HHS Public Access

Author manuscript

Oncogene. Author manuscript; available in PMC 2015 January 10.

Published in final edited form as:

Oncogene. 2014 July 10; 33(28): 3688–3695. doi:10.1038/onc.2013.339.

Role of DNA damage response pathways in preventing carcinogenesis caused by intrinsic replication stress

Marsha D. Wallace[1,2], **Teresa L. Southard**[1,3], **Kerry J. Schimenti**, and **John C. Schimenti**[1,2,4]

[1]Department of Biomedical Sciences, Cornell University, Ithaca, NY 14853, USA

[2]Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY 14853, USA

[3]Section of Anatomic Pathology, Cornell University, Ithaca, NY 14853, USA

[4]Center for Vertebrate Genomics, Cornell University, Ithaca, NY 14853, USA

Abstract

Defective DNA replication can result in genomic instability, cancer, and developmental defects. To understand the roles of DNA damage response (DDR) genes on carcinogenesis in mutants defective for core DNA replication components, we utilized the *Mcm4*$^{Chaos3/Chaos3}$ ("*Chaos3*") mouse model which, by virtue of an amino acid alteration in MCM4 that destabilizes the MCM2-7 DNA replicative helicase, has fewer dormant replication origins and an increased number of stalled replication forks. This leads to genomic instability and cancer in most *Chaos3* mice. We found that animals doubly mutant for *Chaos3* and components of the ATM double strand break response pathway (*Atm*, *p21*/*Cdkn1a*, *Chk2*/*Chek2)* had decreased tumor latency and/or increased tumor susceptibility. Tumor latency and susceptibility differed between genetic backgrounds and genders, with females demonstrating an overall greater cancer susceptibility to *Atm* and *p21* deficiency than males. ATM deficiency was semilethal in the *Chaos3* background and impaired embryonic fibroblast proliferation, suggesting that ATM drug inhibitors might be useful against tumors with DNA replication defects. Hypomorphism for the 9-1-1 component *Hus1* did not affect tumor latency or susceptibility in *Chaos3* animals, and tumors in these mice did not exhibit impaired ATR pathway signaling. These and other data indicate that under conditions of systemic replication stress, the ATM pathway is particularly important both for cancer suppression and viability during development.

Keywords

DNA replication; DNA damage checkpoints; minichromosome maintenance (MCM) proteins; 9-1-1; MCM2-7 helicase; cancer; replication stress

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Corresponding Author: John Schimenti, Ph.D. Cornell University College of Veterinary Medicine Ithaca, NY 14853 jcs92@cornell.edu Tel: 607-253-3636 FAX:.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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INTRODUCTION

Genomic studies have shown that many individual genes are spontaneously mutated or misregulated at low frequencies in cancers, but together comprise disruptions in a few key pathways [1-3]. Alterations in DNA checkpoint and repair pathways are particularly significant. The *BRCA1* and *BRCA2* genes are altered in over 1/3 of serous ovarian and basal type breast cancer cases, highlighting the importance of the homologous recombination (HR) pathway of DSB repair [2, 4]. During HR repair, DSBs are bound by the MRN (MRE11/ RAD50/NBS1) damage sensor complex, the Ataxia Telangiectasia Mutated (ATM) serine/ threonine kinase becomes activated *via* autophosphorylation and, in conjunction with mediator proteins such as BRCA1, signals to downstream transducer and effector kinases to elicit checkpoint and repair responses (reviewed by [5, 6]). DDR pathways are responsible for helping maintain genomic stability and suppressing tumorigenesis [7]. To control cell cycle progression under conditions of DNA damage or replication stress, DDR genes also target components of the DNA replication machinery, including the Minichromosome maintenance 2-7 (MCM2-7) replicative helicase complex. MCM2 is a direct target of ATR (ATM and RAD9-related), and MCM3 is a target of ATM [8, 9].

Whereas the relationship between defects in various DNA repair systems to cancer is well studied, this is not the case for DNA replication - the process during which the greatest opportunity for mutations exists. Accumulating evidence points to associations between deficiencies of the core DNA replication machinery and cancer. For example, mice bearing mutations in the proofreading functions of the major replicative polymerases δ and ε exhibit mutator phenotypes and cancer predisposition [10-13]. Furthermore, Pol ε is frequently mutated in human colorectal cancers [14]. In addition to DNA polymerases, mutations in components of the pre-replication complex (pre-RC) have been linked to cancer susceptibility. These complexes assemble at replication origins during G1 phase (but not during S phase), and a subset of these components constitute the CDC45/MCM2-7/GINS (CMG) replicative helicase complex that unwinds DNA in front of the replisome during S phase [15-17]. The highly conserved MCM2-7 heterohexameric complex is an essential component of the pre-RC and constitutes the core of the replicative helicase (reviewed in [18]). Whereas *Mcm2-7* are essential genes, hypomorphic alleles in mice cause GIN, cancer susceptibility, and cell proliferation defects [19-21], as does overexpression and haploinsufficiency [22-24].

To better understand the *in vivo* impact of the DDR on cancer incidence and tumor latency under conditions of increased replication stress, we utilized the $Mcm4^{Chaos3/Chaos3}$ ("*Chaos3*") mouse model that bears a single amino acid mutation in MCM4 (Phe345Ile). *Chaos3* mice have dramatically elevated GIN, and depending on the strain background, *Chaos3* mice are predisposed to various cancers including mammary tumors, histiocytic sarcoma, lymphoma, and bone tumors [19, 24, 25]. The *Chaos3* mutation destabilizes the MCM2-7 helicase by disrupting MCM4:MCM6 interaction, somehow triggering a post-transcription decrease in the levels of all MCM2-7 mRNA and proteins [24-26]. This reduces the number of dormant replication origins available as backups to replicate DNA near stalled replication forks. These defects contribute to elevated chromosome breakage and segregation defects in *Chaos3* mouse embryonic fibroblasts (MEFs) [25]. Studies of diploid *S.*

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cerevisiae engineered to carry the identical *Chaos3* amino acid change in *MCM4* indicated that the defective helicase causes replication fork collapse, leading to DSBs that require repair by HR [27]. Consistent with replication fork damage leading to DSBs that trigger HR, *Chaos3* MEFs have increased levels of RAD51 and BLM foci [25]. Additionally, they exhibit upregulation of p53/TRP53 and p21, indicative that cell cycle checkpoint responses are activated in these cells [28].

DDR pathways aid proper DNA replication by stabilizing transiently stalled forks to prevent the dissociation of replisome components, promoting replication restart, and facilitating fork movement on difficult-to-replicate templates. The ATM pathway is activated in response to DSBs, while the ATR pathway is activated by RPA-coated ssDNA at stalled replication forks. However, there is clearly overlap and cross-signaling between the pathways [29]. Failure to safeguard genome integrity during DNA replication is associated with increased cancer predisposition [30, 31].

Despite intact DDR pathways, the elevated GIN in *Chaos3* mice eventually result in recurrent segmental copy number alterations that apparently drive carcinogenesis, with a mean latency of 12 months in the case of mammary tumors [19, 32]. Here, we exploit this model, in conjunction with mutations in DDR genes, to better understand cellular responses to endogenous replication stress on an organismal level and the impact on carcinogenesis *in vivo*.

RESULTS

We generated *Chaos3* mice that were also deficient for the ATM pathway (*Atm* or *Chk2*), ATR pathway (*Hus1*), or the cyclin-dependent kinase inhibitor p21 that is downstream of both signaling pathways (Figure 1a). At the time of crossing, *Mcm4*^{Chaos3} (abbreviated hereafter as *Mcm4*^{C3}, or just "C3" in the figures) was congenic in strain C3H/HeBFeJ (C3H), but the other mutations were on different strain backgrounds (see Materials and Methods). C3H-*Mcm4*$^{C3/C3}$ females develop exclusively mammary adenocarcinomas, but males of that genotype and strain background were not reported to be tumor prone [19]. In a mixed genetic background however, other tumor types in females arise (including lymphoma and histiocytic sarcoma) [19, 25]. Additionally, males of mixed strain background were also found to be tumor prone, though the sample size was small and most mice were not aged past 14 months [19]. Here, mutant and control mice of both sexes were aged for eighteen months or until they showed signs of disease, after which a complete necropsy was performed. The results for each set of compound mutants are described below.

ATM deficiency impacts viability, cell proliferation, tumor latency, and tumor susceptibility of *Chaos3* mice

Activation of ATM in response to DSBs triggers several key downstream events. It directly phosphorylates H2AX residing at (and near) the site of DNA breaks. It also phosphorylates downstream targets such as CHK2 to activate the DNA damage checkpoint, leading to cell cycle delay or apoptosis (Figure 1a) [33]. ATM deficiency is associated with the development of lymphomas and leukemias in humans and mice. *Atm*$^{-/-}$ mice develop thymic lymphomas at 2-4 months of age [34, 3536, 37]. We analyzed 648 weaned offspring from mouse crosses

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bearing *Atm* and *Mcm4* genotype combinations, but only 25 of the expected 65 double homozygotes were obtained (P=6.03*10-6) (Supplementary Figure 1a). To investigate the nature of the semi-lethal phenotype, we examined mid-late gestation embryos from timed matings that would yield double mutants and controls. *Mcm4*$^{C3/C3}$ *Atm*$^{-/-}$ embryos were present at expected ratios at and prior to E15.5 (χ^2 P=0.97 and P=0.65, respectively; Supplementary Table 1), but at E18.5 they were smaller than control littermates and/or apparently dead or dying (Supplementary Figure 1b). To better understand the basis for the embryonic lethality at the cellular level, cell proliferation assays were conducted on MEFs of various genotypes. Complete absence of ATM dramatically decreased growth rate regardless of *Chaos3* genotype, but *Atm* heterozygosity also reduced proliferation in *Mcm4*$^{C3/C3}$ but not *Mcm4*$^{C3/+}$ MEFs (Supplementary Figure 1c). The results suggest that reduced cell proliferation is not entirely responsible for the synthetic lethality of *Atm*$^{-/-}$ *Mcm4*$^{C3/C3}$ embryos.

The early-onset lymphoma susceptibility caused by complete ATM deficiency obscured the detection of potential effects on mammary tumorigenesis. Nearly all *Mcm4*$^{C3/C3}$ *Atm*$^{-/-}$ and *Mcm4*$^{C3/+}$*Atm*$^{-/-}$ mice succumbed to lymphoma at ~2-4 months of age (Supplementary Table 2, Supplementary Table 3), compared to much longer tumor latency in *Mcm4*$^{C3/C3}$ animals (Figure 1b, Supplementary Table 2). While several studies have reported that heterozygosity for *Atm* null mutations (alone or in conjunction with *Apc*^{Min} or *p53* mutations) had no effect on mouse spontaneous tumor frequencies [38-41], a role for ATM in mammary tumor prevention was evident in *Mcm4*$^{C3/C3}$ *Atm*$^{+/-}$ and *Mcm4*$^{C3/+}$ *Atm*$^{+/-}$ animals. Females of these genotypes had median mammary tumor latencies of 10.95 and 9.3 months, respectively, both significantly shorter than *Mcm4*$^{C3/C3}$ alone (14.95 months; respectively: LRMCT P=0.001, P=0.0027; GBWT P=0.0031, P=0.0005). *Mcm4*$^{C3/C3}$ *Atm*$^{+/-}$ males neared statistical significance for decreased tumor latency (LRMCT P=0.0751; GBWT P=0.0729), and *Mcm4*$^{C3/+}$ *Atm*$^{+/-}$ male tumor latency was similar to *Mcm4*$^{C3/C3}$ alone (LRMCT P=0.472; GBWT P=0.4339) (Figure 1b, Supplementary Table 2).

Heterozygosity for *Atm* had a striking effect on the spectrum of tumors in mice bearing the *Chaos3* allele. Whereas histiocytic sarcoma was prevalent in *Mcm4*$^{C3/C3}$ mice of mixed strain background (41% in females; 60% in males), its incidence declined in *Mcm4*$^{C3/C3}$ *Atm*$^{+/-}$ mice (5% in females and males). Meanwhile, lymphoma and other cancer types increased (FET P=0.0093; P=0.0001; Figure 1c). The tumor spectrum also differed between genotypes and gender. Nearly all females (98%) of the *Mcm4*$^{C3/C3}$, *Mcm4*$^{C3/C3}$ *Atm*$^{+/-}$, and *Mcm4*$^{C3/+}$ *Atm*$^{+/-}$genotypes developed cancer by the end of the study, vs. 72% of males of the same genotypes (FET P=0.0001). In particular, *Mcm4*$^{C3/+}$ *Atm*$^{+/-}$ females were far more susceptible to cancer than males (FET P=0.0223; Figure 1b, Figure S1). The incidence of mammary tumors was also high in females of these genotypes, but absent in males, influencing overall differences in tumor spectrum.

Chk2 deficiency impacts tumor latency in *Chaos3* females and susceptibility in males

CHK2 is a phosphorylation target of ATM and serves as a downstream effector of the DSB checkpoint response (Figure 1a)[42]. In some circumstances, CHK2 can also be activated by

ATR [43, 44]. When activated, the CHK2 kinase can phosphorylate p53, protecting it from MDM2-catalyzed ubiquitination and degradation [42]. Other targets include BRCA1, which is involved in HR repair [4546]. In sum, CHK2 activation can lead to DNA repair, cell cycle arrest, or apoptotic cell death. Unlike *Atm* or *p53*, several studies have shown that *Chk2* null mice do not spontaneously develop tumors [4748-51]. However, $Mcm4^{C3/C3}$ $Chk2^{-/-}$ females had decreased tumor latency compared to $Mcm4^{C3/C3}$ alone in a mixed C3H x B6 background (LRMCT P=0.0189, GBWT P=0.027; Figure 2, Supplementary Table 2). Interestingly, although the overall tumor incidence was identical, the fraction of mammary tumors in $Mcm4^{C3/C3}$ $Chk2^{-/-}$ females rose significantly from 15% to 50% (Supplementary Figure 2; FET P=.002). $Mcm4^{C3/C3}$ $Chk2^{-/-}$ males did not have a statistically different latency compared to $Mcm4^{C3/C3}$ alone, and their cancer incidence was similar to females of the same genotype (Figure 2). However, $Mcm4^{C3/+}$ $Chk2^{+/-}$ males were more susceptible to cancer (73%) than $Mcm4^{C3/+}$ controls (44%; Supplementary Figure 2).

Hus1 deficiency has no impact on tumor latency or cancer susceptibility in *Chaos3* mice

The study of ATR pathway genes in tumorigenesis is complicated by embryonic lethality that occurs in nulls for *Atr*, *Chk1*, the RAD9-RAD1-HUS1 (9-1-1) complex members *Rad9a* and *Hus1*, and the 9-1-1 clamp loader *Rad17*. The 9-1-1 complex is a PCNA-like clamp that loads onto damage sites and recruits the ATR activator TOPBP1 [52]. Mice with genetically reduced HUS1 levels are viable, normal in appearance, but are not tumor susceptible, and do not experience accelerated tumorigenesis in a *p53*-deficient background [53]. Graded levels of *Hus1* expression can be achieved using the following combinations of null ($Hus1^{1}$) and hypomorphic ($Hus1^{neo}$) alleles: $Hus1^{+/neo}$ (71.4% of WT), $Hus1^{1/+}$ (43.5% of WT), and $Hus1^{1/neo}$ (20.8% of WT) [53]. We used these allele combinations to examine the effects of ATR pathway perturbation upon cancer latency and frequency in $Mcm4^{C3/C3}$ mice. However, none of the *Hus1* mutant genotypes had significantly different cancer susceptibility or latency compared to $Mcm4^{C3/C3}$ $Hus1^{+/+}$ mice (Figure 3a, Supplementary Figure 3, Supplementary Table 2).

The lack of an effect upon cancer phenotypes led us to test whether the hypomorphic *Hus1* genotypes actually impact checkpoint signaling in $Mcm4^{C3/C3}$ mammary tumors. Consistent with previous genomic analyses of $Mcm4^{C3/C3}$ mammary tumors showing that p53 deletions are infrequent in this model [32], p53 levels were robust in most of the 8 tumors tested by Western blotting (Figure 3b), likely reflecting checkpoint-mediated stabilization [54]. There was no correlation between levels of p53 and four genotypes of *Hus1* representing a gradation of HUS1 levels (see above). CHK1 activation, as indicated by phosphorylation of SER345 that is catalyzed by ATR in response to replication or genotoxic stress [55], roughly paralleled the p53 levels in this tumor set. These data indicate that *Hus1* hypomorphism has little impact on ATR axis damage signalling in these tumors. Interestingly however, $Mcm4^{C3/C3}$ $Hus1^{1/neo}$ mice exhibited abnormal craniofacial features (not shown) similar to mice deficient for both *Hus1* and *Atm* [56], suggesting that there is an impact of HUS1 deficiency in some non-tumorigenic cell types during development of $Mcm4^{C3/C3}$ mice.

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p21 deficiency exacerbates tumor frequency and onset in *Chaos3* mice

p21 is a cyclin-dependent kinase inhibitor and downstream target of p53 that halts cell cycle progression when activated (Figure 1a). It functions by blocking the activity of cyclin-CDK complexes (CDK2 and CDC2), and can inhibit proliferating cell nuclear antigen (PCNA) and therefore DNA replication [57]. Despite being a p53 target, mice lacking p21 are not cancer-prone as are *p53* mutants [58]. Mice homozygous for *Chaos3* or the hypomorphic *Mcm2* allele ($Mcm2^{IresCreERT2}$) exhibit modestly elevated p53 phosphorylation and p21 expression. Furthermore, p53 mutation in either of these backgrounds increases embryonic lethality and accelerates cancer formation in survivors [23, 28]. These results are indicative of important cellular roles for the downstream targets of checkpoint pathways in replication-deficient mice.

To explore if p53 signaling to p21 (Figure 1a) is important for tumor prevention in animals with intrinsic replication stress, the effects of p21 deficiency was examined in *Chaos3* mice. While embryonic development of double mutant animals was not affected as are *p53*/$Mcm4^{Chaos3}$ embryos [28], *p21* nullizygosity significantly decreased time to tumor onset of *Chaos3* males and females (Figure 4; Supplementary Table 2), with the predominant tumor class being histiocytic sarcomas in this mixed C3H × B6 background (Supplementary Figure 4). $Mcm4^{C3/C3}$ $p21^{+/-}$ females, but not males, also had significantly decreased tumor latency compared to $Mcm4^{C3/C3}$ alone (Figure 4; Supplementary Table 2). Finally, cancer susceptibility was elevated in $Mcm4^{C3/+}$ $p21^{-/-}$ and $Mcm4^{C3/+}$ $p21^{+/-}$ vs. $Mcm4^{C3/+}$ females (55%, 42% and 21%, respectively; Supplementary Figure 4).

DISCUSSION

Much is known about the molecular biology of the ATM and ATR pathways, their roles in responding to various types of DNA damage, and the impacts upon the cell cycle. However, most of this knowledge is based upon *in vitro* biochemical studies or experiments performed in cultured cells or in yeast. Regarding *in vivo* roles, mouse knockout models have been created for most genes in the ATM and ATR pathways, and phenotypes defined and compared to corresponding human diseases. Especially for the ATM pathway, these mouse models (and cells derived from them) have been exploited to characterize the types of DNA damage to which they primarily respond, such as DSBs. However, certain complications have limited studies on the effects of, and responses to, replication stress *in vivo*, despite the recognition that it is a major driver of genomic instability and tumorigenesis [59, 60]. These complications include the embryonic lethality of null mutations in the *Atr* pathway, and the dearth of suitably relevant models of non-oncogene-associated replication stress.

Here, we utilized the *Chaos3* mouse model to better understand the importance of DDR pathways in whole organisms with intrinsic replication stress, particularly with respect to carcinogenesis. This model is powerful and unique in that the replicative helicase mutation it bears ($Mcm4^{Chaos3}$) is not so disruptive that development is affected. The mutation destabilizes the MCM2-7 hexamer but not its unwinding activity, causes a decrease in dormant replication origins, and triggers multiple fork recovery pathways. These defects ultimately lead to elevated chromosome breaks, chromosome segregation defects and tumorigenesis [19, 25, 26]. Thus, there is opportunity to study the roles of both major DDR

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pathways (ATR and ATM) in cancer susceptibility without applying exogenous agents. Finally, the *Chaos3* model does not involve artificial oncogene overexpression, the most commonly used strategy for inducing and studying replication stress in cancer [59].

Disruption of the ATM pathway via *Atm* or *Chk2* mutation had the effect of exacerbating *Chaos3* phenotypes. Most dramatic was that the $Mcm4^{C3/C3}$ $Atm^{-/-}$ genotype caused semilethality that was traceable to retarded *in utero* growth. One interpretation of this result is that *Chaos3* cells, which sustain elevated DSBs that may arise from collapsed and/or persistently stalled replication forks that fail to be compensated by nearby dormant origin firing (dormant origins are reduced in *Chaos3* mice [25]), accumulate a lethal level of persistent unrepaired DNA damage from the concurrent lack of DDR signaling. Stochastic factors or segregating background genetic variation may underlie the incomplete penetrance of lethality. Although early lymphoma onset in all $Atm^{-/-}$ animals obscured possible effects of *Chaos3* upon other cancer susceptibilities, both $Mcm4^{C3/C3}$ $Atm^{+/-}$ and $Mcm4^{C3/+}$ $Atm^{+/-}$ mice exhibited decreased tumor latency and/or increased tumor susceptibility compared to controls ($Mcm4^{C3/C3}$ and $Mcm4^{C3/+}$, respectively). Heterozygosity for *Atm* alone does not markedly elevate cancer rates or decrease latency in mice [34], but it does render them sensitive to sublethal doses ionizing irradiation [61]. Considering that $Mcm4^{C3}$ heterozygotes have modestly elevated GIN (2-5 increase in erythrocyte micronuclei vs. 20 fold in homozygotes) but are not cancer prone [19], these data indicate that a synthetic phenotype results from the combination of either genetic (*Chaos3* heterozygosity) or environmental (radiation) genomic stresses with a normally benign genetic reduction in ATM signaling. Similarly, heterozygosity for *Chk2* also increased tumor incidence in $Mcm4^{C3}$ heterozygotes. We consider these results as being supportive of the concept that heterozygosity of multiple key genes can drive carcinogenesis [62]. Notably, there is some evidence that human *ATM* mutation carriers are at moderately elevated risk for breast and possibly other cancers (for example, see [63]); it is unclear whether cancer outcome in these individuals is strictly an issue of penetrance or is modified by genetic background or environmental factors.

Chk2 deficiency also increased tumor incidence and decreased tumor latency in *Chaos3* mice, although viability wasn't affected as with the $Mcm4^{C3/C3}$ $Atm^{-/-}$ genotype. We interpret this to indicate that most cells from such animals do not retain a catastrophic level of unrepaired DSBs. The presence of ATM is predicted to allow initial localized responses to DSBs that may occur at collapsed forks, such as H2AX phosphorylation (γH2AX) and subsequent HR repair by RAD51 [64], which may reduce the damage burden below the threshold of cellular lethality or compromised proliferation. It is also possible that in the absence of CHK2, ATM activates CHK1 to stimulate repair responses [65]. Overall, both sets of experiments indicate that perturbation of the ATM pathway, which is involved primarily in the response to DSBs, increases cancer susceptibility in mice with intrinsic replication stress and elevated chromosomal instability/DSBs.

HUS1 was shown to be is critical for CHK1 phosphorylation in response to exogenous genotoxins [66], and genetic reduction of *Hus1* expression was shown to increase genome instability and hypersensitivity to replication inhibitors but not cancer susceptibility [53]. Using this hypomorphic *Hus1* model for putative ATR pathway attenuation, we reasoned

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that *Chaos3* mice might provide a sensitized system for uncovering possible roles of the ATR pathway in tumor suppression in mice with genetically predisposed replication stress. Notably, *Chaos3* MEFs exhibit signs of ATR pathway activation in the form of modestly increased levels of RPA foci, RAD17 phosphorylation, and *Chk1* phosphorylation (the latter in the B6 but not (B6 x C3H)F1 background [25, 28]. However, overall tumor latency and susceptibility were not altered in *Chaos3* mice deficient for *Hus1*. In contrast, depletion of *Atr* in mice has been shown to suppress oncogene-induced tumors that normally exhibit replication stress [67, 68]. These observations contribute to the proposal that while ATR may suppress neoplastic transformation to some degree via its role in DNA damage responses, it may be required for subsequent survival and proliferation of tumors [68, 69]. Interestingly, severe depletion of ATR in a human patient was associated with growth defects and genomic instability but not cancer [70]. In light of those reports, we can offer two interpretations for our observations. One is that ~80% of HUS1 in *Hus1* $^{1/neo}$ mice does not impact the levels of replication stress in *Mcm4*$^{C3/C3}$ cells. Another is that HUS1 may have a more significant role in DNA repair activities distinct from checkpoint signaling [71, 72], a concept not inconsistent with findings that compound deficiency for *Atm* & *Hus1* or *Hus1* & *p53* severely affects animal growth and mammary epithelial maintenance, respectively, without increasing tumorigenesis.

As mentioned earlier, the strong interaction between p53 and MCM deficiency (*Chaos3* or *Mcm*^{IresCreIRCT2} homozygosity) demonstrated that intrinsic replication stresses ultimately trigger p53-dependent damage responses that preserve normal development and inhibit neoplastic transformation [2328]. A previous study suggested that the p21 upregulation observed in (C3HxB6)-*Chaos3* mice was unlikely to contribute to tumor suppression because the mean tumor latency in *Mcm4*$^{C3/C3}$ *p21*$^{-/-}$ was very similar to that of *Mcm4*$^{C3/C3}$ *p21*$^{+/-}$ [28]. However, that study did not include *Mcm4*$^{C3/C3}$ animals as controls. We expanded that study to include both male and female mice and all the relevant control genotypes from related litters. The results indicate a role a tumor suppressive role of p21 in the *Chaos3* model, but that it is probably relevant only in a subset of cells bearing a level of DNA damage that results in p53-mediated *p21* transcription.

During the course of this project, a total of 687 detailed necropsies were performed (Supplementary Table 4). Overall, the results are consistent with previous studies showing that genetic MCM depletion causes extreme cancer predisposition, but that genetic background is the primary determinant of cancer type [19, 23-25]. Because of this strong influence of strain background, possible Mcm- or checkpoint gene-specific alterations in tumor spectrum must be analyzed with caution. With this caveat, the shift towards mammary tumor susceptibility in the *Chk2* -deficient *Chaos3* mice of mixed background is notable. Although overall cancer rates were similar, the mammary tumor incidence in *Mcm4*$^{C3/C3}$ *Chk2*$^{-/-}$ females (50%) was > 3 fold higher than that of *Mcm4*$^{C3/C3}$ relatives (15%, consistent with that in true C3HxB6 F1s [25]). Therefore, rather than a factor of genetic background, the increased mammary tumorigenesis may be attributable to *Chk2* deficiency. Certain *Chk2* alleles (not null alleles) are known to convey a 2-3 fold increased breast cancer risk [73]. Since *Chk2* deficiency alone has not been associated with cancer in mice, the *Chaos3* mutation may bring out a susceptibility that is evident in longer-living humans.

In addition to genetic background effects, we found that tumor latency and susceptibility differed between genders in some of genotypes. Aside from cancers related to sexually dimorphic tissues such as mammary, ovary and prostate, differences in latency or frequency between sexes has been a longstanding puzzle. Differences are often hypothesized to be related to factors such as hormones, immune system differences, and differences in sex chromosome constitution [74]. Here, we observed that females had an overall greater cancer susceptibility to *Atm* and *p21* deficiency than males. Cancer incidence in $Mcm4^{C3/+}$ $p21^{+/-}$ and $Mcm4^{C3/+}$ $Atm^{+/-}$ females was double that of males of the same genotypes. Additionally, p21 nullizygosity increased the cancer incidence of $Mcm4^{C3/+}$ females by 34%, but had no effect on males (Supplemental Fig 4). These results hint at a role for DNA repair pathways in sexual dimorphism in cancer susceptibility, which is not unprecedented in consideration of the consequences of BRCA1/2 deficiencies in female cancers. In humans, certain inherited *Atm* and *p21* polymorphisms (*ATM* Ex1-81G>A, *ATM* D126E, and *CDKN1A* S31R) lead to decreased DDR response and efficiency, which is associated with increased risk of developing lung cancer in African American women [75]. It is possible that further studies in mice can get at the root of cancer susceptibility gender differences and interactions with genetic background.

Overall, this study marks the importance of intact DDR pathways in responding to replication stress, providing protection from carcinogenesis when the DNA replication machinery is defective from birth. It remains unclear if lifelong exposure to exogenous sources of replication stress would benefit from the same DDR genes, but *in vitro* studies indicate this is likely to be so. Our findings also indicate that gender and genetic background significantly impacts cancer susceptibility and tumor latency when DNA replication integrity and DDR pathways are concurrently compromised. DDR pathways are being recognized as potential therapeutic targets in cancer treatment, since tumor cells can be hypersensitized to DNA damaging drugs when both overlapping pathways are inactivated or attenuated [76]. With increasing use of personalized genomics, it may be possible to effectively characterize the status of a tumor's endogenous DDR, and exploit weaknesses in an effective and targeted manner.

MATERIALS AND METHODS

Mice

p21 mice (B6;129S2-$Cdkn1a^{tm1Tyj}$) were purchased from the Jackson Laboratory. *Hus1* mutant mice ($Hus1^{tm2Rsw}$, abbreviated as $Hus1^{neo}$; $Hus1^{tm1Led}$, abbreviated as $Hus1^{\ \ 1}$) were obtained from R. Weiss [77, 78] as were *Atm* mutants (Atm^{tm1Led}, abbreviated as Atm^{-}) [36], and *Chk2* ($Chek2^{tm1Mak}$, abbreviated as $Chk2^{-}$) from Tak Mak [79]. At the time of crossings, *Chk2* and *p21* mutants were congenic in C57BL/6J (B6), *Atm* was congenic in FvB, and the *Hus1* animals were congenic in 129S6. *Chaos3* C3HeB/FeJ (C3H) congenic animals were crossed to DDR mutants to generate double mutant animals that were of mixed genetic background. Progeny were genotyped as described in the original publications or as indicated by The Jackson Laboratory for those mice obtained from that source (http://jaxmice.jax.org). Double mutants and littermates of the same gender were aged to a terminal endpoint of eighteen months or until animals showed clinical signs of disease. Prism

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(GraphPad 5) statistical software was used to analyze survival curves and generate Kaplan-Meier plots.

MEF studies

Timed matings were conducted to collect embryos at embryonic days 12.5, 13.5, and 18.5. MEFs were generated, cultured, and cell proliferation assays performed as previously described [19].

Histopathology

Tumor samples were formalin-fixed and embedded in paraffin for sectioning and histological analysis. Slides were stained with hematoxylin and eosin (H&E) prior to histopathological evaluation.

Statistical analyses

The following tests of significance were performed and abbreviated as follows: LRMCT= Log-rank/Mantel-Cox Test; GBWT= Gehan-Breslow-Wilcoxon Test. LRMCT and GBWT are alternative methods that are applied to the survival curves; the latter gives more weight to deaths at earlier time points. The analysis was performed with Prism software (Graphpad). χ^2 analysis was used to determine statistical significance of observed versus expected genotype ratios. FET was used to examine the significance of the association (contingency) between genotypes and gender to cancer susceptibility/frequency or subtype.

Western Blotting—Tissues were homogenized in T-PER (Pierce), plus complete EDTA-free proteinase inhibitor (Roche). Then, 40 ug of protein was subjected to electrophoresis on a 10% denaturing PAGE gel, transferred to a polyvinylidene difluoride membrane and blocked with 5% milk in Tris-buffered saline with 0.1% Tween 20 (TBST). Membranes were incubated with the following antibodies at 1:1000 in 5% BSA in TBST overnight at 4 deg C: p53 Abcam #26 and CHK1 (SER345) Cell Signaling #2341. Beta-actin (Sigma #A1978) was employed at 1:10,000 in 5% BSA in TBST.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We would like to thank R.S. Weiss and T. Mak for providing mice. We also thank R.S. Weiss and G. Balmus for critical feedback on the manuscript. This study was supported by NIH training grants IT32HDO57854 and 5T32GM007617, and Empire State Stem Cell Fund contract numbers C026442 and C024174 to J.C.S.

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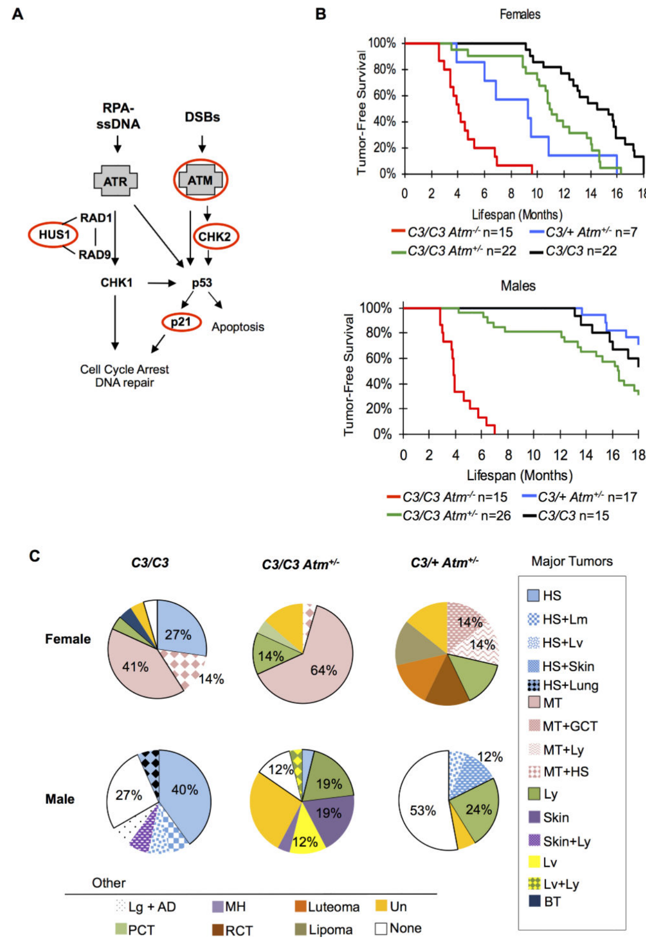

Figure 1. *Atm* deficiency impacts *Chaos3* tumor latency and tumor susceptibility

(A) DNA damage response pathways. Key genes in DDR pathways are shown with the ATR and ATM DNA damage sensors emphasized in gray boxes. Genes perturbed in this study are indicated by red ovals. (**B**) Kaplan-Meier graphs of the indicated genotypes and sexes. $Mcm4^{C3/C3}$ $Atm^{+/-}$ and $Mcm4^{C3/+}$ $Atm^{+/-}$ females have significantly decreased tumor latency compared to $Mcm4^{C3/C3}$ alone (see statistics in Supplementary Table 2). $Mcm4^{C3/C3}$ $Atm^{+/-}$ males neared statistical significance for decreased tumor latency, and $Mcm4^{C3/+}$ $Atm^{+/-}$ male tumor latency was similar to $Mcm4^{C3/C3}$ alone (Supplementary Table 2). C3 = $Mcm4^{C3}$. (**C**) Tumor spectra of selected genotypes. HS=histiocytic sarcoma, MT=mammary tumor, BT=bone tumor, Ly=lymphoma, None=healthy (no detectable cancer), PCT=plasma cell tumor, RCT=round cell tumor, GCT=granulosa cell tumor, Lv=liver, MH=myeloid hyperplasia, AD=adrenal ganglioneuroma, Un=unknown tumor type. Note that tumor spectrum is affected by genotype and gender, and that $Mcm4^{C3/+}$ $Atm^{+/-}$ females are more susceptible to cancer than males. C3 = $Mcm4^{C3}$.

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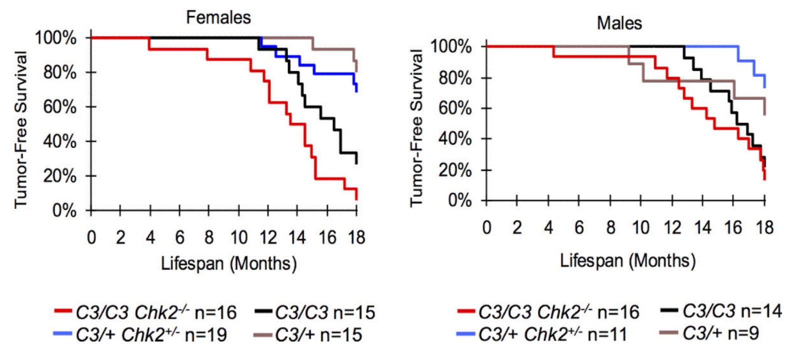

Figure 2. Effects of *Chk2* deficiency upon tumorigenesis in *Chaos3* mice

Kaplan-Meier graphs of the indicated genotypes and sexes are shown. $Mcm4^{C3/C3}$ $Chk2^{-/-}$ female mice have significantly decreased time to tumor onset than $Mcm4^{C3/C3}$ alone. C3 = $Mcm4^{C3}$.

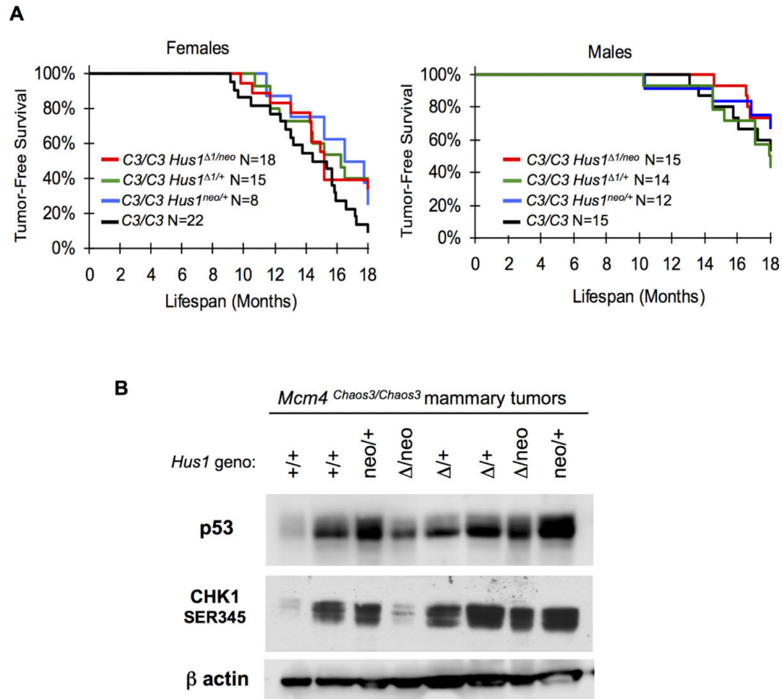

Figure 3. *Hus1* deficiency has no effect upon tumorigenesis or checkpoint signaling in *Chaos3* mice and tumors

(**A**) *Chaos3* x *Hus1* tumor latency. *Mcm4*$^{C3/C3}$ *x Hus1* mice do not have significantly different (see statistics in Supplementary Table 2) time to tumor onset than *Mcm4*$^{C3/C3}$ alone. C3 = *Mcm4*^{C3}. (**B**) Western blot analysis of *Mcm4*$^{C3/C3}$ mammary tumors with a gradation of *Hus1* hypomorphism. The genotypes are abbreviated as follows: " " is a null allele (*Hus1* ^{1}); "Neo" is a hypomorphic (*Hus1*^{neo}) allele; "+" is the WT allele. For levels of HUS1 in these genotypes, see the text. Antibodies used are as indicated to the left of the panels. The results shown are from the same Western blot that was stripped and reprobed sequentially, following verification of effective stripping.

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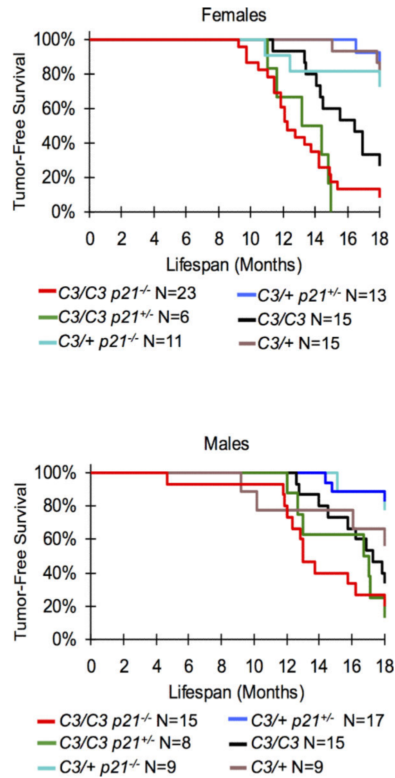

Figure 4. *p21* deficiency impacts *Chaos3* tumor latency in males and females and tumor susceptibility in females

Kaplan-Meier graphs of the indicated genotypes and sexes are shown. $Mcm4^{C3/C3}$ $p21^{-/-}$ male and female mice have significantly decreased time to tumor onset than $Mcm4^{C3/C3}$ alone. $Mcm4^{C3/C3}$ $p21^{+/-}$ females, but not males, also have significantly decreased tumor latency compared to $Mcm4^{C3/C3}$ alone. See statistics in Supplementary Table 2. C3 = $Mcm4^{C3}$.