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XXXII CYCLE

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The metastasis suppressor protein NM23-H1 interacts with PI3K catalytic subunit p110α and impairs PI3K-Akt axis.

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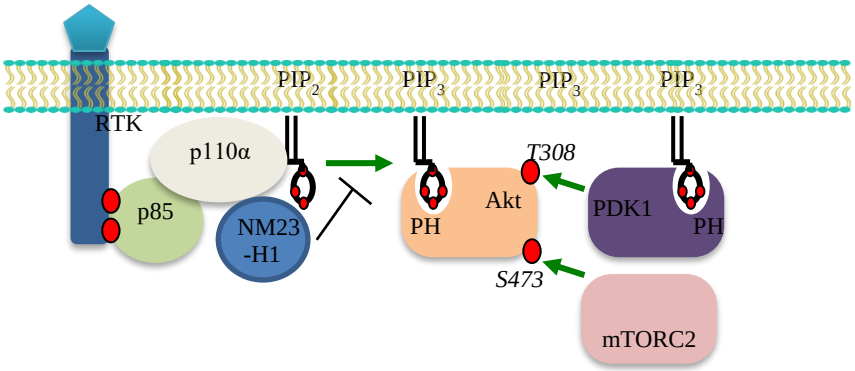

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ABSTRACT. This thesis documents a novel interaction between the catalytic subunit of PI3K class I subunit alpha p110α (PI3KCA) and the anti-metastatic protein NM23-H1 (NDPKA) and analyzes the functional consequences of this interaction. A yeast two hybrid screening of a cDNA library derived from an immortalized lymphoblastoid cell line (LCL) using NM23-H1 as "bait", identified the PI3K Class I catalytic subunit p110α (PI3KCA) as a strong binding partner. The interaction was validated by GST pull down and two-way co-immunoprecipitation experiments.

Among the various components that regulate PI3K signalling, class I p110α is associated with neoplastic progression because it is frequently mutated or overexpressed in different types of tumors. NM23-H1 is a well characterized protein with different enzymatic activities (nucleoside diphosphate kinase, protein histidine kinase, serine/threonine protein kinase, 3'-5' exonuclease) and with metastasis suppressor activity in different tumors. Loss of function NM23-H1 mutants, which determine the Killer of Prune phenotype in Drosophila, abrogate completely the anti-metastatic activity and do not interact with PI3K p110α. HEK293T, MDA-MB435 and MDA-MB231 cell lines stably expressing NM23H1 inhibit Akt phosphorylation induced by the Epidermal growth factor (EGF). Under the same conditions NM23-H1 protein mutants fail to inhibit EGF-induced AKT phosphorylation. *NM23H1* knockdown in HEK293T cell lines stimulates Akt phosphorylation. The main functional consequence of NM23 and p110α interaction is the inhibition of cell motility and clonogenic potential. In fact, in MDA-MB435 cells expressing p110α, wild type *NM23*H1, not the mutants, counteracts the enhancement of motility, invasion, adhesion, and the formation of filopodia (cell motility structures) induced by expression of p110α.

These results provide a new mechanism that explains the NM23-H1anti-metastatic properties during cancer progression and pave the way to a novel translational perspective.

1. INTRODUCTION

1.1 The PI3K History. Since the early 1980s it has been understood that biochemical modifications of inositol lipids play a key role in cellular physiology. It was clear that the activity of growth factor receptors and oncoproteins is associated with an enzyme that phosphorylates phosphatidylinositol (Ptdlns) (Sugimoto, Whitman et al. 1984, Whitman, Kaplan et al. 1985). In 1988 it was shown that the enzymatic activity repeatedly associated with oncoproteins mediates phosphorylation of 30-hydroxyl substituent of the inositol ring to produce phosphatidylinositol-3-phosphate (PtdIns-3-P) and, in 1989, was shown that platelet-derived growth factor (PDGF) stimulates this enzyme to produce phosphatidylinositol-3,4-bisphosphate (PtdIns-3, 4-P2) and phosphatidylinositol-3,4,5-trisphosphate (PtdIns3,4,5-P3) in smooth muscle cells (Whitman, Downes et al. 1988, Auger, Carpenter et al. 1989). These preliminary findings have markedly associated the kinase activity of phosphoinositide 3-kinase (PI3K) with growth factors and tumor transformation. PI3K takes part in important signaling upstream different physiological process in all tissue type. Genetic analysis in model organisms has been fundamental in understanding the genetics of PI3K.The first PI3K gene cloned was S. cerevisiae Vps34, which is required for vacuolar protein sorting; while the human ortholog hVPS34 (encoded by PIK3C3), is involved in vesicular trafficking and autophagy. This underlines that one of the most conserved functions of 3-phosphoinositides is the regulation of vesicular (Herman and Emr 1990, Backer 2016). Chemogenomic analysis in yeast identified TOR as a gene involved in nutrient sensing (Heitman, Movva et al. 1991). Then it was shown that the mammalian target of rapamycin (mTOR) play a key role in integration signal from nutrient and PI3K (Dorman, Albinder et al. 1995, Morris, Tissenbaum et al. 1996). Further studies carried out in *C. Elegans* showed that PI3K is involved in cell metabolism and aging, a study confirmed subsequently in murine models, an interesting study where the authors show that long-term inhibition of the PI3K catalytic subunit p110α has a beneficial effect on the metabolism (Selman, Tullet et al. 2009, Foukas, Bilanges et al. 2013). The first evidence on a transforming potential of PI3K genes was shown by Chang et al in 1997 following the infection of chicken cells with a retrovirus encoding an active form of PI3K catalytic subunit (Chang, Aoki et al. 1997), however, it was already known in the 1980s that the binding and activation of PI3K by the polyoma middle T antigen was necessary for the cellular transformation by this viral oncoprotein (Whitman, Kaplan et al. 1985). Subsequently cancer whole genome analysis revealed that mutations in the PI3K genes are very frequent and particularly affect the PI3KCA gene which encodes the p110α catalytic subunit (Samuels, Wang et al. 2004) (Fig.1).

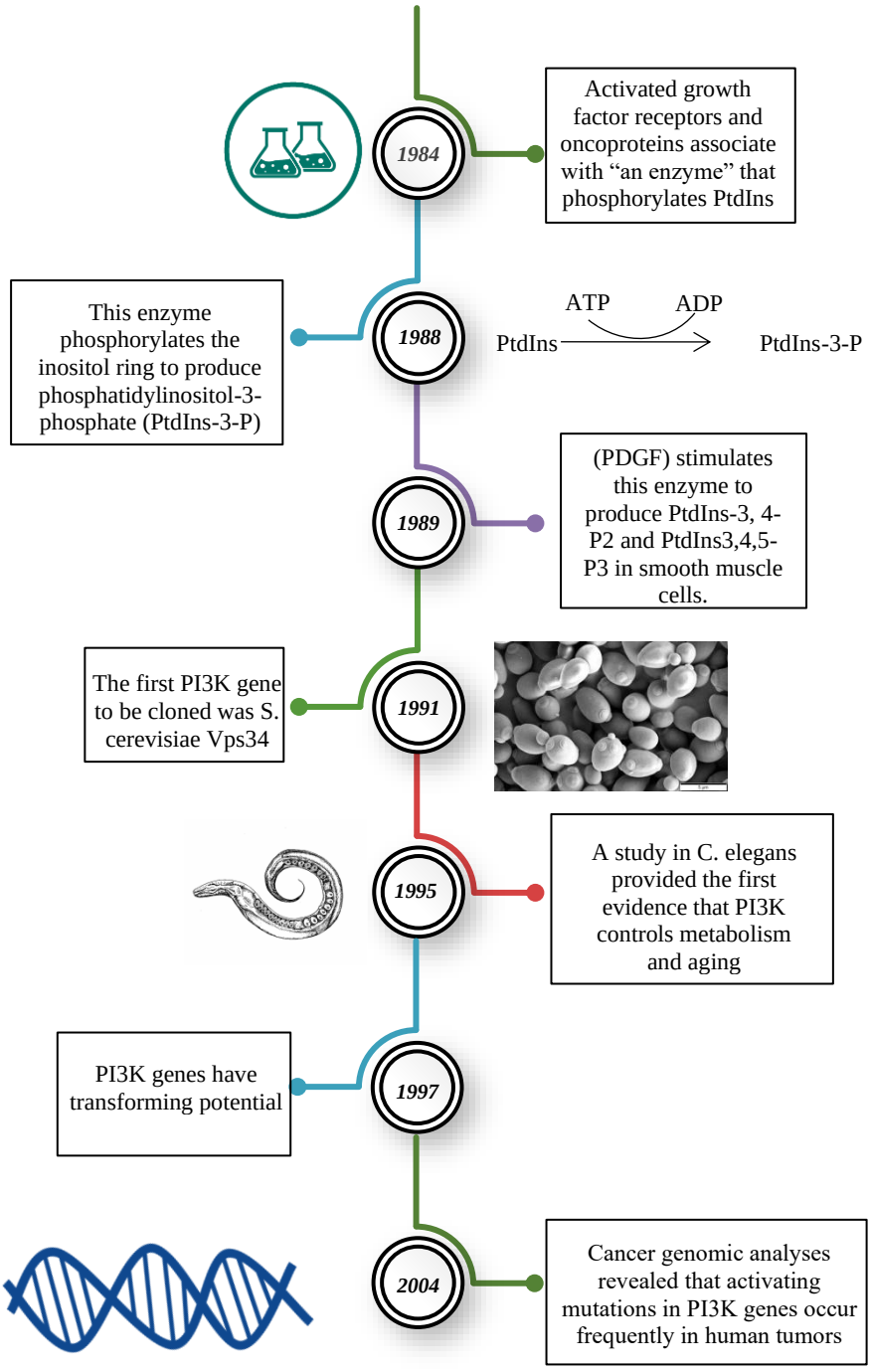


Figure 1: PI3K TimeLine. In twenty years of research we have gone from the characterization of a new kinase whose bioproducts are fundamental for different aspects of cell physiology, to establish PI3K as one of the most important "Hallmark of Cancer".

1.2 The PI3K Family and Signaling. The activity of PI3K is almost entirely connected to the production of phosphorylated phosphoinositide. Human cells express three different PI3K classes (I, II and III) (Fig.2). With the exception of class III PI3K, that is conserved from yeast to human (Auger, Carpenter et al. 1989), the PI3K family is restricted to eukaryotes. All classes can generate phosphatidylinositol 3-phosphate [PtdIns(3)*P*], while class I and class II can also synthesize phosphatidylinositol (3,4)-bisphosphate [PtdIns(3,4)P_2], and only class I can produce phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P_3].(Fig.2). There are four classes I catalytic isoforms (p110α, β, γ, and δ encoded by PIK3CA, PIK3CB, PIK3CG, and PIK3CD), three classes II PI3Ks (PI3K-C2α, β, γ) and a single class III PI3K (hVPS34). The Class I subfamily (PI3KC) acts as heterodimers consisting of one of four catalytic subunits (p110α, β, γ, δ) and one of seven regulatory subunits (p85α, p55α, p50α, p85β, p55γ, p101 or p84) (Vanhaesebroeck, Guillermet-Guibert et al. 2010, Vadas, Burke et al. 2011). The class II PI3K (PI3KC2) subfamily has three members (PI3KC2α, PI3KC2β and PI3KC2γ). This class presents two additional domains at N-terminal and C-terminal extensions. No regulatory subunit is known today for this class. The presence of N-terminal clathrin-binding domain (CB) suggests a possible link with clathrin-coated vescicles. PI3KC2α is involved in clathrin-mediated endocytosis, while PI3KC2β through the N-terminal domain interacts the scaffold protein Intersectin to stimulate PtdIns(3)*P* synthesis. To date, little is known about PI3KC2γ. Jean et al. identified the 3-phosphoinositide phosphatase myotubularin (MTM) as a possible PI3KC2 adaptor (Jean and Kiger 2014). Similar to the Class I, Class II PI3Ks harbors a Ras binding domain, but the signalling input was not characterized for this PI3K isoform. Moreover, all three PI3KC2 subfamily members possess a unique C-terminal signature that carries a C2 domain and a PX domain that binds with high affinity to phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2] (Stahelin, Karathanassis et al. 2006). Multiple upstream signals can activate Class I PI3Ks by coupling different cell surface receptor with specific PI3K isoforms. Activated tyrosine kinase coupled receptors (RTKs), G protein coupled receptors (GPCR), small Ras-related GTPases are all capable to activate PI3K signaling. This is due to the presence of conserved protein-protein interaction domains that allow their involvement in different pathways (Fig.2, see below). p110α, p110γ and p110δ possess a Ras protein interaction domain, but only p110β interacts with the Ras/cdc42 small GTPases. PI3Ks family possesses a signature motif known as the C2 domain, involved in membrane binding, the *Helical domain* that acts as a scaffold domain and a Catalytic kinase domain. The differences between the main classes are based on the presence of additional domains or on the different regulatory subunits (Vanhaesebroeck, Guillermet-Guibert et al. 2010). The p85 regulatory subunit possesses 3 Src homology domains (nSH2, cSH2, iSH2) and constitutively interacts with p110 catalytic subunit α, β, δ through an N-terminal domain named ABD (Adaptor Binding Domain) acting as a bridge between the catalytic subunit p110 and the phosphorylated tyrosine downstream of activated

receptor tyrosine kinases (RTKs).The class I catalytic isoform contains two subgroups. Specifically, the Class II subgroup (p110α, β, Δ) is associated with a regulatory subunit that mediates the binding to a TK receptors or adaptors protein through his SH2 domain which recognizes the phosphotyrosyl residue. P110γ belongs to the Class IB that is associated with the regulatory subunit p101 and p87, which mediates the binding of p110γ to the heterotrimeric G proteins to activate the G protein-coupled receptors (GPCRs). However, the context is much more complex because the RTK and GPCR pathways display a certain plasticity in the activation of different Classes I of catalytic isoforms of PI3K. To better understand the plasticity of this signaling, we will take as an example Class I PI3Ks, which is associated with tumorigenesis and cancer progression. The catalytic subunit p110α forms a heterodimer with one of five different regulatory subunits (p85a, p55a, p50a, encoded by PIK3R1; p85b, PIK3R2; p55g, PIK3R3). These regulatory subunits inhibit the catalytic subunit under basal conditions. In particular, the helical segments, the kinase, and the C2 domains of the catalytic subunit contact the p85-N-SH2 domain; the C2 domain also contacts the p85-iSH2 domain. The binding of the regulatory subunit to phosphotyrosines relieves these inhibitory contacts, positions the heterodimer next the plasma membrane where the catalytic subunit can start his enzymatic activity and receives different inputs from Ras and other signalling components.

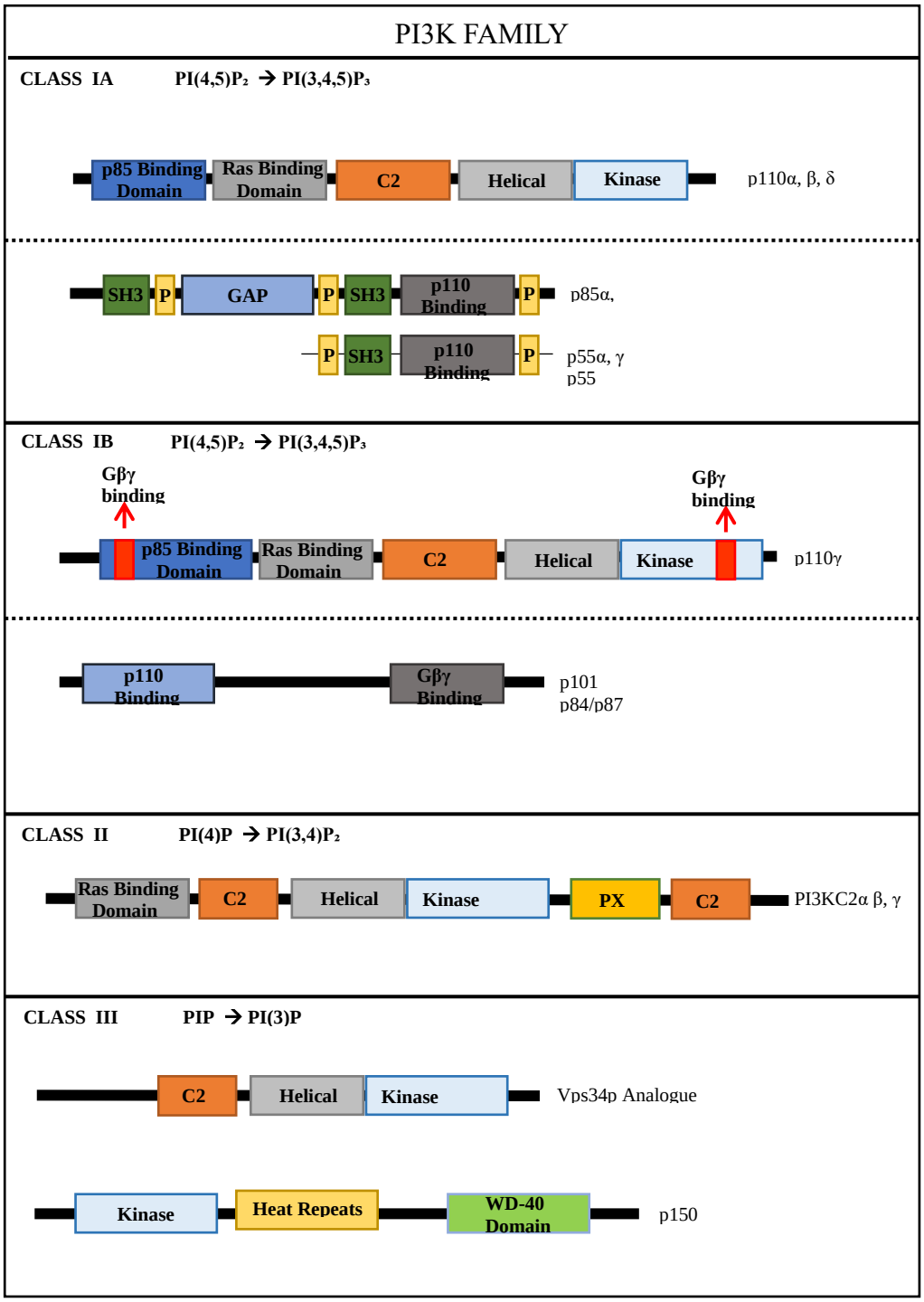


Figure 2. The PI3K family. PI3K Family is composed of 3 classes. Each of these classes has different regulatory and catalytic subunits that take part in the formation of the PI3K complex., only class I can produce phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5) P_3].

The most important and characterized outcome of PtdIns-3,4,5-P3 production by class I PI3Ks is the recruitment of specific proteins that recognized the PIP3 as anchoring point to membrane-signaling complexes. This PI3K effectors protein possess a typical domain named pleckstrin homology (PH) domain which is selective for PtdIns-3,4,5-P3 and/or PtdIns-3,4-P2. The PI3K effectors consist of a subset of proteins with distinct enzymatic activity and signalling function. These include serine/threonine kinases of the AGC kinase family (Fig.3A), tyrosine kinases of the TEC (tyrosine kinase expressed in hepatocellular carcinoma) family (Fig 3B), and modulators of small GTPase activities, termed guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (Fig. 3C).The characterization of these different families of effectors has explained the multitasking role of PI3K in the activation of different downstream signalling. The AKT sub-family of AGC serine/threonine kinases (AKT1, AKT2, AKT3) seemed to be activated more universally downstream of receptors mediated PI3K activation. In fact, the phosphorylation state of Akt can be considered a marker for the PI3K pathway activation. The full activation of Akt requires two key phosphorylation. Phosphorylation of the AKT activation loop (Thr 308 on AKT1) occurs through a straightforward mechanism, involving dual recruitment to the plasma membrane of AKT and its upstream activating kinase, phosphoinositide-dependent kinase-1 (PDK-1) (Manning and Toker 2017). Phosphorylation on Akt Ser 473 by mTOR complex-2 (mTORC2) promotes full AKT activation and seems particularly important for a subset of substrates, including forkhead box, subgroup O (FOXO) transcription factors (Jacinto, Facchinetti et al. 2006). AKT phosphorylates many substrates involved in cell proliferation, metabolism, survival, and motility (Manning and Toker 2017). The importance of Akt's role in the context of the PI3K pathway is preserved in an evolutionary way. In *C.Elegans*, in fact, two AKT orthologs act downstream of an insulin receptor homologue (DAF2) and PI3K (AGE1) to suppress the activity of DAF-16, a transcription factor homologous to human FOXO proteins, similar in human where the response to insulin in mammalian cells involves AKT-mediated inactivation of FOXO-dependent transcription. The Guanine exchange factors (GEF)(of which only a subset bear PH domains with selectivity for PtdIns-3,4,5-P3) for Rho / Rac / cdc42 family small GTPases are less associated with the PI3K pathway but still critical effector in the dynamism of this signalling, and act like effectors of the PI3K Class I enzymes (p110α,β,γ,δ)(Welch, Coadwell et al. 2002, Damoulakis, Gambardella et al. 2014).

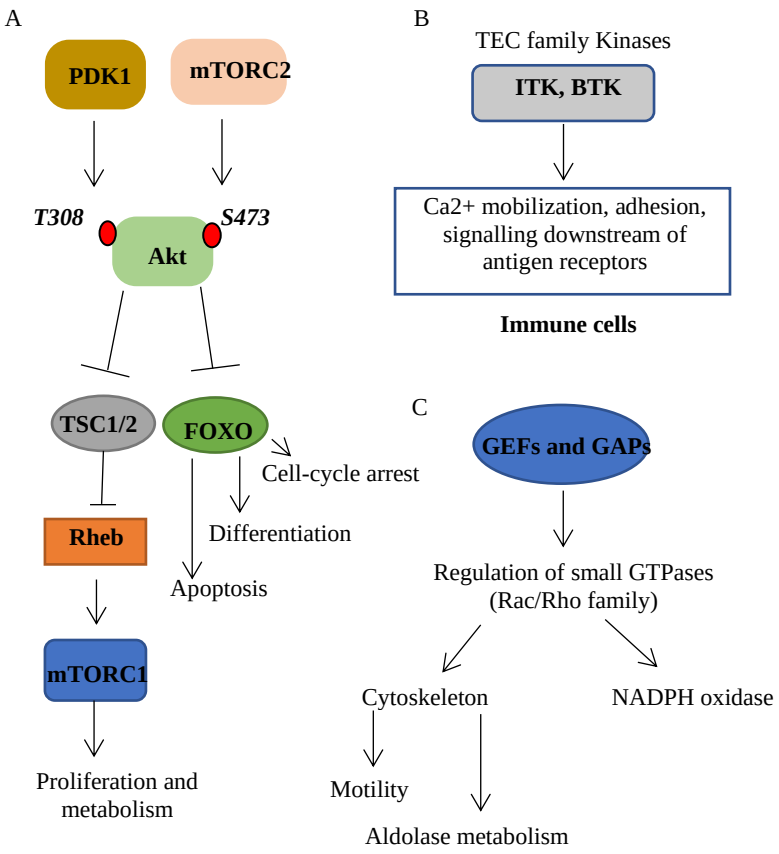


Figure 3. The PI3K effectors. A) Serine/threonine kinases of the AGC kinase family. B) Tyrosine kinases of the TEC (tyrosine kinase expressed in hepatocellular carcinoma) family. C) Modulators of small GTPase activities, guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). The specific GEFs that mediate PI3K-dependent Rac activation to promote motility and aldolase release are not known.

1.3 PI3K is one of the most "Hallmark of Cancer". Over 30 years of research on signalling associated with phosphatidylinositol bioproducts has confirmed the key role of PI3K pathway activity in controlling biological processes important for cell physiology such as growth, proliferation, metabolism, secretion and migration. Furthermore, the importance of this pathway is evident when we find that a wide spectrum of disorders and pathologies such as cancer, immunological disorders, neurological disorders, diabetes, localized tissue overgrowth, and cardiovascular disease are caused by a deregulation in this signalling pathways. This pathway is unique in that every major node is frequently mutated or amplified in a wide variety of solid tumors. Receptor tyrosine kinases upstream of PI3K, the Class I p110α catalytic subunit of PI3K, the downstream kinase, AKT and the negative regulator, PTEN, are all frequently altered in cancer (Table 1). RTKs activate PI3K directly or through adaptor proteins. Only a small percentage of PI3K becomes activated while the majority of PI3K remain in the cytoplasm in an inactive form. Therefore, deregulation in receptor activity can lead to upregulation in PI3K activity. Epidermal growth factor receptor (EGFR, ERBB1), an upstream PI3K activator, is frequently mutated in cancer. EGFR amplification is often reported in Malignant Glioma (Sauter, Maeda et al. 1996) while some gain of function mutation (deletions in exon 19 or L858R substitutions in exon 21; exon 20 insertions and T790M mutations) are specific for non-small cell lung cancer (NSCLC)(Arteaga 2006). Another member of EGFR family is HER2 (EGFR-2, ERBB2) which possesses the strongest catalytic kinase activity of this family of receptors. Overexpression through gene amplification or transcriptional deregulation of HER2 is represented in 25-30% of metastatic breast and ovarian cancer associated with poor prognosis (Moasser 2007). Rare (4% of NSCLC and 10% of lung adenocarcinomas) somatic mutations have been found in the kinase domain of HER2 and occur predominantly in people of Asian descent (Stephens, Hunter et al. 2004, Moasser 2007). Gain of function mutation can occur in some gastrointestinal stromal tumor. However, MET (hepatocyte growth factor receptor) amplifications have been found in 22% of acquired gefitnib-resistant lung cancers and have been shown to restore AKT activity via MET-dependent phosphorylation of ERBB3 and consequent activation of PI3K (Engelman, Zejnullahu et al. 2007). Mutations in the PH domain that promote constitutive cell membrane localization occur frequently in cancer (e.g., AKT1-E17K in 4%–8% of breast cancer patients), suggesting that AKT is an important PI3K effector in oncogenic signalling. This constitutive association with plasma membrane lead to a prolonged activation state that is sufficient to transform cell *in vitro* (Carpten, Faber et al. 2007). Amplifications in different AKT isoforms have been reported

in pancreatic, ovarian, and head and neck cancers (Engelman, Luo et al. 2006, Carpten, Faber et al. 2007). In a meta-analysis of cancer genome sequencing studies, PIK3CA and PTEN were found to be the second and third most highly mutated genes in human cancers (Lawrence, Stojanov et al. 2013). Phosphatase and tensin homolog (PTEN) is a key tumor suppressor gene and mutations of this gene are a step in the development of many cancers. Germline mutations in PTEN cause autosomal dominant hamartoma tumor syndromes, whereas sporadic missense mutations occur frequently in central nervous system (20%), endometrial (39%), colorectal (9%), skin (17%), prostate (14%) and breast (6%) cancers (http://www.sanger.ac.uk/genetics/CGP/cosmic). In the context of other somatic mutations, monoallelic loss of PTEN contributes to tumor growth and PTEN protein levels correlate with disease severity, suggesting that PTEN is functionally haploinsufficient (Salmena, Carracedo et al. 2008).*The gene encoding the class I catalytic subunit p110α (PIK3CA)* was found to be frequently mutated in breast (27%), endometrial (23%), colorectal (14%), urinary tract (17%) and ovarian (8%) cancers (http://www.sanger.ac.uk/genetics/CGP/cosmic). Many PIK3CA - *gain of function* mutation has been identified in human tumors. The most common "Hotspost" mutations are H1047R and E542K/E545K. The H1047R mutation constitutively associates the p110α kinase with plasma membrane without the requirement for association with Ras (Burke and Williams 2015). E542K and E545K mutations affect the inhibitory activity of regulatory subunit through structural change in the N-SH2 domains (Miled, Yan et al. 2007, Burke, Perisic et al. 2012). Cancer-associated mutations in other class I PI3K genes are very rare and transformation by these regulatory subunit variants requires activation of the p110α catalytic isoform, emphasizing the oncogenic potential of this protein (Sun, Hillmann et al. 2010). Not only in cancer, some recurrent mutations in the PIK3CA gene can cause abnormalities during the developmental stages and result in mosaic tissue overgrowth syndromes, venous malformations, and brain malformations associated with severe epilepsy (Kurek, Luks et al. 2012). The recent identification of NT5C as a novel AKT substrate with a role in cytoskeletal remodeling gave useful information to contextualize PI3K signaling in the dynamic processes of cytoskeleton remodeling in collaboration with ARP2 / 3 protein complex (Moniz, Surinova et al. 2017). The responses of fibroblast and cancer cell lines to growth factors are a well-characterized model for studying signals that regulate protrusion. PI3K is required during these early responses, as pharmacological inhibition abrogates Epidermal Growth Factor (EGF) and Platelet –Derived Growth Factor (PDGF)-induced lamellipodium formation (Sossey-Alaoui, Ranalli et al. 2005, Yip, El-Sibai et al. 2007). The

p110α isoform is required for protrusion formation during EGF stimulation, whereas overexpression of p110β, but not p110α, inhibits protrusion and lamellipodium formation (Yip, El-Sibai et al. 2007). This suggests that different PI3K catalytic subunit isoforms have distinct functions in growth-factor responses. It is likely that these early events require Ras, as the activation kinetics of Ras, but not Rac, coincide with accumulation of PtdIns(3,4,5)P_3 at the leading edge, and siRNA (small interfering RNA) knockdown of K-Ras, but not Rac1, inhibits EGF-stimulated protrusion (Yip, El-Sibai et al. 2007). However, Rac1 siRNA-treated cells have reduced motility, due to inhibition of integrin-based adhesion formation, suggesting that PI3K is involved in both protrusion and adhesion formation through signal transduction with both Ras and Rac (Yip, El-Sibai et al. 2007). PI3K may also regulate WAVE proteins during protrusion, as the class 1A regulatory subunit p85 binds WAVE3, and WAVE3 RNAi prevents PDGF-induced lamellipodium formation in a similar way to PI3K inhibition (Sossey-Alaoui, Ranalli et al. 2005). Adhesion and protrusion have been investigated *in vitro* using cultured cells with a controlled and manipulated environment that can be assessed either on surfaces coated with ligand or in co-culture on other cell types, such as endothelial cells. Several different types of surface receptors contribute to adhesion, of which the best characterized in cell migration are integrins (Ridley, Schwartz et al. 2003). PI3Ks are known both to be activated by and contribute to activation of integrins "outside-in and inside-out" signaling respectively (Ginsberg, Partridge et al. 2005). In outside-in signaling, ligand binding to the extracellular domains of integrins causes conformational changes in the cytoplasmic domains that allow signalling partners to bind. In slow-moving cells, such as fibroblasts, this leads to the production of large adhesive contacts, which are called focal adhesions (Worth and Parsons 2008). For example, FAK (focal adhesion kinase) binds to and is activated by the cytoplasmic tails of some integrins, leading to activation of the tyrosine kinase Src, and to stimulation of both Rac via the Rac GEF DOCK180 (180-kDa protein downstream of CRK), and PI3Ks via Ras signalling (Cote and Vuori 2007). In inside-out signals received by other cell-surface receptors activate intracellular signaling pathways that interact with integrin cytoplasmic domains, which in turn modulate ligand binding to the extracellular domain. Inside-out integrin activation is regulates synergistically by several signalling pathways (Ginsberg, Partridge et al. 2005), including Talin binding and signals mediated by PKC, Rap1 and PI3K (Tadokoro, Shattil et al. 2003, Somanath, Kandel et al. 2007, Wegener, Partridge et al. 2007). PI3K regulates integrin-dependent cell motility by modulating integrin responses (Han, Luby-Phelps et al. 1998, Welch, Coadwell et al. 2003). Conversely, activation of Rac

can lead to clustering of integrins at the leading edge (Kiosses, Shattil et al. 2001), thereby increasing integrin avidity. This feedback promotes additional recruitment of integrins and adhesion formation, in turn, reinforcing high Rac activity. Increased PI3K activity leads to up-regulation of the transcription factor Snail, which is known to repress expression of the E-cadherin gene (Grille, Bellacosa et al. 2003), weakening cell-cell contacts and promoting the mesenchymal state. Given the multiple roles of PI3Ks in epithelial cell motility, such as increased migration, loss of polarity, disruption of cell-cell junctions (Gonzalez-Mariscal, Tapia et al. 2008), changes in integrin turnover (Zutter 2007) and expression of MMPs (matrix metalloproteinases) (Dunn, Torres et al. 2001, Ellerbroek, Halbleib et al. 2001), it is likely that *PIK3CA* mutations contribute to cancer invasion and metastasis, although this has not so far been investigated in detail.

Gene	Aberrations	Tumor	Incidence
FGFR2	Mutation	Endometrial	10%
		Breast	2%
		Colorectal	1%
HER2	Amplification	Breast	25%
		Gastric	15%
		Lung	10%
		Ovarian	8%
MET	Amplification	Colorectal	10%
		Lung	10%
PTEN	Mutation	Endometrial	44%
		Breast	<5%
		Colorectal	<5%
		GBM	5%-40%
	Loss	Lung (NSCLC)	75%
		Endometrial	55%
		Ovarian	45%
		Colorectal	20%-40%
		Breast, HNSCC	30%
		GBM	60%-80%
		Prostate	20%
PIK3R1/2	Mutation	Prostate	22%
		Endometrial	20%
		GBM	8%
		Breast	2%
		Colorectal	<1%
PIK3CA	Mutation	Breast	20%-50%
		Endometrial,Ovarian	30%
		Lung (SCLC)	23%
		HNSCC	20%
		Colorectal	14%
		GBM	10%
		Prostate	6%
		Lung (NSCLC)	5%
	Amplification	Lung	43%
		HNSCC	20%
		Breast, Ovarian	5%
AKT1/2	Amplification	HNSCC	5%
		Breast, Ovarian, Pancreatic, Gastric	<5%
		Colorectal	<1%
AKT1	Mutation	Colorectal	6%
		Breast (TNBC)	3%
		Lung (NSCLC)	2%
		Endometrial	1%

Table 1. Common PI3K pathway aberrations found in a variety of solid tumor types. Among the different genes that take part directly or indirectly in the regulation of the PI3K pathway, the PI3KCA gene coding for the catalytic subunit p110 is the most mutated.

1.4 NM23: The first characterized metastasis suppressor gene.

Nucleoside diphosphate kinase (NDPK) was first discovered in yeast (Heitman, Movva et al. 1991) and in pigeon breast muscle (Dorman, Albinder et al. 1995). In human erythrocytes and other mammalian tissues, electrophoretic profiles are complex, suggesting that several isozymes were present (Morris, Tissenbaum et al. 1996, Foukas, Bilanges et al. 2013). The first primary structures for NDP kinases were reported in 1990 for Myxoccus xanthus (Selman, Tullet et al. 2009), Dictyostelium discoideum (Wu, Liu et al. 2013), and rat (Chang, Aoki et al. 1997). This led to the discovery that the product of an independently isolated regulatory gene is NDP kinase (Chang, Aoki et al. 1997). The first nm23 gene was isolated by Steeg et al in 1989, on the basis of its reduced expression in highly metastatic murine myeloma cell lines, as compared with their non-metastatic counterparts, and has been proposed as a metastatic suppressor gene (Steeg, Bevilacqua et al. 1988). They utilized differential colony hybridization to analyze seven cell lines derived from a murine K-1735 melanoma with varying metastatic potential. They found that clone 23, exhibiting the highest RNA levels, was associated with lower metastatic potential. Therefore, this gene was termed as non-metastatic clone 23, which was abbreviated to nm23. The second nm23 gene of mouse was subsequently found by Urano et al and termed nm23-M2 (Brown and Auger 2011). Then the gene, which was found by Steeg et al, was regarded as nm23-M1. In 1989, the first human equivalent was isolated by screening a human fibroblast cDNA library. Rosengard et al identified the nm23 gene, for which RNA levels were reduced in tumor cells of high metastatic potential(Vanhaesebroeck, Guillermet-Guibert et al. 2010). This gene was referred to as nm23-H1. The human Nm23 protein has sequence homology over the entire translated region with a described developmentally regulated protein in Drosophila, encoded by the abnormal wing discs (awd) gene. Thus far, 10 genes have been documented (Boissan, Dabernat et al. 2009) (Fig.4, see below).

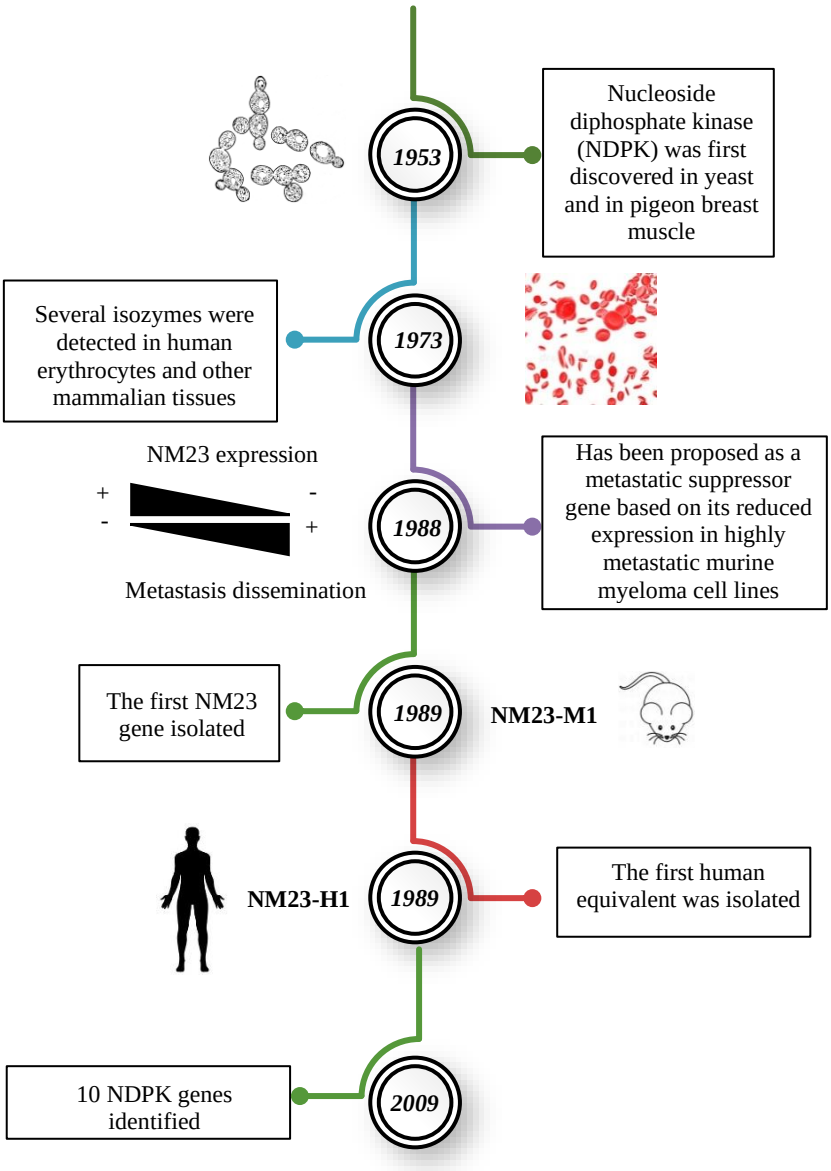


Figure 4: NM23 TimeLine. The first evidence of enzymes with NDPK activity dates back to 1953. To date 10 genes have been identified and of these, thanks to its antimetastatic activity, NM23H1 is the most characterized.

1.5 The NM23-H1 family and enzymatic activities. To date, 10 genes have been identified that make up the NM23 / NDPK family (Boissan, Dabernat et al. 2009). NME genes encode one (NME1 to NME6 and NME9) or several (NME7 and NME8), conserved NDPK domains, either full length or truncated. The NDPK domain occurs individually or associated with extra-domains (NME5, NME7 to NME9, and RP2). Nm23/NDPK family members are divided into two distinct groups (Jean and Kiger 2014) (Fig.5). Group I include four genes encoding proteins sharing 58 to 88% identity and endowed with NDPK activity (NDPK A to D). Group II includes genes encoding more divergent proteins sharing only 22% to 44% identity with group I enzymes and between each other. Most of group II genes are essentially expressed in ciliated structures such as primary cilia and sperm flagella. NME6 is the only group II member whose gene product has NDPK activity (Stahelin, Karathanassis et al. 2006). The NM23/NDPKs have been shown to be involved in cellular events such as proliferation, differentiation, development, apoptosis, and metastasis through mechanisms still largely unknown. Within Group I, all the proteins are enzymatically active and hexameric (Jean and Kiger 2014). NDPK A, encoding Nm23-H1, and B, encoding Nm23-H2, which are 88% identical at the protein level, are the most abundant and the most studied especially because NDPK A is a metastasis suppressor gene (Steeg, Bevilacqua et al. 1988, Manning and Toker 2017). Both are mainly cytoplasmic enzymes, but nuclear and plasma membrane localization has been reported. NDPK C (also named Dr-Nm23) is involved in differentiation and apoptosis of myeloid and neuroblastoma cells (Jacinto, Facchinetti et al. 2006, Facchinetti, Ouyang et al. 2008). It was reported to be, at least partly, localized in mitochondria but only NDPK D possesses the canonical mitochondria localization sequence (Jacinto, Facchinetti et al. 2006, Damoulakis, Gambardella et al. 2014). Besides their role in nucleoside triphosphate synthesis, a variety of other functions have been attributed to NM23/NDPKs. These include DNA binding, transcription modulation, and DNA cleavage (Sauter, Maeda et al. 1996, Welch, Coadwell et al. 2002). It has also been reported that the high-energy phosphate of the intermediate in the NDPK reaction could be transferred to other proteins, suggesting its role as a ‘‘histidine kinase’’(Arteaga 2006, Moasser 2007). Two other members join this group : TXNDC3 (thioredoxin domain containing 3) also named NME8 and TXNDC6, also named TXL2 or NME9 (thioredoxin-like 2) (Stephens, Hunter et al. 2004). The 10th family member is the RP2 gene, which encodes the XRP2 protein involved in X-linked retinitis pigmentosa, an inherited affection of the retina due to photoreceptor degeneration (Tornillo and Terracciano 2006).

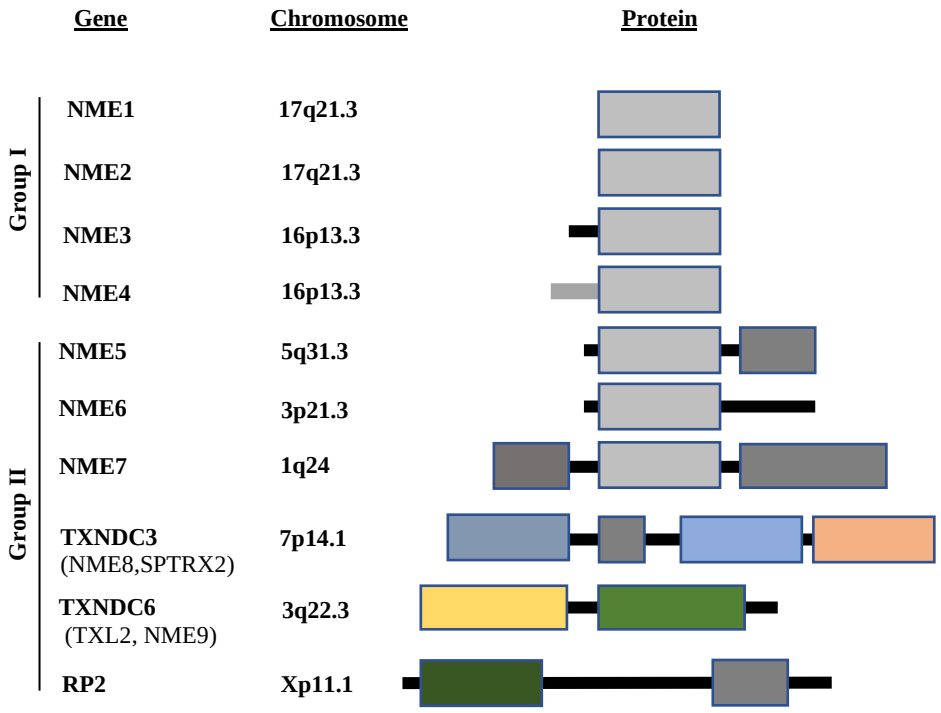


Figure 5. n. 10 genes identify since to date. The NDPK family Group I include four genes encoding proteins sharing 58% to 88% identity and endowed with NDPK activity. Group II includes genes encoding more divergent proteins sharing only 22% to 44% identity with group I enzymes and between each other.

NME8 was first reported to be exclusively expressed in testis, mainly in primary spermatocytes (Tornillo and Terracciano 2006). NME9 is highly expressed in testis but also in lung and other ciliated cell containing tissues. The protein is associated with microtubular structures such as lung airway epithelium cilia and the manchette and axoneme of spermatids. XRP2 contains two major domains. The N-terminal domain acts as a GTPase-activating protein (GAP) for beta-tubulin (Heinrich, Corless et al. 2003) and is also an efficient GAP for the small G protein Arf-like 3. The C-terminal domain of XRP2 is about 25% identical with the N terminal half of NDPK B and is lacking the catalytic histidine (Bauer, Duensing et al. 2007). NDPKs catalyze the transfer of a phosphate from nucleoside triphosphates (NTPs) to nucleoside diphosphates (NDPs), in particular GDP, by a ping-pong mechanism involving the formation of a phospho-histidine intermediate (Engelman, Luo et al. 2006, Carpten, Faber et al.

2007, Engelman, Zejnullahu et al. 2007) (Fig.6). In this reaction, ATP is the main donor of phosphate, mainly provided by the mitochondrial oxidative phosphorylation, since its intracellular concentration is much higher than that of any other nucleoside triphosphate (Lawrence, Stojanov et al. 2013). A 3′-5′ exonuclease activity has also been described for NM23-H1, which shows a preference for DNA substrates containing overhanging 3′-termini (Salmena, Carracedo et al. 2008). 3′-5′ Exonucleases activity has been associated with proofreading activity during DNA repair and replication process (Burke and Williams 2015). This potential role of the 3′-5′ Exonuclease in maintenance of genomic integrity suggests a novel mechanism in metastasis suppression, however this needs still
further investigation.

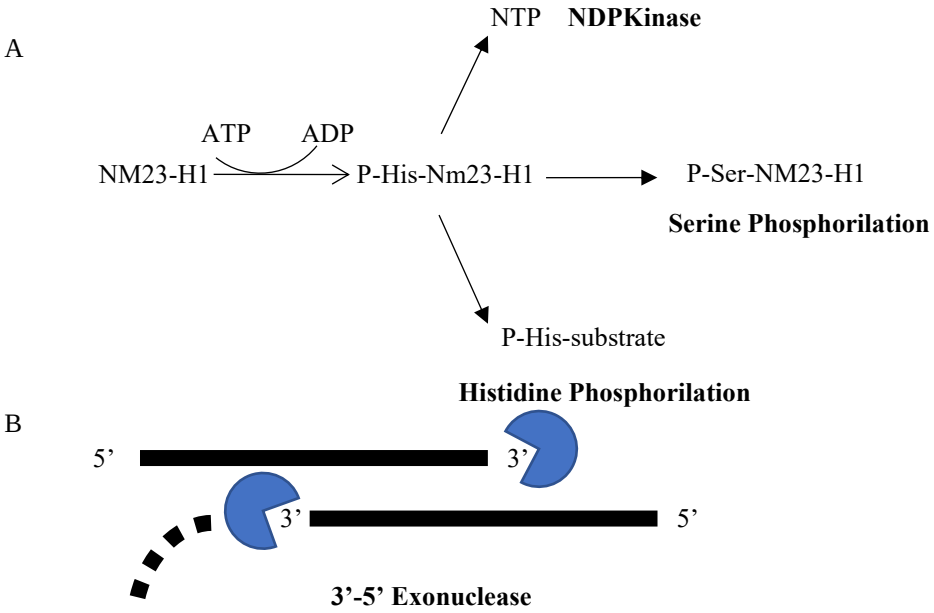


Figure 6. NM23-H1 enzymatic activities. A) NM23-H1 take part to the synthesis of nucleoside triphosphates other than ATP. The ATP gamma phosphate is transferred to the NDP beta phosphate via a ping-pong mechanism, using a phosphorylated active-site intermediate. Possesses nucleoside-diphosphate kinase, serine/threonine-specific protein kinase, histidine protein kinase and B) 3'-5' exonuclease activities.

1.6 The NM23-H1 interactome. Several proteins involved in different cellular functions have been reported as binding partners with NM23-H1. In this section, only the interactions known to be associated with the metastatic process or mechanism related to cell motility will be described (Table 1). The different enzymatic activities and the extended network of interaction can help to explain the ability of NM23-H1 to function as a metastasis suppressor gene (Steeg et al. 1988b). One of the major in vitro phenotypes of NM23-H1 overexpression is the downregulation of cancer cells motility to a variety of chemoattractant (Horak et al. 2007). Rho family GTPases are key mediators of cell transduction pathways that regulate changes to the actin cytoskeleton and are activated by guanine nucleotide exchange factors (GEFs) and negatively regulated by GTPase-activating proteins (GAPs) and GDP dissociation inhibitors. So far, several GEFs for the Rho family have been reported to be NM23-H1 interactors. Tiam1 is a GEF for the Rac1 GTPase (Habets et al. 1994). Tiam1 was first identified in T-lymphoma cells as an invasion and metastasis inducing gene and has been shown to stimulate the activity of c-Jun kinase (JNK) via the production of GTP-loaded Rac1 (Minard et al. 2004). While NM23-H1 has no direct interaction with Rho family GTPases, transiently transfected NM23-H1 and Tiam1 co-immunoprecipitated from 293T cells. NM23-H1 and Tiam1 co-expression in 293T cells was used as a readout of Rac function and the addition of NM23-H1 variably decreased Rac-GTP levels, although it cannot be proven that this resulted directly from Tiam1. A transphosphorylation of Rac by NM23-H1 was also proposed, although subsequent evidence in multiple other biochemical assays indicates that this is likely due to NM23-H1 NDPK activity. Another GEF identified as NM23-H1 binding partner is Dbl-1, involved in the activation of the Rho family member Cdc42 through exchange of GDP to GTP by Dbl-1, which leads to downstream activation of several cellular pathways involved in DNA synthesis, transcription activation, translation regulation, and cytoskeletal reorganization Murakami et al;. . The interaction between NM23-H1 and Dbl-1 could regulate the activation of Cdc42 by blocking the GDP/GTP exchange activity of Dbl-1. This regulation thereby reduced the ability of cells to migrate, which in turn could be rescued by increased expression of Dbl-1 to overcome the inhibitory effects of NM23-H1. In their proposed model system, NM23-H1 would therefore serve as a mechanism to disrupt the activation of Cdc42 in response to chemoattractant by cancer cells by modulating the GDP/GTP exchange by Dbl-1. The identification of GEFs proteins as NM23-H1 interacting proteins suggests that this signaling pathway may be biologically

relevant. One hypothesis is that NM2-H1, through its NDPK activity, alters the GEF activity of these proteins; alternatively binding to NM23 may block their normal function. Constitutively activated ARF6 bound to NM23-H1 and recruited NM23-H1 to cell- cell junctions. ARF6 is a member of the ARF family of Ras related GTPases and has been shown to control endocytic traffic and actin remodeling (D'Souza-Schorey and Chavrier 2006) and in epithelial cells, ARF6 is involved in E-cadherin endocytosis during adherens junction disassembly and cell migration (D'Souza-Schorey 2005). Moreover, increased ARF6-GTP in cells is associated with acquisition of a pro-migratory phenotype (Palacios and D'Souza-Schorey 2003). These data suggest a pro-migratory role for NM23-H1, which may be relevant in explaining the association of high NM23-H1 expression with adverse outcomes in non-Hodgkin lymphomas and neuroblastoma (Hartsough and Steeg 2000). A role for NM23-H1 in cell-cell adhesion has been suggested by Boissan et al. (2010). The authors confirmed the localization and role of NM23-H1 in cell–cell adhesion, they described an anti-motility effect of NM23- H1, compared to the pro-motility described by Palacios et al. Boissan et al. showed that the depletion of NM23-H1 in hepato-carcinoma cells (HepG2) resulted in delocalization of plasma membrane junction proteins (E-cadherin, β-catenin, γ-catenin) without altering their expression levels and increased cellular motility. Therefore, it appears that the function of NM23-H1 in the cell–cell adhesion and its effects on motility are cell line-dependent. The Epstein- Bar nuclear antigen 1-3C (EBNA1-3C) are latent genes coded by the oncogenic Epstein–Barr virus (EBV) involved in lymphoid tumorigenesis. EBNA3C is one of the best characterized interactors of NM23-H1 (Subramanian et al. 2001b). Subsequent work has confirmed this interaction in lymphoblastoid cells (Murakami et al. 2005). The Robertson lab developed the hypothesis that the Nm23-H1/EBNA-3C interaction regulates the expression of Cox-2 and αV integrin; these effects may be indirect, mediated through interactions with the Sp1, Necdin, and GATA transcription factors (Kaul et al. 2009; Choudhuri et al. 2006). EBNA-3C has been demonstrated that it could induce tumor cell motility in vitro and metastasis in vivo, and increased NM23-H1 expression rescues this phenotype (Murakami et al. 2005; Kaul et al. 2007; Subramanian et al. 2001a). Given the dynamic changes to the cytoskeleton involved in tumor cell motility, a key component of tumor metastasis, it is unsurprising that NM23 proteins have been reported to interact with the cytoskeletal machinery. Interestingly, the cytoskeletal interactions of NM23 have been observed in cells from a variety of organisms and tissues, and during development; the ubiquity of this association suggests that it may serve as essential function (Biggs et al. 1990a; Otero 1997). In a work published in 2000 by Otero et al., an interaction between Nm23-H1

and Vimentin has been demonstrated. Vimentin is a member of the intermediate filament family that promotes changes in cell shape, adhesion, and motility during cancer invasion (Mendez et al. 2010). With an assay in which Vimentin was incubated with either BSA or NM23-H1, the author demonstrated that intermediate filaments assembled in the presence of NM23-H1 formed bundles and became more densely packed. If NM23-H1 has such an effect in vivo, over-expression of NM23-H1could reduce cytoskeleton plasticity and cell movement, contributing to an anti-metastatic activity. Plakoglobin, also known as γ-Catenin, is a cytoplasmic protein that binds the intracellular domain of cadherin cell–cell adherence proteins. α-Catenin, also binds actin, linking the cadherin–catenin cell adhesion complex to the actin cytoskeleton (Nelson 2008). Plakoglobin has been associated with tumor and metastasis suppressor activity, although the mechanism of this property is unclear (Kanazawa et al. 2008). Aktary et al. (2010) showed that the stable overexpression of Plakoglobin in SCC9 cells increased the protein levels of both NM23-H1 and -H2, moreover they showed an interaction between Plakoglobin and NM23 proteins. The authors suggest that Plakoglobin's metastatic suppressor activity may be mediated through the interaction and modulation of the stability, degradation, or other posttranslational mechanisms affecting the levels of NM23-H1/H2. Serine-threonine kinase receptor associated protein (STRAP) is a transforming growth factor beta (TGF-β) receptor-interacting protein, which stabilize the association of the TGF-β receptor with Smad proteins. Seong et al. (2007) demonstrated that NM23-H1 interacted with STRAP and this interaction leads to a downregulation of TGF-β signaling suggesting a key mechanism in the negative regulation of the Epithelial to Mesenchymal transition (EMT) process in which the TGF-β pathway is a master regulator. PRUNE1 overexpression in MDA-MB-435 cell line promotes cell motility and correlates with poor prognosis in breast, colorectal, and gastric cancers (D'Angelo et al. 2004; Forus et al. 2001; Kobayashi et al. 2006). Evidences of the interaction between Nme-1 and Prune-1 were first reported in Drosophila melanogaster, where both homozygous and hemizygous Prune (Pn) mutants, responsible for the brownish-purple “prune” eye color, were shown to be lethal in the presence of just a single mutation (i.e., P97S substitution) in the NME-1 protein (the NDPK-A orthologue; alternatively known as *awd*, abnormal wing disks gene). Prune was initially reported to bind NM23- H1 using interaction mating assays, in which the P96S (killer of prune) mutation of NM23-H1 continued to interact, but the S120G mutant protein was impaired (Reymond et al. 1999). Galasso and Zollo (2009) showed that the Prune1-Nm23-H1 complex lead to a downregulation of NM23-H1 anti-metastatic properties and an increase in cell motility. The authors postulate that

(1) at high levels of Prune1 expression, all of the NM23-H1 molecules are involved in the complex formation reducing the possibility of NM23- H1 to interact with other partners mediator of its antimotility function, or, (2) the complex formation enhances the cAMP-PDE Prune1 activity which has been associated with increased cell motility. The Prune1-NM23-H1 interaction was impaired by IC261 treatment, a specific inhibitor of Casein kinase I (CKI) isoforms ε-δ. Garzia et al. (2008) reported that NM23-H1 was phosphorylated on Ser 120, 122, and 125 residues by CKI ε-δ, and that this phosphorylation was necessary for NM23-H1-Prune1 interaction. Blockage of the CKI-mediated phosphorylation of NM23-H1 inhibited cellular motility. These data paved the way for the design of a small mimetic peptide capable of inhibiting the formation of the Prune1-NM23-H1 complex. Indeed, a competitive cell-permeable peptide (CPP) that mimics the minimal region of interaction of Nme-1 and Nme-2 (i.e., amino acids 115–128) was designed to impair the formation of the NM23-H1–Prune-1 complex. This synthetic mimetic peptide has been used in different cancer cell lines (breast cancer, prostate cancer, colorectal cancer, neuroblastoma and Group3 Medulloblastoma) and functional assays demonstrated reduced proliferation and migration in the tested tumor cell lines(Ferrucci, Pennino et al. 2018).

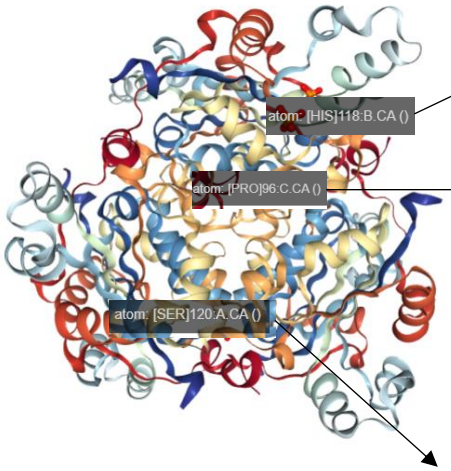
Protein	Assay	Cell lines	Interaction Phenotype	Reference
Tiam1	Co-IP	HEK293T, MDA-MB435	Rac1 pathway inactivation	Otsuki et al. 2001
Dbl-1	Two-Hybrid, GST-pulldown, Co-IP	Yeast, HEK293T	Cdc42 inactivation and inhibition of cytoskeleton remodeling	Murakami et al. 2008a, b
ARF6	Co-IP	MCF-7, MDCK II, NIH3T3	E-cadherin-endocytosis with consequent increase of cell motility	Palacios et al., 2002
EBNA-1–3C	Co-IP	HEK293T	Regulation of EBNA-l-3C-mediated transcription and increased cell motility	Subramanian et al. 2001
Vimentin	GST pull-down, Co-IP	Recombinant protein, frog heart muscle liver lung, C6	Reduced cytoskeleton flexibility	Otero et al. 1997 Otero, 2000
Plakoglobin	IF, Co-IP	SCC9, MCF-10–2-A, MCF-7, SW620, MDCK	Increase of Nm23-Hl stability	Aktary et al. 2010
STRAP	GST pull-down, Co-IP	293T, Hep3B, SK-N-BE (2)C	Cell cycle arrest and p53-dependent apoptosis	Seong et al. 2007
Prune-1	Co-IP	MDA-MB-435	Inhibition of Nm23-Hl NDPK and metastasis suppressor activity	D'Angelo et al., 2004, Timmons and Shearn 1996

Table 2. NM23-H1-binding proteins and the biochemical consequences of the interaction.

1.7 NM23-H1 and its mutant isoforms.

NM23H1 gene has proven to be increasingly a negative "Master Regulator" of the tumor associated migration process over the years. Either through its multiple enzyme activities or through its extensive interactome, Nm23-H1 negatively regulates different signaling that point to a single goal, metastasis. Many tumor cell lines showed a reduction of *in vitro* cell motility and *in vivo* metastasis dissemination when NM23H1 is exogenous overexpressed (Leone, Flatow et al. 1991). The functional characterization of different NM23H1 proteins mutant, as P96S, a site corresponding to the k-pn mutation that is responsible for developmental defects in Drosophila that exhibited normal autophosphorylation and nucleoside-diphosphate kinase (NDPK) but is deficient in phosphotransfer activity; H118F a site crucial for NDPK activity and histidine protein kinase activity that did not produce autophosphorylated proteins, indicating that autophosphorylation of Nm23-H1 mostly occurs at the histidine-118 residue, and S120G a site of mutation found in aggressive Human Neuroblastoma that retain NDPK activity but lack of histidine dependent serine phosphorylation, link the antimotility activity of NM23H1 to its enzymatic activity(Sturtevant 1956, Chang, Zhu et al. 1994, Freije, Blay et al. 1997) (Fig 7).

A



H118F:
"Site-directed Mutation of NM23-H1 mutation lacking motility suppressive capacity upon transfection are deficient in Histidine-dependent protein Phosphotransferase pathways *In Vitro*"
Josè M.P. Freije et al.,1997

P96S:
« Lethal in complementation studies in Drophila Melanogaster»
A.H.Sturtevant.,1955

S120G:
«NM23-H1 mutation in neuroblastoma»
Chang et al.,1994

Picture modified from Protein Data Bank (*Hang et al.; 2010)*

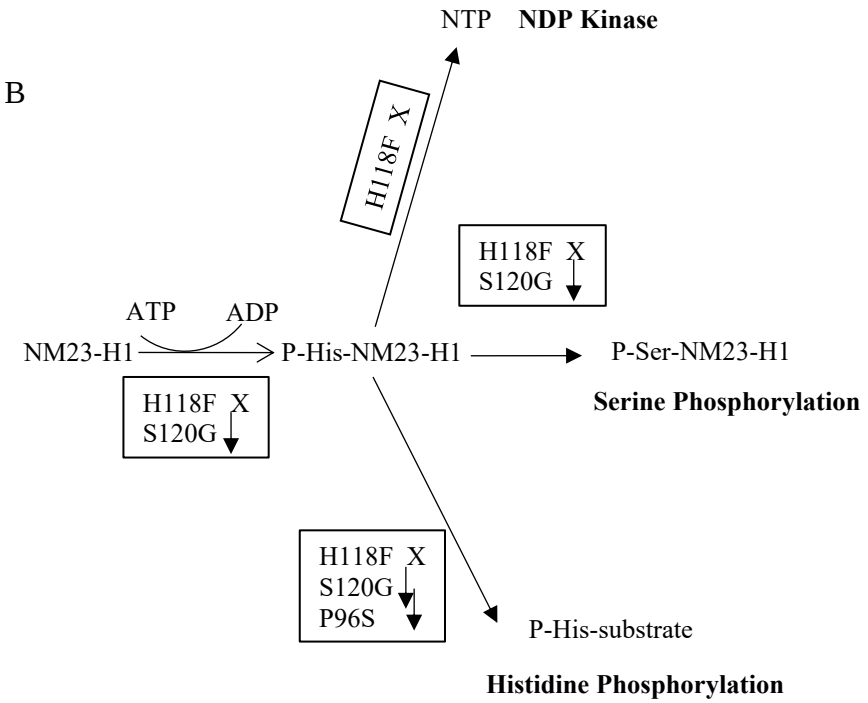


Figure 7. A) Schematic representation of NM23-H1 protein. This protein is structured in 6 alpha domains, 4 beta domains. The alpha 2 and alpha 4 domains take part in the formation of the main domains of NM23-H1, Domain Head and Domain K-pn loop. The most characterized mutations to date (P96S, H118F, S120G) are highlighted in red. Picture modified from PDN. B) The main NM23-H1 enzymatic activities are diagrammed. The mutants inactive in biochemical assays are marked with X, while the mutants with decreased activity are marked with downward arrows.

However, it was shown that P96S and S120G point mutations not only lead to a reduction of NM23H1 enzymatic activities but switch the balance from a hexameric structure to a dimeric structure. So it is not completely clear if the antimotility activity of NM23H1 protein is due to its enzymatic activities or the oligomeric structure (Kim, Park et al. 2003). Of note, it is known that Neuroblastoma, Osteosarcoma and Hematological tumors that show high NM23-H1 protein levels positively correlated with poor prognosis and low overall survival. Interestingly, *NM23-H1* overexpression or the expression of the S120G mutant proteins have been detected in 14% to 30% of advanced Neuroblastoma. This contrasting behavior of NM23-H1 protein activity was partly explained by its different enzymatic activities (a nucleoside diphosphate kinase (NDPK), protein histidine kinase, serine/threonine-specific protein kinase 3'-5' exonuclease activity) but also by its extensive proteins interaction network, (Otero 2000, Otsuki, Tanaka et al. 2001, Seong, Jung et al. 2007, Murakami, Meneses et al. 2008, Ferrucci, Pennino et al. 2018).

2. AIM

2.1 NM23-H1 interacts with PI3K. Preliminary data obtained in the ES Robertson Laboratory (University of Pennsylvania, Philadelphia, USA) provided evidence of interaction between the metastatic suppressor protein NM23-H1 and the PI3K Class I Catalytic subunit p110α. This interaction will be confirmed with other binding assays using cell lysates from cell lines transiently transfected with both interactors.

2.2* NM23-*H1 colocalizes with PI3K in the cell membrane. Analysis of the complex p110α- NM23-H1 will be performed at sub-cellular level to determine the localization of both proteins in specific cell compartments by means of confocal microscopy.

2.3 NM23H1 inhibits PI3K-Akt axis activation. The activation of the PI3K pathway mainly occurs through the activation of the catalytic subunit p110α and phosphorylation of Akt. To analyze the regulation of the PI3K pathway in stable cell lines by *NM23H1*, Akt phosphorylation will be monitored. Several mutants of NM23-H1 (P96S, H118F, S120G) which do not inhibit the metastatic process will be analyzed.

2.4 NM23H1 counteracts p110α induced tumor progression. The PI3KCA gain of function mutations induce tumor progression. Specific mutations affecting the PI3KCA gene have been associated with a promigratory phenotype that further drives cancer progression. By using *NM23* knock-in and knock-down cellular systems and the collection of *NM23* mutants, a functional analysis will be systematically carried out to determine the mechanism of the antimetastatic actions of NM23 during tumor progression.

3.MATERIALS AND METHODS

3.1 Cells and Antibodies. Antibodies: Nm23-H1 antibody (sc-514515) and p110α antibody (sc-293172) used for Western blot, immunoprecipitation and Confocal Microscopy were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit monoclonal antibody p-AKT1 (Ser 473) (#4060) and rabbit monoclonal antibody β-Catenin (#8480) were purchased from Cell Signalling Technology. AKT1 (sc-1618), GAPDH (sc-47724) and GFP (sc-53882) were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). mouse anti Myc and mouse anti-HA were purified in our lab from the hybridoma cell lines Mycl-9E10 and 12CA5 respectively. HEK293T (Human embryonic kidney cell line) was provided by Upenn Core facility. MDA-MB435 (Melanoma cell line), MDA-MB231 (Triple negative breast cancer) were provided by Jon Aster (Brigham and Woman's Hospital, Boston, MA). MDA-MB435, MDA-MB231 and HEK-293T were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 5% Bovine Growth Serum (BGS, HyClone Bovine Growth Serum). MDA-MB435 Sh-p110α and Sh-Ctrl were generated in as previously described (Saha, Halder et al. 2011) and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 5% bovine serum and 1 μg/ml puromycin.

3.2 Transfection, Immuno-precipitation and Western blotting. HEK293T, MDA-MB435 and MDA-MB231 cells were transfected with jetPRIME (Polyplus Transfection, Illkirch, France) according to the manufacturer's instructions. Co-Immunoprecipitation (Co-IP) and Western blotting were performed as described (Lang, Li et al. 2017). Briefly, cells were collected and were lysed in the immunoprecipitation assay (RIPA) buffer (10 mM Tris, 1% NP-40, 2 mM EDTA, 150 mM NaCl [pH 7.5]) with protease inhibitors. For co-IP, lysates were incubated with the antibodies indicated in the figures and 30 μl of a 1:1 mixture of protein A/G Sepharose beads at 4°C overnight. The beads were washed with RIPA buffer for 3 times, boiled and then subjected to SDS-PAGE for Western blotting.

3.3 Lentiviral production and Infection. Sh-RNAs targeted p110α and NM23H1 were constructed by annealing two pairs of primers. The sequences of the primers are
5'tcgagtgctgttgacagtgagcgaCCAGATGTATTGCTTGGTAAA tagtgaagccacagatgta TTTACCAAGCAATACATCTGG gtgcctactgcctcggaa-3'(Sh-p110α-1) 5 '
cgagtgctgttgacagtgagcgaGCATTAGAATTTACAGCAAGAtagtgaagccacagatg taTCTTGCTGTAAATTCTAATGCgtgcctactgcctcggaa-3' (Sh-p110α-2).The sense stranded oligonucleotides were annealed with their respective anti-sense stranded oligonucleotides , and then cloned into Xho I and Mlu I digested pGIPZ vector. A negative control was constructed by the same method with another pair of primers. The sense strand is 5'-

TCTCGCTTGGGCGAGAGTAAG–3' (Dharmacon Research, Chicago, IL). Lentivirus production and transduction have been described previously (Banerjee, Lu et al. 2013, Pei, Banerjee et al. 2017).

3.4 Wound healing assay. MDA-MB-435 cells were seeded at a density of 1.2×10^6 cells/well in 6-well culture plates and allowed to form a confluent monolayer. The layer of cells was scraped with a 20–200 μl micropipette tip to create a wound of ~1 mm width. Pictures of the wound were collected at T0 and T24h under a phase-contrast microscope at x100 magnification.

3.5 Adhesion assay. 96-well plate were coated with 10 μg/mL mouse collagen IV (BD Biosciences) for 30 minutes at 37°C. Wells were then washed with 1 mg/mL BSA DMEM and blocked for 1 h with 5 mg/mL BSA DMEM at 37°C, 5% CO_2. Subsequently, cells were seeded into wells (2×10^4 per well) and allowed to adhere for 30 min. Wells were washed four times with 1 mg/mL BSA DMEM and adherent cells were fixed, stained with crystal violet, and dried overnight. Cells were then counted with ImageJ software (a Java-based image processing program developed at the National Institutes of Health and the Laboratory for Optical and Computational Instrumentation (LOCI, University of Wisconsin).

3.6 Transwell Invasion assay. The Matrigel invasion assays were done using BD Biocoat Matrigel-coated invasion chambers (BD Biosciences). FBS (2%) was applied as a chemoattractant. Cells that had invaded through Matrigel to the other side of the 8-μm porous membrane were fixed and stained with crystal violet. Membranes were then affixed to glass slides and examined microscopically at ×100 magnification. Cells in a representative region of each membrane were counted with ImageJ. MDA-MB435 cell line was assayed in triplicate chambers and in duplicate experiments. Statistical significance was determined with a Student's *t* test.

3.8 Colony formation assay. MDA-MB435 in $10cm_3$ plates were transfected with pA3M, control vector, Myc-p110α alone or together with HA-NM23H1 or HA-NM23H1-P96S, Sh-p110α alone or with HA-NM23H1 or HA-NM23H1-P96S, HA-NM23H1 and HA-NM23H1-P96S alone. The transfected cells were selected in DMEM with 2mg/ml G418 (Sigma-Aldrich, St. Louis, MO, USA). Ten days later, 4% paraformaldehyde was used to fix the cell colonies at room temperature for 30 minutes and stained with 0.05% crystal violet for overnight. The colonies were scanned by BioRAD ChemiDOC MP Imaging system and the relative colony number was measured by Image J software. All assays were repeated three times for reproducibility.

3.9 Soft agar assays. The soft agar assays were performed in MDA-MB435 transfected with the vectors described previously in the colony formation assay. 1 ml 0.8% low melting agar in supplemented with DMEM media was poured

into 6-well plate and set aside to solidify. 1×10^5 cells were mixed with 1 ml 0.7% low melting agar/medium and poured on the top of the 0.8% agar layer. Ten days later, colonies were stained with 0.05% crystal violet overnight and scanned by BioRAD ChemiDOC MP Imaging system. The relative colony numbers were measured by Image J software. All assays were repeated three times for reproducibility.

3.9.1 Confocal microscopy. MDA-MB435 and HEK293T cell lines were grown in 6-well plates on coverslips and transfected with indicated plasmids. 24 h post-transfection, cells were washed with ice-cold PBS three times and fixed with 4% paraformaldehyde.at room temperature for 20 minutes. Then the fixed cells were washed with PBS and permeabilized with 0.2% Triton X-100 for 20 min at room temperature. After blocking by 3% bovine serum albumin for 1 hour at room temperature, the cells were incubated with mouse anti-NM23H1 antibody and/ or rabbit anti-p110α overnight at 4 °C. The cells were washed by PBS for three times and incubated with the Alexa Fluor 594 goat anti-rabbit (Thermo; 1:100) or Alexa Fluor 488 goat anti-mouse (Thermo; 1:1000) secondary antibody for 1 h at room temperature. The same protocol was used to stain f-actin with Phalloidin-iFluor 594 conjugated (ab176757). The cells were stained with DAPI (4', 6-diamidino-2-phenylindole) for 2 minutes at room temperature. After being washed with PBS three times, the coverslips were turned over and put on a glass slide with a drop of mounting media. Confocal images were collected by Fluoview FV300 confocal microscope.

4. RESULTS

4.1 NM23-H1 interacts with PI3K catalytic subunit p110α. A two-hybrid yeast screen was performed to identify potential cellular binding partners of the metastasis suppressor protein NM23-H1. A yeast library containing a LEU-selectable marker and cDNAs originating from EBV infected LCL cells fused to the GAL4 acidic transactivation domain was transformed into the Y190 yeast strain that expresses the GAL4-DNA binding domain-NM23-H1 fusion (Figure 8).

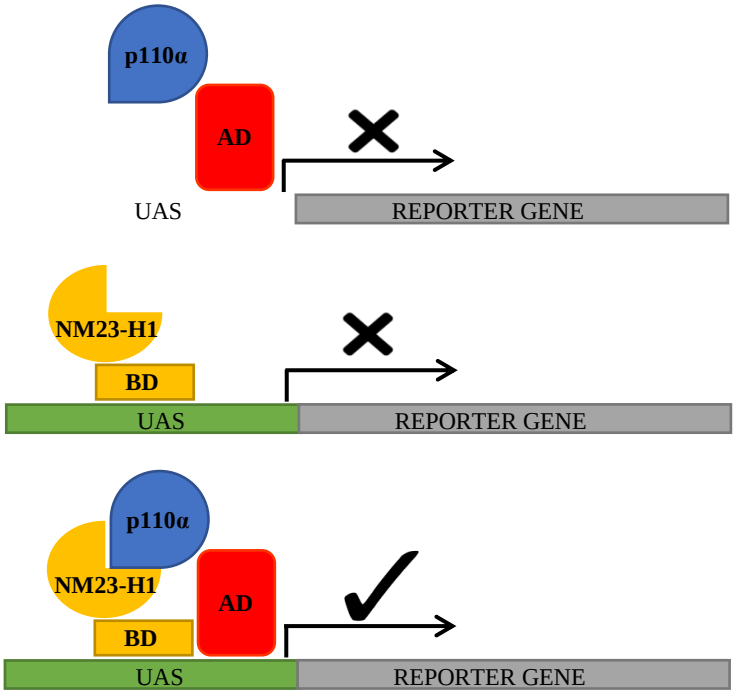


Figure 8. The yeast two hybrid system. A GAL4-DNA binding domain-NM23-H1 fusion construct was used while p110α was linked to a transcription activator domain. Interaction of NM23-H1 with p110α reconstitutes the transcriptional activity of GAL4 and therefore the transcription of the reporter gene.

The positive clones were identified and their cDNA inserts were sequenced, and analyzed for homology with known proteins through the GenBank and EMBL sequence libraries. Sequence analysis of positive interacting clones identified the PI3KCA as a strong binding partner. The construct displayed a significant beta-galactosidase activity in a 30-minut assay. The PI3K is a heterodimer containing a regulatory subunit p85, and a catalytic subunit, p110. The p85 regulatory subunit binds to the p110 catalytic subunit through its inter-SH2

domain and is responsible for docking the PI3K complex to an activated receptor through its SH2 domains (Woscholski, Dhand et al. 1994) (Figure 9).

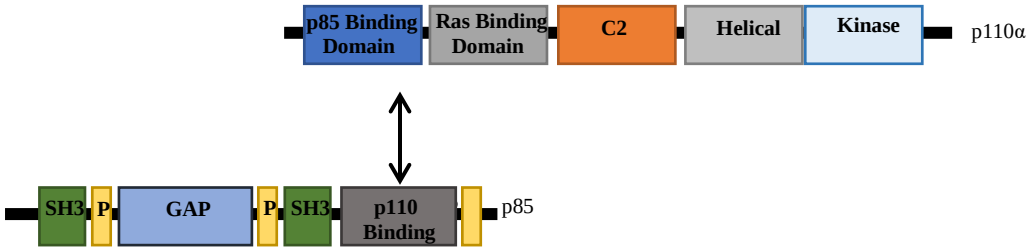


Figure 9. The class I PI3K complex. Schematic representation of the interaction between the p85 regulatory subunit and the p110 catalytic subunit. The interaction between the two subunits takes place between the inter-SH2 domain of p85 and the first domain at the N-terminal of p110.

To exclude the possibility that the interaction between NM23-H1 and p110α could also involve the regulatory subunit p85α, a GST fusion protein of wild-type NM23-H (GST-NM23-H1) was generated and incubated with *in vitro*-transcribed/translated ^{35}S-labeled p85α or p110α. While a GST control protein was unable to bind to either p85α or p110α, the GST-NM23-H1 bound only to the p110α subunit (Fig.10A). To further confirm this finding, a two-direction co-immunoprecipitation assay was performed using MDA-MB435 cells lysate overexpressing Myc-p110α, Myc-p85α and GFP-NM23-H1. Immunoblots against p85α did not show any binding with GFP-NM23-H1 (Fig. 10 B lower side), while p110α immunoblots revealed the binding of GFP-NM23-H1 (Fig. 10 B upper side).

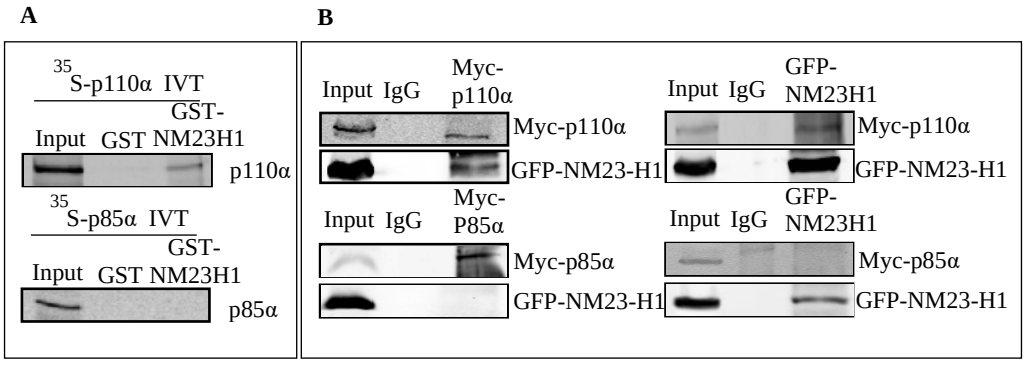


Figure 10. p110α interacts with NM23-H1 in vitro and forms a protein-protein complex in cells. A) [^{35}S] methionine labeled *in vitro* translated p110α and p85α were incubated with either GST or GST-NM23-H1 fusion proteins. Bound proteins were resolved on SDS-PAGE and analyzed by phosphoimager. Lane 1, 5% input labeled lysate; lane 2, pull downs with GST; lane

3, pull down with GST-NM23-H1 fusion protein. B) MDA-MB435 lysates from cells transfected with Myc-p110α (upper panel) or p85α (bottom panel) plasmids were first incubated with protein G beads to preclear the samples for 1h and then incubated with either IgG, anti-Myc or anti-GFP antibody. Bound proteins were resolved on SDS-PAGE and blot membrane were analyzed with odyssey imaging system. Lane 1, 5% MDA-MB435 cell lysates; lane 2, pull downs with mouse IgG; lane 3, pull down with Myc (left panel) or GFP fusion proteins (right panel).

To date, several mutations that affect NM23-H1 folding, oligomerization, enzymatic activity, or DNA binding have been described. Specifically, the NM23-H1-P96S mutation, a *Drosophila* developmental mutation homolog (*awd/K-pn*), exhibits normal autophosphorylation and NDPK function, while it is deficient in phosphotransferase activity and does not fold properly (Sturtevant 1956, D'Angelo, Garzia et al. 2004). The mutation NM23-H1-S120G, associated with stage IV-S neuroblastoma (Chang, Zhu et al. 1994), stimulates tumor cell motility (MacDonald, Freije et al. 1996). This mutant does not assemble properly and accumulates as a molten globule precipitate (Mocan, Georgescauld et al. 2007). Furthermore, there is another mutant of Nm23-H1-H118F which is deficient in histidine kinase and NDPK activities (Freije, Blay et al. 1997). To determine if these NM23-H1 protein mutants were still able to interact with p110α a co-immunoprecipitation assay from MDA-MB435 cell lysate overexpressing Myc-p110alpha, HA-P96S, HA-H118F and HA-S120G was carried out. The immunoblot with antibodies against NM23-H1 protein mutants did not show any binding to Myc-p110α, suggesting that the main aminoacidic residues involved in the NM23-H1 enzymatic activities are crucial for this interaction (Fig.11 A).

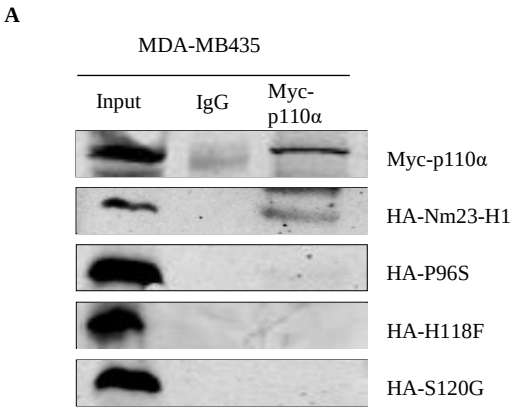


Figure 11. A) Western blot result of co-immunoprecipitation of MDA-MB435 cell lysates transfected with Myc-p110α and HA-P96S, HA-H118F or HA-S120G NM23H1 mutant expressing plasmids. Cell lysates were first incubated with protein G beads to preclear the samples and then incubated with anti-Myc antibody and protein G beads. Bound proteins were resolved on SDS-PAGE and membranes were analyzed by odyssey imaging system and odyssey

software version 1.2. Lane 1, 10% MDA-MB435 cell lysates as input; lane 2, pull down with mouse IgG; lane 3, pull down with Myc-p110α fusion protein.

4.2 NM23-H1 co-localizes with p110α to the cellular membranes. NM23-H1 is a housekeeping enzyme and its expression has many downstream functions distributed among almost all cellular compartments. It is known that NM23-H1 interacts in the cytoplasm with proteins involved in the actin remodeling. For example, NM23 interacts with Gelsolin, a key actin binding protein regulator of the assembly and disassembly of the actin filaments, located in the cytosol or mitochondria (Koya, Fujita et al. 2000, Marino, Marshall et al. 2013). Similarly, NM23 interacts also with STRAP, a protein that interacts with the growth factor receptor β (TGF-β), involved in both proliferation and cell death (Seong, Jung et al. 2007). Although NM23-H1 lacks a nuclear localization signal (NLS), it has been reported that it localizes to the nucleus by interacting with carrier proteins, such as viral proteins EBNA1 and EBNA3C of Epstein Barr Virus (Subramanian, Cotter et al. 2001, Murakami, Lan et al. 2005). NM23-H1 staining shows a diffused signal throughout the cytosol with decreasing intensity from the perinuclear region towards the periphery of the cell. In some cells, granum-like structures were observed (Fig.12). On the other hand, p110α localizes to the cytoplasm and following the interaction with the p85α regulatory subunit, migrates to the plasma membrane (Klippel, Reinhard et al. 1996) (Fig.12).

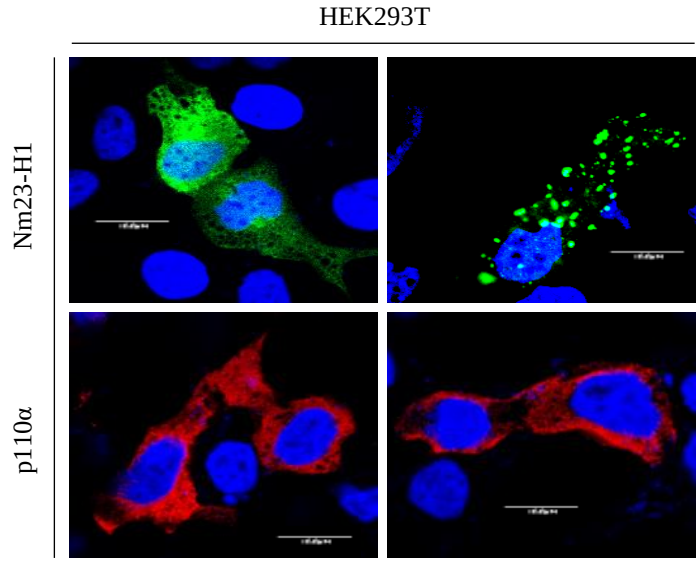


Figure 12. NM23-H1 and p110α distribution in cell compartments. Cells were co-transfected with HA-Nm23-H1 and Myc-p110α. Cells were fixed in 4% PFA. Proteins were detected with NM23-H1 (green) and p110α (red). Nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI, blue). Scale bar 10μm. NM23-H1 shows a predominantly cytoplasmic distribution with a pattern of expression which gradually increases from the perinuclear space to the plasma membrane, while p110α is mostly localized in the cytosol and plasma membrane.

HEK293T and MDA-MB435 cell lines co-expressing Myc-p110α and HA-NM23-H1 display an overlapping signal at the cell edge, which is consistent with a potential role of NM23-H1 as component of the network related to cell shape remodeling and motility activity. Additionally, the p110α signal is found as the PI3K complex in the cytoplasmic site of the cell membrane (Fig. 13 A, B). Of note, the colocalization pattern of p110α in HEK293T begins at the cytoplasmic side, where p110α appears in the plasma membrane, suggesting that p110α interacts with NM23-H1 in both its inactive and active states. Binding analysis has previously shown that the mutated isoforms of NM23-H1 (P96S, H118F and S120G) lacking the antimetastatic activity, do not physically interact with p110α although it is still possible that these mutants share the same cellular compartments. While the NM23-H1 protein mutants show the same distribution pattern as the WT isoform at the level of the plasma membrane, the Z-section and R colocalization coefficient analysis do not show a clear overlap between NM23-H1 mutant proteins and p110α (Fig.13B). Having demonstrated by binding analysis and by confocal microscopy that the mutated isoforms of NM23-H1 do not interact with p110α, these *NM23* mutants will be used as negative controls of the possible biochemical and functional effects that will be analyzed, as a specific consequence of the interaction between NM23-H1 WT and p110α.

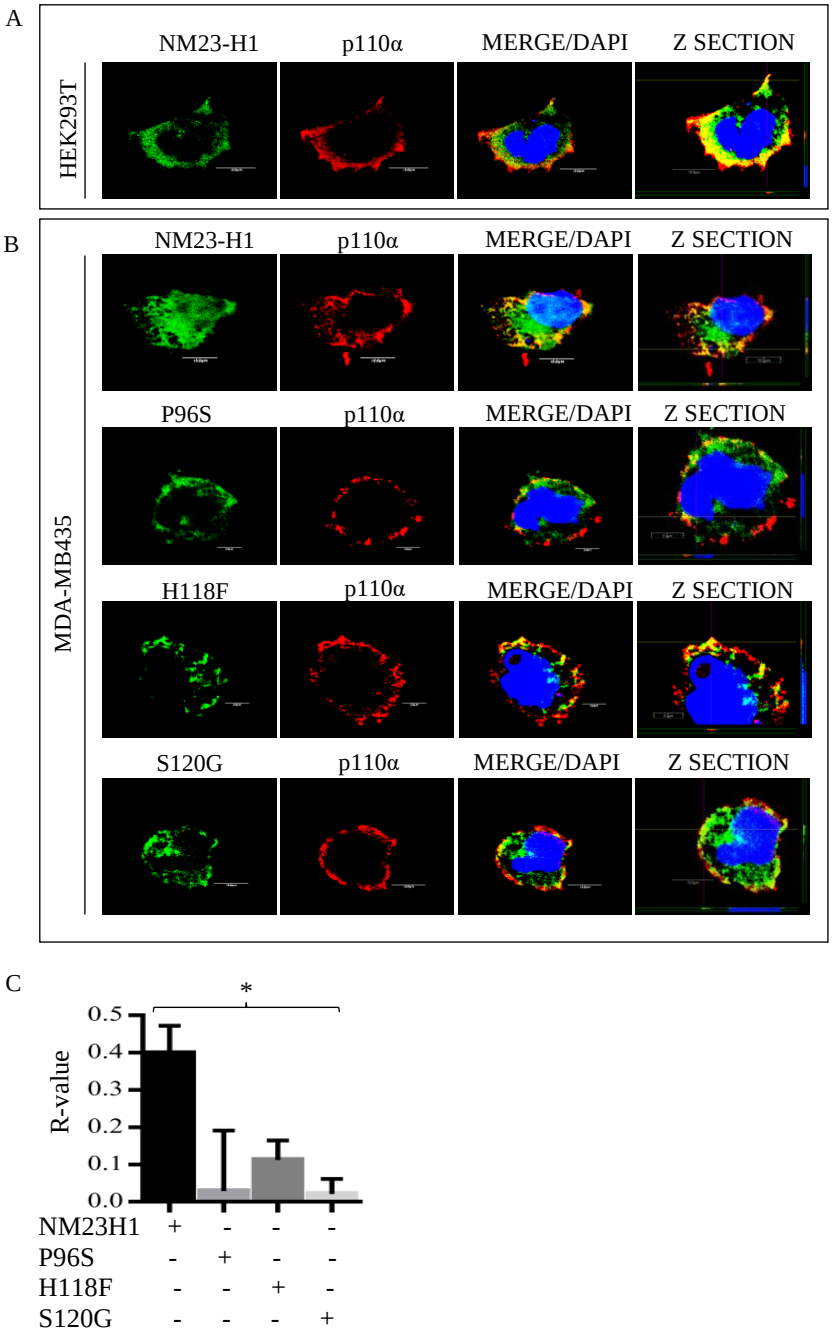


Figure 13. p110α colocalizes with NM23-H1 in human HEK 293T and MDA-MB435 cell lines. Cells were co-transfected with HA-NM23-H1 and Myc-p110α and NM23-H1 mutated isoforms HA-P96S, HA-H118F, HA-S120G constructs. Cells were fixed in 4% PFA. Proteins were detected with NM23-H1, P96S, H118F, S120G (green), and p110α (red). Nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI, blue). Merge images were composed of the 3 independently acquired images. Scale bar 10µm. (* $p < 0.05$)

4.3NM23-H1 expression negatively correlates with Akt phosphorylation. p110α is a class I catalytic subunit involved in the production of phosphatidylinositol 3,4,5-triphosphate (or PIP3) from phosphatidylinositol 4,5-biphosphate (or PI2P) that acts as a docking point for proteins containing the pleckstrin homology (PH) domain. Akt protein, among other proteins containing the PH domain, is the most characterized protein interacting with PIP3. This interaction leads to Akt localization at the cell membrane and allows Akt to be phosphorylated at serine 473 and threonine 308 by the ubiquitously expressed 3-phosphoinositide-dependent protein kinase-1 (PDK1) and PDK2 respectively (Alessi, Deak et al. 1997, Alessi, James et al. 1997, Stokoe, Stephens et al. 1997, Stephens, Anderson et al. 1998) (Fig.14)

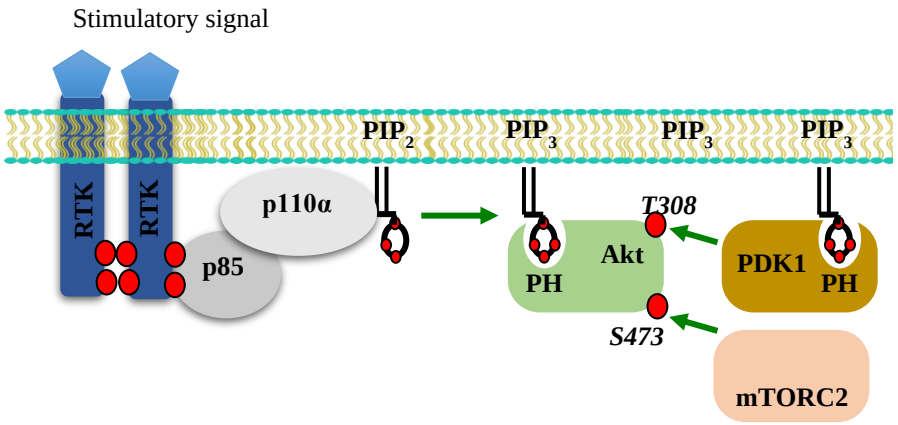


Figure 14. A) The first steps of activating PI3K signaling begin with the interaction between the activated tyrosine kinase receptors and the p85 / p110 complex at the plasma membrane level. B) At this point the catalytic subunit p110 through the phosphorylation of the phosphatidylinositol present in the membrane, produces docking points for proteins that contain the PH domain such as AKT or PDK1(C). Akt phosphorylation, main marker of PI3K activity, starts the cascade of events that characterize the PI3K pathway.

To understand a possible role of NM23-H1 on p110α kinase activity resulting in Akt phosphorylation, a western blot analysis of cell lysate from HEK293T, MDA-MB435 and MDA-MB231 cell lines stable expressing NM23H1 and control vector was carried out (Fig.15 A, B, C). All cell lines analyzed show a significant reduction in p-Akt (Fig.15 D). Decreased p-Akt levels of 1.96-fold was found in HEK293T, 1.92-fold in MDA-MB435 and 3.66-fold in MDA-MB231.

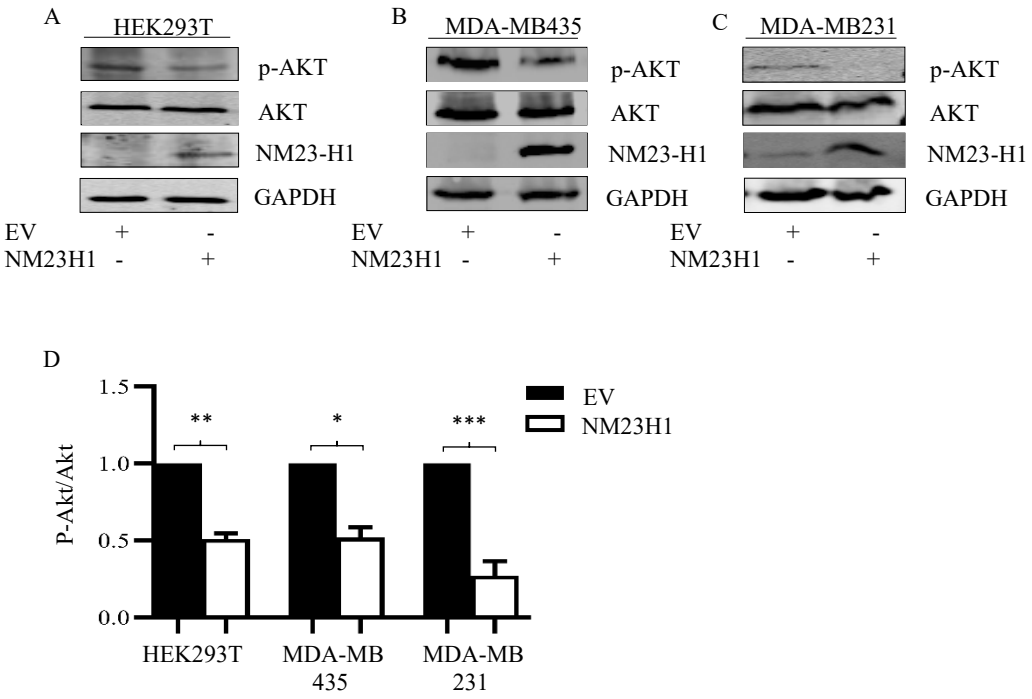


Figure 15. NM23H1 stable expressing cell line show a reduction in p-Akt. In order to assay if NM23H1 expression can downregulate Akt phosphorylation, a western blot analysis on cell lysate from HEK293T, MDA-MB435 and MDA-MB231 was carried out. A, B, C) Phosphorylated Akt were evaluated by immunoblot densitometry analysis and the normalized expression relative to total Akt is represented in the bar graph below the immunoblot (D). Fluorescent secondary antibodies were used and the quantification was performed in a LI-COR system. T-test statistical analysis was performed. n=3. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

NM23-H1 mutant proteins lack enzymatic activity and anti-motility properties of WT isoform. These mutants were selected based on previous findings which characterized these isoforms as the most representative mutations in terms of biochemical activity. For this reason, these mutations will be used as controls. Cells MDA-MB435 expressing the mutant proteins P96S, H118F, S120G (Fig. 16 A) do not show any reduction in p-Akt levels (Fig.16 B).

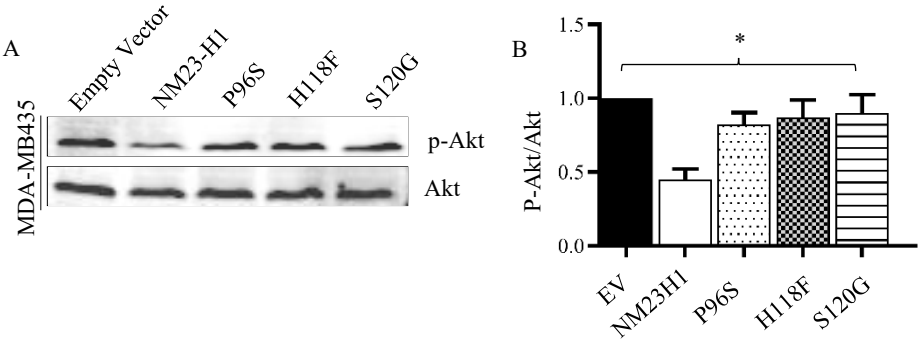


Figure 16. The expression of NM23 mutant proteins does not dowregulate p-Akt. Western blot analysis of cell lysates from MDA-MB435 expressing the mutant proteins P96S, H118F, S120G in (A). p-Akt level were normalized to total Akt protein levels and evaluated by immunoblot densitometry analysis represented in the bar graph (B). Fluorescent secondary antibodies were used and the quantification was performed in a LI-COR system. Anova statistical analysis was performed. n=3. (* $p < 0.05$).

To find out if Nm23H1 knock down rescues p-Akt levels, a western blot analysis on cell lysates from HEK293T silenced for NM23H1 was carried out (Fig. 17A). Protein and mRNA analysis reveal that the short hairpin structures designed to target NM23H1 and p110α transcript downregulate the expression of both genes. p110α knock down leads to a reduction of p-Akt, while NM23H1 knock down increased the levels of Akt phosphorylation (Figure 17B).

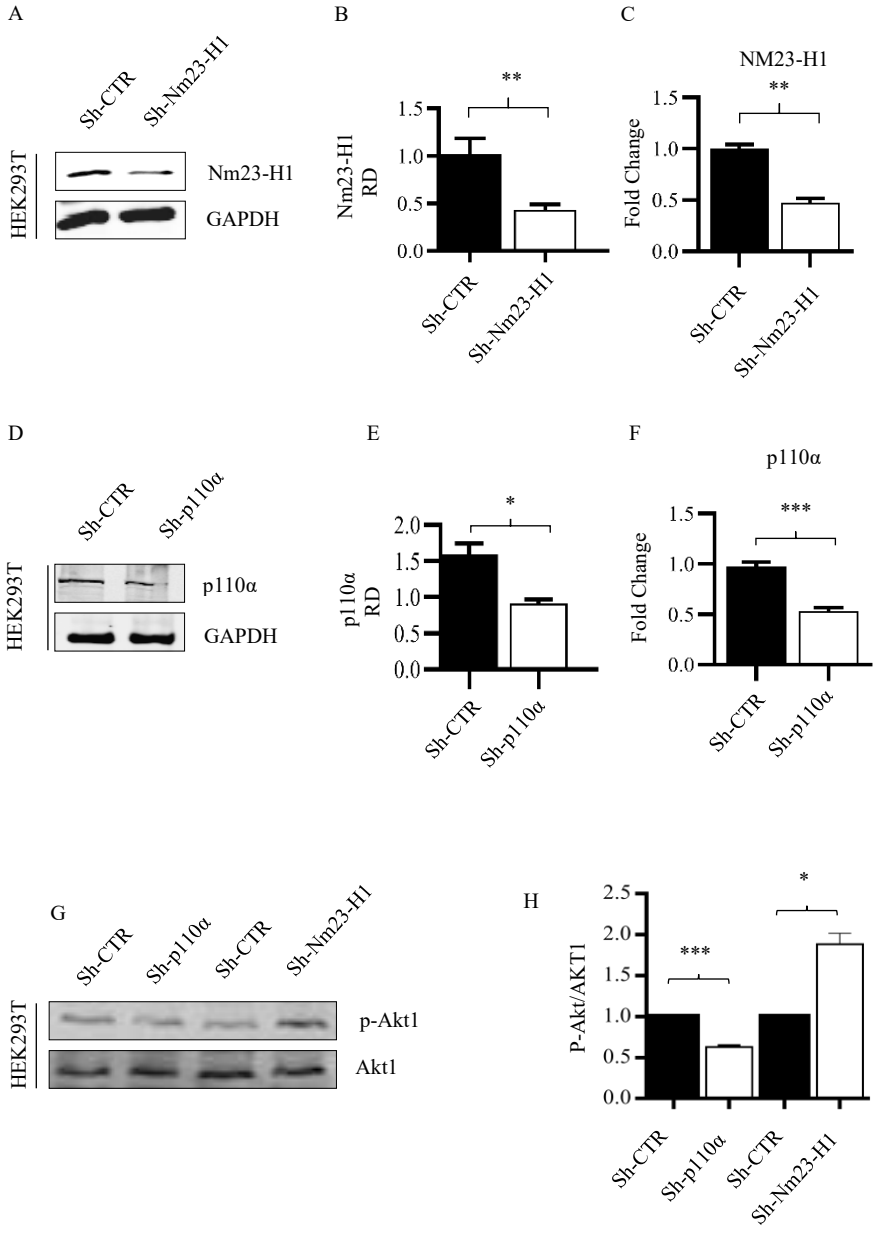


Figure 17. The NM23H1 stable expressing cell line shows a reduction in p-Akt. Western blot and RealTime analysis on cell lysate from HEK293T cell line knockdown for p110α and NM23H1 expression. A, B, C) HEK293T cell line expressing a short hairpin construct targeting NM23-H1 contains reduced NM23-H1 protein and mRNA levels. D, E, F) HEK293T cell line expressing the short hairpin construct targeting p110α contains reduced p110α protein and mRNA levels. G, H). p-Akt levels in HEK293T-Shp110α and HEK293T -ShNm23-H1 cell lines. p-Akt levels were determined by immunoblot densitometry and were normalized to total Akt. Fluorescent secondary antibodies were used and the quantification was performed in a LI-COR system. T-test statistical analysis was performed. n=3. (* $p < 0.05$; ** $p< 0.01$; *** $p< 0.001$).

The point mutations that compromise the structure and enzymatic activity of NM23 protein are contextualized as *loss of function* mutations related to the inhibitory activity of the PI3K (see cartoon as represented on Figure 18).

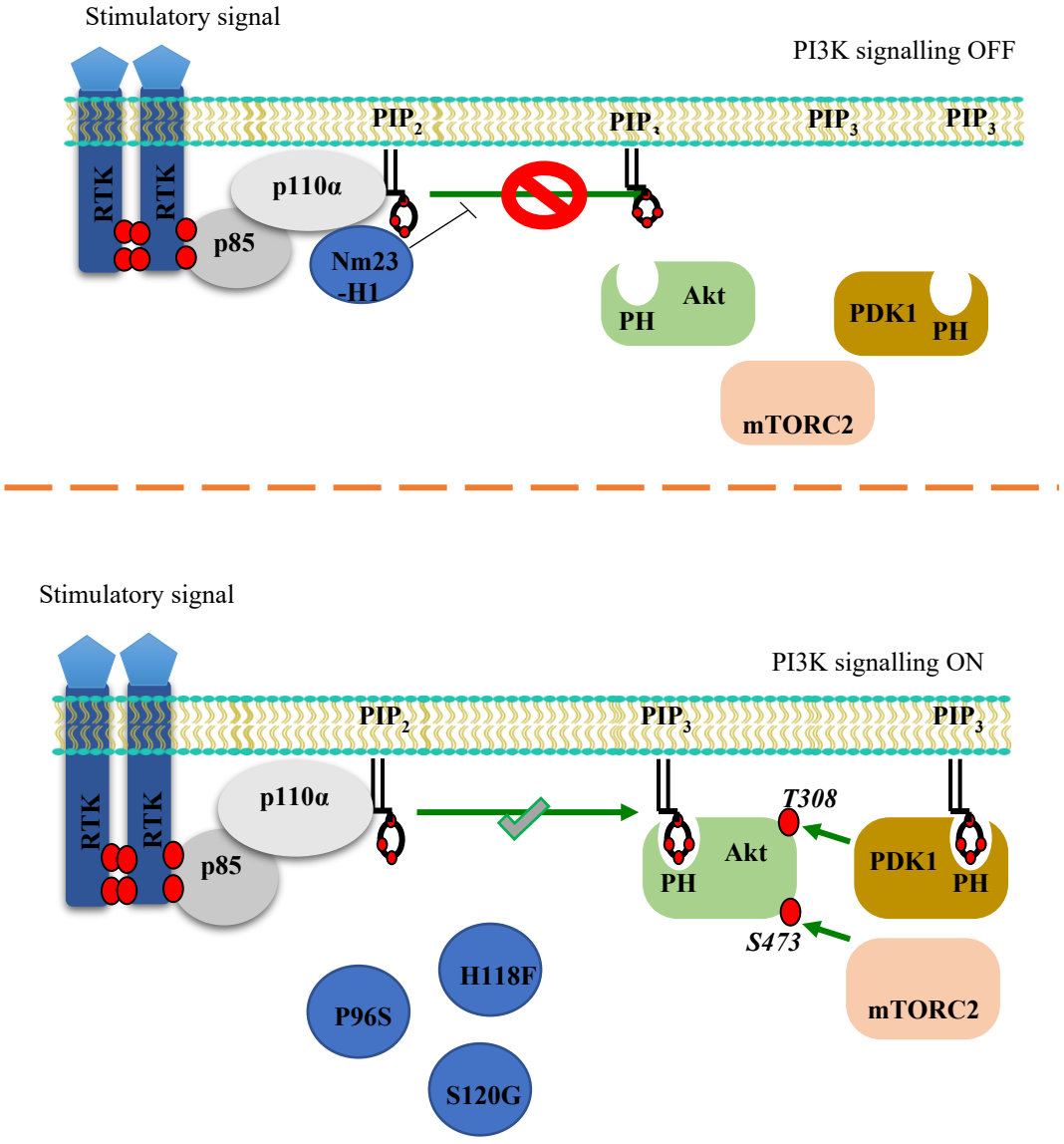


Figure 18. NM23H1 expression negatively correlates with Akt phosphorylation. Schematic model of the inhibitory action exerted by NM23-H1 WT (upper panel), but not by the mutated isoforms (lower panel), on Akt phosphorylation. Nm23-H1 interacts with p110α and reduces phosphorylated Akt levels, probably due to the reduced concentrations of PIP3, which is the docking site for proteins with a PH domain such as Akt and PDK1.

All together these data demonstrate a negative correlation between NM23H1 expression and Akt phosphorylation. These findings suggest that the NM23-H1 / p110α complex inhibits p110α kinase activity that compromises PIP3 production with consequent reduction of docking sites for proteins that present, in their structural domains, the PH domain such as Akt. The reduction of PIP3 production therefore influences Akt localization in the cellular membrane and therefore its activation by phosphorylation, marking the starting point of biochemical events in the PI3K pathway.

4.4 NM23-H1 negatively regulates growth factor mediated PI3K activation.
The main mechanism of PI3K-Akt axis is regulated by different stimuli and growth factors activated by tyrosine kinase receptors. For example, the epidermal growth factor receptor (EGFR) activates PI3K signaling (Diaz, Gonzalez et al. 2012) (Fig. 19). Epidermal growth factor (EGF) - EGFR interaction leads to serine/threonine autophosphorylation of EGFR intracellular domain. This event is necessary for the activation of target proteins such as MAPKs, PKC, STAT and the PI3K-Akt axis that activates downstream signaling involved in cell growth, proliferation, survival and migration (Wee and Wang 2017). Furthermore, in a work published by Veronica Dudu et al.; in 2012, it was shown that EGF stimulated the cell motility of Medulloblastoma Daoy cell line in a dose dependent manner and through the activation of the PI3K / Akt axis (Whitman, Downes et al. 1988). Specifically, EGF induces the actin barbed ends and lamellipodia extension through p85/p110α isoform of PI 3-kinase (Auger, Carpenter et al. 1989). Therefore, we used EGF as an activator of the PI3K pathway because it induces cell motility through activation of the PI3K catalytic subunit p110α.

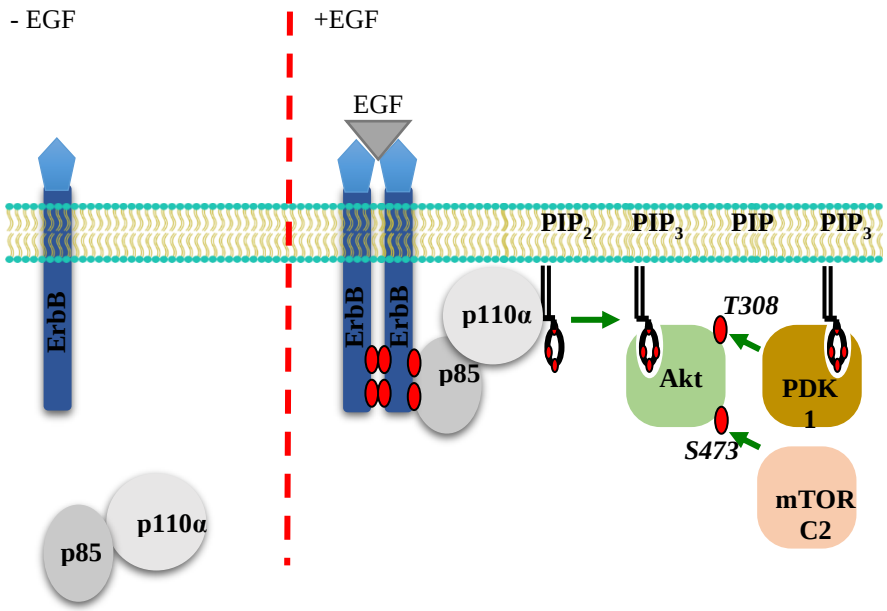


Figure 19. The epidermal growth factor (EGF) activates the phosphatidylinositol 3-kinase (PI3K)-Akt cascade. In the absence of external stimuli, the p85 / p110 complex is inactive and not associated with the plasma membrane. Following stimulation by EGF, the EGFR receptor dimerizes and activates the auto-transphosphorylation mechanisms typical of tyrosine kinase receptors. The regulatory subunit p85 recognizes phosphotyrosine residues as docking points. The p85/ receptor interaction reduces the inhibitory action of p85 on the catalytic subunit p110.

To demonstrate that NM23-H1 is able to downregulate Akt phosphorylation upon EGF stimulation, a western blot analysis of cell lysates from MDA-MB435 and MDA-MB231 NM23H1 stable expressing cell lines treated with EGF was performed (Fig. 20 A, B). Reduction of phospho- Akt levels was observed when NM23-H1 was expressed in MDA-MB435 and MDA-MB231, (Fig. 20 A, B, D). The P96S-NM23-H1 mutant isoform (Fig.20 C and Fig.20 D) did not reduce phospho-Akt.

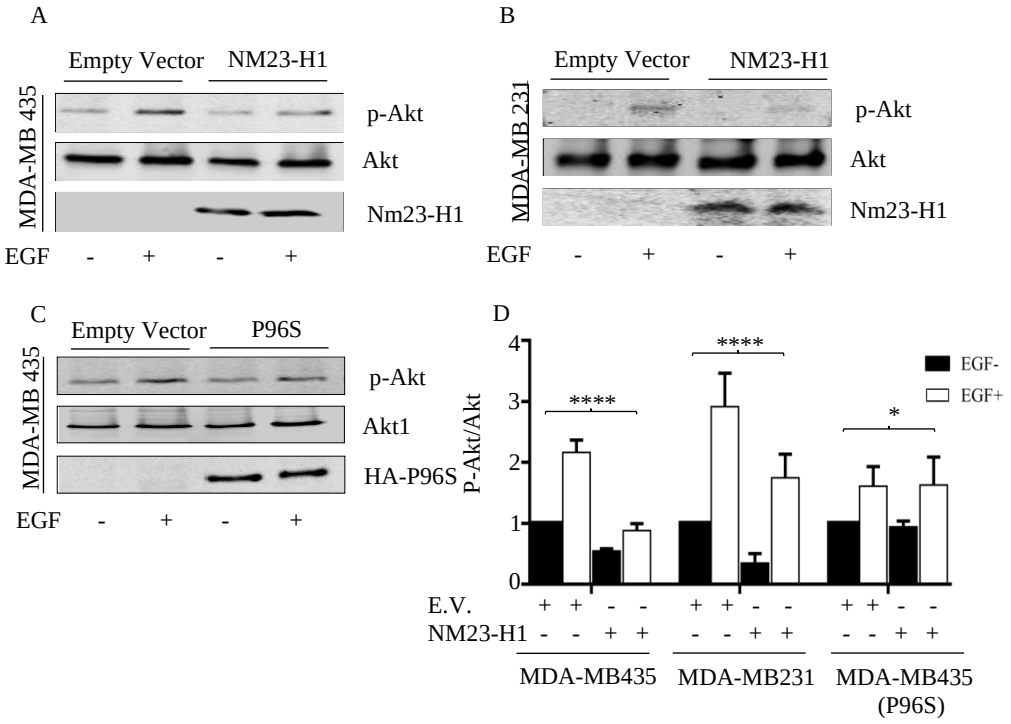


Figure 20 NM23H1 expression impair Akt phosphorilation upon EGF treatment. MDA-MB435 and MDA-MB231 expressing Empty vector, NM23H1 and MDA-MB435 expressing P96S NM23H1 mutant isoform were starved for 16 hours and treated with EGF (20 ng/ml) or vehicle for 10 minutes. A, B, C) p-Akt protein was evaluated by immunoblot densitometry analysis and the normalized expression relative to total Akt is represented in the bar graph (D). Fluorescent secondary antibodies were used and the quantification was performed in a LI-COR system. T-test statistical analysis was performed. n=3. (* $p < 0.05$; ****$p< 0.0001$)

These results demonstrate that NM23-H1 inhibits the PI3K pathway after stimulation with a strong activator of this pathway, such as the growth factor EGF.

4.5 p110α expression increases tumor metastatic potential and NM23H1 counteracts this effect. Metastasis is a multi-stage process characterized by the acquisition of a mesenchymal phenotype that gives cancer cells the ability to migrate. In cancer development and progression, invasion and metastasis occurs when tumor cells disseminate from the primary tumor spreading through the circulatory and lymphatic systems, invade across the basement membranes and endothelial walls and finally colonize distant organs (Herman and Emr 1990, Friedl and Alexander 2011). Cancer cells migration to peripheral organs constitutes a primary cause of cancer-associated morbidity and mortality (Heitman, Movva et al. 1991). Cell motility is the capacity of cells to translocate

onto a solid substratum. There is usually a cell phenotype associated with cell motility. Motile cells are polarized, and the actin cytoskeleton is a key modulator of cell polarity and cell motility. Although cellular polarity is a functional aspect of most cell types, the polarity of the motile cell is associated with the formation of pseudopods whose stabilization determines the directionality of the movement. Pseudopodal structures (eg. lamellipodia or filopodia) are associated with actin cytoskeletal dynamics because the extension of pseudopodia by the motile cell requires the polarized targeting of the proteins involved in the transduction of motility signals, the regulation of actin and microtubule cytoskeleton dynamics, the formation of cell substrate adhesion, and the localized degradation of the extracellular matrix (ECM) (Figure 21).

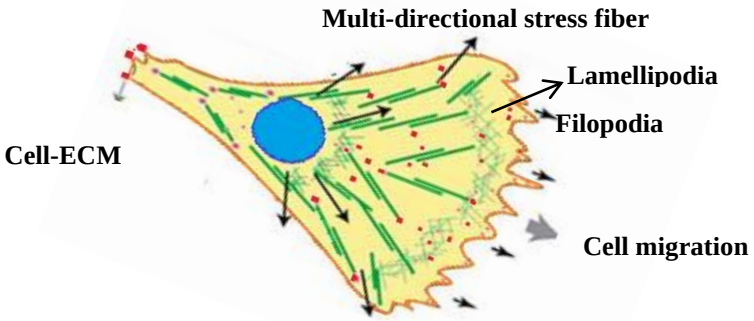


Figure 21 Representation of a polarized cell. Cell migration is a polarized cellular process involving a protrusive cell front and a retracting trailing rear. The dynamic actin cytoskeleton spatially and temporally regulates protrusion, adhesions, contraction, and retraction from the cell front to the rear. Filopodia and lamellipodia are dynamic membrane protrusions of the cell edge responsible for the forward progression of the anterior edge of migrating cells.

The ability of NM23H1 to inhibit metastasis in several tumor cell lines has been widely described (Steeg, Bevilacqua et al. 1988;Vanhaesebroeck, Guillermet-Guibert et al. 2010). Moreover, the pro-migratory phenotype of colon cancer cell lines carrying the p110α gain-of-function mutation underlines the important role of this protein in promoting metastasis spread. As part of this study, we asked whether the expression of NM23H1 was able to reduce cell migration, adhesion and invasion. While antimotility property of NM23-H1 is known, we focused our attention on the analysis on the properties of p110α and NM23H1 co-expressing clones under conditions of p110α silencing. We also monitored under the same conditions the function of NM23-H1 protein mutant P96S. The effects of the short hairpin for p110α is shown in the Figure 22. MDA-MB435 cells

expressing the Sh-p110α show approx 50% reduction of the protein (Fig.22 A-B) and mRNA levels (Fig.22 C).

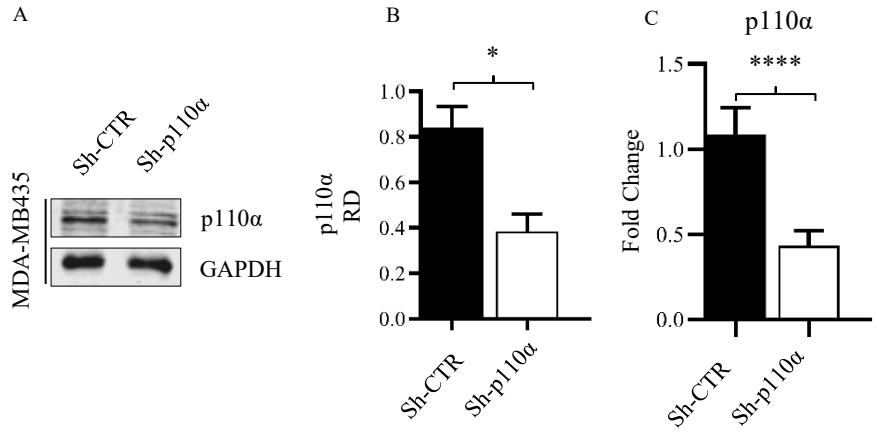


Figure 22. p110α expression. Western blot and PCR analysis on cell lysates from MDA-MB435 cell line silenced for p110α expression. A, B, C) MDA-MB435 cell line expressing a short hairpin construct targeting p110α shows a reduction of p110α mRNA and protein levels.

MDA-MB435 cells expressing p110α migrate faster than the E.V., whereas co-expression with NM23-H1 slowed down this process compared to p110α expressing cells (Fig.23A, C). MDA-MB435-P96S expressing cells expressing p110α migrate faster than NM23H1/p110α expressing cells (Fig. 23 A, C). Silencing p110α with a short hairpin strategy slows down the migration process Fig. 23 B and D. MDA-MB435-Shp110α stable cell line resulted in an increased wound area compared to the Sh-CTR. Furthermore, co-expression of Sh-p110 and NM23H1 led to a reduction of cell motility compared to Sh-CTR and the Sh-p110α. Co-expression of Sh-p110α with NM23H1 mutated isoform P96S showed the same pattern seen with Sh-p110α, indicating that the expression of the mutated isoform does not contribute to the reduction of cell motility as the WT NM23 counterpart (Fig.23 B, D).

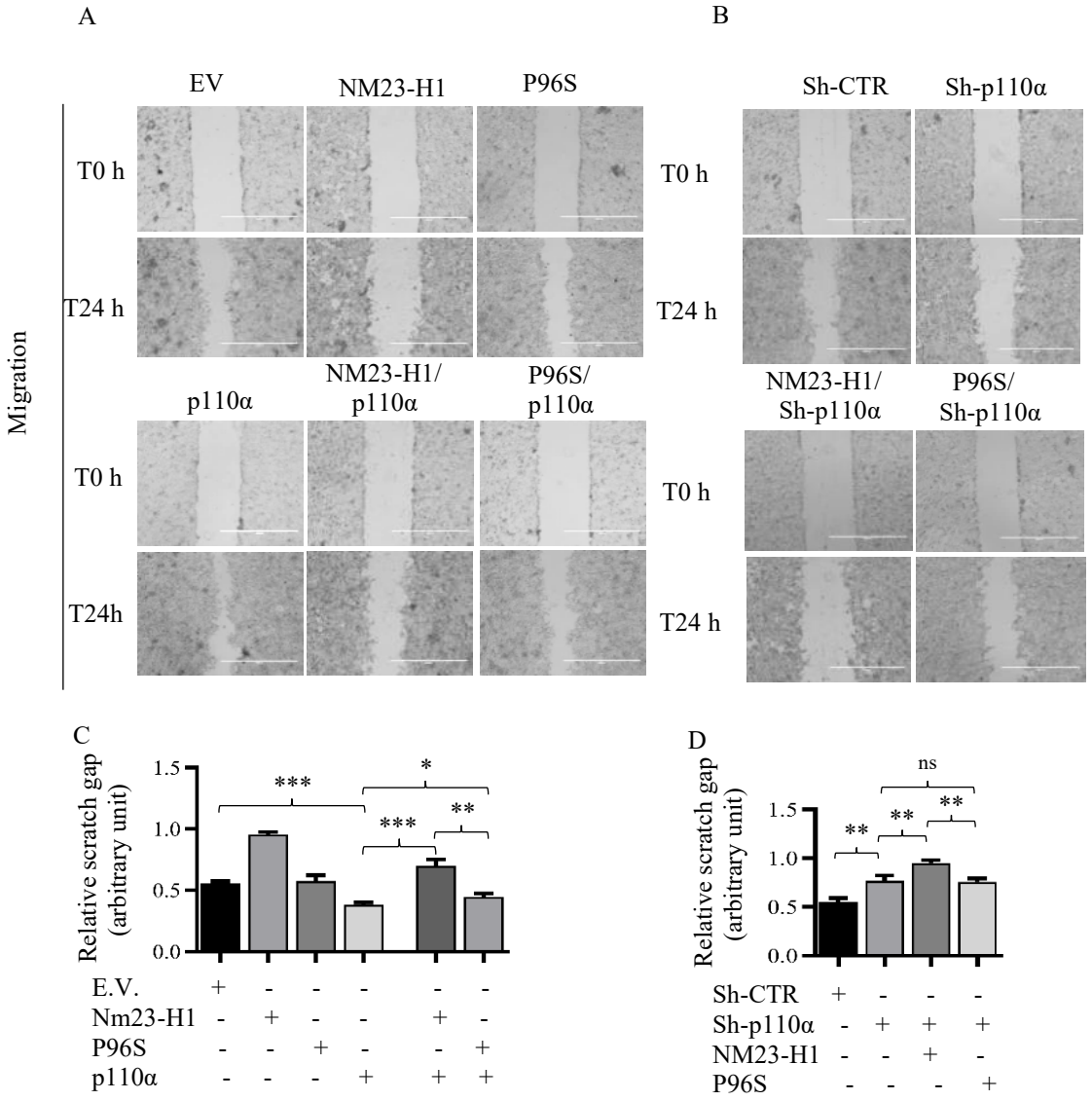


Figure 23. p110α increases motility of MDA-MB435 cell line and NM23-H1 counteracts this process. A, B) Representative images and quantitative analysis of Wound healing assay, which was performed by measuring the surface of the scratch in different time points. The cells were seeded and after reaching an optimal confluence scratches were made using a pipette tip through the entire center of the well. The diagrams show fold of scratch gap surface after T24h on T0h. T-test statistical analysis was performed. n=3. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns = Not Statistically Significant).

In cancer progression and metastasis spread, cell adhesion molecules play a key role in cell-cell interaction with endothelial cells. Therefore, direct interaction with platelets, leukocytes and soluble components is required for significant and

successful cancer cell adhesion, extravasation and establishment of a new metastatic lesion (Bendas and Borsig 2012).

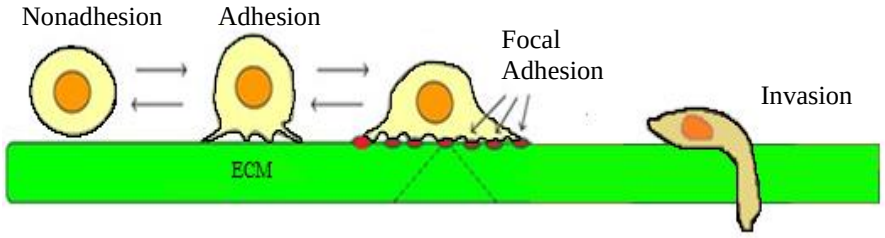


Figure 23. Representative model of Adhesion and Invasion process. Tumor cells inevitably experience alterations in cell-cell and cell-ECM adhesion and the transformation activities of tumor cells are highly influenced by cell adhesion via adhesion receptors. These adhesion receptors, together with extracellular ligands in the tumor microenvironment, couple the extracellular environment to intracellular signals, thereby enhancing cancer cell migration, invasion, proliferation, and survival. Tumor invasion, is the capacity for tumor cells to disrupt the basement membrane and penetrate underlying stroma, a the distinguishing feature of malignancy.

To test if the experimental setup previously showed can affect the adhesion property of MDA-MB435 cell line, an Adhesion assay was performed (Fig.25 A, B). MDA-MB435 cell line expressing p110α shows an increase in adherent cell number compared to the E.V. (0.60-fold). While in Nm23-H1 expressing cells it was observed a reduction of adherent cells compared to the E.V. of 2-fold. The mutated isoform P96S expressing cell line showed no significant reduction of adherent cells (Fig. 25A). Furthermore, p110α-NM23H1 co-expression show a reduction of adherent cells compared to the p110α expressing cells (2.22-fold), while a decrease was observed p110α-P96S expressing cells (0.56-fold) (Fig.25C). Silencing p110α expression slightly reduced adherent cell numbers while a significant reduction of adherent cells was observed in NM23-H1-Sh-p110α-expressing cells (Fig.25D). Overall, the data presented confirm the antimetastatic activity of NM23H1and demonstrate that the activity of Nm23-H1 counterbalances the prometastatic phenotype p110α-expressing cells.
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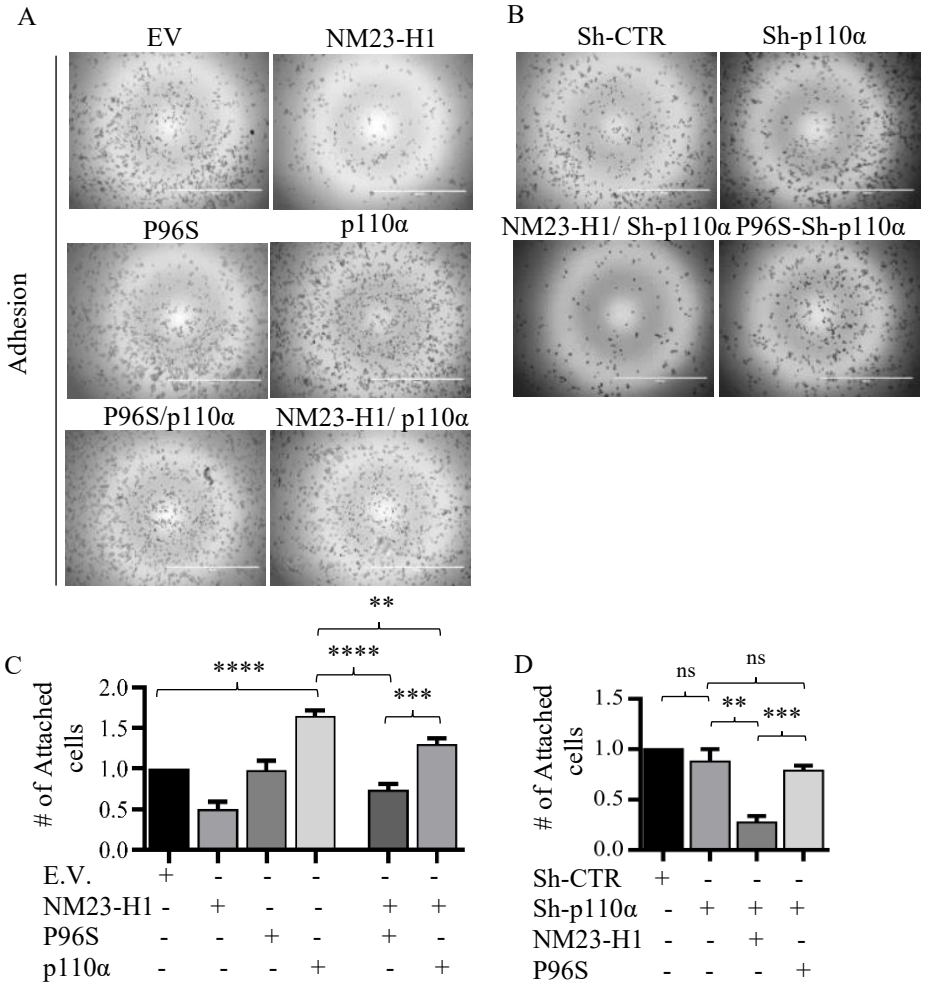


Figure 25. p110α increase adhesion property of MDA-MB435 cell line and NM23-H1 impair this process. Representative images (A) and quantitative analysis of adhesion assay (B). The analysis was performed by counting the number of adherent cells after 3 steps of washing, fixed with Methanol and then stained with 0.05 % Crystal violet**.** T-test statistical analysis was performed. n=3. (* $p < 0.05$; ** $p< 0.01$; *** $p< 0.001$; **** $p< 0.0001$; ns = Not Statistically Significant).

Invasion is a crucial step for metastasis spread. Cancer cells acquire a promotility phenotype and through extracellular matrix they migrate and reach the neighboring tissues. Only invasive cancer cells can disseminate and metastasize in a second site (Friedl and Alexander 2011). To investigate the invasion property of the cell lines analyzed, an invasion assay was carried out with Boyden chamber coated with Matrigel solution that mimics extracellular matrix (Fig. 26 A, B). While p110α expression leads to an increased number of migrated cells compared to E.V. (1.49-fold) (Fig.26 C), NM23H1 expressing cells show

a reduction of migrating cells (8.55-fold) (Horak, Mendoza et al. 2007, Boissan, De Wever et al. 2010, Marino, Marshall et al. 2013) (Fig.26C), while no substantial difference was detected in P96S expressing cells. MDA-MB435 expressing p110α- NM23H1 WT or mutated P96S isoform revealed a reduction of migrating cells compared to the p110α expressing cells (Fig.26C). Silencing p110α leads to a significate reduction of migrating cells (2.54-fold) because p110α, promotes migration (Graupera, Guillermet-Guibert et al. 2008, Wan, Pehlke et al. 2015) (Fig. 26D). Simultaneous p110α silencing and NM23H1 expression further reduce the number of migrating cell (6.65-fold on Sh-p110α expressing cells). No further reduction of migration was found in P96S/Sh-p110α co-expressing cell line (Figure 26D).

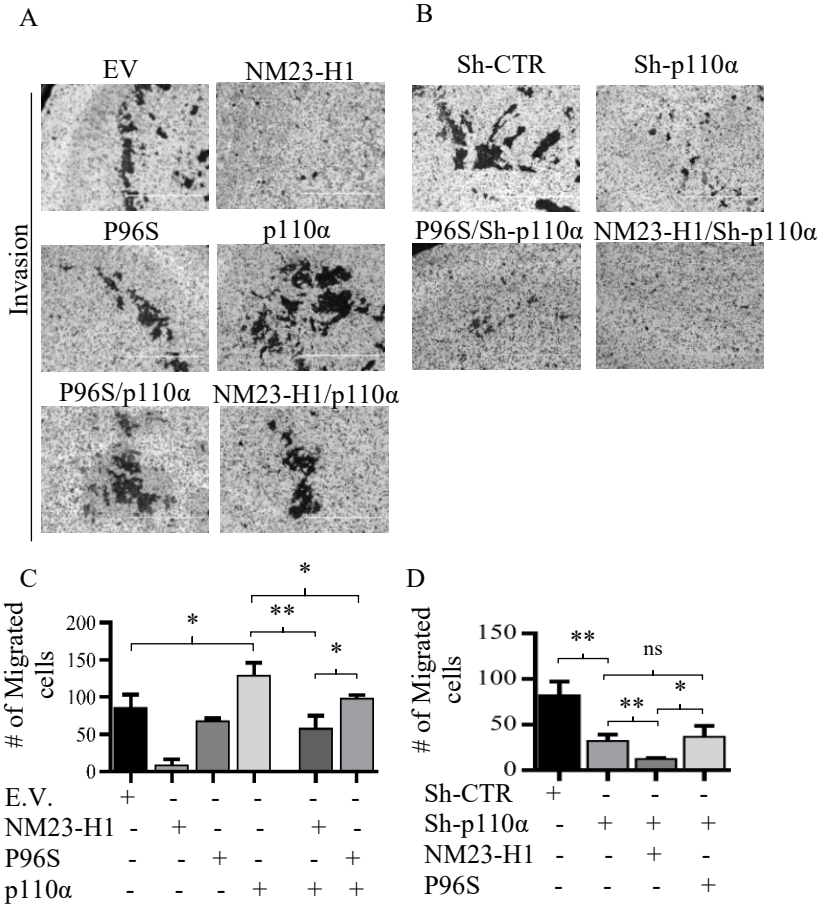


Figure 26. p110α increases the invasiveness of MDA-MB435 cells and NM23-H1 inhibits this process. Representative images (A) and quantitative analysis of Matrigel invasion assay (B). Quantitative analysis was performed by counting the number of migrated cells. 2% FBS was used as chemoattractant. T-test statistical analysis was performed. n=3. (* $p < 0.05$; ** $p < 0.01$; ns = Not Statistically Significant)

4.6 NM23-H1 WT, but not the mutant protein, reduces Filopodia structures.
Cell migration is a complex and dynamic process that requires the formation of specialized structures that permit to the cell the ability to move. In particular, cell membrane protruding structures known as Filopodia are fundamental for migration, invasion, adhesion, angiogenesis and cell-cell contact (Arjonen, Kaukonen et al. 2011)(Fig.27). In this context, it is known that the p110α gain of function mutation (H1047R) increases the number of Filopodia arborization, modifies the cell shape and stimulates migration (Wan, Pehlke et al. 2015) . On the other hand, NM23H1 silencing promotes an increase of invadopodia formation and upregulation of the expression of different matrix metalloproteinases (Boissan, De Wever et al. 2010). Moreover, Masanao Murakami et al.; in 2008 provided evidence of direct interaction between NM23-H1 and the small GTPase of Rho-subfamily Cdc42, a key regulator of cell morphology, migration, endocytosis and cell cycle progression with an important role in the extension and maintenance of actin-rich surface filopodia (Murakami, Meneses et al. 2008).

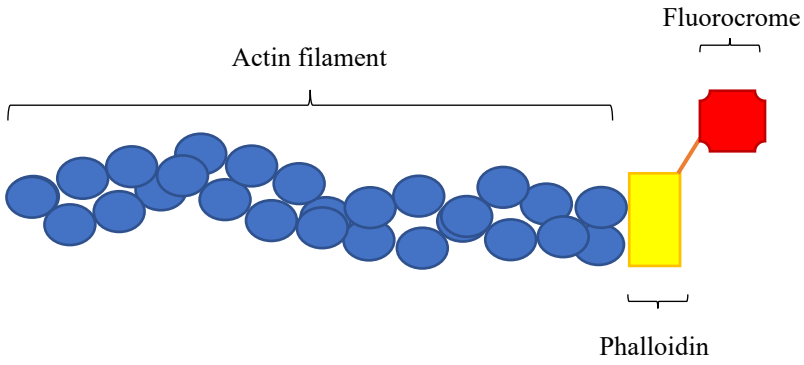


Figure 27. Phalloidin is a mushroom toxin used to stain actin filament. Actin undergoes structural transitions during polymerization. Phalloidin, a fungal toxin that is routinely used to stabilize and label actin filaments, inhibits structural changes in actin, likely influencing its interaction with actin-binding proteins.

We thus asked if p110α expression was able to increase this dynamic process and if NM23-H1 expression counteracts these p100α effects. To analyze this process, an immunofluorescence analysis on MDA-MB435 stained with Fluorocrome- tagged Phalloidin was carried out (Fig.28 A, B). p110α expressing cells show an increase of filopodia number compared to the E.V., while

NM23H1 expressing cells show a reduction of filopodia number (Fig.28 C). No significative differences were found in P96S expressing cells compared to EV. MDA-MB435 expressing p110α. NM23H1 WT expressing cells show a reduction of filopodia compared to p110α alone while coexpression with P96S doesn't show any reduction (Fig.28C). p110α silencing reduced filopodia formation (2-fold decrease then Sh-CTR) as well as in NM23H1/Sh-p110α expressing cells (1.9-fold compared to Sh-p110α) (Fig.28D), while no significative difference in the P96S expressing cells were observed when the mutant isoform was expressed with Sh-p110α (Fig.28D). This data suggests that restoring NM23H1 expression in a metastatic cell lines leads to a reduction of motility structures through inhibition of the PI3K-AKT axis.

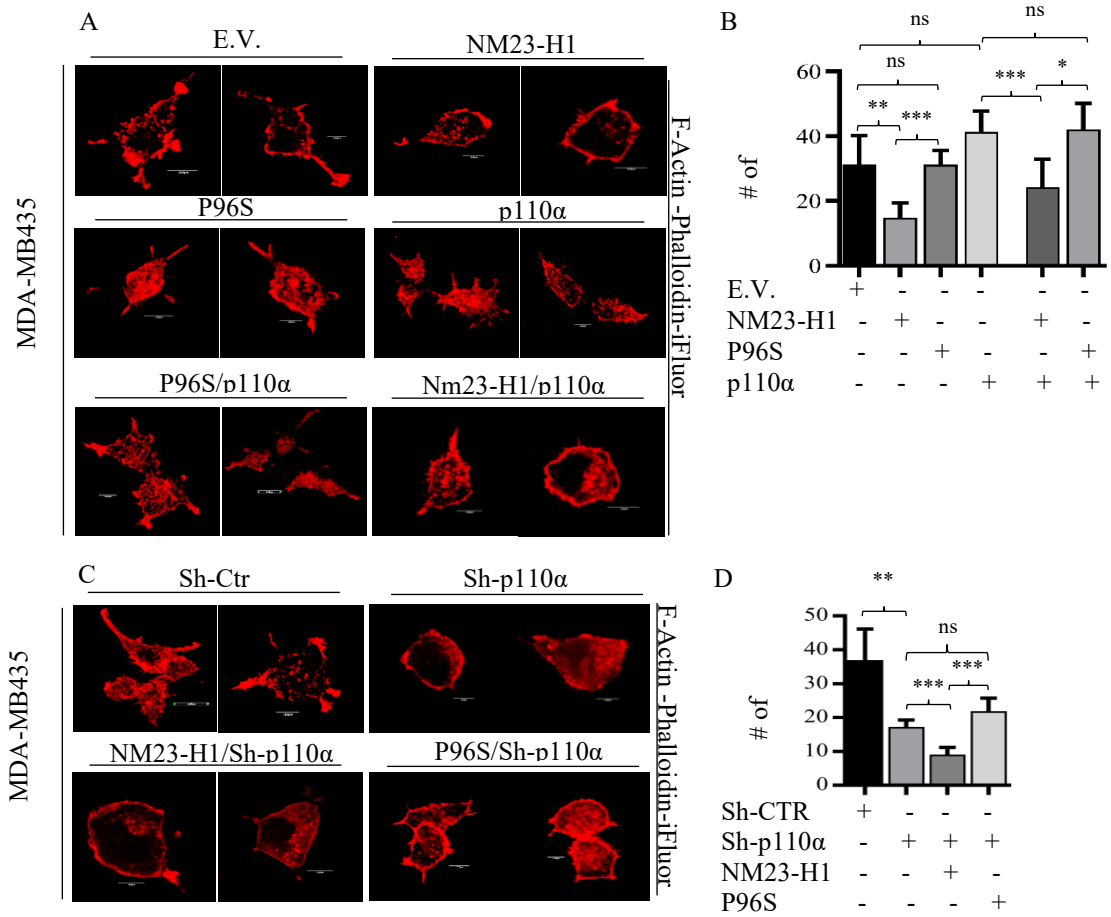
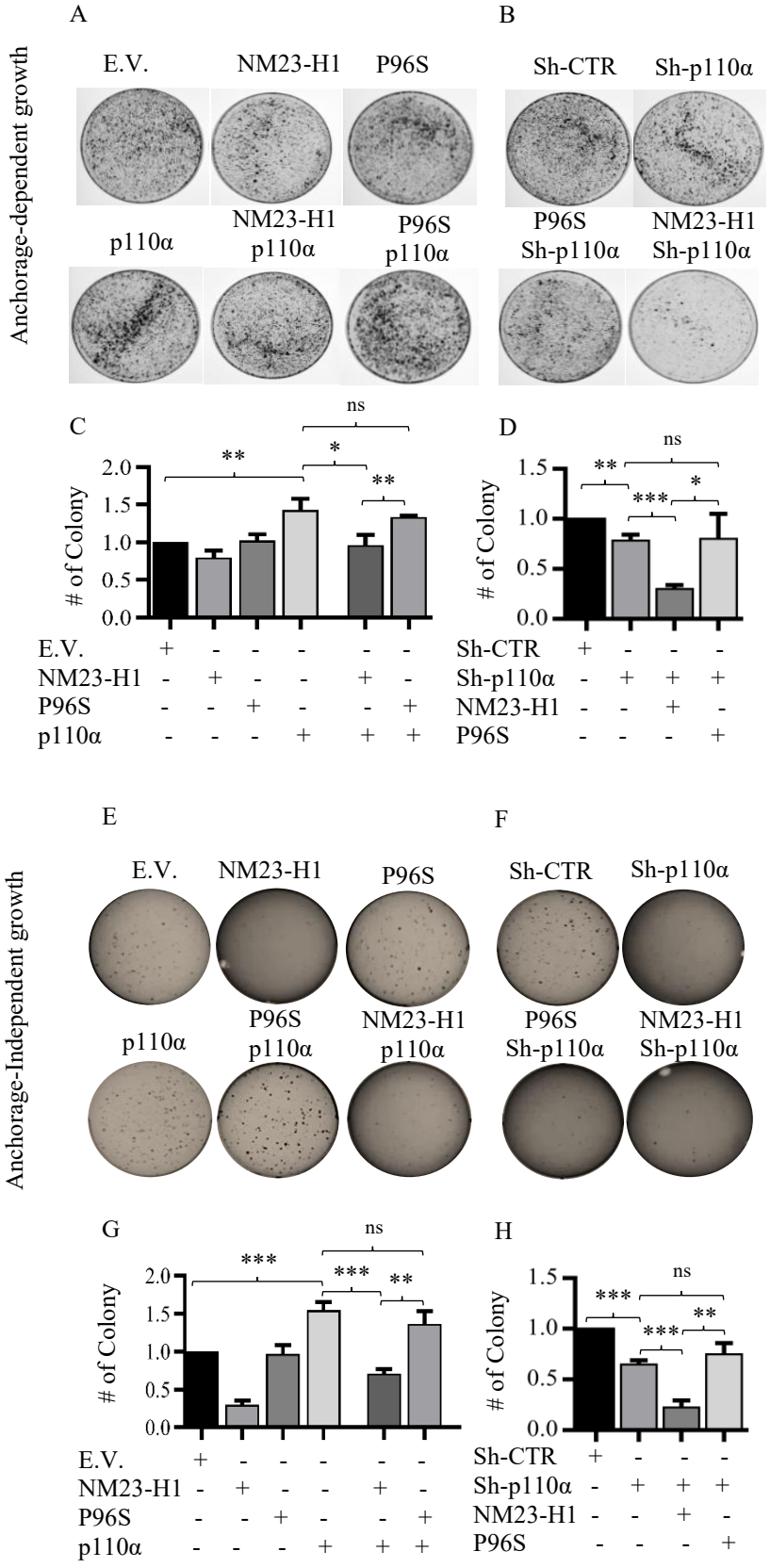


Figure 28. NM23-H1 downregulates p110α induction of Filopodia structures. A, B) Cell morphologies and filopodia structures were analyzed using fluorochrome tagged-Phalloidin to stain f-actin. Confocal Images shown were taken at x600 magnification. Cells were fixed and stained for f-Actin with fluorochrome tagged-Phalloidin. C, D) Filopodia structure were counted and the average of 3 independent experiment is shown. Anova statistical analysis was performed. n=3. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns = Not Statistically Significant).

4.7 NM23-H1 impairs growth and clonogenicity induced by p110α. p110α has been implicated in tumor cells growth and proliferation, because targeting p110α expression pharmacologically or with RNA interference strategy markedly impairs proliferation and survival of tumor cells (Wojtalla and Arcaro 2011). Manfred Volm et al and Leone et al describe downregulation of proliferation, survival and anchorage-independent growth in the NM23H1 expressing cell line (Leone, Flatow et al. 1991) (Volm, Mattern et al. 1998). To evaluate whether NM23-H1 expression impairs p110α-induced proliferation, an anchorage-dependent and independent growth assay was carried out (Fig.29 A, B, E, F). While MDA-MB435-p110α expressing cells show an increased number of colonies of 1.43-fold then the E.V. (Fig.29 C) no significant differences were detected in MDA-MB435 expressing NM23-H1 WT and mutated isoform P96S (Fig.29 C). NM23-H1 -p110α co-expression leads to a reduction of number of colonies compared to the p110α expressing cells (1.56-fold) cells. The expression NM23-H1 was able to counterbalance the proliferative stimulus of p110α (Fig.29 C). NM23H1 mutant P96S show no significant difference in colonies number related to EV (Fig.29 C). MDA-MB435-Sh-p110α cells does not show a clear reduction in the number of colonies and this is consistent with previous analysis on p110α silencing strategy that shows a switch of PI3K activity on the p110β isoform (Foukas, Berenjeno et al. 2010) (Fig.29 D). Surprisingly, simultaneous expression of p110a with NM23H1 drastically reduces the number of colonies by 3.3-fold compared to Sh-CTR and 2.59-fold compared to Sh-p110α cells, while we didn't observe this trend in the P96 / Sh-p110α expressing cells (Fig.29 D). An anchorage-independent growth was also assayed and the relative size of the colonies was analyzed (Fig.29 E, F, I, L). Similar to the previous assay in anchorage-dependent condition, a mild increase in colony number and size was present in MDA-MB435 p110α expressing cells (1.4-fold) while MDA-MB435-NM23-H1 expressing cells showed a reduction of colony number of 3.3-fold compared to the EV (Fig.29 G). No significant differences were detected between E.V. and P96S expressing cells (Fig.29 G). p110α-NM23H1 co-expression led to a reduction in colonies number by 2-fold compared to cells expressing p110α, while no substantial difference was present in P96S- p110α co-expressing cells (Fig.29 G). MDA-MB435-Sh-p110α cells display a reduction of colonies compared to Sh-CTR while co-expression with NM23-H1 WT or P96S shows a reduction of colonies by 4.4 and 1.3-fold respectively (Fig.29 H) confirming that the inhibitory action shown in the anchorage dependent and independent growth was due most probably, to the activity Nm23-H1 in a p110α expressing cells.


A
B
Anchorage-dependent growth
E.V.
NM23-H1
P96S
Sh-CTR
Sh-p110α
p110α
NM23-H1 p110α
P96S p110α
P96S Sh-p110α
NM23-H1 Sh-p110α
C
D
# of Colony
ns
**
*
**
***
E.V.
NM23-H1
P96S
p110α
Sh-CTR
Sh-p110α
NM23-H1
P96S
E
F
Anchorage-Independent growth
G
H

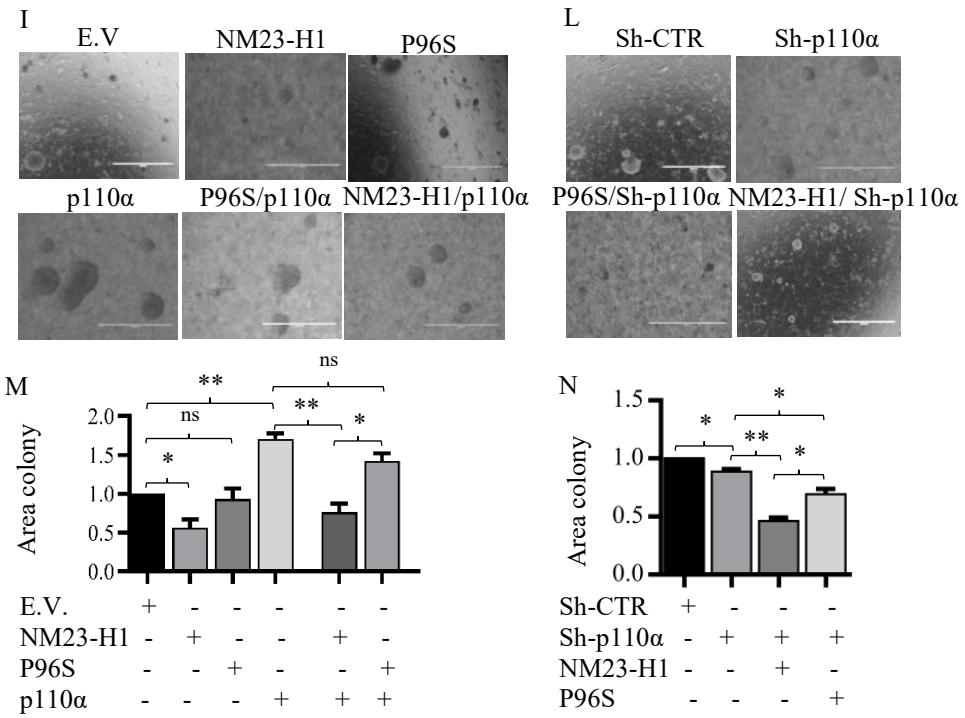


Figure 29 Simultaneous NM23H1 expression and p110α impairs clonogenicity of MDA-MB435 cell line in anchorage-dependent and independent growth. A, B) Clonogenicity of MDA-MB435 in different expression conditions was measured seeding cells at low density ($1x10^4$). After 14 days cells were fixed with 4% PFA and stained with 0.25% Crystal Violet and then counted. Quantitative results are represented by bar graph (C, D). E, F). The same MDA-MB435 clones were tested for anchorage-independent growth in a soft agar assay. Cells were seeded at low density ($1x10^3$). After 14 days cells were stained with 0.05% Crystal Violet O.N. The number and size (I, L) of colony were counted/measured and represented by a bar graph (G, H, M, N). Anova statistical analysis was performed. n=3. (* $p < 0.05$; ** $p< 0.01$; *** $p< 0.001$; **** $p< 0.0001$).

5. DISCUSSION

The PI3K pathway is one of the most important hallmarks of cancer due to its role in regulating numerous processes that control cancer progression. It is not surprising that some of the key genes in the regulation of this pathway, such as PI3KCA, PTEN, and AKT, are among the most mutated genes in certain types of cancer. In fact, the deregulation of the class I p110α catalytic subunit is sufficient for cellular transformation. Characterization of some gain-of-function mutations (H1047R and E542K/E545K) that affect the PI3KCA gene, and rarely other PI3K genes, confirms this characteristic (Burke, Perisic et al. 2012).This is the reason why the catalytic subunit p110α is the target of numerous anticancer drugs that aim to limit the activation of the PI3K pathway. Beyond the interactors that regulate its activity (e.g. p85 or Ras), not much is known about the p110α interactome (Zhao and Vogt 2008, Murillo, Rana et al. 2018).
NM23-H1 suppression of cell migration has been observed in different cancers and numerous evidences gathered over the years highlight how the different enzymatic activities and the extensive network of protein-protein interactions play an important role on suppression of cell motility. It has been shown that the Histidine Kinase activity is fundamental for NM23-H1 antimetastatic activity, however it has also been demonstrated that delocalization operated by Nm23-H1 protein interactors such as PRUNE1 or EBNA3C / EBNA1, negatively affects the antimetastatic activity of Nm23-H1(Freije, Blay et al. 1997, Reymond, Volorio et al. 1999, Subramanian, Cotter et al. 2001, Murakami, Lan et al. 2005). It has been demonstrated that PRUNE1 is highly expressed in metastatic Group3 Medulloblastoma, which is characterized by TGF-β signaling activation, c-MYC amplification and OTX2 expression. In this work, we show that Prune-1, through interaction with NM23-H1, delocalizes NM23-H1 from its inhibitory activity on the TGB-β pathway, operated through interaction with the protein STRAP. This condition leads to the activation of TGF-β pathway with effector Smad2/3 nuclearization, OTX2 upregulation, SNAIL (SNAI1) upregulation, and PTEN inhibition with enhanced EMT process resulting in a downregulation of E-cadherin expression and up-regulation of N-cadherin expression (Ferrucci, de Antonellis et al. 2018). Considering the negative regulation of NM23-H1 on the PI3K / AKT axis shown in this thesis research, the negative regulation of Prune-1 on the antimetastatic activity of Nm23-H1 and PTEN acquires an important new meaning. The biochemical and functional characterization of the mutated isoforms of NM23H1 (P96S, H118F and S120G) has put into frame/context certain aspects of the antimotility activity of NM23H1 associating it mostly with its different enzymatic activities (Steeg, Zollo et al. 2011). However, the biochemical analysis of the mutants has also shown a protein structural instability associated with a reduction of the hexameric complex (typical of the NDPK family) in favor of catalytically inactive dimers. Therefore, it cannot yet be said that the antimetastatic activity of Nm23-H1 is due to its enzymatic activities or its hexameric structure and therefore its ability to interact with different proteins involved, directly or indirectly, in the control

of cell motility mechanisms. However, to date, the main mechanism by which Nm23-H1 acts as an antimetastatic protein is still unclear. It is likely the functional interaction with p110α and NM23-H1 proteins would shed light on new understandings of how NM23-H1 acts an anti-metastatic regulator, and future research should address this question. We show for the first time interaction between these two proteins with opposite cancer progression promoting activities. Yeast two hybrid systems, in vitro translation system and two-way co-immunoprecipitation confirmed this interaction. Binding analysis of the p85α regulatory subunit shows that interaction occurs only with the catalytic subunit and not the entire PI3K protein complex. This interaction has cellular localization signals ranging from the cytoplasmic compartment to the plasma membrane where p110α act as a kinase, suggesting that this interaction can occur in both active and non-active forms of p110α. The main mechanism of action through which p110α activates the PI3K / AKT pathway, is the production of PIP3 starting from PIP2. Several effector proteins containing the PH domain recognize PIP3 as the membrane attachment point and from here the signal cascade typical of the PI3K pathway is activated. Among the known PI3K pathway effectors, AKT is certainly the most characterized/understood and is recognized as the main activation marker of this pathway. p-AKT analysis at the protein level on HEK293T, MDA-MB435 and MDA-MB231 stable expressing NM23H1 or control vector show a clear reduction in PI3K-AKT axis activation. Expression of the mutants does not induce any downregulation of p-Akt, suggesting that mutants are not able to inhibit the PI3K-AKT axis as the WT form does. We still cannot say that the NM23-H1 / p110α interaction leads to a reduction of p110α catalytic activity and PIP3 production, but a biochemical assay that measures the catalytic activity of p110α in the presence of NM23-H1 will be useful to understand more. However, the reduction of p-Akt is an indirect marker of reduced production of PIP3 and therefore of membrane localization and AKT activation. Among the different activation modes of the PI3K pathway, growth factors play an important role through the activation of putative tyrosine kinase receptors (IGFR, PDFR, EGFR). We observe that stable clones for NM23H1 expression show a reduction in Akt phosphorylation even when we stimulate the MDA-MB435 and MDA-MB231 cell lines with EGF, a strong activator of the PI3K pathway also involved in the activation of promotility mechanisms, which however we do not observe in clones expressing the mutated isoform P96S. As expected, the use of a short hairpin for NM23H1 leads to an increase in p-Akt, showing a negative correlation between the expression of NM23H1 and p-Akt, consistent with what has been shown in previous works where over-expressing or knock-down cell lines for NM23H1 gene show alterations of p-Akt levels and other kinases associated with phospholipid signaling (Horak, Mendoza et al. 2007, Hua, Feng et al. 2008). It is not the first time that a negative correlation has been described between the expression of NM23-H1 and the activation of the PI3K pathway. Weak NM23-H1 and high Akt and p-Akt expression was observed in ovarian serous adeno-carcinoma and ovarian clear cell adenocarcinoma. Additionally, the expression of Nm23-H1

was negatively correlated with tumor stage and grade and lymph node metastasis, whereas the expression of Akt/pAkt was positively correlated with these clinic factors (Hua, Feng et al. 2008) estrogen and progestin regulate metastasis through the PI3K/Akt pathway). It is interesting to note that a correlation between NM23H1 expression and PI3K-AKT axis is described for the process of *decidualization* (a process that results in significant changes to cells of the endometrium in preparation for, and during, pregnancy) in mice and humans. This could suggest that the interaction between p110α and NM23-H1 is not only relevant in the context of cancer, but has a precise function in the regulation of biological processes important for development (Zhang, Fu et al. 2016). The antimetastatic activity of Nm23-H1 is well known and has been demonstrated in different tumor cell lines and analyzed in different aspects. NM23-H1, in fact, carries out its anti-migration activity at different levels of regulation. For example, NM23-H1 interacts with Gelsolin, an actin-binding protein with an actin remodeling role, involved in the regulation of changes in shape and cell motility. NM23-H1 also interacts with Cdc42, a small GTPase protein involved in cell cycle progression and control of cell morphology and migration (Murakami, Meneses et al. 2008, Marino, Marshall et al. 2013). Migration assays performed with the MDA-MB435 cell line are already described well in previous literature/research. As a result, we focused our attention on the concomitant expression of p110α and NM23H1, but also simultaneous expression of NM23H1 / knockdown p110α in the same experimental setting but with the mutated form of NM23H1- P96S has been compared. Consistent with the role of p110α in cancer progression, we showed an increased in cancer cells motility and invasiveness as well as adhesion in MDA-MB 435 overexpressing p110α, cell function that decreased when NM23H1 is co-expressed, and markedly reduced when NM23H1 is expressed in MDA-MB435 knockdown for p110α. These results confirm that p110α up-regulation is one of the main stimuli for cancer progression and that restored NM23H1 expression not only counterbalances this prometastatic phenotype but downregulates these mechanisms considerably when p110α expression is affected. NM23-H1 interactome is extended also to proteins directly involved in actin remodeling processes with a key role in cell motility regulation. In the context of the mechanisms that regulate the activation of molecular pathways involved in the remodeling of actin and of those structures that give the cell a promigratory phenotype, it is interesting to note that an increased number of filopodia in Human colon cancer cells HCT116 positive for the p110α gain of function point mutation H1047R has been previously described (Wan, Pehlke et al. 2015). The analysis of the structures associated with cell motility gives important information regarding the dynamic processes that involve actin remodeling in the context of cell migration (Xue, Janzen et al. 2010). We have therefore analyzed filopodia structure rising from cell membrane of metastatic melanoma MDA-MB 435 cancer cell line. We observed an increase in the number of filopodia when p110α is expressed alone or with P96S. While we do not observe this increase when p110 is co-expressed with NM23H1 WT, it

suggests that the WT form is necessary to negatively regulate the formation of filopodia. However. restored NM23H1 expression does not show a considerable effect on the reduction of cell proliferation in an anchorage dependent growth assay while, coherently with what has been shown in several previous works, the expression of NM23H1 affects notably clonogenicity in an anchorage independent growth assay (McDermott, Boissan et al. 2008). In both assays the co-expression of NM23H1 / p110α reduces the clonogenicity induced by p110α expression, this reduction is evident in MDA-MB435 knockdown for p110α expressing NM23H1 and we didn't observe the same trend when P96S NM23H1 is expressed. To contextualize Nm23-H1 activity in the regulation of metastatic process is still very complicated due to the numerous biological processes in which this protein is involved. However, the description of this interaction is consistent with the scientific literature which has demonstrated the existence of this negative correlation between the expression of NM23-H1 and the activity of the PI3K pathway under different research aims. Future studies will focus on the characterization of this interaction from a biochemical and structural point of view. The perspective of a translational approach will also be interesting for future applications. It has been shown that the treatment of MDA-MB 231 with Medroxyprogesterone acetate increases the expression levels of NM23H1 with reduction of metastasis formation in *in vivo* experiments (Palmieri, Halverson et al. 2005), results also obtained with ovarian cancer cell lines in which estrogen treatment has been shown to downregulate NM23-H1 expression and promote cell migration and invasion through activation of the PI3K / AKT pathway, while treatment with Progestin shows opposite results.. The analysis of a combinatorial strategy will therefore be interesting considering that the therapies that use PI3K inhibitors prove to be increasingly promising as anticancer therapies (Yang, Nie et al. 2019).

6. APPENDICS

PI3K: Phosphoinositide 3-kinases
NDPK: Nucleoside-diphosphate kinases
EBV: Epstein–Barr virus
LCL: Lymphoblastoid cell lines
IVT: In Vitro Translation
GST: Glutathione *S*-transferase
EGF: Epidermal growth factor
Ptdlns: Phosphatidylinositol
PtdIns-3-P: phosphatidylinositol-(3)-phosphate
PtdIns-3, 4-P2: phosphatidylinositol-(3,4)-bisphosphate
PtdIns3,4,5-P3: phosphatidylinositol-(3,4,5)-trisphosphate
PDGF: Platelet-derived growth factor
mTor: Mammalian target of rapamycin
RTK: Receptor tyrosine kinases
GPCR: G protein coupled receptor
ABD: Adaptor Binding Domain
PIP3: Phosphatidylinositol (3,4,5)-trisphosphate
PH: Pleckstrin homology
GEF: Guanine nucleotide exchange factors
GAP: GTPase-activating proteins
PDK: Phosphoinositide-dependent kinase
FOXO: Forkhead box subgroup O
PTEN: Phosphatase and tensin homolog
EGFR: Epidermal growth factor receptor
NSCLC: Non-small cell lung cancer
HER2: Human Epidermal growth factor Receptor 2
FAK: Focal adhesion kinase
FGFR2: Fibroblast growth factor receptor 2
GBM: Glioblastomamultiforme
HNSCC: Head and neck squamous cell carcinoma
INPP4B: Inositol polyphosphate 4-phosphatase type II
MET: Hepatocyte growth factor receptor
SCLC: Small-cell lung cancer
TNBC: Triplenegative breast cancer
Awd: Abnormal wing discs
TXNDC3: Thioredoxin domain containing 3
TXL2: Thioredoxin-like 2
XRP2: X-linked retinitis pigmentosa 2
NTP: Nucleoside triphosphates
GTP: Guanosine triphosphate

ATP: Adenosine triphosphate
ARF6: Alternate Reading Frame
EBNA1-3C: Epstein- Bar nuclear antigen 1-3C
SCC9: *Squamous Cell Carcinoma-9*
STRAP: Serine-threonine kinase receptor associated protein
EMT: Epithelial to Mesenchymal transition
CPP: Competitive cell-permeable peptide
GFP: Green fluorescent protein
SDS-PAGE: Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis
NLS: Nuclear localization signal
DAPI: 4′,6′-diamidino-2-phenylindole
WT: Wild Type
PFA: *Paraformaldehyde*
p-Akt: Phosphorylated Akt
MAPK: *Mitogen-activated protein kinase*
ECM: Extracellular matrix

8. ACKNOWLEDGEMENTS

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10. LIST OF PUBLICATIONS DURING THE YEAR OF DOCTORATE PROGRAM

- The metastasis suppressor protein Nm23-H1 interacts with PI3K catalytic subunit p110α and impair PI3K-AKT axis.

Pennino FP, Murakami M, Zollo M, Erle S. Robertson.

In preparation.

- Targeting PRUNE-1 in a GEMM of metastatic medulloblastoma: a potential route of inhibition for new future therapies.

Veronica Ferrucci, Pasqualino de Antonellis, Francesco Paolo Pennino, Fatemeh Asadzadeh, Roberto Siciliano, Antonella Virgilio, Aldo Galeone, Lucia De Martino, Lucia Quaglietta, Maria Elena Errico, Vittoria Donofrio, Daniel Picard, Marc Remke, Louis Chesler, Fredrik Swartling, William Weiss, Michael Taylor, Giuseppe Cinalli, Massimo Zollo. *Neuro-Oncology*, Volume 20, Issue suppl_2, 1 June 2018, Page i139, https://doi.org/10.1093/neuonc/noy059.497. **Published:** 22 June 2018

- A new triple negative breast cancer (TNBC) murine model for in vivo preclinical immunotherapies.

Ferrucci V, Pennino PF, Dassi L, Asadzadeh F, Siciliano R, Carotenuto M, Spano D, Chiarolla MC, Greco A, Cantile M, Di Bonito M, Botti G, Vandenbussche J, Gevaert K and Zollo M.

Journal of Translational Medicine 2018, 16 (Suppl 1):O4

- Metastatic group 3 medulloblastoma is driven by PRUNE1 targeting NME1-TGF-β-OTX2-SNAIL via PTEN inhibition.

Ferrucci V, de Antonellis P, Pennino FP, Asadzadeh F, Virgilio A, Montanaro D, Galeone A, Boffa I, Pisano I, Scognamiglio I, Navas L, Diana D, Pedone E, Gargiulo S, Gramanzini M, Brunetti A, Danielson L, Carotenuto M, Liguori L, Verrico A, Quaglietta L, Errico ME, Del Monaco V, D'Argenio V, Tirone F, Mastronuzzi A, Donofrio V, Giangaspero F, Picard D, Remke M, Garzia L, Daniels C, Delattre O, Swartling FJ, Weiss WA, Salvatore F, Fattorusso R, Chesler L, Taylor MD, Cinalli G, Zollo M.

Brain. 2018 May 1;141(5):1300-1319. doi: 10.1093/brain/awy039.

PMID:29490009

- A competitive cell-permeable peptide impairs Nme-1 (NDPK-A) and Prune-1 interaction: therapeutic applications in cancer.

Ferrucci V, Pennino FP, Siciliano R, Asadzadeh F, Zollo M.

Lab Invest. 2018 May;98(5):571-581. doi: 10.1038/s41374-017-0011-6. Epub 2018 Feb 15. Review.PMID:29449633