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ROLE OF MUTANT P53 AS MECHANOSENSITIVE REGULATOR OF AMINO-ACID METABOLISM IN BREAST CANCER

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ABSTRACT

Amino acids are crucial nutrients for cancer cells since they provide plenty of metabolic and energetic intermediates and promote their survival in challenging environments, thus enabling them to proliferate, disseminate and generate metastases. Tumors are indeed avid for amino acids and, consequently, they aberrantly increase amino acids intake, biosynthesis and catabolism. This metabolic reprogramming represents an Achille's heel of tumors. Thus, unveiling the oncogenic drivers that reprogram amino acid metabolism in cancer is fundamental to understand disease progression and to find therapeutic opportunities.

In this work, we disclosed a new role of mutp53 in regulating amino acid metabolism in breast cancer cells. We demonstrated that mutp53 promotes synthesis of aspartate, serine and glycine through upregulation of amino acids biosynthetic enzymes and increases the expression of specific amino acids transporters. Our findings indicate that mutp53, unleashing this metabolic program, supports metabolic adaptation to environmental stresses, such as nutrient starvation. Indeed, in conditions of amino acids scarcity, mutp53 sustained cancer cells survival and proliferation via upregulation of serine synthesis and BCAAs/bulky amino acids intake. Furthermore, we showed that a stiff ECM cooperates with mutp53 in the induction of such genes, unveiling a novel branch of amino acid metabolism regulated in response to mechanical inputs and fostered by mutp53. Notably, inhibition of either mechanotransduction or serine synthesis or BCAAs/bulky amino acids intake was able to blunt the pro-survival effects exerted by mutp53 on cancer cells proliferation in amino acids deprivation.

1. INTRODUCTION

1.1 METABOLIC REWIRING IN CANCER

Tumors are multifactorial diseases characterized by uncontrolled growth and invasion of normal tissues resulting in the alteration of their functionalities. Tumorigenesis is a multistep process that mainly starts from the accumulation of mutations in the DNA of normal cells which, in some cases, acquire the ability to evade the surveillance mechanisms. The consequent rapid cell replications further promote genomic instability thus generating subclones with several genetic lesions that evolve towards a neoplastic state. Thus, cancer cells acquire some distinct and complementary traits that are considered "*hallmarks of cancer*", such as evasion from anti-proliferative signals, senescence, and programmed cell death[1].

Tumors develop in a host healthy tissue that activates several mechanisms to limit and eradicate the neoplastic lesions. This is achieved by setting up a coordinate response that recruits immune cells, induces inflammation, and promotes matrix deposition that encapsules and fights the growing tumor mass. As a response, cancer cells not only activate several processes to cope with these adverse conditions but also exploit them to get an advantage over the surrounding tissue. Moreover, tumors continuously reshape the host environment by reprogramming stromal, immune, and endothelial cells to sustain tumor fitness and to induce the formation of a tumor-associated neo-vasculature that guarantees nutrients and oxygen. Thus, tumors are not merely constituted by cancer cells but, instead, they are defined as ecosystems characterized by different cellular and non-cellular components whose dynamic interplay is critical for tumor progression.

In this regard, cell metabolism has been demonstrated to act as a central cellular program that sustains cancer cell fitness, alters cell signaling pathways and modulates the tumor microenvironment (TME), influencing the entire tumor ecosystem. Cancer cells exhibit a metabolic rewiring that is emerging not only as an additional *hallmark of cancer* but as a central pillar that underlies and enables almost each *hallmark*[2,3]. Indeed, altered metabolic fluxes are essential for cancer cells to allow their rapid proliferation, to endow them with migratory and invasive abilities, to guarantee their survival in harsh microenvironments, and to establish interactions with stromal cells[4]. To satisfy their metabolic needs, tumors increase the uptake and utilization of nutrients, including glucose, lipids, and amino acids. This ensures availability of energy and macromolecules for anabolic growth during nutrient-replete conditions and sustenance of survival through catabolism during nutrient-deprived conditions[3,4]. In addition, metabolic rewiring is required to provide reducing equivalents fundamental to buffer the

excessive oxidative stress frequently experienced by cancer cells and for epigenetic and post-translational modifications, thus tuning cellular signaling pathways[3,4].

The best example of metabolic reprogramming in tumors is the Warburg effect, a condition in which cancer cells increase the intake and utilization of glucose via glycolysis regardless of oxygen availability[5]. This high glycolytic flux is crucial to sustain the generation of ATP and to supply intermediates for anabolic pathways. Despite an initial observation describing that mitochondria were dysfunctional in cancer cells, it is now well established that tumors enhance both glycolysis and mitochondria functions to fulfill their metabolic demands in terms of ATP production and of precursors for the synthesis of macromolecules such as proteins, lipids, and nucleic acids[6,7]. More recently, it has been demonstrated that a main feature of cancer cells is an extreme avidity for amino acids, used for biomass generation and energy production[8].

Cancer cells display two important metabolic capacities that confers adaptive advantages during tumorigenesis, termed metabolic flexibility and metabolic plasticity[9]. Metabolic flexibility concerns their ability to use different metabolites to meet the same metabolic requirement. Indeed, instead of glucose, cancer cells can use a widespread repertoire of nutrients such as glutamine, acetate, fatty acids, and other amino acids as fuels for their core metabolic functions. This phenomenon is highly advantageous since it allows cancer cells to avoid a rigid dependency on specific nutrients[3]. Therefore, metabolic flexibility is particularly relevant to enable cancer cells metabolic plasticity. This term refers to the capacity of tumors to best align their metabolic activities across tumor states and environments. Indeed, they are able to differentially use the same metabolite to face various metabolic requirements, overcoming the difficulties associated with changing and nutrient-limited conditions[9,10]. For instance, it has been reported that human cancer cells rely on exogenous serine to rapidly proliferate, and they redirect glucose from aerobic glycolysis to serine synthesis pathway, when serine is limiting in the environment, resulting in a compensative increased flux to the tricarboxylic acid cycle (TCA) to provide energy. Furthermore, scarce serine stores are preferentially channeled to glutathione synthesis, rather than to nucleotides synthesis, in order to preserve cellular anti-oxidant capacity [11].

1.1.1 Signaling pathways control cancer metabolism

Metabolic reprogramming in tumors is driven by a combination of genetic alterations and environmental conditions[12]. The discovery of a key set of cancer-associated mutations in genes encoding metabolic enzymes has provided a direct link between altered metabolism and cancer[13]. These specific gene mutations result in the generation of metabolites that accumulate in cells and play a role in cellular transformation and tumorigenesis. These so called “oncometabolites” include the 2-

hydroxyglutarate (2HG), produced as a consequence of mutations in the isocitrate dehydrogenase (IDH), and the TCA cycle metabolites succinate and fumarate, whose levels increased due to loss-of function mutations in genes encoding for fumarate hydratase (FH) and succinate dehydrogenase (SDH) respectively[13]. These metabolites act both as biosynthetic and signaling molecules. This implies that they can dysregulate several cellular processes and alter epigenetic and post-translational modifications impacting on gene expression and promoting development of malignancies[13,14].

However, genetic alterations that lead to oncometabolites production are not so common events and are unable to account for the complex metabolic rewiring exhibited by tumors, including glucose, mitochondria, fatty acids, amino acids and nucleotides metabolism[3]. Indeed, the need of having mutations in metabolic enzymes *per se* is mitigated by a more frequent coordinated metabolic reprogramming that is achieved through alterations in a limited number of major oncogenic pathways[12]. The aberrant regulation of these pathways in tumors is dictated by perturbation of a restricted number of highly connected signaling nodes such as phosphatidylinositol 3-kinase (PI3K)-AKT- mammalian target of rapamycin (mTOR) pathway, hypoxia-inducible factor-1 (HIF1), sterol regulatory element-binding protein (SREBP), MYC and p53 (Fig. 1)[3].

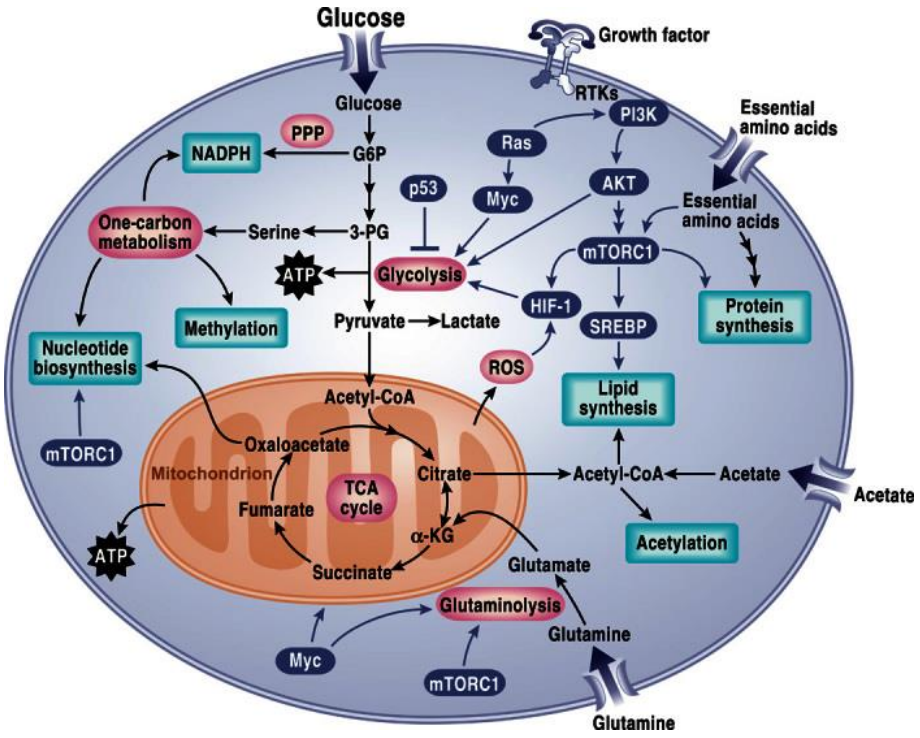


Figure 1. Signaling pathways in the regulation of cancer cells metabolism. Cancer cells enhance the uptake of glucose and amino acids to activate and support biosynthetic programs. The nutritional stimuli together with the regulation mediated by oncogenes and tumor suppressors (in dark blue) determine an aberrant activation of anabolic and catabolic pathways (in light blue and pink respectively)[3].

mTOR sits at the convergence point of this vast signaling network acting both as a metabolic sensor and regulator that integrates a wide range of environmental and intracellular signals to properly control cell growth and survival. In particular, in response to growth factors and in presence of adequate amount of nutrients in the cells, mTORC1 promotes several anabolic processes while dampening catabolic processes such as autophagy[15,16]. Thereby, it is not surprising that the mTORC1 pathway is hyperactivated in tumors: this occurs in more than 80% of malignancies and it is rarely due to mutations on mTOR itself but, instead, it is caused by mutations in upstream regulators, such as PI3K, the tumor suppressor phosphatase and tensin homolog (PTEN) and Ras[15,17]. All these events result in the activation of a stable and robust anabolic growth program. Indeed, mTORC1 signaling leads to activation of several transcription factors including MYC, HIF1α and SREBP, which consequently induce glycolysis, angiogenesis, and metabolic rearrangements in amino acid, lipid, and nucleotide metabolism. Concomitantly, activation of these pathways promotes the expression of transporters for the intake of glucose and amino acids, ensuring an appropriate supply of nutrients required to sustain the chronic activation of mTORC1[16].

In addition, genetic alterations that directly lead to constitutive activation of oncogenes like HIF1, Ras and MYC, and to loss of tumor suppressors like p53 further foster anabolism through transcriptional regulation of several metabolic genes[3]. MYC is the master regulator of cell metabolism and it is often hijacked in cancers by gene amplifications, mutations, and chromosomal translocations. MYC drives aberrant transcription of genes for enzymes crucial for several metabolic pathways and for the membrane transporters to supply nutrients[18]. Indeed, it is well known that MYC-transformed cells display increased uptake and utilization of glucose and glutamine to provide energy and intermediates for shunt pathways[19]. In addition, MYC promotes amino acid metabolism at various levels: it increases the expression of enzymes of the serine synthesis pathway (SSP) and it favors the intake and catabolism of essential amino acids (EAAs)[20–22]. This metabolic reprogramming fosters nucleotide synthesis, generation of reducing equivalents, production of one-carbon donors for epigenetic regulation and activation of mTORC1 pathway[19]. In particular, the increased intake of EAAs promoted by MYC further sustains MYC protein levels in a mTORC1-dependent manner, thus establishing a feed-forward loop[23].

Beside oncogenes, loss of major tumor suppressors like p53, leads to significant metabolic rearrangements that sustain malignant cells proliferation. P53 oncosuppressive function has been attributed to its capability to support cell survival through DNA repair and cell cycle arrest in response to mild stresses or even to promote senescence and cell death when cells are too much damaged. However, in recent years, a central role of p53 in cell metabolism has emerged, through regulation of glucose, lipid, and amino acid metabolism[24]. Of note, a provocative genetic work highlights that

a p53 mutant able to modulate cell metabolism and antioxidant functions but unable to induce cell cycle arrest, senescence, and apoptosis, is sufficient to impede early-onset tumor formation in mice, unlike what happens in p53 null mice[25]. This suggests that unconventional p53-mediated activities are critical to suppress tumorigenesis. Overall, loss of p53 fosters glycolysis resulting in enhanced anabolic capacity and redox balance, key processes that sustain cancer cell survival and growth[24].

1.1.2 Cancer metabolism is influenced by environmental and tissue's features

Cancer cells metabolism is a dynamic process regulated by a plethora of intrinsic and extrinsic factors that fine tune metabolic adjustments according to nutrients' availability and cell requirements. Indeed, the properties of the parental tissue and of the TME strongly influence tumor metabolism[26–28].

The tissue of origin affects cancer metabolism

Malignancies retain some metabolic traits of the normal tissue from which they arose, thus suggesting that in cancers oncogenes hijack the existing tissue metabolism to sustain their aberrant growth. Of note, a study that classified 1100 human tumors in 33 cancer subtypes, reveal that tissue of origin is a major determinant of DNA methylation and thus of gene expression pattern[29]. Focusing on the metabolic network, tumors gene expression signatures are more similar to those of parental normal tissues than to tumors that developed in different organs[30]. This is consistent with the metabolic heterogeneity of tumors and with the fact that some metabolic features, as well as liabilities, are subtype-specific rather than uniform across malignancies[31]. Coherently, distinct metabolic profiles characterize tumors that develop in different tissues, even when they are initiated by the same driver mutation[4,26]. For instance, MYC-induced liver tumors enhance glutamine catabolism while MYC-induced lung tumors synthesize glutamine from glucose[32]. Thereby, although there are metabolic activities chronically activated by genetic alterations in cancer cells, the emerging view is that the resulting metabolic program is highly context specific and flexible.

Nutrient availability in the TME directs cell metabolism

The TME is extremely heterogeneous and dynamic, characterized by challenging conditions such as reduced oxygen availability, acidic conditions and by changes in nutrient availability that continuously modulate cancer cells metabolism.

In general, nutrient availability is strongly influenced by anatomical location of the tumor, tumor type, and host diet[33]. Moreover, when a solid tumor evolves from a small pool of malignant cells to a larger mass, different regions of the tumor experience different accessibility to nutrients and oxygen, depending on the proximity to blood vessels and on the perturbed surrounding tissue architecture[34].

For instance, amino acids used for multiple anabolic processes such as glutamine, serine, asparagine, and aspartate rapidly become depleted in the core regions of the tumor mass compared to the periphery, probably due to their heavy consumption and the poor blood supply of those regions[35]. When nutrients are exceptionally scarce, tumor cells rely on autophagy or on scavenging of proteins and necrotic cell debris from the TME through micropinocytosis, thus deriving elements for the synthesis of macromolecules and for feeding the central carbon metabolism[36,37]. Therefore, to maintain their fitness, cancer cells adapt to the different conditions encountered and acquire different metabolic phenotypes thus creating an intra-tumoral metabolic heterogeneity[9,26].

Metabolic competition and metabolic coupling among cells in the TME modulate cell metabolism

In the tumor ecosystem, cancer, stromal and immune cells coexist and exhibit distinct nutrients preference and utilizations. Recent works raise the possibility that competitive and cooperative relationships may be established between distinct populations of cells in the TME but how different cell types share nutrients and mutually contribute to alter their metabolism is still under investigation (Fig. 2)[27,38].

Fast proliferating cancer cells are extremely avid for nutrients and thus they must compete for those with non-malignant cells present in the TME. Surprisingly, a recent study shows that glucose is not a limiting factor in the TME, since tumor cells are programmed to drive a preferential uptake and consumption of glutamine instead of glucose, which remains available and effectively acquired by myeloid cells and T-cells[39]. This opens the perspective that amino acids, rather than glucose, may be limiting in the TME. Indeed, glutamine, serine, glycine, arginine, alanine, tryptophan, and methionine are strongly needed for both cancer cells proliferation and cytotoxic T cells functions[33,40]. In agreement, cancer cells, compared to cytotoxic T cells, display a competitive advantage for methionine, increasing the levels of its transporter. This leads to reduced methionine availability for T cells and consequent modification of their histone methylation patters thus hampering their functions[40]. Therefore, depletion of amino acids from the extracellular milieu, due to high consumption by cancer cells, may be a way to contribute to evade anticancer immune response. Beside nutrient competition, cancer cells engage other strategies to avoid T cells-mediated killing, including the release of metabolites such as lactate and kynurenine which promotes immunosuppressive cell response[41].

On the contrary, cells in the TME can establish interactions that mutually support their growth. The metabolic coupling, that indicates the capability of different cells to exchange nutrients and metabolites, may occur between different cancer cells in the tumor mass or even between cancer cells and stromal/immune cells. A paradigmatic example is provided by the lactate generated by cancer

cells during glycolysis and secreted in the TME. Lactate, in turn, can be imported by a subpopulation of tumor cells, that exhibit an oxidative phenotype, or by cancer-associated fibroblasts (CAFs), being subsequently converted into pyruvate to foster the TCA cycle[42,43]. Many other examples of symbiotic relationships between cancer cells and stromal cells can be cited and, among these, in pancreatic tumors, stroma-associated pancreatic stellate cells and CAFs are reported to secrete alanine and glutamine respectively, thus supplying TCA cycle in tumor cells[44,45]

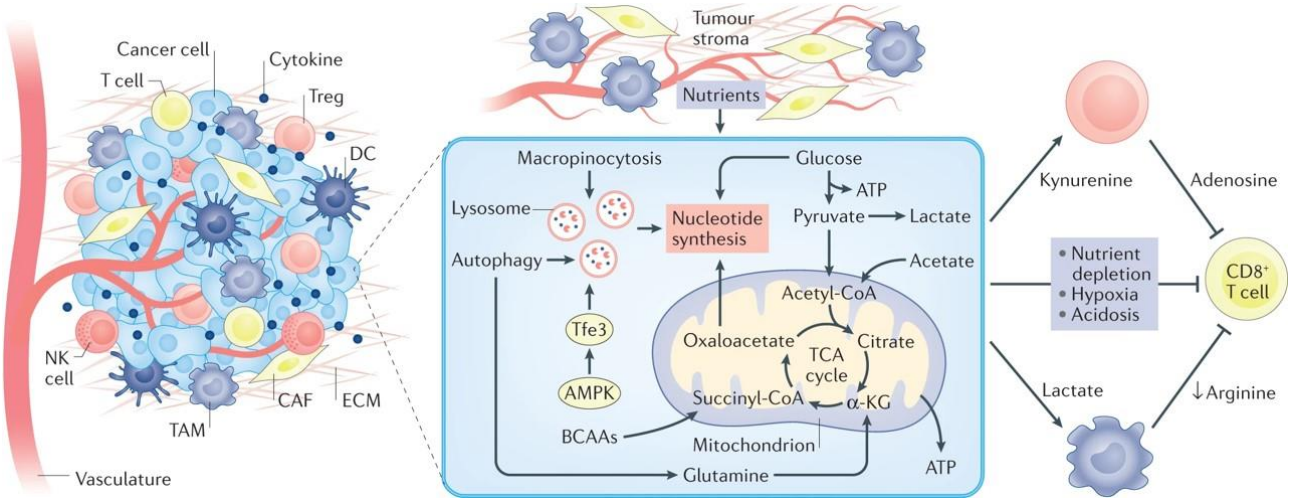


Figure 2. Metabolic interactions among cells in the TME. Exemplified view of the different cell types that coexist in the TME and establish both competitive and cooperative interactions. In details, cancer cells survival in nutrient limited environment is achieved by activating macropinocytosis and autophagy as well as by exploiting nutrients and metabolites provided by stromal cells. In turn, tumor cells release metabolites, such as kynurenine and lactate, that induce an immunosuppressive cell response meanwhile they inhibit T-cells cytotoxic functions mainly by limiting amino acids availability[12].

TME physical properties regulate cancer metabolism

Growing tumors are continuously subjected to different types of mechanical forces generated by increased cell density of cancer cells themselves and of neighboring normal cells, by increased interstitial fluid pressure and by altered extracellular matrix (ECM). The latter is a complex network of proteins, mainly secreted by CAFs, whose aberrant deposition and altered organization are primarily responsible for increasing TME stiffness[46]. The corrupted architecture of the ECM results in a dense and fibrotic niche that embeds and constrains tumors. This, together with the augmented compressive forces of the tumor mass in expansion, generates an increased pressure on the tumor, the so called "solid stress"[47]. Notably, this pressure is not homogeneously distributed within solid tumor, rising towards the core and here leading to partial collapse of blood vessels thereby reducing oxygen and nutrients availability in the inner regions[48,49].

ECM stiffening cannot be considered only as a stress condition for tumors since it plays a pivotal role in promoting cancer growth and progression, sustaining many hallmarks of tumors[50]. Indeed,

malignant cells can sense mechanical inputs from the surrounding environment through specialized modules, such as focal adhesions, that couple signals derived from the ECM outside the cells with the actin cytoskeleton inside the cells. Then, dynamic rearrangements of cytoskeleton enable the conversion of physical stimuli into a biochemical response, in a process known as mechanotransduction. Moreover, mechanical cues lead to activation of crucial oncogenic signaling pathways such as FAK/SRC, PI3K/AKT/mTOR, RAS/RAF/ERK, YAP/TAZ and mutant p53 that in turn foster cancer cells survival, proliferation, and metastatic dissemination[51,52].

Cancer cells metabolism is emerging to be extremely altered by physical forces from the TME. Mechanical cues have a direct effect on several metabolic pathways[53,54], affecting glycolysis[55], mitochondrial shape and metabolism[56,57], lipid synthesis[58,59] and amino acid metabolism[60,61]. Moreover, mechanical inputs globally perturb cell metabolism by modulating the activity of the key metabolic sensor kinases, mTORC1 and AMPK[62,63]. These indeed act as mechanosensitive kinases that directly control anabolic and catabolic cellular processes, thus exponentially amplifying the control by mechanical stimuli over many facets of cellular metabolism.

Of note, the activity of several glycolytic enzymes is directly regulated by the extracellular stiffness and the actin cytoskeleton. A relevant example is the glycolytic enzyme Aldolase, that is sequestered by binding F-actin and released in response to actin remodeling upon increased mechanical cues. This mechanism emerges as particular relevant in cancer cells, in which PI3K activated signaling, leads to Rac-mediated cytoskeleton remodeling and consequent mobilization of aldolase from actin, thus resulting in enhanced glycolysis. This mechanism ensures a coordination between energy consuming actin dynamics and high glycolytic flux to sustain it[64].

It is emerging that also metabolic pathways can influence mechanosignaling and composition/structure of the ECM, thus establishing a reciprocal regulation between mechanical cues and cell metabolism to sustain cancer growth and progression (Fig. 3)[53,54]. Indeed, cell metabolism is essential to provide energy for cytoskeletal dynamics and metabolites for synthesis and remodeling of ECM proteins[65].

Recently, some works unveiled a role of mechanobiology in controlling specific amino acid metabolisms in cancer progression. Indeed, a stiff ECM induces an exchange of aspartate and glutamine between CAFs and cancer cells. CAFs-derived aspartate fuels nucleotides synthesis in cancer cells thus supporting their proliferation, while cancer cell-derived glutamate is imported by CAFs and used to sustain their ECM remodeling activity. This results in a feedforward mechanism in which a stiff TME, coupled with alterations in amino acids metabolism, fosters CAFs functions and further promotes ECM stiffening[60]. In addition, stiff matrix rewires glutamine metabolism to induce microtubule glutamylation and consequent stabilization. Thus, by modulating amino acid

metabolism, breast cancer cells are able to adjust the rigidity of cytoskeleton to the mechanical loads of their environment thereby promoting cell invasion[61]. In sum, the connection between mechanobiology and cell metabolism has been proved to be a determinant for tumors. In particular, these works emphasize the emerging perspective that amino acid metabolism couples mechanobiology and cancer progression.

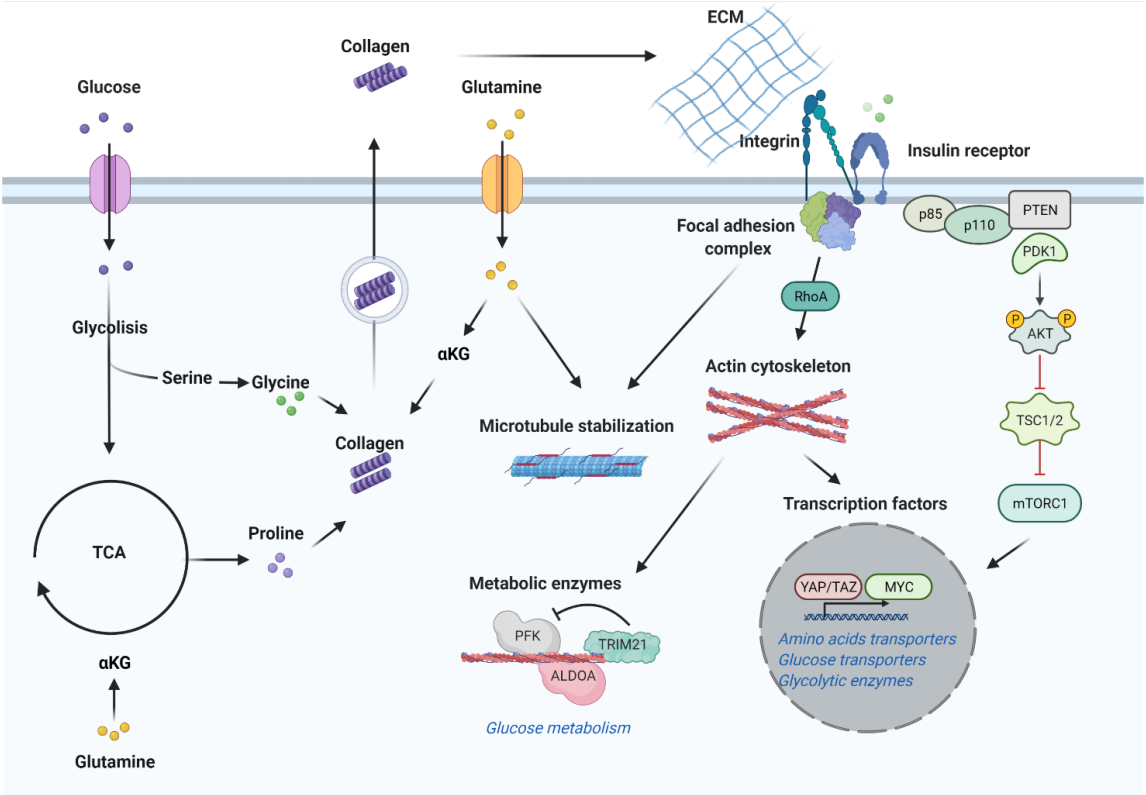


Figure 3. Crosstalk between mechanical cues and cell metabolism. Schematic representation of the crosstalk between mechanical cues and cell metabolism. In particular, cells respond to mechanical inputs from the ECM by activating signaling pathways, transcription factors and metabolic enzymes thus reprogramming cell metabolism. Cell metabolism provides energy and metabolites critical for ECM synthesis and remodelling as well for cytoskeleton-mediated mechanotransduction. Created with BioRender.com.

1.2 ROLE OF AMINO ACIDS IN CANCER

In the last decades, the emerging perspective has been that amino acids are the major fuel for tumor growth favoring tumor dissemination and resistance to therapy. Indeed, two pillars works shed light on the metabolic scenario of tumors, unveiling that cancer cells are the most avid for amino acids which, in turn, constitute the majority of the cell biomass[39,66]. Amino acids (AAs) are also required for energy production, redox balance and signaling pathways regulation. This extreme nutritional avidity of cancer is exploited as strategy for diagnosis, using radiolabeled amino acids, and as a metabolic Achille's heel of tumors, starving cancer to death[67]. Thus, understanding how, when, and why cancer cells increase their AA metabolism is fundamental to find therapeutic opportunity to dampen tumor progression.

AAs isolated in nature are more than 300 but, of these, only 20 are needed for protein synthesis and for this reason they are called proteinogenic. In humans, some of these can be synthesized *de novo* by the cells and they are defined as non-essential amino acids (NEAAs). On the contrary, other AAs are considered essentials (EAAs) since they cannot be produced by the cells and must be introduced with the diet. Consequently, mammalian cells are obliged to derive them from the diet. To this category belong phenylalanine, lysine, methionine, tryptophane, threonine, isoleucine, leucine, and valine. The last three also constitute the branched-chain amino acids (BCAAs)[8,68].

AAs are required for several cellular physiological processes since they provide essential building blocks for the synthesis of proteins and macromolecules. In addition, they can be used as alternative sources of energy and they are fundamental to counteract oxidative stress, to regulate gene expression and to activate signalling pathways, including the mTORC1 pathway (Fig. 4)[8].

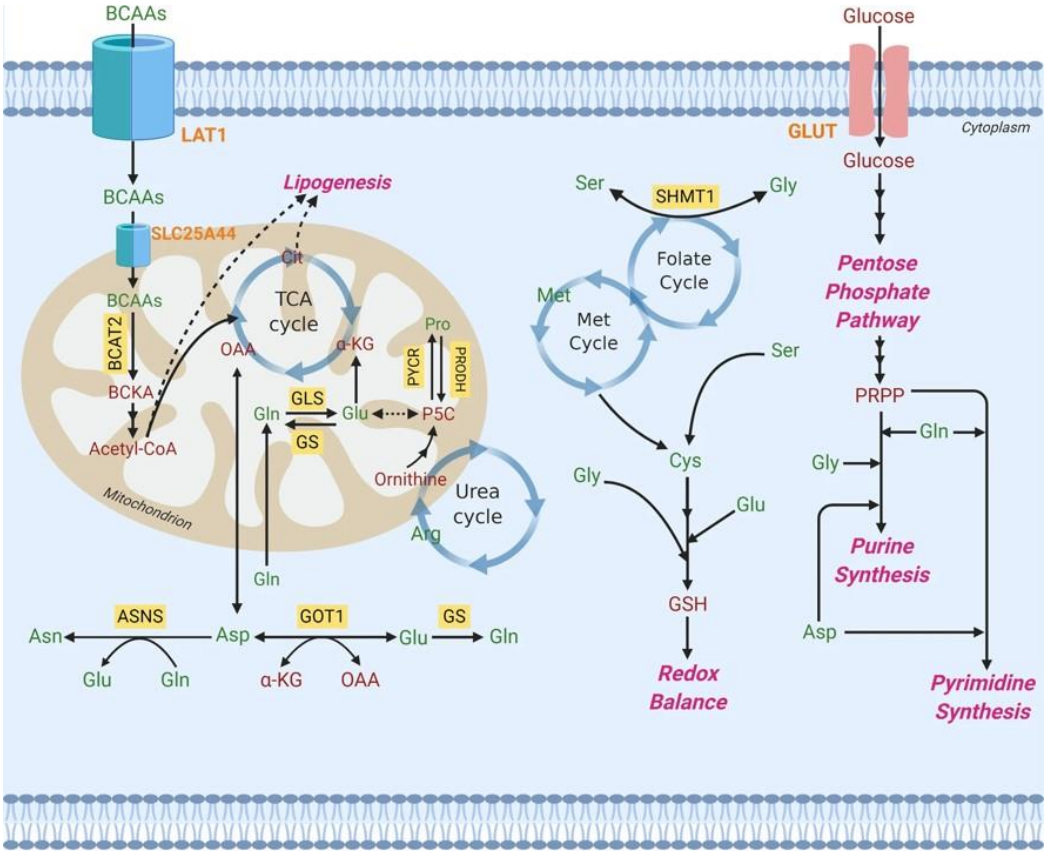


Figure 4. Role of amino acid metabolism in cancer. Amino acids (in green) sustain cell metabolism, growth and homeostasis generating metabolic intermediates (in red), such as Acetyl-CoA, that fuel TCA cycle and provide precursors for the synthesis of nucleotides and lipids, fundamentals for cell growth and proliferation, as well as regulating redox balance via GSH production. In orange are indicated the transporter of essential amino acids LAT1 and of glucose GLUT1, while in yellow are highlighted the enzymes of amino acids metabolism[8].

In term of energy production, despite glucose being the main molecule used to fuel the TCA cycle, AAs may serve as anaplerotic metabolites. In particular, in a process termed glutaminolysis, glutamine is largely used to generate α-ketoglutarate (α-KG) that is then converted into oxalacetate thus fueling the TCA cycle. In addition, also BCAAs can be used as alternative fuels, since their catabolism provide Acetyl-CoA that can be directed towards TCA cycle[68]. Moreover, AAs are essential for the synthesis of macromolecules such as lipids and nucleotides. Indeed, the same BCAAs-derived Acetyl-CoA is the precursor for lipogenesis while glycine, serine, and methionine, through the folate and methionine cycles, generate one-carbon units that support nucleobases synthesis[68–70]. Furthermore, AAs play a pivotal role in maintaining redox balance since they are key elements for glutathione (GSH) synthesis and NADPH generation. GSH is derived from glycine, glutamate, and cysteine and in particular the latter is critical because of its thiol group which has redox functions. Indeed, inhibition of cysteine uptake impairs cellular viability due to uncontrolled oxidative stress[71]. Another distinctive role of AAs is their ability to modulate gene expression and signaling pathways, as key molecules for epigenic regulation and post-translational modifications are

generated from their metabolism. For instance, DNA and histone methylation is mediated by methyltransferases, enzymes that utilize the intermediate S-adenosylmethionine (SAM) produced by methionine cycle as a methyl donor[72].

Given the relevance of AAs for many cellular processes, it is not surprising that cancer cells heavily rely on exogenous supply of AAs. This nutritional dependence is not only related to EAA, as expected, but also to NEAA. Indeed, this increased requirement of AAs may even cause auxotrophy for NEAA, indicating with this term the inability to grow without a given nutrient[67]. A relevant example is of course glutamine: although glutamine is synthesized by cells, it is the most imported and consumed nutrient by cancer cells after glucose. Thus, glutamine is considered as a conditional EAA and its availability in the tumour environment can be limiting for tumour cells proliferation[73]. A recent work provides a quantitative analysis of the main contributors to the mass of proliferating cells. The authors found that other AAs, consumed at lower rates rather than glucose and glutamine, account for the majority of the biomass of cells, while glutamine primarily sustains protein synthesis[66]. Thus, tumour metabolic dependency may involve several AAs and interfering with their availability can be selectively lethal to tumor cells[67].

1.2.1 Amino acid metabolism plays a crucial role in tumorigenesis

Given these premises, it is comprehensible that amino acid metabolism plays a crucial role not only in the early stages of tumorigenesis, but also during tumor progression, contributing to the acquisition of aggressive phenotypes and to the resistance to several anticancer strategies.

Role of amino acid metabolism in tumor onset and growth

A lot of studies show that alteration of AA metabolism is an early event in tumorigenesis and strongly depends on the metabolism of normal tissue from where cancer arose. An illustrative example emerges from pancreatic ductal adenocarcinoma (PDAC) and non-small cell lung carcinoma (NSCLC) that share the same etiology but develop in different tissues. Indeed, both are initiated by activation of K-Ras and loss of p53, but they differently use branched-chain amino acids (BCAAs). In particular, NSCLC tumors increase intake and catabolism of BCAAs that appears essential for nucleotides synthesis whereas PDAC tumors exhibit reduced BCAAs incorporation[74]. The decreased consumption of these AAs from PDAC cells results in augmented levels of free BCAAs in the bloodstream of mouse models and patients. This event is detectable at initial stages of tumorigenesis suggesting the possibility to use elevated plasma levels of BCAAs as a predictive marker for PDAC tumors early diagnosis[75].

The relevance of the tissue of origin in determining the metabolic dependence of tumors is evident in many other cancer types. Breast tumors and melanomas develop in an environment with scarce serine availability and, thus, they rely on the *de novo* serine synthesis. Coherently, these tumors often display gene amplification or overexpression of phosphoglycerate dehydrogenase (PHGDH), the rate limiting enzyme of the SSP[76]. These findings underline how nutritional features of the environment in which tumors develop strongly influence the metabolic program adopted by cancer cells, which thus exhibit different metabolic dependencies according to the tissue of origin.

Role of amino acid metabolism in the metastatic cascade

During the metastatic cascade primary cancer cells need to acquire the ability to invade the basement membrane and to enter the surrounding vasculature or the lymphatic system. Once they intravasated they need to survive in the circulation and finally extravasate and colonize secondary sites[10]. This is an inefficient process since cancer cells must overcome multiple environmental hurdles before successfully reach distal sites, and the majority of the cells succumb during this journey[10]. However, given the primary tumor heterogeneity, some of these may be endowed with metabolic traits that advantage them in surviving in harsh environments. Accordingly, cancer cells exploit their metabolic plasticity and nutrient flexibility during metastases in order to cope with different and challenging conditions they encounter (Fig. 5)[12,26]. Thereby metabolic alterations result crucial for cancer cells survival in each steps of the metastatic cascade[10].

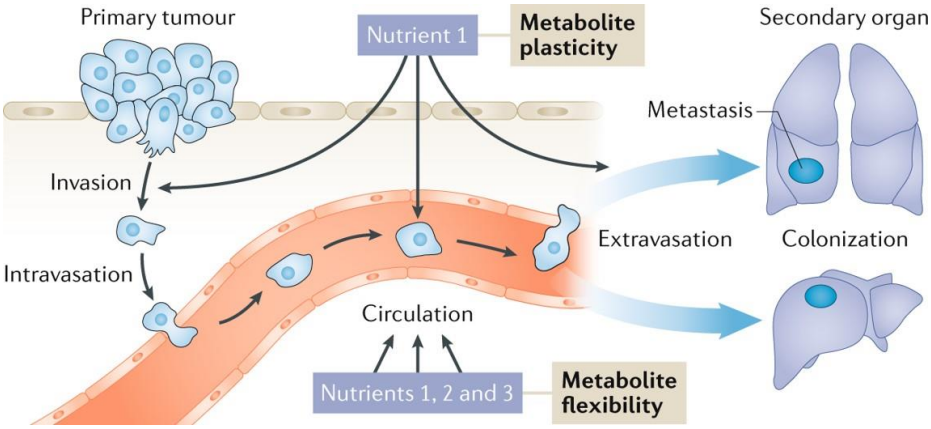


Figure 5. Metabolic adaptations of tumor cells during metastases. Cancer cells dynamically adapt their metabolic profiles to cope with environmental stresses they encounter during the metastatic journey. Indeed, metastasizing cells exhibit metabolic plasticity and nutrient flexibility that allow them to survive and to colonize the distal organs[10].

Colonization of the distal organ requires mandatory metabolic adjustment to a distinct TME in terms of nutrient availability and tissue organization. Indeed, metastases often exhibit a different metabolic profile in respect to primary tumor[77]. Lung metastases, derived from breast cancers, do no longer

depend on glutamine to support anabolism but instead they strongly rely on the uptake of pyruvate, which is abundant in the lungs, and on the *de novo* serine synthesis[78]. Rinaldi et al. found that these metabolites are fundamental to foster mTORC1 signaling and metastatic growth. Coherently, blocking pyruvate uptake or inhibiting the rate limiting enzyme of the SSP strongly impairs mTORC1 pathway in lung metastases but not in the primary tumor[78]. Similar observations have been made for brain metastases arising from breast primary tumors. In fact, they display a massive usage of extracellular acetate and BCAAs as a source of energy. Moreover, given the scarcity of serine and glycine in the brain, they increase expression levels of enzymes involved in the SSP and ensure an adequate supply of these AAs for survival[77,79]. In addition, cancer cells have been shown to be able to modulate and prime the pre-metastatic niche thus creating a more favorable colonizing tissue that partially meets cancer cells metabolic needs[80].

Role of amino acid metabolism in therapy resistance

Recent evidence points out that reprogramming of AA metabolism in cancer cells and in their supportive TME drives resistance to antitumor therapies[81]. Indeed, cancer cells, through adaptations in AA metabolism, acquire the capability to resist to therapies based on both genotoxic and oxidative stress-inducing agents as well as to endocrine therapy (ET)[81]. This is due to the fact that AAs promote nucleotide synthesis to prevent DNA damage-induced cell death or are used to generate reducing equivalents to overcome ROS-induced cell death[81]. Moreover, recent works highlight that alterations in AA metabolism determine resistance to ET, the standard of care for estrogen receptor-positive (ER+) breast tumors. Indeed, tamoxifen (TMX)-resistant ER+ breast cancer cells promote LAT1-dependent leucine uptake and consequent mTORC1 activation leading to cell proliferation[82]. Coherently, inhibition of LAT1 increases breast cancer cells sensitivity to tamoxifen[83].

The efficacy of a therapeutic intervention is importantly influenced by the crosstalk between malignant cells and their TME[84]. AA metabolism is known to play a determinant role in the interplay between tumors and the surrounding environment and, in particular, in the regulation of tumor-induced immunotolerance, thus underscoring its critical involvement in chemotherapy and immune therapy resistance[85]. For instance, acute myeloid leukemia (AML) cells educate mesenchymal stromal cells to increase aspartate production and efflux through the transporter SLC1A3. This allows AML cells to increase pyrimidine and GSH synthesis, protecting them from chemotherapy[86]. Moreover, several examples may be provided regarding how tumor cells exploit AA metabolism to block an effective anti-tumor immune response thus hampering immunotherapy success[41]. It was observed that glutamine availability acts modulating the tumor immune environment. Indeed, high glutamine levels favor a preferential differentiation of monocyte into macrophages with a

protumorigenic M2 phenotype rather than a proinflammatory M1 phenotype[87]. Coherently, impairing glutamine metabolism re-sensitizes tumors to checkpoint blockade therapy by promoting the differentiation of immature myeloid cells into proinflammatory macrophages[88]. Moreover, a recent study reported that treatment with a glutamine antagonist induces tumor regression in mouse models by starving cancer cells and by concomitantly conditioning the TME to become more favorable for effector T cells. In sum, the authors demonstrated that this metabolic intervention, in combination or even in absence of additional immunotherapy, remarkably enhances endogenous anti-tumor immunity leading to tumor immune rejection[89].

1.2.2 Amino acid metabolism is deregulated in cancer

Cancer cells AA metabolism is influenced by tissue of origin, tumor type, mutational background and the TME. However, in general, tumors must guarantee a high intracellular pool of these nutrients and they adopt some common strategies for this purpose. Indeed, while cancer cells display specific utilization of AAs, they generally exhibit upregulation in the expression of AAs transporters and of enzymes involved in their biosynthesis.

Alterations in the intake of amino acids

AAs need a transporter system to cross the membranes. Amino acids transporters may be localized in the plasma membrane or even in intracellular compartments, such as lysosomes and mitochondria, where they mediate intake, exit or exchange of AAs. These membrane-bound transfer proteins belong to the Solute Carrier (SLC) superfamily, and they have been classified into different systems depending on their substrate specificity and transport mechanism. Since in mammalian cells intracellular AAs concentration is higher than that in extracellular fluids, the transfer of AAs into the cells is often ion-coupled and associated with exchange with other AAs[90]. In normal cells, the expression of most AA transporters is modulated based on the tissue and the developmental stage[91]. In tumor cells, instead, there is a general increased expression of one or more of these transporters. Although there are specific upregulations of AA transporter(s) depending on the specific tumor type, overall, four of these, i.e. SLC6A14, LAT1, ASCT2 and xCT have been found highly expressed in cancer. Moreover, these AAs transporters appear to be functionally coupled, thus maximizing their ability to sustain cancer growth (Fig. 6)[92].

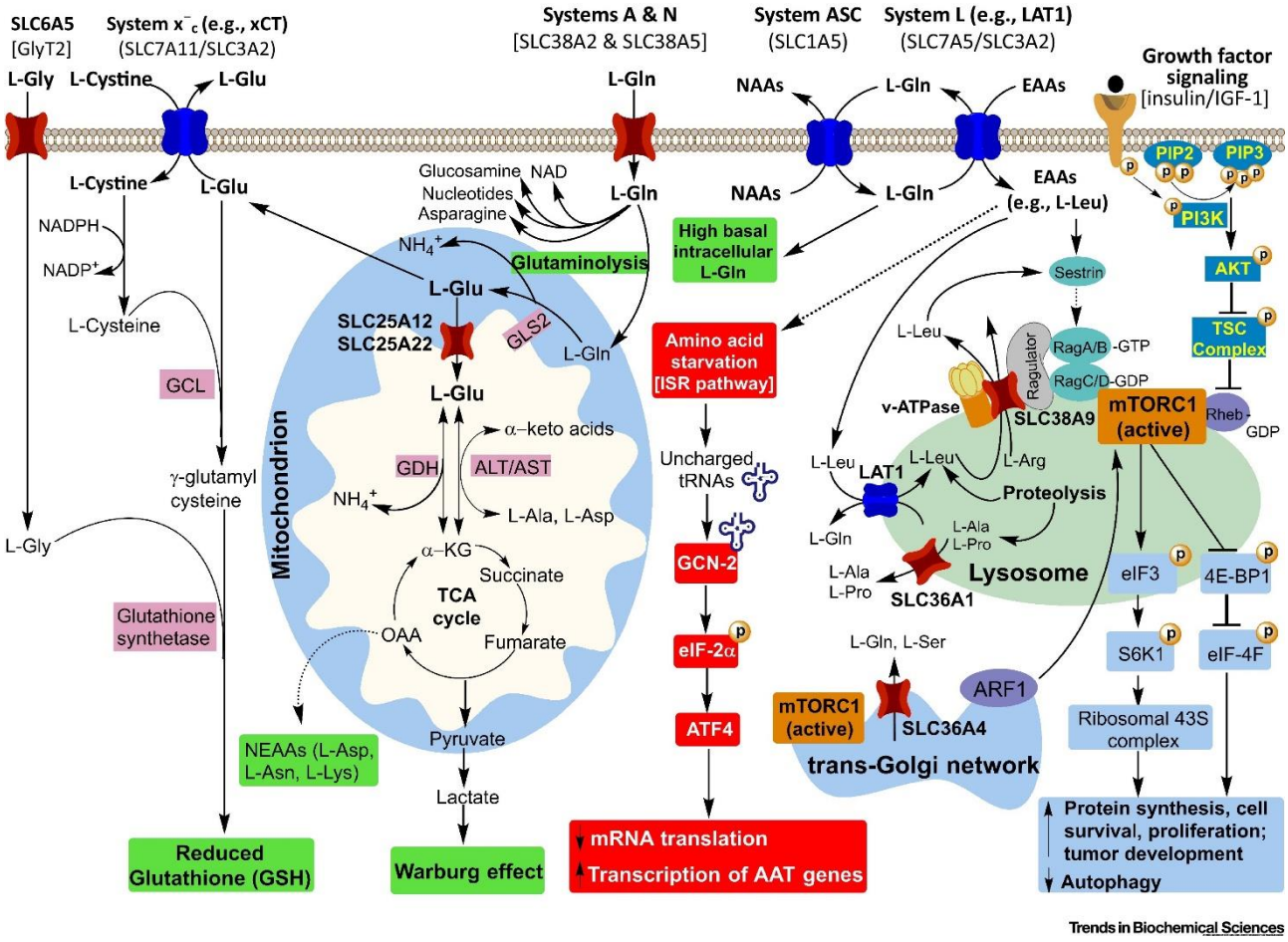


Figure 6. Role of amino acids transporters in energy metabolism, mTORC1 activation, nutritional stress and tumor progression. ASCT2 **(***SLC1A5*), xCT (*SCL7A11*) and LAT1 (*SLC7A5*) are commonly overexpressed in cancer and importantly involved in metabolic rewiring. ASCT2 imports glutamine that is converted into other amino acids or used as a source of energy. Moreover, it can be exchanged by the xCT transporter to allow the intake of cysteine, crucial for the synthesis of GSH, or by LAT1 for the intake of essential amino acids. The latter in turn are required to activate mTORC1 signaling thus promoting anabolic pathways while dampening catabolism[90].

SLC6A14 has unique features compared to other AAs transporters. Indeed, it induces unidirectional influx of AAs into cells and shows the broadest substrate specificity including EAAs and glutamine. It is upregulated in several cancer of epithelial origin, encompassing colon cancer, cervical cancer, and ER+ breast cancer[93].

LAT1, encoded by *SLC7A5* gene, belongs to system L (Leucine-preferring) AA transporters and is an obligatory exchanger. Indeed, it mediates the influx of BCAAs and bulky AAs such as phenylalanine, methionine, histidine and tryptophane, coupled with efflux of other AAs, mainly glutamine[92–94]. Physiologically, it is highly expressed in the placenta, brain, liver and testis while, in malignancies, it is broadly upregulated in various cancer types, comprising bladder, breast, lung, colon, cervical, brain and skin tumors[90,94,95]. Its expression is transcriptionally activated in response to hypoxia through HIF2α and by oncogenic MYC and YAP, whereas some miRNAs, such as miR-126, inhibit its expression[23,96–98]. The functional relevance of LAT1 in cancer mostly relies on its

ability to mediate the intake of leucine, which in turn is a major activator of mTORC1 signaling[16]. Indeed, LAT1 expression is associated with activation of this pathway, increased tumor size, high tumor grade, and consequently poor patient outcome[99]. In agreement, pharmacologic inhibition of this transporter suppresses mTORC1 signaling and tumor growth in different types of tumors[100,101]. LAT1 is often functionally coupled with another amino acid transporter ASCT2, which is encoded by *SLC1A5*.

ASCT2 mediates the influx of neutral AAs and in particular of glutamine in exchange with efflux of other AAs, in a Na^+-coupled dependent manner[92,102]. Glutamine entered via ASCT2 in the cell can be used for anaplerotic reactions, converted into other NEAAs or exchanged by LAT1 for the intake of leucine[103]. Since tumors are strongly dependent on glutamine uptake, this transporter is highly expressed in many tumors, including prostate, gastric and breast cancers[90]. Of note, *SLC1A5* is a target of MYC thereby supporting the idea that the expression of the two transporters may be coordinated by the same oncogenic lesions to optimize AA metabolism and tumor growth[103,104].

xCT, encoded by *SLC7A11* gene, is a Na^+-independent obligatory exchanger that promotes cysteine intake with concomitant release of glutamate out of the cells[92,94]. Its expression is strongly increased in several tumors and it was reported to be controlled by multiple oncogenes, including MYC. The functional role of this transporter in tumors is associated with maintenance of GSH pool since cysteine is the rate-liming AA in its synthesis[105]. The glutamate secreted in the environment acts through metabotropic and ionotropic glutamate receptors on cancer cells, potentiating oncogenic signaling[106].

Although LAT1 and xCT are the actual amino acid transporters, they are associated to the type II transmembrane glycoprotein SLC3A2/4F2hc/CD98hc that recruits them to the plasma membrane. Indeed, both of LAT1 and xCT exist as heterodimers with CD98hc, in which the latter constitutes the heavy chain. CD98hc is ubiquitously expressed in healthy tissues but it is strongly overexpressed in cancers. Of note, beyond the promotion of AAs fluxes in cells, CD98hc was reported to interact with β-integrins and to promote mechanosignaling[107–109]. This function is partially exploited by promoting *de novo* sphingolipids synthesis that potentiates integrin-rigidity sensing in response to stiff ECM thus promoting cancer growth and invasion[109].

The basic AAs transporter CAT1 is critical for cancer, it is encoded by *SLC7A1* gene, and it preferentially imports arginine and lysine in the cells. In cancer setting, it is frequently overexpressed and correlates with tumor grade[110,111]. Interestingly, its ability to transport arginine into the cells, appears to be more relevant for various solid cancers and leukemias that are unable to synthetize an adequate amount of arginine. Thus, these tumors are auxotrophic for arginine, required for the generation of polyamines and nitric oxide (NO)[8]. Coherently, CAT1 downregulation decreases arginine intake and NO production, leading to breast cancer cells death[112].

Since many tumors rely on uptake of extracellular AAs, inhibition of the related transporters has been investigated as a therapeutic approach to target AA metabolism in cancer, leading to the development of new drugs. For instance, V9302 and JPH203, selective inhibitors of ASCT2 and LAT1 respectively, have shown *in vivo* efficacy in reducing tumor growth[113,114]. However, because of the functional redundancy of many transporters, combination therapy must be considered the best approach.

Alterations in amino acids biosynthesis

To satisfy their increased demand of AAs, cancer cells not only promote overexpression of AAs transporters but also enhance expression of enzymes involved in the synthesis of NEAAs. Among these, the most frequently altered in cancer are GLS, ASNS, and enzymes of the serine synthesis pathway (SSP), that are responsible for the synthesis of glutamate, asparagine and serine/glycine respectively[76,115,116].

Given that glutamine is the second most consumed nutrient in cancers, it is not surprising that GLS, which catalyzes the first step of glutamine catabolism into glutamate and ammonia, is crucial for tumor growth. Upregulation of GLS is observed in different tumors including breast, liver, colorectal, brain, cervical, lung tumors and melanomas and its expression and activity correlate with high tumor growth and poor patients' outcome[115]. Differently from the isoform GLS2, that owns antitumor activity and is regulated by p53, GLS is positively controlled by oncogenic MYC and, indeed, MYC tumors are known to exhibit "glutamine addiction"[103,115,117]. Considering its relevance, inhibition of GLS has been proposed as a possible therapeutic approach particularly in those tumors which exhibit glutamine dependence[118,119].

ASNS is a cytoplasmatic enzyme that generates asparagine from glutamine and aspartate in an ATP-dependent reaction. Then asparagine can be converted in oxalacetate and enter in the TCA cycle, or even can be used as exchange AA to allow the intake of other AAs, thus leading to activation of mTORC1 pathway[8,120]. In cancer, ASNS is overexpressed in many tumors such as gastric cancer, colon cancer, hepatocellular carcinoma and metastatic breast cancer[116].

In recent years, several works disclosed that beyond glucose and glutamine, the third consumed metabolite is serine, a NEAA which has a central role in various aspects of tumorigenesis[66]. Serine is a precursor of the NEAAs glycine and cysteine, it supplies folate and methionine cycles, that underpin one-carbon metabolism, and it is a precursor of sphingolipids. This implies that serine is crucial for proteins, nucleotides and lipids synthesis, redox balance, methylation reactions and generation and recycling of functional metabolites, such as SAM and ATP[69,121,122]. Serine is both imported in the cells, via AA transporters (ASCT1 and ASCT2), and synthetized *de novo* from glucose. Despite

extracellular serine alone is sufficient to support cancer cell proliferation in many situations, cancer cells frequently exhibit an increased serine synthesis. Indeed, increased serine biosynthesis is a well-established metabolic hallmark of cancer[123]. In detail, the SSP starts from the glycolytic intermediate 3-phosphoglycerate (3PG) and the enzyme PHGDH that catalyzes the NAD+-dependent conversion of 3PG into 3-phosphoidrossipiruvate (3PHP). This is then converted into phosphoserine by the phosphoserine aminotransferase (PSAT1) in a transamination reaction that uses nitrogen from glutamate and generates αKG. Serine is finally generated by the action of phosphoserine phosphatase (PSPH) (Fig. 7). The SSP is highly regulated and it is induced in response to several metabolic stresses encompassing glucose and glutamine deprivation as well as reduction in the extracellular levels of serine itself[69]. For instance, given that serine is an allosteric activator of pyruvate kinase M2 (PKM2), a drop in serine availability leads to reduced PKM2 activity thus slowing the flux of carbon through the final step of glycolysis whereas promoting the diversion of 3PG into the SSP[124,125].

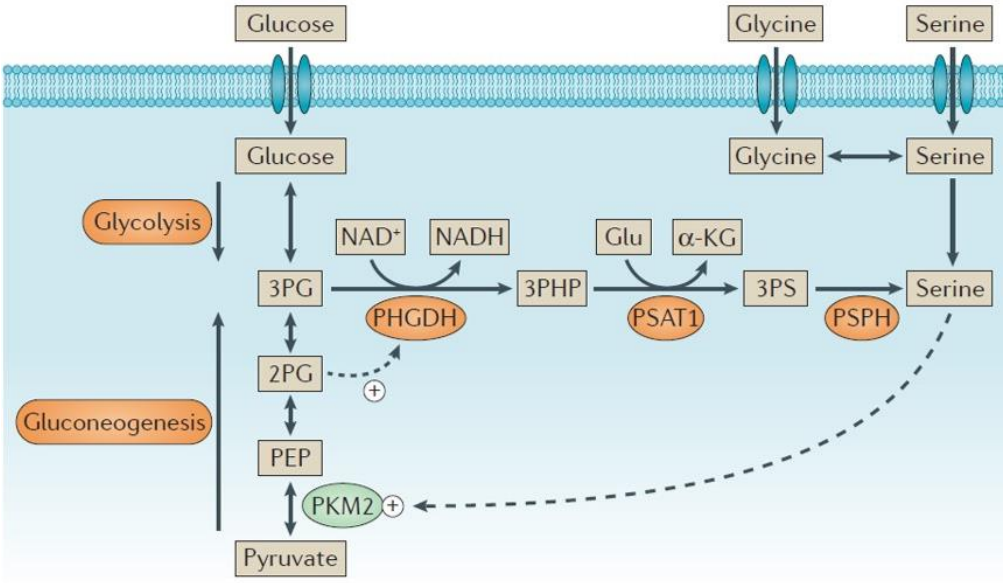


Figure 7. Serine synthesis pathway. Schematic representation of *de novo* serine synthesis pathway (SSP) from the glycolytic or gluconeogenic intermediate 3-phosphoglicerate (3PG). In details, PHGDH catalyzes the NAD^+-dependent oxidation of 3PG to 3-phosphohydrossipyruvate (3PHP). Then, PSAT1 converts 3PHP into 3-phosphoserine (3PS) in a transamination reaction that depends on glutamate and generates αKG. Finally, PSPH catalyzes the last step of serine synthesis via hydrolysis of 3PS. The SSP is allosterically regulated (dashed arrow with "+" symbol that denotes an activator). Serine itself is an allosteric activator of PKM2 and when serine pool is low within the cell, PKM2 activity is reduced, thus leading to 3PG diversion into the SSP. In addition, 2-phosphoglycerate (2PG) is an activator of PHGDH[69].

Many studies demonstrate that the SSP enzymes are frequently overexpressed in different tumor types, including breast cancer, melanoma and colon cancer, thus conferring aggressive properties to those tumors and their over-expression correlate with poor prognosis[126–128]. PHGDH is the only example of metabolic enzyme whose deregulation is achieved by genetic mutation without generating oncometabolites. Indeed, PHGDH elevated expression is due to its amplification found in approximately 70% of TNBC and this enzyme is further overexpressed in brain metastases derived

from breast tumors[79,126]. Pharmacological inhibition of PHGDH reduces brain metastases formation from breast primary tumors[79].

Another enzyme that has been shown to be crucial for cancer progression is PSAT1. While PSAT1 expression is almost undetectable in normal mammary tissue, its levels are increased in several breast cancer subtypes as well as in other aggressive tumors such as colon cancer, head and neck tumor and lung adenocarcinoma. Upregulation of PSAT1 correlates with a variety of aggressive tumor features and, in particular, a recent work reported that in TNBC, its overexpression is associated with invasive and migratory phenotypes through rearrangements in F-actin cytoskeleton and cellular morphology[129]. Moreover, a relevant role of PSAT1 is associated with its involvement in modulation of αKG levels[130]. Indeed, αKG acts as a cofactor for a group of histone demethylases that regulates differentiation of murine embryonic stem cells[131]. Moreover, it was shown to induce growth signaling through mTORC1 activation[78]. Thus, beyond fueling TCA cycle, αKG can model the epigenetic context determining cell fate and to sustain anabolic growth program. Given the relevance of serine in the cellular homeostasis, the expression of SSP genes is finely regulated by proteins that are crucial for both cellular homeostasis and cancer, including MYC, MDM2, NRF2 and ATF4, in response to oxidative stress or AAs deprivation[69].

During tumor growth and progression, cancer cells frequently experience a reduction of amino acid availability, due to their local reduction in the extracellular environment or to high synthesis of macromolecules that consumed their intracellular pool. Cells display a highly conserved system to sense this AAs' reduction through GCN2 kinase thereby activating an elaborate transcriptional program, termed "amino acid response" (AAR), that leads to the increased expression of AAs transporters and biosynthetic enzymes to refill their intracellular pool[132]. In particular, the lack of AAs results in uncharged tRNAs that in turn activate the kinase GCN2. The latter phosphorylates the initial factor eIF2α that dampens cap-dependent protein synthesis, while promoting the preferential cap-independent translation of a subset of transcripts. These foresee the transcription factor ATF4, that is one of the main players in the general induction of AA metabolism genes expression to guarantee cell survival[133].

In tumors, oncogenic signaling that foster synthesis of proteins and of other macromolecules, such as MYC and mTORC1, exacerbate AAs consumption and thus activate the AAR. In particular, mTORC1 directly promotes ATF4 translation independently of GCN2/eIF2α axis thus coupling cellular AAs supply with demand for protein synthesis[134,135]. In general, it is emerging that several oncogenes such as MYC and RAS confer advantage to cancer cells by favoring they survival when they encounter fluctuations of nutrients and in particular a scarcity of amino acids.

1.2.3 mTORC1 pathway sits at the nexus of nutrients availability and cell growth

As described above, mTOR is a central metabolic node in cancer cells, frequently hyper-activated by oncogenes to support cancer cell growth. mTOR is a serine/threonine kinase that constitutes the catalytic subunit of two different complexes, i.e. mTORC1 and mTORC2, characterized by the association of the kinase with different proteins to regulate different substrates[16]. Both complexes directly regulate cell growth and metabolism by modulating the phosphorylation status of key metabolic enzymes and by controlling the activity of downstream effectors. mTORC2 regulates several classes of protein kinases C (PKCs) including PKCα involved in cytoskeleton remodeling and cell motility, the ion transport regulator SGK1 and the oncogene Akt, which mediates the crosstalk with mTORC1 thus promoting a general anabolic metabolism[136]. mTORC1 phosphorylates several substrates to promote protein, lipid and nucleotide synthesis, to sustain mitochondrial biogenesis while limiting autophagic breakdown of cellular proteins and organelles. In details, the major substrates of mTORC1 are the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and the p70 S6 kinase 1 (S6K1). Upon mTORC1-mediated phosphorylation, 4E-BP1 is inhibited and releases the translation initiation factor eIF4E, while S6K1 is activated and phosphorylates the ribosomal protein S6, thus overall promoting the 5'-cap dependent mRNAs translation[16]. This leads to the synthesis of master metabolic regulators including HIF1α, MYC and ATF4[135,137,138]. Moreover, mTORC1 activation induces lipid synthesis by modulating nuclear translocation and processing of SREBPs and promotes mitochondrial biogenesis driving formation of the PGC1α complex[139–141]. Concomitantly, it applies inhibitory phosphorylation to ULK1 and ATG13, two autophagy initiation factors, and to UVRAG, regulator of autophagosome maturation, therefore impeding degradation and recycling of cellular components[142,143].

Since mTORC1 initiates an anabolic program that importantly consumes cell resources, it should only be activated when cells can support it. Thus, mTORC1 tightly senses and integrates a wide range of signals coupling growth factors presence with an adequate energetic and nutritional status of the cell to govern cellular anabolic and catabolic processes[16].

Growth factors such as IGF-1 and EFG, regulate mTORC1 activity through activation of PI3K-AKT pathway that phosphorylates the subunit tuberous sclerosis complex 2 (TSC2), leading to the removal of TSC from lysosomes. Thus, TSC cannot more inhibit Rheb that, in turn, maintains its active state at the surface of the lysosomes where it promotes mTORC1 activation[16]. As previously said, the PI3K-AKT pathway is often mutated in cancer promoting a chronic inhibition of TSC and activation of Rheb independently from growth factors stimulation. However, this condition is insufficient to activate mTORC1 if the mTOR kinase is not localized to the lysosome. mTORC1 recruitment to the

lysosome can be achieved exclusively when AAs are available. This ensures that mTORC1 is activated only if cellular conditions can support a sustained growth. Thus, while displaying growth factors-independent mTORC1 activation, cancer cells remain dependent on exogenous AAs[15].

mTORC1 acts as an amino acid sensor and its activation in response to AAs availability is mediated by Rag GTPases, that are obligated heterodimers configured as RagA/B and RagC/D, anchored to the lysosomes through the complex Ragulator. In sum, presence of AAs promotes the active state of Rag GTPases in which RagA/B is bound to GTP while RagC/D to GDP. This allows the interaction of mTORC1 to Rags and thus its localization to the lysosomes. RAG GTPases are regulated by a complex repertoire of AAs sensors and in particular of those involved in monitoring the cytosolic levels of leucine, arginine, methionine and glutamine (Fig. 8).

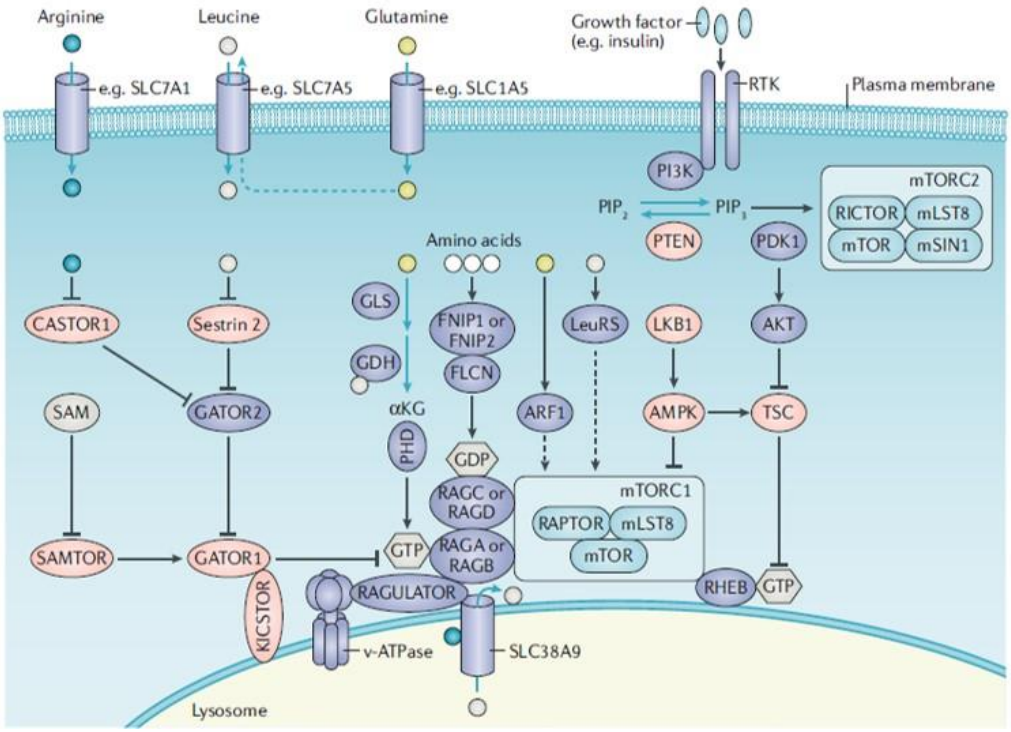


Figure 8. Activation of mTOR signaling pathway. Leucine, glutamine, and arginine are carried into the cell by transporters encoded by *SLC7A5*, *SLC1A5* and *SLC7A1*, respectively. Then, intracellular amino acids availability is detected mainly by the sensors Sestrin2, CASTOR1 and ARF1, that ultimately enable mTORC1 to bind to RagGTPases, thus allowing its lysosomal localization. Once anchored to lysosomes, mTORC1 can be activated by Rheb, but only if growth factors are present. Pink molecules indicate negative regulators and purple molecules indicate positive regulators of mTOR signaling. Turquoise arrows indicate metabolite flux, while black arrows indicate a signaling cascade[15]. In detail, leucine binds to Sestrin2 while arginine to CASTOR1, dissociating these proteins from GATOR2 which hence can block the mTORC1 negative regulator GATOR1. This inhibitory cascade, finally leads to activation of RAG GTPases and thus lysosomal recruitment and activation of mTORC1[144]. Another sensor critical for mTORC1 signaling is SAMTOR which responds to methionine-derived SAM levels in cells. SAM prevents SAMTOR to directly associates and activates the mTORC1 suppressor GATOR1. Glutamine activates mTORC1 signaling in a RAG-dependent manner via enhancing glutaminolysis and consequently producing αKG, which helps the GTP loading of RagB. Moreover, glutamine can also bind the small GTPase ADP-ribosylation factor 1 (ARF1) promoting mTORC1 lysosomal translocation independently from RAGs activity[144].

In agreement with mTORC1 pathway dependency on AAs availability, high expression of AAs transporters such as LAT1, ASCT2 and CAT1 is critical to boost mTORC1 signaling in cancer cells[145]. For instance, a genetic work demonstrated that LAT1 transport activity constitutes a key limiting step for cancer cells proliferation via sustaining leucine uptake and consequent mTORC1 activation[146]. Of note, mTORC1 signaling in turn drives AA metabolism reprogramming, establishing a feed forward loop that sustains tumor growth and survival[15]. Beyond the well-known role of leucine, arginine, methionine and glutamine in modulating the activity of mTORC1, recent evidence highlights that multiple AAs can activate mTORC1[147]. In this regard the role of asparagine and serine appears particularly relevant in the activation of mTORC1[78,120,148]. Indeed, asparagine can indirectly concur to mTORC1 activation serving as exchange factor to favor the intake of serine, histidine and arginine, promoting mTORC1 activation[120]. [148]. In addition, it was observed that in lung metastases derived from mammary carcinoma, sustained serine synthesis promotes mTORC1 activation[78].

The global picture provided by these examples is that mTORC1 is generally activated by an increased availability of AAs; this is coherent with the role of mTORC1 in promoting proteins, nucleotides, and lipids synthesis. Moreover, every environmental condition or oncogenic lesion that increases the pool of AAs in cancer cells, inevitably leads to the activation of mTORC1. This aspect offers a translational opportunity for cancer treatment: indeed, pharmacological and dietary intervention that starves cancer for AAs, can be used as therapeutic strategy to curb cancer growth through mitigation of mTORC1 activation.

1.3 THE TUMOR SUPPRESSOR P53

Metabolic reprogramming in cancer is driven by activation of oncogenes and loss of tumor suppressors that favor nutrient acquisition and assimilation of carbon and nitrogen in macromolecules such as lipids, proteins, and nucleic acids to sustain cell proliferation and growth. Notably, the most frequent genetic alterations found in cancers occur in key regulators of metabolic homeostasis, i.e. the PI3K-AKT-mTOR pathway, the oncogenes MYC and RAS and the oncosuppressor p53.

P53, encoded by *TP53* gene, is one of the main cellular tumor suppressors and is the most commonly mutated gene in cancer, highlighting its pivotal role in prevention and suppression of neoplastic transformation[149]. It is a DNA-binding protein that mainly acts as a tetrameric transcription factor promoting or even repressing the expression of several genes[150]. Moreover, it can also modulate cellular processes directly interacting with cytoplasmatic proteins like apoptotic effectors and metabolic enzymes[151]. P53 is activated by several stress conditions including DNA damage, oncogene activation, hypoxia, ROS, and nutrient fluctuations. In turn, it activates mechanisms to ensure cell homeostasis, by enabling cellular adaptation to a transient stress, or to induce cell elimination when the stresses are unresolved or the damages cannot be repaired, thus determining cell fate in terms of survival or death[24]. In particular, upon stresses, post-translational modifications promote p53 stabilization and induction of its transcriptional activity thus leading to a transient or even permanent cell cycle arrest. Indeed, p53 controls the G1/S and G2/M cell cycle checkpoints through transcriptional activation, of the cyclin-dependent kinase inhibitor CDKN1a/p21 and of GADD45 and SFN, respectively[152,153]. For instance, in case of DNA injuries, this allows to avoid DNA damage propagation while triggering the activity of the DNA repair machineries, inducing genes involved in nucleotide and base excision repair, mismatch repair and recombination. However, when the damage is too severe and cannot be repaired, the prolonged increase of p21 upregulates the CDK inhibitor p16 and consequently activates RB transcriptional program, thus leading to senescence[154]. Moreover, p53 can activate the cell death program by transcriptionally inducing the expression of several pro-apoptotic proteins that belong to the BCL-2 family such as BAX, NOXA and PUMA, while repressing anti-apoptotic ones[155]. In addition, p53 can promote apoptosis in a transcription-independent manner by inducing mitochondrial membrane permeabilization via interaction with BCL-XL and BCL-2 proteins[156,157]. Another critical oncosuppressive function of p53 derives from its ability to activate autophagy by directly modulating the expression of a large set of target genes like *DRAM*, *ULK1* and c*athepsin D*, and also by inhibiting mTOR and the PI3K-AKT axis[158]. This additional mechanism depends on its capacity to increase the transcription of sestrin2 and TSC2, main inhibitors of mTORC1, and of the β1 and β2 subunits of AMPK, an AMP activated kinase that activates TSC[159,160]. However, some evidence suggests that p53 can also limit or inhibit autophagy

thus further complicating the interplay between autophagy and p53. Regarding its role in modulating ROS levels, p53 maintain redox homeostasis in physiological conditions by inducing the expression of antioxidant genes, such as *SESN1*, *GPX1* and *AIF*, and metabolic genes such as *TIGAR*, *SCO2* and *PGM*, in order to control the fluxes responsible for ROS generation[161,162]. Nevertheless, in response to a severe oxidative stress, p53 can promote pro-oxidant genes and repress antioxidant ones, thus further exacerbating the redox imbalance and promoting p53-mediated apoptosis[161].

1.3.1 Role of *TP53* missense mutations in cancer

Loss of tumor suppressive functions of wild-type p53 (wtp53) constitutes a fundamental prerequisite for cell transformation and tumor progression. Indeed, almost 50% of all malignancies harbor p53 mutations and a large proportion of those tumors that do not display mutated p53, still have inactivated or downregulated p53 through other altered mechanisms[149]. However, differently from most of other tumor suppressor genes in cancer, 75% of *TP53* mutations are missense mutations that produce single amino acidic substitutions mainly in the DNA-binding domain of the protein. In particular, six residues in this domain are more frequently affected by substitution and for this reason they are defined "hotspots" (R175H, G245S, R248Q, R249S, R273H and R289W)[163].

These alterations result in mutant p53 forms (mutp53) that are no more able to activate canonical target genes and to interact with canonical partners thus losing wtp53 oncosuppressive activities. Meanwhile, the mutp53 acquires a dominant negative effect over the wild-type form by participating in hetero-oligomerization to form tetramers with wtp53, thus inactivating its function[164]. Moreover, mutp53 gains new oncogenic properties through a complex repertoire of interactions with transcription factors, enzymes and proteins involved in a plethora of cellular processes (Fig. 9)[165]. Consequently, reshaping cancer cell's transcriptome, proteome, and metabolic network, mutp53 acts as driver oncogene and promotes cancer progression, metastasis dissemination, and drug resistance. Similarly to its wild-type counterpart, mutp53 becomes activated in response to several stresses and exerts its functions sustaining cancer cells survival in adverse conditions like in presence of DNA damages caused by high proliferation rate and oxidative stress that characterize malignant cells[164].

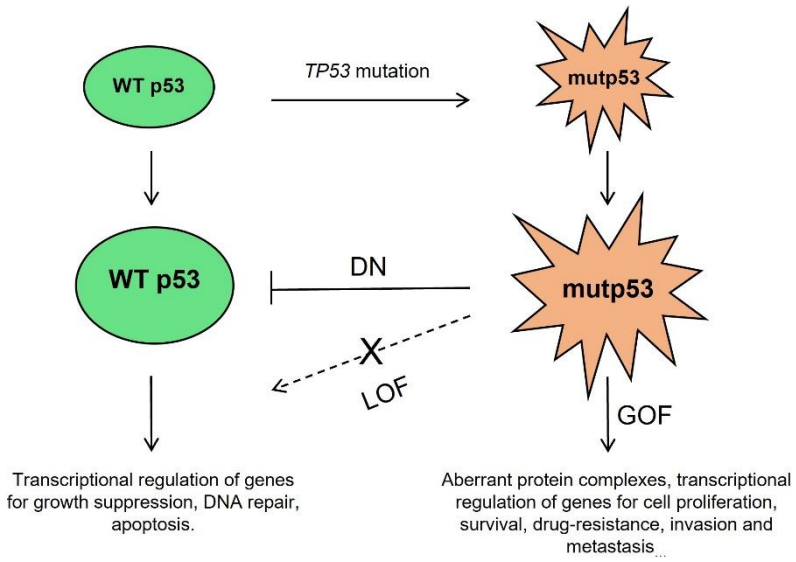


Figure 9. Hijacking of wild-type p53 functions by mutant p53. Schematic representation of the alterations in the functions of p53 upon missense mutations. "DN" indicates dominant negative effect over the wild-type p53, "LOF" indicates loss of oncosuppressive functions and "GOF" indicates gain of oncogenic functions.

1.3.2 Mechanisms of regulation of p53 in cancer

Functions of p53, as well as of mutp53, strongly rely on its cellular levels, thus every condition that alters its expression and stabilization is critical for its activation and for cell transformation.

The expression of p53 is controlled by changes in transcription, translation and alternative splicing events that generate several p53 isoforms which exhibit different activities[166]. In addition, p53 is subjected to a variety of post-translational modifications that importantly modulate its stabilization and its activation in response to stress signals[167]. In unstressed conditions, p53 is maintained at very low levels through proteasomal degradation mediated by the E3-ubiquitin ligase MDM2 and its cofactor MDMX. When cells are exposed to stresses, MDM2 dissociates from p53 which consequently increases its protein levels and oncosuppressive functions. Of note, p53 transcriptionally activates MDM2 itself, thus ensuring a negative feedback mechanism to control p53 response (Fig. 10)[155].

Similarly, in tumor tissues, mutp53 stabilization is required for the execution of its pro-tumoral functions and, interestingly, mutp53 triggers positive feedback loops that feed its own accumulation[164]. This is achieved through the stable association of mutp53 with components of the HSP chaperone machinery that inhibit MDM2 activity towards mutp53[168]. Indeed, pharmacological inhibition of HSP90 or of its activators, elicits mutp53 degradation and impairment of its oncogenic functions both *in vitro* and *in vivo*[169]. In addition, mutp53 directly upregulates HSP90 expression by potentiating stabilization and activation of heat-shock factor-1 (HSF1), master transcription factor of heat-shock proteins, thus sustaining its own accumulation[170].

Moreover, mutp53 stabilization is mediated by altered mechanical inputs displayed by tumor tissues. Indeed, our laboratory demonstrated that a dense and stiff ECM leads to mutp53 activation, in response to focal adhesion signaling and RhoA-dependent actin cytoskeleton remodeling[171]. In agreement, accumulation of mutp53 within tumors is heterogeneous and locally influenced by tissue rigidity, being increased in fibrotic regions[171]. Also in this case, mutp53 sustains its own stabilization through activation of several responses. Indeed, mutp53 promotes RhoA activation by inducing its positive regulators GEF-H1 and RhoGDI and by stimulating the mevalonate pathway (MVP) that drives prenylation and plasma membrane translocation of RhoA, an event that is required for its activation by mechanical inputs[171,172]. Of note our laboratory has also recently demonstrated that mutp53 induces tumor cells secretion of pro-malignant soluble factors in the TME, potentiating ECM deposition and stiffening[173]. This indicates that mutp53 establishes a vicious cycle that leads to its accumulation in response to mechanical inputs.

Other extracellular environmental conditions, such as nutrient availability, importantly modulate mutp53 oncogenic activities. Indeed, it has been shown that glucose restriction induces mutp53 deacetylation and autophagy-mediated degradation. This results in reduced accumulation of mutp53 and impaired growth of tumor xenograft harboring mutp53[174]. Of note, mutp53 increases glucose uptake in the cells by promoting translocation of GLUT1 transporters to the plasma membrane thus counteracting autophagy and enhancing its own protein levels[175].

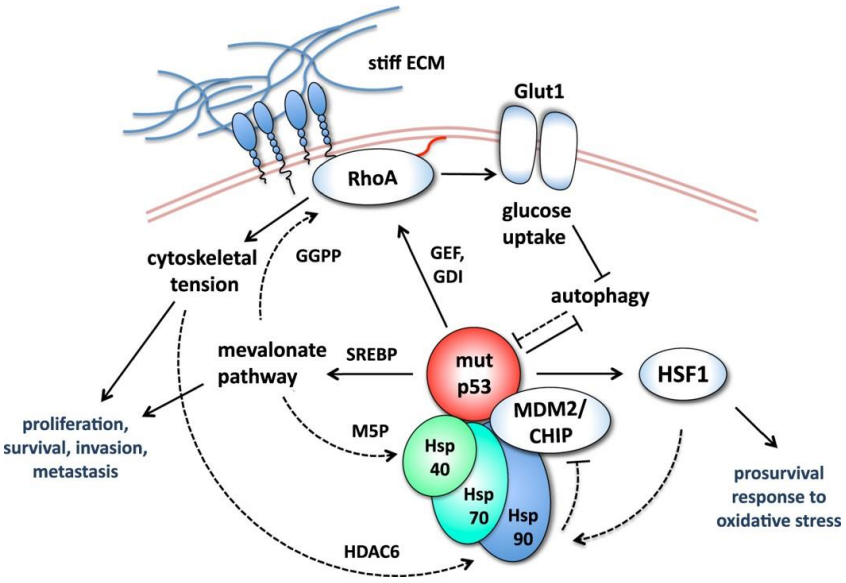


Figure 10. Molecular mechanisms of mutant p53 regulation in cancer cells. Hsp proteins determine mutant p53 stabilization in cancer cells by inhibiting its ubiquitin ligase MDM2 and mutp53 in turn stimulates HSF1 thus increasing HSP90 transcription. Mutant p53 increases GLUT1 membrane translocation and consequently glucose uptake, thus limiting its own autophagic degradation. Moreover, acting on SREBP, it promotes the MVP thus sustaining RhoA geranyl-geranylation, a step required for its stabilization downstream to a stiff ECM[164].

1.3.3 P53 in metabolic reprogramming

The role of p53, both in its wild-type and mutated form, in the regulation of cell metabolism has been widely described in last years and has proved to be a crucial aspect in cancer[176]. Indeed, an intriguing study disclosed the possibility that tumor suppressive functions of wtp53 may largely rely on its regulation of cell metabolism and oxidative stress, rather than on its canonical activities, such as cell cycle arrest and apoptosis[25]. An illustrative example unveiled that wtp53 reshapes cell metabolism thus globally perturbing chromatin modifications and gene expression to prevent the transition from premalignant to undifferentiated malignant lesions. Indeed, in pancreatic tumors wtp53 controls glucose and glutamine metabolism to favor αKG accumulation at the expense of succinate. The increased levels of αKG promote the activity of αKG-dependent epigenetic enzymes thus inducing chromatin de-methylation and tumor differentiation. Therefore, loss of wtp53 reduces αKG levels and determines the transition toward less differentiated and more aggressive tumor phenotypes[177]. This work highlights the relevance of cell metabolism regulation by wtp53 in cancer: indeed, in tumors, the increase in αKG levels is sufficient to induce activation of a transcriptional profile similar to that dictated by wtp53[177]. Many other evidences underline the pivotal role of wtp53 in controlling multiple metabolic axes including glycolysis, mitochondrial metabolism, lipid and amino acid metabolism[176,178,179].

As described above, loss of wtp53 leads to broad metabolic alterations thus favoring cancer growth. However, in cancer, loss of wtp53 function is rarely due to inactivating mutations and it is often ascribed to missense mutations of *TP53* that generate mutp53 oncoproteins. Also the mutp53 forms have been shown of being able to broadly control metabolic pathways. Interestingly, as described in the following paragraphs, both wtp53 and mutp53 control similar metabolic processes but often in an opposite manner. This ability of mutp53 to control cell metabolism is emerging as fundamental for tumorigenesis as well as a potential Achille's heels for cancer treatment.

Role of p53 in the regulation of glycolysis and oxidative phosphorylation

In most cells wtp53 favors oxidative phosphorylation (OXPHOS) rather than glycolysis. Indeed, p53 reduces glucose intake by transcriptional repression of glucose transporters GLUT1 and GLUT4 and slows various steps of the glycolytic flux by increasing expression of TIGAR while reducing the expression of phosphoglycerate mutase PGM[180–182]. In parallel, p53 couples the limitation imposed to the glycolytic rate with an increase in the OXPHOS, by promoting transcription of several genes involved in the mitochondrial respiration such as *COX1*, *SCO2* and *p53R2*[183,184]. In addition, p53 negatively regulates the transcription of pyruvate dehydrogenase kinase isoenzyme-2 (PDK2) thus promoting conversion of pyruvate into Acetyl-CoA instead of lactate, finally fostering TCA cycle[185].

Conversely, mutp53 sustains the Warburg effect in malignant cells. This is mainly achieved by increasing glucose uptake via induction of GLUT1 translocation to the plasma membrane through activation of the RhoA-ROCK signaling[175]. Moreover, mutp53 induces the expression of the glycolytic enzyme HK2 and enhances mTORC1-mediated phosphorylation of PKM2[186,187]. However, it has been reported that mutp53 is also able to promote OXPHOS by binding and fostering PGC1α functions, master transcription factor of mitochondrial biogenesis[188]. This feature appears particularly relevant in the Li Fraumeni syndrome (LFS), a condition characterized by p53 missense mutations in the germinal line, that lead to the expression of mutp53 and increased risk of tumorigenesis in various tissues. In this context, genetic or pharmacological interference with mitochondrial respiration improves cancer-free survival of LFS murine models. Moreover, in LFS patients, metformin treatment decreases mitochondrial respiration and promotes antiproliferative signaling[189]. This only apparently contrasting role of mutp53 in cancer metabolism suggests that mutp53 probably enables cancer cells with metabolic plasticity, favoring their adaptation to constantly challenging conditions[164].

Role of p53 in controlling lipid metabolism

Metabolic regulation directed by p53, importantly involves lipid metabolism which is crucial for example to provide constituents for membranes and to support signaling pathways. In particular, p53 interacts ambivalently with the transcription factors SREBP1 and SREBP2, main regulators of fatty acids biosynthesis and MVP respectively. Indeed, wtp53 transcriptionally represses SREBP1 and impairs maturation of SREBP2[190,191]. Coherently, pharmacological inhibition of MVP strongly limits tumor development due to loss of p53, demonstrating that the repression of the MVP is a crucial component of p53 tumor suppressive functions[191].

On the other hand, mutp53 acts as a transcriptional co-activator of SREBPs thus promoting expression of genes of the fatty acids biosynthesis pathway and MVP[163]. The latter, generates sterols and isoprenoids needed for the membrane synthesis, signal transduction and proteins prenylation. The relevance of this mutp53 activity emerges from the fact that, via MVP, mutp53 favors tumor invasion by disrupting the normal mammary tissue architecture and drives cancer cells aberrant mechano-responsiveness[192]. In this regard, as I previously mentioned, MVP-derived geranylgeranyl-pyrophosphate is crucial to activate RhoA, that in turn induces actin polymerization and activation of several oncoproteins including YAP/TAZ and mutp53 itself[171,193]. Thus, mutp53 simultaneously senses and controls lipid metabolism and mechanosignaling.

Mutp53 has also been reported to control lipid metabolism via inhibition of AMPK, master metabolic sensor in the cells. Indeed, AMPK dampens lipid synthesis and promotes fatty acid oxidation through

inhibitory phosphorylation of SREBP1/2 and acetyl-CoA carboxylase (ACC). Thus, mutp53 association with AMPK results in further enhanced lipid synthesis to sustain tumorigenesis[194].

Role of p53 in amino acid metabolism regulation

In recent years, a dual role of wtp53 has emerged in the regulation AA metabolism in challenging nutritional conditions. Indeed, it was reported that wtp53 can promote cancer cells death by inhibiting AAs transporters and biosynthetic enzymes or can even favor cells adaptation and survival during deprivation of specific AAs, a function that can be beneficial for cancer cells.

Considering its canonical tumor suppressive functions, a recent work links wtp53 to asparagine metabolism, unveiling a mechanism that can at least in part explain the propensity of p53-deficient mice to develop lymphomas. The authors show that wtp53 represses the expression of ASNS and thus dismantles the aspartate-asparagine homeostasis. This results in reduced asparagine production and secretion which is crucial to promote lymphomas proliferation, dampening their growth[195]. Another study discloses a new tumor suppressive activity of wtp53 in sensitizing cancer cells to ferroptosis upon oxidative stress through the regulation of cysteine metabolism. This function depends on the ability of wtp53 to transcriptionally suppress the xCT transporter and cysteine uptake, impairing the generation of GSH[196]. Regarding wtp53 functions in regulating serine metabolism, the situation appears more complicated and context dependent. In general, tumor cells rapidly use exogenous serine and deprivation of this AA triggers the activation of the SSP with consequent suppression of glycolysis and increased flux of the TCA cycle. At a molecular level, wtp53 has been reported to repress *PHGDH* promoter in melanoma cells, promoting apoptosis in serine deprivation conditions. However, independently from p53, it has been shown that its canonical target MDM2 has an opposite role in the transcriptional control of genes coding for the enzymes of the SSP. Indeed, in serine and glycine restriction, MDM2 is recruited to the nucleus where it interacts with ATF4 directly activating the expression of SSP enzymes. Despite MDM2 is a canonical target of wtp53, the authors did not investigate a potential role of wtp53 in cancer cells exposed to serine deprivation or in general to AAs restriction. In this regard many groups unveiled a peculiar role of wtp53 in preserving cancer cell fitness during specific AAs restriction. The group of K. Vousden observed that wtp53 plays a critical role in sustaining colorectal cancer cells survival when serine availability is reduced, firstly revealing an AA metabolism-related p53 function that provides tumors with a survival advantage. In details, p53 induces a transient activation of p21 and consequent G1 cell cycle arrest of colorectal cancer cells during serine starvation; this is an essential step to allow a switch in energy metabolism and a redirection of serine stores to generate GSH instead of nucleotides, thus ensuring antioxidant power. Notably they found that wtp53 had no effect on the SSP expression. Moreover, cancer cells that lack

p53 are no more able to adapt to this condition and show high oxidative stress, reduced viability, and compromised proliferation[11]. The same group published that one specific mutp53 (the R248H), selectively retains wtp53 ability to sustain cell survival in serine deprivation through the induction of p21 and MDM2, and consequent induction of antioxidant response[197].

More recently, other groups demonstrated that p53 is activated in AAs depleted conditions and protects cancer cells from this nutritional stress. For instance, p53 promotes an adaptive response under glutamine removal, transcriptionally inducing an upregulation of the arginine transporter SLC7A3, in order to transiently increase the intracellular level of this AA, thus promoting mTORC1 pathway activation and tumor growth in glutamine starvation[198]. In similar conditions, p53 promotes the expression of aspartate/glutamate transporter SLC1A3. Indeed, aspartate plays a crucial role in glutamine deprivation fueling TCA cycle and promoting *de novo* synthesis of glutamine, glutamate and nucleotides in order to ensure cancer cell viability in glutamine depleted conditions[199].

This evidence unveils the emerging concept that wtp53 activities, which aim at maintaining cellular homeostasis during metabolic stresses, can be usurped by malignant cells. This means that wtp53 may provide unexpected and counterintuitive mechanisms through which it sustains tumorigenesis, therefore disclosing a p53 pro-tumoral behavior. Nevertheless, these observations have been made under total deprivation of a specific amino acid, a condition that is quite far from what might happen *in vivo*. Indeed, tumors frequently experience a more general and global reduction in amino acids availability.

Most importantly, these studies suggested that missense p53 mutations might act merely as a loss of wtp53 functions or, as described only for specific mutp53 forms, similarly to wtp53. Unexpectedly, whether mutp53 may regulate AA metabolism, thus favoring cancer evolution, has not been investigated so far.

2. AIM OF THE THESIS

Metabolic reprogramming is a hallmark of tumors that enables cancer cells to proliferate, to disseminate and generate metastases by proving plenty of metabolic and energetic intermediates and by allowing their survival in challenging environments. In particular, AA metabolism has been shown to be crucial for tumor homeostasis and progression and it enables cancer cells to cope with a variety of cancer related stresses. AAs are indeed involved in several vital processes i.e., energy production, synthesis of macromolecules, maintenance of redox balance, epigenetic and post-translational regulation, and activation of the mTORC1 pathway. Thus, tumors exhibit a particular avidity and dependence on AA metabolism. The massive and coordinated influx, synthesis and consumption of AAs is often achieved by alterations in major oncogenes and tumor suppressors.
mutp53 acts as a guardian of cancer cells since it orchestrates adaptive responses to multiple intrinsic and extrinsic cancer-related stresses by reshaping cancer cell's transcriptome, proteome, and metabolism. In particular, the ability of mutp53 to upset the metabolism of cancer cells is emerging to be particularly relevant for tumorigenesis as well as a potential Achille's heel for cancer treatment. Therefore, in light of the fact that tumorigenesis heavily relies on a robust AA metabolism, we hypothesized that mutp53 might control specific amino acid metabolism's branches in order to favor tumor growth and progression in response to cancer related stresses.

Thus, the aim of this thesis is to investigate the role of mutp53 in the regulation of AA metabolism in breast cancer as well as its relevance for cancer cells fitness. In particular, we aim to investigate whether mutp53, through hijacking AA metabolism, might support metabolic adaptation to environmental stresses, such as nutrient starvation and mechanical cues.

3. RESULTS

3.1 MUTP53 CONTROLS A SPECIFIC PROGRAM OF AMINO ACID METABOLISM IN BREAST CANCER

Invasive breast cancers (BCs) strongly rely on an increased metabolic rate. In particular the metabolic profile of the TNBC greatly diverts from all other BC subtypes and normal tissues, exhibiting a heterogeneous but massive increase in lipid, carbohydrate, nucleotide, and amino acid metabolism[200,201]. Notably, *TP53* mutations, that occur in approximately 25% of primary breast cancers, are predominantly found in TNBC (80% of cases). For these reasons, we decided to perform metabolic analysis in the TNBC MDA-MB 231 cell line, derived from a metastatic mammary adenocarcinoma and expressing the missense *TP53* mutation R280K.

De novo synthesis of AAs, fed by central carbon metabolism, is a key feature of aggressive tumors enabling cancer cell to be independent of environmental nutrient fluctuations. Thus, we monitored the fate of glucose-derived carbon units in AAs biosynthesis by performing metabolic flux analysis. This technique is widely used to properly follow the fate of a specific metabolite. In details, a substrate, e.g. glucose, can be labeled with a stable isotope, such as ^{13}C, that can be internalized and metabolized by the cells; thus, the downstream labeled metabolites can be identified and quantified by mass spectrometry. To assess whether mutp53 may have an impact on the synthesis of NEAAs, we cultured MDA-MB 231 cells in presence of ^{13}C-labeled glucose in control conditions or upon silencing of mutp53 and we traced carbon units from this substrate into amino acids and other metabolites. Unexpectedly, $^{13}C_6$-Glucose incorporation into glycolytic metabolites and TCA cycle intermediates was not altered in the two different conditions (Fig. 11B). Analyzing newly synthesized NEAAs, we observed that cells silenced for mutp53 exhibited a significantly reduced incorporation of ^{13}C-carbon units into aspartate, serine and glycine, compared to control cells (Fig. 11B).

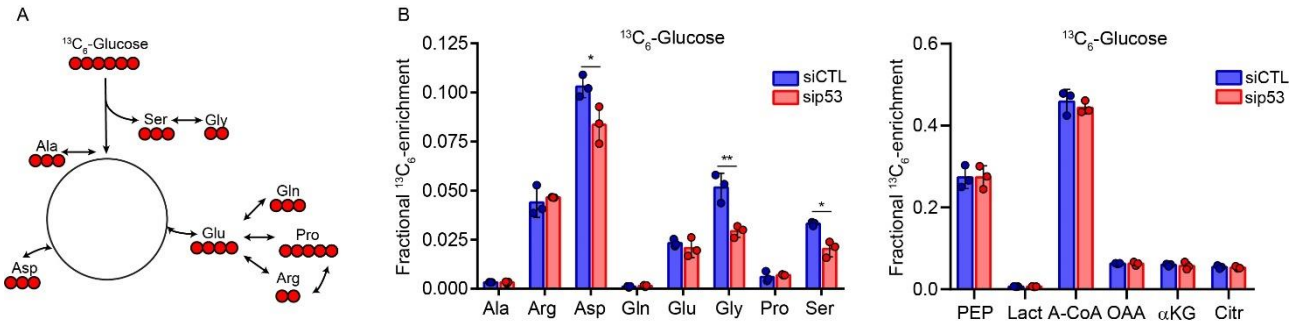


Figure 11. Serine, glycine and aspartate *de novo* synthesis is altered upon silencing of mutp53. A. Schematic depicting synthesis of AAs from labeled $^{13}C_6$-Glucose. Red spots represent ^{13}C units**. B.** Mass isotopomer analysis of AAs (left) and metabolites of glycolysis and TCA cycle (right) from $^{13}C_6$-Glucose in MDA-MB 231 cells upon control (siCTL) or p53 (siP53) silencing. For labeling experiments, cells were transfected with control or p53 siRNAs the day of seeding and cultivated for 24h followed by incubation with $^{13}C_6$-Glucose in medium with dialyzed serum for other 24h. Graph bars represent mean ± s.d. of n=3 biological replicates. P value: P < 0.05 [*], P < 0.01 [**], P < 0.005 [***] by two-tailed Student's t-test.

Given the crucial role of serine and glycine biosynthesis in cancer, since they are frequently limiting in the TME, we focused our attention on these amino acids. For this reason, we performed $^{13}C_6$-glucose tracing upon removal of serine and glycine from the medium, a stressful condition known to foster their synthesis. As expected, to compensate the serine/glycine removal, the amount of serine and glycine newly synthesized by the cells importantly surged. Notably, this increase was significantly reduced in cells silenced for mutp53 (Fig. 12B).

These experiments prove that mutp53 is required to sustain the synthesis of aspartate, serine and glycine from the glycolytic flux. Given that mutp53 did not alter glucose incorporation into glycolytic and TCA cycle metabolites (Fig. 11B), this increased biosynthesis seems not related to an increased glycolytic flux induced by mutp53, suggesting that mutp53 may specifically foster the expression or the activity of AAs related enzymes.

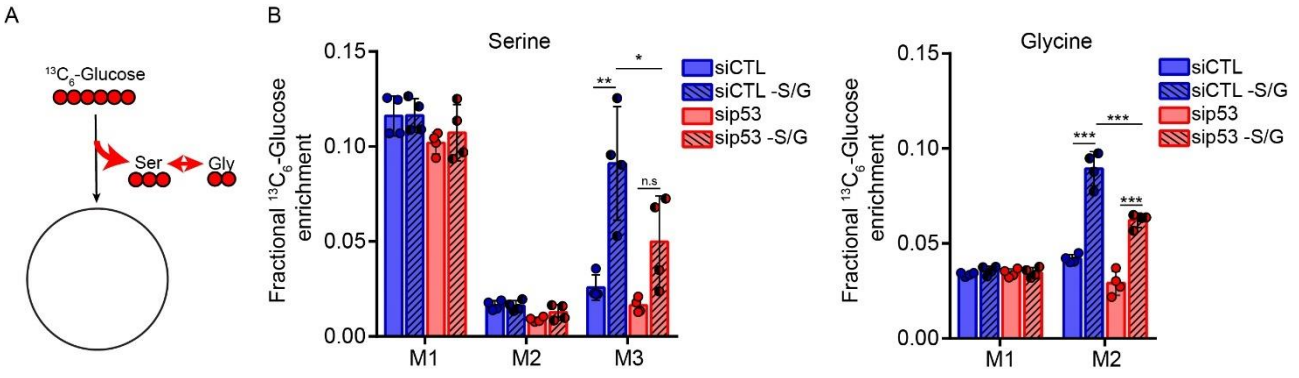


Figure 12. Increased incorporation of glucose into serine and glycine in response to serine/glycine withdrawal is reduced upon mutp53 silencing. A. Schematic depicting synthesis of serine and glycine from labeled $^{13}C_6$-Glucose after serine/glycine removal. Red spots represent ^{13}C units. **B.** Mass isotopomer analysis of serine (left) and glycine (right) from labeled $^{13}C_6$-Glucose in MDA-MB 231 cells upon control (siCTL) or p53 (siP53) silencing in complete medium or medium without serine and glycine (-S/G). For labeling experiments, cells were transfected with control or p53 siRNAs the day of seeding followed by incubation with $^{13}C_6$-Glucose in complete medium or medium without serine and glycine

(-S/G) for other 24h. M1, M2 and M3 represent AA mass with 1, 2 and 3 incorporated ^{13}C, respectively. Graph bars represent mean ± s.d. of n=4 biological replicates. P value: P < 0.05 [*], P < 0.01 [**], P < 0.005 [***] by One-way ANOVA.

3.2 MUTP53 PROMOTES THE EXPRESSION OF SPECIFIC AA METABOLISM ENZYMES AND TRANSPORTERS

3.2.1 Mutp53 sustains high expression of AA metabolism enzymes and transporters in BC cell lines

Given that mutp53 controls several metabolic pathways via interaction with master regulators of cell metabolism and/or by altering the transcription of metabolic enzymes, we wondered if mutp53 may promote the expression of enzymes involved in the AAs synthesis and metabolism. To test this hypothesis, we performed RNAseq analysis in MDA-MB 231 cells upon silencing of mutp53. We found that in mutp53-silenced cells the expression of genes involved in synthesis of aspartate, serine and glycine, including *GOT2*, *PSAT1* and *SHMT1*, were significantly reduced. Moreover, globally observing alterations in genes related to amino acid metabolism we found a reduced expression of several genes encoding for enzymes of folate, cysteine and methionine metabolism as well as for amino acid transporters. In particular, we found reduced expression of *SLC7A5* and *SLC3A2* (encoding for the LAT1/CD98hc heterodimer), *SLC1A5* (encoding for ASCT2) and *SLC7A1* (encoding for CAT1) which are responsible for the intake of BCAAs/bulky AAs, glutamine and arginine respectively (Fig.13A).

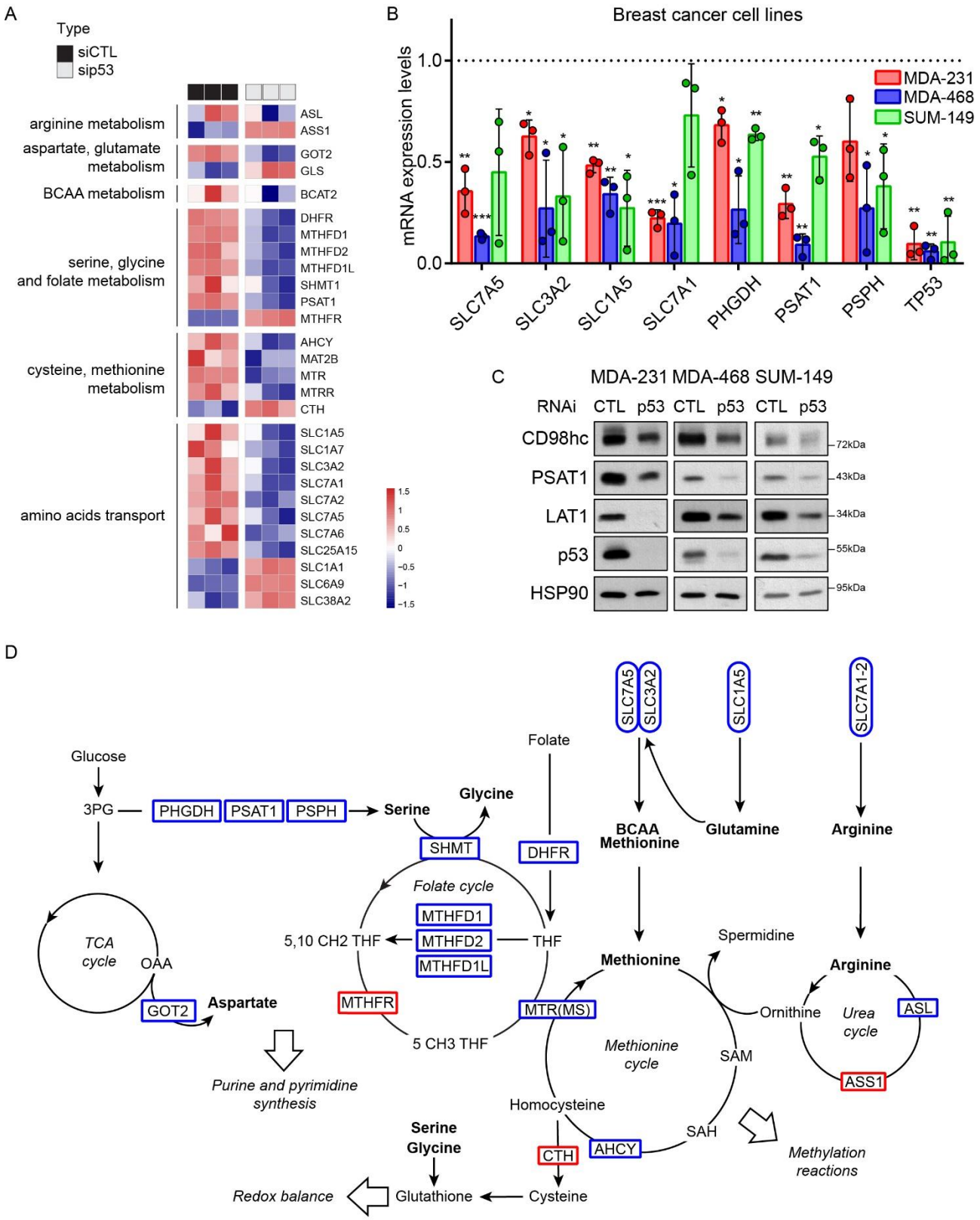


Figure 13. Mutp53 controls a transcriptional program related to specific amino acid metabolisms in BC cell lines. **A.** Heatmap of RNA sequencing data of genes related to amino-acid biosynthesis, intake and metabolism in MDA-MB 231 cells upon control (siCTL) or p53 (siP53) silencing. Three columns for each condition represent n=3 biological replicates. **B.** qRT-PCR of indicated genes in MDA-MB 231, MDA-MB 468 and SUM-149 cells upon silencing of p53. qRT-PCR values are expressed as mRNA levels relative to control condition set at 1 (dotted line). **C.** Western blot analysis of indicated proteins in MDA-MB 231, MDA-MB 468 and SUM-149 cells upon silencing of p53. P53 was shown as silencing control. CD98hc is encoded by *SLC3A2* and LAT1 by *SLC7A5*. **D.** Schematic showing genes that encode for AAs enzymes and transporters downregulated (blue) and upregulated (red) upon silencing of mutp53. The metabolic

pathways to which they belong and their cellular functions are indicated in italic. See Table 1 for gene names. Graph bars represent mean ± s.d. of n≥3 biological replicates. P value: P < 0.05 [*], P < 0.01 [**], P < 0.005 [***] by two-tailed Student's t-test. Blots are representative of n=3 biological replicates.

To verify if mutp53 sustains the expression of genes for the AA metabolism that emerged from the transcriptomic analysis, we performed RNA interference in three TNBC cell lines harboring mutp53. In details, we used the MDA-MB 231, MDA-MB 468 and SUM 149PT cell lines which express the mutated p53 variants R280K, R273H and M237I, respectively. Notably, in BC cells depleted for mutp53 we observed a strong reduction not only of *PSAT1* expression levels, but of all the SSP genes (Fig. 13B-C). Moreover, we found that silencing of mutp53 reduced the levels of several genes encoding for AAs transporters that emerged from the RNAseq analysis (Fig. 13B-C). Notably, overexpression of mutp53 was able to boost *PSAT1*, *SLC7A5* and *SLC3A2* also in less aggressive MCF10DCIS.com cell line (Fig. 14A). To extend the relevance of our results, we decided to engineer the 4T1 metastatic breast cancer cell line, that is functionally null for p53, to express four different inducible mutant p53 variants. The induction of all the four p53 mutated forms strongly increased the expression of *Psat1*, *Slc7a5* and *Slc3a2* after 24h of doxycycline treatment (Fig. 14B).

Overall, we found that presence of different mutp53 forms in aggressive metastatic cell lines are able to sustain high levels of genes encoding for SSP enzymes and transporters of BCAAs/bulky AAs, glutamine and arginine. Increased amino acid metabolism is a feature of aggressive BCs, in particular of TNBC, where mutp53 is frequently observed.

Interestingly, similar results were obtained using cell models derived from mouse models that harbor different germline *TP53* status: $p53^{+/+}$ (p53wt), $p53^{-/-}$ (p53KO), $p53^{R172H/R172H}$ (mutp53). In particular, we generated mouse mammary organoids and mouse embryonic fibroblasts (MEFs) and we observed higher protein levels of Psat1 in presence of mutp53 (Fig. 14C-D).

Overall, these results demonstrate that mutp53 sustains the expression of specific AAs biosynthetic enzymes and transporters related to serine/glycine, BCAAs/bulky AAs, glutamine and arginine (Fig. 13D).

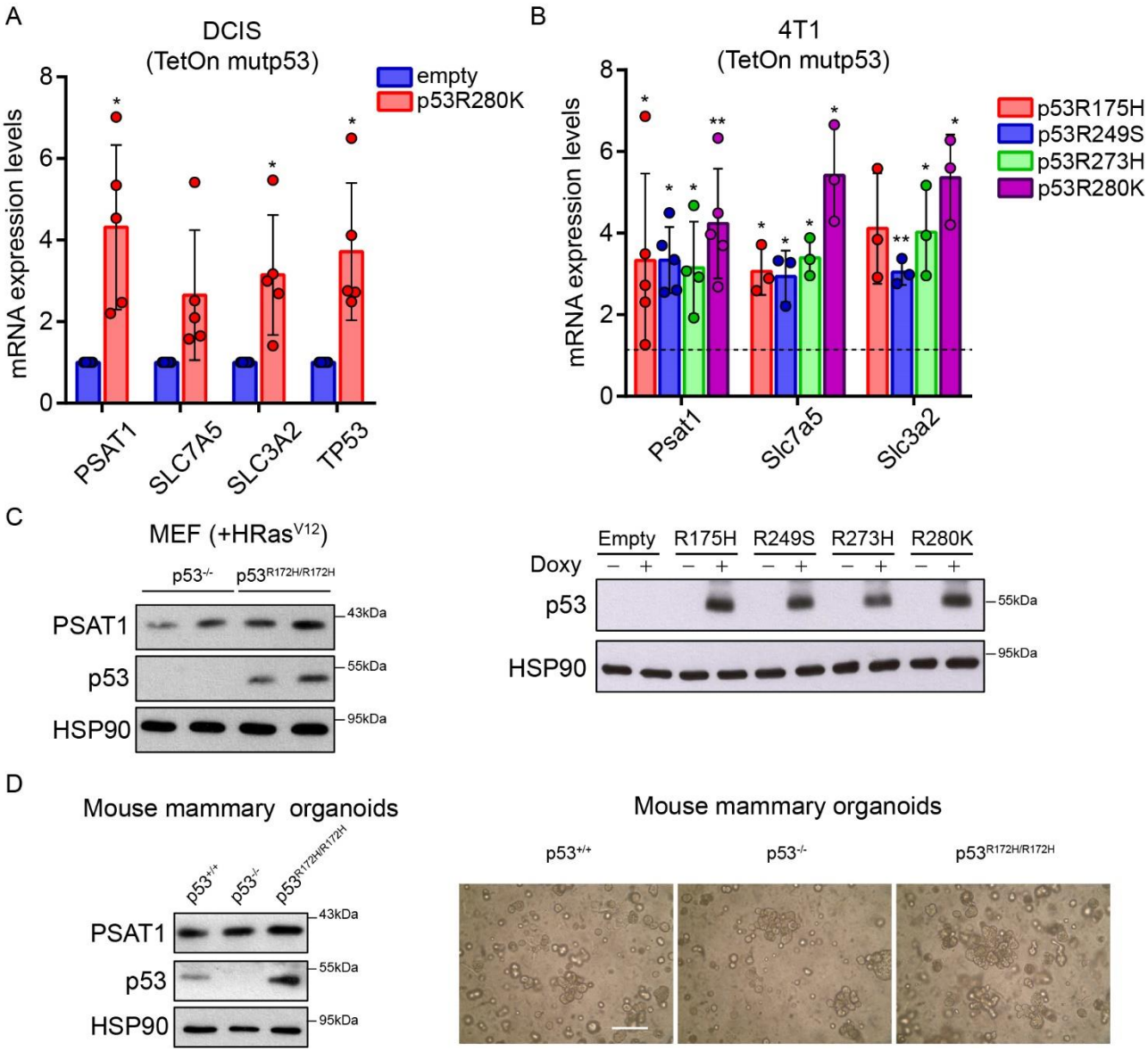


Figure 14. Mutp53 promotes the expression of SSP enzymes and several AAs transporters in BC cell lines. A. qRT-PCR analysis of the indicated genes in MCF10DCIS.com TetOn inducible clones in presence doxycycline 1ug/mL for 48h. qRT-PCR values are expressed as mRNA levels relative to MCF10DCIS.com clone expressing empty vector set at 1. **B.** qRT-PCR analysis of the indicated genes in 4T1 TetOn inducible clones in presence of doxycycline 1ug/mL for 24h. qRT-PCR values are expressed as mRNA levels relative to 4T1 clone expressing empty vector set at 1 (dotted line). Western blot analysis of p53 levels in 4T1 TetOn inducible clones in presence (+) or absence (-) of doxycycline 1ug/mL for 24h. **C.** Western blot analysis of Psat1 levels in mouse mammary organoids derived from $p53^{+/+}$, $p53^{-/-}$ or $p53^{R172H/R172H}$ mice. Representative images of the same organoids are shown. Scale bar 50µm. **D.** Western blot analysis of Psat1 levels in Ras-transformed mouse embryonic fibroblast derived from $p53^{-/-}$ or $p53^{R172H/R172H}$ mice. Graph bars represent mean ± s.d. of n≥3 biological replicates. P value: P < 0.05 [*], P < 0.01 [**], P < 0.005 [***] by two-tailed Student's t-test. Blots are representative of n=3 biological replicates.

3.2.2 Mutp53 sustains mTORC1 activation

mTORC1 is the main cellular sensor of the nutritional and energetic status of the cell and its activation requires the presence of specific AAs. Different amino acids sensors are present in cells and, upon influx of specific amino acids, they mediate mTORC1 translocation to the lysosomes, allowing its activation. Based on previous results demonstrating that mutp53 promotes serine/glycine synthesis

and the expression of the AAs transporters, we hypothesized that mutp53 may induce mTORC1 lysosomal localization and activation in BC cells. To verify this hypothesis, we performed immunofluorescence staining of mTOR and lysosomes. In BC cells we observed that, upon silencing of mutp53, mTORC1 lysosomal localization is reduced (Figure 15A). In addition, we decided to analyze mTORC1 activity by monitoring the phosphorylation level of its downstream target, S6 ribosomal protein (S6RP). Coherently, we found that silencing of mutp53 decreased the phosphorylation level of S6RP and concomitantly increased the LC3-II/I ratio indicating enhanced autophagy (Fig. 15B).

Collectively, this evidence demonstrates that mutp53 promotes mTORC1 lysosomal recruitment and activation in breast cancer cells, potentially by favoring AAs availability.

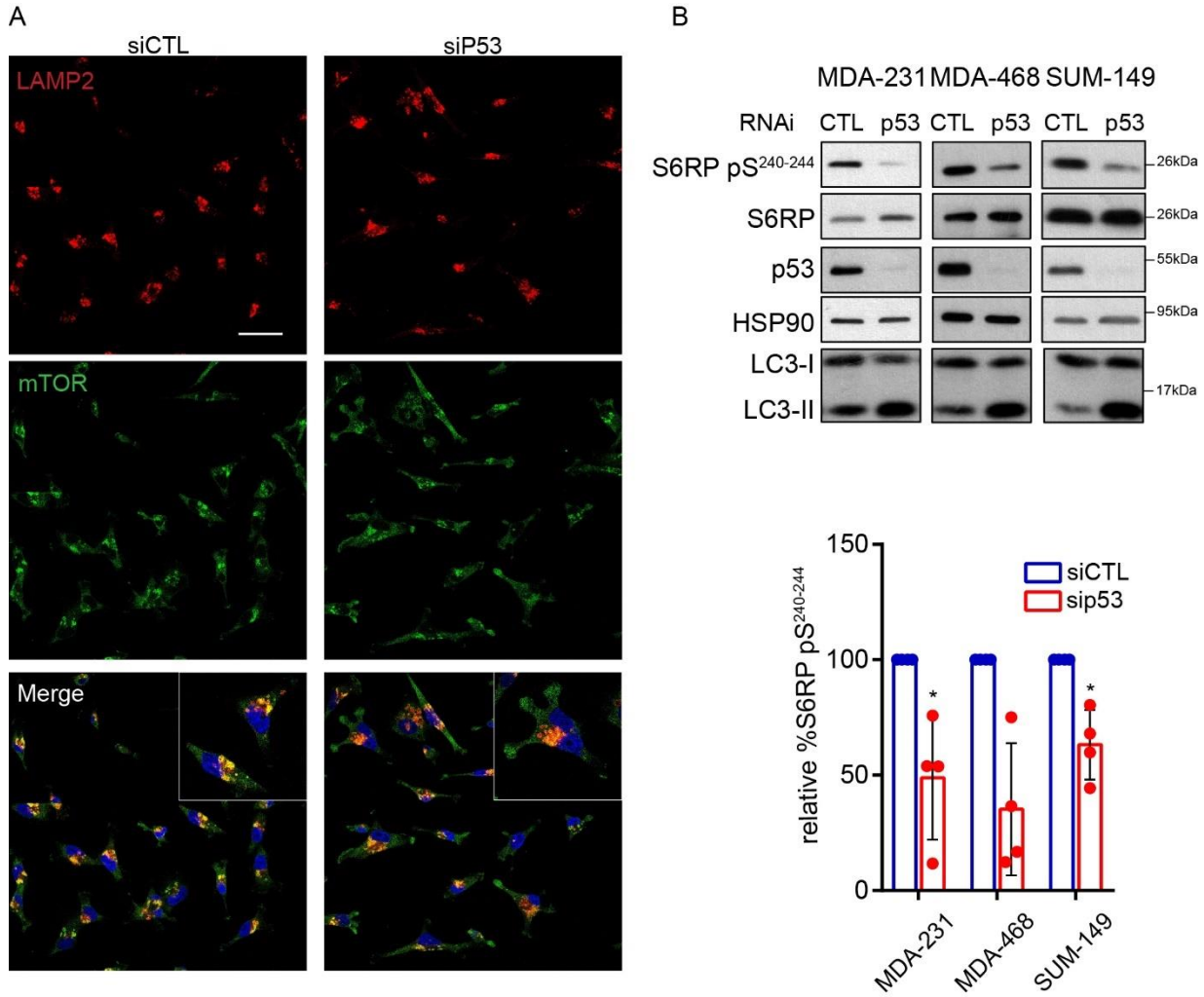


Figure 15. Mutp53 sustains mTORC1 lysosomal localization and activation in BC cell lines. A. Representative images of immunofluorescence analysis of MDA-MB 231 cells grown in control condition (siCTL) or upon silencing of p53 (siP53) stained with anti-mTOR (in green) and anti-LAMP2 (in red), a lysosomal marker. Nuclei were stained with HOECHST (in blue). Scale bar 20 μm. Magnifications of mTOR/LAMP2 staining are also shown. **B.** Western blot analysis of the indicated proteins in MDA-MB 231, MDA-MB 468 and SUM-149 cells upon silencing of p53. P53 was shown as silencing control (upper panel). Graph shows the quantification of depicted western blot bands expressed as percentage of S6RP $pS^{240-244}$ relative to S6RP (lower panel). Graph bars represent mean ± s.d. of n=3 biological replicates. P value: $P < 0.05$ [*], $P < 0.01$ [**], $P < 0.005$ [***] by two-tailed Student's t-test. Blots are representative of n=3 biological replicates.

3.2.4 Mutp53 engages MYC to promote transcription of AAs transporters and biosynthetic enzymes

To evaluate whether mutp53 could be associated to the promoters of these genes, we took advantage of data derived from a ChIP-seq of mutp53 performed in MDA-MB-231 that are available in the public resource ReMap 2022[202]. We found the presence of ChIP-seq peaks of mutp53 on the promoters of *PSAT1*, *SLC7A5*, *SLC3A2* and *SLC1A5* (Fig. 16A). Since mutp53 frequently binds DNA via interaction with specific transcription factors, we inspected public ChIP-seq analyses examining the transcription factors that co-occur in the same regions of mutp53 ChIP-seq peaks (Table 2.). Hypothesizing that mutp53 may be recruited to these genomic loci together with one specific transcription factor, we only selected transcription factors that bind to all these genes. This analysis revealed the presence of 10 putative TFs, including MYC/MAX, BRD4, FOXM1 and c-JUN. Looking for their relevance for amino acid metabolism in cancer, we focused our attention on MYC since it has been reported to transcriptionally regulate several amino acids related genes and to functionally interact with mutp53 in cancer[203,204]. In order to evaluate whether MYC is required for mutp53-dependent induction of *PSAT1*, *SLC7A5* and *SLC3A2*, we performed RNA interference of MYC in MDA10DCIS.com cell line expressing inducible mutp53. We observed that the induction of mutp53 promoted the expression of these genes and that silencing of MYC was able to dampen this induction. These data indicate that MYC is required for the mutp53 dependent induction of *PSAT1*, *SLC7A5* and *SLC3A2* (Fig. 16B).

Taken together, this evidence indicates that mutp53 binds the promoter regions of *PSAT1*, *SLC7A5* and *SLC3A2* and that it depends on the presence of MYC to promote their expression. However, further experiments are required to verify whether MYC is required for mutp53 binding to their promoters.

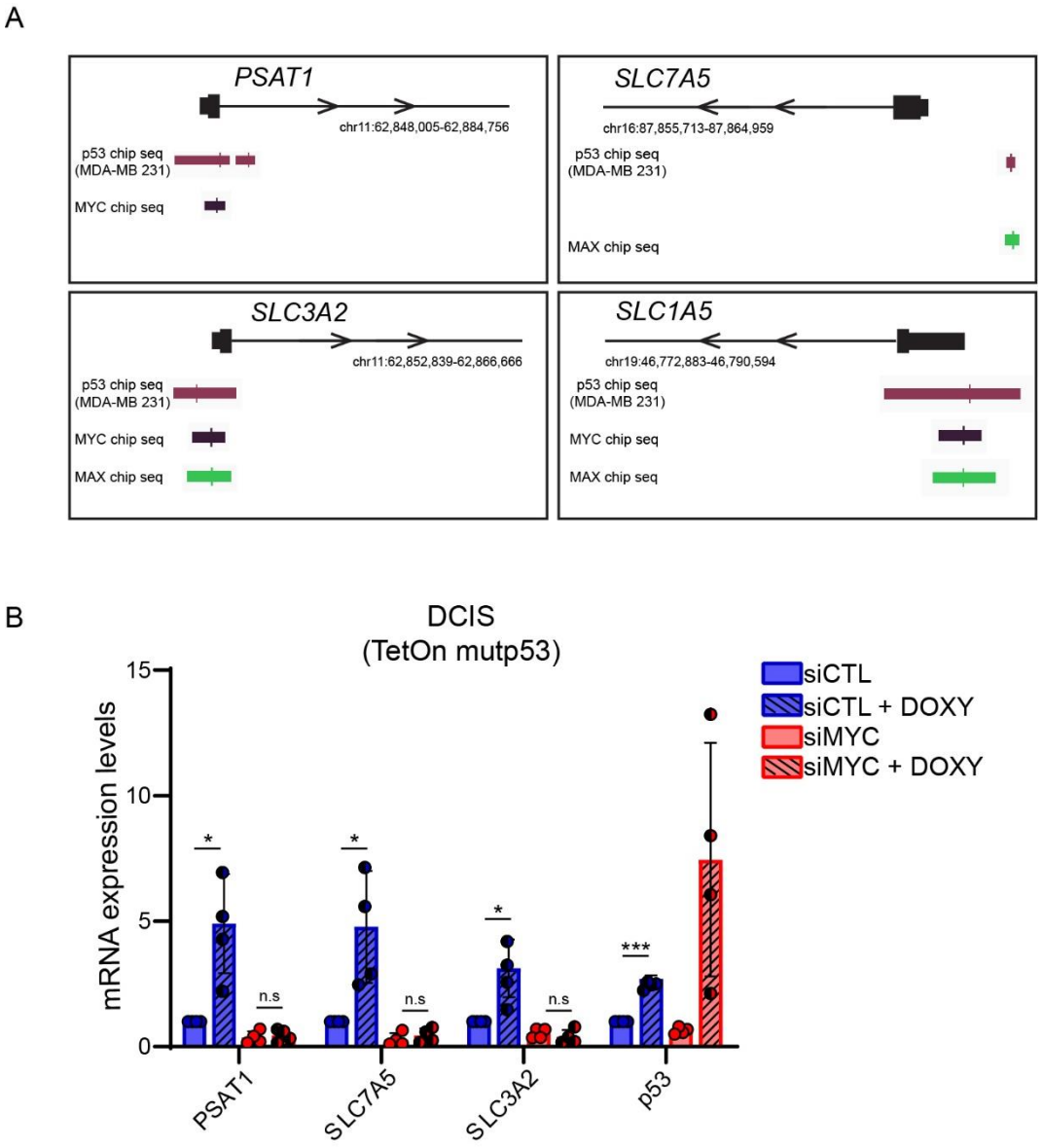


Figure 16. MYC mediates mutp53-dependent induction of PSAT1, SLC7A5 and SLC3A2. A. Schematic representation of *PSAT1*, *SLC7A5*, *SLC3A2*, *SLC1A5* loci comprising the features annotated on the putative promoters**. B.** qRT-PCR of the indicated genes in MCF10DCIS.com TetOn inducible clone p53 R280K in presence (+DOXY) or absence of doxycycline 1ug/mL upon control (siCTL) or MYC (siMYC) silencing for 48h. qRT-PCR values are expressed as mRNA levels relative to MCF10DCIS.com clone in absence of doxycycline in control condition (siCTL) set at 1. Graph bars represent mean ± s.d. of n=4 biological replicates. P value: P < 0.05 [*], P < 0.01 [**], P < 0.005 [***] by two-tailed Student's t-test.

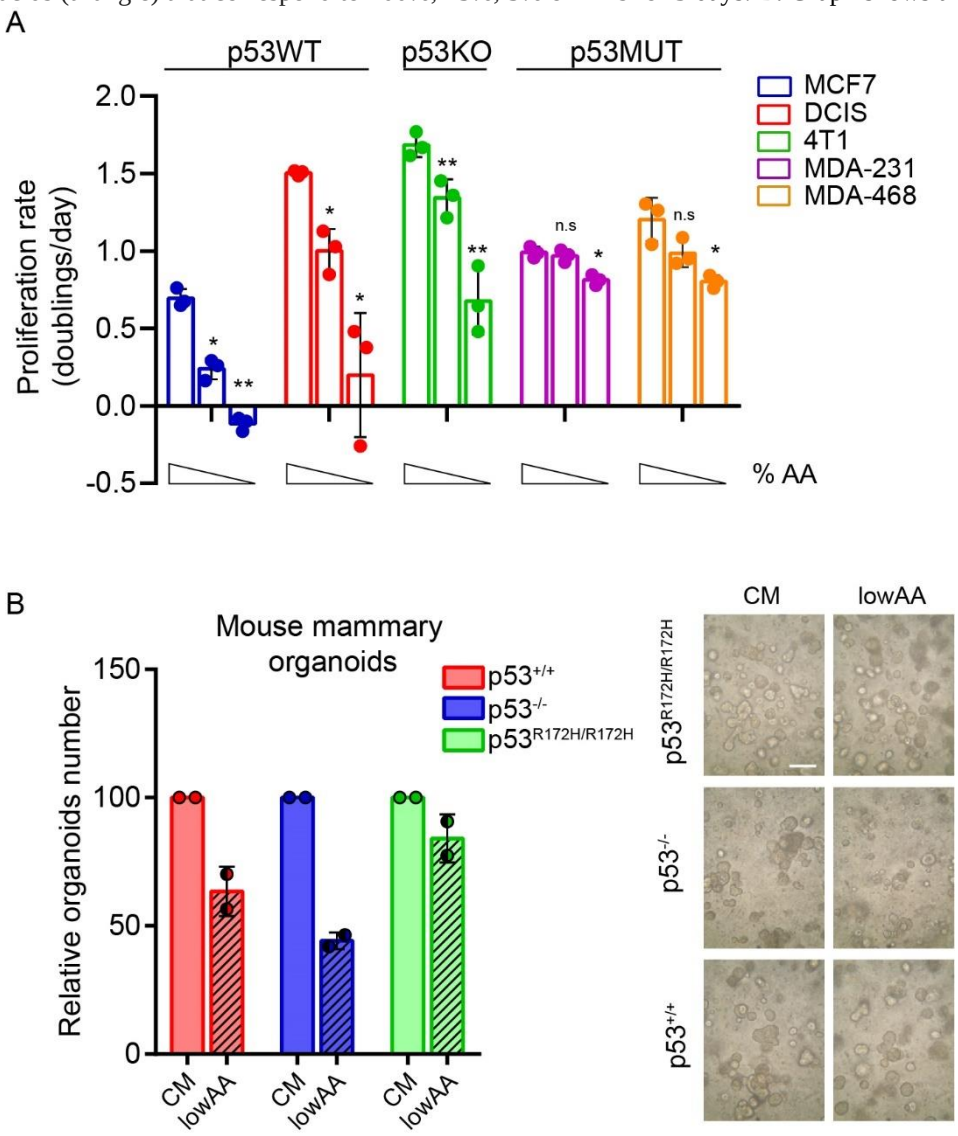
3.3 MUTP53 ENABLES CANCER CELLS TO COPE WITH NUTRIENT DEPRIVATION

3.3.1 Mutp53 confers a proliferative advantage to BC cells during AAs deprivation

The metabolic adaptation of cancer cells to cancer related stresses is crucial to guarantee their fitness. Specifically, tumors frequently encounter reduced amino acids availability and they try to increase amino acids intake and biosynthesis in order to survive. Mutp53 is known to promote adaptive responses to several cancer-related stresses, supporting tumor progression. Given the capacity of

mutp53 to sustain serine/glycine synthesis and to increase the expression of several amino acid transporters, we speculated that mutp53 may support cancer cells fitness when they experience amino acids scarcity. This hypothesis led us to monitor the proliferation rate of different BC cell lines with a different p53 status cultivated in amino acids deprivation. We observed that, despite different basal proliferation rates, BC cell lines expressing mutp53 were still able to proliferate when cultivated in media with reduced amount of AAs, while cells not expressing p53 or expressing wtp53 drastically reduced, or even arrested, their proliferation in the same conditions, supporting the idea that mutp53 may sustain cancer cell fitness during AAs restriction (Fig. 17A).

Figure 17. Cells harboring mutp53 maintain a high proliferation rate even in AAs deprivation. A. Doubling times of MCF7, MCF10DCIS.com, 4T1, MDA-MB 231 and MDA-MB 468 cell lines cultured in medium with a decreasing amount amino acids (triangle) that correspond to 100%, 25%, 5% of AAs for 3 days. **B.** Graph shows the percentage of



proliferating mouse mammary organoids, selected as bigger than 50µm, derived from $p53^{+/+}$, $p53^{-/-}$ and $p53^{R172H/R172H}$ mice grown in complete medium (CM) or medium with low AAs (low AAs). Representative images show the same organoids grown in low AAs relative to the same organoids grown in CM. Scale bar 50µm.Graph bars represent mean ± s.d. of n≥2 biological replicates. P value: P < 0.05 [*], P < 0.01 [**], P < 0.005 [***] by two-tailed Student's t-test.

To further address this point we decided to use a more physiological system, taking advantage of mouse mammary 3D organoids. We grew mouse mammary organoids WT for p53, loss for p53 or expressing mutp53 in complete medium (CM) or with a medium that mimic AAs scarcity (Low AA). We found that in AA deprivation the percentage of proliferating organoids with WT p53 or loss for p53 was reduced while cells expressing mutp53 were still able to proliferate (Fig. 17B).

Given this evidence, we decided to evaluate the impact of mutp53 in sustaining cancer cell proliferation during nutritional stress. To this aim, we assessed the proliferation rate of MDA-MB 231 cells upon silencing of mutp53. As shown in Fig. 18A silencing of mutp53 was most effective in reducing the proliferation rate of the cells when they were exposed to AAs deprivation. Since adaptive response of cancer cells to AAs restriction increases expression of amino acids transporters and enzymes, we investigated whether BC cells depleted for mutp53 might be unable to properly activate this response. As expected, MDA-MB 231 cultivated in medium with low amount of AAs, exhibited the activation of stress response, as detected by an increase in the phosphorylation level of eukaryotic translation initiation factor 2α (eiF2α), as well as a reduction in mTORC1 activation, indicated by a decrease in S6RP phosphorylation (Fig. 18D). Moreover, we observed an increased gene expression and protein levels of several amino acids related genes compared to cells grown in complete medium (CM) (Fig. 18B; D). Notably, in the same experimental condition, we observed that silencing of mutp53 partially prevented this response (Fig. 18B; D). Similar results were obtained in MDA-MB 468 (Fig. 18C) cells confirming that mutp53 sustains the upregulation of the SSP enzymes and of relevant AAs transporters, in particular when cancer cells experience AA scarcity, in order to sustain their proliferation.

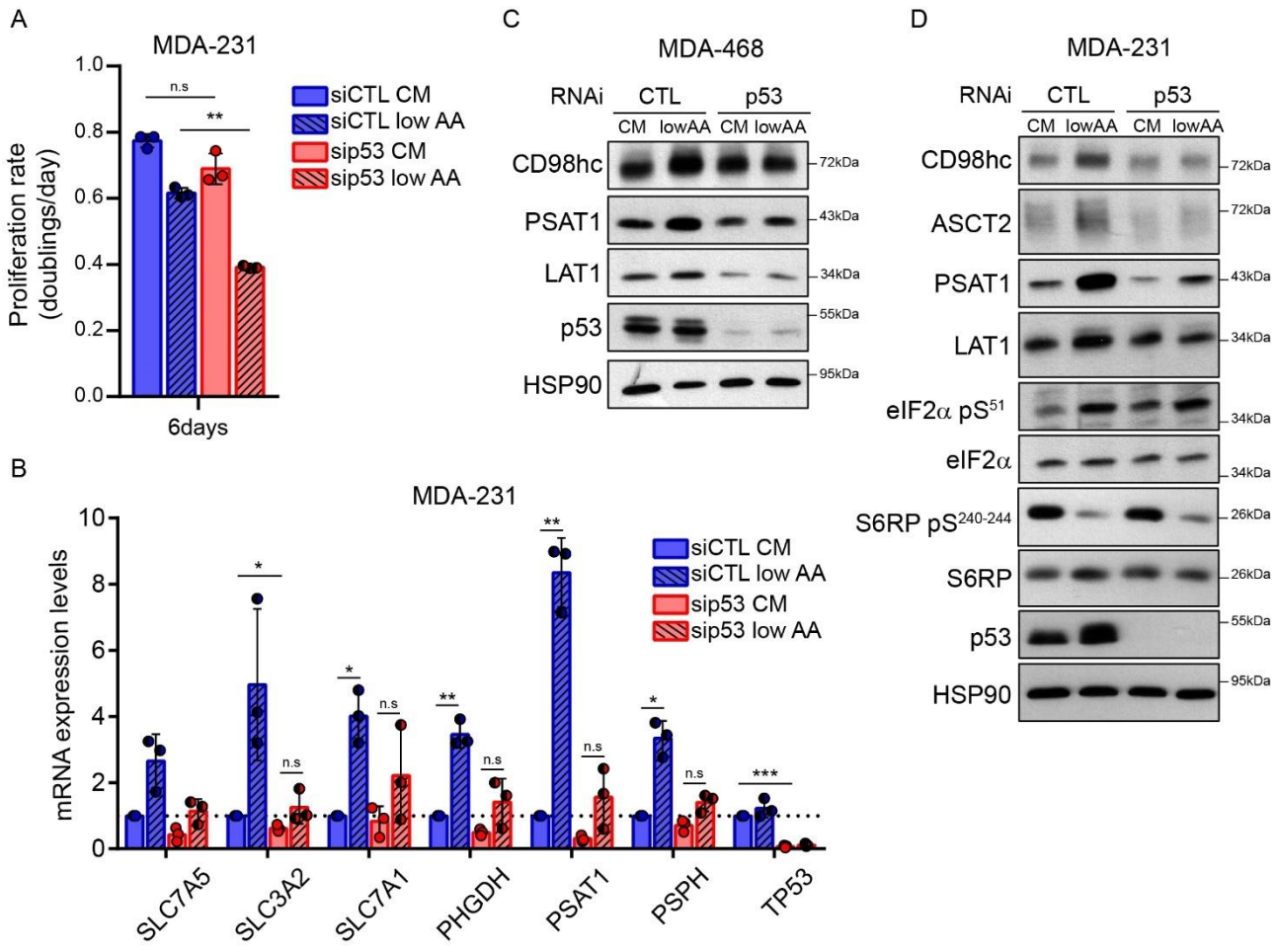


Figure 18. Mutp53 is required for BC cells proliferation and increased expression of AA biosynthetic enzymes and transporters in AAs deprivation. **A.** Doubling times of MDA-MB 231 cells cultured in complete medium (CM) or medium containing 5% of AAs (low AA) upon silencing of p53 for 6 days. **B.** qRT-PCR of indicated genes in MDA-MB 231cells cultivated for 72h in complete medium (CM) or medium containing 5% of AAs (low AA) upon silencing of p53. qRT-PCR values are expressed as mRNA levels relative to control condition set at 1 (dotted line). **C-D.** Western blot analysis of the indicated proteins in MDA-MB 468 and MDA-MB 231 cells cultivated for 72h in complete medium (CM) or medium containing 5% AAs (low AA) upon silencing of p53. CD98hc is encoded by *SLC3A2*, ASCT2 by *SLC1A5* and LAT1 by *SLC7A*. Graph bars represent mean ± s.d. of n=3 biological replicates. P value: P < 0.05 [*], P < 0.01 [**], P < 0.005 [***] by two-tailed Student's t-test. Blots are representative of n=3 biological replicates.

Next, we compared the replication rate of 4T1 cells expressing inducible mutp53 R175H and R280K with parental 4T1, in nutritional stress conditions. We found that 4T1 cells proliferation was progressively reduced by the concomitant deprivation of AAs. Conversely, cells exposed to AAs restriction were still able to survive and proliferate when expressing mutp53 (Fig. 19A). Notably, the induction of mutp53 R280H had favored activation of mTORC1 pathway in these conditions (Fig. 19B).

All these results demonstrate that mutp53 provides an advantage to BC cells when they are exposed to scarcity of AAs.

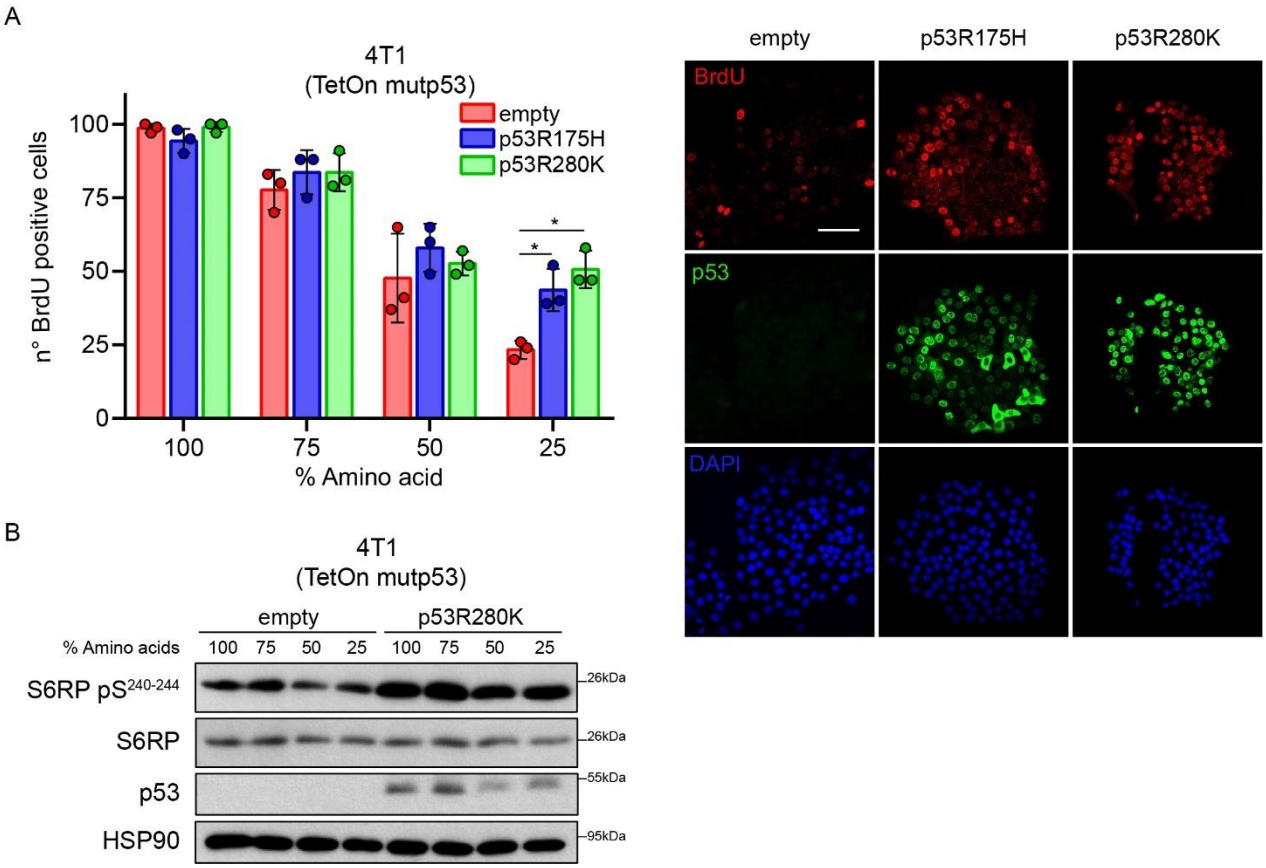


Figure 19. Mutp53 provides a survival and proliferative advantage to BC cells in AAs deprivation. A. BrdU incorporation analysis in indicated 4T1 TetOn inducible clones grown in medium with the indicated percentage of AAs for 48h, in presence of doxycycline 1ug/mL. BrdU was added 12h before the end of the experiment. The number of BrdU positive cells out of 100 nuclei counted for each condition are shown (left panel). Representative images of immunofluorescence analysis of indicated 4T1 TetOn inducible clones grown in medium containing 25% AAs and stained with anti-p53 (in green) and anti-BrdU (in red) antibodies. Nuclei were stained with Dapi (in blue). Scale bar 55 μm (right panel). **B.** Western blot analysis of indicated proteins in 4T1 TetOn inducible clone expressing p53 R280K cultivated in medium with the indicated percentage of AAs for 48h in presence (+) or absence (-) of doxycycline 1ug/mL. Graph bars represent mean ± s.d. of n≥3 biological replicates. P value: $P < 0.05$ [*], $P < 0.01$ [**], $P < 0.005$ [***] by two-tailed Student's t-test. Blots are representative of n=3 biological replicates.

3.2 Serine synthesis and LAT1-mediated AAs transport are required for mutp53 gain of function

In light of the above results, we asked whether inhibition of serine synthesis and LAT1-mediated AAs transport may dampen mutp53-dependent growth advantage of tumor cells. To address this point we used MCF10DCIS.com and 4T1 cell lines in which we observed that induction of mutp53 conferred a proliferative advantage in AAs deprivation (Fig. 20A; C). In the same condition, the effect of mutp53 was lost upon treatment with NCT-503 and JPH203, which selectively inhibit the first enzyme of SSP (PHGDH) and the AAs transporter LAT1, respectively (Fig. 20B-C).

These results demonstrate that mutp53 capability to sustain proliferation and survival of BC cells in nutritional deprivation depends on *de novo* serine synthesis and LAT1 activity.

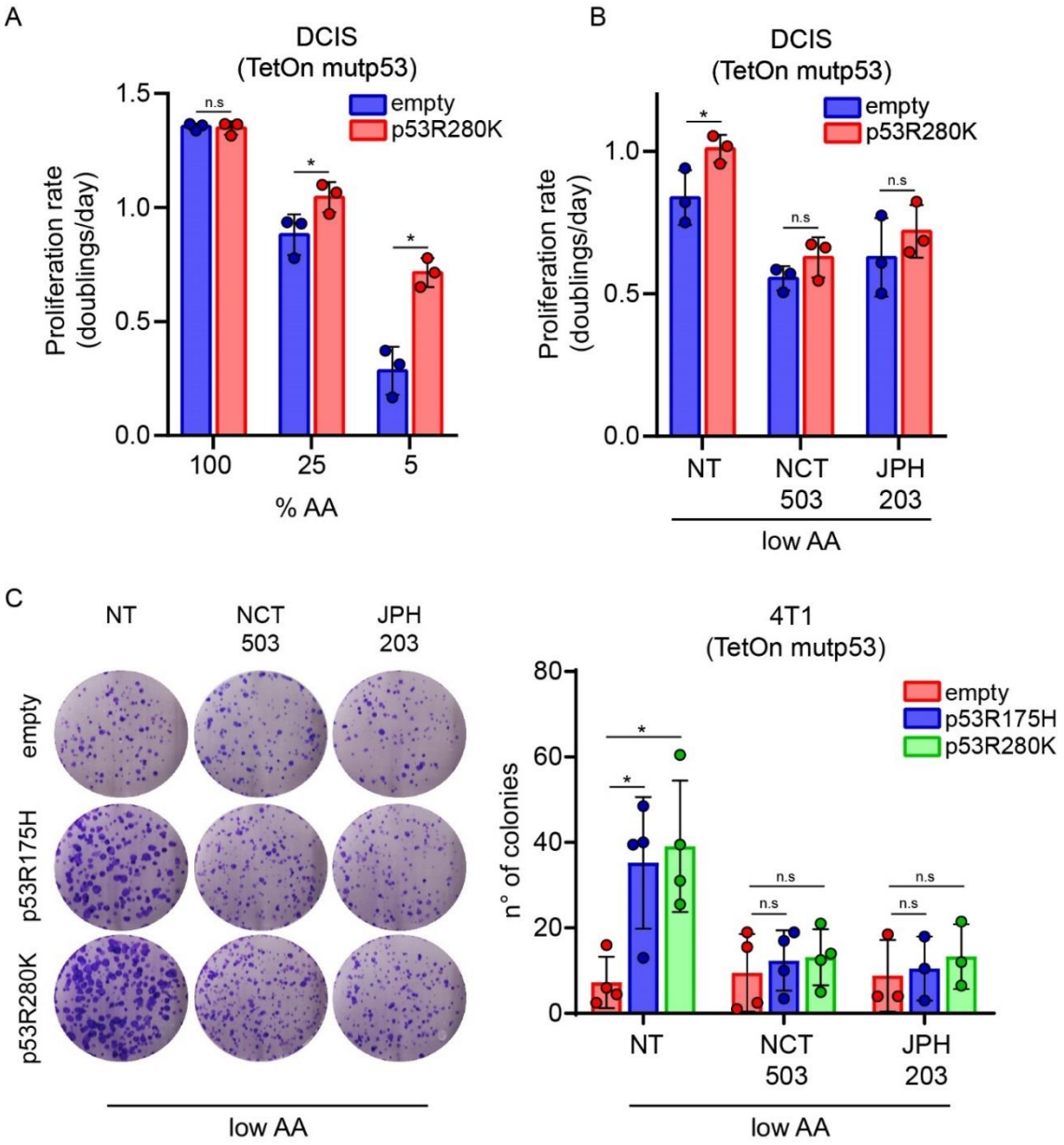


Figure 20. Mutp53 promotes cancer cells proliferation and survival in AAs deprivation via SSP and LAT1-mediated AAs transport. A. Doubling times of MCF10DCIS.com TetOn inducible clones cultured in medium with the indicated percentage of AAs for 3 days in presence of doxycycline 1ug/mL **B.** Doubling times of MCF10DCIS.com TetOn inducible clones cultured in medium with 25% of AAs (low AAs) for 3 days in presence of doxycycline 1ug/mL, treated with DMSO (NT), NCT-503 10μM or JPH203 10μM. **C.** Representative images of colonies formed by the indicated 4T1 TetOn inducible clones grown in medium containing 25 % AAs (low AAs) in presence of doxycycline 1ug/mL, treated with DMSO (NT), NCT-503 1μM or JPH203 1μM (left panel). Graph shows the quantification of the colonies number (right panel). Graph bars represent mean ± s.d. of n≥3 biological replicates. P value: $P < 0.05$ [*], $P < 0.01$ [**], $P < 0.005$ [***] by two-tailed Student's t-test.

3.3.3 Lack of PSAT1 and LAT1/CD98hc regulation by wtP53

It has been reported that wtp53 activates different cellular programs in response to specific AAs depletion by regulating its canonical targets or AA metabolism enzymes and transporters[11,199,205]. Given these premises we wondered whether wtp53 might regulate LAT1, CD98hc and PSAT1. To answer this question, we performed RNAi of p53 in two BC cell lines harboring wtp53 and we expressed inducible wtp53 in 4T1 cell line. We found that silencing or induction of wtp53 did not

alter the expression levels of *SLC7A5*, *SLC3A2* and *PSAT1* with the only exception of MCF10DCIS.com cells in which depletion of p53 reduced *PSAT1* expression (Fig. 21A-B).

Moreover, we cultured BC cells WT for p53 in medium with low amount of AAs and, as expected, we observed increased PSAT1, LAT1 and CD98hc protein levels (Fig. 21C). Nevertheless, silencing of wtp53 did not perturb this response indicating that, differently from mutp53, wtp53 does not play a role in promoting the expression of these genes in these conditions (Fig. 21C).

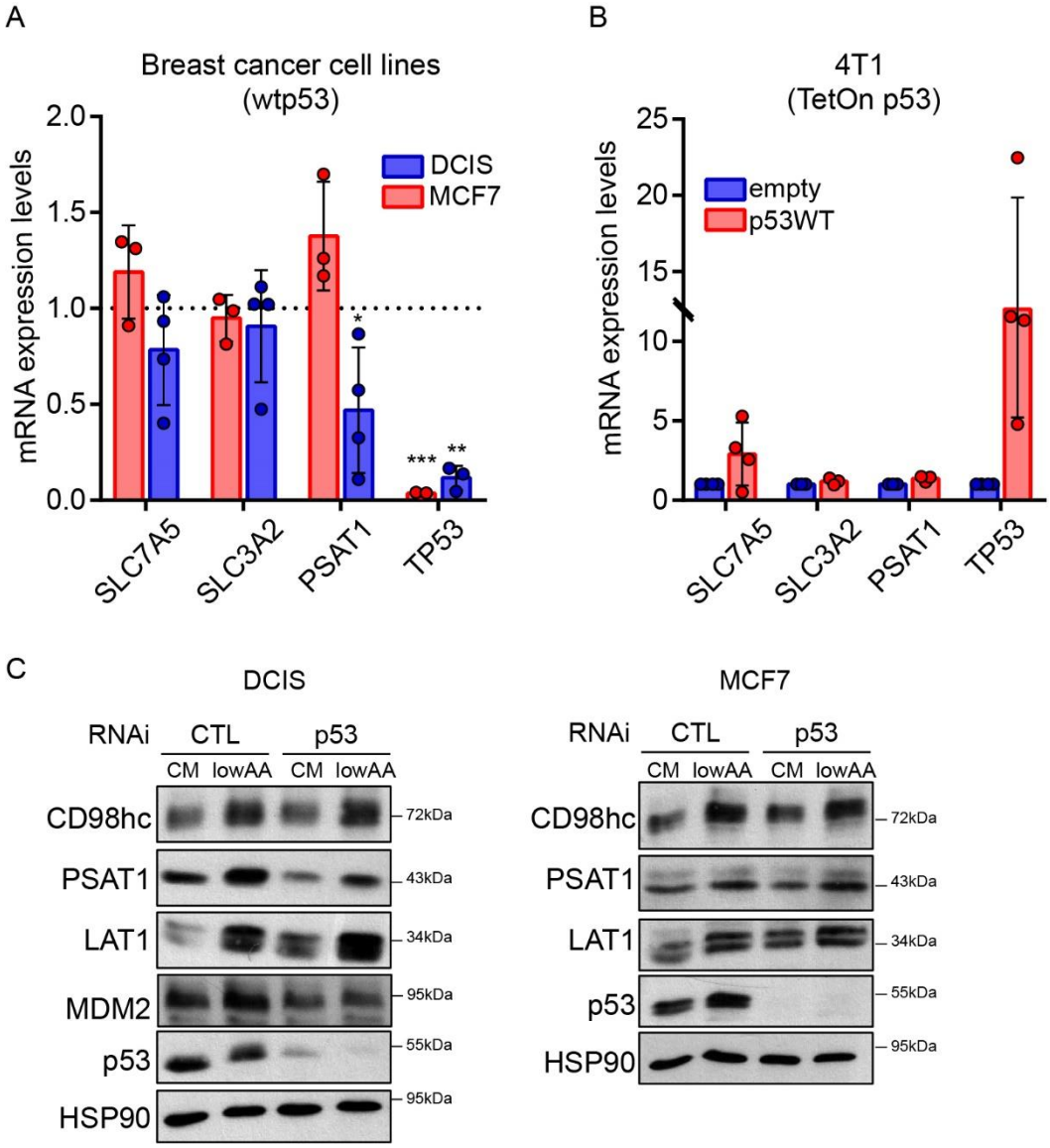


Figure 21. wtp53 does not behave similarly to mutp53 in regulating PSAT1 enzyme and LAT1/CD98hc transporter. **A.** qRT-PCR of the indicated genes in MCF7 and MCF10DCIS.com cell lines upon silencing of p53. qRT-PCR values are expressed as mRNA levels relative to control condition set at 1 (dotted line). **B.** qRT-PCR of the indicated genes in 4T1 TetOn inducible clones expressing empty vector or wt p53. qRT-PCR values are expressed as mRNA levels relative to 4T1 clone expressing empty vector. Doxycycline 1ug/mL was administered to all clones for 24h. **C-D.** Western blot analysis of the indicated proteins in MCF10DCIS.com and MCF7 cell lines cultivated for 72h in complete medium (CM) or in medium containing 25% AAs (low AA) upon silencing of p53. CD98hc is encoded by *SLC3A2* and LAT1 by *SLC7A5*. Graph bars represent mean ± s.d. of n=3 biological replicates. P value: $P < 0.05$ [*], $P < 0.01$ [**], $P < 0.005$ [***] by two-tailed Student's t-test. Blots are representative of n≥3 biological replicates. P53 was shown as silencing control.

3.5 LAT1 TRANSPORTER AND SSP ENZYMES ARE HIGHLY EXPRESSED IN BC PATIENTS HARBORING MUTP53

Based on our results, we hypothesized that BCs harboring mutp53 may present higher levels of the above identified AAs transporters and biosynthetic enzymes, as well as an increased mTORC1 pathway activation. Thus, we analyzed the expression levels of our genes of interest in a panel of 701 breast primary tumors from Metabric, classified based on the p53 status (wt or missense mutations). From this analysis we found that SSP genes (*PHGDH*, *PSAT1* and *PSPH*) and *SLC7A5* are significantly more expressed in BC patients harboring missense mutp53 compared to the ones that harbor wtp53 (Fig. 22A). Similar results were obtained by stratifying BC patients for a specific missense mutp53 signature (Fig. 22B). Accordingly, mTORC1 activation is higher in patients with missense mutp53 (Fig. 22A-B).

Then, we performed immunohistochemistry analyses on BCs samples derived from 6 patients stratified accordingly to p53 protein levels. Indeed, the long half-life of mutp53, compared to wtp53, results in its accumulation in the cells and a strong and diffuse immunolabeling of p53 is commonly considered as a marker of *TP53* missense mutations[206–209]. Interestingly, we observed that the protein levels of PSAT1, LAT1 and CD98hc are higher in BCs tissues with high levels of p53 (Fig. 22C). In agreement, we found that the mTORC1 target 4EBP1 is highly phosphorylated in tumors with higher p53 levels (Fig. 22C). Thus, this evidence indicates that in BC patients harboring mutp53 are present higher levels of PSAT1 and LAT1/CD38hc as well as an increased mTORC1 activation.

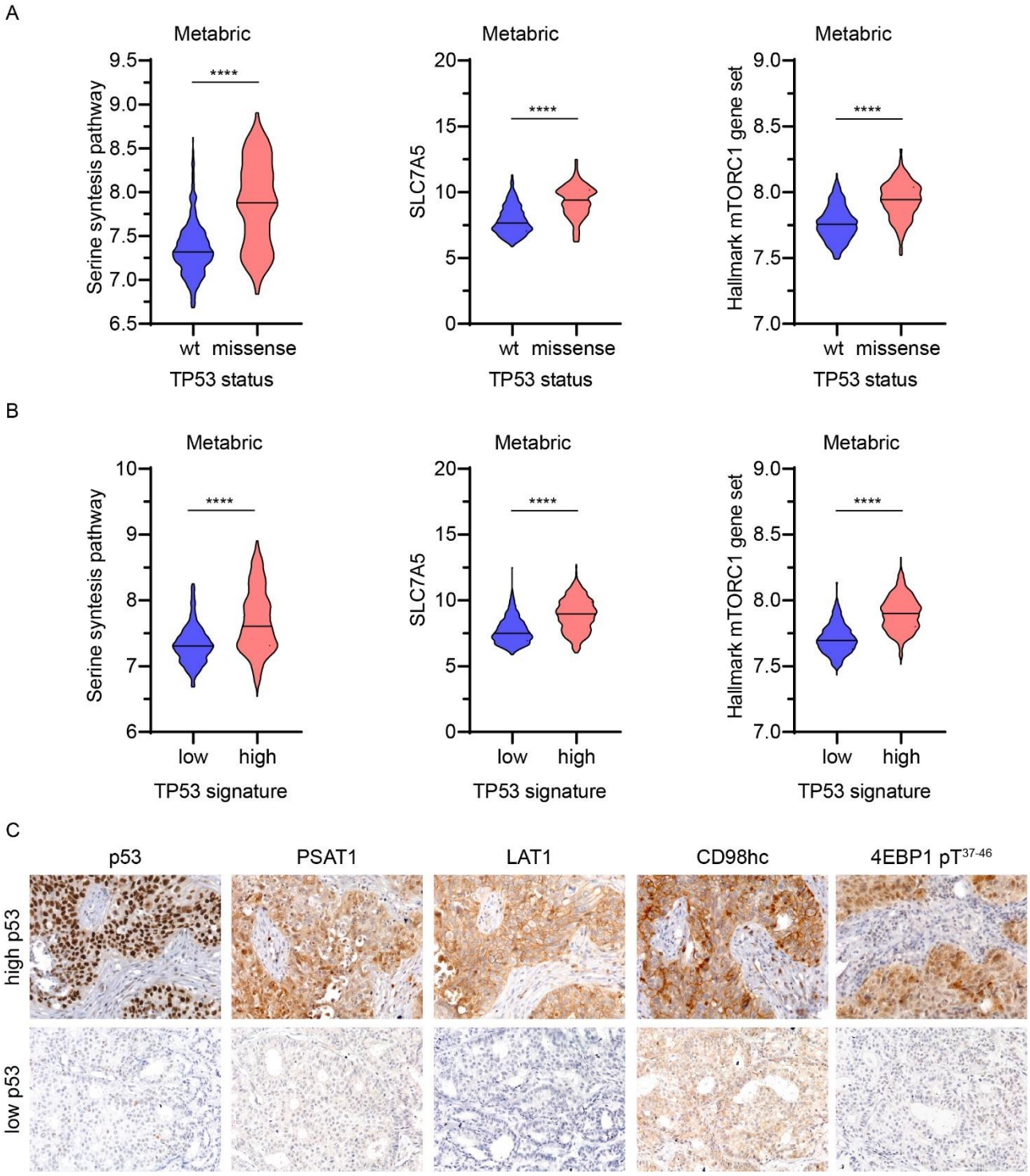


Figure 22. mutp53 correlates with AAs metabolism genes expression and mTORC1 activation in BC patients. A-B. Average expression levels of serine synthesis pathway genes (i.e. *PHGDH*, *PSAT1* and *PSPH*), *SLC7A5* and a gene set of mTORC1 activation in human samples dataset (Metabric) of breast cancer tissue (n=701) classified according to p53 status (wt and missense TP53 mutations) (**A**) or to *TP53* signature[210] (high and low) (**B**). **C.** Representative images of immunohistochemical analysis of 6 breast cancer samples stratified on the basis of p53 protein levels (high and low) (n = 3 for each condition). Samples were stained with anti-p53, anti-PSAT1, anti-LAT1, anti-CD98hc and anti-Phospho-4EBP1 antibodies. CD98hc is encoded by *SLC3A2* and LAT1 by *SLC7A5*. P < 0.05 [*], P < 0.01 [**], P < 0.005 [***], P<0.0001 [****] by two-tailed Student's t-test.

3.4 MECHANICAL CUES SUSTAIN MUTP53 DEPENDENT AA METABOLISM GENES EXPRESSION IN BREAST CANCER CELLS

3.4.1 Matrix rigidity increases mutp53 dependent expression of AA metabolism genes and mTORC1 activation

Tumors are frequently encapsuled in a fibrotic tissue that compress the tumor; this condition obviously reduces tumor perfusion, contributing to depriving it of nutrients, and imposes a massive mechanical load to tumor cells[47]. Tumors are indeed typically stiffer than the surrounding healthy tissues and, particularly well-defined for breast tumors, ECM stiffening has a pivotal role in promoting cancer progression favoring metabolic rewiring and activation of several oncogenic pathways, including mutp53[46,171]. Recent studies have also revealed an important cross-talk between mechanobiology and AA metabolism[60,61]. Thus, we wondered whether mechanical cues and the consequent activation of mutp53 may converge on the induction of genes related to serine synthesis and intake of BCAAs. Notably, we observed that the expression levels of *SLC7A5* and SSP genes (*PHGDH*, *PSAT1* and *PSPH*) as well as mTORC1 activation are higher in BC patients with a high stiffness signature (Fig. 23A). Next, we plated primary epithelial cells derived from the mammary gland of mice harboring mutp53 and MDA-MB 231 cells in hydrogels with a gradient of matrix rigidity recapitulating that of breast tumor progression. In particular, 0,5 kPa corresponds to ECM rigidity of normal mammary tissue, while 4 kPa is an average value associated with tumor tissue. However aggressive breast cancer subtypes can reach stiffness values higher than 5 kPa[211,212]. We found that mutp53 and Psat1 protein levels were increased in mouse epithelial cells plated on 4 kPa hydrogels (Fig. 23B). Moreover, matrix rigidity strongly affected the protein levels of mutp53, of the AAs heterodimeric transporter LAT1/CD98hc, ASCT2 and of the SSP gene PSAT1 (Fig. 23C). Together these results confirm that mechanical inputs modulate the levels of mutp53, PSAT1 and some AAs transporters. However, to understand the contribution of mutp53 in this process, we performed RNA interference of p53 in MDA-MB 231 cells grown in the same experimental condition as above. We observed that downregulation of mutp53 significantly reduced the induction of *SLC7A5* and *PSAT1* genes expression observed at higher matrix rigidity (Fig. 23D). Coherently, phosphorylation of S6RP was increased when BCs cells had been plated on stiffer hydrogels, while ablation of mutp53 significantly impeded this mTORC1 activation (Fig. 23E).

Moreover, observing the effect on *PSAT1* and *SLC7A5* expression levels, we found that the effect of mutp53 is stronger when cells are plated on 8 kPa hydrogels compared to softer hydrogels, suggesting that the increased stiffness had somehow unleashed mutp53 gain of functions over this AA metabolism program.

Overall, we demonstrated that mutp53, downstream to mechanical cues, strongly contributes to induce PSAT1, the heterodimer LAT1/CD98hc and, thus, mTORC1 activation.

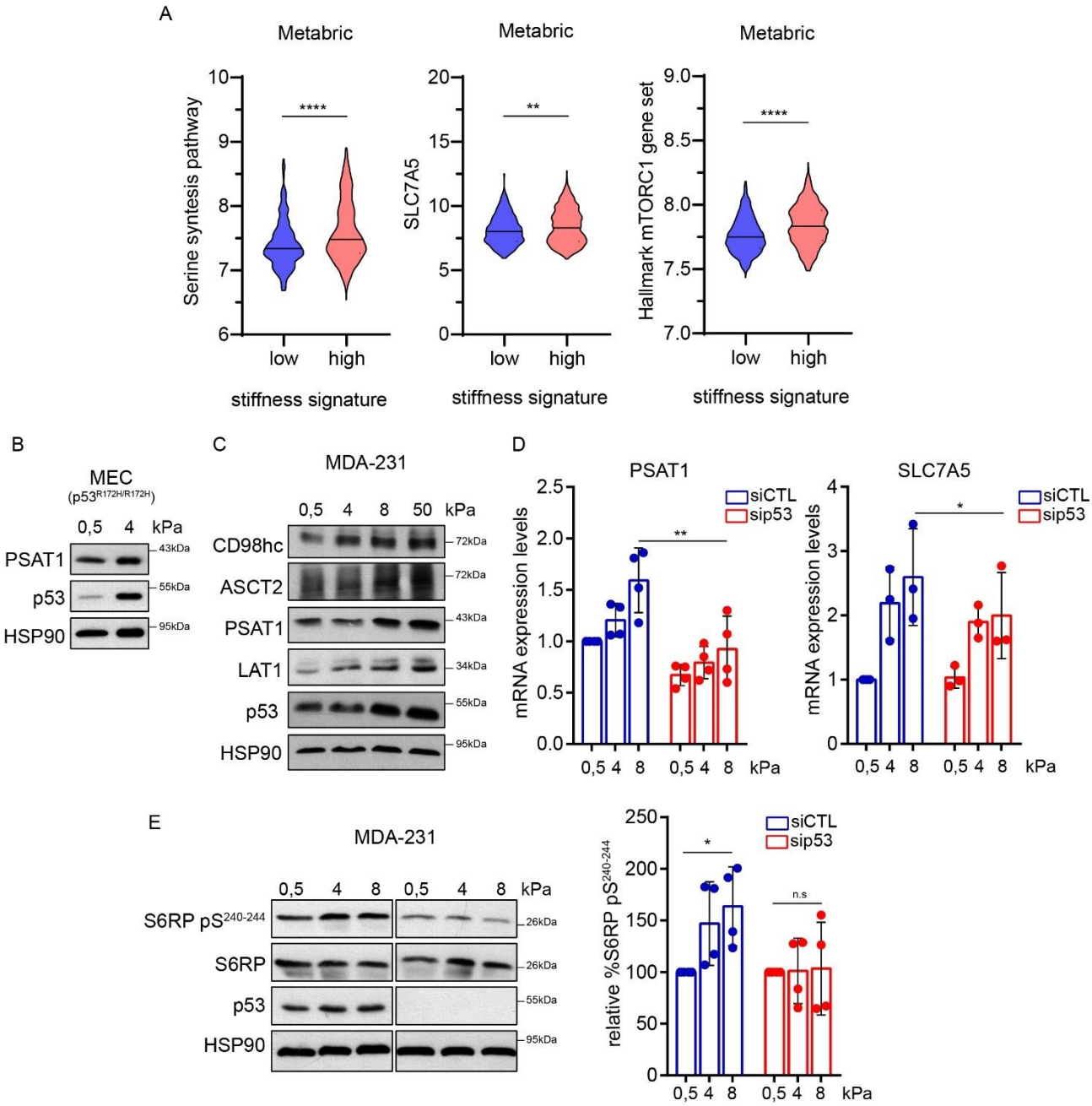


Figure 23. **Mutp53, downstream to mechanical cues from stiff matrix, contributes to induce AA metabolism genes expression and mTORC1 activation. A.** Average expression levels of SSP genes (i.e. *PHGDH*, *PSAT1* and *PSPH*), *SLC7A5* and a gene set of mTORC1 activation in human samples dataset of breast cancer tissue (n=701) classified according to high and low "stiffness" signature determined by MDA-MB 231 array[171]. **B.** Western blot analysis of Psat1 in mammary epithelial cells cultured in fibronectin-coated hydrogels with 0,5 and 4 kPa elastic moduli for 48h. **C.** Western blot analysis of the indicated proteins in MDA-MB 231 cells cultured on fibronectin-coated hydrogels with 0,5, 4, 8 and 50 kPa elastic moduli for 48h. **D.** qRT-PCR of *PSAT1* and *SLC7A5* genes in MDA-MB 231 cells cultured on fibronectin-coated hydrogels with 0,5, 4 and 8 kPa elastic moduli for 48h upon control (siCTL) or p53 (sip53) silencing. **E.** Western blot analysis of indicated proteins in MDA-MB 231 cultured on fibronectin-coated hydrogels with 0,5, 4 and 8 kPa elastic moduli for 48h upon control (siCTL) or p53 (sip53) silencing. P53 was shown as silencing control. Graph shows the quantification of depicted western blot bands expressed as percentage of S6RP $pS^{240-244}$ relative to the S6RP. CD98hc is encoded by *SLC3A2*, ASCT2 by *SLC1A5* and LAT1 by *SLC7A5*. Graph bars represent mean ± s.d. of n ≥3 biological replicates. P value: P < 0.05 [*], P < 0.01 [**], P < 0.005 [***], P<0.0001 [****] by two-tailed Student's t-test. Blots are representative of n=3 biological replicates.

3.4.2 Pharmacological inhibition of mechano-signaling reduces the expression of AA genes and mTORC1 activation

Considering the previous results and that interfering with mechanotransduction can be considered a way to target mutp53 activation, we evaluated whether pharmacological inhibition of mechanotransduction may perturb AA metabolism genes expression and consequent mTORC1 activation. To this end, we treated cells with drugs that inhibit different steps of mechanosignaling i.e. ECM adhesion signaling (the FAK inhibitor PF573 and the SRC inhibitor Dasatinib) and actin polymerization (Latrunculin A and Cythocalasin D) (Fig. 24A). We observed that AA metabolism genes expression was importantly reduced upon mechanotransduction inhibition (Fig. 24B).

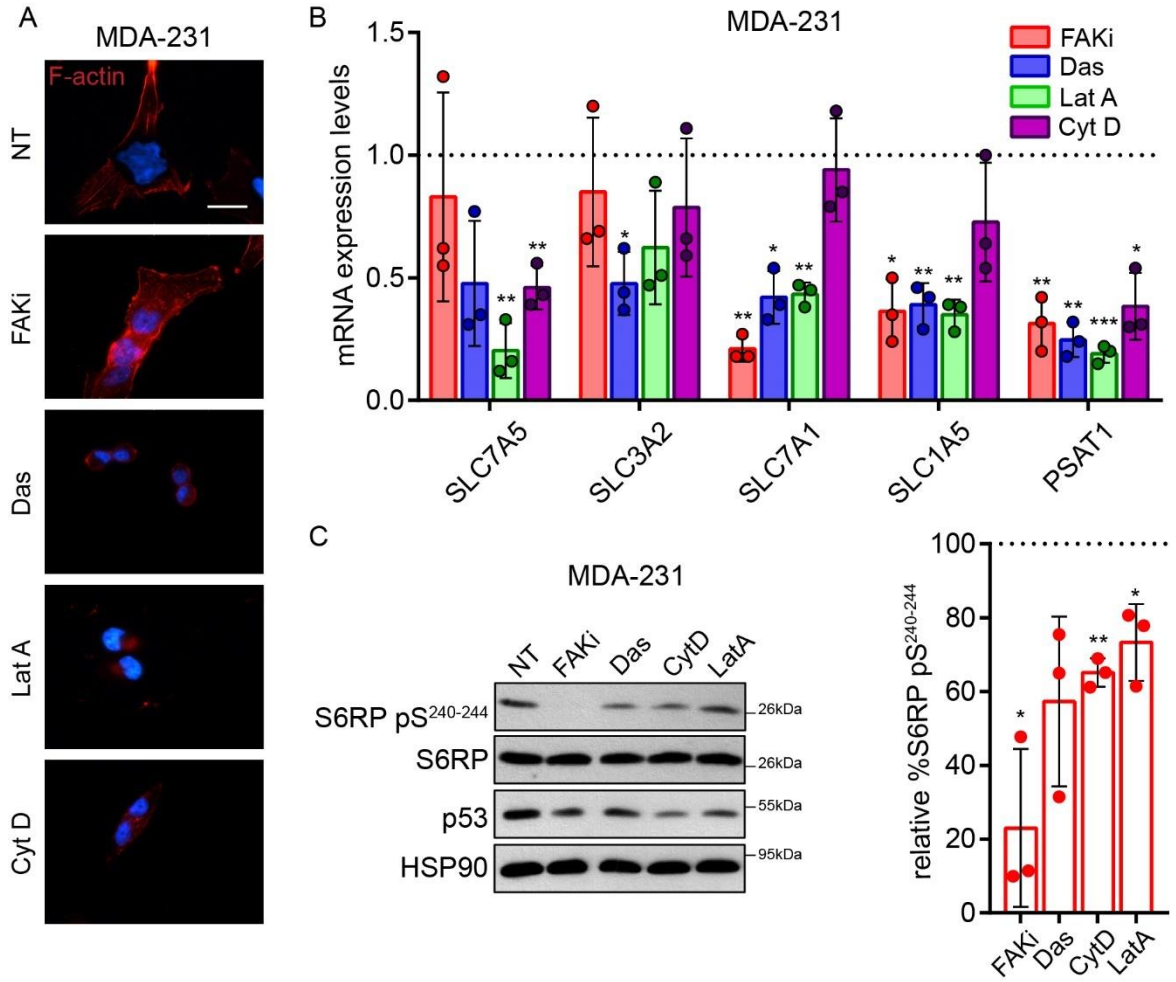


Figure 24. Mechanotransduction inhibitors reduce expression of AA metabolism related genes and mTORC1 activation. A. Representative images of immunofluorescence analysis of MDA-MB 231 cells treated with DMSO (NT), PF573 (FAKi) 10 µM, Dasatinib (Das) 0,5 µM, Latrunculin A (Lat A) 0,5 µM, Cytochalasin D (Cyt D) 1 µM for 48h stained with phalloidin (in red). Nuclei were stained with HOECHST (in blue). Scale bar 20 µm **B.** qRT-PCR of the indicated genes in MDA-MB 231 cells treated as in A. qRT-PCR values are expressed as mRNA levels relative to DMSO treated cells set at 1 (dotted line). **C.** Western blot analysis of the indicated proteins in MDA-MB 231 upon treatment with DMSO (NT), PF573 (FAKi) 10 µM, Dasatinib (Das) 0,5 µM, Cytochalasin D (CytD) 1 µM, Latrunculin A (LatA) 0,5 µM, for 48h (left panel). Graph shows the quantification of depicted western blot bands expressed as percentage of S6RP $pS^{240-244}$ relative to S6RP and referred to not treated condition set at 100 (dotted line) (right panel). Graph bars represent mean ± s.d. of n =3 biological replicates. P value: P < 0.05 [*], P < 0.01 [**], P < 0.005 [***] by two-tailed Student's t-test. Blots are representative of n=3 biological replicates.

In agreement, mTORC1 activation decreased upon treatment with the same mechanosignaling inhibitors (Fig. 24C). In sum, we found that mechanical cues transduced into the cells sustain mutp53 protein levels and its transcriptional metabolic program related to AA metabolism as well as mTORC1 pathway activation.

3.4.3 Targeting mechanosignaling blunts cancer cells adaptation to AAs deprivation

Our data unveil that mutp53 guarantees cancer cells proliferation when they are exposed to AAs deprivation and that an increased mechanical loading further unleashes mutp53 gain of function. Thus, we wondered whether targeting mechanosignaling may offer a potential therapeutic opportunity to dampens mutp53-mediated cancer cell adaptation to nutritional stresses.

To this end, we decided to combine AAs deprivation and mechanosignaling inhibition cultivating MDA-MB 231 cells in AAs restricted conditions together with Dasatinib treatment, an FDA approved drug. We observed that treatment with Dasatinib blunted the induction of *SLC7A5*, *SLC3A2* and *PSAT1* genes in response to AA deprivation (Fig. 25A). Coherently, Dasatinib treatment strongly reduced cancer cells proliferation rate when they were grown in AAs deprivation thus suggesting a potential therapeutic way to target breast cancer cells harboring mutp53 (Fig. 25B).

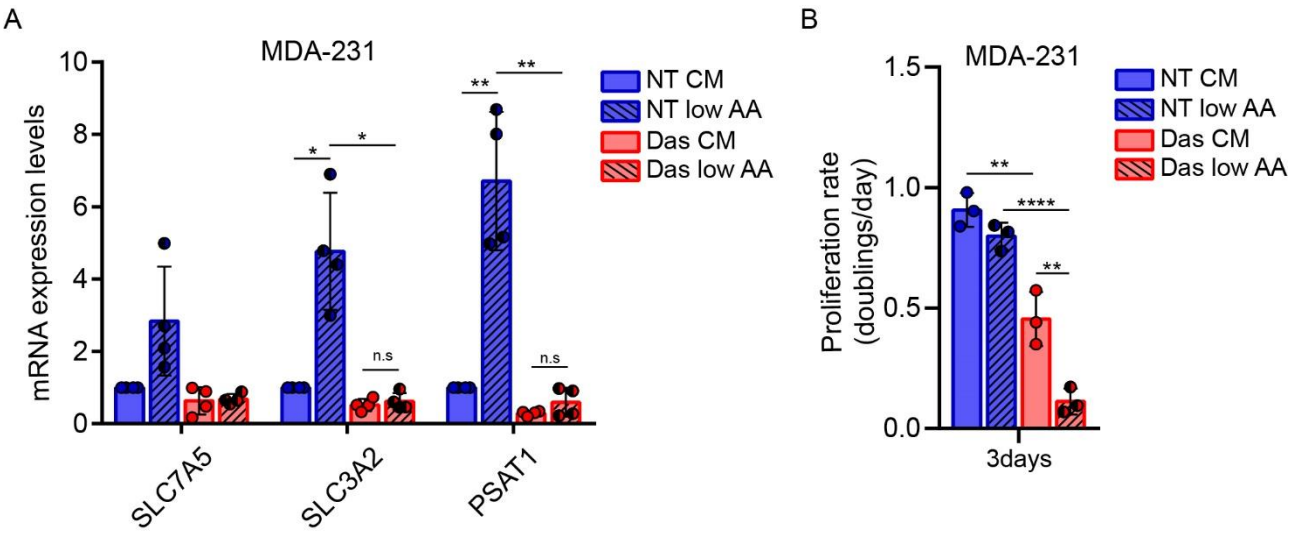


Figure 25. Dasatinib treatment impairs adaptation of BC cells to nutrient deprivation. A. qRT-PCR of the indicated genes in MDA-MB 231 cells cultured in complete medium (CM) or medium containing 5% AAs (low AA) and treated with DMSO (NT) or Dasatinib (Das) 0,5 μM for 72h. qRT-PCR values are expressed as mRNA levels relative to not treated condition in complete medium (NT CM). **B.** Doubling times of MDA-MB 231 cells cultured in complete medium (CM) or medium containing 5% of AAs (low AA) and treated with DMSO (NT) or Dasatinib (Das) 0,5 μM for 3 days. Graph bars represent mean ± s.d. of n =3 biological replicates. P value: P < 0.05 [*], P < 0.01 [**], P < 0.005 [***], P<0.0001 [****] by two-tailed Student's t-test.

4. DISCUSSION

In this thesis, combining a metabolic tracing approach and RNAseq analysis, we unveiled a new role of mutp53 in promoting synthesis of aspartate, serine and glycine and potentially the intake of specific AAs, through upregulation of GOT2, SSP enzymes and selected AAs transporters. This mutp53 metabolic program is particularly relevant when cancer cells experience cancer-related stresses. Indeed, in conditions of AAs scarcity, mutp53 supported cancer cells survival and growth via upregulation of serine synthesis and BCAAs/bulky AAs intake. Moreover, we discovered that a stiff ECM cooperates with mutp53 in the induction of such genes, disclosing a novel branch of AA metabolism regulated in response to mechanical cues and fostered by the presence of mutp53. Notably, inhibition of either mechanotransduction or serine synthesis or BCAAs/bulky AAs intake was able to blunt the pro-survival effects exerted by mutp53 on cancer cells proliferation during nutritional stresses. Given the relevance of these AAs for tumor growth and progression, future experiments will address the impact of this mutp53-dependent metabolic program in breast cancer growth and metastases dissemination (Fig. 26).

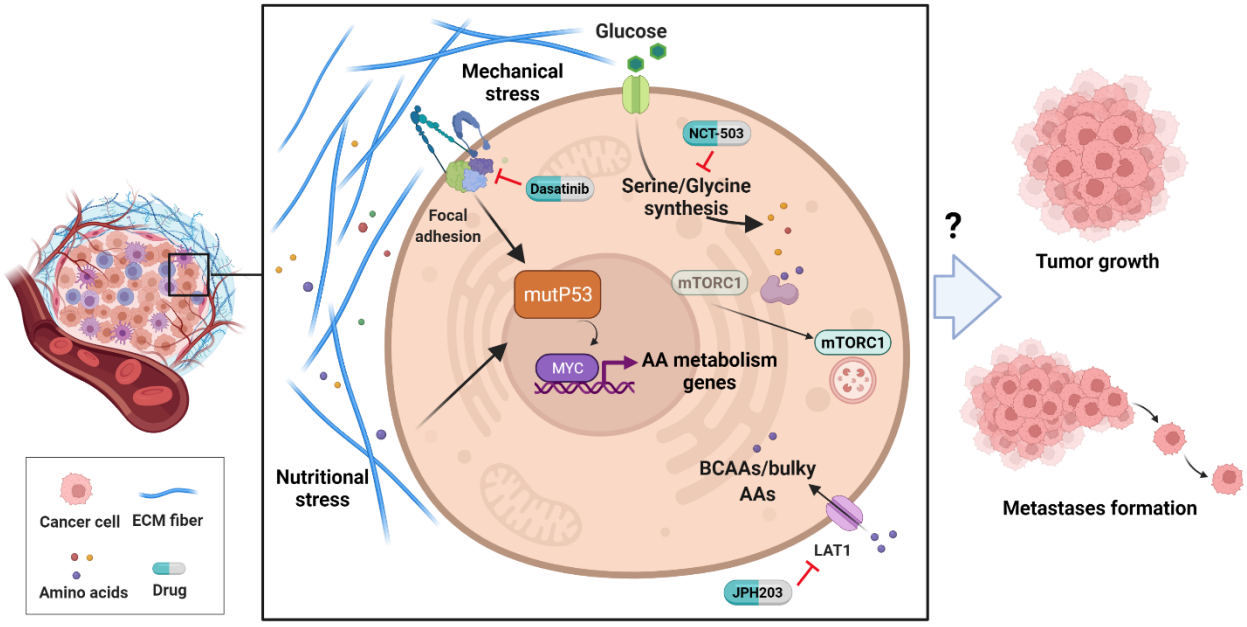


Figure 26. Reprograming of amino-acid metabolism by mutp53 in breast cancer. Working model depicting the role of mutp53 in promoting serine/glycine synthesis and potentially the intake of BCAAs/bulky AAs via increasing the expression of amino-acid metabolism genes in breast cancer cells. This process appears to be relevant when cancer cells face conditions of AAs scarcity and ECM stiffening. This model implies that targeting serine synthesis pathway, LAT1-mediated AAs transport or mechanosignaling might be strategies to hit breast cancer cells harboring mutp53. Future experiments will address the impact of this metabolic reprogramming in breast cancer growth and progression. Created with BioRender.com.

Serine is a NEAA that represents the third most used metabolite by cancer cells after glucose and glutamine and whose metabolic reprogramming is a hallmark of cancer[69,121,213]. Serine is crucial for the synthesis of other NEAAs such as glycine and cysteine as well as for the generation of phospholipids including sphingolipids and phosphatidylserine[213]. Moreover, serine is used as a major donor of one-carbon units to the folate cycle, which is coupled to the methionine cycle. Therefore, through several intertwined biochemical reactions, serine fuels synthesis of amino acids, lipids and nucleotides, and generation/recycling of crucial metabolites such as ATP, SAM, GSH and NAPDH, broadly impacting on several cellular processes[69,121]. Thus, it is not surprising that many tumors display an aberrant upregulation of SSP and alterations in one-carbon metabolism downstream to major oncogenic signaling such as MYC, HIF1α and mTORC1[69].

Here, we demonstrated that mutp53 sustains the expression of all the enzymes of the SSP and thus promotes serine/glycine synthesis. Notably, we found that mutp53 also controls the expression of several genes encoding for metabolic enzymes downstream to SSP belonging to folate, methionine, and cysteine metabolism, potentially impacting on one-carbon metabolism (Fig. 13A, D). Moreover, mutp53 is known to increase glucose intake favoring the membrane translocation of its transporter GLUT1, thus providing cancer cells with increased levels of substrate required for the serine and glycine synthesis [175].

Metabolic adjustments are essential during initial steps of cancer development and growth, but they are also mandatory in metastatic dissemination and colonization of distal organs. Indeed, metastases often exhibit a different metabolic profile in respect to primary tumor[10,26,77]. In this regard, several works unveiled that serine/one-carbon metabolism reprogramming is crucial for the metastatic outgrowth of BC cells in different distal organs including brain, lung, and bone[78,79,214]. This might imply that oncogenic signaling that foster serine/one-carbon metabolism is required to sustain this process. Interestingly, mutp53 is critical for metastasis dissemination and frequently observed in metastatic lesions, especially in brain metastasis from BC[215]. Based on our evidence, we are tempted to speculate that mutp53, by promoting serine/one-carbon metabolism, may provide an advantage to breast cancer cells to reach and colonize this site[215,216]. However, extensive experiments are needed to address this point.

In addition to our findings, considering that mutp53 is also able to promote glucose intake and lipid synthesis[175,192], we hypothesize that mutp53 may generally confer metabolic plasticity and flexibility to cancer cells, features that allow adaptation to challenging circumstances and are essential to guarantee survival during metastases. Notably, we found that the effect of mutp53 on this AA metabolism program was stronger in challenging conditions such as AAs deprivation or stiffer matrix

rigidity. These findings suggest that the protective role of mutp53 on cancer cells might rely on its capability to reprogram cancer cell metabolism.

Regarding the effect of mutp53 on AAs intake, we found that mutp53 controls the expression of specific AA transporters belonging to SLC7 and SLC1 families, whose expression is aberrantly upregulated in cancer[8,92]. These transporters are often functionally coupled to maximize their ability to sustain cancer growth by promoting the intake of non-essential and essential AAs that can fuel TCA cycle, one-carbon metabolism and mTORC1 activation (Fig. 13D)[16,69,92]. Of note, amino acids availability is a prerequisite for lysosomal localization and activation of mTORC1, the sensor kinase that couples cellular nutritional status with cell growth and which is hyper-activated in almost all cancers[144]. In particular, LAT1 transporter has been proved to be essential for mTORC1 activation in cancers strongly contributing to MYC and RAS induced transformation[23,217]. We discovered that mutp53 upregulates such key transporter as well as other AAs transporters and, indeed, BCs expressing mutp53 exhibited increased LAT1, SSP enzymes and mTORC1 pathway activation. In this regard there is evidence that loss of wtp53 or presence of mutp53 favor mTORC1 activation[218]. Our findings suggest that mutp53 could sustain mTORC1 activation also by increasing AAs intracellular pool.
Beyond impacting on AAs intracellular availability, we can speculate that mutp53, by modulating the expression of AAs transporters and enzymes, may perturb the quantity and the quality of the AAs pool also in the TME. Indeed, these AAs transporters act as antiporters, by exchanging AAs between cells and the extracellular space, thus potentially altering the metabolism of both cancer and stromal cells. In this way mutp53 may perturb the metabolic landscape of the tumor ecosystem.

AA metabolism is also under control of wtp53. Indeed, a pro-survival role of wtp53 in cancer cells during complete deprivation of serine and glutamine has been recently described. This effect was dependent on the transcriptional activation of the cyclin dependent kinase inhibitor (CDK1NA) p21 or of some AAs transporters and enzymes[11,195,196,198,199,205]. In particular, in colon cancer cells, after serine/glycine removal, wtp53 redirects the residual intracellular serine storage to buffer the elevated oxidative stress, allowing their survival under this condition without altering serine synthesis pathway[11].
In light of this, we wondered whether and how wtp53 might control SSP and LAT1/CD98hc expression in BCs. We observed that wtp53 did not alter the expression of these genes, except in MCF10DCIS.com where wtp53 silencing reduced *PSAT1* expression levels. Moreover, as expected, AAs deprivation induced an increase in the expression of these genes in all wtp53 BC cell lines;

however, unlike to mutp53, silencing of wtp53 did not alter this response. Accordingly, we found that BC cells expressing wtp53 are more sensitive to AAs deprivation, compared to BC cells expressing mutp53. Therefore, it appears that wtp53 is not able to control SSP activation and LAT1 expression in this context suggesting that this could be a peculiar function of mutp53.

We are aware that all our experiments have been performed in BC cells globally reducing AA availability, while the role of wtp53 has been demonstrated mainly in colon cancer and by totally removing specific AAs; this suggest that the cellular and tissue contexts, as well as the type of stress, might activate different p53-mediated responses.

Interestingly, other works demonstrated that specific mutp53 forms (R248W, R248Q and R273H), by retaining some wtp53 functions, can support adaptation of colon cancer cells to glutamine or serine withdrawal, while R175H variant does not confer any advantage[197,219].

Here we found that, in breast cancer cells, missense gain of function mutp53 forms, including R273H and R175H, are able to promote the serine synthesis pathway and the expression of some AAs transporters both in basal and stressed conditions. This mechanism could be considered a common mutp53 gain of function transcriptional program, via cooperation with MYC, since we have tested different mutp53 variants and since we found that high expression of SSP genes and SLC7A5 correlates with mutp53 signature in patient's dataset (Fig. 22).

Tumors are frequently encapsuled in a fibrotic tissue that compress the tumor contributing to depriving it of nutrients and imposing a massive mechanical load to cancer cells[13,14]. ECM stiffening has a critical role in promoting cancer progression activating several oncogenic pathways and favoring metabolic reprogramming[3].

Recently, two works disclosed a crosstalk between mechanical inputs and AA metabolism[60,61]. In this regard, we found that increased matrix rigidity and activation of mechanotransduction promoted the expression of PSAT1, LAT1/CD98hc and ASCT2 and that this effect is potentiated by the presence of mutp53, which our lab recently discovered as a mechanosensitive oncoprotein[171]. This axis appears to be relevant to sustain cancer cells survival when they are exposed to a nutritional stress: indeed, mechanotransduction inhibition impeded the BC cells adaptive response to cope to AAs deprivation. However, we can't certainly exclude the contribution of other factors responsible of this transcriptional program activated in response to mechanical cues. Since MYC mediates the activity of mutp53 on these genes, it will be relevant to investigate whether matrix stiffness could promote MYC activity. This possibility is conceivable considering that the interaction between cells and the ECM activates focal adhesions kinases thus triggering a signaling cascade that involves the activation of PI3K-AKT-mTOR pathway and the inhibition of GSK3β, known regulators of MYC levels and

activity[17,220]. Therefore, future experiments will assess whether mechanical cues can activate both mutp53 and MYC thus increasing serine synthesis and specific AAs transport. This process might be particularly relevant for aggressive BCs in which mechanical signals are known to boost disease progression and where MYC amplification co-occurs with *TP53* missense mutations[221].

From a clinical point of view, our findings suggest that mutp53 bearing BCs may display vulnerabilities at multiple levels, ranging from focal adhesions complexes to synthesis of serine and LAT1-mediated AAs transport, as well as to mTORC1 activation. Indeed, targeting focal adhesions with SRC inhibitor Dasatinib, is able to blunt mutp53 activity and curb cancer cell adaptive response to AAs deprivation. Given that Dasatinib completed clinical trial phase II for the treatment of advanced BCs, it could be taken in consideration as a potential way to hit BCs in patients harboring mutp53[222,223]. Moreover, the growing interest in AA metabolism reprogramming in tumors led to the development of new drugs that inhibit AAs transporters (e.g. LAT1 inhibitor JPH203) and biosynthetic enzymes (e.g. PHGDH inhibitor NCT-503)[101,114,224]. These drugs revealed *in vivo* efficacy in reducing tumor growth and promising therapeutic potential for patients' treatment[225,226]. Notably, JPH203 recently completed phase I clinical trial in patients with advanced solid tumors and a phase II trial had just started[227]. However, single-agent metabolic treatments are rarely effective against cancer in clinical settings and combination therapy is often considered the best strategy. In this regard, a recent work demonstrated that NCT-503 treatment synergizes with mTORC1 inhibition in osteosarcoma[228]. Given our results, also mTORC1 represents an attractive pharmacological target that could be exploited for combinational therapies with SSP and LAT1 inhibitors.

Our findings, although preliminary, suggest that patients with mutp53 harboring BCs may benefit from combinatorial therapeutic strategies that could disable cancer cell to properly activate metabolic pathway in response to cancer associated stresses, thus impacting on tumor growth and progression.

5. MATERIALS AND METHODS

Cell lines

MDA-MB-231, MDA-MB-468, SUM149PT, MCF7 and MCF-10A.DCIS.COM are human breast cancer cell lines. MDA-MB-231 and MDA-MB-468 were cultured in Dulbecco's Modified Eagle's Medium (DMEM, LONZA) supplemented with 10% Fetal Bovine Serum (FBS) 100U/mL penicillin and 10µg/mL streptomycin. SUM149PT were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12 (LONZA) (1:1) supplemented with 10% Fetal Bovine Serum (FBS), 100U/mL penicillin, 10µg/mL streptomycin, 1%. MCF7 were cultured in Eagle's Minimum Essential Medium (EMEM, Sigma) supplemented with Fetal Bovine Serum (FBS), 100U/mL penicillin, 10µg/mL streptomycin, 1% Minimum essential medium non-essential amino acids (MEM NEAA), and 10µg/ml recombinant human insulin. MCF-10A.DCIS.COM cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12 (LONZA) (1:1) supplemented with 5% Horse Serum (HS), 100U/mL penicillin and 10µg/mL streptomycin, 20 ng/ml recombinant human epidermal growth factor (EGF), 10 µg/ml recombinant human insulin, 500 ng/ml hydrocortisone. HEK-293T are a human embryonic kidney cells cultured in Dulbecco's Modified Eagle's Medium (DMEM, LONZA) supplemented with 10% Fetal Bovine Serum (FBS) 100U/mL penicillin and 10µg/mL streptomycin. 4T1 are a mouse breast cancer cell line cultured in Dulbecco's Modified Eagle's Medium (DMEM)/F12 (LONZA) (1:1) supplemented with 10% Fetal Bovine Serum (FBS), 100U/mL penicillin, 10µg/mL streptomycin and Glutamax 1%.

Human cell lines are from ATCC or other laboratories cooperating on the project. Cells were tested for mycoplasma contamination with negative results.

Mouse embryonic fibroblasts

Mouse embryonic fibroblasts transformed with H-Ras^{V12} were generated and cultured as previously described[229].

Mouse mammary organoids

For organoids generation, tissues were mechanically processed until small portions (1 mm^3) were obtained and then they were enzymatically digested for 3 hours at 37°C with a solution containing collagenase/hyaluronidase (Collagenase/Ialuronidase 10X in DMEM, Stem Cell). At the end of the digestion, the enzymes were inactivated by adding Advanced DMEM/F-12 medium (Ad-DF, Gibco). Red blood cells were eliminated by diluting NH_4Cl (4:1) in Hank's Balanced Salt Solution (HBSS). After 2 washes in HBSS, cells were enzymatically digested for 2 minutes with a solution containing

trypsin (0.25%, TrypLE Express 1X, Gibco) and EDTA (0.01 M in PBS). Next, cell aggregates were incubated for 1 minute with a solution containing Dispase (5 U/mL, Stem Cell) and DNase I (Sigma Aldrich). When tissue processing is completed, approximately 1x10^6 mammary epithelial cells were obtained from a mouse. Cells were resuspended in Matrigel (MATRIGEL® Matrix, Corning) and plated in 24-well multiwells pre-warmed for 1 hour at 37°C. When Matrigel was solidified, Ad-DF medium containing Glutamax 1%, 50 µg/mL Primocin, 100U/mL penicillin, 10µg/mL streptomycin, 10mM Hepes, and supplemented with the growth factors EGF (5ng/mL, Peprotech), B27 50X (1X, Gibco), FGF basic (20 ng/mL, Peprotech) was added.

Mouse mammary organoids were maintained in Matrigel and in Ad-DM medium with the above supplementations. Mouse mammary organoids≥ 50 µm were counted by using brightfield microscopy.

Mouse mammary epithelial cells

Mammary glands from 8 to 12 week old virgin female mice were enzymatically digested and single cell suspensions of purified mammary epithelial cells (MECs) were obtained following a standard protocol[230]. Briefly, mammary glands were digested at 37°C for 1–2 hours in Epi-Cult-B medium (StemCell Technologies Inc) with 600 U/ml collagenase (Sigma Aldrich) and 200 U/ml hyaluronidase (Sigma Aldrich). After lysis of the red blood cells with NH_4Cl, the remaining cells were washed with HBSS/0.02% w/v EDTA. Cells were then dissociated with 0.25% w/v trypsin, 0.2% w/v EDTA for 2 min by gentle pipetting, then incubated in 5 mg/ml Dispase II (Sigma Aldrich) plus 1 µg/ml DNase I (Sigma Aldrich) for 5 minutes, followed by filtration through a 40 µM cell strainer (BD Falcon). MECs were then purified using the EasySep Mouse Mammary Stem Cell Enrichment Kit (StemCell Technologies Inc).

MECs were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100U/mL penicillin and 10µg/mL streptomycin, 20 ng/ml recombinant human epidermal growth factor (EGF), 10 µg/ml recombinant human insulin, 500 ng/ml hydrocortisone.

Amino acids deprivation

Cells were cultivated in media with different percentages of amino acids as indicated in figure legends. These media were obtained by diluting complete medium (depending on the cell line) with medium lacking amino acids. The latter was generated by using Dulbecco's Modified Eagle's Medium (DMEM) without amino acids and glucose or, alternatively, Hank's Balanced Salt Solution (HBSS) supplemented with MEM Vitamin solution 100X (1X, Thermo Fischer). These media were then supplemented with 100U/mL penicillin, 10µg/mL streptomycin, glucose, Fetal Bovine Serum (FBS)

or Horse Serum (HS), and growth factors to reach the same molar concentrations of the original complete medium.
Mouse mammary organoids were grown in Eagle's Minimum Essential Medium (EMEM, Sigma) containing the supplementations of the original complete medium.

Preparation of fibronectin-coated hydrogel matrix

50, 8, 4 or 0.5 kPa Easy Coat hydrogels (Cell guidance system) were coated with 10 μg/ml fibronectin.

Reagents and plasmids

The following compounds and working concentrations were used: Fibronectin (10 μg/ml, Sigma Aldrich F0895), JPH203 (Selleck Chemicals S8667), NCT-503 (Sigma Aldrich SML1659), Dasatinib (Selleck Chemicals S1021), PF-573228 (Selleck Chemicals S2013), Cythocalasin D (Sigma Aldrich C2618) Latrunculin-A (Cayman 10010630) and DMSO (Sigma Aldrich D4540). Treatments lasted as described in figure legends.
pCW57-GFP-P2A-MCS (empty backbone) was bought by Addgene (plasmid #89181). pCW57-GFP-HA-p53 wt, pCW57-GFP-HA-mutp53 R175H, pCW57-GFP-HA-mutp53 R249S, pCW57-GFP-HA-mutp53 R273K or pCW57-GFP-HA-mutp53 R280K were generated by cloning a PCR amplified DNA fragment of the human HA tagged *TP53* sequence (WT or mutated) into the pCW57-GFP-P2A-MCS with MluI and BamHI restriction sites.

Inducible transduced cell lines

4T1 cells overexpressing inducible empty vector, wtp53, mutp53 R175H, mutp53 R249S, mutp53 R273K or mutp53 R280K were obtained by lentiviral transduction with pCW57-GFP-P2A-MCS, pCW57-GFP-HA-p53 wt, pCW57-GFP-HA-mutp53 R175H, pCW57-GFP-HA-mutp53 R249S, pCW57-GFP-HA-mutp53 R273K or pCW57-GFP-HA-mutp53 R280K.
MCF10DCIS.com cells overexpressing inducible empty vector or mutp53 R280K were obtained by lentiviral transduction with pCW57-GFP-P2A-MCS or pCW57-GFP-HA-mutp53 R280K.
Infected cell populations were selected using puromycin (Sigma-Aldrich) 2,5 μg/mL for at least one week.

Transfections

siRNA transfections were performed with Lipofectamine RNAi-MAX (Life technologies) in antibiotic-free medium according to manufacturer instructions. As negative control siRNA the Qiagen AllStars Negative Control was used. Sequences of siRNAs are reported below.

Oligonucleotide	Sequence	Manufacturer
siP53	GACUCCAGUGGUAAUCUAC	Eurofins MWG
siMYC	CGAGCUAAAACGGAGCUUU	Eurofins MWG

For lentiviral production, low confluence HEK-293T packaging cells were transfected using PEI 2X (1mg/ml) with the appropriate plasmids in combination with the pMD2.G and ps-PAX2 packaging vectors. After 48-72h the viral supernatant was collected, centrifuged 5 min at 500g and filtered with 0.45 μm low-protein binding filter to remove cellular debris. Then the virus-containing medium was added to the target cells.

RNA extraction and qRT-PCR

Cells were harvested in Qiazol lyses reagent (Qiagen) for total RNA extraction, and contaminant DNA was removed by DNase treatment. Quantitative real time PCR analyses were carried out on cDNAs retrotranscribed with iScript™ Advanced cDNA Synthesis Kit (Biorad 172-5038) and analyzed with BIORAD CFX96 Touch^{TM} detection system and Biorad CFX Manager software. Experiments were performed at least three times, and each sample is the average of a technical duplicate. The quantification is based on the $2^{-\Delta\Delta Ct}$ method. Histone 3 (*H3*) was used as reference gene in human cell lines while *Rpl13a* were used for mouse cell lines. PCR primer sequences are the following:

Gene target	Primer sequence	Direction
hTP53	CTCCTCTCCCCAGCCAAAGA	FW
hTP53	GGAACATCTCGAAGCGCTCA	REV
hPHGDH	CTGCGGAAAGTGCTCATCAGT	FW
hPHGDH	TGGCAGAGCGAACAATAAGGC	REV
hPSAT1	ACTTCCTGTCCAAGCCAGTGGA	FW
hPSAT1	CTGCACCTTGTATTCCAGGACC	REV
hPSPH	GAGGACGCGGTGTCAGAAAT	FW
hPSPH	GGTTGCTCTGCTATGAGTCTCT	REV
hSLC7A5	TCATCATCCGGCCTTCATCG	FW
hSLC7A5	TCACGCTGTAGCAGTTCACG	REV
hSLC3A2	CCAGGTTCGGGACATAGAGA	FW
hSLC3A2	GAGTTAGTCCCCGAAATCAA	REV
hSLC1A5	TGGTACGAAAATGTGGGCA	FW

hSLC1A5	GTGCCCCAGCAGGCAGCACA	REV
hSLC7A1	ATGGGTGGAAACGCTGATGATAC	FW
hSLC7A1	ACCTTGCCTGTTAAGTCTGGGTG	REV
mPSAT1	CAGTGGAGCGCCAGAATAGAA	FW
mPSAT1	CCTGTGCCCCTTCAAGGAG	REV
mSLC7A5	AGATGGGGAAGGACATGGGA	FW
mSL7A5	GCCAACACAATGTTCCCCAC	REV
mSLC3A2	GAAGATCAAGGTGGCGGAGGAC	FW
mSLC3A2	CAAGTACTCCAGATGGCTCTTCAGACC	REV

Antibodies

The following antibodies and working concentrations were used for western blot: anti-Hsp90 (1:10000, Santa Cruz Biotechnology sc-13119), anti-vinculin (1:10000, Sigma-Aldrich, v4505); anti-p53 DO1 (1:2000 or 1:10000, Santa Cruz Biotechnology, sc-126); anti-p53 pab240 (1:300, Santa Cruz, sc-99); anti-LAT1/slc7a5 (1:2000, Abcam, ab208776); anti-CD38hc/slc3a2 (1:2000, Sigma-Aldrich, HPA017980); anti-ASCT2/slc1a5 (1:2000, Cell Signaling Technology, #8057); anti-PSAT1 (1:2000, Proteintech, 10501-1-AP); anti-eIF2α D7D3 (1:5000, Cell Signaling Technology, #5324); anti-eIF2α phopsho S51 (1:1000, Cell Signaling Technology, #3398); anti-4E-BP1 (1:1000, Cell Signaling Technology, #9452); anti-4E-BP1 phospho T37/46 (1: 1000, Cell Signaling Technology, #2855); anti-S6RP (1:1000, Cell Signaling Technology, #2317); anti-S6RP phospho S240/244 (1:1000, Cell Signaling Technology, #2215); anti-MDM2 (1:1000, Santa Cruz, sc-965); anti-LC3 A/B (1:1000, Cell Signaling Technology, #12741).

The following antibodies and working concentrations were used for immunofluorescence analysis: anti-p53 DO1 (1:100, Santa Cruz Biotechnology, sc-126); anti-LAMP2 (1:500, Santa Cruz, sc-18822); anti-mTOR (1:100, Santa Cruz, sc-136269).

The following primary antibodies and working concentrations were used for immunoistochemistry: anti-p53 (1:50 pH6, Leica Novocastra, NCL-L-p53-DO7), anti-LAT1/SLC7A5 (1:500 pH9, Abcam, ab208776), anti-PSAT1 (1:400 pH6, Proteintech, 10501-1-AP), anti-CD98hc/SLC3A2 (1:2500 pH9, Sigma Aldrich, HPA017980), anti-phospho-4E-BP1 (1:1000 pH9, Cell Signaling, #2855).

Protein extraction

For immunoblotting analyses total cell extracts were lysed in Lysis Buffer (NP40 1%, Tris-HCL pH=8 50mM, NaCl 150mM, EDTA 1mM) solution, supplemented with PMFS 1mM (Sigma-Aldrich), NaF 5mM (Sigma-Aldrich), Na_3VO_4 1mM (Sigma-Aldrich), 10μg/mL CLAP (Sigma-

Aldrich). Protein concentration was determined with Bio-Rad Protein Assay Reagent (Bio-Rad). All the samples were then denatured in Laemmli Sample Buffer 2x or 6X and finally by heating at 95 °C for 5 min.

Western Blot analysis of mammalian cells

Proteins were loaded and separated in SDS-PAGE, followed by transferring on Nitrocellulose membranes (Cytiva). Blocking was performed in Blotto-tween (PBS, 0.2% Tween-20, not fat dry milk 5%) or with TBST (0.2% Tween-20, Tris/HCl 25 mM pH 7.5) plus 5% not fat dry milk or 5% BSA (PanReac Applichem) depending on the antibody. Anti-mouse and anti-rabbit HRPO-conjugated (Sigma-Aldrich) were used as secondary antibodies. Membranes were analyzed by chemiluminescence using Pierce ECL™ Western Blotting Substrate or Pierce ECL™ Plus Western Blotting Substrate.

Immunofluorescence analysis of mammalian cells

Briefly, cells were fixed in 4% paraformaldehyde for 20 minutes, washed in phosphate buffered saline (PBS), permeabilized with 0.1% Triton X-100 for 10 minutes and blocked in 3% Fetal Bovine Serum FBS/PBS for 30 minutes. Antigen recognition was done by incubation with primary antibody at 4°C for 14 h and with secondary antibodies (goat anti-mouse Alexa Fluor 568 and goat anti-rabbit Alexa Fluor 488, Life Technologies) at 4°C for 2 h. Actin stress fibers were stained with Rhodamine phalloidin for 1 h (Thermo Fischer, R415). Nuclei were stained with Hoechst (Life Technologies, 33342) or Dapi (Sigma Aldrich, 32670-F) for 15 min.

Immunohistochemical analysis (IHC)

For IHC, human samples were fixed in 10% buffered formalin and paraffin embedded. 4 mm-tissue sections were deparaffinized and rehydrated. Novocastra Epitope Retrieval Solution (pH6 or pH 9) was used to unmask antigens in a thermostatic bath at 98°C for 30 min. Subsequently, the sections were brought to room temperature and washed in PBS. After neutralization of the endogenous peroxidases with 3% H_2O_2 and Fcblocking by 0.4% casein in PBS (Novocastra), the sections were incubated with primary antibodies for 90 minutes at room temperature. The immunostaining was revealed by a polymer detection method (Novolink Polymer Detection Systems Novocastra Leica Biosystems Newcastle Ltd Product No: RE7280-K) and 3,3'-diaminobenzidine (DAB) substrate-chromogen. Slides were analyzed under a Zeiss Axioscope A1 microscope and microphotographs were collected using a Zeiss Axiocam 503 Color digital camera with the Zen 2.0 Software (Zeiss).

RNA-seq from MDA-MB-231 cell line

For total RNA extraction from the cells, RNeasy Mini kit (Qiagen, 74104) were used with contaminant DNase treatment (RNase-Free DNase set, Qiagen, 79254).

mRNA-seq libraries were obtained by the Illumina TruSeq library construction kit using total RNA from the cell line transfected with control siRNA or p53 siRNA I. mRNA-seq libraries were sequenced using Illumina HiSeq2000 for 100 bp paired-end sequencing. Quality control of mRNA-seq data was performed using Fatsqc (www.bioinformatics.babraham.ac.uk/projects/fastqc). Read files were mapped to the human genome (hg38) and analyzed for differential expression using the Tuxedo software suite implemented in the Galaxy workflow manager. The mapping was performed by Tophat2 and Cufflinks was used to find out differential expressed genes. *P* values are adjusted for multiple testing using the Benjamini–Hochberg correction.

Analysis of RNA-seq data from MDA-MB-231 cell line

Read quality was verified using fastQC (version 0.11.3; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Raw reads were trimmed for adapters, polyA read through, and low-quality tails (quality <Q20) using BBDuk (version 37.02; sourceforge.net/projects/bbmap/). Reads shorter than 35 bp were also removed resulting on average 51 M trimmed reads per sample. Reads were subsequently aligned to the human reference genome (hg38) using STAR (version 2.7.3a;[231]) with default parameters. Raw gene counts were obtained using the *featureCounts* function of the *Rsubread* R package (version 2.0.1;[232]) and the Gencode gene annotation for hg38 genome. Gene counts were normalized to counts per million mapped reads (cpm) using the *edgeR* package (version 3.28.1;[233]); only genes with a CPM greater than 1 in at least 2 samples were further retained for differential analysis. Gene expression heatmaps have been generated using the function *pheatmap* of R *pheatmap* package (https://cran.r-project.org/web/packages/pheatmap/index.html) after row-wise standardization of the expression values. All analyses were performed using R 3.6.3 and publicly available packages explicitly cited in the manuscript. No custom functions were written for the analysis.

Gene expression datasets

The METABRIC collection, comprising gene expression data and clinical annotations for 997 breast cancer samples, has been downloaded from the European Genome-Phenome Archive (EGA, http://www.ebi.ac.uk/ega/) under accession number EGAD00010000210[234]. Original Illumina probe identifiers have been mapped to Entrez gene IDs using the Bioconductor illuminaHumanv3.db annotation package for Illumina HT-12 v3 arrays obtaining log2 intensity values for a total of 19,761

genes. The TP53 status of 701 samples annotated as wild-type (n=584) and 'missense' mutant p53 (n=117) was derived from Silwal-Pandit et al. (2014)[235].

Tumor classification based on signature scores

To identify two groups of tumors with either high or low mutant-p53 gene signature[210], we used a classification rule based on the signature score. Signature scores have been obtained summarizing the standardized expression levels of signature genes into a combined score with zero mean[216]. Tumors were classified as signature 'Low' if the combined score was negative and as signature 'High' if the combined score was positive. Gene set expression levels have been calculated as the average expression of all gene set genes in sample subgroups (e.g., TP53 status or mutant-p53 signature level). Genes up regulated by the activation of mTORC1 complex have been downloaded from the Molecular Signature Database mTORC1 gene set (https://www.gsea-msigdb.org/gsea/msigdb/cards/HALLMARK_MTORC1_SIGNALING).

Analysis of CHIP-seq data

To retrieve mutp53 binding sites, we used ReMap2022[202], a large-scale database comprehending analysis from public sources (ENCODE, GEO, ENA) of DNA-binding experiments for transcriptional regulators in the most studied species such as Homo sapiens, Arabidopsis thaliana, Drosophila melanogaster and Mus musculus. In particular, we choose MDA-MB-231 cell experiments as a cell model, based on GEO GSE95303 data. Consequently, we have searched for TP53 peaks across the genome selecting genomic regions of interest and therefore retrieving mutp53 peaks in our genes of interest (PSAT1, SLC1A5, SLC3A2, SLC7A5). With this information, we have looked for transcription factors (TFs) that bind these specific regions in order to further analyse which TFs interact with TP53, modifying its interactions or binding to the DNA regions encoding for the above-mentioned genes.
We have then manually inspected via IGV (Integrative Genomics Viewer, version 2.11.3)[236], a visual exploration tool of genomic data, TP53 peaks occurring together with our genes of interest, also considering a region extended by ± 1000 nucleotides upstream or downstream the selected genes.

Metabolite extraction and liquid chromatography/tandem mass spectrometry analysis.

For metabolic tracing analyses, cells were exposed for 24 h to 1 mM [U-13C6] glucose (Sigma-Aldrich, 389374). Metabolite extraction and analysis were performed as previously described[237]. Briefly, cells were harvested in 250 µl of ice-cold methanol/acetonitrile 1:1 containing 1 ng/µl [U-13C6] glucose (internal standard, Sigma-Aldrich, 389374) and spun at 20,000 × g for 5 min at 4°C.

Supernatants were then passed through a regenerated cellulose filter, dried, and resuspended in 100 µl methanol for subsequent analysis. Amino acid quantification was performed through previous derivatization. Briefly, 50 µl of 5% phenyl isothiocyanate in 31.5% ethanol and 31.5% pyridine in water were added to 10 µl of each sample. Mixtures were then incubated with phenyl isothiocyanate solution for 20 min at RT, dried under N2 flow, and suspended in 100 µl of 5 mM ammonium acetate in methanol/ H2O 1:1. The identities of all metabolites were confirmed by using pure standards. Quantification of different metabolites was performed with a liquid chromatography/tandem mass spectrometry method using a C18 column (Biocrates) for amino acids and a cyano-phase LUNA column (50 mm × 4.6 mm, 5 µm; Phenomenex) for metabolites, respectively. Methanolic samples were analyzed by 10-min runs in positive (amino acids) and 5- min runs in negative (all other metabolites) ion mode with a 35-multiple-reaction monitoring (MRM) transition in positive ion mode and a 139-MRM transition in negative ion mode, respectively. The mobile phases for the amino acid analysis were phase A: 0.2% formic acid in water; and phase B: 0.2% formic acid in acetonitrile. The gradient was T0 100% A, T5.5 min 5% A, T7 min 100% A with a flow rate of 500 µl/min. The mobile phase for negative ion mode analysis (all other metabolites) was phase A: water; and B: 2 mM ammonium acetate in methanol. The gradient was 90% B for all analyses with a flow rate of 500 µl/min.

Proliferation assay

Cells (1,2x10^4 to 4x10^4 cells/well depending on the cell lines) were plated in 12-well plates in their regular medium. The next day, after washing cells with PBS, the medium was changed with medium with different amino acids concentration as indicated in the figures. At 3 or 6 days following the treatment, cells were trypsinized, suspended in medium and counted. The relative cell number was calculated based on the number of cells initially plated. Proliferation rate was determined using the following formula: Proliferation rate (doublings/day) = [$\log_2$(Final Day 3 cell number/Initial Day 0 cell number)]/3 or 6 days. For each experimental condition, two technical replicates were plated and counted.

BrdU incorporation assay

Cells (1,2x10^4) were plated in 12-well plates. The next day, after washing cells with PBS, the medium was changed with medium with different amino acids concentration as indicated in the figures. At 36 h following the treatment, the DNA precursor bromodeoxyuridine (BrdU) (20 µ M) was added to the medium for 12 h before fixation. Briefly, the cells were fixed in 4% paraformaldehyde for 20 min, washed in PBS, permeabilized with Triton 0.1% for 10 min and washed

three times with NaOH 50 mM solution and washed in PBS. Primary anti-BrdU antibody solution (1:2 dilution), to detect incorporated bromodeoxyuridine (BrdU), was used for 2 h at 37 °C and goat anti-mouse Alexa Fluor 568 (Life Technologies) was used as the secondary antibody for 1 h a 37 °C. Nuclei were counterstained with Hoechst 33342 (Life Technologies). The number of cells incorporating BrdU out of 100 nuclei in immunostained samples were determined by fluorescence microscopy.

Colony formation assay

4T1 cells (5x10^2) were plated in 6-well plates. The next day, after washing cells with PBS, the medium was changed with medium with 25% of amino acids and upon treatments as indicated in the figures. Growth was continued until the appearance of clones (at least 1 week). Briefly, the cells were fixed in 4% paraformaldehyde for 20 min, washed in PBS, and stained with GIEMSA solution (1:10 in water) for 1h. Colonies ≥1000 pixels were counted using countPHICS software after background subtraction.

Statistics and reproducibility

All the experiments are representative of at least three independent repeats. Graph bars represent mean ± s.d. from at least n=3 biological replicates. P values were determined using two-tailed Student's t-test, with a 95% confidence threshold or using One-way ANOVA followed by Tukey's comparison test ($\alpha<0.05$) as indicated in figure legends.

6. TABLES

Table 1. List of genes and proteins abbreviations

4EBP1	Eukaryotic Translation Initiation Factor 4E Binding Protein 1
ACC1	Acetyl-CoA Carboxylase 1
AHCY	Adenosylhomocysteinase
AIF	Apoptosis Inducing Factor Mitochondria Associated 1
AKT	AKT Serine/Threonine Kinase 1
AMPK	AMP-Activated Protein Kinase
ASCT2	Alanine, Serine, Cysteine Transporter 2
ASL	Arginosuccinate Lyase
ASNS	Asparagine Synthetase
ASS1	Arginosuccinate Synthase 1
ATF4	Activating Transcription Factor 4
ATG13	Autophagy Related 13
BAX	BCL2 Associated X, Apoptosis Regulator
BCL-2	BCL2 Apoptosis Regulator
BRD4	Bromodomain Containing 4
CAT1	Cationic Amino Acid Transporter 1
CD98hc	CD98 heavy chain
CDKN1	Cyclin Dependent Kinase Inhibitor 1A
C-JUN	Jun Proto-Oncogene
COX1	Mitochondrially Encoded Cytochrome C Oxidase I
CTH	Cystathionine Gamma-Lyase
DHFR	Dihydrofolate Reductase

DRAM1	DNA Damage Regulated Autophagy Modulator 1
EIF2α	Eukaryotic Translation Initiation Factor 2A
ERK	Extracellular Signal-Regulated Kinase
FAK	Focal Adhesion Kinase
FH	Fumarate Hydratase
FOXM1	Forkhead Box M1
GADD45	Growth Arrest And DNA Damage Inducible Alpha
GCN2	General Control Nonderepressible-2 Kinase
GEF-H1	Rho/Rac Guanine Nucleotide Exchange Factor 2
GLS	Glutaminase
GLUT1/4	Glucose Transporter 1/4
GOT2	Glutaminc-Oxaloacetic Transaminase 1
GPX1	Glutathione Peroxidase 1
HIF1α	Hypoxia Inducible Factor 1 Subunit Alpha
HK2	Hexokinase 2
HSF1	Heat Shock Transcription Factor 1
HSP	Heat Shock Protein
IDH	Isocitrate Dehydrogenase
LAT1	Large Amino Acid Transporter 1
LC3	Microtubule-associated proteins 1A/1B light chain 3B
MAX	MYC Associated Factor X
MDM2	MDM2 Proto-Oncogene
MDMX	MDM4 Regulator of P53
MTHFD1	Methylenetetrahydrofolate Dehydrogenase 1
MTHFD1L	Methylenetetrahydrofolate Dehydrogenase 1 Like

MTHFD2	Methylenetetrahydrofolate Dehydrogenase 2
MTHFR	Methylenetetrahydrofolate Reductase
mTOR	Mechanistic Target Of Rapamycin Kinase
MTR	5-Methyltetrahydrofolate-Homocysteine Methyltransferase
MYC	MYC Proto-Oncogene
NRF2	NF-E2 Related Factor 2
PDK2	Pyruvate Dehydrogenase Kinase 2
PGC1α	PPARG Coactivator 1 Alpha
PGM	Phosphoglucomutase
PHGDH	Phosphoglycerate Dehydrogenase
PI3K	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase
PKC	Protein Kinase C
PKM2	Pyruvate Kinase M2
PSAT1	Phosphoserine Aminotransferase 1
PSPH	Phosphoserine Phosphatase
PTEN	Phosphatase And Tensin Homolog
RAF	Raf-1 Proto-Oncogene
KRAS	KRAS Proto-Oncogene
RHOA	Ras Homolog Family Member A
ROCK	Rho Associated Coiled-Coil Containing Protein Kinase
S6K1	Ribosomal Protein S6 Kinase 1
S6RP	Ribosomal Protein S6
SCO2	Synthesis Of Cytochrome C Oxidase 2
SDH	Succinate Dehydrogrenase
SESN1	Sestrin 1

SFN	Stratifin 1
SHMT1/2	Serine Hydroxymethyltransferase 1/2
SLC	Solute Carrier Family
SRC	SRC Proto-Oncogene
SREBP1/2	Sterol Regulatory Element Binding Transcription Factor 1/2
TIGAR	TP53 Induced Glycolysis Regulatory Phosphatase
TP53	Tumor Protein P53
TSC2	Tuberous Sclerosis Complex Subunit 2
ULK1	Unc-51 Like Autophagy Activating Kinase 1
UVRAG	UV Radiation Resistance Associated
xCT	Cysteine/Glutamate Transporter
YAP	Yes1 Associated Transcriptional Regulator

Table 2. List of p53 peaks, regions and bound TFs from Chip-seq analysis

Gene	Peak	Score	Chrom	Start	End	TF bound
PSAT1	1	26.5	9	78296255	78297415	ZMYND8;BRD9;JUN;CDKN1B;BRD2;BRD4;SOX4;BRD2;ZMYND8;FOXM1;CDKN1B;ESR1;BRD9;JUN;FOSL1;MYC
PSAT1	2	1.0	9	78331264	78331456	No Tfs
SLC1A5	1	43.3	19	46787916	46789019	BRD9;BRD2;CDKN1B;ZMYND8;STAT3;BRD4;MYC;ESR2;ESR1;MAX;FOXM1
SLC1A5	2	16.7	19	46787125	46787503	CDKN1B;BRD9;ZMYND8;FOSL1;FOSL2;JUN;STAT3;BRD2;MYC;BRD4;ESR2;ESR1
SLC1A5	3	32.4	19	46786113	46786961	E2F1;BRD4;MAX;CDKN1B;BRD9;ZMYND8;FOSL1;FOSL2;JUN;STAT3;BRD2;MYC;ESR2;ESR1
SLC1A5	4	5.3	19	46785506	46785808	CDKN1B;E2F1;BRD4;BRD2;BRD9;ESR1;TEAD4;ESR2;SOX4;STAT3;MAX;JUN;YAP1;MYC;FOXM1;FOSL1;ZMYND8;FOSL2
SLC1A5	5	49.1	19	46784250	46785179	CDKN1B;E2F1;BRD4;BRD2;BRD9;ESR1;TEAD4;ESR2;SOX4;STAT3;MAX;JUN;YAP1;MYC;FOXM1;FOSL1;ZMYND8
SLC1A5	6	10.9	19	46781002	46781302	FOSL1;BRD9;JUN;SOX4
SLC3A2	1	70.6	11	62855445	62856510	BRD4;BRD2;MYC;BRD9;FOXM1;CDKN1B;SOX4;MAX;ZMYND8;TP53;YAP1;ESR1;TEAD4;JUN;FOSL1;STAT3
SLC3A2	2	15.6	11	62862708	62862918	BRD4;BRD9;BRD2;E2F1
SLC3A2	3	4.5	11	62879698	62879907	E2F1;CDKN1B;BRD9;MYC
SLC3A2	4	5.4	11	62884259	62884490	No Tfs
SLC3A2	5	9.9	11	62889209	62890454	CDKN1B;E2F1;BRD9;BRD2;BRD4;STAT3;FOXM1;TEAD4
SLC7A5	1	14.0	16	87871659	87871843	JUN;YAP1;BRD4;TEAD4;E2F1;ZMYND8;CDKN1B;BRD2;BRD9;SOX4;STAT3;MAX;FOXM1
SLC7A5	2	5.7	16	87860668	87860916	ESR1;ESR2;STAT3;JUN;CDKN1B

SLC7A5	3	9.7	16	87856207	87856506	JUN;CDKN1B;STAT3;E2F1;ESR1;ESR2
SLC7A5	4	4.7	16	87854315	87854640	BRD2;CDKN1B;E2F1;JUN;STAT3;FOXM1;ZMYND8;SMAD3;BRD9;MAX;MYC;HIF1A;SMAD1-5;ESR1;BRD4;SOX4;ESR2
SLC7A5	5	15.2	16	87853457	87854043	BRD2;CDKN1B;E2F1;JUN;STAT3;FOXM1;ZMYND8;SMAD3;BRD9;MAX;MYC;HIF1A;SMAD1-5;ESR1;BRD4;SOX4;ESR2
SLC7A5	6	6.6	16	87850038	87850338	CDKN1B;BRD4;BRD9;JUN

Table 3. Amino acid concentrations (µM) in the indicated culture media formulations.

	EMEM	**DMEM**	**DMEM/F-12**
L-Alanine			50
L-Arginine	597	398	699
L-Asparagine			50
L-Aspartic Acid			50
L-Cysteine			100
L-Glutamate			50
L-Glutamine	2000	4000	2500
Glycine		400	250
L-Histidine	200	200	150
L-Isoleucine	397	802	416
L-Leucine	397	802	451
L-Lysine	399	798	499
L-Methionine	101	201	116
L-Phenylalanine	194	400	215
L-Proline			150
L-Serine		400	250
L-Threonine	403	798	250
L-Tryptophan	49	78	44
L-Tyrosine	199	399	214
L-Valine	393	803	452

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