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14. ABSTRACT

Several recent studies have indicated that decreased levels of the MCM2-7 DNA replication proteins can lead to genomic instability (GIN) and cancer formation. Interestingly, genetic or RNAi-mediated depletion of one MCM has been demonstrated to cause decreases in other MCMs, presumably as a consequence of MCM heterohexamer destabilization. In the first year of my training grant, my research results show that in cells bearing only the *Mcm4*^{Chaos3} cancer susceptibility allele, the cause for reduced MCM protein levels is related to decreased *Mcm2-7* and *10* mRNA. Despite being present at levels far exceeding that required for DNA replication under normal circumstances, we found that heterozygosity for 2 or more different MCMs caused genomic instability, and in the cases of MCM2, MCM6 and MCM7, synthetic lethality in conjunction with *Mcm4*^{Chaos3} homozygosity. These data suggest that proper stoichemistry of MCM components is carefully regulated, and that relatively minor disregulation or destabilization of MCM levels can have serious consequences for survival or cancer susceptibility in whole animals.

15. SUBJECT TERMS

MCM2-7, Genomic instability, DNA replication, Cancer susceptibility

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Introduction:

In late mitosis to early G1 phase, DNA replication origins are selected and bound by the hexameric origin recognition complex (ORC). ORC then recruits the initiation factors CDC6 and CDT1, which are required for loading MCM2-7, thereby forming the "pre-replicative complex" (pre-RC). The formation of pre-RCs is termed origin "licensing" and this allows origins to gain competency for a single round of DNA synthesis before entering S phase. MCM2-7 is a hexamer of six distinct but structurally-related minichromosome maintenance (MCM) proteins (reviewed in [Lei, 2005;Tye, 1999]). *In vivo* and *in vitro* evidence indicates that the MCM2-7 complex is the replicative helicase [Moyer, 2006; Labib, 2000].

MCM2-7 proteins exist abundantly in proliferating cells, and are bound to chromatin in amounts exceeding that which is present at active replication origins or which is required for complete DNA replication. Although many studies showed that drastic decreases in MCMs are tolerated by dividing cells, there are certain deleterious consequences. A reduction in MCM proteins reportedly causes decreased usage of certain *ARSs* [Lei et al, 1996], precipitating genome instability in yeasts. More recently, it was found that in *Xenopus* extracts and mammalian cells, excess chromatin-bound MCM2-7 complexes occupy dormant or "backup" origins that are activated under conditions of replication stress, compensating for stalled or disrupted primary replication forks. A depletion of these backup licensed origins could potentially lead to incomplete DNA replication and genetic aberrations [Ge et al, 2007; Ibarra et al, 2008].

In previous work, Shima *et al* found that a hypomorphic allele of mouse *Mcm4* (*Mcm4*^{Chaos3}) caused high levels of GIN and extreme mammary cancer susceptibility. The ethylnitrosourea (ENU)-induced *Mcm4*^{Chaos3} point mutation changed PHE to ILE at residue 345 (Phe345Ile). This amino acid is conserved across diverse eukaryotes and is important for interaction with other MCMs. Subsequently, it was reported that mice containing 1/3 the normal level of MCM2 succumbed to lymphomas at a very young age, and had diverse stem cell proliferation defects [Pruitt et al, 2007]. These mice also had 27% reduced levels of MCM7 protein. Working with human cultured cells, Ge *et al* reported that *Mcm5* knockdown decreased the amount of chromatin-bound MCM2,3,6

and 7 (total MCM2 was unchanged; levels of the others were not reported [Ge et al, 2007], and Ibarra *et al* found that RNAi-mediated knockdown of *Mcm2* or *Mcm3* caused a drastic decrease in the levels of all other MCM2-7 proteins. They speculated that elimination of a single MCM component destabilized the hexamer [Ibarra et al, 2008]. In both cases, GIN and proliferation defects were observed under conditions of chemically-induced replicative stress. These studies suggest that in addition to the biochemical consequences of the Phe345Ile alteration, the overall decrease in MCM levels might contribute to GIN and cancer in *Mcm4*^{Chaos3} mice. It also implies that relatively modest decreases in any of the MCMs may be sufficient to cause these and other developmental defects, such as those that affect stem cells in MCM2-deficient mice [Pruitt et al, 2007].

In my first and second year of funding, I showed that genetically-induced reductions of MCM levels, achieved by breeding combinations of MCM2-7 alleles, can have severe consequences for embryonic development and cancer susceptibility in mice. Additionally, I found that heterozygosity for *Mcm3* rescued many of these defects. Consistent with a role in MCM nuclear export possessed by the yeast Mcm3 ortholog, the phenotypic rescues correlated with increased chromatin-bound MCMs, and a lower degree of nuclear MCM2 reduction during S phase. The results were published this year in *PLoS Genetics.*

Body of annual summary:

Based on my proposal and statement of works, I list all of the tasks and the progress nade towards them during this year.

Task 1: Generate MCMs hypomorphic mice and prove that heterozygosity for Mcm2, Mcm6 or Mcm7 in an Mcm4$^{Chaos3/Chaos3}$ background causes partial synthetic lethality, severe growth defects and (for Mcm2) dramatically accelerated cancer onset.

As outlined above, previous studies showed that reductions of particular MCMs in cells or mice reduces the levels of other MCMs, causing GIN, cancer, and developmental defects. However, the reduction in MCM levels required to precipitate these consequences, and whether there is a threshold effect, is unclear. For example, the early-

onset lymphoma and stem cell deficiency phenotypes in mice with a hypomorphic MCM2 allele occur in homozygotes having ~35% of WT MCM2 protein, but not heterozygotes (62% of WT) [Pruitt, 2007]. However, our mutant mice heterozygous for Mcm genes appear to have an intermediate phenotype, in that they are cancer susceptible with a longer latency and lower penetrance. Our data in the *PLoS Genetics* paper are suggestive that a potential gradient of susceptibility, or a critical threshold of MCM levels is required to avoid cancer outcomes. Another indication of a possible threshold effect is that C3H-*Mcm4*$^{Chaos3/Chaos3}$ mice are developmentally normal, but *Mcm4*$^{Chaos3/-}$ animals die *in utero* or neonatally [Shima, 2007].

To further explore the consequences of incremental MCM reductions on viability and cancer, we crossed the *Mcm4*^{Chaos3} and gene trap alleles into the same genome. In the case of *Mcm2*, there was a striking and highly significant shortfall of *Mcm4*$^{Chaos3/Chaos3}$ *Mcm2*^{Gt}/+ offspring at birth (Fig. 1a). Heterozygosity for *Mcm2*^{Gt} itself was not haploinsufficient, as indicated by Mendelian transmission of *Mcm2*^{Gt} in crosses of heterozygotes to WT (119/250; $c^2 = 0.448$). These results demonstrate that there is a synthetic lethal interaction between *Mcm4*^{Chaos3} and *Mcm2*^{Gt} that is related to MCM2 levels. Another obvious phenotype was that the surviving *Mcm4*$^{Chaos3/Chaos3}$ *Mcm2*^{Gt}/+ offspring were severely growth retarded; males weighed ~50% less than *Mcm4*$^{Chaos3/Chaos3}$ siblings (Fig. 1b).

The synthetic interaction between *Mcm4*^{Chaos3} and *Mcm2*^{Gt} might be specific, or it may reflect a general consequence of reduced replication licensing (and consequent elevated replication stress). We therefore tested whether hemizygosity for *Mcm3*, *Mcm6* or *Mcm7* would also cause synthetic phenotypes in an *Mcm4*$^{Chaos3/Chaos}$ background. The *Mcm4*$^{Chaos3/Chaos3}$ *Mcm6*^{Gt}/+ genotype caused highly penetrate embryonic lethality; only 10% of the expected number of such animals survived to birth (Fig. 1a). The *Mcm4*$^{Chaos3/Chaos3}$ *Mcm7*^{Gt}/+ genotype caused both embryonic and postnatal lethality. The number of liveborns was 50% of the expected value, but only 3% of the expected value survived to weaning (Fig. 1a). Additionally, as with *Mcm2*, hemizygosity for *Mcm6*^{Gt} and *Mcm7*^{Gt} in the *Mcm4*$^{Chaos3/Chaos3}$ background caused growth retardation (Fig. 1b). The decrease in male weight was ~20% and ~80% respectively, compared to *Mcm4*$^{Chaos3/Chaos3}$ siblings at the oldest age measured (*Mcm4*$^{Chaos3/Chaos3}$ *Mcm7*$^{Gt/+}$ animals

died before wean, so the oldest weights were taken at 10 dpp). In contrast to the synthetic phenotypes with *Mcm2, 6* and *7*, there was no significant decrease in viability or weight in $Mcm4^{Chaos3/Chaos3}$ $Mcm3^{Gt/+}$ mice. This seeming inconsistency is addressed in the following section.

To determine if hemizygosity of *Mcm2* would impact lifespan or tumor susceptibility in $Mcm4^{Chaos3}$ homozygotes, $Mcm4^{Chaos3/Chaos3}$ $Mcm2^{Gt}$/+ mice (N=26) were aged and monitored. They began dying at 2 months of age, and all were dead (or sacrificed when they appeared moribund) by 7 months (Fig. 2a). Gross necropsy and histopathological analyses revealed or suggested lymphomas/leukemias in 20 of these animals. Six of these had chest tumors that were likely thymic lymphomas. Consistent with previous studies [Shima, 2007] most $Mcm4^{Chaos3/Chaos3}$ mice hadn't yet succumbed from tumors or other causes by 12 months of age. These data show clearly that removing a half dose of MCM2 from $Mcm4^{Chaos3/Chaos3}$ cells is sufficient to produce greatly elevated cancer predisposition. MEFs of this genotype had 47.4% the amount of *Mcm2* mRNA as $Mcm4^{Chaos3/Chaos3}$ cells (data not shown), which already had a 38% reduction compared to WT (data not shown). Thus, *Mcm2* RNA was reduced to ~29% of WT. To determine if elevated GIN might be responsible for the cancer susceptibility phenotype, we measured erythrocyte MN. Whereas the percentage of micronucleated RBCs in $Mcm4^{Chaos3/Chaos3}$ mice was 4.18 $\pm$ 0.26 (mean$\pm$ SEM, N=12), $Mcm4^{Chaos3/Chaos3}$ $Mcm2^{Gt}$/+ mice averaged 5.85 $\pm$ 0.47 (N=16), indicating a synergistic increase ($P< 0.01$) (Fig. 3d).

Task 2: Rescue of phenotypic defects in $Mcm4^{Chaos3/Chaos3}$ and $Mcm4^{Chaos3/Chaos3}$ $Mcm2^{Gt}$/+ mice by reducing Mcm3 genetic dosage.

The data reported here and elsewhere [Shima, 2007; Pruitt, 2007] support a model where phenotypic severity is proportionally related to MCM concentrations. However, our genetic experiments uncovered one notable exception: hemizygosity for *Mcm3* did not cause severe haploinsufficiency phenotypes (increased lethality and decreased weight) as did *Mcm2/6/7* in the $Mcm4^{Chaos3/Chaos3}$ background (Fig. 1a). Since extreme reductions of MCM3 in cultured human cells caused GIN and cell cycle arrest [Ibarra, 2008], the absence of synthetic effects with Mcm^{Chaos3} led us to hypothesize that either mice are more tolerant to lower levels of this particular MCM, or that MCM3 is present

in a stoichiometric excess compared to the other MCMs. To explore these issues we performed additional phenotype analyses, and also sought to uncover potential effects of MCM3 reduction by reducing other MCMs simultaneously.

Strikingly, rather than exacerbating the synthetic lethality in *Mcm4*$^{Chaos3/Chaos3}$ *Mcm2*$^{Gt/+}$ mice, *Mcm3*^{Gt} heterozygosity significantly *rescued* their viability (Fig. 3a). Not only was viability rescued, but also growth (weight) of *Mcm4*$^{Chaos3/Chaos3}$ *Mcm2*$^{Gt/+}$ *Mcm3*^{Gt} survivors compared to *Mcm4*$^{Chaos3/Chaos3}$ *Mcm2*$^{Gt/+}$ animals produced from the same matings (Fig. 3b). *Mcm3* hemizygosity also significantly rescued the near 100% lethality of *Mcm4*$^{Chaos3/-}$ animals (nearly 6 fold increased viability), and doubled the viability of *Mcm4*$^{Chaos3/Chaos3}$ *Mcm6*$^{Gt/+}$ mice (Fig. 3a). Rescue of *Mcm4*$^{Chaos3/Chaos3}$ *Mcm7*$^{Gt/+}$ was not observed (not shown).

The rescue of the reduced growth phenotype by *Mcm3* hemizygosity led us to evaluate the proliferation of compound mutant cells. Whereas *Mcm4*$^{Chaos3/Chaos3}$ and *Mcm4*$^{Chaos3/Chaos3}$ *Mcm3*$^{Gt/+}$ primary MEFs proliferated at identical rates, *Mcm4*$^{Chaos3/Chaos3}$ *Mcm2*$^{Gt/+}$ MEFs showed a severe growth defect beginning ~5 days in culture (Fig. 3c). As with whole animals, MEF growth was partially but significantly rescued by *Mcm3* hemizygosity.

Since the *Mcm4*^{Chaos3} and *Mcm2*^{Gt} alleles causes elevated GIN (micronuclei in RBCs), we considered the possibility that the *Mcm3* rescue effect might be related to an attentuation of GIN. Accordingly, we measured MN levels in *Mcm4*$^{Chaos3/Chaos}$ mice with different combinations of other Mcm mutations. As shown in Fig. 3d, hemizygosity for *Mcm2* and *Mcm7* caused a significant elevation in MN levels, unlike *Mcm3*. However, the increased MN in *Mcm4*$^{Chaos3/Chaos3}$ *Mcm2*$^{Gt/+}$ was not rescued by *Mcm3* hemizygosity. This suggests that the synthetic lethality and mouse/cell growth defects are not related to GIN *per se*. However, in the course of measuring MN in enucleated peripheral blood cells, we noticed that the ratio of CD71+/CD71- cells was significantly higher in both *Mcm4*$^{Chaos3/Chaos3}$ *Mcm2*$^{Gt/+}$ and *Mcm4*$^{Chaos3/Chaos3}$ *Mcm7*$^{Gt/+}$ mice (3.3 and 6.2 fold, respectively; Fig. 3e). This increase in the ratio of reticulocytes (erythrocyte precursors) to mature RBCs is characteristic of anemia. Hemizygosity for *Mcm3*, which alone had no effect on CD71 ratios of Chaos3 mice, corrected this abnormal phenotype in *Mcm4*$^{Chaos3/Chaos3}$ *Mcm2*$^{Gt/+}$animals.

Because MCM2-depleted mice were reported to have stem cell defects {Pruitt, 2007 #2433}, and *Mcm4*$^{Chaos3/Chaos3}$ *Mcm#*$^{Gt/+}$ mice had clear developmental abnormalities, we examined the efficiency of reprogramming mutant MEFs into induced pluripotent stem cells (iPS). The efficiency was quantified by counting iPS-like colony formation and cell number on primary plates of MEFs infected with reprogramming vectors. Both gave similar results. Those reprogrammed iPS cultures cells were confirmed with flow cytometric analysis of with the stemness markers SSEA1 and LIN28. *Mcm4*$^{Chaos3/Chaos3}$ *Mcm2*$^{Gt/+}$ cells were severely compromised in the ability to form iPS cells compared to *Mcm4*$^{Chaos3/Chaos3}$ (~ 200 fold less efficient; Fig. 3f). However, additionally reducing *Mcm3* by 50% increased iPS formation from both *Mcm4*$^{Chaos3/Chaos3}$and *Mcm4*$^{Chaos3/Chaos3}$ *Mcm2*$^{Gt/+}$ MEFs by ~2.5 and 10 fold, respectively.

Finally, we found that reduced MCM3 levels could rescue the cancer susceptibility of two different Chaos3 models. As shown earlier (Fig. 2), *Mcm4*$^{Chaos3/Chaos3}$ *Mcm2*$^{Gt/+}$ mice were highly cancer-prone with an average latency of <4 months. When a dose of Mcm3 was removed from mice of this genotype, lifespan was extended dramatically, in both males (latency of >10 months) and females (latency of >8 months), as a consequence of delayed cancer onset (Fig. 4a). Additionally, hemizygosity of *Mcm3* delayed the onset of mammary tumorigenesis in *Mcm4*$^{Chaos3/Chaos3}$ females by about 3-4 months (Fig. 4b). However, although *Mcm3* hemizygosity rescued viability of *Mcm4*$^{Chaos3/Gt}$ mice (Fig. 3a), these animals remained cancer prone with a shorter latency (by ~ 6 months) and different spectrum (primarily lymphomas) than *Mcm4*^{Chaos3} homozygotes.

Key Research Accomplishments

- Decreased MCM2-7 in *Mcm4*$^{Chaos3/Chaos3}$ cells is due to lower Mcm2-7 mRNA levels, which are regulated posttranscriptionally.

- Decreased Mcm gene dosages cause elevated chromosomal instability in mice

- MCM2-7 are essential for viability of all invertebrate species investigated, and I confirmed that this is true in mammals (note: Mcm5 was not studied).

- Heterozygosity for *Mcm2*, *Mcm6* or *Mcm7* causes partial synthetic lethality, severe growth defects and (for *Mcm2*) early tumor predisposition in $Mcm4^{Chaos3/Chaos3}$ mice.

- There is a minimal MCM threshold that is required for embryonic viability, as demonstrated by the synthetic lethalities I observed when combining homozygosity of $Mcm4^{Chaos3}$ with $Mcm2^{Gt}$, $Mcm6^{Gt}$ or $Mcm7^{Gt}$ heterozygosity, but not in the heterozygous single mutants. Whatever the exact mechanistic cause of these phenotypes, it is clear that the phenotypes are related to reduction of one or more MCMs below a threshold level that is somewhere below 50%.

- Reductions in MCM3 can rescue various phenotypes lends insight into dynamics and regulation of mammalian DNA replication. Our data, lend support to the idea that subcellular re-distribution of MCMs is a very important process in mammals. MCM3 may be a potential drug target for manipulating MCM distribution in cancer cells.

Reportable Outcomes:

Manuscript:

Incremental genetic perturbations to MCM2-7 expression and subcellular distribution reveal exquisite sensitivity of mice to DNA replication stress. **Chuang CH**, Wallace MD, Abratte C, Southard T, Schimenti JC. PLoS Genet. 2010 Sep 9;6(9)

Student Work-In-Progress Presentations:

Incremental genetic perturbations to MCM2-7 expression and subcellular distribution reveal exquisite sensitivity of mice to DNA replication stress. Chen-Hua Chuang and John C. Schimenti. Field of Molecular and Integrative Physiology Student Work-In-Progress. Mar 2010 (orally presented)

Meeting Presentations:

Incremental genetic perturbations to MCM2-7 expression and subcellular distribution reveal exquisite sensitivity of mice to DNA replication stress. Chen-Hua Chuang, Marsha Wallace and John C. Schimenti. The Sixth Annual Center for Vertebrate Genomics Symposium. May 2010 (poster presented)

Incremental genetic perturbations to MCM2-7 expression and subcellular distribution reveal exquisite sensitivity of mice to DNA replication stress. Chen-Hua Chuang, Marsha Wallace and John C. Schimenti. Mechanisms & Models of Cancer meeting. Cold Spring Harbor Laboratory NY. September 2010. (poster presented)

Conclusions

Mutations causing replication stress can lead to genomic instability (GIN). In vitro studies have shown that drastic depletion of the MCM2-7 DNA replication licensing factors, which form the replicative helicase, can cause GIN and cell proliferation defects that are exacerbated under conditions of replication stress. To explore the effects of incrementally attenuated replication licensing in whole animals, we generated and analyzed the phenotypes of mice that were hemizygous for Mcm2, 3, 4, 6, and 7 null alleles, combinations thereof, and also in conjunction with the hypomorphic $Mcm4^{Chaos3}$ cancer susceptibility allele. $Mcm4^{Chaos3/Chaos3}$ embryonic fibroblasts have ~40% reduction in all MCM proteins, coincident with reduced Mcm2-7 mRNA. Further reductions of Mcm2, 6, or 7 in this background caused various phenotypes including synthetic lethality, growth retardation, decreased cellular proliferation, and early onset cancer. Remarkably, heterozygosity for Mcm3 rescued many of these defects. Consistent with a role in MCM nuclear export possessed by the yeast Mcm3 ortholog, the phenotypic rescues correlated with increased chromatin-bound MCMs, and a lower degree of nuclear MCM2 reduction during S phase. The genetic, molecular and phenotypic data demonstrate that relatively minor quantitative alterations of MCM

expression, homeostasis or subcellular distribution, can have diverse and serious consequences for health, viability and cancer susceptibility. The results support the notion that the normally high levels of MCMs in cells are needed not only for activating the basal set of replication origins, but also "backup" origins that are recruited in times of replication stress to ensure complete replication of the genome.

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Supporting Data

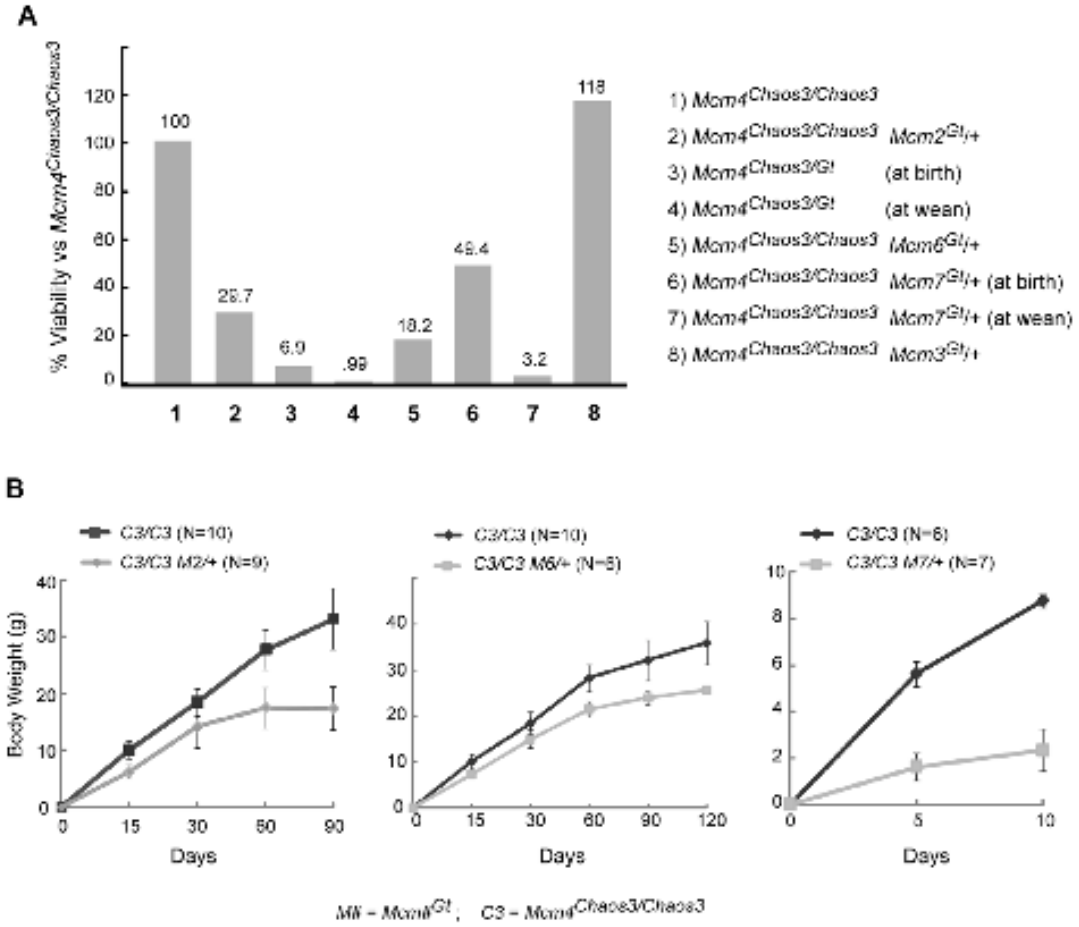


Figure 1. Synthetic lethality and growth retardation between *Mcm4*^{Chaos3} and *Mcm2*, *Mcm6* and *Mcm7*. (A) Graphed are viability data from crosses. Unless otherwise indicated, the values represent expected proportions of indicated genotypes that were present at wean. (B) Weights of surviving animals are graphed over time. SEM bars are shown.

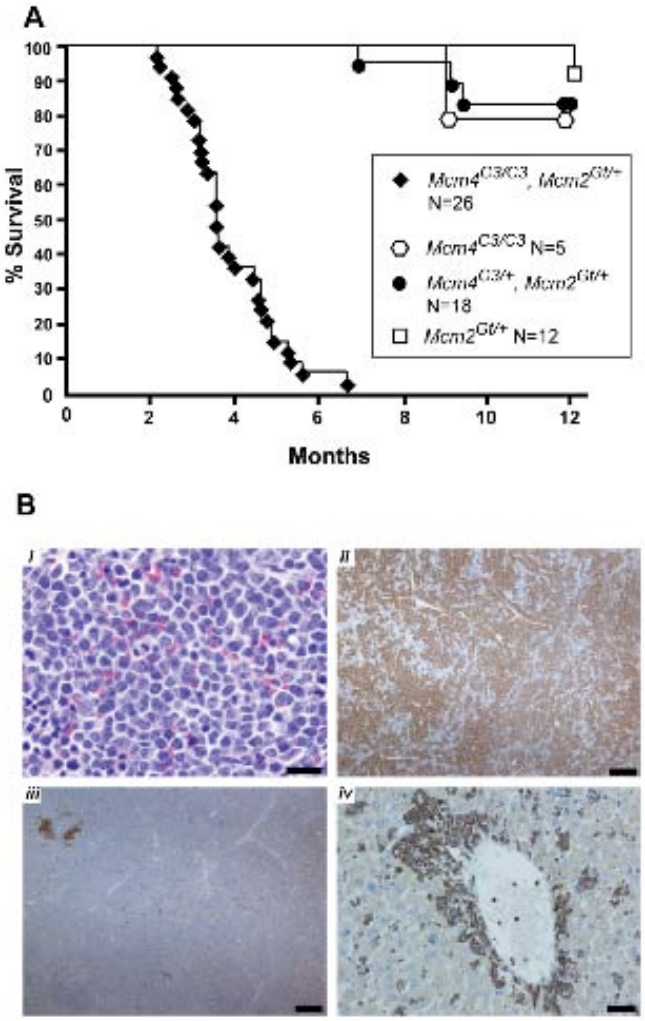
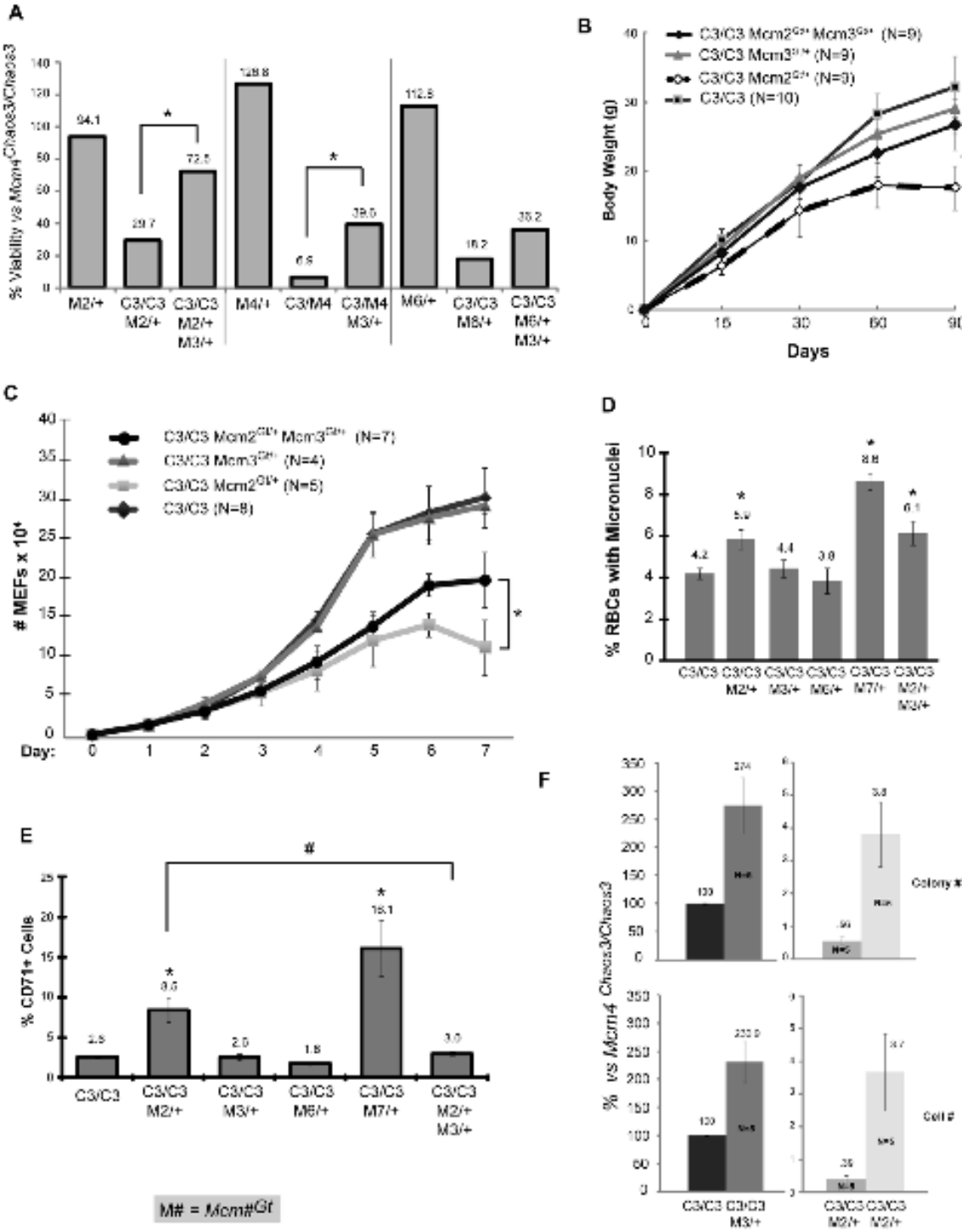


Figure 2. Premature morbidity and cancer susceptibility in *Mcm4*$^{Chaos3/Chaos3}$ *Mcm2*$^{Gt/+}$ mice. (A) Kaplan-Meier survival plot of the indicated genotypes. Animals of both sexes are combined. ‘‘C3’’ = *Chaos3*. (B) Spleen and liver histopathology of a *Mcm4*$^{Chaos3/Chaos3}$ *Mcm2*$^{Gt/+}$ male diagnosed with T cell leukemic lymphoma. i. H&E stained spleen. Neoplastic cells have abundant cytoplasm, 1–2 nucleoli and a high mitotic rate, consistent with lymphoblastic lymphoma. Bar = 20 mm. ii. Neoplastic cells in spleen demonstrate immunoreactivity with anti-CD3 (brown; immunoperoxidase staining with DAB chromogen & hematoxalin counterstain), indicating T lymphocytes. Bar = 200mm. iii. In spleen, immunoreactivity (brown) with anti- PAX-5 (a B cell marker) is limited to follicular remnants and scattered individual cells. Bar = 200 mm. iv. In liver, neoplastic cells surround central veins and expand sinusoids and demonstrate immunoreactivity (brown) with the anti-CD3 T lymphocyte marker. Bar = 50 mm.


A
% Viability vs Mcm4Chaos3/Chaos3
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C3/C3 M2/+
C3/C3 M2/+ M3/+

Figure 3. Rescue of phenotypes by *Mcm3* hemizygosity. (A) Heterozygosity for $Mcm3^{Gt}$ rescues the low viability of various mutant genotypes (asterisk indicates significance at P,0.05 by FET). (B) Male body weights of combination mutant mice. The weights of $Mcm4^{Chaos3/Chaos3}$ $Mcm2^{Gt/+}$ $Mcm3^{Gt/+}$ mice are significantly higher (asterisk; P,0.01, Student's t-test) at 90 days than $Mcm4^{Chaos3/Chaos3}$ $Mcm2^{Gt/+}$ mice. Error bars represent SEM. (C) $Mcm4^{Chaos3/Chaos3}$ $Mcm2^{Gt/+}$ MEF proliferation defects are partially rescued by Mcm3 hemizygosity. The effect is significant after 6 days in culture (P,0.05, Student's t-test; Error bars represent SEM). (D) Micronucleus levels in $Mcm4^{Chaos3/Chaos3}$ mice bearing additional gene trap alleles. At least 5 males were analyzed for each genotype. Error bars represent SEM. Asterisk indicates P,0.05 (student's t-test.) compared to $Mcm4^{Chaos3/Chaos3}$ alone. (E) CD71+ reticulocyte ratios in mutant male mice. At least 5 animals were analyzed from each class. The samples are identical to those in ''D''. All scored cells were anucleate peripheral blood cells Error bars represent SEM. Asterisks and ''#'' indicate P,0.05 (Student's t-test) when compared to $Mcm4^{Chaos3/Chaos3}$ and $Mcm4^{Chaos3/Chaos3}$ $Mcm2^{Gt/+}$cohorts, respectively. (F) *Mcm2* hemizygosity decreases efficiency of reprogramming $Mcm4^{Chaos3/Chaos3}$ MEFs into iPS cells, and *Mcm3* hemizygosity significantly increases reprogramming efficiency. Two methods of quanitifying reprogramming were used as described in Materials and Methods. ''Cell number'' refers flow cytometric quantification of LIN28/SSEA1 double positive cells from primary cultures of reprogrammed MEFs. Relative reprogramming efficiencies were normalized to $Mcm4^{Chaos3/Chaos3}$ MEFs (considered to be 100%). Error bars represent SEM. All samples within quantification class are significantly different from one another (P,0.05, Student's t-test). C3 = $Mcm4^{Chaos3}$; M = *Mcm.*

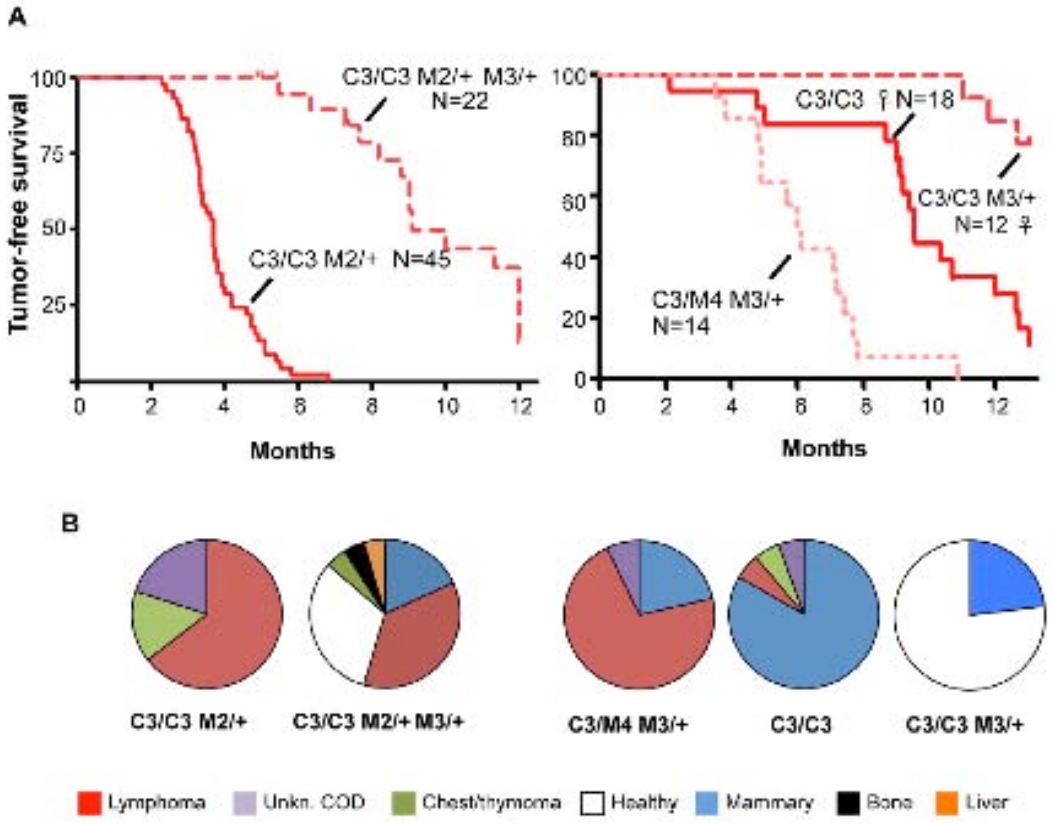

Figure 4. Inhibition of Chaos3 cancers by MCM3 reduction. (A) Kaplan-Meier graphs of cohorts of the indicated genotypes. C3 = *Mcm4*^{Chaos3}; M# = *Mcm#*^{Gt}. In the left panel, the experiment was terminated at 12 months, with ,1/3 animals tumor-free and healthy at the time (see ‘‘B’’). Unless otherwise indicated, the cohorts contained both sexes. (B) Pie charts of cancer types in mice from ‘‘A.’’ COD=cause of death; Unkn=unknown. Classification of cancer types was assigned during necropsy, not from histological analysis.