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

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Introduction

Protein-protein interactions (PPIs) are the basis for human metabolic and signaling systems. Studies of PPIs within living mammalian cells may provide useful information for deeper understanding of the functional relationships and mechanisms between proteins, eventually leading to decoding of complex protein networks. In order to directly visualize PPIs *in vivo* we have used BiFC (Bimolecular Fluorescence Complementation) approach, a noninvasive, fluorescent-based technique crucial for determining the subcellular locations of the interacting proteins. Based upon the reconstitution of a fluorescent protein *in vivo*, we have confirmed the interaction of two proteins in mouse embryo fibroblast (NIH 3T3) and adenocarcinoma (HeLa) cells; SH2 domain-containing protein 3C (SH2D3C) and dipeptidyl peptidase 3 (DPP3). DPP3 is a ubiquitous, intracellular Zn-dependent protease, a cytosolic enzyme known to cleave dipeptides from amino-termini of 3-10 aa long peptides *in vitro*. Through the known interaction with KEAP1, DPP3 is involved in the regulation of oxidative stress response through Nrf2/KEAP1 signaling pathway. SH2D3C protein acts as an adapter protein involved in the regulation of cell adhesion and cell migration, tissue organization, and the regulation of the immune response. The preliminary co-localization analysis of the EGFP-DPP3 and SH2D3C-mCherry in NIH 3T3 and HeLa cells showed that two chimeric proteins co-localize in cytoplasm and on the membrane (in membrane ruffles) indicating the possible involvement in the control of cell migration.

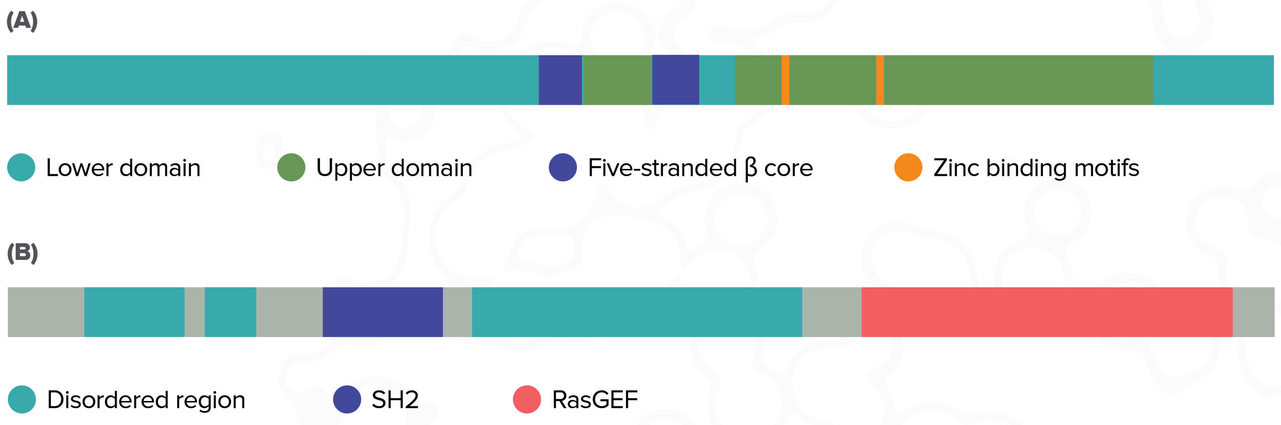
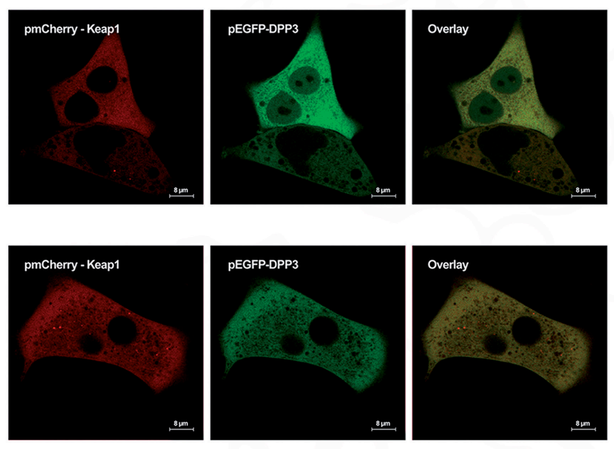


Fig. 1 Schematic of the full length DPP3 (A) and SH2D3C (B) sequence with its most relevant regions. Highly conserved enzyme DPP3, primarily a mammalian cytosolic protein is composed of two domains; upper lobe (mostyl helical, contains the zinc binding motifs) and the lower domain (mix of α- and β secondary structures with a five-stranded β-barrel core) (Malovan et al., 2022). Two domains are separated by a wide cleft that forms the peptide binding site. The encoded SH2D3C protein consists of N-terminal SH2 domain, C-terminal domain with week, but significant homology with CDC25-like Ras GEF domains, and a proline/serine rich region in between with many potential sites for phosphorylation by proline-directed kinases.



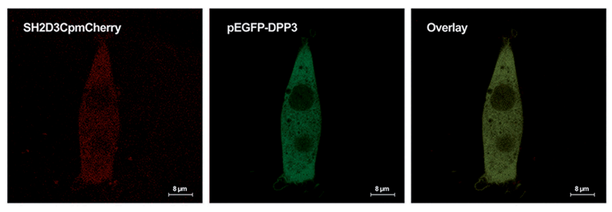


Fig. 2 Subcellular localization.
Co-transfection of pmCherry-Keap1 with pEGFP-DPP3, and SH2D3C-pmCherry with pEGFP-DPP3 in NIH 3T3 cells (after 24 h incubation). Cells were seeded in 4 chamber glass bottom dish (IBL) and analyzed by TCS SP8 X confocal microscopy. The suitable cell confluency was 30–40% at the time of transfection. The ratio of DNA (μg) to LTX (μL) was 1:2.

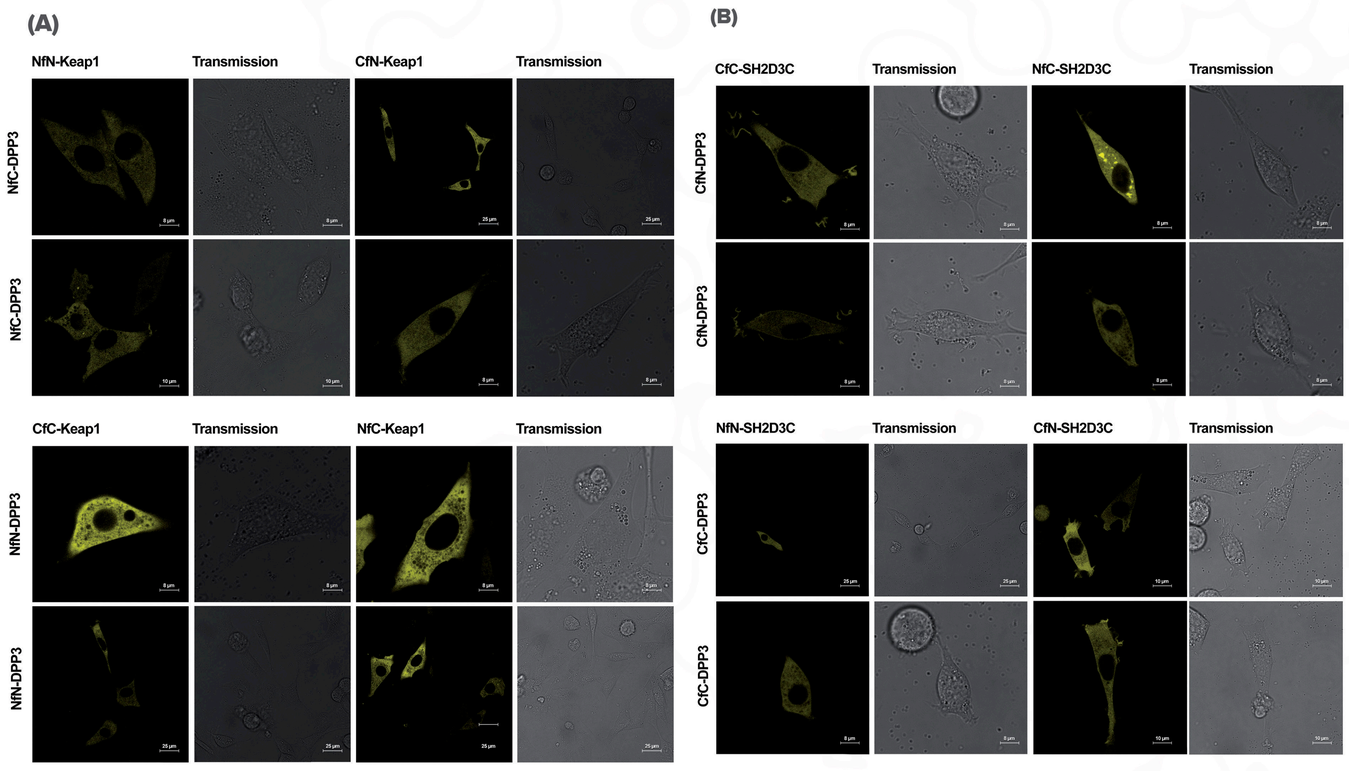


Fig. 3 Confocal photofluoromicrographs of NIH 3T3 cells transiently transfected with BiFC vectors expressing DPP3 with Keap1 (A), and DPP3 with SH2D3C (B). Fluorescence confocal and transmission images are represented. The ratio of DNA (μg) to LTX (μL) were 1:2

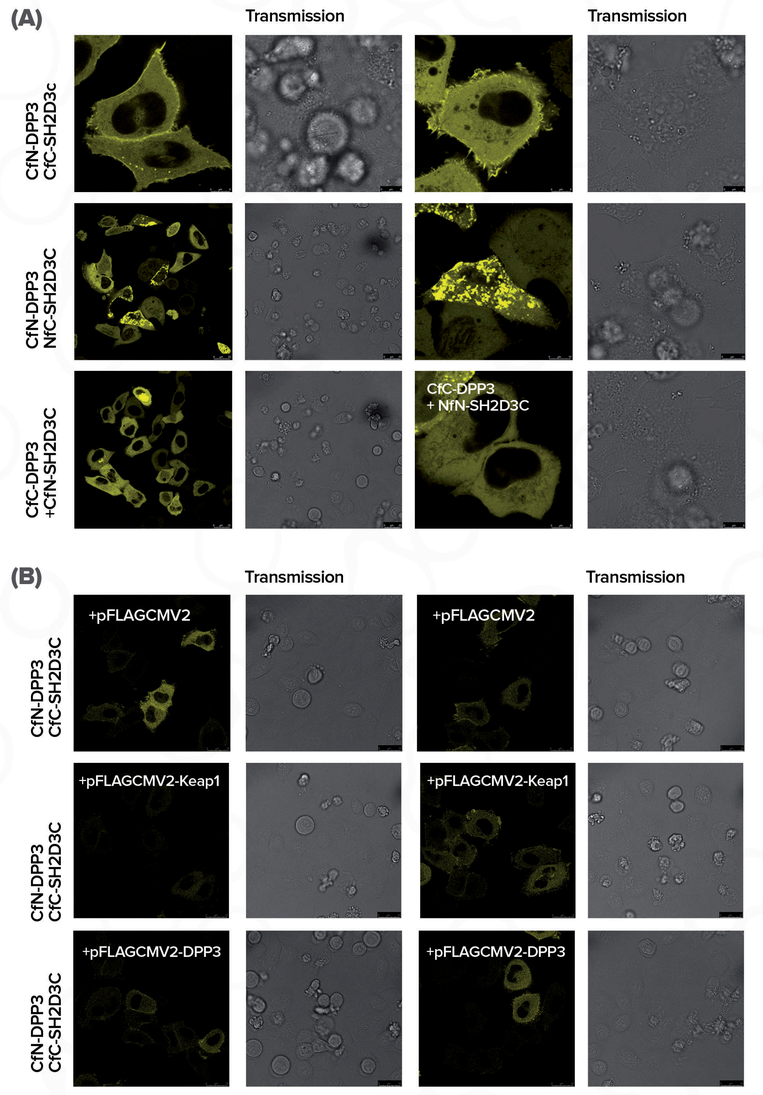


Fig. 4 BiFC protein interaction analysis.
(A) Confocal photofluoromicrographs of HeLa cells transiently transfected with BiFC vectors expressing CfN-DPP3 in combination with CfC-SH2D3C or NfC-SH2D3C; and CfC-DPP3 in combination with NfN-SH2D3C or CfN-SH2D3C. **(B)** Lower intensity BiFC protein interactions were detected with the addition of pFLAGCMV2, pFLAGCMV2-Keap1 and pFLAGCMV2-DPP3 constructs.

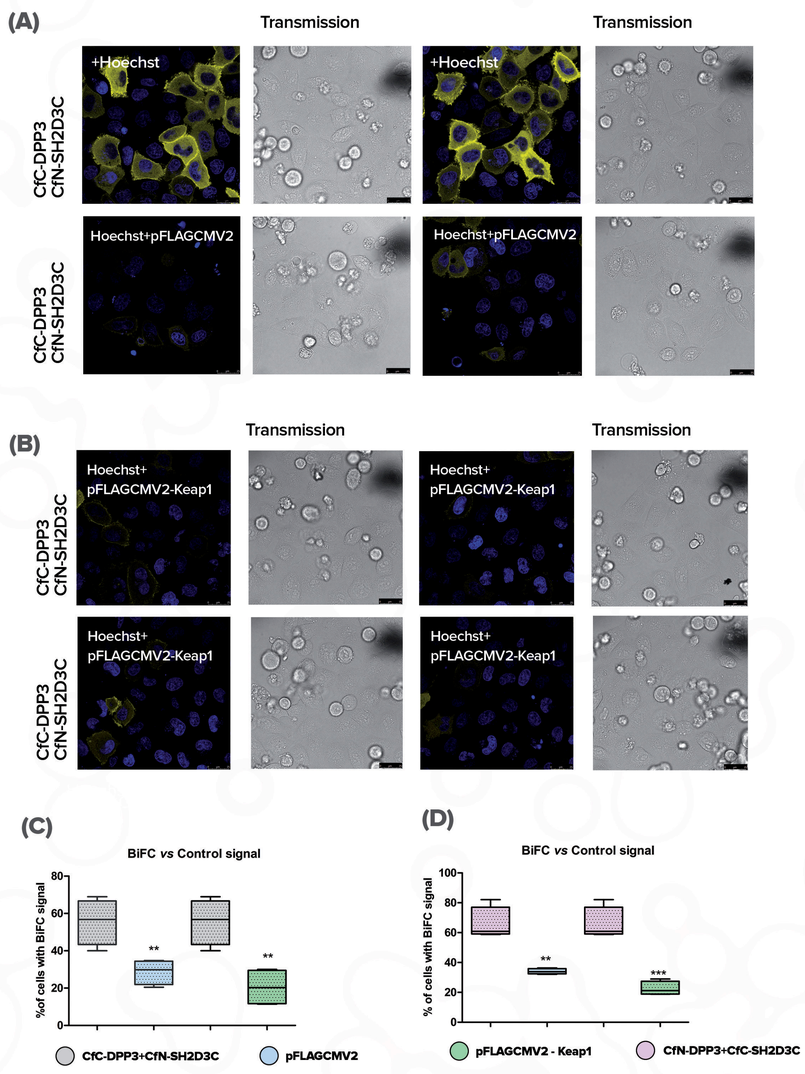


Fig. 5 BiFC screen. (A), (B) Confocal photofluoromicrographs of HeLa cells transiently transfected with BiFC vectors and constructs (same as above) for competition experiments. Nuclei were stained with Hoechst 33342 (5 μg /ml). **(C), (D)** Two-tailed Student t-test was applied and means were found to be statistically significant (P<0.05).

Results

Based on the FACS results (data not shown here), NIH3T3 cells showed not to be appropriate for BiFC assays due to poor transfection efficiency (3.74%), and the experiments were continued with HeLa cells (76.6% of transf. eff.). Considering the lack of the ideal neg. ctrl (proteins with mutations in the interacting region) we have used Keap1 a known binding partner of DPP3 (pFLAGCMV2-Keap1) in the competition experiment or FLAG-DPP3 (pFLAGCMV2-DPP3) that competes for binding to SH2D3C with Venus fragment labeled DPP3. When we analyzed HeLa cells co-transfected with BiFC plasmids and stained with Hoechst 33342 dye by confocal microscopy, the % of cells showing bright BiFC signal was around 60%. In the case of co-transfection of the HeLa cells with both BiFC plasmids and EV, the % cells showing BiFC signal drops to around 30%, and in the case of a competitive inhibitor it drops to 25% assuming weaker BiFC interactions. The % of cells showing BiFC signal was calculated as a ratio of transfected HeLa cells (BiFC plasmids with EV or competitive inhibitors) to a total number of cells (n=100) when stained with Hoechst 33342, and visualized in 4-5 fields. The differences between groups were statistically processed using unpaired two tailed Student t-test (n=100) and were considered to be significant at a P value of <0.05. Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA).

Conclusion

In summary, the BiFC assay allowed us an efficient visualization of the protein interaction between DPP3 and SH2D3C protein as well as the identification of the subcellular location of the protein complex, in cytoplasm and in membrane ruffles of living cells. However, negative controls that we used are not adequate for our experiment as resulting BiFC signal is almost equal in the cells transfected with either EV or with competitive inhibitors (Keap1 or DPP3). Therefore, we need to search for a better negative control.

Acknowledgement

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References:

Malovan, G. et al. FEBS J. (2022) DOI: 10.1111/febs.16429
Mace, P.D. et al. Nat. Struct. Mol. Biol. (2011) 18(12): 1381–1387.
Lepur, A. et al. Methods Mol. Biol. (2018) 1794:259-267.