Quantification of 4EBP1 Phosphorylation by PICO

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Introduction

Among the translation initiation factors, the eIF4E complex stands out as a critical player. This complex, consisting of eIF4E (elongation initiation factor 4E) and eIF4G, has been implicated in various diseases, including cancer (2–4). The activity of eIF4E is tightly regulated by 4EBP1 (eukaryotic translation initiation factor 4E-binding protein 1), thereby playing a pivotal role in regulating protein synthesis by modulating the assembly of the eIF4F complex.

4EBP1 undergoes dynamic phosphorylation events that impact its function. When 4EBP1 is dephosphorylated, it binds tightly to eIF4E, preventing its association with eIF4G and inhibiting translation initiation. Conversely, phosphorylation of 4EBP1 weakens its interaction with eIF4E, allowing eIF4F assembly and cap-dependent translation to proceed (5).

To assess the quantitative difference in 4EBP1's phosphorylation status, a Protein Interaction Coupling (PICO) assay was employed. PICO is an ultra-sensitive and versatile digital immunoassay that uses DNA oligonucleotide-labeled (PICO Label) antibodies to bind protein targets and the readout is performed by digital PCR (dPCR). During the dPCR reaction, the PICO Labels are amplified and together with specific PICO Probes fluorescent signals are generated. The fluorescent signals, arising from individual antibody molecules, are detected and counted by the dPCR instrument. The counting allows the calculation of the couplex concentration, which is the ternary complex of two antibodies and their target. From there, the absolute quantities of the target can be determined (6).

For the quantification of 4EBP1 phosphorylation we employed a 'triangular' PICO assay (**Figure 1**). During the triangular PICO assay three labeled antibodies are employed: two antibodies are specific to the protein target, while a third antibody is specific for the phosphorylation. Measuring all three antibodies simultaneously allows the quantification of the total protein and its phosphorylated version.

Highlights

- Absolute quantification of 4EBP1 phosphorylation.
- Dactolisib treatment resulted in a ~200 fold decrease in 4EBP1 phosphorylation level in BT474 and MCF7 cells.

Here we investigated the effect of Dactolisib on 4EBP1 phosphorylation in MCF7 (HER2 negative) and BT474 (HER2 positive) cell lines, representing different breast cancer subtypes. Dactolisib is an inhibitor of the PI3K/mTOR pathway (7) and dysregulation of the pathway is common in breast cancer, making it a possible target for therapeutic intervention (8). Therefore, investigating dactolisib's effects on these cell lines can provide insights into its potential as a targeted therapy, while assessing its impact on 4EBP1 phosphorylation and translation control can offer mechanistic understanding crucial for personalized treatment strategies and drug development.

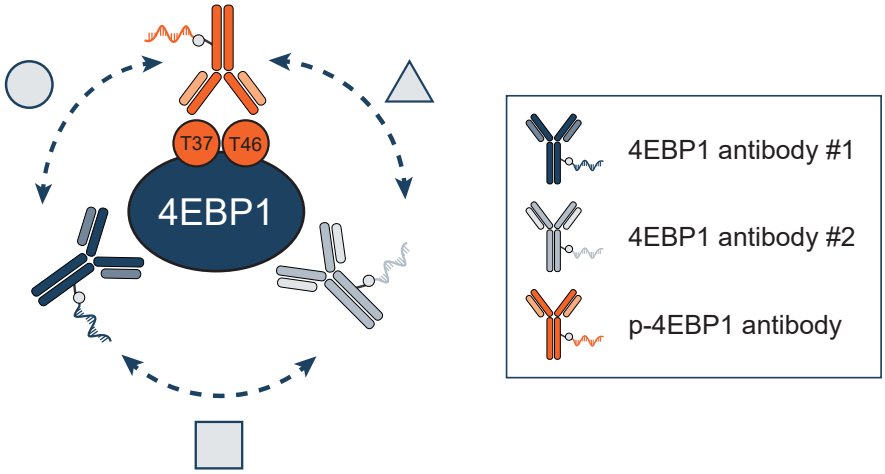

Figure 1: Triangular detection strategy of 4EBP1 and p-4EBP1 by PICO
For the detection and quantification of 4EBP1/p-4EBP1, two anti-4EBP1 antibodies (depicted with blue and gray color) and one anti-p-4EBP1 antibody (depicted with orange color) were employed. The PICO readout is done pairwise (marked with dashed arrows). PICO readout using the blue and gray antibodies (marked with a box) gives quantitative information about the total amount of 4EBP1 protein while PICO readout using the blue and orange (marked with circle) or gray and orange (marked with triangle) antibodies provides quantitative information about the phosphorylated form of 4EBP1.

Results and Discussion

To demonstrate the quantitative capabilities of PICO, we have conducted precise measurements of 4EBP1 protein and its phosphorylated form using the 'triangular' approach described above. BT474 and MCF7 cells were treated for 4 hours with Dactolisib or left untreated (Mock). Dactolisib treatment resulted in a striking reduction of

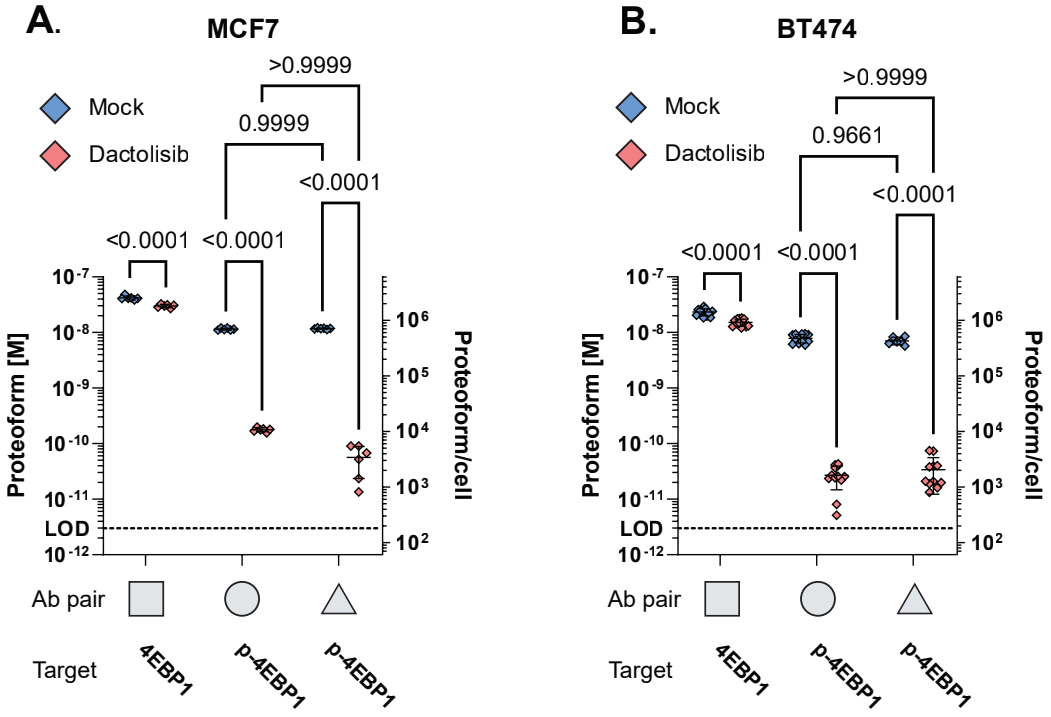

Figure 2: Targeted manipulation of 4EBP1 phosphorylation by Dactolisib treatment
Absolute quantitative measurement of 4EBP1 protein and its phosphorylation levels in MCF7 (A) and BT474 (B) cells using PICO. MCF7 and BT474 cells were treated with Dactolisib (orange diamonds) for 4 h or left untreated - mock (blue diamonds). Dactolisib treatment led to a strong reduction in p-4EBP1 signals. For MCF7 the average molar concentration from left to right are 41.9 ± 3.36 nM, 30.1 ± 2.05 nM, 11.5 ± 4.57 nM, 0.18 E-10 ± 0.014 nM, 11.7 ± 0.32 nM and 0.056 ± 0.033 nM. For BT474 the average molar concentration from left to right are 23.3 ± 3.45 nM, 15.1 ± 2.24 nM, 7.84 ± 1.41 nM, 0.027 ± 0.012 nM, 7.08 ± 1.11 nM and 0.031 ± 0.022 nM.

the phosphorylation signal in both BT474 and MCF7 cells (**Figure 2**). The phosphorylation signal decreased from 30.35% to 0.13% in BT474 cells and from 28.00% to 0.13% in MCF7 cells which translates to a log2 fold change of -2.35 and -2.12, respectively. Notably, the overall 4EBP1 protein levels were also significantly affected by the treatment and decreased by 35.07% in BT474 cells and 28.21 % in MCF7 cells.

These results were further confirmed by western blot which showed a strong reduction of the p-4EPB1 signal in the Dactolisib treated cells (**Figure 3**).

This data demonstrates the versatility of PICO, which is capable of detecting a diverse array of targets with high specificity, including proteins, post-translational modifications and even protein-protein interactions. Moreover we show that PICO delivers high levels of accuracy and precision and allows for absolute quantification of proteoforms with zero background.

4EBP1 emerges as a critical regulator of mitogenic signaling pathways, impacting cap-dependent translation. As we delve deeper into its phosphorylation dynamics, we uncover diagnostic opportunities and potential therapeutic targets. Robust quantification methods will play a pivotal role in realizing these clinical applications. In general altered 4EBP1 phosphorylation is associated with various cancers (2–4). Quantifying its phosphorylation status could serve as a diagnostic marker for disease progression and treatment response. Also resistance to targeted therapies often involves dysregulated translation. Understanding 4EBP1 phosphorylation dynamics can shed light on resistance mechanisms and guide personalized treatment strategies.

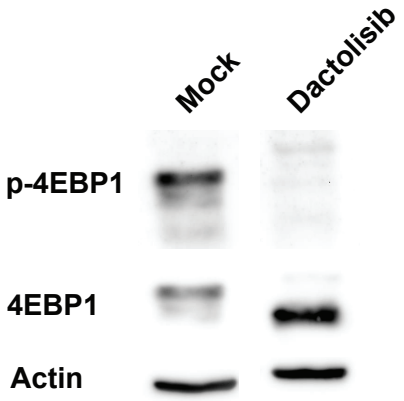

Figure 3: Confirmation of the Dactolisib effect on p-4EBP1 by western blot
U937 cells were treated with Dactolisib or left untreated (Mock) as described above. Dactolisib treatment led to a strong reduction in p-4EBP1 signals.

Materials and Methods

Cell cultivation

BT474, MCF7, and U937 cells were cultivated in Nunc™ EasYFlask™ Nunclon™ Delta Surface (Thermo Scientific). BT474 cells were grown in DMEM/F-12, GlutaMAX Supplement medium (Cat#: 31331028, Thermo Fisher Scientific), MCF7 cells were cultivated in DMEM high glucose, GlutaMAX™ Supplement with pyruvate (Cat#: 31966021, Thermo Fisher Scientific), and U937 cells were grown in RPMI 1640 Medium, GlutaMAX™ Supplement (Cat#61870010, Thermo Fisher Scientific). Each cell culture medium was supplemented with 10% FBS (Gibco) and 1% Pen-Strep (Gibco) and all cells were cultivated at 37˚C and 5% CO2. Cells were grown until they reached a confluency of approximately 50% (BT474) and 80% (MCF7).

Dactolisib treatment

The 4EBP1 phosphorylation was impaired by 5.6 µM Dactolisib (Cat# HY-50673, MedChemExpress) for 4 h during cultivation in the cell culture medium. The results were compared to mock treated (DMSO) cells.

Cell lysis

MCF7 and BT474 cells were harvested by incubation with 5 ml EDTA-based non-enzymatic detachment solution (Versene; Thermo Fisher Scientific) per T75 flask for 5 min at 37°C. The cells were washed twice in PBS and once in 1x PIC-PBS (cOmplete Protease Inhibitor Cocktail; Roche) by centrifugation at 400 g for 5 min and resuspended in Actome's lysis buffer (LB) to achieve a cell concentration of 2 × 10^4 cell/µl. After incubation at 4°C for 3 h the crude lysate was sonicated for 5 min at full-power in a sonicator bath. The lysate was then homogenized using a QIAshredder homogenizer column by centrifugation for 2 min at 20,000 g.

Western blot analysis

For western blot analysis Dactolisib and mock treated U937 cells were harvested by centrifugation at 400 g for 5 min and resuspended in Tris-based lysis buffer, followed by incubation for 20 min on ice. The supernatant was cleared by centrifugation for 20 min at full speed and 4°C. 45 µg of total protein per sample was incubated at 95°C for 5 min in 1x SDS loading buffer and separated by SDS gel electrophoresis. The proteins were transferred in methanol transfer buffer after which the membrane was blocked in 5% milk TBS-T.

For detection the membrane was incubated overnight at 4°C with the primary antibodies: anti-actin and 4EBP1 Monoclonal Antibody 554R16 (AHO1382, Invitrogen) 1:1000 dilution; Phospho-4EBP1 (Thr37, Thr46) Antibody (MA5-36935, Invitrogen) 1:500 dilution. This was followed by three washes with TBS-T and incubation with the secondary antibodies for 1 h (anti-rabbit 1:6000 dilution and anti-goat 1:3000 dilution). The membrane was washed again, three times with TBS-T and once with TBS, developed and imaged.

Antibody mix preparation

The anti-4EBP1 antibody 554R16, (AHO1382, Invitrogen) was labeled with PICOglue BL Label (#PICO-000120, Actome), the anti-4EBP1 antibody 4F3-H2 (H00001978-M01; Abnova) was labeled with PICOglue O7 Label (#PICO-000123, Actome), and the anti-phospho 4EBP1 antibody T37T46-A5 (MA5-27999, Invitrogen) was labeled with PICOglue P8 Label (#PICO-000121, Actome).

PICO assay

2 µl of the antibody mix was combined with 2 µl of the prediluted cell lysate (termed 'binding reaction'), sonicated for 1 min at full-power in a sonicator bath and incubated overnight at 4°C. The binding reaction was prediluted 1:10 and diluted again twice more 1:60 in PBS. 1µl of the diluted binding reaction was used as input for dPCR in a total reaction volume of 42 µl. This equates to a total dilution factor back to the binding reaction of 1562820. The master mix was transferred to the 24-well 26k nanoplates (250001, QIAGEN) of the QIAcuity system (911021, QIAGEN) and the dPCR was conducted as described by the manufacturer. For proper imaging conditions see the PICO Amplification Core Kit protocol (PICO-000010, Actome).

Evaluation

The results of the dPCR were evaluated for the couplexes using Actome's AMULATOR software, allowing for absolute quantification of the proteoforms.

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05/2024