MASARYK UNIVERSITY
FACULTY OF SCIENCE
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Interaction of Class Frizzled receptors with G proteins and Dishevelled

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Thesis for doctoral degree

Supervisor: Professor Gunnar Schulte Brno 2017

ABSTRACT

The developmental processes driving embryogenesis have to be carefully regulated in order to achieve proper development of the embryo. One of the main signalling pathways responsible for the synchronization of these processes is the WNT signalling network. Through ligands of the WNT family, their receptors Frizzleds (FZDs) and various downstream pathway effectors, key developmental events such as cellular proliferation, polarization, migration, cell fate specification or body axis patterning are controlled.

In this thesis, I summarize the results of me and my colleagues obtained during the investigation of the most proximal events in WNT signalling pathway following WNT/FZD binding. The interaction of FZDs with their immediate binding partners Dishevelled (DVL) and heterotrimeric G proteins is scrutinized. I introduce and discuss novel aspects of FZD/G protein and FZD/DVL interaction with regard to the structural features, post-translational modifications, binding selectivity determinants and downstream pathways associated with these interactions.

Our results advocate the formation of WNT-sensitive $FZD_4/G\alpha_{12/13}$ inactive state complexes and therefore support the hypothesis of GPCR/Gα inactive state precoupling, at least within the Class F. Two novel connections linking $FZD_4/G\alpha_{12/13}$ with RHO GTPases and effectors of the YAP/TAZ pathway are described, shedding light on the molecular basis of FZD_4 function in vasculogenesis and proposing partial redundancy of FZD_4- and FZD_{10}-dependent signalling. We report the formation of a conserved intra-molecular network between transmembrane helices 2 and 4 of FZDs, disruption of which results in decreased DVL-binding capacity of FZD_4 and impairs DVL-dependent WNT signalling. Further, we show that conformation stabilized by this intra-molecular network as well as the C-terminus of FZD_4 is dispensable for the assembly of the $FZD_4/G\alpha_{12/13}$ complex. The complex also forms independently of DVL, suggesting a FZD-specific DVL involvement in FZD/Gα-dependent signalling. Our results indicate that FZD_4-mediated recruitment of DVL is enhanced by the multimerization of DVL. Lastly, we report that FZD phosphorylation might not be a prerequisite for FZD/DVL interaction but instead a consequence of such interaction induced by kinases such as CK1δ/ε or GRK2.

G protein-coupled receptors (GPCRs) such as FZDs are promising candidates for drug design. The goal in such case is to target a specific signal transduction cascade without affecting the other pathways. Our results help to understand the selectivity of signal transduction induced by WNT/FZD/DVL and WNT/FZD/G protein complexes and therefore can contribute to developing drugs with higher efficacy and fewer side effects.

LIST OF PUBLICATIONS

(I) Arthofer E, Hot B, Petersen J, **Strakova K**, Jäger S, Grundmann M, Kostenis E, Gutkind JS, Schulte G. (2016) WNT Stimulation Dissociates a Frizzled 4 Inactive-State Complex with Gα12/13. Mol Pharmacol. 90(4):447-59. PMID 27458145

I have contributed to the FRAP measurements determining the WNT-selectivity of FZD_4/Gα dissociation and analyzed my data (total contribution estimated as 5 %)

(II) **Strakova K**, Matricon P, Yokota C, Bernatik O, Rodriguez D, Arenas E, Carlsson J, Bryja V, Schulte G. (2017) The tyrosine Y2502.39 in Frizzled 4 defines a conserved motif important for recruitment of DVL and activation of downstream signaling. Cell Signal. Oct;38:85-96. Epub 2017 Jun 29. PMID: 28668722

I have designed, performed and analysed most of the experiments and wrote most of the paper (total contribution estimated as 75 %)

(III) **Strakova K**, Gybel T, Dhople VM, Bernatik O, Sedova K, Harnos J, Bryja V, Schulte G. Frizzled 6 is phosphorylated in a CK1ε-, GRK- and DVL-dependent manner. *Manuscript for J Biol Chem*

I have designed, performed and analysed most of the experiments and wrote the paper (total contribution estimated as 90 %)

INTRODUCTION

The WNT signalling pathway takes part in many fundamental developmental processes such as cellular proliferation, migration, polarity, cell fate specification or body axis patterning. When WNT signalling is malfunctioning during development, it leads to abortions of the foetus or more or less severe congenital malformations. Deregulated WNT signalling in adults contributes to multiple diseases with cancer in the lead, but including also neurodegenerative disorders or type II diabetes.

The WNT signalling *sensu lato* is historically seen as a number of parallel sub-pathways with distinct cellular functions. However, all the specific sub-pathways share some common transduction steps. All the pathways are activated by WNT proteins binding to receptors from the Frizzled (FZD) family. Then the signal is further transmitted through the binding of a scaffold protein Dishevelled (DVL) or through the action of heterotrimeric G proteins. Different intracellular transducers then confer the signal to the downstream effectors including the transcription factors β-catenin, NFAT or Jun or exert their effects directly on the cytoskeleton.

The best described is the β-catenin-dependent pathway which regulates the levels of β-catenin and is associated mainly with cell fate and proliferation. The planar cell polarity pathway, as the name indicates, commands the polarity of cells within a two-dimensional cell layer, but regulates also more complex events such as convergent extension movements of cells. WNT/calcium pathway is transmitted via calcium, affects processes such as migration or adhesion and is well known for the dependence on heterotrimeric G proteins. Gradually, even more branches of WNT signalling are emerging and evidence is piling for more and more crosstalk between all the branches. Thus, an integrative model of WNT signalling pathway was proposed, merging all the distinct branches into one complex signalling network initiated by WNT-FZD interaction, taking different complicated routes and executed into many different outcomes.

One of the unanswered questions in the WNT field concerns the selectivity of induced signalling pathways. In other words, what factor does determine which effectors will be activated by a WNT? So far, it seems that specificity in WNT signalling might be achieved by a combination of factors including WNT/receptor and receptor/G protein selectivity followed by the subsequent pathway bias as well as meticulous spatio-temporal expression – and thus the availability - of WNTs, receptors and other downstream components of the pathway during the development of metazoans.

Although the FZD/DVL and FZD/G protein interaction lies at the centre of WNT signalling pathways and has immense influence on the pathway selectivity, it is still not fully understood how these proteins interact and how selective is their interaction. In my thesis, I am trying to answer some of these questions in the context of two chosen members of the Class F receptors - FZD_4 and FZD_6.

AIMS

Despite FZD/DVL/Gαβγ being at the centre of signal specification in WNT signalling, the interaction interface and binding selectivity of FZD/DVL and FZD/G proteins is still a matter of debate. Also, the phosphorylation of FZDs is largely unexplored.

My specific aims were defined as follows:

- to determine FZD_4/G protein binding selectivity and to find the downstream pathways mediated by FZD_4 and G proteins
- to explore the involvement of DVL in FZD_4/G protein interaction
- to investigate the significance of FZD_4 IL1/TM2 in the FZD/DVL and FZD/G protein binding interface
- to find out whether phosphorylation of FZD_4 is required for DVL binding
- to examine whether FZD_6 is phosphorylated similarly to GPCRs and explore the circumstances and function of such phosphorylation

RESULTS

My doctoral studies have been dedicated to finding answers to the scientific problems exposed in the previous chapters and narrowed down into specific aims. My experimental efforts combined with the efforts of many of my colleagues brought novel results which were published as two papers in peer-reviewed scientific impact journals. Also, a third study, which has not been published yet, is presented in the form of a manuscript. The papers as well as the manuscript are attached to the end of the thesis. Here, I provide a short summary of all the papers presented in my thesis and then I shortly focus on each of the papers.

Article I - *WNT Stimulation Dissociates a Frizzled 4 Inactive-State Complex with $G\alpha_{12/13}$.*

In the first paper, we have focused on FZD_4 and its role in GPCR signalling. We have found that FZD_4 maintains an inactive state complex with $G\alpha_{12}$ and $G\alpha_{13}$, but not with $G\alpha_i$, $G\alpha_o$, $G\alpha_s$ and $G\alpha_q$. FZD_4-$G\alpha_{12/13}$ interaction is interrupted upon WNT treatment, independent on the FZD_4 C-terminus and independent of DVL. Further, the WNT/FZD_4/$G\alpha_{12/13}$ further signals to recruit p115-RHO-GEF to the membrane, suggesting the small GTPase RHO as a downstream target of the pathway.

Article II - *The tyrosine $Y250^{2.39}$ in Frizzled 4 defines a conserved motif important for recruitment of DVL and activation of downstream signaling.*

The second paper investigates the conserved motif at the border of the IL1/TM2 in human FZD_4, in particular a tyrosine 250 (Y250). Y250 is found to be a part of an important intramolecular network linking TM2 and TM4. Disruption of this interaction results in a receptor defective in both DVL recruitment and induction of downstream DVL-dependent WNT signalling. In contrast, Y250 is dispensable for FZD_4 interaction with $G\alpha_{12}$ and $G\alpha_{13}$ and for inducing the $G\alpha_{12/13}$-mediated YAP/TAZ-dependent transcription.

Article III - *FZD_6 is phosphorylated in a CK1ε-, GRK- and DVL-dependent manner.*

The third paper focuses on the phosphorylation of FZD_6. We have found several phosphorylated residues in the C-terminus of FZD_6 and raised a phospho-selective antibody against serine 648 (S648). S648 in human FZD_6 is phosphorylated in a CK1ε- and GRK-dependent manner, DVL enhances the phosphorylation. FZD_6 phosphorylated at S648 is localized at the plasma membrane and internalized. Further, we report an important role for DVL in the phosphorylation of FZD_6 – DVL overexpression causes FZD_6 hyper-phosphorylation. The DVL-mediated FZD_6 hyper-phosphorylation is dependent on the N-terminal part of the DVL DEP domain, on the ability of DVL to be recruited to the plasma membrane by FZD and the ability to bind CK1.

WNT Stimulation Dissociates a Frizzled 4 Inactive-State Complex with Gα12/13.

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Frizzleds (FZDs) are unconventional G protein-coupled receptors that belong to the class Frizzled. They are bound and activated by the Wingless/Int-1 lipoglycoprotein (WNT) family of secreted lipoglycoproteins. To date, mechanisms of signal initiation and FZD-G protein coupling remain poorly understood. Previously, we showed that FZD_6 assembles with $G\alpha_{i1}/G\alpha_q$ (but not with $G\alpha_s$, $G\alpha_o$ and $Ga_{12/13}$), and that these inactive-state complexes are dissociated by WNTs and regulated by the phosphoprotein Dishevelled (DVL). Here, we investigated the inactive-state assembly of heterotrimeric G proteins with FZD_4, a receptor important in retinal vascular development and frequently mutated in Norrie disease or familial exudative vitreoretinopathy. Live-cell imaging experiments using fluorescence recovery after photobleaching show that human FZD_4 assembles-in a DVL-independent manner with $G\alpha_{12/13}$ but not representatives of other heterotrimeric G protein subfamilies, such as $G\alpha_{i1}$, $G\alpha_o$, $G\alpha_s$, and $G\alpha_q$. The FZD_4-G protein complex dissociates upon stimulation with WNT-3A, WNT-5A, WNT-7A, and WNT-10B. In addition, WNT-induced dynamic mass redistribution changes in untransfected and, even more so, in FZD_4 green fluorescent protein-transfected cells depend on $G\alpha_{12/13}$. Furthermore, expression of FZD_4 and $G\alpha_{12}$ or $G\alpha_{13}$ in human embryonic kidney 293 cells induces WNT-dependent membrane recruitment of p115-RHOGEF (RHO guanine nucleotide exchange factor, molecular weight 115 kDa), a direct target of $G\alpha_{12/13}$ signaling, underlining the functionality of an FZD_4-$G\alpha_{12/13}$-RHO signaling axis. In summary, $G\alpha_{12/13}$-mediated WNT/FZD_4 signaling through p115-RHOGEF offers an intriguing and previously unappreciated mechanistic link of FZD_4 signaling to cytoskeletal rearrangements and RHO signaling with implications for the regulation of angiogenesis during embryonic and tumor development.

Mol Pharmacol. 2016 Oct;90(4):447-59. doi: 10.1124/mol.116.104919. Epub 2016 Jul 25.

The tyrosine Y250$^{2.39}$ in Frizzled 4 defines a conserved motif important for structural integrity of the receptor and recruitment of Disheveled

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Frizzleds (FZDs) are unconventional G protein-coupled receptors, which activate diverse intracellular signaling pathways via the phosphoprotein Disheveled (DVL) and heterotrimeric G proteins. The interaction interplay of FZDs with DVL and G proteins is complex, involves different regions of FZD and the potential dynamics are poorly understood. In the present study, we aimed to characterize the function of a highly conserved tyrosine (Y250$^{2.39}$) in the intracellular loop 1 (IL1) of human FZD_4. We have found Y250$^{2.39}$ to be crucial for DVL2 interaction and DVL2 translocation to the plasma membrane. Mutant FZD_4-Y250$^{2.39}$F, impaired in DVL2 binding, was defective in both β-catenin-dependent and β-catenin-independent WNT signaling induced in *Xenopus laevis* embryos. The same mutant maintained interaction with the heterotrimeric G proteins $G\alpha_{12}$ and $G\alpha_{13}$ and was able to mediate WNT-induced G protein dissociation and G protein-dependent YAP/TAZ signaling. We conclude from modelling and dynamics simulation efforts that Y250$^{2.39}$ is important for the structural integrity of the FZD-DVL, but not for the FZD-G protein interface and hypothesize that the interaction network of Y250$^{2.39}$ and H348$^{4.46}$ plays a role in specifying downstream signaling pathways induced by the receptor.

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Frizzled 6 is phosphorylated in a CK1ε-, GRK- and DVL-dependent manner.

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Frizzleds function as receptors for WNT proteins in the WNT signaling pathway, an important signal transduction network crucial for the development of multicellular organisms. Frizzleds also belong to the superfamily of G protein-coupled receptors which control a wide variety of physiological functions. The G protein-coupled receptors are well known to be regulated by phosphorylation – it can induce receptor desensitization or specify the downstream signaling pathways induced by a ligand. However, the phosphorylation of Frizzleds remains mostly unexplored. Here, we investigated the phosphorylation of Frizzled 6 (FZD_6). We have found several phosphorylation sites in the C-terminus of FZD_6, among others two novel sites – serine 592 and serine 648 (S648). Further analysis of S648 revealed that this residue can be phosphorylated by casein kinase 1 epsilon (CK1ε), that Disheveled (DVL) enhances this phosphorylation and that FZD_6 phosphorylated at S648 is localized at the plasma membrane or internalized in the cytoplasm. Moreover, over-expression of DVL induces a massive hyper-phosphorylation of FZD_6. The DVL-induced FZD_6 hyper-phosphorylation is dependent on the DEP domain of DVL, specifically on the N-terminus of DEP domain and the FZD-binding interface of the DEP domain. Also, it requires the activity of CK1δ/ε and G protein-coupled receptor kinase 2 (GRK2), but not the activity of glycogen synthase kinase 3 (GSK3) or cAMP-dependent protein kinase (PKA).

Manuscript for Journal of Biological Chemistry

CONCLUSIONS

The results of the combined experimental efforts of me and my colleagues can be summarized as follows:

- Tyrosine 250, histidine 348 and tryptophan 352 in FZD_4 form an intra-molecular network which is likely conserved in Class F
- FZD_4 interacts with $G\alpha_{12}$ and $G\alpha_{13}$ heterotrimeric G proteins, the downstream targets include small GTPase RHO and effectors of the YAP/TAZ pathway
- DVL does not affect the binding of FZD_4 to heterotrimeric G proteins
- The C-terminus of FZD_4 is not required for the interaction with heterotrimeric G proteins, same as the conformation stabilized by the Y250/H348/W352 network
- Intact IL1/TM2 border and the Y250/H348/W352 network is required for FZD_4-DVL interaction and downstream DVL-dependent WNT signalling
- FZD_4 more effectively recruits DVL present in WNT signalosomes
- Recruitment of DVL by FZD_4 is probably not dependent on receptor phosphorylation, the only candidates are phosphorylatable residues in IL3
- Several phosphorylation sites were detected in the C-terminus of FZD_6, some of them WNT-3A and WNT-5A-induced
- FZD_6 can be phosphorylated by CK1ε, GRK2 and GRK5; phosphorylated FZD_6 resides more at the plasma membrane
- DVL mediates hyper-phosphorylation of FZD_6 with potential relevance to the PCP signalling pathway

In conclusion, our results have contributed to understanding Frizzleds with regard to their interaction with DVL and heterotrimeric G proteins. We provide evidence for the inactive state assembly model of FZDs and heterotrimeric G proteins, contribute to mapping the FZD/Gα selectivity and explore the downstream effector pathways. Moreover, we inspect FZD_4 structural determinants affecting DVL and G protein binding and investigate the role of FZD phosphorylation in WNT signalling. All the speculations raised in the previous section will need further careful examination to answer the remaining questions.

Dissecting the various aspects of complex WNT signalling network would be highly beneficial for the design of novel drugs. Of utmost importance is studying the function of receptors, which are the most common targets of drug design. When we understand the mechanisms of WNT pathway selectivity, we can treat diseases associated with deregulated WNT signalling, such as neurodegenerative disorders or cancer, by drugs with higher efficacy and fewer side effects.

CURRICULUM VITAE

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PRACOVNÍ ZKUŠENOSTI

2016 – dosud	lektorka, Biologický ústav LF MU
2013 – dosud	odborná pracovnice, Ústav experimentální biologie PřF MU, Oddělení fyziologie a imunologie živočichů, laboratoř WNT signalizace
2012 – 2015	4 pracovní stáže v celkové délce 1,5 roku na Karolinska Institutet, Švédsko, Department of Physiology and Pharmacology, laboratoř Receptor Biology and Signaling (v rámci projektu MŠMT Spolupráce mezi Masarykovou univerzitou a Karolinska Institutet, Stockholm na poli biomedicíny – „KI-MU")

STUDIUM

2012 – dosud	Doktorský studijní program, obor Fyziologie živočichů, PřF MU téma dizertační práce: *Interaction of Class Frizzled Receptors with G proteins and Dishevelled* školitel: Prof. Gunnar Schulte (Karolinska Institutet)
2010 – 2012	Magisterský studijní program, obor Molekulární biologie a genetika, PřF MU závěrečná práce: *Molekulární interakce onkogenů Mdm2 a MdmX* školitel: Mgr. Stjepan Uldrijan, CSc.
2007 – 2010	Bakalářský studijní program, obor Molekulární biologie a genetika, PřF MU závěrečná práce: *Molecular interactions of oncogene Mdm2 in cancer cells* školitel: Mgr. Stjepan Uldrijan, CSc.
1999 – 2007	Gymnázium Pardubice, Dašická 1083

PUBLIKACE

WNT Stimulation Dissociates a Frizzled 4 Inactive-State Complex with Gα12/13. *E. Arthofer, B. Hot, J. Petersen, **K. Straková**, S. Jäger, M. Grundmann, E. Kostenis, J. S. Gutkind a G. Schulte. Molecular Pharmacology, 2016.*

The tyrosine Y250 in Frizzled 4 defines a conserved motif important for structural integrity of the receptor and recruitment of Disheveled. ***K. Strakova**, P. Matricon, C. Yokota, E. Arthofer, O. Bernatik, D. Rodriguez, E. Arenas, J. Carlsson, V. Bryja, G. Schulte. Cellular Signalling, 2017.*

ÚČAST NA KONFERENCÍCH

WNT Meeting 2016, EMBO konference, Brno, Česká republika, 14.-17.9. 2016. **Poster:** Tyrosine 250 in Frizzled-4 intracellular loop 1 provides a mechanism for pathway selectivity in downstream signaling. *Katerina Strakova, Chika Yokota, Pierre Matricon, Ondrej Bernatik, Jakub Harnos, Ernest Arenas, Jens Carlsson, Vitezslav Bryja, Gunnar Schulte*

Cooperation with Karolinska Institute: KI-MU Closing Meeting, Brno, Česká republika, 22.-24.4 2015. **Přednáška:** Phosphorylation of Frizzled-4.

Gordon Research Molecular Pharmacology Conference & Seminar 2015, Ventura Beach Marriott, USA, 30.1.-6.2. 2015. **Poster:** The first intracellular loop of human FZD4 is important for selective recruitment of the phosphoprotein Disheveled. *Katerina Strakova, Gunnar Schulte*

Cooperation with Karolinska Institute: Kick Off Meeting, Brno, Česká republika, 25.-27.10. 2012.

The Student Scientific Conference on Cancer Research, Brno, Česká republika, 7.-8.4. 2011.

ODBORNÉ DOVEDNOSTI

schopnost samostatného plánování a vyhodnocování experimentů
in vitro kultivace buněčných kultur, transfekce, siRNA
reportérové eseje
transformace bakterií, izolace DNA
místně-cílená mutageneze, klonování, PCR, gelová elektroforéza
imunocytochemie, konfokální mikroskopie, FRAP
imunoprecipitace proteinů, SDS-PAGE, western blotting

SEZNAM PUBLIKACÍ

WNT Stimulation Dissociates a Frizzled 4 Inactive-State Complex with Gα12/13.
*E. Arthofer, B. Hot, J. Petersen, **K. Straková**, S. Jäger, M. Grundmann, E. Kostenis, J. S. Gutkind a G. Schulte.*
Molecular Pharmacology, 2016.

The tyrosine Y250 in Frizzled 4 defines a conserved motif important for structural integrity of the receptor and recruitment of Disheveled.
***K. Strakova**, P. Matricon, C. Yokota, E. Arthofer, O. Bernatik, D. Rodriguez, E. Arenas, J. Carlsson, V. Bryja, G. Schulte.*
Cellular Signalling, 2017