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wnt genes encode a large family of secreted signaling molecules essential for development and oncogenesis. wnt-1, the founding member of the wnt gene family, was initially identified as an oncogene which, upon ectopic expression induced by viral insertion, causes mammary tumorigenesis in mice, providing a potential model for studying human breast cancer. However, the Wnt-1 receptor, an essential component mediating Wnt-1 function, has not been identified, and the molecular and biochemical nature of the Wnt signaling pathway is not fully understood. In this proposal for the Career Development Award, we propose experiments combining molecular techniques and the axis duplication assay in the <i>Xenopus</i> embryo to answer the following two critical questions: 1) What is the receptor mediating Wnt-1 oncogenic function? 2) How does the Dishevelled protein, which is an essential Wnt signaling component, transduce Wnt-1 signal? These experiments should provide a better understanding of the molecular nature of Wnt-1 signaling in mammary malignancy.					
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FOREWORD

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Introduction

Wnt genes encode secreted signaling molecules that play important roles in mammary tumorigenesis and embryonic development (Cadigan and Nusse, 1997; Moon et al., 1997). Wnt-1, the founding member of the Wnt gene family, was identified as an oncogene that upon ectopic expression resulted in mammary tumors in mice (Nusse, 1991). Delineation of the mechanisms of Wnt-1 signaling can provide insights into the molecular nature of mammary tumor formation. Despite the recent significant progress in understanding the Wnt signaling pathway, several outstanding issues remain. In this Career Development Award, I proposed two specific aims to begin to address two critical issues:

- 1) What is the receptor mediating Wnt-1 oncogenic function in mammary carcinogenesis?
- 2) How does the Dishevelled protein, which is an essential Wnt signaling component, transduce Wnt-1 signal?

Recent studies suggest that the frizzled (fz) family of proteins may encode the receptors for the Wnt proteins. It was demonstrated that the *Drosophila* Wnt-1 homologue, wingless (wg), was able to bind to the surface of cells transfected with the fz related protein, dFz2 (Bhanot et al., 1996). Recent genetic evidence further suggests that both Fz and dFz2 behave as functionally redundant receptors for Wg (Bhat, 1998; Kennerdell and Carthew, 1998). Our previous studies in *Xenopus* embryos have identified a mammalian fz protein, hFz5, as a receptor for the Wnt-5A ligand (He et al., 1997). Based on these findings it is therefore likely that a member (or members) of the fz family encodes for the receptor (or receptors) for the Wnt-1 molecule.

Direct demonstration of Wnt-Fz binding has been hampered by the difficulty of obtaining soluble Wnt proteins, including the soluble Wnt-1 protein. In order to bypass this

problem, I proposed to identify the Wnt-1 receptor or receptors using the *Xenopus* axis duplication assay (specific aim 1). I also proposed to isolate Dishevelled-interacting gene products from the yeast two-hybrid system, and to test the potential function of these cDNAs in Wnt signaling in the *Xenopus* axis duplication assay (specific aim 2).

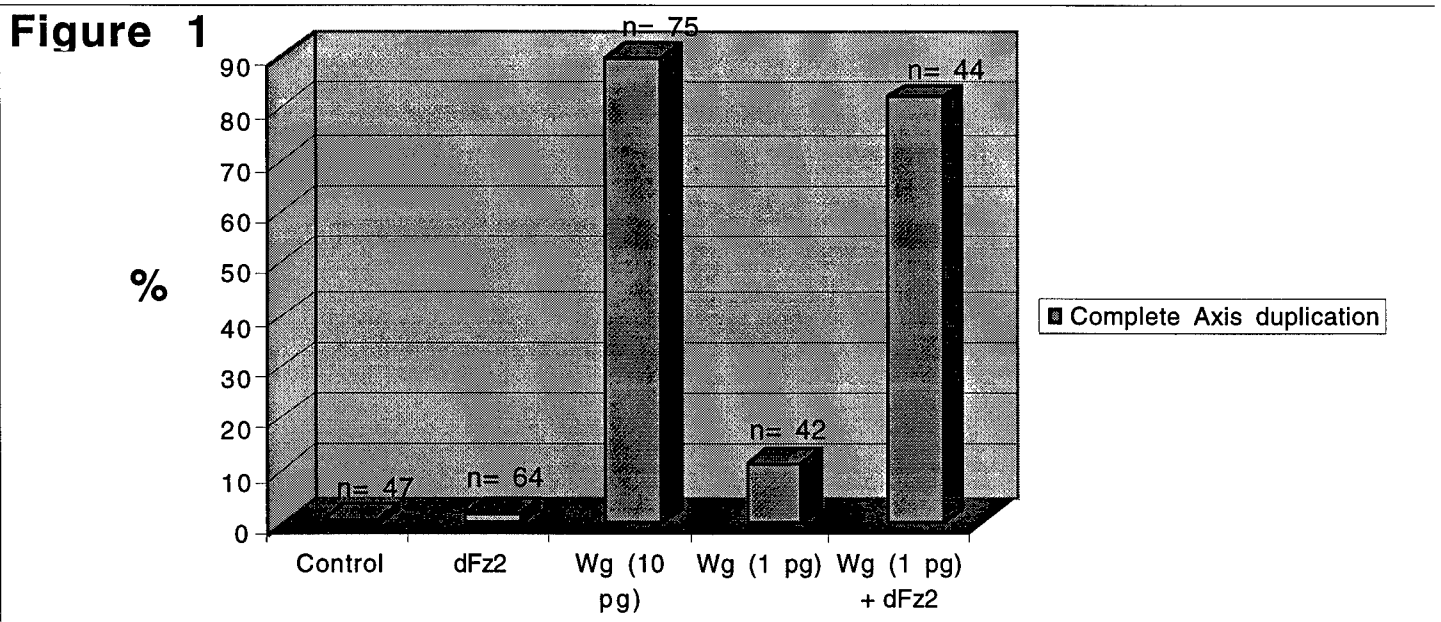
BODY

Aim 1: Identification of candidate Wnt-1 receptor(s)

The *Xenopus* axis duplication assay has proven to be a useful biological assay for Wnt-Fz interaction as demonstrated by the elucidation of a fz family member, hFz5, as the receptor for Wnt-5A (He et al., 1997). This assay involves injection of synthetic Wnt RNA, thereby bypassing the requirement for soluble Wnt proteins. In this assay, injection of Wnt-5A alone does not induce axis duplication but co-injection of Wnt-5A plus hFz5, but not any other fz proteins, induces axis duplication. These results led to the suggestion that hFz5 can function as a receptor for Wnt-5A. Can we use this approach to identify the receptor(s) for Wnt-1?

There needs one important modification of this approach. Unlike Wnt-5A, Wnt-1 has been shown to directly induce secondary axis duplication without any co-injected fz molecules (McMahon and Moon, 1989). This is presumably mediated by an endogenous fz protein(s) already present in the embryo. A modification of the procedure was proposed that involves reducing the quantity of injected Wnt-1 mRNA to suboptimal concentrations where minimal or no secondary axis formation is observed. Such a modification has been tested using the *Drosophila* homologue of Wnt-1, wg, which like Wnt-1 can induce

secondary axis duplication (Chakrabarti et al., 1992). In these experiments, the amount of injected wg RNA was reduced to suboptimal levels so that wg could only inefficiently induce secondary axis duplication. Coinjection of wg with the RNA for dFz2, a known receptor for wg, was shown to significantly enhance axis duplication by the suboptimal concentrations of wg RNA, whereas the same amount of dFz2 RNA alone did not induce secondary axis duplication (Figure 1). This result may be explained by the fact that the ligand-receptor complex formation between the reduced concentration of Wg protein and an endogenous fz protein(s) is suboptimal; Increasing the protein concentration of dFz2 from injected RNA permits effective Wg-dFz2 complex formation and leads to enhanced signaling. By the same principle, I proposed that this modified approach may be applicable to Wnt-1 and its receptor(s).

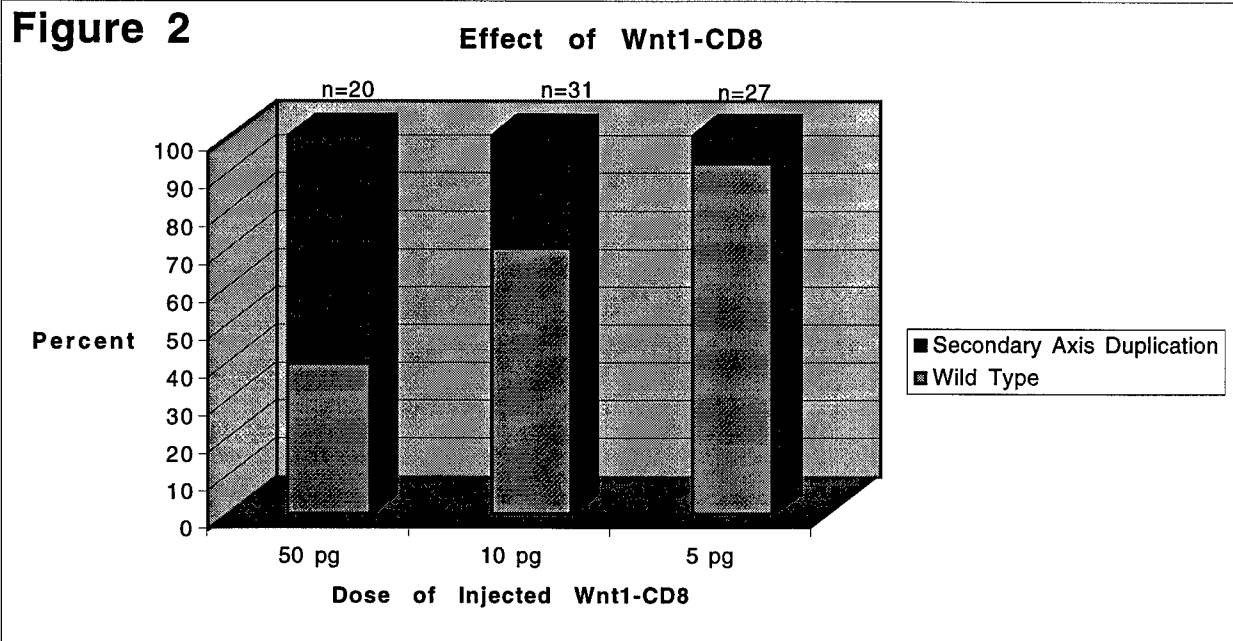


In order to synthesize fz mRNAs in vitro for injection experiments, all known mammalian fz cDNAs, including rfz1, rfz2, mfz3, mfz4, hfz5, mfz6, mfz7, mfz8 and mFZD3, were subcloned into either pRK5 or pCS2+, both of which are suitable for in vitro RNA transcription by SP6 RNA polymerase. Because Wnt-1 induces axis duplication

efficiently with even pico-gram quantity of RNA (data not shown), rendering the adjustment of suboptimal quantity difficult, we used a modified Wnt-1 construct, Wnt-1CD8, which contains the Wnt-1 coding sequence fused in frame with the transmembrane protein CD8 (Parkins et al., 1993). The rationale for the use of this construct was twofold. Firstly it has been demonstrated that Wnt-1CD8 can induce secondary axis duplication but it requires a much higher quantity of injected RNA to achieve this outcome. Almost a twenty-five fold higher concentration of the Wnt1-CD8 RNA was required to achieve the same effect observed with Wnt-1 RNA (Parkins et al., 1993). This lowered activity of the Wnt1-CD8 fusion can therefore allow for a better discrimination of the range of RNA that would provide a suboptimal concentration. Secondly, because the Wnt1-CD8 fusion protein will be tethered to the plasma membrane of cells expressing this construct, this may allow for a more efficient co-localization and interaction of Wnt molecule with its putative injected frizzled molecule (either in autocrine or paracrine fashion). Therefore, we subcloned Wnt-1CD8 into pCS2+ for in vitro mRNA transcription.

To delineate the range of Wnt1-CD8 mRNA for a suboptimal concentration for secondary axis duplication, injections were performed into *Xenopus* embryos in the range of 5 pg to 50 pg RNA per embryo. As shown in Figure 2, Wnt-1-CD8 RNA induced secondary axis formation in a dose-dependent manner. 10 pg Wnt1-CD8 RNA had weak axis inducing activity, with about 30% embryos exhibiting partial axis duplication and no complete axis duplication. Partial or complete axis duplication is defined as without or with anterior structures such as eyes in the secondary axis. As a comparison, 10 pg Wnt-1 RNA induced 95% complete axis duplication in these experiments (data not shown). Although we were primarily interested in Fz proteins that would synergize with Wnt-1, we did not want to rule out the possibility of any Fz proteins that might suppress Wnt-1 function. We reasoned that the level of 30% partial axis duplication would be a reasonable base level to

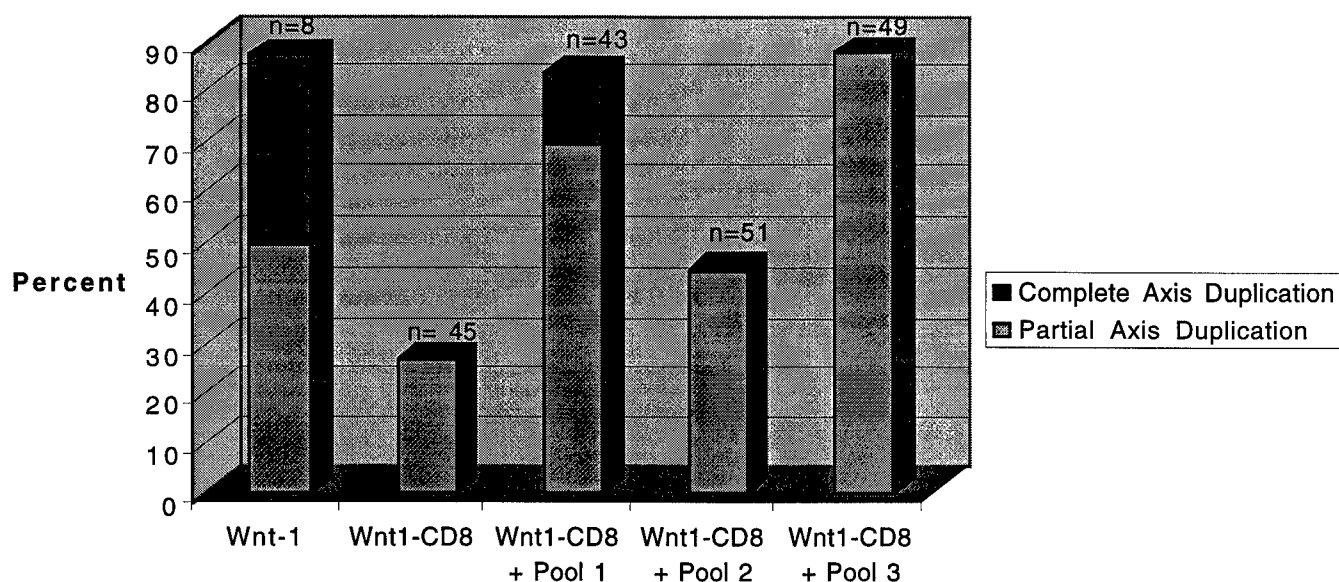
score for either stimulatory or inhibitory Fz function. Thus, 10 pg Wnt1-CD8 RNA was used in all the following injection experiments.



The ten identified mammalian fz RNAs and *Drosophila* dFz2 RNA were tested for their ability to interact with Wnt1-CD8 RNA to induce secondary axis formation. These fz RNAs were subdivided into three pools based on their sequence similarities: Pool 1 (hFz5, mFz8, Dfz2), Pool 2 (mFz3, mFz4, mFz6, FzD3 and mFzD3) and Pool 3 (rFz1, rFz2 and mFz7). Co-injection experiments of these pools of mRNA and 10 pg of Wnt1-CD8 was then performed in *Xenopus* embryos. The total amount of RNA for each fz construct in the individual pools was calculated to be 400 pg. The amount of injected RNA (1.2- 2.0 ng/embryo) injected is calculated to be within 20% of the maximum level (10 ng/embryo), which is considered the maximum amount of RNA the embryo can be injected without non-specific toxic effects. It is known that the fz RNA pools do not by themselves induce secondary axis duplication (He et al., 1997). As a positive control, injection of the optimal amount of Wnt-1 (10 pg) was done to induce secondary axis duplication to test the responsiveness of the embryo.

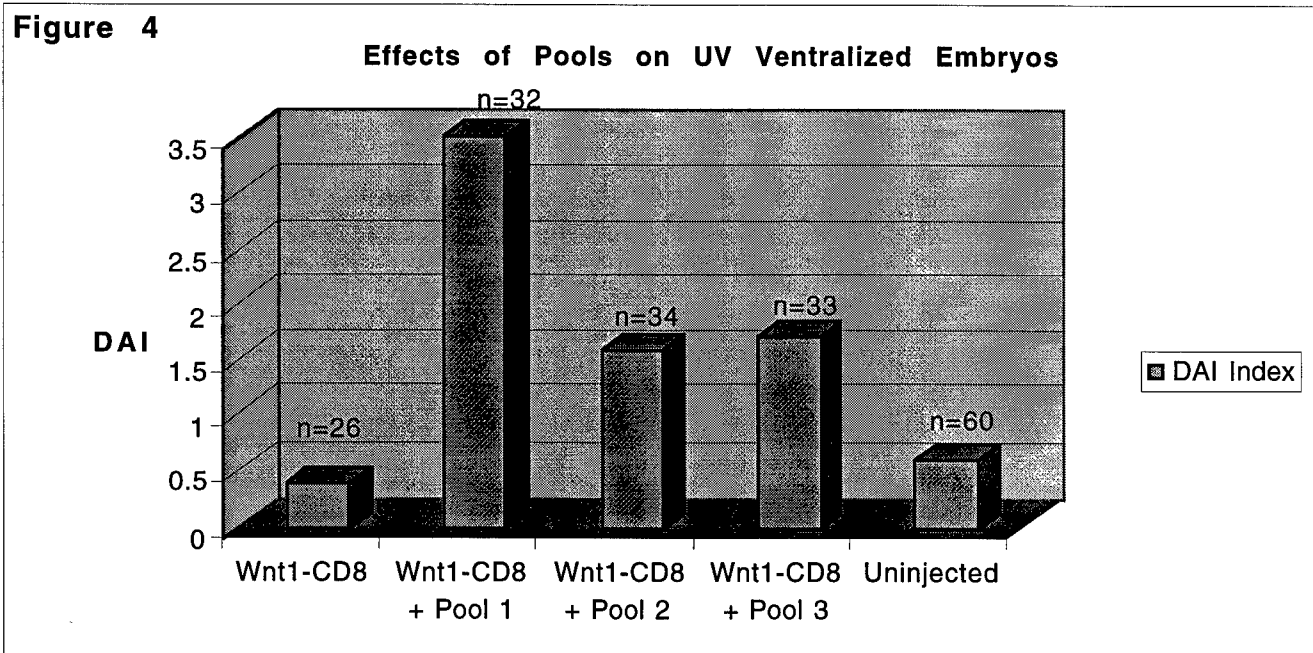
Figure 3

Effects of fz Pools on Axis Duplication



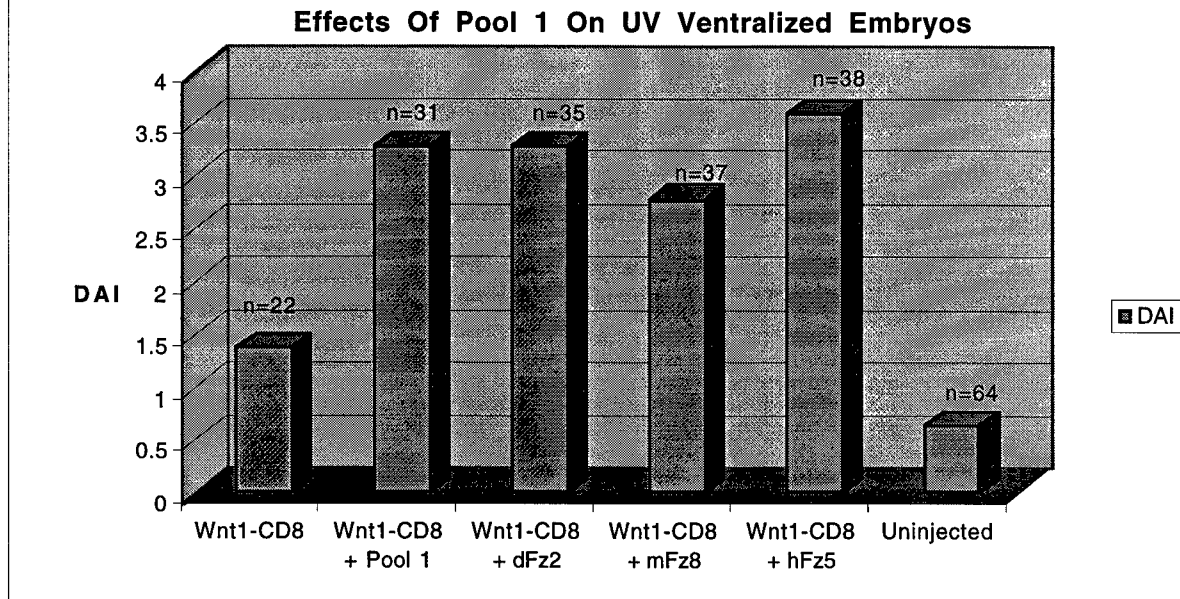
The results of the co-injection experiments with the Wnt1-CD8 RNA and the three pools of fz RNAs revealed that fz Pool 1 and, to a lesser extent fz pool 3, were able to increase the observed percentage of secondary axis formation by Wnt1-CD8 (Figure 3). Wnt1-CD8 plus fz Pool 1 induced significant axis duplication, including complete axis duplication, whereas Wnt1-CD8 plus fz pool 3 only exhibited increased partial axis duplication. fz pool 2 had a small but consistent enhancement of axis duplication in two separate experiments. These results prompted us to use a more quantitative method for assessing potential interaction with Wnt1-CD8 and its potential receptor(s). *Xenopus* embryos treated with ultraviolet (UV) irradiation at the 1-cell stage lack dorsal development (Gerhart et al., 1989). Such ventralized embryos can be rescued by the Wg/Wnt-1 subclass of molecules (Moon et al., 1997), as well as by XWnt-5A plus hFz5 (He et al., 1997). The extent of dorsal development can be quantified by the so-called dorso-anterior index (DAI),

with complete ventralization of DAI 0 and normal development of DAI 5 (Kao and Elinson, 1988). Thus, the strength of Wnt signaling in the rescue of UV-ventralized embryos can be quantified by DAI. We treated 1-cell stage embryos with UV, and then injected these embryos with Wnt1-CD8 or Wnt1-CD8 plus different fz pools. These injected embryos were scored by the DAI scale for rescue of normal development. As shown in Figure 4, Wnt1-CD8 plus pool 1 rescued dorsal development significantly, whereas Wnt1-CD8 plus fz pool 1 or pool 2 also rescued dorsal development to certain extent.



Since fz pool 1 showed most significant enhancement of Wnt1-CD8 in both the axis duplication assay and the UV-rescue assay, we further focused on the investigation of which of the components of Pool 1 (hFz5, mFz8 or dFz2) was responsible for synergizing with Wnt1-CD8. RNA of each of the fz molecules (400 pg each) was co-injected with Wnt1-CD8 (10 pg) into UV ventralized embryos and any rescue of the ventralized phenotype was scored. As shown in Figure 5, Dfz2, hFz5 and mFz8 each was able to enhance Wnt1-CD8 signaling, suggesting that each of these fz molecules can interact with Wnt-1 in this assay.

Figure 5



Aim 2. Dishevelled signaling and function

Dishevelled is a cytoplasmic protein (Cadigan and Nusse, 1997) and functions downstream of Wnt receptors via an unknown mechanism. It is a modular protein, containing the amino-terminal DIX domain, the central PDZ domain and the carboxyl-terminal DEP domain (Cadigan and Nusse, 1997). The DIX domain and the PDZ domain have been implicated as essential for Wnt signaling. In order to understand how Dishevelled protein transduces Wnt signaling, we undertook a yeast two-hybrid screen to search for interacting proteins that may shed light on Dishevelled function.

We used the DIX domain and the PDZ domain of mouse Dishevelled-2 as two separate baits by fusing them each to the Gal4 DNA-binding domain. A cDNA library made from the mouse embryonic day 13 mRNA was used. From screening 5.4 million independent cDNA clones, we identified 86 strong and 33 weak interacting cDNA clones (judged by beta-gal staining) for the DIX domain bait. We also identified 38 interacting cDNA clones for the PDZ domain bait from 2 million independent cDNA screening. We currently are categorizing and sequencing these positives isolates. Because it is well known that putative interacting clones isolated from an yeast two-hybrid screening may not necessarily represent physiologically relevant interactions, we will rely on the axis duplication assay in *Xenopus* embryos to identify Dishevelled-interacting proteins that function in Wnt signaling. We anticipate to use either the wild type or dominant-negative mutant form of the isolated cDNA gene product to test its potential role in Wnt signaling and axis duplication.

Conclusion

We attempted to use the axis duplication assay in *Xenopus* embryos to identify Wnt-1 receptor or receptors. We used this strategy to identify Wnt-5A/hFz5 interaction (He et al., 1997). Our results suggested Dfz2, hFz5 and mFz8 all are capable of synergizing with Wnt-1, thereby functioning in this assay as potential receptors for Wnt-1. In addition, other unknown fz molecules in pool 2 and pool 3 also seem to be able to enhance Wnt-1 signaling, albeit to a lesser extent. This is in sharp contrast with the Wnt-5A case, where only hFz5 can mediate axis duplication by Wnt-5A. This seemingly lack of strong specificity made the interpretation of our results less straight forward. There are at least two possibilities to account for this. The first is the intrinsic difference of Wnt-1 and Wnt-5A in the axis duplication assay. Unlike Wnt-5A, Wnt-1 induces axis duplication very efficiently

without any co-injected Fz, presumably by activating some endogenous Fz molecules in embryos. This endogenous background certainly decreased signal/noise ratio of the assay, despite our effort to use the suboptimal level of Wnt-1 molecules. The second possibility is the redundant nature of Wnt-Fz interaction. It has been shown genetically that in *Drosophila*, both Frizzled and dFz2 proteins serve as redundant receptors for Wg, the *Drosophila* ortholog of Wnt-1 (Bhat, 1998; Kennerdell and Carthew, 1998). In fact, the possibility of a third Wg receptor has been proposed because the phenotype of the fz and dfz2 double null mutant is not as severe as the wg null mutant (Bhat, 1998). Therefore, it is quite possible that Wnt-1 has several fz receptors, and the observation we made in the *Xenopus* axis duplication that several fz molecules can synergize with Wnt-1 may just be a reflection of such a complexity. Considering the above scenarios, while we are encouraged by the fact that we were able to identify some Fz molecules, we also realize that we need to be careful in the interpretation of these results and that we may need to revise some of our strategies to study Wnt-1/Fz interactions.

Our work on Dishevelled-interacting molecules is on-going and is affected to some degree by an unexpected personnel change (a postdoctoral fellow working on the two-hybrid screen left the lab due to heavy clinical duties). Nonetheless, we feel that the Dishevelled protein remains one of several major unanswered issues in the Wnt signal transduction, and we are confident that the combination of the two-hybrid screen and the axis duplication assay will provide us powerful tools to identify functional partners for the Dishevelled protein.

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