Cancer Biology

Mutant mice lacking alternatively spliced p53 isoforms unveil *Ackr4* as a male-specific prognostic factor in Myc-driven B-cell lymphomas

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Abstract

The *Trp53* gene encodes several isoforms of elusive biological significance. Here we show that mice lacking the *Trp53* Alternatively Spliced (AS) exon, thereby expressing the canonical p53 protein but not isoforms with the AS C-terminus, have unexpectedly lost a male-specific protection against Myc-induced B-cell lymphomas. Lymphomagenesis was delayed in $p53^{+/+}$ Eμ-Myc males compared to $p53^{\Delta AS/\Delta AS}$ Eμ-Myc males, but also compared to $p53^{+/+}$ Eμ-Myc and $p53^{\Delta AS/\Delta AS}$ Eμ-Myc females. Pre-tumoral splenic cells from $p53^{+/+}$ Eμ-Myc males exhibited a higher expression of *Ackr4*, encoding an atypical chemokine receptor with tumor suppressive effects. We identified *Ackr4* as a p53 target gene whose p53-mediated transactivation is inhibited by estrogens, and as a male-specific factor of good prognosis relevant for murine Eμ-Myc-induced and human Burkitt lymphomas. Furthermore, the knockout of *ACKR4* increased the chemokine-guided migration of Burkitt lymphoma cells. These data demonstrate the functional relevance of alternatively spliced p53 isoforms and reveal sex disparities in Myc-driven lymphomagenesis.

eLife assessment

This **important** study using engineered mouse models provides a first and **compelling** demonstration of a pathogenic phenotype associated with lack of expression of p53AS, an isoform of the p53 protein with a different C-terminus than canonical p53. The role of this isoform has been elusive so far and this first demonstration represents a substantial advance in our understanding of the complex role(s) of p53 isoforms. The revised manuscript adequately addresses previous concerns.

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Introduction

TP53, the human gene for tumor suppressor p53, encodes several isoforms owing to distinct promoters, alternative splicing and multiple translation initiation sites (Bourdon et al., 2005; Courtois et al., 2002; Flaman et al., 1996; Yin et al., 2002). p53 alternative isoforms can be abnormally expressed in cancer cells and some may regulate the canonical p53 protein (Anbarasan and Bourdon, 2019; Bourdon et al., 2005; Mondal et al., 2013; Senturk et al., 2014). However, aberrant RNA splicing is a common feature of cancer cells (Graubert et al., 2012; Martin et al., 2013; Pajares et al., 2007; Sette and Paronetto, 2022) and to which extent alternative splicing generates functionally relevant proteins is controversial (Abascal et al., 2015; Bardot and Toledo, 2015; Blencowe, 2017; Tress et al., 2017a, 2017b; Ule and Blencowe, 2019; Weatheritt et al., 2016). Thus, the biological importance of many p53 isoforms remains elusive.

Like its human *TP53* homolog, the murine *Trp53* gene encodes multiple isoforms differing in their N- or C-termini (Arai et al., 1986; Marcel et al., 2011). Mouse models to evaluate the role of p53 isoforms differing in their N-terminus revealed that Δ40-p53 overexpression leads to accelerated ageing (Maier et al., 2004; Steffens Reinhardt et al., 2020). However, the potential role of p53 isoforms with an alternative C-terminus was not analyzed *in vivo*. p53 isoforms with distinct C-termini result from the splicing of two mutually exclusive final exons: exon 11, encoding the canonical "α" C-terminal domain, and the Alternatively Spliced (AS) exon, encoding another C-terminus (Arai et al., 1986). In adult mice, isoforms with the canonical C-terminus are predominant in all tissues (Figure S1A). Two models ($p53^{\Delta 31}$ and $p53^{\Delta CTD}$), designed to study the consequences of a loss of the canonical p53 C-terminus, exhibited signs of increased p53 activity leading to a rapidly lethal anemia (Hamard et al., 2013; Simeonova et al., 2013). To determine the role of p53-AS isoforms *in vivo*, we created $p53^{\Delta AS}$, a mouse model with a specific deletion of the AS exon (Figure S1B). In mouse embryonic fibroblasts (MEFs), the $Trp53^{\Delta AS}$ allele prevented the expression of isoforms with the AS C-terminus whereas it did not affect RNA levels for p53 isoforms with the canonical C-terminus (Figure S1C). We previously used this model to show that p53-AS isoforms had no role in the anemia affecting $p53^{\Delta 31/\Delta 31}$ mice (Simeonova et al., 2013). However, a detailed phenotyping of $p53^{\Delta AS/\Delta AS}$ mice remained to be performed. The detailed phenotyping, presented here, yielded surprising information on lymphomagenesis.

Results

Stress responses in WT and $p53^{\Delta AS/\Delta AS}$ cells

We analyzed cellular stress responses in thymocytes, known to undergo a p53-dependent apoptosis upon irradiation (Lowe et al., 1993), and in primary fibroblasts, known to undergo a p53-dependent cell cycle arrest in response to various stresses - e.g. DNA damage caused by irradiation or doxorubicin (Kastan et al., 1992), and the Nutlin-mediated inhibition of Mdm2, a negative regulator of p53 (Vassilev et al., 2004). We first compared thymocytes from irradiated wild-type (WT) and $p53^{\Delta AS/\Delta AS}$ mice. In WT thymocytes, isoforms with the AS C-terminus were 5 times less abundant than isoforms with the α C-terminus at the RNA level (**Figure 1A**), and in western blots the p53-AS protein appeared as a faint band running just ahead of, and often hard to separate from, the band specific for p53-α, the canonical full-length p53 (**Figure 1B**). In $p53^{\Delta AS/\Delta AS}$ thymocytes, mRNA levels for α isoforms were slightly decreased, if at all (**Figure 1A**), whereas p53-α protein levels appeared markedly decreased (**Figure 1B**), raising the possibility that p53-AS isoforms might contribute to p53-α abundance. Nevertheless, the transactivation of classical p53 target genes (**Figure 1C**) and apoptotic response (**Figures 1D** and S1D) were not

significantly altered by the loss of AS isoforms. Likewise, no significant difference was observed between WT and $p53^{\Delta AS/\Delta AS}$ fibroblasts in assays for cell cycle control (**Figures 1E** and S1E), expression of well-known p53 target genes (**Figure 1F** and S1F-G), proliferation under hyperoxic conditions (**Figure 1G**), or the growth of tumor xenografts (**Figure 1H**).

Lymphomagenesis in WT and $p53^{\Delta AS/\Delta AS}$ mice

We compared spontaneous tumor onset in WT and $p53^{\Delta AS/\Delta AS}$ littermates for over 2 years and observed no significant difference in tumor-free survival (**Figure 1I**). Because lymphoma is a common neoplasm in C57BL6/J WT mice (Brayton et al., 2012) and our mouse cohorts resulted from >10 generations of backcrosses with C57BL6/J mice, we searched for evidence of lymphoma in the lymph nodes and spleen, by macroscopic examination at autopsy and histological analyses. B-cell lymphomas were observed in about 30% of mice of either genotype (**Figure 1J**). In $p53^{+/+}$ mice, a higher incidence of B-cell lymphomas was observed in females, in agreement with previous observations (Brayton et al., 2012). By contrast, no obvious sex-specific bias was observed for B-cell lymphomas in $p53^{\Delta AS/\Delta AS}$ mice (**Figure 1J**), raising the possibility that the loss of p53-AS isoforms affected B-cell lymphomagenesis. However, the numbers of lymphoma-bearing mice were too small to be conclusive.

We next used Eμ-Myc transgenic mice, prone to highly penetrant B-cell lymphomas (Adams et al., 1985). $p53^{+/+}$ Eμ-Myc and $p53^{\Delta AS/\Delta AS}$ Eμ-Myc mice developed B-cell lymphomas (Figure S2A) with similar survival curves when sexes were not considered (Figure S2B). Importantly however, death was accelerated, and tumor lymph nodes were larger, in $p53^{\Delta AS/\Delta AS}$ Eμ-Myc males compared to their $p53^{+/+}$ Eμ-Myc male counterparts, whereas no difference in lymphomagenesis was noticeable between $p53^{\Delta AS/\Delta AS}$ Eμ-Myc and $p53^{+/+}$ Eμ-Myc female mice (**Figure 2A-B**). Our data (**Figures 2A-B** and S2C), together with the fact that B-cell lymphomas occur with a higher incidence in WT C57Bl6/J female mice (Brayton et al., 2012), suggested that $p53^{+/+}$ Eμ-Myc male mice are more refractory to B-cell lymphomas, and that p53-AS isoforms might confer this male-specific protection against lymphomagenesis.

Cause for accelerated lymphomagenesis in $p53^{\Delta AS/\Delta AS}$ E_μ-Myc males

We next aimed to determine the mechanisms underlying the accelerated lymphomagenesis in $p53^{\Delta AS/\Delta AS}$ Eμ-Myc males. Inactivating p53 mutations were not more frequent in tumors from $p53^{\Delta AS/\Delta AS}$ Eμ-Myc males than in those from $p53^{+/+}$ Eμ-Myc males, ruling out additional mutations at the *Trp53* locus as potential causes for accelerated lymphomagenesis in $p53^{\Delta AS/\Delta AS}$ Eμ-Myc males (Figures S2D-E). We next analyzed a subset of tumors with no detectable *Trp53* mutation in males of both genotypes and found that Myc was expressed at similar RNA and protein levels in all tumors (**Figure 2C**). No difference in p53-α mRNA levels was observed in tumors from both genotypes, although a decrease at the protein level was detected in most tumors from $p53^{\Delta AS/\Delta AS}$ Eμ-Myc males (**Figure 2D**). Nevertheless, similar transcript levels for classical p53 target genes were observed in tumor cells of both genotypes (**Figure 2E**). To test whether a higher tumor volume in $p53^{\Delta AS/\Delta AS}$ Eμ-myc males might result from lower apoptosis and/or higher cell proliferation, we next analyzed tumors by immunohistochemistry with antibodies against cleaved caspase-3 or ki-67, respectively. Similar apoptotic and proliferation indexes were observed for both genotypes (**Figure 2F-G**). In sum, classical assays for p53 activity in tumors failed to account for differences between the two male genotypes.

The speed of lymphomagenesis in Eμ-Myc mice correlates with the extent of B-cell expansion in the first stages of B cell differentiation (Langdon et al., 1986) and p53 was proposed to control the pool of early B cells (Slatter et al., 2010). Therefore, we determined the levels of the early

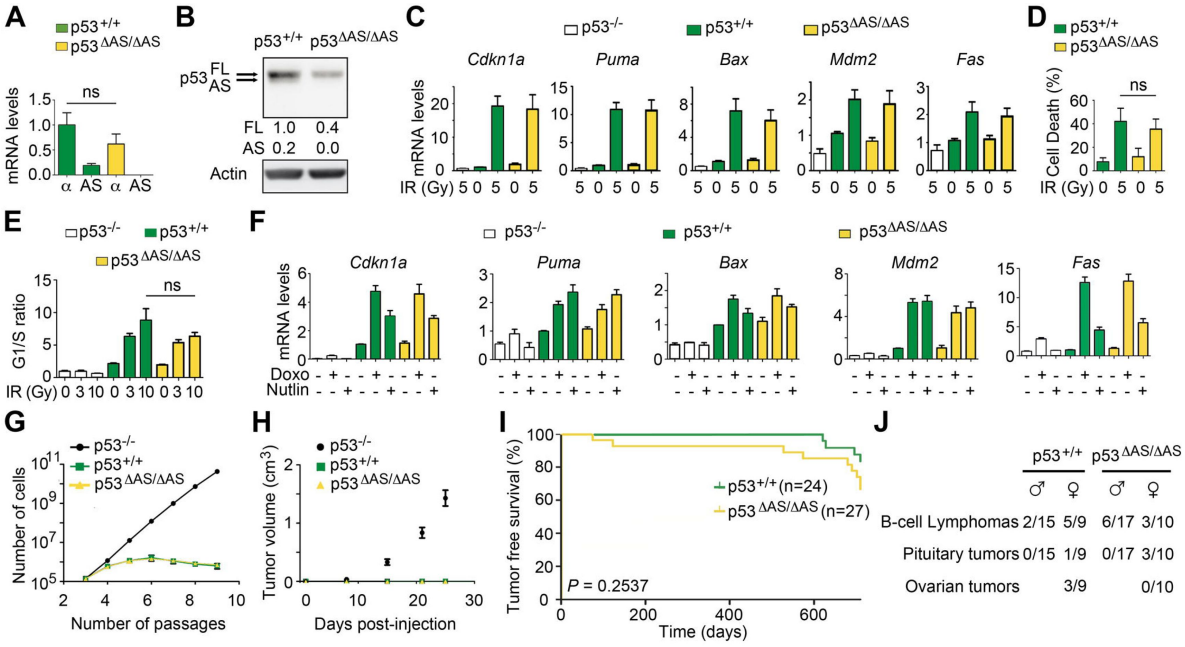

Figure 1.

The loss of p53-AS isoforms does not alter cellular stress responses or survival to spontaneous tumors.

(**A**) mRNAs for p53-α and p53-AS isoforms from thymocytes of irradiated mice were quantified by RT-qPCR, and p53-α levels in $p53^{+/+}$ mice were assigned a value of 1. Means ± SEM (n=3). (**B**) Protein extracts from thymocytes of irradiated mice were immunoblotted with p53 or actin antibodies. After normalisation to actin, full-length (FL) p53-α levels in $p53^{+/+}$ thymocytes were assigned a value of 1. (**C**) mRNA levels of p53 target genes in thymocytes, before or after γ-irradiation. Means ± SEM (n=3). (**D**) Thymocyte apoptotic response to γ-irradiation. Means ± SEM (n=6). (**E**) Cell cycle control in mouse embryonic fibroblasts (MEFs) after γ-irradiation. Asynchronous MEFs were exposed to 0-10 Gy γ-irradiation, and after 24h, cells were labelled with BrdU for 1h and analyzed by FACS. Means ± SEM from >3 independent experiments with at least 2 independent MEF clones per genotype. (**F**) mRNA levels of p53 target genes in MEFs untreated or treated with 0.5 µg/ml of clastogenic Doxorubicin (Doxo) or 10 µM of the Mdm2 antagonist Nutlin. Means ± SEM from > 3 experiments with ≥ 2 independent MEF clones. (**G**) MEFs proliferation under hyperoxic conditions. Cells were grown according to a 3T3 protocol. Each point is the mean from 4 independent MEF clones, the value for each clone resulting from triplicates. (**H**) Growth of tumor xenografts. E1A+Ras-expressing MEFs were injected into the flanks of nude mice and tumor volumes were determined after 1-25 days. Means ± SD (n=4 per timepoint and genotype). (**I**) Tumor-free survival of $p53^{+/+}$ and $p53^{\Delta AS/\Delta AS}$ mice (n=cohort size). (**J**) Incidence of the indicated tumor types, determined at death after macroscopic examination and histological analysis. In A, D, E, ns=non-significant in Student's t-test.

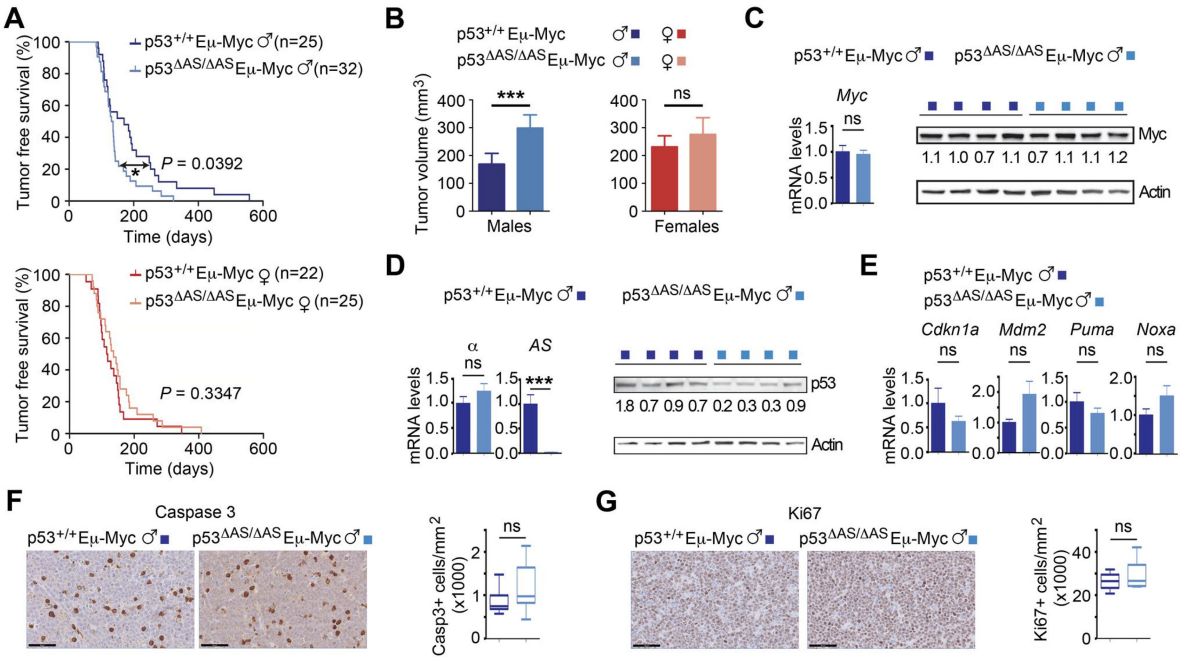

Figure 2.

Male-specific acceleration of Myc-induced B-cell lymphomagenesis in mice lacking p53-AS isoforms.

(**A**) Tumor-free survival of $p53^{+/+}$ Eμ-Myc and $p53^{\Delta AS/\Delta AS}$ Eμ-Myc mice, classified according to sex. (n=cohort size). (**B**) Tumor volumes upon dissection of $p53^{+/+}$ Eμ-Myc and $p53^{\Delta AS/\Delta AS}$ Eμ-Myc mice, classified according to sex. Means ± SEM from 100 lymph nodes from $p53^{+/+}$ Eμ-Myc males, 96 from $p53^{+/+}$ Eμ-Myc females, 148 from $p53^{\Delta AS/\Delta AS}$ Eμ-Myc males and 124 from $p53^{\Delta AS/\Delta AS}$ Eμ-Myc females. (**C**) Myc mRNA and protein levels in lymph node tumors. (**D**) Levels of p53-α and p53-AS transcripts and p53 protein levels in lymph node tumors. (**E**) Transcript levels of the indicated p53 target genes. Means ± SEM (n=6 per genotype). (**F-G**) Apoptosis (F) and cell proliferation (G) in tumor lymph nodes from Eμ-Myc males were determined by immunohistochemistry with antibodies against cleaved caspase-3 and ki67, respectively. Positive cells were counted and normalized to the analyzed areas. Means ± SEM (n=6 mice per assay and genotype). In F, G scale bars=50 μm. Statistical analyses with Mantel-Cox (A) and Student's t (B-G) tests. ***P<0.001, *P<0.05, ns: non-significant.

pre-B / immature B cells in 6 weeks-old mice, before any sign of tumor. We analyzed the spleen, a preferential site of B-cell expansion (Langdon et al., 1986) with a relatively high AS/α isoform ratio (Figure S1A). Flow cytometry with a combination of markers was used to discriminate the pre-B, immature, transitional and mature B subpopulations. As expected (Langdon et al., 1986), we observed high numbers of pre-B and immature B cells in Eμ-Myc mice. In males, pre-B and immature B cells were more abundant in $p53^{\Delta AS/\Delta AS}$ Eμ-Myc animals, while no difference was observed for transitional and mature B cells (**Figures 3A** and S3A). By contrast, in the spleen of $p53^{+/+}$ and $p53^{\Delta AS/\Delta AS}$ 6 weeks-old male mice without the Eμ-Myc transgene, most B cells were mature B cells (Figure S3B). In Eμ-Myc females, the numbers of pre-B and immature B cells were similar between genotypes, as were the numbers of transitional and mature B cells (**Figure 3A**). Interestingly, $p53^{+/+}$ Eμ-myc males, which develop lymphomas less rapidly, exhibited the lowest number of immature B cells (**Figure 3A**), suggesting a direct correlation between the level of immature B cell expansion and the speed of lymphomagenesis. Together, these data suggested that p53-AS isoforms may not be required to control the pool of early B cells under normal conditions, but that in an Eμ-Myc context they would limit the expansion of pre-tumor early B cells, specifically in males.

Transcriptomes from $p53^{+/+}$ E_μ-Myc and $p53^{\Delta AS/\Delta AS}$ E_μ-Myc male spleens

We next performed bulk RNA-seq and differential expression analyses comparing the spleens from 4-6 weeks-old $p53^{\Delta AS/\Delta AS}$ Eμ-Myc males to spleens from age-matched $p53^{+/+}$ Eμ-Myc males. This revealed a limited number of significantly deregulated genes (**Figure 3B**), including 13 protein-coding genes and 11 pseudogenes (**Figure 3C**). Out of the 13 protein-coding genes, we focused on the 10 genes not encoding an immunoglobulin and analyzed the same samples by RT-qPCR (**Figure 3D**). For 6 of the 10 genes, expression levels were too low to be quantified (*Tcstv3*), or differences in expression were not statistically significant (*Masp2*, *Akr1c19*, *Cd300lh*, *Il5ra*, *Slc26a1*). Of note, *Il5ra* belonged to this group, although it is regulated by p53 (Zhu et al., 2022), which illustrates the difficulty to analyze subtle effects in our experiments. Taking this into account, we considered as potentially interesting the 4 remaining genes, exhibiting differences in mRNA levels with statistical significance ($p<0.05$) or borderline statistical significance ($p=0.057$): *Ackr4*, less expressed in $p53^{\Delta AS/\Delta AS}$ Eμ-Myc males, and *Fam132a*, *Mt2* and *Prss50*, with an increased expression in $p53^{\Delta AS/\Delta AS}$ Eμ-Myc males (**Figure 3D**). Importantly, survival curves indicated that the Myc-induced lethality was delayed in $p53^{+/+}$ Eμ-Myc males compared to $p53^{\Delta AS/\Delta AS}$ Eμ-Myc males, $p53^{+/+}$ Eμ-Myc females and $p53^{\Delta AS/\Delta AS}$ Eμ-Myc females (Figure S2C). Thus, we quantified transcripts for *Ackr4*, *Fam132a*, *Mt2* and *Prss50* in the spleen of 4-6 weeks-old $p53^{+/+}$ Eμ-Myc and $p53^{\Delta AS/\Delta AS}$ Eμ-Myc females, then compared mRNA levels in $p53^{+/+}$ Eμ-Myc males versus the 3 other groups. Significantly higher expression of *Ackr4* and lower expression of *Mt2* were found in $p53^{+/+}$ Eμ-Myc males (**Figure 3E**).

Ackr4 (also known as *Ccrl1*) encodes the atypical chemokine receptor 4, a decoy receptor promoting the degradation of chemokines that modulate cancer cell proliferation and metastasis (Chow and Luster, 2014; Marcuzzi et al., 2018; Müller et al., 2001). Our data suggested that *Ackr4* might be a gene transactivated by p53-α and/or p53-AS isoforms. Consistent with this, by extracting data from a transcriptome-wide study in MEFs (Younger et al., 2015) we found evidence for a p53-dependent transactivation of *Ackr4* in response to doxorubicin (**Figure 3F**). Furthermore, ChIP-Atlas, the database of chromatin immunoprecipitation experiments (Oki et al., 2018), indicated p53 binding to sequences within the intron 1 of *Ackr4* in doxorubicin-treated MEFs, and we identified a candidate p53 responsive element in this intron (**Figure 3G**). We next used luciferase assays to show that this p53 responsive element can be bound and regulated by both p53-α and p53-AS (**Figures 3G-H** and S3C). Together, these data show that *Ackr4* is indeed a p53 target gene, although RNAseq data indicated that it is expressed at much lower levels than

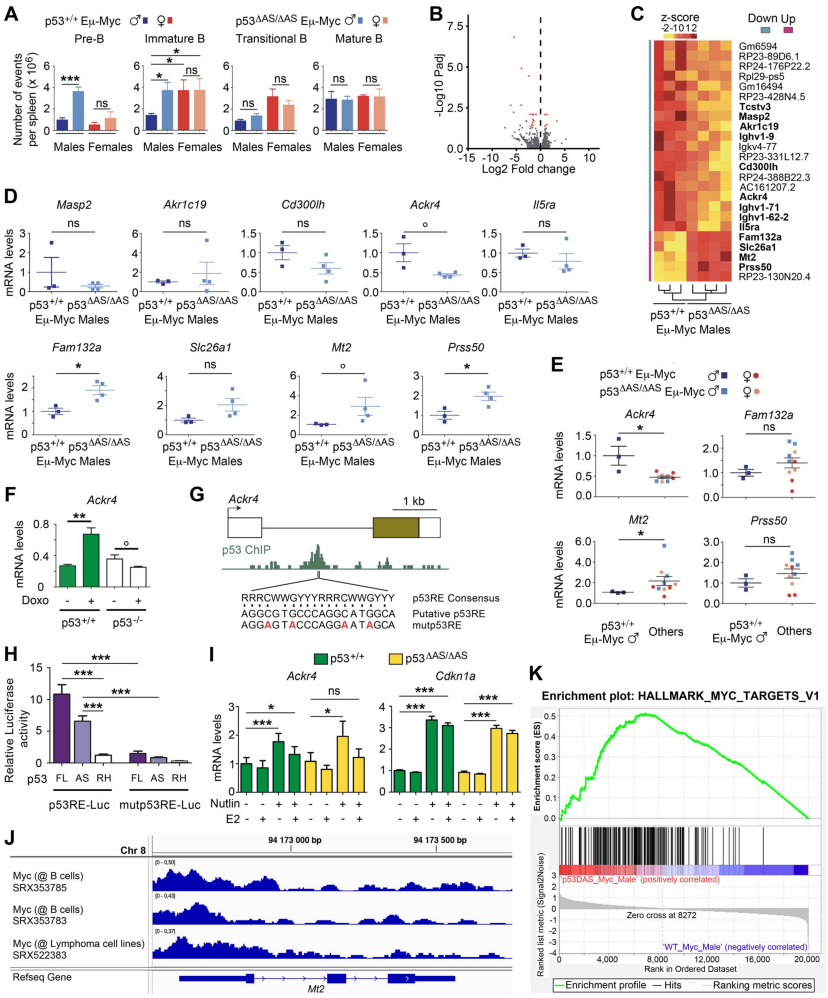

Figure 3.

The loss of p53-AS isoforms affects *Ackr4* expression in E_{μ}-Myc male mice.

(**A**) B-cell subpopulations in spleens of 6 weeks-old $p53^{+/+}$ Eμ-Myc and $p53^{\Delta AS/\Delta AS}$ Eμ-Myc mice. Means ± SEM (n=6 per genotype). (**B-C**) RNAseq analysis of spleens from $p53^{+/+}$ Eμ-Myc (n=3) and $p53^{\Delta AS/\Delta AS}$ Eμ-Myc (n=4) 4-6 weeks-old male mice. Volcano plot (B), with differentially expressed genes (DEGs) in red. Unsupervised clustering heat-map plot (C), with DEGs ranked according to mean fold changes, and protein-coding genes in bold. (**D**) RT-qPCR analysis of candidate DEGs from spleens of $p53^{+/+}$ Eμ-Myc males and $p53^{\Delta AS/\Delta AS}$ Eμ-Myc males. Means ± SEM (n=3-4 per genotype). (**E**) RT-qPCR analysis of indicated DEGs from spleens of 4-6 weeks-old $p53^{+/+}$ Eμ-Myc males, $p53^{\Delta AS/\Delta AS}$ Eμ-Myc males, $p53^{+/+}$ Eμ-Myc females and $p53^{\Delta AS/\Delta AS}$ Eμ-Myc females. Means ± SEM (n=3-4 per sex and genotype). (**F**) *Ackr4* is transactivated by p53 in response to stress. Ackr4 mRNAs in untreated or doxorubicin-treated WT and $p53^{-/-}$ MEFs. Data from 2-3 MEFs per genotype (Younger et al., 2015). (**G**) A putative p53 response element in *Ackr4* intron 1. Top: Map of the *Ackr4* gene. (boxes: exons (brown box: translated region), black line: intron 1); middle: p53 ChIP in doxorubicin-treated MEFs according to ChIP-Atlas (SRX270554) (Oki et al., 2018); bottom: p53 Response Element (p53RE) consensus sequence (R=G or A, W=A or T, Y=C or T), the putative p53RE and its mutated counterpart. (**H**) Luciferase assays of the candidate p53RE. A 1.5 kb fragment containing the WT or mutant p53RE was cloned upstream a luciferase reporter, then transfected into $p53^{-/-}$ MEFs together with an expression plasmid for full length p53 (FL), p53-AS or the DNA-binding mutant $p53^{R270H}$ (RH). Means ± SEM (n=4-6). (**I**) In MEFs, p53 activation leads to an increased *Ackr4* expression attenuated by estradiol. *Ackr4* and *Cdkn1a* mRNAs were quantified by RT-qPCR from $p53^{+/+}$ and $p53^{\Delta AS/\Delta AS}$ MEFs, untreated or treated with 10 μM Nutlin and/or 5 μg/ml 17-β estradiol (E2). Means ± SEM from 4 independent experiments. (**J**) Evidence for Myc binding at the *Mt2* promoter in B-cells. ChIP-Atlas reports Myc binding to *Mt2* promoter sequences in primary B-cells from the lymph nodes of Eμ-Myc mice (SRX353785, SRX353783) and in Eμ-Myc-induced lymphoma cells (SRX522383). Chr: chromosome. (**K**) Gene set enrichment analysis (GSEA). GSEA, performed in $p53^{+/+}$ Eμ-Myc and $p53^{\Delta AS/\Delta AS}$ Eμ-Myc male splenic cells, indicated an enrichment of hallmark Myc targets in $p53^{\Delta AS/\Delta AS}$ Eμ-Myc cells. In A, D, E, F, H, I ***P<0.001, **P<0.01, *P<0.05, °P≤0.057, ns: non-significant in Student's t or Mann-Whitney tests.

classical p53 targets like *Cdkn1a* or *Mdm2* in the splenic cells of Eμ-Myc mice (Table S1). Furthermore, *Ackr4* was shown to be regulated by Foxl2 and estrogen signalling in ovarian cells (Georges et al., 2014) and 17-β estradiol was recently found to regulate *ACKR4* expression in meniscal cells from both sexes, albeit differentially (Knewtson et al., 2020). Accordingly, we observed, in both WT and $p53^{\Delta AS/\Delta AS}$ MEFs, that p53 activation with the Mdm2 antagonist Nutlin led to the transactivation of *Ackr4,* but that a concomitant treatment with 17-β estradiol markedly decreased, or completely abrogated, *Ackr4* transactivation (**Figure 3I**). By contrast, *Cdkn1a* was efficiently transactivated under both conditions in mutant and WT cells (**Figure 3I**). These data indicate that *Ackr4* is a p53 target gene whose p53-mediated transactivation can be inhibited by estrogens.

The *Mt2* gene, encoding the potentially oncogenic metallothionein-2 (Si and Lang, 2018), was less expressed in $p53^{+/+}$ Eμ-Myc male pre-tumoral splenic cells, which raised the possibility of its direct or indirect repression by p53, potentially through the binding of p53 or the DREAM complex at its promoter (Engeland, 2018; Peuget and Selivanova, 2021). However, ChIP-Atlas reported no binding of these proteins at the *Mt2* promoter. Alternatively, evidence that Myc may impact on *Mt2* expression was obtained previously (Qin et al., 2021), and ChIP-Atlas reported Myc binding at the *Mt2* promoter in primary B-cells from lymph nodes of Eμ-Myc mice as well as Eμ-Myc-induced lymphoma cells (**Figure 3J**). This may suggest that the lower expression of *Mt2* in pre-tumoral splenic cells from $p53^{+/+}$ Eμ-Myc males (**Figure 3E**) might result from a subtle difference in Myc signalling. Consistent with this, the comparison of transcriptomes of splenic cells from $p53^{\Delta AS/\Delta AS}$ Eμ-Myc males and $p53^{+/+}$ Eμ-Myc males, when analyzed by gene set enrichment analysis (Subramanian et al., 2005), revealed an enrichment of hallmark Myc target genes in $p53^{\Delta AS/\Delta AS}$ Eμ-Myc male splenic cells (**Figure 3K**, Table S2).

Relevance of *ACKR4* expression in Burkitt lymphomas

Murine and human alternatively spliced p53 isoforms exhibit structural differences (Marcel et al., 2011) and the ChIP-Atlas database (Oki et al., 2018) does not report p53 binding to the human *ACKR4* intron 1. Nevertheless, we found that p53 activation in human cells also led to an increased *ACKR4* expression abrogated by 17-β estradiol, whereas 17-β estradiol had no significant effect on the p53-mediated transactivation of *CDKN1A* (**Figure 4A**). This led us to investigate the potential relevance of *ACKR4* expression in human B-cell lymphomas. We analyzed public databases of B-cell lymphomas patients with clinical and gene expression information. We first analyzed #GSE4475 (Hummel et al., 2006), a dataset of mature aggressive B-cell lymphomas previously used to define Burkitt lymphoma-like specific transcriptomes, comprising 159 patients (91 men, 68 women) with clinical follow-up. Overall, *ACKR4* gene expression was not significantly different between male and female patients (**Figure 4B**, left). However, average mRNA levels appeared higher in males when we considered the 30% patients of each sex with the highest *ACKR4* expression (**Figure 4B**, right). Strikingly, when we compared the survival of 30% patients with the highest ACKR4 mRNA levels to the survival of the 30% patients with the lowest ACKR4 mRNA levels, high *ACKR4* expression correlated with a better prognosis in men, but not in women (**Figure 4C**). By contrast, for *MT2A,* the human homolog of *Mt2*, differences in mRNA levels did not correlate with significant differences in survival for either sex (Figure S4A).

We also analyzed dataset #GSE181063 (Lacy et al., 2020), comprising mostly diffuse large B-cell lymphomas (DLBCL; 613 men, 536 women) and a few Burkitt lymphomas (65 men, 18 women). We found no difference in survival curves of DLBCL patients with low versus high *ACKR4* levels, neither in men nor in women. However, there was again an increased survival for Burkitt lymphoma male patients with high *ACKR4* expression, but not for women (Figure S4B). Next, we analyzed #phs000235 (Morin et al., 2011), a Burkitt lymphoma-specific dataset (65 men, 37 women) comprising mostly patients diagnosed at 0-17 years of age, hence providing cohorts homogeneous for both tumor type and age of onset. Again, *ACKR4* was expressed at higher levels in a subset of male patients (**Figure 4D**) and high *ACKR4* expression correlated with a better prognosis only in males (**Figure 4E**). Finally, we analyzed dataset #GSE136337 (Danziger et al.,

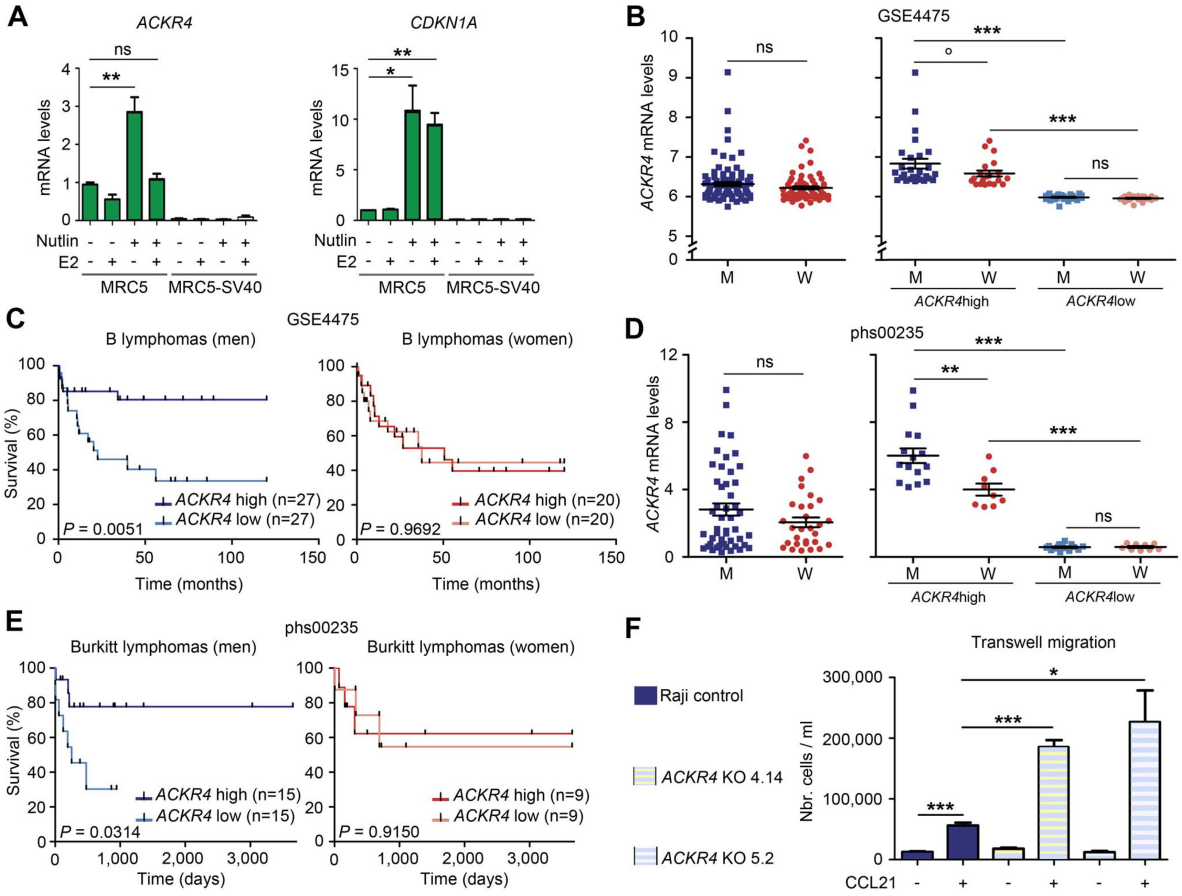

Figure 4.

ACKR4 is a male-specific prognostic factor in Burkitt lymphoma.

(**A**) In human cells, p53 activation leads to an increased *ACKR4* expression abrogated by estradiol. *ACKR4* and *CDKN1A* mRNAs were quantified by RT-qPCR from p53-proficient (MRC5) and p53-deficient (MRC5-SV40) human fibroblasts, untreated or treated with Nutlin and/or estradiol (E2). Means ± SEM from 4 independent experiments. (**B-C**) Analysis of lymphoma dataset #GSE4475. *ACKR4* gene expression was plotted for all lymphoma patients with clinical follow-up (91 men [M], 68 women [W]), classified according to sex (B, left). Gene expression (B, right) or survival curves (C) were plotted for the 30% patients (27 men, 20 women) with the highest or lowest *ACKR4* expression, classified according to sex. (**D-E**) Analysis of Burkitt lymphoma-specific dataset #phs00235. *ACKR4* gene expression was plotted for all patients with a Burkitt lymphoma diagnosed at age 0-17 (48 males, 29 females), classified according to sex (D, left). Gene expression (D, right) or survival curves (E) were plotted for the 30% patients (15 men, 9 women) with the highest or lowest *ACKR4* expression, classified according to sex. (**F**) The knockout of *ACKR4* in Burkitt lymphoma Raji cells increases their CCL21-guided migration. Chemotaxis was assayed by using Boyden chambers with bare polycarbonate membranes as previously described (Calpe et al., 2011). Equal number of cells were deposited on the membrane of a transwell insert, then migration was determined by counting cells in the lower compartment, after 15h of culture with or without CCL21 added to the lower chamber. Statistical analyses by Student's t or Mann-Whitney tests (A, B, D, F) and Mantel-Cox (C, E) test. ***P<0.001, **P<0.01, * P<0.01, °P=0.054, ns: non-significant.

2020), comprising data from the malignant plasma cells of patients with multiple myeloma (260 men, 166 women), and found that *ACKR4* is not a prognostic factor for this cancer type (Figure S4C). Altogether, these analyses led us to conclude that, as in Eμ-Myc mice, *ACKR4* is a male-specific positive prognostic factor in Burkitt lymphoma, the archetype of MYC-driven B-cell lymphomas.

We next considered the possibility that ACKR4 might be more than a biomarker, if it acts as a suppressor of MYC-driven B-cell lymphomagenesis. In support of this hypothesis, ACKR4 scavenges the chemokine CCL21, a ligand of the chemokine receptor CCR7 (Bastow et al., 2021; Ulvmar et al., 2014), and the ACKR4-mediated sequestration of CCL21 may impair the CCR7 signalling cascade, which might lead to decreased MYC activity (Shi et al., 2015). Consistent with this, a *Ccr7* deficiency was shown to delay the lymphomagenesis induced by Eμ-Myc in mice (Rehm et al., 2011). In addition, Ccr7 is required for lymphoma cell lodging to secondary lymphoid organs (Rehm et al., 2011) and ACKR4 expression was inversely correlated with the metastasis capacity of different types of cancer cells (Shi et al., 2015; Zhu et al., 2014). These data suggested that ACKR4 might regulate the behavior of Burkitt lymphoma cells. To test this hypothesis, we designed a CRISPR-Cas9 approach to perform the knockout of *ACKR4* in Raji cells. Raji is a Burkitt lymphoma cell line isolated from a 11 years-old male patient (Pulvertaft, 1964). Although p53 is mutated in Raji cells (Duthu et al., 1992), these cells overexpress MYC (Hamlyn and Rabbitts, 1983) and express both ACKR4 and CCR7 (Ferreira et al., 2014), suggesting that they might be suitable to evaluate the impact of ACKR4 on Burkitt lymphoma cell behavior, particularly their migratory capacities. Raji cells were transfected with a vector expressing Cas9, a puromycin resistance gene and either of two guide RNAs targeting *ACKR4* (or no guide RNA for control), then puromycin-resistant cells were selected and recovered as cellular pools or diluted to isolate cellular clones (Figure S5). Both guide RNAs targeted sequences in *ACKR4* mapping upstream an encoded DRY motif essential for signal transduction (Watts et al., 2013), so that Cas9-induced DNA breaks would generate knockout alleles. With this strategy, we obtained puromycin-resistant clones from Raji cells, two of which were verified to be *ACKR4* KO clones by DNA sequencing (Figure S6). We compared the proliferation and migration capacities of Raji control cells (transfected with the Cas9 expression vector without guide RNAs) and the two independent *ACKR4* KO Raji clones identified, cultured for 15 hours in medium supplemented or not with the CCL21 chemokine. Under these conditions, Raji cells of all genotypes appeared to proliferate similarly (Figure S7). Strikingly however, the KO of *ACKR4* led to a 4-fold increase in CCL21-mediated cell migration, consistent with the hypothesis that ACKR4 may hinder MYC-driven B-cell lymphomagenesis (**Figure 4F**).

Discussion

In this report we analyzed a mouse model with a specific deletion of the alternatively spliced (AS) exon of the *Trp53* gene. Despite a subtle phenotype, this model revealed that a male-specific protective effect against Eμ-Myc-induced B-cell lymphomas is lost in the absence of p53-AS isoforms. $p53^{\Delta AS/\Delta AS}$ males also appeared more prone to develop spontaneous lymphomas, suggesting that the sex-specific protective effect conferred by p53-AS isoforms might not be restricted to the Eμ-Myc model.

Our transcriptomic data from splenic cells of $p53^{+/+}$ Eμ-Myc and $p53^{\Delta AS/\Delta AS}$ Eμ-Myc males disclosed very few differentially expressed genes, and highlighted *Ackr4* as a male-specific positive prognostic factor in Eμ-Myc-induced lymphomas. Mechanistically, we identified *Ackr4*, expressed at low levels in splenic cells, as a p53 target gene that may be transactivated by p53-α and/or p53-AS according to luciferase assays. That *Ackr4* might be regulated by both types of p53 isoforms was expected, because their DNA binding domains are identical. In fact, if one considers that p53-α isoforms appear more abundant than p53-AS isoforms in wild-type cells, and that the loss of p53-AS isoforms correlated with a decrease in p53-α levels in the thymocytes and tumor lymph nodes

of mutant mice, then it seems likely that the reduced transactivation of *Ackr4* in the splenic cells of $p53^{\Delta AS/\Delta AS}$ Eμ-Myc males could mainly result from decreased p53-α levels, rather than the loss of p53-AS isoforms *per se*. In addition, we observed that 17-β estradiol can inhibit the p53-mediated transactivation of *Ackr4*. Together, our data suggest that *Ackr4* may be regulated by p53-α, p53-AS and estrogens, likely accounting for sex-specific and p53-status dependent differences in gene expression.

Our analyses reveal that in both mice and humans, *Ackr4/ACKR4* is a male-specific prognostic factor in Burkitt-like lymphomas. Furthermore, several lines of evidence suggest that Ackr4 might act as a tumor suppressor of Myc-driven B-cell lymphomas. As mentioned before, the ACKR4-mediated sequestration of CCL21 may impair the CCR7 signalling cascade, which might lead to decreased MYC activity (Shi et al., 2015). In $p53^{+/+}$ Eμ-Myc male splenic cells, the observed lower expression of *Mt2*, known to be regulated by Myc (Qin et al., 2021), and of many genes that are hallmark Myc targets (as revealed by GSEA), appear consistent with this hypothesis. In addition, Ccr7 is required for lymphoma cell lodging to secondary lymphoid organs (Rehm et al., 2011) and ACKR4 expression was inversely correlated with the metastasis capacity of different types of cancer cells (Shi et al., 2015; Zhu et al., 2014). Consistent with this, we found that the KO of *ACKR4* in Raji Burkitt lymphoma cells led to a dramatic increase in CCL21-guided cell migration. Finally, Ackr4 regulates B cell differentiation (Kara et al., 2018), which raises the possibility that an alteration of the p53-Ackr4 pathway in $p53^{\Delta AS/\Delta AS}$ Eμ-Myc male splenic cells might contribute to increase the pools of pre-B and immature B cells that may be prone to lymphomagenesis. In sum, a decrease in Ackr4 expression might promote B-cell lymphomagenesis through several non-exclusive mechanisms. Importantly, ACKR4 was previously found to inhibit the growth and metastasis of breast, cervical, colorectal, hepatocellular and nasopharyngeal cancer cells (Feng et al., 2009; Hou et al., 2013; Ju et al., 2019; Shi et al., 2015; Zhu et al., 2014), although no report mentioned any sex-specific bias for cancers occurring in both sexes. Our data provide evidence that sex-specific differences in *Ackr4* expression may have prognostic value. This suggests that measuring *ACKR4* gene expression in male patients with Burkitt lymphoma could be useful to identify the patients at higher risk, for whom specific therapeutic regimens might be required.

Interestingly, our data suggested that *Mt2* might be a male-specific negative prognostic factor in murine Eμ-Myc induced lymphomas, but *MT2A* expression levels had no prognostic value in human lymphomas. A possible explanation for this discrepancy is suggested by the fact that *Mt2* is regulated by Myc. A translocation leading to MYC overexpression drives oncogenesis in all Burkitt lymphomas, but half of them exhibit additional missense MYC mutations enhancing its tumorigenicity (Chakraborty et al., 2015). The transcriptional program of a WT and two lymphoma-associated Myc mutants were recently compared, and we noticed that one of the mutants led to an alteration in *Mt2* expression (Mahani et al., 2021), which would abrogate any potential prognostic value.

Finally, a polymorphism in the *MDM2* gene promoter provided evidence that sex- specific hormones may affect p53 signalling and tumorigenesis (Bond and Levine, 2007). More recently, a higher frequency of *TP53* mutations in men, together with an increased vulnerability to alterations of X-linked genes encoding p53 regulators, were proposed to explain a higher cancer incidence and death in male patients (Haupt et al., 2019). Here, on the contrary, male mice and a subset of male patients were more efficiently protected against Burkitt-like lymphomas, which adds another layer of complexity to sex-specific differences in tumorigenesis. The p53 pathway thus underlies cancer sex-disparities through multiple mechanisms, that may notably include variations in p53 isoforms or Ackr4 expression.

Materials and Methods

Mice

Design and construction of the $p53^{\Delta AS}$ mouse model was previously described (Simeonova et al., 2013). A minimum of 10 backcrosses with C57Bl6/J mice of both sexes (Charles River Laboratories) were performed before establishing the cohorts of $p53^{+/+}$ and $p53^{\Delta AS/\Delta AS}$ littermate mice used in this study. Mouse genotyping with multiple primer sets confirmed >99% C57Bl6/J genetic background after 10 backcrosses (primer sequences available upon request). Cohorts of $p53^{+/+}$ Eμ-Myc and $p53^{\Delta AS/\Delta AS}$ Eμ-Myc mice were established with identical parental origin of the Eμ-Myc transgene. For all experiments, mice housing and treatment were conducted according to Institutional Animal Care and Use Committee of the Institut Curie.

Cells and cell culture reagents

Mouse embryonic fibroblasts (MEFs) were isolated from 13.5 days post-coitum embryos and cultured in a 5% CO_2 and 3% O_2 incubator, in DMEM Glutamax (Gibco), with 15% FBS (PAN Biotech), 100 μM 2-mercaptoethanol (Millipore), 0.1 mM Non-Essential Amino-Acids and penicillin/streptomycin (Gibco) for less than 5 passages, except for 3T3 experiments, performed in a 5% CO_2 incubator for 9 passages. Cells were treated for 24h with 0.5 μg/ml Doxorubicin (Sigma-Aldrich), 15 μM Etoposide (Sigma-Aldrich), 10 μM Nutlin 3a (Vassilev et al., 2004) (Sigma-Aldrich) and/or 5 μg/ml 17β-Estradiol (Merck). At least 3 independent experiments with at least 2 independent littermate MEF clones of each genotype and each sex were performed to measure DNA damage responses. For estradiol assays, four independent experiments with three independent MEF male clones of each genotype were performed. Human lung fibroblast MRC5 and its SV40-transformed derivatives were cultured in a 5% CO2 and 3% O2-regulated incubator in MEM medium without Phenol Red (Gibco), completed with 10% FBS, 2 mM L-glutamine (Gibco), 1 mM pyruvate, 0.1 mM Non-Essential Amino-Acids, and penicillin/streptomycin, and treated for 24h with 10 μM Nutlin 3a and/or 5 μg/ml 17β-Estradiol (Merck). Four independent experiments were performed. Burkitt lymphoma cells (Raji or Raji *ACKR4* KO derivatives) were cultured in a 5% CO2 incubator in RPMI Glutamax (Gibco) supplemented with 10% FBS, 2 mM L-glutamine, 1 mM pyruvate, 0.1 mM Non-Essential Amino-Acids, 0.45% glucose (Sigma-Aldrich), and penicillin/streptomycin. In proliferation and migration assays, Raji control cells and two independent *ACKR4* KO derivatives were treated or not for 15h with 1 μg/ml CCL21 (PreproTech) then counted in triplicates.

Quantitative RT-PCR

Total RNAs were extracted using nucleospin RNA II (Macherey-Nagel), reverse-transcribed using superscript IV (Invitrogen), and real-time quantitative PCRs were performed on an ABI PRISM 7500 using Power SYBR Green (Applied Biosystems) as previously described (Simeonova et al., 2012). For quantification of p53 isoforms in healthy tissues, a forward primer in exon 10 and a reverse primer encompassing the boundary between exons 10 and 11 were used for p53-α amplification, whereas the same forward primer and a reverse primer located in exon AS were used for p53-AS amplification (see Table S3 for primer sequences). To determine the AS/α mRNA ratios, expression levels were compared with a standard curve generated by serial dilutions of a plasmid containing both p53-AS and p53-α cDNAs.

Western blots

Thymocytes were lysed in RIPA buffer (50CmM Tris-HCl pH 8, 150CmM NaCl, 5CmM EDTA, 0.5% deoxycholic acid, 0.1% SDS, 1% NP-40) with a cocktail of protease inhibitors (Roche) and 1CmM PMSF (Sigma). Whole-cell extracts were sonicated three times for 10Cs and centrifuged at

13C000Cr.p.m. for 30Cmin to remove cell debris. MEFs or B-cell lymphomas were lysed in Giordano's buffer (50CmM Tris-HCl pH 7.4, 250CmM NaCl, 5CmM EDTA, 0.1% Triton X-100) with a cocktail of protease inhibitors (Roche) and 1CmM PMSF (Sigma). Protein lysate concentration was determined by BCA assay (Thermo Scientific) and 30Cμg of each lysate was fractionated by SDS–PAGE on a 4-12% polyacrylamide gel and transferred onto PDVF membrane (Amersham). Membranes were incubated with antibodies against p53 (CM5, Novocastra), myc (9E-10, Santa Cruz), p21 (F-5, Santa Cruz) and actin (actin-HRP sc47778, Santa Cruz) and revealed with SuperSignal West femto detection reagent (Thermo Scientific).

Apoptosis Assays

Six weeks-old $p53^{+/+}$ and $p53^{\Delta AS/\Delta AS}$ male mice were whole-body irradiated with 5 Gy of γ-irradiation. Mice were sacrificed 4h later and thymocytes were recovered, stained with AnnexinV-FITC Apoptosis detection kit (Abcam), then analyzed by flow cytometry using FlowJo.

Cell-Cycle assays

Log phase MEFs were irradiated at room temperature with a CS γ-irradiator at doses of 3 or 10 Gy, incubated for 24h, then pulse-labeled for 1h with 10 μM BrdU, fixed in 70% ethanol, double-stained with FITC anti BrdU and propidium iodide, and sorted by flow cytometry using a BD FACSort. Data were analyzed using FlowJo.

Oncogene-induced tumor xenografts

MEFs with the indicated genotypes were sequentially infected with pWZL-E1A12S and pBABE-Hrasv12 viruses as previously described (Toledo et al., 2006). In total, 5×10^6 E1A- and Ras-(E1A+Ras) expressing MEFs of each genotype were injected subcutaneously into the flanks of 7-weeks-old female athymic nude mice (at least 4 mice per genotype) and tumor volumes were determined 1, 8, 15, 21 and 25 days after injection. Importantly, populations of (E1A+Ras)-expressing cells were used to minimize potential differences in expression levels that could result from independent viral insertion sites.

Cell sorting of B-cell sub-populations

Splenic cells were recovered from 6 weeks-old asymptomatic mice and incubated with DAPI and the following antibodies: APC rat anti-mouse CD45R/B220, FITC rat anti-mouse CD43, PE rat anti-mouse IgM and BV605 rat anti-mouse IgD (BD Pharmingen). First, the B220+CD43-cells were selected by flow cytometry from DAPI negative living cells, yielding subsequently 4 different B subpopulations based on IgM and IgD labeling: IgM-/IgD-preB lymphocytes, IgM low/IgD-immature B lymphocytes, IgM high/IgD-transitional B lymphocytes and IgM+/IgD+ mature B lymphocytes.

RNA-Seq analysis

Total RNA was extracted from the spleen of 4-6 weeks-old asymptomatic mice using nucleospin RNA II (Macherey-Nagel). The quality of RNA was checked with Bioanalyzer Agilent 2100 and RNAs with a RIN (RNA integrity number) $\geq$ 6 were retained for further analysis. RNA was depleted from ribosomal RNA, then converted into cDNA libraries using a TruSeq Stranded Total Library preparation kit (Illumina). Paired-end sequencing was performed on an Illumina MiSeq platform. Reads were mapped to the mouse genome version GRCm38 and counted on gene annotation gencode.vM18 with featureCounts (Liao et al., 2014). Differentially expressed genes of C57Bl6/J genetic background with an adjusted p-value < 0.05 were identified using the DESeq2 R package (Love et al., 2014). Gene set enrichment analysis was performed by using the GSEA software with canonical pathway gene sets from the Mouse Molecular Signature Database (MSigDB) (Subramanian et al., 2005).

Luciferase assays

The candidate p53 responsive element (p53 RE) in the *Ackr4* promoter was identified by using the JASPAR database of binding profiles (Fornes et al., 2020) with the position weight matrix scanner PWMscan (Ambrosini et al., 2018). A 1.5 kb fragment from *Ackr4* intron 1, containing a WT or mutant p53 RE at its center, was cloned upstream a SV40 minimal promoter and a luciferase reporter gene in the backbone of a PGL3 plasmid (Promega). We used lipofectamine 2000 to transfect $p53^{-/-}$ MEFs with 2 µg of either luciferase expression vector, 2 µg of an expression vector for $p53^{WT}$, $p53^{AS}$ or the DNA-binding mutant $p53^{R270H}$, and 30 ng of a renilla luciferase expression plasmid (pGL4.73, Promega) for normalization. Transfected cells were incubated for 24 h then trypsinized, resuspended in 75 µl culture medium with 7.5% FBS and transferred into a well of an optical 96-well plate (Nunc). The dual-glo luciferase assay system (Promega) was used according to the manufacturer's protocol to lyse the cells and read firefly and renilla luciferase signals. Results were normalized, then the average luciferase activity in cells transfected with the WT p53RE luciferase reporter and the $p53^{R270H}$ expression plasmid were assigned a value of 1.

Generation of *ACKR4* Knockout Burkitt lymphoma cells

Six guide RNAs (gRNAs) were designed to target the human *ACKR4* gene by using the web tool CRISPOR (Haeussler et al., 2016). For each gRNA, two reverse complementary oligonucleotides were designed (with added backbone sequences, including a 5' G nucleotide for gRNA sequences without a 5' G to improve transcription efficiency from a U6 promoter), annealed and cloned in the Cas9 expression vector pSpCas9(BB)-2A-Puro (PX459) (Addgene). Prelimary tests for efficiency to induce cleavage in the targeted DNA regions were performed in HEK293T cells, which led to select the gRNAs #4 (5'-TGGTAGTGGCAATTTATGCC-3') and #5 (5'-GGGCTGTTAATGCAGTTCAT-3') for further experiments. Raji Burkitt lymphoma cells were transfected with a PX459 vector expressing gRNA #4 or #5 (or no gRNA for control), by using Nucleofector Amaxa kit V (Lonza), and selection was carried out with 1 µg/ml puromycin for 3 weeks to obtain stably transfected cells. Clones were obtained by seeding cells at low density (1 cell/5 wells) in 96-well plates, expanded, then subdivided in two parts, half for storage in liquid nitrogen and half for *ACKR4* genotyping by Sanger DNA sequencing (see Table S3 for primer sequences). For each cell clone, the *ACKR4* target DNA regions were amplified by PCR, PCR products were cloned in a PGL3 plasmid, and 8 transformed bacteria colonies were sequenced (Figure S5). This led to identify two homozygous *ACKR4* KO clones, one (clone 4.14) obtained by using gRNA #4 and one (clone 5.2) by using gRNA #5 (Figure S6).

Cell migration assay

Cell migration assys towards the chemokine CCL21 were performed with Boyden chambers essentially as described (Calpe et al., 2011), by measuring transwell migration across bare polycarbonate membranes with a pore size of 5 µm (Corning). A total of 100 µl of culture medium (RPMI Glutamax, 10% FBS, 2 mM L-glutamine, 1 mM pyruvate, 0.1 mM Non-Essential Amino-Acids, 0.45% glucose, and penicillin/streptomycin) containing 5×10^5 cells was added to a 6.5 mm diameter transwell insert, and 600 µl of culture medium with or without 1µg/ml CCL21 were added to the lower compartment. After 15h at 37°C in 5% CO2, the number of migrated cells in the lower chamber was determined by using a Coulter Counter.

Statistical Analyses

Student's unpaired *t* tests were used in most Figures to analyze differences between WT vs ΔAS values. Log-Rank (Mantel-Cox) tests were used to analyze Kaplan-Meier tumor-free survival curves. Analyses were performed using Graphpad Prism 5, and values of $p<0.05$ were considered significant.

Data availability

RNA sequencing data have been deposited in the Gene Expression Omnibus (GEO) under the accession code GSE209708.

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Author Contributions

F.T, J.C.B. and I.S. initiated the project; F.T., B.B. and A.F. designed the research and analyzed data; B.B., A.F., I.S., J.L., A.M., V.L., F.T., A.H., J.R., W.V.-Z. and E.E. performed the research; M.G., B.B., M.P. and A.M. analyzed RNAseq data; F.T. acquired funding; F.T., A.F. and B.B. wrote the paper.

Declaration of interests

The authors declare no competing interest.

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Editors

Reviewer #1 (Public Review):

Summary:

The authors originally investigated the function of p53 isoforms with an alternative C-terminus encoded by the Alternatively Spliced (AS) exon in place of exon 11 encoding the canonical "α" C-terminal domain. For this purpose, the authors create a mouse model with a specific deletion of the AS exon.

Strengths:

Interestingly, wt or p53ΔAS/ΔAS mouse embryonic fibroblasts did not differ in cell cycle control, expression of well-known p53 target genes, proliferation under hyperoxic conditions, or the growth of tumor xenografts. However, p53-AS isoforms were shown to confer male-specific protection against lymphomagenesis in Eμ-Myc transgenic mice, prone to highly penetrant B-cell lymphomas. In fact, p53ΔAS/ΔAS Eμ-Myc mice were less protected from developing B-cell lymphomas compared to WT counterparts. The important difference that the authors find between WT and p53ΔAS/ΔAS Eμ-Myc males is a higher number of immature B cells in p53ΔAS/ΔAS vs WT mice. Higher expression of Ackr4 and lower expression of Mt2 was found in p53+/+ Eμ-Myc males compared to p53ΔAS/ΔAS counterparts, suggesting that

these two transcripts are in part regulators of B-cell lymphomagenesis and enrichment for immature B cells.

The manuscript integrates an elegant genetic approach with in vivo analyses providing a robust set of data which strengthens the role of p53 isoforms in leukemogenesis.

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Reviewer #2 (Public Review):

Summary:

This manuscript provides a detailed analysis of B-cell lymphomagenesis in mice lacking an alternative exon in region encoding the C-terminal (regulatory) domain of the p53 protein and thus enable to assemble the so-called p53AS isoform. This isoform differs from canonical p53 by the replacement of roughly 30 c-terminal residues by about 10 residues encoded by the alternative exon. There is biochemical and biological evidence that p53AS retains strong transcriptional and somewhat enhanced suppressive activities, with mouse models expressing protein constructs similar to p53AS showing signs of increased p53 activity leading to rapid and lethal anemia. However, the precise role of the alternative p53AS variant has not been addressed so far in a mouse model aimed at demonstrating whether the lack of this particular p53 isoform (trp53ΔAS/ΔAS mice) may cause a specific pathological phenotype.

Results show that lack of AS expression does not noticeably affect p53 the patterns of protein expression and transcriptional activity but reveals a subtle pathogenic phenotype, with trp53ΔAS/ΔAS males, but not females, tending to develop more frequently and earlier B-cell lymphoma than WT. Next, the authors then introduced ΔAS in transgenic Eμ-Myc mice that show accelerated lymphomagenesis. They show that lack of AS caused increased lethality and larger tumor lymph nodes in p53ΔAS Eμ-Myc males compared to their p53WT Eμ-Myc male counterparts, but not in females. Comparative transcriptomics identified a small set of candidate, differentially expressed gene, including Ackr4 (atypical chemokine receptor 4), which was significantly expressed in the spleens of ΔAS compared to WT controls. Ackr4 encodes a dummy receptor acting as an interceptor for multiple chemokines and thus may negatively regulate a chemokine/cytokine signalling axis involved in lymphomagenesis, which is down-regulated by estrogen signalling. Using in vitro cell models, the authors provide evidence that Ackr4 is a transcriptional target for p53 and that its p53-dependent activation is repressed by 17b-oestradiol. Finally, seeking evidence for a relevance for this gene in human lymphomagenesis, the authors analyse Burkitt lymphoma transcriptomic datasets and show that high ACKR4 expression correlated with better survival in males, but not in females

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Author response:

The following is the authors' response to the original reviews.

> ***Reviewer #1 (Recommendations For The Authors):***
>
> *(1) In the first paragraph of the result section it is not clear why the authors introduce the function of p53ΔAS/ΔAS in thymocyte and then they mention fibroblasts. The authors should clarify this point. The authors should also explain based on what rationale they use doxorubicin and nutlin to analyze p53 activity (Figure 1 and figure S1).*

We thank the reviewer for this comment. In the revised manuscript, we corrected this by mentioning, at the beginning of the Results section: “We analyzed cellular stress responses in thymocytes, known to undergo a p53-dependent apoptosis upon irradiation (Lowe et al., 1993), and in primary fibroblasts, known to undergo a p53-dependent cell cycle arrest in response to various stresses - e.g. DNA damage caused by irradiation or doxorubicin (Kastan et al., 1992), and the Nutlin-mediated inhibition of Mdm2, a negative regulator of p53 (Vassilev et al., 2004).”

> *(2) The authors should provide quantification for the western blot in figure 2D because the reduction of p53 protein level in mutant vs wt tumors is not striking.*

In the previous version of the manuscript, the quantification of p53 bands had been included, but quantification results were mentioned below the actin bands, rather than the p53 bands, and this was probably confusing. We have corrected this in the revised version of the manuscript. The quantification results are now provided just below the p53 bands in Figs. 1B and 2D, which should clarify this point. For Figure 2D, the quantifications show a strong decrease in p53 levels for 3 out of 4 analyzed mutant tumors. For consistency purposes, in the revised manuscript the quantification results also appear below Myc bands in Fig. 2C.

> *(3) In the discussion section, the authors propose that a difference in Ackr4 expression may have prognostic value and that measuring ACKR4 gene expression in male patients with Burkitt lymphoma could be useful to identify the patients at higher risk. However the authors perform a lot of correlative analysis, both in mice and in patients, but the manuscript lacks of functional experiments that could help to functionally characterize Ackr4 and Mt2 in the etiology of B-cell lymphomas in males (both in mouse and in human models).*

In the previous version of the manuscript, we proposed that Ackr4 might act as a suppressor of B-cell lymphomagenesis by attenuating Myc signaling. This hypothesis relied on studies showing that Ackr4 impairs the Ccr7 signaling cascade, which may lead to decreased Myc activity (Ulvmar et al., 2014; Shi et al., 2015; Bastow et al., 2021) and that the loss of Ccr7 may delay Myc-driven lymphomagenesis (Rehm et al., 2011). Furthermore, we proposed that the increased expression of Mt2 in p53ΔAS/ΔAS Em-Myc male splenic cells reflected an increase in Myc activity, because Mt2 is known to be regulated by Myc (Qin et al., 2021) and because the Mt2 promoter is bound by Myc in B cells according to experiments reported in the ChIP-Atlas database. However, in the first version of the manuscript this hypothesis might have appeared only partially supported by our data because an increase in Myc activity could be expected to have a more general impact, i.e. an impact not only on the expression of Mt2, but also on the expression of many canonical Myc target genes. In the revised manuscript, we show that this is indeed the case. We performed a gene set enrichment analysis (GSEA) comparing the RNAseq data from p53ΔAS/ΔAS Eμ-Myc and p53+/+ Eμ-Myc male splenic cells and found an enrichment of hallmark Myc targets in p53ΔAS/ΔAS Eμ-Myc cells. These new data, which strengthen our hypothesis of differences in Myc signaling intensity, are presented in Fig. 3K and Table S2.

Importantly, we now go beyond correlative analyses by providing direct experimental evidence that ACKR4 impacts on the behavior of Burkitt lymphoma cells. We used a CRISPR-Cas9 approach to knock-out ACKR4 in Raji Burkitt lymphoma cells and found that ACKR4 KO cells exhibited a 4-fold increase in chemokine-guided cell migration. These new data are presented in Figure 4F and the supplemental Figures S5-S7.

Finally, following a suggestion of Reviewer#2, we now also point out that “Ackr4 regulates B cell differentiation (Kara et al., 2018), which raises the possibility that an altered p53-Ackr4

pathway in p53ΔAS/ΔAS Eμ-Myc male splenic cells might contribute to increase the pools of pre-B and immature B cells that may be prone to lymphomagenesis."

In sum, we now mention in the Discussion that a decrease in Ackr4 expression might promote B-cell lymphomagenesis through three non-exclusive mechanisms.

> ***Reviewer #2 (Recommendations For The Authors):***
>
> *(1) A great addition would be to demonstrate how p53AS specifically contributes to the regulation of Ackr4. In particular, is there evidence that p53AS might be preferentially recruited on p53 RE within that gene as compared to WT? The availability of specific antibodies that distinguish between AS and WT p53 might help to address this (experimentally complex) question. As a note, usage of such antibodies would also strengthen Fig 1B, in which the AS isoform appears as a mere faint shadow under p53, thus making its "disappearance" in trp53ΔAS/ΔAS difficult to evaluate.*

We agree with the referee that efficient antibodies against p53-AS isoforms would have been useful. In fact, we tried a non-commercial antibody developed for that purpose, but it led to many unspecific bands in western blots and appeared not reliable. Importantly however, our luciferase assays clearly show that both p53-a and p53-AS can transactivate Ackr4, a result that might be expected because these isoforms share the same DNA binding domain. Furthermore, because p53-a isoforms appear more abundant than p53-AS isoforms at the protein and RNA levels (Figs. 1B and S1A), and because the loss of p53-AS isoforms leads to a significant decrease in p53-a protein levels (Figs. 1B and 2D), we think that in p53ΔAS/ΔAS cells the reduction in p53-a levels might be the main reason for a decreased transactivation of Ackr4. This is now more clearly discussed in the revised manuscript.

> *(2) A most interesting observation is in Fig3 A and Fig S3, showing that spleen cells of p53ΔAS Eμ-Myc males (but not females) were enriched in pre-B and immature B cells as compared to WT counterparts. This observation points to a possible defect in B cell maturation process. It would be most interesting to determine whether this particular defect is directly mediated by a p53AS-Ackr4 axis. The hypothesis raised by the authors in the Discussion section is that increased Ackr4 expression may delay lymphomatogenesis, but data in Fig 3A and 3S actually suggest that ΔAS increases the pool of immature B-cell that may be prone to lymphomagenesis.*

We thank the reviewer for this useful comment, which we integrated in the Discussion of the revised manuscript. Ackr4 was shown to regulate B cell differentiation (Kara at al. (2018) J Exp Med 215, 801–813), so this is indeed one of the possible mechanisms by which a deregulation of the p53-Ackr4 axis might promote lymphomagenesis. We now mention: "Ackr4 regulates B cell differentiation (Kara et al., 2018), which raises the possibility that an altered p53-Ackr4 pathway in p53ΔAS/ΔAS Eμ-Myc male splenic cells might contribute to increase the pools of pre-B and immature B cells that may be prone to lymphomagenesis." This is presented as one of three possible mechanisms by which decreased Ackr4 levels may promote tumorigenesis, the two others being the impact of Ackr4 on the chemokine-guided migration of lymphoma cells and its apparent effect on Myc signalling.

> *(3) The concordance with a male-specific prognostic effect of Ackr4 is most interesting in itself but is only of correlative evidence with respect to the study. Is there any information on whether p53AS expression is also a prognostic factor in BL? And is there evidence that Ackr4 may also be a male-specific prognostic factor in other B-cell malignancies, e.g. Multiple Myeloma?*

We have now performed the CRISPR-mediated knock-out of ACKR4 in Burkitt lymphoma cells and found that it leads to a dramatic increase in chemokine-guided cell migration, which goes

beyond correlation. This significant new result is mentioned in the revised abstract and presented in detail in Figures 4F and S5-S7.

Regarding p53-AS isoforms, they are murine-specific isoforms (Marcel et al. (2011) Cell Death Diff 18, 1815-1824), so there is no information on p53-AS expression in Burkitt lymphoma. Human p53 isoforms with alternative C-terminal domains are p53b and p53g isoforms, but the datasets we analyzed did not provide any information on the relative levels of p53a (the canonical isoform), p53b or p53g isoforms. We agree with the referee that this is an interesting question, but that cannot be answered with currently available datasets.

Regarding the different types of B-cell malignancies, we had already shown that Ackr4 is a male-specific prognostic factor in Burkitt lymphomas but not in Diffuse Large B cell lymphomas, which indicated that it is not a prognostic factor in all types of B cell lymphomas. For this revision, we also searched for its potential prognostic value in multiple myeloma, and found that, as for DLBCL, it is not a prognostic factor in this cancer type. This new analysis is presented in Figure S4C.

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