

GFP Fusion Proteins: A Solution or a Problem?



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Received: May 31, 2018; Published: June 20, 2018

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Abstract

Using fluorescent proteins as imaging probes is a wide spread and versatile technique in microscopy. GFP tagged proteins can be used to track and examine real time localization, interactions and translocation of proteins of interest as well as to investigate several aspects of cell biology. The Y-box binding protein 1 (YB-1) is a pleiotropic protein involved in a wide number of cellular processes and a substantial amount of knowledge on YB-1 localization and function was produced using YB-1-GFP constructs. Recently, it has been shown that YB-1 plays a critical role in cell stress response, playing an important role in the formation of stress granules (SG). To deeply investigate on this subject, we produced a stable HEK293T cell line constitutively expressing YB-1-GFP. In physiological conditions YB-1-GFP expressing cells behaved like the parental cells and YB-1-GFP protein properly localized to the cytoplasmic compartment. However, upon oxidative insult we observed a strong reduction in cell viability along with the occurrence of unusual GFP positive aggregates. Taken together, our findings suggest to critically revise existing insights obtained with YB-1-GFP construct and, more in general, to beware and be critical in interpreting data obtained with functionally uncharacterized GFP fusion proteins.

Keywords: GFP; Stress Granules; Oxidative Stress; Stable Cell Line

Abbreviations: CSD: Cold Shock Domain; NES: Specific Nuclear Export; NLS: Nuclear Localization; CRS: Cytoplasmic Retention Signals; SG: Stress Granules; RNP: Ribonucleoproteins; CLS: Cell Line Service; mRNPs: Messenger Ribonucleoproteins

Introduction

Y-box binding protein 1 (YB-1) is a member of the evolutionarily conserved cold shock domain (CSD) proteins and was first identified as a DNA/RNA binding protein [1] involved in the control of gene expression at both transcriptional and translational level [2-4]. Given its multiple cellular functions, YB-1 is involved in the control of several biological processes including cell proliferation and migration. To properly perform its functions, YB-1 subcellular localization has to be finely regulated. Specific nuclear export (NES), nuclear localization (NLS) and cytoplasmic retention signals (CRS) contribute and direct the multifunctional tasking of YB-1 [5]. In normal resting cells, YB-1 localizes to cytoplasm where it is a major component of P-bodies and messenger ribonucleoproteins (mRNPs) [6]. Recent studies link YB-1 to the cellular response to oxidative stress and DNA repair mechanisms. Indeed, following acute oxidative stress, YB-1 localizes to cytoplasmic Stress Granules (SGs), organelle-like structures devoid of membranes engaged in mRNA sorting and pro-survival translational reprogramming [7,8]. In particular, YB-1 is recruited in TIA-containing Stress Granules (SGs) where it functions as a component of translationally inactive mRNPs to directly block translational initiation of highly expressed [9]. Following DNA damage, YB-1 translocates to the nucleus and associates with DNA repair protein complexes. Here we report the characterization of a stable pool of HEK293T cells constitutively ex-

pressing YB-1 as a GFP fusion protein enabling sensitive analysis of YB-subcellular localization by confocal microscopy.

Methodology

Cell Culture: HEK293T, human embryonic kidney cells were purchased from Cell Line Service (CLS, Germany). Cells were cultured in humidified incubator at 37°C and 5% CO₂ and maintained in DMEM High Glucose (Gibco) supplemented with 10% Fetal Bovine Serum (Gibco), 1% L-Glutamine (Gibco) and 1% Pen-Strep-solution (Gibco). For HEK293T YB-1-GFP and HEK293T GFP, blasticidin (Sigma-Aldrich, St Louis, MO) and G418 (Sigma-Aldrich, St Louis, MO) antibiotics were added in the medium to a final concentration of 5 µg/ml.

Plasmids: The expression construct pcDNA6V5/HIS-A-YB-1-GFP derived from pcDNATM3.1(+) (Invitrogen); pcDNA 3.1/HIS-C was purchased from Thermo Fischer Scientific (USA). pEGFP-C1 was purchased from Clontech (USA).

Transfections: Cells were transfected using Lipofectamine 2000 (Life Technology) according to the manufacturer's instructions. Briefly, cells were seeded at 70-80% confluence (1.5×10^6) in 100mm dishes and transiently transfected with different concentrations of plasmid (from 800 ng up to 1.5 µg).

Generation of YB1-GFP Stable Clones: HEK293T cells were tested for their sensitivity to blasticidin in order to determine the best concentration for a correct selection. Concentrations of 2,5, 8, 10 and 12mg/ml were tested. Controls start to die from the day after the addition of the higher blasticidin concentration up to 3 days for the lower antibiotic concentration. The concentration chosen for selection was 5mg/ml. HEK293T cells were seeded in 100mm dishes (Corning) and let grow to reach 80% confluence. Cells were transfected by lipofectamine with pcDNA6/V5-HISA encoding human YB-1-GFP bearing the gene for blasticidin resistance or with pcDNA3.1/His C control plasmid. After 48 hours from transfection, blasticidin was added to plates at a final concentration of 5mg/ml. As previously observed, pcDNA3.1/His C transfected cells start to die after 3 days of selection. After one week of selection cells were trypsinized and replated and after two weeks of selection stable clones were obtained.

Immunofluorescence Microscopy: 3×10^4 HEK293T, HEK293T GFP and HEK293T YB-1-GFP cells were seeded on poly-D-lysine pre-treated coverslips (12mm thickness); the day after, cells were treated or not with H_2O_2 (Sigma-Aldrich, St Louis, MO). At the end of treatment cells were gently rinsed with 1X PBS before adding PFA at 3,4% for 10 minutes to fix cells; after 3 washes with 1X PBS cells were permeabilized by adding 0.5% Triton X-100-PBS for five minutes; then, after 3 washes with 1X PBS, cells were blocked to avoid unspecific binding of antibodies with a 3% BSA solution for 20 minutes. Primary antibodies were added for 1 hour (dilution range 1:200); after incubation, three washes with 0.05% Tween PBS were performed; then Alexa Fluor 488 (Life Technologies) and Cy3 (Jackson Immuno Research, USA) secondary antibodies were added for 45 minutes in dark; after 3 washes in 0.05% Tween PBS coverslips were immersed in a glycerol PBS solution and fixed. Direct eGFP signal was acquired without the addition of fluorescent dyes. Images were acquired using a Carl Zeiss LSM700 equipped with an axio Observer Z1 or with a Nikon TE Eclipse 2000. Image processing and analysis were performed with Fiji (ImageJ) software.

Cell Viability Assay: Cell viability was determined by the MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (Sigma-Aldrich, St Louis, MO) as previously described [10]. Cells were seeded in 96-well plate at the cell density of 2×10^3 ; the day after cells were treated or not with H_2O_2 . After treatment, cell medium was substituted with DMEM without phenol-red (GIBCO) and without supplements; MTT solution was added to cells to produce formazan crystals. After 2hrs MTT solution was substituted by acidic isopropanol to solubilize the formazan crystals. The optical

absorbance was determined at 570nm using an iMark microplate reader (Bio-Rad, USA). The experiments were carried out in triplicate for each experimental point and reported as percentage of the untreated control set at 1.0.

Trypan Blue Assay: Cells were seeded on poly-D-lysine pre-treated 12 well plates at $2,5 \times 10^5$. The day after plating, cells were treated or not; after treatment cells were gently rinsed with 1X PBS, trypsinized and collected. An aliquot was diluted 1:1 with trypan blue (Sigma-Aldrich, St Louis, MO). The experiments were carried out in triplicate for each experimental point.

Cell Proliferation Analysis: Cells were seeded on poly-D-lysine pre-treated 12-well plates at $2,5 \times 10^5$. Every 24 hours cells were gently rinsed with 1X PBS, trypsinized and counted in a Burkert chamber. The count was confirmed with analysis by a Scepter 2.0 (Millipore). The experiments were carried out in triplicate for each experimental point.

Immunoblot Analysis: Cells were seeded at 60% confluence (1.5×10^6) in 100mm plates; the day after, plates were gently rinsed with 1X PBS, and collected in Lysis Buffer (50mM Tris-HCl pH 7.5, 5mM EDTA, 150mM NaCl, 1% NP-40, 1M phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate, and protease inhibitors). Cells were then left on ice for 30 minutes, and crude extracts were obtained following centrifugation at 13200rpm for 30 minutes at 4°C. The amount of protein in the samples was determined by the Bio-Rad protein assay (Bio-Rad, Milan, Italy). After the addition of Laemmli buffer (Sigma Chemical Co, St. Louis, MO, USA) the samples were boiled at 100°C for 5min and resolved by SDS- polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred to a polyvinylidene difluoride membrane (PDVF, Millipore) using a Mini trans-blot apparatus (Bio-Rad, Milan, Italy) according to the manufacturer's instructions. The PVDF membrane was blocked in 5% w/v milk buffer (5% w/v non-fat dried milk, 50mM Tris, 200mM NaCl, 0.2% Tween 20) and incubated with primary antibodies diluted in 5% w/v milk or bovine serum albumin (BSA) buffer overnight at 4°C. The blots, washed three times with TTBS (Tris-buffered saline, 0.1% Tween), were incubated for 1 hour at RT with HRP-conjugated secondary antibodies (Sigma-Aldrich). Proteins were visualized by an enhanced chemiluminescence method (ECL, GE-Healthcare) and analyzed by Quantity One W software of ChemiDoc TM XRS system (Bio-Rad, Milan, Italy).

Primary Antibodies: Anti-YB-1 (Abcam, UK); anti-PABP (Sigma-Aldrich, St Louis, MO); anti-GAPDH (Santa Cruz Biotechnology, USA) (Table 1).

Table 1: Statistical analysis.

| Panel Experiment | | Test Method | Significance | n |
|-------------------------------|--------------------|---|---|---|
| Panel c (proliferation curve) | Cell line vs. time | 2 way ANOVA Time(h) | | |
| | | Cell line Interaction Sidak's multiple comparison test | F = 1031; p < 0,00*** F = 0,3737; p = 0,54 ns F = 67,97; p < 0,001*** | 6 |

| | | | | |
|-----------------------------|------------------------------|---|---|---|
| | HEK293T vs. HEK293T YB-1-GFP | | | |
| | 0 h | - | P>0,99 ns | |
| | 24h | - | P=0,25 ns | |
| | 48h | - | P<0.001*** | |
| | 72h | - | P<0,001*** | |
| | 96h | - | P<0,001*** | |
| Panel E (Trypan blue count) | | 2-way ANOVA Time(h) Cell line Interaction Sidak's multiple comparison test | F=695,1; P<0,001*** F=493,3; P<0,001*** F=47,13; P<0,001*** | 3 |
| | HEK293T vs. HEK293T YB-1-GFP | | | |
| | Control | - | P>0,99 ns | |
| | Hydrogen Peroxide 0,5 mM | - | P<0,001*** | |
| | Hydrogen Peroxide 1 mM | - | P<0,001*** | |
| | Hydrogen Peroxide 2,5 mM | - | P<0,001*** | |
| | Hydrogen Peroxide 5 mM | - | P<0,001*** | |
| | Hydrogen Peroxide 7,5 mM | - | P<0,001*** | |
| Panel F (MTT assay) | | 2-way ANOVA Time(h) Cell line Interaction Sidak's multiple comparison test | F=1136; P <0,001*** F=43,62; P<0,001*** F=4,174; P=0,003*** | 6 |
| | HEK293T vs. HEK293T YB-1-GFP | | | |
| | Control | - | p>0,99 ns | |
| | Hydrogen Peroxide 0,5 mM | - | P=0,010** | |
| | Hydrogen Peroxide 1 mM | - | P=0,71 ns | |
| | Hydrogen Peroxide 2,5 mM | - | P=0,29 ns | |
| | Hydrogen Peroxide 5 mM | - | P<0,001*** | |
| | Hydrogen Peroxide 7,5 mM | | P<0,002*** | |

Statistical analyses were performed using GraphPad Prism7 (GraphPad Software Inc). Statistical significance of difference in measured variables between control and treated groups was determined by the two-way analysis of variance (ANOVA) followed by Sidak's multiple comparisons post-test. Difference were considered significant at $P < 0.033$ (*), $P < 0.002$ (**) and $P < 0.001$ (***).

Conclusion

Stable cell lines represent a permanent resource that can be stored under cryogenic conditions for long periods of time, retrieved, and cultured to provide a consistent and reliable level of sustained gene expression. To explore the role of YB-1 localization upon stress stimuli we sought to generate a stable pool of cells constitutively expressing YB-1 as a GFP fusion protein. Preliminarily, we transiently transfected HEK293T cells with YB-1-GFP cDNA cloned in pcDNA6/V5-His, a vector derived from pcDNATM3.1(+) (Invitrogen). 5.0×10^5 cells were seeded in order to reach 80% confluence the day after; lipofection was performed using pcDNA6/V5-HisA-YB-1GFP and peGFP-C1 empty vector as control [10]. 48 hours after transfection cells were analyzed by immuno blot to evaluate the level of YB-1-GFP fusion GFP exogenous expression. Transfected YB-1-GFP was highly expressed in HEK293T cells (Figure 1). Direct fluorescence, acquired by Nikon Eclipse 800 microscope,

showed a prevalent cytoplasmic localization for YB1-GFP (Figure 2). Moreover, viability of YB-1-GFP cells, as assessed by MTT assay, was undistinguishable from HEK293T parental cells and cells expressing GFP alone (data not shown). Next, we produced pools of clones of HEK293T stably expressing YB-1-GFP or GFP alone. YB-1-GFP expressing cells were selected by blasticidin in while G418 was used for selection of peGFP-C1 stable pool. After 4 weeks, 91% of selected cells were GFP positive. Next, we analyzed the proliferation rate of YB-1-GFP expressing pool. Compared to the parental cell line, YB-1-GFP expressing cells grew slightly faster (Figure 3). To examine YB-1 protein localization under oxidative stress we treated HEK293T YB-1-GFP cells with hydrogen peroxide (H_2O_2). By immunofluorescence, we could observe typical stress granules (SGs) in control and GFP expressing cells, using a canonical SGs marker, PABP-1 Figure 4 left panel. Unexpectedly, YB-1 fusion protein was detected in unusual GFP positive aggregates that were ab-

normal in both dimension and shape (Figure 5 right panel arrows). Remarkably, although YB-1-GFP cells grew faster than control cells, their sensitivity to oxidative stress was increased as demonstrated by MTT and trypan blue assays (Figure 6). Taken together, our data show that, even though the GFP fusion protein behaved like the endogenous one and is localized in the right sub cellular compart-

ment, the presence of the huge GFP tag causes a lack in functionality that can be observed only in particular experimental conditions. Therefore, insights obtained so far with YB-1-GFP construct should be critically revised and, more in general, we should beware and be careful in interpreting data obtained with functionally uncharacterized GFP fusion proteins.

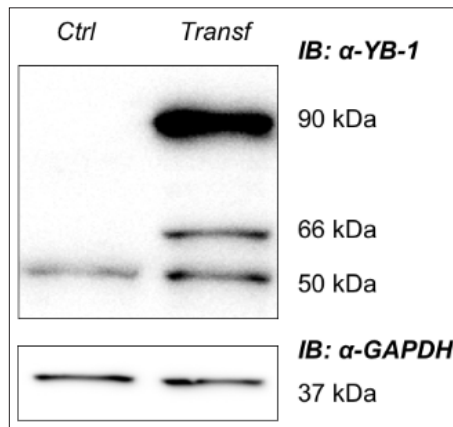


Figure 1: Western blot of total extracts from HEK293T YB-1-GFP and control (untransfected) HEK293T cells, showing the full length endogenous 50 kDa YB-1 band and the YB-1-GFP bands (66-90 kDa); GAPDH was used as loading control.

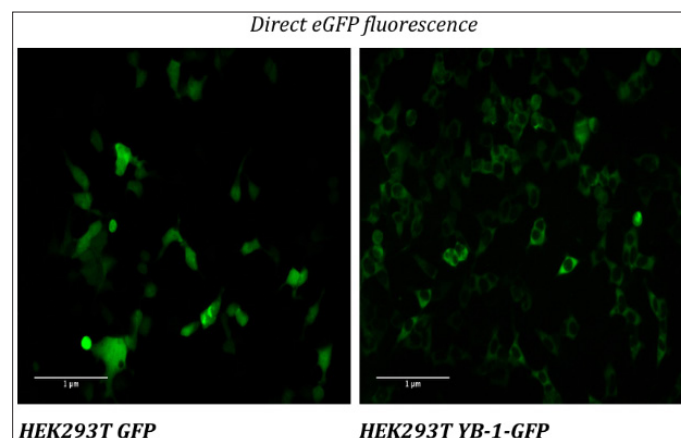


Figure 2: Direct eGFP fluorescence of HEK293T YB-1-GFP and HEK293T GFP in physiological conditions; images were acquired using a Nikon TE Eclipse 2000 (20x objective).

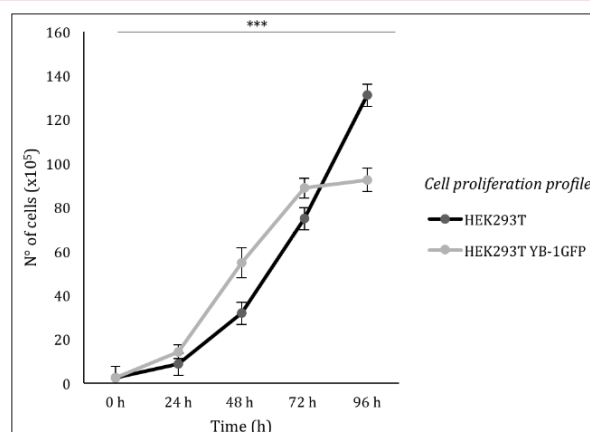


Figure 3: Cell proliferation profile of HEK293T and HEK293T YB-1-GFP from time 0 (seeding time) to 96 hours (2-way ANOVA P<0,001***, for post-hoc tests see Table 1).

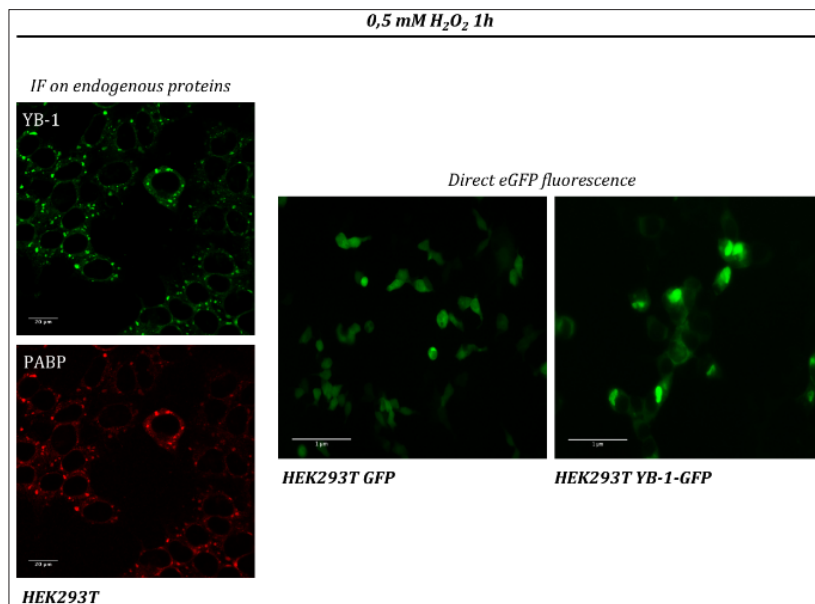


Figure 4: (left panel) confocal immunofluorescence of HEK293T treated with 0,5 mM hydrogen peroxide (H₂O₂) for 1 hour; PABP (red) and YB-1 (green) mark Stress Granules; (right panel) direct eGFP fluorescence of HEK293T, HEK293T GFP and HEK293T YB-1GFP treated with 0,5 mM hydrogen peroxide (H₂O₂) for 1 hour; images acquired using a Carl Zeiss LSM 700 (40x oil objective) (left panel) and Nikon TE Eclipse 2000 (20x objective) (right panel).

| MTT assay | HEK293T | SD | HEK293T YB-1-GFP | SD | *** |
|--------------------------------------|---------|------|------------------|------|-----|
| Control | 100% | 3,75 | 100% | 7,5 | |
| H ₂ O ₂ 0,5 mM | 41,55% | 1,59 | 39,95% | 1,2 | |
| H ₂ O ₂ 1 mM | 36% | 1,62 | 33,73% | 3,54 | |
| H ₂ O ₂ 2,5 mM | 34,33% | 1,66 | 31,03% | 0,5 | |
| H ₂ O ₂ 5 mM | 32,86% | 1,5 | 23,03% | 0,5 | |
| H ₂ O ₂ 7,5 mM | 26,06% | 0,5 | 19,67% | 2,83 | |

Figure 5: Cell viability assay (MTT) of HEK293T and HEK293T YB-1-GFP cells treated with indicated concentrations of hydrogen peroxide (H₂O₂) for 1 hour (2-way ANOVA $P < 0,001^{***}$, for post-hoc tests see Table 1).

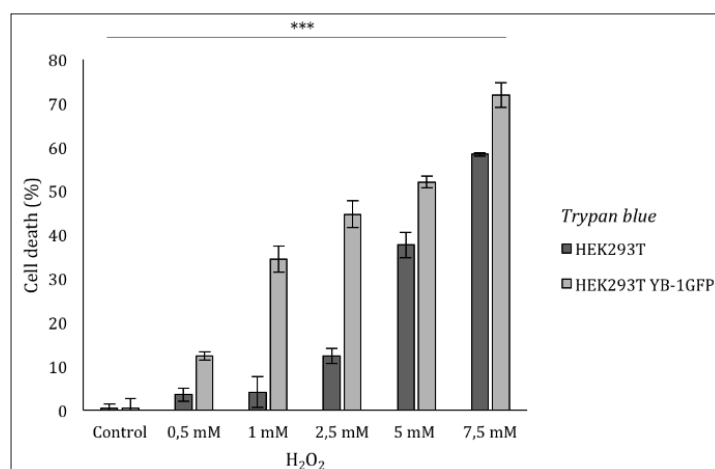


Figure 6: Trypan blue count; representation of the increasing number of dead cells after hydrogen peroxide (H₂O₂) treatments (2-way ANOVA $P < 0,001^{***}$, for post-hoc tests see Table 1).

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