MASARYKOVA
UNIVERZITA

PŘÍRODOVĚDECKÁFAKULTA

The role of RING finger proteins in Wnt signalling

Disertačnípráce

TOMASZ RADASZKIEWICZ

Vedoucí práce: prof. Mgr. Vítězslav Bryja, Ph.D.

Animal physiology, immunology and developmentalbiology

Brno 2020

BIBLIOGRAFICKÝZÁZNAM

Autor: Tomasz Radaszkiewicz
Oddělení Fyziologie a Imunologie Živočichů
Ústavexperimentální biologie
Přírodovědecká fakulta
Masarykova univerzita

Název práce: The role of RING finger proteins in Wnt signalling

Studijní program: Fyziologie, imunologie a vývojová biologie živočichů

Vedoucípráce: prof. Mgr. VítězslavBryja,Ph.D.

Rok: 2020

Klíčová slova: RNF43, ZNRF3,WNT5A,melanoma,targetedtherapy,PCP, VANGL1, VANGL2, dishevelled

BIBLIOGRAPHIC RECORD

Author: Tomasz Radaszkiewicz
Section of Animal Physiology and Immunology
Department of Experimental Biology
Faculty of Science
Masaryk University

Title of Thesis: The role of RING finger proteins in Wnt signalling

Degree Programme: Animal physiology, immunology and developmental biology

Supervisor: prof. Mgr. Vítězslav Bryja, Ph.D.

Year: 2020

Keywords: RNF43, ZNRF3, WNT5A, melanoma, targeted therapy, PCP, VANGL1, VANGL2, dishevelled

ABSTRAKT

Wnt signální dráhy představují komplikovaný systém interakcí, které řídí různé funkce mnohobuněčných organismů. Úsilí vědecké komunity po celém světě vedlo k objevení základních mechanismů, ale mnoho dalších zůstává zatím stále neprobádaných. Patří k nim například řídící uzly mezi jednotlivými Wnt drahami a důsledky jejich aktivace, a proto jsem se v mé práci zaměřil na protein RNF43.

RNF43 je E3-ubikvitin ligáza z enzymatické rodiny R-TM-RING. RNF43 a jeho homolog ZNRF3 jsou podrobně studovány kvůli jejich uplatnění v udržení homeostázy a vzniku onemocnění. Hlavní funkcí RNF43/ZNRF3 v kontextu Wnt signalizace je negativní regulace množství receptorů Frizzled a koreceptorů LRP5/6 na povrchu buňky. Doposud nebylo jasné, zda extracelulární Protease Associated (PA) doména proteinu RNF43 je nezbytná pro jeho působení v lidském organismu. Z tohoto důvodu jsme se rozhodli detailně prostudovat vliv PA na funkci RNF43 proteinu. Pomocí aplikovaných metod jsem dokázali, že tato doména není nezbytná k inhibici kanonické Wnt signalizace zprostředkované RNF43. Předchozí práce naznačují možné zapojení RNF43 v další větvi Wnt signalizace zvané nekanonická Wnt dráha. Proto jsme se zaměřili na úlohu RNF43 v této dráze a sledovali jsme, zda se liší od kanonické větve. Abychom získali odpověď na tyto otázky, využili jsme metodu pro studium protein-proteinových interakcí, která je založena na označení proteinů v blízkosti proteinu zájmu biotinem. Naše poznatky jsme dále ověřili na buněčných modelech. Zapojení nekanonické Wnt dráhy je také zmiňováno v souvislosti s progresí nádorů a vznikem rezistence k cílené terapii melanomu a proto jsme testovali vliv působení RNF43 na buňky melanomu *in vitro* a *in vivo*. Kromě toho jsme také popsali nový mechanizmus RNF43 aktivace zprostředkovaný fosforylací CK1α a nečekané onkogenické vlastnosti zkrácené varianty RNF43, která se objevuje u nádorových buněk různého původu.

Výsledky získané během mého studia poskytují nový a komplexní pohled na mechanizmy RNF43 působící v lidských buňkách. Ustanovili jsme nové *in vitro* a *in vivo* modely, které mohou být využity v následných studiích této problematiky.

ABSTRACT

Wnt signalling pathway is a complicated system of interactions, which controls the various aspects of multicellular organism biology. Efforts of the scientific community worldwide lead to the description of its general action mechanism. Nevertheless, there is still blank space left and we need to understand better i.e. regulation points within multiple Wnt pathways and outcomes of their activity. Thus, in my work I investigated the protein called RNF43.

RNF43 is E3 ubiquitin ligase from the PA-TM-RING enzymatic family. RNF43 and its homolog ZNRF3 are being extensively studied because of their role in the various aspects of homeostasis and disease. The main function of RNF43/ZNRF3 in Wnt pathway is the negative regulation of the surface level of Wnt receptors Frizzleds and LRP5/6 co-receptors. Importantly, it is currently unclear whether extracellular Protease Associated domain of RNF43 is required for its action in human cells. This fact encouraged us to investigate in detail the role of PA fold in the RNF43 function. Thanks to applied approaches, we were able to demonstrate that this domain is not essential for the RNF43-mediated inhibition of the canonical Wnt signalling pathway. Next, the existing data suggest that RNF43 can be also involved in the other branch of Wnt pathway, called the noncanonical Wnt signalling. We were intrigued to ask if RNF43 can modulate it by specific mechanism, different than the one dedicated for the canonical axis. To address this question, we utilized proximity-dependent biotin identification method for protein-protein interactions detection. In the next step, we verified our key findings using cellular models. Further, noncanonical Wnt signalling is known to drive cancer progression and targeted therapy resistance in melanoma. Hence, we tested the impact of RNF43 action on the several aspects of melanoma cell properties *in vitro* and *in vivo*. Lastly, we described the new mechanism of RNF43 activation by CK1α-mediated phosphorylation and unexpected oncogenic properties of RNF43 truncated variant occurring in various cancer types.

Results gathered during my studies provide novel and comprehensive insights into the RNF43 mechanistic mode of action in human cells. We established new *in-vitro* and *in-vivo* models which can be utilised in the follow-up studies.

Contents

1 AIMS

The aim of this study was to describe better the RNF43 E3 ubiquitin ligase. As it was portrayed in the introductory part, RNF43 is relatively well understood protein with important roles in development and cancer. Nevertheless, several key issues are unsolved and in my thesis, together with collaborators, we decided to face the following problems:

- What is the role of the Protease Associated domain of RNF43 in the Wnt signalling suppression in human cells?

- Does RNF43 specifically target and inhibit WNT5A-driven signalling pathway? What is the mechanism behind it? Does it have pathophysiological significance?

- Can we identify protein-protein interaction mediated by the intracellular part of RNF43 and find new targets of this E3 ligase?

- Is RNF43 post-translationally modified? What is the function of these modifications?

2 RESULTS AND DISCUSSION

2.1 LIST OF PUBLICATIONS INCLUDED IN THESIS

1) Article:

Radaszkiewicz T, Bryja V. *Protease associated domain of RNF43 is not necessary for the suppression of Wnt/β-catenin signaling in human cells*. Cell Commun Signal. 2020 Jun 11;18(1):91. doi: 10.1186/s12964-020-00559-0.

Total contribution estimated to 90%.

2) Manuscript:

Radaszkiewicz T., Nosková M., Gömöryová K., Vondálová Blanárová O., Radaszkiewicz KA., Picková M., Gybeľ T., Kotrbová A., Verner J., Kaiser K., Kaiser L., Fedr R., Demková L., Kučerova L., Potěšil D., Zdráhal Z., Souček K., Bryja V. *RNF43 directly inhibits WNT5A driven signaling and suppresses melanoma metastasis.*

Total contribution estimated to 70%.

3) Article (*in press*, Article accepted on 22 June 2020):

Spit M., Fenderico F., Jordens I., **Radaszkiewicz T.**, Lindeboom RGH., Bugter JM., Ootes L., Osch MV., Janssen E., Boonekamp KE., Hanakova K., Potesil D., Zdrahal Z., Boj SF., Medema JP., Bryja V., Koo BK., Vermeulen M., Maurice MM. *RNF43 truncations trap CK1 to drive niche-independent self-renewal in cancer.* The EMBO Journal doi:10.15252/embj.2019103932

Total contribution estimated to 20%

2.2 OTHER PUBLICATIONS

1) Kotrbová A, Ovesná P, Gybel' T, **Radaszkiewicz T**, Bednaříková M, Hausnerová J, Jandáková E, Mírná A, Crha I, Weinberger V, Záveský L, Bryja V, Pospíchalová V. *WNT signaling inducing activity in ascites predicts poor outcome in ovarian cancer*. Theranostics. 2020 Jan 1;10(2):537-552.
2) Harnoš J, Cañizal MCA, Jurásek M, Kumar J, Holle C, Schambony A, Hanáková K, Bernatík O, Zdráhal Z, Gömöryová K, Gybel T, **Radaszkiewicz TW**, Kravec M, Trantírek L, Rynešová J, Dave Z, Fernández-Llamazares AI, Vácha R, Tripsianes K, Hoffmann C, Bryja V. *Dishevelled-3 conformation dynamics analyzed by FRET-based biosensors reveals a key role of casein kinase 1*. Nat Commun. 2019 Apr 18;10(1):1804.
3) Kaiser K, Gyllborg D, Procházka J, Salašová A, Kompaníková P, Molina FL, Laguna-Goya R, **Radaszkiewicz T**, Harnoš J, Procházková M, Pešíl D, Barker RA, Casado ÁG, Zdráhal Z, Sedláček R, Arenas E, Villaescusa JC, Bryja V. *WNT5A is transported via lipoprotein particles in the cerebrospinal fluid to regulate hindbrain morphogenesis*. Nat Commun. 2019 Apr 2;10(1):1498.
4) Mentink RA, Rella L, **Radaszkiewicz TW**, Gybel T, Betist MC, Bryja V, Korswagen HC. *The planar cell polarity protein VANG-1/Vangl negatively regulates Wnt/β-catenin signaling through a Dvl dependent mechanism.* PLoS Genet. 2018 Dec 7;14(12):e1007840.
5) Lee Y, Kim NH, Cho ES, Yang JH, Cha YH, Kang HE, Yun JS, Cho SB, Lee SH, Paclikova P, **Radaszkiewicz TW**, Bryja V, Kang CG, Yuk YS, Cha SY, Kim SY, Kim HS, Yook JI. *Dishevelled has a YAP nuclear export function in a tumor suppressor context-dependent manner*. Nat Commun. 2018 Jun 12;9(1):2301.
6) Janovska P, Verner J, Kohoutek J, Bryjova L, Gregorova M, Dzimkova M, Skabrahova H, **Radaszkiewicz T**, Ovesna P, Vondalova Blanarova O, Nemcova T, Hoferova Z, Vasickova K, Smyckova L, Egle A, Pavlova S, Poppova L, Plevova K, Pospisilova S, Bryja V. *Casein kinase 1 is a therapeutic target in chronic lymphocytic leukemia*. Blood. 2018 Mar 15;131(11):1206-1218.
7) Fatima I, El-Ayachi I, Taotao L, Lillo MA, Krutilina RI, Seagroves TN, **Radaszkiewicz TW**, Hutnan M, Bryja V, Krum SA, Rivas F, Miranda-Carboni GA. *The natural compound Jatrophone interferes with Wnt/β-catenin signaling and inhibits proliferation and EMT in human triple-negative breast cancer*. PLoS One. 2017 Dec 27;12(12):e0189864.
8) Paclíková P, Bernatík O, **Radaszkiewicz TW**, Bryja V. *The N-Terminal Part of the Dishevelled DEP Domain Is Required for Wnt/β-Catenin Signaling in Mammalian* Cells. Mol Cell Biol. 2017 Aug 28;37(18):e00145-17.
9) Bernatik O, **Radaszkiewicz T**, Behal M, Dave Z, Witte F, Mahl A, Cernohorsky NH, Krejci P, Stricker S, Bryja V. *A Novel Role for the BMP Antagonist Noggin in Sensitizing Cells to Non-canonical Wnt-5a/Ror2/Disheveled Pathway Activation*. Front Cell Dev Biol. 2017 May 4;5:47. doi: 10.3389/fcell.2017.00047.
10) Kaucká M, Petersen J, Janovská P, **Radaszkiewicz T**, Smyčková L, Daulat AM, Borg JP, Schulte G, Bryja V. *Asymmetry of VANGL2 in migrating lymphocytes as a tool to monitor activity of the mammalian WNT/planar cell polarity pathway*. Cell Commun Signal. 2015 Jan 28;13:2.

2.3 The role of the Protease Associated domain in human RNF43 function (Article 1)

My work resulted in the demonstration that the absence of PA domain does not impede the ability of RNF43 to inhibition of the β-catenin dependent WNT3A-induced signalling (Fig. 1). Rescue experiments confirmed that PA domain function is to control the activity and plasma membrane level of RNF43 via RSPO-regulated signalling axis. In other words, RNF43 has in-built “switch”, by which canonical Wnt signalling level of activity can be fine-tuned by secreted factors. We also generated toolbox of inducible cell lines that can be used in other studies.

We prepared cellular models inducibly overexpressing RNF43 wild type and RNF43ΔPA mutant in human Hek293 T-REx cell line. RNF43 wild type and variant lacking PA domain efficiently blocked cellular responses to the recombinant WNT3A treatments, as it was shown in various assays to ascertain specificity of the findings. Moreover, we asked if RNF43 and RNF43ΔPA are able to perform their inhibitory actions in following signalling conditions:

- receptors activated by rWNT3A treatment prior to RNF43 and RNF43ΔPA induction
- receptors inactive due to the inhibition of endogenous Wnt secretion, followed by the stimulation of canonical Wnt pathway
- mixed model, where RNF43/RNF43ΔPA expression induction and WNT3A treatment were performed simultaneously

In our studies, we demonstrate that the absence of PA domain does not impede the ability of RNF43 to the β-catenin dependent WNT3A-induced signalling inhibition. Rescue experiments confirmed that PA domain function is to control the activity and plasma membrane level of RNF43 via RSPO-regulated signalling axis. In other words, RNF43 has in-built “switch”, by which canonical Wnt signalling level of activity can be fine-tuned by secreted factors. We also generated toolbox of inducible cell lines that can be used in other studies.

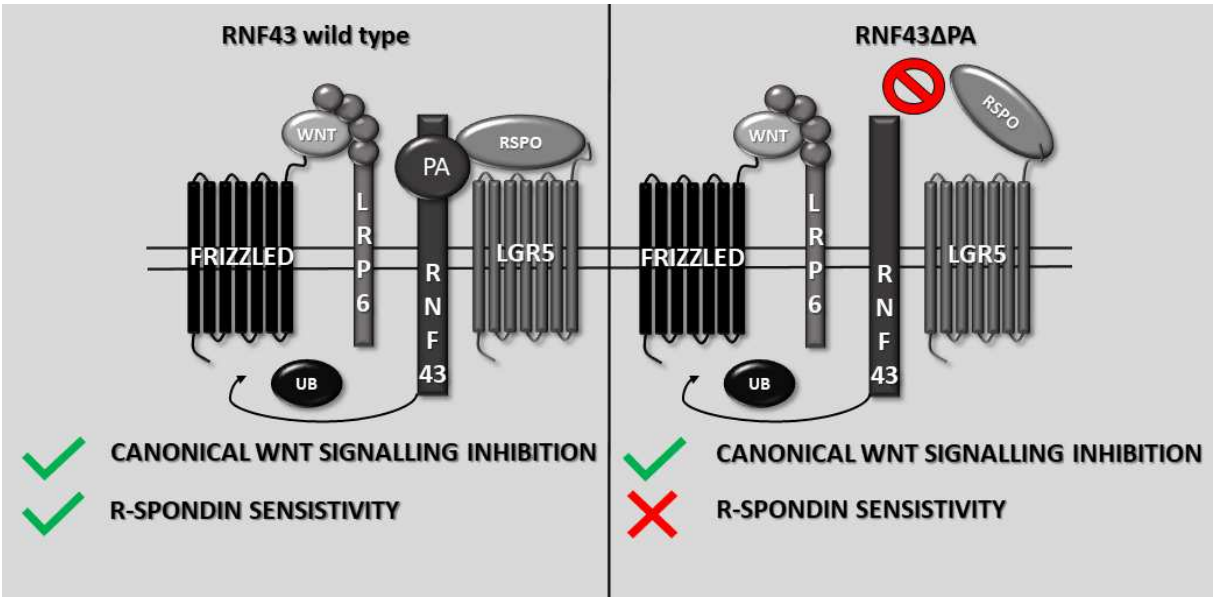

Fig. 1. Protease associated domain of RNF43 binds RNF43 and regulates its cell surface level. RNF43 lacking PA domain inhibits canonical Wnt signalling pathway.

2.4 RNF43 IN WNT5A-DRIVEN WNT SIGNALLING (MANUSCRIPT 2)

We showed that (i) RNF43 interacts with core components of the noncanonical Wnt signalling, (ii) suppresses WNT5A-driven signalling on the multiple levels, (iii) controls the surface level of WNT5A receptor ROR1, (iv) leads to the VANGL2 degradation and DVL isoforms ubiquitination and inactivation. Thereby, we confirm and extend previously described *C. elegans*-, *Xenopus*- and mice- based observations underlying the involvement of RNF43/ZNRF3 in the WNT5A-dependent signalling and Wnt/PCP protein trafficking. WNT5A/RNF43 module involves formation of interactions that are mediated by the intracellular part of RNF43 and RING domain enzymatic activity.

2.5 RNF43 IN MELANOMA (MANUSCRIPT 2)

To validate the importance of our findings in the pathophysiologic conditions, we decided to study the impact of RNF43 on melanoma cell biology. We chose to do that, because database screening showed that low expression of *RNF43* gene is a negative overall survival factor of melanoma patients. Also, its transcript level decreases with cancer progression and Wnt/PCP plays an important role in melanoma. Contrary, high expression of *VANGL1* and *DVL3* genes, encoding direct RNF43 targets, are pro-metastatic factors. These pieces of information encouraged us to perform the further studies. For validation of our hypothesis, we employed broadly used

A375 cells as a model. BioID-mediated analysis of RNF43 interactome (article 3) was performed in T-REx 293 cells, so we needed to validate results from the first part of this manuscript. We generated toolbox of melanoma cell lines overexpressing exogenous *RNF43* and *RNF43*/*ZNRF3* double knockouts. In accordance with the previously descried results (3.2), RNF43 downregulated DVL2 and DVL3 phosphorylation and desensitised cells to the recombinant WNT5A treatment. In functional assays, cells overexpressing RNF43 showed decreased migratory and invasive properties. Lastly, we confirmed the role of the noncanonical Wnt pathway on the melanoma response to the *BRAF* V600E targeted therapy, which is a crucial issue in melanoma treatment. Simply, cells overexpressing RNF43 did not develop resistance to BRAF V600E inhibitor. In summary, RNF43 plays an oncosupressive function in case of this deadly skin cancer.

RNF43 inhibits invasive properties of melanoma cells in vivo

Our in vitro research showed that A375 melanoma cells overexpressing RNF43 had impaired Wnt/PCP pathway, they were not sensitive to the WNT5A treatment and displayed decreased migratory and invasive properties (manuscript 2). Consequently, we postulated that these attributes might be responsible for the oncosuppressive activity of RNF43 in melanoma. To verify our hypothesis and increase the biological meaning of our studies, together with our collaborators from the laboratory of Mgr. Karel Souček Ph.D., we developed in vivo melanoma model. Human melanoma cell line A375 based for the generation of cell line series with different metastatic potential. This includes i.e. the highly metastatic A375M derivate obtained from the lung tumor nodules (Kozlowski et al, 1984). Here, we employed A375 IV-EGFP cell lines with increased ability for formation of lungs metastasis. It was established by the three rounds of subcutaneous injection and cell isolation in the athymic nude mice background (Kucerova et al, 2014). We additionally modified A375 IV-EGFP parental cells to express luciferase for bioluminescence imaging. Also, we transduced these cells with the lentiviral vector allowing doxycycline-controlled expression of RNF43 cDNA fused with HA and FLAG tags. Our novel in vivo orthotopic model of metastatic melanoma mimicking all steps of melanoma progression–tumor formation in the skin, spontaneous dissemination and metastatic outgrowth. Thus, it makes it suitable for our studies and can be used by others as i.e. preclinical drugs screening platform.

2.6 RNF43 FUNCTION IS CONTROLLED BY THE CK1A-MEDIATED PHOSPHORYLATION AND RNF43 R519X TRUNCATED MUTANT SHOWS AND UNUSUAL ONCOGENIC ACTIVITY (ARTICLE 3)

This work is a result of our collaboration with the group of prof. Madelon Maurice from University Medical Center Utrecht. For the purpose of this publication, I prepared BioID assay to identify RNF43 intracellular interactome. Together with colleagues from prof. Zbyněk Zdráhal laboratory, we analysed phosphorylation of RNF43 wild type and RNF43 R519X truncated mutant.

Our study shows important findings helping to extend our knowledge about the canonical Wnt signalling mechanism of action. We presented that CK1α can act also upstream from the destruction complex to mediate negative regulation of the Frizzled receptors at the cell surface by RNF43. Moreover, RNF43 links two mechanisms regulating the β-catenin dependent genes expression – cells desensitisation to Wnt ligands and facilitation of β-catenin degradation. Interaction of the wild type protein with destruction complex requires further studies. These findings are presented in the Figure 2

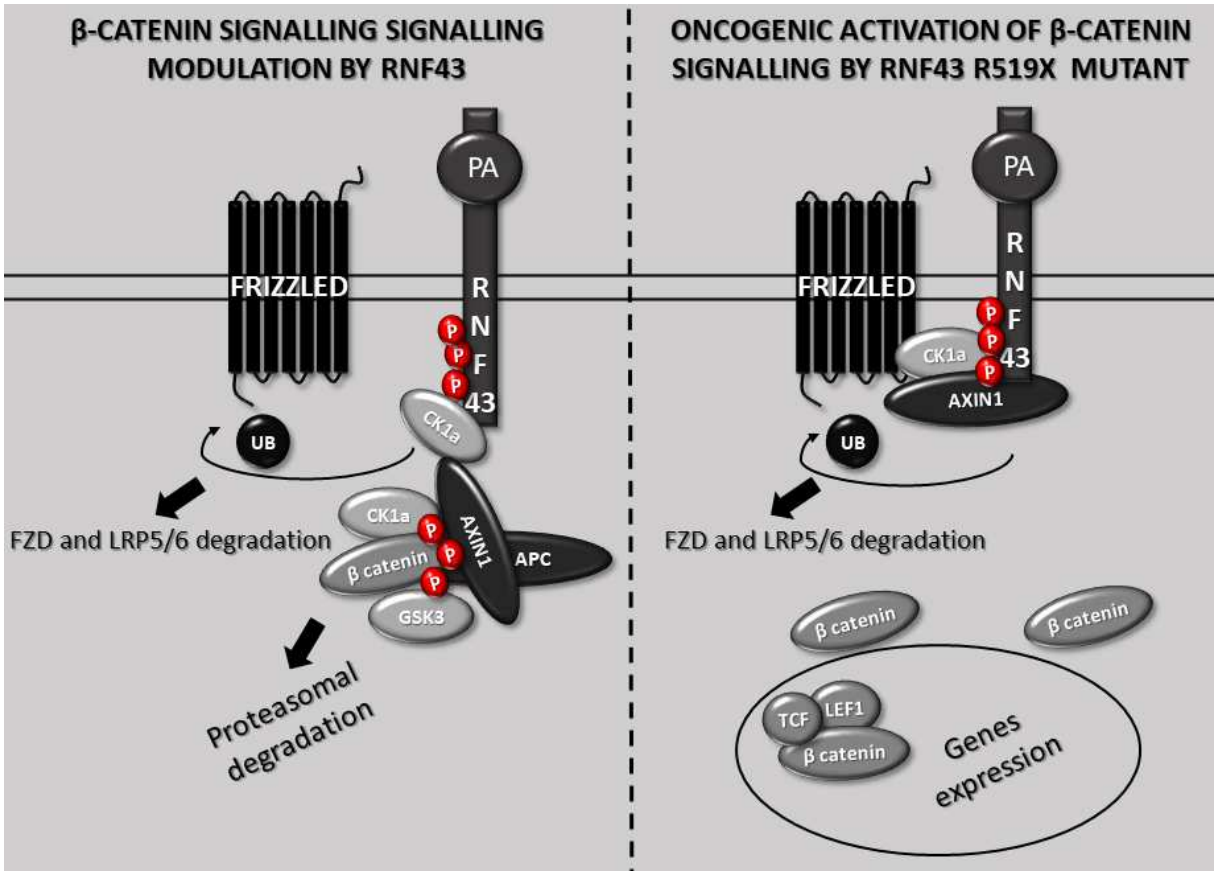

Fig. 2. Novel insight into the RN43 mode of action, in which CK1α plays major regulatory function (left). RNF43 R519 oncogenic mutant binds CK1α more efficiently and traps destruction complex components on the plasma membrane.

3 CONCLUSIONS

We have showed that:

- Protease Associated domain of RNF43 facilitates the RSPO1-mediated cellular surface level regulation of this E3 ubiquitin ligase, but it is dispensable for the direct negative regulation of the canonical Wnt signalling. We believe that this work sorted out long standing discrepancies in the field.

- RNF43 binds the multiple core components of the noncanonical Wnt pathway and desensitises cells to the WNT5A ligand.

- VANGL1, VANGL2, DVL1 and DVL2 are RNF43 substrates

- RNF43 downregulates the ROR1 cell surface protein level

- RNF43 inhibits invasive properties of melanoma cells

- RNF43 blocks acquired resistance to *BRAF V600E* targeted therapy in melanoma

- We have developed new *in vivo* model for melanoma studies, which mimics all steps of this skin cancer progression

- RNF43 R519X truncated mutant activates canonical Wnt signalling in the Wnt ligand independent manner by trapping destruction complex components and RNF43 is phosphorylated by CK1α and CK1ε. These PTMs are needed for RNF43 wild type and truncated variant functions in the canonical Wnt signalling.

Curriculum Vitae

Name, Personal Data

Education

Research Experience

Teaching Experience