</td>
<td>59.4 +/- 23.5</td>
<td>37.9 +/- 21.6</td>
<td>2.6 +/- 1.7</td>
<td>.15 +/- 0.21</td>
<td>771</td>
</tr>
<tr>
<td>4-48</td>
<td>68.4 +/- 12.8</td>
<td>28.9 +/- 13.1</td>
<td>2.4 +/- 0.042</td>
<td>.31 +/- 0.016</td>
<td>750</td>
</tr>
<tr>
<td>66-21</td>
<td>92.1 +/- 1.9</td>
<td>7.2 +/- 1.8</td>
<td>.56 +/- 0.33</td>
<td>.16 +/- 0.23</td>
<td>1129</td>
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**Allele with mutation in Zn finger**

<table>
<thead>
<tr>
<th>Allele</th>
<th>% Wildtype</th>
<th>% Fused</th>
<th>% None</th>
<th>% Collapsed</th>
<th>Total Eggs</th>
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<tr>
<td>100-37</td>
<td>1.8 +/- 2.0</td>
<td>11.5 +/- 3.8</td>
<td>28.2 +/- 4.6</td>
<td>58.6 +/- 10.3</td>
<td>630</td>
</tr>
<tr>
<td>114-33</td>
<td>0.24 +/- 0.34</td>
<td>4.3 +/- 0.99</td>
<td>12.1 +/- 7.6</td>
<td>83.4 +/- 8.3</td>
<td>677</td>
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<tr>
<td>9A9-18</td>
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<td>0</td>
<td>100</td>
<td>410</td>
</tr>
<tr>
<td>4E2-14</td>
<td>0.36 +/- 0.5</td>
<td>2.3 +/- 2.2</td>
<td>6.25 +/- 2.8</td>
<td>91.1 +/- 0.07</td>
<td>414</td>
</tr>
<tr>
<td>616</td>
<td>0.1 +/- 0.14</td>
<td>31.5 +/- 7.8</td>
<td>20 +/- 1.4</td>
<td>48 +/- 9.9</td>
<td>687</td>
</tr>
<tr>
<td>653</td>
<td>4 +/- 0.0</td>
<td>14 +/- 4.2</td>
<td>9.5 +/- 3.5</td>
<td>77 +/- 14.1</td>
<td>414</td>
</tr>
</tbody>
</table>

Dorsal-ventral patterning defects were quantitated by collecting eggs from *spn-Emutant/*spn-E<sup>125</sup>* female flies and determining the percentage of eggs with two dorsal appendages (wild type), fused dorsal appendages, no dorsal appendages, and eggs that were collapsed. Calculations are from two separate experiments, each consisting of three 24 hour egg collections, with the exception of 653 where the data was from two separate experiments, each consisting of two 24 hour egg collections.
Fig 1. Ott et al.
Fig 4. Ott et al.