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Loss of PML cooperates with mutant p53 to drive more aggressive cancers in a gender-dependent manner

Sue Haupt,[1,2,*] Catherine Mitchell,[3] Vincent Corneille,[1,2] Jake Shortt,[1,4] Stephen Fox,[3] Pier Paolo Pandolfi,[5] Mireia Castillo-Martin,[6] Dennis M. Bonal,[6] Carlos Cordon-Cardo,[6] Guillermina Lozano[7] and Ygal Haupt[1,2,8,9]

[1]Research Division; Peter MacCallum Cancer Centre; East Melbourne, VIC Australia; [2]Sir Peter MacCallum Department of Oncology; University of Melbourne; Parkville, VIC Australia; [3]Department of Pathology; Peter MacCallum Cancer Centre; East Melbourne, VIC Australia; [4]Department of Clinical Haematology; Monash Medical Centre; Clayton, Australia; [5]Cancer Genetics Program; Beth Israel Deaconess Cancer Center; Departments of Medicine and Pathology; Beth Israel Deaconess Medical Center; Harvard Medical School; Boston, MA USA; [6]Department of Pathology; Mount Sinai School of Medicine; The Mount Sinai Medical Center; New York, NY USA; [7]The University of Texas; MD Anderson Cancer Center; Houston TX USA; [8]Department of Pathology; University of Melbourne; Parkville, VIC Australia; [9]Department of Biochemistry and Molecular Biology; Monash University; Clayton, VIC Australia

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Abbreviations: PML, promyelocytic leukemia; EMH, extramedullary hematopoiesis; MCV, mean cell volume; WBC, white blood cells; RBC, red blood cells; HGB, hemaglobin; HSC, hematopoietic stem cells; LIC; leukemic initiating cells; IHC, immunohistochemistry

p53 mutations and downregulation of promyelocytic leukemia (PML) are common genetic alterations in human cancers. In healthy cells these two key tumor suppressors exist in a positive regulatory loop, promoting cell death and cellular senescence. However, the influence of their interplay on tumorigenesis has not been explored directly in vivo. The contribution of PML to mutant p53 driven cancer was evaluated in a mouse model harboring a p53 mutation (*p53*$^{wild\text{-}type/R172H}$) that recapitulates a frequent p53 mutation (*p53*^{R175H}) in human sporadic and Li-Fraumeni cancers. These mice with *PML* displayed perturbation of the hematopoietic compartment, manifested either as lymphoma or extramedullary hematopoiesis (EMH). EMH was associated with peripheral blood leucocytosis and macrocytic anemia, suggestive of myeloproliferative-myelodysplastic overlap. In contrast, a complete loss of *PML* from these mice resulted in a marked alteration in tumor profile. While the incidence of lymphomas was unaltered, EMH was not detected and the majority of mice succumbed to sarcomas. Further, males lacking PML exhibited a high incidence of soft tissue sarcomas and reduced survival, while females largely developed osteosarcomas, without impact on survival. Together, these findings demonstrate that PML is an important tumor suppressor dictating disease development in a pertinent mouse model of human cancer.

Key Points: (1) A mutant p53 allele disrupts hematopoiesis in mice, by promoting lymphomas and myeloproliferative/myelodysplastic overlap. (2) Coincidental p53 allele mutation and PML loss shifts the tumor profile toward sarcoma formation, which is paralleled in human leiomyosarcomas (indicated by immunohistochemistry; IHC).

Introduction

The tumor suppressor p53 is the most frequently mutated protein in cancer. p53 gene mutations may not only disable the normal, tumor-suppressive functions of p53, but also confer novel capabilities that promote tumorigenesis. Newly acquired properties promote tumor cell invasion of adjacent tissues, migration from the primary tumor bed, seeding of metastases and drug resistance.[1] These "gain of function" (GOF) properties are strictly imparted by mutant p53 and do not result from p53 loss (refs. 2 and 3 and reviewed in ref. 4). Mutant p53 stabilization is essential for its GOF;[5] however stabilization of mutated p53 protein is not an automatic consequence of *p53* gene mutation. The fundamental observation that mutant p53 does not accumulate in normal, healthy tissues of mice bearing germline *p53* mutations, but can be detected in tumors, implies that like wt p53, mutant p53 is inherently labile.[2,3,5] Mutant p53 accumulation in tumors must therefore result from a breakdown of the mechanisms that normally act to keep levels low. In vitro studies[6,7] and mouse models have identified that the E3 ligase Mdm2 contributes to reduced mutant p53 levels as does the p16^{INK4a} locus;[5] however, a complete delineation of the determinants of mutant p53 lability is yet to

*Correspondence to: Sue Haupt; Email: sue.haupt@petermac.org
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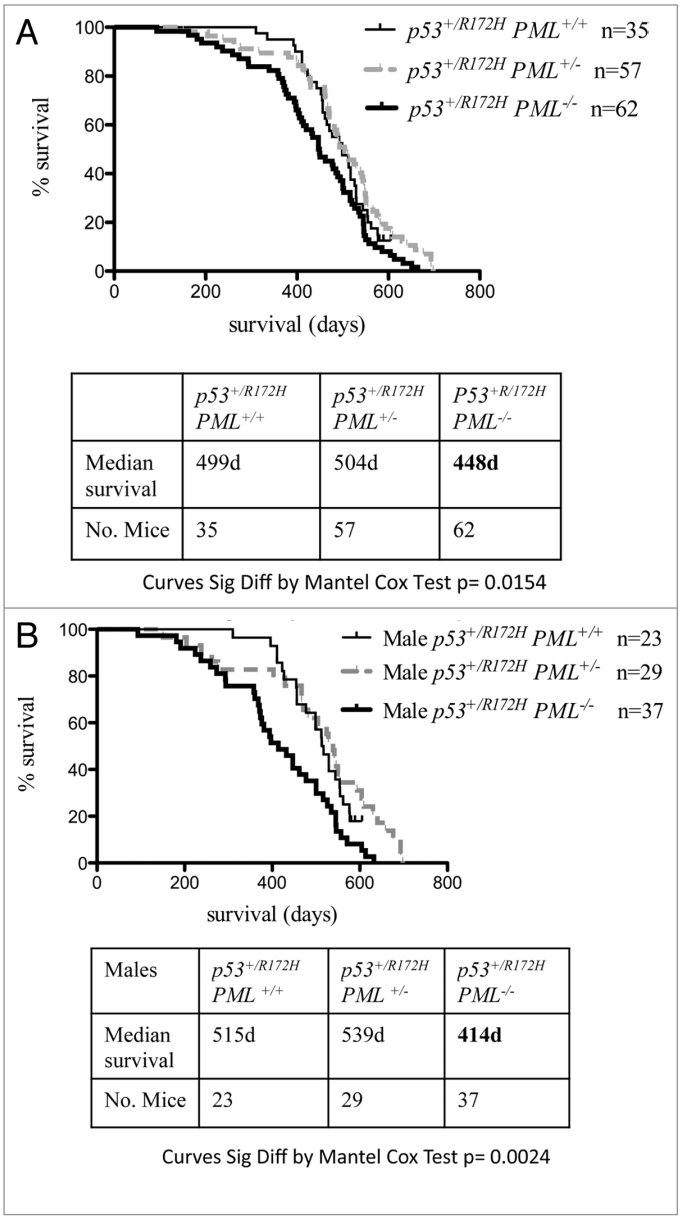

	$p53^{+/R172H}$ $PML^{+/+}$	$p53^{+/R172H}$ $PML^{+/-}$	$P53^{+R/172H}$ $PML^{-/-}$
Median survival	499d	504d	**448d**
No. Mice	35	57	62

Curves Sig Diff by Mantel Cox Test p= 0.0154

Males	$p53^{+/R172H}$ $PML^{+/+}$	$p53^{+/R172H}$ $PML^{+/-}$	$p53^{+/R172H}$ $PML^{-/-}$
Median survival	515d	539d	**414d**
No. Mice	23	29	37

Curves Sig Diff by Mantel Cox Test p= 0.0024

Figure 1. Kaplan-Meir survival curves for $p53^{+/R172H}$ mice. *PML* loss from $p53^{+/R172H}$ mice was associated with significantly lower overall survival (**A**); male mice survival was most profoundly reduced (**B**).

be achieved. Molecules that prevent mutant p53 stabilization are presumed tumor suppressors and represent potential candidates for cancer therapy.

Wt p53 becomes stabilized in response to stress, largely through extrication from Mdm2. Multiple pathways act in concert to execute stress-induced modifications of p53, facilitated by the promyelocytic leukemia (PML) protein.[8-10] PML constitutes a family of at least nine isoforms in humans.[11-14] Collectively, these isoforms are considered to be tumor suppressive, as first surmised from PML dysfunction in acute promyelocytic leukemia [due to t(15:17) translocation and *PML* fusion with the retinoic acid receptor α][11] and further elaborated with the identification of mutant PML isoforms in APL that inhibit proper function of PML and p53.[15] PML tumor-suppressive capacity was been corroborated in a mouse model for leukemia.[12] PML has been identified to impede cell proliferation through both p53-dependent and -independent mechanisms (ref. 13 and review ref. 14). PML protein downregulation or complete loss from human solid human tumors was subsequently observed in immunohistochemical (IHC) studies.[16] In mice, a loss of one or two alleles of *PML* was sufficient to exacerbate Ras- or loss of PTEN-driven specific cancers,[17,18] and PML elimination increased susceptibility to chemically induced carcinogenesis.[19] Together, these observations support a role for PML as a tumor suppressor that is frequently targeted during malignancy onset. However, the contribution of PML to the suppression of tumor onset in a mutant p53 context has not been established and is the subject of this study.

We identified that PML isoform IV interacts through its C terminus with mutant p53,[20] as it does with wt p53 (PML3);[21] however, in contrast to the stress provocation essential for its binding to wt p53 in normal cells, in cancer cells mutant p53 interaction with PML appears constitutive.[20] The contribution of PML to mutant p53 accumulation was therefore rational to interrogate. We chose to adopt the mouse model of the human germline $p53^{R175H}$ mutant [$p53^{R172H}$ mutant knock-in (KI)][3] to perform a novel, in vivo investigation of the consequence of *PML* loss, for mutant p53 accumulation and tumor development and metastasis. Here we demonstrate that in a heterozygous wt and mutant p53 context, the presence of PML prolonged survival, although most of these mice eventually succumbed to the consequences of disrupted hematopoiesis. When p53 mutation was compounded by the absence of *PML,* survival was reduced, and tumor manifestation dominated in the connective tissue, with a gender-dictated tumor spectrum.

Results

***PML* loss reduced survival in $p53^{+/R172H}$ male mice.** Survival of $p53^{+/R172H}$ mice modeling the human Li-Fraumeni syndrome was assessed as an indication of tumor aggression (dictated by the rate of onset and progression). Survival of $p53^{+/R172H}PML^{+/+}$ mice was measured for the combined population of both males and females to be around 500 d (**Fig. 1A**), which is similar to previous reports[2,3] and to $p53^{+/R172H}PML^{+/-}$ mice. However, a significant reduction in survival (by ~50 d) was evident with the loss of two *PML* alleles. Strikingly, separate analysis of male and female survival demonstrated that a complete loss of *PML* exerted a clear gender influence. Median survival of male mice without PML ($p53^{+/R172H}PML^{-/-}$) was diminished by over 100 d (to 414 d; **Fig. 1B**) compared with their male counterparts with PML. Survival in $p53^{+/R172H}$ females in contrast, was little affected by *PML* loss, and although exhibiting slightly reduced median survival to 463–488 d (**Fig. S1**), this was not significantly different from the male counterparts with PML. These data support haplosufficiency of PML function in a gender-independent manner, as loss of a single *PML* allele did not reduce the mean survival of either male or female $p53^{+/R172H}$ mice. In contrast, a gender-discriminating response to *PML* loss was demonstrated for the first time

by these studies, where male survival (but not female) was significantly reduced, in these heterozygous mutant p53 mice.

***PML* loss did not reduce *p53*$^{R172H/R172H}$ male mouse survival.** In contrast to the impact of *PML* loss on the lifespan of male *p53*$^{+/R172H}$ mice, mean survival in *p53*$^{R172H/R172H}$ mice was ~150 d regardless of PML status (**Fig. S2**), which is comparable to published findings for *p53*$^{R172H/R172H}$ mice.[2,3,22] Together these findings suggest that PML is incapable of limiting tumor development in an exclusively mutant p53 context.

The influence of gender on survival of *p53*$^{R172H/R172H}$ mice could not be evaluated due to insufficient female births, and this was not affected by PML status (**Table S1**). Poor survival of female mice lacking p53 has been attributed to female-specific exencephaly[23,24] (with the X-chromosome determining neural tube defects),[25] and a similar phenotype appears in mutant p53 mice.[2,22] This is in contrast with the near equivalent Mendelian ratios of female and male progeny of *p53*$^{wt/wt}$ and mutant *p53*$^{+/R172H}$ mice and is not influenced by PML absence (**Table S1**).

***PML* loss altered the tumor spectrum in *p53*$^{+/R172H}$ mice.** Our study for the first time demonstrates that PML has a significant impact on tumor manifestation in *p53*$^{+/R172H}$ mice. A dose-dependent loss of *PML* (one, then two alleles) led to a reduction in the incidence of lymphomas as a percentage of the total numbers of tumors (in *PML*$^{+/+}$ 52%; *PML*$^{+/-}$ 43% and *PML*$^{-/-}$ 36%) and an increase in sarcomas (from 43% to 52% and 59%, respectively, **Table 1A**), with no significant impact on carcinoma prevalence. Two tumor types were identified in some mice (**Fig. 2A**); however, the proportion of mice with multiple tumors did not change substantially with genotype (**Table 1A**). It should be added, however, that extramedullary hematopoesis (EMH; indicating altered hematopoiesis, possibly associated with a pre-leukemic myeloproliferatative neoplasm) was evident (**Table 1B** and **Fig. 2B**), without histological evidence of transformation to acute leukemia, in many of the mice containing PML, that had been ethically designated to have reached an end-point (largely due to marked hepatosplenomegaly). This EMH phenotype was associated with peripheral blood leucocytosis and macrocytic anemia, also indicative of a myeloproliferative/myelodysplastic overlap (further elaborated below).

It is pertinent to note that mutation of p53 has been suggested as a predictive marker of leukemic transformation in human myeloproliferative neoplasms,[26] and, further, the prognosis of hematological malignancy in patients harboring a p53 mutation is worse than those expressing the wt p53 protein (reviewed in ref. 27). EMH has not been previously reported for this genotype; however, distinct disease manifestations between studies have been attributed to individual genetic backgrounds.[2,3] Specifically, in our study on an advanced C57BL.6 genetic background, disruption of hematopoiesis in *p53*$^{+/R172H}$ mice with *PML* was higher than previously published.[2,3] Importantly, neither male or female *p53*$^{+/R172H}$ mice lacking *PML* were ever identified with EMH. Since females did not exhibit a significantly curtailed lifespan, it suggests that a loss of *PML* offers protection from this phenotype in the context of mutant p53 mice.

Strikingly, when tumor incidence was evaluated per mouse (including those that succumbed to EMH, rather than as a % of tumors), a high incidence of sarcomas was identified to accompany the elimination of *PML* (increasing from *PML*$^{+/+}$ 37%, to *PML*$^{+/-}$ 46% to *PML*$^{-/-}$ 72%, **Table 1B**). When tumors were segregated according to gender, and soft tissue sarcomas were distinguished from osteosarcomas, it became profoundly apparent that males compromised for PML succumbed to soft tissue sarcomas more frequently than females (**Table 1C**). These data therefore indicate that the proportion of males and females in a cohort influence the abundance of tumor types for a particular genotype. Most importantly, the average survival duration of male *p53*$^{+/R172H}$*PML*$^{-/-}$ mice with soft tissue sarcomas was shorter (380 d;

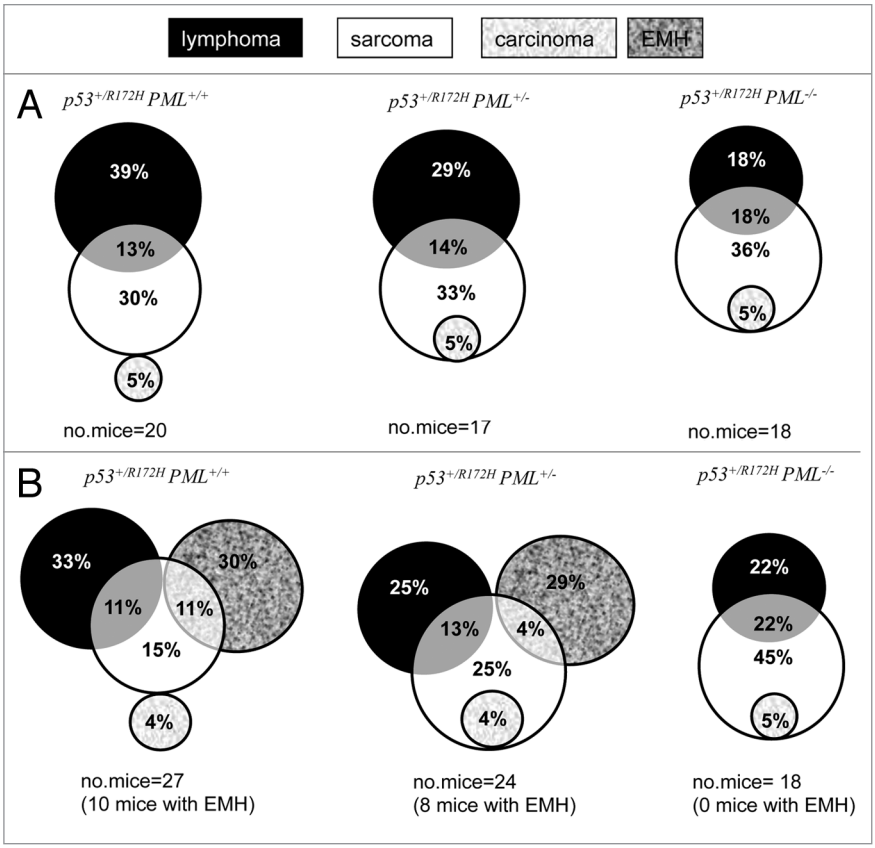

Figure 2. Tumor development in *p53*$^{+/R172H}$ mice. Abundance of lymphomas, sarcomas and carcinomas were dictated by *PML* abundance in *p53*$^{+/R172H}$ mice, as indicated by the proportion of tumors (calculated as the number of tumors of a specific tumor type/total number of tumors and expressed as a percentage) in a Venn Diagram presentation (**A**). Tumor prevalence as determined on an individual mouse basis indicated the extensive perturbation of the hematological niche in mice with *PML*; however, in mice without *PML*, sarcomas dominated (**B**).

Table 1. *PML* influences the disease profiles of $p53^{+/R175}$ mice

(A) *PML* loss influences tumor profiles in $p53^{+/R172H}$ mice (% tumor type)			
Tumor types	$p53^{+/R172H}$ $PML^{+/+}$	$p53^{+/R172H}$ $PML^{+/-}$	$p53^{+/R172H}$ $PML^{-/-}$
Lymphoma	52%	43%	36%
Sarcoma	43%	52%	59%
Carcinoma	4%	5%	5%
No. tumors	23	21	22
No. of mice with tumors	20	17	18
(B) *PML* loss influences disease manifestation $p53^{+/R172H}$ mice (% disease/total no. mice)			
Tumor types	$P53^{+/R172H}$ $PML^{+/+}$	$p53^{+/R172H}$ $PML^{+/-}$	$p53^{+/R172H}$ $PML^{-/-}$
Lymphoma	44%	38%	44%
EMH	41%	33%	0%
Sarcoma	37%	46%	**72%**
Carcinoma	4%	4%	6%
No. tumors	23	21	22
Total no. mice	27	24	18
Mean survival	499d	504d	448d
$p53^{+/R172H}$ female mice			
(C) Tumor spectrum is influenced by gender and *PML* loss in $p53^{+/R172H}$ mice (% disease/total no. mice)			
$p53^{+/R172H}$ male mice			
Tumor types	$p53^{+/R172H}$ $PML^{+/+}$	$p53^{+/R172H}$ $PML^{+/-}$	$p53^{+/R172H}$ $PML^{-/-}$
Lymphoma	50%	33%	44%
EMH	43%	33%	0%
Osteosarcoma	14%	8%	**33%**
Soft tissue sarcoma	14%	**42%**	**44%**
Carcinoma	0%	0%	0%
No. tumors	10	11	11
Total no. mice	**14**	**12**	**9**
$p53^{+/R172H}$ female mice			
Tumor types	$p53^{+/R172H}$ $PML^{+/+}$	$p53^{+/R172H}$ $PML^{+/-}$	$p53^{+/R172H}$ $PML^{-/-}$
Lymphoma	38%	42%	44%
EMH	31%	33%	0%
Osteosarcoma	38%	25%	**56%**
Soft tissue sarcoma	8%	17%	11%
Carcinoma	8%	8%	11%
No. tumors	13	10	11
No. mice	**13**	**12**	**9**

n = 4) than for all other mice, including male $p53^{+/R172H}PML^{+/-}$ mice presenting with soft tissue sarcoma, (443 d; n = 5; which manifested with a similar frequency). Further, while both male and female $p53^{+/R172H}PML^{-/-}$ mice exhibited an elevated incidence of osteosarcomas, these were proportionately more abundant in females but did not alter survival latency. These data support the finding that loss of *PML* in $p53^{+/R172H}$ resulted in an enhanced incidence of osteosarcomas in females and also males, while in males, aggressive soft tissue sarcomas were more abundant.

Hepatosplenomegaly was identified as a prominent feature of $p53^{+/R172H}$ mice, with diminished severity corresponding to *PML* loss (**Fig. 3A and B**; **Table S2A and B**, as measured by percentage body weight, and **Fig. S4**; C57BL.6:$p53^{+/R172H}PML^{+/+}$: $p53^{+/R172H}$ $PML^{+/-}$:$p53^{+/R172H}PML^{-/-}$: for spleens 1:21:9:6.5; and for livers 1:2:1.5:1-fold variation). Interestingly, $p53^{+/R172H}PML^{+/+}$ mice exhibited the lowest body weights (**Fig. S4**) and also the greatest incidence of lymphomas (and correspondingly in humans, unintentional weight loss of > 10% body weight is defined as a "B-symptom" according to the Ann Arbor staging).[28] These findings are consistent with extensive targeting of the hematopoietic system in mice with mutant p53 and PML and support the suggestion that an absence of *PML* results in earlier cancer development at alternative sights (apparently the connective tissue).

Table 2. *p53*$^{+/R172H}$ mice manifested macrocytic anemia, but less frequently with *PML* depletion

mean values	C57BL.6	*PML*$^{-/-}$	*p53*$^{+/R172H+/+}$ *PML*$^{+/+}$	*p53*$^{+/R172H}$ *PML*$^{+/-}$		*p53*$^{+/R172H}$ *PML*$^{-/-}$	
				normal HGB	**low HGB**	normal HGB	**low HGB**
HGB (g/L)	145	141.3	**64.00**	131.2	**65.0**	142.8	**33.5**
p-value		ns 0.7355	s < 0.0001	ns 0.0490	s < 0.0001	ns 0.8304	s < 0.0001
MCV (fL)	51.11	49.90	**59.41**	49.28	**64.28**	50.56	**62.01**
p-value		ns 0.3239	s 0.0307	ns 0.2110	s 0.0027	ns 0.7131	s 0.0252
RBC	9.078	8.775	**4.281**	8.106	**3.583**	8.628	**2.195**
p-value		ns 0.4893	s 0.0002	s 0.0120	s < 0.0001	ns 0.4722	s < 0.0001
WBCs (10^9/L)	3.317	4.138	**9.250**	7.276	**5.700**	6.139	8.215
p-value		ns 0.3578	s 0.0113	ns 0.1583	s 0.0394	ns 0.3062	ns 0.1419
No. mice	9	4	15	17	5	18	10

Normal ranges: HGB 118–149 g/L; MCV 42.2–59.2 fL; RBC 7–10 × 10^{12}/L; WBC 3–13 × 10^9/L ADVIA. *Significance (s) or no significance (ns) was determined using the unpaired t-test; p < 0.05, where all values were compared with C57BL.6.

Anemia and elevated WBC counts in *p53*$^{+/R172H}$*PML*$^{+/+}$ was alleviated with *PML* depletion. Anemia was identified in p53$^{+/R172H}$ mice (**Fig. 3C** and **Table 2**; **Fig. S5**) and was more severe on average in the presence of two *PML* alleles compared with mice with a single or no *PML* alleles, respectively. Intriguingly, with *PML* reduction, two populations emerged, either with normal HGB levels or with elevated levels. Anemia coincided with an elevated mean cell volume (MCV; **Fig. 3D** and **Table 2**), indicative of macrocytic anemia. Leukocytosis was also evident in *p53*$^{+/R172H}$*PML*$^{+/+}$ mice (**Fig. 3E** and **Table 2**). Together with the EMH described above, the constellation of macrocytic anemia with leukocytosis is suggestive of a myeloproliferative/myelodysplastic overlap syndrome according to current diagnostic criteria.[29]

Immunophenotyping of resected lymphomas and spleens from *p53*$^{+/R172H}$ mice identified B-cell lymphomas (**Fig. 3F**) dominating, in contrast to the predominant T-cell lymphomas in *p53*$^{-/-}$ mice.[30] The identification of B-cell lymphomas is similarly consistent with their occurrence in the presence of other p53 mutations.[2] These data are consistent with a single allele of mutant p53 driving B-cell lymphomagenesis, more profoundly in the presence of *PML*. It is interesting then that the loss of *PML* in *p53*$^{+/R172H}$ mice led to an increased abundance of T-cell lymphomas.

Mutant p53 accumulation was enhanced in the absence of *PML*. Immunoblotting of a range of tissues from male *p53*$^{+/R172H}$ *PML*$^{+/+}$, *p53*$^{+/R172H}$ *PML*$^{+/-}$ and *p53*$^{+/R172H}$ *PML*$^{-/-}$ mice demonstrated that an increased accumulation of mutant p53 accompanied *PML* loss, both in mice that had developed lymphomas and sarcomas (**Fig. 4A and B**; quantified in **Fig. S6**). Further, levels of the key oncogenic stress response protein p19^{ARF}, were identified to coincidently accumulate with increased levels of mutant p53, as *PML* diminished. Additional substantiation was provided by the examination of extra mice from each cohort, in which PML levels were verified. Interestingly, c-Myc, a known activator of p19^{ARF}, was also most profoundly accumulated in the absence of *PML*, accompanying p53 mutation in lymphomas (with only very weak detection in sarcomas, **Fig. S7**). These data support the notion that *PML* loss promotes mutant p53 accumulation, which is vital for its "gain of function" capacity.

PML loss and p53 mutation was identified in human leiomyosarcoma. PML and p53 staining of human sarcomas was undertaken to investigate whether the phenomena of PML loss and p53 mutation as identified in mice was a faithful indicator of genetic alterations in humans. PML depletion coincided with p53 mutation in a subset of leiomysarcomas (**Table S3**).

Discussion

Dysfunction of the tumor suppressor p53 network is a near universal hallmark of cancer[31] that in at least 50% of human cancers is attributed to p53 mutations,[32] which may also confer metastatic potential (as demonstrated in mouse models).[2,3] A partial or complete loss of *PML* expression is also frequently observed in multiple types of cancer.[16] In our study we examined for the combined influence of these common genetic alterations, and found that *PML* loss both reduced survival and profoundly altered the spectrum of tumors driven by the mouse equivalent of the human hotspot p53^{R175H} mutation (in heterozygous *p53*$^{+/R172H}$ mice). Mice expressing *PML* predominantly exhibited lymphomas or altered hematopoiesis reminiscent of a myeloproliferative/myelodysplastic phenotype (as evidenced by EMH, **Table 1B**), hepatosplenomegaly (**Fig. 3A and B**, macrocytic anemia and leukocytosis, **Table 2**). Notably, p53 function is critical for restricting the numbers of proliferating hematopoietic stem cells (HSC) (ref. 33 and references within), and its absence leads to greater HSC proliferation.[34,35] Strikingly, loss of *PML* resulted in a marked susceptibility to sarcomas and reduced survival. The more limited involvement of the hematopoietic compartment in the absence of *PML* may be explained, at least in part, by its role in quiescence maintenance of HSC and leukemic initiating cells (LIC; at least in some forms of leukemia).[36] In the absence of *PML*, the leukemic stem cell pool becomes exhausted, which reduces the number of LIC and hence limits tumorigenic capacity (reviewed in ref. 37).

Unexpectedly, the effect of *PML* loss was gender-specific. Explicitly, *PML* absence from male mutant p53 mice resulted in reduced survival, associated with a high prevalence of soft tissue sarcomas; suggesting that in males, cooperation between these pathways drove a more aggressive disease. In this context, it is

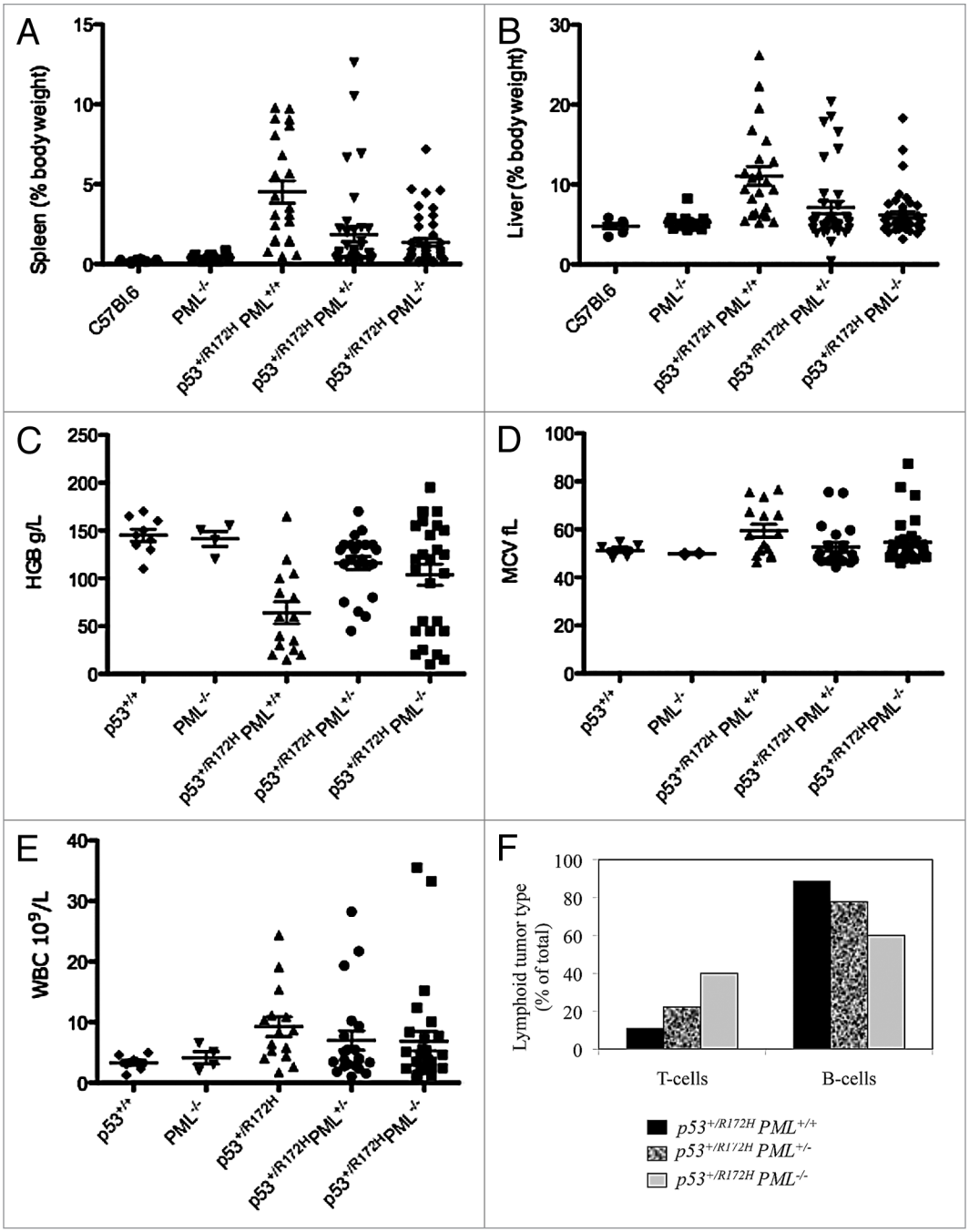

Figure 3. Disease manifestation in *p53*$^{+/R172H}$ mice. Significant splenomegaly (**A**) and hepatomegaly (**B**) were most pronounced in *p53*$^{+/R172H}$ mice with *PML*. Hematological disruption in *p53*$^{+/R172H}$ mice manifested as anemia (**C**), elevated MCV (**D**) and elevated white blood cell levels (**E**) were most pronounced in *p53*$^{+/R172H}$ mice with *PML* but only in subpopulations of *p53*$^{+/R172H}$ mice with reduced *PML*. Immunophenotyping of lymphocytes from terminally resected tumors of *p53*$^{+/R172H}$ mice identified B cell lymphomas to be most abundant in *p53*$^{+/R172H}$ mice with *PML* (**F**).

pertinent to note that significant PML loss in locally advanced and metastatic soft tissue human sarcomas corresponded with reduced time to tumor progression, duration of response and overall survival.[38,39] Clearly it would be important to know the status of p53 in these tumors, where other studies have defined a ~30% incidence of p53 mutations in human soft tissue sarcomas.[32,40] We have addressed this in a small study on selected sarcoma types and grades and identified a subset of leiomysarcomas with coincident PML loss and p53 mutation (**Table S3**). Interestingly, this rare cancer type was identified among our mice bearing mutant p53 and reduced PML; it will be fascinating to investigate this correlation on a larger population of human leiomysarcomas (and also other muscle sarcomas), for which survival data are available, to validate the prognostic value of the identified correlation. Overall, our results support a role for PML loss in the context of p53 mutation in sarcomas.

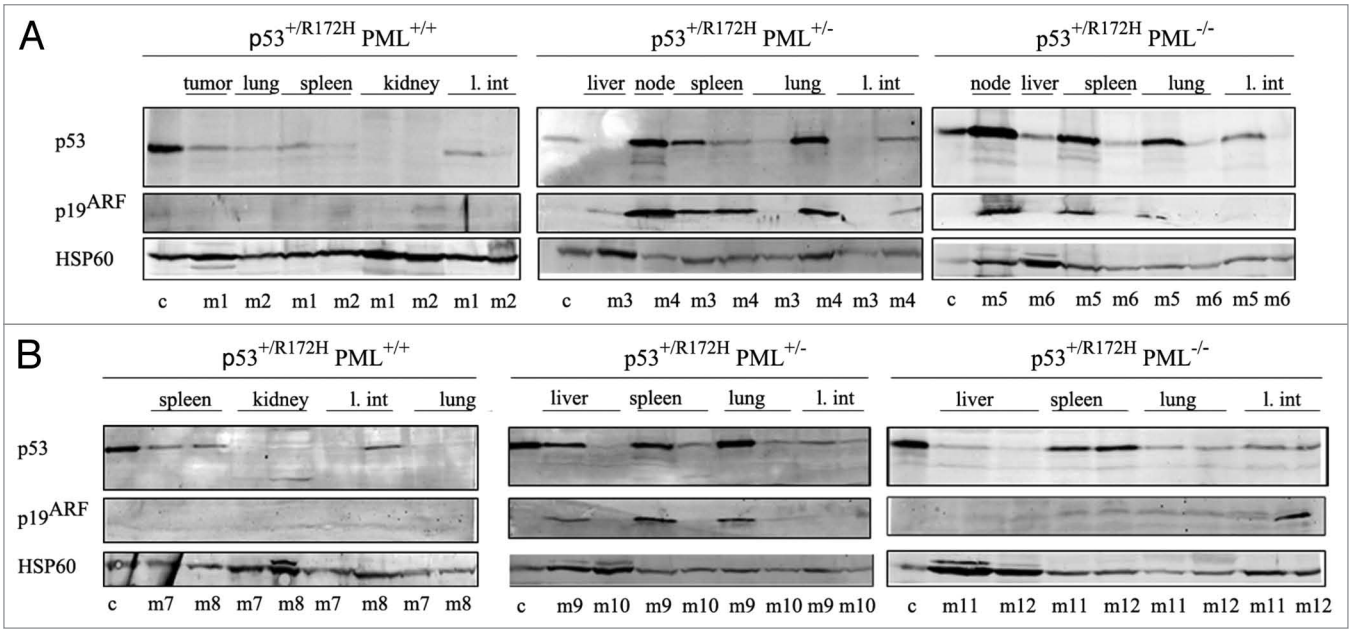

Figure 4. Western immunoblotting of a range of tissues from *p53*$^{+/R172H}$ mice stained for p53, p19^{ARF} and HSP60 diagnosed with lymphomas (**A**) and sarcomas (**B**). Control was *p53*$^{+/+}$ irradiated splenocytes. c, control; m, male.

Intriguingly, *PML* loss had no influence on the survival of mutant p53 female mice in our study. Previous studies have also shown that *PML* loss did not accelerate breast cancer induced by the (MMTV)/neu transgene.[12] Further, while *PML* loss accelerated PTEN-induced invasive colon and prostate carcinomas in males, evaluation in females was preempted by an overriding lethality that resulted from a female dominant autoimmune disease.[17] Together with our findings, it is tempting to speculate the fascinating possibility that PML loss affects males more than females, at least in the context of certain oncogenic events. It would be interesting to re-evaluate previous studies for possible gender differences, to further clarify the role of PML in solid tumors. The basis for the gender effect of PML is not clear. However, clues may lie in the recent finding that coincident mutation of the PML-regulated circadian clock gene Period2 (*Per2*)[41] and p53 mutation resulted in reduced survival of male mice but did not further reduce the diminished female survival.[22]

PML affects multiple apoptotic pathways implicated in tumor suppression (reviewed in ref. 42). Most pertinently, PML is a key partner of wt p53;[8-10] however, its capacity to also interact with mutant p53[20] led us to examine the effect of *PML* loss on the stability of mutant p53. Indeed, we found elevated levels of mutant p53 protein in tumors lacking either one or two *PML* alleles (**Fig. 4**). This is the first study to suggest that *PML* loss can induce mutant p53 accumulation. Previously, mutant p53^{R172H} has been shown to accumulate through the germ line elimination of Mdm2 or through the deletion of p16^{INK4a5} (leading to elevated levels of p19^{ARF}, able to sequester Mdm2 and promote the stabilization of p53: wt[43] and mutant p53[5]). We observed that mutant p53 accumulation in the absence of *PML* was invariably associated with elevated p19^{ARF} expression, without engineered deletion of p16^{INK4a} (**Fig. S7**). Further, an absence of *PML* was also frequently coincident with both p53 and Myc accumulation (**Fig. S7**). Myc levels are likely to have been elevated through mutant p53-driven *myc* transcription,[44] compounded by the absence of PML-driven Myc destabilization.[45] Pertinently, Myc elevation was demonstrated to be promoted by p53^{R172H} in a Ras-induced mouse skin cancer model.[46] At least in the context of wt p53, Myc activates p19^{ARF} and protects p53 from Mdm2.[47] Together with previous findings of the role of PML in the protection of p53 from Mdm2,[8-10,48] these observations support the involvement of Myc/p19^{ARF}/Mdm2 in the accumulation of mutant p53 driven by *PML* loss (**Fig. 5**), but suggest that additional pathways cannot be excluded.

Soft tissue sarcomas are a diverse collection of malignancies that represent a disproportionate abundance (~15%) of cancers in the young, and metastatic disease is very aggressive with a 5-y survival rate of 10–30%.[49] Our study reveals cooperation between *PML* loss and mutant p53, particularly in soft tissue sarcomas in males. These results parallel previous findings, which identified that high p53 levels (suggestive of mutant p53) and coincident low PML levels correlated with reduced patient survival in another malignancy: sporadic gall bladder cancer.[50] Together, these data provide a rational basis for further exploration of mutant p53 and PML as disease markers in certain human hemopoietic malignancies and sarcomas and also predict exciting possibilities for combinatorial treatments of the new era of mutant p53 specific therapies together with PML enhancing drugs.[51]

Materials and Methods

Mouse husbandry and tumor analysis. Mice knocked-in for mutant *p53*^{R172H} (bearing a G-to-A substitution at nucleotide 515 in a single p53 allele) were as previously described.[3]

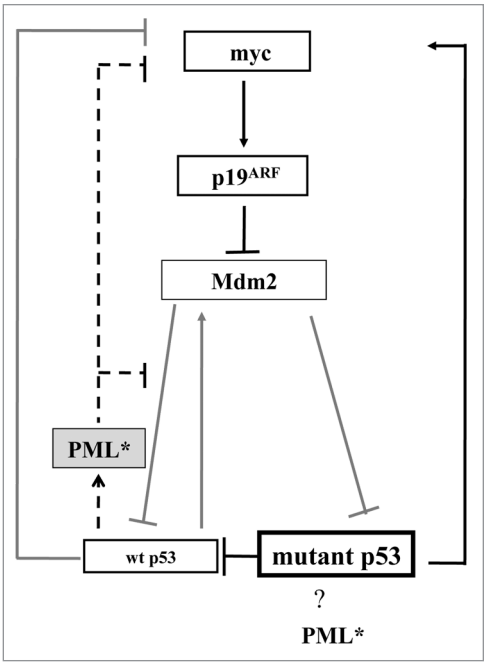

Figure 5. A postulated model mechanism of PML tumor protection in $p53^{+/R172H}$ mice. PML presence promotes wt p53 activity in response to stress provocation and preserves genomic integrity. In the absence of PML, wt p53 function is compromised through the loss of its co-activator and mutant p53 is accumulated and drives tumorigenesis. Dashed line indicates pathways dependent on PML presence. Grey pathways indicate pathways that are anticipated to be less active when PML is absent.

PML-knockout mice ($PML^{-/-}$) mice were also as previously described.[19] Mutant mice were generated on an equivalently advanced C57BL.6 background (99.61% C57BL.6, achieved by backcrossing with C57BL.6 mice for eight generations, N8) and then intercrossed to generate the experimental cohorts ($p53^{+/R172H}PML^{+/+}$; $p53^{+/R172H}PML^{+/-}$; $p53^{+/R172H}PML^{-/-}$). Mice were aged until a tumor burden was evident, or until ethical endpoints were reached, then killed per institutional guidelines. The research was approved by the institute's ethics committee. Necropsies were performed, and soft tissue organs were harvested and divided for protein analysis and microscopy. In preparation for microscopy, soft tissues were fixed in 10% neutral buffered formalin for 24 h. Skeletons were also fixed, then decalcified in hydrochloric acid/formic acid (8.5%:11%; 2 h). Fixed tissues were processed for paraffin embedding, sectioned and stained with hematoxylin and eosin. Histopathological analysis for each organ was performed by an onco-pathologist (CM). Tisssue sections were visualized using a BX-51 microscope (Olympus). Pictures were acquired using SPOT Version 4.7 software (Diagnostic Instruments).

Quantification of tumor burden and metastases. Histopathology of hematoxylin and eosin stained slides was performed on every soft tissue organ of the analyzed mice. Metastases were distinguished from primary tumors by histopathology. Mice with more than two tumor types were scored as containing multiple tumors. Decalcified bones were also analyzed where osteosarcoma was suspected.

The weight of each mouse was measured, and the weight of their livers and spleens were assessed as a percentage of total body weight.

Immunophenotyping. Mice from the $p53^{+/R172H}$ cohorts with enlarged spleens and nodes, suspected to have lymphoid malignancies were terminally resected. Single-cell suspensions of isolated lymphoid tissues were stained with the following antibodies: FITC-conjugated monoclonal anti-mouse Thy-1.2 (CD90.2; eBioscience); APC-conjugated monoclonal anti-mouse B220 (CD45R; eBioscience); FITC-conjugated anti-mouse CD4+ (L3T4; eBioscience); APC-conjugated monoclonal antibody anti-mouse CD8+ (Ly2; eBioscience). Data was collected using BD FACSCanto 2 flow cytometer and analyzed on FlowJo software. Immunophenotyping of lymphoid cells from selected animals enabled a lymphocyte spectrum analysis according to genotype.

Blood analysis. At death, cardiac puncture was performed and blood was immediately diluted 1:5 in heparin-sulfate (50 IU in 5 ml 0.9% NaCl, Pfizer) at collection and analyzed using the Advia blood analyzer for WBC and RBC.

Immunoblot analysis. Immunoblot analysis was performed essentially as described,[52] where lysates of homogenized mouse tissues were loaded for equivalent protein (20–50 μg) and separated by SDS-PAGE. Electrophoresed proteins were transferred to nitrocellulose membranes (Biorad) and probed with antibodies prior to detection using the Odyssey® Imager (LI-COR). Immunodetection of mouse proteins was undertaken using a mouse monoclonal antibody to PML (clone 36.1–104) from Upstate Biotechnology, a rabbit polyclonal antibody to p53 (CM5) from Vector Laboratories, a rabbit polyclonal antibody to CDKN2A/p19Arf (ab80) from abcam and a rabbit polyclonal antibody to c-Myc (9402) from Cell Signaling. Secondary goat ant-rabbit (926–32,211) or goat anti-mouse (926–32,220) antibodies conjugated with infrared dyes (IRDye) were purchased from LI-COR. Densitometry analysis was performed using ImageJ software (NIH) as per the request of the ImageJ developers.

Immunohistochemistry. Human tissue sections (NY; covered by institute ethic approvals) were stained for PML[51] and p53 (Santa Cruz rabbit polyclona antibody sc-6243, diluted 1:200). Staining was visualized using the chromogen diaminobenzidine together with hematoxylin as the nuclear counter stain.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Authorship Contributions

S.H. performed all the experimental work on the mice, with technical assistance from V.C.; S.H. designed the experiments and wrote the manuscript with Y.H.; pathology was assessed by C.M. and S.F.; hematological analysis was undertaken in consultation

with J.S.; mutant p53 mice were provided by G.L., PML KO mice were provided by P.P.P.; human sarcoma samples were provided, stained for IHC and analyzed by M.C-M. D.M.B. and C.C-C.

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/cc/article/24806

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