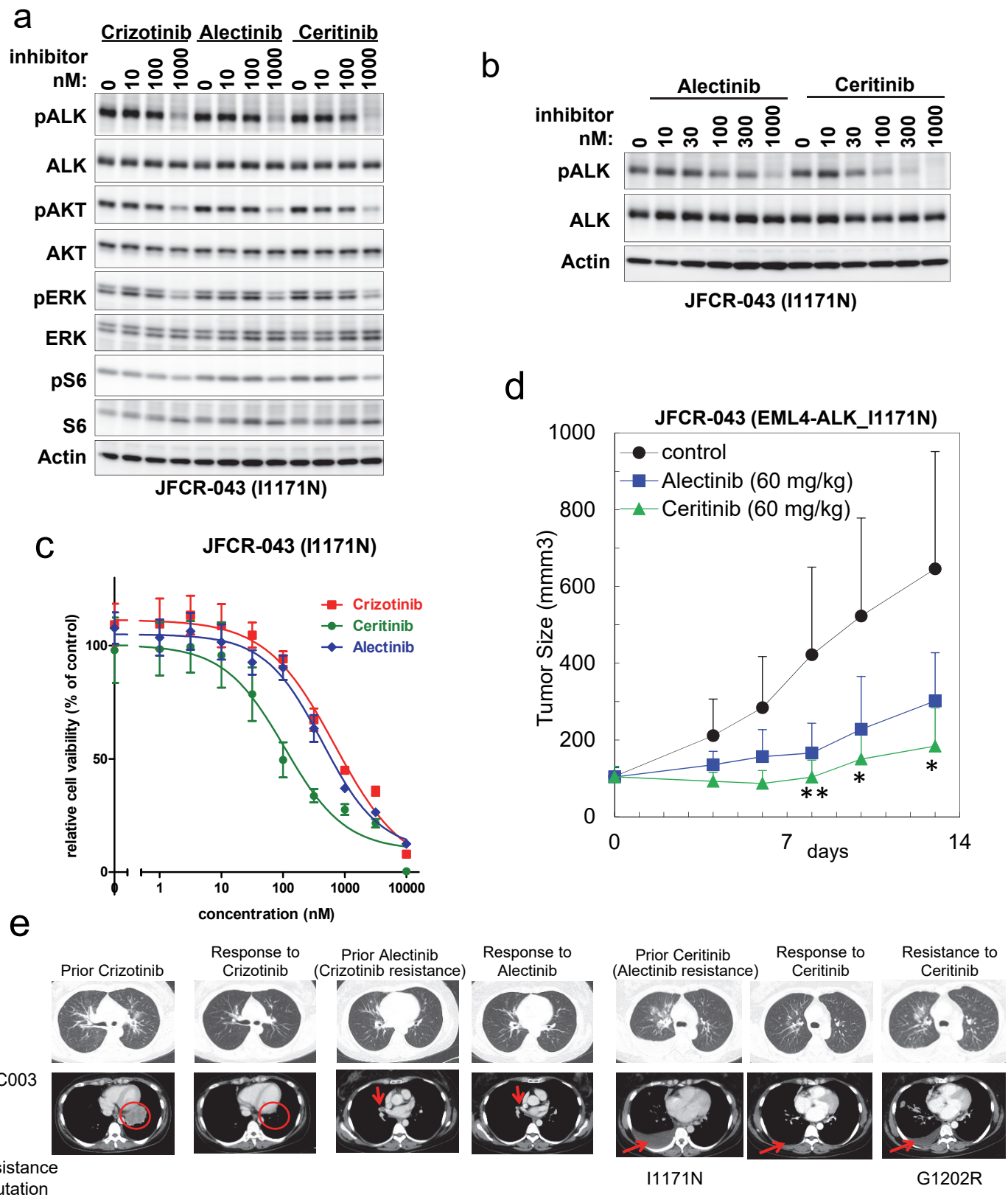
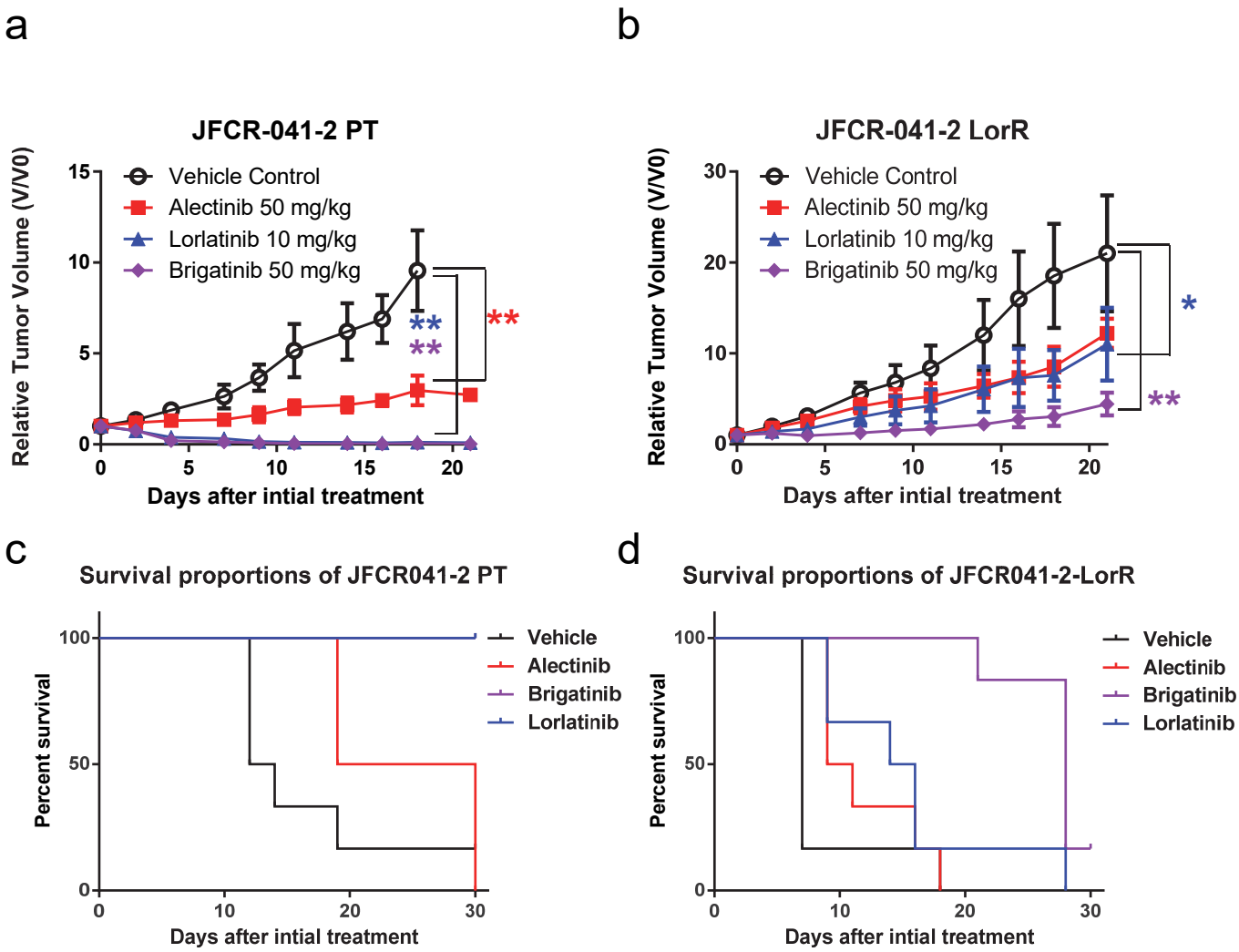


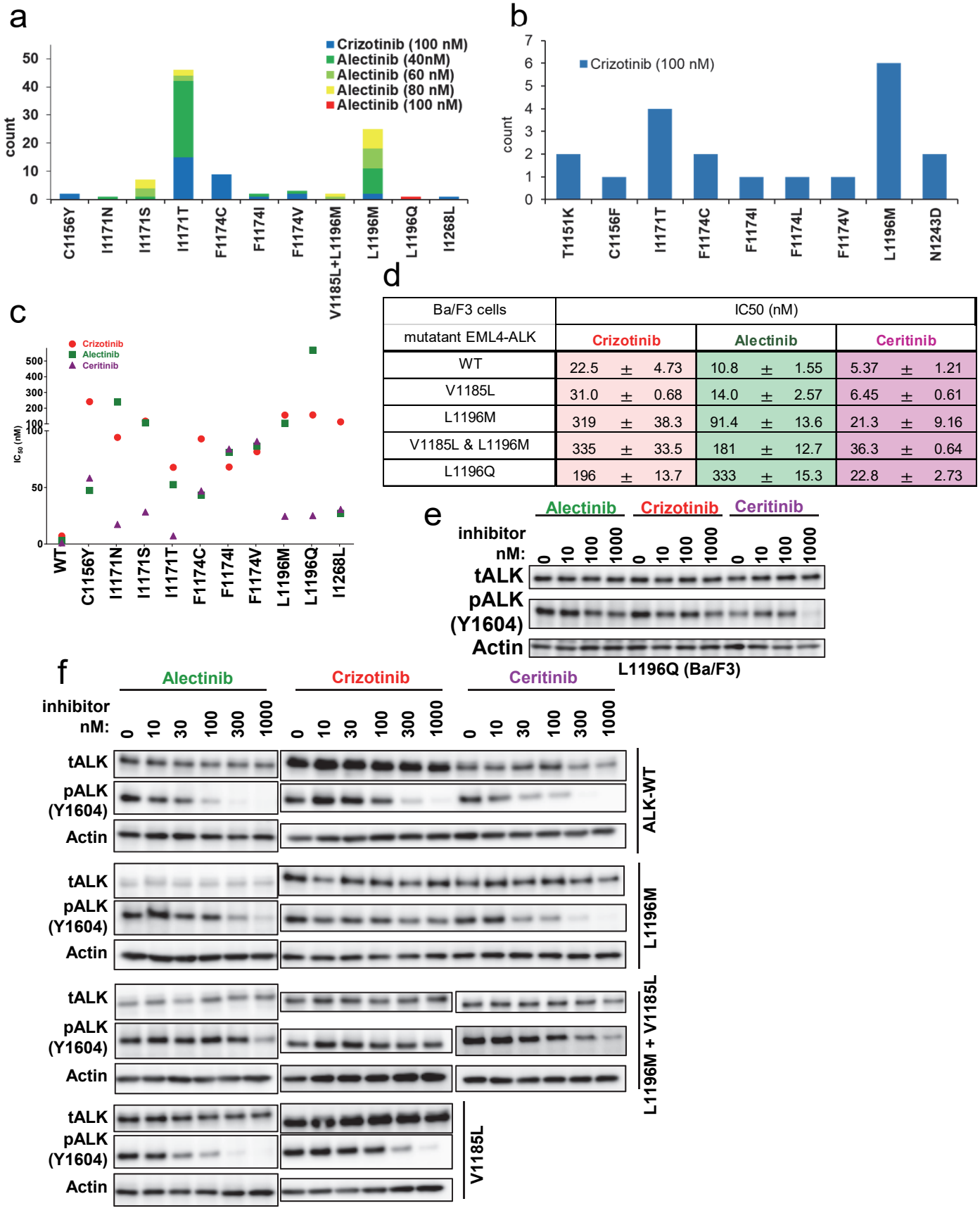
Supplementary Figure S1. Effect of various ALK-TKIs on JFCR-043 cells.

(a) Suppression of phospho-ALK and downstream ALK in patient-derived JFCR-043 cancer cells by crizotinib, alectinib, or ceritinib. Cells were treated with the indicated concentrations of each inhibitor for 3 h. Cell lysates were immunoblotted to detect the indicated proteins. (b) Suppression of phospho-ALK and downstream ALK in patient-derived JFCR-043 cells by alectinib or ceritinib. Cells were treated with the indicated concentrations of each inhibitor for 3 h. Cell lysates were immunoblotted to detect the indicated proteins. (c) JFCR-043 cells were treated with the indicated concentrations of crizotinib, ceritinib, or alectinib for 72 h. Cell viability was analyzed using the CellTiter-Glo assay. (d) JFCR-043 cells were subcutaneously implanted into BALB-c *nu/nu* mice. Once the tumor volumes reached approximately 200 mm^3 in each mouse, the mice were randomized into vehicle, alectinib 60 mg/kg, or ceritinib 60 mg/kg (with n = 6 in each group), and treated once daily by oral gavage for 5 to 6 days/week. The tumor size was measured twice weekly. The relative tumor size was calculated by dividing the tumor size of each day by that at the start of treatment. Results are expressed as mean ± SD. The statistical significance between the mean tumor volumes of vehicle control and ceritinib on day 8, 10, and 13 was calculated using the Mann-Whitney *U* test (** $P < 0.01$, * $P < 0.05$).. (e) Computed tomographic images of MCC-003 patients at baseline and at the time of relapse. Identified resistance mutations are indicated in the figures.



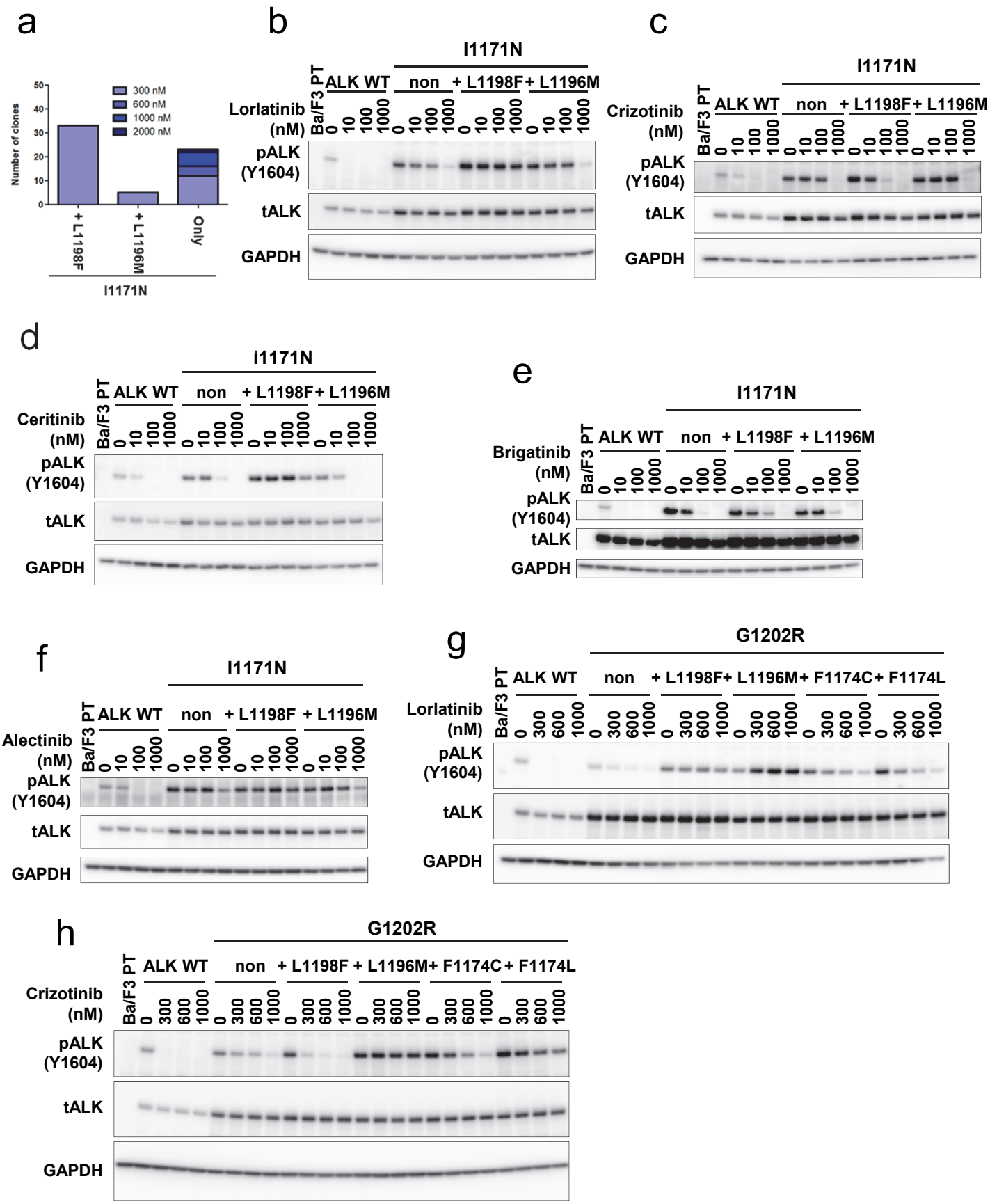
Supplementary Figure S2. Partial response of EML4-ALK-G1202R+G1269A positive JFCR-041-2 LorR tumor to brigatinib in mouse xenograft model.

(a and b) JFCR-041-2 or JFCR-041-2 LorR cells were subcutaneously implanted into BALB-c *nu/nu* mice. Once the tumor volumes in each mouse reached around 200 mm^3, the mice were randomized into vehicle, alectinib, lorlatinib, or brigatinib (with n=6 in each group). The relative tumor size was calculated by dividing the tumor size of each day by that at the start of the treatment. Results are expressed as mean ± SD. The statistical significance between the mean tumor volumes of vehicle control and alectinib, lorlatinib or brigatinib on day 19 for (a) and on day 21 for (b) are calculated using the Mann-Whitney *U* test (** $P < 0.01$, * $P < 0.05$). (c and d) The survival periods of mice in each treatment group were demonstrated using the Kaplan-Meier curve.



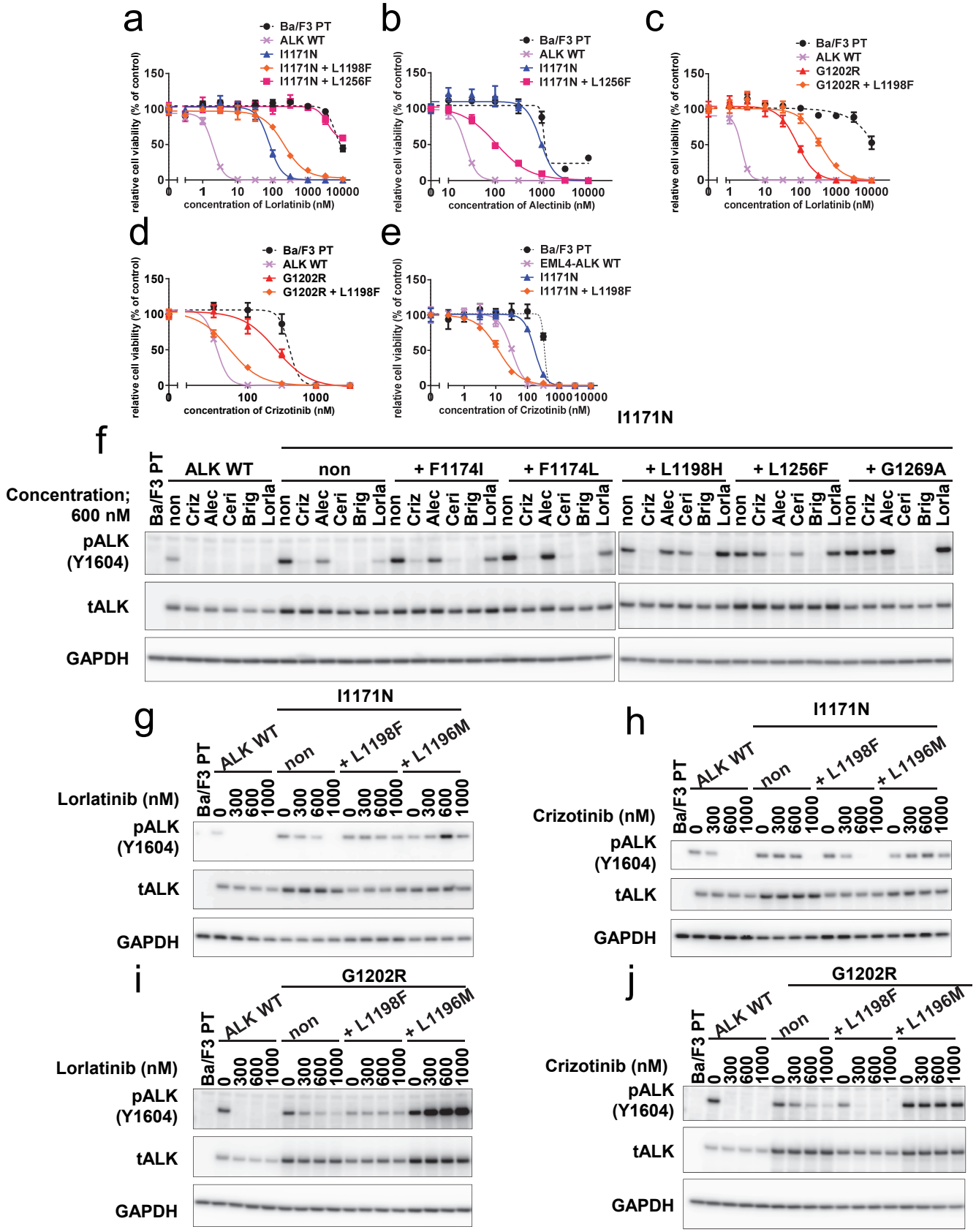
Ba/F3 cells	IC50 (nM)		
mutatant EML4-ALK	Crizotinib	Alectinib	Ceritinib
WT	22.5 ± 4.73	10.8 ± 1.55	5.37 ± 1.21
V1185L	31.0 ± 0.68	14.0 ± 2.57	6.45 ± 0.61
L1196M	319 ± 38.3	91.4 ± 13.6	21.3 ± 9.16
V1185L & L1196M	335 ± 33.5	181 ± 12.7	36.3 ± 0.64
L1196Q	196 ± 13.7	333 ± 15.3	22.8 ± 2.73

Supplementary Figure S3. Alectinib- or Crizotinib-resistant ALK mutations by ENU mutagenesis screening.
(a and b) Number of clones with various mutations in the ALK kinase domain identified as alectinib-, or crizotinib- resistant mutations by ENU mutagenesis screening. (c) Calculated IC_{50} values of alectinib-resistant ALK-mutated Ba/F3 clonal cells obtained from ENU mutagenesis screening. These cells were treated with crizotinib, alectinib, or ceritinib for 72h.
(d) Average IC_{50} values of each Ba/F3 cell group to crizotinib, alectinib, or ceritinib (from three independent experiments) are shown. (e) Suppression of phospho-ALK in Ba/F3 expressing EML4-ALK-L1196Q by alectinib, crizotinib, or ceritinib. Cells were exposed to increasing concentrations (10 nM, 100 nM, and 1000 nM) of each inhibitor for 3 h. Cell lysates were immunoblotted to detect the indicated proteins. (f) Suppression of phospho-ALK in Ba/F3 expressing EML4-ALK-WT, L1196M, L1196M+V1180L, or V1180L by alectinib, crizotinib, or ceritinib. Cells were exposed the increasing concentrations (10 nM, 30 nM, 100 nM, 300 nM, and 1000 nM) of each inhibitor for 3 h. Cell lysates were immunoblotted to detect the indicated proteins.



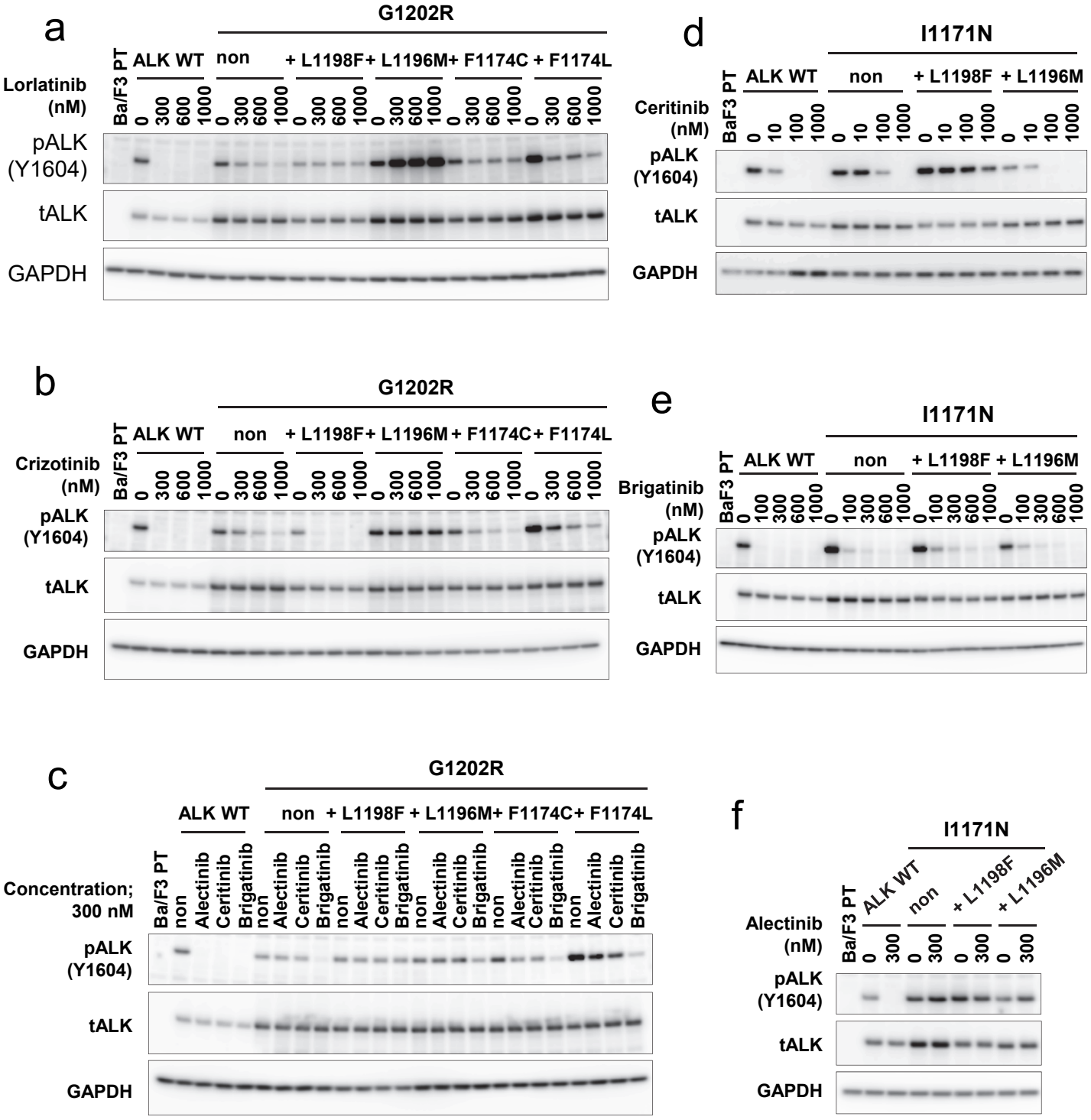
Supplementary Figure S4. Lorlatinib-resistant I1171N+L1198F and I1171N+L1196M mutations appeared frequently in 2nd ENU mutagenesis.

(a) Number of clones with various I1171N double mutations in the ALK kinase domain identified as lorlatinib-resistant mutations in the independent ENU mutagenesis screening. (b-f) Suppression of phospho-ALK in parental Ba/F3 cells, EML4-ALK- WT Ba/F3 cells, or various EML4-ALK I1171N double mutated Ba/F3 ENU clonal cells by lorlatinib (b), crizotinib (c), ceritinib (d), brigatinib (e) or alectinib (f). Cells were exposed the increasing concentrations (10 nM, 100 nM, and 1000 nM) of each inhibitor for 3 h. Cell lysates were immunoblotted to detect the indicated proteins. (g-h) Suppression of phospho-ALK in parental Ba/F3 cells, EML4-ALK-WT Ba/F3 cells, or various EML4-ALK G1202R double mutated Ba/F3 ENU clonal cells by lorlatinib (g) or crizotinib (h). Cells were exposed the increasing concentrations (300 nM, 600 nM, and 1000 nM) of each inhibitor for 3 h. Cell lysates were immunoblotted to detect the indicated proteins.



Supplementary Figure S5. Suppression of double mutated ALK by clinically approved ALK-TKIs.

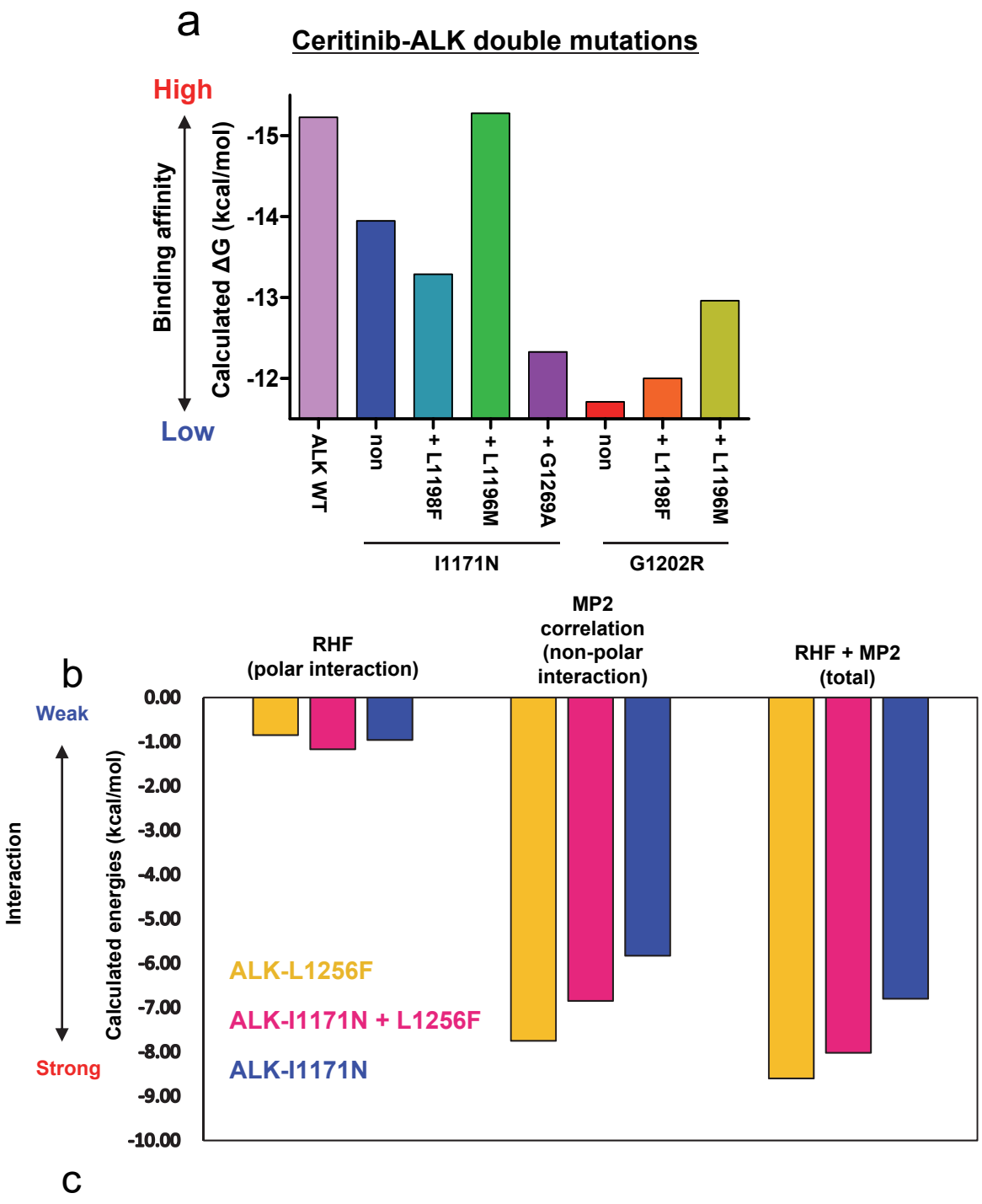
(a-e) Parental Ba/F3 cells, Ba/F3 EML4-ALK wild-type poly clonal cells, Ba/F3 I1171N#12 cells, Ba/F3 G1202R poly clonal cells, and ALK double mutated Ba/F3 cells (I1171N+L1198F and G1202R+L1198F: re-induced cells, I1171N+L1256F: ENU clonal cells) were treated with indicated ALK-TKIs. These cells were treated ALK-TKIs for 72 h. Cell viability was analyzed using the CellTiter-Glo assay. (f) Suppression of phospho-ALK in Ba/F3 EML4-ALK wild-type poly clonal cells, Ba/F3 I1171N#12 cells, and lorlatinib-resistant ENU I1171N double mutated Ba/F3 clonal cells by ALK-tyrosine kinase inhibitor treatment (crizotinib, alectinib, ceritinib, brigatinib or lorlatinib). Cells were exposed to 600 nM of each ALK-TKI for 3 h. Cell lysates were immunoblotted to detect the indicated proteins. (g-j) Suppression of phospho-ALK in Ba/F3 EML4-ALK wild-type poly clonal cells, Ba/F3 I1171N#12 cells, Ba/F3 G1202R poly clonal cells, Ba/F3 cells with re-induced ALK-I1171N+L1198F or +L1196M, and re-induced ALK-G1202R+L1198F or +L1196M. Cells were treated with the indicated concentration of ALK-tyrosine kinase inhibitors, lorlatinib (g and i) or crizotinib (h and j) for 3 h. Cell lysates were immunoblotted to detect the indicatedproteins.



Supplementary Figure S6. Suppression of Ba/F3 re-induced ALK double mutations by various ALK-TKIs.

(a-c) Suppression of phospho-ALK in EML4-ALK-WT Ba/F3 cells, EML4-ALK G1202R Ba/F3 poly clonal cells, or re-induced EML4-ALK G1202R double mutated Ba/F3 cells in various ALK-TKIs treatment. Cells were treated with indicated concentrations of ALK-TKIs for 3 h. Cell lysates were immunoblotted to detect the indicated proteins.

(d-f) Suppression of phospho-ALK in EML4-ALK-WT Ba/F3 cells, EML4-ALK I1171N Ba/F3 #12 cells, or re-induced EML4-ALK I1171N double mutated Ba/F3 cells in ceritinib (d), brigatinib (e) or alectinib (f) treatment. Cells were treated with indicated concentration of ALK-TKIs for 3 h. Cell lysates were immunoblotted to detect the indicated proteins.



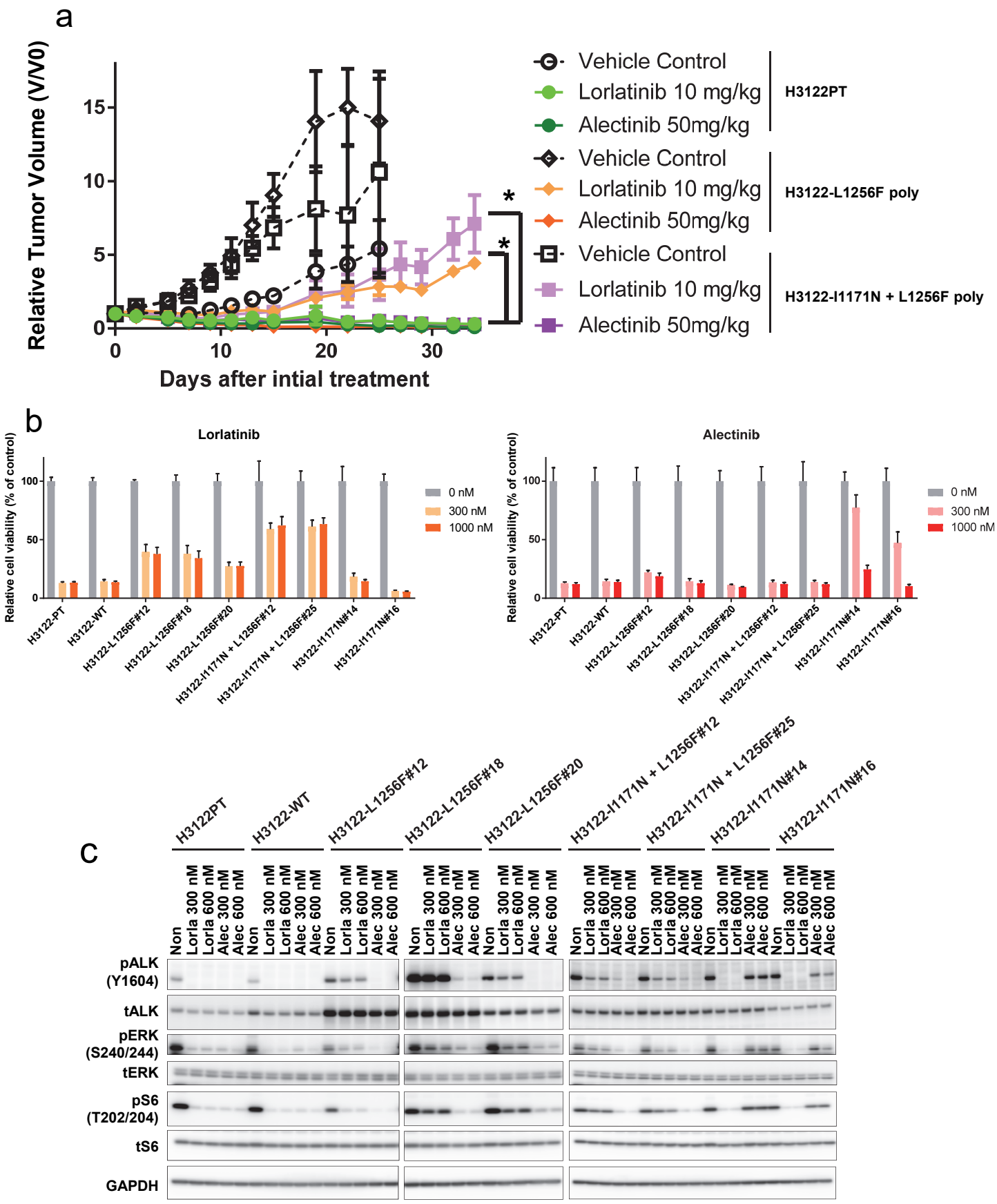
c

	HF	MP2	HF + MP2
ALK-L1256F	-0.85	-7.75	-8.60
ALK-I1171N + L1256F	-1.17	-6.85	-8.02
ALK-I1171N	-0.96	-5.83	-6.80

(kcal/mol)

Supplementary Figure S7. Computational Simulation for lorlatinib resistant ALK mutants (ALK-L1256F single or double mutation) to understand alectinib re-sensitivity of those ALK-L1256F mutants by MP-CAFEE and FMO calculation.

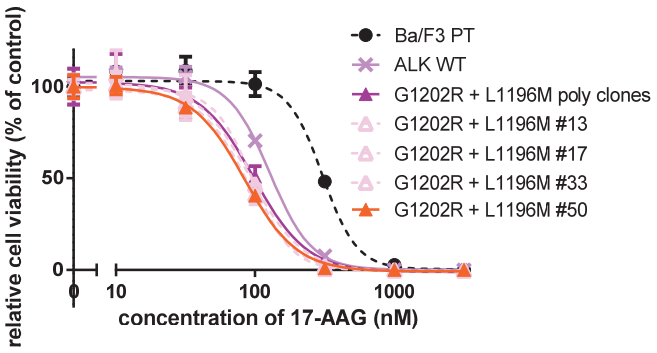
(a) The calculate ΔG values by MP-CAFEE of ceritinib to each ALK double mutant are shown in the bar graph. (b) L/F1256 residue-alectinib interaction energies in these mutated ALK kinase domain estimated by FMO calculation. In this calculation, RHF energy mainly reflects the polar interaction and MP2 energy mainly reflects the non-polar interaction, indicating significant π-π interaction between the phenyl group in F1256 and alectinib. (c) Calculated values of the energies in (b).



Supplementary Figure S8. EML4-ALK-L1256F single mutant demonstrated the lorlatinib resistance, and was sensitive to alectinib similar to H3122 parental cells, or -EML4-ALK-WT overexpressed H3122 cells.

(a) H3122 parental, -EML4-ALK-I1171N+L1256F, and -L1256F overexpressed H3122 poly clonal cells were subcutaneously implanted into BALB-c *nu/nu* mice. Once the tumor volumes in each mouse reached around 200 mm^3, the mice were randomized into vehicle, alectinib, or lorlatinib (with n=3 in each group). The relative tumor size was calculated by dividing the tumor size of each day by that at the start of the treatment. Results are expressed as mean ± SD. The statistical significance between the mean tumor volumes of alectinib and lorlatinib on day 34 are calculated using the Mann-Whitney *U* test (* $P < 0.05$). (b) Parental, EML4-ALK-WT, these L1256F single, or double mutated mono clonal H3122 cells were treated with the indicated concentrations of lorlatinib, or alectinib for 72 h. Relative cell viability was analyzed using the CellTiter-Glo assay. (c) Cells were exposed the increasing concentrations (300, and 600 nM) of each inhibitor for 6 h. Cell lysates were immunoblotted to detect the indicated proteins.

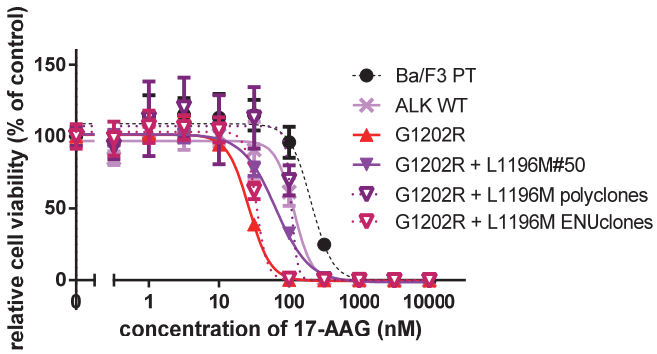
a



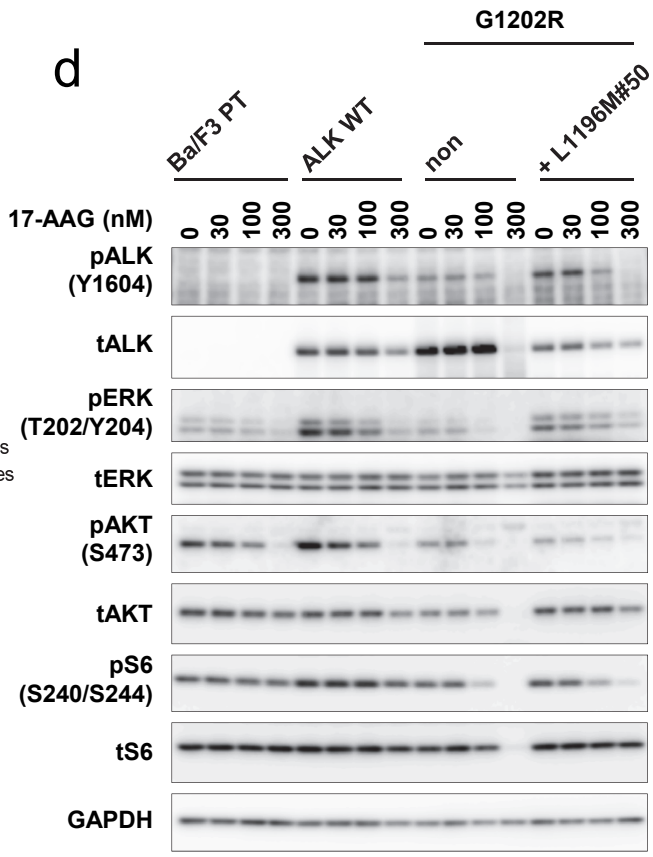
b

Ba/F3cells	IC50 (nM)
ALK WT	130
G1202R + L1196M poly clones	97
G1202R + L1196M #13	96
G1202R + L1196M #17	86
G1202R + L1196M #33	93
G1202R + L1196M #50	84

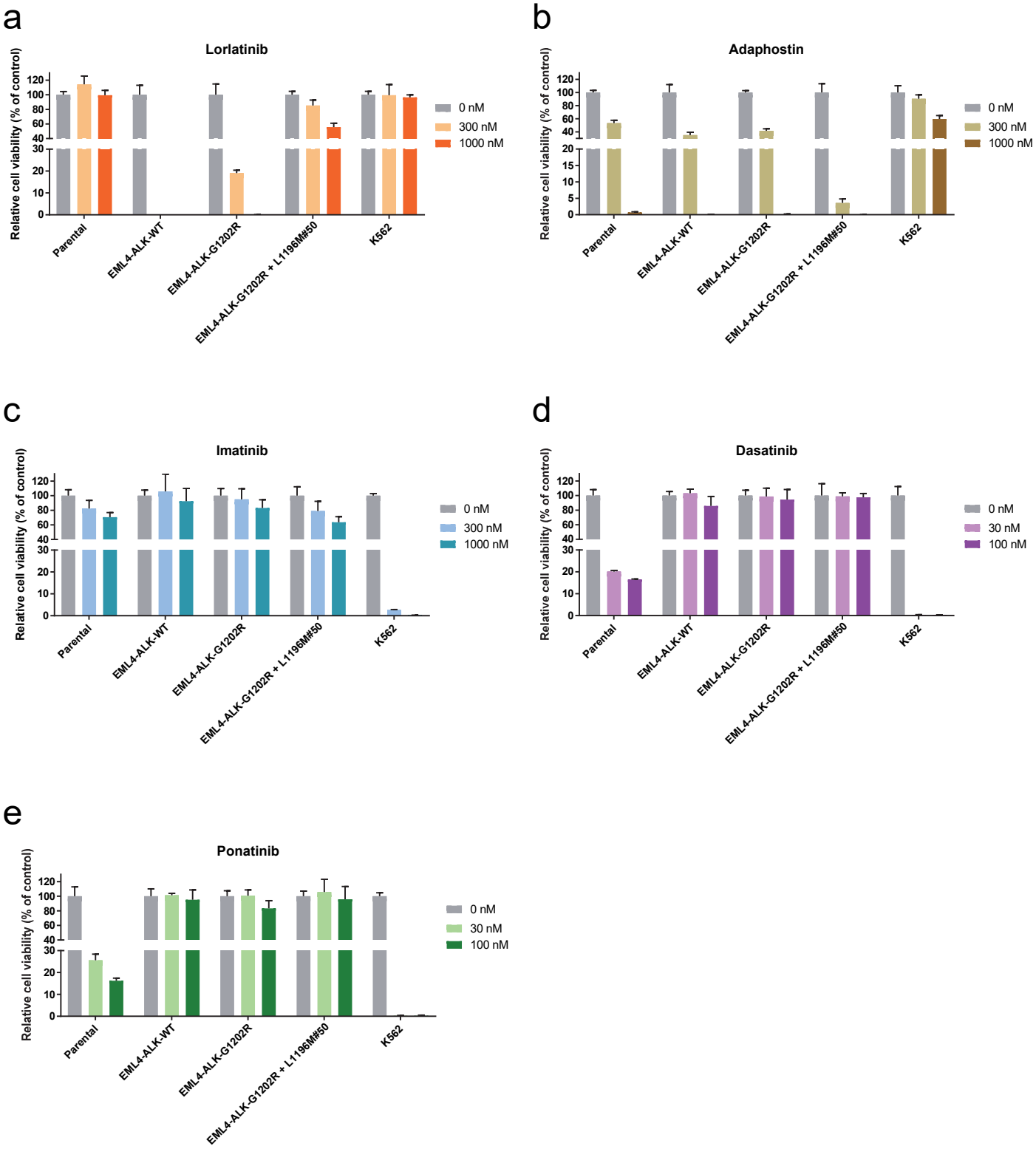
c



d



Supplementary Figure S9. Selection of Ba/F3 EML4-ALK-G1202R+L1196M clonal cells#50.
(a) Parental Ba/F3 cells, EML4-ALK Ba/F3 cells, and various EML4-ALK G1202R+L1196M Ba/F3 clonal cells were treated with the indicated concentrations of 17-AAG for 72 h. Cell viability was analyzed using the CellTiter-Glo assay. (b) IC_{50} values of each EML4-ALK-G1202R+L1196M clonal cell. (c) Parental Ba/F3 cells, EML4-ALK Ba/F3 cells, and EML4-ALK G1202R+L1196M Ba/F3 clonal cells#50, EML4-ALK G1202R+L1196M Ba/F3 polyclonal cells, and EML4-ALK G1202R+L1196M Ba/F3 ENU clonal cells were treated with the indicated concentrations of 17-AAG for 72 h. Cell viability was analyzed using the CellTiter-Glo assay. (d) Suppression of phospho-ALK by 17-AAG in Ba/F3 parental cells, Ba/F3 EML4-ALK-WT polyclonal cells, Ba/F3 EML4-ALK-G1202R polyclonal cells, or Ba/F3 EML4-ALK-G1202R+L1196M clonal cells#50. Cells were exposed to increasing concentrations of 17-AAG for 24 h. Cell lysates were immunoblotted to detect the indicated proteins.



Supplementary Figure S10. Insensitivity of Ba/F3 EML4-ALK-G1202R+L1196M clonal cells#50 to several BCR-ABL inhibitors.

(a-e) Parental, EML4-ALK-WT, -G1202R Ba/F3 cells and EML4-ALK G1202R+L1196M Ba/F3 clonal cells #50 were treated with the indicated concentrations of the BCR-ABL inhibitors for 72 h. Relative cell viability was analyzed using the CellTiter-Glo assay.

Supplementary Tables

A

50 nM ≦ IC50		50 nM < IC50 < 200 nM		200 nM ≦ IC50	
IC50 (±SD) ; nM	Crizotinib	Alectinib	Ceritinib	Brigatinib	Lorlatinib
ALK WT	22 (±4.9)	11 (±0.7)	4.5 (±1.5)	2.4 (±1.1)	1 (±0.2)
G1202R	230 (±24)	640 (±170)	120 (±17)	56 (±2.6)	69 (±14)
G1202R + G1269A	390 (±10)	960 (±190)	120 (±30)	75 (±25)	700 (±210)

B

50 nM ≦ IC50		50 nM < IC50 < 200 nM		200 nM ≦ IC50	
IC50 (±SD) ; nM	Crizotinib	Alectinib	Ceritinib	Brigatinib	Lorlatinib
ALK WT	42 (±17)	12 (±2.8)	9.4 (±2.5)	5 (±0.9)	1.5 (±0.3)
I1171N	220 (±46)	600 (±70)	34 (±7.2)	27 (±1.7)	81 (±19)
I1171N + F1174I	350 (±60)	1300 (±60)	140 (±7.1)	110 (±13)	320 (±60)
I1171N + F1174L	440 (±140)	1600 (±500)	160 (±53)	100 (±6.8)	220 (±67)
I1171N + L1198H	160 (±30)	1600 (±420)	540 (±22)	140 (±24)	670 (±150)
I1171N + L1256F	560 (±53)	73 (±5.4)	400 (±71)	42 (±6.3)	6000 (±3500)
I1171N + G1269A	670 (±38)	1100 (±95)	14 (±2.9)	7.1(±0.4)	470 (±70)

C

50 nM ≦ IC50		50 nM < IC50 < 200 nM		200 nM ≦ IC50	
IC50 (±SD) ; nM	Crizotinib	Alectinib	Ceritinib	Brigatinib	Lorlatinib
ALK WT	28 (±12)	9.8 (±2.6)	4.5 (±2.1)	2.9 (±0.6)	1.2 (±0.2)
I1171N	140 (±59)	400 (±69)	18 (±6.9)	20 (±1.5)	60 (±13)
I1171N + L1198F	21 (±4.8)	660 (±110)	520 (±120)	49 (±3.9)	340 (±15)
I1171N + L1196M	350 (±17)	370 (±41)	11 (±0.8)	32 (±6.1)	320 (±21)

D

50 nM ≦ IC50		50 nM < IC50 < 200 nM		200 nM ≦ IC50	
IC50 (±SD) ; nM	Crizotinib	Alectinib	Ceritinib	Brigatinib	Lorlatinib
ALK WT	45 (±13)	17 (±6.1)	10 (±1.2)	5.4 (±0.9)	1.9 (±0.1)
I1171N	220 (±44)	600 (±290)	36 (±2.1)	28 (±1.2)	81 (±4)
I1171N + L1198F	19 (±8.4)	750 (±180)	660 (±190)	43 (±20)	240 (±63)
I117N + L1196M	460 (±160)	460 (±180)	21 (±2.6)	31 (±9.9)	360 (±22)

E

50 nM ≦ IC50		50 nM < IC50 < 200 nM		200 nM ≦ IC50	
IC50 (±SD) ; nM	Crizotinib	Alectinib	Ceritinib	Brigatinib	Lorlatinib
ALK WT	35 (±3.1)	15 (±1.6)	7.6 (±2.3)	3.8 (±1.2)	2 (±0.2)
G1202R	260 (±24)	580 (±38)	150 (±8.5)	55 (±1.9)	74 (±5.6)
G1202R + L1198F	47 (±7.9)	420 (±47)	620 (±260)	290 (±55)	370 (±67)
G1202R + L1196M	370 (±37)	880 (±4)	690 (±200)	320 (±64)	1400 (±190)
G1202R + F1174C	320 (±25)	700 (±46)	280 (±31)	180 (±12)	300 (±24)
G1202R + F1174L	200 (±33)	560 (±82)	260 (±29)	160 (±34)	110 (±24)

F

50 nM ≦ IC50		50 nM < IC50 < 200 nM		200 nM ≦ IC50	
IC50 (±SD) ; nM	Crizotinib	Alectinib	Ceritinib	Brigatinib	Lorlatinib
ALK WT	34 (±1.4)	23 (±1.4)	6.6 (±5.1)	4.6 (±0.3)	1.8 (±0.1)
G1202R	180 (±45)	990 (±180)	120 (±26)	50 (±9.4)	54 (±11)
G1202R + L1198F	61 (±7.9)	1400 (±210)	3100 (±930)	430 (±210)	580 (±36)
G1202R + L1196M	430 (±90)	1500 (±500)	940 (±69)	350 (±79)	1900 (±650)
G1202R + F1174C	370 (±43)	1400 (±310)	420 (±90)	220 (±18)	280 (±23)
G1202R + F1174L	490 (±89)	2300 (±150)	590 (±120)	200 (±25)	190 (±41)

Supplementary Tables

Results are expressed as mean ± SD. IC_{50} values of G1202R-double mutated Ba/F3 ENU clonal cells to crizotinib, alectinib, ceritinib, brigatinib or lorlatinib. ALK-WT: Ba/F3 EML4-ALK wild-type polyclonal cells, I1171N: Ba/F3 EML4-ALK-I1171N#12 and G1202R: Ba/F3 EML4-ALK-G1202R polyclonal cells. Gray mutants shows ENU clonal cells and White mutant exhibits re-constructed cells, respectively.