Supplemental Data

Activation of Wingless Targets Requires Bipartite Recognition of DNA by TCF

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Supplemental Experimental Procedures

Drosophila Cell Culture, RNAi and Wg-CM Treatment

Kc cells were cultured as described previously [3]. Clone 8 cells were cultured as described (http://flyrnai.org/DRSC-PRC.html#Cl8). RNAi-mediated gene knockdowns were performed as described [4]. For the TCF rescue assays, double-stranded RNA corresponding to the TCF 3`UTR was added to the medium (10μg/10^6 cells). After 4 days, cells were diluted to 10^6 cells/ml and transfected with the plasmids indicated in the figure legends. Three days later, cells were harvested for reporter assays. The primer sequences to generate TCF dsRNA are provided in the supplemental data. Wg-CM and control medium were prepared as described [5]. 10^6 Kc cells were treated with 500 μl of Wg-CM for 24 hr prior to harvesting.

Transient Transfections, Reporter Gene Assays, and Purification of Transfected Cells

Transient transfections and reporter assays were carried out as described [4, 5]. For reporter assays, a mixture of plasmids containing 100 ng of luciferase reporters, 100 ng of pAcArm* and 10 ng of pAcLacZ (Invitrogen) were co-transfected into 10^6 Kc cells. For the TCF rescue assays, 50 ng of luciferase reporters, 2 ng (for 6TCF) or 10 ng (for *nkd*-IntE and *wf-luc*) of pAcArm*, 20 ng of pAc TCF-V5 or pAc mutTCF-V5 and 10 ng of pAcLacZ were co-transfected. For all reporter assays shown, each result represents the mean of two independent transfections, with the standard deviation indicated. The data shown are representative of multiple independent experiments. Luciferase activity in the absence of Arm* was normalized to 1.0 for each construct unless otherwise mentioned in figure legends.

Purification of transfected cells was performed as described [4]. Briefly, cells were co-transfected with 100 ng of pAc-IL2α [6] along with pAc TCF-V5 or pAc mutTCF-V5. Three

days later, transfected cells were harvested with anti-CD25 magnetic beads (Dynabeads) and resuspended in protein loading buffer for western blot analysis using mouse anti-V5 antibody (Invitrogen) and anti-tubulin antibody (Sigma).

Plasmids

The pAcArm* construct was described previously [4]. pAc TCF-V5 was generated by substituting the stop codon of pAc TCF [4] with Alanine (TAA ➔ GCA). Luciferase reporter constructs containing *nkd*-WREs, *Notum*-WREs, slp5-2` and clusters 1-7 are derivatives of pGL3-Basic Vector (Promega) containing a *hsp*70 minimal promoter [4]. In order to mutate TCF sites or Helper sites in these WREs, QuickChange II (Stratagene) was used. Base substitutions were A to C or T to G (or vise versa). Three positions in the TCF binding sites (SSTTTGWW) or seven positions in Helper sites (eg. GCCGCCA) were substituted in all luciferase reporters except UpE2, cluster1 and cluster3. In UpE2, all eight nucleotides of TCF binding sites were mutated. In cluster1 and cluster3, only two positions in Helper sites (eg. GCCGCCA) were mutated. The underlined sequences indicate the positions mutated. Primer sequences to generate the WRE reporters can be found in the supplemental data. Luciferase reporter constructs containing multimerized TCF sites or Helper sites were derivatives of pGL2-Basic Vector containing a *hsp*70 minimal promoter. For analysis in fly tissues, WRE/LacZ reporters were generated by cloning WREs into pH-Pelican [7] and introduced into fly genome by P-element transgenesis (performed by BestGene Inc.).

Immunostaining, In Situ Hybridization, and Microscopy

Immunostaining and in situ hybridization were performed as described [8]. Briefly, rabbit anti-LacZ (1:500) (Abcam Inc.) and mouse anti-Wg antisera (1:100) (Developmental Studies Hybridoma Bank at the University of Iowa) were used. Samples were examined by using Leica confocal miscroscope DM6000 B (Leica) and processed in Adobe Photoshop 7.0. Probes for in situ hybridization of *nkd* transcript were generated by amplifying the genomic DNA. Primer sequences can be found in the supplemental data. Three to five independent transgenic lines of each construct were assayed and typical representative images are shown.

Electrophoretic Mobility Shift Assay

The GST-TCF expression plasmid was generated by replacing the HMG box in GST-HMG expression plasmid [2] with a TCF fragment containing both the HMG box and C-clamp (amino acids 271-408) using the EcoRI and XhoI restriction sites. Primer sequences to clone the TCF fragment are provided in the supplemental data Table I. GST-TCF wild-type and C-clamp mutant proteins were expressed and purified from *Escherichia coli*. EMSA was performed by using the LightShift Chemiluminescent EMSA Kit (Pierce) and the Chemiluminescent Nucleic Acid Detection Module (Pierce) per the manufacturer's instructions. Briefly, proteins in 10% glycerol and DNA oligos were incubated with 50ug/ml poly (dI-dC), 0.05% NP-40, 5 mM $MgCl_2$ and 2µl of 50% glycerol in the presence of binding buffer (10 mM Tris–HCl, pH 7.5, 50 mM KCl, 1 mM DTT). For the competition assay, unlabeled DNA oligos were incubated with reaction mixtures containing proteins for 10 min prior to adding labeled DNA oligos. The concentrations of proteins and oligos used in each experiment were indicated in figure legends.

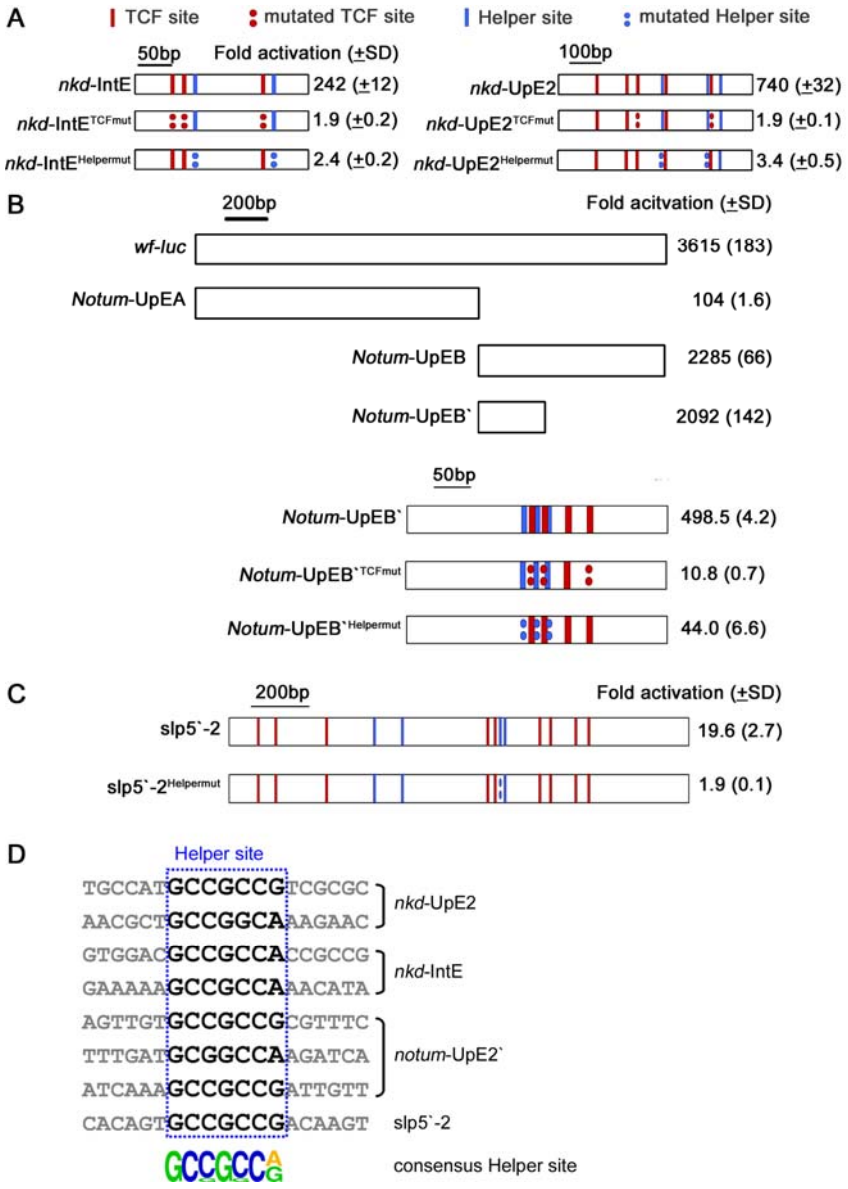

Figure S1. Helper Sites Are Essential for Activation of WREs by Wg Signaling
(A-C) Cartoons of WREs showing predicted TCF sites, Helper sites and their mutations. WREs were cloned upstream of a *hsp70* core promoter/luciferase reporter. These reporters were highly activated by Arm* in Kc cells. This activation was TCF and/or Helper site dependent.
(B) The 2.2 kB *wf-luc* construct previously identified [1] is highly activated by Arm*. This fragment was divided into two halves (*Notum*-UpEA and *Notum*-UpEB). *Notum*-UpEB` is a 3` deletion of *Notum*-UpEB.
(C) slp5`-2 has four predicted Helper sites. Mutation of the third Helper motif, which is adjacent to a functional TCF site [2] caused a large reduction in the responsiveness of slp5`-2-luciferase reporter to Arm* in Kc cells. Mutation of the other Helper sites had no effect (data not shown).
(D) Alignment of functional Helper sites from several WREs with the consensus sequence.

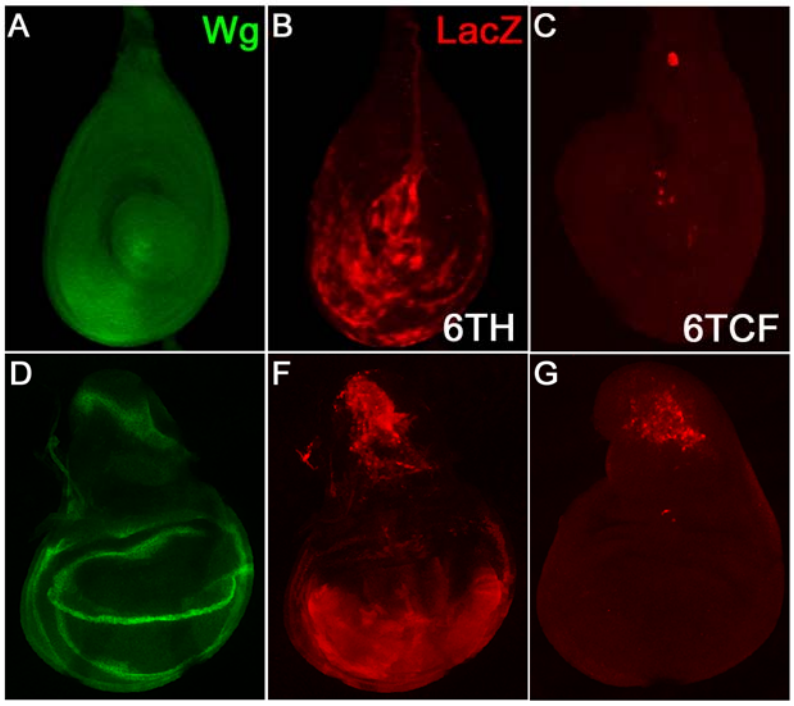

Figure S2. Helper Sites Enable TCF Site to Respond to Wg Signaling in Diverse Tissues
(A-G) Confocal images stained for Wg (green) and lacZ (red) from leg discs (A-C) and wing discs (D-G). Discs from P[6TH-lacZ] flies were double-stained for Wg and lacZ (A, B and D, F) while only the lacZ pattern is shown for discs from P[6TCF-lacZ] flies (C, G). In leg discs, the 6TH reporter was active in the ventral portion of the tissue (B), consistent with activation by Wg (A), with the peripodial membrane expressing high levels of the reporter (B) while 6TCF was barely active in leg discs (C). In the wing disc, 6TH has enhanced expression in the notum (F) compared to 6TCF (G). In the wing pouch, 6TH has strong expression throughout the pouch (F), though the pattern is variable even within a single transgenic line (data not shown). The reason for this variation is not clear, but 6TH can be further activated by Arm* expression in the pouch (data not shown).

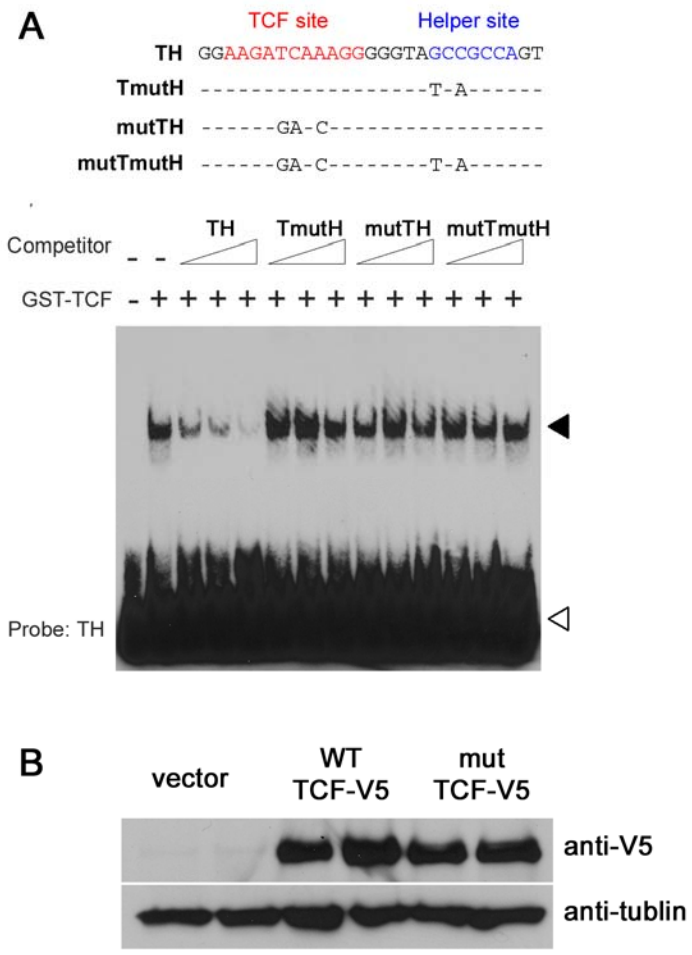

Figure S3. The Interaction between GST-TCF and TH Probe is Specific and the Protein Expression Level of V5-tagged TCFs

(A) The sequences of competitors used are shown. Competition assays where mixtures containing GST-TCF (20nM) and TH probe (4nM) were incubated with unlabeled competitors at 10, 20 and 50 molar excess. The TH competitor dramatically reduces binding to labeled TH, indicating specific binding. Competitors containing mutations in either the TCF sites or Helper sites don't compete with the labeled TH probe, suggesting that both TCF site and Helper site are required for the specific binding of TCF to the tandem TCF-Helper sites.

(B) Western blots with a V5 antibody of whole-cell extracts from transfected cells demonstrating that wild type and mutant TCF were expressed at similar levels.

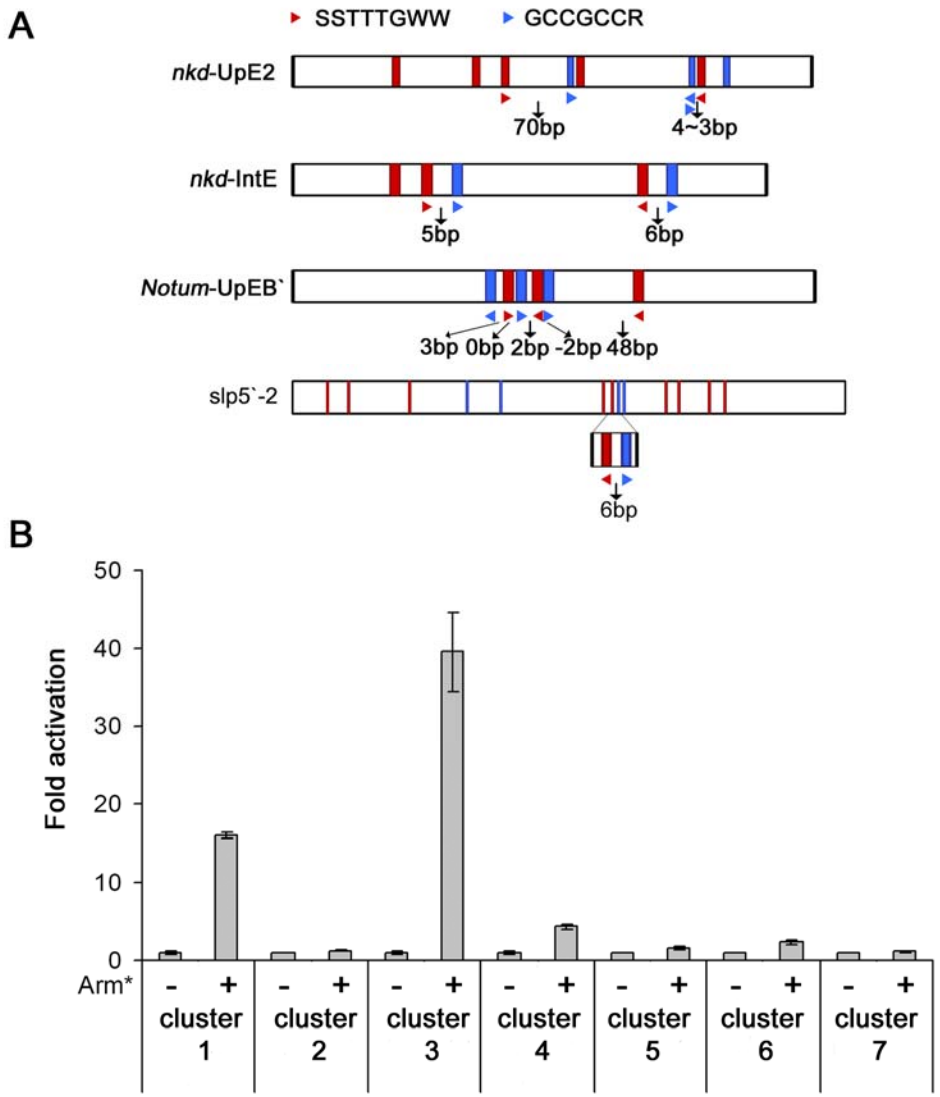

Figure S4. The Inconsistency in the Spacing and Orientation of Functional Helper Sites and a Genome-wide Search for WREs Containing TCF and Helper Site Clusters

(A) The spacing and orientation of functional Helper sites in relation to nearby TCF sites are variable. TCF sites were defined as eight core sequences (SSTTTGWW) and Helper sites were defined as seven consensus sequences (GCCGCCR). In the WREs shown here, the spacing between Helper site and nearby functional TCF sites varies from 0 to 70 bp. The direction of arrowheads indicates the orientation of each Helper site or each TCF site. Functional Helper sites are found in either orientation. The sequence of second Helper site in *nkd*-UpE2 is TGCCGGCA. Therefore, this helper site can be read out in both orientations: TGCCGGC (reverse complement) and GCCGGCA.

(B) Fragments of approximately 500 bp surrounding the clusters identified in a genome-wide search were cloned upstream of a *hsp*70 core promoter/luciferase reporter. Each reporter was co-transfected with an Arm* expression plasmid in Kc cells and luciferase activity was measured. Two reporters containing cluster 1 or cluster 3 were significantly activated by Arm*. These two TCF-Helper site clusters were further characterized.

Table S1. Primer Sequences for dsRNA, WRE Reporters, In Situ Hybridization Probes, and GST-TCF Expression Plasmid

dsRNA	TCF (3`UTR)	TTAATACGACTCACTATAGGGAGACTGATCGCCATGGATTTGTAGAA TTAATACGACTCACTATAGGGAGAGTTTTAGTGTGTATTGTCTGTTT (underlined sequences are T7 promoter sites)
WRE reporters	*hsp70* minimal promoter	ATCTCGAGCTCGAGATCTGAGCGCCGGAGT (XhoI) ATAAGCTTAAGCTTCCCAATTCCCTATTCAGAGTTCTC (HindIII)
	nkd-UpE2	AATAACGCGTCAGGAGGTCTGCCAACTTAAGTAG (MluI) AATTCCCGGGGGGCCGCTGTCGGCCAACTG (XhoI)
	nkd-IntE	GCCACGCGTATAGTTTGTGTATAGTT (MluI) CCCAGCCCGGGTTCCTCAAAGCAACC (XhoI)
	Notum-UpE1	CGATCGGTACCGGCGTTGGTAACC (KpnI) CGATGAAGATCTAGAGGACGGCGAGG (BglII)
	Notum-UpE2	CCTCTGGTACCTCATCGTCATCGTC (KpnI) GCGCTCAAGCTTAGATCTCACCGTA (BglII)
	Notum-UpE2`	CCTCTGGTACCTCATCGTCATCGTC (KpnI) CCAAAAGATCTACATTTTCTTGCGG (BglII)
	slp5``-2	GCGACGCGTAGGATCTCGAATCGCTAATC (MluI) GCGCCCGGGATGTGGATCTCTGGCAATCC (XhoI)
	Cluster 1	CCTGAACGCGTGACCTTCCCCAGCTC (MluI) GAGCGCTCGAGAATAAGCACTCGATG (XhoI)
	Cluster 2	CGCGAAGTACGCGTGCTCATCTC (MluI) CGCGCTCGAGATGCTTTTTGTCT (XhoI)
	Cluster 3	CATTCACGCGTGTCAGCCCAGTG (MluI) CGCTGATCTTCTCGAGAATTTGT (XhoI)
	Cluster 4	AACGGGCACGCGTTTAAAATTGGA (MluI) GATATTTGCTCGAGGCCATTTGA (XhoI)
	Cluster 5	ATAGCACGCGTGAGAGGCATTGG (MluI) GCCCCTCGAGGATGTATTTGATT (XhoI)
	Cluster 6	AAGCGAATTACGCGTAACAATCA (MluI) GCGCAGTTCTCGAGTTCCTTCAA (XhoI)
	Cluster 7	GGAACGCGTTCAATTTGATGCCAACAT (MluI) TCCAGATAAACATGCCCTCGAGTTA (XhoI) (Restriction enzyme sites are underlined.)
in situ hybridization probe	*nkd*	GAATTAATACGACTCACTATAGGGAGAGCTGCTGGTCAGCGAACGTGACAATAA GAATTAATACGACTCACTATAGGGAGACAGACCCGTGGGCAACTTCTTCAGTTT (underlined sequences are T7 promoter sites)
GST-TCF	TCF fragment	AAGAATTCCCCCATATTAAGAAGCCA (EcoRI) GCTCTCGAGTTCGGCGGGCCCATT (XhoI) (Restriction enzyme sites are underlined.)

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