Oncogenesis by sequestration of CBP/p300 in transcriptionally inactive hyperacetylated chromatin domains

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In a subset of poorly differentiated and highly aggressive carcinoma, a chromosomal translocation, t(15;19)(q13;p13), results in an in-frame fusion of the double bromodomain protein, BRD4, with a testis-specific protein of unknown function, NUT (nuclear protein in testis). In this study, we show that, after binding to acetylated chromatin through BRD4 bromodomains, the NUT moiety of the fusion protein strongly interacts with and recruits p300, stimulates its catalytic activity, initiating cycles of BRD4–NUT/p300 recruitment and creating transcriptionally inactive hyperacetylated chromatin domains. Using a patient-derived cell line, we show that p300 sequestration into the BRD4–NUT foci is the principal oncogenic mechanism leading to p53 inactivation. Knockdown of BRD4–NUT released p300 and restored p53-dependent regulatory mechanisms leading to cell differentiation and apoptosis. This study demonstrates how the off-context activity of a testis-specific factor could markedly alter vital cellular functions and significantly contribute to malignant cell transformation.

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Introduction

Malignant cell transformation is associated with a global disruption of genetic and epigenetic mechanisms leading to

both aberrant gene activation and silencing. Although oncogenic gene silencing and activation and/or amplification of oncogenes are well documented, the impact of aberrant activation of normally silenced tissue-specific genes, that is, testis-specific genes, which is known to occur in many somatic cancers, is much less studied (Rousseaux and Khochbin, 2009).

The NUT midline carcinoma (NMC) refers to a group of malignant and highly lethal cancers, occurring in children and adults, which arise from chromosomal translocations systematically involving the *NUT* (NUclear protein in Testis) gene on chromosome 15q14. The function of *NUT* gene is unknown and it is normally expressed in testis (French, 2008). In the majority of NMC cases (two-third), the chromosomal translocation fuses *NUT* to the *BRD4* gene on chromosome 19 (French *et al*, 2003). A detailed analysis of the fusion transcript showed that *BRD4* exon 10b, normally used in a splice variant encoding a large BRD4 isoform, is fused to *NUT* exon 2 (French *et al*, 2008). The *BRD4* gene encodes a double bromodomain-containing protein belonging to a specific family of transcription/chromatin regulators known as BET (Bromodomain and Extra Terminal; Florence and Faller, 2001). In contrast with *NUT*, *BRD4* is ubiquitously expressed in somatic cells (French *et al*, 2003; Wu and Chiang, 2007).

Interestingly, in an additional subset of NMCs, which has recently been characterized, the chromosomal translocation fuses *NUT* to the *BRD3* gene on chromosome 9 (French *et al*, 2008). The *BRD3* gene is a paralogue of *BRD4* and also a member of the BET family function of which is less studied but, similar to BRD4, it preferentially associates with acetylated histones (LeRoy *et al*, 2008). This important finding points to the fusion of *NUT* with a double bromodomain-containing member of the BET family as an important determinant in the oncogenic activity of the fusion protein. Although the molecular basis of the oncogenic activity of *BRD–NUT* fusions remains largely unknown, it is clearly established that their downregulation in NMC cell lines induces squamous differentiation and arrested growth (French *et al*, 2008).

The data presented here explain why and how the fusion of *NUT* with genes encoding double bromodomain-containing factors of the BET family creates a new functional fusion protein, and shed light on a new oncogenic mechanism based on the off-context activity of a testis-specific factor.

Results

The BRD4–NUT fusion protein induces the formation of hyperacetylated chromatin domains

On its ectopic expression, BRD4–NUT forms discrete nuclear foci, which perfectly co-localize with hyperacetylated histone H3 and H4 chromatin domains (Figure 1A; Supplementary

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Figure 1 The BRD4–NUT fusion protein forms nuclear foci containing hyperacetylated chromatin domains. Cos7 cells were transfected with the indicated expression vectors, (A) GFP–BRD4–NUT, (B) HA–sBRD4, (C) HA–NUT and (D) HA–sBRD4 together with GFP–NUT. Ectopically expressed proteins were visualized by GFP or indirect fluorescence after anti-HA detection (A–D) and co-detected with an anti-pan acetylated histone H4 antibody (A–C). Bar: 10 μ m.

Figure S1A and S2A). This particular pattern of nuclear localization is specific to the fusion protein because the expression of BRD4 alone (the short isoform of BRD4, approximately corresponding to the BRD4 part of the fusion protein, named here sBRD4) or that of NUT alone, or the co-expression of both (sBRD4 + NUT), does not induce the formation of hyperacetylated chromatin domains (Figure 1B–D, respectively). Moreover, the expression of the longer BRD4 (fBRD4) isoform with NUT did not allow the formation of distinct nuclear foci containing the two proteins or hyperacetylated chromatin (Supplementary Figure S1B and C).

It has previously been shown that BRD4 bromodomains interact with histone H4 acetylated at different positions and histone H3 acetylated on its lysine 14 (Dey *et al*, 2003; Lee and Chiang, 2009). As expected, these marks and other H4 and H3 acetylated forms were enriched in BRD4–NUT foci (Supplementary Figure S2A; data not shown). The absence of RNA polymerase II, or of its phosphorylated forms, suggests that these BRD4–NUT hyperacetylated foci are not associated with active gene transcription (Supplementary Figure S2B; also see Figure 2G). Consistent with this observation, no accumulation of H3K4me3 was observed in the BRD4–NUT foci (Supplementary Figure S2C, H3K4me3 panel). The absence of H3K9me3 in the foci shows that, despite the absence of RNA pol II, and in agreement with the presence of

hyperacetylated histones, the BRD4–NUT foci are not of a heterochromatic nature (Supplementary Figure S2C, H3K9me3 panel).

The NUT moiety of BRD4–NUT specifically recruits CBP/p300

On the basis of these data, we hypothesized that BRD4–NUT could recruit cellular histone acetyltransferases (HATs), initiating cycles of BRD4–NUT/HAT recruitment and chromatin acetylation, leading to the formation of foci. Antibodies against several known HATs were used to detect their presence in the BRD4–NUT foci on its ectopic expression in Cos7 cells. Among the antibodies tested (P/CAF, Tip60, p300, CBP, HBO1 and HAT1), only anti-p300 and anti-CBP antibodies resulted in a clear accumulation of the endogenous protein in the BRD4–NUT foci (Figure 2A; Supplementary Figure S3; data not shown).

As no HAT had been reported to co-purify with BRD4 complexes (Jang *et al*, 2005; Yang *et al*, 2005), we assumed that NUT could be directing this efficient recruitment of cellular CBP/p300. This hypothesis was confirmed after immunoprecipitation of HA-tagged NUT, BRD4 and BRD4–NUT expressed in Cos7 cells. Endogenous p300 was observed to be associated only with NUT or BRD4–NUT (Figure 2B, lanes 2 and 4). In addition, we observed that NUT alone immunoprecipitates a strong associated HAT activity comparable with that of Gcn5 used as a control (Figure 2C, lanes 2 and 3).

To confirm the relevance of these observations based on the ectopic expression of our proteins of interest, we analysed the intranuclear localization of BRD4–NUT in a cell line derived from an aggressive, metastatic lung cancer arising in a young, non-smoking woman, HCC2429 (Haruki *et al*, 2005). Interestingly, the endogenous BRD4–NUT expressed in these cells (Figure 2D) also formed distinct nuclear foci very similar to the pattern observed after ectopic expression of BRD4–NUT (Figure 2E). In these cells, p300 and CBP were also observed to form foci, which perfectly localized with the BRD4–NUT nuclear domains (Figure 2E; data not shown).

Furthermore, we investigated the signature of p300 activity in the p300-containing foci in these cells. Histone H3K56 was very recently shown *in vivo* to be specifically acetylated by p300 (Das *et al*, 2009). Using a rabbit antibody specific for H3K56ac and a mouse monoclonal antibody against p300, we were able to clearly show a perfect co-localization of H3K56ac with p300 (Figure 2F). Another *in vivo* p300 mark is the acetylation of H3K18 (Ferrari *et al*, 2008; Horwitz *et al*, 2008). This modification also perfectly co-localized with p300 foci that co-localized with the BRD4–NUT foci (Figure 2F). Moreover, to definitely show the occurrence of hyperacetylated chromatin in the BRD4–NUT foci, several acetylated H4 and H3 sites, including H4K8ac, H3K14ac and H3K27ac, against which mouse monoclonal antibodies were available, were co-detected along with BRD4–NUT in the HCC2429 cells (Figure 2F; Supplementary Figure S2D).

Finally, to confirm that the endogenous BRD4–NUT foci are not active transcription sites, nascent RNAs labelled by BrU were detected both in HCC2429 and in a non-BRD4–NUT-expressing lung cancer cell line (A549). As shown in Figure 2G, none of the active transcription foci co-localize with the BRD4–NUT-containing domains, supporting the conclusion that the BRD4–NUT foci are transcriptionally silent chromatin domains.

Figure 2 Specific recruitment of cellular p300 by the NUT moiety of BRD4-NUT to the nuclear foci. (A) Cos7 cells were transfected with GFP-BRD4-NUT and the endogenous cellular p300 was detected by immunofluorescence using an anti-p300 antibody. Both GFP and p300-related fluorescence were recorded and shown individually or together (merge panel). (B) Cos7 cells were transfected with the indicated HA-tagged vectors and, after anti-HA immunoprecipitation, HA-tagged proteins and associated endogenous p300 were visualized using the corresponding antibodies. The 'input' panel shows the amount of cellular p300 in each extract. (C) Cos7 cells were transfected with vectors expressing HA-tagged GCN5 or NUT and the corresponding empty vector (ϕ) and extracts were used to immunoprecipitate HA-tagged proteins. A fraction of immunoprecipitated materials was used to visualize proteins by an anti-HA western blot (anti-HA panel). Another fraction was used to monitor the HAT activity present in the immunoprecipitates using purified histones (lower panel, showing the corresponding autoradiography), which were visualized on a Coomassie-stained gel (middle panel). (D) Detection of endogenous BRD4-NUT with anti-NUT antibody in the HCC2429 cells compared with two other lung cancer cell lines (indicated). As a control, NUT was also detected in a rat total testis extract (lane testis). (E) Endogenous BRD4-NUT foci and p300 were detected in HCC2429 cells using the corresponding antibodies. (F) BRD4-NUT was co-detected with histone H4K8ac. The specific *in vivo* signatures of p300 activity, histones H3K56ac and H3K18ac, were also co-detected with endogenous p300 in HCC2429 cells (indicated). (G) BrU incorporation in nascent RNAs and BRD4-NUT foci were visualized in HCC2429 and A549 cells after a pulse labelling with BrUTP. Bar, 10 μ m.

Domains involved in the direct interaction between NUT and p300

We next explored whether NUT and p300 interact directly and determined which domains are involved. First, a p300 fragment lacking the 870 N-terminal amino acids was observed to interact with NUT as efficiently as the full-length p300 (data not shown). This deletion mutant of p300 (Δ 870) was used to further map the p300-interacting domains in NUT. An advantage of this p300 deletion mutant is that the interaction of both transfected p300 (Δ 870) and endogenous p300 with NUT can be monitored. Figure 3A shows different fragments of both proteins used in these experiments. Co-immunoprecipitation experiments showed that full-length NUT, as well as a NUT deletion mutant containing only the N-terminal half of NUT (F1, see Figure 3A), interacted with both transfected Δ 870 and endogenous p300 (Figure 3B, lanes 2 and 4). No interaction with p300 was observed when fragments of the C-terminal half of NUT were used (Figure 3B, lanes 6 and 8). We then tried to map the p300-interacting domain within the N-terminal half of NUT more precisely. Three fragments

covering the N-terminal 593 amino acids of NUT were cloned (named F1a, b and c, respectively; Figure 3A) and tested as above. Only a limited region of NUT spanning amino acids 346–593 (F1c) interacted specifically with endogenous p300 (Figure 3C, lane 4). An analysis of the amino-acid sequence of NUT from different mammalian species showed that this region is highly conserved (data not shown), indicating its importance for the function of NUT. We further confirmed the interaction between the F1c domain of NUT and p300 by using a GST pull-down assay. GST alone or fused to the F1c fragment was incubated with Cos7 nuclear extracts. Figure 3D shows that the F1c fragment efficiently pulled down endogenous p300 (anti-p300 panel), and that this fraction contained *in vitro* HAT activity (autoradiography panel).

To map the NUT-interacting domain of p300, Cos7 cell extracts expressing different fragments of p300 were used in a pull-down assay with GST alone or the GST-NUT F1c fragment as described above. Figure 3E shows that the CH3 fragment of p300 encompasses the major binding site for

Figure 3 Mapping of domains in NUT and p300 involved in a direct interaction between the two proteins. (A) Schematic representation of domains and fragments used in both p300 and NUT. (B) Cos7 cells were transfected with Myc-tagged $\Delta 870$ p300 and the indicated fragments of HA-tagged NUT-expressing vectors and, after anti-HA immunoprecipitation, the immunoprecipitated proteins were visualized using the indicated antibodies. (C) The indicated HA-tagged NUT fragments were expressed in Cos7 cells and, after anti-HA immunoprecipitation (anti-HA panel), the association of the endogenous p300 was visualized (anti-p300 panel). The input panel indicates the presence of p300 in the different extracts. (D) Glutathione-bound GST or the GST-F1c fragment of NUT were incubated in a nuclear extract from Cos7 cells. Fractions of the bound proteins were either used to detect the presence of endogenous p300 (bottom panel) or to run a HAT assay using purified histones, as described previously. The Coomassie-stained gel (upper panel) shows GST and GST-F1c NUT before and after the pull down. The gel was then dried and used to visualize acetylated proteins after autoradiography (middle panel). (E) GST or GST-F1c were used to pull down either Myc-tagged p300 WT or the HA-tagged CH3 domain of p300. Extracts and proteins used in the pull down are shown after the Coomassie staining of the gel (upper panel). The proteins pulled down were visualized using the indicated antibodies. (F) In an experiment similar to (E), the interaction of GST or GST-F1c with the indicated fragments of p300 was monitored. Please note that as the HA-tagged proteins were of different sizes (B, C), only the corresponding areas of the films are shown and separated by lines. A full-colour version of this figure is available at *The EMBO Journal Online*.

NUT (lane 9). Two sub-domains have been identified in the CH3 domain and are known as ZZ and TAZ2. Pull-down experiments using extracts from cells expressing HA-tagged ZZ, TAZ2 and the p300 TAZ1 domain, showed that binding of NUT was specific to the TAZ2 domain (Figure 3F).

We next wanted to determine whether the interaction between p300 and NUT is direct. Bacterially expressed GST-NUT F1c fragment and two versions of p300 (called p300L for amino acids 324–2094 and p300S for 1045–1666 fragment, respectively with or without the CH3 domain) were purified after expression in a baculovirus system, and their interaction was tested in a GST pull-down assay. Purified p300 directly binds to NUT F1c (Figure 4A, lane 4), whereas no interaction was observed under the same conditions in the absence of the p300 CH3 domain (lane 3).

NUT strongly enhances p300 HAT activity

To investigate whether the NUT–p300 interaction could account for the hyperacetylation phenotype, we tested the effect of NUT on p300 HAT activity. Myc-tagged p300 with or without HA-NUT was transfected into Cos7 cells. Figure 4B shows that for the same amount of immunoprecipitated Myc–p300 (lanes 2 and 4, anti-Myc panel), the HAT activity was much stronger when p300 was co-expressed with NUT (autoradiography panel). We have also immunoprecipitated

NUT using an anti-HA antibody. Figure 4B (anti-HA panel) shows that NUT efficiently co-immunoprecipitates p300 and that, here again, the p300 HAT activity is highly enhanced (compare lane 2, immunoprecipitated p300 without NUT, with lane 8, showing the same amount of p300 with NUT). Quantification of the labelled histones from several independent experiments by PhosphorImager and ImageQuant analysis showed that the presence of NUT increased p300 activity by about 8–10 times (data not shown).

We then took advantage of our purified system to directly test the stimulatory effect of NUT on p300 HAT activity. Increasing amounts of purified GST-NUT F1c fragment were incubated with the purified 324–2094 p300L fragment in an *in vitro* HAT assay. Histone H3 is a preferred target of p300 *in vivo* and was therefore used as a substrate in these assays. Figure 4C shows that purified F1c fragment clearly stimulates H3 acetylation by purified p300 in a dose-dependent manner. Using several modification-specific antibodies, we tested the stimulatory effect of the NUT F1c fragment on the specificity of p300 HAT activity. We observed that p300 alone acetylated all H3 acetyl-acceptor lysines except K56. Addition of GST-F1c enhanced the acetylation of H3K9, K14, K18 and K27 in a dose-dependent manner. Interestingly, in the presence of NUT F1c, p300 was able to acetylate H3K56, indicating that NUT might also specifically stimulate H3K56

Figure 4 Direct enhancement of p300 HAT activity by NUT. **(A)** p300L (amino acids 324–2094 fragment) or p300S, a deletion mutant lacking its CH3 domain (amino acid 1045–1666 fragment) were expressed using a baculovirus system and purified. In parallel, GST and GST-F1c were expressed in bacteria and purified and used in a pull-down assay. The gel shows proteins present in the input (left panel) and after the pull down (right panel). **(B)** Cos7 cells were transfected with vectors expressing the indicated tagged proteins alone or together. After an immunoprecipitation using anti-Myc (p300) and anti-HA (NUT) antibodies, a fraction was used to monitor protein immunoprecipitations (upper panels). Another fraction was used for an *in vitro* HAT assay as described in Figure 2C. Coomassie-stained histones and the corresponding autoradiography are shown (middle and lower panels, respectively). **(C)** Purified p300 was incubated with histone H3 in the absence (0) or presence of increasing amounts of purified GST-F1c fragment of NUT (Coomassie panel). Aliquots corresponding to each incubation conditions were used to monitor site-specific histone H3 acetylation using antibodies recognizing the indicated acetylated sites. Anti-acetylated lysine and anti-H3 antibodies were used to show p300 autoacetylation and H3 loading, respectively. A full-colour version of this figure is available at *The EMBO Journal* Online.

acetylation. The same *in vitro* experiment was repeated using another fragment of NUT, F1b, incapable of interacting with p300 (Figure 3C). In contrast to F1c, this p300 non-interacting region of NUT had no effect on p300 HAT activity (Supplementary Figure S4).

BRD4–NUT mediates histone acetylation propagation through a feed-forward mechanism

The data obtained thus far suggested that the BRD4–NUT fusion could create a unique factor, binding acetylated chromatin through its bromodomains, and providing a platform for the recruitment of p300 and the stimulation of its HAT activity through NUT. An original feed-forward mechanism could then be initiated, through cycles of p300-mediated acetylation of adjacent nucleosomes and additional BRD4–NUT recruitments, leading to the formation of the observed foci. To test this hypothesis, we evaluated several of its testable aspects. First, BRD4–NUT foci formation and the corresponding chromatin acetylation should be abrogated by inactivating BRD4 bromodomains. Figure 5A shows that indeed, BRD4–NUT bearing inactivating mutations in its first bromodomain is unable to form foci and sustain local histone acetylation. Second, we reasoned that ectopic expression of p300 should stimulate the propagation of BRD4–NUT and hence lead to a decrease in the number of small BRD4–NUT foci in favour of the formation of larger foci. Figure 5B

shows an example of two cells expressing BRD4–NUT, one of which also ectopically expresses p300. As predicted, fewer but larger BRD4–NUT foci are observed in the Myc–p300-expressing cells. Although p300 is known to form nuclear foci when expressed in cells (Eckner *et al*, 1994), p300 present in the BRD4–NUT foci, however, seemed to be of a different nature (Figure 5B, compare p300 in the BRD4–NUT foci with p300 alone shown in the inset). To quantify the effect of the ectopic expression of p300, the foci number and the area occupied by these foci were determined and compared with those of cells expressing only BRD4–NUT. This quantitative analysis shows that the ectopic expression of p300 leads to an approximately four-fold enlargement of BRD4–NUT foci mean area per cell, as well as a decrease in the total number of foci (Figure 5B, histograms).

We then used the HCC2429 carcinoma cell line and observed that the overexpression of p300-interacting region of NUT, F1c, or the NUT interaction region of p300, CH3, leads to the dispersion of p300 in the nucleus (Figure 5C). Notably, anti-NUT antibody recognizes the transfected F1c fragment, leading to a more intense NUT labelling in F1c-transfected cells.

We also anticipated that a continuous p300 activity should be required to keep BRD4–NUT concentrated at the foci and, consequently, that the inhibition of p300 catalytic activity should lead to the dispersion of BRD4–NUT foci. Accordingly,

Figure 5 Propagation of chromatin acetylation through a feed-forward mechanism induced by BRD4-NUT. (A) The first BRD4 bromodomain was inactivated by site-directed mutagenesis (BRD4-NUT Δ br1) and the capacity of this mutant to form nuclear foci and acetylated chromatin domains enriched in p300 was analysed after the transfection of Cos7 cells. (B) Co-transfection of p300 together with GFP-BRD4-NUT leads to the enlargement of BRD4-NUT foci. The inset shows a cell expressing Myc-p300 alone. The histogram shows the results of quantitative evaluations (ratios of the values observed in p300-transfected cells to those observed in non-transfected cells) of the mean foci area (left), mean foci number (middle) and mean total foci area per cell (right), obtained as described in Materials and methods section. (C) HCC2429 were transfected either with the NUT interaction domain of p300, CH3 (upper panel), or the p300-interacting domain of NUT, F1c (lower panel), and the formation of foci by the endogenous p300 was monitored. NUT-F1c and p300-CH3 were detected with an anti-HA antibody, endogenous p300 with an anti-p300 antibody and BRD4-NUT with an anti-NUT antibody. Please note that the anti-NUT antibody also recognizes the NUT-F1c fragment. The images are representative of nearly 100% of the cells expressing either fragments. (D) HCC2429 cells were treated with the small p300 inhibitor, C646 (+ p300 inhibitor) or its inactive analogue (+ control inhibitor) and BRD4-NUT and p300 were visualized with the corresponding antibodies. The images are representative of nearly 100% of the cells. Bar: 10 μ m.

a newly developed small molecule inhibitor of CBP/p300, C646 and its inactive analogue (Bowers *et al*, 2010), were used to test this hypothesis. Figure 5D shows that the treatment of cells with the specific CBP/p300 inhibitor, but not with its inactive analogue, leads to a clear dispersion of the BRD4-NUT and p300 foci.

Finally, our model predicts that the activity of cellular HDACs should oppose the p300-dependent propagation of BRD4-NUT foci. Accordingly, the inhibition of cellular HDACs by increasing the genome-wide histone acetylation should also lead to the propagation of BRD4-NUT and the dispersion of the foci. This was indeed the case because the

treatment of HCC2429 with an HDAC inhibitor, TSA, induced complete dispersion of the foci (Supplementary Figure S5A). Besides, monitoring the amounts of BRD4-NUT after TSA treatment, we also noticed that this treatment induces a significant downregulation of BRD4-NUT at the time when an accumulation of the cell cycle regulator p21 was observed (Supplementary Figure S5B). As TSA treatment did not change the amount of BRD4-NUT encoding mRNA in HCC2429 cells (Supplementary Figure S5C), the BRD4-NUT downregulation probably occurred through a post-translational mechanism. This downregulation of BRD4-NUT was also observed after the treatment of cells with other unrelated

class I/II HDAC inhibitors, SAHA (vorinostat) and butyrate, but not with the class III HDAC inhibitor, nicotinamide, nor after a genotoxic treatment by etoposide (Supplementary Figure S5D).

Altogether these data are consistent with a mechanism in which the bromodomains and the NUT moiety of BRD4–NUT act synergistically as a potent histone hyperacetylator, action of which is counteracted by cellular HDACs, leading to the formation of hyperacetylated chromatin foci.

Inactivation of p53 regulatory circuits by BRD4–NUT

Our data show that BRD4–NUT foci titrate out cellular p300 into a limited number of transcriptionally silent hyperacetylated chromatin domains. Accordingly, we predicted that important p300-dependent cellular functions should also be altered in BRD4–NUT-expressing HCC2429 cells. CBP/p300 has an important role in mediating p53-dependent cellular responses by acetylating p53 (for review, see Grossman (2001)). We therefore hypothesized that p53 regulatory activity could be disrupted in the HCC2429 cells. Using an antibody specifically recognizing p53 acetylated by CBP/p300 (K373ac and K382ac), we observed that in HCC2429 cells acetylated p53 is also present in distinct nuclear foci, which disappear after the knockdown of BRD4–NUT (by two independent anti-NUT siRNAs), strongly suggesting that acetylated p53 remains bound to the BRD4–NUT/p300 foci (Supplementary Figure S6A). As both anti-acetylated p53 and anti-NUT antibodies were generated in rabbit, the co-detection of acetylated p53 and BRD4–NUT was not possible in HCC2429 cells; however, in a transfection-based assay using GFP–BRD4–NUT, acetylated p53 indeed accumulated in the BRD4–NUT foci, whereas total p53 was observed all over the nucleus (Supplementary Figure S7A).

On the basis of these observations, we predicted that p53 should be inactive in HCC2429 cells and therefore unable to ensure known p53-dependent cellular responses, such as *p21* induction after a genotoxic assault. To test the activity of p53 in HCC2429 cells, A549 cells expressing wild-type p53, used as a control, and HCC2429 cells, also expressing wild-type p53 (verified by sequencing; data not shown) were treated with etoposide and the activation of one p53 target gene, *p21* was monitored. Although an etoposide treatment efficiently induced *p21* accumulation in A549 cells, it was not associated with a *p21* gene response in HCC2429 cells (Figure 6A), supporting the hypothesis that BRD4–NUT is capable of interfering with p53 functions in these cells. To confirm the inactivating role of BRD4–NUT on p53, we then transiently expressed BRD4–NUT in A549 cells and showed that it severely hindered *p21* activation, normally occurring in these cells after an etoposide treatment (Figure 6B). In addition, we made use of a *p21* promoter reporter system to monitor the p53 transactivator capacity in the absence or presence of increasing amounts of BRD4–NUT. After co-transfection of BRD4–NUT and p53, as expected, acetylated p53 was sequestered in BRD4–NUT foci (Supplementary Figure S7A) and increasing amounts of BRD4–NUT significantly reduced its activator function on the *p21* promoter (Supplementary Figure S7B).

Finally, to show the direct involvement of BRD4–NUT in p53 inactivation, we knocked down BRD4–NUT using two independent anti-NUT siRNAs and showed an efficient restoration of *p21* gene expression (Figure 6C) corresponding

to the dispersion of acetylated p53 in the whole nucleus (Supplementary Figure S6A, siRNA BRD4–NUT panels). We could also demonstrate that the restoration of p53 activity was associated with a spontaneous HCC2429 cell apoptosis as judged by the accumulation of active caspase 3 (Figure 6C; Supplementary Figure S6B), as well as cell differentiation, as judged by the accumulation of epithelial differentiation marker E-cadherin (Figure 6C). Moreover, the release of p300 and acetylated p53 after BRD4–NUT knockdown in HCC2429 cells correlated with an enhanced apoptotic cell response after a genotoxic assault (Supplementary Figure S6B).

As some of the cellular events described here may occur in a p53-independent manner, we focussed our attention on *p21* and showed that *p21* accumulation induced by BRD4–NUT knockdown, was abolished if p53 was also knocked down (Figure 6D). Finally, to extend this study to other p53 target genes, in addition to *p21*, we also considered the expression of *PUMA* and *GADD45* (Harris and Levine, 2005). Figure 6E shows that BRD4–NUT downregulation after the treatment of cells with two independent anti-NUT siRNAs leads to a significant accumulation of mRNA encoded by the three p53 target genes. Interestingly, the treatment of cells with a CBP/p300 inhibitor severely interfered with the induction of these genes, showing that CBP/p300 activity is required to activate p53 target genes after BRD4–NUT knockdown. Accordingly, a ChIP approach showed that the knockdown of BRD4–NUT leads to the recruitment of acetylated p53 and p300 on *p21* promoter concomitant with *p21* gene activation (Figure 6F).

Altogether, these data clearly show a role for BRD4–NUT in the extinction of critical CBP/p300 functions through the sequestration of these enzymes in HCC2429 cells.

Discussion

The testis-specific factor, NUT, is central to the oncogenic activity of the fusion protein in NMCs because it is invariably involved in the resulting oncogenic chromosomal translocations (French, 2008). In addition, the fusion with a double bromodomain protein of the BET family, either BRD4 or BRD3, seems to constitute the second critical element in the pathological activity of the fusion protein (French *et al*, 2008). Here, the functional dissection of the BRD4–NUT protein reveals unique properties for this fusion protein and explains why selection operates towards the fusion of NUT with members of the BET family.

The fact that the fusion of NUT with BRD4 or BRD3, probably with distinct cellular functions (Wu and Chiang, 2007; LeRoy *et al*, 2008; Chiang, 2009), results in the same phenotype strongly suggests that their important functional contribution is related to the presence of their bromodomains that show similar acetylated histone-binding activities *in vivo* (Dey *et al*, 2003; LeRoy *et al*, 2008).

The protein, NUT, is a testis-specific protein of totally unknown function. On the basis of the data presented in this study, it can be speculated that the physiological role of NUT is related to its association with, and stimulation of p300 and/or other yet unknown HATs. This activity of NUT could contribute to the wave of genome-wide post-meiotic histone hyperacetylation that occurs in elongating spermatids just before their replacement by transition proteins (Boussouar

Figure 6 p53 inactivation in BRD4-NUT-expressing cells. (A) HCC2429 and A549 cells were treated with 20 μ M etoposide for 15 h and p21, p53 and p300 were visualized as indicated. (B) A546 cells were transfected with an empty vector (–) or with a BRD4-NUT-expressing vector (+) and treated or not with etoposide as above (+ and –, respectively) and the indicated proteins were detected with the corresponding antibodies. (C) HCC2429 cells were treated with scrambled siRNAs or two specific anti-BRD4-NUT siRNAs and the indicated proteins were visualized as above. (D) HCC2429 cells were treated with the anti-BRD4-NUT siRNAs indicated above as well as with an anti-p53 siRNA and the indicated proteins were visualized using the corresponding antibodies. (E) The expression of *p21*, *PUMA* and *GADD45* were monitored after the knockdown of BRD4-NUT with the two above-mentioned siRNAs in the presence (dark grey histograms) or absence (light grey histograms) of p300 inhibitor. Data represent the mean $\pm$ s.d. values of at least three independent experiments. (F) The association to *p21* promoter of p53, acetylated p53 (p53ac) and p300 was monitored in HCC2429 cells before and after the knockdown of BRD4-NUT by siRNA as above. The PCR amplification shown here is representative of three independent ChIPs.

et al, 2008; Rousseaux *et al*, 2008) in which both NUT and p300 are expressed (data not shown).

Interestingly, the BRD4-NUT-dependent histone hyperacetylation evidenced here is not associated with transcription. This situation is similar to that observed in maturing post-meiotic male germ cells in which, despite histone hyperacetylation, there is a major turn-off in transcriptional activity associated with chromatin compaction. In BRD4-NUT-expressing cells, the absence of transcription, despite p300 recruitment and histone hyperacetylation, could also be associated with an acetylation-dependent compaction of chromatin in the foci. Indeed, an acetylation-dependent chromatin compaction was previously reported under the action of the testis-specific member of the BET family, Brdt (Pivot-Pajot *et al*, 2003; Govin *et al*, 2006; Moriniere *et al*, 2009). It is therefore possible that BRD4-NUT locally mimics a 'Brdt-like' activity in compacting acetylated chromatin domains, which would hinder the access of RNA Pol II to genes.

The dissection of the mechanisms underlying the formation of acetylated BRD4-NUT chromatin foci revealed a unique mechanism leading to the creation of large acetylated chromatin domains. Indeed, a BRD4-NUT mutant with an inactive bromodomain is incapable of forming BRD4-NUT acetylated chromatin domains, suggesting that the initial targeting of acetylated chromatin by the fusion protein is necessary to initiate the process. Once BRD4-NUT is in place, the recruitment of p300 and the stimulation of its HAT activity would allow the propagation step. As shown also for MAML1-p300 interaction, engagement of the CH3 domain by various protein ligands may be a general mechanism for

enhancing the catalytic activity of p300 (Hansson *et al*, 2009). The extent of this propagation is limited by HDAC activity, which, by opposing histone acetylation, could slow down and eventually stop the propagation, thereby creating discrete foci. The presence and activity of p300 are critical for the maintenance of BRD4-NUT foci. Indeed, the overexpression of the p300-interacting domain of NUT, F1c, leads to the displacement of p300 and the dispersion of the BRD4-NUT foci. This experiment also reveals an important role for the CH3 domain of p300 in the maintenance of these foci. Interestingly, this domain of p300 is critical for the 'physiological' p300 foci formation because, according to early investigations, the CH3-interacting viral protein E1A also disrupts these foci (Lill *et al*, 1997). In addition, we show in this study that the continuous activity of p300 is also required for the stability of the BRD4-NUT foci, as its inhibition with a specific small molecule inhibitor disrupts the foci probably due to a dominant HDAC activity. Indeed, in patient-derived HCC2429 cells, the genome-wide histone hyperacetylation induced by TSA and the subsequent downregulation of BRD4-NUT leads to the dispersion of BRD4-NUT foci.

In the absence of TSA treatment, the high affinity binding of p300 to the NUT moiety of the fusion protein leads to the accumulation of the majority of cellular p300 into these transcriptionally inactive BRD4-NUT foci. On the basis of these findings, we also predicted that in BRD4-NUT-expressing cells important cellular functions depending on p300 should be altered. One of the functions of p300 is the control of p53 regulatory activity (Grossman, 2001). Indeed, we show in this study that the release of p300 after BRD4-NUT

downregulation in HCC2429 cells leads to the activation of p53 target genes in a CBP/p300-dependent manner. These studies allowed us to clearly demonstrate one of the oncogenic activities of the BRD4–NUT fusion protein. However, as CBP/p300 and BRD4 are involved in various critical cellular processes, the sequestration of these proteins in limited numbers of nuclear domains certainly affects other cellular functions hindering apoptotic cell response and cell differentiation. For instance, a deficiency of BRD4, which has important roles in polII transcription (Wu and Chiang, 2007), in BRD4–NUT-expressing cells could also be oncogenic. Indeed, it has been shown that Brd4^{+/−} cells present increased chromosomal missegregation (Nishiyama *et al*, 2006).

The most important aspect of this study, however, is that it uncovers a new concept in oncogenesis. Indeed, we show in this study how the off-context activity of a testis-specific factor in a somatic cell becomes oncogenic. As the aberrant expression of testis-specific genes has been reported in many cancer cells, we propose that, at least in some cases, that is NUT, the product of these genes can be major contributors to malignant cell transformation (Rousseaux and Khochbin, 2009). This concept opens a new area of research in cancer biology.

Materials and methods

Plasmids

The BRD4–NUT GFP construction was kindly provided by Dr Dang, and then sub-cloned in a pcDNA 3.1 HA vector. p300 WT and ΔN870 Myc plasmids were generous gifts from Dr H Stunnenberg. The sBRD4 and NUT constructions were obtained after PCR amplification of their corresponding coding sequences from a human testis cDNA bank (Clontech) and cloned in pcDNA 3.1 HA and pEGFP-C vectors. Different NUT fragments (f1, f1a, f1b, f1c, f2 and f3) were amplified using the appropriate sets of primers and cloned in pcDNA 3.1 HA and in pGEX5 GST vectors. p300 CH3, TAZ1, ZZ and TAZ2 fragments were amplified from the p300WT plasmid and cloned into the pcDNA 3.1 HA vector. BRD4–NUT ΔBromo1 construct was obtained by deleting six critical amino acids of bromodomain 1 (81–PFQQPV-88) directly in BRD4–NUT pcDNA HA plasmid, using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Mutations were then confirmed by DNA sequencing.

p53 Flag and p21 promoter luciferase plasmids were purchased from Addgene Organisation (www.addgene.org).

Cell culture, treatments, transfection assays, antibodies and gene reporter assay

Cos7 cells were maintained in DMEM medium, whereas H1299, A549 and HCC2429 cells were maintained in RPMI medium complemented with 10% SVF, 2% glutamine and 1% P/S. When needed, cells were treated with TSA 100 ng/ml (Sigma) for different times or with etoposide 20 μg/ml (Sigma).

p300 inhibitor, C646, or its inactive analogue (Bowers *et al*, 2010) was added to the culture medium from 1 h to complete siRNA incubation time (24–48 h), at 20 μg/ml.

Cells were seeded and grown to 60–70% confluence on the day of transfection. Transfections were performed with Lipofectamine 2000 (Invitrogen), using a ratio of 1 μl of Lipofectamine for 1 μg total quantity of vectors. The BRD4–NUT siRNA1 (forward: 5′-gcacuaaagugagaagacca-3′), BRD4–NUT siRNA2 (forward: 5′-gggaugcagaagaccaa-3′) and scrambled siRNA were purchased from Eurogentec, whereas p53 siRNA was obtained from Qiagen (SI02655170, Hs_TP53_9). Cells were transfected with Lipofectamine RNAimax (Invitrogen) using the company instructions and cells were collected 24–48 h before analysis.

The primary antibodies used were: anti-NUT (Cell Signaling Technology), anti-p300 rabbit (N-15, Santa Cruz Biotechnology), anti-p300 mouse (05–257, Millipore), anti-BrdU (A21300, Invitrogen), anti-HA rat (for IP and IF, Roche), anti-HA mouse (for WB,

Covance), anti-FlagM2 (F3165, Sigma), anti-Myc rabbit (ab9106, Abcam), anti-β-Actin (A5441 Sigma), anti-Histone H4 pan rabbit (05–858, Millipore), anti-H3 rabbit (05–928, Millipore), anti-H3K9ac rabbit (07–352, Millipore), anti-H3K14ac rabbit (07–353, Millipore), anti-H3K18ac rabbit (Millipore), anti-H3K56ac rabbit (2134–1, Epitomics), anti-RNA pol II (8WG16, H5, H14, Covance), anti p53 (DO7, Dako), anti-p53 K373ac-K382ac (06–758, Millipore), anti-p21/WAF1 (ab-10P64, Calbiochem), anti-PARP (Boehringer), anti-cleaved-caspase 3 (9661S, Cell Signaling Technology), anti-E-cadherin (18–0223, Zymed Laboratories) and mouse anti-acetylated histones from H Kimura.

Gene reporter assay was carried out as detailed in the Supplementary data.

Immunofluorescence, BrUTP incorporation, immunoprecipitation, HAT assay, GST pull down, qRT-PCR and ChIP

Cells were grown on two- or four-well glass Lab-Tek (Nunc), transfected with 1 μg plasmid of interest, fixed in 4% paraformaldehyde for 5 min at room temperature and then permeabilized in 4% paraformaldehyde–0.1% Triton X-100 for 1 min at room temperature. Fixed and permeabilized cells were incubated in blocking buffer (PBS containing 5% skim milk) for 30 min at room temperature, then in primary antibody (except for GFP-tagged proteins) for 1 h at 37°C, then washed with PBS and incubated in the secondary antibody for 30 min at 37°C. After three washes in PBS, the cells were counterstained with Hoechst (250 ng/ml) and examined under a confocal microscope.

In situ transcription was monitored by BrUTP (Sigma) incorporation as described previously (Roussel *et al*, 1996) in conditions set up to reveal RNA pol I and RNA pol II transcription (Moore and Ringertz, 1973). Briefly, cells were grown on two- or four-well glass Lab-Tek (Nunc) and slightly fixed and permeabilized for 5 min at 4°C, then incubated for 30 min at 37°C with transcription buffer (100 mM Tris, 4 mM MgCl₂, 2 mM MnCl₂, 100 mM (NH₄)₂SO₄, 0.1% B-mercaptoethanol, 150 mM sucrose, 0.6 mM ATP, GTP and CTP and 0.12 mM BrUTP). Incorporated BrU was then detected with 1/100 anti-BrdU antibody (Invitrogen) incubated for 1 h at 37°C.

For qRT-PCR, RNA was extracted from treated or non-treated cells using TRIzol reagent (Invitrogen) and reverse transcribed into cDNA with SuperScript III First-Strand Synthesis mix (Invitrogen). Human p21 (F: 5-CCTGTCACGTCTGTGTACCT-3, R: 5-TTAGCAGCGGAACAAGGAGT-3), PUMA (F: 5-GACCTCAACGCACACTACGA-3, R: 5-GAGATTGTACAGGACCCTCCA-3), GADD45a (F: 5-TTTGCAATA TGACTTTGGAGGA-3 R: 5-CATCCCCACCTTATCCAT-3) and BRD4–NUT (F: 5-AGGCTGTCATTACCCCTCAA-3, R: 5-CTCTTCCAAGGCCA TGAGTC-3) expressions were then monitored using Brilliant SYBR Green Master mix and MX3005P (Stratagene) and normalized by ACTIN or GAPDH expression.

ChIP experiment was carried out on the basis of X-ChIP protocol from Abcam with modifications. Briefly, 10⁷ cells after siRNA treatment were cross-linked for 10 min at RT with 1% formaldehyde on a shaking platform and final 125 mM glycine was added to stop the reaction. Cells were then collected, washed twice with cold PBS and incubated for 10 min with cell lysis buffer (20 mM Tris–HCl (pH 8.0), 85 mM KCl and 0.5% NP40). Nuclei were re-suspended in Nuclei lysis buffer (50 mM Tris–HCl (pH 8.0), 10 mM EDTA and 1% SDS) and then sonicated to obtain 500-bp fragments of DNA. After centrifugation, the supernatant was diluted five times in ChIP buffer (20 mM Tris–HCl (pH 8.0), 1.1 mM EDTA, 0.01% SDS, 1.1% Triton X-100 and 167 mM NaCl) and incubated overnight at 4°C with appropriate antibodies, and then for 1 h with protein A dynabeads (Invitrogen). After intensive washes, twice in low-salt buffer (20 mM Tris–HCl (pH 8.0), 2 mM EDTA, 0.1% SDS, 1% Triton X-100 and 150 mM NaCl), twice in high-salt buffer (20 mM Tris–HCl (pH 8.0), 2 mM EDTA, 0.1% SDS, 1% Triton X-100 and 500 mM NaCl), once in LiCl buffer (0.25 M LiCl, 1% NP40, 20 mM Tris–HCl (pH 8.0), 1 mM EDTA and 1% deoxycholate) and finally in TE buffer (10 mM Tris–HCl (pH 8.0) and 1 mM EDTA), the DNA was eluted by incubating the beads for 30 min at RT with the elution buffer (1% SDS and 100 mM NaHCO₃). The DNA was then purified by phenol:chloroform and qRT-PCR was performed using primers for the p53-binding site on p21 promoter (F: 5-GCTTGGGCAGCAG GCTG-3, R: 5-AGCCCTGTCGCAAGGATCC-3).

Detailed protocols for immunoprecipitation, HAT assay and GST pull down, as well as for insect cell culture, baculovirus cultivation and protein purifications are provided in the Supplementary data.

Quantitative analysis of BRD4–NUT Foci

Confocal microscopy images from different nuclei along the z-axis were analysed using the Metamorph software. The number of foci was counted in 50 nuclei and normalized with respect to the total nucleus area obtained using Metamorph software functions. The mean foci area was obtained by dividing the total number of BRD4–NUT foci areas in a given nucleus obtained by the Metamorph software as above, by the number of foci counted within the same nucleus.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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