Is eIF3h a regulator of the expression of the transcription factor C/EBPβ?

Multiple studies have now indicated the important role C/EBPβ, and its isoform LAP*, LAP and LIP can play in aggressive cancer types like triple negative breast cancer (TNBC). A high C/EBPβ LIP/LAP(*) isoform ratio results in more aggressive cancers and reverting this ratio has been proven to be a target for reducing proliferation and metastasis. mTORC1 can be used as a target to achieve this goal, however, this has been proven unsuccessful in TNBC. It has been observed that eIF3h is significantly upregulated in different cancers, in particular TNBC. Knowing that eIFs can influence uORF mediated isoform translation by affecting ribosome reinitiation we tested if eIF3h affects the LIP/LAP ratio in different Breast cancer cell lines. From using eIF3h knockdown (KD) and overexpression and a translational reporter we failed to come to definitive conclusion as changes in ratio were marginally in KD and overexpression had failed. Most compelling evidence was obtained from the reporter assay however experiments must be repeated for a definitive conclusion.

Introduction

Countless factors and genetic regulators are being investigated in the light of cancer and ageing with one of those factors being CCAAT/enhancer-binding protein β (C/EBPβ). C/EBPβ is a transcription factor which has been described to affect innate and specific immunity; proliferation, differentiation, regeneration, cell migration and metabolism (Greenbaum et al., 1998; Nerlov, 2007; Ramji & Foka, 2002; Sterken et al., 2022; Tanaka et al., 1995). Moreover, C/EBPβ is a master regulator of mammary gland development and tissue remodeling during lactation, and a key factor in the metastasis of breast cancer (Albergaria et al., 2013; Li et al., 2011; Park et al., 2013; Sterken et al., 2022).

C/EBPβ exerts it functions trough three different main isoforms named LAP*, LAP (also known as LAP1 and LAP2 respectively) and LIP. LAP stands for **L**iver **A**ctivating **P**rotein whereas LIP stands for **L**iver **I**nhibiting **P**rotein(Descombes & Schibler, 1991). LAP(*) is a transcriptional activator consisting of a basic leucine zipper that can bind to DNA and a transcription activation domain that can promote transcription. LIP is a shorter version of LAP(*) containing the DNA binding portion but lacks the transcription activation domain. This explains the mostly opposite effects observed compared to LAP(*) with LIP being a transcriptional repressor. (Descombes & Schibler, 1991; Landschulz et al., 1989; Poli et al., 1990).

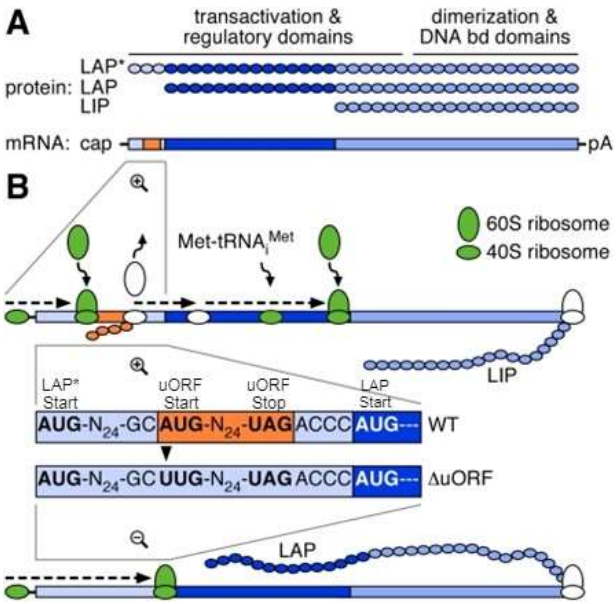


Figure 1: (A) Three protein isoforms LAP, LAP, and LIP are translated from consecutive in-frame initiation codons in the same transcript (Descombes & Schibler, 1991). The C/EBPβ mRNA contains a conserved cis-regulatory small uORF (9 and 11 codons in mice and humans respectively, orange) terminating 4 base pairs upstream of the LAP initiation site in a different reading frame. (bd) Binding; (pA) poly(A) tail. (B) Translation of the uORF serves to strip ribosomes from their initiating Met-tRNAiMet (green to white) and prevents initiation at the proximate LAP initiation codon. Upon reloading of ribosomes with the ternary eIF2–GTP–Met-tRNAiMet complex (white to green), translation reinitiation from the downstream AUG codon generates LIP. In C/EBPβΔuORF mice, an A-to-U point mutation was designed to abrogate ribosomal initiation at the uORF start codon without changing the amino acid sequence of the C/EBPβ isoforms. Most ribosomes will thus initiate at the LAP AUG instead. Figure adapted from* (Wethmar, Bégay, et al., 2010)

These isoforms stem from the same C/EBPβ-mRNA which is possible due to multiple initiation sites (AUG start codons) being present within the same reading frame as can be seen in *figure 1A* (Calkhoven et al., 2000; Descombes & Schibler, 1991; Ossipow et al., 1993) The expression ratio of LIP/LAP is regulated by a dedicated translation control mechanism that requires a cis-regulatory upstream open reading frame (uORF) in the C/EBPβ-mRNA leader sequence (Calkhoven et al., 2000). uORFs are sequence determinants found in mRNA and are important regulatory elements for translation. The coding

sequences of uORFs contain generally fewer than 50 codons, reside in the 5' leader sequence and often inhibit overall translation initiation (Kim et al., 2007). LAP is translated when the C/EBPβ uORF initiation site was not recognised by ribosomes. This explains LAP overexpression when the uORF is removed (Wethmar, Bégay, et al., 2010). When the uORF initiation site is recognised however LAP gets skipped and LIP can be translated (Calkhoven et al., 2000). Close proximity of uORF termination to following start codons (4 base pairs in LAP) causes ribosomes not being able to reinitiate due to not yet occurred reloading of Met-tRNAiMet. Depending on numerous factors, the ribosomes can be stabilised after uORF translation to keep on scanning and can reinitiate downstream at a different start codon (LIP in our case)(Kozak, 1987; Mohammad et al., 2017; Pöyry et al., 2004; Roy et al., 2010) as can be seen in *figure 1B.*

The C/EBPβ uORF controls the expression ratio of the isoforms by sensing the translational status of the cell (Wethmar, Smink, et al., 2010). The nutrient sensing kinase complex mTORC1, for instance, stimulates synthesis of LIP inducing metabolic adaptations. mTORC1 inhibition results in decreased LIP levels which gives rise to a healthier metabolism (Albert & Hall, 2015; Calkhoven et al., 2000; Zidek et al., 2015). In C/EBPβΔuORF mice, where the uORF is genetically absent, the decreased LIP/LAP ratio resulted in an increase in health and life span, and a decreased spontaneous cancer incidence in the mice was observed (Müller et al., 2018). Alternatively, LIP overexpression results in an increased cancer incidence (Bégay et al., 2015).

It has been found that high expression of LIP is associated with breast cancer, ovarian cancer, anaplastic large cell lymphoma, and cellular transformation in cell culture (Calkhoven et al., 2000; Jundt, Raetzel, Muller, et al., 2005; Sterken et al., 2022; Zahnow et al., 1997). Multiple studies have investigated the oncogenic properties of C/EBPβ uncovering that elevated LIP levels induce a switch in cancer-type metabolic re-programming, may inhibit terminal differentiation and lead to proliferation (Ackermann et al., 2019; LaMarca et al., 2010; Robinson et al., 1998; Zahnow et al., 2001). As reverting the high LIP/LAP ratios in triple-negative breast cancer (TNBC) cell lines into low LIP/LAP ratios by overexpression of LAP reduces migration and matrix invasion of these TNBC cells, research into the regulation of C/EBPβ may provide new therapeutic avenues for treating this otherwise difficult to treat cancer variant (Sterken et al., 2022). Targeting mTORC1 with rapamycin has shown potential to decrease LIP and cancer incidence as well as proliferation (Anisimov et al., 2011; Jundt, Raetzel, Müller, et al., 2005; Komarova et al., 2012; Neff et al., 2013). In TNBC, however, targeting mTORC signaling seems to be ineffective in lowering LIP expression and other targets must be identified (unpublished data Calkhoven lab).

In multiple cancers, including breast cancer, an increased expression of eukaryote initiation factor 3 subunit h (eIF3h) has been observed (Gomes-Duarte et al., 2018; Nupponen et al., 1999). Eukaryotic initiation factors (eIFs) are protein complexes necessary for starting translation and altered expression and/or modification can influence the reinitiation in the context of uORFs (Chukka et al., 2021; Lebaron et al., 2012; Olsen et al., 2003; Wethmar, Smink, et al., 2010). eIF3 has been described as the largest and most complex of the mammalian initiation factors being composed of 13 nonidentical subunits, eIF3a-m, as can be seen in figure 2 (des Georges et al., 2015). eIF3 has multiple important functions regarding the initiation of translation of cytoplasmic mRNAs. These functions include translation initiation, by facilitating translation loop formation, termination, ribosomal recycling, and the stimulation of stop codon readthrough (Gomes-Duarte et al., 2018). Notable, eIF3h supports efficient reinitiation and ensures that a fraction of ribosomes retain their competence to resume scanning after uORF translation seen in both plants and humans (Hronová et al., 2017; Kim et al., 2007; Roy et al., 2010; Schepetilnikov et al., 2013). Like LIP, knockdown of eIF3h in breast cancer cells reduces proliferation and growth, and overexpression can induce a malignant phenotype (Zhang et al., 2007, 2008). These observations together with, the apparent correlation between eIF3h and LIP expression in breast cancer, indicate that LIP expression may be increased due to upregulated eIF3h. Here we have quantitated the levels of eIF3h and C/EBPβ-LIP in several breast cancer cell lines using western blots showing a correlation. We have also used short hairpin RNA (shRNA) to create eIF3h knockdowns and have tried to induce overexpression using a lentiviral transfection, but the western blots with quantification of the LIP/LAP ratio failed to show the hypothesized effects. Finally, we used a cellular luciferase-reporter showing increased reinitiation in the presence of lentiviral overexpression vector for eIF3h.

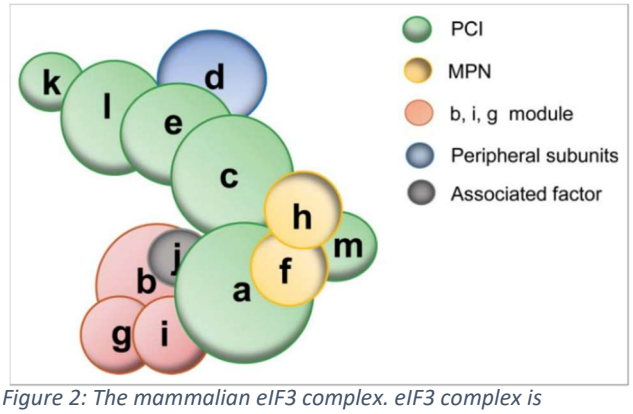


Figure 2: The mammalian eIF3 complex. eIF3 complex is composed of a PCI (Proteasome, COP9, eIF3)/MPN (Mpr1–Pad1 N-terminal) octamer including PCI subunits a, c, e, l, k and m (green) orientated sequentially to form an arc and MPN subunits f and h (yellow) connected with the former through f:m interaction; the b-i-g subunits module (red) is attached to the PCI/MPN octamer by its association with eIF3a carboxy-terminal domain (CTD); the two more peripheral positions are occupied by d and j subunits (blue), respectively. Adapted from(Gomes-Duarte et al., 2018)

Results

C/EBPβ LIP/LAP ratio and eIF3h are increased in TNBC

To test potential correlation of eIF3h expression and LIP expression on breast cancer cell lines, different cell lines from different breast cancer subtypes (triple negative breast cancer, Luminal A, Her2+) were seeded for harvesting. The cells were harvested, and the proteins were extracted. The protein concentration was measured to make sure that the same amount of protein concentration is loaded on the SDS-page gel. SDS-page was used in order to separate the proteins by size. This was done so that the proteins were spread out over the membrane. The membranes were first incubated with skimmed milk containing 0,02% Tween-100, to weaken unspecific antibody interactions. Secondly, the membranes were probed with primary antibodies specific for eIF3h and C/EBPβ and β-Actin. The membranes were probed with secondary antibodies that recognize the animal parts of the primary antibodies and are coupled with horseradish peroxidase. When the membrane is incubated with a luminol-based chemiluminescent peroxidase substate and peroxidase enzyme is present due to the interaction of the secondary with the primary antibodies, there's an emission of light at the positions recognized by the primary antibodies, which was photographed and studied.

It can be observed that both eIF3h and LIP expression is increased in TNBC cell lines. In contrast to this, lower eIF3h levels are observed in the luminal A and HER2 positive breast cancer cell lines (figure 3b). In most cell lines, however the expected increase in LIP/LAP ratio is observed, but notable is also a high eIF3h expression in the untransformed breast cell line MCF10A (figure 3).

eIF3h KD failed to show significant C/EBPβ LIP/LAP ratio alteration

To create a knockdown a vector coding for small hairpin RNA (shRNA) complementary to eIF3h, with eIF3a and -i for control, was created. HEK293T cells were transfected with these shRNA vectors, as well as vectors for lentivirus particle formation. The cells were incubated for 2 days, after which the supernatant virus particles were harvested. The virus particles were placed onto our cell lines, after which transduction of the shRNA vector by lentivirus infection took place. We selected using antibiotics (G418 and puro). Western blots were created to check the results.

First, we sought to determine whether the knockdown was successful. Figure 4 shows the western blots done for our different cell lines. From these blots we concluded that the knockdown was successful for our three cell lines. The knockdown seems to be stronger in MDA-MB-231 compared to BT20 and SKBR3. The blots show the expected effect, as both LIP and eIF3h go down for knockdowns. This seems to point to a regulatory effect. An interesting observation is that for SKBR3 both LAP and LIP go down for the first knockdown.

Figure 3

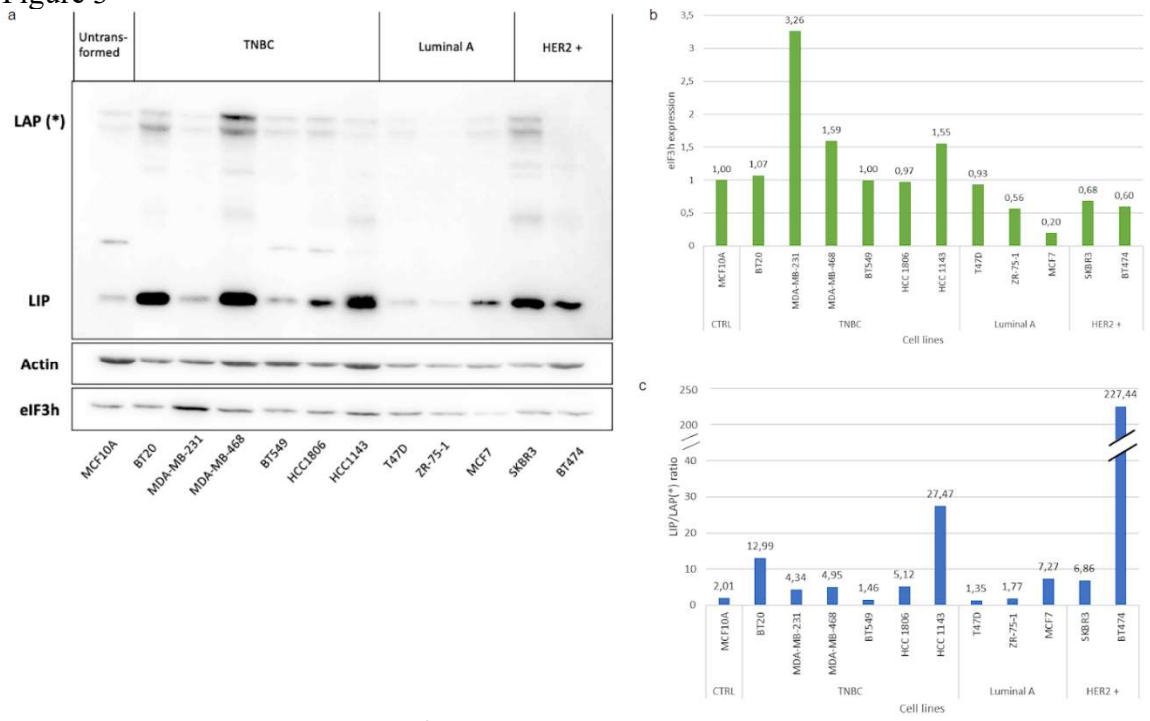


Figure 3: (a) western blots visualizing the C/EBPβ isoforms, actin and eIF3h in different breast cancer cell lines and 1 untransformed cell line (b) quantification of eIF3h from the western blot and (c) the quantification of the LIP/LAP isoform ratio

Figure 4

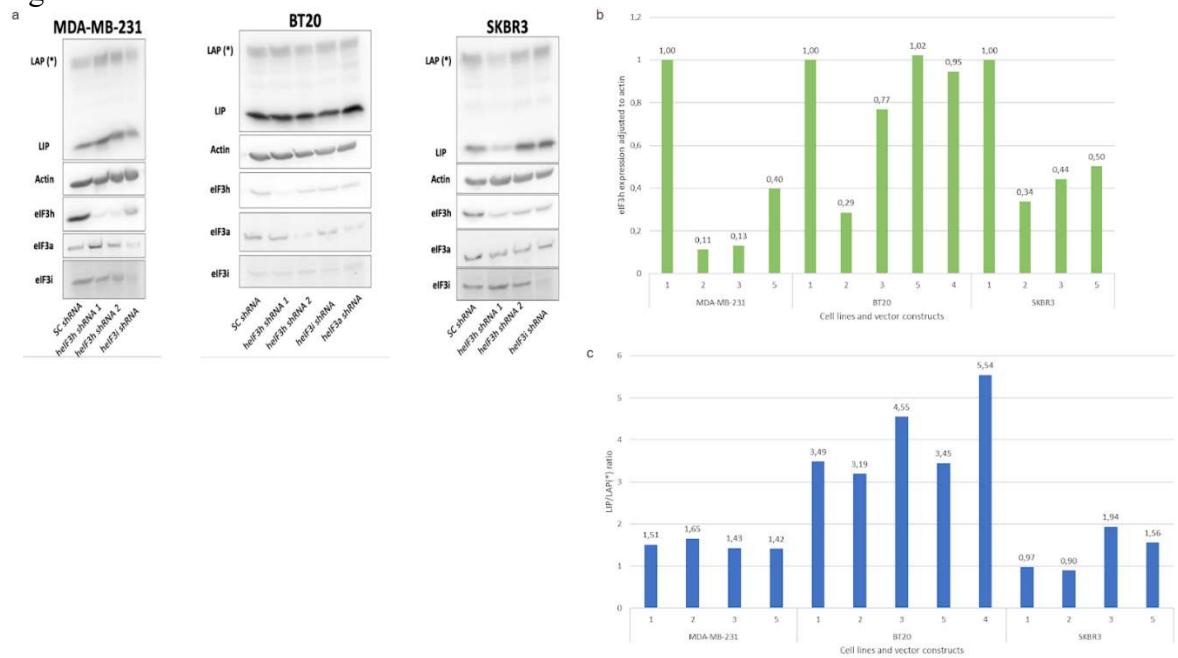


Figure 4: (a) western blots visualizing the C/EBPβ isoforms, actin and eIF3h in different breast cancer cell lines after introducing shRNAs (b) quantification of eIF3h from the western blot and (c) the quantification of the LIP/LAP isoform ratio

Overexpression

To determine the effects of increased eIF3h levels on C/EBPβ LIP/LAP ratios, the same lentivirus transfection/infection protocol was used to introduce an overexpression. Here the cell lines MDA-MB-231, MCF7 and T47D where infected with lentivirus particles containing vectors with eIF3h preceded by a promoter that would drive the expression of eIF3h in these cells. Western blotting was used to determine if eIF3h is indeed overexpressed and to check the LIP/LAP isoform ratio. Unfortunately, the blots and following quantification failed to show a significant increase in eIF3h compared to the factor lacking the eIF3h sequence. We only see a slight increase in the T47D and MCF7 cells but see even a decrease in the MDA-MB-231 cells of the eIF3h levels. Interestingly enough we see also a slight decrease in LIP/LAP isoform ratio in T47D and MCF7 cells and a marginal increase in the MDA-MB-231. The differences are too small however to make any assumptions on the effect, however and can be observed in figure 5.

Figure 5

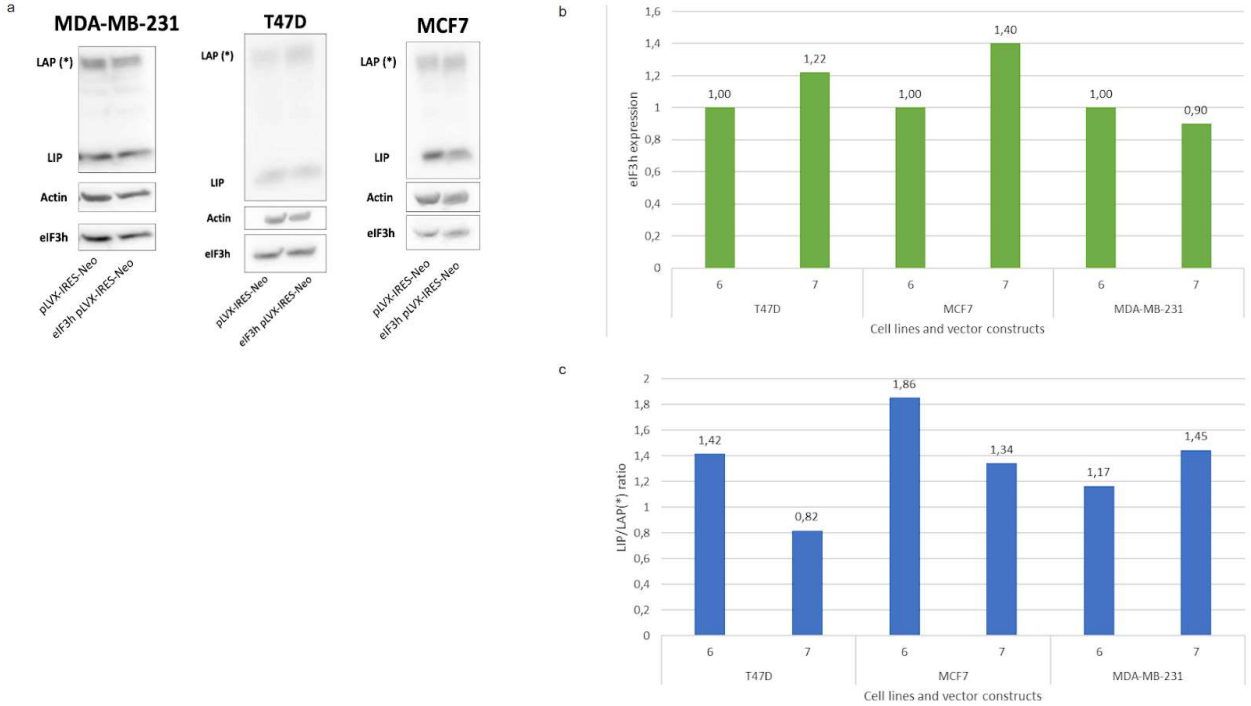


Figure 5: (a) western blots visualizing the C/EBPβ isoforms, actin and eIF3h in different breast cancer cell lines after introducing eIF3h promoting vectors (b) quantification of eIF3h from the western blot and (c) the quantification of the LIP/LAP isoform ratio

Sample	Amount (µL)	Buffer (µL)	Milli-q water (µL)
BT474	7.17	6	10.83
BT20	4.79	6	13.21
MCF10A	7.98	6	10.02
SKBR3	3.30	6	14.70

T47D	5.29	6	12.71
ZR-75-1	2.61	6	15.39
MCF7	3.33	6	14.67
HCC1143	6.53	6	11.47
MDA-MB-231	6.97	6	11.03
BT549	6.29	6	11.71
HCC1806	6.56	6	11.44
MDA-MB-468	6.64	6	11.36
Marker	5	6.25	13.75

Table 1; First SDS-page samples. 60 µg protein was loaded.

Sample	**Amount (µL)**	**Buffer (µL)**	**Milli-q water (µL)**
MDA-MB-231 1	3.74	3.75	7.51
MDA-MB-231 2	2.64	3.75	8.61
MDA-MB-231 3	3.81	3.75	7.44
MDA-MB-231 5	3.10	3.75	8.15
BT20 1	4.64	3.75	6.61
BT20 2	6.64	3.75	4.61
BT20 3	5.26	3.75	5.99
BT20 4	9.41	3.75	1.84
BT20 5	5.25	3.75	6.00
SKBR3 1	2.76	3.75	8.49
SKBR3 2	3.15	3.75	8.10
SKBR3 3	6.96	3.75	4.29
SKBR3 5	2.27	3.75	8.98
T47D 6	3.08	3.75	8.17
T47D 7	2.88	3.75	8.37
MCF7 6	4.18	3.75	7.07

MCF7 7	1,91	3.75	6.62
MDA-MB-213 S.Ctrl	3.46	3.75	7.70
MDA-MB-231 6	3.71	3.75	7.54
MDA-MB-231 7	1.91	3.75	9.34
Marker	2.5	3.75	8.75

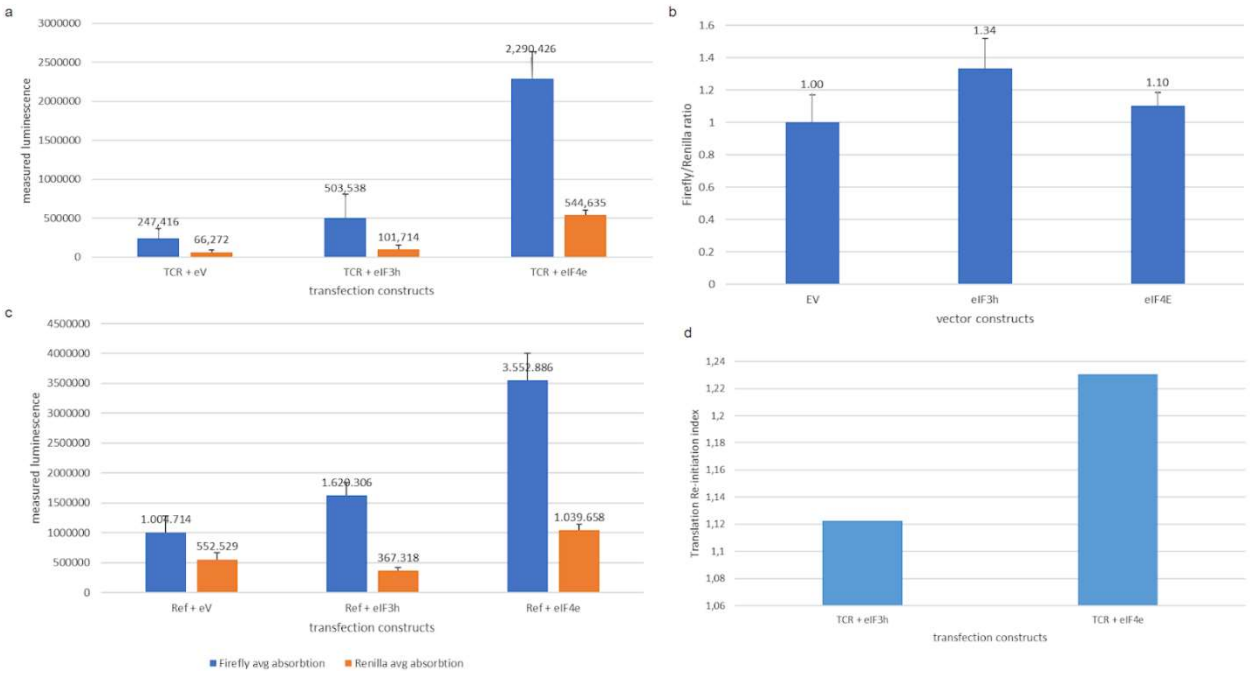
Table 2; Second SDS-page. With 1. Scrambled control shRNA; 2. heIF3h shRNA 1; 3. heIF3h shRNA 2; 4. heIF3a shRNA; 5. heIF3i shRNA; 6. pLVX-IRES-Neo; 7. eIF3h pLVX-IRES-Neo. 60 µg protein was loaded.

eIF3h expression increases ribosomal reinitiation after C/EBPβ uORF translation in HEK293T

In order to identify if eIF3h play a role in the protein isoform ratio modulation of LIP and LAP connected to C/EBPβ-uORF dependent and differential translation of the C/EBPβ-mRNA into the different isoforms we used a reporter system as introduced by Zaini, M. A. et al.(2017).This single dual reporter plasmid was used to test the translation initiation into LAP and the translation re-initiation into LIP under control of the C/EBPβ-5′-leader sequence in presence of eIF3h and eIF4e. In this reporter the renilla luciferase simulates LAP expression, whereas firefly luciferase simulates that of LIP.

To test the assumption that eIF3h increases the amount of reinitiation and thus LIP translation we introduced CDNA3.1 plasmid containing eIF3h into the HEK293T cells as well as eIF4e which is known to increase the LIP/LAP ratio due to increased reinitiation (Zaini et al., 2017). Compared to the cells were an empty pCDNA3.1(eV) was introduced both the eIF3h and the eIF4e group show an increase in ratio as well as calculated TRI. Notable however is that in the eV group the calculated translation reinitiation index (TRI) came out to be around 2 while it would be expected to be around 1(data not shown). Luckily when the reference cassette results are exempted, and the empty vector is used as reference the results still indicate an increase in reinitiation in the case of eIF3h overexpression as well as eIF4e overexpression. When we compare the ratios, we see a non-significant increase in the eIF4e overexpression group and interestingly a significant increase in reinitiation in the eIF3h overexpression group. This data reinforces the idea that eIF3h can increase LIP expression due to facilitating efficient reinitiation after C/EBPβ uORF translation. As this way of comparison is used before, we still included these results and can be seen in figure 6.

Figure 6


a
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2000000
1500000
1000000
500000
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503,538
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TCR + eV
TCR + eIF3h
TCR + eIF4e
transfection constructs
b
Firefly/Renilla ratio
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1.00
1.34
1.10
EV
eIF3h
eIF4E
vector constructs
c
measured luminescence
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4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
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1.629.306
367,318
3.552.886
1.039.658
Ref + eV
Ref + eIF3h
Ref + eIF4e
transfection constructs
Firefly avg absorbtion
Renilla avg absorbtion
d
Translation Re-initiation index
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1,22
1,2
1,18
1,16
1,14
1,12
1,1
1,08
1,06
TCR + eIF3h
TCR + eIF4e
transfection constructs

Discussion

TNBC lacks expression of the hormone receptors for estrogen and progesterone, and the growth factor receptor HER2. While these receptors are used as therapeutic targets their absence makes treatment far more difficult in TNBC. This together with TNBC being an aggressive type of breast cancer the prognosis is far poorer than breast cancer types proficient for at least one of these factors. The expression ratio of the C/EBPβ protein isoforms LIP to LAP determines cell migration and cell invasion behavior and contributes to the regulation of EMT- and ECM-related genes and it was found to be increased in TNBC (Sterken et al., 2022; Zahnow et al., 1997). It is essential for the development of more effective therapies to gain a better understanding of factors that influence this ratio, as lowering the ratio makes the cancer less aggressive (Sterken et al., 2022). With the correlation between eIF3h expression and high C/EBPβ LIP/LAP ratio in mind, together with the knowledge of eIFs subunits affecting uORF mediated reinitiation, we aimed to investigate the potential role of eIF3h as a regulator of the expression of the transcription factor C/EBPβ. The obtained data suggest that there might be a correlation between eIF3h and a high LIP/LAP ratio in TNBC, however the data do not indicate this for other breast cancer types. The knockdown of eIF3h was successful, however the quantification did not show any significant difference in the LIP/LAP ratio. The cell lines infected with lentivirus carrying vectors for overexpression of eIF3h failed to show a very pronounced overexpression as well as a significant LIP/LAP ratio alteration. The reporter assay, however, supports the theory that eIF3h promotes reinitiation after uORF translation.

It is already clear from previous research that both eIF3h and C/EBPβ play important roles in cancer and malignancy. In line with the hypothesis are the results off the reporter assay and correlation between an increased LIP/LAP ratio and increased eIF3h within TNBC cell lines. Contrary our findings in the knockdown experiment are not in line with what we would have expected. It is possible that because a higher LIP/LAP ratio result in more proliferation this has been selected for on the plates while we still were culturing the cells. A possible work-around would be to harvest the cells sooner after the infection to prevent such selection. Another possibility is to use an inducible eIF3h knockdown or even create a full knockout if cells are still viable. Our findings between the different experiments are not consistent with each other which might suggest that eIF3h does not affect C/EBPβ. Next to this, the reporter assay showed unexpected values for the reference cassette that measures general transcriptional and translational effect without affecting specifically reinitiation. This should be comparable between different sample groups but was, however, greatly differing between the different groups. The reason for this is not clear, but it may have something to do with the transfecting or another step within the assay which might have been not optimal and might influence the results and therefore we used comparison between different groups instead. It has also to be acknowledged that the eIF3h levels of our control cell line MCF10-A were higher than expected. A possible explanation for this was uneven loading on the SDS-page, however, an India ink staining showed no significant differences in protein between the lanes suggesting that uneven protein loading could not have been the problem. Another plausible explanation could be, as the used cell line was in culture for a longer period before, that the characteristics of MCF10A cells could have changed and this of course can affect the eIF3h levels or just a unsuitable control for correlation and experiments have to be repeated to account for variability. All data of this study must be interpreted with caution because the experiments where only performed once with very low sample sizes

Overall, this study failed to refute or confirm the theory of eIF3h being a regulator of the C/EBPβ LIP/LAP ratio. To truly test our hypothesis our experiments should be repeated with the use of a fresh batch of control cells and preferably a second control cell line to account for natural high

expression ~~of~~ eIF3h. It would also be advisable to redesign the eIF3h overexpression vectors to create a more pronounced overexpression and in that way allow to test whether eIF3h overexpression will affect the LIP/LAP ratio. Finally, the reporter assay should be repeated with an added selection step to assure a successful transfection. One could also try to create different expression levels of eIF3h to see if there is any dose dependent effect that can further solidify our understanding on the working mechanisms of regulation of ribosome reinitiation after uORF translation. Finally, these future experiments might lead to a new avenue of opportunities to create more effective treatment of TNBC and maybe of other aggressive cancers which show a high LIP/LAP isoform ratio.

Methods

Preparation of protein extracts and protein concentration measurement (Bradford Assay)

The harvested and frozen cell pellets were defrosted using RIPA buffer. The suspended cells went through 10 cycles (30 sec on; 30 sec off) in the sonicator. 1 µl of the cell extract was resuspended in 800µL water in new Eppendorf tubes and mixed with 200 µl Bradford reagent to measure the protein concentration. For the Bradford assay 200 µL of this mixture per sample was transferred to a well of a 96-well plate. The plate was loaded on the machine and the optical density was measured using a wavelength of 600 nm. The concentration of each sample was determined with the help of an already present standard curve.

Cell culture

HEK293T, MDA-MB-231, BT20 were maintained in DMEM. MCF7, SKBR3, T47D, ZR-75-1 cells in RPMI (Gibco). Both media were supplemented with 10% fetal calf serum (FCS) and cells were cultivated at 37 °C with 5% CO2. Cell viability was checked using light microscopy. Cell number was determined using the TC20TM automated cell counter (Bio-Rad) following the manufacturers instruction or a Neubauer counting chamber in the case of the HEK293T cells. For generation of cells stably expressing the eIF3h subunit vector or a eIF3 subunit shRNA, the cells were transfected as described. Cells expressing overexpression or control constructs were selected by addition of 800µg/ml G418 to the medium in the case of MCF7 and T47D, and 1200µg/ml G418 for MDA-MB-23. The knockdown constructs selection of the transduced cells was done using puromycin. For MDA-MB-231 0.5 µg/ml, BT20 1 µg/ml and SKBR3 2 µg/ml puromycin was added to the culture plates.

Harvesting cells

A bucket of ice was prepared containing a metal Eppendorf tube holder and cold PBS (pH ~7.4). Empty tubes were placed in the holder to cool down. The medium was removed from the plate containing the cells. The cells wer washed twice with 10 mL cold PBS. After removing the second wash of PBS, 1 mL of cold PBS was added to the plate and the cells were scraped off from the plate with a cell scraper. The PBS-cell mixture was added to a labelled Eppendorf tube. The tube was centrifuged at 4ºC for 30 seconds. The PBS was removed, and the cell pellet was snap-frozen in liquid nitrogen. The tube was scooped out of the liquid nitrogen and stored in a freezer at -80ºC.

SDS-page

All protein samples were kept in ice for the entire experiment. The same protein amount (~60 µg) of protein extract per sample was transferred to a new Eppendorf tube and mixed with milli-q water and sample buffer (*tables 1 and 2*). The samples were incubated at 97º for 4 minutes and shortly centrifuged to collect liquid from inside the lid.

A separating gel made of 10 mL acrylamide 30%, 168 µL APS, 8.75 mL milli-q water, 6.25 mL SDS Tris pH 8.8 and 34 µL TEMED was loaded into the SDS-page apparatus. A layer of isopropanol was added on top to make sure the separating gel would polymerize with a straight surface. After ~30 minutes, the isopropanol was removed and the stacking gel made of 1.95 mL acrylamide 30%, 150 µL APS, 9.15 mL milli-q water, 3.75 mL SDS Tris pH 6.8 and 30 µL TEMED was added on top of the separating gel. The comb was added, and the gel was set aside to harden for ~30 minutes. After the gel was polymerized, the comb was removed. A running buffer was added to the apparatus. The samples were loaded in the combs and the gels were run (midi-sized gels at 40 mA: mini-sized gels at 150V).

Western Blot

The gels of the SDS-page were transferred onto either PVDF or nitrocellulose membranes using the Bio-Rad Trans-Blot Turbo Transfer System. To do this, after electrophoresis, the gel was transferred into the cassette with the membrane that had previously soaked in a transfer buffer (100 mL transfer buffer, 300 mL milli-q water and 100 mL pure ethanol). PVDF membranes were activated by incubation in methanol for 1 min before the incubation in transfer buffer. The cassette was inserted into the instrument and ran for 10 minutes (high molecular weight program).

After transferring the proteins onto the membrane, the membrane was washed twice in TBS-T buffer, refreshing the TBS-T after each wash, in 5-minute intervals. Then, the membrane stayed in 20 mL milk (5% milk powder) for one hour on the rocking platform to block nonspecific antibody interactions. The milk was removed and 20 mL milk with 1:1000 antibody, except for 1:10.000 for β-actin antibody and 1:400 for eIF3i antibody, was added onto the membrane. The membrane was placed in the cold room (~4ºC) overnight.

The next day, the milk was removed, and the membrane was washed four times with TBS-T in 5-minute intervals, refreshing the TBS-T after each interval. Afterwards, 20 mL milk with 1:5000 secondary rabbit antibody was added onto the membrane. The membrane stayed on the rocking platform for one hour. The antibody mixture was removed, and the membrane was again washed four times as previously described with TBS-T. Then, the membrane was incubated with ECL reagent followed by imaging.

For imaging, the membrane was put on the tray for the imaging machine and Western Lightning Plus-ECL was added onto the membrane. We used the settings of 2 minute intervals for 16 images, except for beta β-actin, where we used 30 seconds intervals and 16 images.

Translational control reporter

For the reporter assay, the luciferase-based cellular reporter system for C/EBPβ was used (Zaini et al., 2017). A total of 40 wells were seeded with 20000 HEK293T cells per well in 96-well plate. The cells were transfected with, pcDNA-FireflyRe-ini/RenillaIni or pcDNA-FireflyRef/RenillaRef together with either an empty vector, a eIF3h expression vector or a eIF4e expression vector in quadruplicates using FUGENE HD (Promega).

Each of the vectors are concentrated at 1.33 µg in 65µl FuGene The next day, luciferase activity was measured in the HEK293T cells using Dual-Glo Luciferase Assay System (#2920, Promega) following the manufacturer's protocol using a GloMax-Multi Detection System (Promega).

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