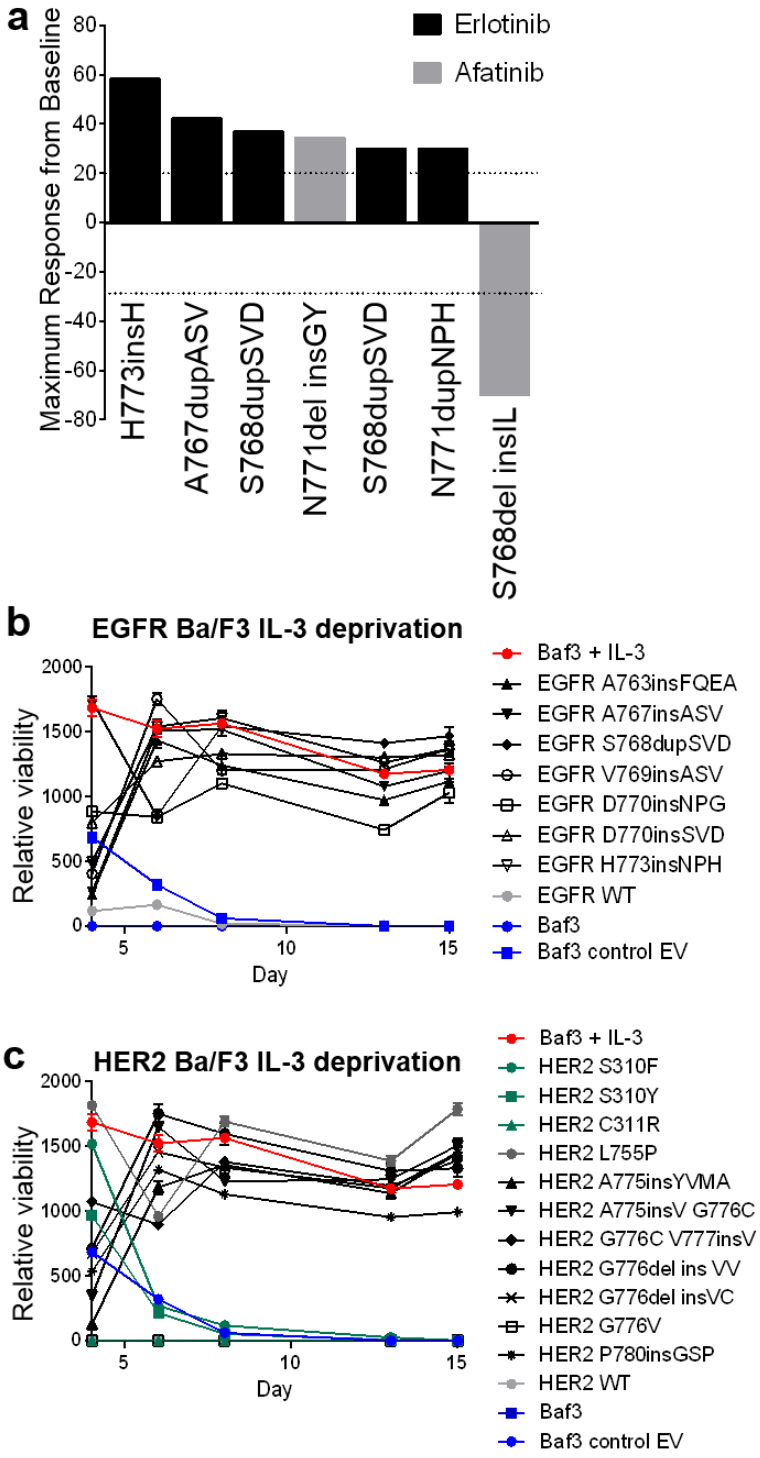
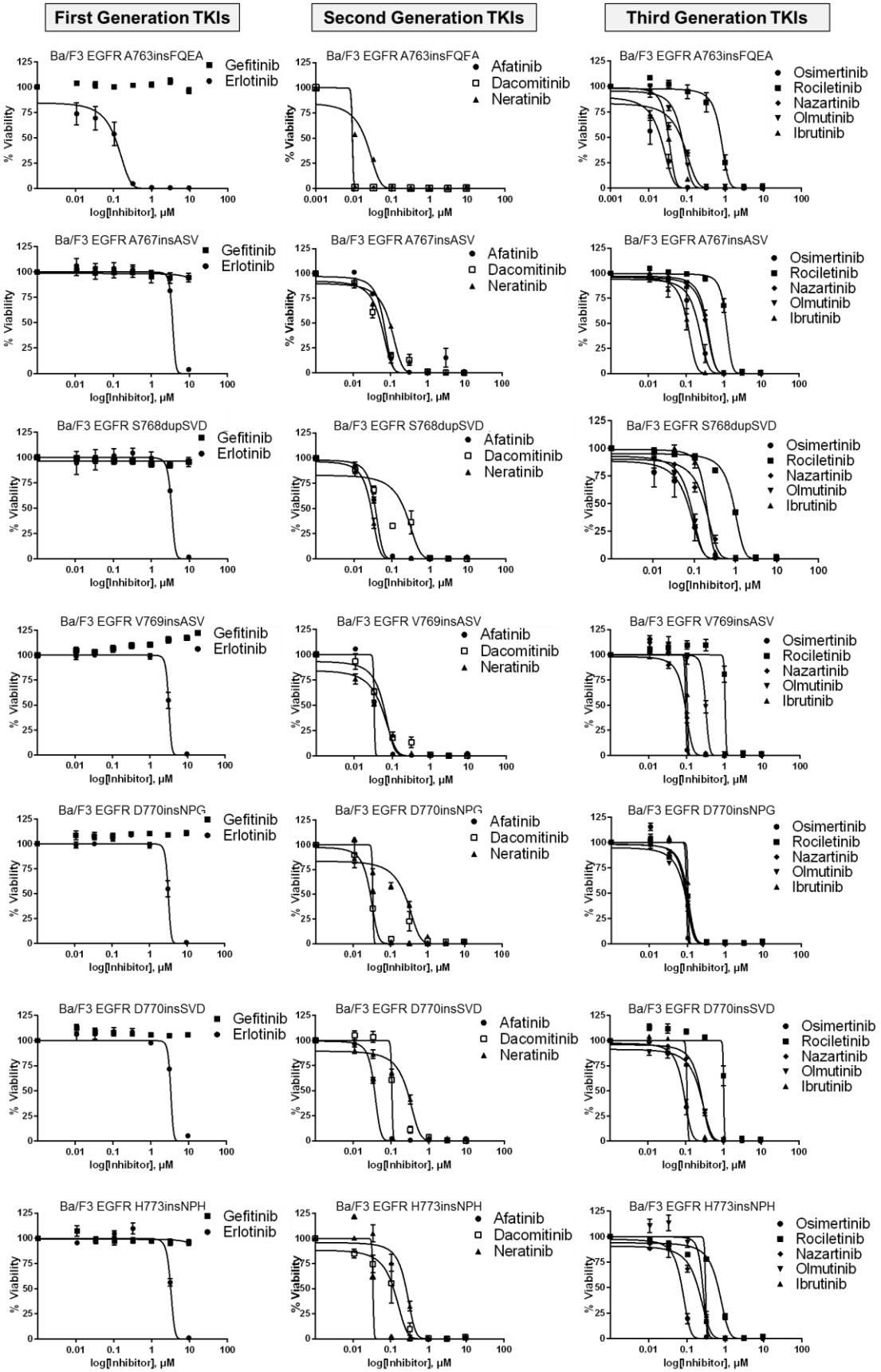
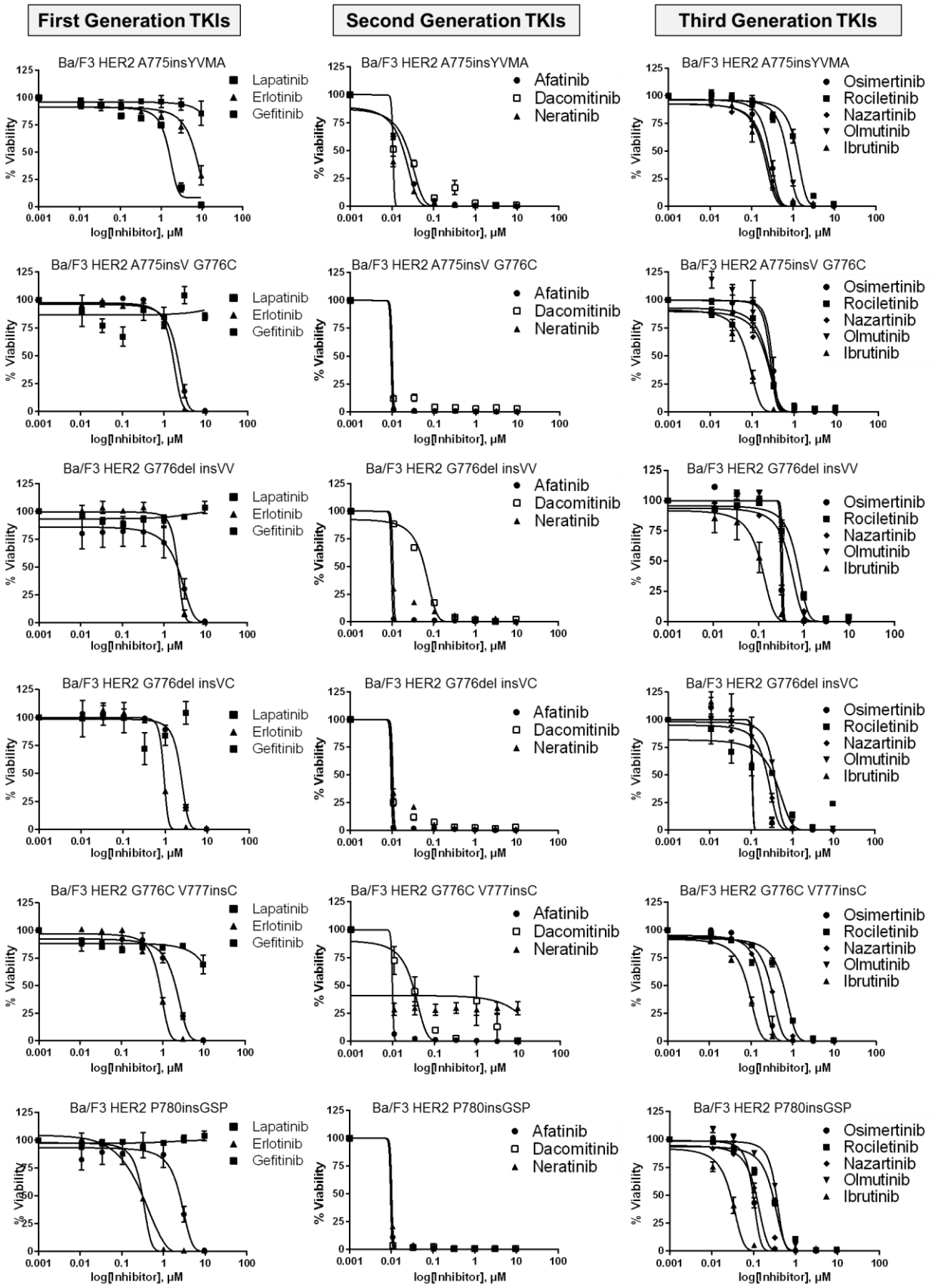


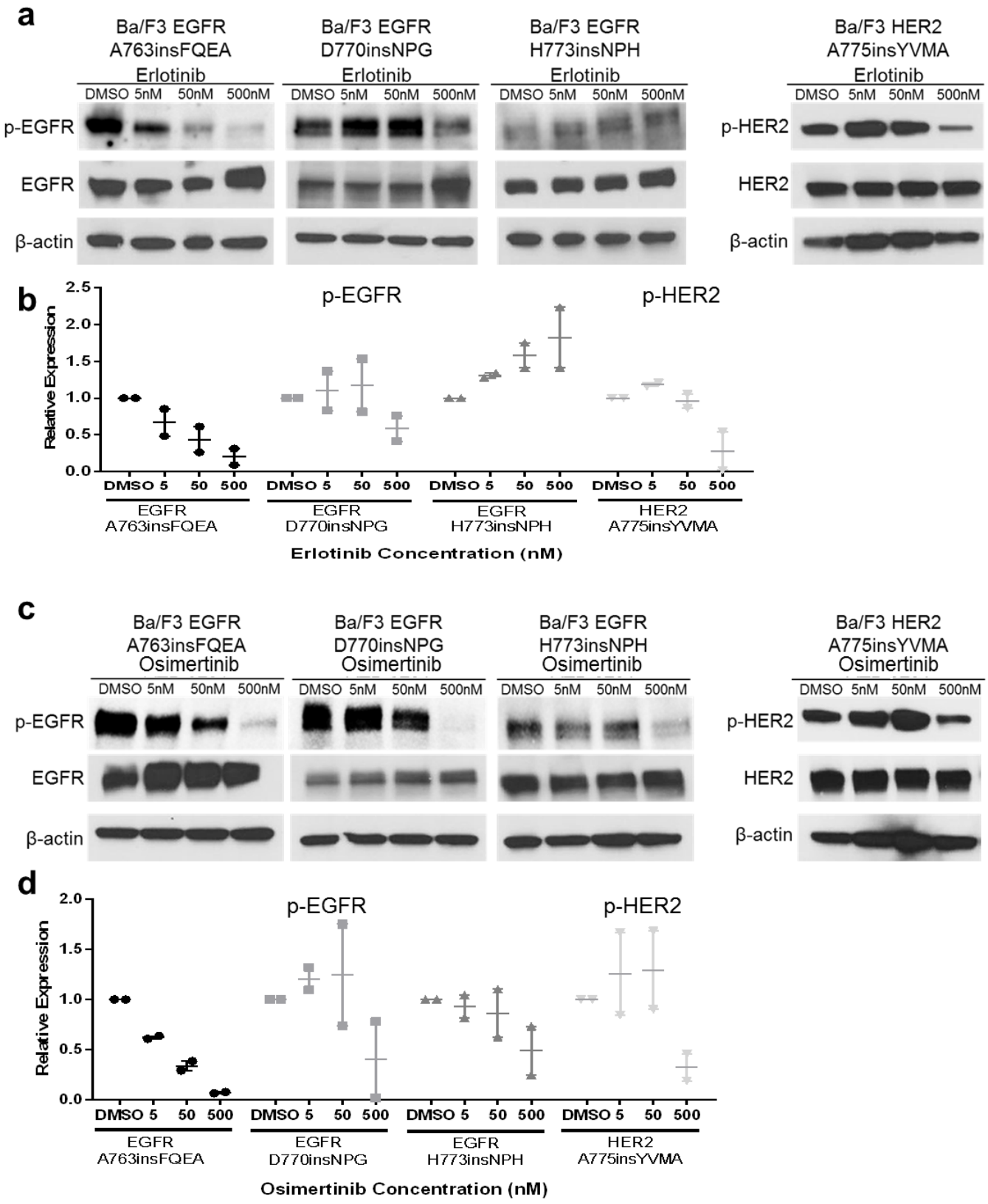
Supplementary Figure 1: EGFR and HER2 exon 20 insertion mutations are activating mutations. a, Waterfall plots of individual patients with *EGFR* exon 20 insertions displays *de novo* resistance to erlotinib, geftinib, or afatinib. Patient mutations are listed below each representative bar. **b,** Stable Ba/F3 cell lines expressing EGFR exon 20 insertion mutations are viable in IL-3 independent conditions, unlike Ba/F3 empty vector expressing cells or EGFR WT expressing Ba/F3 cells, indicating that *EGFR* exon 20 insertions are activating mutations. **c,** IL-3 independent growth of 11 stable Ba/F3 cell lines expressing different HER2 mutations displays that the majority of HER2 activating mutations are within exon 20 of *HER2*. With the exception of L755P, all activating mutations were HER2 exon 20 insertion mutations. **b, c** Cell viability was determined by the Cell Titer Glo assay. The mean ± SEM is plotted for each cell line (n=3 biologically independent experiments).



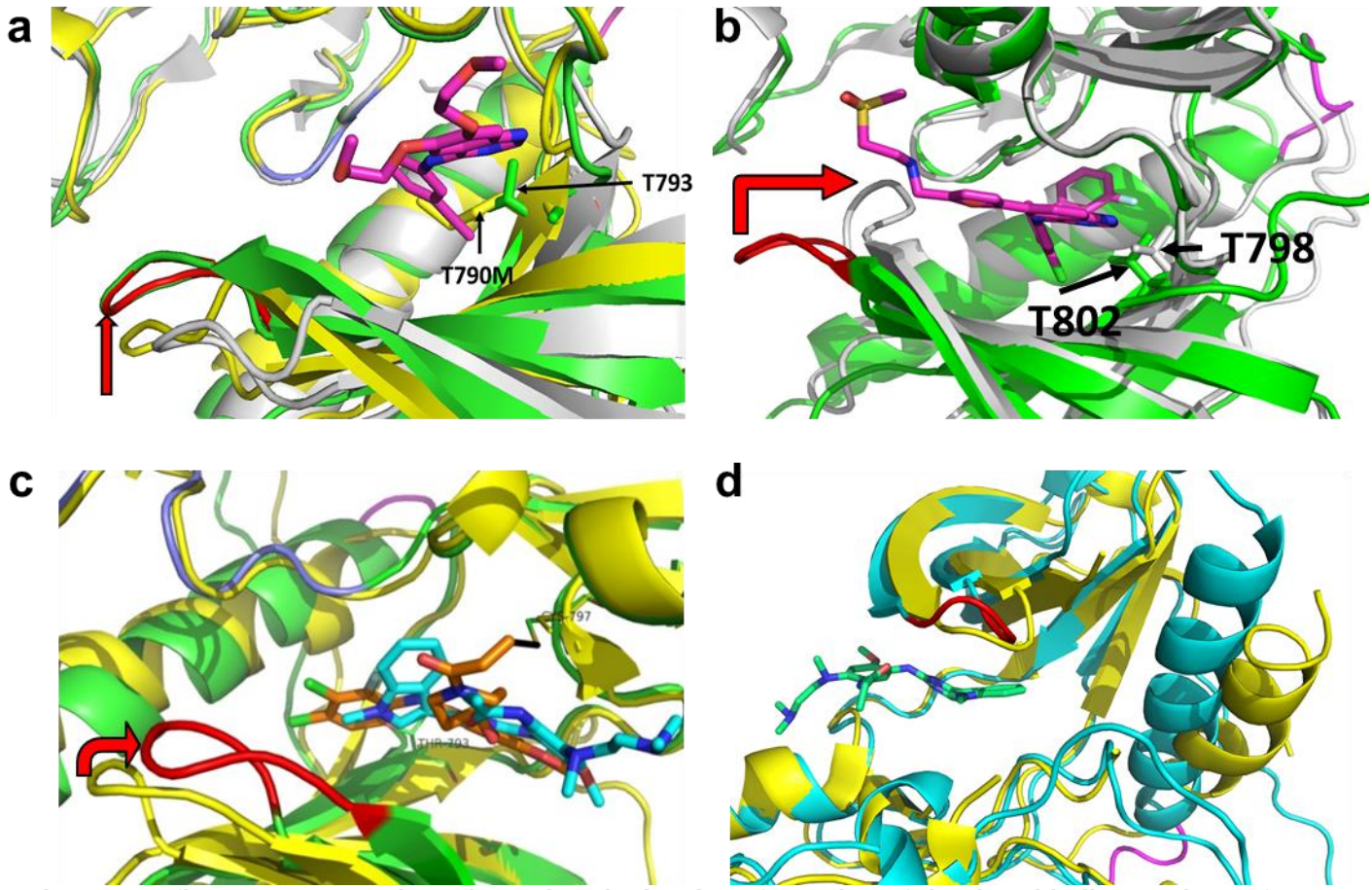
Supplementary Figure 2: Ba/F3 EGFR exon 20 insertion cell lines are resistant to EGFR/HER2 TKIs. Dose response curves of cell viability of individual Ba/F3 cell lines expressing EGFR exon 20 insertion mutations treated with 1st, 2nd, and 3rd generation TKIs for 72 hours. The mean ± SEM is plotted for each concentration (n=3 biologically independent experiments).



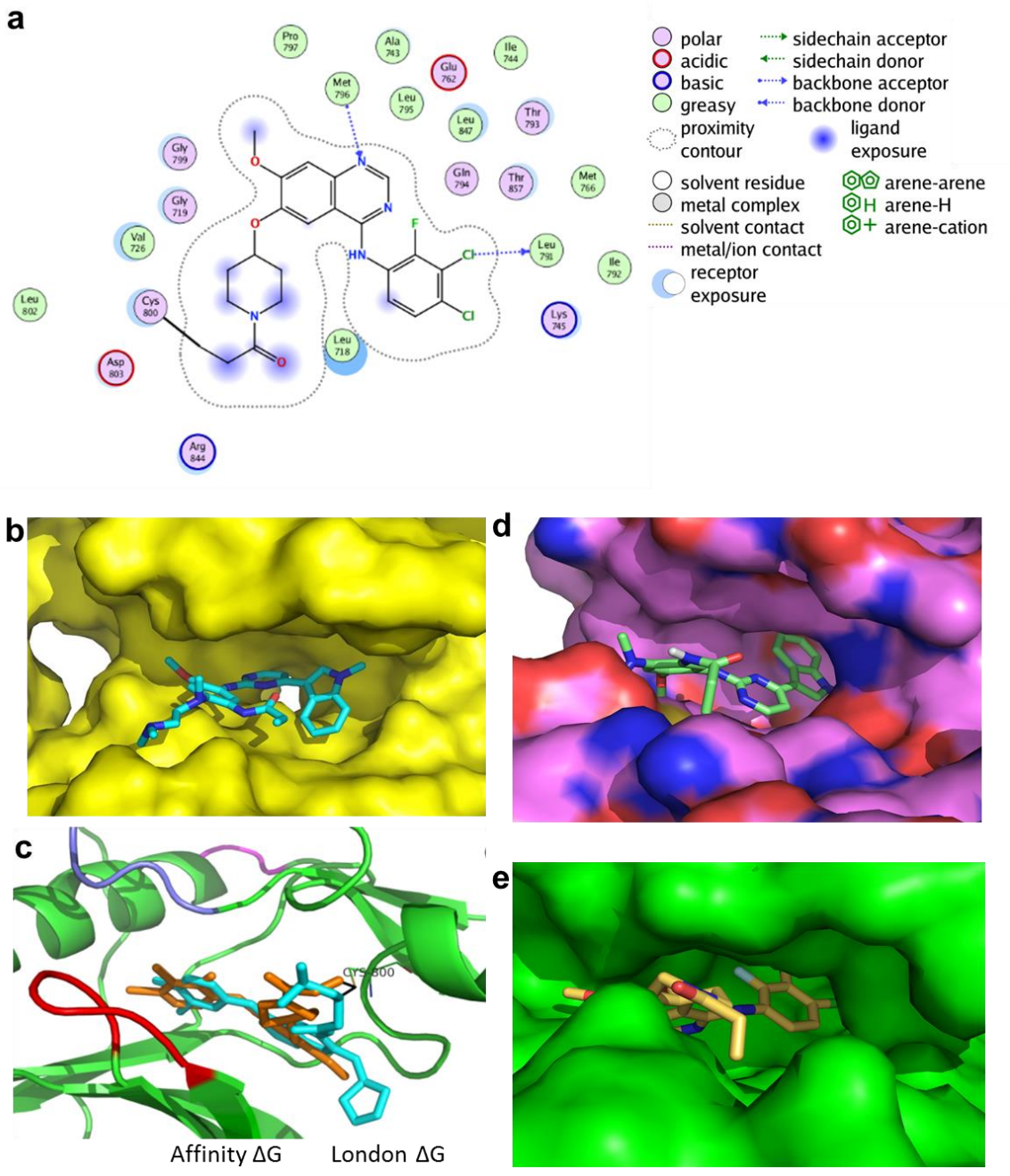
Supplementary Figure 3: Ba/F3 HER2 exon 20 insertion cell lines are resistant to EGFR/HER2 TKIs. Dose response curves of cell viability of individual Ba/F3 cell lines expressing HER2 exon 20 insertion mutations treated with 1st, 2nd, and 3rd generation TKIs for 72 hours. The mean ± SEM is plotted for each concentration (n=3 biologically independent experiments).



Supplementary Figure 4: EGFR and HER2 exon 20 insertions mutations after residue A763 are resistant to 1st and 3rd generation TKIs. Represenative Western blot of Ba/F3 cells with EGFR exon 20 insertions treated with indicated doses of **a,** erlotinib or **c,** osimertinib for 2 hours (N=2 biologically independent experiments). Uncropped western blots are available in supplementary figure 10. p-EGFR and p-HER2 levels after **(b)** erlotinib treatment and **(d)** osimertinib treatment were quantified. Dot plots are representative of mean ±SEM. (N=2 biologically indepenedent experiments).

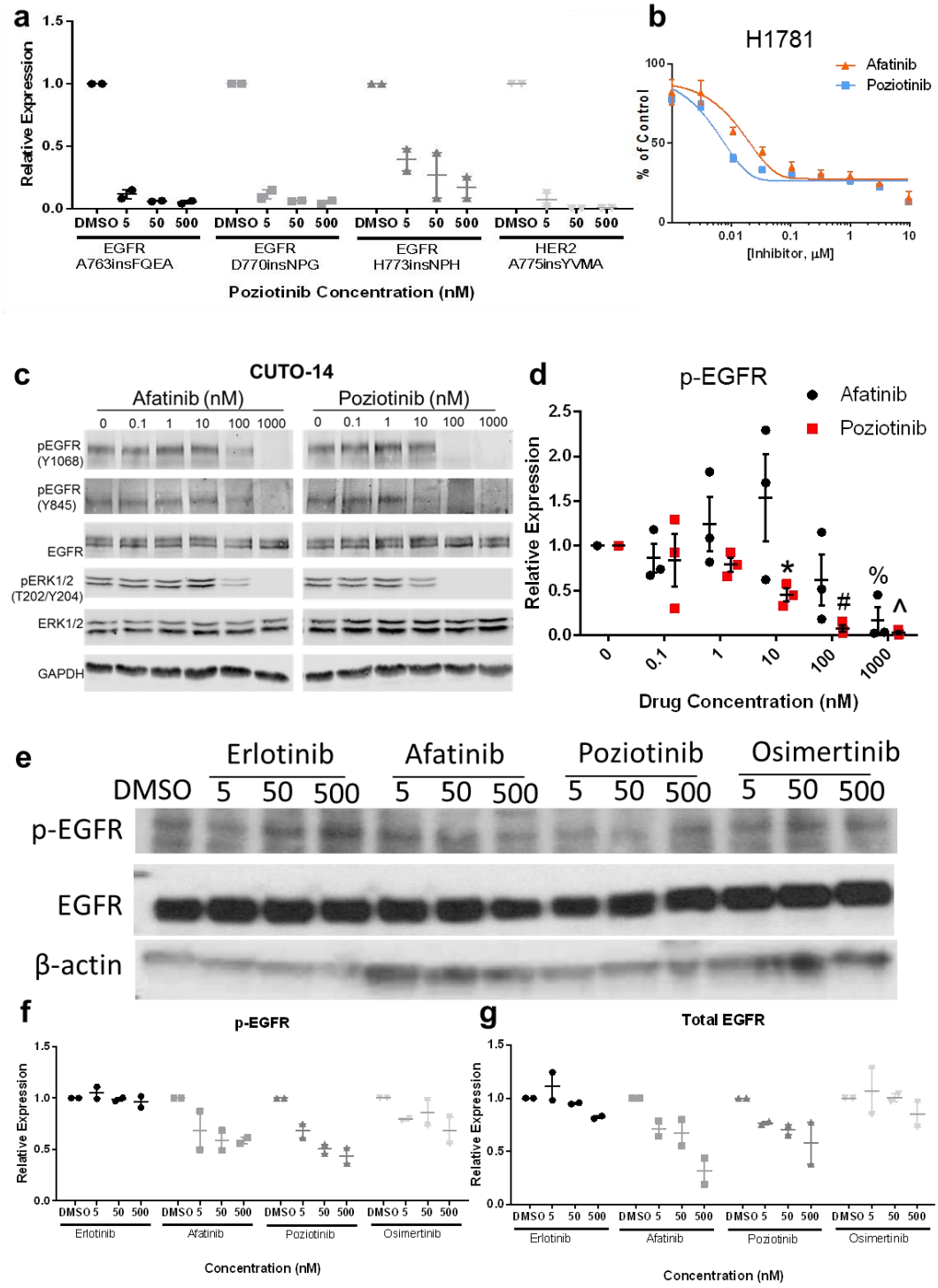


Supplementary figure 5: Exon 20 insertions alter the local conformation of the drug binding pockets of EGFR and HER2 receptors. a, 3-D modeling of EGFR D770insNPG (green), T790M (yellow) and WT (grey) show that the NPG insertion (pink), result in T793 of D770insNPG to reside in the same location as T790M. This shift of the gatekeeper residue is predicted to result in increased affinity to ATP and reduced binding of erlotinib (shown in pink) Moreover, modeling displays the increasing inward shift of the P-loop (red arrow) from the position of WT to the most inward position of the exon 20 insertion. **b**, 3-D modeling of HER2 A775insYVMA (green) and WT (grey) shows that the YVMA insertion (magenta loop) results in shifting of the gatekeeper residue (T798 for wild type and T802 for insertion) as well as constitutive activation of the receptor with rigid placement of the α-c-helix in the inward position, which is predicted to reduce binding of lapatinib (pink) in the HER2 WT (grey) model. Furthermore, modeling displays the substantial shift of the P-loop into the binding pocket (red arrow).**c,** 3-D modeling of EGFR D770insNPG (green) and T790M (yellow). The NPG insertion (pink) induces a substantial shift (red arrow) of the P-loop (red) into the binding pocket thereby pushing osimertinib (blue) out of the binding pocket preventing binding at C797 (black). **d,** 3-D modeling of HER2 A775insYVMA (blue) and WT (yellow). The YVMA insertion (pink) induces a shift of the P-loop (red) and α-c-helix into the drug binding pocket, reducing the size of the binding pocket and preventing larger inhibitors like osimertinib from binding.

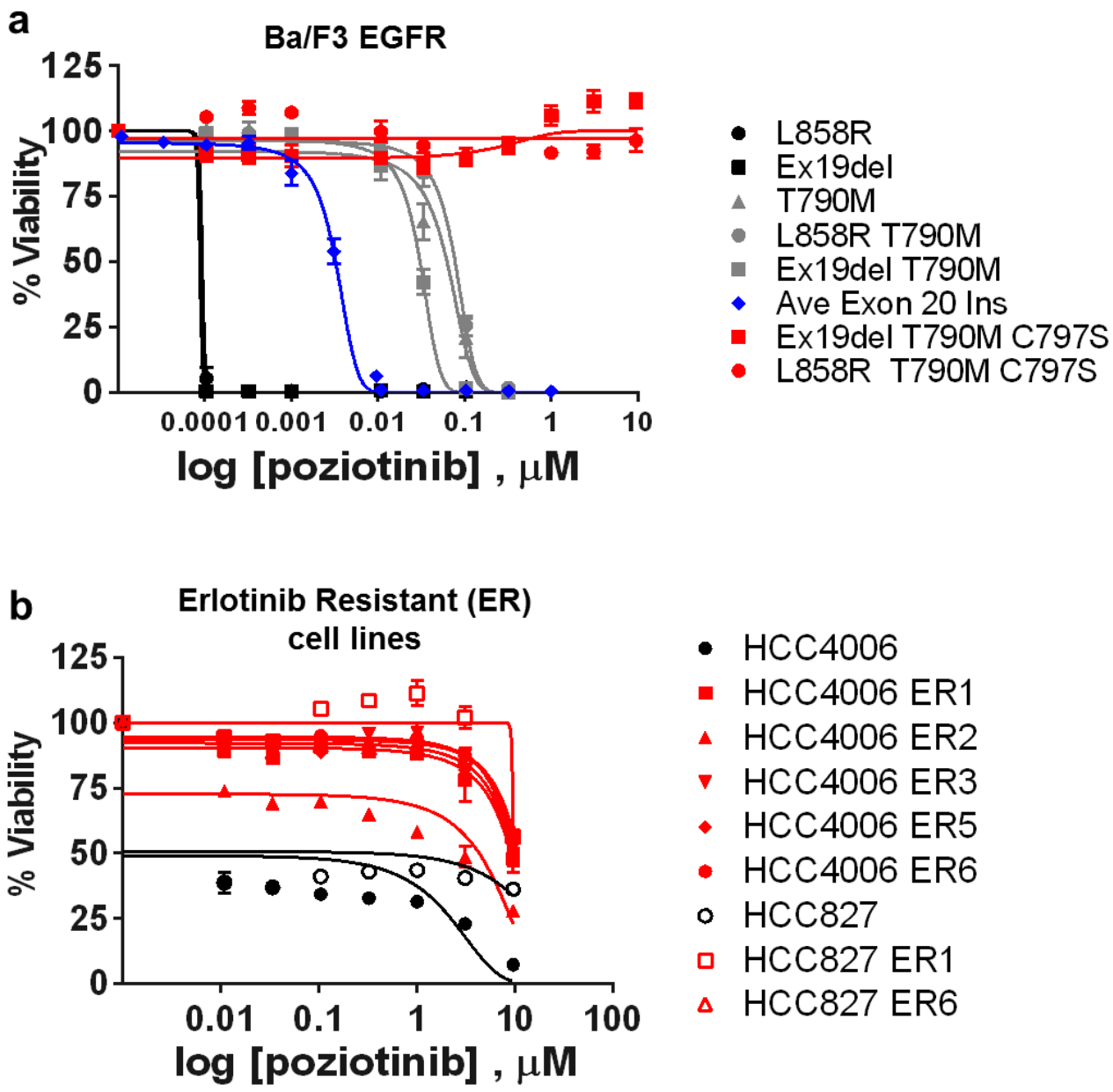


	Affinity ΔG	London ΔG
Afatinib (blue)	9.6494	-9.8287
Poziotinib (orange)	0.7711	-13.0410

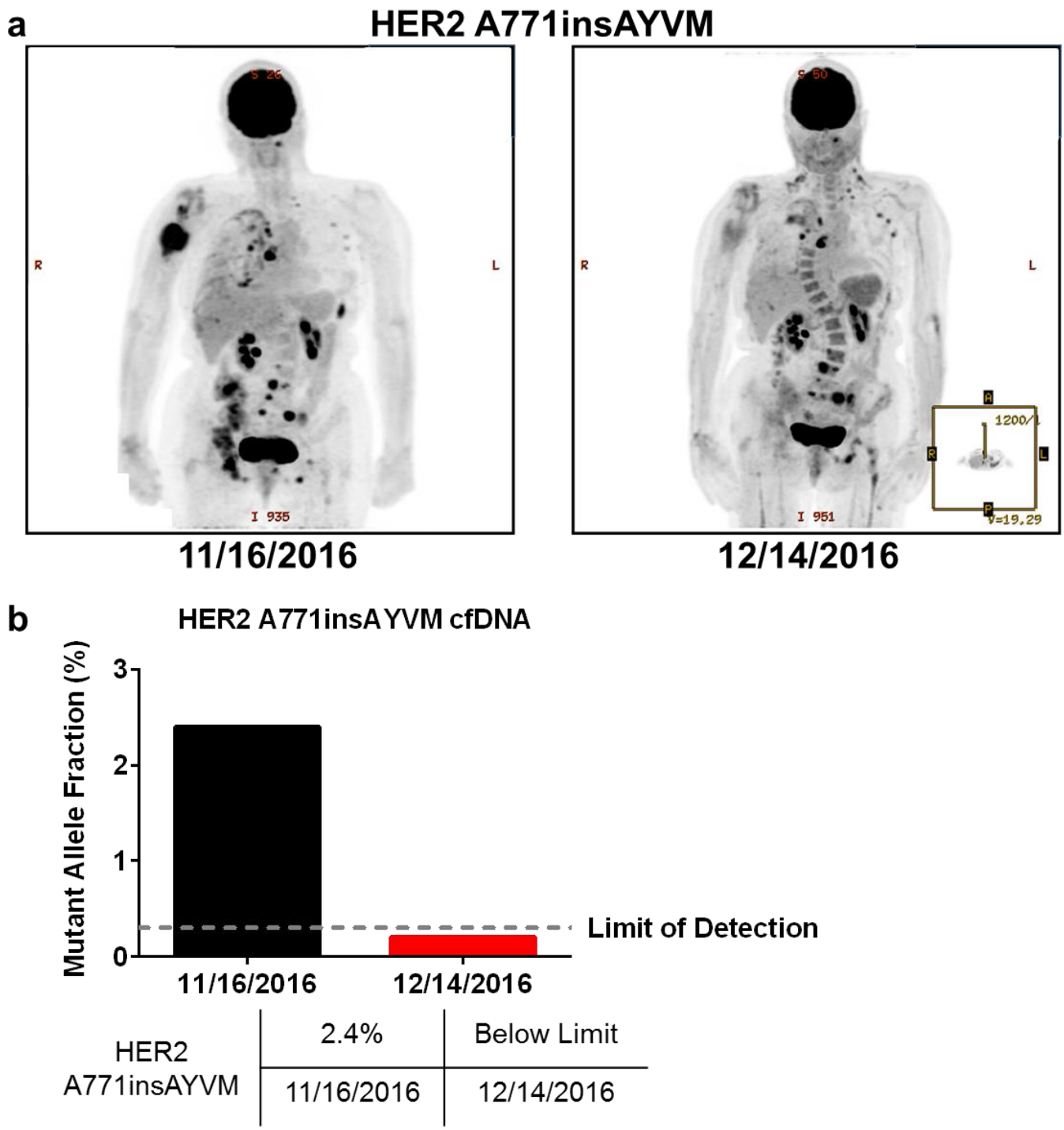
Supplementary figure 6: Poziotinib is predicted to tightly bind EGFR and HER2 exon 20 insertions. a, Illustration of interactions of poziotinib and EGFR exon 20 insertion mutation binding pocket demonstrates that the tri-halogenated terminal benzene ring sits in a hydrophobic pocket formed by residues such as L791, T793 and L802. Also this electron-rich moiety of poziotinib interacts with basic residues including K745 and further stabilizes binding of the inhibitor. On the other end of the molecule, the inhibitor is in a position oriented towards C800 (equivalent to C797 in wild type), rendering the possibility to form covalent bond with the receptor. **b,** Space filling model of EGFR L858R/T790M with osimertinib (blue) has a similar size binding pocket as EGFR T790M single mutant, and a large binding pocket compared to EGFR exon 20 insertion mutations. **c,** 3-D modeling of EGFR D770insNPG (green) with poziotinib (orange) and afatinib (blue) predicts that poziotinib effectively binds C797 more tightly than afatinib. Predicted free energy of binding (London ΔG) is lower for poziotinib and exon 20 insertions than afatinib. **d,** HER2 WT (pink) with osimertinib (green) has a much larger binding pocket compared to **e,** HER2 A775insYVMA (green) with poziotinib (yellow). Poziotinib tightly binds into the smaller binding pocket of HER2 A775insYVMA allowing the potential covalent bond formation as illustrated in black lines at C801.



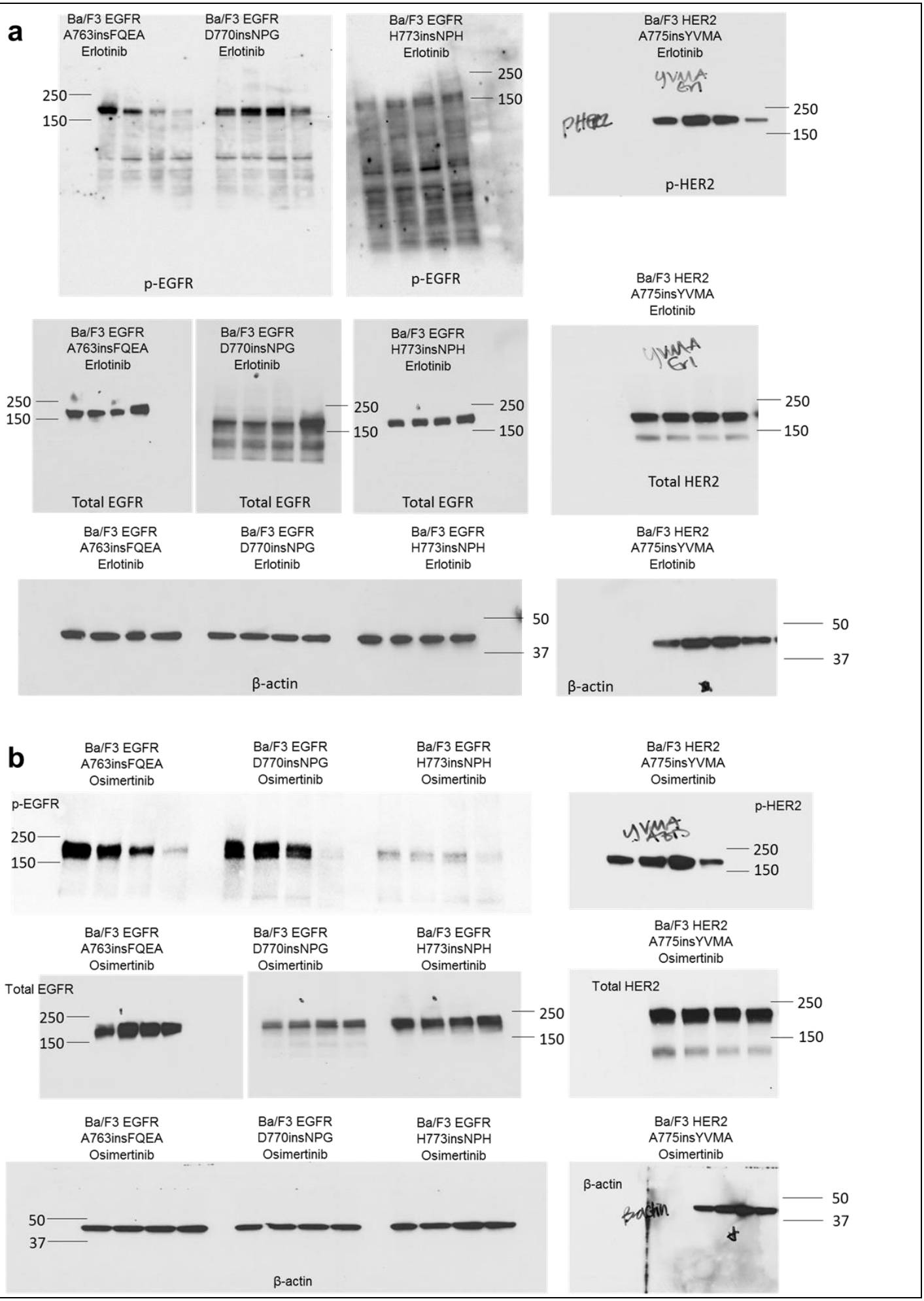
Supplementary figure 7: EGFR and HER2 exon 20 insertions mutations are sensitive to poziotinib *in vitro*. a, Western blots of p-EGFR and p-HER2 after 2 hours of poziotinib treatment in indicated Ba/F3 cell lines were quantified. Dot plots are representative of mean ±SEM. (n=2 biologically independent experiments) **b,** Dose response curves of cell viability of H1781 cell line expressing HER2 G776del insVC treated with poziotinib or afatinib for 72 hours (n=3 biologically independent experiments) **c,** Representative Western blot of CUTO-14 patient-derived cell line after 3 hours of treatment with afatinib or poziotinib (n=3 biologically independent experiments). Uncropped western blots are available in supplementary figure 10. **d,** Quantification of p-EGFR from Western blots after 3 hours of treatment with afatinib or poziotinib in CUTO-14 cell line. Two-way ANOVA with Fisher's test was used to determine statistical differences from DMSO control: p = 0.02 (*), p = 0.006 (#), p = 0.012 (%) p = 0.004 (^). **e**, Representative Western blot of p-EGFR and total EGFR after 18 hours treatment of erlotinib, afatinib, poziotinib, and osimertinib at the indicated doses in EGFR WT NSCLC cell line H292. (n=2 biologically independent experiments.) Uncropped Western blots are available in supplementary figure 10. **f,** Quantification of p-EGFR from Western blots after 18 hours of treatment with erlotinib, afatinib, poziotinib, and osimertinib in H292 cell line. **g,** Quantification of total EGFR from H292 Western blots after 18 hours of treatment with erlotinib, afatinib, poziotinib, and osimertinib.



Supplementary figure 8: C797S and EMT are two distinct mechanisms of poziotinib resistance *in vitro*. a, Dose response curves of cell viability of *EGFR* mutant Ba/F3 cell lines treated with poziotinib for 72 hours. The mean ± SEM is plotted for each concentration (n=3 biologically independent experiments). **b,** Dose response curves of cell viability of erlotinib resistant (ER) and parental NSCLC cell lines treated with poziotinib for 72 hours. The mean ± SEM is plotted for each concentration (n=2 biologically independent experiments).



Supplementary figure 9: Poziotinib induces radiological response in a patient with HER2 exon 20 insertion mutant NSCLC. a, PET scans of patient 1 day before poziotinib treatment and 4 weeks after therapy showed reduction in FDG avid lesions in the right humerus, iliac bone, sacrum, the left 7th rib, as well as the nodule in the right lower lobe. **b,** Bar graph of HER2 A771AYVM cfDNA before and after poziotinib therapy shows a reduction of HER2 A771insAYVM cfDNA to undetectable levels.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Ba/F3 EGFR H773insNPH Erlotinib
Ba/F3 HER2 A775insYVMA Erlotinib
250
150
p-EGFR
p-HER2
Total EGFR
Total HER2
50
37
β-actin
b
Ba/F3 EGFR A763insFQEA Osimertinib
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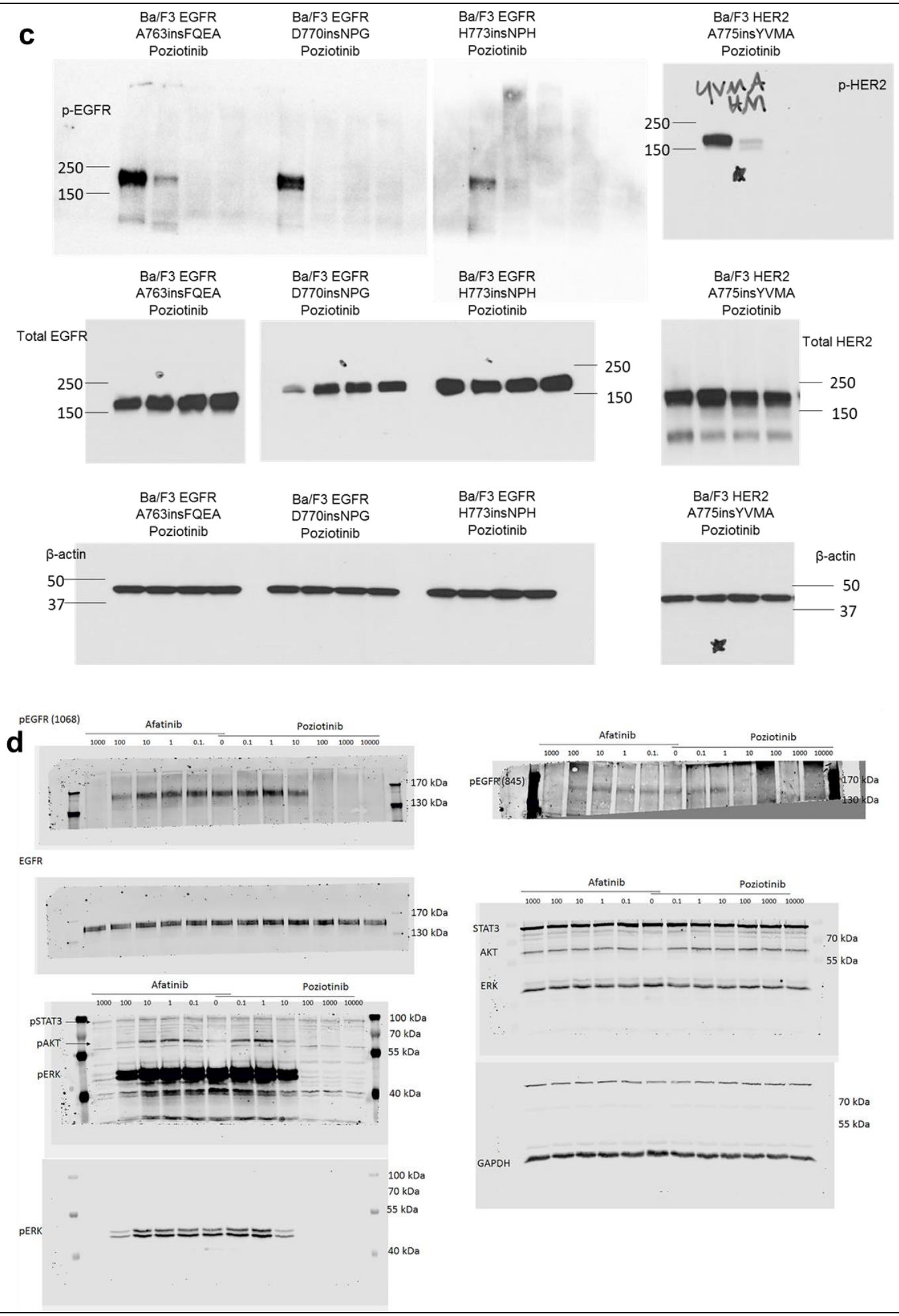
Ba/F3 HER2 A775insYVMA Osimertinib


c
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Ba/F3 EGFR D770insNPG Poziotinib
Ba/F3 EGFR H773insNPH Poziotinib
Ba/F3 HER2 A775insYVMA Poziotinib
p-EGFR
p-HER2
250
150
Total EGFR
Total HER2
β-actin
50
37
d
pEGFR (1068)
Afatinib
Poziotinib
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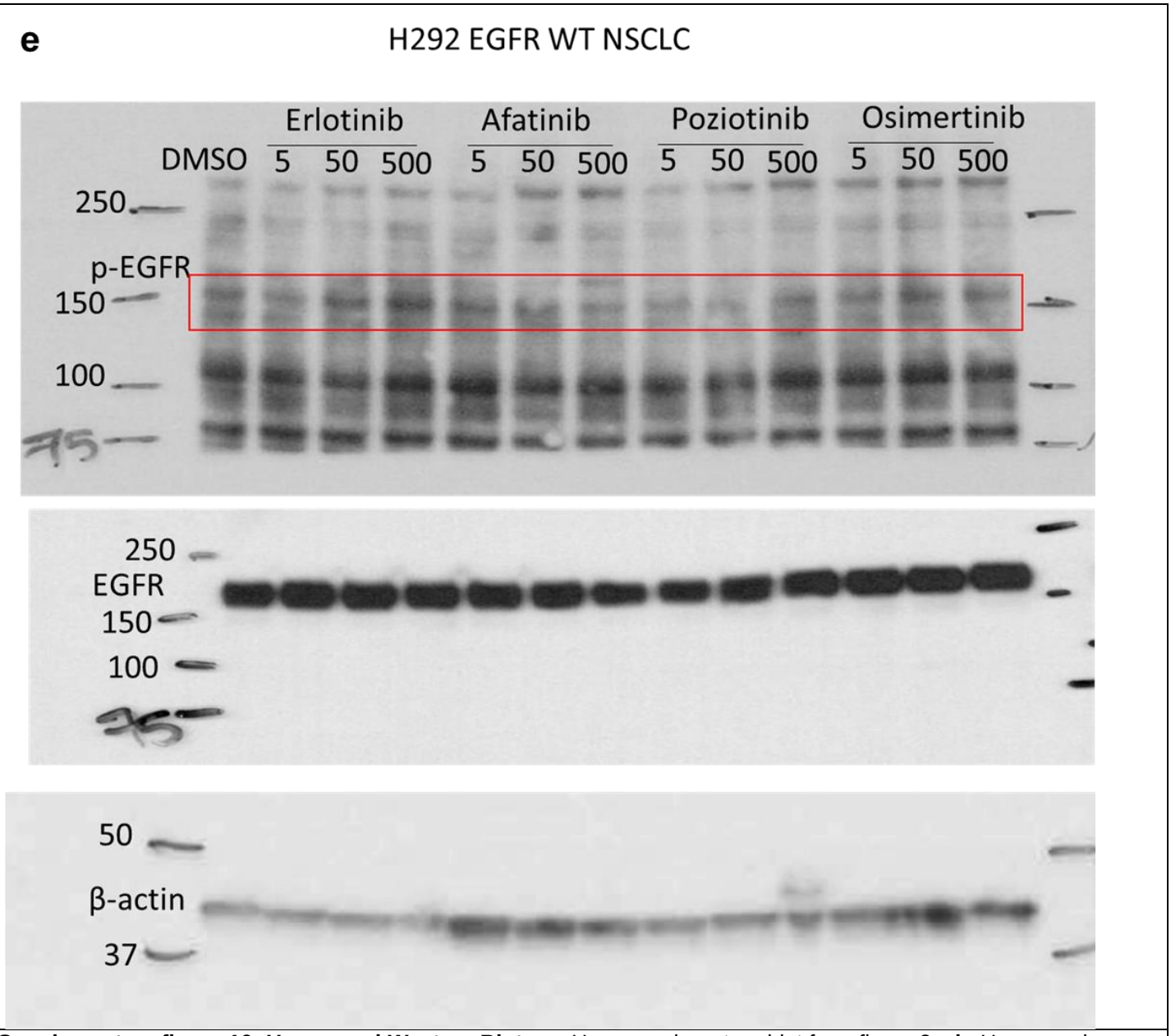

Supplementary figure 10: Uncropped Western Blots. **a,** Uncropped western blot from figure 2c. **b,** Uncropped western blot from supplementary figure 4a. **c,** Uncropped western blot from supplementary figure 4c. **d,** Uncropped western blot from supplementary figure 7c. **e,** Uncropped western blot from supplementary figure 7e.

Supplementary Table 1: IC_{50} values of EGFR and HER2 Exon 20 insertions with EGFR/HER2 TKIs

		Ave EGFR Exon 20 insertions (N=6 cell lines)	Ave HER2 Exon 20 insertions (N=6 cell lines)
1st gen TKI	Erlotinib	3,310 nM	3,250 nM
	Gefitinib	>10,000 nM	12,900 nM
	Lapatinib	-	1,190 nM
	L858R + Erlotinib	17.0 nM	
2nd gen TKI	Afatinib	39.9 nM	11.7 nM
	Dacomitinib	61.1 nM	12.4 nM
	Neratinib	135 nM	10.4 nM
	L858R + Afatinib	0.876 nM	
3rd gen TKI	Osimertinib	103nM	444 nM
	Rociletinib	850nM	505 nM
	Ibrutinib	143 nM	114 nM
	Olumtinib	204 nM	352 nM
	Nazartinib	198 nM	233 nM
	L858R/T790M + Osimertinib	9.00 nM	

Supplementary Table 2: Figure 3g p-values

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
Vehicle Vs. Afatinib (20mg/kg)	NS	NS	NS	NS	NS	NS	5.06E-03	2.84E-03	4.77E-03	1.03E-03
Vehicle Vs. Poziotinib (5mg/kg)	NS	NS	NS	1.30E-03	NS	4.40E-05	1.46E-03	1.20E-05	1.60E-06	7.00E-07
Vehicle Vs. Poziotinib (10mg/kg)	NS	NS	1.15E-02	4.41E-04	1.00E-04	1.16E-05	1.10E-06	2.00E-07	2.00E-07	2.30E-06
Afatinib Vs. Poziotinib (5mg/kg)	NS	1.27E-02	9.16E-03	8.00E-04	NS	5.37E-03	9.79E-03	6.08E-04	7.66E-03	3.57E-03
Afatinib Vs. Poziotinib (10mg/kg)	NS	2.70E-03	8.50E-03	1.51E-04	9.96E-03	3.75E-03	7.60E-04	7.90E-05	9.86E-03	1.10E-02
Poziotinib (5mg/kg) vs Poziotinib (10m/kg)	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

Supplementary Table 3: Patient history

	Mutation	Previous Treatments	Current Dose	Age
1	V769insGSV	Carboplatin, pemetrexed, oncothermia, ASP8273	16mg	55
2	H773insAH	Carboplatin, docetaxel, paclitaxel, pembrolizumab	12mg	29
3	H773dupPR	Carboplatin, pemetrexed, avastin	12mg	52
4	D770insY H773Y	Carboplatin, pemetrexed	12mg	71
5	S768dupSVD	Cisplatin, pemetrexed, nivolumab	12mg	76
6	N771insHH	Nivolumab, WBRT, pemetrexed, avastin, carboplatin	16mg	66
7	D770insG	Carboplatin, pemetrexed, pembrolizumab	12mg	59
8	P772insDNP	Carboplatin, pemetrexed, pembrolizumab	8mg	58
9	A767dupASV	Cisplatin, carboplatin, pemetrexed, pembrolizumab	8mg	46
10	S768I	Cisplatin, pemetrexed, carboplatin, docetaxel, afatinib, erlotinib, radiation	12mg	62
11	D770del insGY	Afatinib, AP32788	16mg	60

Supplementary table 4: Vectors used to generate stable cell lines

Name	Mutation	Vendor
EGFR A763insFQEA	c.2290_2291insTCCAGGAAGCCT	Created from Bioinnovatise from pBabe-puro-EGFR WT from Addgene (#11011)
EGFR A767insASV	c.2302_2303insGCCAGCGTG	Purchased from Addgene (#32066)
EGFR S768dupSVD	c.2303_2304dupAGCGTGGAC	Created from Bioinnovatise from pBabe-puro-EGFR WT from Addgene (#11011)
EGFR V769insASV	c.2308_2309insCCAGCGTGG	Created from Bioinnovatise from pBabe-puro-EGFR WT from Addgene (#11011)
EGFR D770insNPG	c.2310_2311insAACCCCGGC	Purchased from Addgene (#11016)
EGFR D770insSVD	c.2311_2312insGCGTGGACA	Created from Bioinnovatise from pBabe-puro-EGFR WT from Addgene (#11011)
EGFR H773insNPH	c.2319_2320insAACCCCCAC	Created from Bioinnovatise from pBabe-puro-EGFR WT from Addgene (#11011)
EGFR T790M		Purchased from Addgene (#32070)
EGFR T790M L858R		Purchased from Addgene (#32073)
EGFR T790M Ex19del		Purchased from Addgene (#32072)
EGFR T790M L858R C797S	c.2389T>A	Created from Bioinnovatise from pBabe-puro-EGFR L858R/T790M from Addgene (#32073)
EGFR T790M Ex19del C797S	c.2389T>A	Created from Bioinnovatise from pBabe-puro-EGFR Del1/T790M from Addgene (#32072)
HER2 S310F	c.929C>T	Purchased from Addgene (#40991)
HER2 S310Y	c.929C>A	Purchased from Addgene (#40992)
HER2 C311R	c.931T>C	Purchased from Addgene (#40980)
HER2 L755P	c.2263_2264delinsCC	Created by Bioinnovatise from pBabe-puro HER2 WT from Addgene (#40978)
HER2 A775insV G776C	c.2323–2324insTTT	Purchased from Addgene (#40979)
HER2 A775insYVMA	c.2323_2324insTATGTCATGGCT	Purchased from Addgene (#40982)
HER2 G776V	c.2327G>T	Created by Bioinnovatise from pBabe-puro HER2 WT from Addgene (#40978)
HER2 G776C V777insV	c.2326G>T, c.2331_2332insTGT	Created by Bioinnovatise from pBabe-puro HER2 WT from Addgene (#40978)
HER2 G776del insVV	c.2327delinsTTGT	Created by Bioinnovatise from pBabe-puro HER2 WT from Addgene (#40978)
HER2 G776del insVC	c.2326_2328insTCT	Created by Bioinnovatise from pBabe-puro HER2 WT from Addgene (#40978)
HER2 P780insGSP	c.2339_2340insTGGCTCCCC	Created by Bioinnovatise from pBabe-puro HER2 WT from Addgene (#40978)