



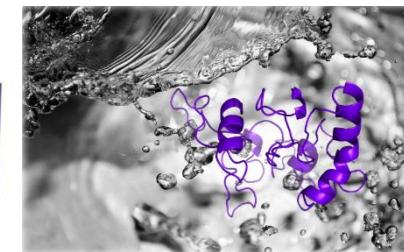
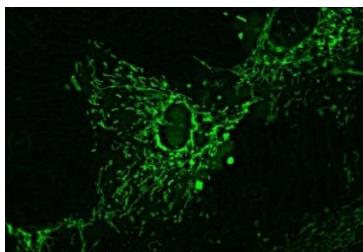
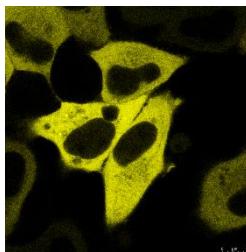
Laboratorij za biokemiju proteina i molekulsко modeliranje

Institut Ruđer Bošković

Ana Tomašić Paić
(viši stručni suradnik)

„Bioimaging of DPP3-SH2D3C Protein Interactions in Living Cells Using BiFC Method”

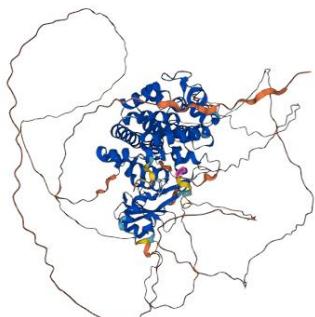
Projekti: „DPP3 BioRe“ (Sanja Tomić)
„OxMiLink“ (Mihaela Matovina)



SH2 domain-containing protein 3C · Homo sapiens (Human) · Q8N5H7; 860 aa



Predviđena struktura SH2D3C
prema AlphaFold-u
(AF-Q8N5H7-F1, UniProt)



*imena: *Cas/HEF1-associated signal transducer 1* ([Chat-H 1](#)); *SH2 domain-containing Eph receptor-binding protein 1* ([SHEP1](#)), *novel Src homology 2-containing protein* ([NSP3](#))

*otkiven analizom interaktoma DPP3 ([SILAC MS](#)); potencijalni interaktor proteina **DPP3**



*ima dvije domene; SH2 (220-319); Ras-GEF (586-854) domenu sličnu domeni faktora razmjene gvanin nukleotida za Ras obitelj GTPaze; te *intrinsically disordered regions* (51-117; 130-180; 335-537); regiju bogatu prolinom/serinom s potencijalnim mjestima fosforilacije pomoću prolin- usmjerenih kinaza

*6 isoformi /UniProt

*proteini iz NSP porodice: SH2D3A, BCAR3 (SH2D3B), SH2D3C ulaze u interakciju s proteinima iz porodice Cas putem svoje RasGEF slične domene

*SH2D3C ulazi u interakciju s EphB2 fosforil. domenom proteina te s proteinima R-Ras i Rap1A (porodica malih Ras GTPaza), no ne pokazuje GTPaznu aktivnost *in vitro*

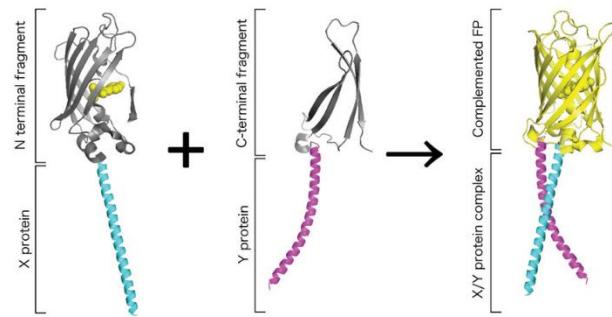
*BCAR3 i SH2D3C: inaktivna Ras GTPaza

*sudjeluje u regulaciji stanične migracije i adhezije, organizacije tkiva i regulaciji imunog odgovora

*SH2D3C protein kodira SH2D3C gen smješten na 9. kromosomu

Komplementacija dviju molekula fluorescencijom

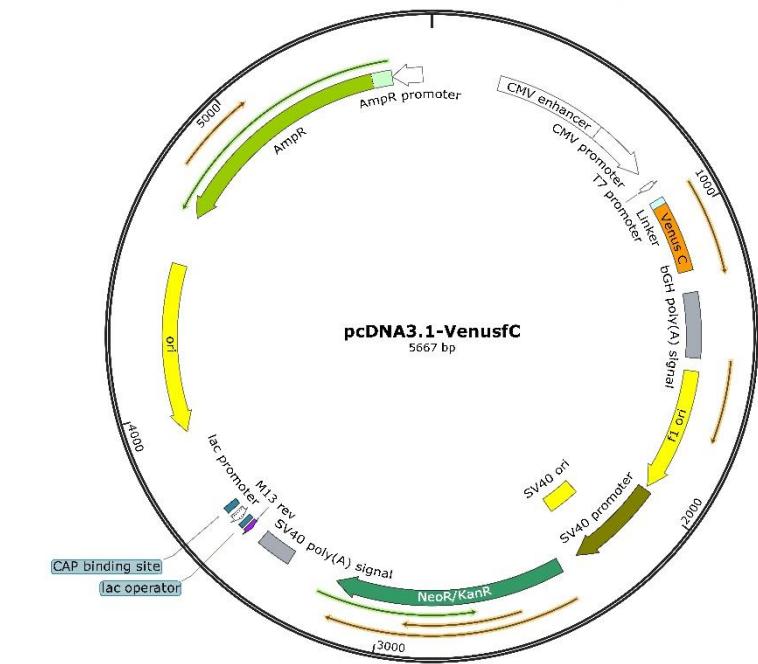
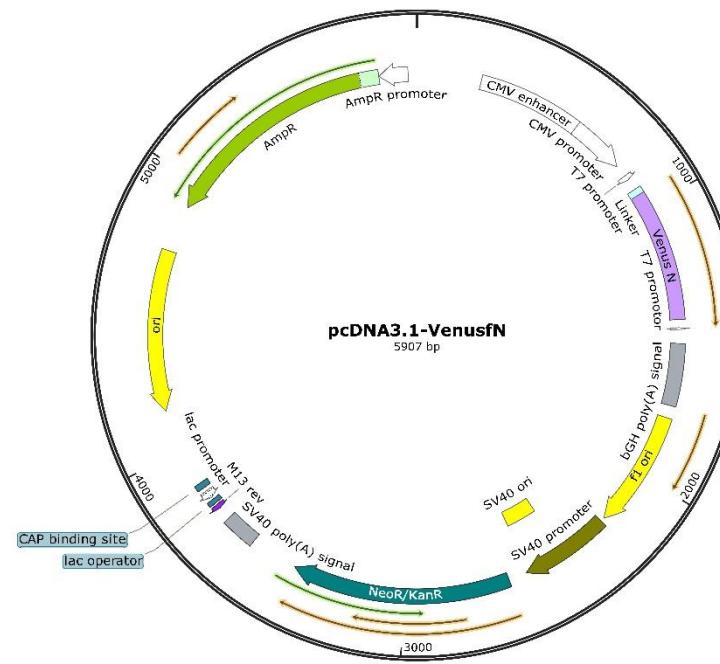
Princip rada BiFC metode



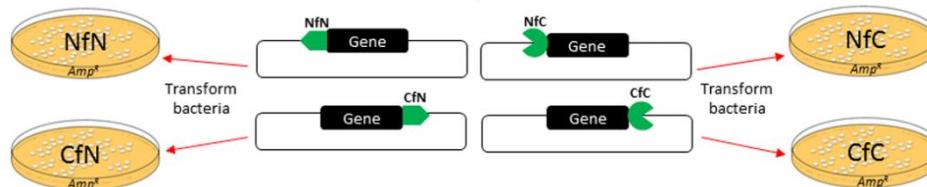
Venus yellow fluorescent protein

(PDB ID: 1MYW from RCSB Protein Data Bank)
Ex=515 nm; Em=528 nm

Ishodišni vektori (za potrebe kloniranja i konstrukciju BiFC plazmida)

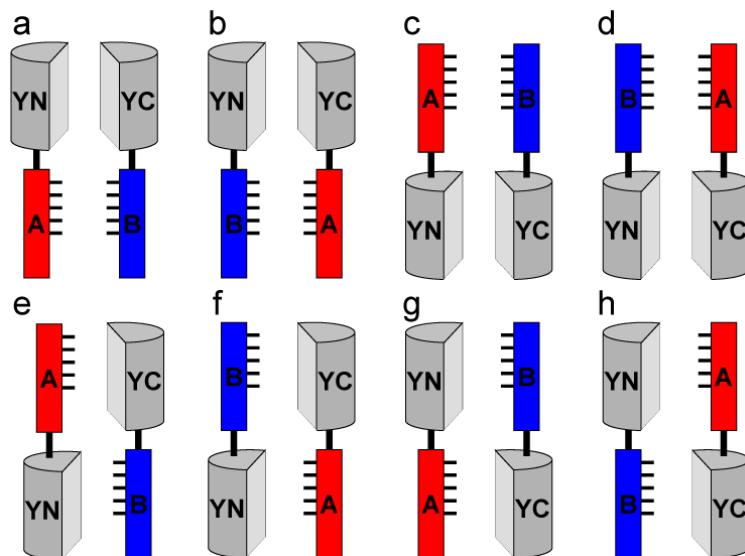


Prikaz mogućih topologija (NfN, NfC, CfN, CfC)



*Lepur A. et al. (2016) *Journal of Biomolecular Screening*

DOI: 10.1177/1087057116659438



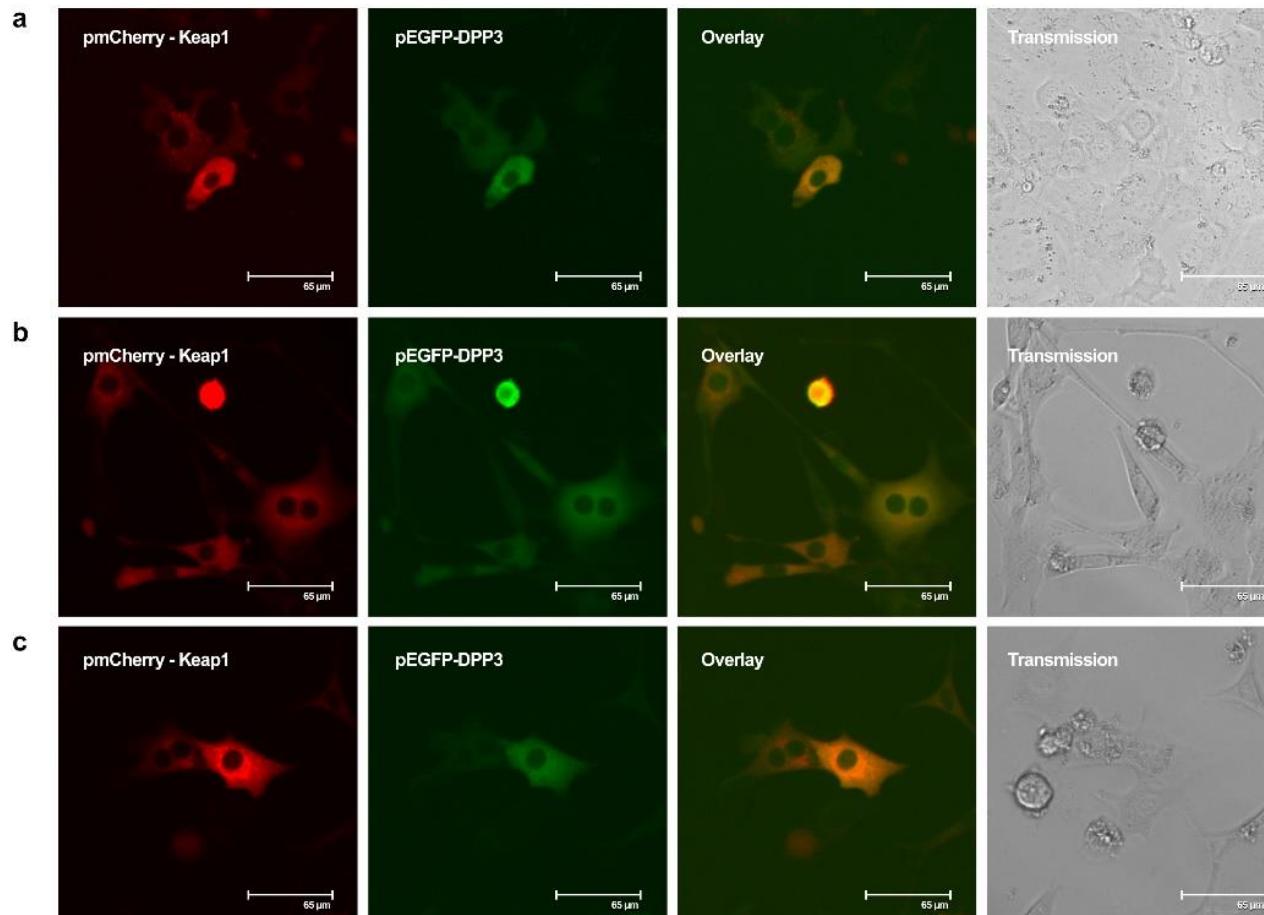
Kombinacije potencijalnih interakcija
(pozitivna kontrola):

VenusfN-DPP3 VenusfC-Keap1
VenusfC-DPP3 Keap1-VenusfC
DPP3-VenusfN VenusfN-Keap1
DPP3-VenusfC Keap1-VenusfN

*Nat Protoc. 2006 ; 1(3): 1278–1286.

Live-cell istraživanja substanične (ko)-lokacije pmCherry-Keap1 i pEGFP-DPP3 plazmida primjenom EVOS Floid uređaja

NIH3T3 cell line

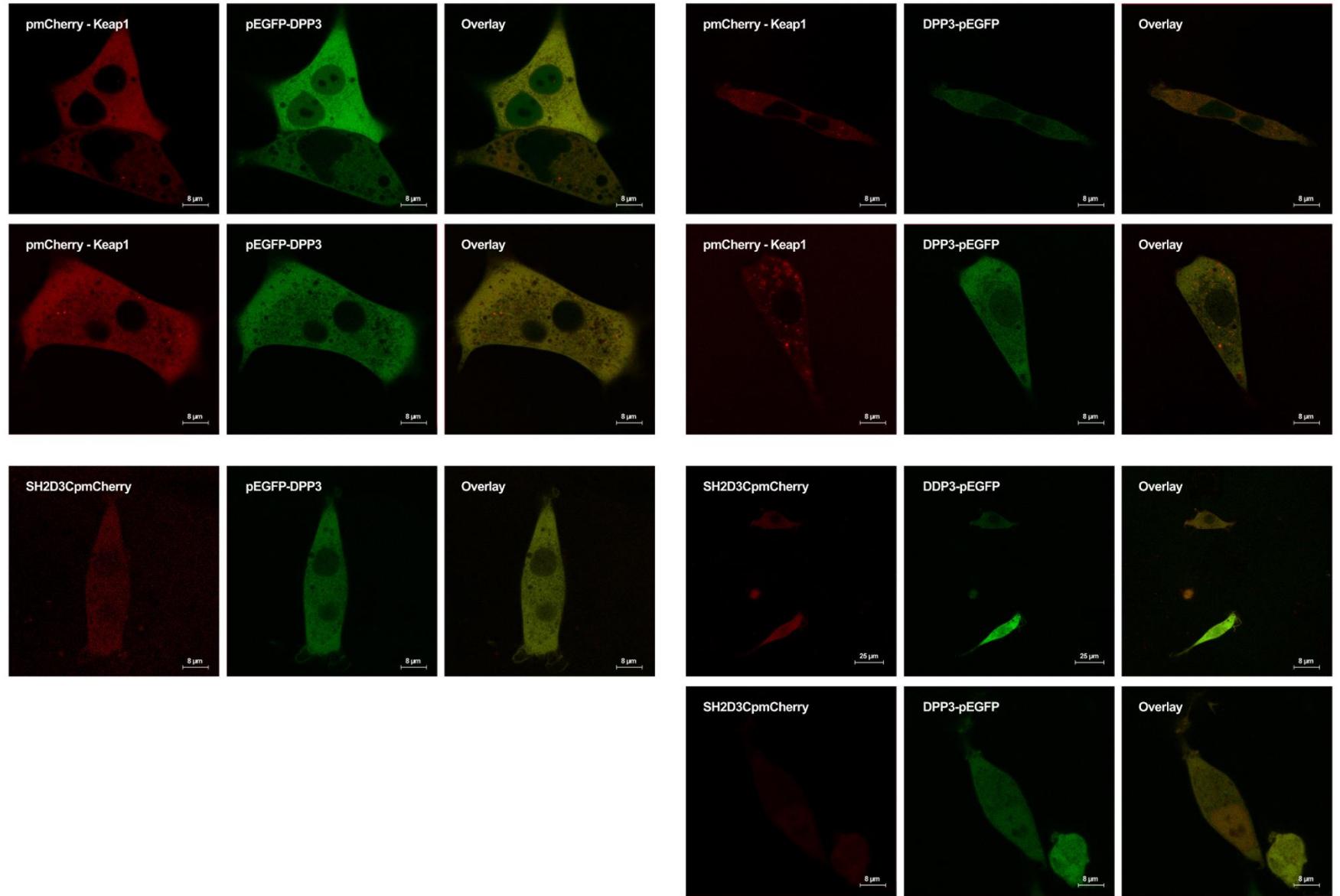


(a) nakon 24 h inkubacije
(b,c) nakon 48 h inkubacije

Live-cell istraživanja
primjenom SP8 X FLIM

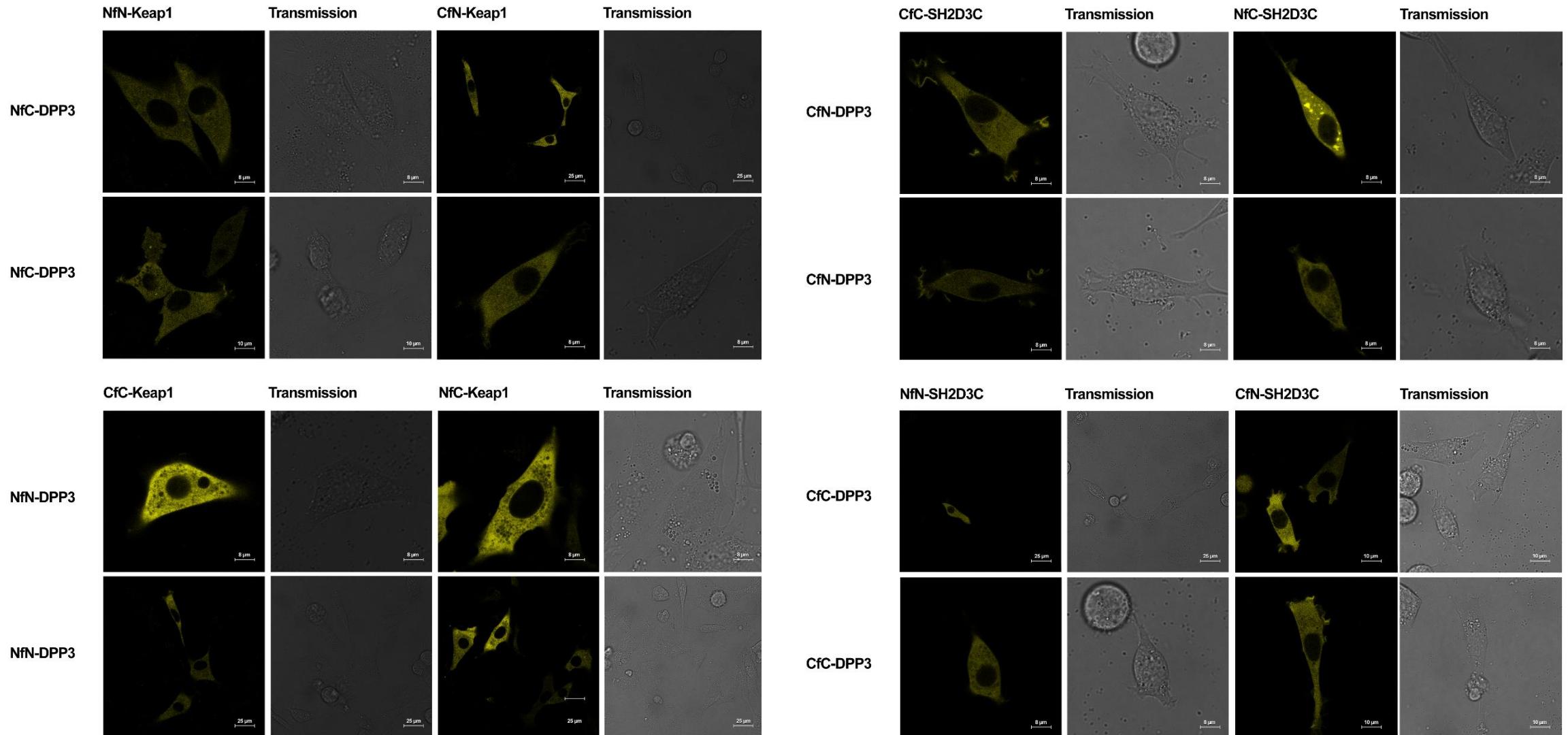
NIH3T3 cell line

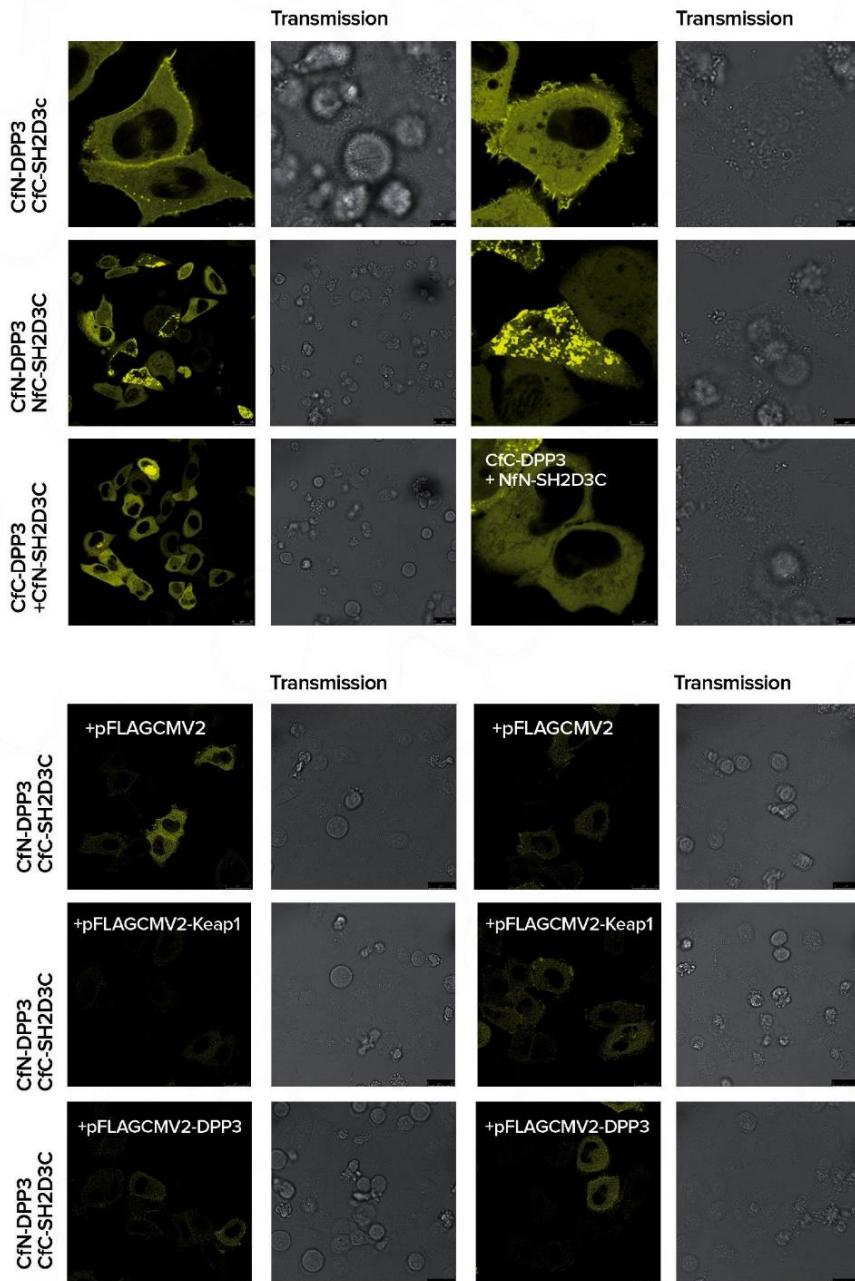
nakon 24 h
inkubacije



IBL, 4 chamber glass
bottom dish, dish size
35mm, well size 20mm

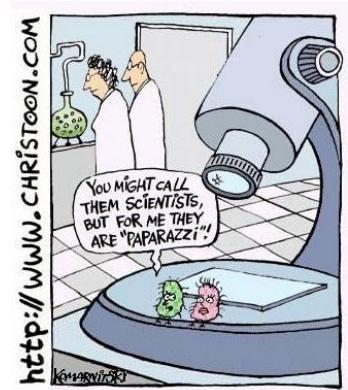
Live-cell istraživanja komplementacije dviju molekula fluorescencijom primjenom SP8 X FLIM

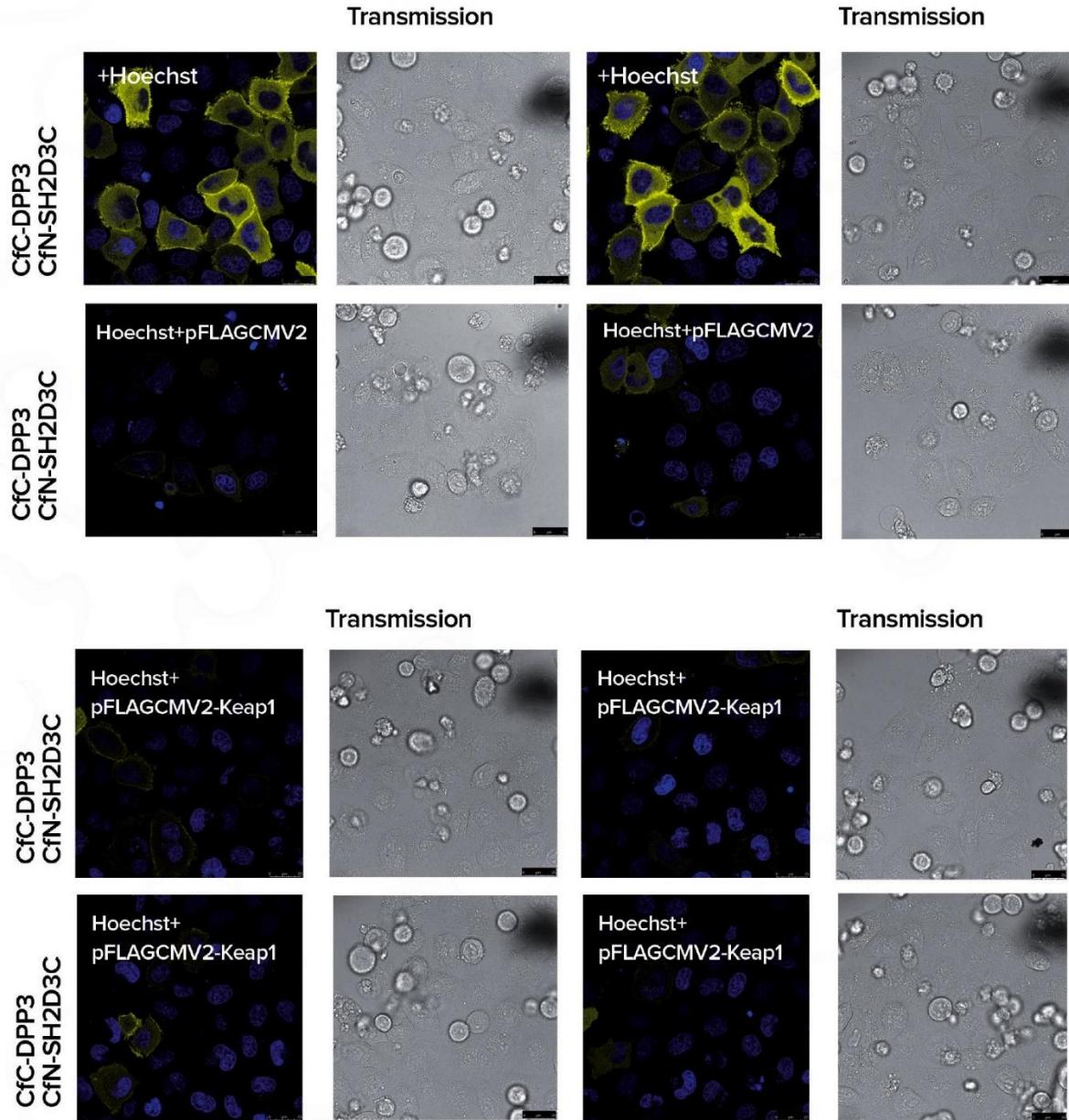




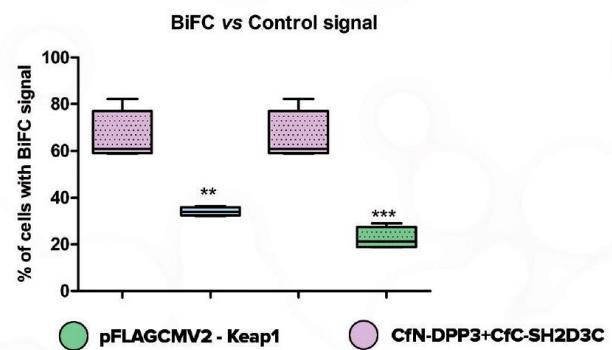
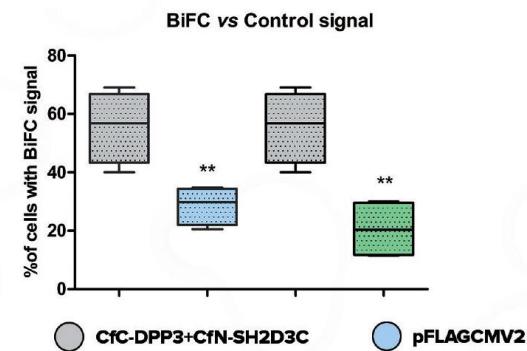
BiFC interakcijska analiza
(kompetitivni eksperiment)
HeLa stanice

Suradnici:
Lucija Horvat
Adriana Lepur
Katja Ester





BiFC screen
(kompeticijski eksperiment)



**ISFMS
2022**

4th ISFMS—Biochemistry, Molecular Biology and Druggability of Proteins

6–9 Sep 2022, Florence, Italy



Live cell imaging of DPP3 and SH2D3C protein interaction using bimolecular fluorescence complementation

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Division of Molecular Biology²

Introduction

Protein-protein interactions (PPIs) are the basis for most biological and signaling systems. Studies of PPIs within living organisms have provided important insights into the mechanisms of disease development, particularly leading to defects of complex membrane proteins. In order to identify co-interacting PPIs in *vitro* we have used BFC (Bimolecular Fluorescence Complementation approach), a non-invasive, fluorescence-based technique usually applied for the detection of protein-protein interactions in living cells or tissues. We have previously shown that the DPP3 protein *in vivo*, we have confirmed the interaction of two proteins in mouse embryo fibroblast (MEF) 3T3 and HEK293T cells using the BFC-domain-containing peptide (BCP) (DPP3-BCP) and recombinant peptide 3 (RCP) (SH2D3C-RCP) as substrates. Interactions were detected by confocal microscopy using fluorescence microscopy images of cells expressing BCP and RCP fusions in cell migration. The primary co-activator analysis of the EGFR-DPP3 and SH2D3C-RCP fusions in NIH 3T3 and HEK 293T cells revealed that the BCP fusion protein interacts with the cytoskeleton and on the membrane (in membrane raft) indicating the possible involvement in the last step of cell migration.

(A)

Lower domain Upper domain Five-stranded β-barrel Zinc binding motifs

(B)

Disordered region S442 Res-GFP

Fig. 1 Structure of the human DPP3 (Q9H3C3). **(A)** Structure of the DPP3 protein. Human carboxyl esterase DPP3, primarily a membrane cysteine protein, is composed of two domains. Upper (blue) hydrophilic, contains the zinc binding motifs and the Res-GFP domain. Lower (green) hydrophobic, contains the disordered region, the S442 residue and a five-stranded β-barrel. **(B)** Domains of the DPP3 protein. Two chemins are shown by a wavy line that marks the possible binding site. The associated SH2D3C-RCP construct has a N-terminal GFP domain, C-terminal domain with a week, but sufficient homology with CD40-like Res-GFP domains, and a proline-rich RA region in between with many potential sites for phosphorylation by protein kinases/kinases.

Fig. 2 Subcellular localization.

NIH 3T3 cells were transiently transfected with BCP-DPP3 and RCP-SH2D3C-mCherry or pDPP3-DPP3 in NIH 3T3 cells [pHEV 24 kDa (lambda)]. Cells were visualized using confocal microscopy. The yellow color indicates the BCP fusion protein, the red color indicates the RCP fusion protein, and the green color indicates the endogenous DPP3 protein. The scale bar is 20 μm. **(A)** Confocal microscopy. The visible cell confluency is 30%. **(B)** Cell fractionation. The ratio of DNA (μg) to LTX (μg) was 10.

Fig. 3 Confocal fluorescence images of HEK293T cells transiently transfected with BFC vectors expressing DPP3 with GFP-tag and DPP3 with SH2D3C. Fluorescence control and transmission images are represented. The ratio of DNA (μg) to LTX (μg) was 12.

Results
Based on the FACS analysis data not shown here, NIH 3T3 cells showed no response for BFC assays due to low transfection efficiency (37%), and the experiments were continued with HEK 293T cells (70% of trans.). Considering the lack of the detectable cell migration with mutations in the interacting regions of the DPP3 protein, we have chosen the BCP fusion protein as a substrate for the BFC peptides or a fusion protein as a known binding partner of one of the surveyed proteins (DPP3). The BCP fusion protein was expressed in NIH 3T3 cells and HEK 293T cells and visualized *in vivo* by confocal microscopy that were co-transfected with BCP fusions and stably transfected HEK 293T cells with a strong bright BPC signal was around 60%. In case of the co-transfected HEK 293T cells with BCP-DPP3 and RCP-SH2D3C protein we observed a significant increase in the BCP signal compared to a control without a fusion protein. The BCP signal was also increased in NIH 3T3 cells when stably transfected with DPP3-S242 and visualized *in vivo* in 4 fields. The difference between the BCP signal in NIH 3T3 cells with DPP3-S242 and without it was statistically significant ($p < 0.05$). The same results were obtained when two stable HEK 293T cells in 100% and were considered to be equivalent at a p value of <0.05 . Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA).

Fig. 4 BFC protein interaction analysis.

NIH 3T3 cells were transiently transfected with BCP-DPP3 and RCP-SH2D3C-mCherry in combination with RCP-SH2D3C or NC-SH2D3C, or DPP3-BCP in combination with NC-SH2D3C or NC-SH2D3C-RCP. The BCP fusion protein was visualized by confocal microscopy. The visible cell confluency was determined with the assistance of phalloidin-stained fibroblasts detected with the assistance of phalloidin-phAGM95-RFP and rFP-MAC202-295B constructs.

(A) NIH 3T3 cells
(B) HEK293T cells

Fig. 5 BFC sensor (BFS) Confocal fluorescence images of HEK293 cells transiently transfected with BFC vectors. **(A)** BCP fusion protein. **(B)** BCP fusion protein with mutations. **(C)** Two stable HEK 293T cells that was apical and means were found to be statistically significant ($P < 0.05$).

Conclusion
Based on the BFC assay data showed us an efficient visualization of the protein interaction between DPP3 and SH2D3C protein system as well as the identification of the protein subcellular location of the protein complex, in cytoplasm and membrane raft of living cells. Thus, we lacked the adequate reagent for the detection of the BCP fusion protein in living cells. The BCP fusion protein was used for BFC assays, and confirmed decline of the BFC signal that were found to be statistically significant.

Acknowledgments

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References:
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Macia PD, et al. (2011) DOI: 10.1002/1365-2427.13837.
Lopez A, et al. Methods Mol Biol. (2016) 1354:229–237.

Projekt Mihaele Matovine: OxMiLink



Hvala na pažnji