Instructions:

The following pages contain six questions. You must answer five out of the six questions, on each of the two days. Do not answer extra questions; indicate which one you are skipping on each day. Any one question may ask for more than one response so please read each question carefully for specific instructions pertaining to that question.

All answers are to be typed on the provided computer and saved to a provided USB drive. Hand-written figure pages will be included with your typed answers when they are processed and distributed to graders. SAVE ANSWERS FREQUENTLY, in case of computer issues.

Start typing your answer after the end of its question. Do not make changes to the format, font, color, etc. Do not change the header or footer. For the question you choose not to answer, just type "SKIP". Use the provided pages for your figures.

Please remember as you answer the questions that you have approximately one hour per question. This means that we expect in-depth answers unless otherwise noted. Use diagrams to illustrate your answer wherever possible. Although each question is worth the same number of points, not all questions will require the same amount of time. Allocate your time wisely. We recommend that you first make an outline of your answer, rather than just making up your answer as you write. Some questions ask you to propose experiments. Choose the most direct and realistic approaches and explain your experimental rationale as clearly as possible. Be sure to include controls, expected outcomes, and possible problems and solutions.

Do NOT put your name on any of the question or answer sheets. To keep the exam anonymous, label your figure pages with the question number (*e.g.,* Question 1) and with a coded name using the code distributed by Susan.

PLEASE MAKE SURE THAT ALL FIGURE PAGES ARE NUMBERED and IDENTIFIED!!!!!

This is a closed book exam. Absolutely no discussion will be allowed between the students while the exam is in progress. Cell phones must be silenced and in a bag – NOT accessible on the desk! You will be held to an honor code by agreeing not to receive or give aid on this exam.

You will have from 8:00 a.m. until 2:00 p.m. to finish the exam. Lunch will be available from (about) 12:00-1:00. No exam materials can leave the room with you during lunchtime or any other time, and you may not refer to outside materials at any time. The exams will be collected no later than 2:00 p.m. sharp.

If you have questions during the exam please contact Sho Ono: (404) 727-3916. In case of emergency or no answer, call his cell (770) 940-9272.

If you need supplies, call Susan: 404 727-1594.

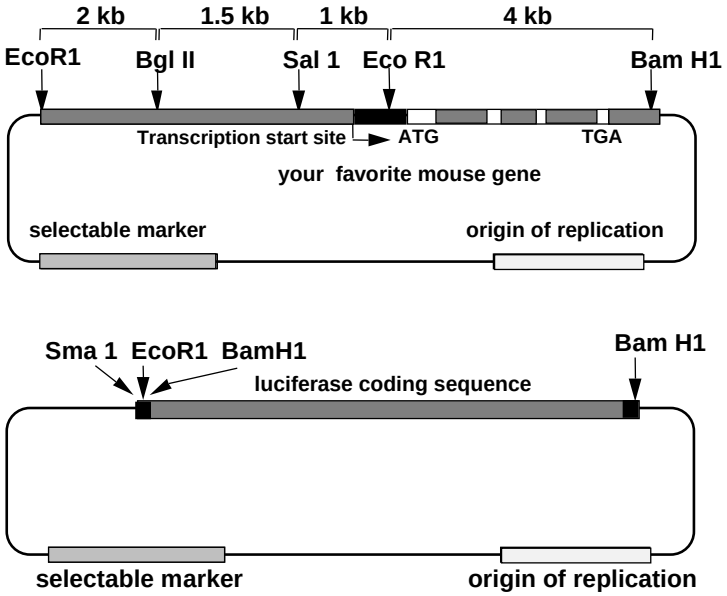
Be sure to save your final exam version to your USB drive. Put the drive and all papers into your envelope. If you have figure papers that are NOT to be used, just draw an X on them, fold them, or tear them in half.

Question 1

You are given tubes of purified DNA representing the two plasmids illustrated below. The top plasmid includes sequence from your favorite mouse gene (*YFG*); the transcription start site and translation start and stop codons are indicated. The bottom plasmid encodes a luciferase reporter sequence but no promoter.

Your goal is to use this luciferase reporter to test whether the upstream *YFG* sequence from the top plasmid has detectable promoter activity in a given cell type that you can grow and transfect in culture.

Both plasmids include recognition sites for the restriction enzymes indicated. The recognition sites and cut positions (scissors icons in the recognition site sequences) for all of the relevant enzymes are also illustrated. Eco R1, Bgl II, Sal 1, and Bam H1 each cut leaving a 5' 4bp overhang; Sma 1 cuts leaving blunt ends. Assume the cut sites indicated on the plasmid maps are the only places these specific enzymes cut in both plasmids.



Eco R1 5' –G ✂ AATT C -3'
3' -C TTAA ✂ G -5'

Bgl II 5' -C ✂ GATC G -3'
3' -G CTAG ✂ C -5'

Sal 1 5' -G ✂ TCGA C -3'
3' -C AGCT ✂ G -5'

Bam H1 5' -G ✂ GATC C -3'
3' -C CTAG ✂ G-5'

Sma 1 5' -CCC ✂ GGG -3'
3' -GGG ✂ CCC -5'

Your goal is to start with the two plasmids illustrated above and make new derivative plasmids to address the questions posed below:

(**A**) First, test whether the 4.5 kb putative *YFG* promoter sequence, bounded by the two Eco R1 sites in the top plasmid, have strong promoter activity. Describe each of **TWO DIFFERENT** strategies (**worth 5 points each**) to create the derivative plasmid needed to answer the question. Explain each strategy, including the purpose of each step and any important controls. Also explain how you will confirm the derivative plasmid you make is correct. (**Total 10 points**)

(**B**) You found that the 4.5 kb sequence you tested in (**A**) had strong promoter activity. Now, you want to test whether the 1 kb proximal region of that sequence (bounded by Eco R1 and Sal 1 sites in the top plasmid) is sufficient to also have promoter activity. Describe each of **TWO DIFFERENT** strategies (**worth 5 points each**) to create the derivative plasmid needed to answer this question. Explain each strategy, including the purpose of each step and any important controls. Also explain how you will confirm the derivative plasmid you make is correct. (**Total 10 points**)

Question 2

Previous studies have shown that the important enzyme Examase, which binds to a specific non-coding RNA (~75 nucleotides; nc1000), is critical for pancreatic tumor growth in mice and involved in regulation of cell growth in response to stress. Using high-throughput screening of a chemical biology library, you have identified a small molecule (C007) that inhibits the cellular activity of Examase and attenuates tumor growth.

(A) You hypothesize that C007 acts *in vivo* by preventing Examase from binding to the nc1000 RNA. How would you test this hypothesis *in vitro* assuming you have sufficient amounts of recombinant Examase, *in vitro* transcribed RNA and C007? (**4 points**)

(B) You have now confirmed that C007 prevents the binding of Examase to the RNA. Design an experiment to test if Examase and C007 bind quantitatively to each other. (**4 points**)

(C) Your quantitative analysis reveals that binding of C007 to Examase is very weak and cannot explain the strong inhibitory effect from C007 on the activity of Examase. You hypothesize that C007 binds to the RNA and this binding induces a conformational change in the RNA that prevents it from binding to Examase. First how would you test that C007 binds to RNA *in vitro*? Then how would you test the hypothesis that the RNA undergoes a conformational change *in vitro*? (**3 points**)

(D) Your previous studies have shown that Examase can adopt multiple oligomeric forms in the absence of RNA. How would you test the effect of C007 on the oligomeric state of Examase in the absence of RNA? (**3 points**)

(E) You have some data that the cellular levels of the nc1000 RNA may change depending upon treatment of cells with C007. Design an experiment to determine if steady state nc1000 RNA levels in cells change in response to different concentrations of C007. (**3 points**)

(F) Finally, your data indicate that Examase may be overexpressed in cells in response to stress. How would you check if the steady state Examase levels differ between normal and stressed cells? (**3 points**)

Question 3

You are studying shear flow-induced inflammatory signaling in endothelial cells. You have conducted experiments with bovine aortic endothelial cells (BAECs) and have assessed inflammatory signaling by measuring activation of NFκB, an important transcription factor for transcription of inflammatory genes. NFκB activation was assessed by either Western blotting lysates with a phosphorylation-specific antibody against the p65 subunit of NFκB or quantifying the percentage of cells with nuclear localization of NFκB by immunofluorescence microscopy.

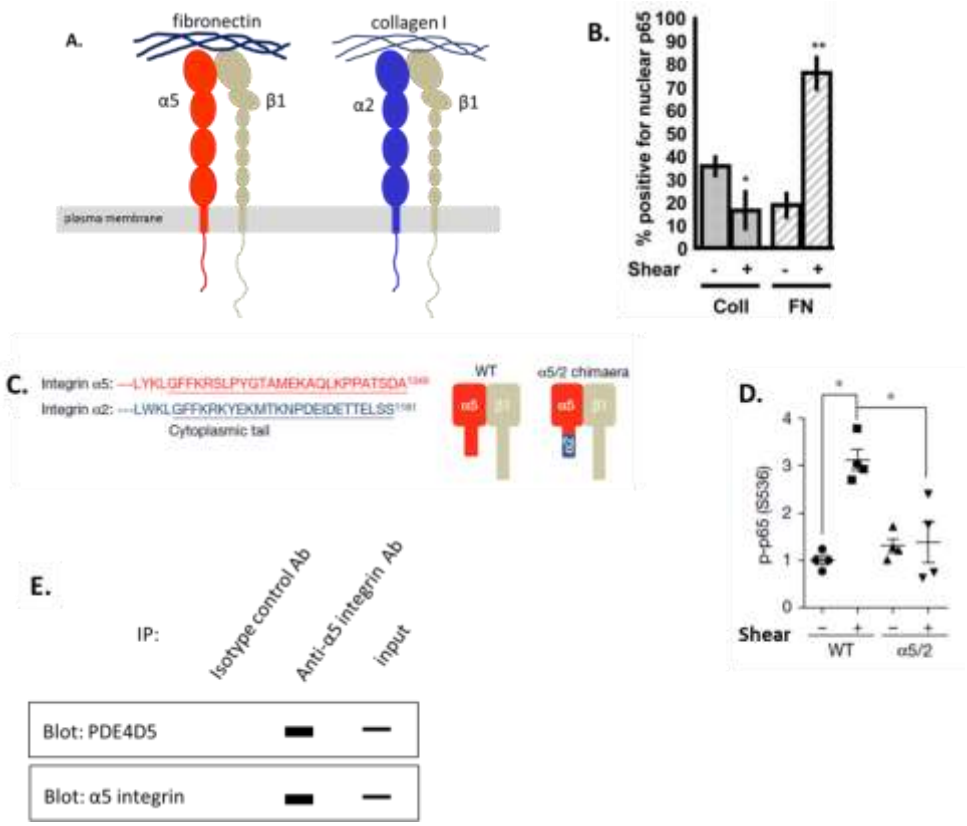


Figure 1. A. Diagramatic representation of the α5β1 and α2β1 integrins. **B**. BAECs were plated onto collagen or fibronectin coated glass and cultured in static or shear flow conditions for 8 hrs at which time the nuclear localization of NFκB was assessed by immunofluorescence microscopy. **C**. Schematic of "WT" and "α5/2 chimera" integrin. The sequences of the cytoplasmic domains of α2 and α5 are shown with the regions that were swapped in the chimera underlined. **D**. BAECs were transfected with the chimeric integrins shown in **C**. Transfected BAECs were plated on fibronectin coated plastic in static or shear flow conditions and 8hrs later activation of NFκB determined by western blotting with a phospho-specific antibody against the p65 subunit of NFκB. Shown is densitometry of the western blot results relative to total p65 protein. **E**. Immunoprecipitation from lysates of BAECs transfected with WT α5β1 integrin with an antibody against the extracellular domain of α5 integrin. Immunoprecipitates were blotted with an antibody against the phosphodiesterase PDE4D5 or α5 integrin.

(A) What do the results in **Figure 1B** and **D** tell you about the role of fibronectin and the fibronectin receptor integrin α5β1 in the shear-induced activation of NFκB? (**2 points**)

(B) Your lab has generated data that indicates that shear-induced activation of NFκB in BAECs transfected with the WT α5β1 integrin on fibronectin (as shown in **Figure 1D**) correlates with decreased cAMP levels. In addition, you know from previous studies that cAMP suppresses NFκB activation. You have performed an immunoprecipitation experiment described in **Figure 1E** to assess interaction of the phosphodiesterase PDE4D5 with α5 integrin.

i. Describe a follow-up immunoprecipitation experiment using the reagents described in **Figure 1** that would test whether α5/PDE4D5 association could play an important role in the matrix-specific effects on shear-induced NFκB activation shown in **Figure 1B**. Be sure to include appropriate controls (**4 points**)

ii. Describe an experiment to test whether PDE4D5 binds directly to α integrin. Be sure to include appropriate controls (**2 points**)

(C) Propose a model of how shear flow induces NFκB activation in BAECs. The model must include integrins, cAMP and PDE4D5. (**4 points**)

(D) Propose an experiment to test the model you proposed in C. Be sure to include appropriate controls (**8 points**)

Question 4

You are working in a lab studying activation and inhibition of a triheteromeric membrane protein complex comprised of four protein subunits: two copies of the A subunit and one copy each of the B and C subunits (**Fig. 1**; Note: A, B and C are homologs and are similar in size, ~60 kDa). Your lab has previously identified the locations of posttranslational modifications in A, B and C, and also raised antibodies specific for each subunit.

Your goal is to determine the structure and organization of the triheteromeric complex, and to define its domain interactions with ligands at the molecular level.

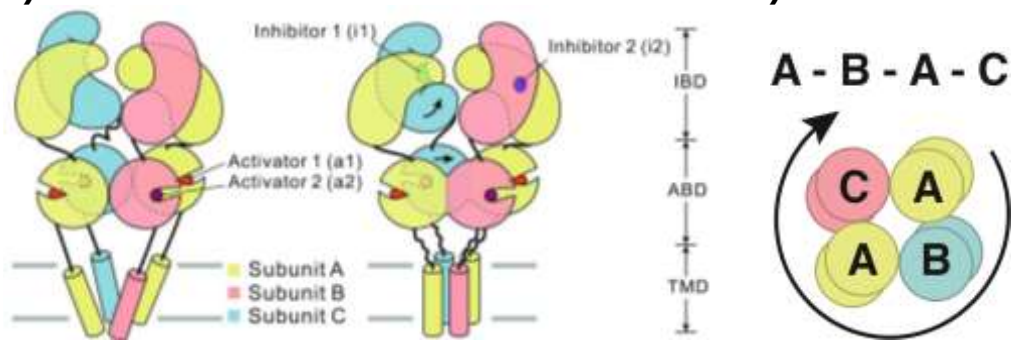


Figure 1: **A potential organization of the triheteromeric A/B/C complex**.
a) A **side view** of the triheteromeric complex. Each A/B/C subunit contains three domains: transmembrane domain (TMD), activator binding domain (ABD) and inhibitor binding domain (IBD). Two activators named activator 1 (*a1*) and activator 2 (*a2*) bind to the ABD, while two inhibitors named inhibitor 1 (*i1*) and inhibitor 2 (*i2*) bind to the IBD.
b) A **top view** of the complex highlighting one potential organization of the four protein domains (clockwise from top right: A-B-A-C).

(A) Fig. 1b shows one possible arrangement of the complex triheteromeric: A-B-A-C.

a. List or sketch the other possible arrangements of the subunits within the complex. (**1 point**)

b. Describe **ONE** approach that would allow you to experimentally define the correct stoichiometry and organization of the complex. (**3 points**)

(B) Having defined the complex composition and organization, your PI sets you the ambitious goal of determining the high-resolution (atomic or near-atomic resolution) structure of this complex and/ or substituent domains. Describe the key steps in determining the structure using ONE potential

approach. Be sure to clearly state your rationale for your choice as well as any potential disadvantages or limitations you identify with the selected approach. (**6 points**)

(C) Briefly describe your strategy for expression and purification of this triheteromeric membrane protein complex for your structure determination. Be sure to justify your choices and note any potential limitations of your chosen approach. (**4 points**)

(D) The fused-ring aromatic ligand MK-801 binds within a hydrophobic pocket in the transmembrane domain (TMD) of the triheteromeric complex to allosterically regulate ligand binding in the ABD and IBD. You determine the structure of the triheteromeric complex structure bound with MK-801 to reveal its binding site in the TMD (**Fig. 2a**). Using an established binding assay (an example is shown in **Fig. 2b**), you also determine binding affinities for a series of variant proteins as shown in **Table 1**.

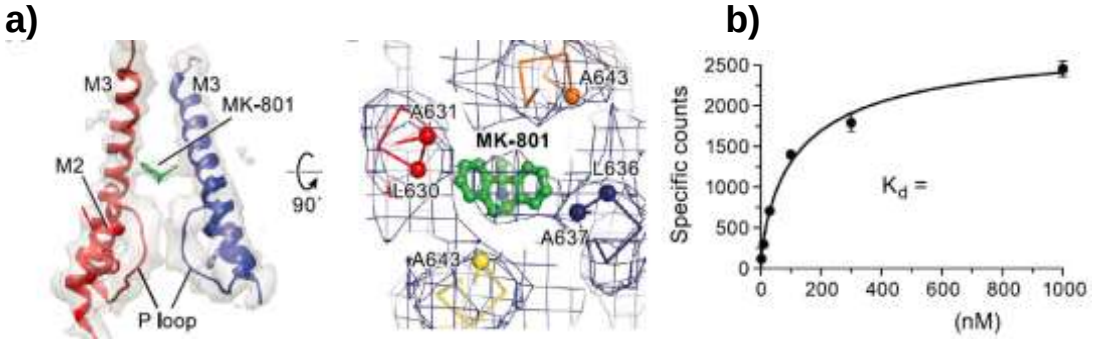


Figure 2. Binding of MK-801. ***a)*** The MK-801 binding site in the TMD of the triheteromeric complex. ***b)*** An example binding experiment for MK-801 interaction with the wild-type triheteromeric complex.

Table 1. MK-801 binding affinity for triheteromeric complex variants.

Substitution	Subunit	Color in Fig. 2	Kd (nM)
Ala 643 to Leu	A	Orange/yellow	1000
Ala 637 to Leu	B	Blue	500
Ala 631 to Leu	C	Red	100
Leu 636 to Trp	B	Blue	5
Leu 630 to Trp	C	Red	No binding

c. From **Fig 2b**, what do you estimate the binding affinity (Kd) to be for MK-801 interaction with the wild-type heterotrimeric complex? (**1 point**)

d. For each variant noted in **Table 1**, provide a potential explanation for the impact on MK-801 binding affinity. (**4 points**)

e. What do you predict would be the effect on binding affinity of an Ala643 to Glu substitution in the A subunit? (**1 point**)

Question 5

You are assigned to investigate function of an uncharacterized protein, ActBP1. To gain rough ideas about its function, you overexpressed full-length ActBP1 as a fusion protein with green fluorescent protein (GFP) in rat fibroblasts and found that thick bundles of actin filaments were significantly increased (**Figure, top panels**). Next, you made two ActBP1 fragments, N-fragment (N-terminal half) and C-fragment (C-terminal half), and performed similar experiments to the one you did for full-length ActBP1. You found that N-fragment localized to actin filaments without increasing bundles (**Figure, middle panels**), and that C-fragment did not localize to actin filaments with no effects on actin bundles (**Figure, bottom panels**).

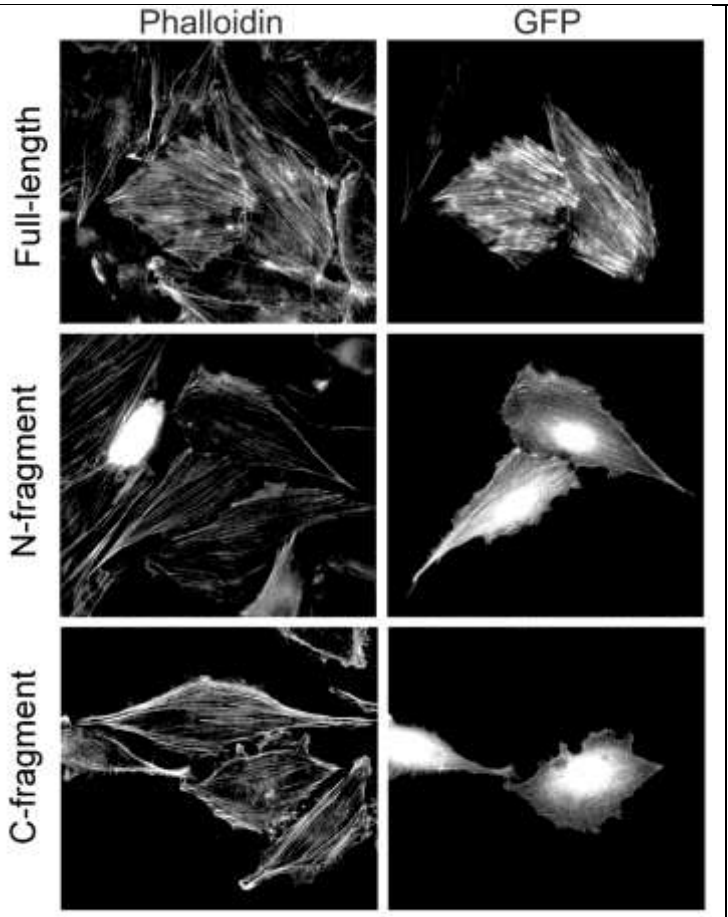


Figure. **Expression of ActBP1 in rat fibroblasts and effects on actin filaments**. GFP-tagged ActBP1 (full-length, N-, or C-fragment) was overexpressed in rat fibroblasts by transient transfection. The cells were fixed and stained with rhodamine-phalloidin (a specific probe for filamentous actin). These were observed by fluorescence microscopy for rhodamine (phalloidin) and GFP.

(**A**) You hypothesized that full-length ActBP1 cross-links two or more actin filaments to induce actin bundles. You have successfully purified full-length ActBP1 protein and actin. Assume that you can add any tags (fluorescent, epitope, etc) or chemical modifications to ActBP1 protein and actin. Describe **ONE** experimental approach to test your hypothesis including appropriate controls. (**4 points**)

(**B**) You have successfully demonstrated that purified full-length ActBP1 can bundle actin filaments. Based on the results shown in the **Figure**, describe **ONE** hypothesis about the mechanism of actin filament bundling by ActBP1 including specific roles of N- and C-fragments. (**3 points**)

(**C**) Describe a set of experimental approaches to test your hypothesis that you proposed in (**B**). If different sets of experimental approaches are needed separately to test functions of N- and C-fragment, feel free to do so. Include appropriate controls. (**7 points**)

(**D**) You presented all these data to your thesis committee and stated your conclusion that ActBP1 is critical for bundling actin filaments. However, one of your committee members criticized that you cannot conclude the function of ActBP1 from the overexpression experiments. You came back to the lab, quantified protein levels by Western blot, and found that GFP-ActBP1 was present five times as much as endogenously expressed ActBP1. State whether you agree or disagree with the committee member's comments, and explain why you think so. Then, propose **ONE** additional experimental approach to strengthen your conclusion. Include appropriate controls. (**6 points**)

Question 6

The diagram below illustrates the affinity of the 5 anti-apoptotic BCL2 proteins with 8 BH3-only BCL2 family members. Red indicates high affinity, orange is intermediate affinity and green is low affinity (not physiologically relevant).

(**A**) Based on what is presented here, which BH3-only proteins would you predict to be BH3-only activator proteins and which ones would be BH3-sensitizer proteins? (**4 points**)

(**B**) Explain the rationale for why you assigned proteins as BH3-only activator proteins. As part of the explanation be sure to include the function of BH3-only activator proteins and how that guided your decision (diagrams are always acceptable as part of your answer). (**6 points**)

(**C**) If you were going to design a drug that mimicked the function of a BH3-only protein to selectively kill cancer cells, would you pick a BH3-only activator or sensitizer protein? Please explain why you picked one over the other and in your explanation be sure to include the molecular basis for why your drug would work to selectively kill cancer cells. (**5 points**)

(**D**) Design an experiment that demonstrates that your drug is working by the mechanism that you predict. Be sure to include what your readout will be and how it will measure drug activity. (**5 points**)

Question 7

The CDC just announced the detection of a new infectious agent surfacing initially in sub-Saharan Africa that has now spread to Atlanta. Long induction periods and developmental phases, typical of a slow acting virus, give an initial phenotype of uncontrolled hair growth, allowing the infection to be vectored by sloughing off hair strands.

Initial results have identified viral-like particles isolated from the hair of symptomatic individuals and these particles induce hair growth in mice. Chemical analyses detect both protein and RNA components, and infectivity is attenuated following RNase treatments, but not eliminated. Proteases are not effective alone, but when the particles are treated with both RNases and proteases, infectivity is destroyed. Interestingly, if the particles are treated with EDTA, they also lose activity, but the activity can be recovered in the presence of excess Cu(II). Protein sequencing by mass spectrometry finds a small 40 residue peptide having strong sequence homology with the Aβ peptide of Alzheimer's disease, but the RNA is heterogeneous and does not obviously code for the peptide.

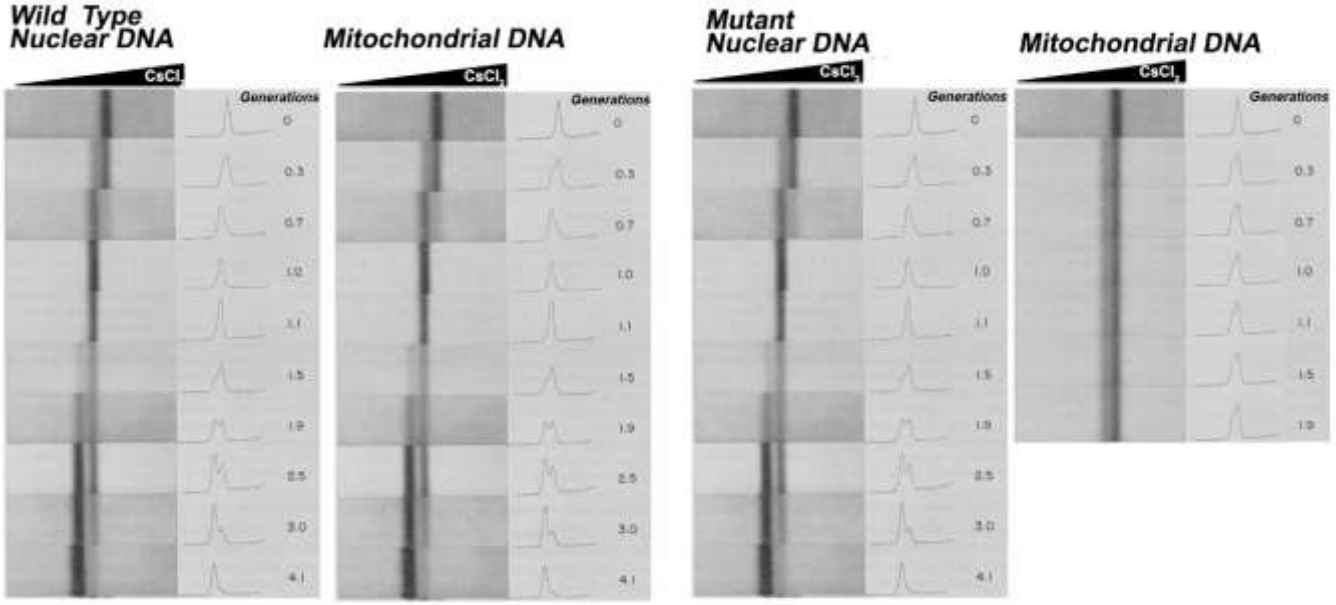
Use magnetic resonance as the principal approach, complemented by other methods as necessary, to address the following:

(A) Describe an experimental design to determine the relative stoichiometry of protein to RNA in the particles. (**6 points**)

(B) Propose one set of experiments necessary to determine whether the Cu(II) binds to the peptide, the RNA, or both. As part of your answer, and in as much detail as possible, sketch the predicted spectra from your proposed experiments. (**7 points**)

(C) Propose an experimental design to determine whether the structure changes when Cu(II) is removed with EDTA, and whether it changes again when Cu(II) is re-added to the RNA/peptide particle. (**7 points**)

Question 8



Meselson and Stahl performed experiments similar to the one depicted here to test the hypothesis of the semiconservative nature of DNA replication [PNAS 46:671 (1958)]. The images present ultraviolet absorption photographs showing DNA bands resulting from density gradient centrifugation of lysates of a wild type and mutant yeast sampled at various times after the addition of an excess of N^{14} substrates to a growing N^{15}-labeled culture.

Yeast were fractioned and nuclear and mitochondrial DNA were isolated. Each photograph was taken after 20 hours of centrifugation. The density of the $CsCl_2$ solution increases to the right. Regions of equal density occupy the same vertical position on each photograph. The time of sampling is measured from the time of the addition of N^{14} in units of the generation time. Microdensitometer tracings of the DNA bands are shown in the adjacent photographs.

Note the contrast in DNA sedimentation between wild type and mutant strain.

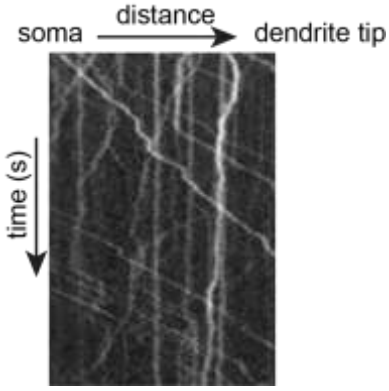
(**A**) Describe the phenotype of the mutant yeast strain. Formulate two molecular hypotheses that could account for the phenotypic differences between these strains. (**8 points**)

(**B**) Choose one of your hypotheses and design an experimental strategy to test your hypothesis. (**8 points**)

(**C**) The gene mutated in this strain encodes for a membrane protein that possesses 8 transmembrane domains. How could a membrane protein account for the phenotype observed in the mutant strain? (**4 points**)

Question 9

You are currently rotating in a lab that studies the role of Cargo X in neurodegeneration. From a mutant screen, your lab previously showed failure to transport Cargo X from the soma into the dendrites is sufficient to cause neurodegeneration. Microtubules are required for the transport of Cargo X into the dendrites, although the mechanisms are unclear. As part of your rotation project, you used live imaging to monitor the dynamics of fluorescent Cargo X. During group meeting, you presented the kymograph shown below.

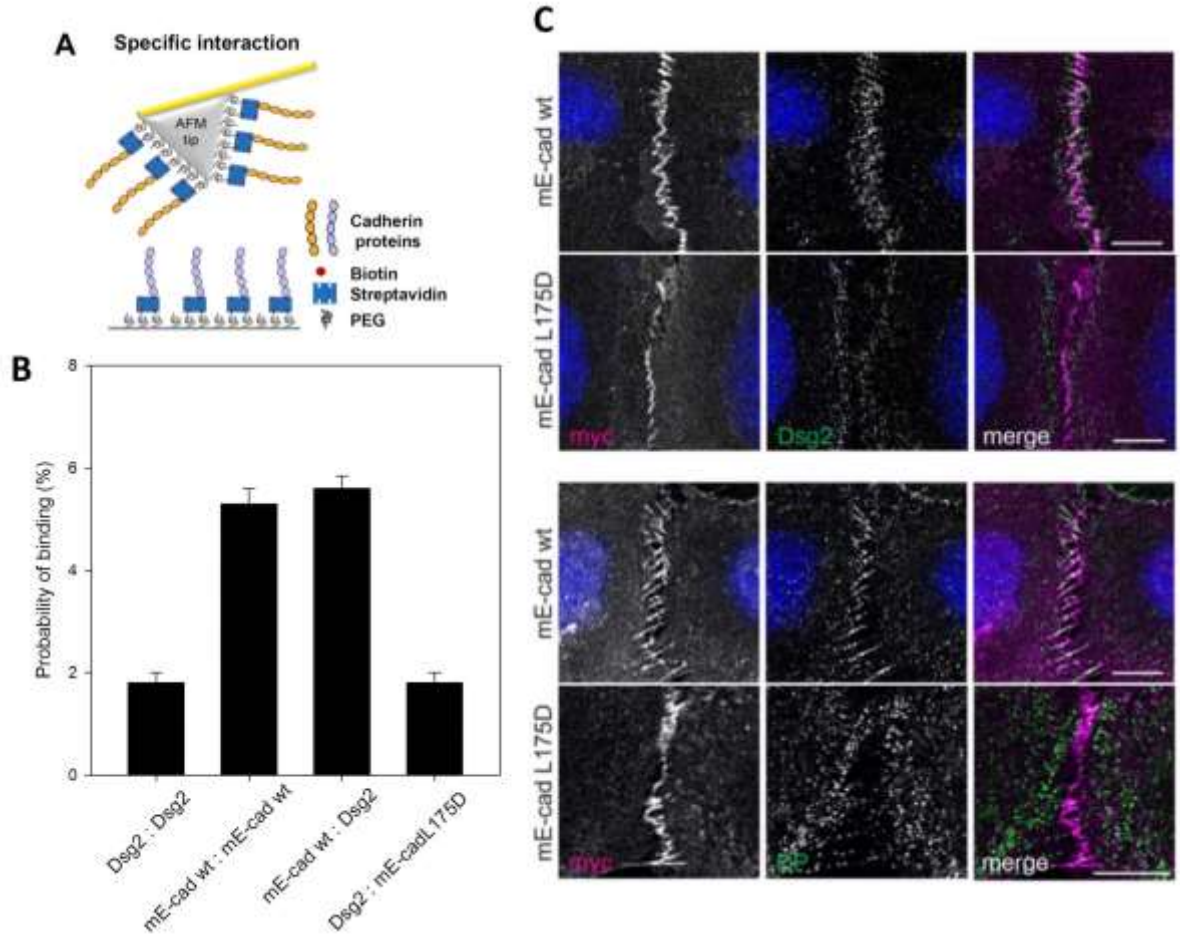


(A) During your presentation, you are asked to briefly describe the motility of Cargo X. In your description, be sure to address whether Cargo X moves in the anterograde or retrograde direction and if the motility is processive. (**6 points**)

(B) Your PI is now eager to submit a grant to the Big Brains Foundation. Write an aim for a grant in which you state a hypothesis for the mechanism for how Cargo X moves the anterograde direction within the dendrites. Describe an experiment with relevant controls to test your model and describe at least two possible outcomes. In discussing the two possible outcomes, mention an alternative hypothesis and how your experiment will allow you to distinguish between these possibilities. (**8 points**)

(C) Dendrite microtubules show mixed polarity. Describe an experiment to determine the orientation of microtubules within dendrites. (**6 points**)

Question 10



You are investigating protein interactions between cadherins using atomic force microscopy (AFM). In these experiments, you are using purified proteins to measure the binding capacity of a classical E-cadherin (mE-cad) and a desmosomal cadherin desmoglein-2 (Dsg2) (**Panel A**). The results you obtain are shown in **Panel B** and are expressed as probability of binding between purified cadherins. In addition to wild type (wt) mE-cad, you also test a point mutant of E-cadherin, mE-cadL175D. You then express wt or the mE-cadL175D mutant with a myc epitope tag in mouse epithelial cells lacking any endogenous classical cadherins and measure the effect on endogenous Dsg2 or desmoplakin (DP), a desmosomal plaque protein using immunofluorescence microscopy (**Panel C**). Note that nuclei are shown in blue in some micrographs

(**A**) What does the AFM assay shown in **Panels A** and **B** demonstrate about the binding capacity of E-cad wt, Dsg2 and mE-cadL175D? (**4 points**)

(**B**) What does the transfected cell data in **Panel C** suggest about desmosome assembly? (**4 points**)

(**C**) State ONE mechanistic hypothesis that could explain the results obtained by AFM and in the transfected cell analysis. (**6 points**)

(**D**) Outline TWO experiments that you could perform to test your hypothesis and how you would interpret the results. (**6 points**)

Question 11

PD-L1, a type I transmembrane protein, is a key molecule regulating anti-tumor immunity and a recently validated drug target for cancer immunotherapy. CMTM6 (CKLF-like MARVEL transmembrane domain-containing protein 6) was recently identified to modulate the expression level of PD-L1 via its interaction with PD-L1 in the plasma membrane. You want to determine how CMTM6 interacts with PD-L1, but crystallization of the PD-L1/CMTM6 complex is not yet feasible. The literature suggests that a MARVEL domain contains 4 transmembrane helices, with an N-in/C-in membrane topology. However, a look at the protein sequence of human CMTM6, shown below, reveals only 3 clear-cut transmembrane helices.

```
         10         20         30         40         50
MENGAVYSPT TEEDPGPARG PRSGLAAYFF MGRLPLLRRV LKGLQLLLSL

         60         70         80         90        100
LAFICEEVVS QCTLCGGLYF FEFVSCSAFL LSLLILIVYC TPFYERVDTT

        110        120        130        140        150
KVKSSDFYIT LGTGCVFLLA SIIFVSTHDR TSAEIAAIVF GFIASFMFLL

        160        170        180
DFITMLYEKR QESQLRKPEN TTRAEALTEP LNA
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(A) Underline the 3 transmembrane helices of CMTM6 in the sequence above. Also, describe the criteria that you use to predict transmembrane helices from amino acid sequences (**6 points**)

(B) Hypothesize the location of the possible 4th transmembrane helix (i.e. which residues) of CMTM6, and explain why. (**4 points**)

(C) You have just successfully cloned the cDNA of CMTM6, and its heterologous expression in the plasma membrane of transfected XYZ cells can be readily detected by an anti-CMTM6 antibody. The XYZ cell is devoid of endogenous CMTM6 expression. Design ONE set of experiments to determine the membrane topology of CMTM6, and the presence and location of the 4th transmembrane helix. Describe (or draw) the anticipated results that would support your hypothesis of the 4th transmembrane helix, and also those that would disprove your hypothesis. (**10 points**)

Question 12

You extracted total RNA from a cultured glioma cell line after treatment with growth hormones or after mock-treatment. On a Northern blot hybridized to a single probe derived from gene X, you found two transcripts of 2.0 kb and 2.5 kb that are drastically increased in response to growth hormone treatment. In particular, the 2.5 kb band is increased 3 fold whereas the 2.0 kb band is increased 10 fold. You are funded by NIH to investigate gene X in tumor cell proliferation. Please answer the following questions. Be sure to provide necessary controls for experimental design in order to obtain definitive answers.

(A) How do you determine whether the two transcripts derived from gene X are long noncoding RNAs (lncRNAs)? (**4 points**)

(B) Provide a hypothesis for how gene X can produce two transcripts with distinct sizes that are differentially up-regulated by growth hormone? What experiments would you do to test your hypothesis? (**8 points**)

(C) Based on proteomic profiling, you found that many up-regulated proteins are predicted targets specific for microRNA Y. Provide a hypothesis for how up-regulation of gene X leads to increased expression of microRNA Y- targeted mRNAs and how you would test your hypothesis. (**8 points**)