Bili Inhibits Wnt/β-Catenin Signaling by Regulating the Recruitment of Axin to LRP6

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Abstract

Background: Insights into how the Frizzled/LRP6 receptor complex receives, transduces and terminates Wnt signals will enhance our understanding of the control of the Wnt/β-catenin pathway.

Methodology/Principal Findings: In pursuit of such insights, we performed a genome-wide RNAi screen in Drosophila cells expressing an activated form of LRP6 and a β-catenin-responsive reporter. This screen resulted in the identification of Bili, a Band4.1-domain containing protein, as a negative regulator of Wnt/β-catenin signaling. We found that the expression of Bili in Drosophila embryos and larval imaginal discs significantly overlaps with the expression of Wingless (Wg), the Drosophila Wnt ortholog, which is consistent with a potential function for Bili in the Wg pathway. We then tested the functions of Bili in both invertebrate and vertebrate animal model systems. Loss-of-function studies in Drosophila and zebrafish embryos, as well as human cultured cells, demonstrate that Bili is an evolutionarily conserved antagonist of Wnt/β-catenin signaling. Mechanistically, we found that Bili exerts its antagonistic effects by inhibiting the recruitment of AXIN to LRP6 required during pathway activation.

Conclusions: These studies identify Bili as an evolutionarily conserved negative regulator of the Wnt/β-catenin pathway.

Introduction

Wnt signaling through β-catenin regulates the expression of genes involved in cell proliferation and cell fate during development, in adult homeostasis, and in diverse diseases [1]. In the absence of a Wnt ligand, steady-state levels of β-catenin are maintained at a low level by a ‘degradation complex’ that promotes the phosphorylation, ubiquitination, and proteasomal degradation of β-catenin [2]. The binding of Wnt ligands to the transmembrane FZD (frizzled) and LRP5/6 (low-density-lipoprotein-related protein 5/6) co-receptors triggers an inhibition of the degradation complex resulting in the stabilization and nuclear accumulation of β-catenin, where it binds to transcription factors of the TCF/LEF family and regulates target gene expression.

Signaling events immediately following Wnt binding to the receptor complex are poorly understood. Previous studies have shown that in the presence of Wnt ligand, Dishevelled (DVL) facilitates phosphorylation of the cytoplasmic tail of LRP5/6 [3] by Casein Kinase 1γ (CSNK1G1) and Glycogen Synthase Kinase-3β (GSK3β) [4,5]. Phosphorylation of LRP5/6 is followed by the recruitment of AXIN away from the degradation complex. As AXIN is likely a rate-limiting scaffold protein required for the degradation of β-catenin [6], the relocation of AXIN to the membrane away from the degradation complex likely promotes stabilization of β-catenin [7].

We performed a genome-wide RNAi screen in Drosophila cells to identify genes that regulate LRP6 mediated activation of a β-catenin reporter. Here we describe Bili (Band4.1 inhibitor LRP interactor), a previously uncharacterized FERM domain containing protein. Analyses of Bili function in Drosophila, zebrafish, and cultured human cells support the conclusion that Bili is an evolutionarily conserved antagonist of β-catenin signaling. Mechanistically, we show that Bili regulates the recruitment of AXIN to LRP6.

Results

RNAi screen in Drosophila identifies Bili as a negative regulator of Wg signaling

Drosophila mutagenesis screens have resulted in the identification of several core Wg signaling proteins [8]. The potential for discovering additional contextually relevant molecular players in the pathway is enhanced by robust read-Outs such as transcriptional reporter assays coupled with genome-wide RNAi libraries.
Bili is a conserved FERM-domain protein

Of the 11 remaining candidate negative regulators, we chose to focus on CG11140 (dBili) due to its predicted plasma membrane localization and the specificity of its effect on the Wg pathway.

More specifically, of the 12 other screens in the DRSC database, dBili was identified only as a weak hit in a MAPK kinase screen (Friedman et al., 2007). Bili, annotated as CG11140 (fly) and FRMD8/FKSG44 (human), also has orthologs in worms, zebrafish and mice (Fig S1A). In human tissue samples, we found Bili mRNA was expressed highest in heart and spleen (Fig S1B). Bili is a predicted 464 amino acid protein with a Band4.1/FERM domain in its N-terminus (amino-acids, 26-272) (Fig S1C). FERM domains (4.1 Ezrin Radixin Moesin) are highly conserved protein domains important for directing plasma membrane association and, in some cases, linking integral membrane proteins to the actin cytoskeleton and modulating signaling [12]. Given that no prior Band4.1 domain proteins have been implicated in Wnt signaling, we focused on Bili to determine whether it functioned in a unique capacity in the Wnt/β-catenin pathway.

Bili dsRNA validates in low throughput assays
dsRNA targeting either dBili or Ran, a previously identified negative regulator of Wg signaling [13], enhanced the activation of δT12 by either δNLRP6 or Wg (Fig 1A). As expected, dBili dsRNA decreased endogenous dBili transcripts compared to control dsRNA (Fig S3A). A second independent dBili dsRNA targeting dBili, dBili-2, also decreased dBili transcript levels and enhanced δNLRP6-activated reporter activity (Fig S3A and B). Additionally, overexpressing dBili in S2R+ or Clone8 Drosophila cell lines inhibits Wg-dependent reporter activation (Fig S3C and D). These data support the conclusion that dBili is a novel negative regulator of Wg signaling.

Bili is coexpressed with Wg during Drosophila development

If dBili is a component of the Wg pathway in Drosophila, then it should be co-expressed with other genes involved in Wg signaling. To test this hypothesis we performed in situ hybridization of dBili in Drosophila embryos (Fig 1B) and larval imaginal discs (Fig 1C). At early stages (stages 5–6) dBili RNA is uniformly expressed in the early embryonic ectoderm at low levels (data not shown), and more highly expressed in the neuroectoderm (Fig 1B, a and b). Additionally, dBili is expressed in cells adjacent to the ventral midline just prior to initiation of the invaginating ventral furrow (black arrows, Fig 1B, a and b). Interestingly, at later stages (stages 13–17), the expression of dBili is evident in the developing central nervous system (CNS, Fig 1B, c and e) and the ventral epidermis in stripes (black arrows, Fig 1B, c, e and g) similar to the expression of Wg (Riggleman et al. 1990). At the end of embryogenesis (stage 16/17), the majority of dBili expression is restricted to the CNS (Fig 1B, d).

dBili and Wg expression patterns also overlap in wing, leg, and the eye-antenna imaginal discs in third instar larvae (Fig 1C). In the wing imaginal disc, dBili is expressed broadly in the wing pouch (black arrows, Fig 1C, panel a) and in the notum (red arrow, Fig 1C, panel a). The expression of dBili in the notum and that of Wingless (Wg) protein (blue arrow, Fig 1C, panel b) appears to be non-overlapping, however they do abut each other. In the leg disc, dBili is expressed in a small discrete domain in the posterior leg disc (red arrow, Fig 1C, panel c) as well as in the ventral wedge (black arrows, Fig 1C, panel c) in a pattern slightly broader yet nearly identical to Wg expression (white arrow, Fig 1C, panel b). In both dorsal and ventral compartments of the eye disc, dBili expression (black arrows, Fig 1C, panel d) partially overlaps with the lateral edge expression of Wg (white arrows, Fig 1C, panel e). In the antennal disc, dBili is expressed as a dorsal wedge (red arrow, Fig 1C, panel d) which also overlaps with that of Wg (blue arrow, Fig 1C, panel c). We conclude that the overlapping and adjacent expression of dBili and Wg are consistent with dBili functioning in the Wg pathway.

dBili negatively regulates Wg signaling during Drosophila embryogenesis

We next investigated the function of dBili during Drosophila embryogenesis. Wg has a well-established role in patterning the ventral epidermis and has been shown to be involved in cell fate determination [14,15]. Epidermal cells with active Wg signaling secrete naked-cuticle, whereas cells lacking Wg signaling activity secrete denticle belts. If dBili is a negative regulator of Wg signaling, reduced dBili function should resemble Wg gain of function phenotypes including ectopic naked cuticle.

dBili levels were reduced during embryogenesis by expressing a short-hairpin RNA (shdBili) in the paired domain (prd-GAL4>UAS-shdBili). Quantitative real time-PCR (qRT-PCR) analysis following expression of dBili shRNA under the control of prd-GAL4 in the Drosophila embryo revealed >75% knockdown of dBili mRNA levels compared to prd-GAL4 control embryos (Fig S2A). As a control for the specificity of shdBili we also quantified levels of armadillo mRNA and found no change (Fig S2A).

Importantly, the expression of shdBili led to the expansion of Wg signaling activity as demonstrated by the secretion of mostly naked cuticle with very few cells secreting cuticle with trichomes (denticles) (Fig 2A and 2B). Furthermore, driving dBili shRNA expression with even skipped stripe 3/7-GAL4, disrupted denticle stripe 2/3 (Fig S2B). Thus, depleting dBili in the Drosophila embryo phenocopies hyperactivated Wg signaling, suggesting that dBili is a negative regulator of the Wg pathway in Drosophila embryos.

Next we assessed the molecular consequences of depleting dBili in the paired domain. During embryonic segmentation, Wg activates and maintains the expression of engrailed (En) and defines para-segment boundaries [16]. Consistent with Wg gain-of-function, immunohistochemistry for En protein revealed a marked and uniform expansion of its expression in shdBili embryos (Fig 2E–I). Wild type embryos at stage 12 (germ band retraction)
and the ventral epidermis at stage 14 (onset of head involution and dorsal closure) have approximately two rows of cells expressing En (Fig. 2, F and G). These regions were expanded to approximately four rows of cells in the presence of shdBili (Fig. 2, H and I). These data further support the conclusion that dBili is an inhibitor of Wg signaling during Drosophila embryonic development.

Figure 1. dBili (cg11848) is a negative regulator of Wg signaling. (A) Secondary reporter assay in clone 8 cells showing gfp, armadillo (arm), ran and dBili dsRNA effects on wingless (wg) (white bars) and ΔNLRP6 (gray bars) activation of β-catenin reporter, dTF12. Individual dsRNA effects on reporter activity in the absence of wg or ΔNLRP6 (inset). (B) Expression pattern of dBili mRNA by in situ hybridization. Embryos are oriented with anterior on the left and posterior on the right. (a & b) Stages 5–6 showing dBili expression in the neuroectoderm and cells adjacent to the ventral midline (black arrows) with high levels of expression in cells adjacent to the ventral furrow (white arrowhead). (c–e). At stages 13–17 the expression of dBili in the developing central nervous system (panel c & e) and the ventral epidermis in stripes (e & e'). At the end of embryogenesis (stage 16/17), the majority of dBili expression is restricted to the CNS (panel d). (C) (a & b) Wing imaginal discs. Panel a, shows dBili expression in the wing pouch (black arrows) and in the notum/hinge area (red arrow). Panel b, staining for Wg protein in wing pouch and notum/hinge (blue arrow). (b & c) Leg imaginal discs. Panel c shows dBili staining in the ventral wedge (black arrows), identical to Wg expression (white arrow, panel b) (d & e) Expression in the eye disc of dBili (black arrows, d) and Wg (white arrows, e). Expression in the antenna disc of dBili (d, red arrow) and Wg (e, blue arrow). doi:10.1371/journal.pone.0006129.g001
Bili is conserved in vertebrates and negatively regulates Wnt/β-catenin signaling in zebrafish

The Wg signaling pathway is highly conserved throughout evolution. Therefore, we next asked if Bili negatively regulates Wnt signaling in vertebrates. To this end, we asked if Bili function is conserved in zebrafish. Ectopic activation of Wnt/β-catenin signaling in early zebrafish development causes dose-dependent anterior truncations and mesodermal defects (Fig. 3A) [17]. If zfBili functions as a negative regulator of Wnt/β-catenin signaling during zebrafish development, then silencing zfBili expression should exacerbate these phenotypes. Overexpression of Wnt8 results in anterior truncation phenotypes of varying severity (Fig. 3A). Silencing Bili with either of two non-overlapping antisense morpholinos targeting zfBili (bilimo or bilimo2) shifted the Wnt8 induced phenotypes toward increasing severity (Fig. 3C, top panel, Fig. S4E). As controls, we first confirmed that zfBili is expressed throughout early zebrafish development (Fig. S4A-D) and second verified that bilimo, but not a control morpholino (como), repressed translation of zfBili (Fig. 3B).

We next investigated the effect of zfBili gain-of-function on zebrafish development. Embryos injected with zfBili RNA, but not control RNA, displayed a slight expansion of anterior structures, which is consistent with diminished Wnt/β-catenin signaling (data not shown) [18]. Importantly, zfBili overexpression rescued Wnt8 gain-of-function phenotypes (Fig. 3C, bottom). Together, these results demonstrate that Bili is an evolutionarily conserved negative regulator of Wnt/β-catenin signaling.

Bili negatively regulates Wnt/β-catenin signaling in human cultured cells

We next tested whether Bili regulates Wnt signaling in human cells. We performed siRNA knockdown or cDNA overexpression of hBili in RKO colorectal carcinoma cells or human embryonic kidney (HEK293T) cells expressing a β-catenin responsive
luciferase reporter and *Renilla* luciferase normalization control. Activation of the reporter in RKO cells following treatment with WNT3A conditioned media was enhanced by transfection of either hBili or AXIN1 siRNA and repressed by β-catenin siRNA (Fig. 4A). Similarly, activation of the reporter in HEK293T cells by transfection of WNT1, a pathway activator, was enhanced by hBili siRNA and repressed by β-catenin siRNA (Fig. S5A).

Consistent with these loss-of-function studies, overexpression of hBili in HEK293T cells inhibited WNT3A-mediated activation of the reporter (Fig. 4B).

A series of additional controls confirm these results and the conclusion that Bili is a negative regulator of the Wnt/β-catenin pathway in human cells. First, hBili siRNA effectively reduced both hBili mRNA and tagged protein levels. By qRT-PCR, hBili siRNA depleted hBili transcript levels by 50% (Fig. S5B), and reduced levels of overexpressed hBili protein (Fig. S5C). Second, four additional independent siRNAs designed against hBili had a similar or greater effect on Wnt-mediated activation of the β-catenin responsive reporter (Fig. S5D and E and data not shown) ruling out the formal possibility that the activity of the first siRNA was due to off-target effects. Third, hBili siRNA synergized with WNT3A conditioned media to induce transcriptional upregulation of the endogenous β-catenin target gene, AXIN2 (Fig. S5F). This effect on expression of an endogenous β-catenin target gene validates the conclusions of the synthetic reporters. Fourth, knockdown or overexpression of hBili had no effect on control β-catenin unresponsive reporters (Fig. S6A andB) or a CREB reporter that was activated with forskolin (Fig. S6C). These data collectively support the conclusion that Bili is a conserved inhibitor of Wnt/β-catenin signaling in diverse species.

**Bili functions upstream of β-catenin stabilization and associates with LRP6**

We next carried out epistasis experiments in HEK293T cells expressing a β-catenin responsive luciferase reporter and *Renilla* luciferase normalization control. hBili or GFP were overexpressed...
in the presence of BIO (6-bromoindirubin-3’-oxime), a GSK3 inhibitor [19], or co-expressed with other pathway activators including WNT1, ΔNRIP6, Dishevelled (DVL) or β-catenin. Bili gain-of-function reduced reporter activation by WNT1, ΔNRIP6, and DVL (Fig. 4C). In contrast, hBili overexpression did not affect reporter activation by overexpression of β-catenin or BIO treatment (Fig. 4C). These results place the function of hBili between the Wnt receptor complex and the β-catenin degradation complex. Consistent with these epistasis studies, and the observed membrane associated localization of other FERM domain containing proteins, we found that dBili localizes in a concentric ring, adjacent to Arm protein at the plasma membrane in fly cells (Fig. S3E and S3E’).

Based on these results we predicted that Bili might regulate signaling from the Wnt co-receptors LRP5/6 or Frizzled. In direct support of this prediction, tagged hBili colocalizes with tagged LRP6 as detected by immunocytochemistry (ICC) (Fig. 4D). Furthermore, hBili-FLAG pulls down tagged-LRP6 in co-immunoprecipitation (co-IP) assays (Fig. 5A). The association of Bili and LRP6 was also confirmed in Drosophila S2R+ cells where immunoprecipitated tagged-ΔNLIP6 pulled down tagged-dBili (Fig. 5B). Collectively, these data suggest that Bili functions upstream of β-catenin stabilization and associates with the LRP6 receptor complex.

**Bili antagonizes the recruitment of Axin to LRP6**

During Wnt signaling, LRP6 is phosphorylated in a Wnt-dependent manner by CSNK1G1 and GSK3B, which then facilitates the binding of AXIN to LRP6 [4,5]. Consistent with this model, overexpression of GSK3B consistently and robustly mimics the ability of WNT to induce the binding of AXIN to LRP6 ([20,21]). Our observations that Bili negatively regulates Wnt/β-catenin signaling upstream of the destruction complex and associates with LRP6 lead us to hypothesize that Bili may regulate the recruitment of AXIN to LRP6.

To address this hypothesis we employed co-immunoprecipitation to monitor the association of AXIN and LRP6 following gain-of-function (Fig. 5C) or loss-of-function (Fig. 5D) of Bili. HEK293T cells stably expressing LRP6-EGFP were transiently transfected with AXIN-MYC. As expected LRP6 co-immunoprecipitated AXIN in the presence of overexpressed GSK3B (Fig. 5C & 5D, lane 1 compared to lane 3). This interaction of LRP6 and AXIN was attenuated approximately 2-fold in the presence of overexpressed hBili (Fig. 5C, lane 2 compared to lane 1) and enhanced more than 2-fold when hBili levels were knocked down (Figure 5D, lane 2 compared to lane 1). Furthermore, even in the absence of GSK3B, knockdown of hBili enhanced the association of AXIN and LRP6 approximately 4-fold (Fig. 5D, lane 4 compared to lane 3). Together these data suggest that Bili negatively regulates the recruitment of AXIN to LRP6 thereby providing a mechanism for how Bili negatively regulates Wnt/β-catenin signaling.

**Discussion**

Many aspects of Wnt/β-catenin signal transduction remain poorly understood. One aspect in particular is the transduction
events occurring at the plasma membrane following Wnt activation of the Frizzled/LRP6 coreceptor complex. Studies over the last several years have shed light on these events by uncovering several kinases that phosphorylate LRP6 resulting in its recruitment of AXIN [4,5]. As AXIN recruitment to LRP6 appears to be a key step in the propagation of a WNT signal, it is likely a hotspot for pathway regulation. Our identification of Bili as a negative regulator of Wnt/β-catenin signaling and inhibitor of AXIN recruitment to LRP6 is the first report of negative regulation at this step in pathway transduction. The specific details of how Bili affects AXIN recruitment will be the focus of future studies. One proposed mechanism is that Bili sterically hinders the recruitment of the kinases required for LRP6 phosphorylation and subsequent AXIN recruitment. A second possibility is that Bili inhibits the formation of LRP6 aggregates or ‘signalosomes’ which have been shown to be the LRP6 population associated with AXIN (Bilic et al, 2007).

We propose that inhibitors of the Wnt/β-catenin pathway fall into two groups: ‘constitutive inhibitors’ that keep basal levels of β-catenin low, such as members of the degradation complex; and ‘inducible inhibitors’ that act outside of the degradation complex and may function only when the pathway has been activated. The ‘constitutive inhibitors’ would include APC and AXIN, while ‘inducible inhibitors’ would include NKD and Bili. Consistent with this categorization, NKD and Bili both antagonize a WNT signal between receptor activation and the degradation machinery as well as having similar phenotypes in both flies [22] and fish [23]. In concert, constitutive and inducible inhibitors allow for rapid pathway activation in response to WNT ligand and tight regulation of the strength and duration of the WNT signal. Continued characterization and identification of both constitutive and inducible inhibitors is key to our understanding of the regulation of Wnt/β-catenin signaling in development and disease.

Materials and Methods

Fly screen

The screen was performed as previously described [10].

Reporter assays

β-catenin reporter assays were carried out in the 24-well or 48-well plate format in HEK293T cells transiently expressing SuperTOPFLASH (1–5 ng) or BAR (β-catenin Activated Reporter) and Renilla luciferase (10–40 ng) for normalization. FOPFlash and fuBAR, which contain mutated response elements and do not respond to WNT/β-catenin signaling, were used as control.
reporters. We used RKO cells that stably express BAR and Renilla [24]. In LOF studies, cells were seeded to 30% confluency before siRNAs were transfected using Lipofectamine RNAiMax (invitrogen) at a concentration of 20 μM. 24 h later the cells were treated with L-cell media or WNT3A media (or transfected with WNT1 (0.1 ng) and TOPFLASH luciferase(10 ng)). Lysis and luminescence (using Promega dual-luciferase assay kit) was carried out 24 h after WNT treatment. For GOF experiments, cells were seeded to 50% confluency after which eDNA GFP (50-100 ng) or Bili-FLAG (25–100 ng), TOPFLASH, WNT1 (0.1 ng), ANLRP6 (5 ng), DVL (5 ng), β-catenin (1 ng) was transfected. BIO (0.5 μM) was also added to the cells at this point. Cells were lysed and luminescence measured 24 h hours later. Bili siRNAs were purchased from Ambion; siRNA ID# 332724 that targeted Exon 8 (sense 5’-ggcgacagcaacgauatt3’), antisense 5’-aucaucaugagcugcagctrr3’ and siRNA ID# 45515 that targeted Exon 7, 8. Control siRNA and Axin 1 & 2 siRNAs have been described [24].

**Drosophila in situ hybridization and immuno-staining**

In situ hybridization and immuno-staining in the fly embryos and larval imaginal discs were performed using standard protocols described in the laboratory manual "Drosophila Protocols" by William Sullivan et al., CHSL press.

**qRT-PCR in Drosophila embryos**

RNA was isolated from Drosophila embryos using Trizol and was further purified using the RNA “clean-up” protocol with the Qiagen RNAeasy kit. This was followed by reverse transcription (Applied Biosystems - High Capacity cDNA Reverse Transcription Kit) and quantitative PCR (SYBR green) using Applied Biosystems 7300 Real-Time PCR Machine. PCR primers used were as follows: Actin: Act Forward 5’- GAA GAT CTC CCA ATC GGC GAA CAA TTC ATA AC – 3’; Act Reverse 5’- GAA GAT CTT TGA ACG CGA CTT GAG AGC GG – 3’; Armadillo: Arm Forward 5’- CAA CTT TCG TCG ATC ATA GCC AC – 3’; Arm Reverse 5’- GCA ATG AGT GGC CCG TTG TG – 3’; C11849/dBili: CG11849 Forward 5’- GCG CCA – 3’, reverse primer sequence 5’- CAA CGT TCC TCG ATA GCC. GAC TG – 3’; C11849/dBili: CG11849 Forward 5’- CAA CTT TCG TCG ATC ATA GCC AC – 3’; Arm Forward 5’- GCA ATG AGT GGC CCG TTG TG – 3’. RNA was isolated following treatment using Qiagen RNAeasy kit. This was followed by reverse transcription (Invitrogen ThermoScript or SuperScript) and quantitative PCR (sybr green) using Roche Light cycler 2.0. PCR primers Bili forward 5’- CCAAGGCACGCGGACACTG’, Bili reverse 5’- CTTGTCGCCCTTCCACGTA3’, GAPDH forward 5’-CCA GCC ATG GAA AAT TCC ATG GCA3’, GAPDH reverse 5’- CTTGACGCGCGTCGTCGAC3’. Actin2 forward 5’- CTTCCGACCTTTGAATGAGA3’, Actin2 reverse 5’- TGCCCTGTTGCAAAGACATAG3’.

**Gene accession numbers.** NM_143085 (Drosophila), NM_682750.2 (Zebrafish), NM_031904 (Human).

**Supporting Information**

**Table S1** Table showing dsRNA targeting eighteen genes with known human orthologs that increased reporter activation two-fold or greater. Found at: doi:10.1371/journal.pone.0006129.s001 (0.01 MB DOC)

**Figure S1** (A) Sequence alignment of Bili protein shows it is well conserved phylogenetically. (B) Human Multi Tissue Northern blot show mRNA expression. (C) Illustration of Bili protein structure and domains. Found at: doi:10.1371/journal.pone.0006129.s002 (2.14 MB EPS)

**Figure S2** (A) qRT-PCR analysis of dBili knockdown. Expression of short-hairpin (shRNA) dBili (#3M) under the control of prd-GAL4 in the Drosophila embryo results in >75% knockdown in message level (Red bar) as compared to prd-GAL4 control embryos (Blue bar). As a control, Armadillo mRNA was not affected in embryos expressing dBili siRNA. (B) Embryos expressing dBili shRNA under the control of even skipped stripe 3’/7-GAL4: These embryos display a partial lack/disruption of the 2nd or 3rd denticle belt which coincides with the eve-stripe 3
expression. This phenotype is consistent with a localized increase in Wingless signaling activity in the region around eve-stripe 3. The same however was not observed for stripe 7 (posterior end of the embryo), perhaps due to differential expression of GAL4 in stripe 3 versus stripe 7.

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Author Contributions

Conceived and designed the experiments: LK TB AK RD RTM. Performed the experiments: LK TB WHC RD. Analyzed the data: LK TB AD WHC RD RTM. Contributed reagents/materials/analysis tools: TB. Wrote the paper: LK TB RTM.

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