"Z4" Complex Member Fusions in NUT Carcinoma: Implications for a Novel Oncogenic Mechanism

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Abstract

Nuclear protein in testis (NUT) carcinoma (NC) is a rare, distinctly aggressive subtype of squamous carcinoma defined by the presence of NUT-fusion oncogenes resulting from chromosomal translocation. In most cases, the NUT gene (NUTM1) is fused to bromodomain containing 4 (BRD4) forming the BRD4-NUT oncogene. Here, a novel fusion partner to NUT was discovered using next-generation sequencing and FISH from a young patient with an undifferentiated malignant round cell tumor. Interestingly, the NUT fusion identified involved ZNF592, a zinc finger containing protein, which was previously identified as a component of the BRD4-NUT complex. In BRD4-NUT–expressing NC cells, wild-type ZNF592 and other associated "Z4" complex proteins, including ZNF532 and ZMYND8, colocalize with BRD4-NUT in characteristic nuclear foci. Furthermore, ectopic expression of BRD4-NUT in a non-NC cell line induces sequestration of Z4 factors to BRD4-NUT foci. Finally, the data demonstrate the specific dependency of NC cells on Z4 modules, ZNF532 and ZNF592.

Implications: This study establishes the oncogenic role of Z4 factors in NC, offering potential new targeted therapeutic strategies in this incurable cancer.

Visual Overview: http://mcr.aacrjournals.org/content/molcanres/16/12/1826/F1.large.jpg.

Introduction

Nuclear protein in testis (NUT) carcinoma (NC, also known as NUT midline carcinoma) is a rare, aggressive cancer of squamous origin affecting patients of all ages, but predominantly children and young adults. NC is one of the most aggressive solid tumors known, with a median survival of 6.7 months (1), and no known effective treatment, despite the emergence of targeted inhibitors (2). NC is characterized by the presence of chromosomal translocation involving rearrangement of the NUT gene (also known as NUTM1) on chromosome 15q14 encoding the NUT (3). In most cases, NUT is fused to BRD4, and its encoded fusion oncoprotein, BRD4-NUT, drives growth through the blockade of differentiation and maintenance of tumor growth (4). BRD4-NUT function...
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depends on its binding to acetylated chromatin through the bromodomains of BRD4, as shown through mutagenesis of key acetyl-histone–binding amino acid residues within the bromodomains of BRD4 (5). Indeed, the antioncogenic activity of the acetyl-histone mimetic prototype bromodomain inhibitor, IQ1, was first demonstrated in NC (6), and has subsequently been shown to have activity in human patients (2). The findings in NC have led to interest in this class of drug in a broad range of cancers; however, its clinical efficacy in NC and other malignancies has been severely limited by toxicity (2). Thus, there remains an urgent need for effective treatment of NC, necessitating a better understanding of the molecular pathway that drives this cancer.

The function of wild-type NUT, whose expression is restricted to the testis (3), is almost completely unknown. Immunoprecipitation of NUT has revealed that NUT interacts with and activates the histone acetyltransferase activity of p300 (7). A study using LacO transgene system with LacI-fused NUT demonstrated that tethering of NUT on the chromatin is sufficient to induce transcriptional activation through recruitment of p300, BRD4, and P-TEFB subunits (8). These findings have led to the hypothesis that BRD4 bromodomains tether BRD4-NUT to chromatin-containing acetylated histones, whereby recruitment of p300 leads to further acetylation, and iterative recruitment of BRD4-NUT and p300 in a feed-forward manner that leads to expansion of BRD4-NUT occupancy across broad regions of chromatin (7, 9). Indeed, comprehensive analysis of genomic regions enriched by BRD4-NUT revealed that BRD4-NUT co-occupies with p300 up to 100 kb to 2 Mb “megadomains” of acetylated chromatin (10). Megadomains drive transcription of underlying coding and noncoding DNA. The targets of BRD4-NUT megadomains critical to the growth and blockage of differentiation of all NCs analyzed include MYC and TP63 (5, 10, 11). Despite these advances, very little is known of what factors determine how and where BRD4-NUT megadomains form, or what their oncogenic functions are.

Although the BET family protein, BRD4, is considered the canonical fusion partner of NUT, several variant NUT fusions have been reported in NC, including BRD3- (4), NSD3- (12), and ZNF532-NUT (11). All of the wild-type counterparts of the variant fusion partners interact with BRD4, and through this interaction are components of the BRD4-NUT oncogenic complex (11). Thus, every known variant fusion causes aberrant association of BET proteins with NUT. Moreover, like BRD4-NUT, the known variant NUT-fusion oncoproteins form megadomains that drive expression of MYC (10, 11) and enforce growth and the blockade of differentiation (4, 12), implicating their key oncogenic role in NC. Hence, identification of variant NUT-fusion partners has proven a powerful means to identify indispensable members of the BRD4-NUT oncogenic complex, a model analogous to the many MLL-fusion genes in infantile leukemia that each plays important roles in the super elongation complex that drives that disease (13).

To illustrate this point, ZNF532 can be fused to NUT to drive the ZNF532-NUT variant of NC, but wild-type ZNF532 is also present as a BRD4-NUT complex interacting protein in classic NC through its association with BRD4 (11). Normally, ZNF532 is a component of a transcriptional coregulator complex, here termed “Z4,” with other zinc finger–containing proteins ZNF592, ZNF687, and ZMYND8 (14, 15). Importantly, all four Z4 factors are present within the BRD4-NUT oncogenic complex (11), and as such may pathologically affect the function of both BRD4-NUT and Z4 complexes. Here, we show direct evidence of the role of Z4 proteins in NC pathogenesis.

Materials and Methods

FISH

Dual-color bring-together and split-apart FISH on 5 μm, formalin-fixed, paraffin embedded (FFPE) sections of surgically removed primary pelvic bone tumor (UNC) was performed as described (16), using the probes described in Supplementary Materials and Methods.

Next-generation sequencing (Archer FusionPlex)

An Anchored Multiplex PCR assay was performed with the Archer FusionPlex solid Tumor Kit (ArcherDX) for detection of targeted fusion transcripts using next-generation sequencing (NGS; ref. 17). Detailed protocol is described in Supplementary Materials and Methods.

Reverse transcriptase PCR and sequencing

Nested RT-PCR was performed as described in Supplementary Materials and Methods. PCR products were identified by 2% agarose gel electrophoresis, and gel-extracted DNA fragments (QIAquick Gel Extraction Kit; Qiagen) were cloned by TOPO TA Cloning Kit (Invitrogen) according to the manufacturer’s instructions and sent for Sanger sequencing (GeneWiz).

Immunoblotting and immunofluorescence

Western blots were performed as described (4). Antibodies used are listed in Supplementary Materials and Methods. Immunofluorescence (IF) was performed as described previously (7) using the antibodies described in Supplementary Materials and Methods.

Histology and immunohistochemistry

Hematoxylin and eosin (H&E) staining was performed using standard procedures. Immunohistochemistry (IHC) was performed on FFPE as described (11) using mAb to NUT (clone C52B1; Cell Signaling Technology).

Cell culture

293T, TC-797 (18), PER-403 (19), 797TRex-FLAG-BRD4-NUT-HA (10), U2OSTRex-FLAG-BRD4-NUT, and 797-ZNF532-NBioTAP cell lines were cultured under conditions described in the Supplementary Materials and Methods.

siRNA transfections

SiRNA (50 nmol/L) was transfected using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer’s instructions. The sequence of siRNA is listed in Supplementary Materials and Methods.

Viability assay

Cells after transfection were plated at 8,000 cells per well on 96-well plates and cultured for 48 or 96 hours (specified in figure legends). The cellular viability was determined by CellTiter-Glo Luminescent Cell Viability Assay (Promega) following the manufacturer’s protocol. Data are shown as mean ± SD from three biological replicates. Statistics used are Student t test (two-tailed), and a P value less than 0.01 was considered as significant.
Results

Identification of ZNF592-NUT fusion

A pelvic tumor in an 18-year-old female was biopsied and revealed an undifferentiated malignant round cell tumor (Fig. 1A) that lacked IHC staining for known epithelial, mesenchymal, or hematolymphoid markers. Because of the undifferentiated characteristics of the tumor and the patient age, we performed IHC with NUT antibody, and this revealed diffuse nuclear speckled staining diagnostic of NC (Fig. 1A). We performed FISH to identify the NUT-fusion partner using probes to the known NUT-fusion partners including BRD4, BRD3, ZNF532, and NSD3, and all failed to demonstrate a fusion (Supplementary Fig. S1A). The patient subsequently developed pulmonary metastases despite intensive chemotherapy and radiotherapy and died 13 months after diagnosis.

We then used an NGS approach in an effort to identify the fusion partner to NUT using RNA extracted from archival FFPE sections of the patient’s tumor. Archer FusionPlex, an NGS derivation of rapid amplification of cDNA ends, was employed and included targeting primers to the 3’ end of known NUT-fusion breakpoints (exon 3). Analysis revealed sequences consistent with a fusion between NUT and a zinc finger protein encoding gene, ZNF592 (Fig. 1B).

RT-PCR on the FFPE-extracted total RNA revealed two products, the larger of which is an in-frame ZNF592-NUT fusion, and the smaller is an out-of-frame sequence corresponding...
with the one detected by Archer FusionPlex (Fig. 1B). Of note, Archer FusionPlex performed on FFPE is often biased toward shorter fragments generated by RNA fragmentation from formalin fixation and does not always identify the in-frame functional product, as in this case. FISH confirmed a ZNF592-NUT fusion (Fig. 1C; Supplementary Fig. S1B).

The identified ZNF592-NUT transcript is 7327 bp and contains the 5′ coding sequence of ZNF592 up to exon 10 fused with exons 2 to 10 of NUT (variant 1, RefSeq_NM_001284292), and it is predicted to encode a 2249 amino acid protein containing amino acids 1 to 1091 of ZNF592 and 3 to 1160 of NUT. The ZNF592 moiety of the fusion protein retains 11 C2H2 zinc finger domains (Fig. 1D). The NUT portion that is essential for interaction between BRD4-NUT and p300 is entirely conserved in ZNF592-NUT; therefore, ZNF592-NUT likely drives aberrant histone hyperacetylation through recruitment of p300, the oncogenic mechanism characteristic of other NCs (7, 10, 11). Indeed, IF performed on the ZNF592-NUT; therefore, ZNF592-NUT likely drives aberrant histone hyperacetylation through the mechanism similar to that of BRD4-NUT and ZNF532-NUT.

Z4 (ZNF592, ZNF352, ZMYND8) components colocalize with and are sequestered by BRD4-NUT

We and others had previously shown that ZNF592 and other zinc finger proteins, ZNF352 (RefSeq_NM_005687), and ZMYND8, collectively termed "Z4," copurify with BRD4/BRD4-NUT chromatin complex proteins (11, 20). We hypothesized that if ZNF592 is associated with BRD4-NUT megadomains, it should form foci similar to those of BRD4-NUT foci observed within the nuclei of NC cells expressing endogenous BRD4-NUT. To determine the pattern of distribution of ZNF592 within the nuclei of NC cells that are driven by endogenous BRD4-NUT, we first performed IF on the NC patient–derived cell line, TC-797. IF revealed that ZNF592 forms discrete foci within the nuclei of TC-797 similar to those of BRD4-NUT (Fig. 2A) and ZNF532-NUT (Fig. 1A). Next, to test whether the nuclear distribution of Z4 factors overlaps with that of BRD4-NUT foci, we used a derivative of TC-797, 797TRex-FLAG-BRD4-NUT-HA (10), that can be induced to express a single dual HA-/FLAG-tagged BRD4-NUT-HA (FLAG-BRD4-NUT-HA) transgene in the presence of 1 μg/ml of tetracycline to allow co-IF staining of tagged BRD4-NUT and ZNF592 or other Z4 factors. Of note, 797TRex also retains expression of endogenous BRD4-NUT; therefore, untagged BRD4-NUT is constitutively expressed regardless of the induction of the tagged protein. We found that FLAG-BRD4-NUT-HA formed overlapping foci with ZNF592, as it did with ZNF532 and ZMYND8 (Fig. 2B). The overlap of these factors with FLAG-BRD4-NUT-HA foci was predicted based on the known association of these factors shown by proteomic and biochemical analyses, and indicates that these Z4 factors colocalize with BRD4-NUT (11, 20). To test whether BRD4-NUT is sufficient to cause recruitment of Z4 factors to BRD4-NUT foci, we expressed FLAG-tagged BRD4-NUT in non-NC U2OSRex cells containing a single isogenic copy of the transgene. We observed strong accumulation of ZNF592 and ZMYND8 at nuclear foci upon induction of the FLAG-BRD4-NUT (Fig. 2C). Of note, induction of FLAG-BRD4-NUT had minimal or no effect on levels of ZNF592 and ZMYND8 (Supplementary Fig. S2).

These findings, together with previous biochemical and proteomic studies (11, 20), suggest that Z4 factors associate with BRD4-NUT within megadomains. Moreover, BRD4-NUT–driven foci formation of Z4 factors suggests a mechanism of pathologic sequestration that may alter the normal function of Z4 proteins while enhancing those of BRD4-NUT, as first proposed for the sequestration of p300 by BRD4-NUT (7, 8, 21).

**Knockdown of ZNF532 or ZNF592 reduces cell viability in NC cells**

Considering the potential functional association of Z4 factors and BRD4-NUT, we tested the effect of siRNA knockdown of the two Z4 factors identified as oncogenic fusions to NUT in NC, ZNF532 and ZNF592, on viability of NC cells. We were unable to obtain sufficient knockdown of ZNF532 using seven siRNAs targeting the gene; therefore, we utilized an alternative approach. Using CRISPR/Cas9, we genomeically inserted a BioTAP tag into the 5′ end of both ZNF532 alleles in TC-797 and knocked the gene down using siRNA targeting the BioTAP-tag (Supplementary Fig. S3A). TC-797s lacking the BioTAP tag were also transfected with siBioTAP as control. siBioTAP efficiently knocked down ZNF532-NBioTAP after 48 hours from the transfection and strongly reduced the viability of 797-ZNF532-NBioTAP cells after 96 hours from transfection, whereas control TC-797 cells were unaffected (Fig. 3A; Supplementary Fig. S3B). Similarly, knockdown of ZNF592 using two siRNAs targeting this gene in TC-797 and another NC cell line, PER-403, caused a strong decrease in viability whereas non-NC 293T cells were unaffected (Fig. 3B). These results indicate that ZNF532 and ZNF592 are critical factors for NC growth and/or viability.

**Discussion**

In this study, we discover a novel ZNF592-NUT fusion in an NC patient and together with previous work demonstrate the importance of Z4 factors ZNF532 and ZNF592 in function of the BRD4-NUT oncogenic complex. Archer FusionPlex analysis is a powerful tool for diagnosis in cancers harboring multiple fusion gene partners, such as NC, where fresh tissue is often not available, and where detection of multiple partner genes is not clinically feasible. The present analysis enabled the identification of a novel NUT–fusion variant, and the method could be used to identify NUT fusions in the future both clinically and as a discovery tool.

Figure 3C shows the working model of the function of NUT complex. In our previous study, we proposed that forced cooperation of NUT and BRD4 (or BRD3), through the BRD4-NUT fusion, or fusion with other subunits in the NUT complex such as ZNF532, drives p300-dependent histone hyperacetylation and megadomain formation (11). The findings in this study add another key factor in this complex, ZNF592, that facilitates linkage between NUT and BET through the ZNF592-NUT fusion. Furthermore, wild-type ZNF32 and ZNF592, two of four Z4 factors found in NUT complex, are essential for growth of BRD4-NUT–driven NC cells. Strikingly, siRNA targeting ZNF592 decreased cell viability specifically in NC cell lines, indicating that ZNF592 plays a key role in BRD4-NUT–dependent growth, and may be a therapeutic target in this disease.

Although understanding of Z4 factor function is limited, some Z4 proteins have been shown to have a role in cancer...
pathogenesis, either as oncogenes or tumor-suppressor genes. RUNX1-ZNF687 and ZMYND8-RELA fusion oncoproteins have been reported in acute myeloid leukemia (22, 23). Moreover, it has been shown that ZMYND8 may function to promote tumor growth through the activation of tumor angiogenesis (24). Conversely, ZMYND8 has been shown to repress the expression of metastasis-linked genes in prostate cancer through combinatorial binding to H3K14ac and H3K4me1 through its PHD finger-bromodomain cassette, and acting as a corepressor of JARID1D, a histone demethylase.

Figure 2. Z4 proteins colocalize with and are sequestered by BRD4-NUT. A, IF of TC-797 cells using anti-NUT and anti-ZNF592 antibodies (magnification, ×1000; scale bar, 10 μm). B, IF of 797Trex cells with (+) or without (-) tetracycline induction of FLAG-BRD4-NUT-HA expression using anti-HA (visualizing FLAG-BRD4-NUT-HA), anti-ZNF532, anti-ZNF592, and anti-ZMYND8 antibodies (magnification, ×1000; scale bar, 10 μm). C, Top: IF of U2OSTrex cells with (+) or without (-) tetracycline induction of FLAG-BRD4-NUT expression using anti-NUT, anti-ZNF592, and anti-ZMYND8 antibodies (magnification, ×1000; scale bar, 10 μm). Bottom: Boxplots showing the number of foci formed by ZNF592 (left) or ZMYND8 (right) in U2OSTrex-FLAG-BRD4-NUT cells treated with ethanol vs. tetracycline. FLAG-BRD4-NUT expression was induced by tetracycline treatment for 36 hours. Up to 40 nuclei were counted. *, P < 0.01, Student t test.
Figure 3. ZNF592 and ZNF532 are critical for NC cell viability. A, Top: Immunoblot of TC-797, 797-ZNF532-NBioTAP at 48 hours after siCTRL or siBioTAP using Peroxidase Anti-Peroxidase (PAP) antibody, which binds the protein A moiety of BioTAP and visualizes ZNF532-NBioTAP protein on the blot. ZNF532-NBioTAP protein band is indicated by a red arrow. Tubulin was used as a loading control. Bottom: Viability assays performed on TC-797 or 797-ZNF532-NBioTAP cells at 96 hours following transfection with siCTRL or siBioTAP. Eight thousand cells were plated per well in a 96-well plate. Error bars represent SD from three biological replicates. *, P < 0.01, Student t test. B, Top: Immunoblot of 293T, PER-403, and TC-797 at 48 hours after transfected with siCTRL, siZNF592-1, or siZNF592-2 with ZNF592 antibody. Tubulin was used as a loading control. Bottom: Viability assay performed on TC-797, PER-403, and 293T cells 48 hours following transfection with siCTRL, siZNF592-1, or siZNF592-2. Eight thousand cells were plated per well in a 96-well plate. Error bars represent SD from three biological replicates. *, P < 0.01, Student t test. C, Mechanistic model of how NUT-fusions function. Associations of NUT, BET protein, and p300 through NUT fusions are essential to drive proliferation and blockade of differentiation by inducing ectopic histone hyperacetylation on broad region of the chromatin ("megadomain"). This association is mediated by interaction of NUT-fusion protein with other associated proteins (BRD4, ZNF532, ZMYND8, ZNF687, and NSD3) or formation of BRD4/4-NUT, NSD3-NUT (not shown here), ZNF532-NUT, or ZNF592-NUT fusion.
often mutated in this malignancy (25). Another important study demonstrated that ZMYND8, together with histone demethylase KDM5C, suppresses overactivation of enhancers/superenhancers of oncogenic genes, including S100, in a breast cancer cell line (26). These studies suggest that ZMYND8 can act either against cancer progression, or in other settings may promote tumor growth. One could speculate that in NC, BRD4-NUT sequesters Z4 and effectively short-circuits its tumor-suppressive function.

The multiple interaction domains of Z4 proteins may also be important in BRD4-NUT function. All components of Z4 contain zinc finger domains known to modulate protein–DNA and/or protein–protein interactions. ZMYND8 contains additional interaction modules that allow for both chromatin reading, DNA-binding, and protein–protein interactions, including PHD, bromodomain, PPWP, and MYND domains. Thus, the multimodality of Z4 suggests a possible function as an interaction hub that could play an important role in the stability of the BRD4-NUT complex. Moreover, the presence of zinc finger DNA-binding modules could impart DNA sequence specificity and influence the genomic localization of BRD4-NUT, which otherwise lacks DNA sequence–specific recognition.

In a clinical context, there is no effective therapy for NC, and there is an immediate unmet need for rational approaches to combating this aggressive cancer. Uncovering the mechanism by which BRD4-NUT may alter Z4 function, and vice versa, is likely to identify targetable factors that are specific for the BRD4-NUT oncogenic pathway.

References


Disclosure of Potential Conflicts of Interest

S.A. Gorman and O. Barbash have an ownership interest (including stock, patents, etc.) in GlaxoSmithKline. C.A. French reports receiving commercial research grant from, and is a consultant/advisory board member for, GlaxoSmithKline. No potential conflicts of interest were disclosed by the other authors.

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