UNIVERSITÀ DEGLI STUDI DI TRIESTE

XXIX CICLO DEL DOTTORATO DI RICERCA IN

BIOMEDICINA MOLECOLARE

GTSE1: a novel TEAD4-E2F1 target gene involved in cell protrusions formation in Triple Negative Breast Cancer

Settore scientifico-disciplinare: BIO/13

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"Meravigliarsi di tutto è il primo passo della ragione verso la scoperta"

Louis Pasteur

Abstract

Dissemination of cancer cells from the primary tumors to distant organs represents the main cause of death in cancer patients. GTSE1 over-expression has been reported as a potential marker for metastasis in various types of malignancies including breast cancer where GTSE1 expression levels associate with tumor grade, enhanced invasive potential and negative prognosis. Given the strong association between GTSE1 deregulation and bad clinical outcome the aim of this work was to clarify how GTSE1 is regulated in triple negative breast cancer and to elucidate the mechanism underlying GTSE1-dependent cell movement. Here, I identified GTSE1 as a novel direct TEAD4 and E2F1 transcription factors target gene, highlighting a role for YAP and TAZ co-activators in GTSE1 transcriptional regulation. Frequently deregulated in cancers, TEAD4 and the co-activators YAP and TAZ have been reported to promote tumorigenesis, invasion and metastasis in breast cancer. I demonstrated that the effect of the TEAD transcription factor on cell migration and invasion is GTSE1-dependent. Moreover, I found that TEAD controls cell protrusions formation, required for cell migration, through GTSE1 protein, unveiling a relevant effector role for GTSE1 in the TEAD-dependent cellular functions.

Table of contents

Introduction

Breast cancer

Breast cancer is one of the most frequently diagnosed form of cancer and the second leading cause of death in Western women (Weigelt et al., 2005). It is a very complex and heterogeneous disease including subtypes with different molecular alterations and clinical outcome (Stingl and Caldas, 2007). According to a classical molecular classification based on the gene expression profiles, breast tumours can be distinguished in five different subtypes: luminal A, luminal B, normal-like, HER2-positive and basal-like breast cancers (Perou et al., 2000; Sorlie et al., 2001).

The most recurring subtype is the luminal A, representing 50-60% of the total cases. It is characterized by the expression of oestrogen receptor (ER), progesterone receptor (PgR), low expression of Ki67 and absence of human epidermal growth factor receptor 2 (HER-2) amplification. The luminal A subtype shows a gene expression pattern similar to that of luminal epithelial mammary cells where ER transcription factor is activated. In general, this subtype of tumour displays low rate of proliferation, low histological grade and is associated with good prognosis (Kennecke et al., 2010).

The luminal B subtype represents about 10-20% of the total breast cancer cases. Its immunohistochemistry (IHC) profile is characterized by ER+/HER2- and high Ki67 or ER+/HER2+ or in about 6% of the cases ER-/HER2-. Respect to the luminal A these tumors present higher proliferation rate, histological grade and worse clinical outcome (Eroles et al., 2012).

There are few studies about the normal-like breast cancer for two reasons: first of all it is a rare form and secondary some groups believe that it is not a real subtype, but a technical artefact due to contamination with normal tissue during microarrays (Weigelt et al., 2010).

Of the total cases of breast tumors, 15-20% is represented by the HER2-positive subtype. Cancers belonging to this subtype show HER2 gene amplification, over-expression of genes related to the cell cycle and high proliferation rate. About 75% of these cancers have high histological grade (Eroles et al., 2012). HER2 positive breast

tumors have been further subdivided in three subtypes, one of which associated with higher invasive potential and worse clinical outcome than the others. 158 genes, identified as differentially expressed between the subtype related to worse prognosis and the other subtypes, have been used to develop a prognostic predictor useful to better stratify the patients identifying those belonging to the more aggressive subgroup (Eroles et al., 2012; Staaf et al., 2010). Although related to poor prognosis (12% ten year survival in patients with the most aggressive subgroup and 50-55% in the other subgroups), the development of a targeted therapy anti-HER2 has enabled progress in the treatment of this breast cancer subtype (Eroles et al., 2012; Gianni et al., 2011; Piccart-Gebhart et al., 2005; Slamon et al., 2001).

Accounting for about 10-20% of all breast carcinomas, the basal-like subtype is characterized by a gene expression pattern similar to that of normal breast myoepithelial cells, expressing cytokeratins CK5 and CK17, CD44, P-cadherin, nestin, EGFR and caveolin 1 and 2 (Eroles et al., 2012). This subtype tends to occur in premenopausal women with the highest incidence among African-American population (Carey et al., 2006; Millikan et al., 2008) and shows high histological grade and frequent lymph node metastasis at diagnosis(Eroles et al., 2012). It is associated with worse prognosis than luminal ones showing a very aggressive relapse pattern affecting visceral organs, such as lung and central nervous system(Kennecke et al., 2010; Smid et al., 2008).

About 55-85% of basal-like tumors are triple negative breast cancers (TNBC), lacking of the expression of ER, PGR and HER2 gene amplification (Seal and Chia, 2010). Basal-like subtype and TNBC are not synonymous, in fact not all TNBC belong to the basal-like subtype.

TNBCs represent about the 15% of all invasive breast tumours (Mayer et al., 2014). These cancers seem to be more aggressive than other breast cancer subtypes, leading to a high recurrence probability and poor survival rates (Stingl and Caldas, 2007).

In 2007 the claudin low subtype has been identified as a novel breast cancer subtype (Herschkowitz et al., 2007). This type of breast carcinoma has derived its name from the low expression of genes involved in tight junctions and intercellular adhesions, including claudin-3, -4, -7 cingulin, E-cadherin and ocludin. These tumours display low expression of genes related to cell proliferation, over-expression of genes associated to EMT and generally are TNBCs and related to poor prognosis (Eroles et al., 2012).

In the last twenty years breast cancer treatment has evolved from a baseline therapy based on chemotherapy to a more target-directed approach. In fact nowadays, patients with hormone receptor positive tumour are generally treated with hormonal therapy, such as selective oestrogen-receptor response modulators (SERM) or aromatase inhibitors. Endocrine therapies slow or stop the growth of HR-positive tumours by preventing the cancer cells from getting the hormone they need to grow in several different ways. Some drugs, like tamoxifen, couple and lock to the receptors in the cancer cells and prevent ligands from binding to the receptor. Other drugs, like aromatase inhibitors, decrease the level of oestrogen in the body. Analogously, patients with HER2-positive tumours typically receive an anti-HER2 targeted therapy. HER2 is a cell membrane tyrosine kinase receptor member of the epidermal growth factor receptor (EGFR) family (Mathew and Perez, 2011). The first commercially available HER2 targeting agent was the monoclonal antibody trastuzumab (Herceptin®). The humanized monoclonal antibody trastuzumab binds to an extracellular segment of the HER2 receptor, leading to inhibition of the proliferation of human tumor cells that overexpress HER2 (Leyland-Jones, 2002).

Breast cancer treatment strategies such as hormonal or targeted therapies are effective only when the corresponding receptors or molecular targets are expressed by the tumour cells.

Of course in the TNBC patients the hormonal therapy and anti-HER2 targeted therapy are ruled out leading to a difficult treatment scheme and to the need of identifying novel therapeutic targets for a more targeted approach.

GTSE1 (G2 and S phase expressed 1)

The murine Gtse-1 gene was discovered in our group in 1998 as a p53-inducible gene (Utrera et al., 1998). In this study, by using a differential subtractive hybridization approach in the murine cell line Val 5 stably transfected with the temperature-sensitive Val 135 allele of murine p53, Gtse-1 was isolated as a novel p53 target gene whose protein was selectively induced in the G2 fraction of the cell population. A p53

responsive element was identified in close vicinity of the Gtse-1 promoter (Utrera et al., 1998).

Further characterization of Gtse-1 expression revealed that it is almost undetectable during the G1, increases in S phase and peaks during the G2 phase, showing that it is tightly regulated during the progression of the cell cycle (Collavin et al., 2000). Wt p53-containing and p53-null murine cells share the same regulation of Gtse-1 during the cell cycle suggesting that other transcription factors may be involved in the control of Gtse-1 expression.

Gtse-1 is mainly a microtubules-associated protein, although clear evidence of nuclear-cytoplasmic shuttling has been observed (Monte et al., 2004; Utrera et al., 1998). Its over-expression leads to accumulation of cells with 4N DNA content indicating a prolonged G2/M phase of the cell cycle. This suggested that Gtse-1 might play specific biological activities in this cell cycle window where it is physiologically expressed (Utrera et al., 1998).

The human homologue of Gtse-1 (GTSE1) shares the same intracellular localization, the ability to undergo to nuclear-cytoplasmic shuttling and identical cell cycle regulated expression pattern (Monte et al., 2000). Unlike its murine homologue, hGtse-1 gene lacks of the p53-responsive element (Monte et al., 2000).

Murine and human GTSE1 amino acid sequences present 60% of amino acid identity and more than 70% of similarity, with N-terminal and C-terminal region highly conserved (Monte et al., 2000). Moreover, more than 85% of Ser-Pro motifs and 50% of Thr-Pro motifs are conserved between mouse and human GTSE1(Monte et al., 2000). GTSE1 harbours a conserved and active NES, located in the C-terminal region and required for the shuttle from the nucleus into the cytoplasm, and three putative NLS (Monte et al., 2000).

hGtse-1 gene includes at least 11 exons and 10 introns stretching over about 33Kb of genomic DNA (Monte et al., 2000). This gene and its murine homologue mapped on chromosome 22 corresponding to band q13.2–q13.3 and chromosome 15 respectively. In addition to Gtse-1, different murine genes, such as Cyp2d, G22p1, Ncf4 and others, have been reported to localize on chromosome 15 and their human homologues on chromosome 22, indicating that these are regions of conserved synteny (Dunham et al., 1999; Huppi et al., 1998; Monte et al., 2000).

GTSE1 biological functions

GTSE1 is mainly an intrinsically disordered protein (IDP), a part from a region of 100 amino acids located in N-terminal domain expected to fold in an ordered secondary structure (Scolz et al., 2012).

Analysis of the amino acids sequences and secondary structure of IDPs revealed that they are characterized by the presence of low complexity of regions, in which there is the repetition of one or few amino acids, coupled with low content of bulky hydrophobic amino acids and high content of hydrophilic and charged amino acids. As result of this, they are unable to spontaneously fold in stable three-dimensional structures under normal conditions (Wright and Dyson, 2015). Their peculiar structure provides to IDPs great flexibility and the ability to bind different partners with high specificity and low affinity, making these regulatory interactions easily reversed.

IDPs transiently interact with multiple different targets in dynamic regulatory networks exerting a key role in the regulation of signalling pathways and cellular processes such as transcription, translation, cell cycle, chromatin remodelling and assembly and disassembly of microfilaments and microtubules (Galea et al., 2008; Guharoy et al., 2013; Iakoucheva et al., 2002; Liu et al., 2006; Wright and Dyson, 2015).

Looking at GTSE1 sequence we identified different protein binding sites such as p53, p21 and EB1 binding domains (Bublik et al., 2010; Monte et al., 2003; Scolz et al., 2012), see fig. A.

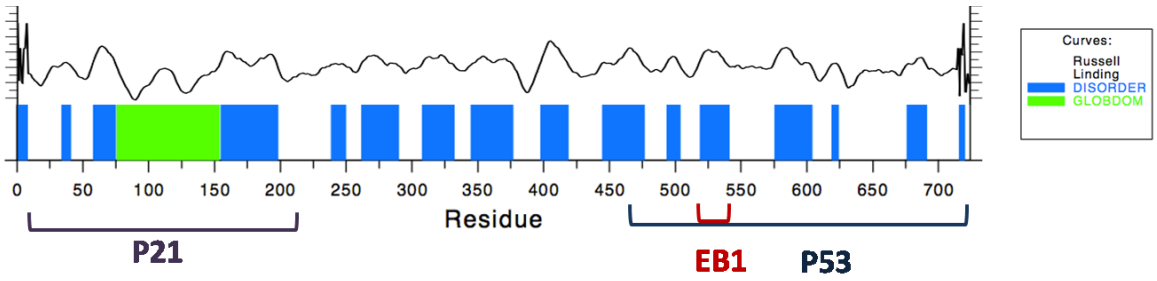


Fig. A Cartoon representing p53, p21 and EB1 protein binding sites in GTSE1 sequence

In a previous work, carried out in our group, we reported that in response to DNA damage GTSE1 is stabilized and accumulates into the nucleus and, controlling p53 stability and function, regulates the DNA damage-induced apoptosis (Monte et al., 2003). In fact, GTSE1 knock-down sensitizes cells to p53-dependent apoptosis after DNA damage. Under stress conditions p53 undergoes to post-translational modifications that lead to its stabilization and accumulation into the nucleus. Instead, in the post-damage recovery phase, the activity of negative regulators is required to down-regulate p53 protein levels and activity and to restore its steady-state functions. Time-course experiments demonstrated that GTSE1 accumulates into the nucleus at slower rate than p53, suggesting that it could play a critical role in the post-damage recovery phase (Monte et al., 2003; Monte et al., 2004). In fact, we demonstrated that GTSE1 interacts with p53 through its C-terminal domain and shuttles it from the nucleus into the cytoplasm promoting its proteasome-dependent degradation(Monte et al., 2003; Monte et al., 2004). It has been reported that p53 cytoplasmic localization enhances during the S and G2 phases of the cell cycle (David-Pfeuty et al., 1996; Shaulsky et al., 1990), coincident with GTSE1 increased expression, suggesting that GTSE1 could play a key role in the control of p53 stabilization and activity also in unstressed cells in physiological conditions (Monte et al., 2003).

It has been reported that GTSE1 has a critical role in cisplatin resistance in gastric cancer cells, in fact, its loss associates positively with increased sensitivity to the treatment. As seen for other DNA-damage inducing agents, cisplatin treatment up-regulates GTSE1 and its nuclear localization. Furthermore, in line with its previously reported function of negative regulator of p53, in cisplatin treated cells, GTSE1 depletion increases p53 expression levels. This, in the end, leads to enhanced sensitivity to p53-mediated cisplatin induced apoptosis (Subhash et al., 2015). Moreover, increased GTSE1 expression consequent to cysplatin treatment has been observed not only in gastric cancer cells, but also in multiple myeloma cell lines, suggesting that the GTSE1-mediated mechanism of acquired resistance to this drug could be shared (Spanswick et al., 2012).

As mentioned above, another partner of GTSE1 is p21. p21 is a p53-responsive gene and a member of the family of CDKs inhibitors (CKI) able to halt the cell cycle progression by binding cyclin-CDKs complexes. Interacting with different partners, it

has a critical role in biological processes such cell cycle arrest in response to stress, apoptosis, differentiation and senescence, and for this reason its expression levels must be tightly regulated (Brugarolas et al., 1995; Kagawa et al., 1999; Li et al., 1999; Parker et al., 1995). It has been demonstrated that p21 stability is regulated by the proteasome system through both ubiquitin-dependent and -independent mechanisms. In a previous work, our group have shown that GTSE1 stabilizes p21 protecting it from the proteasome-dependent degradation (Bublik et al., 2010). GTSE1 interacts with p21 through its N-terminal domain and together with Hsp90 and WISp39 proteins forms a chaperone complex that controls p21 turnover. As functional consequence, GTSE1 up-regulation stabilizing p21 confers chemoresistance to paclitaxel-induced cell death. In fact, GTSE1 silencing sensitizes cancer cells to paclitaxel, its overexpression leads to resistance to paclitaxel-induced apoptosis and p21 knock-down restores the sensitivity to the treatment (Bublik et al., 2010).

GTSE1 and cancer

GTSE1 deregulation has been reported to occur in different types of cancer indicating that this protein has a role in cancer progression (Scolz et al., 2012). We demonstrated that GTSE1 protein levels are very low in non-transformed cell lines and dramatically up-regulated in tumorigenic cell lines. Noteworthy, GTSE1 expression in transformed cells is elevated across all the cell cycle phases, including G1, phase in which its expression levels are almost undetectable in non-transformed cell lines (Scolz et al., 2012). It has been reported that invasion of cancer cells occurs mainly in this cell cycle phase (Iwasaki et al., 1995). In a previous work we demonstrated that GTSE1 not only binds directly the microtubule lattice, but is also localized at the growing microtubules plus ends in an end-binding protein 1-dependent manner (Scolz et al., 2012).

End-binding protein 1 (EB1) recruits many different microtubule plus end tracking proteins (+TIPs) at microtubules (MT), to do this it binds MT through its N-terminal calponin homology domain and +TIPs via the EBH (EB-homology) domain located in its C-terminal region.

The majority of EB1-binding proteins interacts with it through short interaction motifs called SKIP.

We demonstrated that GTSE1 interacts with EB1 through SKIP motifs located in its disordered regions (Scolz et al., 2012). Among the different cellular functions regulated by the EB1-dependent +TIPs there is cell migration. Some +TIPs act at the leading edge of migrating cells promoting microtubules stabilization, actin polymerization and cell adhesion (Kaverina and Straube, 2011; Stehbens and Wittmann, 2012). Moreover, it is known that the disassembly of focal adhesions (FAs), necessary for cell migration, is microtubules-dependent and occurs when MTs physically interact with FAs, indicating that MAPs have a critical role in this process (Ezratty et al., 2005; Kaverina et al., 1999; Rooney et al., 2010).

We have reported that GTSE1 regulates cell migration promoting microtubule-dependent focal adhesions disassembly in EB1-dependent way (Scolz et al., 2012). In fact, serum-starved U2OS cells have few focal adhesions, but GTSE1 knock-down increases their number. The treatment with nocodazole, a drug inhibiting microtubule polymerization, causes persistence of focal adhesions, when the drug is washed out the polymerization of MTs is restored and FAs are disassembled. GTSE1 depletion affects negatively FAs disassembly after nocodazole wash out suggesting a deficiency in their microtubule-mediated dismantlement (Scolz et al., 2012). Moreover, EB1-dependent MT plus ends localization of GTSE1 is required for focal adhesions turnover. In fact, the expression of a RNAi resistant wild-type GTSE1 is able to rescue the reduced FAs disassembly in GTSE1 depleted cells, instead the expression of a GTSE1 construct mutated in the SKIP motifs is not able to produce the same effect (Scolz et al., 2012).

GTSE1 is an interphase specific microtubule plus-ends binding protein. In fact, during prophase/ prometaphase transition GTSE1 +TIP activity stops and restarts in anaphase. De facto, it has been suggested that during mitosis the hyperphosphorylation of CDK1 phosphorylation sites, located around GTSE1 SKIP motifs, leads to the disruption of EB1-GTSE1 interaction and the loss of the MTs growing ends localization of GTSE1 (Scolz et al., 2012).

It has been reported that during mitosis GTSE1 together with clathrin and TACC3 takes part to the formation of a multiprotein complex at the mitotic spindle pole. In particular, during the assembly of this complex, clathrin is first recruited to the spindle, and then

TACC3, after its Aurora A-mediated phosphorylation, interacts with clathrin and allows the recruitment of GTSE1 at the spindle pole (Hubner et al., 2010).

Very recently it has been suggested that the TACC3-Clathrin-GTSE1 complex promotes the microtubules stability through the GTSE1-dependent inhibition of the mitotic centromere associated kinesin (MCAK) (Bendre et al., 2016). Due to its potent microtubules depolymerase activity, MCAK has a critical role in MT stability and must be tightly regulated to avoid defects in chromosome alignment and segregation. Continuous alterations in these two processes lead to an enhanced rate of gain or loss of chromosomes, a phenomenon known as chromosome instability (CIN), frequently observed in cancers. Hyperstabilization of kinetochore-MT attachments has been related to enhanced chromosome missegragation and CIN. GTSE1 interacts with MCAK via its N-terminal domain and this binding abolish MCAK MTs depolymerase activity in vitro. It has been suggested that the up-regulation of GTSE1, frequently observed in tumours, may lead to the inhibition of MCAK activity, enhanced MTs stability and consequently CIN (Bendre et al., 2016). In fact, GTSE1 overexpression in chromosomally stable cell lines induces chromosome missegregation and CIN, instead GTSE1 depletion in highly CIN cancer cell lines reduces the defects in chromosome segregation (Bendre et al., 2016).

The recruitment of GTSE1 to the mitotic spindle is Aurora A-dependent and recently it has been demonstrated that the overexpression of this latter is associated with increased kinetochore-MT stabilization and CIN in cancer (Ertych et al., 2014; Hubner et al., 2010). This has suggested that Aurora A deregulation could enhance GTSE1 localization to the spindle, promoting the inhibition of MCAK activity and the MT stability leading to CIN (Bendre et al., 2016).

GTSE1 is over-expressed in various types of malignancies, including breast cancer (Canevari et al., 2016; Scolz et al., 2012). In a previous study carried out in our lab, we demonstrated that GTSE1 protein levels correlate with invasive potential, indeed higher expression of this protein is found in the most aggressive and invasive breast cancer cell lines (Scolz et al., 2012). Moreover, GTSE1 over-expression enhances the motility of the poor invasive breast cancer cell line MCF7, instead its depletion reduces the ability to migrate of the highly invasive and metastatic breast cancer cell line MDA-MB-231.

Furthermore, GTSE1 protein levels correlate with bad clinical outcome and tumour grade, with patients with higher protein expression levels showing shorter time to distant metastasis and shorter survival time (Scolz et al., 2012).

Interestingly, GTSE1 up-regulation was identified as a potential marker for metastasis not only in breast cancer, but also in hepatocellular carcinoma, gastroenteropancreatic neuroendocrine tumour and oral tongue squamous cell carcinoma (Guo et al., 2016; Lee et al., 2012; Zhou et al., 2006).

It has been reported that GTSE1 is up-regulated in hepatocellular carcinoma (HCC) and, as seen in breast cancer, has a key role in the promotion of cancer cell migration and invasion. Moreover, its expression levels correlate with venous invasion, tumour grade, tumour size and shorter survival time, suggesting that GTSE1 has a proto-oncogenic role in the development of hepatocellular carcinoma and may be considered a therapeutic target in this type of cancer (Guo et al., 2016).

In the study of Lee J and colleagues, GTSE1, together with ATM, CCND2, RBL2, CDKN3 and CCNB1 genes, was identified as differentially expressed in metastatic neuroendocrine tumours (NET) versus the non metastatic ones. In particular, the expression of ATM protein has been reported as down-regulated in more than 80% of metastatic NET and its mRNA expression levels negatively associate with GTSE1, CDKN3 and CCNB1 levels that are, instead, up-regulated in metastatic NET (Lee et al., 2012). Moreover, in head and neck squamous cell carcinomas it has been demonstrated that GTSE1 is differently expressed in patients with lymph-nodes metastasis versus individuals metastasis-free (Zhou et al., 2006). All together these data underline the strong correlation existing between misregulation of GTSE1, tumor invasiveness and bad prognosis. For this reason the goals of the work presented in this thesis were to clarify how GTSE1 is regulated, in particular in TNBC, the pathways involved and the mechanism underlying GTSE1-dependent cell functions.

Taking advantage of a multidisciplinary approach, we unveiled a key role for the TEAD4 and E2F1 transcription factors (TFs) in the regulation of GTSE1 expression in TNBC. The TEAD family TFs are the main partners of the YAP and TAZ transcriptional co-activators in the control of epithelial to mesenchymal transition, cancer cells migration, invasion, tumour progression and metastasis (Lamar et al., 2012; Wang et al., 2014; Zhang et al., 2009). YAP (Yes-associated protein) and its homolog

TAZ (also known as WWTR1, WW domain-containing transcription regulator protein1) are the downstream effectors of the Hippo signaling transduction pathway, a tumour suppressor pathway frequently deregulated in cancers. Here, I demonstrated that the YAP/TAZ-TEAD4 axis plays a pivotal role in the transcriptional regulation of GTSE1, showing that the effect of TEAD on cell migration is, at least partially, GTSE1-dependent. Moreover, I showed that TEAD regulates the formation of cell protrusions necessary for cancer cells migration through GTSE1, providing, for the first time, a mechanistic explanation of how it affects cell migration.

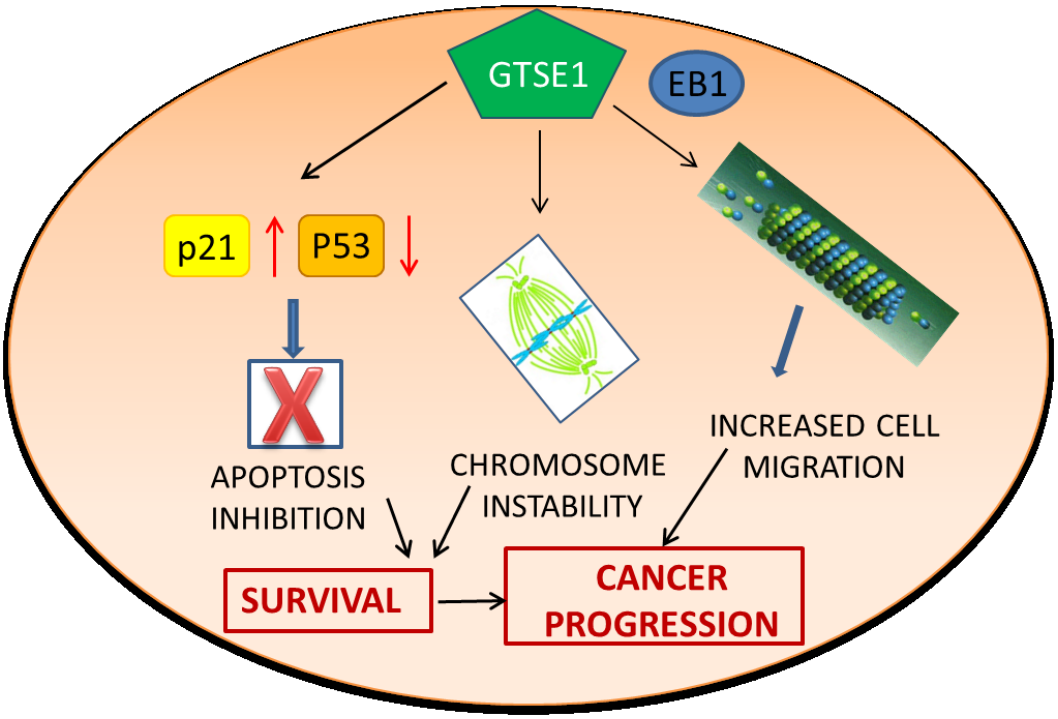


Fig. B Cartoon summarizing the role of GTSE1 protein in cancer

The Family of TEAD Transcription Factors

The TEAD transcription factors were discovered in 1987 by Xiao et al. as nuclear proteins able to bind the B1 domain in the SV40 enhancer and to activate transcription in a cell-specific way (Xiao et al., 1987). In mammals, this family of transcription factors includes four highly conserved proteins, named TEAD1-4, that share a common N-terminal TEA DNA binding domain and a C-terminal immunoglobulin-like ß-sandwich fold transactivation domain (Tian et al., 2010).

Anbanandam et al. described for the first time the structure of TEAD1 as a folded globular protein composed by three α-helices H1,H2,H3, the last of which is the DNA-Recognition helix (Anbanandam et al., 2006). The N-terminal domain recognizes and bind the sequence 5'-GGAATG-3' presents in the SV40 enhancer and in the promoter regions of target genes.

The TEAD TFs lack of a real transcription activation domain and require the interaction with co-activators to promote the transcription of target genes (Xiao et al., 1991). In fact, the co-activators, generally, present an activation domain that allow them to interact with the basal transcription and chromatin remodeling machineries controlling transcription (Pobbati and Hong, 2013). Their interaction with the TEAD TFs is mediated by the TEAD transactivation domain. TEAD-interacting co-activators comprise YAP and TAZ, p160s and vestigial proteins (Vgll) (Pobbati and Hong, 2013).

The co-activators YAP, TAZ and p160s present a TEAD-binding motif located in their N-terminal domain, instead the vestigial proteins interact with TEAD through the Vestigial motif (Vg) whose localization is different in the various isoforms (Pobbati and Hong, 2013).

In mammals it has been reported that about 75% of purified TEAD2 is associated with YAP (Vassilev et al., 2001). In fact, TEAD TFs represent the main platform through which YAP/TAZ interact with DNA at genome-wide level (Zanconato et al., 2015) and are essential for YAP/TAZ-dependent gene expression (Ota and Sasaki, 2008; Zanconato et al., 2015; Zhao et al., 2008).

Li et al. described, for the first time, the three-dimensional structure of the TEAD1-YAP2 complex, showing that YAP2 is wrapped around the globular structure of TEAD

leading to the formation of three highly conserved interfaces, one of which essential for the formation of the complex (Li et al., 2010). Mutations regarding the residues S94 of YAP or Y406 of TEAD1 abolish YAP/TEAD interaction and suppress YAP-mediated gene expression (Zhao et al., 2008).

The role of TEAD TFs in physiological processes

TEAD TFs are expressed in almost all tissue, although they differ for the expression pattern. Some functions of TEAD TFs have been deduced from gene inactivation studies carried out in mice. In particular, TEAD1 has been reported as essential for the differentiation of the cardiac muscle and its null mutation is lethal in embryos (Chen et al., 1994), instead in TEAD4 null embryos the development of the trophoectoderm is impaired and they fail to implant (Yagi et al., 2007). It has been demonstrated that TEAD TFs have a key role in development and differentiation, in fact they are necessary for cardiogenesis, myogenesis and notochord and neural crest development (Pobbati and Hong, 2013). Furthermore, TEAD1 is required for the transcription of E6 and E7 oncogenes of HPV-16 in human keratinocytes (Ishiji et al., 1992).
In the last decade the interest in the study of the TEAD family TFs is notably increased due to multiple evidences that involve these TFs in tumorigenesis and cancer progression.

TEAD TFs and Cancer

It has been reported that TEAD TFs are up-regulated in different types of tumours including breast cancer, gastric cancer, colorectal cancer and prostate cancer in which they are considered prognostic markers (Zhou et al., 2016).
TEAD TFs together with YAP/TAZ transcriptional co-activators regulate the expression of multiple genes involved in tumorigenesis such as survivin, Cyr61, CTGF, Myc and Axl receptor tyrosine kinase (Dong et al., 2007; Schutte et al., 2014; Xu et al., 2011; Zhao et al., 2008).

As mentioned above, YAP and its paralog TAZ are known to be the down-stream effectors of the Hippo tumour suppressor pathway (Hong and Guan, 2012). First discovered in the fruit fly *Drosophila melanogaster*, this signalling pathway has a crucial role in cell proliferation and organ size control (Huang et al., 2005; Justice et al., 1995; Wu et al., 2003; Xu et al., 1995). The first evidence in this direction appeared with the work of Dong et al. showing that YAP overexpression caused an increase in mouse liver size. This effect was reversible, in fact, the liver returned to normal size when YAP overexpression was turned off (Dong et al., 2007). The Hippo pathway exerts its regulatory role also in other organs such as heart, where its inactivation promoting the proliferation of cardiomyocytes causes cardiomegaly, and central nervous system in which it controls the expansion of neural progenitors (Cao et al., 2008; Heallen et al., 2011).

Conserved in mammals, this pathway has a tumour suppressor function (Harvey et al., 2013; Zhao et al., 2010). In mammals, the core of the Hippo pathway is composed by the kinases Mst1/2 and LATS1/2 and by the two adaptor proteins Sav1 and MOB (Zhao et al., 2010). When this pathway is activated, the kinases Mst1/2 interact with Sav1, a scaffold protein, forming a complex that facilitates the phosphorylation of LATS1/2 kinases. The latter are further activated by the adaptor protein MOB and in turn phosphorylate the terminal effectors YAP and TAZ (Hao et al., 2008; Lei et al., 2008; Zhao et al., 2007). The phosphorylated co-activators are sequestered into the cytoplasm and, consequently, the transcription of their target genes is inhibited (Dong et al., 2007; Zhao et al., 2007). When this pathway is not activated, YAP/TAZ co-activators are dephosphorylated and can shuttle from the cytoplasm into the nucleus promoting the expression of target genes, see fig. C.

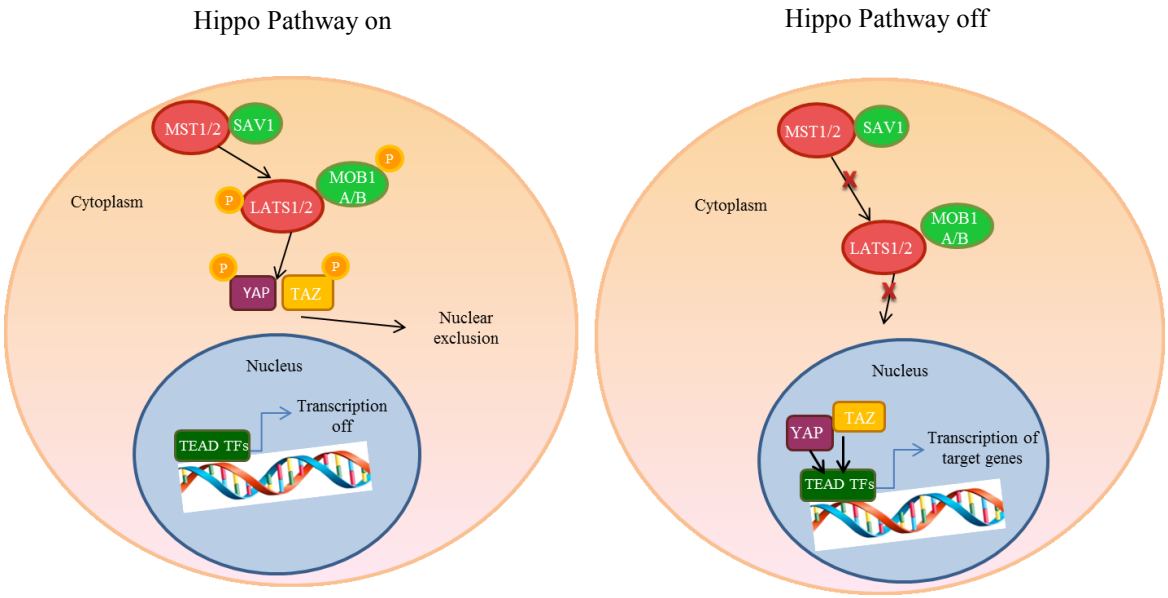


Fig. C Cartoon summarizing the YAP/TAZ regulation by the Hippo Pathway

Evolutionary study revealed that an advanced Hippo pathway, structured as seen in human, appeared concurrently to the emergence of an organized body plan with different tissues and organs in basal metazoans (Hilman and Gat, 2011). The first YAP progenitor was identified in the placozoan *Trichoplax adhaerens*, unlike its paralog TAZ appeared only in vertebrates (Hilman and Gat, 2011). The evolutionary distances of YAP and TEAD orthologs revealed that their genes have coevolved and the residues required for their interaction are evolutionary conserved (Hilman and Gat, 2011), further highlighting the importance of the YAP/TEAD interaction .

As TEAD TFs, YAP and TAZ co-activators are frequently up-regulated in cancers and are considered *bona fide* oncogenes. In mice, sustained YAP over-expression leads to increased liver size and hepatocellular carcinoma (Dong et al., 2007). Also in ovarian cancer the expression levels of YAP and TAZ are up-regulated and correlate with poor prognosis (Chen et al., 2016; Zhang et al., 2011). In breast cancer and melanoma the interaction between YAP and TEAD is required to promote cell proliferation, transformation, migration and invasion, furthermore the enhanced transcriptional activity of TEAD TFs correlates with metastatic potential (Lamar et al., 2012). In addition, it has been demonstrated that RHAMM, a YAP/TEAD4 target gene, regulates breast cancer cell migration in a ERK-dependent manner (Wang et al., 2014).

Enhanced stiffness of extracellular matrix (ECM) is an hallmark of many solid tumours including breast cancer (Paszek et al., 2005). It has been demonstrated that a rigid ECM

promotes the YAP/TAZ nuclear localization and activity. These co-activators are considered not only the "molecular readers" of matrix elasticity, but essential mediators of biological responses to these mechanical cues (Dupont et al., 2011). Moreover, it has been demonstrated that YAP is activated in cancer associated fibroblasts (CAFs) and itself is required to promote CAFs-mediated matrix stiffening, cancer cells invasion and angiogenesis (Calvo et al., 2013). In breast cancer, tumour stiffness correlates with histological grade, with higher stiffness values in the most aggressive subtypes (Chang et al., 2013). In line with this, YAP/TAZ activity is increased in high histological grade breast tumours and it is associated with shorter survival time, enhanced probability to develop metastasis and enrichment in stem cell signature (Cordenonsi et al., 2011).

It is believed that cancer stem cells (CSCs) drive tumour initiation and progression. In fact, the CSCs population is enriched in poorly differentiated tumours than well-differentiated ones supporting this hypothesis (Pece et al., 2010). It has been demonstrated that TAZ expression levels are elevated in CSCs and it is necessary to sustain self-renewal and tumour-initiation abilities in this cell population (Cordenonsi et al., 2011). Moreover, TAZ has been reported as up-regulated in invasive infiltrating ductal breast carcinomas where it is required for breast cancer cell migration, invasion and tumorigenesis. Its over-expression induces the fibroblast-like morphology in MCF10A, an hallmark of cell transformation (Chan et al., 2008). It has been demonstrated that TEAD TFs control the nuclear accumulation of TAZ and its transforming capability, moreover TEAD4 itself is able to induce cell transformation with the same efficiency of TAZ, suggesting that this TF could be considered an oncogene (Chan et al., 2009).

Furthermore, TEAD4, together with KLF, promotes cell proliferation and tumour growth and it has been identified as a prognostic marker and a novel potential therapeutic target in TNBC (Wang et al., 2015). Recently, it has been reported that TEAD4 cooperates with AP1 to carry out a transcriptional program required to promote migration and invasion in cancer (Liu et al., 2016).

91% of YAP/TAZ bound cys-regulatory regions are represented by enhancer elements (Zanconato et al., 2015). The majority of these regulatory elements harbours both the TEAD and AP1 binding sites, suggesting the formation of a transcription factors complex for the promotion of the transcription of target genes (Zanconato et al., 2015).

In fact, the gain of AP1 strongly increases the YAP/TAZ/TEAD mediated gene expression and the YAP-dependent oncogenic growth (Zanconato et al., 2015).
It has been demonstrated that about 67% of the promoters of YAP/TAZ target genes involved in cell proliferation harbours the E2F TFs binding sites and both the YAP/TAZ-TEAD and Rb-E2F1 pathways are required to promote the progression of the cell cycle from G1 to S phase (Zanconato et al., 2015). Zanconato et al. have suggested an interesting model according to YAP/TAZ are recruited on distant enhancers through TEAD and cooperate with E2F TFs bound to the promoters via chromatin looping (Zanconato et al., 2015).
It has been reported that Yki, Scalloped and dE2F1, the homologues of YAP, TEAD and E2F1 in fruit fly respectively, act synergically to induce the activation of a transcriptional program necessary to bypass cell cycle exit and to promote cell proliferation in *Drosophila* (Nicolay et al., 2011). Moreover, in pancreatic ductal adenocarcinoma the YAP1/TEAD2 complex cooperates with E2F1 in the execution of a transcriptional program inducing KRAS-independent tumour relapse (Kapoor et al., 2014), further underlining the importance of a crosstalk between these two pathways.

E2F transcription factors family

The E2F family of transcription factors comprises eight proteins (E2F1-E2F8) playing a key role in processes like cell cycle progression, DNA repair, apoptosis, chromosome stability and development (Chen et al., 2011; Kel et al., 2001; Lazzerini Denchi and Helin, 2005; Li et al., 2003; Luo et al., 2016).
According to their functional proprieties and structure, E2F TFs are divided into three groups: transcriptional activators (E2F1, E2F2, E2F3a), repressors (E2F3b, E2F4, E2F5) and inhibitors (E2F6, E2F7, E2F8). The transcriptional activators control the transcription of target genes essential for the cell cycle progression from G1 to S phase. The E2F TFs repressors accumulate into the nucleus during the G0/G1 transition and induce cell cycle exit and differentiation (Attwooll et al., 2004). E2F6 turns off the transcription of E2F targets during the G1/S phase transition (Bertoli et al., 2013), instead, E2F7/E2F8 inhibit the expression of E2F target genes during the S and G2

phases and are also involved in the repression of cell cycle related genes during DNA damage (Westendorp et al., 2012). Although the E2F TFs inhibitors and repressors play similar functions, their transcriptional activity is regulated in a different way. In fact, with the exception of the E2F TFs inhibitors, all members of the E2F TFs family interact with the pocket proteins family that controls their functions (Zhan et al., 2014). The pocket proteins family includes: the retinoblastoma protein (Rb), p107 and p130. All the pocket proteins family members share a conserved central domain, called pocket domain, required for the interaction with different cellular proteins. It has been reported that the E2F1-E2F3 TFs interact mainly with Rb, E2F4 binds all members of the pocket proteins family and E2F5 associates mainly with p130 (Zhan et al., 2016). In general, the binding of pocket proteins leads to the inhibition of the E2F TFs functions. The cyclin-dependent kinases (CDKs) can regulate this interaction through the hyperphosphorylation of pocket proteins that leads to the E2F TFs release (Zhan et al., 2016).

Moreover, the E2F TFs can be classified in typical (E2F1-E2F6) and atypical (E2F7-E2F8) on the basis of their DNA-binding domain. Typical E2F TFs require the interaction with the dimerization partner proteins (DP) to form a functional DNA-binding domain, instead in atypical members the DNA-binding domain is duplicated and this allow them to bind DNA in a DP-independent manner (Zhan et al., 2014).

E2F1 is the most studied and founding member of this TFs family. The name of this TF derives from its ability to bind the E2 promoter of adenovirus and to act as transcriptional activator (Yee et al., 1987). Its sequence consists of 437 amino acids organized in a N-terminal DNA-binding domain, a dimerization partner binding domain including a leucine zipper (LZ) and a marked box domain (MB) and a C-terminal transactivation domain. The latter contains a pocket protein Rb binding domain. Moreover, the N-terminal region of E2F1 includes the nuclear localization signals necessary for its nuclear accumulation (Zhan et al., 2016).

E2F1 is a crucial regulator of cell cycle. This transcription factor modulates the progression of cell cycle from G1 to S phase through the control of the transcription of its target genes and, for this reason, its expression and activity must be tightly regulated (Ishida et al., 2001; Kel et al., 2001; Muller et al., 2001; Wells et al., 2000). In fact, the deregulation of CDKs/Rb/E2F1 network may result in tumourigenesis. In the last

decade the number of evidence that correlates E2F1 misregulation to tumour progression is notably increased. In fact, it has been reported that E2F1 up-regulation promotes cell proliferation and correlates with poor prognosis in non-small lung cell carcinoma, esophageal squamous cell carcinoma and ovarian cancer (Ebihara et al., 2004; Huang et al., 2007; Zhan et al., 2016). The role of E2F1 in hepatocellular carcinoma is still controversial, in fact it acts as pro-apoptotic or anti-apoptotic factor in different stages of this disease (Zhan et al., 2014). Breast cancer patients with low expression of E2F1 have better clinical outcome (Vuaroqueaux et al., 2007). Moreover, in patients with lymph node positive breast cancer treated with doxorubicin, fluorouracil or cyclophosphamide E2F1 overexpression is associated to poor prognosis (Han et al., 2003).

It has been demonstrated that in melanoma the eugenol, the main component of the oil of clove, inhibits E2F1-dependent cell proliferation (Ghosh et al., 2005) and in breast cancer the eugenol-dependent anti-cancer effect is at least partially E2F1-mediated, suggesting the potential use of E2F1 as therapeutic target (Al-Sharif et al., 2013). Furthermore, in different types of cancer E2F1 expression correlates with metastatic potential. In melanoma cell lines E2F1 down-regulation reduces their invasive potential (Alla et al., 2010). Instead, RHAMM (hyaluronan-mediated motility receptor), a direct E2F1 target gene, acts as co-activator of E2F1 itself and, together with it, stimulates the expression of fibronectin required to promote the cell migration through the endothelial layer (Meier et al., 2014).

The main regulator of E2F1 activity is the retinoblastoma protein (Martelli and Livingston, 1999). Rb acts as transcriptional modulator binding E2F family members and recruiting co-repressors to regulate the transcription of target genes (Bosco and Knudsen, 2007). It is a central regulator of cell cycle progression. In early G1 phase, Rb is hypophosphorylated and by binding to E2F1 inhibits the progression of the cell cycle. In late G1, Rb is sequentially phosphorylated by cyclin D-CDK4/6 and cyclin E-CDK2 complexes, these events lead to E2F1 release and cell cycle progression (Martinsson et al., 2005). Loss of RB expression has been reported in 20-30% of all breast cancers (Bosco and Knudsen, 2007) and its somatic mutations promote the development of different types of tumour including lung carcinoma, osteosarcoma and bladder carcinoma (Hickman et al., 2002). Moreover, cyclin D1 amplification, commonly

reported in cancers, promoting Rb aberrant phosphorylation can further contribute to its negative regulation (Takano et al., 1999; Watts et al., 1995). Currently inactivation of Rb pathway is considered an obligatory step in cancer development (Hanahan and Weinberg, 2000). In fact, Rb-E2F1 axis controls the expression of about 150 genes involved in processes associated with tumour suppression, for this reason the deregulation of this pathway has a critical role in cancer progression (Bosco and Knudsen, 2007).

Aim of the Thesis

In the last years the number of evidence supporting a strong connection between GTSE1 deregulation and cancer progression is notably increased, despite this the GTSE1 transcriptional regulation and the cause of its misregulation are poorly understood. For these reasons the aims of the work presented in this thesis were:

- To unveil the pathways responsible for the control of GTSE1 expression;
- To identify the transcription factors and the co-activators involved;
- To clarify the mechanism underlying GTSE1-dependent cell movement in triple negative breast cancer.

Results

Regulation of GTSE1 expression by the YAP/TAZ-TEAD axis

The first objective of this work was to elucidate how the GTSE1 protein was regulated at the transcriptional level and the transcription factors involved. Hence, taking advantage of published TCGA Breast cancer gene expression data, we performed a bioinformatics analysis in order to identify the transcription factors (TFs) co-expressed with GTSE1. The list of 36 TFs that we obtained should comprise, among others, those that are able to modulate GTSE1 expression. In order to obtain the best candidates to the role of regulators of GTSE1 transcription we overlapped these results with the output generated by the TRANSFAC/Matchtool (Matys et al., 2006), listing the TFs exhibiting at least one binding site (TFBS) in the genomic region corresponding to the GTSE1 promoter. Interestingly, the outcome showed a very short TFs list including TEAD, E2F1 and the chromatin modifier HMGA1(Fig.1a).

Initially, I decided to focus my attention on the regulation of GTSE1 expression by the TEAD family of TFs. We mapped the TEAD consensus binding sequence ("GGAATG") on the GTSE1 promoter, finding that it harbored two putative binding sites located 1162 and 1097bp upstream from the transcription start site (TSS) (Fig.1b).

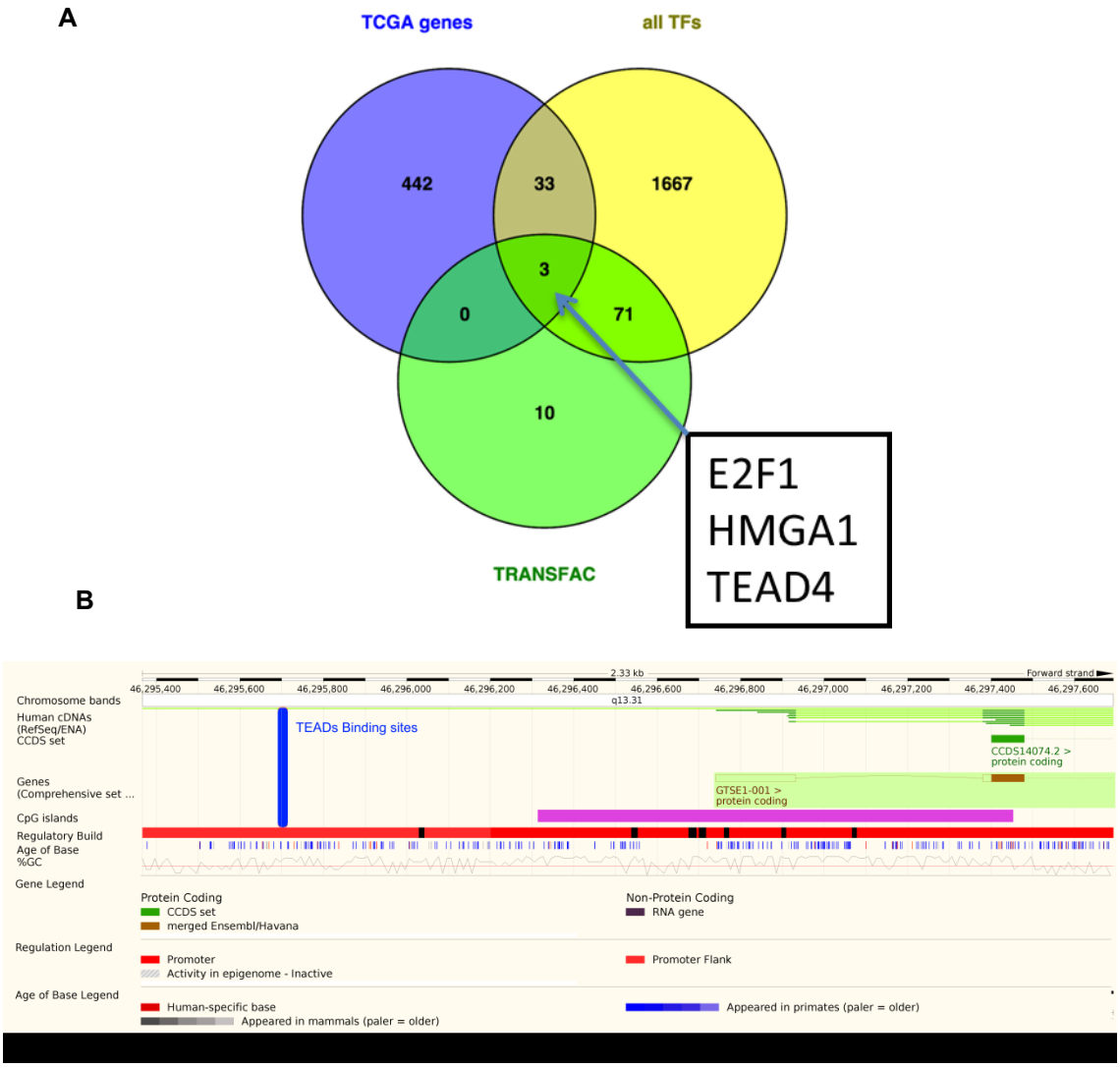


Fig.1 TEAD, E2F1 and HMGA1 transcription factors are the putative regulators of GTSE1 expression. (A) Comparison of the TCGA GTSE1 co-expressed genes, Transfac and "all TFs" predicted transcription factors regulating GTSE1 expression. (B) Mapping of the TEAD4 binding sites in the GTSE1 promoter region. Graphical representation of GTSE1 promoter region from Ensemble database (modified). In particular tracks regarding the conserved regions, GC content, CDSs information and gene structure are shown. The position of TEADs binding sites are shown ; please note that in order to make the TFBS representation clear the corresponding box width is increased.

This strengthened the possibility for the TEAD family of transcription factors to play a role in the regulation of GTSE1 expression. To test this hypothesis, I evaluated the effect of $TEAD_{1/3/4}$ knock-down on GTSE1 expression levels. To do this, I performed

$TEAD_{1/3/4}$ silencing by siRNA in MDA-MB-231 cell line and assessed GTSE1 protein levels after 72 hours. Under these conditions, GTSE1 expression levels dramatically decreased confirming my previous assumption (Fig.2a).

As mentioned above, all members of the TEAD family contain a DNA-binding and a transactivation domain but lack a real transcriptional activation domain, present instead in the structure of the YAP and TAZ transcriptional co-activators. For this reason, the interaction of TEAD with these co-activators is required to promote the transcription of target genes. In line with this, I decided to assess the effect of YAP/TAZ silencing on GTSE1 protein levels. As shown in Fig.2a, GTSE1 expression significantly dropped after the double silencing compared to the control.

To clarify if the YAP/TAZ-TEAD axis exerted its regulation on GTSE1 at the transcriptional level, I silenced $TEAD_{1/3/4}$ and YAP/TAZ by siRNA in the MDA-MB-231 cell line and evaluated GTSE1 mRNA levels after 72 hours.

As shown in picture Fig.2b, GTSE1 mRNA expression levels dramatically diminished in cells silenced for $TEAD_{1/3/4}$ and YAP/TAZ. As positive controls, I measured the mRNA levels of the known YAP/TAZ-TEAD target genes BIRC5, RHAMM and CTGF. The knock-down of YAP/TAZ and TEAD significantly decreased the mRNA levels of all these target genes.

To evaluate if other TNBC cell lines shared the regulation of GTSE1 expression by the YAP/TAZ-TEAD axis, I carried out $TEAD_{1/3/4}$ and YAP/TAZ silencing in MDA-MB-157 cell line and measured GTSE1 protein (Fig.2c) and mRNA levels (Fig.2d). Under this experimental setting, GTSE1 expression levels significantly decreased in silenced cells compared to the control ones confirming the results achieved in MDA-MB-231 cell line. The obtained results suggest that the YAP and TAZ transcriptional co-activators regulate GTSE1 expression at the transcriptional level through their interaction with the TEAD family of transcription factors.

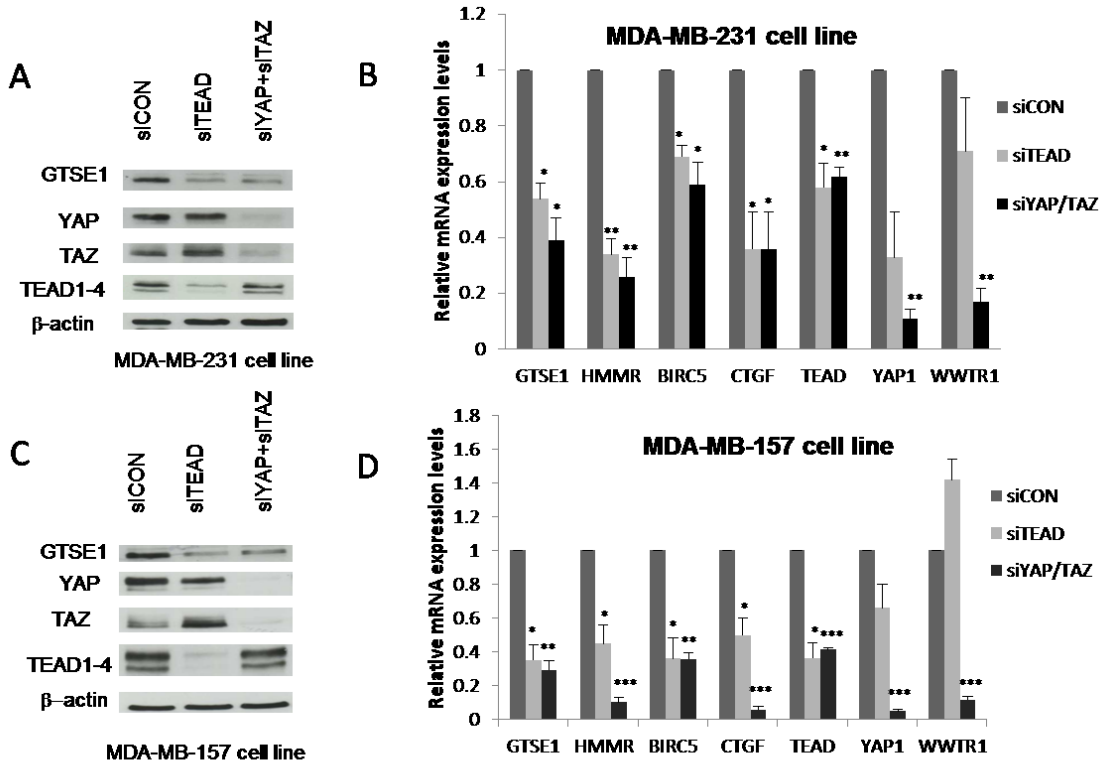


Fig.2 The YAP and TAZ transcriptional co-activators promote GTSE1 transcription through the TEAD family of transcription factors.

GTSE1 protein and mRNA levels decrease after knockdown of TEAD1/3/4 and YAP/TAZ in MDA-MB-231 (A and B) and MDA-MB-157 (C and D) cell lines. Data are shown as mean ± SEM of at least three independent experiments. For the statistical analysis, Student two-tailed T-test was applied. *p-value<0.05; **p-value<0.01; *** p-value<0.001.

GTSE1 is a novel direct target gene of TEAD4 Transcription Factor

As mentioned above, the GTSE1 promoter region harbors two possible TEAD binding sites, named S1 and S2, respectively located 1162 and 1097bp upstream from the transcription start site (Fig.3a). Among the different members of the TEAD family of TFs, TEAD4 has been previously shown up-regulated in breast cancer cell lines, in particular in TNBC, and able to control the expression of genes involved in breast cancer cell migration and invasion like RHAMM (Wang et al., 2015; Wang et al., 2014). For these reasons we decided to perform a ChIP assay to verify its modulatory potential on GTSE1 transcriptional levels, assessing if TEAD4 physically interacted

with GTSE1 promoter and hence identifying its real binding site in the analyzed genomic region. As shown in Fig.3b, TEAD4 occupies both the S1 and S2 binding sites on the GTSE1 promoter region, physically interacting with it. These results demonstrate that GTSE1 is a target gene of TEAD4 that directly promotes GTSE1 transcription through the binding to the GTSE1 promoter region.

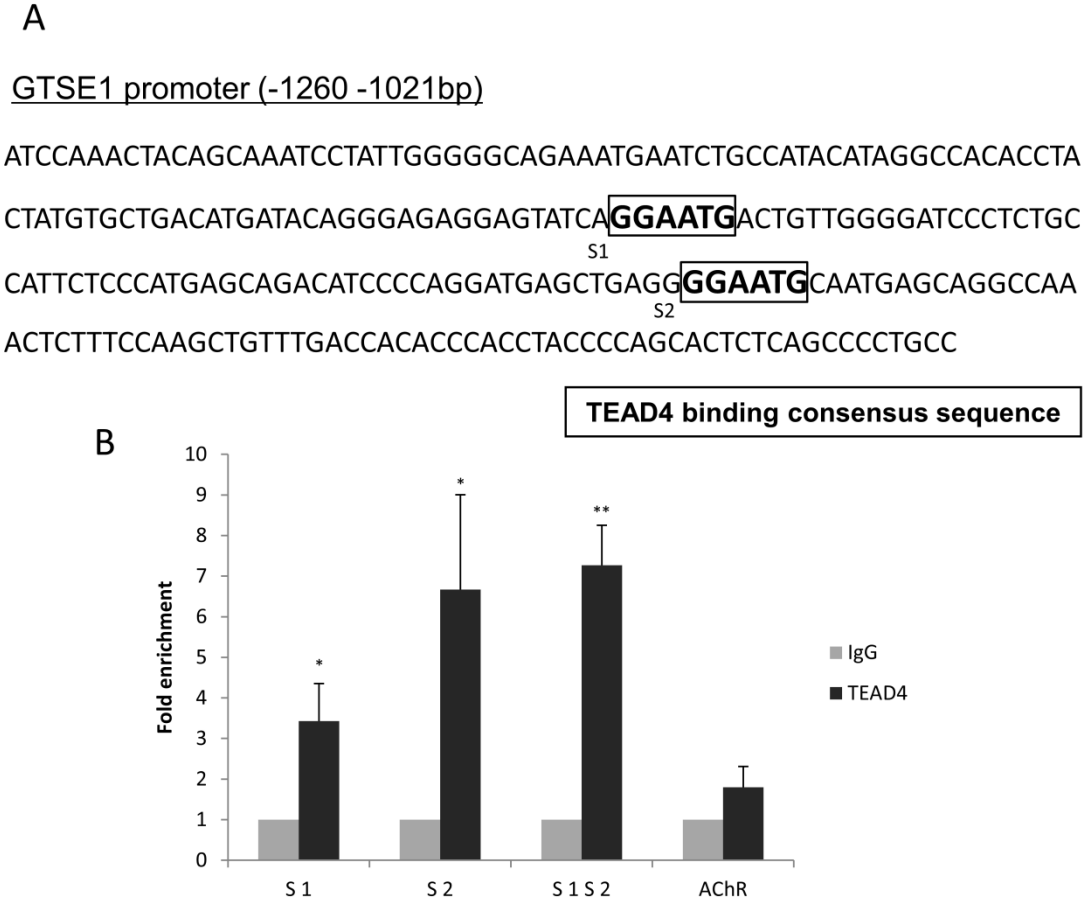


Fig.3 TEAD4 physically interacts with the GTSE1 promoter region.

(A) Position of the TEAD4 consensus binding sequences in the GTSE1 promoter. (B) IgG (control) or TEAD4 antibodies were used in ChIP assays of MDA-MB-231 cell line. The human muscarinic receptor gene was used as negative control (AChR). Data are shown as mean ± SEM of three independent replicates. For statistical analysis Student's t-test was applied. *p-value<0.05; **p-value<0.01.

GTSE1 expression is regulated by the Mevalonate Pathway

Previous studies showed that breast cancer cell migration is inhibited by statins (Mandal et al., 2011), molecules frequently used to treat hypercholesterolemia because of their ability to block the mevalonate pathway and cholesterol biosynthesis. Cerivastatin, a molecule belonging to the statins family, is an inhibitor of the HMG-CoA reductase, an enzyme that catalyzes the synthesis of mevalonate from HMGCoA. The mevalonate pathway regulates YAP/TAZ localization and activity (Sorrentino et al., 2014; Wang et al., 2014). In fact, the inhibition of this pathway promotes YAP/TAZ cytoplasmic retention and in this way stops the transcription of YAP/TAZ target genes. Consequently, I decided to test the effect of the inhibition of the mevalonate pathway on GTSE1 expression. MDA-MB-231 and MDA-MB-157 cell lines were treated with cerivastatin and GTSE1 expression levels were assessed at both the mRNA and protein levels. As shown in Fig.4, GTSE1 protein (Fig.4a,b) and mRNA levels (Fig.4 c, d) dramatically decrease in cerivastatin treated cells with respect to control ones. The addition of mevalonate to cerivastatin treated cells, promoting YAP/TAZ nuclear localization and activity, is able to completely rescue the effect of cerivastatin (Fig.4a,b,c, d).

These results indicate that the mevalonate pathway regulates the expression of GTSE1, as shown for other YAP/TAZ targets, further suggesting the involvement of these transcriptional co-activators in the control of GTSE1 transcription.

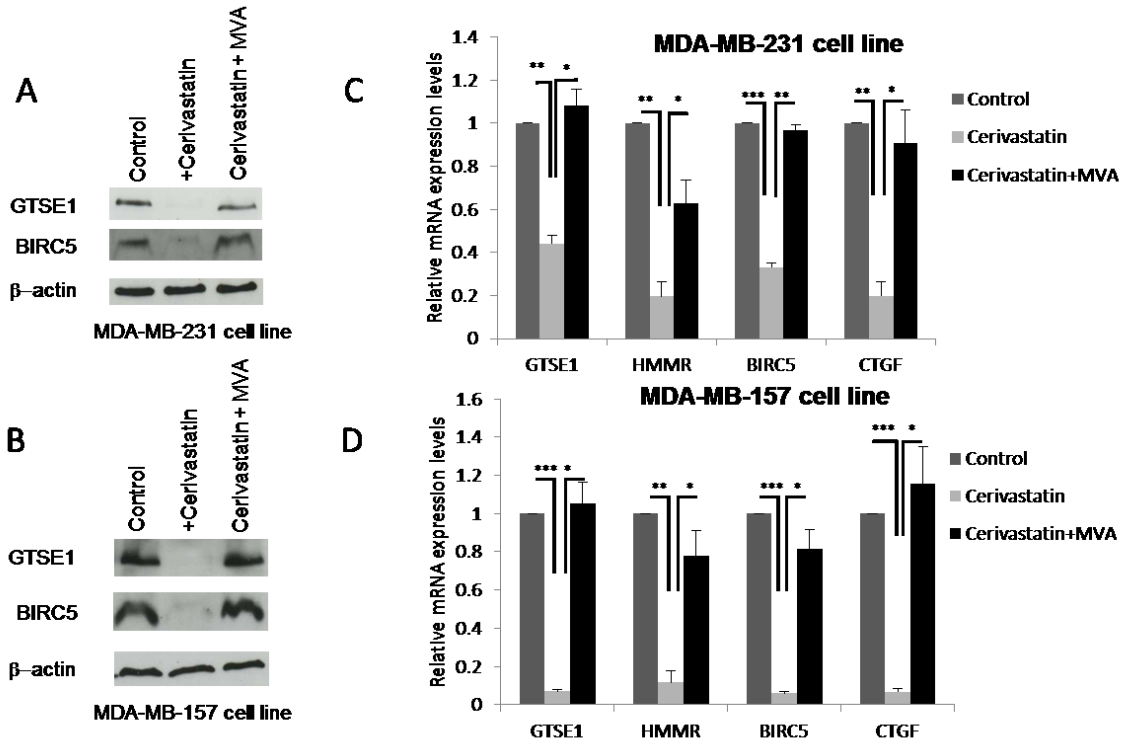


Fig.4 The mevalonate pathway regulates GTSE1 expression.

GTSE1 protein levels after treatment with cerivastatin 1µM alone or in combination with mevalonic acid 0.5mmol (MVA) in MDA-MB-231 for 24 hours(A) and in MDA-MB-157 (B) for 72 hours. (C) and (D) Real-time RT-qPCR analysis of GTSE1 mRNA levels after cerivastatin or cerivastatin and mevalonate treatment. Data are represented as mean ± SEM of three independent replicates. Student two-tailed t-test was applied for the statistical analysis. *p-value<0.05; **p-value<0.01; ***p-value<0.001.

TEAD4 regulates breast cancer cells migration through GTSE1

YAP, TAZ and TEAD4 are well-known regulators of breast cancer cell migration and invasion. In fact, the ability of TNBC cell lines to migrate and to invade decreases after TEAD, YAP and TAZ silencing (Chan et al., 2008; Wang et al., 2014). As previously mentioned, GTSE1 activity is another feature required for breast cancer cells migration. Indeed, the ability of MDA-MB-231 cell line to migrate is reduced after GTSE1 knock-down and GTSE1 up-regulation dramatically increases the migratory capability of the poorly invasive MCF7 breast cancer cell line (Scolz et al., 2012). Consequently, I

decided to investigate whether the effect of TEAD on cell migration and invasion is mediated by GTSE1.

As shown in Fig.5, over-expression of GTSE1 is able to rescue the reduced ability of TEAD-silenced TNBC cell lines to migrate, in wound healing and transwell migration assays (Fig.5a and 5b), and to invade (Fig.5 d), as measured through transwell invasion assay, without any significant effect on cell proliferation in the considered time interval (Fig.6).

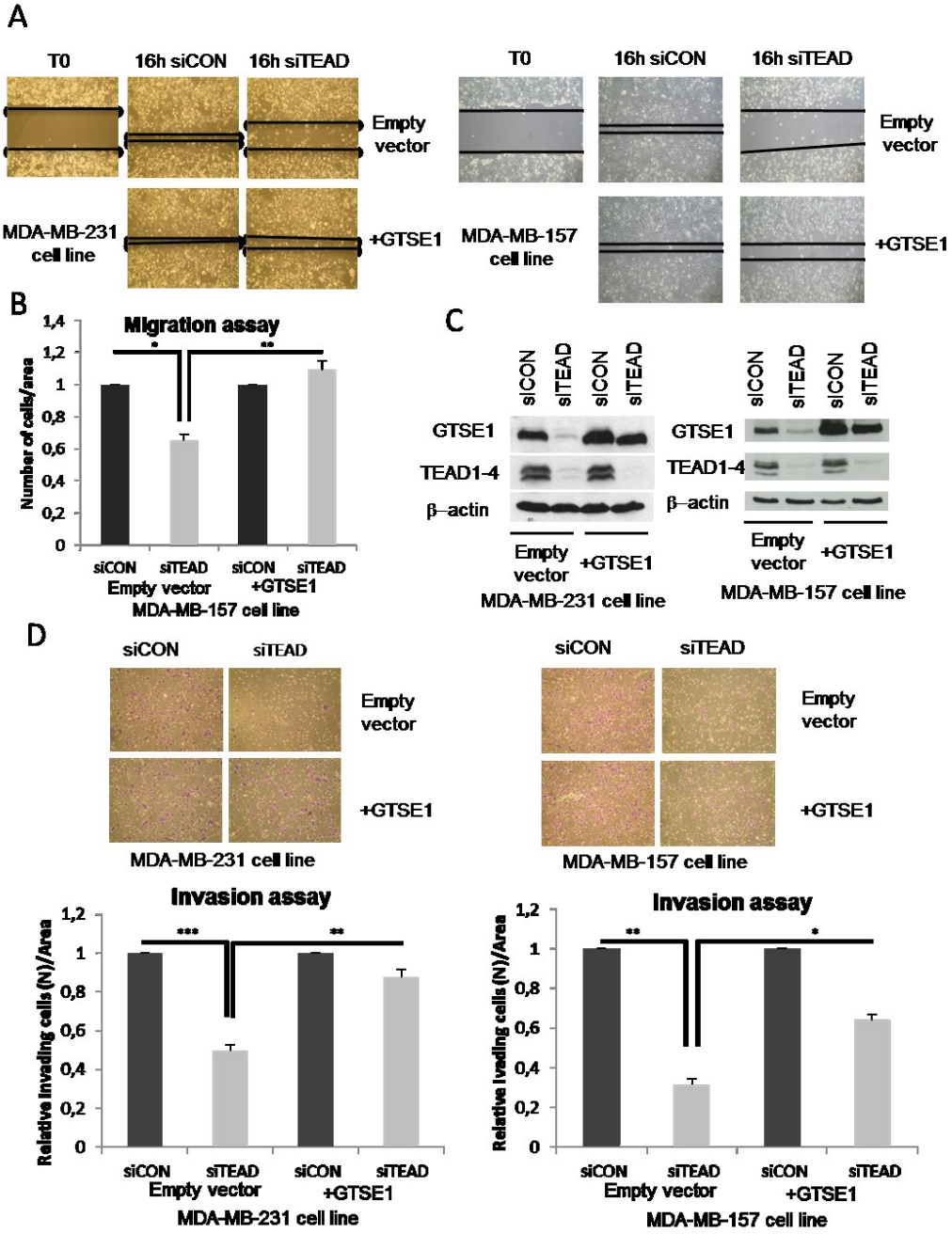


Fig.5 TEAD regulates breast cancer cell migration and invasion through GTSE1.

(A) Wound-healing motility assay showing the capability of GTSE1 to rescue the reduced cell migration followed to TEAD silencing. The scratch assay was carried out in MDA-MB-231 and MDA-MB-157 cell lines containing a stably integrated GTSE1 over-expressing construct (pBABE-GTSE1) or empty vector (pBABE). (B) The transwell migration assay was performed in MDA-MB-157 cell line. Histograms show the mean number of cells/area that migrated through the transwell inserts after 16 h. Error bars represent the standard error of the mean from three independent experiments. Student two-tailed T-test was applied for statistical analysis. *p-value<0.05; **p-value<0.01; ***p-value<0.001. (C) Western blot of the MDA-MB-231 and MDA-MB-157 cell lines containing a stably integrated construct over-expressing GTSE1(pBABE-GTSE1) or empty vector (pBABE). (D) The transwell invasion assay was performed in the same cell lines of wound-healing and tranwell migration assays. Histograms show the mean number of cells/area that invaded through the transwell inserts after 18 h. Error bars represent the standard error of the mean from three independent experiments. Student two-tailed T-test was applied for statistical analysis. *p-value<0.05; **p-value<0.01; ***p-value<0.001.

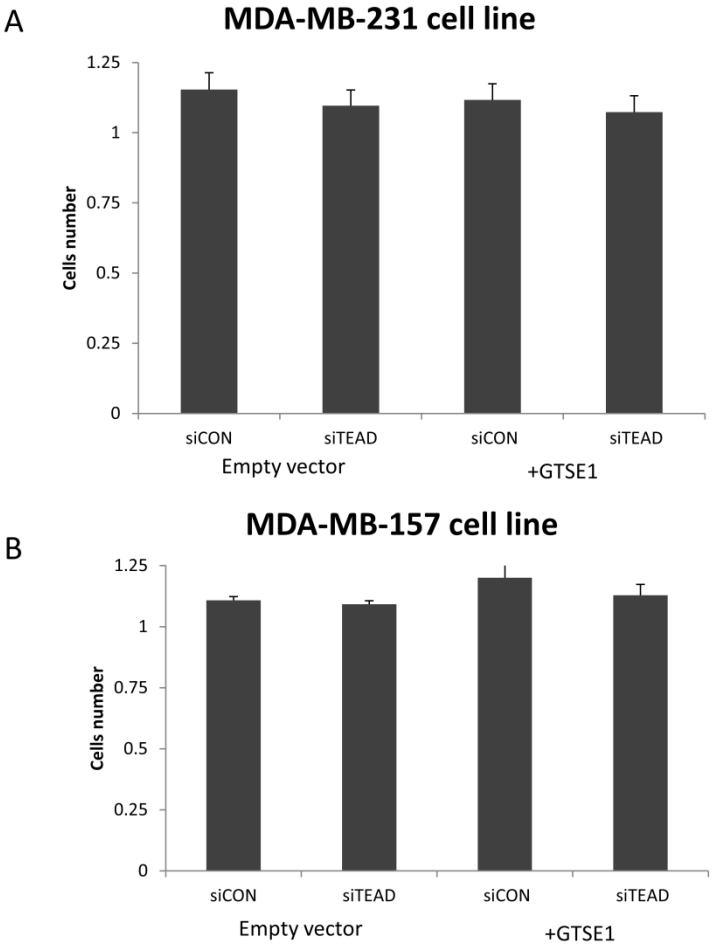


Fig.6 GTSE1 rescues the reduced motility observed after TEAD 1/3/4 silencing without any effect on cell proliferation.

The cell proliferation assays were carried out in MDA-MB-231 (A) and MDA-MB-157 (B) cell lines containing a stably integrated GTSE1 over-expressing construct (pBABE-GTSE1) or empty vector

(pBABE). 48 hours after TEAD1/3/4 silencing, $1X10^5$ cells were plated and counted 16 hours later. Data are shown as mean ± SEM of three independent experiments. For statistical analysis Student two-tailed t-test was applied.

These results indicate that the effect of TEAD on cell migration and invasion is GTSE1-dependent, unveiling a relevant effector role for GTSE1 in TEAD-dependent cellular functions.

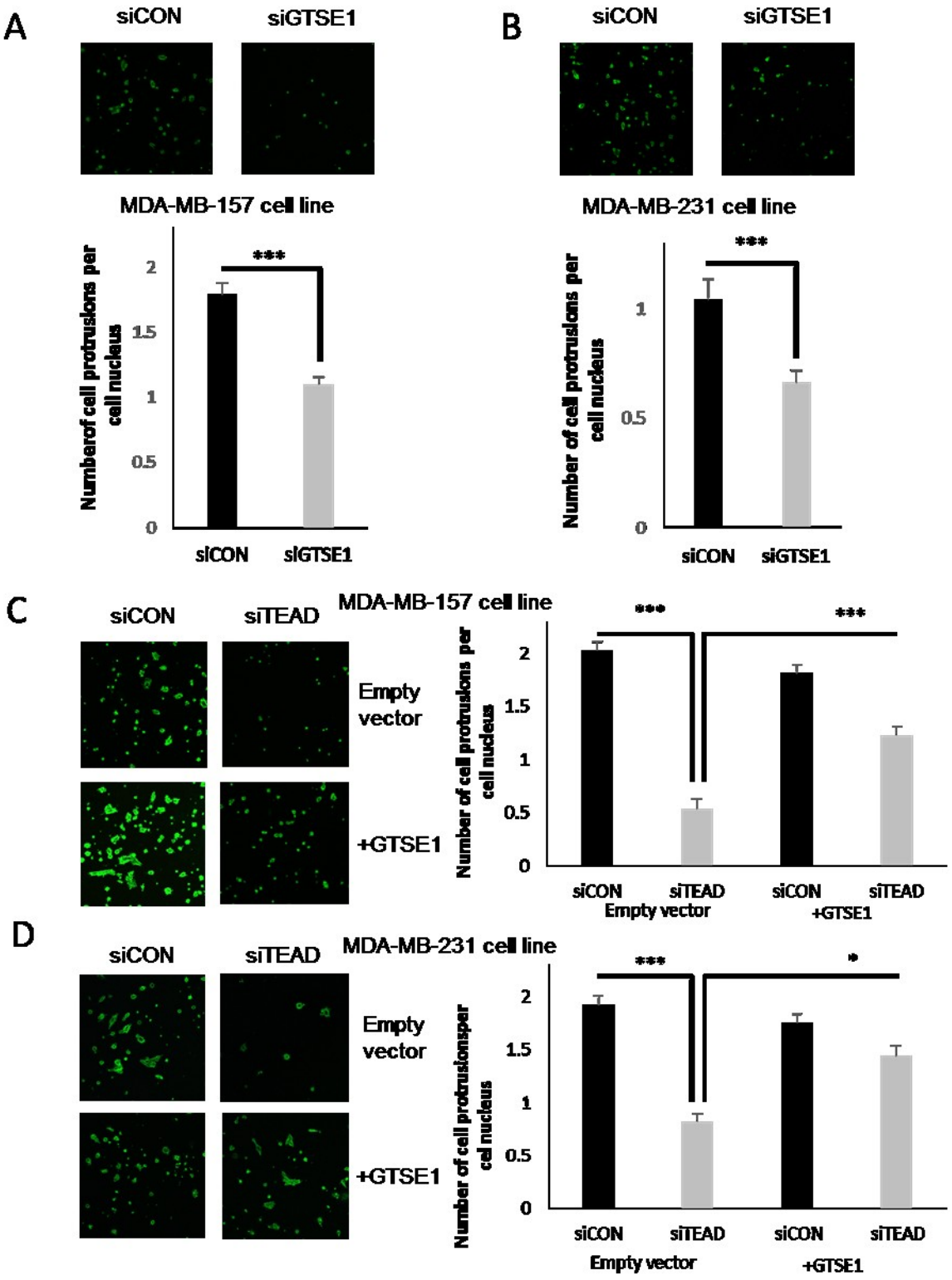
The next step was to elucidate the mechanism by which TEAD controls cell migration through GTSE1.

It is thought that the transition from an epithelial-like to a mesenchymal-like with a higher migratory migratory ability (Thiery, 2002), represents an early step in the development of metastasis. The establishment of a front-back cell polarity is required for cell migration of mesenchymal-like cells (Etienne-Manneville, 2008). The front is characterized by F-actin rich filaments, called cell protrusions, that allow the cell to extend forward to adhere to the substrate, while the rear is retractile and generates the force necessary to push up the cell body in the direction of the movement (Sahai and Marshall, 2003). Cell protrusions are classified on the basis of their shape as: pseudopodia (round), filopodia (needle shape), lobopodia (cylindrical) and lamellipodia (flat veils) (Taylor and Condeelis, 1979). During chemotaxis, fast moving amoeboid cancer cells present F-actin rich pseudopodia that drive them toward blood vessels before intravasation (Condeelis and Segall, 2003; Gligorijevic et al., 2012).

Since cell protrusions represent a common feature of moving cells in tumors, I examined whether GTSE1 controls breast cancer cells migration through the regulation of cell protrusions formation. As shown in Fig.7a and b the knock-down of GTSE1 reduces the number of cell protrusions/cell.

Afterward I evaluated if TEAD regulates the formation of the GTSE1-dependent cell protrusions. As shown in Fig.7c,d and e TEAD silencing impacts negatively on the number of cell protrusions/cell, while GTSE1over-expression is able to rescue this effect.

The obtained results suggest that TEAD controls the formation of cell protrusions through GTSE1, providing, for the first time, a mechanical explanation of how it controls breast cancer cell migration.


A
siCON
siGTSE1
MDA-MB-157 cell line
Numberof cell protrusions per cell nucleus
2
1.5
1
0.5
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siCON
siGTSE1
B
siCON
siGTSE1
MDA-MB-231 cell line
Number of cell protrusions per cell nucleus
1
0.5
0
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siCON
siGTSE1
C
siCON
siTEAD
Empty vector
+GTSE1
MDA-MB-157 cell line
Number of cell protrusions per cell nucleus
2
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1
0.5
0
***
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siCON
siTEAD
siCON
siTEAD
Empty vector
+GTSE1
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siCON
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MDA-MB-231 cell line
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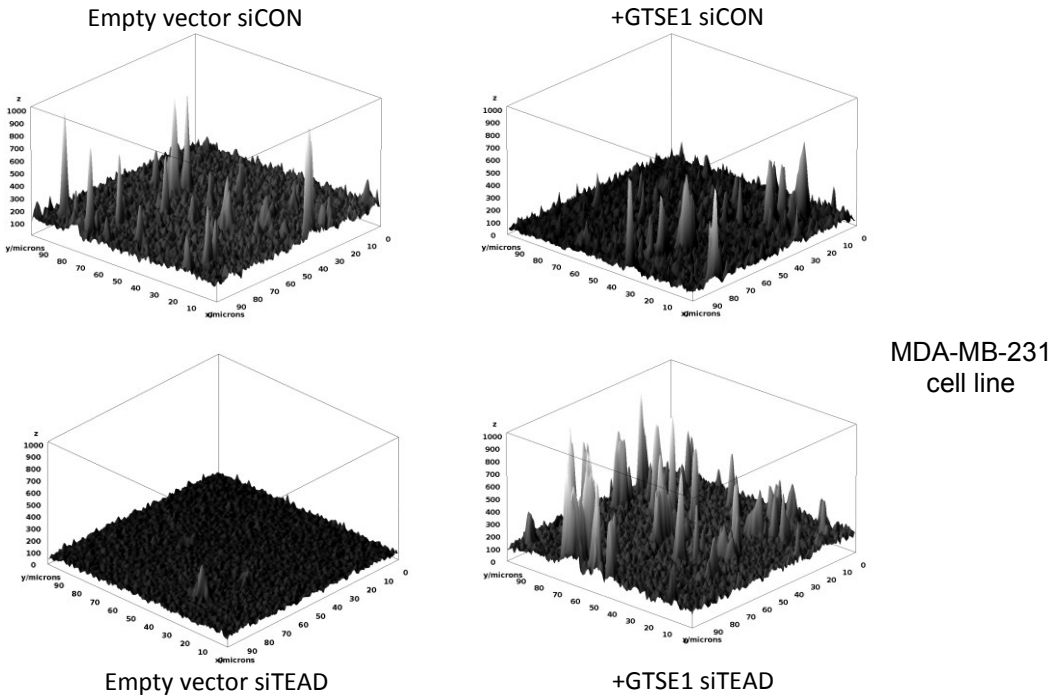

Fig.7 TEAD4 modulates the formation of cell protrusions through GTSE1.

(A)and (B) Histograms represent the number of cell protrusions/cell in TNBC cell lines after control or GTSE1 silencing. (C) and (D) The protrusion assay was performed in MDA-MB-157 and MDA-MB-231 cell lines containing an empty vector (pBABE) or a stably integrated GTSE1 over-expressing construct (pBABE-GTSE1). Histograms show the number of cell protrusions/cell after control or TEAD silencing.Dataare shown as mean ± SEM of three independent experiments. For statistical analysis Student two-tailed T-test was applied. *p-value<0.05; ***p-value<0.001 (E) Three-dimensional representation of the cell protrusions (MDA-MB-231) showing the differences between conditions also in term of cell surface extensions.

The E2F1 transcription factor controls the expression of the GTSE1 protein

As mentioned above, TEAD4 is not the only possible transcription factor regulating GTSE1 expression. In fact, based on the E2F1 consensus binding sequence ("TTTSSCGS", where S = C/G), we identified five possible E2F1 binding sites in the GTSE1 promoter region respectively located 58 (G1), 201 (G2), 344 (G3), 360 (G4) and 389 (G5) nucleotides upstream from GTSE1 transcription start site (Fig.8a).

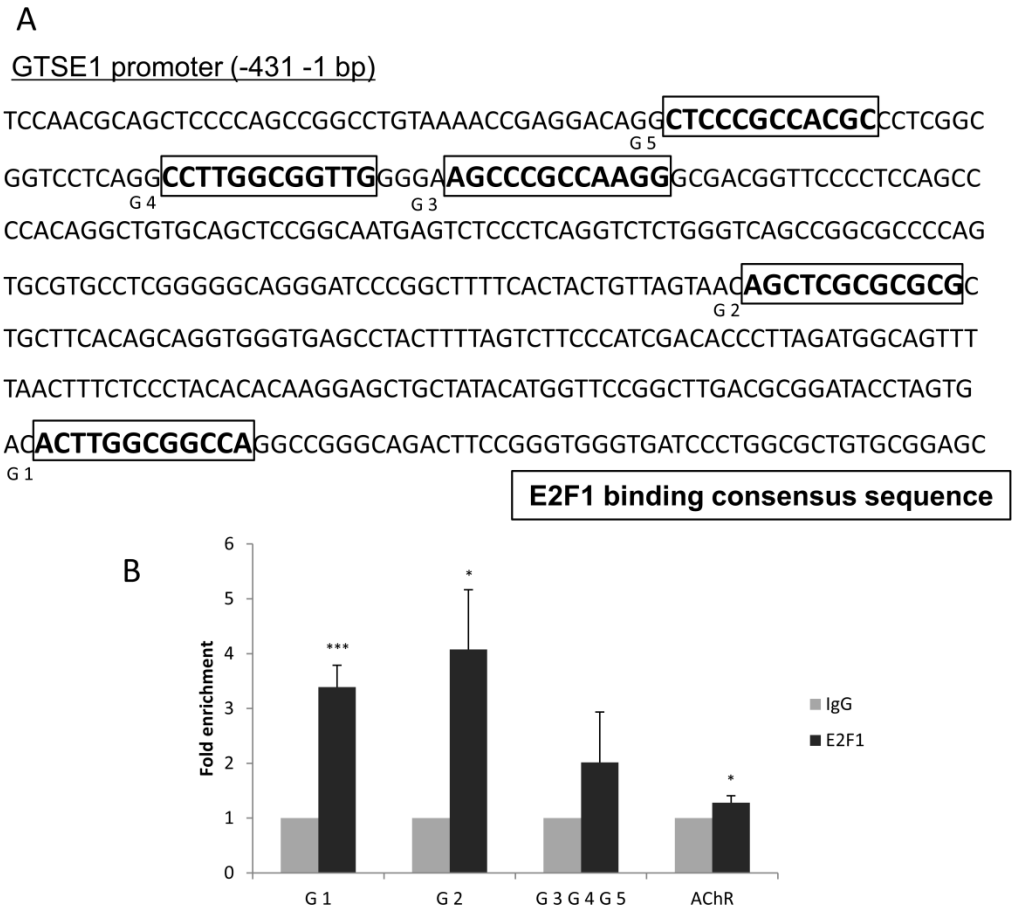

Fig.8 E2F1 physically binds the GTSE1 promoter region.

(A) Picture representing the position of E2F1 binding consensus sequences in the GTSE1 promoter. (B) For the ChIP assays IgG(control) or E2F1 antibodies were used in MDA-MB-231 cell line. The AChR was used as negative control. Data are shown as mean ± SEM of at least three independent replicates. For statistical analysis Student's t-test was applied. *p-value<0.05; ***p-value<0.001.

By performing a chip analysis, I found that E2F1 directly binds the G1 and G2 sites in the GTSE1 promoter region, suggesting its involvement as an additional regulator of GTSE1 transcription (Fig.8b).

Therefore, I decided to test the effect of E2F1 knockdown on GTSE1 expression. As shown in Fig.9a and b, after E2F1 depletion both protein and mRNA levels of GTSE1, similar to other known E2F1 target genes, notably decreased. The obtained results, further confirming the involvement of this TF in the control of GTSE1 expression, identify GTSE1 as a novel E2F1 target gene.

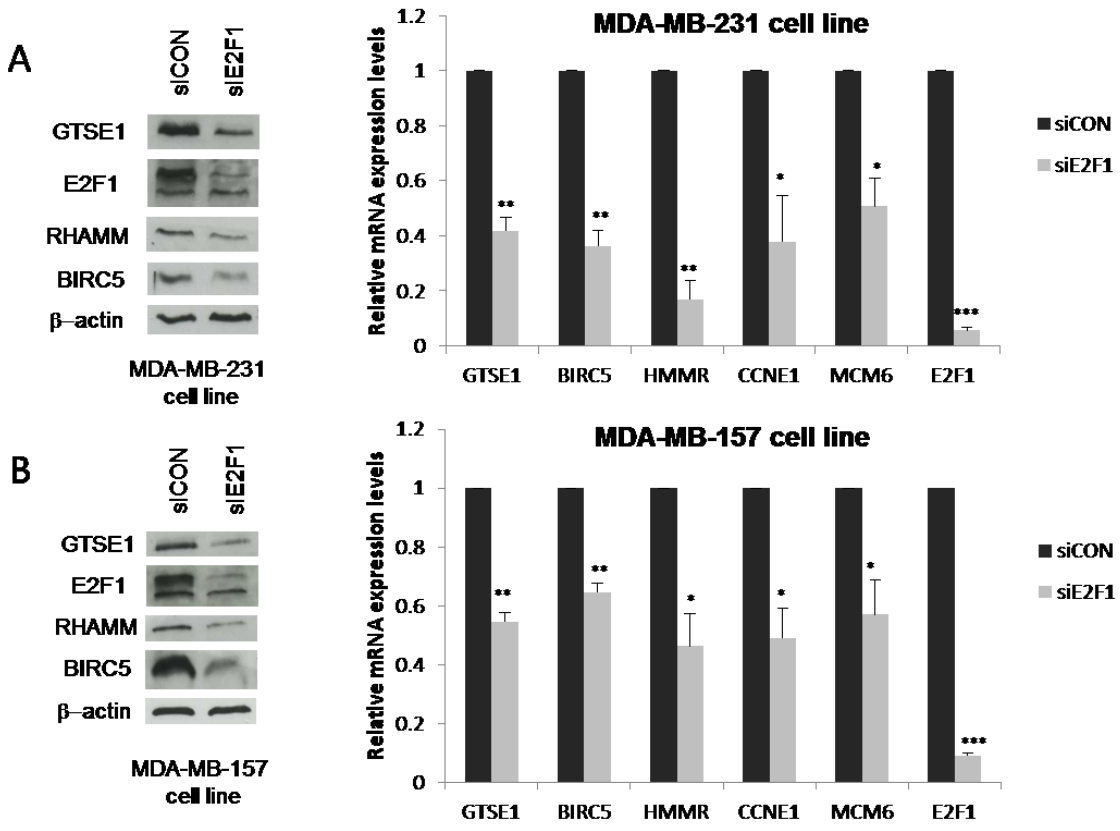

Fig.9 E2F1 modulates the GTSE1 expression.

GTSE1 protein and mRNA levels after knockdown of E2F1 in MDA-MB-231 and MDA-MB-157 cell lines (A) and (B). Data are shown as mean ± SEM of at least three independent experiments. For statistical analysis Student two-tailed T-test was applied. *p-value<0.05; **p-value<0.01; ***p-value<0.001

Similarly, BIRC5 (Baculoviral IAP Repeat Containing 5) and RHAMM (hyaluronan-mediated motility receptor) have been reported to be E2F1 target genes, too (Kan et al., 2013; Meier et al., 2014). In fact, as seen in other cancer cell lines, after E2F1 silencing the expression levels of Birc5 and RHAMM significantly decreased also in my model.

Most of YAP/TAZ target genes promoters harbor both TEAD and E2F TFs binding sites (Nicolay et al., 2011; Zanconato et al., 2015), and it has been demonstrated that these two pathway cooperate synergistically for the implementation of a transcriptional program required to bypass the cell cycle exit and to promote cell proliferation both in fruit fly and human (Kapoor et al., 2014; Nicolay et al., 2011).

Here, I showed that these two transcription factors regulate the expression of genes involved not only in cell proliferation, such as BIRC5, but also of genes (e.g. GTSE1 and RHAMM) involved in other aspects of cancer progression such as migration, invasion and metastasis, further highlighting the importance of E2F1 and TEAD cooperation in cancer.

In order to further functionally corroborate the GTSE1 regulation by E2F1, I tested the effect of the drug Palbociclib (PD0332991), a well-known inhibitor of the Rb-E2F1 pathway (Fry et al., 2004), on GTSE1 expression. Palbociclib is a selective inhibitor of CDK4 and CDK6 that causes Rb hypophoshorylation and block of E2F1 activity (Fry et al., 2004). As shown in Fig.10a and b, after treatment with palbociclib GTSE1 expression levels, as well as other E2F1 target genes, dramatically decrease at both the protein and the mRNA level. Taken together these data further confirm the involvement of the E2F1 TF in the transcriptional regulation of GTSE1 protein. Interestingly, under these conditions and in both cell lines, I observed not only a reduction in Rb phosphorylation, but also a concomitantly decrease in total Rb and E2F1 levels suggesting that the Rb-E2F1 pathway became dramatically altered. These observations are further supported by the recent work of Pollutri D et al. (unpublished data) that demonstrates that the treatment with CDK4/6 inhibitors inhibits this pathway through both Rb hypophosphorylation and E2F1 proteasome-dependent degradation. In fact, the ubiquitin-proteasome system represent a critical regulator of Rb-E2F1 pathway (Sengupta and Henry, 2015). However, further study are required to clarify the mechanism of E2F1 degradation in the model investigated.

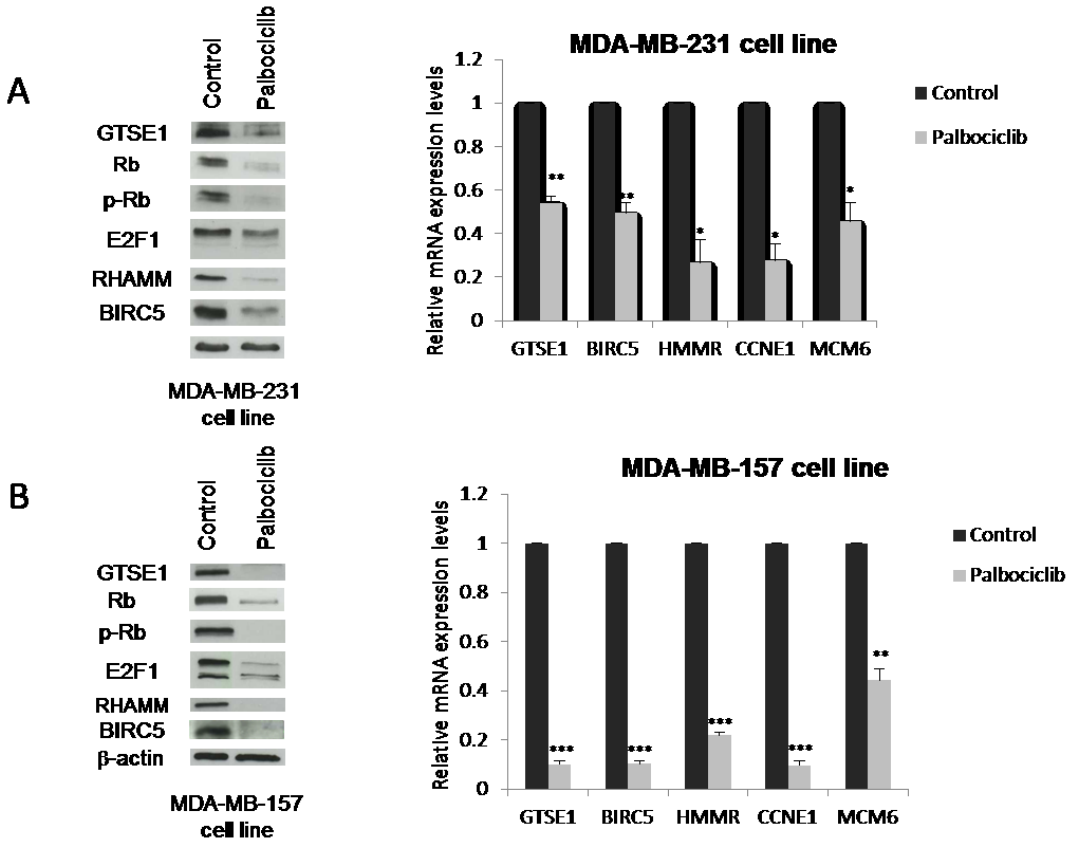

Fig.10 GTSE1 expression levels decrease after palbociclib treatment.

GTSE1 protein and mRNA levels in MDA-MB-231 (A) and MDA-MB-157 (B) treated respectively with palbociclib 0.5μM and 1μM for 72h (B). Data are shown as mean ± SEM of at least three independent experiments. For statistical analysis Student two-tailed T-test was applied. *p-value<0.05; **p-value<0.01; ***p-value<0.001

Discussion

In the last years, the involvement of the TEAD family of transcription factors together with YAP and TAZ co-activators has strongly emerged in the development of different types of tumors (including breast cancer). In fact, both TEAD4 and TAZ over-expression in breast cancer correlates with poor prognosis (Bartucci et al., 2015; Wang et al., 2015). Moreover, the YAP and TAZ co-activators interact with the TEAD TFs to promote epithelial to mesenchymal transition, migration and invasion, events that are critical for cancer progression and metastasis formation(Wang et al., 2014; Zhang et al., 2009).

Despite the great interest and the vast literature, less is known about the YAP/TAZ downstream effectors and how they exert their functions.

In this study, through the use of a multidisciplinary approach, I identified GTSE1 as a novel YAP/TAZ-TEAD4 regulated protein. The YAP/TAZ co-activators and the TEAD4 transcription factor exert their regulation at the transcriptional level promoting GTSE1 transcription. In fact, both the TEAD and the YAP/TAZ knock-downs lead to a decrease of GTSE1 mRNA level. Moreover, TEAD4 was chipped on the GTSE1 promoter indicating that it directly controls GTSE1 expression.

Given its role in tumorigenesis, chemoresistance and metastasis, many studies suggested a promising role for the YAP/TAZ-TEAD axis as therapeutic target in cancer treatment. Different inhibition strategies have been proposed among which: preventing the YAP-TEAD interaction, targeting F-actin, controlling the mevalonate pathway (Guo and Teng, 2015; Johnson and Halder, 2014).

Verteporfin (VP) is a porphyrin compound able to bind YAP and to interfere with the TEAD association (Liu-Chittenden et al., 2012). Vp, already used as photosensitizer in the treatment of neovascular macular degeneration, efficiently suppresses YAP-driven tumorigenesis in liver cancer mouse model (Liu-Chittenden et al., 2012). However, this compound binds YAP at micromolar concentration, higher affinity porphyrin drugs could be more effective in cancer treatment (Johnson and Halder, 2014).

Another known regulator of YAP activity is F-actin. In fact, it has been demonstrated that F-actin accumulation, promoting YAP nuclear localization, sustains the expression

of genes controlling cell proliferation and survival (Fernández et al., 2011; Sansores-Garcia et al., 2011). Cytochalasin D and latrunculin A/B, destabilizing F-actin, support YAP/TAZ nuclear export (Johnson and Halder, 2014). On the other hand the actin cytoskeleton is essential for many cell functions, so the possibility of toxic side effects should be considered (Johnson and Halder, 2014).

Previously it has been demonstrated that the mevalonate pathway promotes the YAP/TAZ transcriptional program and its inhibition is sufficient to block YAP/TAZ nuclear accumulation and activity (Sorrentino et al., 2014; Wang et al., 2014). Rho family of GTPases is a positive regulator of YAP/TAZ functions and the geranylgeranylation of these proteins is essential for their plasma membrane localization and activity (Dupont et al., 2011; Sansores-Garcia et al., 2011; Wada et al., 2011; Yu et al., 2012). Geranylgeranyl pyrophosphate is a metabolic intermediate of mevalonate pathway and the treatment with statins blocking its production leads to the inhibition of both the Rho GTPases activity and YAP/TAZ gene expression program (Sorrentino et al., 2014). In line with this GTSE1 protein and mRNA levels markedly decrease after the cerivastatin treatment, but the addition of mevalonate totally rescues this effect further confirming the involvement of these co-activators in the control of GTSE1 expression.

Recently statin use has been associated with decreased risk of death in a nationwide cohort study of 31,236 Finnish breast cancer patients (Murtola et al., 2014). Moreover, different studies linked statin usage to lowered probability of breast cancer recurrence (Ahern et al., 2011; Chae et al., 2011).

The next step of my work consisted in identifying the TEAD-dependent cellular functions mediated by GTSE1 and the underlying mechanism. We previously reported that GTSE1 is required for TNBC cell migration and it was also demonstrated that YAP, TAZ and TEAD down-regulation impacts negatively on the ability of breast cancer cells to migrate (Wang et al., 2014). For these reasons, I decided to verify if TEAD controls breast cancer cells migration through GTSE1.

Here, I found that GTSE1 over-expression is able to rescue the reduced migration following TEAD silencing in TNBC cell lines, suggesting that the effect of TEAD on cell motility is, at least partially, GTSE1-dependent.

A common feature of migrating cells required for cell migration is the formation of cell protrusions at the leading edge. Here, I highlighted a role for TEAD and GTSE1 in the control of cell protrusions formation. In fact, the number of cell protrusions/cell markedly decreases after TEAD depletion, with GTSE1 expression rescuing, at least partially, this effect. The achieved results suggest that TEAD regulates cell protrusion formation via GTSE1 providing, for the first time, a mechanical explanation on how this pathway regulates cell migration.

TEAD4 is not the only transcriptional regulator of GTSE1 expression; in fact, we found that the GTSE1 promoter region contains not only the TEAD4 consensus sequence, but also the E2F1 and HMGA1 binding sites.

Similarly to TEAD4, E2F1 is required for the transcriptional regulation of GTSE1. Its depletion, in fact, causes the lowering of both GTSE1 mRNA and protein levels. As demonstrated by ChIP assay, E2F1 directly binds the GTSE1 promoter region, further supporting its role in the control of GTSE1 transcription and making GTSE1 a novel direct E2F1 target gene.

The Rb protein is the main regulator of E2F1 activity and its signature includes 159 genes, most of which are E2F regulated genes, that are up-regulated after RB1 deletion or repressed by RB1 activation (Ertel et al., 2010). As reported by Ertel et al., the signature associated with RB1 loss presents the highest expression values in ER-negative tumors, reflecting a deep deregulation of RB1 in this type of cancers. It has been showed that GTSE1 is part of the RB1 loss signature (Ertel et al., 2010), further confirming the involvement of the Rb-E2F1 pathway in the control of GTSE1 expression. Moreover, the treatment with drugs targeting this pathway such as palbociclib decreases the expression of GTSE1 as well as other known targets.

Recently this inhibitor was approved for use in combination with letrozole in postmenopausal women with ER-positive, HER2-negative locally advanced or metastatic breast cancer. Furthermore, it has been demonstrated that the palbociclib treatment has anti-metastatic activity in both ER-positive and –negative breast cancer cell lines suggesting the exploration of its potential use in also this breast cancer subtype (Qin et al., 2015).

GTSE1 expression levels are higher in the most aggressive and invasive breast cancer subtypes. Both Rb-E2F1 and YAP/TAZ-TEAD4 pathways are deregulated in TNBC,

leading us to speculate that these two pathways could cooperate to promote GTSE1 up-regulation, in combination with other genes.

Similarly to GTSE1, RHAMM is regulated by both TEAD4 and E2F1 and has a critical role in breast cancer cell migration (Meier et al., 2014; Wang et al., 2014). These observations suggest that these two transcription factors may cooperate in orchestrating a transcriptional program of genes involved in breast cancer cell migration, invasion and metastasis. An independent regulation of GTSE1 and RHAMM expression by E2F1 and TEAD4 is still possible, although less probable, therefore further studies are required to verify if they act synergistically or not.

Previous studies suggested that breast cancer cells migration is reduced after treatment with palbociclib in a COX2 mediated manner (Qin et al., 2015). However, COX2 up-regulation alone is not able to completely rescue the reduced migration that follows the treatment, suggesting the involvement of other pathways. Here, I demonstrated that the palbociclib treatment leads to a reduction of the expression of both GTSE1 and RHAMM, suggesting that they may contribute to the reduced breast cancer cell migration.

Interestingly, the third regulator of GTSE1 expression that emerged from our bioinformatics analysis is the chromatin modifier HMGA1(high mobility group A). The HMGA1 protein regulates the transcription of target genes through the architectural remodeling of chromatin, allowing the formation of multi-protein complexes on promoter and enhancer regions (Brocher et al., 2010). It was reported that GTSE1 is part of the HMGA1 loss signature and that this TF regulates GTSE1 expression at least at the transcriptional level (Pegoraro et al., 2013). This suggests an attractive hypothesis in which HMGA1 could mediate the chromatin modification required for GTSE1 transcription. In the future, additional studies are required to investigate HMGA1 role in GTSE1 expression regulation.

In conclusion, the work of this thesis allowed to delve deeper into the understanding of GTSE1 transcriptional regulation, identifying the co-activators and transcription factors involved and establishing a role for both the YAP/TAZ-TEAD4 and the Rb-E2F1 pathways in the control of GTSE1 expression. The observation that GTSE1 is not the only protein involved in cell migration regulated by these two pathways suggests that they are profoundly interconnected in the regulation of processes required not only for

cell proliferation but also for other aspects of tumor progression such as invasion and metastasis. Further work is required to validate my hypothesis that these two pathways cooperate synergistically in the promotion of a transcriptional program required for metastasis, if it will be confirmed, the combinatorial use of drugs targeting both the YAP/TAZ-TEAD4 and the Rb-E2F1 pathways could be tested for the treatment of TNBC.

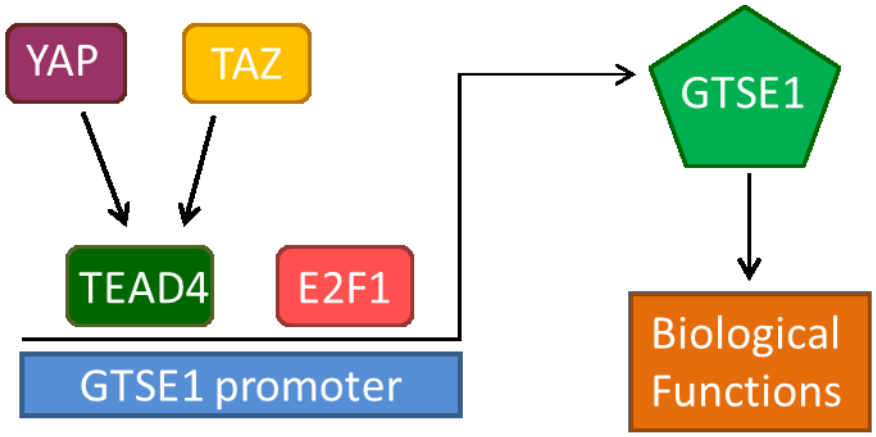

Fig.11 Final model summarizing GTSE1 transcriptional regulation in TNBC

Future perspectives

YAZ/TAZ silencing heavily affects the G1/S transition of the cell cycle, this in turn impacts on E2F1 transcriptional activity (Enzo et al., 2015). To further confirm that the transcriptional regulation of GTSE1 by the YAP/TAZ-TEAD4 axis is direct and not mediated by E2F1 inhibition in the future I'm planning to perform a reporter luciferase assay. I'm going to clone the GTSE1 promoter upstream of the luciferase reporter gene in an expression vector and to introduce mutations in the TEAD4 and/or E2F1 binding sites. It enables us to understand if both these two transcription factors are required to directly control GTSE1 expression.

The *in vivo* recapitulation of the *in vitro* phenotype of reduced migration and invasion after GTSE1 knockdown is also important. Previously the zebrafish model was used to study the YAP dependent cell vascular invasion (Sharif et al., 2015). Therefore, I'm going to use this well-established model to evaluate cancer cells invasiveness upon GTSE1 shRNA. Cancer cells labeled with a color tracer will be injected in the zebrafish circulation and their extravasation and colonization of peripheral tissues monitored.

Moreover, I would test the effect of the combination of drugs targeting both Rb-E2F1 and YAP/TAZ-TEAD4 pathways on metastasis formation. Nude mice represent a validated model to study tumorigenesis. For this reason I'm planning to inject TNBC cells into the tail veins of nude mice and then to compare the presence/absence of metastasis and the tumor growth rate in mice untreated to that of mice receiving the single treatment and the combination of two drugs.

In addition I'm planning to perform a high throughput fluorescent-based drug screening with FDA approved molecules in order to detect drugs able to down-regulate GTSE1 protein levels. This could enable us to identify novel compounds potentially able to control Rb-E2F1 and/or YAP/TAZ-TEAD4 pathways.

Materials and Methods

Cell culture and chemicals

MDA-MB-231 and MDA-MB-157 cell lines were obtained from ATCC. All cell lines were grown in DMEM with 4.5 g/L glucose (Lonza) and L-glutamine, supplemented with 10% fetal bovine serum (Euroclone), 100 U/ml penicillin and 100 U/ml streptomycin (Lonza) at 37°C in a humidified atmosphere of 5%CO_2.
Palbociclib (PD0332991, PZ 0199, Sigma Aldrich) was dissolved in H_2O at a 1mmol/L concentration. Cerivastatin (SML0005, Sigma Aldrich) and DL-mevalonic acid 5-phosphate (79849, Sigma Aldrich,) were prepared in dimethyl sulfoxide at a 10mmol/L concentration and 0,5mol/L, respectively.

Western blot analysis and antibodies

The $TEAD_{1/3/4}$ transcription factors (TFs) and YAP/TAZ silencing was performed by transfecting the siRNA in MDA-MB-231 and MDA-MB-157 cell lines for 72 hours.
For cerivastatin treatment, cells were incubated with DMSO or 1μM cerivastatin alone or with 0,5mmol mevalonic acid (MVA) for 24 hours (MDA-MB-231) or 72 hours (MDA-MB-157).
For palbociclib treatment, cells were treated with 0,5μM (MDA-MB-231) or 1μM (MDA-MB-157) PD0332991 for 72 hours.
Western blot analysis was performed according to the standard procedures.
Briefly, cells were washed twice with PBS and total protein extracted on ice using an home-made lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.1 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonylfluoride, 5 mM EDTA, 0.5mM β-glycerophosphate, 0.5mM NaF and Protease Inhibitor Cocktail, Sigma). Total protein concentration was measured by Bio-Rad protein assay (5000006, Bio-Rad) and 25μg of total lysate separated using SDS-PAGE. Proteins are then transferred on a nitrocellulose membrane and after blocking incubated with the specific antibodies.

Antibodies used: anti YAP/TAZ (sc101199, Santa Cruz Biotechnology), anti-actin (C11, Sigma Aldrich), anti pan-TEAD (13295, Cell Signaling), anti-phospho Rb (ab76298, Abcam), anti-Rb (554136, BD Pharmingen), anti-BIRC-5 (sc-10811, Santa Cruz Biotechnology), anti-E2F1(sc-251, Santa Cruz Biotechnology), anti-RHAMM (ab108339,Abcam), rabbit antibody against GTSE1 was previously described [4]. Bound primary antibodies were visualized using Pierce ECL Plus (Thermo Scientific) after addition of secondary antibodies.

RT-qPCR

Total RNA extraction was performed using QIAzol Lysis Reagent (Qiagen) and Nanodrop spectrophotometer was used to quantify the nucleic acid extracted and to assess its purity. The integrity of total RNA extracted was verified by agarose gel electrophoresis.

500 ng of the total nucleic acid extracted were reverse-transcribed into cDNA using a QuantiTect Reverse Transcription Kit (Qiagen), according to the manufacturer's instructions. Briefly, genomic DNA contamination was removed through the incubation of purified RNA with gDNA Wipeout buffer at 42°C for 2 minutes, subsequently RNA was reverse-transcribed at 42°C for 30 minutes using the Quantiscript Reverse Transcriptase, the Quantiscript RT buffer and the RT Primer mix supplied by the kit.

The enzyme was then inactivated by incubation at 95°C for 3 minutes.

The obtained cDNAs were diluted 1:20 and real-time qPCR was performed with the SYBR Green PCR Master Mix (Applied Biosystems) using a StepOnePlus real time PCR machine (Applied Biosystems).Oligonucleotides were used at 5μM final concentration and their sequences are reported in the table 1. Each experiment was performed at least three times and expression levels were normalized to the B-actin and GAPDH mRNA levels. $\Delta\Delta C_q$ method was used to calculate relative gene expression.

Target gene	Primer sequence
GTSE1	FW 5'-GCCCCGGGTGCTGTCAATGT-3' Rev 5'-GCCCACTGCTGGGGATGTGC-3'
TEAD4	FW 5'-TTGGAACTGGCTTAGCGCAC-3' Rev 5'-CGTCATTGTCGATGGGCTTG-3'
YAP1	FW 5'-GCCGGAGCCCAAATCC-3' Rev 5'-GCAGAGAAGCTGGAGAGGAATG-3'
TAZ	FW 5'-CAGCAATGTGGATGAGATGG-3' Rev 5'-TCATTGAAGAGGGGGATCAG-3'
E2F1	FW 5'-AACATCGATCGGGCCTTGTTTG-3' Rev 5'-GTGGACTCTTCGGAGAACTTTCAG-3'
BIRC5	FW 5'-AGCATTCGTCCGGTTGCGCT-3' Rev 5'-TCGATGGCACGGCGCACTTT-3'
RHAMM	FW 5'-AGAACCAACTCAAGCAACAGG-3' Rev 5'-AGGAGACGCCACTTGTTAATTTC-3'
CTGF	FW 5'-AGGAGTGGGTGTGTGACGA-3' Rev 5'-CCAGGCAGTTGGCTCTAATC-3'
CCNE1	FW 5'-TGAGCCGAGCGGTAGCTGGT-3' Rev 5'-GGGCTGGGGCTGCTGCTTAG-3'
MCM6	FW 5'-ATCCCTCTTGCCAAGGATTT-3' Rev 5'-GAAAAGTTCCGCTCACAAGC-3'
ACTB	FW5'-CCAACCGCGAGAAGATGA-3' Rev 5'-CCAGAGGCGTACAGGGATAG-3'
GAPDH	FW 5'-TCTCTGCTCCTCCTGTTC-3' Rev 5'-GCCCAATACGACCAAATCC-3'

Table 1. Primer sequences

RNAi interference

siRNAs were purchased from Eurofins Genomics and their sequences were reported in the table 2.
The negative control siRNA was the AllStars negative control siRNA(Qiagen 1027281). LipofectamineRNAi-MAX (Invitrogen) was used for siRNA transfections in antibiotic-free medium according to the manufacturer's instructions.

siRNA	Sequence
GTSE1	5'-GAUUCAUACAGGAGUCAAATT-3'
$TEAD_{1/3/4}$	5'-UGAUCAACUUCAUCCACAAGC-3'
YAP	5'-GACAUCUUCUGGUCAGAGA-3'
TAZ	5'-ACGUUGACUUAGGAACUUU-3'
E2F1	5'-CCAACGUCCUUGAGGGCAU-3', 5'-CUGCAGAGCAGAUGGUUAU-3' 5'-GGAAAGUGAGGGAGGGAGA-3'

Table2. siRNAs sequences

ChIP Assay

The ChIP assay was performed by using the ChIP-IT Express Enzymatic Chromatin Immunoprecipitation kit (Active Motif) according to the manufacturer's instructions. Eluted DNA was amplified by qPCR with GTSE1 promoter-specific primers. The sequences of the primers used were reported in the table 3.
We used the anti-TEAD4 (5H3) from Abnova and the anti-E2F1 (KH95 and C-20) from Santa Cruz Biotechnology. For statistical analysis Student t-test was applied. *p-value<0.05; ***p-value<0.001

Target	Primer sequence
TEAD4 ChIP site 1	S1-FW 5'-CCACACCTACTATGTGCTGAC ATG-3' S1-Rev 5'-CCTCAGCTCATCCTGGGGATGT-3'
TEAD4 ChIP site 2	S2-FW 5'-GATCCCTCTGCCATTCTCCCATG A-3' S2-Rev 5'AGGTGGGTGTGGTCAAACAGCT-3'
TEAD4 ChIP site 1 and 2	S1-FW 5'-CCACACCTACTATGTGCTGAC ATG-3' S1S2-Rev 5'TGGAAAGAGTTTGGCCTGCTCA-3'
E2F1 ChIP site 1	ES1-FW 5'CTACACACAAGGAGCTGCTAT-3' ES1-Rev 5'-ATCACCCACCCGGAAGT-3'
E2F1 ChIP site 2	ES2-FW 5'-ATGAGTCTCCCTCAGGTCTC-3' ES2-Rev 5'-TAAGGGTGTCGATGGGAAGA-3'
E2F1 ChIP site 3,4 and 5	ES3,4,5-FW 5'-GCTCTCTCCTCCAACGCA-3' ES3,4,5-Rev 5'AGAGACCTGAGGGAGACTCA T-3'

Table 3. ChIP primer sequences

Cell Migration Assays

For the wound-healing assay, silenced cells were plated in 6-well plates and cultured to confluence. Cell monolayers were scratched using a pipette tip, washed with PBS to remove debris, and incubated for 16 hours in cell culture medium. The wounded areas were imaged immediately after wounding and after 16 hours.

For the transwell migration assay,48 hours after silencing of TEAD $5X10^4$ cells were plated in 24well 8µm PET cell culture inserts(BD Falcon) and allowed to migrate for 16 hours at 37°C.After removing unmigrated cells with a cotton swab, migrated cells were fixed in 3% para-formaldehyde (PFA) and stained with crystal violet 0,5%. The migrated cells were then counted in ten randomized fields.

Transwell invasion assays were performed in 24well 8µm PET cell culture inserts (BD Falcon) coated with Corning Matrigel Matrix. 48 hours after silencing $8X10^4$ cells were

plated in cell culture inserts and allowed to invade for 18 hours at 37°C. The invaded cells were fixed in 3% PFA, stained with crystal violet 0,5% and ten randomized fields were counted.

Cell protrusion assay

The cell protrusion assay was performed as previously described (Shankar and Nabi, 2011). Briefly, TNBC cells were silenced for GTSE1 or TEAD TF and after 72 hours $1x10^6$ cells were plated in 24well 1µm PET cell culture inserts (BD Falcon) for 4 hours at 37°C. Cells were then fixed in 3% PFA, cell protrusions were stained using F-432 (Molecular Probes) and nuclei were stained using a propidium iodide solution (P4864, Sigma Aldrich). Multiple images of stained nuclei and pseudopodia were taken and counted using ImageJ software. The average number of cell protrusions/cell was obtained dividing the number of pseudopodia by the number of cell nuclei. Three-dimensional representation of the cell protrusions was performed using Interactive 3D Surface Plot plugin (https://imagej.nih.gov/ij/plugins/surface-plot-3d.html) for ImageJ. For statistical analysis Student two-tailed T-test was applied. *p-value<0.05; ***p-value<0.001.

Cell proliferation assay

For cell proliferation assay, 48 hours after TEAD1/3/4 silencing $1X10^5$ cells were plated and counted after 16 hours. Data are shown as mean ± SEM of three independent experiments. For the statistical analysis, Student two-tailed T-test was applied.

Abbreviations:

BIRC5 (Baculoviral IAP Repeat Containing 5);

CAFs (Cancer Associated Fibroblasts);

CCNE1 (Cyclin E1);

CIN (Chromosome Instability);

CSCs (Cancer Stem Cells);

CTGF (Connective Tissue Growth Factor);

E2F1 (E2F transcription factor 1);

ECM (Extracellular Matrix);

GTSE1 (G2 and S phase expressed1);

MCAK (Mitotic Centromere Associated Kinesin);

MCM6 (Minichromosome Maintenance Complex Component 6);

MVA (Mevalonic Acid);

Rb (Retinoblastoma protein);

RHAMM (Hyaluronan-Mediated Motility Receptor);

TAZ (Transcriptional co-Activator with PDZ-binding motif);

TEAD (TEA Domain transcription factor);

TF (Transcription Factor);

TFBS(Transcription Factor Binding Site);

TNBC (Triple Negative Breast Cancer);

TSS (Transcription Start Site);

VP (Verteporfin);

YAP (Yes-Associated Protein).

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