Problem Set 2 (**Due January 28th**)

1. The KEGG Pathways website can also be used to investigate signal transduction. For example, we can use this website to learn about the p53 (a central protein in regulating the cell cycle) signaling network. Navigate to the p53 Signaling Pathway page from the KEGG Pathway homepage.
    a. What extracellular stress signals can induce a response from p53?

γ radiation, UV radiation, Genotoxic drugs, Nutrition deprivation, Heat/cold shock

    b. P53 can induce apoptosis in a mechanism that is dependent on communication to the mitochondria AND a mechanism that is independent of the mitochondria.
        i. The mitochondria-dependent pathway can be stimulated by multiple pathways, but they all converge on one mitochondrial protein (which is actually ejected from the mitochondria). What is this protein? Cytochrome C
            1. This protein is specifically implicated in one disease (this is in the disease box, NOT the Pathways box). Name this disease and briefly describe what it is. Thrombocytopenia - a decrease in the number of platelets in circulating blood, resulting in the potential for increased bleeding and decreased ability for clotting.
            2. The next protein in the signal cascade is CASP9. In the definition box, you can learn what kind of enzyme this is. What chemical reaction does this protein catalyze? The Definition box notes that it is a cysteine peptidase. These enzymes are similar to serine proteases (so hydrolysis of a peptide bond), except the nucleophilic residue is a cysteine instead of a serine.
        ii. What two proteins are involved in the mitochondria-independent pathway? IGF-BP3 and IGF
            1. IGF-1 can stimulate PKB (Akt). Please rationalize this observation (the full name of IGF might help you make the connection). IGF stands for Inusulin-like growth factor. As we learned in class, insulin binding to the insulin receptor triggers phosphorylation of IRS-1. p-IRS-1 can subsequently activate PI3 Kinase which phosphorylates $PIP_2 \rightarrow PIP_3$. PIP_3 is an activator or PKB. Insulin-like growth factors are names because they have a similar physiological effect, so they can trigger the same signal cascade.

2. GPCR:
    a. Clearly describe how GPCRs work. In your description, make sure to comment on the different types of alpha subunits (i.e. G_s, G_i, G_q). An extracellular molecule specifically binds to the GPCR. This binding causes a structural change in the intracellular side of the protein (reorientation of a helix by 30º). This structural change causes the release of GDP an subsequent binding of GTP in the alpha subunit of a heterotrimeric G-protein. GTP bound Gα then dissociates from the β and γ subunits (through the movement of 3 switch regions that are stabilized by H-bonding to the γ phosphate of ATP) and can interact with another enzyme. The Gs G-proteins activate Adenylate Cyclase while Gi inhibits the same enzyme. G-proteins can also activate phospholipase C (PLC); these are designated Gq.

    b. Discuss the parallel role of PLC and AC in GPCR nucleated signal transduction cascades. Phospholipase C and Adenylate Cyclase are both plasma membrane bound enzymes that are affected by interaction with the alpha subunit of a heterotrimeric G protein. Adenylate Cyclase catalyzes the production of cAMP from ATP while PLC catalyzes the production of

diacylglycerol (DAG) and inositol-1,3,5-triphosphate (IP_3) from phosphatidylinositol-3,5-diphosphate (PIP_2). Products of each of these reactions serve as secondary messengers that activate one or more enzymes – this is how the extracellular signal gets relayed to cellular response machinery.

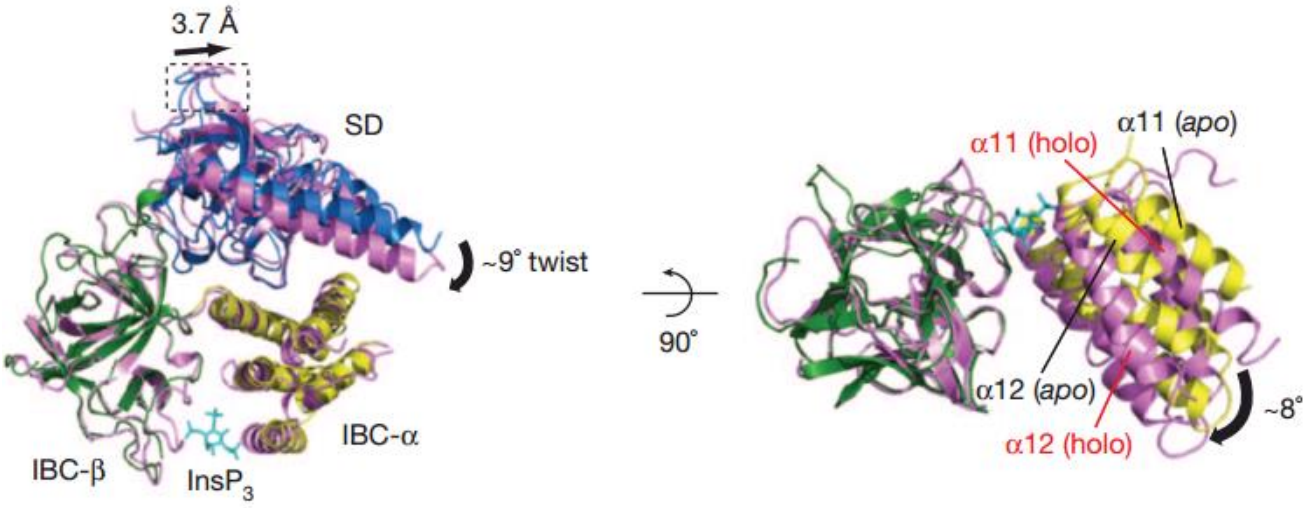
3. Adaptor proteins:
    a. What is the role of adaptor proteins in signal transduction? Adaptor proteins have no catalytic abilities, however they do nucleate supramolecular complexes of enzymes that have complementary functions.
    b. Name the two adaptor proteins that were discussed in lecture and identify what important interaction each make. AKAP = A Kinase Anchoring/Adaptor Protein. This protein makes specific contacts with Protein Kinase A (PKA), Adenylate Cyclase (AC), a GPCR, as well as a protein phosphatase (PP2B).

       Grb2 - this protein facilitates the activation of Ras. It makes specific contacts with phosphorylated IRS-1 (via an SH2 domain) and with the GAP (GTPase activating protein) for Ras, Sos – this interaction occurs via an SH3 domain which interacts with proline rich sequences.

    c. If either of these proteins have important structural domains, identify them and discuss their role. As discussed above – SH2 domains recognize phosphotyrosines and SH3 domains recognize proline rich sequences.

4. Calcium as a secondary messenger:
    a. Calcium is released by an IP3 gated Calcium Channel in the ER. Clearly explain what this means and why it is relevant. Gated ion channels are proteins that facilitate the transport of ions across a membrane down a concentration gradient. Ion transport is only permitted when the protein is in a specific conformation (gate is open). In this specific Calcium Channel, the molecule that can cause the gate to open, hence initiate Ca^{2+} transport, is the secondary messenger IP_3. This is relevant because IP_3 is only present when the PLC is activated by an extracellular signal. When high concentrations of Ca^{2+} are present in the cytosol, a variety of additional enzymes get activated (typically via Ca^{2+} binding to Calmodulin, which subsequently activates the partner enzyme).

    b. Inspect the structures of the IP3 binding domain in the unbound (pdbID 3UJ4) and bound (pdbID 3UJ0) forms. Describe major differences that are important in calcium release. You may find it helpful to read the primary literature citation (available here). Figure 2 from the paper gives a very good idea of what is going on. The IP_3 binding domain undergoes very little structural change when the substrate binds, however IP_3 binding causes a significant structural change in the Suppressor Domain (SD) – α11 and α12 rotate by 8-9º in the holo form relative to the apo form. This structural change is sufficient to open the gate and allow Ca^{2+} transport.

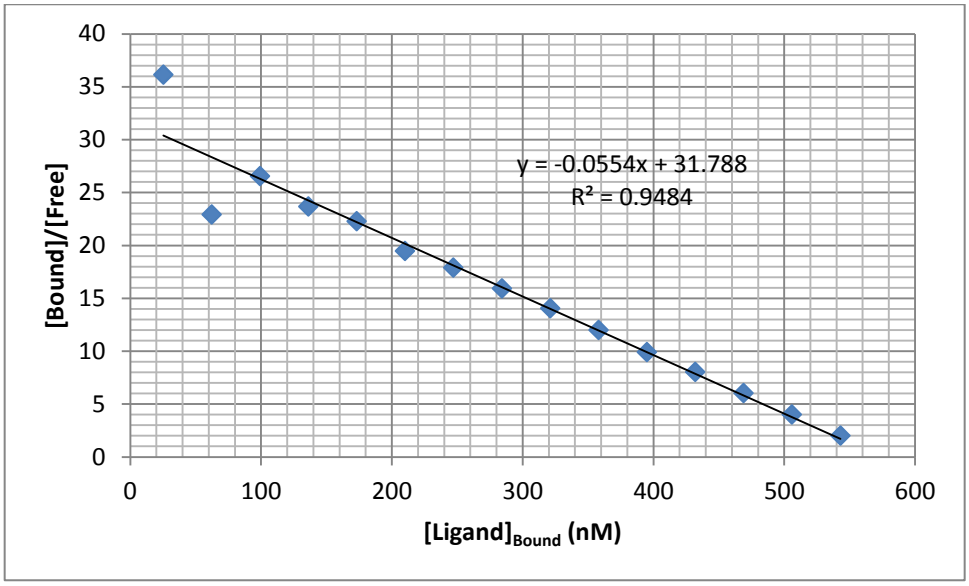


c. Describe how Ca^{+2} is able to activate kinases. In your answer, please discuss the relevant calcium binding motif. Ca^{2+} activates a variety of kinases through Calmodulin, a protein mediator. Calmodulin contains two metal binding domains (2 metals per domain) connected through a long and flexible α-helix linker. Calmodulin presents a motif known as an EF hand domain – two helices (E and F) are oriented roughly orthogonal to each other connected through a loop – taken together, this motif resembles a hand. Ca^{2+} binds in the palm (loop). When fully populated with Ca^{2+}, calmodulin can interact with its target kinase by wrapping around the activating helix.

5. Compare signaling in prokaryotes and eukaryotes. What are the similarities and how do they differ?
Prokaryotes and Eukaryotes both take advantage of phosphate transfer to propagate a signal. Signal propagation is much more complex in eukaryotes, involving many kinases and complex branching between signal transduction pathways. Eukaryotes rely on phosphorylation of Serine, Threonine, and Tyrosine residues. Prokaryotes, on the other hand, primarily make use of a genetically linked 2-protein module known as a Two Component System. The two proteins are a Histidine Kinase, which is a membrane receptor and contains an intracellular Histidine kinase domain that allows autophorphorylation of a solvent exposed Histidine residue upon activation, and a Response Regulator, which is a DNA binding protein that can repress or activate gene expression upon phosphorylation of a conserved aspartic acid residue in the receiver domain. There are other strate

6. A binding study was carried out to determine the binding properties of an adrenergic agonist interacting with the epinephrine receptor. Using the following experimental data, determine the affinity that the receptor has for the agonist. Assume that no competing interactions occur.

$[Ligand]_{total}$ (nM)	$[Ligand]_{bound}$ (nM)	$[Ligand]_{free}$ (nM)	[Bound]/[Free]
26	25.3	0.7	36.1428571
65	62.28	2.72	22.8970588
103	99.26	3.74	26.540107
142	136.24	5.76	23.6527778
181	173.22	7.78	22.2647815
221	210.2	10.8	19.462963
261	247.18	13.82	17.8856729
302	284.16	17.84	15.9282511
344	321.14	22.86	14.048119
388	358.12	29.88	11.9852744
435	395.1	39.9	9.90225564
486	432.08	53.92	8.01335312
547	469.06	77.94	6.01821914
633	506.04	126.96	3.98582231
815	543.02	271.98	1.99654386



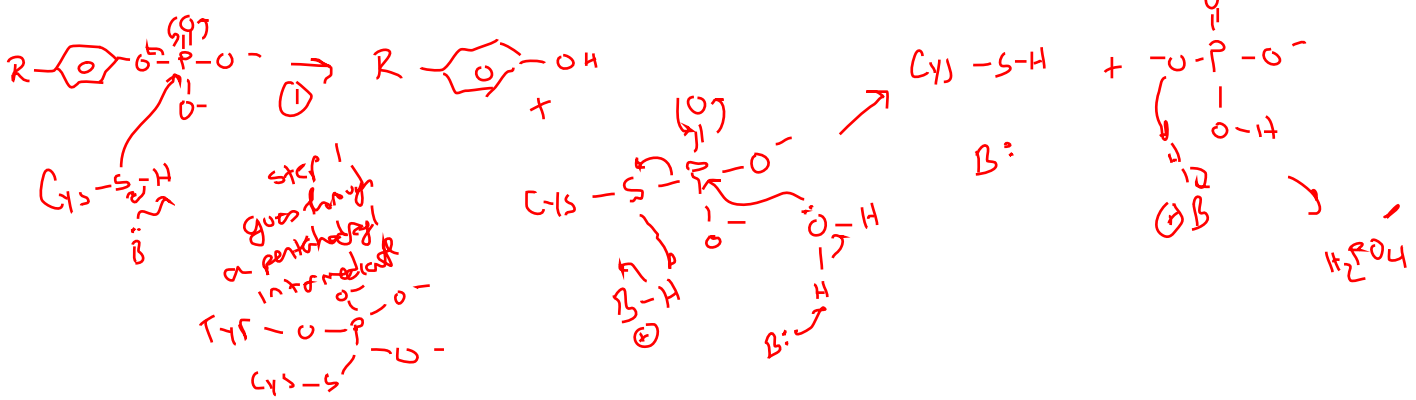
Kd = 18.05 nM

B_{max} = 574 nM

slope = $-0.0554\ nM^{-1} = \frac{-1}{K_D}$

$y_{int} = 574\ nM = \frac{B_{max}}{K_D}$

7. Familiarize yourself with the following article (Huyer, G., et al. *J. Biol. Chem*. **272**, 843-851).
   a. What reaction does PTP1B catalyze? Noting that a phosphocysteine is formed in the active site as an intermediate, propose a mechanism for this reaction. This enzyme is a protein tyrosine phosphatase, so it catalyzes the hydrolysis of a phosphorylated tyrosine.



b. Describe the technique that was used PTP1B Activity assay. Activity was assessed by the ability of an enzyme to catalyze the hydrolysis of fluorescein diphosphate to fluorescein monophosphate. In the diphospho-form, fluorescein does not absorb light at 450 nm. Upon hydrolysis, the absorbance of light at 450 nm can be detected.

c. Figure 1 clearly shows that vanadate affects the enzyme activity. Discuss the purpose of the experiment show in panel B. Upon preincubation of PTP1B with vanadate (or pervanadate), the enzyme is significantly inhibited. The experiment in panel B confirms that it is the addition of inhibitor immediately triggers enzyme response. This experiment also indicates that the two vanadium containing molecules inhibit the enzyme is a different manner.

d. Still referring to Figure 1, comment on the affect EDTA has on the reaction. Why is EDTA influencing the experiment? EDTA is a strong metal chelator; addition of EDTA should prevent inhibition of the enzyme by interacting with vanadium more strongly than the enzyme.

e. Refer back to the mechanism you proposed in 7a. Do the conclusions drawn from the mass spectrometry experiments support your mechanism? Please justify your claim. The mass spec experiment indicates that the catalytic cysteine residue of PTP1B is oxidized from a –SH to SO_3^{2-}. This is an irreversible oxidation and completely removes the nucleophilic capacity of the cysteine residue. If your mechanism shows cysteine acting as a nucleophile (which it should), it should make sense that this modification should prevent the enzyme from initiating its catalytic cycle.

f. Figure 3 establishes that vanadate is a(n) Competitive inhibitor of PTP1B.

g. The article points out that Pervanadate has insulin-mimetic properties. Justify this claim based on what you learned in the article. Pervanadate inhibits PTP1B. This enzyme is responsible for hydrolyzing the phosphorylated tyrosine residues on the PTK domain of the insulin receptor, which will subsequently turn off the insulin triggered signal. If the phosphatase activity is inhibited, the signal will continue unregulated.