Exercise week 3:
Sustained proliferative signaling I – Receptor tyrosine kinases

1) For memorization:

a) What characterizes the growth of "transformed" cells (by definition)?
Answer: Shape changes; Anchorage-independence (i.e. the ability to survive and divide independently of adhesion to extracellular matrix); loss of contact inhibition (i.e. cells continue to divide in a culture dish even after reaching confluence).

b) What are SH domains, and what are their functions?
Answer: Src homology domains share sequence similarity with non-catalytic domains of the cytoplasmic tyrosine kinase Src; they function as adapter proteins.

c) What do geneticists mean if they say that EGFR and Son-of-Sevenless (Sos) are *epistatic*?
Answer: An interaction between two or more gene loci is called “epistatic” if mutations in one of them can hide the phenotypic effect of mutations in one of the others.

2) Exam-style MCQ:

Which one of the following statements about oncogenic mutations is ***false***:

A. CDK4,6 activity induces cell division by phosphorylating cyclin D
B. RTKs can become oncogenic by mutations that delete the extracellular domain
C. EGFR frequently becomes oncogenic by means of gene amplification
D. The mutations that make KRAS oncogenic *inhibit* its GTPase activity, rather than stimulating it
E. G12C mutant KRAS can be targeted by drugs that covalently bind its inactive form

Answer: A. For B, see also the answer to question 3c below.

3) Mechanism-based explanations

40% of GBM cases show mutations in the EGFR gene. Particularly common (26%) is a truncation of the extracellular domain by deletion of exons 2 to 7 (EGFR vIII, **Fig. 1**).

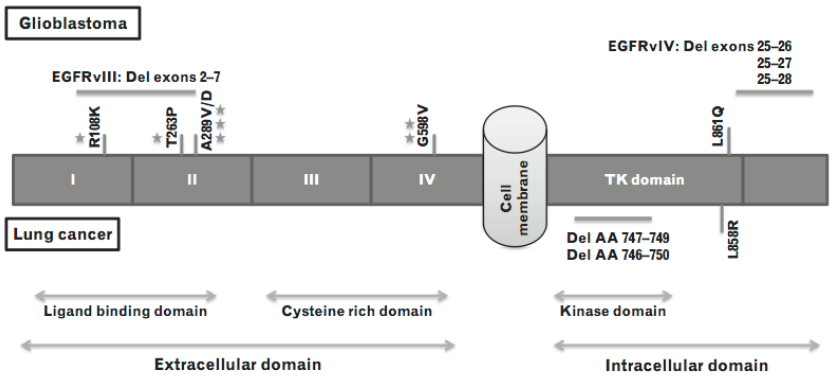


FIGURE 1. Mutations in the epidermal growth factor receptor gene associated with glioblastoma and non-small cell lung cancers (NSCLC). Missense mutations reported from glioblastoma are clustered in the extracellular domain. The most common mutations are annotated and indicated by marks above the gene bar for glioblastoma. In contrast, mutations reported from lung cancer are located in the kinase domain, indicated below the gene bar. (Hegi et al. 2012, Curr Opin Neurol 25:774-9)

a) How does this truncation lead to EGFR hyperactivation?

Answer: Through *ligand-independent autophosphorylation* of *tyrosine* residues within the *cytosolic domain* of EGFR.

b) Oncogenic KRAS mutations are extremely rare in GBM. They also only very rarely co-occur with EGFR mutations NSCLCs (<5% of all all cases examined). If GBMs become resistant to *all* available EGFR inhibitors, would it make sense to consider treating patients with the recently approved KRAS inhibitor Sotorasib? Why or why not?

No, not in GBM patients: Since KRAS is rarely mutated in GBM to begin with, and since Sotorasib specifically targets only a single mutant form (G12>C), the vast majority of GBM cannot possibly respond to such $KRAS^{G12C}$-specific drugs.

By contrast, in lung cancer, oncogenic KRAS mutations (including G12C) are now thought to contribute to intrinsic and acquired EGFRi resistance. Despite the relatively low frequency, the G12C mutation could potentially make such tumors eligible for KRASi for future personalized therapies.

c) Why are only so few mutations in Ras oncogenic, compared to the many different oncogenic point mutations found in EGFR?

Answer: Oncogenic mutations in both Ras and EGFR are so-called *gain-of-function mutations*. In Ras, one of only 2 very specific residues must be mutated to allow stabilization of the GTP-bound form. By contrast, EGFR can be activated by any type of mutation that promotes ligand-independent dimerization, e.g. by inactivating EGFR autoinhibition by its extracellular domain.

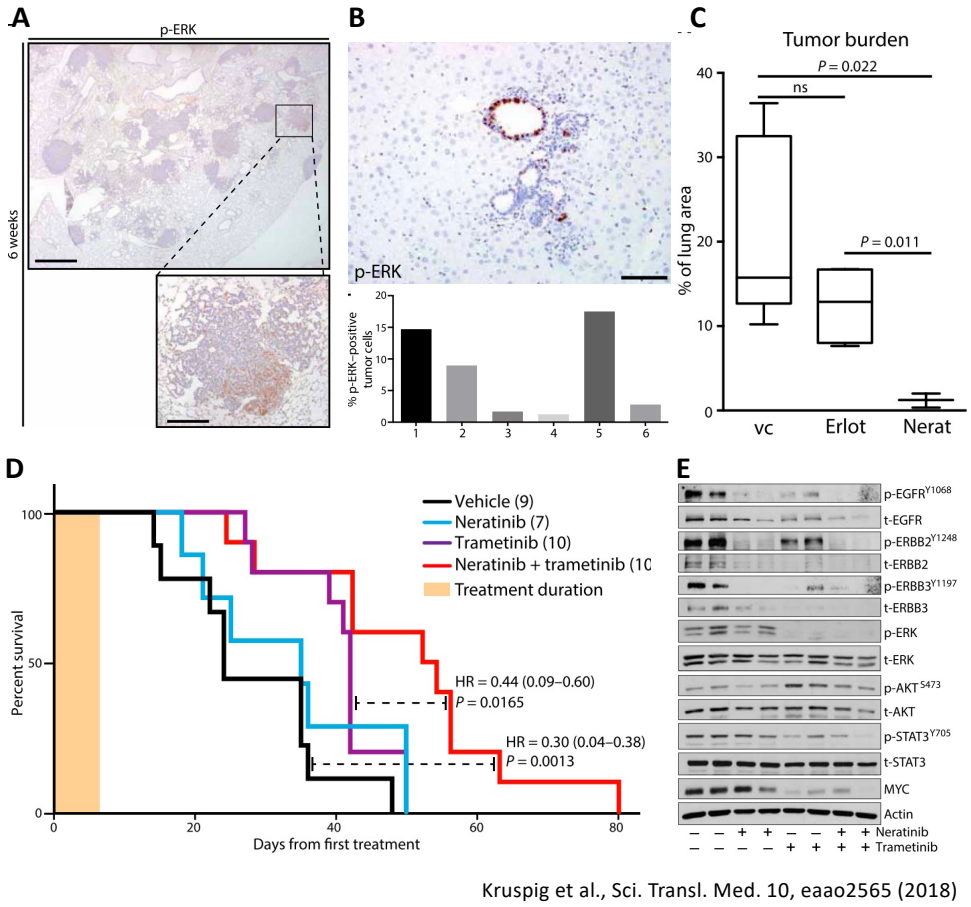
4) Data interpretation: Therapeutic targeting of KRAS in lung cancer

In lung adenocarcinoma (LuAd), activating mutations in either KRAS or EGFR are mutually exclusive. EGFR inhibitors are clinically approved for non-small cell lung carcinoma (NSCLC). However, only 70% of patients respond, and these do so only transiently (<12 months). In a search for strategies to overcome drug resistance, researchers have investigated the effect of inhibiting MEK, a kinase downstream of KRAS that mediates activation of the so-called extracellular signal-regulated kinase ERK1:

RAS/Raf → MEK → ERK1 → cell proliferation & growth

a) In a subset of metastatic NSCLC patients carrying the oncogenic Val600>Glu mutation in the Raf family member B-RAF, a combination treatment with the V600E mutant-specific B-RAF inhibitor (BRAFi) dabrafenib plus the MEK inhibitor (MEKi) trametinib has been approved by the FDA in 2018. Given this synergism with BRAFi, should one also consider combine MEKi with second generation EGFR/ERBB family inhibitors? Why or why not?

A priori, both combination therapies seem counterintuitive: If you compare a signaling pathway to a linear stream of water, erecting a second dam further upstream would seem to only make the barrier downstream obsolete, at least at first glance. However, most drugs inhibit their targets incompletely, and signaling pathways are in reality not linear but rather split into multiple branches. E.g. the RTK/Ras pathway has at least 3 major branches, and the Raf/MEK/ERK branch is only one of them. Therefore, and since treatment with MEKi potentiated BRAFi therapy, researchers also tried to combine MEKi with second generation EGFR/ERBB inhibitors that target EGFR as well as related ERBB family members such as HER2 (ERBB2) and ERBB3.



Kruspig et al., Sci. Transl. Med. 10, eaao2565 (2018)

FIGURE 2. Analysis of tumors in KM mice that were engineered to express KrasG12D and elevated levels of another oncogene, c-Myc, specifically in lung epithelial cells. A) *Multiple papillary lung adenocarcinoma in situ 6 weeks post-induction of oncogenic KrasG12D and c-Myc alleles, stained (brown) for phosphorylated extracellular signal-regulated kinase (p-ERK).* ***B)*** *Immunohistochemical staining of p-ERK in liver metastases 6 months post-induction. Quantification of the fraction of stained cells per metastasis is shown below.* ***C)*** *Histological quantification of tumor burden in lungs of KM mice treated daily with neratinib or erlotinib for 4 weeks, commencing 2 weeks after induction of oncogenic KrasG12D and c-Myc alleles.* ***D)*** *Overall survival of tumor-bearing KM mice treated daily for 1 week (orange bar) with neratinib, or with the MEK inhibitor trametinib, or both, then followed without further intervention. Treatment was commenced at 5 weeks; the number of mice analyzed per group is indicated in brackets). Log-rank hazard ratios (HR ± 95% confidence interval) and P values are shown for the indicated comparisons (dashed lines).* ***E)*** *Lysates of individual tumors from mice treated with neratinib and/or trametinib for 3 days, analysed by Western blotting using antibodies against the indicated proteins (total, t) or specific phosphorylated tyrosine (Y) or serine (S) residues that mark their activation.*

b) The ERBB inhibitor neratinib was administered with or without trametinib to engineered mice that express oncogenic mutant $Kras^{G12D}$ specifically in lung epithelial cells. c-Myc, a transcription factor important for most cancer types, was overexpressed as a transgene in the same cells, allowing $Kras^{G12D}$ to rapidly induce multiple tumors (within ≤6 weeks). Less than 5% of those lesions (**Fig. 2A**, magnified area), and only a fraction of cancerous cells in liver metastases of older mice (6 months) stained positive for p-ERK1 (**Fig. 2B**).

- Why was this unexpected (back in 2018) that $Kras^{G12D}$ activated ERK1 in only 5% of the tumor cells?
- What additional mutations or changes in gene expression would you consider as candidates for increasing p-Erk levels in this subset (5%) of cells?

The observation that KrasG12D activated ERK1 in only a subset of the tumor cells means that those apparently generate a higher signaling threshold. This was unexpected because missense mutations of glycine 12 have long been thought to *maximally* activate KRAS already independently of RTKs. Possibly, some cells in panels A and B further increased the signaling threshold by duplicating the mutant $Kras^{G12D}$ allele, or they inactivated a negative regulator of either $Kras^{G12D}$, Raf, MEK, or of p-ERK1 itself.

None of these formal possibilities were conclusively addressed in this study. Instead, the authors proposed that pERK1 staining marks the subset of cells that found a way to further ratchet up the activation of $Kras^{G12D}$ or of endogenous wild-type Ras family members by upregulating the expression of one or several ligands of multiple ERBB family members.

c) Treatment of *KRAS* mutant lung cancer patients with the EGFR-specific inhibitors Erlotinib or Gefitinib has no benefit. Here, Erlotinib treatment similarly failed to inhibit tumor growth in KM tumor-bearing mice; however, Neratinib which blocks *multiple* ERBB family members largely suppressed tumors during the time window examined (**Fig. 2C**). To distinguish how Neratinib achieved this, your lab project supervisor asked you to treat cultured $Kras^{G12D}$ mutant (KM) cells with Neratinib or with the ERBB ligand neuregulin. How would this experiment allow you to distinguish if Neratinib slows tumor growth in vivo by blocking an **autocrine** or a **paracrine** growth factor signal?

Autocrine means: The tumor cells themselves produce both a receptor and its ligand(s). By contrast, if the ligands are provided by their immediate neighbors or independently of cell-cell contacts from even further away, these are called juxtacrine or paracrine signals, respectively.

If sustained proliferation of KM cells requires autocrine signaling of an ERBB ligand, Neratinib treatment should block its activity also *in vitro* and thereby reduce the KM cell proliferation to the level that is induced by $Kras^{G12D}$ alone. By contrast, if they depend on juxtacrine or paracrine signals from cells in the tumor microenvironment, their proliferation *in vitro* should further increase upon treatment with recombinant ERBB ligand, and only in the absence but not in the presence of Neratinib.

In a related study (published back-to-back with this one), researchers deleted EGFR in a human KRAS mutant LuAd cell line and in $Kras^{G12D}$-induced mouse tumors. Both of these tumor models still responded to Afatanib, another pan-ERBB inhibitor, despite the oncogenic KRAS mutations. Afatinib also impaired cell proliferation and p-ERK1 activation in cultured $KRAS^{G12S}$ mutant cell lines, i.e. in the absence of any other cells, and even if EGFR was deleted. These observations revealed that upregulation of ERBB ligands and their autocrine signaling within the tumor cells further increased cell proliferation beyond the level induced by oncogenic mutant $KRAS^{G12D}$ alone (Moll et al. 2018, Sci. Transl. Med. 10, eaao2301).

d) The Kaplan-Meier plot in panel D reveals no benefit for Neratinib treatment alone. Tumors that had been treated for 3 days with the indicated drugs were lysed and analyzed by Western blot in panel E. How do these blots confirm whether Neratinib and Trametinib each reached their target(s)?

Trametinib alone sufficed to block Erk phosphorylation, a readout of MEK activity. Therefore, it is plausible that Trametinib inhibited its target (MEK).

Neratinib alone blocked the tyrosine phosphorylation of ERBB1 to 3, confirming that it reached the target. However, Erk and Akt clearly remained phosphorylated, which means

that some receptor(s) other than ERBB family members must be responsible for their activation (the authors did not point that out).

Do these Western blot data support the model that ERBB family members are the main RTKs driving MEK/Erk signaling in these tumors? Why or why not?

No: During the time window analyzed, Neratinib clearly inhibited tumor growth, suggesting a role for ERBB signaling. However, at the time point analyzed, Erk signaling was not inhibited. The finding that (only) Trametinib but not Neratinib blocked pErk implies that an RTK other than ERBB contributed to MEK/Erk signal activation.
Of note, Trametinib (unlike Neratinib) also diminished the accumulation of c-Myc: There is evidence that phosphorylation by ERK increases c-MYC protein stability (Cancers 2022, 14, 4840. https://doi.org/10.3390/cancers14194840).