Chapter 5. Modelling the consequences of *Brd4-NUT* oncogenic translocation in mouse ES cells using a conditional knockin strategy

5.1 Introduction

Chromosome translocations and structural aberrations giving rise to fusion oncogenes are some of the most common mechanisms in oncogenesis. However, although these types of chromosome rearrangements have long been identified in hematological and soft-tissue malignancies, they have only rarely been described in the corresponding carcinomas, which are responsible for around 80 % of the human cancer cases. These recurrent oncogenic fusions include the *RET* and *NTRK1* genes involved in papillary thyroid carcinomas (163), *PAX8-PPARG* fusion involved in follicular thyroid carcinoma (164), *MECT1-MAML2* fusion involved in mucoepidermoid carcinoma (165,166), *ETV6-NTRK3* fusion involved in secretory breast carcinomas (167,168) . Similar to hematological and soft-tissue malignancies, the most common types of genes involved in fusion oncogenes in carcinomas are transcription factors and tyrosine kinases. However, the mechanisms behind most of these chromosome fusions in carcinogenesis have far from been clarified, mostly because these

types of somatic fusions have only started to be identified very recently and have not been well studied in human and mice.

One particularly interesting chromosomal translocation in solid tumours is the t(15;19)(q13;p13) which results in the expression of a fusion transcript between the bromodomain transcription factor *BRD4* and a novel gene *NUT*. Expression of the *BRD4-NUT* fusion transcript is diagnostic of a highly lethal carcinoma (average survival after diagnosis in human patients = 28 weeks) called midline carcinoma arise from the epithelia midline structures (169-171). The human *BRD4* gene encodes a 1400 amino acid BET family protein containing two bromodomains in the N terminus and an ET domain in the C terminus. The bromodomain has been shown to interact with chromatin via acetylated histone H3 and H4 (172). BRD4 plays a role at several stages of the cell cycle progression, including during the G_1/S and G_2/M transition (173,174). In contrast, the cellular function of the *BRD4* fusion partner, *NUT*, is largely unknown, but recent studies have identified *NUT* rearranged with other genes in a subgroup of *NUT* midline carcinomas (NMC) (175).

The mechanism for *BRD4-NUT* fusion in the generation of carcinomas is largely unknown. Cytogenetic analysis suggests that the BRD4-NUT fusion protein always binds to chromatin through a NUT mediated interaction, indicating the oncogenic property of BRD4-NUT could be due to this chromatin binding activity associated with NUT (176). Interestingly, rather than other carcinomas which are normally associated with genomic instability, the karyotype(s) associated with all *BRD4-NUT* translocations are remarkably simple, often having the t(15;19) as the sole aberration (177-180) (**Figure 5-1**), suggesting that few additional mutations are required for this fusion to initiate cancer formation. In addition, *NUT* has also been identified to fuse with *BRD3*, a BRD family protein with very similar structure to *BRD4* (176), suggesting the fusion between *BRD4* and *NUT* is not due to a structural bias on the chromosome. Therefore, a mouse model of *BRD4-NUT* fusion is required for modelling this disease and better understanding the molecular mechanism(s) associated with the *BRD4-NUT* translocation.

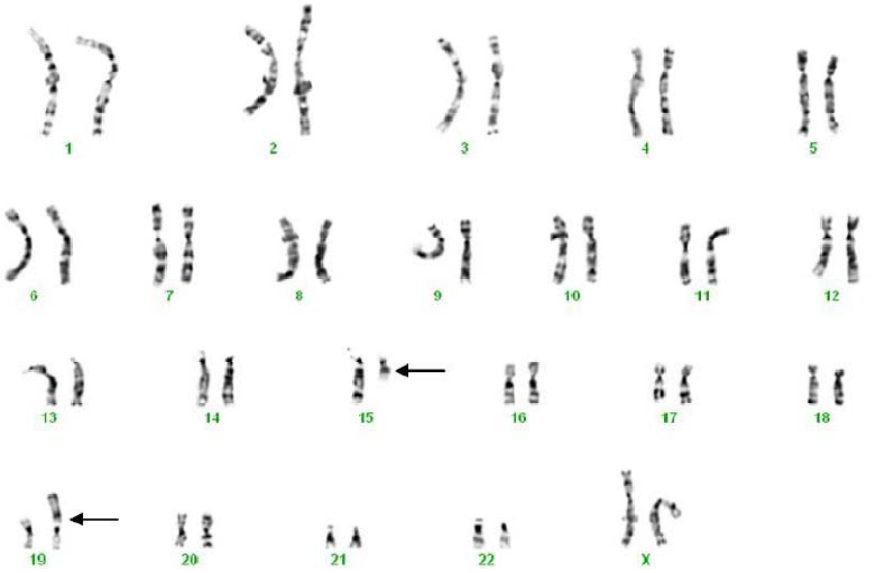

Figure 5-1. Karyotype of t(15;19) BRD4-NUT translocation in a 30-year-old midline carcinoma patient. Cited from Engleson, J, et. al, 2006. (171)

5.2 Aims and summary of the project

This primary aim of this project is generation of a *BRD4-NUT* knockin mouse model for studying the associated phenotype *in vitro* and the tumourigenesis of BRD4-NUT *in vivo*. Specifically the aims for this project are:

1. Evaluation of the phenotype of *BRD4-NUT* expression in a cell culture based assay, specifically with respect to cell cycle and cell growth.
2. Generate conditional knockin mice and tumour watch studies by crossing the *BRD4-NUT* +/- mice with the *CreERt2 +/- mice*.
3. Cross the *BRD4-NUT* +/- mice with the *T2/Onc3* mice - a recently modified *Sleeping Beauty* transposon mouse line developed by *Dupuy et al.* (181) for screening secondary mutations.
4. Establishment of a collaboration with pathologists at Addenbrooke's hospital to characterize human *BRD4-NUT* carcinomas and therefore further understand clinical aspects of midline carcinoma.

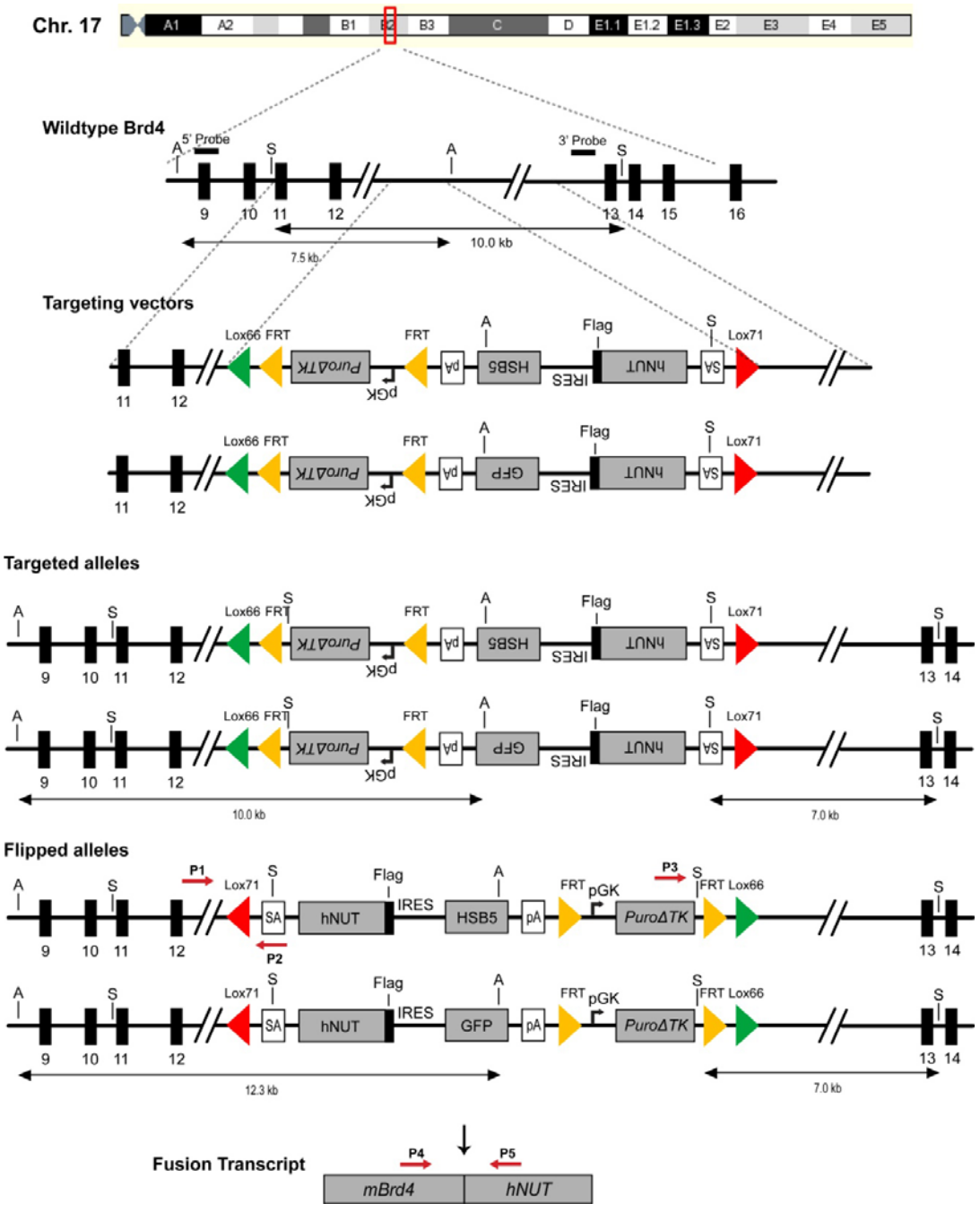

Figure 5-2. Schematic diagrams of Brd4-NUT targeting constructs and targeted alleles.

Both the SB and GFP versions targeting constructs and targeted alleles are shown. The double head arrows under each allele represent southern blot DNA fragments length after enzymatic digestion. S – *Sph*I, A – *Afl*II restriction sites.

Similar to the *Tel-AML1* mouse model described in Chapter 4, to generate the *BRD4-NUT* fusion the human *NUT* cDNA was knocked into mouse *Brd4* locus followed by an IRES and a transposase coding sequence (**Figure 5-2**). As the expression of *BRD4-NUT* has been shown to affect cell cycle progression (161), the knockin cassette is flanked by two *loxP* sites to allow activation of the *BRD4-NUT* in an conditional manner. After the knockin allele is transmitted through the germ line, this line will be crossed with a CreERt2 mouse model (182) to induce BRD4-NUT and transposase expression following treatment with 4-hydroxytamoxifen (4-OHT) (**Figure 5-3**).

After targeting and screening, the targeted ES cell clones for *Brd4-NUT* were sent for blastocyst injection to derive germ line transmissions for the *BRD4-NUT* mouse line. However, although 14 clones were injected and over 30 chimera mice were generated with chimeric rate between 20-90 percent, knockin alleles could not be transmitted through the germ line (more information will be presented in the result section for this part of work). Therefore *in vivo* characterization of this mouse model could not be performed.

In *in vitro* studies, following targeting of this allele into a lab made ES cell line carrying the CreERt2 allele, I have shown that after 4-OHT application, fusion transcript is expressed at a comparable level with the endogenous *Brd4* expressed from the untargeted locus. However, only weak expression of the fusion protein could be identified in the targeted cells using immunoprecipitation, indicating the fusion protein is either not stable in ES cells or expressed at too low level to be identified. Cell culture characterization studies showed the cell growth is severely impaired and the colony formation is completely blocked upon expression of *Brd4-NUT*. Cell cycle analysis using Propidium iodide (PI) staining subsequently indicated the arrest is taking place at the G_2/M phase.

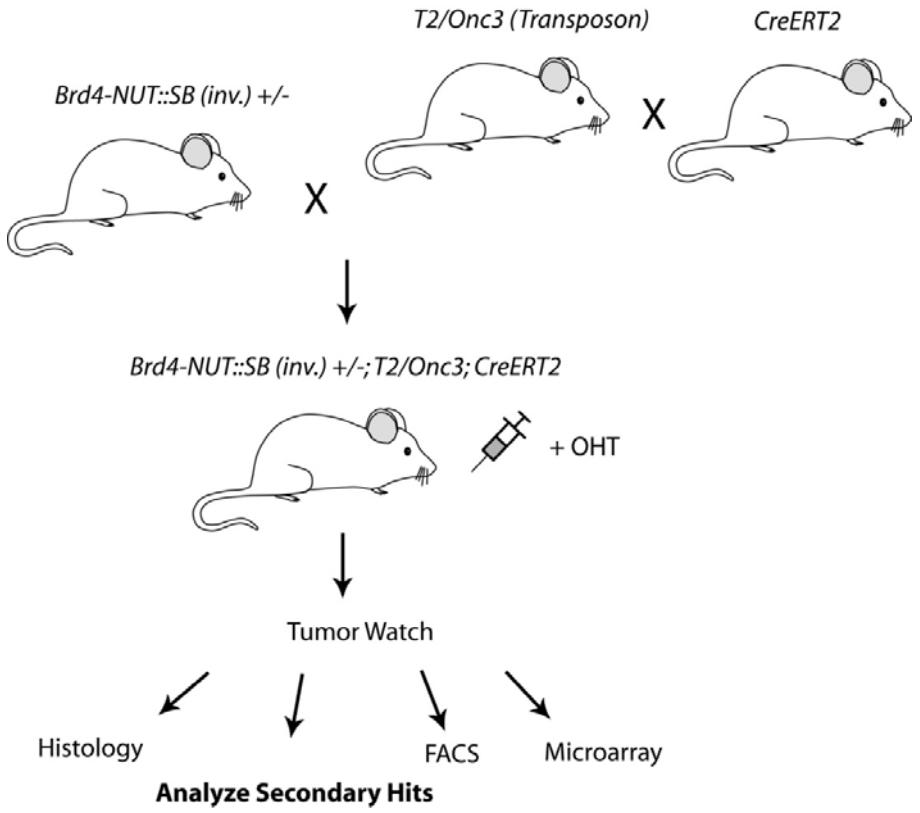

Figure 5-3. Crossing strategy for tumour watch and subsequent characterization studies in *Brd4-NUT* mouse model.

After the F1 mice are derived, the *Brd4-NUT-SB* mouse will be crossed with the *T2/Onc3;;CreERt2* mouse. Tumour watch studies in experimental mice treated with 4-OHT administration at different stage to turn-on BRD4-NUT expression. After tumours are derived, tumours will be identified and characterized. Secondary hits by transposon mutagenesis will also be identified using splinkerette PCR and 454 sequencing.

5.3 Materials and Methods

5.3.1 Targeting vector construction

For generating the *Brd4-NUT* targeting construct (**Figure 5-2**), the human *NUT* cDNA was cloned from the GFP-BRD4-NUT expression vector (161) and was used to exchanged with the *AML1* sequence in the vector described above (see Chapter 4). Two genomic fragments from the *Brd4* locus were amplified by High Fidelity PCR and inserted into pBlueScript SK+ construct as targeting arms (5' arm: FWD 5'-AAAGCGGCCGCGGCCAAAAAGGCCTTGGCT TCAGTCACCAGTCTGGGTTGGTGCCCTATCATACGCA-3', REV 5'-AAAAAAA GGCCGGCCACGCGTAAGCTTGACTGGCAATAAAAGTG AAAAGTCAGTG-3'; 3' arm: FWD 5'- AAAAAATTAATTAAACGCGTAAGCTTAAGAATCCAAGTGTT CAAATGACAATCCCAGAGACTGACCCT-3', REV 5'- AAAAAAAAAAGGCGCG CCTGGCTCCCCAACAGGATCCCAGCTGGTATACTAAGGCTT-3'). The NUT-SB-Puro cassette flanked by the Lox66 and Lox71 sites was inserted into engineered *Mlu*I restriction sites in a reverse orientation relative to *Brd4* transcription. To generate a Flag tag-GFP version construct of the *Brd4-NUT* construct a similar recombineering strategy was used to that described in Chapter 4. The constructs generated were sequenced in full to ensure that PCR had not introduced any mutations.

5.3.2 Immunoprecipitation of FLAG Tagged Proteins

The Dynabeads protein G (Invitrogen, 0.5 ml beads) were first washed three times (buffer: 24.5mM Citric Acid, 51.7 mM Dibasic Sodium phosphate (Na_2HPO4) dehydrate, pH = 5.5). One microgram of anti-Flag M2 antibody (Sigma, F1804) was incubated with the beads in 20 µl of bead wash buffer for 40 min at room temperature. After incubation beads were washed three times with beads wash buffer with 0.1 % Tween-20 (Sigma). To prepare a cell lysate, cells were treated with protein lysis buffer (50 mM Tris pH 8.0, 450 mM NaCl, 0.2 % Nonidet P-40 (Igepal), 1 mM DTT, 1 mM EDTA, 1X Protease inhibitor (Roche) for 15 min on ice, then collected by centrfiguation at maximum speed using a desktop centrifuge for 15 min at 4 ˚C. Cell lysate was collected and incubated with the antibody conjugated beads for 1 hour at 4 ˚C with gentle shaking. The beads were collected after incubation and washed three times with protein wash buffer (same formula as protein lysis buffer except using 150 mM NaCl and 0.1 % NP-40 concentration). For Western blotting, 30 µl loading buffer were added

to the beads after pull down. The beads were then boiled for 10 min at 95 ˚C and supernatants were loaded directly on SDS page gels (5 %, Bio-rad). Western blotting was using anti-Flag M2 antibody (Sigma, F1804) and performed following manufacturer's instructions from Sigma.

5.3.3 Cell proliferation and colony formation assay

Cells were seeded into T25 flasks at a density of $4x10^6$. After two days these cultures had reached 80 % confluence. Cells were then treated with trypsin and counted using a Beckman Coulter cell counter. For cell proliferation experiments, $2x10^4$ cells were plated on each well of a 6 well plate in ES cell culture medium supplemented with 1 μM 4-OHT (Sigma) or the same volume of vehicle solution (95 % EtOH). Triplicate samples were prepared for each cell line. Media was changed every two days and cell number was calculated at day 6. For colony formation assays, 1000 cells were seeded into 10 cm culture dishes at single cell density. Triplicate plates were prepared for each cell line. Cultures were supplemented with 1 μM 4-OHT or 95 % EtOH for 10 days. Media was changed every three days and colonies were stained with methane blue at day 10 and colony numbers counted.

5.3.4 Cell Cycle Analysis by Flow Cytometry

Wild type E14 ES cells and *Brd4-NUT-GFP* targeted ES cells (CE-37) were treated two days with 4-OHT or 95 % EtOH (vehicle) and then subjected to flow cytometry analysis for GFP which is bicistronically expressed from the *Brd4-NUT* fusion transcript. GFP positive and negative cells were sorted by FACS into cold 70 % EtOH and fixed overnight at 4˚C. Unsorted wild type and CE-37 cells were fixed overnight with 70 % EtOH. Cells were washed two times with PBS and stained with 500 μl PI solution (0.1 % Triton X-100 (v/v), 50 μg/ml Rnase A (Sigma), 25 μg/ml Propidium Iodide (Aldrich,)) overnight at 4˚C then analysed by flow cytometry.

5.4 Results

5.4.1 Generating "conditional" *Brd4-NUT* knockin mouse model and characterizing targeted ES cells

The construct design for the *Brd4-NUT* knockin mouse model used a similar approach to the *Tel-AML1* mouse, except the human *NUT* and SB transposase cDNA sequence were

introduced in an inverted orientation into mouse *Brd4* locus flanked by two mutant *LoxP* sites, 66 and 71 (**Figure** 5-2), so that Cre-mediated recombination could be applied to flip the cassette 'on' and thus express *Brd4-NUT* conditionally on application of 4-OHT. To validate this conditional allele, this construct has also been targeted into an ES cell line expressing an inducible CreERt2 so that 4-hydroxytamoxifen (4-OHT) could be used to flip the cassette and study the cellular phenotype of the fusion protein *in vitro*. Verification that the ES cells been successfully targeted and that the flipping of the cassette occurred after induction of Cre via 4-OHT administration was determined through Southern blotting (**Figure 5-4, A)**. Using primer pairs flanking the flipped junction sites (Primers P1 + P2), a 0.5 Kb PCR fragment was detected in targeted ES cells after 4-OHT treatment but not in the absence of 4-OHT (**Figure 5-4, B**), indicating the CreERt2 cell line was tightly controlled and spontaneously flipping events were low. Using primers flanking the transcript junction site (**Figure 5-2**, primer P4 + P5), the fusion transcript could also be detected by RT-PCR and the fusion between *Brd4* and *NUT* transcripts was verified by subsequent sequencing (**Figure 5-4, C and D**).

By immunoprecipitation using an anti-BRD4 antibody described previously (173), a faint band at around 200 kDa could be identified representing the BRD4-NUT fusion protein, which was not identified in either the wild type or untreated *Brd4-NUT* ES cells (**Figure 5-4 E**). Western blotting using the anti-Flag M2 antibody could not detect any fusion protein expression, despite the addition of a Flag tag to the BRD4-NUT fusion protein (**Figure 5-4 E**), the reasons for which are unclear.

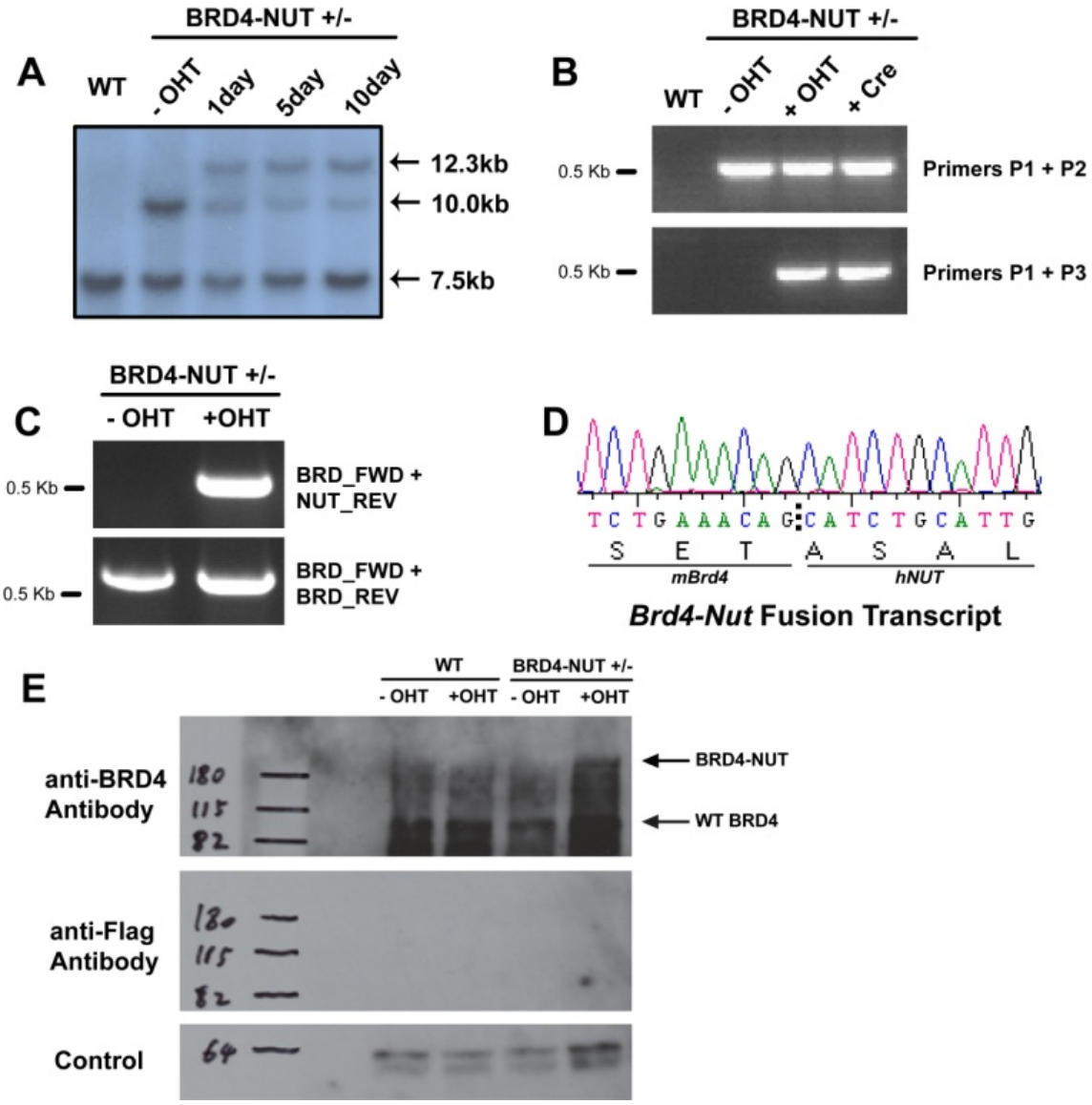

Figure 5-4. Characterization of the *Brd4-NUT* targeted ES cells.

(A) Southern blotting confirmed correct targeting and flipping of the targeted ES cell clone showing a targeted band at 10.0 kb in the targeted cells and 12.3 kb flipped band after treatment with 4-OHT for 1 day, 5 days and 10 days. (B) Genomic PCR using primer pairs flanking the flipping junction site (see Figure 5-2) in genomic DNA for the targeted cells before and after flipping using 4-OHT treatment or Cre-expressing plasmids. (C) RT-PCR showing a PCR band could be amplified using primer pairs flanking the fusion transcript junction site (see Figure 5-2) in the targeted cells treated with 4-OHT. (D) Sequencing trace confirmed the in-frame fusion between Brd4 and NUT transcript. (E) Immunoprecipitation and western blot for the BRD4-NUT fusion protein using an anti-BRD4 or anti-Flag antibody.

5.4.2 Analysis of Brd4-NUT expression using Quantitative PCR

As the BRD4-NUT fusion protein was only detectable at a very low level, a real-time qPCR experiment was setup to validate the expression level of *Brd4-NUT* at the transcriptional level. The *Brd4* gene is expressed in cells as a short and long isoform (161). To compare the relative expression of the two Brd4 isoforms and the fusion transcript, qPCR primers were designed to flank the junction points on three transcripts as indicated in **Figure 5-5**. The Brd4 short isoform was expressed at a much lower level than the long isoform in knockin cells both with or without 4-OHT treatment. The expression level of both isoforms was decreased when cells were treated with 4-OHT, which would be expected as a consequence of the inversion of the knockin allele in response to 4-OHT treatment, thus disrupting one of the Brd4 alleles. In the meantime, the Brd4-NUT transcript was expressed upon 4-OHT treatment, at a lower but comparable level to the Brd4 long isoforms. These results indicate that the conditional *Brd4-NUT* knockin construct is functional.

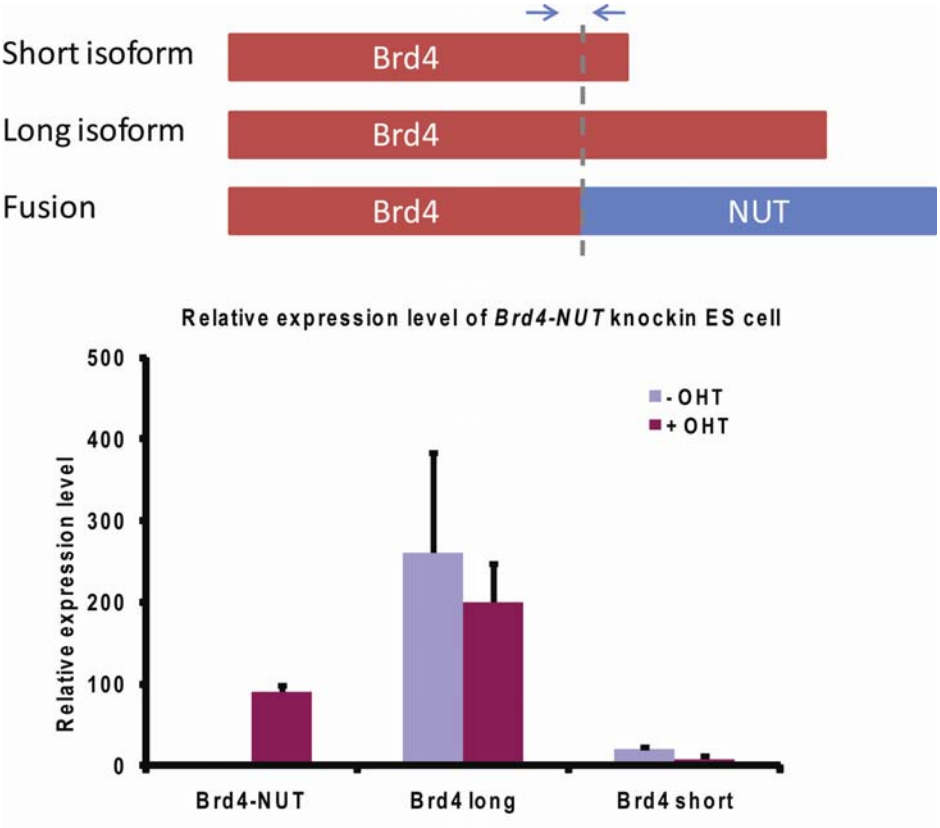

Figure 5-5. Evaluation of the *Brd4-NUT* expression level by qPCR

ES cells were treated with 4-OHT and 95 % EtOH (control) for two days before harvesting and cDNA preparation . For each type of transcript (*Brd4* short, *Brd4* long and *Brd4-NUT*), the expression level of control ES cells was shown in blue and the 4-OHT treated ES cells in purple. The blue arrows represent the primer pairs used for qPCR analysis on each transcript. The experiments were performed in triplicate. Error bars indicate mean expression level ± SD.

5.4.3 Deriving germ line transmission with the Brd4-NUT knockin ES cell lines

14 ES cell clones were injected to derive *Brd4-NUT* knockin lines, however none were successful in obtaining germ line transmission. These ES cell clones were generated from three different genetic backgrounds in over 5 different targeting batches: cell strains E14J – the ES cell strain from the Netherland Cancer Institute (NKI), B6 Blue –the commonly used ES cell strain and JM8.N4 from the mouse knockout project (COMP). Some of these ES cell clones have generated chimeras with quite high chimeric rate (80 - 90 %, as examined by animal facility staff), but none of these chimeras could produce F1 mice with *Brd4-NUT* transmitted through the germ line. Below is a list of all the targeted clones injected for attempted generation of F1 mice.

Table 5-1. Brd4-NUT clone microinjection information

Date	Team 113 ID	Cell Strain	Passage	RSF ID
13/07/2007	AX0015	E14J	15	BRD4-NUT_1
26/07/2007	AX0016	E14J	17	BRD4-NUT_2
14/09/2007	BI0067	E14J	11	BRD4-NUT_3
26/10/2007	BN0005	E14J	9	Cta_BN5
29/10/2007	BK0056	E14J	11	Nut-Flag-Ires-SB-BK56
30/10/2007	BK0004	E14J	11	Nut-Flag-Ires-SB-BK4
21/12/2007	BK0018	E14J	11	Nut-Flag-Ires-SB-BK18
03/03/2008	CB0015	B6 Blu	n/a	Nut-flag-IRES-T
31/03/2008	CB0061	B6 Blu	n/a	Nut-flag-IRES-T
10/06/2008	CB0061	B6 Blu	17	Brd4-NUT-Ires-Transposase
08/01/2009	CL0166	JM8.N4 (Cre-ERt2)	n/a	Brd4_Nut_CL166
13/01/2009	CL0180	JM8.N4 (Cre-ERt2)	n/a	Brd4_Nut_CL180
16/01/2009	CL0180	JM8.N4 (Cre-ERt2)	n/a	Brd4_Nut_CL180
27/01/2009	CL0121	JM8.N4 (Cre-ERt2)	n/a	Brd4_Nut_CL121

5.4.4 Expression of *Brd4-NUT* fusion impaired cell growth in ES cells

Although the efforts to derive a *Brd4-NUT* knockin mouse was unsuccessful, it was observed that during *in vitro* culturing of these *Brd4-NUT* knockin ES cells there was a strong cell growth arrest phenotype associated with 4-OHT induced *Brd4-NUT*. *In vitro* experiments were therefore carried out to evaluate the effects of *Brd4-NUT* expression in ES cells. It has been reported previously that ectopic expression of BRD4-NUT fusion protein could interfere with cell growth and inhibit S phase *in vitro* (161). To test the effects of endogenous *Brd4-NUT* expression in the knockin ES cells, the targeting construct was modified to exchange the SB sequence with a GFP or Neomycin coding sequence (to avoid the possible side-effects of SB transposase expression in cells), and these constructs were subsequently targeted into a CreERt2 ES cell line which has *CreERt2* cDNA targeted into *Rosa26* locus under a CMV promoter. In these cells the *Cre*ERt2 could therefore be activated by 4-OHT treatment to induce *Brd4-NUT* expression.

Four *Brd4-NUT* knockin cell lines derived from targeted ES cells were used for a cell plating assay (see Materials and Methods) to evaluate the effects of *Brd4-NUT* expression on cell growth (**Figure 5-6 A**). At 6 days after plating the original CreERt2 line and the control cell lines (Random 1-3,) which contained a random integrated targeting construct, the cell numbers in the 4-OHT treated samples have dropped to about 60 % of the control cell numbers (grown in vesicle medium with 95 % EtOH). This drop in cell number in 4-OHT treated plates could either be due to a Cre toxicity (183) or 4-OHT toxicity effect, or both. In contrast, all four Brd4-NUT lines (BN-GFP and BN-Neo1-3) treated with 4-OHT to induce expression of the fusion protein had a clear cell proliferation arrest phenotype; producing only 20 % of the number of cells compared with control cells treated in a vesicle medium. (**Figure 5-6 B**). This indicates that the *Brd4-NUT* expression severely impaired cell growth ability of ES cells. In addition, all four targeted ES cells grew slower than control cell lines in the absence of 4-OHT treatment (data not shown); this could be due to disruption of the knockin allele in one *Brd4* allele causing a negative effect on cell growth independent of *Brd4-NUT* expression.

During activation of the *Brd4-NUT* expression by 4-OHT, the flip of the knockin cassette could also disrupt *Brd4* gene transcription from the targeted allele. This has previously been demonstrated to cause haploinsufficiency for the wild type *Brd4*, therefore could be a possible mechanism causing the cell proliferation phenotype observed (184). To confirm this

cell growth arrest phenotype is associated with *Brd4-NUT* expression, the *Brd4* gene was knocked out using a targeting construct obtained from the EUCOMM project which targets exon 5 at the open reading frame of *Brd4*. In this targeting construct the *Brd4* exon 5 is flanked by two *loxP* sites which could be removed by Cre expression. After targeting of this construct into the CreERt2 cell line and validation by Southern blotting (data not show), two targeted ES cells were selected as a control in the cell proliferation assay. The two Brd4 +/- knockout cell lines had very similar cell growth pattern to the Cre-ERT2 and random insertion control cells after 4-OHT treatment (**Figure 5-6 B**), indicating that the disruption of one *Brd4* allele is sufficient reason to cause cell proliferation arrest. As further proof that the phenotype in cell culture is caused by *Brd4-NUT* expression, the knockin targeting construct was modified to truncate ~2700 base pairs from the C-terminal *NUT* cDNA using a *BstE*II restriction site. This construct expressed a truncated BRD4-NUT protein (Brd-ΔNUT), with a 200 amino acid NUT sequence forming a C-terminal fusion to BRD4, in contrast with the over 1000 amino acid NUT sequence of the original construct. Surprisingly, when the knockin ES cells with the truncated NUT cDNA were treated with 4-OHT, the cell growth rate was only slightly slower than the wild type controls but much higher than the growth rate of all four *Brd4-NUT* knockin cell lines (**Figure 5-6 B**). This indicates that full length *Brd4-NUT* is essential for causing the cell proliferation arrest phenotype.

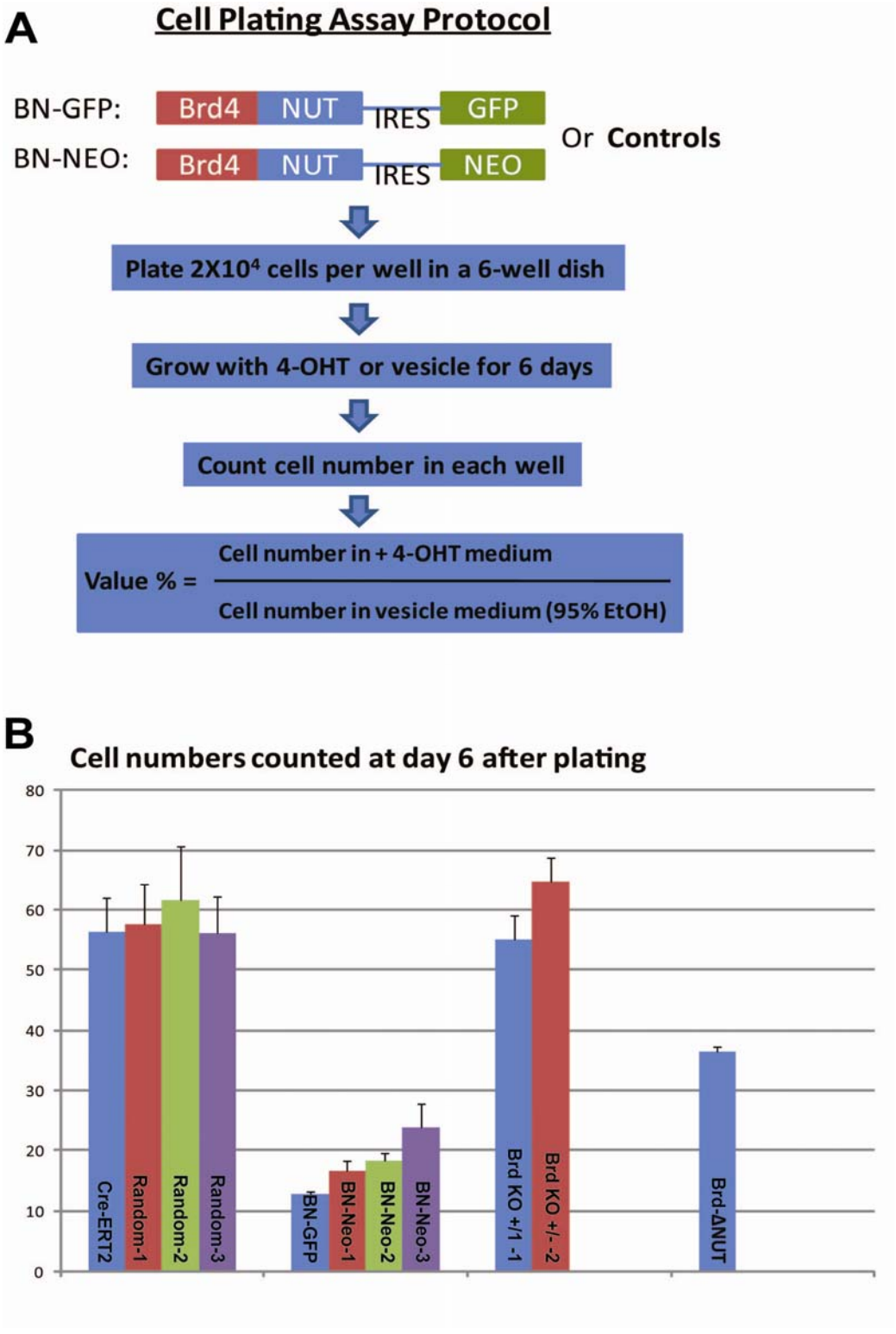
A
Cell Plating Assay Protocol
BN-GFP:
Brd4
NUT
IRES
GFP
BN-NEO:
Brd4
NUT
IRES
NEO
Or Controls
Plate 2X10⁴ cells per well in a 6-well dish
Grow with 4-OHT or vesicle for 6 days
Count cell number in each well
Value % = Cell number in + 4-OHT medium / Cell number in vesicle medium (95% EtOH)
B
Cell numbers counted at day 6 after plating
80
70
60
50
40
30
20
10
0
Cre-ERT2
Random-1
Random-2
Random-3
BN-GFP
BN-Neo-1
BN-Neo-2
BN-Neo-3
Brd KO +/1 -1
Brd KO +/- -2
Brd-ΔNUT

Figure 5-6. Cell plating assay in *Brd4-NUT* ES cells.

(A) Experiment strategy of the cell plating assay. (B) Cell number counting results at day 6 after plating for *Brd4-NUT* knockin ES cells and control groups. The Y-axial indicates the *'*value %*'* for each cell line (*n* = 3) calculated using the cell numbers in medium treated with 4-OHT divided by the cell numbers in medium treated with vesicle solutions (95 % EtOH) at day 6. Means ± SD are shown for three parallel experiments. Cre-ERT2: original ES cell line used for the targeting experiments; Random 1-3: cell line with random integration of the targeting constructs; BN-GFP: *Brd4-NUT* targeted ES cell expressing a GFP protein after IRES site; BN-Neo 1-3: *Brd4-NUT* targeted ES cell expressing a Neomycin drug resistance protein after IRES site; Brd KO: Brd4 conditional knockout cell lines; Brd-ΔNUT: *Brd4-NUT* targeted ES cell expressing a truncated NUT protein fused to the C-terminal BRD4.

5.4.5 Expression of *Brd4-NUT* fusion blocks colony formation ability in ES cells

To further validate the growth arrest phenotype associated with *Brd4-NUT* expression, a colony formation assay (see Material and Methods) was carried out to evaluate the colony formation ability of ES cells expressing *Brd4-NUT*. For this cell lines with or without 4-OHT treatment were plated at single cell density to result in individual colony formation, and colony numbers were counted after 10 days (**Figure 5-7 A**). Treatment with 4-OHT completely blocked colony formation ability in targeted ES cells (BN-GFP and BN-Neo-1, -2, -3) but only caused a moderate drop (~40 %) in control cell lines (Random-1, -2, -3 and the original Cre-ERt2 cell line) (**Figure 5-7 B and C**). Similar to the results from the cell plating assay, the number of colonies formed for the two *Brd4* KO +/- cell lines were similar to the control groups (**Figure 5-7 B and C**), indicating disruption of the *Brd4* allele during *Brd4-NUT* activation is not a cause of cell growth arrest in ES cells. Interestingly, albeit a moderate delay in cell growth was observed in the NUT truncated knockin ES cells (Brd4-ΔNUT), this cell line was able to form some colonies when treated with 4-OHT to induce truncated Brd4-NUT expression, compared with the full length Brd4-NUT lines where cell proliferation was almost completely arrested after treatment with 4-OHT. This demonstrates that the NUT sequence in the BRD4-NUT is essential to cause the growth arrest phenotype in ES cells (**Figure 5-7 B and C**).

In another experiment to derive a flipped *Brd4-NUT* clone (**Figure 5-7 D**), the targeted ES cells (BN-GFP) were treated for two days with 4-OHT so that the cell population contains a mix of flipped and unflipped cells. These cells were seeded to form single colonies and 100 colonies were picked at day 10 for genotyping and southern blot. In alignment with the colony formation results, none of these colonies contains the flipped allele indicating that expression of *Brd4-NUT* transcript has completely blocked colony formation ability.

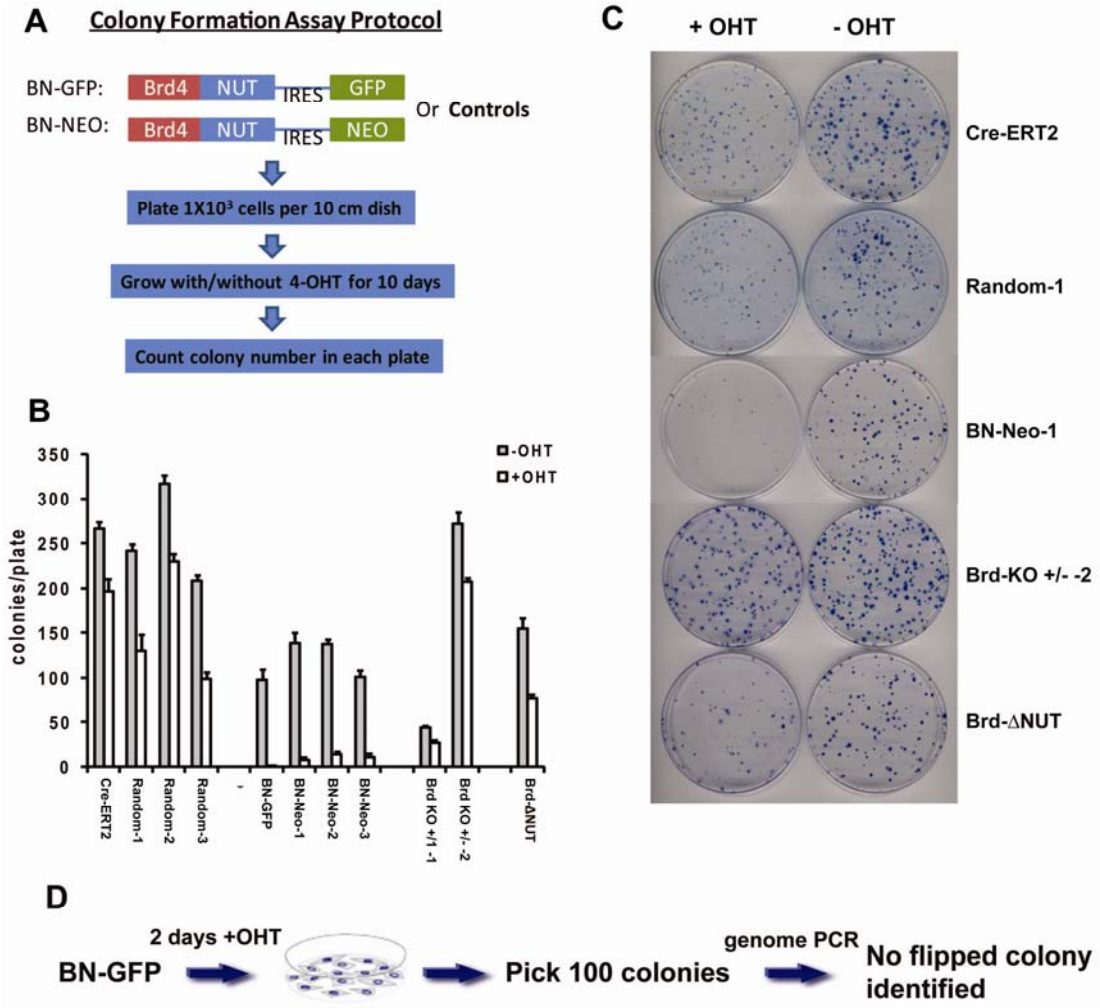

Figure 5-7. Colony formation assay with *Brd4-NUT* ES cells

(A) Experiment strategy for the colony formation assay. (B) Quantification of colony numbers of experiment and control group cell lines without or with 4-OHT treatment. Means ± SD are shown for three parallel experiments. (C) Representing plates from colony formation assay. (D) Procedure for colony plating assay to derive flipped *Brd4-NUT* clone. Cre-ERT2: original ES cell line used for the targeting experiments; Random 1-3: cell line with random integration of the targeting constructs; BN-GFP: *Brd4-NUT* targeted ES cell expressing a GFP protein after IRES site; BN-Neo 1-3: *Brd4-NUT* targeted ES cell expressing a Neomycin drug resistance protein after IRES site; Brd KO: Brd4 conditional knockout cell lines; Brd-ΔNUT: *Brd4-NUT* targeted ES cell expressing a truncated NUT protein fused to the C-terminal BRD4.

5.4.6 Expression of *Brd4-NUT* arrested cell cycle at G2/M phase

Since the targeted BN-GFP cell line also expressed a GFP protein from the targeted allele after treatment of 4-OHT, GFP signal could be used to indicate the *'*switched on*'* or *'*switched off*'* state of the *Brd4-NUT* expression. To further characterize the *Brd4-NUT* induced cell growth arrest, flow cytometry was used to separate green and non-green populations after 4-OHT treatment. Propidium Iodide (PI) staining was performed alongside to analyze progression of the cell cycle (**Figure 5-8 A**). Although the GFP signal is weak due to low expression from the endogenous *Brd4* locus, a broadened GFP spectrum could be seen after 4-OHT treatment in the BN-GFP cell line compare to the CreERt2 control ES cells treated with 4-OHT (**Figure 5-8 B**). After sorting, the green and non-green populations in 4-OHT treated BN-GFP cells were PI stained and analysed to determine cell cycle progression. An obvious change in cell cycle stage was observed in the green cell population with a much stronger peak at G_2/M phase (**Figure 5-8 D**), indicating a strong cell cycle arrest at this stage. However the cell cycle appeared normal in the non-green cells sorted from the same cell population. This G_2/M arrest was only present in the targeted BN-GFP ES cells after 4-OHT treatment but not in the control CreERt2 cell. The G2/M cell population was increased in the 4-OHT treated BN-GFP cells after FACS sorting, which was enriched in the GFP::Brd4-NUT cell population (**Figure 5-8 C**).

This experiment suggested that *Brd4-NUT* expression in ES cells results in a significant cell cycle arrest at G_2/M phase, which could be the cause of cell growth arrest phenotypes observed in the previous experiments. Due to the weak expression of the GFP marker, the construct has been re-engineered by exchanging GFP with the more sensitive *Venus* YFP cell marker (185). However, due to YFP filters not being available, separation experiments based on Venus YFP could not be performed at the present time.

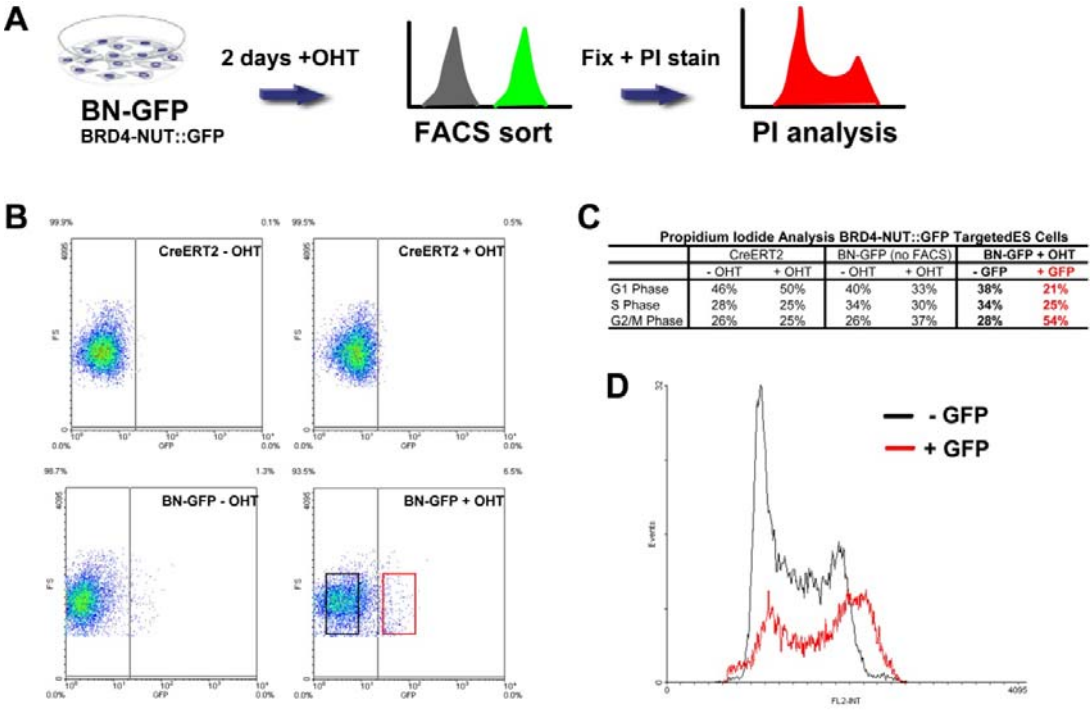

Figure 5-8. Cell cycle analysis of *Brd4-NUT* ES cells

(A) Experimental strategy for cell cycle analysis of *Brd4-NUT* ES cells. (B) FACS sorting for GFP signal of *Brd4-NUT* ES cells without or with 4-OHT treatment. (C) Quantification of cell cycle population in cell samples without or with 4-OHT treatment (Column 1: CreERt2 ES cells, Column 2: BN-GFP ES cells, Column 3: 4-OHT treated BN-GFP ES cells after FACS sorting). (D) PI analysis for FACS sorted green (red) and non-green (black) populations in targeted *Brd4-NUT* ES cells treated with 4-OHT.

5.5 Discussion

5.5.1 Models of choices for characterizing human carcinogenic translocations

This study aimed to generate a *Brd4-NUT* mouse model to study the oncogenic translocation of BRD4-NUT *in vivo*. In contrast to *TEL-AML1* which has been well studied, *BRD4-NUT* is a relatively rare translocation. Only a few clinical cases have been documented although it is likely to be under-reported because karyotype analysis is rarely performed on solid tumours. However, as a recurrent translocation identified in midline carcinoma, *BRD4-NUT* might represent a novel mechanism in the pathogenesis of solid tumours that has been underestimated. The mouse model described in this chapter brings the potential to identify a novel pathway in *BRD4-NUT* induced midline carcinoma, a rare human disease.

The *BRD4* gene is present as a long and a short isoform in both the mouse and human genome. In human patients with *BRD4-NUT* translocation, the break point has been identified in intron 12 of the *BRD4* gene, which disrupted both the short and long isoforms (161). Although the mouse *Brd4* short isoform only has 12 exons in Ensembl database (Transcript ID: ENST00000360016), in-house sequence comparison revealed a 13 exon after the NUT insertion site. The NUT insertion site in our knockin cell line is in mouse *Brd4* intron 12, at a similar position with the genomic break point found in human patients. Therefore, activation the NUT cassette would also disrupt both short and long *Brd4* isoforms in our mouse model, which exactly mimics the events taking place in human *BRD4-NUT* translocation.

5.5.2 Germ line transmission of the *Brd4-NUT* knockin ES cells

In this study I attempted to derive germ line transmission for the *Brd4-NUT* knockin mouse. Although 14 cell clones under three genetic backgrounds were used for microinjection the knockin allele could still not transmit through the germ line. Given that the *NUT* cDNA is knocked-in at a reverse orientation, is it highly unlikely that the problem is caused by *Brd4-NUT* expression in the targeted ES cells without induction. Nevertheless, knocking-in of *NUT* cDNA into the wild type mouse *Brd4* locus could still disrupt the original locus to some extent and cause haploinsufficiency of *Brd4*. However, it has been also shown that the heterozygous *Brd4* knockout could also be generated and be transmitted through the germ line (184). It is worth to notice that in the cell plating assay, the growth rate of all four *Brd4-NUT* knockin ES cell lines were approximately 50 % slower than the growth rate of control cell lines in the medium without 4-OHT. Therefore the difficulties in obtaining *Brd4-NUT*

knockin ES cells for germ line transmission might reasons associated with disruption of the mouse *Brd4* locus which results in a reduction in cell growth rate.

5.5.3 *Brd4-NUT* induced cell growth arrest

The *Brd4-NUT* mouse model had a strong cell growth arrest phenotype in ES cells, which was observed in both cell growth assays and cell cycle analysis. In the *Brd4-NUT* knockin allele, the *NUT* cDNA was inserted into intron 12 on *Brd4* locus (**Figure** 5-2). Activation of the NUT cassette results in expression of the fusion protein but also causes *Brd4* heterozygosity. We believed that the cell growth arrest phenotype is caused by expression of the fusion protein rather than *Brd4* haploinsufficiency, because although the *Brd4* haploinsufficiency has been indicated to slow down cell growth rate (184), it did not cause a strong cell growth arrest in our experiments. In addition, the *Brd4* null allele could be derived by gene trapping (184), which further indicates that the *Brd4* haploinsufficiency could not block colony formation ability. To better discriminate the effects between fusion protein expression and *Brd4* heterozygosity, we generated *Brd4* +/- cell lines in CreERt2 background and demonstrated that the cell growth rate of *Brd4* +/- cell lines was similar to the rate of control cell lines in cell plating and colony formation assays.

The cell cycle arrest was identified to be at the G2/M phase, which is different from the results obtained by Haruki et al. who claimed that ectopic expression of BRD4-NUT in H293T cells caused cell cycle arrest at S phase (161). However, another publication showed that inhibition of wild type BRD4 protein using an anti-BRD4 antibody could inhibit HeLa cells entering mitosis (173). Considering the *BRD4* itself is a multi-stage cell-cycle controlling gene and is affected by haploinsufficiency (184), it is possible that the *Brd4-NUT* expression in different cells and at different levels could result in different phenotypes. Therefore the actual function of *Brd4-NUT* in cancer inducing cells requires the *Brd4-NUT* expression system to be tested in further *in vitro* and *in vivo* studies.