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Role of Chromodomain Helicase DNA binding protein 2 in DNA damage response signaling and tumorigenesis

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Abstract

The chromodomain helicase DNA binding proteins (CHDs) are known to affect transcription via their ability to remodel chromatin and modulate histone deacetylation. In an effort to understand the functional role of the chromodomain helicase DNA binding protein 2 (CHD2) in mammals, we have generated Chd2 mutant mouse model. Remarkably, the Chd2 protein appears to play a critical role in development, hematopoiesis, and tumor suppression. The Chd2 heterozygous mutant mice exhibit increased extra-medullary 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, γ H2AX, 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.

Introduction

Chromatin remodeling serves as an important regulator of various DNA processes including replication, transcription, recombination, and DNA repair (Bernstein et al., 2002; Hasan & Hottiger, 2002; Osley et al., 2007; Qin & Parthun, 2002; Wu, 1997). The remodeling of chromatin has also been implicated in physiological processes as diverse as embryonic development and cancer (Muller & Leutz, 2001; Wang et al., 2007a; Wang et al., 2007b). The chromodomain helicase DNA binding proteins (CHD) were characterized as a distinct family of proteins in the 1990's (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-

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terminal helicase domain (Woodage, 1997). The chromodomain was initially characterized in *Drosophila* HP1 and Polycomb proteins (Paro & Hogness, 1991). Chromodomain containing proteins can self associate and also interact with the heterochromatic regions at centromeres, telomeres and polytene chromosomes (Cowell & Austin, 1997; Singh et al., 1991). 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 via its unique HMG-1 binding domain (Delmas et al., 1993; Stokes & 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 (Lusser et al., 2005; Simic et al., 2003). 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 (NuRD) complexes (Brehm et al., 2000; Targoff & Reichlin, 1985; Tong et al., 1998; Xue et al., 1998; Zhang et al., 1998). Patients with the autoimmune condition dermatomyositis produce antibodies against CHD3 and CHD4 and are prone to increased susceptibility to cancer (Takeda & Dynan, 2001; Targoff & Reichlin, 1985). 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 multi-organ disorder that includes Coloboma, Hear defects, choanal Atresia, mental Retardation, Genital and Ear anomalies and scoliosis (Gao et al., 2007; Lalani et al., 2006; Vissers et al., 2004).

Recent in vitro studies have shown that the chromodomains present in CHD1 bind to specific histone H3 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 high mobility group (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 (Jones et al., 2000; Lusser et al., 2005; Simic et al., 2003; Tran, 2000; Woodage, 1997). 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 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 revealed a drastic reduction in the formation of vascular structures and regions of localized hemorrhaging (Fig. 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 knock down of specific genes have shown that the effectiveness of the gene trap is variable and may lead to the generation of hypomorphic mutants (Fukasawa et al., 2006; Gonzalez-Billault et al., 2000; Voss et al., 1998). 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 RT-PCR assays. Surprisingly, we found that the homozygous mutants did express the wild type mRNA albeit at lower levels (Supplementary Figure 2). The ineffective down regulation of *Chd2* may lead to the generation of a hypomorphic allele or a dominant negative allele (via the interaction of the *Chd2*- β -gal-neomycin fusion peptide with the wild-type protein). To test this possibility of dominant negative effects, we analyzed the inter-molecular interactions between recombinant *Chd2* peptides that contained 6 \times -His and HA epitope tags. As shown in supplementary figure 3, reciprocal immuno-precipitation 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*- β geo 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. Due 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 to the wild type neonates that showed well organized clusters of hematopoietic cells (Fig. 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 analyzed 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 (Fig. 1d). The lowered capacity of erythrocyte differentiation in Chd2 mutants were further confirmed in fluorescence activated cell-sorting (FACS) analyses using flow cytometry. The differentiation of erythroblasts to erythrocytes can be assessed by analyzing and quantitating the expression of cell surface markers Ter119, CD71, and CD117 (Kina et al., 2000; Socolovsky et al., 2001; Spike et al., 2004; Zhang et al., 2003). During erythroid maturation, the expression of CD71 decreases from proerythroblasts to early basophilic erythroblasts while 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 (27.9.0% and 25.0%) in comparison to 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 wt vs. 60.5% and 54.7 in mutants). Similar results were obtained from the FACS 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 (Fig. 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 (Fig. 2 b-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, only 1 out of the 6 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 & Lipman, 1991; Jeganathan et al., 2007; Venkatachalam et al., 1998). In addition to the lymphoid tumor phenotypes, a significant proportion of the mice exhibited extra medullary 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 (Fig. 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 analyzed the cells by fluorescence activated cell sorting (FACS). While 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 the observed hyperplasias and lymphomas were likely unrelated to dysregulation of the B cell compartment (Fig. 3b). However, analysis of T cells in the Chd2 heterozygous

mice revealed significantly increased numbers of activated CD44^{high} CD4 T cells in mice independently confirmed with hyperplasia or lymphomas by histopathology (Fig. 3a and c). In disease-free younger mutants, no differences were observed in the proportion or number of activated CD4 T cells (Supplementary Fig. 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 Fig. 6). The highest expression of Chd2 in the thymus provides additional evidence for the 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 may 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 co-localized with the CHD2 chromosomal locus (15q26). Systematic analysis and categorization of 15q26 chromosomal aberrations 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

Based on 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 γ H2AX) 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 (Banath & Olive, 2003; Rogakou et al., 1998). Furthermore, studies have also shown the recruitment of DNA repair proteins to IR induced γ H2AX foci and mice deficient for H2AX show IR sensitivity (Downs et al., 2000; Kang et al., 2005; Paull et al., 2000). To examine the possibility that the Chd2 mutant cells may have an aberrant DNA damage response with respect to γ H2AX, we compared the levels of γ H2AX in Chd2 mutant and wild-type mouse embryonic fibroblasts (MEFs) treated with low levels of X-ray irradiation. We found several differences in the γ H2AX patterns between the wild type and mutant cells. Firstly, there was a consistent subpopulation of mutant controls (~25%) that exhibited the presence of several γ H2AX foci within their nuclei that was significantly higher in comparison to the wild type controls suggesting the presence of DNA strand breaks even without any external DNA damage induction (Table 2). Secondly, as shown in Figure 4, X-ray induced DNA damage led to a higher increase in γ H2AX foci in Chd2 homozygous mutant cells within 30 minutes in comparison to wild-type littermate control cells. Furthermore, the γ H2AX foci were much

more intense in the Chd2 mutant cells in comparison to the WT cells treated similarly indicative of higher levels of DNA damage (compare the middle panels in Fig. 4, table 2). The initial induction of γ H2AX foci declines within a few hours of DNA damage induction and this reduction is thought to occur due to the resolution of DNA strand breaks (Rothkamm et al., 2003). Comparison of the clearance of γ H2AX foci after 3 hours (data not shown) and 6 hours (last panel) indicated a higher persistence of γ H2AX foci in the mutant cells (Fig. 4, right panels). Semi-quantitative analysis of γ H2AX foci also showed that the heterozygous and homozygous mutant cells were unable to down-regulate the γ H2AX response whereas the wild-type cells showed a robust clearance of γ H2AX foci within 6 hours (Table 2 and Supplementary Fig. 7). Consistent with the defective clearance of γ H2AX 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 to wild type MEFs (Supplementary table 3). These observations indicate that the Chd2 mutant cells have defects in DNA damage induced γ H2AX response and suggest a potential role for Chd2 in either the repair of DNA strand breaks or the attenuation of the γ H2AX 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 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 via 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 RT-PCR analysis used by the other group in comparison to 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 and 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. While 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 (Whiteford et al., 2000; Wilson et al., 1985). 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 lymphoma cell line using array comparative genomic hybridization (aCGH) 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 γ H2AX induced by DNA damage. While several studies have shown that a decrease in the γ H2AX foci often mirrors a decrease in the number of DNA strand breaks, we cannot rule out the possibility that the persistence of γ H2AX foci may relate to the inability of the Chd2 mutant cells to displace γ H2AX subsequent to DNA repair (Banath & Olive, 2003; Jin et al., 2005; Lukas et al., 2004; Rogakou et al., 1998; Rothkamm et al., 2003). Consistent with this notion, a recent study has shown that the removal of γ H2AX 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 γ H2AX (Morrison et al., 2004; Tsukuda et al., 2005; van Attikum et al., 2004). 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 γ H2AX (Lusser et al., 2005). Whether Chd2 plays a functional role in γ H2AX 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- β geo cassette (beta-galactosidase-neomycin fusion gene). ES cell clones are then characterized by 5'RACE to identify upstream exons abutting the β geo sequence. One of the ES cell clones that had been characterized to have a genetrapp 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 analyzed by PCR to confirm *Chd2* disruption by using primers that were specific for *Chd2* exon 27 (5'- TGT GTG TCA GCA ATG CAG GA -3') and the gene-trap sequences (5'- ACC TGG CTC CTA TGG GAT AG -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 germ line 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 employed to generate the *Chd2* mutant ES cells also leads to the generation of a putative truncated *Chd2*- β geo fusion protein containing the first 1,198 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 γ H2AX response in mouse embryonic fibroblasts—MEFs 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 (ADB; 3% BSA, 10% goat serum, 0.05% Triton X-100 in PBS). The slides were incubated with anti- γ H2AX antibodies (Cell Signal Technology) followed with FITC – conjugated anti rabbit antibodies. The slides were counterstained for DNA with DAPI and the γ H2AX foci were visualized using a fluorescent microscope (Zeiss Axioplan) and acquired with Metamorph software (Molecular Devices, Sunnyvale, CA).

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 semi-solid complete methylcellulose medium (Methocult medium supplemented with SCF, IL-3, IL-6 and Epo, Stem Cell Technologies). Burst Forming Unit erythroids (BFU-E) and colony forming unit —granulocyte macrophages (CFU-GM) 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 analyzed with Prism 4 (GraphPad Software).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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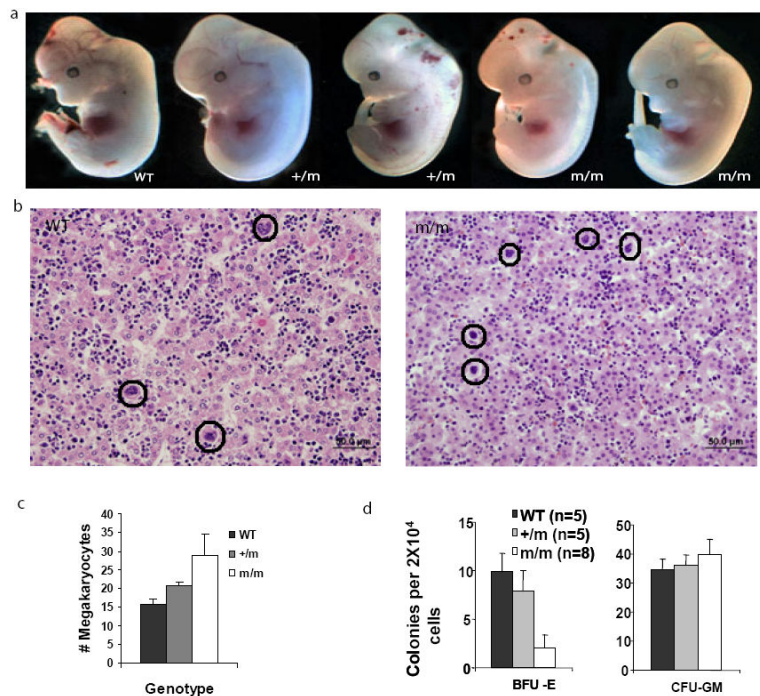


Figure 1. Analysis of developmental phenotypes in Chd2 mutant mice

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 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 hematoxylin and eosin 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.

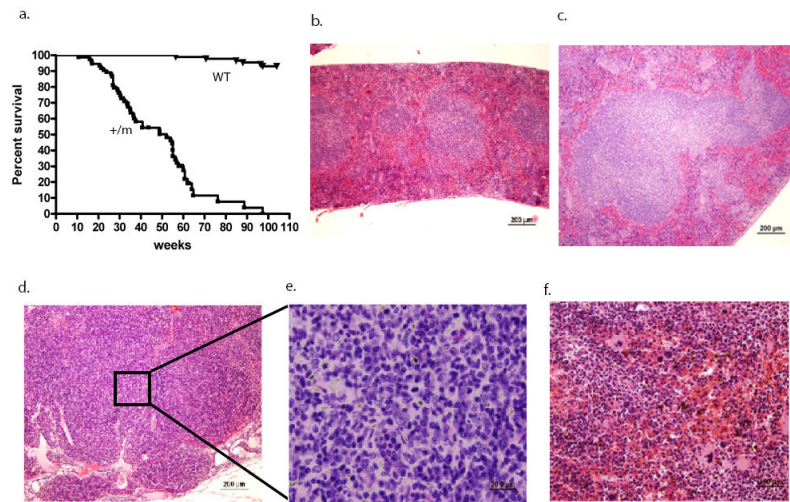


Figure 2. Lymphoid tumor susceptibility and hematopoietic defects in *Chd2* mutant mice
 a, Kaplan-Meier survival curves of *Chd2* mutant and wild-type littermates (n=50). The percentages of survival are plotted as a function of age in weeks. Animals were monitored for tumors, morbidity, or spontaneous death over a period of 105 weeks. Of the 74 animals analyzed for each group, 50 of the heterozygous mutants have died in comparison to 6 for the wild-type controls during a period of two 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 extra medullary hematopoiesis from heterozygous mice (f) is shown.

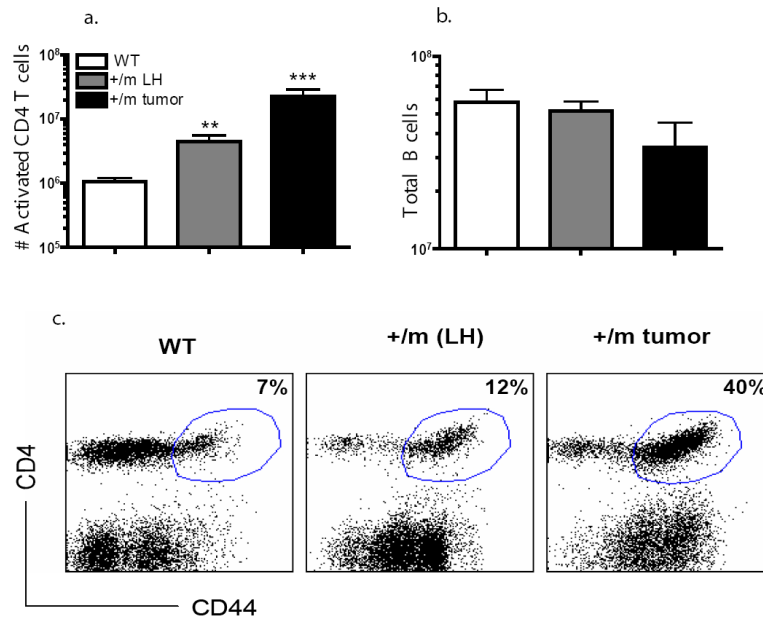


Figure 3. Characterization of lymphoid hyperplasias and lymphomas in *Chd2* mutant mice
 a, Total number of activated T cells from the spleens of age-matched wild type, *Chd2* mutants with hyperplasia, and *Chd2* mutants with lymphoma. b, Total number of B cells from spleens of indicated mice. c, Representative FACS 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 $p < 0.001$.

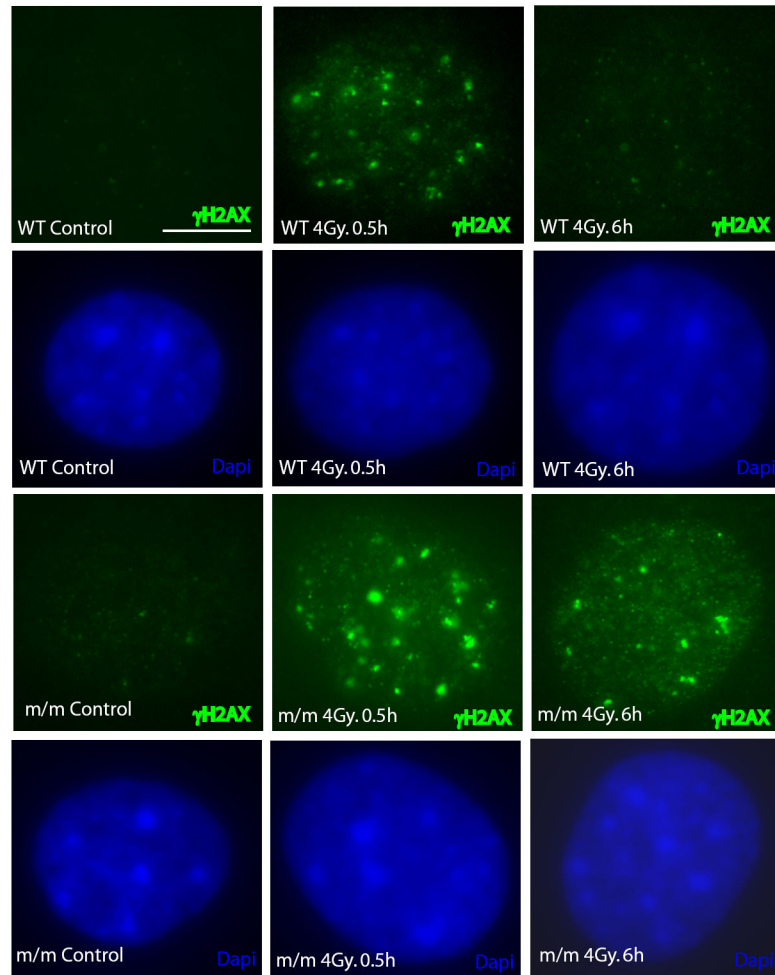


Figure 4. Defective DNA damage response in Chd2 mutant cells

Wild-type and Chd2 homozygous mutant littermate mouse embryonic fibroblasts (MEFs) were treated with 4Gy X-ray irradiation and analyzed for γ H2AX foci formation at 30 minutes, 3 (data not shown) and 6 hours. For immuno-fluorescence 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 analyzed per experiment. All cells were photographed using the same exposure time and microscope settings. Bar equals 10 microns.

Table 1
Distribution of pathological conditions in Chd2 heterozygous mice

Tissues from a total of 48 mice were analyzed to determine the reasons for morbidity. Hearts were examined for 37 mice. To avoid over-estimation of lymphoid hyperplasias, animals exhibiting lymphomas as well as lymphoid hyperplasias (in other organs) were categorized under lymphomas.

Lymphoma	43.7% (21 of 48)
Lymphoid hyperplasia	31.2% (15 of 48)
Extra Medullary Hematopoiesis [*] (EMH)	39.6% (19 of 48)
Glomerulo-nephropathy ^{**}	46.8% (15 of 32)
Inflammation of heart/artery ^{***}	43.2% (16 of 37)
Other cancers ^{****}	4.1% (2 of 48)

^{*} 13 out of 19 animals diagnosed with EMH exhibited either lymphoid hyperplasia or lymphoma.

^{**} 12 out of 15 animals exhibiting nephropathy were diagnosed with either lymphoid hyperplasia or lymphoma.

^{***} 13 out of 16 animals diagnosed with arterial and heart inflammation exhibited either lymphoid hyperplasia or lymphoma phenotypes.

^{****} Two animals were diagnosed with hemangiosarcoma and bronchoalveolar carcinoma.

Table 2
Persistence of γ H2AX foci in Chd2 mutant cells

Wild type and Chd2 homozygous mutant MEFs were treated with X-ray irradiation (4 Gy) and fixed at 30 minutes, 3 hours, and 6 hours post-irradiation. The γ H2AX foci formation was visualized by immunofluorescent staining with anti- γ H2AX antibodies and FITC labeled secondary antibodies. A minimum of 30 cells were analyzed per genotype and the numbers represent mean number of larger γ H2AX foci/cell. The percentage of foci remaining unresolved at 3 hours and 6 hours with respect to the percentage obtained at 30 minutes (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 MEFs that gave similar results. The differences between the wild type and mutants (+/m and m/m) were statistically significant. Bars represent s.d.

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- 3h	10.1±2.57 (55.4)	21.66±3.92 ** (92.3)	23.76±5.08 ** (87.1)
4 Gy -6h	3.43±1.86 (18.8)	14.87±2.87 *** (63.38)	18.0±3.97 *** (66.0)

*
p<0.01

**
p<0.0001

p<0.0002