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IDENTIFICATION AND CHARACTERIZATION OF A NOVEL *EIF3E* TRANSCRIPT: ROLE IN MAMMARY DEVELOPMENT AND CARCINOGENESIS

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SOMMARIO

Il gene *EIF3E* (eukaryotic translation initiation factor 3 subunit e) è stato identificato inizialmente in topo come sito di integrazione del virus MMTV (mouse mammary tumor virus). Nell'uomo *EIF3E* è un gene di 45 Kb localizzato nella banda 22-23 del braccio lungo del cromosoma 8 che codifica per un RNA messaggero di 1516 nucleotidi ed una proteina di 445 amminoacidi (52 KDa). La funzione di *EIF3E* è stata associata ai meccanismi di regolazione dell'espressione genica e alla stabilità ed integrità genomica. Sebbene sia stata individuata l'esistenza di una correlazione tra il tumore mammario e la riduzione dell'espressione di *EIF3E*, non è ancora noto l'esatto ruolo che *EIF3E* svolge nell'insorgenza e nella progressione del tumore.

Questo progetto di dottorato è focalizzato sullo studio di una nuova isoforma del gene *EIF3E*: chiamata *EIF3E*-5/5. Questo trascritto individuato e caratterizzato nel nostro laboratorio è costituito da 771 nucleotidi: di cui 492 corrispondenti alla sequenza del trascritto *EIF3E* (dall'esone 1 all'esone 5) e 279 nucleotidi all' introne 5 del gene *EIF3E* in grado di generare per splicing alternativo un nuovo esone che abbiamo chiamato 5A. Il trascritto *EIF3E*-5/5 codifica per una proteina di 171 aa (20 KDa). Entrambe le isoforme di *EIF3E* (*EIF3E* e *EIF3E*-5/5), sebbene ubiquitarie, sono più espresse nella ghiandola mammaria rispetto ad altri tessuti e la loro espressione diminuisce nella condizione tumorale.

Nella ghiandola mammaria, in condizioni fisiologiche il rapporto tra l'espressione di *EIF3E* ed *EIF3E*-5/5 è circa 100:1. Questo valore nel 75% dei tessuti tumorali analizzati, provenienti da pazienti affetti da carcinoma mammario diminuisce. Questo risultato indica che variazioni nel rapporto tra le due isoforme di *EIF3E* potrebbero essere correlate alla formazione e progressione del carcinoma mammario.

Attraverso esperimenti *in vitro* abbiamo dimostrato che la riduzione del rapporto tra *EIF3E* e *EIF3E*-5/5, ottenuta mediante l'over-espressione di *EIF3E*-5/5, è in grado di indurre un fenotipo tumorale (aumento della proliferazione) sia in cellule primarie normali della ghiandola mammaria sia in linee cellulari continue. Nella linea cellulare MCF7 l'up-regolazione di *EIF3E*-5/5 sembra inoltre svolgere una funzione nel controllo del self-renewal di una sub-popolazione di cellule in essa presenti, dotate di proprietà staminali.

EIF3E-5/5 è coinvolto nella regolazione di mRNAs tradotti mediante il meccanismo cap-indipendente e nella traduzione degli mRNA degli istoni.

Con il nostro lavoro dimostriamo che sia nelle cellule MCF7 sia nelle cellule primarie mammarie normali l'up-regolazione di *EIF3E*-5/5 è in grado di favorire l'espressione di mRNA soggetti a traduzione cap-indipendente. Questo risultato sottolinea come il ruolo di *EIF3E*-5/5 nel carcinoma mammario possa essere associato alla sua funzione di controllo della modalità di traduzione di alcuni mRNA. Infine è stato dimostrato che *EIF3E*-5/5 è coinvolto nella regolazione dei trascritti codificanti gli istoni H3. Questa funzione conferisce ad *EIF3E*-5/5 un importante ruolo nel controllo della replicazione del DNA e spiegherebbe come le sue alterazioni possano essere alla base dell'instabilità genomica e quindi dell'insorgenza e progressione del carcinoma mammario.

ABSTRACT

The *EIF3E* (eukaryotic translation initiation factor 3, subunit e) gene was originally identified in mouse as a integration site of the Mouse Mammary Tumour Virus (MMTV). It is a 45Kb gene localized to the region q22-23 of the human chromosome 8, that encodes a mRNA of 1516 nt and a protein of 445 amino acids, of size 52 KDa. The EIF3E protein is involved in various type of cellular regulation including gene expression, genome integrity and stability.

Altered expression of the *EIF3E* gene occurs in human breast cancers, but the exact nature of its involvement in tumorigenesis is not fully understood.

This Ph.D. work was focused on studying a new ubiquitously expressed alternatively spliced form of the gene *EIF3E* called *EIF3E*-5/5 never described. It is a 771 nt RNA, containing 492 nt of the *EIF3E* mRNA sequence corresponding to sequences from exon 1 to exon 5 and 279 nt of *EIF3E* intron 5 that generate a new exon, that we name exon 5A, by alternative splicing. We assume that the *EIF3E*-5/5 transcript encodes a cytoplasmatic protein of 171 amino acid (20KDa) that until now we have not yet identified in cells.

Both *EIF3E* isoforms (*EIF3E* and *EIF3E*-5/5) while ubiquitously expressed in normal and tumor cells, have higher expression levels in the mammary gland compared to other tissues in the body. We showed that in breast cancer tissues their expression is lower than in healthy breast tissue.

We measured the expression of both *EIF3E* isoforms in the mammary gland and obtained a ratio of expression for *EIF3E* and *EIF3E*-5/5 of about 100:1. The expression level of both transcripts in 75% of breast cancer tissues analyzed was lower and the ratio *EIF3E* and *EIF3E*-5/5 was reduced compared to the ratio obtained in the normal breast tissue. Our results support that variation in the ratio between the two *EIF3E* isoforms correlates with breast cancer initiation and progression.

By *in vitro* experiments we showed that the reduction of the ratio of *EIF3E* and *EIF3E*-5/5, induced by *EIF3E*-5/5 over-expression, produces a tumoral phenotype (increase in cell proliferation) in normal primary mammary cells and in MCF7 cells. The results obtained from *EIF3E*-5/5 up-regulation in MCF7 cells support also that this transcript has a function in controlling the self-renewal potential of cells with stem cells proprieties.

Our results support that *EIF3E*-5/5 has an important function in controlling the expression of specific mRNAs involved in key aspects in the cancer process such as histones and the translation of specific mRNAs in cap-independent manner.

In this Ph.D. work we showed that *EIF3E*-5/5 up-regulation supports the expression of cap-independent mRNAs both in MCF7 cells and human normal primary cells. These transcripts are usually expressed in stress and tumoral conditions. The role of *EIF3E*-5/5 could strongly correlate the expression of this *EIF3E* isoform to breast cancer.

We also showed that *EIF3E*-5/5 is involved in H3 histone mRNAs transcription through its ability to control DNA replication and genomic instability.

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1. INTRODUCTION

1.1. Breast cancer

1.1.1. Breast cancer features

The mammary gland is a unique organ that undergoes extensive remodeling and differentiation even in adult mammals. It reaches a mature functional state only during the pregnancy-lactation cycle (PCL) in adult female and regresses to a resting state upon cessation of lactation. The same cycle of expansion and regression accomplishes in all subsequent pregnancies, during reproductive life. The remodeling of the gland into a milk secretory organ requires hormonal influences that effect drastic modifications in the micro- and macro-anatomy of the gland. The female breast is constituted mainly of lobules (milk-producing glands), ducts (tiny tubes which carry the milk from the lobules to the nipple), and stroma (fat tissue and connective tissue surrounding the ducts and lobules, blood vessels, and lymphatic vessels) (Fig. 1 A, B).

The plasticity of the mammary gland is achieved by the existence of cells that have stem cell-like proprieties. These cells, mammary stem cells (MaSCs), were found in human and mouse breast tissue and they are required for the normal mammary gland development [1, 2]. MaSC has a self-renewal potential, it differentiates into a common progenitor, which gives rise to myoepithelial or luminal progenitor. The progenitors ultimately differentiate into luminal, ductal epithelial and myoepithelial cells (Fig. 1 C). The regulators of these differentiation pathways remain elusive [3]. A sound knowledge of the development, anatomy, histology, and regulation of the breast is integral in the understanding the complete mammary gland biology and its pathologies.

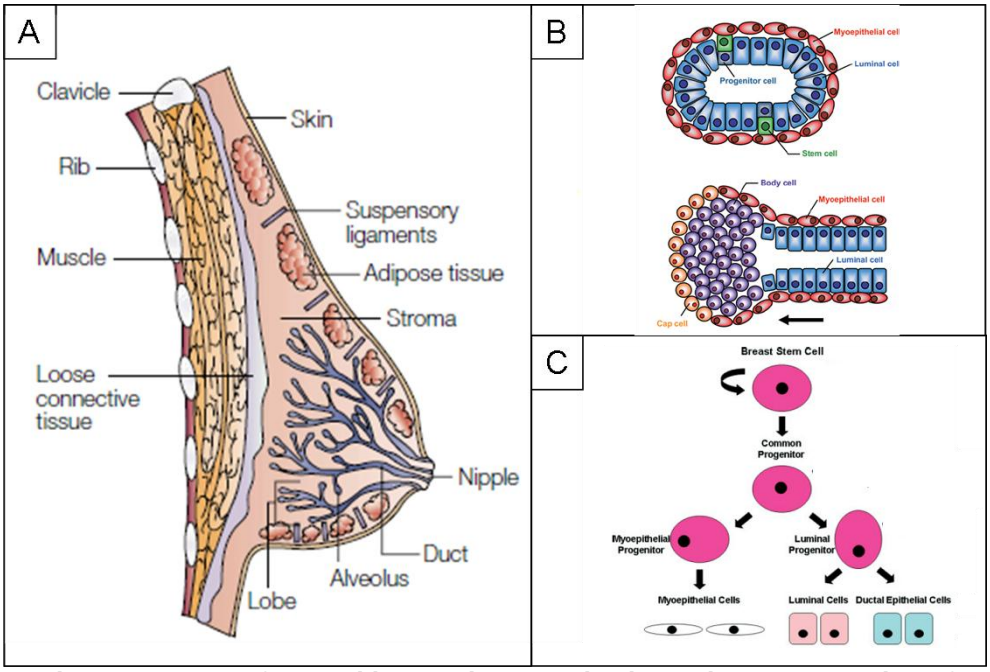

***Fig. 1 Structure of normal breast tissue and schematic representation of MaSCs differentiation**. **A** Anatomy of the human mammary gland. Each mammary gland contains lobes, each lobe containing a series of alveoli and branched ducts that drain into the nipple. **B** Cellular structure of the mammary gland. Each duct has an outer layer of myoepithelial cells (red) surrounding an inner layer of luminal epithelial cells (blue). MaSCs (green) reside in a basal position between these two populations and give rise to progenitor cells. During the developing of mammary gland the ducts invade the empty fat pad generating the terminal end bud (TEB). Stem-like cap cells (orange) lead invasion in the direction of black arrows. Many of the inner body cells (purple) undergo apoptosis during the gland growing. **C** Schematic representation of MaSCs differentiation. MaSCs, with self-renewal potential, differentiates into a common progenitor that gives rise to committed myoephitelial and luminal progenitor, which differentiate into myoepithelial, luminal and ductal epithelial cells. Modified from Ali et Coombes (Nature Reviews Cancer, 2002), Tiede et Kang (Cell Res., 2011) and Bombonati et Sgroi (J Phatol., 2011).*

Breast cancer is a devastating pathology of the mammary gland. It is the most frequent cancer and cancer-related cause of death in women world-wide.
Until now it has not characterized yet appropriate prognostic guidelines and treatment options for patients with breast cancer, despite considerable scientific effort were done into elucidating the primary cause of breast cancer. The main problem to prevent, diagnose and treat breast cancer is associated to lack of complete understanding of the normal mammary gland physiology and to heterogeneity of the breast cancer, at both molecular and clinical level. Knowledge gained in the last years from studying the molecular pathology of human breast

cancer progression, together with integration and implementation in the clinical setting, promise to further reduce breast cancer morbidity and mortality in the next years.

Breast cancer progresses during multiple alterations that affect molecular signatures, functions and structures of cells in the mammary gland [4]. These alterations cause cellular immortality, hyperplasia, tumorigenicity, and invasiveness. Most breast cancers take their origin from the cells that line the ducts (ductal cancers), some from the cells that line the lobules (lobular cancers), while a small number start in other cells. The progression of breast cancer is defined by specific pathological and clinical stages. These stages start with an initial epithelial hyperplasia, which develops into cellular athypia, in situ carcinoma with the progression to a locally invasive carcinoma. The *in situ* carcinoma can metastasize to various organs such as the lung, bone and liver [4, 5, 6] (Fig. 2).

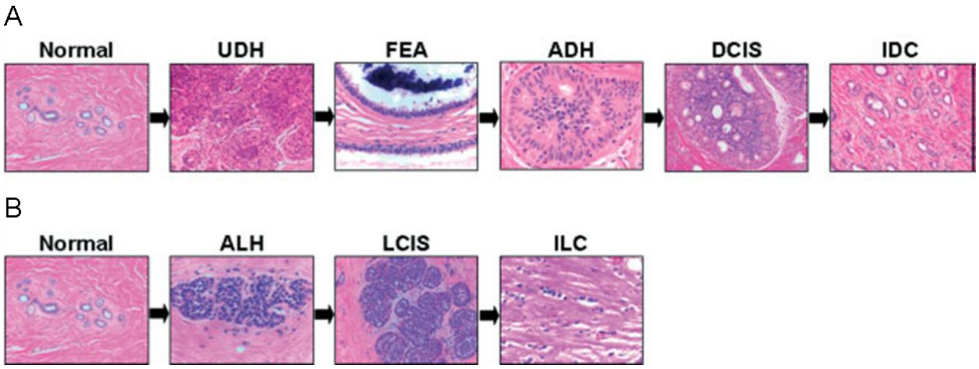

Fig. 2 Model of breast cancer progression. A *Model of the ductal type breast cancer progression. The breast cancer starts in the normal epithelium (normal), progresses to usual ductal hyperplasia (UDH) and proceeds to flat epithelial atypia (FEA), atypical ductal hyperplasia (ADH), ductal carcinoma in situ (DCSI) and culminates into invasive ductal carcinoma.* ***B*** *Model of lobular neoplasia. The multi-progression steps are normal epithelium, atypical lobular hyperplasia (ALH), lobular carcinoma in situ (LCSI) and invasive lobular carcinoma. Modified from Bombonati et Sgroi (J Phatol., 2011).*

The initiation of breast cancer is due to transforming (genetic) events in a single cell. It has been demonstrated that, as well as genetic alteration, also microenvironment and epigenetic changes can influence breast cancer initiation and progression [7]. The accumulation of genetic modifications in breast cancer cells, combined with clonal expansion and selection are the causes of tumor progression. There are two leading models about the cell that gives rise to breast cancer: the cancer stem cells (cSCs) model and the sporadic clonal evolution model [8, 9, 10] (Fig. 3). According to the first model only stem and progenitor cells (estimated to be one out of 2000 epithelial cells) can initiate and maintain tumor progression. In the alternative model any breast cell can be the target of random mutations. The cell with advantageous genetic and epigenetic alterations is selected over time and contributes to tumor progression. The two models are not

mutually exclusive. It has been suggested that stem cells might undergo clonal evolution, providing a dynamic link between the two models [11, 12]. The more aggressive breast cancers originate from mammary stem cell/progenitor, while the less aggressive from more differentiating cell [13, 14]. More cancer stem cells are present in poorly differentiated than in well-differentiated tumors, and are associated with poor survival and high invasiveness [15,16].

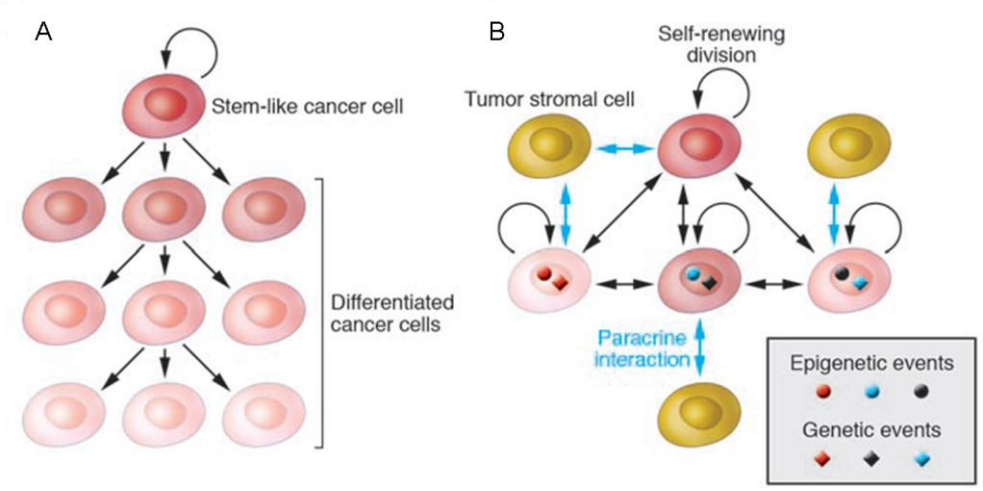

Fig. 3 Model of cell that drive breast cancer. A *Cancer stem cell model. Differentiated cancer cells are progeny of cancer stem cells and they are not able to undergo self-renewing cell division. Thus, only the cancer stem cell can accumulate additional genetic changes that can drive tumor progression and drug resistance.* ***B*** *Clonal evolution model. Genetic and epigenetic alteration can affect each kind of mammary gland cells. Tumor cell phenotypes are based on the combination of cell type of origin of the tumor-initiating cell and paracrine signals from surrounding cells. All tumor cells have the capacity to undergo self-renewing division; thus they have the potential to contribute to tumor progression and drug resistance. Modified by Polyak (J Clin Invest, 2007)*

Although the two models are based on monoclonal origin, most tumors appear to contain a heterogeneous population of cancer cells. This observation increases the evidence for the CSC model. These CSCs, having stem cells proprieties including self-renewal and multi-lineage differentiation, can contribute to cellular heterogeneity. CSCs pluripotency allows these cells to adapt and to be resistant to chemotherapy, radiation therapy, and current molecularly targeted therapies. Furthermore the CSCs represent a rare population of cells; it is believed that many CSC types are preserved in a relatively quiescent phase of cell proliferation. The existence of quiescent cancer stem cells in tumors has implications in cancer therapy since current anticancer therapies predominantly target highly proliferative cells and do not affect low proliferating cells, such as CSCs. These cells do not eradicated can give rise to tumor formation, that reoccurs even years after of cancer therapy treatment. CSCs present yet in the breast tissue are able to

produce a new set of cancer cells. Many researchers and oncologists see in the cancer stem cell model an explanation of how the cancer may be so difficult to prognosticate and treat.
Even thought breast cancer is a very complex and heterogeneous disease, different subtypes are been characterized using their distinct molecular signatures. Each type of breast tumor has varied responses to therapy and shows differences in patient survival [17]. Perou and colleagues, by gene expression profiling, characterized four breast cancer subtypes: two ER-positive (luminal A and luminal B) and two ER-negative (ERBB2 and basal subtype) [17]. Luminal A and luminal B are the most common subtypes usually representing low to intermediate-grade tumors. The ERRB2 tumor subtype represents high grade tumors while the basal subtype usually represents the more aggressive tumor displaying necrosis, lymphocytic infiltrate and a pushing border. It has been suggest that distinct cell types or transformed MaSCs (Mammary stem cells), at different developmental stage are behind each breast cancer subtype [13].

1.1.2. Gene associated to breast cancer

While the exact etiology of breast cancer is unknown, family history is one of the strongest determinants of risk used to do a prognosis of this disease. Only a small percentage of all breast cancers clusters in a hereditary subset and are associated with inherited gene mutations. Variations, that involve gene such as *BRCA1*, *BRCA2* (breast cancer-1,-2), *CDH1* (E-chaderin), *STK11* (serine/threonine kinase 11), and *TP53* (tumor protein p53) are correlated to increase the risk of developing breast cancer. *BRCA1* and *BRCA2* are tumor suppressor genes that play an important role in maintaining the stability of cell genetic information. Mutations in the *BRCA1* and *BRCA2* genes are inherited in an autosomal dominant pattern, which means one copy of the altered gene in each cell is sufficient to increase a person's chance of developing cancer. It is important to note that people inherit an increased risk of cancer, not the disease itself. Not all people who inherit mutations in these genes will develop cancer. An important gene associated to breast cancer is *TP53*. Inherited mutations that involve *TP53* gene account for less than 1 percent of all breast cancer cases. Mutations in this gene greatly increase the risk of developing breast cancer as part of a rare inherited cancer syndrome called Li-Fraumeni. *TP53* is also subject to somatic mutations that are much more common and occur in approximately 20 percent to 40 percent of all breast cancer cases. These types of cancer are typically not inherited and do not occur as part of a cancer syndrome. In other cases of breast cancer, one copy of the *TP53* gene is lost and the remaining copy has a mutation that prevents the cell from producing any p53 protein. Without this protein, DNA damage accumulates and cells divide by uncontrolled way, leading to a cancerous tumor.
The knowledge of genes involved in breast cancer is very important for preventing, diagnose and treat this kind of pathology. Until now the known susceptibility genes, in breast cancer, can only account for around 20-25% of the familial risk of the breast cancer, so more than 75% remains unexplained [18].
Several large-scale studies recently executed have identified such allele variants and SNPs (single nucleotide polymorphisms) in *FGFR2* (fibroblast growth factor receptor 2), *TNRC9* (thymocyte selection–associated high mobility group box 9), *MAP3K1* (mitogen-activated kinase kinase kinase 1), *LSP1* (lymphocyte-specific

protein), *CASP8* (caspase 8), and *TGFB1* as being associated with breast cancer risk [19,20,21]. Although with a few exceptions, the mechanisms by which these variants may influence breast tumorigenesis are largely unknown.
In order to identify other genes involved in the initiation and progression of human breast cancer it has been used a MMTV/mouse model of mammary cancer [22]. MMTV (mouse mammary tumor virus) acts as an environmental mutagen producing random interruptions in the somatic DNA of infected cells by insertion of proviral DNA copies. In addition to disrupting the host genome, the proviral DNA also influences gene expression through its associated enhancer sequences over significant inter-genomic distance. MMTV by insertional mutagenesis induce premalignant lesions and malignant tumors exclusive in the mammary gland [23]. The MMTV/mouse model system has provided, relative to other strategies, a productive and experimentally amenable approach to identify genes and signaling pathways which are essential in the normal mammary gland development and their mutations and/or deregulation leading to mammary tumorigenesis [22]. Some genes and pathways commonly affected by MMTV insertion in multiple individual tumors include, the *Wnt* (wingless), *Fgf* (fibroblast growth factor), *Rspo* (R-spondin) gene families [22,23]. It has been showed as all of these genes, affected by MMTV, are associate with breast tumorigenesis [22]. This mouse model provided to identify different genes, some of them as *Notch4* (Neurogenic locus notch homolog protein 4) are well characterized and correlated to breast caner while others as *Eif3e* (eukaryotic initiation factor subunit e) need more study to better understand its function in the normal mammary gland development and its role in the initiation and progression of breast carcinogenesis [22].
Therefore the characterization and study of genes, which mutation or alteration occur in breast cancer, is very important to identify, by screening, individuals with higher risk of developing breast cancer and to find new specific therapeutic strategies in a personalized manner. Identifying women with specific gene alteration could be the first step for cancer-preventative approach and increased surveillance. The most dramatic effect on breast cancer outcome is linked to lack of early diagnosis, prevention and existence of treatment for advanced-stage tumors.

1.2. EIF3E

1.2.1. *EIF3E* (Eukaryotic translation initiation factor 3 subunit e)

The *EIF3E* (eukaryotic translation initiation factor 3 subunit e) gene was originally identified in mouse as a common integration site of the Mouse Mammary Tumour Virus (MMTV), in two virally induced tumours and in a mammary hyperplastic outgrowth line [24]. Integration of MMTV in one of the introns into *Eif3e* locus, in the opposite transcriptional orientation, results in the expression of truncated RNA species [24]. These altered transcripts terminate with a cryptic termination signal in the sequence of the MMTV LTR [24]. Truncated *Eif3e* mRNA produces a biological active form of *Eif3e*, acting as dominant negative mutant inducing malignant phenotype *in vitro* and tumour formation *in vivo* [25, 26].
The function of *EIF3E* was extensively studied accumulating evidences of its involvement in developing and progression of breast cancer in mice and humans [25,26,27]. EIF3E was proposed as an onco-protein [24,25,27] or as a tumour

suppressor [28, 29], but until now the exact role of *EIF3E* in breast cancer development and progression remains unclear.

1.2.2. *EIF3E* gene and protein structures

EIF3E is a gene highly conserved during evolution, with related proteins found in fission yeast through humans. It is expressed ubiquitously in all cell types and encodes one of the thirteen subunits, p48, of the 800 kDa eukaryotic translation initiation factor 3 (eIF3) [30]. The human *EIF3E* gene was also identified as a target of the Human T-cell Leukaemia Virus type 1 (HTLV-1) transforming protein Tax [31].

EIF3E interacts with other multisubunit complexes (COP9 signalosome, 26S proteasome) performing different functions, some are known and others are not clearly understood.

The human *EIF3E* gene, localized to the region q22-q23 of the chromosome 8, is a 45Kbp gene, containing 13 exons. *EIF3E* mRNA is a transcript (NM_001568) of 1516 nt that encodes a protein of 445 amino acid (52 KDa).

The main functional domain, in the EIF3E protein is the PCI (Proteasome/COP9 signalosome/Initiation of translation) domain, located at the C-terminus (290-395 aa) region (Fig.4). This protein domain was found in proteins of the translation initiation factor 3 (eIF3), in several subunits of 19S regulatory cap of 26S proteasome, which degrades polyubiquitylated proteins and in subunits of the COP9 signalosome (CSN). The CSN is an eight subunits protein complex, predominantly nuclear, which regulates protein degradation by the proteasome [32]. The presence of PCI domain allows EIF3E to interact with these three protein complexes and therefore to control synthesis and turnover of specific proteins.

Three binding domains were identified into EIF3E protein by yeast two-hybrid assay [33,34,35]. The first one, localized in the N-terminal region (4-128aa), is the binding site for HIF-2α/EPAS1 (hypoxia inducible factor 2a) (Fig.4) [33], the second one is the MCM7 (minichromosome maintenance complex component 7) binding domain at amino acids 351-455 (Fig.1) [34] and the third one involves the TRIM27/Rfp (tripartite motif containing 27/ Ret finger protein) interaction (amino acids 9-195 Fig.4) [35].

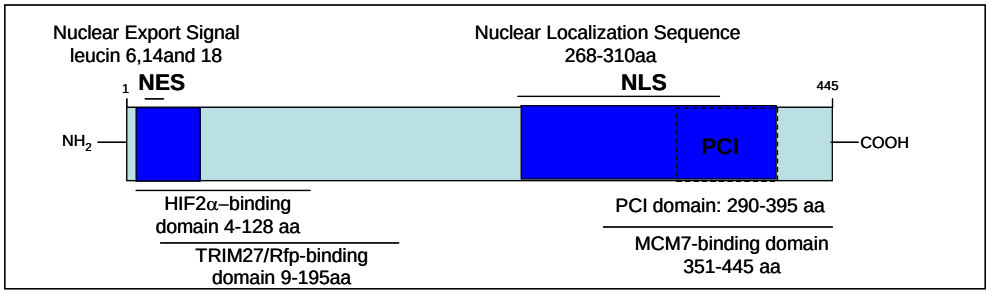

Fig.4 Schematic representation of EIF3E functional domains. *PCI domain (Proteasome/COP9 signalosome/Initiation of translation) at amino acids 290 and 395; MCM7-binding domain in position 351-455 amino acids; HIF 2α-binding domain from amino acid 4 to 128; NES, nuclear export signal leucin 6, 14 and 18; NLS, nuclear localization sequence at amino acids numbers 268 and 310.*

1.2.3. EIF3E subcellular localization

The topological distribution of EIF3E protein in mammalian cells varies among cell types and tissues. EIF3E protein is localized in the nucleus and in the cytoplasm of the cells and potentially shuttling between these two compartments [35]. This double localization is due to a bipartite nuclear localization sequence (NLS) in the C-terminal region (268-310aa) and an N-terminal nuclear export signal (NES) at leucines 6,14 and 18 (Fig.4). EIF3E bipartite localization in the cells gives to the EIF3E protein the ability to interact with different protein complexes, localized in nucleus and/or cytoplasm and performing multiple functions. The translocation of EIF3E in the nucleus of human cells could be due to its interaction with Ret finger protein/TRIM27 (RFP) [35]. This protein is located in the nuclear matrix and has a transcriptional repressor activity. RFP triggers the translocation of EIF3E from cytosol to nucleus and co-localize with it in the nuclear bodies [35]. EIF3E localization in the nuclear bodies was also shown in the primary lymphocytes. In the nucleus of these cells EIF3E proteins is present in the 77% of nuclear bodies containg the PML (promyelocytic leukemia gene product) even though it is also distributed in other nuclear compartments [35,36].
EIF3E subcellular localization seems to be related to cell-cycle [37]. Watkins and colleagues observed that, in the human proliferating fibroblasts, nuclear EIF3E was reduced in 10-20% of cells in the early S phase and this value increases at 40% in synchronized cells [37]. The reason of this EIF3E re-distribution is still unclear. Interestingly, EIF3E localization during S phase was observed only in normal fibroblasts and not in immortalized fibrosarcoma cells. This observation suggests that redistribution of EIF3E localization may be a significant and widespread feature of tumorigenesis in fibroblasts.

1.3. EIF3E functions

1.3.1. *EIF3E* regulates protein expression

1.3.1.1. EIF3E *is one subunit of the eukaryotic translation initiation factor 3 (eIF3)*

EIF3E has been characterized as one of the eIF3 protein complex subunits, called p48, implicated in the translation process. eIF3 is a large, multi proteins complex which is responsible for dissociating the 80S ribosome into subunits and for promoting the binding of methionyl-tRNA and mRNA to the 40S ribosome during the initiation phase of protein synthesis [30] (Fig.5).

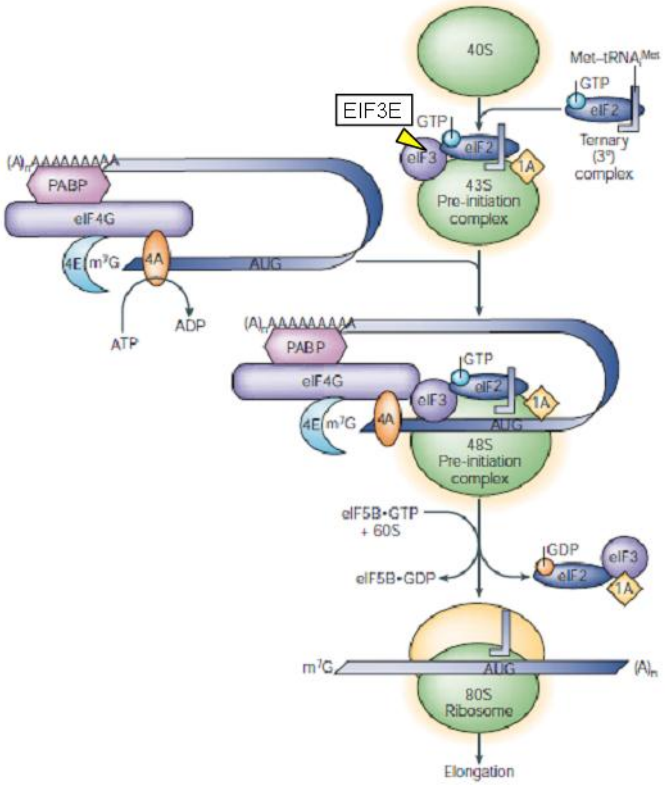

Fig. 5 Pathway of translation initiation in eukaryotes. EIF3E *(yellow triangle) is a subunit of eIF3 complex. eIF3 associated with the ternary complex (eIF2, GTP and methionyl-transfer RNA), the 40S ribosomal subunit and eIF1A generates the 43S pre-initiation complex. Modified from Klann and Dever (Nat Rev Neurosci, 2004).*

Several studies have proved that *EIF3E*, during the translation process, has not a role in bulk translation, but it is important for translation of specific genes involved in breast cancer [38,29,39]. In normal mammalian cells, the content of average EIF3E protein per cell was estimated to be more than 2-fold than other subunits of

eIF3 [40]. This data is consistent with the notion that EIF3E, beyond acting with the eIF3 complex performs other functions in the cell.
The capacity of *EIF3E* to regulate, positively or negatively, the expression of particular genes was supported by Zhou and colleagues observations. They found in fission yeast the existence of two eIF3 complexes: one including *Yin6* (*EIF3E* yeast homologue) and the other one *eIF3m*. Each type of these eIF3 complexes, in yeast, is associated with different mRNA subsets [41].
In mammalian, *EIF3E* is a regulator element rather than a core element in the eIF3 complex [38]. Grzmil and colleagues observed that the *EIF3E* silencing, in MDA-MB-231 cells (tumorigenic breast cancer cell lines) has not significant impact on bulk protein synthesis, measured as ^{35}S-methionine labeling and polysoma profile, but regulates a group of mRNAs involved in cell proliferation, invasion and apoptosis [38]. Some of these mRNAs, which translation is controlled by *EIF3E* are: PLAU/uPA (Urokinase-type plasminogen activator), MAD2L1 (MAD2 mitotic arrest deficient-like 1) and BCL-XL (B-cell lymphoma-extra large). PLAU/uPA is a serine protease that degrades extra cellular matrix, promoting invasion and metastasis of malignant tumors. BCL-XL is an apoptotic regulator. MAD2L1 is a component of the mitotic spindle attachment checkpoint and it is negatively regulated from *EIF3E*. Suo and colleagues characterize another important protein, which translation is regulated by *EIF3E*: the ubiquitin [29]. They showed as the *EIF3E*-reduced expression in the human normal epithelial cells (MCF10A) affects the ubiquitin protein levels in the cells. Ubiquitin has been associated with protein degradation, DNA repair, cell cycle regulation, kinase modification, endocytosis and regulation of other cell signaling pathways. Therefore change in ubiquitin expression, caused by *EIF3E*, could have strong effects on cell physiology.
The *EIF3E* function as a translation regulator element has been supported recently also by Neusiedler and colleagues. They proved that the human *EIF3E* is required for the efficient translation of the histone mRNAs during phase S. They observed that *EIF3E* down regulation by RNA interference approach, in the HeLa cells (human cervical cancer cell line), affects the histone translation [39]. A specific feature of histones mRNA (five classes: H1, H2A, H2B, H3 and H4) is the lacking of poly(A) tail and the presence of a 16-nt stem-loop motif at the 3' end. This sequence binds SLBP (stem-loop binding protein) that controls 3' processing, translation and nuclear export of these cell-cycle-regulated mRNA during phase S. MIF4GD (MIF4G domain containing) interacts with the N-terminus of SLBP and the 3' end of histone mRNA supporting the activation of its translation. In this study has been demonstrated, by two-hybrid assay, the existence of the interaction between the N-terminal region of EIF3E and MIF4GD. EIF3E therefore performs the regulation of histone mRNA translation through its ability to interact with MIF4GD that binds SLBP. Neusieder and colleagues assumed also that EIF3E interacts with SLBP independently from MIF4GD protein [39]. In consequence of this, *EIF3E* through the control of histone mRNAs translation can act as a sensor of DNA replication.
Alteration in this *EIF3E* ability, to regulate translation of specific mRNA, can have strong outcome on cellular state inducing malignant transformation.

1.3.1.2. EIF3E is involved in the quality control of specific mRNAs by NMD

Human *EIF3E* is involved in nonsense-mediate mRNA decay (NMD). NMD is a cellular mechanism of mRNA surveillance. This process works to detect nonsense

mutations (PTC, premature translation termination codon), located more than 50 nucleotides upstream of an exon-exon junction, and to prevent the expression of truncated or erroneous proteins by inducing degradation (Fig. 6). NMD is trigger by exon junction complexes that are deposited during pre-mRNA processing. NMD pathway does not only degrade aberrant mRNAs containing PTCs but it is also implicated in regulating the expression of wild-type transcripts. Human cells defective in NMD has revealed that this surveillance pathway regulates the expression of about 10–20% of the whole transcriptome [42]. It has been showed that NMD can be regulated by wide variety of cellular stresses. Many of the stresses that inhibit NMD, including cellular hypoxia and amino acid deprivation, are events that promote cellular adaptation. Because adaptation to the microenvironment is crucial in tumorigenesis, and because NMD targets many mutated tumor suppressor gene transcripts, the NMD regulation may have particularly important implications in cancer [43].

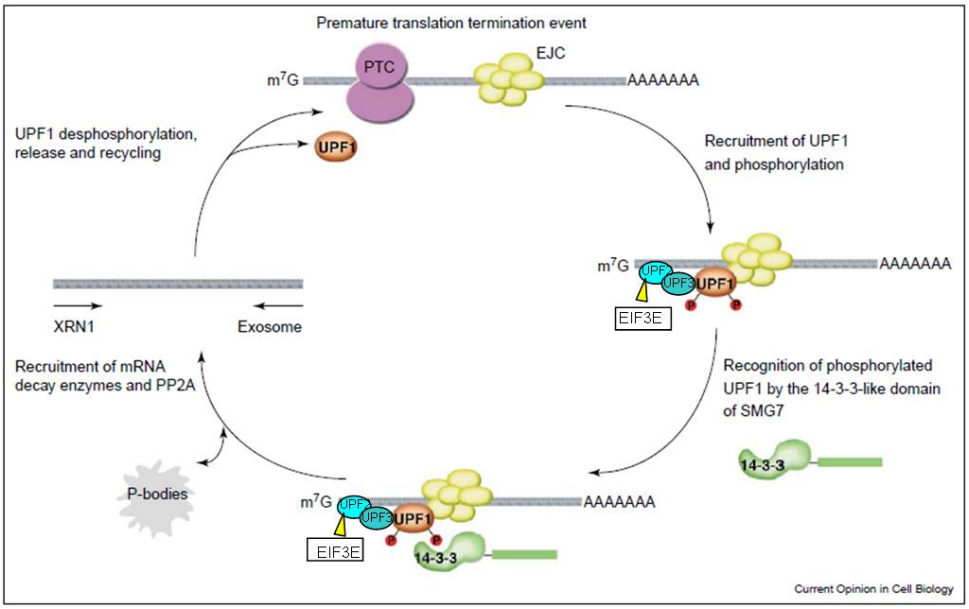

Fig. 6 Schematic representation of NMD in mammals. *Exon junction complexes (EJC) located downstream of a PTC tags for recruitment of UPF1, that phosporilated by UPF2 and UPF3, causes the mRNA's transport out of the nucleus. The RNA degradation has place into cytosol.* EIF3E *(yellow triangle) interacts with UPF2.*
Modified from Conti and Izzaurlande (Curr Opin Cell Biol, 2005).

Morris and colleagues established that *EIF3E* is required in the human cells for the efficiency of NMD. This EIF3E activity is correlated with its ability to interact with specific NMD factors like UPF2 (up frameshift factor 2) [44]. UPF2, together with UPF1 and UPF3, forms a complex that disrupts mRNP (ribonucleoprotein complex) structure to prevent further translation and to trigger mRNA decay.

So *EIF3E* acts in the cap-dependent translation to direct mRNAs containing premature termination codons, towards degradation rather than to active translation. *EIF3E* contribution in NMD process seems to give it an important role in the quality control of mRNAs.

1.3.1.3. EIF3E and proteasome-mediate protein turnover

Another important function of *EIF3E* is its ability to control the stability and degradation of specific cellular proteins such as SRC3, MCM7 and HIF-2α [15,20]. EIF3E interacts with several subunits of the proteasome and COP9 signalosome (CSN) [29,32,45]. The CSN is a conserved protein complex involved in the ubiquitin-proteasome pathway. Suo and colleagues showed that EIF3E forms a stable complex with Rpn5/PSMD12 (proteasome 26S subunit non-ATPase 12), a proteasome regulatory subunit, and it is required for proteasome assembly and functionality [29]. The proteasomal degradation pathway is essential for many cellular processes, including the cell cycle, the regulation of gene expression and response to oxidative stress.

For instance, *EIF3E* is able to control the degradation, via proteasoma, of the oncoprotein SRC3 (steroid co-receptor 3), during mitosis [29]. SRC3 is a nuclear receptor coactivator that interacts with nuclear hormone receptors to enhance their transcriptional activator functions. This protein is frequently over expressed and /or amplified in breast cancer and its level are controlled by protasome activity [46]. In breast cancer high SCR3 levels correlate with larger tumor size, higher tumor grade and poor prognosis [46]. Suo and colleagues showed that *EIF3E* depletion stabilizes SRC3 protein. This function is performed by EIF3E interaction with both SRC3 and its ubiquitin ligase Fbw7 (F-box and WD repeat domain-containing 7). EIF3E seems to mediate the interaction between SRC3/Fbw7 and the proteasome, therefore controlling SCR3 cellular levels. Moreover the SCR3 stabilization induced by *EIF3E* reduction was observed in MCF10A and MCF7 (human mammary epithelial cell lines) but not in HeLa cells (human cervical cell line) where Fbw7 is expressed at low levels [29]. These results suggest that in different cell types *EIF3E* can regulates different set of genes and therefore it can have different functions.

Another important protein, which levels are controlled by *EIF3E*, is MCM7 (minichromosome maintenance complex component 7). EIF3E interacts with polyubiquitylated MCM7, associated to chromatin, through an MCM7 binding domain, located in its C-terminus region between amino acids 351 and 445 (Fig.1) [34]. MCM7 is a subunit of the Mini Chromosome Maintenance complex, which plays a key role in DNA replication licensing to let one replication per cell cycle. MCM7 is necessary for the helicase activity of the complex. The result of EIF3E-MCM7 interaction is increases MCM7 stability protecting it from degradation by proteasome during S phase. EIF3E seems to indirectly contribute to genome stability, assuring the accurate and complete DNA replication, by controlling MCM7 degradation rate. Alterations of *EIF3E* expression could be associated to replication deficiency as confirmed by Buchsbaum and colleagues. As a matter of fact they showed as human *EIF3E*-silecing cells fail to complete the replication and the loss of MCM7 at early step of replication, producing replication fork stress [34].

EIF3E is also involved in HIF-2α/EPAS1 (Hypoxia-Inducibile Factor 2 a) regulation [47]. HIF-2α encodes a transcription factor involved in the activation of genes

regulated by oxygen, which are induced when oxygen level decrease. These genes are involved in glucose transport, glycolysis, erythopoiesis, angiogenesis, vasodilatation and respiratory rate. These genes are involved in the pathogenesis of many cardiovascular diseases and cancer [48,49]. A hallmark of most solid tumors is the presence of hypoxic regions, which are associated with resistance to radiation and chemotherapy. For example in a variety of breast carcinomas and metastasis the oxygen-responsive hypoxia-inducible factor α (HIF-2α) is over expressed and it is able to promote different steps of the metastasis program [50].
EIF3E directly binds, with its domain in N-terminus region (4 to 128 aa), the Int6 binding site (IBS) of HIF2α (571 to 700 aa). This interaction leads to HIF2α proteasome degradation as Li Chen and colleagues demonstrated by *in vitro* studies [47]. The EIF3E over-expression reduces the level of HIF-2α protein via proteasoma, while *EIF3E* silencing makes HIF2α stable and increases the levels of several angiogenesis factors, promoting the formation of functional and stable blood vessels. *EIF3E* through HIF-2α regulation could be a key-element to control angiogenesis and sustain tumor growth and progression.
Lastly, human EIF3E may indirectly influence proteolysis via its interaction with TRIM27/Rfp in the nucleus. This protein is widely present among ubiquitin ligases and localizes to promyelocytic leukemia gene product (PML) nuclear bodies (NBs), which are proteasome-rich [35,51].

1.3.2. *EIF3E* involvement in genome integrity and stability

1.3.2.1. EIF3E role in DNA damage response

Recently it has been shown that *EIF3E* is involved in the pathway of DNA damage response (DDR). The *EIF3E* function in DDR does not involve the translation of mRNA encoding specific DDR components, but it seems to be involved directly in the DDR pathway. Silencing *EIF3E* decreases cell survival after γ-irradiation and impairs the G_2 DNA damage checkpoint [51]. During DNA damages EIF3E is rapidly recruited at the DNA-double strand breaks, interacts directly with ATM (ataxia telangiectasia mutated) protein and controls the level of chromatin-bound ATM (Fig. 6). EIF3E interferes with histones ubiquitilation and promotes the recruitment at DBSs sites of essential repair factors, including BRCA1 (Breast cancer 1, early onset) (Fig. 6).

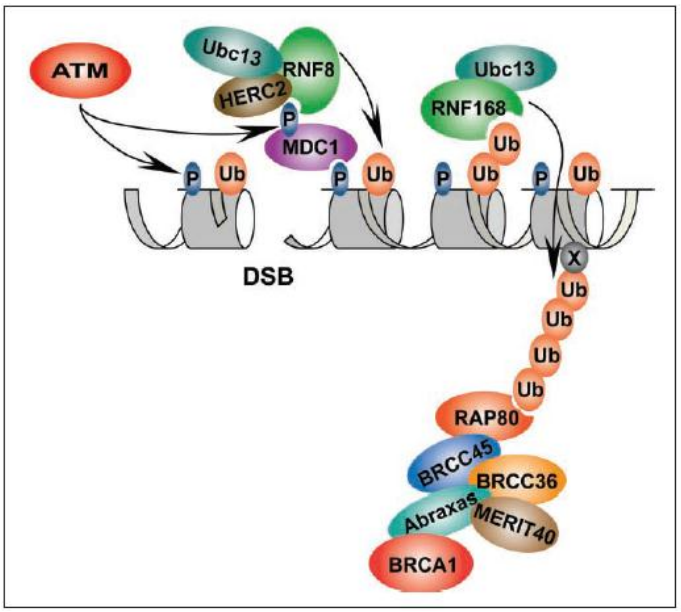

Fig. 6 Schematic representation of the DNA damage response (DDR) pathway. *In response to DNA double-strand breaks (DSBs) ATM relocalizes rapidly to sites of DNA double-strand breaks (DSB) and phosphorylates numerous substrates including histone H2AX. Chromatin marked with phosphorylated H2AX establishes a chromatin domain onto which DDR proteins accumulate, among them MDC1. MDC1 phosphorylated by ATM recruits the RNF8 ubiquitin ligase. And other thereby repairs factors, including BRCA1. Modified from Tang and Greenberg (Genes Cancer, 2010).*

The strictly connection between EIF3E, ATM and BRCA1 suggests a protective function of *EIF3E* in the onset of breast cancer, involving DNA damage response pathway.

1.3.2.2. EIF3E role in mitosis and DNA replication

EIF3E is involved in the control of chromosome segregation and DNA replication. Its alteration results in chromosome instability (CIN) as showed by Morris and colleagues [52]. They demonstrated that EIF3E reduction in HeLa cells (human cervical cancer cell line), as showed in earlier studies in fission yeast [53], causes defects in spindle formation, chromosome degradation and cytokinesis. These abnormalities were correlated to the reduction in the kinase activity of the cyclin B-Cdk1 (cyclin-dependent kinase 1) complex [52].

Mitosis is a complex process relying on an orderly sequence of protein modifications and destructions. Several kinases play crucial roles in cell cycle regulation, in particular Cdk1, which phosphorylate a variety of target substrates leading to cell cycle progression. During G2/M transition the complex Cdk1-cyclin B is activated by Wee1 tyrosine kinase degradation. In the cell in which EIF3E is silenced there is a reduction in the level of inactivated Wee1 protein that prevents the complete activation of Cdk1-cyclin B1 complex causing abnormality in the

mitosis process [52]. These results assert that *EIF3E* is involved in the mitosis process due to its ability to control Cdk1-cyclin B activity.
The function of *EIF3E* in the control of chromosome segregation is also associated which its ability to regulate negatively some transcripts such as MAD2L1 (MAD2 mitotic arrest deficient-like 1) [38]. MAD2L1 is a component of spindle-assembly checkpoint that prevents the onset of anaphase until all chromosomes are properly aligned at the metaphase plate. MAD2L1 dysfunction, through either reduced or increased expression, can cause aberrant chromosome segregation, and hence contribute to malignancy, in mammalian cells [54,55].
An inhibition of *EIF3E* expression was shown to delay mitotic progression in human cells [52]. This function is fulfilled by its ability to control the Cdk1-cyclin B1 complex activation, the expression of MAD2L1 and the translation of histone mRNAs, as explained in the previous section [38,30].

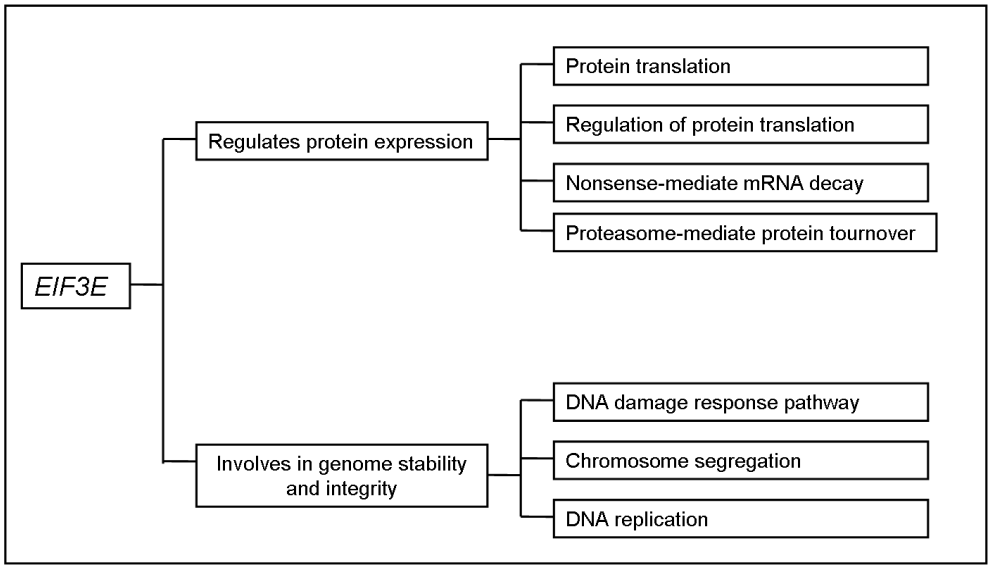

Fig.7 Schematic representation of *EIF3E* functions

1.4. EIF3E in breast tumorigenesis

1.4.1. *EIF3E* and breast cancer

EIF3E is a gene implicated in the development and progression of breast cancirogenesis in humans and mice. Its exact role in breast tumorigenesis, such as its molecular mechanisms remains unclear. In the last years the involvement of *EIF3E* in breast cancer pathogenesis has been investigated mainly in two directions: from one sides the study of altered *EIF3E* transcripts to the other the study of variation in *EIF3E* expression.
Same researchers focused their work on the study of the truncated *EIF3E* transcripts, generated by MMTV integration in mouse EIF3E locus, to identify its functions and roles in carcinogenesis [24,25,27,56]. The *EIF3E* truncated

transcripts seem to have an oncogenic function because of their ability to generate malignant transformation in normal epithelial and mesenchymal cell lines and to initiate and sustain tumor in transgenic mice, as explained in the next sections [1, 2, 24,25,27].
Other works were based on studying the *EIF3E* functions and how changes of its expression are correlated with breast cancer [29,38]. This study brought to two different conflicting conclusions. In one scenario, EIF3E was reported to behave like an oncoprotein [38], while in the other one as a tumor suppressor [29]. Anyway, in both cases variation of *EIF3E* expression are correlated to tumor formation and progression.

1.4.2. Truncated and chimeric *EIF3E* RNA

1.4.2.1. Truncated EIF3E transcripts in breast tumor

The identification in mouse of *Eif3e* as a site of Mouse Mammary tumour virus (MMTV) integration in two MMTV-induced tumors and in a hyperplastic outgrowth line brought many researchers to focus their studies on *Eif3e* as a gene implicated in breast cancer [24]. In all cases the MMTV integration, in the reverse transcription orientation respect to *Eif3e*, generates C-terminally truncated transcripts [24]. The three different MMTV sites of integration were identified, respectively, in intron 5, 9 and 12 of the *Eif3e* locus (Fig. 8). Different studies showed as ectopic expression of the mouse truncated *Eif3e* (*Eif3e-sh*) in normal mammary epithelial cells contributes to generate malignant transformation and causes the tumor formation in transgenic mouse model [25,27].

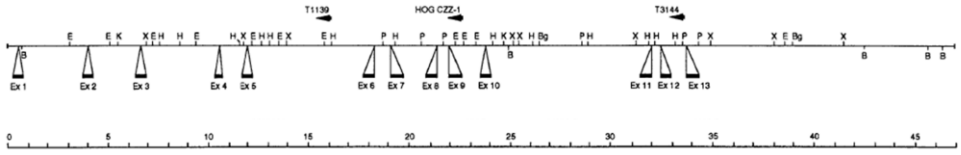

Fig.8 Diagram of the mouse Eif3e locus with MMTV integration sites. *The locations and transcriptional orientations of integrated MMTV proviral genomes are indicated by arrowheads with the tumor or HOG (hyperplastic outgrowth lines) number above the Eif3e locus. The scale of map is given in increments of 1 Kb at the bottom of figure. Modified from Marchetti et al. (J Virol. 1995).*

Rasmussen and colleagues demonstrated by *in vitro* and *in vivo* experiments the implication of truncated *Eif3e* RNA in breast tumorigenesis. They observed that the ectopic expression of the mouse truncated *Eif3e* transcript (*Eif3e-sh*) in normal mammary epithelial cells HC11 (mouse) and MCF10A (human) confers to the cells a malignant phenotype [25]. These cells, expressing *Eif3e-sh*, gain an increased ability to grow in anchorage-independent condition, forming more colonies in soft agar [25]. The malignant transformation acquired by these mammary epithelial cells was also tested *in vivo*. MCF10A cells expressing *Eif3e-sh* engrafted into cleared fat pads of athymic mice led to the development of epithelial nodules in 50% of the cases. HC11-Eif3e-sh cells injected into cleared and filled fat pads of

BALB/c mice produce lobular/alveolar structures, indicating that HC11-transformed cells were able to overcome local growth regulatory control [25]. These *in vitro* and *in vivo* data strongly sustain the hypothesis that *Eif3e-sh* is an oncogenic alteration in mammary epithelium that acts in a dominant negative fashion since it is able to transform the epithelial cells growth proprieties in the presence of the two fully functional copies of wild type gene.
The ability of *Eif3e-sh* to produce a malignant transformation was also proved in mesenchymal cells by Mayeur and colleagues. They showed as the stable *Eif3e-sh* expression in a mouse fibroblast cell line (NIH3T3) increase the ability of these cells to form *foci*, to grow in anchorage-independent condition and to be resistance to serum starvation-induced apoptosis [26]. These features are often correlated with tumorigenesis in mice. Taken together these data suggest that truncated *Eif3e* is able to cause malignant transformation both in mammary epithelial and mesenchymal cells.
The *Eif3e-sh* dominant negative role in breast carcinogenesis was also demonstrated by Mack *et al.* [27]. They showed as the mammary specific expression of the shorter truncated *Eif3e* is sufficient to lead the persistence of alveolar hyperplasia with the accompanying increased predisposition to mammary tumors [27]. They created a transgenic mouse model, in which the expression of *Eif3e*-sh was targeted to the differentiating alveolar epithelium using the whey acidic protein (Wap) promoter. Wap expression occurs during the secretory development of the mammary gland during late pregnancy and during lactation. Using this transgenic model they observed that the ectopic expression of *Eif3e*-sh, in 42% of the mammary epithelium of heterozygous parous females at an average of 18 months, were able to induce mammary tumors compared with 2% in control mice. They inferred that Wap-Eif3e-sh alveolar cells survive involution following the cessation of lactation, and subsequently give rise to the mammary tumors that arise in aging multiparous females [27]. The long tumor latency, in addition with the observation that the tumors arose stochastically one gland per mouse, suggests that other genetic or epigenetic events were required for initiation or progression of tumor growth.
All these studies showed that *Eif3e*-sh has an oncogenic function causing malignant transformation in the normal mammary epithelial and mesenchymal cells and induces tumor formation in a transgenic mouse model [25,26,27]. *Eif3e*-sh seems to act as a dominant negative alteration because its ability to transform phenotype was observed in the presence of both *Eif3e* wild type allele [25,26,27].
An alteration that involves the gene *EIF3E* was identified for the first time in a human breast cell line HCC1954 (epithelial cells obtained from a ductal carcinoma), through high-throughput transcriptome sequencing. In this cell line was identify a translocation event, that involves the chromosome 8 and 5 and (t(5;8)(q23.1;q23.1)) and produces a truncated transcript of *EIF3E* [57].
Until now in the human primary breast cancer samples have not been identified chimeric or truncated *EIF3E* transcript as those characterized in mouse MMTV-induced tumors. These results support the idea that events causing the expression of truncated *EIF3E* could have a low incidence in the breast cancer developing.

1.4.2.2. Potential function of the truncated EIF3E

Though the expression of truncated *Eif3e* is sufficient to cause malignant transformation in mammary and non-mammary cell lines [25,26] and generates

mammary tumors in transgenic mice [27], its mechanism of action is still poorly known.
The only function connected to *Eif3e*-sh was proposed by Chiluiza and colleagues. They demonstrated that the expression of the shorter truncated *Eif3e* (*Eif3e5*), generated by MMTV integration in intron 5 of mouse *Eif3e*, acts in a dominant negative way, causing the shift from cap-dependent to cap-independent translation [56]. Eif3e5 expressed in NIH3T3 cells (mouse fibroblast cell line) from a single-copy gene, is able to inhibit protein synthesis by interfering with the binding between eIF3 and eIF4G1 (Fig.9 A). The eIF3 function is the recruitment of eIF4G, associated with mRNA, to the 43S pre-initiation complex (PCI) to form 48S PIC and initiates mRNA scanning (Fig.9 A). EIF3E5 binds eIF4G1 whit its N-terminal region reducing eIF3-eIF4G1 interaction and causing abnormal gene expression at the translational level. This EIF3E5 interaction causes an inhibition of Cap-dependent translation supporting the cap-independent translation (Fig.9 B). Many genes translated by cap-independent machinery are involved in tumourigenesis such as: *XIAP* (anti-apoptotic protein), c-*MYC* (onco-protein) and *PIM*-1 (proto-oncogene activated in murine T cell lymphomas). The expression of these mRNAs increases when *Eif3e5* is expressed [56]. These data suggest that the putative oncogenic function of *Eif3e5* could be the supporting cap-independent relative to cap-dependent translation.

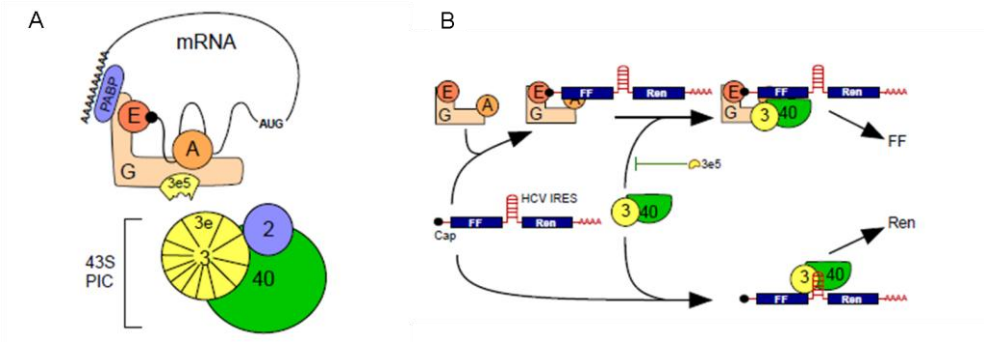

Fig.9* EIF3E5 *antagonizes the interaction* eIF3-eIF4G*, inhibiting cap-dependent translation and favouring cap-independent translation A *Model for competition between* EIF3E*5 (3e5) and* eIF3 *(3) for binding* eIF4G *(G).* EIF3E5 *binds* eIF4G *(G) and prevents the recruitment of mRNAs to 43S preinitiation complex (PIC) inhibiting cap-dependent translation.* ***B*** *Model for the* EIF3E5 *effects of a bicistronic mRNAs separated by the HCV IRES.* EIF3E5 *prevents the binding between* eIF4G *(G) with 43S PIC to form the 48S PIC. The 48S is required directly to IRES, supporting cap-dependent translation. Abbreviations; 2: eIF2; 3e:* EIF3E*; A:* eIF4A*; E:* eIF4E*;* PABP*: the poly(A)-binding protein; 40: 40S ribosomal subunit. Modified from Chiluiza et al. (J Biol Chem, 2011)*

This *Eif3e5* function could be a key element during breast carcinogenesis. Many oncogenes, containing internal ribosome entry site (IRES) sequences, are translated by cap-independent machinery. The expression of these genes is activated in response to stress conditions (hypoxia, serum starvation or gamma

irradiation), in which the cap-dependent machinery is inhibited. So the switch from cap-dependent to cap-independent mRNA translation, induces by *Eif3e5* could support tumour angiogenesis and hypoxia responses in breast cancer [58].

1.4.3. *EIF3E* expression in breast cancers

Despite *EIF3E* is an ubiquitary gene, expressed in all human normal tissues, its expression changes in cancer tissues as demonstrated by different studies [28, 38,59].
Marchetti and colleagues observed, examining *EIF3E* in several human breast carcinomas, a loss of heterozygosis in 21-28% of breast tumors [28]. The analysis of *EIF3E* mRNA levels in cancer tissues revealed that in one third of all human breast carcinomas (37%) there is a reduction in *EIF3E* expression [59]. *EIF3E* alterations were also observed at proteomic level by Grazmil and colleagues through immunohistochimical analysis performed on 81 primary breast cancers tissues. They showed that in the breast cancer there is a positive correlation between levels of cytoplasmic EIF3E protein and poorly differentiated higher-grade tumors, while they did not observe changes in the nuclear EIF3E levels [38].
All these variations in *EIF3E* expression support the idea that this gene has a pivotal role in the human breast cancer. The loss of *EIF3E* activity such as changes in its expression could be important events in cancer initiation and progression. The changes observed in cytosolic EIF3E levels could be related with the ability to promote motility and decrease adhesion: cellular proprieties relevant to cancer invasion and metastasis. *EIF3E* could become for these reasons an important breast cancer prognostic and therapeutic marker, even if its function and contribution to cancer have not been completely elucidated yet.

1.4.4. *EIF3E* is involved in the EMT process

Recently Gills and colleagues characterized a new *EIF3E* function that strictly links *EIF3E* to cancer progression. They showed that *EIF3E* is involved in the Epithelial to Mesenchymal Transition (EMT) [60]. EMT has an important role in cancer metastasis because it is the process underling changes that allow cancer cells to adopt migratory properties leading to the dissemination of tumor cells throughout bloodstream to reach distant location. Cancer cells that have undergone to EMT have increased resistance to apoptosis, oncogene-induced senescence and exhibit increased resistance to chemotherapy [61]. Gills and colleagues demonstrated that the reduction of EIF3E expression causes, in epithelial cells MCF10A morphological changes, loss of epithelial cobblestone morphology and exhibition of spindle-like morphology, similar to fibroblast cells as occur during EMT [60]. These morphological changes, induced by EIF3E reduction, were associated whit loss of the epithelial markers such as E-cadherin, ZO-1 (zonula occludens 1) and an increase of mesenchymal markers like N-cadherin and vimentin. The EMT process observed in MCF10A cells, owing to stable reduction of EIF3E, was also supported by a reduction in proliferation rate, enhanced migration, invasive phenotype and the loss of the ability to form acinar structures in 3D culture [60]. EIF3E down regulation induce the expression at RNA level of two key EMT regulators Snai1 (Snail homolog 1) and Zeb2 (zinc finger E-box binding homeobox 2). These two transcription factors induce the expression of genes required for mesenchymal

properties and repress the expression of genes that are required for the epithelial phenotype [62]. It is not clear how a decrease in EIF3E expression may affect the transcription of Snail1 mRNA, but since EIF3E protein has been implicated in protein regulation as reported in the previous sections, it is possible that reduced *EIF3E* expression may affect the expression of proteins that participate directly in Snail1 transcription or are part of signaling pathways, upstream Snail1 expression. Similarly, *EIF3E* may control the stability of Zeb2 mRNA by directly participating in the processes that mediate Zeb2 mRNA degradation (such as nonsense-mediated decay) or controlling the expression of key proteins in these mRNA degradation pathways.
The finding that reduced *EIF3E* expression induces EMT in breast epithelial cells suggests that a reduction in *EIF3E* abundance or activity alters, at multiple levels, the expression of genes that drives cancer metastasis.
It is important to note that Snai1 and Zeb2 translation can realize throughout the cap-independent machinery, because they have internal ribosomal entry site (IRES). This suggests that the *EIF3E* reduction in breast epithelial cells leads to preferential translation of proteins that utilize IRES-dependent mechanism, such as Snai1 and Zeb2. This *EIF3E* effect on cap-independent translation has been also related to function of C-truncated *EIF3E* transcript (generated by MMTV integration in mouse *EIF3E* locus). As explained better in the next section, the truncated *EIF3E*, which lacks normal *EIF3E* activity, in NIH3T3 cells causes a decrease in global, cap-dependent translation initiation and a concomitant increase in the translation of mRNAs that contain IRES [58]. Together these data suggest that the reduced *EIF3E* activity can promote IRES-dependent translation initiation. This role of *EIF3E* could be very important in breast cancerogenesis due to the fact that many IRES-containing mRNA are key elements in proangiogenic, hypoxia, and survival processes, which favor angiogenesis tumor [58].

1.4.5 *EIF3E* role in breast cancer

1.4.5.1. EIF3E behaves as an oncoprotein

Many studies support the idea that *EIF3E* acts in breast cancer as an oncoprotein [25,27,64]. As analyzed in the previous section alteration in this gene, producing truncated *EIF3E* transcripts, can induce a dominant negative malignant transformation and support tumor formation [25,27].
Also in its un-mutated form *EIF3E* has a role as an ocoprotein, thought all events that could compromise its function in the control of transcription, translation of specific mRNA and in the proteasome pathway. The ability of wild type EIF3E to act as an onco-protein is realized, for example, by its ability to control histone translation, *MCM7* stability and *MDA2L1* expression [38,34,64]. Alterations that involve this protein can cause a direct change in the levels of important cellular proteins, thus making *EIF3E* a key element in the regulation of cell proliferation, genome stability and replication.

1.4.5.2. EIF3E as tumor suppressor

Different studies support the hypothesis that *EIF3E* is a tumour suppressor. In 37% of breast tumor *EIF3E* expression is reduced, so experiments based on silencing *EIF3E* in human cell lines showed as the lower levels of *EIF3E* prevent the degradation of onco-proteins and lead to neoplastic transformation [29].

EIF3E seems to be critical for maintaining proper levels of regulatory proteins. This *EIF3E* function could be accomplished by its dual ability to regulate proteins levels by controlling the translation and proteasome-dependent degradation.
Suo and colleagues studied the role of *EIF3E* as a tumor suppressor. They showed as the reduction of *EIF3E* expression is sufficient to induce malignant transformation in MCF10A cells. These cells are able to form acini in 3D culture. When the *EIF3E* expression was reduced by RNA interference they form abnormal acini [29]. These acini contained proliferating cells in the interior that would otherwise undergo apoptosis in the presence of *EIF3E*. This result suggests that *EIF3E* is required for proper development of human mammary structures. These abnormalities were partially explained by EIF3E ability to bind and regulate proteasome activity in human cells altering some onco-proteins levels. They showed that *EIF3E* down regulation, in human mammary epithelial cells, lead to stabilization of oncoproteins, such as SRC3 (Steroid receptor coactivator-3). SRC3 levels are frequently up regulated and positively correlate with large tumor size, higher tumor grade and poor disease-free survival [46]. The role of *SRC3* as oncogene has been firmly established in mouse models.
Recently Gillis and colleagues established the *EIF3E* tumor suppressor role through its involvement in the EMT process. Reduce *EIF3E* expression affects, *in vitro*, the levels of proteins involved in invasion and migration such as Snal1 and Zeb2 [60]. This work supports that *EIF3E* has an important role in the regulation of EMT in breast epithelial cells and it acts as a tumor suppressor whose loss mediates cancer growth and progression [60].

2. MATERIALS AND METHODS

2.1. 3' Rapid amplification of cDNA end

RNA was extracted from human mammary tumor samples and cell lines using TriZol™ reagent following manufacturer's instructions (Invitrogen). Human mammary tumor samples were provided by Azienda Ospedaliera Treviglio-Caravaggio. All patients signed informed consent forms. The 3' rapid amplification of cDNA ends (3' RACE) was performed with the SMART™ RACE cDNA Amplification Kit (Clonotech Laboratories). For the selective amplification of *EIF3E* transcripts an *EIF3E* Specific Primer (SP EIF3E, 5'-CTCGCATCGCGCACTTTTTGGATCGGC-3'), designed within the 5' region of *EIF3E* mRNA was combined with Universal Primer A Mix (UPM, SMART™ RACE cDNA Amplification Kit). The amplification products obtained were run on 1% agarose gels, extracted from the gels by the QIAquick Gel Extraction kit (Qiagen), cloned into the pCRII construct (TOPO TA Cloning, Invitrogen) and sequenced.

2.2. Bioinformatics analysis

In order to study the cDNA sequences we used the BLAT algorithm (BLAST-like alignment tool) in the UCSC Genome Browser website (http://genome.ucsc.edu/). To study protein sequence we used the ExPASy, Bioinformatics Resource Portal website.

2.3. Cell cultures

2.3.1. Cell lines

2.3.1.1. Culture conditions

MCF7 cells (a human epithelial tumorigenic breast cancer cell line) were cultured in RPMI-1640 medium (Invitrogen, Gibco Laboratories) supplemented with 10 % fetal bovine serum (Invitrogen, Gibco Laboratories), 10 μg/ml insulin (Sigma) and penicillin-streptomycin (Invitrogen, Gibco Laboratories). Cells plated at the density of 5000 cells/ cm^2 were grown in 5% CO_2 at 37 °C.

MCF10A cells (a human no-tumorigenic mammary epithelial cell line) were cultured in DMEM-GlutaMax/F-12 (Invitrogen, Gibco Laboratories) supplemented with 5% equine serum (heat inactivated, Invitrogen, Gibco Laboratories), 50 μM dibutyryl cyclic AMP, 10 μg/ml insulin, 20 ng/ml mouse recombinant EGF, 500 ng/ml hydrocortisone (Sigma), and penicillin-streptomycin (Invitrogen, Gibco Laboratories). Cells plated at the density of 25000 cells/ cm^2 were grown in 5% CO_2 at 37 °C.

Both cell lines were divided three times a week in order to maintain a pre-confluent culture.

HEK293 (human embryonic kidney cell line) and MDA-MB231 (human breast cancer cell line) cells were cultured in DMEM-GlutaMax supplemented with 10 % fetal bovine serum (FBS) and penicillin-streptomycin (Invitrogen, Gibco Laboratories). Cells plated at the density of 5000 cells/ cm^2 were grown in 5% CO_2 at 37 °C.

2.3.1.2. Three-dimensional in vitro culture in rat tail collagen

MCF7 cells were detached from monolayer cultures by trypsinization, mixed with 500 μl of collagen, obtained from rat tail (prepared according to Soriano et al. [62]) and plated (5000 cells/ml in 48-well plates). After the collagen had solidified 300 μl of culture medium was added and changed every two days. The culture medium used was: RPMI-1640 (Invitrogen, Gibco Laboratories) supplemented with 10% fetal bovine serum, 10 μg/ml insulin, 20 ng/ml basic fibroblast growth factor (bFGF), 20 ng/ml mouse recombinant Epithelial Growth Factor (EGF) (Sigma) and penicillin-streptomycin (Invitrogen, Gibco Laboratories).
The cells were observed after 6, 11 and 20 days.

2.3.1.3. Mammospheres assay

The generation of the mammospheres was obtained culturing MCF7 cells in low attachment plates (Bibby Sterilin) at the concentration of 1000 cell/ml with RPMI-1640 media supplemented with B27, penicillin-streptomycin (Invitrogen, Gibco Laboratories), 25 ng/ml bFGF (Sigma), 20 ng/ml mouse recombinant EGF (Sigma), 4μg/ml heparin (Sigma) and 5% KnockOut™ Serum Replacement (Gibco Laboratories).
The re-generation ability of the mammospheres was tested dissociating both mechanically and enzymatically with 0.05% trypsin, 0.53 mM EDTA (Invitrogen, Gibco Laboratories). Single-cell obtained were plated and observed during time.

2.3.2. Human primary breast normal sample processing and culture

2.3.2.1. Tissue dissociation

Mammary tissue from reduction mammoplastic, was collected and mechanically and enzymatically dissociated. The mammary tissue was cutted in small pieces with surgical scissors and incubated for 4 hours at 37°C, 5% CO_2 in DMEM-KO, 10% KnockOut™ Serum Replacement (Gibco Laboratories) supplemented with 300 U/ml Collagenase and 100 U/ml Hialuronidase. After the enzymatic treatment the tissues were collected in a falcon tube, centrifuged at 0.2xg for 10 min, washed once with DMEM-KO and filtered with 40 μm cell strainers (Fisher Scientific). Single cells were collected in the flow throw.

2.1.2.3. Mammosphere culture

The human primary cells, obtained from tissue dissociation, were seeded in low attachment plates (Bibby Sterilin), using a culture medium composed by DMEM-Glutamax/F-12, 10% KnockOut™ Serum Replacement (Invitrogen, Gibco Laboratories), 10 ng/ul EGF, 1 μg/ml Insulin, 0.5 μg/ml Hydrochortisone, 4 μg/ml bFGF (Sigma). After one week the mammospheres were collected by filtration (40 μm strainer, Millipore) and dissociated both mechanically and enzymatically with 0.05% trypsin, 0.53 mM EDTA (Invitrogen, Gibco Laboratories). Single cells were plated in adherent condition

2.3.2.2. Culture conditions

The human primary cells were cultured in adherent conditions using the same medium of mammopshere culture condition. The cells were plated at a density of

4000 cells/cm^2 and divided every 3-4 days by trypsinizzation. The culture dishes were coated with 0.1% gelatin (Sigma) to enhance cell adhesion.

2.4. Lentiviral system

2.4.1. Lentivector production

The *EIF3E*-5/5 sequence was amplified from MCF10A cDNA. The primers used in the PCR were: forward 5'-GACTCCCTTTTCTTTGGCAAGAT-3' and reverse 5'-AGGTCTTTTTCCAACTTCTAGGAT-3'. The product of amplification was cloned into the pCRII construct (TOPO TA Cloning, Invitrogen) generating the vector pCRII-EIF3E-5/5.
The pCDH-CMV-MCS-EF1-copGFP (System Biosciences, SBI) and pCRII-EIF3E-5/5 vectors were digested with XbaI and BamHI (NEB). The pCDH linearized vector and the *EIF3E*-5/5 sequence were purified using QIAquick Gel Extraction Kit (Quiagen). The *EIF3E*-5/5 was therefore cloned into the pCDH-CMV-MCS-EF1-copGFP linearized vector using T4 DNA ligase (Invitrogen). The sequence of pCDH-CMV-EIF3E-5/5-EF1-copGFP obtained was confirmed by sequencing.

2.4.2. Lentiviral production

Lentiviral particles were produced in the HEK-293T cells by transfecting the pCDH-CMV-MCS-EF1-copGFP or pCDH-CMV-EIF3E-5/5-EF1-copGFP vector with the helper vectors, psPAX2 and pMD2.G (System Biosciences, SBI) in a ratio of 3:2:1 using the Lipofectamine™ 2000 Transfection Reagent (Life Technologies). The supernatant of the culture were harvested after 48 to 72 hours. Lentiviral particles were concentrated with the PEG-it^{TM} Virus Precipitation Solution (System Biosciences, SBI).

2.4.3. Lentiviral transduction

MCF7 and human normal primary cells were plated in adherent condition. After 24 hours the cells were infected adding the virus preparation directly to culture medium, supplemented of 8 μg/ml with hexadimethrine Bromide (Sigma). To prevent toxicity, the culture medium was replaced after 24 hours. The cells were transduced with lentivirus expressing *EIF3E*-5/5 and GFP and as control with lentivirus expressing only GFP. After transduction the cells were used for different assays.

2.5. Flow cytometry analysis

MCF7 and human primary breast (29-09) cells after lentiviral infection were cultured in adhesion, detached with trypsin-EDTA 0.25% (Invitrogen), suspended in DPBS (Dulbecco's Phosphate Buffered Saline, Sigma) and maintained at 4°C. The cells were analyzed using FACSCanto II^{TM} cytometer (BD). Twenty thousand events were collected in the cell line and ten thousand events in the human primary sample. Green fluorescence was collected through a 425-to 475-nm wavelength bandpass filter.

Data were presented by using the software FACSDiva Version 6.1.2. The cells not infected and GFP-negative were used to set the gates and establish the GFP-positivity range.

2.6. siRNA technology

For the inhibition of *EIF3E* (NM_001568) were used two siRNAs oligonucleotide double strand (Sigma): SASI_Hs01_00166672 (5'- GUUUAUAUGUUAACUUUGA-3') and SASI_Hs01_00166675 (5'- CAGUGUAUCAGCAUUAACA-3'). These siRNA oligonucleotides were used together at the concentration of 12,5 nM each.
For the inhibition of *EIF3E*-5/5 were synthesized two siRNA doublexes (Sigma): *EIF3E*-5/5 A (5'-GUGAUCCUCCCUCCUCAGC-3') and *EIF3E*-5/5 B (5'-UGCUUUGGGAUUUACAGCC-3'). They were used together at the concentration of 25 nM each.
The negative control siRNA duplexes, MISSION® siRNA Universal Negative Control #1 and MISSION® siRNA Universal Negative Control #2 (Sigma), were used together at the concentration of 25 nM each.
MCF10A cells were plated at the concentration of 25000 cells/cm^2 in 12-well plates. After 24h the siRNA transfection was performed using the Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen). The cells were collected for RNA extraction and RT-qPCR analysis after 48h of treatment.

2.7. Quantitative RT-PCR analyses

For RT-qPCR experiments total RNAs were extracted from MCF7, human normal primary cells and breast tissues using TriZol™ reagent following manufacturer's instructions (Invitrogen). Human pancreas, skeletal muscle and mammary gland polyA+ RNA were provided by Clontech. Human mammary tissues were obtained from Azienda Ospedaliera Treviglio-Caravaggio. All patients signed informed consent forms.
cDNAs were synthesized from 1 μg of total RNA using the High capacity RNA-to-cDNA Master Mix (Applied Biosystems). The sequence of the sense and antisense primers used for quantitative PCR were:

EIF3E sense (NM_001568) : 5'- CAGATGTTGGCCATGAATATTGA-3'
EIF3E antisense (NM_001568) : 5'-GCCCAGTTAGGAGCCTCTGA-3'
EIF3E-5/5 sense: 5'-GCATGGTTTTAGGCAGGAATATTT-3'
EIF3E-5/5 antisence: 5'-CTTCAGGCCAGGAGTTTGAGA-3'
Hprt1 sense (NM_000194): 5'-TTTGCTGACCTGCTGGATTACA-3'
Hprt1 antisense (NM_000194: 5'-GGTCATTACAATAGCTCTTCAGTCTGAT-3'
Cyr61 sense (NM_001554): 5'-CCAGCTCCACCGCTCTGA-3'
Cyr61 antisense (NM_001554): 5'-AAACTTTCCCCGTTTTGGTAGAT-3'
Bcl2 sense (NM_000633): 5'-ATGTGTGTGGAGAGCGTCAACC -3'
Bcl2 antisense (NM_000633): 5'-TGAGCAGAGTCTTCAGAGACAGCC -3'
Pim1 sense (NM_001243186): 5'-TCTCTTCAGAATGTCAGCATCTCATTA-3'
Pim1 antisense (NM_001243186): 5'-GAAGGTTGGCCTATCTGATGGT-3'
Sna1 sense (NM_005985): 5'-CACTATGCCGCGCGCTCTTTC-3'
Sna1 antisense (NM_005985): 5'-GTCCTGCAGCTCGCTGTAGTT-3'

XIAP sense (NM_001167): 5'-TGGATTTTATGCTTTAGGTGAAGGT-3'
XIAP antisense (NM_001167): 5'-TCACTGGGCTTCCAATCAGTT-3'
Rpl32 sense (NM_000994): 5'-TTCATCCGGCACCAGTCA-3'
Rpl32 antisense (NM_000994): 5'-GTCAATGCCTCTGGGTTTCC-3'
Rpl34 sense (NM_000995): 5'-TTTGACATACCGACGTAGGCTTT-3'
Rpl34 antisense (NM_000995): 5'-GGGTTCGGGACAGCCTAGTT-3'
Histone H3 sense (NM_001005464): 5'-TCCGGCGCTACCAGAAGTC-3'
Histone H3 antisense (NM_001005464): 5'-TCTTAAAGTCCTGCGCGATCTC-3'

To quantify c-myc we used a TaqMan assays (Applied Biosystem): Hs00153408_m1.

3. RESULTS

3.1. Characterization of new EIF3E isoforms

3.1.1. Identification of novel *EIF3E* transcripts in human cells

In order to understand the role of *EIF3E* in human mammary breast cancer, we investigated whether aberrant or truncated *EIF3E* transcripts are present in human mammary cancer cells by using the 3' Rapid Amplification of cDNA Ends (3' RACE) method.

To amplify all *EIF3E* polyA transcripts we used an *EIF3E* Specific Primer annealing to the 5' region (exon 1) of *EIF3E* mRNA (SP-EIF3E).

RNA obtained from cells of 9 human breast cancer tissues and 2 human mammary cell lines (MDA231 and MCF10A) were used. In all reactions performed we detected three different amplification products (Fig. 10A) that were cloned and sequenced. By sequence analysis we identified three products: *EIF3E* mRNA (1516 nt, NM_001568) and two novel *EIF3E* transcripts, called respectively *EIF3E*-144 and *EIF3E*-5/5 (Fig. 10A).

The *EIF3E*-144 RNA corresponds to a transcript of 1364 nt, lacking the 144 nt of the 3' UTR (untranslated region) of the *EIF3E* mRNA (Fig. 10B).

The *EIF3E*-5/5 is a 771 nt transcript, corresponding to the shorter amplification product in figure 1A. The *EIF3E*-5/5 transcript is constituted of 492 nt corresponding to the sequences from exon 1 to exon 5 of *EIF3E* and by 279 nt from the fifth intron (ch8: 109245623-109245894) of the *EIF3E* gene (Fig.10B). We assigned the name "exon 5A" to the sequence located into intron 5 and presents in the transcript *EIF3E*-5/5 (Table 1). The sequence shows as "intron 5A" is the region of intron 5, located between the 3' end of exon 5 and the 5' of exon 5A (Tab.1).

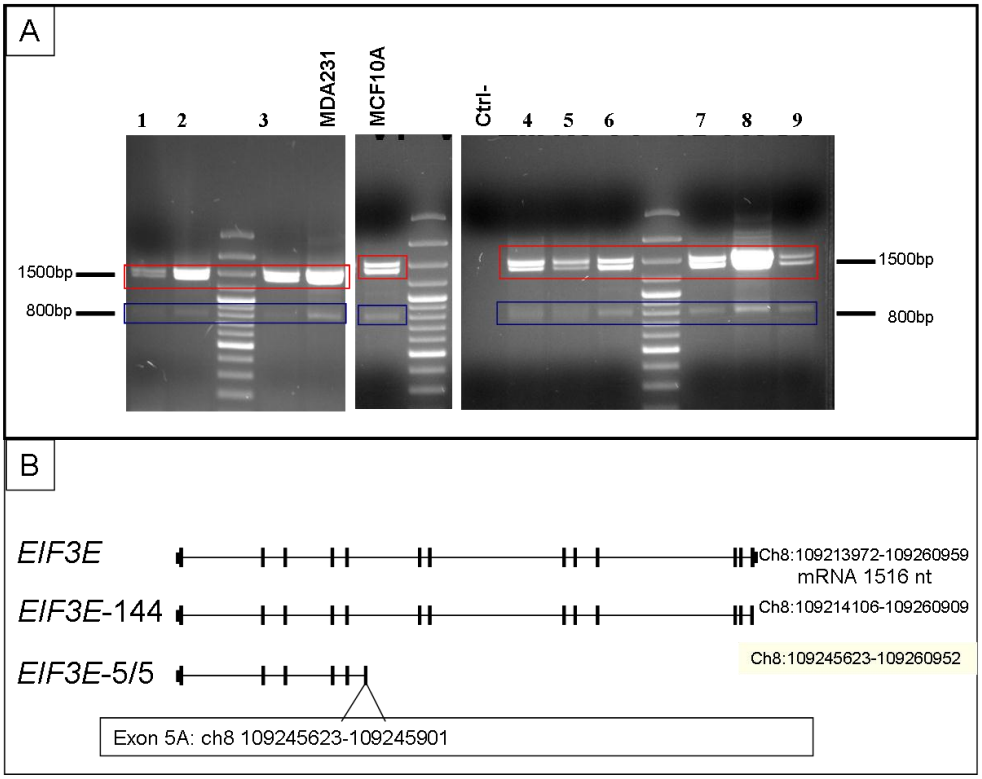

Fig. 10 Electrophoresis analysis and schematic representation of two novel EIF3E transcripts (EIF3E-144 and EIF3E-5/5) identified in cells from human breast cancer tissues and human breast cell lines.

A*. 3' RACE-PCR amplification products detected by electrophoresis in agarose gel (1%). Lanes: from 1 to 9, different human breast cancer samples; MDA231 and MCF10A (human mammary cell lines) and Ctrl (PCR control with no template). In the red box: the longer product of 1516 bp corresponds to EIF3E mRNA (NM_001568) and the shorter product, 1382 bp, is the transcript EIF3E-144. In the blue Box: the product of 771 bp corresponds to EIF3E-5/5 transcript. The DNA ladder is GeneRuler™ 100 bp Plus (Fermentas).*

B*. Schematic representation of transcripts: EIF3E, EIF3E-144 and EIF3E-5/5. Exons are represented by black boxes and introns by black lines. Their localization in the human genome is reported on the right.*

EIF3E mRNA NM_001568					
EXON			INTRON		
Name	Genome interval (5'-3')	Lenght (nt)	Name	Genome interval (5'-3')	Lenght (nt)
1	109260959-109260842	118	1	109260841-109254143	6699
2	109254142-109254028	115	2	109254027-109252305	1723
3	109252304-109252187	118	3	109252186-109248433	3754
4	109248432-109248390	43	4	109248389-109247332	1058
5	109247331-109247227	105	5	**109247226-109241425**	**5802**
6	109241424-109241299	126	6	109241298-109240621	678
7	109240620-109240496	125	7	109240495-109229690	10806
8	109229689-109229563	127	8	109229562-109228743	820
9	109228742-109228641	102	9	109228640-109226946	1695
10	109226945-109226836	110	10	109226835-109215733	11103
11	109215732-109215630	103	11	109215629-109215347	283
12	109215346-109215212	135	12	109215211-109214153	1059
13	109214152-109213972	181			
***EIF3E*-5/5 RNA**					
EXON			INTRON		
name	Genome interval (5'-3')	Lenght (nt)	Name	Genome interval (5'-3')	Lenght (nt)
1	109260952-109260842	86	1	109260841-109254143	6699
2	109254142-109254028	115	2	109254027-109252305	1723
3	109252304-109252187	118	3	109252186-109248433	3754
4	109248432-109248390	43	4	109248389-109247332	1058
5	109247331-109247227	105	**5A**	**109247226-109245902**	**1325**
5A	**109245901-109245623**	**279**			

Table 1 Genomic coordinates of EIF3E mRNA (NM_001568) and EIF3E-5/5 RNA in the human chromosome 8. *In the table are reported the exons, introns of EIF3E and EIF3E-5/5, with their localization in the human genome (chromosome 8) their name and length (nt).*

In one human breast cancer tissue (ITB-CNR 8910), analyzed by 3' RACE-PCR, we obtained a new amplification product of 1169 nt, designed *EIF3E*-8910. Sequence analysis of this transcript reveals that it has the same sequence of the *EIF3E* mRNA, but it lacks exons 8, 9 and 10 (Fig. 11). This altered *EIF3E* transcript was not found in the other eight human breast cancer tissues or in the human mammary cell lines analyzed by 3' RACE. This data supports the idea that *EIF3E*-8910 is an altered transcript exclusive of the breast cancer sample ITB-8910 probably originated from a genomic deletion event.

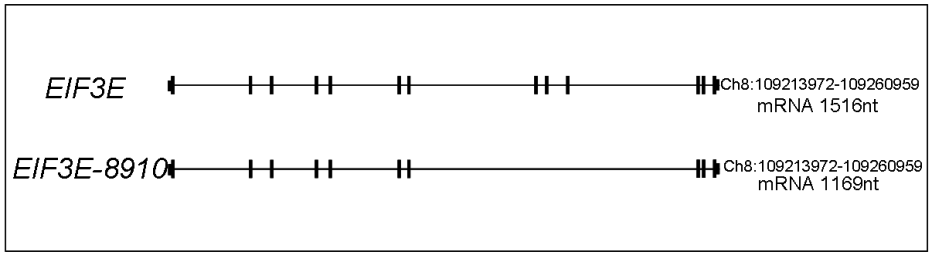

Fig 11. Schematic representation of the transcripts EIF3E and EIF3E-8910.
The EIF3E and EIF3E-8910 transcripts are represented as black boxes (exons) and black lines (introns). Their localization in the human genome is reported on the right.

3.1.2. *EIF3E*-5/5

We focused our work on studying *EIF3E*-5/5. This transcript is very similar to the oncogenic truncated EIF3E transcript that was generated in mouse by integration of the MMTV into the EIF3E gene [24].
EIF3E-5/5 was identified in all the humans samples analyzed (9 breast cancer tissues and 2 human mammary cell lines). Analysis of *EIF3E*-5/5 was performed using BLAT (BLAST-like alignment tool) and the UCSC Genome Browser website (http://genome.ucsc.edu). The BLAT algorithm allows the alignment of the query sequence with sequence obtained from the human genome or with mRNAs. The result of the *EIF3E*-5/5 BLAT search (Fig. 12) shows the alignment of this sequence with spliced ESTs (expressed sequence tags), supporting that the mature transcript contains a region of *EIF3E* intron 5.
The *EIF3E*-5/5 sequence analysis points out the presence of a predicted polyadenylation (polyA) site located in the ending region of exon 5A (ch8: chr8:109245623-109245623) (Fig. 12). The *EIF3E*-5/5 RNA has a polyA tail in the 3' since this transcript was obtained by 3' RACE that amplifies only RNA having a polyadenylated sequence. The presence of a polyA predicted signal and the polyA tail, support the hypothesis that the *EIF3E*-5/5 transcript is a mature coding mRNA because polyadenylation is an essential step for post-transcriptional mRNA maturation, translation by ribosome and protection from cellular nuclease activity.

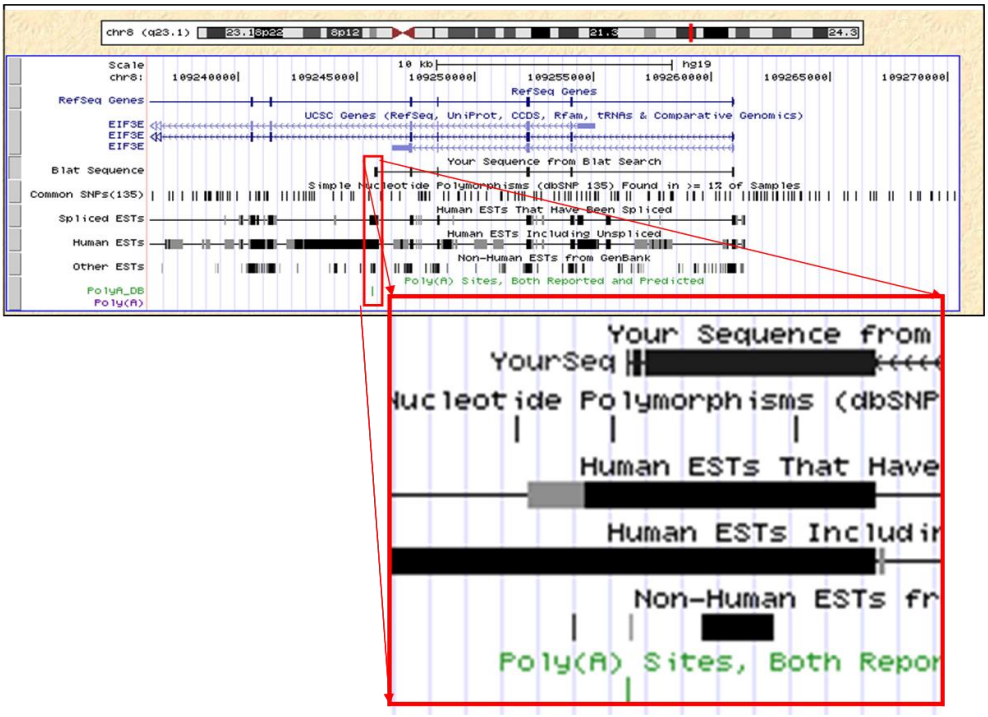

Fig. 12 UCSC Genome Browser representations of EIF3E-5/5 alignment with human chromosome 8 using BLAT. *Alignment of the EIF3E-5/5 sequence with the human genome at chromosome 8, mRNA (EIF3E) and ESTs. Red box displays the alignment of exon 5A to EIF3E intron 5, human ESTs (both spliced and not spliced) and the presence of a predicted polyA signal located in exon 5A in the 3' end.*

A putative transcript, annotated in the gene set of ENCODE/GENCODE version7, which aligns with the *EIF3E*-5/5 sequence supports the existence of this novel 5/5 isoform (Fig. 13). The transcript ID of this predicted transcript is ENST00000521440 and its cellular existence is supported by different sequences reported in figure 13A.
In the figure 13B are reported all ESTs supporting the *EIF3E*-5/5 transcription in human cells.

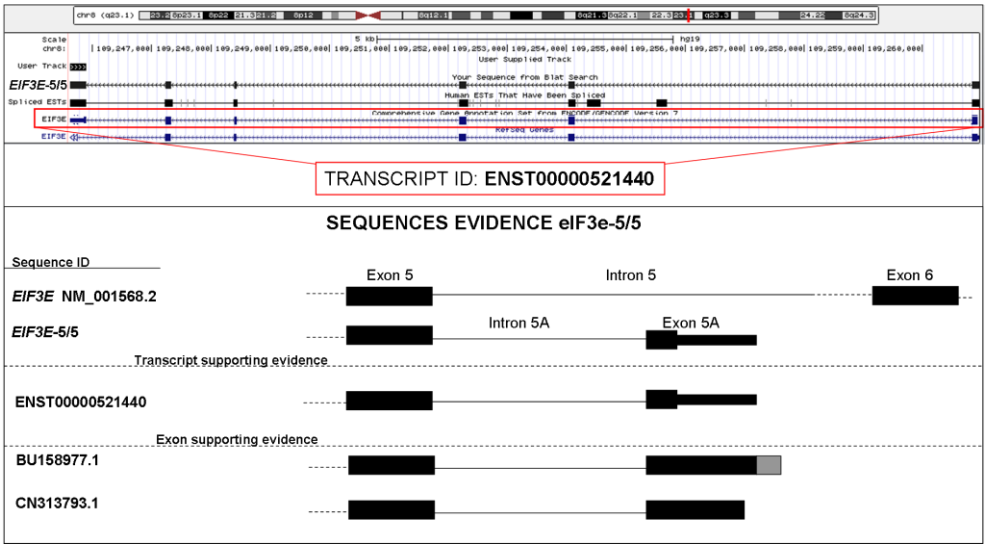

Fig. 13 Alignment of the sequence data set ENCODE/GENCODE version 7 to EIF3E mRNA.

A. *In the red box is pointed out the sequence identified as transcript ID: ENST00000521440 that aligns with EIF3E-5/5.*

B. *Schematic representation of ESTs and transcripts that support existence of human EIF3E-5/5 RNA.*

The analysis of the *EIF3E*-5/5 sequence allows to map into intron 5A (intron sequence located between exon 5 and exon 5A) the specific elements recognized by the spliceosomal complex machinery of cells. Within the intron 5A we can identify a donor site (GU) at the 5' end of intron 5, a branch site near the 3' end of intron 5A, containing a region high in polypyrimidines and an acceptor site (AG) at the 3' end of the same intron (Fig. 14).

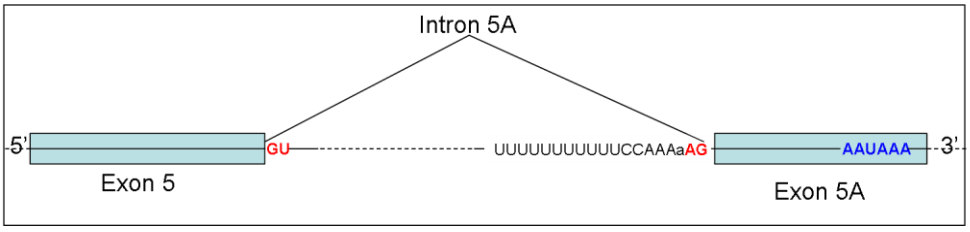

Fig. 14 Schematic representation of the EIF3E-5/5 transcript from exon 5 to exon 5A. *In the intron 5A, located between exon 5 and exon 5A in the EIF3E transcript, there is a donor site (5' end), an acceptor site (3' end) show in red and a region high in polypyrimidines upstream of the acceptor site. A polyA signal was identified in exon 5A.*

This data supports that *EIF3E*-5/5 is a novel previously undetected *EIF3E* splicing form, who's cellular function was never described. When the *EIF3E* translation ends in corresponding to the polyA signal in exon 5A the spliceosome machinery recognizes the acceptor site of intron 5A allows the complete *EIF3E*-5/5 RNA maturation. When transcription goes beyond exon 5A, allowing the transcription of complete *EIF3E* gene, the spliceosome recognizes the branching and acceptor site of the intron 5 allowing the complete maturation of *EIF3E* mRNA (NM_001568).

3.1.3. Characterization of the putative protein generated by *EIF3E*-5/5

The analysis of the *EIF3E*-5/5 RNA sequence reveals the presence of a stop codon in exon 5A in frame with the *EIF3E* mRNA start codon (ATG) (data obtained from ExPASy, Bioinformatics Resource Portal website). The polyA tail and the presence of an open reading frame in the *EIF3E*-5/5 RNA supports the hypothesis that this transcript is a protein coding mRNA.
The putative EIF3E-5/5 protein, constituted by 172 amino acids has a molecular weight of 20 KDa as calculated using the bioinformatics tool, ExPASy, Compute pI/Mw (Fig. 15).

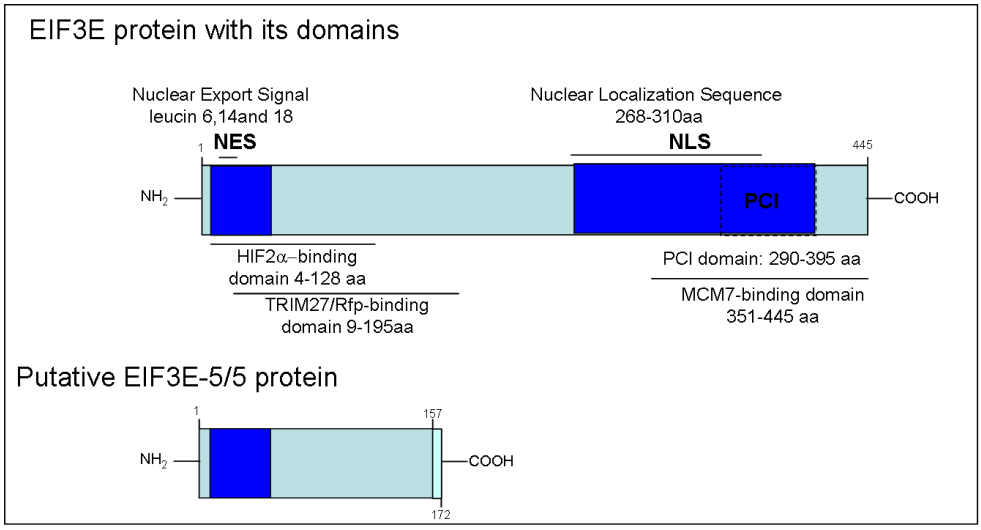

***Fig. 15 Schematic representation of proteins* EIF3E *and* EIF3E-5/5.** *Schematic illustration of the proteins:* EIF3E *(containing the PCI, MCM-7 binding domain, HIF 2α-binding domain and the NE and NL sequences) and the putative* EIF3E-5/5 *protein. The two proteins are identical from amino acids 1 to 157. The 16 amino acids from 157 to 172 of the protein* EIF3E-5/5 *(represented by light blue box) are the contribution of exon 5A to the translated protein.*

As represented in figure 15 the predicted EIF3E-5/5 protein should lack certain functional domains of EIF3E such as the Proteasome/COP9 signalosome/Initiation

of translation (PCI), MCM7-binding domain and Nuclear Localization Sequence (NLS) (Fig. 15). The absence of a NLS should prevent the EIF3E-5/5 protein transport into the nucleus, so its sub-cellular localization should be only in the cytoplasm.

3.2. Characterization of EIF3E-5/5 expression in normal human tissues

We analyzed by RT-qPCR, the expression of the *EIF3E-5/5* transcript in the normal human mammary gland, pancreas and skeletal muscle. The results prove that *EIF3E*-5/5, like for *EIF3E*, is present in all normal tissues examined (Fig. 16). This data supports the idea that *EIF3E*-5/5 is an *EIF3E* isoform, ubiquitously expressed. The two transcripts, *EIF3E* and *EIF3E*-5/5, have different basal levels of expression, in each sample analyzed. In the normal mammary gland the *EIF3E*-5/5 transcript is 100-fold less expressed then the *EIF3E* mRNA.

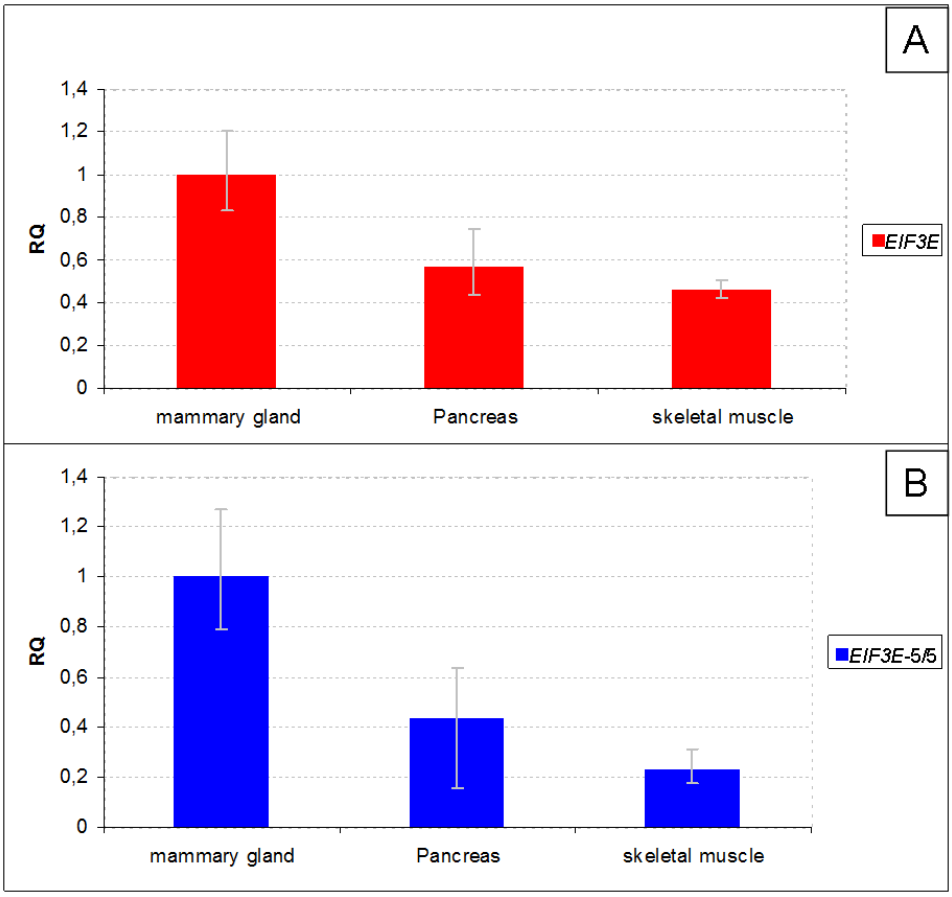

Fig. 16 EIF3E-5/5 and EIF3E expression determined by RT-qPCR in three human normal tissues. *EIF3E (A) and EIF3E-5/5 (B) expression in the normal human mammary gland tissue, pancreas and skeletal muscle. Error bars indicate the 95% confidence interval.*

Analyzing *EIF3E* and *EIF3E*-5/5 expression in the three normal human tissues (Fig. 16) we observed that both transcripts are about 2-fold more expressed in the normal mammary gland compared to the pancreas and skeletal muscle. This data supports the idea that *EIF3E* and its isoforms could have a mammary gland specific type regulation, expression and function.

3.3. EIF3E-5/5 expression in human breast tissues

To assess if the *EIF3E*-5/5 isoform is involved in breast carcinogenesis we estimated its expression level in human normal and cancer breast tissues by RT-qPCR. We tested the expression of EIF3E and EIF3E-5/5 transcripts in human breast cancer samples and we analyzed their ratio of expression in normal and cancer breast samples, obtained from the same patient. In all samples analyzed the RNA, used for RT-qPCR, was extracted directly from human breast surgically tissues.

Our results confirm that in all human samples analyzed, there is a reduced *EIF3E* expression in breast cancer tissue compared to the healthy counterpart (Fig.17A). The same result was obtained for *EIF3E*-5/5 expression (Fig. 17B). In all sample pairs of normal/tumor tissues obtained from patients we observed an *EIF3E*-5/5 reduction in the tumor samples compared to their normal counterparts.

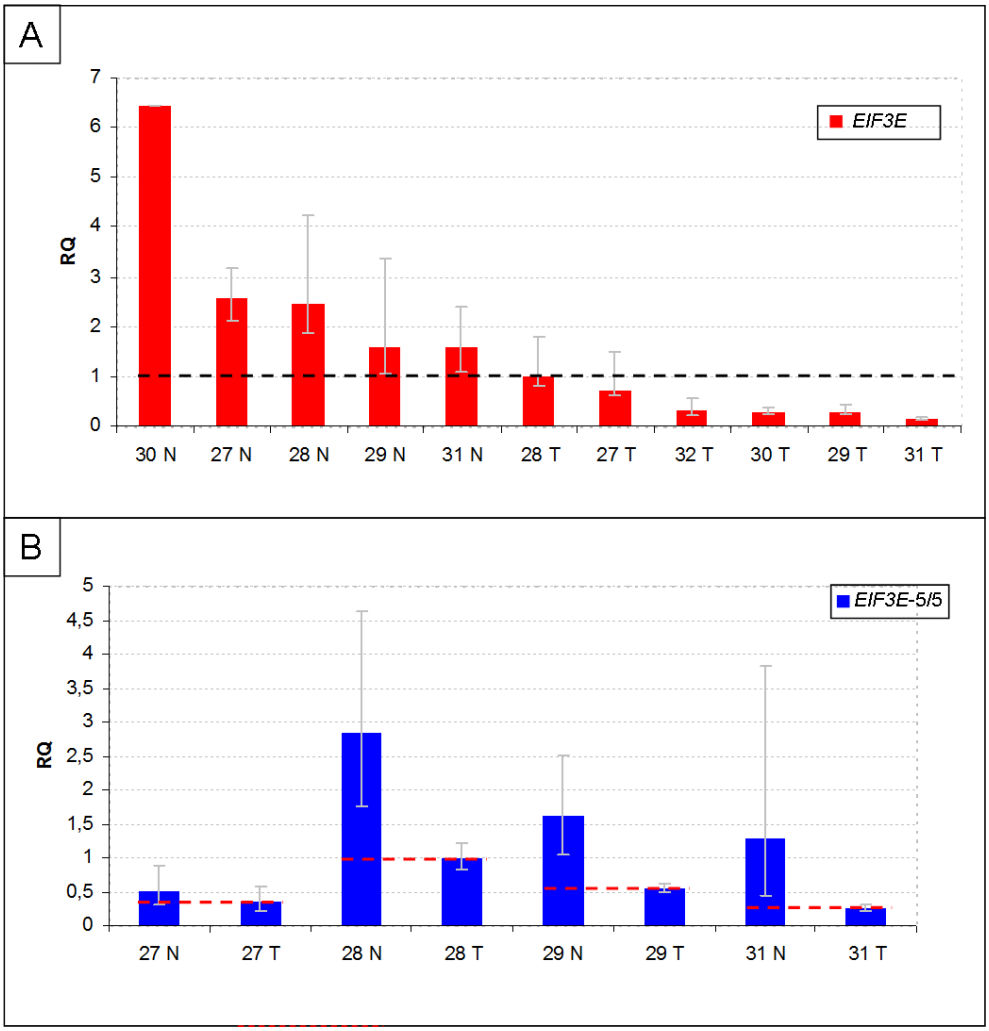

Fig. 17 EIF3E and EIF3E-5/5 expression analysis in human cells from normal and cancer breast tissues.

A. *EIF3E expression in human breast tissues: five normal (N) and six cancer (T) breast tissues. The EIF3E expression is reduced in all breast cancer samples compared to their normal counterpart.*

B. *EIF3E-5/5 expression in human normal and cancer breast samples. In all pairs normal/tumor analyzed, obtained from the same patient, EIF3E-5/5 expression is lower in the tumoral tissue compared to its healthy counterpart.*

Human normal or tumor breast samples are designed with the letter N or T following a number. Hprt1 was used as an endogenous control. Error bars indicate a confidence interval of 95%.

The RT-qPCR analysis revealed that *EIF3E* and *EIF3E*-5/5 are two isoforms differentially expressed in the breast tissue. In order to measure the relative ratio between these two isoforms in the normal and tumor conditions we determined the absolute number of *EIF3E* and *EIF3E*-5/5 molecules per cell. The ratio between the *EIF3E* and *EIF3E*-5/5 molecules was calculated in four pairs of normal and tumor samples obtained from the same patients and represented in figure 18A.
This ratio of *EIF3E* to *EIF3E*-5/5, in three normal tissues out of four is about 100. Moreover this value was confirmed in the breast tissue obtained from a subject that never has been affected by breast cancer (Fig. 18A). In the normal sample 27 this ratio is about 500 (Fig. 18A). The ratios of *EIF3E* to *EIF3E*-5/5 calculated in the breast cancer tissues are smaller than ratios obtained in their corresponding normal tissues (Fig. 18A). We tried to understand what is the cause of this change in the ratio *EIF3E*/*EIF3E*-5/5 analyzing the number of molecules of EIF3E and *EIF3E*-5/5 in the breast normal and cancer tissues. We observed that both *EIF3E* isoforms have a smaller expression in the tumoral condition compare to the normal one. Since their ratios, as shown in figure 18A, do not stay steady in the normal and cancer breast tissue we can assert that the two isofrms decrease in different way in the tumor condition. Exactly, the reduction of EIF3E is more significance than the *EIF3E*-5/5 one. Only in one sample (28) *EIF3E* and *EIF3E*-5/5 in the tumor decrease with the same trend, so their ratio remains unchanged in the cancer tissue (Fig. 18A).
The individual trend reduction in expression of *EIF3E* and *EIF3E*-5/5 in the breast cancer tissues is shown in the figure 18B. In three samples out of four the *EIF3E-5/5* reduction in expression from normal to tumor sample is half if compared to the decrease of *EIF3E*. For example in the sample 31, *EIF3E* expression is reduces 11-fold in the cancer tissue with respect to the normal one while *EIF3E*-5/5 decreases only 5-fold. This may explain why the ratio between the two isoforms changes even though both are down-regulated in the breast cancer tissues.

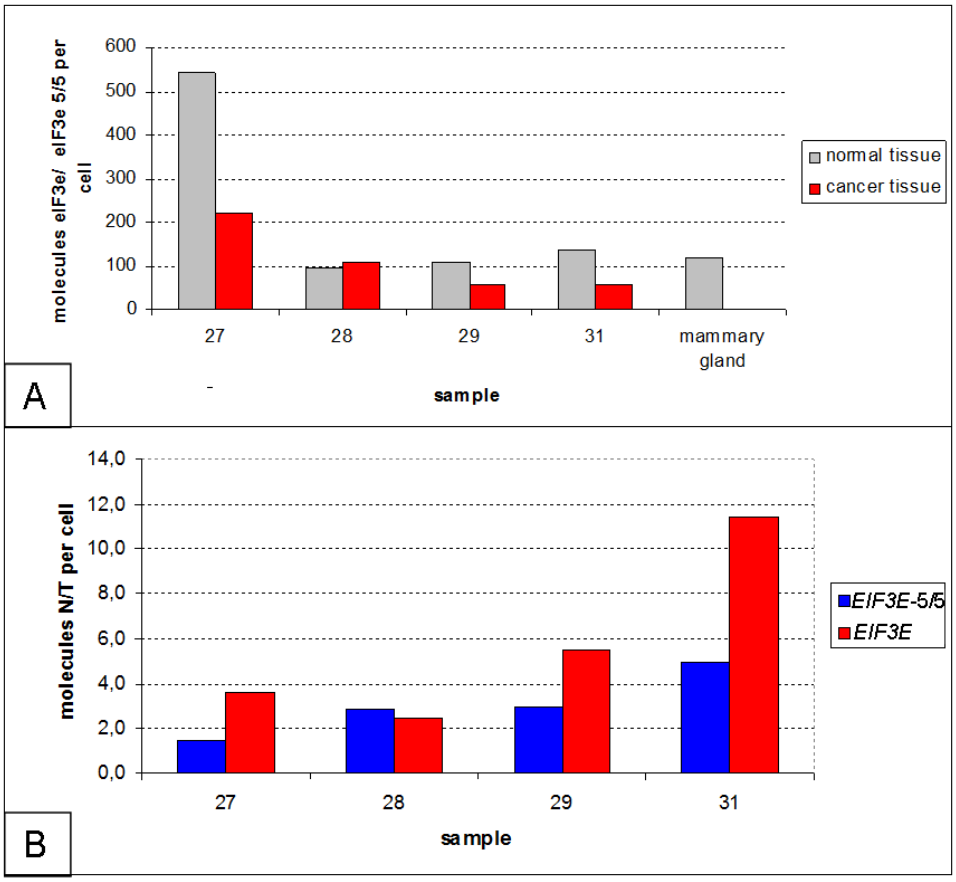

Fig. 18 Ratio of the number of EIF3E and EIF3E-5/5 molecules obtained from normal and cancer tissues.

A. *Ratio between the number of EIF3E and EIF3E-5/5 molecules in human breast samples from different patients. The ratio obtained from the breast normal or tumor tissue for each patient is shown with grey bars or red bars respectively.*

B. *Ratio between the number of EIF3E-5/5 molecules in the normal tissue and EIF3E-5/5 molecules in the cancer tissue (blue bars) of the same patient. Red bars correspond to the number of EIF3E molecules in normal and breast tissue, obtained from the same patient.*

Since *EIF3E* and *EIF3E*-5/5 do not have the same observed reduction trend in the breast cancer tissue we investigated the effects that variation in the ratio between the two isoforms have in the initiation and progression of breast cancer.

3.4. EIF3E-5/5 over expression in human mammary cells

3.4.1. *EIF3E-5/5* over expression in human mammary cell line MCF7

To investigate the function of *EIF3E*-5/5 in cells, we stably over-expressed *EIF3E*-5/5 in a tumorigenic mammary epithelial cell line MCF7, using a lentiviral system approach. We cloned into a lentiviral vector (pCDH-CMV-EIF3E5/5-EF1-copGFP) the *EIF3E*-5/5 coding sequence under the control of the CMV (Cytomegalovirus) promoter. This vector includes a reporter gene copepod green fluorescent protein (cop-GFP) under the control of the elongation factor 1α (EF1) promoter. This lentiviral system allows us to over-express stably *EIF3E*-5/5 in targeted cells and to follow them by fluorescence microscopy or fluorescence-activated cytofluorimetry using the expression of the GFP reporter gene. Lentiviral particles were obtained by packaging both pCDH-CMV-EIF3E5/5-EF1-copGFP (which generates the transgene *EIF3E-5/5*) and pCDH-CMV-MCS-EF1-copGFP (which produce empty lentivirus particles, expressing only cop-GFP) with helper vectors (psPAX2 and pMD2.G). MCF7 cells were infected with lentiviral particles, which integrated into the genomic DNA of target cells providing stable and long-term expression of the transgene. The efficiency of infection was assessed by the expression of GFP through flow cytometry analysis. MCF7 cells not infected were used to set the gates and establish the GFP-positivity range and twenty thousand events were collected for each sample. After two weeks from transduction the MCF7 cells displaying GFP were: 56,9±6,4 %in the MCF7-EIF3E-5/5 cells (cells infected with lentivirus expressing *EIF3E*-5/5) (Fig. 19B) while the MCF7-ctrl cells, infected with lentivirus not expressing the transgene, were 56,4±7,2 % of the total population (Fig. 19C). The efficiency of transduction was calculated in three independent transduction experiments performed in MCF7 cells. We reported the mean value and its standard deviation.

By RT-qPCR we tested the expression level of EIF3E-5/5 and EIF3E in the MCF7-EIF3E-5/5 and in MCF7-ctrl cells. The expression of EIF3E was almost unchanged in the two cell populations while EIF3E-5/5 was expressed 2-fold more in MCF7-EIF3E-5/5 cells compared to MCF7-ctrl cells (Fig. 19D).

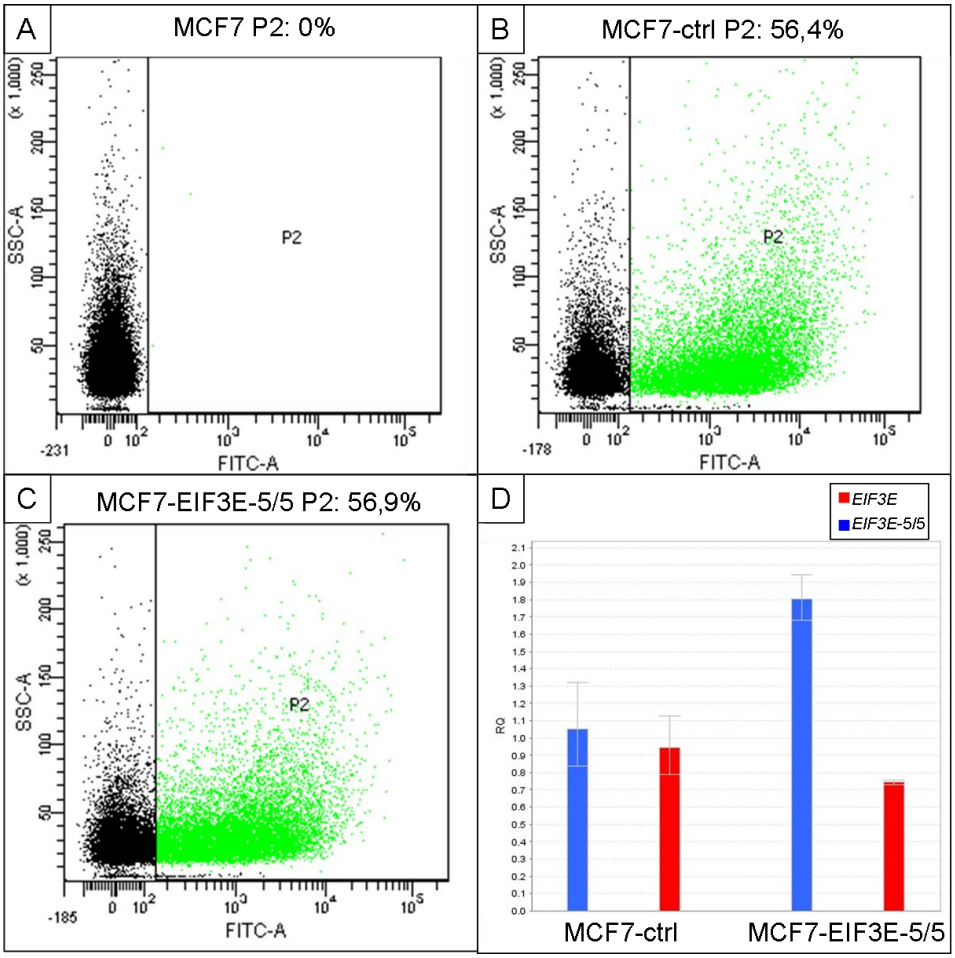

Fig. 19 Efficiency of infection in transduced cells. Cells expressing EIF3E-5/5 (MCF7-EIF3E-5/5) and cells expressing GFP only (MCF7-ctrl) were evaluated for their GFP expression. Validation of EIF3E and EIF3E-5/5 RNA expression in MCF7-EIF3E-5/5 and MCF7-ctrl cells by RT-qPCR.

A. *Flow cytometry analysis of MCF7 cells that were not transduced with population skipped from P2 containing almost 100% of the cells.*

B, C. *Flow cytometry analysis of GFP-positive cells in the MCF7-ctrl and MCF7-EIF3E-5/5. The efficient of transduction is a mean value obtained from three independent experiments.*

D. *EIF3E-5/5 and EIF3E expression in the samples: MCF7-ctrl and MCF7-EIF3E-5/5. The level of both EIF3E isoforms was estimated by RT-qPCR; blue bars corresponding to expression of EIF3E-5/5, red bars to EIF3E. Hprt1 was used as endogenous control. Error bars indicate the 95% confidence interval.*

Phenotypic changes induced by *EIF3E*-5/5 over-expression were examined by culturing MCF7 cells, in three-dimensional (3D) matrix or in non adherent culture conditions to invoke complex 3D structure or sphere formation.
MCF7 cells plated in 3D culture condition (collagen) grow forming in few days large, round and solid clusters of cells. The presence of growth factors in the collagen and in the culture medium induce cell polarity during the 3D growth producing structures containing a lumen that are reminiscent of cysts.
MCF7-EIF3E-5/5 and MCF7-ctrl cells were seeded into collagen, derived from rat-tail, and grown for 20 days. After 11 days we observed that EIF3E-5/5 over-expressing cells were able to grow faster than control cells and to form a larger number of cysts (Fig. 20). After 20 days of culture this effect was strongly evident (Fig. 20).

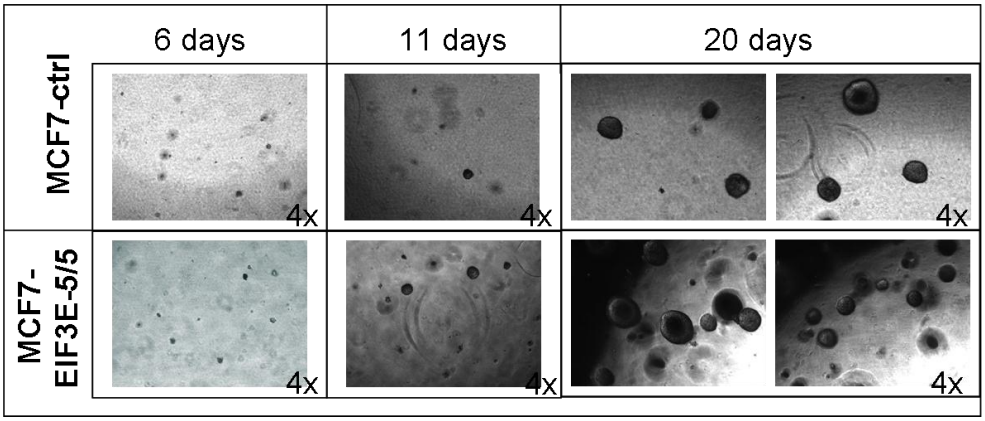

Fig. 20 MCF7-EIF3E-5/5 and MCF7-ctrl cells grown in 3D collagen culture condition. *MCF7-EIF3E-5/5 and MCF7-ctrl cells were seeded into lab-made rat-tail collagen and monitored for 6, 11 and 20 days. Elevated transcript levels of EIF3E-5/5 induce an increase in cyst structure formation, both in number and in size.*

Transduced MCF7 cells were cultured in non-adherent conditions in order to investigate their ability to generate mammospheres. The capacity of cells to form sphere is an indication that cells have stem like features, such as self-renewal potential. In the MCF7 breast cancer cell line these features were associated with cancer stem/progenitor cells previously identified as side populations in this cell line [61].
We observed that *EIF3E*-5/5 over-expression is able to affect the ability of MCF7 cells to generate mammospheres. In particular, the number of spheres obtained from MCF7-EIF3E-5/5 cells was double compared to MCF7-ctrl cells (Fig. 21). We tested the ability of MCF7-EIF3E-5/5 cells to form the second generation spheres in order to investigate if *EIF3E-5/5* is able to increase the number of MCF7 cells with self-renewal potential and/or cells retain stem cell features. Single cells derived from dissociation of first generation spheres were cultured in suspension conditions and allowed to re-form spheres. In second sphere assays it was

observed that MCF7 cells over-expressing *EIF3E*-5/5 were able to form double amount of spheres compared to control cells (Fig.20).
Both in the first and in the second generation sphere formation assays, the spheres generated from MCF7 cells over-expressing *EIF3E*-5/5 were more and larger in volume than in control cells (Fig. 21). We interpret the increase in the number of cells with sphere formation potential in the cultures, as a measure of increase in the rate of self-renewal of the cells with stem cell features.

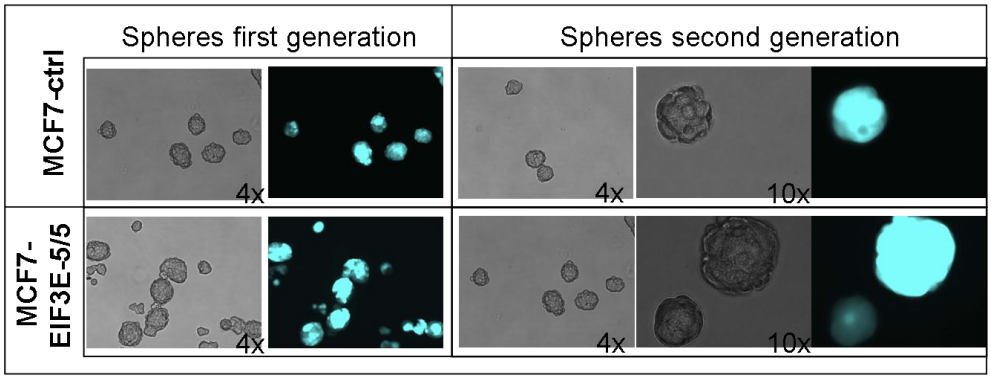

Fig. 21 MCF7-EIF3E-5/5 and MCF7-ctrl cells grown in non-adherent conditions generate mammopheres. *MCF7-EIF3E-5/5 cells (co-expressing EIF3E-5/5 and GFP) and MCF7-ctrl cells (expressing only GFP) cultured in suspension conditions. The second generation spheres were generated from cells obtained from dissociated first generation spheres.*

These *in vitro* results obtained by MCF7 cells suggest that *EIF3E*-5/5 over-expression may promote malignant features in mammary gland cells by its capacity to control self-renewal of the side population cells retaining stem cell properties.

3.4.2. *EIF3E-5/5* over expression in normal human primary mammary cells

We investigated the effects of *EIF3E*-5/5 over expression in normal primary mammary cells (sample 29-09). Single cells obtained from the dissociation of human normal mammary gland tissue were cultured in suspension to generate mammospheres, in order to enrich for cells having stem and progenitor proprieties. The mammospheres, obtained in 7 days of culture in suspension, were dissociated and cultured in adherent conditions. The 29-09 cells obtained in this way were infected with the lentivirus encoding for cop-GFP and *EIF3E*-5/5 (29-09-EIF3E-5/5) or only cop-GFP (29-09-GFP), as control. The efficiency of viral infection was measured by flow cytometry analysis estimating the GFP expression 15 days after infection. Transduction with GFP alone resulted in 86,2% of cell population being GFP-positive while the transduction with *EIF3E*-5/5 resulted in 62% of the cells displaying GFP signal (Fig. 22A, B). For flow cytometry analysis the boundaries of the gate regions for the GFP positive and negative cells were selected using 29-09 cells not infected (Fig. 22C). Ten thousand events were collected.

Up regulation of *EIF3E*-5/5 in 29-09 cells was validated after 15 days from the time of lentiviral infection by qPCR. The *EIF3E*-5/5 transcript was found 25-fold more expressed in these cells compared to the expression level in the 29-09-GFP cells (Fig. 22D).

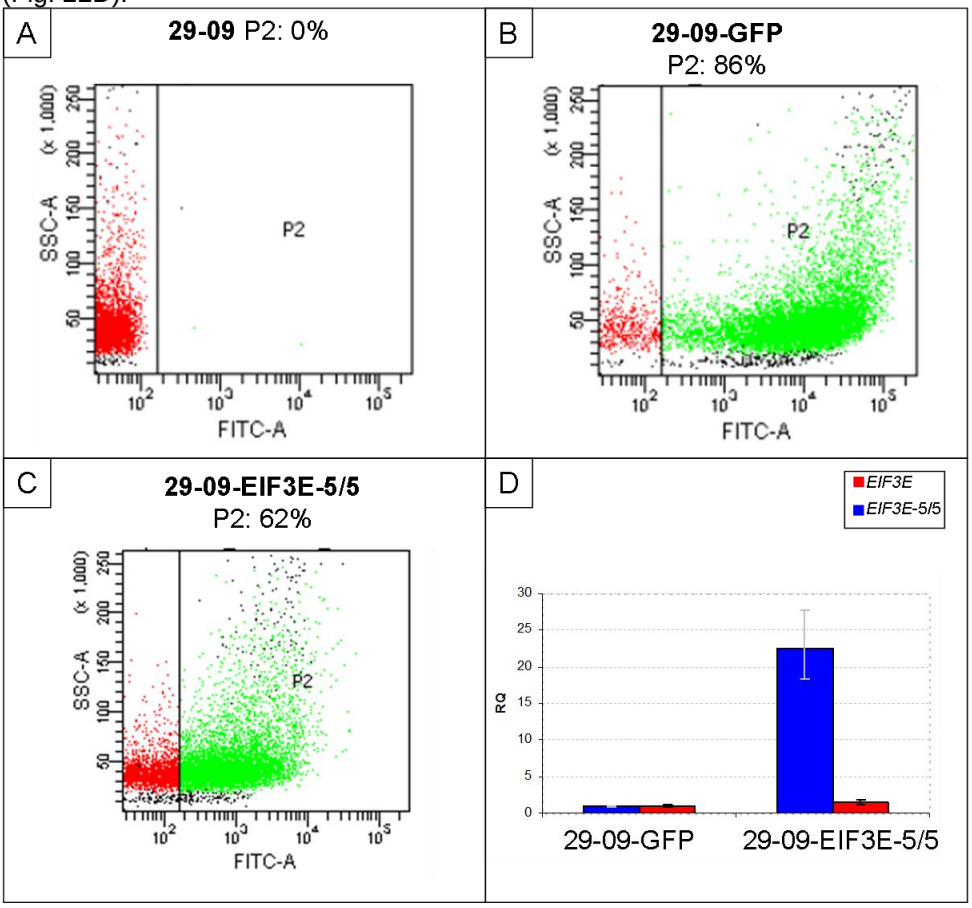

Fig. 22 Efficiency of viral infection in transduced 29-09-EIF3E-5/5 and 29-09-GFP cells evaluated for their GFP expression. Comparative levels of expression of EIF3E and EIF3E-5/5 expression in 29-09-EIF3E-5/5 and 29-09-GFP cells determined by qPCR.

A. *Gated regions were selected to display relative fluorescence intensity of the GFP-positive cells. Non-viral infected 29-09 cells were used to establish the region in which GFP signal is not present in cells.*

B, C. *Flow cytometry analysis indicate the number of GFP-positive cells with certain levels of GFP expression, in 29-09 cells expressing GFP alone (A) or jointly to EIF3E-5/5 in 29-09-EIF3E-5/5 cells (B).*

D. *Relative expression of EIF3E-5/5 and EIF3E expression in 29-09-GFP and 29-09-EIF3E-5/5 cells, determined by qPCR. Blue and red bars indicate EIF3E-5/5 or EIF3E relative expression levels, respectively. Hprt1 was used as endogenous control. Error bars indicate the 95% confidence interval.*

After transduction the 29-09 cells were cultured in adhesion culture conditions for 70 days corresponding to 19 passages (one passage corresponds to 3-4 days of culture and after which the cells were re-plated) (Fig. 23A). Analyzing the cells during time no morphological changes and no changes in the growth rate were observed between the 29-09-EIF3E-5/5 and 29-09-GFP cells. Conversely flow cytometry performed on the cells, collected at different time points after the time of infection, reveals that in the 29-09-EIF3E-5/5 transduced cell population, the number of GFP-positive cells increased during the 70 days of cell growing. While in the control transduced population 29-09-GFP, the number of GFP-positive cells did not change during 70 days of analysis (Fig. 23B).

We developed two hypotheses to explain the increase in the number of 29-09 EIF3E-5/5 cells. In the first one, we assume that the cells over-expressing *EIF3E*-5/5 proliferate faster than the non-infected cells. The increase of cell over time is accurately quantifiable with flow cytometry. Our second hypothesis is that the EIF3E-5/5 over-expressing cells are able to release factors that make non-transduced cells less competitive in terms of cells proliferation.

In both 29-09, GFP and *EIF3E*-5/5, expressing cells we observed a progressive increase in the GFP-intensity over time. This phenomenon can be explained by the store of GFP in the cell that are not able to proliferate. The numbers of these cells increase over time in primary cells.

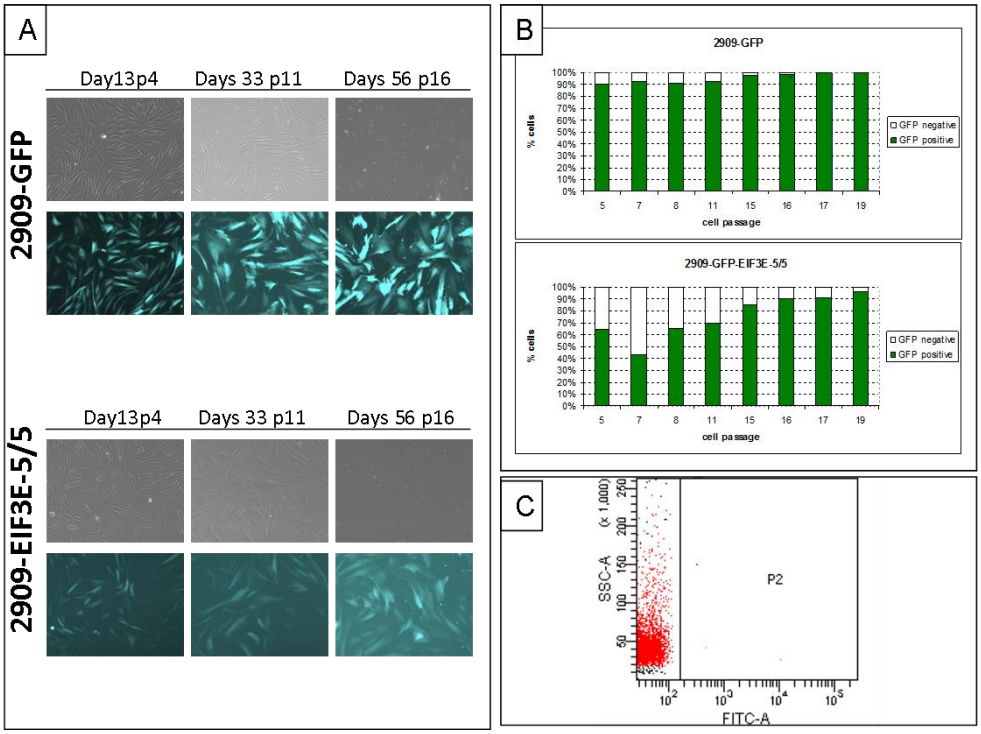

Fig. 23 Characterization of 29-09 cells stably expressing EIF3E-5/5 or GFP alone cultured for 70 days.

A. *29-09-EIF3E-5/5 and 29-09-GFP cells growth in adherent culture conditions at 13, 33 and 56 days.*

B. *Percentage of GFP expressing cells in 29-09-EIF3E-5/5 and 29-09-GFP transduced cell populations at different times and culture passage number in adherent conditions.*

C. *Non-viral infected 29-09 cells were used to establish the region in which GFP signal is not present in cells and cells are GFP negative.*

3.5. Increase of EIF3E-5/5 expression is sufficient to induce a shift from cap-dependent to cap-independent RNA translation

Chiluiza and colleagues showed that the shorter truncated *Eif3e* transcript generated by MMTV integration (*Eif3e5*) interferes with the translation process that promote the mechanism associated with cap-independent translation [56].
Since the *EIF3E*-5/5 RNA sequence is very similar to the truncated form EIF3E5 generated in mouse by viral DNA integration [24] we investigated if elevated expression of *EIF3E*-5/5 is able to support and increase the expression level of mRNAs translated by the cap-independent mechanism.

This putative function of *EIF3E*-5/5 was investigated in MCF7 cells stably expressing the double amount of *EIF3E*-5/5 RNA (MCF7-EIF3E-5/5) and in the control cells (MCF7-ctrl).
By qPCR analysis we measured the expression levels of six representative mRNAs translated by the cap-independent mechanism: CYR61 (cysteine-rich, angiogenic inducer, 61), Bcl2 (B-cell CLL/lymphoma 2), Pim-1 (pim-1 oncogene), Snai1 (snail homolog 1) c-Myc (myelocytomatosis viral oncogene homolog avian) and XIAP (X-linked inhibitor of apoptosis protein), and three representative mRNAs translated by the canonical cap-dependent pathway: Hprt1 (hypoxanthine phosphoribosyltransferase 1), Rpl32 (ribosomal protein L32), and Rpl34 (ribosomal protein L34).
The results, represented in figure 24, show that up-regulation of *EIF3E*-5/5 in the MCF7 cells induces an increase in the expression of five transcripts translated by the cap-independent mechanism (Cyr61, Bcl2, Pim1, Snai1, c-Myc). The *EIF3E*-5/5 over-expression was not observed to change the expression levels of L32 and Hprt1 (Fig. 24).

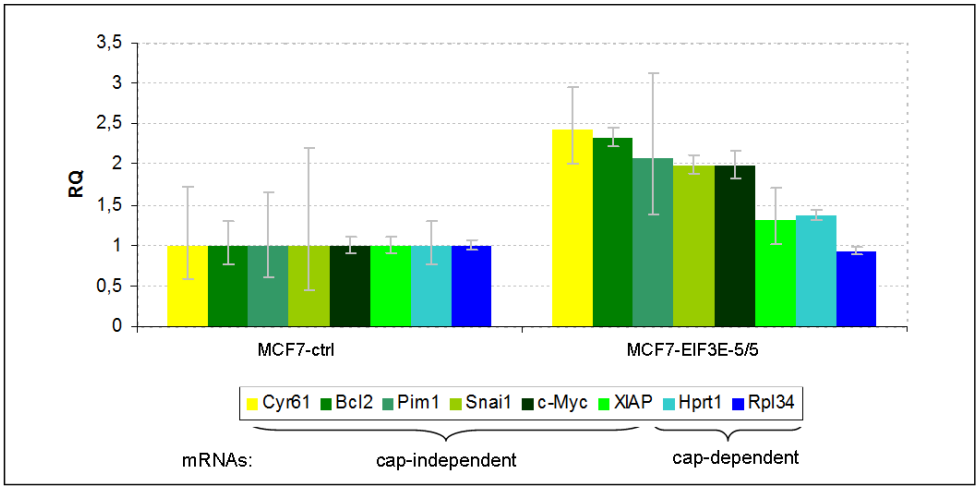

Fig. 24 Expression of mRNA translated by cap-independent or cap-dependent machinery in MCF7cells stably expressing EIF3E-5/5 or GFP alone.
Expression of selected mRNAs translated by cap-independent (Cyr61, Bcl2, Pim1, Snai1, c-Myc and XIAP) or cap-dependent (Hprt1 and Rpl34) mechanisms in MCF7-EIF3E-5/5 and MCF7-ctrl cells. Rpl32 was used as an endogenous control. Error bars indicate the 95% confidence interval.

The ability of *EIF3E*-5/5 to affect the expression of transcripts translated by the cap-independent machinery was also tested in the normal mammary primary 29-09 cells. We assessed in these cells the expression of the same mRNAs evaluated in the MCF7 cells.
The qPCR results, reported in figure 25, show that *EIF3E*-5/5 over-expression induces a detectable increase in the expression of three out of six mRNAs: Cyr61, Bcl2 and XIAP, associated with cap-independent translation. The level of

expression of the cap-dependent transcripts in both samples was unchanged (Hprt1 and Rpl34) (Fig. 25).
Compared to MCF7 we observed a relative low effect of *EIF3E*-5/5 over-expression in the primary mammary sample. We conjectured that the presence in the primary cell cultures of various cell types, making expression profiling quantitatively challenging.

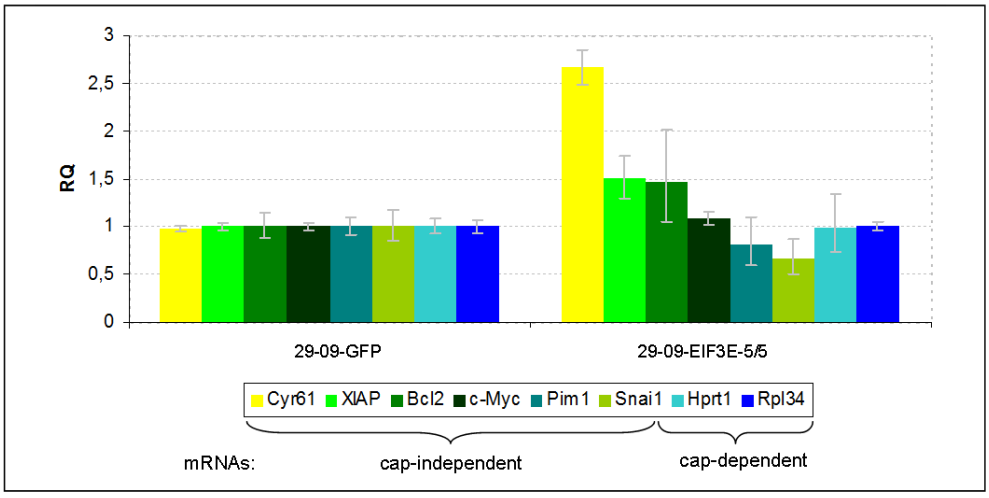

Fig. 25 Expression of mRNAs translated by cap-independent and cap-dependent machinery in 29-09 human primary mammary cells stably expressing EIF3E-5/5 or GFP alone.
Expression of mRNAs translated by cap-independent: Cyr61, XIAP, Bcl2, c-Myc, Pim1 and Snai1) and cap-dependent machinery: Hprt1 and Rpl34 in the 29-09-EIF3E-5/5 and 29-09-GFP cells. The reference RNA Rpl32 was used as an endogenous control. Error bars indicate the 95% confidence interval.

This experimental results obtained suggest that over-expression of *EIF3E*-5/5 is sufficient to support the increase of mRNA translation via to cap-independent machinery without affecting the expression of mRNA translated in a cap-dependent way. Since oncogenes are translated by cap-independent translational machinery we hypothesize that *EIF3E*-5/5 could have an important role during breast cancer initiation and/or progression.

3.6. Control of histone mRNA transcription by EIF3E-5/5

Neusiedler and colleagues recently demonstrated that *EIF3E* is necessary for efficient histone mRNA translation [64]. Since their study was performed without discriminate the role of individual *EIF3E* isoforms, *EIF3E* and *EIF3E*-5/5, we investigated if the efficient histone mRNAs transcription and translation is achieved

through a mechanism that utilizes the novel shorter *EIF3E* isoform (*EIF3E*-5/5) rather than the full-length form (*EIF3E*).
We analyzed by qPCR the mRNA expression of histone 3 in cells in which *EIF3E*-5/5 was modulated. We designed and used a primer forward and a primer reverse that amplify *HIST2H3A*, *HIST2H3C*, *HIST2H3D*, *HIST1H3*, *HIST1H3D* and *HIST3H3*. All these histones belong to class 3 histone family (H3). We decided to amplify in a single reaction different H3 histone mRNAs in order to understand the effect of *EIF3E*-5/5 on histone class 3.
The effect of stable *EIF3E*-5/5 over-expression to control the histone H3 level was assessed in MCF7 cells. The level of H3 mRNAs was determined in MCF7-ctrl cells, which express a basal level *EIF3E*-5/5 and in MCF7-EIF3E-5/5 cells that express the double amount of the 5/5 isoform. The results obtained show that *EIF3E*-5/5 up-regulation causes a significant increase in the histone H3 mRNAs level in the MCF7 cells (Fig.26).

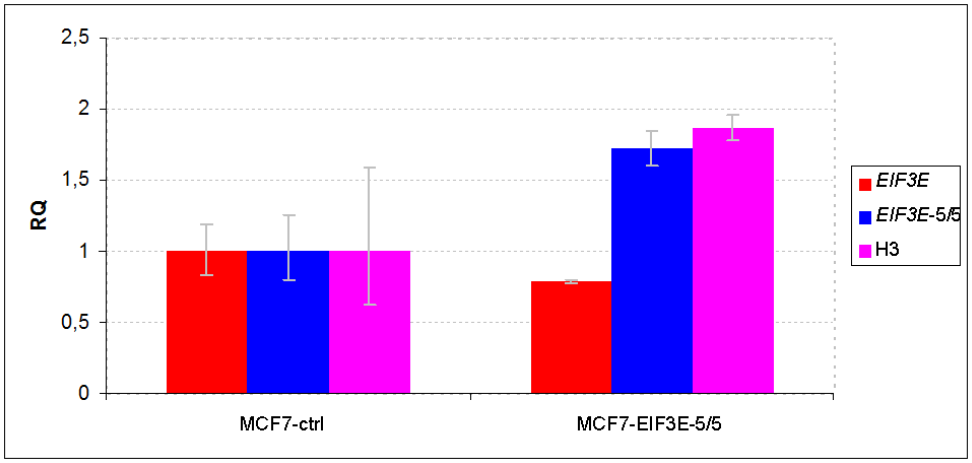

Fig. 26 Analysis of H3 expression in MCF7 stably over-expressing EIF3E-5/5. *The expression of histone mRNAs HIST2H3A, HIST2H3C, HIST2H3D, HIST1H3, HIST1H3D and HIST3H3 belonging to the class H3 family were analyzed by RT-qPCR (pink bars) in MCF7-ctrl and MCF7-EIF3E-5/5 cells. Expression levels of EIF3E and EIF3E-5/5 are shown as red or blue bars, respectively. Rpl32 was used as endogenous mRNA and the error bars corresponding to 95% confidence interval.*

The effect of *EIF3E*-5/5 down-regulation in H3 mRNA levels was also evaluated by qPCR in MCF10A (non-tumorigenic epithelial cell line) cells. To reduce the expression of *EIF3E*-5/5 RNA in cells we used the RNA interference approach. MCF10A cells were transfected with three kinds of siRNAs: siRNA with no targeting sequence (siRNA ctrl), a siRNA directed against *EIF3E* (siRNA *EIF3E*, sequence complementary to 3' *EIF3E* mRNA) and siRNA against *EIF3E*-5/5 (siRNA *EIF3E*-5/5, sequence complementary to exon 5A of *EIF3E*-5/5). After 48h, we determined the expression levels of *EIF3E*, *EIF3E*-5/5 and histone mRNAs by qPCR. The data

obtained shows that each siRNA used is specific for each *EIF3E* isoform it was suppose to target, in fact the expression of the other isoform results in both cases unchanged (Fig.27). The RT-qPCR analysis, reported in figure 27, shows that 50% decrease in *EIF3E*-5/5 expression level in MCF10A cells results in reduction of about 40% of histone mRNA H3 levels, while 90% reduction of *EIF3E* induces only 25% in histone H3 expression.
We calculated that in MCF10A cells the number of *EIF3E*-5/5 RNA molecules per cell is about 40 compared to 1000 of *EIF3E*. This means that a reduction of only 20 *EIF3E*-5/5 molecules in cells causes a reduction of 40% in the histone expression. At the contrary to reduce histone H3 mRNAs only by about 25% we have to silence 900 *EIF3E* mRNA molecules.

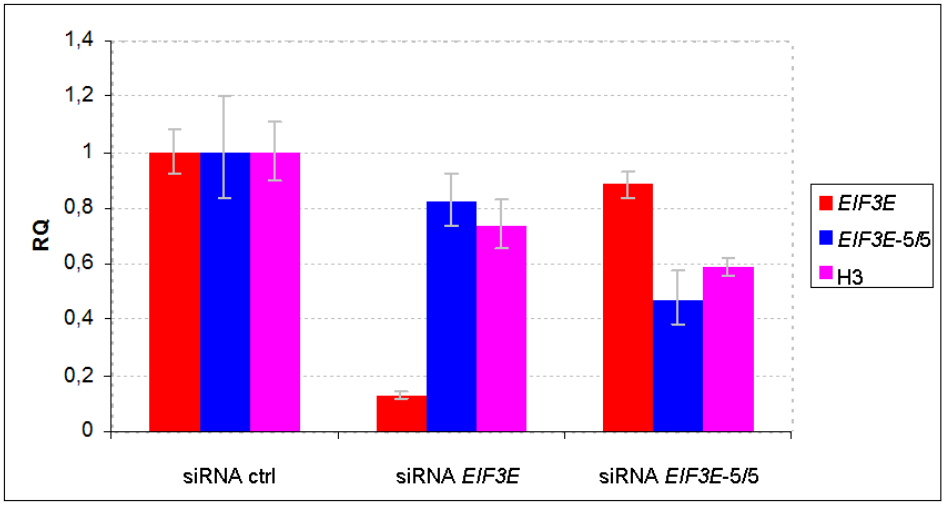

Fig. 27 Analysis of expression EIF3E, EIF3E-5/5 and H3 in MCF10A cells after 48h of siRNA treatment. *Relative expression of EIF3E, EIF3E-5/5 and H3 was determined by qPCR in MCF10A cells transfected with siRNA control (siRNA ctrl) or directed against EIF3E (siRNA EIF3E) or EIF3E-5/5 (siRNA EIF3E-5/5). Hprt1 and MCF10A siRNA ctrl cells are used as an endogenous mRNA control and as reference cells, respectively. Error bars indicate the 95% confidence interval.*

Considering the different expression level of *EIF3E* and *EIF3E*-5/5 in the various mammary cells analyzed and the results in histone H3 expression analysis we can assert that *EIF3E*-5/5 rather than *EIF3E* controls the histone expression and that *EIF3E*-5/5 has an important role as a sensor for DNA replication.

4. DISCUSSION

Breast cancer is the primary and secondary leading cause of cancer death in women between the ages of 15 and 54, 55 and 74 respectively. The high mortally is due to lack of early diagnostics markers for tumor formation, prevention and treatment for metastasis. The identification and characterization of oncogenes and oncogenic associated mechanisms that promote tumor features in cells is essential for early detection.

The MMTV model identified in mouse tumorigenesis has been used extensively to investigate the aetiology of breast, resulting in the identification of novel cancer genes and tumour biomarkers [48,49]. The eukaryotic translation initiation factor 3 subunit e (*Eif3e*) gene has been identified in the MMTV cancer model as a common integration site of the mammary tumor virus. Viral integration causes the expression of truncated and oncogenic transcripts of *Eif3e* gene. Diverse studies have shown that *EIF3E* is involved in the initiation and progression of human breast cancer through its ability to regulate the expression and stability of specific mRNAs [38,34,64] to control protein degradation by proteasome [29,46] and to induce the epithelial to mesenchimal transition (EMT) [60]. Despite this extensive research the specific mechanism of *EIF3E* in human mammary tumorigenesis remains largely unclear.

My Ph.D. project was focused on identifying the functional role of *EIF3E* in normal mammary gland and the mechanicist mode by which *EIF3E* contributes to breast cancer.

We identify by 3' RACE-PCR in breast cancer tissues, three novel alternately transcripts of the gene *EIF3E*: *EIF3E*-8910, *EIF3E*-144 and *EIF3E*-5/5. Our study is the first report of the identification and characterization of these novel transcripts. We determined that *EIF3E*-8910 detected only in one human breast cancer sample, ITB-8910, is an aberrant splicing form of the *EIF3E* transcript of 1169 nt lacking the exons 8, 9 and 10. The *EIF3E*-144 form lacks the 144 nt of the 3' UTR of *EIF3E* and it is expressed in all breast cancer samples analyzed. The third alternative form of *EIF3E* is a polyA RNA of 771 nt, constituted by 492 nt corresponding to the sequence from exon 1 to exon 5 of *EIF3E* and 279 nt of *EIF3E* intron 5 that generates a new exon, that we name exon 5A, by alternative splicing.

We primarily focused our study on the *EIF3E*-5/5 transcript since it is very similar to the shorter truncated and oncogenic *EIF3E* transcript identified in the MMTV model [24].

Sequence analysis of *EIF3E*-5/5 revealed the existence of two ESTs (EMBL ID: BU158977.1 and CN313793.1) and one putative transcript (ID: ENST00000521440, GENCODEv7) that provide support that this RNA is expressed in human cells (Fig. 13). We identified also a predicted polyA site located in exon 5A that could force both the premature ending of EIF3E-5/5 during gene transcription of *EIF3E* and addition of its polyA tail (Fig. 12). We propose that *EIF3E*-5/5 is a mature coding mRNA, in which a stop codon in exon 5A results from an in frame shift of the the *EIF3E* start codon located in exon 1. The putative protein that would be translated by *EIF3E*-5/5 should be a 20 KDa protein (172 amino acids) with a cytoplasmatic localization since, differently from EIF3E, it lacks the nuclear localization sequence (Fig. 15). Identification and characterization of the *EIF3E*-5/5 protein is in progress.

Even though *EIF3E*-5/5 was originally identified in breast cancer tissues and tumorigenic mammary cell lines we determined that this *EIF3E* isoform is also expressed in different human normal tissues such as breast, pancreas and skeletal muscle (Fig. 16). This data suggests that *EIF3E*-5/5 is a ubiquitous and not tissue-specific isoform. We observed that in all normal tissues, the *EIF3E*-5/5 transcript is less expressed than the *EIF3E* mRNA and in particular their ratio in the normal mammary gland is 1:100.

Despite the two isoforms have different levels of expression in different normal tissues we noticed that both are more expressed in the mammary gland than in other tissues. This data supports that *EIF3E* and its alternative transcripts may have a unique function in the mammary gland and aberrant expression could be related to the aetiology of human breast cancer.

Until now, we have not been able to fully elucidate which are the molecular mechanisms that coordinate the ratio of transcription for the two RNAs (*EIF3E* or *EIF3E*-5/5) in different cell types. We assume that when *EIF3E* transcription terminates with a polyadenylation site located into exon 5A, the spliceosomal complex recognizes the splicing acceptor site located at 5' end of exon 5A and allows the complete maturation of *EIF3E*-5/5. In contrast when the transcription of *EIF3E* goes beyond this polyadenylation signal and exon 6 is included in transcription, the spliceosomal complex recognizes the acceptor site of intron 5, causing the removal of the intron 5 (containing exon 5A) generating the mature *EIF3E* mRNA.

We investigated the expression of *EIF3E*-5/5 and *EIF3E* in tumoral and healthy tissues in order to understand their involvement in the oncogenic process. In our studies we used RNA extracted from surgically derived tissues of women affected by breast cancer. By RT-qPCR analysis we confirmed that in all tumoral breast tissues the expression of *EIF3E* is lower compared to their healthy counterparts (Fig. 17) [28, 59]. The same reduction in expression was also observed, in all cohorts of normal and tumour samples derived from each patient, for *EIF3E*-5/5 expression (Fig. 17). The absolute quantification analysis showed that the reduction of *EIF3E*-5/5 and *EIF3E*, in each pair normal tumour, do not have the same decrease tendency. We estimated that in 75% of pairs analyzed, *EIF3E* is reduced by about 80% in the cancer tissue compared to 60% of *EIF3E*-5/5. This different reduction trends observed means that in the breast cancer tissue the ratio between the two isoforms (*EIF3E/EIF3E-5/5*) is reduced compared to the value obtained in the normal tissue (Fig. 18).

We investigated by *in vitro* experiments how variations in the ratio between the two *EIF3E* isoforms, found in human breast cancer can contribute to cancer initiation and/or progression. In order to perform these experiments we over-expressed by a lentiviral approach, *EIF3E*-5/5 in a human tumorigenic mammary epithelial cell line (MCF7) and in primary cells derived from a mammary normal sample (29-09).

We observed that the double amount of *EIF3E*-5/5 expression in MCF7 cells is able to increase the proliferation of cells cultured in a 3D matrix (collagen) and in suspension culture conditions producing larger 3D structures and mammospheres than control cells (Fig. 20, 21). Since MCF7 cells over-expressing *EIF3E*-5/5 increase also the number of 3D structures and spheres (demonstrated with first and second generation spheres assays) we hypothesized that *EIF3E*-5/5 may control the self-renewal potential of cells with stem cell features (Fig. 20, 21).

Analyzing the 29-09 cells after lentiviral infection we observed that in the 29-09-*EIF3E*-5/5 sample the number of infected cells, over-expressing *EIF3E*-5/5, increase over time (70 days) compared to non infected cells (Fig. 23). The number of *EIF3E*-5/5 over-expressing cells was determined by flow cytometry measuring the number of cells with specific GFP intensity since the transgene *EIF3E*-5/5 expression was coupled in the same vector with a GFP coding sequence. We hypothesized that the increase of *EIF3E*-5/5 over-expressing cells over time was due to cells proliferating faster than non infected cells or releasing factors negatively affecting the growth rate of the non transduced cells.
All of our results support that alterations affecting *EIF3E*-5/5 expression and the ratio difference between the two *EIF3E* isoforms are able to induce a tumoral phenotype by increasing cell proliferation and the self-renewal potential of cells with stem cell proprieties.
In my Ph.D. project we demonstrated also that *EIF3E*-5/5 affects the expression of a certain class of mRNAs. In particular we observed that *EIF3E*-5/5 up-regulation in the MCF7 cells promotes the expression of mRNAs translated specifically by the cap-independent machinery of cells (*e.g.* Cyr61, Bcl2, Pim1, Snai1, c-Myc and XIAP). The changes in expression level of mRNAs translate in a cap-dependent manner were not affected (Hprt1, Rpl32 and Rpl34) (Fig. 24). The similar results were observed using cells from a primary normal mammary sample, although the expression results were less robust than obtained in cell line. It was most likely due to different cell types that composed the primary tissue sample (Fig. 25). Collectively, these experiments implicate *EIF3E*-5/5 in controlling mRNAs translated by the cap-independent machinery. The cap-independent machinery has been shown to be important of cancer associated pro-growth, pro-angiogenesis and survival proteins. These transcripts are usually activated in response to stress conditions such as cellular hypoxia, serum starvation or ionizing radiation. Therefore we propose a model by which aberrant cellular ratio between EIF3E and *EIF3E*-5/5 isoforms expression promote breast tumorigenesis by a process that increases the level of cap-independent mRNAs.
Recently it was demonstrated another important function performed by *EIF3E*-5/5, that is the control of histones mRNAs transcription. In agreement with previous research, we observed that variations in *EIF3E*-5/5 expression lead to a change in histone H3 mRNA expression in mammary epithelial cell lines by a mechanism that we have not fully identified yet. More precisely we observed that *EIF3E*-5/5 up-regulation in MCF7 cells induces an increase of 80% of histone mRNA expression, while an *EIF3E*-5/5 reduction of 50% is responsible of 40% decrease in H3 mRNA levels in MCF10A cells (Fig. 26,27). Through our experiments we were able to show that in contrast to the model presented by Neusiedler and colleagues, histone transcription is most likely controlled by *EIF3E*-5/5 expression [64]. These results suggest that *EIF3E*-5/5 functions as sensor in the DNA replication and alteration of protein levels of *EIF3E*-5/5 in cells generate breast carcinogenesis.
During my Ph.D. project we characterized a previously un-described human EIF3E isoform, *EIF3E*-5/5. While *EIF3E*-5/5 is ubiquitously expressed, tight regulation of its expression is essential for normal cell regulation of histone and cap-independent protein translation and for inhibiting the transformation of normal cells to tumor like cells. We observed that this transcript has a positive role in repressing the abundance of mRNAs that maybe associated with tumorigenic process.

The future work we will be focus on understanding the specific function of *EIF3E*-5/5 in the breast cancer and its possible use as a cancer prognostic and diagnostic factor and as a therapeutic target for cancer treatment.

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