Supplemental Materials

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

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pathway	pval	padj	ES	NES	nMoreExtrer	size
HALLMARK_MYC_TARGETS_V2	3.66568914956012e-05	0.000366568914956012	-0.571841185842812	-2.03052920663009	1	58
HALLMARK_TNFA_SIGNALING_VIA_NFKB	1.76211453744493e-05	0.000222190621778236	-0.454736566115785	-1.94272679216512	0	191
HALLMARK_INFLAMMATORY_RESPONSE	1.77374153038419e-05	0.000222190621778236	-0.433177734599041	-1.81281063881849	0	163
HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	1.77386738567425e-05	0.000222190621778236	-0.423535999716905	-1.77713182793385	0	166
HALLMARK_KRAS_SIGNALING_UP	1.77752497422589e-05	0.000222190621778236	-0.425862435413622	-1.7737890280657	0	157
HALLMARK_UV_RESPONSE_DN	0.000304092730394963	0.00253410608662469	-0.407250154110172	-1.66584002362953	16	137
HALLMARK_INTERFERON_ALPHA_RESPONSE	0.00341296928327645	0.0170648464163823	-0.41529577496119	-1.59216161787522	187	90
HALLMARK_GLYCOLYSIS	0.000951592154651347	0.00594745096657092	-0.362629891577758	-1.54595052700103	53	188
HALLMARK_KRAS_SIGNALING_DN	0.00258403789922252	0.0143557661067918	0.373475170892941	1.55131646149907	113	132

Supplemental Table 1. Gene sets altered in PC9GR cells upon incubation with gefitinib.
PC9GR cells were serum-starved for 18h and then treated with vehicle (DMSO) or gefitinib
(1 μM) for 6h. RNA was extracted and analyzed by RNA-seq (n = 3 per condition; FDR <
0.001).
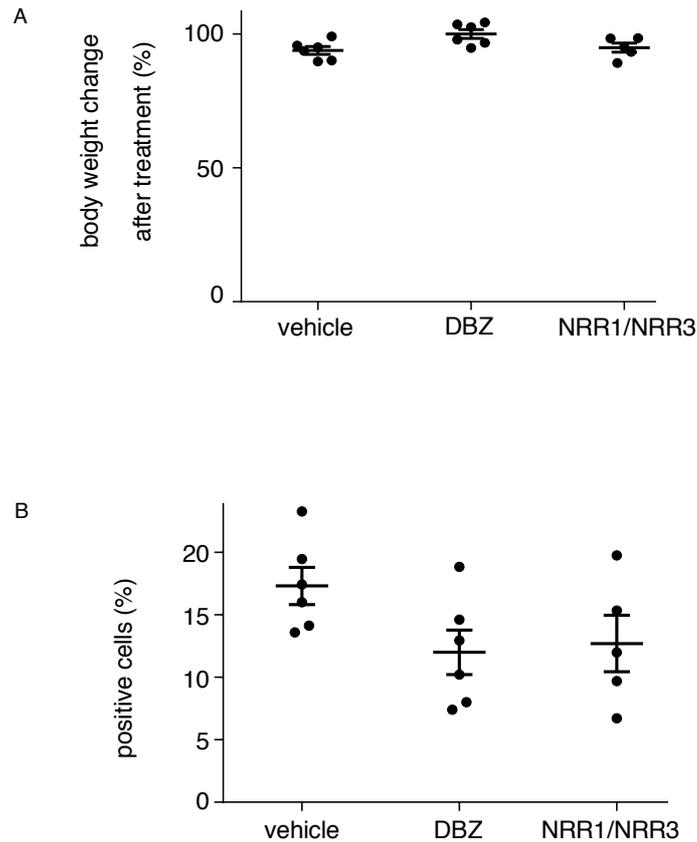
Supplemental Figure 1. Treatment schedules do not affect the weight of animals, and AKT phosphorylation status is not altered by Notch inhibition.

(**A**). Treatment with DBZ and the NRR1/NRR3 antibodies does not affect the weight of mice compared with controls. Animals treated with vehicle (methocel and IgG, n = 6), DBZ (GSI, n = 6), or NRR1/NRR3 (anti-NOTCH1 and anti-NOTCH3 antibodies, n = 5) were weighted the first and last day of treatment and the change in body weight is shown. (**B**) Immunohistochemical analysis of pAKT in lung tumors from mice treated with vehicle (methocel and IgG, n = 6), DBZ (GSI, n = 6), or NRR1/NRR3 (anti-NOTCH1 and anti-NOTCH3 antibodies, n = 5). In **A** and **B** values correspond to the mean ± S.E.M. Statistical significance was determined by one-way ANOVA test followed by Tukey's post hoc test, but results were not significantly different.
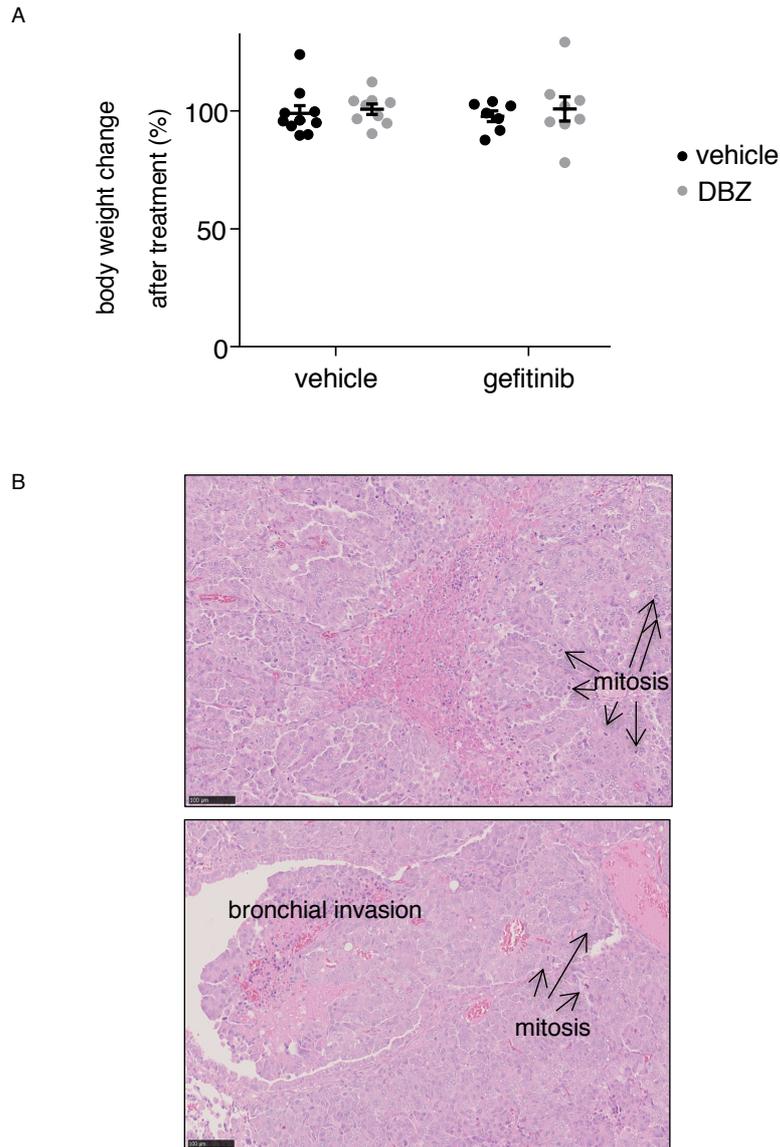
Supplemental Figure 2. Treatment schedules of DBZ and/or gefitinib do not alter the weight of animals, and examples of lung adenocarcinomas in *EGFR^{T790M/L858R}* mice.

(**A**) Effect of the DBZ and gefitinib treatments on the weight of mice. Mice treated with vehicle (methocel, n = 9), DBZ (n = 10), gefitinib (n = 7), and DBZ+gefitinib (n = 8) for 5 weeks were weighted the first and last day of treatment, and the change in body weight is shown. Statistical significance was determined by one-way ANOVA test followed by Tukey's post hoc test, but results were not significantly different. (**B**) Representative images of lung adenocarcinoma in *EGFR^{T790M/L858R}* mice. After H&E staining, lung adenocarcinomas were identified and quantified. Here are two examples of lung adenocarcinomas with classic citoarchitectural histological features, such as invasion and high mitotic rate. Black arrows indicate some mitosis.
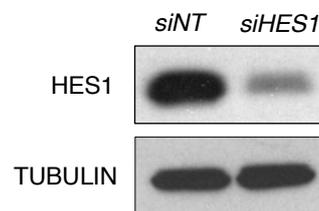
Supplemental Figure 3. *siHES1* **treatment reduces HES1 levels.**

 PC9GR cells were transfected with 20 nM of non-targeting siRNA (*siNT*) or a siRNA targeting *HES1* (*siHES1*). 48h after transfection, proteins were extracted and analyzed by western blotting with antibodies against the indicated proteins. This is a representative image of three different experiments.
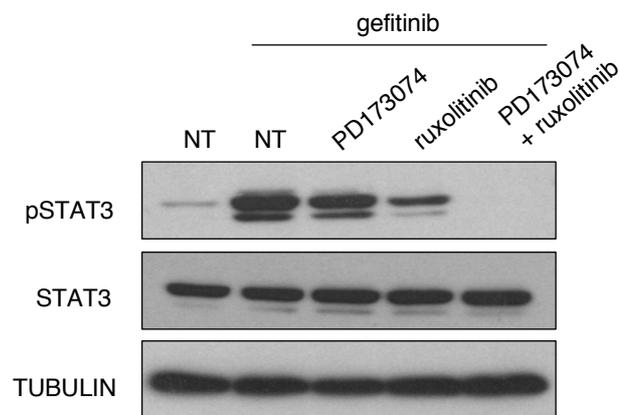
Supplemental Figure 4. Gefitinib induces STAT3 activation through FGFR and JAK.
PC9GR cells were treated with PD173074 (2μM) or/and ruxolitinib (0.25 μM) for 24h. Gefitinib was added (1μM) for the last 6h. Then, proteins were extracted and analyzed by western blotting with antibodies against the indicated proteins. This is a representative image of two different experiments.

A HUMAN HES1 PROMOTER

acgccggccggctgatgtcaaactgcagctcggctggtgtagctcttaaa
gggcccgcgggcgccgggggccgaggccgccccgcggggcgaggagggaga
ggctgcttccttgcccgccacctcaacgcgccgcagaacctaaagcctac
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AAATTCTCTAGAGATAAAAAAAAAAAAAAAAAGGAAAATGCCAGCTGATAT

RBPJ
STAT3
ATG CDS

B MURINE HES1 PROMOTER

caggcgcgccctcgccggccggctgatgtcaaactgcagctcggctggtg
gagctcttaaagggctcgcgcgcggggcgccgaggccgccttggccgccc
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GGGGACTGAGAAGAAAAAAAAAAAAAAAAACGAAAATGCCAGCTGATATAA

Supplemental Figure 5. Human *HES1* and murine *Hes1* promoter sequences.

(**A**) Murine *Hes1* and (**B**) human *HES1* promoter sequences. The consensus binding sites for RBPJ and STAT3 are highlighted in red and light blue, respectively. Dark blue letters indicate where the coding sequences (CDS) of both genes start.
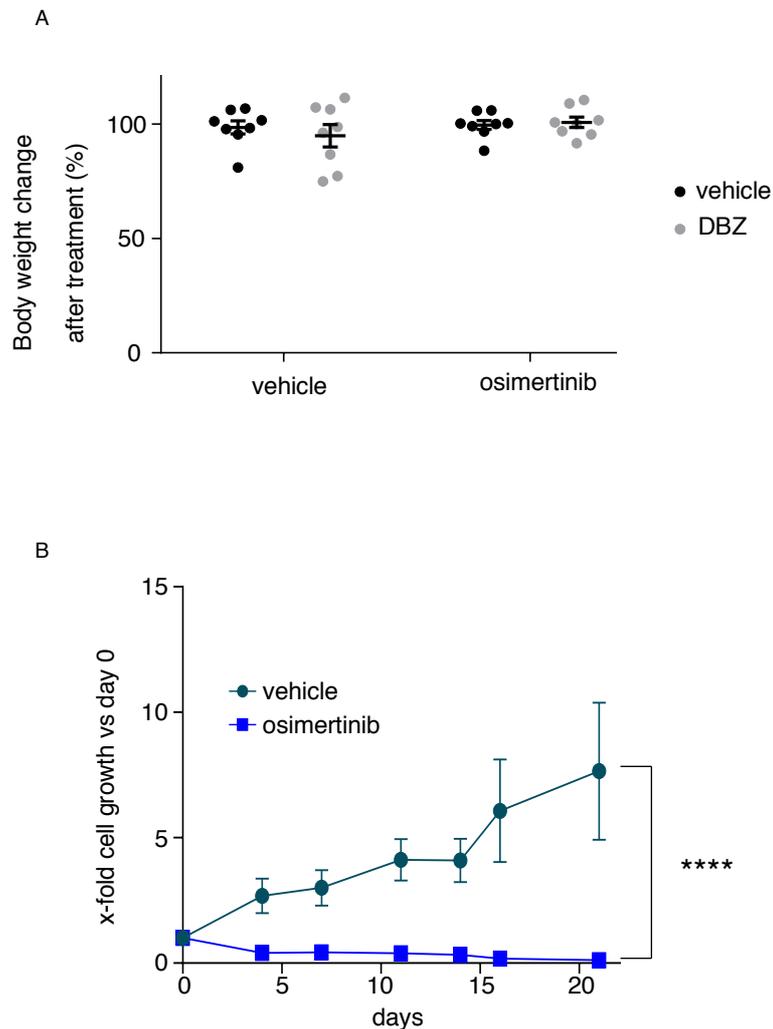
Supplemental Figure 6. Treatment schedules of DBZ and/or osimertinib do not alter the weight of animals.

(A) Mice treated with vehicle (methocel, n = 8), DBZ (n = 8), osimertinib (n = 8), or DBZ/osimertinib (n = 7) for 5 weeks were weighted the first and last day of treatment, and the change in body weight is shown. Values correspond to the mean ± S.E.M. Statistical significance was determined by one-way ANOVA test followed by Tukey's post hoc test, but results were not significantly different. (B) 3.5 x 10^6 PC9GR cells were injected subcutaneously in the right flank of nude mice. Mice were treated with vehicle (methocel, n = 8), or osimertinib (n = 8). The tumor size was measured twice per week. The y-axis shows the tumor growth fold increase versus day 0 in each treatment and time point. Values correspond to the mean ± SEM. Statistical significance was determined by a two-way ANOVA test followed by Tukey's post hoc test: **** $p \leq 0.0001$.
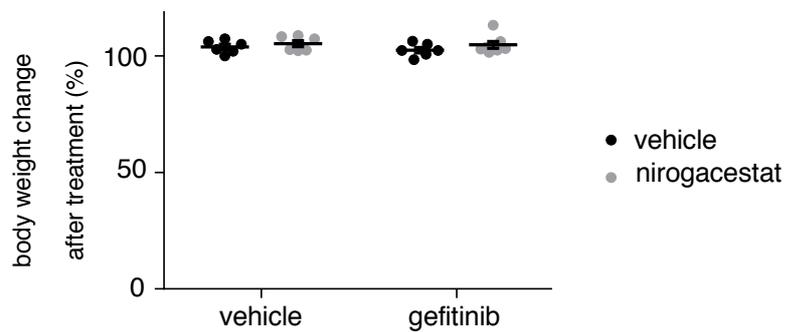
Supplemental Figure 7. Treatment schedules of nirogacestat and/or gefitinib do not impact on the weight of animals.

Mice treated with vehicle (methocel, n = 6), nirogacestat (n = 6), gefitinib (n = 6), or nirogacestat+gefitinib (n = 7) were weighted the first and last day of treatment, and the change in body weight is shown. Values correspond to the mean ± S.E.M. Statistical significance was determined by one-way ANOVA test followed by Tukey's post hoc test, but results were not significantly different.
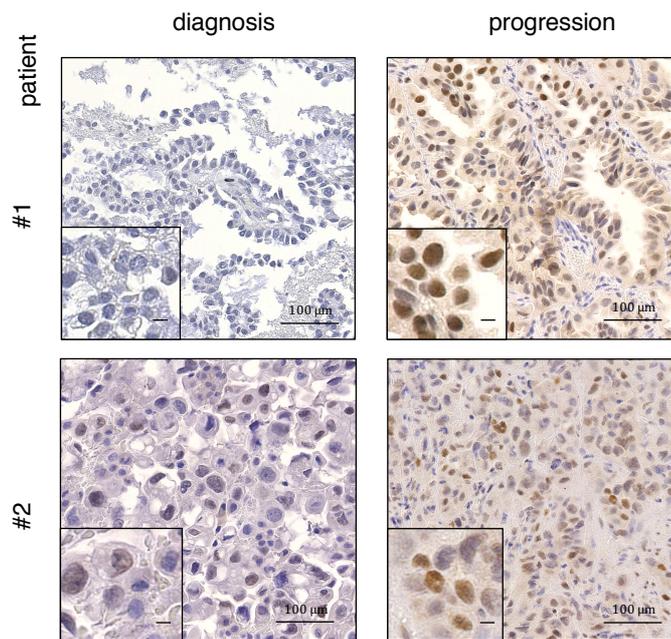
Supplemental Figure 8. Examples of immunohistochemical analysis of HES1 expression in patient biopsies of lung adenocarcinoma.

HES1 staining in lung adenocarcinoma tissue sections from two patients with an EGFR-mutated cancer before TKI treatment (diagnosis) and after relapse. Scale bar at insets = 25µm.