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Notch inhibition overcomes resistance to tyrosine kinase inhibitors in EGFR-driven lung adenocarcinoma

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1 ABSTRACT

2 EGFR mutated lung adenocarcinoma patients treated with gefitinib and osimertinib
3 showed a therapeutic benefit limited by the appearance of secondary mutations, such as
4 *EGFR*^{T790M} and *EGFR*^{C797S}. It is generally thought that these secondary mutations
5 render EGFR completely unresponsive to such inhibitors. Here, we found that gefitinib
6 and osimertinib increased STAT3 phosphorylation (pSTAT3) in lung adenocarcinoma
7 cells harboring the *EGFR*^{T790M} and the *EGFR*^{C797S} mutations. Moreover, concomitant
8 treatment with a Notch inhibitor and gefitinib or osimertinib strongly reduced the
9 expression of the transcriptional repressor HES1, in a pSTAT3-dependent manner.
10 Importantly, we show that tyrosine kinase inhibitor resistant tumors, with *EGFR*^{T790M}
11 and *EGFR*^{C797S} mutations, are highly responsive to treatment with the combination of
12 Notch inhibitors and gefitinib or osimertinib, respectively. Finally, in patients with lung
13 adenocarcinoma harboring EGFR mutations treated with tyrosine kinase inhibitors,
14 HES1 protein levels increased during relapse, and high HES1 expression level
15 correlated with shorter progression-free survival. Our results offer a proof of concept for
16 a novel alternative to treat patients with lung adenocarcinoma after disease progression
17 following osimertinib treatment.

18 INTRODUCTION

19 Lung cancer kills about a million people every year worldwide being the leading cause
20 of death by cancer in the world. There are two main types of lung cancer: small cell
21 lung carcinoma (about 20% of all lung cancers) and non-small-cell lung carcinoma that
22 includes lung adenocarcinoma (40%), lung squamous carcinoma (30%), and large cell
23 carcinoma (about 10% of all lung cancers) (1). *EGFR* gene alterations are detected in
24 about 20% of patients with lung adenocarcinoma in Western countries, and in up to
25 50% in some Asian countries, such as Korea. The most common *EGFR* alterations in
26 lung adenocarcinoma are exon 19 deletion and the activating *EGFR*^{L858R} mutation (2).
27 The life expectancy of patients has improved dramatically thanks to the development of
28 *EGFR* tyrosine kinase inhibitors (TKI) (3). Most of the patients treated with first-
29 generation TKIs (i.e., gefitinib and erlotinib) initially respond well; however, their
30 tumors rapidly develop resistance. This is explained, in about 60% of cases, by
31 acquisition of the so-called ‘gatekeeper’ mutation *EGFR*^{T790M} (4). More recently, third-
32 generation TKIs that target *EGFR*^{T790M}, such as osimertinib, have shown a very good
33 therapeutic effect in patients with this mutation (5). Unfortunately, tumors from patients
34 treated with osimertinib also become resistant to this drug. In about 30% of cases, this is
35 due to appearance of new gatekeeper mutations, such as *EGFR*^{C797S} (6, 7). Thus,
36 treatment of *EGFR*-driven lung adenocarcinoma with a single TKI might have limited
37 value, and strategies based on combinational drug therapies could be more effective for
38 controlling the effects of gatekeeper mutations.

39 The *EGFR*^{T790M} gatekeeper mutation confers resistance to TKIs through different
40 mechanisms, such as weaker drug binding due to steric hindrance and increased ATP
41 affinity of mutated *EGFR* (8). However, gefitinib binding to *EGFR*^{T790} protein is
42 reduced, but not totally inhibited (8). Moreover, X-ray structure analysis indicates that

43 gefitinib binds in a similar manner to wild type EGFR and to EGFR^{T790M} proteins (9).
44 Therefore, we hypothesized that in tumors with gatekeeper EGFR mutations, gefitinib
45 could still affect EGFR downstream signaling pathways, although not reaching
46 therapeutic effect, and that this could be exploited by concomitantly inhibiting other
47 signaling pathways.

48 The Notch signaling pathway is highly conserved among metazoans, and it is
49 important during embryonic development as well as adult tissue homeostasis. In
50 mammals, there are four NOTCH receptors (NOTCH1 to 4) that are activated upon
51 interaction with their transmembrane ligands (DELTA and JAGGED). For this
52 activation to occur, an intramembrane protease called γ -secretase, releases the Notch
53 intracytoplasmic domain (NICD). Upon nuclear translocation and binding to its DNA
54 binding partner RBPJ, NICD modulates the expression of target genes of the canonical
55 Notch pathway, such as HES1 (10). Therefore, the Notch pathway can be inhibited by γ -
56 secretase inhibitors (GSIs), or by antibodies against the Notch ligands and receptors
57 (11).

58 By making use of genetically engineered mouse models, we and others
59 demonstrated that KRAS-driven lung adenocarcinoma is dependent on Notch activity
60 (12-14). Moreover, seminal *in vitro* and *in vivo* (mouse xenografts) studies using lung
61 cancer cell lines showed that the combination of Notch inhibitors and EGFR TKIs gives
62 a better anti-tumor response than single treatments in sensitive cells (15-17). However,
63 the underlying mechanism is not fully understood, and the role of the Notch pathway in
64 lung adenocarcinoma after relapse due to acquisition of gatekeeper mutations in EGFR
65 remains largely unknown.

66 Here, to find some answers to these questions, we first performed a transcriptomic
67 analysis in human lung adenocarcinoma PC9GR cells harboring the *EGFR*^{T790M}

68 gatekeeper mutation, and found that after gefitinib treatment, several pathways,
69 including the KRAS signaling pathway, were downregulated. Therefore, based on our
70 previous results concerning the importance of Notch in KRAS-driven lung
71 adenocarcinoma (14), we tested the combined effect of TKI and Notch inhibition in
72 different preclinical mouse models of lung adenocarcinoma with the *EGFR*^{T790M/L858R}
73 mutations. We found that *in vivo* Notch inhibition overcame resistance to gefitinib in
74 human and murine lung adenocarcinoma cells via pSTAT3 binding to the *HES1*
75 promoter and HES1 decreased expression. Similarly, Notch inhibition re-sensitized also
76 *in vivo* human lung adenocarcinoma cells harboring the *EGFR*^{C797S} mutation, that cause
77 resistance to osimertinib, which might become soon the first-line treatment for patients
78 with EGFR-driven lung adenocarcinoma. Altogether, our data show that concomitant
79 Notch inhibition with TKI, could be a potent strategy to treat EGFR-driven lung
80 adenocarcinoma patients after TKI relapse.

81 **RESULTS**82 **Gefitinib treatment in human lung adenocarcinoma cells with the gatekeeper**
83 **mutation $EGFR^{T790M}$ induces changes in several cancer-associated genetic**
84 **signatures**

85 To identify molecular changes upon gefitinib treatment in lung cancer cells harboring
86 the $EGFR^{T790M}$ mutation that confers resistance to first-generation TKIs, we used the
87 already described human EGFR-driven lung adenocarcinoma PC9GR cell line
88 ($EGFR^{T790M}$) resistant to gefitinib (18). Gene set enrichment analysis using the
89 «Molecular Signatures Database Hallmark Gene Set Collection» (19, 20) of data
90 obtained by RNA-seq of PC9GR cells treated with vehicle or gefitinib showed that
91 among the fifty signatures, only one was upregulated
92 (HALLMARK_KRAS_SIGNALING_DN) (Supplemental Table 1). Accordingly,
93 among the eight downregulated gene sets in gefitinib-treated cells, we found
94 “HALLMARK_KRAS_SIGNALING_UP” (Figure 1A and Supplemental Table 1).
95 This suggests that in PC9GR cells, gefitinib decreases the activity of the KRAS
96 signaling pathway, a well-known EGFR downstream pathway (21).

97 We previously reported that the Notch pathway plays a major role in KRAS-
98 driven lung adenocarcinoma, and that its inhibition fully stops tumor growth in this
99 setting (14). Therefore, we hypothesized that gefitinib effects in PC9GR cells harboring
100 the $EGFR^{T790M}$ gatekeeper mutation could be enhanced by Notch inhibition.

101

102 **Inhibition of Notch signaling hampers tumor growth in $EGFR^{T790M/L858R}$ mice**

103 Before directly testing this hypothesis, we studied the Notch pathway activation in
104 EGFR-driven lung tumors *in vivo*, by crossing $EGFR^{T790M/L858R}$ (22) and lung-specific
105 *CCSP-rtTA* transgenic mice (23) to obtain mice in which $EGFR^{T790M/L858R}$ expression in

106 lungs can be induced by treatment with doxycycline ($EGFR^{T790M/L858R}$ mice, hereafter).
107 After 8 weeks of doxycycline treatment, mice developed bronchial and peripheral
108 $EGFR^{T790M/L858R}$ -driven tumors that are resistant to first-generation EGFR TKIs, such as
109 gefitinib (22). Western blot analysis showed that N1ICD, the processed and active form
110 of NOTCH1, and HES1, a Notch target gene, were strongly expressed in
111 $EGFR^{T790M/L858R}$ -driven tumors compared with normal lung tissue from control mice
112 (either littermates with the same genotype but not treated with doxycycline, or CCSP-
113 rtTA transgenic mice treated with doxycycline) (Figure 1B). This finding is similar to
114 what observed in the $Kras^{G12V}$ mouse model (14), and suggests that the Notch pathway
115 may play a similar role in both tumor types.

116 As the NOTCH1 and NOTCH3 receptors promote $Kras^{G12V}$ -driven lung
117 adenocarcinoma, whereas NOTCH2 has a tumor suppressive role (12, 13, 24), we
118 analyzed their expression in $EGFR^{T790M/L858R}$ -driven lung adenocarcinoma. The
119 transmembrane forms of NOTCH1 and NOTCH3 (i.e., before γ -secretase cleavage)
120 were strongly expressed in tumor samples compared with controls (Figure 1B), whereas
121 NOTCH2 expression was comparable in both groups (Figure 1B). Although the level of
122 the transmembrane forms of NOTCH receptors does not reflect Notch activity, and
123 NOTCH3 can be a direct target of NOTCH1 in some circumstances, this finding
124 suggests that both NOTCH1 and NOTCH3 are mediators of the Notch pathway in
125 EGFR-driven tumors *in vivo*.

126 To test whether Notch pathway activity is necessary for the growth of EGFR-
127 driven tumors, we treated $EGFR^{T790M/L858R}$ mice with doxycycline for 8 weeks to induce
128 tumor formation, and then randomly assigned them to three groups: i) control group,
129 treated with vehicle and IgG antibody control; ii) GSI group, treated with dibenzazepine
130 (DBZ), a potent and selective GSI; and iii) anti-NRR1/NRR3 group, treated with

131 blocking antibodies against NOTCH1 and NOTCH3, according to previously described
132 treatment regimens (25-27). After five weeks of treatment, tumors represented more
133 than 40% of the lung area in the control group, but only 20% and 10% in the DBZ and
134 anti-NRR1/NRR3 groups, respectively (Figure 1C). This indicates that the Notch
135 pathway is required for *EGFR*^{T790M/L858R}-driven tumor growth. Body weight was
136 comparable in the three groups (Supplemental Figure 1A), suggesting the absence of the
137 intestinal toxicity reported by other studies using regimens that led to stronger Notch
138 inhibition (28).

139 As expected, analysis of protein expression by immunohistochemistry (IHC) of
140 tumors from anti-NRR1/NRR3- or DBZ- treated mice showed fewer HES1-
141 positive cells than in the vehicle-treated control group, implying that these treatments
142 effectively inhibited the Notch pathway (Figure 1D). Moreover, the percentage of Ki67-
143 positive cells was lower in tumors from the anti-NRR1/NRR3 and DBZ groups than the
144 control group, indicating that Notch activity promotes cell proliferation in
145 *EGFR*^{T790M/L858R}-driven tumors (Figure 1D). As the MAPK and AKT pathways are
146 crucial downstream players of the EGFR signaling pathway (21), we also analyzed the
147 expression of pERK and pAKT in the same samples. The percentage of pERK-positive
148 cells was similarly reduced by treatment with the anti-NRR1 and -NRR3 antibodies and
149 with DBZ compared control (Figure 1D), consistent with previous observations (13,
150 14). Conversely, the percentage of pAKT-positive cells was comparable in all groups
151 (Supplemental Figure 1B).

152

153 **Notch inhibition overcomes resistance to gefitinib in *EGFR*^{T790M/L858R}-driven lung**
154 **adenocarcinoma**

155 To study whether pharmacological inhibition of the Notch pathway *in vivo* had any
156 impact on the resistance to gefitinib conferred by the gatekeeper mutation $EGFR^{T790M}$,
157 we randomized $EGFR^{T790M/L858R}$ mice (after 8 weeks of doxycycline treatment) in four
158 treatment groups: i) vehicle (control), ii) gefitinib, iii) DBZ, and iv) gefitinib + DBZ.
159 For simplicity we decided to inhibit the Notch pathway hereafter only with a GSI.

160 As before, body weight was comparable in the different groups after the five
161 weeks of treatment, suggesting that these drugs were well tolerated alone or in
162 combination (Supplemental Figure 2A). In agreement with the previous findings (Figure
163 1C), tumor tissue occupied 42% of the lung in the control group, whereas it was
164 decreased to 23% in the DBZ group (Figure 2A). As expected, gefitinib alone did not
165 have any anti-tumor effect in $EGFR^{T790M/L858R}$ mice (52% of lung was tumor tissue).
166 Conversely, the DBZ and gefitinib combination led to a very significant reduction of the
167 tumor area compared with DBZ alone (tumor tissue covered only 10% of the total lung
168 area) (Figure 2A).

169 Histopathological analysis of lung adenocarcinoma samples (i.e., non-benign
170 tumors, Supplemental Figure 2B) showed that the single treatments had no effect on the
171 lung adenocarcinoma number compared with control (i.e., vehicle-treated mice) (Figure
172 2B). Importantly, animals treated with the combination of gefitinib and Notch
173 inhibition had significantly fewer lung adenocarcinomas than vehicle-treated ones (a
174 mean of 10 lung adenocarcinoma per mouse *vs* 31 in the control, Figure 2B).

175 IHC analysis showed that the percentage of HES1-, Ki67-, pERK- and pAKT-
176 positive cells was comparable in tumors from the gefitinib group and from controls
177 (Figure 2C). By contrast, the percentage of HES1-, Ki67- and pERK-positive cells was
178 reduced in tumors from DBZ-treated mice (Figure 2C), as before (Figure 1D), although
179 in this case the difference was not significant for pERK. The percentage of HES1-,

180 Ki67- and pERK-positive cells tended to be lower in mice treated with the gefitinib and
181 DBZ combination compared with DBZ-treated mice, particularly for pERK. Finally, the
182 percentage of pAKT-positive cells was comparable in the DBZ, gefitinib and control
183 groups, but interestingly, it was significantly reduced in the gefitinib + DBZ group
184 compared with control mice (Figure 2C).

185 Altogether, these data demonstrate that inhibition of Notch signaling by DBZ
186 restores sensitivity to treatment with gefitinib in *EGFR*^{T790M/L858R}-driven lung
187 adenocarcinoma *in vivo*.

188

189 **Notch inhibition overcomes resistance to gefitinib in lung adenocarcinoma patient-** 190 **derived xenografts with *EGFR*^{T790M/L858R} mutations**

191 These results were very encouraging; however, it is considered that the best strategy for
192 testing new cancer treatments is the combination of genetic mouse models and patient-
193 derived xenograft (PDX) preclinical models (29). Therefore, we developed a lung
194 adenocarcinoma PDX that harbors the *EGFR*^{T790M/L858R} mutations, like our transgenic
195 mouse model. One week after subcutaneous grafting of the PDX, nude mice were
196 randomized in four groups as before: i) vehicle alone (control), ii) gefitinib, iii) DBZ,
197 and iv) gefitinib + DBZ. Tumor growth was monitored for 30 days (i.e., the treatment
198 duration). As expected, the *EGFR*^{T790M} mutation conferred resistance to gefitinib. On the
199 other hand, DBZ inhibited tumor growth, and strikingly, the DBZ and gefitinib
200 combination almost totally blocked tumor growth (Figure 3A).

201 As before, IHC analysis of tumors showed that DBZ (alone or in combination
202 with gefitinib) efficiently decreased the percentage of HES1-positive cells compared
203 with control (Figure 3B). Tumor cell proliferation (Ki67-positive cells) was reduced by
204 DBZ alone, and this effect was increased by addition of gefitinib. Similarly, the

205 percentage of pERK-positive cells was decreased by treatment with DBZ alone and
206 even more by the DBZ and gefitinib combination compared with control. This indicated
207 that the DBZ and gefitinib combination was more effective in reducing MAPK
208 signaling than Notch inhibition alone. Finally, the percentage of pAKT-positive cells
209 also was efficiently and similarly reduced by DBZ and by the DBZ and gefitinib
210 combination.

211 Altogether, our results provide strong preclinical evidence for the likely
212 therapeutic benefit of Notch inhibition and gefitinib combination in patients with TKI-
213 resistant EGFR-driven lung adenocarcinoma harboring the gatekeeper mutation
214 *EGFR*^{T790M}.

215

216 **Combining EGFR TKIs and Notch inhibitors synergistically decreases HES1** 217 **expression**

218 Our previous analysis showed that the DBZ and gefitinib combination is more efficient
219 than each single treatment in reducing MAPK and AKT pathways. Previous reports,
220 including work from our laboratory, identified HES1 as an important positive MAPK
221 regulator in KRAS-driven lung adenocarcinoma (13, 30). Even more, HES1 has a
222 similar effect on AKT signaling in T-cell acute lymphoblastic leukemia (T-ALL) (31).
223 Therefore, we hypothesized that HES1 could be an important mediator of pERK and
224 pAKT upon treatment with the DBZ and gefitinib combination. As the percentage of
225 HES1-positive cells was similar in tumors from mice treated with DBZ alone and the
226 DBZ and gefitinib combination in both preclinical models (Figure 2C and Figure 3B),
227 we analyzed HES1 signal intensity in the same samples. Importantly, HES1 signal
228 intensity was significantly lower in tumors from mice treated with the DBZ and

229 gefitinib combination than from mice treated with DBZ alone in the PDX model, and
230 followed a similar trend in *EGFR*^{T790M/L858R} mice (Figure 4, A and B).

231 To further validate our data, we analyzed HES1 expression by western blotting
232 in PC9GR cells (previously used for the RNA-seq analysis, Figure 1A and
233 Supplemental Table 1) after incubation with the different drugs alone or in combination.
234 In accordance to our *in vivo* observation, HES1 expression was strongly reduced in cells
235 exposed to the DBZ and gefitinib combination (Figure 4C).

236 Then, to explore HES1 role in PC9GR cells, we depleted HES1 using a pool of
237 siRNAs targeting *HES1* mRNA (*siHES1*) (Supplemental Figure 3). Of note,
238 proliferation of *siHES1*-treated cells was impaired compared with control cells
239 transfected with the non-targeted siRNA (*siNT*), and this effect was potentiated in the
240 presence of gefitinib (Figure 4D).

241 To test whether gefitinib effect was EGFR-mediated, we used the Chinese
242 hamster ovary (CHO) cell line that is a natural null for EGFR, and was previously used
243 for EGFR gain of function analyses (32). Interestingly, HES1 expression was not
244 affected by co-treatment with DBZ and gefitinib in CHO cells transfected with empty
245 vector, but was reduced in CHO cells that express *EGFR*^{T790M/L858R} protein (Figure 4E).
246 We concluded that EGFR is needed for HES1 expression reduction by the DBZ and
247 gefitinib combination.

248 Taken together, our data indicate that the DBZ and gefitinib combination
249 synergistically reduces the expression of HES1, a major driver in lung adenocarcinoma.

250

251 **pSTAT3 directly binds to the *HES1* promoter and inhibits its expression**

252 Previous studies have shown a benefit of combining EGFR TKIs and Notch inhibitors
253 in TKI-sensitive cells, but the underlying mechanism was not fully described (15-17).

254 On the basis of the EGFR-dependent HES1 decrease in EGFR^{T790M/L858R}-expressing
255 CHO cells upon incubation with the DBZ and gefitinib combination, we hypothesized
256 that a common mechanism could be involved in the response to TKI treatment in TKI-
257 sensitive and -resistant lung adenocarcinoma cells. An increase in the phosphorylation
258 of STAT3 protein (pSTAT3), dependent on both JAK and FGFR activities, is reported
259 in sensitive lung adenocarcinoma cells upon treatment with first-generation (erlotinib)
260 and second-generation (afatinib) TKIs (33-35), hence, we investigated whether this
261 occurred also in TKI-resistant cells.

262 Indeed, analysis of STAT3 phosphorylation status in PC9GR cells showed an
263 increase in pSTAT3 levels upon gefitinib treatment (Figure 5A). This effect was
264 partially inhibited by co-treatment with PD173074 or ruxolitinib, pan-inhibitors of
265 FGFR and JAK pathways respectively. Even more, the combination of both inhibitors
266 reduced pSTAT3 to levels lower than in control non-treated cells (Supplemental Figure
267 4). Moreover, we found that in the human *HES1* and mouse *Hes1* gene promoters,
268 consensus binding sites for pSTAT3 (i.e., TTNNNNNAA) (36) are close to RBPJ sites
269 (i.e., where the Notch transcription complex binds) (Supplemental Figure 5, A and B).
270 To test whether pSTAT3 binds directly to the human *HES1* promoter in PC9GR cells,
271 we performed chromatin immunoprecipitation (ChIP) experiments using antibodies
272 against pSTAT3 and against NOTCH1, which is known to bind to the *HES1* promoter
273 (positive control). NOTCH1 bound to the *HES1* promoter, and this interaction was
274 reduced by incubation with DBZ (Figure 5B). Importantly, pSTAT3 bound to the *HES1*
275 promoter only when cells were co-incubated with gefitinib and DBZ (Figure 5B). To
276 determine whether pSTAT3 binding was critical for HES1 downregulation (Figure 4C),
277 we incubated PC9GR cells with the various drug combinations after *siSTAT3* treatment
278 that efficiently reduced both pSTAT3 and STAT3 expression (Figure 5C). Co-

279 incubation with gefitinib and DBZ strongly reduced HES1 protein level in control *siNT*-
280 treated cells (Figure 5C), but strikingly, the same co-treatment kept HES1 levels in
281 *siSTAT3*-treated cells (Figure 5C).

282 Altogether, these findings support that pSTAT3 decreases HES1 protein level by
283 acting as a transcriptional repressor at the *HES1* promoter.

284

285 **Notch inhibition overcomes resistance to osimertinib in human lung** 286 **adenocarcinoma cells harboring the *EGFR*^{C797S} mutation**

287 As various TKIs increase pSTAT3 levels in lung adenocarcinoma cells (33-35), we
288 asked whether the pSTAT3-dependent mechanism observed for gefitinib applied also to
289 osimertinib. To this aim, we used the PC9GROR cell line (previously generated from
290 PC9GR cells) that is resistant to osimertinib and harbor the gatekeeper mutation
291 *EGFR*^{C797S} (18).

292 First, western blot analysis of PC9GROR cells incubated with DBZ and/or
293 osimertinib showed that pSTAT3 levels increased upon osimertinib treatment.
294 Accordingly, the combination of DBZ and osimertinib reduced HES1 protein levels
295 (Figure 6A).

296 To test whether DBZ re-sensitized *EGFR*^{C797S} mutant human lung
297 adenocarcinoma cells to osimertinib *in vivo*, we grafted PC9GROR cells subcutaneously
298 in mice, and two weeks later, we treated them with DBZ and/or osimertinib for 3 weeks.
299 Body weight remained comparable in the different treatment groups (Supplemental
300 Figure 6A). Osimertinib alone had no significant effect on growth of PC9GROR cell
301 xenografts (Figure 6B), while it strongly inhibited the growth of PC9GR xenografts
302 (Supplemental Figure 6B). Similarly, DBZ showed no effect on growth of PC9GROR

303 cell xenografts, but importantly, tumor growth was strongly inhibited in mice treated
304 with the osimertinib and DBZ combination (Figure 6B).

305 This finding demonstrates that treatment with DBZ restores sensitivity to
306 osimertinib in human lung adenocarcinoma cells harboring the *EGFR*^{C797S} mutation,
307 confirming and extending our previous observations that DBZ sensitizes TKI-resistant
308 tumors to TKIs.

309

310 **Nirogacestat overcomes resistance to gefitinib in human lung adenocarcinoma cells**
311 **harboring the *EGFR*^{T790M} mutation**

312 To strengthen the translational impact of our work, we wanted to confirm the
313 Notch inhibitor sensitizing effect using a GSI under clinical trials. We chose
314 nirogacestat because a recently finished phase 2 trial, showed that it has promising
315 effects in patients with desmoid tumors, is well tolerated, and can be used for long-term
316 treatments (37).

317 We randomized mice with subcutaneous PC9GR cell xenografts in six treatment
318 groups: i) vehicle, ii) DBZ, iii) nirogacestat, iv) gefitinib, v) DBZ + gefitinib, vi) and
319 nirogacestat + gefitinib. As gefitinib has some effect in PC9GR cells *in vitro* (Figure
320 4D), we used 10 mg/kg instead of the previously used dose of 20mg/kg. This lower
321 concentration had a mild, non-significant effect on tumor growth compared with
322 vehicle. Like in PC9GROR cells, the GSIs alone (DBZ and nirogacestat) did not have
323 any effect. Conversely, gefitinib in combination with DBZ or nirogacestat strongly
324 inhibited tumor growth (Figure 7A), as observed in mice harboring PDX and
325 *EGFR*^{T790M/L858R}-driven tumors treated with the gefitinib and DBZ combination.

326 Moreover, Kaplan-Meier survival analysis of mice treated or not with
327 nirogacestat and/or gefitinib showed that survival rate was comparable in mice treated

328 with vehicle, nirogacestat or gefitinib alone, although it tended to be higher in the
329 gefitinib group (Figure 7B). By contrast, the nirogacestat with gefitinib combination
330 increased survival compared with all other groups (median survival after treatment
331 started: 24, 26.5, 32, and 39 days for vehicle, nirogacestat, gefitinib, and nirogacestat +
332 gefitinib, respectively). For this analysis, we used only nirogacestat because at the used
333 dose we could administer DBZ only for 5 weeks (26), while nirogacestat is well
334 tolerated in patients for more than 2 years (37). As before, body weight was not
335 significantly different in all groups during the experiment (Supplemental Figure 7).

336 These results show that the combination of gefitinib and nirogacestat increases
337 the survival of mice xenografted with human lung adenocarcinoma cells that carry the
338 *EGFR*^{T790M} mutation conferring resistance to EGFR TKIs.

339

340 **High HES1 protein levels correlate with poor progression-free survival and relapse** 341 **in patients with EGFR mutated lung adenocarcinoma treated with TKIs**

342 Our findings showed that HES1 has a key role in the resistance of EGFR-driven lung
343 adenocarcinoma to TKI therapy. To strengthen this observation, we analyzed the
344 correlation between progression-free survival (PFS) and nuclear HES1 protein levels in
345 75 patients with lung adenocarcinoma harboring *EGFR* mutations and treated with
346 TKIs. We found that patients with low nuclear HES1 expression had a median PFS of
347 14 months, whereas patients with high nuclear HES1 expression had a median PFS of 7
348 months (hazard ratio 2.77, 95% CI [1.4-5.5], p = 0.006) (Figure 7C). Moreover, analysis
349 of HES1 protein in tumor biopsy samples from patients with lung adenocarcinoma
350 harboring *EGFR* activating mutations and treated with TKIs taken at diagnosis and after
351 disease progression showed that HES1 nuclear levels were increased in samples

352 obtained at relapse in six of the seven patients ($p = 0.034$) (Figure 7D and Supplemental
353 Figure 8).

354 These findings extend our previous study (14), and suggest a crucial role for
355 HES1 in the relapse of patients with EGFR-driven lung adenocarcinoma under
356 treatment with TKIs.

357 **DISCUSSION**

358 In this study, we confirmed and extended the observation that HES1 is a crucial
359 mediator of the Notch pathway oncogenic activity in lung adenocarcinoma and
360 uncovered its crucial role in resistance to EGFR TKIs.

361 We first observed that in mouse models of EGFR-driven lung adenocarcinoma,
362 treatment with GSIs leads to decreased HES1 expression and pERK levels. This is
363 consistent with studies in KRAS-driven lung adenocarcinoma mouse models showing
364 that HES1-induced repression of DUSP1 increases pERK levels (13, 14). Therefore, we
365 hypothesize that this is the same mechanism for the antitumor effect of Notch inhibitors
366 when used as single treatment in EGFR-driven tumors.

367 Next, as a proof of concept of sensitizing cells with EGFR gatekeeper mutations
368 to TKIs upon Notch inhibition, we found that murine and human EGFR-driven lung
369 tumors harboring the *EGFR*^{T790M} gatekeeper mutation sensitized to gefitinib when it is
370 administered in combination with the GSI DBZ. Concomitantly, pAKT and pERK were
371 further decreased upon combined treatment with gefitinib and DBZ, compared with the
372 other treatments. It is known that HES1 represses PTEN and increases AKT activity in
373 T-ALL (31), and represses DUSP1 and increases pERK levels in lung adenocarcinoma
374 (30). Interestingly, we found in preclinical mouse models a higher HES1 decreased
375 expression after treatment with the DBZ and gefitinib combination compared with DBZ
376 alone. Moreover, HES1 loss of function sensitized human lung adenocarcinoma cells
377 harboring the *EGFR*^{T790M} mutation to gefitinib through a still unknown mechanism. In
378 T-ALL, HES1 directly represses the *BBC3* gene that encodes PUMA, an inducer of
379 apoptosis (38). Therefore, it is tempting to speculate that in lung adenocarcinoma cells,
380 HES1 could repress *BBC3* or other genes encoding apoptosis-related factors, such as
381 *BCL2L11* (i.e., BIM), crucial for gefitinib-induced cell death (39-41).

382 The *EGFR*^{T790M} mutation does not totally inhibit gefitinib binding to EGFR (8,
383 9), and mutated EGFR is needed in CHO cells to reduce HES1 expression upon co-
384 incubation with gefitinib and DBZ compared with DBZ alone. As the level of active,
385 phosphorylated STAT3 is increased upon treatment with first- and second-generation
386 TKIs (33-35), we hypothesized that this feature could explain the decreased levels of
387 HES1 in our experimental setting. Indeed, incubation of *EGFR*^{T790M} lung
388 adenocarcinoma cells with gefitinib increased pSTAT3 levels in an FGFR- and JAK-
389 dependent manner. Moreover, pSTAT3 directly interacted with the *HES1* promoter by
390 ChIP analysis only when cells were gefitinib and DBZ co-treated. Finally, in PC9GR
391 cells transfected with siRNAs against *STAT3*, HES1 levels were comparable in cells
392 incubated with the combination of gefitinib and DBZ and with DBZ alone. These
393 findings indicate that pSTAT3 represses HES1 expression effectively only when
394 NOTCH processing is concomitantly inhibited, probably because the NOTCH
395 transcriptional complex binds more efficiently to the *HES1* promoter than pSTAT3. A
396 previous study showed that in sensitive EGFR-driven lung adenocarcinoma cells,
397 erlotinib promotes Notch pathway induction after several days (16). We did not observe
398 such induction, and this discrepancy could be due to the different treatment kinetics
399 and/or the resistant background of PC9GR cells. Our data are in accordance with
400 previous studies showing that pSTAT3 acts as a transcriptional repressor (42), and has a
401 tumor suppressive role in prostate cancer (43), glioblastoma (44), and importantly,
402 *KRAS*-driven lung adenocarcinoma (45). On the basis of these findings, the STAT3
403 inhibitors currently in clinical trials (46) should be used with caution, at least in tumors
404 where the Notch pathway and consequently HES1 have a pro-tumorigenic role, as for
405 instance in lung adenocarcinoma.

406 Our findings provided the proof of concept that lung adenocarcinoma cells with
407 gatekeeper mutations conferring resistance to TKIs can be sensitized to such
408 compounds by inhibiting the γ -secretase activity. To extend and validate our findings,
409 we performed additional experiments using the lung adenocarcinoma PC9GROR cell
410 line with the gatekeeper mutation $EGFR^{C797S}$ that confers resistance to the third-
411 generation TKI osimertinib. The relevance of this experiment relies in results from a
412 recent phase 3 clinical trial showing that the PFS of patients with EGFR mutated lung
413 adenocarcinoma was significantly longer in the group with osimertinib as a first-line
414 treatment than in the group with gefitinib or erlotinib (47). This might soon lead to the
415 use of osimertinib as first-line treatment in patients with EGFR-mutated lung
416 adenocarcinoma. Our data shows that osimertinib treatment in lung adenocarcinoma
417 cells harboring the $EGFR^{C797S}$ mutation induced also pSTAT3 and when combined with
418 DBZ further inhibited HES1 expression. Moreover, the DBZ and osimertinib
419 combination strongly inhibited the growth of PC9GROR cell xenografts *in vivo*. This
420 suggests that although osimertinib binding is highly reduced by the
421 $EGFR^{C797S}$ gatekeeper mutation, it can still promote similar changes as observed in
422 $EGFR^{T790M}$ cells treated with gefitinib. Moreover, these effects are exacerbated by
423 Notch inhibition. Our results question the effect of the Notch pathway in the drug-
424 tolerant state (48) in lung adenocarcinoma cells treated with osimertinib and this is
425 currently an important area of study in the laboratory.

426 Overall, our new mechanistic findings highlight the role of HES1 in tumor
427 relapse after EGFR TKI therapy. This was confirmed by the negative correlation
428 between HES1 expression and PFS, as well as HES1 upregulation upon disease
429 progression in patients with EGFR-mutated lung adenocarcinoma treated with TKIs.
430 Our results are in agreement with a recent publication showing the negative correlation

431 between *HES1 mRNA* levels and PFS in a cohort of 64 patients with EGFR mutated
432 non-small cell lung carcinoma treated with TKIs (49).

433 Our findings might be very relevant for patients with EGFR-driven lung
434 adenocarcinoma that becomes resistant to osimertinib through acquisition of gatekeeper
435 mutations, such as EGFR^{C797S}, and for whom treatment possibilities are limited to
436 conventional therapies, because checkpoint inhibitors are mostly ineffective in this
437 context. A phase 1 clinical trial should now be put in place to confirm the efficacy of
438 the GSI–TKI combination in patients. Interestingly, a phase I/II trial in 16 patients with
439 lung adenocarcinoma showed that the TKI erlotinib and GSI RO4929097 (Roche)
440 combination is safe and feasible (50). As the side effects associated with erlotinib are
441 higher than those with osimertinib (47), the osimertinib and GSI combination also might
442 be safe in patients, particularly when using nirogacestat. Indeed, this GSI displays long-
443 term efficacy and is well tolerated by patients (37). Moreover, the present study showed
444 that similarly to DBZ, nirogacestat also overcomes resistance to TKIs in human lung
445 adenocarcinoma cells harboring gatekeeper mutations.

446 MATERIAL AND METHODS

447 Mice

448 Tet-on-EGFR^{T790M/L858R} and CCSP-rtTA mice were described previously (22, 23). For
449 *in vivo* PC9GROR lung adenocarcinoma cell xenograft growth assays, 3.5×10^6 PC9GR
450 or PC9GROR cells were injected subcutaneously in the flank of 6-week-old, female
451 athymic Nude-Foxn1 mice (Envigo). Drug treatments were started when tumors reached
452 the volume of 200 mm³. For Kaplan-Meier analyses, mice were killed when tumors
453 reached the volume of 1200 mm³.

454 Animal procedures were performed according to protocols approved by the
455 French national committee of animal care.

456

457 Western blotting

458 Western blotting was performed as previously described (14). The following antibodies
459 were used for the analysis: anti-N1ICD, -HES1, -NOTCH1, -NOTCH2, -NOTCH3 and
460 -pSTAT3 (Cell Signaling Technology), -STAT3 (BD), and -tubulin (Sigma). Secondary
461 antibodies were horseradish peroxidase-linked anti-rabbit or anti-mouse (Cell Signaling
462 Technology). Tubulin was used as loading control for all blots.

463

464 Treatments in mice

465 Dibenzazepine (DBZ) (Syncom) was administered 4 days per week (3.3 mg/kg/day) by
466 intraperitoneal (IP) injection. Gefitinib and osimertinib (Cliniscience) were
467 administered by gavage 4 days per week (20 mg/kg/day) and 5 days per week (5
468 mg/kg/day), respectively. Antibodies against NOTCH1 (NRR1) and NOTCH3 (NRR3)
469 were administered by IP injection: NRR1 at 5 mg/kg/day every 5 days, and NRR3 at 15
470 mg/kg/day every Monday and Thursday (Genentech).

471

472 **Histopathology and immunohistochemistry**

473 Lung lobes were fixed, embedded in paraffin and stained with hematoxylin and eosin
474 (HE) or used for immunohistochemistry. Tumor area and total lung area were measured
475 using the Image J software. For pathological analysis of HE-stained tissue sections,
476 classical cytological and architectural features (tumor cell invasion and high mitotic
477 rate) were evaluated by our expert pathologist (M.C.). For immunohistochemistry, the
478 following antibodies were used: anti-HES1 (Cell Signaling Technology), -Ki67
479 (Agilent), -pERK (Cell Signaling Technology), and -pAKT (Novus Biologicals). For
480 each tumor, five 10X magnification fields were scored using the Image J software.
481 Signal intensity in murine and human tissue samples was scored as follows: 0 = lowest
482 intensity, and 5 = highest intensity.

483

484 **Cell culture and transfection reagents**

485 PC9GR (resistant to gefitinib), and PC9GROR (resistant to gefitinib and osimertinib)
486 cells were obtained from Yarden's laboratory (18). The control siRNA (non-targeting,
487 *siNT*), and the siRNAs against *HES1* (*siHES1*) and *STAT3* (*siSTAT3*) (Dharmacon) were
488 transfected at 20 nM with Dharmafect1 following the manufacturer's instructions.

489 For western blotting, RNA-seq or ChIP, cells were incubated with DBZ (250
490 nM), gefitinib (1 μ M), osimertinib (250 nM), PD173074 (2 μ M), ruxolitinib (0.25 μ M),
491 or DMSO (vehicle).

492 For the proliferation assays, siRNA-transfected cells were incubated with
493 gefitinib (15 nM) (or DMSO). Then, cells were fixed at various time points and stained
494 with sulforhodamine B (SRB). Absorbance was measured at 560 nm in a microplate
495 reader (Glomax, Promega).

496

497 **RNA sequencing**

498 RNA was sequenced by Fasteris (Switzerland) using Next-Generation DNA sequencing
499 (NGS) based on the Illumina technology. The RNA-seq data were deposited in the
500 National Center for Biotechnology Information Gene Expression Omnibus (accession
501 number GSE117846).

502 Reads were aligned against the Ensembl *Homo sapiens* genome assembly
503 (GRCh38). Read counts were extracted from the STAR output file with HTSeq and
504 only the protein-coding genome features were taken into account in the final count
505 matrix. Sample counts were normalized by summing read counts for each sample
506 ($s_i, i = 1, \dots, 12$), and computing a first factor for each sample
507 $f_i = s_i / \text{median}_{j=1, \dots, 12}(s_j)$. These factors were normalized such that the product of all
508 the normalized factors g_i was equal to 1: $g_i = \frac{f_i}{\sqrt[1/12]{\prod_{j=1, \dots, 12} f_j}}$. Finally, each column
509 (each sample) of the read count matrix was divided by the corresponding g_i . Gene set
510 enrichment analysis (19) was performed using «The Molecular Signatures Database
511 Hallmark Gene Set Collection» (20).

512

513 **Chromatin immunoprecipitation (ChIP)**

514 Chromatin was prepared as described previously (51). The ChIP-Adem-Kit and ChIP
515 DNA Prep Adem-Kit (Ademtech) were used for ChIP and DNA purification,
516 respectively, on an AutoMag robot, according to the manufacturer's instructions. The
517 anti-NOTCH1 antibody was purchased from Abcam and the anti-pSTAT3 antibody
518 from Cell Signaling Technology.

519 The immunoprecipitated DNA was analyzed by PCR using the following
520 primers:

521 Prom*HES1* Fw: GAAGGCAATTTTTCCTTTTTC

522 Prom*HES1* Rev: AAGTTCCCGCTCAGACTTTAC

523

524 **Patient-derived xenograft model**

525 The patient-derived xenograft (PDX) was generated in Paz-Ares's laboratory at the
526 Instituto de Biomedicina de Sevilla (IBIS). The lung adenocarcinoma was classified
527 (TNM) as T2a N1 M0. A tumor piece of 0.5 mm³ was implanted in the right flank of
528 mice, and after two weeks, mice were randomized for treatment.

529

530 **Patients and ethical considerations**

531 Tumors were analyzed from 75 patients with lung adenocarcinoma harboring EGFR
532 mutations and treated with EGFR TKIs at Toulouse University Hospital (52). Four
533 patients were included in the MOSCATO (NCT01566019) or MATCH-R
534 (NCT02517892) clinical trials at the Institut Gustave Roussy, Paris. All patients signed
535 an informed consent form to allow tissue analyses. This study was approved by the
536 Committee for the Protection of Persons of each institution, and by the French National
537 Agency for Medicines and Health Products Safety (ANSM).

538

539 **Statistical analysis**

540 Unless otherwise specified, data are presented as mean \pm S.E.M. One way analysis of
541 variance (ANOVA) was carried out to assess the significance of the different expression
542 levels in IHC, and to determine differences in tumor growth and body weight among
543 animal groups. Correlation of HES1 expression in patient samples and PFS, and

544 Kaplan-Meier survival curves were analyzed with the Gehan–Breslow–Wilcoxon test.
545 Hazard ratios were calculated with the Mantel-Haenszel test. Samples (cells or mice)
546 were allocated to their experimental group according to their pre-determined type (cell
547 type or mouse treatment). Investigators were blinded to the experimental groups for the
548 analysis of data presented in Figures 1C, 1D, 2A, 2B, 2C, 3A, 3B, 4A, 4B, 5D, 7C and
549 7D. For the rest they were not blinded.

550 # $p \leq 0.1$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, **** $p \leq 0.0001$.

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566 L.P., performed some experiments. M.M. M.G. and A.G. performed some *in vivo*
567 treatments. E.F. performed the ChIP experiments. I.F., and L.P-A designed and
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569 experiments on clinical samples. M.C. performed the immunohistochemistry analysis of
570 mouse tumors. H.P.M and E.C. supervised the experiments with STAT3. Y.G. and N.P.
571 performed some IHC experiments. K-K.W. supervised the experiments in
572 *EGFR^{T790M/L858R}* mice. A.T. and J.C. performed the RNA-seq analysis. C.S. supervised
573 the experiments with the anti-NRR1 and NRR3 antibodies. A.M. designed and
574 supervised the study, secured funding, analyzed data, and wrote the manuscript. All
575 authors discussed the results and commented the manuscript.

FIGURES

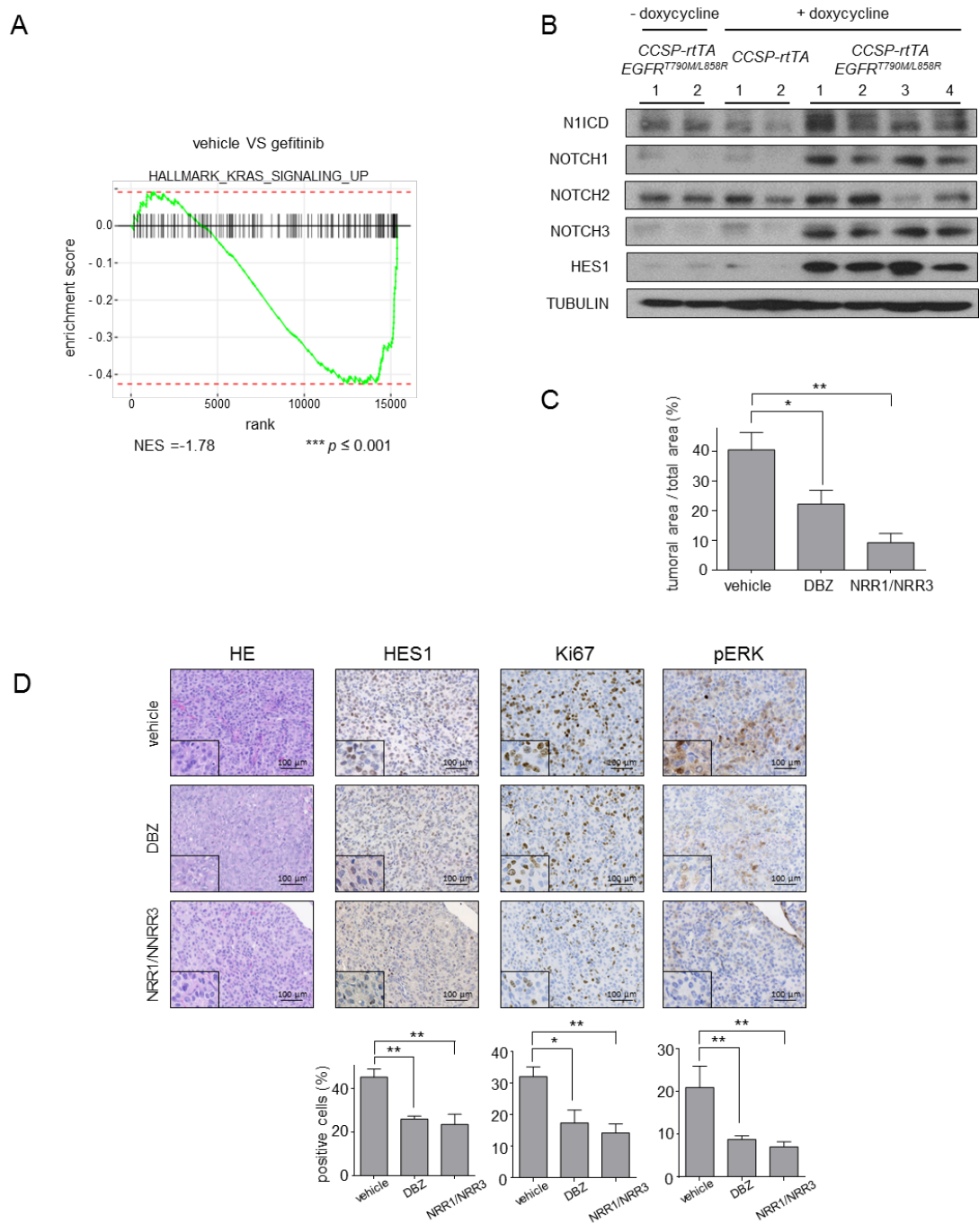


Figure 1. Inhibition of Notch signaling hampers tumor growth in *EGFR*^{T790M/L858R} mice.

(A) PC9GR cells were starved for 18h and then treated with vehicle (DMSO) or gefitinib (1 μ M) for 6h. RNA was extracted from cells and subjected to RNAseq. The KRAS-associated Gene Set was downregulated in PC9GR cells treated with gefitinib

compared with control (n = 3 per condition; FDR < 0.001). **(B)** Immunoblotting of the indicated proteins in lungs from control mice (four lanes on the left) and in mice with *EGFR*^{T790M/L858R}-driven tumors (four lanes on the right). Controls were littermates of *EGFR*^{T790M/L858R} mice without doxycycline induction or *CCSP-rtTA* mice treated with doxycycline. **(C)** The tumor area relative to the total lung area in mice with *EGFR*^{T790M/L858R}-driven tumors treated with methocel and IgG (vehicle; n = 6), with a γ -secretase inhibitor (DBZ; n = 6), or with anti-NOTCH1 and anti-NOTCH3 antibodies (NRR1/NRR3; n = 5) was determined by staining tissue sections with hematoxylin and eosin (H&E). **(D)** H&E and immunohistochemical staining of lung tumor sections from the same mice as in C. The histograms show the staining quantification (percentage of positive cells) in the corresponding sections obtained by analysis of 5 fields (10X) per tumor. Values correspond to the mean \pm SEM; Statistical significance was determined by a one-way ANOVA test: * $p \leq 0.05$, ** $p \leq 0.01$

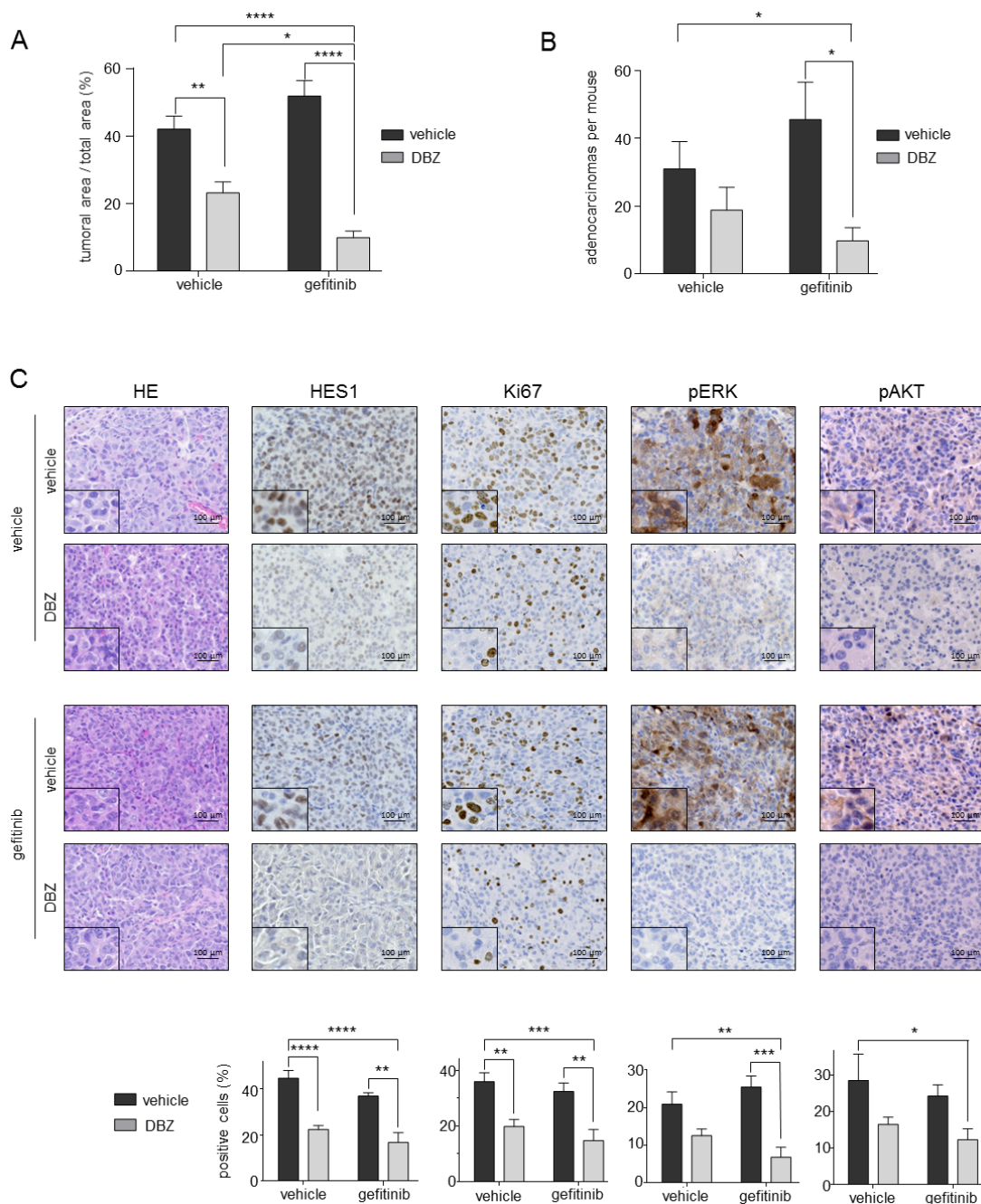


Figure 2. Notch inhibition overcomes resistance to gefitinib in mouse *EGFR* *T790M/L858R*-driven tumors.

(A) The tumor area relative to the total lung area in lung tissue sections from mice with *EGFR*^{T790M/L858R}-driven tumors treated with methocel (vehicle; n = 9), DBZ (n = 10), gefitinib (n = 7), or the DBZ and gefitinib combination (n = 8) was determined by staining tissue sections with H&E. (B) Number of lung adenocarcinomas in the mice

described in A. (C) H&E and immunohistochemical staining of tumors from the mice described in A. The histograms show the percentage of positive cells in the corresponding sections obtained by analysis of 5 fields (10X) per tumor. Values correspond to the mean \pm SEM. Statistical significance was determined by a one-way ANOVA test: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$. In panel 2A, the comparison between gefitinib and DBZ single treatments was significant (****). In panel 2C, HES1 and pERK expression levels in tumors after treatment with gefitinib alone and DBZ alone also were significantly different (* and **, respectively).

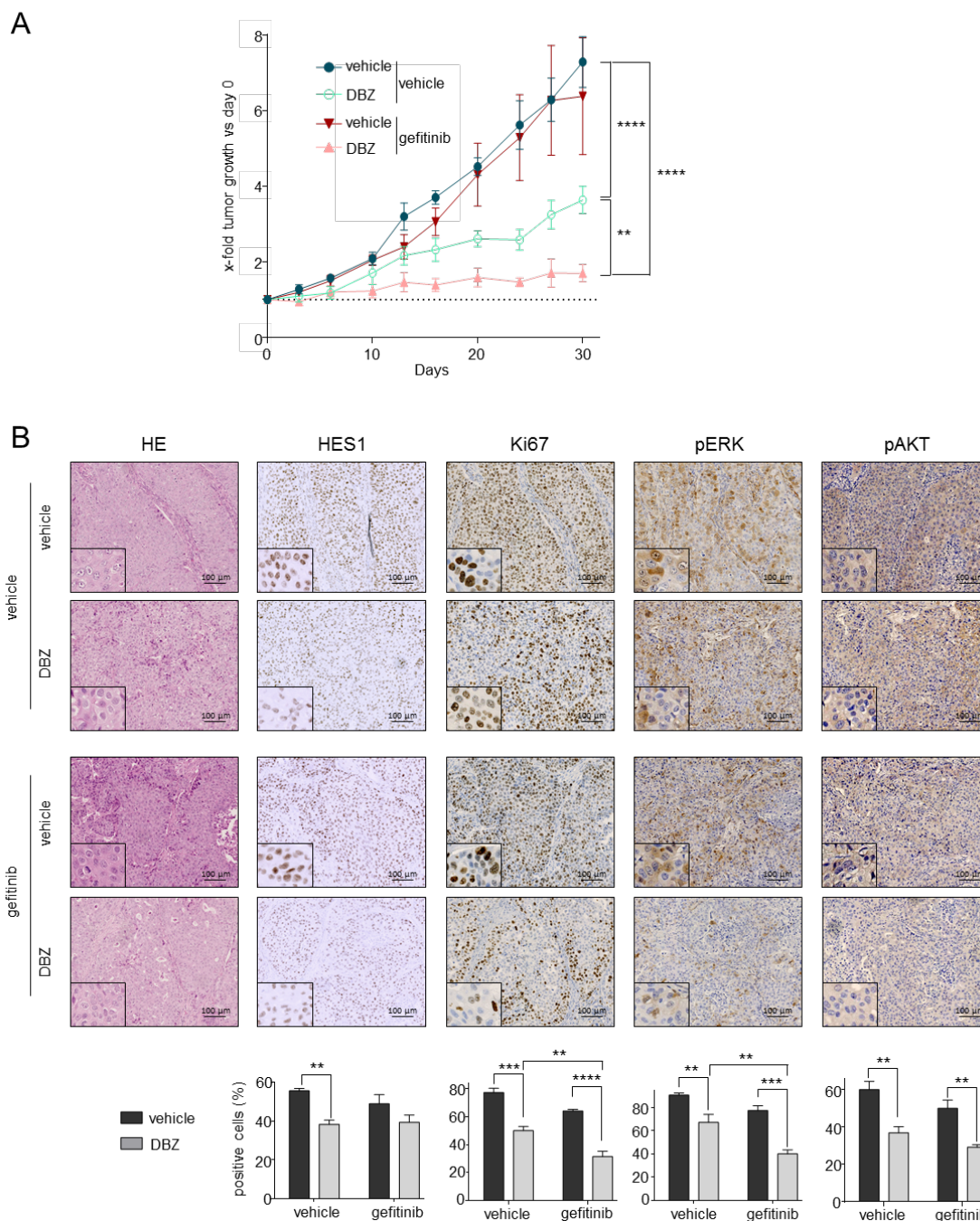


Figure 3. Notch inhibition overcomes resistance to gefitinib in human *EGFR*^{T790M/L858R}-driven lung adenocarcinoma.

(A) Growth of *EGFR*^{T790M/L858R} lung adenocarcinoma PDX implanted in the right flank of nude mice. When tumors were approximately 100 mm³ in volume, mice were treated with vehicle (methocel, n = 5), DBZ (n = 5), gefitinib (n = 4), or the DBZ and gefitinib combination (n = 5). The y-axis shows the fold-increase in tumor size versus day 0. (B)

H&E and immunohistochemical staining of tumor sections from the mice described in A. The histograms show the percentage of positive cells in the corresponding sections. For each treatment, 5 fields (10X) per mouse were analyzed. Values correspond to the mean \pm SEM. Statistical significance was determined by two-way ANOVA in A, and one-way ANOVA in B: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$. In panel 3A, the comparisons between gefitinib and DBZ single treatments and between gefitinib and the combination were also significant (*** and ****, respectively). In panel 3B, the comparison between vehicle and DBZ was also significant for all analyses (** for HES1 and **** for Ki67, pERK and pAKT), as well as the comparison between gefitinib and DBZ for Ki67 expression (*).

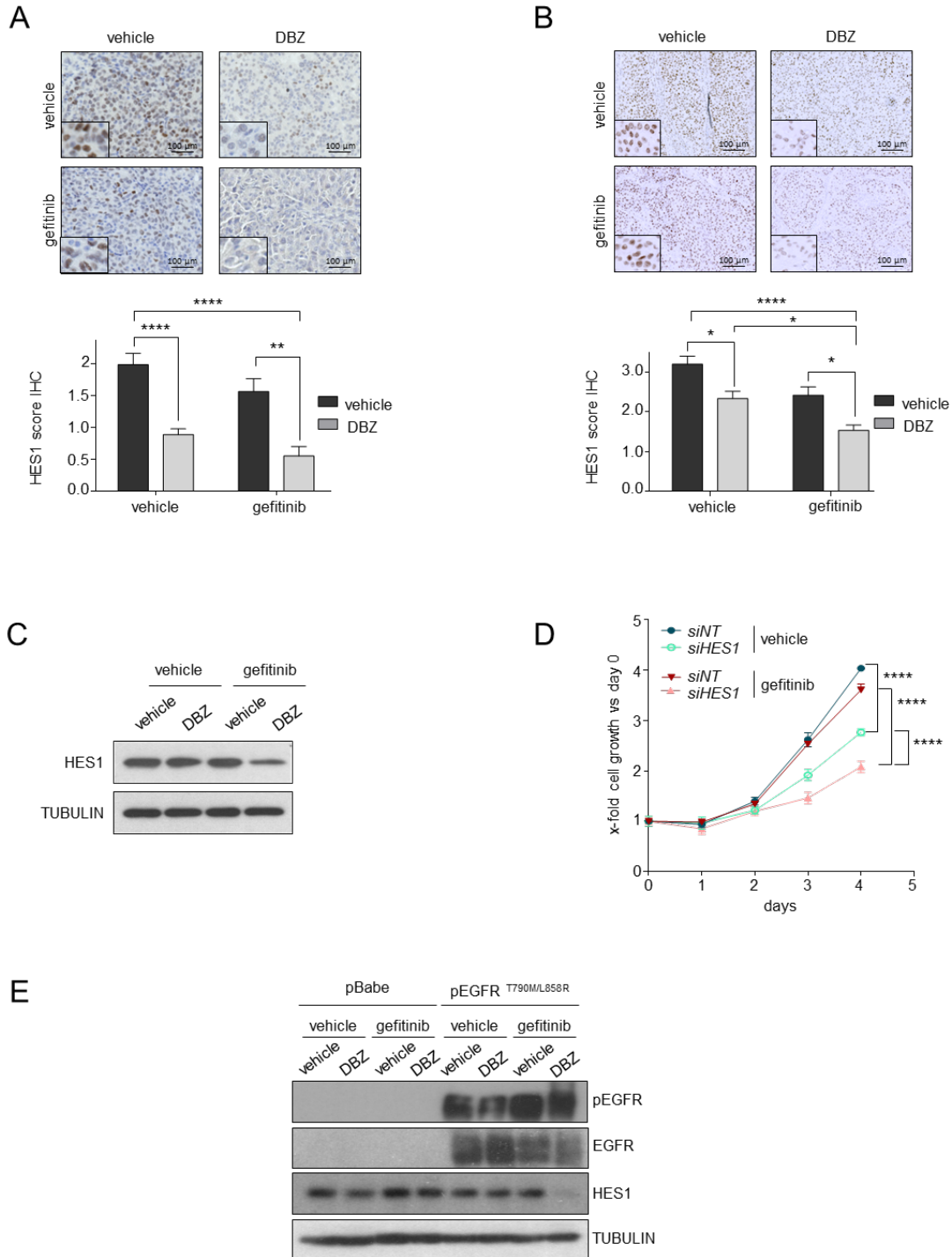


Figure 4. Combining EGFR TKIs and Notch inhibitors synergistically decreases HES1 expression.

(A) Immunohistochemical analysis of HES1 expression in tumors from *EGFR*^{T790M/L858R} mice treated with methocel (vehicle; n = 9), DBZ (n = 10), gefitinib (n = 7), or the DBZ and gefitinib combination (n = 8). The histograms show the quantification of HES1

signal intensity by analysis of 5 tumor fields (10X) per mouse. **(B)** Immunohistochemical analysis of HES1 expression in tumors from *EGFR*^{T790M/L858R} PDX implanted in nude mice treated with vehicle (methocel, n = 5), DBZ (n = 5), gefitinib (n = 4), or the combination (n = 5). **(C)** Immunoblotting of the indicated proteins in PC9GR cells treated with vehicle (DMSO), DBZ (250 nM) or/and gefitinib (1 μ M). **(D)** Proliferation of PC9GR cells transfected with a non-targeting siRNA (*siNT*) or against *HES1* (*siHES1*) and treated with vehicle (DMSO) or gefitinib (15 nM) for 72 h. Data are the mean \pm SEM (n = 3 in all groups). **(E)** Immunoblotting of the indicated proteins in control (pBabe vector) and in *EGFR*^{T790M/L858R} protein expressing CHO cells treated with vehicle (DMSO), DBZ (250 nM) and/or gefitinib (1 μ M). This is a representative image of two different experiments.

Values correspond to the mean \pm SEM. Statistical significance was evaluated with the one-way ANOVA test in A and B, and the two-way ANOVA test in D: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$. In panel 4A, the comparison between gefitinib alone and DBZ alone was also significant (*). In panel 4B, the comparison between vehicle and gefitinib was also significant (*). In panel 4D, the comparisons between *siNT*/vehicle and *siNT*/gefitinib, between *siNT*/vehicle and *siHES1*/gefitinib, and between *siNT*/gefitinib and *siHES1*/gefitinib also were significant (**, ****, and ****, respectively).

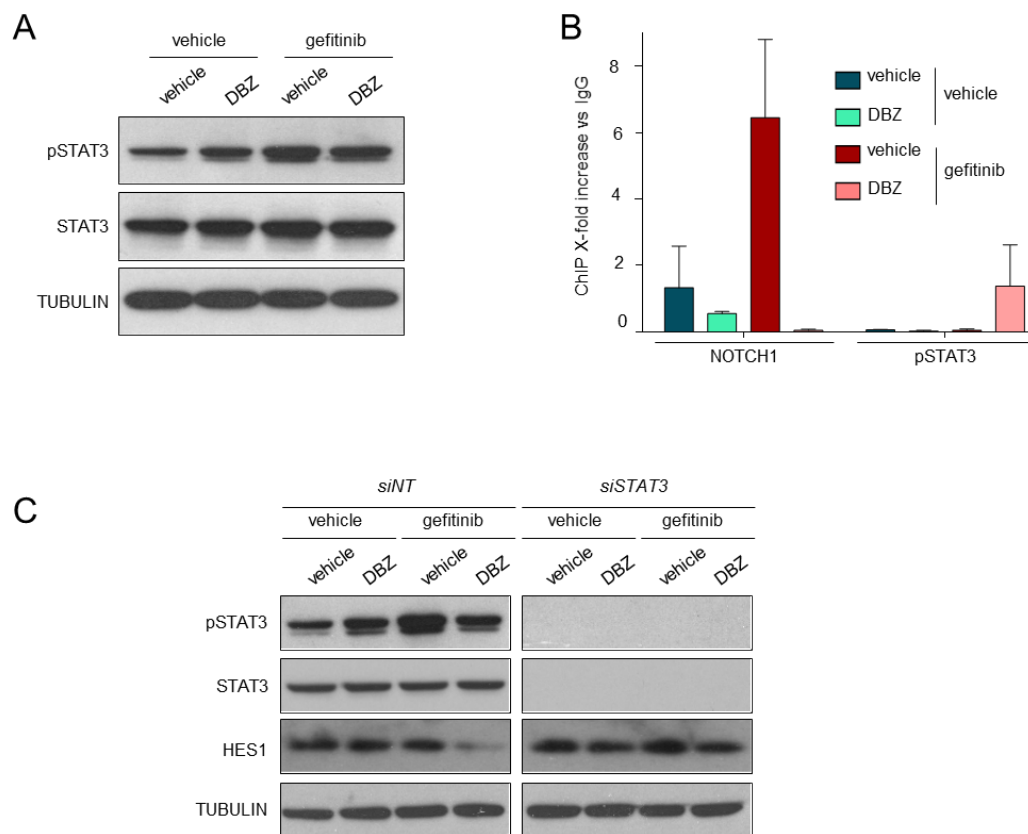


Figure 5. pSTAT3 directly binds to the *HES1* promoter and inhibits its expression.

(A) Immunoblotting of the indicated proteins in PC9GR cells treated with vehicle (DMSO), DBZ (250 nM) and/or gefitinib (1 μ M). (B) ChIP analysis of NOTCH1 and pSTAT3 binding to the *HES1* promoter in PC9GR cells treated as in A. (C) Immunoblotting of the indicated proteins in PC9GR cells transfected with a non-targeting siRNA (*siNT*) or against *STAT3* (*siSTAT3*) and treated with vehicle (DMSO), DBZ (250 nM) and/or gefitinib (1 μ M).

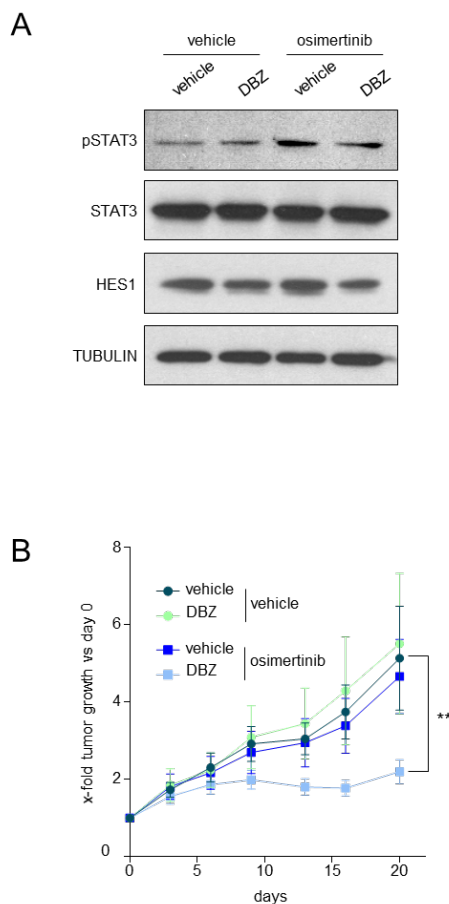


Figure 6. Notch inhibition overcomes resistance to osimertinib in *EGFR*^{C797S} human lung adenocarcinoma cells.

(A) Immunoblotting of the indicated proteins in PC9GROR cells treated with vehicle (DMSO), DBZ (250 nM) and/or osimertinib (250 nM). (B) Nude mice xenografted with PC9GROR cells (subcutaneous injection in the right flank) were treated with vehicle (methocel, n = 8), DBZ (n = 8), osimertinib (n = 8), or the DBZ and osimertinib combination (n = 7). The y-axis shows the tumor growth fold increase versus day 0. Values correspond to the mean \pm SEM. Statistical significance was determined by two-

way ANOVA test: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$. In panel 6B, the comparisons between DBZ alone and the combination, and between osimertinib and the combination also were significant (** and *, respectively).

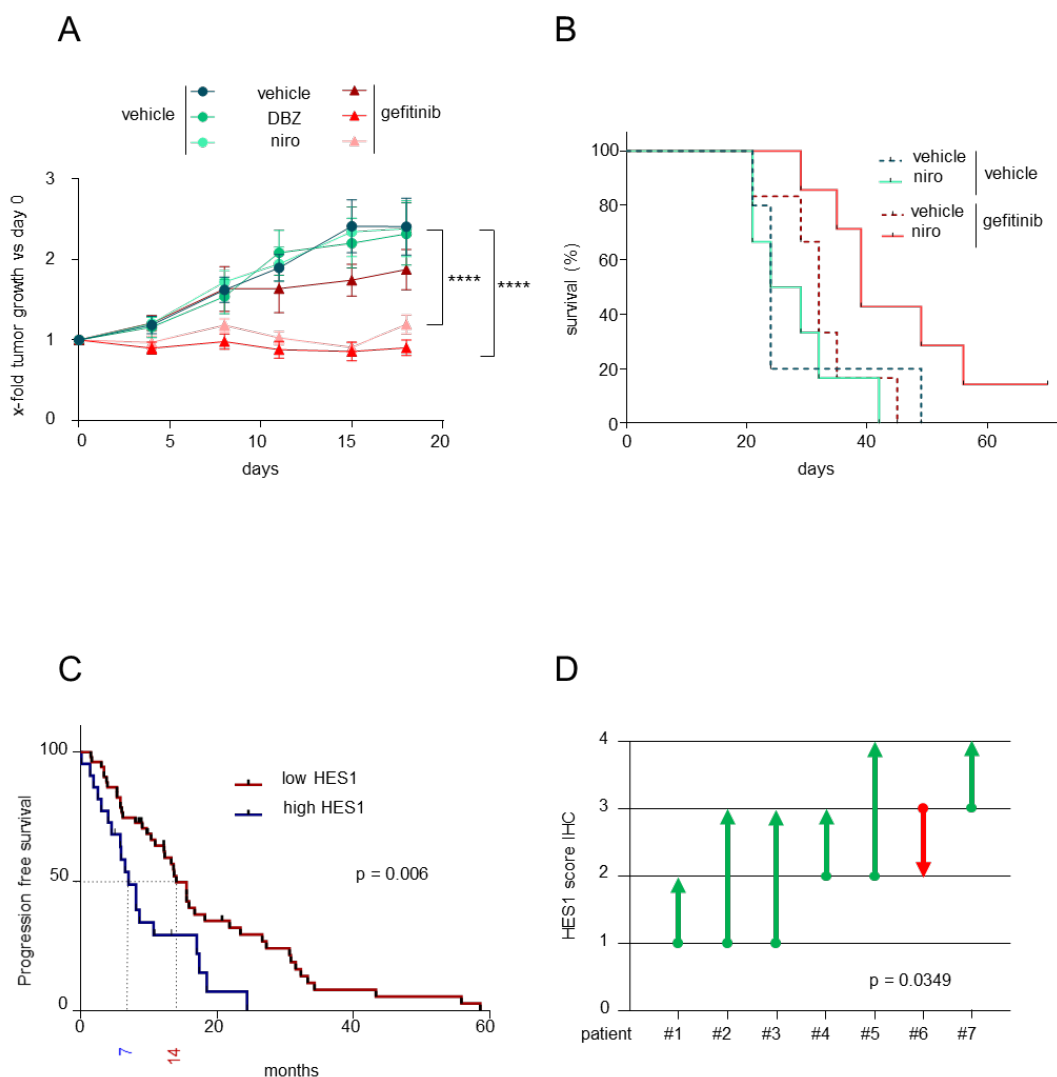


Figure 7. High HES1 protein levels correlate with poor progression-free survival and relapse in patients with EGFR-mutated lung adenocarcinoma treated with TKIs.

(A) Nude mice were xenografted with PC9GR cells and then treated with vehicle (methocel, n = 6), DBZ (n = 6), nirogacestat (n = 6), gefitinib (n = 6), the DBZ and gefitinib combination (n = 7), or the nirogacestat and gefitinib combination (n = 7). The y-axis shows the tumor growth fold increase versus day 0. Values correspond to the mean ± SEM. Statistical significance was determined by a two-way ANOVA test: * p ≤

0.05, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$. The comparisons between DBZ and the DBZ-gefitinib combination and between DBZ and the nirogacestat-gefitinib combination also were significant (**** and ***, respectively), as well as the comparison between nirogacestat and the DBZ-gefitinib or the nirogacestat-gefitinib combination (**** and ****, respectively) and the comparison between gefitinib and the DBZ-gefitinib combination (**). **(B)** Nude mice were xenografted with PC9GR cells and then treated with vehicle (methocel, $n = 5$), nirogacestat ($n = 6$), gefitinib ($n = 6$), or the nirogacestat+gefitinib combination. Statistical significance was determined by Gehan–Breslow–Wilcoxon test: vehicle *vs* gefitinib ($p = 0.3$), vehicle *vs* nirogacestat ($p = 0.93$), vehicle *vs* the combination ($p = 0.021$), gefitinib *vs* the combination ($p = 0.05$), and nirogacestat *vs* the combination ($p = 0.02$). **(C)** Progression-free survival of EGFR TKI-treated patients with *EGFR*-mutated lung adenocarcinoma ($n = 75$) classified according to the tumor HES1 expression assessed by IHC staining (low HES1 = 0–2.50 HES1 score; high HES1 = 2.51–5.00 HES1 score). Statistical significance was determined with the Gehan–Breslow–Wilcoxon test. **(D)** Representation of HES1 staining intensity score change in tumor samples from seven patients with *EGFR*-mutated lung adenocarcinoma before TKI treatment (dot) and after relapse (arrowhead). Statistical significance was determined with the paired two-tailed Student's *t* test.

REFERENCES

1. Herbst RS, Heymach JV, and Lippman SM. Lung cancer. *N Engl J Med*. 2008;359(13):1367-80.
2. Rosell R, Bivona TG, and Karachaliou N. Genetics and biomarkers in personalisation of lung cancer treatment. *Lancet*. 2013;382(9893):720-31.
3. Reck M, Heigener DF, Mok T, Soria JC, and Rabe KF. Management of non-small-cell lung cancer: recent developments. *Lancet*. 2013;382(9893):709-19.
4. Tan CS, Gilligan D, and Pacey S. Treatment approaches for EGFR-inhibitor-resistant patients with non-small-cell lung cancer. *Lancet Oncol*. 2015;16(9):e447-59.
5. Janne PA, Yang JC, Kim DW, Planchard D, Ohe Y, Ramalingam SS, et al. AZD9291 in EGFR inhibitor-resistant non-small-cell lung cancer. *N Engl J Med*. 2015;372(18):1689-99.
6. Thress KS, Paweletz CP, Felip E, Cho BC, Stetson D, Dougherty B, et al. Acquired EGFR C797S mutation mediates resistance to AZD9291 in non-small cell lung cancer harboring EGFR T790M. *Nat Med*. 2015;21(6):560-2.
7. Le X, Puri S, Negrao MV, Nilsson M, Robichaux JP, Boyle TA, et al. Landscape of EGFR -dependent and -independent resistance mechanisms to osimertinib and continuation therapy post-progression in EGFR-mutant NSCLC. *Clin Cancer Res*. 2018.
8. Yun CH, Mengwasser KE, Toms AV, Woo MS, Greulich H, Wong KK, et al. The T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP. *Proc Natl Acad Sci U S A*. 2008;105(6):2070-5.
9. Yoshikawa S, Kukimoto-Niino M, Parker L, Handa N, Terada T, Fujimoto T, et al. Structural basis for the altered drug sensitivities of non-small cell lung cancer-associated mutants of human epidermal growth factor receptor. *Oncogene*. 2013;32(1):27-38.
10. Bray SJ. Notch signalling in context. *Nat Rev Mol Cell Biol*. 2016;17(11):722-35.
11. Takebe N, Miele L, Harris PJ, Jeong W, Bando H, Kahn M, et al. Targeting Notch, Hedgehog, and Wnt pathways in cancer stem cells: clinical update. *Nat Rev Clin Oncol*. 2015;12(8):445-64.
12. Licciulli S, Avila JL, Hanlon L, Troutman S, Cesaroni M, Kota S, et al. Notch1 is required for Kras-induced lung adenocarcinoma and controls tumor cell survival via p53. *Cancer Res*. 2013;73(19):5974-84.
13. Baumgart A, Mazur PK, Anton M, Rudelius M, Schwamborn K, Feuchtinger A, et al. Opposing role of Notch1 and Notch2 in a Kras-driven murine non-small cell lung cancer model. *Oncogene*. 2014.
14. Maraver A, Fernandez-Marcos PJ, Herranz D, Canamero M, Munoz-Martin M, Gomez-Lopez G, et al. Therapeutic Effect of gamma-Secretase Inhibition in Kras(G12V)-Driven Non-Small Cell Lung Carcinoma by Derepression of DUSP1 and Inhibition of ERK. *Cancer Cell*. 2012;22(2):222-34.
15. Konishi J, Yi F, Chen X, Vo H, Carbone DP, and Dang TP. Notch3 cooperates with the EGFR pathway to modulate apoptosis through the induction of bim. *Oncogene*. 2010;29(4):589-96.
16. Arasada RR, Amann JM, Rahman MA, Huppert SS, and Carbone DP. EGFR blockade enriches for lung cancer stem-like cells through Notch3-dependent signaling. *Cancer Res*. 2014;74(19):5572-84.

17. Hu S, Fu W, Li T, Yuan Q, Wang F, Lv G, et al. Antagonism of EGFR and Notch limits resistance to EGFR inhibitors and radiation by decreasing tumor-initiating cell frequency. *Sci Transl Med.* 2017;9(380).
18. Mancini M, Gal H, Gaborit N, Mazzeo L, Romaniello D, Salame TM, et al. An oligoclonal antibody durably overcomes resistance of lung cancer to third-generation EGFR inhibitors. *EMBO Mol Med.* 2018;10(2):294-308.
19. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, et al. Gene set enrichment analysis: A knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences.* 2005;102(43):15545-50.
20. Liberzon A, Birger C, Thorvaldsdottir H, Ghandi M, Mesirov JP, and Tamayo P. The Molecular Signatures Database (MSigDB) hallmark gene set collection. *Cell Syst.* 2015;1(6):417-25.
21. Arteaga CL, and Engelman JA. ERBB receptors: from oncogene discovery to basic science to mechanism-based cancer therapeutics. *Cancer Cell.* 2014;25(3):282-303.
22. Li D, Shimamura T, Ji H, Chen L, Haringsma HJ, McNamara K, et al. Bronchial and peripheral murine lung carcinomas induced by T790M-L858R mutant EGFR respond to HKI-272 and rapamycin combination therapy. *Cancer Cell.* 2007;12(1):81-93.
23. Tichelaar JW, Lu W, and Whitsett JA. Conditional expression of fibroblast growth factor-7 in the developing and mature lung. *J Biol Chem.* 2000;275(16):11858-64.
24. Zheng Y, de la Cruz CC, Sayles LC, Alleyne-Chin C, Vaka D, Knaak TD, et al. A rare population of CD24(+)ITGB4(+)Notch(hi) cells drives tumor propagation in NSCLC and requires Notch3 for self-renewal. *Cancer Cell.* 2013;24(1):59-74.
25. Choy L, Hagenbeek TJ, Solon M, French D, Finkle D, Shelton A, et al. Constitutive NOTCH3 Signaling Promotes the Growth of Basal Breast Cancers. *Cancer Res.* 2017;77(6):1439-52.
26. Rivera-Torres J, Guzman-Martinez G, Villa-Bellosta R, Orbe J, Gonzalez-Gomez C, Serrano M, et al. Targeting gamma-secretases protect against angiotensin II-induced cardiac hypertrophy. *J Hypertens.* 2015;33(4):843-50.
27. Wu Y, Cain-Hom C, Choy L, Hagenbeek TJ, de Leon GP, Chen Y, et al. Therapeutic antibody targeting of individual Notch receptors. *Nature.* 2010;464(7291):1052-7.
28. van Es JH, van Gijn ME, Riccio O, van den Born M, Vooijs M, Begthel H, et al. Notch/gamma-secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells. *Nature.* 2005;435(7044):959-63.
29. Day CP, Merlino G, and Van Dyke T. Preclinical mouse cancer models: a maze of opportunities and challenges. *Cell.* 2015;163(1):39-53.
30. Maraver A, and Serrano M. Notching up a new therapeutic strategy for Non-Small Cell Lung Carcinoma (NSCLC). *Oncotarget.* 2012.
31. Palomero T, Sulis ML, Cortina M, Real PJ, Barnes K, Ciofani M, et al. Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. *Nat Med.* 2007;13(10):1203-10.
32. Lo HW, Hsu SC, Ali-Seyed M, Gunduz M, Xia W, Wei Y, et al. Nuclear interaction of EGFR and STAT3 in the activation of the iNOS/NO pathway. *Cancer Cell.* 2005;7(6):575-89.

33. Kim SM, Kwon OJ, Hong YK, Kim JH, Solca F, Ha SJ, et al. Activation of IL-6R/JAK1/STAT3 signaling induces de novo resistance to irreversible EGFR inhibitors in non-small cell lung cancer with T790M resistance mutation. *Mol Cancer Ther.* 2012;11(10):2254-64.
34. Lee HJ, Zhuang G, Cao Y, Du P, Kim HJ, and Settleman J. Drug resistance via feedback activation of Stat3 in oncogene-addicted cancer cells. *Cancer Cell.* 2014;26(2):207-21.
35. Codony-Servat C, Codony-Servat J, Karachaliou N, Molina MA, Chaib I, Ramirez JL, et al. Activation of signal transducer and activator of transcription 3 (STAT3) signaling in EGFR mutant non-small-cell lung cancer (NSCLC). *Oncotarget.* 2017;8(29):47305-16.
36. Darnell JE, Jr. STATs and gene regulation. *Science.* 1997;277(5332):1630-5.
37. Kummar S, O'Sullivan Coyne G, Do KT, Turkbey B, Meltzer PS, Polley E, et al. Clinical Activity of the gamma-Secretase Inhibitor PF-03084014 in Adults With Desmoid Tumors (Aggressive Fibromatosis). *J Clin Oncol.* 2017;35(14):1561-9.
38. Schnell SA, Ambesi-Impiombato A, Sanchez-Martin M, Belver L, Xu L, Qin Y, et al. Therapeutic targeting of HES1 transcriptional programs in T-ALL. *Blood.* 2015;125(18):2806-14.
39. Gong Y, Somwar R, Politi K, Balak M, Chmielecki J, Jiang X, et al. Induction of BIM Is Essential for Apoptosis Triggered by EGFR Kinase Inhibitors in Mutant EGFR-Dependent Lung Adenocarcinomas. *PLOS Medicine.* 2007;4(10):e294.
40. Costa DB, Halmos B, Kumar A, Schurer ST, Huberman MS, Boggon TJ, et al. BIM Mediates EGFR Tyrosine Kinase Inhibitor-Induced Apoptosis in Lung Cancers with Oncogenic EGFR Mutations. *PLOS Medicine.* 2007;4(10):e315.
41. Cragg MS, Kuroda J, Puthalakath H, Huang DCS, and Strasser A. Gefitinib-Induced Killing of NSCLC Cell Lines Expressing Mutant EGFR Requires BIM and Can Be Enhanced by BH3 Mimetics. *PLOS Medicine.* 2007;4(10):e316.
42. Zhang H, Hu H, Greeley N, Jin J, Matthews AJ, Ohashi E, et al. STAT3 restrains RANK- and TLR4-mediated signalling by suppressing expression of the E2 ubiquitin-conjugating enzyme Ubc13. *Nat Commun.* 2014;5:5798.
43. Pencik J, Schleder M, Gruber W, Unger C, Walker SM, Chalaris A, et al. STAT3 regulated ARF expression suppresses prostate cancer metastasis. *Nat Commun.* 2015;6:7736.
44. Peixoto P, Blomme A, Costanza B, Ronca R, Rezzola S, Palacios AP, et al. HDAC7 inhibition resets STAT3 tumorigenic activity in human glioblastoma independently of EGFR and PTEN: new opportunities for selected targeted therapies. *Oncogene.* 2016;35(34):4481-94.
45. Grabner B, Schramek D, Mueller KM, Moll HP, Svinka J, Hoffmann T, et al. Disruption of STAT3 signalling promotes KRAS-induced lung tumorigenesis. *Nat Commun.* 2015;6:6285.
46. Johnson DE, O'Keefe RA, and Grandis JR. Targeting the IL-6/JAK/STAT3 signalling axis in cancer. *Nat Rev Clin Oncol.* 2018;15(4):234-48.
47. Soria JC, Ohe Y, Vansteenkiste J, Reungwetwattana T, Chewaskulyong B, Lee KH, et al. Osimertinib in Untreated EGFR-Mutated Advanced Non-Small-Cell Lung Cancer. *N Engl J Med.* 2018;378(2):113-25.
48. Sharma SV, Lee DY, Li B, Quinlan MP, Takahashi F, Maheswaran S, et al. A chromatin-mediated reversible drug-tolerant state in cancer cell subpopulations. *Cell.* 2010;141(1):69-80.

49. Codony-Servat J, Codony-Servat C, Cardona AF, Gimenez-Capitan A, Drozdowskyj A, Berenguer J, et al. Cancer Stem Cell Biomarkers in EGFR-Mutation-Positive Non-Small-Cell Lung Cancer. *Clin Lung Cancer*. 2019;20(3):167-77.
50. Gold KA, Byers LA, Fan YH, Fujimoto J, Tse WH, Lee JJ, et al. A phase I/II trial combining erlotinib with gamma secretase inhibitor RO4929097 in advanced non-small cell lung cancer (NSCLC). *Journal of Clinical Oncology*. 2013;31(15_suppl):8104-.
51. Fabbrizio E, El Messaoudi S, Polanowska J, Paul C, Cook JR, Lee JH, et al. Negative regulation of transcription by the type II arginine methyltransferase PRMT5. *EMBO Rep*. 2002;3(7):641-5.
52. Calvayrac O, Mazieres J, Figarol S, Marty-Detraves C, Raymond-Letron I, Bousquet E, et al. The RAS-related GTPase RHOB confers resistance to EGFR-tyrosine kinase inhibitors in non-small-cell lung cancer via an AKT-dependent mechanism. *EMBO Mol Med*. 2017;9(2):238-50.