Supplementary Information for:

Quantitative analysis of tyrosine phosphorylation from FFPE tissues reveals patient specific signaling networks

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Supplementary Materials

Figure S1. Protein extraction and pTyr analysis in single 10-µm FFPE sections.

Figure S2. Phosphotyrosine analysis of FFPE specimens from NSCLC patients from a tumor tissue bank.

Figure S3. Cluster set enrichment analysis for clusters observed in Figure 2c.

Figure S4. Phosphorylation analysis of frozen and FFPE breast cancer tumors.

Figure S5. Barplots with phosphorylation levels of various proteins quantified for each patient based on frozen tissues of breast cancer tumors.

Figure S6. Comparison of phosphoproteomics and proteomics in FFPE and flash frozen tissues.

Figure S7. Phosphotyrosine signaling in response to afatinib treatment in selected proteins belonging to EGFR pathway

Supplementary Information: Step-by-step detailed protocol for protein/peptide extraction from FFPE tissues.

Data file 1: Tables with quantitative data extracted from proteomics experiments.

Data file 2: Clinical and pathological information for breast cancer and NSCLC tumor samples.

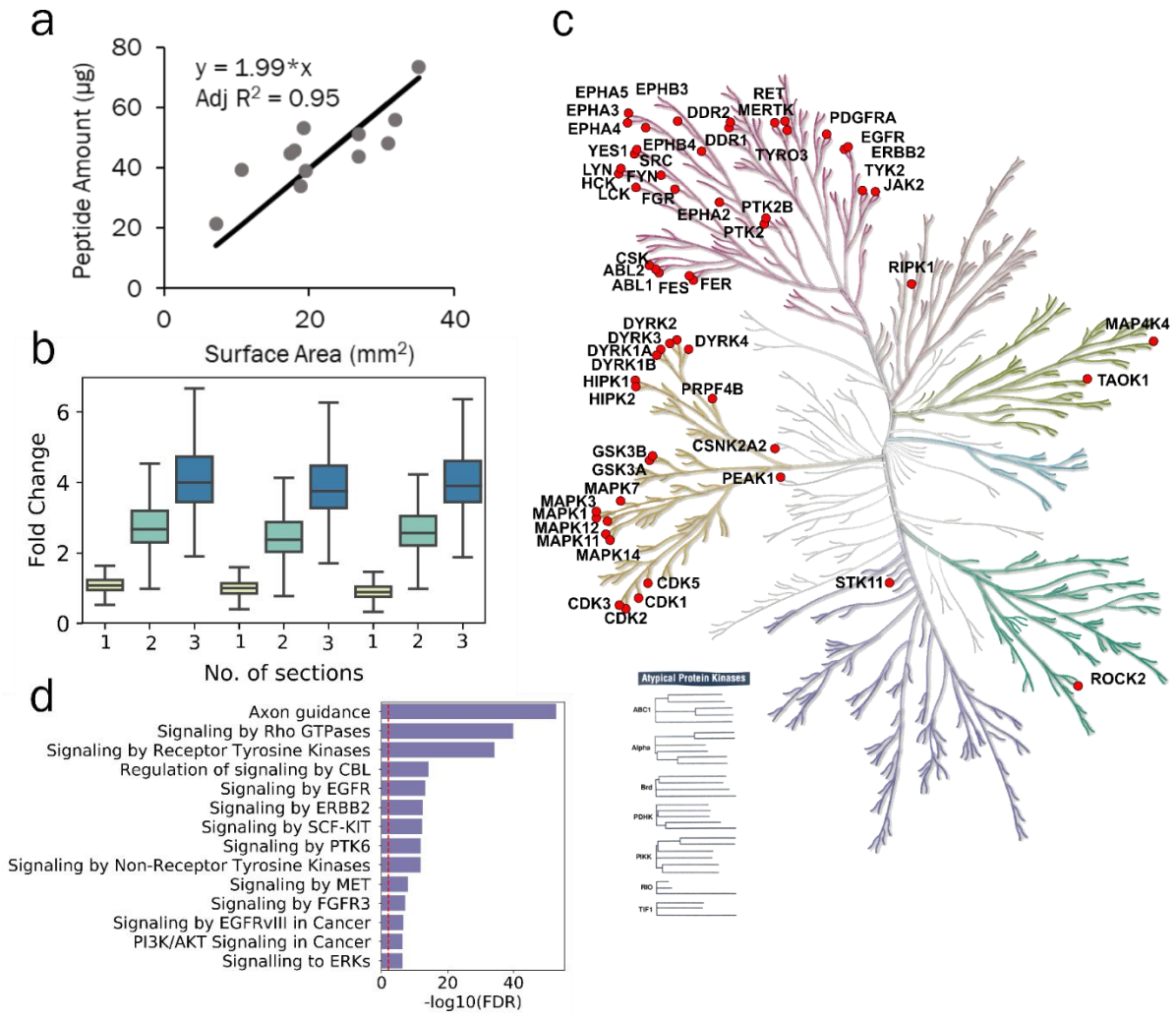

Figure S1. Protein extraction and pTyr analysis in single 10-µm FFPE sections. **a)** Peptide yields from FFPE tissues were proportional to the surface area of tumors. Approximately two micrograms of peptide were derived per mm^2 of FFPE tissue. **b)** Fold change of TMT intensities of peptides quantified in each channel compared to the average of TMT intensities from single sections from crude lysate analysis. Error bars represent interquartile range. **c)** Kinome tree depicting pTyr containing proteins identified in single 10-µm sections of FFPE tissues. **d)** Selected reactome pathways enriched in gene ontology analysis of pTyr-proteins quantified in single 10-µm FFPE sections. Dashed red line depicts FDR q-value = 0.01.

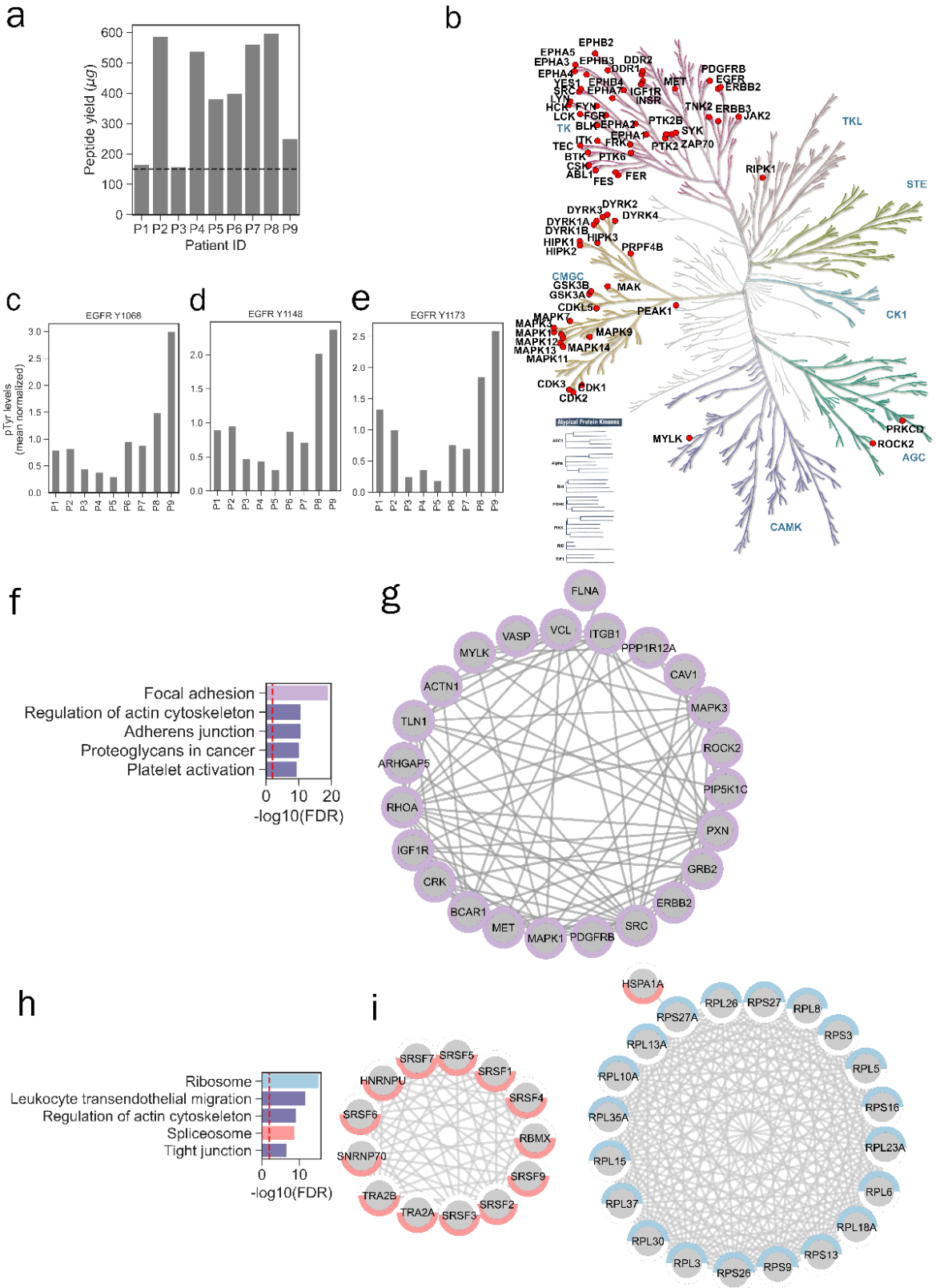
a
Peptide yield (µg)
Patient ID
P1 P2 P3 P4 P5 P6 P7 P8 P9
b
TK
TKL
STE
CMGC
CK1
AGC
CAMK
Atypical Protein Kinases
c
EGFR Y1068
d
EGFR Y1148
e
EGFR Y1173
pTyr levels (mean normalized)
f
Focal adhesion
Regulation of actin cytoskeleton
Adherens junction
Proteoglycans in cancer
Platelet activation
-log10(FDR)
g
h
Ribosome
Leukocyte transendothelial migration
Regulation of actin cytoskeleton
Spliceosome
Tight junction
-log10(FDR)
i

Figure S2. Phosphotyrosine analysis of FFPE specimens from NSCLC patients from a tumor tissue bank. **a)** Peptide yields from 2 10-µm sections of FFPE tissues as measured by BCA assay (average peptide yield = 403 µg). Dashed line depicts peptide amount of 150 µg used for a multiplexed analysis. **b)** Kinome tree depicting pTyr containing proteins quantified in the multiplexed pTyr analysis. **c-e)** Phosphorylation levels of tyrosine sites on EGFR plotted relative to the mean of all 9 tumors. **f)** Top 5 significantly enriched Kegg pathways in pTyr-proteins belonging to cluster 2 from Figure 2c. **g)** Interaction network of proteins belonging to Focal adhesion that were identified in cluster 2. **h)** Top 5 significantly enriched Kegg pathways in pTyr-proteins belonging to cluster 3 from Figure 2c. **i)** Interaction network of proteins belonging to Ribosome (cyan) and Spliceosome (red) that were identified in cluster 3. All of the interactions are highest confidence based on all interaction sources except text mining from STRING database. Dashed red line depicts FDR q-value = 0.01.

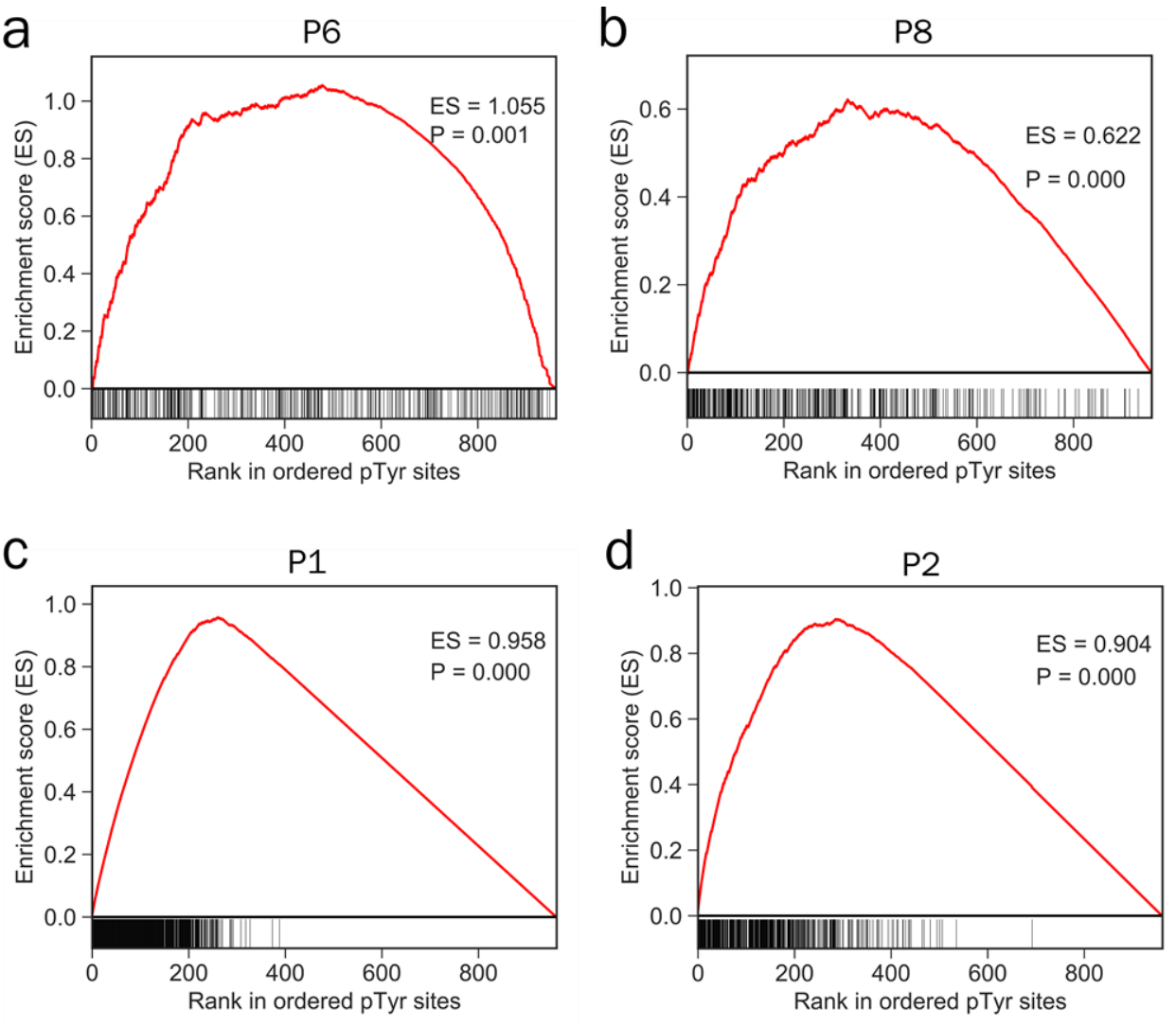

Figure S3. Cluster set enrichment analysis for clusters observed in Figure 2c. Enrichment of cluster 1 in **(a)** P6 and **(b)** P8. Enrichment of **(c)** cluster 2 in P1 and **(d)** cluster 3 in P2. Phosphotyrosine sites were rank ordered according to their mean normalized phosphorylation levels compared to all 9 tumors, and running enrichment score was calculated. Significance (P) of ES was derived from 1000 permutations where ranks of pTyr sites were randomized. P represents fraction of permutations where the maximum ES was greater than the observed one.

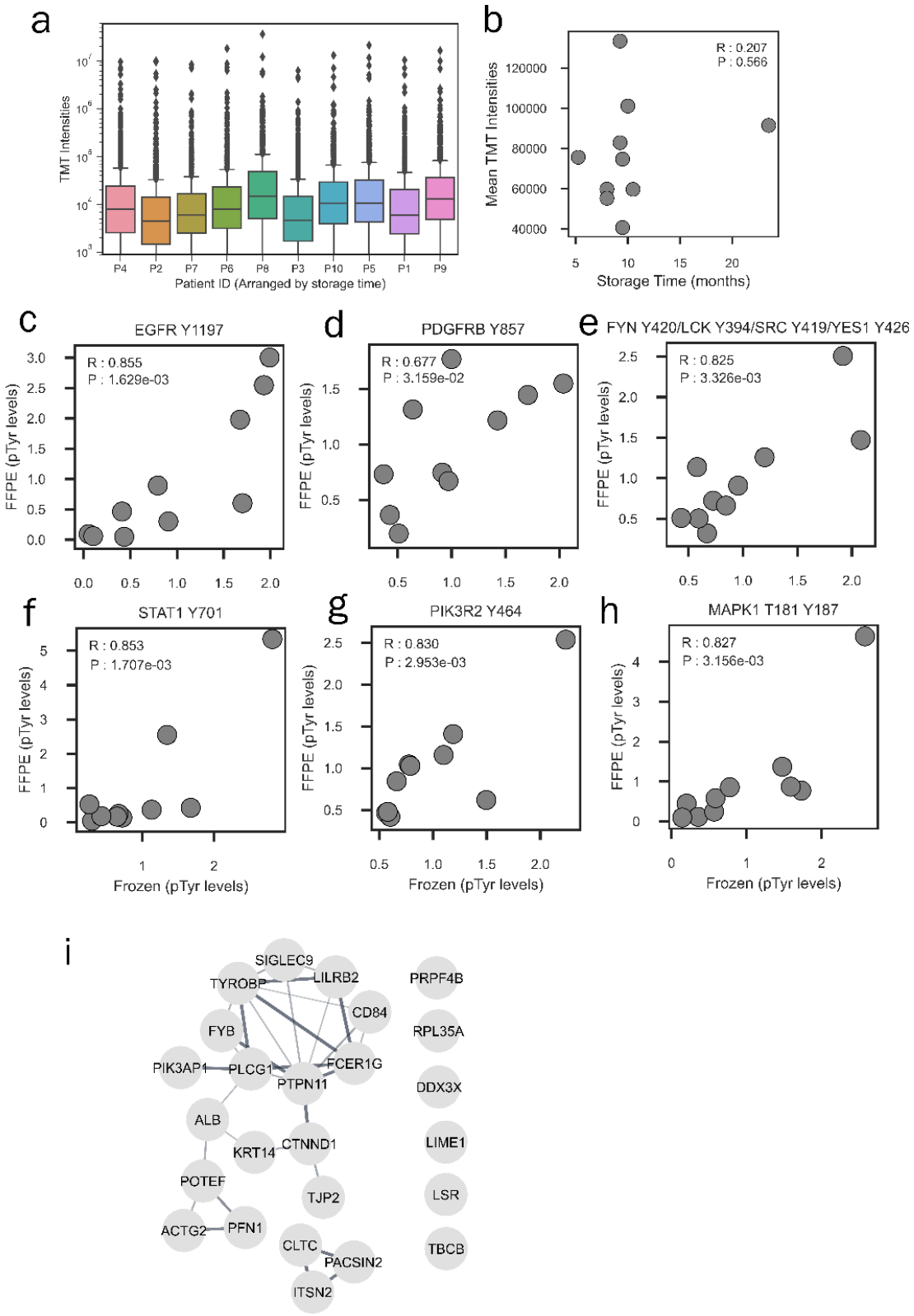
a
TMT Intensities
P4
P2
P7
P6
P8
P3
P10
P5
P1
P9
Patient ID (Arranged by storage time)
b
Mean TMT Intensities
R : 0.207
P : 0.566
Storage Time (months)
c
EGFR Y1197
R : 0.855
P : 1.629e-03
FFPE (pTyr levels)
d
PDGFRB Y857
R : 0.677
P : 3.159e-02
e
FYN Y420/LCK Y394/SRC Y419/YES1 Y426
R : 0.825
P : 3.326e-03
f
STAT1 Y701
R : 0.853
P : 1.707e-03
Frozen (pTyr levels)
g
PIK3R2 Y464
R : 0.830
P : 2.953e-03
h
MAPK1 T181 Y187
R : 0.827
P : 3.156e-03
i
SIGLEC9
TYROBP
LILRB2
CD84
FYB
PIK3AP1
PLCG1
FCER1G
PTPN11
ALB
KRT14
CTNND1
POTEF
TJP2
ACTG2
PFN1
CLTC
PACSIN2
ITSN2
PRPF4B
RPL35A
DDX3X
LIME1
LSR
TBCB

Figure S4. Phosphorylation analysis of frozen and FFPE breast cancer tumors. **a)** Boxplot showing TMT intensities of phosphotyrosine peptides across different FFPE samples. Patient IDs are arranged by the storage time of FFPE samples (in ascending order). **b)** Mean TMT intensities of phosphotyrosine peptides plotted against storage time. P value is derived from Person's correlation test. **c-h)** Correlation plots of selected pTyr sites between FFPE and flash frozen pairs. pTyr levels are plotted relative to the mean of all 10 tumors (mean normalized) within each processing condition. **i)** Proteins with pTyr sites that were poorly correlated between flash frozen and FFPE tissues of breast cancer patient tumors.

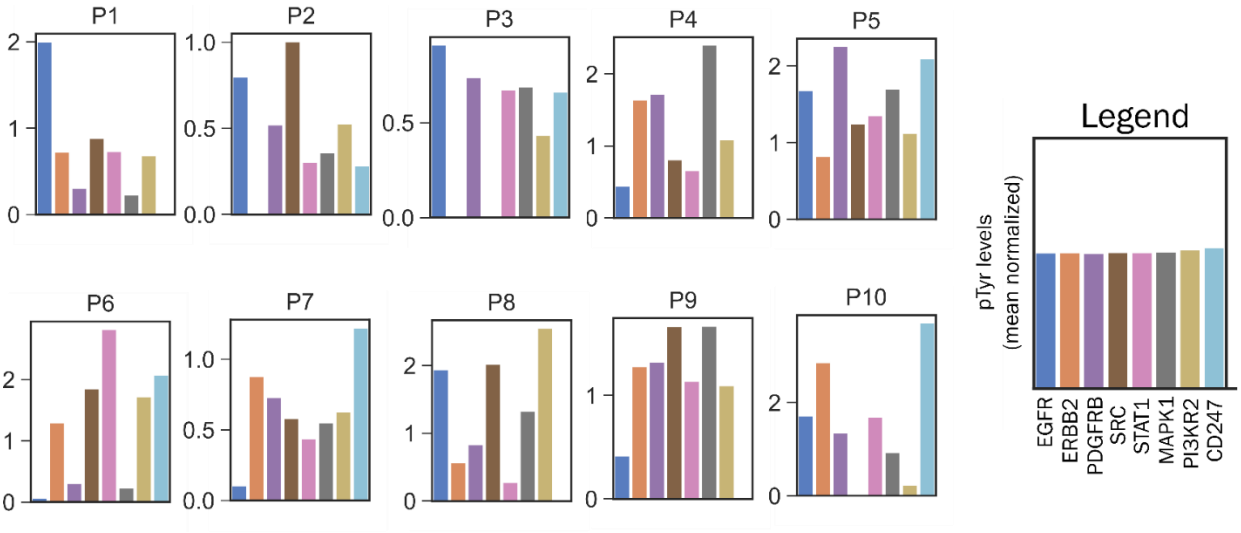

Figure S5. Barplots with phosphorylation levels of various proteins quantified for each patient based on Frozen tissues of breast cancer tumors. Phosphorylation levels represent average phosphorylation across multiple pTyr sites for a given protein target and are plotted relative to the mean of all 10 tumors (mean normalized). ERBB3 and MET were not identified and quantified in Frozen tissues compared to FFPE tissues in Figure 3e.

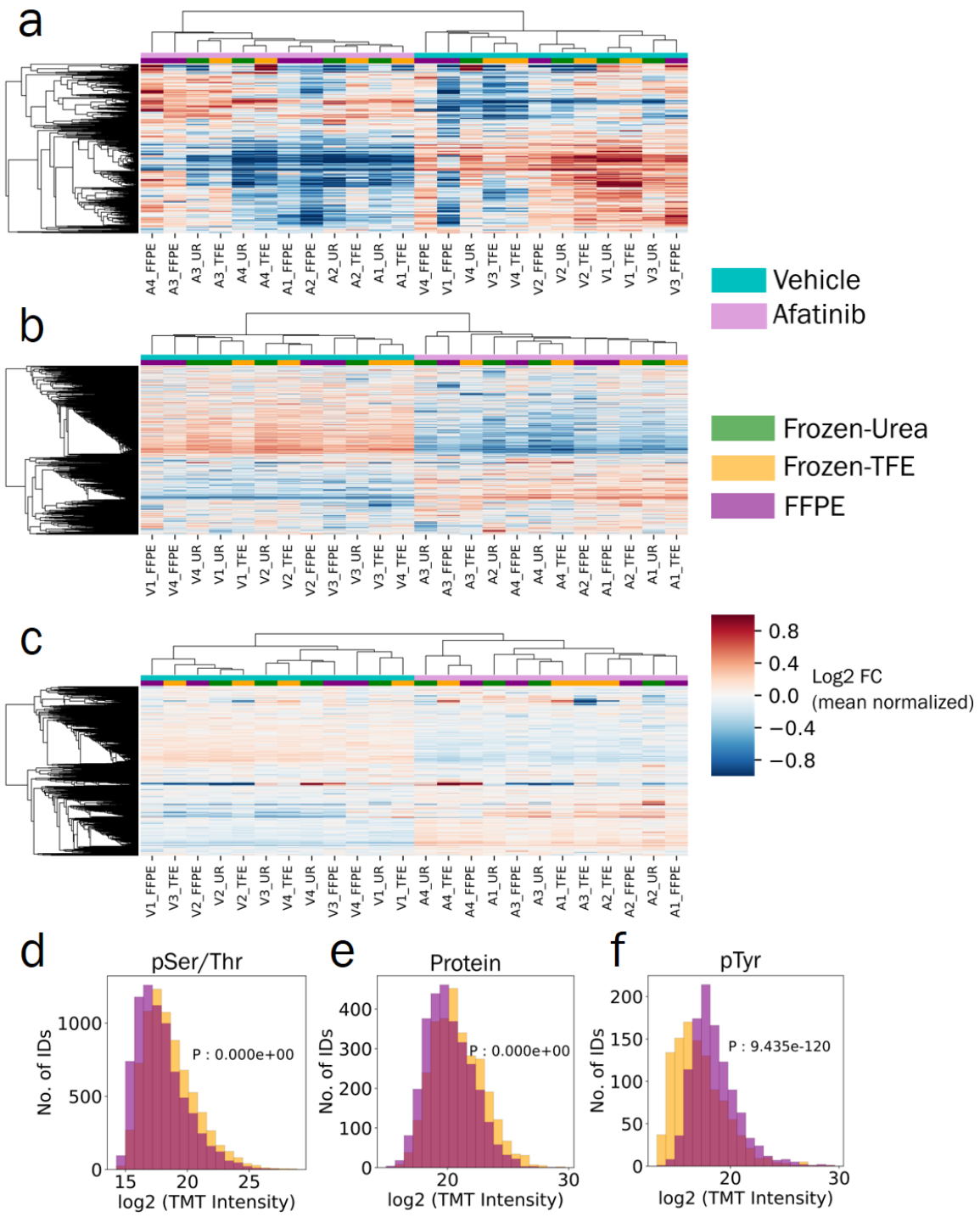

Figure S6. Comparison of phosphoproteomics and proteomics in FFPE and flash frozen tissues. **a-c)** Hierarchical clustering heatmap of **(a)** pTyr, **(b)** pSer/Thr and **(c)** proteins identified and quantified across Frozen-Urea (UR), Frozen-TFE and FFPE workflows. Quantified levels were mean normalized and $\log_2$

transformed within each workflow before concatenating together. **d-f)** Differential TMT intensities observed in Frozen-TFE and FFPE workflows across **(d)** pSer/Thr, **(e)** proteins and **(f)** pTyr. TMT intensities were summed across all channels within the workflow. P values were derived from paired two-sided *t*-test.

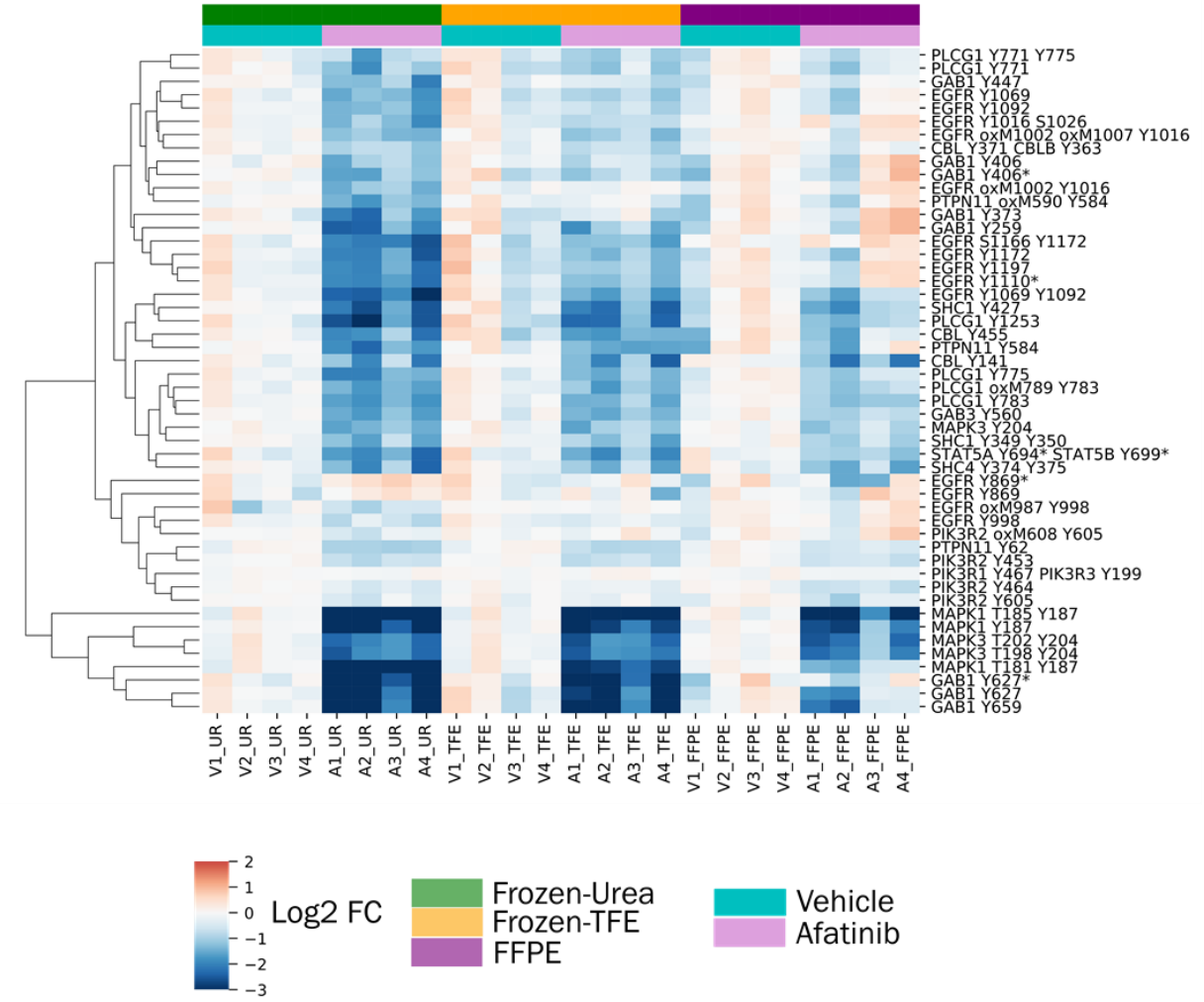

Figure S7. Phosphotyrosine signaling in response to afatinib treatment in selected proteins belonging to EGFR pathway as quantified in Frozen-Urea (UR), Frozen-TFE and FFPE workflows. Quantified levels are presented as $\log_2$ fold change relative to the average of vehicle treated group. Oxidation of methionine is denoted by oxM. Miscleaved peptides are denoted by *.

FFPE Tissue Processing

Materials:

- Xylene (Sigma 534056)
- Ethanol (200 Proof)
- TFE (2,2,2 Trifluoroethanol)
- 50 mM Sodium bicarbonate
- HALT Protease and Phosphatase inhibitor cocktail
- 50 mM Hepes (Sigma H3375) at pH 8.5
- 1M DTT (Sigma D0632) in 50 mM Hepes at pH 8.5
- 800mM Iodoacetamide (Sigma I1144) in 50 mM Hepes at pH 8.5
- Trypsin (Promega V5113)
- Sp3 Beads (GE Healthcare 45152105050250, Thermo 65152105050250)
- Pierce Protein BCA Assay

Lysis Buffer: 50% TFE in 25 mM Sodium bicarbonate with 10mM DTT and 1x Halt protease and phosphatase cocktail, pH 8.5. Heat the lysis buffer at 90 °C before adding to the sample to minimize gradual temperature changes.

  - Make it fresh
  - Make the volume that you need (200 µL per sample, usually 2-3 mL for 10 samples)

Notes:

- SP3 protocol works best for protein amount less than 500 µg. Sample losses have been observed for higher protein input.
- SP3 bead amount should be kept above 0.1 ug/uL during the binding step.
- Expect peptide yields of ~2 µg per mm^2 of a 10-µm section of FFPE tissue from previous experience; however, peptide yields can vary with types of tissues, etc.
- Assume 100 µg protein per 10-µm FFPE section for tissue with cross-sectional surface area of $\leq$ 50 mm^2. Otherwise, you can calculate cross-section area and adjust approximate protein amounts for each sample.

DAY 1:

1. After sectioning on a microtome, place a 10-µm FFPE section in 1.7 mL tube. If using multiple sections from same samples, add them to the same tube.
2. Add 500 µL of xylene and incubate on rotator for 5 minutes. Spin down at 1000 rcf for 30 seconds, and discard xylene supernatant. Be watchful while pipetting so that you don't discard the FFPE tissue.
3. Repeat Step 2 one more time.
4. Add 500 µL of ethanol, and let it sit for 5 minutes to hydrate the tissue section. Discard the ethanol.
5. Leave tube open for residual ethanol to evaporate for few mins.
6. Add 200 µL of lysis buffer to each sample.
7. Incubate the sample at 90°C for 1 hour, vortex every 10 minutes to avoid sample loss from evaporation.
8. Sonicate the samples in ice-cold water bath for 10 minutes.

9. Add IAA solution to sample (final concentration should be 55mM IAA, adjust volumes accordingly).
10. Incubate on rotator for 1 hour in the dark.
11. Prepare SP3 beads for sample.
    I. Warm beads to room temperature.
    II. Amount of beads required: 10 µg of beads per 1 µg of protein. For 500 µg of protein lysate, add 50 µL of each hydrophobic and hydrophilic stock to a new 1.7 ml tube (Stock concentration of beads = 50 µg / µL, Total of 100 µL of beads = 5000 µg). Calculate total amount of beads required for your samples and aliquot accordingly with ~10% extra to account for pipetting loss.
    III. Add 500 µL of milliQ water, mix and place the beads on the magnetic rack for 2 minutes, then remove water. Wash the beads with milliQ water two more times for a total of 3 washes.
    IV. Resuspend beads in total of 50 µL of milliQ water to get **100 µg/uL final concentration**. (Beads contribute to the volume, so adjust the amount of water accordingly). Mix the beads by gently pipetting.
12. Add the beads to samples based on **10 µg of beads per 1 µg of protein**.
13. Add equal volume of 100% ethanol to beads resulting in **50% final ethanol concentration**. (Add up starting lysis buffer + IAA + Bead volume).
14. Vortex sample gently for 2-3 seconds. Do not pipette mix. Samples can be lost via pipette tip.
15. Incubate the sample with beads for 8 minutes at room temperature.
16. Spin down the samples on table top centrifuge for ~6 seconds (at less than 3000g).

17. Put the beads on magnetic rack and incubate for 2 minutes. Remove supernatant without disturbing the beads. Use 200 µL loading pipette tip if required.
18. Wash the beads with 200 µL of 80% Ethanol. Remove the tube from magnetic rack, add ethanol directly onto the beads, and incubate on magnetic rack. Avoid touching the beads with pipette. Repeat ethanol wash twice for a total of 3 washes.
19. Let the beads air dry for 30 seconds to remove residual ethanol.
20. Add appropriate amount of trypsin (1:50 trypsin:protein ratio) (2 µg of trypsin for 100 µg protein or 2 µg of trypsin per 10-µm FFPE section of surface area of $\leq$ 50 mm^2) in 200 µL of 50 mM HEPES buffer for on bead digestion.
21. Sonicate the beads for 1 minute.
22. Incubate the beads on rotator overnight (18-24 Hrs)

Day 2:

23. Elute peptides off of SP3 beads
    I. Spin down the beads on table-top centrifuge for ~6 seconds.
    II. Incubate the beads on magnetic rack for 2 minutes.
    III. Transfer the supernatant into a new tube. Supernatant contains the digested peptides.
    IV. Repeat the elution with 200 µL of HEPES buffer. (Second elution improves the recovery by ~10%)
        a. Add 200 µL of HEPES to the beads.
        b. Sonicate the beads for 1 minute and vortex the beads for 5 seconds.
        c. Incubate the beads at room temperature for 2 mins.

d. Put the beads on magnetic rack for 2 minutes, and transfer the supernatant to the elution tube.

24. Measure peptide concentration by doing Pierce Protein BCA assay.
25. Lyophilize the peptides and store at -80 °C.
26. Peptides can be used directly for label-free analysis or can be labeled with TMT for multiplexed analysis.