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SHP2 is required for growth of KRAS-mutant non-small-cell lung cancer in vivo letter

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(Article begins on next page)

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2 **SHP2 is required for growth of *KRAS* mutant Non Small Cell Lung**
3 **Cancer *in vivo*.**

4

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28 RAS mutations are frequent in human cancer, especially in pancreatic,
29 colorectal and non-small cell lung cancers (NSCLC)^{1,2 3}. Inhibition of the RAS
30 oncoproteins has proven difficult⁴, and attempts to target downstream effectors
31^{5 6 7} have been hampered by the activation of compensatory resistance
32 mechanisms⁸. It is also well-established that *KRAS* mutant tumors are
33 insensitive to inhibition of upstream growth factor receptor signaling. Thus,
34 EGFR antibody therapy is only effective in *KRAS* wild type colon cancers^{9 10}.
35 Consistently, inhibition of the protein tyrosine phosphatase non-receptor type 11
36 (SHP2, encoded by *PTPN11*), which links receptor tyrosine kinase signaling to
37 the RAS-RAF-MEK-ERK pathway^{11 12}, was shown to be ineffective in *KRAS* or
38 *BRAF* mutant cancer cell lines¹³. Our data also indicate that SHP2 inhibition in
39 *KRAS* mutant NSCLC cells under normal cell culture conditions has little effect.
40 In contrast, SHP2 inhibition under growth factor-limiting conditions *in vitro*
41 results in a senescence response. *In vivo*, inhibition of SHP2 in *KRAS* mutant
42 NSCLC also provokes a senescence response, which is exacerbated by MEK
43 inhibition. Our data identify SHP2 inhibition as an unexpected vulnerability of
44 *KRAS* mutant NSCLC cells that remains undetected in cell culture, which can be
45 exploited therapeutically.

46 Activation of members of the EGFR family of Receptor Tyrosine Kinases (RTKs)
47 contributes significantly to the intrinsic resistance to MEK inhibition in *KRAS* mutant
48 lung and colon cancers¹⁴. To study this further, we used phosphorylation of SHP2 as
49 a readout of RTK activation in a panel of *KRAS* mutant lung, colon and pancreatic
50 cancer cell lines treated with the MEK inhibitor AZD6244 (selumetinib) (**Fig. 1a**,
51 **Supplementary Figs. 1a** and **2a**). We found that all 6 MEK inhibitor resistant
52 NSCLC cell lines tested (**Fig. 1b**) showed an initial reduction in phospho-ERK
53 (pERK) levels following MEK inhibition, which was restored within 72 hours,
54 together with an increase in SHP2 activation, as judged by Tyrosine 542
55 phosphorylation¹⁵⁻¹⁷ (pSHP2 Y542, **Fig. 1a**). Similarly, pSHP2 and pERK increased
56 in the *KRAS* mutant pancreatic cancer cell lines Panc10.05 and MiaPaCa2
57 (**Supplementary Fig. 1a**) upon MEK inhibition and in 4 out of 5 *KRAS* mutant colon
58 cancer cell lines (**Supplementary Fig. 2a**). Overall these data support the notion that
59 a feedback loop involving RTKs is activated upon MEK inhibition. The increased
60 RTK signaling subsequently activates the RAS-MEK-ERK pathway through SHP2

61 the extent that the MEK inhibitor is unable to completely block signaling to ERK
62 kinase, thereby maintaining proliferation¹⁴.

63 We next explored the possibility of increasing sensitivity to MEK inhibition by
64 concomitantly inhibiting RTK signaling to RAS-RAF-MEK by using a SHP2
65 inhibitor (compound #57¹⁸). Consistent with earlier results¹³, SHP2 inhibition alone
66 had no or very little effect on proliferation in all cell lines tested, both in a two weeks
67 colony formation assay and in a one week cell proliferation assay (**Fig. 1b**,
68 **Supplementary Figs. 1b, c, 2b, c and 3**). In contrast, combination of SHP2 and MEK
69 inhibitors showed strong synergy in all the *KRAS* mutant lung cancer cell lines tested
70 (**Fig. 1b, Supplementary Fig. 3**). Similar results were obtained with another SHP2
71 inhibitor SHP099¹³ (**Supplementary Fig. 4a, b**). SHP099 treatment resulted in an
72 increase in phosphorylation of the its target STAT1, confirming target engagement of
73 the drug (**Supplementary Fig. 5a**)¹⁹. Similarly, although to different extents, the 2
74 pancreatic and 5 colon cancer cell lines showed increased sensitivity to AZD6244
75 when combined with SHP2 inhibitor (**Supplementary Figs. 1b, c, 2b, c**,
76 **Supplementary Table 1**). Western blot analysis of H2122 and H1944 NSCLC cells
77 confirmed that concomitant SHP2 inhibition prevented pERK reactivation following
78 treatment with AZD6244 (**Fig. 1c**). Overall, our results indicate that SHP2 inhibition
79 is synthetic lethal with MEK inhibition in *KRAS* mutant tumors of different origins,
80 with the strongest effect being observed in NSCLC. We therefore focused on NSCLC
81 for further experiments.

82 We next validated genetically the synthetic lethality between SHP2 and MEK
83 inhibition by generating CRISPR/Cas9 *PTPN11* knockouts in H2122 and H1944
84 NSCLC cells (**Fig. 1e**). In both lines, knockout of *PTPN11* in two independent clones
85 had little effect on cell proliferation, but increased sensitivity to MEK inhibitor (**Fig.**
86 **1d**). Western blot analysis showed that *PTPN11* knockouts are unable to restore
87 pERK levels upon MEK inhibition (**Fig. 1e**). Similar results were obtained in the
88 pancreatic cancer cell line Panc10.05 (**Supplementary Fig 1d, e**). The effects of
89 MEK inhibitor appear due to the absence of phosphatase activity, as restoration of
90 wild type, but not a phosphatase inactive mutant of SHP2, could confer resistance to
91 MEK inhibition in *PTPN11* knockout H2122 cells (**Supplementary Fig. 5b, c**).
92 These results indicate that SHP2 inactivation disables the RTK-mediated feedback

93 loop leading to re-activation of the MAPK pathway in the presence of MEK
94 inhibition.

95 To further test the hypothesis that *PTPN11* suppression uncouples RTK activation
96 from downstream RAS signaling, we measured the activation state of RAS by
97 measuring GTP-bound RAS levels in H2122 and H1944 cells through a RAS GST-
98 RBD pulldown assay²⁰ (**Fig. 2a**). *PTPN11* knockout cells displayed lower RAS-GTP
99 levels compared to their parental counterparts (in H2122), and importantly those
100 levels were not increased 30 minutes after addition of EGF or EGF+AZD6244 (in
101 both H2122 and H1944). Similar results were obtained in Panc10.05 (**Supplementary**
102 **Fig. 1f**). Likewise, treatment with SHP2 inhibitor of parental H2122 and H1944
103 blocked the increase in RAS-GTP levels resulting from MEK inhibition (**Fig. 2b**). It
104 is important to point out that the RAS pulldown assay doesn't allow discrimination
105 between wild type and mutant members of the RAS family. Consequently, we could
106 not ascertain in this experiment whether the oncogenic KRAS protein is affected in its
107 GTP loading, as this effect could be obscured by effects on the wild type RAS
108 proteins present in the cells. To overcome this limitation, we used Rasless murine
109 embryonic fibroblasts (from now on Rasless) reconstituted with either wild type or a
110 *KRAS*^{G12V} expression vector, in order to study the effects of SHP2 inhibition in a
111 mutant *KRAS* only context²¹. *KRAS*^{G12V}-reconstituted Rasless cells show increased
112 sensitivity to the combination of MEK and SHP2 inhibitors (**Fig. 2c**), suggesting that
113 SHP2 inactivation actually inhibits *KRAS*^{G12V} activity. Consistent with this, we find
114 that activation of *KRAS*^{G12V}-GTP levels after MEK inhibition, resulting from
115 feedback activation of RAS through HER-receptors, was blocked by SHP2 inhibition
116 (**Fig. 2d**). Combined SHP2+MEK inhibition also leads to inhibition of pERK,
117 explaining the anti-proliferative effect of the drug combination (**Fig. 2d**).

118 Having established that SHP2 and MEK inhibitors synergize to inhibit proliferation of
119 *KRAS* mutant NSCLC cells *in vitro*, we set out to validate our findings in an *in vivo*
120 context. To do this, we evaluated the ability of *PTPN11* knockout H2122 cells to
121 grow subcutaneously as xenografts in immunocompromised mice, both in the
122 presence and absence of MEK inhibitor. Remarkably, and in contrast to our *in vitro*
123 findings, after the initial engraftment, *PTPN11* knockout tumors failed to grow, even
124 in the absence of MEK inhibitor (**Fig. 3a**). To study this further, we injected H1944
125 NSCLC cells in immunocompromised mice and treated with the SHP2 inhibitor

126 SHP099 when tumors had reached a volume of 200 mm³. **Fig. 3b** shows that SHP2
127 inhibition alone was sufficient to completely stop tumor growth *in vivo*, which was
128 associated with a complete loss of pERK in the tumors after SHP099 treatment (**Figs.**
129 **3c-e, Supplementary Fig. 6a**). Similarly, the *in vivo* growth of Rasless MEFs
130 reconstituted with either *KRAS*^{G12C} or *KRAS*^{G12V} was severely inhibited upon
131 treatment with SHP099 (**Fig. 3f**).

132 One of the major differences between *in vitro* and *in vivo* growth of cancer cells is the
133 paucity of growth factors *in vivo*, whereas in fetal calf serum (used for *in vitro*
134 culturing) these growth factors are plentiful. We therefore asked whether reduction of
135 the serum concentration in the *in vitro* culture medium would impair the proliferation
136 rate of *PTPN11* knockout NSCLC cells. **Fig. 3g, h** show that H2122 cells had a
137 slower proliferation rate when the serum concentration in the culture medium was
138 reduced, but this effect was more pronounced in *PTPN11* knockout derivatives of
139 these cells. Biochemically, knockout of *PTPN11* resulted in a more complete
140 inhibition in pERK and phospho-RB levels in 1% serum conditions (**Fig. 3i**).

141 Morphologically, the *PTPN11* knockout H2122 cells cultured in reduced serum
142 conditions had a senescence-like appearance, which is supported by the notion that
143 these cells stain positive for senescence-associated β-galactosidase (SA-β-gal, **Fig. 3j**)
144 and have reduced phospho-RB (**Fig. 3i**). An increase in SA-β-gal was also seen in
145 H2030 NSCLC cells grown in low serum and a trend of increasing SA-β-gal was seen
146 in 4 other NSCLC cell lines (**Supplementary Fig. 6e**). To ask whether the *in vivo*
147 growth defect of *PTPN11* knockout H2122 cells was also due to a senescence-like
148 response we used tumor sections derived from H2122 wild type and *PTPN11*
149 knockout tumors obtained from the nude mice tumors shown in **Figure 3a**. **Figure 3k**
150 shows that tumors derived from *PTPN11* knockout H2122 cells stained strongly for
151 SA-β-gal, whereas the parental cells, or the tumors treated with AZD6244, failed to
152 stain. SHP099-treated H1944 tumors also showed increased senescence as compared
153 to the vehicle-treated (**Fig. 3l**). This suggests that *in vivo* the availability of growth
154 factors and other signaling molecules can be limiting, thus revealing a latent weakness
155 of *PTPN11* knockout lung cancer cells.

156 Senescent cells are known for their “senescence associated secretory phenotype”,
157 consisting of a multitude of inflammatory cytokines, which contributes to their

158 clearing through recruited immune cells ²²⁻²⁴. Indeed, *PTPN11* knockout H2122
159 tumors appeared to be massively infiltrated with inflammatory cells, especially at the
160 tumor periphery, with CD3-positive T-cells being the major component and PAX5-
161 positive B-cells being relatively rare (**Supplementary Fig. 7a, b**). We speculate that
162 although lymphocytes are still partly present in the immune system of CD1 nude
163 mice, and likely can be attracted by senescent tumor cells, their compromised
164 maturation prevents them from clearing out the cancer cells.

165 To study the mechanism of growth inhibition of mutant *KRAS* cells by SHP2
166 inhibitors as a function of growth factor availability, we obtained from the NCI RAS
167 Initiative a panel of Rasless cells reconstituted with mutant *KRAS* alleles ²¹.
168 **Supplementary Fig. 6b** shows that neither Rasless cells having wild type *KRAS*, nor
169 cells reconstituted with the *KRAS* G13D, G12C, G12D, G12V and Q61R mutants
170 showed a decrease in pERK following treatment with SHP099 in high serum
171 conditions. In contrast, all isogenic cell lines except Q61R cells, showed a decrease in
172 pERK after SHP099 treatment when cultured in 3% serum (**Supplementary Fig. 6b**).
173 It has recently been reported that the various *KRAS* mutant proteins differ in their
174 intrinsic RAS GTPase activity, with *KRAS* G13D having the highest and Q61
175 mutants having the lowest intrinsic GTPase activity ^{25,26}. In this context, it is
176 noteworthy that the mutant RAS reconstituted Rasless cells respond to SHP099
177 approximately according to their intrinsic GTPase activity: the cells having the
178 highest intrinsic GTPase activity have the highest sensitivity to SHP099, the Q61R
179 with lowest GTPase activity is the most resistant to SHP099 (**Supplementary Fig.**
180 **6c**). This effect is only seen in 3% serum and much less pronounced in 10% serum, in
181 agreement with the notion that these cells display a reduction on pERK levels only in
182 3% serum and not in 10% serum upon SHP099 treatment. These data are compatible
183 with a model in which mutant *KRAS* proteins still depend on upstream signals to
184 become GTP bound, but that this dependency is relative: the mutations with the
185 lowest intrinsic GTPase activity require less upstream signal to remain GTP bound.
186 Consistent with this model, we find that MEFs expressing *KRAS* G12C or G12V do
187 not reduce their RAS GTP levels upon SHP2 inhibition in 10% FCS, but do have a
188 drop in RAS-GTP in 3% serum upon SHP2 inhibition (**Supplementary Fig. 6d**).

189

190 Next, we established patient-derived xenograft (PDX) models and treated them with
191 SHP099. SHP2 inhibition was able to significantly reduce the growth of
192 subcutaneously implanted PDX2 *KRAS* mutant NSCLC (**Fig. 4a, b**) which was
193 associated with a decrease in pERK levels in the tumor (**Fig. 4e**). Also in the PDX2
194 induced tumors, SHP2 inhibition induced a senescence-like state as judged by
195 staining for SA- β -gal (**Fig. 4f**). Furthermore, RNAseq analyses of vehicle and
196 SHP099 treated tumors revealed an increase in three established “senescence
197 signatures”²⁷⁻²⁹ and an increase in a SASP signature²⁹ (**Fig. 4h**). SHP099 also
198 partially reduced the growth of an additional *KRAS* mutant NSCLC PDX3 implanted
199 subcutaneously, which was also associated with staining for SA- β -gal (**Fig. 4c, d, g**).
200 Importantly, SHP099 was able to prolong the survival of two different NSCLC
201 patient-derived orthotopic xenografts (PDOX)³⁰, generated by implanting the two
202 previously described patient-derived specimen in the lungs of nude mice (**Fig. 4i, j**).
203 Of note, both SHP099 and AZD6244 monotherapies were well tolerated by the mice,
204 as demonstrated by the lack of weight loss reported in **Supplementary Figure 8 a-d**.
205 In agreement with this, we found that in a genetically engineered conditional mouse
206 model of p53^{fl/fl}, *KRas*+/*LSLG12D* induced NSCLC³¹, SHP099 was able to control
207 orthotopically growing lung tumors for the duration of the treatment (**Supplementary**
208 **Fig. 9**). Following our *in vitro* observation of a synergistic effect of SHP2 and MEK
209 inhibition in *KRAS* mutant NSCLC cell lines, we tested the effect of the double
210 treatment in a subcutaneously implanted (PDX3) PDX model. Notably, combined
211 administration of SHP099 and AZD6244 was able to induce tumor regression even at
212 the lowest AZD6244 dose (25 mg/kg) (**Fig. 4 k, l** and **Supplementary Fig. 8f**),
213 whereas AZD6244 monotherapy was mainly ineffective (**Fig. 4c, d**). Importantly, the
214 double treatment showed no major toxicity using a 5 days on/ 2 days off schedule, as
215 reported in **Supplementary Figure 8e**.

216 Finally, we asked whether SHP2 inhibition is also effective in *KRAS* wild type
217 NSCLC. We transplanted two PDX models (PDX4 and PDX5) having wild type
218 EGFR, BRAF and *KRAS* genes into nude mice. **Supplementary Fig. 9a-c** show that
219 SHP099 inhibited their growth almost completely, with little if any additional effect
220 when combined with MEK inhibition. These data agree well with our observations in
221 Rasless cells reconstituted with various mutant *KRAS* alleles, in which we observed a

222 correlation between high intrinsic RAS GTPase activity and favorable response to
223 SHP2 inhibition under growth factor limiting conditions.

224 Overall our data together with those from the accompanying manuscript by Ruess et
225 al.³², demonstrate that genetic or pharmacological inactivation of the SHP2
226 phosphatase in *KRAS* mutant tumors can interfere with RAS signaling to the
227 downstream MAPK signaling cascade. This is unexpected, given that SHP2 acts
228 upstream of RAS in signal transduction. SHP2 inhibition alone could be sufficient, at
229 least in some contexts, to induce tumor senescence³³ which in turn can trigger
230 clearance of the cancer cells by the immune system. Our data as well as those of
231 Ruess et al., indicate that co-treatment with MEK inhibition may further enhance the
232 effect of SHP2 inhibition. Our findings suggest that inhibition of SHP2 could have
233 clinical utility for *KRAS* mutant NSCLC.

234

235 **References**

236

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321 **Figure legends:**

322 **Fig. 1. SHP2 inactivation sensitizes *KRAS* mutant lung cancer cells to MEK**

323 **inhibition (a)** Western blot analysis of 6 *KRAS* mutant, AZD6244-resistant, lung
324 cancer cell lines (H358, H2122, A549, H2030, H23, H1944), treated with 1 μ M
325 AZD6244 and collected for lysis at the indicated time points. Protein extracts were
326 probed with specific antibodies against SHP2 (total and phosphorylated), ERK (total
327 and phosphorylated) and GAPDH (as a loading control). The blots are representative
328 of at least three independent experiments. Full blots are shown in **Supplementary**
329 **Figure 11. (b)** Colony formation assay of the above cell lines treated either with
330 AZD6244, SHP2 inhibitor (compound #57) or a combination. The 6 *KRAS* mutant
331 lung cancer cell lines were cultured in medium containing the indicated
332 concentrations of drugs for two weeks. After this, cells were fixed and stained.
333 Images are representative of at least three independent experiments. (c) Western blot
334 analysis of H2122 (left panel) and H1944 (right panel) cells treated either with 10%
335 FCS, 1 μ M AZD6244, 1 μ M compound #57 or combinations as indicated for 72 hours.
336 Protein extracts were probed with specific antibodies against ERK (total and
337 phosphorylated) and HSP90 (as a loading control). The blots are representative of at
338 least three independent experiments. Full blots are shown in **Supplementary Figure**
339 **11. (d)** Colony formation assay of H2122 (left panel) and H1944 (right panel)
340 parental (WT) and *PTPN11* KO clones. The cells were cultured in medium containing
341 the indicated concentrations of AZD6244 for two weeks. After this they were fixed
342 and stained. Images are representative of at least three independent experiments. (e)
343 Western blot analysis of H2122 (left panel) and H1944 (right panel) parental (WT)
344 and *PTPN11* KO clones. The cells were treated with 1 μ M AZD6244 and lysates
345 collected at the indicated time points and probed with specific antibodies against
346 phosphorylated SHP2, ERK (total and phosphorylated), and HSP90 or GAPDH (as a
347 loading control). The blots are representative of at least three independent
348 experiments. Full blots are shown in **Supplementary Figure 11.**

349

350 **Fig. 2. SHP2 inhibition affects *KRAS*^{G12V} GTP loading status. (a) RAS-GST-RBD**

351 pulldown assay to measure the activity of RAS proteins in *PTPN11* proficient and KO
352 H2122 (right panel) and H1944 (left panel) cells. Cells were serum-starved for 24
353 hours before being stimulated with 50 ng/ml EGF and/or 1 μ M AZD6244 as indicated.

354 After 30 minutes cells were collected and lysed. Active RAS was affinity-precipitated
355 and detected by western blot analysis. Total RAS, SHP2 and phospho-ERK levels
356 were also detected in total lysates from the same samples. The blots are representative
357 of at least two independent experiments. Full blots are shown in **Supplementary**
358 **Figure 11. (b)** RAS-GST-RBD pulldown in H1944 (right panel) and H2122 (left
359 panel), performed as described above. Cells were serum-starved for 24 hours before
360 being stimulated with 50 ng/ml EGF and/or 1 μ M AZD6244 and/or 1 μ M of SHP099
361 for 72 hours as indicated. The blot is representative of three independent experiments.
362 Full blot is shown in **Supplementary Figure 11. (c)** Dose-response curves of
363 KRAS^{WT}-reconstituted (left panel) or KRAS^{G12V}-reconstituted (right panel) Rasless
364 MEFs. The cells were treated with increasing concentrations of AZD6244, either
365 alone or in combination with SHP099, as indicated. Data is shown as mean of three
366 technical replicates. **(d)** RAS-GST-RBD pulldown assay in KRAS^{G12V}-reconstituted
367 Rasless MEFs, performed as described above. Phospho-SHP2, total RAS, ERK (total
368 and phosphorylated) and HSP90 levels were detected in total lysates from the same
369 samples. The blot is representative of two independent experiments. Full blot is
370 shown in **Supplementary Figure 11.**

371 **Fig. 3. SHP2 inactivation induces senescence and impairs tumor growth in**
372 **xenograft models of KRAS mutant tumors. (a)** H2122 parental (WT) and *PTPN11*
373 knockout clones were grown as tumor xenografts in CD1 nude mice. After tumor
374 establishment (200-250 mm³), mice were randomized and treated with either vehicle
375 or AZD6244 (25 mg/kg) for the indicated period of time. Mean tumor volumes \pm
376 SEM are shown (n=7 mice per group). **(b)** H1944 cells were grown as tumor
377 xenografts in NODSCID mice. After tumor establishment (200-250 mm³), mice were
378 either left untreated (n=6) or treated with vehicle (n=6) or SHP099 (82,5 mg/kg)
379 (n=7) for the indicated period of time. Mean tumor volumes \pm SEM are shown. **(c-d)**
380 Representative pictures of Hematoxilin & Eosin (HE) **(c)** and p-ERK
381 immunohistochemistry **(d)** staining of formalin-fixed paraffin-embedded (FFPE)
382 sections from H1944 vehicle and SHP099-treated xenografted tumors. Scale bars
383 represent 200 μ m. n= 3 mice per group with 2 pictures per mouse. **(e)** p-ERK
384 quantification in immunohistochemistry. Vehicle: n= 6 mice. SHP099: n= 3 mice.
385 One section per mouse was scored blindly by the assigned pathologist. The height of
386 the bars represents the mean value, error bars represent standard deviations. Data

387 points are shown as dots to indicate the distribution of the data. Statistical significance
388 (P value) was determined by an unpaired, two-tailed Student's T-test. **(f)** KRAS^{G12C}
389 (upper panel) and KRAS^{G12D} (lower panel) –reconstituted Rasless MEFs were grown
390 as xenografts in NODSCID mice. After tumor establishment (200-250 mm³), mice
391 were randomized and treated with either vehicle or SHP099 (82,5 mg/kg) for the
392 indicated period of time. Mean tumor volumes ± SEM are shown. n=7 mice per
393 group, except KRAS^{G12C} vehicle group (n=6). **(g)** Colony formation assay of H2122
394 parental (WT) and *PTPN11* KO cells cultured in medium containing 10%, 3%, 1% or
395 0.1% FCS during two weeks. Images are representative of three independent
396 experiments. **(h)** IncuCyte growth curves of H2122 parental (WT) and *PTPN11* KO
397 cells cultured in medium containing 10% or 1% FCS for 5 days. The curves are
398 representative of two independent experiments. **(i)** Western blot analysis of *PTPN11*
399 proficient (WT) and KO H2122 cells cultured in medium containing 10%, 3% or 1%
400 FCS for 72 hours (after 24 hours serum-starvation). UT = cells collected after 24
401 hours serum-starvation. The blots are representative of two independent experiments.
402 Full blots are shown in **Supplementary Figure 11**. **(j)** Senescence-associated beta-
403 galactosidase staining of H2122 parental (WT) and *PTPN11* KO cells cultured in
404 medium containing 10% or 3% FCS as indicated during 2 days. Scale bars represent
405 200 μm. The pictures are representative of two independent experiments. **(k-l)**
406 Representative pictures of senescence-associated beta-galactosidase stainings.
407 Cryosections were obtained from H2122 parental and *PTPN11* KO **(k)** (n= 3 mice per
408 group with 2 pictures per mouse) as well as from H1944 vehicle and SHP099-treated
409 **(l)** (n= 6 mice per group with 2 pictures per mouse) xenografted tumors. Sections
410 were counterstained with Nuclear Fast Red. Scale bars represent 200 μm.

411 **Fig. 4. SHP2 inhibition induces senescence and impairs tumor growth in PDX**
412 **models of KRAS mutant NSCLC.** **(a)** Tumor growth curve of patient-derived PDX2
413 *KRAS* mutant NSCLC tumor subcutaneously implanted in 12 Crl:NU-Foxn1nu mice.
414 Mice were randomized to be treated (Vehicle n=6; SHP099 n=6) daily during 19
415 days. The growth curve was generated measuring tumors with caliper. Mean tumor
416 volumes ± SEM are shown. **(b)** Tumor weight of PDX2 at sacrifice after 19 days of
417 treatment (n= 6 mice per group). The height of the bars represents the mean value,
418 error bars represent standard deviations. Data points are shown as dots to indicate the
419 distribution of the data. Statistical significance (P value) was determined by an

420 unpaired, two-tailed Student's T-test. **(c)** Tumor growth curve of PDX3 *KRAS* mutant
421 NSCLC tumor subcutaneously implanted in 11 CrI:NU-Foxn1nu mice. Mice were
422 randomized to be treated (Vehicle n=4; SHP099 n=4; AZD6244 n=3) daily during 19
423 days. Mean tumor volumes \pm SEM are shown. **(d)** Tumor weight of PDX3 at sacrifice
424 after 19 days of treatment. Vehicle: n=4 mice; SHP099: n=4 mice; AZD6244: n=3
425 mice. The height of the bars represents the mean value, error bars represent standard
426 deviations. Data points are shown as dots to indicate the distribution of the data.
427 Statistical significance (P value) was determined by an unpaired, two-tailed Student's
428 T-test. **(e)** Representative pictures of H&E staining and p-ERK
429 immunohistochemistry performed on consecutive FFPE sections from vehicle (left
430 panel) and SHP099-treated (right panel) subcutaneous PDX2. The necrotic area in the
431 right panel is marked by a dashed line for better visualization. Scale bars represent
432 200 μ m. n= 6 mice per group with 2 pictures per mouse. **(f)** Representative pictures of
433 senescence-associated beta-galactosidase staining performed on cryosections from
434 subcutaneous PDX2 tumors treated either with vehicle or SHP099. Sections were
435 counterstained with Nuclear Fast Red. Areas marked by a dashed square are amplified
436 in the lower left corner for better visualization. Scale bars represent 200 μ m. n= 6
437 mice per group with 2 pictures per mouse. **(g)** Representative pictures of senescence-
438 associated beta-galactosidase staining performed on cryosections from subcutaneous
439 PDX3 tumors treated either with vehicle, AZD6244 or SHP099. Sections were
440 counterstained with Nuclear Fast Red. Areas marked by a dashed square are amplified
441 in the lower left corner for better visualization. Scale bars represent 200 μ m. n= 3
442 mice per group with 2 pictures per mouse. **(h)** Gene set enrichment analysis (GSEA)
443 of senescence (FRIDMAN_SENESCENCE_UP; PURCELL_SENESCENCE_UP and
444 HERNANDEZ_SENESCENCE_UP) and SASP (HERNANDEZ_SASP_UP) gene
445 sets signatures, performed on RNAseq data obtained from 5 vehicle and 7 SHP099-
446 treated PDX2 tumors. Enrichment scores (ES), normalized enrichment scores (NES)
447 and p-values are reported. **(i-j)** Survival curves of orthoxenograft PDOX2 **(i)** and
448 PDOX3 **(j)** *KRAS* mutant NSCLC tumors implanted in the lungs of CrI:NU-Foxn1nu
449 mice. Mice were randomized to be treated daily during 14 **(i)** or 18 **(j)** days. Vehicle
450 n=6; SHP099 n=9. **(k)** Tumor growth curve of PDX3 *KRAS* mutant NSCLC tumor
451 subcutaneously implanted in 20 CrI:NU-Foxn1nu mice. Mice were randomized to be
452 treated (Vehicle n=4; SHP099 + 25 mg/kg AZD6244 n=8; SHP099 + 50 mg/kg
453 AZD6244 n=8) during 3 weeks following a 5 days on/ 2 days off treatment schedule.

454 The growth curve was generated measuring tumors with caliper. Mean tumor volumes
455 \pm SEM are shown. (I) Tumor weight at sacrifice after 3 weeks of treatment. Vehicle:
456 n=6 mice; SHP099 + AZD6244 25 mg/kg: n=8 mice; SHP099 + AZD6244 50 mg/kg:
457 n=8 mice. The height of the bars represents the mean value, error bars represent
458 standard deviations. Data points are shown as dots to indicate the distribution of the
459 data. Statistical significance (P value) was determined by an unpaired, two-tailed
460 Student's T-test.

461

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482

483 **AUTHORS CONTRIBUTION**

484 R.B. supervised the work. R.B., S.M. and A.P. designed experiments. S.M., A.M-S,
485 A.P. and A. Bosma performed experiments and analyzed data. C.L. analyzed data.

486 P.K. designed GEMM experiments. J.D.S and N. d. W. acquired and analyzed MRI
487 data. A. Bardelli designed and G.G. carried out xenograft experiments. A.V., E.N. and
488 S.G.R designed and carried out PDX and PDOX experiments. R.B. and S.M. wrote
489 the paper.

490

491 **ONLINE METHODS**

492 **Cell lines and cell culture, inhibitors and antibodies**

493 All the human cell lines used in the study were purchased from American Type
494 Culture Collection (ATCC), and they were cultured in RPMI 1640 medium
495 supplemented with 10% fetal calf serum, glutamine and Penicillin/Streptomycin
496 (Gibco) at 37 °C in 5% CO₂, unless differently stated. The panel of reconstituted
497 Rasless MEFs was obtained from the NCI RAS Initiative under an MTA agreement.
498 MEFs were cultured in DMEM medium supplemented with 10% fetal calf serum,
499 glutamine and Penicillin/Streptomycin (Gibco) at 37 °C in 5% CO₂, unless differently
500 stated. HEK 293T cells were used for lentivirus production and Phoenix-Ampho cells
501 were used for retrovirus production. Both lines were maintained in DMEM medium
502 supplemented with 10% fetal calf serum, glutamine and Penicillin/Streptomycin
503 (Gibco) at 37 °C in 5% CO₂.

504 AZD6244/Selumetinib (S1008) was purchased from Selleck Chemicals. SHP2
505 inhibitors were synthesized as described in Fortanet et al.³⁴ (SHP099) and
506 WO 2015/107495A1 patent¹⁸ (compound #57).

507 Antibodies against SHP2 (3752), ERK 1/2 (137F5), GAPDH (5174), STAT-1 (9172),
508 p-STAT-1 (9167) and p-RB (9308) were purchased from Cell Signaling Technology.
509 Antibodies against SHP2 (SHPTP2) (C-18), p-ERK1/2 (E-4), ERK1 (C-16), ERK2
510 (C-14) and HSP90 (H-114) were purchased from Santa Cruz Biotechnology. p-SHP2
511 (Y542) (ab62322) was obtained from Abcam. Anti-RAS antibody was obtained from
512 Thermo Fisher Scientific as included in the Active RAS Pulldown and Detection Kit
513 (16117). Alternatively, RAS10 from Millipore (05-516) was used.

514 **Protein lysate preparation and western blot**

515 Cells were plated in complete medium. After 24 hours incubation, cells were grown in
516 the absence of serum (starvation) overnight. After the starvation, cells were stimulated

517 with medium containing 10% serum (unless differently stated) and drugs of interest.
518 At the desired time points, the cells were washed with PBS and lysed in RIPA buffer
519 supplemented with Complete Protease Inhibitors (Roche) and Phosphatase Inhibitor
520 Cocktails II and III (Sigma). Protein quantification was performed with the BCA
521 Protein Assay Kit (Pierce). The lysates were then resolved by electrophoresis in
522 Bolt™ 4-12% Bis-Tris Plus Gels (Thermo Fisher Scientific) and followed by western
523 blotting.

524 **Long-term cell proliferation assays (colony formation)**

525 Cells were cultured and seeded into 6-well plates at a density of $1-4 \times 10^4$ cells per
526 well, depending on growth rate and were cultured in medium containing the indicated
527 drugs for two weeks (medium was changed twice a week). After this, cells were fixed
528 with 4% formaldehyde in PBS and stained with 0.1% crystal violet in water.

529 **Short-term cell proliferation assay**

530 Indicated cells were cultured and seeded into 96-well plates at a density of 500-2000
531 cells per well, depending on growth rate. 24 hours later, drugs were added at indicated
532 concentrations using HP D300 Digital Dispenser (HP). Cells were imaged every 4
533 hours in IncuCyte ZOOM (Essen Bioscience). Phase-contrast images were analyzed
534 to detect cell proliferation based on cell confluence.

535 **Determination of active RAS (RAS-GTP) levels**

536 Active RAS (RAS-GTP) was pulled down from the indicated cell lines after 24 hours
537 serum starvation followed by stimulation with either EGF (50ng/ml, BD Biosciences
538 354052) for 30 minutes or FCS (10%) for 72 hours and/or AZD6244 (1 μ M) and/or
539 SHP099 (1 μ M). Active RAS was pulled down based on affinity for the glutathione *S*-
540 transferase (GST)-tagged RAF1-RAS binding domain (RBD), using an active RAS
541 pulldown and detection kit from Thermo Fisher Scientific (16117). RAS was detected
542 by Western blot analysis.

543 Alternatively, levels of activated RAS-GTP were determined using the RAS GTPase
544 ELISA Kit (Abcam), according to the manufacturer's instructions. Briefly, cells were
545 plated in 10 cm dishes in complete medium. After 24 hours incubation, cells were
546 grown in the absence of serum (starvation) overnight. After the starvation, cells were
547 stimulated with medium containing either 10% or 3% serum with or without 1 μ M
548 SHP099 for 72 hours. Subsequently, cells were washed in ice-cold PBS and

549 immediately lysed in ice-cold Complete Lysis/Binding buffer. Following protein
550 extraction and quantification, 600µg proteins per sample were used to determine the
551 levels of RAS-GTP using a plate-based ELISA as described in the manufacturer's
552 protocol. Data in figures represent mean +/- standard deviation of triplicates from
553 representative experiments.

554 **CRISPR/Cas9 mediated gene knockout**

555 CRISPR/Cas9-based *PTPN11* knockout clones were obtained as previously described
556 ³⁵. Briefly, a dual vector doxycycline inducible CRISPR/Cas9 system was made on
557 the basis of FH1tUTG ³⁶, composed by a pLenti-Cas9-T2A-Neo and a pLenti-gRNA-
558 tetR-T2A-BSD. To prevent premature activation of CRISPR/Cas9 due to the time the
559 cell needs to build up enough of the tet repressor to efficiently dampen gRNA
560 expression, cells were always first infected with pLenti-gRNAtetR-T2A-BSD,
561 encoding the repressor, and at least three days later with pLenti-Cas9- T2A-Neo.

562 **Reconstitution of WT or phosphatase-dead SHP2**

563 WT or phosphatase dead (C459S) mutant of SHP2 were reconstituted
564 into *PTPN11* knockout H2122 cells (B17 clone), using a pBabe-puro (pbp) expression
565 construct, (Addgene). Vector-carrying viruses were produced using Phoenix-Ampho
566 cells and used for 3 subsequent rounds of infection.

567 **Xenografts**

568 H2122 parental (WT) and *PTPN11* knockout clones #B17 and #B33 were injected
569 (5×10^6 cells per mouse) subcutaneously in the right flank of 8-week-old
570 immunocompromised CD1 nude female mice (from Charles River Laboratory).
571 Tumor volume was monitored once a week by digital caliper and quantified by the
572 modified ellipsoidal formula (tumor volume = $1/2(\text{length} \times \text{width}^2)$). Mice were
573 randomized when they reached a volume of approximately 200-250 mm³ and treated
574 for a 34-day period. AZD6244 (25 mg/Kg) was dissolved in 0.2% Tween 80 and 1%
575 methylcellulose (Sigma) and administered daily by oral gavage. Control groups were
576 treated at the same schedule with the vehicle of AZD6244. H1944 cells were injected
577 (5×10^6 cells per mouse) subcutaneously in the right flank of 8-week-old NODSCID
578 female mice and tumor volume was monitored twice a week by digital caliper. Mice
579 were randomized when they reached a volume of approximately 200-250 mm³ and
580 treated for a 21-day period. SHP099 (82.5 mg/kg) was dissolved in 0.5% (W/V)

581 hydroxypropyl methylcellulose (Sigma) and administered daily by oral gavage.
582 Control group was treated at the same schedule with the vehicle of SHP099.

583 Rasless MEFs reconstituted either with KRAS^{G12C} or KRAS^{G12V} (5×10^6 cells per
584 mouse) were subcutaneously injected in the right flank of 8-week-old NODSCID
585 female mice and tumor volume was monitored twice a week by digital caliper. When
586 they reached a volume of approximately 200-250 mm³ mice were randomized to be
587 treated for 18 (G12C) or 20 (G12V) days either with SHP099 (82.5 mg/kg) or with its
588 vehicle daily by oral gavage.

589 All animal procedures were approved by the Ethical Commission of the University of
590 Turin and by the Italian Ministry of Health and they were performed in accordance
591 with institutional guidelines.

592 **Patient-derived xenografts (PDX) and orthoxenografts (PDOX)**

593 Primary tumors were obtained from Bellvitge Hospital (HUB) and the Catalan
594 Institute of Oncology (ICO) with approval by the Ethical Committee (CEIC Bellvitge
595 Hospital no. PR265/13 and PR036/14), and ethical and legal protection guidelines of
596 human subjects, including informed consent, were followed. Tumors were isolated
597 and implanted either subcutaneously or orthotopically in CrI:NU-Foxn1nu mice by
598 following previously reported procedures³⁷. PDOX were inspected daily and
599 monitored for the presence of breathing problems. Tumor volume was monitored
600 every 2 days by digital caliper in subcutaneous experiments, and the mice were
601 randomized when they reached a volume of approximately 200-1000 mm³ (depending
602 on the experiment) and treated for the indicated periods. SHP099 (75 mg/Kg) was
603 dissolved in 0.5% (w/v) hydroxypropyl methylcellulose in water (Sigma) and
604 administered daily by oral gavage. Control group was treated at the same schedule
605 with the vehicle of SHP099. For combined SHP2 and MEK inhibition, AZD6244 (25
606 mg/Kg or 50 mg/Kg) was dissolved in 0.2% Tween 80 and 1% methylcellulose
607 (Sigma). Drugs were administered by oral gavage following a 5 days-ON/ 2 days-
608 OFF schedule.

609 **Genetically engineered mouse models**

610 All experiments involving genetically engineered mice were performed in accordance
611 with Dutch and European regulations on care and protection of laboratory animals
612 and have been approved by the local animal experiment committee at Netherlands

613 Cancer Institute (CCD licence number AVD301002016407, NKI protocol 1.2.8060).
614 Mice were housed under standard conditions of feeding, light, and temperature, with
615 free access to food and water. KRas^{LSLG12D} and p53^{fl} alleles have been previously
616 described^{31,38}. Initially, 28 mice were injected with 1x10⁶ cfu (20 µl) AdenoCre virus
617 intratracheally. After 6 weeks mice were scanned by MRI for the presence of tumors.
618 Subsequent scans were performed every 2 weeks for the whole duration of the
619 experiment. 26 out of 28 mice showed MRI-positive areas bigger than 3 mm³ and
620 where therefore randomized in one of the treatment arms (vehicle, SHP099 75 mg/kg,
621 AZD6244 25 mg/kg, combination: AZD6244 25 mg/kg + SHP099 75 mg/kg).
622 Subsequent scans revealed that only 12 out of 26 mice had developed actual tumors,
623 while others most likely displayed inflammation-related lesions. Only 12 mice were
624 therefore considered for the study shown in Supplementary Fig.8.

625 **MRI imaging**

626 Mice were scanned on a Bruker Biospec 7T magnetic resonance imaging (MRI)
627 scanner. After the localizer, a T1-weighted fast low angle shot (FLASH) (TR/TE =
628 200/2.9 ms, flip angle = 40°, acquisition matrix size = 256 × 256, voxel size = 0.12 ×
629 0.12 × 1.0 mm³) and a T2-weighted Rapid Acquisition with Refocused Echos
630 (RARE) (TR/TE = 2000/24 ms, echo train length = 8, matrix size = 256 × 256, voxel
631 size = 0.12 × 0.12 × 1.0 mm³) were performed.

632 The T1- and T2-weighted scans were used for identification of tumors, and
633 volumetric measurements of the tumors were determined from the images acquired
634 with T2-weighted RARE sequence. The volume was calculated by drawing regions of
635 interest (ROI) around the tumor in each slice using Medical Image Processing,
636 Analysis and Visualization (MIPAV) and then calculating the volume within the
637 defined ROIs.

638 **Senescence-associated beta-galactosidase staining**

639 SA-β-Gal staining was performed either in 6 well plates (for *in vitro* studies) or on
640 10µm thick cryosections from xenografted tumors, using a commercial kit (Cell
641 Signaling Technology), following the manufacturer's instructions.

642 Alternatively, beta-galactosidase activity was quantified using the luminescence-
643 based Beta-Glo assay system from Promega following the manufacturer's
644 instructions. Briefly, 1000 cells/well were seeded in complete medium in duplicated

645 96 well plates and incubated for 24 hours. After that, the cells were switched to
646 serum-free medium and incubated overnight. Subsequently, the cells were stimulated
647 either with 10% or 3% FCS, in the presence or absence of SHP099 (1 μ M). After 48
648 hours incubation, one plate per cell line was used to assess cell viability using
649 CellTitre-Glo assay (Promega) while the duplicate plate was used to determine the
650 beta-galactosidase activity using Beta-Glo. The beta-gal activity was normalized for
651 the cell viability and relative beta-galactosidase activity of the treated condition
652 (compared to the untreated) was calculated. The data in the figure represent mean +/-
653 standard deviation of duplicates from representative experiments.

654 **Immunohistochemistry**

655 For immunohistochemical analysis, tumors from xenografted mice were snap frozen
656 in liquid nitrogen and subsequently sectioned (10 μ m thick) using a cryostat.
657 Sections were probed with CD3 (RM-9107, Neomarkers), or PAX5 (31R-15, Cell
658 Marque), antibodies. Alternatively, sections were obtained from FFPE samples from
659 xenograft or PDX tumors and probed with p-ERK1/2 (T202/Y204) antibodies from
660 Cell Signaling (4370). Following incubation with the primary antibodies, positive
661 cells were visualized using 3,3-diaminobenzidine tetrahydrochloride plus (DAB+) as
662 a chromogen.

663 **RNA sequencing**

664 For each snap frozen tumor (5 from vehicle and 7 from SHP099-treated PDX2) 30
665 cryosections of 30 μ m thickness were cut, RNA was extracted using RNeasy Mini Kit
666 from Qiagen and analyzed using an Agilent 2100 Bioanalyzer system. Sequencing
667 was performed using an Illumina TruSeq system. The sequences were aligned against
668 the human genome (hg38) and gene set enrichment analysis was performed using
669 GSEA software. FRIDMAN_SENESCENCE-UP; PURCELL_SENESCENCE_UP
670 and HERNANDEZ_SENESCENCE_UP gene sets were used to assess the enrichment
671 of senescence-associated genes in the treated versus vehicle group.
672 HERNANDEZ_SASP_UP gene set was used to infer the presence of a senescence-
673 associated secretory phenotype.

674 Raw and processed data from the next generation RNA sequencing of samples have
675 been deposited to NCBI Gene Expression Omnibus (GEO) under accession number
676 GSE109270.

677 **Statistics**

678 All *in vitro* data are expressed as averages from at least 2 technical replicates \pm
679 standard deviations, unless differently stated, and they have been independently
680 reproduced at least twice with similar results. All *in vivo* data are expressed as
681 averages \pm standard deviations, unless differently stated. Statistical significance (P
682 value) was determined by an unpaired, two-tailed Student's T-test. Analyses were
683 performed either with Prism 6 software from GraphPad (San Diego, CA, USA) or
684 with Microsoft Excel (version 14.7.2)

685 **Data availability**

686 The data from this study are available from the corresponding author upon reasonable
687 request.

688
689 Further detailed information on experimental design and reagents is available in the
690 Life Sciences Reporting Summary associated to this paper.

691

692

693 Methods-only References:

694

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709

Figure 1







