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Supplemental Data

Chk2 Suppresses the Oncogenic Potential of DNA Replication-Associated DNA Damage

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Supplemental Experimental Procedures

Immunoreagents and Western Blotting

Antibodies for mMre11 and mNbs1 were described (Stracker et al., 2007; Theunissen et al., 2003; Williams et al., 2002). Antibodies were purchased for Chk2 (Upstate, 1:500), p53 (Vector, CM5 1:1000), p53-S15 (R&D, 1:1000), Actin (Sigma, 1:2000), Atm-S1981 (Cell Signaling, 1:1000), BID, S61 and S78 (Bethyl Labs, 1:1000), Histone H3-S10 (Upstate, 1:200), BrdU-FITC (BD Biosciences, 1:200). Anti-Atm (MAT3, 1:3000) monoclonal antibody was a gift from Yossi Shiloh. Cells were lysed in TNG lysis buffer with 150 mM NaCl (Kitagawa et al., 2004). Equal amounts of protein (Biorad DC assay) were run on SDS-PAGE or 3-8% Tris-acetate gels (Invitrogen) and transferred to PVDF membranes (Millipore). Blocking and incubation with antibodies was done in 5% dry milk in PBS-T at 4°C overnight. The ECL-plus kit (GE Healthcare) was used for detection after incubation with the appropriate secondary antibodes for 1 hour at room temperature in PBS-T w/ 5% dry milk. MG132 (Sigma) was used at 10 μM and dexamethasone (Sigma) was used at 1 mM.

Pathology, Histology, and Immunohistochemistry

Moribund mice were sacrificed, tissues fixed in 10% neutral buffered formalin, and embedded in paraffin in accordance with standard procedures. For immunohistochemistry (IHC) analysis representative sections were deparaffinized, rehydrated in graded alcohols, and processed using the avidin-biotin immunoperoxidase method (Trotman et al., 2006). Antigen retrieval by microwave oven treatment was performed using standard procedures. Diaminobenzidine was used as the chromogen and hematoxylin to counterstain nuclei and Ki-67 (NovoCastra), CD3 (DAKO), B220 (BD Biosciences-PharMingen), and MAC2 (Cedarlane) antibodies were used for IHC. Apoptosis was determined by TUNEL assay in tissue sections as previously described (Garcia-Barros et al., 2003). Pathological analysis was carried out in the MSKCC Genetically Engineered Mouse (GEM) Phenotyping core facility by Krista M.D. La Perle and Suzana S. Couto.

Sequencing of p53 and p19^{ARF}

For amplification and sequencing of *p53* exons 5-10 from genomic DNA the following primers were utilized for amplification and priming of the sequencing reaction: mp53-5F TACTCTCCTCTCAATAAG, mp53-I6R CGGGTTGCTAGAAAGTCAAC, mp53-I6F TGCCGAACAGGTGGAATATC, mp53-I7R TGGAACAGAAACAGGCAGAA, mp53-I8F TCTGTGGCTTCTCGGGGTTCCT, mp53-9R ACCTTGAGGGTGAAATACTCTCC, mp53-10F GTGCTTCCATCTCACTTC, mp53-10R GGTAGAGCACCACAGGCA. For cDNA sequencing, the primers ATGGAGGAGTCACAGTCGGAT and AGTCAGGCCCCACTTTCTTGAC

encompassing exons 2-11 of *p53* were used for PCR amplification and sequencing. The primers p19Ex1- TTCTCACCTCGCTTGTCACAGT and p19Ex3-CCACATGCTAGACACGCTAGCATC were used for the amplification and sequencing of $p19^{ARF}$.

Microarray analysis

Data was analyzed using the Bioconductor packages (www.bioconductor.com) for the R statistical system. Raw Affymetrix CEL files were normalized and quantitated using the GC-RMA algorithm to give the signal level (log base 2 transformed in subsequent analysis). To determine genes that are differentially expressed between the various sample types, the LIMMA package (Bioconductor) was used to perform a variant of linear models (t-test or ANOVA) where the variance is corrected to deal with small sample numbers. To account for the multi-testing issue, the False Discovery Rate (FDR) method was used and genes filtered at an FDR level of 0.05. The heatmap in Figure 4 was generated using Genespring (Silicon Genetics).

Supplemental References

Garcia-Barros, M., Paris, F., Cordon-Cardo, C., Lyden, D., Rafii, S., Haimovitz-Friedman, A., Fuks, Z., and Kolesnick, R. (2003). Tumor response to radiotherapy regulated by endothelial cell apoptosis. Science *300*, 1155-1159.

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Theunissen, J. W., Kaplan, M. I., Hunt, P. A., Williams, B. R., Ferguson, D. O., Alt, F. W., and Petrini, J. H. (2003). Checkpoint failure and chromosomal

instability without lymphomagenesis in Mre11(ATLD1/ATLD1) mice. Mol Cell *12*, 1511-1523.

Trotman, L. C., Alimonti, A., Scaglioni, P. P., Koutcher, J. A., Cordon-Cardo, C., and Pandolfi, P. P. (2006). Identification of a tumour suppressor network opposing nuclear Akt function. Nature *441*, 523-527.

Williams, B. R., Mirzoeva, O. K., Morgan, W. F., Lin, J., Dunnick, W., and Petrini, J. H. (2002). A murine model of nijmegen breakage syndrome. Curr Biol *12*, 648-653.

Supplemental Figures and Tables

Table S1. Metaphase Aberrations in Proliferating Splenocytes.
Untreated or IR treated (1 Gy) splenocytes stimulated with IL-4 and LPS were analyzed for metaphase aberrations after Giemsa staining. Cells were recovered for 1 hour after IR treatment and incubated in colce Scored chromosomal aberrations are listed. *Chk2* deficiency did not cause chromosome instability or enhance that observed in *Mre11*$^{ATLD1/ATLD1}$ cultures.

Genotype	N	IR (Gy)	Cd	Frag	Rearrg	# abnormal	% abnormal
WT	30	0	1	0	0	1	3.33
Chk2$^{-/-}$	30	0	0	0	0	0	0.00
Mre11$^{ATLD1/ATLD1}$	80	0	3	2	0	5	6.25
Mre11$^{ATLD1/ATLD1}$ *Chk2*$^{-/-}$	84	0	5	1	0	6	7.14
WT	31	0.5	6	0	0	6	19.35
Chk2$^{-/-}$	30	0.5	7	0	0	7	23.3
Mre11$^{ATLD1/ATLD1}$	30	0.5	9	4	0	13	43.33
Mre11$^{ATLD1/ATLD1}$ *Chk2*$^{-/-}$	35	0.5	10	0	1	15	42.86

Supplemental Figure S1

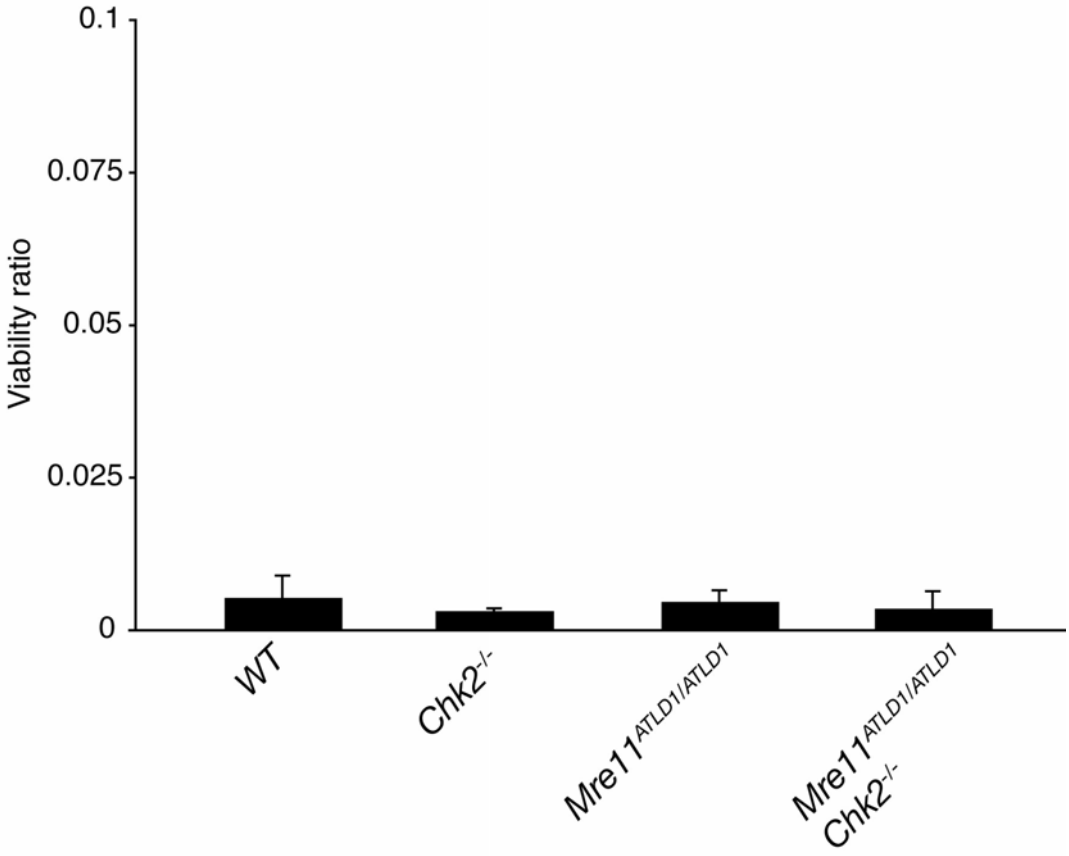

Figure S1. Apoptosis in response to dexamethasone.
Thymocytes were treated with 1 mM dexamethasone for 20 hours and apoptosis was analyzed as described in the experimental procedures section. Viability ratios are plotted for each genotype.

Supplemental Figure S2

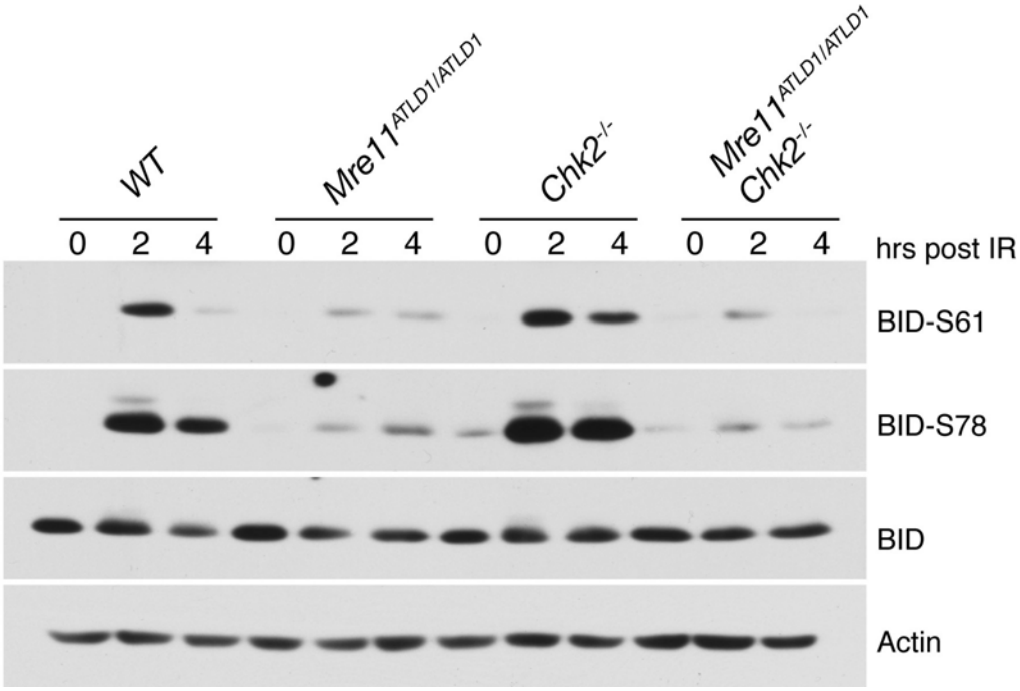

Figure S2. BID phosphorylation in splenocytes.
Western blots from mock or irradiated splenocytes were probed with antibodies specific for phosphorylated or total BID. BID phosphorylation after irradiation is attenuated by Mre11 hypomorphism but not by loss of Chk2.

Supplemental Figure S3

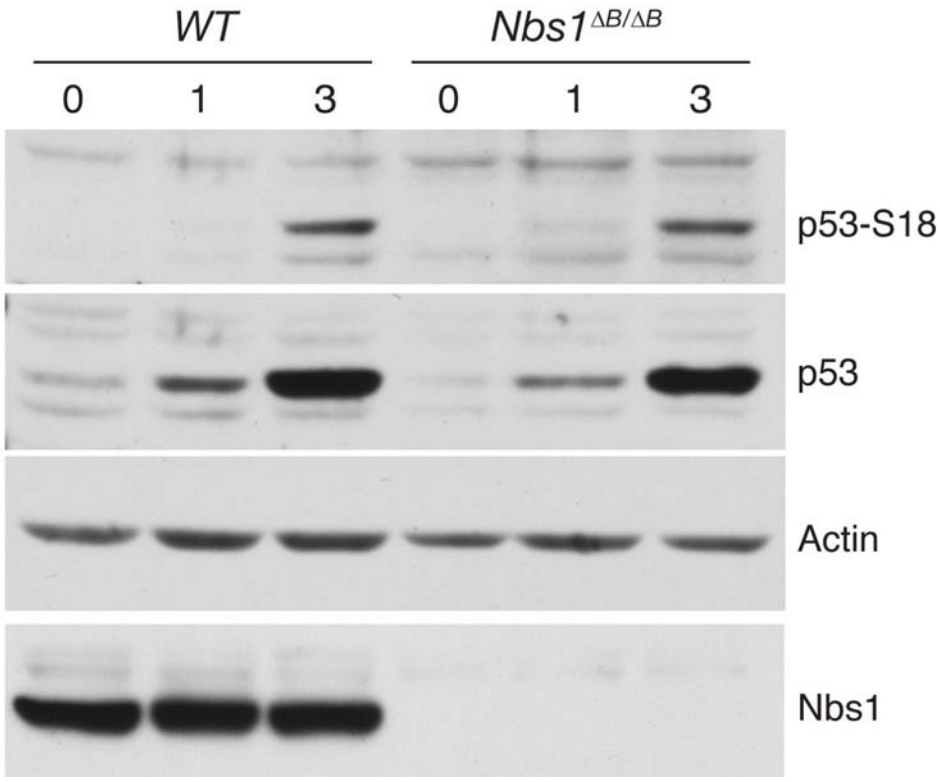

Figure S3. Stabilization and phosphorylation of p53 in *Nbs1*$^{\Delta B/\Delta B}$ thymocytes.
Western blots from mock or irradiated (IR) thymocytes at the indicated time points post treatment were probed with antibodies specific for phosphorylated (S18) or total p53. p53 responses and apoptosis appear normal in cell cultures from *Nbs1*$^{\Delta B/\Delta B}$ animals.

Supplemental Figure S4

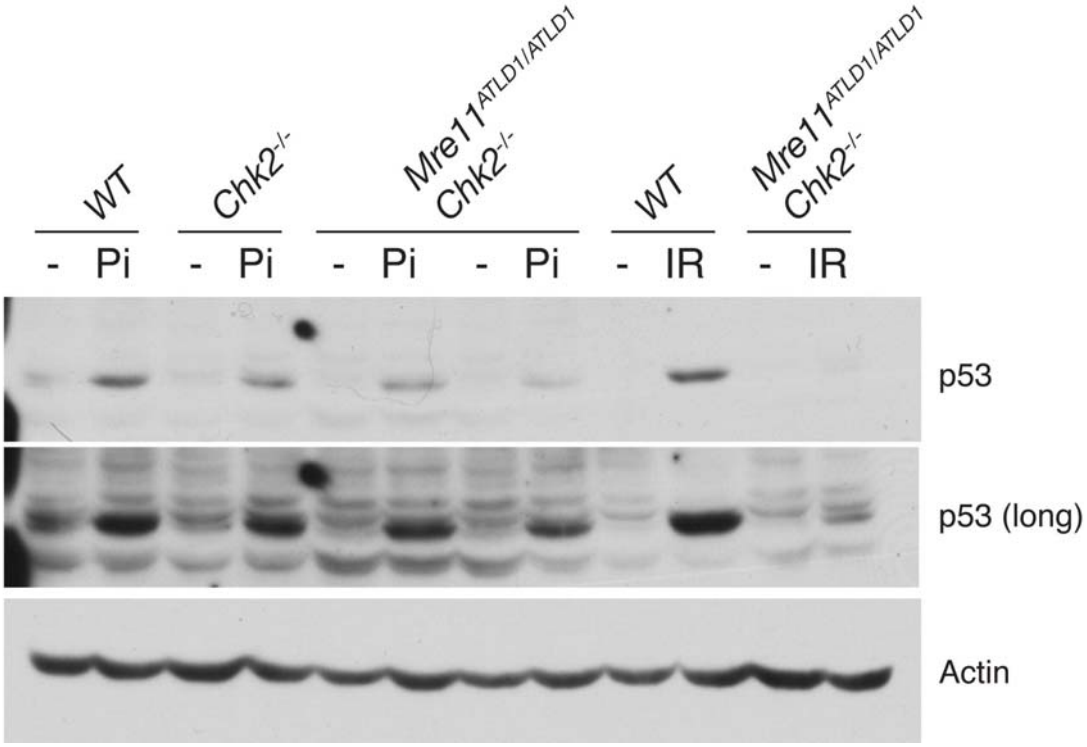

Figure S4. Stabilization of p53 in MG132 treated thymocytes.
Lysates from mock treated, 10 µM MG132 treated (Pi) or irradiated (IR, 5 Gy) thymocytes were harvested 4.5 hours post treatment and Western blots were probed with antibodies for p53 and Actin (loading control). p53 is stabilized to similar levels in wild type and *Mre11*$^{ATLD1/ATLD1}$ *Chk2*$^{-/-}$ cell cultures in response to MG132 but not IR treatment.

Table S2 is included as separate excel file.

Table S2. Statistical analysis of microarray data.
Raw affymetrix .CEL files were analyzed as described in the materials and methods. Lists of statistically significant changes after IR treatment of WT and *Atm*$^{-/-}$ thymocytes are presented. Column header abbreviations: Gene symbol (SYM), false discovery rate (FDR), and fold change (FC). No significant changes were identified between mock and irradiated *Chk2*$^{-/-}$ samples.

Supplemental Figure S5

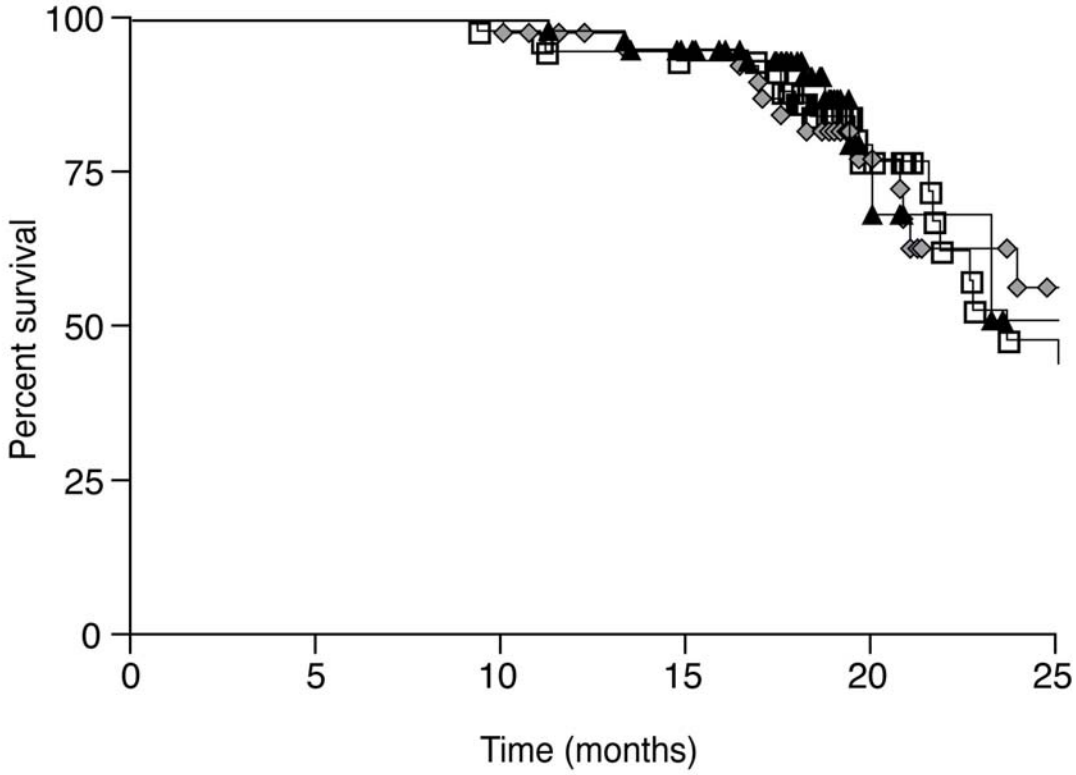

Figure S5. Cohort survival.
Individual Kaplan Meier survival curves for wild type (*WT*, ◆, n=62), *Nbs1*$^{\Delta B/\Delta B}$ (□, n=61), and *Mre11*$^{ATLD1/ATLD1}$ (▲, n=49) animals (these curves were combined in Figure 5A).

Supplemental Figure S6

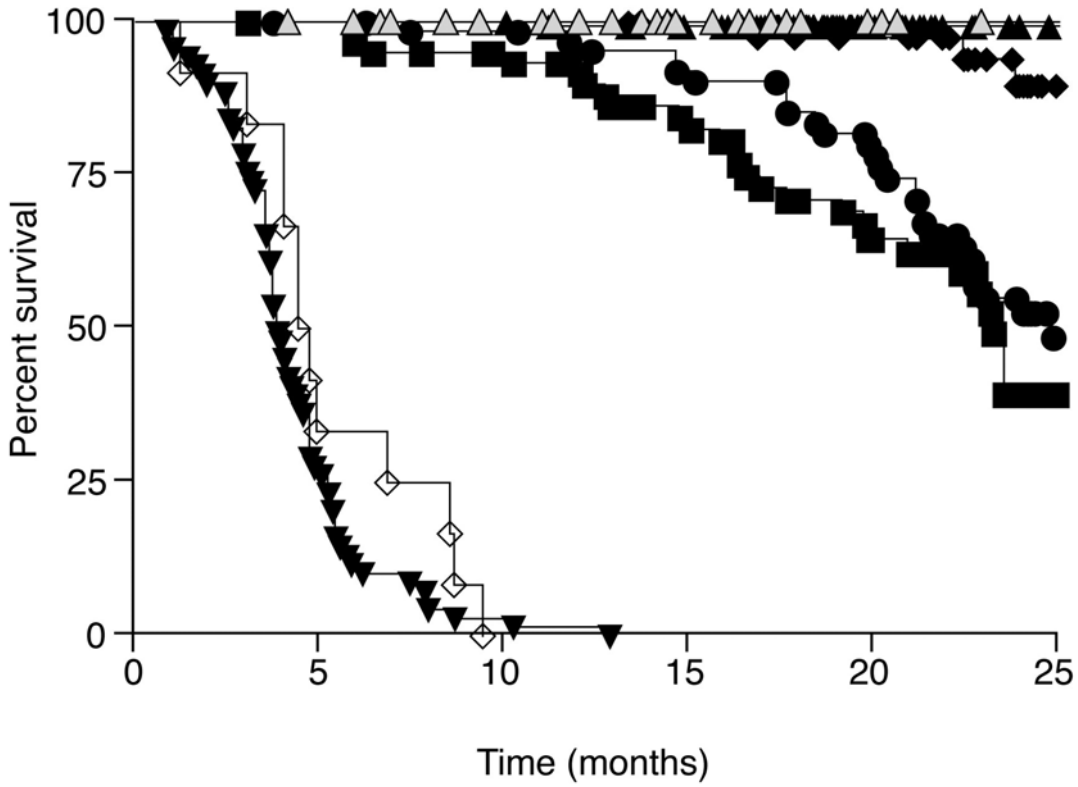

Figure S6. Tumor free survival.
Kaplan Meier tumor free survival curves for WT/*Nbs1*$^{\Delta B/\Delta B}$/*Mre11*$^{ATLD1/ATLD1}$ (▲, n=172), *Chk2*$^{-/-}$ (◆, n=41), *Nbs1*$^{\Delta B/\Delta B}$ *Chk2*$^{-/-}$ (■, n=41), *Mre11*$^{ATLD1/ATLD1}$ *Chk2*$^{-/-}$ (●, n=41), *Atm*$^{-/-}$ (▼, n=71), *Atm*$^{-/-}$ *Chk2*$^{-/-}$ (◇, n=19), *Prkdc*$^{scid/scid}$ *Chk2*$^{-/-}$ (▲, n=34)

Table S3. Statistical analysis of survival curves. Analysis of statistically significant differences between survival curves and tumor free survival curves. P-values were generated using the logrank test (Prism software).

Overall cohort survival (logrank test)

	Chk2$^{-/-}$	*Mre11*$^{ATLD1/ATLD1}$ *Chk2*$^{-/-}$	*Nbs1*$^{\Delta B/\Delta B}$ *Chk2*$^{-/-}$	*Prkdc*$^{scid/scid}$ *Chk2*$^{-/-}$
WT	*0.7711*	*0.0078*	*<0.0001*	*0.0058*
Chk2$^{-/-}$	*-*	*0.0700*	*<0.0001*	*0.0829*

Tumor free cohort survival (logrank test)

	Chk2$^{-/-}$	*Mre11*$^{ATLD1/ATLD1}$ *Chk2*$^{-/-}$	*Nbs1*$^{\Delta B/\Delta B}$ *Chk2*$^{-/-}$	*Prkdc*$^{scid/scid}$ *Chk2*$^{-/-}$
WT	*0.1161*	*<0.0001*	*<0.0001*	*0.2015*
Chk2$^{-/-}$	*-*	*0.0002*	*<0.0001*	*0.5168*

Supplemental Figure S7

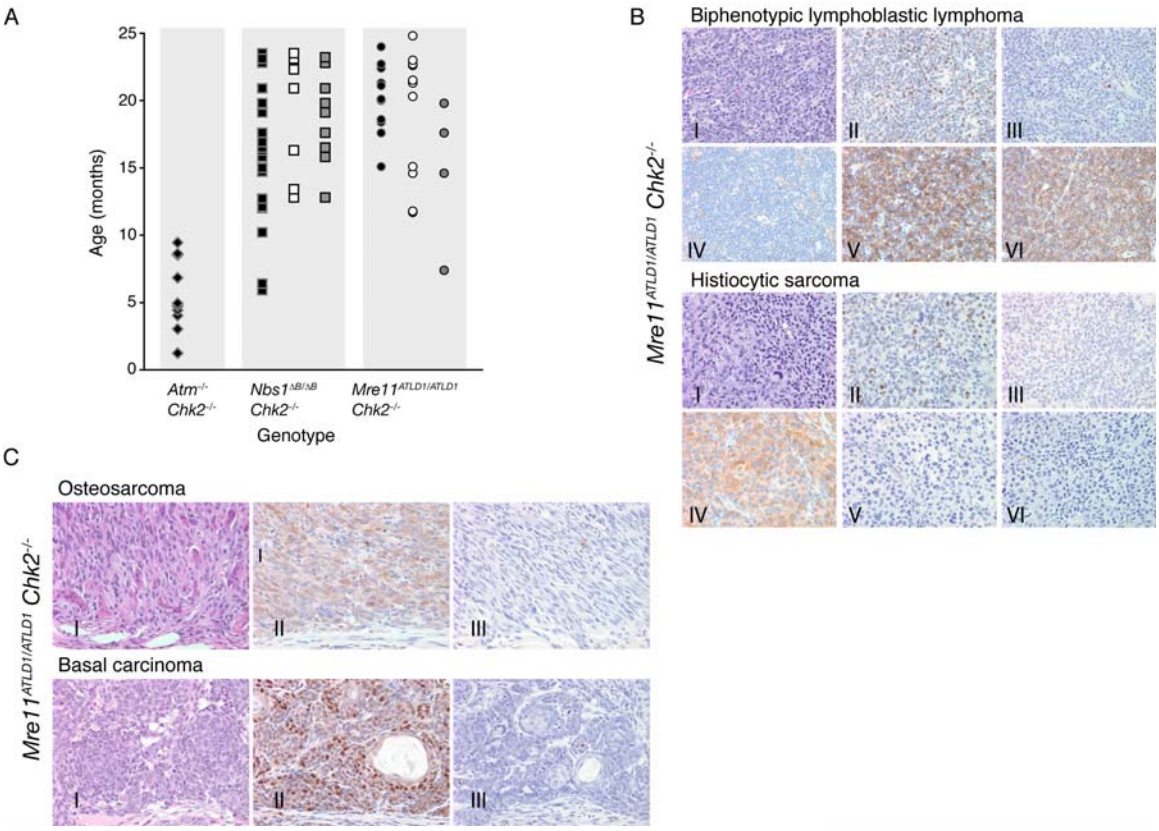

Figure S7. Tumor onset and histology.
(A) Graph depicting age of tumor onset in the indicated genotypes. Lymphomas are represented in black, carcinomas and sarcomas in white, and benign tumors in gray. No pattern emerged regarding tumor type and animal age.
(B) Examples of lymphoma histology stained with H&E (I), Ki67 (II), TUNEL (III), MAC2 (IV), CD3 (V), B220 (VI). A biphenotypic lymphoblastic lymphoma (top) and a histiocytic sarcoma (bottom) from *Mre11*^{ATLD1ATLD1}*Chk2*$^{-/-}$ animals are shown.
(C) Examples of tumor histology stained with H&E (I), Ki67 (II), or TUNEL (III). An osteosarcoma (top panels) and a basal carcinoma (bottom panels) from *Mre11*^{ATLD1ATLD1}*Chk2*$^{-/-}$ animals are shown. Diffuse Ki67 staining in the top panel is a result of decalcification.

Table S4. Pathology of *Nbs1*$^{\Delta B/\Delta B}$*Chk2*$^{-/-}$ and *Mre11*^{ATLD1ATLD1}*Chk2*$^{-/-}$ mice.

The genotypes, age, sex, and pathological assessment are listed for the double mutant cohorts.

ID	Genotype	Sex/age	Pathology
44883	*Nbs1*$^{\Delta B/\Delta B}$*Chk2*$^{-/-}$	F/5.9	Thymic lymphoma
25143	*Nbs1*$^{\Delta B/\Delta B}$*Chk2*$^{-/-}$	M/5.9	Lymphoma involving the thymus, lymph node, kidney, spleen, and lung.
23647	*Nbs1*$^{\Delta B/\Delta B}$*Chk2*$^{-/-}$	F/6.4	Enteric lymphosarcoma involving jejunum and pancreatic lymph node
26362	*Nbs1*$^{\Delta B/\Delta B}$*Chk2*$^{-/-}$	F/10.2	Thymic lymphoma
24071	*Nbs1*$^{\Delta B/\Delta B}$*Chk2*$^{-/-}$	F/12	Thymic lymphoma
24369	*Nbs1*$^{\Delta B/\Delta B}$*Chk2*$^{-/-}$	F/12.1	Histiocytic sarcoma, spleen
26013	*Nbs1*$^{\Delta B/\Delta B}$*Chk2*$^{-/-}$	F/12.7	Thymic lymphoma
23649	*Nbs1*$^{\Delta B/\Delta B}$*Chk2*$^{-/-}$	F/12.8	Mammary carcinoma w/ squamous differentiation Uterine hemangioma
30472	*Nbs1*$^{\Delta B/\Delta B}$*Chk2*$^{-/-}$	F/13.4	Mammary gland adenosquamous carcinoma
23323	*Nbs1*$^{\Delta B/\Delta B}$*Chk2*$^{-/-}$	F/14.6	Hepatic and splenic lymphoma
	Nbs1$^{\Delta B/\Delta B}$*Chk2*$^{-/-}$	F15	Histiocytic sarcoma Hemangiosarcoma
	Nbs1$^{\Delta B/\Delta B}$*Chk2*$^{-/-}$	F/15.8	Histiocytic sarcoma Mammary gland adenocanthoma
30235	*Nbs1*$^{\Delta B/\Delta B}$*Chk2*$^{-/-}$	M/16.3	Hepatocellular carcinoma
30236	*Nbs1*$^{\Delta B/\Delta B}$*Chk2*$^{-/-}$	M/16.3	Lymphoma of the submandibular and mesenteric lymph nodes
30237	*Nbs1*$^{\Delta B/\Delta B}$*Chk2*$^{-/-}$	M/16.5	Lymphoma involving the thymus and mesenteric lymph node, Pituitary gland pars intermedia adenoma with compression of overlying brain parenchyma
26527	*Nbs1*$^{\Delta B/\Delta B}$*Chk2*$^{-/-}$	F/16.9	Lymphoma involving the lung, thymus, kidney, lymph nodes, liver, spleen, pancreas and bone marrow Histiocytic sarcoma of liver
26137	*Nbs1*$^{\Delta B/\Delta B}$*Chk2*$^{-/-}$	M/17.6	Bronciolo/alveolar adenoma
23646	*Nbs1*$^{\Delta B/\Delta B}$*Chk2*$^{-/-}$	F/19.1	Lymphoma involving the liver, spleen, kidneys, lungs, lymph nodes, pancreas, adrenal gland, ovary, uterus, urinary bladder and brown adipose tissue Basophil adenoma of the pars distalis
24378	*Nbs1*$^{\Delta B/\Delta B}$*Chk2*$^{-/-}$	F/19.7	Histiocytic sarcoma
23321	*Nbs1*$^{\Delta B/\Delta B}$*Chk2*$^{-/-}$	M/19.8	Hepatoma and glycogenosis
24098	*Nbs1*$^{\Delta B/\Delta B}$*Chk2*$^{-/-}$	F/20.9	Thymic lymphoma Pituitary gland pars distalis adenoma Basal carcinomas w/ squamous differentiation
25428	*Nbs1*$^{\Delta B/\Delta B}$*Chk2*$^{-/-}$	M/22.3	Unilateral pheochromocytoma Hemangiosarcoma
21872	*Nbs1*$^{\Delta B/\Delta B}$*Chk2*$^{-/-}$	M/22..8	Histiocytic Sarcoma involving the lungs, kidneys, spleen, lymph nodes, pancreas and liver Bronchiolo/alveolar adenoma
23828	*Nbs1*$^{\Delta B/\Delta B}$*Chk2*$^{-/-}$	F/23.1	Lymphoma of lymph node

			Granulosa cell tumor Endometrial carcinoma with endometrial hyperplasia
23825	*Nbs1*$^{\Delta B/\Delta B}$*Chk2*$^{-/-}$	F/23.2	Lymphoma involving the thymus, epicardium, lungs, liver, lymph nodes, and small intestine (Peyer's patch) Bronchioloalveolar adenoma
25094	*Nbs1*$^{\Delta B/\Delta B}$*Chk2*$^{-/-}$	M/23.5	Uterine leiomyosarcoma with necrosis Pars distalis basophilic hyperplasia
25098	*Nbs1*$^{\Delta B/\Delta B}$*Chk2*$^{-/-}$	F/23.5	Lymphoma, granulosa cell tumor Bronchioalveolar carcinoma
23663	*Mre11*$^{ATLD1/ATLD1}$*Chk2*$^{-/-}$	M/7.4	Bronchoalveolar adenoma
30262		F/8.6	Lymphoma of lymph node
21500	*Mre11*$^{ATLD1/ATLD1}$*Chk2*$^{-/-}$	F/11.7	Mammary adenocarcinoma
22790	*Mre11*$^{ATLD1/ATLD1}$*Chk2*$^{-/-}$	F/11.8	Basal carcinoma w/ squamous differentiation
25135	*Mre11*$^{ATLD1/ATLD1}$*Chk2*$^{-/-}$	F/14.6	Basal cell tumor
25440	*Mre11*$^{ATLD1/ATLD1}$*Chk2*$^{-/-}$	M/14.6	Osteoblastic osteosarcoma Prostatic intraepithelial neoplasia
22024	*Mre11*$^{ATLD1/ATLD1}$*Chk2*$^{-/-}$	M/15.1	Histiocytic sarcoma Hepatocellular carcinoma
22705	*Mre11*$^{ATLD1/ATLD1}$*Chk2*$^{-/-}$	M/17.6	Multicentric lymphoma
23180	*Mre11*$^{ATLD1/ATLD1}$*Chk2*$^{-/-}$	M/17.6	Hepatoma
25129	*Mre11*$^{ATLD1/ATLD1}$*Chk2*$^{-/-}$	M/17.6	Hepatic histiocytic sarcoma
22185	*Mre11*$^{ATLD1/ATLD1}$*Chk2*$^{-/-}$	F/18.4	Thymic lymphoma
23141	*Mre11*$^{ATLD1/ATLD1}$*Chk2*$^{-/-}$	F/18.6	Lymphoma in the lungs, spleen, lymph nodes and Peyer’s patch
23665	*Mre11*$^{ATLD1/ATLD1}$*Chk2*$^{-/-}$	M/19.8	Pituitary gland pars intermedia adenoma with dorsal expansion and compression of brain Mesenteric lymph node lymphoma
22435	*Mre11*$^{ATLD1/ATLD1}$*Chk2*$^{-/-}$	M/20	Histiocytic sarcoma involving the lungs, lymph nodes, kidneys, spleen, liver and bone marrow
23896	*Mre11*$^{ATLD1/ATLD1}$*Chk2*$^{-/-}$	M/20.1	Small intestinal lymphoma with ulceration, rupture and necrosuppurative enteritis
23178	*Mre11*$^{ATLD1/ATLD1}$*Chk2*$^{-/-}$	F/20.3	Poorly differentiated spindle sarcoma
24285	*Mre11*$^{ATLD1/ATLD1}$*Chk2*$^{-/-}$	M/21.1	Lymphoma of the lymph nodes and spleen
24301	*Mre11*$^{ATLD1/ATLD1}$*Chk2*$^{-/-}$	F/21.1	Lymphoma involving the thymus, pulmonary interstitium, epicardium, kidneys, liver, spleen, lymph nodes, pancreas, ovary, oviduct and bone marrow Histiocytic sarcoma involving the kidneys, liver, gastric serosa, lymph node, ovary, oviduct, bone marrow
23905	*Mre11*$^{ATLD1/ATLD1}$*Chk2*$^{-/-}$	M/21.3	Adenocarcinoma of the Haderian gland, poorly differentiated, unilateral with osteolysis, extension rostrally to the nasal cavity and caudally to compress the brain and pulmonary metastasis Hemangiosarcoma
24170	*Mre11*$^{ATLD1/ATLD1}$*Chk2*$^{-/-}$	M/21.3	Lymphoma involving the small intestinal Peyer's patch and lymph node
21950	*Mre11*$^{ATLD1/ATLD1}$*Chk2*$^{-/-}$	M/21.5	Bronchiolo/alveolar carcinomas
23903	*Mre11*$^{ATLD1/ATLD1}$*Chk2*$^{-/-}$	F/22.4	Thymic lymphoma

23182	*Mre11*$^{ATLD1/ATLD1}$*Chk2*$^{-/-}$	F/22.6	Osteosarcoma of hindlimb with liver metastasis
23899	*Mre11*$^{ATLD1/ATLD1}$*Chk2*$^{-/-}$	M/22.7	Lymphoma involving the spleen and thymus
23186	*Mre11*$^{ATLD1/ATLD1}$*Chk2*$^{-/-}$	F/22.7	Adeno/squamous carcinoma
23902	*Mre11*$^{ATLD1/ATLD1}$*Chk2*$^{-/-}$	F/23	Basal carcinoma with squamous differentiation Stromal sarcoma of uterus
25002	*Mre11*$^{ATLD1/ATLD1}$*Chk2*$^{-/-}$	M/24	Lymphoma with evidence of leukemia involving the lungs, lymph nodes, liver, spleen, pancreas, abdominal adipose tissue and urinary bladder
21473	*Mre11*$^{ATLD1/ATLD1}$*Chk2*$^{-/-}$	M/24	Dermal/subcutaneous spindle sarcoma