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CircPVT1 as non-coding mediator of breast cancer metabolism

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1.SUMMARY

The role of circular RNAs in cancer has started to be deeply investigated in order to identify new molecular targets for future personalized anti tumoral therapies.

Here, we investigated the pivotal role of a peculiar circRNA called circPVT1 demonstrating for the first time that it is able to act as miRNA sponge for the well-known metabolic miRNA, miR-33a-5p leading to the upregulation of its targets among them c-Myc, located in the same locus of PVT1 gene.
Previous studies showed that c-Myc could regulate indirectly through miRNAs expression Glutaminase, the first enzyme involved in glutaminolysis, the metabolic pathway deregulated in breast cancer. Notably, we demonstrated for the first time that c-Myc acts as transcriptional factor directly regulating Glutaminase expression. These data provide new insights about the role of non-coding RNAs as upstream regulators of cell metabolism which contributes to cancer onset and progression.

2. Glossary

TNBC: Triple Negative Breast Cancer

circRNAs: Circular RNAs

miRNAs: micro RNAs

lncRNAs: long non-coding RNAs

lncPVT1: long non-coding Plasmacytoma variant translocation 1

circPVT1: circular RNA Plasmacytoma variant translocation 1

GLS1: Glutaminase 1

GLS2: Glutaminase 2

KGA: kidney glutaminase

GAC: mitochondrial enzyme glutaminase C

BPTES: (bis-2-(5-phenylacetamido-1,3,4-thiadiazol-2-yl)ethyl sulfide)

PDOs: patients derived organoids

ChIP: chromatin immunoprecipitation

3. INTRODUCTION

1. Breast Cancer

Breast cancer is one of the most common cancers worldwide and the principal cause of death among women. Intriguingly, according to Globocan statistics, there is an estimated 2.3 milion new cases, representing the 11.7% of all cancer cases [1]. Moreover, the increased incidence found in countries with high HDI (human development index) is related to the presence of a majority of lifestyle risk factors such as alcohol intake, weight and physical inactivity and also to the presence of reproductive and hormonal risk factors such as early age at menarche, later age at menopause, advanced age at first birth, hormone therapy or use of oral contraceptives [2]. From a molecular point of view, breast cancer subtypes are classified into Luminal A and B, HER2 positive and Triple Negative breast cancer (TNBC) [3]. Beyond the intra-tumoral heterogeneity there is evident heterogeneity even among the various subtypes. TNBC represents the most aggressive breast cancer subtype

with the poorest prognosis due to its molecular characteristics [4]. Indeed, it lacks expression of estrogen (ER), progesterone (PgR) and human epidermal growth factor receptor 2 (HER2) thus preventing the effectiveness of any targeted therapy currently used for the other subtypes such as hormonal or anti-HER2 therapies [5]. Currently, TNBC is treated with chemotherapy in the absence of specific molecular targets. In the last years, several studies have been focused on the identification of promising therapy, this is the case of Kwapisz D. group that proposed the use of immunotherapy with pembrolizumab and atezolizumab for patients with advanced or metastatic TNBC with high programmed cell death ligand 1 (PD-L1) expression [6] [7]. In 2017, the US Food and Drug Administration (FDA) approved the use of atezolizumab and paclitaxel as first-line therapy while in 2019 also the use of pembrolizumab was approved [8]. The major issue of all these treatments is the development of drug resistance leading to relapse. The identification of novel biomarkers and molecular targets represents the crucial aim of the TNBC

studies in order to provide novel beneficial therapeutic treatment.

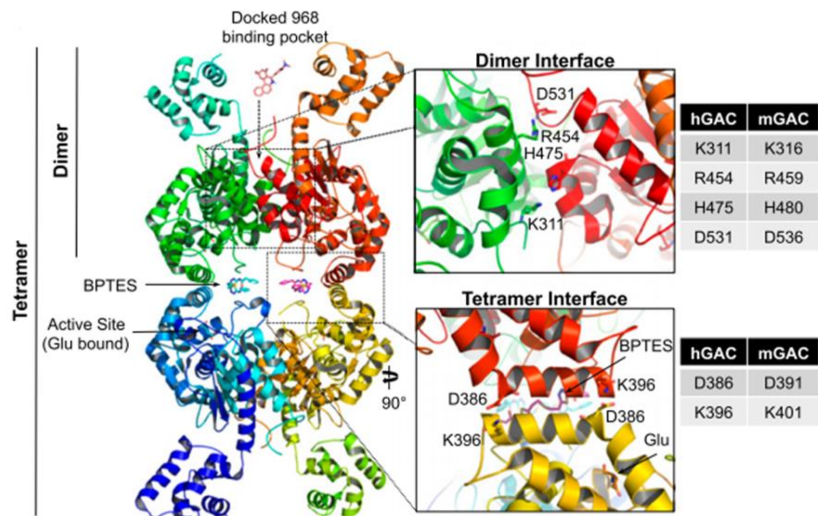
2. Cancer metabolism

One of the new hallmarks of cancer is the metabolic reprogramming triggered by the process of tumorigenesis due to the direct or indirect involvement of oncogenic mutations. The first process described by Otto Warburg in cancer metabolism is the increase of glycolytic fermentation even in the presence of oxygen (Warburg Effect) because cancer cells require a more rapid generation of ATP to sustain cell proliferation [9] [10]. Indeed, this process is triggered by upregulation of pivotal genes of this pathway such as the glucose transporter GLUT1 or the glycolytic enzyme hexokinase (HK) [11] [12] [13]. Another important pathway that results altered in cancer and in particular in breast cancer is the glutamine metabolism [14]. Glutamine, even if synthesized by mammalian cells is highly required during cell transformation. It represents an important substrate for carbon and reduced nitrogen involved in the *de novo* biosynthesis of macromolecules. The key enzyme of

glutaminolysis, the first of this pathway, is Glutaminase (GLS) that converts glutamine into glutamate allowing the entrance in the tricarboxylic (TCA) cycle after glutamate dehydrogenase-1(GLUD1) has converted it into α-ketoglutarate [15]. There are two paralogous genes in mammals for glutaminase GLS1 and GLS2 originated presumably from gene duplication of the same ancestor. They show sequence similarity but exerts different roles in tumor development. GLS2, localized in the liver and regulated by p53 was proposed as a tumor suppressor gene in several tumors such as hepatocellular carcinoma and glioblastoma despite recent reports of M. M. Dias and colleagues reported that in breast cancer cells including also TNBC, GLS2 shows a pro-tumorigenic role highlighting how GLS2 elicits a context-specific role [16] [17] [18] [19]. On the other hand, GLS1 found in the kidney, is a well-known tumorigenic gene since it is often upregulated in highly proliferating cancer cells [20]. GLS1 is located on chromosome 2 and through alternative splicing originates 2 variants, KGA and GAC isoforms that share same N-

terminal but different C-terminal structures. Moreover, the GAC isoform, that express a higher catalytic activity is the one more upregulated and expressed in cancer. The altered expression of GLS1 is regulated by other oncogenes such as c-Myc that directly controls the expression of glutamine transporters SLC1A5 and SLC38A5 and negatively regulates the expression of the antisense lncRNA GLS-AS that has GLS1 as target. Moreover, c-Myc triggers GLS1 expression through the regulation of miR-23a and miR-23b highlighting the pivotal role that this oncogene has in glutamine metabolism [21] [22] [23]. Furthermore, also hypoxia-inducible factor 1 (HIF-1) is able to control GLS1 expression in hypoxia condition by binding the hypoxia-responsive element (HRE) present on GLS gene while inorganic Pi is necessary for the induction of the GLS active form [24]. The active form, is a tetramere that can be exploited for the use of small inhibitor molecules which can bind the allosteric pocket triggering the inactive form [25]. Intriguingly, this is the mechanism of action of the small inhibitor Bis-2-(5-

phenyl-acetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES) which showed in vivo a favourable safety profile [26].



hGAC	mGAC
K311	K316
R454	R459
H475	H480
D531	D536

hGAC	mGAC
D386	D391
K396	K401

The use of these inhibitors such as BPTES or all its further derivatives compounds represent a promising tool to target cancer cells with high glutamine addiction thus restoring a normal phenotype.

3. Non-coding RNAs

Thanks to the Encyclopaedia of DNA Elements (ENCODE) project that analysed all the human transcriptome, it was demonstrated that only 2.94% of human genome encodes for proteins highlighting that the remaining part described as non-coding RNAs (ncRNAs), previously considered "junk DNA" have actually an important role within cells [27]. Indeed, in the last decades, studies have focused on the understanding of their roles in cellular development, gene expression, metabolism or diseases. NcRNAs can be divided into 2 categories: the housekeeping ncRNAs, those that are expressed in a constitutive manner such as ribosomal (rRNAs) or small nuclear and nucleolar RNAs (snRNAs), and the second group, the regulating ncRNAs including the main ones: long non-coding RNAs (lncRNAs) that are more than 200 nucleotides (nt) long, microRNAs (miRNAs) and circular RNAs (circRNAs) with less than 200 nt long.

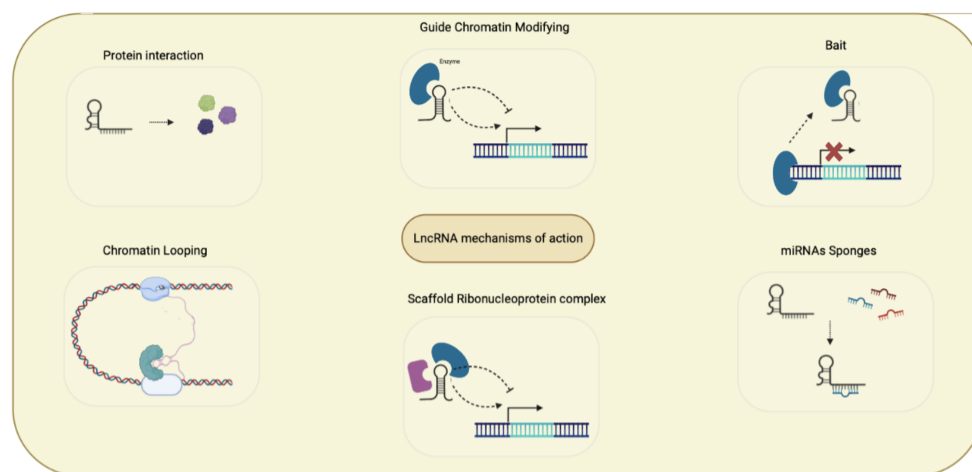
4. miRNAs

miRNAs are small RNA molecules with 22-23 nt in length that exert a pivotal role in the regulation of gene expression. Indeed, their main function is related to the messenger RNAs (mRNAs) sponge ability. They are localized in the cytoplasm where they can directly bind the 3'UTR of the mRNA targets inhibiting translation or leading also to degradation. The first miRNA described was lin-4 and the Ambros and Ruvkun groups identified its sponge activity on lin-14 mRNA. [28]. miRNAs exert a pivotal role also in tumorigenesis since their expression results often deregulated. Intriguingly, those miRNAs that sustain cell growth and pathways related to tumorigenesis are called onco-miRNAs while those that are frequently downregulated in cancer are considered onco-suppressors [29] [30].

5. Long non-coding RNAs

Long non-coding RNAs are RNA molecules with more than 200 nt. They are transcribed by RNA polymerase II and are

very similar to mRNAs but apparently do not have coding potential; indeed, they are 5′ capped with methyl-guanosine, are 3′ polyadenylated and also are subjected to alternative splicing. LncRNAs show high tissue specificity while regarding their main functions can be mentioned their role in gene expression regulation, the scaffold or guide activity as well as the decoy activity through which lncRNAs can sequester other factors preventing the binding with their interactors. LncRNAs are also involved in epigenetic regulation, in chromatin remodelling and also in diseases including cancer [31].



LncRNAs mechanisms of action.

Peculiar lncRNA involved in tumorigenesis is lncPVT1 that originates from Plasmacytoma variant translocation 1 (PVT1) gene located on chromosome 8q24.21. LncPVT1 was frequently described as upregulated in tumors such as gastric cancer, pancreatic cancer, lung cancer and also breast cancer [32] [33] [34] [35] . The peculiarity of PVT1 locus is its localization just 53kb downstream MYC, one of the most upregulated genes in all cancers, highlighting a possible interaction between Myc and lncPVT1 [36] [37]. Jin K. and colleagues reported that Myc stability is controlled by lncPVT1, indeed the lncRNA molecule by binding Myc prevents its degradation through proteasome pathway. Intriguingly, this positive feedback loop needs particular attention and further investigations because it could represent a promising molecular target for future personalized cancer therapy [36].

6. Circular RNAs (circRNAs)

Circular RNAs are particular ncRNAs due to their characteristic covalently closed structure that allows them to be resistant to the action of exonucleases RNase R [38].

Initially they were considered as splicing errors, but further studies revealed their important involvement in several pathways within cells. Moreover, recent studies discovered that circRNAs expression is often altered in cancer highlighting that circRNAs can exert a role as oncogene or oncosuppressor. Among the three main mechanism from which they originate, the most frequent one is called backsplicing where the downstream splice donor site binds an upstream splice acceptor site promoting circularization [39]. This process is also facilitated by the presence of ALU elements in the flanking regions or by the presence of RNA binding proteins (RBP) such as Quaking (QKI) and FUS [40]. CircRNAs functions involve a variety of pathways: the most studied and debated one is the miRNAs sponge activity thus modulating their expression and preventing their inhibitory action on the mRNAs targets; circRNAs are also able to interact with proteins also with RNA polymerase II and they can favour protein interaction as in the case of circFOXO3 that in breast cancer facilitates the interaction between MDM2-mediating degradation and mutp53 [41] [42] [43].

CircRNAs can be involved also in cell cycle regulation or autophagy process but also can be packaged into exosomes. Interestingly, recent studies revealed that some circRNAs can undergo cap-independent translation leading to the production of small functional peptides [44] [45] [46]. Considering their characteristics and in particular their presence in body fluids, circRNAs are promising candidates for liquid biopsy [47]. Indeed, the analysis of their expression in blood or saliva represents a non-invasive method to monitor tumor progression, the therapeutic responsiveness and recurrency. A particular example of circRNA molecule is circPVT1 derived from circularization of exon 2 of PVT1 gene that encodes also for a lncRNA. It was identified as oncogene in gastric cancer and further analysis revealed that its expression results upregulated in several tumors hystotipes such as head and neck squamous cell carcinoma, hepatocellular or esophageal cancer but also in ovarian cancer [48] [49] [50] [51] [52]. Interestingly, circPVT1 oncogenic activity has been reported also in breast cancer even if further studies are needed in this field in order

to better understand the circPVT1 involvement and the mechanisms of action [53] [54] [55].

4. AIM OF THE PROJECT

According to Globocan, despite the improvements in the last decade in early detection and cancer therapies, in 2020 was registered 685000 breast cancer deaths globally and at the end of the year the new cases diagnosed were about 7.8 million establishing breast cancer as the most prevalent type of cancer in the world [1]. Between them, TNBC continues to be the subtype of breast cancer with the highest rates of recurrence and mortality and with a higher therapeutic need. The discovery of molecular biomarkers to be used as targets for personalized therapies could represent a promising opportunity to fight cancer progression. Intriguingly, they could help in monitoring a therapy response and also they could exert an important role in preventing relapse. Our aim is to investigate the molecular mechanisms of a peculiar oncogenic circular RNA called circPVT1 in breast cancer wondering if it could have an

impact also on cancer metabolism. First, we will study the expression and the cellular localization in TNBC cell lines and in our cell system. We will focus on the mechanism of action and we will investigate the involvement in glutamine metabolism. Therefore, we will also use small metabolic inhibitors in order to provide insights about their use as anticancer therapy both in cells and in patients derived organoids that recapitulate the original tumor.

5. RESULTS

1. Aberrant expression of circPVT1 elicits pro-tumorigenic effects in breast cancer cells.

We found in The Cancer Genome Atlas (TCGA) data set that circPVT1 expression was higher in breast cancer patients tissues compared to non-tumoral ones and the higher expression correlated also with advanced stage III-IV compared to stage I-II (Fig.1 A-B). Moreover, patients with higher circPVT1 levels expressed shorter overall free survival (Fig.1 C). We performed dPCR analysis using primers specifically spanning the circPVT1 circularization junction and we found that circPVT1 expression levels were higher in breast cancer tissues when compared to matched non-tumoral ones (Fig.1D). To investigate the role of circPVT1 in breast cancer, we performed overexpression and silencing analysis on two triple negative breast cancer cell lines, SUM-159PT and MDA-MB-468. We found that

ectopically expression of circPVT1 increased migration and colony formation abilities (Fig.1 E-J) while depletion of circPVT1 led to a reduction in the ability to migrate and to form colonies in both cell lines (Fig.1 G-J). To better investigate the oncogenic role of circPVT1 we used a non tumorigenic breast cell line, MCF-10A, in which we overexpressed the circRNA molecule creating stable clones (Fig.1 K). After checking the efficiency of circPVT1 plasmid expression using convergent primers for the linear form (Fig.1 L), we found that circRNA overexpression was sufficient to make these cells to acquire neoplastic features such as the ability to migrate and to form colonies (Fig.1 M-N) highlighting the oncogenic role of circPVT1.

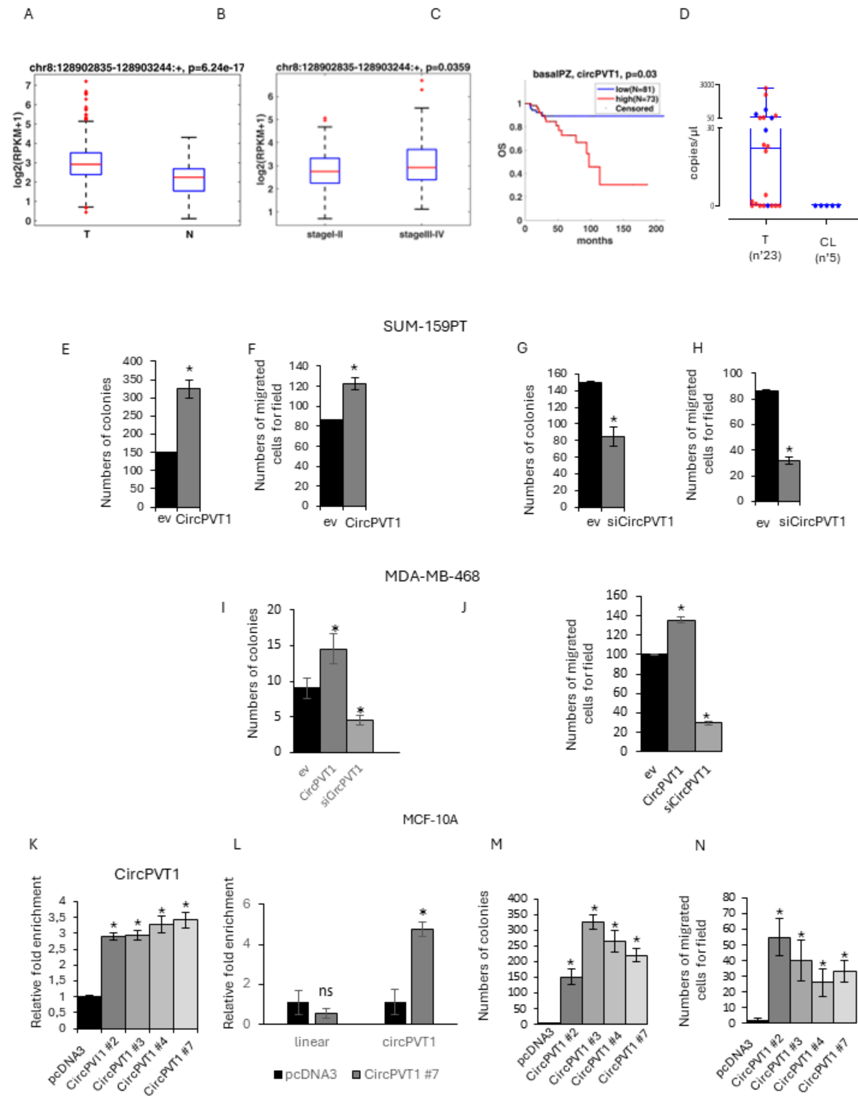


Figure 1. A-B. Log2 expression levels of chromosome interval containing circPVT1 in tumor and non-tumoral samples (A) and in

stage I-II and III-IV tumor samples (B). C Kaplan Meier curves indicating the overall survival of patients basing on the expression level of circPVT1. D Boxplots show the copies/μl of circPVT1 in 23 TNBC tumors and in 5 controlateral breast tissues. E-G Histograms show the number of colonies of SUM-159PT cells either expressing high (E) or low levels (G) of circPVT1. F-H. Histograms show the number of migrated SUM-159PT cells treated as in E-G. I. Histograms show the number of colonies of MDA-MB-468 cells either expressing high or low levels of circPVT1. J. Histograms show the number of migrated MDA-MB-468 cells treated as in I. K. CircPVT1 RNA relative enrichment levels measured in MCF-10A stably expressing pcDNA3 or circPVT1. Numbers (#) indicated the different clones of MCF-10A stably expressing high circPVT1 levels. L. Histograms show the relative enrichment of the linear and circular form of circPVT1. M-N. Histograms show number of colonies (M) or of migrated cells (N) count in MCF-10A treated as in K.

2. CircPVT1 sponges the metabolic miR-33a-5p

We proceed our analysis with the investigation on the mechanism through which it elicits the pro-tumorigenic effect and first we looked for the subcellular localization. We found that circPVT1 is expressed mainly in the cytoplasm (Fig.2 A) thus accordingly, we searched for a possible sponge activity. In silico analysis, reported a circPVT1 binding site for miR-33a-5p a well-known metabolic miRNA (Fig.2 B), indeed all MCF10A-clones generated after circPVT1 overexpression showed a reduced expression of this miRNA molecule strengthening the possible circPVT1 sponge activity (Fig.2 C). Previously, we demonstrated the anti-cancer effect of metformin, an anti-diabetic drug, which in tumors is able to regulate several miRNAs expression such as miR-33a-5p and miR-21-5p and we wondered if it could have an impact on circPVT1 expression. We demonstrated that TNBC cell line SUM-159PT treated with increased concentrations of metformin downregulated circPVT1 expression (Fig.2 D). Moreover, also SUM-159PT in the circRNA overexpression or silencing condition or after

metformin treatment, showed a modulation of the miR-33a-5p levels but not of miR-21-5p which was only modulated by metformin (Fig.2 E). Intriguingly, overexpression of circPVT1 in MCF10A cells, decreased the expression of two other miRNAs, miR-145-5p and miR-203-5p, known targets of circPVT1 (Fig.2 F-G). We further performed chromatin immunoprecipitated analysis on MCF-10A clone7 and on SUM-159PT to demonstrate the direct binding between circPVT1 and miR-33a-5p and also the other 2 already known targets miR-145 and miR-203 (Fig.2 H-I).

Figure 2

A. Upper part, representative protein gel blot of nucleus/cytosol cell lysates obtained from MCF-10A cells stably expressing

pcDNA3 or circPVT1 (clone #7) stained with the indicated antibodies. Lower part, histograms show the levels of circPVT1 between nucleus/cytosol obtained from MCF-10A treated as in the upper part. B. Predictive site of binding interaction between miR-33a-5p and circPVT1. C. Histograms show the expression levels of miR-33a-5p in MCF-10A cells treated as in Figure 1K. D. Relative fold enrichment of circPVT1 levels measured in SUM-159PT cells treated with increased concentration of Metformin. E. Histograms show the expression levels of miR-33a-5p and miR-21-5p measured in SUM-159PT expressing endogenous or high or low levels of circPVT1 or treated with 0.5 mM of metformin. F-G. Histograms show the miR-203a-5p and miR-145-5p relative fold enrichment in MCF-10A clone#7. H-I Histograms show the miR-33a-5p, miR-145-5p and miR-203 relative fold enrichment in SUM-159PT (H) and MCF-10 circPVT1#7 cells (I) measured in total RNA immunoprecipitated with circPVT1-capture probes.

3. Oncogenic CircPVT1 impacts on cell metabolism

Considering the sponge activity of circPVT1 on a metabolic miRNA, we wondered if the pro-tumorigenic effect of circPVT1 could pass through the alteration of metabolism which is often encountered in breast cancer.

Intriguingly, we performed 1H-NMR spectroscopy metabolic profiling of culture media of circPVT1 depleted SUM-159PT cells (SUM_sicircPVT1) compared to that of control cells (SUM_siSCR) (Fig. 3A) and from the analysis of the released metabolites we noticed an increase in glutamine levels (Fig. 3B) highlighting the higher glutamine consumption in SUM-159PT untreated cells. Same trend was found also in MDA-MB-468 cell line (Fig. 3C-D). We further performed 1H-NMR spectroscopy metabolic analysis in MCF-10A clones overexpressing circPVT1 (Fig. 3E-F) and from the metabolites released in culture media we found an increase in glutamine consumption that was more evident in MCF-10A clone #7 which was chosen for further investigations (Fig. 3G-H).

Figure 3

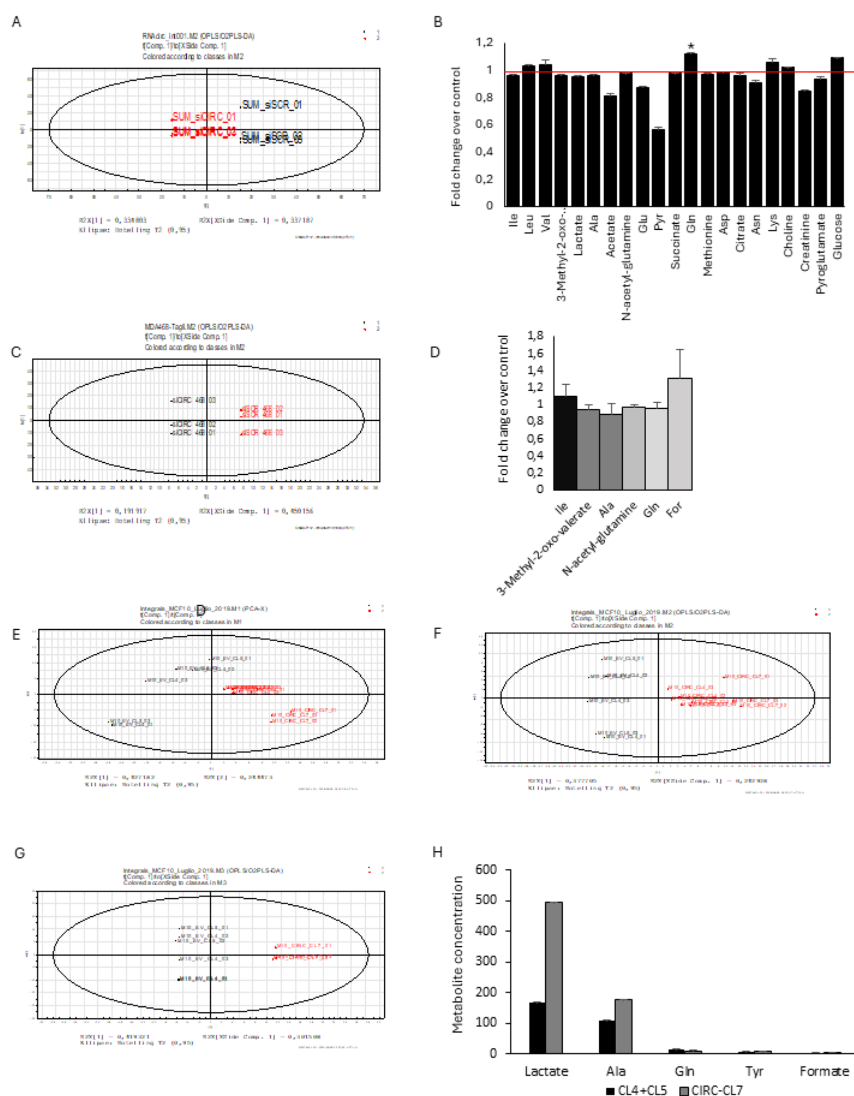


Figure 3. A. PCA models built on the 1H-NMR dataset of media samples cell extracts from SUM-159PT cell cultures either

expressing endogenous or low levels of circPVT1. B. Histograms show the fold changes of the most discriminant metabolites between the two groups from the PCA models. C PCA models built on the 1H-NMR dataset of media samples cell extracts from MDA-MB-468 cell cultures either expressing endogenous or low levels of circPVT1. D Histograms show the fold changes of the most discriminant metabolites between the two groups from the PCA models. E-F PCA models built on the 1H-NMR dataset of media samples cell extracts from MCF-10A clones expressing high levels of circPVT1. G OPLS-DA model built on the 1H-NMR dataset of media samples cell extracts from MCF-10A cell clone # 7 either expressing endogenous or high levels of circPVT1. H Histograms show the fold changes of the most discriminant metabolites between the different groups from the PCA model.

4. CircPVT1 sponge activity leads to MYC overexpression.

Among miR-33a-5p targets, we focused our attention on MYC considering its localization near PVT1 locus and its already established involvement in the regulation of key metabolic enzymes. Analysis on breast cancer TGCA dataset reported a direct correlation between circPVT1 and MYC which was recapitulated also by our MCF-10A clones overexpressing circPVT1 (Fig. 4 A, B). Indeed, MCF-10A clones presented higher levels of MYC mRNA and protein expression levels (Fig.4 C, D). Moreover, metabolic analysis performed on SUM-159PT cells depleted for MYC (Fig.4 E, F) showed a stronger metabolic impact except on only glutaminolysis (Fig.4 G, H).

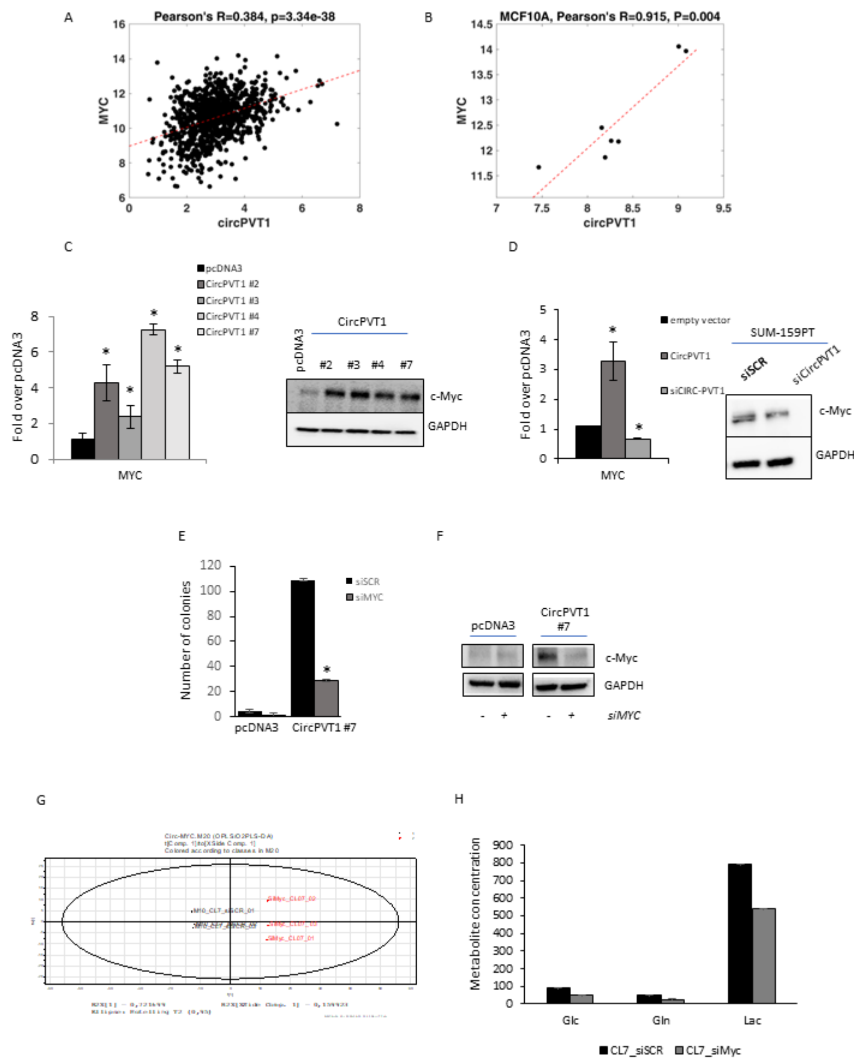


Figure 4. A-B Pearson positive correlation between MYC and circPVT1 expression levels from TCGA breast data set (A) or from

seven different clones of MCF-10A ectopically expressing high levels of circPVT1 (B). C Histograms show the MYC expression levels measured in MCF-10A treated as in Figure 2K and representative protein gel blot of whole cell lysates extracted from MCF-10A treated as in Figure 2K. D Histograms show the MYC expression levels measured in SUM-159PT expressing endogenous or high or low levels of circPVT1 and representative protein gel blot of whole cell lysates extracted from SUM-159PT cells silenced or not for circPVT1. E Histograms show the MYC expression levels measured in MCF-10A clone #7 after c-Myc silencing. F Representative protein gel blot of whole cell lysates extracted from MCF-10A cells silenced or not for c-Myc. G PCA models built on the 1H-NMR dataset of media samples cell extracts from MCF-10A cell cultures either expressing endogenous or stably expressing high levels of circPV1 followed silencing of MYC or SCR. H Histograms show the fold changes of the most discriminant metabolites between the four groups from the PCA models (G).

5. c-Myc directly regulates GLS1 expression levels

We proceeded our investigations in order to assess if c-Myc could regulate the transcription of the key enzyme of glutaminolysis, Glutaminase (GLS1). Indeed, analysis on breast cancer TGCA dataset showed a positive correlation between c-Myc and GLS1 and the same result was obtained by the same analysis considering our MCF-10A clones overexpressing circPVT1 (Fig.5 A-B). Moreover, MCF-10A clone#7 depleted for c-Myc showed also a decrease in the GLS1 expression levels (Fig.5 C-D). By using Lasagna software, we identified a putative binding site for c-Myc on GLS1 promoter (4850-4811 upstream GLS1 transcriptional starting site) (Fig.5 E) and further analysis of c-Myc ChIP-seq data deposited on the CistromeDataBase highlighted the presence of a c-Myc protein binding site on the GLS1 promoter region (Fig. 5F). Accordingly, we demonstrated through chromatin immunoprecipitation assays the c-Myc direct binding on the GLS1 promoter region both in MCF-10A clone and in SUM-159PT (Fig. 5 G, H). Moreover, we also demonstrated the presence of active Polymerase II

(ser5p) thus indicating the active GLS1 transcription mediated by c-Myc binding.

Altogether these findings indicate the critical role of c-Myc in the metabolic enzyme GLS1 transcriptional regulation as a direct consequence of the circPVT1/miR-33a-5p axis.

Figure 5

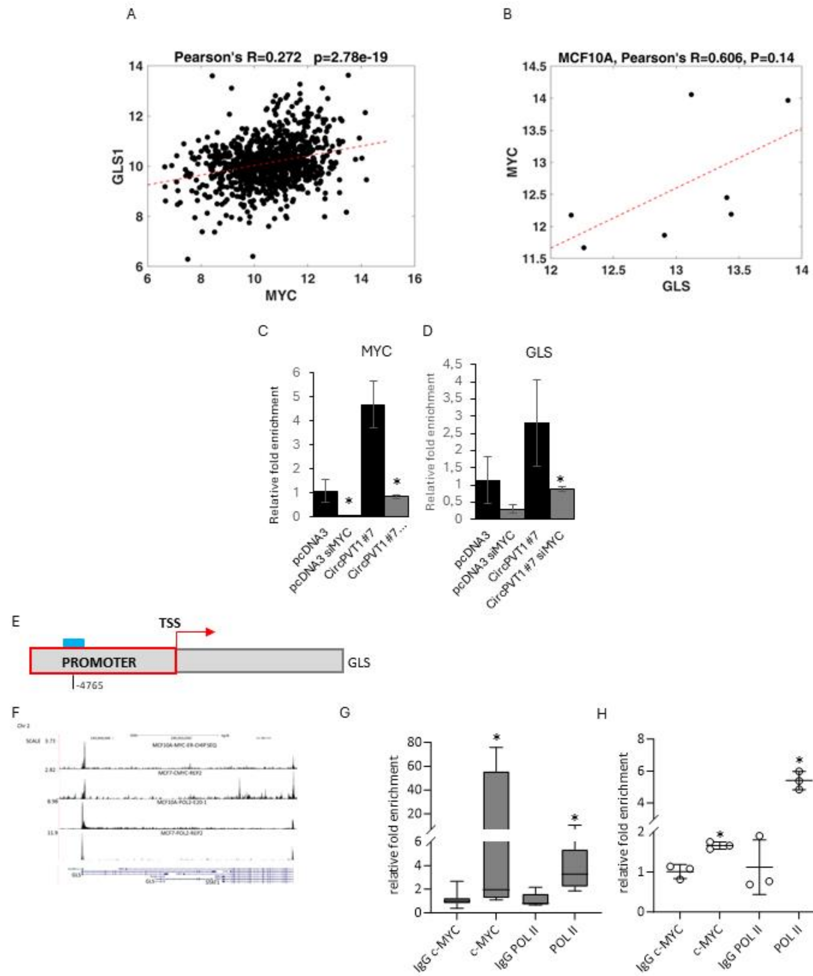


Figure 5. A-B Pearson positive correlation between MYC and GLS1 expression levels from TCGA breast data set (A) or from

seven different clones of MCF-10A ectopically expressing high levels of circPVT1 (B). C-D Histograms show the relative MYC (C) and GLS1 (D) expression levels measured in MCF-10A pcDNA3 and circPVT1 #7 silenced for SCR or MYC. E Predictive binding region of c-Myc on GLS1 promoter region. F c-Myc protein enrichment on GLS1 promoter region in MCF-10A cells from ChIP-seq data deposited on the CistromeDataBase. G-H Relative enrichment of the occupancy of c-Myc p-Ser62 on the regulatory regions of GLS1 (-4850: -4811 upstream GLS1 transcriptional starting site) assessed by Chromatin Immunoprecipitation in MCF-10A circPVT1 #7 (G) or SUM-159PT (H).

6. GLS1 inhibitors impair cell and PDOs viability.

Recent evidence in literature indicates the promising roles of small inhibitor molecules as good candidates for anti-cancer therapy [56].
Indeed, we tested the use of small GLS1 inhibitors such as BPTES and CB839 in SUM-159PT cells in circPVT1 depleted condition and after metformin treatment in order to evaluate the small inhibitors response (Fig.6 A-C). Intriguingly, the absence of circPVT1 rendered the cells more sensitive to the killing effect induced by BPTES and CB839 (Fig. 6 B, C).

We also tested BPTES effects on patients derived organoids, PDOs, that recapitulated the original tumor (Fig.6 D) showing a dose- dependent effect thus highlighting the pivotal role of glutaminase in breast cancer (Fig.6 E-F).

Collectively, we demonstrated that circPVT1 mainly localized in the cytoplasm, acts as a sponge for the metabolic miR-33a-5p leading to the upregulation of its target MYC that in turn directly enhances GLS1 transcription supporting the altered glutamine metabolism of breast cancer (Fig.6 G).

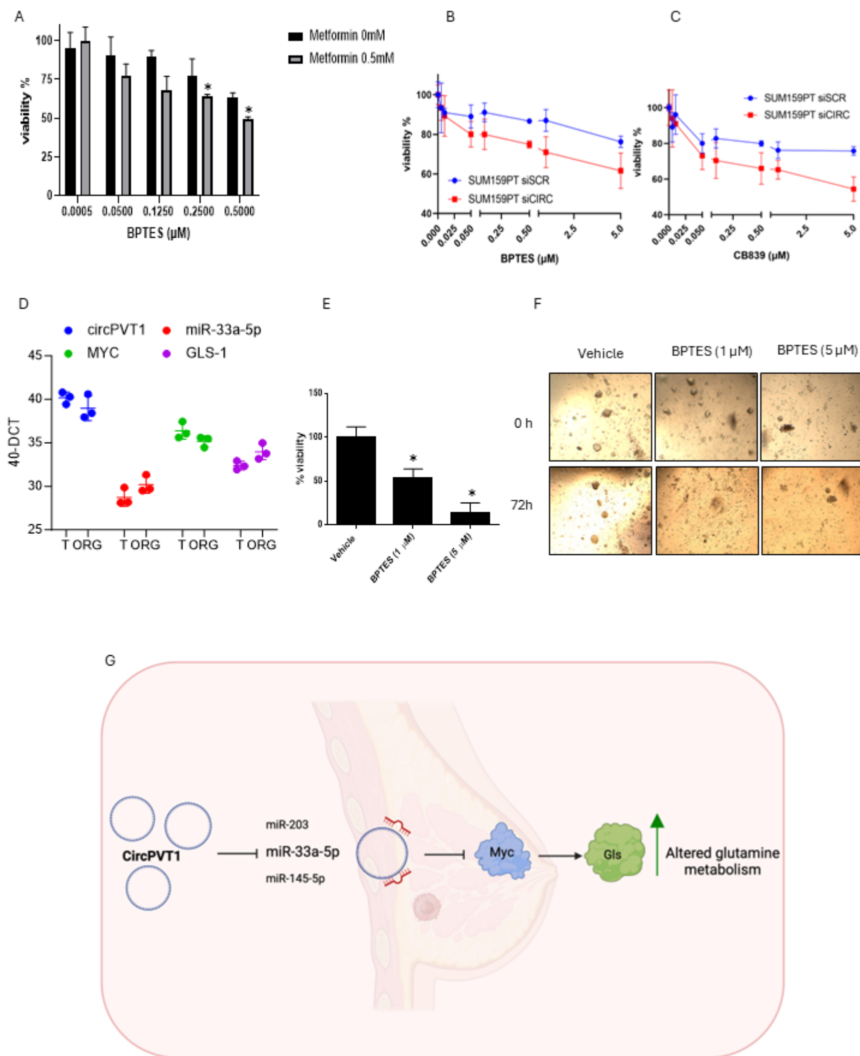


Figure 6. A. Histograms show the viability rate of SUM-159PT pre-treated or not for 24 hrs with 0.5 mM of metformin and treated for

72 hrs with increasing doses of BPTES (0 – 5 uM). B-C Viability curves obtained by measuring ATP levels in SUM-159PT cells silenced or not for circPVT1 and treated for 72 hrs with increasing doses of BPTES (0 – 5 uM) (I) or CB839 (0 – 5 uM). D Relative fold enrichment of circPVT1, miR-33a-5p, MYC and GLS1 among tumoral and matched patients derived organoids (ORG) (n=3). E Histograms show PDO#243 viability after treatment with BPTES 1uM and BPTES 5uM. F Representative images of PDO #243 treated as in E. G CircPVT1 alters glutamine metabolism by sponging metabolic miR-33a-5p sustaining c-Myc translation that in turn promotes GLS1 transcription.

5. DISCUSSION

CircularRNAs role in tumorigenesis has been already demonstrated. However, there are few evidence about their role in regulating metabolic processes resulted altered in breast cancer. These peculiar covalently closed molecules without 3′ and 5′ ends are more resistant than their linear counterparts highlighting a promising role as biomarkers in liquid biopsy. Here we investigated the role of a peculiar circRNA in breast cancer, highlighting its role in regulating tumor metabolism. Indeed, we demonstrated that circPVT1 results upregulated in two triple negative breast cancer cell lines, SUM-159PT and MDA-MB-468 and in particular its overexpression was sufficient to make non tumorigenic cells, MCF-10A, to acquire neoplastic features such as the ability to migrate and to form colonies. We found, for the first time that circPVT1, mainly localized in the cytoplasm, acts as a miRNA sponge for the well-known metabolic RNA miR-33a-5p. Intriguingly, this miRNA, has been previously shown to play as oncosuppressor downregulating several target genes involved in tumorigenesis such as MYC and HIF-1a [57] [58].

Moreover, it has also been previously demonstrated by Blandino' group that metformin, an anti-diabetic drug, exerts an anti-cancer activity by restoring DICER expression and consequently changing miRNAs profiling expression among which upregulating miR-33a-5p [57]. Interestingly, here we demonstrated the positive correlation between circPVT1 and c-MYC and also between c-MYC and GLS1 (glutaminase), the first enzyme involved in glutaminolysis. Indeed, it is already known that one of the hallmarks of cancer is the altered metabolism and recent studies highlighted the increased glutamine metabolism association with cancer [59]. Moreover, it has been reported the role of c-MYC on GLS1 expression trough the suppression of miR-23a/b in order to sustain glutamine metabolism but no evidence until now was reported regarding a possible direct regulation of GLS1 expression by c-Myc [23]. Further analysis of c-Myc ChIP-seq data deposited on the CistromeDataBase revealed a c-Myc binding site on GLS1 promoter. Notably, we then found for the first time in breast cancer that c-Myc acts as a transcription factor by binding GLS1 promoter region and

the presence also of active Polymerase II (ser5p) in that region sustains the active transcription mediated by c-Myc binding. Considering the emerging clinical importance of patients derived organoids PDO as tool to predict the patient's response to therapy, we analysed circPVT1, c-Myc and GLS1 expression in three breast cancer patients considering tumor tissue, peritumoral tissue and contralateral one. We found that PDOs recapitulated the originating breast cancer tissues and we tested the effect of two GLS1 small inhibitors BPTES and CB839 that have been already proposed as tools to fight glutamine addiction found in TNBC [60] [61]. Intriguingly, we found in SUM-159PT cell line after metformin or after siRNA CircPVT1 depletion, that cells expressing low levels of circPVT1 showed a higher sensitivity to the killing effect of BPTES. Moreover, we found a dose-dependent response effect also on PDOs cell viability highlighting the critical role of glutaminase in the survival of breast cancer cells.

Collectively, our data indicate that circPVT1 in TNBC acts as an upstream non-coding regulator of altered glutaminolysis

found in breast cancer by controlling the miR-33a-5p/c-Myc/GLS1 axis. This proposed molecular mechanism might have clinical implications and could be proposed as new therapeutic target.

6. Materials and methods

1. Cell culture and transfection

Human breast cancer cell lines SUM-159PT, MDA-MB-468 and normal human breast epithelial cell line MCF-10A were purchased from the American Type Culture Collection (ATCC, Manassas, VA). SUM-159PT and MDA-MB-468 cells were grown in DMEM/F12 Glutamax medium (Invitrogen, Carlsbad,CA) supplemented with 10% fetal bovine serum, 100 units/mL Pen/Strep antibiotic and Insulin 5 μg ml−1 (Sigma) at 37 °C in a balanced air humidified incubator with 5% CO_2. MCF-10A cells were grown in DMEM/F12 Glutamax (Invitrogen, Carlsbad,CA) supplemented with 10% horse serum and 100 μL of EGF 20 ng/ml, 500 μL of Antibiotic 100X, 500 μL of HC 500 ng/ml and 500 μL of Human insulin 0.01 mg/ml at 37 °C in a balanced air humidified incubator with 5% CO_2.

Lipofectamine RNAimax (Invitrogen) was used in accordance with the manufacturer's instruction for

transfection with siRNAs and miRNA mimics. SiRNAs were used at the final amount of 300 pmol in 100 mm dish. si-circPVT1 5′-CUUGAGGCCUGAUCUUUUA-3′ was used for functional in vitro experiments. For mature miR-33a-5p overexpression, we used the mirVana miR-33a-5p mimic (Ambion) at a final concentration of 5 nM and as control we used the mirVana miRNA mimic, Negative Control #1 (Ambion), at the same concentration. The circPVT1 overexpression in MCF-10A cells was performed using 4 μg pcDNA3-circPVT1 (cit Verduci) and 4 μg pcDNA3 vector as control. Plasmids were transfected with Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer's instruction at a final concentration of 1 μg in a 60 mm dish. Cells were collected 48–72 h post transfection for subsequent analyses.

2. RNA processing and qPCR

The total RNA was extracted with TRizol (Thermo Fisher Scientific) following the manufacturer’s instructions and the concentration, purity, and quality of total RNA were

assessed using a Nanodrop TM 1000 spectrophotometer (Nanodrop Technologies).

3. cDNA synthesis and qRT-PCR

One microgram of total RNA was reverse transcribed at 37 °C for 60 min in the presence of random hexamers and Moloney murine leukemia virus reverse transcriptase (Invitrogen). Specific oligonucleotide primers for

ACTIN Fw: 5′-GGCATGGGTCAGAAGGATT-3′ and Rv: 5′-CACACGCAGCTCATTGTAGAAG-3′.

CircPVT1 Fw:5′CGACTCTTCCTGGTGAAGCATCTGAT-3′ and Rv:3′ TACTTGAACGAAGCTCCATGCAGC-5′;

C-MYC Fw: 5′-CTCCTGGCAAAAGGTCAGAG-3′ and Rv: 5′-TCGGTTGTTGCTGATCTGTC-3′;

GLS1 Fw: 5′-TTCCAGAAGGCACAGACATGGTTG-3′ and Rv: 5′-GCCAGTGTCGCAGCCATCAC-3′ were used for PCR analyses. Gene expression levels were measured by quantitative real-time PCR using the SYBR Green assay (Applied Biosystems) on a StepOne instrument (Applied

Biosystems). Small amounts of RNA (10 ng) were reverse transcribed using the TaqMan microRNA Reverse Transcription Kit (Applied Biosystems) in a final volume of 10 μl using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). The PCR reactions were initiated with a 10-min incubation at 95 °C followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. qPCR quantification of miRNA expression was performed using TaqMan MicroRNA® Assays (Applied Biosystems) according to the manufacturer’s protocol. RNU48 and RNU44 were used as an endogenous control to normalize miRNA expression. All reactions were performed in triplicate. For circPVT1, PVT1, MYC, GAPDH and GLS1 gene expression analysis, reverse transcription and RT-qPCR were performed using MMLV RT (Invitrogen) and SYBR Green® Assays (Applied Biosystems), respectively, according to the manufacturers’ instructions.

4. PDOs cultures

Patient Derived Organoids (PDOs) were obtained according to published protocols with no modifications [62]. Briefly, breast cancer biopsies were minced into < 1 mm pieces, and

enzymatically and mechanically digested. Cells freed from tissue were filtered and suspended in extracellular matrix drops. Human organoid growing medium (hOGM) (Stem Cell technologies, Vancouver, CA) was added to the jellified drops. PDO cultures were passaged every 5-7dd by mechanical-enzymatic disaggregation as mentioned.

5. Cell and PDOs viability assay

Viability of treated cells and PDOs was assessed using ATPlite assay (Perkin Elmer, Massachusset, USA) accordingly to the manufacturer's instructions. Cells (8x102cells) and PDOs were seeded in 96 well-plates and cultured for 24hrs and treated for 72 hrs with BPTES or CB839. Each plate was evaluated immediately on a microplate reader (EnSpire Technology, Perkin Elmer).

6. Clonogenic assay

Transfected BC cells at a density of 1,000 were seeded in six-well plates. Cell colonies were subsequently washed, fixed, and stained until the colonies were visible. Then, colonies were counted and imaged.

7.Transwell invasion assay

Transfected BC cells were added into the upper chamber with 200 μL of serum-free medium. After culturing for 24 h for the SUM-159PT, MDA-MB-468 and MCF-10A cell lines, cells that migrated to the opposite side of the filter were fixed, stained, imaged (Leica Microsystems, Germany), and counted.

8. Protein extracts and western blot analysis

Cells were homogenized on ice for 30 min in a lysis buffer composed by 50 mM, Hepes pH 7.5, 5 mM EDTA pH 8.0, 10 mM MgCl2, 150 mM NaCl, 50 mM NaF, 20 mM β-glicerophosphate, 0.5% NP40, 0.1 mM sodium orthovanadate, 1 mM PMSF, 1 mM dithiothreitol (DTT), and protease inhibitor cocktail (Roche). Lysates were clarified by centrifugation for 10 min, max speed, at 4 °C. Proteins (30 μg/lane) were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Immunoblots

were probed with the following primary antibodies: rabbit monoclonal anti-c-Myc (DO1; Oncogene Science Uniondale, NY, USA), and mouse monoclonal anti-GAPDH (Calbiochem). Immunostained bands were detected by a chemiluminescent UVITEC Alliance 4.7 instrument (Cambridge, UK). ECL solution (entry-level peroxidase substrate for enhance chemiluminescence) (Thermo Scientific) was loaded on the membrane in order to allow the chemiluminescent reaction between horseradish peroxidase (HRP) labelled on the secondary antibody and the peroxidase substrate of ECL solution. The reaction generates energy that is released in the form of light and in this way the protein signal is detected by the camera.

9. Chromatin Immunoprecipitation (ChIp)

ChIP Assay Kit (Millipore, Bedford, MA) was used according to manufacturer's instructions. In brief, the 1% formaldehyde cross-linked chromatin was sonicated into fragments and then immunoprecipitated using MYC and POL2(ser5p) antibodies. IgG was used as negative control. DNA fraction was analyzed by qRT-PCR.

10. MagIC Beads RNA pull down

Samples of total, unfragmented RNA from SUM-159PT and MCF-10A #7 cells were incubated with MagIC Beads targeting human circPVT1 transcript according to manufacturer's instructions (https://elementzero.bio/magic-beads-rna-enrichment/). RNA attached to the beads was washed, eluted and subjected to cDNA synthesis. Levels of miR-33a-5p, miR-145 and miR-203 were measured in the input and enriched samples with RT-qPCR.

11. Subcellular fractionation

Nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific) were used for subcellular fractionation of BC cells. We used H3 as the nuclear control and Tubulin as the cytoplasmic control.

12. Analysis of GLS1 promoter

Lasagna 2.0 web-tool to analyse GLS1 promoter. The promoter sequences are related to the human genome GRch38/hg38.

13. 1H-NMR spectroscopy

All 2D 1H J-resolved (JRES) NMR spectra were acquired on a 500 MHz VNMRS Varian/Agilent spectrometer (Agilent, Santa Clara, CA) at 25 °C using a double spin echo sequence with pre-saturation for water suppression and 16 transients per increment for a total of 32 increments. These were collected into 16 k data points using spectral widths of 8 kHz in F2 and 64 Hz in F1. Each free induction decay (FID) was Fourier transformed after a multiplication with sine-bell window functions in both dimensions. JRES spectra were tilted by 45°, symmetrized about F1, referenced to lactic acid at δH = 1.33 ppm and the proton-decoupled skyline projections (p-JRES) exported using Agilent VNMRJ 3.2 software. The exported p-JRES were aligned, corrected for baseline offset and then reduced into spectral bins with

widths ranging from 0.02 to 0.06 ppm by using the ACD intelligent bucketing method (1D NMR Manager software, ACD/Labs, Toronto, Canada). This method sets the bucket divisions at local minima (within the spectra) to ensure that each resonance is in the same bin throughout all spectra. The area within each spectral bin was integrated and in order to compare the spectra, the integrals derived from the bucketing procedure were normalized to the total integral region. Metabolites were identified using an in-house NMR database and literature data and confirmed by 2D homo- and hetero-nuclear NMR spectroscopy.

14. NMR spectra pre-processing treatment

The 1D skyline projections exported were aligned and then reduced into spectral bins with ranging from 0.01 to 0.02 ppm by using the ACD intelligent bucketing method (1D NMR Manager software (ACD/Labs, Toronto, Canada). To compare the spectra, the integrals derived from the binning procedure were normalized to the total integral region, following exclusion of bins representing the residual water peak (4.33–5.17 ppm) and the TSP peak (0.5–0.5 ppm).

The resulting data was used as input for multivariate analysis: Principal Component Analysis (PCA and Orthogonal projections to latent structures discriminant analysis (OPLS-DA) were performed using SIMCA-P + version 12 (Umetrics, Umea, Sweden).

15. Statistical analysis

The resulting data was used as input for univariate and multivariate analysis PCA34 and OPLS-DA35. PCA and OPLS-DA were conducted using SIMCA-P+ version 12 (Umetrics, Umea, Sweden). For microRNA analysis, Pearson's correlation coefficient was calculated to assess quality of replicates. Generally, Student's t-test was used to assess significance of the data and P-values ≤0.05 were considered statistically significant.

7. Appendix

(list of pubblications)

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