

**THE C-TERMINAL DISORDERED  
REGION OF THE RNA  
HELICASE DEAD IS REQUIRED FOR  
COLD SHOCK GROWTH IN E. COLI**

by

Arwen Portilla

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Degree in Major with Distinction

Spring 2025

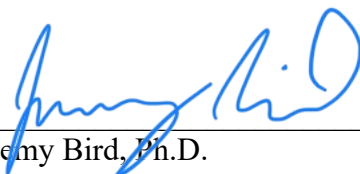
© 2025 Arwen Portilla  
All Rights Reserved

**THE C-TERMINAL DISORDERED  
REGION OF THE RNA  
HELICASE DEAD IS REQUIRED FOR  
COLD SHOCK GROWTH IN E. COLI**

by

Arwen Portilla

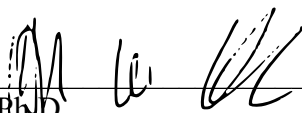
Approved:

  
\_\_\_\_\_  
Jeremy Bird, Ph.D.  
Professor in charge of thesis on behalf of the Advisory Committee

Approved:

  
\_\_\_\_\_  
Molly Sutherland, Ph.D.  
Committee member from the Department of Department Name

Approved:

  
\_\_\_\_\_  
Chi Keung Lam, Ph.D.  
Committee member from the Board of Senior Thesis Readers

Approved:

\_\_\_\_\_  
Sarah Trembanis, Ph.D.  
Chair of the University Committee on Student and Faculty Honors

## ACKNOWLEDGMENTS

I would like to thank everyone who supported me in my research goals and in completing my senior thesis. I would also like to acknowledge Dr. Chi Keung Lam for agreeing to be my third reader. Graduate students Kaustuv Das and Laura Marvin were instrumental in helping me achieve my research goals. I would like to thank Kaustuv for supporting me in building a new project using the DeaD protein and for allowing me to reference his data. I hope that what I have done will contribute to his work on DeaD. I would like to thank Laura for serving as both a mentor and a role model in research and scientific success. Finally, I would like to thank Dr. Bird and Dr. Sutherland for all they did to ensure my success in undergraduate research and senior thesis. The Bird Lab has given me an invaluable experience, and I will be forever grateful.

## TABLE OF CONTENTS

LIST OF TABLES .....	vi
LIST OF FIGURES .....	viii
ABSTRACT .....	ix
1.1 INTRODUCTION.....	1
DeaD interaction with NAD <sup>+</sup> Capped RNA.....	1
1.1.1 Cold Shock Function in DeaD.....	2
1.1.2 C-terminal RNA Binding Domain.....	3
1.1.3 DeaD implicated in RNA Condensates .....	4
1.1.4 Plasmids with Linearly Inducible Promoters .....	6
2.1 MATERIALS AND METHODS .....	8
2.1.1 Bacterial Cell Overnight Growth.....	8
2.1.2 Transduction $\Delta$ deaD Cells Using P1 Phage .....	8
2.1.3 Competent Cells .....	10
2.1.4 Transformation Protocol.....	10
2.1.5 Molecular Cloning.....	10
2.1.6 Spot Assay Protocol .....	12
2.1.7 Growth Curves.....	15
2.1.8 Fluorescence Microscopy .....	15
2.2 Modifications for different experiments:.....	16
2.2.1 Wild Type Phenotype and $\Delta$ deaD Phenotype .....	16
2.2.2 DeaD Phenotype and DeaD 1-533 C-terminal Truncation Phenotype .....	17
2.2.3 DAPI Amendments .....	17
2.2.4 2-Hour DeaD Phenotype and DeaD 1-533 C-terminal Truncation Phenotype.....	17
2.2.5 Confocal Amendments .....	17
1 RESULTS.....	18
3.1 Spot Assays .....	18
3.1.1 Wild type Vs. $\Delta$ deaD phenotype .....	18
3.1.2 The C-terminal Truncation vs. Full-length DeaD protein .....	18
3.2 Microscopy .....	22

3.2.1	Wild Type Vs. $\Delta$ DeaD Localization.....	22
3.2.2	DeaD Localization is Dependent on Temperature and Presence of C-terminal Region.....	23
3.2.3	Temperature Experiment Repeated at 2 Hours with DAPI.....	25
2	DISSCUSSION .....	27
	REFERENCES.....	32

## LIST OF FIGURES

Figure 1: Using NAD capped RNA Affinity Purification (NcRAP) to identify novel NAD cap interacting proteins. a) SDS-PAGE of proteins that interact with triphosphate or NAD-capped RNAs b) Volcano plot of proteins identified that interact with triphosphate or NAD-capped RNAs as determined by LC mass spectrometry (Das and Bird, unpublished data) .....	2
Figure 2: Alphafold structure of DeaD with important domains outlined along the C-terminal end. ....	4
Figure 3: Representation of different expression constructs under the control of linearly inducible promoter constructs on pAJM011 plasmids. Plasmids contain a kanamycin resistance gene for selection. ....	7
Figure 4: Figure outlining the experimental method of performing the spot assays....	14
Figure 5: Figure outlining the experimental method of prepping samples from microscopy imaging. ....	16
Figure 6: The <i>deaD</i> gene is necessary for growth at low temperature (Cold Shock). Spot assays were performed using serial dilutions of WT MG1655 and $\Delta$ <i>deaD</i> strains, followed by incubation at 37°C for 16 hours or 16°C for 96 hours. ....	18
Figure 7: Plasmid expression of full length DeaD but not a truncation mutant without the C-tail IDR can rescue the $\Delta$ <i>deaD</i> cold shock phenotype (a) Spot assays were performed at 37°C and 16°C with $\Delta$ DeaD cells transformed separately with plasmids containing full-length DeaD and a truncated form of DeaD with the C-terminal end deleted. The plates at 37°C were incubated for 24 hours on plates with and without the ATC inducer (b). These plates were transformed using the aforementioned procedure at 16 °C for 96 hours. ....	20
Figure 8: A spot assay showing all different cell types transformed with all of the experimental and control plasmids. The assay was done with varying concentrations of ATC (inducer) for the linearly inducible plasmids. The plates were then incubated at 16°C an 37°C for 96 and 24 hours, respectively.....	21

Figure 9: DeaD localizes to distinct puncta in E. coli. GFP DeaD fusion microscopy was performed on fixed  $\Delta$ deaD and MG1655 cells incubated at 37°C. Both strains were also transformed with a plasmid encoding only GFP as a control. This experiment was replicated with mCherry-tagged DeaD. .... 23

Figure 10:  $\Delta$ DeaD cells transformed with a plasmid with a GFP-DeaD fusion expression cassette. The cells were incubated at 37°C and 16°C for 1 hour..... 24

Figure 11:  $\Delta$ DeaD cells transformed with a plasmid with a GFP-DeaD 1-553 fusion expression cassette. The cells were incubated at 37°C and 16°C for 1 hour..... 25

Figure 12: Deletion of the DeaD C-terminal IDR reduces the number of DeaD puncta at low temperature.  $\Delta$ deaD cells transformed with plasmids encoding GFP-DeaD or GFP-DeaD 1-553. The cells were fixed after 2 hours of protein induction. GFP fluorescence is shown in green, and DAPI staining is used to show DNA localization in blue. .... 26

Figure 13: Confocal airyscan imaging of  $\Delta$ deaD cells transformed with a plasmid expressing the truncated form of deaD with a GFP tag at 16°C at 2 hours. .... 31

## LIST OF TABLES

Table 1: Plasmid identification chart with antibiotic resistance and expression cassette.....	8
Table 2: Primers used for cloning plasmids in spot/growth assays and fluorescence microscopy.....	11

## ABSTRACT

DeaD, a protein belonging to the dead box family of RNA helicases in *Escherichia coli* (*E. coli*), has many cellular functions. The known functions of DeaD include its function as an RNA helicase, a role in ribosome biogenesis and enabling growth at cold shock temperatures. DeaD expression is upregulated in cold temperatures, which has led to it being given an alternative name: cold shock Dead box protein A (CsdA). We want to explore the function of DeaD during *E. coli* growth under cold shock conditions. Most of the protein's structure has been attributed to the RNA helicase function. In addition to the RNA helicase domains, DeaD has two intrinsically disordered regions (IDRs) and a DbpA domain. The function of these other regions is less well researched. Using epifluorescence microscopy and growth assays, I have been able to outline the significance of the C-terminal IDR to DeaD's primary functions. In addition to assessing the function of DeaD as a cold shock protein, we also want to characterize its localization within the cell.

Dead and other Deadbox helicases have been studied for their role in forming RNA condensates. This phase-phase separation is important to cellular organization and has been observed in both prokaryotic and eukaryotic cells. Since the C-terminal IDR and DbpA domain have been implicated as RNA-binding domains, I wanted to see if DeaD could form condensates without the C-terminal IDR. By fusing the DeaD protein with a fluorescent protein, we can use microscopy to track protein localization inside the cell. A *deaD* deletion strain of *E. coli* was transformed with tetracycline

inducible plasmids encoding either the full-length dead or the truncated form tagged with a fluorescent protein. Full-length DeaD was able to form puncta at both preferred temperature and cold shock, while the truncation was not entirely successful. Based on our results, we have concluded that functioning DeaD is necessary for cells to survive cold shock conditions and for this localization to occur.

## Chapter 1

### 1.1 INTRODUCTION

#### Dead interaction with NAD<sup>+</sup> Capped RNA

RNA is a crucial macromolecule that is integral to the central dogma of gene expression. It is well known that most eukaryotic RNA is post-transcriptionally modified to have a Guanosine cap<sup>1</sup>. These guanosine caps protect the RNA from degradation before translation. While prokaryotes like *E. coli* don't have these caps, recent research has shown that prokaryotic RNA carries non-canonical RNA caps. These caps are made up of metabolites such as Nicotinamide Adenine dinucleotide (NAD<sup>+</sup>) and Coenzyme A (CoA)<sup>2</sup>. NAD<sup>+</sup> caps are added to an RNA by being used as initiating nucleotides by RNA polymerase during transcription initiation<sup>2</sup>. The existence of NAD<sup>+</sup> RNA caps suggests that metabolites may be potential regulators of transcription. As a result, we in the Bird lab are interested in understanding the function of NAD<sup>+</sup> RNA caps, particularly identifying proteins that interact with NAD<sup>+</sup> caps that may be involved with their role in transcription. In previous work, we performed NAD cap RNA Affinity Purification (NcRAP) to identify proteins that specifically interact with NAD<sup>+</sup> capped RNAs. Using this method, we identified

DeaD, an RNA Helicase that preferentially binds to NAD<sup>+</sup> capped RNA instead of triphosphate RNA (Fig. 1).

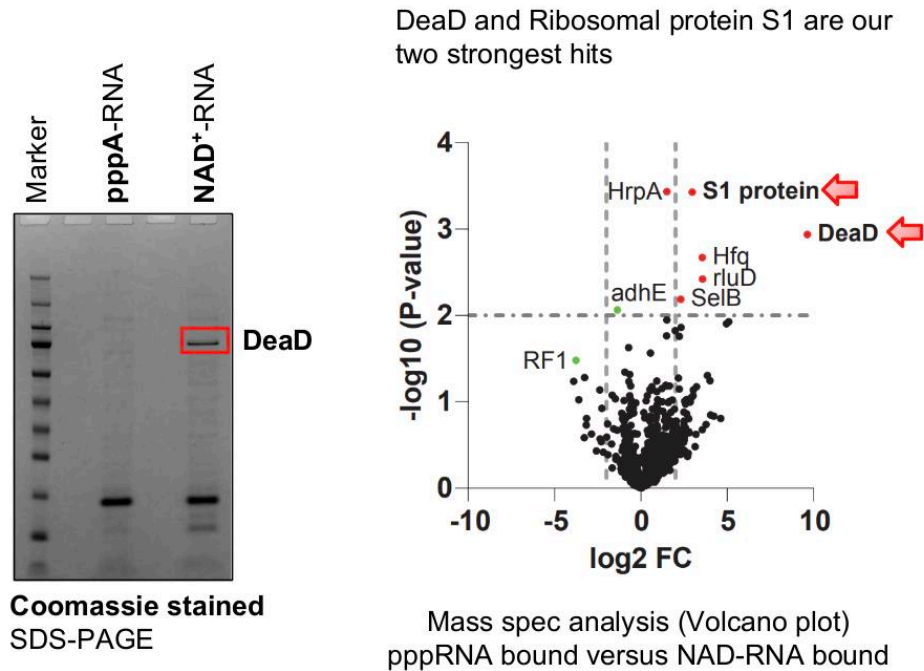


Figure 1: Using NAD capped RNA Affinity Purification (NcRAP) to identify novel NAD cap interacting proteins. a) SDS-PAGE of proteins that interact with triphosphate or NAD-capped RNAs b) Volcano plot of proteins identified that interact with triphosphate or NAD-capped RNAs as determined by LC mass spectrometry (Das and Bird, unpublished data)

### 1.1.1 Cold Shock Function in DeaD

*Escherichia coli*, commonly found in the lower digestive tract of mammals, prefers conditions similar to body temperature, 37°C<sup>3</sup>. Many proteins are responsible for regulating the cell's stress response when exposed to cold shock conditions. DeaD

is one of these cold shock proteins that are upregulated at low temperatures<sup>4</sup>. As a result, Dead is also known as CsdA (Cold Shock DEAD-box Protein A). *E. coli* carrying a deletion of the *deaD* gene demonstrates normal cell growth at 37°C but poor growth at 16°C<sup>5</sup>. The known functions of DeaD include its role in assembling the large subunit of the ribosome<sup>5</sup>, involvement in RNA degradation, as an RNA helicase<sup>6</sup>, and responding to cold shock temperature. Which of DeaD's functions are impacted by cold shock is still unclear. This is significant since translation initiation is substantially affected cell function at cold shock<sup>7</sup>. Prior research has indicated that at cold temperatures mRNA respond with formation of secondary structures<sup>7</sup>. For effective translation a RNA helicase would be required to unwind the secondary structures formed<sup>7</sup>. The role of cold shock proteins in reestablishing low-temperature translation is essential to understanding *E. coli* as a model organism.

### **1.1.2 C-terminal RNA Binding Domain**

Intrinsically disordered regions or IDRs are portions of the protein that have no secondary structure. These disordered regions are often involved in protein-protein and intermolecular interactions<sup>8</sup>. While most of the DeaD protein structure is attributed to the helicase function, the C-terminal IDR is less well characterized. It is this C-terminal IDR that is implicated in RNA binding as an RNA-binding domain<sup>9</sup>. Many other proteins share a similar disordered region on their C-terminal or N-

terminal ends<sup>10</sup>. While not all functions have been characterized, some have also been implicated in RNA binding and RNA condensate formation.

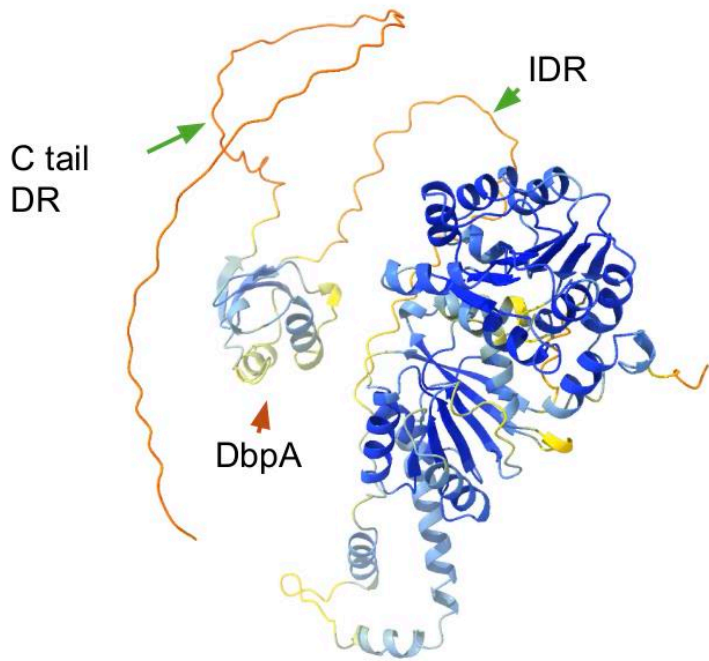


Figure 2: AlphaFold structure of DeaD with important domains outlined along the C-terminal end.

### 1.1.3 DeaD implicated in RNA Condensates

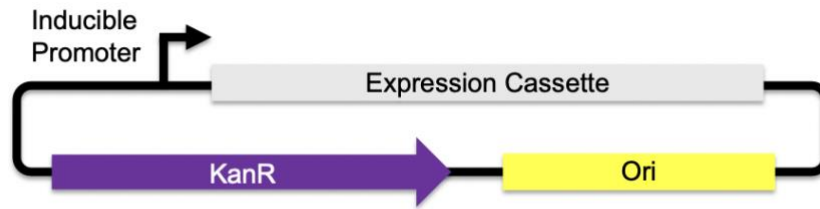
Molecular condensates are significant for prokaryotic subcellular organization, representing phase-phase separation. This condensate formation insinuates that both prokaryotes and eukaryotes use this condensate separation to organize RNA at the subcellular level. RNA condensate forming dead box RNA ATPases like DeaD are present in humans, yeast, and prokaryotes. Since the DeaD protein is implicated in

these RNA condensates, it is essential to understand how this function is related to cold shock. The implication that C-terminal IDR is an RNA-binding domain adds significant evidence that this domain has a function tied to condensate formation. These IDR regions have been involved in RNA condensate formation in other proteins. Dr. Jared Schrader's laboratory at Indiana University has observed that *Caulobacter crescentus* RNAaseE forms RNA condensates through the activity of a c-terminal IDR. Determining if the DeaD C-terminal IDR affects condensate formation by DeaD, its helicase ability, or both is crucial to understanding the protein's function. Using fluorescently tagged protein fusions, the localization of DeaD can be observed in cells. Localization of a protein can offer many insights into a protein's function, even in prokaryotic cells.

#### **1.1.4 Plasmids with Linearly Inducible Promoters**

To replace the *deaD* gene within the cell with our fluorescent tags, we used plasmids, small circular pieces of DNA that can be modified to include our gene of interest on an expression cassette. The benefit of the particular plasmids I used in this study is the presence of a linearly inducible promoter. These “Marionette Promoters” maintain constant protein expression across control and experimental groups by controlling the amount of inducer present. These plasmids provided benefits over the standard overexpression plasmids, which “overexpress” or make much more protein than the cell needs. For these studies, we needed to induce DeaD at a lower level than standard protein expression plasmids would allow. These lower expression levels more consistent with normal cellular protein levels. This is important because too much of many proteins can be toxic to the cells, and too little may not rescue the cold shock phenotype of the *E. coli*  $\Delta$ *deaD* deletion strain. In the case of these promoters, the plasmids are activated for expression by introducing a small molecule. This molecule is called the inducer. The plasmids used in this study are specifically induced by Anhydrous tetracycline (ATC).

## Marionette linearly inducible expression vector



- Meyer *et al.* (2018) *Nat. Chem Bio*

Inducer	Plasmid	Expression Cassette
	pAJM.011-GFP	GFP
	pJB206	DeaD
ATC	pJB212	GFP + DeaD
	pAEP15	DeaD 1-553 trunc.
	pAEP14	GFP + DeaD
	pAJM.773-mCherry	mCherry
Vanillic Acid	pJB215	DeaD
	pJB221	mCherry + DeaD

Figure 3: Representation of different expression constructs under the control of linearly inducible promoter constructs on pAJM011 plasmids. Plasmids contain a kanamycin resistance gene for selection.

## Chapter 2

### 2.1 MATERIALS AND METHODS

Table 1: Plasmid identification chart with antibiotic resistance and expression cassette.

Plasmid	Antibiotic Resistance Gene	Genes Added
pJB212	Kanamycin	GFP-deaD
pJB206	Kanamycin	deaD
pAP14	Kanamycin	GFP-deaD 1-553
pAP15	Kanamycin	Dead 1-553
pAJM.O11 GFP	Kanamycin	GFP
PAJM.733-mCherry	Kanamycin	mCherry
pJB215	Kanamycin	deaD
pJB221	Kanamycin	mCherry-deaD

#### 2.1.1 Bacterial Cell Overnight Growth

Single *E. coli* colonies of the appropriate strain were chosen for overnight cell culture in Luria-Bertani broth (LB Typtone, NaCl, yeast extract, agar, water) with 50µg/ml of kanamycin. The cells are then incubated in a shaker incubator for 12-16 hours at 37°C.

#### 2.1.2 Transduction $\Delta$ deaD Cells Using P1 Phage

An overnight culture of the bacterial donor strain, *E. coli* BW25113  $\Delta$ deaD:KanR, is set up. After growing the cells according to the protocol above, they

are diluted 1:50 in 5 mL of medium. They are grown in a shaking incubator at 37 °C until they reach an optical density (OD) of 0.2. 100 µL of 0.25 M CaCl<sub>2</sub> and incubated for 5 minutes at room temperature. At the same time, an aliquot of P1 phage stock was incubated at room temperature in an open Eppendorf tube to evaporate the chloroform. Add volume to the phage stock. The phage stock was added to the bacterial donor strain.

To allow for phage absorption, the strain is incubated at room temperature for 2 minutes. It is then placed at 37 °C and shaken until bacterial lysis occurs, which takes approximately 90-180 minutes. After 250 µL of chloroform is added to the strain, it is incubated for 5 minutes. The culture is then spun down for 2 minutes to pellet the cellular debris. The supernatant is saved in polypropylene snap-cap tubes with 250 µL of chloroform and stored at 4°C. *E. coli* K-12 strain MG1655 was grown overnight according to the protocol above as the recipient strain. Two milliliters of the cell culture were spun in an Eppendorf tube for 5 minutes at 7800 rpm. The pellet was resuspended in 1 mL of 10 mM MgSO<sub>4</sub> and 5 mM CaCl<sub>2</sub>. The recipient strain suspension and approximately 20 µL of P1 vir phage prepared on the donor strain were incubated at 37°C in a sterile test tube. After incubation, 1 mL of LB containing 50 mM sodium citrate is added. The culture is transferred to a glass test tube and vortexed before being incubated on a roller drum at 37°C for 1 hour. 100 µL of sodium citrate was added to LB plates containing kanamycin using sterile technique. After incubation, the cells are centrifuged and resuspended in 100 µL of LB

containing 50 mM sodium citrate. The culture is then spread onto the kanamycin-containing LB plates and incubated overnight.

### **2.1.3 Competent Cells**

The cells are grown overnight at 37°C according to the overnight procedure.

They are then back diluted 1:100 in new LB broth. The cells are shaken again at 37 °C until the optical density (OD) reaches 0.6. Once the OD has been reached, 12.5 mL of culture is placed into tubes and centrifuged at 8,000 rpm for 15 minutes at 4 °C. All future steps are performed on ice. Once the supernatant is removed, 625 µL of Solution A (10 mM MES, pH 6.3, 10 mM MnCl<sub>2</sub>, 50 mM CaCl<sub>2</sub>, 15% Glycerol) is added and the sample is resuspended. The cells are then aliquoted into Eppendorf tubes and stored at -80°C until needed.

### **2.1.4 Transformation Protocol**

*E. coli* K-12 strain MG1655 WT or  $\Delta$ deaD cells are incubated on ice with 1 µL of the chosen plasmid for 30 minutes. They are then heat-shocked at 42°C for 1 minute. After heat shock, the cells are resuspended in 975 µL of LB and incubated at 37 °C for 45 minutes. 50 µL of cell culture is plated on specific antibiotic LB plates and spread with glass beads. The plates are then incubated overnight until colonies form.

### **2.1.5 Molecular Cloning**

DeaD and Fluorescent protein (GFP and mCherry) fusion expression plasmids were generated using the New England Biolabs HiFi assembly method. First,

pAJM.011-GFP and pAJM.773-mCherry plasmid backbones (gift of Karl Schmitz and Patrick Beardslee) were PCR amplified using DreamTaq DNA polymerase (Thermo Scientific) and primer sets: JB836 and JB837 for insertion of *deaD* alone, JB836 and JB840 for generating an N-terminal fluorescent protein fusion, and JB850 and JB837 for generating a C-terminal fluorescent protein fusion. The resulting PCR products were gel-purified using the Monarch Gel extraction kit (New England Biolabs). *E. coli* genomic DNA was PCR amplified using DreamTaq DNA polymerase (Thermo Scientific) and the following primer sets to generate *deaD* and *deaD* truncation fragments for insertion into plasmid backbones: JB843 and JB844 for generating *DeaD* alone, JB846 and JB844 for N-terminal fusions, JB845 and 844 for C-terminal fusions, JB843 JB876 for generating *DeaD* 1-553 truncation, JB846 and JB876 for *DeaD* 1-553 N-terminal fusions, JB845 JB877 for *DeaD* 1-553 C-terminal fusions. The resulting PCR products were purified using a PCR cleanup kit (Qiagen). Equal nanogram quantities of linear plasmid PCR products and *deaD* or *deaD* truncation PCR products were mixed in 1X HiFi Assembly Mix (New England Biolabs) per manufacturer's instructions and incubated at 55 °C for 10 minutes. Assembly mixes were then transformed into NEB Turbo chemically competent cells (New England Biolabs). The resulting colonies were grown in LB media overnight. Plasmids were isolated using the Plasmid Miniprep Kit from Qiagen. Correct plasmid sequences were confirmed by whole plasmid sequencing by Plasmidsaurus, Inc.

Table 2: Primers used for cloning plasmids in spot/growth assays and fluorescence microscopy.

Primers For Cloning	Sequence
JB836 - pAJM plasmid fwd primer	ctcgtaccaaatccagaaaagaggc
JB837 - pAJM plasmid rev primer	ctagtatttcccctctttctctagtattaaa
JB840 - pAJM N-term GFP fusion rev primer	GCTACCGCCGGAACCCCC
JB850 - pAJM C-term GFP fusion fwd	atgTACCCATACGATGTTCCAGATTACG
JB843 - deaD pAJM cloning fwd	ttaataactagagaaagaggggaaatactagatggctgaattcgaaccacttttgc
JB844 - deaD pAJM cloning rev	gcctcttttctggaatttggtaccgagttacgcatcaccaccgaaacgac
JB845 - deaD pAJM c-term GFP fusion rev	CTGGAACATCGTATGGGTAcatcgcacaccaccgaaacgac
JB846 - deaD pAJM N-term GFP fusion fwd	GGGGGTTCCGGCGGTAGCgctgaattcgaaccacttttgc
JB876 - deaD trunc pAJM rev	gcctcttttctggaatttggtaccgagTTActtggtgagaatgcgagtg
JB877 - deaD trunc pAJM c-term GFP fusion rev	CTGGAACATCGTATGGGTAcatttgggtgagaatgcgagtg

### 2.1.6 Spot Assay Protocol

MG1655  $\Delta$ deaD was transformed with the pJB206 and pAP15 plasmids and grown as detailed in "Bacterial Cell Overnight Growth." After 12 hours of shaking incubation at 37 °C, the culture is back diluted 1:100 in LB with 50  $\mu$ g/ml of kanamycin. Serial dilutions were made using LB with decreasing concentrations from  $10^{-2}$  to  $10^{-7}$ . 5  $\mu$ L of each dilution is spotted onto both LB agar plates and LB agar

plates with 10  $\mu\text{g}/\text{mL}$  ATC inducer to induce plasmid expression. Half of the technical replicates are incubated at 37°C for 24 hours, and the others at 16°C for 96 hours. Untransformed MG1655 and MG1655  $\Delta\text{deaD}$  were grown overnight at a 1:100 dilution in LB and then back-diluted as detailed above, without kanamycin. Serial dilutions were made using LB with decreasing concentrations from  $10^{-2}$  to  $10^{-7}$ . 5  $\mu\text{L}$  of each dilution is spotted onto plain LB agar plates.

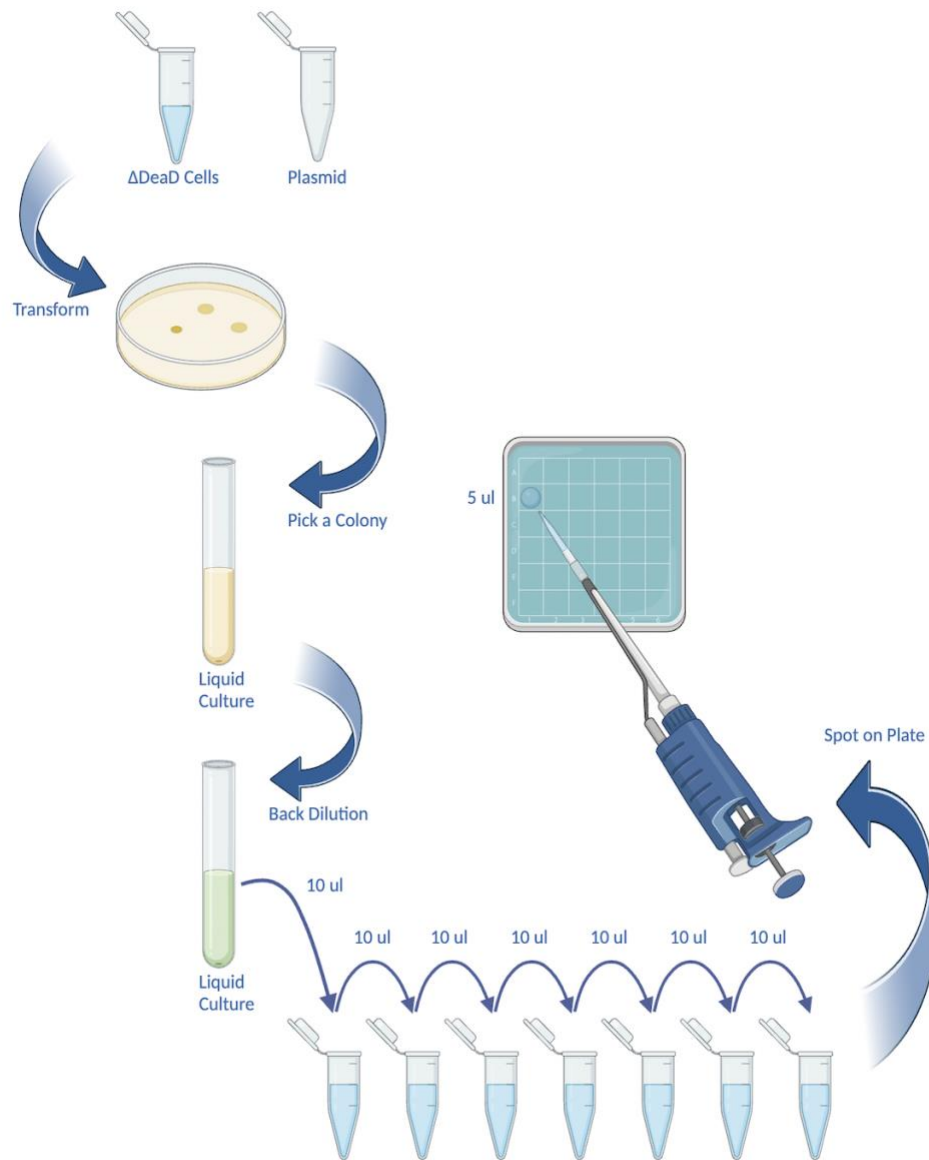


Figure 4: Figure outlining the experimental method of performing the spot assays. See Section 2.1.6 for detailed step by step explanation.

### **2.1.7 Growth Curves**

MG1655  $\Delta$ deaD was transformed with all plasmids and grown as detailed in Bacterial Cell Overnight growth. 200 microliters of water are added to the perimeter of a 96-well plate. 195 microliters of LB are added to all experimental wells. 5 microliters of culture is then added, with 5 replicates of each. The plate is then incubated and shaken in a growth curve reader, with OD readings taken every 30 minutes for 24 hours at the designated temperature.

### **2.1.8 Fluorescence Microscopy**

MG1655  $\Delta$ deaD were transformed with plasmids containing the gene of interest and grown as detailed in Bacterial Cell Overnight growth. After 12 hours of shaking incubation at 37 °C, the culture is back diluted 1:100 in LB with 50  $\mu$ g/ml of kanamycin. The back-diluted culture is grown for 2 hours or until it reaches an optical density (OD) of 0.6. The cultures are induced with 10  $\mu$ g/mL of ATC and incubated at 37°C for 30 minutes to acclimate to the presence of the inducing agent. Half of the culture is moved to 16 °C to induce the appropriate hours, while the other half stays at 37 °C for 30 minutes to serve as a control. After the cultures are induced appropriately, the cells are fixed using formaldehyde. The cells are spun down at 9000 rpm for 2 minutes to form a pellet. The supernatant is discarded, and the pellet is resuspended in 997 microliters of 1x PBS. After adding three microliters of 37% formaldehyde, the cells are incubated, shaking gently for 12 minutes. 100 microliters of 2.5 M of Glycine are added and incubated for 5 minutes. The cells are then spun

down as before and resuspended in 1x PBS. 5 microliters of the fixed cells are added to the microscope slide for imaging.

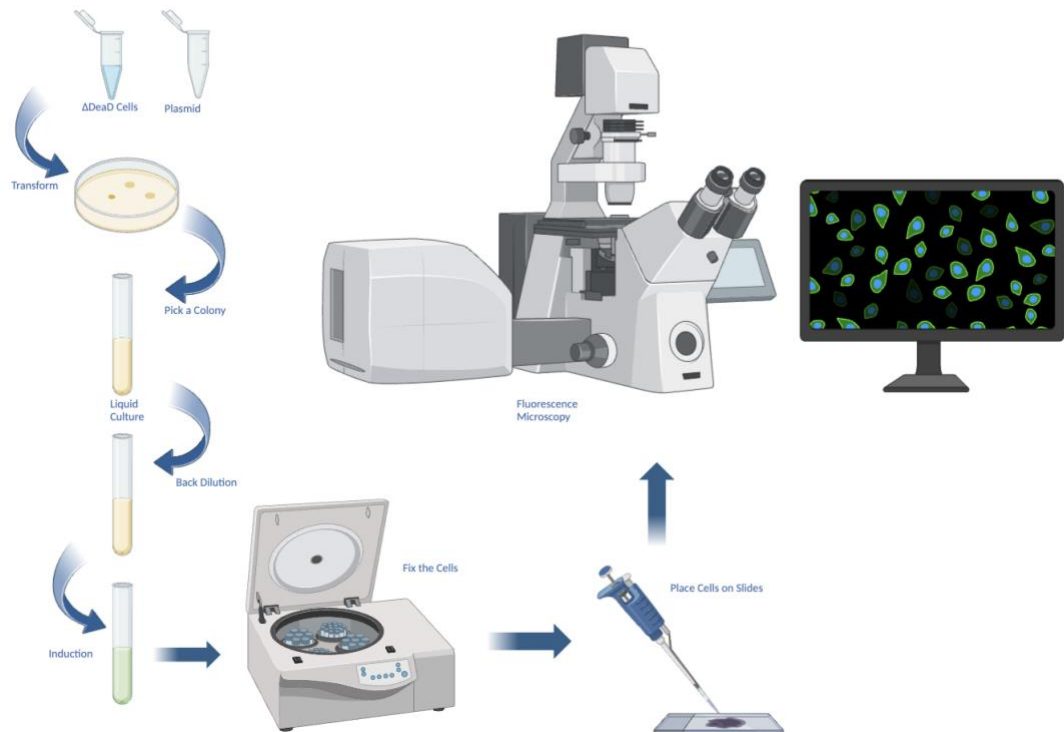


Figure 5: Figure outlining the experimental method of prepping samples from microscopy imaging.

## 2.2 Modifications for different experiments:

### 2.2.1 Wild Type Phenotype and $\Delta$ deaD Phenotype

The same protocol was followed for the pictures in Figure 2 without the low-temperature replicates. MG1655 and MG1655  $\Delta$ deaD were transformed with pAJM011 and pJB212.

### **2.2.2 DeaD Phenotype and DeaD 1-533 C-terminal Truncation Phenotype**

The same basic protocol was followed for the pictures in Figure 3. In this case, MG1655  $\Delta$ deaD cells were transformed with pAP14 and pJB212. The cold shock (16°C) incubation time was 1 hour.

### **2.2.3 DAPI Amendments**

After following the basic protocol, the cells were resuspended in a 1x PBS DAPI solution.

### **2.2.4 2-Hour DeaD Phenotype and DeaD 1-533 C-terminal Truncation Phenotype**

The same basic protocol was followed for the pictures in Figure 3. In this case, MG1655  $\Delta$ deaD cells were transformed with pAP14 and pJB212 plasmids. The cold shock (16°C) incubation time was 2 hours.

### **2.2.5 Confocal Amendments**

Different coverslips were used to accommodate the microscope. Otherwise, all other sample preparation was the same as for standard epifluorescence microscopy.

## Chapter 3

### RESULTS

#### 3.1 Spot Assays

##### 3.1.1 Wild type Vs. $\Delta$ deaD phenotype

To demonstrate the cold shock phenotype of *deaD*, the wild-type and  $\Delta$ deaD *E. coli* cells were grown at 37°C and 16°C. The wild-type strain has the chromosomal *deaD* gene and can grow at both temperatures. The  $\Delta$ deaD cells, which have the *deaD* open reading frame (ORF) deleted, can grow at 37 °C but not at 16 °C. This result is consistent with previous work that has shown this phenotype.

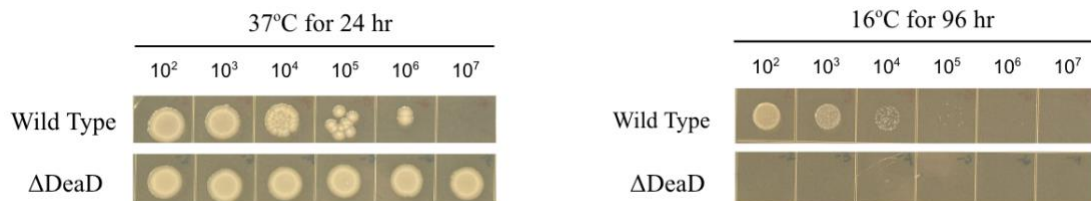


Figure 6: The *deaD* gene is necessary for growth at low temperature (Cold Shock). Spot assays were performed using serial dilutions of WT MG1655 and  $\Delta$ deaD strains, followed by incubation at 37°C for 16 hours or 16°C for 96 hours.

##### 3.1.2 The C-terminal Truncation vs. Full-length DeaD protein

To determine the effect of the C-terminal IDR on growth under cold shock, MG1655  $\Delta$ deaD cells were transformed with plasmids expressing full-length DeaD (pJB206) or a C-terminal truncation missing the IDR (DeaD 1-553; pAP15). The C-terminal IDR is an RNA-binding domain and likely regulates DeaD formation of RNA condensates. Because of this, we designed expression constructs that delete this region to see if they can rescue the cold growth phenotype. Expression of the plasmid constructs was induced by the addition of Anhydrous Tetracycline (ATC). As a positive control, all the cells were grown at the permissive temperature of 37°C. At 37°C, all of the cell types were able to grow despite the presence of ATC, the inducer of the plasmid. At 16°C with ATC, expression of full-length DeaD rescued growth in the  $\Delta$ deaD strain, while expression of the DeaD 1-553 truncation could not. This was compared to cells grown with either plasmid, but without the inducing agent, which could not grow at 16 °C.

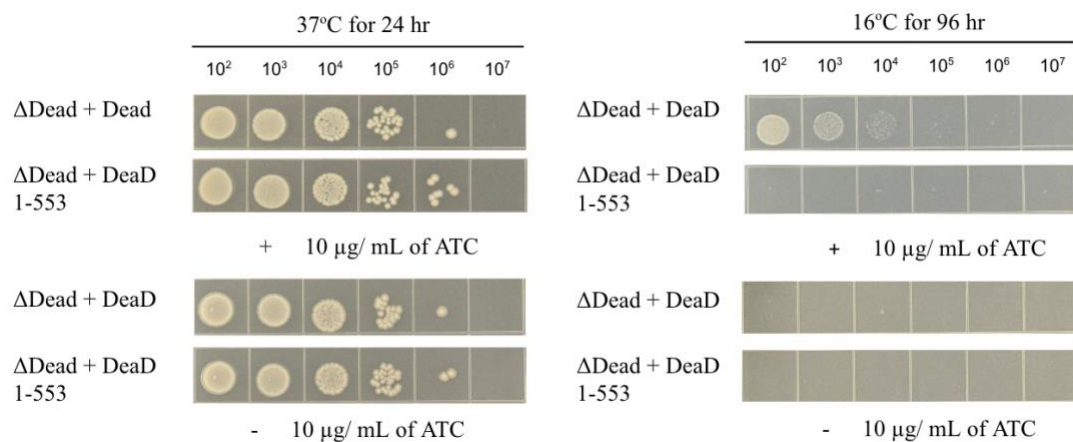


Figure 7: Plasmid expression of full length DeaD but not a truncation mutant without the C-tail IDR can rescue the  $\Delta$ deaD cold shock phenotype (a) Spot assays were performed at 37°C and 16°C with  $\Delta$ DeaD cells transformed separately with plasmids containing full-length DeaD and a truncated form of DeaD with the C-terminal end deleted. The plates at 37°C were incubated for 24 hours on plates with and without the ATC inducer (b). These plates were transformed using the aforementioned procedure at 16 °C for 96 hours.

The plates with no inducer show the same results as outlined in the previous section. All cells can grow at 37°C, independent of which plasmids they are transformed with. At 16 °C, only the wild type cells are able to grow, since none of the plasmids are induced without ATC present. With the standard concentration of ATC present (50 µg/mL), the phenotype is easily recovered by a copy of the deaD gene encoded by the plasmid; however, the results at 37°C are not consistent with normal growth. Neither of the samples transformed with plasmids encoding the full-length deaD were able to grow normally at 37°C. It is most likely that the level of DeaD or DeaD fusion protein present in the cells incubated with the standard concentration of ATC was toxic to cell growth due to overexpression. When I plated cells on plates containing ¼ of the standard concentration of ATC (12.5 µg/mL), we observed normal growth at 37°C and phenotypic recovery from all cells containing a functioning full-length DeaD protein. No growth is seen with the C-terminal truncation under any conditions; therefore, using 12.5 µg/mL ATC has no impact on the C-terminal growth either. As a result, this concentration was used for all spot assays and microscopy.

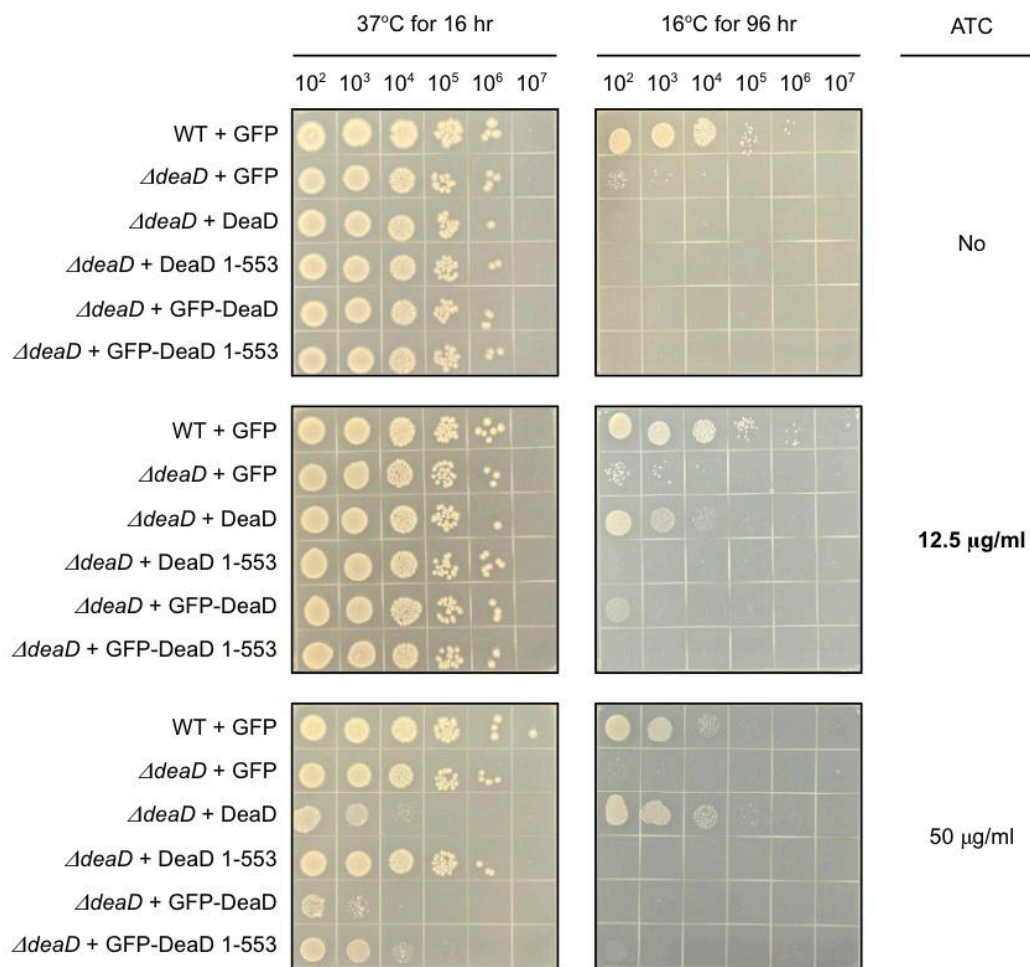


Figure 8: A spot assay showing all different cell types transformed with all of the experimental and control plasmids. The assay was done with varying concentrations of ATC (inducer) for the linearly inducible plasmids. The plates were then incubated at 16°C and 37°C for 96 and 24 hours, respectively.

## 3.2 Microscopy

### 3.2.1 Wild Type Vs. $\Delta$ DeaD Localization

One concern with using a plasmid to express a fluorescently tagged protein is that the cell now has two copies of the gene. The strain with two copies of the gene could affect the expression level, function, and localization of the protein in cells. To address this, we transformed our fusion protein expression plasmids into the *deaD* deletion strain,  $\Delta$ *deaD*, so that the only copy of *deaD* was expressed on the plasmid. Second, our expression plasmids are linearly inducible so that we can ensure low levels of expression compared to the overexpression common in most *E. coli* expression plasmids.

Wild type and  $\Delta$ *deaD* *E. coli* cells were transformed with the plasmid containing a *deaD* GFP fusion expression cassette to test if the localization remained similar. They were compared to cells containing a GFP expression construct. Both were then imaged using epifluorescence microscopy using a Zeiss Axio Observer microscope. The localization in both strains showed puncta formation concentrated at the poles and the middle of the cell. While the  $\Delta$ *deaD* cells had similar localizations in their puncta to the wild type, the  $\Delta$ *deaD* cells had more distinct puncta overall. One possible explanation for this is the existence of non-fluorescently labeled *deaD* in the wild type cells due to the two copies of the gene. To ensure that the particular fluorescent protein was not interfering with localization, the experiment was replicated using a different fluorescent protein, mCherry. Since the localization pattern is

consistent with the GFP data, GFP was the fluorescent tag for the rest of the experiments.

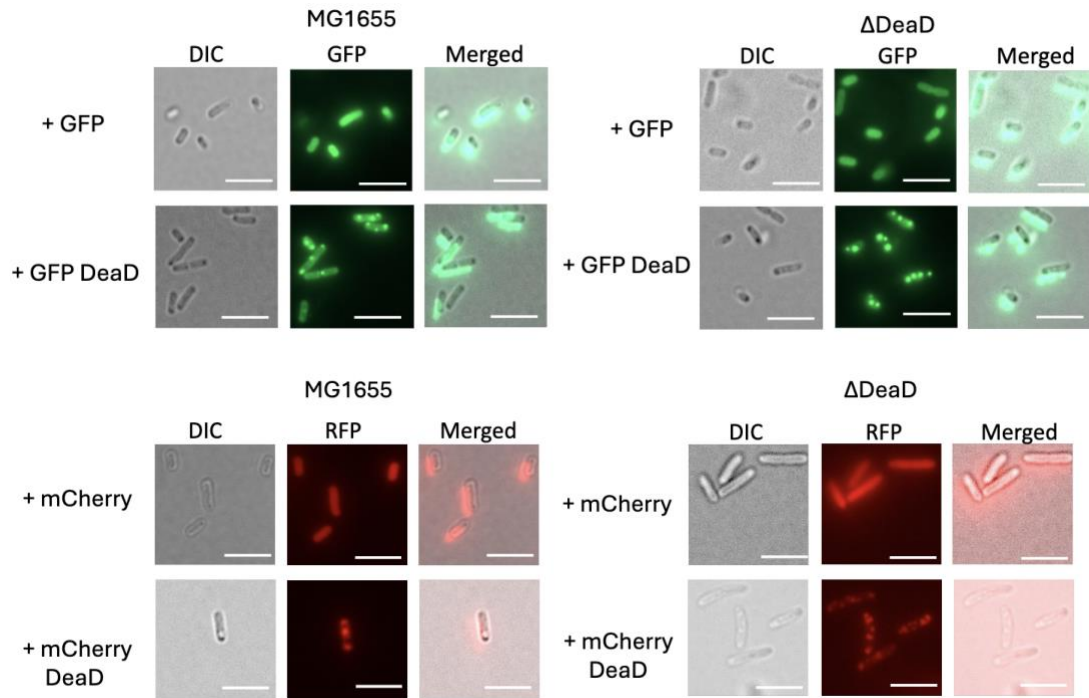


Figure 9: DeaD localizes to distinct puncta in *E. coli*. GFP DeaD fusion microscopy was performed on fixed  $\Delta$ deaD and MG1655 cells incubated at 37°C. Both strains were also transformed with a plasmid encoding only GFP as a control. This experiment was replicated with mCherry-tagged DeaD.

### 3.2.2 DeaD Localization is Dependent on Temperature and Presence of C-terminal Region.

Similar to the spot assays performed earlier, localization of the C-terminal DeaD truncation was compared to the full-length deaD protein. Cells were also imaged after being incubated at 37 °C and 16 °C to determine any differences in localization due to cold shock. The  $\Delta$ deaD cells containing the full-length deaD fusion plasmid could form distinct puncta at 37° C, and some could form puncta at 16°C. At the same time,

the  $\Delta$ deaD cells with the truncated deaD protein could not form distinct puncta at 37°C or 16°C. At 37°C, they appear to have the beginning of localization, as there is a more concentrated signal instead of the pure, even diffuse signal observed, like the signal from GFP alone.

DeaD 1-553 is localized to the cytoplasm but is excluded from the central region of the cytoplasm that is most likely occupied by the chromosome. While we did not stain DNA in this experiment, we used DAPI to stain DNA in the confocal microscopy afterwards. We saw that DeaD 1-553 localized to the cytoplasm but was excluded from the DAPI-stained region, indicating that it was not present in the space occupied by the chromosomes.

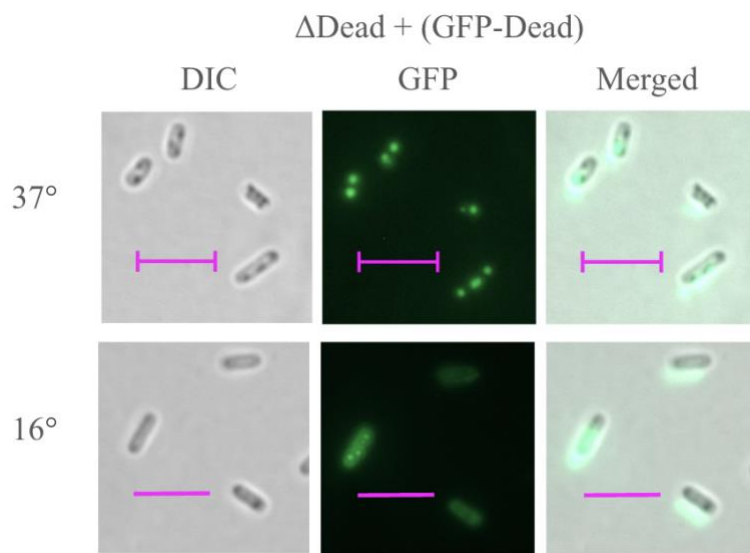


Figure 10:  $\Delta$ DeaD cells transformed with a plasmid with a GFP-DeaD fusion expression cassette. The cells were incubated at 37°C and 16°C for 1 hour.

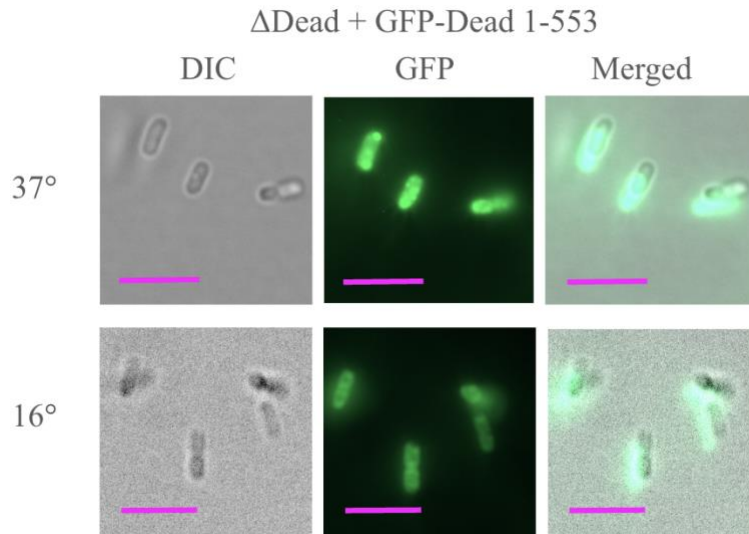


Figure 11:  $\Delta$ DeaD cells transformed with a plasmid with a GFP-DeaD 1-553 fusion expression cassette. The cells were incubated at 37°C and 16°C for 1 hour.

### 3.2.3 Temperature Experiment Repeated at 2 Hours with DAPI

Since the temperature-related difference in protein localization might be time-sensitive,  $\Delta$ deaD with the plasmid expressing full-length protein and  $\Delta$ deaD with the plasmid expressing the truncated form of the protein were imaged again after a longer induction time of two hours. I also used DAPI to stain the chromosome so it could be used for reference. After two hours of incubation, the  $\Delta$ deaD cells with the full protein have many distinct puncta in the center and the poles. The  $\Delta$ deaD cells with the tagged c-terminal truncation have fewer puncta.

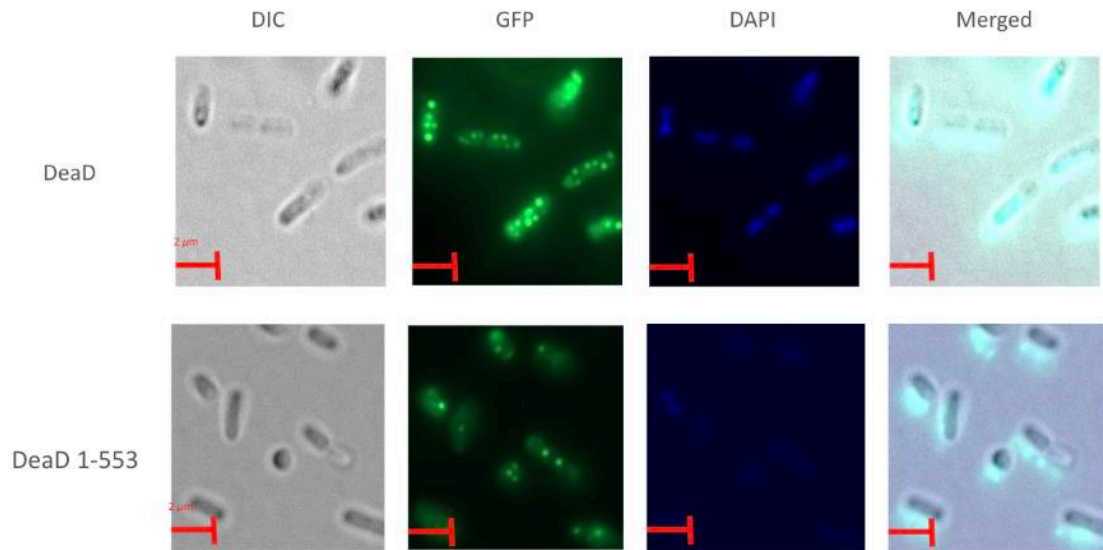


Figure 12: Deletion of the DeaD C-terminal IDR reduces the number of DeaD puncta at low temperature.  $\Delta$ deaD cells transformed with plasmids encoding GFP-DeaD or GFP-DeaD 1-553. The cells were fixed after 2 hours of protein induction. GFP fluorescence is shown in green, and DAPI staining is used to show DNA localization in blue.

## Chapter 4

### DISCUSSION AND CONCLUSIONS

#### 4.1.1 Cold Shock Growth Phenotype:

Previous literature has outlined DeaD's necessity during cold shock growth in *E. coli*, which we have replicated<sup>5</sup>. Because the DeaD 1-553 truncation construct, which does not contain the C-terminal IDR, cannot rescue the cold shock phenotype like the wild type, it implies that the IDR is necessary for DeaD's function during cold shock. Wild type MG1655 was able to grow at both temperatures. This phenotype was also rescued in  $\Delta$ deaD cells expressing DeaD from a plasmid.  $\Delta$ deaD cells with no copy of the deaD gene could not grow at 16°C. The results for these three cell types acted as controls for the truncated deaD mutant. In addition to replicating the existing phenotype, the spot assay results implicated the importance of the C-terminal IDR in rescuing growth at cold temperatures. The  $\Delta$ deaD cells with the truncated protein could not grow at a cold shock temperature. Their phenotype directly mirrors that of the  $\Delta$ deaD cells. These phenotypes display the same pattern with fluorescent tag proteins present. The changes in DeaD localization and condensate formation indicate that DeaD needs to be present in specific loci inside the cell for it to allow growth at low temperatures.

#### 4.2 Condensate Formation:

Distinct localization patterns seen in imaging with fluorescent-tagged DeaD can give insight into its ability to form RNA condensates and what function these condensates might serve during bacterial growth. Previous work has shown that DDXs

(DEAD-box ATPases) in *E. coli* and yeast, which contain IDRs show distinct puncta when tagged with fluorescent proteins. These other DDXs have also been used in *in vitro* condensate assays to confirm their ability to form condensates<sup>10</sup>. Using epifluorescence microscopy can approximate this ability to form condensates for DeaD and its truncated form, which lacks a complete RNA-binding domain. As stated before, both the C-terminal IDR and DbpA domains are implicated in RNA binding<sup>9</sup>; this activity is crucial to the formation of the RNA condensates. Cells with the full-length DeaD protein were able to localize and form puncta under all conditions. The cells with the truncation could only form puncta after two hours of incubation at 16°C. As a result, we conclude that the C-terminal IDR is necessary for condensate formation. The fewer puncta indicate that deleting the C-terminal IDR most likely reduces DeaD's ability to form condensates. However, we need the *in vitro* data to demonstrate this definitively. Also, while the truncated form of the protein lacks the C-terminal IDR, it still contains the DbpA domain. Retaining this domain could explain the existence of puncta with the truncation, although it is substantially hindered when compared to the full-length. Future experiments with a C-terminal IDR and DbpA domain deletion would hopefully prevent puncta formation.

#### **4.3 Ribosome Biogenesis:**

One of DeaD's primary functions is its involvement in ribosome biogenesis. Ribosomes are comprised of protein and RNA. Organizing these macromolecules into a functional protein requires the intervention of many helper proteins. For example,

DeaD is crucial in assembling the large subunit of 50S ribosomes. Deleting the *deaD* gene from cells has been shown to result in a deficit in 50S ribosomes<sup>5</sup>. The current literature shows that despite *E. coli* containing seven ribosomal operons, ribosome transcription and biogenesis occurs at one specific loci in the cell associated with the chromosomes. It also shows more puncta in doubling cells<sup>11</sup>. The DeaD localization pattern in  $\Delta$ *deaD* cells show a similar pattern to the ribosome biogenesis labeling experiments in the doubling cells in previous literature. The truncation on the other hand does not show the same localization pattern. Using this information DeaD's function in ribosome biogenesis during cold shock is hindered by the deletion of the C-terminal tail. Colocalization studies with tagged rRNA and tagged DeaD with and without the C-terminal domain could provide more concrete evidence of this.

#### **4.4 Conclusions**

RNA Condensate formation has previously been shown to be important to RNA function and localization in eukaryotes<sup>7</sup>. My data shows that RNA condensates also have a significant role in the proper functioning of *E. coli* under stress conditions such as cold shock. Based on my results, this role in cold shock growth could be looked at in other model organisms to see if the function is conserved. Additionally, these results underline the significance of IDRs in protein function since, most of the time, the protein's wound-up structure is viewed as more significant to its function than the IDR.

## 4.5 Significance

Deadbox RNA helicases are prevalent across all arms of the tree of life. Many of these enzymes are responsible for crucial functions in the human body. Deadbox helicases have been found to participate in all aspects of RNA metabolism—for example, transcription, translation, and degradation<sup>12</sup>. In recent studies, the activity of deadbox helicases have been shown to play a role in the development of gastrointestinal cancers<sup>13</sup>. As a result, insight into the cellular functions of these deadbox helicases could have clinical impacts for cancer treatment. The DeaD protein in *E. coli* shares similar characteristics with some deadbox helicases in humans. For example, DDX21, a human deadbox helicase, has a similar IDR domain on its N-terminal end. The N-terminal IDR on DDX21 is also implicated in forming phase-separated condensates and has been demonstrated to influence migration in colorectal cancer cells<sup>13</sup>. Understanding the impact of DeaD's C-terminal IDR in *E. coli* will give us insight into the general function of these IDR-containing dead box RNA helicases and allow us to better understand how these functions can influence human disease and health.

## 4.6 Future Directions

### 4.6.1 In Vitro Condensate Assay

While, the *in vivo* microscopy data has shown strong evidence of disrupted condensate forming ability in the absence of the C-terminal IDR, this phenotype will need to be corroborated by *in vitro* condensate assays. These assays are already being performed by our collaborators which we should have the data for soon.

### 4.6.2 Confocal Microscopy

To quantify the puncta more definitively to display the *in vivo* data and *in vitro* data simultaneously we would like to replicate the data with better resolution. Using the Airyscan confocal microscopy the puncta can be clearly distinguished from each other and quantify for statistical significance. This project is also already in progress.

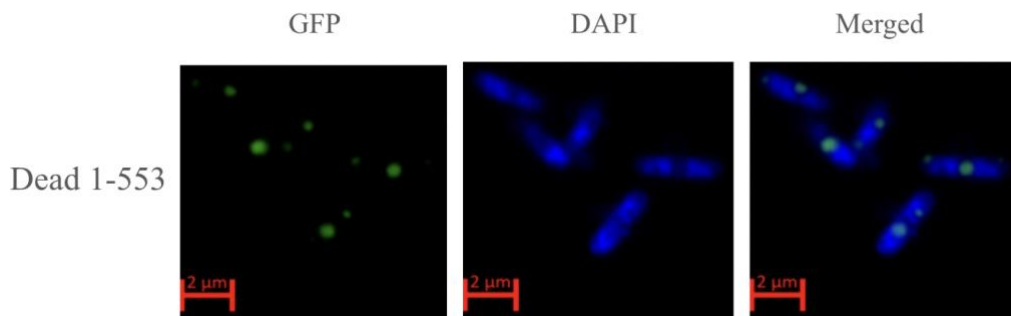


Figure 13: Confocal airyscan imaging of  $\Delta$ deaD cells transformed with a plasmid expressing the truncated form of deaD with a GFP tag at 16°C at 2 hours.

## REFERENCES

- (1) Furuichi, Y.; Muthukrishnan, S.; Tomasz, J.; Shatkin, A. J. Mechanism of Formation of Reovirus mRNA 5'-Terminal Blocked and Methylated Sequence, m7GpppGmpC. *J. Biol. Chem.* **1976**, *251* (16), 5043–5053. [https://doi.org/10.1016/S0021-9258\(17\)33218-0](https://doi.org/10.1016/S0021-9258(17)33218-0).
- (2) Bird, J. G.; Zhang, Y.; Tian, Y.; Panova, N.; Barvík, I.; Greene, L.; Liu, M.; Buckley, B.; Krásný, L.; Lee, J. K.; Kaplan, C. D.; Ebright, R. H.; Nickels, B. E. The Mechanism of RNA 5' Capping with NAD<sup>+</sup>, NADH and Desphospho-CoA. *Nature* **2016**, *535* (7612), 444–447. <https://doi.org/10.1038/nature18622>.
- (3) Escherich, Th. The Intestinal Bacteria of the Neonate and Breast-Fed Infant. *Rev. Infect. Dis.* **1988**, *10* (6), 1220–1225. <https://doi.org/10.1093/clinids/10.6.1220>.
- (4) *Cold shock induces a major ribosomal-associated protein that unwinds double-stranded RNA in Escherichia coli.* <https://doi.org/10.1073/pnas.93.1.76>.
- (5) Charollais, J.; Dreyfus, M.; Iost, I. CsdA, a Cold-shock RNA Helicase from Escherichia Coli, Is Involved in the Biogenesis of 50S Ribosomal Subunit. *Nucleic Acids Res.* **2004**, *32* (9), 2751–2759. <https://doi.org/10.1093/nar/gkh603>.
- (6) Prud'homme-Généreux, A.; Beran, R. K.; Iost, I.; Ramey, C. S.; Mackie, G. A.; Simons, R. W. Physical and Functional Interactions among RNase E, Polynucleotide Phosphorylase and the Cold-Shock Protein, CsdA: Evidence for a 'Cold Shock Degradosome.' *Mol. Microbiol.* **2004**, *54* (5), 1409–1421. <https://doi.org/10.1111/j.1365-2958.2004.04360.x>.
- (7) Zhang, Y.; Burkhardt, D. H.; Rouskin, S.; Li, G.-W.; Weissman, J. S.; Gross, C. A. A Stress Response That Monitors and Regulates mRNA Structure Is Central to Cold Shock Adaptation. *Mol. Cell* **2018**, *70* (2), 274-286.e7. <https://doi.org/10.1016/j.molcel.2018.02.035>.
- (8) Musselman, C. A.; Kutateladze, T. G. Characterization of Functional Disordered Regions within Chromatin-Associated Proteins. *iScience* **2021**, *24* (2), 102070. <https://doi.org/10.1016/j.isci.2021.102070>.
- (9) Tsu, C. A.; Kossen, K.; Uhlenbeck, O. C. The Escherichia Coli DEAD Protein DbpA Recognizes a Small RNA Hairpin in 23S rRNA. *RNA* **2001**, *7* (5), 702–709. <https://doi.org/10.1017/S1355838201010135>.
- (10) Hondele, M.; Sachdev, R.; Heinrich, S.; Wang, J.; Vallotton, P.; Fontoura, B. M. A.; Weis, K. DEAD-Box ATPases Are Global Regulators of Phase-Separated Organelles. *Nature* **2019**, *573* (7772), 144–148. <https://doi.org/10.1038/s41586-019-1502-y>.
- (11) Gaal, T.; Bratton, B. P.; Sanchez-Vazquez, P.; Sliwicky, A.; Sliwicky, K.; Vogel, A.; Pannu, R.; Gourse, R. L. Colocalization of Distant Chromosomal Loci in Space in E. Coli: A Bacterial Nucleolus. *Genes Dev.* **2016**, *30* (20), 2272–2285. <https://doi.org/10.1101/gad.290312.116>.
- (12) Ma, X.; Lu, T.; Yang, Y.; Qin, D.; Tang, Z.; Cui, Y.; Wang, R. DEAD-Box Helicase Family Proteins: Emerging Targets in Digestive System Cancers and

Advances in Targeted Drug Development. *J. Transl. Med.* **2024**, *22* (1), 1120.  
<https://doi.org/10.1186/s12967-024-05930-0>.

- (13) Gao, H.; Wei, H.; Yang, Y.; Li, H.; Liang, J.; Ye, J.; Zhang, F.; Wang, L.; Shi, H.; Wang, J.; Han, A. Phase Separation of DDX21 Promotes Colorectal Cancer Metastasis via MCM5-Dependent EMT Pathway. *Oncogene* **2023**, *42* (21), 1704–1715. <https://doi.org/10.1038/s41388-023-02687-6>.