Development 138, 1759-1769 (2011) doi:10.1242/dev.058420

The *Drosophila* STUbL protein Degringolade limits HES functions during embryogenesis

Kevin C. Barry[1,*], Mona Abed[2,*], Dorit Kenyagin[2], Timothy R. Werwie[1], Olga Boico[2], Amir Orian[2,†] and Susan M. Parkhurst[1,†]

SUMMARY

Degringolade (Dgrn) encodes a *Drosophila* SUMO-targeted ubiquitin ligase (STUbL) protein similar to that of mammalian RNF4. Dgrn facilitates the ubiquitylation of the HES protein Hairy, which disrupts the repressive activity of Hairy by inhibiting the recruitment of its cofactor Groucho. We show that Hey and all HES family members, except Her, interact with Dgrn and are substrates for its E3 ubiquitin ligase activity. Dgrn displays dynamic subcellular localization, accumulates in the nucleus at times when HES family members are active and limits Hey and HES family activity during sex determination, segmentation and neurogenesis. We show that Dgrn interacts with the Notch signaling pathway by it antagonizing the activity of E(spl)-C proteins. *dgrn* null mutants are female sterile, producing embryos that arrest development after two or three nuclear divisions. These mutant embryos exhibit fragmented or decondensed nuclei and accumulate higher levels of SUMO-conjugated proteins, suggesting a role for Dgrn in genome stability.

KEY WORDS: STUbL, RING finger, RNF4, Genome stability, HES, PNS, Sex determination, *Drosophila*

INTRODUCTION

The HES family of basic-helix-loop-helix (bHLH) proteins function as dedicated transcriptional repressors that are important for regulating many biological processes, including sex determination, segmentation, myogenesis, somitogenesis, vasculogenesis, mesoderm formation and neurogenesis (for reviews, see Davis and Turner, 2001; Kageyama et al., 2007). Misregulation of HES family members has been linked to developmental defects and oncogenesis (Davis and Turner, 2001).

In *Drosophila*, the HES family of proteins consists of 11 proteins, including Hairy, seven members of the Enhancer of Split complex [E(spl)-C; -m8, -m7, -m5, -m3, -mβ, -mδ, -mγ], Deadpan (Dpn), Similar to Deadpan (Side) and HES-related (Her) (Davis and Turner, 2001). A closely related clade encodes Hairy/E(spl)-related with YRPW motif (Hey) (Moore et al., 2000). Dpn acts as an autosomal counting protein that represses the transcription of the master sex regulator gene *Sex lethal* (*Sxl*) (Younger-Shepherd et al., 1992; Estes et al., 1995). Recently, Hey and Her were also shown to have roles in controlling the expression of Sxl (Lu et al., 2008). Hairy has been shown to mimic the activity of Dpn when ectopically expressed even though it normally does not have a role in sex determination (Parkhurst et al., 1990; Parkhurst and Ish-Horowicz, 1992). Endogenous *hairy* functions as a primary pair-rule gene that controls reiterative patterning in *Drosophila* as well as in other invertebrates and vertebrates (Carroll and Scott, 1986; Howard and Ingham, 1986; Davis and Turner, 2001). In addition to segmentation, Hairy is also required for peripheral nervous system development, where it negatively regulates the expression of *achaete* (*ac*), a proneural bHLH activator gene (Ohsako et al., 1994; Van Doren et al., 1994). Dpn and the E(spl)-C proteins play important roles in neurogenesis. A subset of the E(spl)-C genes are activated by Notch signaling and, along with the Notch-independent proteins Hairy and Dpn, block neuronal differentiation by inhibiting the proneural activity of a number of bHLH activators, such as Daughterless and those of the Achaete-Scute complex (Wurmbach et al., 1999; Davis and Turner, 2001; Fischer and Gessler, 2007; Krejcí et al., 2009).

HES and Hey proteins are sequence-specific DNA binding proteins that, like most transcriptional repressors, recruit cofactors to facilitate transcriptional repression (Davis and Turner, 2001; Bianchi-Frias et al., 2004). HES family members recruit different classes of cofactors. Some cofactors, such as the WD domain-containing cofactor Groucho (Gro), are recruited by all HES family members; whereas others, including C-terminal Binding Protein (CtBP), Silent Information Regulator 2 (Sir2), and Topoisomerase I-Interacting Protein (Topors), are recruited by a subset or specific family members (Paroush et al., 1994; Poortinga et al., 1998; Rosenberg and Parkhurst, 2002; Secombe and Parkhurst, 2004; Buscarlet and Stifani, 2007).

Recently, a new regulator of Hairy function was identified named Degringolade (Dgrn; meaning 'to deteriorate rapidly'), which encodes a RING finger protein with E3 ubiquitin ligase activity that is a member of a subclass of E3 ubiquitin ligases called SUMO-targeted ubiquitin ligase (STUbL) proteins (Abed et al., 2011). STUbL proteins recognize the small ubiquitin-like modifier (SUMO) moiety of SUMOylated proteins via their SUMO interaction motif (SIM) domains and facilitate the transfer of ubiquitin to the SUMOylated protein (c.f. Perry et al., 2008; Wilson and Heaton, 2008). Dgrn binds physically to the basic domain of Hairy, a domain that is well conserved in Hey and all of the HES family members. Consistent with this, Dgrn interacts genetically with Hairy and has been proposed to inhibit Hairy-mediated repression by ubiquitylating Hairy such that it can no longer recruit its cofactor Gro and by targeting SUMOylated Gro for sequestration (Abed et al., 2011).

[1]Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA. [2]Cancer and Vascular Biology Research Center, Faculty of Medicine, Technion-Israel Institute of Technology, Haifa 31096, Israel.

*These authors contributed equally to this work
†Authors for correspondence (mdoryan@techunix.technion.ac.il; susanp@fhcrc.org)

Accepted 1 February 2011

Here, we show that Dgrn limits the activity of Hey and all HES family members, except Her, in vivo. Dgrn/RNF4 interacts physically with the Hairy and Dpn HES proteins and they are substrates for its E3 ubiquitin ligase activity. A null mutation for Dgrn is female sterile with embryos from homozygous null mothers arresting after only two or three nuclear divisions and exhibiting accumulation of SUMOylated proteins, probably owing to a conserved role for Dgrn in genome stability. Genetic experiments using this null mutation indicate that Dgrn is essential for sex determination, neurogenesis and segmentation, all of which require the activity of HES family members. As shown for the interaction of Dgrn with Hairy during segmentation, Dgrn interferes with Gro-mediated repression during sex determination, thereby de-repressing transcription of Sxl. Additionally, Dgrn interacts genetically with the Notch signaling pathway and, consistent with Dgrn's roles in segmentation and sex determination, directly antagonizes the E(spl)-C proteins, direct effectors of Notch signaling.

MATERIALS AND METHODS

Fly strains and genetics

Flies were cultured and crossed on yeast-cornmeal-molasses-malt extract medium at 25°C. The alleles used in this study were: w, pCog-GAL4::VP16; p[GAL4-nos.NGT40]40; p[GAL4::VP16-nos.UTW]MVD1 (Grieder et al., 2000), PBac{WH}CG10981^{f05920} (Exelixis Collection at Harvard), His2AvD (Clarkson and Saint, 1999), sGMCA (Kiehart et al., 2000), SxlPe-LacZ$_{3.0kb}$ (Estes et al., 1995), N^{1}, N^{AX1682}, and P{w[+mW.hs]=GawB}C253 (Bloomington Stock Center). The $dgrn^{DK}$ deletion was made by the imprecise excision of the EY09862 element inserted in the first exon, 150 bp before the translation start site of Dgrn. The $dgrn^{DK}$ deletion is a 1368 bp deletion starting 150 bp upstream of the ATG and was confirmed by sequencing. GFP-Dgrn transgenic flies contain a GFP-Dgrn fusion gene that is expressed under the control of the endogenous Dgrn promoter. A 2822 bp *Kpn*I/*Xba*I genomic fragment was cloned into the pCaSpeR4 transformation vector and GFP was fused in-frame at the ATG of Dgrn. The resulting GFP-Dgrn fusion constructs were used to make germline transformants. UAS-Dgrn (1-319aa) transgenic flies were made by cloning the full length Dgrn ORF (960 bp) into pUASp as an *Xba*I/*Xho*I fragment. UAS-Dgrn^{I268A} and UAS-Dgrn$^{HC/AA}$ (H300A+C302A) point mutations were generated from the full length Dgrn construct using primers that substitute alanines for the amino acids indicated and were cloned into pUASp as *Xba*I/*Xho*I fragments. UAS-Dgrn$^{\Delta SIMs}$ was generated by deletion of 75-87 aa, 181-184 aa, 202-210 aa and 239-242 aa from the wild-type Dgrn cDNA and cloned into the UASp vector. All pUASp and pCaSpeR4 constructs were used to make germline transformants as described previously (Spradling, 1986). All transformant lines used in this study were mapped to a single chromosome and shown to have non-lethal insertions.

Plasmids and constructs

The following constructs were used: Dgrn (1-319 aa), Her (1-149 aa), Hey (1-425 aa) and Side (1-507 aa). Dgrn^{I268A}, Dgrn$^{HC/AA}$ and UAS-Dgrn$^{\Delta SIMs}$ were generated as described above. These constructs were cloned into pCite, pGEX and/or pUASp vectors using standard PCR cloning techniques.

Protein expression and GST pull-down assays

Protein expression and GST pull-down assays were performed as described previously (Linardopoulou et al., 2007; Poortinga et al., 1998; Rosales-Nieves et al., 2006).

Northern analysis

Northern blot analysis was performed as described previously (Poortinga et al., 1998). RNA was isolated from ovaries and 0- to 4-hour-old embryos.

Antibody production and characterization

Polyclonal mouse antiserum against Dgrn was generated by immunizing BALB/c BYJ Rb(8.12) 5BNR/J mice (Jackson Labs) with a GST fusion to Dgrn (1-319 aa). Western blotting was used to test antibody specificity against endogenous protein in whole-cell, nuclear and S2 cell extracts (Fig. 1). Wild-type *Drosophila* whole-cell (from 0- to 2-hour-old embryos) and nuclear (from 0- to 12-hour-old embryos) extracts were gifts from Toshi Tsukiyama (Fred Hutchinson Cancer Research Center, Seattle, WA, USA).

Immunofluorescence and western blots

Immunofluorescence, confocal microscopy and live imaging of embryos were performed as described previously (Rosales-Nieves et al., 2006; Liu et al., 2008). The following antibodies were used in this study: anti-Bcd (1:100; J. Reinitz, Stony Brook University, Stony Brook, NY, USA); anti-Kr (1:100, J. Reinitz); anti-Kni (1:100, J. Reinitz); anti-Hb (1:300, J. Reinitz); anti-Hairy (1:200, J. Reinitz); anti-Ftz (1:100, J. Reinitz); anti-Dgrn (1:200); anti-En [1:10, Developmental Studies Hybridoma Bank (DSHB)]; anti-Cnn (1:200; T. Kaufman, University of Indiana, Bloomington, IN, USA); anti-γ-tubulin (1:1000, Sigma); FITC-anti-tubulin (1:1000, Sigma); anti-Lamin/ADL67.10 (1:10, DSHB); 22C10 (1:20, DSHB); anti-SUMO2 (1:100, Invitrogen); anti-actin (1:1000, MP Biomedicals); and anti-Sxl/M114 (1:10, DSHB). DAPI was used at 1 μg/ml.

Reporter and in vitro ubiquitylation assays

The Sxl-P$_E$ reporter assay system was described previously (Hoshijima et al., 1995). Luciferase and Renilla (control) activity were assayed using the Dual Reporter Assay (Promega) 48 hours after transfection of *Drosophila* Kc cells as described (Abed et al., 2011). In vitro ubiquitylation assays with ubiquitin were carried out as described previously (Ben-Saadon et al., 2006).

RESULTS

Dgrn interacts physically with Hey and all HES family members except Her

Dgrn interacts directly with the basic domain of bHLH protein Hairy (Abed et al., 2011). As the basic domain is highly conserved among Hey and all HES family members (Fig. 1A), we examined the ability of Dgrn to interact with Hey and the remaining HES family members using GST pulldown assays. As expected, we found that Dgrn interacts physically with Hey and all HES family members, with the exception of HES-related (Her) (Fig. 1B,D). Dgrn also bound to a subset of activator bHLH proteins (see Fig. S1 in the supplementary material). Consistent with these interactions, Hey and all HES family members except Her were substrates for the E3 ubiquitin ligase activity of Dgrn (see Fig. S1 in the supplementary material; data not shown). This activity is functionally conserved as the mammalian ortholog of Dgrn, RNF4, can ubiquitylate *Drosophila* Hey and HES proteins (see Fig. S1 in the supplementary material; data not shown) (Abed et al., 2011).

Dgrn displays dynamic subcellular localization, but is present in the nucleus at times when Hey and HES family members are known to be active

Northern blot analysis indicated that a Dgrn transcript is expressed in both the adult ovary and the developing embryo (Fig. 1F). To investigate the biological role of Dgrn in *Drosophila* embryogenesis we generated a mouse polyclonal antibody using a GST-tagged full length Dgrn protein. This polyclonal antibody recognizes specifically Dgrn protein in nuclear and whole-cell extracts, as well as full-length protein in embryo or S2 cell extracts (Fig. 1G,H). Wild-type embryos stained with this antibody exhibited cytoplasmic subcellular Dgrn localization in the early embryo (Fig. 2A-I″″). In addition, Dgrn also accumulated in the nucleus at specific stages, including nuclear cycles 5, 9, 12 and 14A (Fig. 2A-A″″,C-C″″,F-F″″,H-H″″). In general, stages in which Dgrn was present in the nucleus correspond to times when HES family members are known to be active. At nuclear cycle 12, the

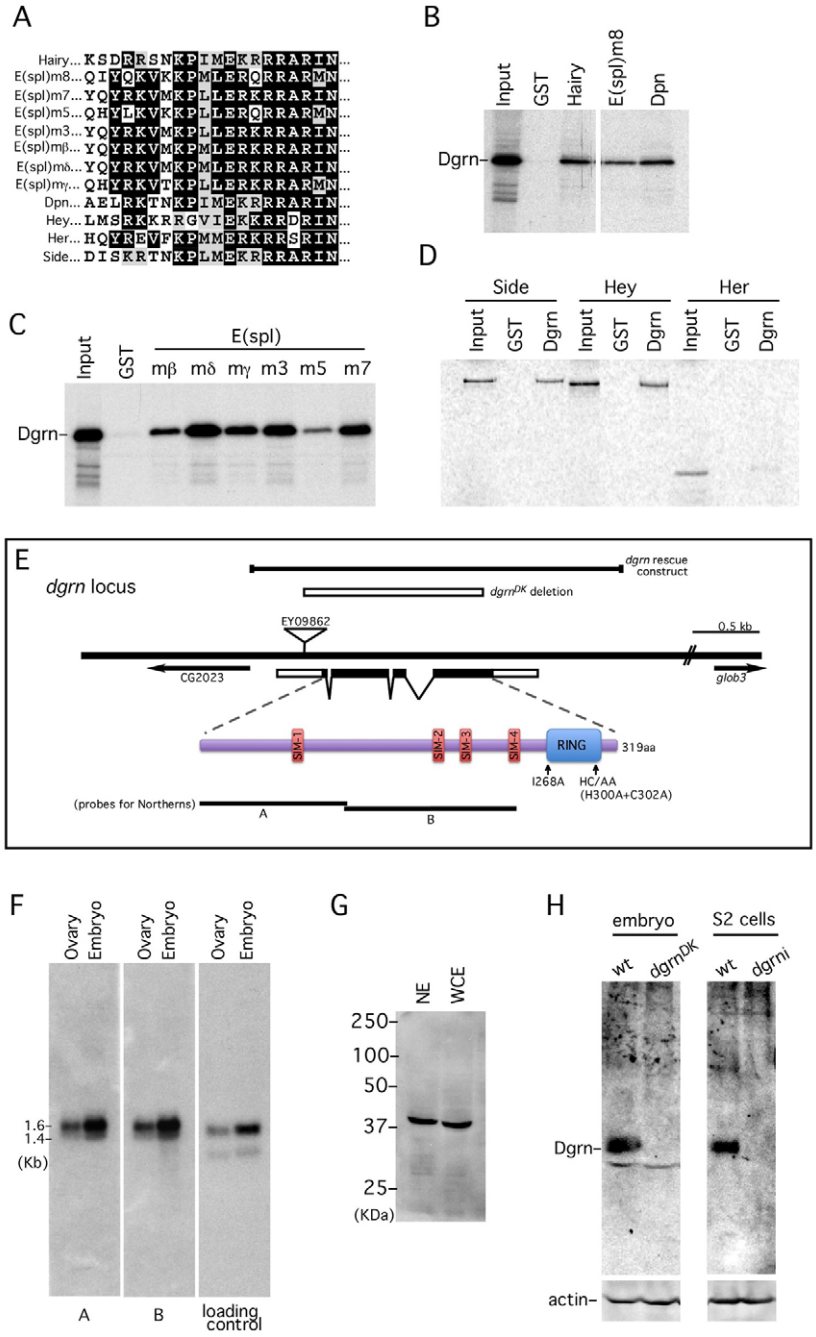

Fig. 1. Dgrn interacts physically with Hey and all members of the HES family of bHLH transcription factors, except Her. (**A**) Alignment of the basic regions of Hey and all fly HES family members showing the conserved nature of this domain. (**B-D**) Dgrn binds to Hey and all HES family members except Her. GST pulldown assays with ^{35}S-labeled in vitro translated proteins on the left and GST fusion proteins or GST alone across the top. (**E**) Map of the *dgrn* locus indicating the size of the deletion in the *dgrn*^{DK} mutation as well as the Dgrn protein structure and pieces of Dgrn used for northern blot analysis. (**F**) Northern blots using probes made to regions A and B of the Dgrn protein demonstrating that *dgrn* mRNA is found in the fly ovary and in 0- to 4-hour-old embryos. (**G**) Western blots using the polyclonal antibody generated to GST-Dgrn showing that this antibody recognizes Dgrn in both nuclear extracts (NE) and whole-cell embryo extracts (WCE). (**H**) Western blots of wild-type and *dgrn*^{DK} mutant embryos and wild-type and *dgrn* RNAi cell lysates (S2 cells) showing the specificity for the Dgrn antibody for Dgrn.

HES family members Dpn, and possibly Hey and Her, are required for sex determination (Erickson and Quintero, 2007; Lu et al., 2008). At nuclear cycle 14A, the HES family member Hairy is required for proper segmentation. The nuclear staining of Dgrn seen at nuclear cycle 9 (Fig. 2C-C‴) does not correspond to a known time when HES family members are active, and thus indicates either a previously undescribed Hey or HES family activity, or a Hey- or HES-independent activity of Dgrn.

Following cellularization, Dgrn expression is predominantly cytoplasmic. However, Dgrn does continue to exhibit nuclear localization coincident with HES family activity. In particular, we observed accumulation of Dgrn in the nuclei of neuroblasts, development of which is dependent on E(spl)-C activity (Fig. 2J-L′). Interestingly, Dgrn accumulation also prefigured morphogenetic furrows in gastrulating embryos: cells adjacent to morphogenetic furrows showed a higher accumulation of Dgrn that persists throughout gastrulation (see Fig. S2 in the supplementary material). None of the patterns described above was observed when staining embryos with pre-immune sera (data not shown).

dgrn null mutants are female sterile with embryos arresting after two or three nuclear divisions

To study the role of Dgrn in vivo, a *dgrn* null mutation was generated using imprecise P-element excision (Fig. 1E; see Materials and methods). The original P-element allele (*dgrn*^{17615}), the resulting imprecise P-element excised null mutation (*dgrn*^{DK}), as well as an additional P-element insertion allele (*dgrn*^{f05920}) were female sterile (100%, *n*=1006; 100%, *n*=1059; and 99% penetrant, *n*=1171; respectively) and embryos from homozygous *dgrn*^{DK} females displayed a severe early phenotype whereby they arrested

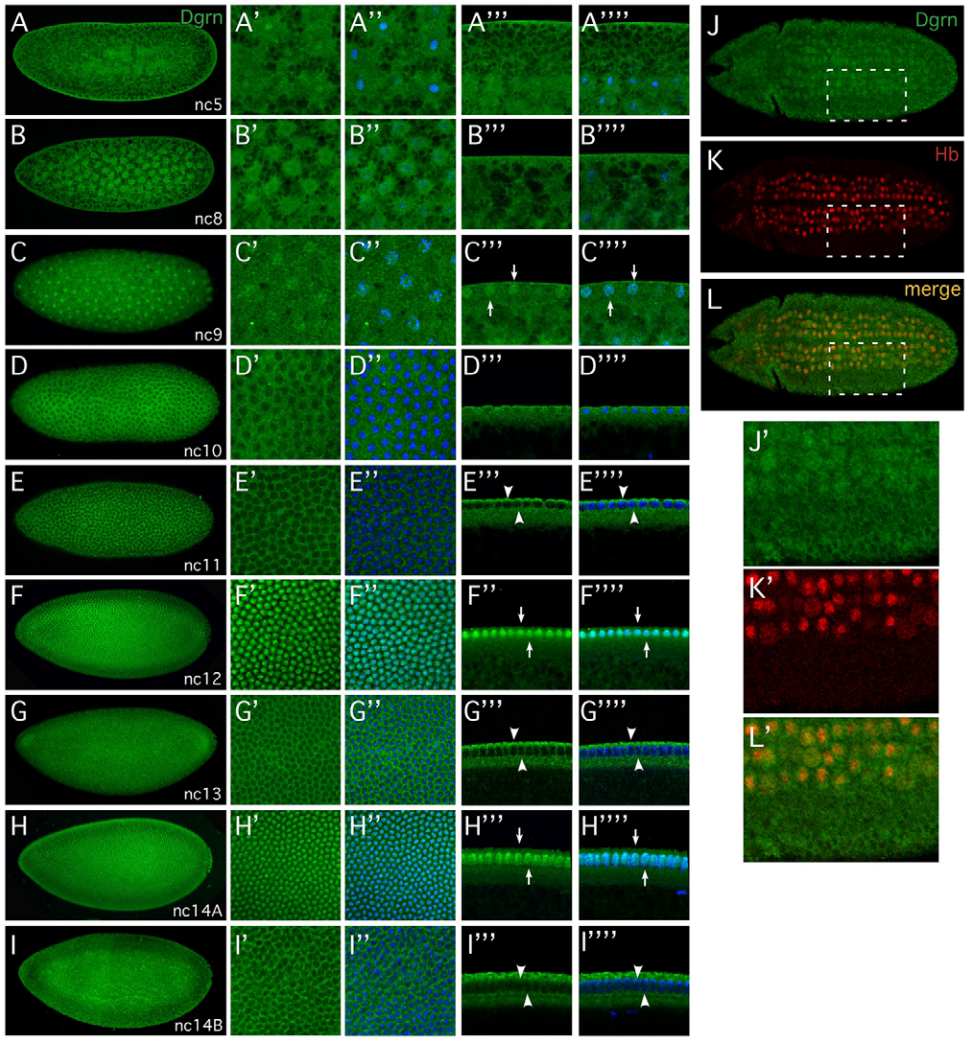

Fig. 2. Dgrn exhibits dynamic cellular and subcellular localization. (**A-I''''**) Surface projections of progressively older wild-type embryos stained with anti-Dgrn (green). Nuclear division cycle (nc) is indicated. Higher magnification sections (A′-I″) and tangential sections (A‴-I⁗) of the corresponding embryos A-I are shown. DAPI staining (blue; visualizing nuclei) is included in panels A″-I″ and A⁗-I⁗. Note the alternating nuclear accumulation (arrows) and nuclear exclusion (arrowheads) of Dgrn. (**J-L**) Surface projections of wild-type stage 9 embryos stained with anti-Dgrn (green) and anti-Hb (red; to indicate neuroblasts). Note nuclear Dgrn in developing neuroblasts. Dashed boxes indicate region of higher magnification projections (**J′-L′**) of corresponding embryos J-L.

after two or three nuclear divisions (88% penetrant, *n*=736) (Fig. 3A-C′). Staining *dgrn*^{DK} null embryos with anti-α-lamin to visualize the nuclear envelope showed that although nuclear envelope can form in these mutants, it does so around chromosome pieces rather than as a single nucleus (Fig. 3D,D′).

Late arrest *dgrn*^{DK} null embryos display abnormal nucleus localization

Twelve percent of *dgrn*^{DK} null mutant embryos overcame the early arrest phenotype and developed further; however, they still arrested prior to gastrulation with a phenotype in which nuclei make it to the surface in patches and many of the nuclei that do reach the surface subsequently fall inwards (referred to as late arrest *dgrn*^{DK} null mutants; Fig. 3E-N). These nuclei fell from the surface of the mutant embryos and formed a halo just under the surface, as well as aggregating in the center of the embryo (Fig. 3M,N). Only an occasional nucleus was seen to fall from the surface of wild-type embryos, and no central nuclear aggregation was observed. Anti-Lamin staining in these mutant embryos showed that most nuclei could form a single nuclear envelope; however, distortions in nuclear shape and size were common (Fig. 3O-P′). To characterize further this nuclear arrest phenotype, we performed time-lapse confocal microscopy on wild-type and *dgrn*^{f05920} mutant embryos using a histone-GFP reporter (His2AvDGFP) (Clarkson and Saint, 1999). Wild-type embryos expressing histone-GFP showed evenly distributed nuclei, synchronous nuclear divisions and normal development (Fig. 3Q-Q‴; see Movie 1 in the supplementary material). *dgrn*^{f05920} mutant embryos expressing histone-GFP exhibited non-uniform nuclear distribution with patches of nuclei making it to the surface of the embryo (Fig. 3R). Many of the nuclei that reached the surface of the embryo subsequently fell inwards. At the time corresponding to gastrulation, massive nuclear movements occurred and the nuclei at the surface of the embryo coalesced (Fig. 3R-R‴; see Movie 2 in the supplementary material).

Previous studies have shown that centrosomes reach the surface of an embryo with the nuclei and often remain there even when the nucleus falls from the embryo's surface (Sullivan et al., 1990). To determine whether the nuclei in the center of *dgrn* mutant embryos never reached the surface or reached the surface then fell back inside, we stained *dgrn*^{DK} null embryos with γ-tubulin or Centrosomin (Cnn), markers of centrosomes. *dgrn*^{DK} null embryos had regions where no nuclei reach the embryo surface, as well as regions where the nuclei properly associated with two centrosomes at the surface (Fig. 3S,S′; data not shown). Consistent with the time-lapse movie analyses, some regions of *dgrn*^{DK} null embryos had centrosomes not associated with nuclei, indicative of nuclei reaching the surface and subsequently falling inward (Fig. 3S′). Co-staining *dgrn*^{DK} null embryos with γ-tubulin and α-tubulin demonstrated that centrosomes free of nuclei are still able to act as microtubule organizing centers (Fig. 3T,T′).

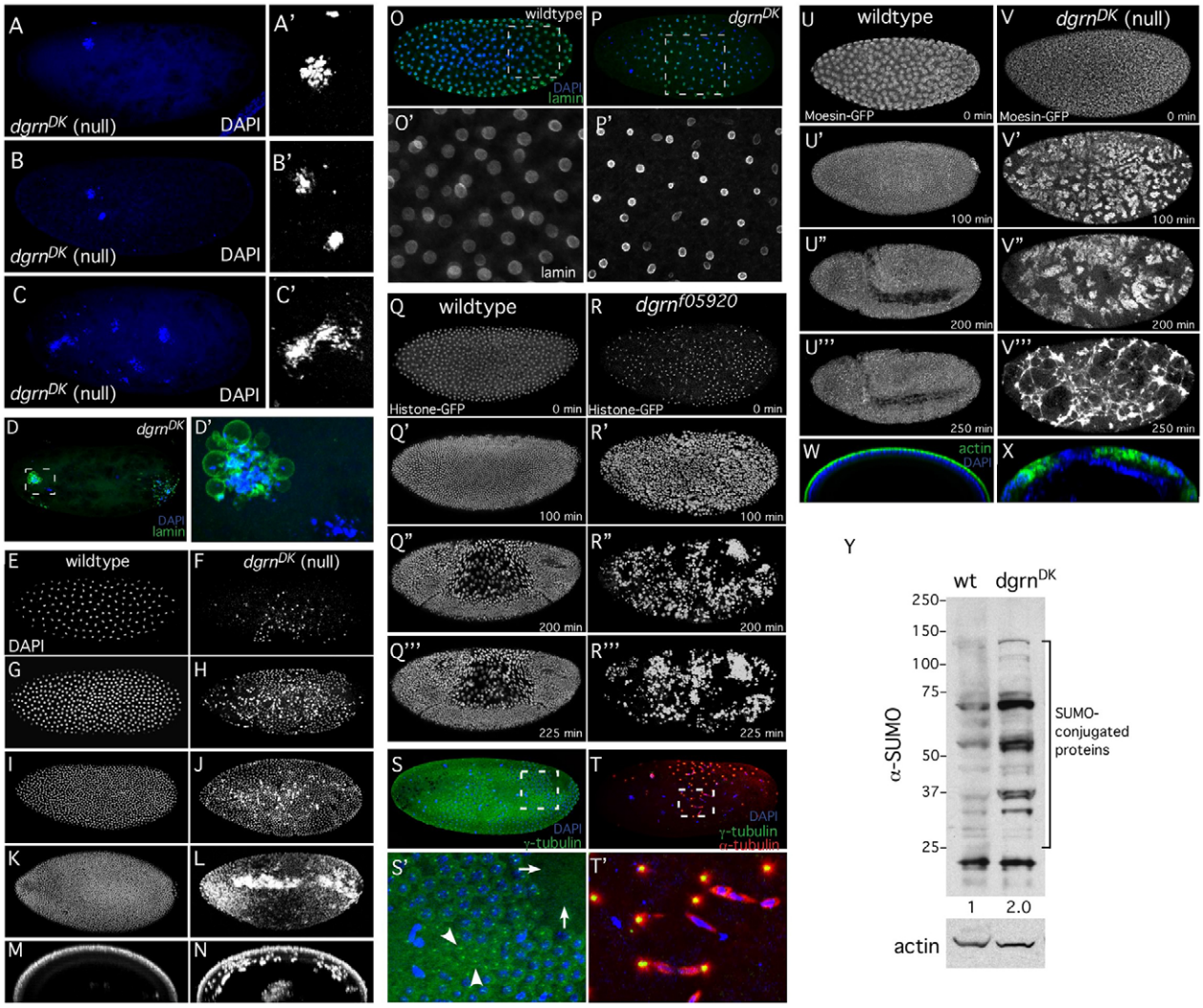

Fig. 3. Nuclei fall from the surface of *dgrn* null embryos and subcortical actin forms abnormal figures. (**A-C'**) $dgrn^{DK}$ null mutant embryos stained with DAPI showing arrest of mutants at nuclear cycle 2-3. A′-C′ show higher magnification projections of corresponding embryos A-C. (**D**,**D'**) $dgrn^{DK}$ null embryo stained with anti-Lamin (green) to visualize nuclear envelope and DAPI (blue) to visualize nuclei. Boxed region in D is shown at higher magnification in D′. (**E-N**) DAPI staining of progressively older wild-type and $dgrn^{DK}$ null embryos that make it past the early arrest phenotype. (M,N) Projections of cross-sections of wild-type (M) and $dgrn^{DK}$ (N) null embryos. Note the accumulation of nuclei in the center and just below the surface of the *dgrn* mutant embryo. (**O-P'**) Surface projections of wild-type (O,O′) and late arrest $dgrn^{DK}$ null (P,P′) embryos stained with anti-Lamin (green) and DAPI (blue). Boxed regions in O and P are shown at higher magnification in O′ and P′, respectively. Note the abnormal mitotic figures and mis-shapen nuclear envelopes in *dgrn* mutant embryos (P′). (**Q-R'''**) Still images from a time lapse of wild-type (Q-Q‴) and $dgrn^{DK}$ mutant (R-R‴) embryos expressing a histone-GFP fusion reporter where nuclei fall from the surface and aggregate in the center of the *dgrn* mutant embryo (R‴). (**S**,**S'**) Surface projection of a $dgrn^{DK}$ null embryo stained with the centrosome marker anti-γ-tubulin (green) and DAPI (blue). Boxed region in S is shown at higher magnification in S′ and shows regions where no nuclei or centrosomes are at the surface (arrows) and where centrosomes are on the surface but nuclei have fallen inwards (arrowheads). (**T**,**T'**) Surface projection of a $dgrn^{DK}$ null embryo stained with anti-α-tubulin (red) to visualize microtubules, anti-γ-tubulin (green) to visualize centrosomes and DAPI (blue). Boxed region in T is shown at higher magnification in T′ and shows the proper association of free centrosomes with microtubules, as well as abnormal mitotic figures. (**U-V'''**) Still images from a time lapse of wild-type (U-U‴) and $dgrn^{DK}$ null (V-V‴) embryos expressing the sGCMA reporter to visualize actin. (**W**,**X**) Cross-sections of wild-type (W) and $dgrn^{DK}$ null (X) embryos expressing the sGCMA reporter (green) and stained with DAPI (blue). Note the actin accumulation at the periphery of the embryo and the accumulation of nuclei in the center of the embryo. (**Y**) Western blot analysis of SUMOylated proteins in embryo extracts from wild-type (wt) and $dgrn^{DK}$ null embryos. Note that *dgrn* mutants show a 2-fold increase in SUMO expression compared with wild type.

dgrn mutants exhibit cytoarchitecture defects

Defects in the actin cytoskeleton have also been linked to loss of surface nuclei (Sullivan et al., 1993; Rothwell et al., 1998). To determine whether loss of Dgrn affects the actin cytoskeleton, we expressed a reporter line in which the actin-binding domain of Moesin is fused to GFP (sGMCA) (Kiehart et al., 2000) in a $dgrn^{DK}$ null background and performed time-lapse confocal microscopy. Although $dgrn^{DK}$ mutant embryos initially developed normally (Fig. 3U-V‴; see Movies 3 and 4 in the supplementary material), at roughly the time gastrulation should commence actin began to move in the plane of the embryo's periphery and eventually coalesced into abnormal structures

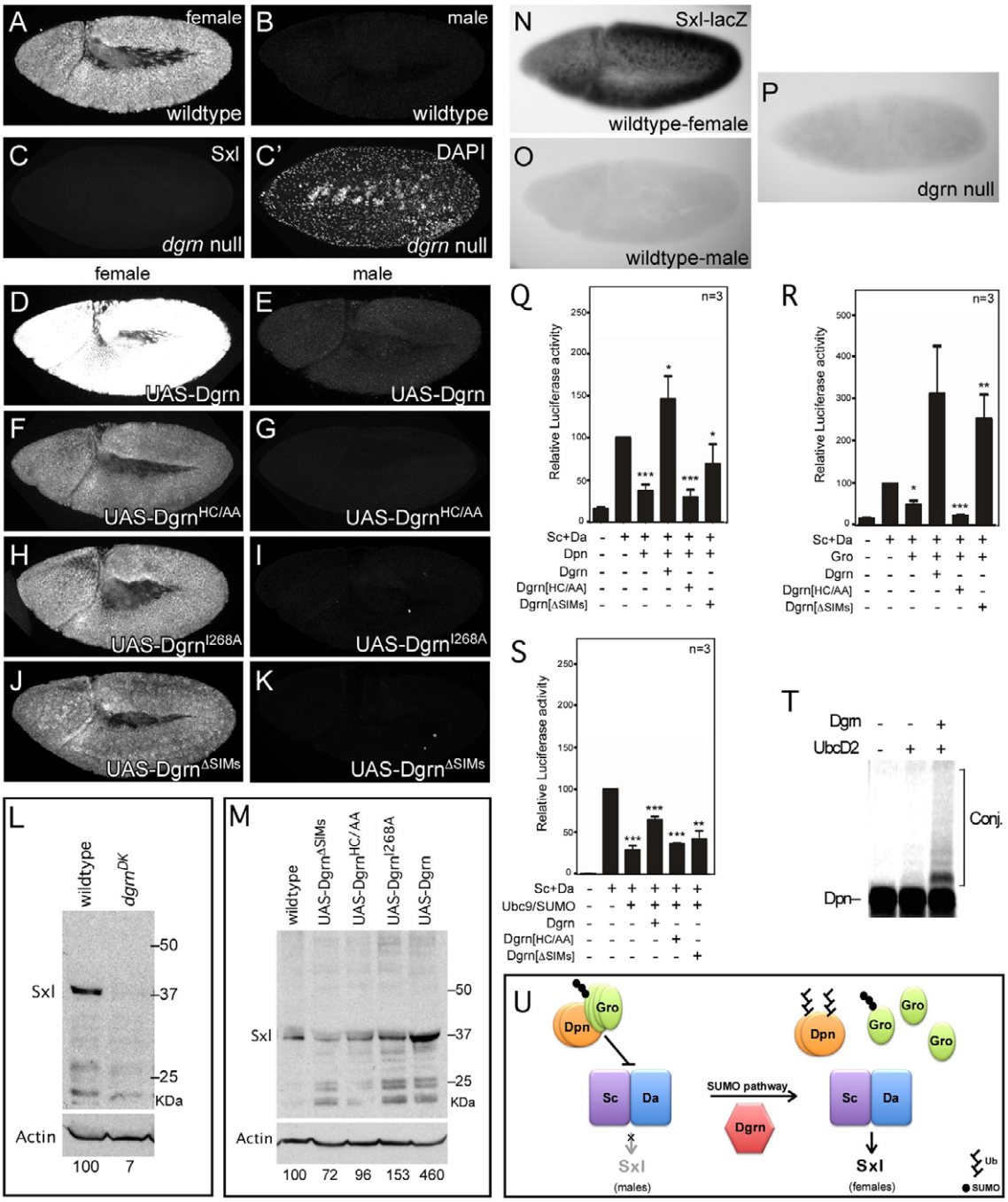

Fig. 4. Dgrn antagonizes Dpn and Gro in controlling the transcriptional regulation of the master sex determinant protein Sex Lethal. (**A**,**B**) Female wild-type embryos express Sxl (A), whereas males do not (B). (**C**,**C′**) A representative *dgrn*^{DK} null mutant embryo showing no Sxl expression (C) and stained with DAPI (C′). (**D-K**) Sxl staining of embryos ectopically expressing Dgrn constructs. (D,E) Ectopic expression of wild-type Dgrn leads to an overexpression of Sxl protein in females (D) and a misexpression of Sxl in males (E); however, ectopic expression of mutant forms of Dgrn does not affect Sxl expression (F-K). (**L**) Western blot analysis of Sxl expression in embryo extracts from wild-type and *dgrn*^{DK} null embryos. Note that *dgrn*^{DK} null mutants show an 93% decrease in Sxl expression compared with wild type. (**M**) Western blot analysis of Sxl expression on embryo extracts from wild type and the four Dgrn overexpression lines: UAS-Dgrn, UAS-Dgrn$^{HC/AA}$, UAS-Dgrn^{I268A} and UAS-Dgrn$^{\Delta SIMs}$. Note that UAS-Dgrn embryos show a 4.6-fold increase in Sxl expression compared with wild type. (**N-P**) A transgenic Sxl-P_E-lacZ reporter was incorporated into a wild-type (N,O) and *dgrn*^{DK} null (P) background. This in vivo Sxl-lacZ reporter is not activated in *dgrn*^{DK} null embryos. (**Q-S**) Transcription assays using the Sxl-P_E promoter-luciferase fusion that is activated by Da and Sc. Dpn (Q), Gro (R) and Ubc9 (S) repress transcription of Sxl, which is alleviated by the co-expression of wild-type Dgrn. By contrast, Dgrn$^{HC/AA}$, and to a lesser extent Dgrn$^{\Delta SIMs}$, are unable to antagonize this repression of Sxl. Error bars represent s.e.m. **P*<0.01, ***P*<0.001, ****P*<0.0001. (**T**) In vitro ubiquitylation assay using in vitro translated ^{35}S-Methionine-Dpn protein and bacterially expressed then purified recombinant ubiquitin activating enzyme (E1), UbcD2 (E2) and His_6-Dgrn. Conj., Ub-protein conjugates. (**U**) Model for Dgrn function in sex determination. Dgrn binds to Dpn via its RING domain and simultaneously associates with SUMOylated-Gro via its SIM domains. Ubiquitylation of Dpn by Dgrn prevents association of Dpn with Gro, allowing Da-Sc to activate Sxl expression. SUMO-Gro and its associated Gro-oligomers are then inactivated by sequestration.

Table 1. Dgrn affects sex determination, antagonizes E(spl) activity during neurogenesis and rescues Notch male lethality

A. Overexpression of Dgrn results in male lethality

Female genotype	Male genotype	% Female progeny	% Male progeny	*n*
+/+	+/+	47	53	760
MatGal4/MatGal4*	+/+	49	51	2364
MatGal4/MatGal4	UAS-Dgrn/UAS-Dgrn	99	1	777
MatGal4/MatGal4	UAS-Dgrn$^{HC/AA}$/UAS-Dgrn$^{HC/AA}$	58	42	647
MatGal4/MatGal4	UAS-Dgrn^{I268A}/UAS-Dgrn^{I268A}	54	46	1057
MatGal4/MatGal4	UAS-Dgrn$^{\Delta SIMs}$/UAS-Dgrn$^{\Delta SIMs}$	53	47	721

*MatGal4=w; pCog-Gal4::VP16; p[Gal4-nos.NGT40]40; p[Gal4::VP16-nos.UTR]MVD1

B. Expression of Dgrn alleviates E(spl)m8-dependent repression during Notal bristle formation

Transgene present*	% Adults with <15 Notal bristles	*n*
UAS-GFP/+	0	57
UAS-E(spl)m8/+	100	53
UAS-E(spl)m8/+; UAS-Dgrn	48	90
UAS-E(spl)m8/+; UAS-Dgrn$^{HC/AA}$	95	106
UAS-E(spl)m8/+; UAS-Dgrn$^{\Delta SIMs}$	100	53
UAS-Dgrn	0	58
UAS-Dgrn$^{HC/AA}$	0	55
UAS-Dgrn$^{\Delta SIMs}$	0	62

*C253-Gal4 driver used in all cases.

C. Dgrn rescues Notch male lethality

		Female progeny		Male progeny	
Female genotype	Male genotype	N^1	FM7†	N^1	FM7†
N^1/FM7; +/+	+/¬; +/+	128	143	0	112
N^1/FM7; +/+	+/¬; *dgrn^{17615}*/*dgrn^{17615}*	128	150	11*	79
N^1/FM7; +/+	+/¬; *dgrn^{DK}*/*dgrn^{DK}*	48	45	1	21

*Genotype confirmed by PCR.
†FM7 is an X-chromosome balancer chromosome.

(Fig. 3V-V‴; see Movie 4 in the supplementary material). Consistent with actin remaining at the surface of the embryo, cross-sections through the middle of these embryos showed that actin accumulation occurs at the periphery (Fig. 3W,X).

dgrn^{DK} mutant embryos accumulate SUMO conjugated proteins

The yeast homologs of Dgrn have been shown to be involved in genome stability, with this role being dependent on their STUbL activity (c.f. Burgess et al., 2007). Mutating the yeast or human homologs of Dgrn results in an increase of SUMOylated proteins and inability to cope with genotoxic stress (c.f. Prudden et al., 2007; Sun et al., 2007). If Dgrn is a bona fide STUbL protein, we would expect to see higher SUMO levels in *dgrn^{DK}* null embryos. As expected, Western blot analysis showed a 2-fold increase of SUMO-conjugated proteins in *dgrn^{DK}* null embryo lysates (Fig. 3Y). This increase in SUMOylated proteins was also seen in embryos (see Fig. S3 in the supplementary material).

Dgrn is involved in the proper transcriptional control of the master sex determinant gene *Sex lethal*

HES family repressors have been shown to play a pivotal role in *Drosophila* sex determination by controlling the expression of Sxl (Parkhurst et al., 1990; Younger-Shepherd et al., 1992; Lu et al., 2008). Sxl activity is required for female development and is expressed in all female cells, whereas males do not require Sxl activity and do not express Sxl protein. However, misexpression of Sxl in males results in lethality due to aberrant dosage compensation (Cline, 1978; Maine et al., 1985). As Dgrn interacts physically with the subset of HES proteins affecting sex determination, we examined Sxl expression in *dgrn^{DK}* null mutant embryos and found that Sxl protein was not expressed (Fig. 4A-C′). Sxl protein expression was also not detected in Western blot analysis of lysates generated from *dgrn^{DK}* null mutant embryos (Fig. 4L). Conversely, overexpressing full-length Dgrn maternally using the UAS-Gal4 conditional expression system (Brand and Perrimon, 1993) led to the overexpression of Sxl in females and the misexpression of Sxl in males (Fig. 4D,E). Consistent with this altered Sxl expression, only <1% of expected male progeny were recovered when wild-type Dgrn was ectopically expressed (99% of progeny were female, *n*=777; Table 1A). Both Dgrn's E3 ubiquitin ligase and STUbL activities were required for this phenotype as overexpressing point mutations disrupting its RING finger domain structure (Dgrn$^{HC/AA}$), disrupting its E3 ubiquitin ligase activity (Dgrn^{I268A}) or deleting its conserved SIM domains (Dgrn$^{\Delta SIMs}$), did not affect Sxl expression (Fig. 4F-K) or adult sex ratios (Table 1A). Western blot analysis of lysates generated from embryos ectopically expressing wild-type Dgrn or the Dgrn point mutants reflected these changes in Sxl expression (Fig. 4M).

Consistent with Dgrn's effects on *Sxl* expression in vivo, Dgrn counteracted the repressor activity of the HES family member Dpn on an in vivo Sxl reporter in embryos. Transcription of *Sxl* is activated by Scute (Sc)-Daughterless (Da) heterodimers and this activation is repressed by Dpn or Gro. A transgenic line with the Sxl-P$_E$ promoter region fused to the *lacZ* gene (Estes et al., 1995) was placed in a *dgrn* mutant background. Loss of Dgrn led to loss of expression for this SxlP$_E$-lacZ reporter (Fig. 4N-P), indicating

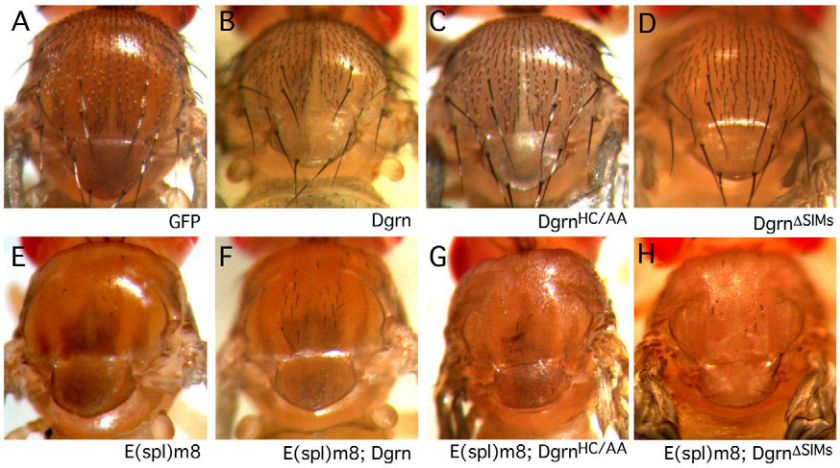

Fig. 5. Ectopic Dgrn expression suppresses E(spl) activity during *Drosophila* neurogenesis. (**A-D**) Overexpression of UAS-GFP (A), UAS-Dgrn (B), UAS-Dgrn$^{HC/AA}$ (C) or UAS-Dgrn$^{\Delta SIMs}$ (D) does not affect adult thoracic bristle development. (**E**,**F**) Ectopic expression of UAS-E(spl)m8 leads to a loss of thoracic bristles in the adult fly (E); however, this 'bald' phenotype is suppressed by co-expression of UAS-Dgrn (F). Note the increase of thoracic bristles. (**G**,**H**) Co-expression of UAS-E(spl)m8 along with either UAS-Dgrn$^{HC/AA}$ (G) or UAS-Dgrn$^{\Delta SIMs}$ (H) does not suppress the E(spl)m8 bald phenotype.

that Dgrn controls the expression of Sxl protein at the level of transcriptional regulation. Owing to genetic incompatibilities, we have been unable to generate lines in which to examine potential *dgrn-dpn* genetic interactions.

To define further the role that Dgrn plays in HES-mediated transcriptional repression, we examined the ability of Dgrn to influence Dpn repression of Sxl in transcription assays using the same Sxl-P_E promoter in *Drosophila* Kc cells (Fig. 4Q-S; see Fig. S1 in the supplementary material) (Estes et al., 1995; Hoshijima et al., 1995). Sxl transcription activated by Da-Sc heterodimers was repressed when Dpn is co-expressed (Fig. 4Q). Dpn recruits the co-repressor Gro, which is required to repress *Sxl* expression (Paroush et al., 1994). Co-overexpression of Gro with Da-Sc also repressed transcription from the *Sxl* promoter (Fig. 4R). Importantly, activation of the *Sxl* promoter by Da-Sc could be repressed by co-overexpression of Ubc9 (Lwr – FlyBase; SUMO ligase) (Fig. 4S). In this assay, Dgrn alleviated the repression of the *Sxl* promoter induced by either Dpn or Gro (Fig. 4Q,R). Similar to the molecular interaction of Dgrn with Hairy and Gro on the *achaete* promoter (Abed et al., 2011), the loss of Dgrn's ubiquitin ligase activity (Dgrn$^{HC/AA}$), blocked the ability of Dgrn to alleviate Dpn, Gro and Ubc9 repression. Importantly, whereas disruption of its STUbL activity (Dgrn$^{\Delta SIMs}$) only reduced Dgrn's ability to counteract repression of the *Sxl* promoter by Dpn and Gro, it blocked its ability to alleviate Ubc9-mediated repression (Fig. 4Q-S). Consistent with the ability of Dgrn overexpression to repress Da-Sc activation at the *Sxl* promoter, we found that Dgrn efficiently ubiquitylated Dpn in an in vitro reconstituted system (Fig. 4T). Thus, Dgrn controls the expression of Sxl protein at the level of transcriptional regulation (Fig. 4U).

Dgrn antagonizes E(spl)-C activity during *Drosophila* neurogenesis

In addition to their roles in sex determination and segmentation, HES family members play pivotal roles in the specification and development of the peripheral and central nervous systems (c.f. Davis and Turner, 2001). As *dgrn* mutant embryos do not survive past the onset of gastrulation, we assessed Dgrn's role in neurogenesis using the UAS-Gal4 system to ectopically express wild-type Dgrn and two Dgrn mutations (Dgrn$^{HC/AA}$ and Dgrn$^{\Delta SIMs}$). Overexpressing GFP, Dgrn, Dgrn$^{HC/AA}$ or Dgrn$^{\Delta SIMs}$ using the C253 Gal4 driver (Orian et al., 2007) led to adult flies with wild-type thoracic bristles (indicators of underlying neurogenesis; Fig. 5A-D; Table 1B). By contrast, ectopically expressing E(spl)m8 (a member of the E(spl)-C) or Hey led to a bald phenotype in which adult flies do not have bristles (100%, n=53; Fig. 5E; Table 1B; data not shown). We found that ectopic Dgrn expression suppressed the activity of E(spl)m8 and Hey in bristle development (48%, n=90, P<0.001; data not shown), and that this suppression was dependent on functional RING and SIM domains (Fig. 5F-H; Table 1B). Consistent with the role of Dgrn in adult neurogenesis, we found that ectopically expressing Dgrn maternally disrupted PNS development and the resulting adult females were unable to expand their wings (see Fig. S4 in the supplementary material).

Dgrn interacts genetically with the Notch signaling pathway

As HES family repressors are induced by Notch and are substrates for Dgrn, we wanted to test whether Dgrn modulates Notch activity. In addition, a recent study showed that the master sex determinant protein Sxl negatively regulates Notch signaling (Penn and Schedl, 2007). As we found that Dgrn regulates the expression of Sxl, we explored whether Dgrn interacts genetically with the Notch signaling pathway using a sex specific allele of *Notch* (N^1). N^1 leads to viable heterozygous females with notched wings (Fig. 6B), but is lethal to males (100%; Table 1C). When *dgrn* mutations were put in trans with N^1, male lethality was partially rescued (7% for $dgrn^{17615}$, P=0.0001; 2% for $dgrn^{DK}$, P=0.0003) (Fig. 6C; Table 1C). This partial rescue of the *N* male lethality when Dgrn was reduced in this *Notch* mutant is probably due to an interaction between Dgrn, Sxl levels and the negative regulation of Notch signaling by Sxl, as females still showed a notched wing phenotype (Fig. 6D) (Penn and Schedl, 2007).

We found that reducing Dgrn also rescued the loss of veins associated with the *N* gain-of-function allele N^{AX1682}. The wings of adult wild-type flies consist of a conserved vein organization where longitudinal vein 4 (L4) and longitudinal vein 5 (L5) run to the periphery of the adult wing (Fig. 6E). *N* is normally involved in refining the longitudinal veins; however, the N^{AX1682} gain-of-function allele led to the loss of L4 and L5 at the periphery of the wing (Fig. 6E,F). The $dgrn^{DK}$ null mutation placed in trans to the N^{AX1682} allele rescued the truncation of L4 (100% penetrance, n>50; Fig. 6G). Thus, Dgrn appears to interact genetically with multiple facets of Notch signaling, depending on the stage of development of the embryo.

DISCUSSION

A common theme among DNA-bound transcriptional regulators is the recruitment of co-activators and/or co-repressors to carry out their function. An important aspect of all HES family regulation is their recruitment of the co-repressor Groucho (c.f. Buscarlet and Stifani,

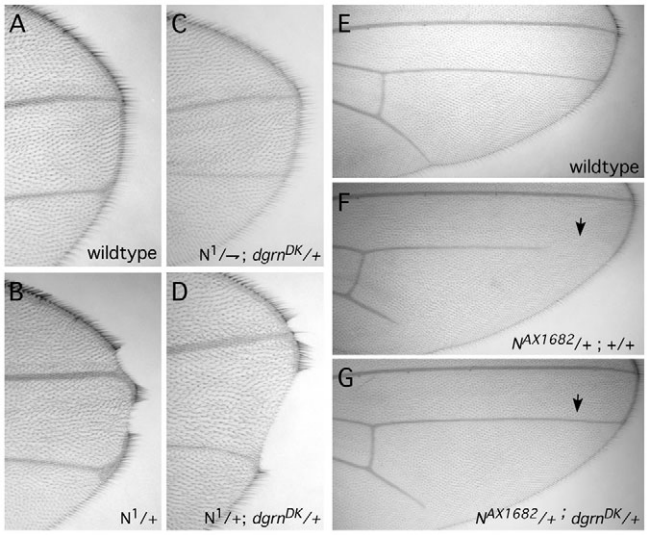

Fig. 6. Dgrn interacts genetically with the Notch signaling pathway. (**A**,**B**) Tip of adult wing in wild type (A) and in an N^{1}/+ female (B) displaying a notched adult wing phenotype. (**C**,**D**) Reducing the dose of *dgrn* in the N^{1} background results in a rescue of the male lethality with the adult males displaying wild-type wings (C), whereas females are not affected and continue to display a notched wing phenotype (D). (**E-G**) Adult wing showing longitudinal vein 4 (L4) and 5 (L5) from wild type (E) and heterozygous N^{AX1682} (F). Note that these veins are truncated in the N^{AX1682} mutant (arrow in F indicates L4 truncation). Reducing the dose of *dgrn* in trans to the heterozygous N^{AX1682} allele partially rescues the vein phenotype caused by this *N* gain-of-function allele (arrow in G).

2007). Dgrn, a new Hey and HES family regulator, encodes the *Drosophila* homolog of the mammalian SUMO-targeted ubiquitin ligase (STUbL) protein RNF4 (Abed et al., 2011). Dgrn binds the basic domain of Hey and all of the HES family members, except Her, and facilitates the ubiquitylation of these factors. Abed and colleagues (Abed et al., 2011) have shown that the ubiquitylation of Hairy does not lead to its degradation, but rather interferes with the ability of Hairy to recruit Gro, thereby antagonizing Hairy's repressive activity. Consistent with this, we find that *dgrn* mutant embryos show defects in segmentation (see Fig. S5 in the supplementary material). We suggest that, similar to Dgrn's interaction with Hairy in segmentation, the ubiquitylation of other HES family members or Hey leads to their inability to recruit the cofactor Gro and thus antagonizes the repressor activity of this protein family. Consistent with this, we find that loss of Dgrn function affects known Hey and HES family early functions, including sex determination and nervous system development. It was surprising that Dgrn is female sterile rather than exhibiting zygotic lethality. Another, as yet unidentified, STUbL protein might function redundantly to Dgrn postzygotically. As the early *Drosophila* embryo develops essentially as a closed system running on maternally provided mRNA and proteins, the early syncytial embryo relies heavily on translational and post-translational modifications to control protein activity. Both Dgrn and Gro are maternally contributed and ubiquitously distributed. Thus, Dgrn might be recruited to the nucleus at different times during these early developmental stages to attenuate Gro's ability to be a potent co-repressor in the absence of active transcription, thereby modulating Hey and HES family activity.

dgrn mutant embryos exhibit early arrest phenotypes consistent with a conserved role for Dgrn in genome stability

Dgrn's human homolog is the transcriptional cofactor and STUbL protein RNF4. Indeed, human RNF4 acts as a functional homolog of Dgrn (see Fig. S1D,E in the supplementary material) (Abed et al., 2011). RNF4 has also been shown to be a functional ortholog of the Rfp1/Rfp2-Slx8 heterodimer (from now on referred to as Rfp-Slx8) in *S. pombe* (Kosoy et al., 2007; Prudden et al., 2007) and the Slx5-Slx8 heterodimer in *S. cerevisiae* (Uzunova et al., 2007). RNF4 and the yeast Rfp-Slx8 and Slx5-Slx8 heterodimers have been shown to be important for DNA repair, kinetochore assembly and genome stability, with the loss of these proteins leading to fragmented chromosomes, elongated nuclei, asymmetric positioning of the nuclei and an accumulation of SUMOylated proteins (Burgess et al., 2007; Kosoy et al., 2007; Prudden et al., 2007; Sun et al., 2007; Mullen and Brill, 2008; Cook et al., 2009; Mukhopadhyay et al., 2010).

The budding yeast Slx5-Sx8 proteins were identified as a complex of proteins required for the viability of *SGS1* (a gene encoding the only RecQ helicase involved in genomic integrity in *S. cerevisiae*) mutant cells (Mullen et al., 2001). In *Drosophila*, loss of RecQ5 function leads to the loss of synchronous divisions in the syncytial embryo, an increased number of double strand breaks and a slight increase in the number of abnormal nuclei falling from the surface of the embryo (Nakayama et al., 2009). Mutations of the RecQ family member DmBlm (mus309 – FlyBase; the *Drosophila* ortholog of human BLM, which leads to the human disorder Bloom Syndrome when mutated) are female sterile with severe defects in embryogenesis: syncytial embryos frequently include anaphase bridges, gaps in the normally uniform monolayer of nuclei and asynchronous mitoses (McVey et al., 2007).

Recently, *smt3* (SUMO) mutant embryos were shown to display embryonic nuclear cycle defects, including irregular size and distribution of nuclei, chromosome clustering, chromosome bridges, fragmentation and reduced number of nuclei in relation to the centrosome pairs (Nie et al., 2009). Nie et al. (Nie et al., 2009) identified several cell cycle factors that are substrates for SUMOylation and propose that SUMOylation of these factors is important for controlling the cell cycle. The fragmented and de-condensed nuclei observed in the early arrest phenotypes of $dgrn^{DK}$ null embryos (Fig. 3A-C′) are reminiscent of the Slx8-Slx5 mutant phenotypes, fly RecQ mutant phenotypes and *smt3* mutant phenotypes, suggesting that the role of STUbL proteins in genome stability and DNA repair might be a conserved function.

Alternatively, mutations in actin cytoskeleton and cell cycle checkpoint components in *Drosophila* have also been shown to exhibit nuclear arrest phenotypes. Defects in cell cycle checkpoint proteins, including Pan Gu, Plutonium and Giant Nuclei affect the S-phase checkpoint in the early embryo such that mutation of any of these genes leads to unregulated S-phase, resulting in giant polyploid nuclei (Freeman and Glover, 1987; Elfring et al., 1997; Fenger et al., 2000; Lee et al., 2003). Disruption of the actin cytoskeleton can also lead to nuclear division abnormalities of cortical nuclei. For example, the *scrambled* and *nuclear fallout* mutants exhibit severe abnormalities in the appearance and localization of cortical nuclei (Rothwell et al., 1998; Sullivan et al., 1993). Further experiments will be needed to determine the molecular mechanism(s) underlying Dgrn's early arrest phenotype. However, regardless of the mechanism, this represents a new function for Hey or HES family proteins or a function for Dgrn that is not HES-dependent.

Dynamic Dgrn nuclear localization suggests possible novel Hey or HES family activities

Interestingly, nuclear cycles during which Dgrn accumulates in the nucleus correspond to times when HES family members are active, which would be necessary for Dgrn to interact physically with HES proteins and subsequently affect their functions. One exception to this is Dgrn nuclear localization at nuclear cycle 9. There are no known HES family activities at nuclear cycle 9; however, several HES family members are yet to be characterized molecularly and genetically. Dgrn also exhibits a novel accumulation pattern during the gastrulation stages where it prefigures morphogenetic furrows, suggesting a possible role for Hey or HES family members in morphogenesis. Chromatin profiling experiments identifying direct transcriptional targets of Hairy identified a number of targets important for morphogenesis, suggesting that Hairy might play a role in morphogenesis (Bianchi-Frias et al., 2004). Consistent with this, a new *hairy* allele (h^{674}) was reported to affect the early stages of salivary gland morphogenesis (Myat and Andrew, 2002; Abrams et al., 2003). Thus, although Dgrn might work with Hey, Hairy and/or other HES family members during these times, it also remains possible that these Dgrn activities are Hey- and HES-independent.

Dgrn antagonizes the activities of Hey and HES family members in sex determination

Dpn is a negative regulator of Sxl (Younger-Shepherd et al., 1992; Barbash and Cline, 1995). *dpn* mutants have a modest effect on Sxl in males leading to ectopic expression from the Sxl-Pe promoter that is sufficient to induce the inappropriate female fate in some cells (Barbash and Cline, 1995). The Hey and HES family co-repressor Gro has also been shown to act as a negative regulator of Sxl, as the loss of maternal Gro results in severe misexpression of Sxl in males leading to female fate (Paroush et al., 1994). The relatively mild effect of Dpn on Sxl regulation compared with Gro led Lu and colleagues (Lu et al., 2008) to search for additional HES family proteins involved in Sxl regulation. They identified Hey as a maternal repressor of Sxl-Pe, albeit in a spatially variable pattern in males (Lu et al., 2008). Unlike the mammalian homologs of Hey, which are unable to bind Gro presumably owing to its C-terminal YRPW domain (c.f. Fischer and Gessler, 2007), we find that *Drosophila* Hey binds Gro in GST pulldown assays (see Fig. S1B,C in the supplementary material). Our data suggests that Dgrn is an important player in sex determination where it interferes with the repressive activities of Dpn and Gro; we find that both Dpn and Hey are substrates for Dgrn's E3 ubiquitin ligase activity (see Fig. S1 in the supplementary material). In addition, Sxl protein staining and in vitro transcription assays demonstrate that Dgrn antagonizes the repression of Sxl. As proposed for the interaction of Dgrn with Hairy during segmentation (Abed et al., 2011), we find that Dgrn provides a new level of control over the activity of Hey and the HES family members in sex determination. This control is mediated by ubiquitylation that probably disrupts the ability of these repressors to recruit Gro, thereby antagonizing their ability to repress transcription of Sxl-Pe in males.

Erickson and colleagues have recently proposed that sex in *Drosophila* is not determined by the ratio of X-chromosomes to sets of autosomes (X:A ratio), but rather by X chromosome dose (Erickson and Quintero, 2007; Lu et al., 2008). The authors speculate that a feedback mechanism in females is caused by the acetylation of chromatin, which inhibits Gro-mediated repression. Interestingly, our finding that Dgrn antagonizes Gro activity via the ubiquitylation of Hey and HES family repressors and targets SUMOylated Gro for sequestration provides an alternate scenario for this feedback mechanism in females.

Dgrn antagonizes Notch signaling

Notch (N), through the E(spl) proteins (its downstream targets), heads one of the major developmental signaling pathways that functions in progenitor cell fate determination and differentiation (Bray, 1998; Fischer and Gessler, 2007). Recently, Sxl has been shown to inhibit Notch RNA translation and to negatively regulate the Notch signaling pathway in females (Penn and Schedl, 2007). The notched wing phenotype of *N* was shown to be sensitive to *Sxl*, such that reducing the dose of *Sxl* suppressed the lethal effects of *N* hypomorphic alleles. We find that reducing the dose of *dgrn* can also partially rescue the lethal effects of *N* hypomorphic alleles suggesting that Dgrn antagonizes N signaling. More specifically, we hypothesize that the rescue of N^{1} male lethality is due to a decrease in Sxl expression. Dgrn heterozygosity also suppresses the vein patterning phenotype associated with N^{AX1682}, suggesting that it is required for N signaling in this context also. Interestingly, Dgrn could be antagonizing N by two distinct mechanisms (or a combination of the two): the first an indirect antagonization of N signaling through Dgrn's control of Sxl expression, and the second by direct inhibition of the repressor activities of the E(spl)-C protein by ubiquitylation, thus blocking the repressive arm of the N pathway. The second mechanism has implications in regulating crosstalk between N and EGFR signaling pathways. Further studies will be required to determine the role of Dgrn's STUbL activity and whether Dgrn's activity on E(spl)-C proteins is redundant to EGFR signaling or whether both of these activities are required to antagonize Notch signaling.

Acknowledgements

We thank Dan Gottschling, Hector Rincon, Limor Broday and members of the lab for their interest, advice and comments on the manuscript. We thank T. Phippen and D. Metzger for their help during the initial stages of this work. We also thank T. Kaufman, J. Reinitz, T. Tsukiyama, the Developmental Studies Hybridoma Center and the Bloomington Stock Center for cell lines, antibodies, DNAs, flies and other reagents used in this study. This work was supported by ISF grants (FIRST 1215/07 and 418/09), a GIF grant (918/07), and the Rappaport Institute Research Fund (to A.O.) and NIH grant GM073021 (to S.M.P.). Deposited in PMC for release after 12 months.

Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.058420/-/DC1

References

Abed, M., Barry, K. C., Kenyagin, D., Koltun, B., Phippen, T. M., Delrow, J. J., Parkhurst, S. M. and Orian, A. (2011). Degringolade, a SUMO-targeted ubiquitin ligase, inhibits Hairy/Groucho-mediated repression. *EMBO J.* (in press).

Abrams, E. W., Vining, M. S. and Andrew, D. J. (2003). Constructing an organ: the Drosophila salivary gland as a model for tube formation. *Trends Cell Biol.* **13**, 247-254.

Barbash, D. A. and Cline, T. W. (1995). Genetic and molecular analysis of the autosomal component of the primary sex determination signal of Drosophila melanogaster. *Genetics* **141**, 1451-1471.

Ben-Saadon, R., Zaaroor, D., Ziv, T. and Ciechanover, A. (2006). The polycomb protein Ring1B generates self atypical mixed ubiquitin chains required for its *in vitro* Histone H2A ligase activity. *Mol. Cell* **24**, 701-711.

Bianchi-Frias, D., Orian, A., Delrow, J. J., Vazquez, J., Rosales-Nieves, A. E. and Parkhurst, S. M. (2004). Hairy transcriptional repression targets and cofactor recruitment in Drosophila. *PLoS Biol.* **2**, E178.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Bray, S. (1998). Notch signalling in Drosophila: three ways to use a pathway. *Semin. Cell Dev. Biol.* **9**, 591-597.

Burgess, R. C., Rahman, S., Lisby, M., Rothstein, R. and Zhao, X. (2007). The Slx5-Slx8 complex affects sumoylation of DNA repair proteins and negatively regulates recombination. *Mol. Cell. Biol.* **27**, 6153-6162.

Buscarlet, M. and Stifani, S. (2007).The 'Marx' of Groucho on development and disease. *Trends Cell Biol.* **17**, 353-361.

Carroll, S. B. and Scott, M. P. (1986). Zygotically active genes that affect the spatial expression of the fushi tarazu segmentation gene during early Drosophila embryogenesis. *Cell* **45**, 113-126.
Clarkson, M. and Saint, R. (1999). A His2AvDGFP fusion gene complements a lethal His2AvD mutant allele and provides an in vivo marker for Drosophila chromosome behavior. *DNA Cell Biol.* **18**, 457-462.
Cline, T. W. (1978). Two closely linked mutations in Drosophila melanogaster that are lethal to opposite sexes and interact with daughterless. *Genetics* **90**, 683-698.
Cook, C. E., Hochstrasser, M. and Kerscher, O. (2009). The SUMO-targeted ubiquitin ligase subunit Slx5 resides in nuclear foci and at sites of DNA breaks. *Cell Cycle* **8**, 1080-1089.
Davis, R. L. and Turner, D. L. (2001). Vertebrate hairy and enhancer of split related proteins: transcriptional repressors regulating cellular differentiation and embryonic patterning. *Oncogene* **20**, 8342-8357.
Elfring, L. K., Axton, J. M., Fenger, D. D., Page, A. W., Carminati, J. L. and Orr-Weaver, T. L. (1997). Drosophila PLUTONIUM protein is a specialized cell cycle regulator required at the onset of embryogenesis. *Mol. Biol. Cell* **8**, 583-593.
Erickson, J. W. and Quintero, J. J. (2007). Indirect effects of ploidy suggest X chromosome dose, not the X:A ratio, signals sex in Drosophila. *PLoS Biol.* **5**, e332.
Estes, P. A., Keyes, L. N. and Schedl, P. (1995). Multiple response elements in the sex-lethal early promoter ensure its female-specific expression pattern. *Mol. Cell. Biol.* **15**, 904-917.
Fenger, D. D., Carminati, J. L., Burney-Sigman, D. L., Kashevsky, H., Dines, J. L., Elfring, L. K. and Orr-Weaver, T. L. (2000). PAN GU: a protein kinase that inhibits S phase and promotes mitosis in early Drosophila development. *Development* **127**, 4763-4774.
Fischer, A. and Gessler, M. (2007). Delta-Notch-and then? Protein interactions and proposed modes of repression by Hes and Hey bHLH factors. *Nucleic Acids Res.* **35**, 4583-4596.
Freeman, M. and Glover, D. M. (1987). The gnu mutation of Drosophila causes inappropriate DNA synthesis in unfertilized and fertilized eggs. *Genes Dev.* **1**, 924-930.
Grieder, N. C., de Cuevas, M. and Spradling, A. C. (2000). The fusome organizes the microtubule network during oocyte differentiation in Drosophila. *Development* **127**, 4253-4264.
Hoshijima, K., Kohyama, A., Watakabe, I., Inoue, K., Sakamoto, H. and Shimura, Y. (1995). Transcriptional regulation of the sex-lethal gene by helix-loop-helix proteins. *Nucleic Acids Res.* **23**, 3441-3448.
Howard, K. and Ingham, P. (1986). Regulatory interactions between the segmentation genes fushi tarazu, hairy, and engrailed in the Drosophila blastoderm. *Cell* **44**, 949-957.
Kageyama, R., Ohtsuka, T. and Kobayashi, T. (2007). The Hes gene family: repressors and oscillators that orchestrate embryogenesis. *Development* **134**, 1243-1251.
Kiehart, D. P., Galbraith, C. G., Edwards, K. A., Rickoll, W. L. and Montague, R. A. (2000). Multiple forces contribute to cell sheet morphogenesis for dorsal closure in Drosophila. *J. Cell Biol.* **149**, 471-490.
Kosoy, A., Calonge, T. M., Outwin, E. A. and O'Connell, M. J. (2007). Fission yeast Rnf4 homologs are required for DNA repair. *J. Biol. Chem.* **282**, 20388-20394.
Krejcí, A., Bernard, F., Housden, B. E., Collins, S. and Bray, S. J. (2009). Direct response to Notch activation: signaling crosstalk and incoherent logic. *Sci. Signal*. **2**, ra1.
Lee, L. A., Van Hoewyk, D. and Orr-Weaver, T. L. (2003). The Drosophila cell cycle kinase PAN GU forms an active complex with PLUTONIUM and GNU to regulate embryonic divisions. *Genes Dev.* **17**, 2979-2991.
Linardopoulou, E. V., Parghi, S. S., Friedman, C., Osborn, G. E., Parkhurst, S. M. and Trask, B. J. (2007). Human subtelomeric WASH genes encode a new subclass of the WASP family. *PLoS Genet.* **3**, e237.
Liu, R., Woolner, S., Johndrow, J. E., Metzger, D., Flores, A. and Parkhurst, S. M. (2008). Sisyphus, the Drosophila myosin XV homolog, traffics within filopodia transporting key sensory and adhesion cargos. *Development* **135**, 53-63.
Lu, H., Kozhina, E., Mahadevaraju, S., Yang, D., Avila, F. W. and Erickson, J. W. (2008). Maternal Groucho and bHLH repressors amplify the dose-sensitive X chromosome signal in Drosophila sex determination. *Dev. Biol.* **323**, 248-260.
Maine, E. M., Salz, H. K., Cline, T. W. and Schedl, P. (1985). The sex-lethal gene of Drosophila: DNA alterations associated with sex-specific lethal mutations. *Cell* **43**, 521-529.
McVey, M., Andersen, S. L., Broze, Y. and Sekelsky, J. (2007). Multiple functions of Drosophila BLM helicase in maintenance of genome stability. *Genetics* **176**, 1979-1992.
Moore, A. W., Barbel, S., Jan, L. Y. and Jan, Y. N. (2000). A genomewide survey of basic helix-loop-helix factors in Drosophila. *Proc. Natl. Acad. Sci. USA* **97**, 10436-10441.
Mukhopadhyay, D., Arnaoutov, A. and Dasso, M. (2010). The SUMO protease SENP6 is essential for inner kinetochore assembly. *J. Cell Biol.* **188**, 681-692.
Mullen, J. R. and Brill, S. J. (2008). Activation of the Slx5-Slx8 ubiquitin ligase by poly-small ubiquitin-like modifier conjugates. *J. Biol. Chem.* **283**, 19912-19921.
Mullen, J. R., Kaliraman, V., Ibrahim, S. S. and Brill, S. J. (2001). Requirement for three novel protein complexes in the absence of the Sgs1 DNA helicase in Saccharomyces cerevisiae. *Genetics* **157**, 103-118.
Myat, M. M. and Andrew, D. J. (2002). Epithelial tube morphology is determined by the polarized growth and delivery of apical membrane. *Cell* **111**, 879-891.
Nakayama, M., Yamaguchi, S., Sagisu, Y., Sakurai, H., Ito, F. and Kawasaki, K. (2009). Loss of RecQ5 leads to spontaneous mitotic defects and chromosomal aberrations in Drosophila melanogaster. *DNA Repair (Amst.)* **8**, 232-241.
Nie, M., Xie, Y., Loo, J. A. and Courey, A. J. (2009). Genetic and proteomic evidence for roles of Drosophila SUMO in cell cycle control, Ras signaling, and early pattern formation. *PLoS One* **4**, e5905.
Ohsako, S., Hyer, J., Panganiban, G., Oliver, I. and Caudy, M. (1994). Hairy function as a DNA-binding helix-loop-helix repressor of Drosophila sensory organ formation. *Genes Dev.* **8**, 2743-2755.
Orian, A., Delrow, J. J., Rosales-Nieves, A. E., Abed, M., Metzger, D., Paroush, Z., Eisenman, R. N. and Parkhurst, S. M. (2007). A Myc-Groucho complex integrates EGF and Notch signaling to regulate neural development. *Proc. Natl. Acad. Sci. USA* **104**, 15771-15776.
Parkhurst, S. M. and Ish-Horowicz, D. (1992). Common denominators for sex. *Curr. Biol.* **2**, 629-631.
Parkhurst, S. M., Bopp, D. and Ish-Horowicz, D. (1990). X:A ratio, the primary sex-determining signal in Drosophila, is transduced by helix-loop-helix proteins. *Cell* **63**, 1179-1191.
Paroush, Z., Finley, R. L., Kidd, T., Wainwright, S. M., Ingham, P. W., Brent, R. and Ish-Horowicz, D. (1994). Groucho is required for Drosophila neurogenesis, segmentation, and sex determination and interacts directly with hairy-related bHLH proteins. *Cell* **79**, 805-815.
Penn, J. K. and Schedl, P. (2007). The master switch gene sex-lethal promotes female development by negatively regulating the N-signaling pathway. *Dev. Cell* **12**, 275-286.
Perry, J. J., Tainer, J. A. and Boddy, M. N. (2008). A SIM-ultaneous role for SUMO and ubiquitin. *Trends Biochem. Sci.* **33**, 201-208.
Poortinga, G., Watanabe, M. and Parkhurst, S. M. (1998). Drosophila CtBP: a Hairy-interacting protein required for embryonic segmentation and hairy-mediated transcriptional repression. *EMBO J.* **17**, 2067-2078.
Prudden, J., Pebernard, S., Raffa, G., Slavin, D. A., Perry, J. J., Tainer, J. A., McGowan, C. H. and Boddy, M. N. (2007). SUMO-targeted ubiquitin ligases in genome stability. *EMBO J.* **26**, 4089-4101.
Rosales-Nieves, A. E., Johndrow, J. E., Keller, L. C., Magie, C. R., Pinto-Santini, D. M. and Parkhurst, S. M. (2006). Coordination of microtubule and microfilament dynamics by Drosophila Rho1, Spire and Cappuccino. *Nat. Cell Biol.* **8**, 367-376.
Rosenberg, M. I. and Parkhurst, S. M. (2002). Drosophila Sir2 is required for heterochromatic silencing and by euchromatic Hairy/E(Spl) bHLH repressors in segmentation and sex determination. *Cell* **109**, 447-458.
Rothwell, W. F., Fogarty, P., Field, C. M. and Sullivan, W. (1998). Nuclear-fallout, a Drosophila protein that cycles from the cytoplasm to the centrosomes, regulates cortical microfilament organization. *Development* **125**, 1295-1303.
Secombe, J. and Parkhurst, S. M. (2004). Drosophila Topors is a RING finger-containing protein that functions as a ubiquitin-protein isopeptide ligase for the hairy basic helix-loop-helix repressor protein. *J. Biol. Chem.* **279**, 17126-17133.
Spradling, A. C. (1986). P element-mediated transformation. In *Drosophila, A Practical Approach* (ed. D. B. Roberts), pp.175-197. Oxford: IRL Press.
Sullivan, W., Minden, J. S. and Alberts, B. M. (1990). Daughterless-abo-like, a Drosophila maternal-effect mutation that exhibits abnormal centrosome separation during the late blastoderm divisions. *Development* **110**, 311-323.
Sullivan, W., Fogarty, P. and Theurkauf, W. (1993). Mutations affecting the cytoskeletal organization of syncytial Drosophila embryos. *Development* **118**, 1245-1254.
Sun, H., Leverson, J. D. and Hunter, T. (2007). Conserved function of RNF4 family proteins in eukaryotes: targeting a ubiquitin ligase to SUMOylated proteins. *EMBO J.* **26**, 4102-4112.
Uzunova, K., Gottsche, K., Miteva, M., Weisshaar, S. R., Glanemann, C., Schnellhardt, M., Niessen, M., Scheel, H., Hofmann, K., Johnson, E. S. et al. (2007). Ubiquitin-dependent proteolytic control of SUMO conjugates. *J. Biol. Chem.* **282**, 34167-34175.
Van Doren, M., Bailey, A. M., Esnayra, J., Ede, K. and Posakony, J. W. (1994). Negative regulation of proneural gene activity: hairy is a direct transcriptional repressor of achaete. *Genes Dev.* **8**, 2729-2742.
Wilson, V. G. and Heaton, P. R. (2008). Ubiquitin proteolytic system: focus on SUMO. *Proteomics* **5**, 121-135.
Wurmbach, E., Wech, I. and Preiss, A. (1999). The enhancer of split complex of Drosophila melanogaster harbors three classes of Notch responsive genes. *Mech. Dev.* **80**, 171-180.
Younger-Shepherd, S., Vaessin, H., Bier, E., Jan, L. Y. and Jan, Y. N. (1992). deadpan, an essential pan-neural gene encoding an HLH protein, acts as a denominator in Drosophila sex determination. *Cell* **70**, 911-922.