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Report

Activation of Wingless Targets Requires Bipartite Recognition of DNA by TCF

Mikyung V. Chang,[1] Jinhee L. Chang,[1] Anu Gangopadhyay,[1] Andrew Shearer,[1] and Ken M. Cadigan[1,]*
[1]Department of Molecular, Cellular, and Developmental Biology
University of Michigan
Ann Arbor, MI 48109-1048
USA

Summary

Specific recognition of DNA by transcription factors is essential for precise gene regulation. In Wingless (Wg) signaling in *Drosophila*, target gene regulation is controlled by T cell factor (TCF), which binds to specific DNA sequences through a high mobility group (HMG) domain [1]. However, there is considerable variability in TCF binding sites [2–5], raising the possibility that they are not sufficient for target location. Some isoforms of human TCF contain a domain, termed the C-clamp, that mediates binding to an extended sequence in vitro [6]. However, the significance of this extended sequence for the function of Wnt response elements (WREs) is unclear. In this report, we identify a *cis*-regulatory element that, to our knowledge, was previously unpublished. The element, named the TCF Helper site (Helper site), is essential for the activation of several WREs. This motif greatly augments the ability of TCF binding sites to respond to Wg signaling. *Drosophila* TCF contains a C-clamp that enhances in vitro binding to TCF-Helper site pairs and is required for transcriptional activation of WREs containing Helper sites. A genomewide search for clusters of TCF and Helper sites identified two new WREs. Our data suggest that DNA recognition by fly TCF occurs through a bipartite mechanism, involving both the HMG domain and the C-clamp, which enables TCF to locate and activate WREs in the nucleus.

Results and Discussion

Helper Sites Are Crucial for Wg Signaling Activation of WREs in the *nkd* Locus

The Wg target gene *naked cuticle* (*nkd*) contains numerous clusters of potential T cell factor (TCF) binding sites. However, chromatin immunoprecipitation studies revealed that TCF preferentially binds to two regions: in the first intron of *nkd*, approximately 5 kb downstream of the transcription start site (TSS) (*nkd*-IntE) [7], and in a region 10 kb upstream of the TSS (*nkd*-UpE) [5]. These regions contain functional WREs, but several other TCF-site clusters in the *nkd* locus not bound by TCF do not act as WREs [7] (data not shown). These data argue that TCF binding sites are not sufficient for TCF binding and WRE function.

To identify additional sequence information necessary for WRE function, we performed systematic mutagenesis of the entire *nkd*-IntE with nonoverlapping 10 bp substitutions in Kc cells. Besides the three TCF sites that were already known [5], two other motifs, adjacent to the TCF sites, which are also required for full activation of the WRE were identified (data not shown). Additional mutagenesis revealed that both motifs (hereafter termed "Helper sites") consist of seven nucleotides (GCCGCCA). Simultaneous mutation of both motifs resulted in a 100-fold decrease in responsiveness of *nkd*-IntE when the Wg pathway was activated by expression of a stabilized form of Armadillo (Arm*; Figure S1A, available online).

When the *nkd* locus was searched with Target Explorer (http://luna.bioc.columbia.edu/Target_Explorer/) for additional clusters of TCF and Helper sites [8], a second cluster was identified 10 kb upstream of the TSS in a WRE known as *nkd*-UpE2. As seen for *nkd*-IntE, mutation of these two Helper sites in *nkd*-UpE2 resulted in a drastic reduction in activation by Arm* in Kc cells (Figure S1A).

In transgenic fly reporter assays, both the *nkd*-IntE and *nkd*-UpE2 WREs are active in patterns similar to that of endogenous *nkd* [5] (Figures 1A, 1B, 1E, and 1F). These reporters are activated by Wg signaling, and mutation of their TCF sites abolishes activity [5] (Figures 1C and 1G). Strikingly, mutation of the Helper sites also completely abolished reporter-gene expression (Figures 1D and 1H). Similar to the data shown for *nkd*-UpE2 in the wing imaginal disc (Figures 1A–1D) and for *nkd*-IntE in the eye disc (Figures 1E–1H), Helper sites were also required for the activity of these WREs in other imaginal discs (data not shown). These results demonstrate that Helper sites are indispensable for Wg responsiveness of *nkd*-WREs in a broad range of tissues.

Functional Helper Sites Are Also Present in WREs from Other Wg Targets

A Target Explorer search of the Wg target *Notum* (also called *wingful* or *wf*) revealed TCF-Helper site clusters in a previously identified 2.2 kb WRE called *wf-luc* [9], upstream of the *Notum* TSS. Deletion analysis of *wf-luc* revealed two separable WREs (Figure S1B). Site-directed alterations of the predicted TCF or Helper sites in one of these WREs (*Notum*-UpEB′) greatly compromised Wg-pathway responsiveness in Kc cells (Figure S1B).

In transgenic flies, the *Notum*-UpEB′ WRE directed LacZ expression in a pattern consistent with activation by Wg signaling in a broad range of tissues (Figures 1I and 1J and data not shown). These expression patterns were largely TCF site- and Helper site-dependent (Figures 1K and 1L and data not shown).

Like *nkd*, *Notum* encodes a Wg-feedback antagonist whose expression is activated by Wg signaling in many fly tissues throughout development [10–12]. However, most Wg target genes are regulated in a cell-specific manner at particular developmental stages [13, 14]. This raises the possibility that Helper sites are found only in broadly activated WREs. To address this issue, we examined a WRE from the *sloppy paired* (*slp*) locus (slp5′-2). This WRE is directly activated by Wg signaling in the embryonic ectoderm and mesoderm (Figure 1N) [3]. slp5′-2 has four predicted Helper sites (Figure S1C). Mutation of the third Helper site in slp5′-2 caused a large reduction in reporter-gene expression in Kc cells (Figure S1C) and in the epidermis and mesoderm of embryos (Figure 1P). These results extend the importance of Helper sites to tissue-specific targets of Wg signaling.

*Correspondence: cadigan@umich.edu

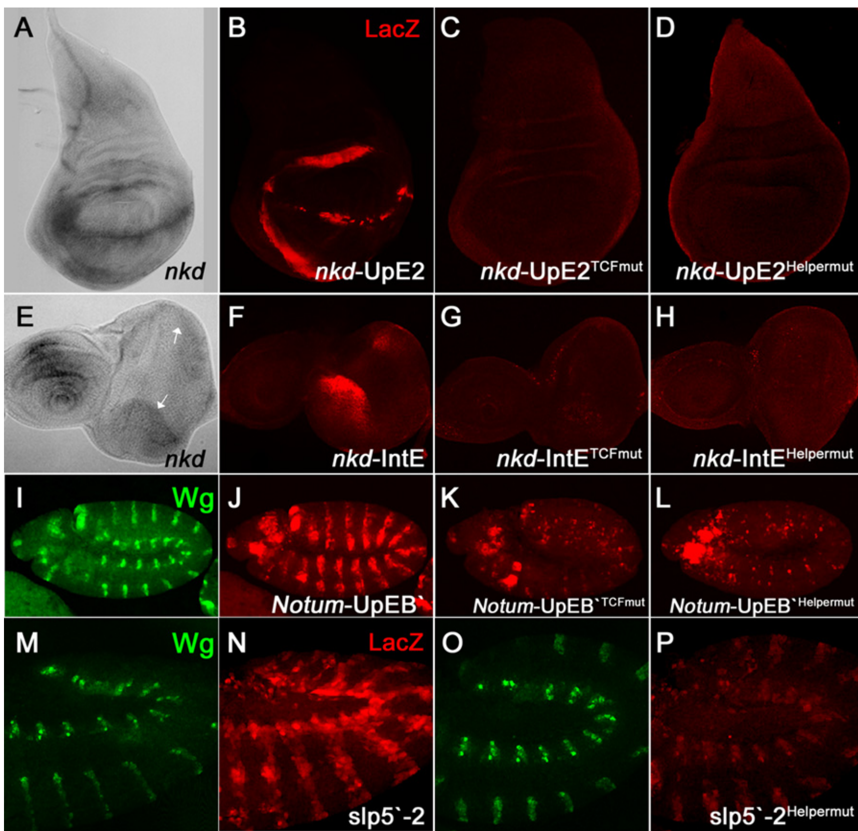


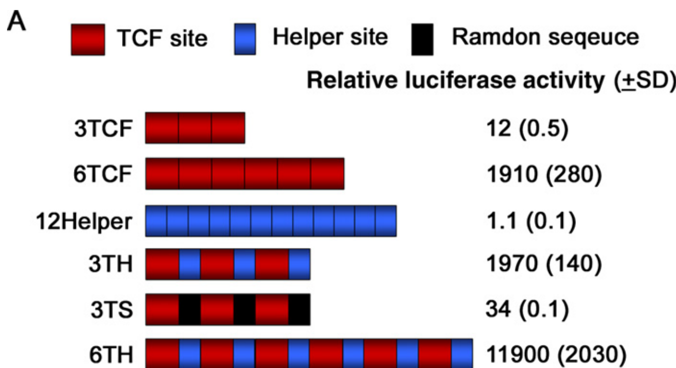
Figure 1. Helper Sites Are Crucial for Activation of Several WREs

Endogenous *nkd* transcripts in the wing (A) and eye-antennal (E) imaginal discs of the third instar larvae detected by in situ hybridization. The white arrows (E) indicate the dorsal and ventral regions of the presumptive eye, where *nkd* is expressed. Confocal images of wing imaginal discs from P[*nkd*-UpE2-lacZ] flies (B–D), eye-antennal discs from P[*nkd*-IntE-lacZ] flies (F–H), and stage 11 embryos from P[*Notum*-UpEB′-lacZ] flies (I–L) or P[slp5′-2-lacZ] flies (M–P) immunostained for Wg and LacZ. The WT reporters are active in patterns similar to those of the endogenous genes and are activated by Wg signaling (data not shown). Mutation of the TCF sites (C, G, K) or Helper sites (D, H, L, P) in these WREs significantly reduced the LacZ expression. Three to five independent transgenic lines for each construct were analyzed, with similar results.

The use of Helper sites may be a common strategy for WRE function in *Drosophila*. The consensus of the Helper site motif is GCCGCCR (Figure S1D). If one allows for one substitution from this consensus, then Helper sites are present near functional TCF sites in WREs from the *Ultrabithorax* (*Ubx*) and *even-skipped* (*eve*) loci [2, 4] as well as *nkd*-UpE1 [5]. Functional analysis of these putative Helper sites should help to refine the criteria of what constitutes a Helper site.

Helper Sites Augment TCF Site-Mediated Transcriptional Activation in Response to Wg Signaling

To learn more about the mechanism behind Helper sites' functioning, we constructed a series of synthetic reporters (Figure 2A). Consistent with previous reports, multimerized TCF sites (3TCF or 6TCF) were substantially activated by Arm* (Figure 2A) [1, 15]. In contrast, Helper sites alone (three, six, or 12 copies) had no response to Arm* (Figure 2A and data not shown).



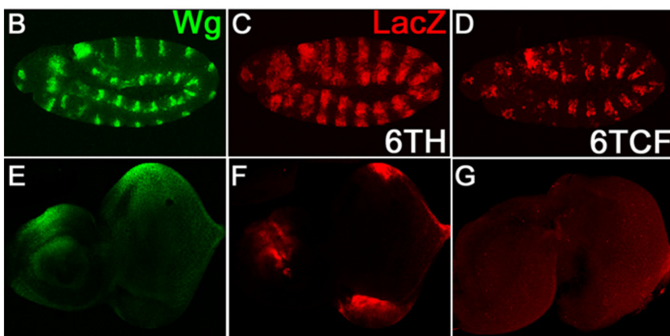


Figure 2. Helper Sites Have No Activity By Themselves but Augment TCF-Site-Mediated Transcriptional Activation by Wg Signaling

(A) TCF sites or Helper sites were multimerized and cloned upstream of a *hsp*70 core promoter-luciferase reporter and tested for activation by Arm*. TCF sites (3TCF and 6TCF) are responsive to Arm*, but Helper sites alone (12Helper) are not. However, Helper sites adjacent to TCF sites greatly enhance activation by Arm* (3TCF versus 3TH, 6TCF versus 6TH). This effect is not observed when the Helper sites in 3TH are replaced by random sequence (3TS). Luciferase activity in the absence of Arm* was normalized to 1.0 for each reporter.

(B–G) Confocal images immunostained for Wg (green) and LacZ (red) from early stage 11 embryos (B–D) and late third instar eye-antennal imaginal discs (E–G). Embryos and discs containing a six TCF-Helper site tandem (6TH) cloned upstream of a *hsp70* core promoter-lacZ reporter were double stained for Wg and LacZ (B, C, E, F), whereas only the LacZ pattern is shown for the construct with six TCF sites (D and G). As with cultured cells, the presence of Helper sites greatly increased the ability of the reporters to be expressed in a pattern consistent with activation by Wg signaling.

However, Helper sites potently augmented the ability of TCF sites to respond to Wg signaling. TCF-Helper site pairs in three (3TH) or six (6TH) copies showed a much greater activation by Arm* in Kc cells than in constructs with the same number of TCF sites (Figure 2A). Replacing the Helper sites in 3TH with random sequences (3TS) reduced the fold activation 50-fold, arguing that the Helper-site effect was not due to the spacing of the TCF sites.

In transgenic flies, a reporter construct containing six TCF sites (6TCF) had some expression in the embryonic epidermis (Figure 2D) and no detectable expression in eye-antennal imaginal discs (Figure 2G). A reporter with six copies of the Helper site did not exhibit significant expression in any tissue examined (data not shown). However, when Helper sites flanked the TCF sites (6TH), a dramatic enhancement of reporter-gene expression was observed in embryos and eye-antennal discs (Figures 2C and 2F). Similar results were observed in other imaginal discs (Figure S2). The results suggest that the presence of Helper sites markedly enhances the ability of TCF sites to respond to Wg signaling in many tissues.

Helper Sites Physically and Functionally Interact with the C-clamp Domain of TCF

Drosophila TCF contains a Cysteine-rich domain, called the C-clamp, downstream of the high mobility group (HMG) domain (Figure 3E). The presence of the C-clamp in human isoforms TCF-1E and TCF-4E enables them to bind in vitro to the classic TCF site and to an extended sequence (RCCG) [6]. This extended sequence is somewhat similar to the Helper site, raising the possibility that it interacts with the C-clamp. A fragment of TCF containing the HMG and C-clamp domains, fused to glutathione-S-transferase (GST-TCF), was found to bind to an oligonucleotide containing a classic TCF site and a Helper site (TH in Figure 3A) in a electrophoretic mobility shift assay (EMSA; Figure 3B). An oligonucleotide containing only a TCF site (TS) was bound much less efficiently by GST-TCF (Figure 3B). No binding was observed with GST alone (data not shown). Competition assays showed that both the TCF site and the Helper site are required for the specific binding of TCF to the TH probe (Figure S3A). A mutation in the C-clamp of GST-TCF (Figure 3E) weakened the affinity of the protein for the TH probe (Figure 3C), indicating that the enhanced binding of GST-TCF to the TH probe is C-clamp-dependent.

Can TCF bind to the Helper site independent of an adjacent TCF site? Under conditions containing high concentrations of both protein and probe, a weak interaction between GST-TCF and a Helper-site-only probe (SH) was observed (Figure 3D). Mutations in the C-clamp of GST-TCF abolished the interaction, implying that the C-clamp interacts directly with the Helper site. Consistent with this weak binding, multiple copies of the Helper site cannot mediate Wg-pathway activation of transcription (Figure 2A). These results support a model in which high-affinity DNA binding of TCF occurs through simultaneous HMG domain–TCF site and C-clamp–Helper site interactions.

To determine whether the C-clamp is required for the activation of WREs containing functional Helper sites, Kc cells were depleted of endogenous TCF by RNAi and subsequently transfected with WRE reporter constructs and expression vectors for Arm* and either wild-type (WT) TCF or TCF containing the C-clamp mutation (the exogenous TCFs are not targeted by the TCF dsRNA used for RNAi). For all reporters examined, expression of WT TCF rescued the defect in Arm* responsiveness caused by TCF RNAi (Figure 3F). In stark contrast, the TCF C-clamp mutant rescued the activation of the 6TCF reporter (containing only TCF sites) but not that of the *nkd*-IntE and *wf-luc* reporters (Figure 3F). Western blots show that WT and C clamp mutant TCFs were expressed at similar levels (Figure S3B). These results demonstrate that the C-clamp of TCF is necessary for the activation of Helper site-dependent WREs by Wg signaling. These results are further supported by the previous finding that a missense mutation in the fifth position of the C-clamp (A374V; see Figure 3E) causes Wg signaling defects in fly embryos [1].

New WREs Identified by Genome-wide Search for Clusters Containing Both TCF Sites and Helper Sites

For the identification of new WREs through computational methods, Fly enhancer (http://opengenomics.org) [16] was used for searching the entire fly genome for clusters of TCF sites and Helper sites. To reduce the number of hits, we employed stringent parameters: the presence of two TCF sites (SSTTTGW) and two Helper sites (GCCGCC) within 100 bases. Ninety-seven clusters were identified. These positives were further prioritized by organization (alternating TCF and Helper sites), proximity of the TCF sites to the Helper sites, and phylogenetic conservation. After these secondary screens, seven clusters were selected for reporter-assay analysis in Kc cells (Figure S4). Two positives out of seven clearly activated the expression of a reporter gene in response to Arm* (Figures S4B).

One positive (cluster 1) is located within the first intron of *ladybird late* (*lbl*) (Figure 4A), a gene known to be regulated by Wg signaling in muscle progenitors of *Drosophila* embryos [17]. Cluster 3 is found 15.2 kb upstream of *pxb* (Figure 4B), a gene that is expressed in an embryonic pattern consistent with activation by Wg signaling [18, 19]. Mutations in the TCF or Helper sites of both clusters resulted in a large reduction in Wg responsiveness (Figure 4C). These results further highlight the functional importance of Helper sites in WREs and illustrate how they can be used to facilitate identification of WREs in silico.

Bipartite Recognition of DNA by TCF

Target location by transcription factors is critical for precise gene regulation. In order to find their appropriate targets among the vast excess of genomic chromatin, these proteins employ various strategies for the enhancement of DNA recognition. For example, the p53 tumor suppressor binds DNA as a homotetramer, with each subunit contacting a quarter site. Thus, a typical p53 binding site contains 20 bases of specific DNA information to mediate the recognition [20]. Many transcriptional factors enhance their specificity for DNA through cooperative binding with other transcription factors; e.g., HOX proteins and Extradenticle/Pbx [21, 22]. *Drosophila* TCF solves this problem by bipartite recognition of DNA. The binding of both the HMG domain and the C-clamp to their respective DNA sites would effectively double the size of the TCF-recognition site, enabling TCF to achieve high-affinity and high-specificity binding to WREs. In this way, fly TCF is similar to POU proteins, which contain two distinct DNA binding domains: a POU-specific domain and a homeodomain [23].

In all of these abovementioned cases, a single DNA binding domain is not sufficient for recognition of transcriptional targets. This raises the question of how vertebrate TCFs solve the specificity problem, given that only a couple of TCF isoforms (TCF-1E and TCF-4E) contain a C-clamp [6]. For these isoforms, the presence of the C-clamp is necessary for activation of an alternative promoter of the *Lef-1* gene and for

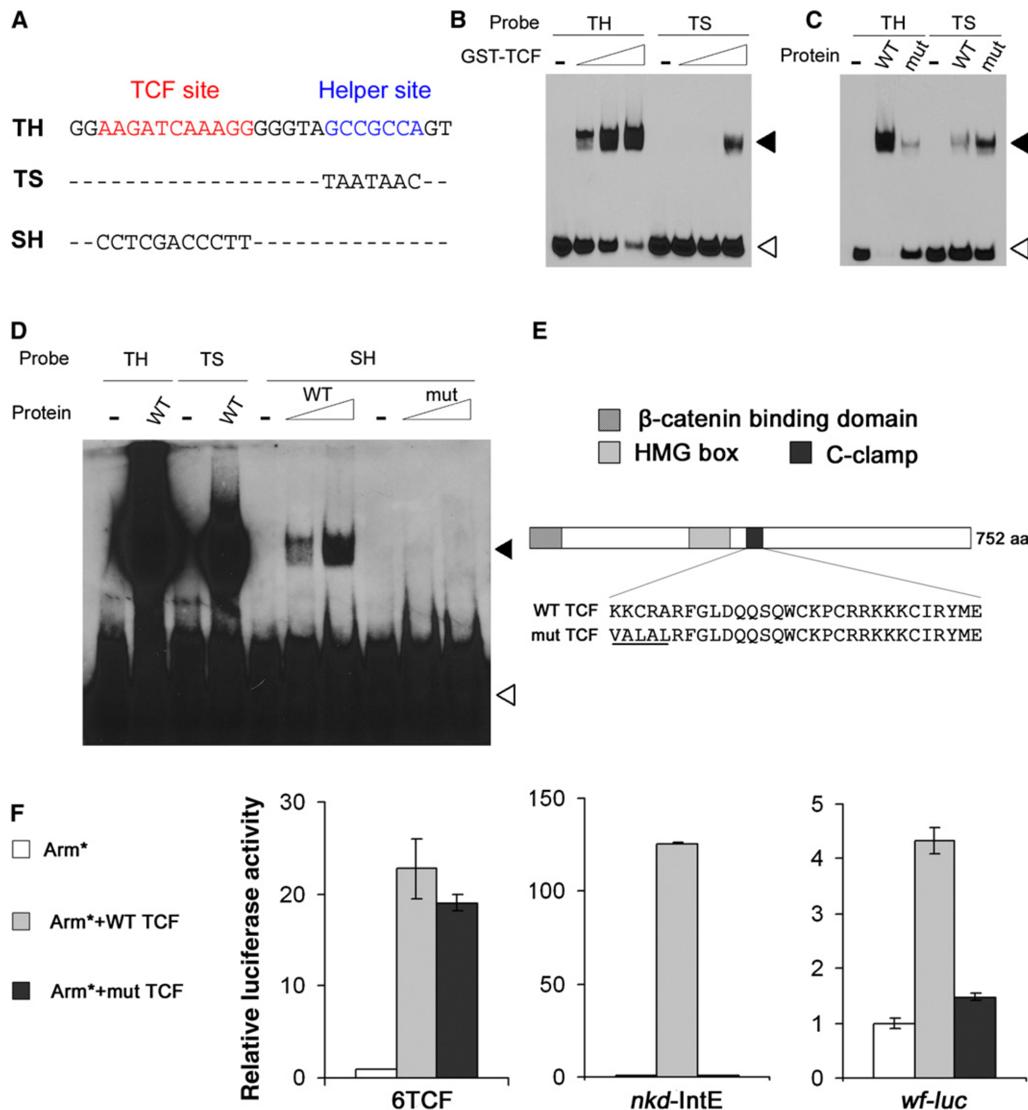


Figure 3. Helper Sites Physically and Functionally Interact with the C-clamp Domain of TCF

(A) The sequences of oligonucleotide probes used for the EMSA assays.

(B) Increasing concentrations of GST-TCF (0.1, 0.3, and 1 μM) were incubated with DNA probes (4 nM). GST-TCF binds to the TH probe better than to the TS probe. White arrowheads indicate free probe, and black arrowheads indicate the protein-DNA complexes.

(C) Binding of TH and TS oligonucleotides (4 nM) to WT GST-TCF or C-clamp mutant proteins (1 μM; mutated residues indicated in Figure 3E). The C-clamp mutant displays weaker affinity for the TH probe.

(D) TH and TS probes (20 nM) were incubated with WT GST-TCF (1 μM). SH probes (20 nM) were incubated with WT or C-clamp mutant GST-TCF (1 and 5 μM). WT protein binds to SH with low affinity, and this binding is C-clamp-dependent.

(E) Cartoon of *Drosophila* TCF indicating the position of the HMG and C-clamp domains. The underlined amino acid sequence of the C-clamp indicates the five residues altered in the mutant.

(F) TCF rescue assays in Kc cells in which endogenous TCF was depleted with dsRNA corresponding to the TCF 3′UTR. Each WRE reporter was cotransfected with Arm* and V5-tagged TCF-expression plasmids. The activity of a 6TCF-luciferase reporter was efficiently rescued by both WT and C-clamp mutant TCF. However, the C-clamp mutant didn't rescue the activity of the *nkd*-IntE-luciferase reporter and *wf-luc* reporters. Luciferase activity in the presence of Arm* but without TCF expression was normalized to 1.0 for each reporter.

dominant-negative versions of TCF to suppress growth of a colorectal cancer cell line [6]. For all of the TCF family members and isoforms that lack a C-clamp, we suggest the likelihood that additional mechanisms exist to enhance HMG-domain binding to the classic TCF site for the achievement of high-specificity DNA recognition.

Our knowledge of the Helper site consensus allowed us to locate WREs within target genes already known to be activated by Wg, such as the *nkd*-UpE2 and *Notum*-UpEB′ WREs (Figure 1 and Figure S1). In addition, we were able to identify two new WREs in silico. However, several questions about the relationship between TCF sites and Helper sites remain unsolved. For example, the spacing and orientation of Helper sites in relation to nearby functional TCF sites vary significantly among the known TCF-Helper site pairs (Figure S4A). Additional studies are needed for full elucidation of the mechanism by which this motif enhances TCF binding and transcriptional activation in response to Wg signaling.

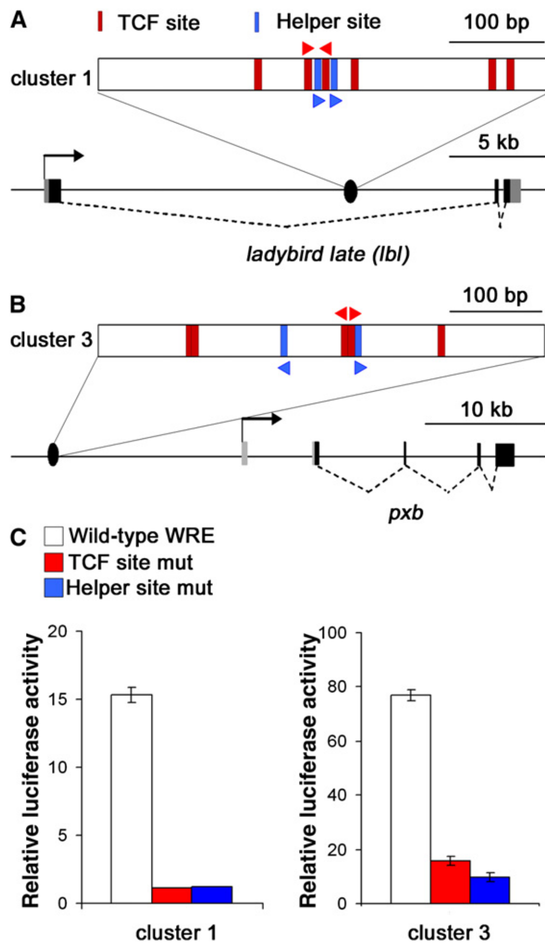


Figure 4. A Genome-wide Search for TCF-Helper Site Clusters Identified New WREs

(A and B) Schematic diagram of the *lbl* (A) and *pxb* loci (B), showing the locations of the identified clusters. The gene structure for *pxb* was drawn on the basis of the *pxb*-PB isoform. Red and blue triangles indicate the location and orientation of mutated TCF sites and Helper sites, respectively (see Figure S4A).

(C) The fragments containing cluster 1 (478 bp) or cluster 3 (484 bp) were cloned upstream of a *hsp*70 core promoter-luciferase reporter. In Kc cells, both clusters activate luciferase expression when cotransfected with an Arm*-expression plasmid. Mutation in the Helper sites or adjacent TCF sites significantly reduces the Arm* responsiveness of both reporters. Luciferase activity in the absence of Arm* was normalized to 1.0 for each reporter.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, four figures, and one table and can be found with this paper online at http://www.current-biology.com/supplemental/S0960-9822(08)01416-4.

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