Physical and functional interaction between MNT and CCDC6

Author – **Sandra Lastra Aristín**

Director - **Dr. Javier León Serrano/ Dra. M. Dolores Delgado Villar**

Máster Interuniversitario de Biología Molecular y Biomedicina

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ABBREVIATIONS

aa	Amino acids
BSA	Bovine Serum Albumin
bHLH-LZ	Basic-helix-loop-helix-leucine zipper
CCDC6	Coiled Coil domain containing 6
cDNA	Complementary DNA
CML	Chronic Myelogenous Leukemia
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
FBS	Fetal bovine serum
IMDM	Iscove's Modified Dulbecco's Medium
IP	Immunoprecipitation
IR	Ionizing radiation
kDa	Kilodalton
LZ	Leucine Zipper
mA	Milliamps
MARP	Mitogen-activated protein kinase
N-terminal	Amino-terminal
O/N	Overnight
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate Buffer Saline
PEI	Polyethylenimine
Polybrene	Hexadimethrine bromide
PTC	Papillary thyroid carcinoma
rpm	Revolutions per minute
sh	Short hairpin
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
V	Volts
wt	Wild type
WB	Western blot

ABSTRACT

CCDC6 gene was first isolated fused to *RET*, forming the *PTC1* oncogene in papillary thyroid carcinomas. The gene product, termed H4(D10S170) or *CCDC6*, is a 65 kDa phosphoprotein located in both cytoplasm and nucleus, that contains a coiled coil domain for protein-protein interactions. Our preliminary results from proteomic screenings showed that CCDC6 interacts with MNT, a protein containing a basic-helix-loop-helix-leucine zipper (bHLH-LZ) protein from the MXD family. MNT is known by its role as an antagonist of the MYC oncogene in several model systems. In this project, we have confirmed the CCDC6-MNT interaction in the HEK293T cell line derived from human embryonic kidney. We aimed to delimitate the interaction domain transfecting HEK293T cells with CCDC6 and MNT mutants and performing co-immunoprecipitation assays. The results suggest that the C-terminal domain of CCDC6 and that both the LZ together and the N-terminal domains of MNT are necessary for the interaction. We have also studied the nuclear or cytoplasmatic localization of the *M*NT-CCDC6 complex in HeLa cells (derived from human cervical carcinoma), and we found that they interact mainly in the cytoplasm. In order to evaluate the effects of CCDC6-MNT on cell proliferation, we transfected HeLa and HeLa shCCDC6 cells (CCDC6 silenced) with an MNT overexpressing vector and performed clonogenic assays. Furthermore, to study the role of MNT-CCDC6 in DNA damage, a viability analysis was performed in HAP1 (derived from Chronic Myelogenous Leukemia) and HAP1 MNT KO cells treated with cisplatin and etoposide. Data suggest that MNT confers partial resistance against etoposide. Finally, we studied the effects of CCDC6 silencing on apoptosis in HAP1 MNT KO versus HAP1 control. For this purpose, we silenced CCDC6 with short-hairpin RNA (shRNA) lentiviral constructs, and the results showed that the silencing seems to play an antiapoptotic role, mostly in the absence of MNT. In summary, our work has shed light into different biochemical and biological functions of these two important oncoproteins.

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1. INTRODUCTION

1.1. MNT, a member of the MYC –MAX-MXD network

MYC (c-MYC) belongs to the human *MYC* gene family and is one of the most frequently active oncogenes in cancer. MYC protein is a transcription factor that regulates proliferation and promotes tumor formation when deregulated (1). MYC presents at the C-terminal end a basic helix-loop-helix leucine zipper (bHLH-LZ) DNA binding domain that allows heterodimerization with the same motive in MAX protein (Figure 1). This heterodimerization allows MYC to bind to the E-box sequences of the target genes (1, 2).

MAX also interacts with the proteins from the MXD family, which also present the bHLH-LZ domain. MNT is a member of the MXD family (Figure1). MNT use MAX as a cofactor for DNA binding at E-box sequences (3, 4). This heterodimer seems to perform a function of transcriptional repressor (2, 5). The repression in mediated by the co-repressors proteins Sin3A and Sin3B that are able to recruit proteins such as histone deacetylases (HDAC), involved in chromatin modification (1, 2, 5).

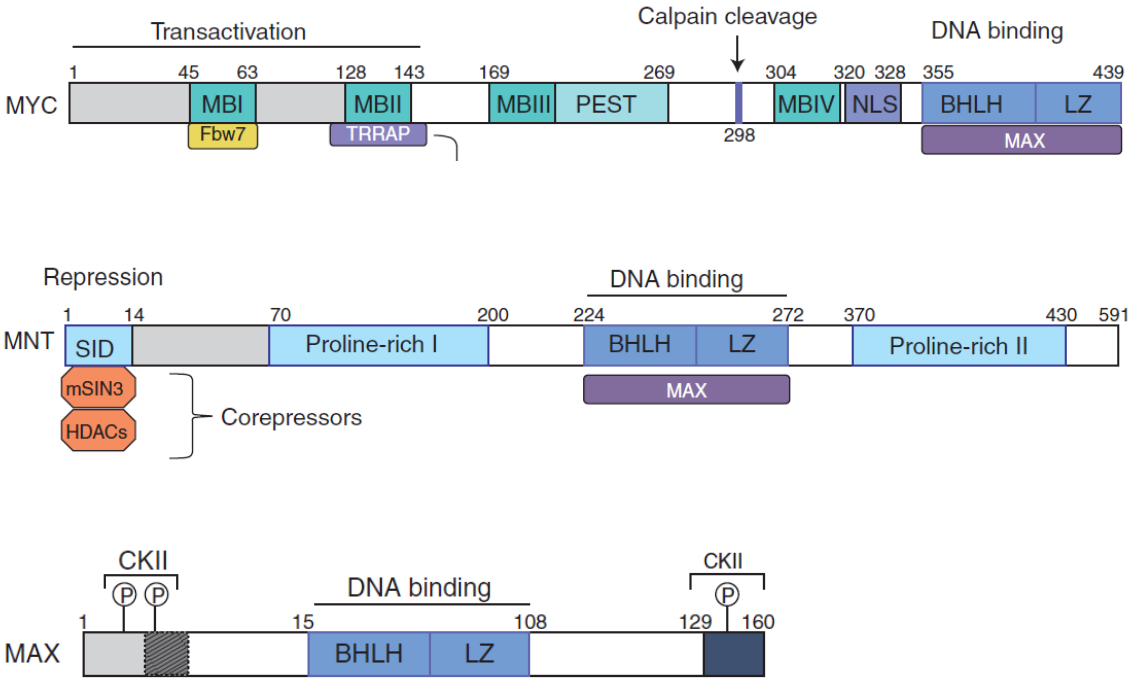


Figure 1. MYC, MNT and MAX structures. MYC-MAX and MNT-MAX interact through the bHLH-LZ domain (5).

MYC regulation is critical for the control of cell proliferation. The members of MXD family, including MNT, are considered antagonist of MYC. First, because of their competition for the available MAX to form heterodimers (MAX can also form homodimers, that could be another point of regulation). Second, for the competition of MYC-MAX and MXD-MAX dimers for the binding to the E-box of the shared target genes and finally because of their antagonist functions on cell proliferation, differentiation etc. in determined cell types (Figure 2)(2) (3) (5).

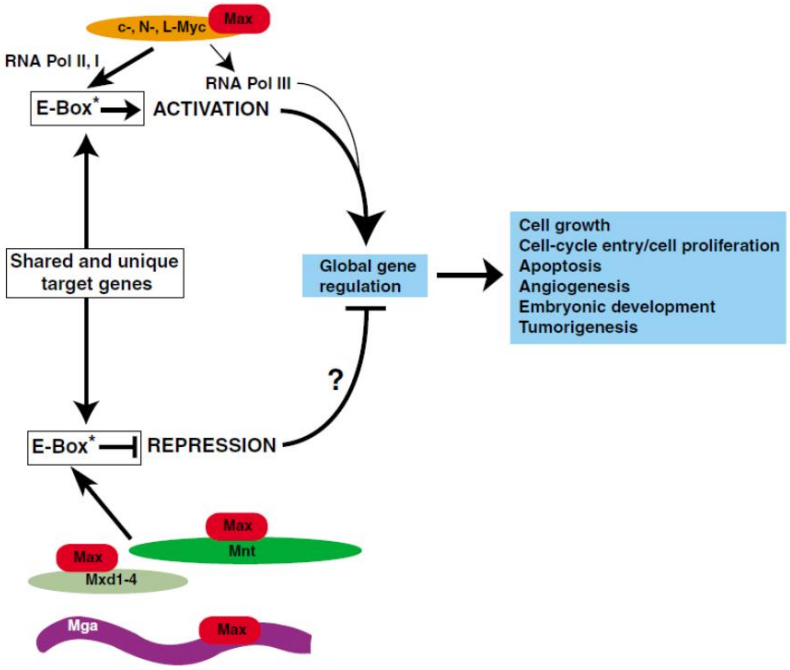


Figure 2. The figure shows the relationship between activating MYC-MAX dimers and repressive MXD-MAX complexes (2)

MNT is an important member of *MXD* family. It is localized in the 17p13.3 human chromosome, that is a region of genomic instability. Therefore, MNT function may be altered and consequently be involved in some diseases. This chromosome loss is observed in many adenocarcinomas and it has been shown that other tumor suppressor genes are also located in that region (6).

MNT is considered an essential protein due to its several roles in the cell. It is a ubiquitously expressed protein which does not fluctuate during cell cycle. MNT-MAX heterodimers are found in proliferating cells that contain MYC-MAX heterodimers, which suggests a possible important role of MNT during proliferation. MNT expression lost led to adenocarcinomas due to their accelerated entry in the cell cycle, leading to a faster proliferation and avoiding senescence (3, 6).

MNT protein contains 582 amino acids. At the N-terminal end it can be found the SID domain, involved in repression (see Figure 1). The central region of the protein contains the bHLH-LZ DNA binding domain. Previous and following this domain, there can be found high proline content regions, that are usually involved in transcriptional activation. At the C-terminal end, histidine-rich regions can be found, but their function is unknown (7).

During years, our group has been investigating on the functional interactions of the MYC-MAX-MXD network and their role in cells proliferation, differentiation and cell cycle, mainly in hematopoietic cells (for reviews see (8, 9)).

Recently, we have focused in new biological functions of MNT (Lafita, M.C. and Liaño, J Doctoral Theses). Searching for new MNT partners in collaboration with Alex von Kriegsheim's group (Systems Biology Ireland, Conway Institute, Dublin) we analyzed proteins by mass spectrometry after MNT immunoprecipitation. From the new identified MNT interacting proteins, in this work we have focused in the CCDC6 oncoprotein.

1.2. CCDC6 protein structure and functions

CCDC6 gene product is a 65 kDa nuclear and cytosolic pro-apoptotic phosphoprotein. Its amino acid sequence contains a long coiled-coil region that allows protein-protein interaction and a putative SH-3 binding domain at the carboxy-terminus that acts as an adaptor domain for regulatory mechanism (Figure 3) (10).

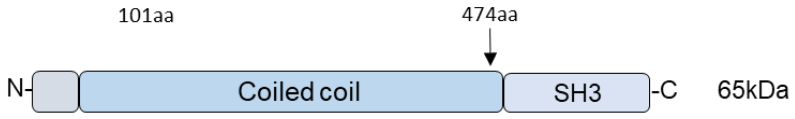


Figure 3. CCDC6 structure showing the coiled coil sequence and SH3 domain.

The CCDC6 phosphorylation occurs following serum stimulation and depends on the mitogen-activated protein kinase (MAPK) ERK1/2 activity. This phosphorylation (at Ser244 residue) is responsible of the relocation of CCDC6 from cytosol to nucleus (10). Under DNA damage conditions, induced with etoposide or ionizing radiation (IR), CCDC6 suffers ATM-mediated phosphorylation (at T434 residue) making it more nuclear and leading to apoptosis. In ATM deficient cells, this phosphorylation does not occur correctly, and the endogenous CCDC6 is

localized in the cytoplasm and excluded from the nucleus and cells do not suffer genotoxic stress-induced apoptosis (11).

CCDC6 was first isolated (with the name of H4(D10S170)) fused to the protooncogene RET, generating the RET/papillary thyroid carcinoma (PTC) oncogene (12, 13)(14). The rearrangement of the two genes derived from the paracentric inversion of the right arm of chromosome 10, that join the tyrosine kinase-encoding domain of RET (mapped at 10q11.2) to the 101 amino acids of CCDC6 gene, necessary for the activation (mapped at 10q21) (Figure 4) (10).

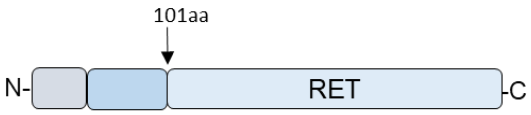


Figure 4. PTC oncoprotein (RET/papillary thyroid carcinoma) structure.

The fusion of these two genes let the idea that CCDC6 1-101 deleting mutant could interact with other proteins such as CREB1 (described as CCDC6 partner), but the mutant seems not to be enough for the interaction (10). CCDC6 is found fused to several genes (Figure 5), showing its high susceptibility for recombination. Most of these genes are oncogenes that promote proliferation (Figure 5) (15). CCDC6 alterations have been described in a variety of human cancers (16, 17) (Figure 5)

CCDC6 alteration	Tumor type	Ref.
CCDC6/RET	*Thyroid*	*1987 Nature*; 1990 Cell[1,2]
CCDC6/PDGFR	*NSCLC*	*2012 Nat Med*[29]
CCDC6/PTEN	*Colon*	*2015 Oncotarget*[34]
CCDC6/ANK3	*Leukemia (CML)*	*2000 Can Res; 2001 Blood*[38,39]
CCDC6/UBE2D1	*Thyroid*	*2005 Mutat Res*[40]
FGFR2/CCDC6	*Ovarian epithelial tumor*	*2007 Nat Rev Cancer*[41,42,44, 73,74]
CCDC6/LIPI	*Breast cancer*	
CCDC6/CTNNA3	*Breast cancer; iCCA*	
CCDC6/ROS1	*NSCLC*	
KITLG/CCDC6	*NSCLC*	
	NSCLC	
	NSCLC	
CCDC6 mut. 0,1-1%*	*Breast, Endometrium, Ovary* *Large intestine, Liver, Lung* *Pancreas, Prostate, Thyroid etc.*	*cBIOPortal.org*
CCDC6 gene expression	*Breast, Endometrium, Cervix* *Large intestine, Liver, Lung* *Pancreas, Prostate, Thyroid* *Stomach etc.*	*cBIOPortal.org*
CCDC6 protein levels are reduced in 20% of cases	*NSCLC* *SCLC* *Prostate*	*2015 Int J of Cancer*[76] *2017 Lung Cancer*[79] *2017 Oncotarget*[80]

Figure 5. CCDC6 alterations and mutation in cancer (12).

CCDC6 depletion or mutation contributes to the oncogenic activity by increasing growth rates of the affected cells, reducing apoptosis and affecting DNA damage repair (15). CCDC6 is also able to interact directly with proteins and transcription factors such as CREB1 that regulates the transcription of proteins involved in cell proliferation. CCDC6 is able to repress CREB1 transcriptional activity (18). CCDC6 is also described as a negative regulator of serine-threonine protein phosphatase 4 (PP4c) that dephosphorylates H2AX avoiding the DNA repairing process (12, 15). A summary of the different functions described for CCDC6 is shown in the Figure 6.

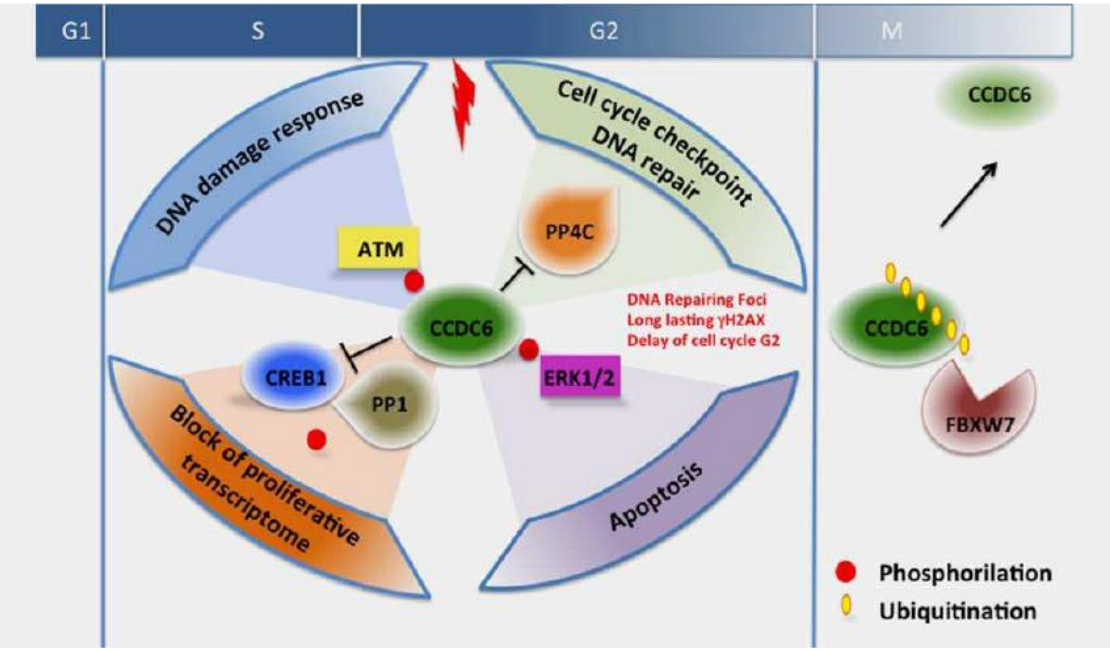


Figure 6. CCDC6 activity in cells proliferation, DNA damage and apoptosis. Following genotoxic stress, ATM activation induces CCDC6 phosphorylation and stabilization in the nucleus where it interacts with the catalytic subunit of the PP4C phosphatase that desphosphorylates H2AX, with the final result of promoting apoptotic cell death. In presence of serum CCDC6 is phosphorylated by ERK1/2 and maintained in the nucleus. CCDC6 degradation upon ubiquitilation by FBXW7 occurs before entrance in mitosis (12).

2. PROBLEM, HYPOTHESIS AND AIMS

CCDC6 and MNT are two important proteins that have been found altered in a variety of human cancers. By mass spectrometry analysis we showed, for the first time, that CCDC6 and MNT proteins could physically interact (unpublished results). On the other hand, it is thought that CCDC6 and MNT could be located in both nucleus and cytoplasm and play different roles in the control of cell proliferation, DNA damage and apoptosis.

Our Hypothesis is that CCDC6 and MNT form complexes in the nucleus and/or the cytoplasm, and these complexes can regulate relevant cellular processes such as cell proliferation or DNA damage response.

Taking this information in account, the aims of this project were:

1. Confirm MNT-CCDC6 interaction and delimitate the interaction domains
2. Study the nuclear or cytoplasmatic localization of CCDC6-MNT complex
3. Evaluate the effects of CCDC6-MNT on cell proliferation and DNA damage

3. MATERIALS AND METHODS

3.1. CELL CULTURE

3.1.1. Cell line maintenance

Cell lines used in this study are shown in Table 1. Cells were grown in either IMDM (Iscove's Modified Dulbecco's Medium) or DMEM (Dulbecco's Modified Eagle Medium) media (Lonza) supplemented with 10% FBS (Fetal Bovine Serum) (Gibco-Life technologies), and with antibiotics, 80 µg/ml of gentamicin (Laboratories Normon) and 2 µg/ml of ciprofloxacin (Sigma-Aldrich). Cells were maintained below 80% confluency in a humidified atmosphere at 37ºC and 5% CO_2. In order to maintain the confluence cells were sub-cultured: cell culture media was aspired, then the plate was washed with 1X PBS (Phosphate Buffered Saline) and the cells were detached using Trypsin-EDTA (Sigma-Aldrich) and re-cultured.

Table 1. List of the human cell lines used in this study.

HUMAN CELL LINE	CELL TYPE AND TISSUE	ORIGIN	CULTURE MEDIUM
293T	Epithelial. Embryonic Kidney	ATCC	DMEM 10% FBS
HeLa	Epithelial. Cervical carcinoma	ATCC	DMEM 10% FBS
HAP1	Bone Marrow. Chronic Myelogenous Leukemia (CML)	HORIZON	IMDM 10% FBS
K562	Bone Marrow. Chronic Myelogenous Leukemia (CML)	ATCC	RPMI 10% FBS
RAMOS	B Lymphocyte. Burkitt's Lymphoma	ATCC	RPMI 10% FBS
SH-SY5Y	Nerve. Neuroblastoma	ATCC	DMEM 10% FBS
T98G	Brain. Glioblastoma Multiforme	ATCC	DMEM 10% FBS
H1299	Lung. Lymph node (lung carcinoma)	ATCC	DMEM 10% FBS
LU165	Lung. Small cell lung cancer	M. Sanchez-Cespedes	RPMI 10% FBS
TPC1	Gland papillary. Thyroid carcinoma	ATCC	DMEM 10% FBS

3.1.2. PEI-mediated transient transfection

In order to express a new vector in the cells, transient transfections were achieved using polyethylenimine (PEI). PEI transfection reagent (Polysciences, Inc.) is able to condensate DNA into positively charged particles that bind to negatively charged cell surfaces and, consequently, go inside the cell by endocytosis. Plasmids were purified with a Plasmid Midi Kit from Qiagen, following manufacturer's indications. The list of plasmids used in this study is shown in Table 2.

Table 2. Plasmids used in this work

NAME	INSERT	ORIGIN
pcDNA4-CCDC6-myc	CCDC6 gene myc-tagged	A. Celetti (Merolla F. et al, 2012)
pcDNA4-CCDC6 1-223-myc	1-223 amino acids of CCDC6 gene fragment myc-tagged	A. Celetti (Merolla F. et al, 2012)
pcDNA4-CCDC6 1-101-myc	1-101 amino acids of CCDC6 gene fragment myc-tagged	A. Celetti (Merolla F. et al, 2012)
pCMV/Sport 6- wt MNT	Human wt MNT	Commercial (Origene)
pcDNA 3.1-wt MNT-HA	Mouse wt MNT (600aa) Ha-tagged	P. Hurlin lab, Oregon University, Portland USA
pcDNA 3.1 Zeo/CAT - ΔCt 1 MNT-HA	Deletion of 276 aa from mouse wt MNT. Ha-tagged	P. Hurlin lab, Oregon University, Portland, USA
pcDNA 3.1 Zeo/CAT - ΔCt 2 MNT-HA	Deletion of 211 aa from mouse wt MNT. Ha-tagged	Laboratory collection
pcDNA 3.1 Zeo/CAT- ΔNt1 MNT-HA	Deletion of 300 aa from mouse wt MNT Ha-tagged	Laboratory collection
pcDNA 3.1 Zeo/CAT- ΔNt2 MNT-HA	Deletion of 270 aa from mouse wt MNT Ha-tagged	Laboratory collection
pmax- GFP	GFP gene	Commercial (Amaxa)
pCDNA3.1/pCEFL	empty vector	Laboratory collection

For transfecting a 150 mm Ø plate, 7µg of DNA were added to 500 µl of medium and mixed with 2.5 times more PEI than DNA, diluted also in 500 µl of medium. Medium without FBS was used in order to eliminate possible charges that could interfere with the process. After 30 min of incubation at room temperature, the plate medium was replaced by medium without FBS and the DNA-PEI solution was added in a drop wise manner to the plate. After 24 h, complete medium was added to the plate and transfection efficiency was tested by GFP expression under a fluorescence microscope.

3.1.3. Lentivirus production and infection

In order to silence CCDC6 in HAP1 cells, lentiviral particles were produced in HEK293T cells. Firstly, HEK293T cells were transfected with PEI, as described before, with three different constructs: pCMV-VSV-G and psPAX2 (virus packaging) plus the plasmid containing a short hairpin sequence against CCDC6, in a 1:3:4 proportion (50 µg of total DNA for a 150 mm plate). Two days after transfection, supernatants were collected, centrifuged for 3 min at 1500 rpm and filtered through a 45µm pore size sterile syringe filter (Merck Millipore). Then, 40 % PEG8000 1 X PBS (Sigma-Aldrich) stock solution was added at a final concentration of 15 % and kept at 4ºC for at least 4 h. Finally, the medium was centrifuged for 30 min at 1500 rpm at 4ºC and precipitated. Lentiviral particles were resuspended in 600 µl of medium.

For lentivirus titering, HeLa cells were seeded in a 6-well plate ($2 \cdot 10^5$/well). Once attached, the complete medium was replaced with half the usual volume of medium, in this case without FBS and supplemented with 5 µg/ml of hexadimethrine bromide (Polybrene, Sigma-Aldrich). Polybrene is used for increasing the transduction efficiency. Then, different volumes of the lentiviral preparation (10, 5, 1, 0.5, and 0.1 µl) were added to the plate. After 12 h, 1.5 ml of complete medium was added to the cells. 48 h after infection, the medium was changed and supplemented with puromycin (1 µg/ml) for selecting the cells. 48 h of selection were usually enough for colonies to grow. Finally, colonies were stained with a crystal violet solution (1 % acetic acid, 1 % methanol, 1 % (w/v) crystal violet dye) scanned. Finally, they were dissolved in a 10 % acetic acid solution and their absorbance was measured at 620 nm.

3.2. CELL PROLIFERATION AND VIABILITY ANALYSIS

3.2.1. Clonogenic assays

HeLa and HeLa shCCDC6 cells were transfected with the pcDNA and MNT-HA vectors. The empty vector pcDNA3 and the MNT WT-HA construct confer the cells resistance to zeocine. Moreover, we co-transfected a pCEFL vector with a Gentamicin (G418) resistance gene. Cells were selected in medium supplemented with antibiotics (100 μg/ml of zeocin or 50 μg/ml of G418), which was refreshed every 2-3 days. After 10 days of selection, cells were washed with 1 X PBS and stained for 10 minutes with a 0.15 % crystal violet solution and scanned. Finally, they were dissolved in a 10 % acetic acid solution and their absorbance was measured at 620 nm. The results of three independent experiments for the selection with G418 and five for the selection with zeocin, were analyzed by Student's T Test. The groups that had a p-value lower than 0.05 were considered to have significant differences.

3.2.2. Viability analysis upon drug treatment

In order to perform a viability analysis, HAP1 and HAP1 MNT KO ($4x10^4$ cells) were seeded in a 96-well tissue culture plate and etoposide (Sigma Aldrich) (that break the double DNA strand and avoid their repair leading them to apoptosis) and cisplatin (Sigma Aldrich) (that creates new bonds intra and inter-DNA strand) drugs were added. After two days of incubation, the cell proliferation reagent WST-1 (Sigma Aldrich) (1:10 final dilution) was used and absorbance of the samples at 405 nm was measured. This reagent allows the quantification of the number of viable cells by the cleavage of the tetrazolium salt to formazan dye.

3.3. PROTEIN ANALYSIS

3.3.1. SDS-PAGE and Western Blot

Protein levels were analyzed by western blot. Cells were lysed using 1% NP40 lysis buffer 2% of SDS. After the aspiration of the growing media and the washing of the cells with 1X PBS, lysis buffer was added directly in the plate (100 μl for p60 plate) on ice and cells were scratched and collected to a 1.5 ml tube. We kept them during 30 minutes in ice pippetting up and down every 5 minutes. Then, protein samples were sonicated using the Bioruptor® Plus sonication device (Diagenode) set at high power setting for 10 cycles (30 s ON, 30 s OFF). After sonication, samples

were centrifuged for 10-20 minutes at 14000 rpm at 4ºC. The supernatant containing the proteins was collected and transferred to a new tube and stored at -80°C until used.

In order to quantify the protein concentration, we used the Qubit®Protein Assay Kit in a Qubit® 2.0 Fluorometer. We diluted the samples at 1:5 in water and 1 µl was added to a dilution 1:200 of the Qubit® working solution in Qubit® Protein Buffer. Samples were incubated 10 minutes protected from light at room temperature and then, samples were read in the Qubit® 2.0 Fluorometer.

80 µg of total protein were used, we mixed the corresponding volume of protein with water until complete 16 µl and 4 µl of Laemmli 5X were added. Then, samples were heated at 95ºC for 5 minutes to denaturalize the proteins, and the samples were loaded and separated according to their molecular weight in a polyacrylamide-SDS (SDS-PAGE) gel. The percentage acrylamide solution used for each gel (10%, 12% or 15%) depended on the molecular weight of the proteins that were going to be analyzed. Electrophoresis was carried out in a Mini Protean III cuvette (Bio-Rad) with running buffer (10 % SDS, 1 X TG (10 X TG: Tris-Glycine Buffer, Bio-Rad)) at 120-160 V. Once electrophoresis finished, proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell) in a Mini-Trans Blot cell (Bio-Rad) at 400 mA for 40 min, using transfer buffer (10 % methanol, 1 X TG (Bio-Rad)). Next, the membrane was incubated with TBS-T (20 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.05 % Tween 20 (v/v)) supplemented with 1 % of non-fat dry milk (w/v) for 1 h at room temperature (RT) for blocking. After three washes of 10 min with TBS-T, the membrane was incubated with the primary antibody, that had been previously diluted 1:500 or 1:1000 in TBS-T supplemented with 1 % BSA (w/v)) overnight (O/N) at 4ºC. Then, other three washes of 10 min with TBS-T were accomplished and the membrane was incubated with secondary antibody conjugated to IRDye680 or IRDye800 fluorochromes (Li-COR Biosciences), diluted 1:15000 in TBS-T 1 % BSA (w/v). Finally, the membrane was washed three times for 10 min with TBS-T and scanned using an Odyssey Infrared Imaging Scanner (Li-COR Biosciences). The antibodies used for immunoblot analysis are shown in Table 3 and Table 4.

3.3.2. Immunoprecipitation assays

Immunoprecipitation assays were carried out in order to study protein-protein interactions. As the cells are adherent, they were lysed directly in the plate after aspiring the growing media and washed twice with 8 ml of 1X PBS. 1 ml of lysis buffer was added to each 150 mm ∅ plate and cells were scratched and were transferred to a 1.5 ml tube. They were kept with rotation at 4ºC for 30 minutes.

During this time, Gammabind Sepharose Beads (20µl/IP) (Amersham Pharmacia Biotech) used for capture the protein-antibody immunocomplexes, were washed in 1 ml of washing buffer (x3), maintained for 5 minutes in rotation at 4ºC and then they were centrifuged at 4000 rpm for 1 minute. 200µl of lysis buffer were added to the sepharose beads to distribute them between the samples.

After centrifugation at 14000 rpm for 10-20 minutes, the supernatants containing the proteins were transferred to a new tube. In order to measure the protein concentration, the Qubit® Protein Analysis Kit was used. We kept 50 µl of each sample for the input and distributed the rest for the incubation with the appropriate antibody (2 µg/IP) and the Gammabind Sepharose Beads overnight at 4ºC with rotation.

The following day, 1 ml of washing buffer was used in order to wash the mixture (x3) maintaining it for 5 minutes in rotation at 4ºC and then they were centrifuged at 4000 rpm for 1 minute. 50µl of Laemmli 2x were added to the inputs and 20 µl of Laemmli 2x plus 20 µl of water were added to the rest of the samples, and the western blot assay was performed.

3.3.3. Nuclear-cytoplasmic fractionation

For the nuclear-cytoplasmic fractionation, we used 3-4x10^6 adherent growing HeLa cells that were first washed twice with 8 ml of 1X PBS. Then, a soft lysis was performed adding 500 µl per each 150 mm Ø plate containing cells of Lysis buffer 1 (10 mM HEPES pH 7; 10 mM KCl; 0.25 mM EDTA pH 8; 0.125 mM EGTA pH 8; 0.5 mM Spermidin, 0.1 % (v:v) IGEPAL (Sigma-Aldrich); 1 mM DTT; 1:100 of proteases and phosphatases inhibitors). Cells were scratched and transferred to a 2 ml tube and maintained for 30 minutes in rotation at 4ºC and then were centrifuged at 1500 rpm for 5 minutes. Supernatant containing cytoplasmic fraction was collected and kept on ice until use.

Nuclear fraction pellet was then resuspended in 750 µl of NP40 Lysis buffer and maintained for 30 minutes in rotation at 4ºC. Then, nuclei and cytoplasm lysates were centrifuged for 10 min at 4ºC maximum speed. Supernatant were transferred to a new tube and protein concentration were measured with Qubit®Protein Assay Kit (described in 3.3.1). IP assay was then performed in order to study where do MNT and CCDC6 interact.

Table 3. Primary antibodies used in this study.

PRIMARY ANTIBODYES			
ANTIGEN	**TYPE**	**REFERENCE**	**USE / DILUTION**
β-Actin	Mouse monoclonal	SantaCruz biotechnology (C4-SC47778)	WB 1:3000
BCL-XL	Rabbit polyclonal	54-H6	WB 1:1000
CCDC6	Mouse monoclonal	SantaCruz biotechnology (SC-100309)	WB 1:500, IP
Cyclin A	Rabbit polyclonal	SantaCruz biotechnology (SC-751)	WB 1:1000
HA	Mouse monoclonal	CGAB-HA-0050	WB 1:1000, IP
IgG	Mouse monoclonal	SantaCruz biotechnology (SC-2025)	IP
Max	Rabbit polyclonal	C-17 SantaCruz biotechnology (sc-197)	WB 1:1000
MYC	Mouse monoclonal	9-E10 SantaCruz biotechnology (SC-40)	WB 1:500, IP
MNT	Rabbit polyclonal	Novus (NBP2-20453)	WB 1:1000, IP
Sin 3B	Rabbit polyclonal	A-20 SantaCruz biotechnology (SC-996)	WB 1:500
PARP	Rabbit polyclonal	SantaCruz biotechnology (SC-7150)	WB 1:1000
Rho GDI	Rabbit polyclonal	A-20 SantaCruz biotechnology (SC-360)	WB 1:500

Table 4. Secondary antibodies used in this study

SECONDARY ANTIBODIES			
ANTIBODY	**TYPE**	**REFERENCE**	**USE/ DILUTION**
Anti-Rabbit IRDye®800	Donkey polyclonal	Li-Cor Biosciences (926-32213)	WB 1:10000)
Anti-Rabbit IRDye®680	Donkey polyclonal	Li-Cor Biosciences (926-68073)	WB (1:10000)
Anti-Mouse IRDye®800	Donkey polyclonal	Li-Cor Biosciences (926-32212)	WB (1:10000)
Anti-Mouse IRDye®680	Donkey polyclonal	Li-Cor Biosciences (926-68072)	WB (1:10000)
Anti-RatIRDye®800	Donkey polyclonal	Li-Cor Biosciences (926-32219)	WB (1:10000)
Anti-Rat IRDye®680	Donkey polyclonal	Li-Cor Biosciences (926-68076)	WB (1:10000)

4. RESULTS

4.1. MNT and CCDC6 interaction

Previous results from our group by proteomic assays showed that MNT could interact with CCDC6. Therefore, our first objective was to prove that this interaction takes place and to try to determine the protein domains involved in such interaction.

First, HEK293T cells were transfected with MNT and CCDC6 overexpressing constructs. For CCDC6 we used the wild-type form and two mutants, one that only presents the first 223 amino acids of the coiled coil domain of the protein and another one that only presents the first 101 amino acids, all of them containing a myc-tag (structures of these proteins can be observed in Figure 7). Then the possible interactions of the proteins were studied by co-immunoprecipitation assays.

A co-IP with the two wild type proteins was first performed to confirm that the interaction occurs, as it is shown in the figure 8a. In order to determine the proteins interaction domains, the same analysis was performed with the CCDC6 mutants, but there was no co-immunoprecipitation between none of the mutants and MNT wt (figure 8a and 8b).

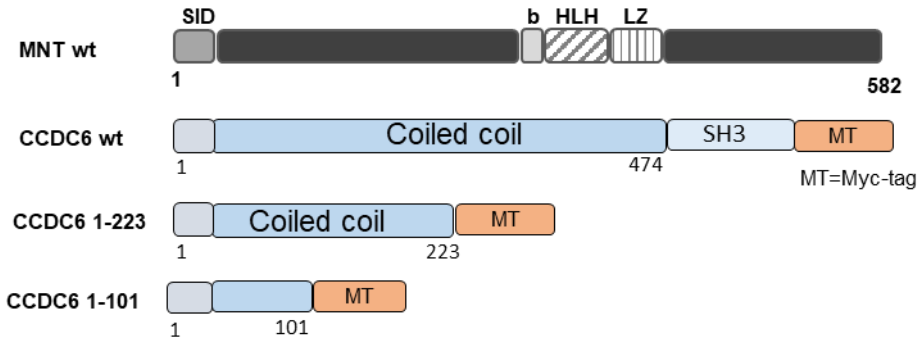
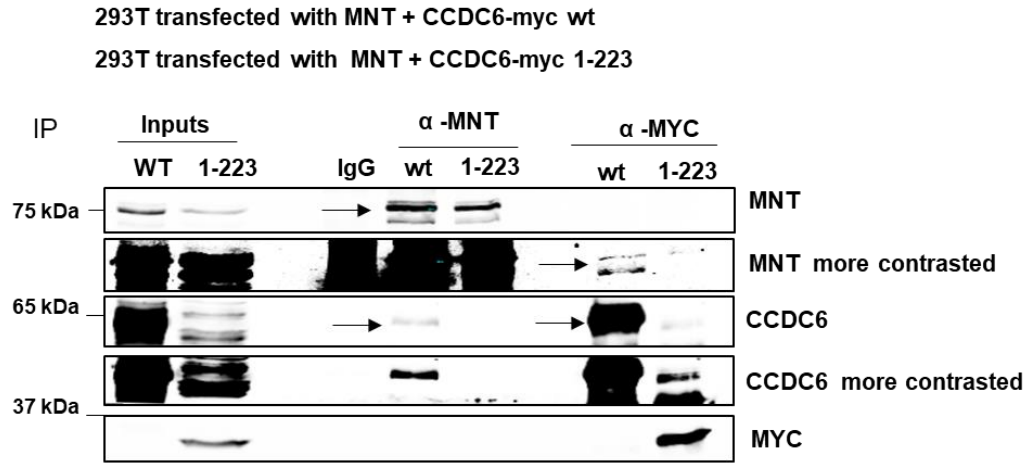


Figure 7. Structure of MNT wt and CCDC6 wt, and structure of the two CCDC6 mutants used for the first interaction assay.

a)



b)

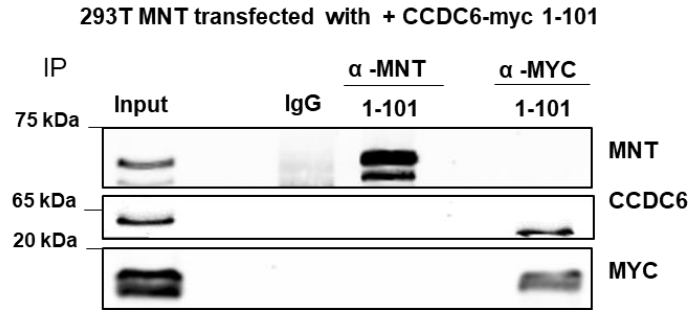


Figure 8. a) IP between wt MNT with an HA-tag and wt CCDC6 with a myc-tag, together with the IP between MNT and the 1-223 CCDC6 mutant. Interaction can only be observed between the wt proteins. Co-IP indicated with the arrows b) MNT wt and 1-101 CCDC6 mutant interaction was studied by IP assay but no co-immunoprecipitation could be observed.

Then, we tried a similar experiment but, in this case, using the wild type form of human CCDC6, the wt form of mouse MNT (figure 9a) and different MNT mutants, which lack different parts of the MNT protein. HEK293T cells were transfected with the different constructs and the IPs assays performed.

First, as the wt MNT is now from mouse (the mutants were provided with an HA-tag), IP between the complete proteins MNT-HA and CCDC6-myc was performed in order to assure the interaction (figure 9b).

a)

b)

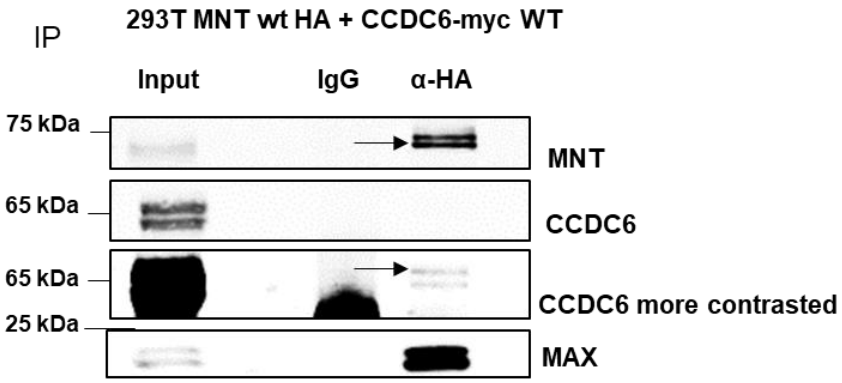
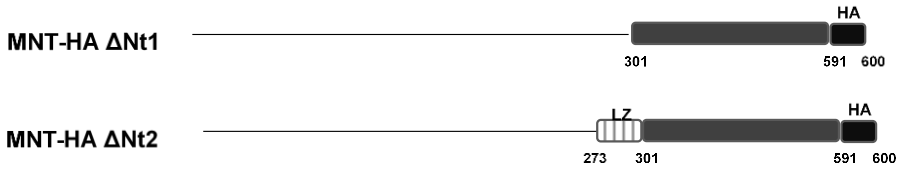


Figure 9. a) mouse MNT-HA wt protein structure. b) IP between MNT-HA wt and CCDC6-myc, confirming the interaction between the proteins. Co-IP indicated with the arrows.

Once the interaction was confirmed, the co- IPs were performed with the wt CCDC6-myc and the different MNT mutants. Using the MNT-HA ΔNt1 and MNT-HA ΔNt2 mutants (Figure 10a), the interaction was only observed between the second one and CCDC6 (Figure 10b).

a)



b)

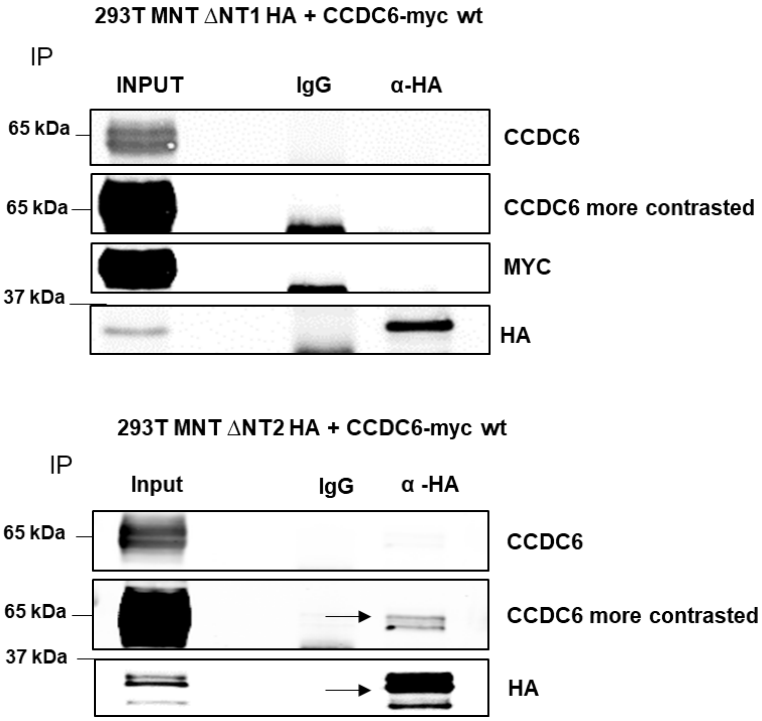
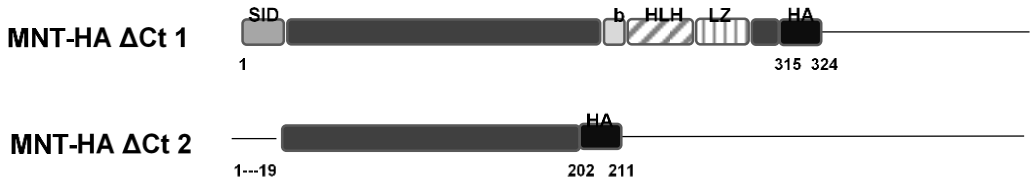


Figure10. a) Structures of MNT-HA ΔNt1 and MNT-HA ΔNt2 mutants. b) IP between MNT-HA mutants and CCDC6-myc wt. As it can be observed, interaction only occurs with MNT-HA ΔNt2 mutant. Co-IP indicated with the arrows.

Next, the IP with MNT-HA ΔCt1 and MNT-HA ΔCt2 mutants and wt CCDC6 was carried out. As it can be observed in Figure 11b, both mutants are able to interact with wt CCDC6.

a)



b)

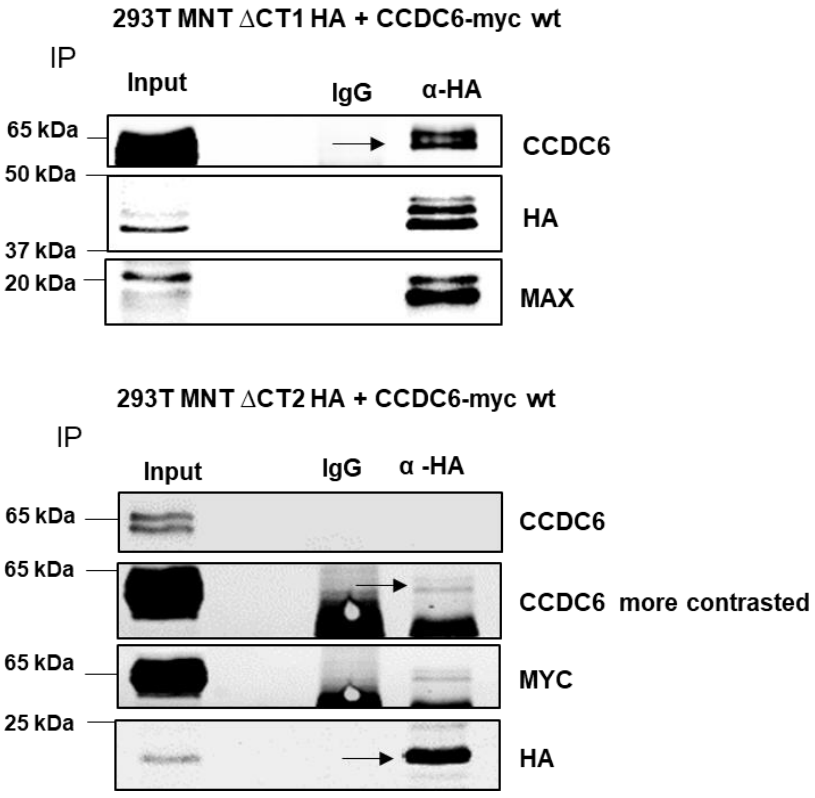


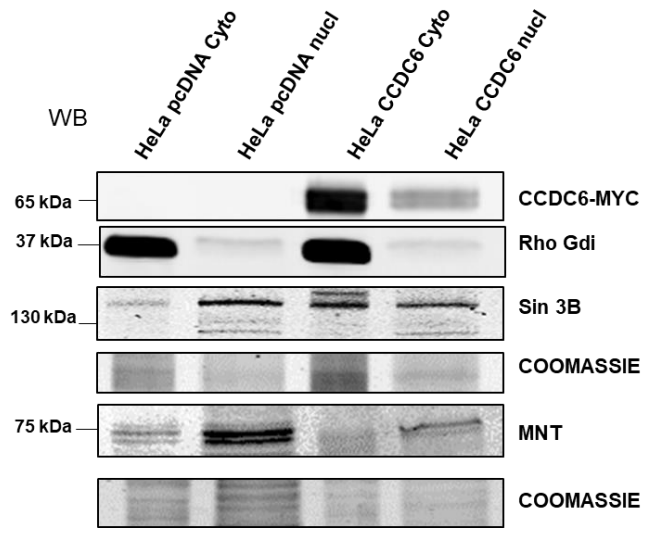
Figure11. a) Structure of MNT-HA ΔCt1 and MNT-HA ΔCt2 mutants. b) IP between CCDC6-myc and the MNT mutants, showing that there is an interaction between the two mutants and the wt protein. Co-IP indicated with the arrows.

4.2. **MNT and CCDC6 interacts in the cytoplasm**

The next step for studying the interaction between MNT and CCDC6 was to search where this interaction occurs inside the cells. It was described that MNT and CCDC6 can be located in both nucleus and cytoplasm (see Introduction) therefore we performed cellular fractionation experiments in HeLa cell line. We first tried to detect the endogenous proteins, but the amount of proteins was very low after the fractionation and we decided to overexpressed CCDC6 and MNT proteins. Overexpression was checked by Western Blot analysis (Figure 12a). In the WB and then in the IP, the markers of cytoplasm and nucleus (Rho GDI and Sin 3B, respectively) were also included to confirm that the cellular fractionation was correct.

After being sure that the proteins were correctly overexpressed, the interaction of the proteins in cytoplasm and nucleus was studied by IP. Results showed that the interaction between MNT and CCDC6 occurs mainly in the cytoplasm (Figure 12b). In the IP and WB analysis can be also observed that MNT appears more in the nucleus while CCDC6 seems to be more cytoplasmatic.

a)



b)

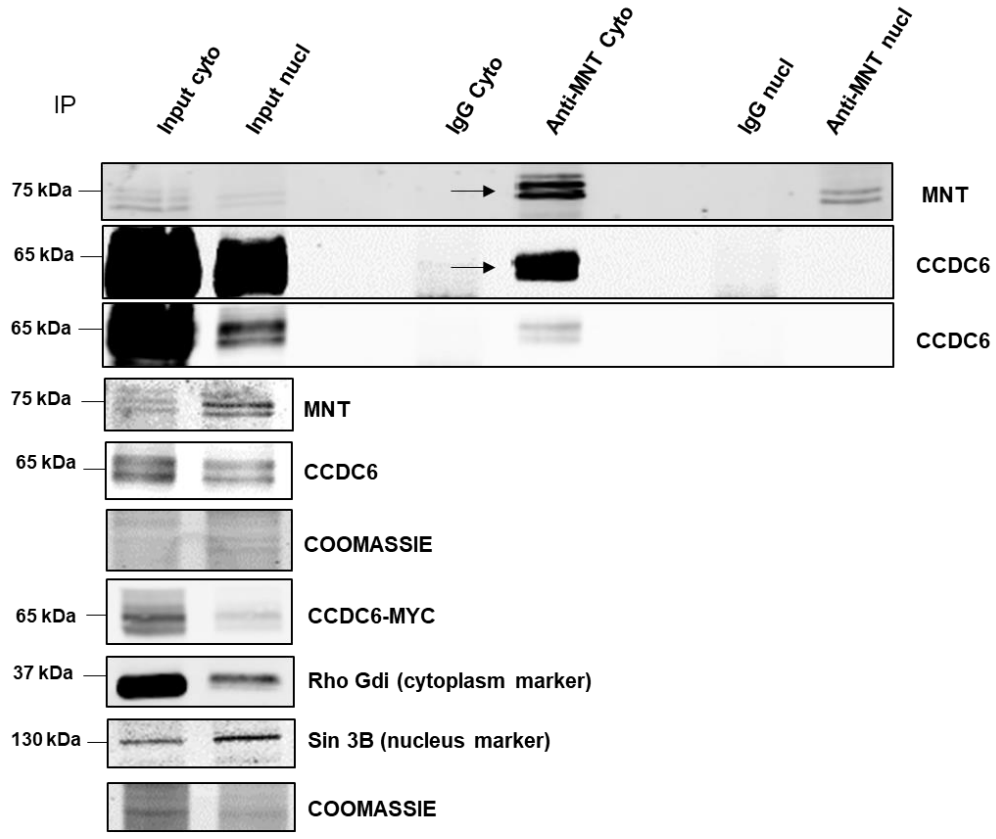


Figure12. a) WB performed in order to check that the overexpression of CCDC6 protein in nucleus and cytoplasm was correctly. Cytoplasm and nucleus markers (Rho GDI and Sin 3B, respectively) were also added. b) IP immunoprecipitating MNT from cytoplasm and nucleus showing that the interaction between MNT and CCDC6 mainly occurs in cytoplasm. Co-IP indicated with the arrows.

4.3 **Effects of CCDC6 and MNT on cell proliferation**

HeLa and HeLa shCCDC6 were used to study the effects of the over expression of MNT and the silencing of CCDC6 on cell proliferation. The over expression of MNT was checked by WB (Figure 13a). The empty vector pcDNA3 and the MNT WT-HA construct confer the cells resistance to zeocine. Moreover, we co-transfected a pCEFL vector with a Gentamicin (G418) resistance gene. Transfected cells were selected adding the antibiotics, and after ten days the results were measured by clonogenic assays (Figure 13b). The results of three independent experiments for the selection with G418 and five for the selection with zeocin, were analyzed by Student's T Test obtaining the following p-values:

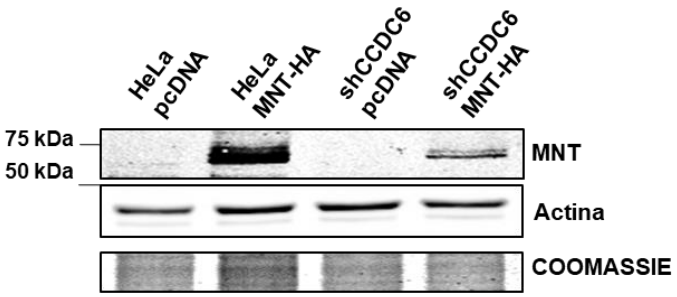
For the cells selected with Zeocin, we obtained a p-value of 0,17501 for HeLa pcDNA MNT-HA cells compared to HeLa pcDNA cells and a p-value of 0,16736 for shCCDC6 pcDNA MNT-HA cells compared to shCCDC6 pcDNA cells.

Moreover, for the cells selected with gentamicin, we obtained a p-value of 0,17432 for HeLa pcDNA MNT-HA cells compared to HeLa pcDNA cells and a p-value of 0,16736 for shCCDC6 pcDNA MNT-HA cells compared to shCCDC6 pcDNA cells.

We considered that the groups that had a p-value lower than 0,05 have significant differences, so, as none of our results have a p-value lower than 0,05 we cannot affirm that the silencing of CCDC6 together with the over expression of MNT affect the proliferation in HeLa cells.

However, we could always observe a tendency of proliferation inhibition in cells transfected with MNT and upon CCDC6 silencing. More experiments are therefore necessary to clarify this possible effect on cells proliferation inhibition.

a)



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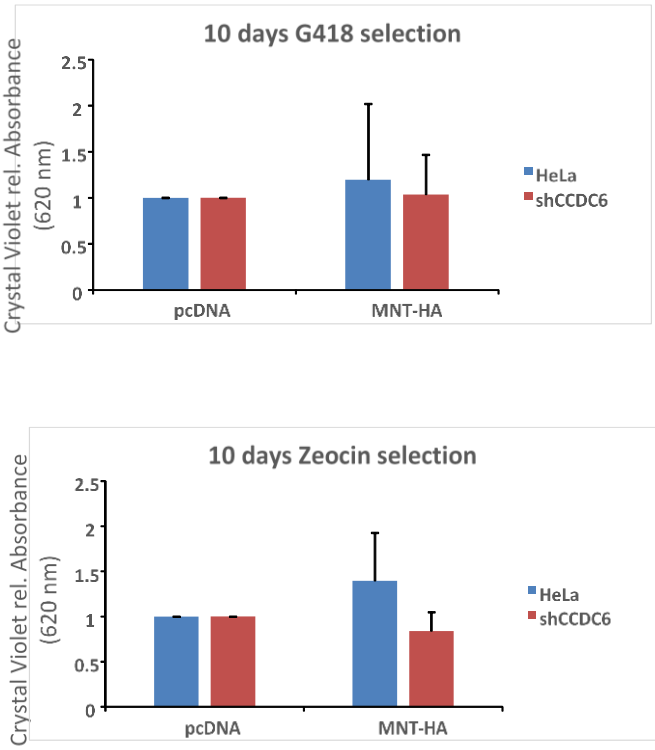


Figure13. a) Over expression of MNT checked by WB b) Graphics showing the clonogenic assays results after 10 days selection. No significant differences on proliferation were obtained.

4.4 **Effects of MNT and CCDC6 on DNA damage**

In order to study the role of MNT and CCDC6 in DNA damage, we used HAP1 and HAP1- MNT KO cells. These are commercial cells (from Horizon Discovery) with a knock-out of MNT achieved by the CRISPR/Cas9 technology. The cells were incubated with chemotherapy drugs. Etoposide (that breaks double DNA strand and gets inside the break, avoiding the reparation and leading to apoptosis) and cisplatin (that creates new intra and inter DNA brands creating DNA

damage) were added. After the incubation with the drugs during 48h, results were analyzed with a WST-1 Cell Proliferation Assay.

Cisplatin seems to cause no effect on the cells. However, HAP1 MNT KO cells are more sensitive to etoposide than HAP1 control cells (Figure 14). The experiments to determine the possible effects of CCDC6, by using shCCDC6 lentivirus (see next section) are in progress.

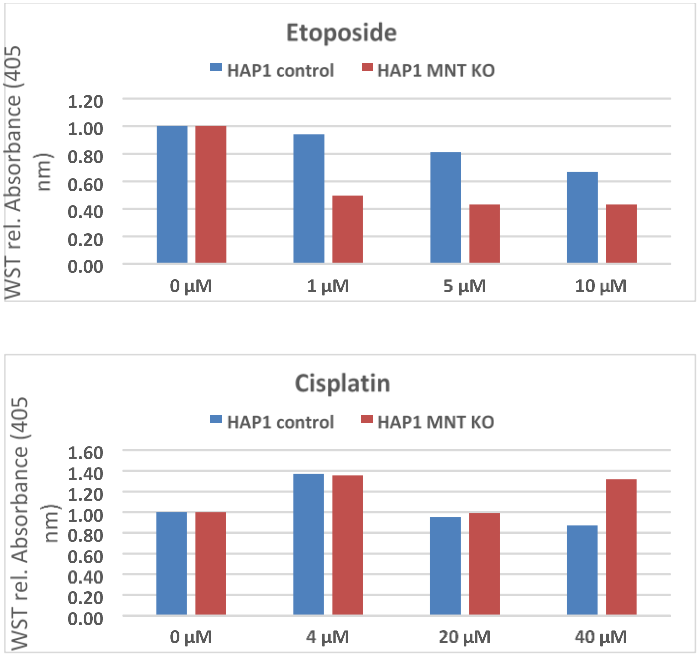


Figure14. Graphics show the results obtained after of the incubation with the indicated doses of etoposide and cisplatin, analyzed with WST-1 assay. MNT-KO cells proliferation seems affected, comparing with the control, upon treatment with etoposide

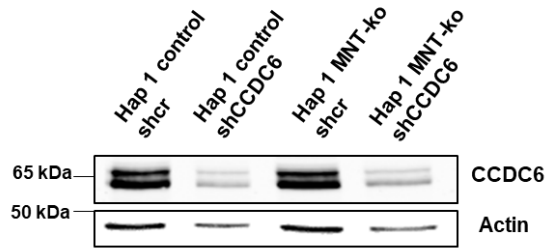
4.5 Effects of CCDC6 silencing on apoptosis

In order to study the role of CCDC6 in apoptosis, silencing it was necessary. For silencing CCDC6 in HAP1 and HAP1-MNT KO cells, we produce lentiviral particles containing a short hairpin sequence for CCDC6. Then cells were infected and the silencing was checked by WB (Figure 15a).

Western Blot (figure 15b) was performed in order to analyze the apoptosis PARP and BCL-XL markers. PARP is a 116 kDa nuclear poly (ADP-ribose) polymerase, and its cleaved fragment (89 kDa) serves as a marker for detecting cells undergoing apoptosis. In our results there were no changes when detected with PARP. However, it can be observed an increase in the levels of

BCL-XL (antiapoptotic protein from the BCL2 family) in MNT-KO shCCDC6 cells, suggesting a role of them in apoptosis.

a)



b)

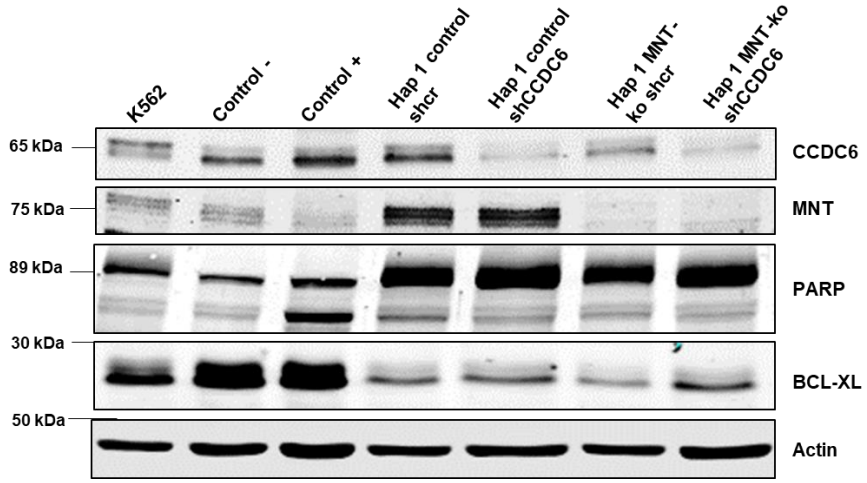


Figure15. a) Silencing of CCDC6 in HAP1 and HAP1-MNT KO cells was checked by WB b) A WB analysis was performed to study apoptosis markers. Ramos cells were used as positive control of PARP cleavage. Results show that, despite PARP does not seem to be altered, BCL-XL (anti-apoptotic marker) is increased after the silencing of both proteins.

In summary, our results showed that CCDC6 and MNT proteins interact and that they do it in the cytoplasm. CCDC6 mutants are no able to interact with MNT wt, but some mutants of this last protein (ΔNt2-HA, ΔCt1-HA and ΔCt2-HA) are able to interact with CCDC6 wt leading us to suggest (in the "discussion" section) some possible interaction domains. Moreover, after studying the proliferation effects of silencing CCDC6 and over expressing MNT, we could not observe a significant difference comparing with the proliferation of normal cells. In DNA damage conferring assays, it was observed that HAP1 MNT-KO cells seems to be more resistant to

etoposide, while cisplatin does not seem to affect them. Finally, after apoptosis analysis, we observed that the silencing of CCDC6 and MNT seems to affect HAP1 cells apoptosis.

5. DISCUSSION

CCDC6 and MNT are two important oncoproteins that have been found altered in a variety of human cancers (12, 19). A mass-spec analysis showed, for the first time, that CCDC6 and MNT proteins could physically interact (unpublished results from our group). On the other hand, CCDC6 and MNT can be located in both nucleus and cytoplasm and play different roles in the control of cell proliferation, DNA damage and apoptosis (see Introduction). Taking this information into account, in this work we have performed biochemical and biological studies in order to clarify:

- MNT and CCDC6 interaction, trying to delimit the interaction domains in 293T cells
- The effects of CCDC6 silencing together with the over expression of MNT on proliferation in HeLa cells
- The role of MNT and CCDC6 in cells exposed to genotoxic drugs in HAP1 cells
- The effect of CCDC6 and MNT silencing on apoptosis in HAP1 cells

5.1. MNT and CCDC6 interaction

In order to study the interaction of MNT and CCDC6, we analyzed the interaction of both proteins in their wild-type form and then tried to determine the interaction domains using deletion mutants. First, a co-immunoprecipitation assay was performed overexpressing MNT wt and CCDC6 wt in 293T cells and their physical interaction was confirmed. CCDC6 had been found to interact with several proteins which can form complex with DNA (14, 18). A similar interaction was found between CCDC6 and CREB1, that form complexes in cultured B-CPAP cells (18). These authors also tried the experiment with the CCDC6 (1-101 aa) deleted mutant, as occurs in *PTC1* oncoprotein, but the mutant protein was not no able to interact with CREB1, indicating that the N-terminal region of CCDC6 was not sufficient for the binding. In our work, we perfomed co-immunoprecipitation analysis with the same mutant, in order to analyze the interaction with MNT in 293T cells. We also assayed a longer mutant CCDC6 (1-223 aa) but any of them were able to interact with MNT, suggesting that the C-terminal of the protein is required for the binding.

To investigate the domains of MNT responsible for the interaction with CCDC6, co-immunoprecipitation assays were performed transfecting different MNT mutants (ΔNT1-HA, ΔNT2-HA, ΔCT1-HA and ΔCT2-HA). Results suggest that two interaction domains are involved:

The C-terminal of the protein plus the leucin zipper domain and the N-terminal of the MNT protein.

Immunofluorescence through regular fluorescence and confocal studies in different cell lines have shown that CCDC6 is a nuclear and cytosolic protein (10). The intracellular location is associated with the phosphorylation induced by serum, being more nuclear upon serum stimulation, and more cytoplasmatic in absence of serum. Furthermore, MNT can also be located in both nucleus and cytoplasm (6). With this information, we asked for the subcellular localization of the MNT-CCDC6 complexes. We were not able to detect the endogenous proteins; therefore, we overexpressed the wt proteins by transfection in 293T cells and performed cellular fractionation. Once the overexpression was confirmed and markers for nucleus and cytoplasm analyzed, co-immunoprecipitation assays were carried out. Our results showed that interaction of MNT and CCDC6 proteins takes place mainly in the cytoplasm. In the case of CCDC6-CREB1 interaction, *in vitro* experiments showed that CCDC6 represses CREB1 activity (18), but this report did not analyze the subcellular localization of the complexes.

5.2. Role of CCDC6 and MNT in proliferation and DNA damage

The group of Celetti and cols. (11, 15) inhibited the expression of CCDC6 in HeLa cells using small interfering RNAs. Then, by clonogenic survival analysis they showed that CCDC6 silencing confers the cells growth advantage by increasing mitosis. On the other hand, MNT-MAX heterodimers are found in proliferating cells that contain MYC-MAX heterodimers, which suggests a possible important role of MNT during proliferation (6).

In our work we wanted to find out what happens in HeLa and HeLa shCCDC6 after transfecting exogenous MNT in terms of proliferation and viability. After the clonogenic survival analysis, we could not find any significant change on the proliferation of these cells. However, we always observed a tendency of proliferation inhibition upon MNT over expression in the absence of CCDC6. This result suggest that MNT could play a role in the control of cell proliferation when CCDC6 is silenced. Further experiments using different proliferation and viability tests are necessary to confirm such effect.

CCDC6 is involved in the DNA damage response (see Introduction). Cells that have been exposed to genotoxic agents undergo that produce DNA damage apoptotic cell death. Studies in cultured cells treated with etoposide showed an increase of CCDC6 levels (11). The mechanism proposed

by the authors is that DNA damage induced the ATM kinase, phosphorylation of CCDC6 and nuclear stabilization of the protein, leading to apoptosis (11).

In this study, we wanted to analyze the role of MNT and CCDC6 under the presence of DNA damage agents by using the HAP1 cell model. For it, we exposed HAP1 and HAP1 MNT-KO cells to two well-known genotoxic drugs: etoposide and cisplatin. While cisplatin does not seem to affect the survival, MNT-KO cells survival seems to be more affected by etoposide than HAP1 control cells, suggesting that MNT confers the cells resistance to etoposide. This is an interesting new finding that requires further confirmation. In addition, experiments with shCCDC6 lentiviral constructs to determine the role of CCDC6 in this cellular model are currently in progress in our laboratory.

5.3. CCDC6 knockdown effect in apoptosis

CCDC6 overexpression is able to induce apoptosis and its mutation or depletion protects cells from apoptotic stimuli (11). It was also observed a "reversion of an antiproliferative effects" after the exposure CCDC6 silenced cells to hydrogen peroxide (20).

In our project, we produced and purified CCDC6 lentiviral particles containing short hairpin sequences against CCDC6. Once inside the cells, these short-hairpin constructs are expressed into siRNA that silence the targeted gene. After lentiviral infection of HAP1 and HAP1 MNT-KO cells with the shCCDC6, we performed western blot to analyze apoptosis markers. We didn't find any cleavage of the PARP protein suggesting that apoptosis was not induced upon silencing of CCDC6, MNT or both. However, we could observe an increase in the expression of BCL-XL (an antiapoptotic protein from the BCL2 family) especially in cells with MNT and CCDC6 silenced. This suggest that these two proteins are important for cell stability.

In summary, we can conclude that CCDC6 and MNT interact in the cytoplasm and that CCDC6 mutants that we used (1-101 and 1-223 aa) were no sufficient for the binding, so in agreement with our results, the CCDC6 C-terminal domain (beyond the 223 position) seems necessary for the interaction. In the case of MNT, C-terminal plus the leucin zipper domain and the N-terminal of the protein were found involved in the interaction. Next, we found that the proliferation of HeLa cells seems not to be significantly affected by the silencing of CCDC6 together with the overexpression of MNT. We also found that MNT seems to confer more resistance to etoposide

in HAP1 cells. Apoptosis of that HAP1 cells seems to be affected by both CCDC6 and MNT silencing.

The results presented here open a new pathway by which the MNT-MYC network regulate cell functions, i.e., the CCDC6-mediated response to DNA damage and apoptosis.

6. **CONCLUSIONS**

1. MNT-CCDC6 interaction is confirmed. The C-terminal domain of CCDC6 seems to be required.
2. MNT ΔNt2-HA interacts with CCDC6 but not MNT ΔNt1-HA, suggesting that the LZ domain is important for the interaction.
3. MNT ΔCt1-HA and ΔCt2-HA interact with CCDC6, suggesting that the N-terminal domain is important for the interaction.
4. MNT-CCDC6 interaction seems to occur mainly in the cytoplasm.
5. The silencing of CCDC6 together with the overexpression of MNT do not affect significantly the proliferation of HeLa cells.
6. HAP1 MNT KO cells are more sensitive to etoposide than HAP1 control.
7. There is an increase of the anti-apoptotic BCL-XL protein levels upon CCDC6 and MNT knockdown, suggesting an effect of these proteins on apoptosis.

FUTURE RESEARCH

MNT and CCDC6 interaction have been shown. It would be interesting to study the exact roles of these proteins and of their interaction, tackling the following points:

1. Elucidate the exact interaction domains in MNT-CCDC6 complexes
2. Confirm the effects of silencing of CCDC6 and overexpressing MNT in proliferation
3. Clarify the obtained results in HAP1 and HAP1-KO cell line after their exposure to cisplatin and etoposide
4. Determine the role of CCDC6 in HAP1 cellular model performing new experiments with shCCDC6 lentiviral constructs

REFERENCES

1. Dang CV. MYC on the path to cancer. Cell. 2012;149(1):22-35.

2. Hooker CW, Hurlin PJ. Of Myc and Mnt. Journal of cell science. 2006;119(Pt 2):208-16.

3. Hurlin PJ, Queva C, Eisenman RN. Mnt: a novel Max-interacting protein and Myc antagonist. Current topics in microbiology and immunology. 1997;224:115-21.

4. Nilsson JA, Cleveland JL. Mnt: master regulator of the Max network. Cell cycle. 2004;3(5):588-90.

5. Conacci-Sorrell M, McFerrin L, Eisenman RN. An overview of MYC and its interactome. Cold Spring Harbor perspectives in medicine. 2014;4(1):a014357.

6. Hurlin PJ, Zhou ZQ, Toyo-oka K, Ota S, Walker WL, Hirotsune S, et al. Deletion of Mnt leads to disrupted cell cycle control and tumorigenesis. The EMBO journal. 2003;22(18):4584-96.

7. Popov N, Wahlstrom T, Hurlin PJ, Henriksson M. Mnt transcriptional repressor is functionally regulated during cell cycle progression. Oncogene. 2005;24(56):8326-37.

8. Bretones G, Delgado MD, Leon J. Myc and cell cycle control. Biochimica et biophysica acta. 2015;1849(5):506-16.

9. Delgado MD, Leon J. Myc roles in hematopoiesis and leukemia. Genes & cancer. 2010;1(6):605-16.

10. Celetti A, Cerrato A, Merolla F, Vitagliano D, Vecchio G, Grieco M. H4(D10S170), a gene frequently rearranged with RET in papillary thyroid carcinomas: functional characterization. Oncogene. 2004;23(1):109-21.

11. Merolla F, Pentimalli F, Pacelli R, Vecchio G, Fusco A, Grieco M, et al. Involvement of H4(D10S170) protein in ATM-dependent response to DNA damage. Oncogene. 2007;26(42):6167-75.

12. Cerrato A, Merolla F, Morra F, Celetti A. CCDC6: the identity of a protein known to be partner in fusion. International journal of cancer. 2018;142(7):1300-8.

13. Nakaoku T, Kohno T, Araki M, Niho S, Chauhan R, Knowles PP, et al. A secondary RET mutation in the activation loop conferring resistance to vandetanib. Nature communications. 2018;9(1):625.

14. Leone V, Langella C, Esposito F, Arra C, Palma G, Rea D, et al. Ccdc6 knock-in mice develop thyroid hyperplasia associated to an enhanced CREB1 activity. Oncotarget. 2015;6(17):15628-38.

15. Merolla F, Luise C, Muller MT, Pacelli R, Fusco A, Celetti A. Loss of CCDC6, the first identified RET partner gene, affects pH2AX S139 levels and accelerates mitotic entry upon DNA damage. PloS one. 2012;7(5):e36177.

16. Morra F, Luise C, Merolla F, Poser I, Visconti R, Ilardi G, et al. FBXW7 and USP7 regulate CCDC6 turnover during the cell cycle and affect cancer drugs susceptibility in NSCLC. Oncotarget. 2015;6(14):12697-709.

17. Morra F, Luise C, Visconti R, Staibano S, Merolla F, Ilardi G, et al. New therapeutic perspectives in CCDC6 deficient lung cancer cells. International journal of cancer. 2015;136(9):2146-57.

18. Leone V, Mansueto G, Pierantoni GM, Tornincasa M, Merolla F, Cerrato A, et al. CCDC6 represses CREB1 activity by recruiting histone deacetylase 1 and protein phosphatase 1. Oncogene. 2010;29(30):4341-51.

19. Yang G, Hurlin PJ. MNT and Emerging Concepts of MNT-MYC Antagonism. Genes. 2017;8(2).

20. Staibano S, Ilardi G, Leone V, Luise C, Merolla F, Esposito F, et al. Critical role of CCDC6 in the neoplastic growth of testicular germ cell tumors. BMC cancer. 2013;13:433.

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