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Investigating KEAP1-DPP3 Interaction in Live Cells: Challenges and Considerations in BiFC Assay Design

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Abbreviations:
BiFC – bimolecular fluorescence complementation
DPP3 – dipeptidyl peptidase 3
KEAP1 – Kelch-like ECH-associated protein 1
NIH 3T3 – mouse embryonic fibroblast cells
NRF2 – Nuclear factor erythroid 2-related factor 2

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Abstract

Background and purpose: *Kelch-like ECH-associated protein 1 (KEAP1) is a repressor of the transcription factor Nuclear factor erythroid 2-related factor 2 (NRF2), a key regulator of the oxidative stress response. Under basal conditions, the NRF2 monomer is bound by a KEAP1 dimer in a complex with Cullin-3 (CUL3) and E3 ubiquitin-protein ligase RBX1 (RBX1), leading to its ubiquitination and subsequent degradation by the proteasome. One mechanism of NRF2 activation involves competitive protein interactors binding to KEAP1, preventing NRF2 ubiquitination. One such interactor is dipeptidyl peptidase 3 (DPP3), whose binding to KEAP1 reduces NRF2 degradation and promotes its transcriptional activity. We developed bimolecular fluorescence complementation (BiFC) assay for live-cell analysis of DPP3-KEAP1 interaction in order to investigate its subcellular localization and determine if BiFC is an appropriate method for the investigation of DPP3's protein interactions.*

Materials and methods: *We constructed BiFC vectors containing N- and C-terminal fragments of the Venus fluorescent protein and cloned WT DPP3, KEAP1 and DPP3-ΔETGE in all possible topologies.*

Results: *All eight WT DPP3 and KEAP1 vector combinations produced positive BiFC signals and the localization of the signal was in the cytosol; however, three out of eight negative control vector combinations (DPP3-ΔETGE-VenusfN/KEAP1-VenusfC, VenusfC-DPP3-ΔETGE/VenusfN-KEAP1 and VenusfN- DPP3-ΔETGE/VenusfC-KEAP1) also yielded false positive signals.*

Conclusion: *BiFC is a useful method for studying protein-protein interactions and their subcellular localization, but careful selection of vector combinations is crucial to avoid false-positive signals.*

INTRODUCTION

Dipeptidyl peptidase 3 (DPP3; Uniprot: Q9NY33) is a cytosolic, zinc-metallopeptidase that cleaves dipeptides from the amino-termini of 4-8 amino acids long peptides with broad specificity. It is ubiquitously present in organisms from bacteria to higher eukaryotes, suggesting a role in the final stages of protein turnover in cells, however, it also cleaves a number of bioactive peptides in vitro, indicating potential involvement in the regulation of blood pressure and pain *(1)*. Apart from the roles related to its peptidase activity, DPP3 is also involved in the regulation of oxidative stress response through the interaction with Kelch-like ECH-associated protein 1 (KEAP1; Uniprot: Q14145) *(2)*. KEAP1 is a repressor that controls the levels of Nuclear factor erythroid

2-related factor 2 (NRF2; Uniprot: Q16236), a transcription factor responsible for the expression of over 250 genes encoding proteins that protect cells from oxidative and electrophilic stress. Under basal conditions, NRF2 levels are kept low by KEAP1-mediated proteasomal degradation. KEAP1 binds NRF2 in the complex with Cullin-3 (CUL3) ubiquitin-ligase resulting in NRF2 ubiquitination and subsequent degradation in the 26S proteasome *(3)*. The NRF2 monomer binds to the KEAP1 dimer through two binding motifs: a higher affinity ETGE motif and a lower affinity DLG motif. When NRF2 is bound to the KEAP1 dimer at both sites it adopts a conformation suitable for ubiquitination *(4)*. Electrophilic and oxidative stress induce conformational changes of the complex, inhibiting NRF2 ubiquitination and degradation, and leading to the translocation of newly synthesized NRF2 to the nucleus. While NRF2 protects cells from oxidative stress, it is also often activated in cancer through several different mechanisms, including binding of other protein interactors (proteins containing ETGE or ETGE-like motifs) to KEAP1. This binding results in the detachment of the lower affinity NRF2 DLG site from KEAP1 and inhibition of NRF2 ubiquitination *(5)*. DPP3 is an ETGE-containing competitive interactor of KEAP1, its overexpression activates NRF2 transcription and decreases NRF2 ubiquitination. DPP3 gene copy number and mRNA expression correlate with high NRF2 activity in squamous cell lung carcinoma *(2)*, while in oestrogen receptor positive breast cancer, DPP3 mRNA overexpression correlates with increased expression of NRF2 controlled genes and poor prognosis *(6)*.

We have developed bimolecular fluorescence complementation (BiFC) assay for analysing DPP3 interactions. BiFC enables direct visualization of complex formation in living cells and allows identification of the subcellular localization of the complex. The principle of BiFC is based on the formation of a fluorescent complex between two non-fluorescent fragments (N- and C-fragment) of a fluorescent protein. Each fragment is expressed as a fusion with proteins under investigation for interaction. If these proteins interact, the two fragments of the fluorescent protein will come into close proximity and emit fluorescence *(7)*. We used yellow fluorescent protein Venus *(8)*, split into an amino-terminal fragment Venus-fN (consisting of 157 amino acids) and a carboxy-terminal fragment Venus-fC (81 amino acids). Both fragments were fused to the gene of interest either N- or C-terminally generating four possible topologies for each fusion protein (Figure 1) and eight possible combinations of BiFC reactions *(9)*. Positive BiFC signals were detected in the cytosol of live NIH 3T3 cells with all combinations of fusion proteins. No signal was detected in the nucleus. These results indicate that KEAP1 and DPP3 interact specifically and exclusively in the cytosol and that BiFC is appropriate methods for the investigation of DPP3's interactions in live cells. Constructed vectors could also be used for the investigation of other protein-protein interactions of KEAP1 and DPP3, respectively, and for the investigation of small molecule inhibitors of DPP3-KEAP1 interaction.

MATERIAL AND METHODS

Cloning of BiFC vectors for the expression of fusion proteins

BiFC vectors pcDNA3.1-VenusfN and pcDNA3.1-VenusfC were cloned as previously described *(10)*. Briefly, expression cDNA inserts for DPP3 and KEAP1 were amplified from previously cloned pFLAG-CMV2-DPP3 and pFLAG-CMV2-KEAP1 expression vectors. Vector pFLAG-CMV2-DPP3 was previously described (*11*), while pFLAG-CMV2-KEAP1 was constructed by recombination cloning of pFLAG-CMV2 vector linearized with BamHI restriction enzyme (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and KEAP1 cDNA insert amplified from pFLAG-CMV-KEAP1vector, kindly provided by Koraljka Husnjak from the Institute of Biochemistry II (IBC2), Medical Faculty of Goethe University, Frankfurt, Germany. DPP3-ΔETGE was amplified from pcDNA3.1-Venus-DPP3-ΔETGE vector, kindly

Table 1. *Primers used for cloning of DPP3, DPP3ΔETGE and KEAP1 cDNA into BiFC vectors for the expression of fusion proteins.*

Fusion protein	Forward primer	Reverse primer
DPP3-VenusfN[1]	CTTAAGCTTGCCACCATGGCGGACACCCAGT	ACTCCGACCGGTATCAGCTTGGCCAGATGGG
DPP3-VenusfC[1]	CTTAAGCTTGCCACCATGGCGGACACCCAG	CCGACCGGTATCCATAGCTTGGCCAGATGGG
VenusfN-DPP3*	ATAGCTCTCTCCCTAATGGCGGACACCCAGTAC	TCGAGCGGCCGCCTAAGCTTGGCCAGATGGG
VenusfC-DPP3*	GCGGCCGCTCGAGTCATGGCGGACACCCAG	TAAACGGGCCCTCTAAGCTTGGCCAGATGGG
Keap1-VenusfN	CTTAAGCTTGCCACCATGCAGCCAGATCCCAGG	CCGACCGGTATCCATACAGGTACAGTTCTGCTGGTC
Keap1-VenusfC	CTTAAGCTTGCCACCATGCAGCCAGATCCCAGG	CCGACCGGTATCCATACAGGTACAGTTCTGCTGGTC
VenusfN-Keap1	ATAGCTCTCTCCCTAATGCAGCCAGATCCCAGG	TCGAGCGGCCGCCTAACAGGTACAGTTCTGCTGGTC
VenusfC-Keap1	GCGGCCGCTCGAGTCATGCAGCCAGATCCCAGG	TAAACGGGCCCTCTAACAGGTACAGTTCTGCTGGTC

*Same primers were used for both DPP3 and DPP3ΔETGE cloning.

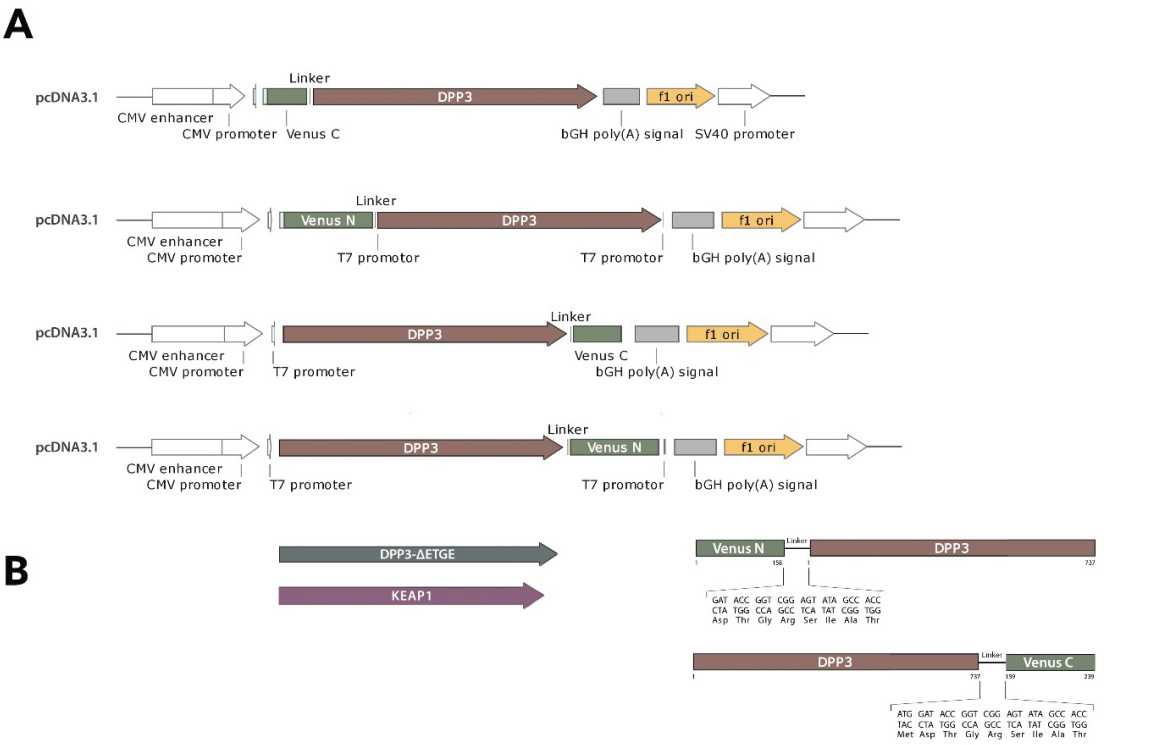

Figure 1. *(**A**) Schematic representation of Venus-based BiFC constructs used in our study; (**B**) The brown DPP3 region can be replaced by specific gene of interest DPP3-ΔETGE (grey) or KEAP1 (purple) fused to the N- or C-terminal fragment of Venus protein, respectively; relevant linker sites in the expression cassettes between the gene of interest and the protein fragments of Venus are indicated.*

provided by Prof. Ben Major from the University of North Carolina at Chapel Hill, NC, USA, using specific primers (Table 1), and cloned on 5' or 3' sides of VenusfN fragment in pcDNA3.1-VenusfN and VenusfC fragment in pcDNA3.1-VenusfC, respectively, to prepare BiFC vectors with 4 different topologies of Venus fragments fused to KEAP1, DPP3 and DPP3-ΔETGE, respectively (Figure 1). All cloning reactions were performed with In Fusion Cloning kit (Takara Bio, Otsu, Shiga, Japan).

Cell culture and Transfections

Mouse fibroblast embryo (NIH 3T3) cells, kindly provided by Maja Sabol from Ruđer Bošković Institute (RBI), Zagreb, Croatia, were grown in Dulbecco's Modified Eagle Medium (Sigma) supplemented with 10% foetal calf serum (Sigma-Aldrich, Saint Louis, MO, USA), 1% non-essential amino acids and 1% antibiotic/antimycotic solution (Capricorn Scientific GmbH, Ebsdorfergrund, Germany) at 37 °C in a humidified atmosphere of 5% CO_2 in Heracell 150 humidified incubator (Heraeus, Hanau, Germany). NIH 3T3 cells were seeded in 4-chamber 35 mm glass bottom dishes (Cellvis, Mountain View, CA, USA) at about 8000 cells/well (in a total volume of 500 µl) for subcellular localization experiments and BiFC screening using confocal microscopy. Cells were previously counted on LUNA-II Automated Cell Counter (Logos Biosystems, Dongan-gu Anyang-si, Gyeonggi-do, South Korea). The cell confluency was estimated at the time of transfection and was around 40-45%. NIH 3T3 cells were transiently transfected with appropriate Venus constructs using Lipofectamine® LTX & PLUS™ Reagent (Thermo Fischer Scientific, Waltham, MA, USA), at 1:2 ratio of DNA (500 ng of each plasmid, i.e., 1 µg of total DNA) to LTX (2 µl) reagent.

Confocal microscopy

Live cells transiently transfected with different combinations of BiFC vectors were analysed 24 or 48 hours post transfection on confocal laser scanning microscope Leica TCS SP8X (Leica Microsystems, Wetzlar, Germany) using an HC PL APO CS2 63/1.4 NA oil-immersion objective, a 405 nm diode laser, and a supercontinuum white-light laser.

Excitation wavelength for Venus was 514 nm and detection range for emission was 520-560 nm. Images were acquired in LAS X Leica Microsystems software, and subsequently processed using Photoshop CS5.

For images used to analyse BiFC signal intensity, imaging settings were determined using a negative control prepared during the same experiment, and kept at constant values (laser power at 20%, detection value 345).

RESULTS

KEAP1 and DPP3 interact specifically in the cytosol of live NIH 3T3 cells

We used BiFC assay to analyse the subcellular localization of the DPP3-KEAP1 interaction. This interaction was confirmed previously by several groups; however, the subcellular localization of the interaction was not investigated previously. We have created BiFC vectors covering all possible topologies of fusion between Venus fragments VenusfN and VenusfC, and KEAP1 and DPP3 proteins, respectively (Figure 1). All 8 possible combinations of fusion proteins (DPP3-VenusfC/ KEAP1-VenusfN, DPP3-VenusfC/VenusfN-KEAP1,

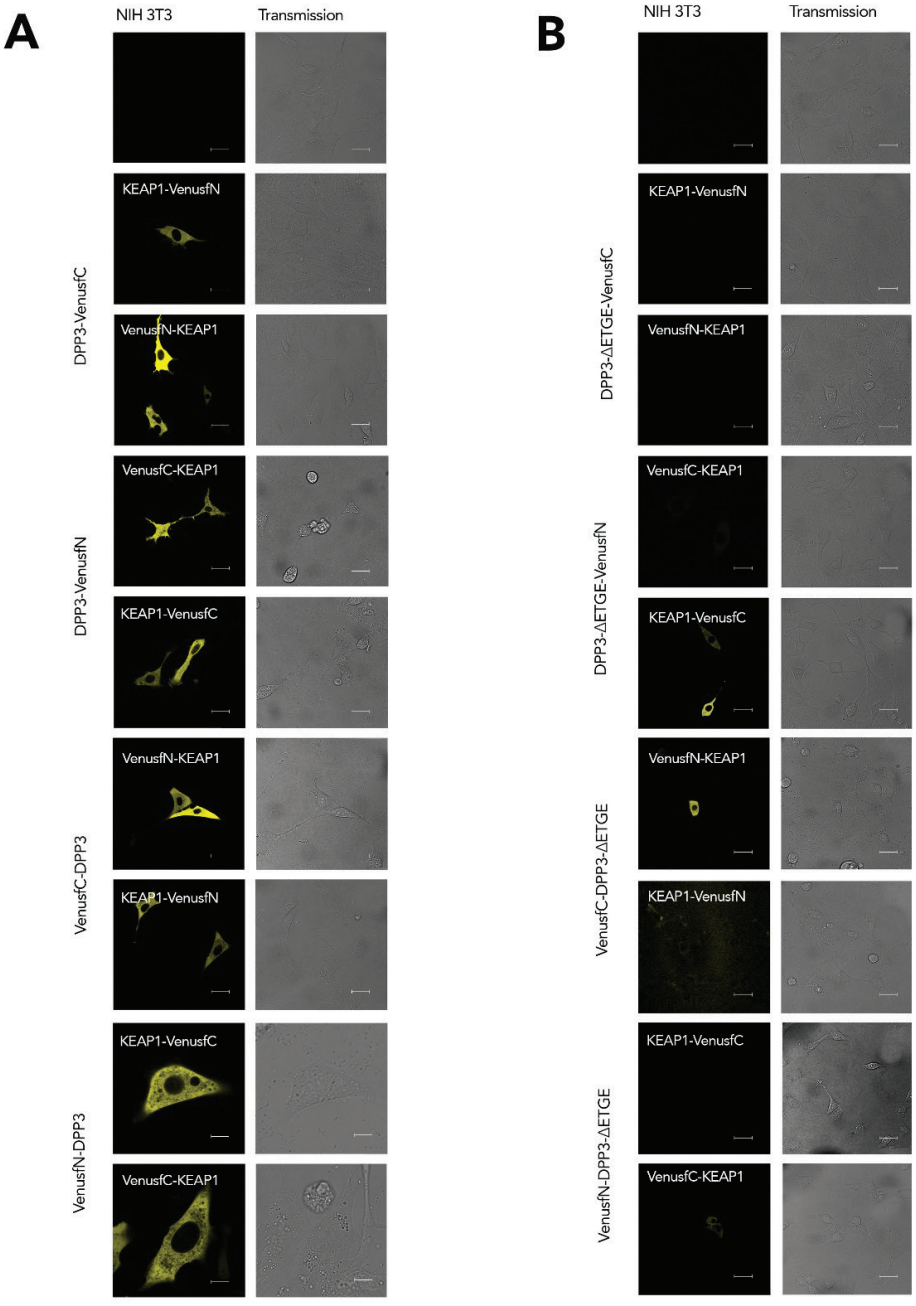

Figure 2. *Live-cell imaging of NIH 3T3 cells transiently transfected with vectors expressing (**A**) all combinations of WT DPP3 and KEAP1 fusions with VenusfN and VenusfC, respectively (positive signals were detected in all orientations); (**B**) all combinations of DPP3-ΔETGE and KEAP1 fusions with VenusfN and VenusfC, respectively, as negative controls (positive signals were detected in 3 orientations, DPP3-ΔETGE-VenusfN/KEAP1-VenusfC, VenusfC-DPP3-ΔETGE/VenusfN-KEAP1, VenusfN- DPP3-ΔETGE/VenusfC-KEAP1). All BiFC experiments were performed in at least two biological and two technical replicates and the representative images are displayed. Scale bar on low magnification = 25 μm; scale bar on high magnification = 10 μm.*

DPP3-VenusfN/VenusfC-KEAP1, DPP3-VenusfN/KEAP1-VenusfC, VenusfC-DPP3/ KEAP1-VenusfN, VenusfC-DPP3/VenusfN- KEAP1, VenusfN-DPP3/KEAP1-VenusfC and VenusfN-DPP3/VenusfC-KEAP1) were expressed in NIH 3T3 cells and positive signals were detected in the cytosol of live cells expressing all 8 combinations of BiFC fusion proteins (Figure 2A).

We also prepared BiFC vectors for negative controls, which are essential in BiFC experiments to ensure that the observed fluorescence is due to the protein-protein interaction and not to non-specific interactions. Several studies confirmed that ETGE motif is indispensable for DPP3 binding to KEAP1, so we constructed BiFC vectors with DPP3-ΔETGE insert, and tested all 8 possible combinations of DPP3-ΔETGE and KEAP1 fusion proteins. We did not detect BiFC signal in 5 fusion combinations, however, in 3 combinations (DPP3-ΔETGE-VenusfN/KEAP1-VenusfC, VenusfC-DPP3-ΔETGE/ VenusfN-KEAP1 and VenusfN-DPP3-ΔETGE/VenusfC-KEAP) BiFC signal, that was somewhat reduced in comparison to the signal of the same combinations of WT DPP3 and KEAP1 fusion proteins, was observed (Figure 2B). We have tried decreasing the amount of the BiFC expression vectors for cell transfections from 500 ng to 250 ng per plasmid, but still detected the same, unexpected false-positive results in the negative control. The reasons for false positive interactions can be manifold, however, most likely it is a result of occasional, spontaneous and non-specific self-assembly of N- and C-terminal Venus (*12*).

DISCUSSION

DPP3, an ETGE-containing protein, is involved in the regulation of the KEAP1-NRF2 oxidative stress response pathway. It competes for binding to KEAP1 with NRF2, and its overexpression activates NRF2 transcription and decreases NRF2 ubiquitination. The interaction of DPP3 and KEAP1 is augmented under oxidative stress conditions induced by hydrogen peroxide treatment *(6)*. Analysis of the subcellular localization of endogenous KEAP1 shows that it is mostly cytosolic, perinuclear protein that regulates the entrance of NRF2 to the nucleus *(13-15)*. Human DPP3 is primarily considered a cytosolic enzyme, however, some reports suggest its association with membranes in other species, despite the lack of exporting signals in its primary structure *(1, 16)*, and its nuclear localization was induced in the livers of mice by growth in hyperoxic conditions or by oestrogen treatment *(17)*. There are reports of increased nuclear localization of endogenous KEAP1 induced by serum starvation *(18)* and oxidative stress *(19)*, and overexpressed KEAP1 was detected in the nucleus even in unstressed conditions *(6, 18, 19)*, however, some authors argue that could be the result of the high levels of overexpressed protein which fail to bind to cytoskeletal scaffold and enter the nucleus *(13)*.

We developed a BiFC assay to investigate the subcellular localization of the DPP3-KEAP1 interaction, which had not been explored previously. BiFC is an advantageous method for the investigation of weak and transient interactions, since it was found to stabilize protein-protein interactions *(20, 21)*. It is also a good method for live cell investigation of subcellular localization of the interaction, since it does not require high levels of the expression of fusion proteins *(7)*. We observed positive BiFC signals, with all combinations of fusion proteins, exclusively in the cytosol of live NIH 3T3 cells (Figure 2A), consistent with them being predominantly cytosolic proteins. We have also tested negative controls for all possible combinations of fusion proteins, which are essential in BiFC experiments to ensure that the observed fluorescence is due to protein-protein and not to non-specific interactions. The best negative controls are proteins with mutations in the amino acids indispensable for binding *(12)*.

Given the known role of the ETGE motif in DPP3-KEAP1 binding, we investigated the DPP3-ΔETGE-KEAP1 interactions as negative controls in BiFC experiments. Expected results (no BiFC signal) were acquired in five negative control fusion protein combinations confirming that deletion of the ETGE motif abrogates the interaction in living cells, while false positive BiFC signals in three orientations are most likely the result of the spontaneous, non-specific self-assembly of N- and C-terminal Venus fragments.

Our findings underscore the utility of BiFC for investigating protein-protein interactions, with proper fusion protein orientations being crucial to avoid false-positive results. Given the correlation between DPP3 overexpression and NRF2 activation in breast and lung cancers, BiFC could facilitate the study of small molecules inhibiting DPP3-KEAP1 binding, potentially offering therapeutic benefits of inhibition of NRF2 in breast and lung cancers with DPP3 overexpression.

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