Wingless Directly Represses DPP Morphogen Expression via an Armadillo/TCF/Brinker Complex

Heidi Theisen1,4*, Adeela Syed1,4, Baochi T. Nguyen2, Tamas Lukacsovich1,4, Judith Purcell1,4, Gyan Prakash Srivastava4a, David Iron2b, Karin Gaudenz2,4c, Qing Nie2, Frederic Y. M. Wan2, Marian L. Waterman3, J. Lawrence Marsh1,4

1 Department of Developmental and Cell Biology, University of California Irvine, Irvine, California, United States of America, 2 Department of Mathematics, University of California Irvine, Irvine, California, United States of America, 3 Department of Microbiology and Molecular Genetics, University of California Irvine, Irvine, California, United States of America, 4 Developmental Biology Center, University of California Irvine, Irvine, California, United States of America

Background. Spatially restricted morphogen expression drives many patterning and regeneration processes, but how is the pattern of morphogen expression established and maintained? Patterning of Drosophila leg imaginal discs requires expression of the DPP morphogen dorsally and the wingless (WG) morphogen ventrally. We have shown that these mutually exclusive patterns of expression are controlled by a self-organizing system of feedback loops that involve WG and DPP, but whether the feedback is direct or indirect is not known. Methods/Findings. By analyzing expression patterns of regulatory DNA driving reporter genes in different genetic backgrounds, we identify a key component of this system by showing that WG directly represses transcription of the dpp gene in the ventral leg disc. Repression of dpp requires a tri-partite complex of the WG mediators armadillo (ARM) and dTCF, and the co-repressor Brinker (BRK), wherein ARM–dTCF and BRK bind to independent sites within the dpp locus. Conclusions/Significance. Many examples of dTCF repression in the absence of WNT signaling have been described, but few examples of signal-driven repression requiring both ARM and dTCF binding have been reported. Thus, our findings represent a new mode of WG mediated repression and demonstrate that direct regulation between morphogen signaling pathways can contribute to a robust self-organizing system capable of dynamically maintaining territories of morphogen expression.


INTRODUCTION

Numerous studies have demonstrated that WNT signaling (WG in Drosophila) mobilizes a nuclear β-catenin/TCF complex that can activate transcription of WNT target genes [1–4]. WNT signaling typically leads to the stabilization and nuclear accumulation of β-catenin ARM (Armadillo), which forms an activating complex with the DNA binding WNT effector TCF (Pangolin or dTCF in Drosophila) [5]. However WNT signaling can also repress gene expression, even within the same cell where WNT activation occurs. In most cases it is unclear if repression is direct or indirect and the molecular mechanisms involved are unknown.

Development of the Drosophila leg imaginal disc requires maintaining complementary territories of dorsal dpp and ventral wg morphogen expression. We and others have noted that WNT/WG signaling activates wg expression and represses dpp expression in the ventral territory of the Drosophila leg imaginal disc, and this is critical for normal patterning of the disc [6–11], but whether WNT/WG directs ARM–dTCF complexes to activate expression of repressor proteins or whether ARM–dTCF complexes bind directly to the dpp gene to repress transcription is unclear. Here we investigate the mechanism of WG mediated repression of dpp and the basis of the self-organizing behavior of the wg and dpp expression territories (Theisen et al., 1996).

Studies with cultured cells using the WNT activated TOPFLASH promoter have identified many components that contribute to WNT mediated gene activation. However, the response to WG signaling in vivo is often repression of gene expression e.g. the dpp, df2, stripe (sr), engrailed (en), ovo/shavenbaby (svb), and Ubx genes are all repressed upon WG signaling [12–18]. It is not known if repression is direct or indirect and little is known about the co-effectors that produce an inhibitory signal versus an activating signal in response to WG signaling. To determine whether repression by WG signaling is direct or indirect and to better understand the factors that allow a WG signal to be inhibitory, we investigated whether dTCF binds to the dpp gene and whether dTCF and/or ARM are required for WG directed repression.

Here, we show that a novel WG dependent repressing complex that includes ARM–dTCF and the co-repressor Brinker binds

To whom correspondence should be addressed. E-mail: jmarsh@uci.edu

* These authors contributed equally to this work.

a Current address: Department of Computer Science, University of Missouri-Columbia, Columbia, Missouri, United States of America
b Current address: Computer Science Department, University of Missouri-Columbia, Columbia, Missouri, United States of America
c Current address: Stowers Institute, Kansas City, Missouri, United States of America

d Current address: Computer Science Department, University of California-Irvine, Irvine, California, United States of America

Academic Editor: Carl-Philipp Heisenberg, Max Planck Institute of Molecular Cell Biology and Genetics, Germany

Received September 29, 2006; Accepted December 8, 2006; Published January 3, 2007

Copyright: © 2007 Theisen et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by NIH grants RO1 HD36081 and RO1 HD36049 to JLM and NIH CA83982 to MLW, by NIH GM067247 and GM75309 to QN and QN through the Joint NSF/NIGMS Initiative to Support Research in the Area of Mathematical Biology, by NSF SCREMS Grant #DMSS012416, P2000M60501 JLM, QN, FW and by the Chao Family Comprehensive Cancer Center Functional Genomics Program. HT was supported in part by a PHS training grant ST32 GM07311-17. This work was made possible, in part, through access to the confocal facility and Bioaxon facility of the Optical Biology Shared Resource of the Cancer Center Support Grant (CA-62203) at the University of California, Irvine.

Competing Interests: The authors have declared that no competing interests exist.
directly to the dpp enhancer region to provide a key component of a self organizing regulatory loop.

RESULTS
Identifying a WG response element in the dpp regulatory domain

The wg and dpp genes are expressed in non-overlapping ventral and dorsal domains respectively in the leg imaginal disc of Drosophila. Loss of WG signaling leads to ectopic transcription of dpp and an engineered gain of WG signaling can suppress dpp transcription [6–11]. To determine if repression of dpp by WG is direct or indirect, we identified WG-responsive sequences within the dpp gene. The dpp gene is regulated by an extensive set of enhancers some of which are located approximately 30 kb downstream of the dpp coding region (Fig. 1A; [19]). A 10 kb fragment from this region (BS3.0; 106.9–116.9; Fig. 1A; [19]) directs β-galactosidase expression in the normal pattern of dpp expression in imaginal discs (Fig. 1B,C). In the leg disc, expression occurs in a stripe along the anterior/posterior (A/P) compartment boundary, except that extension of the stripe into the ventral region is prevented by WG-dependent repression (Fig. 1B,C) [6–11,20,21]. Since WG signaling is mediated via ARM/dTCF complexes, we scanned the 10 kb dpp enhancer fragment and found 8 potential dTCF binding sites [22], 5 of which fell into two clusters within 2kb of each other in a region that is able to direct expression in leg imaginal discs (Fig. 1A; APRD). A proximal cluster (P) is located around map coordinates 110 and is contained within fragments that activate dpp along the entire A/P boundary. Based on the location of these sites, we analyzed a series of dpp enhancer fragments in transgenic flies (Fig. 1A). At least 4 independent transformant lines were examined for each construct; and the expression patterns were the same for each line tested.

The smallest reporter construct that contains all the elements necessary to mimic the normal dpp expression pattern is a 2.8 kb dpp enhancer fragment that includes an activating region (A), the proximal dTCF cluster (P), a co-repressor binding region (R), and a distal cluster of dTCF sites (D) (APRD; 109.5–112.3) (Fig. 1D). We designate these four functional regions of the 2.8 kb enhancer as APRD with dashes to denote deletion of particular regions and lower case italics to denote regions in which specific dTCF binding sites have been mutated.

An 800 bp fragment containing both the activating region (A), and the proximal cluster of dTCF sites (P) [[BS3.1, AP–] [19]; 109.5–110.3] activates transcription along the A/P boundary but does not exhibit ventral repression (Fig. 1F). The downstream 2 kb region (–RD), containing the putative corepressor binding element (R), and the distal cluster of dTCF sites (D), is required for repression but cannot itself activate expression [BS3.2; 110.5–112.3; Fig. 1A; data not shown]. Deleting the 1.4 kb R region of DNA between the dTCF clusters (AP-D) (Fig. 1A,G) or removing a 500 bp fragment that contains the distal cluster of dTCF sites (APR–) (Fig. 1A,E), results in loss of ventral repression. These data show that repression requires at least two regions in the adjacent 2 kb, namely the distal cluster of dTCF sites (D) and a corepressing region (R) that does not contain dTCF sites. Genomic fragments that lack the 800 nucleotide AP fragment (Fig. 1A, –RD, BS3.2 of Blackman) are not expressed at all and hence repression cannot be evaluated e.g. Blk2.5; 106.9–109.3, and BS3.2, [19] Fig. 1A; data not shown). Thus, the minimal region necessary for proper dpp regulation in the leg disc is the 2.8 kb APRD fragment that contains distinct activating (A) and repressing sequences (RD).

The 2.8 kb dpp enhancer, APRD, responds to WG signaling

To determine if the dpp reporter constructs are responsive to WG signaling, we examined reporter gene expression in animals where WG signaling is blocked at the level of the ligand and at the level of ARM/dTCF. A temperature sensitive wg allele, wg<sup>U114</sup> [23], was used to test the effect of WG signaling on the expression of both the 10 kb (BS3.0) and the 2.8 kb dpp enhancer (APRD) fragments (Fig. 2A,B). Repression of both the 10 kb and 2.8 kb (APRD) dpp reporters is lost in the ventral region of wg<sup>u</sup> discs within 24 h of a temperature shift, indicating that the APRD region of the dpp enhancer is responsive to WG directed repression (Fig. 2A,B and data not shown).

To block the nuclear response to WG signaling, we expressed dominant negative dTCF (DNdTCF), which lacks the ARM binding domain [22], and therefore acts as a nuclear repressor of the WG pathway. If repression of dpp by WG requires an ARM/dTCF complex, then over-expression of DNdTCF should block repression of dpp transcription and result in dpp expression in the ventral region. Expression of UAS>DNdTCF was driven with the HS>Gal4 driver and expression of the BS3.0 and APRD enhancer fragments was monitored. Within 2.5 hrs of activating DNdTCF by shifting to 25°C, expression of the dpp reporter increased dramatically in the ventral region (compare Fig. 2D vs C). The cell cycle time at this stage was ~6–10 hrs [24,25], therefore, the change in gene expression occurred over the course of ~1 cell division, suggesting that the regulation of dpp gene expression by ARM/dTCF is not an indirect consequence of a regenerative response. To confirm that the endogenous dpp gene also responds to DNdTCF, dpp expression was monitored in animals where the dpp<sup>ADI14</sup>Gal4 driver was used to drive DNdTCF expression in a pattern that overlaps both the dorsal region of dpp expression and the ventral region of wg expression in leg discs [26]. Repression of endogenous dpp is lost in these discs (not shown). Thus, blocking WG signaling either at the level of ligand activity or at the level of ARM/dTCF complex formation, leads to a rapid loss of dpp repression in ventral cells of the leg discs, indicating that repression of dpp transcription requires the formation of ARM/dTCF complexes.

Repression of the dpp enhancer requires dTCF binding

To evaluate whether the rapid de-repression in response to DNdTCF reflects competition for dTCF binding sites within the dpp locus or an indirect effect being mediated through other factors, we sought to map and mutate the putative dTCF binding sites in the dpp regulatory region. DNase I footprinting analysis with both recombinant dTCF protein and with human LEF-1 protein showed that both the Drosophila and human proteins protect all 5 putative TCF binding sites in the APRD dpp fragment (Fig. 3A, B and data not shown). We also performed electrophoretic mobility shift assays to confirm that these sites were the only bona fide dTCF binding sites and that there were no other dTCF binding sites within the APRD region (data not shown).

To test whether direct binding of dTCF to the 2.8 kb dpp enhancer fragment is required for dpp repression, we engineered specific inactivating mutations in all 5 dTCF binding sites (AP/Rd) or only in the distal cluster of 3 dTCF sites (APRd). Gel shift experiments with recombinant dTCF demonstrated that the introduced mutations eliminated dTCF binding (data not shown). We compared the expression of the dpp reporter gene with the dTCF sites intact is mutated. Loss of binding sites either in both clusters or in only the distal cluster (AP/Rd or APRd), caused
a dramatic loss of repression in the ventral leg disc (Fig. 3C–E). As described earlier, the two dTCF sites in the Proximal Cluster of the APRD fragment are not sufficient to cause measurable repression when the distal complex is absent nor are TCF sites required for activation since fragments with all TCF sites mutated still drive expression (not shown). These data demonstrate that binding of dTCF to the distal sites is necessary to inhibit dpp transcription. 

Figure 1. A 2.8 kb fragment of the dpp enhancer is sufficient for activation and repression of dpp in the leg disc.

A: Schematic representation of the dpp locus and the 6 enhancer fragments used in this study. The dpp transcription unit is centered around 86 kb (arrow). [Map coordinates (in kilobases) from [19,52,53]. The leg disc enhancer is located between 20–30 kb downstream of the dpp coding region. Filled stars represent dTCF-binding sites confirmed by footprinting, open stars are predicted sites and pentagons are BRK binding sites. Arrowheads indicate fusion to the ß galactosidase reporter gene. APRD refers to the 4 relevant domains A (region required for Activation), P (proximal TCF sites), R (repressor domain), D (distal TCF sites). B–E: 3rd instar leg imaginal discs with dorsal up and anterior to the left. B: Normal dpp mRNA expression detected by in situ hybridization. Bracket indicates ventral region, where dpp is repressed. C: A 10 kb dpp enhancer fragment (BS3.0) drives expression of lacZ in a stripe that recapitulates normal dpp expression including ventral repression (bracket). D: Expression driven by the 2.8 kb APRD dpp enhancer fragment mimics dpp mRNA and BS3.0 expression. Again, note ventral repression (bracket). E: Ventral repression is lost (bracket) in the 2.3 kb APR- fragment which has a 500 bp region of APRD that contains the distal cluster of dTCF binding sites (D) deleted. F: An 800 bp fragment (AP-, BS3.1) containing the proximal cluster of dTCF sites (P) is not sufficient for ventral repression (bracket). G: The AP-D fragment does not show ventral repression (bracket). Sequences in the 1.4 kb between the proximal and distal dTCF sites do not contain dTCF sites but are required for ventral repression.

doi:10.1371/journal.pone.0000142.g001
the dTCF sites leads to ventral expression that is unresponsive to WG, ARM and dTCF overexpression (Fig. 4A, B and data not shown). Thus, functional dTCF binding sites in the APRD dpp enhancer fragment are required for ventral repression in vivo.

Brinker is required for WG dependent repression of dpp

How is it that dTCF binding in response to WG signaling inhibits expression of dpp but activates other genes? The AP-D construct, which contains 5 intact dTCF sites but has an internal deletion (Fig. 1G), has lost repression in the ventral region of the leg disc. This suggests that the deleted region contains an element that cooperates with dTCF to repress dpp transcription. A scan of this co-repressor region (R) for potential binding sites of known repressors of dpp identified two potential Brinker (BRK) sites. BRK is a sequence-specific transcription factor that is repressed by DPP signaling. Furthermore, the expression pattern of brk complements that of dpp in the leg disk; there is lower expression along the A/P boundary in the dorsal region, but strong expression in the

Figure 2. The dpp enhancer responds to WG signaling
A–D: 3rd instar leg imaginal discs. Dorsal is up, anterior is to the left. Expression of the 2.8 kb APRD reporter fragment is monitored by β-galactosidase activity. A: In wild type leg discs (mesothoracic shown), APRD–LacZ expression is repressed in the ventral region (bracket). B: WG signaling is required for ventral repression. In a pair of everting prothoracic leg discs from a wgts larva, ventral repression of APRD–LacZ is lost after shifting to restrictive temperature (brackets). C: Expression of the APRD reporter is repressed ventrally in Hs–Gal4; UAS–DNdTCF animals reared at 18°C (bracket). DNdTCF is a dominant negative form of dTCF that cannot bind ARM. These animals and their discs are small compared to their non DNdTCF bearing sibs even when maintained continuously at low temperature, presumably due to low level expression of Hs–Gal4. However, these control animals maintained at low temperature do survive as viable, morphologically intact adults. D: When heat shocked in late third instar, repression is lost within 2.5 hours (bracket). At least 6 animals of each genotype were examined and all legs exhibited the same responses.

doi:10.1371/journal.pone.0000142.g002

Figure 3. Identification of dTCF binding sites required for dpp ventral repression
A,B: dTCF binding sites in the dpp regulatory region from 109.4–112.8 kb were mapped by DNase I footprinting using dTCF protein as described in the methods section [22]. The approximate positions of the protected sites are indicated by stars. DNase I footprinting of the region containing the distal cluster (D) reveals 3 protected sites (sites 3, 4; 5) indicated by the bars in A and B. Similar footprints identified two sites in the proximal cluster (sites 1; 2 = P) and no footprints or gel shifts were detected in the A or R regions (not shown). Duplicate lanes represent independent reactions. Lanes 1; 7 are the GA sequencing ladder. All lanes utilize a 1:1 dilution of bacterial extract containing empty expression vector or protein expressing vector and the same concentration of DNase except lane 4. Lanes 2 and 6 are no protein controls. Lane 3 uses an extract expressing human LEF1 protein. Lanes 4 and 5 use an extract expressing dTCF with lane 4 containing a 3 times higher concentration of DNase. C–E: 3rd instar leg imaginal discs. Dorsal is up, anterior is to the left. dpp lacZ expression is monitored by immunofluorescence. C: The 2.8 kb APRD dpp enhancer fragment with all 5 dTCF sites intact is repressed ventrally (bracket). D: Mutation of all 5 dTCF sites (ApRd) eliminates ventral repression (bracket). E: Mutation of just the 3 distal dTCF sites (ApRd) is sufficient to eliminate ventral repression (bracket). 

doi:10.1371/journal.pone.0000142.g003
anterior and posterior regions, and intermediate expression along the A/P boundary in the ventral leg disk [27–30].

To test whether BRK binds to both of the potential sites in the R region, we used surface plasmon resonance (SPR) with immobilized recombinant BRK protein in a DNA binding assay (Fig. 5A). The SPR sensogram shows that BRK can bind to the R region when at least one of the BRK binding sites is intact, but when both BRK sites are mutated, no binding is observed.

If BRK is specifically required for WG mediated repression of \(dpp\), then introducing either or both mutations into the BRK sites (AP\(\text{Brk}^{1D}\), AP\(\text{Brk}^{2D}\), and AP\(\text{Brk}^{12D}\)) should lead to increased \(dpp\) expression in the ventral region of the leg disk. Indeed, mutation of either BRK site 1 or both sites, results in increased \(dpp\) expression that is restricted to the region of WG signaling (Fig. 5 B,C,D).

To determine whether BRK binding is an essential component of WG mediated \(dpp\) repression, we tested the ability of WG signaling to repress reporter constructs when the BRK sites are mutated. While ectopic \(wg\) expression is able to extinguish all APRD expression (Fig. 4A), ectopic WG cannot repress APRD when the BRK sites are mutated (AP\(\text{Brk}^{D}\)) (Fig. 4C). This suggests that under normal cellular conditions, loss of BRK binding sites prevents repressor complex formation but that experimental induction of high levels of BRK may allow repressor complexes to form that are anchored to the DNA by dTCF-ARM complexes. Taken together these data suggest that at normal factor concentrations both BRK and dTCF sites are necessary for WG mediated repression of \(dpp\) transcription but neither alone is sufficient.

**DISCUSSION**

**Active Repression of \(dpp\) by WG defines a novel mode of WG mediated repression**

TCF is emerging as a multifunctional transcriptional modulator that can act as both an activator and a repressor in multiple environments. In the absence of WNT signaling, LEF/TCFs become default repressors [4,31–33] of genes because they recruit corepressors such as GRO and CtBP [13,34–36]. WNT signaling relieves this repression by causing \(\beta\)-catenin/ARM to accumulate in the nucleus and convert dTCF to a transcriptional activator, possibly by displacing or overriding the default corepressor(s) [37]. This default repression can be further modulated by processes that antagonize the interaction of \(\beta\)-catenin with TCF.

Less well understood is the mechanism whereby TCF can repress genes in response to Wnt signaling. Expression of several genes is repressed in response to WNT signaling, including, \(E\)-cadherin, \(dpp\), \(Ubx\), osteocalcin, stripe, scr, daughterless [14–17,38–43].
Thus far, one mechanism for WG/WNT dependent repression has been described namely, Competitive Repression [44]. In this case, dTCF represses gene activation by displacing other activating proteins through competition for the DNA binding site. For example, WG signaling represses stripe gene expression when dTCF binds to sites that overlap with the sites for the activator (CI) [15]. TCF has also been shown to mask the DNA binding domain of another transcription activator Runt and inhibit its binding to the osteocalcin promoter [42]. In both these cases, repression occurs in response to the WG/WNT signal and requires ARM. Here, we provide evidence of a second mechanism of WG/WNT directed repression, namely Direct Repression [44]. We show, for the first time, that WNT signaling can direct formation of a co-R*ARM+TCF complex that represses transcription. In the case of dpp repression, this co-R is BRK and the formation of a BRK+ARM+TCF complex is required to actively repress dpp gene expression. Other genes, including ovo/svb, da and dfz2 in Drosophila, are actively repressed by WG signaling and contain physically separated activating and repressing enhancer elements [12,14,38], but since the putative regulatory DNA regions necessary for repression of these genes have not been identified, it is not yet possible to tell if repression in these cases also requires an ARM+TCF complex.

Our studies show that BRK can interact with the dTCF-ARM complex to repress target genes. The behavior of the complex in response to altered levels of individual components, especially to altered levels of the non-DNA binding component, ARM, is not monotonic (e.g. repression is lost with both low and artificially high levels of ARM), suggesting a mechanism whereby both TCF and BRK can be titrated out by excess ARM which might be achieved by either direct or indirect interaction of ARM with both DNA binding components. Although, the specific molecular interactions that dictate the behavior of this complex remain to be determined, one can imagine several scenarios. To better understand the potential implications of these different scenarios, we constructed mathematical models that differ primarily in the nature of the interactions between DNA binding and non-binding components (Fig. S1–S5). This modeling analysis suggests distinct functional responses to different biochemical mechanisms that will be the subject of future studies. The biological responses described here and our analysis by modeling using reported values for the biophysical parameters [54–61], [Supporting Text S1; Figs. S1–S6.

**Figure 5. BRK binding is required to suppress dpp expression**

BRK binding sites are located in the R domain of APRD (filled pentagons). SPR analysis shows BRK binding to the intact R domain (R). Mutation of BRK site 1 [r(brk1)] reduces binding incrementally, mutation of BRK site 2 [r(brk2)] reduces binding still further while mutation of both sites [r(brk1,2)] abolishes binding completely. The biophysical binding of BRK to its DNA sites correlates well with the biological responses caused by the same mutations. B: dpp expression is ventrally repressed in the intact APRD fragment (arrow). C: Mutation of both BRK sites leads to loss of repression and ventral expression of dpp (arrow). D: Mutation of a single BRK site leads to ventral expression of dpp (arrow).
and Table S1) suggest a possible interaction mechanism in which a single ARM protein interacts either directly or indirectly with both TCF and BRK.

Since the 


expression were also monitored in a temperature sensitive wg background. The temperature sensitive wg allele, 


expression was monitored in legs from at least 6 animals. The same changes in gene expression were observed in all animals with a particular genotype.

In situ hybridizations


expression were fixed as for in situ and incubated overnight at 4°C with rabbit anti β-galactosidase antibody diluted 1:1000 with PBT (PBS+0.1% Triton×100)+3%BSA. A Cy3 or FITC conjugated donkey secondary antibody (Jackson Immunological Laboratory) was used at a 1/200 dilution. Images were analyzed on a Zeiss 510Meta confocal microscope. In each experiment, gene expression was monitored in legs from at least 6 animals each from 4 transgenic lines. The same changes in gene expression were observed in all animals with a particular genotype.

Protein Preparation and DNAse I footprinting

The DNA binding domain of dTCF was amplified by PCR using primers 5'CGCGGATCCGGAGAAGCTGACGACATCA, and 5'CGCGGATCCGCGGACCACTGT ACTCTGTTG, and cloned into pET15b (Novagen). Bacterial extracts were prepared as described in [49]. Recombinant hLEF-1 [50] and dTCF were incubated with double-stranded DNA probes (5 to 15 fmol per reaction; single end-labeled on the 5' end with [32P]ATP) for 1 minute on ice in a 50 μl reaction containing TM buffer (50 mM Tris pH 7.9, 12.5 mM MgCl2, 1 mM EDTA, 20% glycerol, 0.1% NP-40, 50 mM KCl). DNAse I work-up procedures are described in [51]. Human LEF-1 footprinted to the same sites as dTCF as expected from the highly similar DNA binding domains of these proteins [22]. All gels were analyzed with a PhosphorImager (Molecular Dynamics).
Mutation of dTCF and Brk binding sites

Site-directed mutants were made using the *Pfu* mutagenesis kit (Stratagene) with two complementary 30 nucleotide primers containing the new sequence. Approximately two-thirds of the colonies picked were the correct mutant. The sites were mutated as listed, wild type sequence is underlined, and mutated sequence is in capitals: (site 1) aactttcgaacgtcatggatccccacgttcc; (site 2) aactttcgaacgtcatggatccccacgttcc; (site 3) ctaatgcgcagcatTcatggatccccacgttcc; (site 4) cgggatccggacgtcatggatccccacgttcc; (site 5) cgggatccggacgtcatggatccccacgttcc.

To mutate the BRK binding sites the following mutagenic oligonucleotides were used (the BRK site or its complement is in bold letters with the altered nucleotides underlined):

\[
ggattcgggacgtcatggatccccacgttcc >
ggattcgggacgtcatggatccccacgttcc
\]

and

\[
ggattcgggacgtcatggatccccacgttcc >
ggattcgggacgtcatggatccccacgttcc
\]

The first mutation eliminates an *NcoI* site (ccatgg) while the second mutation creates a *PstI* site (tagctg) making the detection of the mutations easier.

Surface Plasmon Resonance

Computational scanning of 2.8 kb APRD region revealed two consensus BRK binding sites. These were functionally confirmed by SPR on a Biacore 3000. Carboxymethylated dextran (CM5) coated sensor chips (Biacore AB, Uppsala, Sweden) were coated with 800 response units of anti-Flag antibody (Sigma) using NHS/EDC chemistry. HBS buffer (10 mM HEPES pH 7.4, 150 M NaCl, 3 mM EDTA, 0.005% (v/v) Surfactant P20; Biacore AB) was used as the running buffer with a flow rate of 10 μl/min. A fusion protein of the BRK-DNA binding domain with a FLAG epitope tag was purified [16] and captured onto the anti-Flag antibody. A 560 bp fragment spanning both putative BRK sites was tested for binding to immobilized BRK protein and binding was demonstrated. The role of the specific BRK sites was tested for binding to immobilized BRK-DNA and are identical to binding events shown for other models above.

**SUPPORTING INFORMATION**

**Supporting Text S1**

Found at: doi:10.1371/journal.pone.0000142.s001 (0.82 MB DOC)

**Table S1**

Descriptions, values, and references of parameters used.

Found at: doi:10.1371/journal.pone.0000142.s002 (6.76 MB PDF)

**Figure S1**

Computational analysis activation/repression responses of wg and dpp under different possible modes of action A: Cartoon key for the 3 proteins and DNA binding sites involved. The Dpp enhancer (e2) serves to activate wg expression, while the dpp enhancer (e1e2) contains both TCF (e1) and BRK (e2) binding sites and is repressed by WG signaling. Both TCF and BRK bind DNA while ARM does not. B: (i) Depicts the TCF based activation complex formed at the wg enhancer (ii) depicts 3 possible models of complexes involving TCF, BRK and ARM that might contribute to repression. Model 1 requires concurrent binding of an ARM-TCF complex and BRK, but no physical interaction. Model 2 postulates that repression of dpp requires a bridge between TCF and BRK that requires ARM (bridging model). Model 3 proposes a direct binding between TCF and BRK. C(i) Examples of non-productive complexes that might form in the presence of high levels of A under the bridging model (1) or that might form in the presence of high levels of T in the direct binding model (2) (ii) examples of the possible sequences of binding events under model 1. There are several possible intermediates on the way to productive complexes (ATc3 or e1TABe2): D: System is experimentally manipulated by increasing or decreasing the production rates (VT, VA, or VB) of T, A, or B. The computationally predicted response of wg activation (dashed line) and dpp repression (solid line) to changing levels of T, A or B expression is plotted over a wide range of production rates. The experimentally observed response of wild type dpp (e) and wg (f) expression to increased levels of ARM production (g, h) and TCF production (i, j) is shown in the bottom panels. The qualitative behavior predicted by the computational analysis disagrees with the concurrent binding and direct T-A binding models but is consistent with the bridging model when non-productive complexes are considered.

Found at: doi:10.1371/journal.pone.0000142.s003 (6.41 MB PDF)

**Figure S2**

All possible protein-protein and protein-DNA interactions for activation of wg and repression of dpp by models (1) and (2) are shown. Cartoons illustrate the interactions in question and the corresponding binding equations are listed to the right. A. Reactions leading to activation of wg are shown. B. Binding reactions for the concurrent binding model (model 1) are shown where the T-A complex does not bind B. C. Additional binding reactions describing events corresponding to the bridging model (model 2) are shown in a dashed box that correlates with equations in Fig. S3. These binding reactions together with those in B comprise the full set of reactions for the bridging model (2) without formation of NPCs. D. The binding reactions shown in the solid-box describe the formation of all possible NPCs. Together with the reactions shown in B and C, they comprise the full set of reactions for the bridging model with non-productive complexes. Transcriptionally active complexes are shown in bold.

Found at: doi:10.1371/journal.pone.0000142.s004 (6.24 MB PDF)

**Figure S3**

The equations governing activation and repression models (1) and (2) are shown. The unboxed, dash-boxed, and solid-boxed equations/terms correspond to the unboxed, dash-boxed, and solid-boxed interactions in Fig. S2. Model 1 (concurrent binding) is described by the set of equations not enclosed in the dashed and solid-boxes. Model 2 (ARM bridging) is described by the full set of equations. Omitting the terms in the solid-box describes the bridging model (2) in the absence of the formation of NPCs.

Found at: doi:10.1371/journal.pone.0000142.s005 (6.24 MB PDF)

**Figure S4**

All possible protein-protein and protein-DNA interactions for activation of wg and repression of dpp by the direct binding model (models 3) are shown. Several binding reactions in this model are possible intermediates enroute to final complexes and are identical to binding events shown for other models above.
A. Describes the wg activation reactions as in Fig. S2). B. Describes intermediate reactions that are the same as the concurrent binding reactions. C. Binding reactions unique to the T+B binding model are shown in the dashed box. D. The binding reactions leading to non-productive complexes in the T+B binding scenario are shown in the solid box. Transcriptionally active complexes are shown in bold.

Found at: doi:10.1371/journal.pone.000142.s006 (6.24 MB PDF)

Figure S5 Equations governing repression by direct T+B binding (model 3) are shown. The complete set of equations describes the behavior of the direct T+B binding reactions in Fig. S4 with the inclusion of non-productive complexes. Omitting the terms in the solid-box describes the behavior under this model (3) in the absence of the formation of NPCs.

Found at: doi:10.1371/journal.pone.000142.s007 (6.24 MB PDF)

Figure S6 Comparison of the response of T and B to increasing production rates. Why is the response to increased production rate of T to squelch T mediated regulation while increasing production rate of B has little effect? The lack of a known feedback on production of T leads to rapid change in the T:A ratio while the known feedback loops governing levels of B tend to maintain a steady ratio of B:A.

Found at: doi:10.1371/journal.pone.000142.s008 (6.24 MB PDF)

ACKNOWLEDGMENTS

We are indebted to J. Mingqua, T. Li, L. Banchik, S. Sanchez and M. Bahadori for excellent technical assistance, and to O. Marcu for critical reading of the manuscript. We thank Marc Peifer (Univ. North Carolina) for generously sharing stocks and information and K. Matthews and the National Drosophila Stock Center in Bloomington, IN for stocks. We also thank Ron Blackman for sharing flies and DNA constructs, which were used for this project.

Author Contributions

Conceived and designed the experiments: JM TL HT AS MW. Performed the experiments: FW QN TL JP HT AS BN GS DJ KG MW. Analyzed the data: JM FW QN TL JP HT AS BN GS DJ KG MW. Wrote the paper: JM FW QN TL HT AS BN GS DJ MW. Other: Designed and analyzed the computational model: FW QN DI GS BN.

REFERENCES


