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SHORT COMMUNICATION

BRD–NUT oncoproteins: a family of closely related nuclear proteins that block epithelial differentiation and maintain the growth of carcinoma cells

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An unusual group of carcinomas, here termed nuclear protein in testis (NUT) midline carcinomas (NMC), are characterized by translocations that involve *NUT*, a novel gene on chromosome 15. In about 2/3rds of cases, *NUT* is fused to *BRD4* on chromosome 19. Using a candidate gene approach, we identified two NMCs harboring novel rearrangements that result in the fusion of *NUT* to *BRD3* on chromosome 9. The *BRD3–NUT* fusion gene encodes a protein composed of two tandem chromatin-binding bromodomains, an extra-terminal domain, a bipartite nuclear localization sequence, and almost the entirety of NUT that is highly homologous to BRD4–NUT. The function of NUT is unknown, but here we show that NUT contains nuclear localization and export sequences that promote nuclear-cytoplasmic shuttling via a leptomycin-sensitive pathway. In contrast, BRD3–NUT and BRD4–NUT are strictly nuclear, implying that the BRD moiety retains NUT in the nucleus via interactions with chromatin. Consistent with this idea, FRAP studies show that BRD4, BRD4–NUT and BRD3–NUT have significantly slower rates of lateral nuclear diffusion than that of NUT. To investigate the functional role of BRD–NUT fusion proteins in NMCs, we investigated the effects of siRNA-induced BRD3–NUT and BRD4–NUT withdrawal. Silencing of these proteins in NMC cell lines resulted in squamous differentiation and cell cycle arrest. Together, these data suggest that BRD–NUT fusion proteins contribute to carcinogenesis by associating with chromatin and interfering with epithelial differentiation.
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Nuclear protein in testis (NUT) midline carcinoma (NMC) is defined by chromosomal rearrangements involving *NUT* a novel gene on chromosome 15 (French *et al*., 2001, 2004). NMCs are undifferentiated or poorly differentiated squamous cell carcinomas that arise in the mid-line structures of children or adults. With a single exception (Mertens *et al*., 2006), NMCs have proven fatal within 6 months of diagnosis, even in patients treated with multimodality therapy. In 2/3rds of NMCs, essentially the entire coding region of *NUT* is inserted into the 3′ end of *BRD4*, creating a *BRD4–NUT* fusion gene (French *et al*., 2003). In the remaining cases, *NUT* is rearranged, but the fusion partners are unknown (French *et al*., 2004).

NUT midline carcinomas typically have simple karyotypes; often the only aberration is the rearrangement involving *NUT*. Such simple karyotypes are unusual in carcinomas, which typically have complex chromosomal abnormalities, but common in certain leukemias, lymphomas and sarcomas. Based on precedents in these other tumor types, NUT-fusion proteins may initiate malignant transformation within epithelial cell precursors and require relatively few collaborative mutations or epigenetic changes to produce an NMC.

Proteins of the BRD family contain two bromodomains (BD) that bind transcriptionally active chromatin through associations with acetylated histones H3 and H4 (Dey *et al*., 2003). The BRD4 BD and the flanking extra-terminal (ET) domain also participate in interactions with other proteins, including P-TEF-b (Jang *et al*., 2005; Yang *et al*., 2005), through which BRD4 stimulates RNA-polymerase II transcriptional activity; and LANA-1 proteins (Ottinger *et al*., 2006), which may facilitate the attachment of human herpesvirus 8 to chromosomally associated BRD4 during mitotic segregation. The ET domain of BRD3, which is closely related to BRD4 in structure, also binds LANA-1 (Ottinger *et al*., 2006). Overexpression of BRD4 perturbs cell cycle progression, a phenotype that may be related to additional interactions with SPA-1, a Rap GTPase-activating protein (Farina *et al*., 2004), and replication factor C (Maruyama *et al*., 2002).

Detection and characterization of a *BRD3–NUT* fusion gene

To better understand the structural requirements for epithelial transformation by NUT oncoproteins, we screened NMCs for rearrangements involving other members of the bromodomain gene family. Studies were initiated with three *NUT*-variant NMCs with sufficient paraffin-embedded archival tissue (French *et al*., 2004). Dual-color 'bring-together' FISH (fluorescent *in situ* hybridization) assays were developed for 11 genes encoding bromodomain proteins (Supplementary Data, Supplementary Table 1). In 1 of 3 cases (case 1, a well-differentiated squamous cell carcinoma; Figure 1a), nuclear co-localization of *NUT* and *BRD3* hybridization signals was observed (Figure 1c), suggesting the presence of a *BRD3–NUT* fusion gene. In the other 2 cases, none of the 11 genes encoding bromodomain proteins were rearranged (data not shown). Subsequently, fresh frozen material and viable cells were obtained from a poorly differentiated carcinoma with a three-way 2;9;15 chromosomal translocation with breakpoints near the locations of *BRD3* (9q34.2) and *NUT* (15q14) (case 2; Figure 1b). FISH performed on metaphase and interphase cells from this tumor showed the presence of an apparent *BRD3–NUT* fusion gene (Figure 1d). In line with prior experience, the two patients with BRD3–NUT carcinoma lived 148 weeks (case 1) and 8 weeks (case 2) following initial diagnosis.

Reverse transcriptase–PCR amplification of RNA obtained from case 2 using 5′ *BRD3*- and 3′ *NUT*-specific primers gave a single product, which proved to be a fusion cDNA created by splicing of *BRD3* exon 9 to *NUT* exon 2 (Figure 1e). The *BRD3–NUT* mRNA is predicted to encode a polypeptide comprised of the two bromodomains and the ET domain of BRD3 fused to all but the first 6 amino acids of NUT (Figure 1f). This polypeptide is highly homologous to BRD4–NUT, which in previously analysed tumors (French *et al*., 2003; Haruki *et al*., 2005) was the product of a fusion transcript created by splicing of *BRD4* exon 10b to *NUT* exon 2 (Figure 1f). In the region of structural overlap, BRD3 and BRD4 are 54% identical and 64% similar,

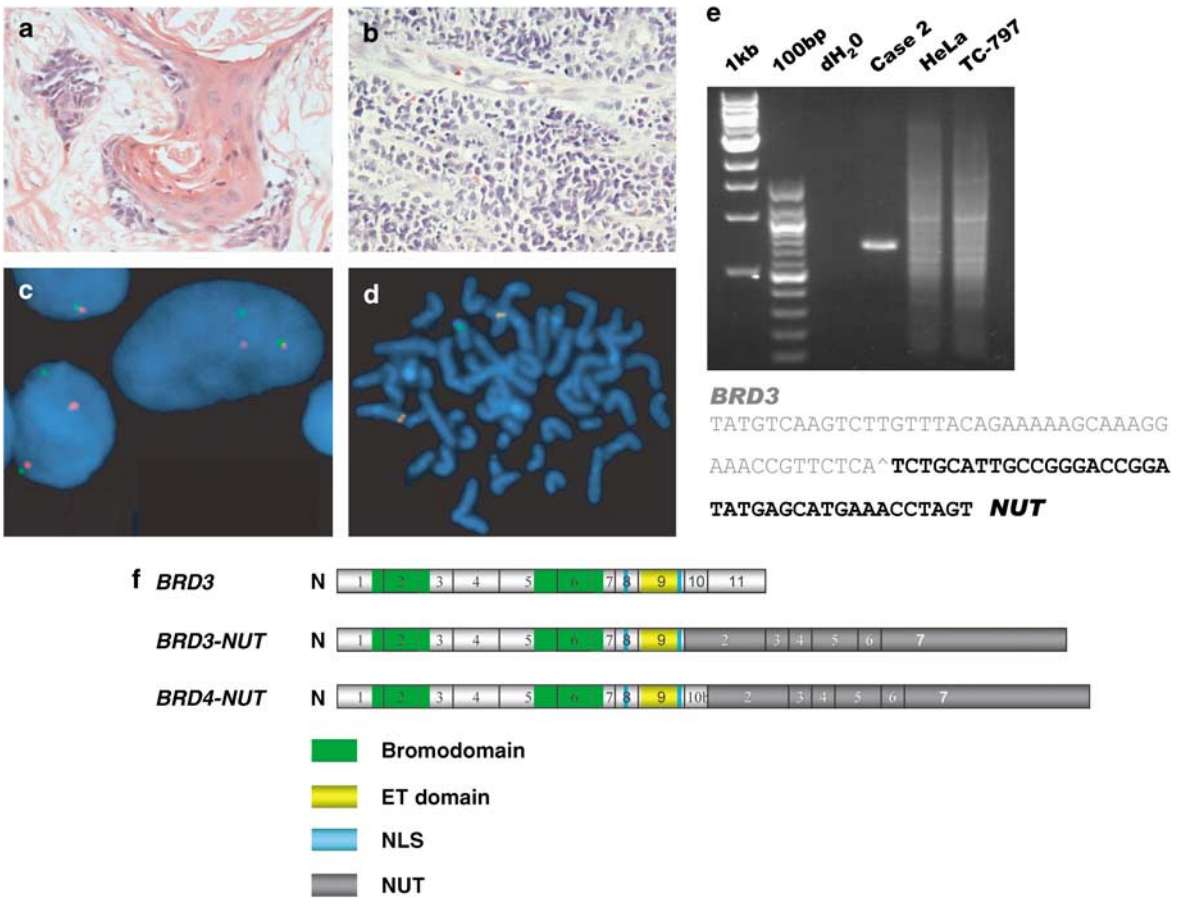


Figure 1 Characterization of *BRD3–NUT*. (**a** and **b**) Histology *BRD3–NUT* carcinomas. Case 1 (**a**), well-differentiated squamous cell carcinoma. Case 2 (**b**), poorly differentiated carcinoma. (**c** and **d**) Fluorescent *in situ* hybridization (FISH) demonstrating fusion of *BRD3* and *NUT*. Fusion signals (arrowheads) are seen in the interphase nuclei of the tumor cells from case 1 (**c**) and a metaphase chromosome preparation of tumor cells from case 2 (**d**). Green signal, probe lying telomeric (3′) of *NUT*; red signal, probe lying telomeric (5′) of *BRD3*. (**e**) Reverse transcriptase–PCR amplification of a ~750 bp *BRD3–NUT* fusion transcript. Case 2, input cDNA from case 2; dH_2O, no input cDNA; HeLa and TC-797, negative control input cDNAs from cell lines lacking *BRD3–NUT* fusion genes. Sequencing of case 2 product revealed an in-frame fusion of *BRD3* exon 9 to *NUT* exon 2. (**f**) Comparison of the predicted structures of BRD3–NUT and BRD4–NUT.

with the highest degree of homology occurring in the BRD and ET domains. Exon 10b of *BRD4* is present normally in an alternatively spliced mRNA transcript that encodes a large BRD4 isoform (French *et al*., 2003). In the *BRD4–NUT* transcript, exon 10b contributes the coding sequence for a short low complexity sequence that is rich in serine residues; based on its absence from *BRD3–NUT*, this sequence is probably not required for oncogenesis. In contrast, attempted RT–PCR with multiple different 5′ *NUT* and 3′ *BRD3* primer pairs failed to yield products (data not shown). Thus, as in translocations involving *BRD4* and *NUT* (French *et al*., 2003; Haruki *et al*., 2005), it appears that the reciprocal *NUT-BRD3* fusion gene is transcriptionally silent.

Identification of BRD3–NUT and BRD4–NUT fusion proteins in NUT carcinoma cells

We developed NUT antibodies to verify expression of BRD3–NUT and BRD4–NUT polypeptides in NMCs. Polyclonal antibodies raised against NUT recognized a single polypeptide of ~215 kDa in lysates prepared from case 2, about the expected size of BRD3–NUT (191 kDa); and a single polypeptide of ~230 kDa in lysates prepared from endogenous BRD4–NUT-expressing TC-797 cells, about the expected size of BRD4–NUT (200 kDa) (Figure 2a). Upon immunohistochemical (IHC) staining, all NMCs (including 2 of 2 tumors with BRD3–NUT rearrangements; 11 of 11 tumors with BRD4–NUT rearrangements; and 3 of 3 evaluable tumors with NUT-variant rearrangements) displayed finely speckled nuclear staining with NUT antiserum (Figures 2b–g, and insets Figures 2b and d). In contrast, poorly differentiated non-small cell carcinomas lacking *NUT* rearrangements (based on FISH analysis showing that *NUT* was intact) demonstrated no staining or weak staining in <10% of tumor cells ($N=10$) (Figure 2h). Although this focal reactivity could indicate variable expression of NUT in typical non-small cell carcinomas, attempts to detect *NUT* mRNA in such tumors has been unsuccessful (data not shown), suggesting that it more likely stems from cross-reactivity with another antigen.

Nuclear retention of BRD–NUT

We sought to determine whether the subcellular localization of NUT differs from that of BRD–NUT fusion proteins. Nuclear localization sequence (NLS), BLASTX, Swiss-Prot, PROSITE and SWISS-MODEL programs identified two predicted nuclear localization signal sequences (NLSs) at the C-terminal end of NUT. However, when expressed transiently, GFP–NUT showed either cytoplasmic or nuclear localization, suggesting that it is subject to nuclear/cytoplasmic shuttling (Figure 3a). Consistent with this possibility, treatment with leptomycin B, an inhibitor of CRM1-dependent nuclear export (Ossareh-Nazari *et al*., 1997) resulted in re-distribution of GFP–NUT to the nucleus (Figure 3b). Inspection of NUT

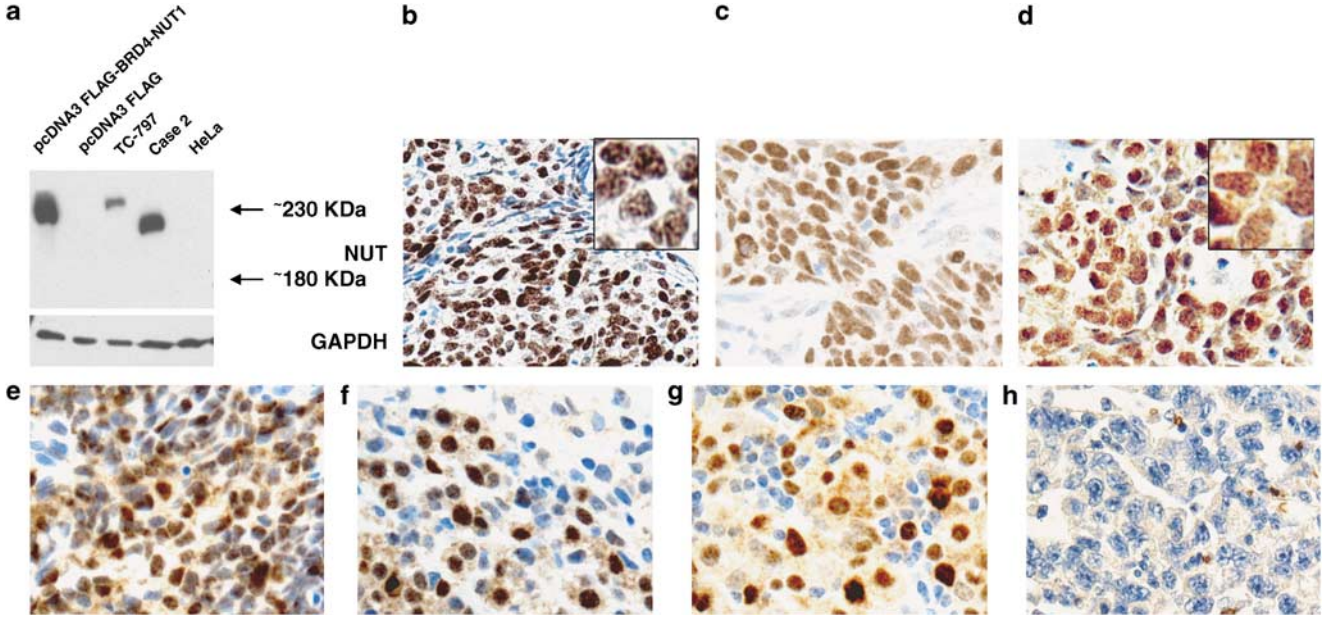
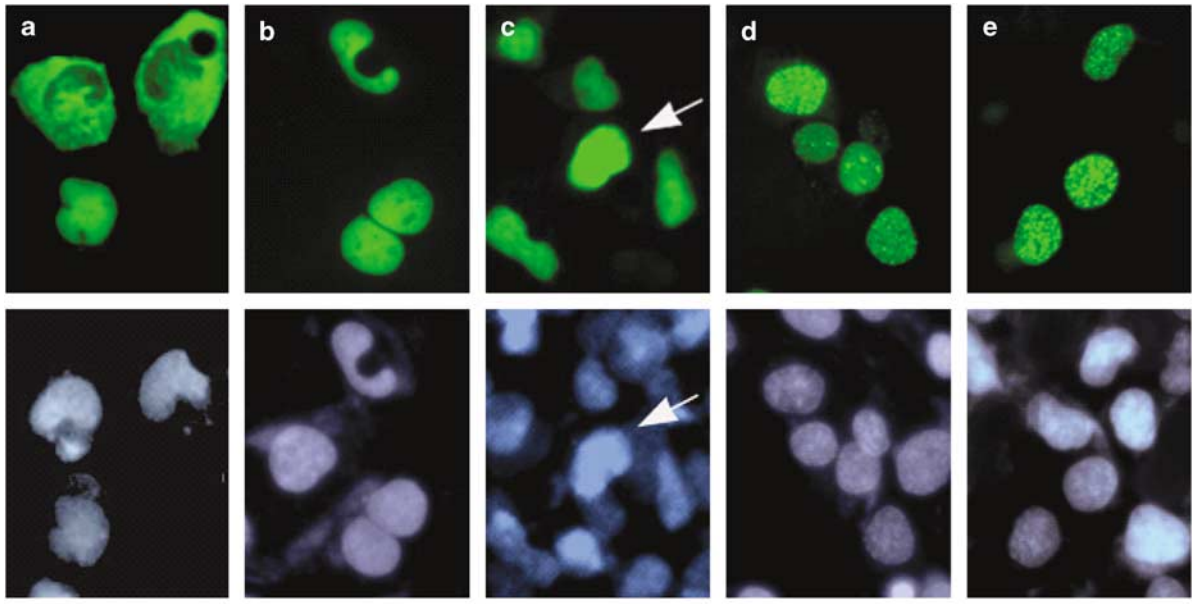


Figure 2 Detection of BRD4–NUT and BRD3–NUT polypeptides. (**a**) western blot analysis with NUT antibody. TC797, cell lysate from a BRD4–NUT cell line (Toretsky *et al*., 2003); case 2, lysate from a BRD3–NUT tumor. Lysates from HeLa cells and 293T cells transfected with pcDNA3 FLAG-BRD4–NUT or empty pcDNA3 vector are included controls. Staining for GAPDH (Ambion, Austin, TX, USA) serves as a loading control. (**b**–**h**) Immunohistochemistry. Representative results of staining with anti-NUT are shown for: (**b**) a NMC with a BRD4–NUT fusion gene; (**c** and **d**) two NMCs with BRD3–NUT fusion genes (case 1, **c**; and case 2, **d**); and (**e**–**g**) three NMCs with uncharacterized NUT-variant fusion genes. (**h**) Staining of a representative squamous cell carcinoma of the lung without a NUT rearrangement. (**b** and **d** insets) A speckled nuclear pattern of NUT antibody staining is characteristic of NMCs.



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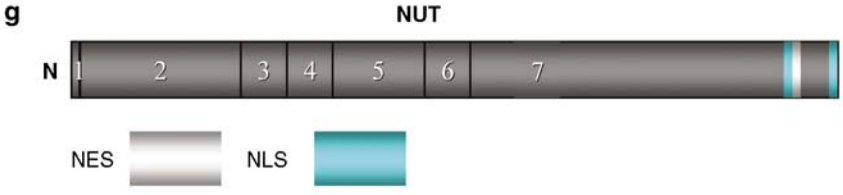

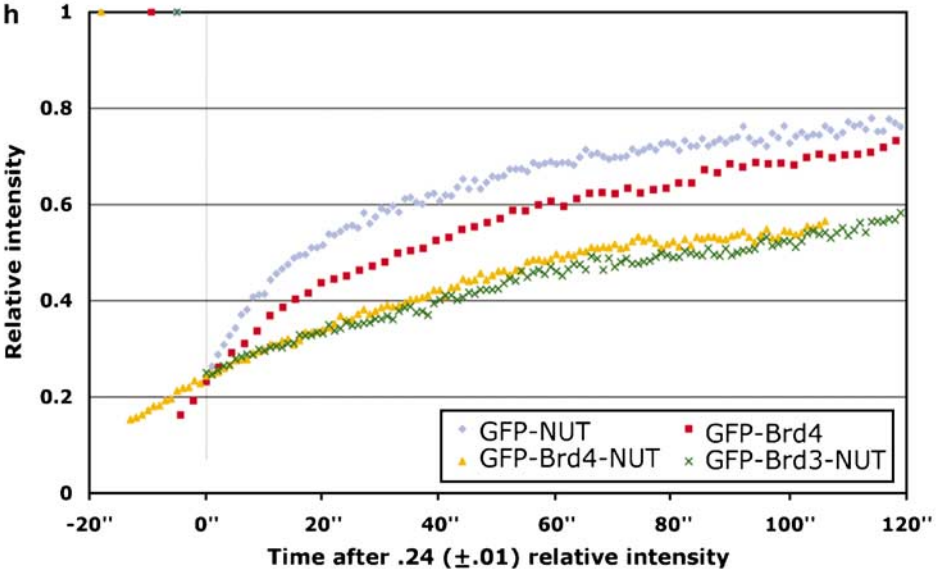

Figure 3 Subcellular localization and nuclear mobility of GFP–NUT and GFP–BRD–NUT fusion proteins. (**a**) GFP–NUT localizes to either the nucleus or the cytoplasm. (**b**) GFP–NUT localizes only to the nucleus of cells treated with leptomycin B. (**c**) GFP–NUT-S1026A/S1029A/S1031A localizes to the nucleus. (**d**) GFP–BRD4–NUT and (**e**) GFP–BRD3–NUT localize to the nucleus in a distinctively speckled pattern. In (**a**–**e**) representative images of transiently transfected U2OS cells are shown: GFP, top; DAPI nuclear counterstain, bottom. (**f**) Sequence of the putative NUT nuclear export signal sequence. S residues affecting export are underlined. (**g**) Schematic of NUT. (**h**) Effect of BD moieties on the distribution and nuclear mobility of NUT. The recovery time after photobleaching is depicted for representative cells. FRAP was performed in triplicate, and findings were similar in all cells tested.

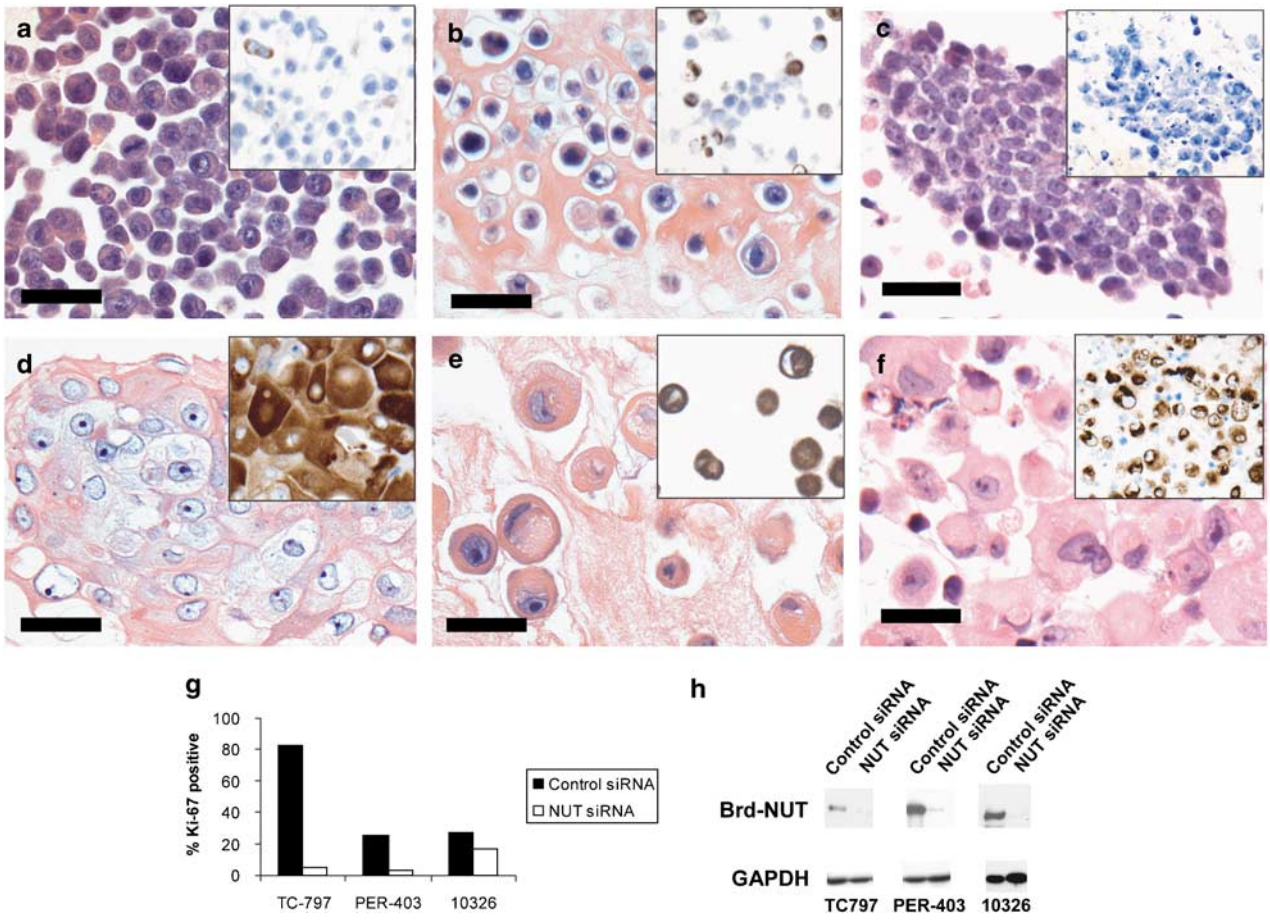

Figure 4 Knockdown of BRD–NUT results in squamous differentiation and growth arrest. (**a**–**c**) Control siRNA has no effect on the TC797, PER-403 (both BRD4–NUT-expressing) and 10326 (BRD3–NUT-expressing) cell lines (hematoxylin and eosin stain, × 400, 96 h post-transfection). PER–403 has been described (Kees *et al*., 1991). (**d**–**f**) Morphologic changes induced by siRNA knockdown of endogenous BRD4–NUT in TC797 and PER-403 cells, and BRD3–NUT in 10326 cells (hematoxylin and eosin stain, × 400, 96 h post-transfection). The pink amorphous extracellular material (**b** and **e**) is fibrin used to produce cell blocks. (**a**–**f**, insets) BRD3/4–NUT knockdown induces keratin expression, as evidenced by increased staining with the pan-keratin monoclonal antibody (clone MNF116, DAKO). (**g**) Knockdown of BRD3/4–NUT decreases proliferation. Staining for Ki-67 + (a marker of cell cycle progression) was performed on sections of cell blocks prepared 96 h post-siRNA transfection. Bars = 50 μm, (**a**–**f**). (**h**) *NUT*-specific siRNA reduces BRD–NUT protein levels in the cell lines TC797, PER-403 and 10326. Protein extracts obtained 24 h after siRNA transfection were analysed on a western blot stained with a polyclonal NUT antibody.

revealed a C-terminal sequence (amino acids 1017–1042) similar to known nuclear export sequences (NES) (Figure 3f), which are often regulated by phosphorylation (Fornerod *et al*., 1997). Consistent with the presence of a functional NES, forms of GFP–NUT with deletions that remove this sequence (not shown) or alanine substitutions at residues S1026, S1029 and S1031 (Figure 3f) localized only to the nucleus (Figure 3c). GFP–NUT polypeptides bearing single or double S to A substitutions of the same three residues demonstrated both nuclear and cytoplasmic staining (data not shown), suggesting that these three S residues influence shuttling of NUT coordinately. We also performed fluorescence recovery after photobleaching (FRAP) on rare cells where GFP–NUT was localized to both the cytoplasm and the nucleus. Fluorescence rapidly recovered in the bleached nucleus and was depleted in the cytoplasm, indicating that in these cells GFP–NUT is being actively transported into the nucleus (Supplementary Figures S1a and b). Conversely, washout of leptomycin resulted in redistribution of GFP–NUT from the nucleus to the cytoplasm (Supplementary Figures S1c and d).

In contrast, GFP–BRD4–NUT and GFP–BRD3–NUT were found only in the nucleus (Figures 3d–e) in a speckled pattern resembling that seen in NMC cells (Figures 2b and d, insets). BRD4 is known to be constitutively nuclear and to associate with chromatin (Dey *et al*., 2003; Haruki *et al*., 2005). Because the BDs of BRD4 are homologous to those of BRD3, it seemed likely that the nuclear retention of BRD–NUT polypeptides was mediated through BD–chromatin interactions. In support of this idea, FRAP revealed that the intranuclear diffusion rates of GFP–BRD3–NUT and GFP–BRD4–NUT were equivalent to one another and significantly less than that of GFP–NUT (Figure 3h; see Supplementary Figures S2a and b for representative images). In addition, the mobilities of GFP–BRD3–NUT and GFP–BRD4–NUT were approximately twofold less than that of GFP–BRD4, suggesting that additional protein–protein interactions involving NUT

contribute to the relatively slow nuclear mobility of BRD–NUT fusion proteins.

BRD–NUT knockdown causes NMC cell differentiation

To investigate the functional role of BRD3–NUT and BRD4–NUT fusion proteins in NMCs, we used siRNA to knock down BRD4–NUT in PER-403 and TC797 cells, and BRD3–NUT in 10326 cells (Figure 4h). Starting on day two following knockdown, all three cell lines exhibited profound morphologic changes consistent with the induction of squamous differentiation, including increased cellular cohesion, stratification, flattening, and enlargement of the cells; and increased keratin production (as indicated by morphologic changes and markedly increased immunohistochemical staining for keratins, Figures 4a–f, and insets). These changes were accompanied by decreases in cellular proliferation, as indicated by reduced staining for Ki-67 (Figure 4g). Overall, siRNA against NUT reduced the Ki-67+ fraction by 9-, 17- and 1.6-fold in TC797, PER-403 and 10326 cells, respectively ($P<0.004$).

BRD4 influences a number of cellular activities through protein–protein interactions, including the transcription of specific target genes. These activities appear to be dependent on the binding of BRD4 to acetylated chromatin through the dual bromodomains, as well as additional protein–protein interactions mediated through the BDs and the flanking ET domain (Dey *et al*., 2003; Farina *et al*., 2004; Jang *et al*., 2005; Yang *et al*., 2005), which are retained in BRD3– and BRD4–NUT fusion proteins. In this paper, we provide the first evidence that BRD–NUT fusion proteins contribute to the maintenance of the transformed state, based on siRNA knockdown studies showing that withdrawal of BRD3– and BRD4–NUT results in squamous differentiation and growth arrest. We propose that tethering of NUT to transcriptionally active, acetylated chromatin through the bromodomains of BRD3 and BRD4 perturbs gene expression and maintains NMC cells in a relatively undifferentiated state. These effects may be exacerbated by the retention of BRD-fused NUT in the nucleus.

To the best of our knowledge, the phenotype observed when NUT-fusion proteins are withdrawn from NMC cell lines is unique among human carcinomas, and serves to further highlight the unusual biology of NMCs. This proposed oncogenic mechanism is a common theme in other cancers, such as acute leukemias, where many fusion oncoproteins act by interfering with transcriptional programs that drive differentiation. In leukemias, many of the involved genes, even those affected by rare chromosomal aberrations, have proven to be of central importance in understanding normal hematopoiesis. Although NMC is a rare tumor, it seems likely that BRD–NUT oncoproteins will provide new insights of some general relevance to understanding normal and pathophysiologic epithelial cell growth and differentiation.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc).