QUESTION 1

You are starting your post-doc fellowship and developing your own project. You have become intrigued with a human disease that has pleiotropic phenotypes including: obesity; loss of sight; lack of smell; renal anomalies; and behavioral complications. While the disease has been known for some time and fourteen genetic loci have been linked to the disease, the function of the proteins encoded by these genes is not known. You decide to tackle this problem with your underlying hypothesis being that some of these genes encode proteins that form a physical complex.

a) What experimental approach would you use to test your hypothesis? Please be sure to describe the experimental approach, the rationale for your choice, what data you would obtain, how you would analyze the data. Include appropriate controls to ensure the validity of your results. Describe what outcome you would expect if your hypothesis is correct and if it were incorrect. (*5 points*)

b) If your first line of experimentation did not work, what alternative approach would you propose? Explain why this is your alternative approach and not your first choice; compare advantages/disadvantages of the two approaches. (*2 points*)

c) Given the pleiotropic phenotypes of this disease, state a hypothesis about what defect you think could underlie this condition? (*1.5 points*)

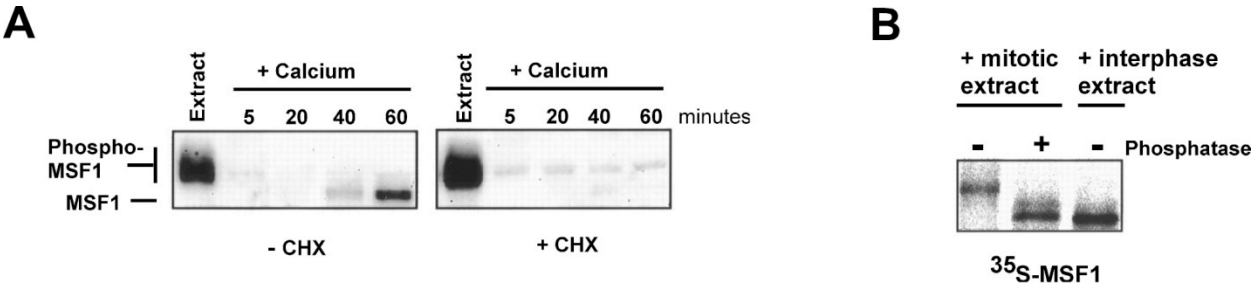
d) Briefly (one or two sentences), describe one experiment that you could use to test your hypothesis. (*1.5 points*)

Assume for all experiments that you have access to the sequence of the genes, knowledge of the mutations, patient tissues, and any reagents you require including clones, antibodies etc…

QUESTION 2

Before fertilization, *Xenopus* eggs are stably held in mitosis by an activity termed Cytostatic Factor (CSF). Upon fertilization of an egg, a wave of intracellular calcium release occurs and leads to rapid inactivation of CSF and exit from mitosis. Although well understood as a mitotic stabilizing "activity", CSF remained ill-defined molecularly for many years. CSF activity and calcium sensitivity can be recapitulated in extracts made from Xenopus eggs, which facilitates the study of this activity. When calcium is added to the extracts, CSF is inactivated. The data below are taken from experiments to investigate the potential role of a protein, MSF1 (Mitotic Stabilizing Factor 1) in CSF activity.

Figure 1



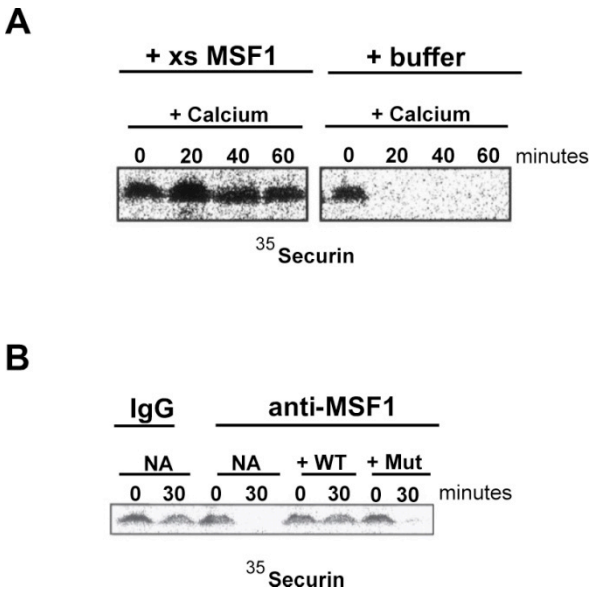
Panel A. Extracts were made from Xenopus eggs under conditions that preserve CSF activity. Calcium was then added and aliquots were taken at specific time points after addition, as indicated, and immunoblotted for MSF1 using a specific antibody. CHX stands for Cycloheximide, a protein synthesis inhibitor. **Panel B.** MSF1 was translated in vitro and labeled with ^{35}S-methionine. Labeled MSF1 was then added to mitotic extract in the presence or absence of a Phosphatase. Aliquots were then analyzed by gel electrophoresis and autoradiography.

a) What happens to MSF1 after calcium is added and why was inclusion of Cycloheximide (CHX) critical to assess what happens to MSF1? (*1 point*)

b) Explain why the behavior of MSF1 is compatible with a role in CSF activity and what these results suggest about the regulation of MSF1 in interphase and mitosis. (*2 points*)

Figure 2

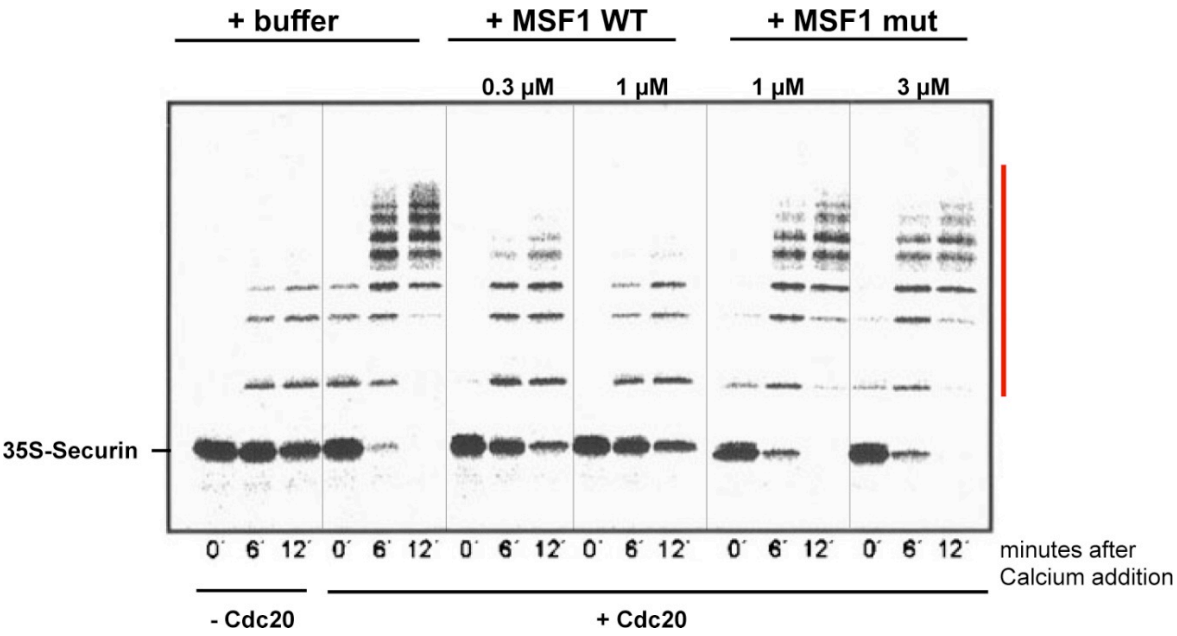
Panel A. Extracts were made from *Xenopus* eggs under conditions that preserve CSF activity and either excess MSF1 (xs MSF1) or buffer was added along with ^{35}S methionine labeled Securin. Calcium was then added to the extracts and aliquots were collected at time points as indicated for analysis by gel electrophoresis and autoradiography. **Panel B.** Extracts were made from Xenopus eggs and then immunodepleted using an excess of either antibody to MSF1 or a control, non-specific IgG. ^{35}S-labeled Securin was added, along with no addition (NA) or addition of either wild type recombinant MSF1 (WT) or MSF1 carrying a single point mutation, C583A (Mut). Aliquots were taken at the indicated time points and analyzed by gel electrophoresis and autoradiography.



c) Why was Securin used in these experiments? What do these results suggest about the activity of MSF1? (*2 points*)

d) In Figure 2B, no calcium was added to these extracts and yet, in some lanes, Securin has been degraded. What does this result suggest about the role of MSF1? (*1 point*)

Figure 3



Extract was prepared from *Xenopus* eggs under conditions that preserve CSF activity. Buffer, recombinant MSF1 (WT), or recombinant MSF1 C583A (mut) was added at the concentrations indicated, along with labeled Securin, and in the presence or absence of His tagged-Cdc20, as indicated. Calcium was added and aliquots were taken at the indicated time points after addition. Samples were analyzed by gel electrophoresis and autoradiography.

e) The band corresponding to Securin is indicated in the figure. With clear justification, explain the origin of the additional bands indicated by the red line and explain why much shorter time points are used in this experiment. (*2 points*)

In a subsequent figure (not shown), the authors repeated the experiment of Figure 3 above but this time utilized purified APC/C in place of the Xenopus egg extract. The results of this experiment are essentially identical to those shown in Figure 3.

f) Taking all of the above results into consideration, propose a model for the contribution of MSF1 to mitotic stabilization in the extract. (*2 points*)

QUESTION 3

Lysozyme, which is highly enriched in the white of chicken eggs, catalyzes the hydrolysis of certain glycosidic bonds.

a) How would you measure the volume of egg white in an average chicken egg? (*0.5 points*)

b) Lysozyme is a protein that is expressed in egg whites. Its molecular weight is 14.4 kDa, and its isoelectric point (pI) is 11.0. Describe a strategy to purify this protein away from other proteins in the egg white (including avidin, which is 69 kDa, with a pI = 10.0)? Provide the rationale for your choice(s) for the purification strategy. (*5.5 points*)

c) Suppose that you discovered that a chicken egg contains 30 mL of egg white, and that you purified 5 micrograms of lysozyme per egg. Assume that your yield was 100%. What was the molar concentration of lysozyme in the intact egg white? (*2 points*)

d) Suppose that you did not know the structure of the lysozyme substrate, and hypothesized that it was a disaccharide (molecular weight = 360Da). Suppose that it was present at a 10-fold molar excess over the enzyme. Assuming 100% yield, how much of that substrate (in grams) would you expect to be able to purify from the white of a single egg? (*2 points*)

Be sure to show your work to obtain any partial credit.

QUESTION 4

You have become interested in the expression of the QE1 gene which is ubiquitously expressed in vertebrate tissues. Several studies from the Oncomine (cancer expression database) suggest and you have experimentally confirmed that the steady-state level of the QE1 protein is significantly decreased in highly invasive metastatic tumors as compared to normal neighboring tissues. This expression pattern is recapitulated in a tumor cell line (low QE1 protein levels) and control (normal, non-cancerous) cell line (high QE1 protein levels) that you can grow in the laboratory. You hypothesize that loss of QE1 protein may contribute to cellular transformation so you are interested in determining whether restoring QE1 protein expression could be a therapeutic approach to combat such tumors. In order to address this question, you will need to understand how the expression of QE1 is regulated. Assume that any reagents that you desire for the study of QE1 gene and protein are readily available.

a) Drawing on your knowledge of the central dogma, name four possible points in the gene expression pathway that could be affected to result in the observed decrease in the steady-state level of the QE1 protein in tumor cells as compared to control cells? (*1 point*)

b) Considering the possible steps affected in (a), state two distinct hypotheses for specific mechanisms that could explain the observed decrease in the steady-state level of the QE1 protein in tumor cells as compared to control cells. (*2 points*)

c) Taking advantage of the cell lines that you have in the laboratory, describe an experimental approach to distinguish between the hypotheses that you developed in part (**b**). Be sure to describe the experimental approach in detail including critical controls, what result you would expect to obtain that would distinguish between your two hypotheses, how the data would look for each hypothesis, and how the data would be analyzed and interpreted. (*5 points*)

d) Assume that loss of QE1 expression does contribute to the phenotype of the invasive tumor cells. Suggest one possible cellular function for the QE1 protein in normal cells. Provide rationale for why the loss of this function might contribute to the observed phenotype of the tumor cells. (*2 points*)

QUESTION 5

You found that a new protein X promotes actin polymerization. Protein X promotes only elongation and has no effect on nucleation or the steady-state level of polymerized actin. Microscopic observation of fluorescently labeled actin showed that addition of protein X led to elongated unbranched actin filaments (Fig. 1).

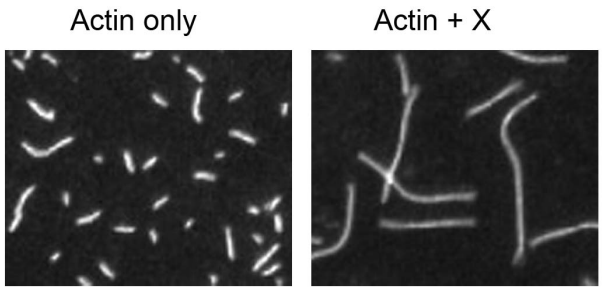


Fig. 1. Micrographs of fluorescently labeled actin filaments under elongation before reaching the steady state. Protein X promoted elongation of unbranched actin filaments

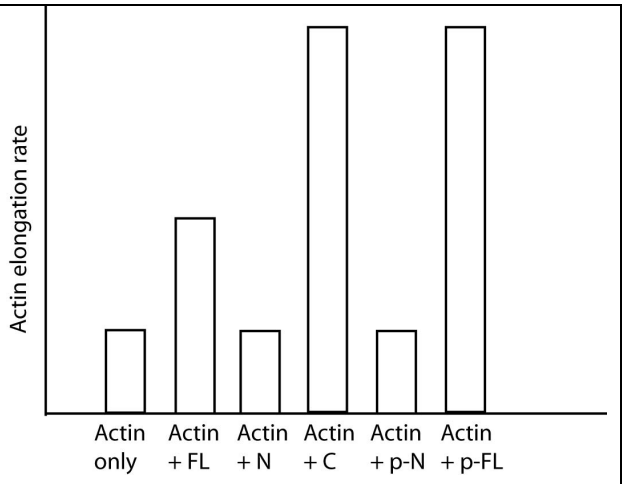


Fig. 2. Effects of protein X variants on actin elongation. FL: full-length; N: N-terminal half; C: C-terminal half; p-N: phosphorylated N-terminal half; p-FL: phosphorylated full-length.

a) Describe an experiment to determine from which end(s) of actin filaments protein X promotes elongation. Assume that you have access to any type of microscopes and can label actin with probes without changing the properties of actin and its interaction with protein X. (*2 points*)

Protein X does not have recognizable domains or motifs, so you made three versions of recombinant protein X: full-length, N-terminal half, and C-terminal half. The N-terminal half did not promote actin polymerization (Fig. 2). Full-length protein X moderately promoted actin polymerization, whereas the C-terminal half had much stronger activity than full-length protein X (Fig. 2).

b) State your hypotheses to describe the function of the N-terminal half and the C-terminal half of protein X. (1.5 points) and
c) describe experimental approaches to test the hypotheses from part (**b**). (*2.5 points*)

You identified one phosphorylation site in the N-terminal half of protein X and confirmed that this is the only phosphorylation site both *in vitro* and in cells. Phosphorylated N-terminal half of protein X did not have actin elongation activity, while phosphorylated full-length protein X enhanced activity in a manner similar to the isolated C-terminal half (Fig. 2).

d) State and describe your hypothesis for the mechanism of how phosphorylation enhances the actin elongation activity of full length protein X (*1.5 points*) and
e) describe an experimental approach to test your hypothesis. (*2.5 points*)

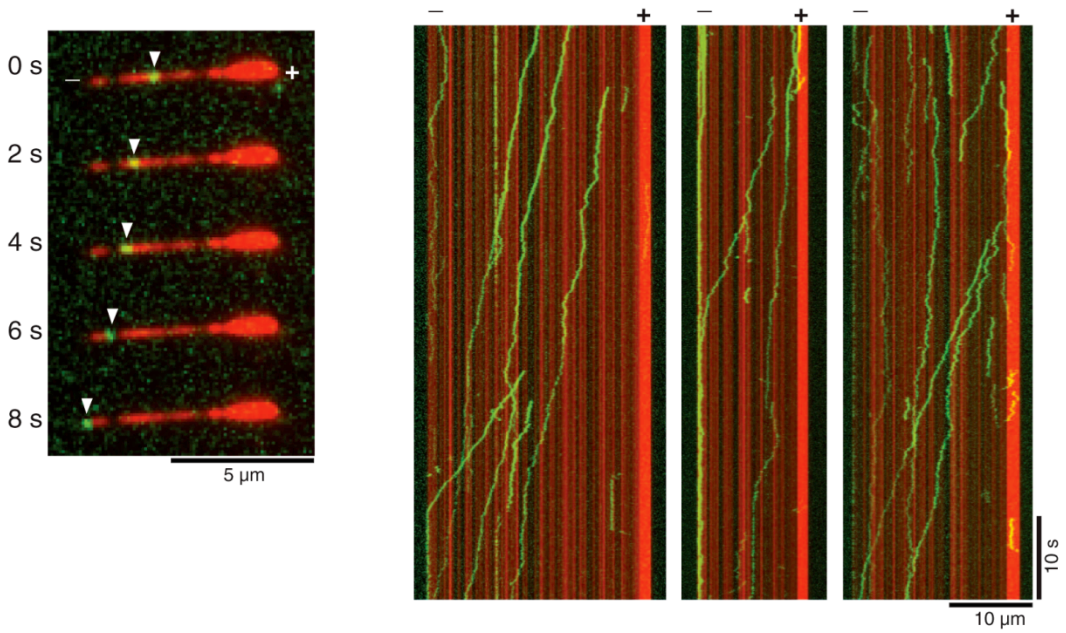
QUESTION 6

DNA methylation and histone modification are two major epigenetic regulatory mechanisms in mammalian genomes that interplay to regulate transcriptional activity. In fact, genome-wide DNA methylation profiles suggest that DNA methylation patterns are correlated with histone methylation patterns, particularly the absence of histone H3 lysine 4 methylation and the presence of histone H3 lysine 9 methylation. However, the fundamental question of how DNA methylation patterns are set up in the first place (that is, how the DNA methylation machinery targets some regions of the genome for DNA methylation while avoiding others) remains incompletely understood. The budding yeast *Saccharomyces cerevisiae* is a model organism whose genome is packaged into chromatin containing conserved histones, but lacks DNA methylation.

a) Using *S. cerevisiae* as a model system, design an experiment to study the mechanism of *de novo* DNA methylation by mammalian DNA methyltransferases. Describe your experimental approach and be sure to include your rationale, approach to data analysis, and how you would interpret the data obtained. (*6 points*)

b) In the same experiment exploiting budding yeast, how could you determine whether specific histone modifications contribute to directing *de novo* DNA methylation? Describe what additional experimental information you would need to collect to address this question including your approach to the experiment and interpretation of the data obtained. (*4 points*)

QUESTION 7



The experiments shown above depict an *in vitro* reconstitution of an Alexa488 (green) fluorescently-labeled and purified motor of unknown function moving on immobilized microtubules labeled in red (Alexa568). The left panel illustrates an example of successive frames of a TIRF microscopy time-lapse movie showing (green; white arrowheads) the motor movement on the surface of immobilized, polarity-marked microtubules with a bright plus end (+) and a dim minus end (-). The right panel shows representative kymographs of the motor (green) movement on the Alexa568-labeled, polarity-marked microtubules (red). Note, for each kymograph, time zero is at the top edge.

Based on this data:

a) Describe the polarity of movement of this motor and propose its molecular identity – illustrate as required. (*2 points*)

b) Propose two experimental strategies to establish the identity of the motor's cellular cargo. (*2 points*)

c) The microtubules in the left panel have an assigned polarity based on Alexa568-labeled polarity-marking. Explain this labeling strategy and how it leads to preferential labeling of on one end of the microtubule (*1 point*). Propose an alternative method to determine the polarity of microtubules in this *in vitro* assay. (*2 points*)

d) Based on only the data presented above, describe what happens to this motor when it reaches the end of a microtubule. Does this motor release itself from the end of a microtubule? Please explain. (*1 point*) If not, propose a mechanism to explain why the motor does not fall off when it reaches the end of the microtubule. (*1 point*) What type of mechanism could be required for motor release? (*1 point*)

QUESTION 8

You are studying a knockout mouse in which the gene for a regulatory subunit of Protein Phosphatase 4 (PP4) has been knocked out. Analysis of the progeny from female $PP4^{+/+}$ x male $PP4^{+/-}$ (hemizygous) crosses reveals the following frequency of genotypes: 30% female $PP4^{+/+}$, 20% female $PP4^{+/-}$, 30% male $PP4^{+/+}$, and 20% male $PP4^{+/-}$.

a) What frequency of genotypes would you have expected if knockout of PP4 had no effect? (*0.5 point*)

b) Propose a hypothesis to explain the frequencies actually observed for the different genotypes. Your model should incorporate a potential role for PP4 in a process that might explain the observed deviation from expected frequencies. (*1.5 points*)

c) As you investigate your hypothesis further you identify a protein called SM that you suspect may be regulated by PP4. You can assume that you have a cell line expressing endogenous SM and PP4, antibodies to SM that immunoprecipitate and immunoblot, and the cDNA for SM. You also know that SM becomes phosphorylated in this cell line when you activate Protein Kinase A (PKA). Be sure to include any controls necessary to make firm conclusions, including but not limited to distinguishing phosphorylation of SM from phosphorylation of a possible SM-associated protein. Describe the two experiments that you could perform to test whether SM is a direct substrate of PP4. (*4 points*)

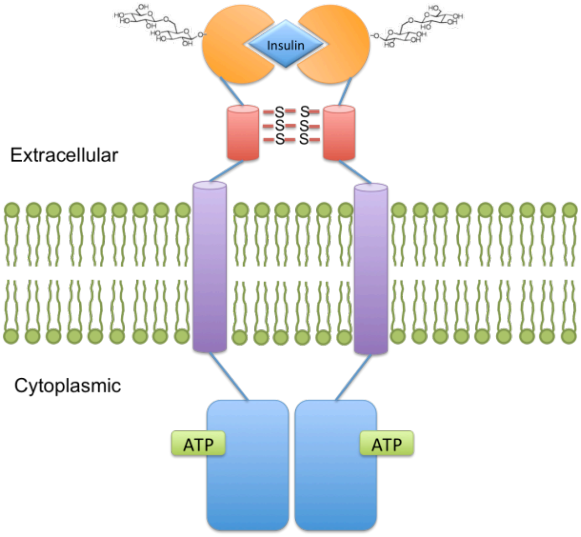
d) Based on the information provided, you do not yet know whether the phosphorylation of SM in cells in which PKA has been activated is a direct effect (activated PKA directly phosphorylated SM) or an indirect effect (e.g., activated PKA phosphorylated and inactivated the phosphatase for SM). Design an experiment to test whether PKA can directly phosphorylate SM. For this experiment, assume that purified PKA can be purchased from a reputable company. Be sure to include appropriate controls. (*2 points*)

e) You hypothesize that SM binds to microtubules and regulates microtubules function. You further hypothesize that upon phosphorylation by PKA, SM dissociates from microtubules in the cell. You wish to test this hypothesis. Assume you know that PKA phosphorylates a single residue on SM, serine 92 and that your polyclonal antibody to SM works in immunofluorescence. You also have the cDNA encoding SM on a plasmid amenable to transfection as well as a cell line that does not express endogenous SM. Briefly describe one experiment that could be performed to support the hypothesis that phosphorylation of SM on serine 92 by PKA causes SM to dissociate from microtubules IN CELLS. Be sure to include all necessary controls and interpretation. (*2 points*)

QUESTION 9

You work for a lab focused on discovering compounds to combat the symptoms of diabetes. While sipping on your green tea, you felt a little crazy (which can happen) and decided to include green tea extract in your high throughput screen for compounds that stimulate the Insulin Receptor. Remarkably, you discover a small molecule that binds to and stimulates the insulin receptor.

The insulin receptor (see Figure) is a multidomain transmembrane kinase that uses the binding of insulin to trigger a conformational change on the cytoplasmic side of the receptor. This conformational change leads to dimerization of the kinase domains which then bind to ATP and phosphorylate downstream targets. You show that your green tea molecule stimulates both the full length receptor and the isolated kinase domain. You surmise that your molecular binds to the cytoplasmic kinase domain but you do not know where it binds. Since it activates the receptor it cannot compete for the ATP binding site or the substrate binding site. You decide to use biochemical and structural studies to identify 1) the binding site of the green tea compound and 2) the conformational change induced upon green tea compound binding.



a) Briefly, describe two structural techniques that could be used to observe: 1) the global and 2) atomic level changes induced upon green tea compound binding. Explain the rationale for your choices, which should include the advantages and disadvantages of each method. (*2 points total*)

b) For these structural studies, describe your strategy for expressing, purifying and assessing the quality (could be folding, function etc..) of final protein for: 1) the (soluble) kinase domain fragment; 2) the disulfide linked/glycosylated extracellular domains; and 3) the full-length receptor. (*4.5 points- 1.5 points/construct*) (Note- you can assume that protein constructs containing the transmembrane domain are easily solubilized by detergent.)

c) Describe two experiments with appropriate controls that would allow you detect direct binding of the ligand to the soluble kinase insulin receptor domain *in vitro*. Be sure to include a rationale for the methods you choose. (Hint: you may assume that the green tea compound is easily synthesized and amenable to functionalization if needed). (*3.5 points*)

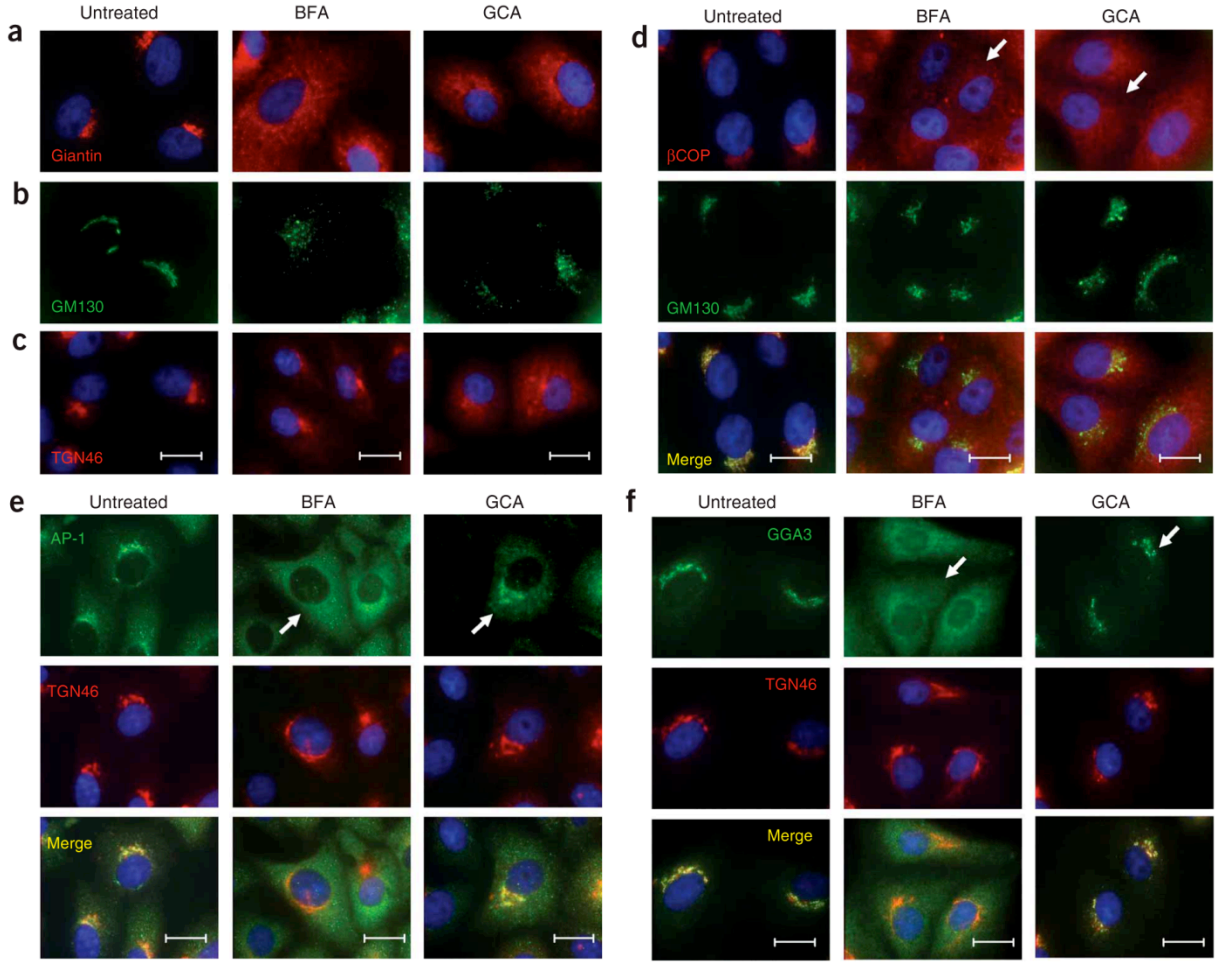
QUESTION 10

a) Identify a stable protein-nucleic acid interaction that you would find in a cell. Name the molecules as specifically as possible. (*2 points*)

b) Design an experiment to demonstrate that the protein and nucleic acid interact DIRECTLY. Mention the source of all molecules in your assay and all other major reagents necessary to carry it out. Provide an appropriate negative AND positive control. Describe how you would quantify the results. (*4 points*)

c) Design an experiment to test that your nucleic acid-protein interaction takes place *IN VIVO*.
Again, detail the source of molecules in the assay.
Again, provide a positive and negative control.
Again, describe how you would quantify the results. (*4 points*)

QUESTION 11



High throughput screening for small molecules that inhibit the effects of Shiga toxin on mammalian cells in culture identified the compound Golgicide A (GCA). GCA disperses medial- and cis-Golgi, inhibits COPI recruitment and maintains localization of the coats AP-1 and GGA3 to trans-Golgi network (TGN). (a–c) Vero cells were either left untreated or treated with brefeldin A (BFA; 10 μg/ml) or GCA (10 μM) for 1 h, and the localization of giantin (medial-Golgi) (a), GM130 (cis-Golgi) (b) and TGN46 (TGN) (c) was determined. The distribution of medial- and cis-Golgi was similar in BFA- and GCA-treated cells, while GCA did not induce tubulation of the TGN as observed in BFA-treated cells. (d) Vero cells treated for 5 min with BFA (10 μg/ml) or GCA (10 μM) show dispersed COPI staining (arrows) that does not colocalize with the Golgi (green) as in untreated cells. (e,f) Unlike BFA, GCA maintains AP-1 (e) and GGA3 (f) association with the TGN after 5 min of treatment (see arrows). Blue = nuclei staining. White scale bars, 20 μm.

a) Describe the phenotype induced by GCA in no more than two sentences and propose a hypothesis to explain this phenotype. (*2 points*)

b) Describe two experimental strategies to determine the cellular mechanisms of action of GCA. Provide the logic for your experimental approach, include technique you will use, expected outcome and the key controls required in your methods. (*4 points*)

c) Describe two experimental strategies to identify the molecular target of GCA. Provide the logic for your experimental approach; include techniques you will use, expected outcomes and key controls. (*4 points*)

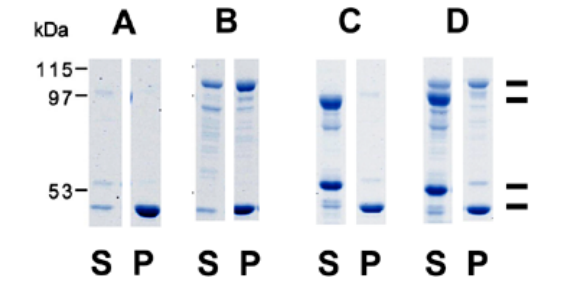
QUESTION 12

In class we discussed a paper by Ozawa, et al [PNAS, 87:4246-4250 (1990)] which provided key observations which formed the basis of the classical model linking cadherins to the actin cytoskeleton.

a) In the classical model, what role(s) do the cytosolic proteins alpha-catenin and beta-catenin play in linking E-cadherin to the cytoskeleton? (*1 point*)

Using purified protein preparations, you perform two experiments to further define how alpha-catenin, beta-catenin and E-cadherin interact with the cytoskeleton. In both experiments, the proteins are mixed *in vitro* in the presence of 1% Triton X-100 at 4°C and then separated into supernatant (S) and pellet (P) fractions by centrifugation. All of the co-factors needed for the proteins to form a complex are present in the reaction mixtures and the proteins were detected using SDS-PAGE and Coomassie blue. In Experiment 1, GST-E-cad-C-tail is a fusion protein consisting of GST and the cytoplasmic C terminus of E-cadherin. Lines next to panel D indicate the approximate migration of proteins added in experiments. In Experiment 2, a diagram showing a beta/alpha-catenin fusion protein examined is shown. The chimera mimics the interaction of the two proteins by covalently linking the alpha-catenin binding site of beta-catenin to the beta-catenin binding domain of alpha-catenin and extends to the C terminus of alpha-catenin including the actin binding site (*).

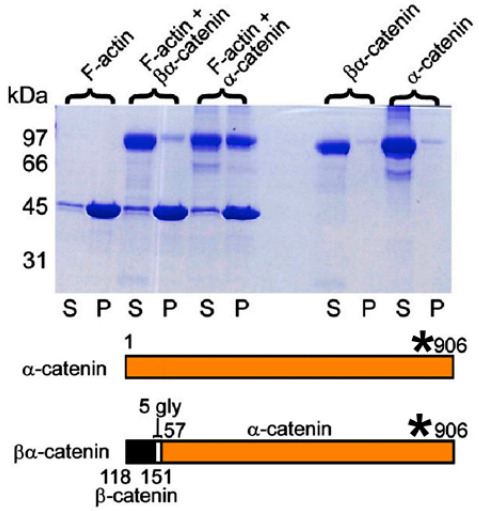
Experiment 1:



A - F-actin alone (46 kD)
B - F-actin + alpha-catenin (105 kD)
C- F-actin + GST-E-cad-C-tail (53 kD) + beta-catenin (94 kD)
D- F-actin + alpha-catenin + GST-E-cad-C-tail + beta-catenin

S- supernatant, P - pellet

Experiment 2:



S - supernatant, P - pellet

b) In Experiment 1, which proteins associate with F-actin? Does this finding fit with the classical model you presented in part **a** above? Explain the reasoning underlying your response- why or why not, including strengths and pitfalls of the approach used in Experiment 1. (*5 points*)

c) In Experiment 2, how does the beta/alpha-catenin chimera differ from normal alpha-catenin in the ability to interact with the cytoskeleton? Provide a model to explain this difference. Be sure to carefully consider structural motifs in your model. (*4 points*)