BCDB Qualifying Exam Part I: May 23 & 24, 2017

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 to not 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

Proper signal transduction requires a progression through a series of activated states, usually in a precise order and with a finite lifetime. For instance, treatment of cells with cytokines, receptors, etc., leads to ubiquitination and phosphorylation of proteins, degradation of an inhibitor of the NF-kB transcription factor and eventually new transcription from kB-responsive sites (see Figure on page 2).

(**a**) How would you determine which proteins are covalently modified upon Tumor necrosis factor (TNF)-treatment? Pick **ONE** type of modification and describe what techniques you would use including necessary controls? (**5 points**)

(**b**) How could you determine if the modification you answered in (**a**) was necessary for signal transduction or simply a side product of the activation events? (**5 points**)

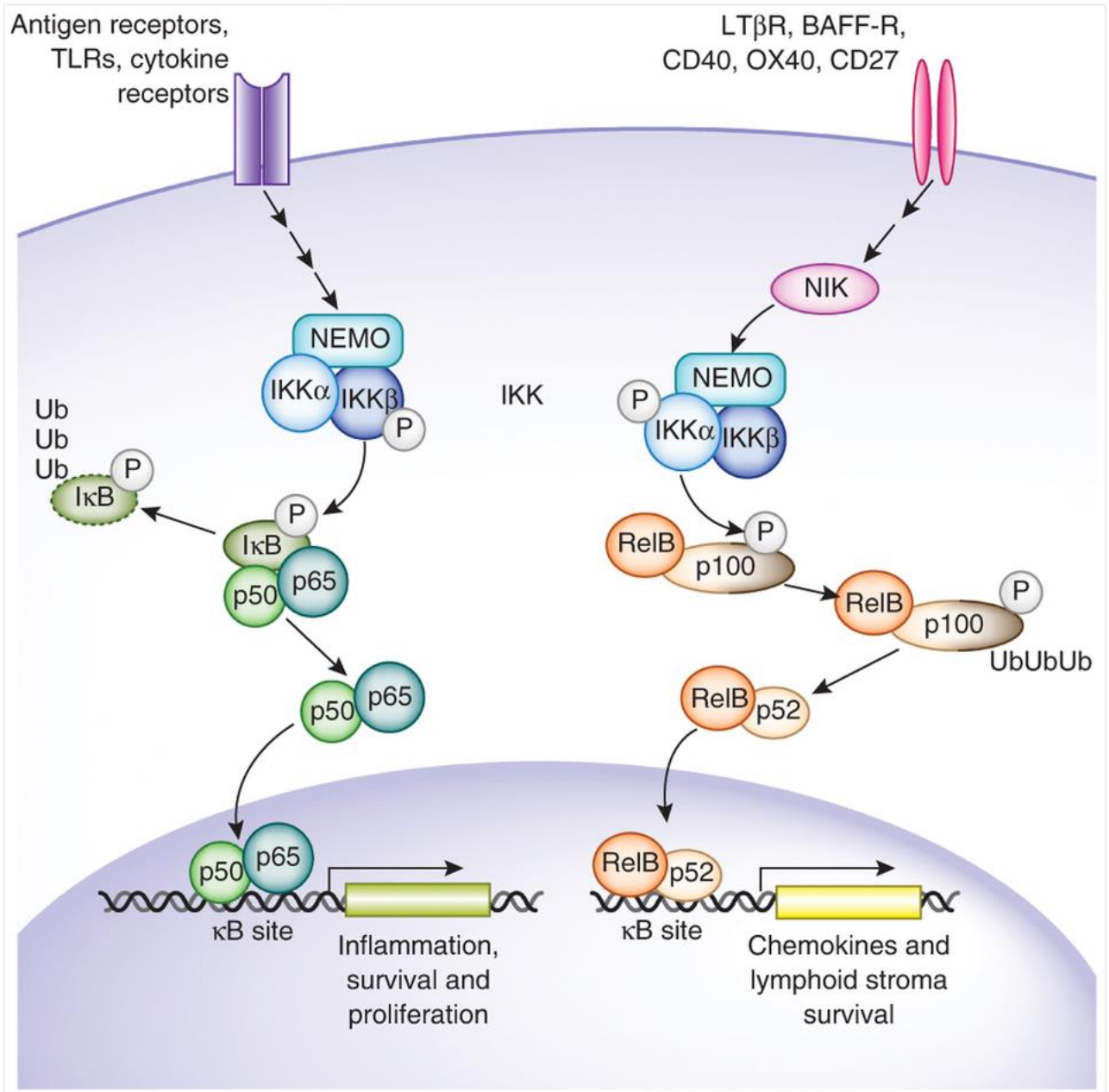
(**c**) How would you use kinetic techniques to establish the order of modification required and the time course of modification? Exactly what would you measure and over what period of time? (**5 points**)

(**d**) How could you demonstrate that phosphorylation of IκB (see Figure) was required for signaling to occur? Describe a positive control you could use to show that phosphorylation was sufficient to allow normal signaling? (**5 points**)

(**Continue on next page**)

Question 1 (Continued from the previous page)

From
NF-κB control of T cell development
Steve Gerondakis, Thomas S Fulford, Nicole L Messina & Raelene J Grumont
Nature Immunology **15**, 15–25 (2014) | doi:10.1038/ni.2785
Received 13 October 2013 | Accepted 12 November 2013 | Published online 18 December 2013

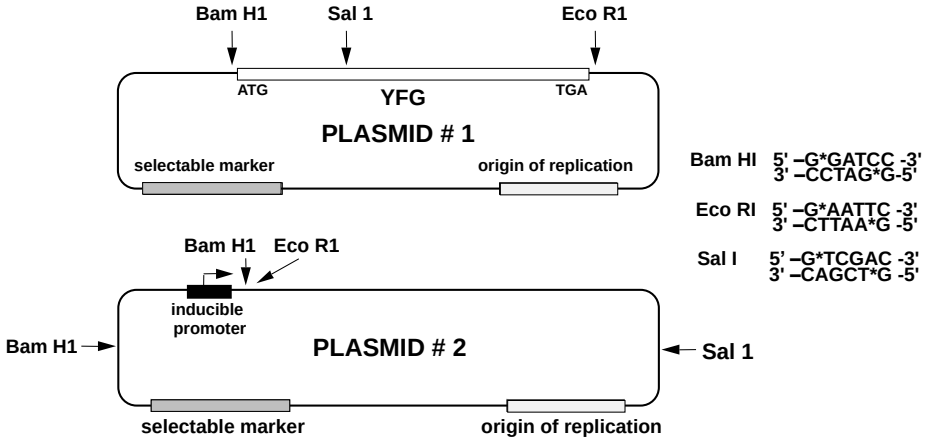


The canonical NF-κB pathway (left) induced by signals including antigens, TLR ligands and cytokines such as TNF uses a wide variety of signaling adaptors to engage and activate the IKKβ subunit of the IKK complex. IKKβ phosphorylation of classical IκB proteins bound to NF-κB dimers such as p50-p65 results in ubiquitination (Ub) of IκB and proteasome-induced degradation. This allows NF-κB to enter the nucleus where it binds specific DNA sequences (κB sites) involved in controlling the transcription of genes encoding functions as diverse as inflammation, cell survival and cell division. The noncanonical pathway (right) engaged by members of the TNF-like family of cytokines requires NIK to activate IKKα, which then phosphorylates p100 (NF-κB2), triggering its proteosomal processing needed for the activation of p52-RelB dimers. Among its functions, this specific NF-κB heterodimer controls gene expression crucial for lymphoid organogenesis.

Question 2

You are given tubes of purified Plasmid #1 and Plasmid #2, illustrated below. Plasmid #1 includes the cDNA sequence from your favorite mouse gene (*YFG*) but no promoter to drive expression of the encoded gene product in bacteria. Plasmid #2 includes an inducible bacterial promoter but not *YFG*.

Both Plasmids #1 and 2 include recognition sites for three restriction enzymes: BamH1, EcoR1, and Sal1. The recognition sites and cut positions (asterisks in the recognition site sequences) for all 3 enzymes are also illustrated below. Assume the cut sites indicated are the only places these three enzymes cut in both plasmids.



Your goal is to start with Plasmids #1 and #2 and make a 3rd plasmid (called Plasmid #3) that will include both the inducible promoter and the *YFG* open reading frame positioned to enable inducible expression of the YFG protein product in transformed host bacteria.

You begin by cutting some of each plasmid and running the restriction products on an ethidium bromide stained agarose gel to see how big the resulting fragments will be. You obtain the results presented in the table below:

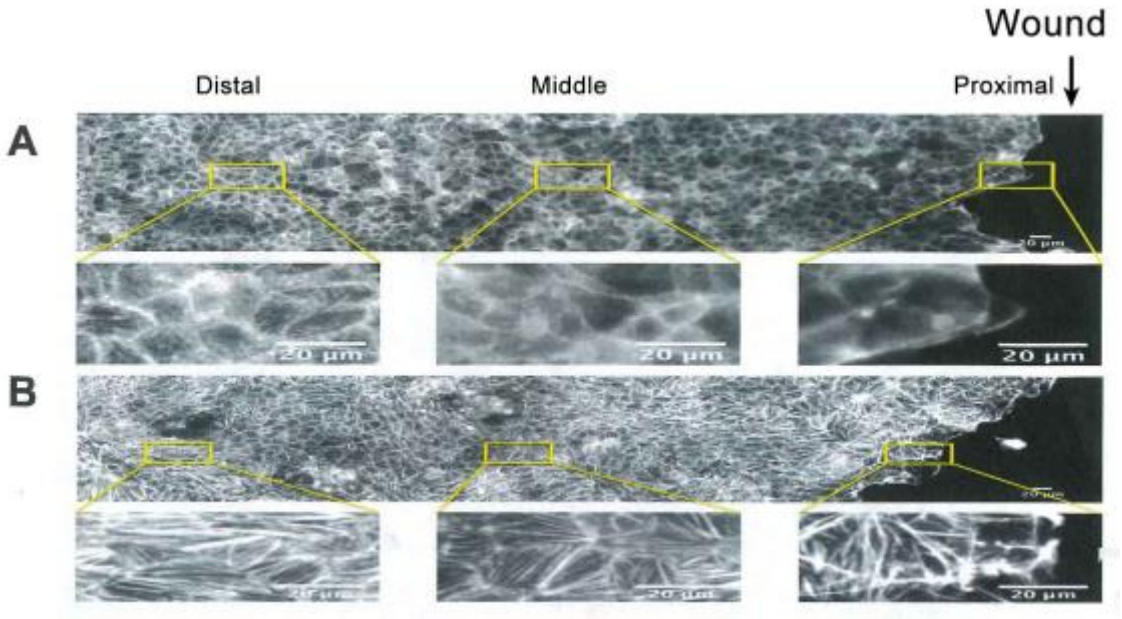
	Plasmid cut with Bam H1	Plasmid cut with Eco R1	Plasmid cut with Sal1	Plasmid cut with Bam H1 and Eco R1	Plasmid cut with Bam H1 and Sal 1	Plasmid cut with Eco R1 and Sal 1	Plasmid cut with Eco R1, Bam H1, and Sal 1
Plasmid #1	6.0kb	6.0kb	6.0kb	2.0kb + 4.0kb	0.5kb + 5.5kb	1.5kb + 4.5kb	0.5kb + 1.5kb + 4.0kb
Plasmid #2	0.8kb + 5.0kb	5.8kb	5.8kb	0.8kb + 5.0kb	0.8kb + 2.0kb + 3.0kb	2.0kb + 3.8kb	0.8kb + 2.0kb + 3.0kb

Question 2 (Continued from the previous page)

(**a**) Given these plasmids and the information provided, describe each of TWO DIFFERENT strategies (**worth 8 points each**) to create Plasmid #3. For each strategy, explain each of the steps you will need to accomplish, including the purpose of each step and any important controls. (**16 points**)

(**b**) Explain each of TWO DIFFERENT approaches you could use to confirm that the Plasmid #3 you have made is correct. (**4 points**)

Question 3



When epithelial cells form a monolayer, they stop migration. However, when a wound is created by a scratch, these cells resume migration toward the wound until the cells reform a monolayer. This process is called "wound healing" and often used as an assay for directional cell migration. You established kidney epithelial cell lines from wild-type mouse strain (**A**) and a mouse strain with a mutation in a novel gene called WH1 (**B**). You performed preliminary wound healing experiments using these two cell lines (see micrographs shown above). Wounds were created on the right sides of the pictures. These cells were fixed after 2 hours and stained with rhodamine-labeled phalloidin (a probe for actin filaments (F-actin)). Representative cells in three regions (proximal, middle, and distal to the wound) are magnified. You immediately noticed that the mutant cells healed the wound much more slowly than wild-type cells.

(**a**) Describe your observations from the micrographs about how actin filaments are organized in these cells at three representative regions. Include statements regarding the following points: (**1**) any differences among cells in proximal, middle, and distal regions; (**2**) any differences between wild-type and mutant cells. (**4 points**)

(**b**) Based on your observations in part (**a**), describe your hypothesis about why wild-type cells heal wounds efficiently and why mutant cells do not. Include your idea about how the difference in actin dynamics (turnover) might contribute to the described cell behaviors. (**4 points**)

(**c**) Describe **ONE** experimental approach to determine whether subcellular actin filament dynamics are different in wild-type and mutant cells. Include appropriate controls, and state **ONE** strength and **ONE** weakness of your selected approach. (**4 points**)

(**d**) You found that the WH1 protein bound directly to actin *in vitro* and in cells. You have confirmed that expression of GFP-tagged WH1 in the mutant cell line can rescue the wound healing

Question 3 (Continued from the previous page)

phenotype to the level of wild-type cells. Based on your observations and hypothesis in (**a**) and (**b**), describe (**1**) your hypothesis for the function of WH1 as an actin-regulatory protein and (**2**) ONE experimental approach to test your hypothesis including controls. (**8 points**)

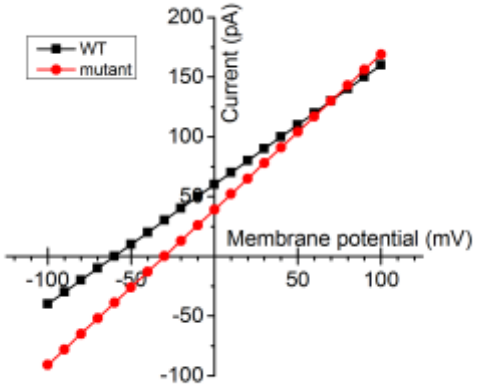
Question 4

Genotyping a family that with an inherited form of epilepsy revealed a mutation in a ion channel gene. The mutation co-segregates with the disorder, with roughly 25% of the family having the disorder. The family's physician arranged to collect skin fibroblasts from affected and non-affected individuals in her family. From the fibroblasts, we made induced pluripotent stem cells that were then differentiated into neurons in culture. We voltage clamped to determine if the mutations affected the electrophysiology of the neuron.

(**a**) The cells were placed into a bathing solution of the following composition: 10 mM KCl, 100 mM NaCl, 10 mM Tris-HEPES pH 7.4. The cells were whole-cell voltage clamped with a pipet solution (intracellular) containing 100 mM KCl, 10 mM NaCl, 10 mM Tris-HEPES pH 7.4.

Calculate E_K _____, E_{Na} _____, and E_{Cl}_____. (**4 points**)

(**b**) Current-voltage relationships from the cells isolated from affected and non-affected individuals are shown in the figure below. What are the reversal potentials for WT and mutant (include units)? (**4 points**)



WT_____

Mutant _____

(**c**) What are the whole-cell conductances for WT and mutant (include units)? (**4 points**)

WT_____

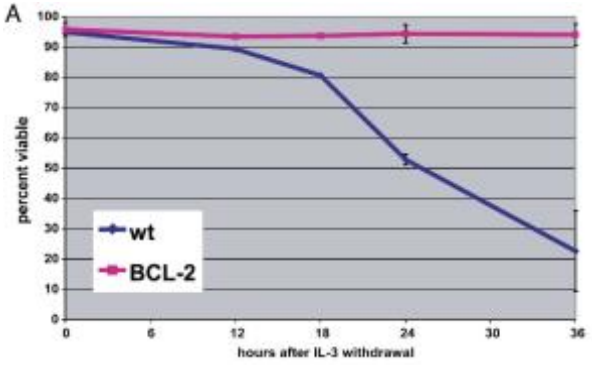
Mutant _____

(**d**) Assume that Tris-HEPES is not permeant through any channel and that these cells could have multiple types of ion channels. How do you interpret the current-voltage relationship for the WT in the Figure? What type(s) of channel (K^+, Na^+, Ca^{2+}, Cl^- etc.) are most likely to explain this current-voltage relationship? How does the current-voltage relationship of the mutant differ from WT? Can you explain this by a loss-of-function or a gain-of-function mutation? Propose a specific hypothesis to explain the result. (**4 points**)

(**e**) Design a patch clamp experiment to test your hypothesis. Draw the experimental setup, the solutions you would use, explain precisely which variables you would change to test your hypothesis, and draw a current-voltage relationship showing the result. Label the axes, label tick marks accurately, and label your plots fully. (**4 points**)

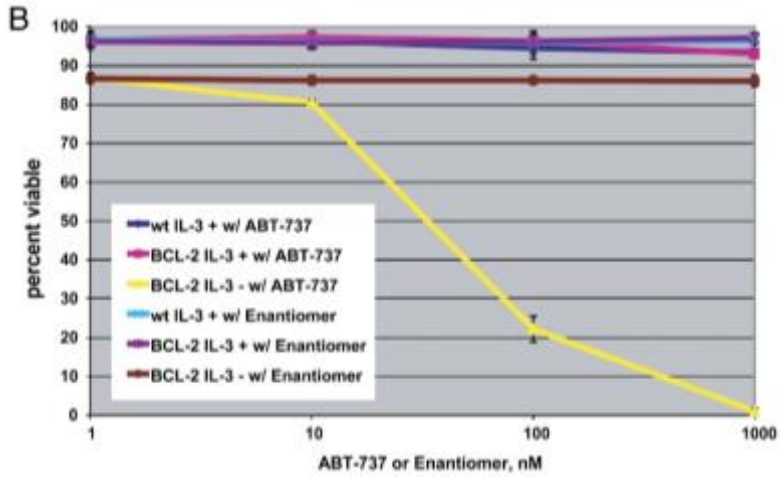
Question 5

In the following experiment, an IL-3-dependent cell line is transfected with an expression vector that results in BCL2 over-expression (BCL-2) or empty vector (WT). The cells are washed and replated in media lacking IL-3 and viability is measured every 12 hours by propidium iodide exclusion.



(**a**) Based on the data, what is the consequence of IL-3 withdrawal in the WT cell line? Please outline the pathway that is activated (feel free to draw as a diagram), and be sure to show how BCL-2 over-expression would block this pathway. (**5 points**)

(**b**) The experiment is repeated, however this time the BH3-mimetic, ABT-737, or an enantiomer of the drug is included and the following results are observed:



Based on these results, answer the following questions. (**1**) Why does the drug only kill the cells that express BCL2 when IL3 is removed? (**2 points**), (**2**) Based on the type of drug, please diagram the molecular mechanism and how this would result in cell death. (**2 points**), (**3**) What do these data tell you about how IL3 regulates cell survival? (**1 point**)

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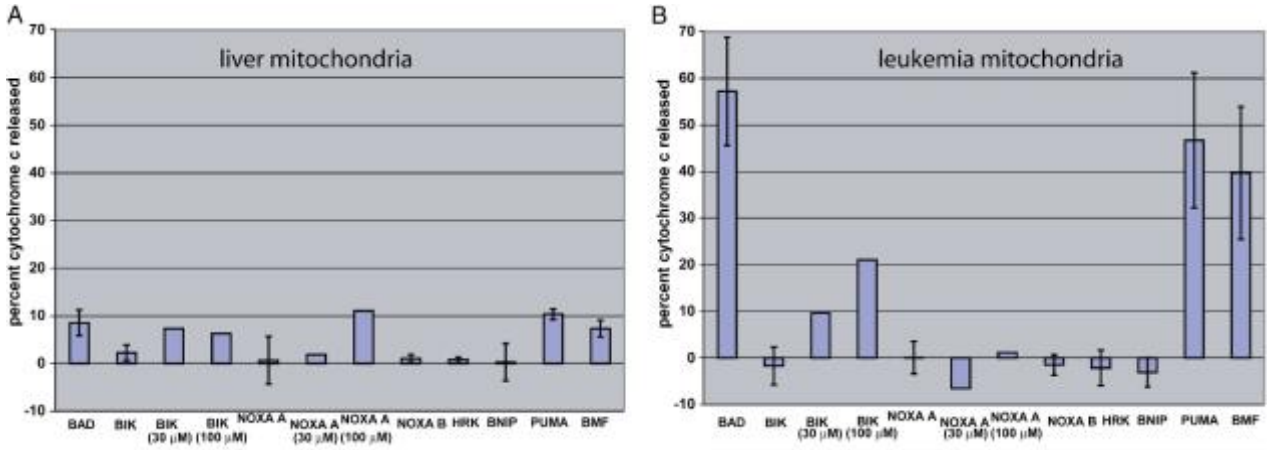
Question 5 (Continued from the previous page)

(**c**) Since you now have a better feel for how one might use this drug, you decide that it might be useful as a cancer therapeutic. However, you aren't sure which cancers that it might work best against or even if it will selectively kill cancer cells. In your first experiment, you compare the profiles of normal mouse liver to mouse leukemias. In this assay, you isolate mitochondria from cells, incubate the mitochondria with a peptide containing the BH3 domain (10 μM unless indicated) from the different BCL2 family members, and then measure the amount of cytochrome c released. The data are presented as percent of the total cytochrome c available to be released (i.e.



that which is released if you lyse the mitochondria).

This table provides information on the binding patterns of BH3-only proteins to anti-apoptotic members (Red=strong affinity, Orange=moderate affinity, Green=little to no affinity):

	BIM	BID	BAD	BIK	NOXA	HRK	PUMA	BMF
BCL-2								
BCL-XL								
BCL-w								
MCL-1								
BFL-1								

(**1**) Based on these findings, which BCL2 family member are liver cells mostly likely dependent on for survival? (**1 point**); (**2**) How about the leukemia cells? (**1 point**);
(**3**) Why is there a difference in the dependence between the normal and tumor cells? (**3 points**)

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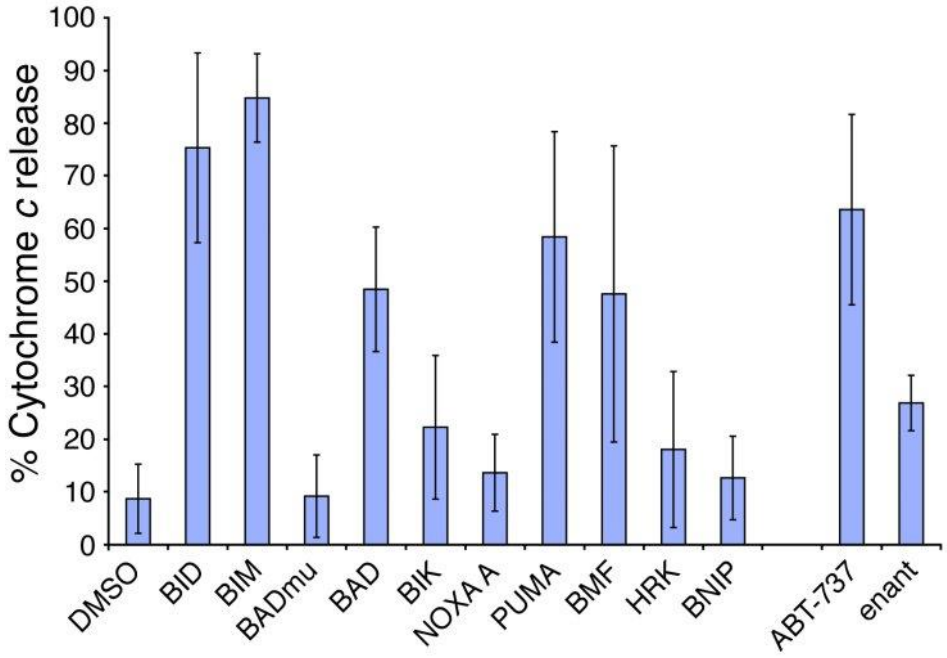
Question 5 (Continued from the previous page)

(**d**) Finally you test your drug, its enantiomer, and the peptides using the same assay on a series of samples from patients with leukemia (BADmu is a version of the BAD peptide with a mutated BH3).



(**1**) Based on the activity of the peptides in these samples, what are the 3 mostly likely targets of the drug? (**2 points**). (**2**)You also do the exact same experiment on a few samples of lymphocytes from donors that do not have leukemia and find that only the BIM and BID peptides cause cytochrome c release in the normal cells. Why is this the case? (**3 points**)

Question 6

Pantothenic acid (vitamin B_5) is the substrate for the biosynthesis of coenzyme A, an essential cofactor in energy metabolism. Because mammals obtain vitamin B_5 from their diet, the biosynthesis of pantothenic acid in pathogenic bacteria has been identified as a promising antibacterial drug target. Ketopantoate reductase (KPR) catalyzes the NADPH-dependent reduction of ketopantoate (KP) to pantoate, the essential precursor of pantothenic acid. You decide to investigate the structure and kinetics of this enzyme from *Staphylococcus aureus*, an important human pathogen.

(**a**) To begin this project, you have cloned the gene encoding KRP into a bacterial expression vector containing an N-terminal 6xHis Tag followed by a protease cleave site. You next need to express and purify protein for both kinetic and structural studies. Briefly describe your strategy to purify KPR and validate that the protein is of suitable quality for your planned experiments. Be sure to define the purification requirements for each application. (**6 points**)

(**b**) Having obtained purified protein, you are able to crystallize and determine the crystal structure of the *S. aureus* enzyme at 1.8 Å resolution, the first description of a dimeric KPR (**Figure 1**).

(**b-1**) How would confirm the oligomeric state of this protein in solution? (**2 points**).

(**b-2**) How would you confirm that the dimeric interface observed in the crystal is indeed the protein dimerization interface present in solution? (**2 points**).

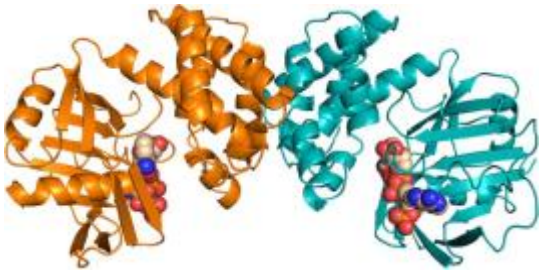


Figure 1. Cartoon representation of KPR which crystallized as a dimer. Protomer 1 is colored orange and protomer 2 is colored cyan. The bound NADPH is shown as spheres (C=white; O=red; N=Blue).

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Question 6 (Continued from the previous page)

(**c**) Several mutations have been identified that affect KPR catalysis. You are particularly interested in variants of residue A181 which show interestingly altered kinetics. To understand how each of the A181 substitutions may affect KPR activity your incredibly talented undergraduate student has crystallized and collected x-ray diffraction data for three different mutant proteins: A181L, A181K, and A181P. Using your wild-type KPR structure, your student performs molecular replacement to obtain phases and next determines and refines all three mutant protein structures.

As the student's mentor on this project, you must decide if the data and structural refinement are of sufficiently high quality (relevant statistics are shown in **Table 1**, below). Where problems exist, how do you suggest that your mentee improves the data and/or structural model? Give your rationale for your advice on each of the three structures. (**4 points**)

Table 1. X-ray data collection and structure refinement for KPR

	KPR_{A181K}	KPR_{A181L}	KPR_{A181P}
	Data Collection		
space group	$P2_12_12_1$	$I121$	$I121$
unit cell dimensions			
a, b, c (Å)	42.2, 85.2, 177.1	123.0, 66.8, 88.7	123.0, 66.8, 88.7
α, β, γ (˚)	90.0, 90.0, 90.0	90.0, 112.0, 90.0	90.0, 112.0, 90.0
resolution (Å)	1.81 (1.87–1.84)	2.62 (2.82–2.72)	2.5 (2.62–2.50)
completeness (%)	99.9 (100)	89.2 (65.8)	99.2 (99.8)
redundancy	13.8 (12.6)	3.6 (2.4)	10.6 (3.4)
no. of reflections	820276	214018	214018
$I/\sigma(I)$	24.84 (2.19)	12.88 (2.80)	12.88 (2.80)
R_{meas}[b] (%)	10.8 (136.2)	15.8 (102.0)	15.8 (102.0)
	Refinement		
R_{work}/R_{free}	0.173/0.211	0.222/0.268	0.222/0.368
no. of protein/water atoms	4740/411	4289/42	4289/90
B factor (Å^2) for protein	29.4	62.3	162.3
bond lengths (Å^2)	0.008	0.002	0.040
bond angles (˚)	1.07	0.665	2.665
Ramachandran space, % residues			
preferred	95.60	94.79	94.79
additionally allowed	4.03	4.83	0.93
disallowed	0.37	0.39	4.83

[a]Values in parentheses are for the highest-resolution shell based on an I/σ cutoff of 2.
[b]R_{meas} is the redundancy-independent merging R factor.

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Question 6 (Continued from the previous page)

(**d**) You next analyze the functional impact of the A181L substitution and find that it increases the K_m of ketopantoate 844-fold, without affecting k_{cat}.

(**d-1**) Does this suggest that the A181L mutation affects binding or maximal enzymatic rate? (**1 point**)

(**d-2**) From a structure of the A181L KPR you solve with your undergraduate student (**Figure 2**), what might explain how this mutation is driving the observed effect on enzyme kinetics? (**2 points**).

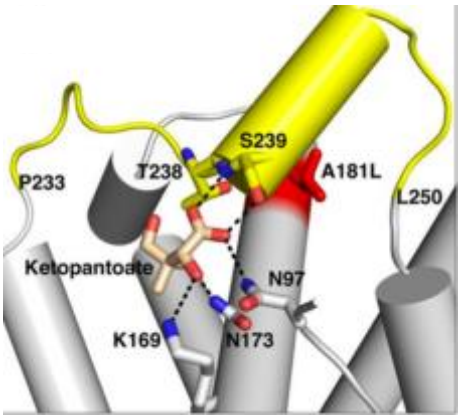


Figure 2. Structure of the A181L KPR mutant enzyme showing a cartoon representation of the ketopantoate binding site. KPR residues interacting with the ketopantoate are depicted as sticks and labeled. The A181L substitution is colored red.

(**d-3**) As a control for your mechanistic studies, you would like to develop a catalytically dead KPR mutant. How might you accomplish this using a structure-guided approach? Clearly state the rationale for your strategy. (**3 points**)

Question 7

You are performing a western blot. You have a stock of your primary antibody but no information about it is available because of a recordkeeping error. It will be important to figure out if it is a monoclonal or a polyclonal antibody. In this regard:

(**a**) Explain succinctly the difference between a monoclonal and polyclonal antibody. (**5 points**)

(**b**) Identify **TWO** benefits a monoclonal antibody has over a polyclonal antibody and **ONE** drawback of a monoclonal antibody. (**3 points**)

(**c**) Describe how monoclonal antibodies are made. What do you need to make a monoclonal antibody that limits the species of animal that generates it? (**3 points**)

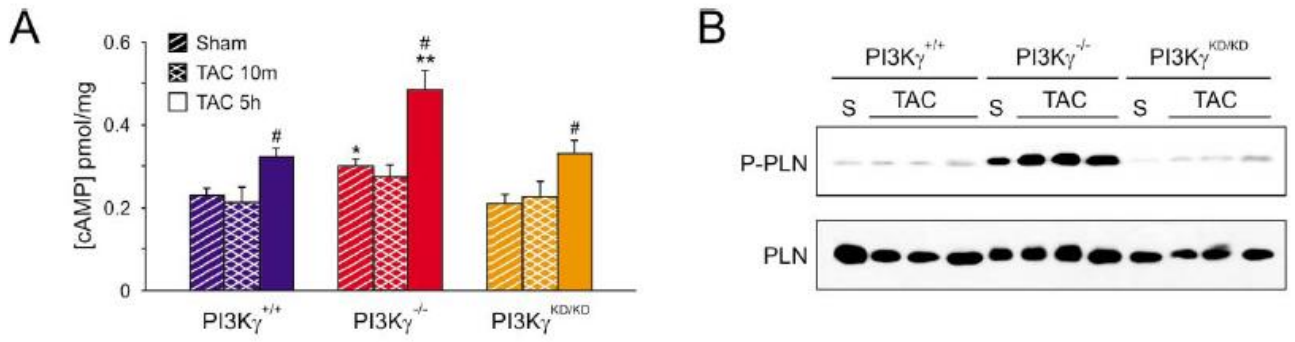
(**d**) Design **ONE** experiment to figure out if your antibody preparation is monoclonally- or polyclonally-derived. (**3 points**)

(**e**) Describe all of the steps required to generate a publication-quality western blot that would include your primary antibody. Include controls to validate specificity of your antibody. Be specific about the reagents/materials you would use, and explain the principles of each step of the procedure. (**6 points**)

Question 8

You are studying phosphoinositide 3-kinase γ (PI3Kγ) signaling in the heart and want to test the hypothesis that PI3Kγ signaling regulates the cAMP-dependent kinase PKA (Protein Kinase A). Previous studies have shown that constricting the aorta in mice (TAC, transaortic constriction) causes a robust increase in PI3Kγ activity within 2 minutes. You perform TAC in 3 strains of mice: wildtype ($PI3K\gamma^{+/+}$), PI3Kg KO ($PI3K\gamma^{-/-}$), or a PI3Kγ KD (kinase dead) mutant that abolishes PI3Kγ kinase activity ($PI3K\gamma^{KD/KD}$). Mice in which surgery was performed but the aorta was not constricted served as controls and referred to as "Sham".

Figure A shows measurements of cAMP that you make from heart samples either 10 minutes or 5 hours after TAC or Sham surgery. # indicates $p<0.05$, TAC vs. Sham. *indicates $p<0.05$ and **indicates $p<0.01$, $PI3K\gamma^{-/-}$ vs. $PI3K\gamma^{+/+}$ and $PI3K\gamma^{KD/KD}$. Figure B shows western blots on heart protein lysates from either a single Sham (S) mouse or 3 mice per TAC group after 5 hours. Membranes were blotted with antibodies against PLN, a protein known to be phosphorylated in a PKA-specific manner. The top panel was blotted with a phosphorylation-specific PLN antibody and the bottom panel with an antibody that recognizes total PLN.



(a) Briefly describe the results of the experiments shown in Figures A and B. Do not make conclusions about the underlying mechanisms here, simply state the results of the experiments. (**4 points**).

(b) Make a conclusion about this data. What does it tell you about how PI3Kγ may be regulating PKA signaling? (**4 points**)

(c) As you remember from the Cell Signaling minicourse, cAMP levels can be regulated through phosphodiesterase and adenylyl cyclase activity. With this in mind and taking into account the data in Figures A and B, develop **ONE** model that describes **ONE** mechanism of how PI3Kg could regulate PKA activity. (**4 points**).

(d) Based on the model you described in Part (**c**), describe **ONE** experiment that would test your hypothesis. Include rationale for the choice of a technique, experimental setup, controls, and how you would interpret your results. You have all the reagents that were used in Figures A and B. You

Question 8 (Continued from the previous page)

also have access to a drug that inhibits PI3Kg kinase activity and assays to measure phosphodiesterase activity and adenylyl cyclase activity. (**8 points**).

Question 9

Type II restriction endonucleases (REases) recognize and cleave specific sequences in double-stranded DNA with extraordinary specificity. They have been among the workhorses of molecular biology for decades.

There are many subclasses of Type II REases, which are drawn from far-flung corners of the prokaryotic kingdom. The more familiar laboratory REases cleave within symmetric DNA recognition sequences, and are termed Type IIP, for palindromic.

Members of another subclass cut at defined positions outside the binding site and are termed Type IIS, for shifted. The best-studied Type IIS REase is Fok I, which binds as a monomer to an asymmetric recognition site and cleaves 9 bp outside the site on one strand and 13 bp outside the site on the other. Remarkably, although Fok I binds as a monomer, it cleaves only as a dimer, that is, when the catalytic domain of a second molecule of Fok I is recruited to form an active complex. The original DNA-bound FokI cleaves one strand, and the recruited FokI cleaves the other.

Although cleavage can occur when the second Fok I is provided at high concentration in solution (as in the figure), it occurs much more readily when the two Fok I molecules are each bound to separate sites in the same DNA molecule. The sites do not have to be nearby or in a specific relative orientation, but they must be on the same DNA.

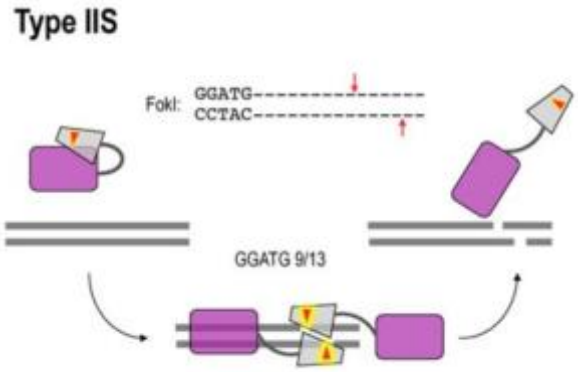


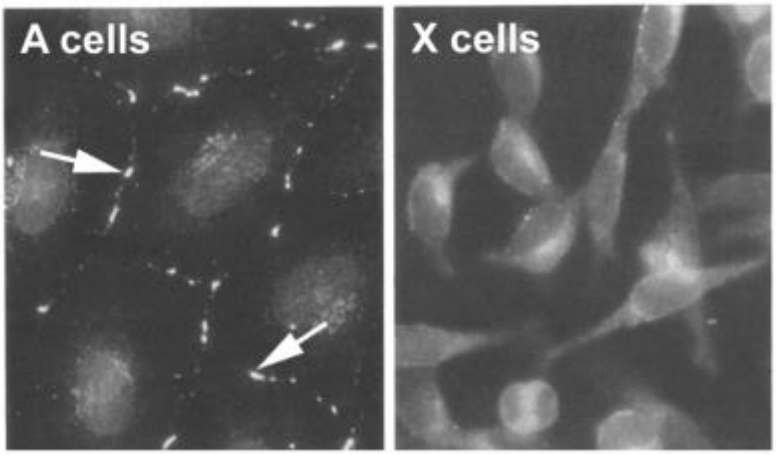
Figure from Pingoud et al., Nucleic Acids Res 42:7489-7527 (2014)

(a) Propose a hypothesis to explain this behavior, and specifically why a distant FokI site on the same DNA facilitates the reaction. (**5 points**)

(b) Propose **THREE** experiments to test this hypothesis. **TWO** of these should use single molecule approaches discussed in Foundations (using different technologies) and **a THIRD** should be a genetic or ensemble (conventional) biochemical approach. Include rationale, setup, and controls for each experiment (**15 points**)

Question 10

You have two different cell lines that you know express the gap junction protein connexin43 (Cx43) by immunoblot. When you examine them by immunofluorescence, you see the following patterns of staining:



The arrows in the image of "A cells" show sites of cell-cell contact staining positively (plaques) for Cx43. "X cells" have predominantly intracellular Cx43.

(**a**) You hypothesize that A cells can communicate by gap junctions and X cells cannot. Propose your mechanistic model for the hypothesis, and describe how you would test this model. Specify a negative control as part of your protocol. (**6 points**)

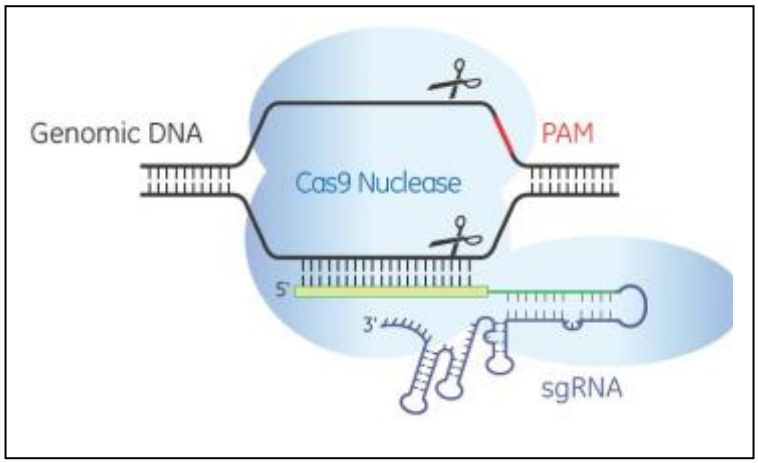
(**b**) Describe **ONE** experimental approach to identify the intracellular compartments containing Cx43 in X cells. Be sure to include details, and positive and negative controls. (**4 points**)

(**c**) You perform RNAseq on both A cells and X cells and find several differences in gene expression between the two cell types. Of particular interest, you find that there was a single pass transmembrane protein expressed by A cells that was not expressed by X cells. When you immunostain A cells for this protein, it shows uniform localization at the plasma membrane surrounding the entire cell. Why did you focus on this protein and what is its likely function? (**4 points**)

(**d**) You hypothesize that the unique protein identified in part 3 and expressed by A cells, but not X cells, is required for Cx43 plaque formation and potentially gap junctional communication. Provide two methods to test this hypothesis. (**6 points**)

Question 11

The CRISPR/Cas9 gene editing technology has emerged as a powerful technique with many applications in molecular biology and beyond. The illustration shows a general overview of the mechanism by which Cas9 functions together with a synthetic single-guide RNA (sgRNA).



(**a**) There have been other genome editing mechanisms used throughout the years such as zinc finger nucleases that showed promise. What is it about the CRISPR/Cas9 system that offers the potential for such exquisite site-specific editing that was not a component of prior approaches to genome engineering (**2 points**)?

(**b**) A modified version of the Cas9 protein exists where a single nuclease domain (indicated by the scissors) is inactivated. What would be the possible advantage of using such a form of Cas9 (**2 points**)?

(**c**) Another modified version of the Cas9 protein exists where both nuclease domains (indicated by the scissors) are inactivated, so called dead Cas9 (dCas9). Suggest an application that this modified dCas9 protein could be used for (**2 points**)?

(**d**) Name two specific challenges or concerns about using CRISPR/Cas9 as a potential therapeutic tool for genome editing. Why are these challenges/concerns (**4 points**)?

(**e**) If you were treating patients with CRISPR/Cas9, would you choose to target a disease of the blood or a muscle disease? Provide the rationale for your decision? (**4 points**)

The first attempt to use CRISPR/Cas9 genome editing for treating patients was carried out by Chinese scientists in the fall of 2016. These scientists isolated T-cells from a patient with cancer and used CRISPR/Cas9 genome editing to target the PD-1 gene and remove PD-1 protein in these cells. These cells, which are the patient's own cells, were re-injected into the patients. The PD-1 protein suppresses the immune response of the T-cells so these modified T cells should have the ability to mount a strong immune response against the cancer in these patients.

(**f**) Suggest a therapeutic approach where you could implement the power of CRISPR/Cas9. You need to provide details of what gene you would target, how you would edit the genome, and how you would actually introduce the modification to your patients. What cells are you targeting and why? Provide rationale for why you have opted to target this specific disease/condition. (**6 points**)

Question 12

What follows is an Abstract of a recent paper in *Science*:
Development of vertebrate embryos involves tightly regulated molecular and cellular processes that progressively instruct proliferating embryonic cells about their identity and behavior. Whereas numerous gene activities have been found to be essential during early embryogenesis, little is known about the minimal conditions and factors that would be sufficient to instruct pluripotent cells to organize the embryo. Here, we show that opposing gradients of bone morphogenetic protein (BMP) and Nodal, two transforming growth factor family members that act as morphogens, are sufficient to induce molecular and cellular mechanisms required to organize, in vivo, uncommitted cells of the zebrafish blastula animal pole into a well-developed embryo.

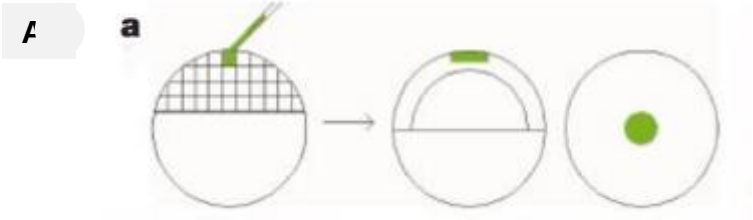
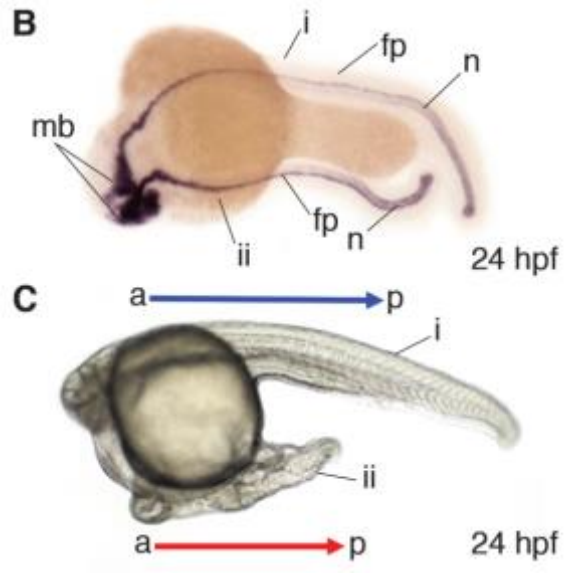


Figure A. This diagram from the Chen and Schier paper on Nodal is provided as a reminder of the experimental set-up used to manipulate and mark uncommitted cells of the zebrafish blastula animal pole.



Figures B and **C** depict actual results from the experiments described in the Abstract above. These are provided as a helpful hint to guide your answers to the questions below. Note: fp, floorplate; n, notochord; mb, midbrain; i, primary axis; ii, secondary axis

(Continue on the next page)

Question 12 (Continued from the previous page)

(a) Based on the Abstract above, what reagents/tools would be minimally required to carry out the experiments that prove that these morphogens are ***sufficient*** to induce the described effects? **(4 points)**

(b) How would you use the tools in Part (**a**) to demonstrate the conclusions stated in the Abstract? Please make sure you describe the experiments in some detail and possible expected results. Include necessary controls needed to confidently arrive at the conclusion, including proving that the gradients need to be *opposing*. **(12 points)**

(c) All of the above experiments are carried out in an artificially manipulated embryo system. What approach would you propose to show that opposing BMP and Nodal gradients actually exist in zebrafish embryos? **(4 points)**