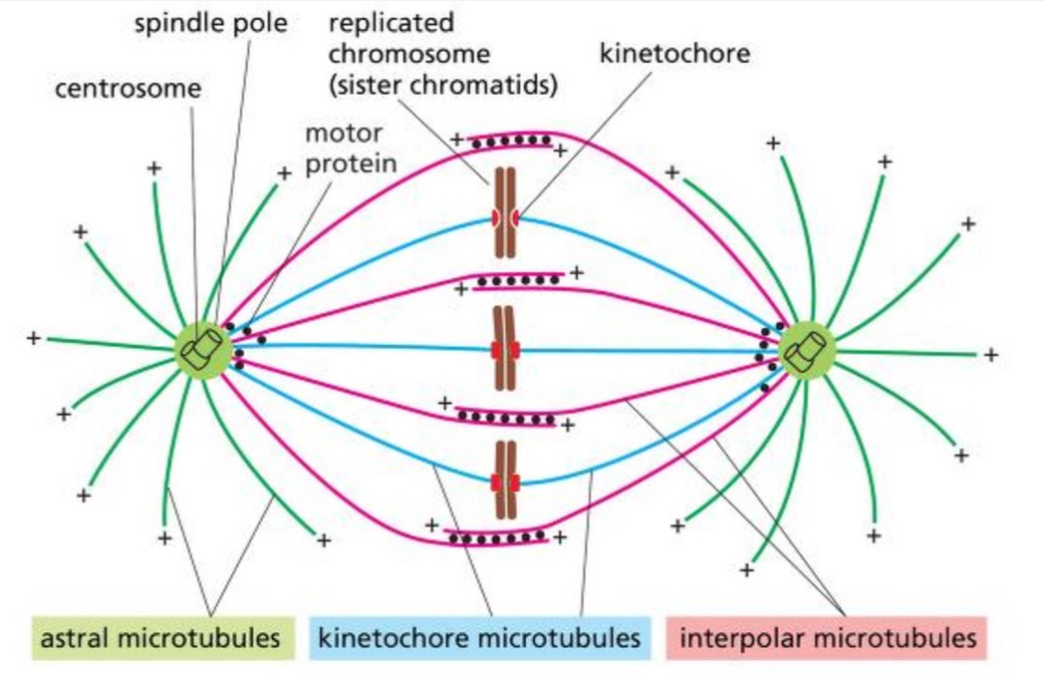
Characterization of Motor Functions and Nuclear Localization of Truncated CtCIN8

By: Daniel Kim Jiarong Liang

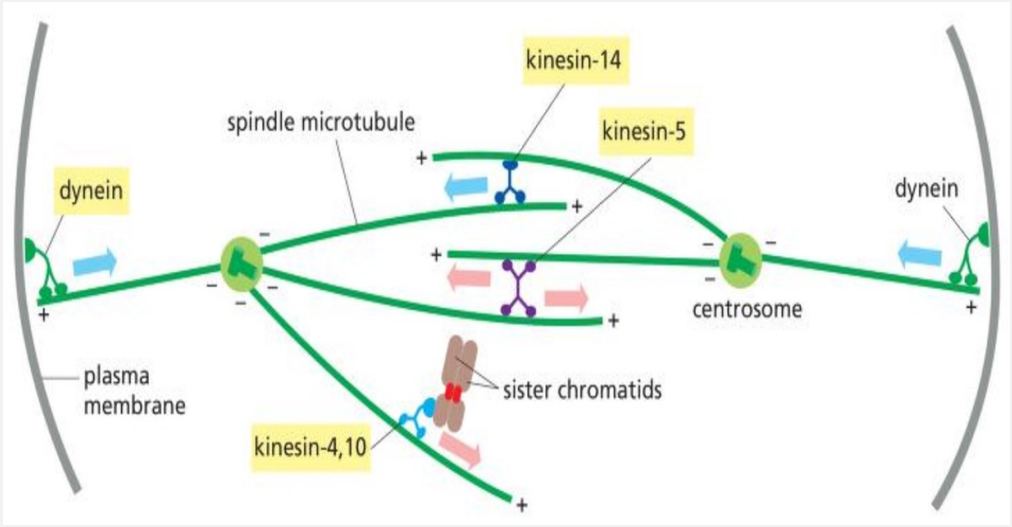
Kinesin-5 Motor Proteins are critical for mitotic spindle assembly & elongation during Mitotic Anaphase

Anaphase



Panel 17-1, Bruce Albert, et. al., Molecular Biology of the Cell, 5/d, Garland Science, 2008, Page 1073

Homotetrameric Cin8 facilitates the anti-parallel movement of Microtubules



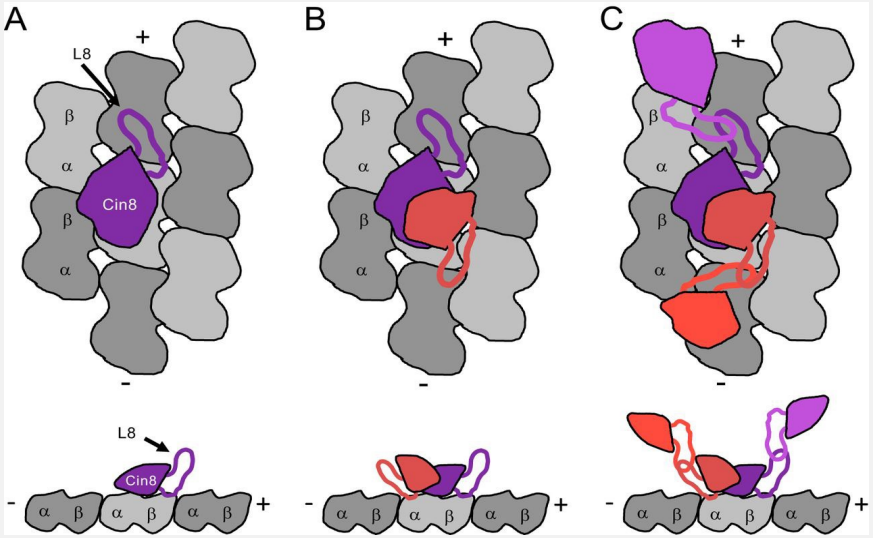
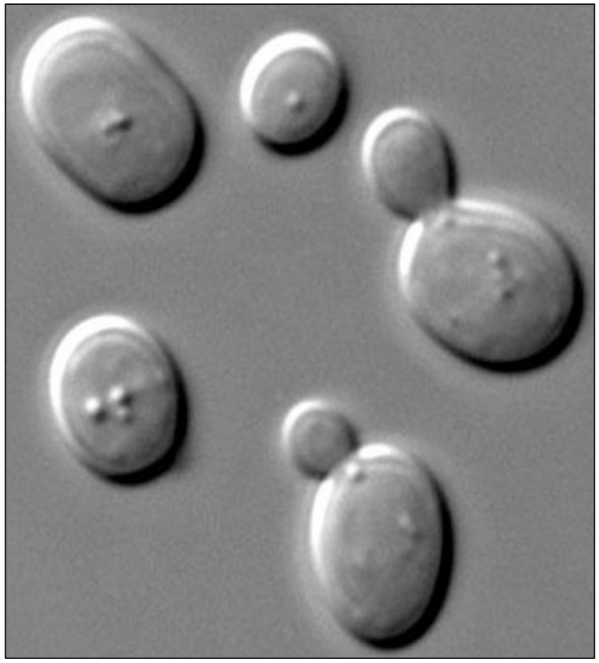
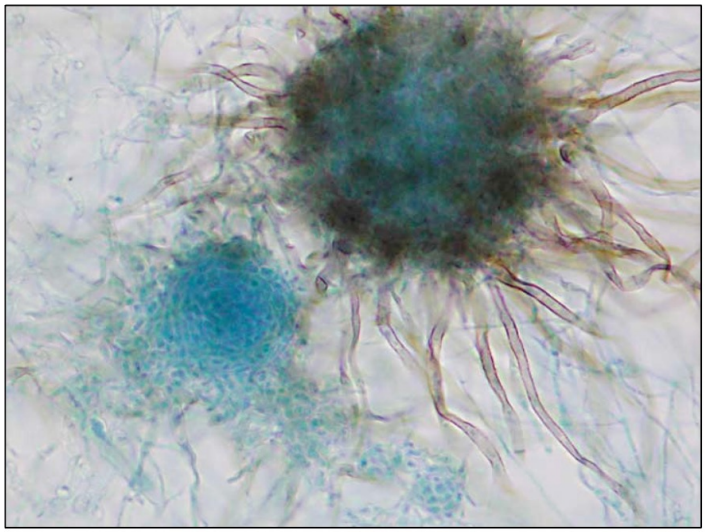


Figure 17-30, Bruce Albert, et. al., Molecular Biology of the Cell, 5/d, Garland Science, 2008, Page 1077
Figure 8. K. Bell, et. al. *Jol. Bio. Chem.* Vol 292 (35), 2007

Structural and Functional Conservation of Kinesin-5 *CIN8* in *Saccharomyces Cerevisiae & Chaetomium Thermophilum*

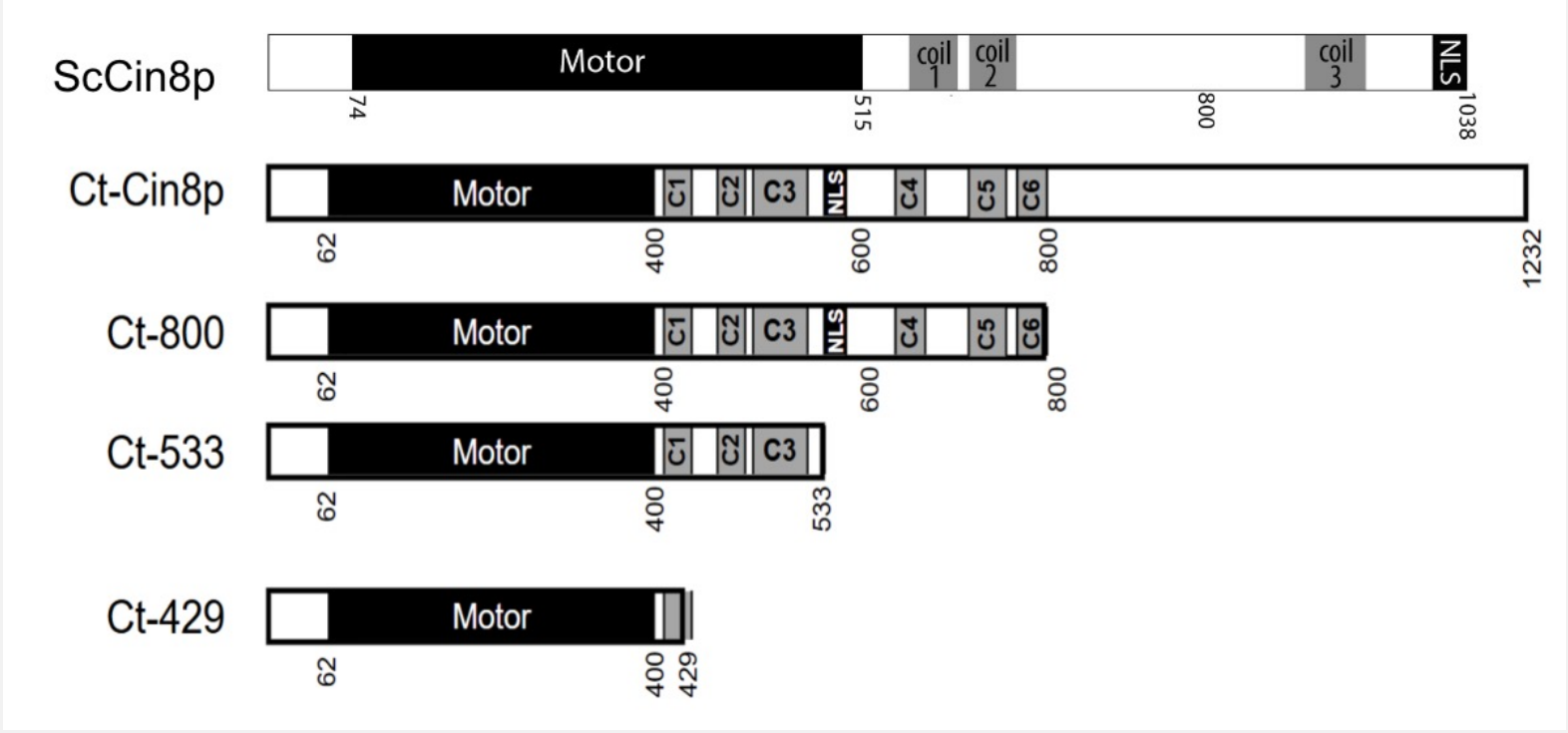


Saccharomyces cerevisiae
Budding yeast



Chaetonium thermophilum
Filamentous fungus

Nuclear Localization Signal located in different domains of CtCin8 & ScCin8

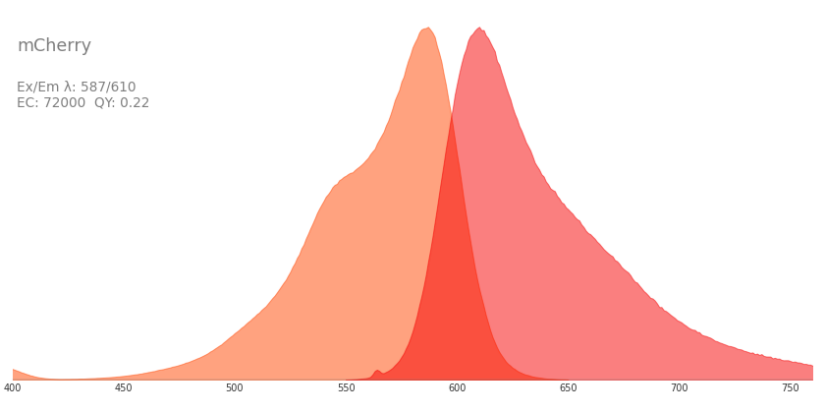


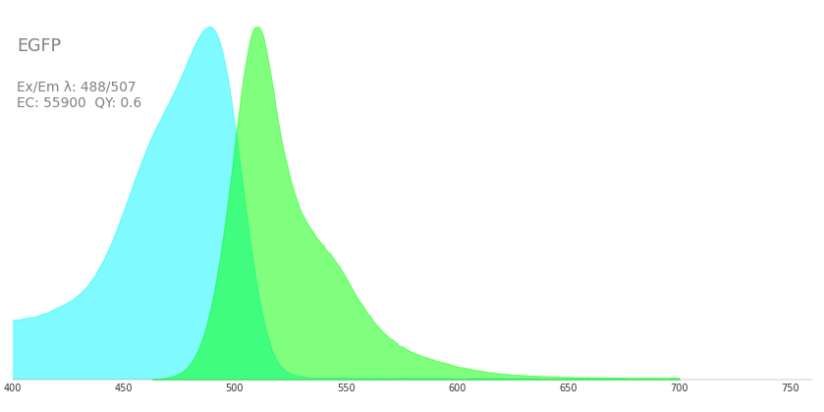
Prof. Isabelle Leblanc, Fa2024 MCB C110L, Lecture 1 & 2, 2024.

Visualizing Protein Localization

DDY904 Strain Contains mCherry (RFP) – Tagged Tub Gene

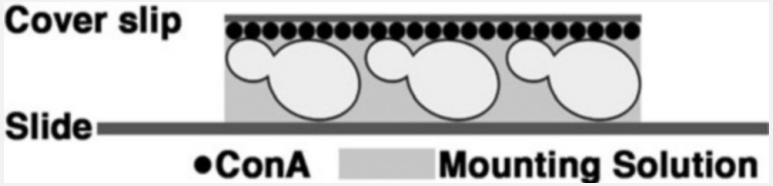
- **DDY904:**
  - *MATα ura3-52 leu2-3,112 his3Δ200 lys2-802 mCherry (RFP)-tagged tubulin*



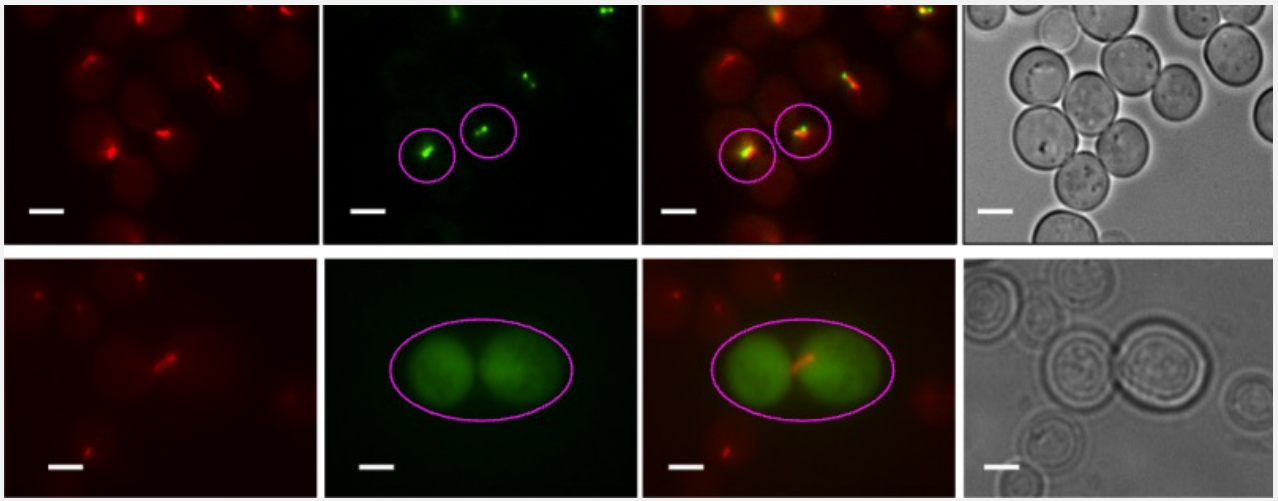


RFP - mCherry, FBBase.org, 2004.
GFP - EGFP, FBBase.org, 1996

Visualizing Subcellular Localization



Tub1 - mCherry | Cin8 - GFP | Merge | Bright-Field

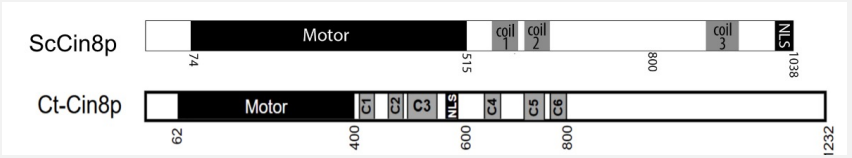


Localized Protein Expression

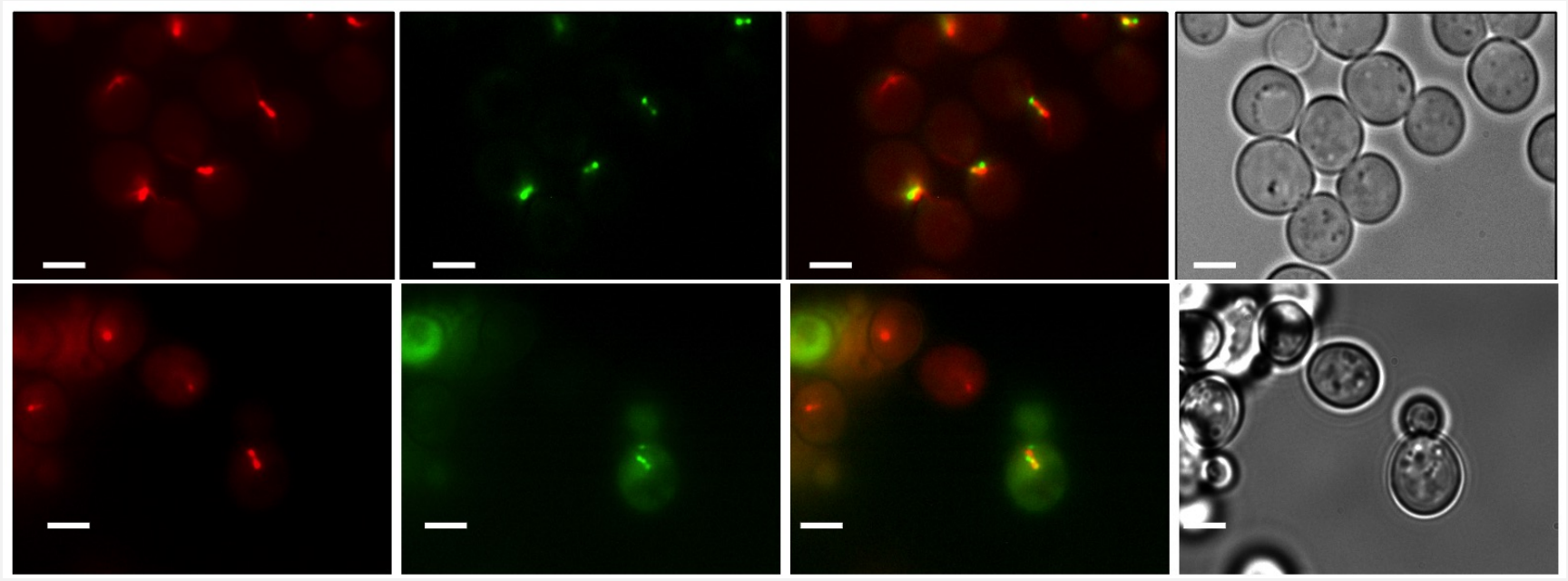
Unlocalized Protein Expression

Figure 1, *Cytoskeleton*, C.Sing, et. al., *Research Gate,* Jan. 2022, Page 88

Full-Length CtCin8 exhibits high degree of localization.



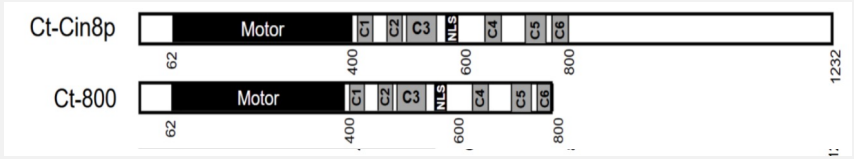
Tub1 - mCherry | Cin8 - GFP | Merge/Superimposed | Bright-Field



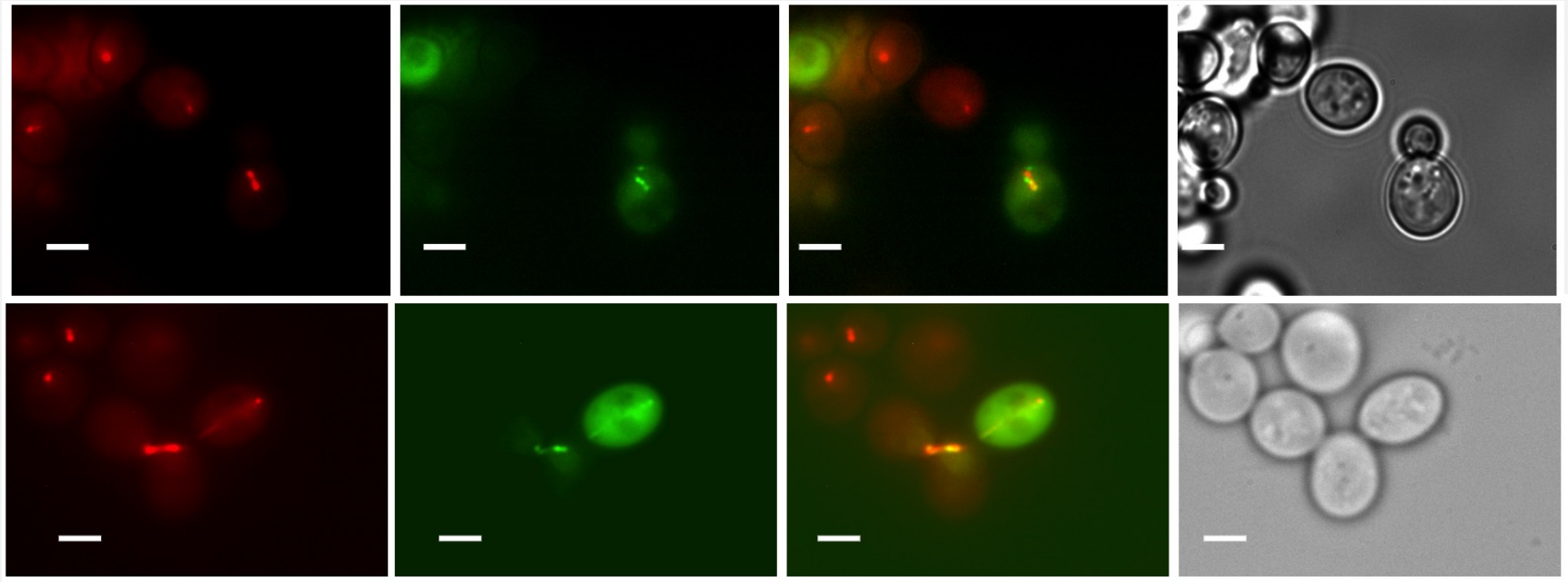
ScCin8

CtCin8

Ct800 demonstrates a slightly lower degree of localization than Full-Length Cin8.



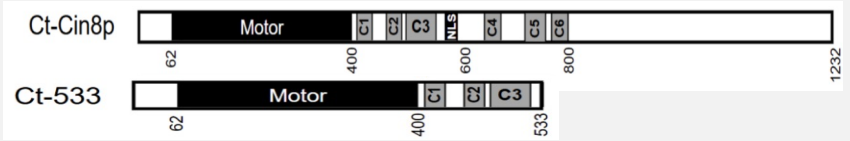
Tub1 - mCherry | Cin8 - GFP | Merge/Superimposed | Bright-Field



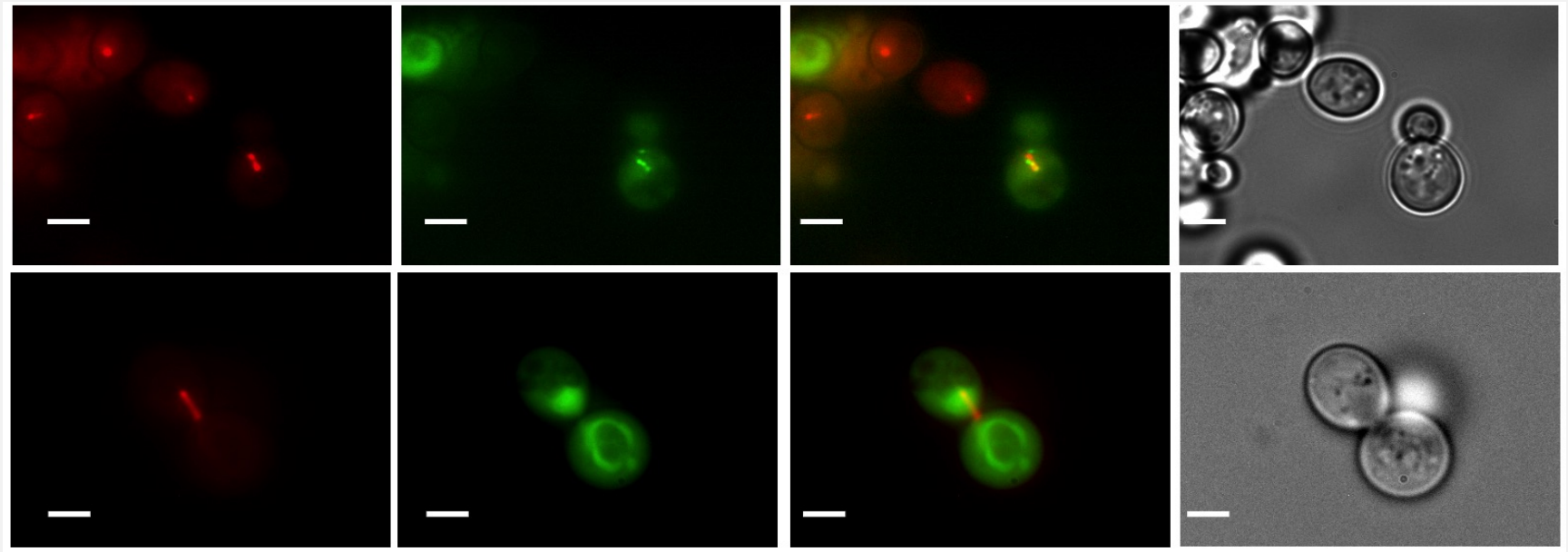
CtCin8

Ct800

Ct533 exhibits much lower levels of Cin8-GFP localization than full-length CtCin8.



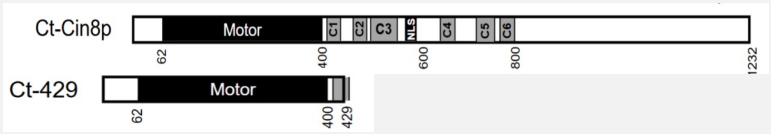
Tub1 - mCherry | Cin8 - GFP | Merge/Superimposed | Bright-Field



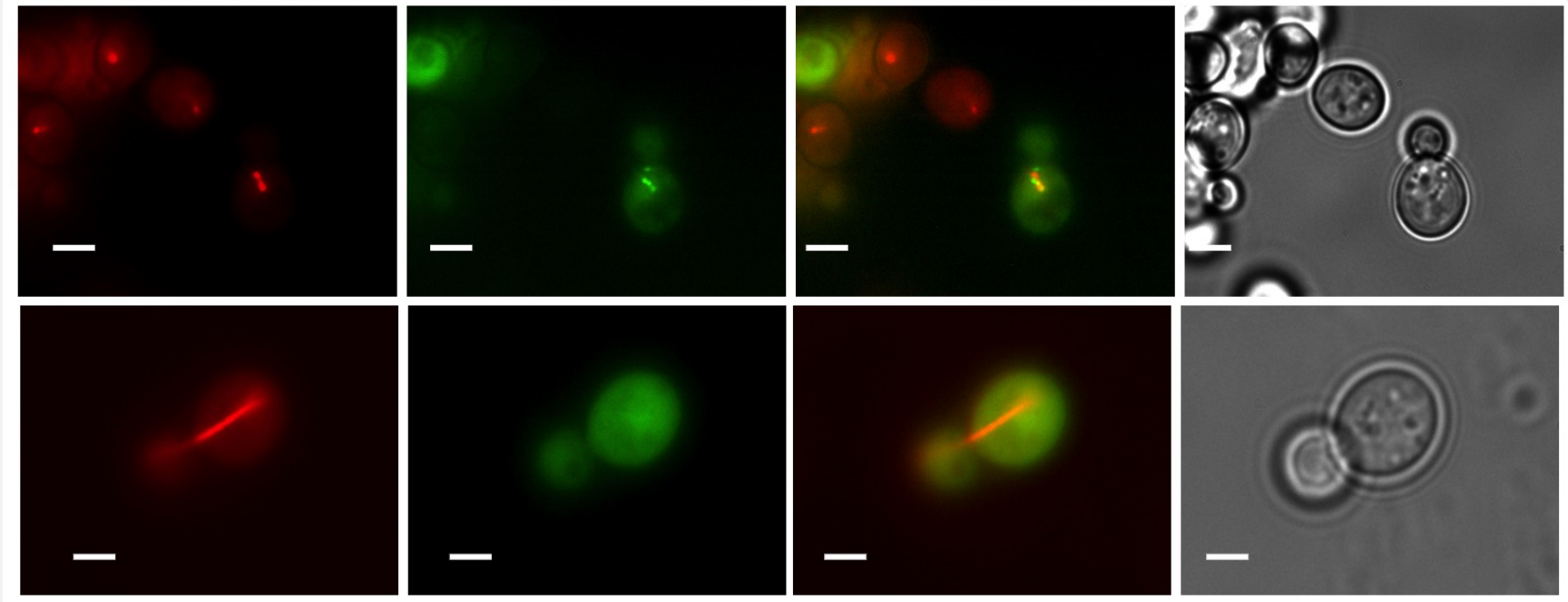
CtCin8

Ct533

Ct429 displays virtually no Cin8-GFP localization



Tub1 - mCherry | Cin8 - GFP | Merge/Superimposed | Bright-Field



CtCin8

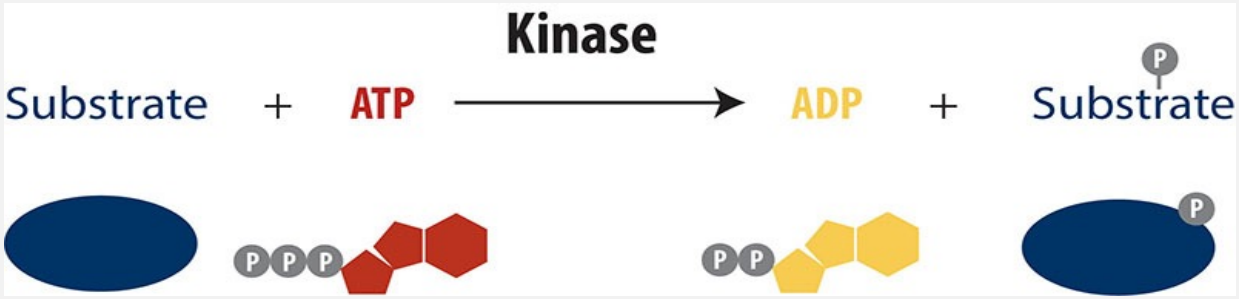
Ct429

Malachite Assay to measure enzyme Kinetics

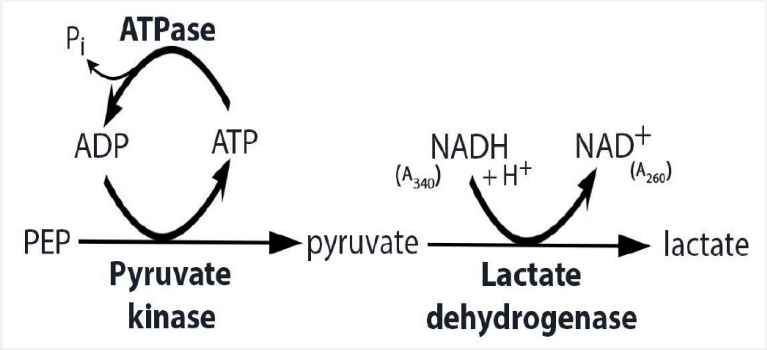
Measuring Kinetic Properties of Cin8

- 1)
  - A) ATPase Activity
  - B) NADH Coupled Assay
- 2) Temperature Dependence Assay

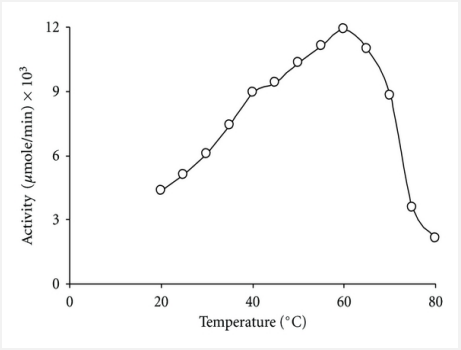
1A)



1B)



2)



BellBrookLabs, Activity Assays, Dec. 2018
R. Yadav., et. al., L. aegytiaca, ResearchGate.com, Jan. 2011
K. Sozanski, et. al., Motor Domain Diffusion, ResearchGate.com, Nov. 2015

Column Chromatography

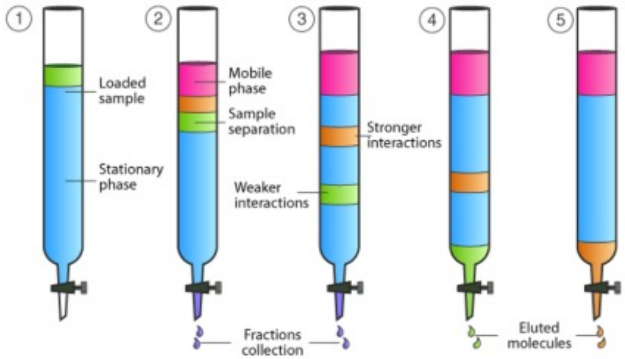
Purification Wash Buffer (W1, W3, & W4):

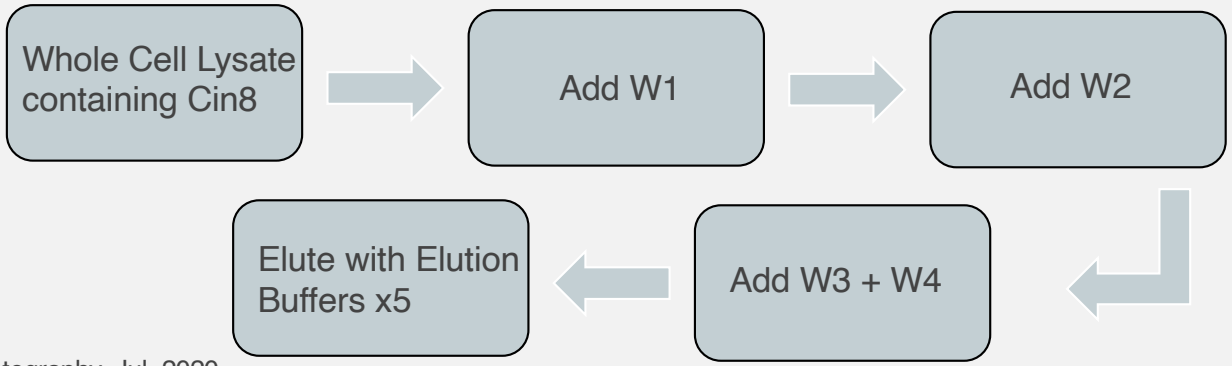
50 mM Hepes pH: 7.5
150 mM KCl
5 mM $MgCl_2$
20 mM Imidazole
10 % Glycerol
1 mM DTT
0.1 mM ADP pH 7.0

Purification Elution Buffer:

50 mM Hepes pH: 7.5
150 mM KCl
5 mM $MgCl_2$
250 mM Imidazole
10 % Glycerol
1 mM DTT
0.1 mM ADP

Wash Buffer (W2): except 1 mM **ATP replacing ADP** (same as Wash buffer above)





Byjus.com, Column Chromatography, Jul. 2020.

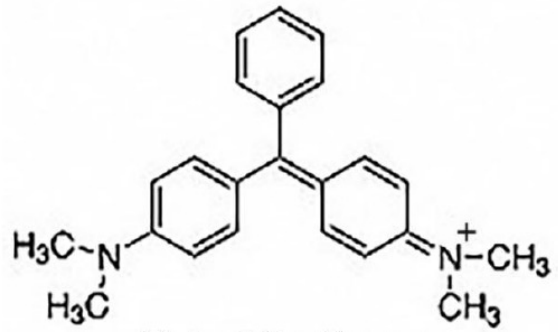
Malachite Green as a Phosphate Sensitive Dye

$$ATP + Mg2+ + MT + Kinesin \xrightarrow{\text{Kinesin (Cin8)}} ADP + Pi + Mg2+ + MT + Kinesin$$

Upon addition of Malachite Green, the presence of Phosphate makes initially yellow dye turn green.

$12\ H_2MoO_4$ + Malachite green = Yellow (λ_{max} = 446nm)

$H_2PO_4(MoO_3)_{12}$ + Malachite green = Green (λ_{max} = 650nm)



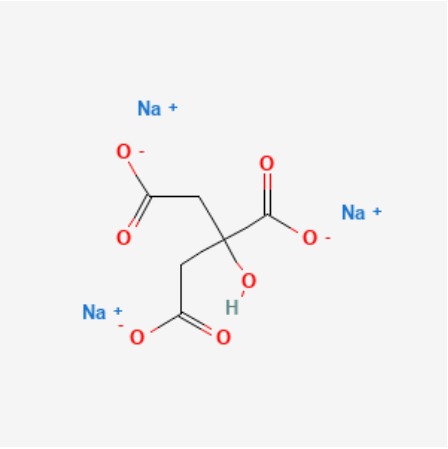
Malachite Green

Prof. Isabelle Leblanc, Fa2024 MCB C110L, Lecture 1 & 2, 2024.

Quenching Continuous Assay EA & SA

34% Sodium Citrate

Prevents further color development of the continuous Malachite Green Assay



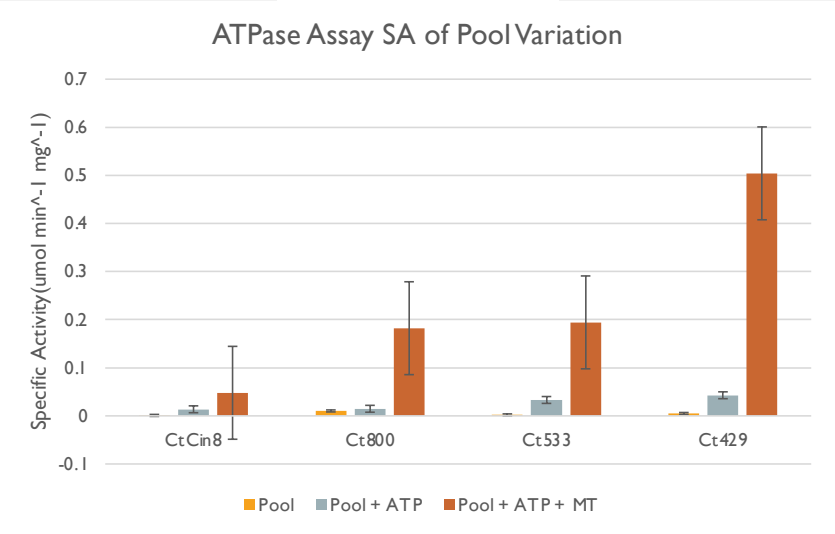
Enzyme Activity (**EA**) = **U = A/l*ε* * V / time**

(***ε***_Malachite Green = 99.4 M^-1 * C^-1)

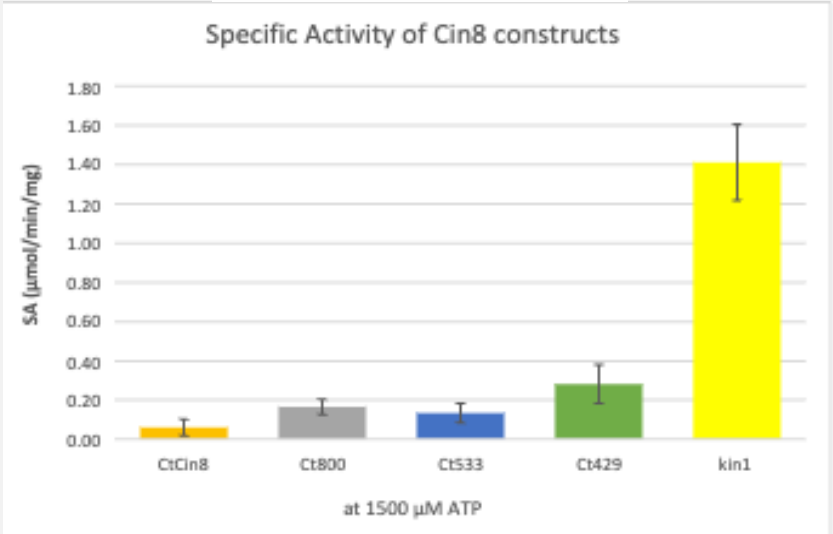
Specific Activity (**SA**) = EA/mass

ATPase/NADH Assay

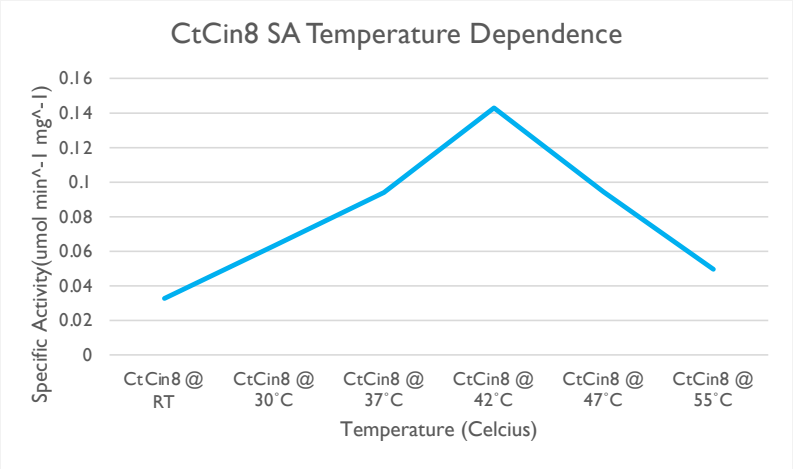
ATPase Assay

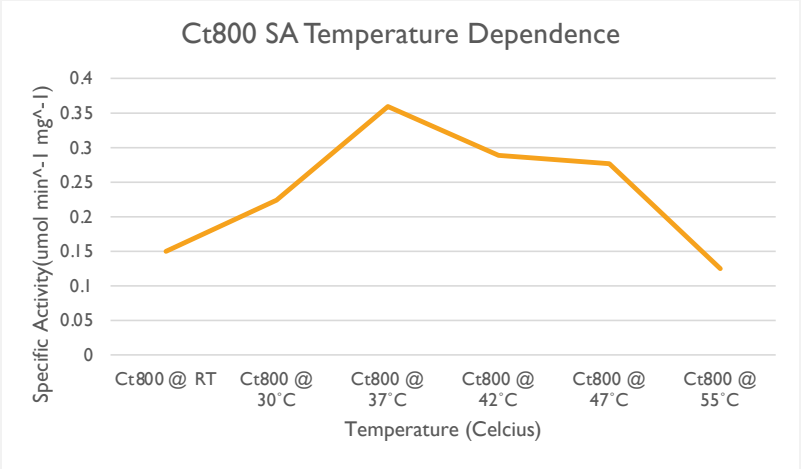


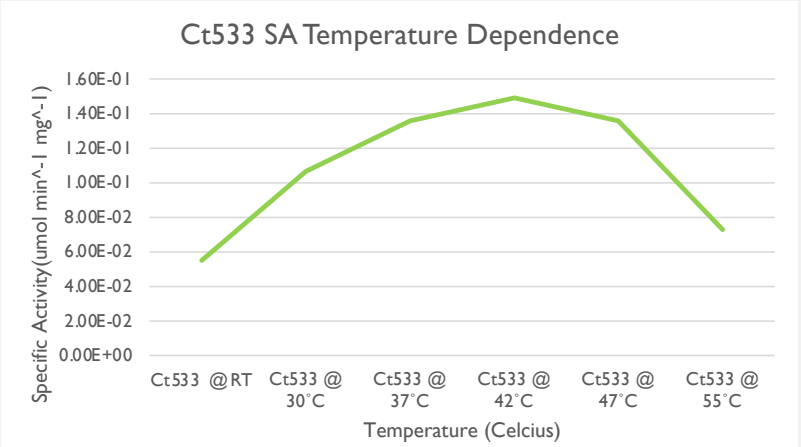
NADH Coupled Assay

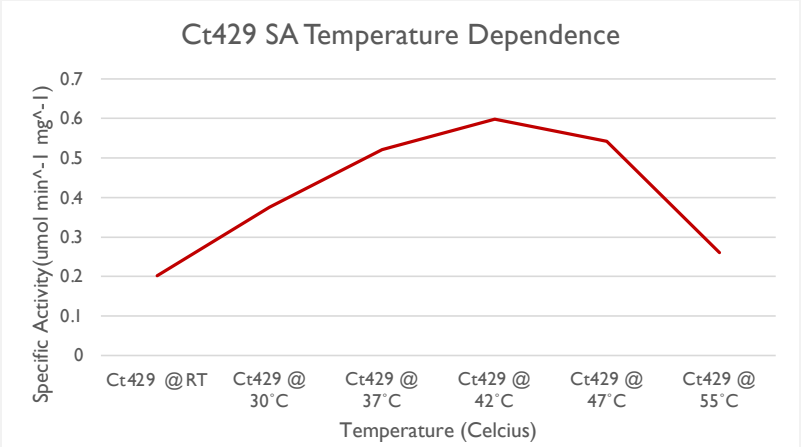


Temperature Binding Assay









Rescuing Growth in Auxin and Cincreasin Conditions

Y119 contained Aid-6Flag Tag and Kip1Δ

Y119

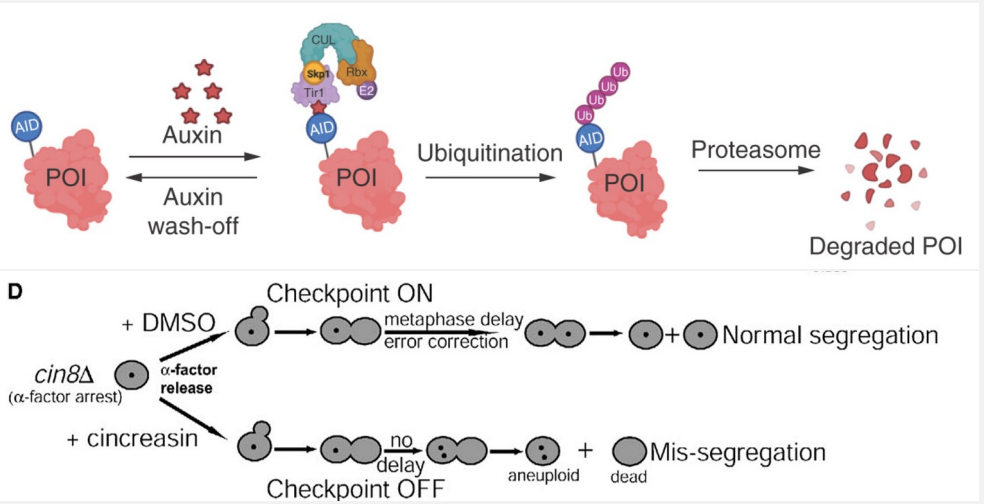
- *MATα* CIN8-AID-6FLAG::pHyg TIR1::LEU2 kip1Δ::HIS3 ura3-52 leu2-3, 112, lys1-801 his3Δ200

Auxin

- Recognizes & Binds to *AID sequence* to induce degradation via ubiquitination

Cincreasin

- Removes the cell cycle checkpoint for spindle assembly



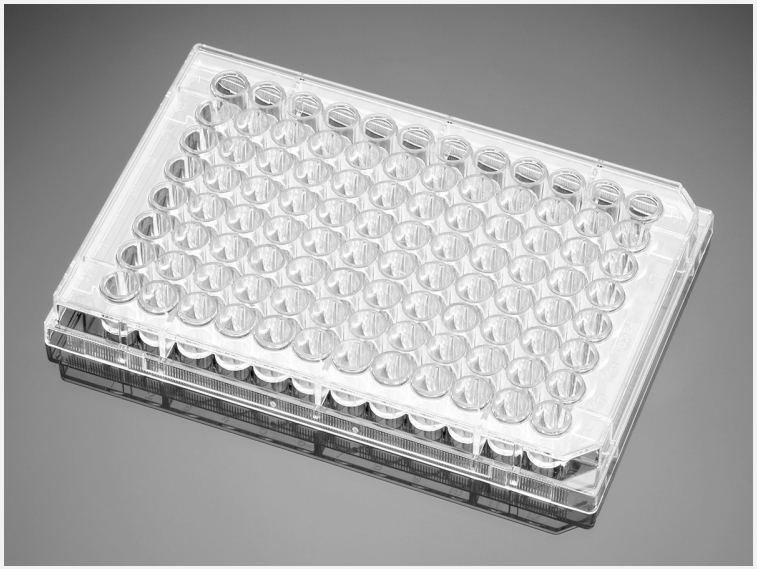
K. Phanindhar, et. al., Biotechniques, Vol 74. (4), 2023
R. Dorer, et. al., Current Biology, Vol. 15, Jun. 2005, Pg 1073

Phenotype Analysis of Cin8 Predictions

+ : Growth
– : No Growth
? : Uncertain Growth

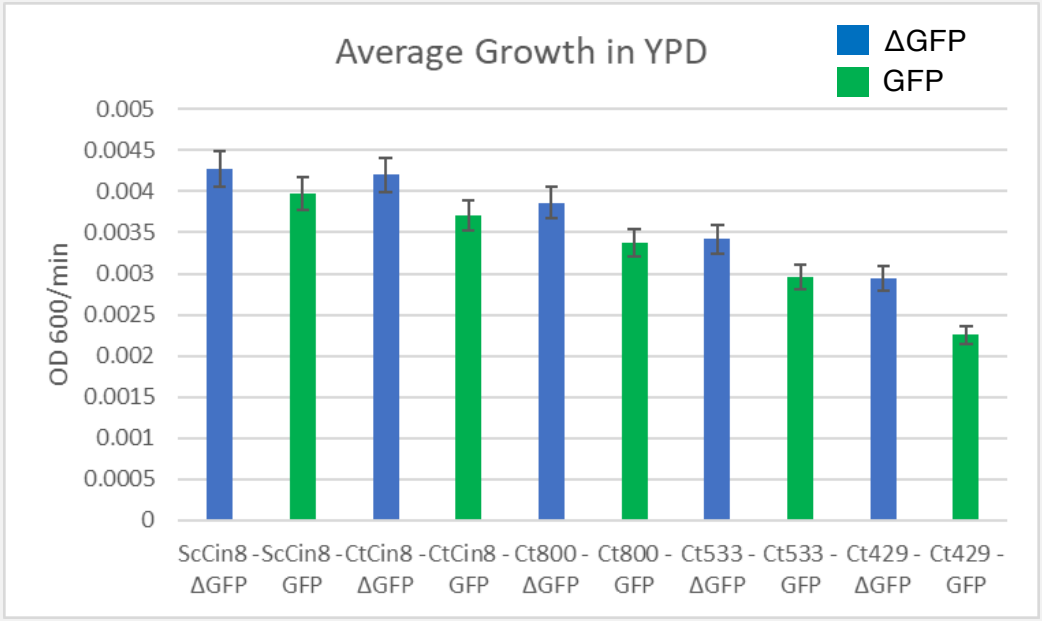
Plasmid	YPD	Auxin	Cincreasin	Auxin + Cinc.
ScCin8	+	+	+	+
CtCin8	+	?	?	?
Ct800	+	?	?	?
Ct533	+	?	?	?
Ct429	+	-	?	-

Plate Reader's Results



- GFP/ΔGFP - Cin8 - Incubated in YPD
- Experiments done in Triplicate
- Reading Taken per 15 minutes:
- Average Growth per 2hr of the fastest growth period

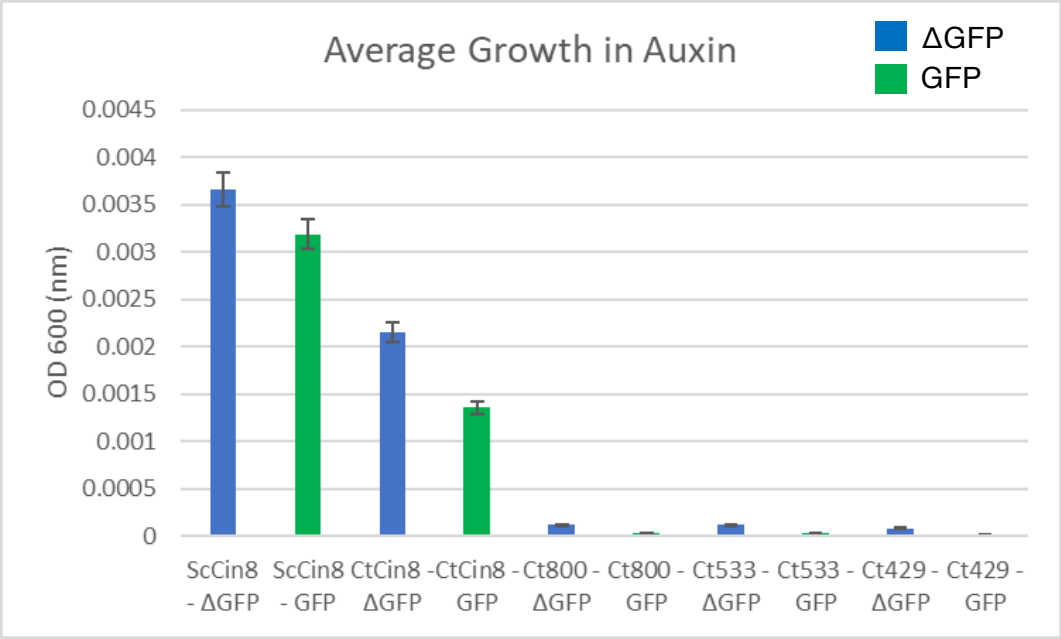
Average Growth in YPD exhibits Size Dependent Growth



In YPD setting, Cin8 Constructs demonstrate size dependent growth

↑ Genome Size = ↑ Growth

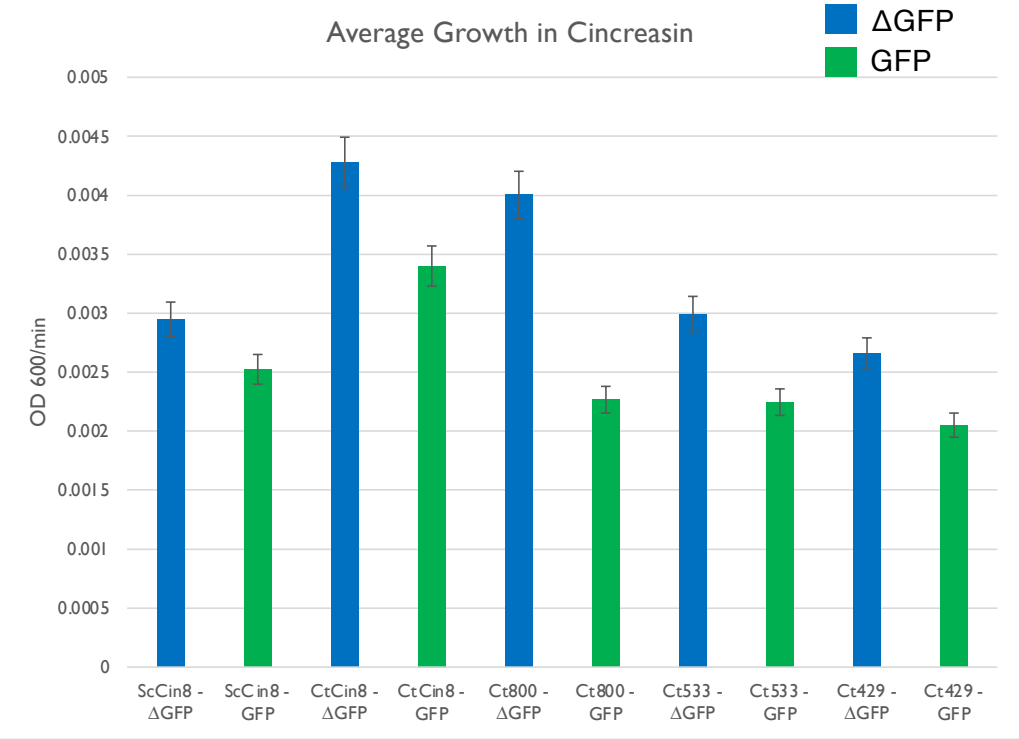
Auxin influences growth of full length ScCin8 & CtCin8 less than YPD



No significant impact to full length constructs of ScCin8 and CtCin8

Truncated constructs exhibit less growth in YPD

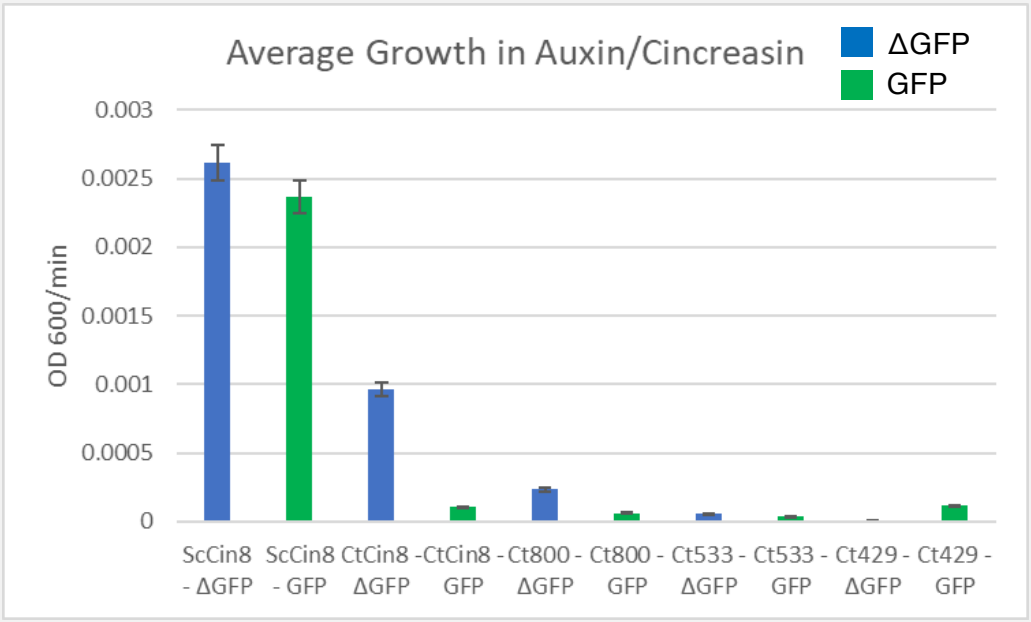
Cincreasin influences growth of full length ScCin8 & CtCin8 less than Auxin



No significant impact to full length constructs of ScCin8 and CtCin8

Truncated constructs exhibit less growth in YPD but more than Auxin

Average Growth in Aux/Cin exhibits Similar Growth Pattern as Auxin-only conditions



No significant impact to full length constructs of ScCin8 and CtCin8

Truncated constructs similar growth to Auxin only conditions

Future Direction

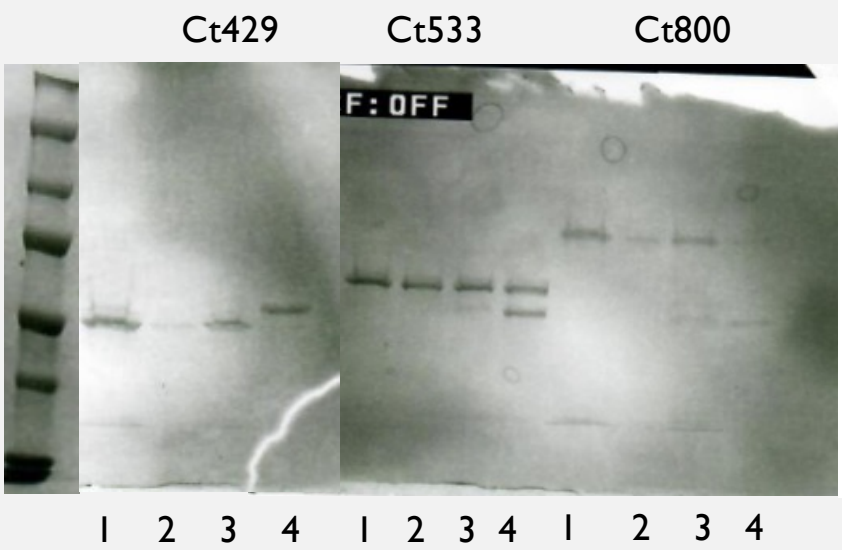
- **3D Crystallization of Truncated CtCin8**
  - **How do the missing coiled domains affect CtCin8 folding?**
  - **Are there any conserved regions to ScCin8?**

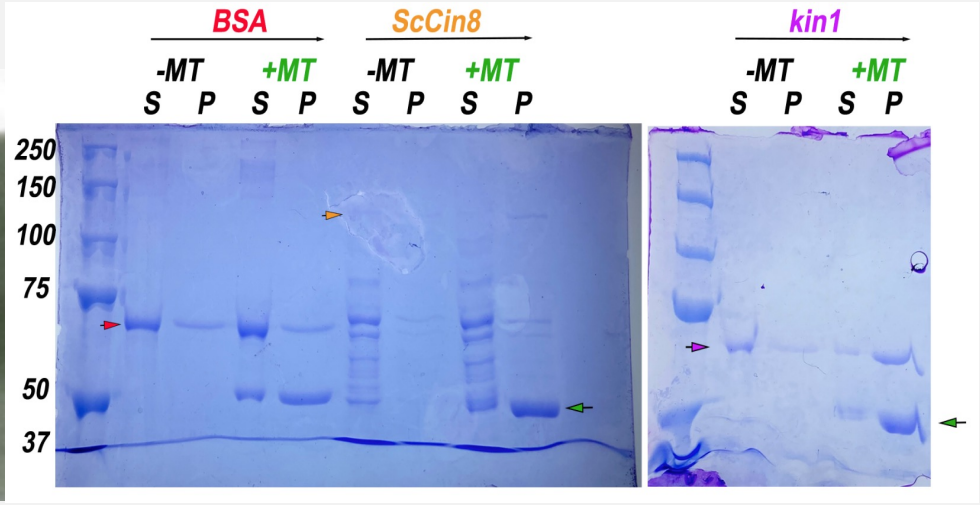
Q/A

APPENDIX

MT Binding Assay

Legend
1) -MT (S)
2) -MT (P)
3) +MT (S)
4) -MT (P)





Note: No strong representation of Full Length CtCin8