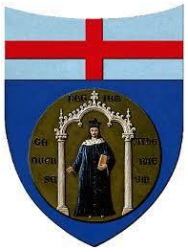

University of Genoa

Ph.D. program in "Biotechnologies in Translational Medicine"

THE BIOLOGICAL ROLE OF 45A ncRNA IN NEUROBLASTOMA

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Abstract

45A non-coding RNA (ncRNA) overexpression induces modifications of neuroblastoma (NB) cell cytoskeleton leading to a cascade of reactions that interferes with proliferation control, cell migration, tumorigenic potential, and cell adhesion properties. In detail, 45A-overexpressing cells show non-specific metastatic engraftment whereas the silencing of its expression drives to the production of liver-specific metastasis.

We investigated by Real-time RT-PCR, western blot, and immunofluorescence analysis the different expression and/or localization of GTSE1, MCAK, Aurora B, p53, EGFR, and the altered organisation of tubulin in different neuroblastoma cell (SKNBE2) models stably overexpressing or downregulating 45A ncRNA. Indeed, the proper regulation of these proteins' expression and function is fundamental for cytoskeleton organization as their impairment leads to the particularly dangerous condition of chromosomal instability (CIN).

We demonstrate that 45A ncRNA not only directly regulates the expression of aforementioned proteins but can even affect GTSE1 subcellular localization: in 45A-overexpressing cells, the protein is accumulated in nuclei, while 45A-downregulation leads to a significant GTSE1 cytoplasm relocation, together with simultaneous cytoplasmatic sequestration of p53, driven by GTSE1/p53 interaction. This shuttling of the oncosopressor decreases the apoptotic potential of 45A-downregulating cells, justifying the resistance to toxoids that we observed. Furthermore, 45A-overexpression leads to an increased number of abnormal spindles, thus promoting CIN, and possibly explaining the increased tumorigenic potential exhibited by 45A-overexpressing cells.
Lastly, we also observed EGFR nucleolar sequestration in 45A overexpressing cells, a mechanism observed in several kind of tumors and correlated with malignancy. These data highlight the role of 45A ncRNA in the functional regulation of several proteins expression involved in microtubules dynamics, causing different drugs responses and suggesting its possible relevance in prognosis.

Introduction

Neuroblastoma

Neuroblastoma (NB) is the most common extracranial tumor occurring in childhood and it can generate during embryonic or early post-natal life from sympathetic cells derived from the neural crest [1,2]. Indeed, NB represents about 8% of all malignant tumors diagnosed in pediatric patients younger than 15 years of age and 15% of pediatric cancer deaths [3]. Every year the new diagnoses formulated in Italy are 130-140 and the incidence is not different between males and females.

The main sites of NB development are in the abdominal region (65% of cases), especially in the adrenal glands; however, it can arise also in the chest (20%, neck (5%) or along the spinal cord (5%), but 1% of patients have a not detectable tumor [2]. Generally, patients can be asymptomatic, but someone presents a growing mass, pain, abdominal distension, or respiratory distress. The metastasis could spread through the bloodstream or lymphatics, seeding bone marrow, liver, and bone. If dissemination occurs to the skin, patients develop blue subcutaneous nodules, a condition called blueberry muffin syndrome. Surprisingly, this is associated with a favorable prognosis with likely spontaneous tumor regression [4].

Different genomic alterations have been identified in NBs leading to several patterns of clinical behavior. Historically, NB subtypes were classified into different stages based on multiple factors such as genetic alterations, age of the patient, presence of metastasis, histopathology of the tumor, etc. This classification is called International Neuroblastoma Staging System (INSS) and includes 5 different stages of classification. Stages 1, 2 and 4S described tumors with little or no risk and favorable prognosis; stage 4S indicates tumors that undergo spontaneous regression. Instead, stages 3 and 4, known as high-risk NBs (HR-NBs), are characterized by aggressiveness, low response to therapy, and poor prognosis [5,6]. Nowadays, researchers and investigators developed and used the International Neuroblastoma Risk Group (INRG) classification system, replacing the INSS one: indeed, this provides an efficient way to classify the stages of NBs and, consequently, relevance for patient outcome [7].

Stage	Definition
L1	Localized tumor not involving vital structures
L2	Localized tumor with the presence of one or more image-defined risk factors
M	Metastatic disease
MS	Children ≤18 months of age at diagnosis with metastases limited to the skin, liver, and/or bone marrow

Adapted from [5].

Recently, a new NB classification was proposed by Ackermann et al., who found that alterations in telomere maintenance mechanisms as well as in RAS or p53 pathways better discriminate between high-risk or low-risk NBs than the previous classification [8].

Telomeres are responsible for genomic integrity in normal cells, and telomere length and telomerase activity are crucial for cancer initiation and tumor survival. In particular, Ackermann et al. [8] showed that survival rates were lowest for NB patients whose tumors harboured telomere maintenance mechanisms in combination with *RAS* and/or *p53* mutations. On the other hand, low-risk NBs did not show telomere maintenance mechanisms, in the absence of which the possible mutations in *RAS* or *p53* genes seem to not affect patient outcome [8].

The real etiology of NB is still unknown, but the sporadic form represents most of the cases, whereas only 1%–2% of affected children present a genetic autosomal dominant inheritance pattern [9]. This tumor can show a broad range of chromosomal abnormalities, but the most common genetic alteration is the amplification of the oncogene *MYCN*, which is observed in 20%–25% of cases and in 50% of high-risk tumors [10]. Another genetic aberration, found in 9% of primary NB, is the activation of the anaplastic lymphoma kinase (*ALK*) gene [11]. All these mutations are associated with poor clinical outcomes, but, in a few cases, they represent possible therapeutic targets.

The treatment for patients with localised disease consists of surgical resection and chemotherapy to prevent possible relapse [7]. Instead, children with high-risk NB have poor outcomes and are the most challenging to treat. The standard regimens continue to have four components: induction chemotherapy, local control, consolidation, and maintenance therapy. Briefly, during induction chemotherapy, a combination of anthracyclines, platinum-containing

compounds, alkylating agents, and topoisomerase II inhibitors are used. After 4-6 cycles of induction therapy, local control is performed through surgical resection and radiotherapy. The consolidation therapy includes myeloablative chemotherapy and autologous stem cell rescue, which are generally collected after 2-3 cycles of induction chemotherapy. Finally, during the maintenance phase patients are treated with retinoic acid derivates, that are able to induce differentiation in NB cells, preventing possible relapses [7].

In addition to the classical protocol used for NB treatment, novel therapeutical approaches have been studied for the last years. Important molecules used for NB therapy act on the membrane targets GD2 and B7-H3. GD2 is a disialoganglioside expressed on the membrane of numerous cancer cells, such as brain tumors, retinoblastoma, osteosarcoma, and NB [12]. Anti-GD2 monoclonal antibodies are currently used in therapy to improve standard treatments for HR-NBs, but further studies are necessary to confirm the effectiveness of this immunotherapy and to optimize it [13]. However, in 12% of patients with bone marrow relapse, NB cells lose GD2 expression, thus rendering the use of this treatment impossible [14]; for this reason, Dondero et al. [15] developed a multiparametric flow cytometry to observe GD2 surface expression, suggesting B7-H3 targeting therapy for those patients in which GD2 is missed. B7-H3 is a transmembrane glycoprotein overexpressed in NB cells (particularly in bone marrow aspirates) [16], as well as in melanomas, gliomas, and breast and pancreatic cancers [17]. B7-H3 is a member of the B7 family that may down-regulate natural killer (NK) cell cytotoxicity through binding to NK receptors, leading to the activation of inhibitory signals. Recently, a murine IgG1 antibody against B7-H3 (omburtamab) was tested for NB therapy, showing significant effectiveness in NB patients with central nervous system involvement [17]. A phase II/III study is still ongoing [18]. Despite the therapeutic approach advancement in recent years, NB still represents 15% of all pediatric cancer deaths [19], and a comprehensive and detailed view of molecular and genetic mechanisms that bring to NB development is not available yet. For this reason, NB represents a significant unmet medical need and a challenge in terms of prevention and treatment, highlighting the importance of exploring new molecular pharmacological targets, such as non-coding RNA, especially for HR-NBs [20].

Non-coding RNA 45A

Non-coding RNA (ncRNA) 45A is a 78 nucleotides transcript that was discovered in our laboratory by a bioinformatics search for Proximal Sequence Element / TATA containing snRNA-like elements in the human genome.

It is transcribed from intron 1 of Amyloid Beta Precursor Protein Binding Family B Member 2 (APBB2) (OMIM: 602710) gene by polymerase III, in antisense configuration [21]. APBB2 is a gene of the FE65 protein family, and it was studied because of its possible role in neurodegeneration, in particular in Alzheimer's disease (AD). Indeed, this gene product is an adaptor protein that binds to the cytoplasmic domain of β-amyloid precursor protein (βAPP), regulating this protein processing [22,23]. When APBB2 is overexpressed, γ-secretase activity is increased, leading to improved processing of βAPP and, consequently, to the β-amyloid formation (Aβ) [24]. Since Aβ is the main constituent of senile plaques, characteristics of AD pathology, the researchers studied APBB2 as a new target for AD therapy during the last years.

45A ncRNA perturbs the maturation of APBB2 pre-mRNA, leading to different APBB2 transcripts and, consequently, to the synthesis of alternative protein variants. Since APBB2 forms protein complex with APP, ensuring APP cleavage by γ-secretase, the synthesis of different protein variants affects this phenomenon, leading to a decreased Aβ secretion and consequently perturbation of the Aβ-42/ Aβ-40 *ratio* [21]. It has been reported that in 45A-overexpressing cells the alternative protein variants have 63 bp-long exon 8, not present in the classical variant. This exon 8 encodes for 21 amino acids (most of which are hydrophobic), generating a difference of 2.2 kDa between the alternative variants: for this reason, these isoforms are clearly discernible with Real-time RT-PCR and not with SDS-PAGE. Moreover, the production of alternative protein variants changes the subcellular localization of APBB2: in 45A-overexpressing cells, the protein is detectable even in the nucleus, while in control cells APBB2 is localized in the cytoplasm.

Since it has been demonstrated that the overexpression of canonical variant of APBB2 affects proliferation rate in fibroblasts [25], Penna et al [21] evaluated if the expression of different isoforms could modulate proliferation in 45A-overexpressing cells. The proliferation rate of 45A-overexpressing and control cells was evaluated by analyzing cell lines of different origins: HEK-293 (epithelial), HeLa (epithelial), SH-SY5Y (neuroblastoma – no-MYCN amplified), and SKNBE2

(neuroblastoma – MYCN-amplified). The results confirmed that 45A ncRNA overexpression enhanced the proliferation rate in all the cell lines, independently from their origin. These data suggest 45A ncRNA as a controller for cellular proliferation through an APBB2 splicing variant shift. In order to better characterize the role of 45A, the authors evaluated the expression of different genes involved in cell cycle regulation in 45A-overexpressing (called 45A cells) and control (Mock) cells, finding out 9 genes whose expression is significantly modulated by 45A. Indeed, in 45A-overexpressing cells up-regulation is seen in those genes that promote proliferation, while the down-regulated genes are correlated with differentiation and cell cycle arrest in response to DNA damage.
Since the increased proliferation rate and the down-regulation of genes involved in DNA repair were observed, Mock and 45A cells were treated with chemical and physical agents to activate different DNA repair pathways. These cytotoxicity experiments demonstrated a higher sensitivity of 45A cells toward double or single-strand break inducers (Adriamycin and methyl methanesulfonate, respectively), but this result is not accompanied by a significant increase of micronuclei or multinucleated cells, that are synonymous with uncorrected chromosomal segregation. This might be associated with a different cell cycle progression due to 45A ncRNA regulation.

Nevertheless, the most interesting aspect of this work is related to the process by which 45A ncRNA could regulate malignant progression in neuroblastoma. Indeed, the 45A ncRNA overexpression in neuroblastoma cells increases the colony-forming potential, while in other cell lines it is not observed, suggesting 45A specific role in neuroblastoma. Moreover, the 45A-overexpressing colonies are less organized and tend to disperse throughout the surrounding space, proposing that this transcript could promote malignant potential. To confirm this hypothesis *in vivo*, we evaluated tumor formation capacity injecting subcutaneously 45A over-expressing HEK-293 and SKNBE2 and their control cells in NOD-SCID mice. In both cases, the overexpression of 45A significantly increases the tumor progression rate and the growth speed of nodules, especially for SKNBE2 cells [21].

In a second work [26] the authors described the effects of the downregulation of 45A ncRNA in neuroblastoma. To this aim, different clones transfected with a plasmid harboring 45A in antisense configuration were generated. As expected,

these cells (called Anti45A) showed a decreased proliferation rate. Concerning the DNA damage response, evaluated also in 45A-overexpressing cells, the sensitivity of Anti45A cells to Adriamycin and MMS was higher with respect to the control cells.

Moreover, phenotypic characteristics were also studied. Colonies formed by Anti45A cells exhibit a spread, strongly adherent shape, while control cells are organized in rounded poorly adherent colonies. Anti45A cells change their shape, showing a characteristic stretched form and adhesion proprieties, probably due to different organisations in cytoskeleton structure. Indeed, these cells show a significantly increased migration ability than mock cells and a reduced capability to form colonies in non-adhesive conditions.

To confirm these observations, the tumor formation of Anti45A cells was evaluated also *in vivo*, finding out that 45A downregulation doesn't affect tumor initiation, but tumor growth rate. The Anti45A nodules present histological differences, such as more compact collagen fibers and lower levels of KI-67 expression (a cell proliferative marker). Moreover, the formation of metastasis was similar in mice injected with SKNBE2-Anti45A or -Mock cells; however, Anti45A cells spread preferentially to the liver, while Mock mainly in lung and lymph nodes, demonstrating an alteration of cytoskeleton and adhesiveness.

Since it was observed a significant variation in cell morphology, adhesion, migration, and *in vivo* tumor growth ability in Anti45A cells, we evaluated if modulation of the expression of several genes involved in cell cycle control was present. Results showed that only one gene was strongly downregulated in 45A downregulating cells: G2 and S phase-expressed-1 (GTSE1, NM_016426).

GTSE1

GTSE1 is a microtubule (MT)-associated and EB1-dependent plus-end tracking protein [27,28]. Its regulation is fundamental to guarantee the G2/M phase progression during the cell cycle [27]. During the interphase, GTSE1 is required to grow MT plus-ends by EB1 for cell migration. Interestingly, GTSE1 overexpression increases cell migration, while its depletion leads to the reduction of MT growth velocity [28]. GTSE1 is also required for focal adhesion disassembly induced by MTs, promoting mitosis event [28]. Focal adhesions are sites in which different molecules, such as integrin and proteoglycan, mediate adhesion links to the cytoskeleton structure. Focal adhesion turnover and disassembly must be correctly regulated to lead to cell migration and cellular division [29]. Scolz et al

demonstrated that GTSE1 is required for focal adhesion disassembly induced by MTs, and the interaction between GTSE1 and EB1 is fundamental in this process [28].

During mitosis, GTSE1 is phosphorylated and linked by TACC3 protein, changing its localisation from MT plus ends to the mitotic spindle [28,30]. GTSE1 is involved in MT regulation during mitosis thanks to its ability to interact with Mitotic Centromere-Associated Kinesin (MCAK) [31], a MT depolymerase protein that controls kinetochore-MT stability, promoting the correct chromosome segregation during anaphase and ensuring MT stability in mitosis [31–33]. Indeed, the deregulation of MCAK may impact chromosomal instability (CIN) development because it leads to poor coordination of chromosome movement; however, in cancer cells MCAK protein levels are not generally downregulated [34], suggesting that its activity rather than its expression could be compromised in tumors. Instead, the overexpression of MCAK mRNA and protein levels was observed in breast, colorectal and gastric cancers and it is correlated with lymphatic invasion, lymph node metastasis, and poor prognosis [35]. Moreover, MCAK overexpression confers resistance to paclitaxel by modulating cytoskeleton dynamics [36,37].

GTSE1 is normally localized at MT level, but after DNA damage it could re-localize from cytoplasm to the nucleus, due to the presence of nuclear export signals (NES) in its C-terminal region [38]. GTSE1 is a p53-inducible gene [39], but its protein can negatively regulate p53 levels and activity, preventing its pro-apoptotic activity after DNA damage [40]. Indeed, p53 is a tumor suppressor that acts as a transcription factor, activating several genes in response to different types of stress, such as oncogene activation, DNA damage, or hypoxia condition [41]. In response to p53 activation, p53-inducible genes can induce growth arrest, the repair of damaged DNA, or apoptosis [42]. The regulation of p53 by GTSE1 appeared to be restricted to the S and G2 phases of the cell cycle, which are the two moments when GTSE1 is expressed [40]. Moreover, GTSE1 controls DNA damage response inhibiting p53 function [40] thanks to the binding at the C-terminal domain of p53, enhancing p53 cytoplasmic localization in presence of another protein (Mdm2) and preventing its pro-apoptotic activity [38].

Another protein regulated by GTSE1 is Aurora B, a serine/threonine-protein kinase involved in mitosis. It is the enzymatic component of the chromosomal

passenger complex (CPC), that acts as a key regulator in mitosis [43]. Aurora B expression and activity are cell cycle regulated: indeed, its expression peaks at G2-M transition, while its activity is maximal during mitosis [44]. Its function is important to regulate chromosome condensation, MT-kinetochore attachment, and chromosomal condensation, alignment, and segregation [45]. The alteration of Aurora B expression or activity could induce aneuploidy and consequently CIN development, promoting malignancy in cancer cells [46]. Indeed, Aurora B results overexpressed in several human cancers, such as non-small cell lung carcinoma [47,48], mesothelioma [49], glioblastoma [50], oral cancer [51], ovarian cancer [52], colon [Tatsuka 1998], and prostate cancer [53]. Given these observations, different clinical trials proposed the use of Aurora B inhibitors as a new antitumoral treatment. Furthermore, preclinical data predicted that the association of these inhibitors with chemotherapy leads to synergistic anticancer effects, thus opening new possibilities from cancer treatment [46].

GTSE1 was reported to promote the localization of Aurora B to chromosome arms, increasing MT dynamics during mitosis [54].

Different drug response due to 45A expression levels

Since increased levels of 45A ncRNA lead to deep modifications of cell cytoskeleton organisation, promoting proliferation rate and migration ability, we recently investigated if 45A ncRNA overexpressing or downregulating cells could respond differently to anticancer drugs targeting cytoskeleton polymerization/depolymerization activity (i.e. taxol, vinblastine, vincristine) [Calderoni et al, in prep]. These mitotic spindle poisons inhibit the mitotic division acting at MT level: indeed, paclitaxel (the generic name for taxol) induces MT polymerization and stabilization, promoting metaphase event but preventing the consequent depolymerization needed during telophase and cytokinesis [55]; vinblastine and vincristine are vinca alkaloids that prevent GTP absorption and consequently MT polymerization [56].

We performed two different experiments to evaluate mitotic spindle poisons response in 45A and Anti45A cells: MTT and xCELLigence RTCA system. We found out that 45A overexpressing cells are more sensitive than control cells, while 45A down-regulating cells show resistance to the treatments [Calderoni et al, in prep]. Moreover, we characterized the cytoskeleton organisation during these treatments, finding out that the response is completely different due to 45A ncRNA expression. In particular, during taxol treatment, we saw that in 45A cells,

MT collapsed, and tubulin accumulated near to nucleus, while in Anti45A cells the cytoskeleton did not undergo changes, except an accumulation of tubulin near the cellular membrane (figure 1). Using vinca alkaloids, we observed that in Anti45A tubulin formed crystals, something not detectable in 45A cells, confirming the different organisation due to 45A expression.

These results suggested us the importance of studying 45A ncRNA in order to propose this transcript as a new marker for NB.

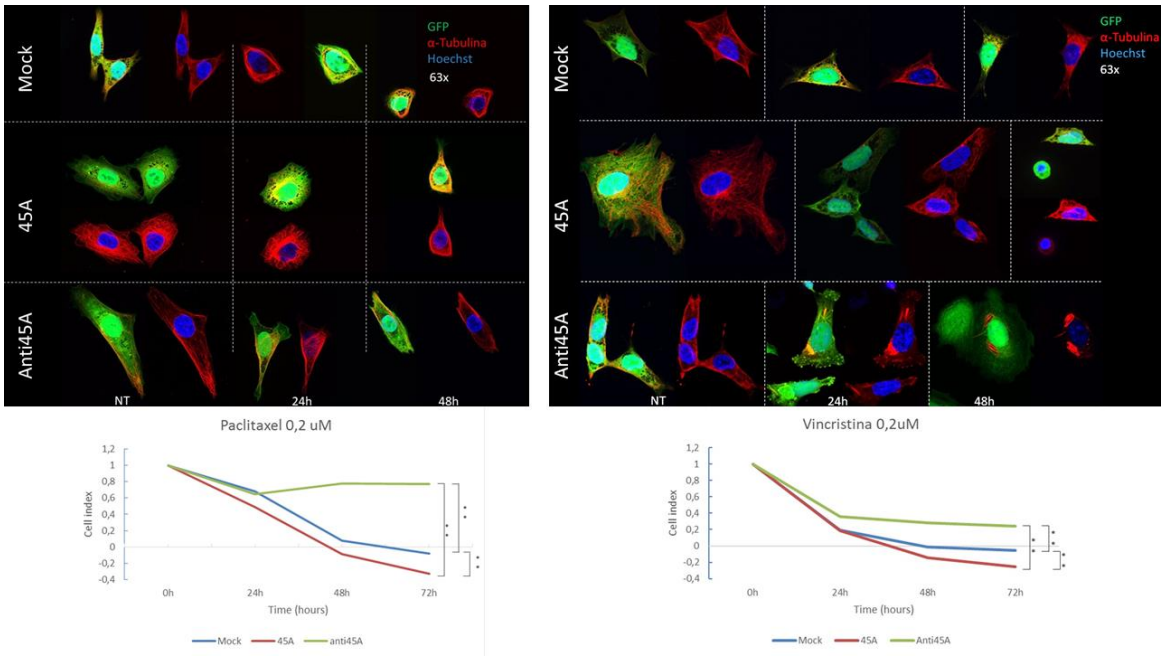


Figure 1. Evaluation of mitotic spindle poisons on SKNBE2 Mock, 45A, and Anti45A cells. Left: Representative images of cytoskeleton organization during Paclitaxel treatment (top) and drug response evaluated with xCELLigence system. Right: Representative images of cytoskeleton organization during Vincristine treatment (top) and drug response evaluated with xCELLigence system.

Aim of this work

The purpose of this work was to determine the biological role of 45A ncRNA in the functional regulation of fundamental proteins for the cytoskeleton organisation. 45A ncRNA is a transcript whose overexpression or downregulation is correlated with different expression levels of GTSE1, an important regulator of microtubules dynamics. Moreover, GTSE1 controls the activity of several proteins, whose dysregulation could lead to errors in chromosome segregation, promoting CIN onset. In turn, CIN is a hallmark in human cancer, and it is associated with poor prognosis and therapeutic resistance [57]. In NB the association between CIN and risk variants on genes that code for proteins involved in chromosomal segregation or centrosome segregation has yet to be investigated [58].

Since in previous works we observed differences in MT organization due to alternative expression of 45A ncRNA, we analysed the different expression levels and/or localization of GTSE1, MCAK, Aurora B, p53, EGFR, and the altered organisation of pericentrin and tubulin in different neuroblastoma cell (SKNBE2) models stably overexpressing or downregulating 45A ncRNA. GTSE1, a MT-associated protein, is able to regulate the activity of MCAK, Aurora B, and p53, and its activity should be finely controlled to lead to correct mitotic divisions and chromosome segregation, preventing possible mutations.

Given the important role of some of these proteins in regulating cytoskeleton organization and chromosome segregation, we characterized the role of 45A ncRNA in this pathway, in order to disclose its possible role in CIN development. Characterizing this tuned regulation is important to understand how CIN is established in NB cells and which proteins could be considered as new therapeutic targets, with the final goal of identifying novel therapeutical approaches.

Materials and methods

Cell cultures

SKNBE2 (Human Neuroblastoma) cells were maintained on RPMI 1640 medium (ECB9006L EuroClone, Milan, Italy), 10% FBS (Euro Clone, Milan, Italy), L-glutamine (2 mM; EuroClone, Milan, Italy), penicillin-streptomycin (100 U/ml/100 µg/ml; Euro Clone, Milan, Italy) (standard medium). The cells were selected in 200 µg/ml G-418 (Geneticin; Invitrogen) in standard culture conditions.

Immunofluorescence

Cells were cultured directly on coverslips of glass coated with poly-lysine. Cells were fixed with methanol:acetone (1:1) for 10min at -20°C, followed by 10 min methanol at -20°C, and then were blocked with 3% BSA (bovine serum albumin) containing 0.1 % Triton X-100.

The following primary antibodies were used:

- Rabbit anti-α-tubulin (Abcam, Cambridge, UK) 1:300
- Mouse anti-α-tubulin (Sigma-Aldrich, USA) 1:1000
- Mouse anti-Aurora B (Abcam, Cambridge, UK) 1:50
- Mouse anti-p53 (Santa Cruz, USA) 1:200
- Mouse anti-MCAK (Santa Cruz, USA) 1:50
- Rabbit anti-GTSE1 (Abcam, Cambridge, UK) 1:50
- Rabbit anti-pericentrin (Abcam, Cambridge, UK) 1:500
- Rabbit anti-EGFR (Epitomics, USA) 1:1000
- Rabbit anti-c-Myc (Epitomics, USA)
- Rabbit anti-MYCN (Epitomics, USA)
- Rabbit anti-APBB2 (Sigma-Aldrich, USA) 1:1000
- Rabbit anti-fibrillarin (Santa Cruz, USA) 1:200

After 1 h incubation with the antibodies diluted in PBS/1% BSA at 37°C and 3 × 5 min washing in PBS, the cells were incubated with secondary antibodies Alexa Fluor 568 goat anti-rabbit IgG and Alexa Fluor 594 goat anti-mouse IgG (Molecular Probes), used at 1:500. The coverslips were then incubated with Hoechst 33342 to detect nucleus counterstaining.

Images were acquired with a Zeiss Axiovert 200-M inverted microscope equipped with ApoTome slide module (Zeiss), through a ×63 objective, and processed by using Zeiss AxioVision 4.8 software (Zeiss).

Quantification of fluorescence intensity

To determine the fluorescence levels from the microscopic images, it was used the tool Analyze → Measure of ImageJ software. After selecting the regions of interest in the image and the parameters to be measured (Analyse → Set Measurements → Area, Integrated density, mean grey value), the correct total fluorescence intensity (CTCF) was calculated, both in the whole cell and in the nucleus only, with the following formula:

CTCF = Integrated density - (Area of selected cell × Mean fluorescence of background area)

The background was obtained by selecting a region of the image without fluorescence. Finally, the fluorescence intensity in the cytoplasm alone was calculated by subtracting the CTCF in the nucleus from the total one.

Giemsa staining

The cells grown on slides were washed twice with PBS, fixed with methanol:acetic acid (3:1) for 30 min at 4°C, and stained in 4% buffered Giemsa stain (pH 7.2) for 25 min. At least 200 consecutive mitotic figures per sample were examined by light microscopy, and images were acquired with Zeiss Axiovert 200-M at 63× optical magnification.

Quantitative Real-Time RT-PCR Analysis

Total RNAs from samples were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and subjected to reverse transcription by Transcriptor First-Strand cDNA Synthesis Kit (Roche, Germany), with random hexamer as primers, following the manufacturer's instructions. The total RNA from samples was measured by real-time quantitative RT-PCR using ABI PRISM® 7700 Sequence Detection System (Perkin Elmer Corp./Applied Biosystems, Foster City, CA) and SYBR Green method following the manufacturer's instructions.

The forward and reverse primer sequences used were:

45A	5'-CATCTATAATGGCTGAATTGGAA-3' and 5'-ATGAACTTTCCAACAAATGTTGTT-3';
Aurora B	5'-CCCTTTCTCTCTAAGGATGG -3' and 5'-TATTCTCCATCACCTTCTGG-3'
Aurora A	5'-GAGATTTTGGGTCAGTAGATG-3' and 5'-TAGTCCAGCGTGCCACAGAGA -3'

EGFR	5′-TGCGTCTCTTGCCGGAAT-3′ and 5′-GGCTCACCCTCCAGAAGGTT -3′
MCAK	5′-CTGTTTCCCGGTCTCGCTATC -3′ and 5′-TCTGGGTTTATTGCAGCCACA -3′
c-MYC	5′-CGTCTCCACACATCAGCACAA-3′ and 5′-GACACTGTCCAACTTGACCCTCTT-3′

For endogenous control, the expression of glyceraldehyde 3 phosphate dehydrogenase (GAPDH) gene was examined. The sequences for human GAPDH primers were 5′-GAAGGTGAAGGTCGGAGTC-3′ and 5′-GAAGATGGTGATGGGATTTC-3′. Relative transcript levels were determined from the relative standard curve constructed from stock cDNA dilutions and were divided by the target quantity of the calibrator, following the manufacturer's instructions.

Western blot

Proteins were extracted from cells using RIPA buffer and a cocktail of protease and phosphatase inhibitors (Roche). Samples were then clarified by centrifugation at 12000 x g for 10 min at 4°C and the supernatant was recovered. Protein concentrations were determined using the colorimetric Bradford assay (Bio-Rad). Nuclear proteins were obtained with another protocol: the cells were washed with PBS and then frozen into -80°C fridge. Then, they were incubated with a specific buffer (buffer A – see below) containing proteinase cocktail inhibitors. After centrifugation, the pellets containing nuclear extract were dissolved in buffer B (see below) and centrifugate to obtain the nuclear extract in the supernatant.

Buffer A:

- Tris-HCl 20mM
- EGTA 2mM
- EDTA 2mM
- Sucrose 250mM
- Proteinase inhibitor containing, add freshly 1:10 from stock
- Fill in ddH20
- pH 7.5

Buffer B:

- Tris-HCl 20mM
- EGTA 1mM

- EDTA 1mM
- NaCl 0.4M
- Proteinase inhibitor containing, add freshly 1:10 from stock
- pH 7.5

Western blotting was performed with SDS-PAGE Electrophoresis System. Protein samples were electrophoresed on 4-12% polyacrylamide gels under reducing conditions and blotted to nitrocellulose membrane (Whatman, Sigma-Aldrich). After the transfer, membranes were blocked with 5 % non-fat dry milk for 1 h at room temperature and incubated either overnight at 4°C with the following primary antibodies:

- mouse anti-Aurora B (Abcam, Cambridge, UK) 1:1000,
- rabbit anti-EGFR (Epitomics) 1:1000,
- rabbit anti-c-MYC (Epitomics) 1:1000,
- mouse anti-APBB2 (Sigma-Aldric, USA) 1:500,
- mouse anti-α-tubulin (Sigma-Aldrich, USA) 1:5000
- mouse anti-laminin (Santa Cruz, USA) 1:100
- mouse anti-H2AX (Santa Cruz, USA) 1.500.

After three washes with TBST, membranes were incubated for 1 h at room temperature with goat anti-mouse IgG (Fc specific)–Peroxidase antibody (1:12000, Sigma-Aldrich, USA). Bands were revealed with the ECL chemiluminescence detection system (Thermo Scientific). The densitometric analysis of protein bands was performed using the ImageJ software system.

Gene expression dataset

One publicly available dataset has been used for gene expression analysis. It contains the gene expression profile of 88 tumors measured by the Affymetrix Human Genome U133 Plus 2.0 platform (GSE16476). The database is called "R2: Genomics Analysis and Visualization Platform".

Statistical analysis

Results are expressed as mean ± Standard Deviation. Statistical significance of observed differences among different experimental groups was calculated using One-way Anova with post-hoc Tukey HSD. A P value of less than 0.05 was considered statistically significant. In the figures, * and ** indicate statistical

significance at $p < 0.05$ and 0.01, respectively. The statistical calculations were performed with GraphPad Prism 8.0 for Windows.

Results

45A ncRNA affects spindle organization

45A up or down-regulation leads to deep modifications of cytoskeleton organization, adhesion, and migration of neuroblastoma cells. These effects are correlated with alterations in the expression of several genes involved in cell cycle control, including GTSE1 [26]. Since GTSE1 is an important regulator for mitotic division and correct chromosome segregation, we investigated the effects of different expression levels of 45A ncRNA on mitotic division events, in order to determine its involvement in tumorigenesis and impact on CIN development.

Before starting with our investigation, we wanted to confirm that the used cell model had the attended expression of 45A ncRNA. We evaluated its expression by a real-time RT-PCR and the results confirmed that 45A cells overexpress the transcript, while Anti45A cells downregulate it [data not shown].

We performed immunofluorescence (IF) staining for tubulin and pericentrin in SKNBE2-Mock, -45A, and -Anti45A cells to detect differences in the number of the mitotic figures. After having counted cells in every mitotic phase (prophase, metaphase, anaphase, and telophase), we didn't find significant differences between 45A overexpressing or downregulating cells, suggesting that there is not an abnormality in phases population due to different 45A ncRNA expression levels. This data was also confirmed by the GIEMSA staining which allows observing the state of chromosomes. Even in this case, no phase differences due to 45A expression levels were detected. Instead, we noted differences in the morphology and organization of the mitotic spindle: we observed a significant mitotic spindle shortening in 45A cells (fig. 2A and B), measurable thanks to the presence of pericentrin that localizes to the centrosome, defining the beginning and the end of this structure; in quantitative terms, these mitotic spindles are halved with respect to the other cells. Moreover, an increase of abnormal spindles (monopolar or multipolar figures) number is observable in 45A cells compared to other cells (fig. 2C), suggesting that 45A ncRNA could be involved in CIN development. This data was confirmed also by GIEMSA staining, by which we observed an increase of lagging of chromosomes in 45A cells.

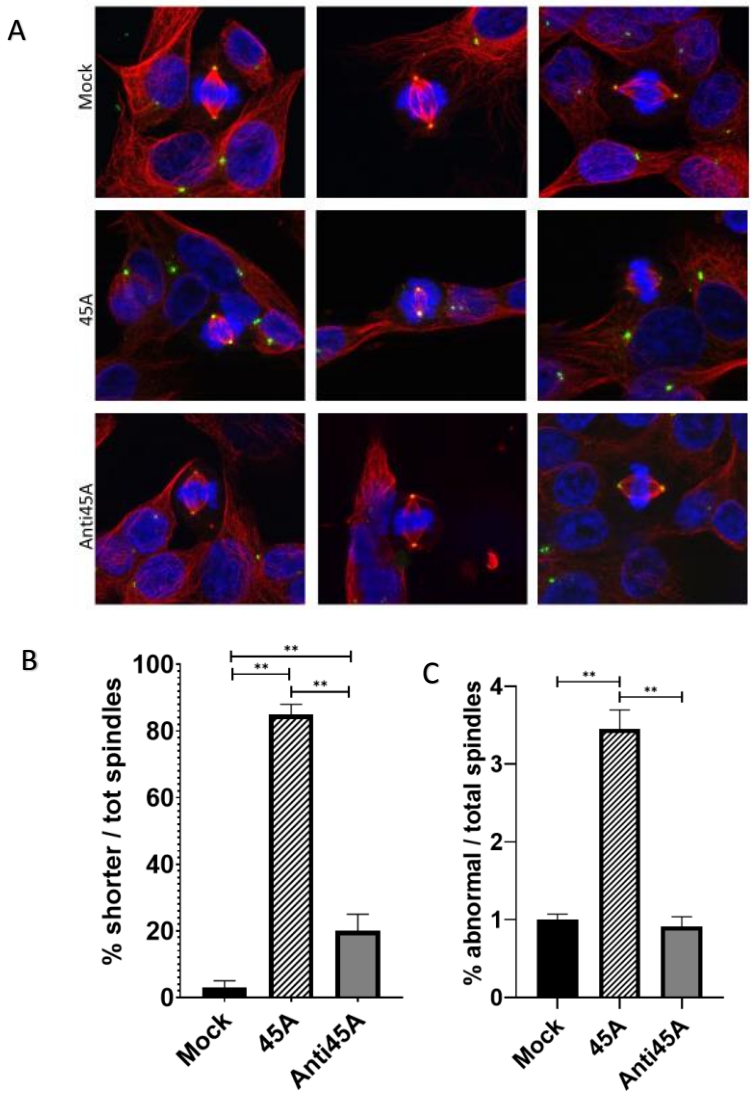


*Figure 2. Centrosome and mitotic spindle immunofluorescence, representative images (A). Blue=Hoechst, red=tubulin, green= pericentrin. Distribution of spindle mitotic shortening (B) and of abnormal spindles (C) in the studied cell model. Data represent mean ± SD, p**<0.01.*

Another observation obtained is that in Anti45A cells, the interzonal fibers are missing during anaphase (fig. 3), important elements for determining the correct cell division. Significant differences were already observed at the level of microtubule organization during cell division due to the different 45A expression levels.

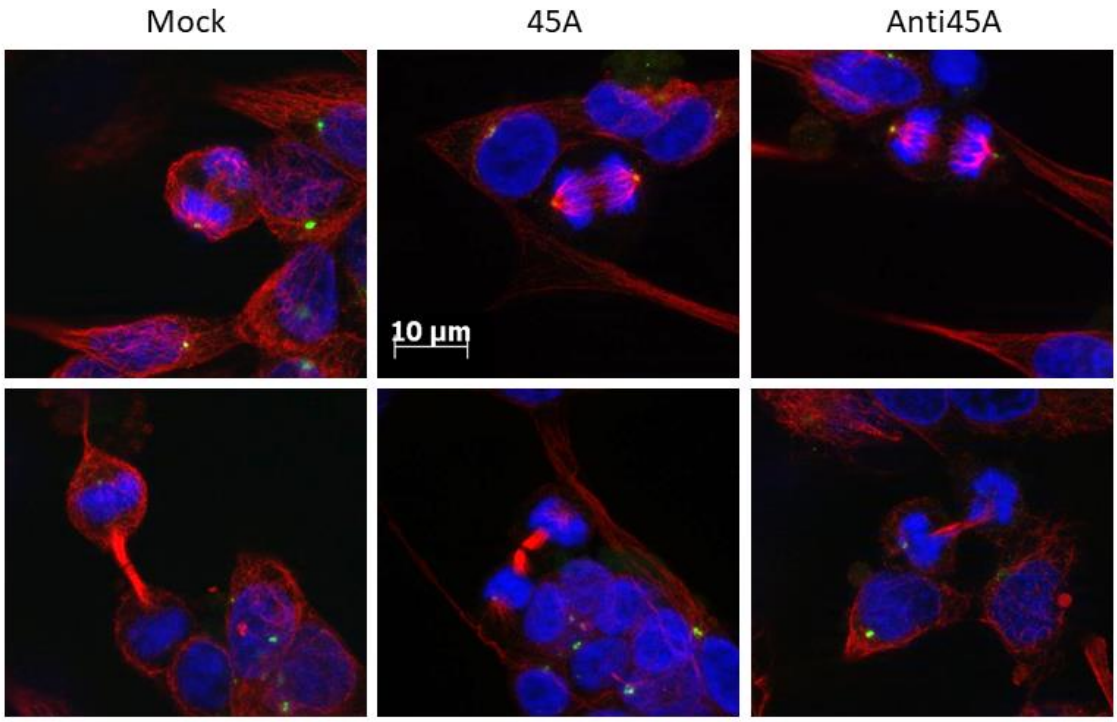
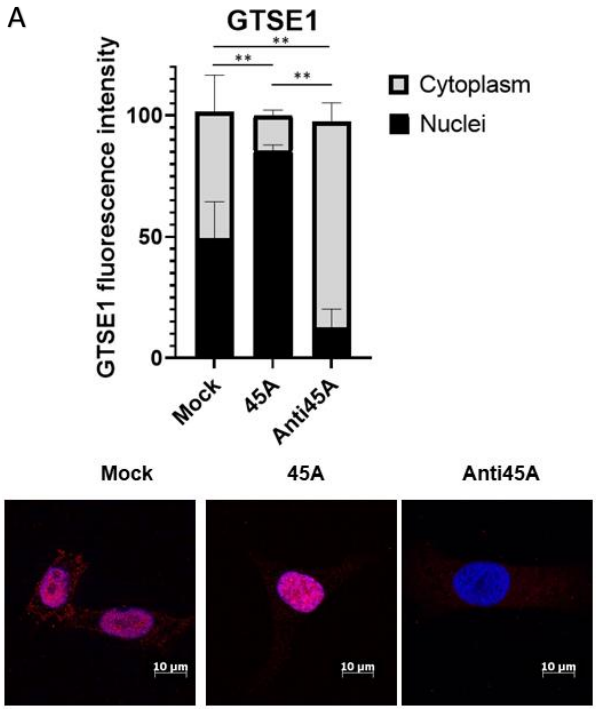


Figure 3. Representative images of anaphase (up) and telophase (bottom) in SKNBE2 - Mock, -45A, and -Anti45A cells. Blue=Hoechst, red=tubulin, green= pericentrin.

Subcellular localization of GTSE1 and p53 changes according to 45A ncRNA expression

A recent work of our laboratory demonstrated that 45A ncRNA expression directly regulates the levels of both mRNA and synthesis of GTSE1 [26]. Since it is known from the literature that its activity could be different with respect to its position inside the cells, we evaluated if 45A ncRNA could determine also a different subcellular localization of GTSE1. We performed IF analysis on SKNBE2-Mock, -45A, and -Anti45A cells to measure the intensity of GTSE1 fluorescence both in the nucleus and in the cytoplasm. Despite the expression of GTSE1 being lower in Anti45A cells, we could quantify its localization thank to a specific equation called correct total fluorescence intensity (see Materials and methods). As reported in figure 2A, in 45A cells GTSE1 is mainly localized in the nucleus and less in the cytoplasm; in the Anti45A, on the contrary, it is prevalent in the cytoplasm and little present in the nucleus. In control cells, GTSE1 is present in both subcellular compartments in an equal way.

Given the role of GTSE1 as a regulator of p53 both at the transcriptional and localization level, we hypothesized that its displacement between the nucleus and the cytoplasm could determine changes also in p53 shuttling. To verify this, we labelled p53 and quantified its presence in the nucleus and in the cytoplasm in Mock, 45A, and Anti45A cells. The results show that the presence of p53 in the cytoplasm is more marked in Anti45A, while in Mock and 45A cells the amount of protein is higher in the nucleus (fig. 2B). Given that the role of GTSE1 in binding and relocating p53 is already known in the literature [59], it is therefore significant that the shuttling of both GTSE1 and p53 in cytoplasm occurs only in 45A down-regulating cells.



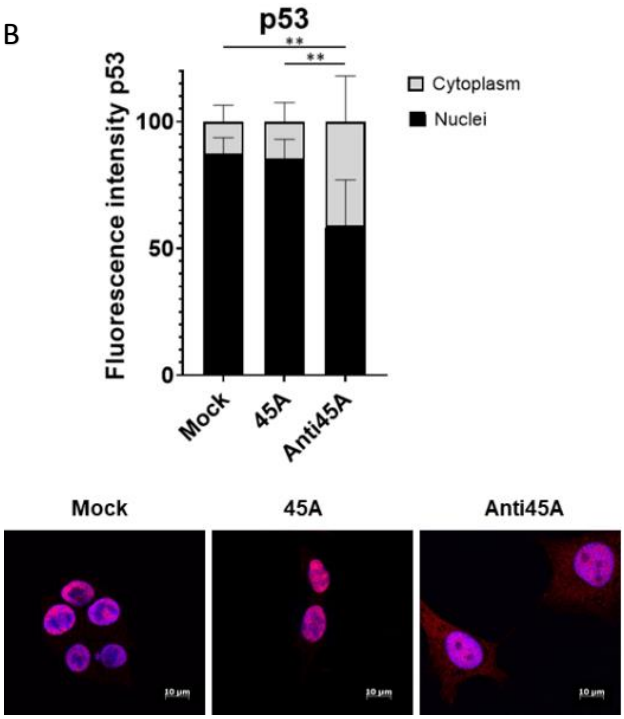


*Figure 4. Quantification of GTSE1 (A) and p53 (B) immunoreactivity both in nucleus and cytoplasm in SKNBE2 Mock, 45A and Anti45A cells. Blue=Hoechst, red=GTSE1 (A) or p53 (B). Data represent mean ± SD, p**<0.01.*

MCAK is not affected by 45A ncRNA expression

Since 45A ncRNA regulates GTSE1 expression level and subcellular localization and, in turn, this controls the MCAK depolymerization activity [31], the next step was to clarify the role of 45A in the microtubule regulation mechanism, by studying the link between the expression of GTSE1 and MCAK and the levels of 45A. We performed IF analysis on SKNBE2 Mock, 45A, and Anti45A cells, labelled both GTSE1 and MCAK, and we observed the presence of these proteins during interphase and mitosis.

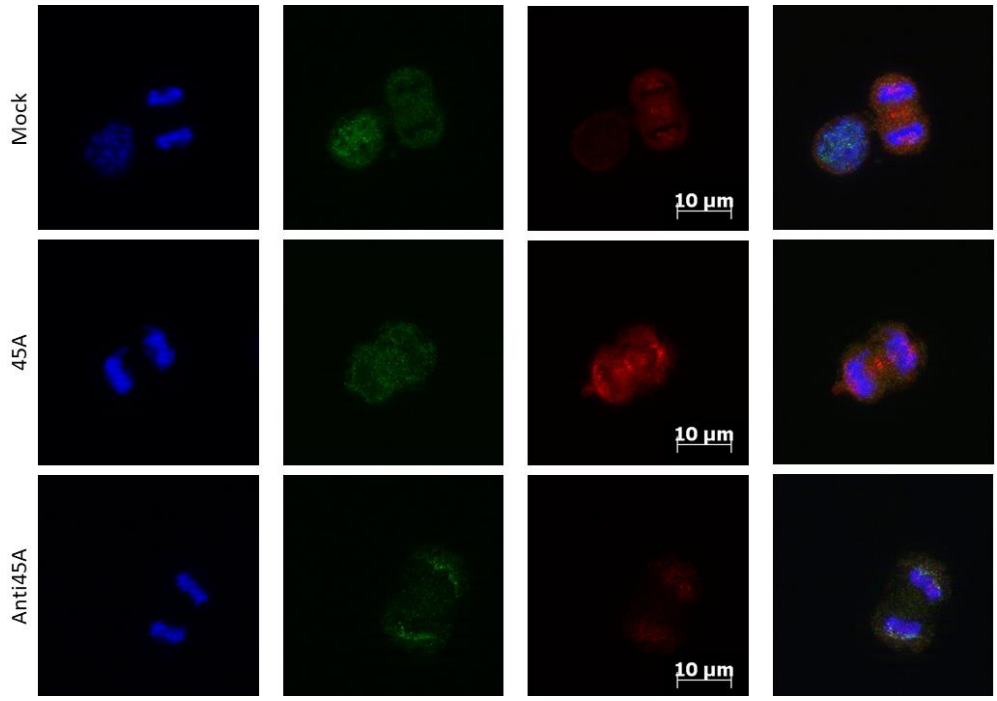


Figure 5. GTSE1 and MCAK immunofluorescence, representative images. Blue= Hoechst, green= GTSE1, red= MCAK.

The levels of 45A ncRNA seem not to affect the expression of MCAK during the early stages of mitosis (data not shown). During anaphase and telophase, on the other hand, the expression of MCAK appears lower in 45A down-regulating cells (fig. 5). To confirm this hypothesis, we performed real-time RT-PCR and WB analysis to quantify MCAK mRNA and protein in our model. As shown in figure 5, there is not a statistically different modulation of expression due to 45A ncRNA expression levels, suggesting that GTSE1 may act binding MCAK and regulating its activity at the post-translational level. These data confirmed that MCAK does not represent a molecular prognostic marker in NB, but it could represent a therapeutic target.

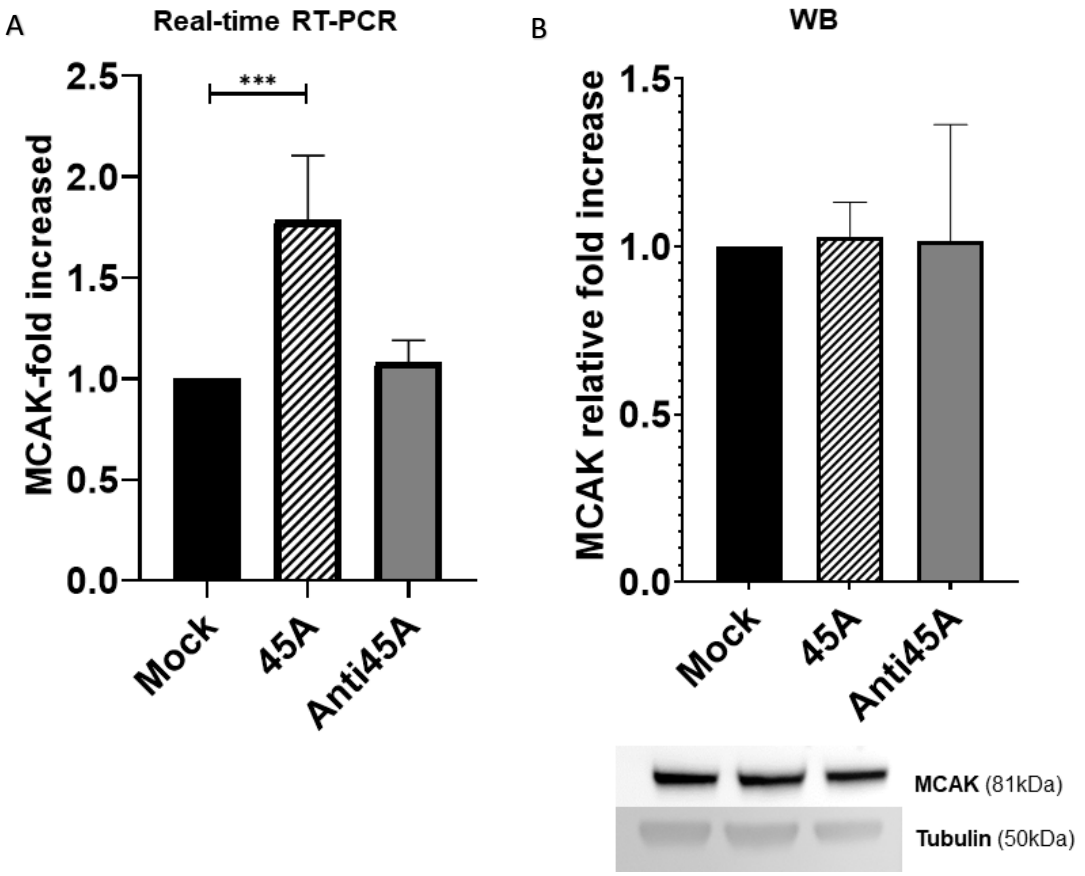


*Figure 6. MCAK real-time RT-PCR (A) and western blotting (B) analysis of Mock, 45A, and Anti45A cells. Data represent mean ± SD. ***p<0.001.*

Downregulation of 45A affects Aurora B expression

After having demonstrated the tuned regulation of GTSE1, p53, and MCAK by 45A ncRNA levels, we hypothesized that the expression of this ncRNA might modulate another important protein involved in chromosome segregation control, Aurora B. To test this hypothesis, we analyzed the different localization of this protein during mitosis phases in SKNBE2 Mock, 45A, and anti45A by IF staining.

Aurora B kinase is the catalytic subunit of the chromosome passenger complex (CPC), which is found at the chromosome arms, the inner centromere, and the midzone. The CPC regulates many events in mitosis, including chromosome congression, kinetochore-microtubule attachments, spindle checkpoint control, and chromosome segregation. In particular, CPC is localized in chromosomes arms at the level of the centromeres in prophase. Instead, during anaphase and telophase CPC, and Aurora B, are found at the midzone [60].

Our results demonstrated that both in Mock and 45A cells Aurora B is expressed correctly in all mitosis phases, while in Anti45A cells the protein immunoreactivity

was practically absent during anaphase, but then reappeared in telophase (fig. 5). This can be explained because Aurora B can resume its activity thanks to the change in the balance between dynamic/stable microtubules, typical of these phases [61].

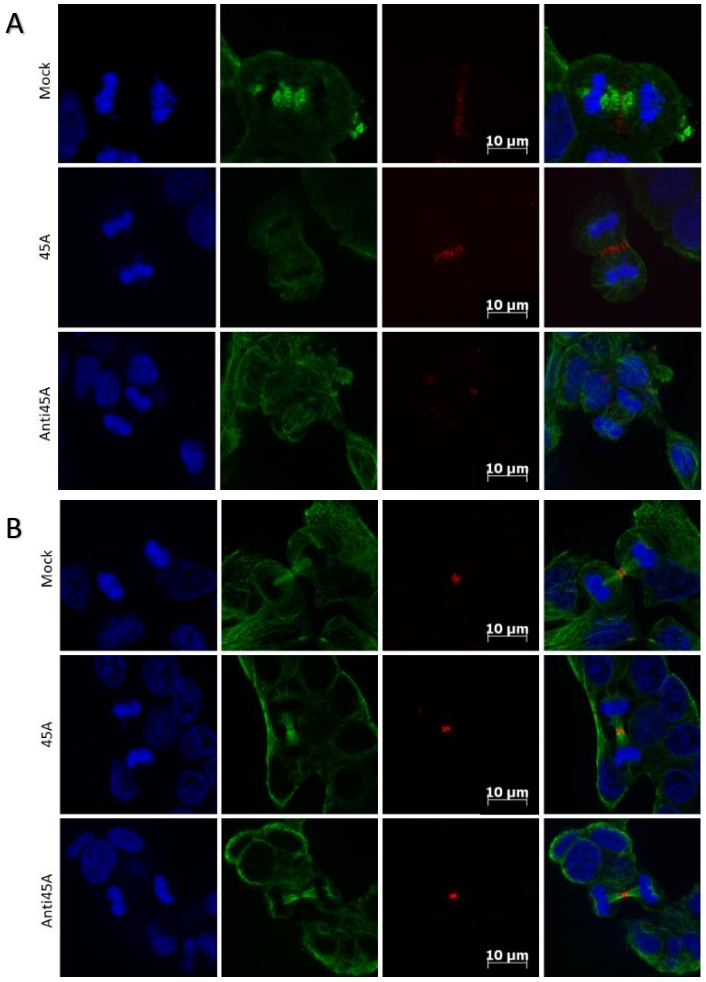


Figure 7. Cytoskeleton and Aurora B immunofluorescence, representative images of anaphase (A) and telophase (B). Blue= Hoechst, green= tubulin, red= Aurora B.

In order to confirm these observations, we measured by Real-Time RT-PCR and Western Blot the expression level of Aurora B transcript and protein. At the transcriptional level, AurB appears to be overexpressed in 45A cells, while in Anti45A cells no significant differences with respect to the control cells were shown. Instead, the quantification of protein levels demonstrated that AurB is significantly down-regulated in Anti45A cells, as we observed from IF analysis (fig. 8).

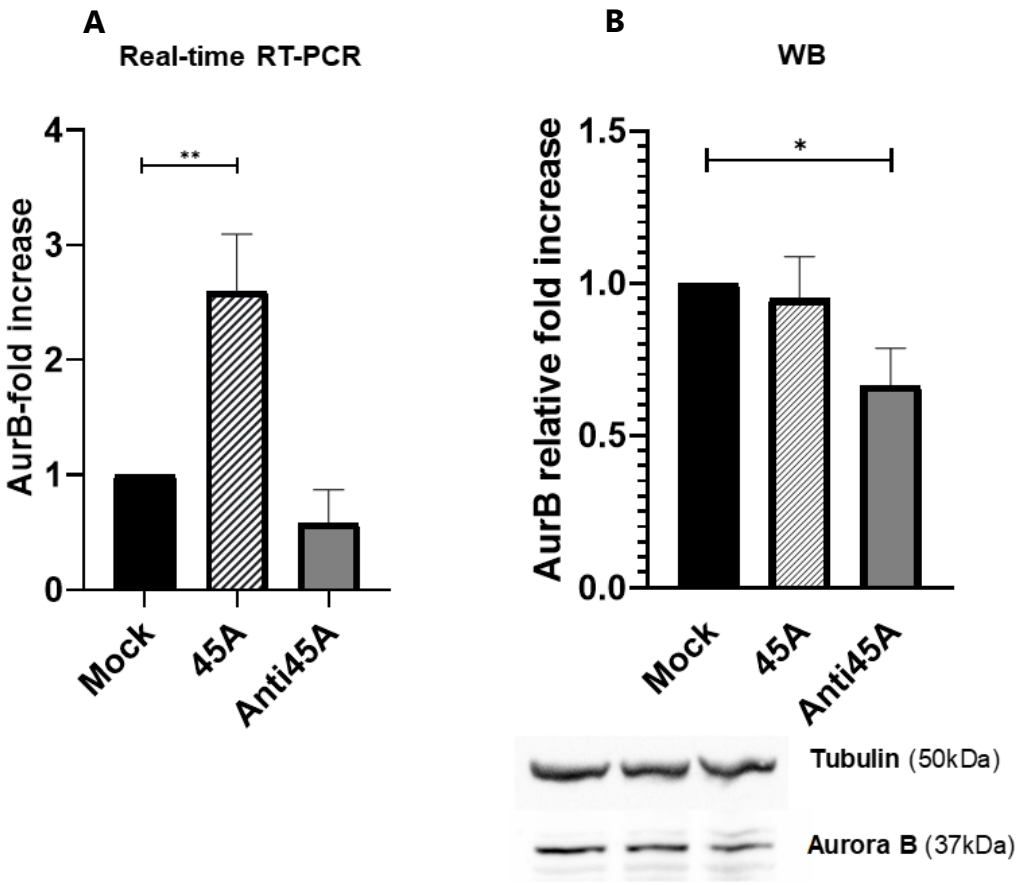


*Figure 8. Aurora B Real-time RT-PCR (A) and Western blotting (B) analysis of SKNBE2 Mock, 45A and Anti45A cells. Equal loading of proteins was ensured by tubulin expression. Data represent mean ± SD, p**<0.01.*

Nucleolar EGFR in 45A cells

Since we discovered that 45A ncRNA is derived from intron 1 of APBB2 protein, another important point that we would like to highlight is the regulation between 45A ncRNA and the pathway described above. In particular, we know that the expression of 45A leads to alternative protein variants of APBB2, modifying their localization to the nuclei. We hypothesize that this phenomenon might perturb the expression levels of p53 and this, consequently, affects GTSE1 expression.

We studied all the possible interactions between APBB2 and other proteins described *in silico* up to date. We considered EGFR because of its ability to shuttle between nucleus and cytoplasm, such as APBB2. Indeed, EGFR is a transmembrane receptor but it can translocate to the nucleus and acts as a transcriptional co-activator for seven cancer-promoting genes: cyclin D1, nitric oxide synthase (NOS), MYBL2 (B-Myb), Aurora Kinase A (AurA), cyclooxygenase-2 (COX-2), c-MYC, and Breast Cancer Resistance Protein (BCRP) [62].

In order to examine the role of EGFR in our cellular model, we performed real-time qPCR and western blotting analysis of Mock, 45A, and Anti45A cells.

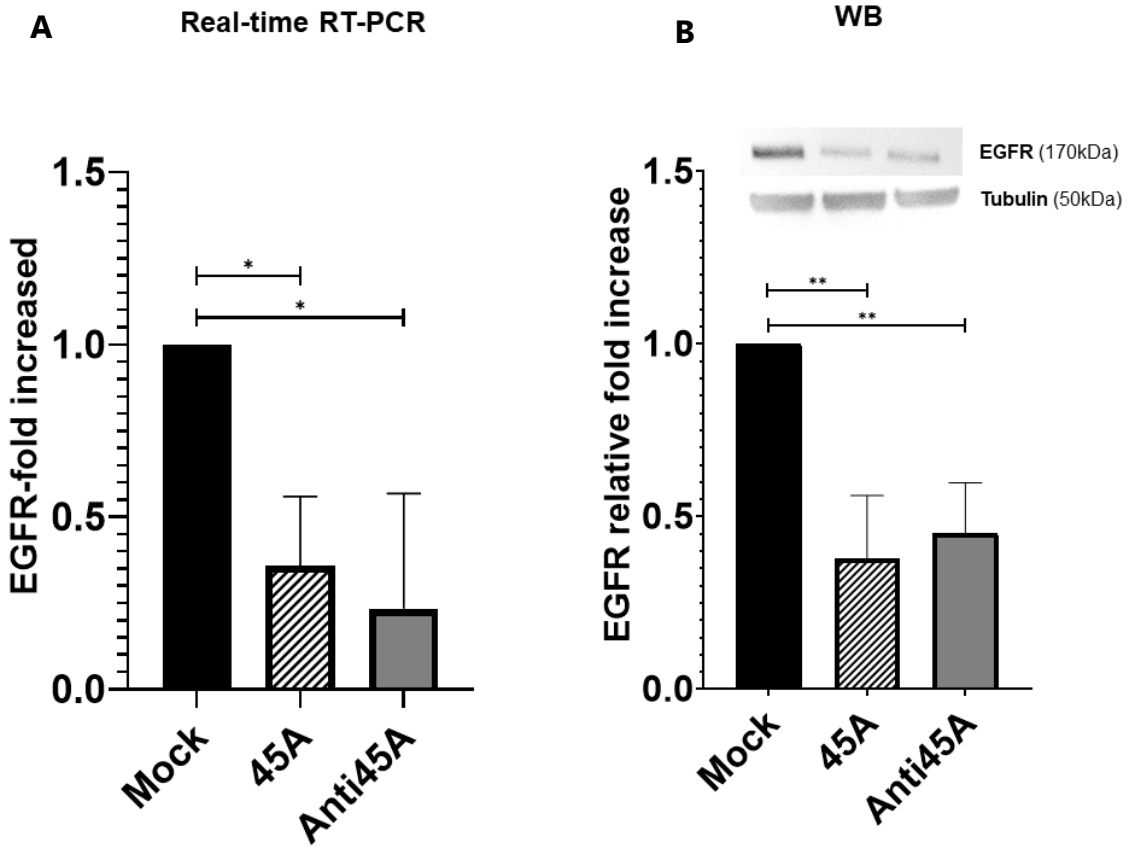


Figure 9. EGFR real-time RT-PCR (A) and western blotting (B) analysis. Data represent mean ± SD, p<0.05, p**<0.01.*

As reported in figure 9, we observed a significant reduction of both EGFR transcript and protein levels in 45A over-expressing and down-regulating cells. As reported in the literature, in NB EGFR is not considered a suitable therapeutic target because its expression is generally down-regulated. In this cellular model, EGFR is significantly downregulated with respect to control cells, confirming that this protein could not represent a strategical target to improve NB therapies.

Since we hypothesize that EGFR could participate in p53 and GTSE1 regulation, we investigated its subcellular localization performing IF analysis to disclose its possible correlation with this pathway.

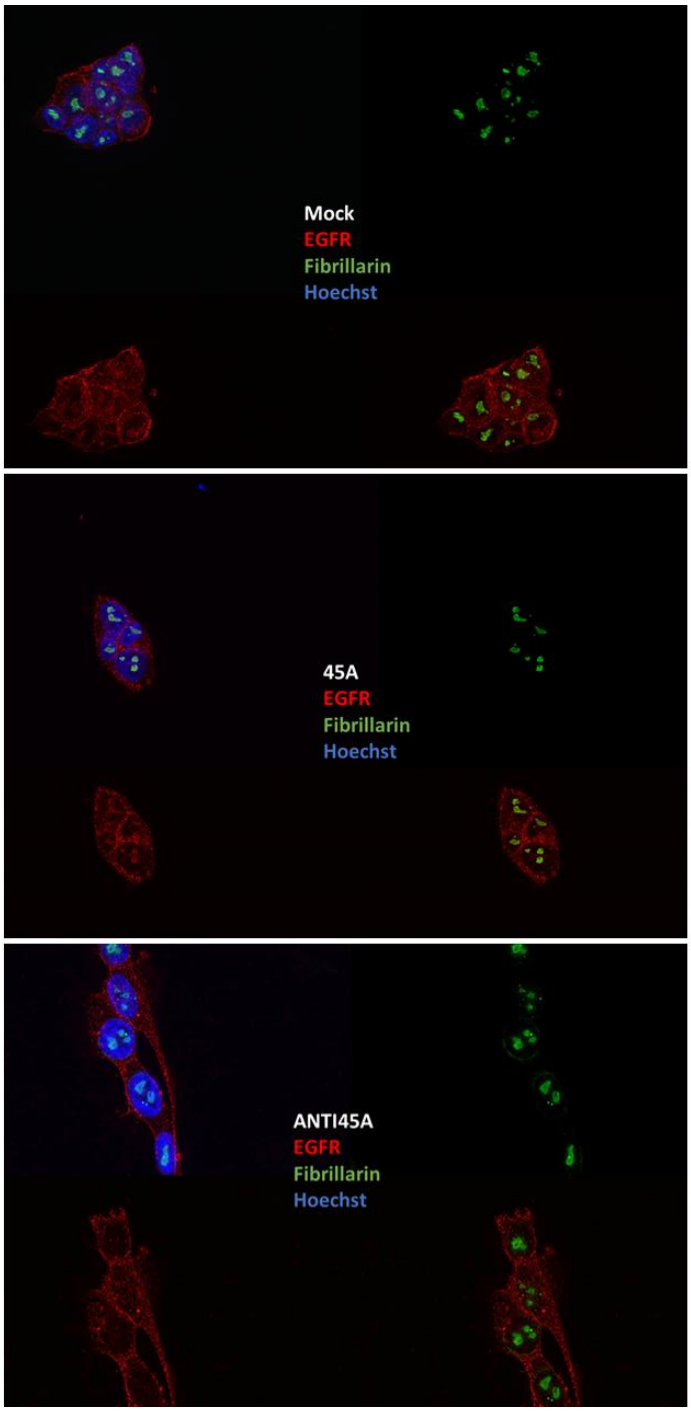


Figure 10. IF analysis of SKNBE2 Mock, 45A and anti45A cells. 63x. Blue= Hoechst, red= EGFR, green= fibrillarin.

We observed a different localization of EGFR in Mock, 45A, and Anti45A. In particular, in Mock e Anti45A cells, EGFR is present at the cellular membrane, as expected. In 45A over-expressing cells, we noted that EGFR localization was similar to the other cells, but it was also concentrated in the nucleolus, as confirmed by co-localization with fibrillarin, a typical nucleolar marker. Instead, in Anti45A this situation is not observable, confirming once again the importance of 45A ncRNA in regulating different pathways. However, we still don't know why

EGFR translocates to the nucleolus. In literature, it is known that EGFR can be sequestrated in the nucleolus, losing its activity as a transcription co-factor, in some tumors, such as non-small cell lung cancer [62].

To confirm the observed different localization of EGFR, we performed WB analysis on nuclear proteins extract of Mock, 45A, and Anti45A cells. In this experiment, the cytoplasmic contamination (resulting from the experimental procedure) was considered thanks to the tubulin quantification, and removed from the results obtained. As reported in figure 11, EGFR quantification is higher in 45A nuclear extraction, and this result is statistically significant with respect to Mock and Anti45A cells. Interestingly, EGFR was totally not detectable in Anti45A cells.

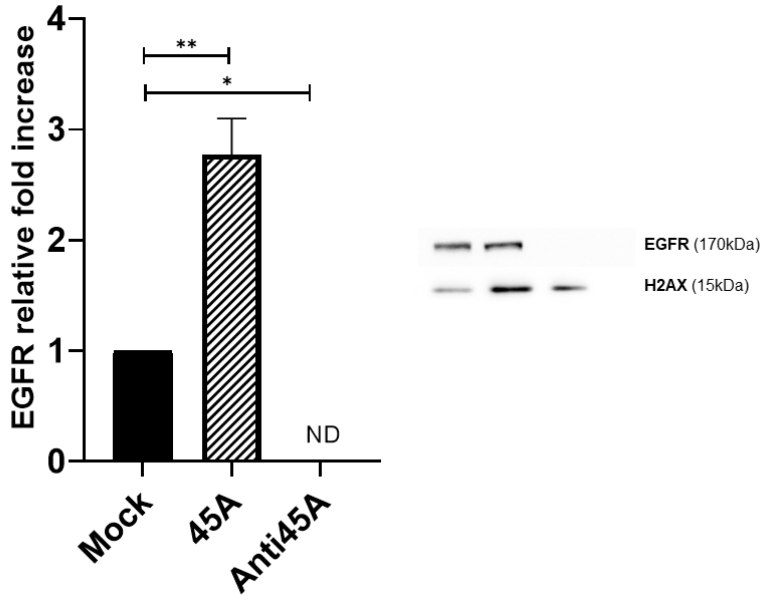


*Figure 11. WB analysis of nuclear extraction of SKNBE2 Mock, 45A and Anti45A cells. Data represent mean ± SD. *p<0.05, **p<0.01.*

Given the activity of nuclear EGFR as a transcription co-factor, as reported above, we wanted to understand how two of the genes co-activated from this protein were regulated in our cell model. We measured Aurora A and c-MYC mRNA levels in SKNBE2 Mock, 45A, and Anti45A cells.

We noticed that where EGFR is present in the nucleus, or better nucleolus, both Aurora A and c-MYC were up-regulated, while in anti45A c-MYC was not detectable (figure 12). This mechanism was not attended, because the nucleolar sequestration of EGFR should inactivate its role as a transcription co-factor, while from this experiment we can assume that it still has this activity.

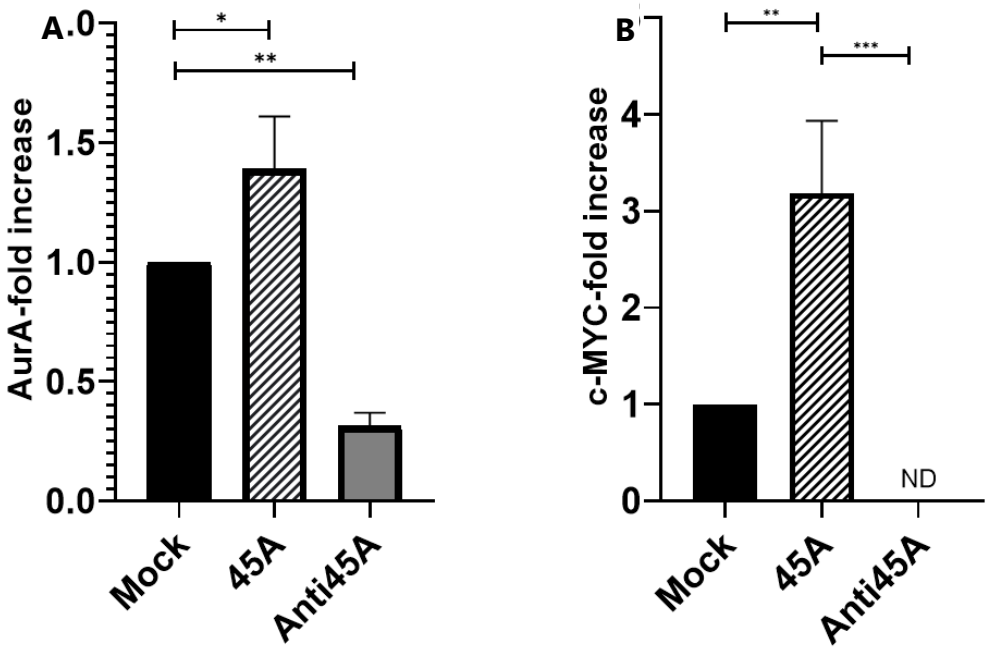


*Figure 12. Real-time PCR analysis to evaluate Aurora A (A) and c-MYC (B) expression levels in mock, 45A, and anti45A cells. Data represent mean ± SD. *p<0.05, **p<0.01.*

Evaluation of GTSE1 and AurB expression in NB patients

The pathway regulated by 45A ncRNA identified GTSE1 as a key protein in NB CIN development and, in turn, it can control AurB activity. In order to consolidate the data obtained *in vitro*, we decided to evaluate the expression of these two genes in NB patients.

We performed a univariate survival analysis using one publicly available gene expression dataset relative to 88 NB patients, called "R2: Genomics Analysis and Visualization Platform" [63]. We found that both GTSE1 and AurB expression display a significant prognostic value as far as overall survival ($p^{**} < 0.01$) (fig. 12A). A higher expression of these genes was associated with a higher risk of succumbing to the disease (fig. 12B) because they resulted in more expressed in NB patients with high-risk NB (stage 4). Moreover, we analysed also MCAK expression in this dataset: the data showed that this gene is not differentially regulated according to NB stages, suggesting that the results that we obtained *in vitro* could recapitulate the conditions present *in vivo*. Altogether, these results highlighted that GTSE1 and AurB are novel prognostic markers for NB, and we are proposing their use to improve prognosis in these patients.

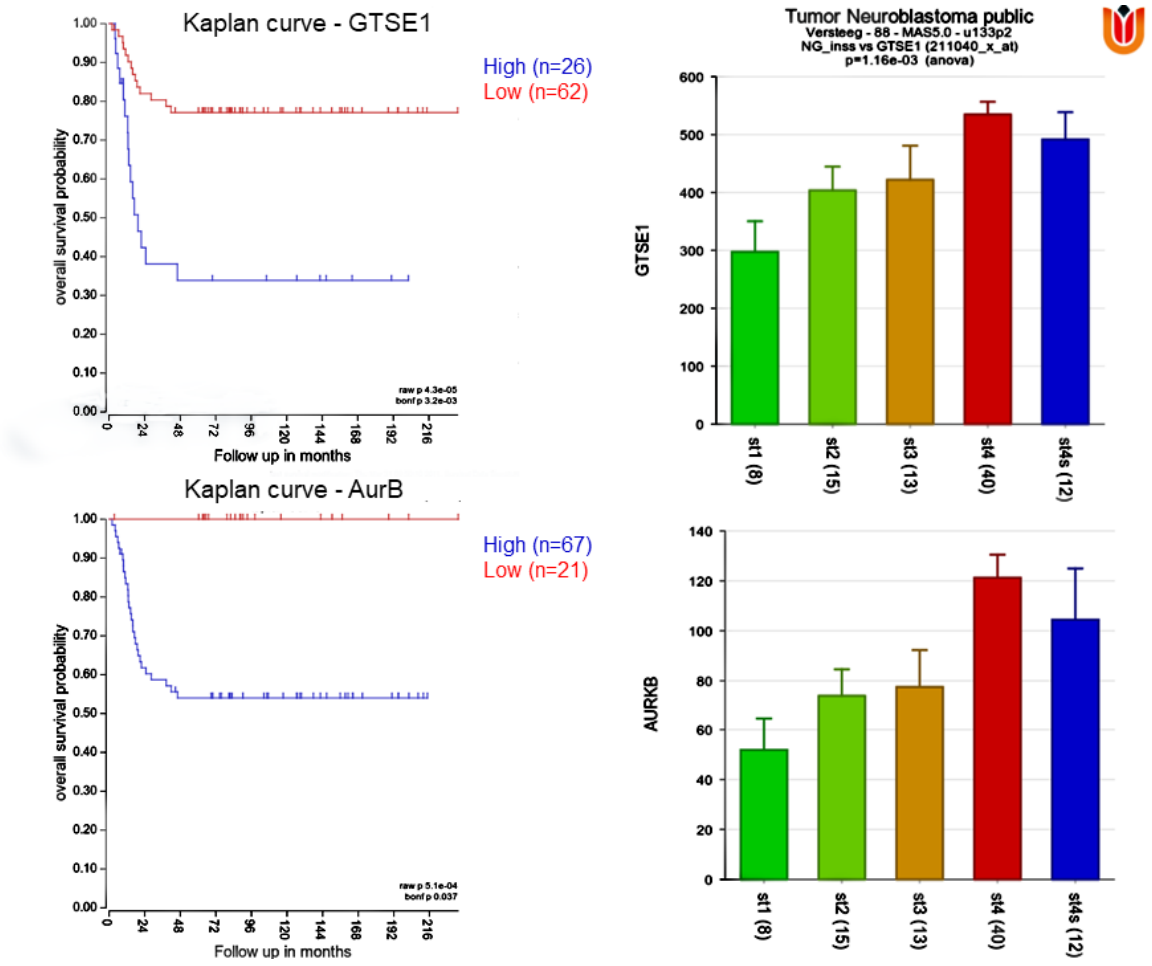


Figure 13. The panel shows the Kaplan Meier for 88 NB patients divided into two clusters (A). On the top GTSE1 curve is represented, while on the bottom AurB one. Curves are relative to the patient's overall survival expressed in years. Blue and red curves represent good and poor prognosis patients, respectively. (B) Expression of GTSE1 (top) and AurB (bottom) in patients divided into NB stages. Images are taken by R2 Neuroblastoma dataset.

Discussion

Up to this point, we highlight the important role of 45A ncRNA in controlling cytoskeleton dynamics during mitosis. Mitotic cells must precisely regulate the dynamics of their spindle microtubules, particularly those that are attached to chromosomes via kinetochores. Kinetochore microtubules must be stable enough to capture chromosomes and align them at the equator of the spindle, yet not so stable that attachment errors cannot be corrected. Cancer cells often have hyper-stable kinetochore microtubules, leading to an increase in attachment errors and chromosome missegregation. This, in turn, causes cancer cells to frequently gain or lose chromosomes, a phenomenon known as chromosome instability (CIN) [58].

The different expression of 45A ncRNA controls the onset of CIN in NB cells, due to its ability to influence proteins fundamental for the control of microtubules during cell division. In particular, a previous work of our laboratory demonstrated that GTSE1 synthesis is directly regulated by 45A ncRNA. Moreover, different 45A expression affects also the localization of GTSE1: indeed, we observed that 45A-overexpressing cells accumulated GTSE1 in nuclei, while in Anti45A it localizes also at the cytoplasmic level. GTSE1 is known to regulate p53 at the transcription and localization level [64]. Therefore, in Anti45A the GTSE1 displacement determines p53 shuttling in the cytoplasm, where it is degraded, and its pro-apoptotic activity is avoided, justifying the observed resistance of 45A-downregulating cells to spindle poisons. Actually, this drug resistance could be explained also by the observed reduction of AurB protein levels that we found out in 45A-downregulating cells [48].
Indeed, we observed that a reduced expression of 45A ncRNA leads to the decrease of the Aurora B levels. In Anti45A cells, we observed that Aurora B is missed during the anaphase, probably due to the absence of regulation by GTSE1, since it is down-regulated in these cells. Despite this, Aurora B returns to be expressed during telophase because the changes between the stable/dynamic microtubule ratio can restore its activity [54].

Since it is known the role of GTSE1 in regulating MCAK, we analysed the expression of these two proteins in 45A-cell model. MCAK mRNA and protein levels did not appear regulated by different expressions of 45A ncRNA and, in turn, of GTSE1. The precise regulatory mechanism of MCAK is still largely

unknown. It was proposed that the inhibition of MCAK activity depends on its phosphorylation, and several phosphorylation sites have been identified in its aminoacidic sequence, but how this event controls MCAK regulation is to demonstrate. Bendre et al [31] observed that depletion of GTSE1 in cells enhances the depolymerization activity of MCAK, leading to defects in mitotic spindles organization and confirming the regulatory role of MCAK by GTSE1. With our experiments, we demonstrated that different levels of GTSE1 (driven by 45A ncRNA expression levels) did not affect the synthesis of MCAK, suggesting that the regulation proposed by Bendre et al might be at a post-translational level [31].

GTSE1 and AurB are two proteins that appear to be fine regulated by 45A ncRNA. Since we characterized this mechanism *in vitro*, we proposed to determine if it could be found also *in vivo*. We evaluated their expression in NB patients thanks to an analysis of a dataset containing data from 88 different patients. We discovered that both GTSE1 and AurB expression reveal a signature for low survival prognosis, thus strengthening the validity of this model. Furthermore, the analysis showed the association between these genes' expression and NB stages, suggesting their possible role as new prognostic markers.

Finally, 45A ncRNA participates also in the regulation of the EGFR pathway: we observed that in 45A over-expressing cells EGFR is present not only on the cellular membrane but also inside the nucleus. In particular, we found out that it localizes in the nucleolus, while in 45A down-regulating cells it remains on the cellular membrane. The presence of EGFR at nuclear level have been observed in different cancer types, such as breast cancer, ovarian cancer, and oropharyngeal and esophageal squamous cell carcinomas. This observation generally correlates with bad prognosis and resistance to various cancer therapies. Moreover, in literature nucleolar sequestration is described as an inhibitory mechanism, since the transcription activity of EGFR is generally lost. However, in our cellular model it seems to have a different role. Indeed, two of the genes of which EGFR is a transcription co-activator are overexpressed in 45A cells, right where it is localized in the nucleolus. Surely, we will explore better this pathway in the future.

In conclusion, the differences we observed between 45A and Anti45A cells demonstrate the importance of this ncRNA in regulating the activity of proteins

involved in the cytoskeleton organization. In this scenario, therefore, the expression level of 45A ncRNA could become prognostic for CIN in different types of cancer and predict drugs response.

Future perspectives

In this work, we characterized the role of 45A ncRNA in association with several proteins involved in microtubules organization. Our data suggest that this non-coding RNA could be fundamental to understand CIN in cancer cells, especially in neuroblastoma. In turn, this important role could be the key to propose novel pharmaceutical treatments for cancer patients. However, different aspects of this project should be consider to consolidate these data. Since we did not demonstrate how 45A ncRNA mechanistically affects microtubules organisation and CIN, it would be important to study the subcellular localization of this ncRNA and evaluate its own activity. This lack of information could be fill with several experiments: first, fluorescence in situ hybridization technique should be used to localize this transcript. Based on the subcellular localisation, different hypothesis could be explored to understand its activity. Revealing the mechanism of action by which 45A ncRNA may act could be relevant to discover its possible targets or interactors, disclosing its regulatory network. This means that a deeper understanding of its specific role in GTSE1 regulation could consolidate the model that we proposed and simplify its use in the prognosis field.

In the future, it could be interesting to evaluate the expression of 45A ncRNA in biopsies from NB patients, to correlate its levels with stages of tumors and/or overall survivance. In addition, the same analysis could be performed also with blood samples from patients, with the final goal of correlating its expression with tumor malignancy. This evaluation could be performed by searching for free RNA in blood or analysing the presence of 45A ncRNA inside extracellular vesicles (EVs) since it is a short ncRNA and a good candidate to be found in EVs.

Finally, we could suppose that this transcript could be important in different kinds of tumors, and this association should be very useful in the diagnostic field for cancer treatment.

Collaborations

During these years, under the supervision of my tutor, I started some collaborations in parallel whit 45A ncRNA project.

1) I participated in a new project to evaluate GTSE1, MCM2, and CA9 expression levels in melanoma, breast cancer, and osteosarcoma samples derived from dogs in collaboration with dr. Paola Modesto (Istituto Zooprofilattico Sperimentale). MCM2 and CA9 are two genes of which implication in tumorigenesis and therapy is studied in our laboratory. We aim to improve both prognosis and therapy in canine cancers and to obtain a new model of study to strengthen our data. We propose two drugs that are inhibitors of MCM2 and CA9 in association with carboplatin as a new therapeutical approach. I evaluated the efficacy of this combo-therapy with the xCELLigence RTCA system.
2) Evaluation of novel molecules that are selective inhibitors of CA9. These molecules are synthesized by the lab of prof. Supuran, from the University of Florence. I collaborated with Dr. Elisabetta Palamà to set up a new protocol by which we generate NB spheroids to test the action of these different molecules.
3) I also performed real-time RT-PCR analysis of drug transporters genes for dr. Sanja Aveic. In particular, we wanted to evaluate if the overexpression of Lin28B, an RNA-binding protein, could regulate the expression of these genes in neuroblastoma. The results suggested us a new possible target for NB treatment, a drug transporter called ABCB5.
4) Development of a novel real-time RT-PCR protocol to detect even traces of *Peganum harmala* DNA presence in food supplement.
5) Toxicity evaluation of several microalgae located in Ligurian Sea.

Scientific production

I am a contributor in the following papers:

- Brizzolara A., Garbati P., Vella S., **Calderoni M**., Quattrone A., Tonini G., Capasso M., Longo L., Barbieri R., Florio T., Pagano A. "Co-Administration of Fendiline Hydrochloride Enhances Chemotherapeutic Efficacy of Cisplatin in Neuroblastoma Treatment", Molecules 2020.
- Garbati P., Barbieri R., Cangelosi D., Zanon C., Costa D., Eva A., Thellung S., **Calderoni M**., Baldini F., Tonini G., Modesto P., Florio T., Pagano A. "MCM2 and Carbonic Anhydrase 9 Are Novel Potential Targets for Neuroblastoma Pharmacological Treatment", Biomedicines 2020.
- **Calderoni M**., Altare M., Mastracci L., Grillo F., Cornara L., Pagano A. "Potential Risks of Plant Constituents in Dietary Supplements: Qualitative and Quantitative Analysis of Peganum harmala Seeds", Molecules 2021.
- Baldini F.*, **Calderoni M**.*, Vergani L., Modesto P., Florio T., Pagano A. "An Overview of Long Non-Coding (lnc)RNAs in Neuroblastoma", Int. J. Mol. Sci. 2021 - * First co-authors.
- Garbati P., Barbieri R., **Calderoni M**., Baldini F., Nizzari M., Modesto P., Florio T., Pagano A. "Efficacy of a Three Drug-Based Therapy for Neuroblastoma in Mice", Int. J. Mol. Sci. 2021.
- 45A ncRNA regulates chromosomal instability onset in neuroblastoma (in preparation)
- EGFR nucleolar sequestration driven by 45A ncRNA expression is prognostic in neuroblastoma (in preparation)

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