A Dominantly Acting Murine Allele of Mcm4 Causes Chromosomal Abnormalities and Promotes Tumorigenesis

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Abstract

Here we report the isolation of a murine model for heritable T cell lymphoblastic leukemia/lymphoma (T-ALL) called Spontaneous dominant leukemia (Sdl). Sdl heterozygous mice develop disease with a short latency and high penetrance, while mice homozygous for the mutation die early during embryonic development. Sdl mice exhibit an increase in the frequency of micronucleated reticulocytes, and T-ALLs from Sdl mice harbor small amplifications and deletions, including activating deletions at the Notch1 locus. Using exome sequencing it was determined that Sdl mice harbor a spontaneously acquired mutation in Mcm4 (Mcm4D573H). Mcm4 is part of the heterohexameric complex of MCM2–7 that is important for licensing of DNA origins prior to S phase and also serves as the core of the replicative helicase that unwinds DNA at replication forks. Previous studies in murine models have discovered that genetic reductions of MCM complex levels promote tumor formation by causing genomic instability. However, Sdl mice possess normal levels of Mcm4, and there is no evidence for loss-of-heterozygosity at the Mcm4 locus in Sdl leukemias. Studies in Saccharomyces cerevisiae indicate that the Sdl mutation produces a biologically inactive helicase. Together, these data support a model in which chromosomal abnormalities in Sdl mice result from the ability of Mcm4D573H to incorporate into MCM complexes and render them inactive. Our studies indicate that dominantly acting alleles of MCMs can be compatible with viability but have dramatic oncogenic consequences by causing chromosomal abnormalities.

Introduction

Mouse models have been invaluable tools for studying human cancer. Many mouse models used for this purpose are reverse genetic, in that they involve genetically modified mice engineered to have lost a specific tumor suppressor gene (tsg) or to overexpress a specific proto-oncogene. More rarely, spontaneous or mutagen induced mouse models that result in tumor formation have been used to study tumorigenesis. Given the contribution of mouse models to understanding tumorigenesis, when a spontaneous mouse mutant that developed T-ALL arose in our colony, we pursued studies to both characterize the disease in these mice and to identify the causal mutation. The mutation was spontaneous and the phenotype dominant, so we named the mutant Spontaneous dominant leukemia (Sdl). We have identified a mutation in the Mcm4 gene as the likely causative genetic lesion in these mice.

Mcm4 is part of the MCM2–7 heterohexameric complex that is involved in licensing origins of DNA replication prior to S phase. The MCM complex has ATPase activity and serves as the core of the replicative helicase that unwinds duplex DNA and drives progression of the replication fork [1]. Improper fork progression can lead to stalled forks, the potential for incomplete DNA replication and even fork collapse which may lead to double strand break (DSB) formation [2]. Therefore, the MCM proteins play important roles in maintaining genomic integrity, however their roles in tumorigenesis are just beginning to be elucidated.

Previous studies of murine Mmm genes have involved hypomorphic or gene-trap null alleles. Gene-trap alleles are heterozygous viable and homozygous lethal [3,4]. Mice harboring hypomorphic alleles of Mcm2 (Mcm2<sup>JAC-JET</sup>) [5] or Mcm4 (Mcm4<sup>JAC-JET</sup>) [3] show reductions in MCM protein levels and develop tumors only
Author Summary

Our study investigated a spontaneous mouse model for dominantly inherited T-cell leukemia/lymphoma. Using genetic methods, we identified a mutant allele of Mcm4 (Mcm4<sup>chaos3</sup>) in this model. Interestingly, this Mcm4 allele promotes the accumulation of focal chromosomal gains and losses, including aberrations at the Notch1 locus that drive the formation of T-cell leukemia/lymphoma. Previous studies of hypomorphic Mcm alleles have demonstrated that a decrease in MCM levels can cause tumorigenesis. However, total and chromatin bound MCM levels were similar to wild-type in our model, indicating that Mcm4 alleles that do not drastically impact MCM levels can cause genomic aberrations that drive tumor formation.

in the homozygous state. Mcm4<sup>dhaus3</sup> was discovered in a screen for mutations that cause increased micronucleus formation in reticulocytes and therefore promote chromosome instability. Mcm4<sup>dhaus3</sup> results from a Phe345Ile substitution in MCM4, which is a residue that is involved in the interaction of MCM4 with MCM6 in the heterohexameric complex [3]. In Mcm4<sup>dhaus3/chaus3</sup> mouse embryonic fibroblasts (MEFs), total and chromatin bound levels of MCM4 and other MCM proteins are reduced compared to wild-type [3,6]. This leads to a loss of backup origins that normally fire during replicative stress which is hypothesized to be the mechanism by which low levels of MCM proteins promote genomic instability [6,7]. Mcm4<sup>dhaus3/chaus3</sup> mice develop tumors with long latency. Although the tumor spectrum varies with genetic background, Mcm4<sup>dhaus3/chaus3</sup> mice have not been reported to develop T-ALL [3,6]. We have accumulated evidence that the early-onset T-ALL phenotype in Sdl mice results from a novel allele of Mcm4 (Mcm4<sup>D573H</sup>) that in the heterozygous state promotes chromosomal abnormalities that cause highly penetrant tumor formation.

Results

Sdl causes primarily early onset T-ALL

The Sdl mutation arose in our colony in the germline of a breeder on the C57BI/6 genetic background. We therefore pursued a recombination mapping strategy by utilizing out-crosses and backcrosses to the FVB/N and 129S1/SvImJ genetic backgrounds. A whole genome scan using simple sequence length polymorphisms (SSLPs) was performed and it was determined that mice of backcross generations that inherited C57BI/6 markers at D16MIT131 and D16MIT4 on proximal Chr 16 rapidly became moribund (Figure 1A) indicating linkage to this chromosomal location. Therefore, Sdl carriers can be identified by the presence of C57BI/6 markers at these two SSLPs. Phenotypically, 94.2% (180 of 191) of moribund Sdl mice necropsied had signs of hematologic malignancy including mediastinal masses, splenomegaly and/or lymphadenopathy. Histologically, neoplastic cells filled hematopoietic tissues (Figure 1B) and infiltration of neoplastic lymphocytes into non-hematopoietic organs was frequently observed (Figure 1C). Leukemic cells are also found in the blood (Figure 1C) and bone marrow (Figure S1A). Sdl-induced disease was transplantable as tumors (Figure S1B) developed with an average latency of 29 days in four of four immunocompromised nude/nude mice that received cells isolated from mediastinal masses from moribund Sdl mice.

Flow cytometry was used to determine the phenotype of hematologic tumors from four Sdl mice. Three mice developed disease early in life that was phenotypically T-ALL (Figure 1D–1F and Table S1). The fourth mouse developed leukemia/lymphoma late in life, which expressed few lineage markers (Figure 1G and Table S1). Southern analysis of early-onset leukemias/lymphomas from Sdl mice detects rearrangements of the T cell receptor (TCR) β locus [8] (Figure S1). The majority of Sdl leukemias/lymphomas express TdT (Figure S1) that, together with the surface phenotypes, indicates that most Sdl mice develop T-ALL with an immature phenotype.

Inter-crosses of Sdl heterozygotes were performed to determine the phenotype of Sdl homozygotes. No Sdl/Sdl mice were present at weaning, so embryos from timed pregnancies of Sdl inter-crosses were examined. No Sdl/Sdl embryos were detected even as early as 8.5 dpc (n = 69; 20 wild-type, 49 Sdl/+; 0 Sdl/Sdl; p<0.0001 Chi square test), indicating that Sdl is homozygous lethal early during embryonic development. Therefore, all carrier mice utilized for the experiments described here are Sdl/+.

Sdl causes subtle defects during thymic development

To further characterize the molecular basis of leukemogenesis in Sdl mice, microarray analysis was performed to detect mRNA expression differences between wild-type thymuses and thymuses from pre-leukemic Sdl carriers. To detect differentially expressed genes, the false discovery rate was controlled at 5%. Specifically, transcripts with a posterior probability of differential expression >95% and a q-value <0.05 were considered to be significantly differentially expressed (see Methods). No transcripts were found to be significantly differentially expressed between wild-type and pre-leukemic Sdl carrier thymuses. Tests for common function did identify Gene Ontology (GO) sets enriched for differential expression between wild-type and carrier thymuses (Table 1), indicating some molecular differences between wild-type and carrier thymuses. However, a similar analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) failed to detect any differences between wild-type and carriers.

To determine if Sdl impacts T cell development, flow cytometry was performed to characterize T cell developmental stages in Sdl carriers. Thymocytes were analyzed from Sdl carrier (n = 4) and wild-type siblings at 3.5 weeks of age (n = 4). Analysis of more mature thymocyte populations (CD4, CD8 and CD4/8 double positive) revealed a trend toward decreased levels of CD4+ cells in Sdl mice, however this did not reach statistical significance (p<0.064 Table 2). Lineage markers as well as CD44 and CD25 were then utilized to further analyze more immature double negative (DN) populations. There was a statistically significant decrease in the percentage of DN cells at the DN1 stage of development in Sdl mice (Table 2). Although no statistically significant differences in other DN cell populations were observed, flow cytometry profiles from individual mice revealed inter-animal differences, particularly in the DN3 population, in Sdl mice (Figure S2). Taken together, these data indicate that Sdl does cause subtle defects in thymocyte development; with some mice more severely affected than others. However, it is unlikely that Sdl causes T-ALL by directly promoting a block in thymocyte differentiation.

Sdl mice harbor a mutation in Mcm4 that causes chromosomal abnormalities

To identify the affected gene in Sdl mice, the chromosomal location of the Sdl mutation was further narrowed utilizing single nucleotide polymorphic (SNP) markers to analyze mice with recombinant events in proximal Chr 16. Using this approach, the Sdl mutation was mapped to a 1.4 Mb candidate region (Figure 2A) that contains 30.5 kb of annotated protein-coding sequence. No differences in expression levels of genes in the interval were detected by quantitative RT-PCR (qRT-PCR)
Mice depicted in D–F succumbed to early onset-disease that is Sdl
haplotype at D16MIT131 and D16MIT4 on proximal Chr 16) are denoted by black squares, while sibling non-carriers are denoted by grey circles. Examples of flow cytometry analysis of lymphomas from four moribund mice. A full summary of flow-cytometry data is available as Table S1.

To further investigate if Sdl mice harbor phenotypes indicative of replicative stress, chromosomal aberrations were examined in both reticulocytes and mouse embryonic fibroblasts (MEFs) isolated from Sdl mice. Sdl mice harbor an ~18-fold increase in spontaneous micronucleated reticulocytes compared to non-carrier siblings (Figure 2C and 2D). This is similar to the ~20-fold increase reported for Mcm4D573H/+ mice studied on a different strain background [3]. MEFs from Sdl carriers and non-carrier siblings were analyzed cyto genetically for chromosome breaks in the presence and absence of the DNA replication inhibitor aphidicolin (APH) (Figure 2E–2F). More chromosome breaks were found in APH-treated Sdl MEFs compared to wild-type (p<0.02). Together, these observations indicate that Sdl causes chromosomal aberrations and increased sensitivity to exogenous replication stress, a phenotype that is consistent with McM dysfunction [3,5]. Therefore, all evidence suggests that the Sdl phenotype is caused by Mcm4D573H.

Mcm4D573H acts in a dominant manner to promote tumorigenesis

Previously studied hypomorphic or gene trap null alleles of Mcm have indicated that minimum thresholds of MCM levels are needed for normal development and for tumor suppression in adults; and reductions in protein levels of other members of the MCM2–7 complex have been detected in mutant Mcm4D573H/+ mouse tail tumors (Figure 3A) or total or chromatin bound protein levels in 21-day-old wild-type thymuses and 21-day-old Sdl carrier thymuses. No reductions in mRNA levels for Mcm2–7 subunits in eukaryotes (Figure S5). This residue is conserved not only in MCM4 but also across all MCM2–7 subunits in eukaryotes (Figure S5). Therefore, all evidence suggests that the Mcm4D573H allele is a loss-of-function allele that acts in a dominant manner to promote tumorigenesis.

Although genetically it acts dominantly, Mcm4D573H could actually promote tumor formation in a recessive manner if loss-of-heterozygosity (LOH) or epigenetic silencing at the Mcm4 locus occurs during tumor formation. To address these possibilities, RT-PCR followed by re-sequencing was used to examine if both wild-type and mutant Mcm4 alleles are expressed at the mRNA level in Sdl tumors. Peak heights of Sanger sequencing traces indicated that both alleles are expressed at similar levels in both Sdl tumors and in 21-day-old thymuses from Sdl carrier mice (Figure 3G). As stromal cells are present in bulk tumors, Mcm4 allele expression was also examined in cell lines that were established from Sdl T-
ALLs. Both alleles were expressed at similar levels as they are in thymuses from pre-leukemic Sdl carrier mice (Figure 3C). Therefore, tumorigenesis in Sdl mice does not require LOH, and the Mmc4^D573H allele acts dominantly to cause T-ALL.

The Sdl mutation generates a biologically non-functional helicase

To determine the impact of the Sdl mutation on MCM function, complementation studies in *Saccharomyces cerevisiae* were performed. These studies utilized a haploid strain that harbors a deletion of the chromosomal *mcm4* locus in which viability is maintained by a URA3-*mcm4* plasmid [9]. This strain was transformed with a TRP1 plasmid harboring *mcm4* with the Sdl mutation engineered into the analogous yeast residue (mcm4^D573H), hereafter referred to as mcm4^Sdl^*. Cloning into the TRP1 vector added a HA/10XHis tag, which has been shown to not compromise Mcm4 protein function in complementation tests [9] and allowed verification of Mcm4^Sdl^ protein expression by Western blotting (not shown). If the mcm4^Sdl^ allele expressed by the TRP1 plasmid complements the mcm4 genomic deficiency, then growth on -TRP+5-Fluoroorotic Acid (FOA) (restrictive conditions) will occur due to the ability to lose the wild-type mcm4 copy on the URA3 plasmid. TRP1-mcm4^WT^ wild-type and empty TRP1 vectors served as positive and negative controls, respectively. For each TRP1 vector, multiple individual colonies were analyzed for growth under restrictive conditions (example shown in Figure 4A). As expected, no empty TRP1 vector colonies (n = 37) grew while all TRP1-*mcm4* colonies (n = 37) grew. Surprising, mcm4^WT^ showed an intermediate phenotype as 10 of 38 colonies grew. To further examine this phenomenon, mcm4^Sdl^ colonies were examined for the presence of mcm4^Sdl^ sequences (Figure 4B). As expected, freshly isolated mcm4^Sdl^ colonies grown under permissive conditions (–URA –TRP) harbored both wild-type mcm4 and mcm4^Sdl^ sequences due to the presence of both URA3-*mcm4* and TRP1-*mcm4* plasmids. However, mcm4^Sdl^ colonies that grew under restrictive conditions (–TRP +FOA) only harbored mcm4 wild-type sequences, indicating that a reversion or gene conversion involving

| GO Term                                      | # of genes | Z score |
|----------------------------------------------|------------|---------|
| protein localization in mitochondrion        | 18         | 12.612  |
| protein targeting to mitochondrion           | 18         | 12.612  |
| C-C chemokine binding                        | 17         | 10.805  |
| C-C chemokine receptor activity              | 17         | 10.805  |
| positive regulation of endothelial cell proliferation | 16 | 10.403  |
| mitochondrial transport                       | 39         | 9.838   |
| chemokine binding                            | 22         | 9.334   |
| chemokine receptor activity                  | 22         | 9.334   |
| G-protein chemoattractant receptor activity  | 22         | 9.334   |
| regulation of endothelial cell proliferation | 20         | 9.272   |
| endothelial cell proliferation                | 22         | 8.863   |
| regulation of immunoglobulin production      | 29         | 8.26    |
| positive regulation of angiogenesis          | 33         | 7.651   |
| nucleoside diphosphate kinase activity       | 9          | 7.358   |
| UTP metabolic process                         | 9          | 7.358   |
| CTP metabolic process                         | 9          | 7.358   |

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Table 2. T cell development in wild-type and Sdl carrier mice.

| OP Term                                      | # of genes | Z score |
|----------------------------------------------|------------|---------|
| DN1                                         | 12.75#/-2.04 | 6.11#/-2.61^ |
| DN2                                         | 4.35#/-0.82  | 4.04#/-0.78   |
| DN3                                         | 40.65#/-2.41 | 49.75#/-12.67 |
| DN4                                         | 42.28#/-1.98 | 40.08#/-10.52 |

Abbreviations used: WT = wild-type, C = Sdl carrier, SP = single positive, DP = double positive, DN = double negative. ^t-test p value <0.064. 1DN cells are defined as negative for all lineage markers (see Materials and Methods). 2t-test p value <0.014. doi:10.1371/journal.pgen.1003034.t002

Sdl leukemias are characterized by intragenic deletions at the Notch1 locus

To further characterize the molecular basis of leukemogenesis in Sdl mice, microarray analysis was performed to compare expression in overt thymic tumors from Sdl mice to wild-type thymus. Utilizing the same criteria described above for analysis of pre-leukemic Sdl carriers, 3027 genes were found to be differentially expressed, of which 745 had ≥2 fold change in expression levels. The 20 significantly differentially expressed genes with the largest fold changes of increased and decreased expression in Sdl leukemias compared to wild-type thymuses are outlined in Table 3.

Microarray data indicated that the Notch1 pathway is activated in Sdl tumors as Notch1 itself and several Notch1 target genes including Myc, Hes1, Dlx1, Adam19, Hey, Heyl and Il2ra [10–13] were transcriptionally up-regulated in Sdl tumors compared to normal thymus (Table 2 and microarray data available at GEO). Expression levels of the Notch1 targets Hes1 and Myc were investigated by qRT-PCR (Figure 5A), which revealed that they were expressed at equivalent levels in wild-type and carrier thymuses, but were approximately 2 fold up-regulated in tumors. As NOTCH1 activating mutations are present in >50% of human T-ALL [14], exons 26, 27 and 34 which are the common sites of Notch1 mutational activation in murine leukemias [15] were sequenced from Sdl tumors. Only one point mutation in exon 26 was detected out of 13 tumors sequenced. Therefore, activation of Notch1 by point mutation is not a common mechanism in this model.

To further investigate the mechanism of Notch1 activation in Sdl T-ALL, Notch1 transcript levels were investigated by qRT-PCR
with primer pairs spanning several exon-exon boundaries (Figure 5B). Five of five Sdl tumors examined showed higher levels of expression of 3' exons than 5' exons, and in four of five tumors 3' exons were expressed at higher levels than in normal thymus. These results were consistent with the presence of intragenic deletions removing 5' regions of the Notch1 locus that have been recently reported in murine leukemias. These deletions result in truncated or chimeric transcripts that produce NOTCH1 proteins that are constitutively active [16,17]. Two types of intragenic Notch1 deletions have been reported in murine T-ALLs. Both types of transcripts were shown to be translated beginning at M1727 in exon 28, produce intracellular NOTCH1 (ICN1) and activate a Notch1 reporter [16]. Type 1 were more common, had specific break points that occur immediately adjacent to sequences similar to RAG-signal sequences (RSSs) and had features consistent with being driven by RAG activity. Type 2 deletions were more rare (3 of 10 cell lines examined, two of which were sub-clones of the same tumor) and did not have evidence of RSS-like sequences at their breakpoints [16]. Type 1 deletions break at specific chromosomal locations, so genomic PCR can be used to detect them. No such deletions were detected in Sdl tumors. Type 2 deletions have varying breakpoints as they are not limited to RSS-like sequences, so they are difficult to detect via genomic PCR. However, RT-PCR can be used to detect the resulting abnormal chimeric transcripts. Such transcripts were detected in 12 of 15 tumors examined (Figure 5C). Sequencing of the primary RT-PCR product from three separate tumors revealed splicing from exon 1 to exon 28. To attempt to clone the breakpoints in Notch1 in Sdl tumors, genomic PCR on a separate cohort of tumors was performed with various forward primers spanning exon 1

Figure 2. Sdl mice harbor a mutation in Mcm4 that causes chromosomal abnormalities. A) Recombination events in Chr 16 that define the Sdl interval. Only the relevant Sdl haplotype is shown. White squares: FVB/N alleles, Grey squares: 129S1/SvImJ alleles, Black squares: C57Bl/6 alleles. The Sdl mutation must map within regions harboring C57Bl/6 alleles in leukemic mice, but must be excluded from regions harboring 129S1/SvImJ alleles in non-leukemic mice. The Sdl mutation likely lies distal to 14.56 and proximal to 15.91. The two non-leukemic mice were bred and non-carrier status was verified. B) Sanger sequencing traces demonstrating the G to C substitution present in all confirmed Sdl carriers but absent from all wild-type strains examined. Arrow indicates dual G/C peak, asterisk indicates wild-type G peak. C) Sdl mice harbor increased numbers of spontaneous micronuclei. Example flow cytometry plots for wild-type (WT) and Sdl carrier (C) mice for micronuclei detection. Micronucleated normochromatic erythrocytes are propidium iodide (PI) positive but CD71 negative (lower right quadrant) and are expressed as a percentage of total erythrocytes. D) Quantification of micronuclei for sex and aged-matched wild-type (solid bar, WT) (n = 8) and Sdl carriers (striped bar, C) (n = 5). Mean for wild-type = 0.0815 and for Sdl carriers = 1.503 (p < 0.0005). Error bars represent standard deviation. E) Sdl (striped bars) and wild-type (solid bars) MEFs were treated with Dimethyl sulfoxide (DMSO) as vehicle control or 0.15 μM aphidicolin (APH) and the percent of metaphases with chromosome breaks determined. No statistically significant difference between Sdl and wild-type was observed when cells were treated with vehicle only (p > 0.26). In the presence of APH, Sdl MEFs did harbor more chromosome breaks (average 18.3%) than wild-type MEFs (average 5.8%) (p < 0.02, t-test, asterisk). Error bars represent standard deviation. F) Example metaphase spreads without chromosome breaks (WT, APH treated) and with chromosome breaks (Sdl, APH treated). Arrows indicate chromosome breaks, one example shown as inset.

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A Dominantly Acting Mcm4 Allele

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through intron 2 in combination with an exon 27 or exon 28 reverse primer. In 3 of 13 tumors, products were cloned and it was verified that the breakpoints do not possess evidence of RSS-like sequences. Two of three breakpoints had 2–4 bp microhomology (Figure 5D). Therefore, it is hypothesized that the tumor spectrum of Sdl mice is, at least in part, due to the propensity to develop type 2 non-RAG driven deletions at the Notch1 locus.

To determine if Sdl T-ALLs harbor additional genomic aberrations, array CGH was performed on genomic DNA isolated from Sdl thymic tumors compared to DNA isolated from a non-carrier mouse. Although whole chromosome gains and losses were not detected, many small deletions and amplifications averaging 110 kb in size were present in tumors (Figure 6). Therefore, the Sdl mutation promotes focal copy number changes and not aneuploidy.

Discussion

We have been studying a novel spontaneous mouse cancer model, Sdl, in which an early-onset T-ALL phenotype is inherited in a dominant manner. We have accumulated evidence that Mcm4D573H is the causative tumor-causing genetic lesion in this model. The dominant inheritance of the cancer phenotype observed in Sdl thymic tumors compared to wild-type thymuses (solid bars) as analyzed by qRT-PCR. Values for wild-type thymus are normalized to 1. N = 3 for wild-type, 6 for carrier. Error bars represent standard deviation. There is a trend toward increased expression of Mcm3 and Mcm5 in Sdl carrier thymuses compared to wild-type thymuses (p = 0.07 and 0.09, respectively); all other p values >.2. B) Western analysis on total thymus protein extract as well as purified chromatin bound (c.b.) fractions indicate that Sdl carrier thymuses harbor similar levels of MCM2 and 4 proteins as do wild-type thymuses. TUBULIN and Ponceau S membrane staining were utilized to demonstrate equal loading for whole cell lysates and chromatin bound fractions, respectively. C) Sanger sequencing traces of RT-PCR products demonstrate that both wild-type (G) and mutant (C) Mcm4 alleles are expressed in Sdl tumors and tumor-derived cell lines. RT-PCR products from 21-day-old wild-type and Sdl carrier thymuses are shown for reference. Arrow indicates dual G/C peak, asterisk indicates wild-type G peak.

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Figure 3. Mcm4D573H acts in a dominant manner to promote tumorigenesis. WT = wild-type C = carrier. A) Mcm2–7 transcript levels are not decreased in Sdl carrier thymuses (striped bars) compared to wild-type thymuses (solid bars) as analyzed by qRT-PCR. Values for wild-type thymus are normalized to 1. N = 3 for wild-type, 6 for carrier. Error bars represent standard deviation. There is a trend toward increased expression of Mcm3 and Mcm5 in Sdl carrier thymuses compared to wild-type thymuses (p = 0.07 and 0.09, respectively); all other p values >.2. B) Western analysis on total thymus protein extract as well as purified chromatin bound (c.b.) fractions indicate that Sdl carrier thymuses harbor similar levels of MCM2 and 4 proteins as do wild-type thymuses. TUBULIN and Ponceau S membrane staining were utilized to demonstrate equal loading for whole cell lysates and chromatin bound fractions, respectively. C) Sanger sequencing traces of RT-PCR products demonstrate that both wild-type (G) and mutant (C) Mcm4 alleles are expressed in Sdl tumors and tumor-derived cell lines. RT-PCR products from 21-day-old wild-type and Sdl carrier thymuses are shown for reference. Arrow indicates dual G/C peak, asterisk indicates wild-type G peak.

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Figure 4. S. cerevisiae mcm4 engineered with the Sdl mutation at the equivalent residue (D632H) generates a non-biologically active helicase. A) Examples of genetic complementation tests of a mcm4 deletion haploid strain in which viability is maintained by an URA3-mcm4 plasmid. This strain was transformed with TRP1 plasmids expressing mcm4<sup>Δ6</sup> mutation (S), mcm4 wild-type (W) or empty TRP1 vector (V). A) Growth on permissive conditions (−TRP) demonstrates that all colonies analyzed harbor the expected TRP1 plasmids. B) Growth under restrictive conditions (−TRP+ FOA) occurs only if viability can be maintained by the allele on the TRP1 plasmid. All mcm4 wild-type colonies grew under restrictive conditions and empty vector colonies do not, as expected. A fraction of colonies expressing mcm4<sup>Δ6</sup> mutation grew under restrictive conditions. C) A restriction fragment polymorphism was utilized to distinguish mcm4<sup>Δ6</sup> from wild-type (WT) mcm4 sequences in the yeast strains described above. One mcm4 wild-type (W) colony and 10 mcm4<sup>Δ6</sup> (S) colonies are shown. All freshly isolated mcm4<sup>Δ6</sup> colonies grown under permissive conditions (−URA −TRP) harbor both mcm4<sup>Δ6</sup> and mcm4 wild-type sequences due to the presence of TRP1-mcm4<sup>Δ6</sup> and URA3-mcm4 plasmids. All mcm4<sup>Δ6</sup> colonies that grew under restrictive conditions lost mcm4<sup>Δ6</sup> sequences, indicating that growth occurred due to a reversion or gene conversion event involving mcm4<sup>Δ6</sup> sequences on the TRP1 plasmid and not due to the ability of mcm4<sup>Δ6</sup> to complement the mcm4 genomic deletion.

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backgrounds, genetic modifiers could contribute to the observed phenotypic differences. Alternatively, the recessively acting hypomorphic Mcm4<sup>R573H</sup> and the dominantly acting Mmc4<sup>D632H</sup> may have different consequences on origin licensing and DNA replication. In addition, a recent report found that MCM 2, 3, 5 and 7 regulate HIF1 activity and this function is likely independent from their function in the heterohexamer. A similar activity was not detected for MCM4 or 6 [18]. Therefore, the reduction in levels of total MCMs seen in mcm4<sup>R573H</sup> and mcm4<sup>D632H</sup> may influence HIF1 activity and have phenotypic consequences. A study of tumor and DNA replication phenotypes for both alleles on the same genetic background will be required to address the reasons for phenotypic differences between the two alleles.

Analysis of T cell differentiation in Sdl carriers revealed subtle defects, with some animals being more severely affected than others. One potential interpretation is that T cell differentiation is mostly normal in Sdl mice until genomic mutations due to replicative stress start to accumulate. In support of this, microarray data failed to detect any transcripts that are significantly differentially expressed in Sdl carrier thymuses compared to wild-type thymuses. Tests for common function did identify differences in genes with common gene functions including protein localization or targeting to mitochondria, chemokine binding or receptor activity and endothelial cell proliferation.

In contrast, many expression differences were detected between wild-type thymuses and Sdl leukaemias. Many of the mostly profoundly down-regulated genes in Sdl leukaemias are genes such as Prss16 and Tbeta that are expressed in thymic epithelial cells [19,20]. This observation likely results from a lower ratio of T cells to thymic epithelial cells in normal thymus than in thymic lymphoma. Although non-T lineage cells are the minority of cells in the developing thymus, they nevertheless impacted our ability to identify genes that are down-regulated during T-ALL formation in Sdl mice. The transcripts with the greatest fold up-regulation in Sdl leukaemias compared to normal thymus include genes with unknown function, metabolic genes, genes expressed during T cell activation and Notch1 target genes. RT-PCR in Sdl tumors demonstrated the presence of an aberrant Notch1 transcript splicing from exon 1 to exon 28 in 12 of 15 Sdl leukaemias. Genomic PCR on a separate cohort of Sdl T-ALLs was able to clone genomic breakpoints in the Notch1 locus in 3 of 13 tumors. These breakpoints occurred in introns 2 and 27, introns 1 and 27, and introns 2 and 26. It is possible that the exon 1 to 28 splice is favored even when deletions leave more internal exons intact, or that our RT-PCR conditions failed to robustly amplify transcripts containing other aberrant splice variants. Alternatively, the genomic re-arrangements present at the Notch 1 locus may be more complex than can be detected by our genomic PCR. Nevertheless, the detected Notch1 transcript and lack of RSS-like sequences at the cloned breakpoints are both consistent with the presence of type 2 deletions at the Notch1 locus in Sdl T-ALLs. The vast majority of murine T-ALLs previously examined have harbored type 1 RAG-mediated deletions, while type 2 deletions were more rare. A predisposition to T-ALL has also been observed for Mem2 hypomorphic mice [5,7] and array CHG detected deletions at the Notch1 locus in 4 of 8 of T-ALLs in Mem2 mice [21]. One possibility to explain the tumor spectrum in Sdl mice and Mem2 hypomorphic mice is that the integrity of the murine Notch1 locus is sensitive to replicative dysfunction in developing T cells and that replicative stress promotes the formation of type 2
Table 3. Top 20 genes with increased and decreased expression in Sdl leukemias.

| Gene Symbol | q value | PP.LNNMV.DE | fold change |
|-------------|---------|-------------|-------------|
| A730037C10Rik | 0.022 | 1 | 51.653 |
| Susd4 | 0.032 | 1 | 41.487 |
| Cd5l | 0.024 | 1 | 39.086 |
| I22 | 0.045 | 1 | 38.299 |
| Fbp1 | 0.008 | 1 | 34.682 |
| Arg1 | 0.049 | 1 | 34.535 |
| Emx2 | 0.026 | 1 | 33.934 |
| Drd5 | 0.02 | 1 | 30.812 |
| Aldh1b1 | 0.017 | 1 | 30.350 |
| Dtx1 | 0.012 | 1 | 28.659 |
| Gm11428 | 0.043 | 1 | 27.660 |
| Drd5 | 0.019 | 1 | 27.146 |
| Ace | 0.038 | 1 | 23.650 |
| Hmx1 | 0.009 | 1 | 22.894 |
| Wdr25 | 0.027 | 1 | 22.461 |
| Gpnmb | 0.034 | 1 | 21.205 |
| Heyl | 0.015 | 1 | 20.901 |
| Adam19 | 0.036 | 1 | 19.015 |
| Hdghfrp3 | 0.006 | 1 | 18.947 |
| Slc16a3 | 0.005 | 1 | 18.561 |
| Ube2l6 | 0.023 | 1 | 18.476 |
| Igfbp5 | 0.001 | 1 | 18.466 |
| Krt8 | 0 | 1 | 18.436 |
| Scl46a2 | 0.002 | 1 | 14.050 |
| Psmb11 | 0.009 | 1 | 13.977 |
| Dgat2 | 0.008 | 1 | 13.788 |
| Gpd1 | 0.013 | 1 | 13.666 |
| Krt18 | 0 | 1 | 13.565 |
| Hpgd | 0.001 | 1 | 13.310 |
| Akr1c18 | 0.003 | 1 | 13.302 |
| Car3 | 0.005 | 1 | 13.300 |
| Snca | 0.035 | 1 | 12.585 |
| Coxl8b | 0.016 | 1 | 12.433 |
| Alas2 | 0.03 | 1 | 12.237 |
| Ccxl11 | 0.004 | 1 | 11.922 |
| Skint10 | 0.008 | 1 | 11.700 |
| Ucp1 | 0.016 | 1 | 11.599 |
| Cdc25 | 0.001 | 1 | 10.101 |
| Tbeta | 0.002 | 1 | 10.101 |
| Prss16 | 0.003 | 1 | 0.072 |

1 posterior probabilities of differential expression (see Materials and Methods for details).

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deletions at the Notch1 locus. As the majority of T cell development is completed by young adulthood, Mcm4\footnote{Path:Chaos/Chaos} mice may not experience sufficient replicative stress to cause Notch1 deletions in developing thymocytes, which would allow them survive longer to develop other late-onset tumor types.

Previous array CGH studies of Notch1-driven mouse T-ALLs failed to detect tumor-specific chromosomal aberrations, indicating that chromosomal instability is not a general characteristic of mouse T-ALLs. In contrast, array CGH data of Sdl tumors did detect small amplifications and deletions but not whole chromosome gains and losses. This data is consistent with previous observations that an improved growth phenotype found in mcm4\footnote{Path:Chaos/Chaos} diploid yeast is due to mutations in a few genes and not due to aneuploidy. In addition, recent array CGH experiments on T-ALLs from Mcm2 hypomorphic mice also detected small genomic aberrations. However, aberrations in T-ALLs in Mcm2 mice were primarily deletions, while both amplifications and deletions were found in Sdl T-ALLs. It is possible that functional differences between MCM helicase activity in Sdl and Mcm2 hypomorphic mice could explain this difference. However, it is also possible that strain specific modifiers can impact the types of aberrations generated by replicative dysfunction or selected for during tumorigenesis. Nevertheless, studies in yeast, Mcm2 hypomorphic mice and Sdl mice all support a model that replicative stress can contribute to tumorigenesis by generating smaller chromosomal aberrations and not by causing aneuploidy.

The role of MCM proteins in promoting genomic instability during human cancer initiation and progression remains unclear. Immunohistochemistry detects MCM protein expression in many human tumor samples, as would be expected for rapidly dividing cells. Knockdown of MCM2, 3 or 7 in medulloblastoma cell lines caused inhibition of anchorage-dependent and independent growth; while their over-expression promoted cell migration, invasion and increased anchorage-independent growth. Although mutations in genes involved in DNA damage checkpoints and DNA damage repair are known to contribute to sporadic and hereditary tumorigenesis, it is unclear if genetic changes in the actual components of the helicases are stable, yet functionally inactive.
the *Sdl* model indicate that dominantly acting *Mcm* alleles can be compatible with viability but cause chromosomal abnormalities and highly penetrant tumor formation. Therefore, *Mcm* mutations with different functional consequences on MCM levels and activity have the potential to act as driver mutations during tumorigenesis.

**Materials and Methods**

**Ethics statement**

Mouse experiments were performed according to the institutional guidelines for animal care under the approval of the IACUC of the University of Minnesota and the University of Wisconsin.
Animals

Sdl arose in the germline of a Rosa-SB11 mouse maintained on the C57Bl/6 background [32]. Wild-type mice were purchased from Jackson Labs or Charles River. Non-carrier sibling mice were utilized as controls. Mcm4chaos3 mice on the FVB/N genetic background were generously provided by Naoko Shima.

DNA capture and exon re-sequencing

The SureSelect XT Mouse All Exon Kit (Agilent Technologies) was used to capture exonic sequences from genomic DNA purified from a tail clip of a Sdl carrier. Sequencing was performed on the Illumina Genome Analyzer 2 platform as paired-end 76-bp reads. One lane of sequence was generated. Reads were aligned to the MM9 reference genome with BWA v0.5.5 [33]. The GATK (v1.0.4771) [34] was then used to do local realignment of the reads around all indel sites called in the mouse genomes project [35]. The base qualities of the BAM file were recalibrated with the GATK v1.0.4771 by masking all SNP and indel positions called in the mouse genomes project. SNP calling was carried out using SAMtools mpileup/bcftools (v0.1.16) [36]. For SAMtools mpileup, the following options were used: -d 500 -C50 -m3 -F0.002 -aug. The raw sequence data is available under ERA accession number ERP000474.

Array CGH

DNA was purified from thymic tumors from three Sdl mice on the 129S1/SvImJ genetic background. Tail clip DNA from a non-carrier also on the 129S1/SvImJ genetic background was utilized as reference DNA. Hybridizations were performed by WiCell research institute according to manufacturer’s recommendations to the mouse CGH 3×720 K Whole-Genome Tiling Arrays (NimbleGen). NimbleScan, CGH Fusion (RBS v1.0) (Inforquant) software was utilized for analysis and data visualization. Gains and losses were called with the following parameters; average log-ratio threshold of 0.2, a minimum aberration length of 5 probes and maximum p-value of 0.001.

Southern analysis

DNA was purified from thymic tumors, and TCR β re-arrangement was detected as described [8].

Microarray analysis

RNA was isolated from female mice by Trizol (Invitrogen) extraction followed by further purification using the RNeasy kit (Qiagen). RNA from three animals for each group (21-day-old wild-type thymus, 21-day-old Sdl carrier thymus and Sdl overt thymic tumors) was pooled per array. Four RNA pools were analyzed per group. Amplified sense-strand cDNA was generated using the Ambion WT expression kit (Applied Biosciences), fragmented and labeled with the GeneChip WT Terminal Labeling Kit (Affymetrix) and hybridized to GeneChip Mouse Exon 1.0 ST Arrays (Affymetrix) by the University of Wisconsin.

Western analysis

Whole cell protein extracts were purified using RIPA buffer and protease inhibitors (Fierce). Cell fractionation was performed using the Qproteome Nuclear Protein Kit (Qiagen). Proteins were run on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane. Membranes were incubated with primary antibodies, followed by incubation with the secondary antibody and visualized using Western Lighting Plus-ECL (PerkinElmer).

RT–PCR and quantitative RT–PCR (qPCR)

RNA was isolated by Trizol (Invitrogen) and further purified using the RNeasy Mini Kit (Qiagen). All wild-type and Sdl carrier thymuses used for RNA extraction were from 21-day-old mice. Wild-type thymuses were isolated from non-carrier littermates. First strand cDNA was generated using a 20:80 mix of polyT:random decamer primers (Ambion Retroscript). Real-time PCR was completed using the LightCycler (Roche Applied Science). Briefly, LightCycler FastStart DNA Master PLUS SYBR Green I kit (Roche Applied Science) was used with 5 μl cDNA from the reverse transcriptase reaction. Relative mRNA expression levels were calculated as described previously [38]. Go taq (Promega) was used for non-quantitative PCRs. t-tests or ANOVA followed by Tukey post hoc analysis were performed using Prism software.

Southern analysis

DNA was purified from thymic tumors, and TCR β re-arrangement was detected as described [8].

Microarray analysis

RNA was isolated from female mice by Trizol (Invitrogen) extraction followed by further purification using the RNeasy kit (Qiagen). RNA from three animals for each group (21-day-old wild-type thymus, 21-day-old Sdl carrier thymus and Sdl overt thymic tumors) was pooled per array. Four RNA pools were analyzed per group. Amplified sense-strand cDNA was generated using the Ambion WT expression kit (Applied Biosciences), fragmented and labeled with the GeneChip WT Terminal Labeling Kit (Affymetrix) and hybridized to GeneChip Mouse Exon 1.0 ST Arrays (Affymetrix) by the University of Wisconsin.

Figure 6. Array CGH profiles of thymic tumors from three Sdl mice. Probes with copy number gains and losses in tumors compared to reference are shown in green and red, respectively. doi:10.1371/journal.pgen.1003034.g006
Biotechnology Center Gene Expression Center. The data were normalized using ma [39] as implemented in the xps system, available at Bioconductor (www.bioconductor.org). Summaries at the transcript level using probes given a core level ranking were analyzed, as this annotation level is most conservative [40].

With EBarrays, an empirical Bayes hierarchical modeling approach, was used to identify differentially expressed (DE) genes [41,42]. With EBarrays, a gene was considered significantly DE if the posterior probability of DE under the log-normal Normal moderated variance (PP.LNNMV.DE) model exceeded 0.95, as this threshold controls the posterior expected false discovery rate (FDR) at 5% [42]. We also considered results from gene-specific t-tests with q-values calculated from the t-test p-values [43]. A list of genes with q-values <0.05 has expected FDR of 5%. To help ensure that results were robust to the statistical method used, we define a gene to be significantly DE if its PP.LNNMV.DE>0.95 and its q-value <0.05. Tests for enrichment of common function among sets of differentially-expressed genes were carried out using data from the Gene Ontology (GO) annotations and the Kyoto Encyclopedia of Genes and Genomes (KEGG). The R package allez was used to perform tests of enrichment for each GO category and KEGG pathway [44]. In general, the interpretation of p-values resulting from enrichment tests is not straightforward due to the many dependent hypotheses tested. Furthermore, the enrichment test tends to result in small p-values when groups with few transcripts are considered. The statistical methods underlying allez adjust for these factors, allowing increased power and sensitivity for identifying sets that are biologically meaningful.

Yeasts studies

The mcm4 deletion strain and TRP1 plasmids were described previously [9] and were generously provided by Anthony Schwacha. The Sdl mutation in the equivalent residue (D632H) was generated by site-directed mutagenesis utilizing PhlUltra Hotstart taq (Agilent) followed by DpnI digestion and verified by sequencing. Cloning into the TRP1 plasmid added a HA/H10X tag that allowed Mcm4 D632H protein expression to be verified by Western blot. The engineered Sdl mutation creates a restriction fragment polymorphism that can be detected by PCR amplification followed by BsaI digestion.

MEF isolation and analysis

MEFs were isolated from 12.5–15.5 dpc embryos on the 129S1/ SvImj genetic background. All MEFs were utilized at p3 or lower. For chromosome break analysis, MEFs were treated with aphidicolin or DMSO as vehicle only control for 24 hours. Colemid was added for the final five hours prior to harvest. Three separate cultures were analyzed per genotype. Harvested cells were pelleted and re-suspended in 0.075 M KCl solution and incubated at room temperature for 20 minutes, pelleted, and re-suspended in 3:1 (vol/vol) methanol/acetic acid for 10 minutes twice. Cells were spread on a slide and stained with Giemsa stain (Sigma GS500) in a 1:20 dilution in water. Slides were washed briefly with 1X PBS and observed.

Histopathology

Tissues were fixed in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin by the University of Wisconsin RARC comparative pathology laboratory. Analysis was performed by the UWCC Experimental Pathology Core.

Flow cytometry

Cryopreserved tumor cell samples were briefly thawed at 37°C and then incubated for 15 min at 37°C in DMEM containing 20% FBS, 10 U/ml Heparin (Sigma), and 0.25 mg/ml DNase 1 (Roche). Cells were pelleted and re-suspended in HBSS without Ca/Mg, 2% FBS, 2.5% cell dissociation buffer (GBCO, Invitrogen), 100 U/ml Penicillin G, and 100 μg/ml streptomycin. Tumor cell suspensions were pre-incubated with antibodies to CD3/CD4/CD8 APC to examine CD4, CD8 and CD4/8 double positive T cells. To examine double negative (DN) populations, dissociated thymocytes were stained with a lineage cocktail (anti Gr-1-Biotin, phycoerythrin (PE), or TRI-COLOR (TC) for 20 min at 4°C. After washing, streptavidin-allophycocyanin (APC) was added for 20 min at 4°C to provide fluorescent signal for biotinylated antibodies. Staining was assessed with a FACSCalibur flow cytometer (Becton Dickinson) in four-color mode using CellQuest Pro and FlowJo software for analysis. For thymocyte analysis, dissociated cells were incubated with anti-CD4 FITC and anti-CD8 APC to examine CD4, CD8 and CD4/8 double positive T cells. To examine double negative (DN) populations, dissociated thymocytes were stained with a lineage cocktail (anti Gr-1-Biotin, CD4/8 APC to examine CD4, CD8 and CD4/8 double positive T cells. To examine double negative (DN) populations, dissociated thymocytes were stained with a lineage cocktail (anti Gr-1-Biotin, CD4/8 APC to examine CD4, CD8 and CD4/8 double positive T cells.

Supporting Information

Figure S1 Sdl mice primarily develop T-ALL. A) H&E stain of bone marrow in the sternum of a Sdl mouse showing the presence of neoplastic cells (40X magnification, scale bar = 50 μM) B) H&E stain of a kidney from a nude/nude mouse that received T-ALL cells from a Sdl mouse showing infiltration of transplanted neoplastic lymphocytes (40X magnification, scale bar = 50 μM). C) Southern analysis of Sdl tumors detects re-arrangements at the TCR β locus. Asterisk indicates germine line band, arrowhead indicates an example of a re-arrangement. D) Western analysis detects tT expression in Sdl leukemias (top). Tubulin is used to demonstrate equal protein loading (bottom).

Figure S2 Inter-animal differences in T cell developmental defects in Sdl carriers. CD44 and CD25 staining of Lin-thymocytes was used to examine DN stages of T cell development in four non-carrier (wild-type, WT) and four Sdl carrier (C) thymuses. Differences in the percent of cells at the DN1 (CD25− CD44+) stage of development were statistically significantly decreased in Sdl carriers compared to wild-type (See Table 2), with animals C3 and C4 more severely affected than animals C1 and C2. Animals C3 and C4 also appear to have a defect at the DN3 (CD25+ CD44−) to DN4 (CD25− CD44−) transition.

Figure S3 qRT-PCR detects no differences in expression levels of genes in the Sdl interval between wild-type (solid bars) and carrier thymuses (striped bars). Wild-type thymus is normalized to 1. N = 3 (except for Mcm4 carrier thymus where N = 6), error bars indicate standard deviation, P values are all >0.29. Expression of F830005R03Rik was not detected. Mcm4 data is also depicted in Figure 3.
Figure S4  Histogram showing sequencing depth coverage of exons in the Sdl interval. The number of base pairs (frequency) is plotted against sequencing depth coverage.

(TIF)

Figure S5  A) Alignment of MCMs around the residue equivalent to murine MCM4 573 D (indicated in bold). Murine and yeast MCM 2–7 are shown, as are MCM4 from several eukaryotes. The residue impacted by the Sdl mutation is invariant across MCMs. B–D) The structure of an archaeal (Sulfolobus solfataricus) MCM helicase [45] with the equivalent D residue highlighted (B) was utilized to model the impact of the D to H Sdl mutation (C) and the D to A mutation (D) that was previously shown in complementation tests to not impact the biologic activity of S. cerevisiae mcm4 [9]. The D to H substitution is predicted to have a greater impact on protein structure than the D to A substitution. Structures were visualized using PyMol [46].

(TIF)

Figure S6  Total and chromatin bound levels of MCM4 are similar in Sdl and wild-type MEFs. Western analysis on total MEF protein extract as well as purified chromatin bound (c.b.) fractions indicate that Sdl carrier MEFs (C) harbor similar levels of MCM4 protein as do wild-type (WT) MEFs. TUBULIN and Ponceau S membrane staining were utilized to demonstrate equal loading for whole cell lysates and chromatin bound fractions, respectively.

Table S1  Summary of flow data for Sdl leukemias. Mouse 1 corresponds to panel D, Mouse 2 to panel E, Mouse 3 to panel F and Mouse 4 to panel G in Figure 1. LN = lymph node, SPL = spleen, THY = thymus. + positive staining. – no staining.

(XLSX)

Table S2  Summary of exon and splice site sequences with fewer than 10x coverage by exon-capture re-sequencing that were examined by PCR amplification followed by Sanger sequencing.

(XLSX)

Table S3  Non-synonymous changes identified by exon capture on proximal chromosome 16. * The change in Top3b falls outside of the Sdl interval (see Figure 2A) and was not confirmed by traditional Sanger sequencing in genomic DNA from the mouse utilized for exon capture or other carrier Sdl mice. Visual inspection indicates that the base lies adjacent to a low-complexity polyG tract and that the alternate call is likely due to read misalignment.

(XLSX)

Text S1  Supplemental methods.

(DOC)

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Author Contributions

Conceived and designed the experiments: BNB RTC CK DJA LSC. Performed the experiments: BNB TMK VJM RAI MNC ARP LEB RAB RTC LSC. Analyzed the data: BNB TMK VJM RTC CK DJA LSC. Wrote the paper: BNC LSC.

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