**This is for Git test.**

**Do it like a speech, a talk, not a reciting of my memorized stuff?**

**Aging model/ RLS, then other systems including cell culture in kennedy lab and other model system in neighbor labs.**

**Many genes and pathways are studied and found to be conserved, but I will go into a different angle to understand the mechanisms of degeneration and dysfunction that featuring aging, not just the pathways.**

**Slow down and think carefully what they really asking. Do not answer without**

**Do you think bacteria also do the same thing?** This is a good example of questions that need to be answered indirectly. The real answer is that, mitochondria is not bacteria any more, it evolved for billion years and loss control of itself, it need to import proteins from cytosol, that's not happening in bacteria.

**Be confident and slow, first smile when you have this question, then think about it. Be a thinker, talk about the question behind of the question raised by reviewer. That’s critical to show that this candidate is ready to run an independent lab, not just doing experiments.**

**Also, although the projects sound like depend on each other, but really they are not and all of them have their own preliminary data that not depends on previous aim. Breaking such dependence allow you to be success if one of them is not working. For example, the loss of cellular compartments are not depends on the hypothesis and I can search for other mechanisms if my hypothesized mechanism is not working; Also the metabolism link also has its own preliminary data. The same for two aims of rejuvenation: I can search for proteostasis independent mechanism for rejuvenation.**

**General questions:**

**How to get public audience interested about your research in couple sentence**? Protein damages and mitochondrial dysfunctions are featured in aging and many aged related diseases, but how these two features connected are not well understood. This work will investigate the biological consequences of protein aggregation on the integrity of mitochondria and other organelles during aging, as well as the related rejuvenation mechanisms using budding yeast as a model. The outcomes of this work will significantly improve our understanding of these hallmarks of aging and our ability to intervene in age-related diseases.

Although proteostasis dysfunction during aging has been explored in the context of cytosolic protein aggregation, a largely unknown consequence of proteostasis dysfunction is the alteration of organelle composition and function.

**Why do you think you can bypass postdoc training?**

My exceptional PhD training has shaped my scientific independence and intuition for projects after working autonomously for five years on the new direction I initiated for Rong Li lab. Starting from scratch, I was able to successfully make a series of exciting discoveries that allowed me to publish two first-author Cell articles. In the last year of my PhD, I conceived a new project and independently achieved significant progress in my preliminary studies at the Stowers Institute after my PhD mentor Dr. Li moved to Johns Hopkins University. Through my three successful projects I have demonstrated the creativity and ability in commanding the directions of my research. These experience honed my skills in mentoring graduate students, managing collaborations, writing manuscripts, conference presentations, and grant applications. In addition, I have a broad range of experimental expertise that enables me to pursue the proposed work comprehensively. These three Aims use budding yeast as the model and I am fully experienced with yeast, having routinely employed genetic and cell biology techniques used in this model system during my graduate work. I have extensive experience in genetic screening, cutting-edge imaging technologies, automated image analysis, proteomics, biochemistry, and computer simulation that used in my two completed works and the one under revision.

**About previous studies:**

**what do you think about the evolutionary perspective of the mito import? why do mito import nonmito proteins?** I should really use the model figure to explan any questions related to mito import, that will ease a lot of understanding and also keep me from saying bullshit.

**How this connect to proteostasis, autophagy, protesome defect etc during aging**?.

**About the proposed studies**:

**1. how you gonna test your hypothesized model of mislocalization?** overexpression of chaperones and proteosomes.it is really a traffic jam, and the accumulating damaged proteins compete with the chaperones that required for proper sorting. Use overexpression of some mito proteases whose expression level decreased over aging, such as the LON protease which we know are the strongest protease in degrading imported cytosolic proteins, LON proteases decrease during aging. So in this way we can clean the accumulated proteins in mitochondria. Or, remove the cytosolic damaged proteins by increase the competing pathways, such as proteasomes and chaperones to remove misfolded proteins, or increase the importing ability through expressin of the key importing components. Most telling experiments will be in vitro competition assay using young mito. Using old mito, we can test whether we can detect decrease of protease function with the in vitro importing assay we developed and see the time required to show the degraded luciferase.

**What if the mislocalization of mitochondrial proteins are not depends on the competing process?** **What else the mechanism you can think of and how you can test them?** for example, the mitochondrial may just damage import machineries so they fail to import mitoproteins. But this may predict that all mito proteins are mislocalized, which is not the case, so I favor the competition one. In addition, the **mRNA binding proteins**, which are metastable proteins required for localized translation and import of organelle proteins, may modify mitochondrial composition in response to cytosolic proteostasis stress during aging.

Or, protein expression level difference that drive the overloading and mislocalization of some mito proteins? **Why do you think some mito protein are mislocalized while other are not?**

**What if the hypothesis that unfolded state-proximity is not working?** First of all, the hypothesis is a very simplified version and many other cellular factors may be involved, so I will test this by adding yeast cytosol extract to the importing assay and identify the cytosolic factors involved. Secondary, if none of them works, then I will focus on establishing the importing route of misfolded proteins using in vitro import assay we already established in our previous paper by clogging the specific importing channel or removal of components of importing channels before mixing the mito with aggregates. In this way, we can also establish the importing mechanism and route for the non-mitochondrial proteins.

The competition subaim of aim 1, **how can distinguish the competition in Tom70/40 level from the trapping of PiC by aggregates?** In that case, I will just use another mito protein which is known to bind tom70, then test whether there is competition. Then, I will use tom20 substrate which will have the same problem with tom70 substrate (trapping by aggregates), but will not have problem with receptors as aggregates doesn’t seems to bind tom20. **There seems to be only a few aggregates, how can there be competition as billions of tom70 are there on mitochondria**? Sure, in young cells, only a few aggregates are there, but in old cells, there are a lot of damaged proteins that are not really aggregating. These damaged proteins may resemble the case we observed in young cells that some unstable proteins are improted into mito without aggregating. So big aggregates are not necessary for importing, as long as you have some misfolding, you may get access to it. **How will you test the prediction from the bioinformatics analysis of the imported non-mito proteins**? For example, if we need to test the domain size or protein size, we can just use arrays of a domain/protein. **What if there is no difference you can find by proteomics**?

For the subaim3 of aim1, **what if you couldn’t find a good proteins that faithfully targeted to one organelle**? In this case, then I need to combine the APEX with traditional purification of organelles to reduce the contamination of other organelles. For cytosol, then I can just use saponin to open plasma membrane specifically. Also, the split GFP assay will allow you to see the mislocalization of each protein. If some of the cytosolic-GFP1-10 is mislocalized to other organelle, that’s fine, as we can use different cytosolic-GFP1-10 and find the ones that common between different cytosolic-GFP1-10, based on the assumption that different cytosolic proteins will have different mislocalization pattern. Also, the level of mislocalization of any given cytosolic-GFP1-10 can be found based on the distribution of the library of mito-GFP11: it will be the base line if all of the mito-GFP11 has some split GFP signal as the mislocalized portion will be relatively small compare to the cytosolic pool.

**So, how you can pin down the mechanism if the proposed competition mechanism is not working?** **The same question for the metabolic dysfunction, what if there is no protein accumulation in the mitochondria in old cells**? But this is not valid as I already observe mito localize to cytosol. If you think that although there are mislocalized proteins to cytosol, but the mito composition may be still perfect. Then the problem must be from the upstream of the metabolism in cytosol, one scenario I covered in my research proposal. This can be test if I separate old mito from old cells and fix with cyto from young cells.

I will first characterize the feature of protein mislocaliztion and see if any specific group of proteins are mislocailzed, then I will use the representative protein as model to study their expression and import. for example, I will purifiy mito from old cells and study their import efficiency. Then add the misfolded proteins to see whether that compete. If nothing works, then the problem must be upstream in translation. (**this should be the way I evolve question and answers, level by level step by step**).

**what's the connection between this mislocalization and proteostasis defects in aging**? I would say proteostasis defect is a very broad term and you can say all other aging hallmarks are just because of proteins not functioning well. so I have to say, this is related to proteostasis and a new kind of proteostasis defect we don't know yet. Also regarding the proteostasis mechanisms, such as UPS and chaperones, they are possible mechanisms behind this loss of cellular compartmentalization. So I think this phenotype may indicate the loss of fitness of a cell and indicate a widespread dysfunctions, and this basic mechanism may well conserved in animals. then talk about the aging genes, this is the mechahnism behind the aging related dysfunction and degeneration.

**Do you think mislocalization also happen to other organelle? Evidence?** I should use the conversation that some transcriptional factors are mislocalized to cytosol as evidence.

**how you test this in other model systems, what's the plan**? **Why use budding yeast?**

**How can you distinguish the case that mitochondrial dysfunction is independently from cytosolic dysfunction? Or, whether the dysfunction is caused by mislocalization of mito proteins or the accumulation of cyto proteins**.

**What about the damages in mitochondria itself?** I need to say that my previous study raised a new connection between mito dysfunction and proteostasis problem, it is not clear which defect comes first. According to previous studies, the damaged proteins begin to accumulate in early life, so it is possible the sorting is damaged because of the traffic jam in cytosol.

**Why do you think this is a conserved aging phenotype**? Because all the components in this picture are conserved: the mito import of misfolded proteins is conserved in mammalian cells, the mitochondrial composition is very conserved and the import mechanism is conserved. The accumulation of protein damages and aggregation is conserved in animals during aging. Importantly, many aging pathways identified in budding yeast are very well conserved in higher model system.

**2. How you gonna do the metabolism project?**

When facing the question regarding metaolism, I will resort to my proposal for the protein aggregation-respiration in young cells and the proposed studies. Mention that I found a significant fraction of aggregated proteins are metabolic enzymes and signaling proteins involved in regulating metabolism. Then when talk about the decoupling in old cells and its mechanism. So first study how protein aggregation can activate mito respiration in young cell, then from this mechanism, we will have more idea what happen to old cells. It is possible that the signaling upstream of mitochondria is still working, but the mitoproteins are not in place, so no response. But I need to confess that protein mislocalization could be part of the problem (that’s how the fig 5 of proposal shows), and the upstream signaling could also be damaged.

**How to test the mechanism**, I need to use the idea described above to test cytosol and mito separately. Also, purify mitochondria from old cells that overexpressing protease in mitochondria to resolve accumulation of damaged proteins will be a good way to separate cytosolic factors and mitochondrial factors on mito functions such as respiration. Or even use the young mitochondria to test whether the binding of misfolded proteins cause mitoimport defect and respiration activation.

As I know the respiration is uncoupled in old cells, one working hypothesis is that some cytosolic proteins are accumulated in old mito, which can be tested with first aim. If this is the case, we can purified mito will be tested in their respiration when protease OE is used to get rid of damaged proteins in mito. Also, test the deletion or change expression level with young cell can also help to test whether, out of hundreds of proteins that mislocalized (accumulation in mito or mislocalize to cytosol), certain proteins play a key role in such dysfunction. In addition to these possible mechansims (mislocalization), so I want to see whether this is upstream in the cytosol or downstream in the mito, so the mechanism of coupling studied in young cell will provide many clues and hypothesis than just the mislocalization. Also knowing the composition will allow us to predict what else is dysfunctioning? For example, if the key enzyme of lipid synthesis is mislocliazed, then certain lipid composition will change over time, which is known, such as cardiolipin, which decrease in aging brain.

In the experiment that I discussed about glycolysis, I said that inhibition of ETC has minor effect on aggregate dissolution and it could be due to the overexpression of glycolysis as a feed-back. So the mito response can be a quick response, while if this is not happening, the glycolysis need to fix it self. This can be tested with petite cells which doesn’t have mito respiration from beginning. Then all ATP comes from glycolysis and the cell has to fix glycolysis. This is done by myself as I know petite does not affect agg dissolution. Alternatively, this could be that mitochondria supply other metabolites other than ATP for proteostasis, which can be detected with profiling of metabolomics.

**For the subaim2 of the this aim, how do you know if respiration is a good indicator of mito metabolism?** Alternatively, I will analyze the metabolic profile and transcriptional profile to get a map of response to protein aggregation in the cytosol, this will help me to narrow down and identify the key metabolic pathways get involved and the possible upstream regulators. ***Then I will study the role of specific metabolic pathway on the proteostasis of cytosolic proteins, namely the aggregation and dissolution of cytosolic aggregates with specific metabolites***. As long as I am not trying to understand everything, focus on one pathway is easy to design experiments. **Try to reduce the complexity over the pipeline of project and use one example to tell the story or illustrate the idea**.

**3. What does rejuvenation mean here? How this is different from other studies that inhibit or delay aging process?** This project will be alternative to the previous two aims as all these mechanisms studies we talked about are the ways to rejuvenate the mitochondrial composition and function with specific manipulations/experiments. The third aim this really looks into the naturally occurring rejuvenation process which resolve the aging problem in daughter cell, we can learn from daughter cells how to solve these problem. We expect to uncover multiple solutions the daughter cell has to resolve the mislocalization problem and also the dysfunction problem. So this will also guide our study in the other two aims and help us to understand what’ the problem during aging.

Previous genetic studies, such as mutations in insulin pathways or mTOR pathways, they are able to delay aging process and usually expressed from the beginning of the life. While rejuvenation will be the opposite, we will wait until the cell get old and stop dividing or its proper function, then we test whether we can revive the cell. Also the mislocalization of proteins could serve as a functional status of the cell and used as marker to see whether a method is able to rejuvenate the old cells.

Also, we lacking a good aging marker that allow us to estimate the cellular status and its age. Often time we need to culture the animal or cells until they die. This significantly hampers the research of aging and often make the aging studies hard to be done in single labs. So if the protein misloclaization turns out to *be a good general aging mark, then we may be able to quantify protein mislocalization as an alternative way to evaluate the effect* of genetic mutation or other methods aiming to extend life span. For example, we can test whether caloric restriction is able to fix the problem of mislocalization. *Try to find a correlation between the life span extension and resolution of mislocalization problem*.

Also this aim has two subaims. Study how the daughter cell resolve protesostasis problem and study restoration of mislocalization problem. These two aims are tightly relevant as the mislocalization problem may rise from the chronic protein damages and aggregation in the cytosol. So resolve proteome damage problem in daughter cell may lead to the resolution of mislocalization problem. Also I have strong preliminary data that the daughter cells seems to have stronger disaggregation ability and proteasome activity. *People may ask that both dissolution and mg132 experiment saying the same thing as higher dissolution could be due to the higher proteasome activity, but not the chaperone activity*. So I will do in vitro dissolution assay.

**What if the in vitro dissolution assay or proteasome activity assay is not working**? Then we couldn’t rule out the contribution from the influx of damaged proteins. Then use in vivo assay such as FRET. Also we can rely on the genomic or proteomics screen to identify the regulators and mechanisms. These regulators may modify the chaperones system by expression, posttranslational modification etc. So then we can identify these modification and play with them to see whether that cancelled the higher activity in daughter cell.

**What if none of these daughter specific factors show effect on the chaperone activity or proteasome activity**? Then I will try combinations of them in case one factor is not enough to rejuvenation them (just like the yamanaka factors we need 4). I will select the ones showing small effects if none of them is major, then do the combination study. **When I face this kind of question, instead of answer directly by proposing alternative method, I can also propose the alternative hypothesis that I am planning to test, such as the PTM instead of expression difference**. Everyone understand that nothing is for sure, there is alternative after alternative. But as long as you can propose alternative, either alternative method or hypothesis, it shows you are good and prepared.

**How you will distinguish from your previous mentor?** my mentor work on asymmetric segregation of protein damages and that's her grant. I will work mechanisms of aging and the process of rejuvenation, which is very different from her focus. Also, the aim to study loss of cellular compartment during aging is completely different from my previous study and also I will have the unique opportunity on this as my lab will be surrounded by aging experties. I have the full support from Dr. Kennedy’s lab, a physically adjacent yeast lab led by a world-leading expert in aging and metabolism. We have no competing interests and our different expertise in aging that will stimulate mutual beneficial collaborations.