UNIVERSITÀ DEGLI STUDI DI TRIESTE

XXX CICLO DEL DOTTORATO DI RICERCA IN BIOMEDICINA MOLECOLARE

A mutant p53 gain of function in the oncogenic response to insulin

Settore scientifico-disciplinare BIO/13

DOTTORANDA *Elena Valentino*

COORDINATORE *Prof.ssa Germana Meroni*

SUPERVISORE DI TESI *Prof. Licio Collavin*

ANNO ACCADEMICO 2016/2017

TABLE OF CONTENTS

ABSTRACT

Obesity and type 2 diabetes, with associated chronic inflammation and metabolic syndrome, are important risk factors for malignancies. In particular, meta-analyses of clinical studies suggest that hyperinsulinemia is a major cancer risk factor. Increased levels of circulating insulin may promote tumor progression by activating insulin (INSR) and IGF1 (IGF1R) receptors, that are frequently overexpressed in cancer cells. Moreover, in obese patients, insulin can synergize with inflammation to promote proliferation, survival, and dissemination of cancer cells.

The kinase AKT/PKB is a key mediator of the proliferative and survival effects of insulin on tumor cells. In addition, Akt directly links hyperinsulinemia to inflammation through activation of NF-kB signaling. The tumor suppressor DAB2IP (Disabled homolog 2 interacting protein), also known as AIP1 (ASK1 interacting protein), binds and inhibits PI3K-p85, limiting AKT activation in response to various stimuli. DAB2IP also binds directly to AKT1, possibly contributing to its inhibition.

In addition to its action on AKT, DAB2IP modulates multiple extracellular signals involved in cancer progression, thus acting as a tumor suppressor. Its expression is frequently reduced by methylation in tumors, but other molecular mechanisms of DAB2IP inactivation in cancer have been identified. One of such mechanisms is interaction with mutant p53 proteins.

Point mutation of p53 is a frequent event and a significant factor in cancer development and progression. Mutant p53 proteins (mutp53) are very stable, and acquire oncogenic properties (gain of function) that can increase proliferation, survival and metastatic potential of tumor cells.

In this thesis, I demonstrate that mutant p53, by binding DAB2IP, promotes insulin-induced AKT activation in triple negative breast cancer and androgen-independent prostate cancer cell lines, with cell-autonomous effects on proliferation and survival. Using a decoy protein to displace the mutp53-DAB2IP interaction, I showed that formation of this cytoplasmic complex is necessary for the enhanced response to insulin observed in mutant p53-bearing cancer cells.

Together, the evidence reported in this Thesis underline a specific gain of function of mutp53 in the response of cancer cells to insulin stimulation, offering an additional perspective to understand the complex relationship between hyperinsulinemia and cancer evolution.

INTRODUCTION

1. CANCER, OBESITY AND HYPERINSULINEMIA

1.1 The insulin-cancer connection

Excess body weight (EBW) in the overweight and obese is a well recognized risk factor for the development of cardiovascular disease and diabetes mellitus, but less well appreciated as a risk factor for several common adult cancers (Renehan and Howell, 2005). Only in 2002 the International Agency for Cancer Research stated that there is "now sufficient evidence that excess body weight is an avoidable cause of excess cancers including colorectal, endometrial, post-menopausal breast, kidney, pancreatic, oesophagealand, aggressive prostate cancers" (Vainio, H. and Bianchini, 2002).

Tumorigenesis is a multistep process involving the accumulation of genetic and epigenetic changes in several genes (Bernards and Weinberg, 2002), as well as multiple alterations in the regulatory circuits that finely modulate proliferation and homeostasis of normal cells (Hanahan and Weinberg, 2000). Thus, in addition to various pathological events, tumorigenesis and cancer progression strongly depend on extrinsic factors secreted by cancer cells themselves or by stromal cells. Those secreted factors, i.e. cytokines/chemokines, proteases, growth and angiogenic factors, as well as other molecules having an oncogenic effect, may regulate the crosstalk between stroma-cancer cells and tumor microenvironment promoting cancer initiation and progression. Several biological pathways, such as Smad, PI3K, JAK/STAT, NF-kB, MAPK, CXCR2 and IL-1 have been indicated as able to orchestrate this complex crosstalk system (Coppè et al., 2011) (He and Karin, 2011).

Insulin, insulin-like growth factors (IGFs), sexual hormones, and cytokines can boost cancer growth, linking obesity to cancer (Roberts et al., 2010). Inflammatory cytokines, such as adipokines, play an important role in tumor progression (Palomer et al., 2005). The amount of adiponectin, adipokine mainly produced by adipose tissue, is inversely proportional to the body mass index (BMI), it has an antiproliferative, anti-angiogenic and pro-apoptotic role, so low levels of this cytokine, predominant condition in obese individuals, correlates with a high risk of developing cancer (Roberts et al., 2010). Insulin and IGF-1-induced pathways are strictly linked to obesity and work to prohibit apoptosis and promote cell proliferation. Hyperinsulinemia might contribute to cancer development through the growth-promoting effect of elevated levels of the

hormone. Indeed, the excessive caloric intake of individuals with obesity leads to desensitization of tissues against insulin, resulting in insulin resistance, leading to increased production of insulin to restore normal levels of glucose. The tissues fail to metabolize the excess produced glucose and the pancreas secretes large amounts of insulin, promoting the hyperinsulinemic condition (Wysocki and Wierusz-Wysocka, 2010). Moreover, hyperinsulinemia in obese subjects is fueled by the catabolism of fatty acids. Obesity is, in fact, characterized by an increase in the levels of non-esterified fatty acids released by adipose tissue, and these are mainly catabolized to produce glucose and energy, causing hyperglycaemia (Karpe et al., 2011). Hyperinsulinemia triggers the insulin-IGF pathway, a complex pathway that includes insulin, IGF-1, and IGF-2 (three ligands), along with six receptors (insulin receptor IR α and β, IGF-1 receptor, IGF-2 receptor, hybrid IGF-1R/IR α, and hybrid IGF-1R/IR β) and seven IGF-binding proteins (IGFBPs) (Sridhar and Goodwin, 2009). It is hypothesized that the increased level of insulin reduces the amount of IGFBPs, which leads to an increase in the level of IGF-1 and a change in cell environment which promotes tumor growth (Renehan et al., 2006). Indeed, growth hormone, which is regulated by insulin, stimulates production of IGF-1 and IGFBP-3 (Clemmons, 1998). Insulin controls many aspects of metabolism, growth and survival in a wide range of mammalian tissues (Nakae et al. 2001). Insulin signalling also contributes to regulation of lifespan (Narasimhan et al., 2009), and has been implicated in cancer (Pollak et al., 2008). High circulating levels of insulin and other insulin-like growth factors promote cellular proliferation and affect programmed cell death (apoptosis), increasing the risk of cancer (Giovannucci et al., 2010). Indeed, recent papers report that patients with hyperglycemia show decreased cancer-specific survival respect to patients with normoglycemic plasma levels (Joost, 2014). Compensatory hyperinsulinemia, also consequent to insulin resistance, is the major common factor characterizing a variety of metabolic diseases such as obesity, type 2 diabetes mellitus, and metabolic syndrome. Indeed, several epidemiological studies reveal that patients with these disorders show increased risk to develop a variety of malignancies (Joost, 2014)(Cantiello et al., 2015).

1.2 Insulin receptors

Several studies have convincingly shown that the insulin receptor (IR) pathway is more directly and intimately involved in cancer development and progression than previously thought. Overactivation of this pathway by both insulin and IGF-II is common in cancer cells, particularly in dedifferentiated/stem-like cells, and may represent an important factor of resistance to various anti-cancer drugs (Belfiore, 2007). Additionally, cancer cells are often more responsive to insulin

due to Insulin Receptors (IRs) overespression, obtaining a selective advantage relative to non-malignant cells (Malaguarnera and Belfiore, 2011).

The insulin receptor (IR) is a tetrameric protein that consists of two extracellular α-subunits and two intracellular β-subunits. The α subunit of the receptor contains the ligand binding sites, usually located at a cysteine-rich domain on the extracellular region (Kristensen et al., 1995) (Whittaker and Whittaker, 2005). Insulin binding to the α-subunit leads to increase flexibility of the activation loop to allow ATP to enter the catalytic site and stabilize the activation loop in the active conformation by auto-phosphorylation of the β-subunits in various sites. The β subunits of IR include a large cytoplasmic region with tyrosine kinase activity. Auto-phosphorylation leads to interaction and phosphorylation of insulin receptor substrates (IRS-1 to IRS-4), which are generally viewed as the most specific for insulin signaling; and Shc proteins (Pelicci, G, et al. 1992), Gab-1 (Holgado-Madruga et al., 1996) and $p62^{dok}$ (Carpino et al., 1997). Indeed, activated IRS proteins interact with Grb-2 (growth factor receptor binding protein 2), the tyrosine phosphatase SHP-2 and with the p85 regulatory subunit of phosphoinositide 3-kinase (PI3K). A typical IRS protein contains a PH domain, which targets the protein in plasma membrane (Yenush et al., 1996), and a PTB domain, which recognizes the phosphotyrosine in the amino acid sequence asparagine-proline amino acid-phosphotyrosine (NPXpY), often presents in tyrosine kinase receptors, including β-subunit of insulin receptor (Eck et al., 1996)(Wolf et al., 1995). The specificity of the Grb-2, SHP-2 or PI3K binding is regulated by the few amino acids COOH-terminal to the phosphotyrosine. The SH2 domains of PI3-kinase recognize at least four pYMXM motifs in tyrosine-phosphorylated IRS-1. The SH2 domain of the adaptor protein Grb-2 and the SH2 domain of the phosphotyrosine phosphatase SHP-2 bind other sequences, including pYVNI, pYIDL, and pYASI sequences (Virkamäki et al., 1999).

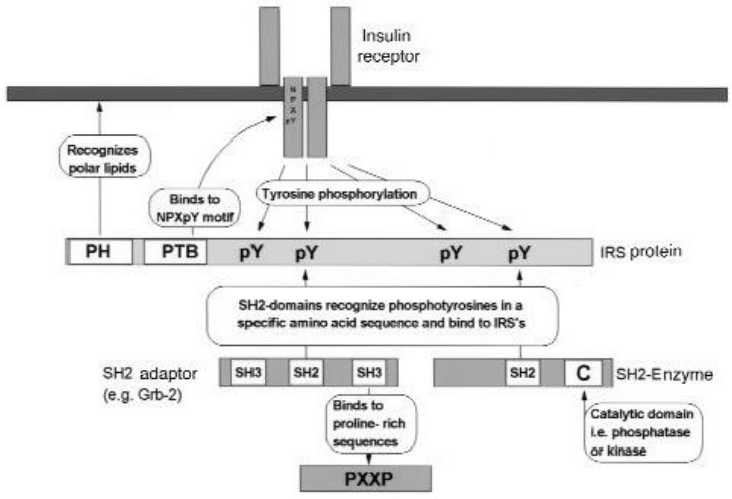
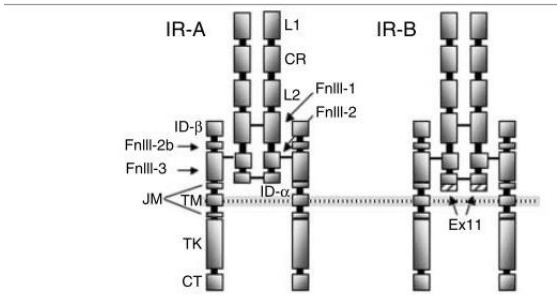


Figure 1: Protein–protein interaction domains involved in insulin signal transduction.

IRS protein contains a PH domain and a PTB domain, which binds to NPXpY motif in the β subunit of the insulin receptor. SH2 domains of SH2 adaptors and SH2 enzymes bind to multiple phosphotyrosines of IRS proteins. SH2 adaptors frequently possess SH3 domains that recognize PXXP motifs of other intracellular proteins, leading to further downstream signal transduction (Virakami et al., 1999).

Alternative splicing of the IR exon 11 results in the expression of two isoforms: IRA (lacking exon 11) and IRB (including exon 11) (Seino and Bell, 1989). The relative abundance of IRA and IRB isoforms displays tissue specificity in both humans and rats (Moller et al., 1989)(Goldstein and Dudley, 1990) and is also regulated during development and cell differentiation, with IRA representing the predominant isoform in fetal and highly proliferative tissues (Frasca et al., 1999). In breast, colon, lung, ovary and thyroid carcinomas, the A isoform is more present than the B isoform (Belfiore and Malaguarnera, 2011).



		IR-A	IR-B
	Predominant expression	Spleen Brain Lymphocytes Placenta Fetus Cancer	Liver Fat Muscle
IC_{50}	Insulin (nM)	0.2 – 0.9	0.5 – 1.6
	IGF2 (nM)	2.2 – 2.5	10 – 1.6
	IGF1 (nM)	9.0 – 41	90 – 390

Figure 2: Structure, tissue distribution, and ligand binding affinity of the IR-A and IR-B. Both receptors (IR-A and IR-B) show high homology in their molecular structure (L1, large domain 1; CR, cysteine-rich domain; L2, large domain 2; Fn, fibronectin type III domains; TM, transmembrane domain; JM, juxta membrane domain; TK, tyrosine kinase domain; CT, C-terminal domain; ID, insert domain). The hatched fragment on the bottom of the ID-α of IR-B (arrows) is encoded by exon 11 and is present in IR-B but not in IR-A. Below, the predominant tissue distribution of each receptor is shown. At the bottom of the figure, the different ligand binding affinity of each receptor is expressed as IC50 values (nM) of Insulin displacement (Frasca et al., 1999).

Some studies have indicated that IR-B may signal more efficiently to metabolic endpoints, and IR-A to mitogenic endpoints (Belfiore et al., 2009). It has also been suggested that IR-A and IR-B localise to different lipid raft microdomains, within which distinct signalling complexes are assembled (Leibiger et al., 2010). Tyrosine phosphorylation of IRS, as described above, creates binding sites for SH2 domains of various proteins, notably the regulatory subunits of class Ia PI3Ks and the adaptor Grb2. Indeed, the IR activation by insulin binding leads to the activation of two main cascades: the MAPK and the PI3K/Akt pathways.

1.3 Insulin activated pathways

1.3.1 Ras–mitogen-activated protein kinase (MAPK) cascade

IR-A activation by insulin leads to the predominance of growth and proliferative signals through the phosphorylation of tyrosine residues into cytoplasmic IRS1/2 and Shc proteins. Shc

activation leads to the recruitment of Grb2 docking protein, which in turn recruits SOS with subsequent activation of the Ras→RAF→MEK→ERK1/2 signaling cascade that phosphorylates a plethora of cytosolic and nuclear proteins (SRC1, Pax6, STAT3, cFos, c-myc, and Elk1). Elk1 kinase translocates to the nucleus and induces the transcription of several genes involved in cell proliferation and survival (Chang et al., 2003).

1.3.2 Phosphatidylinositol 3- kinase (PI3K) – protein kinase B (AKT/PKB) cascade

Insulin binding to the IR-B induces preferential activation of metabolic signals. This cascade starts with the phosphorylation of IRS1/2 and the activation of PI3K.

Phosphatidylinositol 3-kinases (PI3Ks) are a family of lipid kinases involved in the regulation of cell growth, metabolism, proliferation, glucose homeostasis and vesicle trafficking (Engelman et al., 2006). There are three classes in the family (Jean and Kiger, 2014): class I, II and III PI3K. All PI3Ks possess a 'PI3K signature motif ' that is composed of a C2 domain, which likely binds membranes, a helical domain and the catalytic kinase domain (Vanhaesebroeck and Alessi, 2000). The classification of PI3Ks into the three different classes is based mainly on the presence of additional protein domains and their interactions with regulatory subunits. In vitro, all classes can generate phosphatidylinositol 3-phosphate (PtdIns(3)P), class I and II can synthesize phosphatidylinositol (3,4)-bisphosphate (PtdIns(3,4)P2), and only class I can produce phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P3) (Jean and Kiger, 2014) (Katso et al., 2001). In vivo, there is significant support for class I PI3K synthesis of PtdIns(3,4,5)P3 and indirectly, PtdIns(3,4)P2, class III PI3K synthesis of PtdIns(3)P, and to a lesser extent, PI3K class II utilizes PtdIns(3)P to generate PtdIns(3,4)P2 and can also produce PtdIns(3)P from PtdIns (Jean and Kiger, 2014). Class I PI3K is further divided into IA and IB groups, activated by receptor tyrosine kinases (RTKs, PI3K1A) and G-protein-coupled receptors (GPCRs, PI3K1B). Class IA and IB PI3Ks have different regulatory subunits, p85a/p85b/p55 for IA and p101/p84/p87PIKAP for IB. Class II PI3K does not require a regulatory subunit to function, and comprises three different isoforms PI3K-C2α, PI3K-C2β, and PI3K-C2γ, (respectively encoded by PIK3C2A, PIK3C2B, and PIK3C2G genes), that diverge in the N terminus and present different domains within the C terminus. This class is involved in membrane trafficking and receptor internalization and can be activated in response to RTKs, integrins, and cytokine receptors (Engelman et al., 2006). Class III PI3K (Vps34), which was first identified in the budding yeast (Herman and Emr, 1990), is involved in vesicle trafficking and cross-talks with class I PI3K through the regulation of mTORC1 signaling (Backer, 2008). Class I PI3K is the

most studied among the three members of the family, and I will refer to it as PI3K unless otherwise specified.

IRS proteins via a specific phosphotyrosine (P) sequence, (pY)MXM and (pY)XXM motif, bind the heterodimer phosphatidylinositol 3- kinase (PI3K) at its two SH2 domains in the regulatory subunit. After dimerization of the different regulatory and catalytic subunits on IRS, PI3K catalyses the production of phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P3) from PtdIns(3,4) P2 at the plasma membrane. The lipid product of PI3Ks, PtdIns(3,4,5)P3, induces the activation of protein serine kinase cascades by co-recruitment to membranes of phosphoinositide-dependent kinase-1 (PDK1) and its substrate kinases AKT/PKB and atypical protein kinase Cs (aPKCs), via their respective PH domains (Mora et al., 2004).

AKT propagates metabolic signals targeting substrates mostly involved in glucose and lipid homeostasis such as GLUT4, PDE3B, Foxa2, GSK3, and AMPK. Besides its role in metabolic effects, AKT leads to the activation of effectors involved in the control of apoptosis and survival (BAD, Mdm2, FKHR, NFkB and JNK) and protein synthesis and cell growth (mTOR).

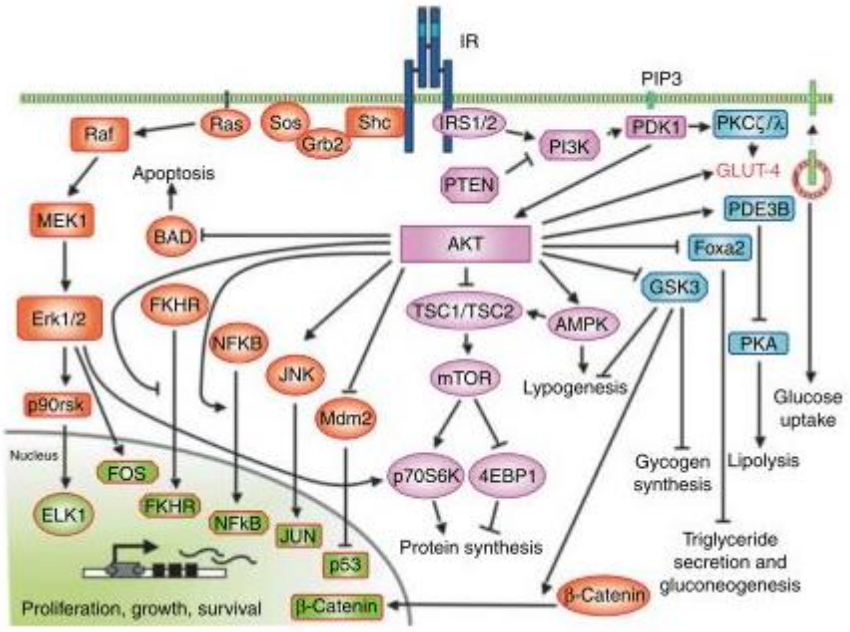


Figure 3: Schematic representation of IR signaling. Ligand binding to the α-subunit of IR stimulates the tyrosine kinase activity intrinsic to the β-subunit of the receptor, which in turn phosphorylates several substrates including IRS1/2 and Shc/Grb2/Sos complexes. IRS proteins interact with the regulatory subunit of PI3K leading to the activation of AKT. Phosphorylated IRS1/2 are also able to recruit the Grb2/Sos complex, which triggers the RAS/RAF/MEK/ERK pathway (Belfiore and Malaguarnera, 2011).

1.4 Cross-talk between PI3K and MAPK pathways

Importantly, the two main pathways regulated by insulin receptor are not totally independent, as they can regulate each other. Indeed, PI3K pathway impacts on Ras–MAPK at multiple levels. Ras activation by PI3K has been well characterized in lysophosphatidic acid (LPA)-induced MAPK activation, where PI3K pharmacological inhibitors blunt the activation of ERK kinase. This activation partially relies on G-beta gamma subunits and Shc-Grb2-Sos activation as well as in PtdIns (3,4,5)P3-mediated PKCz activation. Nevertheless, the specific requirement of PI3K for Ras activation might be tissue-specific and involve additional signaling molecules (Yart et al., 2002). Similarly, somatostatin receptor (Lahlou et al., 2003), as well as insulin and low doses of EGF (Wennström and Downward, 1999), can drive Ras activation through PI3K, which may be due to the ability of PtdIns(3,4,5)P3 to recruit GAP/Shp2 in some circumstances (Sampaio et al., 2008). On the other hand, some PI3K inhibitors such as the cytokine b-galactosidase-binding protein (bGBP) inhibit MAPK (Wells et al., 2007). The cellular context in which PI3K signals to Ras is not yet elucidated, and further research on the role of PI3K-Ras binding, PtdIns species production and involvement of the different PI3K isoforms is required for the proper delineation of this signaling cross-talk. Morevoer, PI3K pathway can directly modulate the Ras target Raf therefore bypassing the GTPase. AKT phosphorylates c-Raf in Ser259 (Zimmermann and Moelling, 2016) and B-Raf (Nadimpalli et al., 2000). This inhibitory phosphorylation on Raf reduces the activity of MAPK pathway (Zimmermann and Moelling, 2016). This action of AKT on MAPK pathway is crucial for proper regulation of differentiation in some tissues and for cell survival (Rommel et al., 1999) and may impact on the response to growth factors (Nadimpalli et al., 2000). Another protein kinase with similar catalytic domain to AKT, SGK has also been suggested to phosphorylate Raf in an AKT site (Zhang et al., 2001). In which circumstances AKT inhibits Raf is still unclear; and the strength of PI3K activation may regulate this cross-talk, as AKT phosphorylation of Raf is more prominent at higher PI3K activity (Moelling et al., 2002).

The expression of a mutant dominant-negative form of p85 (Rodriguez-Viciana et al., 1997) or mutation of PI3K-IA (Gupta et al., 2007) reduces Ras-mediated transformation and the aggressiveness of lung tumors in Ras mutant transgenic mice. On the other hand, Ras influeces PI3K regulation. Indeed, class I and II PI3K isoforms have a Ras Binding Domain (RBD) by which Ras facilitates PI3K anchorage to the membrane and thus its full activation (Engelman et al., 2006). Ras binding to PI3K is required to transmit EGF and FGF2 extracellular signals, which accounts for a primary role of Ras in the regulation of PI3K signal transduction (Ramjaun

and Downward, 2007). Moreover, the oncogenic ability of Ras is linked to its ability to activate this pathway (Rodriguez-Viciana et al., 1997)(Gupta et al., 2007).

With regards to the interaction between Ras and PI3K, it has been demonstrated to be critical for correct cell polarity and migration. Studies carried out in Dictyostelium and hippocampal neurons show that the proper cross-talk of these two pathways is essential for axon formation and cell polarity and migration (Funamoto et al., 2002)(Sasaki et al., 2007)(Fivaz et al., 2008). Moreover, in Dictyostelium, cytoskeleton components such as F-actin filaments play an important role in the signaling between PI3K and Ras, promoting their correct localization and interaction in the plasma membrane (Sasaki et al., 2007). Therefore, the PI3K pathway plays a critical role in cell polarity, chemotaxis and migration, which requires a tightly regulated cross-talk with Ras pathway. The biological consequences of MAPK and PI3K cross-talk are being actively investigated as they may selectively impact in some cellular responses but not others.

2. AKT KINASES: THREE DIFFERENT TEAM PLAYERS IN CANCER?

2.1 Protein kinase B (PKB)/ AKT

Protein kinase B (PKB, also termed Akt or RAC) is a 57 kDa serine/threonine kinase belonging to the "AGC " (related to AMP/GMP kinase and protein kinase C) superfamily of protein kinases (of which there are over 80 members) that includes PKA, PKC, PDK1, and the p70 and p90 S6 kinases (Coffer and Woodgett, 1991). This group of kinases shares similarity within their catalytic domain structure and in their mechanism of activation, and many are frequently deregulated in human diseases including cancer. The catalytic domain of AKT is structurally similar to that of other protein kinases of the AGC family including PKA (Yang et al., 2002). Mammals have three closely related AKT genes, encoding the isoforms AKT1 (PKBα/RAC-PKα), AKT2 (PKBβ) and AKT3 (PKBγ).

AKT1 and AKT2 are ubiquitously expressed, although at varying levels, while AKT3 is restricted to some tissues (Masure et al., 1999). Specifically, AKT3 is highly expressed in brain and testis, with low levels in pancreas, skeletal muscle, and kidney (Nakatani et al., 1999).

AKT2 and AKT3 show 81 and 83% amino acid identity with AKT1 respectively.

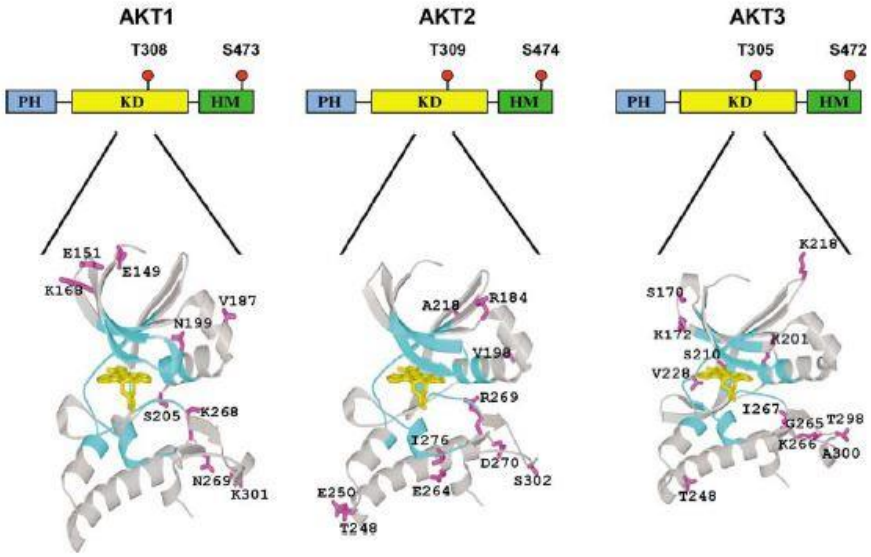


Figure 4: Domain structure of the AKT family members and homology models of the ATP binding regions. All AKT family members contain an N-terminal pleckstrin homology domain, a catalytic kinase domain, and a C-terminal regulatory hydrophobic region. Computer-derived homology models of the ATP binding regions of the three AKTs are shown. ATP binding site is shown in blue. Residues unique to the AKT family members are colored in magenta and labeled. Staurosporine is in yellow (Bellacosa et al., 2005).

All AKT isoforms consist of an N- terminal PH domain, a kinase domain and a C-terminal regulatory tail (Vahaesbroneck and Alessi, 2000). The N-terminal PH domain is common to numerous signaling proteins and provides a lipid binding module to direct AKT to PI3K-generated phosphoinositides PtdIns(3,4,5)P3 and PtdIns(3,4)P2 (Manning and Cantley, 2007). The second important domain of AKT is the C-terminal HM domain presents in many other AGC kinases (Vahaesbroneck and Alessi, 2000) (Pearson et al., 1995). Studies on AKT and other AGC kinases including protein kinase C, p70 ribosomal S6 kinase, serum and glucocorticoid-activated kinase (SGK) and RSK indicate that the HM provides a role in regulating kinase activity. Indeed, the HM domain provides a docking site for the upstream activating kinase, such as phosphoinositide-dependent kinase-1 (PDK1) (Balendran et al., 2000). Two specific sites, one in the kinase domain (Thr 308 in AKT1, Thr 309 in AKT2, Thr 305 in AKT3) and other in the C-terminal regulatory region (Ser 473 in AKT1, Ser 474 in AKT2, Ser 472 in AKT3), need to be phosphorylated for full activation of these kinases. These phosphorylations are performed by the serine/threonine kinases PDK1 and PDK2 or mTORC2 respectively (Alessi et al., 1997) (Jacinto et al., 2006). PDK1 activates AKT/PKB and PKCα by phosphorylation of serine/threonine residues in their kinase regulatory loops (Pearce et al., 2010). Activation of AKT/PKB additionally requires phosphorylation of a C-terminal

hydrophobic motif, catalysed by a distinct enzyme, most probably mTORC2 or DNA-PK (Bozulic and Hemmings, 2009). Indeed, Rictor-mTOR complex can also phosphorylate AKT1 on S473, facilitating T308 phophorylation by PDK1 (Sarbassov, 2005).

As with other AGC kinases, AKT is regulated by upstream, second messengers and secondary, activating enzymes. For AKT, this activating process involves multiple inputs that strictly control the location, duration and strength of response (Balendran et al., 2000).
The duration and amplitude of AKT signalling are controlled by PP2A and PHLPP phosphatases that specifically dephosphorylate AKT on the hydrophobic phosphorylation motif (Brognard et al., 2007). Indeed, several reports have indicated that AKT can be activated in cells by a mechanism independent of PI3K activation, for example in response to heat shock, or increases in intracellular Ca5+ or cAMP (Moule et al., 1997)(Filippa et al., 1999).

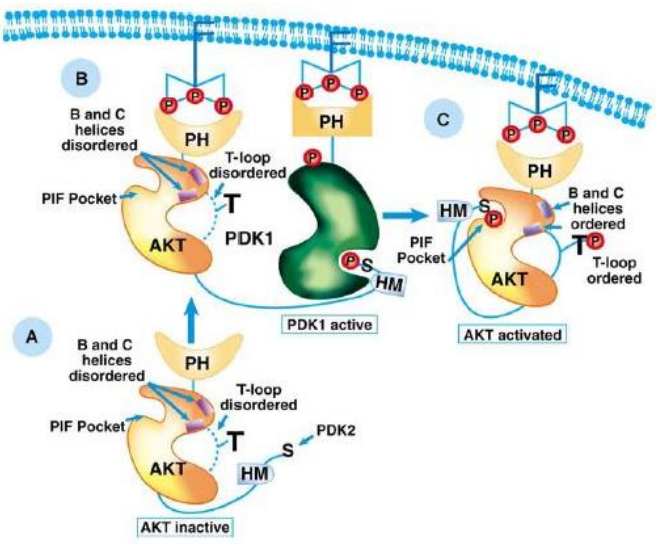


Figure 5: Schematic depicting the conformational changes accompanying AKT activation and the role of the phosphorylated hydrophobic motif in the activation cycle. (A) AKT in its unphosphorylated state remains cytosolic and is inactive. In unphosphorylated AKT, the activation (or T-loop), αB, and αC helices and the hydrophobic motif (HM) remain disordered. PDK2 phosphorylates AKT on Ser in the HM. (B) PI3K-generated PtdIns(3,4,5)P3 and PtdIns(3,4)P2 recruit AKT and PDK1 to the plasma membrane. Phosphorylated HM stabilizes and activates PDK1, which then phosphorylates AKT on Thr. (C) The HM of AKT associates with and stabilizes the kinase domain, leading to ordered structures for αB and αC helices and the activation (T-loop). PH indicates the plekstrin homology domain; red circles denote phosphate groups (Bellacosa et al., 2005).

Activated AKT/PKB phosphorylates multiple substrates, the majority characterized by the RXRXX consensus sequence (S / T) (Fayard, 2005), and controls a variety of downstream responses depending on cell type (Manning and Cantley, 2007). In some cases, phosphorylation of targets itself regulates activity, while in other cases binding of 14-3-3 protein also plays a role (Johnson et al., 2010). Well-established AKT/PKB substrates include GSK-3, regulating glycogen synthesis; the Rab GTPase activating protein AS160/TBC1D4, regulating glucose

transport; the Rheb GTPase activating complex TSC1/2, regulating mTOR and protein synthesis; FOXO transcription factors, regulating expression of gluconeogenic and other genes; BAD and Mdm2, regulating apoptosis (Manning and Cantley, 2007). Both AKT-dependent and -independent mechanisms have been implicated in regulation of adipose tissue lipolysis by insulin (Choi et al., 2010).

New AKT substrates continue to be validated in vivo; it is currently less clear, however, to what extent AKT1, AKT2, and AKT3 are functionally redundant, or each kinase carries out a specific functional role (Bellacosa et al., 2004).

Since the AKT signaling cascade is frequently disrupted in many human cancers, and in light of the wide-ranging biologic consequences, this pathway is considered a key determinant of tumor aggressiveness and an attractive target for therapeutic intervention.

2.2 "AKTivation" in cancer

The three AKT kinases are now known to represent central nodes in a variety of signaling cascades that regulate normal cellular process such as cell size/growth, proliferation, survival, glucose metabolism, genome stability, and neo-vascularization (Bellacosa et al., 2004)(Dummler and Hemmings, 2007) (Vara et al., 2004).

A large body of literature has documented frequent hyperactivation of AKT kinases in a wide assortment of human malignancies (Bellacosa and Kumar, 2005), and a series of elegant studies using animal models has demonstrated that aberrant AKT signaling can, either alone or by cooperating with certain other genetic perturbations, induce malignancy or contribute to a more malignant phenotype (Bellacosa et al., 2004)(Bjornsti and Houghton, 2004). Many human cancers, including carcinomas, glioblastoma multiforme, and various hematological malignancies, exhibit AKTs hyperactivation (Altomare and Testa, 2005). AKT1 is often hyperactivated in prostate, breast, ovarian cancers and in gastric adenocarcinomas (Staal, 1987) with the exception of kidney, liver, and spleen (Altomare et al., 1998). Amplification of AKT2 has been detected in ovarian cancer, breast cancer, pancreatic cancer, non-Hodgkin's lymphoma, and hepatocellular carcinoma (Bellacosa and Kumar, 2005)(Cheng et al., 1992)(Cheng et al., 1996)(Xu et al., 2004). Increased AKT3 protein levels and activity were detected in estrogen receptor deficient breast cancer cells and androgen-independent prostate cancer cells (Nakatani et al., 1999).

Numerous investigators have reported correlations between tumor, AKT activity and various clinicopathologic parameters (Bellacosa and Kumar, 2005). Moreover, Joy's group show that AKT3 mRNA is inversely associated with grade and survival in GBM, AKT3 mRNA is elevated

in the less aggressive subtype of GBM and AKT3 overexpression increases survival in an orthotopic rodent xenograft model of GBM, decreasing colony forming efficiency in glioma cells. In addicition silencing of AKT3 slows cell cycle progression and increases apoptosis (Joy et al., 2016). In particular, AKT activation has been shown to correlate with advanced disease and/or poor prognosis in some tumor types.

AKT pathway is a central node in a signaling pathway consisting of many components that have been implicated in tumorigenesis, including upstream phosphatidylinositol 3-kinase (PI3K), PTEN (Phosphatase and Tensin homologue deleted on chromosome Ten), NF1 and LKB1, and downstream tuberous sclerosis complex 2 (TSC2), Forkhead Box Class O (FOXO) and eukaryotic initiation factor 4E (eIF4E). Several of these proteins (AKT, eIF4E, and both the p110α catalytic and p85α regulatory subunits of PI3K) can behave as oncoproteins when activated or overexpressed, while others (PTEN, FOXO, LKB1, TSC2/TSC1, NF1, and VHL) are tumor suppressors. Somatic genetic and/or epigenetic changes involving genes encoding these AKT pathway components are among the most frequent alterations reported in sporadic cancers.

Indeed, perturbation of the upstream PTEN and PI3K contributes to activation of the AKT pathway in human tumors (Simpson and Parsons, 2001). Moreover, other mechanism such as activation of PI3K due to autocrine or paracrine stimulation of receptor tyrosine kinases (Yuan et al., 2000)(Tanno et al., 2001)(Sun and Steinberg, 2002), overexpression of growth factor receptors such as the epidermal growth factor receptor in glioblastoma multiforme (Schlegel et al., 2002) and HER-2/neu in breast cancer (Bacus et al., 2002), and/or Ras activation (Liu et al., 1998) promotes AKT activation. In some instances, AKT activation may result from overexpression of a wild-type growth factor receptor. In such cases, the abundance of a given receptor may enable a tumor cell to become hypersensitive to ambient levels of growth factors (Hanahan and Weinberg, 2000). Mutant receptors, on the other hand, can give rise to constitutive activation of the AKT signal transduction pathway (Sordella et al., 2004). Furthermore, in certain hematological malignancies, constitutive activation of AKT can result from a chromosomal translocation that triggers permanent activation of an upstream tyrosine kinase (Slupianek et al., 2001).

Although AKT kinases are often hyperactivated throught different mechanisms, mutation of this kinases is a rare event in cancer. A somatic mutation in human breast, colorectal and ovarian cancers that results in a glutamic acid to lysine substitution at amino acid 17 (E17K) in the lipid-binding pocket of AKT1 has been identified. Lysine 17 alters the electrostatic interactions of the pocket and forms new hydrogen bonds with a phosphoinositide ligand. This mutation activates

AKT1 by means of pathological localization to the plasma membrane, stimulates downstream signalling, transforms cells and induces leukaemia in mice (Carpten et al., 2007). The single hotspot mutation G49A:E17K occurs most often in AKT1, but the corresponding E17K mutation has also been found in AKT2 and AKT3 (Yi and Lauring, 2016). This mechanism indicates a direct role of AKTs in human cancer, and adds to the known genetic alterations that promote oncogenesis through the PI3K/AKT pathway.

AKTs play a relevant role in many cellular processes that, when deregulated, can contribute to the development or progression of cancer. Activated AKT is a well-established survival factor, exerting antiapoptotic activity, in part by preventing the release of cytochrome c from the mitochondria (Zhou et al., 2000). AKT also phosphorylates and inactivates the proapoptotic factors BAD and procaspase-9 (Datta et al., 1999)(Downward, 1998). Moreover, AKT-dependent phosphorylation and inactivativation of the FOXO transcription factors, inhibits the expression of genes critical for apoptosis, such as the Fas ligand gene (Brunet et al., 1999)(Zhang et al., 2011)(Hay, 2011). Moreover, nuclear AKT2 selectively inhibits FOXO inhibition and its downregulation results in a gain of FOXO functions. AKT2 nuclear signaling opposes lymbal keratinocyte stem cells (LKSCs) maintenance by selective regulation of a FOXO-mTORC1 signaling pathway In addiction, in limbal karatinocyte stem cell population individual Akt isoforms play opposite roles where AKT1 promotes while AKT2 opposes LKSC self-renewal (Saoncella et al., 2004).

AKT also activates IkB kinase (IKK), a positive regulator of NF-kB, which results in the transcription of antiapoptotic genes (Pommier et al., 2004). In another mechanism to thwart apoptosis, AKT promotes the phosphorylation and translocation of Mdm2 into the nucleus, where it downregulates p53 and thereby antagonizes p53-mediated cell cycle checkpoints (Mayo and Donner, 2002)(Zhou et al., 2001). VEGF effects on cell survival have also been shown to be mediated by the Flk1/VEGFR2-PI3K-AKT pathway (Shiojima and Walsh, 2002).

AKT activation stimulates cell cycle progression by phosphorylation and consequent inhibition of glycogen synthase kinase 3β to avert cyclin D1 degradation (Liang and Slingerland, 2003). AKT also directly antagonizes the action of the cell cycle inhibitors p21WAF1 and p27Kip1 by phosphorylating a site located near the nuclear localization signal to induce cytoplasmic retention of these cell cycle inhibitors (Testa and Bellacosa, 2001)(Bellacosa and Kumar, 2005). Moreover, AKT-induced phosphorylation of mTOR kinases also results in increased translation of cyclin D1, D3, and E transcripts (Muise-Helmericks et al., 1998). AKT activates the downstream mTOR kinase by inhibiting a complex formed by the tumor suppressor proteins TSC1 and TSC2, also known as hamartin and tuberin (Astrinidis and Henske, 2005).

mTOR broadly mediates cell growth and proliferation by regulating ribosomal biogenesis and protein translation (Ruggero and Sonenberg, 2005) and can regulate response to nutrients by restricting cell cycle progression in the presence of suboptimal growth conditions (Plas and Thompson, 2005). Finally, AKT has also been shown to phosphorylate human telomerase reverse transcriptase, which stimulates telomerase activity and replication (Liu, 1999).

Tumor cell migration is also linked to AKT signaling (Lefranc et al., 2005). AKT has been shown to contribute to tumor invasion and metastasis by promoting the secretion of matrix metalloproteinases (Thant et al., 2000), the induction of epithelial-mesenchymal transition (EMT) and by sustaining the remodelling of actin filaments via Girdin (an ubiquititous acting-binding protein) phosphorylation (Enomoto et al., 2006) (Larue and Bellacosa, 2005). In human lung cancer cell Non-Small Cell Lung Cancer cells LNM3, depletion of AKT1 decreases cellular invasion at least in part via COX-2 inhibition, with no effect on cell motility, proliferation, colony formation, and angiogenesis (Attoub et al., 2015). Unexpectedly, AKT1 can also decrease breast cancer cell migration through phosphorylation and activation of the E3 ubiquitin ligase Mdm2, promoting ubiquitination and proteosomal degradation of NFAT transcription factor (Yoeli-Lerner et al., 2005). Activation of AKT2 in ovarian and breast cancers under nutrient-poor conditions results in migration, with increased attachment and invasion through collagen IV (Arboleda et al., 2003)(Chau and Ashcroft, 2004). Moreover, silencing of Akt2 also reduced cellular motility and invasion in vitro, presumably via COX-2 inhibition (St-Germain et al., 2004). In addition, AKT2 is necessary for migration of glioma cell lines (Zhang et al., 2010). Interestingly, AKT3 is required for the migration of transformed astroytes (Endersby and Baker, 2008), but can inhibit cell migration and metastasis formation in triple negative breast cancer (Grottke et al., 2016).

AKT signaling also contributes to other cellular processes considered to be cancer hallmarks (Hanahan and Weinberg, 2000), further confirming the key role of AKT pathway upregulation in many aspects of tumorigenesis.

3. MUTANT P53 AND ITS GAIN OF FUNCTION (GOF) IN CANCER

3.1 p53: from tumor suppressor to oncogene

Wild type p53 (wt) is a sequence specific transcription factor (Laptenko and Prives, 2006) with a relevant tumor suppressive role that makes it the “guardian of the genome” and one of the most important players in cancer biology (Freed-pastor and Prives, 2012). In normal cells, p53 can be activated by numerous cellular stressors, including DNA damage, oncogenes activation and

hypoxia, and its low levels rise dramatically, resulting in the transcription of many genes with important roles in cell cycle arrest, senescence, apoptosis, DNA repair, metabolism, autophagy and differentiation (Meek, 2009). Tumors arise upon loss of p53 function through various mechanisms, including deletion or mutation of the p53 gene itself, overproduction of the p53 inhibitors, Mdm2 and Mdm4, or viral inactivation (Vogelstein et al., 2000)(Wasylishen and Lozano, 2017). Not surprisingly, the TP53 gene is the most frequent target for mutation in human cancer: 96% in ovarian serous carcinoma, 54% in invasive breast carcinoma, and 75% in pancreas cancer (Kim et al., 2014). Loss of p53 function is often a prerequisite for tumor initiation and progression, which has been most clearly demonstrated by the increased cancer risk in Li–Fraumeni syndrome patients (LFS) with germline p53 mutations and p53 knockout mouse models (Lang et al., 2004)(Donehower et al., 1992). Frameshift or nonsense mutations result in the loss of p53 protein expression, as seen with other tumor suppressors (Kim et al., 2014). Regardless of the mechanism of p53 inactivation, loss of tumor suppressive activities exerted by p53 may result in unrestricted cell proliferation and the permissive accumulation of genomic infractions that culminate in tumor growth. Moreover, among cancers with TP53 mutations, ~ 60% show concomitant deletion of the other allele, termed loss of heterozygosity (LOH). In cancer cells that do not undergo LOH of the wild-type TP53 allele, a dominant negative mechanism may inhibit wild-type p53 (Muller and Vousden, 2014). Moreover, the loss of normal p53 function is likely coupled with the adoption of new biologic functions exerted by mutant p53 proteins with additional, deleterious effects.

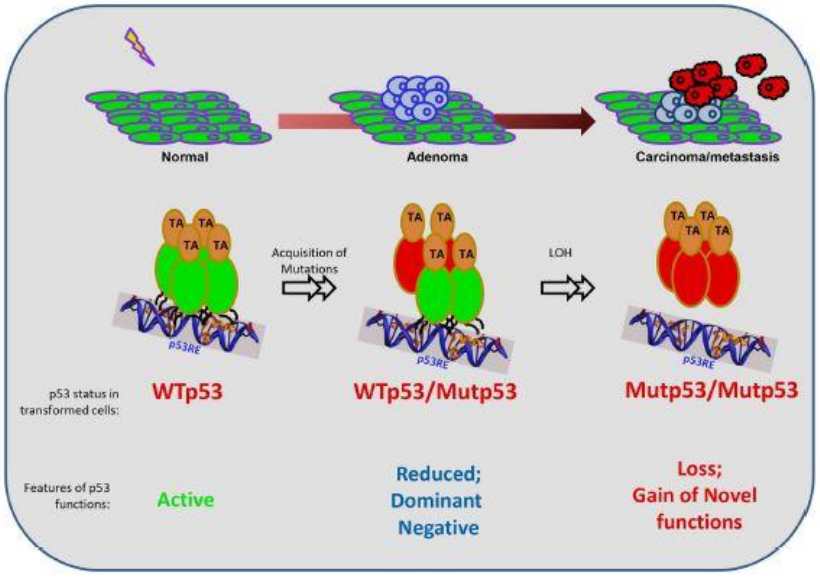


Figure 6: Mutant p53 functions during the evolution of a cancer cell. The picture represents the general evolution of a normal cell into a transformed cell (carcinoma), and the contexts in which mutant p53 exerts its functions (Sabapaty 2015).

In contrast to other tumor suppressors, that are commonly inactivated by frameshift and nonsense mutations leading to production of truncated proteins or complete elimination of the corresponding gene products, the majority of p53 alterations are missense mutations, leading to the substitution of a single amino acid in the p53 protein (Kim et al., 2014)(Muller and Vousden, 2014), resulting in the expression of full-length mutant p53 (mutp53) proteins (Hussain and Harris, 1998).

The full-legnth mutated p53 proteins become highly expressed in human cancers due to its prolonged half-life, reaching levels far above those observed in normal cells expressing wild-type p53 (wtp53) (Freed-pastor and Prives, 2012)(Strano et al., 2007).

Single aminoacid substitutions have been detected throughout the p53 protein, but the great majority clusters within the central DNA binding domain (in about 90% of cases) (Kim et al., 2014). Six "hotspot" residues are the most frequently substituted, including amino acid residues R175, G245, R248, R249, R273, and R282.

Mutations of p53 can affect its transcriptional activity or can also have significant consequences to the folding of p53 and to the capability to establish new interactions. Therefore, they are broadly categorized as those that alter the structure of the binding domain, defined as "conformational mutant" (e.g. R175, G245, and R249), or those that diminish the ability of mutant p53 to bind DNA, known as "contact mutant" (e.g. R248, R273 and R282) (Muller and Vousden, 2014). Conformational mutations usually result in a more dramatic alteration of p53 protein structure compared with DNA contact mutants.

More important, the changes in p53 structural stability may be crucial to the acquisition of new oncogenic properties (Muller and Vousden, 2014). Indeed, numerous mouse models, in vitro and clinical studies have demonstrated that in addition to simple loss of the tumor suppressor function of p53, many mutant p53 (mutp53) proteins gain neomorphic oncogenic activities, termed as gain-of-function (GOF) (Dittmer et al., 1993). Indeed, several studies have found that the median age of cancer onset in Li-Fraumeni Syndrome patients with mutp53 missense mutations is 9–20 years earlier than in LFS patients with loss of p53 expression (Zerdoumi et al., 2013)(Bougeard et al., 2008). Functionally, mutant p53 GOF activities contribute to malignant transformation by enhancing cells proliferation, invasion, metastatic ability, and chemoresistance (Muller and Vousden, 2014).

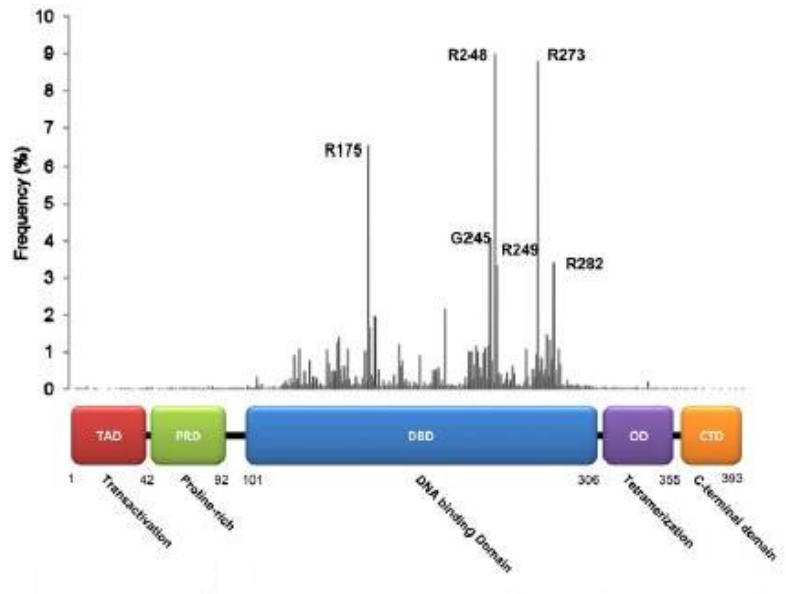


Figure 7: TP53 mutational spectrum in human cancer. Schematic representation of the p53 protein with domains structure illustrated: TAD: transactivation domain; PRD: proline-rich-domain; DBD: DNA-binding domain; OD: oligomerization domain (also contains a nuclear export signal); CTD: C-terminal regulatory domain (also contains three nuclear localization signals). The six hotspot mutations are highlighted (Freed-Pastor and Prives, 2012).

3.2 Mechanisms of mutant p53 gain of function

The vast majority of missense mutations in TP53, as described above, occurs at hotspots located within the DNA-binding domain. Ineffective binding of p53 to consensus DNA sequences in target gene promoters attenuates tumor suppressive mechanisms and reduces barriers to cell proliferation. Although mutant p53s have generally lost the ability to bind consensus p53 DNA binding regions in target gene promoters, their activity appears to reflect an ability to regulate gene expression directly (Weisz et al., 2007). Various different mutant p53s can bind directly to DNA with some degree of selectivity (Brázdová et al., 2013)(Göhler et al., 2005); and may thereby directly control the transcription of some genes (Weisz et al., 2007). Nevertheless, a consensus for mutant p53-specific binding has so far not been identified (Muller and Vousden, 2014). Moreover, cytoplasmic and mitochondrial activities of mutant p53 in regulating apoptosis and autophagy have also been described (Chee et al., 2013)(Frank et al., 2011)(Morselli et al., 2008). Indeed, the relative inability of mutant p53 to directly bind DNA suggests that the mutant p53 GOF is the product of functional partnerships between mutant p53 proteins and available interacting proteins that together mediate changes in cell phenotypes through altered gene expression. Importantly, numerous studies have demonstrated an intact p53 transactivation domain as requisite for specific mutant p53 GOF activities, again indicating codependence on other transcriptional regulators for exertion of GOF (Bullock and Fersht, 2001)(Vousden and Prives, 2009). GOF properties were better studied with the generation of the p53 mutant knock-in mice (Caulin et al., 2007)(Morton et al., 2010). Moreover, the spectrum and extent of GOF

activities endowed by p53 missense mutations change with mutation type. Knock-in p53 alleles with point mutations at codons R172H and R270H (equivalent to R175 and R273 in humans, respectively) showed different tumor spectra: mice heterozygous for the p53R270H mutation have a higher incidence of carcinomas and B-cell lymphomas compared with mice with one null p53 allele (Vogelstein et al., 2000). Similarly, p53R172H mutant mice develop more osteosarcomas with higher rates of metastasis than the R270H mutant (Vogelstein et al., 2000). Humanized p53 knock-in (HUPKI) mice express human coding sequences of the p53 DNA-binding domain and hotspot mutations at codons R248Q and G245S (Toledo et al., 2006). Homozygous p53R248Q mutant mice demonstrated a shortened overall survival owing to accelerated time-to-tumor formation (TTF) relative to p53-null mice. However, homozygous p53G245S mice had similar overall survival and tumor spectrum to p53-null mice, indicating altered GOF activities of mutant p53 proteins with R248Q versus G245S mutations. In addition, mice with mutant p53R248Q showed increased early expansion of the hematopoietic and mesenchymal stem cell pools relative to mutant p53G245S and p53-null mice. Based on the site and nature of missense mutations – conformation versus structural – mutant p53 proteins likely possess different conformations that affect the spectrum of interacting proteins. Indeed, mutant p53 proteins acquire a plethora of gain-of-function (GOF) effects that depend on its ability to bind other proteins and alter gene expression, and all of these mechanisms provide advantage to the cancer cell.

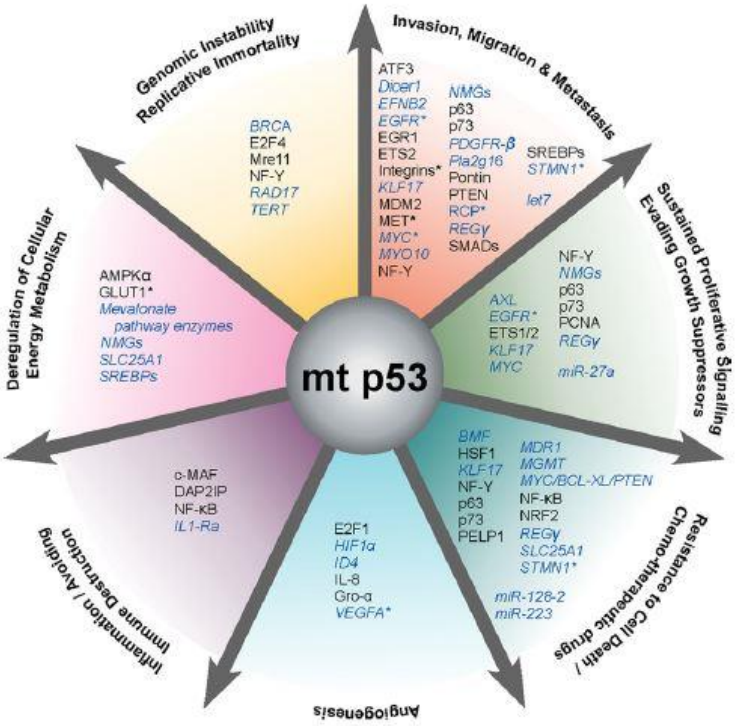


Figure 8: Molecules that are associated with mtp53 GOF. Mutant p53 GOF contributes to cancer through direct interaction with proteins altering their activity or through transcriptional activation or repression of target genes and downstream molecules (Ashauer and Muller 2016).

3.2.1 Interactions mediating mutant p53 transcriptional activities

Mutant p53 gain of function includes both transcriptional and non-transcriptional mechanisms. Regarding transcriptional activities, mutp53 can bind transcription factors (TFs) to transactivate target genes or attenuate target gene expression. In addition, mutp53 may also function to increase chromatin accessibility and drive the expression of genes contained within distinct regions of the genome. The partnership with transcription factors has been recognized as a central mechanism through which the impact of mutant p53 GOF is mediated and amplified. In fact, mutant p53 interacts with transcription factors and cofactors to control gene expression.

Interactions of mutant p53 with different TFs, such as NF-Y, p63, p73, YAP, SREBP; ETS2, VDR, Sp1 and NRF2 (Hamroun et al., 2006)(Xu et al., 2011) (Di Agostino et al., 2006) (Di Agostino et al., 2015)(Haupt et al., 2016)(Do et al., 2012)(Stambolsky et al., 2010)(Chicas et al., 2000)(Tung et al., 2015), have been demonstrated to sustain cancer progression.

Different GOF mutant p53 proteins (p53R280K, p53R175H, p53A193T, p53R248L, p53R273H, p53L194F, and p53P309S), but not wild-type p53 protein, can physically interact with YAP. The protein complex YAP/mutant p53 can form with the transcription factor NF-Y a large multi-protein complex that is recruited onto the regulatory regions of its target genes (Di Agostino et al., 2015). In addition mutant p53 directly interacts with NF-Y to recruit p300 acetyltransferase on the promoters of NF-Y target genes (*CCNA*, *CCNB1*, *CDK1*, and *CDC25C*) resulting in increased cell proliferation (Di Agostino et al., 2006). Mutant p53 proteins also bind p63 and p73 to repress activation of their respective target genes, leading to increased cell motility, invasion, and metastasis (Li and Prives, 2007). Mutant p53R175H and R273H bind to p73 and prevent its binding to NF-Y on the PDGFRβ promoter, allowing PDGFRβ expression (Blandino et al., 1999). By inhibiting TAp63, mutant p53 can regulate a pro-invasive transcription program that includes regulation of the expression of Dicer, DEPDC1, Cyclin G2, and Sharp1 (Muller et al., 2014)(Adorno et al., 2009) (Girardini et al., 2011). Moreover, in a more recent study it has become apparent that mutant p53 can bind to different Smad proteins, both promoting and inhibiting TGF*β* signalling (Ji et al., 2015).

Additionally, mutant p53 proteins can up-regulate genes that inhibit apoptosis or promote chemoresistance. For example, mutant p53 promotes *NF-κB* transcription and activation, inducing a pro-survival signalling (Weisz et al., 2007). Vadlamudi's group also demonstrated that Proline-, Glutamic acid-, and Leucine-rich Protein-1 (PELP1) PELP1 plays an important role in the modulation of mutant p53 anti-apoptotic functions. Indeed, PELP1 interacts with mutant p53, regulates its recruitment on DNA, and alters epigenetic marks at target gene

promoters. This interaction could influence mutant p53 oncogenic functions with potential implications in chemotherapy response, and overall prognosis of patients (Krishnan et al., 2015).
Finally, mutant p53 has been demonstrated to transactivate multiple genes involved in cancer cell proliferation and survival. For istance mutant p53 can transactivate CXCL1 (Yan and Chen, 2009), PCNA (Deb et al., 1992), MYC (Frazier et al., 1998), MAP2K3 (Bossi et al., 2008), ASNS (Scian et al., 2004), E2F5, MCM6 (Scian, 2005), STMN1 (Singer et al., 2007), IGF1R (Werner et al., 1996), and EGFR (Ludes-Meyers et al., 1996), all of which can promote proliferation of cancer cells. Additionally, mutant p53 proteins can transactivate EGR1(Weisz et al., 2004), ABCB1 (also known as MDR1 or P-glycoprotein) (Sampath et al., 2001), IGF2 (Lee et al., 2000), DUT (Pugacheva et al., 2002), BCL2L1 (also known as Bcl-xL) (Bossi et al., 2008), TIMM50 (Sankala et al., 2001), and LGALS3 (Lavra et al., 2009) all of which can inhibit cell death.
Conceptually, a single mutant p53 variant may interact with multiple TFs, and TFs complexed with mutant p53 may transactivate genes normally regulated by such TFs, therefore the number of genes potentially affected by mutant p53 can be very large.

3.2.2 Interactions mediating mutant p53 transcription-independent activities

Mutant p53 can form complexes with many proteins that are not involved in transcription, and this also contributes to its gain-of-function. Song et al. identified interactions between mutant p53 and Mre11 that result in impaired Ataxia-telaniectasia mutated activation, resulting in the disruption of DNA damage response elements and genetic instability (Song and Xu, 2007). Moreover, mutant p53 was also reported to interact with topoisomerase 1, which maintains DNA topology, resulting in hyperrecombination and genomic instability (Restle et al., 2008). Prolyl isomerase Pin1, which regulates conformational changes of proteins to affect protein stability and activity, was reported to be an additional mutant p53-binding protein. In breast cancer cells, Pin1 enhances the oncogenic activity of mutant p53 to promote aggressiveness through mutant p53-dependent inhibition of p63 and induction of a mutant p53 transcriptional program (Girardini et al., 2011).

3.3 Cytoplasmic interactions of mutant p53

In addition to nuclear functions, mutant p53 has cytoplasmic functions that contribute to its GOF. The stability of mutant p53 proteins facilitates their accumulation in the cytosol, and mutant p53 is easily detected in purified cytoplasmic fractions in various cancer cell lines (Di Minin et al., 2014)(Morselli et al., 2008)(Green and Kroemer, 2009). Thus, mutant p53 has

the opportunity to form aberrant complexes with cytoplasmic proteins, potentially altering their functions.

For istance, under condition of energy stress, mutant p53, but not wild-type p53, directly binds to the AMP-activated protein kinase alpha (AMPKα) subunit via the DBD, while its N terminus blocks AMPKα-LKB1(liver kinase β 1) interaction, thus leading to inhibition of AMPK activity. AMPK is the major cellular energy sensor and a master regulator of metabolic homeostasis. GOF mutant p53s negatively regulates the metabolic effects of AMPK signaling, leading to increased lipid production, aerobic glycolysis, and invasive cell growth in head and neck cancer cell lines (Zhou and Myers, 2014).

We have found that mutant p53 binds DAB2IP in the cytoplasm, interfering with its physiological interactions. DAB2IP is a cytoplasmic Ras-GTPase activating protein that also functions as a signaling scaffold to modulate the cell's response to multiple oncogenic extracellular signals, including TNFα (Di Minin et al., 2014) and Insulin (Valentino et al., 2017a) (this thesis). The interaction between mutant p53 and DAB2IP is the main focus of this Thesis and it will be discussed in more detail in a following chapter.

Finally, Yue and collegues recently demonstrated that mutant p53 binds members of the monomeric GTPase family of proteins (Yue et al., 2017). Specifically, mutant p53 interactes with and activates Rac1. Rac1 is a small GTPase that regulates many cellular functions, including cell proliferation, cytoskeletal reorganization, and cell mobility. As all GTPases, Rac1 cycles between the inactive GDP-bound (Rac1-GDP) and the active GTP-bound (Rac1- GTP) forms (Bid et al., 2013)(Heasman and Ridley, 2008). Rac1 SUMOylation is critical to maintain the active GTP-bound form, and thus its activity. The sumo-specific protease SENP1 de-SUMOylates Rac1, leading to its inactivation (Castillo-Lluva et al., 2010). Mechanistically, mutant p53 inhibits the interaction of SENP1 with Rac1 and its de-SUMOylation, thereby activating Rac1, in turn promoting tumor growth and metastasis (Yue et al., 2017).

3.4 Mutant p53 modulates multiple pathways in cancer

Mutant p53 activities interact with multiple pathways involved for cancer progression. Indeed, mutant p53 has been shown to inhibit BTG2 anti-proliferative protein promoting H-Ras activation (Solomon et al., 2012) and to cooperate with RAS to drive MET receptor recycling (Muller et al., 2013), enhancing MAPK activation and accelerating cell proliferation, scattering, and invasion. Similarly, mutant p53 up-regulates platelet-derived growth factor receptor β (PDGFR-β)(Weissmueller et al., 2015), and reprograms the response of cancer cells to TGF-β (Adorno et al., 2009). In addiction, mutant p53 cooperates with the SWI/SNF complex to

remodel the chromatin architecture of the VEGFR2 promoter, leading to enhanced VEGFR2 expression and pathway activity (Pfister et al., 2015). Finally, mutant p53 enhances the cell response to an inflammatory microenvironment, amplifying NF-κB activation and transcriptional function, leading to an increased rate of transformation (Cooks et al., 2014)(Weisz et al., 2007).

3.5 Mutant p53 promotes PI3K/AKT pathway activation

The PI3K/AKT (PKB) pathway is a fundamental signaling axis that controls cell responses to metabolic, survival, and growth signals. Not surprisingly, as described above, dysfunctional PI3K/AKT cascade is commonly observed in metabolic diseases and in multiple tumors. Indeed, PI3K/AKT pathway hyperactivation confers an enormous advantage to cancer.

In this context, mutant p53 gains from its activation to sustain cancer development. Indeed, it has been reported that mutant p53 can enhance PI3K/AKT pathway activation in human endometrial cancer cells (EC), promoting invasion ability. In particular, over-expression of p53-R175H leds to enhanced EGFR phosphorylation, and subsequently activation of PI3K/AKT pathway, but has no effect on the MEK/ERK pathway. Moreover, abolished p53-R175H-induced cell invasion and migration and attenuated activation of AKT upon treatment with EGFR inhibitor, suggest that GOF effects of this mutant could drive invasive characteristics in EC cells through EGFR/PI3K/AKT-dependent pathway (Dong et al., 2009). Overall, the results of this study show the possibility that GOF activity of p53-R175H contributes to an aggressive phenotype to EC cells, at least in part through the ability of p53-R175H to associate with active EGFR, subsequently resulting in the activation of its downstream PI3K/AKT signaling pathway. Moreover, it has been demonstrated that RCP-dependent α5β1/EGFR trafficking augments EGFR phosphorylation, leading to increased AKT signaling (Caswell and Norman, 2008). Consistent with this, Vousden's group showed that mutant p53 drives constitutive activation of EGFR, and its communication with AKT but not ERK. Indeed, upon expression of mutant p53, p63 transcriptional activity is suppressed, resulting in enhanced RCP-driven recycling of α5β1 integrin and EGFR. Moreover, IHC staining of primary human colons showed a correlation between high expression of p53 (an indicator of the presence of mutant p53) and strong phospho-AKT staining. These results suggest that mutant p53 specifically drives RCP-dependent recycling of integrin and EGFR resulting in alteration of AKT signaling, which contributes to increased invasion and metastasis (Muller et al., 2009).

Finally, Tan's group demonstrated that p53-R273H contact mutant suppresses BMF expression in a way that is dependent on PI3K/AKT signaling pathway promoting cancer cell survival and anoikis resistance (Tan et al., 2015). In particular, silencing of endogenous p53-R273H contact

mutant, but not p53-R175H conformational mutant, reduces AKT phosphorylation, induces BCL2-modifying factor (BMF) expression, sensitizes BIM dissociation from BCL-XL and induces mitochondria-dependent apoptosis in cancer cells. Importantly, cancer cells harboring endogenous p53-R273H mutant are inherently resistant to anoikis and lack BMF induction following culture in suspension. However, how exactly mutant p53 contributes to PI3K/AKT activation remains an open question.

4. DAB2IP: GUARDIAN OF CYTOPLASMIC SIGNAL TRANSDUCTION

4.1 The tumor suppressor DAB2IP

DAB2IP is a tumour suppressor gene associated with ovarian (Fazili et al., 1999), prostate (Wang et al., 2002) and mammary cancer (Schwahn and Medina, 1998) as well as choriocarcinoma (Fulop et al., 1998). DAB2IP modulates several signal cascades controlling cell proliferation, survival, apoptosis and metastasis (Liu et al., 2016)(Bellazzo et al., 2016).

DAB2IP is frequently down-regulated in tumours; however, its loss does not induce cancer formation per se, but strongly supports cell transformation induced by driver mutations in various oncogenic pathway (Bellazzo et al., 2016).

DAB2IP was initially identified as a novel member of the RAS GTP-ase (RAS GAP) protein family, cloned specifically as a protein interacting with the tumor suppressor DOC-2/DAB2 (differentially expressed in ovarian carcinoma-2/Disable-2). Concomitantly, DAB2IP has been identified as AIP1, ASK1-interacting protein. Playing as a competitor, AIP1 facilitates dissociation of 14-3-3 from ASK1 and it enhances ASK1-JNK pathway activation in response to TNF (Zhang et al., 2004)(Zhang et al., 2003). In addition to acting as a cytoplasmic Ras GAP, DAB2IP also functions as a signaling scaffold to modulate the cell's response to multiple extracellular signals.

4.2 The human DAB2IP gene

The DAB2IP gene is located on human chromosome 9q33.1–q33.3 and spans approximately 96 kb with 15 exons and 14 introns (Chen et al., 2002). It is highly polymorphic, and several reports indicate that genetic variation of DAB2IP is associated with the risk of cancer (Duggan et al., 2007)(Hsieh et al., 2007). The gene is rather complex, and can express multiple transcripts and proteins. Two human transcripts and proteins are annotated in Ref Seq: hDAB2IP transcript variant 1 (NM_032552.3), and hDAB2IP transcript variant 2 (NM_138709.1). They are transcribed by different promoters, and encode predicted proteins that differ in their N-terminus

(variant 1 being 96 amino acids longer) and have an alternative C terminal exon (Chen et al., 2002)(Chen et al., 2006). The hDAB2IP gene is ubiquitously expressed in different tissues and organs. During development, hDAB2IP is expressed in most human fetal tissues, in a gestational age- and tissue-type specific manner. Low levels of its transcript are found only in seminal vesicle, ventral prostate, epididymis, and bladder (Wang et al., 2002). hDAB2IP transcript variant 1 seems to be the dominant isoform expressed in normal tissues (Qiu et al., 2007).

4.3 DAB2IP protein structure and interactions

DAB2IP consists of several conserved structural domains, involved in the interaction with many different proteins, thus affecting the outcome of multiple cellular inputs.

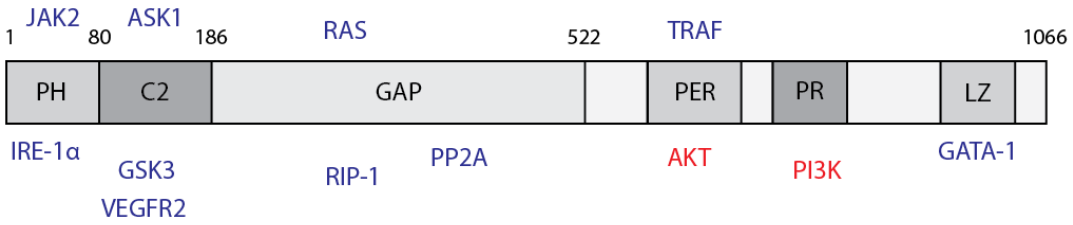


Figure 9: DAB2IP protein structure and interactors. Schematic representation of DAB2IP (isoform 2) domains.

Pleckstrin homology domain (*PH domain*): an approximately 100-residue module, localized on the N-terminal of DAB2IP. This domain results in DAB2IP membrane targeting via the binding phosphatidylinositol lipids. Thus, PH domain is involved in the recruitment of different protein associated to membrane, driving their physiological localization and activity (Musacchio et al., 1993).

Protein kinase C conserved region 2 (C2 domain): a Ca2+ binding motif that in DAB2IP spans from 21 to 110 aa (Chen et al., 2002). This domain displays the remarkable property of binding a variety of different ligand and substrates such as Ca2+, phospholipids, inositol polyphosphates, and intracellular proteins. Not all proteins containing C2 domains are regulated by Ca2+, suggesting that some C2 domains may play a purely structural role (Nalefski and Falke, 1996). DAB2IP C2 domain interacts with several proteins involved in signal transduction such as ASK1 (Zhang et al., 2003), GSK3β (Xie et al., 2010), VEFGR2 (Zhang et al., 2008). Indeed C2 domain binds mutant p53, counteracting the binding of DAB2IP with ASK1, preventing JNK phosphorylation and enhancing NF-κB activation in response to TNF (Di Minin et al., 2014).

PH-C2 domain: This domain is required for the regulation of the JAK/STAT pathway. DAB2IP binding to JAK2 inhibits its phosphorylation, STAT1 and STAT3 phosphorylation and activation upon IFNγ treatment functions, strongly reducing proliferation and migration of vascular smooth muscle cells (VSMC) (Yu et al., 2011).

RasGTPase-activator protein (RasGAP domain): it is localized in the N terminus of DAB2IP and spans from 135 to 438 aa. The GAP domain is critical for the binding for Ras (Chen et al., 2002), RIP (Zhang et al., 2007), HIPK1 (Li et al., 2005) and PP2A (Min et al., 2008).

Proline rich domain (*PR domain*): it is a domain implicated in protein-protein interactions, in particular it is a docking site for proteins with SH3 domains (Feller et al., 1994). PR domain is involved in the binding with c-Src (Wu et al., 2013). Moreover, DAB2IP via its PR domain sequesters p85-p110 complex and inhibits PI3K activity and AKT activation (Xie et al., 2009).

Period-like domain (*PER domain*): a non-described structure, involved in protein-protein interaction. PER domain binds TRAF2 (Zhang et al., 2004) and 14-3-3 proteins (Zhang et al., 2007). Indeed, PER domain is involved in the binding of DAB2IP to AKT1 counteracing its activation (Xie et al., 2009).

Leucine zipper domain: it is a hydrophobic motif, usually found within the DNA-binding domain of transcription factors. It is involved in the formation of protein dimers and is involved in DAB2IP binding to GATA-1 (Yun et al., 2014).

4.4 DAB2IP: a broad modulator of multiple oncogenic pathways

In various cell models, DAB2IP was shown to affect many signaling pathways relevant for cancer almost invariably acting to restrain their oncogenic outcomes.

.

For instance, DAB2IP can down-modulate canonical WNT signaling. DAB2IP sustains activation of GSK- 3β by reducing Serine 9 phosphorylation, with the help of PP2A recruited by DAB2IP in a ternary complex. The C2 domain interacts with both GSK-3β and PP2A, and facilitates GSK-3β activation to decrease nuclear β-catenin accumulation and its transcriptional activity (Min et al., 2008) (Xie et al., 2010). Perhaps the best characterized fucntion of DAB2IP is in TNF-α signaling. DAB2IP acts as a scaffold in TNF-induced recruitment of PP2A to ASK1 complex, leading to dephosphorylation of ASK1 at pSer967 and activation of ASK1-JNK

signaling by the pro-apoptotic kinase (Min et al., 2008). Moreover, RIP in the DAB2IP complex mediates TNF-induced DAB2IP phosphorylation at 14–3–3-binding site (Ser604). Indeed, RIP1 associates with the GAP domain of DAB2IP and synergizes DAB2IP-mediated JNK/p38 MAPK activation (Zhang et al., 2007). More importantly, DAB2IP associates with the effector domain (the RING finger) of TRAF2 through its PER domain and inhibits IKK- NF-κB signaling (Zhang et al., 2004).

Another condition in which DAB2IP promotes ASK1-JNK/p38 signaling is during the Unfolded Protein Response (UPR). DAB2IP binds directly to a key mediator of the UPR, the inositol-requiring enzyme-1 (IRE1α), facilitates its dimerization, behaving as a scaffold protein to mediate JNK phosphorylation by the kinase IRE1α (Luo et al., 2008). DAB2IP also inhibits JAK kinase activity, thus limiting JAK-dependent STAT1/3 and PI3K/AKT phosphorylation and activation in vascular smooth muscle cells. In non-muscle invasive bladder cancer, DAB2IP-dependent inhibition of STAT3 has been reported to limit expression of Twist1 and P-glycoprotein, crucial factors for chemoresistance (Wu et al., 2015). In prostate cancer cells, DAB2IP was found to directly bind STAT3, suppressing transactivation and expression of the anti-apoptotic target survivin. Finally, DAB2IP modulates constitutively active androgen receptor (AR) via PP2A-mediated inhibition of AR phosphorylation and nuclear translocation, thereby inhibiting AR transcriptional activities (Wu et al., 2014). Indeed, the unique PR domain in DAB2IP is capable of competing with AR to form complex with c-Src in PCa cells, inhibiting c-Src and ERK signaling pathway.

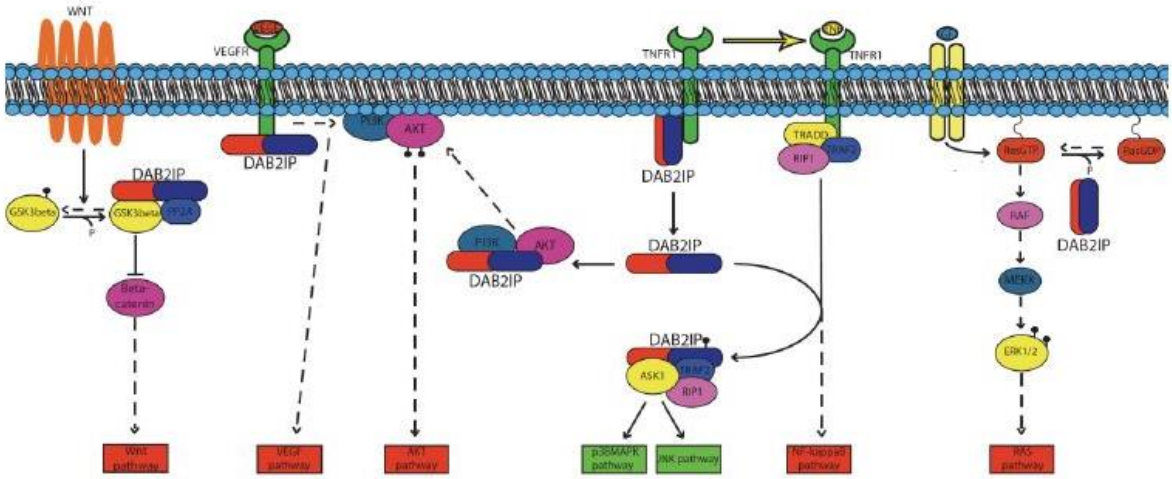
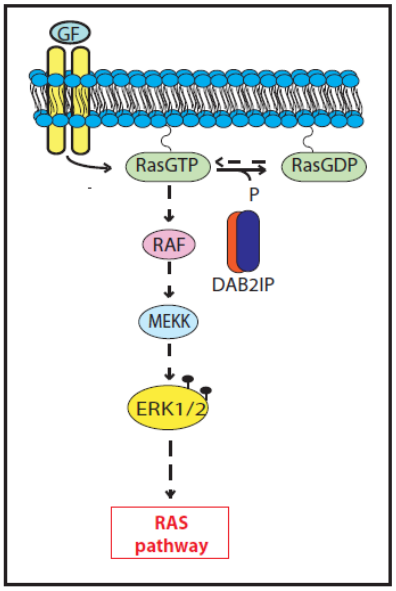


Figure 10: DAB2IP modulates multiple signaling pathways. Schematic rappresentation of the main molecular pathways regulated by DAB2IP

4.4.1 DAB2IP modulates the Ras-MAPK pathway.

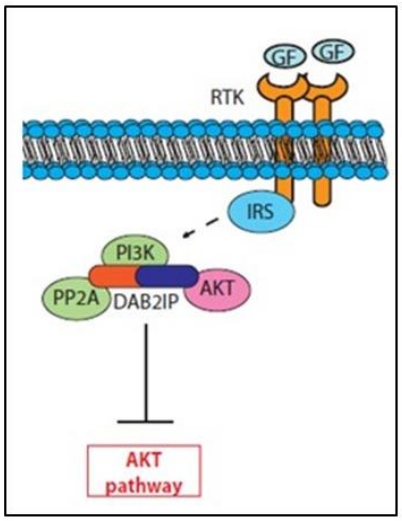
One of the most important activities of DAB2IP is its function as a GTPase-Activating Protein for Ras. Ras proteins are monomeric GTPases strongly involved in cancer. Physiological or oncogenic activation of Ras induces a wide range of downstream effects; the best characterized is the RAF-MEK-ERK cascade, stimulating cell growth, proliferation, survival, and differentiation (Moodie et al., 1993)(Pylayeva-Gupta et al., 2013). In addition to the MAPKs, Ras proteins can also activate PI3K, mediating proliferative and survival signals (Castellano and Downward, 2011).



The GAP domain of DAB2IP is homologous to other Ras-GAPs, such as GAP120 and neurofibromin (NF1), and can stimulate the GTPase activity of Ras proteins both in vitro and in cancer cell lines. For istance, Wang et al. showed that DAB2IP could interact with the N-terminal domain of DAB2 protein directly and functions as a Ras GAP in vivo and in vitro (Wang et al., 2002). Through this activity, DAB2IP can also dampen Ras-induced activation of PI3K, contributing to inhibition of the PI3K–AKT signaling axis.

4.4.2 DAB2IP regulates PI3K/Akt pathway

Another important activity of DAB2IP, particularly relevant for this Thesis, is its function as an inhibitor of the PI3K/AKT signaling axis. The phosphatidylinositol 3-kinase/protein kinase-B/ mammalian target of rapamycin (PI3K/AKT/mTOR) signaling cascade is one of the most important intracellular pathways and is frequently activated in diverse cancers (Janku et al., 2012). It has a major impact on cell metabolism, size, proliferation, survival, and motility, and is frequently activated in cancer either by gain-of-function mutations, or by loss of function of the critical regulator PTEN (Fruman et al., 2014). As previous described, class I PI3K is divided into class I A group with its 110-kDa catalytic subunit (p110) and an 85-kDa regulatory subunit (p85) (Kok et al., 2009). The PR domain within DAB2IP can bind to the p85 regulatory subunit of class IA PI3K kinase, thus inhibiting its activation and



downstream signaling. In addition, DAB2IP binds to AKT via its PER domain, and such interaction inhibits Akt activation and function (Xie et al., 2009)(Valentino et al., 2017b). The GAP activity of DAB2IP can further enforce inhibition of the PI3K–AKT axis by reducing Ras-dependent activation of the PI3K p110α subunit (Castellano and Downward, 2011)(Mendoza et al., 2011). Noticeably, DAB2IP dissociates ASK1 inhibitor 14-3-3 from its C terminus and then facilitates ASK1 activation (Zhang et al., 2003). Moreover, DAB2IP-mediated binding and inhibition of PI3K-AKT also contributes to ASK1 activation, under TNF-α treatment (Xie et al., 2009). Indeed, DAB2IP interacts with either p85α or ASK1; however, a dramatic increase of complex formation was detected under either PI3K inhibitor or TNF-α treatment. In the ASK1-DAB2IP-PI3K complex, the active form of ASK1 (T845) became more predominant than its inactive form (S83). In this complex, AKT activation is reduced, suggesting that DAB2IP might inhibit PI3K via a direct protein interaction with both PI3K and AKT. In a feedback loop, ASK1 increases S604 phosphorylation in DAB2IP, which significantly enhances the binding ability of DAB2IP to AKT and PI3K complex. Interestingly, DAB2IP is also a target of AKT: by scanning the protein sequence, Dai et al. identified two consensus AKT sites (RxRxxpS/T) in the C terminus of DAB2IP at Serine-847 and Serine-907 (Dai et al., 2014). Phosphorylation by AKT reduces DAB2IP activity towards H-Ras and TRAF2, altering DAB2IP regulation of downstream effector pathways (Dai et al., 2014).

Thus, enhanced AKT activation would inhibit DAB2IP, which would further fuel AKT activation in a potentially oncogenic positive feedback loop.

4.5 DAB2IP inactivation enhances multiple oncogenic phenotypes

DAB2IP has been implicated in the regulation of diverse biological processes including proliferation (Wang et al., 2002), apoptosis (Xie et al., 2009), survival (Luo et al., 2008)(Zhang et al., 2007), epithelial-to-mesenchymal transition (EMT) (Xie et al., 2010), cancer stem cell (CSC) (Yun et al., 2015), autophagy (Yu et al., 2012), and angiogenesis (Zhang et al., 2008).

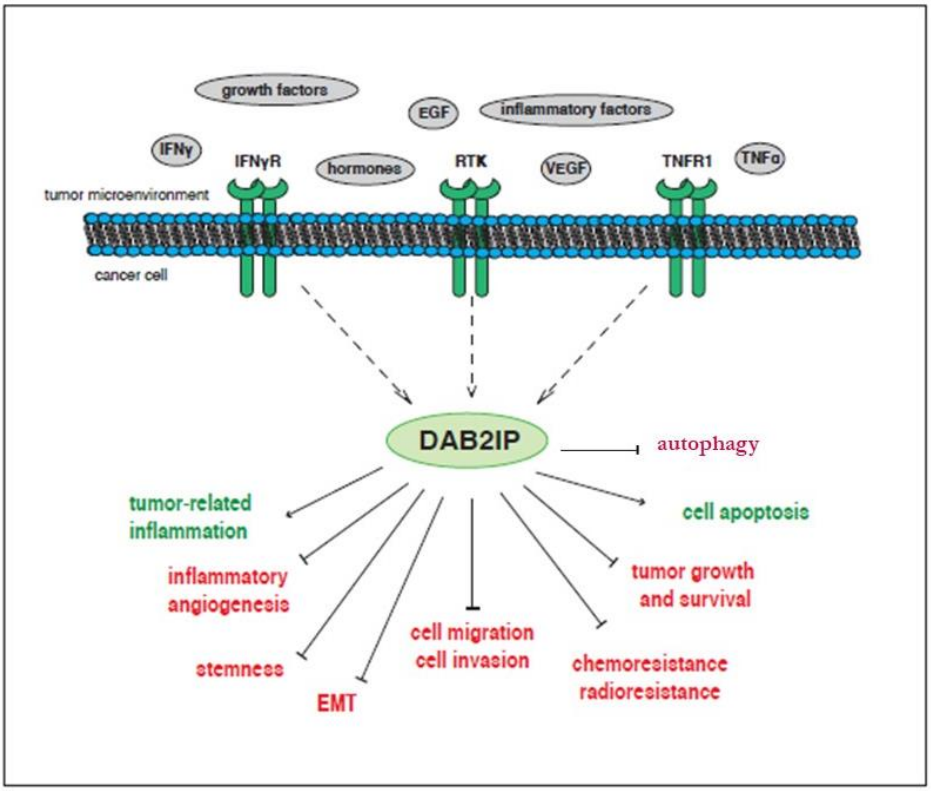


Figure 11: DAB2IP influences the cancer cell's response to a plethora of extracellular stimuli.

DAB2IP protein modulates different cytoplasmic signaling pathways, regulating cancer cell response to inflammatory cytokines, growth factors and hormones that are present in the tumor microenvironment and are secreted from stromal and immune cells, and the tumor cell itself (Adapted from Bellazzo et al., 2017).

Alterations of these processes are common features in cancer and DAB2IP functions as a bona fide tumor suppressor.

Indeed, in human normal prostate epithelial and prostate carcinoma cells, as well as in clinical PCa specimens, loss of DAB2IP expression initiates EMT (Xie et al., 2010). Indeed, DAB2IP controls EMT and cell invasion by coordinating both the WNT/β-catenin and NF-κB pathways (Xie et al., 2009)(Min et al., 2010)(Min et al., 2015). DAB2IP loss enhances chemoresistance in prostate cancer cells by upregulation of the anti- apoptotic protein clusterin (CLU) (Wu et al., 2013), maybe linked to the loss of DAB2IP inhibitory action on EGF1 expression (Wu et al., 2013). Similarly, loss of DAB2IP facilitates survival of prostate cancer cells after androgen deprivation therapy, by augmenting STAT3 activation and survivin expression (Yu et al., 2012).

Moreover, DAB2IP inactivation may promote a hypoxia-like response, with upregulation of HIF target genes and increased expression and activity of VEGF proteins, affecting the cancer cell and its microenvironment, and promoting tumor vascularization (Li et al., 2015). Notably,

DAB2IP directly binds to VEGFR-2 and limits PI3K activation, thus functioning as endogenous inhibitor of adaptative angiogenesis (Zhang et al., 2008).

DAB2IP knock out mice show enhanced inflammation in models of ischemic hind limb, graft arteriosclerosis, and inflammatory angiogenesis (Zhang et al., 2008) (Zhang et al., 2016)(Yu et al., 2011)(Huang et al., 2013). In hyperlipidemic ApoE−/− mice, DAB2IP KO increases secretion of inflammatory cytokines and augments macrophage infiltration, thereby inducing endothelial dysfunction and early phases of atherosclerosis (Huang et al., 2013).

Interestingly, DAB2IP-depleted cells have increased mTOR-S6K pathway activation and autophagy, suggesting that in normal cells DAB2IP inhibits autophagy, and loss of such inhibition in cancer contributes to radiation resistance (Yu et al., 2012).

Finally, Yun and colleagues demonstrated that loss of DAB2IP enriches CSCs characteristics in human PCa cells, facilitating the binding of GATA-1 to CD117 gene promoter while repressing CD117 transcriptional activity (Yun et al., 2015). In addition, loss of DAB2IP may further support PI3K-AKT-mTOR signaling activation, promoting acquisition of CSC phenotypes.

4.6 Mechanisms of DAB2IP inactivation in cancer

The tumor suppressor DAB2IP is rarely mutated in human cancers. Indeed, different mechanisms for DAB2IP inactivation have been identified.

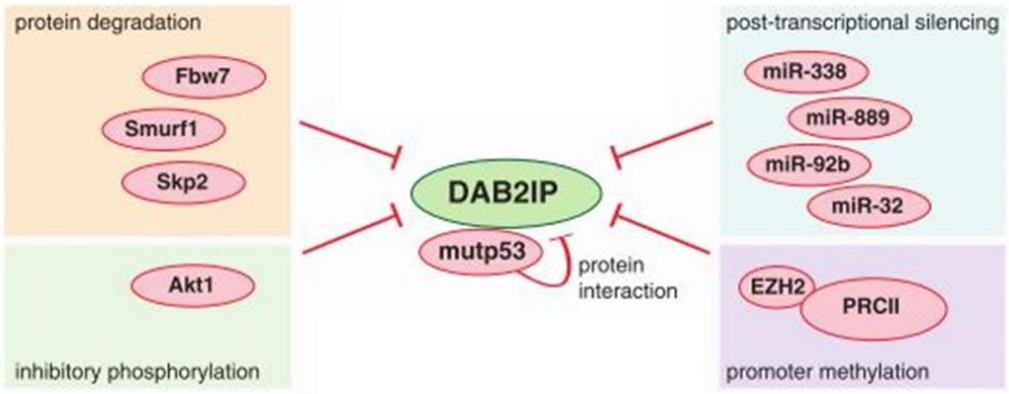


Figure 12: DAB2IP inactivation mediated by various mechanisms. Epigenetic DAB2IP inactivation relies on promoter hypermethylation mediated by the EZH2–PRC2 complex. Various microRNAs have been described that can target the DAB2IP 3′ UTR reducing protein expression. DAB2IP protein levels can be negatively regulated by the activity of the E3-ubiquitin ligases Fbw7, Skp2, and Smurf1. Phosphorylation by AKT1 can also inhibit DAB2IP functions. Finally, direct binding with p53 mutant proteins interferes with other DAB2IP interactions (Adapted from Bellazzo et al., 2016).

Not surprisingly, DAB2IP expression is frequently reduced by methylation in tumors (Chen et al., 2005)((Yano et al., 2005). The main factor controlling DAB2IP gene methylation appears to be the PRC2 (polycomb-repressive complex-2)– EZH2 (Enhancer of Zeste homolog) complex (Chen et al., 2005). Recently, it has been reported that the transcription factor Snail can repress DAB2IP expression in colon cancer cells (Wang et al., 2015). Nonetheless, in many cancers the gene is not methylated, and post-transcriptional mechanisms of DAB2IP inactivation have been reported. Indeed, recent studies identify Fbw7, Smurf1 and Skp2 E3 ubiquitin ligases involved in the regulation of DAB2IP turnover, promoting its degradation (Dai et al., 2014)(Li et al., 2016)(Tsai et al., 2014). Inhibitory phosphorylation of DAB2IP is an other mechanism of inactivation. The kinase AKT1 can phosphorylates DAB2IP on Ser 847 within the proline-rich domain, inhibiting its functions (Dai et al., 2014). Since DAB2IP has a relatively long 3′ UTR sequence, it represents a good candidate for post-transcriptional silencing by micro-RNAs (miRNAs). MiRNAs 338, 889, 92b and 32 have been described to reduces DAB2IP levels in different tissues (Barik, 2008)(Xu et al., 2015)(Liao et al., 2015)(Huang et al., 2016). Finally, DAB2IP can be functionally inhibited by the binding with mutant p53 in the cytoplasm of cancer cells. For istance, mutant p53 inhibition of DAB2IP interferes with its functions, thus reprogramming the way of cancer cells to respond to inflammatory cytokines, in particular TNF-α (Di Minin et al., 2014) and Insulin (Valentino et al., 2017a), inducing a pro-oncogenic behavior.

AIM OF THE THESIS

DAB2IP/AIP1 is a Ras GAP that functions as cytoplasmic adaptor in signal transduction. In particular, DAB2IP modulates TNFα signaling, promoting activation of the Ask1/JNK pathway while counteracting activation of NFkB (Xie et al., 2010). Moreover, DAB2IP modulates other oncogenic pathways such as PI3K/AKT and WNT/beta-catenin, and inhibits Epithelial-Mesenchimal Transition (EMT) in cancer cells (Bellazzo et al., 2017). Not surprisingly, its expression is frequently reduced by methylation in tumors (Chen et al., 2003)(Yano et al., 2005). Nonetheless, in many cancers the gene is not methylated, and we and others have reported post-transcriptional mechanisms of DAB2IP inactivation (Dai et al., 2014)(Xu et al., 2015). One of such mechanisms is interaction with mutant p53.

Point mutation of p53 is a frequent event and a significant factor in cancer development and progression (Muller and Vousden, 2013). Mutant p53 proteins (mutp53) are very stable, and acquire oncogenic properties (gain of function) that increase metastasis, proliferation, and cell survival. Through transcriptional and non-transcriptional activities, mutant p53 fosters tumor progression by reinforcing and up-regulating multiple oncogenic pathways, including RAS-MAPK, PI3K-AKT, β-catenin, and NF-κB (Aschauer and Muller, 2016).

Our group recently discovered that gain-of-function mutp53 proteins can bind DAB2IP in the cytoplasm and interfere with its functions, thus reprogramming the way by which cancer cells respond to inflammatory cytokines, in particular TNF, and inducing a pro-oncogenic behavior (Di Minin et al., 2014). We are convinced that the mutant p53/DAB2IP interaction has even broader implications, modulating the response to additional extrinsic inputs; in fact, DAB2IP modulates other pathways directly or indirectly linked to inflammation. Due to its broad clinical impact, in this Thesis I decided to focus on insulin and the PI3K-AKT axis.

As detailed in the introduction, DAB2IP binds and inhibits PI3K-p85, limiting AKT activation in response to various stimuli (Xie et al., 2009). DAB2IP also binds directly to AKT1, possibly contributing to its inhibition, and is also phosphorylated by AKT1 in a negative feedback loop (Dai et al., 2014). It is therefore legitimate to hypothesize that mutant p53, by binding DAB2IP, modifies the response to insulin, with effects on proliferation and survival.

The aim of my Thesis is to define the relevance of the mutant p53/DAB2IP interaction in this context, and explore possible strategies to target this molecular axis to restore DAB2IP functions

in cancer cells. Elucidating the role of insulin in TNBC and in androgen-independent PCa initiation and progression, and identifying the molecular pathways involved in the response of cells to insulin, may improve the development of effective diagnostic and treatment strategies to provide benefits to specific patients subgroups.

RESULTS

5.1 Mutant p53 amplifies the oncogenic activity of insulin in hormone-independent breast and prostate cancer cells

To explore the potential role of mutant p53 in the response of cancer cells to insulin, we performed BrdU incorporation, cell proliferation, and matrigel invasion assays with two human cancer cell lines bearing different missense TP53 mutations: MDA-MB231 (triple negative breast cancer, p53R280K) and DU145 (androgen-independent prostate cancer, p53V274F/P223L). We performed our experiments using a supraphysiological concentration of insulin (0,5ug/ml), to mimic an hyperinsulinemic condition (De León and Stanley, 2013).

We observed that insulin increases proliferation and enhances the invasive capabilities of these cells. Strikingly, depletion of endogenous mutant p53 prevented this effect, suggesting that such increase is strictly dependent on mutant p53 expression (Figure 13).

Colony formation assays confirmed that mutant p53 enhances proliferation of cancer cells chronically exposed to insulin. Using Ras-transformed embryonic fibroblasts (MEFs) derived from p53 knock-out or mutant p53 (R172H) knock-in mice, we verified that insulin triggers an increase in cell proliferation only in MEFs expressing mutant p53. Together, these data indicate that mutant p53 can be a determinant of the pro-oncogenic response to insulin, potentially defining a novel mutant p53 gain of function (Figure 14).

5.2 Mutant p53 mediates insulin-induced proliferation and invasion by enhancing activation of AKT

To define a mechanism of action for mutant p53 in the response to insulin, we first verified expression of the insulin receptor (INSR) in our cell lines (Figure 15A). Next, we asked if mutant p53 may affect INSR expression: by RT-PCR we observed that mutant p53 depletion does not affect INSR levels (Figure 15 B,C). Moreover, under certain conditions such as increase in concentration, insulin can stimulate the insulin-like growth factor 1 receptor (IGF1R)(Novosyadlyy et al., 2011); so we also checked IGF1R expression in our cell models; interestingly, mutant p53 depletion reduced IGF1R levels in MDA-MB231 but not in DU145 cells (Figure 15 B, C). The results in MDA-MB231 are consistent with previous studies reporting that mutant p53 promotes IGF1R transcription (Werner et al., 1996). However, the results in DU145 imply that IGF1R downregulation is not relevant for the observed phenotypes - a concept further supported by other evidences (Figure 20D and Figure 27C).

We next focused on insulin-induced activation of the PI3K/AKT pathway. Using the specific AKT inhibitor MK2206, we found that the increase in proliferation and invasion triggered by insulin is strictly dependent on AKT activity (Figure 16). This result proves that AKT is a key effector in this context, and implies that other pathways potentially activated by INSR (e.g. Ras/MAPK) are not sufficient for the observed phenotypes.

Since a large body of literature shows that AKT2 activation enhances cell invasiveness and metastasis, while AKT1 plays a major role in cell proliferation and survival (Irie et al., 2005)(Chau and Ashcroft, 2004), we decided to explore the distinct role of the two kinases in the observed phenotypes.

We thus used specific siRNAs to knock-down either AKT1 or AKT2 in our model cell lines. Interestingly, AKT1 depletion abrogated insulin-induced cell proliferation while knockdown of AKT2 strongly reduced insulin-dependent invasion in breast cancer MDA-MB-231 cells (Figure 17A, B). In contrast, AKT2 depletion abrogated insulin-induced cell proliferation, whereas both AKT1 and AKT2 were required for cell invasion in prostate cancer DU145 cells (Figure 17C, D). These results corroborate the notion of a distinct role for the two AKTs, possibly displaying tissue specific activities. In addition, they suggest that the mutant p53 could promote insulin-induced activation of both AKT isoforms, dictating specific oncogenic features of different tumor types.

Previous studies reported evidence of correlation between mutant p53 and enhanced AKT phosphorylation in breast, endometrial, and colon cancer (Dong et al., 2009)(Tan et al., 2015). We therefore asked whether mutant p53 might affect AKT activation in the specific context of insulin stimulation. Given the availability of reagents in our laboratory, we initially focused on AKT1; therefore, in the following chapters we will always refer to AKT1, except when specifically indicated. As shown in Figure 18A, knockdown of mutant p53 clearly reduced insulin-induced AKT phosphorylation in MDA-MB231 cells. Identical results were obtained with DU145 (Figure 18B). Similarly, insulin induced a stronger and prolonged AKT phosphorylation in mutant p53 knock-in versus p53 null MEFs (Figure 18C).

In line with the mitogenic and migratory response, insulin-induced AKT activation correlated with phosphorylation of GSK3β and increased Cyclin D1 levels. Upon mutant p53 depletion, insulin still triggered a moderate and transient AKT phosphorylation, but p-GSK3β and Cyclin D1 were significantly lower at longer times (Figure 19 A,B), consistent with reduced cell

proliferation and invasion. Insulin-induced phosphorylation or accumulation of additional AKT targets was likewise impaired by mutant p53 depletion (Figure 19C).

Since insulin can also trigger activation of the Ras-MAPK cascade (Belfiore and Malaguarnera, 2011) (Taniguchi et al., 2006), we monitored ERK phosphorylation as a readout of this pathway. We found no significant change in ERK activation upon insulin treatment and/or mutant p53 knockdown, thus confirming that mutant p53 affects specifically insulin-induced AKT activation in these cells (Figure 20 A,B).

To verify if mutant p53 is sufficient to promote insulin-induced AKT activation, we stably expressed the p53R280K and p53R175H mutants in p53-null H1299 cells. As shown in Figure 20C, mutant p53 knock-in cells displayed stronger and prolonged insulin-induced AKT activation. No relevant changes were detected in INSR or IGF1R mRNA (Figure 20D), thus excluding an effect on receptor levels. We conclude that mutant p53 establishes a pro-oncogenic response to insulin by specifically favoring or enhancing the activation of AKT.

5.3 Mutant p53 enhances insulin-induced AKT activation by inhibiting DAB2IP

We previously discovered that mutant p53 proteins can bind the tumor suppressor DAB2IP in the cytoplasm, interfering with its functions (Di Minin et al., 2014). Since DAB2IP can modulate both PI3K and AKT (Xie et al., 2009), we hypothesized that the mutant p53-DAB2IP interaction may also affect the response to insulin. We therefore tested if depletion of DAB2IP could rescue the effects of mutant p53 knockdown in our cell models. Indeed, silencing of DAB2IP fully restored insulin-induced proliferation and invasion in mutant p53-depleted MDA-MB231 cells (Figure 21A). Similarly, depletion of DAB2IP restored the kinetics of insulin-induced AKT phosphorylation after mutant p53 knockdown (Figure 21B and 21C). Notably, knockdown of DAB2IP had a negligible impact on insulin-induced phenotypes in the presence of mutp53, thus confirming the epistatic relationship between the two proteins. In the same experimental settings, DAB2IP overexpression reduced insulin-dependent AKT phosphorylation (Figure 21D), and fully abolished the increase in proliferation and invasion triggered by insulin (Figure 21E).

If the effects of mutant p53 are exerted by binding and inhibiting DAB2IP, they should be independent of its nuclear functions. Accordingly, overexpression of a cytoplasmic variant of mutant p53 (p53R280K ΔNLS) (Di Minin et al., 2014) fully restored insulin-induced proliferation and invasion in MDA-MB231 cells previously depleted of endogenous nuclear mutant p53 (Figure 22A), or stably overexpressing DAB2IP (Figure 22B).

The functional impact of the mutant p53/DAB2IP axis was also confirmed in HBL-100, a triple negative breast cancer cell line with wild-type (wt) p53. In these cells, DAB2IP knockdown significantly increased insulin-induced proliferation and invasion (Figure 23A), correlating with enhanced AKT activation (Figure 23B), confirming its modulatory role on this pathway.
Intriguingly, also depletion of wild-type p53 augmented insulin-induced AKT phosphorylation (Figure 23C), as well as proliferation and invasion in HBL-100 cells (Figure 23D), suggesting that loss of wild-type p53 can promote this signaling axis.

Nonetheless, expression of mutant p53 proteins (p53R175H or p53R280K) in HBL-100 cells had a much stronger effect than wild-type p53 depletion (Figure 24) on insulin-induced oncogenic responses, thus formally confirming a proper mutant p53 gain of function. Most importantly, the same was observed with expression of nuclear-excluded p53 mutants (Figure 24), confirming that this phenotype does not involve transcriptional activities of mutp53.

5.4 Cytosolic wild type p53 can interact with DAB2IP

The observation that wild-type p53 depletion enhances insulin-induced responses in HBL-100 cells (Figure 23) raised the question as to whether this effect might also involve DAB2IP. In fact, our group previously reported that DAB2IP can interact with wild-type p53 when co-transfected in recipient cells (Lunardi et al., 2010). To address this point, we first evaluated if a cytosolic version of wild type p53 could promote insulin signaling, thus acting to some extent as a "mutant p53". This also because in tumors there are occasional C-terminal mutations that are predicted to translate a truncated p53 protein lacking the NLS sequences.
We thus generated an expression vector encoding wtp53 ΔNLS (Delta-NLS), and transfected this construct in p53-null H1299 cells. Interestingly, wtp53 ΔNLS appreciably increased insulin-induced AKT activation, although not as efficiently as cytoplasmic mutant p53 R280K ΔNLS. However, cytoplasmic wtp53 did not affect insulin-induced phosphorylation of GSK3β, which instead was strongly enhanced by cytoplasmic mutant p53 (Figure 25A).
Moreover, we found that wtp53 ΔNLS often bound with less efficiency/stability than mutp53 ΔNLS, despite comparable expression levels and cellular localization (Figure 25B and C).
Thus, it appears that mutant p53 might have a higher affinity for DAB2IP than wild-type p53, although this kind of simple co-IP experiments cannot formally prove it.
Based on these preliminary results, we conclude that high levels of cytoplasmic wtp53 can promote a transient insulin-induced AKT activation, possibly by interacting with DAB2IP.

5.5 Disruption of the mutant p53-DAB2IP interaction reduces the aggressive behavior of cancer cells exposed to insulin

DAB2IP can bind both PI3K-p85α and AKT1 to negatively regulate AKT activation (19). On this premise, we decided to test two hypotheses: i) that DAB2IP might also bind AKT2; ii) that mutant p53 might stimulate insulin-induced AKT activation by interfering with DAB2IP-PI3K and DAB2IP-AKT interactions.

To this aim, we performed transient overexpression experiments: we found that increasing amounts of mutant p53 caused a progressive reduction in the fraction of DAB2IP that could be co-immunoprecipitated with PI3K, AKT1, and AKT2 (Figure 26 A,B and C). Analyzing endogenous proteins, we observed that depletion of mutant p53 in MDA-MB-231 cells increased the fraction of DAB2IP co-immunoprecipitated with AKT1 (Figure 26D), further supporting the notion that mutant p53 binds and inhibits DAB2IP in this cell line.

We previously found that mutant p53 binds to the N-terminus of DAB2IP, and we demonstrated that a chimeric decoy protein in which the first 186 amino acids of DAB2IP are fused to GFP (eGFP-KA2) can disrupt the mutant p53-DAB2IP interaction, restoring endogenous DAB2IP functions in cancer cells (Di Minin et al., 2014). We therefore repeated proliferation and invasion experiments using MDA-MB231 cells stably expressing the eGFP-KA2 protein. The eGFP-KA2 decoy had no obvious effects on basal cell proliferation and motility, but clearly abolished the increase in proliferation and invasion triggered by insulin (Figure 27A), also significantly reducing AKT activation (Figure 27B). Expression of eGFP-KA2 did not influence INSR or IGF1R transcription (Figure 27C), so its effect is not linked to reduced receptor levels. Importantly, depletion of DAB2IP rendered the eGFP-KA2 construct ineffective, demonstrating that the inhibitory action of the KA2 peptide is strictly dependent on DAB2IP action (Figure 27D).

Together, these data provide a mechanism by which mutant p53 proteins, blocking DAB2IP, can enhance insulin-induced AKT activation in cancer cells, potentially determining a more aggressive behavior under hyperinsulinemic conditions (Figure 28).

5.6 Increased AKT activation correlates with p53 mutation in breast cancers from obese patients

The above results predict that hormone-independent tumors with mutant p53 should have stronger or prolonged AKT activation under conditions of hyperinsulinemia. To investigate such potential association, we analyzed triple-negative breast cancers from obese and non-obese patients, assuming an obesity-linked hyperinsulinemia in clinically overweight (BMI >30) subjects (Rothman, 2008). The presence of potential p53 gain-of-function mutations was inferred by strong p53 staining in immunohistochemistry, while AKT activation was determined by immunoreactivity to a phospho-AKT (S473) antibody. In line with previous reports (Tan et al., 2015)(Muller et al., 2009), we confirmed a general correlation between p-AKT and mutant p53. In addition, we found a strong correlation between elevated p53 staining (i.e. p53 mutation) and high levels of phospho-AKT specifically in obese patients (Figure 29). These observations are consistent with our model, and support the notion that p53 mutation may amplify AKT activation in cancers that develop under obesity-linked conditions. Moreover, we did not evaluate INSR expression in these samples. Considering that wtp53 represses INSR promoter, affecting its expression in breast cancer cells (Webster et al., 1996), we can not exclude an increase in INSR expression due to the loss of wtp53 function.

The described results have been recently published (E. Valentino, A. Bellazzo, G. Di Minin, D.Sicari, M. Apollonio, G. Scognamiglio, M. Di Bonito, G. Botti, G. Del Sal, L. Collavin. " Mutant p53 potentiates the oncogenic effects of insulin by inhibiting the tumor suppressor DAB2IP ". PNAS, 2017, vol 114, 7623-7628.

Figure 13

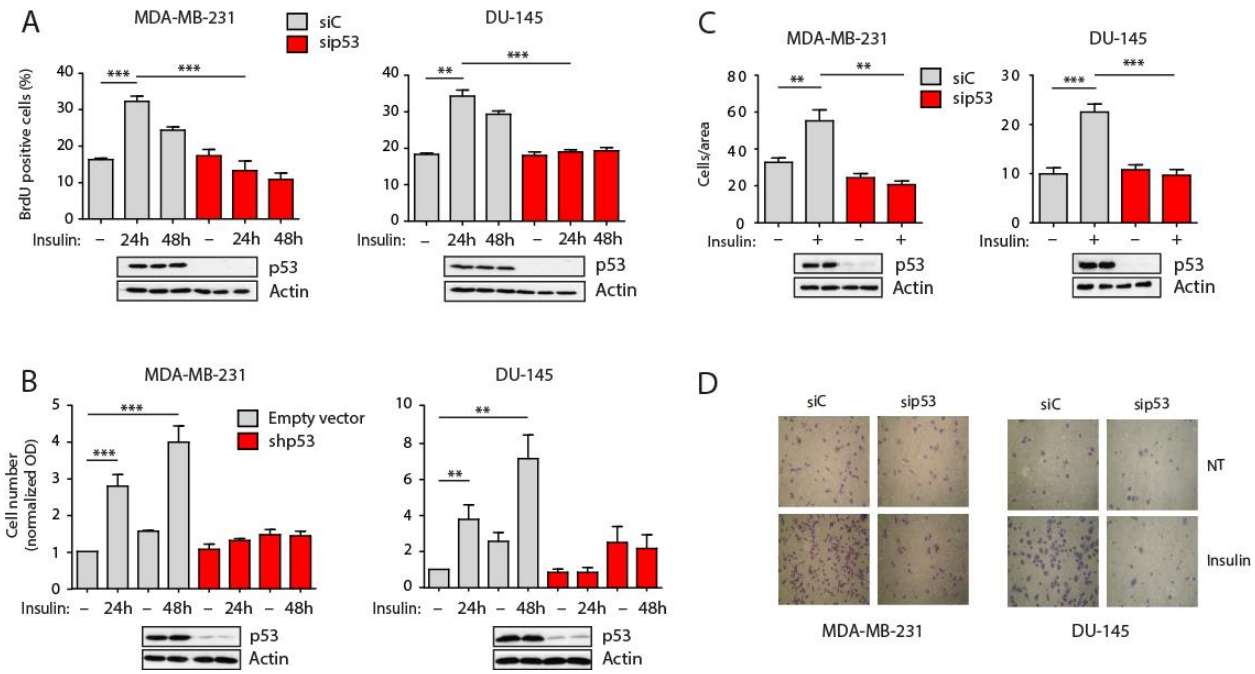


Figure 13: Mutant p53 dictates an oncogenic response to insulin in hormone-independent cancer cells.

A-B) Mutant p53 is required for insulin-induced proliferation in cancer cells. A) MDA-MB231 and DU145 cells were transfected with indicated siRNAs for 48 hours, serum starved, treated with 0.5 ug/ml insulin for 24 or 48 hours, and labeled with BrdU for 2 hours. Graphs summarize the percentage of BrdU positive nuclei (mean ± SEM; n=3; ** P<0.01; *** P<0.001). Depletion of endogenous p53 was checked by western blot. B) MDA-MB231 and DU145 cells stably silenced for endogenous mutant p53 (shp53) were serum starved, treated with 0.5 ug/ml insulin for 24 or 48 hours, and then colored with Crystal Violet. Graphs summarize normalized optical density at 570 nm (mean ± SEM; n=3; ** P<0.01, *** P<0.001). Depletion of endogenous p53 was checked by western blot.

C-D) Mutant p53 is required for insulin-induced invasion. Cells were transfected with indicated siRNAs for 48h, serum starved for 24h, and treated with insulin (0.5 ug/ml) for additional 24h. Invasion assays were performed in low serum plus 0.5 ug/ml insulin. C) Graphs summarize migrated cells per area (mean ± SEM; n=3; ** P<0.01, *** P<0.001). Depletion of endogenous p53 was checked by western blot. D) Representative images of migrated cells from C, fixed and stained with Crystal Violet.

Figure 14

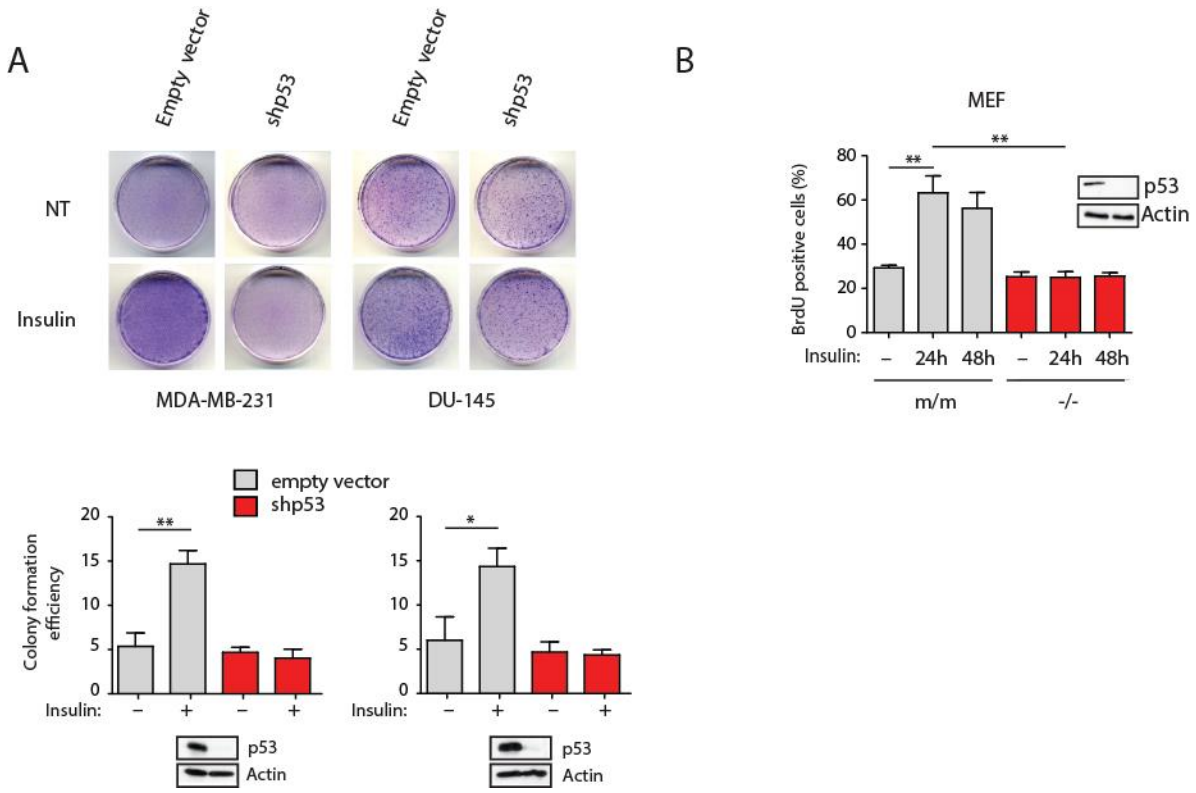


Figure 14: Mutant p53 depletion reduces insulin-induced cell proliferation.

A) Mutp53 sustains insulin-induced cell proliferation. MDA-MB231 and DU145 cells stably silenced for endogenous mutant p53 (shp53) were plated at low density and treated with 0.5 ug/ml insulin every 48 hours for 10 days. Petri dishes were photographed after Giemsa staining (representative pictures are shown), and colony formation efficiency was quantified using ImageJ (mean ± SEM; n=3; * P<0,1 ; ** P<0.01).

B) Mutp53 drives insulin-induced proliferation in Ras-transformed mouse embryo fibroblasts (MEF). MEFs derived from p53-null (-/-) or p53R172H knock-in (m/m) mice were treated and analyzed as in Fig. 1A (mean ± SEM; n=3; ** P<0.01). Expression of p53 was checked by western blot.

Figure 15

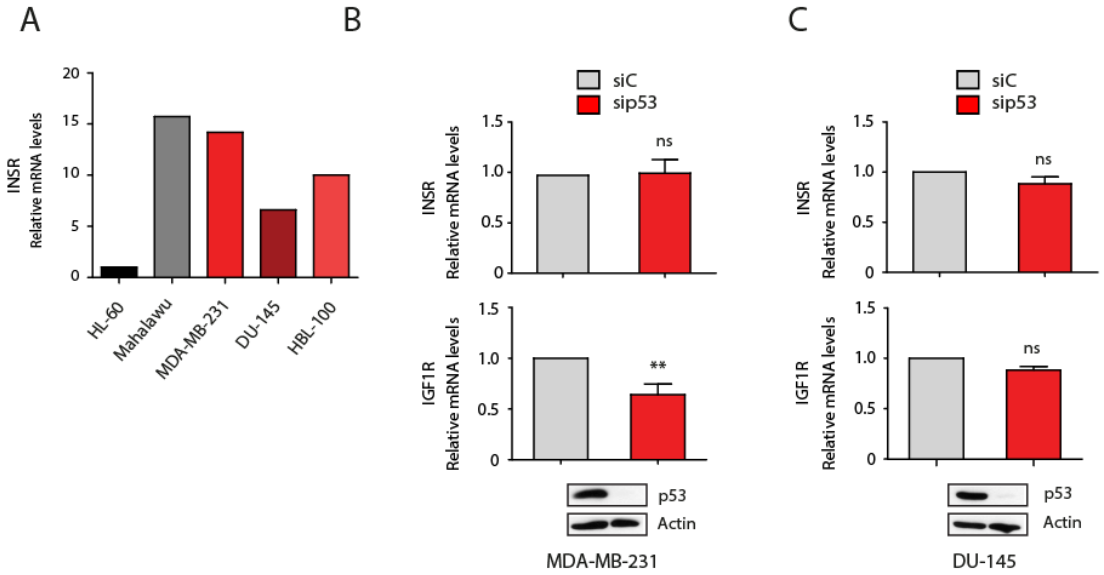


Figure 15: Mutant p53 depletion reduces insulin-induced cell proliferation without affecting expression of insulin receptors.

A) Expression of INSR in various human cancer cell lines. Expression of INSR A/B was measured by RT-qPCR (n=1) in the indicated cell lines. HL-60 and Malahawu cell lines were analyzed respectively as negative (low INSR expression) and positive (high INSR expression) controls.

B-C) Effects of mutant p53 depletion on expression of insulin receptors. MDA-MB-231 and DU-145 cells were transfected with indicated siRNAs. Expression of INSR A/B and IGF1R was measured by RT-qPCR (mean ± SEM; n = 3; ** P<0.01). Immunoblotting confirmed depletion of endogenous mutant p53.

Figure 16

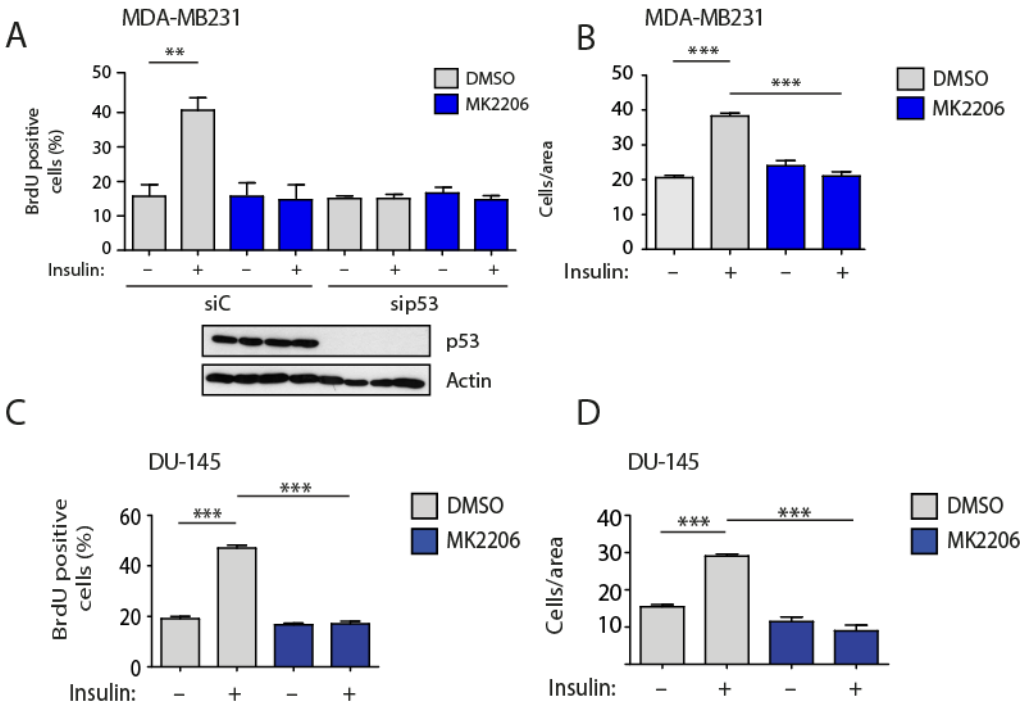


Figure 16: Mutant p53 increases insulin-induced proliferation and migration through AKT activation.

A-B) Insulin-induced cell proliferation and invasion require AKT activity. A) MDA-MB231 cells were transfected with indicated siRNAs for 48 hours, serum starved, and treated with insulin (0.5 ug/ml) for 24 hours, with or without the specific AKT inhibitor MK2206 (5 uM). B) Cells were serum starved and treated with insulin (0.5 ug/ml) for 24 hours, with or without the specific AKT inhibitor MK2206 (5 uM). Proliferation and invasion assays were performed as in Figure 13 (mean ± SEM; n=3; **P<0.01, ***P<0.001). C-D) Insulin-induced cell proliferation and invasion require AKT activity. DU-145 cells were serum starved and treated with insulin (0.5 ug/ml) for 24 hours, with or without the specific AKT inhibitor MK2206 (5 uM). Proliferation and invasion assays were performed as in Figure 1 (mean ± SEM; n=3; ***P<0.001).

Figure 17

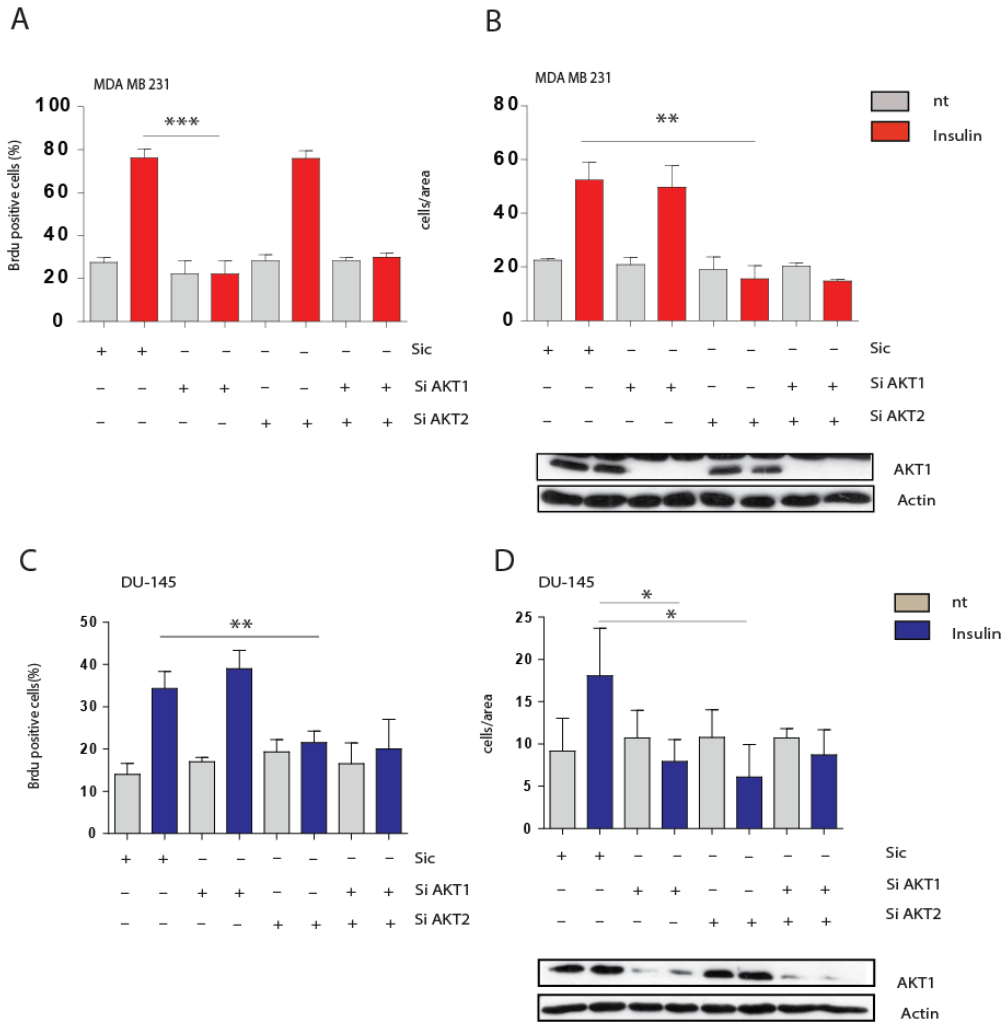


Figure 17: Insulin-induced cell proliferation and invasion can be driven by different AKT family members.

A and C) Insulin-induced cell proliferation. MDA-MB231 and DU-145 cells were treated with indicated siRNA for 48h, serum starved for 24h, treated with insulin (0.5 ug/ml) for additional 24h and labeled with BrdU for 2 hours. Graphs summarize the percentage of BrdU positive nuclei (mean +/- SD; n=3; *** P<0.001). B and D) Insulin-induced matrigel invasion. MDA-MB231 and DU-145 cells were treated with indicated siRNA for 48h, serum starved for 24h, and treated with insulin (0.5ug/ml) for additional 24h. Invasion assays were performed in low serum plus 0.5 ug/ml insulin. Graphs summarize migrated cells per area (mean +/- SD; n=3; ** P<0.01). Depletion of endogenous AKT1 and AKT2 was checked by western blot and RT-qPCR analysis (not shown).

Figure 18

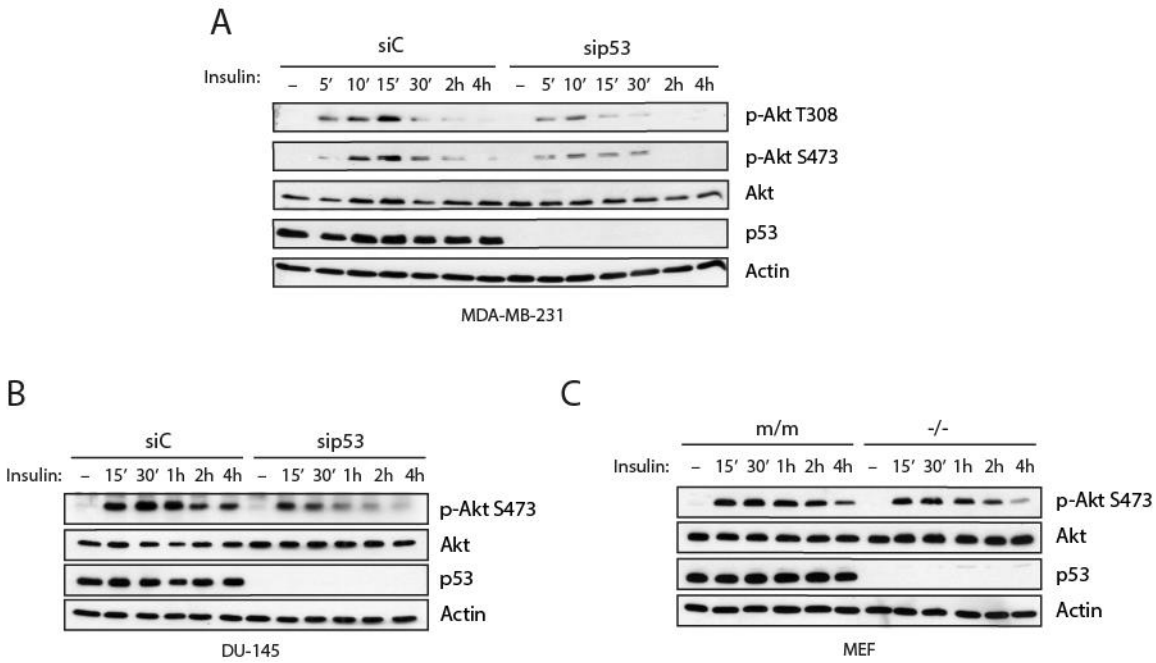


Figure 18: Mutant p53 increases AKT activation.

A) Depletion of mutant p53 reduces insulin-induced AKT activation in cancer cells. MDA-MB231 cells were transfected with indicated siRNAs for 48 hours, serum starved for 24 h, and treated with insulin (0.5 ug/ml) for the indicated times. Phosphorylated (p-T308 and p-S473) and total AKT1 were detected by immunoblotting. p53 was blotted to control knockdown efficiency, with Actin as a loading control.

p-AKT S473 is taken from a different gel, loaded in parallel with the same amount of lysate.

B) Depletion of mutant p53 reduces insulin-induced AKT activation in DU-145. Cells were transfected with control or p53 siRNA for 48 hours, serum starved for 24 h, and treated with insulin (0.5 ug/ml) for the indicated times. Phosphorylated and total AKT1 were detected by immunoblotting. p53 was blotted to verify knockdown efficiency, with Actin as a loading control.

C) Insulin-induced AKT activation is enhanced in mutant p53 knock-in MEFs. Ras-transformed mouse embryo fibroblasts derived from p53-null (-/-) or p53R172H knock-in (m/m) mice were used to analyze Insulin-induced AKT (p-S473) activation. Phosphorylated and total AKT1 were detected by immunoblotting. p53 was blotted to verify knock-in efficiency, with Actin as a loading control.

Figure 19

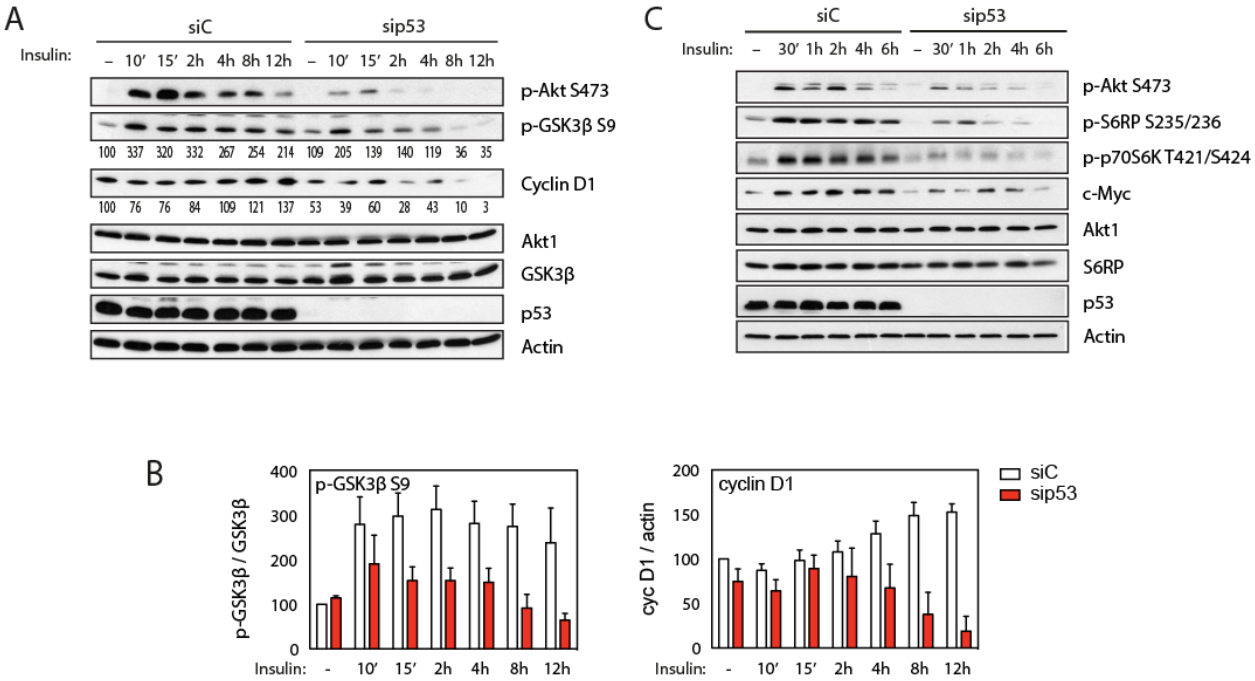


Figure 19: Mutant p53 increases AKT pathway activation.

A) Depletion of mutant p53 affects prolonged AKT-dependent responses to insulin. MDA-MB231 cells were treated as in Figure 18A. Phosphorylated and total AKT1, GSK3β and CyclinD1 were detected by immunoblotting as above. p53 was blotted to control knockdown efficiency, with Actin as a loading control. Total AKT is taken from a different gel, loaded in parallel with the same amount of lysate.

B) Depletion of mutant p53 reduces insulin-induced phosphorylation of GSK3β and accumulation of cyclin D1. Graphs summarize relative p-GSK3β S9/GSK3β and CycD1/actin ratios in MDA-MB231 cells treated with insulin for the indicated times (representative blots are in A), as measured by densitometry on autoradiography film (average ± SEM, n=3). C) Depletion of mutp53 affects insulin-induced AKT-dependent molecular responses. MDA-MB-231 cells were treated as in Figure 18A. Phosphorylated and total Akt1, S6RP, p70S6K and c-Myc were detected by immunoblotting. p53 was blotted to control knockdown efficiency, with Actin as a loading marker.

Figure 20

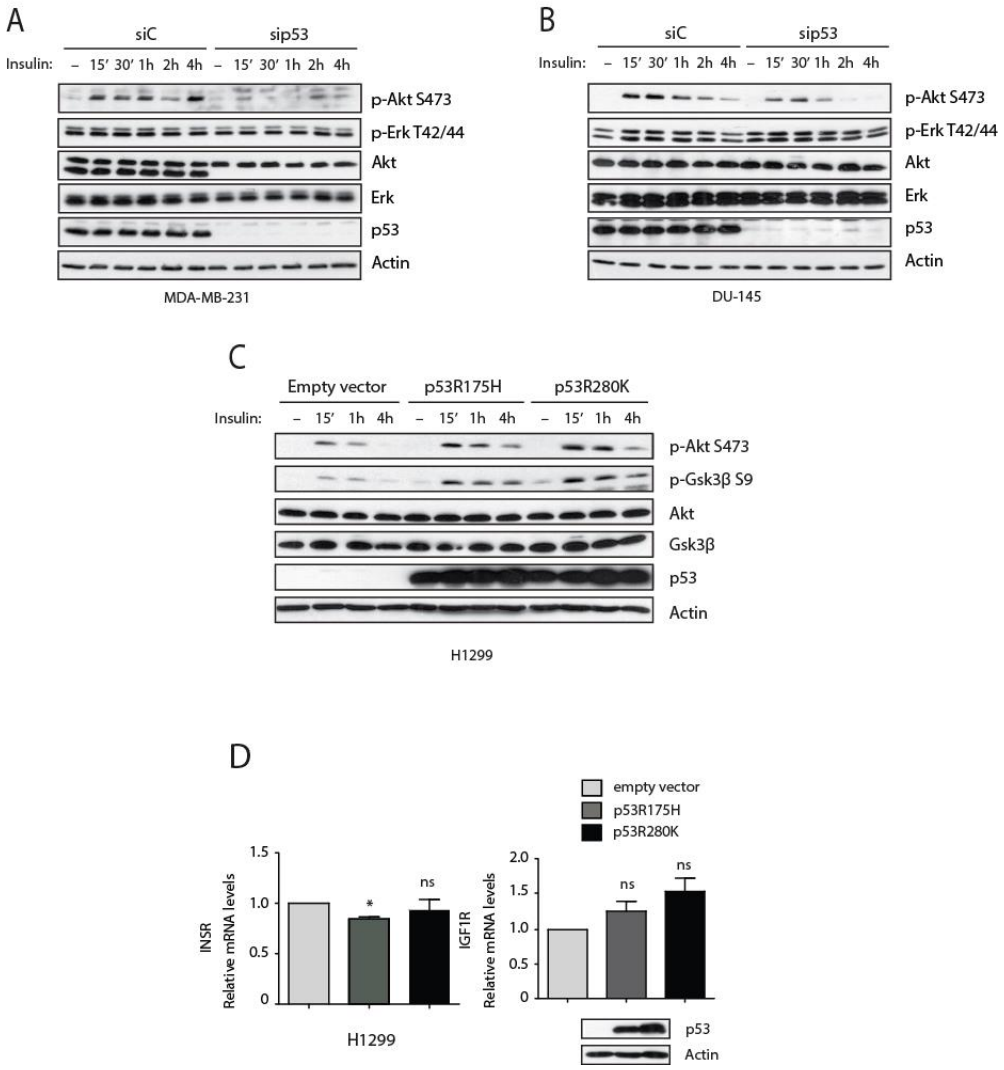


Figure 20: Mutant p53 increases AKT pathway activation without affecting INSR expression.

A-B) Depletion of mutant p53 has no detectable effects on MAPK activation in insulin-treated cancer cells. MBA-MD231 and DU-145 cells were treated as in figure 18A. Phosphorylated and total AKT1 and ERK were detected by immunoblotting. p53 was blotted to verify knockdown efficiency, with Actin as a loading control.C) Mutant p53 enhances insulin-induced AKT activation. H1299 cells (p53 null) were infected with retroviruses encoding the indicated p53 mutants. Cells were treated with insulin (5ug/ml) for the indicated time. Phosphorylated and total AKT1 and GSK3β were detected by immunoblotting. p53 was blotted to verify expression levels, with Actin as a loading control. D) Effects of mutant p53 overexpression on INSR and IGF1R mRNA levels. H1299 p53-null cells were infected with retroviruses expressing the indicated mutant p53 proteins. Expression levels of INSR A/B and IGF1R were measured by RT-qPCR (mean ± SEM; n = 3; * P<0,1). Immunoblotting confirmed expression of exogenous mutant p53 proteins.

Figure 21

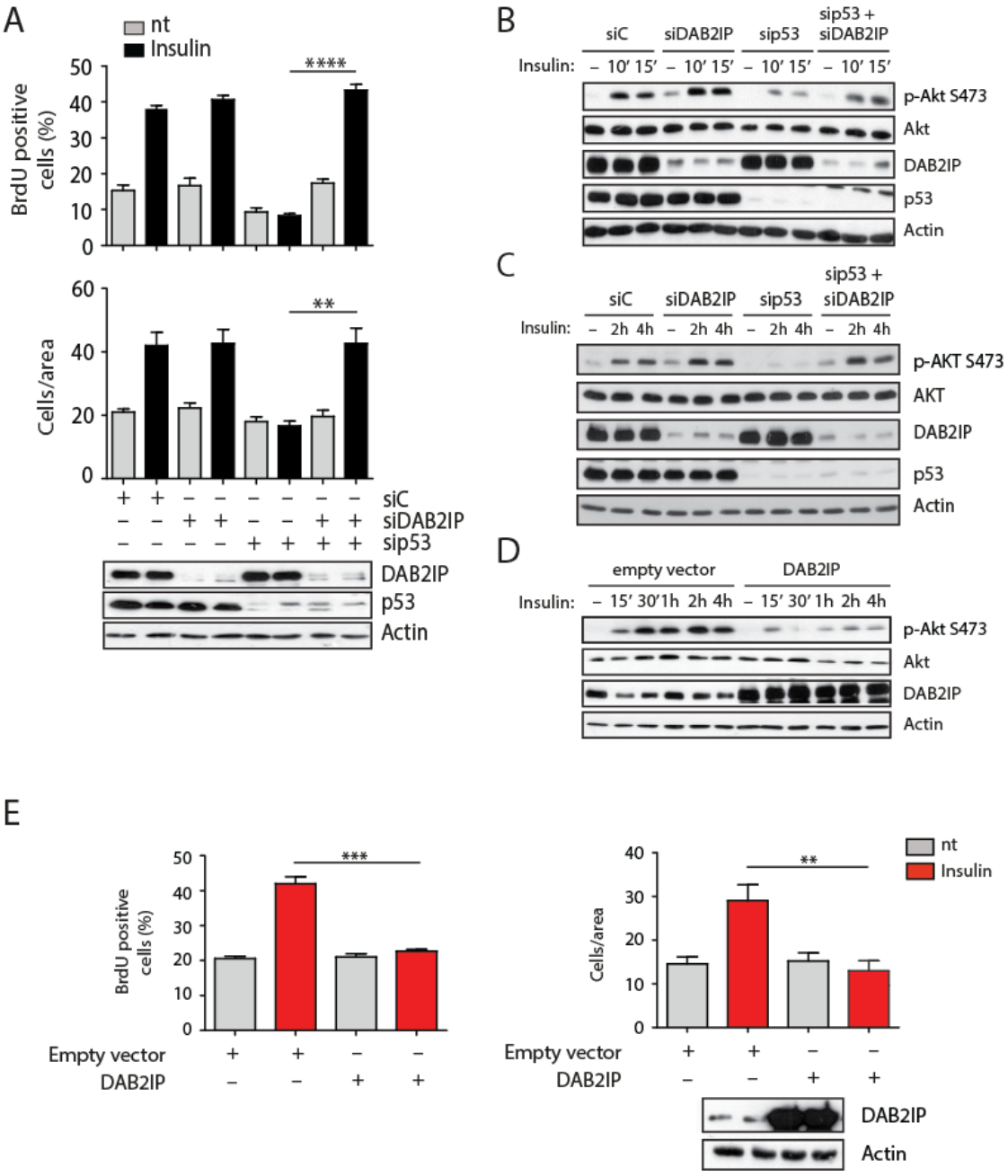

A
nt
Insulin
BrdU positive cells (%)
****
Cells/area
**
siC
siDAB2IP
sip53
DAB2IP
p53
Actin
B
siC
siDAB2IP
sip53
sip53 + siDAB2IP
Insulin: – 10′ 15′ – 10′ 15′ – 10′ 15′ – 10′ 15′
p-Akt S473
Akt
DAB2IP
p53
Actin
C
siC
siDAB2IP
sip53
sip53 + siDAB2IP
Insulin: – 2h 4h – 2h 4h – 2h 4h – 2h 4h
p-AKT S473
AKT
DAB2IP
p53
Actin
D
empty vector
DAB2IP
Insulin: – 15′ 30′1h 2h 4h – 15′ 30′ 1h 2h 4h
p-Akt S473
Akt
DAB2IP
Actin
E
BrdU positive cells (%)
***
Empty vector
DAB2IP
Cells/area
**
nt
Insulin
Empty vector
DAB2IP
DAB2IP
Actin

Figure 21: Functional interaction between mutant p53 and DAB2IP in the response of cancer cells to insulin.

A) Epistasis between mutant p53 and DAB2IP in insulin-induced proliferation and invasion. MDA-MB231 cells were silenced for mutant p53 and/or DAB2IP for 48 hours, and treated with 0.5 ug/ml insulin for 24h. Proliferation (top) and invasion (bottom) assays were performed as in Figure 13 (mean ± SEM; n=3; ** P<0.01; **** P<0.0001).

B and C) Epistasis between mutant p53 and DAB2IP in insulin-induced AKT activation. MDA-MB231 cells were silenced for mutant p53 and/or DAB2IP for 48h, serum starved for 24h, and treated with insulin (0.5 ug/ml) for the indicated times. Phosphorylated and total AKT1 were detected by immunoblotting.

D) DAB2IP overexpression reduces insulin-induced AKT activation in mutant p53 cancer cells. MDA-MB231 cells stably transduced with a retrovirus expressing DAB2IP were serum starved and treated with insulin (0.5 ug/ml) for the indicated times. Phosphorylated and total AKT1 were detected by immunoblotting. Total AKT is taken from a different gel, loaded in parallel with the same amount of lysate. E) DAB2IP overexpression reduces insulin-dependent proliferation and invasion in mutant p53 cancer cells. MDA-MB-231 cells stably transduced with a DAB2IP retrovirus were serum starved and treated with insulin (0.5 ug/ml) for 24 hours. Proliferation (left) and invasion (right) assays were performed as in Figure 13 (mean ± SEM; n=3; ** P<0.01; *** P<0.001). Expression of endogenous and exogenous DAB2IP was checked by western blot.

Figure 22

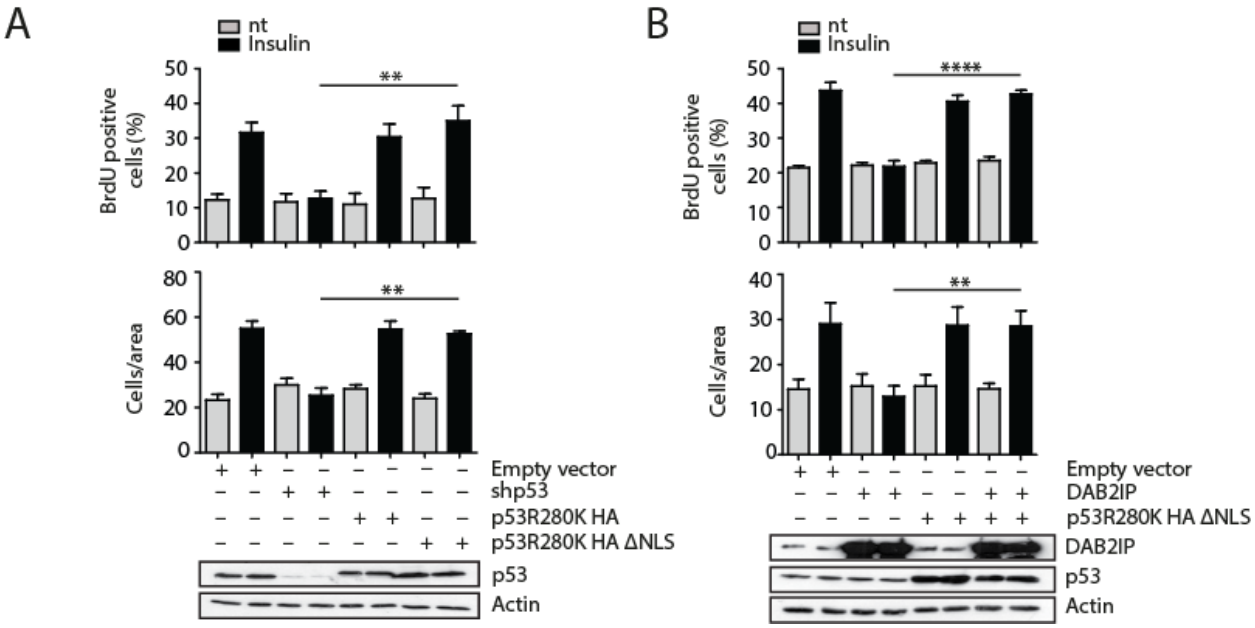


Figure 22: A cytoplasmic mutant p53 drives the oncogenic response to insulin.

A-B) Insulin-induced proliferation and invasion are mediated by cytoplasmic mutant p53. A) MDA-MDA-MB231 cells stably silenced for endogenous mutant p53 (shp53) were infected with retroviruses encoding shRNA-resistant versions of p53(R280K) or its cytoplasmic variant p53(R280K)ΔNLS. Proliferation (top) and invasion (bottom) assays were performed as in Figure 13 (mean ± SEM; n=3; ** P<0.01). B) MDA-MB231 cells stably overexpressing DAB2IP were infected with a retrovirus expressing HA-p53(R280K)ΔNLS or an empty retrovirus as a control. Proliferation (top) and invasion (bottom) assays were performed as in Figure 13 (mean ± SEM; n=3; ** P<0.01, **** P<0.0001).

Figure 23

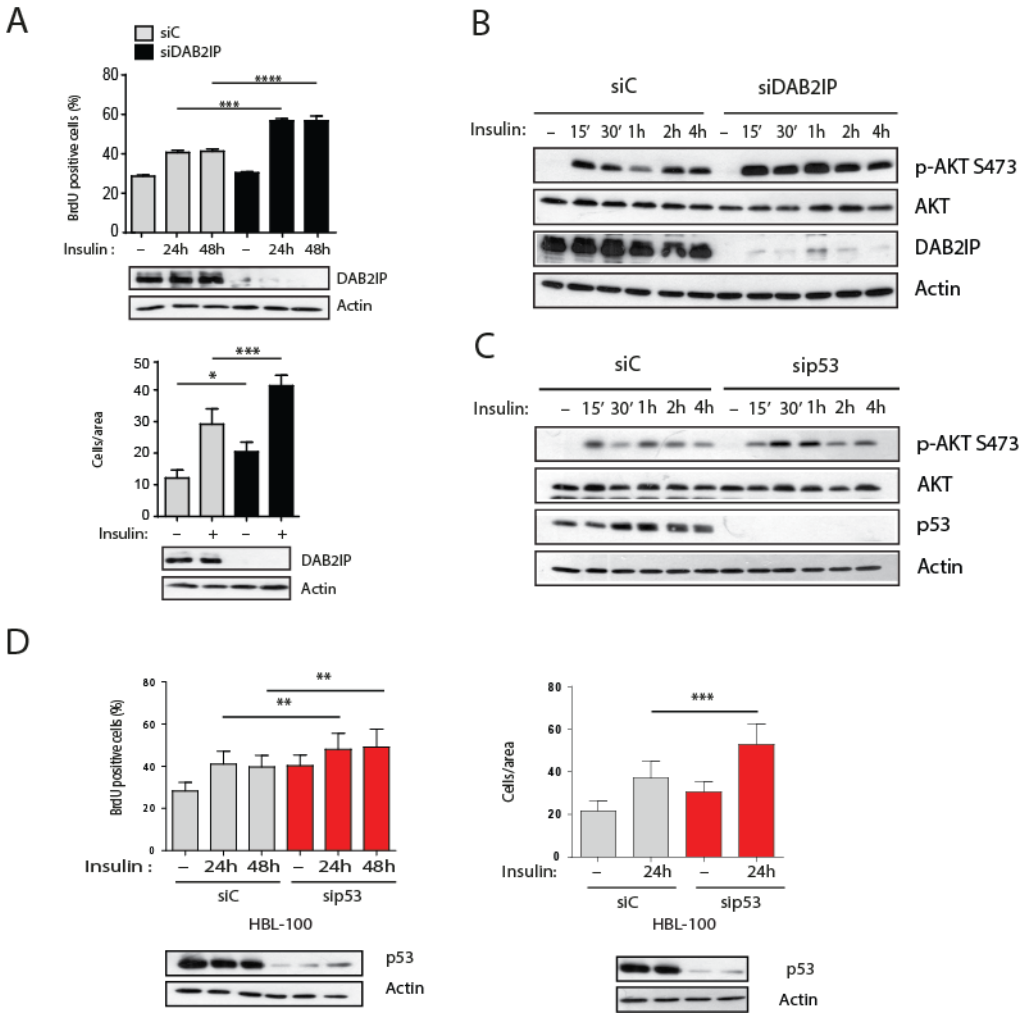

A
siC
siDAB2IP
BrdU positive cells (%)
Insulin : – 24h 48h – 24h 48h
DAB2IP
Actin
Cells/area
Insulin: – + – +
B
siC
siDAB2IP
Insulin: – 15′ 30′ 1h 2h 4h – 15′ 30′ 1h 2h 4h
p-AKT S473
AKT
DAB2IP
Actin
C
siC
sip53
Insulin: – 15′ 30′ 1h 2h 4h – 15′ 30′ 1h 2h 4h
p-AKT S473
AKT
p53
Actin
D
BrdU positive cells (%)
Insulin : – 24h 48h – 24h 48h
siC
sip53
HBL-100
p53
Actin
Cells/area
Insulin: – 24h – 24h
siC
sip53
HBL-100
p53
Actin

Figure 23: Functional interaction between p53 and DAB2IP in the response to insulin of breast cancer cells with wild-type p53.

A) DAB2IP knockdown enhances insulin-induced proliferation and migration in wt p53 cancer cells. HBL-100 were transfected with indicated siRNAs for 48 hours. Proliferation (top) and invasion (bottom) assays were performed as in Figure 13 (mean ± SEM; n=3; *P<0.1 ; *** P<0.001; ****P< 0.0001). Efficiency of endogenous DAB2IP depletion was checked by western blot.

B) DAB2IP knockdown enhances insulin-induced AKT activation in wt p53 cancer cells. HBL-100 cells were transfected with control or DAB2IP siRNA for 48 hours, serum starved for 24 h, and treated with insulin (0.5 ug/ml) for the indicated times. Phosphorylated (p-S473) and total AKT1 were detected by immunoblotting. DAB2IP was blotted to verify knockdown efficiency, with Actin as a loading control.

C) wt p53 depletion enhances insulin-induced AKT activation in HBL-100. Cells were transfected with control or p53 siRNA for 48 hours, serum starved for 24 h, and treated with insulin (0.5 ug/ml) for the indicated times. Phosphorylated (p-S473) and total AKT1 were detected by immunoblotting. p53 was blotted to verify knockdown efficiency, with Actin as a loading control.

D) wt p53 depletion promotes insulin-induced proliferation and migration in HBL-100. Cells were transfected with control or p53 siRNA for 48 hours, serum starved for 24 h, and treated with insulin (0.5 ug/ml) for the indicated times. Proliferation (left) and invasion (right) assays were performed as in Figure 13 (mean ± SEM; n=3; ** P<0.01; *** P<0.001; ****P<0.0001). Expression of endogenous and exogenous p53 proteins was verified by immunoblotting, with Actin as a loading control.

Figure 24

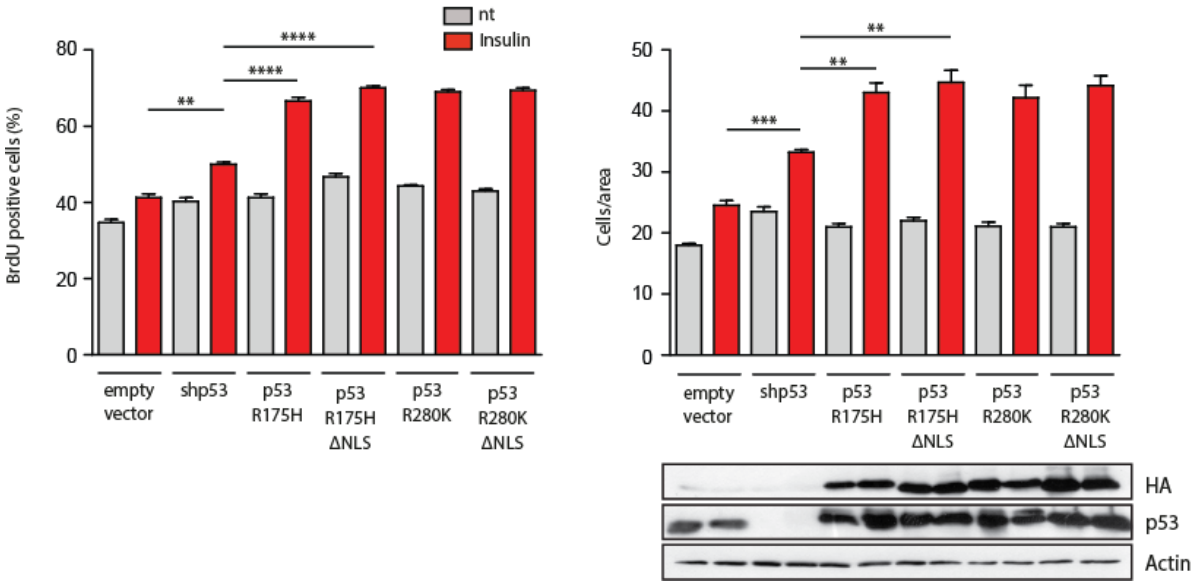


Figure 24: Mutant p53 dominantly enhances insulin-induced proliferation and migration in wt p53 cancer cells via a nuclear-independent activity.

HBL-100 cells were infected with retroviruses encoding p53(R175H) and p53(R280K), and their respective cytoplasmic variants (ΔNLS). As a control, cells were infected with a retrovirus expressing a p53-specific shRNA. Proliferation (left) and invasion (right) assays were performed as in Figure 13 (mean ± SEM; n=3; ** P<0.01; *** P<0.001; ****P<0.0001). Expression of endogenous and exogenous p53 proteins was verified by immunoblotting, with Actin as a loading control.

Figure 25

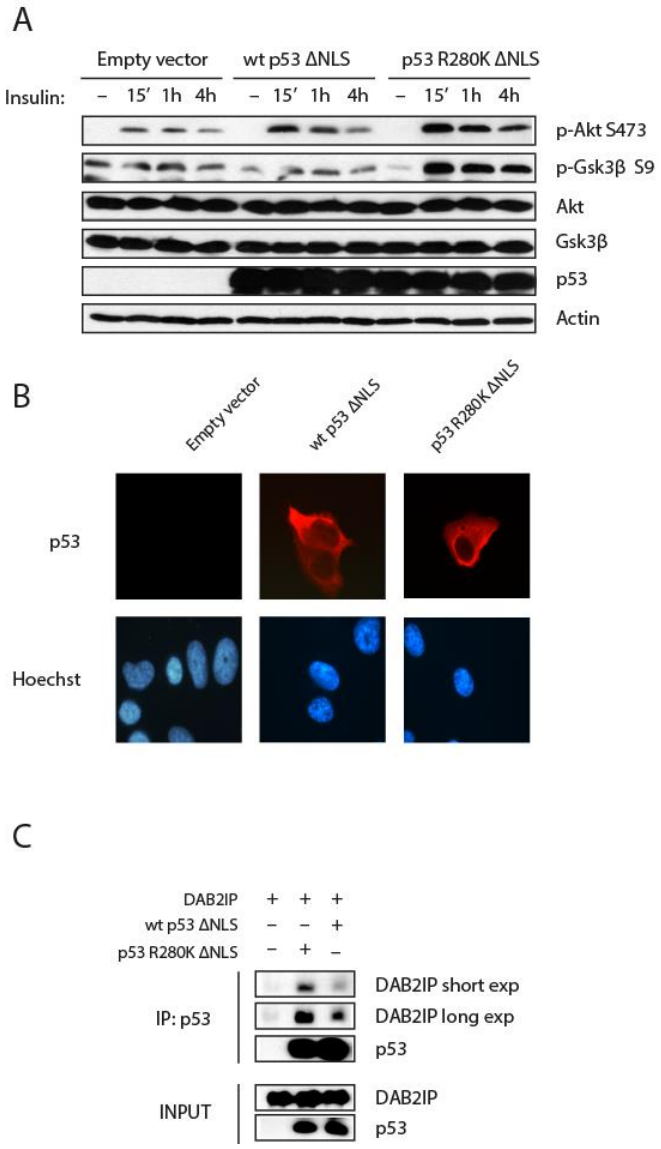


Figure 25: Effects of cytoplasmic wild-type p53 on insulin-induced AKT activation.

A) H1299 (p53-null) cells were transfected with expression vectors encoding nuclear-excluded (ΔNLS) variants of wild-type and mutant p53 as indicated. After starvation, cells were treated with insulin (5ug/ml) for the indicated times. Phosphorylated and total AKT1 and GSK3β were detected by immunoblotting. Exogenous p53 was blotted to verify expression levels. Actin provided a loading control. B) Cytoplasmic localization of nuclear-excluded wt and mutant p53 (red) in transfected H1299 cells was confirmed by immunofluorescence. DNA was counterstained with Hoechst 33342. C) H1299 cells were co-transfected with the indicated expression plasmids. The fraction of DAB2IP bound to different p53 variants was analyzed by western blot of p53 immunoprecipitates.

Figure 26

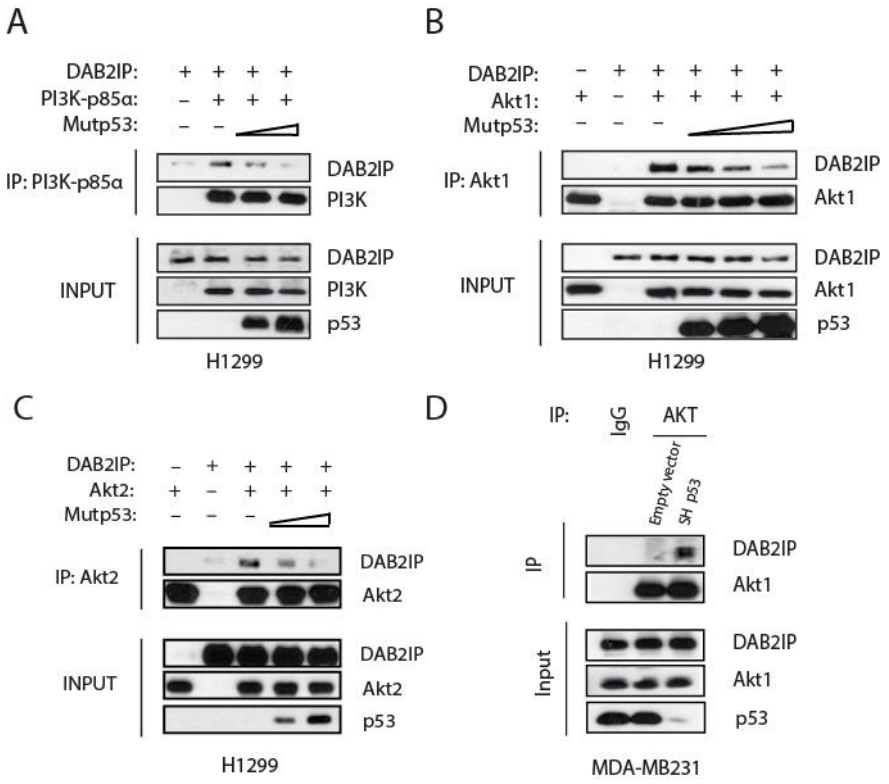


Figure 26: Mutant p53 hinders the interaction of DAB2IP with PI3K-p85α and AKTs.

A-B-C) Mutant p53 displaces the inhibitory binding of DAB2IP with PI3K-p85α and AKTs. A) H1299 cells were co-transfected with plasmids expressing DAB2IP, FLAG-PI3K-p85α and increasing amounts of p53 (R280K). The fraction of DAB2IP bound to PI3K was analyzed by western blot of PI3K immunoprecipitates. B) H1299 cells were co-transfected with plasmids expressing DAB2IP, HA-AKT1, and increasing amounts of p53 (R280K). The fraction of DAB2IP bound to AKT1 was analyzed by western blot of AKT immunoprecipitates. C) H1299 cells were co-transfected with plasmids expressing DAB2IP, HA-AKT2, and increasing amounts of p53 (R280K). The fraction of DAB2IP bound to AKT2 was analyzed by western blot of AKT immunoprecipitates. D) MDA-MB231 cells were stably silenced for endogenous mutant p53 (shp53); endogenous DAB2IP bound to AKT1 was analyzed by co-immunoprecipitation with anti-AKT1 antibody.

Figure 27

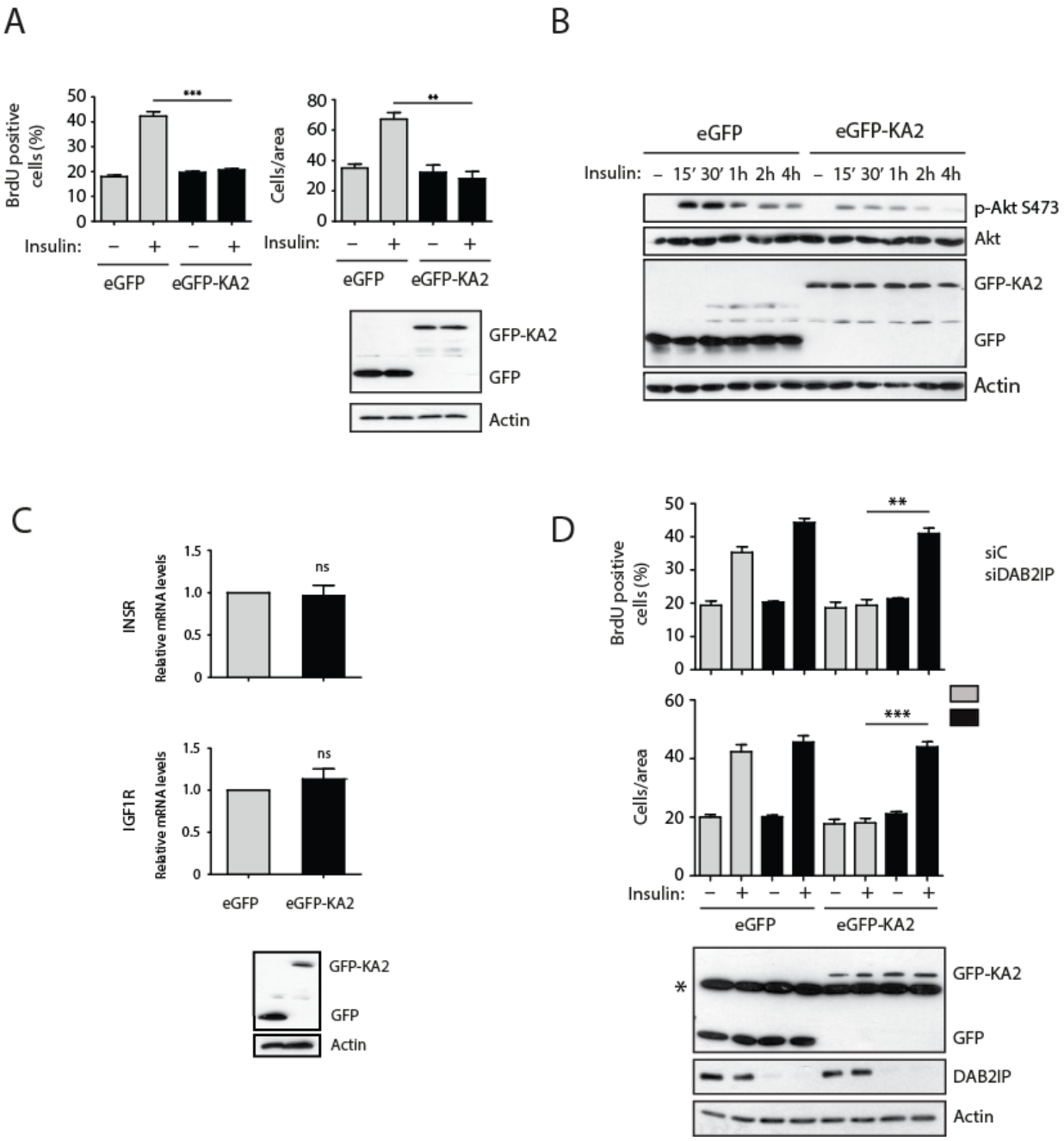

A
BrdU positive cells (%)
50
40
30
20
10
0
***
Insulin: – + – +
eGFP
eGFP-KA2
Cells/area
80
60
40
20
0
**
Insulin: – + – +
eGFP
eGFP-KA2
GFP-KA2
GFP
Actin
B
eGFP
eGFP-KA2
Insulin: – 15′ 30′ 1h 2h 4h – 15′ 30′ 1h 2h 4h
p-Akt S473
Akt
GFP-KA2
GFP
Actin
C
INSR
Relative mRNA levels
1.5
1.0
0.5
0
ns
IGF1R
Relative mRNA levels
1.5
1.0
0.5
0
ns
eGFP
eGFP-KA2
GFP-KA2
GFP
Actin
D
BrdU positive cells (%)
50
40
30
20
10
0
**
siC
siDAB2IP
Cells/area
60
40
20
0
***
Insulin: – + – + – + – +
eGFP
eGFP-KA2
*
GFP-KA2
GFP
DAB2IP
Actin

Figure 27: Blocking mutant p53-DAB2IP interaction inhibits the oncogenic response to insulin without affecting the expression of insulin receptors.

A) MDA-MB231 cells were stably transduced with retroviruses expressing the eGFP-DAB2IP (1-186) KA2 fusion protein (eGFP-KA2) or eGFP alone. Proliferation assays (left) and invasion assays (right) were performed as in Figure 13 (mean ± SEM; n=3; ** P<0.01, *** P<0.001). Expression of eGFP proteins was analyzed by western blot.

B) MDA-MB231 cells stably transduced as in A were treated with insulin (5ug/ml) for the indicated times. Phosphorylated and total AKT1 were detected by immunoblotting. Expression of eGFP proteins and endogenous DAB2IP were verified by western blot. Asterisk indicates a non-specific reactive band.

C) MDA-MB231 cells were stably transduced as in A. Expression of INSR A/B and IGF1R was measured by RT-qPCR (mean ± SEM; n = 3). Expression of eGFP proteins was analyzed by western blot.

D) MDA-MB231 stably transduced as in A were transfected with the indicated siRNAs. Proliferation assays (top) and invasion assays (bottom) were performed as in Figure 13 (mean ± SEM; n=3; ** P<0.01, *** P<0.001). Expression of eGFP proteins and endogenous DAB2IP were verified by western blot.

Figure 28

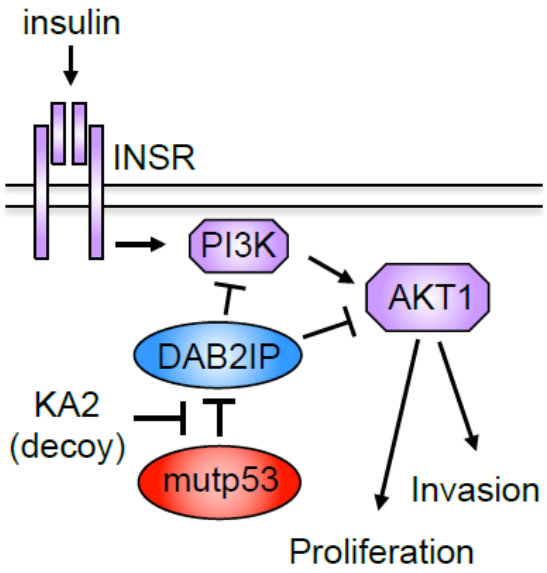
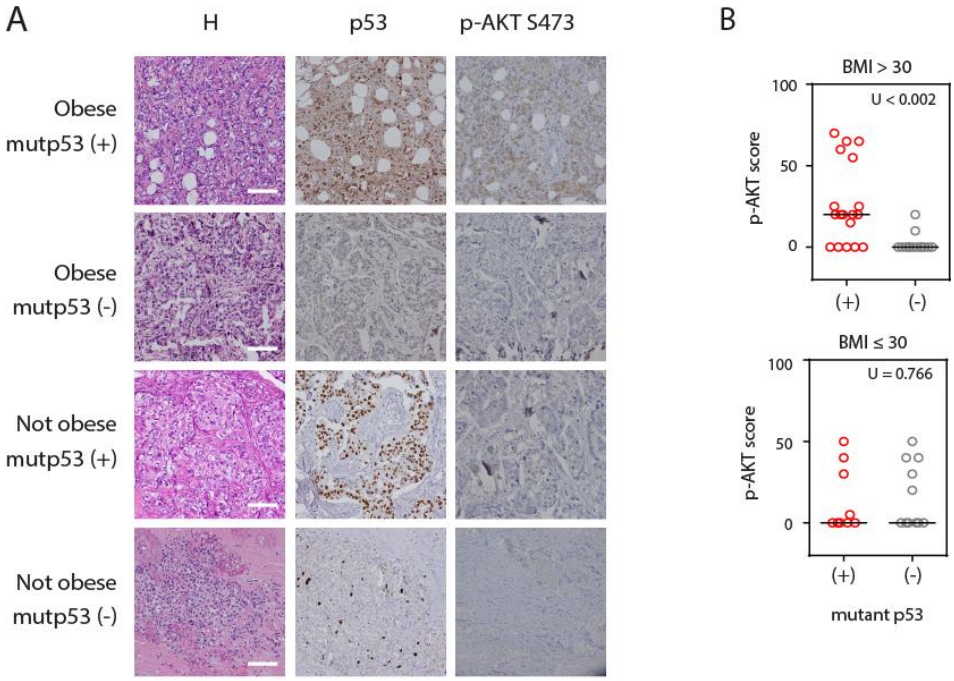


Figure 28: Proposed model for the gain of function of mutant p53 in the response to insulin.
Mutant p53 gain of function is mediated by its cytoplasmic interaction with DAB2IP, promoting an oncogenic response to insulin. A competitor decoy protein (KA2) can restore DAB2IP inhibitory functions in cells with mutant p53.

Figure 29



C

			p-Akt (+)	p-Akt (-)	Pearson P-val
BMI > 30	Mut p53	(+)	13	5	< 0.001
		(-)	2	12	
BMI ≤ 30	Mut p53	(+)	3	6	0.492
		(-)	4	7	
total	Mut p53	(+)	16	11	< 0.001
		(-)	6	19	

Figure 29: AKT activation correlates with p53 mutation in TNBC of obese patients.

A) Immunostaining with antibodies against p53 and p-AKT S473 of representative triple negative breast cancer sections derived from obese and non obese patients (H = Hematoxylin staining. Scale bar = 100 µm). B) Graphs summarize p-AKT S473 staining in tumor samples from 32 obese patients (BMI >30) and 20 non obese patients, sorted according to p53 status. Horizontal bar indicates the median. Correlations were analyzed by non-parametric Mann-Whitney U-test. C) Table summarizes the same tumor samples as in B, grouped according to p53 status and p-AKT S473 staining. Tumors with a p53 staining > 80% have been classified as “mutant p53”. Tumors with p-AKT staining > 15% have been classified as “positive”. Correlations were analyzed by parametric Pearson’s Chi-square test (P-val).

DISCUSSION

Pointing out a correlation among metabolic syndrome, obesity, diabetes, and increased incidence of various malignancies, several epidemiological studies demonstrated that hyperinsulinemia represents an important factor of risk in cancer progression (Giovannucci et al., 2010)(Wolin et al., 2010).

Obese and type 2 diabetic patients display high-doses of circulating insulin in the plasma (Cantiello et al., 2015)(Joost, 2014). Insulin, and host circulating hormones, in general, can have a dramatic impact on cancer growth and dissemination, acting directly on transformed cells as well as indirectly affecting the tumor microenvironment and the immune system (Gupta and Tikoo, 2013).

We found that p53 mutation can significantly increase proliferation and invasion of cancer cells exposed to insulin. This was observed with different missense mutations, and could be recapitulated using a nuclear-excluded mutant p53. Therefore, this phenotype defines a novel cytoplasmic gain of function for mutant p53. This phenotype also indicates that a significant part of the oncogenic potential of mutant p53 – i.e. its gain-of-function - involves distorting the cell's response to various extracellular inputs. This may be exerted at the transcriptional level, changing the transcriptional response to a multitude of pathway, and also – notably – at the cytoplasmic level, affecting various signal transduction steps.

We found that cancer cells with high levels of mutant p53 have a stronger PI3K/AKT response to insulin because DAB2IP functions are limited by aberrant interaction with mutant p53. This discovery provides a molecular mechanism for previously reported correlations between p53 mutation and AKT hyperactivation in cancer (Muller et al., 2009)(Tan et al., 2015). Intriguingly, it also suggests a mechanism by which TP53 mutation could surrogate for PI3K mutation, at least under some conditions. This would be in line with evidence that PI3K and TP53 alterations tend to be mutually exclusive in various carcinomas, including breast (Ciriello et al., 2016)(Boyault et al., 2012).

Moreover, other possible explanation for the observed enhancement of insulin signaling by mutant p53 need to be considerated.

Independent studies demonstrated that mutant p53 can induce the expression of IGF1-R (Werner et al., 1996) and wtp53 represses INSR promoter, regulating its expression in breast cancer cells (Webster et al., 1996). Moreover, at supraphysiological concentrations, insulin and IGF-1 cross-react with each other's receptors (Muniyappa and Sowers, 2012). Indeed, we evaluated mutant

p53 action on INSR and IGF1-R expression. We found that INSR mRNA levels are not affected by mutant p53 depletion. In contrast, IGF1R mRNA levels decreased after mutant p53 silencing in MDA-MB231 cells, in line with Werner et al.

However, IGF1R mRNA levels were not affected by mutant p53 depletion in DU145 cells. We don't know the basis of this discrepancy; it may be due to different levels/activity of other p53-family members, p63 and p73, that can also repress IGF1R transcription (Werner et al., 1996), or it may reflect specific activities of the different p53 mutations in the two cell lines. In any case, this observation argues against a relevant impact of IGF1R in the observed phenotypes, since mutant p53 depletion in DU145 strongly inhibits insulin-induced p-AKT when IGF1R levels are not reduced. Additional evidences indicate that down-regulation of IGF1R is not relevant for our phenotypes: first, both INSR and IGF1R are not significantly increased by mutant p53 overexpression in H1299, while insulin-induced AKT phosphorylation is augmented. Second, both INSR and IGF1R are not affected by overexpression of the eGFP-KA2 decoy protein in MDA-MB231 cells, while insulin-induced AKT phosphorylation is clearly reduced. These evidences can not exclude an action of hight insulin concentration on IGF1-R, moreover it is extremely unlikely that mutantp53-dependent regulation of IGF1R contributes significantly to our phenotypes.

From a molecular point of view, our data raise interesting questions regarding the biochemical nature of the inhibitory action of DAB2IP on the PI3K/AKT axis, which is likely based on physical interaction but still needs to be defined.

Another interesting question concerns the possible reciprocal modulation of DAB2IP by AKT. In fact, AKT1 can phosphorylate DAB2IP in the C-terminal proline-rich (PR) domain, and this phosphorylation reduces DAB2IP interaction with Ras and TRAF2, thus limiting its functions (Dai et al., 2014). Notably, the PR domain is also the site of interaction with p85, so phosphorylation by AKT may reduce the inhibitory action of DAB2IP on PI3K, further increasing AKT activation. Although not formally proved, it is plausible that a reciprocal regulatory circuit between DAB2IP and AKT indeed exists.

We and others confirmed that the signaling protein DAB2IP negatively regulates the PI3K/AKT signaling pathway. In this perspective, therefore, DAB2IP is somehow similar to PTEN, a lipid phosphatase that dephosphorylates PtdIns (3,4,5)P3, or to PP2A, a ser/thr phosphatase that dephosphorylates AKT. In several cancers, downregulation or loss of function of these tumor suppressors can foster a pro-oncogenic response to insulin (Blanco-Aparicio et al., 2007)(Włodarski et al., 2006)(Brognard et al., 2007).

It is tempting to speculate that DAB2IP may act as a buffer to limit activation of the PI3K/AKT axis in cancer cells that bear PI3K or PTEN mutations. Different levels of DAB2IP may thus explain differences in aggressiveness and response to therapy in cancers bearing identical PTEN or PI3K mutations.

In the past, it has been reported that DAB2IP can also bind wild-type p53, at least in vitro or in overexpression (Lunardi et al., 2010), and this raises the question as to whether such interaction might have a biological impact on PI3K/AKT activation. We tried, but could not detect interaction of the endogenous proteins in multiple cell lines (Di Minin et al., 2014), so this hypothesis is unlikely. However, we cannot exclude that wt p53 might bind to DAB2IP under specific conditions that strongly increase cytoplasmic p53 levels. Clearly, additional studies will be required to test this possibility and its potential implications on the PI3K/AKT axis.

Finally, we must consider that humans have three related AKT genes, encoding structurally related kinases with similar mechanism of activation and partially overlapping but distinct biological effects (Dillon and Muller, 2011); in this Thesis we focused on AKT1, but we performed some experiments and confirmed that DAB2IP can efficiently bind AKT2, and that mutant p53 interferes with this interaction.

We also uncovered that AKT1 and AKT2 have a different action in mediating oncogenic responses to insulin in breast cancer (MDA-MB231 cells), while they share similar activity in prostate cancer (DU145 cells). The reasons for such differences are unknown, but are possibly linked to the presence or absence of specific AKT substrates differentially expressed in the tested cell lines. Indeed, how the three AKT proteins differ in their regulation, localization, and substrate utilization remains largely unknown.

In any case, it is legitimate to assume that the cellular phenotypes reported in this Thesis are possibly mediated by both AKT1 and AKT2, hyper-activated in the presence of mutant p53.

Several epidemiological studies reveal a direct correlation between obesity and breast and prostate cancer initiation and progression (Rostoker et al., 2013)(Weinstein et al., 2014). Interestingly, it has been observed that ER-positive breast cancer cells that develop resistance to Tamoxifen become more sensitive to INSR depletion or pharmacologic inhibition (Chan et al., 2016), revealing the importance of insulin signaling to surrogate the mitogenic activity of estrogen. Indeed, tumors deprived from hormones, or other growth-factors, can potentially benefit from the oncogenic action of insulin.

Considering that mutp53 also impacts the response of cancer cells to inflammatory cytokines (Di Minin et al., 2014)(Cooks et al., 2014), it is plausible that p53 mutation might significantly increase growth and dissemination of endocrine-resistant metastatic cancer cells in patients with clinical, metabolic, or dietary conditions leading to hyperinsulinemia. Especially in conditions of obesity, where hyperinsulinemia is frequently coupled to chronic inflammation.

From a clinical point of view, our results suggest that mutation of p53 could potentially affect the outcome of tumors that arise - or progress - under conditions of hyperinsulinemia. By looking at a set of clinical samples from the "Fondazione Pascale" in Napoli, we found a strong correlation between p53 mutation and AKT phosphorylation, specifically and selectively in breast tumors from obese patients. Assuming a hyperinsulinemic context related to obesity, p53 mutation would increase proliferation and dissemination of such tumors by promoting insulin-induced AKT activation.

Regarding the possible implications of this observations, our group in previous studies designed an interfering GFP fusion protein, or decoy, capable of displacing the mutant p53-DAB2IP interaction; in tissue culture and in mouse xenografts, this decoy could efficiently reduce the invasive behavior of cancer cells exposed to inflammation (Di Minin et al., 2014). Here, we verified that such decoy can also efficiently inhibit insulin-induced cell proliferation and invasion.

These results provide a crucial proof of principle that peptide or nucleotide aptamers designed to interfere with the mutant p53/DAB2IP interaction might have a potential application in targeted therapy of mutant p53 cancers. In particular, they point out the possibility to recover DAB2IP suppressive action on insulin-induced oncogenic phenotypes in cancers with mutant p53.

In conclusion, the data presented in this Thesis uncover a molecular mechanism by which mutation of p53 might change the behavior of cancer cells exposed to insulin, suggesting a novel perspective for clinical management of obese, hyperinsulinemic patients.

These data also provide yet another indication that, for those tumors where p53 is mutated, pharmacologic efforts aimed to tackle the gain of function of p53 bear a strong therapeutic potential.

MATERIALS AND METHODS

Cell Culture, Transfections, Retroviral Transductions and drug treatments.

The following cell lines were used: breast cancer MDA-MB-231 (p53R280K) and HBL-100 (wt p53), prostate cancer DU-145 (p53V274F/P223L), H1299 (p53 null), HEK 293GP (wt p53) and Ras-immortalized mouse embryonic fibroblasts (MEF) derived from p53 knock-out and p53R172H knock-in mice. MDA-MB231, HBL-100, HEK 293 GP and MEF were cultured in DMEM medium (Sigma) supplemented with 10% FBS (ECS0180L, Euroclone), and antibiotics (DE17-602E, Lonza). DU145 and H1299 cells were cultured in RPMI medium (Sigma) supplemented with 10% FBS and antibiotics. All cell lines were subjected to STR genotyping with PowerPlex 18D System and confirmed in their identity comparing the results to reference cell databases (DMSZ, ATCC, and JCRB databases), where possible. MEFs were collected from 13.5 d.p.c. embryos of p53 knock-out and p53R172H knock-in mice, and immortalized by retroviral transduction of H-RasV12 (1). Transfections of H1299 were performed with Lipofectamine 2000 (Invitrogen), following manufacturer's instructions. For siRNA experiments, cells were transfected with 50 nM siRNA oligonucleotides using Lipofectamine RNAiMax (Invitrogen), following manufacturer's instructions. After 48 hr of silencing, Cells were processed after 48 hours, unless when differently specified.

Sequence of siRNAs used:

siRNA	**Sequence**	**Purchased from**
Control siRNA	Unknown	All star negative control (1027281, Qiagen).
sip53 ORF	GACUCCAGUGGUAAUCUAC	Eurofins MWG
siDAB2IP A	GGAGCGCAACAGUUACCUG	Eurofins MWG
siDAB2IP B	GGUGAAGGACUUCCUGACA	Eurofins MWG
siDAB2IP 3'UTR	GUAAUGUAACUAUCUCACC	Eurofins MWG

For retrovirus production, low confluency HEK 293GP packaging cells were transfected by calcium phosphate precipitation. After 48–72 hr the virus-containing medium was filtered and

added to target cells. Cells were selected with blasticidin (2 µg/ml) and/or puromycin (0.5 µg/ml) and kept under selection for the entire experiment. Expression plasmids and retroviral constructs encoding DAB2IP, mutant p53 variants, and the eGFP-KA2 decoy construct have all been described previously (15). pcDNA3-flag-HA-AKT1 and was a gift from W. Sellers (Addgene plasmid # 9021). pcDNA3-Hygro-HA-AKT2 was a gift from M. Birnbaum (Addgene plasmid # 16000). pCMV6-p85alpha-Flag was a gift from L. Cantley (Addgene plasmid # 1399). For insulin treatment, cells were serum-starved for 24 h before addition of 0.5µg/ml human recombinant Insulin (Sigma, I2643). The AKT inhibitor MK2206 (Santa Cruz) was used at 5µM.

BrdU incorporation assay

For BrdU incorporation assay, cells were serum starved for 24 hr and after treated with insulin(0.5ug/ml) for 24 hr. Proliferating cells were labeled with 20uM 5'-bromo-2'-deoxyuridine (BrdU) in vitro for 2hr. Cells were fixed in 4% paraformaldehyde at room temperature, permeabilized in PBS plus 0.1% Triton X-100, and washed with NaOH (50 mM). Permeabilized cells were incubated with specific monoclonal antibody (GE Healthcare), followed by Alexa Flour® 568 conjugated secondary antibodies (Life Technologies). Nuclei were stained with Hoechst. Proliferating cells were scored by counting BrdU positive over total cell nuclei, in 25 random microscope fields (at least 100 cells were counted per each sample). The total number of BrdU-labeled cells was counted directly under a Leica DM4000B epifluorescence microscope.

Crystal violet cell proliferation assay

Cells were seeded in a 96-well plate at a density of 5000 cells/well., grown in serum-fee medium for 24h and treated with insulin (0.5 ug/ml) for additional 24h and 48h. Subsequently, cells were fixed in 4% PFA and stained with 0.05% Crystal Violet. Wells filled with only medium provided the background absorbance of the dye. Optical density of each well was measured at 570 nm (OD570) with an Enspire multimode plate reader (PerkinElmer).

Colony formation assay

Cells were seeded at a density of 5000 (MDA-MB-231) or 3000 (DU-145) cells per 6 cm diameter plate, and incubated for 24 hours in 10% FBS-supplemented DMEM culture medium. Cells were then treated with insulin (0.5 ug/ml) every 48 hours. After 10 days, cells were fixed in 4% PFA and stained with Giemsa (Sigma) for 2 hours. Plates were photographed and colonies ≥ 50 pixels were counted using ImageJ software after background subtraction (2).

Matrigel invasion Assays

Cells were grown in serum-fee medium for 24h and treated with insulin for additional 24h. Subsequently, cells were trypsinized, counted, and plated in 24-well PET inserts (8 µm pore size, Falcon), coated with BD Matrigel (BD Bioscience). The lower chamber was filled with high serum medium (10% FBS) without insulin. After 16h, cells passed through the filter were fixed in 4% PFA, stained with 0.05% crystal violet, and counted. Invasion was scored by counting cells in 22 random non-overlapping microscope fields at 40X magnification.

RNA expression analysis

Total RNA was extracted with QIAzol (QIAGEN). For RT-qPCR, 1 ug of total RNA was reverse transcribed with QuantiTect Reverse Transcription kit (QIAGEN). Real-time PCR was performed using SsoAdvanced SYBR Green Master Mix (Biorad) on a CFX96 Real-Time PCR System (Biorad). Primer sequences were as follows:

INSR A/B	Forward	5'-CAG CGA GAA ACT GCA TGG T-3'
	Reverse	5'-CAT TGG ACA TGG TAG AGT CG-3'
IGF1-R	Forward	5'-TCT GGC CGA CGA GTG GAG-3'
	Reverse	5'-CTC GGT AAT GAC CGT GAG CTT-3'
Histone H3	Forward	5'-GAA GAA ACC TCA TCG TTA CAG GCC TGG T-3'
	Reverse	5'-CTG CAA AGC ACC AAT AGC TGC ACT CTG GAA-3'

Protein expression analysis

Total cell extracts were prepared in RIPA buffer without SDS (150mM NaCl, 50mM Tris-HCl pH8, 1mM EDTA, 1% NP-40, 0.5% Na-deoxycholate) supplemented with 1 mM PMSF, 5 mM NaF, 1 mM Na3VO4, 10µg/ml CLAP, 1µM TSA and 5µM nicotinamide. Protein concentration was determined with Bio-Rad Protein Assay Reagent (#500-0006, Bio-Rad). Lysates were resolved by SDS/PAGE and transferred to nitrocellulose (Millipore).

Western blot analysis was performed according to standard procedures. When blots were probed with multiple antibodies, either proteins had markedly different electrophoretic migration, or were detected with primary antibodies raised in different species; in the latter case, signals from previous secondary antibodies were extinguished by sodium-azide treatment. When proteins

could not be detected on the same blot, a second gel was run in parallel with exactly the same amount of lysate.

List of primary antibodies used:

Target	Antibody
P53 (DO1)	sc-126 (Santa Cruz)
P53 (DO-7)	DAKO
Actin	#A9718 (Sigma)
AKT1	#2967 (Cell Signaling)
p-AKTS473	#9271 (Cell Signaling)
p-AKT S473	#4060 (Cell Signaling)
p-AKT T308	#9275 (Cell Signaling)
p-GSK3β S9	#9323 (Cell Signaling)
GSK3β	#9832 (Cell Signaling)
CyclinD1	#2926 (Cell Signaling)
c-Myc (9E10)	Sc-40 (Santa Cruz)
p-p70 S6 kinase T421/S424	#9204 (Cell Signaling)
p-S6RP S235/236	#2211 (Cell Signaling)
S6RP	#2217 (Cell Signaling)
HA(Y11)	Sc-805 (Santa Cruz)
FLAG M2	F3165 (Sigma)
GFP	self-produced rabbit polyclonal
DAB2IP	A302-440A (Bethyl)
p-P44/42 MAPK (ERK1/2) T202/Y204	#9101 (Cell Signaling)
P44/2 MAPK (Erk1/2)	#9107 (Cell Signaling)

Protein interaction studies.

For co-immunoprecipitation, expression plasmids were transfected in human H1299 cells. 24 hr after transfection cells were lysed in Co-IP buffer (NaCl 120mM, Tris-HCl pH7.5 20mM, EDTA 1mM, NP40 0.5%) with protease inhibitors. Samples were cleared by centrifugation for 30 min

at 13000g at 4 °C and incubated overnight at 4 °C with specific antibody. After 1 hr incubation with protein G-Sepharose (GE Healthcare), immunoprecipitates were washed three times in Co-IP buffer, resuspended in sample buffer, and analyzed by immunoblotting. Mouse IgGs were used as negative control.

Immunohistochemical analysis

A cohort of 52 cases of TNBC, including 32 from obese patients (BMI>30) and 20 from non obese patients (BMI <30), were collected from the Department of Pathology of National Cancer Institute "Fondazione G. Pascale", Naples. All cases were reviewed according to WHO classification criteria, using standard tissue sections and immunohistochemical analysis. Informed consent for the scientific use of biological material was obtained from all patients. ER status, PR status, and HER2 status had been routinely recorded at the hospital. Immunohistochemical staining on formalin-fixed, paraffin-embedded tumor tissue was performed on slides (4 μm) in order to evaluate expression of p53 (DO-7, DAKO) and p-AKTS473 (sc-4060, Cell Signaling). The endogenous peroxidase activity was blocked by incubating the sections with about 150 μl of Novocastra Peroxidase Block (RE7101; 3% H2O2) for 10 min at room temperature, followed by 2 washes (5 min/each) in TBS/Tween buffer. The formation of non-specific binding between the antibodies and endogenous proteins was reduced by incubating the sections with about 150 μl of Novocastra Protein Block (10% FBS) for 20 min at room temperature, followed by 2 washes (5 min/each) in TBS/Tween buffer. After protein block, slides were incubated with about 150 μl of primary antibodies for over night at 4°C, followed by two washes in TBS/tween buffer (5 min/ each), secondary antibody (Novocastra Streptavidin- HRP, Leica Microsystems, Milan, Italy) for 40 min at room temperature, two washes in TBS/Tween (5 min/ each) and then visualized using a 3,3′-diaminobenzidine. The following negative controls were performed: (a) omission of the primary antibody; (b) substitution of the primary anti-serum with non-immune serum diluted 1:150 in blocking buffer; no immunostaining was observed in both cases. Sections were counterstained with hematoxylin/eosin and mounted. Stained tissue sections were evaluated independently by two clinical pathologists using uniform criteria. Discrepancies were resolved through simultaneous inspection and discussion of the results.

Samples were scored as "mutant p53" when positive nuclear and cytoplasmic p53 staining was detectable in > 80% of tumor cells (3). Samples were scored as "p-AKT positive" when distinct cytoplasmic staining was detected in > 15% of tumor cells (4). For non-parametric analysis, the actual percentage of cells with distinct cytoplasmic p-AKT staining was used.

Statistical analysis

In all graphs data are expressed as mean ± SEM of at least three independent experiments, except when otherwise indicated. Differences were analyzed by Student's t test using Prism 5 (GraphPad). P-values < 0.05 were considered significant. Immunohistochemical data were analyzed using SPSS 17.0 software (Inc., Chicago, Ill., USA); both the Mann-Whitney non-parametric test and the Pearson's chi-square parametric test were used to evaluate correlations between p53 mutation and p-AKT(S473) expression.

BIBLIOGRAPHY

Adorno, M., Cordenonsi, M., Montagner, M., Dupont, S., Wong, C., Hann, B., Solari, A., Bobisse, S., Rondina, M.B., Guzzardo, V., et al. (2009). A Mutant-p53/Smad Complex Opposes p63 to Empower TGF??-Induced Metastasis. Cell *137*, 87–98.

Di Agostino, S., Strano, S., Emiliozzi, V., Zerbini, V., Mottolese, M., Sacchi, A., Blandino, G., and Piaggio, G. (2006). Gain of function of mutant p53: The mutant p53/NF-Y protein complex reveals an aberrant transcriptional mechanism of cell cycle regulation. Cancer Cell *10*, 191–202.

Di Agostino, S., Sorrentino, G., Ingallina, E., Valenti, F., Ferraiuolo, M., Bicciato, S., Piazza, S., Strano, S., Del Sal, G., and Blandino, G. (2015). YAP enhances the pro-proliferative transcriptional activity of mutant p53 proteins. EMBO Rep. *17*, 1–14.

Alessi, D.R., James, S.R., Downes, C.P., Holmes, A.B., Gaffney, P.R.J., Reese, C.B., and Cohen, P. (1997). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Bα. Curr. Biol. *7*, 261–269.

Altomare, D.A., and Testa, J.R. (2005). Perturbations of the AKT signaling pathway in human cancer. Oncogene *24*, 7455–7464.

Altomare, D. a, Lyons, G.E., Mitsuuchi, Y., Cheng, J.Q., and Testa, J.R. (1998). Akt2 mRNA is highly expressed in embryonic brown fat and the AKT2 kinase is activated by insulin. Oncogene *16*, 2407–2411.

Arboleda, M.J., Lyons, J.F., Kabbinavar, F.F., Bray, M.R., Snow, B.E., Ayala, R., Danino, M., Karlan, B.Y., and Slamon, D.J. (2003). Overexpression of AKT2/protein kinase Bbeta leads to up-regulation of beta1 integrins, increased invasion, and metastasis of human breast and ovarian cancer cells. Cancer Res. *63*, 196–206.

Aschauer, L., and Muller, P.A.J. (2016). Novel targets and interaction partners of mutant p53 Gain-Of-Function. Biochem. Soc. Trans. *44*, 460–466.

Astrinidis, A., and Henske, E.P. (2005). Tuberous sclerosis complex: Linking growth and energy signaling pathways with human disease. Oncogene *24*, 7475–7481.

Attoub, S., Arafat, K., Kamel Hammadi, N., Mester, J., and Gaben, A.M. (2015). Akt2 knock-down reveals its contribution to human lung cancer cell proliferation, growth, motility, invasion and endothelial cell tube formation. Sci. Rep. *5*, 1–14.

Backer, J.M. (2008). The regulation and function of Class III PI3Ks: novel roles for Vps34. Biochem. J. *410*, 1–17.

Bacus, S.S., Altomare, D.A., Lyass, L., Chin, D.M., Farrell, M.P., Gudkov, A., and Testa, J.R. (2002). AKT2 is frequently upregulated in HER-2/neu-positive breast cancers and may contribute to tumor aggressiveness by enhancing cell survival. Oncogene *21*, 3532–3540.

Balendran, A., Biondi, R.M., Cheung, P.C.F., Casamayor, A., Deak, M., and Alessi, D.R. (2000). A 3-phosphoinositide-dependent protein kinase-1 (PDK1) docking site is required for the phosphorylation of protein kinase Cζ (PKCζ) and PKC- related kinase 2 by PDK1. J. Biol. Chem. *275*, 20806–20813.

Barik, S. (2008). An intronic microRNA silences genes that are functionally antagonistic to its host gene. Nucleic Acids Res. *36*, 5232–5241.

Belfiore, A. (2007). The Role of Insulin Receptor Isoforms and Hybrid Insulin / IGF-I Receptors in Human Cancer. 671–686.

Belfiore, A., and Malaguarnera, R. (2011). Insulin receptor and cancer. Endocr. Relat. Cancer

18, 125–147.
Belfiore, A., Frasca, F., Pandini, G., Sciacca, L., and Vigneri, R. (2009). Insulin receptor isoforms and insulin receptor/insulin-like growth factor receptor hybrids in physiology and disease. Endocr. Rev. *30*, 586–623.
Bellacosa, a, and Kumar, C. (2005). Activation of AKT kinases in cancer: implications for therapeutic targeting. Adv. Cancer ….
Bellacosa, A., Testa, J.R., Moore, R., and Larue, L. (2004). A portrait of AKT kinases: Human cancer and animal models depict a family with strong individualities. Cancer Biol. Ther. *3*, 268–275.
Bellazzo, A., Di Minin, G., and Collavin, L. (2016). Block one, unleash a hundred. Mechanisms of DAB2IP inactivation in cancer. Cell Death Differ. 1–11.
Bellazzo, A., Di Minin, G., and Collavin, L. (2017). Block one, unleash a hundred. Mechanisms of DAB2IP inactivation in cancer. Cell Death Differ. *24*, 15–25.
Bernards, R., and Weinberg, R.A. (2002). A progression puzzle. Nature *418*, 823.
Bid, H.K., Roberts, R.D., Manchanda, P.K., and Houghton, P.J. (2013). RAC1: an emerging therapeutic option for targeting cancer angiogenesis and metastasis. Mol. Cancer Ther. *12*, 1925–1934.
Bjornsti, M.A., and Houghton, P.J. (2004). Lost in translation: Dysregulation of cap-dependent translation and cancer. Cancer Cell *5*, 519–523.
Blanco-Aparicio, C., Renner, O., Leal, J.F.M., and Carnero, A. (2007). PTEN, more than the AKT pathway. Carcinogenesis *28*, 1379–1386.
Bossi, G., Marampon, F., Maor-Aloni, R., Zani, B., Rotter, V., Oren, M., Strano, S., Blandino, G., and Sacchi, A. (2008). Conditional RNA interference in vivo to study mutant p53 oncogenic gain of function on tumor malignancy. Cell Cycle *7*, 1870–1879.
Bougeard, G., Sesbou, R., Vasseur, S., Martin, C., Tinat, J., Chompret, A., and Paillerets, B.B. (2008). Molecular basis of the Li – Fraumeni syndrome : an update from the French LFS families. 535–538.
Bozulic, L., and Hemmings, B.A. (2009). PIKKing on PKB: regulation of PKB activity by phosphorylation. Curr. Opin. Cell Biol. *21*, 256–261.
Brázdová, M., Navrátilová, L., Tichý, V., Němcová, K., Lexa, M., Hrstka, R., Pečinka, P., Adámik, M., Vojtesek, B., Paleček, E., et al. (2013). Preferential Binding of Hot Spot Mutant p53 Proteins to Supercoiled DNA In Vitro and in Cells. PLoS One *8*.
Brognard, J., Sierecki, E., Gao, T., and Newton, A.C. (2007). PHLPP and a Second Isoform, PHLPP2, Differentially Attenuate the Amplitude of Akt Signaling by Regulating Distinct Akt Isoforms. Mol. Cell *25*, 917–931.
Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J., and Greenberg, M.E. (1999). Akt promotes cell survival by phosphorylating and inhibiting a forkhead transcription factor. Cell *96*, 857–868.
Bullock, A.N., and Fersht, A.R. (2001). Rescuing the function of mutant p53. Nat. Rev. Cancer *1*, 68–76.
Cantiello, F., Cicione, A., Salonia, A., Autorino, R., De Nunzio, C., Briganti, A., Gandaglia, G., Dell'Oglio, P., Capogrosso, P., and Damiano, R. (2015). Association between metabolic syndrome, obesity, diabetes mellitus and oncological outcomes of bladder cancer: A systematic review. Int. J. Urol. *22*, 22–32.
Carpino, N., Wisniewski, D., Strife, A., Marshak, D., Kobayashi, R., Stillman, B., and Clarkson, B. (1997). p62(dok): A constitutively tyrosine-phosphorylated, GAP-associated protein in chronic myelogenous leukemia progenitor cells. Cell *88*, 197–204.
Carpten, J.D., Faber, A.L., Horn, C., Donoho, G.P., Briggs, S.L., Robbins, C.M., Hostetter, G., Boguslawski, S., Moses, T.Y., Savage, S., et al. (2007). A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. Nature *448*, 439–444.

Castellano, E., and Downward, J. (2011). Ras interaction with PI3K: More than just another effector pathway. Genes and Cancer *2*, 261–274.
Castillo-Lluva, S., Tatham, M.H., Jones, R.C., Jaffray, E.G., Edmondson, R.D., Hay, R.T., and Malliri, A. (2010). SUMOylation of the GTPase Rac1 is required for optimal cell migration. Nat. Cell Biol. *12*, 1078–1085.
Caswell, P., and Norman, J. (2008). Endocytic transport of integrins during cell migration and invasion. Trends Cell Biol. *18*, 257–263.
Caulin, C., Nguyen, T., Lang, G.A., Goepfert, T.M., Brinkley, B.R., Cai, W.W., Lozano, G., and Roop, D.R. (2007). An inducible mouse model for skin cancer reveals distinct roles for gain-and loss-of-function p53 mutations. J. Clin. Invest. *117*, 1893–1901.
Chang, F., Steelman, L.S., Lee, J.T., Shelton, J.G., Navolanic, P.M., Blalock, W.L., Franklin, R.A., and McCubrey, J.A. (2003). Signal transduction mediated by the Ras/Raf/MEK/ERK pathway from cytokine receptors to transcription factors: Potential targeting for therapeutic intervention. Leukemia *17*, 1263–1293.
Chau, N.-M., and Ashcroft, M. (2004). Akt2: a role in breast cancer metastasis. Breast Cancer Res. *6*, 55–57.
Chee, J.L.Y., Saidin, S., Lane, D.P., Leong, S.M., Noll, J.E., Neilsen, P.M., Phua, Y.T., Gabra, H., and Lim, T.M. (2013). Wild-type and mutant p53 mediate cisplatin resistance through interaction and inhibition of active caspase-9. Cell Cycle *12*, 278–288.
Chen, H., Pong, R.C., Wang, Z., and Hsieh, J.T. (2002). Differential regulation of the human gene DAB2IP in normal and malignant prostatic epithelia: Cloning and characterization. Genomics *79*, 573–581.
Chen, H., Toyooka, S., Gazdar, A.F., and Hsieh, J.-T. (2003). Epigenetic Regulation of a Novel Tumor Suppressor Gene (*hDAB2IP*) in Prostate Cancer Cell Lines. J. Biol. Chem. *278*, 3121–3130.
Chen, H., Tu, S.W., and Hsieh, J.T. (2005). Down-regulation of human DAB2IP gene expression mediated by polycomb Ezh2 complex and histone deacetylase in prostate cancer. J. Biol. Chem. *280*, 22437–22444.
Chen, H., Karam, J. a, Schultz, R., Zhang, Z., Duncan, C., and Hsieh, J.-T. (2006). Cloning of mouse Dab2ip gene, a novel member of the RasGTPase-activating protein family and characterization of its regulatory region in prostate. DNA Cell Biol. *25*, 232–245.
Cheng, J.Q., Godwin, a K., Bellacosa, a, Taguchi, T., Franke, T.F., Hamilton, T.C., Tsichlis, P.N., and Testa, J.R. (1992). AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. Proc. Natl. Acad. Sci. U. S. A. *89*, 9267–9271.
Cheng, J.Q., Ruggeri, B., Klein, W.M., Sonoda, G., Altomare, D.A., Watson, D.K., and Testa, J.R. (1996). Amplification of AKT2 in human pancreatic cells and inhibition of AKT2 expression and tumorigenicity by antisense RNA. Proc. Natl. Acad. Sci. U. S. A. *93*, 3636–3641.
Chicas, A., Molina, P., and Bargonetti, J. (2000). Mutant p53 forms a complex with Sp1 on HIV-LTR DNA. Biochem. Biophys. Res. Commun. *279*, 383–390.
Choi, S.M., Tucker, D.F., Gross, D.N., Easton, R.M., DiPilato, L.M., Dean, A.S., Monks, B.R., and Birnbaum, M.J. (2010). Insulin Regulates Adipocyte Lipolysis via an Akt-Independent Signaling Pathway. Mol. Cell. Biol. *30*, 5009–5020.
Ciriello, G., Gatza, M.L., Beck, A.H., Wilkerson, M.D., Rhie, K., Pastore, A., Zhang, H., Mclellan, M., Yau, C., Chen, Y., et al. (2016). Cancer. *163*, 506–519.
Clemmons, D.R. (1998). the action of GH results in a major improvement in insulin sensitivity. 25–27.
Coffer, P.J., and Woodgett, J.R. (1991). Molecular cloning and characterisation of a novel putative protein-serine kinase related to the cAMP-dependent and protein kinase C families. Eur. J. Biochem. *201*, 475–481.

Cooks, T., Pateras, I.S., Tarcic, O., Solomon, H., Schetter, A.J., Wilder, S., Lozano, G., Pikarsky, E., Forshew, T., Rozenfeld, N., et al. (2014). NIH Public Access. *23*, 634–646.
Copp??, J.P., Rodier, F., Patil, C.K., Freund, A., Desprez, P.Y., and Campisi, J. (2011). Tumor suppressor and aging biomarker p16 INK4a induces cellular senescence without the associated inflammatory secretory phenotype. J. Biol. Chem. *286*, 36396–36403.
Dai, X., North, B.J., and Inuzuka, H. (2014). Negative regulation of DAB2IP by Akt and SCF Fbw7 pathways ABSTRACT : *5*.
Datta, S.R., Brunet, A., and Greenberg, M.E. (1999). Cellular survival: A play in three akts. Genes Dev. *13*, 2905–2927.
Deb, S., Jackson, C.T., Subler, M.A., and Martin, D.W. (1992). Modulation of cellular and viral promoters by mutant human p53 proteins found in tumor cells. J. Virol. *66*, 6164–6170.
Di Minin, G., Bellazzo, A., Dal Ferro, M., Chiaruttini, G., Nuzzo, S., Bicciato, S., Piazza, S., Rami, D., Bulla, R., Sommaggio, R., et al. (2014). Mutant p53 Reprograms TNF Signaling in Cancer Cells through Interaction with the Tumor Suppressor DAB2IP. Mol. Cell *56*, 617–629.
Dillon, R.L., and Muller, W.J. (2011). progression. *70*, 4260–4264.
Dittmer, D., Pati, S., Zambetti, G., Chu, S., Teresky, A.K., Moore, M., Finlay, C., and Levine, A.J. (1993). Gain of function mutations in p53. Nat. Genet. *4*, 42–46.
Do, P.M., Varanasi, L., Fan, S., Li, C., Kubacka, I., Newman, V., Chauhan, K., Daniels, S.R., Boccetta, M., Garrett, M.R., et al. (2012). Mutant p53 cooperates with ETS2 to promote etoposide resistance. Genes Dev. *26*, 830–845.
Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A., Butel, J.S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. Nature *356*, 215–221.
Dong, P., Xu, Z., Jia, N., Li, D., and Feng, Y. (2009). Elevated expression of p53 gain-of-function mutation R175H in endometrial cancer cells can increase the invasive phenotypes by activation of the EGFR/PI3K/AKT pathway. Mol. Cancer *8*, 103.
Downward, J. (1998). Mechanisms and consequences of activation of protein kinase B/Akt. Curr. Opin. Cell Biol. *10*, 262–267.
Duggan, D., Zheng, S.L., Knowlton, M., Benitez, D., Dimitrov, L., Wiklund, F., Robbins, C., Isaacs, S.D., Cheng, Y., Li, G., et al. (2007). Two genome-wide association studies of aggressive prostate cancer implicate putative prostate tumor suppressor gene DAB2IP. J. Natl. Cancer Inst. *99*, 1836–1844.
Dummler, B., and Hemmings, B. a (2007). Physiological roles of PKB/Akt isoforms in development and disease. Biochem. Soc. Trans. *35*, 231–235.
Eck, M.J., Dhe-Paganon, S., Trüb, T., Nolle, R.T., and Shoelson, S.E. (1996). Structure of the IRS-1 PTB domain bound to the juxtamembrane region of the insulin receptor. Cell *85*, 695–705.
Endersby, R., and Baker, S.J. (2008). PTEN signaling in brain: neuropathology and tumorigenesis. Oncogene *27*, 5416–5430.
Engelman, J.A., Luo, J., and Cantley, L.C. (2006). The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nat. Rev. Genet. *7*, 606–619.
Enomoto, A., Ping, J., and Takahashi, M. (2006). Girdin, a novel actin-binding protein, and its family of proteins possess versatile functions in the Akt and Wnt signaling pathways. Ann. N. Y. Acad. Sci. *1086*, 169–184.
Fayard, E. (2005). Protein kinase B/Akt at a glance. J. Cell Sci. *118*, 5675–5678.
Fazili, Z., Sun, W., Mittelstaedt, S., Cohen, C., and Xu, X.X. (1999). Disabled-2 inactivation is an early step in ovarian tumorigenicity. Oncogene *18*, 3104–3113.
Feller, S.M., Ren, R., Hanafusa, H., and Baltimore, D. (1994). SH2 and SH3 domains as molecular adhesives: the interactions of Crk and Abl. Trends Biochem. Sci. *19*, 453–458.
Filippa, N., Sable, C.L., Filloux, C., Hemmings, B., and Van Obberghen, E. (1999). Mechanism of protein kinase B activation by cyclic AMP-dependent protein kinase. Mol. Cell. Biol. *19*,

4989–5000.
Fivaz, M., Bandara, S., Inoue, T., and Meyer, T. (2008). Robust Neuronal Symmetry Breaking by Ras-Triggered Local Positive Feedback. Curr. Biol. *18*, 44–50.
Frank, A.K., Pietsch, E.C., Dumont, P., Tao, J., and Murphy, M.E. (2011). Wild-type and mutant p53 proteins interact with mitochondrial caspase-3. Cancer Biol. Ther. *11*, 740–745.
Frasca, F., Pandini, G., Scalia, P., Sciacca, L., Mineo, R., Costantino, A., Goldfine, I.D., Belfiore, A., and Vigneri, R. (1999). Insulin Receptor Isoform A, a Newly Recognized, High-Affinity Insulin-Like Growth Factor II Receptor in Fetal and Cancer Cells. Mol. Cell. Biol. *19*, 3278–3288.
Frazier, M.W., He, X., Wang, J., Gu, Z., Cleveland, J.L., and Zambetti, G.P. (1998). Activation of c-myc gene expression by tumor-derived p53 mutants requires a discrete C-terminal domain. Mol Cell Biol *18*, 3735–3743.
Freed-pastor, W. a, and Prives, C. (2012). Mutant p53 : one name , many proteins Mutant p53 : one name , many proteins. 1268–1286.
Fulop, V., Colitti, C. V, Genest, D., Berkowitz, R.S., Yiu, G.K., Ng, S.W., Szepesi, J., and Mok, S.C. (1998). DOC-2/hDab2, a candidate tumor suppressor gene involved in the development of gestational trophoblastic diseases. Oncogene *17*, 419–424.
Funamoto, S., Meili, R., Lee, S., Parry, L., and Firtel, R.A. (2002). Spatial and temporal regulation of 3-phosphoinositides by PI 3-kinase and PTEN mediates chemotaxis. Cell *109*, 611–623.
Giovannucci, E., Harlan, D.M., Archer, M.C., Bergenstal, R.M., Gapstur, S.M., Habel, L.A., Pollak, M., Regensteiner, J.G., and Yee, D. (2010). Diabetes and cancer: A consensus report. Diabetes Care *33*, 1674–1685.
Girardini, J.E., Napoli, M., Piazza, S., Rustighi, A., Marotta, C., Radaelli, E., Capaci, V., Jordan, L., Quinlan, P., Thompson, A., et al. (2011). A Pin1/Mutant p53 Axis Promotes Aggressiveness in Breast Cancer. Cancer Cell *20*, 79–91.
Göhler, T., Jäger, S., Warnecke, G., Yasuda, H., Kim, E., and Deppert, W. (2005). Mutant p53 proteins bind DNA in a DNA structure-selective mode. Nucleic Acids Res. *33*, 1087–1100.
Goldstein, B.J., and Dudley, a L. (1990). The rat insulin receptor: primary structure and conservation of tissue-specific alternative messenger RNA splicing. Mol. Endocrinol. *4*, 235–244.
Green, D.R., and Kroemer, G. (2009). Cytoplasmic functions of the tumor suppressor p53. Nature *458*, 1127.
Grottke, A., Ewald, F., Lange, T., Nörz, D., Herzberger, C., Bach, J., Grabinski, N., Gräser, L., Höppner, F., Nashan, B., et al. (2016). Downregulation of AKT3 increases migration and metastasis in triple negative breast cancer cells by upregulating S100A4. PLoS One *11*, 1–16.
Gupta, C., and Tikoo, K. (2013). High glucose and insulin differentially modulates proliferation in MCF-7 and MDA-MB-231 cells. J. Mol. Endocrinol. *51*, 119–129.
Gupta, S., Ramjaun, A.R., Haiko, P., Wang, Y., Warne, P.H., Nicke, B., Nye, E., Stamp, G., Alitalo, K., and Downward, J. (2007). Binding of Ras to Phosphoinositide 3-Kinase p110α Is Required for Ras- Driven Tumorigenesis in Mice. Cell *129*, 957–968.
Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57–70.
Haupt, S., Raghu, D., and Haupt, Y. (2016). Mutant p53 Drives Cancer by Subverting Multiple Tumor Suppression Pathways. Front. Oncol. *6*, 1–7.
Hay, N. (2011). Interplay between FOXO, TOR, and Akt. Biochim. Biophys. Acta - Mol. Cell Res. *1813*, 1965–1970.
He, G., and Karin, M. (2011). NF-κB and STAT3- key players in liver inflammation and cancer. Cell Res. *21*, 159–168.
Heasman, S.J., and Ridley, A.J. (2008). Mammalian Rho GTPases: New insights into their functions from in vivo studies. Nat. Rev. Mol. Cell Biol. *9*, 690–701.

Herman, P.K., and Emr, S.D. (1990). Characterization of VPS34, a gene required for vacuolar protein sorting and vacuole segregation in Saccharomyces cerevisiae. Mol. Cell. Biol. *10*, 6742–6754.
Holgado-Madruga, M., Emlet, D.R., Moscatello, D.K., Godwin, A.K., and Wong, A.J. (1996). A Grb2-associated docking protein in EGF- and insulin-receptor signalling. Nature *379*, 560–564.
Hsieh, J.T., Karam, J.A., and Min, W. (2007). Genetic and biologic evidence that implicates a gene in aggressive prostate cancer. J. Natl. Cancer Inst. *99*, 1823–1824.
Huang, J., Wang, B., Hui, K., Zeng, J., Fan, J., Wang, X., Hsieh, J.T., He, D., and Wu, K. (2016). miR-92b targets DAB2IP to promote EMT in bladder cancer migration and invasion. Oncol. Rep. *36*, 1693–1701.
Hussain, S.P., and Harris, C.C. (1998). Molecular Epidemiology of Human Cancer : Contribution of Mutation Spectra Studies of Tumor Suppressor Genes of Human Cancer : Contribution of Mutation Spectra Studies of Tumor Suppressor Genes. Cancer Lett. *58*, 4023–4037.
Irie, H.Y., Pearline, R. V., Grueneberg, D., Hsia, M., Ravichandran, P., Kothari, N., Natesan, S., and Brugge, J.S. (2005). Distinct roles of Akt1 and Akt2 in regulating cell migration and epithelial-mesenchymal transition. J. Cell Biol. *171*, 1023–1034.
Jacinto, E., Facchinetti, V., Liu, D., Soto, N., Wei, S., Jung, S.Y., Huang, Q., Qin, J., and Su, B. (2006). SIN1/MIP1 Maintains rictor-mTOR Complex Integrity and Regulates Akt Phosphorylation and Substrate Specificity. Cell *127*, 125–137.
Janku, F., Wheler, J.J., Naing, A., Stepanek, V.M., Falchook, G.S., Fu, S., Garrido-Laguna, I., Tsimberidou, A.M., Piha-Paul, S.A., Moulder, S.L., et al. (2012). PIK3CA Mutations in Advanced Cancers: Characteristics and Outcomes. Oncotarget *3*, 1566–1575.
Jean, S., and Kiger, A.A. (2014). Classes of phosphoinositide 3-kinases at a glance. J. Cell Sci. *127*, 923–928.
Ji, L., Xu, J., Liu, J., Amjad, A., Zhang, K., Liu, Q., Zhou, L., Xiao, J., and Li, X. (2015). Mutant p53 promotes tumor cell malignancy by both positive and negative regulation of the transforming growth factor β (TGF-β) pathway. J. Biol. Chem. *290*, 11729–11740.
Johnson, C., Crowther, S., Stafford, M.J., Campbell, D.G., Toth, R., and MacKintosh, C. (2010). Bioinformatic and experimental survey of 14-3-3-binding sites. Biochem. J. *427*, 69–78.
Joost, H.-G. (2014). Diabetes and cancer: Epidemiology and potential mechanisms. Diabetes Vasc. Dis. Res. *11*, 390–394.
Joy, A., Kapoor, M., Georges, J., Butler, L., Chang, Y., Li, C., Crouch, A., Smirnov, I., Nakada, M., Hepler, J., et al. (2016). The role of AKT isoforms in glioblastoma: AKT3 delays tumor progression. J. Neurooncol. *130*, 43–52.
Karpe, F., Dickmann, J.R., and Frayn, K.N. (2011). Fatty acids, obesity, and insulin resistance: Time for a reevaluation. Diabetes *60*, 2441–2449.
Katso, R., Okkenhaug, K., Ahmadi, K., Timms, J., and Waterfield, M.D. (2001). 3-K INASES : Implications for Development , Class I PI3Ks. Annu. Rev. Cell Dev. Biol. *17*, 615–675.
Kim, M.P., Zhang, Y., and Lozano, G. (2014). Mutant p53: Multiple Mechanisms Define Biologic Activity in Cancer. Front. Oncol. *5*, 249.
Kok, K., Geering, B., and Vanhaesebroeck, B. (2009). Regulation of phosphoinositide 3-kinase expression in health and disease. Trends Biochem. Sci. *34*, 115–127.
Kristensen, C., Andersen, a S., Hach, M., Wiberg, F.C., Schäffer, L., and Kjeldsen, T. (1995). A single-chain insulin-like growth factor I/insulin hybrid binds with high affinity to the insulin receptor. Biochem. J. *305 (Pt 3*, 981–986.
Lang, G.A., Iwakuma, T., Suh, Y.A., Liu, G., Rao, V.A., Parant, J.M., Valentin-Vega, Y.A., Terzian, T., Caldwell, L.C., Strong, L.C., et al. (2004). Gain of function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome. Cell *119*, 861–872.
Laptenko, O., and Prives, C. (2006). Transcriptional regulation by p53: One protein, many

possibilities. Cell Death Differ. *13*, 951–961.
Larue, L., and Bellacosa, A. (2005). Epithelial-mesenchymal transition in development and cancer: Role of phosphatidylinositol 3??? kinase/AKT pathways. Oncogene *24*, 7443–7454.
Lee, Y.I., Lee, S., Das, G.C., Park, U.S., and Park, S.M. (2000). Activation of the insulin-like growth factor II transcription by aflatoxin B1 induced p53 mutant 249 is caused by activation of transcription complexes; implications for a gain-of-function during the formation of hepatocellular carcinoma. Oncogene *19*, 3717–3726.
Lefranc, F., Brotchi, J., and Kiss, R. (2005). Possible future issues in the treatment of glioblastomas: Special emphasis on cell migration and the resistance of migrating glioblastoma cells to apoptosis. J. Clin. Oncol. *23*, 2411–2422.
Leibiger, B., Moede, T., Uhles, S., Barker, C.J., Creveaux, M., Domin, J., Berggren, P.-O., and Leibiger, I.B. (2010). Insulin-feedback via PI3K-C2α activated PKBα/Akt1 is required for glucose-stimulated insulin secretion. FASEB J. *24*, 1824–1837.
De León, D.D., and Stanley, C.A. (2013). Determination of insulin for the diagnosis of hyperinsulinemic hypoglycemia. Best Pract. Res. Clin. Endocrinol. Metab. *27*, 763–769.
Li, Y., and Prives, C. (2007). Are interactions with p63 and p73 involved in mutant p53 gain of oncogenic function? Oncogene *26*, 2220–2225.
Li, X., Zhang, R., Luo, D., Park, S.J., Wang, Q., Kim, Y., and Min, W. (2005). Tumor necrosis factor α-induced desumoylation and cytoplasmic translocation of homeodomain-interacting protein kinase 1 are critical for apoptosis signal-regulating kinase 1-JNK/p38 activation. J. Biol. Chem. *280*, 15061–15070.
Li, X., Dai, X., Wan, L., Inuzuka, H., Sun, L., and North, B.J. (2016). Smurf1 regulation of DAB2IP controls cell proliferation and migration. Oncotarget *7*.
Liao, H., Xiao, Y., Hu, Y., Xiao, Y., Yin, Z., and Liu, L. (2015). microRNA-32 induces radioresistance by targeting DAB2IP and regulating autophagy in prostate cancer cells. Oncol. Lett. *10*, 2055–2062.
Liu, a X., Testa, J.R., Hamilton, T.C., Jove, R., Nicosia, S. V, and Cheng, J.Q. (1998). AKT2, a member of the protein kinase B family, is activated by growth factors, v-Ha-ras, and v-src through phosphatidylinositol 3-kinase in human ovarian epithelial cancer cells. Cancer Res. *58*, 2973–2977.
Liu, L., Xu, C., Hsieh, J.-T., Gong, J., and Xie, D. (2016). DAB2IP in cancer. Oncotarget *7*, 3766–3776.
Ludes-Meyers, J.H., Subler, M.A., Shivakumar, C. V, Munoz, R.M., Jiang, P., Bigger, J.E., Brown, D.R., Deb, S.P., and Deb, S. (1996). Transcriptional activation of the human epidermal growth factor receptor promoter by human p53. Mol. Cell. Biol. *16*, 6009–6019.
Lunardi, A., Di Minin, G., Provero, P., Dal Ferro, M., Carotti, M., Del Sal, G., and Collavin, L. (2010). A genome-scale protein interaction profile of Drosophila p53 uncovers additional nodes of the human p53 network. Proc. Natl. Acad. Sci. U. S. A. *107*, 6322–6327.
Luo, D., He, Y., Zhang, H., Yu, L., Chen, H., Xu, Z., Tang, S., Urano, F., and Min, W. (2008). AIP1 is critical in transducing IRE1-mediated endoplasmic reticulum stress response. J. Biol. Chem. *283*, 11905–11912.
Malaguarnera, R., and Belfiore, A. (2011). The insulin receptor: A new target for cancer therapy. Front. Endocrinol. (Lausanne). *2*, 1–16.
Manning, B., and Cantley, L. (2007). AKT/PKB Signalling: Navigating Downstream. Cell *129*, 1261–1274.
Masure, S., Haefner, B., Wesselink, J.J., Hoefnagel, E., Mortier, E., Verhasselt, P., Tuytelaars, A., Gordon, R., and Richardson, A. (1999). Molecular cloning, expression and characterization of the human serine/threonine kinase Akt-3. Eur.J Biochem *265*, 353–360.
Mayo, L.D., and Donner, D.B. (2002). The PTEN, Mdm2, p53 tumor suppressor-oncoprotein network. Trends Biochem. Sci. *27*, 462–467.

Meek, D.W. (2009). Tumour suppression by p53: A role for the DNA damage response? Nat. Rev. Cancer *9*, 714–723.
Min, J., Zaslavsky, A., Fedele, G., Mclaughlin, S.K., Reczek, E.E., Raedt, T. De, Guney, I., Strochlic, D.E., Beroukhim, R., Bronson, R.T., et al. (2010). NIH Public Access. *16*, 286–294.
Min, J., Liu, L., Li, X., Jiang, J., Wang, J., Zhang, B., Cao, D., Yu, D., Tao, D., Hu, J., et al. (2015). Absence of DAB2IP promotes cancer stem cell like signatures and indicates poor survival outcome in colorectal cancer. Sci. Rep. *5*, 1–12.
Min, W., Lin, Y., Tang, S., Yu, L., Zhang, H., Wan, T., Luhn, T., Fu, H., and Chen, H. (2008). AIP1 recruits phosphatase PP2A to ASK1 in tumor necrosis factor-induced ASK1-JNK activation. Circ. Res. *102*, 840–848.
Moelling, K., Schad, K., Bosse, M., Zimmermann, S., and Schweneker, M. (2002). Regulation of Raf-Akt cross-talk. J. Biol. Chem. *277*, 31099–31106.
Moller, D., Yokota, A., Caro, J., and Flier, J. (1989). Tissue-specific expression of two alternatively spliced insulin receptor mRNAs in man. Mol Endocrinol *3*, 1263–1269.
Moodie, S.A., Willumsen, B.M., Weber, M.J., and Wolfrnan, A. (1993). Complexes of Ras . GTP with Raf-1 and Mitogen-Activated Protein Kinase Kinase. Science (80-.). *260*, 1658–1661.
Mora, A., Komander, D., Van Aalten, D.M.F., and Alessi, D.R. (2004). PDK1, the master regulator of AGC kinase signal transduction. Semin. Cell Dev. Biol. *15*, 161–170.
Morselli, E., Tasdemir, E., Maiuri, M.C., Galluzzi, L., Kepp, O., Criollo, A., Vicencio, J.M., Soussi, T., and Kroemer, G. (2008). Mutant p53 protein localized in the cytoplasm inhibits autophagy. Cell Cycle *7*, 3056–3061.
Morton, J.P., Timpson, P., Karim, S.A., Ridgway, R.A., Athineos, D., Doyle, B., Jamieson, N.B., Oien, K.A., Lowy, A.M., Brunton, V.G., et al. (2010). Mutant p53 drives metastasis and overcomes growth arrest/senescence in pancreatic cancer. Proc. Natl. Acad. Sci. *107*, 246–251.
Moule, S.K., Welsh, G.I., Edgell, N.J., Foulstone, E.J., Proud, C.G., and Denton, R.M. (1997). Regulation of protein kinase B and glycogen synthase kinase-3 by insulin and beta-adrenergic agonists in rat epididymal fat cells. Activation of protein kinase B by wortmannin-sensitive and -insensitive mechanisms. J Biol Chem *272*, 7713–7719.
Muise-Helmericks, R.C., Grimes, H.L., Bellacosa, A., Malstrom, S.E., Tsichlis, P.N., and Rosen, N. (1998). Cyclin D expression is controlled post-translationally via a phosphatidylinoslitol 3-kinase/AKT dependent pathway. J Biol Chem *273*, 29864–29872.
Muller, P.A.J., and Vousden, K.H. (2013). P53 Mutations in Cancer. Nat. Cell Biol. *15*, 2–8.
Muller, P.A.J., and Vousden, K.H. (2014). Mutant p53 in cancer: New functions and therapeutic opportunities. Cancer Cell *25*, 304–317.
Muller, P.A.J., Caswell, P.T., Doyle, B., Iwanicki, M.P., Tan, E.H., Karim, S., Lukashchuk, N., Gillespie, D.A., Ludwig, R.L., Gosselin, P., et al. (2009). Mutant p53 Drives Invasion by Promoting Integrin Recycling. Cell *139*, 1327–1341.
Muller, P.A.J., Trinidad, A.G., Timpson, P., Morton, J.P., Zanivan, S., Van Den Berghe, P.V.E., Nixon, C., Karim, S.A., Caswell, P.T., Noll, J.E., et al. (2013). Mutant p53 enhances MET trafficking and signalling to drive cell scattering and invasion. Oncogene *32*, 1252–1265.
Muller, P.A.J., Trinidad, A.G., Caswell, P.T., Norman, J.C., and Vousden, K.H. (2014). Mutant p53 regulates dicer through p63-dependent and -independent mechanisms to promote an invasive phenotype. J. Biol. Chem. *289*, 122–132.
Muniyappa, R., and Sowers, J.R. (2012). Endothelial insulin and IGF-1 receptors: When yes means NO. Diabetes *61*, 2225–2227.
Musacchio, A., Gibson, T., Rice, P., Thompson, J., and Saraste, M. (1993). The PH domain: a common piece in the structural pathcwork of signalling proteins. Trends Biochem. Sci. *18*, 343–348.
Nadimpalli, R., Yalpani, N., Gurmukh, S.J., and Simmons, C.R. (2000). JBC Papers in Press. Published on June 26, 2000 as Manuscript M004371200.

Nakatani, K., Thompson, D. a, Barthel, A., Sakaue, H., Liu, W., Weigel, R.J., and Roth, R. a (1999). CELL BIOLOGY AND METABOLISM : Up-regulation of Akt3 in Estrogen Receptor-deficient Breast Cancers and Androgen-independent Prostate Cancer Lines Up-regulation of Akt3 in Estrogen Receptor-deficient Breast Cancers and Androgen-independent Prostate Cancer L. *274*, 3–8.
Nalefski, E.A., and Falke, J.J. (1996). The C2 domain calcium-binding motif: Structural and functional diversity. Protein Sci. *5*, 2375–2390.
Narasimhan, S.D., Yen, K., and Tissenbaum, H.A. (2009). Converging Pathways in Lifespan Regulation. Curr. Biol. *19*, R657–R666.
Novosyadlyy, R., Lann, D.E., Vijayakumar, A., Rowzee, A., Lazzarino, D.A., Fierz, Y., Carboni, J.M., Gottardis, M.M., Patricia, A., Molinolo, A.A., et al. (2011). Insulin-mediated acceleration of breast cancer development and progression in a non-obese model of type 2 diabetes. Cancer Res. *70*, 741–751.
Palomer, X., Pérez, A., and Blanco-Vaca, F. (2005). Adiponectina: un nuevo nexo entre obesidad, resistencia a la insulina y enfermedad cardiovascular. Med. Clin. (Barc). *124*, 388–395.
Pearce, L.R., Komander, D., and Alessi, D.R. (2010). The nuts and bolts of AGC protein kinases. Nat. Rev. Mol. Cell Biol. *11*, 9–22.
Pearson, R.B., Dennis, P.B., Han, J.W., Williamson, N.A., Kozma, S.C., Wettenhall, R.E., and Thomas, G. (1995). The principal target of rapamycin-induced p70s6k inactivation is a novel phosphorylation site within a conserved hydrophobic domain. EMBO J. *14*, 5279–5287.
Pelicci, G, Lanfrancone, L, Grignani, F, McGlade, J, Cavallo, F, Forni, G, Nicoletti, I, Grignani, F, Pawson, T, Pelicci, P. (1992). A novel trans- forming protein (SHC) with an SH2 domain is implicated in mito- genic signal transduction. Cell *70*, 93–104.
Pfister, N.T., Fomin, V., Regunath, K., Zhou, J.Y., Zhou, W., Silwal-, L., Freed-Pastor, W.A., Laptenko, O., Neo, S.P., Bargonetti, J., et al. (2015). Mutant p53 cooperates with the SWI / SNF chromatin remodeling complex to regulate VEGFR2 in breast cancer cells. 1–18.
Plas, D.R., and Thompson, C.B. (2005). Akt-dependent transformation: There is more to growth than just surviving. Oncogene *24*, 7435–7442.
Pommier, Y., Sordet, O., Antony, S., Hayward, R.L., and Kohn, K.W. (2004). Apoptosis defects and chemotherapy resistance: Molecular interaction maps and networks. Oncogene *23*, 2934–2949.
Pugacheva, E.N., Ivanov, A. V., Kravchenko, J.E., Kopnin, B.P., Levine, A.J., and Chumakov, P.M. (2002). Novel gain of function activity of p53 mutants: Activation of the dUTPase gene expression leading to resistance to 5-fluorouracil. Oncogene *21*, 4595–4600.
Pylayeva-Gupta, Y., Grabocka, E., and Bar-Sagi, D. (2013). RAS oncogenes:weaving a tumorigenic webss. Nat Rev Cancer *11*, 761–774.
Qiu, G.H., Xie, H., Wheelhouse, N., Harrison, D., Chen, G.G., Salto-Tellez, M., Lai, P., Ross, J.A., and Hooi, S.C. (2007). Differential expression of hDAB2IPA and hDAB2IPB in normal tissues and promoter methylation of hDAB2IPA in hepatocellular carcinoma. J. Hepatol. *46*, 655–663.
Ramjaun, A.R., and Downward, J. (2007). Ras and phosphoinositide 3-kinase: Partners in development and tumorigenesis. Cell Cycle *6*, 2902–2905.
Renehan, A.G., and Howell, A. (2005). Preventing cancer, cardiovascular disease, and diabetes. Lancet *365*, 1449–1451.
Renehan, A.G., Frystyk, J., and Flyvbjerg, A. (2006). Obesity and cancer risk: the role of the insulin-IGF axis. Trends Endocrinol. Metab. *17*, 328–336.
Restle, A., Färber, M., Baumann, C., Böhringer, M., Scheidtmann, K.H., Müller-Tidow, C., and Wiesmüller, L. (2008). Dissecting the role of p53 phosphorylation in homologous recombination provides new clues for gain-of-function mutants. Nucleic Acids Res. *36*, 5362–5375.

Roberts, D.L., Dive, C., and Renehan, A.G. (2010). Biological Mechanisms Linking Obesity and Cancer Risk: New Perspectives. Annu. Rev. Med. *61*, 301–316.
Rodriguez-Viciana, P., Warne, P.H., Khwaja, A., Marte, B.M., Pappin, D., Das, P., Waterfield, M.D., Ridley, A., and Downward, J. (1997). Role of phosphoinositide 3-OH kinase in cell transformation and control of the actin cytoskeleton by Ras. Cell *89*, 457–467.
Rostoker, R., Bitton-Worms, K., Caspi, A., Shen-Orr, Z., and LeRoith, D. (2013). Investigating new therapeutic strategies targeting hyperinsulinemia's mitogenic effects in a female mouse breast cancer model. Endocrinology *154*, 1701–1710.
Rothman, K.J. (2008). BMI-related errors in the measurement of obesity. Int. J. Obes. *32*, S56–S59.
Ruggero, D., and Sonenberg, N. (2005). The Akt of translational control. Oncogene *24*, 7426–7434.
Sampaio, C., Dance, M., Montagner, A., Edouard, T., Malet, N., Perret, B., Yart, A., Salles, J.-P., and Raynal, P. (2008). Signal Strength Dictates Phosphoinositide 3-Kinase Contribution to Ras/Extracellular Signal-Regulated Kinase 1 and 2 Activation via Differential Gab1/Shp2 Recruitment: Consequences for Resistance to Epidermal Growth Factor Receptor Inhibition. Mol. Cell. Biol. *28*, 587–600.
Sampath, J., Sun, D., Kidd, V.J., Grenet, J., Gandhi, A., Shapiro, L.H., Wang, Q., Zambetti, G.P., and Schuetz, J.D. (2001). Mutant p53 Cooperates with ETS and Selectively Up-regulates Human MDR1 Not MRP1. J. Biol. Chem. *276*, 39359–39367.
Sarbassov, D.D. (2005). Phosphorylation and Regulation of Akt/PKB by the Rictor-mTOR Complex. Science (80-.). *307*, 1098–1101.
Sasaki, A.T., Janetopoulos, C., Lee, S., Charest, P.G., Takeda, K., Sundheimer, L.W., Meili, R., Devreotes, P.N., and Firtel, R.A. (2007). G protein-independent Ras/PI3K/F-actin circuit regulates basic cell motility. J. Cell Biol. *178*, 185–191.
Schwahn, D.J., and Medina, D. (1998). p96, a MAPK-related protein, is consistently downregulated during mouse mammary carcinogenesis. Oncogene *17*, 1173–1178.
Scian, M.J. (2005). Tumor-Derived p53 Mutants Induce NF- B2 Gene Expression. Mol. Cell. Biol. *25*, 10097–10110.
Scian, M.J., Stagliano, K.E.R., Deb, D., Ellis, M.A., Carchman, E.H., Das, A., Valerie, K., Deb, S.P., and Deb, S. (2004). Tumor-derived p53 mutants induce oncogenesis by transactivating growth-promoting genes. Oncogene *23*, 4430–4443.
Seino, S., and Bell, G. (1989). Alternative splicing of human insulin receptor messenger RNA. Biochem. Biophys. Res. Commun. *159*, 312–316.
Shiojima, I., and Walsh, K. (2002). Role of Akt signaling in vascular homeostasis and angiogenesis. Circ. Res. *90*, 1243–1250.
Simpson, L., and Parsons, R. (2001). PTEN: Life as a Tumor Suppressor. Exp. Cell Res. *264*, 29–41.
Singer, S., Ehemann, V., Brauckhoff, A., Keith, M., Vreden, S., Schirmacher, P., and Breuhahn, K. (2007). Protumorigenic overexpression of stathmin/Op18 by gain-of-function mutation in p53 in human hepatocarcinogenesis. Hepatology *46*, 759–768.
Slupianek, A., Nieborowska-skorska, M., Hoser, G., Lymphomagenesis, L.K., Morrione, A., Majewski, M., Xue, L., Morris, S.W., Wasik, M. a, and Skorski, T. (2001). Role of Phosphatidylinositol 3-Kinase-Akt Pathway in Nucleophosmin/Anaplastic Lymphoma Kinase-mediated Lymphomagenesis Role of Phosphatidylinositol 3-Kinase-Akt Pathway in Nucleophosmin/Anaplastic. Cancer Res. *61*, 2194–2199.
Solomon, H., Buganim, Y., Kogan-Sakin, I., Pomeraniec, L., Assia, Y., Madar, S., Goldstein, I., Brosh, R., Kalo, E., Beatus, T., et al. (2012). Various p53 mutant proteins differently regulate the Ras circuit to induce a cancer-related gene signature. J. Cell Sci. *125*, 3144–3152.
Song, H., and Xu, Y. (2007). Gain of function of p53 cancer mutants in disrupting critical DNA

damage response pathways. Cell Cycle *6*, 1570–1573.
Sridhar, S.S., and Goodwin, P.J. (2009). Insulin-insulin-like growth factor axis and colon cancer. J. Clin. Oncol. *27*, 165–167.
Staal, S.P. (1987). Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma. Proc. Natl. Acad. Sci. U. S. A. *84*, 5034–5037.
Stambolsky, P., Tabach, Y., Fontemaggi, G., Weisz, L., Maor-, R., Shiff, I., Kogan, I., Shay, M., Kalo, E., Blandino, G., et al. (2010). NIH Public Access. *17*, 273–285.
St-Germain, M.-E., Gagnon, V., Parent, S., and Asselin, E. (2004). Regulation of COX-2 protein expression by Akt in endometrial cancer cells is mediated through NF-kappaB/IkappaB pathway. Mol. Cancer *3*, 7.
Strano, S., Dell'Orso, S., Di Agostino, S., Fontemaggi, G., Sacchi, A., and Blandino, G. (2007). Mutant p53: An oncogenic transcription factor. Oncogene *26*, 2212–2219.
Sun, S., and Steinberg, B.M. (2002). PTEN is a negative regulator of STAT3 activation in human papillomavirus-infected cells. J. Gen. Virol. *83*, 1651–1658.
Tan, B.S., Tiong, K.H., Choo, H.L., Fei-Lei Chung, F., Hii, L.-W., Tan, S.H., Yap, I.K., Pani, S., Khor, N.T., Wong, S.F., et al. (2015). Mutant p53-R273H mediates cancer cell survival and anoikis resistance through AKT-dependent suppression of BCL2-modifying factor (BMF). Cell Death Dis. *6*, e1826.
Taniguchi, C.M., Emanuelli, B., and Kahn, C.R. (2006). Critical nodes in signalling pathways: insights into insulin action. Nat. Rev. Mol. Cell Biol. *7*, 85–96.
Tanno, S., Tanno, S., Mitsuuchi, Y., Altomare, D.A., Xiao, G.H., and Testa, J.R. (2001). AKT activation up-regulates insulin-like growth factor I receptor expression and promotes invasiveness of human pancreatic cancer cells. Cancer Res. *61*, 589–593.
Testa, J.R., and Bellacosa, a (2001). AKT plays a central role in tumorigenesis. Proc. Natl. Acad. Sci. U. S. A. *98*, 10983–10985.
Toledo, F., Krummel, K.A., Lee, C.J., Liu, C.W., Rodewald, L.W., Tang, M., and Wahl, G.M. (2006). A mouse p53 mutant lacking the proline-rich domain rescues Mdm4 deficiency and provides insight into the Mdm2-Mdm4-p53 regulatory network. Cancer Cell *9*, 273–285.
Tsai, Y.-S., Lai, C.-L., Lai, C.-H., Chang, K.-H., Wu, K., Tseng, S.-F., Fazli, L., Gleave, M., Xiao, G., Gandee, L., et al. (2014). The role of homeostatic regulation between tumor suppressor DAB2IP and oncogenic Skp2 in prostate cancer growth. Oncotarget *5*, 6425–6436.
Tung, M.-C., Lin, P.-L., Wang, Y.-C., He, T.-Y., Lee, M.-C., Yeh, S.D., Chen, C.-Y., and Lee, H. (2015). Mutant p53 confers chemoresistance in non-small cell lung cancer by upregulating Nrf2. Oncotarget *6*, 41692–41705.
Valentino, E., Bellazzo, A., Di, G., Sicari, D., Apollonio, M., and Scognamiglio, G. (2017a). Mutant p53 potentiates the oncogenic effects of insulin by inhibiting the tumor suppressor DAB2IP. 124.
Valentino, E., Bellazzo, A., Di, G., Sicari, D., Apollonio, M., and Scognamiglio, G. (2017b). Mutant p53 potentiates the oncogenic effects of insulin by inhibiting the tumor suppressor DAB2IP. *0*, 124.
VANHAESEBROECK, B., and ALESSI, D.R. (2000). The PI3K–PDK1 connection: more than just a road to PKB. Biochem. J. *346*, 561.
Vara, J.Á.F., Casado, E., de Castro, J., Cejas, P., Belda-Iniesta, C., and González-Barón, M. (2004). PI3K/Akt signalling pathway and cancer. Cancer Treat. Rev. *30*, 193–204.
Virkamäki, a, Ueki, K., and Kahn, C.R. (1999). Protein-protein interaction in insulin signaling and the molecular mechanisms of insulin resistance. J. Clin. Invest. *103*, 931–943.
Vogelstein, B., Lane, D., and Levine, A.J. (2000). Surfing the p53 network. Nature *408*, 307–310.
Vousden, K.H., and Prives, C. (2009). Blinded by the Light: The Growing Complexity of p53.

Cell *137*, 413–431.
Wang, J., Zhu, X., Hu, J., He, G., Li, X., Wu, P., Ren, X., Wang, F., Liao, W., Liang, L., et al. (2015). The positive feedback between Snail and DAB2IP regulates EMT, invasion and metastasis in colorectal cancer. Oncotarget *6*, 27427–27439.
Wang, Z., Tseng, C.P., Pong, R.C., Chen, H., McConnell, J.D., Navone, N., and Hsieh, J.T. (2002). The mechanism of growth-inhibitory effect of DOC-2/DAB2 in prostate cancer. Characterization of a novel GTPase-activating protein associated with N-terminal domain of DOC-2/DAB2. J. Biol. Chem. *277*, 12622–12631.
Wasylishen, A.R., and Lozano, G. (2017). Attenuating the p53 Pathway in Human Cancers : Many Means to the Same End.
Webster, N.J.G., Resnik, J.L., Reichart, D.B., Strauss, B., Haas, M., and Seely, B.L. (1996). Repression of the Insulin Receptor Promoter by the Tumor Suppressor Gene Product p53 : A Possible Mechanism for Receptor Overexpression in Breast Cancer Repression of the Insulin Receptor Promoter by the Tumor Suppressor Gene in Breast Cancer1. Cancer Res. *56*, 2781–2788.
Weinstein, D., Sarfstein, R., Laron, Z., and Werner, H. (2014). Insulin receptor compensates for IGF1R inhibition and directly induces mitogenic activity in prostate cancer cells. Endocr. Connect. *3*, 24–35.
Weissmueller, S., Manchado, E., Saborowski, M., Iv, J.P.M., Wagenblast, E., Davis, C.A., Moon, S., Pfister, N.T., Darjus, F., Kitzing, T., et al. (2015). NIH Public Access. *157*, 382–394.
Weisz, L., Zalcenstein, A., Stambolsky, P., Cohen, Y., Goldfinger, N., and Oren, M. (2004). Transactivation of the EGR1 Gene Contributes to Mutant p53 Gain of Function Transactivation of the EGR1 Gene Contributes to Mutant p53 Gain of Function. 8318–8327.
Weisz, L., Oren, M., and Rotter, V. (2007). Transcription regulation by mutant p53. Oncogene *26*, 2202–2211.
Wells, V., Downward, J., and Mallucci, L. (2007). Functional inhibition of PI3K by the ??GBP molecule suppresses Ras-MAPK signalling to block cell proliferation. Oncogene *26*, 7709–7714.
Wennström, S., and Downward, J. (1999). Role of phosphoinositide 3-kinase in activation of ras and mitogen-activated protein kinase by epidermal growth factor. Mol. Cell. Biol. *19*, 4279–4288.
Werner, H., Karnieli, E., Rauscher, F.J., and LeRoith, D. (1996). Wild-type and mutant p53 differentially regulate transcription of the insulin-like growth factor I receptor gene. Proc. Natl. Acad. Sci. U. S. A. *93*, 8318–8323.
Whittaker, J., and Whittaker, L. (2005). Characterization of the functional insulin binding epitopes of the full-length insulin receptor. J. Biol. Chem. *280*, 20932–20936.
Włodarski, P., Grajkowska, W., Łojek, M., Rainko, K., and Jóźwiak, J. (2006). Activation of Akt and Erk pathways in medulloblastoma. Folia Neuropathol. *44*, 214–220.
Wolf, G., Trub, T., Ottinger, E., Groninga, L., Lynch, A., White, M.F., Miyazaki, M., Lee, J., and Shoelson, S.E. (1995). PTB domains of IRS-1 and Shc have distinct but overlapping binding specificities. J. Biol. Chem. *270*, 27407–27410.
Wolin, K.Y., Carson, K., and Colditz, G.A. (2010). Obesity and Cancer. Oncologist 556–565.
Wu, K., Xie, D., Zou, Y., Zhang, T., Pong, R.C., Xiao, G., Fazli, L., Gleave, M., He, D., Boothman, D. a., et al. (2013). The mechanism of DAB2IP in chemoresistance of prostate cancer cells. Clin. Cancer Res. *19*, 4740–4749.
Wu, K., Liu, J., Tseng, S.-F., Gore, C., Ning, Z., Sharifi, N., Fazli, L., Gleave, M., Kapur, P., Xiao, G., et al. (2014). The role of DAB2IP in androgen receptor activation during prostate cancer progression. Oncogene *33*, 1954–1963.
Wu, K., Wang, B., Chen, Y., Zhou, J., Huang, J., Hui, K., Zeng, J., Zhu, J., Zhang, K., Li, L., et al. (2015). DAB2IP regulates the chemoresistance to pirarubicin and tumor recurrence of non-muscle invasive bladder cancer through STAT3/Twist1/P-glycoprotein signaling. Cell. Signal.

27, 2515–2523.
Wysocki, P.J., and Wierusz-Wysocka, B. (2010). Obesity, hyperinsulinemia and breast cancer: novel targets and a novel role for metformin. Expert Rev. Mol. Diagn. *10*, 509–519.
Xie, D., Gore, C., Zhou, J., Pong, R.-C., Zhang, H., Yu, L., Vessella, R.L., Min, W., and Hsieh, J.-T. (2009). DAB2IP coordinates both PI3K-Akt and ASK1 pathways for cell survival and apoptosis. Proc. Natl. Acad. Sci. U. S. A. *106*, 19878–19883.
Xie, D., Gore, C., Liu, J., Pong, R.-C., Mason, R., Hao, G., Long, M., Kabbani, W., Yu, L., Zhang, H., et al. (2010). Role of DAB2IP in modulating epithelial-to-mesenchymal transition and prostate cancer metastasis. Proc. Natl. Acad. Sci. U. S. A. *107*, 2485–2490.
Xu, G., Zhang, W., and Bertram, P. (2004). Pharmacogenomic profiling of the PI3K / PTEN-AKT-mTOR pathway in common human tumors. Int. J. Oncol. 893–900.
Xu, J., Reumers, J., Couceiro, J.R., De Smet, F., Gallardo, R., Rudyak, S., Cornelis, A., Rozenski, J., Zwolinska, A., Marine, J.C., et al. (2011). Gain of function of mutant p53 by coaggregation with multiple tumor suppressors. Nat. Chem. Biol. *7*, 285–295.
Xu, Y., He, J., Wang, Y., Zhu, X., Pan, Q., Xie, Q., and Sun, F. (2015). MiR-889 promotes proliferation of esophageal squamous cell carcinomas through DAB2IP. FEBS Lett. *589*, 1127–1135.
Yan, W., and Chen, X. (2009). Identification of GRO1 as a critical determinant for mutant p53 gain of function. J. Biol. Chem. *284*, 12178–12187.
Yang, J., Cron, P., Thompson, V., Good, V.M., Hess, D., Hemmings, B.A., and Barford, D. (2002). Molecular mechanism for the regulation of protein kinase B/Akt by hydrophobic motif phosphorylation. Mol. Cell *9*, 1227–1240.
Yano, M., Toyooka, S., Tsukuda, K., Dote, H., Ouchida, M., Hanabata, T., Aoe, M., Date, H., Gazdar, A.F., and Shimizu, N. (2005). Aberrant promoter methylation of human DAB2 interactive protein (hDAB2IP) gene in lung cancers. Int. J. Cancer *113*, 59–66.
Yart, A., Roche, S., Wetzker, R., Laffargue, M., Tonks, N., Mayeux, P., Chap, H., and Raynal, P. (2002). A function for phosphoinositide 3-kinase β lipid products in coupling βγ to Ras activation in response to lysophosphatidic acid. J. Biol. Chem. *277*, 21167–21178.
Yenush, L., Makati, K.J., Smith-Hall, J., Ishibashi, O., Myers, M.G., and White, M.F. (1996). The pleckstrin homology domain is the principal link between the insulin receptor and IRS-1. J. Biol. Chem. *271*, 24300–24306.
Yi, K.H., and Lauring, J. (2016). Recurrent AKT mutations in human cancers: functional consequences and effects on drug sensitivity. Oncotarget *7*, 4241–4251.
Yoeli-Lerner, M., Yiu, G.K., Rabinovitz, I., Erhardt, P., Jauliac, S., and Toker, A. (2005). Akt blocks breast cancer cell motility and invasion through the transcription factor NFAT. Mol. Cell *20*, 539–550.
Yu, L., Qin, L., Zhang, H., He, Y., Chen, H., Pober, J.S., Tellides, G., and Min, W. (2011). AIP1 prevents graft arteriosclerosis by inhibiting interferon-γ- dependent smooth muscle cell proliferation and intimal expansion. Circ. Res. *109*, 418–427.
Yu, L., Tumati, V., Tseng, S.-F., Hsu, F.-M., Kim, D.N., Hong, D., Hsieh, J.-T., Jacobs, C., Kapur, P., and Saha, D. (2012). DAB2IP Regulates Autophagy in Prostate Cancer in Response to Combined Treatment of Radiation and a DNA-PKcs Inhibitor. Neoplasia *14*, 1203–IN36.
Yuan, Z.Q., Sun, M., Feldman, R.I., Wang, G., Ma, X., Jiang, C., Coppola, D., Nicosia, S. V, and Cheng, J.Q. (2000). Frequent activation of AKT2 and induction of apoptosis by inhibition of phosphoinositide-3-OH kinase/Akt pathway in human ovarian cancer. Oncogene *19*, 2324–2330.
Yue, X., Zhang, C., Zhao, Y., Liu, J., Lin, A.W., Tan, V.M., Drake, J.M., Liu, L., Boateng, M.N., Li, J., et al. (2017). Gain-of-function mutant p53 activates small GTPase Rac1 through SUMOylation to promote tumor progression. Genes Dev. *31*, 1641–1654.
Yun, E.J., Baek, S.T., Xie, D., Tseng, S.F., Dobin, T., Hernandez, E., Zhou, J., Zhang, L., Yang, J., Sun, H., et al. (2015). DAB2IP regulates cancer stem cell phenotypes through modulating

stem cell factor receptor and ZEB1. Oncogene *34*, 2741–2752.
Yun, E.-J., Baek, S.T., Xie, D., Tseng, S.-F., Dobin, T., Hernandez, E., Zhou, J., Zhang, L., Yang, J., Sun, H., et al. (2014). DAB2IP regulates cancer stem cell phenotypes through modulating stem cell factor receptor and ZEB1. Oncogene 1–12.
Zerdoumi, Y., Aury-Landas, J., Bonaïti-Pellié, C., Derambure, C., Sesboüé, R., Renaux-Petel, M., Frebourg, T., Bougeard, G., and Flaman, J.M. (2013). Drastic Effect of Germline TP53 Missense Mutations in Li-Fraumeni Patients. Hum. Mutat. *34*, 453–461.
Zhang, B.H., Tang, E.D., Zhu, T., Greenberg, M.E., Vojtek, A.B., and Guan, K.L. (2001). Serum- and Glucocorticoid-inducible Kinase SGK Phosphorylates and Negatively Regulates B-Raf. J. Biol. Chem. *276*, 31620–31626.
Zhang, H., Zhang, R., Luo, Y., D'Alessio, A., Pober, J.S., and Min, W. (2004). AIP1/DAB2IP, a novel member of the Ras-GAP family, transduces TRAF2-induced ASK1-JNK activation. J. Biol. Chem. *279*, 44955–44965.
Zhang, H., He, Y., Dai, S., Xu, Z., Luo, Y., Wan, T., Luo, D., Jones, D., Tang, S., Chen, H., et al. (2008). AIP1 functions as an endogenous inhibitor of VEGFR2-mediated signaling and inflammatory angiogenesis in mice. J. Clin. Invest. *118*, 3904–3916.
Zhang, H., Fagan, D.H., Zeng, X., Freeman, K.T., Sachdev, D., and Yee, D. (2010). Inhibition of Cancer Cell Proliferation and Metastasis by Insulin Receptor Downregulation. Oncogene *29*, 2517–2527.
Zhang, J., Zhou, H.J., Ji, W., Min, W., First, T., Hospital, A., and Haven, N. (2016). Arteriosclerosis. *17*, 1–11.
Zhang, R., He, X., Liu, W., Lu, M., Hsieh, J.T., and Min, W. (2003). AIP1 mediates TNF-α-induced ASK1 activation by facilitating dissociation of ASK1 from its inhibitor 14-3-3. J. Clin. Invest. *111*, 1933–1943.
Zhang, R., Zhang, H., Lin, Y., Li, J., Pober, J.S., and Min, W. (2007). RIP1-mediated AIP1 phosphorylation at a 14-3-3-binding site is critical for tumor necrosis factor-induced ASK1-JNK/p38 activation. J. Biol. Chem. *282*, 14788–14796.
Zhang, X., Tang, N., Hadden, T.J., and Rishi, A.K. (2011). Akt, FoxO and regulation of apoptosis. Biochim. Biophys. Acta - Mol. Cell Res. *1813*, 1978–1986.
Zhou, G., and Myers, J.N. (2014). Mutant p53 exerts oncogenic functions by modulating cancer cell metabolism. Mol. Cell. Oncol. *1*, e963441.
Zhou, B.P., Liao, Y., Xia, W., Zou, Y., Spohn, B., and Hung, M.C. (2001). HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. Nat. Cell Biol. *3*, 973–982.
Zhou, H., Li, X.M., Meinkoth, J., and Pittman, R.N. (2000). Akt regulates cell survival and apoptosis at a postmitochondrial level. J. Cell Biol. *151*, 483–494.
Zimmermann, S., and Moelling, K. (2016). Phosphorylation and Regulation of Raf by Akt (Protein Kinase B) Published by : American Association for the Advancement of Science Stable URL : http://www.jstor.org/stable/2900102 REFERENCES Linked references are available on JSTOR for this article : Ph. *286*, 1741–1744.