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HMGA1-p27-STATHMIN AXIS PROMOTES MIGRATION IN TRIPLE-NEGATIVE BREAST CANCER CELLS

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DOTTORANDO / A

MICHELA SGUBIN

COORDINATORE

PROF. GERMANA MERONI

SUPERVISORI DI TESI

PROF. GUIDALBERTO MANFIOLETTI

PROF. GUSTAVO BALDASSARRE

CO-SUPERVISORE DI TESI

SILVIA PEGORARO, PhD

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ABSTRACT

My research project focused on Triple Negative Breast Cancer (TNBC), which is the most aggressive breast cancer subtype, characterized by the absence of ER, PR and HER2 receptors. Up to now, no targeted therapeutic opportunities for patients are available. One of the key players in the promotion of TNBC aggressiveness is the oncofetal protein HMGA1, an architectural chromatin factor involved in the regulation of gene transcription. In this work we aimed to identify the molecular pathways through which HMGA1 could promote invasiveness in TNBC. By bioinformatic analysis in a cohort of TCGA breast cancer samples, we found an inverse correlation between HMGA1 expression and p27 protein, a well-known CDK inhibitor that has CDK-independent cytoplasmic functions such as the modulation of cell migration. Moreover, in patients with high levels of HMGA1, we observed an enrichment in the expression of a molecular partner of p27, the microtubule-destabilizing protein stathmin. Then, we confirmed the functional relationship observed by TCGA analysis demonstrating that p27 was upregulated at the protein level following the silencing of HMGA1 in two TNBC cell lines (MDA-MB-231 and MDA-MB-157), while stathmin was down-modulated. Moreover, via qRT-PCR, we observed that p27 mRNA level was not modulated by HMGA1, implying a post-translational level of regulation. In fact, by cycloheximide assay, we showed that p27 protein was stabilized after HMGA1 silencing. Moreover, we found that p27 localized in the cytoplasm and, after HMGA1 depletion, was phosphorylated in the cytoplasmic-retaining sites S10 and T198. Looking at stathmin involvement in TNBC, we determined that it is implicated in the promotion of MDA-MB-231 microtubule dynamicity and migration. By silencing the expression of HMGA1 in the same cell line, we demonstrated that stathmin diminishes its interaction with tubulin and it is responsible of motility promotion downstream HMGA1. Thus, we co-silenced HMGA1, p27 and stathmin in MDA-MB-231 cells and we analysed the trans-well migratory abilities of cells showing that HMGA1 promotes the migration through the regulation of p27 and stathmin. One of the chemotherapeutic drugs in the first line treatment for TNBC is paclitaxel, an antineoplastic drug that acts interfering with microtubule function. In the literature, both HMGA1 and stathmin have been shown to be involved in paclitaxel chemoresistance. Therefore, we tried to sensitize TNBC cells by targeting the HMGA1-p27-stathmin axis. Upon HMGA1-silencing of MDA-MB-231 cells, we were able to reduce the motility of cells treated with paclitaxel, sensitizing them to the treatment. Finally, we determined that the HMGA1 depletion in MDA-MB-231 cells injected in the mammary fat pad of nude mice, was able to sensitize the primary tumor volume to paclitaxel treatments.

Overall, we demonstrate that the HMGA1/p27/stathmin axis promotes breast cancer cell migration and may be considered as a possible target in combination with paclitaxel for the treatment of TNBC patients.

INTRODUCTION

1. BREAST CANCER

Breast cancer is the most common cancer in women worldwide, both in developed and less developed countries[1]. Data reported in a study conducted by the Global Burden of Disease Cancer Collaboration for 28 cancers in 188 countries from 1990 to 2013, evidenced that breast cancer was the cancer with the highest incidence and was present in the top three causes of cancer-related death in women. In 2013, there were 1.8 million incident cases and 464 thousand deaths[2]. Breast cancer is a group of diseases, in fact, it is characterized by high degree of heterogeneity, which makes it a challenging solid tumor to diagnose and treat[3]. Therefore, the role of grouping breast cancers in different subtypes is of particular importance for therapeutic decision making[4]. Classical immunohistochemical (IHC) markers such as oestrogen receptor (ER), progesterone receptor (PR) and HER2, together with traditional clinical-pathological variables, e.g., tumor size, tumor grade and lymph node involvement, are conventionally used for patient prognosis and management[5]. However, the most important variable that accounts for much of the biological diversity of human cells and tumors is the variation in transcriptional programs. Thanks to high-throughput technologies, recent advances based on the variations in genes expression patterns and DNA copy number, identified new classifications of breast cancer[6–8]. First Perou et al. and then Sorlie et al., identified novel subtypes of breast cancer that have been shown to have distinct gene signatures and prognostic outcomes[7,8]. Five subtypes derive from hierarchical clustering of breast samples and are named: normal-like, luminal A, luminal B, ERBB2+ and Basal-like breast cancers (fig. 1). Then, Sorlie characterized these subgroups with the following molecular features. The luminal A exhibited the highest expression of the ER α gene, GATA binding protein 3, X-box binding protein 1, trefoil factor 3, hepatocyte nuclear factor 3α, and oestrogen-regulated LIV-1. It is the most common breast cancer subtype, it can be treated with endocrine therapy and, in general, it has a good prognosis. The luminal B subtype is characterized by low or moderate expression of genes luminal-specific, including ER cluster. It has a significant worse prognosis than luminal A and benefits more from a combination of chemotherapy and hormone therapy in respect to the sole endocrine therapy, due to its high expression of proliferation genes. The ERBB2 subtype is characterized by high expression of several genes in the ERBB2 amplicon located at 17q22.24. 40% to 80% of this type of tumors harbour TP53 gene mutations and have poor prognosis. Moreover, they are sensitive to anthracycline and taxane-based neoadjuvant chemotherapy[4,9,10]. Finally, the Basal-like subtype is characterized by high expression of keratins 5 and 17, laminin, and fatty acid

binding protein 7[8]. This subtype is highly aggressive in comparison to the other subtypes: it presents higher tendency to form metastasis to visceral organs[11], it is likely to be of grade three and tends to show a rapid growth[12]. Among Basal-like subtype, we can define a subgroup named Triple-Negative Breast Cancer (TNBC), which comprises largely of the basal subtype and is the most heterogeneous and aggressive. It is characterized, as its name suggests, by the lack of oestrogen, progesterone and HER2 receptors. The absence of possible therapeutic targets leads to not amenable conventional targeted therapies, leaving chemotherapy as the only mainstay of treatment[10]. A further complication for therapy decision making is the intratumor heterogeneity, that is the spatial heterogeneity between different regions of the primary tumor, between the primary tumor and the metastatic lesion or between metastatic lesions. To add complexity to the picture, beyond the spatial heterogeneity, accumulating evidences underline the temporal evolution of tumor progression during the disease of cancer, supporting the theory that the cancer behaves as an evolving ecosystem[13].

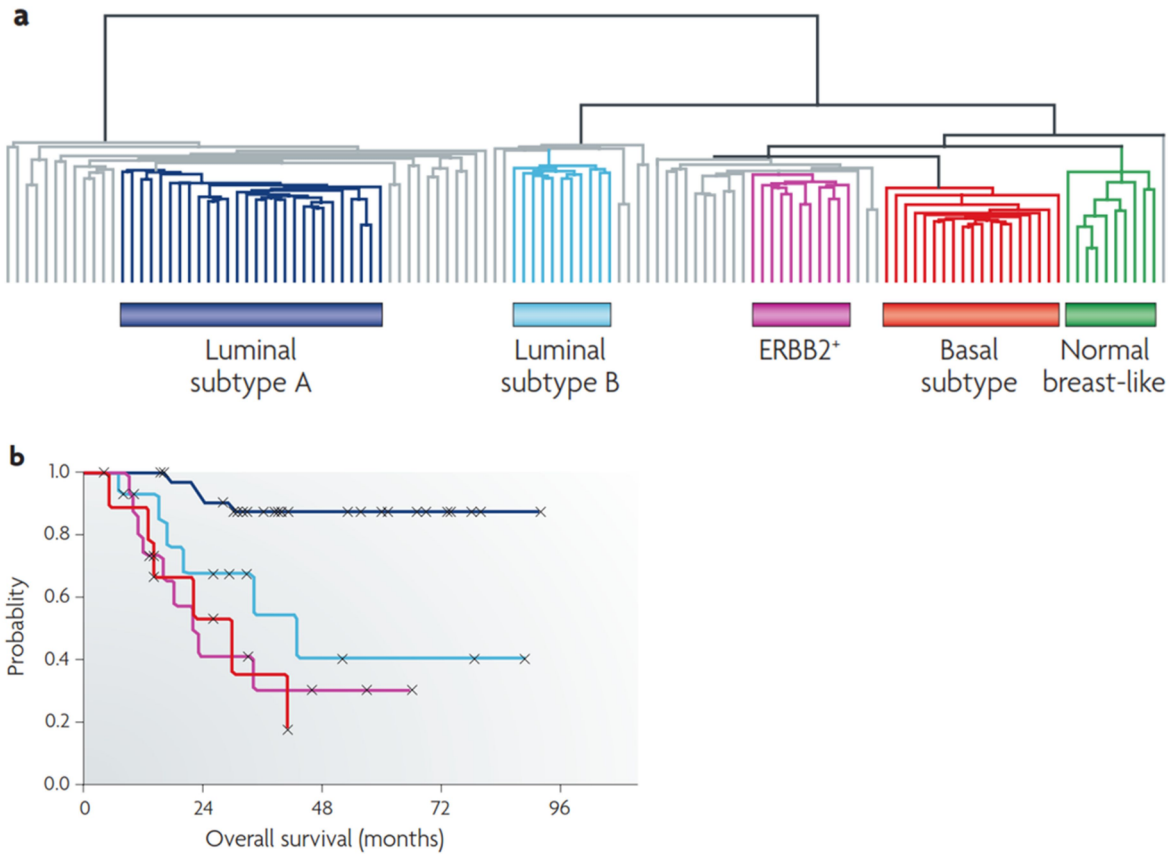


Fig.1. Molecular subtypes identified by gene expression profiling. a) The dendogram shows the cluster of breast cancer samples into five different subtypes: Luminal A, Luminal B, ERBB2+, basal-like and normal-like. b) Prognostic outcome for each of the five subtypes. Adapted by Vargo-Gogola and Rosen, Nature Reviews 2007.

1.1.Triple negative breast cancer

Triple Negative Breast Cancer (TNBC) accounts for 12 to 17% of women with breast cancer[14] and it is more common among Hispanic women and women with metabolic disease. Moreover, lower socioeconomic status was a risk factor for survival[15]. Women with TNBC are diagnosed with larger tumors, more aggressive histology, and at a more advanced stage, which contributes to faster progression to metastasis and poorer prognosis. Furthermore, the lack of oestrogen receptor (ER), progesterone receptor (PR) and HER2, gives no opportunity of specific treatments for TNBC and the only mainstay of medical treatment is chemotherapy. Women with TNBC have a higher pathological complete response (22-45%) for pre-surgery chemotherapy if compared with luminal and normal-like breast cancer subtypes. The higher response rate may be due to frequent high grade tumors and thus high mitotic indexes[15]. Unfortunately, those women who still have residual disease after treatment are at high risk of relapse and the outcome is relatively poor[16]. In TNBC, the conventional chemotherapy used is based on DNA-damaging anthracyclines (such as doxorubicin) or on microtubule-stabilizing taxanes. However, the side effects produced by the use of chemotherapeutic agents are significantly dose-limiting and for this reason there is an urgent need to find new specific targets for therapy[14].

1.2.Chemotherapy in TNBC: paclitaxel

In the first line therapy for TNBC we can find taxane chemotherapy. Taxanes, docetaxel and paclitaxel, are a novel class of antineoplastic drugs that act interfering with microtubule function, leading to mitotic arrest and cell death. Paclitaxel was originally extracted from the bark of the Pacific yew tree (*Taxus brevifolia*) thanks to a screening study of natural compounds. In 1994, Paclitaxel was approved for the treatment of metastatic breast cancer[17] and it was introduced in combination with anthracyclines and alkylating agents, improving better prognosis in terms of overall survival and disease-free survival of metastatic breast cancer patients[18]. Taxanes have also been introduced in the management of early-stage breast cancer, in combination with anthracyclines and trastuzumab[19]. The action of Paclitaxel consists in the binding of β-tubulin on a site present only when tubulin is in a polymerized conformation, causing lateral polymerization and stabilization of microtubules[20]. This action blocks mitosis at the anaphase-metaphase stage, due to the stabilization of microtubule spindle dynamics and causing apoptosis-mediated cell death. However, the major factor hampering the taxanes applicability in clinic is drug resistance, either intrinsic or acquired[21]. There are several players investigated so far that have been found to be implicated in drug resistance. The first ones identified were the drug efflux pumps, such as P-glycoprotein and Multidrug Resistance-associated Protein 1 (MRP1), whose overexpression is

responsible of pumping drugs outside the cell, reducing drastically the effect of therapies[22]. Moreover, several reports demonstrated that mutations in α- and β-tubulin, composition of different β-tubulin isotypes and, finally, alterations of microtubule-associated proteins expression were implicated in drug resistance[23]. Nevertheless, functional alterations in multiple cellular pathways, such as cell cycle, can contribute to chemoresistance[24]. Among the different factors involved, are of particular interest the Microtubule Associated Proteins (MAPs) which are responsible of the regulation of microtubule dynamicity and that play a key role in the resistance of microtubule interacting drugs[23]. Moreover, stathmin, a microtubule-destabilizing phosphoprotein, is implicated in microtubule catastrophe, decreasing polymerization of microtubules and causing resistance to paclitaxel[25,26]. Experiments with stathmin overexpression in breast cancer cell lines demonstrated how the microtubules are more dynamic and have a markedly reduced paclitaxel binding capacity. On the other hand, targeting stathmin with siRNA leaded to microtubule stabilization and sensitization to paclitaxel[25,26].

2. HMGA PROTEINS

The High Mobility Group A (HMGA) proteins are chromatin architectural factors that regulate transcription by either enhancing or suppressing transcription factors activity[27]. Their name comes from their characteristic high electrophoretic mobility both on SDS and acetic acid/urea PAGE. HMGA proteins are widely expressed during embryogenesis, while absent or expressed at low levels in adult tissues. However, HMGA proteins are re-expressed at high levels in some pathological contexts, such as cancer, hence their designation as oncofetal proteins[27].

2.1.HMGA protein structure

The HMGA family includes three protein members: HMGA1a and HMGA1b (107 and 96 amino acid residues respectively) that originate from the same gene through alternative splicing, and HMGA2, 108 amino acids[27]. HMGA1a and HMGA1b have high amino acid sequence homology (55%) with HMGA2. The major feature of HMGA proteins is the presence of three so called “AT-hooks”, conserved protein motifs responsible for the binding of HMGA to DNA stretches enriched in AT in the minor groove of B-form DNA. The AT-hooks are composed of a positively charged stretch of 9 amino acids containing the repeated Arg-Gly-Arg-Pro residues, flanked by other positive amino acids, usually Arg and Lys[28]. When free in solution, the HMGA proteins have no secondary structure, however, upon binding to both DNA or proteins, they undergo conformational changes from a disordered to ordered structure. This endows them with the capability to interact with a multitude of molecular partners regulating a variety of biological processes[28]. In addition, the

more AT-hooks bind to their DNA-target sequence, the strongest is the interaction of HMGA1 with DNA[29]. Moreover, HMGA proteins have an acidic C-terminal tail, whose role is not well understood yet[28].

2.2.HMGA molecular mechanisms of action

HMGA proteins are master regulators of gene expression, but they do not have any transcriptional activity *per se*. However, through the ability of binding to DNA and to other proteins, they can modulate the transcription of several genes. Three are the main mechanisms by which HMGA proteins regulate transcription: they can bind both DNA and proteins forming the enhanceosomes, they can also directly bind transcription factors, enhancing their affinity to the DNA; finally, they can modulate the chromatin structure. The complexes called enhanceosomes are nucleoprotein aggregates that assemble over a regulatory region of a gene. HMGA is able to promote the formation of these complexes by binding and bending DNA, favouring cooperative interactions between the factors of the transcriptional machinery. A well-known enhanceosome studied by Yie J et al. in 1999, is that of the virus inducible enhancer of the human β-interferon gene (IFN-β)[30]. Under basal conditions, the IFN-β enhancer DNA has not a favourable position to interact with its transcription factors, while after viral infection, HMGA1 binds and revers the curvature of DNA, allowing the binding of transcriptional factors and all the machinery necessary for transcriptional initiation[30]. It is to note that HMGA is also able to bend DNA and allow long-range enhancer-promoter interactions at thousands of bases of distance, as it is the case of β-globin gene[31]. The second molecular mechanism of action of HMGA is achieved by protein-protein binding with other nuclear proteins, mostly transcription factors. HMGA is able to induce conformational changes in their bound protein substrates, modulating in this way their transcriptional activity. For example, it has been demonstrated that HMGA can bind the serum response factor (SRF), enhancing its binding to DNA at the level of several promoters[32]. Finally, HMGA, acting at a general level, is able to alter the chromatin structure. Specifically, HMGA proteins bind to matrix and scaffold associated regions (MARs/SARs) leading to the complete de-repression of transcription from these sites by displacing histone H1[33].

2.3.The role of HMGA1 proteins in cancer

The overexpression of HMGA proteins in cancer was observed for the first time several years ago, when they were isolated from Hela cells[34]. Moreover, the functional correlation between HMGA and cancer was initially demonstrated in rat thyroid cells transformed with the Kirsten murine sarcoma virus (KiMSV)[35]. Then, the causal role of HMGA in cancer onset and progression has been shown by the development of highly malignant tumors in nude mice injected with transgenic cells

overexpressing HMGA proteins[36]. Furthermore, thanks to an inducible system driving the expression of HMGA1 transgenes, Reeves et al. (2001) demonstrated that breast epithelial cells developed metastatic tumors in nude mice only when the transgene was transcribed[37]. Among the cancer types, overexpression of HMGA1 was observed in a plethora of human cancers, such as prostate, colorectum, lung, breast, pancreas, bladder, uterine corpus, uterine cervix, kidney, head and neck, nervous system, stomach, liver, hematopoietic system and thyroid, all characterized by malignant epithelial features and by a correlation between HMGA1 protein levels and the aggressiveness of the tumor[38]. HMGA1 has a causal role in tumor progression modulating different cancer hallmarks such as proliferation, promotion of EMT, anchorage-independent growth, migration, invasion, metastasis and cell self-renewal (fig. 2)[38,39]. Several important pathways have been described to be modulated by HMGA1. Liau S.S. and colleagues, reported that HMGA1 promotes cellular invasiveness through PI3K/AKT-dependent promotion of MMP-9 expression and activity in pancreatic adenocarcinoma[40]. Very recently, Penzo et al., demonstrated that HMGA1 induced the activation of RSK2, a component of the MAPK signalling pathway, in TNBC cells[41]. Another fundamental pathway involved in protein synthesis, cell growth, cell proliferation and cell motility is the mTOR pathway, which has been reported to promote the activation of both AKT and SGK1 in melanoma[42]. HMGA1 has been described to constrain the activity of mTOR pathway, negatively regulating the expression of its downstream effector ULK1 and leading to promotion of cell proliferation and survival[43].

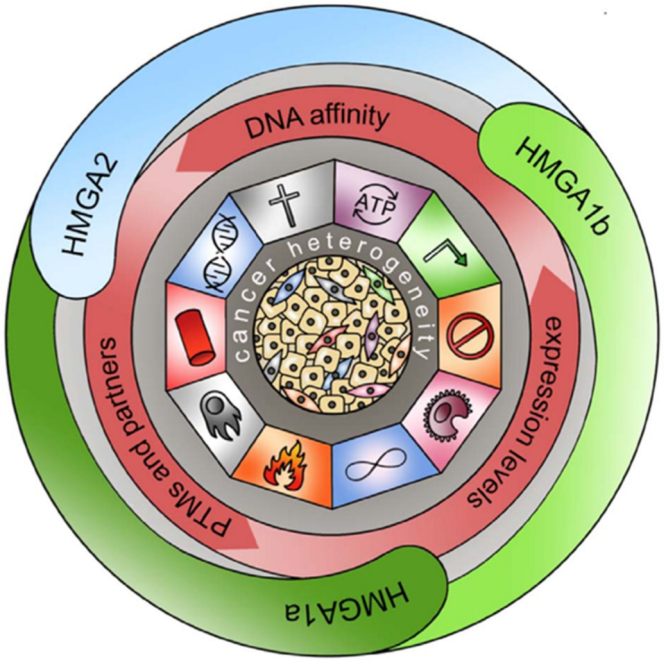


Fig. 2. HMGA proteins in tumor development. Picture that describes the fundamental role of HMGA proteins in cancer, pointing out their contribution in neoplastic transformation and cancer development through the modulation of all cancer hallmarks.

2.4.HMGA1 in breast cancer

Several studies underlined a prominent role for HMGA1 in breast cancer and in particular in the development of breast cancer aggressiveness. First, Ram and colleagues in 1993 showed a strong correlation between high HMGA1 expression level and the tumor transformation grade of mouse mammary epithelial cells[44]. Another study conducted to find genes differently expressed and responsible of tumor progression among primary breast cancers, metastatic cancers and normal-like breasts, reported that HMGA1 was strongly overexpressed in breast tumors respect to normal breast[45]. Later, in 2003, Flohr and colleagues performed an HMGA1 immunohistochemistry screening on 170 breast cancer samples revealing a strong correlation between tumor grade and HMGA1 expression[46]. Moreover, HMGA1 was detected to be overexpressed in 60% of ductal carcinomas by another immunohistochemistry study considering 212 breast tissue specimens[47], thus indicating HMGA1 as a potential prognostic marker for diagnosis of breast cancer. Nowadays, with the advent of the high-throughput technology, a much more precise quantitative information is available in a larger number of datasets. For example, the online tool GOBO (Gene expression-based Outcome for Breast cancer Online) collects 1881 breast cancer samples and allows to assess gene expression levels, identification of co-expressed genes, and association with the outcome for each gene, gene sets or gene signatures. In accordance with the previous data, enriched HMGA1 expression has been detected in the most aggressive breast cancer subtypes, such as the basal-like and the HER2-overexpressed subtypes, in the ER-negative patients and in breast tumors with the highest grade[48]. Furthermore, HMGA1 was shown to regulate different cancer hallmarks and the analysis of the global gene expression profile upon modulation of HMGA1 in breast cancer cells revealed that HMGA1 exerts a key role in the regulation of genes involved in cell proliferation, stemness, cell motility and invasion. First, HMGA1 is implicated in the promotion of tumor cell proliferation. In fact, MCF-7 cells overexpressing HMGA1a have a significant increased sensitivity to the activation of the Ras/ERK signalling pathway through the regulation of factors at or near the cell surface[49]. HMGA1 is also implicated in cellular metabolism. Indeed, HMGA1 is involved in the assembly of a transcriptionally active multiprotein-DNA complex responsible of the Insulin Receptor (IR) overexpression in breast cancer cells[50]. The IR is overexpressed in several breast cancers and it is responsible of increased tumorigenicity[50]. Moreover, HMGA1 has been individuated to have a causative role in breast cancer invasiveness and metastasis. Recently, Pegoraro et al. described a molecular pathway composed by the HMGA1-CCNE2-YAP axis, that has been shown to promote aggressiveness of breast cancer. Mechanistically, HMGA1, by binding to CCNE2 promoter, promotes its expression and in turn deactivates the Hippo Pathway, leading to YAP nuclear localization and promotion of YAP-target genes transcription, by binding to TEAD

transcription factors[51]. Moreover, HMGA1 positively regulates other molecules implicated in invasion, metastasis and proliferation such as SERPINE1 and PLAU[52]. These factors are part of the plasminogen activator system, involved in the metastasis process through the remodelling of the extracellular matrix. Finally, HMGA1 has been shown to be implicated in the promotion of angiogenesis. HMGA1 forms a complex with FOXM1, promoting its nuclear localization and increasing its transcriptional activity. FOXM1 is a key factor in the angiogenesis process, and, in cooperation with HMGA1, its activity is promoted, causing tumor angiogenesis both *in vitro* and *in vivo*[53].

2.5.HMGA1 in cancer cell chemoresistance

HMGA1 proteins have been shown to be involved in chemoresistance to several drugs. For example, it has been demonstrated that HMGA1 proteins are responsible of chemoresistance to gemcitabine in pancreatic and lung adenocarcinoma[54]. Furthermore, also chemotherapy treatment with cisplatin[55], ionizing radiations[56], 5-fluorouracil and doxorubicin[57] is less effective when HMGA1 is overexpressed. More recently, the use of chemotherapy agent temozolomide to treat brain tumor stem cells was more effective upon HMGA1 suppression[58]. Thus, HMGA1 expression has been shown to have a great impact on tumor resistance. Furthermore, there are several miRNAs known to be regulated by HMGA1 that have been shown to be involved in tumor chemoresistance. By miRNACHIP microarray studies on v-ras transformed rat thyroid cells expressing or not HMGA1, Mussnich and colleagues found a list of miRNAs downmodulated or upregulated by HMGA1 expression. Of our particular interest, miR-125b was among the miRNAs downmodulated together with HMGA1 suppression[59]. MiR-125b is implicated in paclitaxel resistance, in fact it has been found to be upregulated in breast cancer cells resistant to the drug, reducing cytotoxicity and apoptosis. miR-125b in this context is implicated in the targeting and consequent downmodulation of Bak1, a pro-apoptotic BCL-2 protein family member and function to induce apoptosis[60]. Another study investigated the role of HMGA1 in Paclitaxel resistance showing that the knockdown of HMGA1 expression in ovarian cancer cells reduced the resistance to paclitaxel and doxorubicin[61].

3. p27

$p27^{Kip1}$ belongs to the CIP/KIP family of CKI proteins and it was first discovered as an inhibitor of cyclin E-CDK2 complex in cells arrested by TGF-β[62], by contact inhibition[63] or by lovastatin[64]. p27 is encoded by the CDKN1B gene, located at 12p13.1 locus and codify for a 22 kDa protein. It belongs to the family of Intrinsically Unstructured Proteins (IUPs), lacking for almost the entire length of stable secondary/tertiary structures. However, upon binding with putative interactors, p27 has the ability to assume a folded structure[65]. p27 is composed by two main regions, the N-terminal

domain and the C-terminal domain. The N-terminus (residues 22-104) presents the so called "Kinase Inhibitor Domain" (KID), by which it can bind and inhibit the activity of cyclin-CDK complexes. In 1996, Russo and colleagues determined the crystal structure of p27 N-terminal domain bounded to cyclin A-CDK2 complex, showing how p27 binds first to cyclin A, shifting conformation upon binding, and then to CDK2[66]. The role of C-terminus of p27 (residues 105-198) is still not well elucidated. Interestingly, this domain is involved in processes not related with cell cycle control, like control of migration, interacting with several proteins, such as RhoA[67], Rac[68], Stathmin[69], GRb2[70] and 14-3-3[71] in different cellular compartments.

3.1.p27 regulation during cell cycle and tumor development

In normal cells, p27 expression is high during quiescence phase, while it rapidly decreases after mitogenic stimuli and cell cycle re-entry. In detail, in early G_1 phase, p27 promotes the assembly of cyclinD-CDK4 complex and its nuclear import[72]. Through this binding, p27 is sequestered from its principal target cyclinE-CDK2 complex, favouring its activation. The subsequent pRB hyperphosphorylation causes the release of E2F, determining further accumulation and activation of cyclinE-CDK2 complex, which in turn phosphorylates p27 in T187, causing its targeting to the proteasome[73]. Different stimuli that inhibit proliferation, such as cell-cell contact, loss of adhesion, induction of differentiation, TGFβ, IFN-γ, c-AMP, rapamycin and lovastatin treatments, have been described to have an impact on p27 up-regulation[74].

The role of p27 in cancer has emerged by the analysis of knock-out mice, in which it has been demonstrated that p27 is a well-defined example of gene haploinsufficiency, that is a selective advantage carried out by the functional loss of only one allele. In fact, animals lacking one copy of Cdkn1b (the gene coding for p27) tend to develop tumors spontaneously late in life and to be highly sensitive to tumor induction due to the use of carcinogens, while $p27^{-/-}$ mice displayed a further increase in tumor rate[75]. For these reasons, p27 has been defined as "dosage-dependent" tumor suppressor gene[74].

p27 is present at high levels in the cell nuclei of normal adult tissues because of the large presence of terminally differentiated cells. Whereas, a large amount of literature data describes that p27 protein is expressed at low levels in tumor tissues[74]. This is due mainly by regulation at post-translational levels, in fact silencing or mutations of p27 gene in human tumors are very rare[76]. In a wide variety of tumors such as colon, breast, lung, prostate, ovary and brain, p27 protein has been found to be reduced or lost. Moreover, in many of these tumors, multivariate analysis showed that reduced p27 levels are considered an independent prognostic factor for tumor diagnosis[74].

3.2.p27 post-translational modifications

p27 is highly modified at post-translational level by phosphorylations, acetylations, glycosylations, ubiquitinations and methylations[77]. These post-translational modifications are essential for protein commitment, interaction with other factors and localization. In particular, 2D analysis on gel electrophoresis revealed that p27 is widely phosphorylated at multiple sites[78]. The main phosphorylation sites present on p27 are: serine 10, tyrosines 74, 88, and 89, and threonines 157, 187, and 198[77] (fig. 3).

3.2.1. p27 phosphorylation on S10

In 2000, Nakayama and colleagues individuated for the first time that p27 is phosphorylated on S10[79]. This is the most abundant post-translational modification of p27, representing the 70-75% of phosphate incorporations. p27-S10ph has been connected to p27 cytoplasmic localization and degradation. In fact, it has been demonstrated that during the early G_1 and the G_1-S phases of the cell cycle p27-S10ph is forced to translocate to the cytoplasm and degraded trough a KPC-dependent manner[80–82]. Different kinases can phosphorylate p27 in S10ph, depending on the cell context. The major kinases described in the literature to be involved are: MAPK, hKis, AKT/PKB and Mirk/Dirk1B[78]. Foster and colleagues reported that this phosphorylation is required for p27 nuclear export, in fact, the oestrogen stimulation of MCF-7 cells leads to the activation of the MAPK pathway which in turn phosphorylates p27 in S10, re-localizing p27 in the cytoplasmic compartment[83]. As regard hKis, it phosphorylates p27 in S10 causing p27 re-localization in the cytoplasm with subsequent degradation and the promotion of cell cycle progression to S phase[84]. Cell cycle progression of muscle cells from G_1-G_0 of the cell cycle involves Mirk/dyrk1B complex, that it has been reported to phosphorylate p27 in S10 leading to its stabilization[85]. Finally, also AKT has been described to phosphorylate p27 in S10. Interestingly, this phosphorylation is fundamental for the sequential phosphorylation on other sites such as T157 and T198[86].

3.2.2. p27 phosphorylation on Y74, Y88 and Y89

Y74, Y88, Y89 are located in the kinase inhibitor domain of p27, they usually occur in G_0-G_1 phase transition and cause a decrease in the CDK-inhibitor activity of p27. These phosphorylations push the p27 protein tail away from the catalytic cleft of CDK4/CDK6[66,87]. It has been demonstrated that, in breast cancer cells, the phosphorylations on these residues are due to different kinases such as ABL, SRC and LYN[82].

3.2.3. p27 phosphorylation on T157 and T198

The phosphorylations on T157 and T198 have been described to prevent nuclear import, resulting in cytoplasmic localization of p27. Regarding p27-T157ph, it resides inside the nuclear localization signal (NLS) of p27[88]. Several studies reported that AKT is the major kinase responsible of T157 and T198[78], even if also RSK, SGK, AMPK and PIM have been described to catalyse these modifications in early G_1 phase[89]. While SGK1 contributes to T157 phosphorylation and cytoplasmic mis-localization of p27[42], RSK1 appears to phosphorylate p27 predominantly at T198[90]. Sekimoto et al. described that T157 phosphorylation is recognized by 14-3-3 protein and sequestered in the cytosol of HeLa and Hek cells[88]. In early G_1 phase of the cell cycle it has been shown that T157 and T198 phosphorylations are necessary for p27-cyclinD-CDK4 complex assembly and stability. Furthermore, p27-T198ph is important for protein stabilization[91,92]. The LKB1-AMPK energy sensing pathway is responsible of p27-T198ph leading to p27 stabilization and linking sensing of nutrient and bioenergetics to cell cycle progression[92].

3.2.4. p27 phosphorylation on T187

One of the p27-degradation pathways is mediated by CyclinE/A-CDK2 and CyclinB-CDK1 that phosphorylate p27 on T187[74]. T187ph is recognized by SKP2, the F-box protein component of SCF ubiquitin ligase E3 (SCF^{SKP2}) complex and leads to p27 ubiquitination and subsequent proteasomal degradation in the nucleus[93,94]. It has been described that another protein is necessary for the formation of the SCF^{SKP2} complex: the cyclin dependent kinase regulatory subunit 1 (CKS1)[95]. Furthermore, evidences described that SCF^{SKP2} mediated degradation of p27 can occur independently from T187 phosphorylation, indicating a further level of complexity[96].

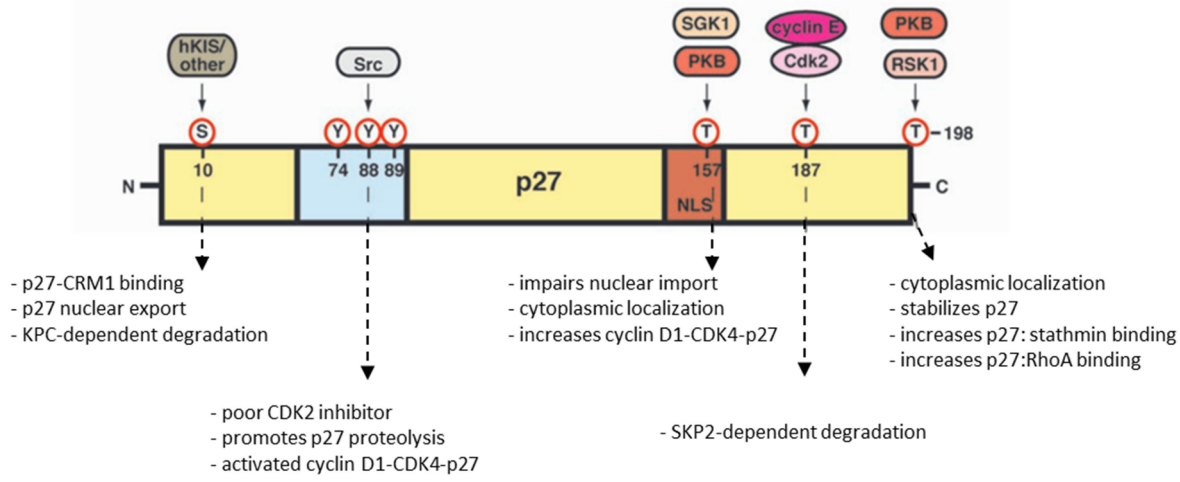


Fig. 3. p27 phosphorylation sites. Highlighted in red circles are the main phosphorylation residues of p27. Above each residue, the putative kinases that are involved in p27 phosphorylation are indicated. Dashed arrows point the different

consequences of phosphorylating these sites. Adapted by Michelle D. Larrea, Seth A. Wander and Joyce Slingerland, Cell Cycle 2009.

3.3.CDK-independent roles of p27

Beside the well-defined roles of p27 in cell-cycle regulation, in the last two decades, several studies have revealed novel CDK-independent functions of p27[97]. In fact, p27 has been demonstrated to have important roles in cytoskeleton dynamics modulation, cell motility control, and apoptosis.

3.3.1. p27 is involved in the actomyosin reorganization

The Rho family of GTPases, which includes Rho, Rac and Cdc42 regulate cell morphology, cytokinesis and cell motility through the reorganization of the actin cytoskeleton. RhoA-GTP activates the RhoA kinases ROCK1 and ROCK2, leading to LIMK activation that in turn phosphorylates and inactivates cofilin. This pathway result in the stability of actin stress fibers and focal adhesions assembly[90]. In hepatocellular cells, it has been demonstrated that the translocation of transduced TAT-p27 protein to the cytoplasmic compartment of cells is sufficient to rearrange actin cytoskeleton, filopodium formation and cell migration[68]. Interestingly, p27-null fibroblasts have been reported to present higher levels of RhoA-GTP (the active form of RhoA) together with more focal adhesions, stress fibers and decreased motility compared with wild-type cells[67,98]. Another study demonstrated that the C-terminal part of p27 is responsible of RhoA binding, preventing RhoA interaction and activation by Rho-GEFs[99]. Moreover, in mammary epithelial cells, it has been demonstrated that migration is promoted by cyclin D1 stimulation, which in turn causes SKP2 repression and subsequent p27 stabilization by the inhibition of proteasome degradation[100]. The interaction of p27 with RhoA has been also observed after RSK1 phosphorylation of p27 on T198 in WM-35 melanoma cells, which promoted p27 mislocalization to the cytoplasm and interaction with RhoA. p27-RhoA interaction leaded to its inactivation and subsequent actomyosin instability[90]. The modulation of RhoA activity by p27 has been reported also in the context of neuronal migration during corticogenesis[101].

3.3.2. p27 involvement in microtubule dynamics

Baldassarre and colleagues reported that p27 affects the microtubule stability following cell adhesion of sarcoma cells on fibronectin extracellular matrix (ECM)[69]. In detail, they demonstrated that p27 in the cytoplasm can bind the microtubule-destabilizing protein stathmin, sequestering stathmin from microtubules and causing stability of microtubule cytoskeleton. In this way, cytoplasmic p27 is able to reduce cell migration[69]. By migration experiments through fibronectin-

coated membranes, it was demonstrated that non-migrated HT-1080 cells which express EGFP-p27 vector had p27 mainly cytoplasmic, while migrated cells through the membrane presented p27 almost always nuclear. They identified the last 28 amino acids as responsible of migration inhibition and stathmin interaction. Moreover, $p27^{wt}$ was demonstrated to counteract stathmin activity on microtubules, in a dose dependent manner, by in vitro tubulin polymerization assay[69]. Later, the same group, has been shown in fibrosarcoma cells that the phosphorylation on T198ph of p27 is responsible of p27 stability and stathmin interaction[102]. Moreover, p27 has a causal role in modulating cell shape of mouse embryonic fibroblasts and in reducing their motility when immersed in three dimensional matrices. In fact, Belletti et al. showed how p27-null cells presented microtubule instability and mesenchymal-ameboid transition in motility due to increased lipid-raft trafficking and RhoA activation[103]. Indeed, lipid-raft trafficking is essential for migration due to integrin and receptor recycling. Moreover, RhoA activation is important for microtubule cytoskeleton modulation during motility because it is necessary for tubulin assemble at the leading edge of migrating cells. In this work, the rescue of the phenotype was observed with stathmin ablation, reinforcing the correlation between p27 and stathmin[103]. Furthermore, p27 can control microtubule dynamics in a stathmin-independent manner. In fact, in cortical neurons, it has been shown to directly bind microtubules via multiple binding sites both in N- and C-terminal regions. The binding of p27 with microtubules increases microtubule stability in interneurons during corticogenesis and it is important for neurite extensions during migration[101].

3.3.3. p27 regulates cell motility

Cell motility is a feature involved in a plethora of processes, necessary for development, tissue repair, immune responses, angiogenesis and tumor metastasis. As widely reported in the previous paragraphs, p27 in the cytoplasm has been demonstrated to have CDK-independent functions through the modulation of actomyosin and microtubule cytoskeleton. Depending on the cell context, it has been demonstrated that p27 can behave as a positive or negative regulator of cell motility. In fact, the works regarding the modulation of actin filaments through RhoA binding and inhibition by cytoplasmic p27, all reported that p27 leads to enhanced migration. In detail, motility could be promoted by RSK1 or PI3K pathways activation, leading in turn to p27 phosphorylation on T198 and subsequent binding of RhoA[104]. On the other hand, the modulation of microtubules by p27 have been reported to inhibit motility. As previously reported, the restore of p27 in sarcoma cells' cytoplasmic compartment is able to bind stathmin and promote microtubule stabilization, thus reducing migration[69]. Therefore, the controversial effects of cytoplasmic p27 are probably cell-type specific or due to differences in the migration assays used in the various studies[90].

3.3.4. p27 action in apoptosis process

Apoptosis has been suggested to accrue in G_1 phase of the cell cycle. p27, regulating cyclins/CDKs, affects apoptosis modulating their activity. In the literature, there are contrasting data about p27 involvement in the apoptotic process, high p27 protein levels have been reported to protect from apoptosis on one hand. However, on the other, it has been shown that p27 can have pro-apoptotic effects[105]. For example, studies on mesangial cells and fibroblasts showed high apoptosis levels concomitantly to absence of p27. The low p27 levels are responsible of an increase in CDK2 activity which in turns leads to apoptosis promotion[106]. On the other hand, p27-positive oropharyngeal tumors have been reported to present higher spontaneous apoptosis if compared with p27-negative ones[107]. Moreover, p27 transfection in lung cancer caused cell death through apoptosis[108]. In studies in cortical neurons, p27 has been reported to stabilize cyclinD1-CDK5 complexes and promote apoptosis cell death[109].

4. STATHMIN

Stathmin is a cytosolic microtubule-destabilizing phosphoprotein, the most prominent member of a conserved family of microtubule accessory proteins[110]. Stathmin has been described by several studies to be overexpressed in a plethora of cancers, thus deserving the name of Oncoprotein 18 (Op18). The phosphoprotein family includes stathmin 1 (Op18), stathmin-like 2 (SCG10), stathmin-like 3 (SCLIP) and stathmin-like 4 (RB) proteins and all of them are structurally and functionally related[111]. Stathmin family is characterized by the presence of a *stathmin-like domain*, an α-helical structure which possesses tubulin-binding activity. Stathmin is composed by 149 amino acids and has been described to be an intrinsically disordered protein which lacks a well-defined tertiary structure[112]. The N-terminus, a relatively unstructured region[113], includes four serine phosphorylation sites, located at residues 16, 25, 38 and 63, whose phosphorylation mediates stathmin inhibition[114]. Moreover, the very N-terminal part of stathmin has been described to dictate the subcellular localization[115]. The C-terminus of stathmin contains heptad repeats throughout its length, a feature of α-helical coiled-coil interactions[116]. It is well established that one stathmin molecule (S) sequesters two α/β-tubulin heterodimers (T) to form a ternary T_2S complex[117]. Two models of stathmin binding to tubulin have been proposed. On one hand, stathmin indirectly promotes microtubule catastrophe, sequestering α/β-tubulin heterodimers; on the other hand, stathmin directly de-stabilizes growing microtubules[118]. Gupta et al., in 2013, well described the multiple ways by which stathmin impinges on the microtubule cytoskeleton (fig. 4): a) stathmin, laterally binding the microtubule protofilaments, can inhibit lateral interactions between them by steric inhibition or by inducing a curvature; b) moreover, by binding to tubulin subunits, stathmin

can promote GTPase activity, leading to catastrophe promotion; c) the stathmin N-terminal region can insert between heterodimers causing severing of portions of GTP cap; d) the N-terminal tail of stathmin can cap the α-tubulin present at the minus end of microtubules thus preventing the incorporation of new tubulin dimers; e) furthermore, stathmin can bind microtubules grid causing microtubule catastrophe[118].

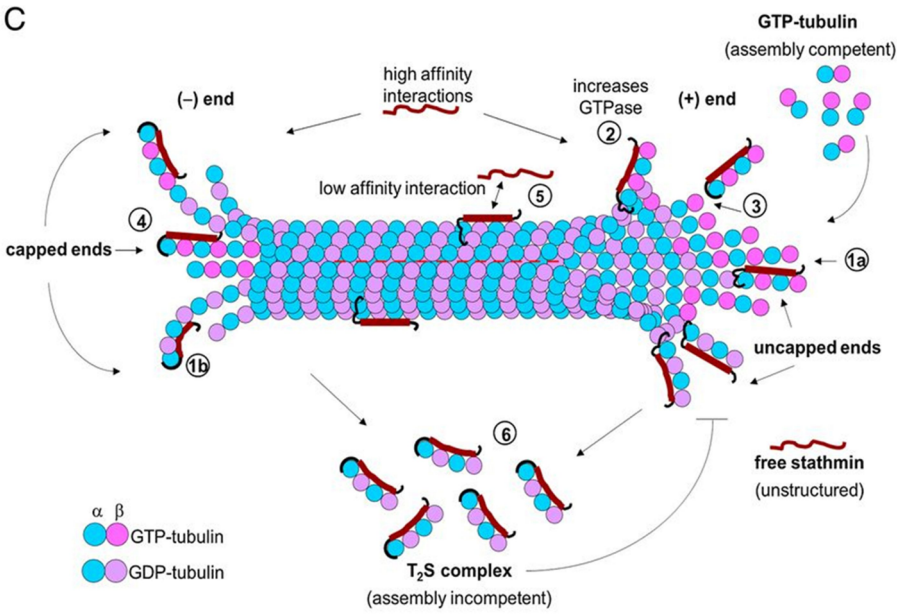


Fig. 4. Conceptual model for the catastrophe promotion activity of stathmin on microtubules. Adapted by Gupta KK et al., PNAS 2013.

4.1.Stathmin roles in normal cells

In normal tissues stathmin is highly expressed in embryonic cells, while its presence decreases during differentiation, being present at very low levels in terminally differentiated cells, with the exception of nervous system[111]. During cell cycle phases of synchronized cells, stathmin mRNA has been described to be present at high levels during S and G_2/M phases, decreasing in G_1 phase of the cell cycle[119]. Stathmin has a fundamental role in the regulation of cell-cycle progression: its activity is inhibited by extensive phosphorylation during the prophase of mitosis to allow the microtubule spindle assembly. Then, when the spindle has to be disassembled, stathmin undergoes a de-phosphorylation process and its activity is again turned on[120]. Stathmin has been shown to be involved also in apoptosis. In fact, its ablation induces cell cycle arrest and promotes apoptosis in *in vitro* models. Moreover, stathmin presence has been proved to protect from apoptosis induced by arsenic, paclitaxel and TGF-β inducible early gene 1[115].

4.2.The role of stathmin in cell motility

Several evidences revealed a critical role of stathmin in cell motility[69,121,122]. In fact, this process is intimately associated with microtubule dynamics and thus to stathmin depolymerization activity. Belletti et al. (2008) showed how stathmin is involved in the modulation of normal motility of fibroblasts and how the enhanced stathmin activity can foster migration[122]. *In vitro* it has been shown that stathmin overexpression promotes migration in and through the extracellular matrix, while it increases metastatic potential of sarcoma cells *in vivo*. A mutant of stathmin, which resulted less phosphorylated, has been shown to be more active and to promote rounded cell shape coupled with amoeboid-like cell motility in three-dimensional matrices, fostering invasive potential of sarcoma cells[122]. This mechanism has been demonstrated to be mediated by p27, when it loses its inhibitory interaction with stathmin[69]. The involvement of stathmin in cancer motility promotion was observed also in different type of tumors such as hepatocellular carcinoma tissue sections, breast cancer, colorectal cancer, non-small-cell lung carcinoma and glioblastoma cells[115]. In xenotrasplanted tumors, the knock down of the pro-apoptotic protein SIVA has been shown to promote stathmin activity, increasing cell mobility and migration of leukaemia cells[123]. Mechanistically, in breast and osteosarcoma cells, SIVA has been demonstrated to inhibit stathmin via their direct interaction, or indirectly, by regulating CaMK II and leading to stathmin phosphorylation on S16[124]. The inhibition of stathmin leads to microtubule stabilization, impeding focal adhesion assembly, cell migration and EMT. Moreover, the Merkel cell polyomavirus small tumor antigen has been demonstrated to de-phosphorylate stathmin, leading to microtubule destabilization followed by increased cell migration and metastasis formation of skin cell carcinoma[125]. Stathmin has a fundamental role also in nervous system and in particular in neurite outgrowth and in determining the behaviour of neuronal growth cone[126]. Furthermore, stathmin is important in the regulation of T-cell migration, because transient TCR signalling inhibits T-cell migration polarity through stathmin phosphorylation[127].

4.3.The role of stathmin in cancer

Several studies demonstrated that stathmin is generally overexpressed in different types of cancers, such as: lung cancer, breast cancer, hepatocellular carcinoma, gastric cancer, pancreatic cancer, cervical carcinomas, colorectal cancer and glioblastoma[128]. Furthermore, it associates with poor survival and local to distant metastasis formation[129–133]. Moreover, stathmin has been defined to be associated with increased invasion and metastatic lesions independently of the primary tumor histopathology, suggesting that it could be a valuable marker of poor prognosis[115]. Also stathmin phosphorylations play a prognostic role in cancer: in breast cancer and sarcomas, it has been

demonstrated that lower stathmin phosphorylation on serine 16 correlates with tumor development and recurrence. This indicates that the coupling of increased stathmin expression together with its increasing in activity is fundamental for local invasion and distant dissemination[122].

Moreover, overexpression of stathmin has been shown to have a role in drug resistance[26,134–136]. Several studies had evaluated the role of stathmin in resistance and sensitization to different chemotherapeutic drugs, demonstrating that high levels of stathmin expression associated with chemoresistance, whereas its down-regulation conferred sensitivity to different drugs. In breast cancer, stathmin is involved in resistance to paclitaxel and Herceptin, due to its higher expression fostered by the transcription factor FoxM1[134]. Alli et al. reported that, mechanistically, the resistance to paclitaxel is leaded by stathmin-mediated decrease of paclitaxel binding to microtubules, thus decreasing the activity of the drug[25]. In hepatocarcinogenesis, stathmin has been shown to confer resistance to paclitaxel, vinblastine and cisplatin (DNA cross-linker agent). The authors demonstrated that stathmin up-regulation can be mediated by gain-of-function mutations in p53[135]. In different epithelial cancer cell lines, it has been demonstrated that stathmin had a causal role in paclitaxel resistance[137], showing that ERK is an upstream regulator of stathmin-induced resistance, responsible of the phosphorylation of stathmin on S25. Moreover, blocking ERK with its specific inhibitor PD98059, leaded to the sensitization of the drug, promoting apoptosis and growth inhibition[138]. Therefore, stathmin expression levels are crucial in moving from tumor resistance to sensitization to different drugs. In fact, in esophageal squamous carcinoma cells, silencing of stathmin has been shown to be a great way to achieve paclitaxel and vincristine sensitization, through G_2-M phase block[139–141]. In lung cancer cells, it has been shown that the inhibition of stathmin together with expression of Bcl-2 is a good strategy to sensitize lung cancer cells to paclitaxel[142]. Moreover, also in breast cancer cells stathmin silencing sensitizes cells to paclitaxel and vinblastine (microtubule targeting drugs)[26]. Stathmin silencing through adenovirus-mediated gene transfer of anti-stathmin ribozyme was able to sensitize to taxol and etoposide (DNA strand-brake inducer) treatments in prostate cancer cells[136]. In extrahepatic cholangiocarcinoma cells, stathmin inhibition led to p27 release and promotion of paclitaxel sensitivity; in this study, the authors also determined that stathmin expression contributes to poor prognosis and cancer progression of patients with extrahepatic cancer[143]. Finally, silencing stathmin in endometrial carcinoma[144], retinoblastoma[145] and glioma stem cells[146] has been reported to enhance sensitivity to paclitaxel and temozolomide.

Thus, taken together all the data, stathmin expression assessment in the choice of chemotherapy treatment should be taken in consideration. Moreover, there is an urgent need to strength the

research on stathmin relationship with chemoresistance, in order to find new potential markers and new molecular targeted drugs[128].

AIM OF THE THESIS

This thesis focuses on Triple-Negative Breast Cancer (TNBC), which is known to have the worst outcome of all clinically recognized breast cancer subtypes. It is characterized by high aggressiveness and invasiveness and there are no specific therapeutic targets discovered so far. Indeed, there is an urgent need to find possible targeted agents. Among the molecular factors that have been discovered to have a causal role in the promotion of TNBC aggressiveness, HMGA1 proteins are promising candidates. HMGA1 proteins are chromatin architectural factors that sustain cancer hallmarks and, in particular, they have been demonstrated to promote breast cancer cell motility, EMT and metastasis formation. Therefore, the aim of this thesis was to investigate the HMGA1-molecular network involved in the promotion of TNBC aggressiveness, in order to identify new molecular targets for therapy. To achieve this result, we used TNBC cell lines depleted or not of HMGA1 and we explored a new HMGA1-p27-stathmin axis involved in the regulation of cell migration.

MATERIALS AND METHODS

1. Cell culture and treatments

The human triple negative breast cancer cell lines MDA-MB-231, MDA-MB-157 and MDA-MB-468 were routinely grown in high glucose Dulbecco's Modified Eagle Medium (DMEM, Euroclone), with 10% tetracycline-free Fetal Bovine Serum (FBS, Euroclone), 2mM L-Glutamine (Euroclone), 100 U/ml Penicillin (Euroclone), 100 µg/ml Streptomycin (Euroclone). The MDA-MB-231 TetR clones with shCTRL and shHMGA1 vectors stably transfected were kept in selection with 1 mg/ml of G418 (Sigma) and 5 µg/ml blasticidin (Sigma) and treated with 1µg/ml doxycycline to induce the shRNA (see the reference for the design of the inducible shRNA cell lines[48]). For routine cell culture passage, cells were dissociated with Trypsin-EDTA (0.05% trypsin, 0.02% EDTA in PBS, Euroclone). For long-term maintenance, cells were collected in FBS 10% (v/v) DMSO (AnalaR BDH) and stored in liquid nitrogen. For cycloheximide experiment, cells were treated with 50 µM Cycloheximide (Sigma) for 1.5, 6 and 9 hours. For the inhibition of the proteasome, MG132 (Sigma) was used at the concentration of 10 µM for 8 hours. For kinase inhibitors screening, cells were treated for 8 hours with LY294002 (10 µM, Selleckchem), U0126 (10 µM, Selleckchem), SNS-032 (0.1 µM, Selleckchem), Temsirolimus (0.1 µM, Wyeth), Palbociclib (1 µM, Selleckchem), PF4708671 (10 µM, Sigma), S3I-201 (50 µM, Selleckchem), GSK-650394 (10 µM, Tocris Bioscience). The drug concentrations used have been described in literature to be the ideal working concentrations that inhibit the specific kinases in human tumor cells. For microtubule-free tubulin separation, paclitaxel (Sigma) was used at 4 µM concentration for 1h. For MTS, Colony, Wound Healing and *in vivo* experiments, paclitaxel (Taxol®, Actavis) was used.

2. siRNA Transfection

The day before the siRNA transfection, cells were seeded in antibiotics-free DMEM at a density of $2*10^5$ cells/35mm dish (Corning). The Lipofectamine™ RNAimax reagent (Invitrogen/Thermo Fisher Scientific) was used for transfection of 30 pmol of siRNA/35mm dish, following the manufacturer instructions. For co-silencing experiments, 30 pmol of each specific siRNA and 60 pmol of siCTRL in the control condition were transfected. The cells were processed after 72 hours of silencing.

siRNAs and shRNAs used in this work are listed in the following table:

TARGET GENE	*siRNA*	*SEQUENCE (5'-3')*
	siCTRL	ACAGUCGCGUUUGCGACUG
HMGA1 a/b	siHMGA1	ACUGGAGAAGGAGGAAGAG
HMGA1 a/b	siHMGA1_1	GACAAGGCUAACAUCCCACTT
STMN	siSTMN	CGUUUGCGAGAGAAGGAUA
	shCTRL	shc002 (non-mammalian shRNA control plasmid RNA, Sigma)
p27	shp27	GCGCAAGTGGAATTTCGATTT (TRC0000039930, Sigma)

3. Preparation of viral particles and viral transduction

For viral particles preparation, we seeded $8*10^6$ HEK293FT (Invitrogen) cells/15cm dish in low antibiotics media (AQMedia (Sigma), with 10% FBS, 0.1% penicillin/streptomycin, 1% L-glutamine, 1% non-essential AA, 1% sodium pyruvate). The day after, cells were transfected by calcium phosphate with 8µg of plKO plasmid with the shRNA for p27 plus viral particles components (pVSV-G, pLP1, pLP2; Invitrogen). The media was changed after 16h with fresh Harvest Media (complete AQMedia with 1% BSA) that was collected after 24h and 48h from transfection and conserved at -80°C.

For viral transduction, MDA-MB-231 cells were seeded $2*10^5$ cells/35mm dish in complete medium and the day after cells were transduced with shp27 by adding 2ml of viral Harvest Medium at the cell culture. The media was then changed after 16h from transduction and the samples collected or processed at the desired time.

4. Protein extraction and western blot analysis

Cells were washed in ice-cold PBS1X and then were lysed in SDS sample buffer (62.5 mM Tris, pH 6.8; 2% SDS; 10% glycerol) with 200 mM DTT, 1 mM Na_3VO_4, 5 mM NaF and mammalian protease inhibitor cocktail (PIC) (Sigma). Afterwards, DNA was disrupted with passing the sample through an insulin syringe and boiled for 5 minutes. Total lysates were separated by SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) in SDS-PAGE gel composed by the stacking and the running gel: Stacking gel: 5%: 5% acrylamide/bis-acrylamide (29:1), 0.125 M Tris-HCl pH 6.8, 0.1% (w/v) SDS, 0.05% (w/v) APS and 0.05% (v/v) TEMED. Running gel: 7.5%: 7.5% acrylamide/bis-acrylamide (29:1), 0.3 M Tris-HCl pH 8.8, 0.1% (w/v) SDS, 0.05% (w/v) APS and

0.05% (v/v) TEMED; 10%: 10% acrylamide/bis-acrylamide (29:1), 0.5 M Tris-HCl pH 8.8, 0.1% (w/v) SDS, 0.05% (w/v) APS and 0.05% (v/v) TEMED; 15%: 15% acrylamide/bis-acrylamide (29:1), 0.375 M Tris-HCl pH 8.8, 0.1% (w/v) SDS, 0.05% (w/v) APS and 0.05% (v/v) TEMED. Gel thickness: 0.75 mm, length of stacking gel: 0.5 cm, length of running gel: 8 cm, gel width: 8 cm. The electrophoresis was carried out in running buffer composed by 25 mM Tris, 200 mM Glycine, 3.5 mM SDS (w/v) and performed at 50V for the accumulation step and then at 200V for the protein separation, till the complete separation of the molecular markers (Pre Stand, Thermo Fisher Scientific). Subsequently, the gel could be stained in solution of methanol/water/acetic acid solution (in a volume ratio of 5/4/1) containing 0.05% (w/v) Coomassie Brilliant Blue R 250, and then de-stained in a solution of 10% Acetic Acid in H_2O, in order to perform the protein quantification. Alternatively, the proteins were transferred to nitrocellulose membrane Ø 0.2 µm (GE Healthcare, WhatmanTM) using a wet transfer system (Transfer buffer: 25 mM Tris, 200 mM Glycine, 20% methanol) at 4°C for 16 hours at 40 V. After the transfer step, membranes were then stained with Red Ponceau solution (0.2% red ponceau S, 3% trichloroacetic acid, 3% sulfosalicylic acid). Western blot analyses were performed according to standard procedures. To reveal horseradish peroxidase conjugated to secondary antibody on autoradiography films (GE Healthcare), Liteablot Extend ECL (Euroclone) or TPro Lumilong Plus (T-Pro Biotechnology) were used.

The antibodies used are listed in the following table:

Primary Antibody	*Antibody dilution*
α-HMGA1 (made in the laboratory)	1:500
α-p27 (BD-TL)	1:750
α-p27-S10ph (Santa Cruz)	1:500
α-p27-T157ph (R&D systems)	1:200
α-p27-T198ph (Santa Cruz)	1:200
α-STMN (Cell Signalling, Santa Cruz)	1:1000
α-CDK2 (Santa Cruz)	1:200
α-H3 (Abcam)	1:2000
α-Tubulin (T4026, Sigma)	1:1000

α-β-actin (A2055, Sigma)	1:2000
α-Vinculin (sc-7679, Santa Cruz)	1:5000
α-PSF (Sigma)	1:1000

Secondary Antibody	*Antibody diluition*
α-rabbit IgG peroxidase conjugate (A0545, Sigma)	1:5000
α-mouse peroxidase conjugate (A9044, Sigma)	1:5000
α-mouse True Blot (Rockland)	1:2000

4.1.Immunoprecipitation analysis

Immunoprecipitation experiments were performed incubating 0.7-1 mg of total cell lysate in HNTG buffer (20 mM HEPES, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100) with the specific primary antibody, incubating them overnight at 4°C. Then, protein A or protein G Sepharose 4 Fast Flow (Amersham Biosciences) was incubated for 1h and 30 min at 4°C with the lysate-antibody formulation. Immunoprecipitates were washed several times in HNTG buffer and resuspended in 3X Laemmli sample buffer with 50 mM dithiothreitol, boiled for 10 minutes and loaded on the gel for Western Blot assay.

4.2.Cytoplasmic and nuclear protein fractionation

Cell monolayers were trypsinized, collected in cold medium, washed with cold PBS1X and resuspended in Reticulocyte Standard Buffer (RSB; 10 mM Tris pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$) for 3 minutes on ice. Cells were subjected to centrifugation at 2900 g, 3 minutes, 4°C and the pellet was resuspended gently in RSB with glycerol (RSBG40; RSB with 10% glycerol). Then, cells were centrifuged at 2800 g, 3 minutes, 4°C and the supernatant collected as soluble cytoplasmic fraction. The remaining pellet was then resuspended in RSGB40 with detergent (RSGB40 plus 0.3% sodium deoxycholate and 0.7% tween40), vortexed and incubated 5 minutes on ice. Then the samples were centrifuged at 2800 g, 3 minutes, 4°C and the supernatant (non-soluble cytoplasmic fraction) was combined with the cytoplasmic fraction. The pellet was washed

two times with RSGB40 and then centrifuged at 18000 g, 5 minutes, 4°C. The supernatant was removed and the pellet nuclei lysed in complete SDS lysis buffer. The fractions were then subjected to normalization and quantification through Blu Coomassie gel staining. The normalized lysates amounts were then subjected to standard Western Blot analysis.

4.3.Microtubule-free tubulin separation

The separation of microtubules from free tubulin was performed when cells reached sub-confluence. Cells were washed once with PBS1X at 37°C and then lysed with Microtubule Stabilizing Buffer (MSB; 20 mM Tris/HCl pH 6.8, NaCl 0.14 M, 2M glycerol, 1mM $MgCl_2$, 2 mM EGTA, 0.5% Triton X-100, PIC, 4 µM Taxol). Cells were then centrifuged 12,000 g for 10 minutes, 4°C. The supernatant was collected as free-tubulin fraction, while the pellet included the polymerized microtubules. The fractions were then loaded on a gel for Western Blot analysis on β-tubulin amount in each fraction. The total tubulin for each sample was calculated as the sum of microtubule and free-tubulin fractions.

5. Migration Assays

For wound healing assay, MDA-MB-231 cells were seeded in antibiotics-free DMEM on glass coverslips at a density of $2*10^5$ cells/well in a 6-multiwell dish (Corning) in biological triplicates and were silenced for HMGA1 as previously described. The cells were cultured to 90% confluence and scraped with a 200-µl tip, thereafter medium was changed and supplemented with 1 nM paclitaxel or DMSO then wound closure was followed for 5 hours. For each condition four pictures were taken. The areas of the scratch were analysed with ImageJ software.

For trans-well migration and invasion assays, 24-well PET inserts were used (8.0 mm ∅, Falcon) and 40,000 cells were seeded on the top. After 18-24h, migrated cells were fixed in PFA 4% and stained with Crystal Violet 0.5% (Sigma).

6. Cell viability assay

Cells were dispensed in 96-well plates at a density of 5,000 cells per well and, the day after, treated with increasing concentrations of Paclitaxel (from $1*10^{-6}$ to 20 µM) for 72h. The percentage of cell death was determined using the CellTiter 96 AQueous cell proliferation assay (MTS) (Promega). The absorbance of the solution was measured at 492 nm using the Tecan microplate reader. The percentage of viability was calculated as (OD of drug treated sample/OD control sample) x 100.

7. Colony formation assay

For colony assay, cells were pre-plated and treated at sub-confluence with 7.5 nM paclitaxel. At 18h from treatment, cells were trypsinized, counted, 600 cells/60 mm dish were seeded and incubated for two weeks in complete medium. Plates were then stained with crystal violet 0.5% and colonies counted. The results were expressed as percentage of growth inhibition comparing the colony number of the paclitaxel-treated conditions with their respective untreated control.

8. Immunofluorescence

Cells were grown on glass coverslips and treated for each experiment; they were then washed in PBS1X and fixed in a solution of PBS 1X 4% Paraformaldehyde (pH 7.2). Cells were incubated with a solution of PBS1X with 0,1 M Glycine (Sigma-Aldrich/Merck) in order to reduce autofluorescence background and then incubated with PBS1X 0.3% Triton 100X (Sigma-Aldrich/Merck) to permeabilize cells. Between each passage, the cells were washed three times in PBS1X. We then proceeded with a 45 minutes blocking step with a blocking solution of PBS1X 5% Bovine Serum Albumin (BSA-Sigma-Aldrich/Merck). Subsequently, we incubated with the appropriate primary antibody diluted in blocking solution added with 0.01% Triton for 90 minutes in a wet environment; we washed the cells in PBS1X and incubated them with the secondary antibody in PBS1X for 45 minutes. We washed the cells in PBS1X, incubated them in a solution of PBS1X 1:1000 Hoescht to detect nuclei and finally we mounted the coverslips on glass supports with the Vectashield mounting medium (Vector Laboratories). The primary and secondary antibodies used in this project are enumerated in the following table:

Primary antibody	*Dilution*
α-p27 (Santa Cruz)	1:100
α-STMN (Cell Signalling)	1:500
α-VIMENTIN (mouse-Dako)	1:200

Secondary antibody	*Dilution*
α-rabbit IgG Alexa 594 (Thermo Fisher Scientific)	1:500
α-mouse IgG Alexa 488 (Thermo Fisher Scientific)	1:500

The images were visualized using a Nikon Eclipse e800 microscope and acquired using Nikon ACT-1 software, then analysed by the ImageJ software analyser.

9. RNA extraction

Cells were washed in ice-cold PBS1X and lysed in 1 ml of TRIzol (Thermo Fisher Scientific). Total RNA was isolated following the manufacturer's instructions of the TRIzol reagent and resuspended in UltraPure™ DNase/RNase-Free Distilled Water (Thermo Fisher Scientific). The isolated RNA was subjected to DNase-I (Deoxyribonuclease I Amplification Grade, Invitrogen) treatment as indicated in the datasheet and to a subsequent phenol/chloroform purification. The RNA quality was checked by running an aliquot of the RNA on a denaturing agarose gel MOPS 1% (w/v) agarose (Sigma), 6.67% (v/v) formaldehyde (Sigma) in MOPS buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7). In addition, RNA was quantified at NanoDrop 2000 (ThermoFisher Scientific).

10. Gene expression analysis

1 μg of total RNA was reverse transcribed with Random primer by the Superscript III (Invitrogen), according to the manufacturer's instructions. The quantitative RT-PCR (qRT-PCR) was carried out using diluted cDNA combined with iQ^{TM} SYBR Green Supermix (BIO-RAD) and specific primers listed below. The reaction was performed in technical duplicates. The PCR reaction was performed in BIO-RAD CFX instrument and the protocol used was the following: an activation step at 95°C for 3 minutes followed by 40 cycles of a denaturation step at 95°C for 5 seconds and an amplification and elongation step at 60°C for 30 seconds. The data obtained were analysed with BIO-RAD CFX Manager software and the relative gene expression was calculated by ΔΔCt method, using the GAPDH as a normalizer. The genes evaluated in qRT-PCR are enumerated below with the primer sequence used:

Target gene/primer name	Forward primer (5'- 3')	Reverse Primer (5'- 3')
hGAPDH	TCTCTGCTCCTCCTGTTC	GCCCAATACGACCAAATCC
hHMGA1/HMGA-3'UTR	ACCAGCGCCAAATGTTCATCCTCA	AGCCCCTCTTCCCCACAAAGAT
hp27	AGCAATGCGCAGGAATAAGG	TTCTGAGGCCAGGCTTCTTG

hSTMN	AATGGCTGCCAAACTGGAAC	TCTCGTCAGCAGGGTCTTTG

11. *In vivo* experiments

Female athymic (nude) mice, 6 to 7 weeks old, were purchased from Charles River Laboratories. The animals were allowed to acclimate for 7 days before the study initiation. All of the animals were housed under pathogen-free conditions and were given water and chow ad libitum. Animal care and use were in accordance with Institutional and NIH guidelines.

MDA-MB-231 shCTRL and shHMGA1 cells ($2*10^6$ cells/0.1ml) were subcutaneously injected into the mammary fat pad of female athymic mice. To induce shRNA, mice were administered drinking water supplemented with 2 % sucrose plus 1 mg/ml doxycycline. When tumors reached 50-100 mm^3 the treatment with 12 mg/kg of paclitaxel started. Treatments were given 3 times a week for 15 times, time by which mice were killed. During the treatments, tumor volumes were measured 3 times/week with a caliper, and the tumor weights were calculated as follows: [length (mm) x width $(mm)^2$]/2. Tumor tissues were collected and fixed in buffered formalin.

12. Breast cancer datasets

To evaluate the enrichment of p27 and stathmin in breast cancer samples according to HMGA1 expression levels, we collaborate with the bioinformatic unit at LNCIB (Trieste). They investigate the TCGA (The Cancer Genome Atlas) dataset on the Memorial Sloan Kettering Cancer Genomic Portal (http://www.cbioportal.org/public-portal). To evaluate the correspondence between the p27 and stathmin expression levels among breast cancer clinical data, we employed the Gene expression-based Outcome for Breast cancer Online (GOBO) web tool.

13. Statistical Analysis

Data were analysed by a two-tailed Student's t test, and results were considered significant at a p-value <0.05. The results are presented as the mean and standard deviation (±SD). Specifically, a p-value<0.05 is indicated with *, a p-value<0.01 with ** and a p-value<0.001 with ***.

14. RNA-seq analysis

RNA-seq analysis was performed by dott. S. Piazza at the LNCIB in Trieste. Demultiplexed raw reads (fastq) generated from the Illumina HiSeq were checked using FASTQC tool (Version 0.11.3). All samples passed the quality standards. Then we aligned them to the reference genome (UCSC-hg19) using STAR, version 2.0.1a using recommended options and thresholds. HTSeq-

count (version 0.6.1) was used to generate gene counts. Transcript abundance, expressed in $\log_2$ count, is used to plot gene expression values in Fig. 3a.

RESULTS

1. p27 AND STATHMIN PROTEIN EXPRESSION ARE DIFFERENTLY ENRICHED AMONG BREAST CANCER SAMPLES EXPRESSING HMGA1

HMGA1 is a master regulator of chromatin architecture, orchestrating the transcription of several genes. A gene signature of 130 genes down-modulated after HMGA1 silencing in a TNBC cell line has been demonstrated to be an independent predictor of poor clinical outcome in breast cancer patients[48]. Moreover, HMGA1 has been shown to play a key role in breast cancer malignancy and in the progression of the metastatic disease[48]. Thus, we wanted to better depict the HMGA1 molecular networks responsible of the TNBC aggressive features, investigating with particular attention that involved in the regulation of motile abilities of cells. To address this issue, we explored the enrichment of specific proteins and/or protein phosphorylation levels in breast cancer patient samples based on HMGA1 expression levels. In collaboration with the bioinformatic unit at LNCIB (Trieste), we performed bioinformatic analysis on a cohort of 406 breast cancer patients present in The Cancer Genome Atlas (TCGA) project, employing the expression data from RNA-Seq and the proteomic data from Reverse Phase Protein Array (RPPA). We categorized patients based on HMGA1 expression levels (low, medium and high) and ranked the differential changes obtained from RPPA. Among the most significant differential protein expression, we found that high p27 protein levels are present in the group of low HMGA1-expressing tumors and that p27 levels gradually decrease among the groups of medium and high HMGA1-expressing tumors (fig.1a), indicating an inverse relationship between HMGA1 and p27 expression. Moreover, we also found a differential protein expression of stathmin (STMN), a known p27-molecular partner, among HMGA1-categorized patients (fig. 1b), indicating that stathmin protein levels are directly associated with HMGA1-expression.

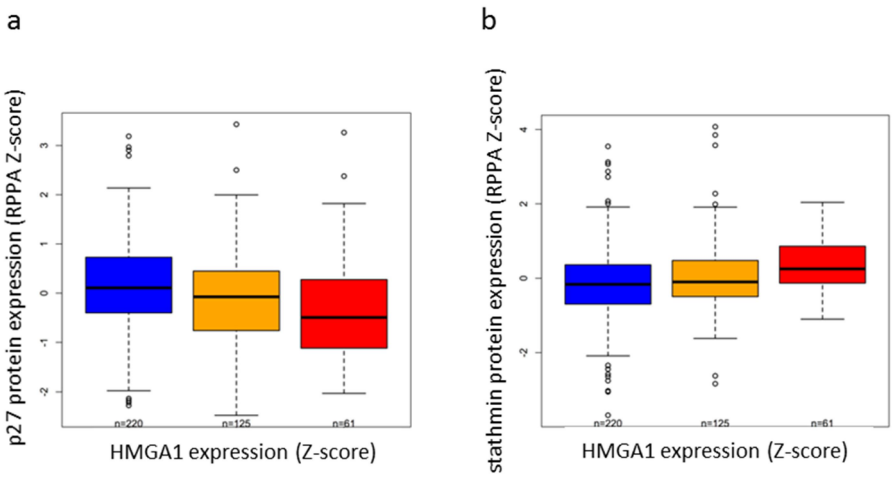


Fig.1. **p27 AND STATHMIN PROTEIN EXPRESSIONS ARE DIFFERENTLY ENRICHED AMONG BREAST CANCER SAMPLES EXPRESSING HMGA1.** Boxplot analysis showing the expression of HMGA1 and the expression of p27 (a) and stathmin (b) proteins in a cohort of 406 TCGA breast cancer patients. The samples were stratified based on HMGA1 mRNA expression. In *blue* are highlighted the tumor samples of patients with low HMGA1 expression levels, in *yellow* the tumor samples with medium HMGA1 expression levels and in *red* the tumor samples with high HMGA1 expression levels.

2. p27 AND STATHMIN ARE DIFFERENTIALLY EXPRESSED AMONG BREAST CANCER SUBTYPES

Pegoraro et al., in 2013, analysed a public microarray data collection investigating 1881 cancer samples thanks to the Gene expression-based Outcome for Breast Cancer Online (GOBO) tool and they found that high expression of HMGA1 in primary breast tumors is associated with the worst tumor subtype, the oestrogen receptor negative subtype and the highest histological tumor grade (fig. 2a)[48]. Thus, we asked how p27 and stathmin expressions are enriched among breast cancer subtypes and how they associate with aggressive features. Thus, we first exploited the GOBO tool to analyse p27 expression levels and we found that among the breast cancer subtypes, p27 mRNA levels were lower in the basal-like and the HER2+ subtypes respect to luminal A, luminal B and normal-like subtypes (Fig. 2b). Furthermore, the p27 mRNA levels were associated with the oestrogen receptor positive subgroup of breast cancer and its expression decreases as the tumor grade become higher (Fig. 2b). These data indicate that p27 expression is specular to that of HMGA1, and suggest that high levels of p27 are an indication of a better prognosis in breast cancer patients. Then, we assessed stathmin expression levels in breast cancer patients. First, we observed an enrichment of stathmin expression in the basal-like and HER2+ subgroups compared to luminal A and normal-like tissues (fig. 2c). Moreover, stathmin mRNA is associated with oestrogen receptor negative tumor and the more advanced tumor grade (fig.2c), resembling the same pattern of what we found for HMGA1 mRNA. Taken together, these data suggested that p27 and stathmin

could have opposite roles in breast cancer patients with aggressive tumor subtypes: the first is expressed at lower levels, while the second is abundantly present. These data indicated an inverse relationship between HMGA1 and p27 and a direct one with stathmin in breast cancer patients.

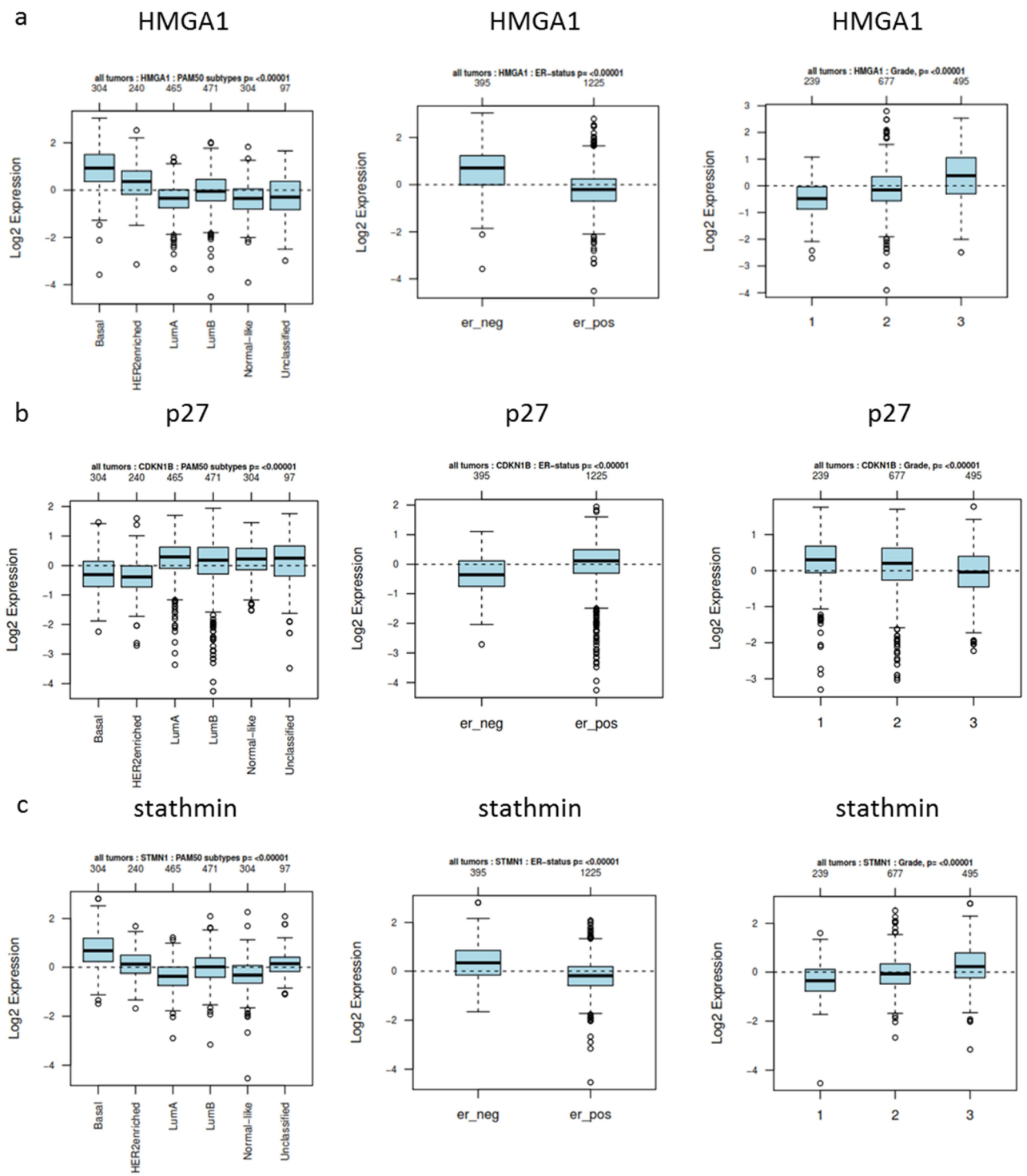


Fig.2. **p27 AND STATHMIN ARE DIFFERENTIALLY EXPRESSED AMONG BREAST CANCER SUBTYPES.** Expression of a) HMGA1, b) p27 and c) stathmin among breast cancer subtypes, ER-status and tumor

grade. The analyses were performed investigating the Gene expression-based Outcome for Breast cancer Online (GOBO) tool.

3. HMGA1 REGULATES p27 AND STATHMIN AT POST-TRANSCRIPTIONAL LEVEL

Starting from these evidences, we asked if HMGA1 regulates the expression of p27 and stathmin in TNBC cells. From literature data, p27 and stathmin are mainly regulated at post-transcriptional levels in cancer[76,115]. On the other hand, HMGA1 acts mainly as master regulator of gene expression. Thus, we first analysed if p27 and stathmin were modulated at mRNA level after HMGA1 silencing in MDA-MB-231. Looking at their mRNA expression levels in an experiment of RNA-Seq performed at 24, 48 and 72 hours after HMGA1 silencing in MDA-MB-231, we observed that p27 and stathmin mRNA levels were not significantly modulated in all the different time points (Fig.3a). Then, we confirmed these results via qRT-PCR by analysing p27 and stathmin mRNA levels in MDA-MB-231 cells after 24 and 72h of HMGA1 silencing. Specifically, we found that after 24h of HMGA1 depletion p27 expression does not change, while at 72h p27 mRNA slightly increases (Fig. 3b). Moving on the analysis of stathmin expression, we observed no significant variation at mRNA levels at 24h, and a slight downregulation at 72h (Fig. 3b).

Interestingly, we found that after HMGA1 silencing p27 and stathmin were modulated at protein level. We performed a time-course analysis of p27 and stathmin protein levels at 24, 48 and 72h of HMGA1 silencing in MDA-MB-231 cells, and we observed a very early increase of the p27 protein levels at 24h after HMGA1 silencing, which remained higher levels also after 48 and 72h (Fig. 3c). On the other hand, we noticed a more complex regulation of stathmin protein levels. In fact, stathmin protein level was higher after 24h of HMGA1 silencing respect to the control condition, but then, it seemed to return to control condition at 48h and finally it reached lower levels at 72h. Taken together, these data suggested that p27 and stathmin were mainly regulated at post-transcriptional levels by HMGA1.

Moreover, we confirmed the modulation of p27 and stathmin protein expression by HMGA1 also in other two triple-negative cell lines, the MDA-MB-157 and MDA-MB-468 cells (Fig. 3d). In fact, we found an upregulation of p27 concomitantly with a down modulation of stathmin after 72h of HMGA1 silencing. In addition to these data, we obtained the same result silencing HMGA1 with another siRNA (siHMGA1_1), proving the specific action of HMGA1 (Fig. 3e). Through these experiments, we demonstrated that HMGA1 silencing in triple negative cell lines induces an up-regulation of p27 and down-regulation of stathmin confirming a functional relationship between HMGA1 and both p27 and stathmin.

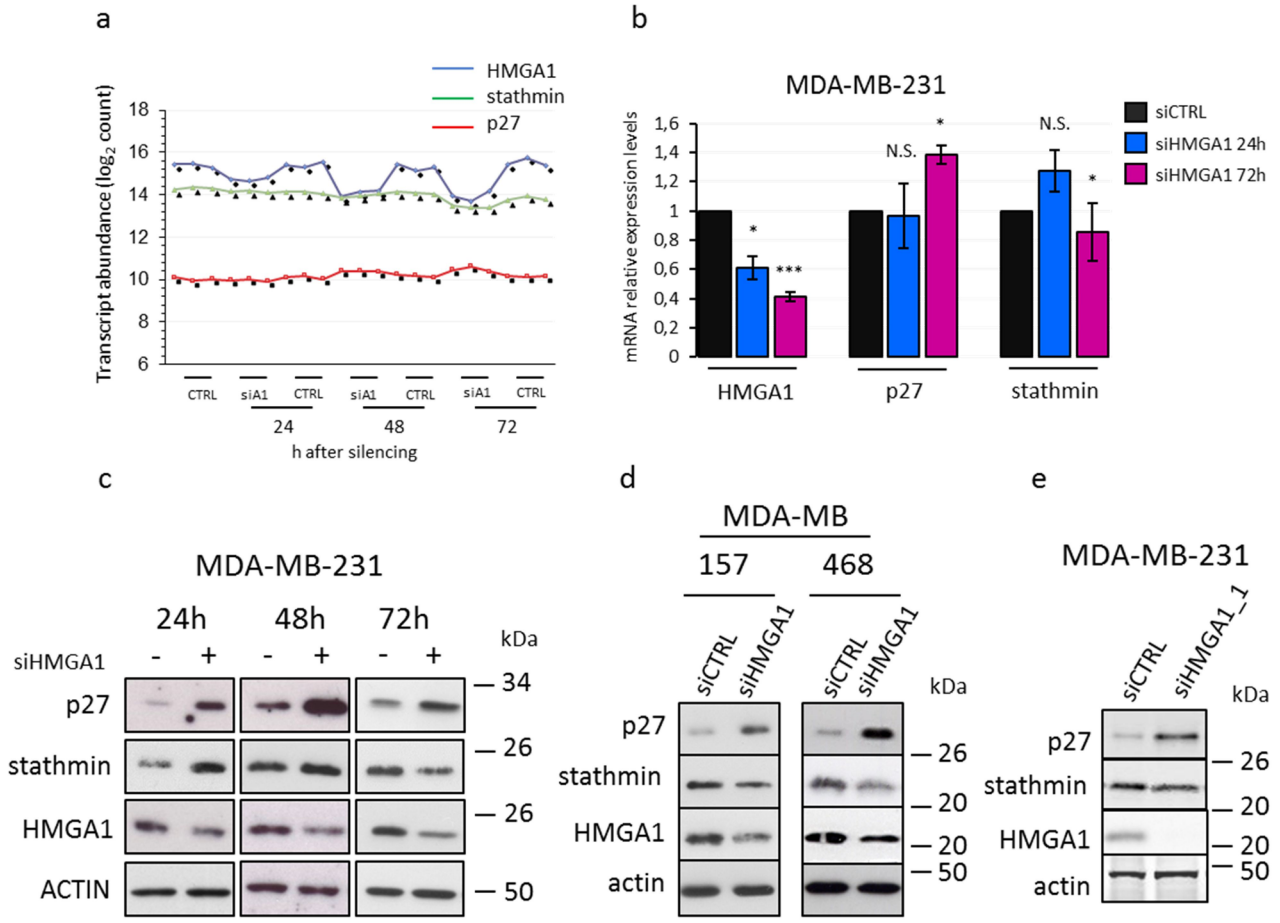


Fig. 3. HMGA1 REGULATES p27 AND STATHMIN AT POST-TRANSCRIPTIONAL LEVEL. a) Graph reporting the mRNA expression levels of HMGA1 (blue), stathmin (green) and p27 (red) obtained by RNA-Seq analysis on MDA-MB-231 cells after 24, 48 and 72h of HMGA1 silencing. b) qRT-PCR of p27 and stathmin in MDA-MB-231 cells after 24 and 72h of HMGA1 silencing (blue and pink bars respectively). GAPDH was used for normalization. The data are compared to control condition and are presented as the mean±SD (n=3). *p<0.05, ***p<0.001; two-tailed Student's t-test. c) p27 and stathmin representative western blot in MDA-MB-231 cells upon 24, 48 and 72h from HMGA1 silencing. β-actin is used as a loading control (n = 3). d) p27 and stathmin representative western blot in MDA-MB-157 and MDA-MB-468 cells after 72h from HMGA1 silencing. β-actin is used as a loading control (n = 3). e) p27 and stathmin representative western blot in MDA-MB-231 cells after 72h of HMGA1 silencing using siHMGA1_1 siRNA. β-actin is used as a loading control (n = 3).

4. HMGA1 REGULATES p27 AT POST-TRANSLATIONAL LEVEL

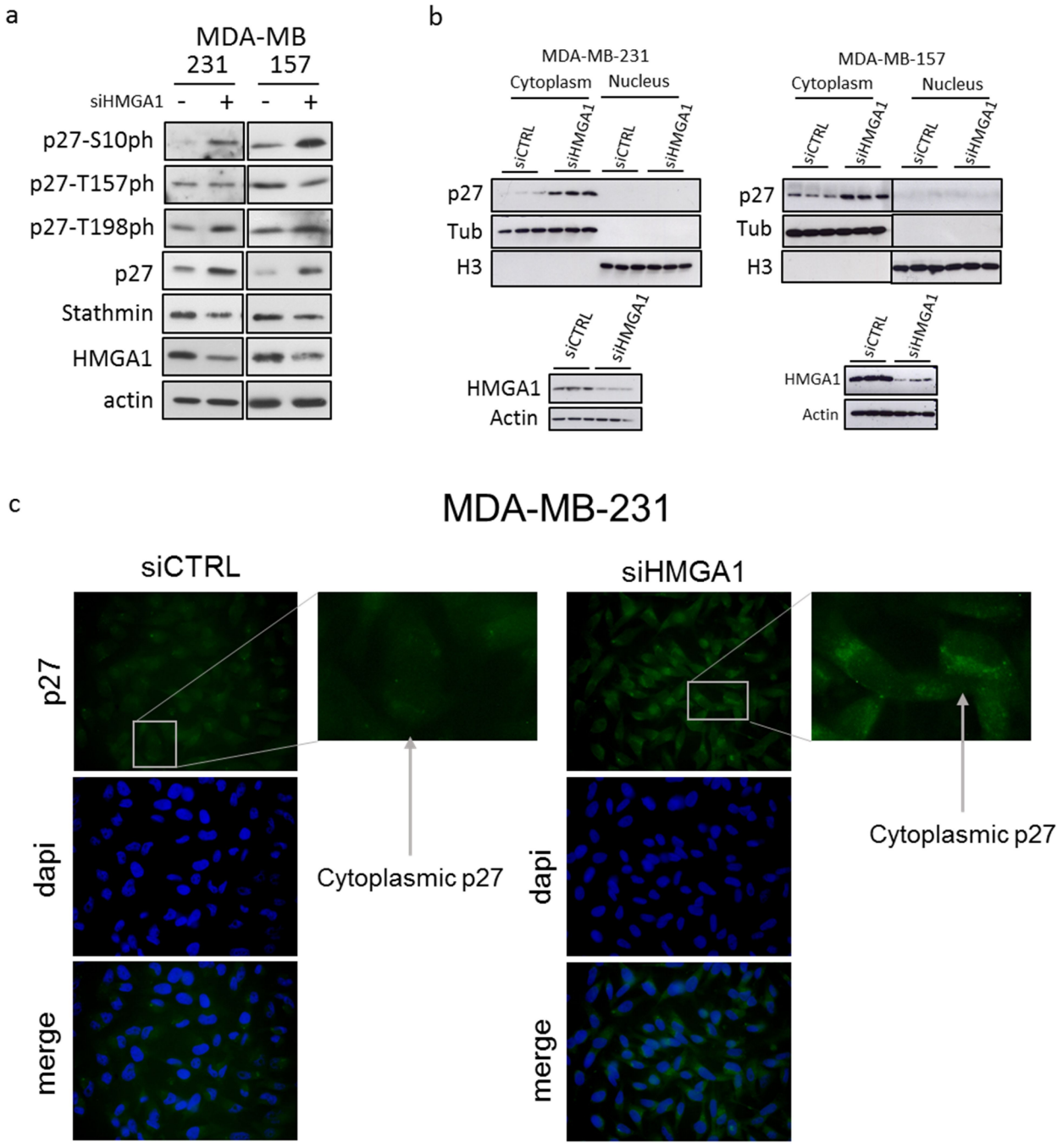
In the literature, p27 activity and stability is described to be mainly regulated at post-translational level[74]. The most important post-translational modification is the phosphorylation, which can occur on multiple sites of p27[78]. Therefore, we analysed the degree of phosphorylation on three sites described in the literature to modulate the localization and the activity of p27: the phosphorylation on S10 which induces p27 stabilization and its localization in the cytoplasmic compartment[79,147];

the phosphorylation on T157, due mainly to AKT activity[78], which is responsible for the retaining of p27 in the cytoplasm; and the phosphorylation on T198 that increases the stability and the cytoplasmic localization of p27[148]. Moreover, this modification is determinant for the binding of p27 with stathmin and modulates its activity on cell migration[69]. Thus, we silenced HMGA1 in two TNBC cell lines and we analysed the p27 phosphorylation levels on S10, T157 and T198. As shown in fig. 4a, concomitantly to higher p27 protein levels, we detected increased levels of p27-S10ph in HMGA1-silenced cells respect to control cells, suggesting that p27 could be localized in the cytoplasm of TNBC cells and that the silencing of HMGA1 could led to the accumulation of p27 protein levels in this cell compartment. On the other hand, we revealed a decrease in T157 phosphorylation status (fig. 4a). Interestingly, this modification is mediated by Akt which is a known HMGA1-downstream protein in pancreatic and hepatocellular cancers[40,149] and melanoma[150]. Therefore, HMGA1 silencing could decrease Akt activity causing a reduction in p27-T157ph. Finally, we detected an increase in p27-T198ph levels after HMGA1 silencing (fig. 4a), suggesting that p27 could be more stable and localized in the cytoplasm. Moreover, this phosphorylation is linked to its ability in binding stathmin protein. Taken together, these data indicate that after HMGA1 silencing p27 could be more stable and localized in the cytoplasmic compartment of MDA-MB-231 and -157 cells, where it is in a favourable condition for binding stathmin.

Given these results, we proceeded by determining the localization of p27 in both MDA-MB-231 and -157 cell lines. We performed a nuclear and cytoplasmic proteins fractionation on both cell lines silenced or not for HMGA1 and we detected the amount of total p27 in each fraction. We were not able to detect p27 in the nuclear fraction, but we found that p27 was present at low levels in the cytoplasm of control condition, and that it increased and localized exclusively in the cytoplasmic compartment of HMGA1-depleted cells (Fig. 4b). Then, we further confirmed the cytoplasmic localization of p27 by immunofluorescence analysis on MDA-MB-231 and -157 cells (Fig.4 c-d), where we detected a more intense and mostly cytoplasmatic p27-staining in HMGA1-depleted cells respect to control cells.

Then, to examine if HMGA1 affected p27 protein stability we performed a cycloheximide assay, treating MDA-MB-231 cells with cycloheximide in order to block protein translation for 1.5, 6 and 9h, and then analysing p27 protein levels in a control and HMGA1-silenced condition (Fig. 4e). We found that in control cells p27 was degraded more rapidly over the time respect to what observed in HMGA1-depleted cells (see graph in Fig. 4e).

These results indicate that HMGA1 influences phosphorylation status on S10 and T198, cellular localization and stability of p27, suggesting a post-translational mechanism of regulation.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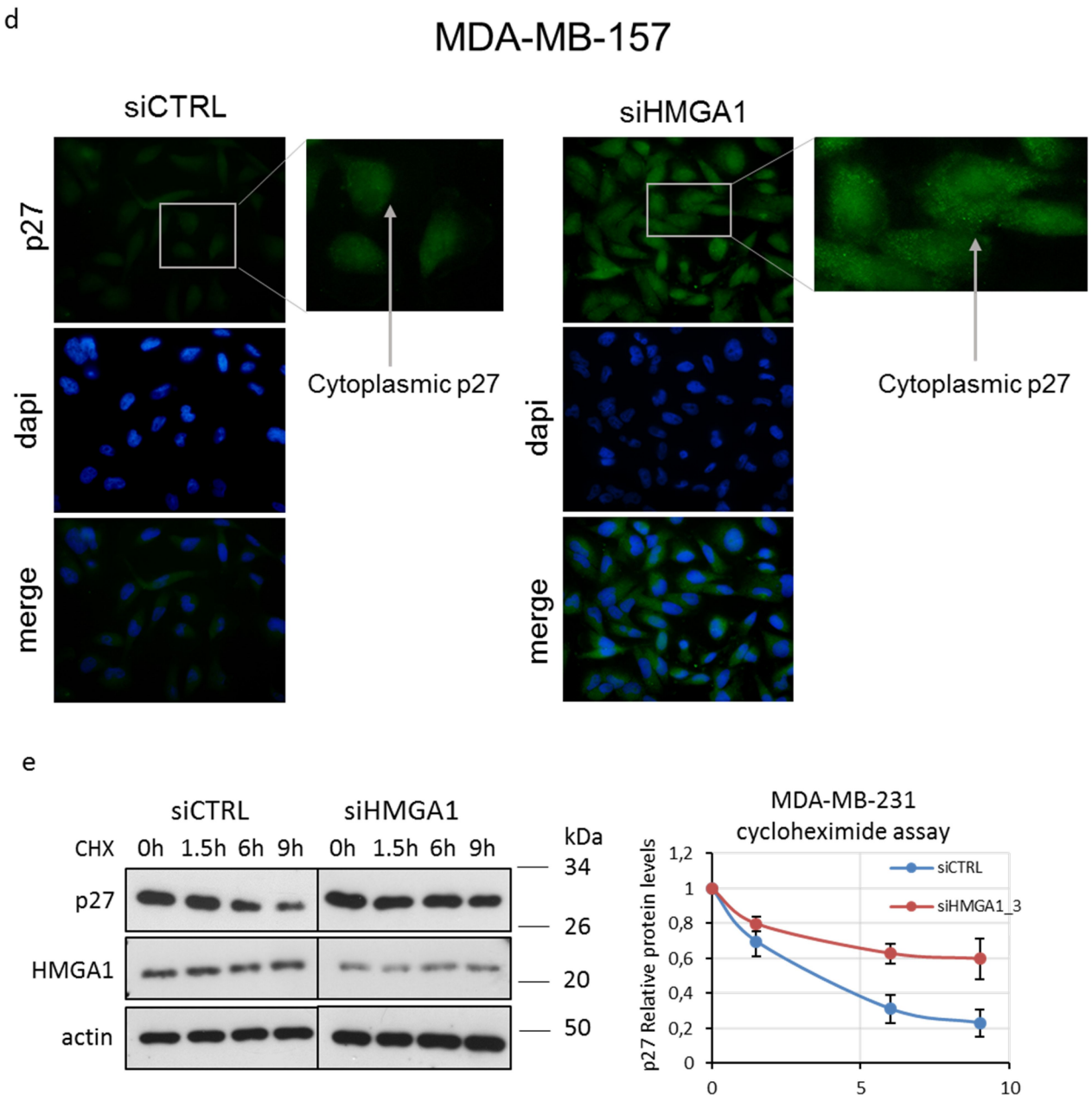


Fig. 4. HMGA1 REGULATES p27 AT POST-TRANSLATIONAL LEVEL. a) Representative western blot of p27 phosphorylations on S10, T157 and T198 after 72h of HMGA1 silencing. β-actin is used as a loading control (n = 3). b) Western Blot analysis of p27 expression in cytoplasmic and nuclear fractions of MDA-MB-231 and MDA-MB-157 after 72h of HMGA1 silencing. Tubulin was used as cytoplasmic control protein and histone H3 as nuclear control protein. c and d) Representative immunofluorescence analysis of p27 localization in MDA-MB-231 (c) and MDA-MB-157 (d) cells after 72h of HMGA1 silencing. On the right magnification of p27 staining. Images were taken at 60X magnification. e) Representative western blot of p27 expression in MDA-MB-231 cells silenced or not for HMGA1 (48h) and treated with Cycloheximide (CHX) for 1.5, 6 and 9 hours. Densitometric analysis of p27 expression (normalized respect to actin expression) are reported on the right.

5. INVESTIGATION OF A POSSIBLE HMGA1-DEPENDENT REGULATION OF p27

We next asked which could be the HMGA1-mediated mechanism through which p27 is kept at low levels in TNBC cells. p27 protein stability is mainly regulated by post-translational modifications[74,76] and several kinase-dependent pathways are known to be involved in this process[90]. Moreover, different studies described the participation of HMGA1 in the modulation of the activity of some of these pathways during the promotion of tumor aggressiveness, such as 1) the ERK pathway[49]; 2) the AKT pathway[40] and 3) the CDK2 pathway[51].

Interestingly, CDK2 is one of the most important kinases involved in the induction of p27 degradation via the proteasome pathway. Therefore, first we analysed if CDK2 was involved in p27 degradation in our TNBC cell model, silencing CDK2 expression via siRNA and then looking at p27 protein levels. We found that CDK2 silencing induced an up-regulation of p27 (Fig. 5a), indicating that it is involved in p27 protein degradation and that it could be a possible candidate for p27 regulation downstream of HMGA1. Then, to determine if the activity of HMGA1 on p27 stability is mediated by CDK2, we co-silenced CDK2 and HMGA1 in MDA-MB-231 cells and we analysed p27 protein levels comparing its levels in cells silenced for HMGA1 and CDK2 alone. As shown in figure 5a, the silencing of both CDK2 and HMGA1 induced an up-regulation of p27 similar to that obtained by the silencing of the single proteins, suggesting that CDK2 acts downstream of HMGA1 in the p27-stability regulation.

Since it is known that the CDK2-dependent p27 degradation pathway is mediated by the proteasome we investigated if the HMGA1 regulation of p27 protein stability occurred via proteasomal degradation. To this end, we first blocked the degradation via proteasome in MDA-MB-231 cells using MG132, an inhibitor of the proteasome, and we found an increase in p27 protein levels, indicating that p27 regulation involves the proteasome (Fig. 5b). Then, we treated cells silenced for HMGA1 with MG132 inhibitor and we found a further increase of the p27 levels (Fig. 5b), suggesting that HMGA1 could regulate p27 degradation also in a proteasome-independent way.

Next, with aim of identifying additional HMGA1-mediators of p27 regulation, we performed a screening. Summarizing, we used the following inhibitors:

- LY294002 (inhibitor of PI3K)
- U0126 (inhibitor of ERK)
- SNS-032 (inhibitor of CDK2 and CDK9)
- TEMSIROLIMUS (inhibitor of mTOR)
- PALBOCICLIB (inhibitor of CDK4/6)
- PF4708671 (inhibitor of S6K1)

- S3I-201 (inhibitor of STAT3)
- GSK-650394 (inhibitor of SGK1/2)

Therefore, we treated MDA-MB-231 cells with these kinase inhibitors and we looked at p27 protein levels (fig. 5c). We found that LY294002, U0126, Palbociclib and PF4708671 were able to induce an increase of the p27 protein levels, indicating that the pathways of PI3K, ERK, CDK4/6 and S6K1 are involved in maintaining p27 at low levels in the MDA-MB-231 cell line. They could be possible candidates of a downstream pathway of HMGA1 in the regulation of p27 and further analyses are needed to clarify this aspect.

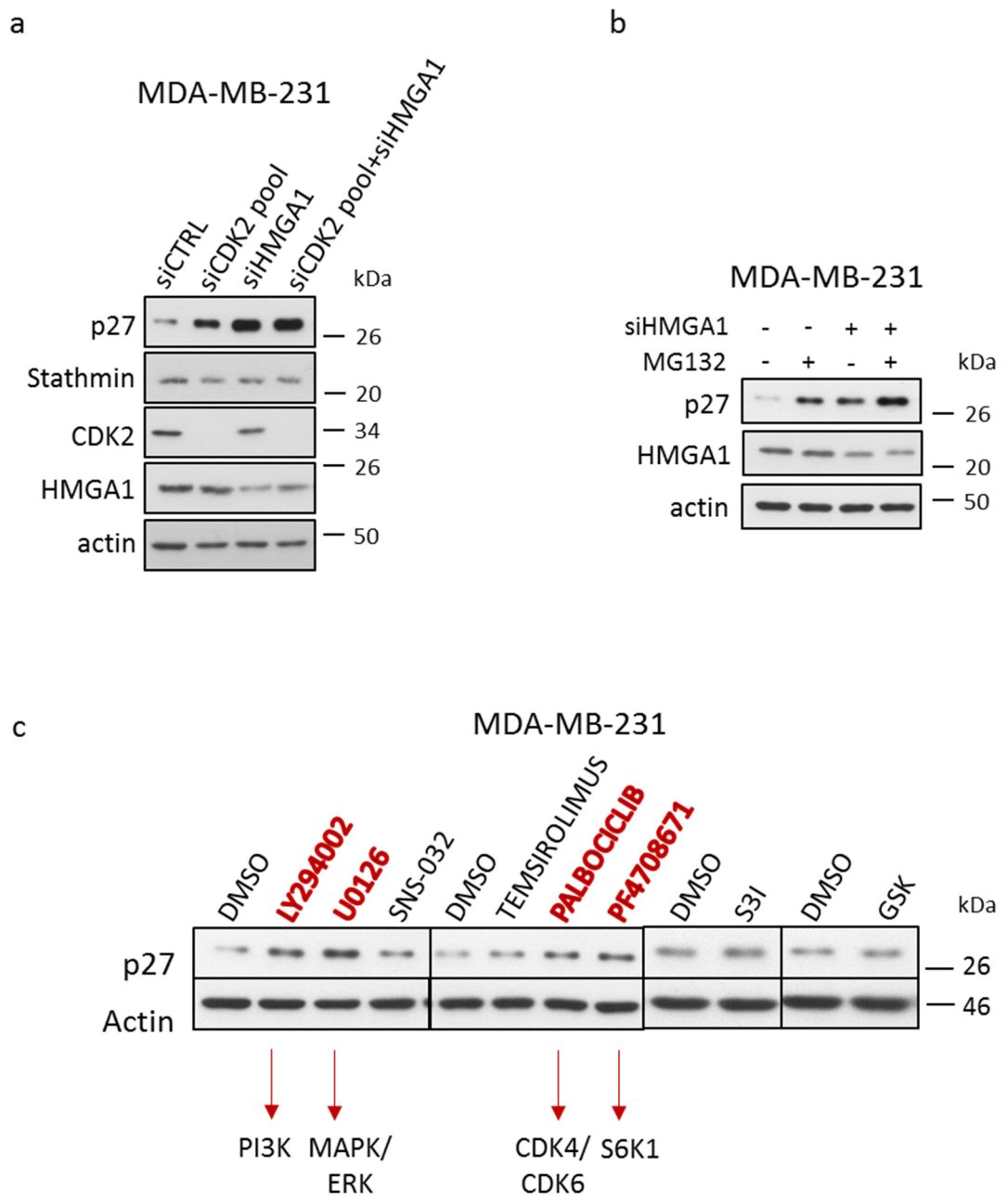


Fig. 5. INVESTIGATION OF A POSSIBLE HMGA1-DEPENDENT REGULATION OF p27. a) Representative western blot of p27 protein levels after 72h of CDK2 silencing and 48h of HMGA1 silencing in MDA-MB-231 cells. β-actin was used as a loading control, (n = 3). b) Representative western blot of p27 after 48h of HMGA1 silencing in MDA-MB-231 cells and then 8h of MG132 treatment. β-actin was used as a loading control. (n = 3). d) Representative western blot of p27 protein levels after treatment with kinase inhibitors LY294002, U0126, SNS-032, Temsirolimus, Palbociclib and PF4708671 for 8h in MDA-MB-231 cells. Drugs evidenced in red are able to upregulate p27 protein levels. β-actin was used as a loading control. (n = 3).

6. STATHMIN REGULATES MICROTUBULE DYNAMICITY AND MIGRATION OF MDA-MB-231 CELLS

Since we showed that HMGA1 regulates p27 and stathmin expression, we investigated more in detail the role of stathmin in TNBC cells, analysing its involvement in the regulation of some aggressive properties of MDA-MB-231 cells. As stathmin plays a critical role in the regulation of microtubule dynamicity acting by destabilizing microtubules, we first analysed its role in this context. For this purpose, we silenced stathmin expression in MDA-MB-231 cells and we analysed the stability of the microtubule cytoskeleton through a microtubule separation assay (Fig. 6a). We found that in MDA-MB-231 cells, the ratio of the portion of polymerized microtubules over the free tubulin was near 3:2, whereas when we silenced stathmin, the presence of free tubulin was strongly reduced and the ratio of polymerized over free tubulin increased to 4:1 (Fig. 6a). As positive control we employed paclitaxel, since it is known to induce the polymerization of all the free tubulin present in the cells. This result demonstrates that in MDA-MB-231 cells, stathmin is deeply involved in the promotion of microtubule dynamicity, an important feature of motile cells.

Second, we analysed the modulation of vimentin after stathmin silencing. Vimentin is a type III intermediate filament and well-accepted EMT marker responsible of the maintenance of cell and tissue integrity. Moreover, it is known that vimentin interacts with microtubules and that its organization contributes to modulate cell polarization and migration properties[152]. In breast cancer, vimentin is highly expressed in high grade ductal breast carcinoma and/or in tumors with low ER levels[153,154]. Thus, upon silencing or not MDA-MB-231 cells for stathmin, both the presence and localization of stathmin and vimentin were detected by immunofluorescence (fig. 6b). In a control condition, both stathmin and vimentin were present along the cell's cytoplasmic body. Whereas, when we silenced stathmin, we found that stathmin signal was very low, confirming the effectiveness of the knock-down, together with a lower signal of vimentin. Therefore, these results indicate that stathmin regulates both vimentin and microtubule dynamicity, causing in this way changes in the cytoskeleton that could have an impact on cell migration properties. Thus, we proceeded by analysing the role of stathmin in conferring migration abilities, performing a trans-well migration assay in MDA-MB-231 cells depleted or not for stathmin (fig. 6c). The MDA-MB-231 cells are highly motile and when we silenced stathmin via siRNA, we observed a strong reduction in the number of cells migrated through the porous membranes of the trans-well (fig. 6c), meaning that stathmin is crucial in conferring motile features to TNBC cells.

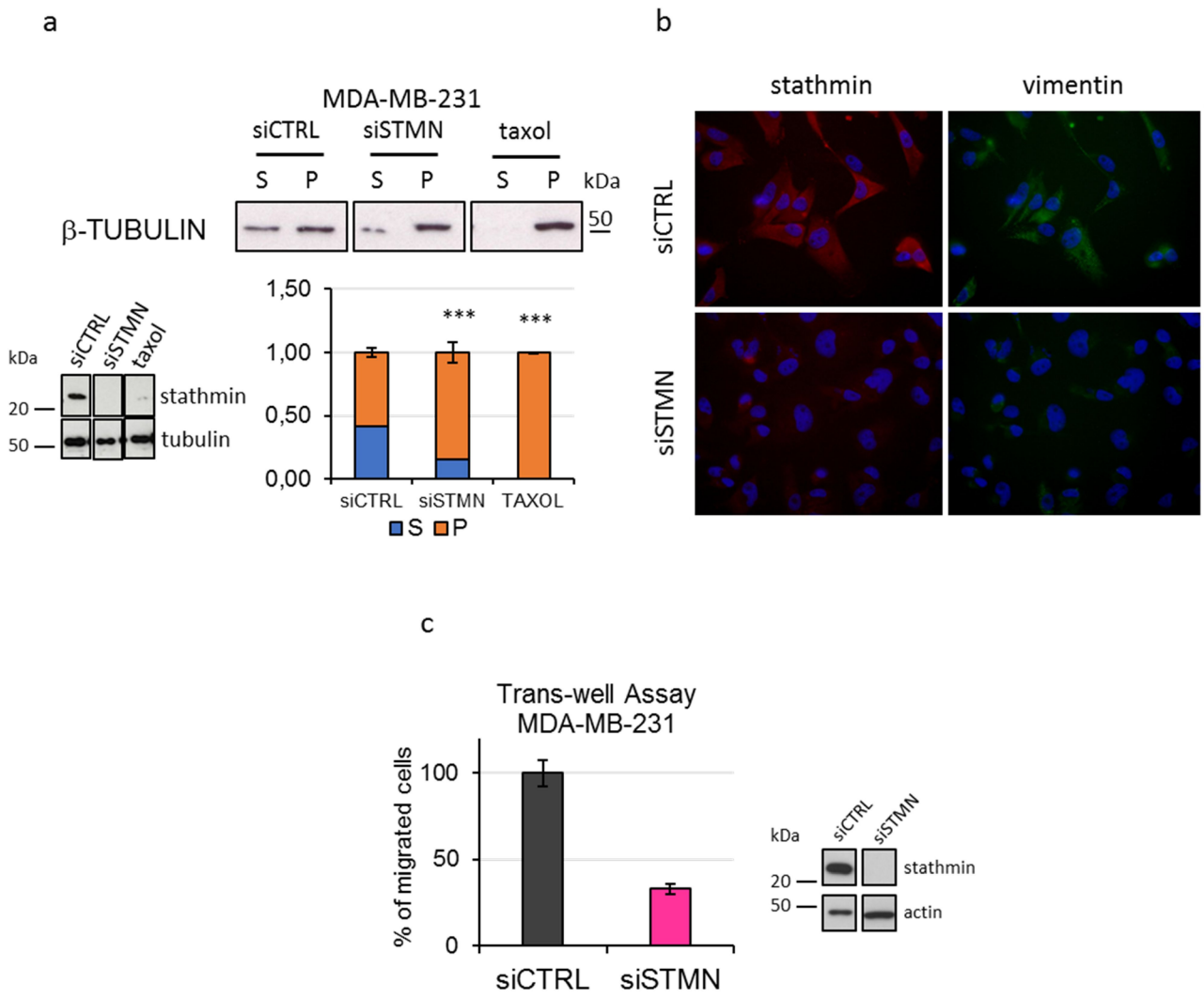


Fig.6. STATHMIN REGULATES MICROTUBULE DYNAMICITY AND MIGRATION OF MDA-MB-231 CELLS. a) Western Blot analysis of tubulin protein levels in soluble-tubulin (S) and polymerized-tubulin (P) fractions after microtubule separation assay. Densitometric analysis of tubulin expression (normalized on total tubulin amount of each sample) are reported in the bottom graphs and represent the mean (± SD) of n = 3 replicates. b) Representative immunofluorescence images of stathmin and vimentin in MDA-MB-231 cells silenced or not for stathmin. Images were taken at 60X magnification. c) Graph representing the percentage of stathmin silenced cells in respect to control condition that migrated trough the trans-well membranes. Results represent the mean (± SD) (n = 3).

7. HMGA1 REGULATES STATHMIN ACTIVITY IN MDA-MB-231 CELLS

Given the critical role of stathmin in increasing the motility of cells and in modulating the cytoskeleton, we asked whether stathmin could be an HMGA1-downstream factor in conferring aggressive features of TNBC cells. Therefore, we first analysed the relationship between stathmin and tubulin after HMGA1 silencing and compared it to the control conditions. To this end, we investigated stathmin and tubulin localization at the migratory front of the cells (Fig. 7a). A scratch was performed on HMGA1 silenced or not MDA-MB-231 cells, and allowed the cells to migrate

for 4 hours. Stathmin and tubulin were analyzed by immunofluorescence focusing the attention on the edges of the migrating cells at the front. We found that stathmin and tubulin mainly localized in the periphery of moving cells. On the contrary, in HMGA1 depleted cells, stathmin and tubulin localized in different cell portions (Fig. 7a), suggesting that HMGA1 is essential for the activity of stathmin on microtubules.

Moreover, we asked if the different localization of stathmin and tubulin after HMGA1 silencing could reflect a lower binding of stathmin to tubulin. Therefore, we immunoprecipitated stathmin in MDA-MB-231 cells silenced or not for HMGA1 and looked for the presence of tubulin in the immunoprecipitated fraction. As expected, we found that stathmin formed molecular complexes with tubulin in MDA-MB-231 cells. Moreover, upon HMGA1 depletion, a reduced amount of tubulin could be detected in the molecular complexes (Fig. 7b), confirming that HMGA1 is able to regulate stathmin binding to tubulin and microtubules.

Altogether these results indicated that HMGA1 silencing reduces the activity of stathmin on cytoskeleton dynamicity. Then, we asked if this regulation was involved in the migration abilities of cells mediated by HMGA1. Thus, we performed a trans-well migration assay, silencing both HMGA1 and stathmin. We found that HMGA1 and stathmin silencing alone was able to strongly reduce the passaging of cells through the membrane pores of the trans-well. Moreover, the HMGA1 and stathmin co-silencing induced the same extent of reduction in cell migration respect to the single silencing conditions (fig. 7c), suggesting that stathmin is an actual HMGA1-downstream factor in the regulation of cell motility.

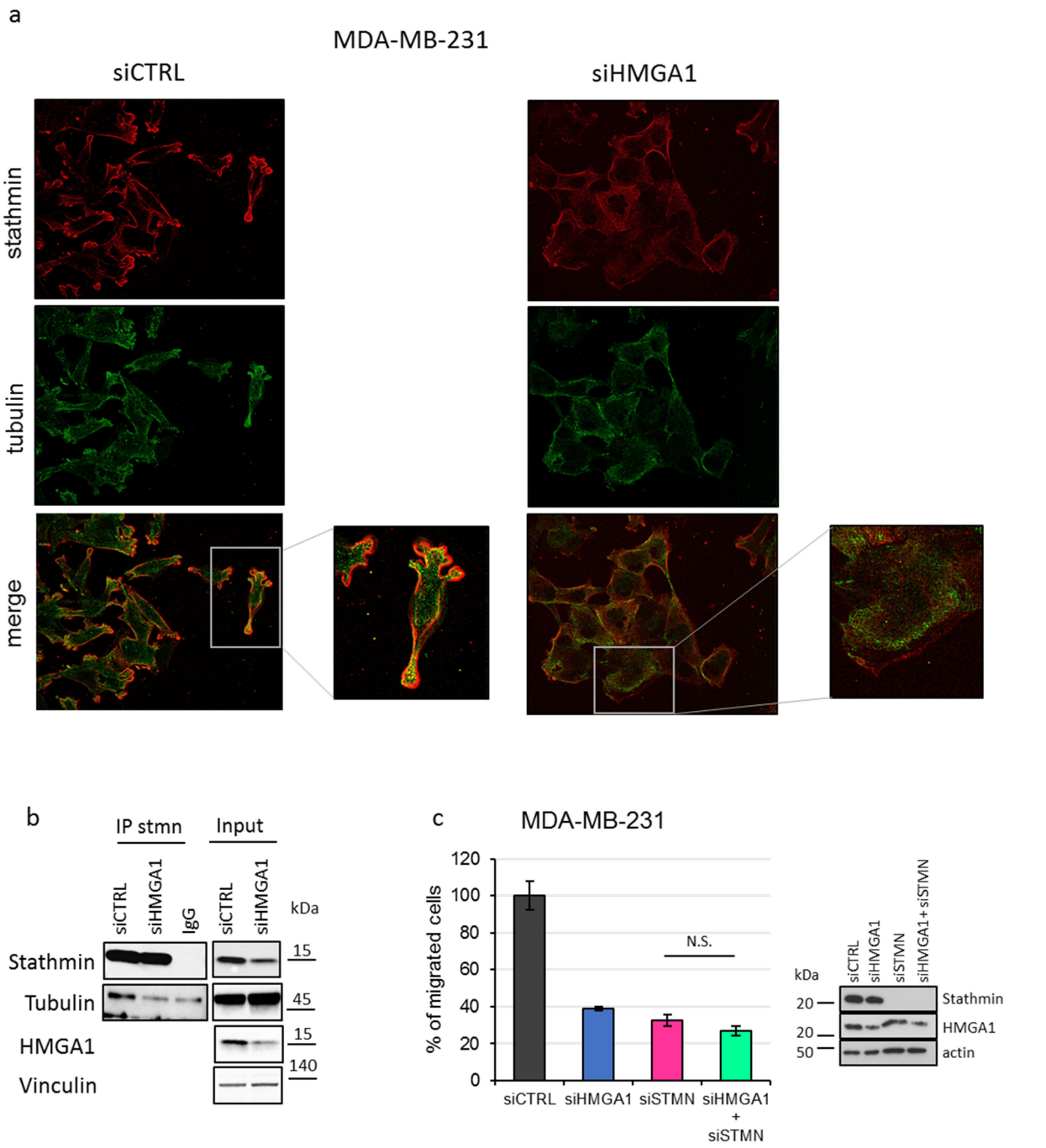


Fig. 7. HMGA1 REGULATES STATHMIN ACTIVITY IN MDA-MB-231 CELLS. a) Representative immunofluorescence analysis of stathmin (red) and tubulin (green) at the migratory front of MDA-MB-231 cells silenced or not for HMGA1. After 72h from silencing, a scratch was performed on the cell culture. Cells were allowed to migrate for 4h and then fixed and stained with specific antibodies. The ImageJ was used to focus the staining on the edges of the cells. For each condition a further magnification of the picture is reported on the right of the merged staining. Images were taken at 60X magnification. b) Lysates from MDA-MB-231 cells transfected with siRNA against control (siCTRL) and HMGA1 (siHMGA1) were immunoprecipitated with stathmin and non-specific IgG antibody. The amount of co-immunoprecipitated tubulin is reported by Western Blot analysis. c) Graph representing the percentage of silenced cells in respect to control condition that migrated through the pore membranes of the trans-well. Results are presented as the mean ± SD (n=3).

8. THE HMGA1/p27/STATHMIN AXIS REGULATES THE MIGRATION OF MDA-MB-231 CELLS

As shown above, p27 and stathmin are both regulated by HMGA1 at post-translational level. In detail, our results indicated that p27 was retained at low levels in the cytoplasm of TNBC cells by HMGA1-regulated pathways, and at the same time stathmin activity on microtubules was promoted. Moreover, our results demonstrate that stathmin plays a crucial role in the promotion of motility downstream HMGA1. Therefore, we hypothesized that after HMGA1 silencing the increased amount of p27 protein could be able to bind stathmin, inhibiting its activity on microtubules and cell migration (schematic representation of the working axis in fig. 8a). Thus, we hypothesized the presence of an HMGA1/p27/stathmin molecular axis as a key regulator of TNBC motility. To verify the presence of this axis, we performed a trans-well migration assay, silencing the expression of the proteins of interest via siRNA or shRNA in MDA-MB-231 cells. As shown in figure 8b, we first confirmed that HMGA1 and stathmin depletion induced a strong reduction of cell motility. Then, we found that p27 silencing via shRNA was not able to modulate cell migration. Since we hypothesized that the increased p27 levels induced by HMGA1-silencing were implicated in the downregulation of cell motility via stathmin inhibition, we analysed if the silencing of p27 in HMGA1-depleted cells could lead to a rescue of cell motility. Interestingly, we found a significant rescue of migration, indicating that p27 was part of an HMGA1-mediated signalling pathway involved in the regulation of cell motility. Finally, to analyse if the observed rescue in cell migration was determined by stathmin activity, we simultaneously silenced HMGA1, p27 and stathmin. Stathmin depletion reduced again the migration rescued by p27 silencing to the level obtained by HMGA1 silencing condition. This result confirmed the presence of an HMGA1/p27/stathmin axis playing an important role in the regulation of cell motility.

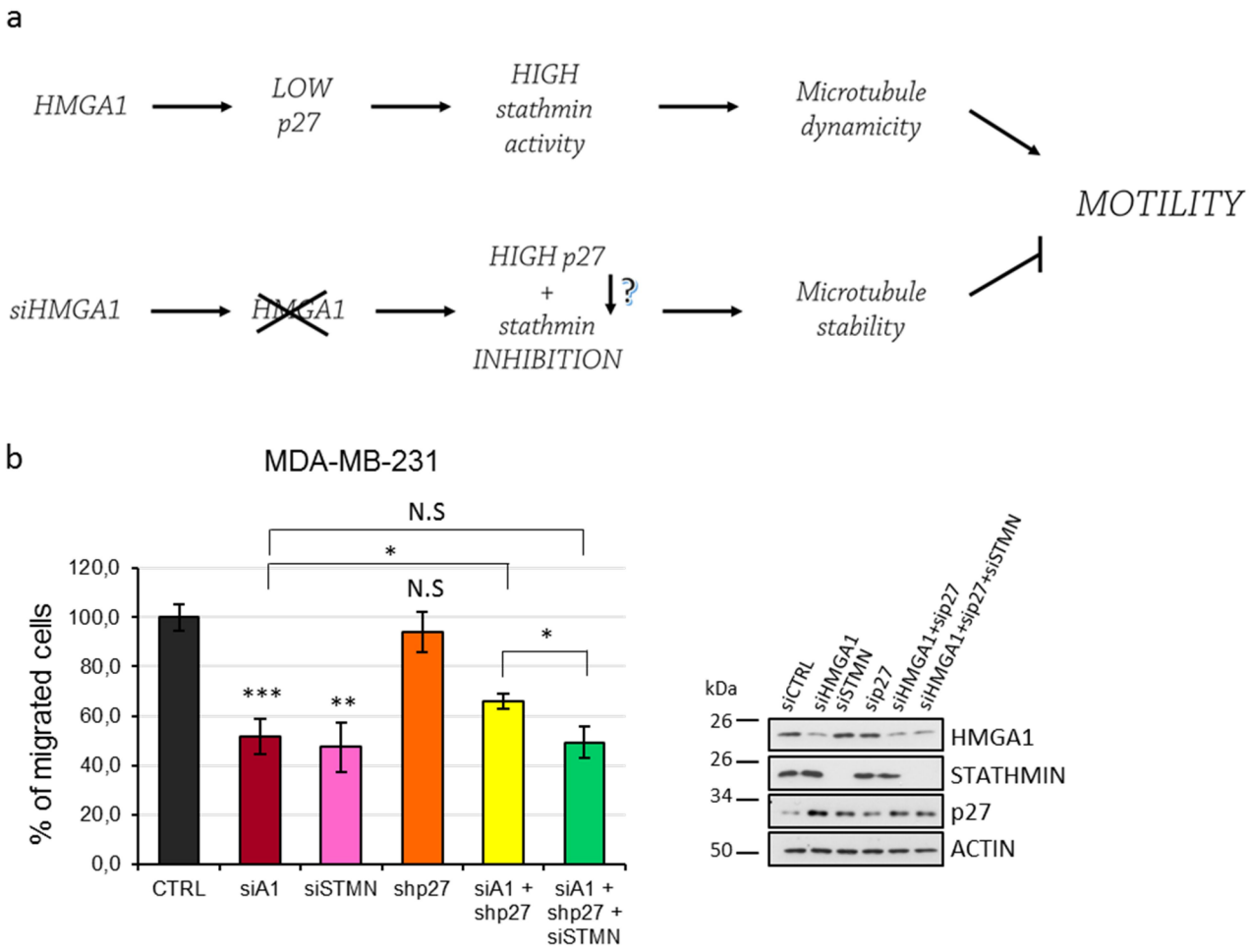


Fig. 8. HMGA1/p27/STATHMIN AXIS REGULATES MIGRATION IN MDA-MB-231 CELLS. A) Schematic representation of the axis model in migration: HMGA1 keeps p27 protein at low levels in the cytoplasm of MDA-MB-231 cells; stathmin, not inhibited by p27, in turn promotes microtubule dynamicity, enhancing cell motility. Upon HMGA1 silencing, p27 protein levels increase in the cytoplasm, stathmin activity is inhibited (probably due to p27 binding) and microtubules are more stable, reducing the motility of cells. b) Quantification of the Trans-well migration assay performed upon MDA-MB-231 silencing for HMGA1 (siA1), stathmin (siSTMN) and p27 (shp27). The results are presented as the mean of the percentage of migrated cells, comparing silenced conditions in respect to control (n = 3). A representative Western Blot of the silencing of HMGA1, stathmin and p27 is reported on the right. *p<0.05, ***p<0.001; two-tailed Student's t-test.

9. TARGETING HMGA1-p27-STATHMIN AXIS CHEMOSENSITIZES CELLS TO PACLITAXEL

The aim pursued by this thesis is to identify new signalling pathways in TNBC, in order to find specific therapeutic targets. In TNBC, the use of standard chemotherapy, such as paclitaxel or doxorubicin, is the mainstay of treatment because of the absence of other possible therapeutic opportunities and the higher pathological complete response if compared with other subtypes.

However, one of the major drawbacks of the use of chemotherapy is the uprising resistance. As reported under the introduction, there are evidences that high stathmin protein levels are responsible of paclitaxel resistance in breast cancer. Paclitaxel acts on microtubule stability modulation, in fact it binds microtubules, stabilizing them and protecting them from disassembly. On the other hand, stathmin is defined as a microtubule-destabilizing protein, therefore its action on microtubules is opposite to the of paclitaxel, thus impairing drug efficacy[25,26]. Our results demonstrated that HMGA1 silencing reduced the level and the activity of stathmin on microtubules. Therefore, we wanted to test whether the targeting of HMGA1-p27-stathmin axis was able to sensitize cells to paclitaxel administration.

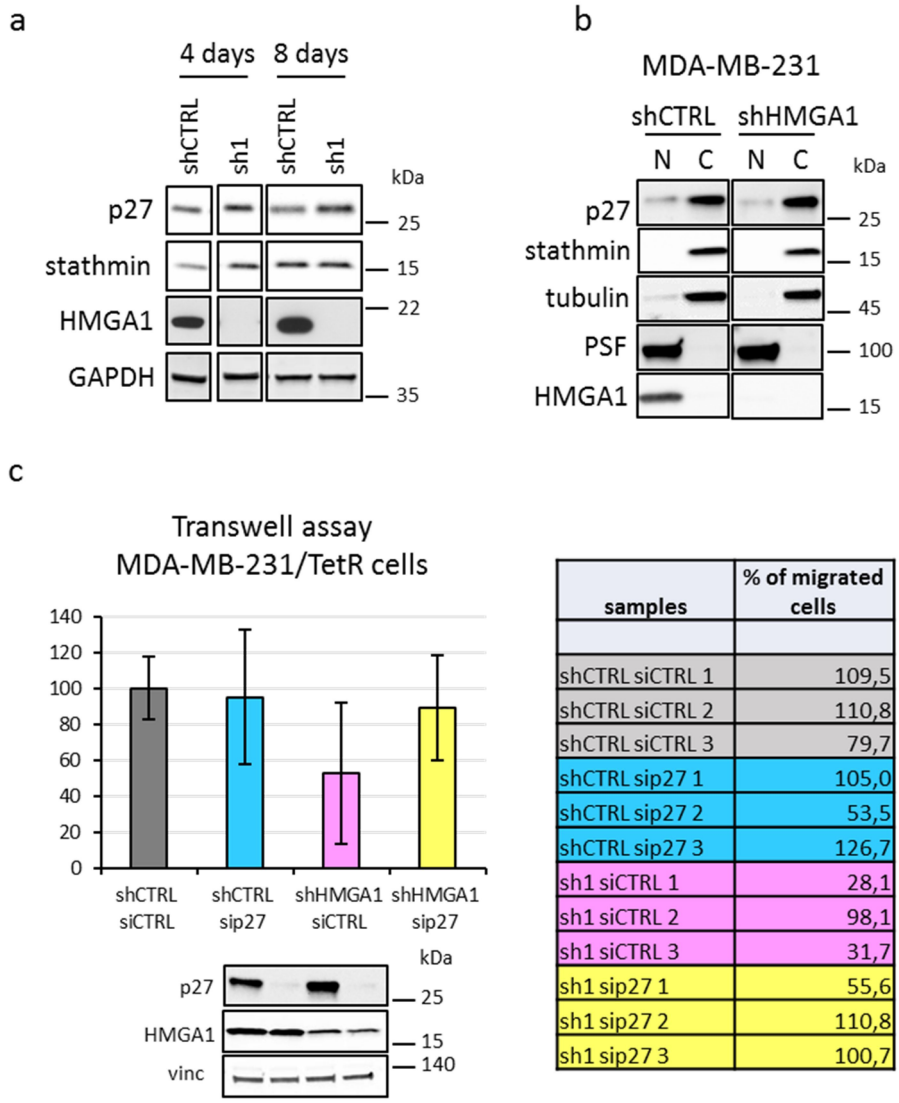
We employed MDA-MB-231 cell line stably silenced for HMGA1, to perform further experiments, which require a long-term silencing, such as *in vivo* studies. As shown in figure 9, these cells recapitulated most of the features observed in transiently silenced cells. At 4 and 8 days after shHMGA1 induction via doxycycline, we were not able to see variations in stathmin levels, while we observed the upregulation of p27 protein (fig. 9a). Moreover, we determined that p27 was mainly localized in the cytoplasm in both shCTRL cells and shHMGA1 cells (fig. 9b) and that the silencing of p27 was able to rescue the reduction of motility observed in shHMGA1 cells (fig. 9c). Taken together, these results indicate that stably HMGA1 silenced cells resembling the molecular traits of transiently silenced cells, represent a valid tool to verify the effects of HMGA1 loss for longer periods of time.

Therefore, we measured p27 and stathmin protein levels after paclitaxel treatment and we detected no modulation of p27 but a down-regulation of stathmin protein if compared with the control condition (Fig. 10a), suggesting that stathmin was implicated in paclitaxel response.

Next, we verified if HMGA1 silencing affected the decrease of MDA-MB-231 cells' viability induced by paclitaxel. Both control and HMGA1 silenced MDA-MB-231 cells displayed a similar paclitaxel IC_{50} (Fig. 10b). Next, we tested the possibility that HMGA1 inhibition could influence the colony formation in the presence of paclitaxel. Also in this case no differences were found respect to the control (Fig. 10c). These results suggested that HMGA1 did not play a role in sensitizing MDA-MB-231 cell proliferation to paclitaxel treatment, not in the formation of colonies from dispersed single cells. Next, we verified if HMGA1 could affect the migration sensitization induced by paclitaxel. To this end we performed a wound-healing assay using control or HMGA1 silenced cells challenged with paclitaxel (fig. 10d). Interestingly, we obtained a strong sensitization to the drug in HMGA1-silenced cells. In fact, treatment of HMGA1-depleted cells with paclitaxel induced a stronger reduction of cell migration respect to the control upon paclitaxel treatment. This result is in line with the role of the HMGA1-p27-stathmin axis in migration of TNBC, proving that

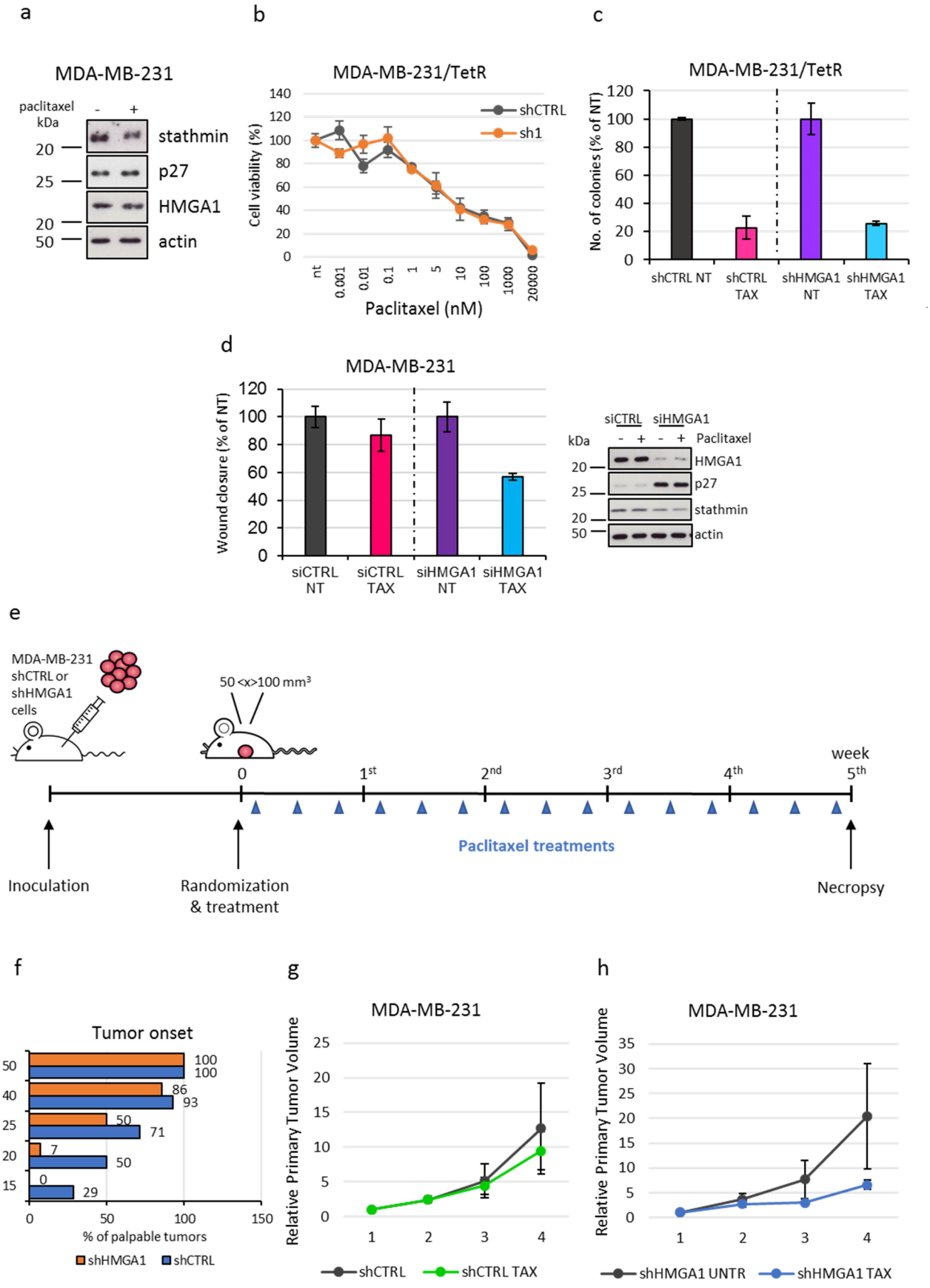
the targeting of the axis together with paclitaxel chemotherapy could be a good option for the patients in order to hinder metastasis formation.

An important step for the study of possible targeted therapeutic opportunities in TNBC, is to test the efficacy of the compounds *in vivo*. Thus, we wanted to test the efficacy of paclitaxel treatment in combination with the silencing of HMGA1 on tumor burden. Therefore, we established the MDA-MB-231/TetR inducible shCTRL and shHMGA1 xenograft mouse model, injecting MDA-MB-231/TetR inducible shCTRL and shHMGA1 clones into the mammary fat pad of nude mice and, when tumors reached a mass of 50-100 mm^3, we started to treat mice with paclitaxel 3 times a week for 5 weeks (fig. 10e). The tumors generated by shCTRL and shHMGA1 MDA-MB-231 cells had different onsets: the shCTRL MDA-MB-231 cells, on average, started to generate palpable masses (50-100 mm^3) earlier than shHMGA1 MDA-MB-231 cells (fig. 10f). To assess the efficacy of the combination of paclitaxel treatment with HMGA1 depletion on mice tumor burden, we measured every week the volume of the tumor till the end point of the treatment, when mice were sacrificed. From the results obtained, we found that paclitaxel treatment did not affect the tumor volume in mice injected with shCTRL MDA-MB-231 cells (fig. 10g), while it strongly reduced the tumor volume in mice injected with shHMGA1 MDA-MB-231 cells (fig. 10h), indicating that taxol-based treatments may be more efficacious in reducing the tumor burden when tumor cells express low levels of HMGA1. In fact, comparing the effect of paclitaxel treatment in shCTRL and shHMGA1 MDA-MB-231 injected mice (fig. 10i), we revealed that HMGA1 depletion was able to sensitize tumors to the treatment.



samples	% of migrated cells
shCTRL siCTRL 1	109,5
shCTRL siCTRL 2	110,8
shCTRL siCTRL 3	79,7
shCTRL sip27 1	105,0
shCTRL sip27 2	53,5
shCTRL sip27 3	126,7
sh1 siCTRL 1	28,1
sh1 siCTRL 2	98,1
sh1 siCTRL 3	31,7
sh1 sip27 1	55,6
sh1 sip27 2	110,8
sh1 sip27 3	100,7

Fig. 9. shCTRL and shHMGA1 MDA-MB-231/TetR INDUCIBLE CLONES RESEMBLE THE TRANSIENT SILENCING OF HMGA1 IN PARENTAL MDA-MB-231 CELLS. a) Western Blot analysis of p27 and stathmin protein levels in shCTRL and shHMGA1 MDA-MB-231/TetR inducible cells after 4 and 8 days from doxycycline induction. GAPDH was used as loading control. b) Western Blot analysis of p27 and stathmin in the nuclear and cytoplasmic fractions of the inducible clones. c) Quantification of Trans-well migration assay performed on shCTRL and shHMGA1 inducible MDA-MB-231 clones. Below, a representative western blot of p27 and HMGA1 silencing is reported. The data are presented as the mean of the percentage of the number of cells relative to the control ±SD (n = 3).


a
MDA-MB-231
paclitaxel
kDa
-
+
20
25
20
50
stathmin
p27
HMGA1
actin
b
MDA-MB-231/TetR
Cell viability (%)
shCTRL
sh1
nt
0.001
0.01
0.1
1
5
10
100
1000
20000
Paclitaxel (nM)
c
MDA-MB-231/TetR
No. of colonies (% of NT)
shCTRL NT
shCTRL TAX
shHMGA1 NT
shHMGA1 TAX
d
MDA-MB-231
Wound closure (% of NT)
siCTRL NT
siCTRL TAX
siHMGA1 NT
siHMGA1 TAX
siCTRL
siHMGA1
Paclitaxel
HMGA1
p27
stathmin
actin
e
MDA-MB-231 shCTRL or shHMGA1 cells
50 <x>100 mm³
week
Paclitaxel treatments
Inoculation
Randomization & treatment
Necropsy
f
Tumor onset
days
% of palpable tumors
shHMGA1
shCTRL
g
MDA-MB-231
Relative Primary Tumor Volume
shCTRL
shCTRL TAX
h
MDA-MB-231
Relative Primary Tumor Volume
shHMGA1 UNTR
shHMGA1 TAX

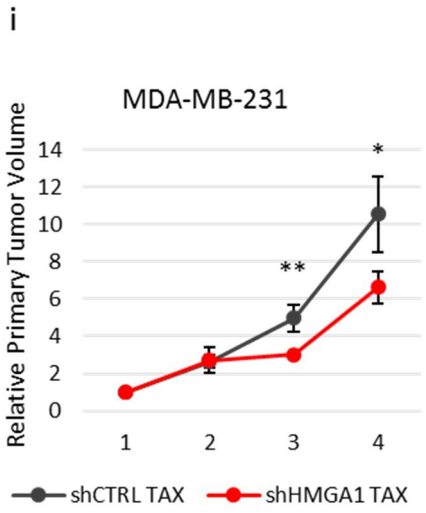


Fig. 10. TARGETING HMGA1-p27-STATHMIN AXIS CHEMOSENSITIZES CELLS TO PACLITAXEL. a) Representative western blot of stathmin and p27 after 1h of paclitaxel treatment at 1 µM concentration. (n = 3). b) Non-linear regression analysis of cell viability assay in MDA-MB-231/TetR shCTRL and shHMGA1 clones treated with increasing doses of paclitaxel for 72h. Data are expressed as percentage of viable cells with respect to the untreated cells and represent the mean (±SD) of three biological replicates. c) Graph showing the percentage of colonies formed in treated (7.5nM paclitaxel for 18h) with respect to untreated cells in shCTRL and shHMGA1 (n = 3). d) Quantification of wound healing assay of MDA-MB-231 cells silenced or not for HMGA1 and treated with 1 nM Paclitaxel for 5h during the migration after the scratch. Data are expressed as percentage of wound closure in treated with respect to untreated cells. (n = 3). HMGA1, p27 and stathmin Western Blot analysis are reported on the right of the graph. e) The scheme of the experimental protocol used for *in vivo* experiment. Mice were inoculated with MDA-MB-231/TetR shCTRL or shHMGA1 inducible clones and, when the tumor masses reached 50-100 mm^3 of tumor volume, they were randomly grouped and the treatment started as described by Materials and Methods section. f) Evaluation of tumor onset. The graph shows the different timing of tumor onset (tumor mass of 50-100mm^3) in MDA-MB-231 shCTRL and MDA-MB-231 shHMGA1 injected cells. g) Graph representing the relative volume masses of the shCTRL tumors (black) and shCTRL tumors treated with Paclitaxel (green). 4 time points during 5 weeks of treatments are reported for each condition. (n > 3 tumors). h) Graph representing the relative volume masses of shHMGA1 tumors (black) and shHMGA1 tumors treated with Paclitaxel (blue). 4 time points during 5 weeks of treatments are reported for each condition. (n > 3 tumors). i) Graph representing the relative volume masses of shCTRL and shHMGA1 treated tumors (black and red respectively). 4 time points during 5 weeks of treatments are reported for each condition. (n = 3 tumors).

DISCUSSION

This study was aimed to explore novel HMGA1-mediated pathways involved in the promotion of aggressive features of TNBC in order to find new possible therapeutic targets.

It has been previously shown that HMGA1 promotes the metastatic process in basal-like breast cancer cells by regulating EMT and stemness via the activation of a specific gene signature[48]. Moreover, investigating this gene signature, the HMGA1/CCNE2/YAP axis was found to play an important role in the regulation of breast cancer cell migration[51]. Given the crucial role of HMGA1 in conferring TNBC aggressiveness through the modulation of specific gene networks and pathways, with this thesis, we dissected other HMGA1-mediators of cell motility, demonstrating the existence of an HMGA1-p27-stathmin axis in TNBC which is responsible of the promotion of cell migration.

In this work, we found that HMGA1 expression in breast cancer patients has an inverse relation with p27 protein and a direct one with stathmin. Moreover, p27 and stathmin have an opposite expression among breast cancer subtypes and in respect to aggressive features. In fact, in basal-like subtype and in breast tumors belonging to the third grade, p27 is expressed at low level whereas stathmin is expressed at high levels. These data are in accordance with the notion that the p27 expression levels are usually associated with good prognosis[74], while stathmin is associated with poor survival and distant metastasis formation[129–133]. Moreover, we found that the expression levels of p27 and stathmin are affected by HMGA1 presence. In fact, in our TNBC cell lines, we found high levels of both HMGA1 and stathmin and low levels of p27, whereas, when we silenced HMGA1 we observed a strong up-regulation of p27 and a down-regulation of stathmin, revealing a new HMGA1-dependent gene network.

Thus, we demonstrated that HMGA1 regulates p27 mainly at post-translational level in TNBC cells. In fact, the HMGA1 silencing leads to a strong up-regulation of p27 protein already at 24 hours after HMGA1 silencing whereas at the mRNA levels we observed only a slight mRNA modulation of p27 at 72h from HMGA1 depletion. This finding sustains the notion that in cancer p27 is a gene poorly mutated but widely regulated at post-transcriptional level[76,78]. Indeed, it is well known that p27 is extensively modified by phosphorylations. Depending on the phosphorylation site, p27 can assume different intracellular localizations, functions and can be modulated at protein stability[77]. Interestingly, we found that, when HMGA1 is depleted, p27 is up-regulated and concomitantly phosphorylated in S10 and T198 in TNBC cells. These PTMs indicate that p27 could i) localize in

the cytoplasm, ii) have higher protein stability and iii) interact with stathmin. Thus, we confirmed that p27 is mainly localized in the cytoplasm and that it is more stable, demonstrating that HMGA1 regulates p27 at post-translational level. These evidences proved for the first time a functional relationship between HMGA1 and p27 protein. In addition, this HMGA1-mediated mechanism of p27 regulation better describes HMGA1 aggressiveness in TNBC: in fact, cells depleted for HMGA1 display epithelial features and reduced migration and invasion[48], and are characterized at molecular level by higher levels of p27 in the cytoplasm, a feature linked to less aggressiveness.

To better elucidate the HMGA1-dependent regulation of p27, we investigated the ways by which HMGA1 could regulate p27 protein. We observed that HMGA1 regulates p27 through CDK2 and that several other kinases are putative HMGA1-downstream effectors in p27 regulation. HMGA1 is a chromatin architectural factor that can modulate several molecular pathways regulating the transcription of different genes through i) the binding with both DNA and proteins forming the enhanceosomes, ii) the binding of transcription factors, enhancing their affinity to the DNA, or iii) acting on the modulation of the chromatin structure[27]. Recently, HMGA1 has been shown to promote aggressiveness in TNBC, and in particular the angiogenic process, through the cooperation with the transcription factor FOXM1 and the regulation of a common gene network[53]. Moreover, HMGA1 is responsible of the regulation of the activity of different kinases. The work of Pegoraro et colleagues showed as HMGA1 is responsible of the direct transcriptional regulation of CCNE2, a known interactor of CDK2, leading to cyclinE2-CDK2 complex activation and promotion of breast cancer aggressiveness[51]. Moreover, HMGA1 has been shown to regulate other important kinases in cancer: for example, it constitutively activates AKT, promoting anoikis resistance[155] and it has been shown to promote epigenetically RSK2 activity affecting the epigenetic status of tumor cells[41]. Interestingly, here we found a novel link between HMGA1 and the regulation of kinases activity. In fact, we detected CDK2 as an intermediate between HMGA1 and the regulation of p27. Therefore, CDK2 not only is involved in HMGA1-CCNE2-YAP axis[51] for the regulation of TNBC cells migration, but also in the regulation of p27 stability downstream HMGA1. CDK2 activity on p27 has been described in the literature to promote its degradation through a proteasome-dependent pathway[93]. In fact, CDK2 regulates p27 by phosphorylating it on T187, leading to its subsequent enrolment in the proteasome-dependent degradation pathway. However, we observed that HMGA1 regulation of p27 stability in MDA-MB-231 cells is not fully proteasome-dependent, thus opening a new potential regulation of p27 by CDK2 and the possibility of a new regulatory mechanism of p27 stability that could be elucidated in the future.

Beside these investigations, we took in consideration other important pathways that have been shown to regulate p27 protein and/or that have been demonstrated to be regulated by HMGA1. Using specific inhibitors of kinase pathways, we found that inhibiting PI3K, ERK, CDK4/6 and S6K1 kinases, we were able to obtain an up-regulation of p27 protein levels, indicating that p27 protein is finely regulated by multiple signals. This sustains the crucial role of p27 in different cancer hallmarks such as the cell proliferation and migration. Further analysis will be necessary to understand their possible involvement in the regulation of p27 downstream HMGA1. Moreover, we noticed that one inhibitor, LY294002, that inhibits the PI3K pathway, is currently used as an inhibitor of autophagy. Because we determined that other pathways than proteasome could be involved in the regulation of p27 downstream HMGA1, we can speculate that autophagy could be a possible way of regulation of p27 protein levels. HMGA1 has already been shown to regulate the autophagic process[43] and little and contradictory data are known about p27 regulation by autophagy[77]. For these reasons it will be interesting to determine the role of autophagy in HMGA1-mediated p27 regulation. A future perspective could be to inhibit these pathways simultaneously silencing HMGA1 and, under these conditions, assess the p27 levels verifying if they are similar to those obtained only in the presence of the inhibitors (meaning that HMGA1 and the kinases are on the same axis) or lead to additive effects (meaning that they act independently). Precisely defining the kinases mediating the effect of HGMA1 on p27, will grant the possibility to target the putative kinase(s) and develop new therapeutic approaches for TNBC patients.

Furthermore, we demonstrated that HMGA1 modulates stathmin activity. We showed that stathmin in MDA-MB-231 cells has a pro-tumorigenic role: it is involved in the alteration of the cytoskeleton dynamicity, modulating the rate of tubulin polymerization and the expression of vimentin, and in the promotion of cell motility. Then, we found that these stathmin-mediated aggressive features are modulated by HMGA1. In fact, we demonstrated that HMGA1 expression modulates stathmin activity on tubulin and promotes motility through this action, thus linking for the first time HMGA1 with stathmin activity on microtubules in TNBC.

It is known that stathmin action can be modulated by two main routes: phosphorylation on serine residues[115] or by protein interactions[69,102,156,157]. The phosphorylations lead to stathmin inhibition and detachment from microtubules, while the binding with other proteins leads to stathmin sequestration from microtubules, inhibiting its activity. Among the proteins that have been reported to bind stathmin affecting cell migration, p27 and STAT3 are important players. Baldassarre and colleagues demonstrated that the stathmin-p27 interaction results in microtubule cytoskeleton stability with consequent reduction of cell migration[69]. While the stathmin-STAT3 binding leads to

microtubule stabilization and motility promotion[156]. In our context, we showed that stathmin inhibition caused a reduced TNBC cell migration, thus suggesting its interaction with p27 rather than STAT3. Moreover, we demonstrated that HMGA1 silencing caused p27 up-regulation together with its phosphorylation on T198, a well-known phosphorylation site involved in the p27-stathmin interaction[102]. Therefore, we reasonably hypothesize that HMGA1 depletion could inhibit TNBC aggressiveness through the up-regulation of p27, which in turn could bind and sequestrate stathmin from microtubules, leading to cytoskeleton stability and motility inhibition. To clearly depict this molecular mechanism, it is of fundamental importance to demonstrate the p27-stathmin interaction. This notion could bring novelty to the molecular mechanisms driving TNBC aggressiveness and could be a further important data in the study of the p27 non-canonical functions in cancer. Therefore, it will be reasonable to look if HMGA1 depletion leads to p27 and stathmin co-immunoprecipitation in MDA-MB-231 cells or if we can detect them at a jointly localization by confocal analysis.

Next, we identified an axis composed by HMGA1-p27-stathmin, involved in the regulation of MDA-MB-231 cell motility. In the literature, p27 has been described to have two opposite roles in motility when located in the cytoplasm: first of all p27 by binding and inhibiting RhoA, can foster migration[99,104]; on the other hand, cytoplasmic p27 is reported to inhibit migration through the binding of stathmin[69]. Therefore, the detection of the HMGA1-p27-stathmin axis supports the second regulatory mechanism of motility in TNBC, further sustaining the possibility that p27 and stathmin can interact with each other. Moreover, the identification of this molecular axis could be exploited to design new possible therapeutic strategies aimed at reducing metastasis formation.

Finally, we demonstrated that HMGA1 confers resistance to paclitaxel. So far, the TNBC mainstay of treatment is chemotherapy, and paclitaxel is a microtubule stabilizing drug used in the first line treatment. HMGA1 has an important role in conferring chemoresistance to several drugs, such as DNA-interfering agents, alkylating agents and mitotic inhibitors[158]. Interestingly, HMGA1 has been shown to regulate the expression of miRNA-125b, involved in the resistance of breast cancer cells to paclitaxel[60]. On the other hand, stathmin has been exhaustively studied via its inhibition or overexpression and demonstrated to cause sensitization or resistance to paclitaxel, respectively[25,26]. In fact, it is well-known that stathmin is involved in chemoresistance to paclitaxel treatment because it promotes microtubule dynamics[25]. We demonstrated by *in vitro* experiments that the cell motility-response to paclitaxel is sensitized upon HMGA1 silencing in MDA-MB-231 cells. Moreover, by *in vivo* experiments we demonstrated that HMGA1 depleted tumors are more sensitive to paclitaxel than those expressing HMGA1. With these findings, we were able to

strengthen the relationship between HMGA1 and paclitaxel, determining for the first time that HMGA1 depletion sensitize cells to the drug. Therefore, by these results we show how targeting HMGA1 axis could be a good option to reduce cell motility and the primary tumor volume.

In conclusion, in the present work we demonstrated these major findings:

- HMGA1 regulates p27 at post-translational level in TNBC cells, regulating p27 protein phosphorylation, localization and stability.
- HMGA1 promotes stathmin activity on MDA-MB-231 cells' microtubules fostering migration; the HMGA1 silencing leads to a different localization of stathmin and tubulin at the migratory front, less stathmin-tubulin binding and reduced cell motility via stathmin inhibition.
- The HMGA1-p27-stathmin axis regulates motility of MDA-MB-231 cells.
- HMGA1 depletion sensitizes MDA-MB-231 cells to paclitaxel, leading to *in vitro* reduction of cell motility and *in vivo* reduction of the primary tumor volume.

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