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LIGAND RECEPTOR INTERACTIONS IN THE WNT SIGNALING PATHWAY IN *DROSOPHILA*

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Running Title
Wnt-receptor interactions

Summary

Secreted Wnt proteins have numerous signaling functions during development, mediated by Frizzled molecules that act as Wnt receptors on the cell surface. In the genome of *Drosophila*, seven *Wnt* genes (including *wingless*; *wg*), and five *frizzled* genes have been identified. Relatively little is known about signaling and binding specificities of different Wnt and Frizzled proteins. We have developed an assay to determine the strength of binding between membrane-tethered Wnts and ligand binding domains of the Frizzled receptors. We found a wide spectrum of binding affinities, reflecting known genetic interactions. Most Wnt proteins can bind to multiple Frizzleds and vice versa, suggesting redundancy *in vivo*. In an extension of these experiments, we tested whether two different subdomains of the Wg protein would by themselves bind to Frizzled and generate a biological response. While these two separate domains are secreted from cells, suggesting that they form independently folded parts of the protein, they were only able to evoke a response when co-transfected, indicating that both are required for function.

In addition to the Frizzleds, members of the LRP family (represented by the arrow gene in *Drosophila*) are also necessary for Wnt signal transduction and have been postulated to act as co-receptors. We have therefore examined whether a soluble form of the Arrow molecule can bind to Wingless and Frizzled, but no interactions were detected.

Introduction

Among the many signaling events occurring during animal development, the interactions between members of the secreted Wnt protein family and their receptors, the Frizzleds, are prominent (1-5). In *Drosophila*, there are seven *Wnt* genes (including *wingless; wg*) and five members of the *frizzled* (*fz*) family (6,7). Given that there are multiple genes for ligands and receptors, the phenotypic consequences of Wnt signaling *in vivo* are probably determined not only by where the genes are expressed but also by the strength of binding between these molecules. However, very few studies have examined to what extent different Wnt proteins bind to their receptors (1,8-10).

Structurally, each Frizzled protein is composed of an extracellular cysteine-rich domain (CRD), a seven-transmembrane region, and a cytoplasmic tail (11). The CRD domain, when over-expressed with a cell surface anchoring sequence, is necessary and sufficient for Wnt binding (1,12). CRDs are also found as the Wnt binding modules in secreted Frizzled Related Proteins (sFRP or FRP), a group of secreted Wnt inhibitors (8,13-15). The crystal structure of the CRD has been obtained recently (16). The structures are predominately α-helical with all cysteines forming disulfide bonds. The ligand-binding surface of the CRD has been defined by combining the information from the crystal structure with mutagenesis studies (9,16).

With respect to the genetics of *frizzled* genes in *Drosophila*, there are mutants in four of the five genes – only *Dfz*4 remains to be mutated. Special attention has been given to *fz*, the first *frizzled* gene found. This gene is essential in tissue polarity formation (11). *fz* does not have phenotypes similar to *wg* mutants (17,18), but when combined with mutants in *Dfz*2, the resulting embryos are identical to *wg* mutants (19-21). Tissue culture experiments demonstrated that over-expressing *fz* or *Dfz*2 in S2 cells allows the cells to respond to Wg protein (1). *DFz3* mutants do not have obvious developmental defects (22,23) but absence of the *Dfz*3 gene can modify some *wg* phenotypes (22). Finally, *smoothened* (smo), a distantly related *fz* gene is required for transducing Hedgehog (Hh) signals (24,25).

Additional complexity in Wnt-receptor interactions arose when Arrow (Arr), an LDL-receptor-related protein (LRP), was found to be required for Wnt signaling

(26,27). The suggestion has been made that LRP can bind to Wnt directly (28), but this important aspect of Wnt-receptor interactions has not always been confirmed(29). The role of LRP in Wnt signaling became more intriguing after several groups showed that LRP is a receptor for Dickkopf (Dkk), a secreted Wnt inhibitor (29-31).

In this work, we analyze the binding between most of the *Drosophila* Wnts and all of the Frizzled proteins in a quantitative manner. We find significant differences, which reflect genetic interactions between *Wnt* and *Frizzled* genes in the fly. We also report on attempts to detect direct binding between Arrow and Wg, using various assays.

Experimental procedures

Generation of CRDs-AP Fusion Constructs, Cell Culture, Transfection, and CRDs-AP Fusion Protein Production

We initially found that chimeric proteins made by fusing various Frizzled CRDs (from the first methionine until the tenth conserved cysteine) to alkaline phosphatase were not secreted into the medium after over-expression in 293T cells. However, the alkaline phosphatase fusion for the secreted Frizzled-related Protein-3 (sFRP-3) which contains the conserved CRD followed by a tail of hydrophilic amino acids (8), is secreted well in 293T cells. Therefore, all CRD-AP fusion constructs were made by replacing the CRD region of the psFRP3-AP construct (8) with CRDs of fz, DFz2, DFz3, DFz4 or Smo.

To easily swap CRDs for these frizzled constructs, MluI sites were generated after the 10th conserved cysteines using QuickChange Site-Directed Mutagenesis kit (Stratagene). After MluI sites were created in the cDNAs of sFRP3-AP, fz, DFz2, DFz3, Dfz4 and smo constructs, the CRD of sFRP3-AP was replaced by the CRDs of Drosophila frizzleds. Changes of DNA and amino acid sequences are as follows (changed sequences are bold),

sFRP3-AP: TGC ATC T**AC GCG T**TG GCC, CI**YAL**A,
fzCRD-AP: TGC GTG G**AC GCG TTG GCC**, CV**DALA**,
Dfz2CRD-AP: TGC ATG G**AC GCG TTG GCC**, CM**DALA**,
Dfz3CRD-AP: TGC ATG C**AC GCG TTG GCC**, CM**HALA**,
Dfz4CRD-AP TTC ACA A**AC GCG TTG GCC**, FT**NALA**,
SmoCRD-AP: TGT TTA A**AC GCG TTG GCC**, CL**NALA**.

All CRD-AP fusion constructs were cloned into the pRK5 vector (1) for expression in 293T cells. 293T cells were cultured with DMEM medium supplemented with 10% FBS (HyClone), penicillin, and streptomycin. Transfection was done using calcium phosphate precipitation. Four days after transfection, AP fusion proteins were collected from the conditioned medium and concentrated using centriprep-50 columns (Amicon). To confirm the expression and concentration of the CRD-AP fusion proteins, concentrated conditioned medium was immunoprecipitated with anti-alkaline phosphatase antibody (Genzyme) and the immunocomplexes were resolved on an SDS-

PAGE. The gel was then stained with Coomassie Blue.

Generation of the Arr-Ig Fusion Construct and Arr-Ig Fusion Protein

The Arr-Ig fusion construct was made by replacing the Lrp6 portion in a Lrp6-Ig fusion construct for IP-Western experiments (28). The construct contains the extracellular domain of Arr, which starts from the first amino acid to the 1447th amino acid. A HindIII site at the 5' and a XbaI site at the junction between Lrp6 and Ig were used for swapping. After replacing the Lrp6 with the extracullar domain of Arr, the sequences of the new junction site between arr and Xba site become: **RMAPAT**SLG (Arr sequences are bold).

Arr-Ig and Ig were produced in 293T cells that were transiently transfected using Lipofectamine (GIBCO/BRL). One day after transfection, cells were transferred to serum-free DMEM, and the secreted proteins were harvested after an additional 24 hours. Control conditioned medium was obtained from untransfected 293T cells. These conditioned medium were then concentrated through Centriprep (Amicon) columns. Concentrated proteins were immunoprecipitated with protein G sepharose (Amersham Pharmacia) and resolved on SDS-PAGE gels. Proteins were transferred to a nitrocellulose filter and the filter was probed with the HRP conjugated horse anti-human antibody (BioRad) overnight. The blot was detected using ECL western blot detecting reagent (Amersham).

Generation of Nrt-Wnts Fusion Constructs, Insect Cell Culture and Stable Line Selection

Nrt-wnt fusion constructs were made by swapping the Wg portion in the Nrt-Wg construct (32) with other wnts. The Nrt-Wg construct has HA sequences between the Nrt and Wg cDNA. The wnt cDNAs excluding the signal sequences were cloned by PCR. For DWnt3, only the Wnt homology region was cloned into the fusion construct (starting from amino acid 559th to 1010th). The sequences around the regions linking HA and Wnts are (Wnt sequences are bold),

Nrt-DWnt2: WEDEEAS**MEIRLVS**,

Nrt-DWnt3: WEDEEAS**MLHLTAR**,

Nrt-DWnt4: WEDEEAS**AGGQGLP**,

Nrt-DWnt8: WEDEEAS**VLEPMSY**.

All Nrt-wnt fusions were cloned into the pMK33HS vector (33) for expression in Schneider 2 (S2) insect cells. Transfections were done by calcium phosphate precipitation. Transfected S2 cells were cultured in Schneider 2 medium (Gibco-BRL) supplemented with 15% FBS (Sigma), penicillin, streptomycin and 125 μg/ml hygromycin (Sigma) for selection.

Antibody Staining

S2 cells were heat shocked at 37^{o}C for 40 minutes and then cultured at 25^{o}C for 2 hours. Cells were fixed in 2% methanol-free formaldehyde in PBS for 20 minutes and then probed with a mouse monoclonal antibody against HA (Boeringer Mannheim) overnight as a 1:25 dilution supplemented with normal donkey serum as the blocking reagent. Cells were washed 3 times in PBS before hybridized with donkey anti-mouse antibodies labeled with Cy3. Cells were again washed with PBS then mounted with Vectashield (Vector). A confocal microscope (BioRad) was used for observing and taking images of the stained cells.

Binding Assay for AP Fusion Proteins and S2 Cells Expressing Nrt-Wnts

The protocol for the binding assay was performed as previously published (34,35). CRD-AP fusion proteins produced from conditioned medium were first checked for their specific activities. All CRD-AP fusions have similar specific activities compared to alkaline phosphatase at the same assay condition (34).

S2 cells over-expressing Nrt-Wnts were heat shocked at 37^{o} for 45 minutes then cultured at 25^{o} for 2 hours. Different concentrations of CRD-AP fusions were incubated with S2 cells for 90 minutes at room temperature. Cells were washed with Hanks Balanced Salt Solution (HBSS) 3 times before lysed in 1% Triton with vortexing. The cell lysate was centrifuged at 1000g for 15 minutes to remove nuclei. The supernatant of the lysate was heated at 85^{o} for 15 minutes to inactivate background phosphatases

activities. The assay was then performed by measuring the OD_{405} after incubating the lysate with 1 M diethanolamine (pH 9.8), 0.5 mM $MgCl_2$, 10 mM L-homoarginine, 0.5 mg/ml BSA (Sigma), and 12 mM p-nitrophenyl phosphate (Sigma).

Affinities Measurement

The total AP activities added to each experiment and the bound AP activities on the S2 cells can be measured as OD_{405} changes over an hour. Saturable binding curves were plotted by fitting the bound AP activities and free AP activities in each experiment with the saturation binding curve, which is $Y = A \cdot X/(B+X)$. Scatchard analysis was done by plotting the ratios of the bound AP activity to the free AP activity against the concentrations of the bound AP in each experiments. All the data were analyzed in Excel and KaleidaGraph.

Cell Surface Binding between Arr-Ig and Nrt-Wg/S2 Cells

Arr-Ig conditioned medium from 293T cells were concentrated with Centriprep (Amicon). Concentrated Arr-Ig was then added to Nrt-Wg/S2 cells after the cells were heat-shocked at 37^oC for one-hour then cultured at 18^o for 2 hours. The Ig fusion protein was incubated with S2 cells for 2 hours at room temperature. S2 cells were then washed with HBSS, lysed, and assayed for the AP activity according to the protocol described in the previous section.

Generation of WgA and WgB Constructs

The WgA construct was generated by inserting two continuous stop codons at the AatII site of the Wg cDNA. DNA oligomers 5'-CGTTGATAAGCTTACGT-3' and 5'-AAGCTTATCAAGCACGT-3' were annealed and then ligated with the Wg cDNA construct cut with AatII. This construct generates the amino-terminal part of Wg, which contains the first amino acid to 359th amino acid in the coding region.

In order to generate the WgB construct, which makes a secreted version of the carboxyl-terminal part of the Wg protein, the Wg cDNA construct was cut with NarI and NdeI enzymes to remove the amino-terminal part of Wg. DNA oligomers containing HA

tag sequences flanking with NarI and NdeI sites (sense 5'-CGCCATGCATTACCCATATGATGTTCCAGATTACGCTTCCGC-3' , anti-sense 5'- TAGCGGAAGCGTAATCTGGAACATCATATGGGTAATGCATGG-3') were used in ligating with the Wg cDNA cut with NarI and NdeI. Therefore, the amino acid sequences at the junction site becomes (Wg sequences are bold and HA sequences are italic): **VKG**AMHYPYDVPDYASA**MPD**.

In order to express WgA and WgB in Schneider 2 (S2) cells , WgA and WgB cDNAs were cloned into the pMK33HS plasmid which contains the hygromycin marker for selecting cells expressing the constructs.

Tissue Culture, Transfection, Conditioned Medium Collection

S2 cells were cultured in Schineider's Drosophila medium (Gibco RRL) supplemented with 12.5% fetal bovine serum (Sigma), penicillin, and streptomycin. pMK33HS-WgA, pMK33HS-WgB, pMK33-Dfz2, pMKHS-Wg were introduced into S2 cells by calcium phosphate mediated transfection followed by selection with 125μg/ml hygromycin B (Sigma) until hygromycin-resistant cell lines were established.

Transgenes in the stable S2 cells were induced by a 45 minutes heat shock at 37^{o}C and then cultured for 2 hours before lysate preparation. For WgA or WgB conditioned medium collection, after heat shock, S2 cells were rested at 25^{o}C for 30 minutes before being transferred into serum free Schineider's Drosophila medium. S2 cells were then cultured in serum free medium for 4 hours. This serum free conditioned medium containing WgA or WgB was collected by first spinning down S2 cells and then centrifuging the soluble medium at 10,000g for 20 minutes.

Cell Lysate Preparation and Immunoblotting

Cells were first washed with phosphate buffered saline and then lysed on ice with lysis buffer (50mM Tris-HCl, 150 mM NaCl, 1% NP-40, pH 8) supplemented with protease inhibitors (0.5μg/ml leupeptin, 1μg/ml pepstatin A and 100 μg/ml PMSF). Protein concentration of the lysates were determined using the Bio-Rad Protein assay dye reagent.

Sample buffer for SDS-PAGE was added to cell the lysate or the conditioned medium before samples were resolved by SDS-PAGE. Proteins were then transferred to a nitrocellulose membrane, and blots were blocked in blocking buffer (3% nonfat dry milk, 1% BSA in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.2% Tween-20, pH 8)), and then incubated overnight at 4^{o}C in blocking buffer containing antibodies. The rabbit anti-Wg antibody (1:1000 dilution) and the mouse monoclonal antibody against HA (Boehringer Manheim) (1:1000 dilution) were used for immunoblotting. Proteins were detected using HRP-conjugated secondary antibodies (Bio-Rad) with the ECL western blot detection reagents (Amersham).

Results

While some progress has been made to produce soluble Wnt molecules (9,36), in particular Wg(37), it is problematic to purify significant amounts of Wnt proteins. This complication limits the possibility of doing conventional binding experiments in which saturating quantities of ligands are necessary. Our approach to measure binding between *Drosophila* Wnts and Frizzleds is therefore based on the "reverse binding" assay we developed, in which Wnts are presented on the cells surface in the form of type II transmembrane proteins, i.e. with the COOH-terminus outside the cells (32). The cells are then incubated with the ligand-binding domain of Frizzled (the CRD) tagged with alkaline phosphatase (10).

Generation of S2 Cells Expressing Neurotactin-Wnt Fusion Proteins

Neurotactin (Nrt) is a single transmembrane type II cell surface molecule (38,39). As we described previously (10), we utilized a clone in which Wg (without its own signal sequence) was fused to the carboxyl-terminal end of the Nrt cDNA to make a membrane-tethered form of Wg (32). Expression from this construct is capable of inducing *wg* target genes and rescuing *wg* mutant phenotypes *in vivo* (32). Other Nrt-Wnt fusions were generated by exchanging the coding regions of the Wnts with the Wg region in the Nrt-Wg construct. The DWnt3 protein, however, has an extension at the amino-terminus that is cleaved to generate the mature and secreted protein (40). To eliminate possible cleavage of the fusion protein, we used only the region where DWnt3 is homologous to other Wnts.

The Nrt-Wnt fusion constructs were tagged with HA sequences to confirm the correct expression of the fusion proteins in S2 cells. All Nrt-Wnt fusion proteins were detected at the predicted sizes (data not shown). We also stained the S2 cells with the anti-HA antibody, detecting cell surface staining for each of the fusion proteins indicating that they are correctly presented on the cell surface (Figure 1). We successfully generated

these fusions for five of the seven *Drosophila* Wnts, but we were unable to synthesize complete cDNAs for DWnt6 and DWnt10 (41), which therefore remain to be examined. Because these genes as presented in the Drosophila genome (7) lack good signal sequences, we suspect that the amino-terminal sequences are incorrect.

Generation of Frizzled CRD-AP Fusion Proteins

To measure binding between the cell surface bound Wnt molecules and the Frizzled CRDs, we had to tag the CRD in such a way that its concentration could be established. We used the alkaline phosphatase (AP) protein to do so (34,35). All of the CRD-AP fusion proteins were secreted into the medium as shown by immunoprecipitation experiments (Figure 2).

By incubating the CRD-AP fusion proteins with a substrate for alkaline phosphatase, specific activities of these fusion proteins were obtained (Table 1). Considering the variations in sizes of different CRDs, the specific activities of the various CRD-AP fusion proteins are not significantly different from non-fused AP on a molar basis.

Binding Affinities between Wnt and Frizzled Molecules in *Drosophila*

We previously presented the binding affinities of FzCRD-AP and Dfz2CRD-AP to Nrt-Wg (10). By using the same technique, we measured binding affinities of other Wnts and Frizzleds in *Drosophila* (Figure 3. and table 2). Wg binds to Fz, DFz2, and DFz3 with the highest affinity for Dfz2 (10). DWnt2 binds to the same set of three Frizzleds with approximately the same affinity, while DWnt3 does not bind any of the Frizzleds. DWnt8 binds to DFz4 only. Figure 4 summarizes the affinities (1/Kd) between different Wnts and Frizzleds.

Separate domains of Wg can be secreted but do not bind to Frizzled or evoke a response.

There are several natural *wg* alleles in which the Wg protein is truncated. Most of these mutant proteins are misfolded and retained in the endoplasmic reticulum. (42-45). However, some of these mutations lead to a shortened protein that is secreted.

Interestingly, those mutations truncate Wg close to a region that is uniquely present in Wg, an 85 amino acid insert that is dispensable for Wg function and evolutionary not conserved (44). This region also contains strong antigenic determinants and has served as an epitope for several anti-Wg antibodies (43,46). The fact that the Wg protein contains a large insert and can be truncated at the site of the insert to generate a folded and secreted variant may be taken as evidence that there are separately folding domains on Wg, perhaps even functionally different. Hays and Bejsovec have reported that such a truncated version of Wg, when overexpressed, can partially rescue *wg* mutant embryos, indicating that this domain has a function by itself (43,44).

We were therefore interested in generating different domains of the Wg protein *in vitro*, to test whether any of them would be able to bind to the Frizzled CRD and would be functional. Thus, the insert region was used as the dividing point for making WgA (the amino-terminal part of Wg) and WgB (carboxyl-terminal part of Wg) constructs (Figure 5A). The WgB construct included the signal sequence of Wg to allow secretion. An HA sequence was added between the signal sequence and the first cysteine for detection of expression.

When we co-transfected plasmids independently generating the WgA and the WgB domains, together with the Dfz2 protein in S2 cells, the cells accumulated the Armadillo protein, just like when the full length Wg was co-transfected with he Dfz2 receptor (Figure 5B). This indicated that the WgA and WgB expression plasmids were functional. When those same plasmids were transfected separately (WgA or WgB), with Dfz2, no response was seen. We did observe that both the WgA and the WgB domain were secreted into the medium (data not shown). However, the secreted forms were not active in eliciting a response in Dfz2-expressing S2 cells or in clone-8 cells, either by themselves or when mixed together (data not shown). Similarly, we were unable to detect binding of either Wg domain to Dfz2 in assays in which full length Wg did bind (data not shown).

Thus, our data support the view that these two portions of the Wg protein can fold separately, but we did not obtain evidence for separate functions, since both the WgA and the WgB domain are required to generate a response when transfected. The lack of a

response when these two domains were jointly added to cells in a soluble form might be due concentration effects - the local concentrations of WgA and WgB in the ER of transfected cells is probably higher than in the medium.

No detectable binding Between Wg And Arr.

Genetic evidence has implicated *arrow* (*LRP5* and *LRP6* in vertebrate species) as being required for Wnt signaling in *Drosophila* and in mice (26,27). Moreover, it has been reported that the LRP6 extracellular domain, as a fusion with the constant region of immunoglobulin, is able to bind to mammalian secreted Wnt1 and form a ternary complex with a Frizzled CRD (28). This has led to a model in which Arrow/LRP acts as a co-receptor for Wnt (28,31). However, other workers failed to see direct interactions between Arrow/LRP and secreted vertebrate Wnts (29). In vertebrates, such differences between experimental results might be explained by the use of different family members and not having the right cognate molecules. In *Drosophila* however, genetic evidence has strongly implicated *wg* and *arrow* to be a matching pair and we therefore set out to detect binding between those molecules.

We generated an Arr-Ig construct similar to the LRP6-Ig fusion used by Tamai, et al to obtain the fusion protein from the medium of transiently transfected 293T cells (Figure 6C). To determine whether Arr-Ig can bind to cell surface-bound Wg, the fusion protein was added to Nrt-Wg/S2 cells, followed by a conjugated mouse anti-human Ig antibody. In comparison to the binding of Dfz2CRD-AP to Nrt-Wg/S2 cells, we did not detect any binding of Arr-Ig (Figure 6A).

To test whether soluble Wg could bind to soluble Arrow, we mixed S2 conditioned medium containing Wg with medium containing the secreted Arr-Ig protein. Protein G Sepharose was added to immunoprecipitate the Arr-Ig fusion. As shown in figure 6B, Wg was not detected in this immunocomplex, while Wg could be pulled down by the Dfz2CRD-AP, as expected. Finally, to test whether a ternary complex could be formed, we mixed Arrow-Ig, Dfz2CRD-AP and Wg. However, a ternary complex could not be

detected (Figure 6B).

Discussion

Implications of the interactions between different Wnts and Frizzleds.

The quantitative measurements of the binding between Wnts and Frizzleds are generally in good agreement with available genetic data in the fly. For example, we have not found any of the five *Drosophila* Wnt molecules tested here to bind to Smo. Smo is a distant member of the Frizzled family and is implicated in Hedgehog rather than in Wnt signaling. In the Hedgehog pathway, Smo is regulated not by an extracellular ligand, but by an inhibitory interaction with the multi-pass membrane protein Patched, which serves as the Hh binding receptor (47,48). When Patched is inactive, Smo generates a signal constitutively. (49-52).

With respect to Wg, the best understood member of the Wnt gene family, we found that it binds to three of the five Frizzleds: Fz, Dfz2 and Dfz3. This suggests that these receptors may act redundantly and indeed, *wg* like phenotypes in *Drosophila* embryos and other tissues are only seen when *fz* and *Dfz*2 are removed (19-21). *DFz3* mutants are viable with no obvious phenotypes (22). While this suggests that *Dfz*3 is not genetically required for Wg signaling, it should be noted that *Dfz3* expression is dependent on active Wg signaling (23). Hence, in flies mutant for *fz* and *Dfz2*, where Wg signaling is blocked, *Dfz*3 is not expressed and the phenotype of the first two receptors may be caused by functional absence of all three of them.

In addition to *wg*, there are mutants in two other *Drosophila* Wnt genes: *DWnt*2 and *DWnt*4. *DWnt2* is required for pigment cell and direct flight muscle formation in the *Drosophila* testes and wings, respectively (53,54). According to our data, DWnt2 binds to Fz, DFz2 and DFz3. The double mutant *fz, Dfz2* dies in embryogenesis, making it impossible to examine a possible later phenotype in the testis, but it is likely that the multiple receptors are redundant in DWnt2 signaling, just as they are for Wg. With respect to DWnt4, we find that it binds to Fz, Dfz2 and Dfz4. This agrees with the phenotypes of *DWnt*4 and *Dfz*2 in the ovary: a cell migration defect seen for both the ligand and the receptor (55).

There are two Wnts tested here for which there are no mutants available, *DWnt*3/5 (40,56) and *DWnt*8. Surprisingly, DWnt3 did not bind to any of the Frizzleds. It is possible that the fusion protein we used, although expressed on the cell surface, was not functional. Because of the proteolytic processing of the amino-terminal part of the DWnt3 protein, we fused the "Wnt homology region" of DWnt3 to Nrt, but there are no assays to demonstrate that Nrt-DWnt3 encodes a functional protein.

DWnt8 is unique in the sense that it binds to only one Frizzled, DFz4. Both genes are expressed in the embryonic CNS ((41), C.-H Wu and Y. K. Xu, unpublished results) and the fact that they interact biochemically suggests that they are likely to share a function, to be explored when mutants are available.

Is There a Wnt Ligand for Frizzled in the Planar Polarity Pathway?

frizzled was originally found as a gene involved in planar polarity pathway, but it has yet to be shown that it interacts with a Wnt during that process. Our binding data indicate that a number of different Wnts, including Wg, DWnt2 and DWnt4, can bind to Frizzled, with approximately equal affinity. All of these Wnt genes are expressed in tissues during the time when planar polarity is established ((57) C. Logan unpublished results). Whether they are functional as ligands for Fz remains to be seen, but as with other Wnt-Frizzled interactions, it seems likely that genetic redundancy will be an important consideration.

Do Wnts Interact with Arrow/LRP?

In contrast to the claim that soluble forms of LRP can bind to Wnt1 and form a ternary complex with a Frizzled CRD, we were unable to detect any direct interactions between the *Drosophila* LRP homolog Arr and Wg. This discrepancy might be explained by technical differences between our experiments and the ones reported by Tamai et al (28). However, we note that Mao et al (29) also failed to see any direct binding between LRP6 and Wnt, in experiments in which robust direct binding between Dickkopf and LRP was detected. At present, we cannot rule out that LRP does act as a co-receptor for Wnt as a direct binding partner, but our experiments do suggest that other mechanisms should also be considered.

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Figure legends

Figure 1.

Nrt-Wnt Fusion Constructs Are Localized on the Cell Surface.

S2 cells stably expressing different Nrt-Wnt constructs (as labeled on each panel) tagged with the HA sequences were stained with an anti-HA antibody. No detergent was applied during the antibody staining to keep the plasma membrane intact. Compared with untransfected S2 cells, HA antibody generates cell surface staining in all Nrt-Wnt transfected cells.

Figure 2.

CRD-AP Fusion Proteins Containing AP Activities Are Secreted into the Conditioned Medium of 293T cells.

Conditioned medium from 293T cells transiently transfected with the CRD-AP fusion constructs was collected and concentrated for immunoprecipitation with the anti-AP antibody. Immunocomplexes from mock transfected (lane1), AP (lane 2), FzCRD-AP (lane 3), Dfz2CRD-AP (lane 4), Dfz3CRD-AP (lane 5), SmoCRD-AP (lane 6) and Dfz4CRD-AP (lane 7) were resolved by SDS-PAGE and the gel was stained with Coomassie blue.

Figure 3.

FzCRD-AP Binds to Nrt-DWnt2/S2 Cells

Nrt-DWnt2/S2 cell were incubated with different concentrations of FzCRD-AP conditioned medium generated from 293T cells. The cells were lysed and the bound AP activities were measure colorimetrically at OD405 after incubating with the AP substrate. A saturable binding curve is observed when bound AP activities was plotted against the total AP activities added into each experiment (panel A). The data was transformed into the Scatchard plot as shown in panel B. The Kd between FzCRD and DWnt2 is 6.918×10^{-8} M.

Figure 4.

Diagram displaying the relative strength of the binding affinities (1/Kd) between Wnts/Frizzleds tested in the assay. The scale is arbitrary.

Figure 5. WgA and WgB Domains Complement Each Other's Activities.
A. Diagrams of WgA (containing the amino-terminal region of Wg) and WgB (containing the carboxyl-terminal region of Wg) and the insert region of Wg.
B. Combinations of DFz2, WgA, WgB or Wg were transfected into S2 cells. S2 cells stably expressed DFz2 (lane1), WgA (lane2), WgB (lane3), WgA and DFz2 (lane4), WgB and DFz2 (lane5), WgA, WgB and DFz2 (lane6), Wg and DFz2 (lane7),were lysed for a Western blot probed with an anti-Armadillo antibody. As shown in lanes 6 and 7, Armadillo proteins are stabilized in WgA WgB DFz2 triple transfected cells and Wg DFz2 double transfected S2 cells.

Figure 6. Dfz2CRD But Not Extracellular Domain of Arr Binds Wg.
A. S2 cells and Nrt-Wg/S2 cells were heat-shocked and then incubated with concentrated mediums containing Alkaline Phosphatase (negative control), Ig, Arr-Ig or Dfz2CRD-AP proteins. Cells incubated with Ig or Arr-Ig were then washed, incubated with the alkaline phosphatase conjugated mouse anti-human Ig antibody. All cells were then lysed and the bound alkaline phosphatase activity was assayed after incubated with the AP substrate. Dfz2CRD-AP is shown to bind to S2 cells expressing Nrt-Wg. However, Arr-Ig does not bind detectable to Nrt-Wg.
B. Conditioned medium containing Ig, Arr-Ig, Wg, Alkaline Phosphatase, or Dfz2CRD-AP were used in this immunoprecipitation-Western experiment. Protein G sepharose was incubated with Ig and S2 conditioned medium (lane 1), Ig and Wg conditioned medium (lane 2), Arr-Ig and S2 conditioned medium (lane 3), Arr-Ig and Wg conditioned medium (lane 4). Protein A sepharose conjugated with the anti-AP antibody was incubated with conditioned medium containing Alkaline Phosphatase (lane 5) or Dfz2CRD-AP (lane 6). The immunocomplexes were then mixed with Wg conditioned medium (lane 5 and lane 6). Protein G sepharose was incubated with Ig, Dfz2CRD-AP

and Wg (lane 7) or Arr-Ig, Dfz2CRD-AP and Wg (lane 8). All the immunocomplexes were separated by SDS-PAGE before transferred to a Western blot probed with an anti-Wingless antibody. Wg is shown to bind to Dfz2CRD-AP (lane 6) but not to Arr (lane 4). After mixing Dfz2CRD-AP, Arr-Ig and Wg, there are no detectable trimeric complexes (lane 8).

> The band co-migrating with Wg in lane 5 is a background band caused by reaction with the Ig heavy Chain from the anti AP antibody (The Wg protein and the Ig heavy chain are both around 50 kD)

C. The blot in above was probed with the anti-Human Ig antibody. Ig and Arr-Ig are seen at the predicted size.

Tables

Table 1

Specific Activities of CRD-AP Fusion Proteins.

Specific activities of fusion proteins and the non-fused alkaline phosphatase are listed here. Activities of CRD-AP fusion proteins generated from 293T cells were assayed colorimetrically after incubated with the AP substrate. Concentration of the fusion proteins were determined by SDS-PAGE as shown in figure 2. Considering the differences in the size of CRDs, the specific activities of the CRD-AP fusion proteins are indistinguishable from the AP on a molar basis.

Fusion Proteins	Alkaline Phosphatase Activity (Units/mg)
AP	1480
FzCRD-AP	1030
Dfz2CRD-AP	780
Dfz3CRD-AP	800
Dfz4CRD-AP	920
SmoCRD-AP	612

Table 2.

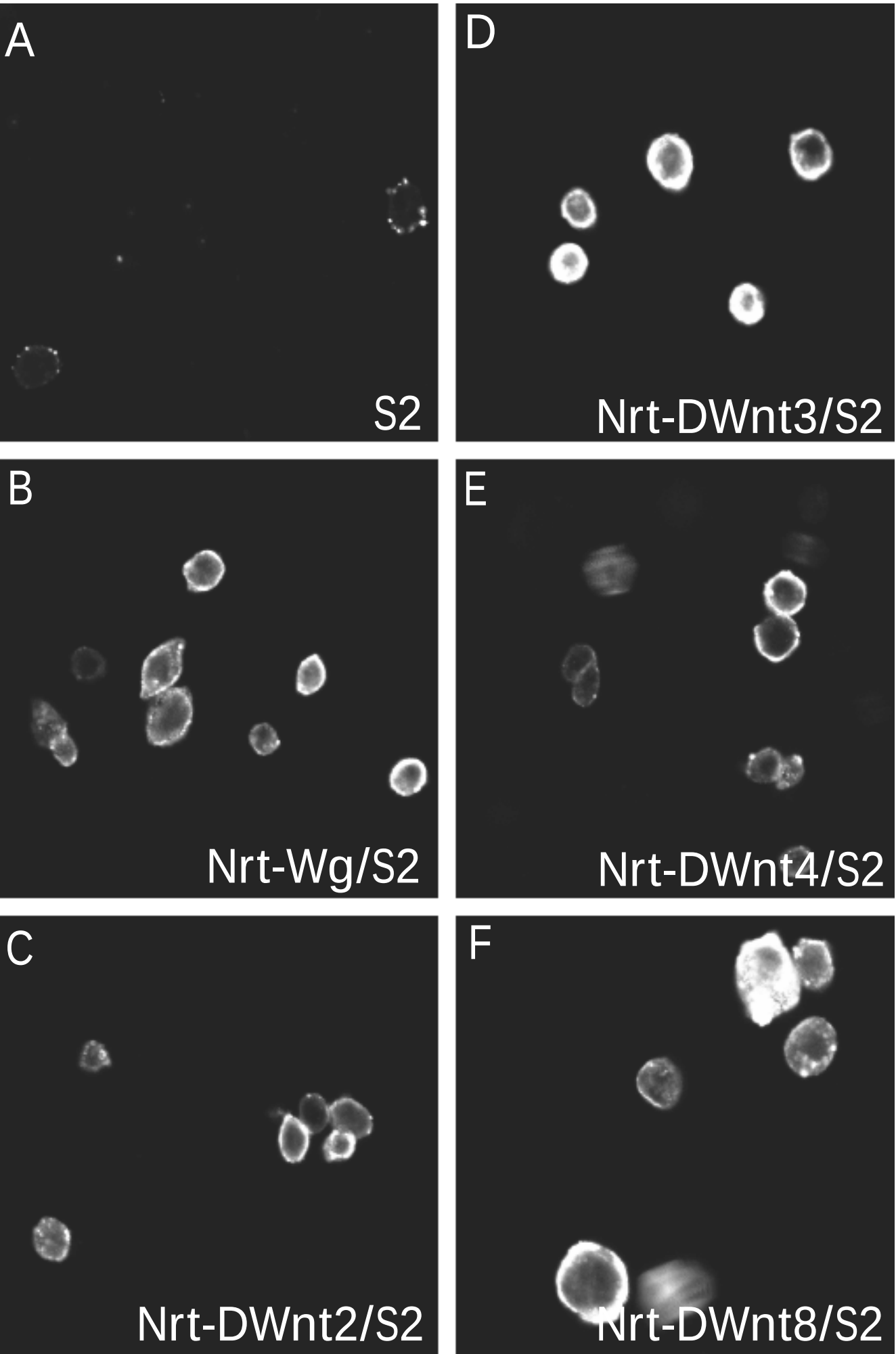
Binding Affinities between Drosophila Wnts and CRDs of Drosophila Frizzleds.
CRD-AP fusion proteins were used for testing their bindings to S2 cells over-expressing Nrt-Wnts.. Affinities of the binding were measured as shown before for DFz2 vs. Wg and Fz vs. Wg (10). Binding affinities for other combinations measured in the binding assay is summarized in this table.

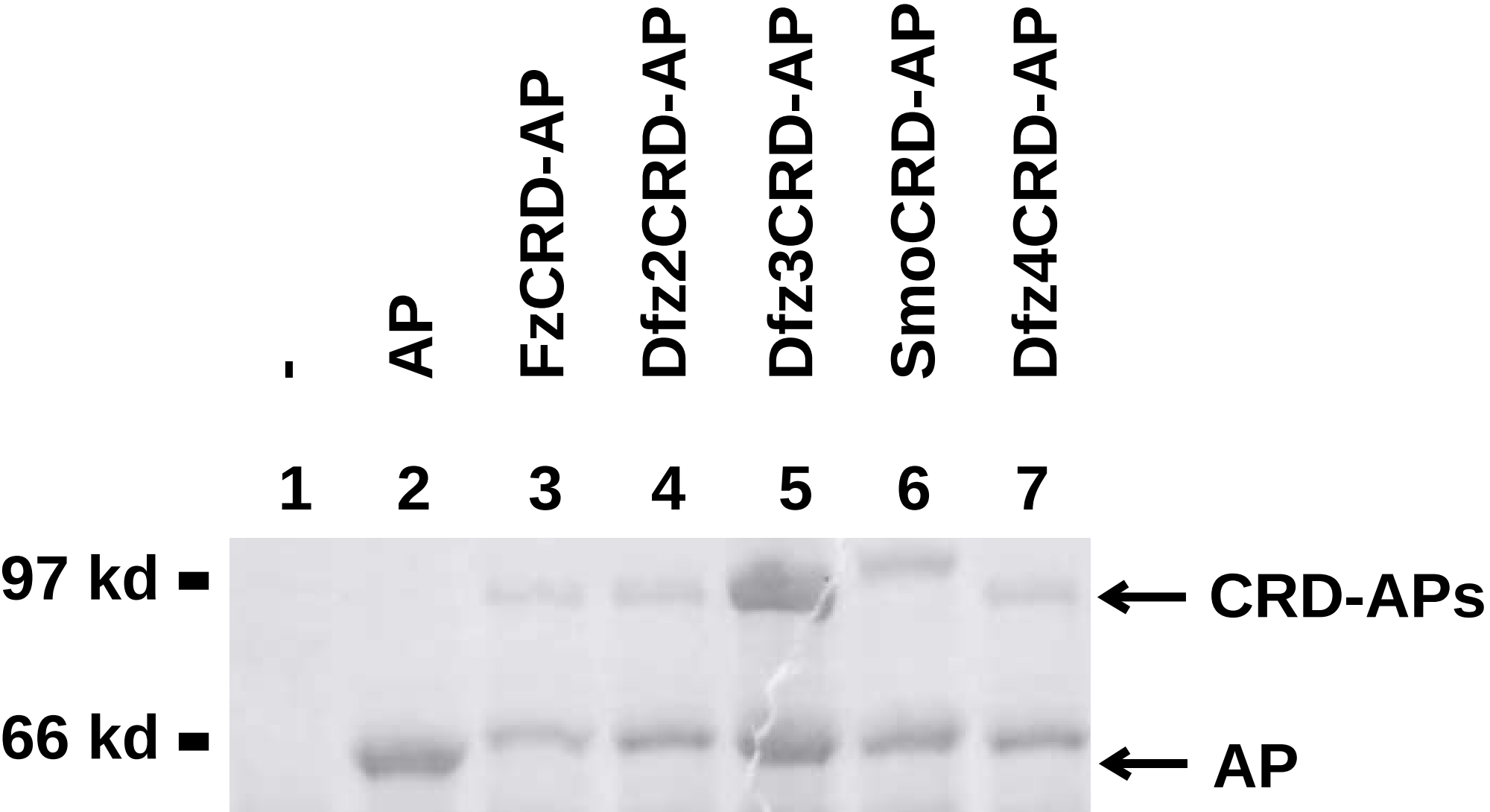
	Wg	DWnt2	DWnt3	DWnt4	DWnt8
Fz	$4.51_{\pm 0.25} \times 10^{-8}$ M	$6.91_{\pm 0.36} \times 10^{-8}$ M	NB	$1.63_{\pm 0.30} \times 10^{-8}$ M	NB
DFz2	$5.44_{\pm 0.26} \times 10^{-9}$ M	$6.79_{\pm 0.31} \times 10^{-8}$ M	NB	$1.59_{\pm 0.26} \times 10^{-8}$ M	NB
DFz3	$5.25_{\pm 0.19} \times 10^{-8}$ M	$5.85_{\pm 0.29} \times 10^{-8}$ M	NB	NB	NB

DFz4	NB	NB	NB	$5.78_{\pm 0.42} \times 10^{-8}$ M	$5.12_{\pm 0.45} \times 10^{-8}$ M
Smo	NB	NB	NB	NB	NB

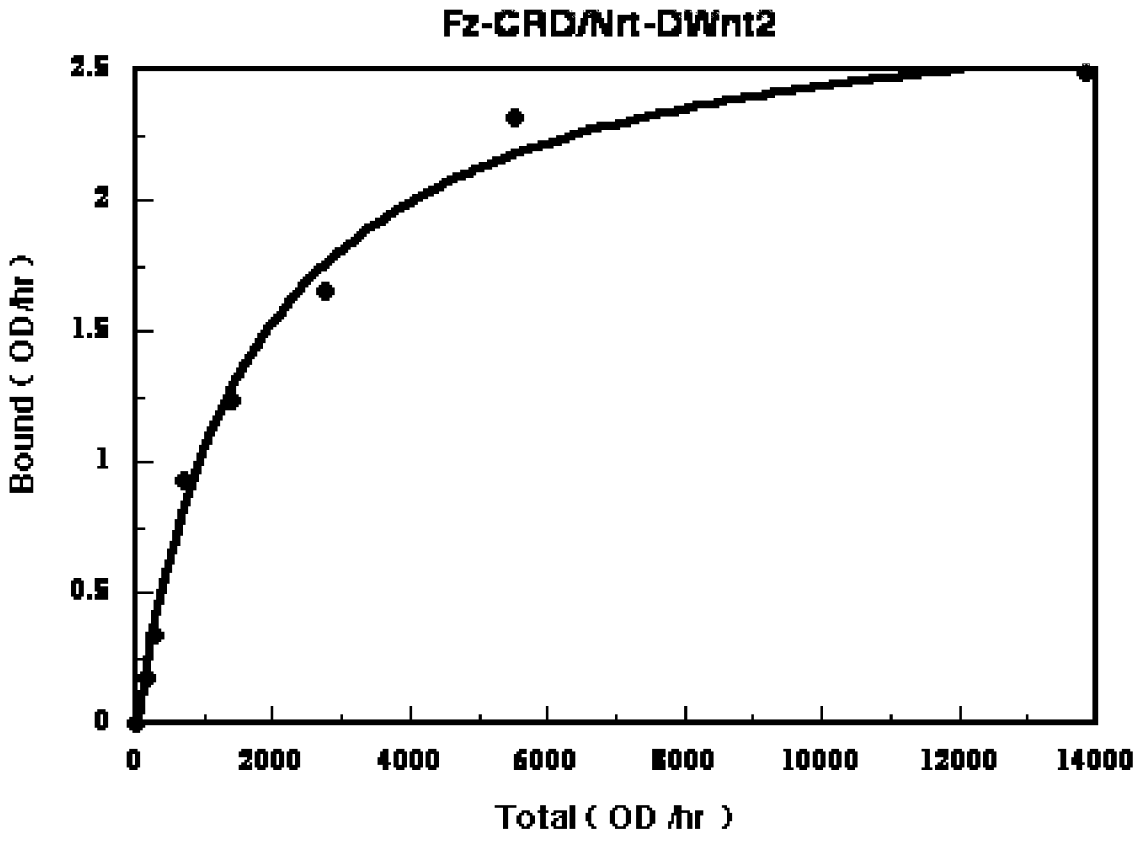
NB: no detectable binding.

Experiments were performed at least twice.


A
S2
D
Nrt-DWnt3/S2
B
Nrt-Wg/S2
E
Nrt-DWnt4/S2
C
Nrt-DWnt2/S2
F
Nrt-DWnt8/S2


-
AP
FzCRD-AP
Dfz2CRD-AP
Dfz3CRD-AP
SmoCRD-AP
Dfz4CRD-AP
1
2
3
4
5
6
7
97 kd
66 kd
CRD-APs
AP

A


Fz-CRD/Nrt-DWnt2
Bound (OD/hr)
Total (OD /hr)
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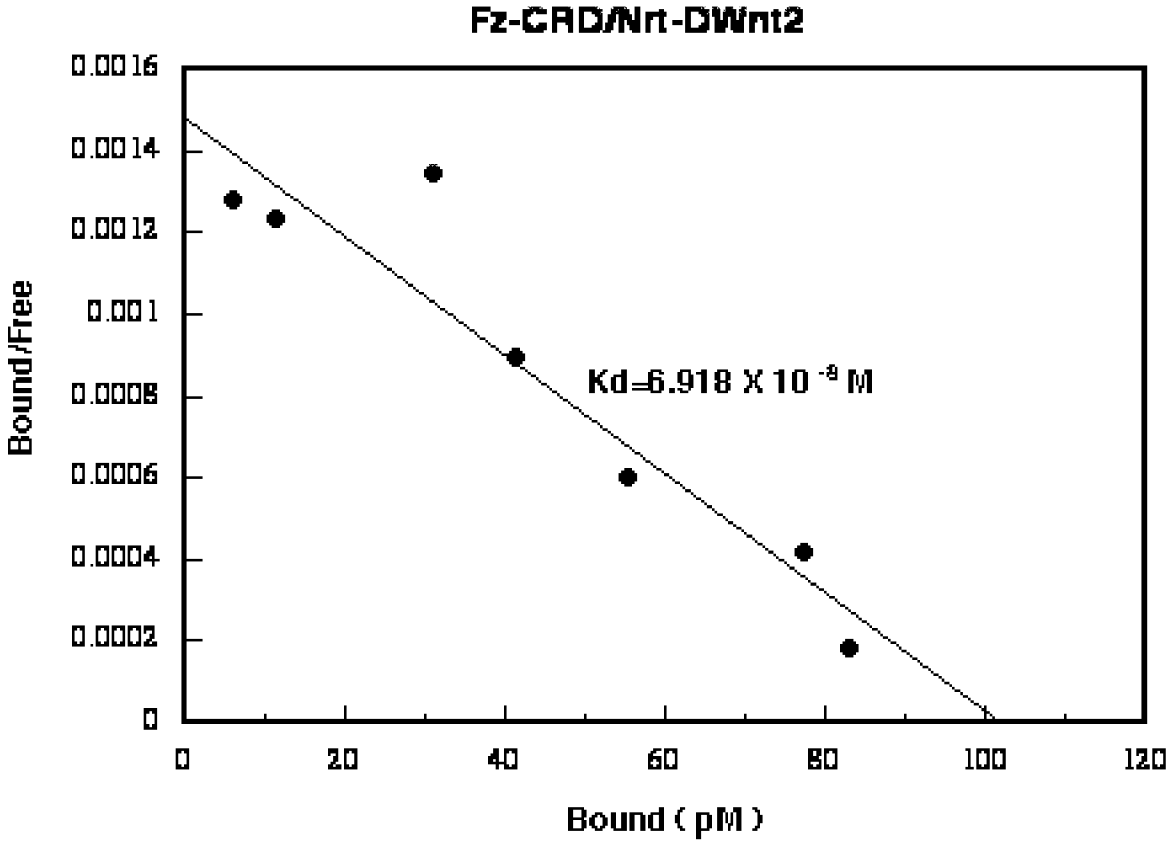
0.5
1
1.5
2
2.5
0
2000
4000
6000
8000
10000
12000
14000

B


Fz-CRD/Nrt-DWnt2
Bound/Free
Bound (pM)
Kd=6.918 X 10 -9 M
0
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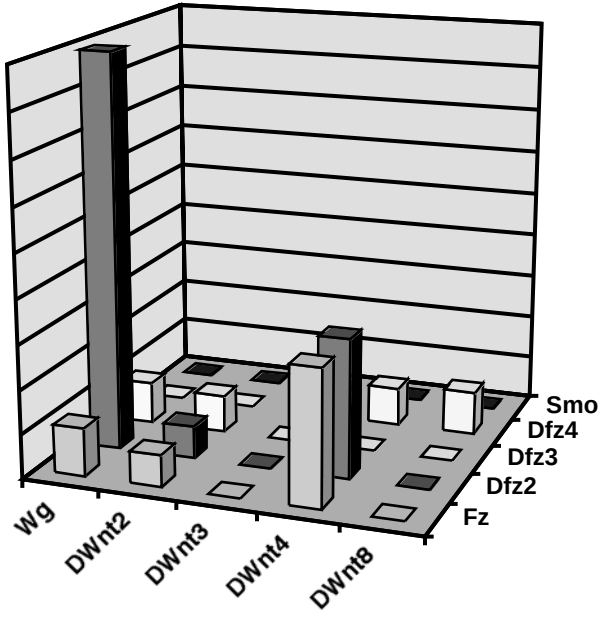
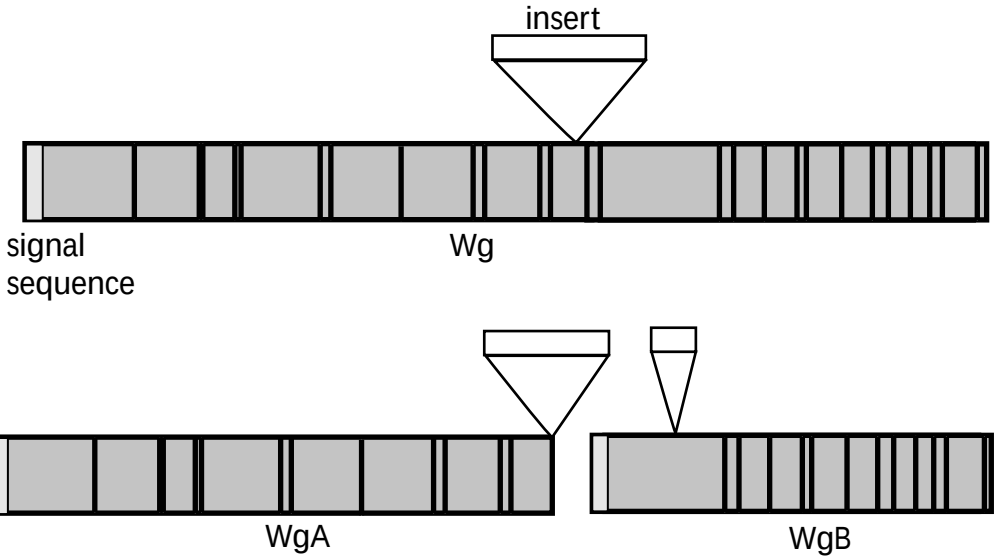
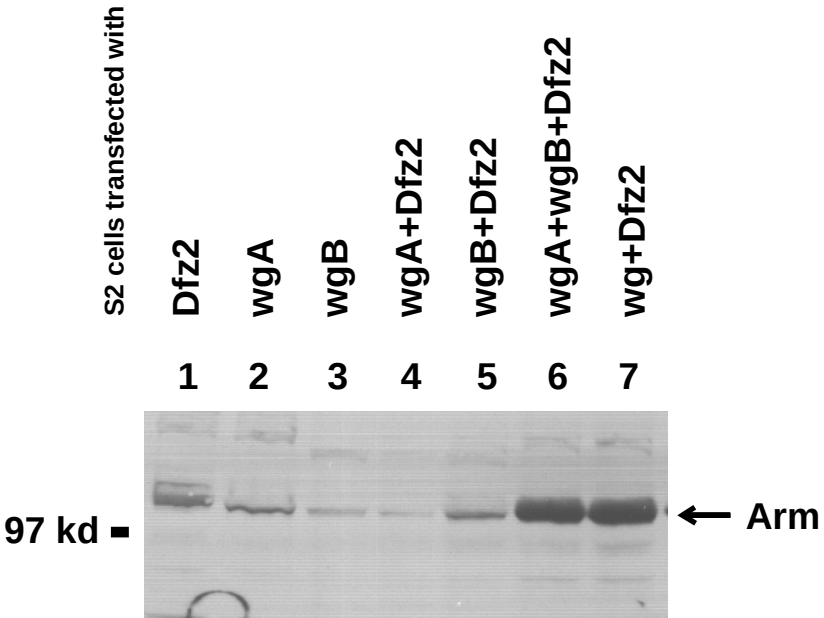
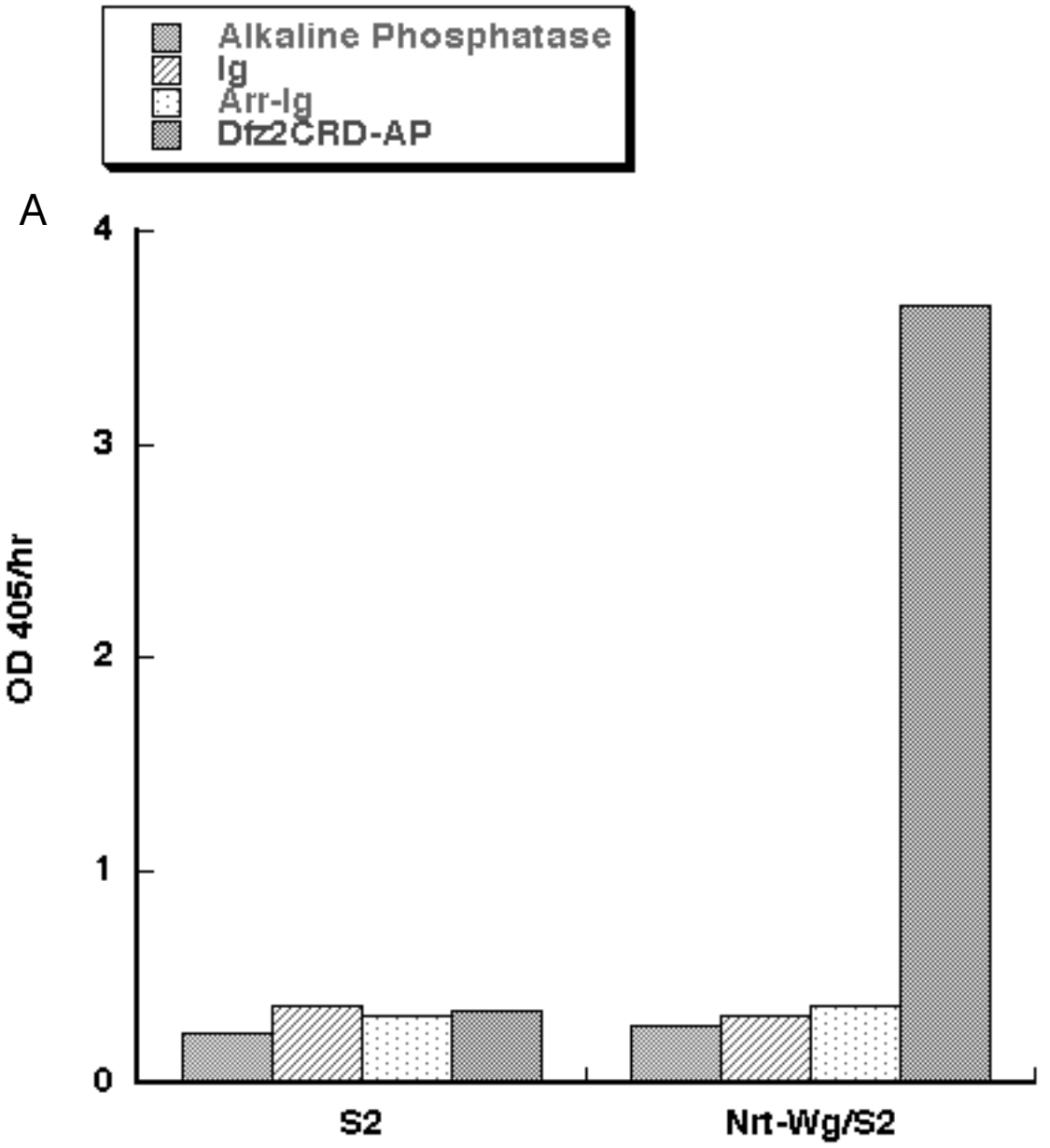
0.0004
0.0006
0.0008
0.001
0.0012
0.0014
0.0016
0
20
40
60
80
100
120

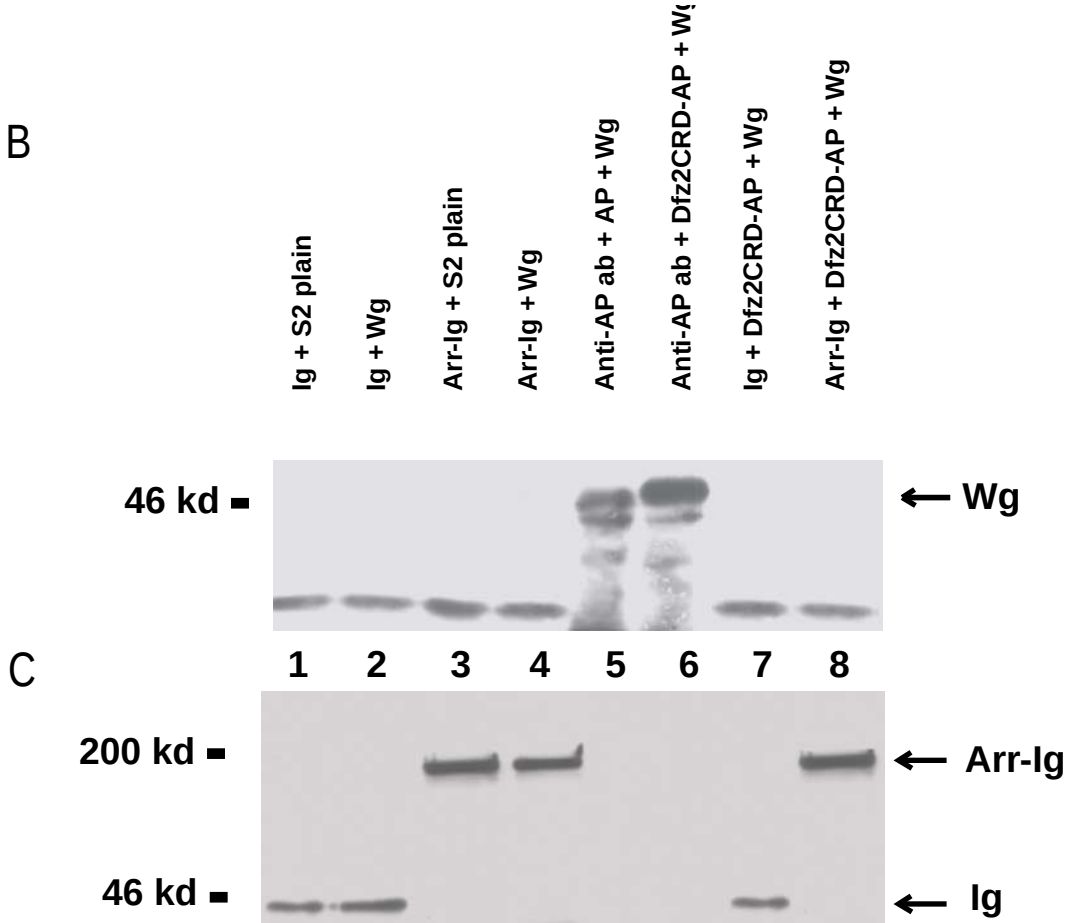

Wg
DWnt2
DWnt3
DWnt4
DWnt8
Fz
Dfz2
Dfz3
Dfz4
Smo


insert
signal
sequence
Wg
WgA
WgB

B


S2 cells transfected with
Dfz2
wgA
wgB
wgA+Dfz2
wgB+Dfz2
wgA+wgB+Dfz2
wg+Dfz2
1
2
3
4
5
6
7
97 kd
Arm


Alkaline Phosphatase
Ig
Arr-Ig
Dfz2CRD-AP
A
OD 405/hr
4
3
2
1
0
S2
Nrt-Wg/S2


B
Ig + S2 plain
Ig + Wg
Arr-Ig + S2 plain
Arr-Ig + Wg
Anti-AP ab + AP + Wg
Anti-AP ab + Dfz2CRD-AP + W
Ig + Dfz2CRD-AP + Wg
Arr-Ig + Dfz2CRD-AP + Wg
46 kd
Wg
C
1 2 3 4 5 6 7 8
200 kd
Arr-Ig
46 kd
Ig