

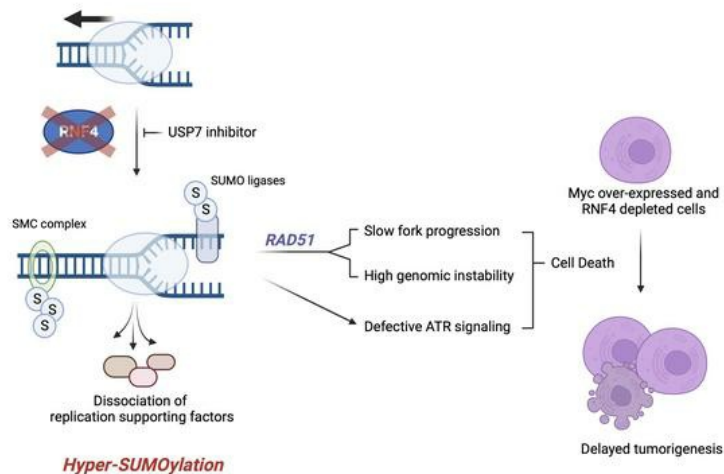
# RNF4 sustains Myc-driven tumorigenesis by facilitating DNA replication

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## Graphical abstract



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1 RNF4 sustains Myc-driven tumorigenesis by facilitating DNA replication

2

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21

22

23 Abstract

24

25 The mammalian SUMO-targeted E3 Ubiquitin Ligase, *Rnf4*, has been reported to act as a regulator  
26 of DNA repair, but the importance of RNF4 as a tumor suppressor has not been tested. Using a  
27 conditional-knockout mouse model, we deleted *Rnf4* in the B cell lineage to test the importance of  
28 RNF4 for growth of somatic cells. Although *Rnf4* conditional-knockout B cells exhibited  
29 substantial genomic instability, *Rnf4* deletion caused no increase in tumor susceptibility. In  
30 contrast, *Rnf4* deletion extended the healthy lifespan of mice expressing an oncogenic *c-myc*  
31 transgene. *Rnf4* activity is essential for normal DNA replication, and in its absence, there was a  
32 failure in ATR-CHK1 signaling of replication stress. Factors that normally mediate replication  
33 fork stability, including members of the Fanconi Anemia gene family and the helicases, PIF1 and  
34 RECQL5, showed reduced accumulation at replication forks in the absence of RNF4. RNF4  
35 deficiency also resulted in an accumulation of hyper-SUMOylated proteins in chromatin, including  
36 members of the SMC5/6 complex, which contributes to replication failure by a mechanism  
37 dependent on RAD51. These findings indicate that RNF4, which shows increased expression in  
38 multiple human tumor types, is a potential target for anti-cancer therapy, especially in tumors  
39 expressing *c-myc*.

40

41

42 Introduction

43

44 Healthy cell growth requires post-translational modification of proteins by addition of ubiquitin  
45 (Ub) or the Small Ubiquitin-like Modifier (SUMO) (1, 2). Ubiquitination and SUMOylation  
46 regulate almost every aspect of cellular physiology by modifying the function and stability of  
47 cellular proteins. DNA replication and repair are examples of the importance of SUMOylation  
48 and ubiquitination, which act in coordination to ensure productive outcomes in each case (3, 4).

49

50 In mammalian cells, RNF4 (RING Finger Protein 4), is a key SUMO-targeted E3 ubiquitin ligase  
51 (STUbL), which has a central importance in linking protein regulation by SUMOylation and  
52 ubiquitination. RNF4 contains four SUMO-Interacting Motifs (SIMs), which target the protein to  
53 SUMOylated substrates. The E3 ubiquitin ligase activity of homodimeric RNF4 subsequently  
54 transfers Ub from one of a number of different E2 conjugating enzymes to the substrate (5).

55 RNF4 is known to target the PML:RARA fusion protein that is frequently found in acute  
56 promyelocytic leukemia (APL) (6). In response to arsenic trioxide treatment, PML undergoes  
57 increased SUMOylation, making it an improved target for RNF4 STUbL activity, leading to  
58 degradation of the PML-RARA fusion protein and successful treatment outcomes even without  
59 use of conventional cytotoxic chemotherapy (7).

60

61 In addition to regulation of PML stability, RNF4 is implicated in a range of cellular processes.  
62 Several studies, using siRNA knockdown, mutant cell lines, or DT40 cells, reported defects in  
63 the cellular response to DNA damage after loss of RNF4 activity (8-12). In particular, efficiency  
64 of the homologous recombination (HR) pathway was reported to be reduced in *siRNF4* cells, as a

65 consequence of a failure to load RPA and RAD51 at resected DNA double-strand breaks (13,  
66 14). RNF4 also promotes normal chromosome segregation, based on its ability to remove toxic  
67 DNA-protein crosslinks that interfere with mitosis (15). Mutations affecting other components of  
68 the HR machinery, such as *BRCA1* or *BRCA2*, increase the rate of genetic mutations, leading to  
69 substantially increased tumor risk (16). Around 6% of primary cancer cases are estimated to be  
70 affected by HR deficiency (17), and identifying these cases is of substantial importance for  
71 treatment, because HR-deficient cancers show greatly increased sensitivity to Poly(ADP-Ribose)  
72 Polymerase (PARP) inhibitors and platinum-based cross-linking agents (18). Human *RNF4*  
73 mutations that might predispose affected individuals to cancer have not yet been reported,  
74 however, and the importance of *RNF4* as a tumor suppressor is unknown.

75

76 A challenge to evaluating the importance of RNF4 as a tumor suppressor is the lack of suitable  
77 animal models to test whether loss of RNF4 increases the risk of malignant cell growth.  
78 Homozygous deletion of the mouse *Rnf4* gene causes embryonic lethality prior to E15 (19). We  
79 now report the use of a conditional-knockout *Rnf4* mouse model to test the importance of *Rnf4*  
80 for DNA repair, and to evaluate how *Rnf4* deficiency contributes to tumor susceptibility. We find  
81 that deletion of *Rnf4* causes genomic instability and reduced cellular viability. These phenotypes  
82 of *Rnf4* deficiency are an outcome of defective DNA replication, and can be partially rescued by  
83 inhibiting the ubiquitin isopeptidase, USP7. The increased genomic instability of *Rnf4*-deficient  
84 cells does not lead to a higher frequency of malignant neoplasms, however, even on a p53-  
85 deficient background. *Rnf4* deficiency instead significantly delayed the rate of tumor formation  
86 in a mouse model of *c-myc* overexpression, indicating that *Rnf4* contributes to the growth of

87 incipient tumors. These results demonstrate that RNF4 is an essential factor for DNA replication  
88 and a potential novel target for anti-cancer therapy.

89

90

91 Results

92

93 *Rnf4* deletion causes genomic instability and cell death in primary B cells

94

95 To test the importance of RNF4 for growth of somatic cells, we first attempted to rescue  
96 embryonic lethality in *Rnf4*<sup>-/-</sup> mice by crossing to *Trp53*<sup>-/-</sup> or *Trp53bp1*<sup>-/-</sup> mouse lines. Co-  
97 deletion of p53 or 53BP1 in these lines rescues embryonic lethality in *Brca1*<sup>Δ11/Δ11</sup> mice (20), but  
98 we were not able to obtain any viable *Rnf4*<sup>-/-</sup> pups by this approach, indicating that *Rnf4* has an  
99 indispensable role in normal embryonic development (Supplemental Table 1). We therefore  
100 generated a novel, conditional *Rnf4*<sup>fl/fl</sup> allele by introducing LoxP sites around *Rnf4* exon 6 (Figure  
101 1A). *Rnf4*<sup>fl/fl</sup> mice were crossed to *CD19-Cre* animals to delete *Rnf4* selectively in the B  
102 lymphocyte lineage (21). The spleens of these *Rnf4* conditional-knockout (*Rnf4*<sup>Δ/Δ</sup>) mice showed  
103 a near-normal population of mature B220<sup>+</sup> B cells, but RNF4 was undetectable in protein lysates  
104 from these cells (Figure 1B and C). *Rnf4*<sup>Δ/Δ</sup> B cells showed a substantially increased proportion  
105 of high-molecular-weight SUMOylated species, consistent with the known role of RNF4 in  
106 ensuring turnover of SUMOylated proteins (6).

107

108 B cells lacking RNF4 showed clear signs of cell stress after 48 hrs of in vitro culture, with  
109 stabilization of p53, and increased cleavage of caspase-3 (Figure 1C). Phosphorylation of KAP1,  
110 and H2AX was also increased (Figure 1D), indicating activation of ATM and ATR after DNA  
111 damage (22). Markers of DNA damage and apoptosis became increasingly apparent after three  
112 days of in vitro B cell culture, indicating an underlying stress that became more acute after a  
113 longer period of B cell activation. Analysis of metaphase chromosomes revealed a significant

114 increase in chromosome aberrations in *Rnf4*<sup>Δ/Δ</sup> B cells (Figure 1E). This increase was apparent  
115 after 24 hrs of in vitro culture, and reached an even greater level after 48 hrs of culture. There  
116 was additionally an increase in the frequency of sister chromatid exchanges in *Rnf4*<sup>Δ/Δ</sup> cells,  
117 indicating increased use of HR-mediated DNA repair (Supplemental Figure 1A). *Rnf4*<sup>Δ/Δ</sup> B cells  
118 also showed a disrupted cell cycle, with a slightly reduced proportion of S phase cells, and an  
119 increase in the proportion of cells in G2 phase (Figure 1F).

120

121 Consistent with the increased induction of apoptotic markers, the majority of *Rnf4*<sup>Δ/Δ</sup> B cells  
122 became non-viable after three days in culture (Figure 1G). Cell death and increased genomic  
123 instability was only observed in homozygous *Rnf4*<sup>Δ/Δ</sup> cells, and not in heterozygotes (Figure 1H  
124 and I). Although p53 is clearly stabilized in *Rnf4*<sup>Δ/Δ</sup> cells (Figure 1C), deletion of *Trp53* only  
125 partially rescued cell viability and had no significant impact on chromosome instability  
126 (Supplemental Figure 1 B and C). RNF4 therefore plays an essential role for ensuring the  
127 viability of proliferating primary B cells, and in its absence, cells undergo apoptosis by a  
128 combination of p53-mediated and p53-independent processes.

129

130 *Rnf4-knockout cells show defects in DNA replication*

131

132 To evaluate the importance of RNF4 for DNA repair, we challenged *Rnf4*<sup>Δ/Δ</sup> cells with a range of  
133 compounds that cause DNA damage of various kinds (Supplemental Figure 2A). These agents  
134 included olaparib, a PARP inhibitor that produces DNA double-strand breaks in replicating cells;  
135 ionizing radiation (IR), which produces DNA double-strand breaks in all phases of the cell cycle;  
136 the DNA crosslinking agents, mitomycin C (MMC), and cisplatin (CDDP); and methyl



137 methanesulfonate (MMS), a DNA alkylating agent. In each case, *Rnf4*<sup>ΔΔ</sup> cells showed an  
138 increase in chromosome instability after treatment, but the increase in *Rnf4*<sup>ΔΔ</sup> cells was not  
139 significantly greater than that observed in WT cells after subtracting the rate of chromosome  
140 breaks and rearrangements in the untreated condition (Supplemental Figure 2B). A high  
141 frequency of chromosome aberrations was observed after olaparib or cisplatin treatment of  
142 *Brca1*<sup>Δ11/Δ11</sup> cells, which express a hypomorphic form of BRCA1, and are HR-deficient (23)  
143 (Figure 2A). In contrast, *Rnf4*<sup>ΔΔ</sup> cells showed only a very mild increase in the frequency of  
144 chromosome breaks and rearrangements after treatment with olaparib or cisplatin, despite having  
145 a higher rate of chromosome instability in the absence of drug treatment. The relatively mild  
146 sensitivity of *Rnf4*<sup>ΔΔ</sup> cells after treatments to induce DNA damage suggests that defects in  
147 additional cellular processes may contribute to the genomic instability and poor growth of these  
148 cells.

149  
150 The proliferation and viability of *Rnf4*<sup>ΔΔ</sup> cells was significantly impacted by treatment with  
151 either MMS, or the DNA polymerase inhibitor, aphidicolin (APH) (Figure 2B, and Supplemental  
152 Figure 2C and D). These agents also induced strong caspase-3 cleavage in *Rnf4*<sup>ΔΔ</sup> cells (Figure  
153 2C). MMS produces cytotoxicity through production of single-stranded DNA breaks and by  
154 inhibition of DNA replication (24-26). Although we did not detect an increase in single-strand  
155 breaks in *Rnf4*<sup>ΔΔ</sup> cells (Supplemental Figure 2E), we observed that *Rnf4*<sup>ΔΔ</sup> cells were  
156 hypersensitive to the replication inhibitors hydroxyurea, aphidicolin, and gemcitabine (Figure  
157 2D). *Rnf4*<sup>ΔΔ</sup> cells also showed an increase in 53BP1 G1 nuclear bodies, a hallmark of cells that  
158 are encountering problems with DNA replication (Figure 2E) (27, 28). *Rnf4*<sup>ΔΔ</sup> cells consistently  
159 incorporated lower levels of the deoxythymidine analog, EdU, indicating a lower rate of DNA

160 replication (Figure 2F). To directly measure the activity of individual replication forks, we  
161 performed a DNA combing analysis after pulsing cells with CldU and IdU, which revealed that  
162 *Rnf4<sup>Δ/Δ</sup>* cells showed a significant reduction in replication fork velocity (Figure 2G). *Rnf4<sup>Δ/Δ</sup>* cells  
163 also showed reduced stability of newly-replicated DNA after hydroxyurea treatment (Figure 2H).  
164 ATR-mediated CHK1 phosphorylation is essential for signaling the presence of replication stress  
165 (29), but phosphorylation of CHK1 was defective after induction of replication stress in the  
166 absence of RNF4 (Figure 2I). Although replication forks in *Rnf4<sup>Δ/Δ</sup>* cells appeared to restart  
167 normally after HU treatment (Supplemental Figure 2F), and showed only marginal levels of fork  
168 asymmetry (Supplemental Figure 2G), the reduction in replication fork progression induced by  
169 MMS treatment in WT cells is not observed in RNF4-conditional-knockout cells (Supplemental  
170 Figure 2H). A failure to adequately signal replication stress therefore correlates with reduced  
171 replication efficiency in *Rnf4<sup>Δ/Δ</sup>* cells.

172

173 *Replication defects and cell death in Rnf4<sup>Δ/Δ</sup> cells are a consequence of deregulated*

174 *SUMOylation*

175

176 Transduction of *Rnf4<sup>Δ/Δ</sup>* B cells with a construct expressing WT RNF4 resulted in a rescue of cell  
177 viability and EdU uptake (Fig 3A-B). This effect was not seen with RNF4 constructs containing  
178 mutations in the E3 ubiquitin ligase active site, or in the SUMO-interaction motifs. The STUbL  
179 activity of RNF4 is therefore essential for normal cell viability. To test whether the defective  
180 DNA replication, genomic instability, and cell death observed in *Rnf4<sup>Δ/Δ</sup>* cells are specifically  
181 caused by the accumulation of high-MW SUMOylated proteins, we used inhibitors of Ubiquitin  
182 Specific Protease 7 (USP7), a ubiquitin isopeptidase that removes ubiquitin from SUMOylated

183 substrates (30). Short-term treatment with USP7 inhibitor produced an increase in the amount of  
184 ubiquitination of SUMOylated proteins (Supplemental Figure 3), indicating that other cellular E3  
185 ubiquitin ligases target SUMOylated proteins at some level in the absence of RNF4. Continuous  
186 treatment with the USP7 inhibitors, P22077 or P5091, substantially reversed the accumulation of  
187 SUMOylated proteins in *Rnf4*<sup>ΔΔ</sup> cells, presumably by stabilizing ubiquitin chains on those  
188 proteins to enable proteasomal targeting (Figure 3C). This reduction in the quantity of  
189 SUMOylated proteins was associated with a rescue in the speed of replication forks in *Rnf4*<sup>ΔΔ</sup>  
190 cells (Figure 3D), reduced cell death (Figure 3E and F), and a reduction in the frequency of  
191 chromosome aberrations (Figure 3G and H). These results suggest that the slow replication forks,  
192 genomic instability, and increased cell death observed in *Rnf4*<sup>ΔΔ</sup> cells arise because of a failure  
193 to properly degrade SUMOylated proteins. Inhibition of SUMOylation by 2-D08, which blocks  
194 transfer of SUMO from UBC9 to potential substrates (31), or by ML-792, which inhibits the  
195 SUMO-Activating enzyme, SAE1/2 (32), also produced a partial rescue of chromosome  
196 instability and cell death in *Rnf4*<sup>ΔΔ</sup> cells (Figure 3I and J), showing that accumulation of poly-  
197 SUMOylated proteins in *Rnf4*<sup>ΔΔ</sup> cells is causative of reduced cell viability.

198

199 *Rnf4* deletion leads to defective recruitment of proteins required for replication stress responses

200

201 To identify changes in cellular protein expression that might account for the genomic instability  
202 and reduced viability of *Rnf4*<sup>ΔΔ</sup> cells, we performed quantitative isobaric labeling mass  
203 spectrometry with resting B cells (Day 0) and cells that were stimulated to divide in vitro for 48  
204 hrs (Day 2). In resting B cells, there was no substantial difference in protein expression between  
205 WT and *Rnf4*<sup>ΔΔ</sup> cells (Figure 4A, Supplemental Table 2). After activation of B cells and the

206 initiation of cell division, there were substantial changes in protein abundance in both the WT  
207 and *Rnf4<sup>Δ/Δ</sup>* samples (Figure 4B). Activated *Rnf4<sup>Δ/Δ</sup>* B cells showed a modest, but statistically-  
208 significant increase in the abundance of 455 proteins encompassing a broad range of protein  
209 classes (Supplemental Table 3, Supplemental Figure 4A and B). Increases in the abundance of  
210 several of these proteins were validated by Western blotting (Figure 4C). The broad changes in  
211 protein expression in *Rnf4<sup>Δ/Δ</sup>* B cells are consistent with previous proteomic studies, which  
212 revealed that RNF4 targets a wide range of substrates (33, 34).

213

214 As *Rnf4<sup>Δ/Δ</sup>* cells appear to have specific problems with DNA replication, we next used iPOND  
215 (isolation of Proteins On Nascent DNA) (35) to identify proteins with altered abundance at  
216 replication forks (Supplemental Table 4). Protein enrichment analysis on candidate proteins that  
217 showed substantially altered abundance at replication forks in *Rnf4<sup>Δ/Δ</sup>* cells revealed a cluster of  
218 proteins around FANCD2 (Supplemental Figure 4C), including Fanconi Anemia family proteins  
219 with known functions in ensuring stable replication (36). Several of these proteins show reduced  
220 abundance at replication forks in *Rnf4<sup>Δ/Δ</sup>* cells (Figure 4D), potentially accounting for the  
221 reduced replication efficiency seen in the absence of RNF4. The helicases PIF1 and RECQL5,  
222 which are required for DNA replication through difficult substrates (37, 38), also show  
223 diminished abundance in iPOND samples from RNF4-deficient cells. These results demonstrate  
224 that *Rnf4<sup>Δ/Δ</sup>* cells show a deficiency in recruitment of proteins necessary for replication fork  
225 stability and progression.

226

227 *Rnf4<sup>Δ/Δ</sup>* cells accumulate poly-SUMOylated proteins in chromatin (Supplemental Figure 4D). We  
228 performed immunoprecipitation of SUMO2/3 on chromatin samples from WT and *Rnf4<sup>Δ/Δ</sup>* cells,

229 to attempt to identify hyper-SUMOylated chromatin proteins in *Rnf4<sup>Δ/Δ</sup>* samples, which might  
230 contribute to defective replication fork progression. Mass spectrometry revealed a set of proteins  
231 with two clusters including proteins involved in regulation of SUMOylation, which are known  
232 targets of RNF4 (34), and several members of the SMC5/6 complex (Supplemental Table 5,  
233 Supplemental Figure 4E and F). The SMC5/6 complex is regulated by SUMOylation, and  
234 contributes to replication fork stability and regulation of recombination (39). SUMOylated  
235 SMC5 and SMC6 were significantly enriched in chromatin from *Rnf4<sup>Δ/Δ</sup>* cells relative to WT  
236 (Figure 4E). Three Non-SMC Element (NSMCE) subunits of the SMC5/6 complex were  
237 additionally present at increased abundance in SUMO2/3 immunoprecipitated proteins (Figure  
238 4F). The SMC5/6 complex therefore shows significant deregulation and hyper-SUMOylation in  
239 chromatin of *Rnf4<sup>Δ/Δ</sup>* cells, which may contribute to deficiencies in responding to replication  
240 stress.

241

242 *Cell death and genomic instability in RNF4-deficient cells are dependent on RAD51*

243

244 The SMC5/6 complex is known to prevent the accumulation of toxic recombination  
245 intermediates that appear at stalled replication forks (39, 40). We observed that knockdown of  
246 *RNF4* using multiple different siRNAs in U2OS EJ-DR reporter cells caused an increase in the  
247 rate of HR-mediated repair of DNA double-strand breaks (Supplemental Figure 5A). Ionizing  
248 radiation-induced foci of RAD51 formed normally in the nuclei of *Rnf4<sup>Δ/Δ</sup>* B cells (Supplemental  
249 Figure 5B). To test whether a potential increase in DNA recombination in RNF4-deficient cells  
250 might contribute to apoptosis and defective replication, we used the RAD51 inhibitors, RI-1 and  
251 B02 (41, 42). At high concentrations, these agents can block formation of ionizing-radiation

252 induced RAD51 foci, and reduce HR efficiency in reporter assays (Supplemental Figure 5C and  
253 D). Treatment with lower doses did not have a measurable impact on the cell cycle of WT cells  
254 (Supplemental Figure 5E), but substantially improved the growth of RNF4-deficient B cells (Fig  
255 5A-H). RAD51 inhibition significantly reduced the p53 activation and caspase-3 cleavage  
256 normally observed in *Rnf4<sup>ΔΔ</sup>* cells, reduced the frequency of chromosome aberrations, and  
257 improved cell proliferation and viability. Improved growth of *Rnf4<sup>ΔΔ</sup>* cells after RAD51  
258 inhibition was associated with a rescue of replication fork velocity (Figure 5I). RAD51 did not  
259 show an increased abundance at replication forks in iPOND data sets from *Rnf4<sup>ΔΔ</sup>* cells,  
260 however, nor is the overall abundance or level of ubiquitination of RAD51 substantially changed  
261 (Supplemental Figure 5F-H). RAD51-mediated toxicity may arise because of dysregulation of  
262 some other RNF4 target, as USP7 inhibition also reduced the hyper-recombinogenic phenotype  
263 of siRNF4-treated cells (Supplemental Figure 5I). Targeting other proteins involved in  
264 homologous recombination did not achieve a similar rescue of the phenotypes of RNF4  
265 deficiency as was achieved with RAD51 inhibition. In particular, deletion of either *Brca1* or  
266 *Brca2* did not result in an improvement in the proliferation, viability, or genomic integrity of  
267 *Rnf4<sup>ΔΔ</sup>* cells (Supplemental Figure 6A-F).

268

269 *Deletion of Rnf4 delays tumor formation in a c-myc cancer model*

270

271 As B cells from *Rnf4*-conditional-knockout mice showed substantial genomic instability (Figure  
272 1E), we performed a longitudinal study to determine if *Rnf4*-conditional-knockout mice are at  
273 increased risk of cancer. Despite elevated genomic instability in *Rnf4<sup>ΔΔ</sup>* B cells grown in culture,  
274 there was no difference in tumor susceptibility caused by deletion of *Rnf4* in the B cell lineage

275 (Figure 6A). The overall survival of conditional knockout and control mice also showed no  
276 significant difference (Supplemental Figure 7A). Tumor susceptibility in *Brca1*- and *Brca2*-  
277 deficient mice is more apparent in a p53-hemizygous background (43-45). We therefore crossed  
278 *Rnf4*-conditional-knockout mice to a *Trp53*<sup>+/-</sup> background, to test whether loss of RNF4 causes  
279 tumor outgrowth in cells lacking normal expression of p53. We observed that *Rnf4* deficiency  
280 did not accelerate tumor formation, even on a *Trp53*<sup>+/-</sup> background (Figure 6B), or affect the  
281 overall survival rate of *Trp53*<sup>+/-</sup> mice (Supplemental Figure 7B). As in the control group,  
282 *Rnf4*<sup>Δ/Δ</sup>;*Trp53*<sup>+/-</sup> animals developed tumors characteristic of p53 loss-of-function, with a large  
283 proportion of thymomas (46). Finally, we tested whether *Rnf4* deficiency causes accelerated  
284 tumor formation in *Eμ-myc* mice, a transgenic model for overexpression of the oncogene, *c-myc*,  
285 in B cells (47). As expected, mice carrying the *Eμ-myc* transgene had a very high incidence of B  
286 cell lymphoma with a median survival rate of 123 days (Figure 6C). In contrast, mice expressing  
287 *Eμ-myc* with conditional deletion of *Rnf4* in the B cell lineage showed a significant delay in  
288 tumor formation, with a median tumor-free survival of 215 days. The overall lifespan of *Eμ*-  
289 *myc*;*Rnf4*<sup>Δ/Δ</sup> mice showed an equivalent extension (Supplemental Figure 7C). These studies show  
290 that *Rnf4* deletion does not predispose mice to tumor formation, and loss of *Rnf4* delays the onset  
291 of tumors in cells with *c-myc* overexpression.

292

293 We considered whether defects in DNA replication might limit the growth of incipient *myc*-  
294 dependent malignancies in mice carrying the *Eμ-myc* transgene. Consistent with the well-known  
295 ability of *c-myc* to induce cellular proliferation, we observed EdU uptake by freshly-isolated *Eμ*-  
296 *myc*;*Rnf4*<sup>+/+</sup> and *Eμ-myc*;*Rnf4*<sup>Δ/Δ</sup> B cells, whereas WT and *Rnf4*<sup>Δ/Δ</sup> B cells showed almost no EdU  
297 incorporation (Supplemental Figure 7D). A proportion of *Eμ-myc* splenic B cells are therefore

298 dividing, even in the absence of any exogenous mitogenic stimulus. As was the case in *Rnf4*<sup>Δ/Δ</sup>  
299 cells, we measured an increased frequency of chromosome aberrations in *Eμ-myc;Rnf4*<sup>Δ/Δ</sup> B cells  
300 relative to *Eμ-myc;Rnf4*<sup>+/+</sup> cells (Fig 6D). *Eμ-myc;Rnf4*<sup>Δ/Δ</sup> B cells also showed a significantly  
301 reduced ability to proliferate in culture as compared to *Eμ-myc;Rnf4*<sup>+/+</sup> cells (Figure 6E). We  
302 tested the effect of knocking down RNF4 on the growth of U2OS cells with a stably-integrated,  
303 doxycycline-inducible *c-myc* construct (48). After knockdown of *RNF4*, these U2OS-iMYC cells  
304 showed significantly reduced proliferation compared to cells transfected with control siRNA  
305 (Figure 6F). In *Eμ-myc*-transgenic cells, deletion of *Rnf4* also led to reduced replication fork  
306 velocity, higher levels of  $\gamma$ -H2AX in S phase, and an increased number of 53BP1 G1 nuclear  
307 bodies (Figure 6G and H, and Supplemental Figure 7E). These results are consistent with the  
308 concept that the increased propensity of *myc*-transgenic cells to proliferate makes them acutely  
309 sensitive to loss of RNF4, which is required for normal DNA replication.

310 To consider whether *RNF4* expression might impact the growth of tumors in humans, we  
311 analyzed survival of B cell acute lymphoblastic leukemia patients studied as part of the NCI  
312 TARGET Phase II initiative. Patients with tumors expressing high levels of *RNF4* had  
313 significantly worse survival outcomes than those expressing normal levels of *RNF4* (Fig 6I).  
314 Elevated *RNF4* expression is a feature of several tumor types in TCGA data sets (Supplemental  
315 Figure 7F), and high *RNF4* expression correlates with poor survival in multiple tumor types,  
316 including breast adenocarcinoma, and lung adenocarcinoma (Supplemental Figure 7G-H). These  
317 findings suggest that RNF4 may have a general role in supporting tumor outgrowth, by  
318 facilitating DNA replication in malignant cells.

319



320 Discussion

321

322 Our observation that deletion of *Rnf4* does not lead to malignancy in B lymphocytes, even in the  
323 absence of p53, is potentially surprising considering the high rate of genomic instability observed  
324 in RNF4-deficient cells. Mice with deletions in known DNA repair genes, such as *Brca1* or  
325 *Brca2*, show tumor susceptibility, especially on a p53-deficient background (43, 45). The rate of  
326 chromosome instability in untreated *Rnf4*<sup>Δ/Δ</sup> B cells is significantly higher than that in  
327 *Brca1*<sup>Δ11/Δ11</sup> B cells (Fig 2A), and yet no tumor susceptibility was observed in *Rnf4* conditional-  
328 knockout mice. This outcome is potentially similar to the situation with cells lacking RAD51, the  
329 central mediator of HR (49). Repression of *RAD51* expression leads to accumulation of  
330 chromatid and chromosome breaks (50), but *RAD51* mutations do not correlate strongly with  
331 cancer incidence (51). Mouse knockout studies have shown that loss of RAD51 causes a  
332 complete block in cellular proliferation (52, 53), therefore it is likely that cancer cells cannot  
333 survive in the absence of RAD51. Based on our results, we propose that a similar effect is caused  
334 by loss of RNF4, in that knockout cells show chromosome instability, but are also unable to  
335 replicate and divide normally, leading to cell death instead of malignancy.

336

337 We find that the block on cell growth caused by conditional inactivation of *Rnf4* is so strong as  
338 to limit the outgrowth of *c-myc* dependent lymphomas. Tumors driven by *myc* activity are  
339 dependent on SUMOylation, and SUMO regulators are upregulated by *myc* overexpression (54).  
340 Our work further underscores the importance of SUMO regulation for tumor cell growth, and  
341 reveals the importance of RNF4 in this pathway. The dependence of incipient tumor cells from  
342 Eμ-*myc* mice on RNF4 for survival and tumor outgrowth is an example of ‘non-oncogene

343 addiction' (55). Increased proliferative signaling arising from *myc* overexpression leads to rapid  
344 DNA replication, which can only be sustained if RNF4 is available to ensure appropriate  
345 ubiquitination in replicating chromatin domains. A similar effect is seen with loss of ATR and  
346 CHK1, which signal the presence of DNA replication stress, including that induced by oncogene  
347 expression (29). CHK1 inhibition compromises the growth of tumors overexpressing oncogenic  
348 Ras or Myc, and tumor formation is significantly reduced in *Eμ-myc* mice expressing mutant  
349 ATR (56, 57).

350

351 Several studies in mammalian cells have supported a role for RNF4 in regulation of DNA  
352 replication. In ATR-deficient cells, RNF4 promotes SLX4-mediated cleavage of replication  
353 forks, leading to fork collapse and the appearance of DNA double-strand breaks (58). RNF4  
354 knockdown also reduces the ability of replication forks to restart after persistent replication  
355 stress, based on the ability of RNF4 to ubiquitinate BLM helicase (59). Another recent study  
356 identified RNF4 as the factor responsible for removal of the ZAPP-TOP2a-PICH complex from  
357 replication forks (33, 60). This finding is consistent with earlier studies, which showed that  
358 RNF4 targets trapped TOP1- and TOP2-cleavage complexes for proteasomal degradation (61,  
359 62). Several studies have identified PARP1 as a target of the E3 ubiquitin ligase activity of  
360 RNF4 (63, 64). PARP1 is active at replication forks, and the cytotoxic effect of PARP inhibitors  
361 is partially dependent on their ability to trap PARP1 on chromatin, creating a barrier to  
362 replication fork progression (65). Although we observed modest increases in the abundance of  
363 PARP1, TOP2A, and TOP2B in whole cell extracts from *Rnf4<sup>Δ/Δ</sup>* cells (Figure 4B and C), we did  
364 not observe significant changes in these factors in iPOND-MS studies of proteins at active  
365 replication forks. We did find reductions in the abundance of FANCD2, FANCI, and FANCM at

366 replication forks in RNF4-deficient cells (Fig 4D). Each of these factors is known to function in  
367 ensuring replication fork progression and stability (66-68). The helicases PIF1 and RECQL5,  
368 which are required for replication through challenging substrates such as G quadruplexes and  
369 transcription-replication conflict sites, are also present at reduced abundance at replication forks  
370 of *Rnf4*<sup>Δ/Δ</sup> cells (37, 38).

371  
372 Work from the Fernandez-Capetillo lab has supported a model in which chromatin around active  
373 DNA replication forks is marked by high levels of SUMOylation and low levels of ubiquitination  
374 (69). According to this model, chromatin outside of active replicating regions is marked by lower  
375 levels of SUMOylation, and high levels of ubiquitination. Disruption of the normal balance of  
376 ubiquitin- and SUMO-conjugated proteins in replicating chromatin domains leads to a reduction  
377 in replication fork progression (30). We find that SUMOylation is broadly increased in  
378 chromatin in *Rnf4*<sup>Δ/Δ</sup> cells (Supplemental Figure 4D) and this increase correlates with reduced  
379 replication fork velocity and cell survival. In particular, we identified five out of eight  
380 components of the mammalian SMC5/6 complex as hyper-SUMOylated chromatin constituents  
381 in *Rnf4*<sup>Δ/Δ</sup> cells (Figure 4E and F). The SMC5/6 complex, like other SMC complexes, is a multi-  
382 protein assembly that encircles DNA and facilitates loop extrusion (70). The SMC5/6 complex  
383 facilitates DNA replication by helping to relieve chromosome-wide superhelical tension that  
384 arises around active replisomes (71). This process involves the prevention of intertwining of  
385 sister chromatid molecules behind the replication fork. Depletion of SMC5/6 in yeast causes a  
386 block in replication at the ribosomal DNA array (39). The SMC5/6 complex furthermore  
387 physically interacts with FANCI and FANCD2 in mammalian cells (72), therefore defects in the  
388 regulation of SMC5/6 may account for the reduced accumulation of FANCI and FANCD2 at

389 replication forks in *Rnf4*<sup>Δ/Δ</sup> cells. The processivity of replication forks in budding yeast is  
390 dependent on the SMC5/6 complex, which limits MPH1-mediated fork regression, and promotes  
391 error-free bypass of DNA lesions (73, 74). The ability of SMC5 to promote lesion bypass is  
392 dependent on SUMOylation, therefore it is an attractive candidate for regulation by RNF4.  
393  
394 Toxic recombination intermediates accumulate in cells with deficiencies in the SMC5/6 complex  
395 (39, 40, 75). The cytotoxic effects of RNF4 deletion are notably rescued by RAD51 inhibitors,  
396 indicating that RAD51-dependent recombination or fork regression is causative of cell death in  
397 the absence of RNF4. It is possible that toxic RAD51-dependent structures arise because of a  
398 failure to adequately regulate or localize the SMC5/6 complex in RNF4-deficient cells.  
399 Mutations in *slx5*, the yeast homolog of RNF4, are known to produce gross chromosomal  
400 rearrangements caused by aberrant recombination between homeologous sequences (76).  
401 Although the importance and details of specific mechanisms for RNF4-dependent regulation of  
402 DNA replication remain to be resolved, it is clear that RNF4 is required for efficient replication,  
403 and may therefore represent an attractive target for cancer cases driven by *myc*, or other  
404 oncogenes that drive rapid cellular proliferation.  
405

406 Methods

407

408 *Sex as a biological variable*

409

410 Sex was not considered as a biological variable in this study.

411

412 *Generation of Rnf4 conditional knockout mice*

413

414 Cas9:sgRNA ribonucleoprotein complexes were prepared by incubating eSpCas9 (Millipore-

415 Sigma) with sgRNA and then adding single-stranded oligodeoxynucleotides, which contained

416 homology arms flanking LoxP sequences. This mixture was microinjected into C57BL/6J

417 embryos. The 5' LoxP sgRNA spacer sequence was CAATCACTTGCCTTATAAGA

418 (Millipore-Sigma) and the 5' LoxP oligo donor sequence was 5'-

419 CAAGATCTTAGGCAGGAAGATGGATGCCTTTGGTCAGACATGTTGGTTTTACTGAAA

420 ATCTCTGCCATTGTACCATGGGCTGAGCCTTCTATA**ACTTCGTATAGCATA**CATTA

421 **TACGAAGTTATTATAAGGCAAGTGATTGGCCTCAGTGCCTGGACAGGACCTTCTGA**

422 G-3' (LoxP in bold). For the 3' LoxP, the sgRNA spacer sequence was

423 TTGGGTTTCAGCTGTCTGCTC and the 3' LoxP donor oligo sequence was 5'-

424 ACAAGTATCTTCCAAGCAGCTAGGTAGGCATATTTGGAATACAGGGTTGGGTTTCAGC

425 TGTCTGa**ATAACTTCGTATAGCATA**CATT**TACGAAGTTATCTCAGGATAGACCAA**

426 TTGACAGACAGACAGGGCCATACATACTGACCAGGGTGTGCTTTTCGCT-3' (LoxP in

427 bold, sequence in lower case added to create XmnI restriction site for screening). Three founders

428 were determined to have both LoxP sites correctly inserted flanking *Rnf4* exon 6. A single line  
429 used for the study was backcrossed four times to C56Bl/6.

430

#### 431 *Cell culture*

432

433 Primary B cells were isolated from mouse spleen and activated for in vitro culture with  
434 lipopolysaccharide and interleukin 4 as previously described (77). U2OS Dox-inducible Myc-  
435 GFP (U2OS-iMyc) cells (48) were seeded in triplicate with or without 0.5 µg/ml doxycycline  
436 treatment in a 24-well plate after siRNA transfection. Eight days after seeding, proliferation was  
437 assayed using CellTiter-Glo® Luminescent Cell Viability assay (#G7572, Promega) according to  
438 the manufacturer's instructions. PLAT-GP cells (Cell Biolabs) were cultured in DMEM  
439 containing 10% fetal bovine serum and 0.5% Penicillin-Streptomycin. For the in vivo  
440 SUMOylation assay, FLAG-Rad51 plasmid was transfected into siRNA-treated U2OS cells  
441 using Lipofectamine 2000. Cells were processed 24 hr after transfection as described (78).

442

#### 443 *Antibodies, chemicals and plasmids*

444

445 Custom anti-RNF4 antibody was generated as previously described (79). Commercial antibodies  
446 used in this study include: SUMO2/3 (ab3742; Abcam), p53 (2524; CST), Cleaved caspase-3  
447 (9661; CST), Tubulin (T8238, Sigma), KAP1-pS824 (A300-767A; Bethyl), CHK1-pS345 (2341;  
448 CST), CHK1 (sc-8408; Santa Cruz), γ-H2AX (05-636; Millipore), γ-H2AX-Alexa 488 (20304S;  
449 CST), Histone H3 (ab1791; Abcam), TOP2a (sc-365916; Santa Cruz), TOP2b (MAB6348;  
450 Novus), PARP1 (9542; CST), RNF10 (16936-1-AP; Proteintech). Antibody specific for mouse

451 POLD4 was provided by Marietta Lee. The following chemicals were used: olaparib (S1060;  
452 Selleckchem), hydroxyurea (H8627; Sigma Aldrich), aphidicolin (A4487; Sigma Aldrich),  
453 cisplatin (S1166; Selleckchem), MMS (129925; Sigma Aldrich), gemcitabine (G6423; Sigma  
454 Aldrich), P22077 (662142; Sigma Aldrich), P5091 (2277; Biovision), 2-D08 (SML1052; Sigma  
455 Aldrich), ML-792 (407886, MedKoo), RI-1 (SML1294; Sigma Aldrich), B-02 (SML0364;  
456 Sigma Aldrich). For plasmids, human *RAD51* cDNA was inserted into pRK5-FLAG and human  
457 *RNF4* cDNA was inserted into pMX-PIE-IRES-eGFP. Amino acid substitutions for *RNF4-CS*  
458 and *RNF4-ΔSIM* were performed as described (78, 80).

459

#### 460 *Chromatin fractionation and immunoprecipitation*

461

462 For chromatin fractionation, harvested cells were gently resuspended in Buffer A (10 mM  
463 HEPES [pH7.9], 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.05% NP-40, 0.5 mM DTT, 10 mM NEM, 50  
464 mM glycerol-2-phosphate, supplemented with protease-inhibitor cocktail, Roche) and incubated  
465 for 10min on ice. Cell nuclei were collected by centrifugation (5 min, 1,300 x g, 4°C) and  
466 incubated in Buffer B (3 mM EDTA, 0.2 mM EGTA, 0.5 mM DTT, 10 mM NEM, 50mM  
467 glycerol-2-phosphate, and protease-inhibitor cocktail) for 30min. The chromatin component was  
468 collected by centrifugation (4 min, 1,700 x g, 4°C) and lysed with Buffer C (20mM HEPES  
469 [pH7.9], 500 mM NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2mM EDTA, 0.5 mM DTT, 125 U / ml benzonase, 10  
470 mM NEM, 50 mM glycerol-2-phosphate, and protease-inhibitor cocktail). Lysates were  
471 incubated for 5 min at 37°C on a shaking heat block followed by 20 min incubation on ice. After  
472 centrifugation at 16,000 g for 20 min, supernatant containing chromatin-bound protein was  
473 collected. Anti-SUMO2/3 immunoprecipitation of chromatin fractions was performed after 10-

474 fold dilution of chromatin samples in dilution buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl,  
475 0.5% NP-40, 10 mM NEM, 50mM glycerol-2-phosphate, and protease-inhibitor cocktail), with  
476 protein G-Agarose used for collection.

477

478 *Double-strand break repair reporter assay*

479

480 The EJ-DR assay was performed as previously described (81). To induce incorporated I-Sce1,  
481 Shield1 (632189; Clontech) and triamcinolone (T6510; Sigma Aldrich) ligands were added to the  
482 media for 24 h. Where necessary, RAD51 inhibitors were added together with the Shield1 and  
483 triamcinolone. NHEJ and HR repair activity was assessed by quantification of DsRed- and GFP-  
484 positive cells on BD FACS Calibur system and analyzed on FlowJo (Tree Star) 48hrs after  
485 induction.

486

487 *siRNA depletion*

488

489 Cells were transfected with siRNA using Lipofectamine RNAiMAX (13778; Invitrogen). The  
490 following siRNA oligonucleotides were used in this study: MISSION siRNA Universal Negative  
491 Control #1 (SIC001, Sigma-Aldrich), siGENOME Human RNF4 siRNA set of 4 (M-006557-03;  
492 Dharmacon), siRNF4-1 (D-006557-03), siRNF4-2 (D-006557-04), siRNF4-3 (D-006557-05 ),  
493 siRNF-UTR (GGGCAUGAAAGGUUGAGAA), siGENOME Human BRCA1 siRNA set of 4  
494 (M-003461-02; Dharmacon), siGENOME Human BRCA2 siRNA set of 4 (M-003462-01;  
495 Dharmacon).

496



497 *Flow cytometry*

498

499 For the CFSE proliferation assay, purified B cells were resuspended at  $1 \times 10^7$  cells/ml in RPMI  
500 medium, labeled with 5  $\mu$ M CFSE for 10 min at 37°C, then cultured in RPMI medium with or  
501 without IL-4 and LPS for 72 h. The CFSE fluorescence of unstimulated cells was taken as 100%  
502 and the number of additional cell divisions was calculated according to reduced CFSE  
503 fluorescence as the cells divided. Number of cell divisions was calculated as  $\log_2(\text{median}$   
504  $\text{intensity of unstimulated cells} / \text{median intensity of indicated cells})$ . For EdU incorporation, B  
505 cells were pulsed with 30  $\mu$ M EdU for 30 min, and fixed with ice-cold methanol for 20 min. EdU  
506 was detected using the Click-iT EdU Alexa Fluor-647 imaging kit. Cells were stained with DAPI  
507 to measure DNA content. For flow cytometry analysis of splenocyte populations, mouse  
508 splenocytes were treated with ACK lysis buffer for 5 min, then blocked with anti-CD16/CD32  
509 antibody for 10 min at room temperature and labeled with B220-A647 (557683; BD) and CD43-  
510 PE (12-0431-82; Invitrogen) for 1 hr at 4°C. Cytometry was performed using a BD FACSCalibur  
511 or Cytex Aurora with analysis in FlowJo.

512

513 *Alkaline Comet assay*

514

515 Alkaline comet assays were performed using CometAssay kit (Trevigen) according to the  
516 manufacturer's protocol. Splenic B cells were cultured for 48 hrs in vitro and then treated with  
517 mock or 100  $\mu$ M MMS treatment for 3 hrs. Images were acquired by Nikon Eclipse E800  
518 epifluorescence microscope and analyzed with OpenComet software.

519

520 *DNA combing and DNA fiber analysis*

521

522 To measure fork speed, cells were incubated with 20  $\mu$ M 5-chlorodeoxyuridine (CldU; C6891;  
523 Sigma-Aldrich) for 20 min, followed by 20 min of incubation with 100  $\mu$ M 5-iododeoxyuridine  
524 (IdU; I7125; Sigma-Aldrich). Genomic DNA purification and combing on glass coverslips was  
525 performed using materials and a protocol from Genomic Vision. DNA fiber analysis was  
526 performed as described (82). The DNA was stained with anti-CldU antibody (ab6326; Abcam)  
527 and anti-IdU antibody (347580; BD) for 1 h, followed by incubation with secondary antibodies  
528 for 45 min. Images were acquired by Nikon Eclipse E800 epifluorescence microscope using a  
529 40x objective and analyzed with ImageJ software.

530

531 *iPOND*

532

533 iPOND was performed as previously described (35).

534

535 *Retroviral transduction*

536

537 To express the human *RNF4* gene in B cells, retroviral vectors were transfected into PLAT-GP  
538 cells using Lipofectamine 2000. B cells were cultured for 36 hrs of in vitro culture and  
539 spinoculated with retroviral supernatants at 1150 g for 1.5 hr in the presence of 10  $\mu$ g/ml  
540 polybrene. After 4 hr at 37°C, supernatants were replaced with normal medium. The percentage  
541 of GFP<sup>+</sup> and DAPI<sup>-</sup> B cells was measured with flow cytometry on Day 2 and Day 3 to check the  
542 infection rates and viability.

543

544 *Preparation of metaphase spreads and FISH*

545

546 Telomere DNA FISH analysis was performed and analyzed as previously described (77). Images  
547 were acquired by an AxioImager.Z2 microscope (Zeiss) using a 60x objective with MetaSystems  
548 automatic stage. At least 50 metaphases were analyzed for each experiment.

549

550 *Mass spectrometry*

551

552 Thirty micrograms of each sample were digested in-gel with trypsin (1:50 ratio, sequencing  
553 grade, Thermo Scientific Cat#90058) and peptides extracted as described previously (83, 84).  
554 Peptides were labeled with Thermo TMTpro 16 Plex (Lot #: WC320807) and prefractionated by  
555 high-pH RPLC. Mass spectrometry was conducted using SPS-MS3 to measure reporter ion  
556 intensities on an Orbitrap Eclipse Tribrid Mass Spectrometer (Thermo Fisher). Instrument  
557 settings were for MS1: Orbitrap resolution 120,000, scan range from 350–1600 Th, automatic  
558 gain control (AGC) target 1E6, maximum injection time 100 ms; for MS2, Top 5 (3 sec) duty  
559 cycle, quadrupole ion trap, AGC 2E4, collision energy 35, maximum injection time 35 ms,  
560 isolation window was set at 0.7; and for MS3, 10 MS2 fragment ions captured as MS3 precursors  
561 using isolation waveforms with multiple frequency notches, Orbitrap AGC 1.5E5, maximum  
562 injection time 54 ms in the higher-energy collisional dissociation (HCD) cell and fragmented  
563 with HCD with collision energy of 55% and scanned in the Orbitrap with scan range 100-500  
564 m/z. Peak lists were generated by Thermo Proteome Discoverer (v. 2.1) as MASCOT Generic  
565 Format (MGF) files and combined MS2-MS3 peak lists searched against the Uniprot mouse

566 database using an in-house version of X!Tandem (GPM Fury) (85). TMT MS3 reporter ion  
567 intensities were extracted with correction for isotope channel crossover using custom in-house  
568 software and exported to Excel. For each protein, the sum of TMT MS3 intensities for all  
569 corresponding peptides were first normalized to the sum of the TMT intensities for each channel  
570 to account for differences in labeling efficiency or amount of protein labeled for each channel.  
571 The mean of the four replicates for each genotype/time-point was used to generate ratios for  
572 selected comparisons. Proteins were filtered for those with 2 or more assigned spectra. Proteins  
573 showing an increase of >10% in *Rnf4*<sup>Δ/Δ</sup> cells relative to WT cells with a P value < 0.05 (t-test)  
574 were considered for further analysis.

575

576 For data-independent acquisition, instrument settings were: MS scan range set to 400-1200 m/z,  
577 with resolution 12,000, AGC 3E6 and ion time set as auto. Eight m/z windows were used to  
578 sequentially isolate (AGC 4E5 and ion time auto) and fragment ions in the C-trap using a relative  
579 collision energy of 30. MS/MS spectra were recorded with a resolution of 30,000. Data were  
580 analyzed using DIA-NN 1.8.1 (86) with default settings for FASTA digest for library-free search  
581 / library generation and deep learning-based spectra and retention time prediction using Uniprot  
582 reference proteomes UP000000589 (mouse). Data were normalized using the MaxLFQ  
583 algorithm (87) and total normalized precursor intensities for each protein group were filtered for  
584 both PEP (an estimate of the posterior error probability for the precursor identification, based on  
585 scoring with neural networks) filter <0.01 and PG.Q (Protein Group Q Value) filter <0.01. T-  
586 tests were calculated as above.

587

588 *Statistics*

589

590 Statistical tests were performed as described.  $P < 0.05$  was considered to be statistically  
591 significant. Where shown, n.s. indicates not significant. \* indicates a P value  $< 0.05$ . \*\* indicates  
592 a P value  $< 0.01$ . \*\*\* indicates a P value  $< 0.001$ . \*\*\*\* indicates a P value  $< 0.0001$ . Data points  
593 on graphs represent results from a minimum of  $n=3$  biological replicates. For beeswarm plots,  
594 small data points represent individual values from  $n=3$  experiments, separately colored, and large  
595 data points indicate the mean of each experiment.

596

### 597 *Analysis of genomic data sets*

598

599 Functional categorization and measurements of statistical over-representation of protein classes  
600 was performed using PANTHER (panther.db). STRING analysis was performed using tools at  
601 <https://string-db.org>. cBioPortal (<https://www.cbioportal.org>) was used to access data on 72  
602 pediatric acute lymphoblastic leukemia patients studied as part of the Therapeutically Applicable  
603 Research to Generate Effective Treatments initiative, phs000218 (TARGET Phase II). The data  
604 used for this analysis are available at <https://portal.gdc.cancer.gov/projects>. Overall survival of  
605 patients with tumors with high *RNF4* mRNA expression (RNASeq z-score vs RSEM  $\geq 2.0$ ) was  
606 compared to patients with *RNF4* mRNA in the unaltered range. cBioPortal was additionally used  
607 to retrieve data on survival of patients with above-median and below median *RNF4* expression in  
608 tumor biopsies, based on samples from 1,082 breast adenocarcinoma patients and 501 lung  
609 adenocarcinoma patients studied as part of the TCGA PanCancer Atlas project (88). Analysis of  
610 *RNF4* expression in multiple tumor types was based on TCGA data accessed with the University  
611 of Alabama Cancer Data analysis portal (89)

612

613 *Study Approval*

614

615 Vertebrate animal studies were carried out under an animal protocol approved by the Rutgers  
616 University Institutional Animal Care and Use Committee.

617

618 *Data availability*

619

620 Proteomic data sets were deposited to the MassIVE database with accession numbers  
621 MSV000090142, MSV000093660 and MSV000093661. All data used for figures is available in  
622 a Supporting Data Values file.

623

624

625 Author Contributions

626

627 Experiments were conceived by J.H. and S.F.B. and performed by JH, except mass spectrometry,

628 which was performed by H.Z. The paper was written by S.F.B.

629

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639 POLD4 antibody.

640



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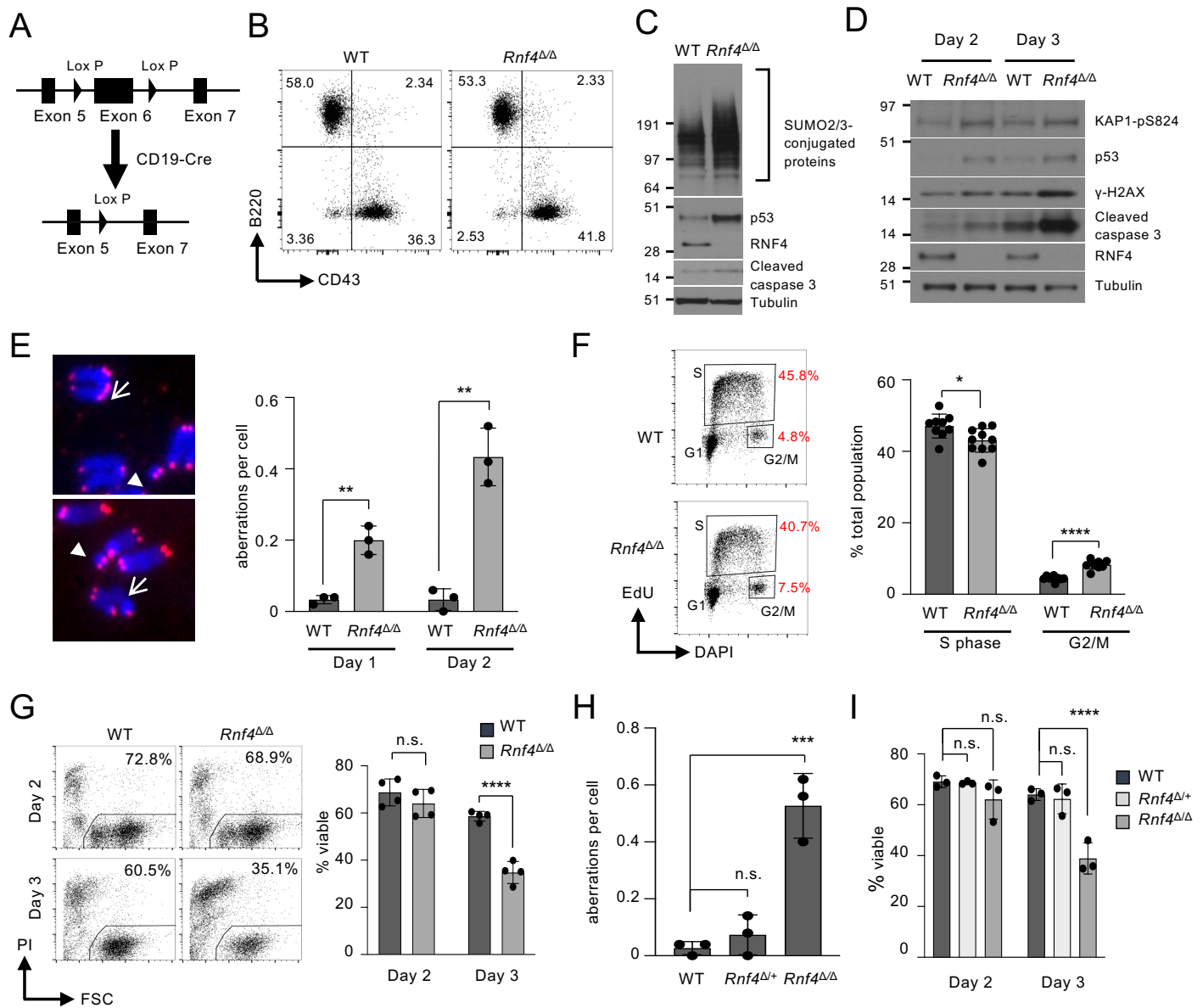
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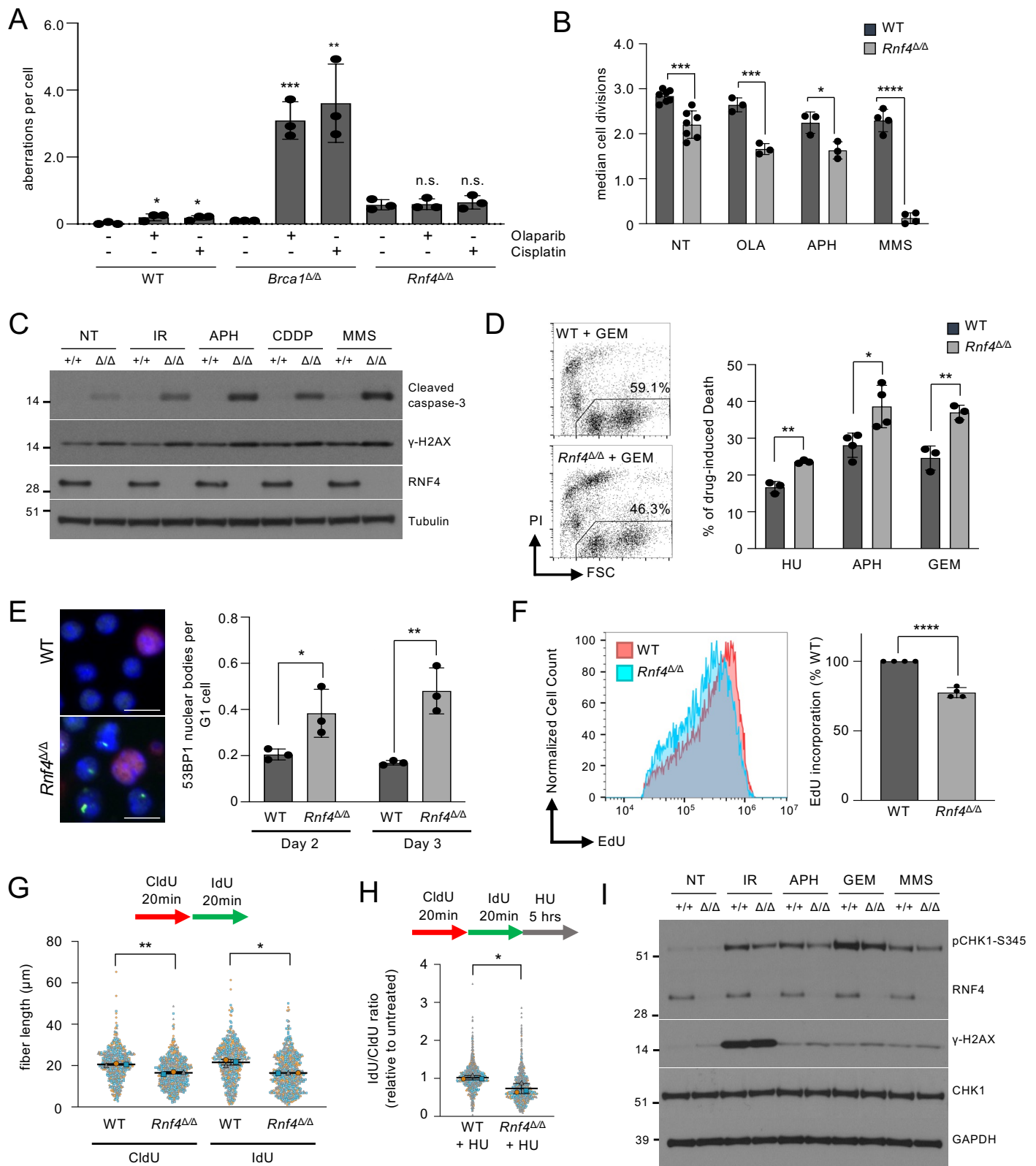
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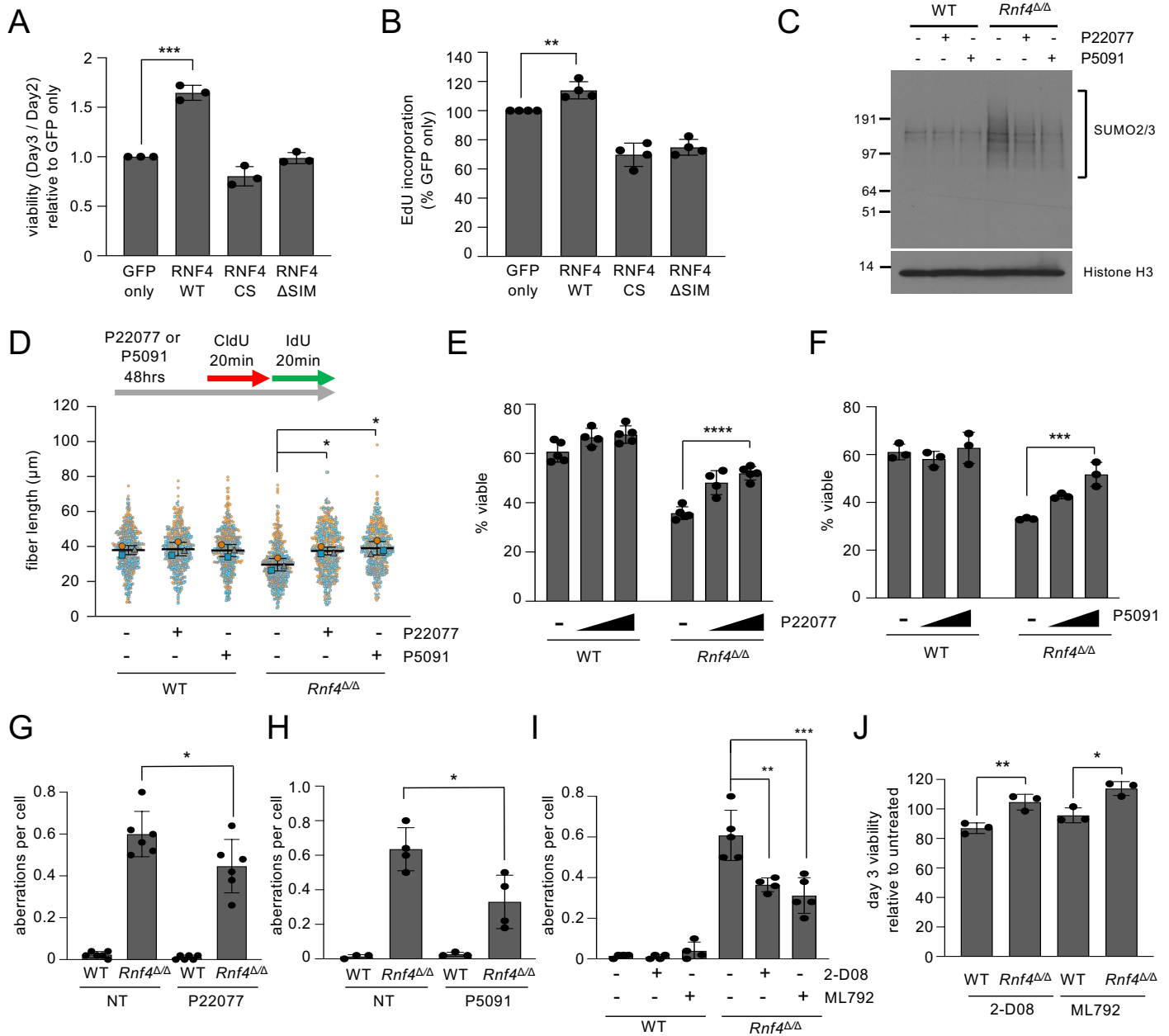


**Figure 1. Genomic instability and apoptosis in RNF4 conditional-knockout B cells.** (A) Schematic representation of Cre-induced deletion of *Rnf4* exon 6. (B) Flow cytometry analysis of cell populations in the spleens of WT and *Rnf4*-conditional-knockout (*Rnf4*<sup>Δ/Δ</sup>) mice. (C) Western blot analysis of protein expression in *Rnf4*<sup>Δ/Δ</sup> B cells after 2 days activation in vitro. (D) Western blot to measure induction of markers of DNA damage signaling in activated WT and *Rnf4*<sup>Δ/Δ</sup> B cells after 2 days and 3 days of in vitro culture. (E) Analysis of chromosome aberrations in metaphase spreads prepared from WT and *Rnf4*<sup>Δ/Δ</sup> B cells after one or two days of in vitro culture. White arrows show examples of chromosome rearrangements and solid arrowheads show examples of chromosome breaks. (F) Cell cycle analysis of fixed B cells after 48 hrs activation in vitro. (G) Flow cytometry analysis of cell death in splenic B cells cultured for 2 or 3 days in vitro. Left, figures indicate the percentage of cells that remained viable, based on propidium iodide exclusion. (H) Chromosome aberrations in metaphase spreads from WT, *Rnf4*<sup>Δ/+</sup>, and *Rnf4*<sup>Δ/Δ</sup> B cells. (I) Viability of B cells after 2 and 3 days of in vitro culture. Error bars where shown indicate S.D. of the mean. P values were calculated with unpaired t-test (E, F, G), one-way ANOVA with Dunnett's multiple comparisons test (H), and two-way ANOVA with Dunnett's multiple comparisons test (I).

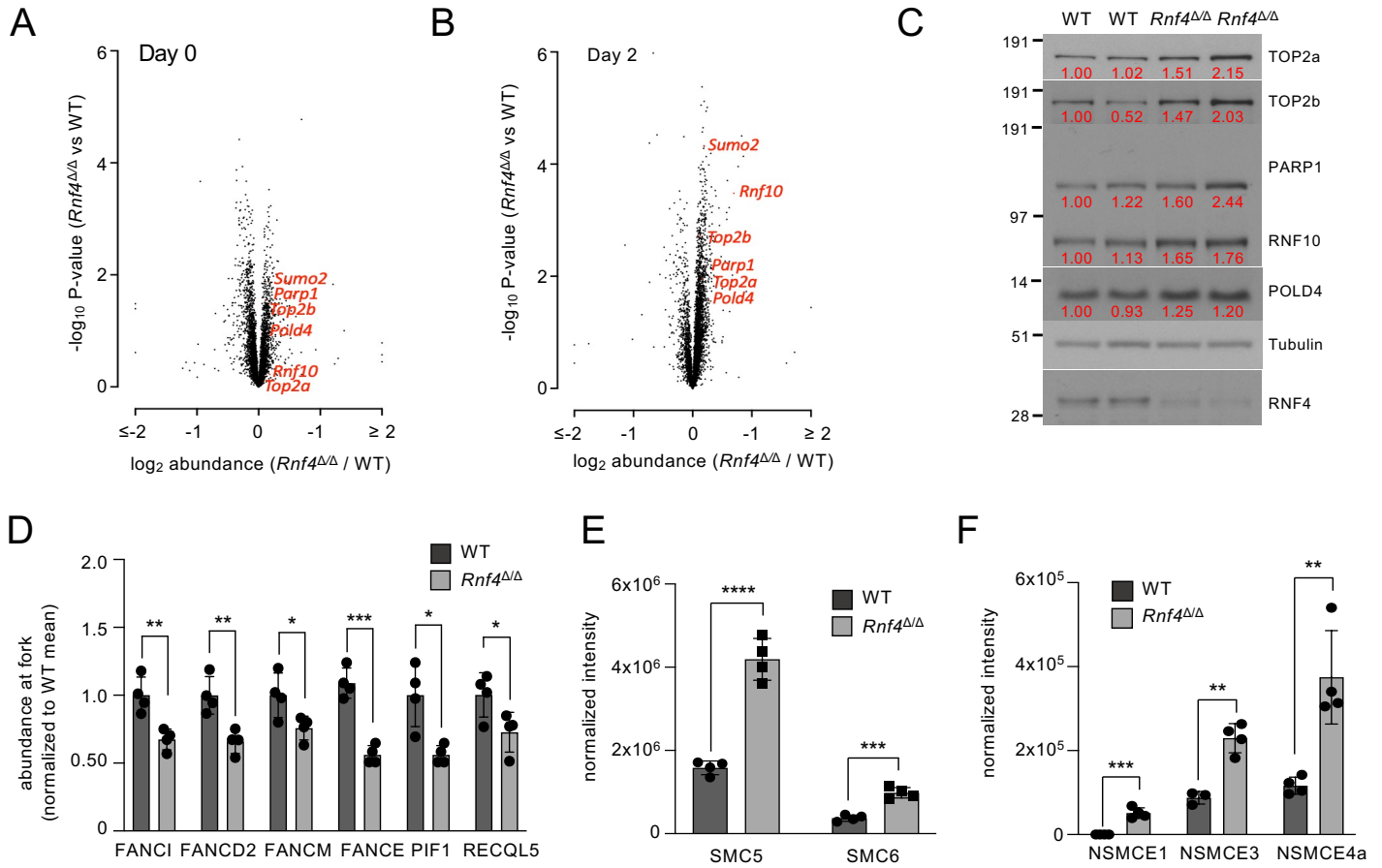


**Figure 2. *Rnf4* $\Delta\Delta$  B cells show defects in DNA replication.** (A) Analysis of chromosome aberrations in B lymphocytes treated overnight  $\pm$  2  $\mu$ M olaparib or 1  $\mu$ M cisplatin. Statistical differences between the means of the treated and non-treated samples are shown. (B) Flow cytometry analysis of CFSE dilution to measure B cell growth over 72 hrs. Chart shows quantification of cell doublings based on CFSE fluorescence. (C) Western blot analysis of caspase-3 cleavage and  $\gamma$ -H2AX after no treatment (NT), or after overnight recovery from treatment with 2 Gy of ionizing radiation (IR), 0.4  $\mu$ M aphidicolin (APH), 2.5  $\mu$ M cisplatin (CDDP), or 100  $\mu$ M MMS. (D) Sample flow cytometry data quantifying cell viability after treatment with gemcitabine (GEM). Gated population shows the viable, PI-negative population. Graph shows proportion of cells that became non-viable 24 hrs post-treatment with either hydroxyurea (HU) (4 mM, 3 hrs), APH (40  $\mu$ M, 2 hrs), or GEM (250 nM, 2 hrs). (E) Immunofluorescent detection of 53BP1 G1 nuclear bodies (green) in B cells after in vitro culture. Cyclin A staining (red) reveals S/G2-phase cells. (F) EdU uptake measured by flow cytometry. (G) Analysis of nascent DNA tract length in WT and *Rnf4* $\Delta\Delta$  splenic B cells by DNA combing. Mean  $\pm$  S.D. of n=3 experiments shown. (H) Stability of nascent DNA measured by fiber analysis after 4mM HU treatment. Mean  $\pm$  S.D. of n=3 experiments shown. (I) Western blot to show induction of pCHK1 in WT and *Rnf4* $\Delta\Delta$  cells after IR (30Gy, 2hrs recovery), APH (0.4  $\mu$ M, overnight), GEM (100 nM, 2 hrs), MMS (200  $\mu$ M, 3 hrs). Error bars in Parts A, B, D, E, and F show S.D. of the mean, with P values calculated by unpaired t-test. P values in Parts G and H calculated by paired t-test.

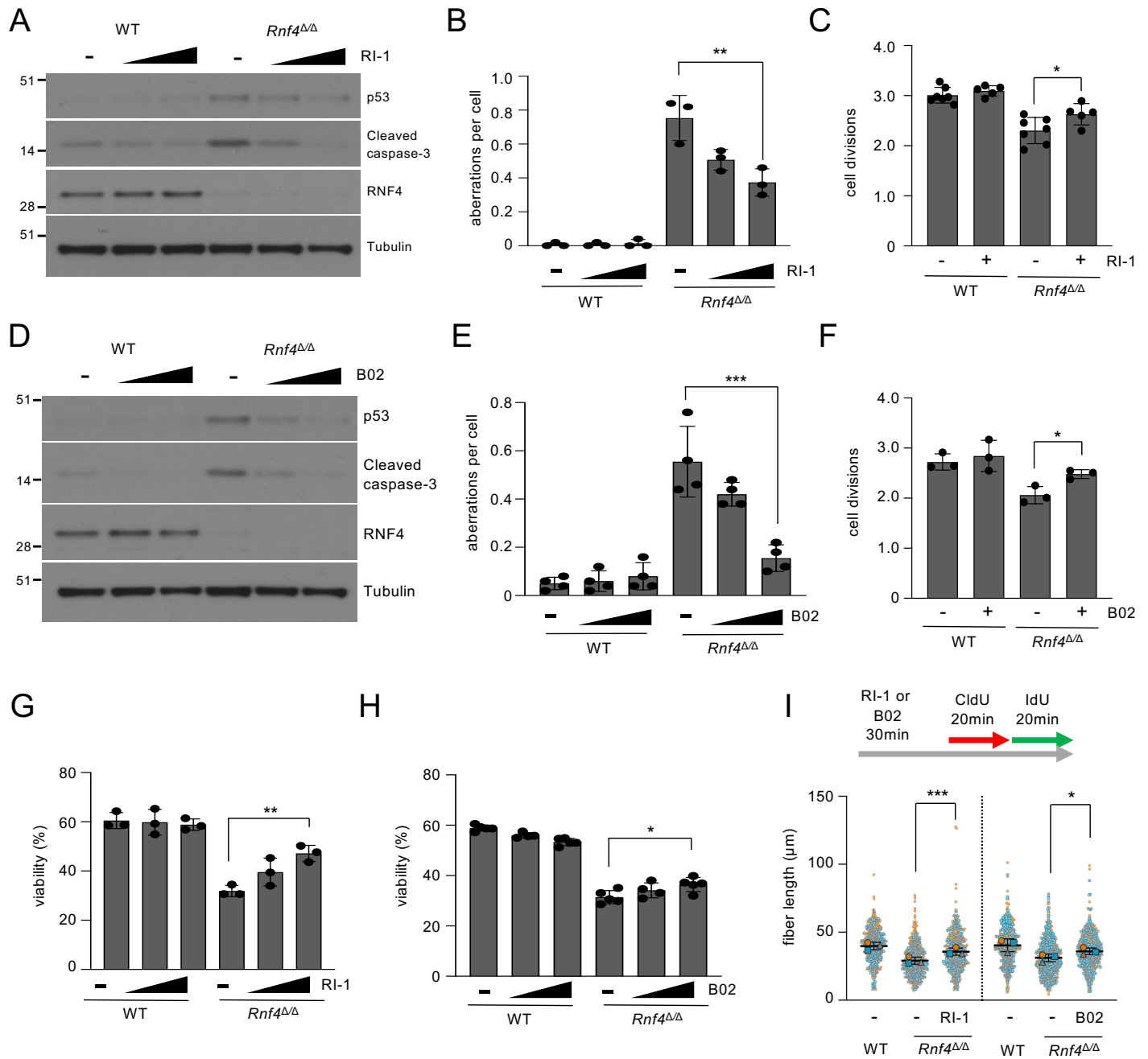




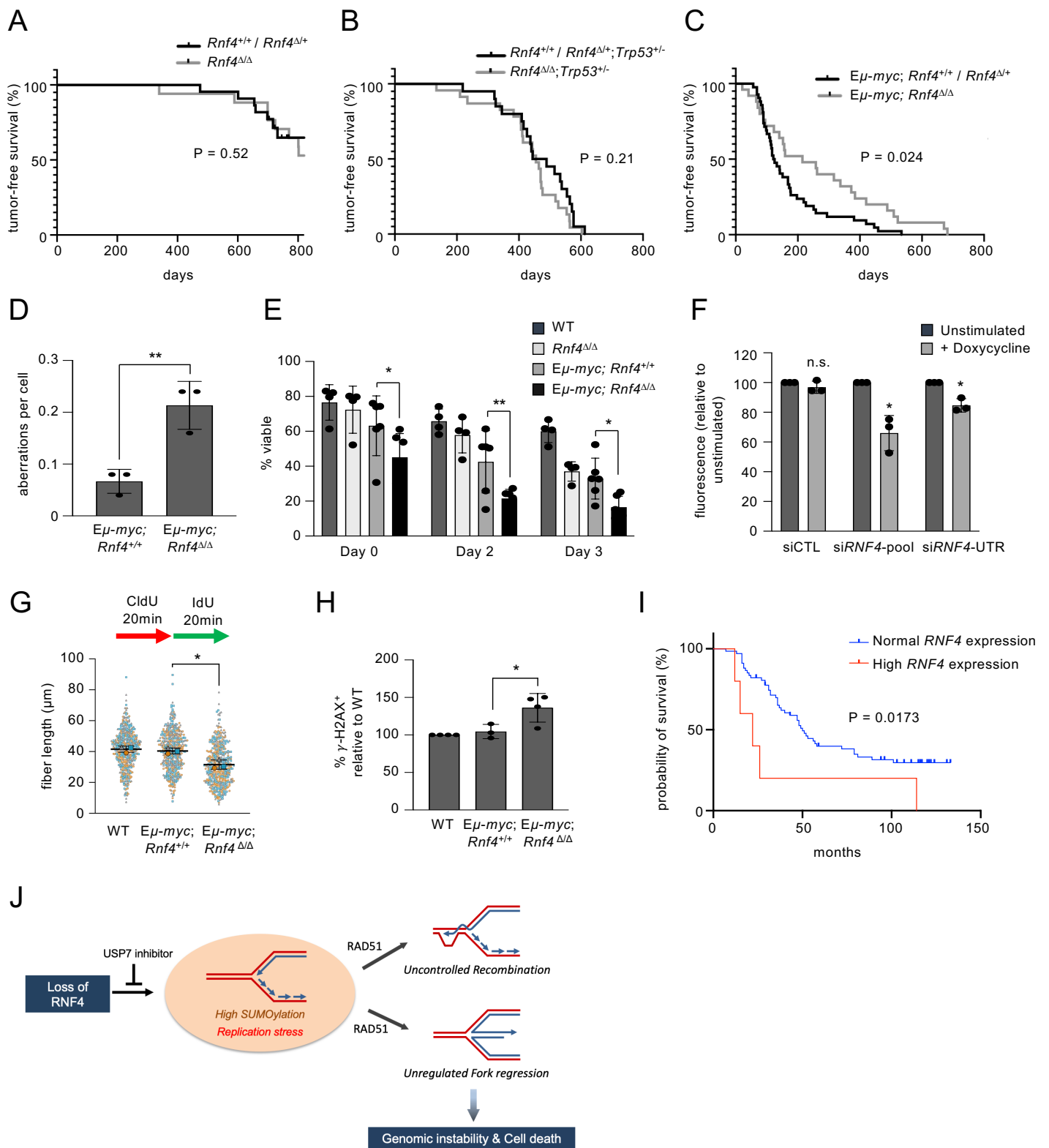
**Figure 3. Phenotypes of *Rnf4*<sup>ΔΔ</sup> cells are caused by accumulation of SUMOylated proteins** (A) Analysis of viability of *Rnf4*<sup>ΔΔ</sup> cells transduced with constructs expressing GFP, WT RNF4, a catalytic mutant of RNF4 (RNF4-CS) or RNF4 defective for SUMO binding (RNF4-ΔSIM). (B) Quantification of EdU uptake by *Rnf4*<sup>ΔΔ</sup> cells transduced with GFP or RNF4 cDNAs. (C) Western blot of cells grown in vitro for two days ± continual treatment with USP7 inhibitors, P22077 (2 μM) or P5091 (1 μM). (D) Analysis of nascent DNA tract length in WT and *Rnf4*<sup>ΔΔ</sup> B cells. Cells were cultured in vitro for 48 hrs ± P22077 (2 μM) or P5091 (1 μM), then incubated with CldU for 20 min, followed by IdU for 20 min. CldU and IdU tract lengths were analyzed after DNA combing. Mean ± S.D. of n=3 experiments shown. (E) Cell viability after 72 hrs culture ± P22077 (1 μM, 2 μM). Viable cells were identified based on ability to exclude DAPI. (F) As (E), using P5091 (0.5 μM, 1 μM). (G) Analysis of chromosome aberrations in B cells cultured 48 hrs ± P22077 treatment (2 μM). (H), as (G), using P5091 (1 μM). (I) Analysis of chromosome aberrations in B cells cultured 48 hrs ± continuous treatment with the SUMO inhibitors, 2-D08 (40 μM) or ML-792 (20 nM). (J) Cell viability measured by quantification of DAPI-negative cells after 72 hrs culture ± 2-D08 (40 μM) or ML-792 (20 nM). Error bars in parts A, B, and E-J, show S.D. of the mean. P values were calculated with unpaired t-test (A, B, J), one-way ANOVA with Dunnett's multiple comparison test, (D, I), two-way ANOVA with Dunnett's multiple comparison test (E, F), and one-way ANOVA with Tukey's multiple comparison test (G, H).



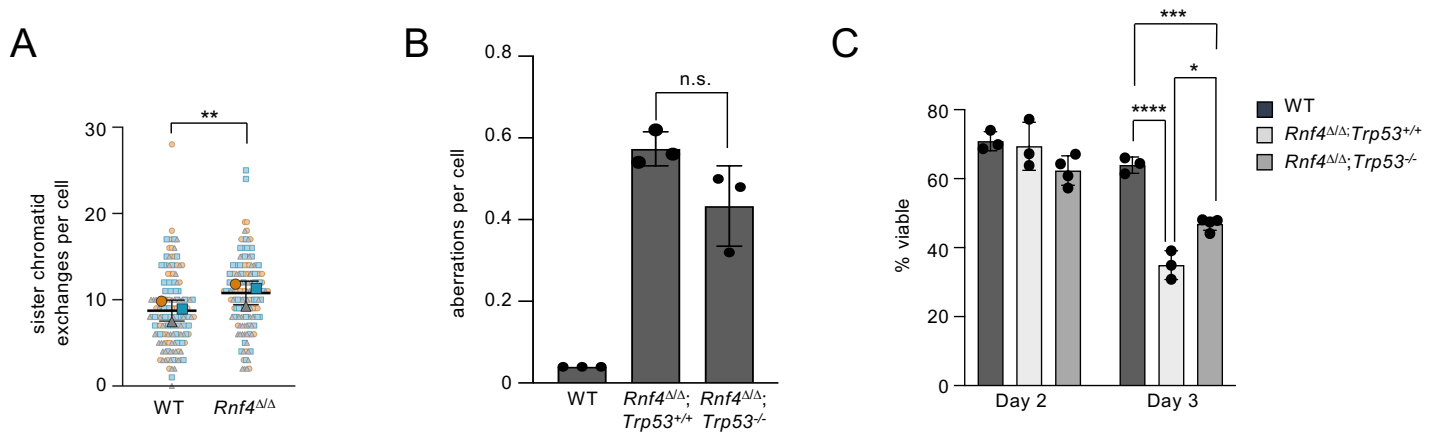
**Figure 4: RNF4 deficiency leads to reduced accumulation of proteins needed for replication stress responses at replication forks.** (A) Volcano plot showing protein abundance in resting *Rnf4*<sup>ΔΔ</sup> B cells relative to WT controls. Each data point represents the average of four samples. Six proteins that showed increased abundance after B cell activation are labeled. (B) Volcano plot of B cells after 48 hrs in vitro culture with cell proliferation induced by addition of LPS. (C) Western blot showing expression of proteins in WT and *Rnf4*<sup>ΔΔ</sup> B cells after 48 hrs culture. Two samples are shown in each case. Figures in red indicate band intensities measured with ImageJ. (D) Abundance of proteins required for replication stress responses at replication forks, measured by iPOND-MS, in WT and *Rnf4*<sup>ΔΔ</sup> B cells. (E) Increase in abundance of SUMOylated SMC5 and SMC6 in *Rnf4*<sup>ΔΔ</sup> cells, measured by mass spectrometry after SUMO2/3 immunoprecipitation of chromatin from WT and *Rnf4*<sup>ΔΔ</sup> cells. (F) Increase in abundance of SUMOylated Non-SMC Element (NSMCE) subunits of the SMC5/6 complex in chromatin of *Rnf4*<sup>ΔΔ</sup> cells. Error bars in parts D-F show S.D. of the mean of n=4 replicates. P values were calculated by unpaired t-test. P < 0.05 was considered significant.



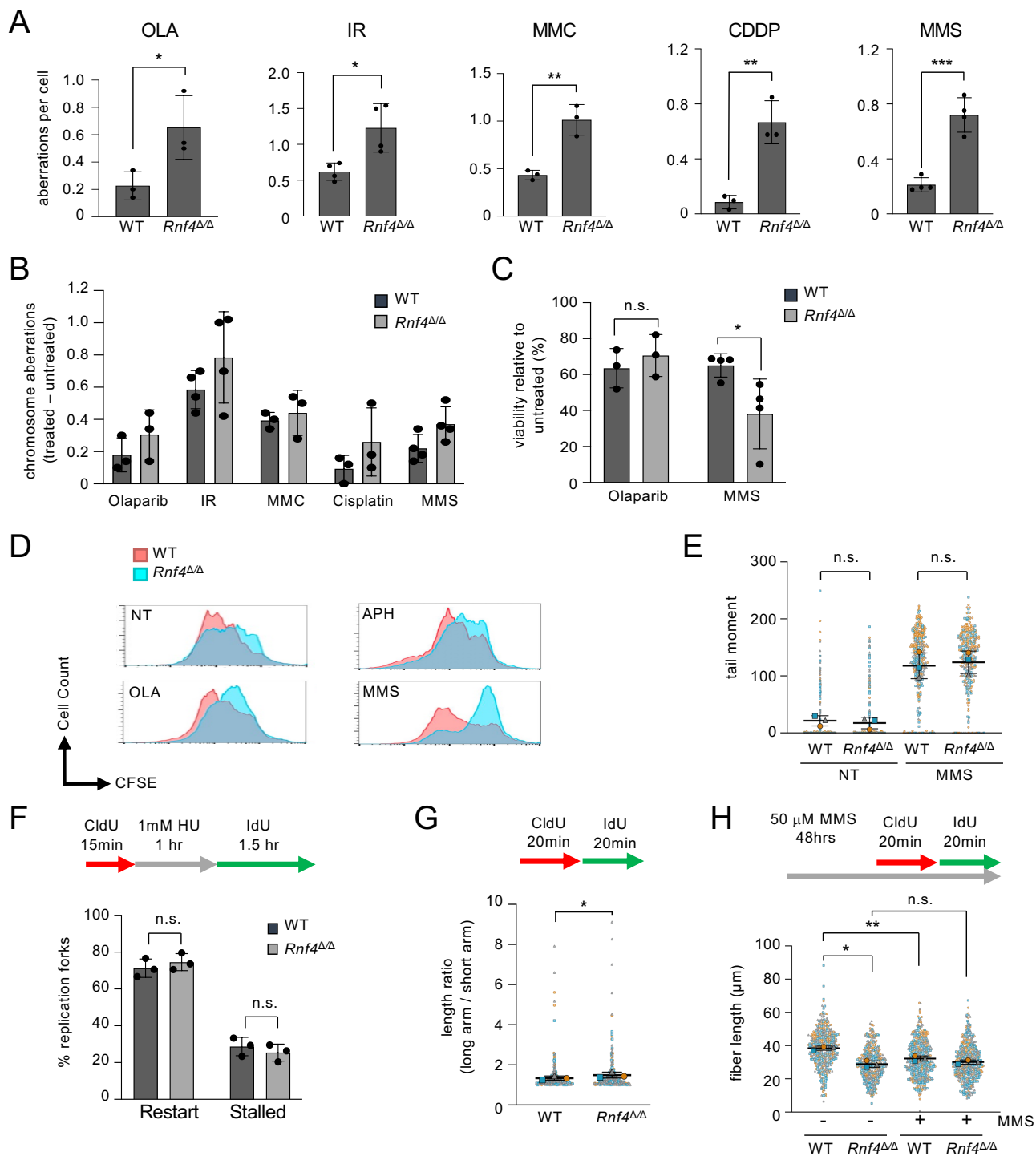
**Figure 5: Genomic instability and cell death in RNF4-deficient cells is dependent on RAD51.** (A) Western blot analysis of WT and *Rnf4* $\Delta\Delta$  B cells cultured in vitro for 72 hrs with mock treatment (-) or with the RAD51 inhibitor, RI-1 (5  $\mu$ M, 10  $\mu$ M). (B) Analysis of chromosome aberrations in Day 2 cultured B cells  $\pm$  RI-1 treatment (5  $\mu$ M, 10  $\mu$ M). (C) Analysis of CFSE FACS to measure cell proliferation during 72 hrs growth  $\pm$  RI-1 treatment (5  $\mu$ M). (D) As (A) with the RAD51 inhibitor, B02 (5  $\mu$ M, 10  $\mu$ M). (E) As (B), with B02 (5  $\mu$ M, 10  $\mu$ M). (F) As (C) with B02 (5  $\mu$ M). (G) Viability of B cells, measured by DAPI exclusion, after treatment with RI-1 (5  $\mu$ M, 10  $\mu$ M) on Day 3. (H) As (G) with B02 (2.5  $\mu$ M, 5  $\mu$ M). (I) Measurement of newly-replicated DNA by DNA combing after pulsing cells with CldU and IdU  $\pm$  RI-1 or B02 (10  $\mu$ M in each case). Mean  $\pm$  S.D. of n=3 experiments shown. Error bars in parts B-C and E-H represent S.D. of the mean. P values were calculated by one-way ANOVA with Dunnett's multiple comparison test (B, E, G, H), unpaired t-test (C, F), and one-way ANOVA with Tukey's multiple comparison test (I). P < 0.05 was considered significant.



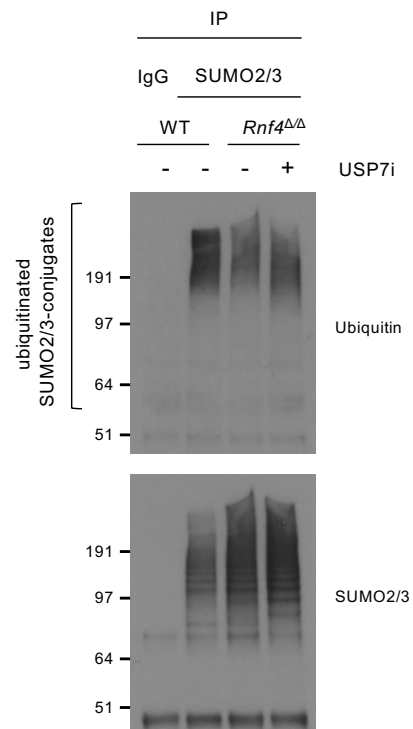
**Figure 6: RNF4 deficiency extends tumor-free lifespan of *Eμ-myc* transgenic mice.** (A) Tumor-free survival of *Rnf4*<sup>+/+</sup>/*CD19-Cre* or *Rnf4*<sup>Δ/Δ</sup>/*CD19-Cre* (n = 38), and *Rnf4*<sup>Δ/Δ</sup>/*CD19-Cre* (n = 44). (B) Tumor-free survival of *Rnf4*<sup>+/+</sup>/*Trp53*<sup>+/-</sup>/*CD19-Cre* or *Rnf4*<sup>Δ/Δ</sup>/*Trp53*<sup>+/-</sup>/*CD19-Cre* (n = 30), and *Rnf4*<sup>Δ/Δ</sup>/*Trp53*<sup>+/-</sup>/*CD19-Cre* (n = 30). (C) Tumor-free survival of *Eμ-myc*;*Rnf4*<sup>+/+</sup>/*CD19-Cre* or *Eμ-myc*;*Rnf4*<sup>Δ/Δ</sup>/*CD19-Cre* (n = 47), and *Eμ-myc*;*Rnf4*<sup>Δ/Δ</sup>/*CD19-Cre* (n = 27). (D) Analysis of chromosome aberrations of B cells of the indicated genotypes after two days in culture. (E) Analysis of cell viability of B cells of the indicated genotypes during 72 hrs of in vitro growth. (F) Growth of U2OS-iMYC cells after doxycycline-induced expression of *c-myc*, measured by quantification of CellTiterGlo fluorescence. P values show difference between the means of unstimulated and stimulated cells. (G) Analysis of replication fork velocity measured by DNA combing. Total CldU + IdU tract length is shown. Mean ± S.D. of n=3 experiments shown. (H) % of S-phase (EdU<sup>+</sup>) cells staining positively for γ-H2AX. (I) Survival of pediatric B cell acute lymphoblastic leukemia (B-ALL) patients with tumors expressing either normal or increased levels of *RNF4*. (J) Model for steps leading to cell death in *RNF4*-deficient cells. Absence of *RNF4* activity causes increased abundance of SUMOylated proteins in chromatin, leading to replication stress and accumulation of non-productive intermediates, dependent on *RAD51*, which prevents successful completion of DNA replication and further cell proliferation. Error bars in parts D-F, and H show S.D. of the mean. P values were calculated by log-rank test (A-C, I), unpaired t-test (D, F), two-way ANOVA with Dunnett's multiple comparison test (E), and one-way ANOVA with Tukey's multiple comparison test (G, H). P < 0.05 was considered statistically significant.



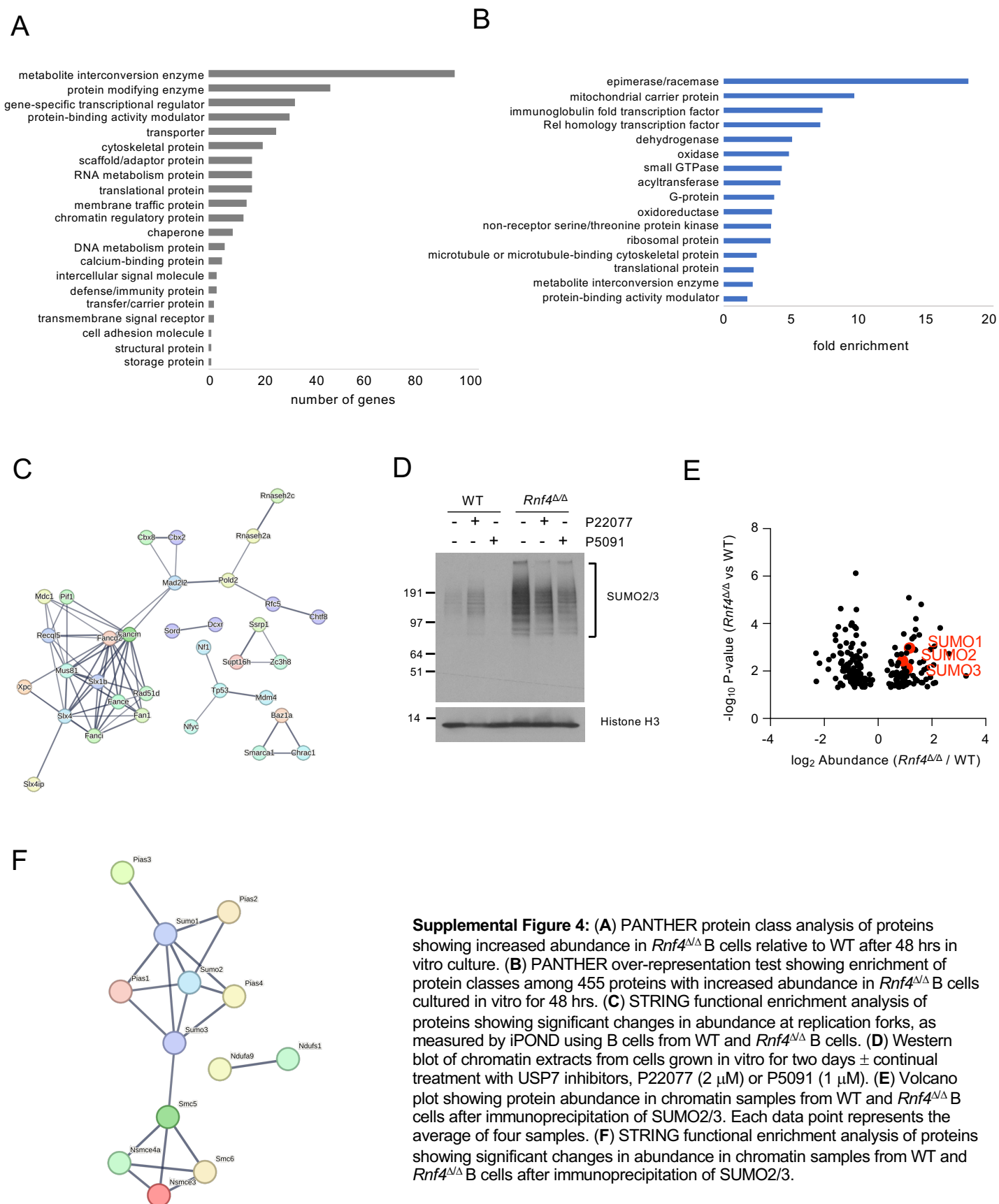
**Supplemental Figure 1:** (A) Quantification of sister chromatid exchanges in WT and *Rnf4*<sup>ΔΔ</sup> B cells after 48 hrs growth in vitro. Mean ± S.D. of n=3 experiments shown. (B) Quantification of metaphase chromosome aberrations from B cells of the indicated genotypes. Error bars show S.D. of the mean. (C) Viability of B cells of the indicated genotypes after two or three days in culture. Viable cells were identified by FACS on the basis of PI exclusion. P values were calculated by paired t-test (A), unpaired t-test (B), and two-way ANOVA with Tukey's multiple comparison test (C). P < 0.05 was considered significant.



**Supplemental Figure 2:** (A) Chromosome aberrations in WT and *Rnf4*<sup>ΔΔ</sup> B cells cultured for 36 hrs in vitro followed by overnight treatment with either olaparib (2 μM), ionizing radiation (2 Gy), mitomycin C (MMC, 250 nM), cisplatin (1 μM), or methyl methanesulfonate (MMS, 100 μM). (B) Total chromosome aberrations induced by treatments as shown in (A), after subtracting the number of chromosome aberrations present in untreated WT and *Rnf4*<sup>ΔΔ</sup> B cells. The differences between the means did not meet the threshold for statistical significance ( $P < 0.05$ ) in any case. (C) B cell viability after induction of DNA damage. B cells were grown for 72 hrs in culture  $\pm$  olaparib (1 μM) or MMS (100 μM). Viability was measured by flow cytometry based on DAPI exclusion. (D) Representative data for Figure 2B, flow cytometry analysis of CFSE dilution to measure B cell growth in vitro over 72 hrs. (E) Alkaline comet assay to measure single-strand DNA breaks in cells of the indicated genotypes after 2 days in culture. MMS treatment was 100 μM, 3 hrs. Mean  $\pm$  S.D. of  $n=3$  experiments shown. (F) DNA fiber analysis to measure fork stalling and restart after hydroxyurea treatment. (G) Measurement of asymmetry of replication from single replication origins, measured by DNA fiber analysis. The ratio of the longer replication tract compared to the shorter tract was quantified for each origin. Mean  $\pm$  S.D. of  $n=3$  experiments shown. (H) Measurement of replication fork velocity by DNA combing in untreated cells and after treatment with MMS. Mean  $\pm$  S.D. of  $n=3$  experiments shown. For parts A-C and F, error bars represent the S.D. of the means. P values were calculated by unpaired t-test (A-C, F), paired t-test (E, G), and one-way ANOVA with Tukey's multiple comparison test (H).  $P < 0.05$  was considered statistically significant.

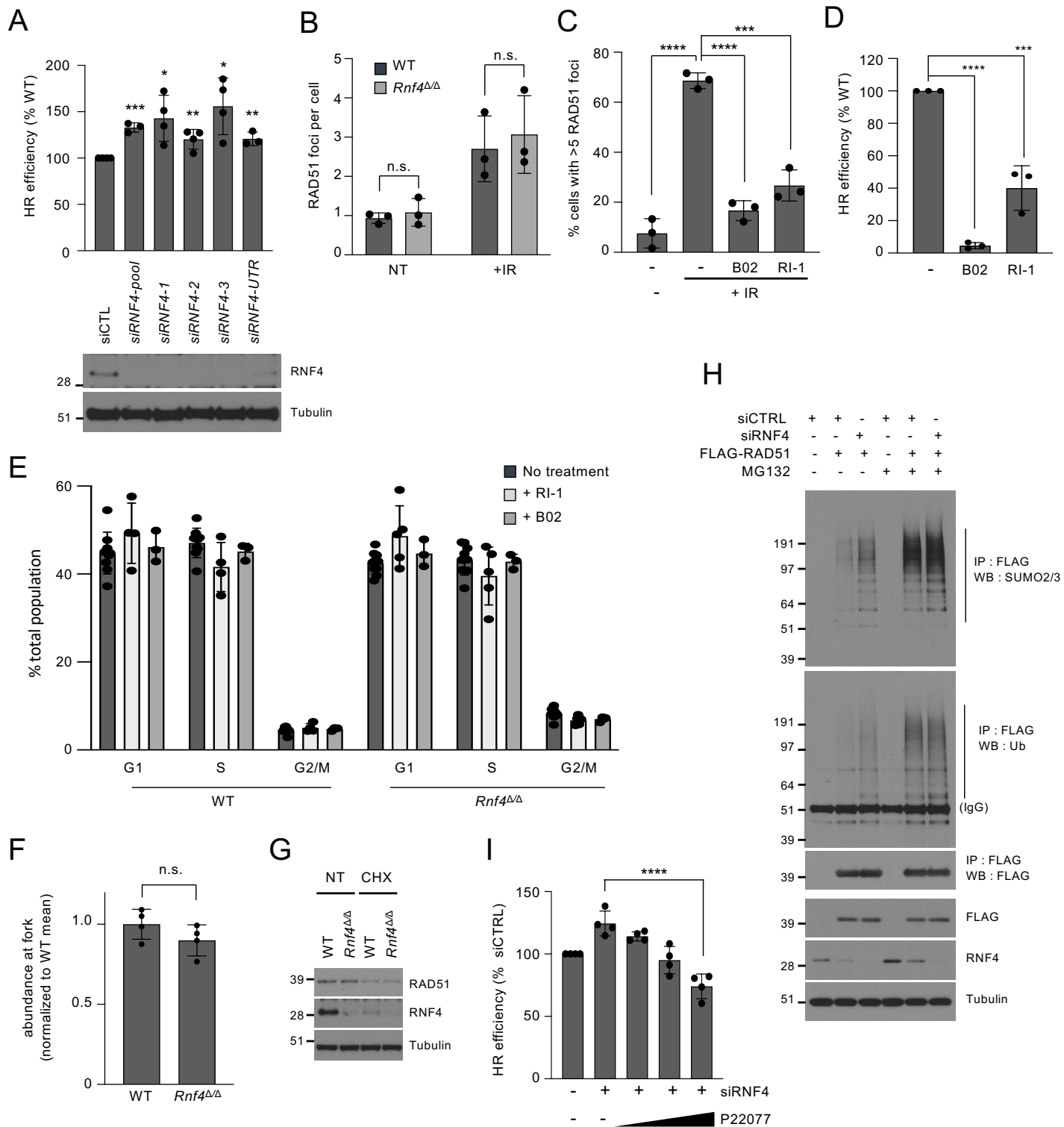


**Supplemental Figure 3:** Western blot to measure levels of ubiquitination of SUMO-conjugated proteins on chromatin in *Rnf4*<sup>ΔΔ</sup> B cells after short-term treatment with USP7 inhibitor. B cells cultured in vitro for 48 hrs were either untreated (-) or treated with 50 μM P22077 for 4 hrs. SUMO2/3-conjugates were immunoprecipitated from the chromatin fraction of B cells using an anti-SUMO2/3 antibody, and subsequently used for detection of ubiquitin.

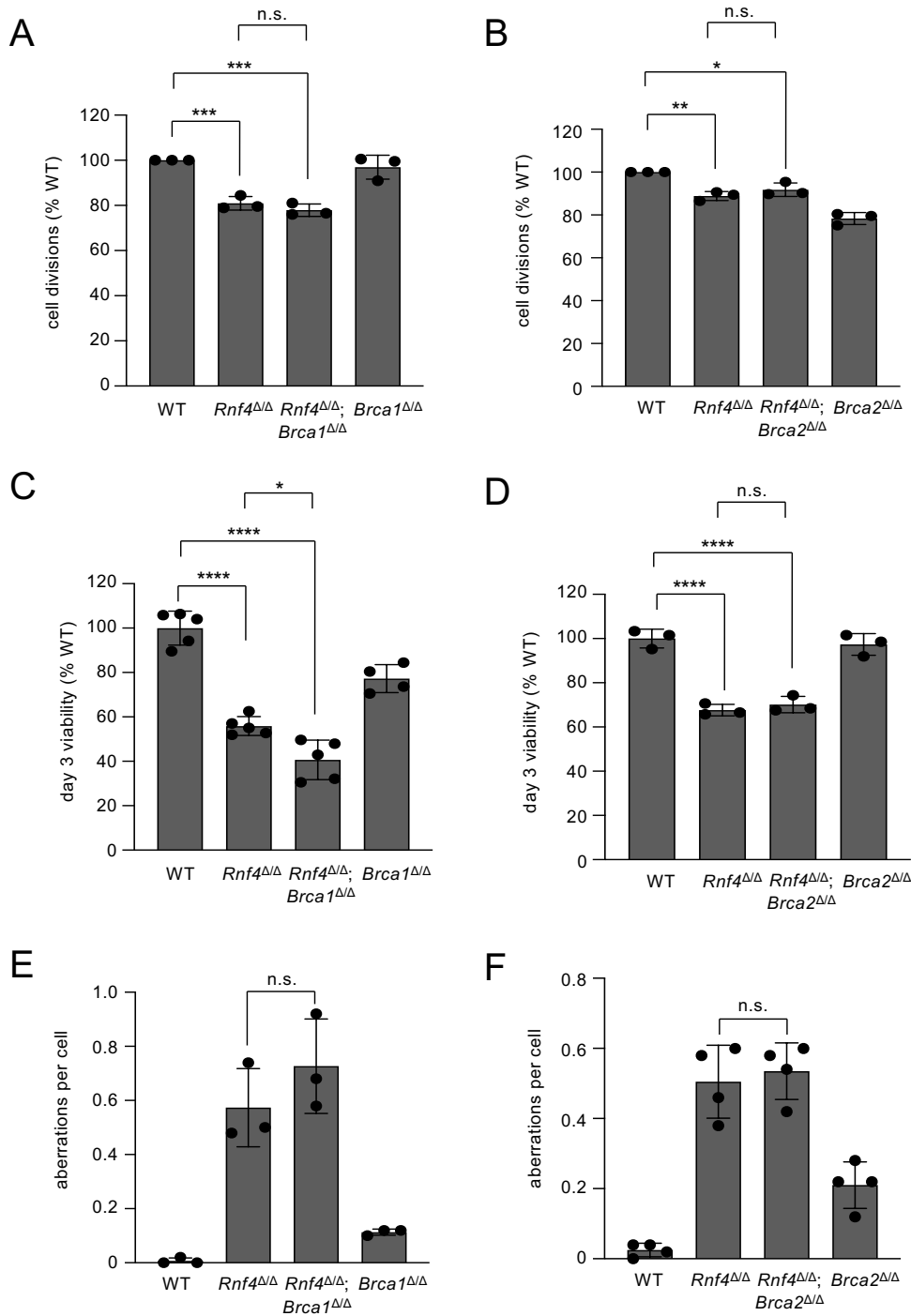


**Supplemental Figure 4:** (A) PANTHER protein class analysis of proteins showing increased abundance in *Rnf4 $\Delta\Delta$*  B cells relative to WT after 48 hrs in vitro culture. (B) PANTHER over-representation test showing enrichment of protein classes among 455 proteins with increased abundance in *Rnf4 $\Delta\Delta$*  B cells cultured in vitro for 48 hrs. (C) STRING functional enrichment analysis of proteins showing significant changes in abundance at replication forks, as measured by iPOND using B cells from WT and *Rnf4 $\Delta\Delta$*  B cells. (D) Western blot of chromatin extracts from cells grown in vitro for two days  $\pm$  continual treatment with USP7 inhibitors, P22077 (2  $\mu$ M) or P5091 (1  $\mu$ M). (E) Volcano plot showing protein abundance in chromatin samples from WT and *Rnf4 $\Delta\Delta$*  B cells after immunoprecipitation of SUMO2/3. Each data point represents the average of four samples. (F) STRING functional enrichment analysis of proteins showing significant changes in abundance in chromatin samples from WT and *Rnf4 $\Delta\Delta$*  B cells after immunoprecipitation of SUMO2/3.

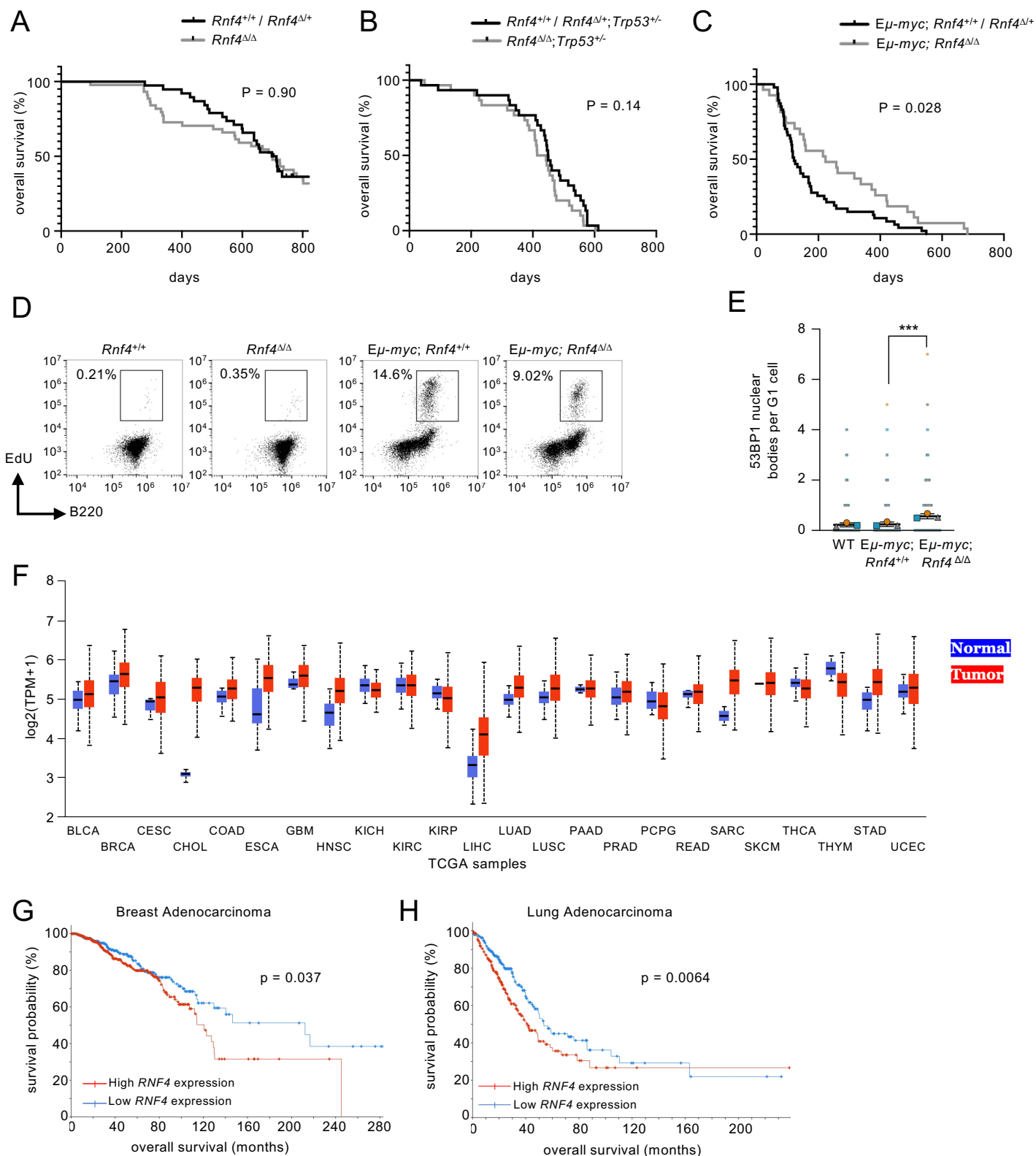




**Supplemental Figure 5: (A)** Measurement of homologous recombination frequency by DR-GFP assay after transfection with either control siRNA (siCTRL) or siRNA oligonucleotides targeting *RNF4*. Efficiency of knockdown is shown by Western blot in the lower panel. **(B)** Quantification of RAD51 foci in cells after no treatment (NT), or after 4 hrs recovery from treatment with 10 Gy of ionizing radiation (IR). **(C)** Analysis of proportion of WT cells showing >5 ionizing radiation-induced nuclear foci after either no treatment (-) or with the RAD51 inhibitors, B02 and RI-1 (1 hr, 50  $\mu$ M). **(D)** Homologous recombination efficiency measured by DR-GFP assay in cells that were either untreated (-) or treated for 1 hr with B02 (20  $\mu$ M) or RI-1 (50  $\mu$ M). **(E)** Measurement of cell cycle by quantification of EdU incorporation and DAPI binding in splenic B cells after 48 hrs growth in vitro  $\pm$  10  $\mu$ M RI-1 or 5  $\mu$ M B02. **(F)** Abundance of RAD51 at active replication forks, measured by iPOND-MS in n=4 replicate samples. **(G)** RAD51 abundance measured by Western blot in cells that were either not treated (NT) or treated with 25  $\mu$ g/ml cycloheximide (CHX) for 4 hrs. **(H)** Analysis of ubiquitin present in FLAG-RAD51 pull-down samples in cells treated with either control siRNA (siCTRL) or siRNA targeting RNF4 (siRNF4). Some samples were treated with the proteasome inhibitor, MG132 (10  $\mu$ M, 4 hrs). **(I)** Homologous recombination efficiency measured by DR-GFP in cells that were treated  $\pm$  siRNF4 and/or the USP7 inhibitor, P22077 (2, 5, 10 $\mu$ M). Error bars in **A-F**, and **I** represent the S.D. of the mean. P values were calculated by unpaired t-test (**A, B, F**), one-way ANOVA with Tukey's multiple comparison test (**C, I**), and one-way ANOVA with Dunnett's multiple comparison test (**D**). P < 0.05 was considered significant.



**Supplemental Figure 6:** (A) Quantification of proliferation of B cells growing in vitro for 72 hrs, measured by flow cytometry analysis of CFSE dilution. *Rnf4* and *Brca1* were deleted either singly or in combination in the B cell lineage by breeding to a CD19-Cre transgenic line. (B) as (A) but with conditional deletion of *Brca2* on a WT or *Rnf4*<sup>ΔΔ</sup> background. (C) Quantification of cell viability of cells of the indicated genotypes, after three days of in vitro B cell culture. Cell viability was measured by DAPI exclusion, as detected by flow cytometry. (D) As (C) but with deletion of *Brca2*. (E) Quantification of chromosome aberrations of B cells of the indicated genotypes. (F) As (E) but with conditional deletion of *Brca2*. Error bars show the S.D. of the mean in each case, with P values calculated by one-way ANOVA with Tukey's multiple comparison test. A P value < 0.05 was considered statistically significant.



**Supplemental Figure 7: (A)** Kaplan-Meier analysis showing overall survival of *Rnf4*<sup>+/+</sup>/*CD19-Cre* or *Rnf4*<sup>Δ/Δ</sup>/*CD19-Cre* (n = 38), and *Rnf4*<sup>Δ/Δ</sup>/*CD19-Cre* (n = 44). **(B)** Kaplan-Meier analysis of overall survival of *Rnf4*<sup>+/+</sup>;*Trp53*<sup>+/-</sup>/*CD19-Cre* or *Rnf4*<sup>Δ/Δ</sup>;*Trp53*<sup>+/-</sup>/*CD19-Cre* (n = 30), and *Rnf4*<sup>Δ/Δ</sup>;*Trp53*<sup>+/-</sup>/*CD19-Cre* (n = 30). **(C)** Kaplan-Meier analysis of overall survival of *Eμ-myc*;*Rnf4*<sup>+/+</sup>/*CD19-Cre* or *Eμ-myc*;*Rnf4*<sup>Δ/Δ</sup>/*CD19-Cre* (n = 47), and *Eμ-myc*;*Rnf4*<sup>Δ/Δ</sup>/*CD19-Cre* (n = 27). **(D)** Flow cytometry analysis showing EdU uptake by B220<sup>+</sup> resting B cells from the spleens of WT, *Rnf4*<sup>Δ/Δ</sup>, and *Eμ-myc* transgenic mice. **(E)** Quantification of 53BP1 G1 nuclear bodies, measured by anti-53BP1 immunofluorescence in G1 cells of the indicated genotypes after two days of in vitro culture. P value calculated with Mann-Whitney U-test. Mean ± S.D. of n=3 experiments shown. **(F)** Expression of *RNF4* in different tumor types according to TCGA data sets. **(G)** Survival of breast adenocarcinoma patients with tumors expressing either above-median (n = 541) or below-median (n = 541) levels of *RNF4*. Data from TCGA PanCancer Atlas retrieved through cBioPortal. **(H)** Survival of patients with lung adenocarcinoma expressing either above-median (n = 249) or below-median (n = 252) levels of *RNF4*. P values in **A-C** and **G-H** calculated by log-rank test. P value in **E** calculated by paired t-test. P < 0.05 was considered significant in each case.