Reading: Paulsen et al "Peroxide-dependent sulfenylation of the EGFR catalytic site enhances kinase activity" Nat Chem Biol 8 57 2012. An example of the importance of sulfenylation in regulation of Y-kinase activity. For more details if interested see Yang et al "Global, in situ, site-specific analysis of protein S-sulfenylation" Nature Protocols 10, 1022 (2015).

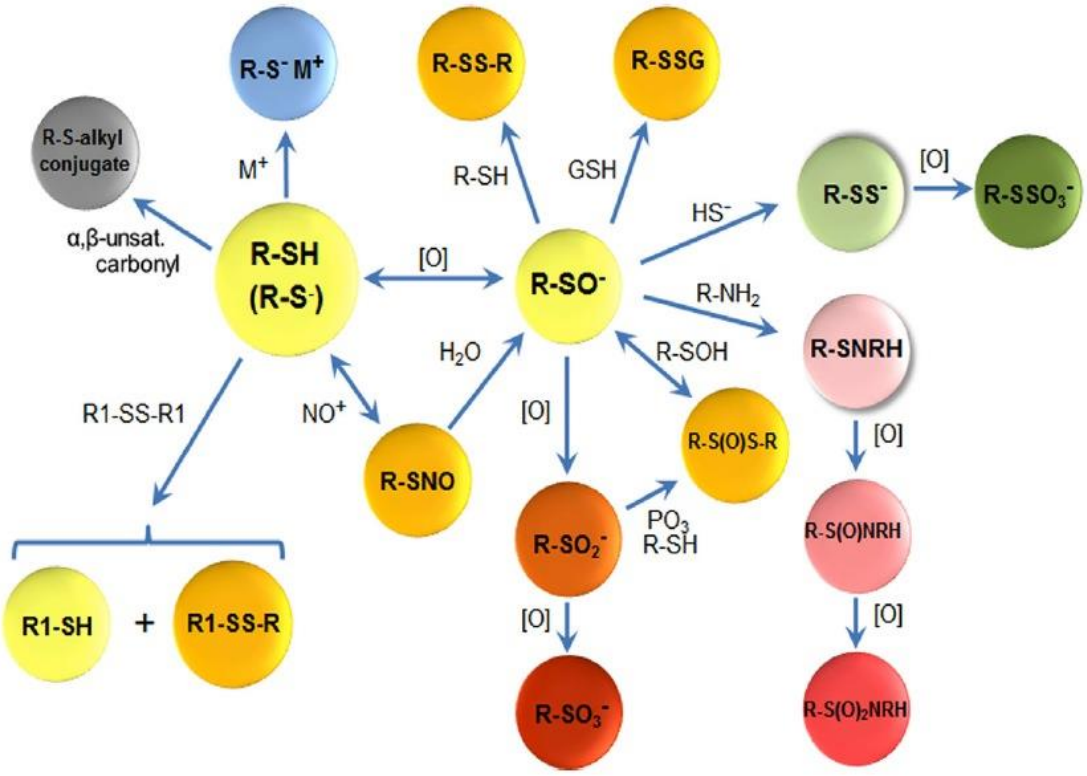
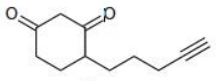


Figure 1. The cysteine proteome and functional modification. The cysteineome undergoes a range of enzymatic and non-enzymatic modifications often mediated by pH-dependent ionization to the cysteine thiolate. Thiolates (RS^-) are common ligands for metals (often found in short binding motifs), and in catalytic sites of enzymes. In addition, cysteines can be oxidized by redox imbalance inside the cells based on environmental changes (ROS and RNS) outside the cells and these cysteines can be involved in regulatory networks. Above are representatives of some of the modifications that have been detected. The cysteine proteome is a subset of the proteome that has recently received much attention. [Ref: "The cysteine proteome" 2015 Free Radicals Biol and Med 84, 227-245; "Quantitative reactivity profiling predicts functional cyssteines in proteomes" 2010 Nature 468 790. NOTE RSO- should be RSOH Those interest in S chemistry including RSOH can read BBA 2013 Gupta and Carroll for a detailed review.

From recitation 11 I. Dm reagent for use in cells: For reagents (Dm or I-Dm, recitation 11) to be useful inside the cell, they need to have a "handle" (an acetylene or an azide, see cartoon below) to allow affinity purification of the protein of interest (POI), that is, to enrich the POI and remove the cellular mess. Note this was described briefly at the end of the Recitation 11 handout. In the Nat Chem Biol paper, the reagent found to be most useful was DYn-2 (**2**). This reagent is membrane permeable. Of course you also need to worry about the length and bulk of the labeling arm (these parameters were varied to optimize the reagent) and whether access to the cysteine to be modified is allowed. For general

labeling of sulfenylated proteins in cells, this remains an issue as each sulfenylated cysteine will be in a distinct environment.



2
DYn-2

The acetylene can react with a biotin-linker-azide construct via click chemistry (Figure 2) to give a biotin tagged-POI that can then be purified by affinity chromatography with a streptavidin bead. Alternatively, Carroll et al made dimedone derivatives with azide linkers that could react with a biotin-linker-alkyne reagent. The chemistry between the azide and acetylene, can be copper catalyzed. The copper catalyzed chemistry (CuACC) is slow and cannot be used inside cells as the copper is toxic. An alternative method involving NO metal and the strained cyclooctyne can be used inside the cell.

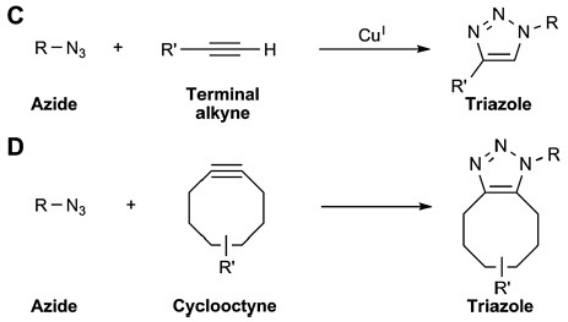


Figure 2. Click chemistry using CuACC (C) or cyclooctyne (D) with the derviatized reagent. The R group in C and D above can be a biotin-linker or a fluorophore-linker. The former is used in the whole cell experiments in the Nat Chem Biol paper.

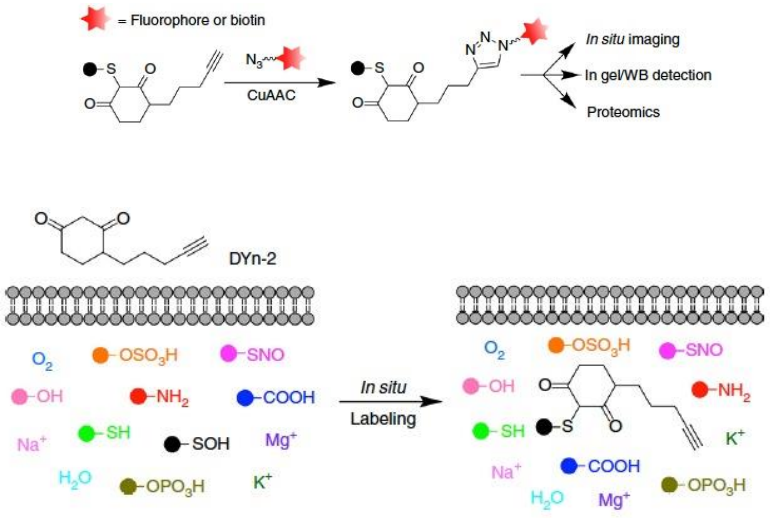


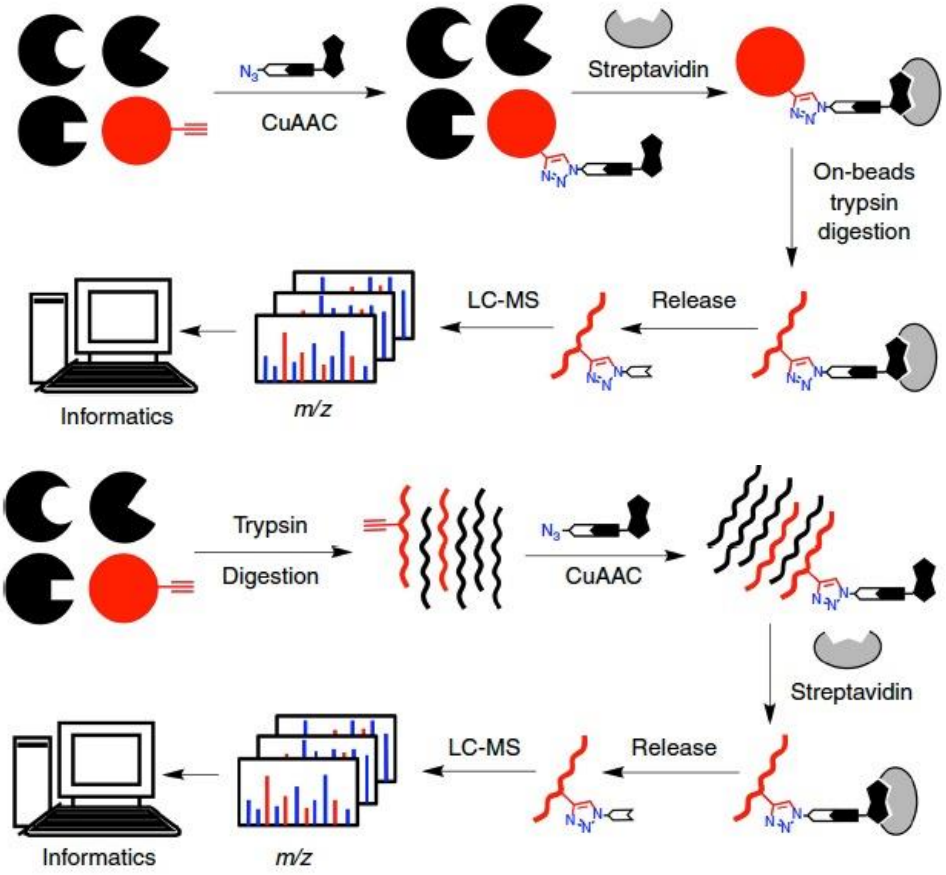
Figure 3 Selective labeling and detection of protein S-sulfenic acid in cells based on the discussion in Recitation 11. The DYn-2 probe selectively reacts with protein sulfenic acid modifications in cells. Importantly the probe does not change cell viability and redox balance.

Dimedone is a reagent "specific" for sulfenic acids. As shown in Figure 1 and Figure 3, there are many reported modifications of cysteines. Reagents for the direct probing and

detection of each distinct redox modification is a very active area of research. [See Martin et al JACS 138 1852-9 (2016) focused on new reagents for cys-S-nitrosation and cys-S-sufination; Tannebaum et al JACS 135 7693-704 (2013) using triarylphosphine-esters probes to capture of RSNOs.]

We are focused on sulfenic acids. The general protocol was recently reported by Liebler and Carroll and the use of this methodology is highlighted in the signaling with EGFR and NOX2.

A.



B.

Figure 4. The workflow of proteome-wide identification of targeted sites of alkyne-tagged probes. Two distinct approaches are shown. A. Proteins captured with the alkynyl probe is first conjugated with an azido biotin with a cleavable linker via the CuAAC chemistry. The biotin tagged proteins are enriched with streptavidin beads and subjected to sequential on–bead tryptic digestion and then released by an enzymatic or a chemical cleavage mechanism. The resulting peptides are analyzed by MS-based proteomics for the site of localization. B. The proteome labeled with the alkynyl probe is in this workflow: first the sample is digested into tryptic peptides and then conjugated with an azido biotin with a photocleavable linker via CuAAC. The resulting peptides are then enriched with streptavidin beads and released by enzymatic or chemical cleavage.

Issues encountered in the protocols described above.

1. Biotinylated proteins are usually eluted from streptavidin only by harsh conditions (denaturants, heat, organic solvents). Non-specifically bound species are also eluted under these conditions. Biotin can alter the ionization of the peptides of interest

and/or interfere with site identification. The analytical chemistry of each step including recoveries, need to be thought about in some detail. As examples, one often observes incomplete click chemistry and instability of the photocleavable linker. Enrichment protocols have a very large, positive effect on recoveries. Each problem is different and the method with the best recovery and quantitation will require experimentation.

2. Additional problems/limitations encountered involve: Chemical labeling that misses sulfenylated targets due to steric factors and differences in reaction rates. The requirement for a large amount of protein and substantial amounts of reagents is often under appreciated as MS in general is thought to be very sensitive. As you saw in recitation 11, **deuteriated probes** and **ratioing** of labeled and unlabeled samples, is essential for quantitation. It is not appreciated that the deuteration (vs hydrogen substitution) has marked shifts in retention times on LC analysis due to the differences in mass. One observes isotope effects on chromatography.

The data presented below is from SI Figure 4 from the Carroll, Nat Chem Biol paper and was already discussed in recitation 11. Their studies suggested that DYn-2 was the most effective Dm probe for labeling that they examined.

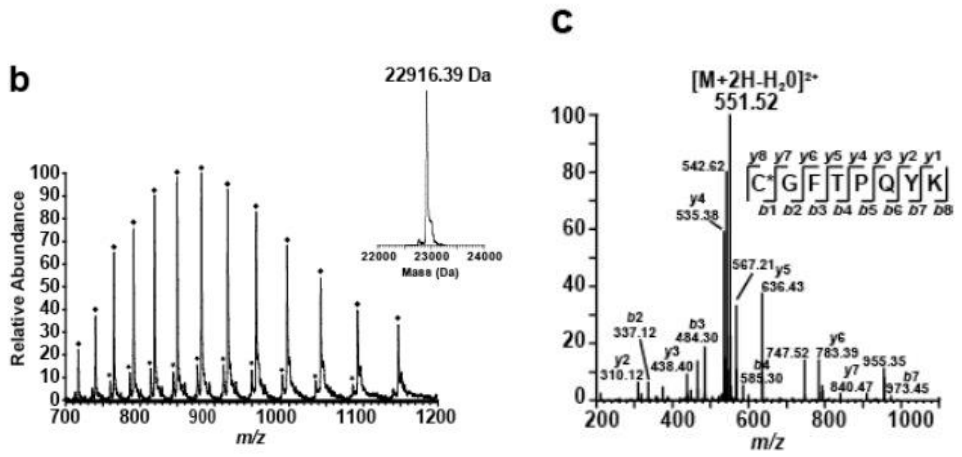


Figure 5. SI Figure 4 from Nat Chem Biol paper: b. ESI-LCMS intact mass analysis shows the covalent attachment of a single DYn-2 molecule Gpx3 (22916.39 Da). The inset shows the deconvoluted mass spectrum. The observed mass shift is 177.48 Da and the expected mass shift for a single DYn-2 adduct is 176.08 Da. C. ESI-LC/MS/MS analysis of the reaction using collison-induced dissociation (CID) MS of the precursor ion m/z 551.52 $[M + 2H- H_2O]^{2+}$ corresponding to Dyn-2-tagged peptide (CGFTPQYK-OH) derived from a trypsin digest and analysis of each of the peptide fragments.

The basis for the fragmentation patterns in Figure 5 (right) was worked out by the Bieman lab at MIT many years ago. In Figure 6, the proposed mechanism for the fragmentation process is shown as are the resulting b and y ions reported in many of the papers you will read.

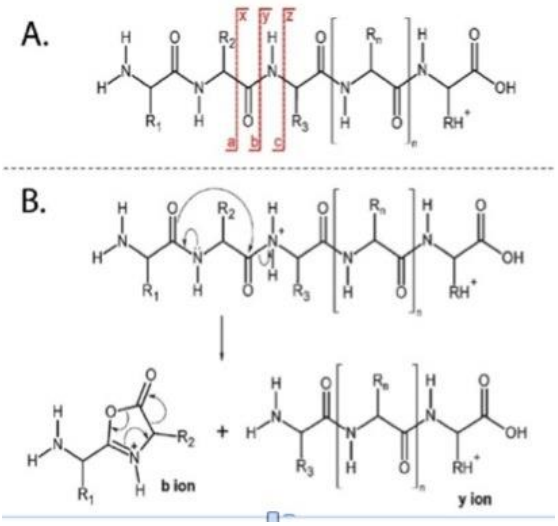


Figure 6. Fragmentation of peptides and proposed mechanism for this process.

Now let us turn to the Carroll, Nat Chem Biol, data to examine the evidence for the importance of NOX2 in EGFR tyrosine kinase activation and phosphatase inhibition. In the latter case the role of SHP2 was identified for the first time . A model for thiol-based redox modulation of signaling is shown in Figure 7. EGF (epidermal growth factor) binds to the extracellular domain of the EGFR and causes it to dimerize. Nox2 is the NADPH oxidase that in the presence of the appropriate complex including the protein Rac1, causes the NADPH reduction of O_2 to $O_2^{\bullet-}$. NOX2 is one of the few enzymes that generates superoxide as its normal product. $O_2^{\bullet-}$ in the presence of H^+ rapidly disproportionates to form H_2O_2 and O_2. The H_2O_2 is proposed to move through the aquoporin channel (AQP) and deliver a "bolus" of H_2O_2 (high effective molarity) that can then target the intracellular tyrosine kinase domain associated with EGFR and the phosphatase that is also involved in modulation of the tyrosine kinase activity. In this model the Y-kinase domain is activated by phosphorylation and from this paper, sulfenylation. The phosphatase that deactivates the Y-kinase activity is itself deactivated by sulfenylation.

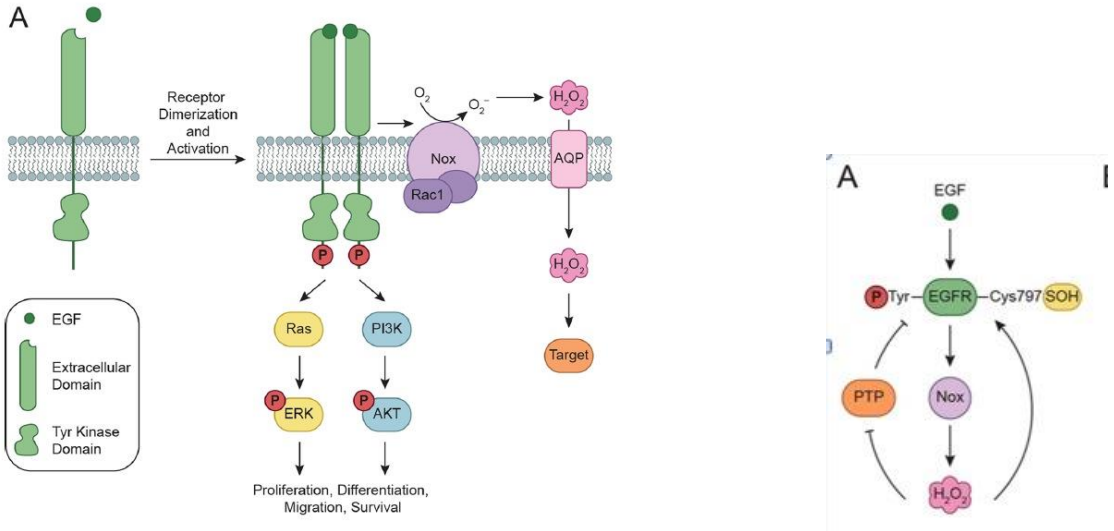


Figure 7 A. The model for EGF signaling via the EGFR dimerization and the role of Nox2 in production of H_2O_2 that results in modification of the tyrosine kinase domain of the EGFR itself and the phosphatase, shown in this paper to be SHP2.

We will briefly examine a few of the experiments have been carried out to support the model in Figure 7: activation the Y-kinase activity of the EGFR and the inactivation of the phosphatase by sulfenylation (Figure 8-11).

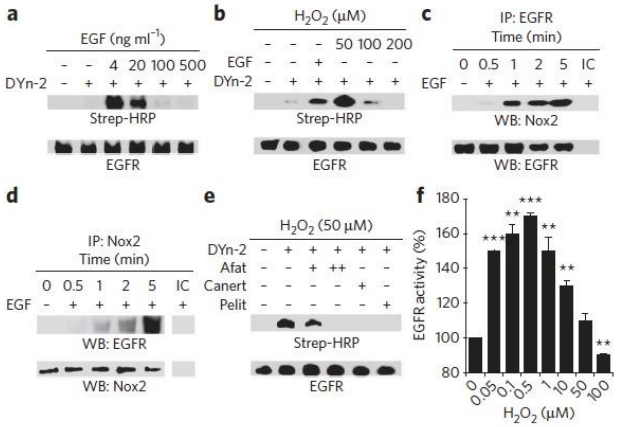


Figure 8 Western blots showing sulfenylation and total EGFR. A431 cells were stimulated with EGF at the indicated concentations or vehicle for 2 min **(a)** or H_2O_2 for 10 min **(b).** Sulfenic acids are detected with Strep-HRP western analysis where Strep-HRP is an antibody generated to dimedone. Western blots (WB) with antibodies to Nox2 (**c**) and EGFR (**d**) have also been used. They show colocalization of Nox2 and EGFR. In (**e**) in the presence of H_2O_2, small molecules Afat, Canert and Pelit were added and the amounts of sulfenylation were measured. These small molecules are EGFR irreversible or reversible inhibitors that are in clinical trials in the treatment of cancer. Their effects on sulfenylation in the presence of H_2O_2 is hwon. (**f**) EGFR tyrosine kinase activity was measured in vitro.

Conclusions from data:

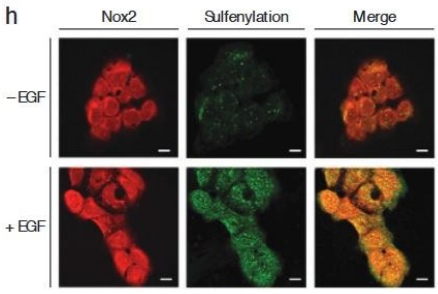


Figure 9. A431 cells were stimulated with 100 ng/mL or vehicle for 0.5 min and treated with dimedone. Cells were then stained for the dimedone-protein adduct (green) and Nox2 (red). Scale bar is 10 microns.

Conclusions from data:

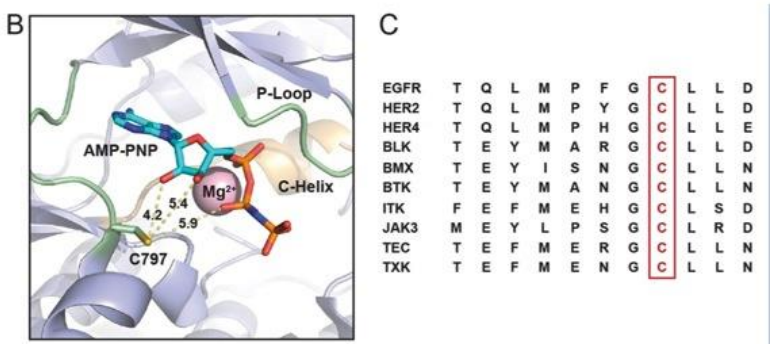


Figure 10 Crystal structure of EGFR kinase domain with the irreversible inhibitor 13-JAB and the non-covalently with the ATP analog ADPNP-Mg^{2+}. Only the ATP analog is shown. The sulfenylation is conserved in any growth factors at the Cys in red.

Conclusions from data:

Phosphatases and their response and localization:
Note: SHP2 has a response at the lowest concentration of EGF added

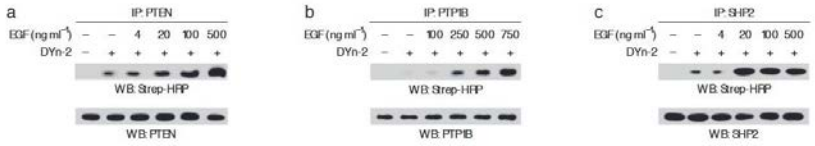


Figure 11 Differential sulfenylaltion of protein tyrosine phosphatases in EGF-treated cells. a-c Western blots showing sulfenylation and total immunoprecipitation of PTEN, PTP1B, and SHP2. A431 cells were stimulated with EGF or vehicle for 2 min at the indicated concentrations and then incubated with 5 mM DYn-2 or vehicle for 1h at 37 C. Lysates were immunoprecipitated with antibodies to mouse PTEN (a), mouse PTP1b (B) and rabbit SHP2 (c).

Conclusions from data:

5.08J Biological Chemistry II

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