FACULTAT
DE CIÈNCIES, TECNOLOGIA
I ENGINYERIES
UVIC | UVIC·UCC

VHIO VALL D'HEBRON Institute of Oncology

Final Degree Project

THE EFFECT OF *BRD4-NUT* FUSION ON REPLICATION STRESS STATUS OF THE NUT CARCINOMA

GABRIEL RUIZ ALÍAS

Bachelor's Degree in Biotechnology

VHIO tutors: Dr. Tian Tian and Carmen Escudero

UVIC-UCC tutor: Dr. Mireia Olivella

Vic, June 2022

Agraïments

En primer lloc, m'agradaria donar-li les gràcies a la Carmen Escudero i al Tian Tian, per la seva ajuda i supervisió durant tot el projecte i per afavorir el meu aprenentatge de la millor manera possible. Gràcies a tots els integrants del grup de recerca, per acollir-me com un més i fer d'aquests mesos una experiència tan agradable i enriquidora. I en concret, a l'Iker Benavides per la cooperació durant la realització dels nostres TFG respectius.

A més, voldria agrair a la Mireia Olivella pels seus consells i orientació com a tutora acadèmica, així com a la Universitat de Vic i al Vall d'Hebron Institut d'Oncologia per permetre als estudiants aquestes estades tan enriquidores per a la nostra formació.

Finalment, m'agradaria donar gràcies a la meva família i amics, per la seva confiança incondicional i recolzament tan essencials al llarg de tots els meus estudis.

Resum

Títol: L'efecte de la fusió *BRD4-NUT* en l'estat d'estrès replicatiu al carcinoma NUT

Autor: Gabriel Ruiz Alías

Tutors VHIO: Dr. Tian Tian, Carmen Escudero

Tutora UVic: Dra. Mireia Olivella

Grup hoste: Noncolorectal Gastrointestinal Cancer Translational Research Group – Vall d'Hebron Institute of Oncology (VHIO)

Data: Juny 2022

Paraules clau: NUT carcinoma, *BRD4*, *NUT*, Replication Stress

El carcinoma NUT (NC) és un tipus de càncer humà rar i molt agressiu provocat per reordenacions genòmiques, que resulten en fusions entre el gen específic de testicles *NUT* i gens codificants per reguladors epigenètics, generalment *BRD4.* La proteïna de fusió BRD4-NUT genera megadominis d'histones acetilades, comportant un augment significatiu de la transcripció. L'agressivitat, diagnosi incorrecte i una mala resposta als tractaments convencionals estan associats amb la reduïda supervivència dels pacients de NC: al voltant de nou mesos des del diagnosi inicial. Tot i que s'ha aconseguit avançar en el coneixement de l'oncogènesi de NC, encara no es disposa d'una estratègia terapèutica efectiva pels pacients. Estudis previs en el laboratori hoste van mostrar que les cèl·lules NC eren sensibles a un conjunt d'inhibidors de 'DNA damage response' (DDRi) *in vitro* i *in vivo.* Donat que les proteïnes DDR són necessàries per resoldre l'estrès replicatiu (RS) trobat al càncer, hem tractat d'estudiar si la proteïna de fusió BRD4-NUT està associada amb RS a les cèl·lules NC. Els nostres estudis poden ajudar a entendre perquè les cèl·lules NC són sensibles a DDRi.

Summary

Title: The effect of *BRD4-NUT* fusion on replication stress status of the NUT carcinoma

Author: Gabriel Ruiz Alías

VHIO tutors: Dr. Tian Tian, Carmen Escudero

UVic tutor: Dr. Mireia Olivella

Host group: Noncolorectal Gastrointestinal Cancer Translational Research Group – Vall d'Hebron Institute of Oncology (VHIO)

Date: June 2022

Keywords: NUT carcinoma, *BRD4*, *NUT*, Replication Stress

NUT carcinoma (NC) is a rare and highly aggressive human cancer type driven by genomic rearrangements, resulting in fusions between testis-specific gene *NUT* and genes encoding for epigenetic regulators, mostly *BRD4*. The fusion protein BRD4-NUT generates megadomains of acetylated histones, entailing a significant transcription increase. The aggressiveness, misdiagnosis, and poor response to conventional treatments are associated with dismal survival of NC patients: approximately nine months after the initial diagnosis. Although progress has been achieved in understanding NC oncogenesis, an effective therapeutic strategy for NC patients is still missing. Interestingly, previous work in the host laboratory showed that NC cells are sensitive to a set of DNA damage response inhibitors (DDRi) both *in vitro* and *in vivo*. Given that DDR proteins are required to resolve the replication stress (RS) found in cancer cells, we sought to study here whether BRD4-NUT fusion protein is associated with the RS in NC cells. Our results may help to understand why NC cells are sensitive to DDRi.

Index of contents

FIGURES LIST

TABLES LIST

INTRODUCTION

NUT carcinoma

NUT carcinoma (NC) is an extremely rare and aggressive human squamous cell carcinoma subtype. Unlike other cancer types, NC is not organ-specific and can be found affecting any epithelia as a monomorphic low differentiated squamous cell carcinoma, but also in leukemia[1]. Even though it had been initially believed that NC involved almost exclusively young adults, recent studies showed that it could appear at any stage of life and was not associated with any known carcinogen exposure[2]. Although less than 100 cases were reported worldwide until August 2021[3], it is believed that the number of patients would be higher because NC is frequently misdiagnosed due to its rarity and lack of histological features[4]. Nowadays, the diagnosis of NC relies on multiple approaches such as CT scan, immunohistochemistry, hematoxylin and eosin (H&E) staining, and molecular tests (e.g., Next-generation sequencing). The aggressiveness of this human carcinoma and its poor response to conventional systematic therapy (e.g., chemotherapy) lead NC patients to a short survival rate: around only nine months after the initial diagnosis[1][3].

Molecular pathology of NC

NC is characterized by the presence of chromosomal translations. Around 70-80% of NC cases present gene fusions between the testis-specific *NUT* gene on chromosome 15q14 and the gene encoding for epigenetic regulator BRD4 (Bromodomain protein 4) on chromosome 19p13 (Fig. 1)[4]. It has been shown that the fusion protein BRD4-NUT is a driver of NC oncogenesis by promoting cellular growth and blocking cell differentiation[5]. In addition, *NUT* can also fuse with other genes encoding for epigenetic or transcription regulators, such as *BRD3* (Bromodomain containing 3)[6], *NSD3* (Nuclear receptor binding SET domain protein 3)[7], *CIC* (Capicua transcriptional repressor)[8] and *ZNF532* (Zinc finger protein 532)[9] in few NC patients.

In normal conditions, NUT is specifically expressed in the human testis and restricted to post-meiotic spermatogenic cells, where it acts as a critical protein in the histone to protamine transition[1]. The NUT part in the fusion BRD4-NUT corresponds to almost the whole intact NUT protein. The BRD4 protein is a chromatin reader protein that recognizes and binds acetylated histones[10]. It contains two tandem bromodomains (BD) and one extra terminal domain (ETD). It plays an essential role in transcription regulation and preserves histone acetylation marks across cell divisions[11]. Moreover, BRD4 protein has two isoforms: BRD4L and BRD4S. Whereas these isoforms share two BD and an ETD, the long isoform BRD4L harbors an extended disordered proline-rich region absent in the short isoform BRD4S[12]. Of note, the BRD4 part of the fusion BRD4-NUT corresponds to BRD4S (Fig. 1).

In NC cells, the oncogenic BRD4-NUT fusion protein can recognize and bind to acetylated histones thanks to the BET domains of the BRD4-NUT fusion protein[1]. Once the fusion protein binds to the acetylated histones, the NUT part of the fusion protein can recruit the histone acetyltransferase (HAT) EP300 (E1A binding protein p300) to acetylate surrounding histones, thus resulting in broad histone

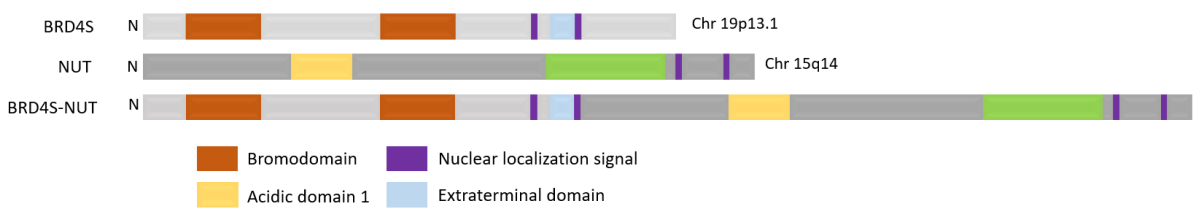

Fig. 1 | BRD4S, NUT, and BRD4-NUT fusion domains. (Adapted from C. French, *10.1146/annurev-pathol-011811-132438*)

domains with acetylation. In addition, these acetylated histones can be recognized by more fusion proteins, generating a positive feedback loop process (Fig. 2)[13].

Interestingly, it has been found that the transcription activities inside the acetylated megadomains are significantly higher than those found in other acetylated loci[14]. Genes associated with NC oncogeneses, such as *MYC*[15] and *p63*[16], were found inside the acetylated megadomains.

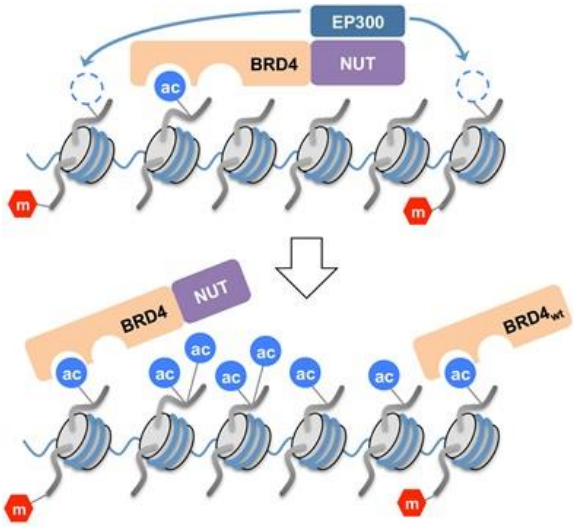

Fig. 2 | Acetylated histone megadomains formation model associated with BRD4-NUT fusion. (Zee BM, et al., *10.1371/journal.pone.0163820*)

R-loops

R-loops are structures consisting of an RNA-DNA duplex and an unpaired DNA strand. They can form upon nascent RNA hybridization with the DNA duplex during transcription to displace the non-template strand[17]. R-loops are widespread in the genome playing important roles in chromatin structure and gene expression regulation[18]. A homeostasis emerges from the constant formation and removal of R-loops throughout the genome. Both R-loop level and distribution in the genome are tightly controlled, and R-loop misregulation is associated with genomic instability leading to neurodegenerative disorders and cancers[19].

Replication stress

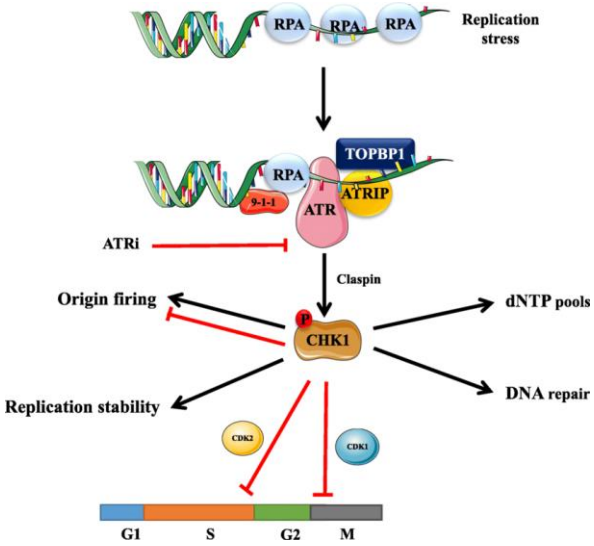

Fig. 3 | ATR-CHK1 activation induced by replication stress. (Mei, L. et al., *10.1186/s13045-019-0733-6*)

Replication stress (RS) is a significant source of genomic instability and a hallmark of cancer cells, and misregulation of R-loop homeostasis is associated with RS[20]. When a single-strand break occurs on DNA, Replication protein A (RPA) binds to single-strand DNA (ssDNA) to protect it and activates the downstream ATR (ATR serine/threonine kinase) signaling pathway[21]: ATR kinase phosphorylates CHK1 (Checkpoint kinase 1), which phosphorylates WEE1 (WEE1 G2 checkpoint kinase) and CDC25 (Cell division cycle 25A) subsequently. The activation of the ATR signaling pathway results in cell cycle arrest and checkpoint activation, preventing cells from entry into mitosis with unreplicated or damaged genomes[22] (Fig. 3).

In an attempt to identify novel therapeutical strategies for NC patients, the host laboratory previously performed a drug screen using NC cell lines. They found that NC cells were sensitive to a set of DDRi, including inhibitors of ATR, CHK1, WEE1, and PARP (Poly ADP-ribose polymerase), all of which are important components for RS-response. These results suggest that these proteins are essential for NC cells to survive due to presumably high RS levels in these cells.

Given that 1) BRD4-NUT fusion is the causal factor of the megadomains of acetylated histone in NC cells[1], 2) the transcription activity is higher inside these megadomains[14], 3) R-loop formation is associated with aberrant transcription process[20] and 4) misregulation of R-loop formation can cause RS that need DDR protein to resolve, we hypothesized that NC cells, characterized by BRD4-NUT fusion, relies on DDR proteins to overcome high RS levels.

OBJECTIVES

In this study, we aim to understand why NC cells are sensitive to inhibitors of ATR, WEE1, CHK1, and PARP1. A hypothesis is that BRD4-NUT fusion protein can generate hyperacetylated histone megadomains in which the aberrant transcription may be associated with R-loop dysregulation and RS. Consequently, NC cells rely on ATR, WEE1, CHK1, and PARP1 proteins to overcome the high levels of RS to survive. Specifically, this TFG project includes three objectives:

1- Generation of cell models in which fusion protein expression can be modulated (loss-of-function and gain-of-function models).
2- Validation of these models by western blot and immunofluorescence.
3- Analysis of RS status and the quantity of R-loop upon fusion protein modulation by immunofluorescence.

METHODS

Cell lines and culture conditions. PER403 cells were maintained in RPMI (Biowest; Ref.: FT.L0501an [23]) with 10% fetal bovine serum (FBS) (Gibco™; Ref.: 10270106 [24]), 1% L-glutamine (Biowest, Ref.: FT.X0550an [25]) and 1% penicillin/streptomycin (pen/strep) (Gibco ™; Ref.:15140122). The NC1015, and HEK293T cells[26] were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Biowest; Ref.: FT.L0106-500 [27]) with 10% fetal bovine serum (FBS) (Gibco™; Ref.: 10270106 [24]), 1% L-glutamine (Biowest, Ref.: FT.X0550an [25]) and 1% penicillin/streptomycin (pen/strep) (Gibco ™; Ref.:15140122).

Cells were cultured at 37ºC and 5% CO_2. Cell culture maintenance was performed twice a week. The cell splits consisted of medium removal, cellular wash with phosphate-buffered saline (PBS), and trypsinization with 0.05% trypsin (Cytiva, Ref.: SH30236.02[28]) for 4 min approximately. Then the fresh medium was added, and cells were passed for subsequent cultures.

Protein extraction and quantification. The medium was collected and put in different falcons from the cell culture plates because detached cells, which probably were those that suffered the highest effect of the treatment, were interesting for our studies. After a PBS wash, coverslips were separated to use at immunofluorescence assay, and attached cells were scrapped and added at respective medium falcons. Falcons were centrifuged at 1,250 rpm for 5 minutes. After that, the supernatant was discarded, and the cell pellets were resuspended with 0.5 mL of PBS, transferred to 1mL Eppendorf tubes, and centrifuged to separate the PBS. Afterward, the corresponding lysis buffer was added to the cell pellets and sonicated at 1.5 power in a Sonicator 3000 (Misonix, Ref.: S-3000-010) for 7-15 seconds and centrifuged at 10,000 rpm for 1 min to eliminate the cell debris. Finally, supernatants were transferred to adequately labeled Eppendorf tubes and stored at -20ºC.

Once the protein extraction had been performed, samples were quantified by Lowry quantification assay with DC Protein Assay kit (Bio-Rad, Refs.: Reagent B: 5000114, Reagent A: 5000113, Reagent S: 5000115). In a 96-well plate, triplicates were made for each sample and blank. In each well, 5uL of sample or blank control were added with 25uL A' reagent (20uL S reagent + 1mL A reagent) and 200uL B reagent. After 10 min of incubation at room temperature, the plate was read in an Infinite® 200 PRO (Tecan, Ref 30050303) plate reader for absorbances. Data analysis consisted of inferring the concentration of the samples and calculating the amount of each sample necessary to load the same protein amount for each condition.

1% SDS Lysis buffer (Cold Spring Harbor): 50mM Tris-HCl pH=8.1, EDTA 10mM, 1% SDS, proteases inhibitors 1:200, phosphatases inhibitors 1:100.

NUT lysis buffer**:** 10 mM Tris-HCl pH=7.5, 150mM NaCl, 1mM EDTA, 1% SDS, proteases inhibitors 1:200, phosphatases inhibitors 1:100.

Western Blot. Western blots were performed according to standard procedures. Once samples were lysed, they were mixed with 5x loading buffer and boiled at 95ºC for 5 min. Protein extracts and Protein marker (Precision Plus Protein™ Standards Dual Color, BIO-RAD) were loaded in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with indicated polyacrylamide percentages ranging from 8% to 15% (Table 1). Electrophoresis gels were run in Tris-glycine-SDS (TGS) buffer for 1.5 hours between 120 and 150mV. After that, proteins were transferred to PVDF (Immobilon-P 0.45µm Transfer membrane) or nitrocellulose membrane (Amersham Protran 0.45µm pore size nitrocellulose, GE Healthcare) in a tray filled with Transfer Buffer. Protein transfers were performed with 40mA overnight at 4ºC or 400mA for 70 min at 4ºC. Membranes were then incubated with Ponceau S staining to verify the protein transference. Next, membranes were blocked for 30 min

with 5% non-fat milk or 5% bovine serum albumin (BSA) in Tris-buffer saline-Tween (TBS-T), depending on where the antibodies were diluted. Primary antibodies (Table 2) were added to a fresh blocking solution and incubated for 1.5 hrs at room temperature or overnight at 4ºC. Three TBS-T washes of 5 min were performed before 1 hr of incubation with corresponding secondary antibodies (Table 3) at room temperature. Three TBS-T washes of 5 min were performed. Finally, membranes were developed with SuperSignal West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific, Ref.: 34580), an enhanced chemiluminescent (ECL) Western blotting detection reagent in an Amersham™ Imager 600 (GE Healthcare Bio-Sciences Corp.)

6X Laemmli SDS sample buffer: 0.3 M DTT

Tris-glycine-SDS (TGS) buffer: 25 mM Tris-OH pH=8.3, 192 mM glycine, 5% SDS

Transfer buffer: 50 mM Tris-OH, 396 mM glycine, 0.1% SDS, 20% methanol

Tris-buffer saline-Tween (TBS-T): 25 mM Tris-HCl pH=7.5, 137 mM NaCl, 0.1% Tween

Ponceau S: 0.5% Ponceau, 1% acetic acid

Table 1 | Electrophoresis gels components depending on polyacrylamide concentration.

	8%	10%	12%	15%	Stacking gel 4%
H_2O	4.6mL	4 mL	3.3 mL	2.3 mL	2.85 mL
Tris-HCl 1.5M pH = 8.8	2.5 mL	2.5 mL	2.5 mL	2.5 mL	-
Tris-HCl 0.5M pH = 6.8	-	-	-	-	1.25 mL
SDS 10%	50 uL	50 uL	50 uL	50 uL	25 uL
Acrylamide 30%	2.7 mL	3.3 mL	4 mL	5 mL	850 uL
APS 10%	100 uL	100 uL	100 uL	100 uL	50 uL
TEMED	10 uL	10 uL	10 uL	10 uL	5 uL

Table 2 | References of primary antibodies.

Antibody	Origin	Reference	Company	Dilution	Experiment
NUT	Rabbit	36255	Cell signaling	1:5,000	Wb
CHK1 pS317	Rabbit	2344T	CST	1:1,000	WB
CHK1 Total	Mouse	2360S	Cell signaling	1:1,000	WB
RPA pS33	Rabbit	A300-246A	Bethyl	1:1,000	WB
RPA pT21	Rabbit	Ab61065	Abcam	1:1,000	WB
RPA total	Mouse	Ab2175	Abcam	1:1,000	WB
Vinculin	Rabbit	Ab129002	Abcam	1:2,000	WB
H3	Rabbit	Ab1791	Abcam	1:10,000	WB
Tubulin	Mouse	T9026	Sigma	1:10,000	WB
S9.6	Mouse	ENH001	Kerafast	1:600	IF
Flag	Mouse	F7425	Sigma	1:200	IF

Table 3 | References of secondary antibodies.

Antibody	Origin	Reference	Company	Dilution	Experiment
Anti-rabbit	Donkey	711-035-152	Jackson Immunoresearch	1:2,000	WB
Anti-mouse	Goat	G21040	Invitrogen	1:2,000	WB
Alexa fluor 555 anti-mouse	Goat	A32727	Invitrogen	1:1,000	IF
Alexa fluor 555 anti-rabbit	Goat	A32732	Invitrogen	1:1,000	IF
Alexa fluor 488 Anti-mouse	Donkey	20014	Biotium	1:1,000	IF
Alexa fluor 488 anti-rabbit	Donkey	20015	Biotium	1:1,000	IF

Immunofluorescence assay. Cells were grown on coverslips and were fixed with 4% PFA for 10 min and permeabilized with PBS-triton for 5 min. Then, cells were blocked with PBS – BSA 1% for 30 min to 1 hr before incubating with primary antibodies (Table 2) for 1 hr at room temperature. Three PBS washes were performed before the incubation with corresponding secondary antibodies. Secondary antibodies (Table 3) were conjugated with fluorescent dyes (Alexa Fluor®). After two PBS washes, a PBS-DAPI (0.25ug/mL) incubation was performed to stain the nuclei, and the coverslips were mounted with Fluoromount-G ® (SouthernBiotech). Fluorescent images were accomplished with a Nikon C2+ Confocal Microscope and were analyzed with ImageJ software.

Lentiviral infection. For the overexpression model, we used a two-component Tet-on system. First, lentivirus containing rtTA construct and hygromycin resistance was used to infect HEK293T cells. Then rtTA expressing HEK293T cells were subsequently infected with lentivirus containing puromycin resistance and NUT, BRD4, BRD4-NUT, or GFP constructs, respectively. The infection was performed when HEK293T cells were at 60-70% confluency in a 6-well plate. First, the medium was changed, and 1mL of the virus-containing medium and 1uL polybrene was added. The plate was centrifuged at 32ºC for 1.5 hrs at 1000 G to increase infection efficiency. After centrifugation, fresh medium was added to each well. The next day, cells were infected again with the same protocol. After 48 hrs of the second infection, cells were selected with 200ug/mL hygromycin and 1ug/mL puromycin.

Statistical analysis. An unpaired two-tailed student t-test was performed in all immunofluorescence data analyses. This procedure compares averages of two unrelated groups to determine if there is a significant difference between them.

RESULTS

MZ1 rapidly eliminates BRD4-NUT fusion protein in NC cells. Proteolysis targeting chimeric (PROTAC) are compounds that bind ubiquitin ligase to degrade proteins[29]. MZ1, a novel PROTAC compound specific to BRD4 protein, has been used in different studies[29][30]. We chose to treat NC cells with MZ1 to eliminate BRD4-NUT fusion. To confirm MZ1's BRD4 degradation activity and define an appropriate time exposure in NC cell lines, we first treated two NC cell lines, NC1015 and PER403 cells, with 100nM of MZ1 for 24 hours and collected proteins lysates at 0h, 1hr, 2hrs, 4hrs, 8hrs and 24hrs (Fig. 4a). To detect BRD4-NUT fusion protein expression, we used an antibody specifically against NUT protein. Because NUT protein is typically expressed in testis but not in other tissues, this antibody can detect specifically fusion protein expression in cancer cells. Of note, this antibody has also been clinically validated to detect BRD4-NUT fusion protein in patients[31]. Western blot analysis showed that in NC1015 cells, MZ1 successfully eliminated BRD4-NUT fusion protein over time. In addition, we also confirmed that a 4-hour treatment of MZ1 can eliminate fusion protein expression by immunofluorescence (Fig. 4b,c). Similar results were also observed in PER403 cells (Fig. 4d,e). Thus, we found that 100nM MZ1 is sufficient to induce fusion degradation within 4hrs in NC1015 and PER403 cell lines.

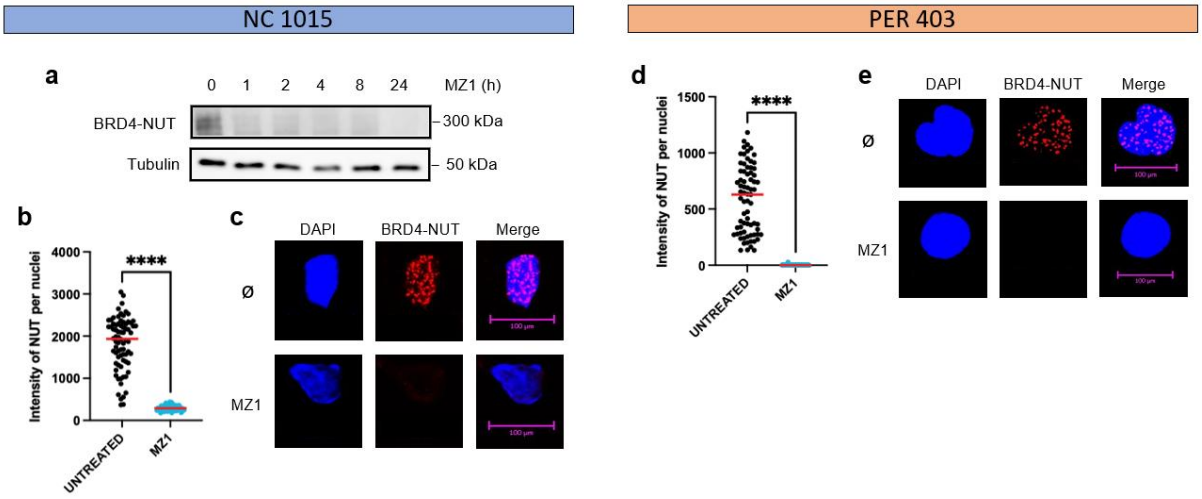

Fig. 4 | MZ1 treatment eliminates BRD4-NUT fusion protein in NC cell lines. a, Western blot analysis of BRD4-NUT expression in NC1015 cell line treated with 100nM MZ1 at different time points. Tubulin was used as a loading control. **b,** Immunofluorescence assay of BRD4-NUT expression in NC1015 treated with 100nM MZ1 for 4hrs. Untreated cells were used as controls. Each dot represents the immunofluorescence intensity measured per nuclei. **c**, Representative BRD4-NUT expression and DAPI staining in both MZ1-treated and -nontreated NC1015 cells analyzed in **b**. **d**, Immunofluorescence assay of BRD4-NUT expression in PER403 treated with 100nM MZ1 for 4hrs and untreated. Untreated cells were used as controls. Each dot represents the immunofluorescence intensity measured per nuclei. **e**, Representative BRD4-NUT expression and DAPI staining in both MZ1-treated and -nontreated PER403 cells analyzed in **d.** Statistics were performed using two-tailed t-student tests. **** $p<0.0001$.

R-loop formation decreased upon MZ1 treatment in NC cell lines. To detect the R-loop, we have used R-loop specific antibody S9.6. Using immunofluorescence staining, we tried to study if the downregulation of the fusion is associated with R-loop homeostasis in NC cells. With this experiment, we found that, in both NC cell lines, the R-loop signal significantly decreased upon MZ1 treatment (Fig. 5). These results suggest that eliminating fusion proteins in NC cells is associated with decreased R-loop formation.

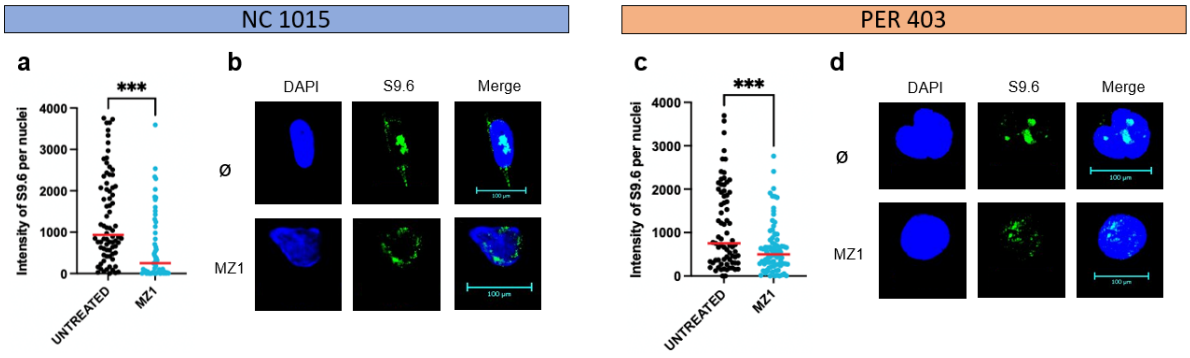

Fig. 5 | MZ1 treatment leads to decreased R-loop formation in NC cells. a, Immunofluorescence assay of R-loop presence with R-loop specific antibody S9.6 in NC1015 cells treated with 100nM MZ1 for 4 hrs compared to those untreated cells. Each dot represents the immunofluorescence intensity measured per nuclei. **b**, Representative S9.6 and DAPI staining in both untreated and MZ1 treated NC1015 cells analyzed in **a**. **c**, Immunofluorescence assay of R-loop presence with R-loop specific antibody S9.6 in PER403 cells treated with 100nM MZ1 for 4 hrs compared to those untreated cells. Each dot represents the immunofluorescence intensity measured per nuclei. **d**, Representative S9.6 and DAPI staining in both untreated and MZ1 treated PER403 cells analyzed in **c.**

MZ1 treatment is associated with decreased RS markers in NC cells. To test whether there is a relationship between fusion protein and RS, PER403 and NC1015 cell lines were treated with MZ1, and RS markers were examined by either Western blot or Immunofluorescence. We first verified both total CHK1 and phosphorylated CHK1 expression by western blot. In fact, CHK1 phosphorylation in the serine 317 (S317), as an intermediate in the ATR signaling pathway, is a marker of replication stress[22]. We found that in PER403 cells, there is a significantly decreased expression of total CHK1 and phosphorylated CHK1 (pS317) upon MZ1 treatment (Fig. 6a). This effect was not observed in NC1015 cells.

Moreover, we also analyzed replication protein A (RPA) by immunofluorescence. Of note, the phosphorylation of serine 33 (pS33) on RPA is also an RS marker[22]. Immunofluorescence in NC1015 showed a significant decrease in pS33 RPA levels after MZ1 treatment, suggesting that BRD4-NUT fusion elimination is associated with a decreased replication stress in NC cells (Fig. 6b,c).

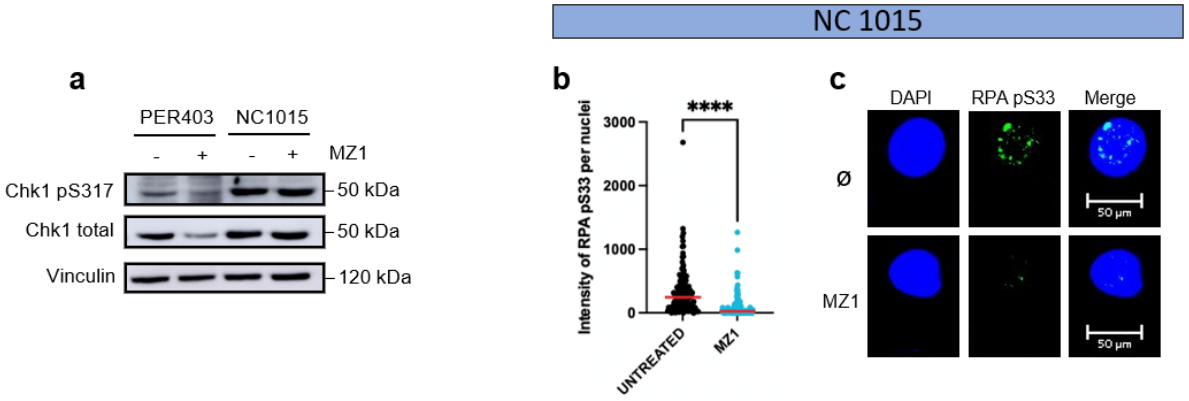

Fig. 6 | MZ1 treatment is associated with decreased RS marker expression levels in NC cells. a, Western blot analysis of pS317 CHK1 and total CHK1 expression in PER403 and NC1015 treated with 100nM MZ1 for 4 hours. Untreated cells were used as controls. Vinculin was used as a loading control. **b**, Immunofluorescence assay of pS33 RPA in CSK-treated NC1015 treated with 100nM MZ1 for 4 hours compared to those untreated cells. Each dot represents the immunofluorescence intensity of pS33 RPA measured per nuclei. **c**, Representative pS33 RPA and DAPI staining in both untreated and MZ1 treated NC1015 cells analyzed in **b**.

Overexpression models were partially validated. To strengthen the conclusion obtained with the previously discussed loss-of-function model, we also generated gain-of-function models in which GFP (control), BRD4S, NUT, and BRD4-NUT can be expressed in a doxycycline-inducible manner in fusion negative cells. First, fusion negative HEK293T cells were engineered to express rtTA stably. Then these cells were infected with lentivirus containing constructs with rtTA binding site (TRE sequence) before the coding sequences of proteins of interest (Fig. 7a). Of note, GFP (control), BRD4S, NUT, and BRD4-NUT were tagged with 3XFLAG and HA to facilitate protein detection. We first checked by immunofluorescence whether the Doxycycline can induce GFP expression in HEK293T cells expressing GFP construct. As shown in Fig. 7b, upon 48 and 72 hours of doxycycline treatment, all cells became GFP positive, whereas control cells (untreated) were GFP negative. Next, a western blot was performed to validate the expression of the different proteins using the FLAG antibody, as all the constructs contained a 3XFLAG tag. We found that only GFP and BRD4S were detected with 72-hour doxycycline treatment (Fig. 7c).

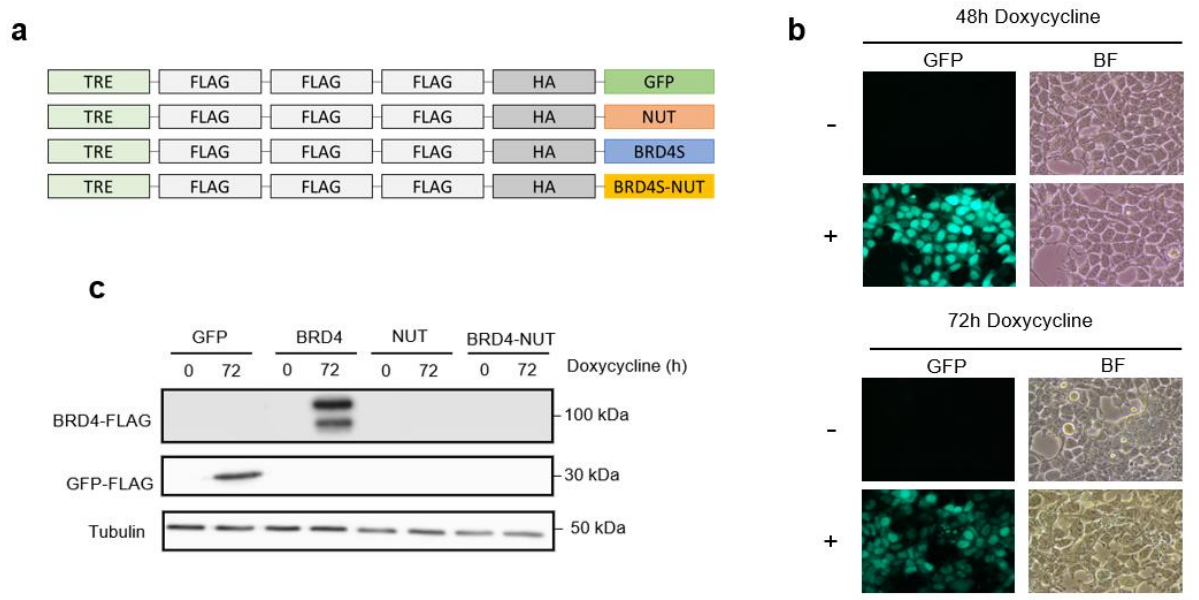

Fig. 7 | Overexpression of GFP, NUT, BRD4S, or BRD4-NUT in HEK293T cells. a, Schematic illustration of the constructs ectopically expressed in HEK293T cells. **b**, GFP expression by immunofluorescent in HEK293T cells expressing doxycycline-inducible GFP construct. Representative immunofluorescent and bright-field images of cells treated with doxycycline for 48 or 72 hrs. Untreated cells were used as controls. **c**, Western blot analysis to evaluate the expression of the constructs. Tubulin was used as a loading control.

DISCUSSION

MZ1 is able to degrade BRD4-NUT fusion protein in NC cells. MZ1 was confirmed to be a good BRD4-NUT degrader in NC cells, as shown in other studies[10][29][30]. We found that the treatment of 100nM of MZ1 for only 4 hours is sufficient to degrade BRD4-NUT protein in NC1015 and PER403 cells. Therefore, we have used this experimental condition for subsequent studies. However, we should note that in NC1015 and PER403 cells, there is also a non-translocated *BRD4* locus. Thus, MZ1 treatment can lead to not only BRD4-NUT degradation but also wild-type BRD4 elimination. Consequently, the results obtained in this "loss-of-function" model should be interpreted with precautions. Any conclusive claims should be made in combination with the "gain-of-function." Nonetheless, as the expression of BRD4-NUT protein was found in approximately 80% of NC patients [5], MZ1 has potential application for NC treatment.

BRD4-NUT fusion and R-loop formation in NC cells. Increased R-loop formation can be found when transcription is dysregulated. In this circumstance, collisions between replication and transcription complexes frequently occur, resulting in replication stress[18][32]. We found a decreased R-loop formation in MZ1-treated NC cells, suggesting the relationship between fusion protein and R-loop formation. However, these results are preliminary. We are not sure whether or not the decreased R-loop formation upon fusion protein elimination is dependent on the transcription process. To test this, we could use inhibitors specific to transcription, such as actinomycin D, to test whether transcription inhibition could "rescue" the phenotype observed in NC cells treated with MZ1. Another limitation we should consider in interpreting these results is that the specificity of the S9.6 antibody was not yet controlled in these experiments. We could include, as a control, RNAseH, an endonuclease that catalyzes RNA cleavage in RNA-DNA hybrids during replication and repair[33]. This experiment will allow us to distinguish "real" R-loops that will be eliminated upon RNAseH treatment from the background.

BRD4 and RS markers levels. Many studies report the implications of RS in many cancer types[20][34]. When RS is detected, the ATR signaling pathway is essential in cell cycle arrest to avoid further DNA damage[22][35]. We observed that RS markers (e.g., pCHK1 and pRPA) decreased in NC cell lines upon MZ1 treatment, suggesting a potential association between the fusion protein and RS regulation. Notably, we observed a statistically significant decrease of pS33 RPA by immunofluorescence in NC1015 and a reduction of pS317 CHK1 in PER403 by western blot. However, we did not observe an apparent reduction of pS317 CHK1 in NC1015 treated with 100nM MZ1 for 4 hours. It is possible that in NC1015, a longer treatment of MZ1 may be needed to detect the CHK1 changes. Therefore, more experimental optimizations will be required to detect pCHK1 expression change in NC1015 cells. Moreover, RS primarily occurs during the S phase[35]. We could synchronize the cells with serum starvation before MZ1 treatment to evaluate the RS in a more controlled manner.

Overexpression model validation. A Tet-On gene expression model allows us to control the expression of a protein of interest upon doxycycline treatment. This project sought to use this system to ectopically express BRD4S, NUT, BRD4-NUT, and GFP (control) in fusion-negative HEK293T cells. Before studying whether ectopic expression of the fusion protein can induce R-loop formation and RS, we needed to validate these doxycycline-inducible overexpression models. To date, we could validate GFP and BRD4S models. In the BRD4 expression model, we could observe two different bands around the desired molecular weight in the western blot: the expected weight corresponds to the lower band, and the upper band may correspond to the protein with post-translational modifications. On the other hand, we could not detect the expression of NUT and BRD4-NUT in the corresponding overexpression models. We think two possible reasons to explain this: 1) transgene silencing and 2) methods to detect these proteins with high molecular weight need to be further optimized (e.g., extraction buffer, transfer protocol).

CONCLUSIONS

The paucity of treatment strategies to fight against NC is one of the reasons for the NC patients' short lifespan. Here, in a cellular model, we tried to study whether BRD4-NUT fusion is associated with RS, which could explain the sensitivity of NC cells to DDRi previously observed in the host laboratory.

Although most of the results are not conclusive yet, there are several promising results. We found that eliminating BRD4-NUT fusion protein in NC cells by MZ1 treatment is associated with decreased R-loop formation and reduction of RS markers. However, this needs to be verified in overexpression models in the future.

Understanding the biology underlying NC tumorigenesis and progression is crucial for designing novel therapeutic strategies for NC patients.

REFERENCES

1. Gu B, Hakun MC, Kawauchi K, Tanaka N, Okada M. Challenges and Opportunities in NUT Carcinoma Research. 2021;
2. Salati M, Baldessari C, Bonetti LR, Messina C, Merz V, Cerbelli B, et al. NUT midline carcinoma: Current concepts and future perspectives of a novel tumour entity. Crit Rev Oncol Hematol 2019;144(October):102826.
3. Zhang Y, Han K, Dong X, Hou Q, Li T, Li L, et al. Case Report and Literature Review: Primary Pulmonary NUT-Midline Carcinoma. Front Oncol 2021;11:3332.
4. Moreno V, Saluja K, Pina-Oviedo S. NUT Carcinoma: Clinicopathologic Features, Molecular Genetics and Epigenetics.
5. Alekseyenko AA, Walsh EM, Wang X, Grayson AR, Hsi PT, Kharchenko P V, et al. The oncogenic BRD4-NUT chromatin regulator drives aberrant transcription within large topological domains. 2015;
6. French CA, Ramirez CL, Kolmakova J, Hickman TT, Cameron MJ, Thyne ME, et al. BRD–NUT oncoproteins: a family of closely related nuclear proteins that block epithelial differentiation and maintain the growth of carcinoma cells. Oncogene 2008 2715 2007;27(15):2237–42.
7. French CA, Rahman S, Walsh EM, Kühnle S, Grayson AR, Lemieux ME, et al. NSD3-NUT Fusion Oncoprotein in NUT Midline Carcinoma: Implications for a Novel Oncogenic Mechanism. Cancer Discov 2014;4(8):928–41.
8. Schaefer IM, Dal Cin P, Landry LM, Fletcher CDM, Hanna GJ, French CA. CIC-NUTM1 fusion: A case which expands the spectrum of NUT-rearranged epithelioid malignancies.
9. Alekseyenko AA, Walsh EM, Zee BM, Pakozdi T, Hsi P, Lemieux ME, et al. Ectopic protein interactions within BRD4-chromatin complexes drive oncogenic megadomain formation in NUT midline carcinoma.
10. Otto C, Schmidt S, Kastner C, Denk S, Kettler J, Müller N, et al. Targeting bromodomain-containing protein 4 (BRD4) inhibits MYC expression in colorectal cancer cells. Neoplasia (United States) 2019;21(11):1110–20.
11. UniProt. UniProtKB - O60885 (BRD4_HUMAN) [Internet]. Available from: https://www.uniprot.org/uniprot/O60885
12. Han X, Yu D, Gu R, Jia Y, Wang Q, Jaganathan A, et al. Roles of the BRD4 short isoform in phase separation and active gene transcription. Nat Struct Mol Biol
13. Zee BM, Dibona AB, Alekseyenko AA, French CA, Kuroda MI. The Oncoprotein BRD4-NUT Generates Aberrant Histone Modification Patterns. 2016;
14. Rosencrance CD, Ammouri HN, Yu Q, Ge T, Rendleman EJ, Marshall SA, et al. Chromatin Hyperacetylation Impacts Chromosome Folding by Forming a Nuclear Subcompartment Molecular Cell Chromatin Hyperacetylation Impacts Chromosome Folding by Forming a Nuclear Subcompartment. Mol Cell 2020;78:112-126.e12.
15. Grayson AR, Walsh EM, Cameron MJ, Godec J, Ashworth T, Ambrose JM, et al. MYC, a downstream target of BRD-NUT, is necessary and sufficient for the blockade of differentiation in NUT midline carcinoma. Oncogene 2014;33:1736–42.

16. Zhou L, Yong X, Zhou J, Xu J, Wang C. Clinicopathological Analysis of Five Cases of NUT Midline Carcinoma, including One with the Gingiva. 2020;

17. Zhu YS, Zhu J. Molecular and cellular functions of long non-coding RNAs in prostate and breast cancer. Adv Clin Chem 2022;106:91–179.

18. Petermann E, Lan L, Zou L. Sources, resolution and physiological relevance of R-loops and RNA–DNA hybrids. Nat Rev Mol Cell Biol 2022;0123456789.

19. Allison DF, Wang GG. R-loops: formation, function, and relevance to cell stress. Cell Stress | Febr 2019;3(2).

20. Bowry A, Kelly RDW, Petermann E. Hypertranscription and replication stress in cancer. Trends in Cancer 2021;7(9):863–77.

21. Vassin VM, Anantha RW, Sokolova E, Kanner S, Borowiec JA. Human RPA phosphorylation by ATR stimulates DNA synthesis and prevents ssDNA accumulation during DNA-replication stress. J Cell Sci 2009;122(22):4070–80.

22. Lecona E, Fernández-Capetillo O. Replication stress and cancer: It takes two to tango. Exp Cell Res 2014;329(1):26–34.

23. Biowest. Technical data sheet RPMI 1640 FT.L0501an. 2014;

24. Gibco. Gibco™ Fetal Bovine Serum.

25. Biowest. Technical data sheet L(+)-Glutamine 100X, 200mM.

26. Graham FL, Smileyt J, Russell WC, Nairn R. Characteristics of a Human Cell Line Transformed by DNA from Human Adenovirus Type 5. J gen Virol 2977;36:59–66.

27. Biowest. Technical data sheet DMEM High Glucose FT.L0106an. 2021;

28. Cytiva. HyClone Trypsin Protease [Internet]. [cited 2022 Apr 28];Available from: https://www.cytivalifesciences.com/en/us/shop/cell-culture-and-fermentation/reagents-and-supplements/cell-disassociation-reagents/hyclone-trypsin-protease-p-00445#related-documents

29. Del Mar Noblejas-López M, Nieto-Jimenez C, Burgos M, Gómez-Juárez M, Montero JC, Esparís-Ogando A, et al. Activity of BET-proteolysis targeting chimeric (PROTAC) compounds in triple negative breast cancer.

30. Zengerle M, Chan KH, Ciulli A. Selective Small Molecule Induced Degradation of the BET Bromodomain Protein BRD4. ACS Chem Biol 2015;10:29.

31. Haack H, Johnson LA, Fry CJ, Crosby K, Polakiewicz RD, Stelow EB, et al. Diagnosis of NUT Midline Carcinoma Using a NUT-Specific Monoclonal Antibody. Am J Surg Pathol 2009;33(7):984.

32. Crossley MP, Bocek M, Cimprich KA. R-Loops as Cellular Regulators and Genomic Threats. Mol Cell 2019;73(3):398–411.

33. Cerritelli SM, Crouch RJ. Ribonuclease H: the enzymes in eukaryotes.

34. Brickner JR, Garzon JL, Cimprich KA. Walking a tightrope : The complex balancing act of R-loops in genome stability. Mol Cell 2022;

35. Gaillard H, García-Muse T, Aguilera A. Replication stress and cancer. Nat Rev Cancer 2015;15(5):276–80.