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Evidence for a pro-oncogenic role of Sirtuin 6 in breast tumorigenesis

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Alla mia famiglia e ai miei colleghi

che mi hanno sostenuto in questi anni….

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ABSTRACT

Background: Sirtuin 6 (SIRT6) is a member of the sirtuin family, NAD+-dependent deacetylases with key roles in cell metabolism, DNA repair and inflammation. High SIRT6 levels in breast tumors confer an adverse prognosis. However, the underlying mechanism for such observations have remained unclear in so far. Here I sought to define the effect of a heterozygous Sirt6 deletion on polyoma middle T antigen-induced mouse mammary tumorigenesis and to establish the biochemical and molecular effects of overexpressing vs. reducing SIRT6 in different breast cancer (BC) models.

Methods: SIRT6 was overexpressed in either wild type or catalytically inactive (H133Y) form, or silenced in BC cell lines (MDA-MB-231 and MCF7), and we monitored oxygen consumption rate, mitochondrial complex I, III, IV, and ATP synthase activity, cell migration and invasion in Matrigel and in transwell assays, matrix metalloproteinase 9 (MMP9) expression and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). In vivo, we monitored the growth of MDA-MB-231 xenografts in which SIRT6 was silenced vs. control tumors. In addition, we crossed Sirt6+/- mice with MMTV-PyMT+/- mice and comparatively monitored tumor latency and overall survival in MMTV-PyMT+/-;Sirt6+/- vs. MMTV-PyMT+/-;Sirt6+/+ mice.

Results: In cultured BC cell lines, overexpression of a catalytically active SIRT6 (but not of the catalytically inactive isoform) boosted OXPHOS and the ATP/AMP ratio. Opposite effects were obtained by SIRT6 silencing. Modulating SIRT6 profoundly affected MMP9 expression and $[Ca^{2+}]_i$. Namely, in MDA-MB-231, SIRT6 overexpression increased, while SIRT6 silencing reduced MMP9 production. $[Ca^{2+}]_i$ was increased in WT-SIRT6 overexpressing MDA-MB-231 and such an effect appeared to reflect Ca^{2+} freeing from its thapsigargin-sensitive stores. Consistent with these data, SIRT6-overexpressing MDA-MB-231 were more invasive than their control cells *in vitro* assays. *In vivo*, subcutaneous xenografts of SIRT6-silenced MDA-MB-231 cells were found to grow more

slowly than the control tumors. MMTV-PyMT+/-;Sirt6+/- mice exhibited a markedly increased tumor latency and an increased overall survival as compared to the control MMTV-PyMT+/-;Sirt6+/+ animals. The metabolic features of the tumor masses isolated from MMTV-PyMT+/-;Sirt6+/- mice resembled those observed in BC cell lines with silenced SIRT6, showing decrease mitochondrial complexes activity and impaired energy status. The anticancer effects of Sirt6 heterozygous deletion did not reflect reduced glucose levels in Sirt6+/- mice, as the latters had normal blood glucose concentrations.

Conclusions: Our data show that reducing Sirt6 levels has significant antitumor activity in *in vivo* BC models. SIRT6 enhances OXPHOS and energy status in BC cells. In addition, by virtue of its ability to enhance MMP9 expression and $[Ca^{2+}]_i$, SIRT6 could be a potential target for countering invasion and metastasis. Future studies should assess which molecular features predict the potential benefit of SIRT6 inhibition in BC and test the anticancer activity of SIRT6 inhibitors in BC models.

BREAST CANCER

Breast cancer is the fifth cause of cancer-related deaths but the most common cause considering only women, indeed breast cancer is 100 times more common in women than in men (although men tend to have poorer outcomes due to delays in diagnosis) and is responsible for over 500 000 deaths annually worldwide (WHO, Anastasiadi Z. et al 2017).

Incidence rates vary greatly from 19% in Eastern Africa to about 80% in Western Europe but the lowest incidence rates of the developing countries are increasing. Also the survival rates vary greatly, ranging from 80% or over in the developed countries to below 40% in low-income countries (National Collaborating Center for Cancer, 2009). Since the 90s there has been a net reduction in breast cancer mortality rates attributed to the introduction of national screening and by improvements in treatment (Sant M et al. 2006). This can also explain the difference between survival rates in developed and developing countries where there is a lack of early detection programmes, resulting in a high proportion of women presenting with late-stage disease, as well as a lack of adequate diagnosis and treatment facilities.

ETIOLOGY

Age, gender and race

The primary risk factors for breast cancer are female sex and older age. Sporadic breast cancer is relatively uncommon among women younger than 40 years but increases significantly thereafter with a peak occurring at about 50 years (Reeder J.G. et al. 2008). Statistical surveys have also shown that white women have a higher risk of developing breast cancer than women from other ethnic groups, probably due to lifestyle factors.

Lifestyle

Smoking tobacco and the use of alcohol are clearly linked to an increased risk of developing breast cancer, the earlier in life smoking and drinking began and the greater the amount, the higher the risk. Among women long-term smokers who started smoking before the age of 20 or before the birth of their first child the risk is increased from 35% to 50% (Johnson K.C. et al. 2009), while there is an increment in risk of about 7 to 12% with every extra unit of alcohol consumed per day (Boffetta P. et al. 2006).

Being overweight or obese after menopause has been associated with an increased risk of developing breast cancer due to the fat tissue production of estrogen, however the connection between weight and breast cancer risk is complex. The risk appears to be increased for women who gained weight as an adult but may not be increased among those who have been overweight since childhood (Blackburn G.L. and Wang K.A. 2007). Moreover physical activity seems to reduce breast cancer risk. A study from the Women's Health Initiative evidence that 1.25 to 2.5 hours per week of brisk walking reduced a woman's risk by 18%. Walking 10 hours a week reduced the risk a little more (Lee I.M. et al. 2012).

There may be an association between use of oral contraceptives and the development of premenopausal breast cancer, women using oral contraceptives have a slightly greater risk than

women who have never used them. This risk seems to go back to normal over time once the pills are stopped (Kahlenborn C. et al. 2006). Women who have had no children or who had their first child after age 30 have a slightly higher breast cancer risk. Having many pregnancies and becoming pregnant at a young age reduce breast cancer risk (Russo J. et al. 2005). Some studies also suggest that breastfeeding may slightly lower breast cancer risk, especially if it is continued for 1½ to 2 years. But this has been a difficult area to study, especially in countries such as the United States, where breastfeeding for this long is uncommon. These reductions could be due to the fact that both pregnancy and breastfeeding reduce a woman's lifetime number of menstrual cycles, and thus her cumulative exposure to endogenous hormones. Other risk factors include the exposure to a number of chemicals as polychlorinated biphenyls, polycyclic aromatic hydrocarbons, organic solvents and pesticides (Brody J.G. 2007).

Genetic risk factor

Genetics is believed to be the primary cause of 5–10% of all cases of breast cancer. These cancers are thought to be hereditary, meaning that they result directly from gene mutations inherited from a parent, and they are referred to as hereditary breast–ovarian cancer syndromes (HBOC). The first breast cancer genes involved in HBOC to be found were BRCA1 and BRCA2. Between women carrying BRCA genes mutations the risk of developing inherited forms of breast cancer is between 45% and 90% (Cipollini G. et al. 2004). Other genes that, if mutated, significantly increase a woman's risk of breast cancer are TP53, PTEN, ATM, CDH1, STK11, CHEK2 (De Silva et al. 2019).

Family history

Breast cancer risk is higher among women whose close blood relatives have this disease. Having one first-degree relative with breast cancer approximately doubles a woman's risk, while having 2 first-degree relatives increases the risk about 3-fold. Also having a male relative with breast cancer seems to increase the risk but the exact increment is not well known. The National Institute for

Health and Clinical Excellence (NICE) has guidelines that identify family histories that could increase your risk of developing breast cancer. These guidelines classify women into 3 groups: moderate risk of breast cancer, a high risk, or the same risk as the general population of women (a low risk).

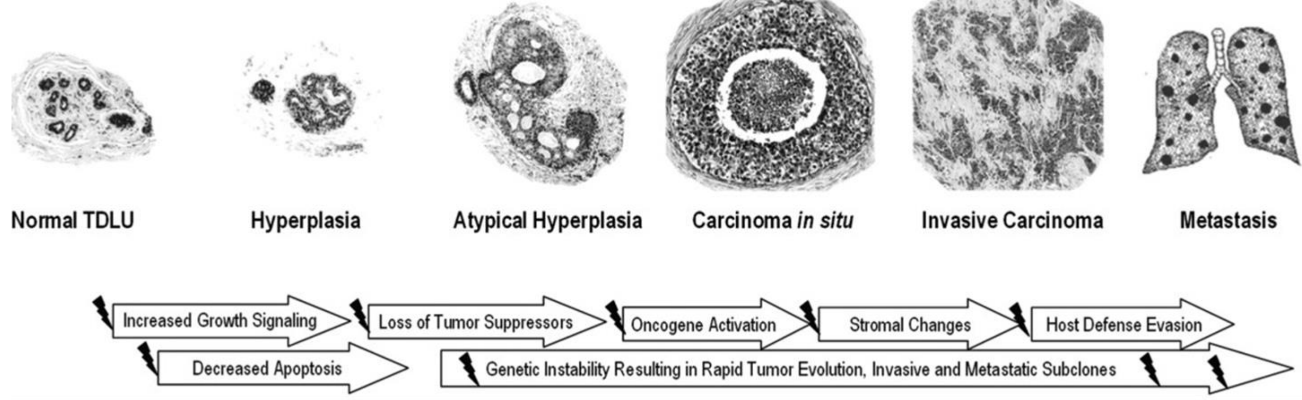
Certain benign breast conditions

Women diagnosed with certain benign breast conditions might have an increased risk of developing breast cancer. Some of these conditions are more closely linked to breast cancer risk than others. Proliferative lesions without atypia cause excessive growth of cells in the ducts or lobules of the breast tissue and raise a woman's risk of breast cancer slightly (1½ to 2 times normal). Otherwise proliferative lesions with atypia cause an overgrowth of cells in the ducts or lobules of the breast tissue, with some of the cells no longer appearing normal, and raise the risk 3-5 times higher than normal.

PATHOGENESIS

Clinico-pathological alterations

The combination of genetic and epigenetic alterations is fundamental in the genesis of breast cancer (Sadikovic B. et al. 2008). As the result of multiple alterations, mammary cancer progresses through multiple stages in a multistep process that drives normal breast cells into highly malignant cells. The resulting tumor cells can then invade through surrounding tissues and metastasize to distal organs, particularly to bones, brain, liver, and lungs. The pathogenesis involves an initial benign lesion called hyperplasia in which cells grow bigger and increased their number. Hyperplasia usually develops naturally as the breast changes with age and can occur in the ducts (ductal hyperplasia) or the lobes (lobular hyperplasia) and graded as mild, moderate or florid, according to how the cells look under the microscope. Hyperplasia doesn’t increase the risk of developing breast cancer, but it can progress through cellular atypia, cells in the breast increase in number and also develop an unusual pattern or shape. It can occur in the ducts (atypical ductal hyperplasia or ADH) or the lobules (atypical lobular hyperplasia or ALH) and it has been shown that atypia slightly increase the risk of developing breast cancer (Beckmann M.W. et al. 1997). Indeed from these cells could develop an in situ carcinoma, later becoming invasive carcinoma and gaining the capability of generating metastasis.



Damonte *et al*. Breast Cancer Research 2008 Jun 10(3)

Molecular biological alterations

The molecular alterations that lead to the development of breast cancer follow the "hallmarks of cancer" described by Hanahan and Weinberg (Hanahan D. and Weinberg R.A. 2011):

- *Self-sufficiency in growth signals.*

In promoting self-sufficiency in the growth of breast cancer, are particularly important the cellular signaling pathways of EGFR, Her2 and Ras.

The epidermal growth factor receptor **EGFR** (also known as ErbB-1) is a membrane receptor which binds the epidermal growth factor (EGF). This binding leads to the activation of the receptor, which homodimerizes or heterodimerizes with other proteins of the family (EGFR, Her2 (ErbB-2), Her 3 (ErbB-3) e Her 4 (ErbB-4)). **Her2** is a member of the same family but for its activation is not necessary a ligand but it follows the law of mass action. The dimerization stimulates their intrinsic tyrosine kinase activity that triggers the mutual auto-phosphorylation of several tyrosine residues in the C-terminal domain and the subsequent activation of downstream pathways principally the MAPK, Akt and JNK pathways. The rate of overexpression of EGFR is particularly high in a group of breast cancer defined as triple negative, while Her2 is overexpressed in a group defined as Her2 positive (Masuda H. et al.2012; den Hollander P. et al. 2013).

Ras is a small protein, member of a superfamily of GTPase, there are three genes encoding for extremely similar proteins designated H-Ras, N-Ras and K-Ras4A and K-Ras4B (from alternative splicing of the gene) (Santos E. and Nebreda A.R. 1989). Ras activates several pathways that regulate cell growth, differentiation, and survival by interacting with multiple effectors, including those in the mitogen-activated protein kinase (MAPK), signal transducer and activator of transcription (STAT), and phosphoinositide 3-kinase (PI3K) signaling cascades (Karachaliou et al 2013). Genetic Ras mutations are infrequent in breast cancer but Ras pathway may be pathologically activated by overexpression of growth factor receptors which signal through Ras

(Von Lintig F.C. et al. 2000).

Myc is a proto-oncogene coding for a transcription factor with a very important role in regulating cell proliferation and cell growth, apoptosis, differentiation and stem cell self-renewal. Myc family of transcription factors includes c-Myc, N-Myc and L-Myc genes. Normally Myc is activated upon various mitogenic signals such as Wnt, Shh and EGF (via the MAPK/ERK pathway) but it is found mutated and constitutively expressed in many types of cancers (Nilsson J.A. and Cleveland J.L. 2003). Indeed Myc overexpression stimulates gene amplification and cell proliferation and this results in the formation of cancer (Denis N. et al. 1991). Triple-negative tumors exhibit elevated MYC expression, as well as altered expression of MYC regulatory genes, resulting in increased activity of the MYC pathway, and that correlates with poor prognosis.

- *Resistance to cell death.*

The ability of tumors to escape cell death is crucial for the progression of the tumor.

p53 is a transcription factor, also known as "the guardian of the genome" because of its role in conserving genome stability by preventing its mutation and it plays a role also in the regulation of the apoptotic process and in the inhibition of angiogenesis (Kern S.E. et al. 1991). In unstressed cells, p53 levels are kept low through its continuous degradation mediated by Mdm-2, a protein capable of targeting p53 for proteasome degradation. p53 becomes activated in response to myriad stressors as DNA damage, oxidative stress, osmotic shock, ribonucleotide depletion and deregulated oncogenes expression. Once activated it can arrest growth by holding the cell cycle at the G21/S regulation point on DNA damage recognition, then it can induce DNA repair, when DNA has sustained damage, or can initiate apoptosis, if DNA damage is irreparable (Rodriguez R. and Meuth M. 2006). Mutations of p53 are associated with more aggressive disease and worse overall survival and are present in 15%–30% of ER+ breast carcinomas and up to 70-80% in HER+ and basal like breast cancer (Turner N et al.2013; Cancer Genome Atlas Network 2012).

- *Escape from the block of cell growth.*

Tumor cells can acquire the capacity of gradually disengaging from the block of cell growth imposed by tumor suppressor genes. Often the expression of these genes is inhibited in tumor cells, and this can lead to genetic instability, alteration in the cell cycle and uncontrolled proliferation. The tumor suppressor genes can be mutated, lost (whenever one of these genes is lost or inactivated by mutation in both alleles is speaks of loss of heterozygosity) or their promoter can be methylated and this result in the silencing of genes and in a functional profile virtually identical to the loss of heterozygosity (Brock M.V. et al. 2008). Studies on lung cancer have highlighted several tumor suppressor alteration.

Rb is is a tumor suppressor protein that prevent excessive cell growth by inhibiting cell cycle progression until a cell is ready to divide. Normally it can prevents cell replication and progression through G1 phase into S phase in cells where there is damaged DNA (Das S.K. et al. 2005). Rb regulates the expression of Cyclin D1, another protein involved in cell cycle regulation. This cyclin forms a complex with and functions as a regulatory subunit CDK4 or CDK6 (Cyclin-dependent kinase), whose activity is required for cell cycle G1/S transition. Deregulation of RB pathway are associated with fast growing tumors with an intrinsically poor prognosis (Ertel A et al. 2010) 20-30% of basal tumors show a loss of Rb while Cyclin D1 and CDK4 are amplified in 30-50% of breast cancers.

- *Capability of inducing angiogenesis.*

Tumor induced angiogenesis is the process of new capillary growth from an existing vasculature, induced by tumor cells that secrete vascular endothelial growth factors (VEGF). **VEGF** is a signal protein produced by cells that stimulates vasculogenesis and angiogenesis, its normal function is to create new blood vessels during embryonic development, after injury, or new vessels (collateral circulation) to bypass blocked vessels. VEGF has been implicated with poor prognosis in breast

cancer (Delle Carpini et al, 2010). Numerous studies show a decreased overall survival and disease-free survival in those tumors overexpressing VEGF. The overexpression of VEGF may be an early step in the process of metastasis, although its exact mechanism of action in the progression of tumors remains unclear (Price D.J. et al 2001).

- *Insensitivity to Antigrowth Signals*

PTEN is a tumor suppressor gene of PI3K/Akt pathway, which acts through the phosphatase activity of its protein product. PTEN specifically catalyses the dephosporylation of PIP3 in PIP2 and that results in inhibition of the AKT signaling pathway. PTEN functions normally prevent uncontrolled cell growth and it is one of the most commonly lost tumor suppressors in human cancer. PTEN is mutated or inactivated in about 30% of primary breast tumors and in 25% of the metastases (Gonzalez-Angulo A.M. et al.2011).

- *Genome Instability and Mutation*

The extraordinary ability of genome maintenance systems to detect and resolve mutations in the DNA ensures that rates of spontaneous mutation are usually very low. Cancer cells have an increment in the rates of mutation due to the breakdown of one or several components of the genomic maintenance machinery and a compromised surveillance systems that normally monitor genomic integrity induce apoptosis (Hanahan D and Weinberg R.A.2011).

BRCA1-2 are tumor suppressor genes involved in DNA double-strand breaks repair. To repair a double-strand brake, the complex BRCA is involved in utilizes the intact sequence from the homologous chromosome as a template in a process called homologous recombination. Mutations in BRCA1-2 genes lead to an increased risk for breast cancer as part of a hereditary breast-ovarian cancer syndrome. Researchers have identified hundreds of mutations in these genes, many of which are associated with an up to 80% increased risk of developing breast cancer (Balmana J. et al 2009). Women having inherited a defective BRCA1 or BRCA2 gene have risks for breast and

ovarian cancer that are so high and seem so selective that many mutation carriers choose to have prophylactic surgery.

CHK2 is a protein kinase and putative tumor suppressor activated in response to DNA damage and involved in cell cycle arrest in G1 phase, preventing entry into mitosis (Matsuoka S. et al 1998). A deletion-mutation of the CHEK2 gene is associated with an increased 2-3 fold in breast cancer risk, particularly in the European population (Meijers-Heijboer H. et al 2002).

Ataxia telangiectasia mutated (**ATM**) is a serine/threonine protein kinase, recruited and activated by DNA double-strand breaks. It phosphorylates several key proteins that initiate activation of the DNA damage checkpoint, leading to cell cycle arrest, DNA repair or apoptosis. Recent epidemiological and molecular studies have clarified the role of ATM in breast cancer and have shown that mutations in this gene confer susceptibility to breast cancer and there is a 2-fold increased risk of developing it (Ahmed M. and Rahman N. 2006).

- *Gained replicative immortality.*

With the repetition of the cell cycles DNA replication enzymes cannot replicate the sequences present at the ends of the chromosomes and these sequences and the information they carry may get lost. **Telomerase** is an enzyme that normally adds repeated sequences of nucleotides (telomeres) to the end of chromatid to maintain their length which should be shortened after each replication cycle. As a result, every time the chromosome is copied, only 100–200 nucleotides of telomeres are lost, which causes no damage to the organism's DNA. This process determines the physiological cellular senescence and when the cell loses all telomerase sequences dies (Cohen S.B. et al. 2007). From this we can deduce that cells with a strong telomerase activity , then with constantly elongated telomeres , may be able to escape to the normal aging processes proceeding towards a state of immortality. Overactive telomerase has been well documented in 70-90% of breast tumors (Papadopoulou A. et al. 2003).

- *Invasion and metastasis generation.*

Integrins are transmembrane receptors, composed of α and β chain heterodimers, that mediate cell-cell interactions and the attachment between a cell and the extracellular matrix (Humphries M.J. 2000). In addition to their role as cell adhesion molecules, they are involved in cell signaling and in the regulation of cell cycle, cell shape, differentiation, proliferation and apoptosis. Integrin upregulation in human cancers frequently indicates poor prognosis and, although breast cancer is a heterogeneous form of cancer, integrins have been identified as prognostic markers. Increased β1-integrin level, which was also linked to the level of its ligand fibronectin, is associated with significantly decreased 10-year overall survival and recurrence-free survival in patients with early-stage invasive breast cancer (Yao E.S. et al. 2007).

E-cadherin is a calcium-dependent glycoprotein which mediates cell-cell adhesion, encoded by the tumor suppressor gene CDH1. Loss of E-cadherin function or expression has been implicated in cancer progression and metastasis indeed its downregulation decreases the strength of cellular adhesion within a tissue, resulting in an increase in cellular motility. This in turn may allow cancer cells to cross the basement membrane and invade surrounding tissues. Consistent with this role in breast cancer progression, partial or complete loss of E-cadherin expression has been found to correlate with poor prognosis in breast cancer patients (Jeschke U. et al. 2007). E-cadherin is also used by pathologists to diagnose different kinds of breast cancer. When compared with invasive ductal carcinoma, E-cadherin expression is markedly reduced or absent in the great majority of invasive lobular carcinomas.

DIAGNOSIS

More than 80% of breast cancer cases are discovered whit the detection of a lump in the breast tissue or in lymph nodes located in the armpit. This is one of the first noticeable symptoms, other symptoms are variation in one breast dimension, skin puckering or dimpling, discharge from nipple/s or nipple changing position, shape or becoming inverted, constant pain and swelling of part of the breast or armpit. Unexplained weight loss, fevers or chills can occasionally herald an occult breast cancer, while bone or joint pains, jaundice or neurological symptoms can sometimes be manifestations of metastatic breast cancer. However these symptoms are non-specific and they could be manifestations of many other illnesses.

Physical examination of the breasts and mammography are the two most commonly used diagnosis methods and they can offer an approximate confirmation that a lump is cancer, and may also detect some other lesions, such as a simple cyst (Saslow D. et al. 2004). If these examinations are inconclusive, the patient can undergoes a fine needle aspiration and cytology (a sampling of the fluid the lump for microscopic analysis) to help establish the diagnosis. A finding of clear fluid makes the lump highly unlikely to be cancerous, but bloody fluid may be sent off for microscope inspection searching for cancerous cells. Other diagnostic procedures include a core biopsy or vacuum-assisted breast biopsy, procedures in which a section of the breast lump is removed, or an excisional biopsy, in which the entire lump is removed (Yu Y.H. et al. 2010). Very often the results of physical, mammography and additional tests, such as imaging by ultrasound or MRI, are sufficient to warrant excisional biopsy as the definitive diagnostic and primary treatment method.

CLASSIFICATION

In order to select the best treatment, breast cancers can be classified according to different schemes. Description of a breast cancer would optimally include all of these classification aspects: histopathological type, grade, stage (TNM), receptor status, and the presence or absence of genes as determined by DNA testing.

Histopathology

The breast is a mass of glandular, fatty and connective tissue and it's made up of lobules (glands producing milk) and ducts (tubes that carry milk from the lobules to the nipple) surrounded and protected by fatty and connective tissue, areola (area around the nipple that contains small sweat glands, which secrete moisture as a lubricant during breast-feeding) and nipple. Theoretically all types of breast tissue can generate a tumor, but the considerable majority are derived from the epithelium lining the ducts or lobules and are classified as mammary ductal carcinoma or mammary lobular carcinoma. Both tumors can be *in situ*, a noninvasive condition where the uncontrolled growth of cells is in its original site, or invasive, when it invades the surrounding tissue (Sinn H.P. and Kreipe H. 2013).

- *Ductal carcinoma in situ*

Ductal carcinoma in situ (DCIS) is a noninvasive condition with malignant cells still confined to the lactiferous ducts. It can progress and become invasive cancer, but estimates of the likelihood of this vary widely. Very few cases of DCIS present a palpable mass, they almost never produce symptoms and are diagnosed by mammography as very small specks of calcium known as microcalcifications. As screening mammography has become more widespread, DCIS has become one of the most commonly diagnosed breast conditions, now accounting for 20% of breast cancers and pre-cancers that are detected through screening mammography (Ernster V.L. et AL. 2002). DCISs have been classified into several subtypes based primarily on architectural pattern (micropapillary, papillary, solid, cribriform), tumor grade (high, intermediate, and low grade), and the presence or absence of comedo histology (Fonseca R. et al. 1997). Comedo-type DCIS consists of cells that appear cytologically malignant, with the presence of high-grade nuclei, pleomorphism, and abundant central luminal necrosis. Comedo-type DCIS appears to be more aggressive, with a higher probability of associated invasive ductal carcinoma.

- *Invasive ductal carcinoma*

Invasive ductal carcinoma (IDC) is the most common form of invasive breast cancer. It accounts for 80% of breast cancer incidence upon diagnosis (Eheman C.R. et al. 2009). On a mammogram, it is usually visualized as a mass with fine spikes radiating from the edges. On physical examination, this lump usually feels much harder or firmer than benign breast lesions such as fibroadenoma. On microscopic examination, the cancerous cells invade and replace the surrounding normal tissues. Although invasive ductal carcinoma can affect women at any age, it is more common as women grow older. There are four types of invasive ductal carcinoma that are less common:

- medullary ductal carcinoma (tumor usually does not feel like a lump, rather it can feel like a spongy change of breast tissue, only 3-5% of breast cancers are diagnosed as medullary ductal carcinoma),
- mucinous ductal carcinoma (cancer cells within the breast produce mucous and it combines with cell to form a tumor, it's rare but has a better prognosis than more common types of IDCs),
- papillary ductal carcinoma (cancer looks like tiny fingers under the microscope, common among women age 50 and older, this kind of cancer is treated like DCIS, despite being an invasive cancer),
- tubular ductal carcinoma (usually small, ER+ tumors with the aspect of tiny tubes, only 2% of tumors are diagnosed as tubular ductal carcinoma) (Colleoni M. et al. 2011).

- *Lobular carcinoma in situ*

Lobular carcinoma in situ (LCIS) is usually not considered cancer, but it can indicate an increased risk of developing invasive breast cancer (Afonso N. and Bouwman D. 2008) and most of the subsequent cancers are ductal rather than lobular. LCIS only accounts for about 15% of the in situ (ductal or lobular) breast cancers. LCIS generally leaves the underlying architecture intact and recognisable as lobules. Tumor cells are small with oval or round nuclei, small nucleoli detached from each other, estrogen and progesterone receptors are present while HER2/neu

overexpression is almost absent (Cotran R.S.K. et al. 2005).

- *Invasive lobular carcinoma*

Invasive lobular carcinoma (ILC) accounts for 5-10% of invasive breast cancer (Boughey J.C. et al. 2009) and is the second most common type of breast cancer. Although invasive lobular carcinoma can affect women at any age, it is more common as women grow older and tends to occur later in life than invasive ductal carcinoma. There are 4 different subtypes ILC, divided according to the shape of the tumor cells:

- classical (round or ovoid cells with little cytoplasm in a single-file infiltrating pattern, it's the most common type, about 55% of ILCs are classical),
- solid (cells grow in large sheets with little stroma in between them, about 10% of ILCs are solid),
- alveolar (cells form aggregates, about 15% of ILCs are alveolar),
- tubulolobular (cells has the "single-file" growth pattern of classic subtype but they form microtubules, about 5% of ILCs are tubulolobular).

Often it's possible to find different subtypes mixed without a predominant pattern (Iorfida M. et al. 2012).

Grade

Comparing the normal architecture of breast tissue with the altered structure of cancerous tissue, it’s possible to classify tumors based on the grade of differentiation of their cells. Indeed tumor cells start to lose their differentiation and the tissue, that would normally have cells line up in an orderly way, becomes disorganized. As the cells progressively lose the normal features, pathologists describe 3 grade of a breast cancer:

- low grade (cells are still well differentiated),
- intermediate grade (cells are moderately differentiated),
- high grade (cells are poorly differentiated).

High grade cancers have a worse prognosis.

Stage

The initial evaluation of breast cancer staging uses the TNM classification, a system that describes the dimension of the tumor, the lymph node involvement and the eventual presence of distant metastasis. It contemplates 5 different stages:

- Stage 0: is a pre-cancerous or marker condition, either ductal carcinoma in situ (DCIS) or lobular carcinoma in situ (LCIS).
- Stage 1: stage 1 is divided into two sub-stages: 1A and 1B. The mass is not larger than 2cm and the cancer is contained within the breast (stage 1A). Clusters of breast cancer cells smaller than 2mm are found in the lymph nodes (stage 1B).
- Stage 2: stage 2 is divided into two sub-stages: 2A and 2B. The tumor is 2-5cm or cluster larger than 2mm spread to nearby lymph nodes (stage 2A). The tumor is larger than 5cm or the tumor is 2-5cm but with cells spreading to nearby lymph nodes (stage 2B).
- Stage 3: stage 3 is divided into two sub-stages: 3A and 3B. The tumor is larger than 5cm and tumor cells spread to nearby lymph nodes (stage 3A). The tumor may be any size and cancer has

spread to the chest wall and/or to the skin of the breast and caused swelling or an ulcer (stage 3B).

- Stage 4: In stage IV, cancer has spread to other organs of the body, most often the bones, lungs, liver, or brain.

Receptor status

On their surface cells present receptors that, binding to chemical messengers, cause activation of certain signaling pathway. Breast tumors can be classified in three groups based on the presence or absence of different types of receptors:

- hormone receptor positive
- Her2 positive
- triple negative

This classification provides valuable information about tumor aggressiveness and what treatments may be more effective, indeed drug treatments can be targeted to the specific type of cancer.

- *Hormone receptor positive*

Epidemiological, biological, and clinical data strongly implicate the role of sex hormones, primarily estrogens but also androgens, in breast cancer. The sex hormone receptors, which belong to the steroid/thyroid superfamily of nuclear receptors, mediate the genomic action of estrogens by acting as ligand-dependent transcription factors (Mangelsdorf D.J. et al. 1995). The hormone receptors activation result in the stimulation of cancer cells proliferation, in the disruption of cell cycle and in the inhibition of apoptosis and DNA repair (Deroo B.J. and Korach K.S. 2006). Estrogen receptors (ER) and progesterone receptors (PR) are overexpressed in about 70% and 65% respectively of breast cancer cases and these tumors grow in response to hormones. Hormone receptor positive tumors can be divided in two subtypes: luminal A and luminal B. Both highly expressing genes normally associated with breast luminal cells, luminal A are ER-and/or PR + and Her2- while luminal B are ER-and/or PR +, Her2+ and more often associated with larger tumor size,

poorer tumor grade and p53 mutations so they tend to have a poorer prognosis than luminal A (Haque R. et al. 2012). However patients with tumors that are ER- and/or PR-+ have lower risks of mortality after their diagnosis compared to women with ER- and/or PR-negative disease because they respond very well to endocrine therapy and the five-year survival is about 10 % better than for women with hormone receptor negative tumors (Higa G.M. and Fell R.G. 2013).

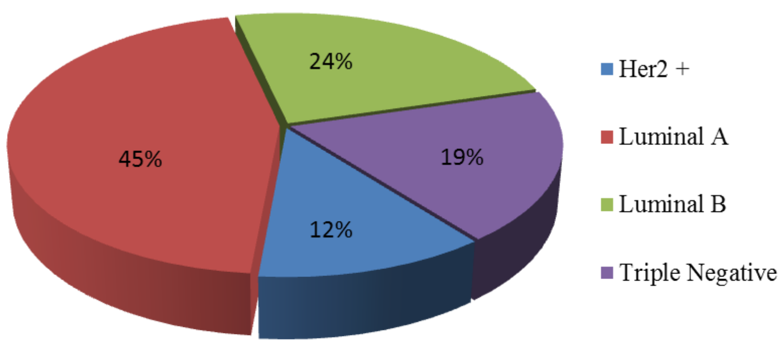
- *Her2 positive*

HER2 is a receptor tyrosine-protein kinase, member of the epidermal growth factor receptor (EGFR/ERBB) family. Dimerisation of Her2 results in the autophosphorylation of tyrosine residues within the cytoplasmic domain of the receptors and initiates a variety of signaling pathways that regulate cell proliferation and oppose apoptosi. Amplification or overexpression of this oncogene has been shown to play an important role in the development and progression of about 20-25% of breast cancer cases (den Hollander P. et al 2013) and it is strongly associated with fast-growing, increased disease recurrence and a poor prognosis (Eroles P. et al. 2012). However, effective targeted therapies have been developed to treat HER2 positive breast cancer. Targeting HER2 through different molecular mechanisms, particularly HER2 inhibitors (including trastuzumab and the dual EGFR/HER2 inhibitor lapatinib), inhibits tumor growth, induces apoptosis and improves the outcome, dramatically reducing the risk of recurrence.

- *Triple negative*

Triple negative breast cancers (TNBC) lack estrogen and progesterone receptors and do not overexpress the HER2 protein. TNBC accounts for approximately 15%-25% of all breast cancer cases and the overall proportion is very similar in all age groups even if seems to occur more often in younger women and African American women. These tumors are often aggressive and have the poorest prognosis (at least within the first five years after diagnosis) compared to the ER+ and Her2+ tumors because these tumors cannot be treated with target therapy, but usually treated

with some combination of surgery, radiation therapy and chemotherapy. BRCA1 mutation is associated with a higher risk for TNBCs. TNBCs comprise a very heterogeneous group of cancers (Hudis C.A. and Gianni L. 2011). One subtype is referred to as basal-like because the tumors have cells with features similar to those of the outer (basal) cells surrounding the mammary ducts. Most basal-like tumors contain p53 mutations (Cancer Genome Atlas Network 2012) while some of them overexpress EGFR, highly express proliferation markers Ki67 and cyclin E (Arnedos M et al. 2012). The genes linked to basal-like tumors are not well understood at this time and thus, targeted therapies do not yet exist, however, potential targets for future therapies include the EGF receptor, aB-crystallin and cyclin E. Clinical trials studying treatment options for triple negative/basal-like tumors are underway.



Incidence of breast cancer subtypes.

Kumar R. *et al.* J Pharm Bioallied Sci. 2012 Jan;4(1):21-6

TREATMENT

The medical therapies used in breast cancer treatment include surgery, which may be followed by chemotherapy, radiotherapy or other adjuvant therapy, depending on several criteria as cancer stage and type, patient's prognosis and the risk of recurrence of the cancer after the treatment. Breast cancer is usually treated with surgery to remove the tumor with some surrounding normal tissue and one or more lymph nodes that may be biopsied starting from the sentinel lymph node. The sentinel lymph node is the first lymph node to receive lymphatic drainage from a tumor, if cancer cells are not found in it, it may not be necessary to remove more lymph nodes (Moncayo V.M. et al. 2013). Standard surgeries include the whole breast removal (mastectomy) or the removal of smaller part of the breast (lumpectomy and partial mastectomy) (Agarwal S. et al. 2014). Once the tumor has been removed, the patient's breast can be reconstructed with plastic surgery, or, alternatively, women can use breast prostheses to simulate a breast under clothing. Chemotherapy can be used as a neoadjuvant therapy, in order to shrink large cancers so that they are small enough to be removed with less extensive surgery, as adjuvant therapy after surgery, to reduces the risk of breast cancer recurrence, or when the tumor is yet in an advanced stage. The chemotherapy medications are usually administered in combinations, one of the most common regimens combines anthracyclines and taxanes, which may be used in combination with certain other drugs, like fluorouracil (5-FU) and cyclophosphamide (Alken S. and Kelly C.M. 2013). Radiotherapy is usually given after surgery as adjuvant therapy. It may also have a beneficial effect on tumor microenvironment, reducing the risk of recurrence by 50–66% when delivered in the correct dose (Belletti B et al. 2008) Radiation can also be given at the time of operation on the breast cancer- intraoperatively. The largest randomised trial to test this approach was the TAR-GIT-A Trial which compared standard radiation therapy, given over several weeks after surgery, with a risk-adapted approach using single dose of TARGeted Intraoperative radioTherapy (TARGIT)

given at the time of surgery. The trial underlined found that TARGIT was equally effective at 4-years as the usual several weeks' of whole breast external beam radiotherapy (Vaidya J.S. et al. 2010).

Breast cancers ER+ and PR+ need estrogen to promote growth (Higa G.M. and Fell R.G. 2013). Ovaries are the main source of estrogen until menopause. After menopause, smaller amounts are still made in the body's fat tissue, where a hormone made by the adrenal gland is converted into estrogen. For ER+ and PR+ tumors can be used hormone therapy. Hormone therapy is often used as an adjuvant therapy to help reducing the risk of cancer recurrence after surgery, but can be used as neoadjuvant treatment as well (Jones K.L. and Buzdar A.U. 2004) . Hormone therapy can interfere with estrogen-induced tumor growth by either stopping phisically the binding between hormone and cell or lowering estrogen levels (Uramoto H. et al. 2006). Tamoxifen is an antagonist of the estrogen receptor which competitively binds to estrogen receptors on tumor cells and other tissue targets, producing a nuclear complex that decreases DNA synthesis and inhibits estrogen effects (Jordan V.C. 2006). Fulvestrant is another estrogen receptor antagonist and is used as second-line therapy for the treatment of postmenopausal women with ER+ and PR+ advanced breast cancer who have progressed following prior endocrine therapy (Croxtall J.D. and McKeage K. 2011) . Between treatments which lower estrogen levels, aromatase inhibitors (AIs) are widely used. They work by inhibiting the action of the enzyme aromatase, which normally converts androgens into estrogens in fat tissue, but they can not block ovarian production of estrogen, so AIs are used in post-menopausal women. Indeed when AIs are used in pre-menopausal women, the decrease in estrogen activates the hypothalamus and pituitary axis to increase gonadotropin secretion, which in turn stimulates the ovary to increase androgen production. The heightened gonadotropin levels also upregulate the aromatase promoter, increasing aromatase production in the setting of increased androgen substrate (Macedo L.F. et al. 2009). In pre-menopausal women,

removing (surgical ovarian oblation) or pharmacologically interfering with ovaries, the main source of estrogens, may allow some other hormone therapies to work better and is most often used to treat metastatic breast cancer, but is being studied in patients with early-stage disease (Pater J.L. and Parulekar W.R. 2003).

Thanks to the identification of molecular targets typical of cancer cells and not of normal cells it has been possible to develop target therapy for breast cancer. Opposite to chemotherapy which has a nonselective activity and causes death also in normal cells, targeted agents are designed to be selective, modulating the activity of proteins necessary and essential for oncogenesis and maintenance of the malignant phenotype, causing fewer toxic effects on normal cells (Dempke W.C. et al 2010). There are several classes of agents that are now being used in breast cancer treatment:

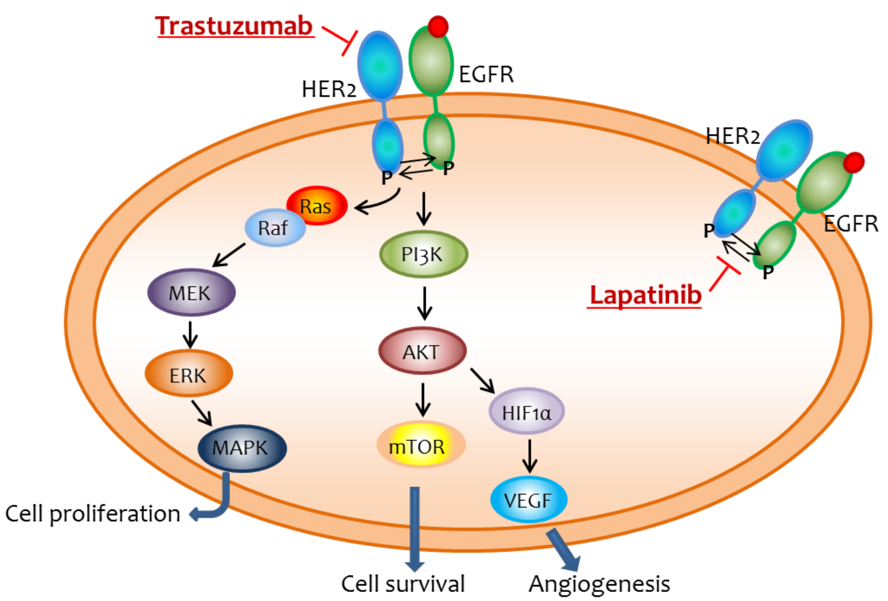
- Inhibitors of Her2 protein: both monoclonal antibodies against Her2 and tyrosine kinase inhibitors. Trastuzumab is a monoclonal antibody which binds the extracellular domain of the Her2 receptor, inducing some of its effect by leading to disruption of receptor dimerization and so interfering with the activation of downstream signaling cascade (as PI3K and MAPK pathway). In addition, it suppresses angiogenesis both by induction of antiangiogenic factors and repression of proangiogenic factors (Nahta R. and Esteva F.J. 2006). Trastuzumab has major impact in the treatment of HER2-positive metastatic breast cancer and its combination with chemotherapy has been shown to increase both survival and response rate, in comparison to trastuzumab alone (Nahta R. and Esteva F.J. 2003). . Unfortunately patients with metastatic breast cancer who initially respond to trastuzumab can develop resistance within one year of treatment initiation, and in the adjuvant setting 15% of patients still relapse despite trastuzumab-based therapy (Nahta R. and Esteva F.J. 2006).

Trastuzumab emtansine (T-DM1) is a novel antibody–drug conjugate that is composed of

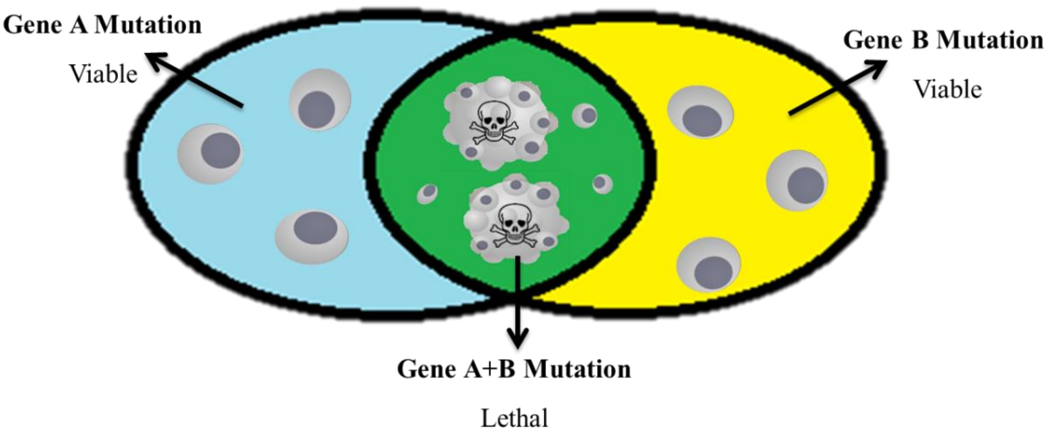
trastuzumab linked to DM1, a cytotoxic antimicrotubule agent derived from maytansine. T-DM1 retains all of the modes of action of trastuzumab, and also delivers the highly potent cytotoxic agent into HER2-overexpressing cells. Preclinical data suggest that T-DM1 has greater activity than trastuzumab while maintaining selectivity for HER2-positive cells (Krop I.E. et al. 2012)

There are other monoclonal antibodies, like pertuzumab, that target different epitope of the extracellular domain of HER2, distinct from that which binds to trastuzumab. A phase II study on women with metastatic HER2 + breast cancer, who developed resistance to trastuzumab, showed a complete response in 6.1 % of the patients and a partial response in about 20 % of the patients to a combination of pertuzumab and trastuzumab, supporting the concept that dual anti-HER2 therapy appears to be better than monotherapy (Tolaney S. 2014).

Lapatinib is a small molecule, dual Her2 and EGFR tyrosine kinase inhibitor that binds to the ATP-binding pocket of the EGFR/HER2 protein kinase domain, preventing self-phosphorylation and subsequent activation of downstream pathway (Nelson M.H. and Dolder C.R. 2006). It is approved in combination with endocrine therapy and with chemotherapy for women with metastatic breast cancer progressed while receiving chemotherapy and trastuzumab treatment (Geyer C.E. et al. 2006) . Preclinical studies suggest synergy between trastuzumab and lapatinib and the combination was assessed in a phase III trial which underlined in a significant prolongation of progression-free survival and overall survival (Blackwell K.L. et al. 2012).



Utilizing the concept of synthetic lethality has provided new opportunities for the development of targeted therapies. Synthetic lethality arises when the simultaneous mutations in two or more genes leads to cell death, while the mutation in only one of these genes is viable (Dedes K.J. et al. 2011).



The concept of Synthetic Lethality.

(3)The loss of function of BRCA1 and BRCA2 result in an increased sensitivity to poly(ADP-ribose) polymerase (PARP)-1 silencing or chemical inhibition (Farmer H et al. 2005). The rationale for this synthetic lethal interactions comes from the fact that BRCA1 and BRCA2 are tumor suppressor genes involved in the repair of DNA double strand breaks (DSBs), while PARP1 is responsible for the base excision repair of DNA single strand breaks. Inhibition of PARP activity thus leads to an

accumulation of unrepaired single strand breaks that, in proliferating cells, result to DSBs. In normal cells these DSBs are repaired by BRCA, but in cells with loss of function of BRCA the DSBs cannot be repaired, resulting in increasingly high levels of genetic instability and, eventually, cell death (Dedes K.J. et al. 2011). A significant proportion of TNBCs carry BRCA1 mutations and have gene expression profiles that are similar to those of BRCA-deficient tumors, so TNBCs may be sensitive to therapeutic strategies that target DNA repair mechanisms. Phase II clinical trials with olaparib (PARP inhibitor) are ongoing in breast cancer patients but early phase I trials were promising (Dent R.A. et al. 2013).

CANCER METABOLISM: Warburg effect vs. OXPHOS

Neoplastic transformation involves a metabolic reprograming that is reminiscent of highly proliferative normal cells during embryogenesis, wound healing, and immune response (Zacksenhaus E et al. 2017). Specifically, cancer cells acquire mutations in oncogenes and tumor suppressor that induce glycolysis and anabolic metabolism in the absence of external signals (Warburg effect). However, in contrast to Warburg's initial hypothesis, mitochondria are intact and actually play a key role in cancer cells. While the glycolysis/OXPHOS ratio is increased in cancer, both glycolysis and OXPHOS are elevated in malignant vs. healthy cells. OXPHOS enhances cancer cell anabolic metabolism, cell proliferation, cancer stemness and metastasis. Loss of the tumor suppressor retinoblastoma (RB1) in breast cancer was shown to induce mitochondrial protein translation and to boost OXPHOS. In addition, this is an emerging class of oncogenes and tumor suppressors that promote OXPHOS, such as mitochondrial STAT3, FER and cancer-specific variant, FerT, as well as CHCHD2. Thus, while aggressive tumors acquire mutations that promote aerobic glycolysis and anabolic metabolism, mounting evidence suggests that OXPHOS-activating mutations may be just as important, particularly to support cancer stem cell pools, cancer cell migration and invasion.

SIRT6

Sirtuin 6 is a member of the sirtuin family, NAD+-dependent deacetylases. In mammals, the sirtuin family includes seven members, from SIRT1 to SIRT7, with different subcellular localization, catalytic activity, targets, and functions.

SIRT6 is a multifunctional nuclear protein, involved in different physiological processes such as genome stability, longevity, glucose metabolism, neurodegenerative and heart diseases, diabetes, liver disease, inflammation, and bone-related issues. Considering that SIRT6 is involved in multiple processes there are also several studies to investigate the role of SIRT6 in tumorigenesis.

Structure of Sirt6 and its activities

The sirtuin family contains a conserved catalytic core region composed of about 275 amino acids. Their different length and sequence is due to the variable N- and C- terminal extensions, labelled as NTE and CTE respectively.

SIRT6 deacetylates the lysine through its coupling with NAD^+ hydrolysis yielding O-acetyl-ADP (adenosine 5'-diphosphoribose), nicotinamide, and a deacetylated substrate (Tanner K.G. et al. 2000). Contrary to all other sirtuins, SIRT6 can bind NAD^+ in the absence of an acetylated substrate therefore SIRT6 acts as an NAD^+ sensor while the nicotinamide products inhibits SIRT6 activity. SIRT6 contains 355 amino acids and a conserved catalytic core composed of two subdomains; a Rossmann fold domain at one end for NAD^+ binding, and a smaller, more variable, zinc-binding domain at the opposite end. It exists in an open conformation where the zinc-binding motif is divided from the Rossman-fold domain. There is a hydrogen bond that connects the zinc-binding motif to the Rossmann fold stabilizing thus the conformation of SIRT6 (Patricia W.P. et al, 2011).

The CTE of SIRT6 contains the nuclear localization signal with the following amino acid sequence ^{345} PKRVKAK ^{351} that is fundamental for appropriate sub-cellular targeting but not essential for enzymatic activity. On the contrary, the NTE of SIRT6 is necessary for chromatin association and intrinsic H3K9 and H3K56 deacetylase activity in cells. Deletion of the NTE decreases the deacetylase activity through impaired enzymatic activity. Moreover, the NTE and CTE of SIRT6 are crucial for nucleosome binding (Tennen R.I et al. 2010).

SIRT6 is tightly bound to chromatin and the principal substrates of SIRT6 are the acetyl groups on Lysine 9 and 56 of histone H3, acetyl-H3K9 and acetyl-H3K56 respectively. As a result of histone deacetylation, the chromatin is in a closed conformation and its accessibility is decreased.

Among the activities of SIRT6, has also been identified the capacity to remove long-chain fatty acyl groups (myristoyl and palmitoyl) from lysine residues in addition to the single acetyl groups. This process is known as lysine deacylation. In this case, SIRT6 use NAD^+ to produce O-myristoyl-ADP, the deacetylated substrate and nicotinamide. The demyristoylation activity is about 300-fold higher than the deacetylation activity in vitro (Jiang, H 2013). Moreover, it was seen that the free fatty acids (FFAs) can act as endogenous activators of SIRT6 deacetylase activity in vitro. It is not known if FFAs could influence the deacetylase activity in vivo (Feldman, J.L 2013).

Another activity of this protein is the ADP-ribosylation, indeed SIRT6 has been reported to mediate mono-ADP ribosylation of PARP1 (poly-adenosine diphosphate ribose polymerase 1). SIRT6 physically interacts with PARP1 and activates its poly-ADP-ribose polymerase activity. This function enhances double-strand break repair under oxidative stress (Mao, Z. et al. 2011).

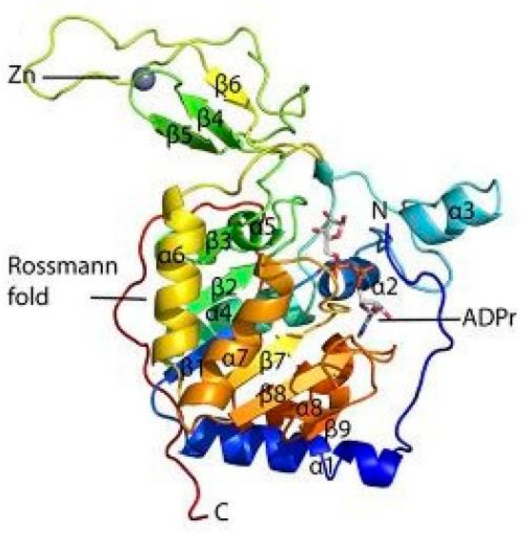


Figure A: Structure of a SIRT6 monomer (from Rubayat Islam Khan et al. 2018)

Roles of SIRT6

Sirt6 is implicated in different processes such as genomic stability, glucose metabolism, ageing, cardiovascular diseases, inflammation and cancer.

Roles of SIRT6 in the maintenance of genomic stability

Ageing is characterized by an accumulation of mutations and gene rearrangements, decreased response to stress, increased DNA damage from impaired DNA repair and further mutations. Since DNA has an important role in aging, the involvement of SIRT6 in DNA repair mechanisms, especially, in DNA double-strand breaks (DBS), in base excision repair and in the maintenance of telomeres, is of particular interest (Gorbunova V, Seluanov A, et al. 2007).

DNA Double Strand Breaks Repair

Deacetylation and mono-ADP-ribosylation activities of SIRT6 are required in double-strand DNA breaks (DSBs) repair mechanisms. Overexpression of SIRT6 has been correlated with improved homologous recombination (HR) and non-homologous end joining (NHEJ). SIRT6 interacts with a ADP-ribosylate PARP1 and stimulate its poly-ADP ribosylation activity. This stimulation was seen only in presence of DNA damage caused by oxidative stress (Mao, Z. et al. 2011).

The evidence that SIRT6 is involved in DSB repair, in particular in HR, was the finding that SIRT6 interacts and deacetylates CtIP [C-terminal binding protein (CtBP) interacting protein] (Kaidi, A. et al 2010). The HR is promoted by the resection of DSB ends, mediated by the action of CtIP together with BRCA1 (breast cancer 1). Therefore, these two proteins generate single-stranded DNA (ssDNA) that is bound by replication protein A (RPA), leading to the formation of a ssDNA-

RAD51 nucleoprotein filament responsible for homologous recombination (Jackson S.P. et al. 2009).

In the absence of SIRT6 is impaired the presence of RPA and ssDNA at DNA damage sites, the rates of HR is reduced and the cells are sensitized to DSB-inducing agents.

Moreover, SIRT6 also interacts with the DNA-dependent protein kinase (DNA-PKc) holoenzyme macromolecular complex, which includes repair factors such as DNA-PKc and Ku70/80. This complex promotes DNA DSB repair by non-homologous end joining in mammalian cells.
In presence of DSBs, SIRT6 interacts and stabilizes the chromatin and thanks to its deacetylase activity removes the acetyl groups on Lysine 9 of histone H3 (H3K9). In addition, SIRT6 is also required for the mobilization and stabilization of the DNA-PK catalytic subunit (DNA-PKcs) to chromatin (McCord R.A. et al 2009).

Among the roles of SIRT6 in DSB repair was found that the deacetylation on lysine 56 of histone H3 (H3K56) and its interaction with SNF2H (SWI/SNF-related matrix-associated actin-dependent regulator of chromatin; also known as SMARCA5) permit the localization of SNF2H to sites of DSB damage. SNF2H promotes chromatin accessibility at DNA breaks and facilitates DNA damage repair mechanisms (Toiber D. et al. 2013) (Figure B). Overall, due to SIRT6 involvement in DNA double-strand breaks repair mechanisms, it is required for the maintenance of genomic integrity.

Base excision repair

After studies in SIRT6 knockout mouse models was also identified a role in base excision repair (BER) through two different mechanisms. In one SIRT6 regulates the chromatin to increase DNA accessibility to BER factors; in the second the loss of SIRT6 causes an increase in oxidative stress levels given its function in activating poly-(ADPribose) polymerase 1 (PARP1) in response to oxidative damage (Mostoslavsky R et al. 2006).

Telomeres maintenance

Loss of SIRT6 leads to the formation of dysfunctional telomeres with random replication-associated telomere sequence loss, accumulation of telomeric DNA damage foci, and genomic instability with chromosomal end-to-end fusions that help to drive the cell into premature senescence. SIRT6 deacetylases telomeric H3K9 (Michishita E. et al. 2008) and H3K56 residues (Michishita E. et al. 2009) during S-phase for efficient association of the Werner syndrome (WRN) protein with telomeric chromatin (Figure B). The WRN protein plays a major role in genome stability, particularly during DNA replication and telomere metabolism (Chen L. et al. 2003). WRN may be required for correct capping of telomeres by the telosome/shelterin complex, as well as for replication of lagging telomeric DNA (Multani A.S et al.2007).

Finally, the genomic instability observed when SIRT6 is lost could partly be caused by a loss of association between WRN and chromatin.

Glucose Metabolism

SIRT6 in Glycolysis

SIRT6 is important for the maintenance of glucose homeostasis influencing both glycolysis and gluconeogenesis. SIRT6-deficient mice initially develop normally but then tend to exhibit hypoglycemia that causes death at about 1 month of age (Mostoslavsky R et 2006). This phenotype is not due to defects in glucose absorption in the intestine or increased glucose secretion by the kidney but is due to an evident increase in glucose uptake in adipose and muscle tissue. In vitro and in vivo studies using different cell types have shown that this increase in uptake of glucose may be linked to lack of SIRT6 (Zhong L. et al. 2010). In particular, the increase in glucose uptake is related with an increase in membrane glucose transporter-1 (GLUT1) expression and improved glycolysis to the detriment of mitochondrial respiration.

SIRT6 is able to suppress directly the expression of multiple glucose-metabolic genes such as pyruvate dehydrogenase kinase-1 (PDK1), lactate dehydrogenase (LDH), phosphofructokinase-1 (PFK1), and GLUT1. This inhibitory action is possible thanks to the deacetylation of H3K9 by SIRT6 on the promoter of hypoxia-inducible factor-1α (HIF1α) target genes (Figure B).

HIF1α promotes the expression of multiple genes that activate glycolysis and repress mitochondrial respiration.

SIRT6 in Gluconeogenesis

In the liver of SIRT6-deficient mice was found a higher expression of gluconeogenesis genes to try to compensate the hypoglycemia caused by a lower expression of SIRT6.

SIRT6 modulates gluconeogenesis modifying the activity of peroxisome proliferator-activated receptor-α coactivator 1α (PGC-1α), responsible for stimulating the expression of gluconeogenesis enzymes (Puigserver P. et al. 2003). In particular, SIRT6 interacts and deacetylases the protein GCN5 (general control non-repressed protein 5) increasing its acetyltransferase activity. GCN5 catalyzes acetylation of PGC-1α which in its acetylation form is removed from the promoters of its gluconeogenic enzyme target genes. Through this mechanism, SIRT6 is able to suppress hepatic glucose production (Dominy J.E. Jr et al. 2012).

Another study showed that increased levels of SIRT6 reduced gluconeogenic gene expression in the liver of wild type but not in liver-specific Forkhead box O1 (FOXO1) knockout mice. One hypothesis is that SIRT6 regulates hepatic gluconeogenesis both PGC-1α and FOXO1 (Xiong X et al. 2013).

Lipid Metabolism

A role of SIRT6 in lipid metabolism was also described, indeed lack of SIRT6 causes an accumulation of triglycerides (TG) which is correlated with fatty liver disease or hepatic steatosis. Without SIRT6 is evident an increase in expression of genes for hepatic long-chain fatty acid (FA) uptake and a decrease in expression of genes for β-oxidation. SIRT6 is also involved in different steps of TG synthesis acting as a negative regulator, indeed in the absence of SIRT6, there is an increase in the expression of genes for TG synthesis (Kim H.S. et al 2010).

SIRT6 is also important in the regulation of the expression of the proprotein convertase subtilisin/kexin type 9 (PCSK9) gene and low-density lipoprotein (LDL)–cholesterol homeostasis. PCSK9 is an important protein in LDL cholesterol metabolism for its role in LDL receptor degradation.

Hepatic knockout of SIRT6 determines an increased PCSK9 gene expression and elevated LDL-cholesterol levels. The PCSK9 gene is controlled by FoxO3, the transcription factor that recruits SIRT6 to the PCSK9 gene promoter and through H3K9 and H3K56 deacetylation is able to repress its expression (Figure B). Therefore, SIRT6 and PCSK9 cooperate in cholesterol homeostasis and their lack causes elevated Pcsk9 gene expression and LDL-cholesterol levels. On the contrary, when SIRT6 is overexpressed, PCSK9 is inhibited and serum LDL-cholesterol levels are low (Tao R. et al. 2013).

Moreover, a function of SIRT6 in cholesterol biosynthesis was also identified. In particular, SIRT6 suppresses sterol-regulatory element binding protein (SREBP), a fundamental protein for cholesterol biosynthesis, through three different mechanisms (Tao R, Xiong X et al. 2013). In the first, as described above for PCSK9, FoxO3 recruits SIRT6 to the SREBP gene promoter and deacetylating H3K9 and H3K56 suppresses the transcription of SREBP and its target genes (Figure B). In the second, SIRT6 prevents the activation of SREBP1/SREBP2 by reducing the transcription of

the protease complex responsible for the cleavage in their active forms. Thus the mature form does not translocate into the nucleus to promote a lipogenic pathway in the liver.

In the third mechanism, SIRT6 activates AMPK (by increasing the AMP/ATP ratio), which phosphorylates SREBP1 on Ser372 thus blocking its cleavage and nuclear translocation (Elhanati S. et al. 2013). The microRNA miR-33a is formed from the intron of SREBP1 while miR- 33b is generated from the intron of SREBP2. This microRNAs bind to the 3' untranslated region (3'-UTR) of SIRT6 mRNA and reduce SIRT6 expression (Najafi-Shoushtari S.H. et al. 2010).

Inflammation

SIRT6 has both proinflammatory and anti-inflammatory properties depending on the cell type involved. The activity of SIRT6 as lysine deacylase has a proinflammatory role. In particular, SIRT6 catalyzes the hydrolysis of myristoylated lysine 19 and 20 of tumor necrosis factor-α (TNF-α), thus allowing its secretion from the cell (Jiang H. et al. 2013) (Figure B).

TNF-α is an important proinflammatory cytokine implicated in different inflammatory diseases.

In vivo, it was seen that TNF-α had lower lysine fatty acylation and was more easily secreted in SIRT6 wild type macrophages compared to SIRT6 knockout macrophages.

The intracellular NAD^+ concentration promotes TNF-α synthesis by activated immune cells in a SIRT6-dependent manner (Van Gool F. et al. 2009).

The anti-inflammatory role of SIRT6 is shown when it acts downstream of TNF-α. TNF-α is able to activate nuclear factor kB (NF-kB), a potent proinflammatory cytokine. Notably, SIRT6 is recruited to promoters of NF-kB target genes, physically interacting with the NF-kB subunit RELA (v-rel avian reticuloendotheliosis viral oncogene homolog A) and deacetylating H3K9 silences NF-kB target genes. Therefore, SIRT6 plays a role as corepressor of NF-kB and also reduces NF-kB-dependent apoptosis and senescence (Figure B).

In SIRT6-deficient mice, it was observed that NF-kB-dependent gene expression causes a shortened lifespan and degenerative symptoms (Kawahara T.L. et al. 2009).

Another evidence of the anti-inflammatory role of SIRT6 was found in SIRT6 null 129/BlackSwiss/FVB, mice characterized by progressive chronic inflammation of the liver leading to fibrosis. This phenotype is determined by SIRT6 deficiency in the lymphocytes and myeloid-derived cells. Normally, SIRT6 interacts with c-JUN and deacetylates H3K9 at the promoter of proinflammatory genes such as monocyte chemotactic protein-1 (MCP-1), interleukin 6 (IL-6), and TNF-α. The lack of SIRT6 leads to hyperacetylation of H3K9 at the promoter of these genes and the activation of c-JUN, which allows their elevated expression (Xiao C. et al. 2012).

Lifespan

SIRT6 plays a role in life expectancy, studies have shown that an overexpression of SIRT6, in male transgenic mice, was correlated with a 15% increase in life expectancy. This phenomenon probably was caused by a reduction in insulin-like growth factor signaling in adipose tissue (Kanfi Y et al. 2012).

In response to stress, the cell produces stress granules (SGs), RNA/protein complexes important in prolonging life and usually impaired with aging-related processes (Anderson P. et al. 2009). SIRT6 may localize to SGs in the cytoplasm and support in recovery from stress that may arise from oxidative damage, heat shock, or deprivation of nutrients (Jedrusik-Bode M. et al. 2013). In particular, SIRT6 promotes dephosphorylation of G3BP [GTPase activating protein (SH3 domain) binding protein] at serine 149 (Ser149) (Figure B). G3BP is a positive regulator of SG assembly, and SIRT6 and RAS signaling contribute to this process by regulating G3BP dephosphorylation. Therefore, a loss of SIRT6 may be associated with the disruption of these SGs and the acceleration of aging-related processes. SIRT6 can be a crucial player in human aging-related diseases such as

heart disease, diabetes, obesity, inflammation, and cancer through control of genomic stability and metabolism.

Recently, studies have demonstrated that a homozygous inactivating mutation in the histone deacetylase SIRT6 results in severe congenital anomalies and perinatal lethality in four affected fetuses (Ferrer CM et.al 2018). In addition, another work suggests that SIRT6 is involved in regulating development in non-human primates, and may provide mechanistic insight into human perinatal lethality syndrome (Zhang W et al. 2018).

Heart disease

A downregulation in SIRT6 levels and activity was observed in both human and mouse failing hearts. At about 8-12 weeks of age, SIRT6 knockout mice spontaneously developed cardiac hypertrophy, whereas SIRT6 overexpression blocks the cardiac hypertrophic response. Notably, SIRT6 inhibits the expression of Insulin-like growth factor 1 (IGF) signaling by interacting with c-JUN and deacetylating H3K9 at IGF downstream targets (Figure B). Under stress conditions, SIRT6 in cardiac tissue is reduced leading to an increase in IGF signaling and in cardiac hypertrophy. Inhibition of c-JUN or IGF signaling blocks hypertrophy of SIRT6-deficient hearts. In conclusion, SIRT6 functions as a negative regulator of cardiac hypertrophy and SIRT6 activators could give therapeutic benefits (Sundaresan N.R. et al. 2012).

Cancer

Considering that SIRT6 is involved in multiple processes there are also several studies to investigate the role of SIRT6 in tumorigenesis.

SIRT6 has been proposed to act both as a tumor suppressor and as an oncogene, depending on the type of cancer, thus suggesting a tissue–dependent function.

Tumor cells have especial metabolic requirements indispensable to support the biosynthesis of macromolecules needed for cell division and growth (Metallo C.M et al. 2013). An example of metabolic reprogramming observed in cancer cells was described by Otto Warburg and defined as the Warburg Effect, (Warburg O. et al. 1927). According to Warburg, enhanced glycolysis in aerobic condition was responsible for this reprogramming. SIRT6 inhibits the activation of HIF1α, the transcription factor that promotes the expression of glycolytic genes, inhibits mitochondrial respiration and enhances the uptake of glucose in the cell (Zhong, L. et al. 2010). Studies have showed that, in mouse embryonic fibroblast (MEFs), the loss of SIRT6 led to tumor growth that was independent of oncogene activation and that primarily relied on enhanced aerobic glycolysis (Sebastian C. et al. 2012). Inhibition of glycolysis through PDK1 knockdown suppressed tumorigenesis in SIRT6 knockout cells. Moreover, to suppress glycolysis, SIRT6 was also found to co-repress MYC transcriptional activity of ribosomal genes (Figure B).

In vivo model of colon cancer, the loss of SIRT6 led to three times higher number of adenomas compared to wild type mice. These tumors showed enhanced glycolysis and pharmacological inhibition of PDK1 inhibited tumor formation in mice lacking SIRT6. In colon cancer, levels of SIRT6 correlated with tumor progression and survival. Patients with low levels develop relapse before and were more likely to relapse than patients with high levels.

SIRT6 expression is downregulated in human pancreatic ductal adenocarcinoma and colorectal carcinomas compared to normal samples and glycolytic genes GLUT1, LDH and PFK1 are upregulated.

In hepatocellular carcinoma (HCC), the high levels of SIRT6 that are frequently found at the early stages of dissease also provide a tumor suppressive mechanism. c-JUN and c-FOS are the components of the transcription factor AP-1, responsible for the liver cancer initiation. In HCC, c-FOS induces SIRT6 transcription that reduces histone H3K9 acetylation and the activation of NF-kB, the transcription factor responsible for survivin expression (Figure B). Thus, increasing the level of SIRT6 or targeting the anti-apoptotic activity of survivin is possible to slow down cancer development (Min L. et al. 2012). The inhibition of this pathway may provide preventive strategies to treat premalignant liver lesions.

In contrast to the role of SIRT6 as tumor suppressor, SIRT6 seems to act as an oncogene in other types of malignancies or, possibly, in defined stages of a certain tumor. In squamous cell carcinoma, SIRT6 is upregulated because of the downregulation of miR-34a which usually decreases the expression of SIRT6, and it promotes keratinocyte de-differentiation. In addition, another mechanism whereby SIRT6 would contribute to skin carcinogenesis is the expression of COX2, which seems to accurr via inhibition of AMPK activity (Mei Ming et al. 2014).

Studies by our group show that SIRT6 promotes cell migration and the secretion of pro-inflammatory and pro-angiogenic cytokines (TNF-α and CXCL8) by pancreatic cancer cells by a mechanism that relies on the production of o-acetyl-ADP-ribose by SIRT6 and on the consequent activation of Ca^{2+} signaling and of NFAT via TRPM2 (Bauer I et al. 2012). SIRT6 levels were found to be particularly high in pancreatic cancer cell lines compared to healthy epithelial cells, suggesting that highly aggressive forms of pancreatic malignancies, such as those cancer cell lines are typically generated from, may indeed exploit this type of SIRT6 function.

In chronic lymphocytic leukemia (CLL), high levels of SIRT6 were found to confer poor prognosis (Wang J.C. et al.2011). The role of SIRT6 as oncogene was also studied in acute myeloid leukemia (AML). Here, SIRT6 is frequently upregulated in tumor cells compared to normal $CD34^{+}$hematopoietic progenitors. SIRT6 loss causes genomic instability and consequently triggers hypersensitivity to clinically used DNA-damaging agents. Hematologic cancers including AML have constitutive ongoing DNA damage as well as a regularly activated DNA repair response. Therefore, interfering with the DNA damage response of AML cells, thereby accentuating their DNA damage, by SIRT6 inhibition has the potential to decrease tumor growth and improve patients outcome (Cea M et al. 2016; Cagnetta A et al. 2018).

In breast cancer (BC), high levels of SIRT6 were associated to a poor prognosis. SIRT6 was shown to enhance BC cell resistance to chemotherapeutics (Khongkow M et al. 2013), but also to promote BC cell survival, migration and invasion, in part via a mechanism that involves the expression of CCND1, NFκB, β-catenin, and of matrix-metalloproteinase 9 (MMP9) (Jun Sang Bae et al. 2016). Despite these findings, a recent reports indicates that SIRT6 suppresses cancer stem-like capacity in tumors with PI3K activation independently of its deacetylase activity, suggesting that pro-oncogenic functions are possibly also exerted by SIRT6 in the mammary tissue.

Thus, overall, SIRT6 has a context-dependent role in cancer and defining its potential as a target in BC treatment remains a priority.

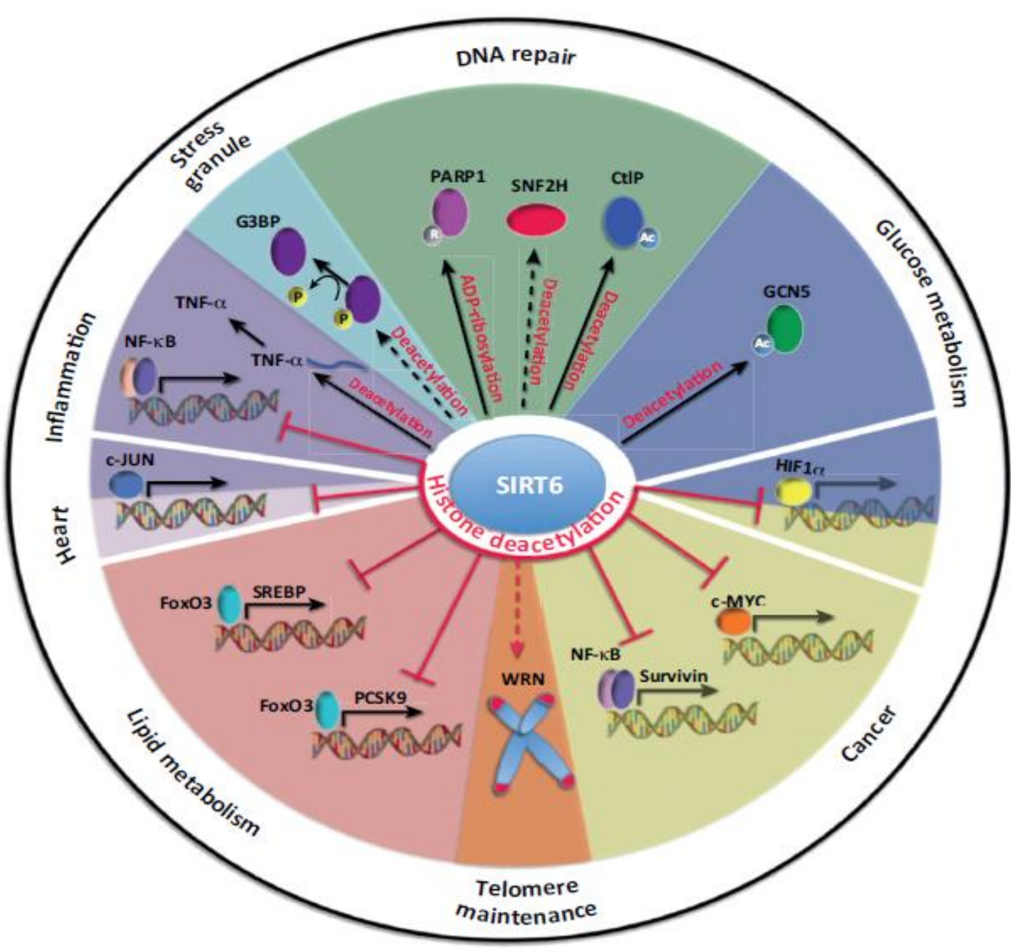


Figure B: The multitasking role of SIRT6 (from Sita Kugel and and Raul Mostoslavsky 2014)

MMTV-PyMT MOUSE MODEL

A transgenic animal is an animal whose genome has been modified through the insertion of one or more genes belonging to organisms of different species, altering its genetic composition with an insertion of exogenous DNA, transmissible to the progeny.

Obtaining transgenic mice is a fundamental step for the creation of models useful for the study of human genetic diseases.

For the creation of transgenic mice, there are three mainly used techniques:

- exploitation of viral vectors
- microinjection in the male pronucleus
- manipulation of embryonic stem cells.

Several retroviral promoters can be used to drive the expression of transgenes in mammary gland, and many well known oncogenes have been expressed under their control to induce breast carcinogenesis in mice, including, polyoma middle T antigen (PyMT).

Polyomaviruses (PyV) have proven to be invaluable models, since their discovery in the 1950s, and they led to better understand basic mechanisms that regulate cell growth and the impact of their deregulation on transformation and tumorigenesis. PyV can cause a wide variety of tumors in different types of cells. The neoplastic transformation is due to three protein named large, middle, and small tumor antigen (LT, MT, and ST, respectively). These viral oncoproteins are produced by differential splicing and mouse PyV and hamster PyV are the only two family members that have MT, which is the most important PyV transforming protein. Mammary gland-specific expression of PyMT under the control of the Mouse Mammary Tumor Virus promoter (MMTV) results in the particularly widely studied mouse model MMTV-PyMT, who shows a widespread transformation of the mammary epithelium, a development of multifocal mammary adenocarcinomas and metastatic lesions in the lymph nodes and in the lungs.

Tumor formation and progression in these mice is characterized by four stages:

-hyperplasia,

-adenoma/mammary intra-epithelial neoplasia

- early carcinoma

- late carcinoma

Summary of tumor progression in the MMTV-PyMT mouse model of breast cancer

Stage	Hyperplasia	Adenoma/MIN	Early carcinoma	Late carcinoma
Gross				
H&E				
Cellular morphology	Basement membrane Lumen Epithelial cells Myoepithelium	Tumor cells Focal infiltration of leukocytes		

The MMTV-PyMT mouse model of breast cancer is characterized by short latency, at 5 weeks of age mice have palpable tumors that involved the whole mammary fat pad, high penetrance, and a high incidence of lung metastasis occurring at 3 months of age independently of pregnancy and with a reproducible kinetics of progression (Guy, Cardiff, and Muller, 1992). It provides a relatively good model for the human disease (Lin et al., 2003; Namba et al., 2004), even though, there are significant differences from the human situation. The mouse tumors are largely ERα negative, while 50% of human tumors are positive, and the mouse tumors metastasize to the lung, whereas human metastases are more broadly distributed.

Genetic analysis indicates both the activation of PI3K and Shc is thought to be important for MT tumorigenesis (Webster et al., 1998). Tumor formation defects with a mutant MT can be enhanced by overexpression of either Shc or Grb2 (Rauh et al., 1999). Induction of metastasis seems to depend on the production of osteopontin (Jessen et al., 2004), which is regulated by MT at the transcriptional level through both Shc and PI3K (Whalen et al., 2008).

Is not totally understood if MT works as “one-hit” tumor inducer, or if it initiates a series of events that lead to other genetic changes required for tumor formation. The high frequency and short latency seem to indicate that MT is all that is needed for tumors development, but, on the other hand, there is obviously progression of the lesions (Lin et al., 2003), and it is possible to isolate mammary intraepithelial neoplasias that have different properties (Maglione et al., 2004) and show different patterns of expression (Namba et al., 2006; Namba et al., 2004).

It has long been known that the transformed phenotype varies with the amount of MT antigen expression and with differences in the nature of the immune response (Velupillai, Carroll, and Benjamin, 2002), so it is quite obvious that the strain of mice being used makes a great difference, affecting latency and metastatic potential (Winter and Hunter, 2008)

An increased metastatic potential has been shown to depend on the presence of macrophages in primary tumors and on the establishment of a chemoattractant paracrine loop of colony-stimulating factor-1 (CSF-1) and EGF ligands between macrophages and tumor cells. MMTV-PyMT mice lacking CSF-1 have delayed tumor progression and metastasis formation, that are restored by the overexpression of CSF-1 in the mammary gland (Fantozzi et al, 2006).

The crucial role of macrophages in sustaining tumor progression was further demostrated depleting plasminogen, a downstream effector of CSF-1, resulting in significantly reduced number of metastasis.

The study of MT give a great contribution to our knowledge of signaling and transformation of cells. The ability of MT transgenes to drive tumor formation in so many tissues and the vast store of mouse lines combining MT with other genetic lesions should enable MT transgenics to be excellent models to decipher tissue and tumor specific roles of signaling molecules. However genetics of MT suggest that there are important aspects of the signaling in malignant transformation that are not yet understood.

RATIONALE FOR THE STUDY

High level of Sirt6 was correlated with shorter overall survival and relapse free survival in breast carcinoma patients. In addition, knockdown of SIRT6 inhibited migration and invasion of breast cancer cells. Such an effect was linked with a decreased expression of MMP9 (Jun Sang Bae et al. 2016). Drawing from these findings, we decided to study a potential pro-oncogenic role of SIRT6 in mammary tumorigenesis. We focused on SIRT6 role in BC cell energy status, AMPK activity, Ca^{2+} signaling and in BC cell invasiveness. We investigated the role of SIRT6 in an *in vivo* model of mammary tumorigenesis, by utilizing the MMTV-PyMT mouse model (w/ or w/o an heterozygous deletion of the *Sirt6* gene) as well as MDA-MB-231 xenografts (expressing an anti-SIRT6 shRNA or a control shRNA).

In conclusion, our data show that reducing SIRT6 levels has antitumor effects in mouse breast cancer models and point to SIRT6 as a promising therapeutic target for treating breast cancer.

MATERIALS AND METHODS

CELLS LINES AND REAGENTS

MCF7, MDA-MB-231 and Phoenix cells were purchased from ATCC and were cultured in RPMI 1640 medium supplemented with 10% FBS, penicillin (50 units/ml), and streptomycin (50 µg/ml) (LifeTechnologies, Italy). Puromycin, protease inhibitor and phosphatase inhibitor mixture were purchased from Sigma Aldrich S.r.l. (Milan, Italy).

PLASMIDS

Empty pBABE-puro (PBP), and empty pRS-puro (PRS) were purchased from Addgene (Cambridge, MA, USA). pRS SIRT6 sh2, pBP SIRT6 WT, and pBP SIRT6 H133Y were a kind gift from Dr. Katrin F. Chua (Department of Medicine, Stanford University School of Medicine, Stanford, CA).

RETROVIRAL TRANSDUCTION

For retroviral transductions, 1 x 10^6 Phoenix cells were plated in 4 ml medium in 6-cm dishes and allowed to adhere for 24 h. Thereafter, cells were transfected with 4 µg of plasmid DNA using TransIT-293 (Mirus Bio, Madison, WI) according to the manufacturer's instructions. Viral supernatants were harvested after 36, 48, 60, and 72 h and used to infect MCF 7 cells ($5x10^5$), MDA-MB-231 ($3x10^5$) cells in 10 cm dishes in the presence of 5 µg/ml protamine sulfate. Successfully infected cells were selected using 1,5 µg/ml puromycin (MCF7) or 1 µg/ml puromycin (MDA-MB-231).

3D SANDWICH MATRIGEL MATRIX ASSAYS

A 24 well plate was prepared adding 150 µl of growth factor reduced matrigel (Corning). Then the plate was incubated for 30 min at 37°C. 100 MDA-MB-231 (PBP, WT, H133Y) cells per well were plated and incubated at 37°C until adhesion was reached. Subsequently we removed the medium and we added 150 µl of matrigel and incubated again for 30 min at 37°C. Finally, we added 1 ml of medium and cells were incubated for 10 days. Cell growth was monitored every 2 days until formation of spheroids. The images were acquired by an optical microscope (Leica DMI 3000 B).

FLUORIMETRIC DETERMINATION OF INTRACELLULAR $Ca2^{+}$ LEVELS

PBP, SIRT6 WT MDA-MB-231 cells (34 X 10^3 cells/well) were seeded on glass bottom cell culture dishes (Greiner Bio-One, Frickenhausen, Germany) and stimulated or not with phytohemagglutinin (PHA) (5 µg/ml). Cells were then incubated with Fura-2AM for 45 min and washed with HBSS, Hanks' balanced salt solution. HBSS was added to Fura-2-loaded cells. Alternatively, in some experiments, cells were washed and resuspended in $Ca2^{+}$-free HBSS before thapsigargin (TG) addition**.** $[Ca2^{+}]_i$ measurements and calibrations were performed with a microfluorimetric system (Cairn Research, Faversham, Kent, UK).

IMMUNOBLOTTING

$6x10^5$ MCF 7 cells and $4x10^5$ MDA-MB-231 were plated in 10 cm dishes and allowed to adhere for 48 hours. Thereafter, cells were lysed in lysis buffer (50mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, and protease inhibitor mixture) and protein concentration was determined according to a standard Bradford assay. Proteins (35 µg) were separated by SDS-PAGE, transferred to a PVDF membrane (Immobilon-P, Millipore, Vimodrone, Italy), and detected with the

following antibodies: anti-SIRT6 (#2590, Cell Signaling Technology, Danvers, MA, USA), anti-Phospho-AMPK (PA5-17831, ThermoFisher), anti-AMPK (PA5-29679, ThermoFisher), anti-GAPDH (#3683, Cell Signaling Technology, Danvers, MA, USA), anti-Vinculin H-300 (sc-5573, Santa Cruz Biotechnology), anti-β-actin (Santa Cruz Biotechnology), and anti-SIRT6 mouse (ab62739, abcam, Cambridge, UK). Band intensities were quantified by Quantity One SW software (Bio-Rad Laboratories, Inc) using standard ECL.

Tumor masses were excised from xenograft mice and homogenized, in ice cold lysis buffer, with an electric homogenizer. The samples were maintained in constant agitation for 2 h at 4°C, on an orbital shaker in the fridge. Finally, the samples were centrifuged for 20 min at 12000 rpm at 4° in a microcentrifuge, the supernatant containing the proteins was removed and Bradford assay was performed.

QUANTITATIVE REAL TIME PCR (qPCR)

Total RNA was extracted from cells using the ReliaPrep RNA ™ Cell Miniprep System kit (catalog no. Z6012, Promega, Milan, Italy) according to the instructions of the manufacturer. 1 µg of RNA was reverse-transcribed in a final volume of 50 µl using a high-capacity cDNA reverse transcription kit (Invitrogen). 5 µl of the resulting cDNA was used for QPCR with a 7900 HT fast real-time PCR instrument (Applied Biosystems by Invitrogen) to confirm SIRT6 overexpression or deletion. (Tab.1).

Tab.1

Name	Primer
HUMAN SIRT6 FW	CCT CCT CCG CTT CCT GGT C
HUMAN SIRT6 REV	GTC TTA CAC TTG GCA CAT TCT TCC
HUMAN ACTIN FW	CGG GAA ATC GTG CGT GAC ATT AAG
HUMAN ACTIN REV	TGA TCT CCT TCT GCA TCC TGT CGG
MOUSE ACTIN FW	GAT GTA TGA AGG CTT TGG TC
MOUSE ACTIN REV	TGT GCA CTT TTA TTG GTC TC
MOUSE SIRT6 FW	GGC TAC GTG GAT GAG GTG AT
MOUSE SIRT6 REV	GGC TCA GCC TTG AGT GCT AC

mRNAlevels were detected using SYBR Green GoTaq^R qPCR Master Mix (Promega, Milan, Italy) according to the protocol of the manufacturer.

Gene expression was normalized to housekeeping gene expression (β-actin). Comparisons in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ method.

ANIMAL EXPERIMENTS

All in vivo experiments were conducted in accordance with the laws and institutional guidelines for animal care, approved by the Institutional Animal Care and Use Committee of the Scientific Institute for Research and Healthcare (IRCCS) University Hospital San Martino–National Institute for Cancer Research (IST, Genoa, Italy; protocol #453). Mice were housed in temperature- and light-controlled conditions (12-h light cycle) with food and water ad libitum.

Six to eight week old female BALB/c athymic (nu+/nu+, n=24) mice were acquired from Charles Rivers Laboratories (Paris, France). Mice were acclimatized for 2 weeks. 2 x 10^6 MDA-MB-231 control cells (PRS) and MDA-MB-231 silenced for SIRT6 (sh SIRT6) were injected subcutaneously. The tumor growth was monitored over time measuring tumor sizes with a caliper and we registered the tumor volume twice a week. Mice were sacrificed when the tumor reached a

volume of about 1,5 cm^3. To further investigate the role of SIRT6 in vivo, we also used a genetically modified model that spontaneously develops breast tumors, on a 129 background. Females mice heterozygous for SIRT6 (SIRT6+/-) were a kind gift from Prof. Raul Mostoslavsky (MGH Cancer Center, Boston, MA, USA). Sirt6 knockout mice were not used for this study, because they are known to develop acute metabolic syndrome and die before 4 week of age (Mostoslavsky R et al.2006).

Male mice heterozygous for MMTV-PyMT (MMTV-PyMT +/-) were provided from Prof. Thorsen Berger (The Campbell Family Institute for Breast Cancer Research Ontario Cancer Institute). The MMTV-PyMT colony will be maintained through heterozygous males because the heterozygous females develop tumors early and are therefore unable to breastfeed any puppies.

Three- to 9-months-old Sirt6+/- female mice were bred with MMTV-PyMT male mice +/- to generate two different groups of interest (n=45): control mice, WT for SIRT6 and heterozygous for PyMT (Sirt6 +/+, PyMT +/-), and experimental mice, heterozygous both for SIRT6 and PyMT (Sirt6 +/-, PyMT +/-). We monitored both groups palpating the mammary glands and when the tumors appear as palpable masses we recorded the age of the mice to study tumor latency and survival. In both studies we controlled daily the tumor volume and mice were sacrificed when one of the tumor reached a volume of about 1,5 cm3. Tumor volume was calculated using the formula: tumor volume= (w2 × W) x π/6, where "w" and "W" are "minor side" and "major side" (in mm), respectively.

DNA EXTRACTION AND GENOTYPING OF MICE Sirt6 +/+, PyMT +/- AND Sirt6 +/-, PyMT +/-

Extraction of DNA from mouse tails was performed according to the following protocol: digest about 4 mm mouse tail with 500 µl tail lysis buffer [100 mM TrisHCl, pH 8.0, 5 mM EDTA, 0,2 % sodium dodecyl sulfate (SDS), 200 mM NaCl] with 5 µl proteinase K (20 mg/ml), in a 1.5 ml tube at 56 °C overnight with agitation (1000 rpm on a heated shaker). Next day samples were centrifuged at 14000 rpm for 10 min at RT to pellet debris, the supernatant was transferred to a clean tube with 500 µl of isopropanol and inverted until DNA precipitation. Subsequently, samples were centrifuged at 14000 rpm for 5 min at RT, the supernatant was discarded and the DNA pellet was washed by adding 300 µl of 70 % ethanol and centrifuged at 14000 rpm for 5 min at RT. The ethanol solution was removed and the DNA pellet was dried at RT or in dessicator. Finally, the DNA pellet was resuspend in 35-100 µl of DNase free water.

Once the DNA is extracted we proceeded with genotyping using Multiplex PCR Master Mix 2x (catalog no. BR0200801, Biotechrabbit, Germany) to determine the presence of WT or KO allele of SIRT6 gene and of PyMT gene. For SIRT6 gene we used three oligonucleotides, two specific for WT or KO allele and one in common for the two different alleles (Tab.2).

WT allele primer pair (WT and COMMON) amplify fragment of 422 bp spanning across exon 8 while KO allele primer pair (KO and COMMON) amplify fragment of 265 bp spanning across exon 8. For PyMT gene we used two oligonucleotides (PyMT 1, PyMT 2) to amplify fragment of 600 bp (Tab.1). The cycling program used in Multiplex PCR was 3 min at 94°C to denaturate DNA, 30 cycles of amplification (30 sec 94°C, 1 min 59°C and 1 min 72°C) and 10 min at 72°C of final extension. PCR products were analyzed by gel electrophoresis with 2% precast agarose gels (catolog no. 54813, 2% seakem gold agarose, Lonza) at 100 V costant. The amplification products were visualized with ChemiDoc XRS (Biorad) instrument.

Tab. 2

Name	Primer
SIRT6 WT	TTT CGT ATA AGT CCA AGC CC
SIRT6 COMMON	GGA AGG ACC TGG ACA AG
SIRT6 KO	GCA ATA GCA TCA CAA ATT TCA C
PyMT 1	GGA AGC AAG TAC TTC ACA AGG
PyMT 2	GGA AAG TCA CTA GGA GCA GGG

MAMMARY TISSUE PREPARATION FOR MORPHOLOGIC ANALYSIS

For preparing whole mounts, the fourth inguinal mammary gland was excised from a female mouse at the appropriate age (30 and 90 days) and spread out on a pre-cleaned microscope slide. The gland was fixed in Carnoy's solution (75% ethanol: 25% glacial acetic acid) overnight at RT, hydrated in 70% ethanol and water and stained overnight with carmine alum (catalog no. 07070, STEMCELL Technologies). After staining, slides were rinsed in water, dehydrated in increasing concentrations of ethanol and cleared in histolemon (catalog no. 454915, Carlo Erba). Photographic images of the whole mounts were acquired by Nikon SMZ1270 microscope using the X-Entry software. Terminal end buds (TEB) were counted in the whole mammary gland at 30 days. Statistical evaluations were performed with a two-tailed Student's t-test.

TUMOR DISSOCIATION AND CELL ISOLATION FROM PyMT MICE MODEL

Masses were collected and dissociated using Tumor Dissociation Kit mouse (cat. no 130-096-730, Macs Miltenyi Biotec) following manufacturer's instructions. Tumor cells were isolated using Tumor Cell Isolation Kit mouse (cat. no 130-110-187, Macs Miltenyi Biotec) following manufacturer's instructions. Cell purity was determinated by flow cytometric analysis on FACS Calibur (BD Bioscence). Non tumor cells were stained with Anti-Ter-119 (130-117-538, Macs

Miltenyi Biotec), CD 31 (130-102-519, Macs Miltenyi Biotec), CD 45 (130-116-500, Macs Miltenyi Biotec) antibodies conjugated with FITC, which are markers of normal cells.

ASSAY OF INTRACELLULAR ATP AND AMP LEVELS

Quantification of ATP and AMP was based on the enzyme coupling method. 20 µg of total protein were used. Briefly, ATP was assayed spectrophotometrically at 340 nm, following NADP reduction. Medium contained 50 mM Tris-HCl pH 8.0, 1 mM NADP, 10 mM MgCl2, and 5 mM glucose in 1 ml final volume. Samples were analysed before and after the addition of 4 µg of purified hexokinase plus glucose-6-phosphate dehydrogenase. AMP was assayed spectrophotometrically at 340 nm, following NADH oxidation. Medium contained 100 mM Tris-HCl pH 8.0, 75 mM KCl, 5 mM MgCl2, 0.2 mM ATP, 0.5 mM phosphoenolpyruvate, 0.2 mM NADH, 10 IU adenylate kinase, 25 IU pyruvate kinase, and 15 IU of lactate dehydrogenase.

OXYMETRIC ANALYSIS

Oxygen consumption was measured with an amperometric oxygen electrode in a closed chamber, magnetically stirred at 37 °C. For each assay, 200, 000 cells were used. After permeabilization with 0.03 mg/ml digitonin for 10 minutes, samples were suspended in a medium containing 137 mM NaCl, 5 mM KH2PO4, 5 mM KCl, 0.5 mM EDTA, 3 mM MgCl2 and 25 mM Tris–HCl, pH 7.4. To activate the pathway composed by Complexes I, III and IV, 5 mM pyruvate + 2.5 mM malate were added. To activate the pathway composed by Complexes II, III and IV, 20 mM succinate was used.

EVALUATION F1Fo-ATP SYNTHASE ACTIVITY

F1Fo-ATP synthase activity was detected by measuring ATP production by the highly sensitive luciferin/luciferase method. Assays was conducted at 37°C, over 2 min, by measuring ATP

produced from di-adenosine-5'penta-phosphate (ADP). 100,000 cells were added to the incubation medium (0.1 ml final volume), which contained 10 mM Tris-HCl pH 7.4, 50 mM KCl, 1 mM EGTA, 2 mM EDTA, 5 mM KH2PO4, 2 mM MgCl2, 0.6 mM ouabain, 0.040 mg/ml ampicillin, 0.2 mM ADP and the metabolic substrates (5 mM pyruvate + 2.5 mM malate or 20 mM succinate). Cells were equilibrated for 10 min at 37°C, then ATP synthesis was induced by addition of 0.2 mM ADP. ATP synthesis was measured using the luciferin/luciferase ATP bioluminescence assay kit CLSII (Roche, Basel, Switzerland), on a Luminometer (GloMax® 20/20 Luminometer – Promega, Wisconsin, USA). ATP standard solutions (Roche, Basel, Switzerland) in the concentration range 10-10 - 10-7 M were used for calibration.

RESPIRATORY COMPLEXES ACTIVITY ASSAY

The activity of the redox complexes I, II, III and IV was measured on 50 µg of protein.

Complex I (NADH-ubiquinone oxidoreductase) was assayed following the reduction of ferricyanide at 420 nm; the reaction mixture was composed by: 10 mM phosphate buffer pH 7.2, 30 mM NADH, 40 mM ferricyanide, 40 µM antimycin A.

Complex II (Succinic dehydrogenase) activity was measured at 600 nm, in 2 mM EDTA, 0.2 mM ATP, 20 mM succinate, 0.5 mM cyanide, 80 µM dicloroindophenol (DCIP), 50 µM decylubiquinone, 40 µM antimycin A, 10 µM rotenone and 10 mM phosphate buffer, pH 7.2. Complex III (Cytochrome c reductase) activity was measured at 550 nm followed the reduction of oxidized Cytochrome c. The reaction mixture containing: 10 mM phosphate buffer pH 7.2, 0.03% oxidized cytochrome C and 0.5 mM KCN. Complex IV (Cytochrome c oxidase) was assayed following the oxidation of ascorbate-reduced Cytochrome c at 550 nm, in a solution containing 10 mM phosphate buffer pH 7.2, 0.03/ reduced cytochrome C and 40 µM antimycin A.

STATISTICS

Data are expressed as mean ± st.dev. and p value < 0.05 was considered significant. Multiple comparisons were performed using the analysis of variance (two-way ANOVA) followed by Bonferroni post hoc test. Analyses were performed by means of SigmaStat (Systat Software, Inc., San Jose, CA, USA) software.

RESULTS

SIRT6 DOWNREGULATION SLOWS BREAST CANCER PROGRESSION IN MMTV-PyMT MOUSE MODEL

To define the role of SIRT6 in mammary carcinogenesis, we made use of the MMTV-PyMT mouse model of breast cancer. Both the MMTV-PyMT$^{+/-}$ mice and the Sirt6$^{+/-}$ mice that we used in our experiments were in a 129 background. Sirt6 heterozygous deletion indeed resulted in a blunted Sirt6 expression in the mammary tumor masses isolated from the animals as shown in Figure 1A, B. MMTV-PyMT$^{+/-}$; Sirt6$^{+/+}$ and MMTV-PyMT$^{+/-}$; Sirt6$^{+/-}$ mice were monitored for tumor latency and for overall survival. As shown in Figure 1C-D, mice carrying a heterozygous deletion exhibited a marked increase in tumor latency and a consistent increase in their overall survival.
Previous studies suggested that the heterozygous deletion of another sirtuin family member, Sirt1, would increased mammary tumor latency by interfering with the normal development of the mammary gland, we monitored mammary gland development in MMTV-PyMT$^{+/-}$; Sirt6$^{+/+}$ and MMTV-PyMT$^{+/-}$; Sirt6$^{+/-}$ mice by generating mammary glands whole mounts, by staining them with carmine alum and by analysing ductal development with a stereomicroscope. In particular, we quantified the number of terminal end buds (TEB), the key structures regulating elongation and branching of the mammary gland into the fat pad. With the onset of puberty, in response to the higher local and systemic reproductive hormones, TEB drive ductal morphogenesis. When comparing 4-weeks old MMTV-PyMT$^{+/-}$; Sirt6$^{+/+}$ and MMTV-PyMT$^{+/-}$; Sirt6$^{+/-}$ mice no significant variation in the number and size of TEB was detected (Figure 2A), indicating that the observed effect of *Sirt6* deletion on mammary tumorigenesis does not reflect a defect in ductal morphogenesis.

We also compared the mammary gland of 90-days old mice to see if differences in the onset of the tumor appeared.

As shown in Figure 2B, in control mice, the primary tumor (PT) was clearly evident and there were multiple small nodules forming along the more distal ducts (2nd). Distinct histopathological changes representing morphological events of tumor progression from benign to malignant were normally observed in the primary tumor (Lin EY et al.2001). On the other hand, in MMTV-PyMT$^{+/-}$; Sirt6$^{+/-}$ mice the primary tumor was typically smaller, consisting of a single mass or, occasionally, of a few apposed masses, developing around the main collecting duct just beneath the nipple. Secondary tumor foci were also less visible in the presence of *Sirt6* heterozygous deletion.

The homozygous deletion of Sirt6 was previously reported to blunt circulating glucose levels (Mostoslavsky et al. Cell 2006). Thus we sought to assess whether such an effect also occurs as a result of a heterozygous Sirt6 deletion. However, we could not detect any reduction in blood glucose levels in MMTV-PyMT$^{+/-}$; Sirt6$^{+/-}$ vs. MMTV-PyMT$^{+/-}$; Sirt6$^{+/+}$ mice (Figure 2C). Therefore, reduced circulating sugar levels do not account for the anticancer effect observed in response to *Sirt6* deletion in this BC model.

Overall, our results clearly indicate that reducing Sirt6 expression slows mammary cancer development in the mouse MMTV-PyMT breast cancer model. These results are also consistent with those of studies attributing a negative prognostic significance to high SIRT6 levels in BC.

SIRT6 SILENCING REDUCE TUMOR GROWTH IN MDA-MB-231 XENOGRAFT

Since in MMTV-PyMT$^{+/-}$; Sirt6$^{+/-}$ mice, Sirt6 deletion affects all bodily tissues, the observed delay in tumor development and the corresponding enhancement of mouse survival could in principle reflect cell non-autonomous anticancer effects or Sirt6 deletion, such as an effect on the tumor microenvironment and or an effect on circulating growth factors. To rule out this possibility we silenced SIRT6 by RNA interference in the BC cell line MDA-MB-231, injected these cells (or cells harbouring a control shRNA) into the flank of nude mice and monitored tumor growth. As shown in Figure 3A, SIRT6-silenced MDA-MB-231 cells exhibited a markedly reduced. These results are consistent with reduced SIRT6 levels slowing BC growth via a cell-autonomous mechanism (Figure 3B). In addition, sh SIRT6 cells generated smaller masses than control cells, data confirmed even when we excised the masses and weighted them (Fig. 3 C-D).

SIRT6 ENHANCES OXPHOS AND ENERGY STATUS AND BLUNTS AMPK ACTIVITY IN BC CELLS

Studies show that tumor growth requires active mitochondrial function and OXPHOS.

OXPHOS enhances anabolic metabolism, cell proliferation, cancer stemness and metastasis (Zacksenhaus E et al. 2017, Jones RA et al. 2016). Consistent with this notion, agents targeting mitochondrial complexes, such as metformin (with blocks mitochondrial complex I activity) show promising anticancer properties.

Previous studies suggested that mitochondrial function may be reduced as a consequence of Sirt6 deletion. Thus we hypothesized that blunting Sirt6 expression in BC cells could affect OXPHOS and cellular energy status.

To address this hypothesis, we used retroviral transduction to overexpress, in either wild type (WT) or in catalytically inactive form (H133Y) and silence SIRT6 in MDA-MB-231 (Figure 4 A-D). In addition, we both silenced and overexpressed SIRT6 (again in either WT or catalytically inactive from) in MCF7 cells (a second BC cell line) (Figure 5 A-D). In these cells, we measured the activity of mitochondrial complexes I, III and IV, O2 consumption, ATP synthase activity, ATP and AMP content and ATP/AMP ratio.

In both cell lines, we found that the overexpression of WT, but not of catalytically inactive SIRT6 enhanced mitochondrial complexes activity (Figure 6 A, C), O2 consumption (Figure 6 B, D), ATP synthase activity (Figure 7A,C), ATP content and ATP/AMP ratio (Figure 7B, D). Opposite effects were obtained by SIRT6 silencing (Figure 6, 7). Consistent with these data, we found that Sirt6$^{+/-}$ tumors from MMTV-PyMT mice exhibited decreased complex I, III, and IV activity as well as reduced ATP stores and ATP/AMP ratio as compared to Sirt6$^{+/+}$ tumor masses (Figure 8A, B). Notably, such an effect of Sirt6 heterozygous deletion was not unique of malignant mammary tissues, since standard Sirt6$^{+/-}$ mice (that did not express the MMTV-PyMT) also exhibited

decreased OXPHOS and lower ATP/AMP rations in their mammary glands (Figure 9A,B). This indicates that this metabolic effect of Sirt6 depletion is a general consequence of the depletion of this deacetylase in mammary tissues and that such a metabolic tissue profile likely poses a major obstacle to the development of primary tumors in response to PyMT activity.

Overall, these findings indicate that SIRT6 depletion blunts OXPHOS and energy status in breast cancer cells of murine and human source, indicating a probable mechanism that could contribute to the reduced tumor growth observed in response to SIRT6 depletion.

The ATP/AMP ratio is the main determinant of AMP-activated protein kinase (AMPK) activation. In turn, activated AMPK is responsible for orchestrating a tumor suppressive response that includes mammalian target of rapamycin (mTOR) inhibition and autophagy initiation. We found that SIRT6-silenced MCF7 and MDA-MB-231 cells exhibited increase levels of AMPK phosphorylation (Figure 10A, B). Higher AMPK activity was also observed in MDA-MB-231 cells that were isolated from mouse xenografts (Figure 10C) and in tumor cells isolated from a MMTV-PyMT$^{+/-}$; Sirt6$^{+/-}$ mouse (as compared to a control animal) (Figure 11C). Therefore, these findings are in line with SIRT6 depletion causing and energetic stress in BC cells and consequently increasing AMPK activity.

SIRT6 REGULATES INVASIVENESS, MMP9 EXPRESSION AND INTRACELLULAR Ca^{2+} LEVELS IN MDA-MB-231 CELLS

Increased OXPHOS and increase ATP availability have been linked to migration, invasion and metastasis (Zacksenhaus E et al. 2017). Thus we performed additional experiments to determine whether SIRT6 would also regulate additional features of aggressiveness in our BC models. We performed 3D colony formation assays in matrigel with control MDA-MB-231 cells (PBP) and with WT- or H133Y-SIRT6 overexpressing MDA-MB-231. Overexpression of WT SIRT6 increased MDA-MB-231 cell propensity to grow and to invade in Matrigel compared to PBP and to SIRT6 H133Y cells (Figure 12A). We also performed migration/invasion transwell assays and found that, consistent with the Matrigel assays, WT SIRT6-overexpressing cells had an increased invasiveness (Figure 12B). Overexpression of the catalytically active SIRT6 was also associated with an increase expression of MMP9, which we also confirmed in ELISA assays (not shown). Vice versa, SIRT6 silencing reduced MMP9 mRNA levels (Figure 12C, D). These results clearly link SIRT6 catalytic activity to BC cell invasiveness.

Finally, we also measured the concentration of intracellular calcium $[Ca^{2+}]_i$, a cation which can also support cancer cell propensity to invade and to metastasize. Consitent with our previous findings in pancreatic cancer cells (Bauer et al. 2012), we found $[Ca^{2+}]_i$ to be increased in WT SIRT6-expressing MDA-MB-231 WT cells (Figure 12E). To investigate the reason for such an increased $[Ca^{2+}]_i$, we performed experiments evaluating the intracellular Ca2+ stores (which are primarily represented by the endoplasmic reticulum) by thapsigargin stimulation (Mekahli D et al. 2011). As reported in Figure 12F, in WT SIRT6-expressing MDA-MB-231 treated with TG, the $[Ca^{2+}]$ were lower than those found in the control cells, thus indicating that SIRT6 acts to promote Ca2+ release from the thapsigargin-sensitive intracellular Ca2+ stores, to increase $[Ca^{2+}]_i$.

Therefore, SIRT6 shows the ability to stimulate BC cell migration and invasion, possibly via a mechanism that foresees the increase in intracellular ATP and $[Ca^{2+}]_i$ (via Ca^{2+} release from intracellular Ca^{2+} stores) and the expression of MMP9.

DISCUSSION

Here we show that a heterozygous deletion of the *Sirt6* gene increases tumor latency and improves overall survival in the MMTV-PyMT mouse model. Such an effect is recapitulated by SIRT6 silencing in MDA-MB-231 xenografts, indicating a cell autonomous anticancer effect of SIRT6 depletion in BC cells. We show that SIRT6 boosts OXPHOS and enhances energy status in both healthy and transformed mammalian tissues and that this translates into dampened AMPK activity. Finally, through it catalytic activity, SIRT6 is shown to promote BC cell invasiveness and MMP9 expression, and to increase $[Ca^{2+}]_i$ in BC cells.

Our *in vivo* data, showing delayed mammary tumor development and increased survival in MMTV-$PyMT^{+/-}$;$Sirt6^{+/-}$ mice compared to MMTV-$PyMT^{+/-}$;$Sirt6^{+/+}$ animals are fully in line with the previously reported adverse prognostic significance of high SIRT6 expression in BC (Jun Sang Bae et al 2016). The two studies that previously highlighted a “pro-oncogenic” role of SIRT6 in BC attributed it to the ability of SIRT6 to promote DNA repair in BC cells and, consequently, to mediate resistance to chemotherapeutics, but also to increase the expression of MMP9, β-catenin, CCND1, and of NF-κB. Our data point out a new “metabolic” function of this NAD-dependent deacetylase in BC, which consists in SIRT6 ability to boost OXPHOS and to increase ATP availability in mammary tissues and in BC cells.

Studies show that, despite early models proposing that cancer cells would primarily rely on aerobic glycolysis for their survival, mitochondrial function is actually key in many neoplasms. Loss of the tumor suppressor RB1, but also several proto-oncogenes, such as mitochondrial STAT3, FER and its variant, FerT, and CHCHD2, induce OXPHOS. The latter in turn enhances anabolic metabolism, promotes cancer stemness and/or metastatic spread (Zacksenhaus E et al. 2017). OXPHOS inhibitors, such as metformin, tigecyclin or salinomycin hold promise for preventing or treating different forms of cancer, including breast cancer. Our study indicates SIRT6 as a new,

druggable target to be exploited for blunting OXPHOS in mammary tissues and in BC, thereby achieving cancer preventive but also, possibly, therapeutic effects.

In our hands, SIRT6 depletion resulted in increased AMPK activity as detected by its phosphorylation on threonine 172. These findings, which we attribute to the low ATP/AMP ratio that is typically observed in tissues with reduced SIRT6, are consistent with those of those of Ming and colleagues, who also found such an increase in AMPK phosphorylation upon skin cancer cell treatment with SIRT6 siRNAs (Mei Ming et al. 2014). On the other hand Elhanati and colleagues found that, in the liver, SIRT6 overexpression, rather than its downregulation, decreases ATP/AMP ratio and activates AMPK (Elhanati S et al. 2013). The possibility that SIRT6-mediated metabolic effects may be different from tissue to tissue has recently been suggested in a study by Sociali et al. (Sociali G et al. 2018).Thus, these opposing effects of SIRT6 on AMPK activity could indeed reflect the differences in tissues where these effects were investigated and/or different outcomes of SIRT6 activity in normal vs. cancer cells.

Based on our data, in addition to modulating OXPHOS and BC cell energy status, SIRT6 also appears to boost MMP9 expression and to increase $[Ca2+]_i$ in BC cells. Increased MMP9 expression via SIRT6 was previously reported by Bai et al. (Bai L et al. 2016), by Lin et al. (Lin et al. 2017) and by Bae at el. (Bae JS et al. 2016). Thus, our findings in MDA-MB-231 cells are in line with those of these researchers and strengthen the notion that SIRT6 could contribute to BC invasiveness by increasing the secretion of this matrix metalloproteinase. In pancreatic cancer cells, we previously found that SIRT6 overexpression results in higher $[Ca2+]_i$ via gating of the cation channel TRPM2 by the SIRT6-derived second messenger o-acetyl-ADPribose (Bauer I et al. 2012). We found that WT SIRT6-overexpressing MDA-MB-231 cells also exhibited higher $[Ca2+]_i$ than their control cells. Intracellular thapsigargin-sensitive Ca2+ stores, were depleted in SIRT6-overexpressing BC cells, suggesting that $Ca2^{+}$ release from its intracellular stores, rather than Ca^{2+} entry, could be the

primary mechanism underlying the higher $[Ca2+]_i$ observed in response to high SIRT6 levels in MDA-MB-231 cells. Since Ca^{2+} is a critical regulator of cell migration in different cell types, including cancer cells, this finding could also explain the increased aggressiveness of SIRT6 overexpressing BC cells.

We have recently demonstrated that SIRT6 affects the enzymatic activity of the NAD-biosynthetic enzyme nicotinamide phosphoribosyltransferase (NAMPT), by directly deacetylating this enzyme on its lysine 53, which is located at the "cleft" containing the catalytic sites in the active dimeric form (Sociali G et al. 2018). As a result, SIRT6 was shown to boost NAD(P)(H) levels and to protect cancer cells against oxidative stress. Our finding that Sirt6 dletion slows mammary tumor development and extends survival in the MMTV-PyMT mammary tumor model and that SIRT6 silencing blunts the *in vivo* growth of the aggressive MDA-MB-231 BC cells are in line with these findings, but also with those of Khongkow et al. (Khongkow M et al. 2013) and of Bae et al. (Bae J.S. et al. 2016), and, overall, suggest a potential for this NAD^+-consuming enzyme as a cancer target. To this end, chemical SIRT6 inhibitors could be exploited, some of which have already shown interesting anticancer effects. This is the case for instance of SIRT6 inhibitors with quinazolinedione structure or with salicylate-like structure, which our group has previously identified (Damonte P et al. 2017, Sociali Get al. 2015) and which were found to sensitize cancer cells to chemotherapeutics and to PARP inhibitors.

In conclusion, based on our data, we proposed a model for a pro-oncogenic role of SIRT6 in breast tumorigenesis (Figure 13). SIRT6 causes an increase in the activity of the mitochondrial respiratory complexes and ultimately raises intracellular ATP/AMP ratio. As a result, in the absence of energetic stress, the activation of AMPK is decreased and more ATP is available to cancer cells to grow, and to carry out anabolic processes, but also migration and invasion. Therefore, studies of SIRT6 inhibitors either as cancer preventive agents or as therapeutic for BC should be considered.

Acknowledgments

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FIGURES AND LEGENDS

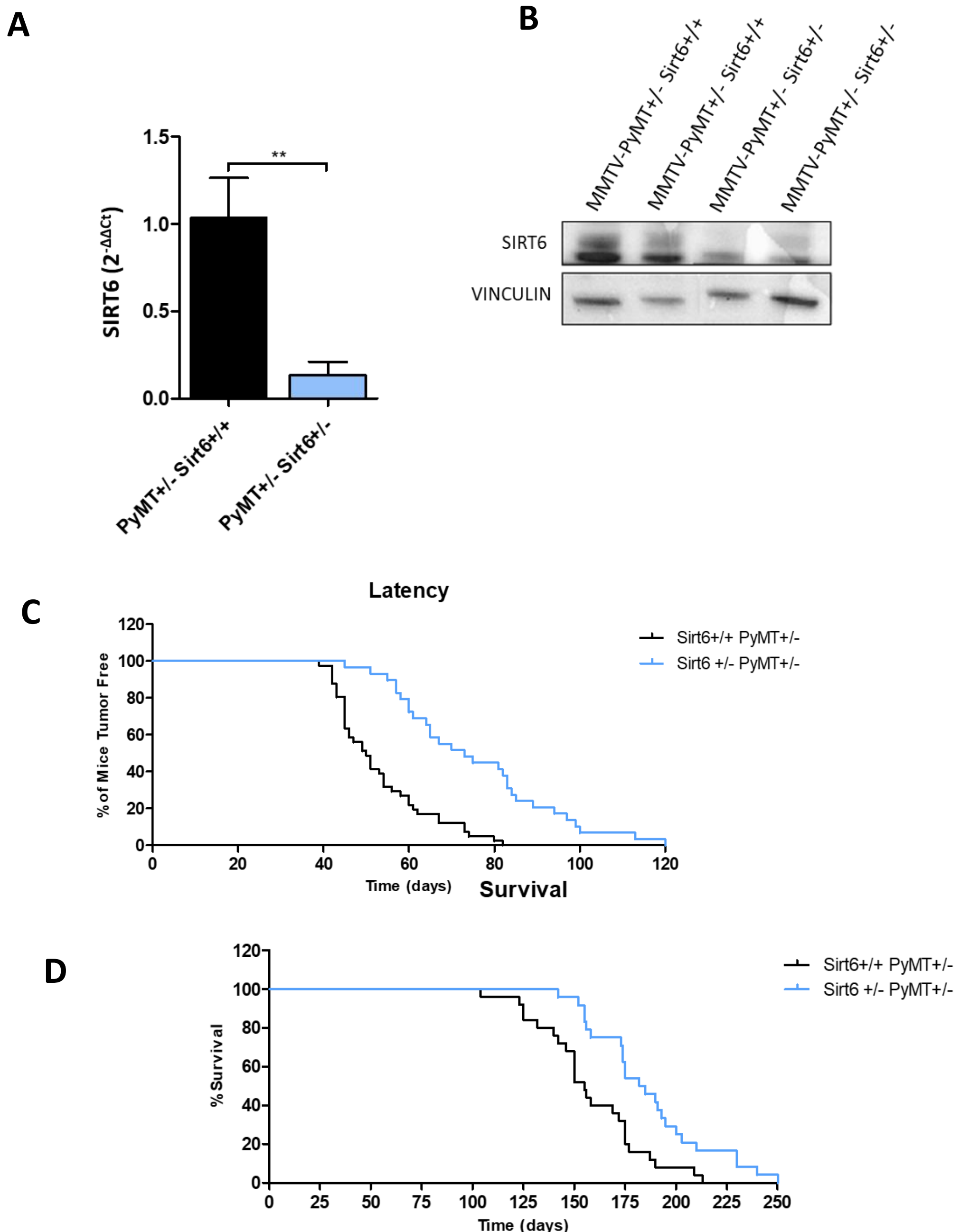


Figure 1A. SIRT6 downregulation slows breast cancer progression in MMTV-PyMT mouse model. SIRT6 expression was evaluated in qPCR **(A)** and by immunoblotting **(B)** on control and experimental masses. Tumor latency **(C)** and overall survival **(D)** in MMTV-PyMT+/-; Sirt6+/+ and in MMTV-PyMT+/-; Sirt6+/- mice.

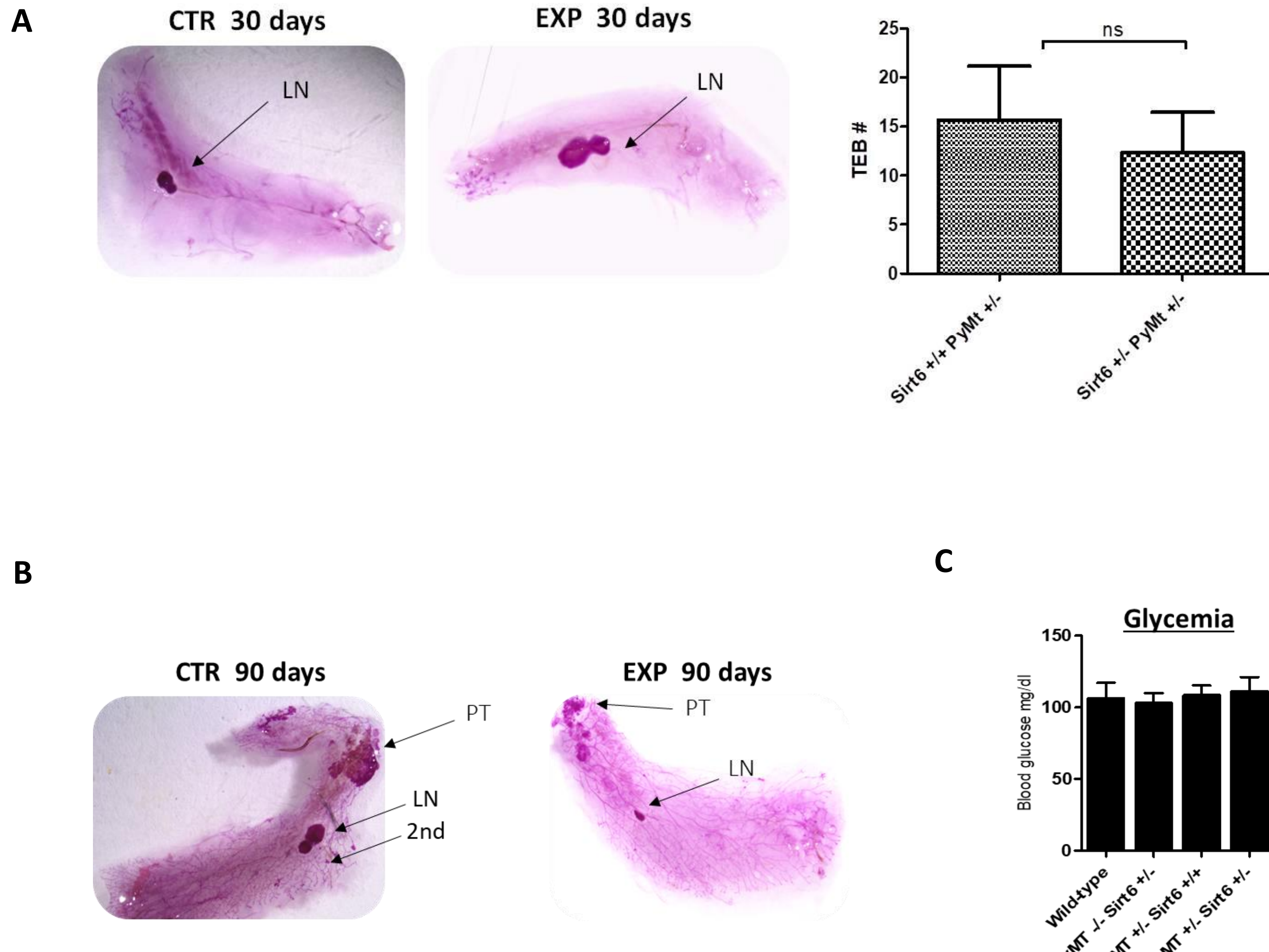


Figure 2. Whole mount staining of mammary inguinal gland from control mice (Sirt6 +/+, PyMT +/-) and experimental mice (Sirt6 +/-, PyMT +/-). (A) Carmine red-stained whole-mount preparation of mammary gland from CTR and EXP mice at 4 weeks of age. LN, lymph node. **(B)** Carmine red-stained whole-mount preparation of mammary gland from CTR and EXP mice at 12 weeks of age. PT, primary tumor; second, foci growing on the distant ducts; LN, lymph node. **(C)** Blood glucose levels from MMTV-PyMT+/-; Sirt6+/+ and from MMTV-PyMT+/-; Sirt6+/- mice were quantified by standard glucose sticks.

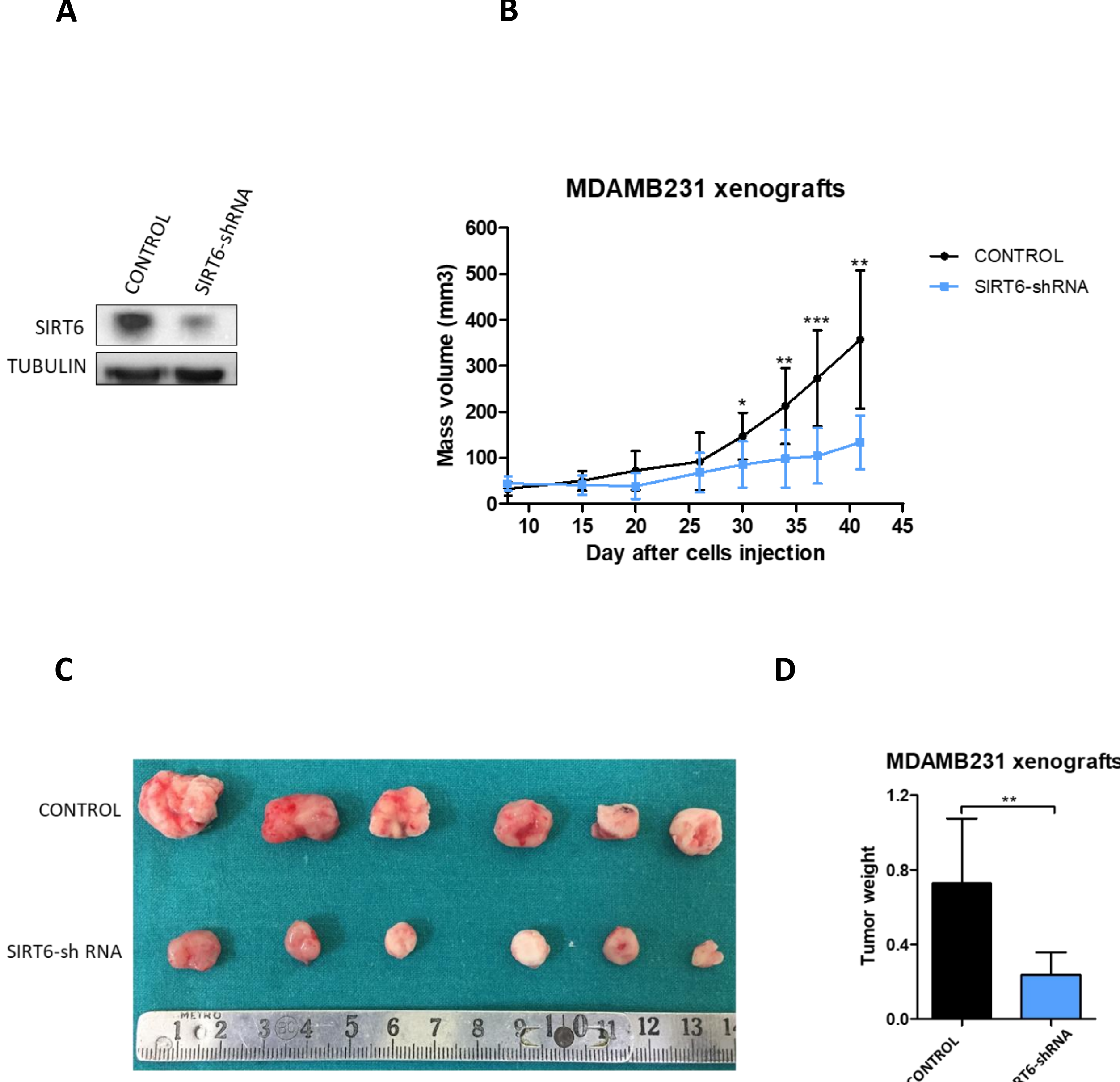


Figure 3. SIRT6 downregulation slows BC growth in subcutaneous model. 2 x 10^6 MDA-MB-231 transduced with either a SIRT6 shRNA or the vector PRS were injected s.c. into nude mice. SIRT6-silenced MDA-MB-231 cells exhibited a markedly reduced **(A)**, tumor volume was monitored over time from the day of tumor inoculation **(B)**, after 50 days mice were sacrified, masses were collected **(C)** and weighted **(D)**.

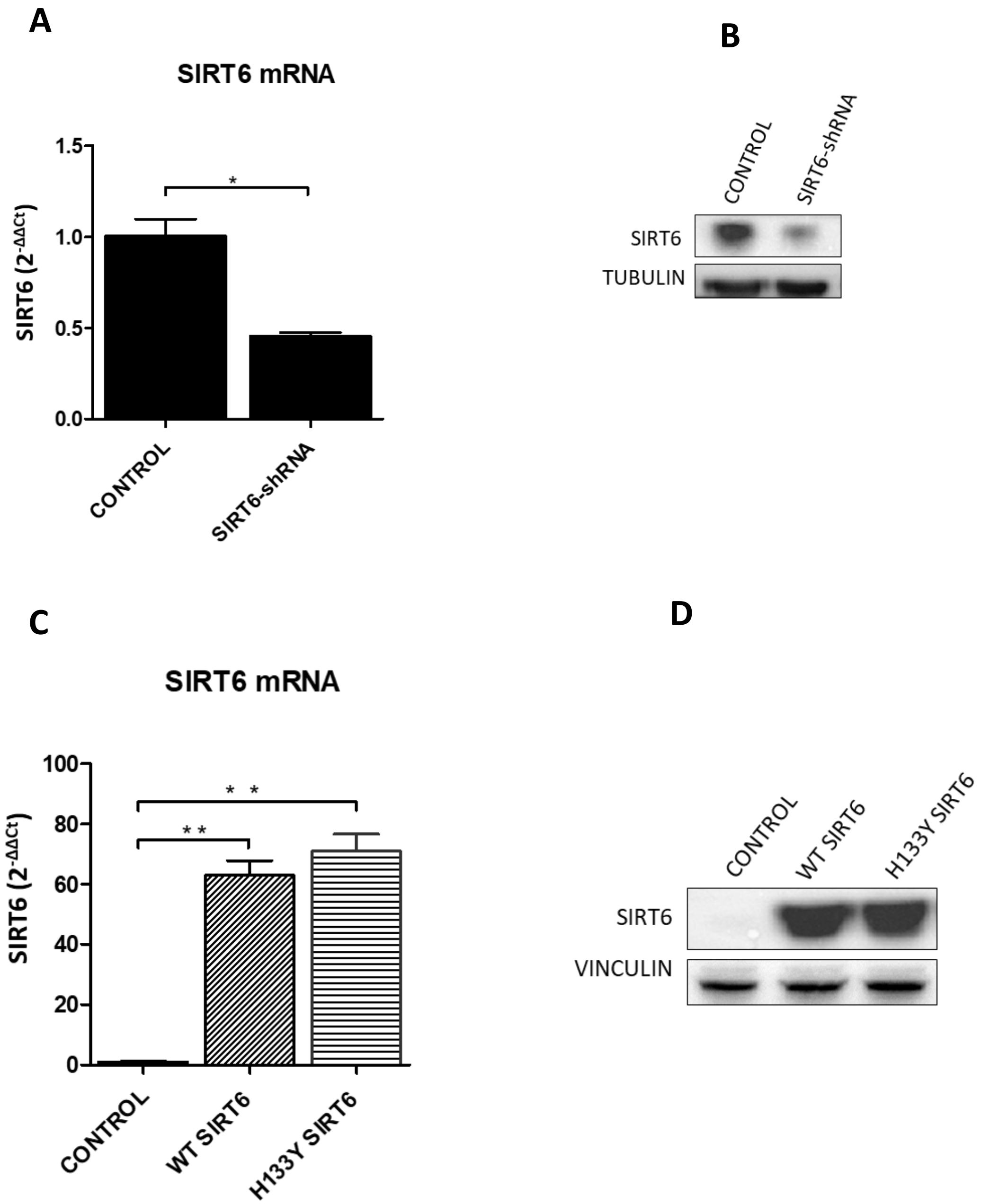


Figure 4. MDA-MB-231 transduced for silencing and over-expression of SIRT6. Cells were transduced with a SIRT6 shRNA or with the control vector PRS, the expression level of SIRT6 was evaluated by qPCR **(A)** and by immunoblotting **(B).** Alternatively, they were transduced with human WT or catalytically inactive (H133Y) SIRT6, or with a control vector (pBABEPURO, PBP). The expression level of SIRT6 was evaluated by qPCR **(C)** and by immunoblotting **(D).**

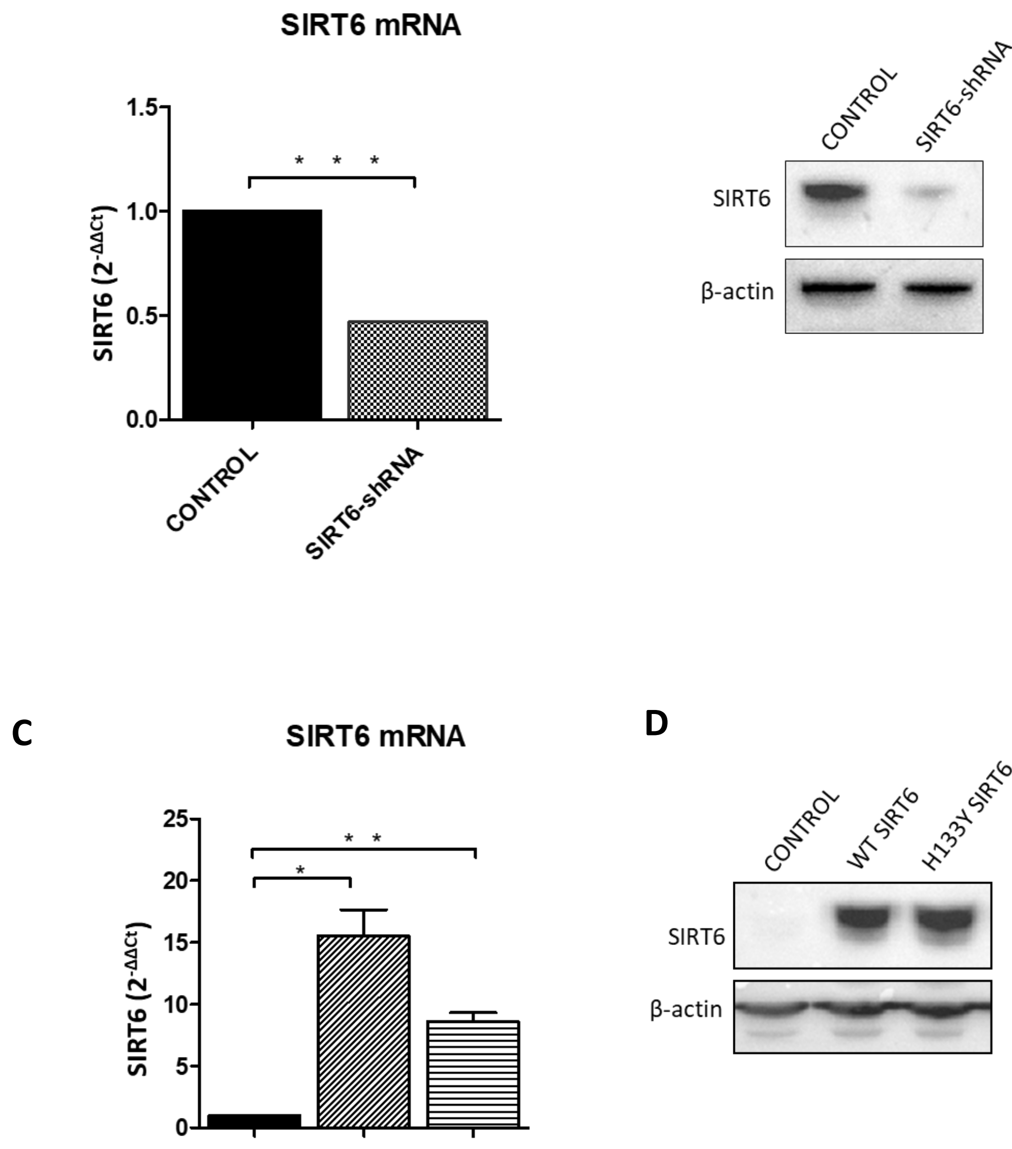
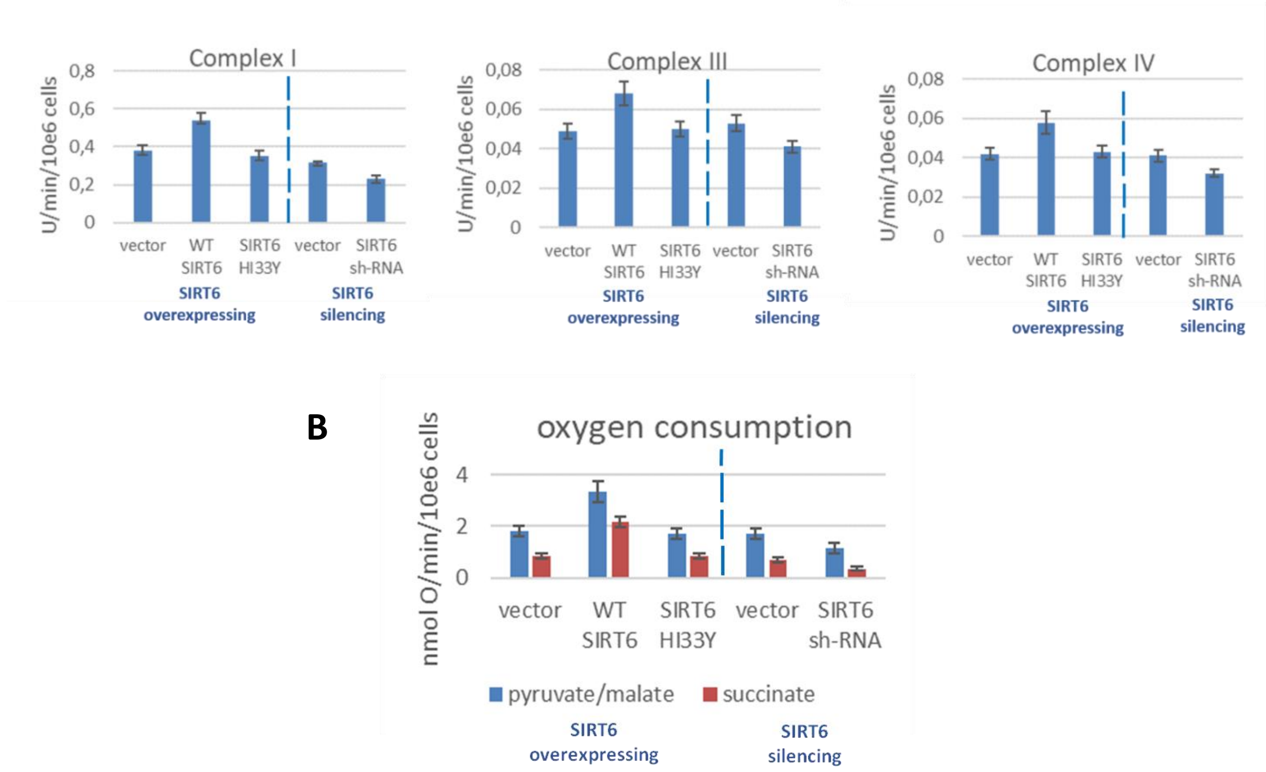


Figure 5. MCF7 transduced for silencing and over-expression of SIRT6. Cells were transduced with a SIRT6 shRNA or with the control vector PRS, the expression level of SIRT6 was evaluated by qPCR **(A)** and by immunoblotting **(B).** Alternatively, they were transduced with human WT or catalytically inactive (H133Y) SIRT6, or with a control vector (pBABEPURO, PBP). The expression level of SIRT6 was evaluated by qPCR **(C)** and by immunoblotting **(D).**

A MCF7



MDA-MB-231

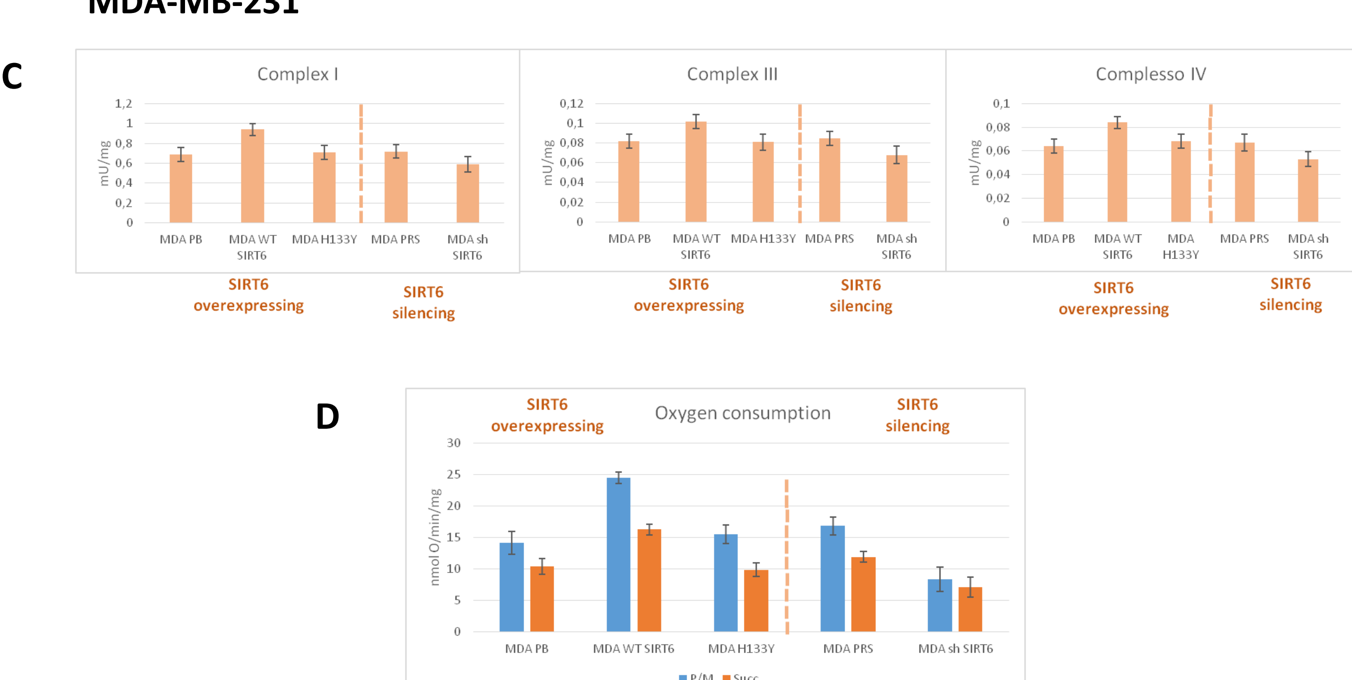
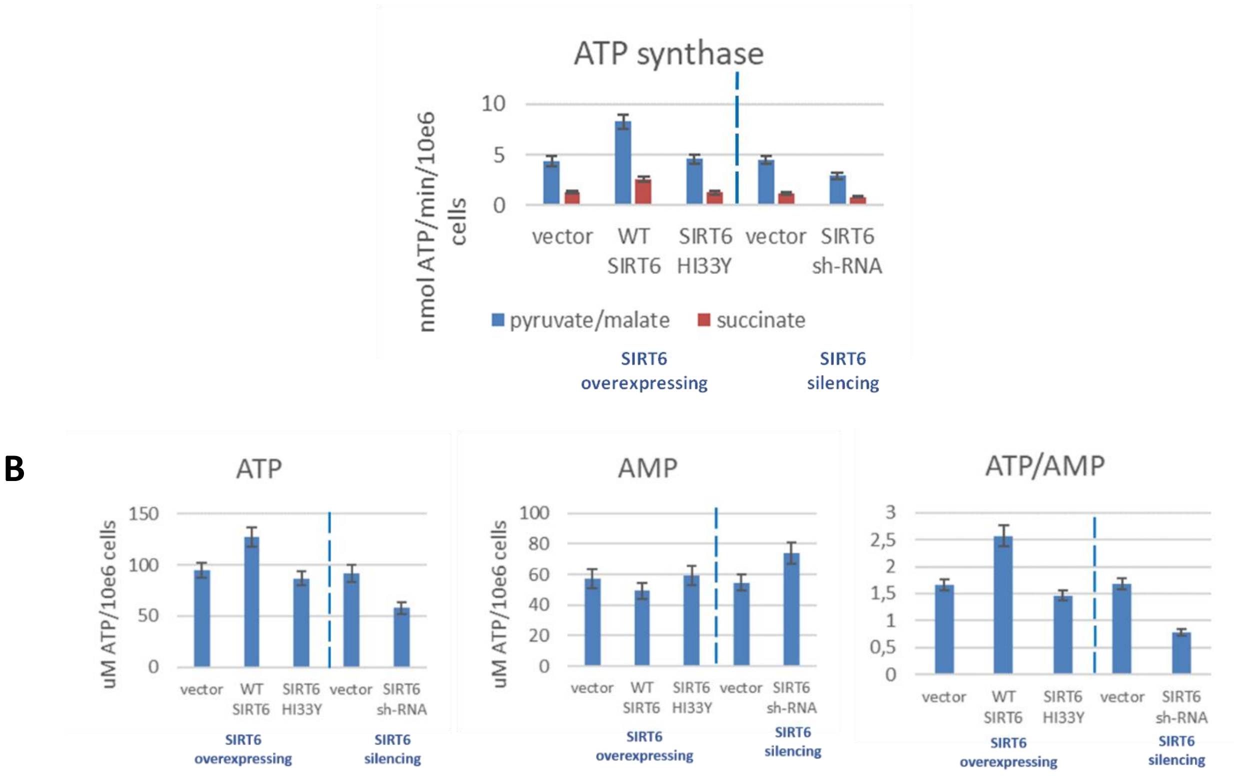


Figure 6. SIRT6 regulates OXPHOS in breast cancer cell lines. Cells were transduced with human WT or catalytically inactive (H133Y) SIRT6, or with a control vector; alternatively they were transduced with a SIRT6 shRNA or with PRS. Mitochondrial complex activity **(A, C)**, O2 consumption **(B, D)** was determined in MCF7 and MDA-MB-231**.**

A MCF7



MDA-MB-231

C

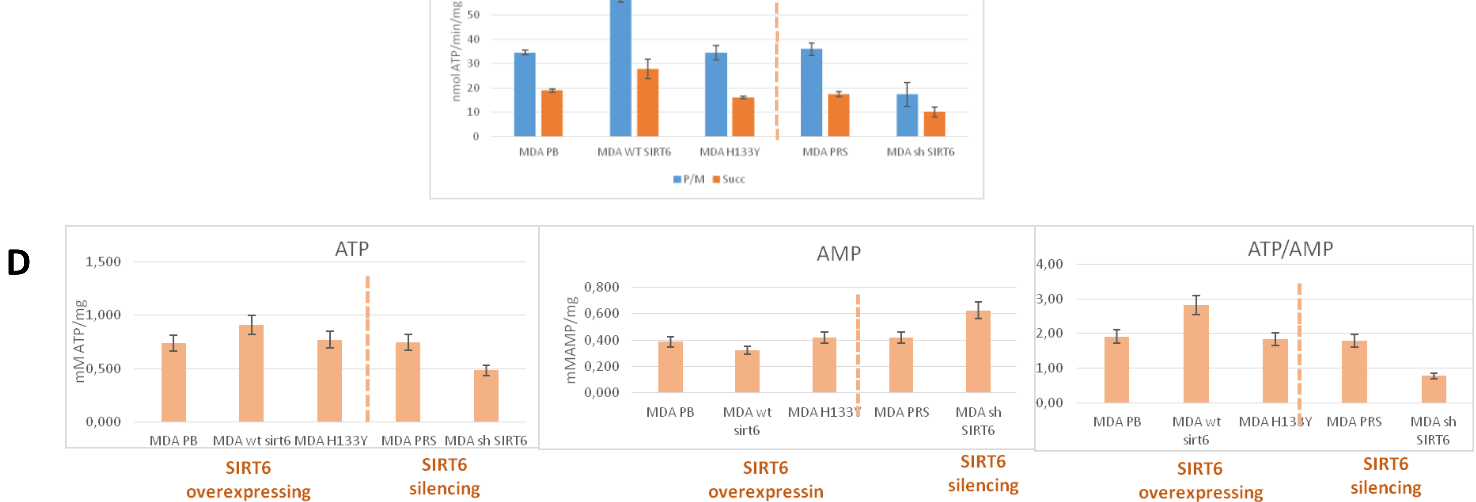


Figure 7. SIRT6 expression regulates ATP production in breast cancer cell lines. MCF7 and MDA-MB-231 cells were transduced with human WT or catalytically inactive (H133Y) SIRT6, or with a control vector; alternatively they were transduced with a SIRT6 shRNA or with PRS. Subsequently, activity of the Fo-F1 ATP synthase **(A, C)**, quantification of ATP, AMP and ATP/AMP ratio concentrations was determined **(B, D)**.

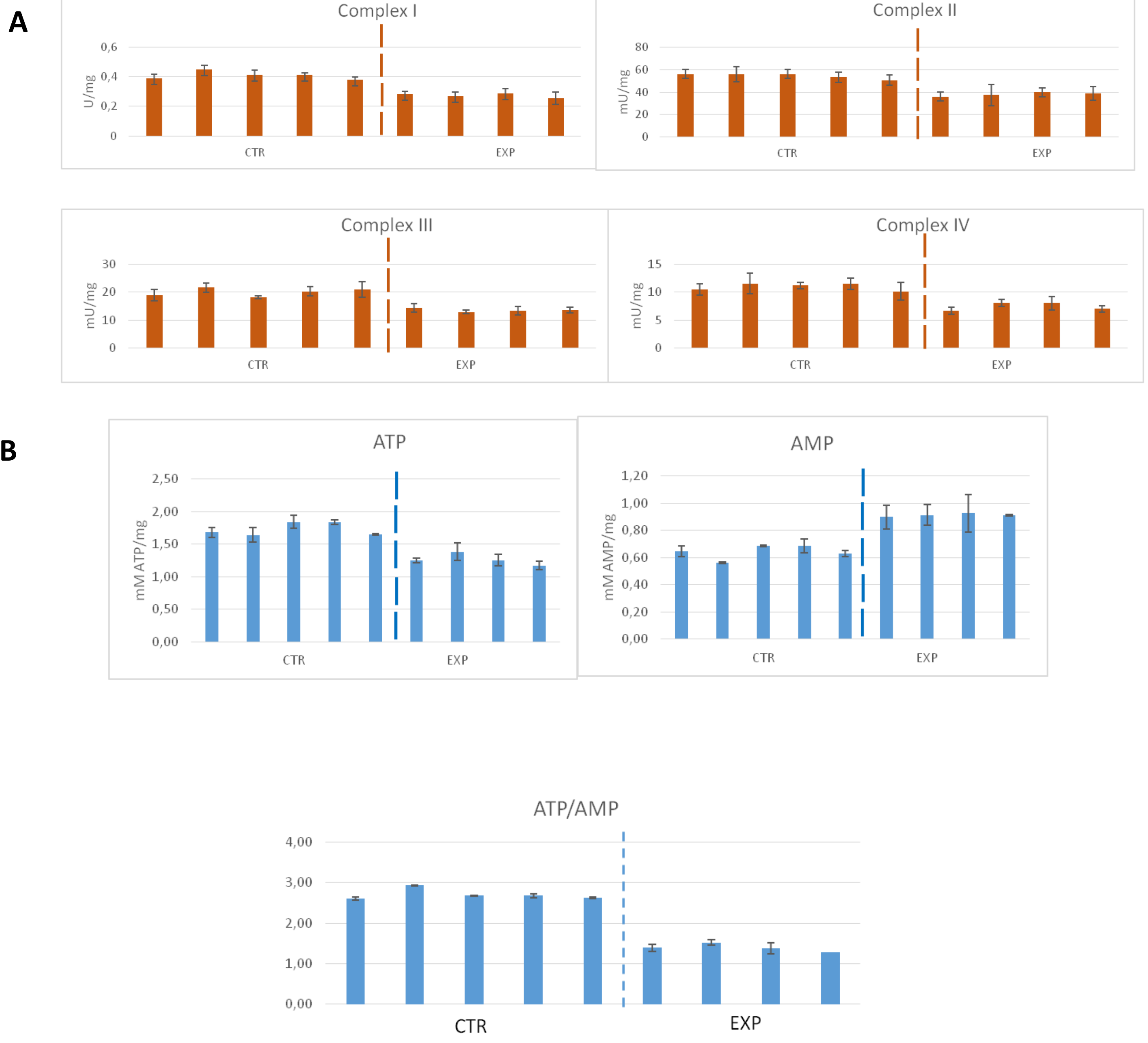


Figure 8. SIRT6 regulates ATP production in PyMT mouse model. Mitochondrial complex activity **(A)**, ATP, AMP and ATP/AMP ratio concentrations **(B)** were assayed on masses isolated from control and experimental mice (MMTV-PyMT+/- Sirt6+/+; MMTV-PyMT+/-; Sirt6+/-).

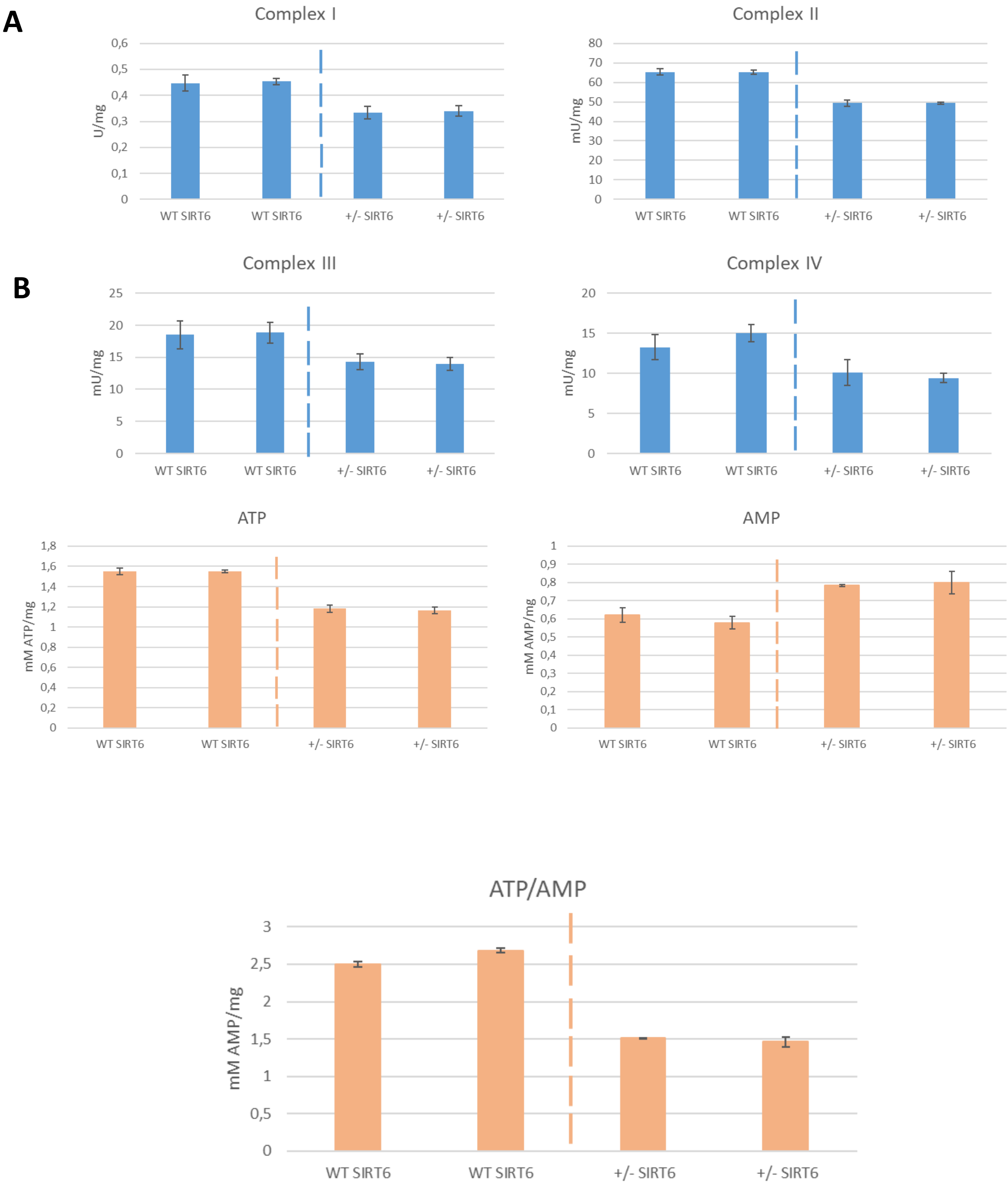


Figure 9. SIRT6 regulates ATP production in mammary tissues. Mitochondrial complex activity **(A)**, ATP, AMP and ATP/AMP ratio concentrations **(B)** were assayed in mammary glands of mice WT and heterozygous for SIRT6 (+/-).

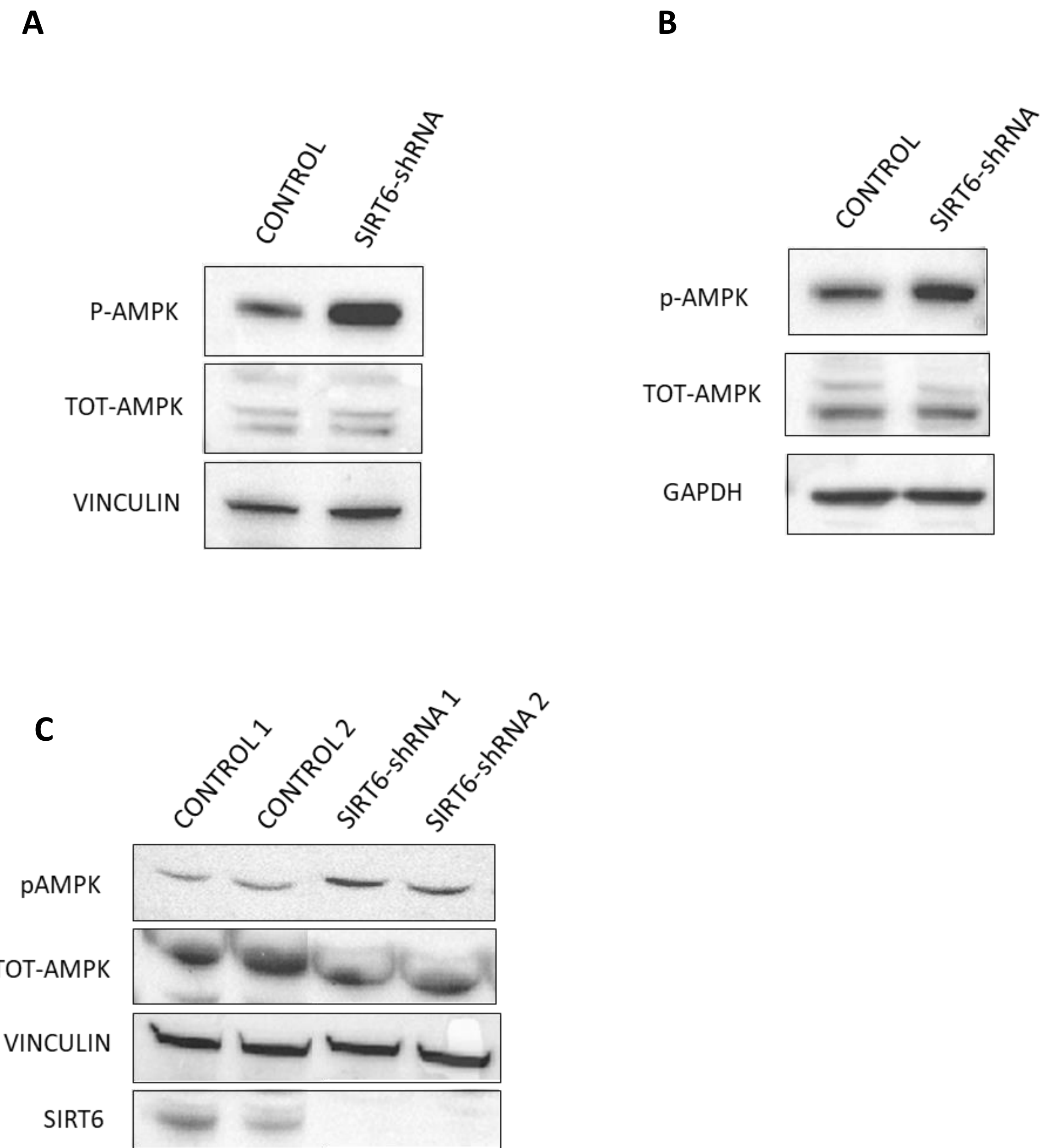


Figure 10. SIRT6 expression regulates AMPK. MCF7 **(A)** and MDA-MB-231 **(B)** cells were transduced with a SIRT6 shRNA or with PRS. pAMPK, total AMPK, SIRT6, GAPDH and Vinculin levels were quantified in western blot. The same protein were quantified also on xenograft masses lysate **(C)**.

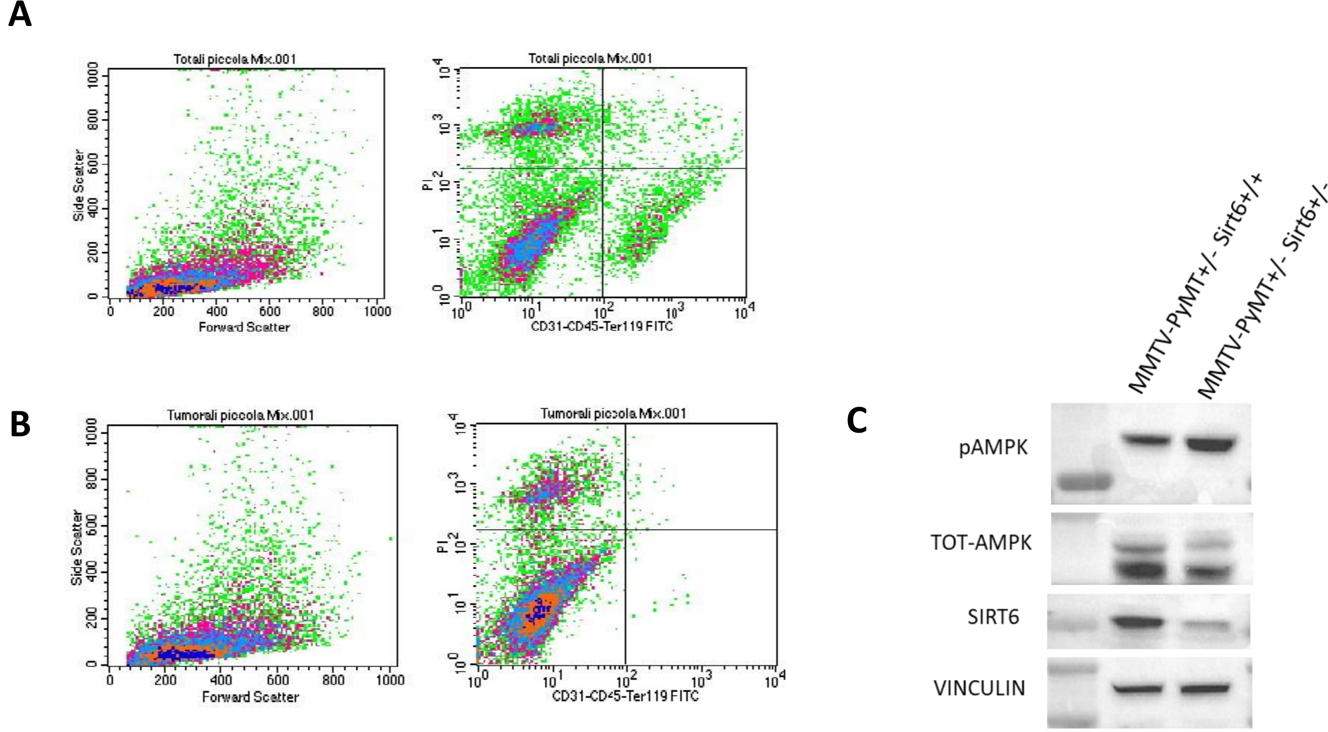


Figure 11. SIRT6 expression regulates AMPK in vivo. Tumor cells were isolated from PyMT +- SIRT6 ++ and PyMT +- SIRT6 +- masses. To verify the result of tumor cells isolation a staining with anti CD31, CD45, Ter 119 (non-tumor cell markers) conjugated with FITC was performed on cells before **(A)** and after the isolation procedure **(B).** pAMPK, total AMPK, SIRT6 and Vinculin levels were quantified in western blot on isolated tumor cells lysates **(C)**.

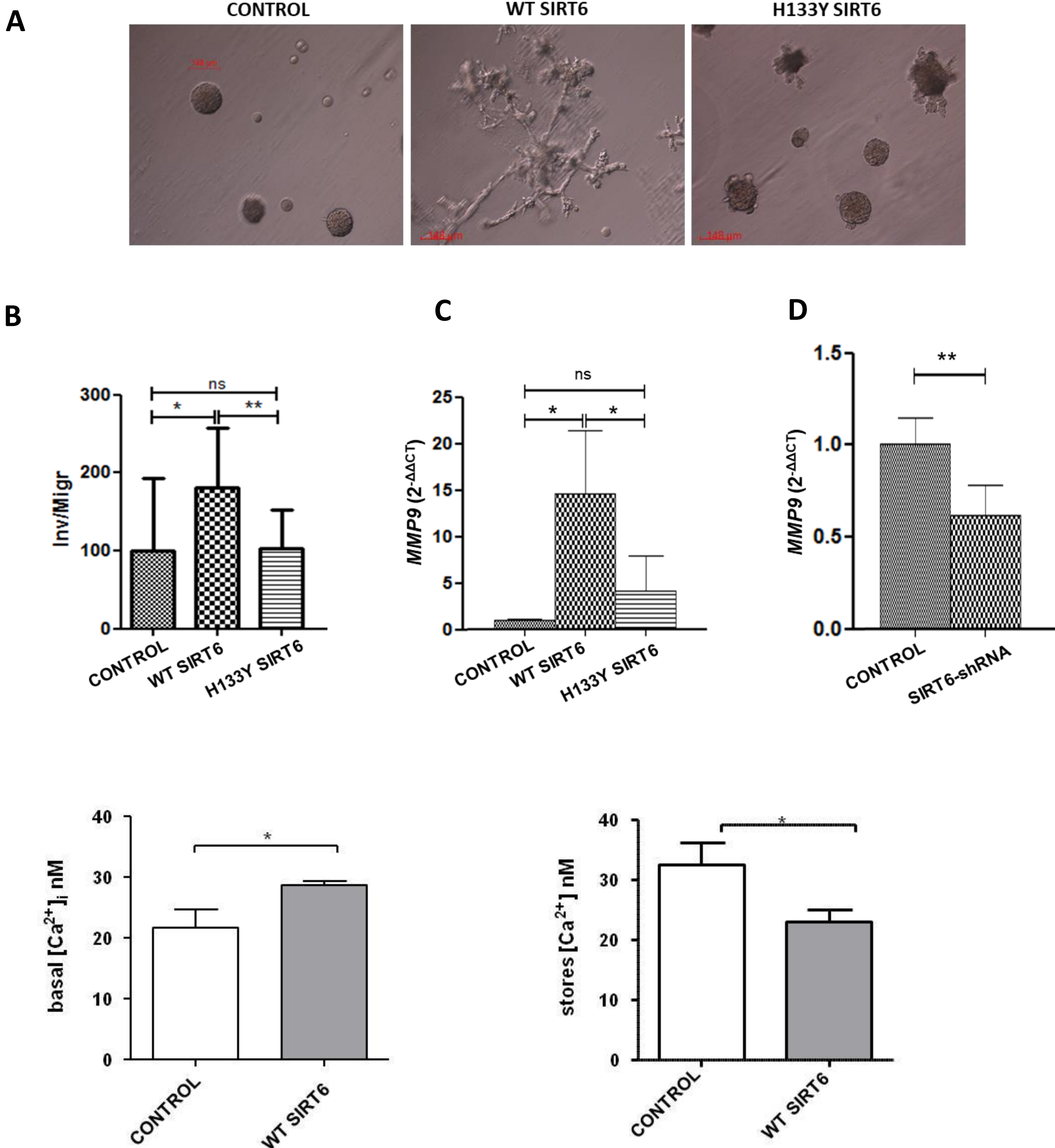


Figure 12. SIRT6 regulates invasiveness and intracellualr $[Ca2^{+}]_i$ in MDA-MB-231 cells. MDA-MB-231 cells were transduced with human WT or catalytically inactive (H133Y) SIRT6, or with a control vector (PBP). 3D sandwich Matrigel matrix assays was performed **(A)**, invasion/migration assays **(B)**, RNA isolation and quantification of MMP9 mRNA levels by qPCR in MDA-MB-231 overexpressed **(C)** and silenced for SIRT6 **(D)**, intracellular $[Ca2^{+}]_i$ was quantified as basal $[Ca2^{+}]_i$ **(E)** and store $[Ca2^{+}]$ **(F).**

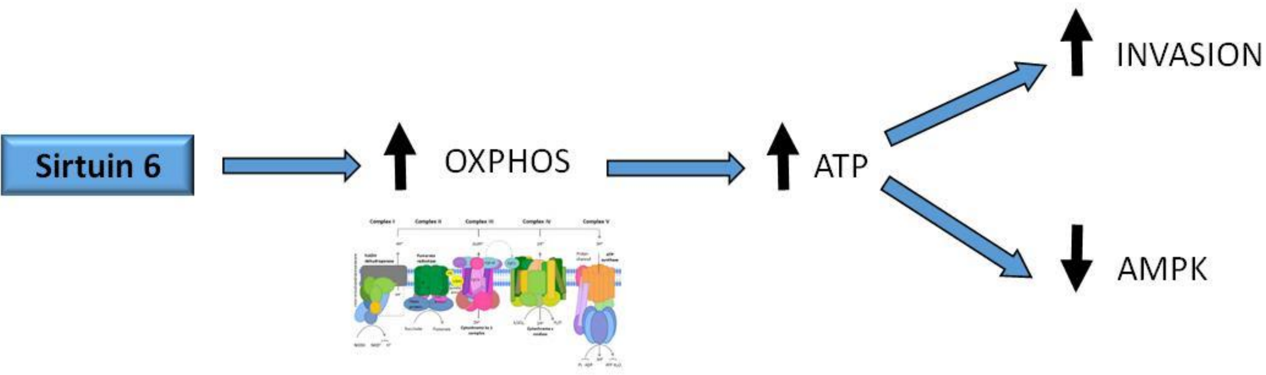


Figure 13. Putative model of SIRT6 pro-oncogenic role in breast cancer tumorigenesis.

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