Molecular Interactions in the Norrin Receptor Complex

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I. Abstract

The retina has the highest oxygen consumption rate of any tissue type within the body and thus, requires extensive vasculature to supply its needs. The blood vessels supplying this tissue form in a distinct and highly characterized manner, known to be under the control of the Norrin/Frizzled4 signaling pathway. Tetraspanin12 (TSPAN12), a membrane protein, is one of the proteins mediating this pathway, but its role in signaling initiation is largely unknown. I hypothesized that TSPAN12 may positively regulate Norrin/Frizzled4 signaling by promoting protein-protein interactions in the Norrin receptor complex. This study utilized the iDimerize system to survey the function of possible protein interactions among components of the Norrin receptor complex. The goal was to gain insight into which interactions promote signaling and to thereby help identify candidate interactions that may be promoted by TSPAN12. I show that forcing the interactions of Frizzled4 and the co-receptor LRP5 promotes two components of signaling: one is Norrin dependent signaling and the other component is Norrin independent. A point mutation in Frizzled4, M105V, impairs only the Norrin-dependent component of signaling and is rescued by TSPAN12. Along with other evidence, this suggests that TSPAN12 may rescue signaling defects of Frizzled4 M105V by promoting Norrin binding to Frizzled4.

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II. Introduction

Vascularization of the retina is a highly characterized and important process for eye development. An incomplete vasculature means the retina will not receive oxygen and vital nutrients, leading to vision impairment or blindness. It has been well established that the Norrin/Frizzled4 signaling pathway mediates retinal vascular development and is an important player in retinal diseases (Xu et al., 2004; Ye et al., 2010). Mutations in proteins controlling this pathway lead to a spectrum of inherited diseases (i.e., Familial Exudative Vitreoretinopathy (FEVR), Norrie disease, and Osteoporosis Pseudoglioma) all of which disrupt the retinal vasculature and potentially vision (Robitalille et al., 2002; Gong et al., 2001). This pathway is also essential for the formation of the blood-retina-barrier, therefore, it is pertinent in other more prevalent diseases such as diabetic retinopathy and age-related macular degeneration (Ye et al., 2010). Understanding the Norrin/Frizzled4 signaling pathway may hold the key for treating vascular diseases affecting the retina and vision.

The Norrin/Frizzled4 pathway uses canonical ß-catenin to regulate genes within endothelial cells in the retina (Ye et al., 2009). Activation of plasma membrane proteins recruits the ß-catenin destruction complex to the plasma membrane, allowing ß-catenin to accumulate within the cytosol. It can then act as a transcriptional transactivator, associating with T cell factor and lymphoid enhancer factor (TCF/LEF) (and other transcriptional regulators including SOX) in the nucleus to regulate transcription of genes involved in the regulation of cell proliferation, cell fate specification, adult tissue homeostasis, and disease development (MacDonald et al., 2009; Kormish et al., 2010). There are other non-canonical ß-catenin forms of signaling but they are not required for

vascular development in the retina and thus not important for this study. ß-catenin signaling is used extensively in developmental signaling pathways such as those that regulate blood vessel formation in the central nervous system, bone growth, and cancer formation (Liebner et al., 2008, MacDonald et al., 2009).

Recently, proteins controlling the Norrin/Frizzled4 pathway have been characterized. The Norrin/Frizzled4 pathway utilizes three membrane molecules called Frizzled4 (FZD4), Low-density lipoprotein receptor-related protein 5 (LRP5), Tetraspanin 12 (TSPAN12), and a small secreted ligand known as Norrin (Xu et al., 2004; Xia et al., 2010; Junge et al., 2009; Luhmann et al., 2005). As shown in Figure 1, Norrin is thought to act as a dimer or multimer, binding Frizzled4 with high affinity leading to downstream canonical ß-catenin signaling (Figure 1) (Xu et al., 2004). The Norrin receptor, Frizzled4, is a seven-pass trans-membrane protein and is postulated to also act as a dimer to initiate signaling (Ke et al., 2013; Kaykas et al., 2004). LRP5 is a

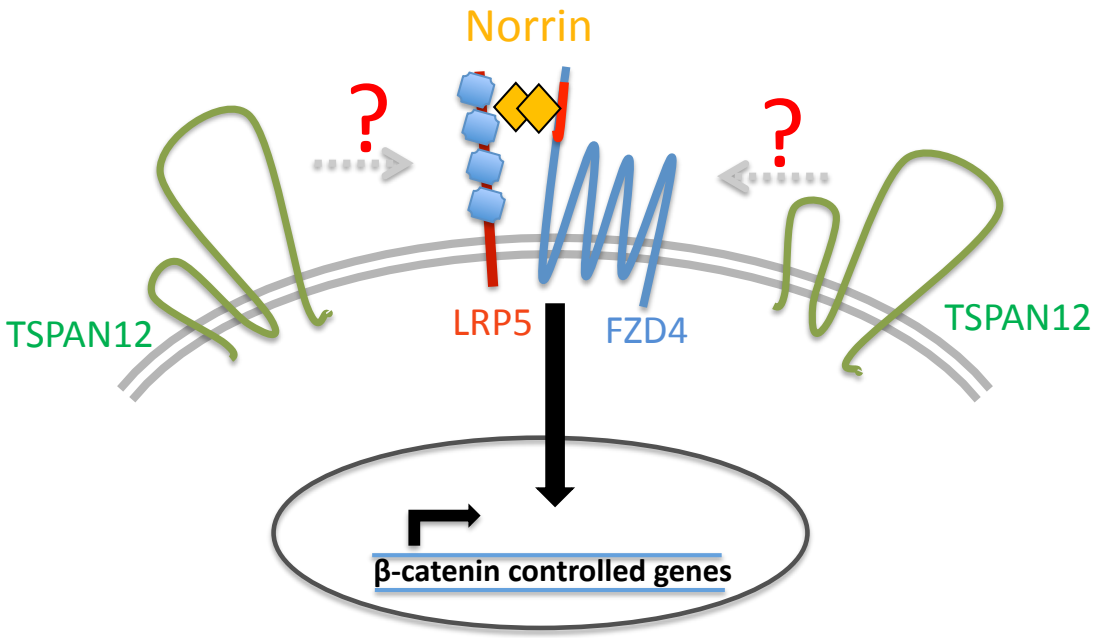

Figure 1: Depiction of the Norrin/Frizzled4 signaling pathway. Figure provided by Maria Lai

single pass trans-membrane protein characterized by four β-propeller regions in its extracellular domain (Cheng et al., 2011). LRP5 has been demonstrated to be required for signaling and evidence has suggested that it may act as a co-receptor and bind Norrin with low affinity (Xia et al., 2010; Ke et al., 2013).

The role of TSPAN12 is of particular interest. Mouse genetic studies have shown that TSPAN12 knockout mice phenocopy retinal vascular defects shown in FZD4, LRP5, and Norrin gene deficient mice, suggesting that it too is required for signaling (Junge et al., 2009). Interestingly, Wnt/Frizzled signaling, a pathway also required for central nervous system vascular development and strikingly similar to the Norrin/Frizzled4 pathway, does not require TSPAN12 (Liebner et al., 2008; Junge et al., 2009). In vitro evidence has also shown that TSPAN12 enhances Norrin/Frizzled4 signaling but not Wnt/Frizzled signaling in TOPflash reporter assays (Junge et al., 2009). This begs the question: What is TSPAN12 doing in Norrin/Frizzled4 signaling?

This study surveyed the function of protein interactions of the Norrin/Frizzled4 signaling complex to gain information on what interactions are required for signaling and how TSPAN12 may regulate them. We tested this by using the iDimerize system to force interactions between components of the receptor complex with or without Norrin (Norrin-dependent or Norrin-independent) to see if we could activate signaling. The iDimerize system is a commercial system of protein tags (encoding for FKBP12, and FRB domains), which can be induced to interact with appropriate chemicals. DmrA and DmrC tags (or tandem DmrAA and DmrCC tags) can be used to force hetero-interactions, and DmrB tags can be used to force homo-interactions. It was observed that when FZD4-DmrAA was forced to interact with LRP5-DmrCC, there was an increase in both Norrin-

dependent and Norrin-independent levels of signaling. A FEVR point mutation in Frizzled4, M105V, was also used to see if signaling could be rescued through forced interactions. It was found that FZD4-M105V decreased only Norrin-dependent but not Norrin-independent components of signaling. Signaling defects of FZD4-M105V were rescued by TSPAN12. This raises the possibility that the M105V mutation impairs Norrin Frizzled4 binding and that TSPAN12 can rescue this mutation by promoting Norrin binding to Frizzled4.

III. Materials and Methods

Plasmid Construction

Vectors containing V5-FZD4, 3xFlag-LRP5, and HA-TSPAN12 were in pcDNA3.3 backbones under a CMV promoter. Vectors also contained an ampicillin resistance gene allowing for the selection of positive transformants. Sequences encoding Dmr domains were obtained by gene synthesis (Biobasic). Sequences for in-frame expression of the DmrAA, DmrCC, or DmrBB domains were added to the 3' end of V5-FZD4, 3xFlag-LRP5, or HA-TSPAN12 genes through PCR cloning with engineered primers. Various restriction sites were used depending on the gene and type of domain. Disease associated point mutations were generated through site-directed mutagenesis. The reporter plasmids used in TOPflash reporter assays were kindly provided by Dr. Michael Klymkowsky

Cell Culture of HEK293T cells

HEK293T cells were cultured at 37º C and 5% CO_2. The cells were plated in high glucose DME medium plus 10% FBS. Cultures were split at 80% confluency using Trypsin/EDTA and replated.

iDimerize system

The iDimerize system was used to force interactions between proteins and to determine if the interactions were important for signaling. A/C and B/B inducer were obtained from Clontech. Inducer was diluted to 500 mM in 200 proof ethanol and stored at -20° C. Serial dilutions were then preformed in 200 proof ethanol to obtain the desired concentrations then added to cells in culture. TOPflash reporter assay system was then used to determine levels of signaling (Molenaar et al., 1996).

TOPflash Reporter Assays

TOPflash reporter assays were utilized to determine levels of Norrin/Frizzled4 signaling in HEK293T cells expressing DNA plasmids of interest. 48-well plates were seeded with a cell density of 120,000 cells/well and allowed to grow for 4 hours. Next, the cells were transiently co-transfected with 4 ng of V5-FZD4, 8 ng of 3xFlag-LRP5, and 8 ng of HA-TSPAN12 or 8 ng of GFP in TransfectaGRO with TransIT-LT1 (Mirus). These constructs were replaced with Dmr tagged constructs depending on the condition desired. Cells were also transfected with 120 ng of reporter mix (for 1mg/ml stock, 700 μg of TOPflash, 200 μg of CMV-Renilla, and 100 μg of pCDNA3.3 hLef-1 in 1 ml Tris 10 mM pH 8.0). Each condition was set up in three different wells allowing for the

calculation of an average. pcDNA 3.3 EGFP was used as a negative control in place of pcDNA 3.3 HA-TSPAN12. Cells were allowed to express the proteins for 6 hours. 5 μL of iDimerize inducer at varying concentrations were added to generate final concentrations of 500 nM, 100 nM, 10 nM, or 1 nM inducer in the assay. 5 μL of 25 μg recombinant Norrin (R+D Systems) were then added to generate a final concentration of 125 ng/ul (10 nM based on the molecular weight of the Norrin monomer) and allowed to induce signaling for 12 hours. After 12 hours, the cells were lysed in a 3:1 mix of 10 nM HEPES pH 8.0 and firefly luciferase substrate in lysis buffer (Promega, Dualglo). Wells were then read for firefly luciferase activity in a synergy 2 multi-mode plate reader (Biotek) generously provided by Dr. Tin Tin Su. Firefly activity was stopped and the renilla luciferase reaction was enabled by adding Renilla substrate mix (Promega, Dualglo). After 15 min incubation renilla luminance was read out. Reporter activity was calculated as firefly/renilla in each well. Data was normalized to the first bar in each data plot.

Western Blots

Immunoblotting was used to examine the expression levels of constructs containing the Dmr domains and FEVR point mutations. HEK293T cells were plated in 24-well plates and then transiently transfected with 400 ng of DNA in TransfectaGRO with TransIT-LT1 (Mirus). The cells were allowed to express the plasmids for 36 hours. Cells were then lysed with lysis buffer and protein samples were prepared with SDS. The samples were then run on a 4-12% BisTris gel (Life sciences) for 3-4 hours at 100 V. After, they were transferred onto a NT membrane in 1x transfer buffer (1.2 mM Bicine,

1.2 mM Bis-Tris, .05 mM EDTA and 15% methanol, pH 7.2) for 2-3 hours at 30 V and blotted for 3 hours in 5% milk in TBST. Finally, LRP5 proteins were probed for with anti Flag HRP conjugated antibody while FZD4 proteins were probed for with anti V5 HRP conjugated antibody for 12 hours at 4º C (1:1000 antibody to 5% milk/TBST). After washing, the blots were developed using Pierce Pico chemiluminescence substrate. ß-actin was probed with anti ß-actin HRP conjugated antibody. This was used as a loading control. In between probes, the NT membrane was stripped with stripping buffer (Restore Plus) for 5 min then washed 3x with TBST for 5 min and 2x with TBS for 1 min.

Statistics

2-tailed student T tests assuming unequal variance were used to compare two groups of data. P values < 0.05 were considered significant.

IV. Results

FZD4-DmrBB induces higher levels of Norrin/Frizzled4 signaling

When 293T cells were co-transfected with wild-type FZD4 and LRP5 in the presence of Norrin, I observed a low level of ß-catenin-dependent TOPflash reporter activity (Bar 2 Figure 2B). However, when TSPAN12 was also co-transfected, there was up to a five-fold increase in luciferase activity, consistent with previous results (Junge et al., 2009) (Bar 4 Figure 2B). As expected, when no Norrin was present, there were low levels of signaling. Together with other genetic and biochemical data (Junge et al., 2009), this result reiterates the point that TSPAN12 is an important component of Norrin/Frizzled4 signaling.

Previous biochemical data suggested that Frizzled4 dimerizes or oligomerize to initiate Norrin/Frizzled4 signaling (Ke et al., 2013; Kaykas et al., 2004). To test this assumption, I constructed vectors containing DmrBB domains linked to the C-terminus of Frizzled4. FZD4-DmrBB can enhance dimerization as well as multimerization depending on the orientation of the Dmr tag (Figure 2A). I next co-transfected 293T cells with FZD4-DmrBB and LRP5 with or without TSPAN12. These cells were then cultured in the presence or absence of 10 nM recombinant Norrin. Because the presence of DmrBB domains appeared to have functional effects even without the presence of B/B inducer, I surmised that the system did not strictly depend on presence of inducer molecule. This property may be exacerbated by the fact that untagged FZD4 has self-interaction sites in the extracellular domain which may synergize with the DmrB interaction interface. When I compared enhanced self-interactions of Frizzled4 (enhanced due to the presence of the DmrBB domain in the absence of B/B inducer) to non-forced interactions, there was a two-fold increase in TOPflash reporter activity under Norrin-dependent, TSPAN12-independent conditions. This result is highly reproducible but was not always statistically significant. The same enhanced self-interaction had no significant effect on signaling in the presence of Norrin and TSPAN12 (Figure 2B). There was also a small TSPAN12-dependent increase in TOPflash activity in Norrin-independent conditions when there were Frizzled4 enhanced self-interactions. The nature of TSPAN12 effects that are seemingly independent of Norrin is currently under investigation, experiments include the removal of endogenous sources of Norrin in the assay (i.e., serum, 293T cells).

Additional stimulation with B/B homo-inducer seemed to have a negative effect on TOPflash activity (not shown), perhaps due to poorly understood desensitization

mechanisms. I tested the toxicity of the inducer and it had no effect on signaling levels of cells overexpressing wild-type Norrin/Frizzled4 proteins, indicating that the inducer was not generally toxic to cells or affected the ability to induce Norrin/Frizzled4 signaling (Figure 7, Appendix A). There also seemed to be little difference in expression levels between Frizzled4 construct with or without DmrBB domains, indicated by a western blot (Figure 9, Appendix B). Tagging Frizzled4 with DmrBB increases Norrin-dependent (and to a smaller degree Norrin-independent) levels of signaling, but the effects of enhanced Frizzled4 interaction and the effects mediated by TSPAN12 are not additive. Altogether, I was able to study the effects of enhanced Frizzled4 self-interaction only in the absence of B/B inducer and the effects were comparably minor (i.e., not always reaching statistical significance).

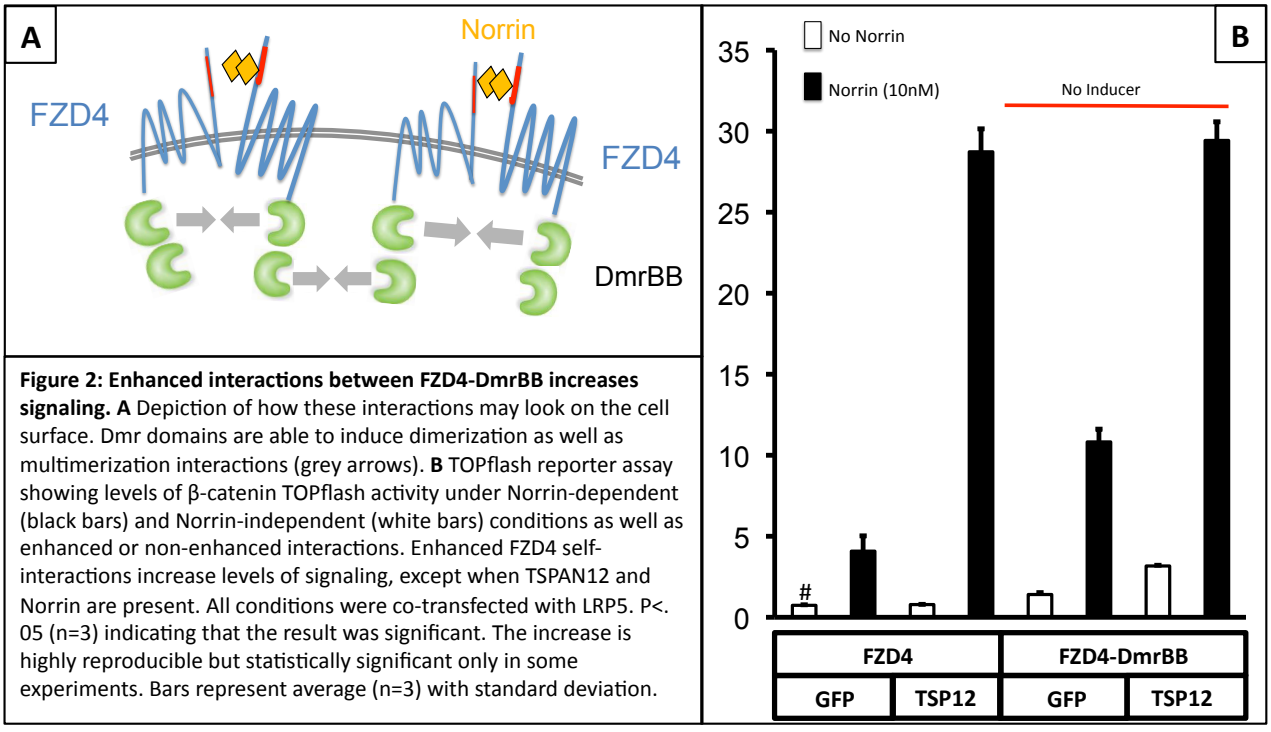

Figure 2: Enhanced interactions between FZD4-DmrBB increases signaling. A Depiction of how these interactions may look on the cell surface. Dmr domains are able to induce dimerization as well as multimerization interactions (grey arrows). **B** TOPflash reporter assay showing levels of β-catenin TOPflash activity under Norrin-dependent (black bars) and Norrin-independent (white bars) conditions as well as enhanced or non-enhanced interactions. Enhanced FZD4 self-interactions increase levels of signaling, except when TSPAN12 and Norrin are present. All conditions were co-transfected with LRP5. P<. 05 (n=3) indicating that the result was significant. The increase is highly reproducible but statistically significant only in some experiments. Bars represent average (n=3) with standard deviation.

Forced interactions between FZD4-DmrAA and LRP5-DmrCC increase TOPflash reporter activity

We next wanted to determine what effects forcing Frizzled4 to interact with LRP5 had on Norrin/Frizzled4 reporter activity. To do this, we once again utilized the iDimerize system but this time to force hetero-interactions controlled by A/C inducer. A/C inducer was tested for toxicity in a similar manner as B/B inducer and signaling levels were not affected, indicating that the inducer is not toxic or affects Norrin/Frizzled4 signaling (Figure 8, Appendix A). Hetero-interactions required Frizzled4 to be tagged with DmrAA and LRP5 tagged to DmrCC, so constructs expressing these modified proteins were created. Expression levels of Frizzled4 and LRP5 constructs with and without Dmr tags were similar (Figure 9 and 10, Appendix B). These constructs were co-transfected into 293T cells with or without TSPAN12 and TOPflash reporter assays were performed. Tagging Frizzled4 and LRP5 with DmrAA and DmrCC tags, respectively, resulted in significantly increased signaling even without A/C inducer (compare result groups A and B, Figure 3). When A/C inducer was present, there was a greater increase in TOPflash reporter activity, except when Norrin and TSPAN12 were present (compare result groups B to C and D, Figure 3). This forced interaction was able to increase TOPflash activity even without the presence of the required signaling ligand Norrin (Norrin-independent conditions) (compare white bars in results groups A and D, Figure 3). Forcing FZD4-DmrAA and LRP5-DmrCC also increased Norrin-dependent signaling, except when TSPAN12 was also present (compare black bars in results groups A and D, Figure 3). There was no statistically significant increase in TSPAN12, Norrin-dependent components of signaling, and the effect of TSPAN12 was not additive with this forced interaction. In conclusion, forcing Frizzled4

to interact with LRP5 increased both the Norrin-dependent and-independent components of signaling.

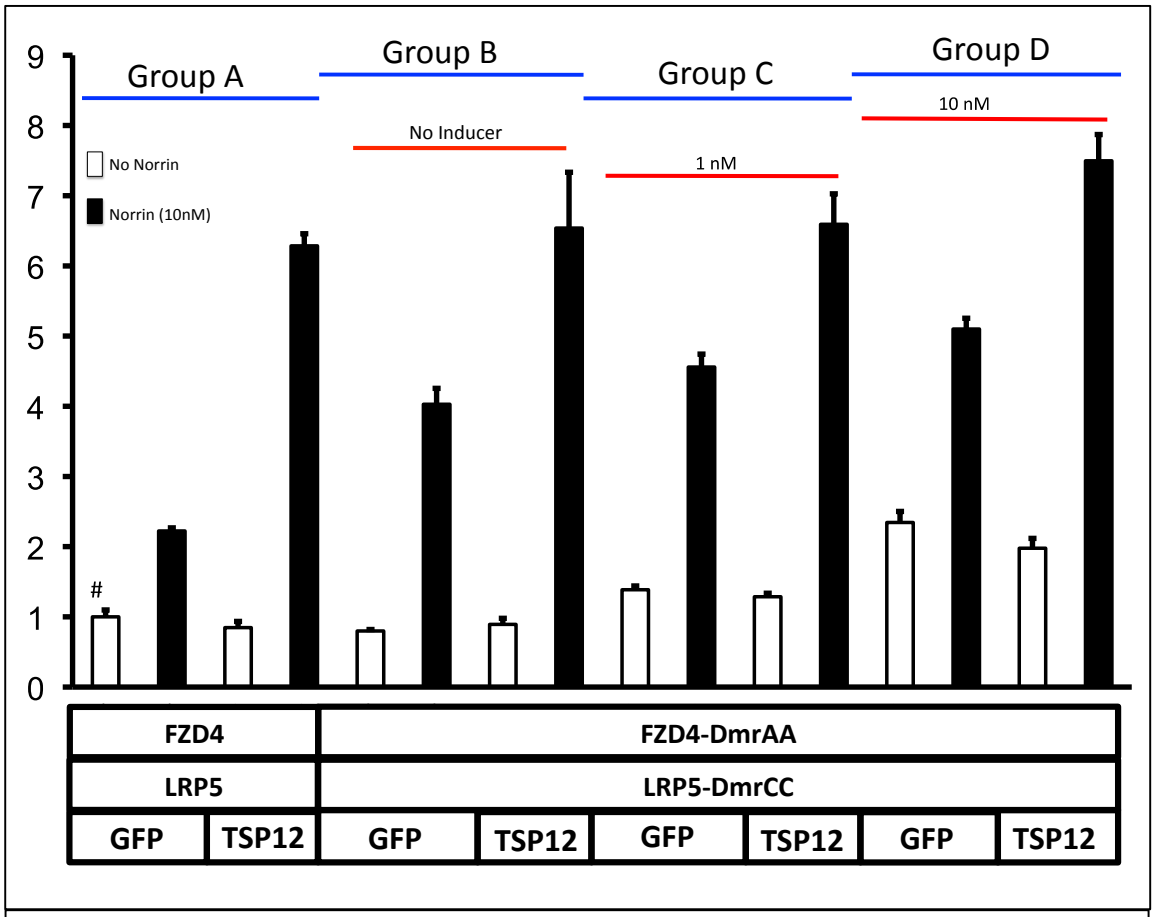

Figure 3: Increase in TOPflash reporter activity due to forced interactions between FZD4-DmrAA and LRP5-DmrCC. 293T cells were co-transfected with FZD4 and LRP5 or FZD4-DmrAA and LRP5-DmrCC with or without TSPAN12 at varying concentrations of A/C inducer. There was an increase in both Norri- dependent and independent levels of signaling when FZD4 and LRP5 were forced to interact (compare bars 1 with 13 and 2 with 14). This result had a P<.05 and was reproducible. Bars represent average (n=3) with standard deviation.

FZD4-M105V impairs Norrin/Frizzled4 signaling

Genetic studies of patients affected by FEVR show that some families contain a missense mutation within the Frizzled4 gene that encodes for a Methionine replaced by Valine at the 105th amino acid residue (Xu et al., 2004). The M105V mutation strongly impairs Norrin/Frizzled4 signaling in TOPflash assays and is located in the extracellular domain of Frizzled4 within its cysteine-rich region (Smallwood et al., 2007). Analysis of

this mutation shows that it is expressed at wild-type levels and localizes to the plasma membrane (Xu et al., 2004). The region that this mutation is located in could be important for Norrin binding or Frizzled4 dimerization (Figure 4A). I thought it would be informative to force interactions of Frizzled4 harboring this mutation to see if signaling defects of Frizzled4 M105V could be rescued. First, however, I needed to better characterize the signaling defects in the presence and absence of TSPAN12. To do this we constructed a FZD4 vector containing the M105V mutation through site-directed mutagenesis. Next, TOPflash reporter assays were performed. In Norrin-dependent, TSPAN12-independent conditions, the M105V mutation caused a 65% reduction in

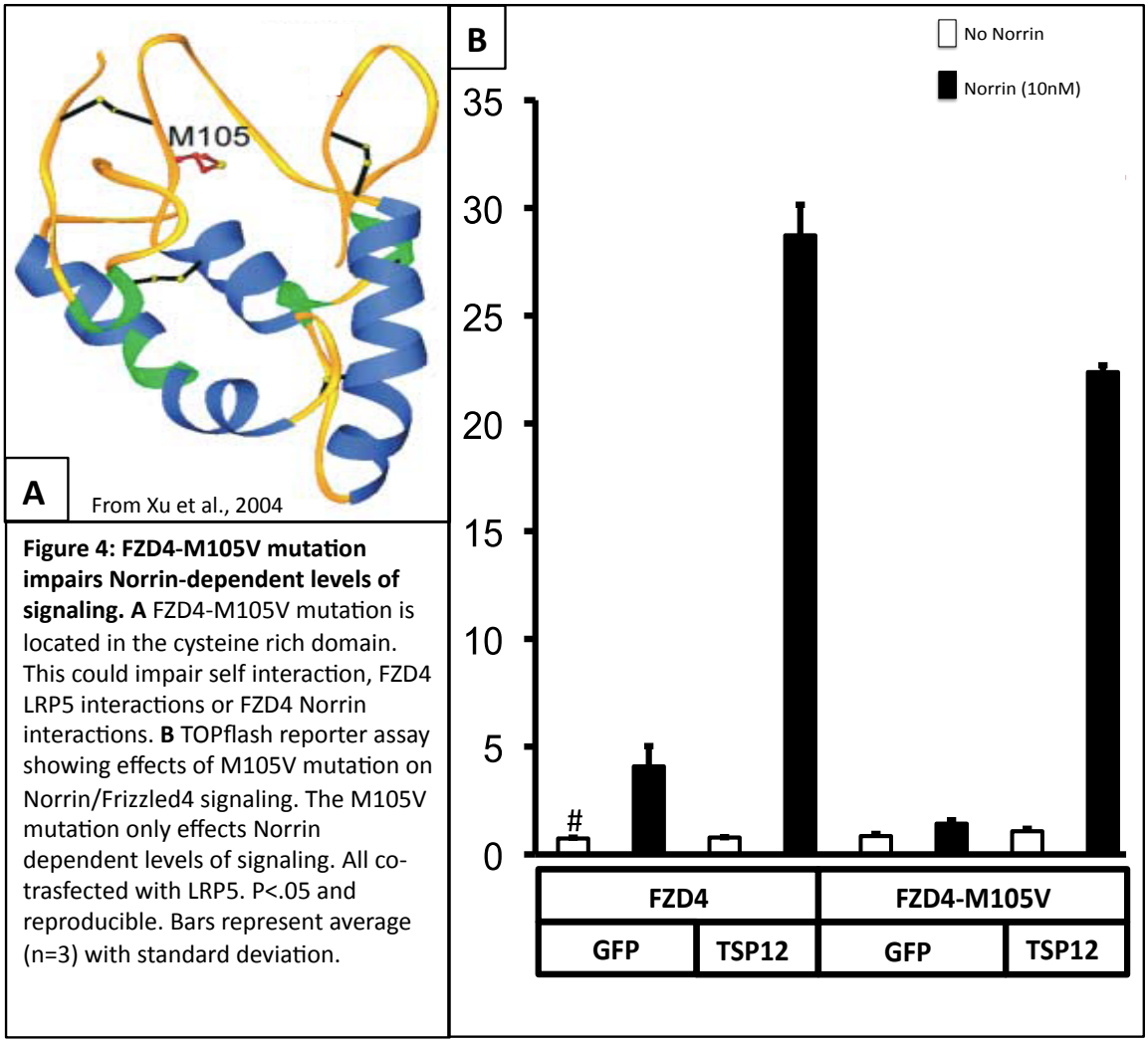

Figure 4: FZD4-M105V mutation impairs Norrin-dependent levels of signaling. A FZD4-M105V mutation is located in the cysteine rich domain. This could impair self interaction, FZD4 LRP5 interactions or FZD4 Norrin interactions. **B** TOPflash reporter assay showing effects of M105V mutation on Norrin/Frizzled4 signaling. The M105V mutation only effects Norrin dependent levels of signaling. All co-trasfected with LRP5. P<.05 and reproducible. Bars represent average (n=3) with standard deviation.

signaling compared to control. TSPAN12 was able to largely rescue the signaling defects caused by the M105V mutation. Baseline TOPflash activity was not affected by the Frizzled4 M105V mutation.

M105V mutation causes similar defects in FZD4-DmrBB

Since the M105V mutation could affect FZD4 dimerization, I tested enhanced self-interactions between FZD4-M105V-DmrBB to see if signaling defects could be rescued. I constructed the FZD4-M105V-DmrBB vector by cutting a section, containing the M105V mutation, of the FZD4 gene and ligating it into the FZD4-DmrBB vector. TOPflash reporter assays were then conducted. Under Norrin-dependent, TSPAN12-independent conditions, there was a 65% reduction in TOPflash activity for enhanced

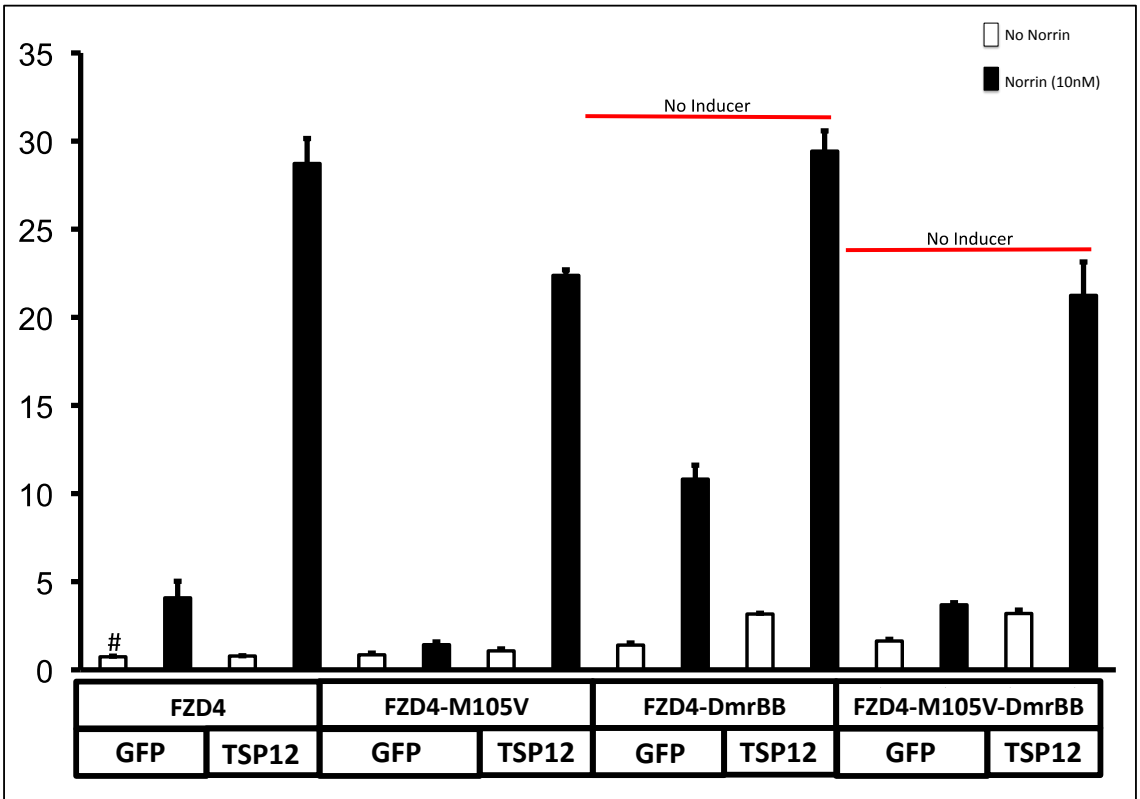

Figure 5: Enhanced interactions between FZD4-M105V-DmrBB still shows deficient TOPflash activity. TOPflash reporter assay showing the effects of enhancing Frizzled4 self-interactions with the M105V mutation. There is a 65% reduction for Norrin dependent, TSPAN12 independent conditions in both enhanced and non-enhanced interactions. This Indicates that M105V does not effect Frizzled4-Frizzled4 interactions. All co-transfected with LRP5. Result met significance criteria and was reproducible. Bars represent average (n=3) with standard deviation.

FZD4-M105V-DmrBB interactions compared to FZD4-DmrBB enhanced interactions (compare 10th and 14th bar in Figure 5). This is the same percent reduction in signaling that I observed when the mutation was in Frizzled4 without Dmr domain (2nd and 6th bar in Figure 5). Again, TSPAN12 was able to largely rescue the signaling defects of the M105V mutation in both enhanced and non-enhanced conditions. Since the M105V mutation caused a 65% reduction in both non-enhanced and enhanced Frizzled4 self-interactions, it appears unlikely that the M105V mutation affects Frizzled4 dimerization.

M105V affects the Norrin-dependent but not the Norrin-independent components of FZD4-DmrAA/LRP5-DmrCC signaling

Having evidence that suggests the M105V mutation could not be rescued by enhanced Frizzled4 self-interactions, I sought to test if forcing FZD4/LRP5 hetero-interactions could rescue this mutation. To test this, the M105V mutation was put into the FZD4-DmrAA vector through molecular cloning, and TOPflash reporter assays were performed. Since I had previously shown that forcing FZD4 and LRP5 to interact affected two components of signaling (Norrin-dependent and Norrin-independent components, Figure 3), I studied the signaling defects of FZD4-M105V with respect to both components of signaling. As shown in figure 6, there was once again an increase in the two components of signaling when FZD4-DmrAA was forced to interact with LRP5-DmrCC, compared to the control condition with untagged FZD4 and LRP5 (Figure 6). When FZD4-M105V-DmrAA was forced to interact with LRP5-DmrCC, a 45% reduction in the Norrin-dependent component of signaling was observed compared to FZD4-DmrAA and LRP5-DmrCC forced interactions. However, the Norrin-independent component of signaling was largely unaffected. This result indicates that forcing

Frizzled4 to interact with LRP5 cannot rescue the defects in TOPflash activity caused by the M105V mutation.

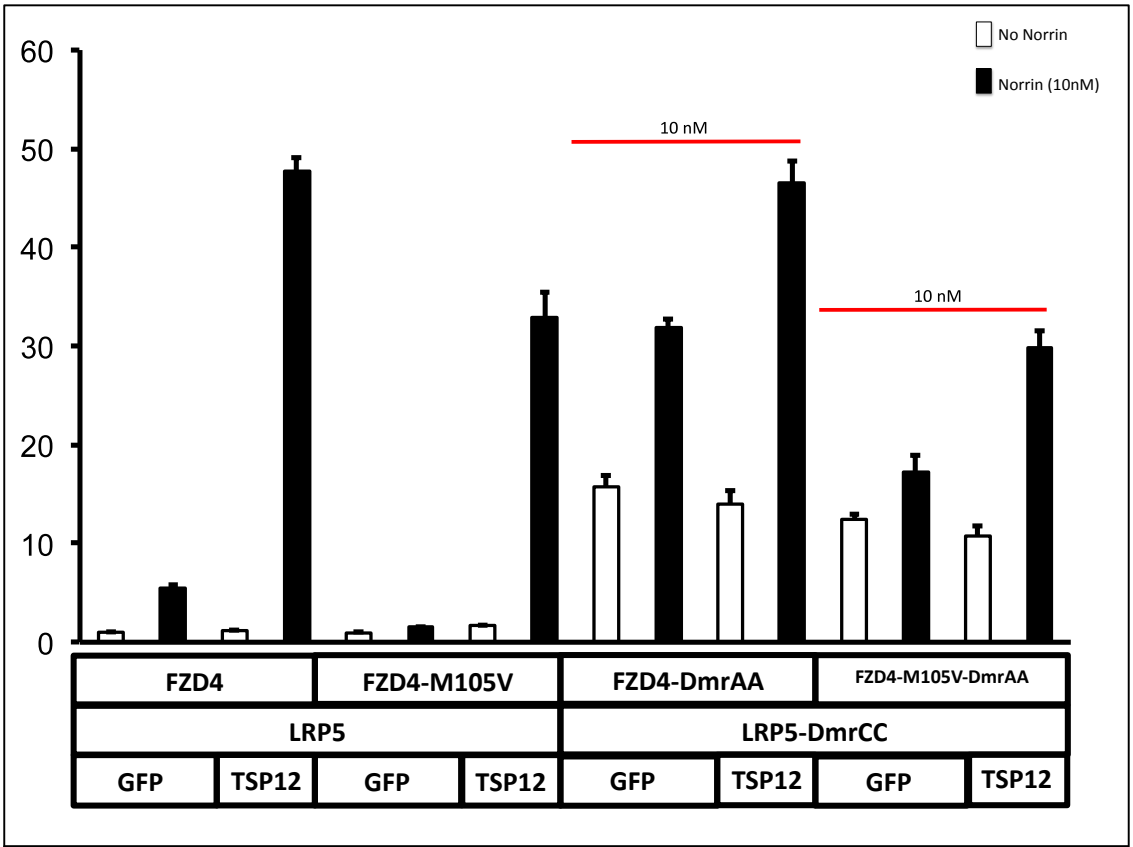

Figure 6: Forced interactions between FZD4-M105V-DmrAA and LRP5-DmrCC still show Norrin dependent deficient TOPflash activity. TOPflash reporter assay showing the effects of forcing FZD4-M105V-DmrAA to interact with LRP5-DmrCC. There was a 45% reduction when the mutant form of Frizzled4 was forced to interact with LRP5 compared to non-mutant form under Norrin dependent TSPAN12 independent conditions. This result met the significance criteria and was reproducible. Bars represent average (n=3) with standard deviation.

V. Discussion

Results from previous data and my own suggest that TSPAN12 is an important regulator of Norrin/Frizzled4 signaling, however, its role within the signaling complex remains to be clarified (Junge et al., 2009 and Figure 2). By utilizing the iDimerize system, I was able to determine interactions that enhance signaling and potential candidates for interactions promoted by TSPAN12. I found that i) forced interactions between FZD4-DmrAA and LRP5-DmrCC increased both Norrin-dependent and-

independent levels of signaling, ii) a FEVR mutation, M105V, abolishes Norrin-dependent TOPflash reporter activity and iii) enhanced or forced interactions between FZD4-M105V and FZD4-M105V or LRP5 could not rescue the defect in Norrin-dependent signaling, however, TSPAN12 could.

When FZD4-DmrAA and LRP5-DmrCC co-transfected cells were incubated with A/C inducer, I observed an increase in both Norrin-dependent and-independent levels of TOPflash reporter activity. It is interesting that there was an increase in Norrin-independent levels of activity because signaling should not be initiated unless Norrin is present. It is possible that this increase in activity is caused by events unrelated to Norrin/Frizzled4 signaling initiation. Forcing FZD4-DmrAA to interact with LRP5-DmrCC could partially inactivate the ß-catenin destruction complex allowing for accumulation of ß-catenin and thus, activation of TOPflash activity. Signaling may also be initiated by endogenous Wnts in the serum used to culture cells or from 293T cells secreting them. I also found it interesting that the increase in the Norrin-dependent TSPAN12-independent component of signaling due to forced interactions was not additive to the Norrin and TSPAN12-dependent component (Figure 3). We may not see this additive effect because forcing the interactions of FZD4 and LRP5, as well as TSPAN12, all impinge on increasing the avidity of the receptor complex for Norrin (see in the following).

When I enhanced interactions between FZD4-DmrBB I observed an increase in Norrin-dependent and to a small degree Norrin-independent components of signaling. The increase due to enhanced Frizzled4 self-interactions in the Norrin-dependent, TSPAN12-independent component of signaling was not additive to the Norrin

TSPAN12-dependent component of signaling. Perhaps this effect in enhanced self-interactions is because FZD4-DmrBB causes oligomerization of Frizzled4, once again causing a change in the avidity of Norrin-Frizzled4 interactions.

From the findings of this study I speculate that the M105V mutation in Frizzled4 affects Norrin binding to its receptor. Previous structural data suggest that this mutation is located in the Norrin-Frizzled4 binding domain (Smallwood et al., 2007). The observation that this mutation only affected the Norrin-dependent levels of TOPflash reporter activity provides some evidence that this mutation affects Norrin-Frizzled4 interactions (Figure 4). It was still possible, however, that M105V could affect other interactions within the receptor complex. From the data I generated, I demonstrated that enhancing self-interactions of Frizzled4 or forcing Frizzled4 to interact with LRP5 increased Norrin-dependent levels of signaling, thus, providing potential interactions M105V could affect (Figure 2 and 3). However, when I tested the effects of enhancing FZD4-M105V self-interactions or Frizzled4-M105V LRP5 hetero-interactions, there was still a reduction in TOPflash reporter activity. This indicates that promoting these interactions could not fully rescue the signaling defect of M105V (Figure 5 and 6). Thus, it appears more likely that the M105V mutation affects the interface between Norrin and Frizzled4 (or another interaction not yet tested).

If M105V affects Norrin-Frizzled4 interactions and TSPAN12 rescues this mutation, I further speculate that TSPAN12 enhances Norrin-Frizzled4 interactions. My results do not directly show that TSPAN12 increases Norrin-Frizzled4 binding. However, since TSPAN12 can rescue the M105V mutation that putatively affects the Norrin-Frizzled4 interface, it is a reasonable direction to test if TSPAN12 increases the affinity

between Norrin and Frizzled4. Further experiments would have to be conducted to substantiate this hypothesis.

In order to provide evidence to support this hypothesis, I propose to utilize the M105V mutation I have characterized. It would first be important to provide direct evidence that the M105V mutation impairs Norrin binding to Frizzled4. If this can be shown, we could then begin identifying whether or not TSPAN12 affects Norrin binding to Frizzled4 and Frizzled4-M105V. If TSPAN12 does promote Norrin-Frizzled4 interactions, it would be important to investigate if TSPAN12 enhances Norrin-Frizzled4 interactions by binding directly to Norrin and acting as a co-receptor.

In summary, this study utilized a disease-causing mutation (M105V) to understand molecular interactions of the Norrin/Frizzled4 receptor complex. We found that forcing interactions between different components of this complex could not fully rescue Norrin-dependent defects in signaling caused by the mutation. However, the presence of TSPAN12 can rescue M105V signaling defects, alluding to its potential role in Norrin/Frizzled4 signaling by enhancing the binding of Norrin to Frizzled4. Understanding TSPAN12 and its role in Norrin/Frizzled4 signaling will help us better understand how the retinal vasculature forms and potentially aid in developing therapeutic interventions in retinal vascular disease.

VI. Acknowledgements

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VII. Appendix

Appendix A: Inducer toxicity

B/B inducer is not toxic to Norrin/Frizzled4 signaling in 293T cells

A TOPflash reporter assay in 293T cells was employed to determine if the B/B inducer was toxic to the cell and Norrin/Frizzled4 signaling. Cells were co-transfected with Frizzled4 and LRP5 with or without TSPAN12 at varying concentrations of B/B inducer. Compared to the control (first four bars), there was no statistically significant change in TOPflash activity for cells incubated with homo-inducer. This indicates that the B/B inducer was not toxic to both Norrin-dependent and-independent TOPflash activity as well as 293T cells.

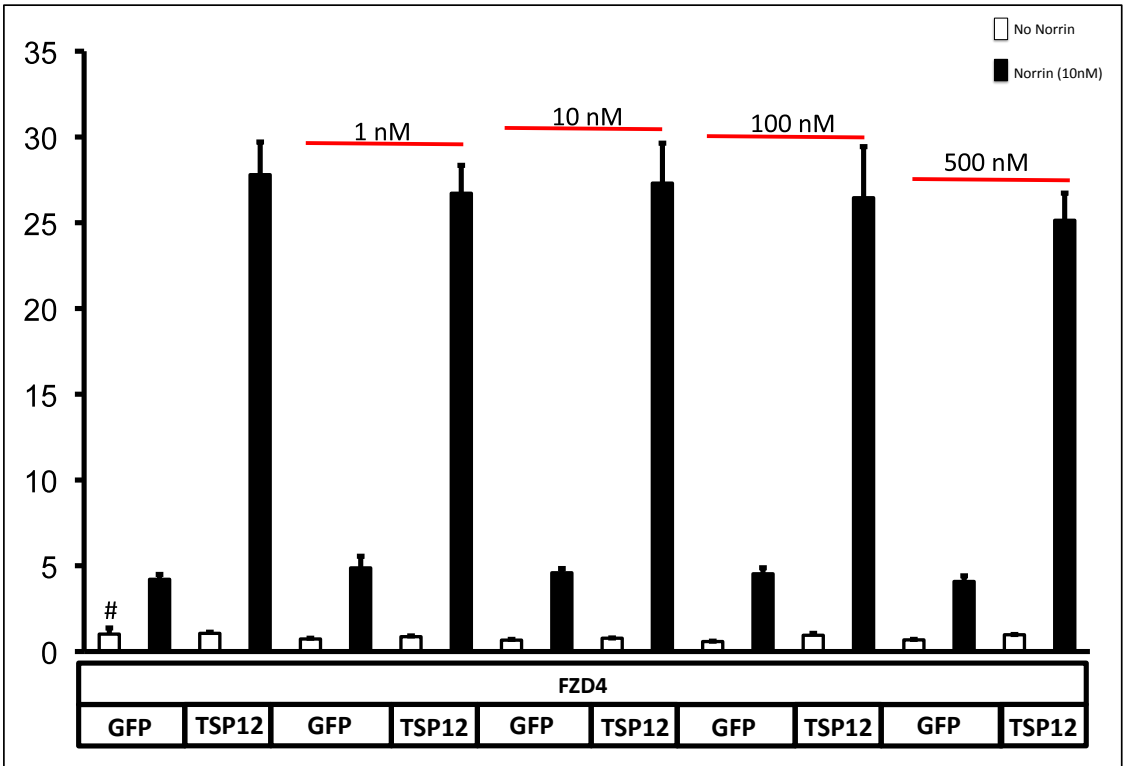

Figure 7: B/B inducer is not toxic to 293T cells or Norrin/Frizzled4 signaling. 293T cells were co-transfected with Frizzled4 and LRP5 with or without TSPAN12. They were then incubated with or without 10 nM recombinant Norrin and varying concentrations of B/B inducer. There was no significant decrease in TOPflash activity indicating the B/B inducer is not toxic. All co-trasfected with LRP5. Bars represent average (n=3) with standard deviation.

A/C inducer is not toxic to Norrin/Frizzled4 signaling in 293T cells

To determine if A/C inducer is toxic to 293T cells or Norrin/Frizzled4 signaling, a TOPflash reporter assay was employed, similar to the B/B inducer toxicity. Cells were co-transfected with Frizzled4 and LRP5 with or without TSPAN12. The cells were then incubated at varying concentrations of A/C inducer. The nature of the effect of TSPAN12 on Norrin independent signaling is under investigation. Compared to the control (first four bars), there was no significant change in TOPflash activity for cells incubated with hetero-inducer. This indicates that the A/C inducer is not toxic. Maria Lai provided these results.

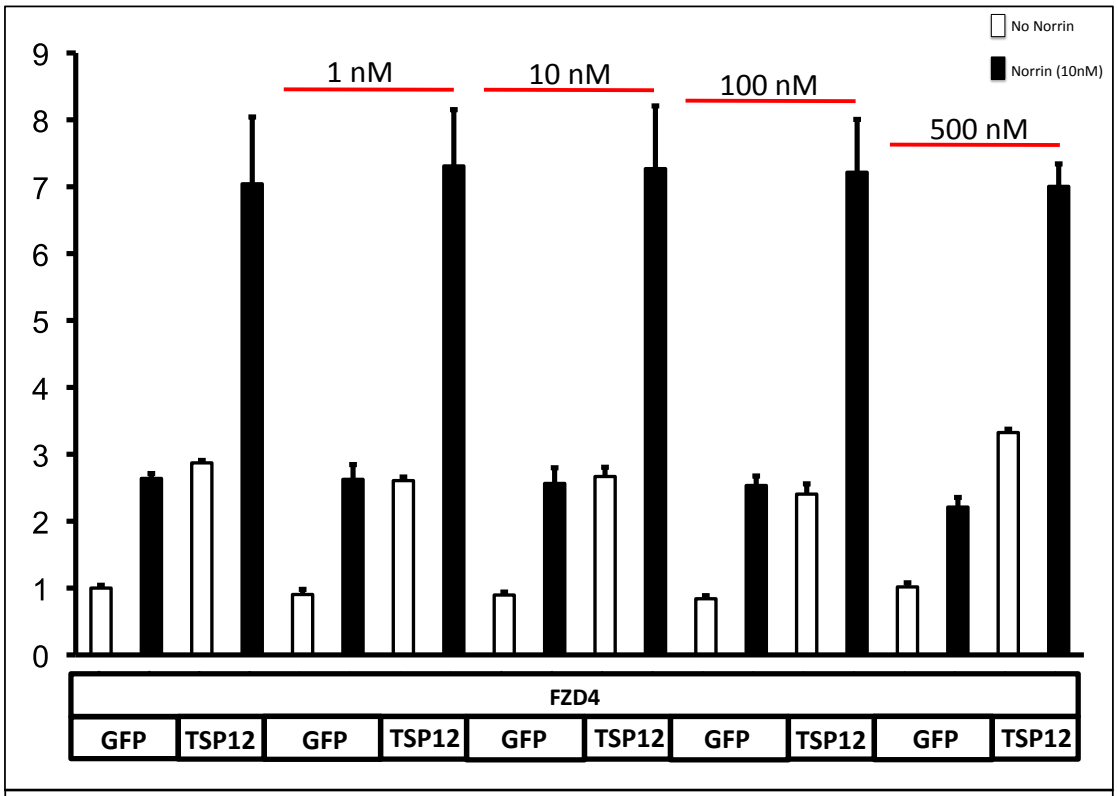

Figure 8: A/C inducer is not toxic to 293T cells or Norrin/Frizzled4 signaling. 293T cells were co-transfected with Frizzled4 and LRP5 with or without TSPAN12. The cells were then incubated at varying concentrations of A/C inducer and in the presence or absence of Norrin. The vectors were in a pcDNA3.1 vector backbone compared to the normal pcDNA3.3 vector. This is believed to be the cause of the higher levels of Norrin independent, TSPAN12 dependent levels of TOPflash activity. Otherwise, there was no affect of A/C inducer on the cells or signaling. All co-transfected with LRP5. Bars represent average (n=3) with standard deviation. Results provided by Maria Lai

Appendix B: Expression of constructs created

Frizzled4 vector expression

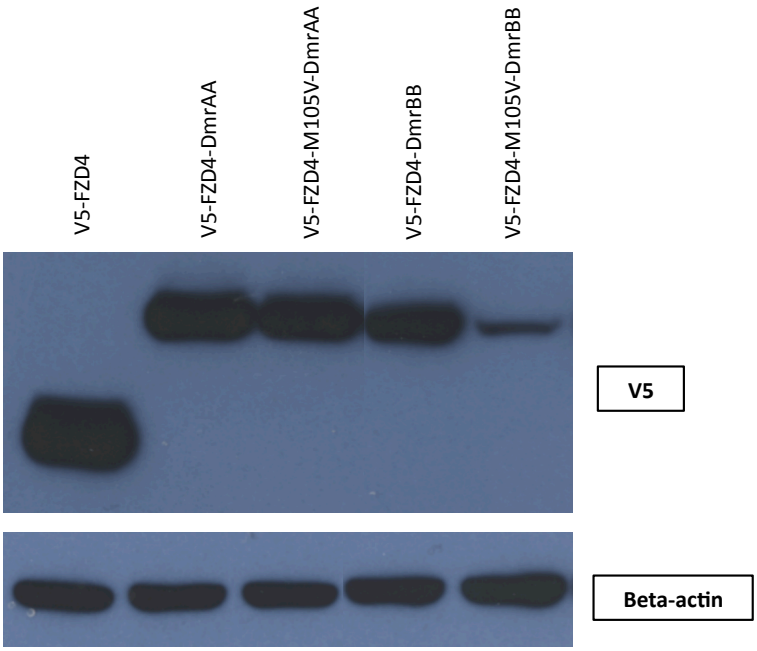

Figure 9: Expression levels of Frizzled4 constructs used. Western blot showing the expression levels of the Frizzled4 constructs used. Frizzled4 proteins were tagged with V5 and probed for with anti-V5. Beta-actin was used as a loading control. All lanes were from the same blot but I omitted unrelated lanes between lane three and four.

LRP5 vector expression

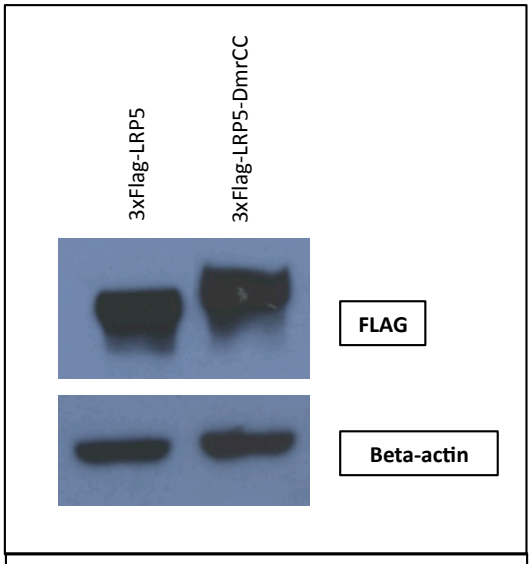

Figure 10: Expression levels of LRP5 constructs used. Western blot depicting the expression levels of LRP5 constructs used. LRP5 proteins were tagged with 3xFlag and probed with anti-Flag. Beta-actin

VIII. References

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