Name _____

7.06 Cell Biology
Exam #3

This is a closed book exam. You are allowed only two pages of notes, but not computers or any other types of electronic devices.

Please write your answers to the questions in pen (not pencil) in the space provided.

Be sure to put your first and last name on each page in case they become separated.

There are **12** pages to the exam. Make sure that you have a complete copy.

Remember that we will photocopy all of the exams before returning them to you.

Good luck.

Question 1. 6 points

Question 2. 20 points

Question 3. 12 points

Question 4. 10 points

Question 5. 12 points

Question 6. 15 points

Question 7. 25 points

Total. 100 points

Name _____

Question 1 (6 points)
You have identified a gene that encodes a candidate for a new Cyclin-Dependent Kinase Inhibitor (CKI). When you transfect and overexpress this gene in mammalian cells, the cells arrest: 1) They ***stop*** growing, 2) do ***not*** replicate their DNA, and 3) do ***not*** divide.

A. Which Cyclin/CDK complex is this CKI likely to affect? (2 points)

Because growth is blocked as well as the onset of the cell cycle, it must be Cyclin D/CDK4 or 6

B. Using an antibody that can immunoprecipitate this CKI, how could you test your hypothesis from part A? What ***two*** criteria would you test to show that this is a CKI and that it acts on this Cyclin/CDK complex? What other reagents would you need to test these two properties? (4 points)

Use the antibody to immunoprecipitate the putative CKI.
If it is a CKI it must bind to a Cyclin/CDK and inhibit the kinase activity.

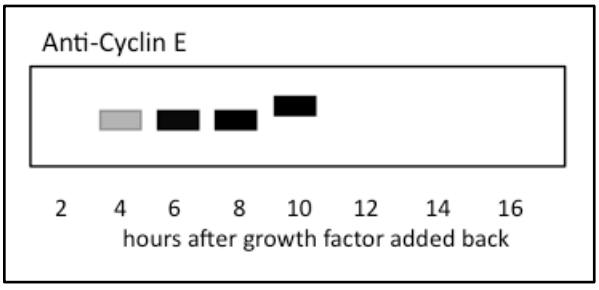
The other reagents needed are antibody to Cyclin D (or CDK4 or 6) to do a Western after immunoprecipitating the CKI to test if these are bound. Then ^{32}P gammaATP and H1 are needed to test if the complex with the CKI bound has inhibited kinase activity (note, for this would need a comparison of kinase activity from immunoprecipitation of Cyclin D/CDK4 or 6 as the control).

Question 2 (20 points)

A. What are two proteins phosphorylated by Cyclin E/CDK2 that can account for the role of Cyclin E/CDK2 in triggering the ***onset*** of S phase? (2 points)

Sld2
Sld3

B. You synchronize human Hela cells in early G1 by growth factor withdrawal. When growth factor is added back to the cells, they proceed synchronously through the cell cycle, spending 5 more hours in G1 before entering a 10-hour S phase. You observe the following levels of Cyclin E on a Western blot.

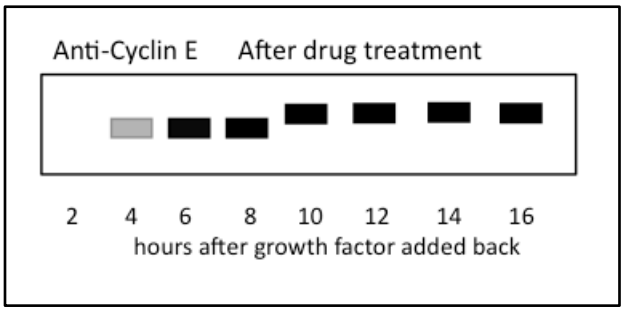


Explain why Cyclin E protein is undetectable after 10 hours. (2 points)

In these cells under these experimental conditions S phase starts after 5 hours. Cyclin E is being degraded after 10 hours, 5 hours into S phase.

Name _____

C. You do the same synchronization and release experiment, except this time you add a drug. Now you observe the following on a Cyclin E Western blot. (2 points)



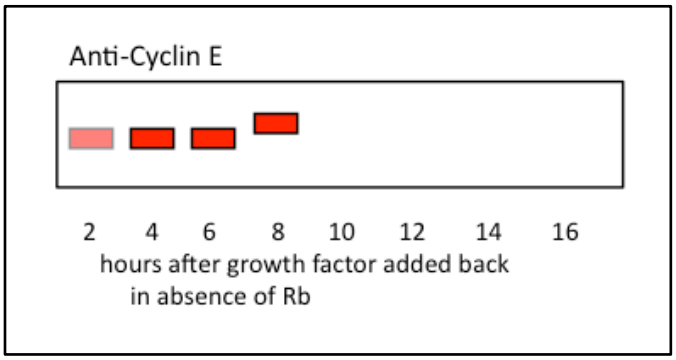
What must the drug be targeting?

The drug must be inhibiting SCF.
(give full credit for "inhibiting the proteasome", although this would also result in an additional shift due to the presence of a ubiquitin ladder)

What is producing the minor mobility shift of this protein in the SDS-PAGE gel?

Cyclin E becomes phosphorylated before it is degraded in S phase.

D. On the blot below, draw what you predict you would observe if you inactivated *Rb* (using a homozygous conditional allele) at the same time as growth factor is added back. (4 points)



Without Rb, E2F will become immediately active, inducing expression of Cyclin E. So the Cyclin E protein will appear earlier, S phase entry will be accelerated, and Cyclin E will be degraded earlier.
2pts for showing immediate appearance of Cyclin E. Okay if show it just as strong as later.
2pts for recognizing that Cyclin E will be degraded earlier, reflecting the earlier onset of S phase.

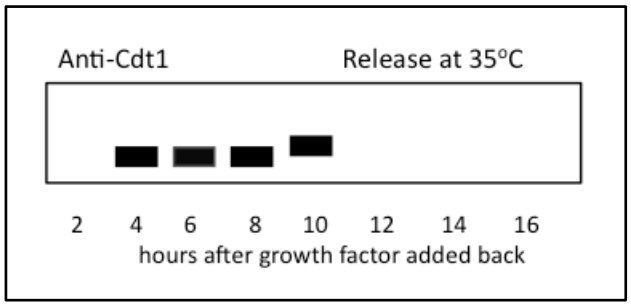
E. You have a human HeLa cell line in which both gene copies of *cyclin E* have a conditional allele that is inactive at 40°C, but active at 35°C. You can measure whether these cells undergo DNA replication based on BrdU incorporation. You synchronize the cells by growth factor withdrawal and release them back into G1 by adding back the growth factor, as in part (A). If you release them at 35°C, you observe BrdU incorporation after 6 hours. If you release them at 40°C and maintain them at this temperature, you never observe BrdU incorporation. **Explain**. (2 points)

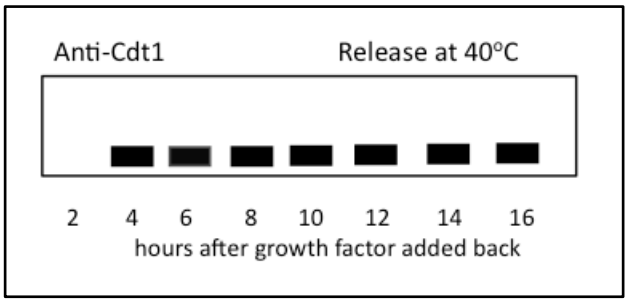
Release at 35°C results in active Cyclin E/CDK2 that triggers the onset of S phase, observed by BrdU incorporation. This takes an hour longer to start than for wild type. There is not Cyclin

Name _____

E/CDK2 activity at 40°C, so under these conditions S phase does not start and there is no BrdU incorporation.

F. You do the same synchronization and release experiment with the *cyclin E* mutant HeLa cells described in (E), but now you monitor Cdt1 levels by Western blot.

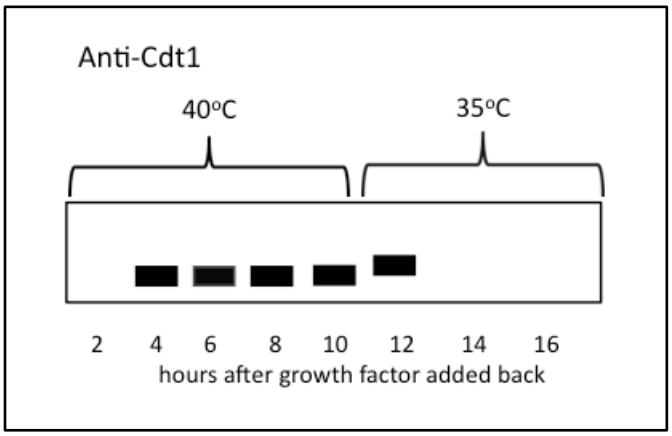




Explain what is occurring with Cdt1, the difference between the two blots, and what you conclude about Cyclin E function. (4 points)

After release at 35°C, Cdt1 becomes phosphorylated, making it a substrate of SCF, and targeting it for degradation. At 40°C Cyclin E/CDK2 is inactive, and Cdt1 is not phosphorylated or degraded. Cyclin E is required for phosphorylation and thus degradation of Cdt1.

G. Now you synchronize and release the *cyclin E* mutant Hela cells at 40°C, but after 10 hours shift down to 35°C. You observe BrdU incorporation only after 10 hours and see the following on a Cdt1 Western blot. (4 points)



i) What does this tell you about when DNA replication starts under these conditions?

1pt This shows you that DNA replication begins immediately after the shift down to 35°C, even though it is later in the cell cycle than when S phase would normally start.

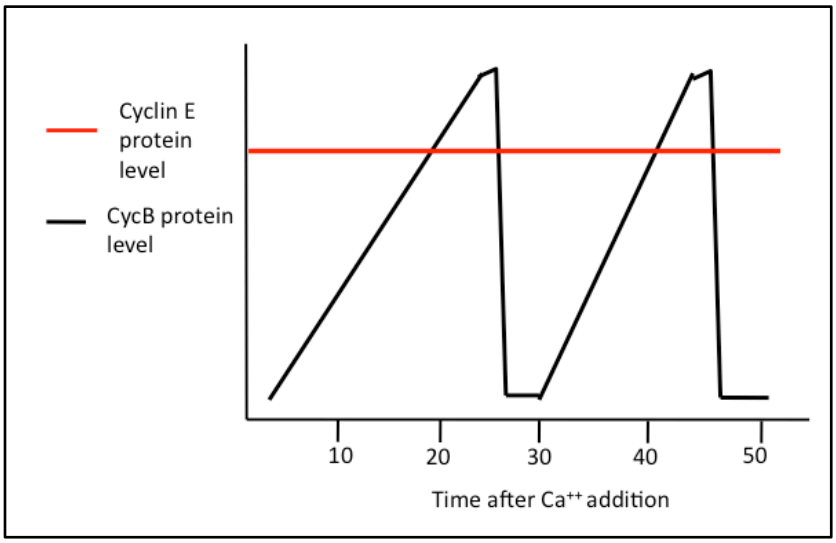
ii) What does this tell you about the timing of Cyclin E's control of S phase?

3 pts This tells you that when Cyclin E becomes active it can trigger the start of S phase, DNA replication, and phosphorylation of Cdt1, even if it does not become active until a point of the cell

cycle that should normally be in the middle of S phase when Cyclin E would normally be degraded. Thus if present, it can act outside of the G1-S window when it normally acts.
[2 points were given for indicating what Cyclin E controls, and 1 point for discussing the timing]

Question 3 (12 points)
Using extracts from Xenopus eggs that cycle, it is possible to observe rapid S-M cycles of added nuclei. If ***all*** the mRNAs in these extracts are degraded by RNAse treatment, no cycling occurs. However, adding back ***solely*** the mRNA for Cyclin B is sufficient to restore cycling.

A. On the graph below, draw the levels of Cyclin E protein in these S-M cycling extracts. (3 points)



Note: it does not matter how high they draw the line. It needs to be constant and clearly high enough to designate activity.

B. What is the ***paradox*** between the regulation of S phase and the initiation of DNA replication in the normal cell cycle versus these embryonic cycles? (4 points)

In the normal cycle Cyclin E protein levels and Cyclin E/CDK2 activity oscillates, and this is key to limiting DNA replication to one round per cell cycle. The paradox is how in the embryonic cycle DNA replication can initiate and be limited when there are constant levels of Cyclin E protein.

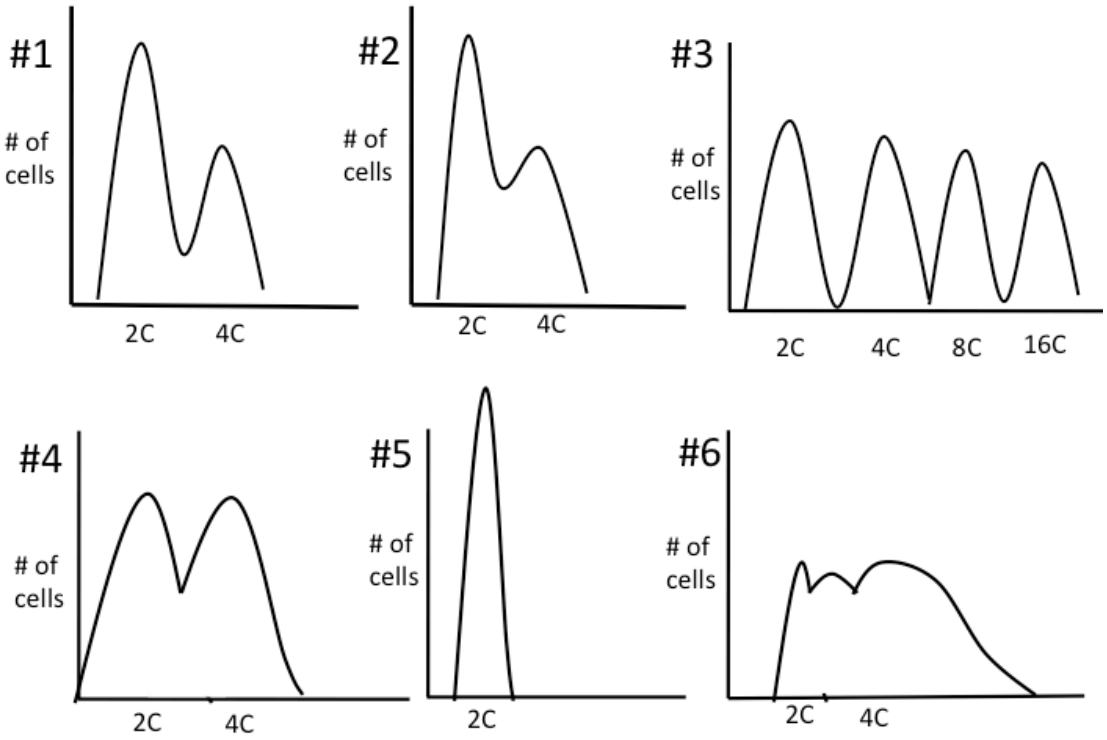
C. In the embryos as well as in these extracts, reformation of the nuclear envelope and nuclear structure is essential for DNA replication to occur, and Cyclin E is observed to be concentrated in the reformed nuclei. ***Explain*** how this resolves the paradox**.** What additional protein that regulates origin firing would you expect to be concentrated in the nuclei? (5 points)

This resolves the paradox by explaining that although total levels of Cyclin E protein are constant, the levels get concentrated in the nucleus, thus becoming locally high to activate S phase. This pool of Cyclin E inside the nucleus would then be degraded to limit DNA replication to one round. Cdt1 also would need to be concentrated in the nuclei to make origins that could be activated.

Name _____

Question 4 (10 points)

For each perturbation below, assume that you can synchronize a mammalian cell culture in G1, replace the endogenous wild type protein with the indicated mutants, and release the cells into the cell cycle. Profile #1 is what would be observed with this synchronization and release, but no perturbation. **For each mutant, indicate the FACs profile you would expect to observe after 48 hours. Briefly explain your reasoning.** (2 points each)



A. Sld2 with serine phosphorylated by Cyclin E/CDK2 changed to aspartic acid

#1

Both Sld2 and Sld3 have to be phosphorylated to activate the helicase, so Sld2 changed to a phosphomimetic form alone will not change the cell cycle from wild type.

B. CAK loss of function

#5

The cells are being released from G1, and with no CAK, Cyclin E/CDK2 won't be active, so there will be G1 arrest.

C. SCF loss of function

#6

Cyclin E and Cdt1 won't be targeted for destruction, so replication origins will keep refiring to give extra amounts of DNA, but not integral doublings of DNA content.

D. A triple mutant of loss of function Cyclin A, Cyclin B, and CDK1

#3

There will be no Cyclin/CDK activity once Cyclin E becomes degraded in S phase, so the cells will be in a gap phase, will reset replication origins, and once Cyclin E accumulates again will go through another round of DNA replication. So the amount of DNA will keep doubling, cyclically with a gap phase.

E. SMC2 loss of function

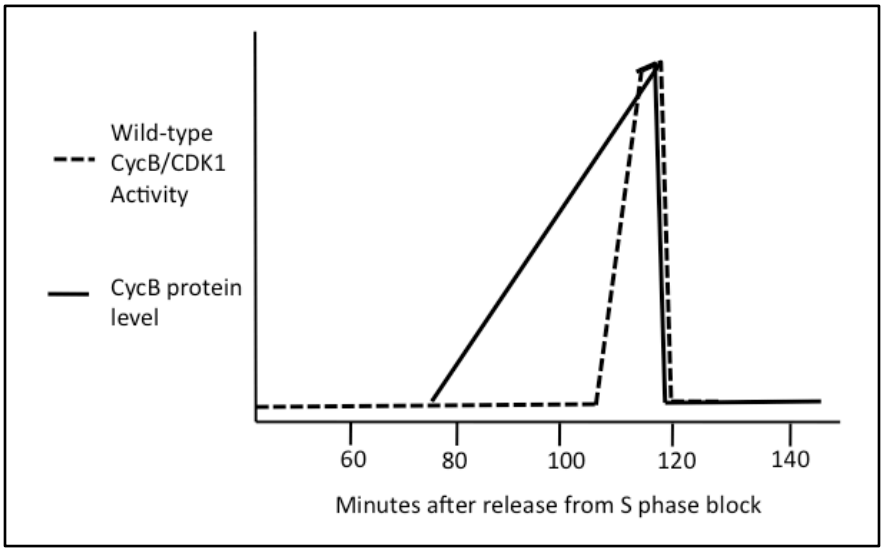
#4

Name _____

The condensin complex won't be functional, so chromosomes will segregate incorrectly in M. This will cause aneuploidy, changes in chromosome number. In FACS plot #4 there are cells with less than 2C content, ones with values between 2C and 4C, and ones with DNA content greater than 4C, reflecting the differences in chromosome number due to segregation errors in mitosis.

Question 5 (12 points)

Fission yeast has a cell division cycle of about 3 hours, and this yeast does most of its growth in G2. You have a conditional mutant that arrests in S phase at the non-permissive temperature and you use this mutant to synchronize the culture. You then shift the culture to the permissive temperature and measure Cyclin B protein levels and Cyclin B/CDK1 kinase activity.

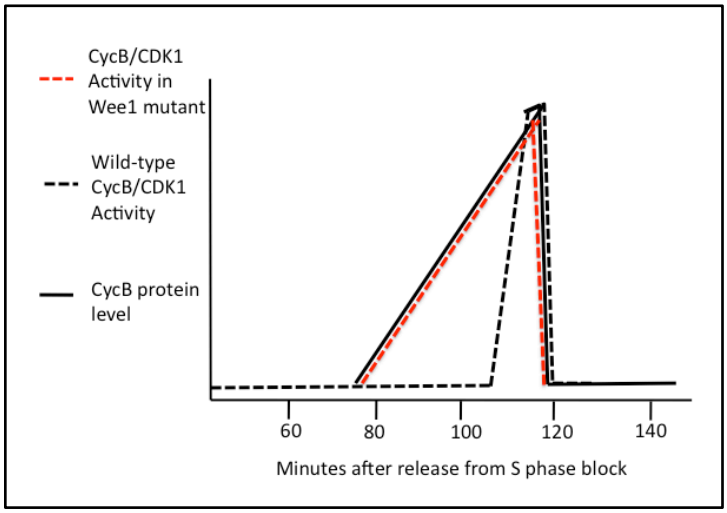


A. Why aren't the two curves the same? (3 points)

Because Cyclin B/CDK1 requires the presence of Cyclin B protein but it also is controlled by phosphorylation: inhibited by phosphorylation by Wee1 and activated by dephosphorylation by Cdc25. Because Cyclin B/CDK1 activates Cdc25 and inhibits Wee1, there is a feedback loop that acts like a switch to give an abrupt onset of activation of Cyclin B/CDK1 kinase activity.

B. On the graph below, ***draw*** what the CyclinB/CDK1 kinase activity will look like in a *wee1* loss of function mutant. (3 points)

Name _____



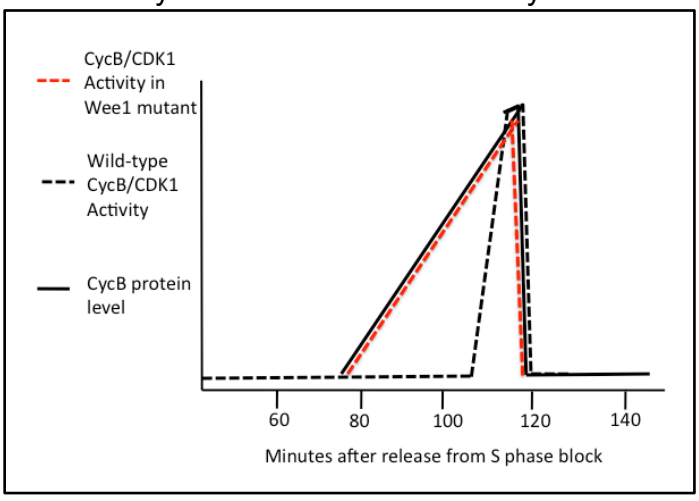
Because Wee1 is inactive Cyclin B/CDK1 kinase activity will appear as Cyclin B protein becomes present.
(What matters is the slope. It is fine if they show the levels of kinase activity being higher than Cyclin B protein levels. But both kinase activity and Cyclin protein levels must drop coincidently.)

C. You have a Wee1 mutant in which Serine 123 (the residue phosphorylated by Cyclin B/CDK1) is changed to aspartic acid. (3 points)

• State your predictions for how this mutation will affect Wee1 activity.

If Serine 123 is changed to asparatic acid, Wee1 will be in an inhibited state, so the activity curve for Cyclin B/CDK1 kinase will look the same as in a *wee1* loss-of-function mutant.

• On the graph below, ***draw*** what the CyclinB/CDK1 kinase activity will look like in this mutant:



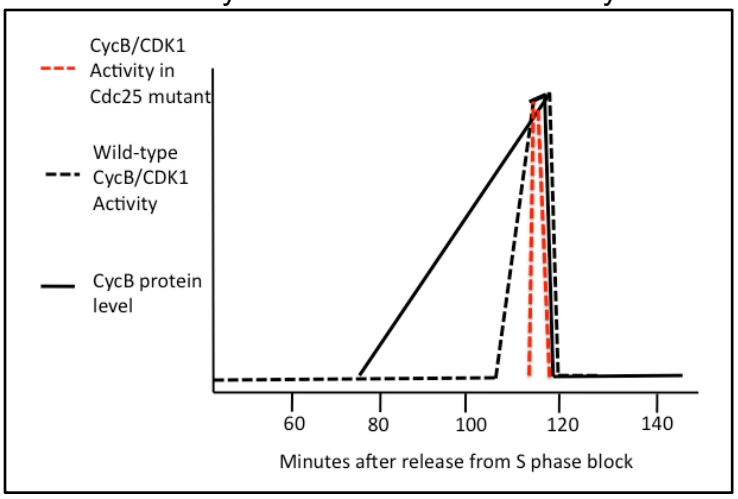
D. Cdc25 is subject to multiple regulatory inputs. You have a Cdc25 mutant in which Threonine 48 and 67 (the two residues phosphorylated by Cyclin B/CDK1) are changed to alanine. (3 points)

• Given that Cdc25 is subject to multiple regulatory inputs, state your prediction for how these mutations will affect Cdc25 activity

With these threonines changed to alanine, Cdc25 will not be able to be activated by Cyclin B/CDK1. Cdc25 will still have activity, but the switch on of Cyclin B/CDK1 will be delayed.

Name _____

• On the graph below, ***draw*** what the Cyclin B/CDK1 kinase activity will look like in this mutant:



Name _____

Question 6 (15 points)
In class, we discussed three different assays that measure aspects of actin assembly:

1. The bulk pyrene actin assembly assay
2. A TIRF-based assay to visualize actin assembly
3. Listeria motility in cell extracts.

For each of the proteins listed below, **indicate "Yes" or "No"** for whether you would be able to visualize their activities in these assays. For each "Yes", additionally **describe the nature of the change**.

A. Pyrene Assay. For the pyrene assay, indicate (Yes/No) for whether the ***addition*** of the protein to an assembly reaction containing ATP, actin, buffer, and ActA would have a detectable effect. For any "Yes", indicate whether polymerization will be "Increased" or "Decreased".

	Pyrene Assay	If "Yes", indicate nature of the change
Arp2/3 complex	**Yes** / No	**Increased** / Decreased
Capping protein	**Yes** / No	Increased / **Decreased**
Fimbrin	Yes / **No**	Increased / Decreased
Formin	**Yes** / No	**Increased** / Decreased
Myosin	Yes / **No**	Increased / Decreased

B. TIRF Assay. For the TIRF assay, indicate (Yes/No) for whether the ***addition*** of the protein to an assembly reaction containing ATP, actin, buffer, and ActA would have a detectable effect. For any "Yes", describe any visual changes to the actin filaments in their length and/or organization

	TIRF Assay	Nature of visual change to actin filaments
Arp2/3 complex	**Yes** / No	Shorter filaments (due to increased nucleation), more total polymer, highly branched
Capping protein	**Yes** / No	Shorter filaments (and less polymer)
Fimbrin	**Yes** / No	Bundled/Crosslinked Filaments
Formin	**Yes** / No	Longer filaments, more of them (more nucleation)
Myosin	Yes / **No**	(if yes and considering a tetrameric myosin, okay to say bundled)

C. Listeria Motility Assay. For the Listeria motility assay, indicate (Yes/No) whether ***depletion*** (removal) of the protein from cell extract would have a detectable effect. For any "Yes", indicate whether the rate of movement will be "Increased" or "Decreased".

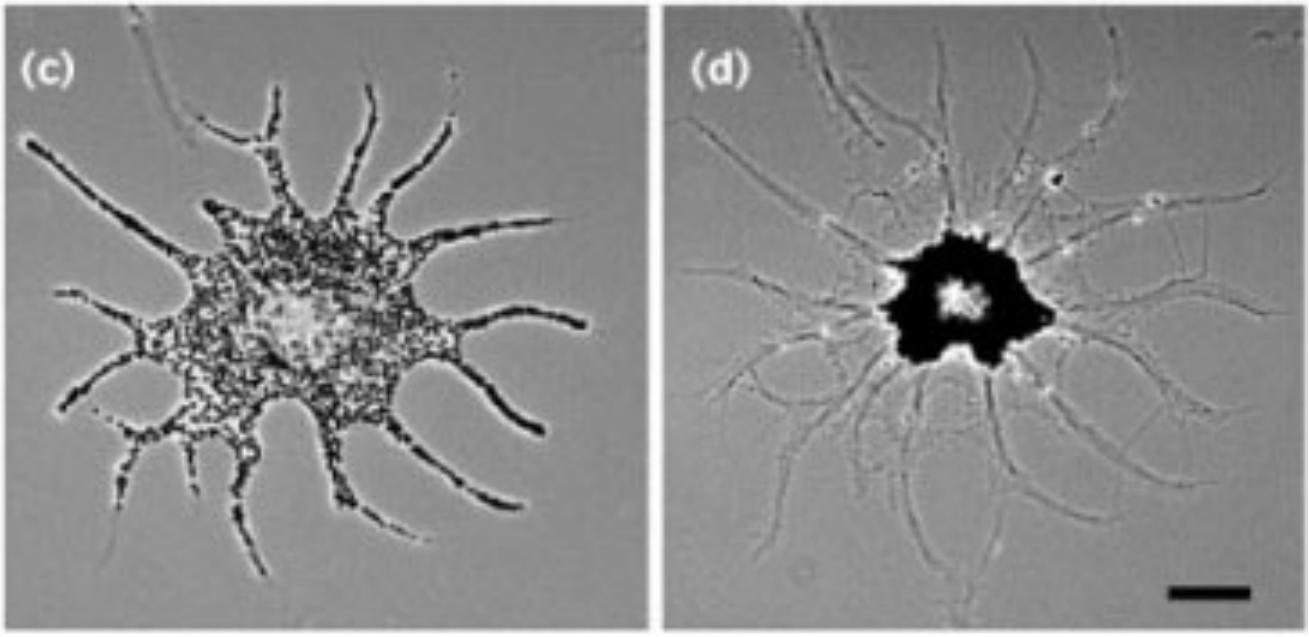
	Listeria Motility	If "Yes", indicate nature of the change
Arp2/3 complex	**Yes** / No	Increased / **Decreased**
Capping protein	**Yes** / No	Increased / **Decreased (counterintuitive, but needed to focus polymerization at the surface)**
Fimbrin	Yes / **No**	Increased / Decreased
Formin	Yes / **No**	Increased / Decreased
Myosin	Yes / **No**	Increased / Decreased

Name _____

Question 7 (25 points)

Melanophores are cells found in some frogs and fishes that allow the organism to dynamically change its coloration for camouflage. This change requires the movement of pigment-containing vesicles, called melanosomes, around an *interphase* cell. For the cell to become darker, the melanosomes must move over longer distances outward from the center of the cell. To become lighter, the melanosomes move inward to the center of the cell.

Melanosomes dispersed throughout the cell **Melanosomes aggregated in the cell center**



A. Previous work on these melanosomes indicated that they ***primarily*** move along microtubule polymers, but not actin (in cells lacking microtubules, melanosomes no longer move). Based on what you know about the properties and organization of these cytoskeletal elements, explain why this would make sense. (3 points)

Microtubules are organized with their minus ends embedded in the centrosome, which is located near the nucleus (near the center of the cell). This provides a network of organized filaments with an appropriate polarity to trafficking these vesicles. They are also typically much longer polymers, allowing the long range transport. Partial credit was given for indicating that motors can walk in both directions along the microtubule. However, full credit needs to highlight microtubule organization.

B. Describe two potential mechanisms by which the melanosomes could move outward towards the periphery of a cell. Be as specific as possible for the mechanisms of force production and the identity of any proteins involved. (2 points)

Polymerization-based pushing and motor-driven motility (using a plus end directed kinesin)

C. Describe two potential mechanisms by which the melanosomes could move inward towards the center of a cell. Be as specific as possible for the mechanisms of force production and the identity of any proteins involved. (2 points)

Depolymerization-based pulling and motor driven motility (using a minus end directed dynein)

Name _____

D. Propose ***two*** experiments that would each (independently) allow you to quickly and unambigiously distinguish between the models you proposed above for the mechanism of force production for ***inward*** movement from part C (for example, with a perturbation affecting one mechanism, but not the other). (4 points)

Inhibit microtubule dynamics using taxol (would prevent from seeing depolymerization based force)

Use non-hydrolyzable ATP or deplete the cell (quickly) of ATP (would disrupt motor driven movement) OR deplete the cells of the dynein motor using RNAi or a small molecule inhibitor

E. To change an organism's color and appearance, the distribution of the melanosomes within the cell must change in response to upstream signals. Assume that the parameters of microtubule dynamics are ***not*** altered by these signals. Propose a model for how these vesicles would switch the directionality of their motility is response to specific signals. (3 points)

Both kinesin and dynein could associate with the same vesicles to move it in different directions (in or out). These signals must either differentially affect to alter the activity of these motors that are simultaneously bound to the vesicle, or it would alter which motor has the capacity to bind to the vesicle.

F. You are analyzing the requirements for melanosome movement in a cell in which the melanosomes are initially distributed to the cell periphery (edge). At the beginning of the experiment, signaling is altered to cause the melanosomes to move to the center. At the start of the experiment, you also overexpress and activate **Katanin** (assume that this occurs instantaneously). Indicate the most likely effect on melanosome distribution. ***Briefly*** explain your reasoning. (3 points)

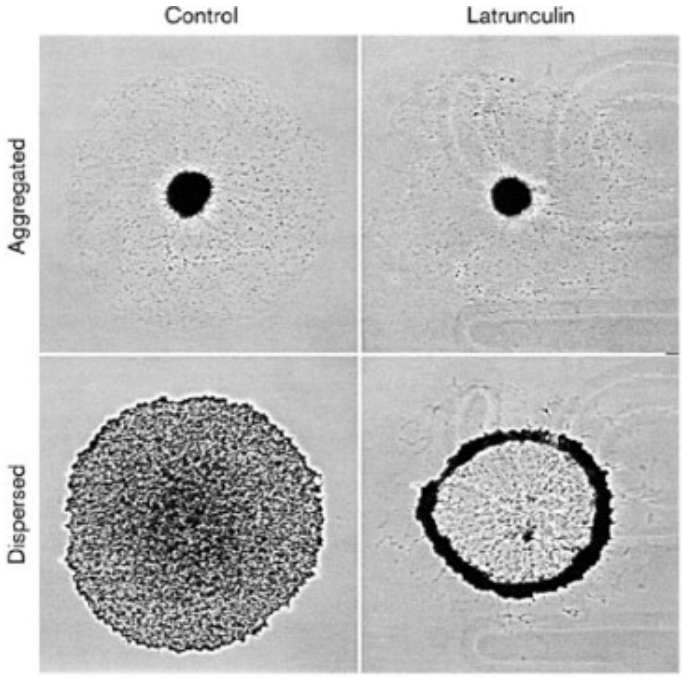
Katanin will chop up the microtubule tracks internally, and therefore would prevent the vesicles from being able to move along the microtubules (will not aggregate or move internally)

G. In the same experiment as Part F, you instead overexpress and activate the tetrameric kinesin-5 (Eg5). Indicate the most likely effect on melanosome distribution. ***Briefly*** explain your reasoning. (3 points)

This will affect anti-parallel microtubules, but would have no real affect on parallel microtubules. Given the organization of the interphase array with all microtubules emanating from the centrosome, this won't change anything.

In cells lacking microtubules, melanosomes no longer move, consistent with a primary role for microtubules in their distribution. However, some researchers have proposed a role for actin in observed final melanosome distribution. For the experiment below, cells were first induced to either

aggregate or disperse their melanosomes (left column). They were next treated with the actin depolymerizing drug Latrunculin A. Note that Latrunculin treatment will decrease cell size slightly.



H. Based on these data, what is the effect of latrunculin treatment on melanosome distribution? (2 points)

Instead of being dispersed throughout the cell, the melanosomes are able to move all the way to the cell periphery.

I. Provide a model to explain this behavior. (3 points)

There are two possibilities. First, the actin could actually inhibit processive outward movement. In the absence of actin, the vesicles would move all the way to the exterior of the cell because they are now more processively able to move along the microtubules. Second, the actin would play a role in dispersion by taking the vesicles off the microtubule and moving them in a directional manner to an intermediate location.