January 2009

Role of chromodomain helicase DNA-binding protein 2 in DNA damage response signaling and tumorigenesis.

Thandi M. Onami
University of Tennessee - Knoxville, tonami@utk.edu

P. Nagarajan

S. Rajagopalan

S. Kania

R. Donnell

See next page for additional authors

Oncogene (2009) 28, 1053–1062

www.nature.com/onc

ORIGINAL ARTICLE

Role of chromodomain helicase DNA-binding protein 2 in DNA damage response signaling and tumorigenesis

P Nagarajan[1,4], TM Onami[2], S Rajagopalan[1], S Kania[3], R Donnell[3] and S Venkatachalam[1]

[1]*Department of Biochemistry and Cellular and Molecular Biology, University of Tennessee, Knoxville, TN, USA;* [2]*Department of Microbiology, University of Tennessee, Knoxville, TN, USA and* [3]*Department of Pathobiology, University of Tennessee, Knoxville, TN, USA*

The chromodomain helicase DNA-binding proteins (CHDs) are known to affect transcription through their ability to remodel chromatin and modulate histone deacetylation. In an effort to understand the functional role of the CHD2 in mammals, we have generated a Chd2 mutant mouse model. Remarkably, the Chd2 protein appears to play a critical role in the development, hematopoiesis and tumor suppression. The Chd2 heterozygous mutant mice exhibit increased extramedullary hematopoiesis and susceptibility to lymphomas. At the cellular level, Chd2 mutants are defective in hematopoietic stem cell differentiation, accumulate higher levels of the chromatin-associated DNA damage response mediator, γH2AX, and exhibit an aberrant DNA damage response after X-ray irradiation. Our data suggest a direct role for the chromatin remodeling protein in DNA damage signaling and genome stability maintenance.
Oncogene (2009) **28,** 1053–1062; doi:10.1038/onc.2008.440; published online 12 January 2009

Keywords: chromatin remodeling; lymphoma; hematopoiesis; mutant mice; tumor suppressor and DNA damage

Introduction

Chromatin remodeling serves as an important regulator of various DNA processes including replication, transcription, recombination and DNA repair (Wu, 1997; Bernstein *et al*., 2002; Hasan and Hottiger, 2002; Qin and Parthun, 2002; Osley *et al*., 2007). The remodeling of chromatin has also been implicated in physiological processes as diverse as embryonic development and cancer (Muller and Leutz, 2001; Wang *et al*., 2007a, b). The chromodomain helicase DNA-binding proteins (CHDs) were characterized as a distinct family of proteins in the 1990s (Woodage, 1997). The *CHD* genes are evolutionarily conserved, and at least nine genes have been identified in vertebrates ((Delmas *et al*., 1993; Schuster, 2002) and (NCBI, assembly: 36)). The various protein domains in these proteins are the chromodomain (Chromatin organization modifier), SNF2-related ATP-dependent helicase domain, specific DNA-binding domains, PHD Zn-finger domains and the C-terminal helicase domain (Woodage, 1997). The chromodomain was initially characterized in *Drosophila* HP1 and Polycomb proteins (Paro and Hogness, 1991). Chromodomain-containing proteins can self-associate and also interact with the heterochromatic regions at centromeres, telomeres and polytene chromosomes (Singh *et al*., 1991; Cowell and Austin, 1997). The CHD1 protein was initially characterized as a protein that bound to immunoglobulin promoter sequences and later analyses showed that the protein bound to decondensed chromosomes and A + T-rich sequences by its unique high mobility group (HMG)-1-binding domain (Delmas *et al*., 1993; Stokes and Perry, 1995). In addition to its DNA-binding properties, CHD1 was also shown to function as a chromatin assembly factor that has the ability to transfer histones to DNA *in vitro* and interact with transcription elongation factors (Simic *et al*., 2003; Lusser *et al*., 2005). Substantial data on the CHD family of proteins have come from biochemical studies on human CHD3 and CHD4, in which they were shown to be components of nucleosome remodeling and histone deacetylase complexes (Targoff and Reichlin, 1985; Tong *et al*., 1998; Xue *et al*., 1998; Zhang *et al*., 1998; Brehm *et al*., 2000). Patients with the autoimmune condition dermatomyositis produce antibodies against CHD3 and CHD4 and are prone to increased susceptibility to cancer (Targoff and Reichlin, 1985; Takeda and Dynan, 2001). More recently, the *CHD5* gene has been identified as a tumor suppressor gene residing in 1q locus of human chromosome (Bagchi *et al*., 2007). Interestingly, mutations in another CHD family member, CHD7, have been shown to lead to CHARGE syndrome, a complex multiorgan disorder that includes Coloboma, Heart defects, choanal Atresia, mental Retardation, Genital and Ear anomalies and scoliosis (Vissers *et al*., 2004; Lalani *et al*., 2006; Gao *et al*., 2007).

Recent *in vitro* studies have shown that the chromodomains present in CHD1 bind to specific histone H3

Correspondence: Dr S Venkatachalam, Department of Biochemistry and Cell and Molecular Biology, University of Tennessee, M407, Walters Life Science Building, Knoxville, TN 37996, USA.
E-mail: sundar@utk.edu
[4]Current address: Department of Molecular and Cellular Biochemistry, The Ohio State University, Columbus, OH, USA.
Received 28 April 2008; revised 20 October 2008; accepted 30 October 2008; published online 12 January 2009

methylated sites associated with activated transcription (Flanagan *et al*., 2005; Pray-Grant *et al*., 2005; Sims *et al*., 2005). Unlike any other CHD family member, the CHD1 and CHD2 proteins contain the A + T-hook motif (also known as HMG1 domain) that is also present in the HMG proteins (Thomas, 2001). Furthermore at the protein level, the CHD2 protein shares a high degree of homology with CHD1, which has been implicated in transcription and chromatin assembly (Woodage, 1997; Jones *et al*., 2000; Tran, 2000; Simic *et al*., 2003; Lusser *et al*., 2005). However, the physiological role of CHD2 and its *in vivo* effects on transcription are yet to be discerned, and recent studies suggest a role for Chd2 in mammalian development and survival (Marfella *et al*., 2006). In this study, we show that the Chd2 protein functions as a tumor suppressor gene and plays a potential role in modulating DNA damage responses at the chromatin level.

Results

Chd2 is an essential gene in mice

In an effort to understand the role of Chd2 in mammalian development and physiology, we generated a Chd2 mutant mouse model using the Baygenomics gene trap embryonic stem (ES) cell resource (Supplementary information and Supplementary Figure 1) (Stryke *et al*., 2003). Genotype analysis of the embryos and offspring obtained from F1 heterozygous intercrosses indicated that the *Chd2* mutation led to embryonic and perinatal lethality (data not shown). Interestingly, the proportion of heterozygotes obtained from the intercrosses was also less than the expected 2:1 ratio of the total offspring and this was further confirmed in crosses between heterozygous males and wild-type females (Supplementary Table 1). Morphological analysis of the heterozygous and homozygous mutant embryos at E12.5 showed a drastic reduction in the formation of vascular structures and regions of localized hemorrhaging (Figure 1a). Further analysis of the reduced vasculature of the Chd2 homozygous mutant embryos showed a substantial decrease in PECAM-1 staining in the peripheral vascular structures, indicative of defective vascular wall integrity in a subset set of mutants (data not shown). Interestingly, the hemorrhages were also present in some of the heterozygous mutants and this could explain the partial lethality of the heterozygous animals.

The developmental phenotypes of the heterozygous and the homozygous mutant animals strongly suggested that the *Chd2* gene is essential for development. However, studies by other groups that have used gene trap-based knockdown of specific genes have shown that the effectiveness of the gene trap is variable and may lead to the generation of hypomorphic mutants (Voss *et al*., 1998; Gonzalez-Billault *et al*., 2000; Fukasawa *et al*., 2006). To ascertain the effectiveness of the gene trap on knocking down the expression of wild-type *Chd2* (by affecting the splicing between exons 27 and 28) and rule out any leaky expression of the wild-type gene in the homozygous mutants, we determined the expression of wild-type *Chd2* in homozygous mutant cells and embryos in reverse transcription–PCR assays. Surprisingly, we found that the homozygous mutants did express the wild-type mRNA albeit at lower levels (Supplementary Figure 2). The ineffective downregulation of *Chd2* may lead to the generation of a hypomorphic allele or a dominant negative allele (by the interaction of the Chd2-β-gal-neomycin fusion peptide with the wild-type protein). To test this possibility of dominant negative effects, we analysed the inter-molecular interactions between recombinant Chd2 peptides that contained 6x-His and HA epitope tags. As shown in Supplementary Figure 3, reciprocal immunoprecipitation analysis of recombinant Chd2 peptides showed that they were able to interact with each other. These results suggest that the Chd2-β-gal-neomycin fusion protein may either sequester the wild-type Chd2 protein to the cytoplasm or compete with the native protein for its binding partners. However, our results do not rule out the possibility that the Chd2-βgeo fusion protein could also function as a gain of function mutant.

Chd2 affects hematopoietic stem cell differentiation

Extensive histological examination of the neonates did not reveal any gross anatomical differences between the wild-type and mutant animals, except for the occasional atrial enlargement in the mutants. Owing to the extremely limited quantities of peripheral blood available in the neonates, we were unable to perform a complete blood analysis of the newborn pups. However, examination of the neonatal livers of the mutants indicated that the hematopoietic cell distribution was different in the mutants that showed an increase in the number of megakaryocytes (Figure 1c). Furthermore, the hematopoietic cell islands were less organized in the mutants in comparison with the wild-type neonates that showed well-organized clusters of hematopoietic cells (Figures 1b and c). The localized hemorrhages and increased megakaryocytes in the mutant animals suggested a role for the Chd2 protein in hematopoietic stem cell differentiation. To test this possibility, we analysed the lineage-specific differentiation potential of hematopoietic stem cells and found that the Chd2 mutant cells were defective in their ability to differentiate into the erythroid lineage (Figure 1d). The lowered capacity of erythrocyte differentiation in Chd2 mutants were further confirmed in fluorescence activated cell-sorting analyses using flow cytometry. The differentiation of erythroblasts to erythrocytes can be assessed by analysing and quantitating the expression of cell surface markers Ter119, CD71 and CD117 (Kina *et al*., 2000; Socolovsky *et al*., 2001; Zhang *et al*., 2003; Spike *et al*., 2004). During erythroid maturation, the expression of CD71 decreases from proerythroblasts to early basophilic erythroblasts, whereas the expression of the Ter119 remains high (Socolovsky *et al*., 2001). As shown in Supplementary Figure 4, there was an increase in the percentage of $CD71^{+}Ter119^{-}$ cells in the mutants (27.9

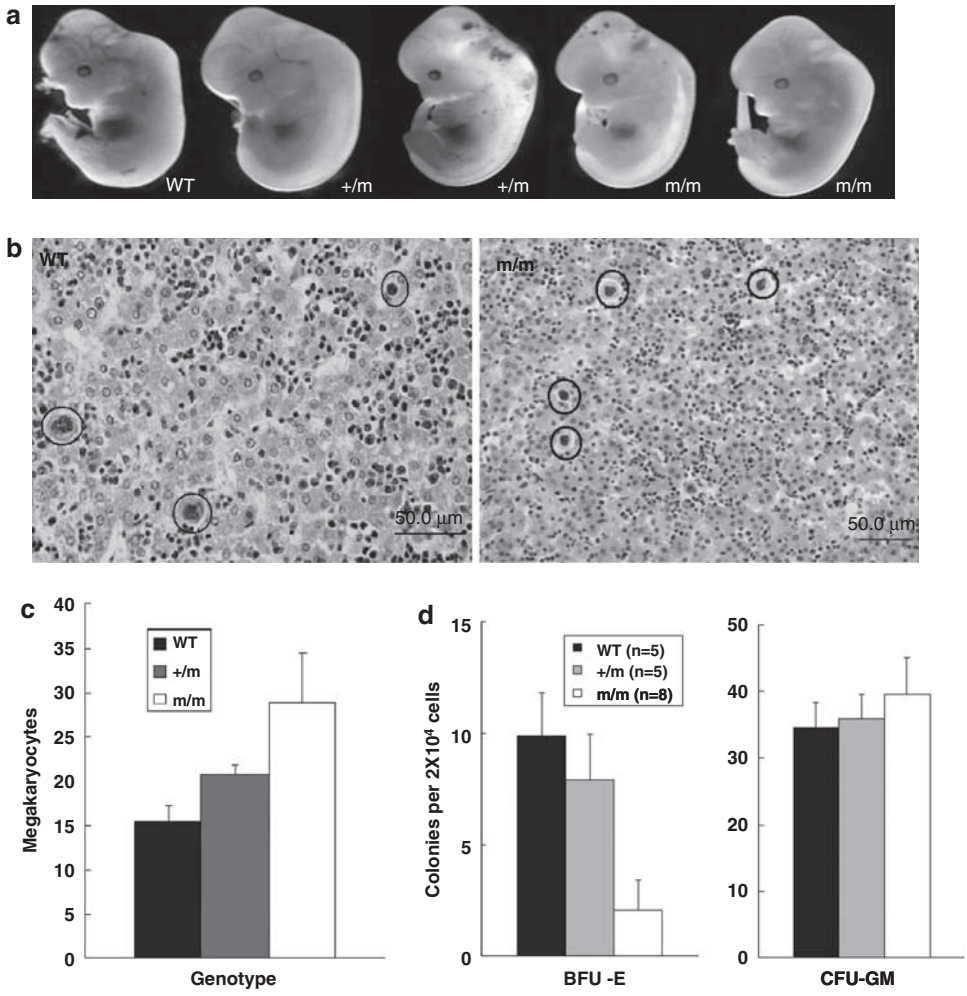

Figure 1 Analysis of the developmental phenotypes in Chd2 mutant mice. (**a**) Morphological phenotypes of Chd2 mutant embryos. E12.5 embryos were harvested and photographed. (**b**) Hematopoietic cell distribution and organization defects in mutant neonates. Representative images of hematoxylin and eosin (H&E)-stained sections of wild type (WT) and homozygous mutant neonatal liver sections are shown. The megakaryocytes are circled. (**c**) Increased megakaryocytes in Chd2 mutant mice. A total of 10 different fields were counted from H&E-stained sections of neonatal fetal livers from each group ($n=7$). The differences between the wild-type and the mutants were statistically significant as determined by single-tailed *t*-test (WT v. +/m, $P<0.002$ and WT v. m/m, $P<0.005$). Error bars represent s.e. (**d**) Hematopoietic stem cell differentiation defects in Chd2 mutants. Burst-forming unit-erythroid (BFU-E) and colony-forming unit-granulocyte macrophage (CFU-GM) formation in wild-type ($n=5$), $Chd2^{+/m}$ ($n=5$) and $Chd2^{m/m}$ ($n=8$) were assayed using E13.5 fetal liver progenitor cells as described. The differences between the wild-type and the homozygous mutants were statistically significant as determined by single-tailed *t*-test (WT v. m/m, $P<0.00005$). Error bars represent s.e.

and 25.0%) in comparison with the wild-type fetal livers (9%). A concomitant reduction in the number of double-positive $CD71^{+}Ter119^{+}$ cells is also observed in the mutants (87.6% in wild type versus 60.5 and 54.7% in mutants). Similar results were obtained from the fluorescence activated cell-sorting analysis profiles of erythroid progenitors stained with Ter119 and CD117 (Supplementary Figure 4, bottom panel).

Chd2 deficiency leads to lymphomas

The heterozygous offspring that survived beyond the perinatal stage did not show any overt developmental abnormalities except for an apparent reduction in size at birth that was less pronounced in mice that were 3–4 months old. However, after 8–10 months of age the heterozygous mice began to exhibit weight loss, lordokyphosis (hunch-back spine) and loss of vitality. Interestingly, the lordokyphosis phenotype of the Chd2 mutant mice resembles the vertebral abnormalities found in human patients with mutations in *CHD7* and *CHD2* (Gao *et al*., 2007; Kulkarni *et al*., 2008). Survival analysis showed drastic reduction in the lifespan of the Chd2 heterozygous mutant mice (median lifespan of 52.3 weeks (Figure 2a)). Histological examination of organs harvested from morbid mice showed that a majority of the mice were succumbing to splenic, lymphnode and thymic lymphomas as well as lymphoid hyperplasias (Figures 2b–e, and Table 1). The earliest incidence of lymphomas in the mutant mice was at 26 weeks of age and a majority of the mice (14/21) had succumbed to lymphomas within 58 weeks. In comparison,

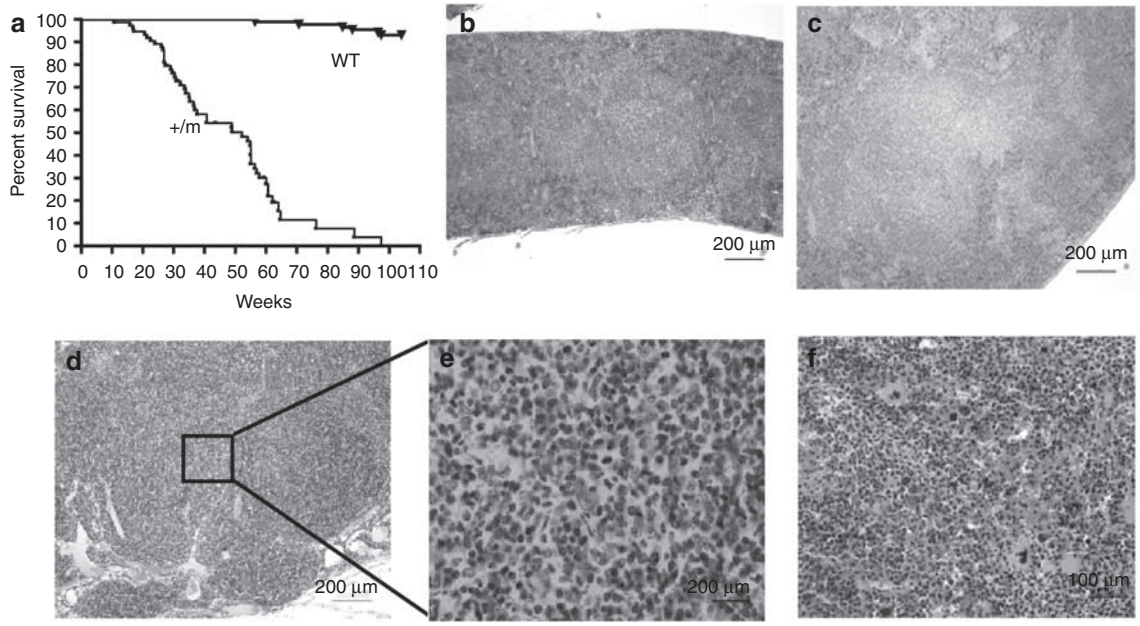

Figure 2 Lymphoid tumor susceptibility and hematopoietic defects in Chd2 mutant mice. (**a**) Kaplan–Meier survival curves of Chd2 mutant and wild-type littermates ($n = 50$). The percentages of survival are plotted as a function of age in weeks. Animals were monitored for tumors, morbidity or spontaneous death over a period of 105 weeks. Of the 74 animals analysed for each group, 50 of the heterozygous mutants died in comparison with 6 for the wild-type controls during a period of 2 years. All mice were of mixed inbred C57BL/6X129/Sv background. (**b**–**f**) Representative images of hematoxylin and eosin-stained sections of a normal spleen from an age-matched wild-type mouse (**b**), lymphoid hyperplasia (**c**), lymphoma (**d** and **e**) and extramedullary hematopoiesis from heterozygous mice (**f**) are shown.

Table 1 Distribution of pathological conditions in Chd2 heterozygous mice

Lymphoma	43.7% (21 of 48)
Lymphoid hyperplasia	31.2% (15 of 48)
Extramedullary hematopoiesis[a] (EMH)	39.6% (19 of 48)
Glomerulo-nephropathy[b]	46.8% (15 of 32)
Inflammation of heart/artery[c]	43.2% (16 of 37)
Other cancers[d]	4.1% (2 of 48)

Tissues from a total of 48 mice were analysed to determine the reasons for morbidity. Hearts were examined for 37 mice. To avoid overestimation of lymphoid hyperplasias, animals showing lymphomas as well as lymphoid hyperplasias (in other organs) were categorized under lymphomas.
[a]13 out of 19 animals diagnosed with EMH showed either lymphoid hyperplasia or lymphoma.
[b]12 out of 15 animals showing nephropathy were diagnosed with either lymphoid hyperplasia or lymphoma.
[c]13 out of 16 animals diagnosed with arterial and heart inflammation showed either lymphoid hyperplasia or lymphoma phenotypes.
[d]Two animals were diagnosed with hemangiosarcoma and bronchoalveolar carcinoma.

only one out of the six wild-type mice was diagnosed with lymphoma during the analysis period. Wild-type mice also develop lymphomas as a function of age, and such tumors account for about 5–20% incidence as reported for mice of various genetic backgrounds by others (Bronson and Lipman, 1991; Venkatachalam *et al*., 1998; Jeganathan *et al*., 2007). In addition to the lymphoid tumor phenotypes, a significant proportion of the mice exhibited extramedullary hematopoiesis and lymphoma-related pathologies that included nephropathy and inflammation of the heart/artery, as a majority of these animals exhibited a concomitant lymphoid phenotype (Figure 2f and Table 1). To determine whether the hyperplasias or lymphomas were due to increased T or B cells, we isolated lymphocytes from spleen and lymph nodes and stained with antibodies specific for T and B cells. In addition, we also used antibodies against the T-cell activation marker CD44 and analysed the cells by fluorescence activated cell-sorting analysis. Although the total lymphocyte numbers were increased in the Chd2 heterozygous mice in spleen and lymph nodes, there were no differences in the total number of B cells, indicating that the observed hyperplasias and lymphomas were most likely unrelated to dysregulation of the B-cell compartment (Figure 3b). However, analysis of T cells in the Chd2 heterozygous mice showed significantly increased numbers of activated CD44 high CD4 T cells in mice independently confirmed with hyperplasia or lymphomas by histopathology (Figures 3a and c). In disease-free younger mutants, no differences were observed in the proportion or number of activated CD4 T cells (Supplementary Figure 5 and data not shown). These data suggest that the hyperplasias and lymphomas observed in Chd2 mice are due to dysregulation of activated T cells. Furthermore, expression analysis of *Chd2* transcripts in organs harvested from wild-type adult mice showed a diverse expression pattern with the highest expression in thymus followed by lungs, kidneys, spleen, heart, testis and liver (Supplementary Figure 6). The highest expression of Chd2 in the thymus provides additional evidence for the

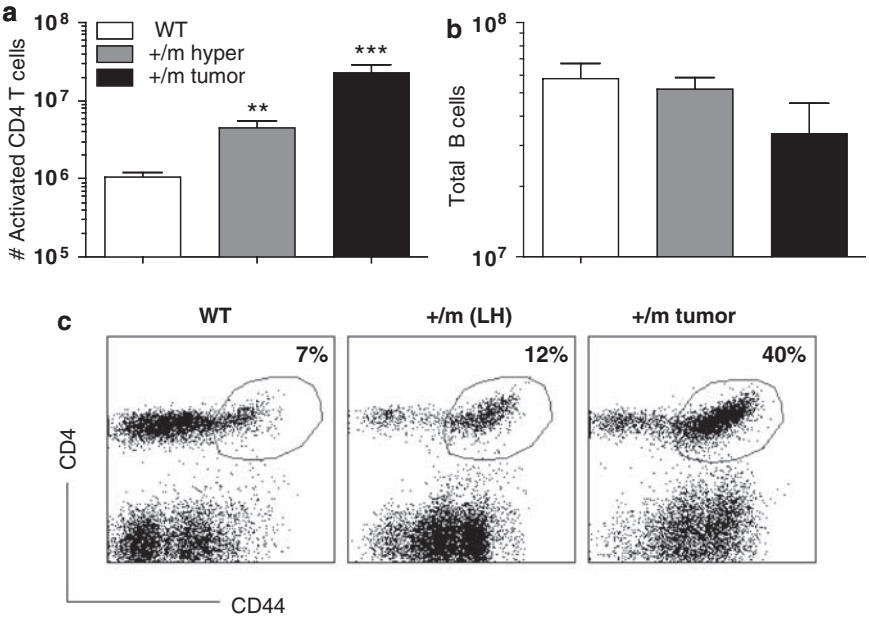

Figure 3 Characterization of lymphoid hyperplasias and lymphomas in Chd2 mutant mice. (**a**) Total number of activated T cells from the spleens of age-matched wild type, Chd2 mutants with hyperplasia, and Chd2 mutants with lymphoma. (**b**) Total number of B cells from spleens of indicated mice. (**c**) Representative fluorescence activated cell-sorting profile showing CD4 and CD44 expression of cells from the lymph nodes of wild type, Chd2 heterozygous mutant with lymphoid hyperplasia and Chd2 heterozygous mutant with lymphoma. In each group 3–4 mice were used; **indicates $P<0.01$ and ***indicates $P<0.001$.

tissue-specific induction of lymphomas in the Chd2 mutants. However, we cannot rule out the role of Chd2 on the induction of other phenotypes that occur in the absence of the lymphoid phenotypes. Future studies that utilize conditional inactivation of Chd2 in a tissue-specific manner would allow us to dissect the role of Chd2 in specific organs.

The preponderance of lymphomas in the Chd2 mutant mice indicated that the loss of the *CHD2* gene might also be important in the development of human cancers. We used the Mitelman database of Chromosomal aberrations in Cancer (Cancer Genome Anatomy Project: http://cgap.nci.nih.gov/chromosome/Mitelman) to determine if there are any chromosomal aberrations in human cancers that colocalized with the *CHD2* chromosomal locus (15q26). Systematic analysis and categorization of 15q26 chromosomal abberations showed that a substantial fraction (128/265, 48.3%) of the reported human cancers with chromosomal aberrations in 15q26 were either lymphomas or leukemias (Supplementary Table 2). Among the various chromosomal aberrations found in lymphomas and leukemias, ~15% of the aberrations were deletions.

Chd2 modulates DNA damage responses

On the basis of the observed susceptibility of the Chd2 mutant mice to lymphomas and the chromatin-binding ability of Chd2, we hypothesized that the Chd2 protein might affect genomic stability by regulating DNA damage responses at the chromatin level. Phosphorylation of the histone variant, H2AX, at serine 139 (also known as γH2AX) is one of the earliest events that occurs at the chromatin level in response to DNA-strand breaks and this modification usually parallels the extent of DNA damage (Rogakou *et al*., 1998; Banath and Olive, 2003). Furthermore, studies have also shown the recruitment of DNA repair proteins to ionizing radiation-induced γH2AX foci and mice deficient for H2AX show ionizing radiation sensitivity (Downs *et al*., 2000; Paull *et al*., 2000; Kang *et al*., 2005). To examine the possibility that the Chd2 mutant cells may have an aberrant DNA damage response with respect to γH2AX, we compared the levels of γH2AX in Chd2 mutant and wild-type mouse embryonic fibroblasts (MEFs) treated with low levels of X-ray irradiation. We found several differences in the γH2AX patterns between the wild-type and mutant cells. First, there was a consistent sub-population of mutant controls (~25%) that exhibited the presence of several γH2AX foci within their nuclei that was significantly higher in comparison with the wild-type controls suggesting the presence of DNA-strand breaks even without any external DNA damage induction (Table 2). Second, as shown in Figure 4, X-ray-induced DNA damage led to a higher increase in γH2AX foci in Chd2 homozygous mutant cells within 30 min in comparison with wild-type littermate control cells. Furthermore, the γH2AX foci were much more intense in the Chd2 mutant cells in comparison with the wild-type cells treated similarly indicative of higher levels of DNA damage (compare the middle panels in Figure 4, Table 2). The initial induction of γH2AX foci declines within a few hours of DNA damage induction and this reduction is thought to occur

Table 2 Persistence of γH2AX foci in Chd2 mutant cells

Treatment/genotype	*WT*	*+/m*	*m/m*
Untreated control	0.88 ± 1.45	1.65 ± 1.73*	2.8 ± 2.3*
4 Gy, 0.5 h	18.23 ± 3.54 (100)	23.46 ± 3.83** (100)	27.26 ± 5.08** (100)
4 Gy, 3 h	10.1 ± 2.57 (55.4)	21.66 ± 3.92** (92.3)	23.76 ± 5.08** (87.1)
4 Gy, 6 h	3.43 ± 1.86 (18.8)	14.87 ± 2.87*** (63.38)	18.0 ± 3.97*** (66.0)

Wild-type (WT) and Chd2 homozygous mutant MEFs were treated with X-ray irradiation (4 Gy) and fixed at 30 min, 3 h and 6 h post-irradiation. The γH2AX foci formation was visualized by immunofluorescent staining with anti-γH2AX antibodies and fluorescein isothiocyanate-labeled secondary antibodies. A minimum of 30 cells were analysed per genotype and the numbers represent mean number of larger γH2AX foci per cell. The percentage of foci remaining unresolved at 3 and 6 h with respect to the percentage obtained at 30 min (set independently at 100% for each group) is shown within parenthesis. Experiments were performed with two separate sets (WT, +/m and m/m) of independently derived mouse embryonic fibroblasts that gave similar results. The differences between the WT and mutants (+/m and m/m) were statistically significant (*$P<0.01$, **$P<0.0001$ and ***$P<0.0002$). Bars represent s.d.

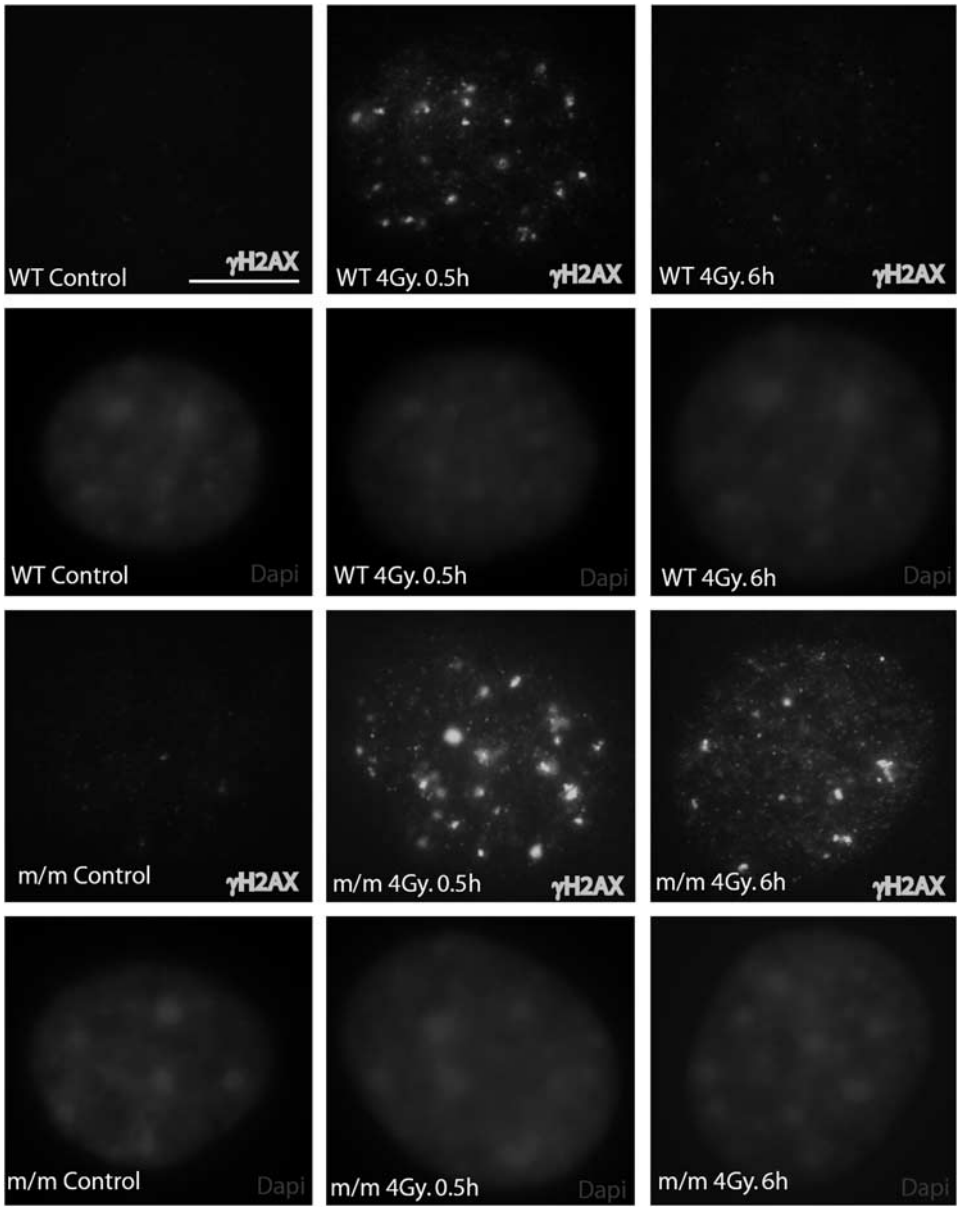

Figure 4 Defective DNA damage response in Chd2 mutant cells. Wild-type and Chd2 homozygous mutant littermate mouse embryonic fibroblasts (MEFs) were treated with 4 Gy X-ray irradiation and analysed for γH2AX foci formation at 30 min, 3 (data not shown) and 6 h. For immunofluorescence analysis, wild-type and mutant cells were grown in different chambers on the same slide. Results were confirmed with four experiments using two sets of independently derived cell lines. At least 30 cells were analysed per experiment. All cells were photographed using the same exposure time and microscope settings. Bar = 10 μ.

due to the resolution of DNA-strand breaks (Rothkamm *et al*., 2003). Comparison of the clearance of γH2AX foci after 3 h (data not shown) and 6 h (last panel) indicated a higher persistence of γH2AX foci in the mutant cells (Figure 4, right panels). Semiquantitative analysis of γH2AX foci also showed that the heterozygous and homozygous mutant cells were unable to downregulate the γH2AX response, whereas the wild-type cells showed a robust clearance of γH2AX foci within 6 h (Table 2 and Supplementary Figure 7). Consistent with the defective clearance of γH2AX foci, the Chd2 mutants were highly sensitive to a variety of DNA-damaging agents (Supplementary Figure 8). The Chd2 mutant MEFs also exhibited moderately higher increase in the G1-S phase of the cell cycle in response to X-ray-induced DNA damage in comparison with wild-type MEFs (Supplementary Table 3). These observations indicate that the Chd2 mutant cells have defects in DNA damage-induced γH2AX response and suggest a potential role for Chd2 in either the repair of DNA-strand breaks or the attenuation of the γH2AX signal after repair.

Discussion

The CHD proteins have distinct structural motifs that implicate specific functional roles in a variety of DNA transactions that include replication, transcription and DNA repair. To further study the physiological role of Chd2 in a mammalian model, we generated a Chd2 mutant mouse model using the Baygenomics gene-trap ES cell resource. Characterization of the gene trap used in the generation of the Chd2 mutant mouse model indicated that it was not completely effective in disrupting the expression of the *Chd2* gene, suggesting the possibility of a hypomorphic or a dominant negative Chd2 mutant mouse model. We have shown through protein interaction studies that the truncated Chd2-β-gal-neomycin has a potential to interact with the native Chd2 protein. However, our studies do not rule out the possibility that the mutant Chd2 fusion protein can either act as a dominant negative mutant or a gain of function mutant. Future studies aimed at identifying functional partners of Chd2 will allow us to determine the effect of this mutation. The expression of wild-type *Chd2* mRNA in the homozygous mutant cells is in contrast to the results reported earlier (Marfella *et al*., 2006) and we believe that the discrepancy is due to the low number of cycles in the reverse transcription–PCR analysis used by the other group in comparison with ours (26 cycles versus 30 cycles in this study). Our results indicate that the *Chd2* mutation leads to pleiotropic effects that impinge on hematopoietic and lymphoid development pathways in mammals. We have shown that the CHD2 protein is involved in the regulation of hematopoietic stem cell differentiation, and its loss may lead to an imbalance in the various downstream compartments that include the erythroid, myeloid and lymphoid compartments. Interestingly, earlier studies have shown the importance of differentiation and cell type-specific transcriptional programming during the terminal differentiation of hematopoietic cells and our studies point to the role of chromatin remodeling and its effects on transcription on hematopoietic stem cell differentiation (Heyworth *et al*., 2002; Iwasaki *et al*., 2003; Ney, 2006).

More importantly, the Chd2 mutant mice develop primarily lymphomas and lymphoid hyperplasias. A few of the phenotypes we have described in this report are similar to the ones reported in a recent study involving the phenotypic characterization of the Chd2 mutant mouse model (Marfella *et al*., 2006). However, the earlier study has not reported the susceptibility of lymphomas in the Chd2 mutant mice and the differences between the two studies may relate to the fact that our study is more extensive that involved the analysis of a larger set of mutant animals. Although the other study does report the presence of lymphoid hyperplasia (a precursor for lymphomas) in the mutants, the reasons for the differences in lymphoma diagnoses between the two studies are yet to be ascertained. Interestingly, the human *CHD2* chromosomal locus (15q26.2) is also implicated in a rare genetic disorder that leads to growth retardation, cardiac defects and early post-natal lethality (Wilson *et al*., 1985; Whiteford *et al*., 2000). The data we have compiled on human chromosomal aberrations provide preliminary evidence that the Chd2 protein may play a role in the etiology of human lymphoid tumors. Furthermore and consistent with our observations, the recent characterization of a T-cell Hodgkin's lymphoma cell line using array comparative genomic hybridization analysis has also shown the homozygous loss of the *CHD2* chromosomal locus (Feys *et al*., 2007). The above mentioned data and the enhanced tumor susceptibility of the Chd2 heterozygous mice raise the possibility that *CHD2* is a potential tumor suppressor gene involved in the suppression of lymphomas.

Our data also show that the Chd2 protein affects DNA damage signaling and processing at the chromatin level by modulating the levels of γH2AX induced by DNA damage. Although several studies have shown that a decrease in the γH2AX foci often mirrors a decrease in the number of DNA-strand breaks, we cannot rule out the possibility that the persistence of γH2AX foci may relate to the inability of the Chd2 mutant cells to displace γH2AX subsequent to DNA repair (Rogakou *et al*., 1998; Banath and Olive, 2003; Rothkamm *et al*., 2003; Lukas *et al*., 2004; Jin *et al*., 2005). Consistent with this notion, a recent study has shown that the removal of γH2AX after DNA damage is mediated by the Tip60 chromatin remodeling complex (Kusch *et al*., 2004). In addition, DNA damage processing in lower eukaryotes is mediated by the INO80 complex and this complex requires the HMG1 domain-containing Nhp10 subunit protein for its interaction with the γH2AX (Morrison *et al*., 2004; van Attikum *et al*., 2004; Tsukuda *et al*., 2005). Interestingly, the Chd2 protein contains a similar domain, and the ability of Chd1 to transfer histones to DNA also suggests a parallel and mutually exclusive role for CHD2 in the removal of γH2AX (Lusser *et al*., 2005). Whether Chd2 plays a functional role in γH2AX removal during the attenuation of DNA damage

response or directly affects DNA repair processes remains to be seen.

The functional roles of CHD family members and other chromatin remodeling proteins in transcriptional regulation have been well established. However, our data suggest that the CHD2 protein may play an additional role in DNA damage signaling besides affecting transcription. Determining the tissue-specific transcriptional targets and the role of Chd2 in downstream DNA damage response pathways will provide further insights on its functions in development, hematopoietic stem cell differentiation and tissue-specific tumor suppression.

Materials and methods

Generation of Chd2 mutant mice

The Baygenomics insertional mutagenesis strategy involved the use of a gene-trap cassette consisting of a splice-acceptor-βgeo cassette (β-galactosidase-neomycin fusion gene). Embryonic stem cell clones are then characterized by 5′RACE to identify upstream exons abutting the βgeo sequence. One of the ES cell clones that had been characterized to have a gene trap insertion within the *Chd2* gene was represented in the Baygenomics ES cell library. The *Chd2*-trapped ES cells were obtained and characterized further. Genomic DNA isolated from ES cells were analysed by PCR to confirm *Chd2* disruption by using primers that were specific for *Chd2* exon 27 (5′-TGTGTGTCAGCAATGCAGGA-3′) and the gene-trap sequences (5′-ACCTGGCTCCTATGGGATAG-3′). Sequencing of the PCR product indicated that the gene trap was integrated within intron 27 (1563 base pairs from the beginning of the intron) of the *Chd2* gene. Chd2-targeted ES cells were used for blastocyst injections using the microinjection services at the University of Massachusetts Medical School, Worcester. The colonies from two germline founders were expanded further for the analysis of the mutant offspring. The insertion of the gene trap was determined to be downstream of the HMG-1 DNA-binding domain and upstream of three putative nuclear localization signals of Chd2. The gene-trap strategy used to generate the Chd2 mutant ES cells also leads to the generation of a putative truncated Chd2-βgeo fusion protein containing the first 1198 amino acids of the wild-type protein. To confirm the absence of the Chd2 protein in the nucleus, we used antibodies against the β-galactosidase part of the fusion and found that the Chd2-β-gal-neomycin fusion gene product was localized only in the cytoplasm (not shown). All protocols and procedures involving the analysis of mutant mice were approved by the University of Tennessee IACUC committee.

Analysis of γH2AX response in MEFs

Mouse embryonic fibroblasts were grown on glass chamber slides and exposed to 4 Gy X-ray irradiation and incubated for the indicated time intervals. The cells were fixed with acetone-methanol (1:1) and blocked with 10% antibody dilution buffer (3% bovine serum antigen, 10% goat serum, 0.05% Triton X-100 in phosphate-buffered saline). The slides were incubated with anti-γH2AX antibodies (Cell Signal Technology, Beverly, MA, USA) followed with fluorescein isothiocyanate—conjugated anti-rabbit antibodies. The slides were counterstained for DNA with DAPI and the γH2AX foci were visualized using a fluorescent microscope (Zeiss Axioplan) and acquired with Metamorph software (Molecular Devices, Sunnyvale, CA, USA).

Fetal liver colony assays

Single-cell suspensions (2×10^4) prepared from fetal livers of E13.5 embryos were plated on 35 mm culture dishes with semisolid complete methylcellulose medium (Methocult medium supplemented with stem cell factor, interleukin-3, interleukin-6 and erythropoietin, Stem Cell Technologies, Vancouver, BC, Canada). Burst-forming unit-erythroids and colony-forming unit-granulocyte macrophages were counted after 8–10 days using morphological criteria.

Statistical analyses

Standard error, mean and *P*-values were determined using the statistics software from Microsoft Excel. Kaplan–Meir survival curves were generated and analysed with Prism 4 (GraphPad Software).

Acknowledgements

We are thankful to Dr Bruce McKee for critically reading the manuscript. This work was supported by the University of Tennessee startup funds, PMERF seed funds, and NIH funds (00293621) to SV and funding from NIH (AI05771901) to TMO.

References

Bagchi A, Papazoglu C, Wu Y, Capurso D, Brodt M, Francis D *et al*. (2007). Chd5 is a tumor suppressor at human 1p36. *Cell* **128**: 459–475.

Banath JP, Olive PL. (2003). Expression of phosphorylated histone h2ax as a surrogate of cell killing by drugs that create DNA double-strand breaks. *Cancer Res* **63**: 4347–4350.

Bernstein BE, Humphrey EL, Erlich RL, Schneider R, Bouman P, Liu JS *et al*. (2002). Methylation of histone h3 lys 4 in coding regions of active genes. *Proc Natl Acad Sci USA* **99**: 8695–8700.

Brehm A, Langst G, Kehle J, Clapier CR, Imhof A, Eberharter A *et al*. (2000). Dmi-2 and iswi chromatin remodelling factors have distinct nucleosome binding and mobilization properties. *EMBO J* **19**: 4332–4341.

Bronson RT, Lipman RD. (1991). Reduction in rate of occurrence of age related lesions in dietary restricted laboratory mice. *Growth Dev Aging* **55**: 169–184.

Cowell IG, Austin CA. (1997). Self-association of chromo domain peptides. *Biochim Biophys Acta* **1337**: 198–206.

Delmas V, Stokes DG, Perry RP. (1993). A mammalian DNA-binding protein that contains a chromodomain and an snf2/swi2-like helicase domain. *Proc Natl Acad Sci USA* **90**: 2414–2418.

Downs JA, Lowndes NF, Jackson SP. (2000). A role for saccharomyces cerevisiae histone h2a in DNA repair. *Nature* **408**: 1001–1004.

Feys T, Poppe B, De Preter K, Van Roy N, Verhasselt B, De Paepe P *et al*. (2007). A detailed inventory of DNA copy number alterations in four commonly used Hodgkin's lymphoma cell lines. *Haematologica* **92**: 913–920.

Flanagan JF, Mi LZ, Chruszcz M, Cymborowski M, Clines KL, Kim Y *et al*. (2005). Double chromodomains cooperate to recognize the methylated histone h3 tail. *Nature* **438**: 1181–1185.

Fukasawa M, Morita S, Kimura M, Horii T, Ochiya T, Hatada I. (2006). Genomic imprinting in dicer1-hypomorphic mice. *Cytogenet Genome Res* **113**: 138–143.

Gao X, Gordon D, Zhang D, Browne R, Helms C, Gillum J *et al*. (2007). Chd7 gene polymorphisms are associated with susceptibility to idiopathic scoliosis. *Am J Hum Genet* **80**: 957–965.

Gonzalez-Billault C, Demandt E, Wandosell F, Torres M, Bonaldo P, Stoykova A *et al*. (2000). Perinatal lethality of microtubule-associated protein 1b-deficient mice expressing alternative isoforms of the protein at low levels. *Mol Cell Neurosci* **16**: 408–421.

Hasan S, Hottiger MO. (2002). Histone acetyl transferases: a role in DNA repair and DNA replication. *J Mol Med* **80**: 463–474.

Heyworth C, Pearson S, May G, Enver T. (2002). Transcription factor-mediated lineage switching reveals plasticity in primary committed progenitor cells. *EMBO J* **21**: 3770–3781.

Iwasaki H, Mizuno S, Wells RA, Cantor AB, Watanabe S, Akashi K. (2003). Gata-1 converts lymphoid and myelomonocytic progenitors into the megakaryocyte/erythrocyte lineages. *Immunity* **19**: 451–462.

Jeganathan K, Malureanu L, Baker DJ, Abraham SC, van Deursen JM. (2007). Bub1 mediates cell death in response to chromosome missegregation and acts to suppress spontaneous tumorigenesis. *J Cell Biol* **179**: 255–267.

Jin J, Cai Y, Li B, Conaway RC, Workman JL, Conaway JW *et al*. (2005). In and out: histone variant exchange in chromatin. *Trends Biochem Sci* **30**: 680–687.

Jones DO, Cowell IG, Singh PB. (2000). Mammalian chromodomain proteins: their role in genome organisation and expression. *Bioessays* **22**: 124–137.

Kang J, Ferguson D, Song H, Bassing C, Eckersdorff M, Alt FW *et al*. (2005). Functional interaction of h2ax, nbs1, and p53 in atm-dependent DNA damage responses and tumor suppression. *Mol Cell Biol* **25**: 661–670.

Kina T, Ikuta K, Takayama E, Wada K, Majumdar AS, Weissman IL *et al*. (2000). The monoclonal antibody ter-119 recognizes a molecule associated with glycophorin a and specifically marks the late stages of murine erythroid lineage. *Br J Haematol* **109**: 280–287.

Kulkarni S, Nagarajan P, Wall J, Donovan DJ, Donell RL, Ligon AH *et al*. (2008). Disruption of chromodomain helicase DNA binding protein 2 (CHD2) causes scoliosis. *Am J Med Genet A* **146A**: 1117–1127.

Kusch T, Florens L, Macdonald WH, Swanson SK, Glaser RL, Yates III JR *et al*. (2004). Acetylation by tip60 is required for selective histone variant exchange at DNA lesions. *Science* **306**: 2084–2087.

Lalani SR, Safiullah AM, Fernbach SD, Harutyunyan KG, Thaller C, Peterson LE *et al*. (2006). Spectrum of chd7 mutations in 110 individuals with charge syndrome and genotype-phenotype correlation. *Am J Hum Genet* **78**: 303–314.

Lukas C, Melander F, Stucki M, Falck J, Bekker-Jensen S, Goldberg M *et al*. (2004). Mdc1 couples DNA double-strand break recognition by nbs1 with its h2ax-dependent chromatin retention. *EMBO J* **23**: 2674–2683.

Lusser A, Urwin DL, Kadonaga JT. (2005). Distinct activities of chd1 and acf in atp-dependent chromatin assembly. *Nat Struct Mol Biol* **12**: 160–166.

Marfella CG, Ohkawa Y, Coles AH, Garlick DS, Jones SN, Imbalzano AN. (2006). Mutation of the snf2 family member chd2 affects mouse development and survival. *J Cell Physiol* **209**: 162–171.

Morrison AJ, Highland J, Krogan NJ, Arbel-Eden A, Greenblatt JF, Haber JE *et al*. (2004). Ino80 and gamma-h2ax interaction links atp-dependent chromatin remodeling to DNA damage repair. *Cell* **119**: 767–775.

Muller C, Leutz A. (2001). Chromatin remodeling in development and differentiation. *Curr Opin Genet Dev* **11**: 167–174.

Ney PA. (2006). Gene expression during terminal erythroid differentiation. *Curr Opin Hematol* **13**: 203–208.

Osley MA, Tsukuda T, Nickoloff JA. (2007). Atp-dependent chromatin remodeling factors and DNA damage repair. *Mutat Res* **618**: 65–80.

Paro R, Hogness DS. (1991). The polycomb protein shares a homologous domain with a heterochromatin-associated protein of drosophila. *Proc Natl Acad Sci USA* **88**: 263–267.

Paull TT, Rogakou EP, Yamazaki V, Kirchgessner CU, Gellert M, Bonner WM. (2000). A critical role for histone h2ax in recruitment of repair factors to nuclear foci after DNA damage. *Curr Biol* **10**: 886–895.

Pray-Grant MG, Daniel JA, Schieltz D, Yates III JR, Grant PA. (2005). Chd1 chromodomain links histone h3 methylation with saga- and slik-dependent acetylation. *Nature* **433**: 434–438.

Qin S, Parthun MR. (2002). Histone h3 and the histone acetyltransferase hat1p contribute to DNA double-strand break repair. *Mol Cell Biol* **22**: 8353–8365.

Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. (1998). DNA double-stranded breaks induce histone h2ax phosphorylation on serine 139. *J Biol Chem* **273**: 5858–5868.

Rothkamm K, Kruger I, Thompson LH, Lobrich M. (2003). Pathways of DNA double-strand break repair during the mammalian cell cycle. *Mol Cell Biol* **23**: 5706–5715.

Schuster E. (2002). Chd5 defines a new subfamily of chromodomain-swi2/snf2-like helicases. *Mamm Genome* **13**: 117–119.

Simic R, Lindstrom DL, Tran HG, Roinick KL, Costa PJ, Johnson AD *et al*. (2003). Chromatin remodeling protein chd1 interacts with transcription elongation factors and localizes to transcribed genes. *EMBO J* **22**: 1846–1856.

Sims III RJ, Chen CF, Santos-Rosa H, Kouzarides T, Patel SS, Reinberg D. (2005). Human but not yeast chd1 binds directly and selectively to histone h3 methylated at lysine 4 via its tandem chromodomains. *J Biol Chem* **280**: 41789–41792.

Singh PB, Miller JR, Pearce J, Kothary R, Burton RD, Paro R *et al*. (1991). A sequence motif found in a *Drosophila* heterochromatin protein is conserved in animals and plants. *Nucleic Acids Res* **19**: 789–794.

Socolovsky M, Nam H, Fleming MD, Haase VH, Brugnara C, Lodish HF. (2001). Ineffective erythropoiesis in stat5a(−/−)5b(−/−) mice due to decreased survival of early erythroblasts. *Blood* **98**: 3261–3273.

Spike BT, Dirlam A, Dibling BC, Marvin J, Williams BO, Jacks T *et al*. (2004). The rb tumor suppressor is required for stress erythropoiesis. *EMBO J* **23**: 4319–4329.

Stokes DG, Perry RP. (1995). DNA-binding and chromatin localization properties of chd1. *Mol Cell Biol* **15**: 2745–2753.

Stryke D, Kawamoto M, Huang CC, Johns SJ, King LA, Harper CA *et al*. (2003). Baygenomics: a resource of insertional mutations in mouse embryonic stem cells. *Nucleic Acids Res* **31**: 278–281.

Takeda Y, Dynan WS. (2001). Autoantibodies against DNA double-strand break repair proteins. *Front Biosci* **6**: D1412–D1422.

Targoff IN, Reichlin M. (1985). The association between mi-2 antibodies and dermatomyositis. *Arthritis Rheum* **28**: 796–803.

Thomas JO. (2001). Hmg1 and 2: architectural DNA-binding proteins. *Biochem Soc Trans* **29**: 395–401.

Tong JK, Hassig CA, Schnitzler GR, Kingston RE, Schreiber SL. (1998). Chromatin deacetylation by an atp-dependent nucleosome remodelling complex. *Nature* **395**: 917–921.

Tran H. (2000). The chromo domain protein chd1p from budding yeast is an atp-dependent chromatin-modifying factor. *EMBO J* **10**: 2323–2331.

Tsukuda T, Fleming AB, Nickoloff JA, Osley MA. (2005). Chromatin remodelling at a DNA double-strand break site in saccharomyces cerevisiae. *Nature* **438**: 379–383.

van Attikum H, Fritsch O, Hohn B, Gasser SM. (2004). Recruitment of the ino80 complex by h2a phosphorylation links atp-dependent chromatin remodeling with DNA double-strand break repair. *Cell* **119**: 777–788.

Venkatachalam S, Shi YP, Jones SN, Vogel H, Bradley A, Pinkel D *et al*. (1998). Retention of wild-type p53 in tumors from p53 heterozygous mice: Reduction of p53 dosage can promote cancer formation. *EMBO J* **17**: 4657–4667.

Vissers LE, van Ravenswaaij CM, Admiraal R, Hurst JA, de Vries BB, Janssen IM *et al*. (2004). Mutations in a new member of the chromodomain gene family cause charge syndrome. *Nat Genet* **36**: 955–957.

Voss AK, Thomas T, Gruss P. (1998). Compensation for a gene trap mutation in the murine microtubule-associated protein 4 locus by alternative polyadenylation and alternative splicing. *Dev Dyn* **212**: 258–266.

Wang GG, Allis CD, Chi P. (2007a). Chromatin remodeling and cancer, part i: covalent histone modifications. *Trends Mol Med* **13**: 363–372.

Wang GG, Allis CD, Chi P. (2007b). Chromatin remodeling and cancer, part ii: Atp-dependent chromatin remodeling. *Trends Mol Med* **13**: 373–380.

Whiteford ML, Baird C, Kinmond S, Donaldson B, Davidson HR. (2000). A child with bisatellited, dicentric chromosome 15 arising from a maternal paracentric inversion of chromosome 15q. *J Med Genet* **37**: E11.

Wilson GN, Sauder SE, Bush M, Beitins IZ. (1985). Phenotypic delineation of ring chromosome 15 and russell-silver syndromes. *J Med Genet* **22**: 233–236.

Woodage T. (1997). Chracterization of the chd family of proteins. *Proc Natl Acad Sci* **94**: 11472–11477.

Wu C. (1997). Chromatin remodeling and the control of gene expression. *J Biol Chem* **272**: 28171–28174.

Xue Y, Wong J, Moreno GT, Young MK, Cote J, Wang W. (1998). Nurd, a novel complex with both atp-dependent chromatin-remodeling and histone deacetylase activities. *Mol Cell* **2**: 851–861.

Zhang J, Socolovsky M, Gross AW, Lodish HF. (2003). Role of ras signaling in erythroid differentiation of mouse fetal liver cells: functional analysis by a flow cytometry-based novel culture system. *Blood* **102**: 3938–3946.

Zhang Y, Leroy G, Seelig H, Lane W, Reinberg D. (1998). The dermatomysitis-specific autoantigen mi2 is a component of a complex containing histone deacetylase and nucleosome remodeling activites. *Cell* **95**: 279–289.

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