PPP2R4 dysfunction promotes *KRAS*-mutant lung adenocarcinoma development and mediates opposite responses to MEK and mTOR inhibition

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Running title: *PPP2R4* in *KRAS*-mutant NSCLC etiology and therapy outcome

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1 **Abstract**

2 *KRAS*-mutant lung adenocarcinomas represent the largest molecular subgroup of non-small

3 cell lung cancers (NSCLC) and are notorious for their dismal survival perspectives. To gain

4 more insights in etiology and therapeutic response, we focused on the tumor suppressor

5 Protein Phosphatase 2A (PP2A) as a player in KRAS oncogenic signaling. We report that the

6 PP2A activator PTPA (encoded by *PPP2R4*) is commonly affected in NSCLC by heterozygous

7 loss and low-frequent loss-of-function mutation, and this is specifically associated with

8 poorer overall survival of *KRAS*-mutant lung adenocarcinoma patients. Reduced or mutant

9 *PPP2R4* expression in A549 cells increased anchorage-independent growth *in vitro* and

10 xenograft growth *in vivo*, correlating with increased Ki67 and c-MYC expression. Moreover,

11 *KrasG12D*-induced lung tumorigenesis was significantly accelerated in *Ppp2r4* gene trapped

12 mice as compared to *Ppp2r4* wild-type. A confined kinase inhibitor screen revealed that

13 *PPP2R4*-depletion induced resistance against selumetinib (MEK inhibitor), but unexpectedly

14 sensitized cells for temsirolimus (mTOR inhibitor), *in vitro* and *in vivo*. Our findings

15 underscore a clinically relevant role for PTPA loss-of-function in *KRAS*-mutant NSCLC etiology

16 and kinase inhibitor response.

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18 Keywords: PPP2R4, PP2A, KRAS, NSCLC, temsirolimus, selumetinib

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23   **1. Introduction**

24   Non-small cell lung cancer (NSCLC) is the most common type of cancer worldwide and the

25   number one cause of cancer-related death (*1*). While the advent of targeted therapies has

26   benefited patients that harbor specific targetable driver mutations in EGFR and ALK genes,

27   they only represent a fraction of NSCLC patients. For *KRAS*-mutant NSCLC – which represents

28   the largest molecular subgroup of NSCLC – the clinical implementation of tailored therapies

29   has proven to be notoriously difficult and largely disappointing (*2*). Due to the hard to target

30   nature of KRAS itself, more easily targetable downstream kinase effectors in the

31   RAF/MEK/ERK and PI3K/AKT/mTOR pathways were long considered as suitable alternatives

32   (*2, 3*). However, most of the clinical trials focusing on downstream kinase inhibition have

33   proven to be unsuccessful, in part due to an incomplete understanding of mechanisms

34   determining therapy response and resistance (*2, 3*).

35   Although protein phosphatases, which counteract protein kinases, exhibit equally important

36   roles in determining net phosphorylation states of their substrates, their function in

37   tumorigenesis and therapy resistance only more recently started to gain attention. Members

38   of the multi-subunit Serine/Threonine Protein Phosphatase 2A (PP2A) family have a wide

39   range of functions in cellular and organismal homeostasis, and are considered to be major

40   tumor suppressors (*4-6*). PP2A exists mainly as trimeric holoenzymes, where a scaffolding A

41   subunit tethers the catalytic C subunit to any of a wide range of regulatory B subunits. The

42   identity of the incorporated B subunit determines substrate specificity, holoenzyme activity

43   and subcellular localization (*4*). Assembly and activation of PP2A as a trimeric holoenzyme is

44   a prerequisite for proper PP2A function and is a closely monitored process (*7*). Here, Protein

45   Phosphatase Two A Activator (PTPA), encoded by *PPP2R4*, plays a central role through

46   activation of the PP2A AC core dimer, which is followed by incorporation of the regulatory B

47   subunit (*8-11*).

48   Inactivation of PP2A is observed in many cancer types, both through genomic and non-

49   genomic mechanisms. Multiple PP2A subunits exhibit decreased expression, loss-of-function

50   mutations and deletions in a range of cancers (*12-14*). Moreover, overexpression of cellular

51   PP2A inhibitors CIP2A, SET or PME-1 are considered to be an important mechanism of non-

52   genomic PP2A inactivation, next to heterozygous loss of the PP2A activator PTPA (*12-16*).

53   For lung cancer specifically, recurrent mutations in the Aα subunit gene *PPP2R1A* are found

54   at low frequency (*17*), while SET and CIP2A overexpression are commonly observed events,

55   associated with decreased lung cancer patient survival (*18, 19*).

56   PP2A acts on several points in the signaling pathways downstream of RAS, making it an

57   important regulator of RAS signaling output (*6, 20*), e.g. by direct dephosphorylation of AKT,

58   ERK1/2, mTOR, GSK-3ß and c-MYC, among others (*4, 12-14*). Notably, interference with

59   specific PP2A holoenzymes is a prerequisite for full-blown malignant conversion of

60   oncogenic RAS-dependent immortalized cells (*21, 22*). Moreover, inhibition of PP2A by

61   Okadaic Acid or genetic knockout promotes tumorigenesis in mice (*16, 23-26*), in part

62   associated with hyperactivation of downstream effectors of RAS (*16, 25, 26*). In addition,

63   knockin of a lung cancer-associated *PPP2R1A* mutation in *Kras*-mutant lung cancer mouse

64   models promotes tumorigenesis and decreases murine survival (*27*).

65   Importantly, PP2A inactivation was recently found to induce therapy resistance against a

66   wide range of targeted anti-cancer compounds, including MEK inhibition (*28, 29*). Moreover,

67   direct pharmacological activators of PP2A were identified as novel classes of promising anti-

68   cancer drugs in a broad range of cancers (*28-35*), including *KRAS*-mutant NSCLC (*29*). A

69   common denominator in several of these studies seemed to be the involvement of the

70   oncogenic transcription factor c-MYC (*30*), of which the stability is closely regulated by a

71   specific subset of PP2A holoenzymes (*36-38*).

72   Here, we specifically investigated the role of the PP2A activator PTPA in *KRAS*-mutant NSCLC

73   cancer etiology and therapeutic response. Our results identify PTPA as an important tumor

74   suppressor and modifier of targeted therapy outcomes in this hard-to-treat disease.

75 **2. Materials & Methods**

76 *2.1 Cloning, site directed mutagenesis and recombinant His-PTPA production.*

77 Wild-type *PPP2R4* cDNA (isoform α; (*39*)) was cloned into 3XFLAG-CMV10 (Sigma, E7658)

78 and pET15b (Sigma, 69661). *PPP2R4* FLAG-tag lentiviral expression vector (pLA CMV-N-Flag)

79 and *PPP2R4* 3'UTR targeting pLKO.1 (shPTPA) and control pLKO.1 (shGFP) were generated as

80 described in (*22*). PCR-based site-directed mutagenesis to generate PTPA point mutants was

81 performed with Pwo polymerase (Roche, 11644955001) and complementary DNA primers

82 (IDT DNA Technologies) containing the desired mutations (**Supplementary Table S1**).

83 Expression and purification of recombinant His-tagged PTPA was performed as described in

84 (*16*).

85 *2.2 Cell culture, transfections and lentivirus production*

86 HEK293T (ATCC), A549 (Sigma, ECACC 86012805) and A427 (ATCC) cell lines were frozen at

87 early passage upon receipt and were routinely tested for *Mycoplasma* (Venor[TM] GeM,

88 Minerva Biolabs, 11-1050). Cell lines were cultured at 37°C/5% CO_2 in DMEM (Sigma, D6546)

89 (+ 10% fetal bovine serum (FBS; Sigma, F7524), 2 mM L-glutamine (Sigma, G7513) and

90 penicillin/streptomycin (100 units/mL and 10 mg/mL, respectively) (Sigma, P0781)).

91 Transfections were performed using polyethyleneimine (MW 25,000) (Polysciences Europe,

92 23966-2). Lentiviral infections were performed as described in The RNAi Consortium

93 Protocols

94 (https://portals.broadinstitute.org/gpp/public/resources/protocols).

95 *2.3 Protein extraction, immunoprecipitation and western blotting*

96 Protein extraction and immunoprecipitation were performed as in (*16*), except that

97 NuPAGE[TM] 4-12%, Bis-Tris Midi Protein Gels (Invitrogen) were used for protein separation.

98 For FLAG-immunoprecipitations, ANTI-FLAG® M2 affinity gel (Sigma, A2220) was used.

99 Primary and secondary antibodies are indicated in **Supplementary Table S2**. Densitometric

100 analysis was performed using Image Studio™ Lite (LiCOR; RRID: SCR_013715).

101 *2.4 Phosphorylase a PP2A reactivation assay*

102 The serine/threonine phosphatase activation by PTPA mutants was measured using P^{32}-

103 labeled phosphorylase *a* as a substrate for PP2A core dimer isolated from GST-PME-1

104 overexpressing HEK293T cells, as described in (*16*).

105 *2.5 Anchorage-independent growth assays*

106 10,000 cells were seeded in triplicate in 0.35% Noble agar (Sigma, A5431) on top of a 0.5%

107 Noble agar base layer in 6-well plates (Greiner Bio-One, 65716) and fed twice weekly with

108 300 μL complete DMEM. After 4 weeks, colonies were stained with 0.01% crystal violet in

109 20% methanol. 4x images were acquired at 5 standard coordinates and analyzed using

110 ImageJ (RRID:SCR_003070).

111 **_2.6 Kinase inhibitor library screen and dose-response curves_**

112 A549 shGFP and shPTPA cells were seeded in 96-well plates at a density of 2.000 cells/well

113 and treated for 72h. Cell viability was determined by Thiazolyl Blue Tetrazolium bromide

114 (MTT) (Alfa Aeser, L11939.06). Kinase inhibitors were selected based upon the known

115 regulation of their targets by PP2A and/or c-MYC, or regulation of PP2A and/or c-MYC by

116 their targets (**Supplementary Table 3A,3B**). Validations were performed in A427 cells, which

117 were seeded at 4000 cells/well. Dose-response curves were fitted using 3- or 4-parameter

118 logistic regression in GraphPad Prism 7.0 (GraphPad Software; RRID: SRC_002798).

119 **_2.7 Animal experiments_**

120 For xenografts, 10^6 cells were subcutaneously injected in the flanks of 5-6 week old female

121 Rj:NMRI-nu mice (Janvier). Tumor measurements started when tumors became palpable and

122 were performed at least twice weekly using a digital caliper. Volumes were calculated by

123 _lengthxwidth^2/2_. Mice were sacrificed when tumors reached 2000 mm^3, or in case of drug

124 treatments at treatment day 15. Drug treatments (**Supplementary Table 3A**) were initiated

125 at a mean tumor size of 150-300 mm^3 after randomization. For lung tumorigenesis, _Ppp2r4_

126 gene-trapped mice (_16_) were crossed with LSL-_Kras_G12D mice (_40_). At 6-12 weeks of age,

127 male and female _Ppp2r4$^{+/+}$_;LSL-_Kras_G12D$^{+/-}$, _Ppp2r4$^{+/gt}$_;LSL-_Kras_G12D$^{+/-}$ and _Ppp2r4$^{gt/gt}$_;LSL-

128 _Kras_G12D$^{+/-}$ were intratracheally infected with 2,5*10^7 Cre-recombinase-encoding

129 adenoviral particles, as described in (_41_). 8 weeks weeks post-infection, anaesthetized mice

130 were exsanguinated and perfused with 0.9% NaCl. Lungs were dissected, fixated overnight at

131 4°C in 4% PFA and embedded in paraffin for sectioning (4 μm thick) and H&E staining.

132 Images of 3 sections (100 μm apart) were acquired (Axio Scan.Z1, Zeiss) at 20x magnification

133 and lesions were quantified in a blinded fashion using QuPath software (RRID: SCR_018257)

134 (_42_). All animal experiments were designed according to ARRIVE guidelines and approved by

135 the Animal Ethics Committee of the KU Leuven (project numbers P109/2013, P213/2017,

136 P087/2018).

137 **_2.8 Immunohistochemical and immunofluorescence stainings_**

138   Immunohistochemical (IHC) and immunofluorescence (IF) stainings of 4µm thick FFPE lung

139   sections and OCT preserved xenografts were performed with antibodies summarized in

140   **Supplementary Table 2** at indicated dilutions. For transgenic mice, proliferative index (PI)

141   was determined as number of Ki67+ CD45- cells, normalized to the number of nuclei (DAPI)

142   at 40X magnification of all observed lesions, in their entirety, for two *Ppp2r4*$^{+/+}$;LSL-

143   *Kras*G12D and two *Ppp2r4*$^{+/-}$;LSL-*Kras*G12D mice. For xenografts, Ki67 PI was determined as

144   number of positive nuclei normalized to the total number of detected nuclei per 20x

145   magnification fields of 3-6 random fields per tumor. Quantifications were performed using

146   QuPath software (RRID: SCR_018257).

147   ***2.9 Cancer databases and survival analysis***

148   The Lung adenocarcinoma (TCGA, Firehose Legacy) study in cBioPortal (*43, 44*) was queried

149   for "PTPA: HOMLOSS; HETLOSS; MUT; CNA" and "KRAS", "BRAF" or "EGFR". Genomic and

150   clinical data were extracted for further stratification of patient groups based on *KRAS, BRAF*

151   *or EGFR* mutational status and *PPP2R4* genetic status or mRNA levels. The Catalog Of

152   Somatic Mutations In Cancer (COSMIC) was queried for "*PPP2R4*" and consulted for NSCLC-

153   associated *PPP2R4* somatic mutations. Kaplan-Meier survival analyses were performed with

154   GraphPad Prism 7.0 (Graphpad Software; RRID: SRC_002798).

155   ***2.10 Statistics***

156   Statistical tests as indicated in the figure legends were performed using GraphPad Prism 7.0

157   (Graphpad Software; RRID: SRC_002798). *P*-values below 0.05 were considered significant.

158

159 ### 3. Results

160 ***3.1 In vitro recapitulation of PPP2R4 alterations underscores a clinical impact of PPP2R4***

161 ***loss-of-function, specifically in KRAS-mutant NSCLC***

162 In the lung adenocarcinoma TCGA (Firehose Legacy) study, nearly half of the patients display

163 heterozygous loss of *PPP2R4,* both in *KRAS* wild-type and mutant patient subsets (**Figure**

164 **1A,1B**). Strikingly, for the *KRAS*-mutant NSCLC patient subset, we observed that *PPP2R4*

165 heterozygous loss was associated with worse patient survival (**Figure 1C**). As *PPP2R4*

166 genotype correlated with mRNA expression (**Figure 1A,1B**), the lower survival was also

167 observed when stratifying on *PPP2R4* mRNA (**Figure 1D**). Notably, for *EGFR* and *BRAF*

168 mutated patients, *PPP2R4* heterozygous loss rather showed a tendency to be associated

169 with increased survival (**Suppl. Figure S1**).

170 Moreover, NSCLC studies included in the cBioPortal and COSMIC cancer databases revealed

171 low frequency heterozygous *PPP2R4* point mutations, of which six – all in the most

172 abundantly expressed α-isoform – were predicted to be detrimental for PTPA function

173 (**Figure 1E**) (*45*). Indeed, while PTPA^{E225*} could not be detected at physiologically relevant

174 levels (**Figure 1F**), binding to PP2A-C was diminished or abrogated for PTPA^{Y234C}, or PTPA^{H306L}

175 and PTPA^{V316F}, respectively (**Figure 1F,1G**). Although PP2A-C binding was not affected for

176 PTPA^{G240S} and even increased for PTPA^{R183L} (**Figure 1F,1G**), these mutants could not

177 reactivate inactive PP2A-C to the same extent as PTPA^{WT}. PTPA^{R183L} did so to the same extent

178 as the previously described PTPA^{G243V} mutant (*16*), while PTPA^{G240S} almost completely lacked

179 PP2A-C reactivating potential (**Figure 1H, Figure S2A,S2B**).

180 To verify the survival disadvantage for the *KRAS*-mutant PTPA$^{HETLOSS/low}$ patient subset, we

181 stably knocked down PTPA in the *KRAS*-mutant (G12V) A549 lung cancer cell line (**Figure 1I**).

182 This increased anchorage-independent growth as compared to shGFP-transduced control

183 (**Figure 1J-1L**) and was rescued by re-introduction of PTPA^{WT}, but not by expression of PP2A-

184 C non-binding PTPA^{V316F} (**Figure 1J-1L**). These findings underscore the relevance of NSCLC-

185 associated *PPP2R4* dysfunctions in the ill-treatable *KRAS*-mutant NSCLC patient subset.

186

187 ***3.2 In vivo reduction of PTPA expression aggravates KRAS-mutant NSCLC growth***

188 To provide *in vivo* evidence, shGFP and shPTPA A549 cells were subcutaneously injected into

189 nude mice, and tumor growth was monitored. At endpoint, shPTPA A549 xenografted

190 tumors were significantly larger and heavier as compared to shGFP control tumors (**Figure**

191 **2A-2C**). In line with our previous findings in spontaneous lymphomas of *Ppp2r4* gene
192 trapped mice (*16*), shPTPA A549 xenografts harvested at endpoint showed an
193 overexpression of the oncogenic transcription factor c-MYC (**Figure 2D-2G**), which could not
194 be attributed to changes in GSK-3ß S9 phosphorylation or ERK1/2 T202/Y204
195 phosphorylation (**Figure S3**).

196 In addition, we crossed *Ppp2r4* gene trapped mice (*16*) with a conditional LSL-*Kras*G12D
197 mouse model (*40*), yielding orthotopic lung adenocarcinoma development upon conditional
198 activation of the *KrasG12D*-allele by intratracheal instillation of Cre-encoding adenovirus
199 (*41*). We found a significantly increased number of early lung lesions (=atypical adenomatous
200 hyperplasia, AAH) in heterozygous as well as homozygous *Ppp2r4* gene trapped mice as
201 compared to wild-type at 8 weeks post tumor initiation (**Figure3A,3B**). Moreover, we
202 observed a larger total size of lesions per lung area (**Figure 3C**), while the average lesion size
203 remained the same (**Figure 3D**). Similar TTF1 positivity across genotypes (data not shown)
204 indicated that loss of PTPA did not change the histology of mutant *KRAS*-driven lung
205 tumorigenesis. Importantly, Ki67 staining showed a higher proliferative index (PI) of the
206 lesions in PTPA-depleted mice (**Figure 3E**). Thus, we conclude that decreased PTPA
207 expression accelerated orthotopic mutant *KRAS*-driven lung tumor development *in vivo*.

208

209 ***3.3 PTPA reduction affects the response of A549 cells on several kinase inhibitor therapies***
210 ***acting downstream of KRAS***

211 PP2A dysfunction has recently been implicated in therapeutic resistance of endometrial and
212 lung cancer cells to kinase inhibition (*28, 29*). Together with the PTPA loss-associated
213 increase of c-MYC expression, another mediator of cancer therapy resistance (*30*), this
214 prompted us to assess the role of PTPA depletion on the therapeutic outcome of a range of
215 kinase inhibitor therapies. We assembled a confined library of 18 kinase inhibitors, of which
216 their targets are regulated by and/or regulate activity of either PP2A or c-MYC (**Supp. Table**
217 **3A,3B**). While most kinase inhibitor treatments remained equally potent (**Figure 4A,4B**), we
218 found that loss of PTPA led to increased resistance to selumetinib (MEK inhibition) or MK-
219 2206 (AKT inhibition) (**Figure 4A-4D**). On the other hand, temsirolimus (mTOR inhibition) and
220 barasertib (AURKB inhibition) both resulted in an increased growth inhibition of PTPA-
221 depleted cells as compared to wild-type (**Figure 4A,4B,4E,4F**).

222 While knockdown of PTPA increased c-MYC levels in xenografts (**Figure 2F,2G**), this appeared

223 not the case in basal 2D anchorage-dependent conditions (**Figure S4A**). Nevertheless,

224 shPTPA A549 cells were more resistant to reduction of c-MYC levels after treatment with

225 selumetinib, as compared to control cells (**Figure 4H,4L**). On the other hand, barasertib

226 reduced c-MYC levels more efficiently after PTPA knockdown (**Figure 4I,4M**). Treatment with

227 MK2206 or temsirolimus did not alter c-MYC levels regardless of PTPA expression (**Figure**

228 **4G,4J,4K,4N**). For each treatment, equal target inhibition was achieved in shGFP and shPTPA

229 A549s, as witnessed by P-ERK1/2 (selumetinib), P-HH3 (barasertib), GSK-3ß P-S9/total

230 (MK2206) and P-p70S6K (temsirolimus) (**Figure S4B-S4E**). Summarized, loss of PTPA induced

231 changes in therapeutic responses towards several kinase inhibitors *in vitro*, which for

232 selumetinib and barasertib correlated with changes at the level of c-MYC.

233 To validate the responses to the former four kinase inhibitors in a second human lung

234 adenocarcinoma cell line, dose-response curves were acquired in A427, another *KRAS*-

235 mutant cell line with much lower endogenous PTPA expression than A549 (**Figure 5A**). While

236 the responses to MK2206 or barasetib did not significantly differ between both cell lines

237 (**Figure 5C, 5E**), PTPA-low expressing A427 cells were more resistant to selumetinib (**Figure**

238 **5B**) and more sensitive to temsirolimus (**Figure 5D**) compared to PTPA-high expressing A549

239 cells, the latter two in line with the data from isogenic shGFP and shPTPA A549 cells.

240

3.4 *In vivo* validation *of kinase inhibition outcomes*

242 To further verify these findings in a biologically more relevant context, we treated mice

243 exhibiting shGFP and shPTPA A549 xenografts with selumetinib, MK2206, barasertib and

244 temsirolimus. While selumetinib and MK2206 were able to reduce growth both in shGFP and

245 shPTPA conditions, the growth of PTPA-depleted tumors was inhibited less dramatically as

246 compared to the shGFP controls at the endpoint of treatments (**Figure 6A, Figure S5A,S5C**),

247 confirming increased resistance to both compounds upon PTPA loss. Conversely, PTPA-

248 depleted xenografts were clearly more sensitive to temsirolimus treatment than shGFP

249 controls (**Figure 6B, Figure S5B**). On the other hand, barasertib treatments did not

250 significantly differ between shPTPA and shGFP tumors at endpoint (**Figure S5D**). Importantly,

251 Ki67-based proliferation index was significantly higher in the untreated shPTPA xenografts as

252 compared to shGFP controls (**Figure 6C-6F, Figure S5E,S5F**), remained higher in the

253 selumetinib- or MK2206-treated groups (**Figure 6C,6E, Figure S5E,S5F**), but was no longer

254 significantly different between the temsirolimus-treated groups (**Figure 6D,6F**).

255 To further underscore these findings, we analyzed our data by adapted RECIST criteria,

256 which use 'change from baseline' as parameters to determine 'outcome' (= 'partial

257 response', 'stable disease', 'progressive disease'). Once again, our findings clearly point

258 towards the more refractory behavior of shPTPA tumors as compared to shGFP when

259 treated with MK2206 or selumetinib (**Figure S6A,S6C**), and to the higher sensitivity of

260 shPTPA tumors, when treated with temsirolimus (**Figure S6B,S6D**).

261 In summary, we demonstrated for at least two kinase inhibitors that the results from long-

262 term in vivo xenograft growth assays are consistent with short-term cell viability results in

263 two independent *KRAS*-mutant cell lines: PTPA-depletion or low PTPA expression clearly

264 resulting in increased resistance for selumetinib, while increasing sensitivity for

265 temsirolimus.

266

267 ***3.5. Loss-of-PTPA-associated responses to selumetinib correlate with changes in c-MYC***

268 ***expression, while loss-of-PTPA-associated responses to temsirolimus correlate with***

269 ***increased dephosphorylation of p70 S6 kinase***

270 Selumetinib treatment resulted in nearly undetectable phospho-ERK1/2 levels in both shGFP

271 and shPTPA A549 xenografts (**Figure 7A,7B**), while c-MYC expression, although decreased

272 upon treatment in both groups, still remained higher in the shPTPA tumors compared to the

273 shGFP group (**Figure 7C,7D**) – providing a potential mechanism of selumetinib resistance

274 (29).

275 Although there was no significant difference in basal phospho-p70 S6K levels in shGFP versus

276 shPTPA A549 xenografts (**Figure 8A,8B**), temsirolimus treatment resulted in significantly

277 lower p70 S6 kinase phosphorylation levels in shPTPA as opposed to shGFP xenografts

278 (**Figure 8A,8B**). With p70 S6 kinase as a major mTOR target, this provides a potential

279 mechanism for the increased sensitivity for temsirolimus upon loss of PTPA.

280
281   **4. Discussion**

282 *KRAS*-mutant lung adenocarcinomas represent the largest subgroup of non-small cell lung

283 cancer (NSCLC), the most common and deadly cancer worldwide (*1, 2*). As no targeted

284 therapy tailored on *KRAS* driver mutation status has been clinically approved so far,

285 understanding mechanisms of therapy resistance against inhibition of oncogenic kinases
286 downstream of *KRAS* remains important (*2, 3*). We focused on a role for the Protein
287 Phosphatase 2A (PP2A) Activator PTPA in *KRAS*-mutant NSCLC, due to the implication of
288 PP2A in the regulation of oncogenic kinases downstream of RAS (*4, 12, 13*), PP2A
289 inactivation in a broad range of cancers (*12, 13*), and the emerging role for PP2A inactivation
290 as an important mechanism of cancer therapy resistance (*28, 29*).

291 Across COSMIC and cBioPortal lung adenocarcinoma studies, we retrieved a low number of
292 PTPA point mutations. All PTPAα isoform mutants were found to be defective, either
293 through loss of binding to the PP2A catalytic subunit, or through attenuation of PP2A
294 reactivating potential. These findings are in line with their localization in the proximity of
295 functionally important domains, based on structure-function and crystallographic studies of
296 PTPA (*9, 11*). More importantly, in the lung adenocarcinoma (TCGA, Firehose Legacy) study,
297 we observed a wide distribution of *PPP2R4* mRNA, which was directly proportional to loss or
298 gain of its gene locus. Heterozygous loss (44% of patients) of *PPP2R4* and low *PPP2R4* mRNA
299 was significantly associated with worse survival in *KRAS*-mutant patients, but not in *EGFR*- or
300 *BRAF*-mutant patients. This further expands the overall number of cancer types where PTPA
301 status impacts patient survival (*16*). PTPA dysfunction thus joins other clinically-relevant
302 PP2A inactivating mechanisms previously described in lung cancer, such as overexpression of
303 the cellular PP2A inhibitors CIP2A (*18*) and SET (*19*), and low-frequency inactivating
304 mutations in the Aα subunit gene *PPP2R1A* (*17*).

305 Mimicking the clinically observed loss of PTPA by shRNA-mediated knockdown increased
306 anchorage-independent and xenograft growth of *KRAS*-mutant A549 lung adenocarcinoma,
307 and was associated with a higher proliferation index (Ki67 expression). Moreover, the
308 increased number of early lung lesions (atypical adenomatous hyperplasia), the larger total
309 size of lesions per lung area and the higher proliferation index of lesions observed in
310 *Ppp2r4*$^{+/gt}$;LSL-*Kras*G12D and *Ppp2r4*$^{gt/gt}$;LSL-*Kras*G12D mice as compared to *Ppp2r4*$^{+/+}$;LSL-
311 *Kras*G12D mice further supported the role of PTPA as a tumor suppressor in *KRAS*-mutant
312 NSCLC. In xenografts, PTPA reduction resulted in overexpression of c-MYC, a transcription
313 factor deregulated in a multitude of cancers, and an important facilitator of RAS-driven
314 tumorigenesis (*36, 46*). This is in line with findings from the RAS-driven HEK-TER system,
315 where loss of PTPA induced full transformation of immortalized HEK-TER cells, associated
316 with overexpression of c-MYC (*22*). Moreover, we previously observed c-MYC

317 overexpression in spontaneous lymphomas of *Ppp2r4* gene trapped mice (*16*). As loss of

318 PTPA induces a selective reduction of PP2A-B' holoenzymes (*16*), we speculate the increase

319 in c-MYC levels might at least in part be attributed through loss of B'α containing

320 holoenzymes that are known to destabilize c-MYC through dephosphorylation of Serine-62

321 (*37*). Unfortunately, however, we could not solidly prove this hypothesis, due to suboptimal

322 quality of commercially available phospho-c-MYC antibodies in our hands. In any case, the

323 lack of significant changes in P-ERK1/2 and GSK-3ß activity (two upstream c-MYC kinases)

324 rules out their involvement in increasing c-MYC after PTPA knockdown.

325 MYC overexpression is a well-known mediator of therapy resistance (*30*), and recent

326 publications have identified PP2A dysfunction as a new partner in crime (*28, 29*). We have

327 now identified kinase inhibitors of which the efficiency is influenced by decreased PTPA

328 expression in *KRAS*-mutant cells. While selumetinib and MK2206 proved to be less efficient

329 to suppress growth after PTPA knockdown, temsirolimus and barasertib did so to a better

330 extent in our initial A549 screen. However, subsequent *in vivo* validation experiments only

331 confirmed the altered responses of shPTPA cells for selumetinib, MK2206 and temsirolimus.

332 A second, short-term validation experiment in another *KRAS*-mutant lung adenocarcinoma

333 cell line further confirmed the higher resistance of PTPA-low expressing A427 cells for

334 selumetinib, as well as the increased sensitivity for temsirolimus, when compared with

335 PTPA-high expressing A549 cells. Mechanistically, we found a correlation between

336 maintained higher c-MYC expression levels in shPTPA cells upon selumetinib treatment *in*

337 *vitro* (**Figure 4H,4L**) and *in vivo* (**Figure 7B,7D**), an observation which had previously been

338 reported in the same cell line (A549) as the causal mechanism of increased resistance to

339 MEK inhibitors upon PP2A inhibition by siRNA-mediated knockdown of the Aα subunit (*29*).

340 Thus, we deem it very likely that PP2A inhibition upon knockdown of PTPA may mediate

341 MEK inhibitor resistance by the same mechanism. In contrast, the increased sensitivity of

342 PTPA-depleted cells for mTOR inhibitors seemed independent of any alterations in c-MYC

343 expression (**Figure 4J,4N**), but instead could be correlated to a stronger decrease in

344 phospho-p70 S6 kinase (**Figure 8A,8B**). Exactly how the specific PP2A dysfunction(s) caused

345 by decreased PTPA expression would contribute to these observations, remains to be further

346 determined.

347 Altogether, our findings underscore that heterozygous loss of PTPA, as a novel mechanism of

348 PP2A dysfunction in *KRAS*-mutant NSCLC, contributes to increased malignancy and altered

therapeutic outcomes upon use of kinase inhibitors. Most significantly, while PTPA depletion in *KRAS*-mutant NSCLC causes resistance to the MEK inhibitor selumetinib, it creates an unexpected window of treatment opportunity for the mTOR inhibitor temsirolimus. These findings could become of clinical importance upon implementation of the *PPP2R4* status as an additional stratification marker in *KRAS*-mutant NSCLC.

5. Acknowledgments

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492 **7. Figure legends**
493
494 <u>Figure 1</u>: ***PPP2R4*, the gene encoding the protein phosphatase 2A activator PTPA, displays**
495 **non-conservative point mutations and clinically relevant heterozygous loss in non-small**
496 **cell lung adenocarcinoma, particularly in the *KRAS*-mutant subgroup.**
497 **A,B:** Distribution of *PPP2R4* mRNA expression across *PPP2R4* genotypes in complete Lung
498 adenocarcinoma (TCGA, Firehose Legacy) cohort (A) and *KRAS*-mutant subcohort (B). Data
499 are represented as mean +/- SD. Statistics: One-way ANOVA. *: $p<0.05$; ***: *$p<0.0001$;*
500 ****: $p<0.0001$. **C,D:** Kaplan-Meier analysis of *KRAS*-mutant patients in the lung
501 adenocarcinoma TCGA (provisional study) stratified according to their *PPP2R4* genotype (C)
502 and *PPP2R4* mRNA expression (50 highest vs. 50 % lowest) **(D).** Statistics: Log-rank test. *:
503 $p<0.05$. **E**: Lung adenocarcinoma-associated PTPA isoform α variants are predicted to be
504 loss-of-function. Numbers indicate amino acid position of the mutations based on the
505 longest human PTPA isoform (beta). D92 is unique to PTPA isoform β. **F:** Assessment of
506 PP2A-C binding of PTPA mutants by immunoprecipitation and Western blotting. Lysates of
507 FLAG-PTPA wild-type and mutant overexpressing HEK293T were FLAG-immunoprecipitated,
508 and IPs were counterstained for endogenous PP2A-C subunit. **G:** Quantifications of panel **F**.
509 Statistics: One-way ANOVA. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ****: $p<0.0001$. **H:** PME1-
510 bound PP2A reactivation capacities of PTPA mutants as compared to wild-type PTPA.
511 Activities are expressed as relative CPM (counts per minute) values compared to PTPA wild-
512 type at 5 minutes (=100%). N=3. Statistics: Two-way ANOVA. *: $p<0.05$; **: $p<0.001$; ****:
513 $p<0.0001$ **I:** PTPA knockdown in the *KRAS*-mutant A549 non-small cell lung cancer cell line
514 and rescue with FLAG-tagged wild-type and mutant PTPA. VINC: Vinculin. **J:** Representative
515 images of anchorage-independent growth of stable A549 cell lines in soft agar. **K,L:**
516 Quantifications of colony number (**K**) and area (**L**). Statistics: One-way ANOVA. ***: $p<0.001$,
517 as compared to shGFP_EV.
518

Figure 2: Xenograft mouse models further corroborate evidence for PTPA as a tumor suppressor in *KRAS*-mutant lung adenocarcinoma

A: Images of A549 shGFP and shPTPA xenografted tumors at endpoint. Weights and volumes are indicated per individual tumor. **B:** Tumor weights (mg) at endpoint. Statistics: Two-sided student's t-test. *: $p<0.05$. **C:** Tumor volumes (mm^3) over time. Statistics: Two-way ANOVA. *: $p<0.05$. Western blots (**D,F**) and quantifications (**E,G**) of A549 shGFP versus A549 shPTPA xenografts. VINC: Vinculin. Statistics: Two-sided student's t-test. **: $p<0.01$.

Figure 3: Loss of *Ppp2r4* in a LSL-*Kras*G12D;*Ptpa*-genetrap transgenic mouse model induces lung tumor initiation.

A: Representative images of H&E stainings displaying *Kras*(G12D)-induced lung tumorigenesis in *Ppp2r4*$^{+/+}$ (=WT), *Ppp2r4*$^{+/gt}$ (=HE) and *Ppp2r4*$^{gt/gt}$ (=HO) mice. **B:** Mean number of lesions per lung section. **C:** Quantification of atypical adenomatous hyperplasia (AAH) and total size relative to the lung area in *Ppp2r4*$^{+/+}$ (= WT), *Ppp2r4*$^{+/gt}$ (=HE) and *Ppp2r4*$^{gt/gt}$ (=HO) mice, 8 weeks post AdCre infection. **D:** Mean size of AAH lesions. **E:** Proliferative index Ki67 staining of two *Ppp2r4* wild-type (WT) and two *Ppp2r4* heterozygous (HE) LSL-*Kras*G12D mice, 8 weeks post AdCre infection. **Statistics**: One-way ANOVA. **: $p<0.01$; ***: $p<0.001$. WT: wild-type; HE: heterozygotes; HO: homozygotes.

Figure 4: A confined kinase inhibitor screen reveals PTPA loss-induced changes in growth response of A549 cells towards different targeted kinase inhibitors.

A, B: Fold-changes (shPTPA/shGFP) in EC50s for 18 kinase inhibitors, as measured by MTT after 72 hours of treatment. Compounds to which loss of PTPA induced resistance are depicted in red, while compounds with improved responses are depicted in green. Each point represents a biological replicate (**A**). Significance levels were determined using ratio-paired t-tests (**A,B**), and are represented as -$\log_{10}$(p-value) in (**B**). **C-F:** Representative dose-response curves of significantly affected compounds. EC50s and *p*-values were determined by 4-parameter non-linear regression. **G-J**: Representative Western blot analyses of c-MYC responses of cells treated at indicated doses for 48 hours. **K-N**: Quantifications of c-MYC levels 48 hours after treatment at indicated doses of at least 3 biological replicates. Statistics: One-way ANOVA. **: $p<0.01$.

551 **Figure 5: Low PTPA expression is associated with differential response towards**

552 **selumetinib and temsirolimus for A427 and A529 KRAS-mutant NSCLC cell lines.**

553 **A:** Western blots of cell lysates of A427 and A549 cells, developed with the indicated

554 antibodies. **B**: Dose-response curves of selumetinib: $EC50_{A549}$ = 24.56 nM vs. $EC50_{A427}$ = 265.9

555 nM (p<0.0001). **C**: Dose-response curves of MK2206 (n.s.). **D**: Dose-response curves for

556 temsirolimus: $EC50_{A549}$ = 1.260 nM vs. $EC50_{A427}$ = 0.1410 nM (p=0.0002). **E**: Dose-resposne

557 curves of barasertib (n.s.). Error bars represent mean +/- SEM.

558

559 **Figure 6:** *In vivo* **treatment outcomes of A549 xenografts against selumetinib and**

560 **temsirolimus are affected by loss of PTPA.**

561 **A,B:** Growth increment (% as of baseline volume) represented across treatment duration,

562 which was initiated at mean tumor volumes between 150 mm^3 and 300 mm^3. Selumetinib

563 and and temsirolimus treatment cohorts were compared to independent shGFP and shPTPA

564 vehicle control groups. Selumetinib was administered at 25 mg/kg daily, for 15 days

565 continuously. Temsirolimus was administered at 5 mg/kg for 16 days (intermittent dosing

566 schedule). Statistics: Two-way ANOVA. *: $p<0.05$; ***: $p<0.001$; ****: $p<0.0001$. Data are

567 shown as mean +/- SEM. **C,D**: Quantifications of Ki67 proliferative index across in

568 selumetinib (**C**) and temsirolimus (**D**) treated and control shGFP and shPTPA xenografts.

569 Statistics: One-way ANOVA. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ^: border-line significance

570 ($p=0.09$). **E,F**: Representative images of Ki67-stainings of xenografts from the indicated

571 genotypes, and treated with indicated kinase inhibitors. Scale bars represent 100 μm.

572

573 **Figure 7: Loss of PTPA is associated with changes in c-MYC expression upon selumetinib**

574 **treatment** *in vivo*.

575 **A, C**: Western blot anlysis of P-ERK and c-MYC after treatment with selumetinib at day 15 of

576 treatment (25mk/kg). **B,D**: Quantifications of P-ERK1/2 levels (**B**) and total c-MYC levels (**D**)

577 normalized to vinculin. Data are shown as mean +/- SEM. Statistics: One-way ANOVA. *:

578 $p<0.05$; **: $p<0.01$; ***: $p<0.001$; ****: $p<0.0001$. VINC: vinculin.

579

580 **Figure 8: Sensitivity towards temsirolimus upon loss of PTPA can be traced back to**

581 **differential mTOR signaling.**

582   **A**: Western blot analysis of p70S6K and P-p70S6K at day 16 of temsirolimus treatment

583   (5mk/kg). VINC: vinculin. **B-D**: Quantifications of P-p70S6K levels (**B**), total p70S6K (**C**), and

584   phopsho/total p70S6K, normalized to vinculin. Data are shown as mean +/- SEM. Statistics:

585   One-way ANOVA. *: $p<0.05$; **: $p<0.01$; ~: $p = 0.07$ (**C**) and 0.13 (**D**).

586

A

Lung adenocarcinoma
(TCGA, Firehose Legacy)

PPP2R4, mRNA expression
(RNA Seq V2 RSEM)

****
**
*
****

	shallow deletion	diploid	gain	amplification
n:	101	103	24	2
%:	43,91	44,78	10,43	0,87

B

KRAS mutant patient population

PPP2R4, mRNA expression
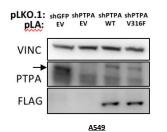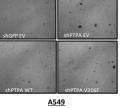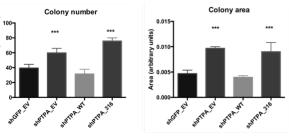(RNA Seq V2 RSEM)

****
*

	shallow deletion	diploid	gain
n:	29	38	8
%:	38,67	50,67	10,67

C

Overall survival
KRAS[mutant] patient population
PPP2R4 genotype

Patient survival (%)
Time (months)

PPP2R4[gain]
PPP2R4[diploid]
PPP2R4[shallow deletion]
*

D

Overall survival
KRAS[mutant] patient population
PPP2R4 mRNA

Patient survival (%)
Time (months)

PPP2R4 mRNA[low]
PPP2R4 mRNA[high]

p=0.0175

E

AA substitution	PROVEAN score	prediction (cut-off = -2.5)
D92N	-0.17	Neutral
R183L	-6.63	Deleterious
E225*	-481.09	Deleterious
Y234C	-8.75	Deleterious
G240S	-5.83	Deleterious
H306L	-10.11	Deleterious
V316F	-4.73	Deleterious

F

3xFLAG CMV10: EV WT 183 225* 234 240 306 316

Lysate
FLAG
PP2A C

IP: FLAG
FLAG
PP2A C

HEK293T

G

Binding PTPA-PP2A C

Binding PTPA-PP2A C (%)

*
**
**** ****

WT R183L Y234C G240S H306L V316F

H

PP2A reactivation PTPA mutants

Activity (%)

WT
R183L *
G243V **
G240S ****

5' 15'

I

pLKO.1: shGFP shPTPA shPTPA shPTPA
pLA: EV EV WT V316F

VINC
PTPA
FLAG

A549

J

shGFP EV shPTPA EV
shPTPA WT shPTPA V316F

A549

K

Colony number

Number of colonies (> 0,01 AU)

*** ***

shGFP_EV shPTPA_EV shPTPA_WT shPTPA_316

L

Colony area

Area (arbitrary units)

*** ***

shGFP_EV shPTPA_EV shPTPA_WT shPTPA_316

A shGFP shPTPA

B Xenograft weights at endpoint

C Xenograft size

D shGFP shPTPA
Mouse ID: 1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8
VINC
PTPA

E PTPA

F shGFP shPTPA
Mouse ID: 1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8
VINC
c-MYC

G c-MYC

A

$Ppp2r4^{+/+}$ $Ppp2r4^{+/gt}$ $Ppp2r4^{gt/gt}$

B

**Number of AAH lesions
(8 weeks post infection)**

Mean number of lesions/section

*** , **

WT   HE   HO

C

Sum of AAH area /lung area

Normalized size (fraction)

** , *

WT   HE   HO

D

**Mean size of
AAH lesion**

Size (μm^2)

WT   HE   HO

E

Proliferative Index

Ki67 proliferative index (%)

*

WT   HE

A

A427 A549

VINC

MYC

PTPA

Ponceau

B

Selumetinib

Relative viability
(% of control)

Selumetinib (Log(nM))

A549
A427

C

MK2206

Relative viability
(% of control)

MK2206 (Log(nM))

A549
A427

D

Temsirolimus

Relative viability
(% of control)

Temsirolimus (Log(nM))

A549
A427

E

Barasertib

Relative viability
(% of control)

Barasertib (Log(μM))

A549
A427

A A549 xenograft - selumetinib

shGFP ctrl
shPTPA ctrl
shGFP + selumetinib (25 mg/kg)
shPTPA + selumetinib (25 mg/kg)

B A549 xenograft - temsirolimus

shGFP ctrl
shPTPA ctrl
shGFP + temsirolimus (5 mg/kg)
shPTPA + temsirolimus (5 mg/kg)

C

D

E
vehicle   selumetinib
shGFP
shPTPA

F
vehicle   temsirolimus
shGFP
shPTPA

A

Vehicle | Selumetinib (25mg/kg)
shGFP | shPTPA | shGFP | shPTPA

VINC

P-ERK

B

P-ERK1/2

P-ERK1/2/VINC

2.0
1.5
1.0
0.5
0.0
-0.5

shGFP ctrl ERK1
shPTPA ctrl ERK1
shGFP ctrl ERK2
shPTPA ctrl ERK2
shGFP + sel ERK1
shPTPA + sel ERK1
shGFP + sel ERK2
shPTPA + sel ERK2

*

n.s

C

Vehicle | Selumetinib (25mg/kg)
shGFP | shPTPA | shGFP | shPTPA

VINC

c-MYC

PTPA

D

c-MYC

% of shGFP mean normalized intensity

400
300
200
100
0

shGFP
shGFP + selumetinib
shPTPA
shPTPA + selumetinib

****
**
***
*

A

Vehicle — Temsirolimus (5mg/kg)
shGFP shPTPA shGFP shPTPA

VINC
p70S6K
VINC
P-p70S6K
PTPA

B

P70S6K phospho

Normalized intensities (phospho/vinc)

*

shGFP control
shPTPA control
shGFP temsirolimus
shPTPA temsirolimus

C

P70S6K total

Normalized intensities (total/vinc)

~   **

shGFP control
shPTPA control
shGFP temsirolimus
shPTPA temsirolimus

D

P70S6K phospho/total

Normalized intensities (phospho/total)

~

shGFP control
shPTPA control
shGFP temsirolimus
shPTPA temsirolimus

9. Supplementary Figures and Tables
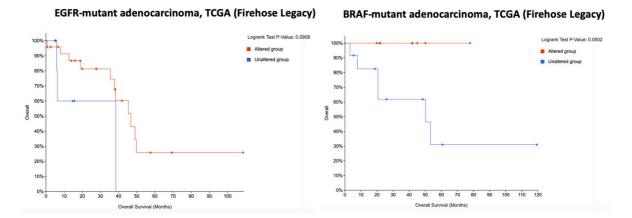
Supplementary Figure 1:

Figure S1: Kaplan-Meier survival data for EGFR-mutant (left panel) and BRAF-mutant non-small cell lung adenocarcinoma. Altered group: patients with PTPA heterozygous loss; Unaltered group: patients without PTPA heterozygous loss.
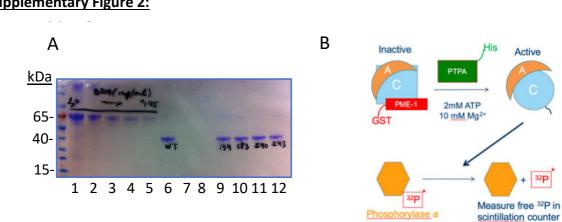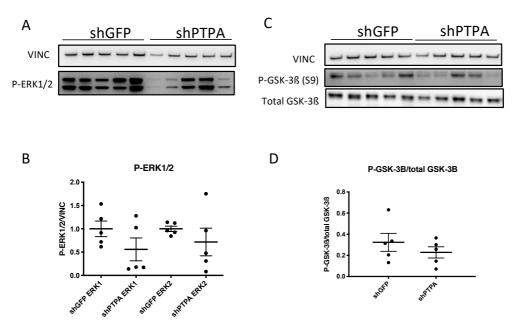
Supplementary Figure 2:

Figure S2: Purified recombinant His-PTPA wild-type and mutants used in phosphorylase *a* PP2A reactivation assay. A: Lane 1-5: 1/2 dilution series (20-1.25 µg) of BSA. Lane 6: WT PTPA; Lane 9: R134W PTPA (not included in this manuscript). Lane 10: R183L PTPA. Lane 11: G240S PTPA. Lane 12: G243V PTPA. **B:** Schematic of PP2A reactivation assay. Inactive PP2A is isolated from HEK293T cells by PME-GST pull down using glutathione-Sepharose beads. Incubation with recombinant His-PTPA in the presence of ATP/Mg^{2+} activates PP2A, which can dephosphorylate a single radio-actively labeled phosphorylated residue on phosphorylase *a*.

Free radioactive phosphate detected by scintillation counting is a measure of PP2A activity and thus of PTPA functionality.

Supplementary Figure 3:

Figure S3: **P-ERK1/2 and P-GSK-3ß levels show no significant shPTPA-induced differences in A549 xenografts. A,C**: Western blot analyses of P-ERK1/2 (**A**) and P-GSK-3ß and total GSK-3ß (**B**) in shGFP and shPTPA A549 xenografts. VINC: Vinculin. **B,D**: Quantifications of panel A (**B**) and panel C (**D**). Statistics: One-way ANOVA (**C**) and two-sided Student's t-test (**D**). Data are represented as mean +/- SEM. Differences in expression are non-significant for all conditions.
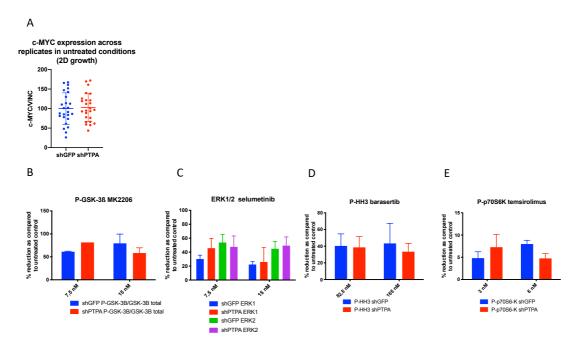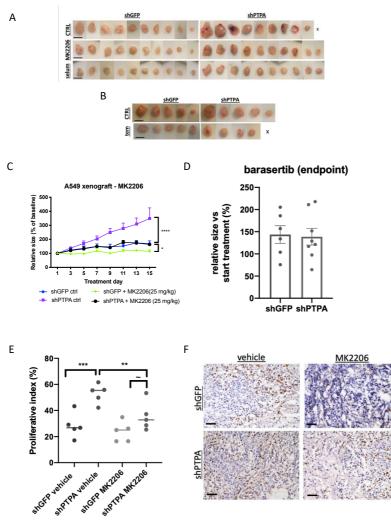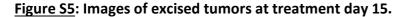
Figure S4: **Quantifications of basal c-MYC levels, and P-GSK-3ß, P-ERK1/2, P-HH3 and P-p70S6K after treatments. A**: Western blot analysis quantifications of c-MYC levels in untreated controls across technical and biological replicates. **B:** Western blot analysis quantifications of P-GSK3-ß/total levels after MK2206 treatments (48 h) at indicated doses. **C:** Quantifications of Western blot images of P-ERK1/2 levels after selumetinib treatments (48 h) at indicated doses. **D:** Quantifications of Western blot images of P-HH3 levels after barasertib treatments (48 h) at indicated doses. **E:** Quantifications of Western blot images of P-p70S6K after temsirolimus treatment at indicated doses. Statistics: **A**: Two-sided Student's t-test. Data are represented as mean +/- SEM. **B-E**: Two-way ANOVA. Data are shown as mean +/- SEM. All statistical tests returned non-significant differences.
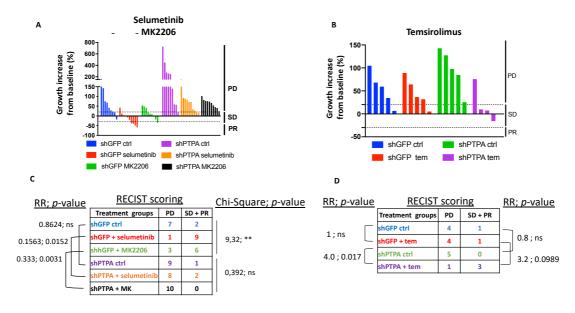
Supplementary Figure 5:

Supp fig S5:

Figure S5: Images of excised tumors at treatment day 15.

A, B: Excised A549 shGFP and shPTPA xenografts after 15 days (25 mg/kg daily) for selumetinib and MK2206 and 16 days (5 mg/kg daily for 4 consecutive days followed by 2 days of rest). **A**: MK2206 and selumetinib treatment cohorts. **B**: Temsirolimus treatment cohorts. Scale bars represent 10 mm. CTRL: vehicle control; selum: selumetinib; tem: temsirolimus. **C**: MK2206 (25 mg/kg) tumor responses over time. Tumor sizes were normalized to size at treatment initiation. Statistics: 2-way ANOVA. *: $p < 0.05$; ****: $p < 0.0001$. **D**: Barasertib tumor responses after 15 days of treatment (50 mg/kg daily). No significant difference between shGFP and shPTPA was observed (p=0.9). Tumor sizes were normalized to size at treatment initiation. **E**: Ki67 proliferative indices of MK2206 treatment and control cohorts. Statistics: One-way ANOVA. **: $p < 0.01$; ***: $p < 0.001$; ~: $p = 0.054$. **F:** Representative images of (E).

Figure S6: Adapted RECIST criteria further underscore the influence of PTPA on therapeutic outcomes against kinase inhibitors. A, B: Waterfall plots representing relative increase in size at endpoint, relative to treatment initiation. **A**: selumetinib and MK2206; **B**: temsirolimus. Responses were subdivided in Progressive Disease (>20% growth increase; **PD**), Partial Response (>30% growth reduction, **PR**) or Stable Disease (in between PD and PR; **SD**).

C,D: Contingency analysis of therapeutic responses against selumetinib and MK2206 (**B**) and temsirolimus (**C**). **RR**: relative risk. **C**: Loss of PTPA significantly deteriorated treatment outcomes against selumetinib and MK2206 (RR=0.15 and RR=0.333, resp.). **D**: Loss of PTPA improved outcomes against temsirolimus to a near significant extent (RR=3.2, p=0.0989), while it was the only therapy to significantly improve RECIST outcomes in the shPTPA depleted xenografts (RR=4.0). On the contrary, selumetinib and MK2206 were more effective in the PTPA wild-type setting as compared to temsirolimus (**C**). Statistics: Fisher-exact (C, D) and Chi-Square (C) test. SD and PR were pooled (SD+PR) in order to obtain sufficiently large groups for proper statistical analysis.

I.   Supplementary Table 1: List of site-directed mutagenesis primers

Mutation	Forward primer sequence	Reverse primer sequence
R148L (183)	5' ggg gaa ctc cac gct cat tga cta cgg cac 3'	5' gtg ccg tag tca atg agc gtg gag ttc ccc 3'
Y199C (234)	5' g aaa ctc cag aaa aca tgc agg atg gag cca gcc 3'	5' ggc tgg ctc cat cct gca tgt ttt ctg gag ttt c 3
G205S (240)	5' gat gga gcc agc cag cag cca ggg agt g 3'	5' cac tcc ctg gct gct ggc tgg ctc cat c 3'
G208V (243)	5' cag ccg gca gcc agg tag tgt ggg gtc tgg at 3'	5' atc cag acc cca cac tac ctg gct gcc ggc tg 3'
E225*(E190*)	5' ttc aat cgg tac ctt tag gtt atg cgg aaa ctc 3'	5' gag ttt ccg cat aac cta aag gta ccg att gaa 3'
H306L(H271L)	5' ggc cca ttt gca gag ctc tcc aac cag ctg tgg 3'	5' cca cag ctg gtt gga gag ctc tgc aaa tgg gcc 3'
V316F(V281F)	5' gg aac atc agc gcc ttc cct tcc tgg tcc aaa 3'	5' ttt gga cca gga agg gaa ggc gct gat gtt cc 3'

II.   Supplementary Table 2: list of antibodies

Target	Brand	Product number	Dilution
VINCULIN	Sigma	V9131	1:5000
PTPA	Santa Cruz	sc-81607	1:500
anti-PP2A C	Gift S. Dilworth	clone F2.5G4	1:1000
anti-FLAG	Sigma	F3165	1:10.000; 1:50 (IP)
c-MYC	Cell Signaling Technologies	#5605	1:1000
c-MYC P-S62	Abcam	ab78318	1:500
c-MYC P-T58	Abcam	ab185655	1:1000
ERK1/2 P-T202/Y204	Cell Signaling Technologies	#9101	1:1000
p70S6K P-T389	Cell Signaling Technologies	#9205	1:1000
Total p70S6K	Cell Signaling Technologies	#9202	1:1000
AKT P-S473	Cell Signaling Technologies	#9271	1:100
GSK-3ß	Cell Signaling Technologies	#9315	1:1000
GSK-3ß P-S9	Cell Signaling Technologies	#9323	1:1000
Secondary, anti-Mouse IgG	Agilent Technologies	P026002-2	1:3000
Secondary, anti-Rabbit IgG	Cell Signaling Technologies	#7074	1:3000
Ki67, human specific	Abcam	EPR3610	1:500 (IHC)
Ki67	Cell Signaling Technologies	#9129	1:80 (IHC)
CD45	BD Biosciences	Clone 30-F11	1:50 (IHC)
AlexaFluor488, anti-rabbit	Invitrogen		1:300 (IHC)
AlexaFluor647, anti-Rat	Jackson's Laboratories		1:300 (IHC)

III. Supplementary Table 3A: Kinase inhibitor information

Compound	Alias	Vendor	Category number	Target	Applied concentrations (µM), *in vitro*	Applied dose, *in vivo*
MK2206		Selleckchem	S1078	AKT	0,3 - 0,00004572474	25mg/kg
Selumetinib	AZD6624	Selleckchem	S1008	MEK	0,3 - 0,00004572474	25mg/kg
Palbociclib	PD-0332991	Selleckchem	S1116	CDK4/6	3,33 - 0,00152416	n/a
Volasertib	BI 6727	Selleckchem	S2235	PLK1,2,3	50 - 0,00762079	n/a
Dactolisib	BEZ235	Selleckchem	S1009	PI3K/mTOR	10 - 0,00152416	n/a
Ralimetinib	LY2228820	Selleckchem	S1494	p38	10 - 0,00152416	n/a
GSK461364		MedChemExpress	HY-50877	PLK1	0,1 - 0, 0001524	n/a
Buparlisib	BKM120	Selleckchem	S2247	PI3K	10 - 0,00152416	n/a
Sorafenib	BAY 43-9006	Selleckchem	S7397	Raf-1, others	10 - 0,00152416	n/a
Alisertib	MLN8237	Selleckchem	S1133	AURKA	3,33 - 0,00152416	n/a
SNS032	BMS-387032	Selleckchem	S1145	CDK2/7/9	10 - 0,00152416	n/a
Erlotinib	CP358774	Selleckchem	S1023	EGFR	3,33 - 0,00152416	n/a
Crizotinib	PF-02341066	Selleckchem	S1068	c-MET, ALK	10 - 0,00152416	n/a
BI2536		Selleckchem	S1109	PLK1,2,3	50 - 0,00762079	n/a
Ro3306		Tocris	4181	CDK1	30 - 0,004572474	n/a
Adavosertib	MK1755	Selleckchem	S1525	Wee1	10 - 0,00152416	n/a
Barasertib	AZD1152	MedChemExpress	HY-10127	AURKB	3,33 - 0,00152416	50 mg/kg
Temsirolimus	CCI-779	Selleckchem	S1044	mTOR	0,120 - 0,01828989	5 mg/kg

IV. Supplementary Table 3B: kinase inhibitors and relevant references

Compound	Target	References (PMID)	
		PP2A	**MYC**
MK2206	AKT	18042541; 30132971; 27531894	23331925; 11018017; 16788862; 33259779; 30132971
Selumetinib	MEK	16456541; 30021885;	30021885; 24685132; 16899113; 11018017; 28179307; 23639941; 21628402
Palbociclib	CDK4/6	23634261	28978620; 32934206; 31727874; 28978620; 24444383; 12070150; 10373516; 21885567;
Volasertib	PLK1,2,3	17121863; 21874008; 21252232	31571905; 29383095; 30217967; 23887393; 27699933; 32468878
Dactolisib	PI3K/mTOR	28199842; 27485451; (26459601) ; 26310906; 26118661 ; 25438055; 20227368; 19553685; 18056704; 11948686; 10200280; 30132971	21876152; 22363436; 27913436; 16788862; 22340590;
Ralimetinib	p38	15569672; 24015987 ; 15972258; 11259586 ; 18039929	28460458; 27913436; 15078869; 12819782; 11408569; 15634685
GSK461364	PLK1	17121863; 21874008; 21252232	31571905; 29383095; 30217967; 23887393; 27699933;
Buparlisib	PI3K		21876152; 22363436; 27913436; 16788862; 32068318; 22340590
Sorafenib	Raf-1, others	28161506; 19933846;16239230; 15664191; 12932319; 11494123; 10801873; 21822300	19647225; 9464539; 2557616;31980175; 29212027; 24934810; 24469106; 22836754; 21822300; 18679422; 11466616; 25128497; 24309997; 32559715
Alisertib	AURKA	24825897	30226440; 25284017; 20519624
SNS032	CDK2/7/9	28137908; 26253406; 18276582; 15122341; 16048649	28665315; 20818171; 19966300; 20010815; 20445224; 20713526; 18206647; 18206647; 11060032; 10409725; 9188852; 9163430; 9119229; 8386381; 25490451; 8065309; 29507396; 32645016; 31311847; 31243099; 29588524; 23776131; 21885567
Erlotinib	EGFR	30830869;27821484; 24954871; 23178652; 19825976;	24922639; 30770740
Crizotinib	c-MET, ALK	30224486; 15075332;	29507657; 31776900; 31462708; 30290287; 12213716
BI2536	PLK1,2,3	17121863; 21874008; 21252232	31571905; 29383095; 30217967; 23887393; 27699933;
Ro3306	CDK1	32900880; 32591484; 26053095; 24616226; 19793917; 18056802; 23672858; 18056802; 23790971; 7622588	28665315; 31822694; 24444383;18206647; 29512702; 28665315; 23776131; 21885567; 17589519;
Adavosertib	Wee1	33108758; 21849476; 18056802;23790971	32195191; 31919076; 32049046; 32195191
Barasertib	AURKB	31527146; 26906715; 12082625; 25892238; 23789096; 23746640; 23345399; 23079597; 21874008;	25739120; 32049046; 25411027; 20519624; 19287963; 30540594
Temsirolimus	mTOR	28199842; 27485451; (26459601) ; 26310906; 26118661 ; 25925585;20227368; 19553685; 18056704; 11948686; 10200280; 30132971	25925585; 33443202; 33259779; 32615570; 30733194; 30705387; 30568499;30389701; 30132971; 30061153; 28978620; 28370287; 28174256; 23887393; 25537515; 26459601; 25999153; 27822418; 26459601; 25797247; 25537515; 24140020; 23242809; 21135252; 19773438; 19342893: 15634685; 14576155; 9715279

Searched Pubmed for: "PP2A" AND "target", "PP2A" AND "compound"; "MYC" AND "target", "MYC" AND ''compound"