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Degringolade, a SUMO-Targeted Ubiquitin Ligase, inhibits Hairy/Grouchomediated repression

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1st Editorial Decision 20 August 2010

Thank you for submitting your manuscript for consideration at The EMBO Journal. I apologise that it has taken so long to have your manuscripts reviewed but I had to extend the deadline for the referees and wanted to have the reports on both manuscripts before making a decision. Two referees have evaluated the study and I enclose their reports below. The referees find it potentially interesting but require further experimental analysis to support several aspects of the study. These include improved interaction data and also additional line of evidence to GST-pulldowns such as Co-IPs and if the function of Dgrn is specific to Hairy or also target the basal transcription machinery. Referee #1 would also like to see a comparison of the genome-wide binding of Dgrn to that of Hairy. Referee #2 requires a more detailed explanation of the genetic data. Should you be able to address these concerns, which are central to the manuscript, I would be happy to re-evaluate a revised version of the manuscript.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your

revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

In this manuscript, the authors present important and interesting new information regarding the regulation of Hairy- and Groucho-mediated processes in Drosophila by Dgrn, a SUMO-targeted ubiquitin ligase. The authors identified a physical interaction between Dgrn and Hairy in a yeast two-hybrid screen, and show compelling evidence for both physical interaction between the two proteins and genetic interactions in the fly. Overexpression or loss of function gro and hairy phenotypes can be suppressed by expression of Dgrn, and dgrn mutants affect total protein levels of Groucho in embryos. The basic/DNA binding portion of Hairy is shown to be important for Dgrn interaction in vitro, and a mutation in this domain blocks Dgrn-mediated ubiquitylation of Hairy, although interestingly repression by this mutant Hairy protein is still interfered with by excess Dgrn expression, indicating that it can directly regulate the Groucho
corepressor independently of Hairy binding.

The experiments that seek to define a molecular basis for the antagonistic interactions between Dgrn and Hairy/Groucho produce less clear-cut results, as discussed below. In addition, the manuscript is replete with typographical errors that in some cases interfere with understanding, and should be corrected.

Overall, this paper would be of great interest to readers of EMBO J., after suitable attention to the following points:

1. The model suggests that Dgrn touches both Hairy and Groucho. The data presented here make a clear case for Hairy-Dgrn physical
interaction, but the only place where Dgrn-Gro physical interactions are tested is in S4E. This experiment tests interaction between Groucho on beads and Dgrn, but controls are lacking, so it is impossible to judge whether the interactions shown are strong, weak, specific, or meaningful at all. This experiment is too preliminary to
make any solid conclusion.

2. In S4F, the levels of SUMOylation of Groucho are tested in cells where Dgrn is overexpressed An immunoprecipitation (or is it NTA-Ni affinity purification? The IP: a-His nomenclature is a bit unclear) against his-tagged SUMO is used to purify proteins, and Groucho proteins are then detected with anti-HA. It is puzzling that unmodified Groucho protein is apparently the most abundant protein, showing up even in lane 2, where no SUMO-his is expressed. Why is Groucho directly purified by the anti-his step? This question also applies to Figure 5B.

3. This paper provides evidence that in cell culture, the
overexpression of Dgrn affects Groucho "extractability" but not
overall levels, while in embryos, the total levels of Groucho are
inversely proportional to Dgrn levels. It would be useful to try to interpret why the cell culture results are so different from those obtained with genetically manipulated embryos.

4. The paper presents results from a DAM-ID genomic mapping of Dgrn, but surprisingly, no correlation is made between Dgrn binding and Hairy binding. The physical Dgrn-Hairy interactions described would indicate that these proteins may cooccupy a gene, so the authors should correlate which binding sites for Dgrn are also known to bind Hairy (data from Bianchi-Frias, as well as Biggin's group - Li et al. 2008 and MacArthur et al. 2009). If there is no correlation, this would influence the model presented in Figure 6L; perhaps Dgrn modifies Hairy off of the DNA, and interacts with Groucho indirectly or off the DNA.

5. Relevant to the former point, in the Abstract, they note that "Concomitantly Dgrn specifically targets SUMOylated-Gro for sequestration and antagonizes Gro functions in vivo genome-wide." The paper does not provide any evidence for genome-wide functions of Groucho, however.

6. Supplemental Figure 3A tests whether Dgrn and Hairy can bind to DNA in vitro. The EMSA shows that Hairy can shift a probe, but the results are not at all compelling that Dgrn is part of this complex. A supershift experiment would indicate whether Dgrn is present.

7. In Figure 3C, the activity of the Dgrn HC/AA mutant protein is tested, and found to be inactive. The authors should test whether the protein is even expressed, because a trivial explanation would be that the loss of activity is simply due to protein instability, not loss of RING finger binding.

8. In Fig. 3B and C, RNAi is used to deplete Hairy and Groucho, to test the effect of Dgrn on reporter gene expression. The Dgrn effect is lost when Gro is targeted, but not Hairy, leading to the suggestion that there may be Hairy-independent pathways. This interpretation rests on the assumption that the knockdowns are complete, as suggested in the western blot in 3D, E. However, if the Hairy depletion were 90% effective, one would likely obtain the result shown in 3E and F, yet the residual protein would still provide activity. Thus, the authors should note that this suggestion of an alternative path is still speculative, due to the experimental design.

9. Why does the mobility of Hairy change under chase conditions shown in Fig. 2G? This is evident with both RNAi to nonspecific GFP and to Dgrn.

10. Figure 3 examines the negaive effect of extra Dgrn on transcriptional repression by Hairy. The specificity of such action is examined in S3B, which indicates that when activator sites are removed, the overall gene activity is reduced (even there, it appears that a qualitatively similar Dgrn effect is present). Yet Dgrn still might act nonspecifically downstream of activator action, by stimulating a component of the basal machinery. This possibility would be easily tested by examining transcription of other reporter genes in the presence of ectopic Dgrn - if all functional promoters are stimulated by extra Dgrn, it might point to a more global role for the protein. That result might detract from the Hairy-centric model in 6L, but it would be important to know.

11. This sentence may be misinterpreted - the data shown simply indicate that SUMO and Dgrn are in the nucleus, but the level of co-localization is not any more detailed than that. "We find that Dgrn co-localizes with SUMO or SUMOylated proteins in vivo in nuclei of developing embryos (Figure 1B-F)." They should revise this sentence to more accurately state their findings.

12. A major point is that ubiquitylated Hairy doesn't bind well to Groucho, but can bind to CtBP. In Fig. 2H, I, the fraction of Hairy that is not ubiquitylated does not preferentially bind to the GST-Groucho column. It appears that something that happened to the protein during the in vitro ubiquitylation reaction has interfered with Groucho binding - perhaps proteolysis of the terminal amino acids necessary for Groucho binding? This point is important for the model presented in Fig. 6L, where after modification, Hairy is suggested to lose Groucho interaction.

In sum, the genetic interactions clearly point to something important happening to Hairy and Groucho, mediated by Dgrn. An alternative reading of the data presented here is that Dgrn reduces the levels of Groucho in embryos, making Hairy a less potent repressor. This simpler model is not consistent with all of the data presented here, but in a number of cases, the data are too preliminary to make a strong interpretation - does Dgrn bind Groucho, is Dgrn acting with Hairy at a promoter, is the Dgrn effect mainly through Groucho, or perhaps through the basal machinery too? I suggest that the authors sort out which pieces of the many preliminary experiments are ready for publication, strengthen those aspects that are clear, and streamline the paper.

Typos and wording suggestions:

Why are some figures labeled "UAS-Gro" and others "Gro" to indicate overexpression of the protein? The lack of consistency is distracting.

Figure 5B neglects to indicate that Gro is being overexpressed, which it clearly is.

The labeling of S3B,C,D is mixed up. The figures to not correspond to the indicated letters in the figure legend.

The legend of Fig. 3 should say "antagonism of" not "antagonizes" to be clear.
Figure 3. Dgrn antagonizes Hairy and Gro repression inversely
correlates with the effects of SUMO pathway and is partially dependent on SUMOylation.

Additional spelling/grammar points:

Mechanistically Dgrn function as a molecular selector;

bHLH-orange [jargon] repressor

"Bianchi-Frias" is misspelled in several places. e.g. "Biachi-Frais/
Bianch-Frias"

and greatly [to a large extent] restored the pattern of the compound eye

as well as conjugating Hairy, although to a lesser extent than
wildtype [what?].

As for Gro, albeit extensive efforts we could not identify

Gro and its mammalian orthologs TLE proteins represse transcription

denaturizing buffers

(Fig. legend 1) (I) Bacterially purified recombinant His6-Hairy binds to GST-Dgrn

In Table S1, "severe" is misspelled sever in several places; "Eyless-Gal4" is also incorrect

% of adults with less than 3 [fewer than]

isolated from E. Coli

aloneor

SUMOylated-Gro was immuno-preciptated

Jenings & Ish-Horowicz 2008

Fig legend 4 suppresses Hairy transcriptional repression of Ftz [ftz]

SF1 "ubquitylation of the Hairy" "notrequired for Dgrn binding"
"sepharose-gluthatione beads"

SF5 - independent of priming RTK priming phosphorylation.

Referee #2 (Remarks to the Author):

"The STUbL protein Degringolade is a negative regulator of Hairy-mediated Groucho dependent repression"

Abed et al., demonstrate that Dgrn interacts with Drosophila Hairy and Groucho to antagonize Hairy/Gro-mediated repression during development. They report that ubiquitylation of Hairy by Dgrn specifically interferes with Hairy's ability to interact with Groucho (but not dCtBP). Finally the authors show that Dgrn specifically targets SUMOylated-Gro and that Dgrn and Groucho co-localize to a subset of loci using DamID analysis in Drosophila cell culture. The interactions described in this manuscript are novel and provide insight into regulation of two important families of transcriptional regulators (Hes and Gro), thus potentially of interest to a broad audience. However, the finding that ubiqitylation of Hairy's basic domain can interfere with Groucho recruitment is unexpected and needs to be better reconciled with established data in the field.

Major points
1) The authors rely heavily on the GST pulldown assay to demonstrate interactions between proteins in this paper. This is an in vitro assay that looks at binding of proteins that are relatively pure (not in the complexes that they may form in a real cell) and at reasonably high concentrations and thus pulldown assays can be misleading. Although GST pulldowns provide a very useful indication of whether proteins can interact, putative protein-protein interactions need to be validated in additional assays which reflect more biological conditions. For example, it is noted that Dgrn was found to interact with Hairy in a yeast two hybrid screen - thus yeast two hybrid data showing the interaction should be shown. The interaction between Dgrn and the basic domain of Hairy should also be validated by alternative assays (e.g. yeast two-hybrid, co-immunoprecipitation).

2) The authors show that Dgrn ubiquitylates the N-terminal basic domain of Hairy (at least in vitro), which inhibits interaction with the Groucho co-repressor protein. This finding is unexpected since previously it has been shown that Hairy interacts with Groucho via its C-terminal WRPW motif (right up the other end of the protein!). It has also been shown in several different assays that the WRPW motif alone is sufficient to interact with Groucho (e.g. in Fisher et al., 1996, MCB, vol 16:2670-2677 and Jennings et al., 2006, Mol Cell, vol 9:645-55). The authors do not acknowledge these conflicting observations in the manuscript. The authors must comment on how their findings can be reconciled to these observations - how does ubquitylation of the N-terminal domain of Hairy prevent binding via the C-terminal WRPW motif?

3) Genetic interactions between hairy and dgrn are examined, however the rationale behind these experiments is not at all clear and needs to be spelt out more explicitly in the text. For example, the nature of the hairy alleles used is not described in the text (or materials and methods), and this is very important for interpretation of the results. The authors appear to be testing for rescue of the phenotype of hairy loss of function alleles by an loss of function allele of dgrn. Thus, the model tested appears to be that Dgrn reduces Hairy's repressive function, so if you remove Dgrn, any residual Hairy will have increased function and you will get some rescue of the hairy phenotype. However, in a hairy null mutant background (where there is no residual Hairy function) you would not expect to see any rescue of the phenotype. Thus the nature of the hairy alleles used must be known to interpret the results.

More specifically in the second paragraph on page 12 the authors state "Since hairy null embryos die during embryogenesis, we tested if reducing the dose of dgrn can rescue the embryonic lethality associated with hairy mutants." If there is no Hairy in the embryos for Dgrn to interact with, why would you expect to see any modification of the hairy phenotype? Presumably the hairy7C/hairy12C is not a null Hairy background (but we currently don't know, as the nature of the 7C and 12C alleles is not stated), in which case the text is misleading and this is just repeating the previous experiment that looked at rescue of the segmentation phenotype. Alternatively the data does not support a direct interaction between Dgrn and Hairy, and indicates that Dgrn functions in segmentation independently of Hairy. The model and rationale behind the genetic interactions needs to be greatly clarified.

Minor points
Figure 1C - why is there an asterisk by "Basic" ?

1st Revision - authors' response 17 November 2010

Response to the Reviewer's comments: Abed *et al.*

We address the Reviewer's specific comments in detail below. Below we present a summary of the data that we have added to the manuscript:

1) We show that Dgrn and Hairy interact *in vivo* using a yeast two-hybrid assay (Figure 1K). We also used this assay to demonstrate that this *in vivo* interaction is mediated via the basic domain of Hairy (similar to the interaction we observe *in vitro* with purified proteins). A point mutation within the isolated Hairy bHLH ($bHLH^{R33E}$) abrogates binding. We also show that binding of Dgrn is independent of the WRPW, and that neither Gro nor dCtBP bind to Hairy's bHLH.

2) We present comparison of the direct genomic loci bound by Hairy and its associated co-factors dCtBP, Gro, Sir2, and Dgrn (Figure 6L). One of the major conclusions from our previous work is that the binding of Hairy to its targets is context-dependent (Bianchi-Frias et al, 2004). Here, we show that in Kc cells most of Hairy targets are shared with CtBP and Sir2, but not with Gro. We now show that 38% of the genomic sites bound by Gro are shared with Dgrn (Figure 6K). Additionally, in the new Figure 6L we compare the binding sites of the above co-factors including Dgrn to that of Hairy. Consistent with our previous findings, we see that no gene overlap between Dgrn and Hairy in this experimental setting. Thus, the binding landscape depicted in the context of Kc cells reflects the situation "post" co-factors selection: Hairy associates with genomic regions that preferentially recruit Sir2 and CtBP, but not either Gro and Dgrn. Together with the *in vitro* observations in Figure 2 our data support a model in which Dgrn is a dedicated Gro antagonist and functions as a selector for co-factor recruitment.

3) We provide additional evidence that Dgrn is required to sequester Gro *in vivo.* In new Figure 5B, we show that Dgrn is required to sequester endogenous Gro protein using protein extracts derived from embryos. Using western-blot analysis comparing Dgrn-null to wild-type embryos, we find higher levels of Gro protein are observed in Dgrn null mutants only if protein extraction is performed using RIPA buffer, but not when proteins are extracted in a harsher extraction method using 4% SDS. This loss of function *in vivo* experiment is highly complementary to the experiment performed in S2R *Drosophila* cells shown in Figure 5A and immunostaining in embryos shown in Figure 5E-G.

4) Using EMSA we show that Dgrn associates with Hairy on DNA (Supplementary Figure 3B). The Hairy-Dgrn complex results in a slower migrating form of the DNA-Protein complex than that generated by Hairy alone ("super shift").

5) We show that Dgrn's activity in transcription is highly selective. Our new Supplementary Figure S4D shows that changes of Dgrn protein levels (by either over-expression or RNAi) affect the transcription of the *ac* reporter, but do not affect other reporter systems such as Dorsal-mediated activation of the Drosomycin reporter.

6) Reviewer 2 suggested that ubiquitylation of Hairy may trigger a novel endo-peptidase event: clipping between the WRPW and the PLSLV motif within Hairy that would thereby abolish Hairy's interaction with Gro, but not CtBP. In new Supplementary Figure 1H we show that this is not the case. We tagged the C-temini of Hairy with HA-tag, thus generating Hairy-HA. Subsequently we performed an ubiquitylation assay followed by HA-IP and were able to detect Ubiquitin-Hairy-HA conjugates. Thus, this experiment shows that an intact C-terminal Hairy is part of Ubiquitin-Hairy conjugates, and that ubiquitylation does not clip off Hairy's WRPW during ubiquitylation. Furthermore, all ubiquitylation experiments in our work are performed in the presence of both proteasome (MG132) and protease inhibitors cocktail, and no protease cleavage site is located in the 10 amino acid spacer located between the dCtBP binding site and the Gro binding site.

Point-by-point answers to the Reviewers' comments follow:

Reviewer #1

1. The model suggests that Dgrn touches both Hairy and Groucho. The data presented here make a clear case for Hairy-Dgrn physical interaction, but the only place where Dgrn-Gro physical interactions are tested is in S4E

(New figure S5). This experiment tests interaction between Groucho on beads and Dgrn, but controls are lacking, so it is impossible to judge whether the interactions shown are strong, weak, specific, or meaningful at all. This experiment is too preliminary to make any solid conclusion.

The Reviewer is concerned about the lack of controls in the *in vitro* assay that we performed to test Gro binding to Dgrn. In the revised Supplementary Figure 5B we now show a set of GST-pulldown assays using GST-Dgrn with IVT-Gro, alongside IVT-Hairy and -Hairy$^{\Delta Basic}$ (that serve as positive and negative controls respectively). In contrast to IVT-Hairy which binds to GST-Dgrn, unmodified Gro and Hairy lacking a basic domain show no binding at all. We do find that Dgrn targets specifically SUMOylated Gro (Figure 5). Together our data suggest that Dgrn does not interact directly with its SUMOylated substrate, but rather it interacts loosely and non-covalently with the poly-SUMO chains. Indeed, Geoffroy *et al.* (2010) recently showed that this is the case for PML.

2. In S4F (New Figure S5F), the levels of SUMOylation of Groucho are tested in cells where Dgrn is over-expressed an immunoprecipitation (or is it NTA-Ni affinity purification? The IP: a-His nomenclature is a bit unclear) against his-tagged SUMO is used to purify proteins, and Groucho proteins are then detected with anti-HA. It is puzzling that unmodified Groucho protein is apparently the most abundant protein, showing up even in lane 2, where no SUMO-his is expressed. Why is Groucho directly purified by the anti-his step? This question also applies to Figure 5B.

We needed a method that would be aggressive enough to preserve the SUMOylated moieties of Groucho and therefore used a buffer containing guanidine hydrochloride to lyse the cells. This is a very harsh buffer and therefore cannot be used for IP's with an antibody because of its aggressive denaturizing nature. NTA-Ni beads on the other hand still are able to bind His-tagged proteins in this environment. We therefore over expressed a His-tagged version of SUMO in cells along with HA-tagged Gro, incubated the lysates with NTA-Ni beads and blotted for HA. The results show that unmodified HA-Gro has a tendency to bind to NTA-Ni beads, explaining the band of HA Gro when no "His-tagged SUMO" is expressed, (Fig 5C, lane 1). Aware of this problem, we also set up an opposite approach where we IP'ed HA-tagged Gro with an anti-HA Ab (lysates were prepared in boiling SDS buffer which was then diluted in a less harsh buffer to allow the IP process) and blotted with anti-His tag Ab to identify the conjugates. In this case a His-signal (SUMO) was detected only in cells co-transfected with both HA-Gro and His-SUMO, but not with cells transfected with HA-Gro alone. The same results were observed using both Co-IP protocols. Therefore, we decided to present the hydroguanidine data regardless of the background binding of HA-Gro to NTA-Ni beads. We agree with the Reviewer that the IP nomenclature was unclear and have revised it to NTA-Ni in the relevant figures (Fig 5C, Fig S5F).

3. This paper provides evidence that in cell culture, the overexpression of Dgrn affects Groucho "extractability" but not overall levels, while in embryos, the total levels of Groucho are inversely proportional to Dgrn levels. It would be useful to try to interpret why the cell culture results are so different from those obtained with genetically manipulated embryos.

We now provide additional evidence that Dgrn is required to sequester Gro *in vivo*. In new Figure 5B, we show that Dgrn is required to sequester endogenous Gro protein using protein extracts derived from embryos. Using western-blot analysis comparing *dgrn* null to wild-type embryos, we find higher levels of Gro protein are observed in *dgrn* null mutants only if protein extraction is performed using RIPA buffer, but not when proteins are extracted by 4% SDS. This loss of function *in vivo* experiment is highly complementary and in agreement to the experiment performed in S2R *Drosophila* cells shown in Figure 5A and immunostaining in embryos shown in Figure 5E-G.

4. The paper presents results from a DAM-ID genomic mapping of Dgrn, but surprisingly, no correlation is made between Dgrn binding and Hairy binding. The physical Dgrn-Hairy interactions described would indicate that these proteins may cooccupy a gene, so the authors should correlate which binding sites for Dgrn are also known to bind Hairy (data from Bianchi-Frias, as well as Biggin's group - Li et al. 2008 and MacArthur et al. 2009). If there is no correlation, this would influence the model presented in Figure 6L (New Figure 6M); perhaps Dgrn modifies Hairy off of the DNA, and interacts with Groucho indirectly or off the DNA.

The Reviewer's point is well taken. Accordingly, we have added new Figure 6L and modified Figure 6K. We now present a comparison of the direct genomic loci bound by Hairy and its associated co-factors dCtBP, Gro, Sir2 and Dgrn. One of the major conclusions from our previous work is that the binding of Hairy to its targets is context-dependent (Bianchi-Frias *et al*., 2004). Here, we now show that in Kc cells most of Hairy targets are shared with CtBP and Sir2, but not with Gro (Figure 6L). We show that 38% of the genomic sites bound by Gro are shared with

Dgrn (Figure 6K). In addition, we compare the binding sites of the above co-factors including Dgrn to that of Hairy. Consistent with our previous findings (Bianchi-Frias *et al*., 2004), no gene is shared between Dgrn and Hairy and only a single gene is shared with Dgrn and dCtBP. Thus, the binding landscape depicted in the context of Kc cells reflects the situation "post" co-factors selection: Hairy associates with genomic regions that preferentially recruit Sir2 and CtBP, but not either Gro and Dgrn. This clearly fits with previous observations that the set of Hairy's direct targets during embryogenesis is different than the set of Hairy targets in Kc cells (Bianch-Frias 2004, MacArthur 2009, that is now cited in the paper). Together with our *in vitro* observations our data support a model in which Dgrn is a dedicated Gro antagonist and functions as a selector for co-factor recruitment.

The Reviewer also suggests that maybe Dgrn's modification of Hairy does not occur on the DNA itself. In this regard in new Supplementary Figure S3B we show that Dgrn forms a complex on the DNA with Hairy. We would also like to note that marking DNA with DAM requires a very close proximity to chromatin.

5. Relevant to the former point, in the Abstract, they note that "Concomitantly Dgrn specifically targets SUMOylated-Gro for sequestration and antagonizes Gro functions in vivo genome-wide." The paper does not provide any evidence for genome-wide functions of Groucho, however.

We have taken the Reviewer's comment into account and rephrased the sentence to omit "genome-wide". The sentence now reads: " Concomitantly Dgrn specifically targets SUMOylated-Gro for sequestration and antagonizes Gro functions *in vivo* ".

6. Supplemental Figure 3A tests whether Dgrn and Hairy can bind to DNA in vitro. The EMSA shows that Hairy can shift a probe, but the results are not at all compelling that Dgrn is part of this complex. A super shift experiment would indicate whether Dgrn is present.

We now have included Supplemental Figure 3B that shows such a super-shift. The EMSA shown in our former version (and is now presented in Supplemental Figure 3A), illustrates that bacterially expressed Hairy binds to the *ac* promoter-derived Hairy binding site and results in a significant shift of the probe. Since the initial shift of the probe with full length Hairy is already localized to the very top of the gel, it is hard to notice the super-shift by Dgrn. To resolve this issue we set up another EMSA experiment using the bacterially expressed Hairy bHLH region (1-48aa; the region that binds Dgrn). As predicted by the Reviewer, and as shown in Fig S3B, this Hairy-Dgrn complex results in a slower migrating form of the DNA-Protein complex than that generated by the bHLH region of Hairy alone ("supershift").

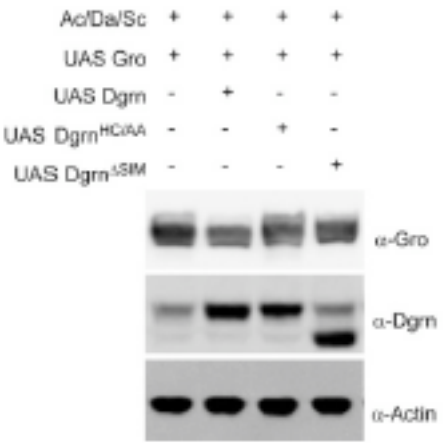
7. In Figure 3C, the activity of the Dgrn HC/AA mutant protein is tested, and found to be inactive. The authors should test whether the protein is even expressed, because a trivial explanation would be that the loss of activity is simply due to protein instability, not loss of RING finger binding.

In Fig5C shows a western blot of cells over-expressing Gro and Dgrn or the mutant $Dgrn^{HC/AA}$. The blot clearly shows that the mutant Dgrn is expressed in cells at the same levels as the wild-type form. We therefore can conclude the HC/AA mutation of Dgrn disrupts the RING domain yet does not affect its expression. However to confirm that this is also the case in our reporter assays, we examined the expression of the $Dgrn^{HC/AA}$ mutant in the *ac* reporter system. To this end we blotted the lysates used in the reporter assay for Dgrn and Gro (as shown in "Figure for reviewer" below). Again, in the *ac* reporter system, Dgrn and its mutants are expressed at similar levels.

In the case that the Editor/ Reviewers find that this analysis should be part of the manuscript we can add it to the Supplementary Figures.

Abed et al. Figure for Reviewer/Editor

Legend: Western blot analysis of S2R cell transfected with the indicated plasmids. The protein level of Dgrn, its derived mutants, and Gro is shown. Actin serves as a loading control.



8. In Fig. 3B and C (Now figure 3F), RNAi is used to deplete Hairy and Groucho, to test the effect of Dgrn on reporter gene expression. The Dgrn effect is lost when Gro is targeted, but not Hairy, leading to the suggestion that there may be Hairy-independent pathways. This interpretation rests on the assumption that the knockdowns are complete, as suggested in the western blot in 3D, E.
However, if the Hairy depletion were 90% effective, one would likely obtain the result shown in 3E and F, yet the residual protein would still provide activity. Thus, the authors should note that this suggestion of an alternative path is still speculative, due to the experimental design.

We agree with the Reviewer that our RNAi is not 100% effective and that the alternative interpretation is valid. Our results show that RNAi targeted against Hairy depletes Hairy at the protein level (at least to levels that are no longer detectable by our α-Hairy Ab). However, we set up a qPCR analysis of the Hairy RNAi samples and found that when the relative expression of Hairy mRNA in GFPi cells is set to 100%, the mRNA expression level of Hairy in Hairy RNAi cells is reduced, but not completely eliminated (10%, new Supplemental Figure S3C). Although our RNAi targeting is significant, the reduction is not 100% and as the Reviewer mentioned, the residual 10% may still provide activity and allow Gro repression. We have rephrased our findings and taken into account that residual Hairy may still allow Gro repression. We now state in the text (page 11): "However, Gro was still able to repress transcription in the absence of detectable Hairy protein, suggesting that this activity is dependent on either residual (trace-amounts) of Hairy, or that factors other than Hairy are involved in Gro recruitment and *ac* repression, and may be the target of Dgrn action".

9. Why does the mobility of Hairy change under chase conditions shown in Fig.2G? This is evident with both RNAi to nonspecific GFP and to Dgrn.

The mobility of Hairy does not change under chase conditions, but rather we have observed that Hairy is detected in several isoforms in cells when extracted with RIPA buffer, one isoform of which has a slower migration than the other. The isoforms are also apparent in Fig2E when endogenous levels of Hairy were analyzed. Our pulse chase results indicate that the more slowly migrating band has a longer half-life. We do not know the nature of the modification (we tested for phosphorylation, ubiquitylation, or SUMOylation). Importantly, the kinetics of both isoforms is similar in GFP-i or in Dgrn-i treated cells so we are able to conclude that Dgrn doesn't affect the stability of either isoforms. Therefore, while interesting by itself, the nature of this slower migrating form is out of this paper's scope.

10. Figure 3 examines the negative effect of extra Dgrn on transcriptional repression by Hairy. The specificity of such action is examined in S3B (New Figure S4A), which indicates that when activator sites are removed, the

overall gene activity is reduced (even there, it appears that a qualitatively similar Dgrn effect is present). Yet Dgrn still might act nonspecifically downstream of activator action, by stimulating a component of the basal machinery. This possibility would be easily tested by examining transcription of other reporter genes in the presence of ectopic Dgrn - if all functional promoters are stimulated by extra Dgrn, it might point to a more global role for the protein.That result might detract from the Hairy-centric model in 6L (New Figure 6M), but it would be important to know.

As requested by the Reviewer, we show that Dgrn activity in transcription is highly specific and selective. Our new Figure S4D shows that changes of Dgrn protein level (by either over-expression or RNAi) affect the transcription of *ac* reporter, but do not affect other reporter systems such as Dorsal-mediated activation of the Drosomycin reporter (Supplementary Figure 4D). As shown, in the absence of Dgrn protein (Dgrni), which we previously showed to cause a significant increase of Sc/Da activation of the *ac* reporter (Fig3I), the Drosomycin activation by Dorsal remains unaffected. Furthermore, over-expression of the mutant $Dgrn^{HC/AA}$ that has a dominant negative effect on the *ac* reporter (FigS4B) shows no effect on the Drosomycin reporter either. Taken together, the data presented in Figure S4 supports the notion that Dgrn activity in transcription is highly selective and specific.

11. This sentence may be misinterpreted - the data shown simply indicate that SUMO and Dgrn are in the nucleus, but the level of co-localization is not any more detailed than that. "We find that Dgrn co-localizes with SUMO or SUMOylated proteins in vivo in nuclei of developing embryos (Figure 1B-F)." They should revise this sentence to more accurately state their findings.

We take the point of the Reviewer and have rephrased our statement: Page 6: "We find that Dgrn localizes with SUMO or SUMOylated proteins *in vivo* in nuclei of developing embryos (Figure 1B-F)" .

12. A major point is that ubiquitylated Hairy doesn't bind well to Groucho, but can bind to CtBP. In Fig. 2H, I, the fraction of Hairy that is not ubiquitylated does not preferentially bind to the GST-Groucho column. It appears that something that happened to the protein during the in vitro ubiquitylation reaction has interfered with Groucho binding – perhaps proteolysis of the terminal amino acids necessary for Groucho binding? This point is important for the model presented in Fig. 6L (New Figure 6M) , where after modification, Hairy is suggested to lose Groucho interaction.

Our data support a model in which the multi or mono poly-ubiquitylation of Hairy prevents the association with Gro, but not dCtBP. Please note that the Gro and CtBP recruitment sites (WRPW and PLSLV, respectively) are separated by only 10 amino acids. The Reviewer suggested that ubqiuitylation of Hairy may trigger a novel endo-peptidase event (clipping between the WRPW and the PLSLV motif within Hairy) that abolishes Hairy's interaction with Gro but not CtBP. To test this possibility we tagged Hairy at its C-termini with HA-tag, thus generating Hairy-HA. Subsequently, we performed an ubiquitylation assay followed by a HA-IP, and were able to detect Ubiquitin-Hairy-HA conjugates (see Supplementary Figure 1H). Thus, this experiment shows that an intact C-terminal Hairy is part of the Ubiquitin-Hairy conjugates, and that ubiquitylation does not clip off Hairy's WRPW during ubiquitylation. Furthermore, all ubiquitylation experiments are performed in the presence of both proteasome (MG132) and protease inhibitors cocktail, and no protease cleavage site is located in the 10 amino acid spacer located between the CtBP and the Gro binding site. Thus, collectively our data supports the notion that binding of Dgrn requires Hairy's basic region, and that poly-ubiquitilation of Hairy interferes with Gro binding, likely by creating a modified Hairy protein that is not accessible for Gro. However, this ubiquitylation does not interfere with CtBP recruitment and does not involve clipping of Hairy's WRPW.

Typos and wording suggestions:

– Why are some figures labeled "UAS-Gro" and others "Gro" to indicate overexpression of the protein? The lack of consistency is distracting. This has been corrected.

– Figure 5B neglects to indicate that Gro is being over-expressed, which it clearly is.
We corrected this omission in the text and in the figure.

– The labeling of S3B,C,D is mixed up. The figures to not correspond to the indicated letters in the figure legend.
This has been corrected.

– The legend of Fig. 3 should say "antagonism of" not "antagonizes" to be clear. Figure 3. Dgrn antagonizes Hairy and Gro repression inversely correlates with the effects of SUMO pathway and is partially dependent on SUMOylation.
This has been corrected.

– Additional spelling/grammar points raised by the Reviewer have all been corrected.

Reviewer #2

1) The authors rely heavily on the GST pull down assay to demonstrate interactions between proteins in this paper. This is an in vitro assay that looks at binding of proteins that are relatively pure (not in the complexes that they may form in a real cell) and at reasonably high concentrations and thus pull down assays can be misleading. Although GST pull downs provide a very useful indication of whether proteins can interact, putative protein-protein interactions need to be validated in additional assays, which reflect more biological conditions. For example, it is noted that Dgrn was found to interact with Hairy in a yeast two-hybrid screen - thus yeast two hybrid data showing the interaction should be shown. The interaction between Dgrn and the basic domain of Hairy should also be validated by alternative assays (e.g. yeast two-hybrid,co-immunoprecipitation).

As suggested by the Reviewer, we now show that Dgrn and Hairy interact *in vivo* using a yeast two-hybrid assay (Figure 1K). We also used this assay to demonstrate that similar to the interaction we observe *in vitro* with purified proteins, the interaction *in vivo* is mediated via the basic domain of Hairy. A point mutation within an isolated Hairy bHLH ($bHLH^{R33E}$) abrogates binding. We also show that binding of Dgrn is independent of the WRPW, and that neither Gro nor dCtBP bind to Hairy's bHLH.

2) The authors show that Dgrn ubiquitylates the N-terminal basic domain of Hairy (at least in vitro), which inhibits interaction with the Groucho co-repressor protein. This finding is unexpected since previously it has been shown that Hairy interacts with Groucho via its C-terminal WRPW motif (right up the other end of the protein!). It has also been shown in several different assays that the WRPW motif alone is sufficient to interact with Groucho (e.g. in Fisher et al., 1996, MCB, vol 16:2670-2677 and Jennings et al., 2006, Mol Cell, vol 9:645-55). The authors do not acknowledge these conflicting observations in the manuscript. The authors must comment on how their findings can be reconciled to these observations - how does ubquitylation of the N-terminal domain of Hairy prevent binding via the C-terminal WRPW motif?

We show that Dgrn binds to Hairy's basic domain, a binding that is required to initiate ubiquitylation of Hairy. However, the basic domain is not the target of ubiquitylation, and the Lys residues required for ubiquitylation are scattered along the protein. Ubiquitylation is clearly not limited to the N-terminal portion of Hairy, and we could not detect a single critical Lys residue. By using Me-Ub (a Ubiquitin derivative that can not undergo elongation, and therefore results in mono-ubiquitination at the initial conjugation site along the target protein), we show in FigS2D that Hairy is ubiquitylated at a number of different sites. We propose that this ubiquitylation of Hairy prevents the association of Gro with the WRPW domain. We do not find that our observations conflict with the findings by Ish-Horowicz lab (Paroush *et al* or Jennings *et al*). On the contrary, we reproduced these findings in Figure 1, which shows that Gro binding to Hairy requires the WRPW.

3) Genetic interactions between hairy and dgrn are examined, however the rationale behind these experiments is not at all clear and needs to be spelt out more explicitly in the text. For example, the nature of the hairy alleles used is not described in the text (or materials and methods), and this is very important for interpretation of the results. The authors appear to be testing for rescue of the phenotype of hairy loss of function alleles by an loss of function allele of dgrn. Thus, the model tested appears to be that Dgrn reduces Hairy's repressive function, so if you remove Dgrn, any residual Hairy will have increased function and you will get some rescue of the hairy phenotype. However, in a hairy null mutant background (where there is no residual Hairy function) you would not expect to see any rescue of the phenotype. Thus the nature of the hairy alleles used must be known to interpret the results.

More specifically in the second paragraph on page 12 the authors state "Since hairy null embryos die during embryogenesis, we tested if reducing the dose of dgrn can rescue the embryonic lethality associated with hairy mutants." If there is no Hairy in the embryos for Dgrn to interact with, why would you expect to see any modification of the hairy phenotype? Presumably the hairy7C/hairy12C is not a null Hairy background (but we currently don't know, as the nature of the 7C and 12C alleles is not stated), in which case the text is misleading and this is just repeating the previous experiment that looked at rescue of the segmentation phenotype. Alternatively the data does not support a direct interaction between Dgrn and Hairy, and indicates that Dgrn functions in segmentation independently of Hairy. The model and rationale behind the genetic interactions needs to be greatly clarified.

The Reviewer's point is well taken. All the *hairy* mutant alleles used in the paper were hypomorphic alleles with varying phenotypical strengths – not null alleles. We have corrected and rephrased our statements throughout the paper and now include a detailed description of the *hairy* alleles used in the assays in the supplemental information.

Minor point:
– Figure 1C - why is there an asterisk by "Basic" ?
This was a typo and it is now corrected.

2nd Editorial Decision 18 December 2010

Your revised manuscript has been reviewed once more by both of the original referees. Both now support publication in The EMBO Journal, however, referee #1 does request a significant number of revisions to be incorporated in the final version of the manuscript, I suggest that all the issues are clearly discussed in a new version. In addition, I believe the comment made by referee #2 about the title is valid and would make the title more accessible to the general readership.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to reading the revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

Referee #1 (Remarks to the Author):

The authors have revised the manuscript to address previous concerns with data and presentation. Particularly useful are additional control experiments to test the specificity of Dgrn action on transcription, showing that Dgrn does not interfere with a Dorsal-activated reporter, the extractability of Groucho from wild-type and Dgrn mutant embryos, and the ability of Dgrn to form a complex on DNA with a Hairy DNA binding domain. The authors also provide more information on the results of the DAM-ID experiments, compared to other binding studies, as well as Dgrn-Hairy yeast two-hybrid interaction data.

As before, the most compelling part of the story is the evidence that Dgrn interacts with Hairy and Groucho genetically, while the mechanistic data are still somewhat contradictory and confusing in part. Nonetheless, the manuscript is improved, and this overall body of data merits publishing in EMBO J. if the authors can address some facets of the data that are still not adequately addressed, or in some cases confusingly interpreted, as noted below.

1) The basic model proposed here suggests that Dgrn ubiquitylates Hairy, interfering with Gro binding, and somehow "sequesters" Gro that has been SUMOylated to downregulate it. Individual experiments do support each of these pieces of the picture, but the reader would not appreciate the fuzzy areas from such a short statement, and the paper consistently takes only a single tack, without noting possible problems that are evident in the data. For example, in Fig. 5B, the level of Groucho protein in dgrnDK mutant embryos is unchanged from wild-type embryos, while in Fig. 5E and G, it

appears to be upregulated. The manuscript doesn't actually comment on the differences, but notes that it appears that Dgrn has a negative role in each case. The differences must be addressed in the manuscript. In Fig. 2E and G, it appears that Dgrn-RNAi leads to loss of Hairy protein, but the manuscript only notes that no upregulation of Hairy was observed. In fact, it appears that Dgrn is somehow stimulating or protecting the levels of Hairy in these cells, contrary to the model presented. This too, is confusing and has to be addressed.

2) The best information on Dgrn binding to Hairy appears to come from in vitro assays (or yeast two-hybrid, which is largely a proxy for in vitro touching). Consistent with this work, a RING finger mutant HC/AA (which the authors show is expressed) that does not bind Hairy is also ineffective at blocking its activity on the ac promoter. Yet in vivo data, such as a ChIP of Dgrn on Hairy-bound sites, is missing, and the DAM-ID work suggests that Hairy and Dgrn are not found at the same genomic locations. Hairy is not observed to be ubiquitylated in vivo. It may be that Dgrn was initially identified because of an artifactual interaction with Hairy, and its real role in the Hairy/Gro pathway was confirmed by genetic tests. I would not suggest that the authors throw out their model, merely comment on where the data is still soft (no actual evidence that Dgrn physically interacts with Hairy in vivo), and where we can have better confidence in its predictions.

3) Some of the interpretations of the data are rather surprising, and should be reconsidered. In Supplementary Figure 4A, a mutant ac reporter construct missing several binding sites has overall lower activity, but responds to transfected Dgrn in a manner similar to the wild-type promoter. Somehow this is interpreted as meaning that Dgrn requires the presence of sequence-specific transcription factors to mediate its effect. The experiment does not show that at all. If one completely killed the promoter, one presumably would not be able to measure an effect on transcription, but even in this weakened state, the pattern is similar to that seen with the wild-type promoter. In Figure 4B, the effect of Dgrn on the promoter in the absence of extra Sc/Da activators is tested - here, Dgrn again can lead to an increase in overall activity, presumably by counteracting some endogenous repressors/corepressors. In the presence of Sc/Da, without added Hairy, no stimulatory effect is noted, presumably because any repressors are unable to compete. Instead, the incomprehensible conclusion is provided that "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." This makes no sense, because their data shows a clear response. The apparent repressive activity of the HC/AA mutant in the absence of added Hairy, though ignored in the discussion, is puzzling and should be interpreted.

The interpretation of Supplemental Figure 5 is also misleading. The legend title notes "Dgrn binds weakly to Gro and targets SUMOylated Gro". What is actually shown is NO specific binding of Dgrn to Gro, whether SUMOylated or not, can be detected. That is not the same as detection of weak binding! The "targeting" concept is also troubling: the experiments show that there is less SUMOylated Groucho to be detected in extracts overexpressing Dgrn; their model would like to propose that Dgrn is reacting to SUMO-Gro, but the data only show that less of this modified form is present. Dgrn may block SUMOylation of Gro, or block the interaction of NTA-Ni with SUMOylated proteins. The one-dimensional interpretation of the data hurts the impact of the overall study.

4) Some typos and confusing sections that still need work:

The authors tried to modify this sentence to accurately represent their findings, but still fell short of the mark: "We find that Dgrn localizes with SUMO or SUMOylated proteins in vivo in nuclei of developing embryos (Figure 1B-F)." What they actually found was "Dgrn is a nuclear protein, as are SUMO or SUMOylated proteins in developing embryos (Figure 1B-F)."

["Since expression of DgrnHC/AA significantly reduced Sc/Da activation even in the absence of exogenous repressor, DgrnHC/AA may be functioning in a "dominant negative" capacity (Supplementary Figure S4B)."] It is not clear what the authors mean by "dominant negative" in this case. Acting through a yet-uncharacterized pathway? Dominant would usually mean that the mutant protein somehow takes over the role/contacts of the wild-type protein to interfere with a process; how that operates in this context is not clear.

In Fig. 6K,L, a generic set of Venn diagrams shows overlap between binding sites for Dgrn and

other factors. The paper does not supply any further information about which genes are involved, limiting the usefulness of this figure.

A major point is made in the sequestration of Groucho. Although the response letter describes different methods for extract preparation, there is no description of the methods used for IPs and westerns in the manuscript - what are the buffers note in the Materials and Methods? Amounts of guanidine, detergents in RIPA? This section is inadequately described; it would be difficult to reproduce these experiments.

In figure 2B, they note R33E, but they show a mutation that is R32E. Which is the actual mutant assayed?

Typos/grammar:
"Dorsomycin"
"This is highly complimentary to previous reports"

Referee #2 (Remarks to the Author):

This version of the manuscript is a vast improvement on the original one and all my previous concerns have been adequately addressed. The genetic data is now solid and provides compelling evidence that the interactions detected in vitro have biological relevance.

I suggest revision of the title to remove the abbreviation "STUbL", which will be unfamiliar to much of the target audience.

"Degringolade, a member of the SUMO Targeted Ubiquitin Ligase family, is a negative regulator of Hairy-mediated Groucho dependent repression"

2nd Revision - authors' response 20 January 2011

Referee #1:

1A) *The basic model proposed here suggests that Dgrn ubiquitylates Hairy, interfering with Gro binding, and somehow "sequesters" Gro that has been SUMOylated to down regulate it. Individual experiments do support each of these pieces of the picture, but the reader would not appreciate the fuzzy areas from such a short statement, and the paper consistently takes only a single tack, without noting possible problems that are evident in the data. For example, in Fig. 5B, the level of Groucho protein in dgrnDK mutant embryos is unchanged from wild-type embryos, while in Fig. 5E and G, it appears to be upregulated. The manuscript doesn't actually comment on the differences, but notes that it appears that Dgrn has a negative role in each case. The differences must be addressed in the manuscript.*

The protein levels of Gro in Figure 5B indicate that Dgrn mediates the sequestration of Gro. Gro that is sequestered can be extracted only in a very harsh buffer (embryos are lysed in 4%SDS buffer, then boiled and sonicated). Therefore, the detected protein levels of Gro in this experiment are dependent on Dgrn activity (or lack of it in the *dgrnDK* embryos) and the extraction method of the proteins.

We show that when WT or *dgrnDK* embryos are lysed in RIPA buffer the detected Gro protein level in WT is lower than *dgrnDK* embryos. This difference is likely because Gro is not sequestered in the absence of Dgrn (*dgrnDK* embryos) and hence the apparent increase in detectable Gro protein levels. In contrast, when WT or *dgrnDK* embryos are lysed in 4%SDS buffer, any sequestered Gro protein is released such that the total Gro protein levels in WT and *dgrnDK* embryos is equal.
Gro immunostaining of embryos (Figure 5G) is highly complementary to the

milder RIPA extraction conditions described above. *dgrnDK* embryos show an increased Gro signal compared with the wild-type embryos, likely resulting from an increase Gro available for detection by the antibody as less Gro is being sequestered in the mutant.

These experiments are also highly complementary with our experiments in S2R cells shown in Figure 5A where over-expression of Dgrn results in reduction of HAGro detection in RIPA extracts, but not when the extraction is performed using 4% SDS. Together, our data suggests that Dgrn expression is associated with Gro sequestration and that loss of Dgrn "liberates" sequestered Gro. We have now addressed this in more detail in the Discussion.

1B) *In Fig. 2E and G, it appears that Dgrn-RNAi leads to loss of Hairy protein, but the manuscript only notes that no upregulation of Hairy was observed. In fact, it appears that Dgrn is somehow stimulating or protecting the levels of Hairy in these cells, contrary to the model presented. This too, is confusing and has to be addressed.*

The Reviewer is correct and we have now specifically addressed this observation in the text (page 19). However, importantly, the rate of Hairy's degradation in pulse chase or CHX chases is identical in control or Dgrn RNAi cells. Thus, this is likely an indirect effect of Dgrn at the level of transcription or translation of Hairy. It is also important to note that in not one case (steady state, CHX chases or 35S-Met labeled pulse chase) does Dgrn enhance the degradation rate of Hairy. Therefore we believe that the important take home message is that unlike "conventional" ubiquitylation, Dgrn activity does not mediate Hairy's degradation.

2) *The best information on Dgrn binding to Hairy appears to come from in vitro assays (or yeast two-hybrid, which is largely a proxy for in vitro touching). Consistent with this work, a RING finger mutant HC/AA (which the authors show is expressed) that does not bind Hairy is also ineffective at blocking its activity on the ac promoter. Yet in vivo data, such as a ChIP of Dgrn on Hairy-bound sites, is missing, and the DAM-ID work suggests that Hairy and Dgrn are not found at the same genomic locations. Hairy is not observed to be ubiquitylated in vivo. It may be that Dgrn was initially identified because of an artifactual interaction with Hairy, and its real role in the Hairy/Gro pathway was confirmed by genetic tests. I would not suggest that the authors throw out their model, merely comment on where the data is still soft (no actual evidence that Dgrn physically interacts with Hairy in vivo), and where we can have better confidence in its predictions.*

Following the Reviewer's suggestion we now specifically comment on these specific points in the text. In addition, we now include data in 293T cells showing that Dgrn, but not its RING mutant, ubiquitylates Hairy (Supplementary Figure S2F).

While Dgrn was initially identified in a Y2H assay using Hairy as bait, this interaction has also been demonstrated *in vitro* using bacterially expressed affinity purified proteins. Under the same conditions, Dgrn does not bind to Her or dMnt, which are both bHLH repressors. We have also shown specificity by demonstrating that the interaction and ubiquitylation is dependent on R33, and that this mode of interaction holds for all of the HES proteins with the exception of HER (Barry et al., *Development* under revision). Finally, we show that Dgrn suppresses Hairy mutant phenotypes.

The interactions between ubiquitin ligases and their substrates are known to be transient in nature. This transience can be usually bypassed in interaction assays by using a RING mutant that binds to the substrate but does not release it. In the case of Hairy, Dgrn's RING domain is required for binding and therefore its RING mutant cannot be assayed in this way. Taken together, our data suggest that the interaction of Dgrn with Hairy is highly specific and biologically relevant.

The Reviewer also commented on the lack of ChIP assays. Others and us have shown that DamID and Chip assays are highly complementary. When comparing the binding of two proteins, both types of assays are correlative in nature, and as such, do not prove the formation of protein complexes. To partially address this we showed that Hairy and Dgrn associate on DNA (Figure S3B). Thus, one of the main messages from our previous and current work is that context dependent recruitment is important, and that in Kc cells Dgrn is not associated with sites that are associated with Hairy.

3) Some of the interpretations of the data are rather surprising, and should be reconsidered. In Supplementary Figure 4A, a mutant ac reporter construct missing several binding sites has overall lower activity, but responds to transfected Dgrn in a manner similar to the wild-type promoter. Somehow this is interpreted as meaning that Dgrn requires the presence of sequence-specific transcription factors to mediate its effect. The experiment does not show that at all. If one completely killed the promoter, one presumably would not be able to measure an effect on transcription, but even in this weakened state, the pattern is similar to that seen with the wild-type promoter In Figure 4B, the effect of Dgrn on the promoter in the absence of extra Sc/Da activators is tested - here, Dgrn again can lead to an increase in overall activity, presumably by counteracting some endogenous repressors/co repressors. In the presence of Sc/Da, without added Hairy, no stimulatory effect is noted, presumably because any repressors are unable to compete. Instead, the incomprehensible conclusion is provided that "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." This makes no sense, because their data shows a clear response. The apparent repressive activity of the HC/AA mutant in the absence of added Hairy, though ignored in the discussion, is puzzling and should be interpreted.

We also noticed that DgrnHC/AA in the absence of Hairy can repress activation by Sc and Da (Figure S4B). One possibility is that this is due to activity of Dgrn with activators, or alternatively due to Dgrn action on endogenous repressors and corepressors. We now explain both possibilities in the discussion.

We also take the point of the Reviewer that the “trend” of Dgrn and its mutants is observed in the absence of Da/Sc or in a reporter where the Sc/Da binding sites have been mutated or in the absence of activator (Figure S4A-B). However, while this trend is seen visually, it is statistically insignificant (and annotated “ns”; nonsignificant). We have now revised the text to fully address this issue.

4A) *The interpretation of Supplemental Figure 5 is also misleading. The legend title notes "Dgrn binds weakly to Gro and targets SUMOylated Gro". What is actually shown is NO specific binding of Dgrn to Gro, whether SUMOylated or not, can be detected. That is not the same as detection of weak binding!*

We agree with the Reviewer that weak binding is not “no binding”. Our data strongly suggest that Dgrn does not bind directly to full length Gro. However, since in our invitro binding assays (Figure S5), the WD domain still binds weakly but a bit more than the background, we phrased our finding as “weakly binds”. Reconsidering our data and the Reviewer’s comment, we have revised the title and text in Figure S5 and the manuscript. Likely the interaction with SUMOylated-Gro is very transient and is mediated by interaction with the SUMO chain and not directly Gro. A similar mode of interaction was recently shown for the interaction between SUMOylated PML and RNF4 (Geoffroy MC, Hay RT et al. 2010, *Mol Biol Cell*).

4B) *The "targeting" concept is also troubling: the experiments show that there is less SUMOylated Groucho to be detected in extracts over expressing Dgrn; their model would like to propose that Dgrn is reacting to SUMO-Gro, but the data only show that less of this modified form is present. Dgrn may block SUMOylation of Gro, or block the interaction of NTA-Ni with SUMOylated proteins. The one-dimensional*

interpretation of the data hurts the impact of the overall study.

We do agree that our results may be a result of indirect effects of Dgrn beyond what we have suggested in the model. We have now addressed these alternatives in the text.

The Reviewer is specifically concerned about the inability to detect SUMOylated-Gro when Dgrn is expressed that is due to technical interference with the binding of His-SUMO to the Ni-NTA beads. While this method has been used by others to test the fate of SUMOylated proteins with RNF4 (see below), to further control our experiment we performed a parallel experiment shown in Fig 5D. In this experiment we IP'd Gro with an anti-HA antibody (bypassing the need for NTA-Ni beads) and blotted for SUMOylated proteins with an anti-His antibody. This experiment shows similar results to Fig. 5C: Dgrn specifically targets the SUMOylated forms of Gro. Furthermore, Dgrn is highly similar to RNF4 that is a known SUMO targeted ubiquitin ligase. Tatham *et al* previously showed that RNF4 ubiquitinates PML only if it is conjugated with SUMO, indicating that it is only the SUMOylated portion of PML that is recognized by RNF4 and sent off for degradation. To the best of our knowledge, Dgrn and RNF4 have no SUMO ligase activity, and other enzymes within the SUMO pathway or SUMO peptidases are not target genes of Dgrn in our DamID mapping. There is no work we know of that demonstrates that STUbL proteins impact the SUMO pathway or SUMO-peptidases. Therefore, the possibility that Dgrn directly affects SUMO peptidases or the SUMO machinery is less likely.

Typos and confusing sections that needed work:

A) *The authors tried to modify this sentence to accurately represent their findings, but still fell short of the mark: "We find that Dgrn localizes with SUMO or SUMOylated proteins in vivo in nuclei of developing embryos (Figure 1B-F)." What they actually found was "Dgrn is a nuclear protein, as are SUMO or SUMOylated proteins in developing embryos (Figure 1B-F)."*

We have modified the text as suggested.

B) *"Since expression of DgrnHC/AA significantly reduced Sc/Da activation even in the absence of exogenous repressor, DgrnHC/AA may be functioning in a "dominant negative" capacity (Supplementary Figure S4B)."] It is not clear what the authors mean by "dominant negative" in this case. Acting through a yet-uncharacterized pathway? Dominant would usually mean that the mutant protein somehow takes over the role/contacts of the wild-type protein to interfere with a process; how that operates in this context is not clear.*

We take the point of the Reviewer and have re-evaluated our data. While we think that Dgrn may exhibit some dominant negative effects, further analysis is required to support such a statement. We now omit this statement.

C) *In Fig. 6K,L, a generic set of Venn diagrams shows overlap between binding sites for Dgrn and other factors. The paper does not supply any further information about which genes are involved, limiting the usefulness of this figure.*

Supplemental Table S2 details all the genes bound by both Gro and Dgrn and the single gene that overlaps between Dgrn and dCtBP. Full description of the genes bound by dSIR2, dCtBP, Gro and Hairy can be found in Bianchi et al. 2004.

D) *A major point is made in the sequestration of Groucho. Although the response letter describes different methods for extract preparation, there is no description of the methods used for IPs and westerns in the manuscript - what are the buffers note in the Materials and Methods? Amounts of guanidine, detergents in RIPA? This section is inadequately described; it would be difficult to reproduce these experiments.*

This has now been added in the Supplementary Information. We now include the RIPA, Guanidine-HCl and “hot lysis buffer” protocols.

E) *In figure 2B, they note R33E, but they show a mutation that is R32E. Which is the actual mutant assayed?*

The figure has been corrected to read R33E.
Please note that in *Drosophila* there are two anti-bacterial peptides with similar names: Drosomycin and Drosocin. We have tested the role of Dgrn in the transcription of Drosmycin (CG10801, Drs).

We have corrected the other typos/grammar mistakes pointed out by the Reviewer.

Referee #2:

I suggest revision of the title to remove the abbreviation "STUbL", which will be unfamiliar to much of the target audience.
"Degringolade, a member of the SUMO Targeted Ubiquitin Ligase family, is a negative regulator of Hairy-mediated Groucho dependent repression"

We have amended the title similar to the what suggested by the Reviewer in order to fit the EMBO required 100ch limit: “Degringolade, a SUMO-Targeted Ubiquitin Ligase, inhibits Hairy/Groucho- mediated repression”

