SUPPLEMENTAL INFORMATION ABED *ET AL*.

-Supplemental Tables S1, S2

-Description of *hairy* alleles used in this study.

-Protein extraction methods.

- Supplemental Figures S1, S2, S3, S4, S5, S6.

Table S1: Dgrn suppresses Gro related phenotypes

A. Expression of a functional Dgrn alleviates Gro small eye phenotype (P<0.01)

Genotype	% Severe Small Eye phenotype	n
Ey-Gal4/+;UAS-GFP/+	0	55
Ey-Gal4/+;UAS-Gro/+	100	50
Ey-Gal4/+;UAS-Dgrn/+;UAS-Gro/+	4	47
Ey-Gal4/+;UAS-Dgrn$^{HC/AA}$/+;UAS-Gro/+	90 (10 with a less severe phenotype)	61
Ey-Gal4/+; UAS-Dgrn^{I268A}; UAS-Gro/+	55 (45 with a less severe phenotype)	60
Ey-Gal4/+;UAS- Dgrn$^{\Delta SIMS}$;UAS-Gro/+	98	70
Ey-Gal4/+UAS-Dgrn	0	60
Ey-Gal4/+UAS-Dgrn$^{HC/AA}$/+	0	56
Ey-Gal4/+ UAS-Dgrn^{I268A}/+	0	70
Ey-Gal4/+UAS-Dgrn$^{\Delta SIMS}$/+	Die at pupal stage- headless	0

Eyeless-Gal4, Ey-GAL4;

B. Expression of Dgrn alleviates Gro dependent repression during the formation of notal bristles (P<0.01).

Genotype	% of adults with fewer than 3 scutellar bristles	n
C253-Gal4/+; UAS-GFP/+	0	211
C253-Gal4/+; UAS-Gro/+	53	294
C253-Gal4/UAS-Dgrn; UAS-Gro/+	30	208
C253-GAL4/UAS- Dgrn$^{HC/AA}$: UAS-Gro/+	59	216
C253-GAL4/UAS- Dgrn$^{\Delta SIMS}$:UAS-Gro/+	41	115
C253-Gal4/UAS-Dgrn	0	218
C253-GAL4/UAS-Dgrn$^{HC/AA}$	0	215
C253-GAL4/UAS-Dgrn$^{\Delta SIMS}$	0	112

Genotype	% of adults with ectopic bristle	n
UAS-GFP/+ ;Pnr-Gal4/+	0	130
Pnr-Gal4/ UAS-Gro-i	100	108
Pnr-Gal4/ UAS-Gro-i,UAS Dgrn-i	0	112
Pnr-GAL4/UAS- Dgrn-i	0	109

Gro-i, RNAi Gro transgene; Dgrn-i, RNAi Gro transgene

Table S2: 59 Dgrn and Gro shared targets identified using DamID in *Drosophila* Kc cells.

Gene	Designation	Cytological location	Biological process
sm	CG9218	56D15-56E1	Axon Guidance
Mical	CG33208	85F5-85F7	Axon Guidance
Con	CG7503	64C2-64C4	Cell adhesion
Scrib	CG5462	97B9-97C1	Cell polarity
Cbp53E	CG6702	53E4-53E4	Cellular homeostasis
Fpps	CG12389	47F1-47F1	Cholesterol metabolism
cid	CG13329	50A11-50A11	Chromatin structure
HP4	CG8044	66A21-66A21	Chromatin structure
Msp-300	CG33715	25C7-25C10	Cytoplasmic transport
Htt	CG9995	98E2-98E2	Cytoskeleton
Tun	CG8253	52C8-52C8	Learning
—	CG5976	77C4-77C4	Metabolism-Proteolysis
—	CG32700	8E4-8E7	Metabolism
—	CG7077	94A5-94A5	Metabolism
—	CG10184	95A4-95A4	Metabolism
—	CG7544	51E2-51E2	Methylation
mei-S328	CG5303	58B9-58B10	Mitosis
Smc5	CG32438	78D6-78D7	Mitosis
Kirre	CG3653	3C3-3C7	Myogenesis
MRE10	GM08676		ncRNA
MRE20	LD03248	3A3-3A4	ncRNA
MRE30	CR32028	66C13-66C13	ncRNA
dpr11	CG33202	83F1-83F4	Neuronal behavior
dpr9	CG33485	88E1-88E1	Neuronal behavior
cta	CG17678	2L	Signaling
igl	CG18285	51E3-51E5	Signaling Ca^{+2} binding
Amon	CG6438	97C4-97C5	Signaling-Hormone processing
nAcR&beta-64B	CG11348	64B6-64B6	Signaling-Ion transport
—	CG8422	50F8-50F9	Signaling-Neuronal
CSN7	CG2038	44C6-44C6	Signaling-Signalosome
—	CG30078	51F1-51F1	Signaling-Thermotaxis
Bi	CG3578	4C3-4C4	Transcription
Ci	CG2125	102A1-102A3	Transcription
Tio	CG12630	40D3-40D3	Transcription
Sox100B	CG15552	100B2-100B2	Transcription
—	CG9705	73C4-73C4	Transcription
zfh2	CG1449	102C3-102C4	Transcription
Bari1			Transposable element
—	CG32633	12A4-12A4	Unknown
—	CG9106	13B4-13B4	Unknown
—	CG14033	25D2-25D2	Unknown
—	CG7251	25F1-25F1	Unknown
—	CG18480	35D3-35D3	Unknown
—	CG15233	42C1-42C1	Unknown
Coilin	CG8710	44B3-44B3	Unknown
—	CG30069	50E2-50E3	Unknown
—	CG12424	51D6-51D6	Unknown
—	CG42365	57F7-57F7	Unknown
—	CG7049	61B2-61B2	Unknown
—	CG32333	61F4-61F5	Unknown
—	CG14965	63B10-63B11	Unknown
—	CG12213	87A3-87A3	Unknown
—	CG5060	92D9-92E1	Unknown
—	CG7084	94A4-94A4	Unknown
beat-IV	CG10152	95A2-95A2	Unknown
—	CG5515	95E8-95E8	Unknown
—	CG34354	98C2-98C2	Unknown
—	CG32850	102B3-102B4	Unknown
—	GM03205		Unknown

* CG8669, cryptocephal (crc), location 39C2-3 is the only shared target between CtBP and Dgrn.

Hypomorphic *hairy* alleles used in the study:

hairy^{7H} and *hairy*^{12C} are well-characterized *hairy* hypomorphic alleles that have been used to study Hairy function during segmentation and PNS specification, as well as in interactions with Groucho, CtBP, Sir2 and other hairy cofactors (see for example: Poortinga *et al,* 1998*;* Phippen *et al*, 2000). *hairy*^{7H} is an EMS-generated point mutation that leads to a premature stop codon being introduced just after the basic region (L72*; referred to in FlyBase as *h*^{26}). *hairy*^{12C} is an EMS-generated point mutation that mutates the Proline residue of Hairy's C-terminal WRPW motif (P336L; referred to in FlyBase as *h*^{30}). *hairy*^{l} is a viable *hairy* allele that exhibits ectopic bristles.

Protocols for SUMOylation assay in cells :

In vivo SUMOylation assays was similar to Tatham *et al.* (Nature protocols 4(9):1363:1371). SUMOylation assays were performed by either one of the following methods as stated in the manuscript:

1) Guanidine HCl method: S2R cells were transfected with combinations of Gal4-driven expression vectors for Gro, Dgrn, Dgrn$^{HC/AA}$ and Dgrn$^{\Delta SIMs}$, and a His-Sumo2 expression plasmid. 48 h after transfection, cells were harvested, lysed in buffer A (6 M guanidinium-HCl, 0.1 M sodium phosphate buffer pH 8.0, 250 mM sodium chloride, 10 mM imidazole, 0.4% Triton X-100 and 0.1 mM *N*-ethyl maleimide), sonicated, and cellular debris was removed by centrifugation. The lysate was incubated with Ni-NTA beads (Qiagen) overnight at 4ºC with gently spinning to purify His-tagged proteins. The beads were washed twice with buffer A, twice with a 1:4 mixture of buffer A and buffer B respectively (25 mM Tris–HCl, pH 6.8, and 20 mM imidazole), and twice with buffer B. Bound proteins were eluted by boiling in SDS sample buffer containing 200 mM imidazole, and eluted proteins were analyzed by Western blotting.

2) Hot Lysis Buffer method: S2R cells were transfected as mentioned above. 48 h after transfection, cells were harvested and lysed in boiling hot lysis buffer (2% SDS, 20mM EDTA, 50mM Tris-HCl pH 8.0, 20mM DTT, 20mM *N*-ethyl maleimide), boiled, sonicated and cellular debris was removed by centrifugation. The lysate was diluted 1:10 with TNN buffer (50mM Tris-HCl pH 7.5, 120mM sodium chloride, 5mM EDTA, 0.5% NP-40) and incubated with EZviewTM Red anti-HA Affinity Gel (Sigma Aldrich) overnight at 4ºC with gently spinning. Sepharose beads were washed three times with TNN buffer and eluted by boiling in SDS sample buffer. Eluted proteins were analyzed by Western blotting.

3) RIPA buffer: 10mM Tris-HCl pH 7.4, 150mM sodium chloride, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS.

Abed et al._Figure S1

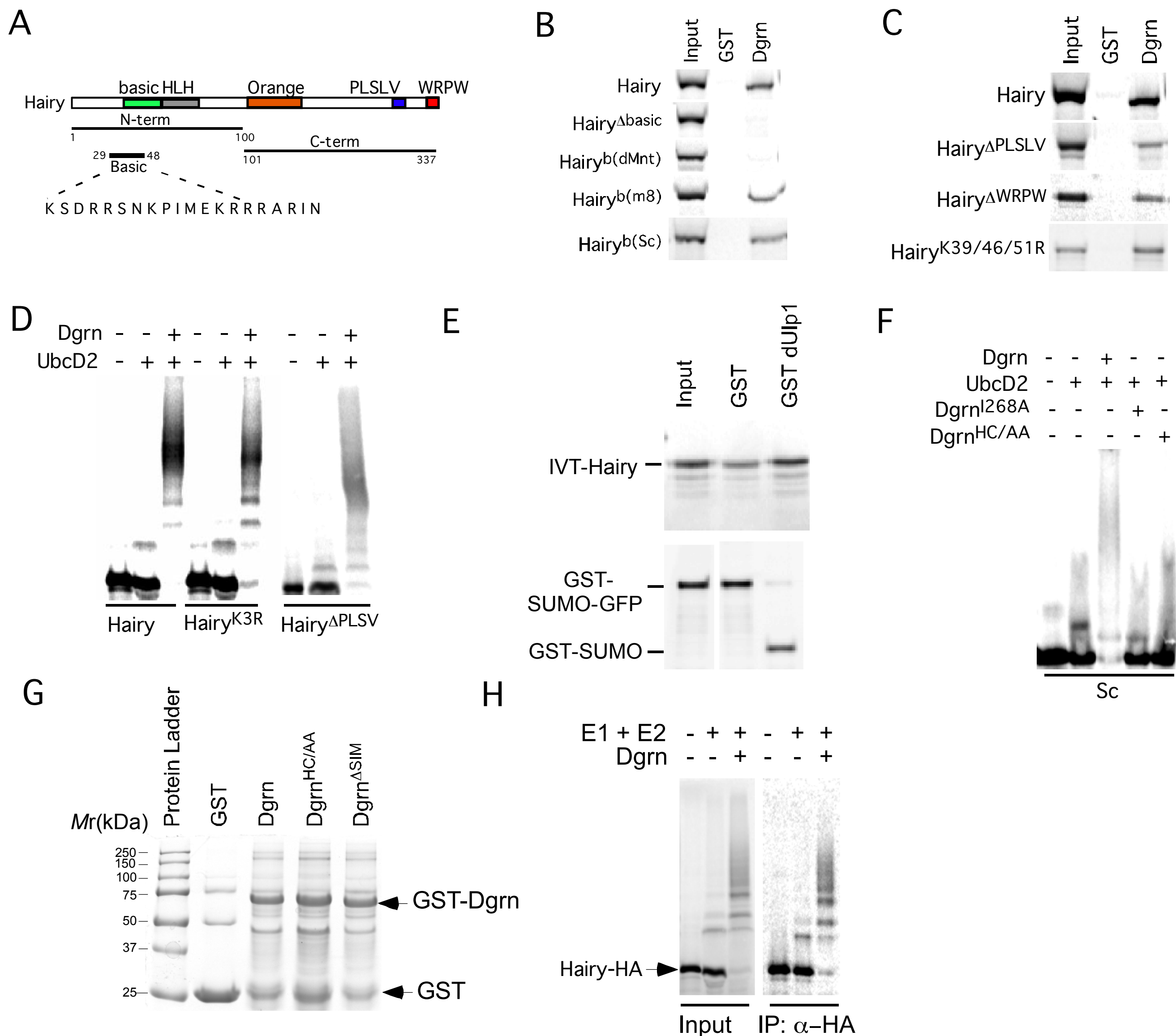


Figure S1. Molecular recognition and ubiquitylation of Hairy and Scute proteins by Dgrn.
(A) Diagram depicting Hairy domains. N-terminal (1-100aa) and C-terminal (101-338aa) Hairy fragments were used initially to map the interaction region between Dgrn and Hairy. Dgrn binds to 20 amino-acids derived from Hairy's basic domain (see also Figure 1).
(B) Binding of Hairy to Dgrn in GST pulldown assays requires Hairy's basic domain. Substituting the basic domain with the basic domains derived from other Dgrn binding proteins [Espl(m8) or Sc], but not from the non-binding bHLH repressor dMnt, restores binding.
(C) GST pulldown assays with the indicated Hairy mutants show that the CtBP recruitment motif (PLSLV), as well as the Gro recruitment motif (WRPW), are not required for Dgrn binding. Replacement of Lys residues with Arg within a putative SUMOylation site (K39,46,51R), does not alter binding or ubiquitylation (not shown).
(D) Dgrn efficiently ubiquitylates *in vitro* Hairy proteins that are either mutated in a putative SUMOylation site located between the dCtBP and Gro recruitment motifs (Lys 325, 326, 328; designated $Hairy^{K3R}$), or that lacks the CtBP recruitment motif ($Hairy^{\Delta PLSLV}$).
(E) Treatment with the SUMO specific isopeptidase Ulp does not alter Hairy's mobility in SDS-PAGE (upper panel). In contrast, treatment of GST-GFP-SUMO with Ulp results in a faster migrating GST-SUMO species (lower panel), suggesting that IVT-Hairy is not SUMOylated. Ulp treatment was performed as described (1).
(F) A functional Dgrn but not RING mutants, ubiquitylates the bHLH activator Scute (Sc) *in vitro* in a partially purified reconstituted system.
(G) Comassie-blue staining of the proteins bound to sepharose-glutathione beads used in Figure 1J (12.5% SDS-PAGE gel).
(H) To test if ubiquitylation of Hairy results in clipping of its WRPW domain we tested if Hairy that is tagged with HA at its C-termini retains its tag upon ubiquitylation and subsequent anti-HA IP. As shown ubiquitylated Hairy has an intact c-termini.

(1) Bhaskar, V., Valentine, S.A., and Courey, A.J. (2000). A functional interaction between dorsal and components of the Smt3 conjugation machinery .J. Biol. Chem. *275*, 4033-4040.

Abed et al._ Figure S2

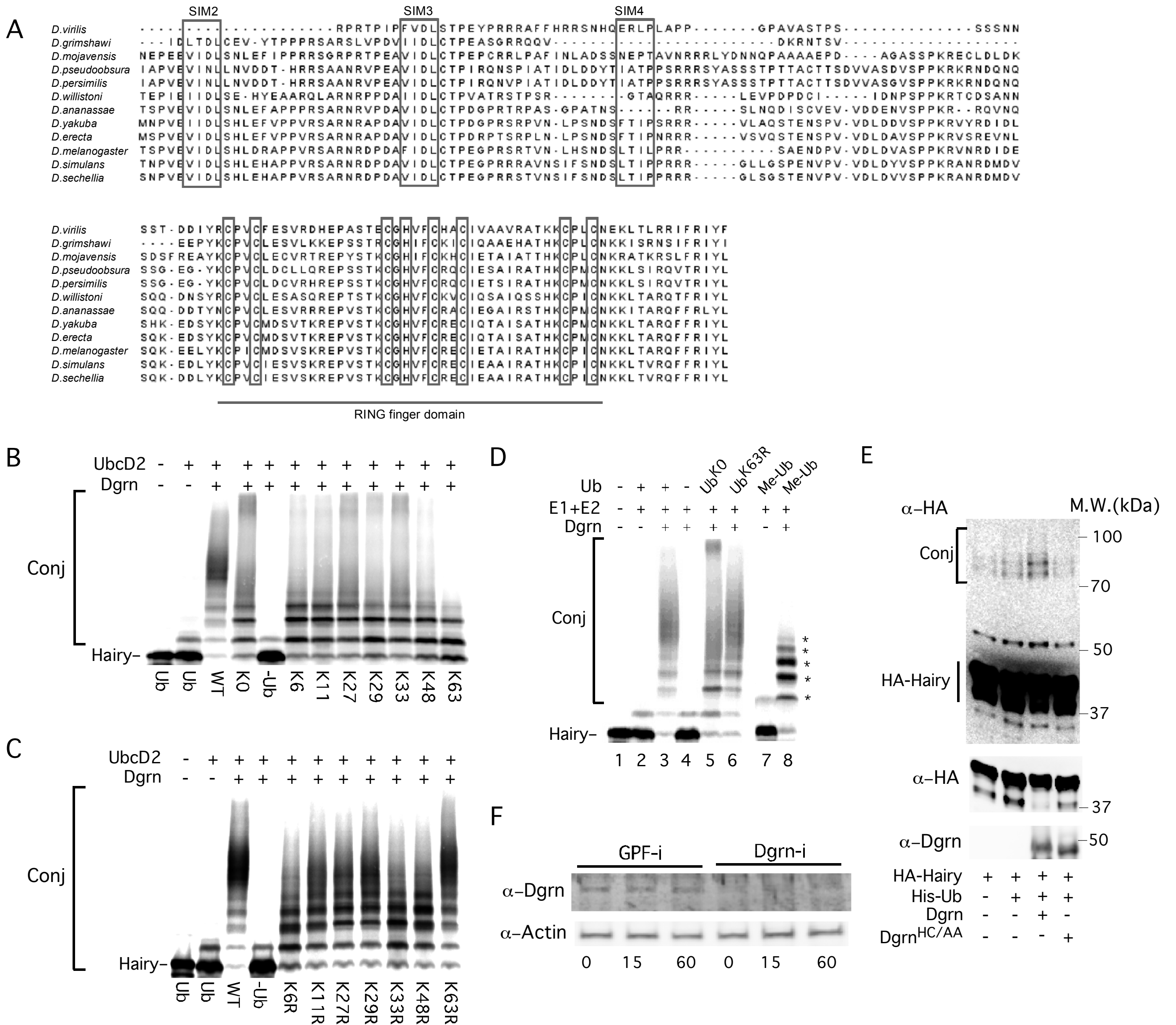
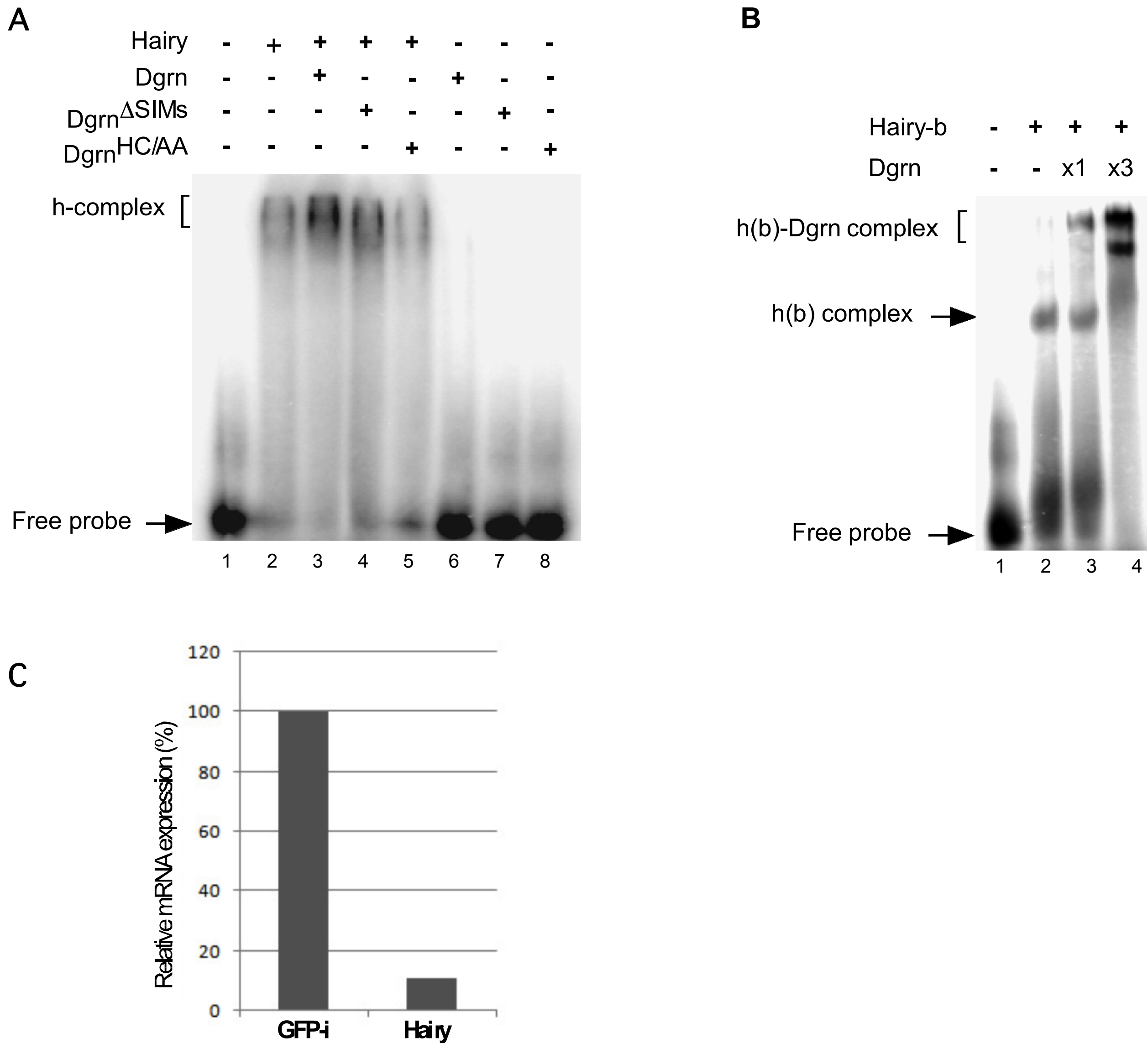


Figure S2. Dgrn protein is well conserved between *Drosophila* species and Dgrn catalyzes the generation of a mixed poly-ubiquitin tree.
(A) Protein sequence alignment of Dgrn orthologs from twelve *Drosophila* species. Three of the putative SIM domains are boxed and labeled (the first SIM is less conserved and is not shown).
The zinc coordinating residues within the RING finger domain are boxed.
(B) Ubiquitylation with ubiquitin derivatives, which contain only a single internal Lys residue (as indicated), is sufficient to support conjugation of Hairy. Note that Lys-48 and -63 are the least efficient.
(C) No single Lys residue is critically required for Hairy ubiquitylation. Ubiquitin derivatives where single Lys residues are replaced with Arg efficiently support Hairy's ubiquitylation.
(D) *In vitro* ubiquitylation assays with and without ubiquitin (lanes 3 and 4), or with ubiquitin derivatives Ub^{K63R} and Ub^{K0} (lanes 5 and 6). Since high molecular weight Hairy-Ub conjugates are detected in all cases, the chains are likely of mixed types. Hairy ubiquitylation using the Me-Ub derivative (methylated-ubiquitin; lanes 7 and 8) reveals that at least 5-6 independent Lys residues are involved (indicated by "*"), and that the Ub linkage is not solely made of linear N-terminal poly-ubiquitylation.
(E) Dgrn ubiquitylates Hairy in cells. 293T cells were transfected with the indicated pCDNA3-based vectors 48h after transfection proteins were extracted using guanidine-HCl buffer were prepared and visualized using western-blot analysis and the indicated antibodies. The middle pane is a short exposure, note the disappearance of the lower Hairy band. Conj. denotes Hairy-Ub conjugates.
(F) Western-blot analysis of S2R *Drosophila* cell extract. Dgrn protein levels are reduced in Dgrn RNAi (Dgrn-i) during a 60 minute cyclohexamide (CHX) chase experiment, whereas it remains unaffected when control RNAi (GFP-i) is applied.

Abed et al._Figure S3



(A-B) Purified Dgrn and Hairy form a complex on DNA *in vitro*.
(A) Dgrn does not displace Hairy from DNA. Electro-Mobility Shift Assay (EMSA) showing that the binding of full length bacterially expressed Hairy to the *ac* promoter-derived Hairy binding site is not abolished by Dgrn or its mutant derivatives as indicated (compare lane 2 to 3-5). Dgrn and its mutants show minimal or no binding to the Hairy binding site on their own (lanes 6-8). EMSA was performed as described in (1). The free probe is shown at the bottom. “h-complex” denotes the slow migrating Hairy-DNA complex.
(B) Dgrn binds to Hairy’s basic domain and results in a supershift of the Hairy(basic)-DNA complex. EMSA shows that bacterially expressed Hairy basic domain (1-48aa, Hairy-b) binds to the ac promoter-derived Hairy binding site (lane 2). In addition, increasing amounts of bacterially expressed Dgrn (x1 and x3 correspond to 100ng and 300ng of Dgrn protein respectively) which also binds Hairy’s basic region domain, forms a slower migrating complex (compare lanes 3 and 4 to lane 2). h(b) denotes Hairy-basic.

(C) qPCR analysis of RNAi-mediated targeting of Hairy. To quantify the extent of Hairy-RNAi targeting in S2R cells the levels of Hairy’s mRNA were compared between cells with specific Hairy targeting (Hairy-i) to that of cells targeted with control RNAi (GFP-i) using qPCR. Relative mRNA expression is shown.

(1) Bianchi-Frias *et al*. (2004). Hairy-mediated transcriptional repression and cofactor recruitment in *Drosophila* *PLoS Biology* **2:**975-986.

Abed et al._Figure S4

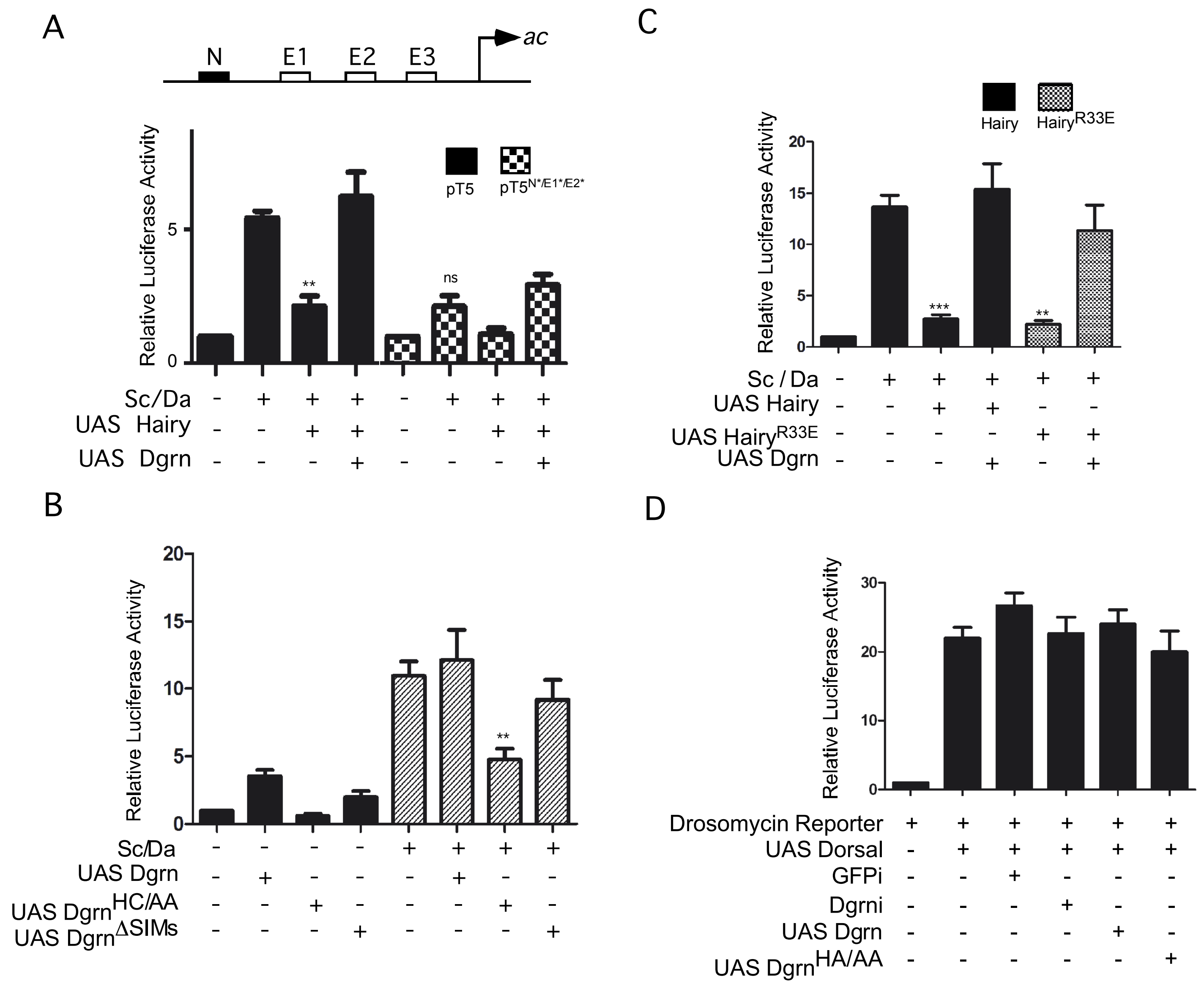


Figure S4. Dgrn's transcriptional activity is specific to the *ac* reporter and requires Dgrn's ligase activity.
Shown are Luciferase reporter assays with the indicated plasmids in S2R *Drosophila* cells.
(A, B) Dgrn activity requires specific binding sites within the *ac* promoter. **(A)** Upper panel denotes the specific binding sites within the *ac* reporter required for Da, Sc, and Hairy transcriptional activity. Analysis of the impact of point mutations within the *ac* reporter (N*/E1*/E2*), show that inactivating even part of these sites is sufficient to inhibit transcriptional activity. **(B)** Dgrn and its RING and SIM mutants require the activity of sequence specific transcription factors and have an insignificant effect on their own in the absence of Sc and Da. **(C)** Dgrn successfully alleviates $Hairy^{R33E}$ mediated repression of the *ac* promoter in S2R *Drosophila* cells. Similar to Hairy, the $Hairy^{R33E}$ mutant effectively represses transcription from the *ac* luciferase reporter. Dgrn alleviates *ac* repression regardless of Dgrn's incapability to bind or ubiquitylate $Hairy^{R33E}$. **(D)** In contrast to Dgrn's effect on the *ac* reporter, expression of Dgrn, or its RING mutant as well as reduction of Dgrn levels via RNAi have no effect on Dorsal dependent activation of the Drosomycin reporter (1). Expression of Dorsal, Dgrn expression vectors and RNAi efficiency were confirmed by western-blot (not shown).In all experiments, 4 independent experiments were performed. Statistical analysis including SEM and t-test comparisons were performed using Prism5 Anova software. ***= P<0.001; **= P<0.01. ns; not significant.

(1) Goto A, Matsushita K, Gesellchen V, El Chamy L, Kuttenkeuler D, Takeuchi O, Hoffmann JA, Akira S, Boutros M, and Reichhart JM (2008) Akirins are highly conserved nuclear proteins required for NF-kB-dependent gene expression in drosophila and mice *Nature Immunology* 9: 97-104

Abed et al._ Figure S5

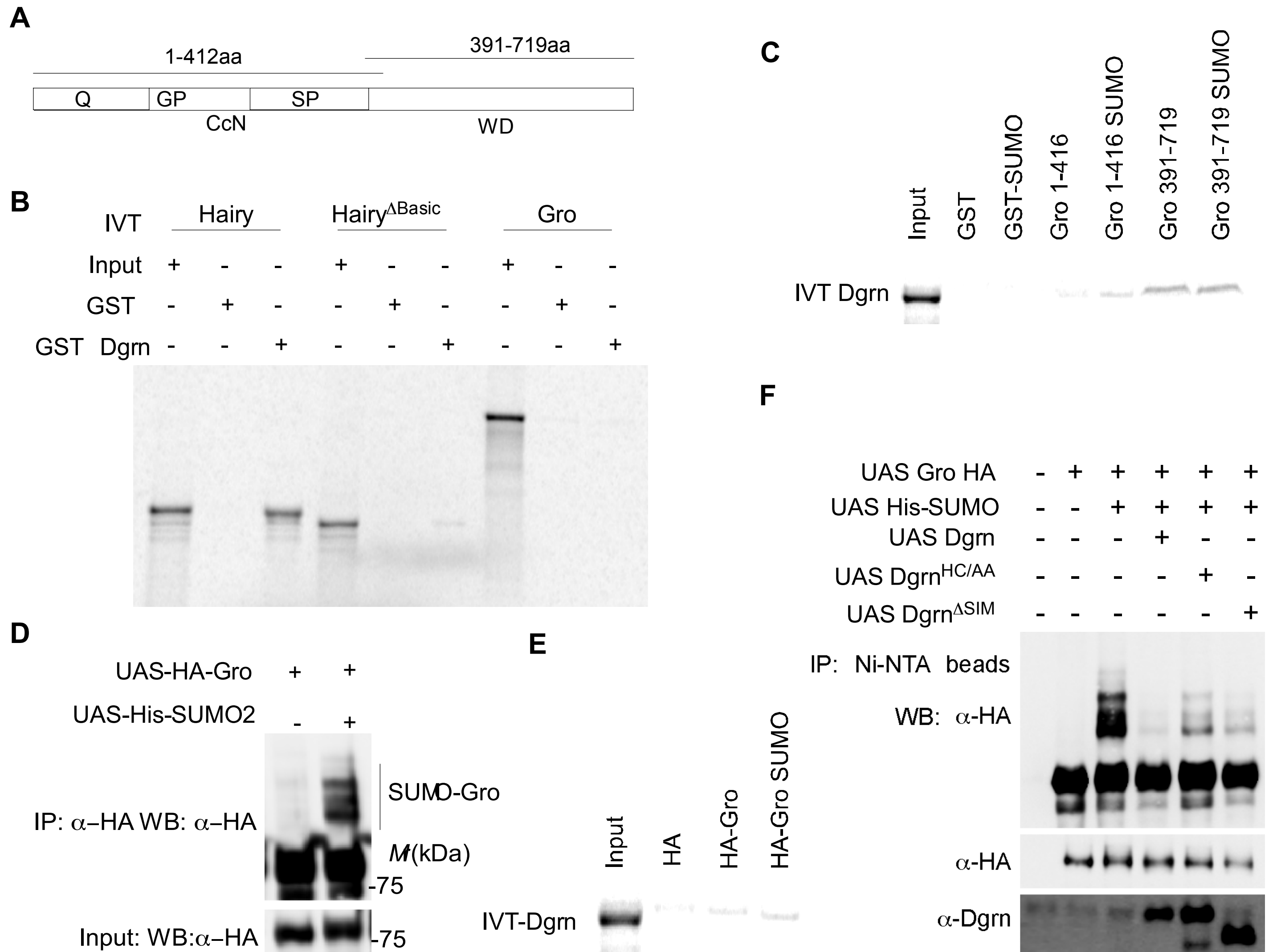


Figure S5. Dgrn does not bind Gro directly, but targets SUMOylated Gro.

(A) Schematic diagram of Gro, indicating Gro's protein domains.

(B) GST pulldown assay with GST-Dgrn. Dgrn binds to IVT-Hairy but not to unmodified IVT-Gro or IVT-Hairy lacking the basic domain .**(C)** Dgrn binds weakly to the WD40-domain fragment of Gro in its naive form or when it is SUMOylated in bacteria. Shown is a GST pull-down assay using GST and GST-Gro fragments that were isolated from *Escherichia coli* bacteria with or without a SUMO expressing system (1). SUMOylation of the GST-fusion protein was verified using α–SUMO antibodies(not shown).

(D-E) Gro and SUMOylated-Gro isolated from S2R *Drosophila* cells bind weakly to IVT-Dgrn. HA-Gro was transfected into S2R cells in the absence or along with His-SUMO. Subsequently naive or SUMOylated-Gro was immunoprecipitated (IP is shown in D). Beads bound by either Gro or SUMOylated-Gro were used in an *in vitro* binding assay with IVT-Dgrn. Regardless of the SUMOylation status of Gro, only weak binding was detected. **(F)** Dgrn selectively targets SUMOylated Gro in a RING and SIM dependent manner. Western blot analysis of S2R cell-derived extracts. Cells were transfected with the indicated plasmids. 48h post transfection, cells were harvested and subsequently SUMOylated proteins were precipitated using Ni-NTA beads. Gro and SUMOylated-Gro were detected using HA-antibody. Note that since expression of Dgrn affects Gro protein levels, the amount of material used for IP was adjusted to have equal amounts of naive Gro. Under these conditions, it is apparent that Dgrn specifically targets SUMOylated-Gro.Targeting SUMOylated-Gro requires intact RING and SIM domains.
The expression of endogenous and transfected Dgrn is shown in the bottom panel.

(1) Uchimura Y, Nakamura M, Sugasawa K, Nakao M, Saitoh H. (2004). Overproduction of eukaryotic SUMO-1- and SUMO-2-conjugated proteins in Escherichia coli. *Anal Biochem.* **331**:204-206.

Abed et al._ Figure S6

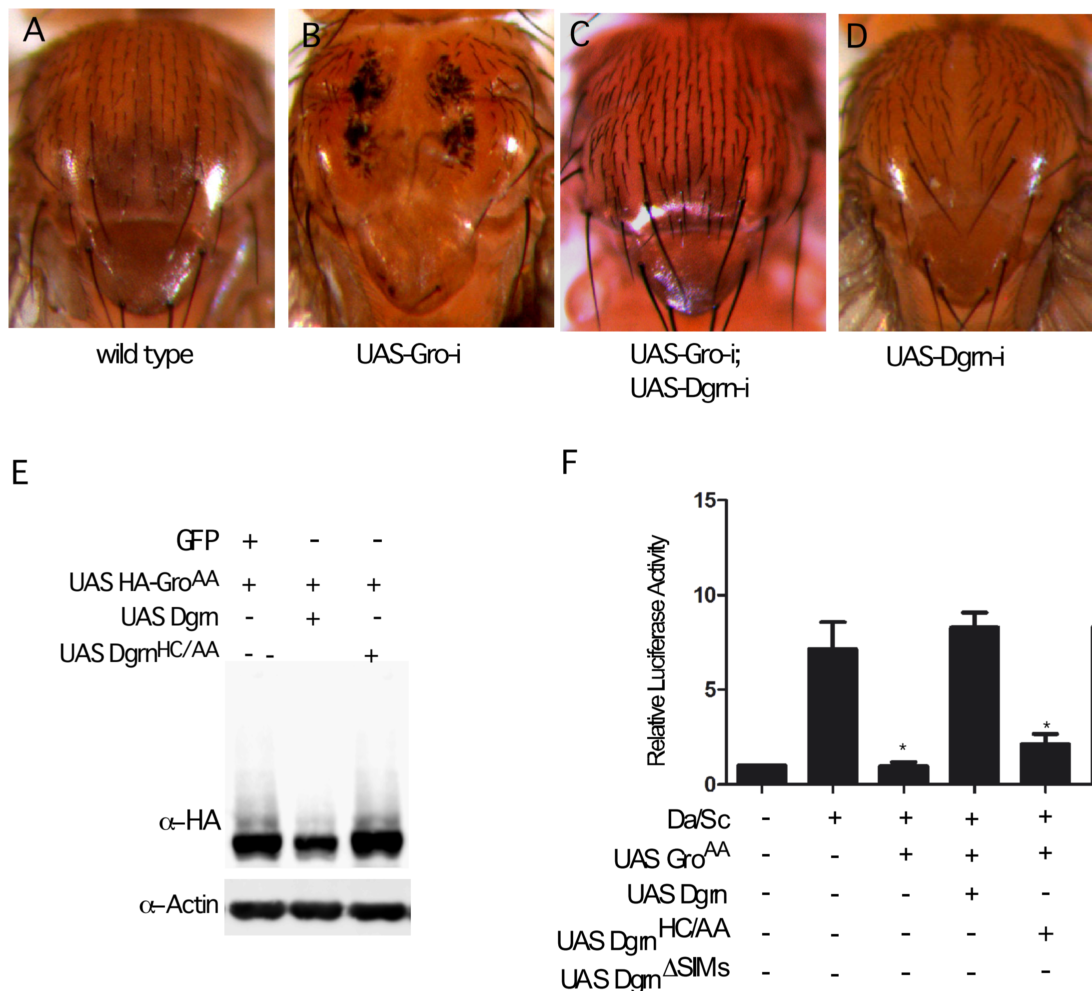


Figure S6. Targeted reduction of Dgrn levels suppresses the Gro accessory bristle phenotype and is independent of priming RTK phosphorylation.

(A-D) Reduction of Dgrn levels suppresses the Gro phenotype during sensory bristle specification. Reduction of Gro levels using UAS-RNAi transgene (Gro-i), under the control of Pnr-Gal4 results in ectopic bristle formation lateral to the midline [Compare (A) to (B)]. Co-expression of Dgrn RNAi-transgene (Dgrn-i) suppresses this Gro phenotype (C). Note that expression of Dgrn-i or the Pnr-Gal4 driver alone results in only a mild bristle hypoplasia (D, and not shown).

(E-F) Dgrn function is independent of priming RTK phosphorylation of Gro. Western blot analysis of RIPA derived S2R extracts (E). Cells were transfected with the indicated plasmids, and RIPA derived protein extract were prepared. The protein levels of HA-Gro^{AA} that can not undergo RTK mediated phosphorylation are reduced upon expression of functional Dgrn, but not by Dgrn$^{HC/AA}$. (F) HA-Gro^{AA} mediated repression of the *ac* luciferase reporter is alleviated by co-expression of Dgrn.

Data was calculated from 3 independent experiments. Statistical analysis including SEM and t-test comparisons were performed using Prism5 software. *= P<0.1