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# Investigating the Helicase Activity of Methylated vs Unmethylated Ded1

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# of Methylated vs Unmethylated Ded1

by

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**Abstract**

Ded1 is an RNA helicase protein of the DEAD-box subfamily in eukaryotic organisms (Sharma & Jankowsky, 2014) which can act as an activator or initiation factor, during translation (Hilliker et al., 2011). Ded1 has several functions in yeast including assembly of translational initiation factors, scanning the mRNA for the start codon, and unwinding any double stranded segments of mRNA with its helicase ability. Ded1 was discovered to be methylated at four arginine sites in vivo (Low et al., 2013), with a fifth methylation site being discovered recently (Low et al., 2020), however the purpose of such post-translational modifications is still unknown. Our goal is to determine whether the methylation of Ded1 has an effect on the resulting helicase ability of the protein, by comparing the helicase activity of unmethylated to methylated WT Ded1 using an *in vitro* helicase assay. So far, troubleshooting has identified favorable conditions for RNA duplex annealing, and the current work is optimizing the subsequent unwinding kinetics of the helicase assay. This investigation of the helicase ability of Ded1 will provide us with more knowledge of the downstream effects of Ded1 methylation.

**Introduction**

Cellular translation refers to the process where transcribed messenger RNA (mRNA) is decoded by ribosomes and transfer RNA (tRNA) molecules to yield an amino acid chain, which can then fold to form a protein. Translation is a crucial process in both eukaryotic and prokaryotic cells, and translational control plays an essential role in the regulation of gene expression (Hersey et al., 2012). Translational regulation refers to the control mechanisms by which cells localize, use, conserve, and/or degrade RNA prior to protein synthesis. This type of cellular regulation is especially important in responding to environmental changes, maintaining homeostasis, and, controlling cell growth, proliferation, and development. Many diseases or

illnesses are the result of irregular translation, such as neurodegenerative disorders (Kapur & Ackerman, 2018) or cancers (Robichaud et al., 2019). Understanding translation regulation is important for understanding the most energy intensive processes in the cell, which may possibly inform us of the abnormal translational mechanisms which lead to a particular disease or propose drug candidates for countering such.

Ded1 is an ATP-dependent RNA helicase found in yeast, which is an essential translational factor for survival. Ded1 has a highly conserved human homolog, DDX3, and both proteins are of the DEAD-box subfamily of helicases in eukaryotic organisms (Sharma & Jankowsky, 2014), which have important cellular roles in translational regulation (Cordin et al., 2006). The mutation or degradation of such proteins have been linked to many diseases, including cancers. DEAD-box proteins fall under helicase subfamily 2 (SF2), meaning they contain two recombinant protein A (RecA)-like domains, which act as nucleotide triphosphate binding sites, and a Walker B motif (motif II) sequence consisting of D-E-A-D in single amino-acid nomenclature, which is part of the active site of these DEAD-box proteins (Linder & Jankowsky, 2011).

In yeast, Ded1 has multiple functions related to translation. It serves as a translation initiation factor, in conjunction with eIF4E, eIF4G, and eIF4A proteins, to correctly assemble the ribosomal subunits onto mRNA (Gupta & Hinnebusch, 2018). Then Ded1, with the other translation initiation factors and the small ribosomal subunit, scans the highly complex 5’ UTR regions of mRNA to move the small subunit of the ribosome to the AUG start codon (Sen et al., 2015). During this scanning, Ded1 utilizes its helicase ability to unwind any double stranded segments of mRNA. Ded1 scans and separates RNA strands, by opening the helix locally, through hydrolysis of ATP. This local helix opening can occur anywhere in the duplex, which

then destabilizes the complex and accelerates further strand separation (Raj et al., 2019). Unlike previously characterized DNA and viral RNA helicases, Ded1, and all proteins in the DEAD-box helicase family, do not undergo translocation; instead they separate only the nucleotides closest to the duplex-helicase interaction and then dissociate to bind again (Yang et al., 2007). At this site of interaction, in the presence of bound RNA and ATP, the two RecA-like domains form a "closed" conformation with high affinity for RNA (Linder & Jankowsky, 2011). The RecA-like domain 1 is involved in steric hinderance to disrupt the helix and unstack the terminal bases, while the RecA-like domain 2 interact with RNA through an A-form helix. Together, the two domains coordinate to unwind RNA duplexes, however the exact mechanism and order of which these interactions occur, is still unknown. After hydrolysis, when ADP is now present, the RecA-like domains adopt a "open" conformation with low affinity for RNA, leading the complex to disassociate from the duplex (Linder & Jankowsky, 2011). This complex process shows that Ded1's helicase activity requires specific coordination between RNA binding, ATP binding, ATP hydrolysis, and duplex disruption. If something were to alter any of those steps, the overall helicase activity of Ded1 could be affected.

Initiation, during which the needed machinery assembles correctly on an mRNA strand, is the rate-limiting step of translation. This means that even a small change in the rate of initiation will have an amplified effect on overall protein synthesis in the cell, and because of this cascade, translation initiation is a heavily targeted process for cellular regulation. One form of control is posttranslational modification (PTM) of proteins. This generally refers to the covalent attachment of function groups to an amino acid side chain, or the larger C- or N- termini of a protein. PTM is a widespread regulation mechanism utilized for many eukaryotic proteins (Xu et al., 2018), and since these PTMs can critically influence numerous biological processes,

abnormal PTMs are associated with many diseases, such as cancers (Jin & Zangar, 2009), diabetes (Morino, et al., 2005), and neurodegenerative diseases (Gong, et al., 2005). Therefore, understanding PTMs, especially those associated with translation initiation factors, is essential for analyzing their cellular function, and how they impact critical mechanisms of biological processes (Yang & Park, 2017).

Ded1 is heavily involved in yeast translation initiation, and has, importantly, known to be the target of several PTMs, including methylation at five arginine sites (Low et al., 2013 & 2020). Three of these methylations occur in the N-terminus, and the other two in the C-terminus. Arginine methylation is a common post-translational modification that is used to regulate signal pathways in cells, and has been observed to occur with many proteins whose function involves the translation or storage of mRNA (Khoury et al., 2011). Commonly, methylation changes the shape and thus the function or stability of a protein which can, in some cases, increase or decrease the rate of enzymatic activity. Methylation of an arginine will not affect the overall charge of the residue; however, it adds bulk and hydrophobicity to the side chain that is likely to either promote or inhibit molecular interactions (Lee et al., 2005). Arginine methylation of translation initiation factors has been discovered to either sterically hinder the association between the protein and RNA (Blackwell & Ceman, 2012), or strengthen this interaction due to an increase in hydrophobicity which can facilitate stacking with the RNA bases (Bedford & Richard, 2005). However, the reason for this arginine methylation in Ded1, specifically, is unknown, as there is no clear correlation between productivity of Ded1 and the methylation occurring. Since Ded1 has numerous functions in yeast, any one of them could be affected by this methylation, which could prove to be a key control mechanism for yeast translation.

Our goal is to determine whether this arginine methylation impacts Ded1's helicase ability, specifically, by comparing the activity of recombinant methylated versus unmethylated wild-type (WT) Ded1 using a fluorophore-quencher helicase assay. In this work we discover the optimal conditions for fluorophore-quencher RNA duplex association, which will be used in the future to investigated the unwinding kinetics of Ded1.

## Materials and Methods

### *Annealing the RNAs*

The RNA duplexes were annealed via either a three oligo or two oligo protocol. The three oligo procedure involved the annealing of the 500 nM fluorophore (Cy3) RNA oligo (rAKH002; Table 1) and the 500 nM quencher (BHQ2) RNA oligo (rAKH003; Table 1) to the 500 nM loading RNA oligo (rAKH004; Table 1). While the two oligo procedure entailed the annealing of the 500 nM fluorophore (Cy3) RNA oligo (rAKH002; Table 1) to the 500 nM quencher (BHQ2) RNA oligo (rAKH009; Table 1). Both RNA annealing protocols are based on the eIF4A procedure developed by Özes et al., 2014. Either annealing occurred in Reaction Buffer (40 mM Tris-HCl pH 8.0, 50 mM NaCl, 0.1% NP-40, 20 mM DTT; based on Yang & Jankowsky, 2005) with 2.0 mM additional MgOAc added, and the mixture was heated to either 80˚C or 92˚C for 20 minutes, gradually cooled to room temperature (RT) via one of three methods, and placed in an ice bath for 10 minutes, with the goal of having at least 80% RNA duplex annealing (Equation 1.1). The three cooling methods were (1) in a PCR machine which decreased in temperature 0.5˚C every minute, resulting in ~ 2 hours of cooling, (2) the heat block being removed from the machine and placed on a benchtop, while covered by foil to prevent bleaching, resulting in ~ 1 hour of cooling, and (3) the heat block being removed from the machine and placed in an ice bath, while covered in foil to prevent bleaching, resulting in ~ 20 minutes of cooling.

$$\% \text{ annealing} = \frac{(Cy3-BHQ)-(Experimental-BHQ)}{(Cy3-BHQ)} \times 100 \qquad (1.1)$$

**Table 1**: Oligonucleotides used in fluorophore-quencher assay.

| Oligo Name | Nucleotide Sequence (5' to 3') | Description |
|---|---|---|
| rAKH002 | A GCA CCG UAA AGA CGC Cy3 | Fluorophore (Cy3 tag) labeled RNA |
| rAKH003 | BHQ2 GGC CCC ACC GGC CCC UCC G | Quencher (BHQ2 tag) labeled RNA (three oligo version) |
| rAKH004 | C GGA GGG GCC GGU GGG GCC U GCG UCU UUA CGG UGC UUA AAA CAA AAC AAA ACA AAA CAA AA | Loading strand for the three oligo procedure |
| rAKH009 | BHQ2 GCG UCU UUA CGG UGC UUA AAA CAA AAC AAA ACA AAA CAA AA | Quencher (BHQ2 tag) labeled RNA (two oligo version) |
| oAKH838 | GCG TCT TTA CGG TGC T | DNA competitor strand |

***Helicase reaction and tracking fluorescence***

To monitor the efficiency of annealing, mock helicase reactions were made with 107 nM RNA duplex, either Cy3, BHQ2, or experimental, in Reaction Buffer (40 mM Tris-HCl pH 8.0, 50 mM NaCl, 0.1% NP-40, 20 mM DTT; based on Yang & Jankowsky, 2005) with 2.0 mM additional MgOAc added, and Ded1 Storage Buffer (version 8, Hilliker at al., 2011). The fluorescence of which was recorded at an excitation wavelength of 540 nm using a Jasco Spectrofluorometer in a black wall, quartz cuvette, to ensure (a) the Cy3 control showed little bleaching and detectable fluorescence, (b) the BHQ2 control did not exhibit fluorescence contamination and (c) the percent annealing for the experimental condition exceeded 80%, calculated via equation 1.1.

For unwinding kinetics, complementary DNA oligo (oAKH838; Table 1) was added to prevent the rebinding of the fluorophore oligos to the RNA duplex after helicase activity (Figure 1), by instead forming a blunt-ended DNA-RNA duplex that Ded1 *cannot* bind, and the mixtures were kept on ice for an additional 10 minutes. Then either 10 µM of methylated or unmethylated WT Ded1 was mixed with 50 nM RNA duplex in Reaction Buffer (40 mM Tris-HCl pH 8.0, 50 mM NaCl, 0.1% NP-40, 20 mM DTT; based on Yang & Jankowsky, 2005) with 2.0 mM additional MgOAc added. 4 mM buffered ATP was added to begin the unwinding and the change in fluorescence, at excitation wavelength 540 nm and emission wavelength 560 nm using a Jasco Spectrofluorometer in a black wall, quartz cuvette, was recorded over 5 minutes after 100 second of equilibration at RT. This change was proportional to the number of RNA strands which had been unwound over that same period of time. Utilizing enzyme kinetics, the efficiency of methylated vs unmethylated WT Ded1 helicase activity could be compared based on their duplex unwinding rates.

**Results**

The exact purpose of Ded1's post-translational methylation at five arginine sites is unknown, however we hypothesize that this methylation likely helps the enzyme with one or many of its functions regarding translation and the regulation of such in yeast. Since methylation is known to alter RNA binding affinity for other RNA binding proteins, we wanted to test whether methylation affected Ded1 its helicase activity. To determine whether the methylation effects the helicase activity of WT Ded1, a fluorophore assay was utilized. Fluorophore assays are a quantification technique utilizing fluorescence, where oligos containing a fluorophore tag bind closely with those with a quencher tag. When the fluorophore and quencher are bonded in close proximity, within the space of a few nucleotides, no fluorescence is observed, but when the

fluorophore is free floating in solution, it emits fluorescence which can be measured with a fluorometer.

We initially utilized a three oligo protocol which is most common for these types of fluorescent assays. This involved the annealing of a Cy3 fluorophore RNA oligo (rAKH002), which has an excitation wavelength of 550 nm, and a BHQ2 quencher RNA oligo (rAKH003), which can suppress fluorescence when in close proximity to a fluorophore, to a loading RNA oligo (rAKH004) (Figure 1b). The RNA sequences of the Cy3 oligo (rAKH002) and loading strand (rAKH004) was adapted from Özes et al., 2014 to match the model RNAs used in Gao et al., 2016, while maintaining the 3' overhang that is preferred by Ded1. We tested three different combinations of RNA oligos: "Cy3", "BHQ2-3", and "Experimental-3" (Table 2). The Cy3 condition contains only the fluorophore RNA oligo and showed maximum fluorescence. The BHQ2-3 group contains the quencher RNA oligo and the loading strand to show background fluorescence. The Experimental-3 group contains both the Cy3, BHQ2, and loading oligos, which will act as the substrate when moving on to enzyme kinetics. An annealing percent exceeding 80% is needed to accurately monitor the unwinding kinetics of Ded1.

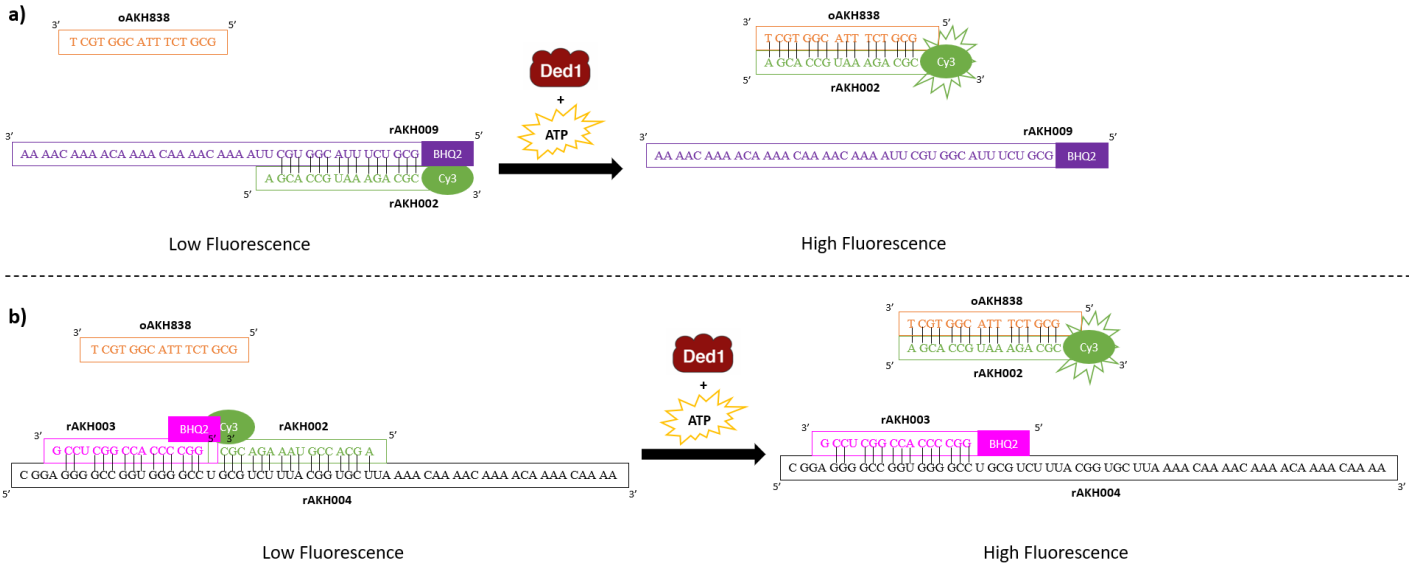


**Figure 1**. Two possible duplex structures for the fluorescence helicase assay utilizing either (a) two or (b) three RNA oligos. Cy3 is a fluorophore which has an excitation wavelength of 550 nm and emission wavelength of 570 nm. BHQ2 is a quencher which will suppress the fluorescence of Cy3 when in close proximity. A loading strand is a longer RNA oligo to which other oligos can bind in a specific orientation. DNA competitor strands are not involved in the RNA duplex annealing, but will bind to any free floating fluorophore oligos, preventing reannealing following the unwinding. Based on Figure 1 of Ösez et al., 2014.

**Table 2**: Fluorescence assay oligo combinations.

| Reaction Type | RNA oligos included | Purpose |
|---|---|---|
| Cy3 | Cy3 (rAKH002) | Will show max fluorescence |
| BHQ2 - 3 | BHQ2 (rAKH003), Loading (rAKH004) | Will show background fluorescence for the three oligo procedure |
| BHQ2 - 2 | BHQ2 (rAKH009) | Will show background fluorescence for the two oligo procedure |
| Experimental - 3 | Cy3 (rAKH002), BHQ2 (rAKH003), Loading (rAKH004) | Duplex which will be used for the unwinding kinetics in the three oligo procedure |
| Experimental - 2 | Cy3 (rAKH002), BHQ2 (rAKH009) | Duplex which will be used for the unwinding kinetics in the two oligo procedure |

The RNA oligos had to be heated to a high temperature before they annealed to one another, because mRNA forms intramolecular base pairing. By heating the oligos, these intramolecular bonds were broken allowing for other oligos to access the sequence. We tested both a high temperature of 80ºC and 92ºC (Figure 2), which resulted in annealing percentages of 6.02% and 4.90%, respectively. However, at either temperature, the annealing percentage did not exceed 80%.

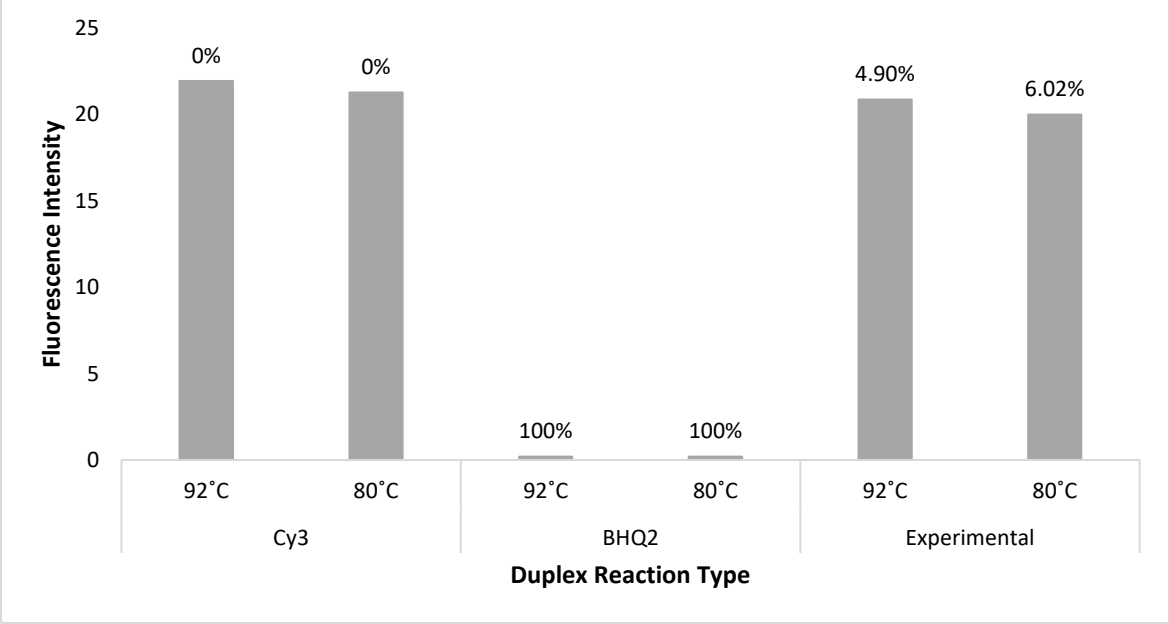


**Figure 2**. Fluorescence intensity at different annealing temperatures when annealing three, 107 nM RNA oligos. The temperatures (in ˚C) used to unwind intramolecular structure are listed under the x-axis. Cy3 was the control showing maximum fluorescence and contained only the Cy3 oligo (rAKH002). BHQ2 showed background fluorescence and contained both the BHQ2 oligo (rAKH003) and the loading strand (rAKH004). Experimental was the duplex reaction containing the Cy3 oligo (rAKH002), BHQ2 oligo (rAKH003), and the loading strand (rAKH004) (Table 2). The fluorescence intensity of these solutions was determined at an excitation wavelength of 540 nm using a Jasco Spectrofluorometer. Percent annealing is displayed above each bar and was calculated via equation 1.1.

Length of cooling can greatly affect oligo annealing, because a too rapid cooling often results in incorrect base pairing, while a too slow cooling can increase the presence of intramolecular RNA folding. Three lengths of cooling were tested: extended ~ 2 hours, intermediate ~ 1 hour,

and quick ~ 20 minutes. The optimal length of cooling for the RNA duplex mixture from 80ºC to RT during the annealing step was determined to be intermediate ~ 1 hour. A higher annealing percentage was observed with an intermediate length of cooling (17.60%), than for an extended cooling of about 2 hours (4.53%) or a quick cooling of about 20 minutes (7.50%) (Figure 3). However, even 17.60% annealing at the intermediate length did not exceed 80%.

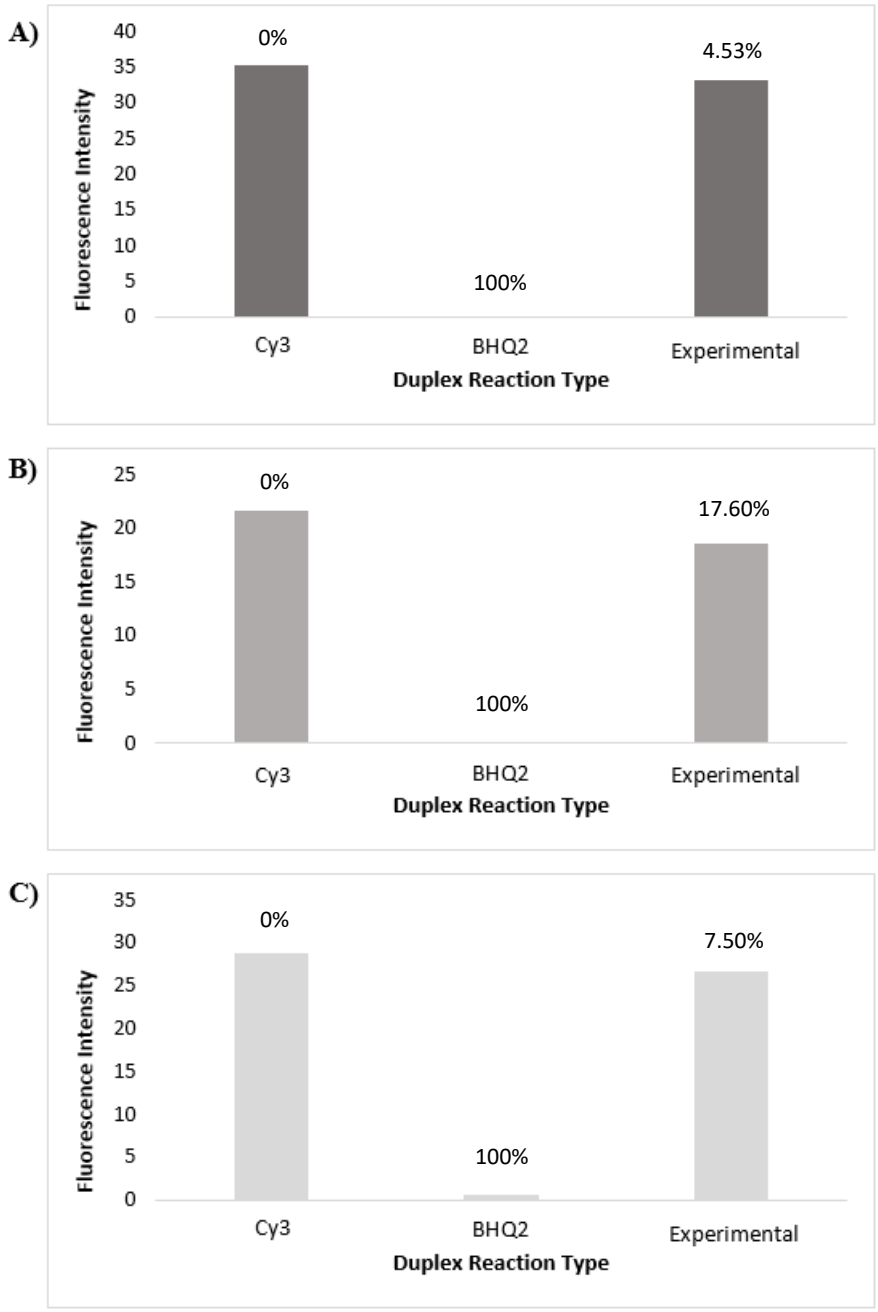


**Figure 3**. Fluorescence intensity at different lengths of cooling when annealing three, 107 nM RNA oligos. In each condition, intramolecular structure was removed via a 20 minute incubation at… A) Extended cooling ~2 hours. B) Intermediate cooling ~1 hour. C) Quick cooling ~20 minutes. The duplex reaction types are the same as in Figure 2. The fluorescence intensity of these solutions was determined at an excitation wavelength of 540 nm using a Jasco Spectrofluorometer. Percent annealing is displayed above each bar and was calculated via equation 1.1.

Magnesium ions serve to stabilize the folded RNA duplex through the attraction between the positively charged cations and the negatively charged backbone of the nucleic acids. A range of final $Mg^{++}$ concentrations was tested from 0.5 mM to 6.0 mM, which resulted in annealing percentages between 2.88% and 14.33%, however, no annealing percentages exceeded 80%.

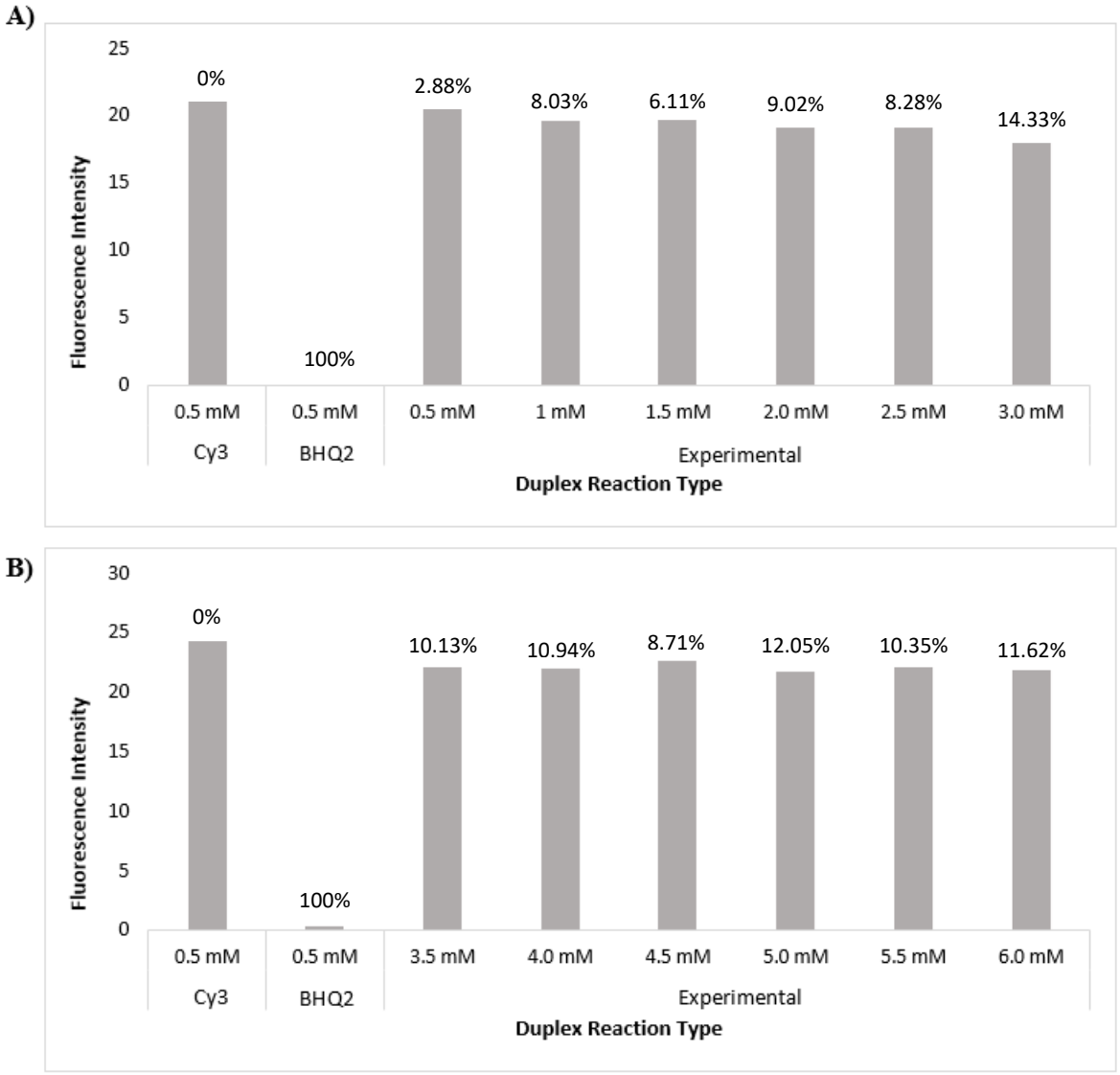


**Figure 4**. Fluorescence intensity at varying final magnesium concentrations when annealing three, 107 nM RNA oligos. The $Mg^{++}$ concentrations (in mM) of the reactions during annealing are listed under the x-axis. A) $Mg^{++}$ concentration range from 0.5 mM to 3.0 mM B) $Mg^{++}$ concentration range from 3.5 mM to 6.0 mM. The duplex reaction types are the same as in Figure 2. The fluorescence intensity of these solutions was determined at an excitation wavelength of 540 nm using a Jasco Spectrofluorometer. Percent annealing was calculated via equation 1.1.

Özes et al., 2014 also tested a two oligo method for the helicase assay with eIF4A and showed that the fluorophore and quencher could be annealed directly to each other, and that these molecules would still be in close enough proximity for fluorescence to be suppressed when on opposite sides of the duplex (Figure 1a). Additionally, it is likely much easier for two oligos to orient themselves and bind properly than it would be for three. This new two oligo procedure involved the annealing of a Cy3 fluorophore RNA oligo (rAKH002), which has an excitation wavelength of 550 nm, directly to the BHQ2 quencher RNA oligo (rAKH009) (Figure 1b). We tested three different combinations of RNA oligos: "Cy3", "BHQ2-2", and "Experimental-2" (Table 2). Through the use of this new annealing procedure, and by testing a range of final $Mg^{++}$ concentrations from 0.5 mM to 6.0 mM, annealing percentages of between 77.89% and 91.01% were observed. The best final $Mg^{++}$ concentration was identified as 2.0 mM with 89.59% annealing, as all higher concentrations exhibited little change to the annealing (Figure 5), and greater than 80% annealing was achieved.

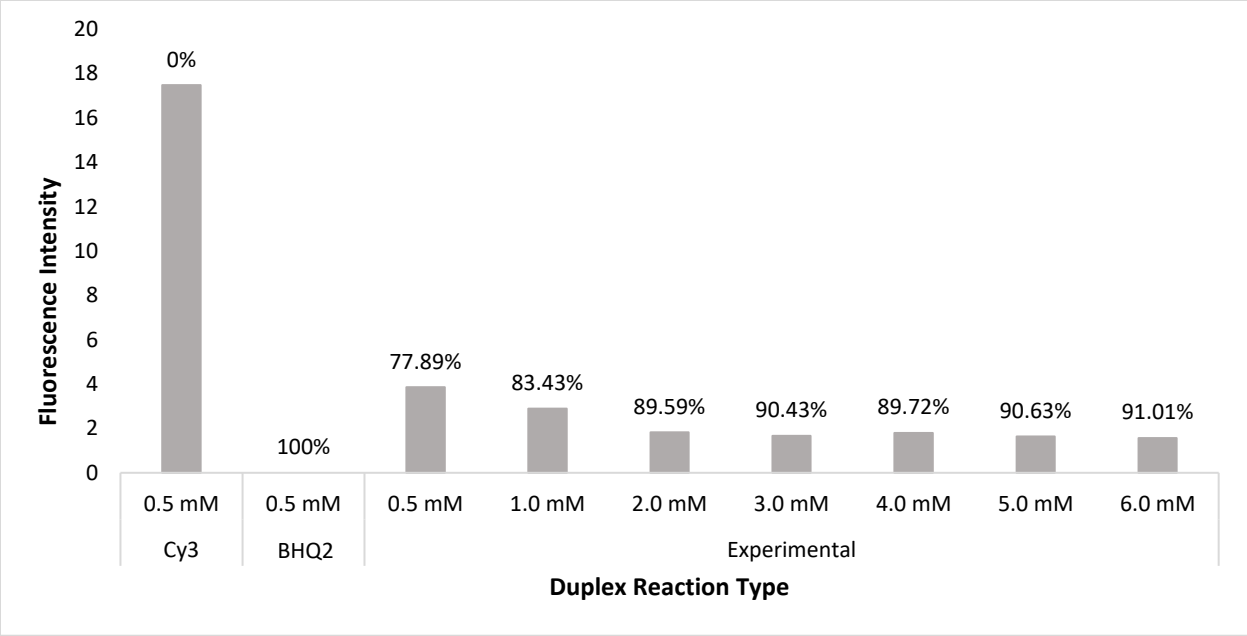


**Figure 5**. Fluorescence intensity at different magnesium concentrations when annealing two, 107 nM RNA oligos. The $Mg^{++}$ concentrations (in mM) of the reactions during annealing are listed under the x-axis. Cy3 was the control showing maximum fluorescence and contained only the Cy3 oligo (rAKH002). BHQ2 showed background fluorescence and contained only the BHQ2 oligo (rAKH009). Experimental was the duplex reaction containing the Cy3 oligo (rAKH002), and the BHQ2 oligo (rAKH009) (Table 2). The fluorescence intensity of these solutions was determined at an excitation wavelength of 540 nm using a Jasco Spectrofluorometer. Percent annealing was calculated via equation 1.1.

However, it was important to ensure that the Ded1 storage buffer (version 8, Hilliker at al., 2011) and addition of competitive DNA (oAKH838; Table 1) did not disrupt the RNA duplex if added after annealing. Four conditions were tested… (1) the absence of both Ded1 storage buffer and competitive DNA, (2) the presence of Ded1 storage buffer, but absence of competitive DNA, (3) the absence of Ded1 storage buffer and the presence of competitive DNA, and (4) the presence of both Ded1 storage buffer and competitive DNA. The annealing percentages for these conditions ranged from 91.43% to 100% (Figure 6), suggesting that the presence of Ded1 storage buffer and competitive DNA does not disrupt the RNA duplex, and greater than 80% annealing was achieved.

The optimal conditions for this RNA duplex annealing were determined to involve a high temperature of either 80˚C or 92˚C, an intermediate cooling length of about 1 hour, two oligos, and a magnesium concentration of 2.0 mM, with the presence of Ded1 storage buffer and competitive DNA (oAKH838) causing no detectable disruption to the duplex. Due to this success in annealing, the unwinding kinetics of methylated vs unmethylated WT Ded1 can now be investigated.

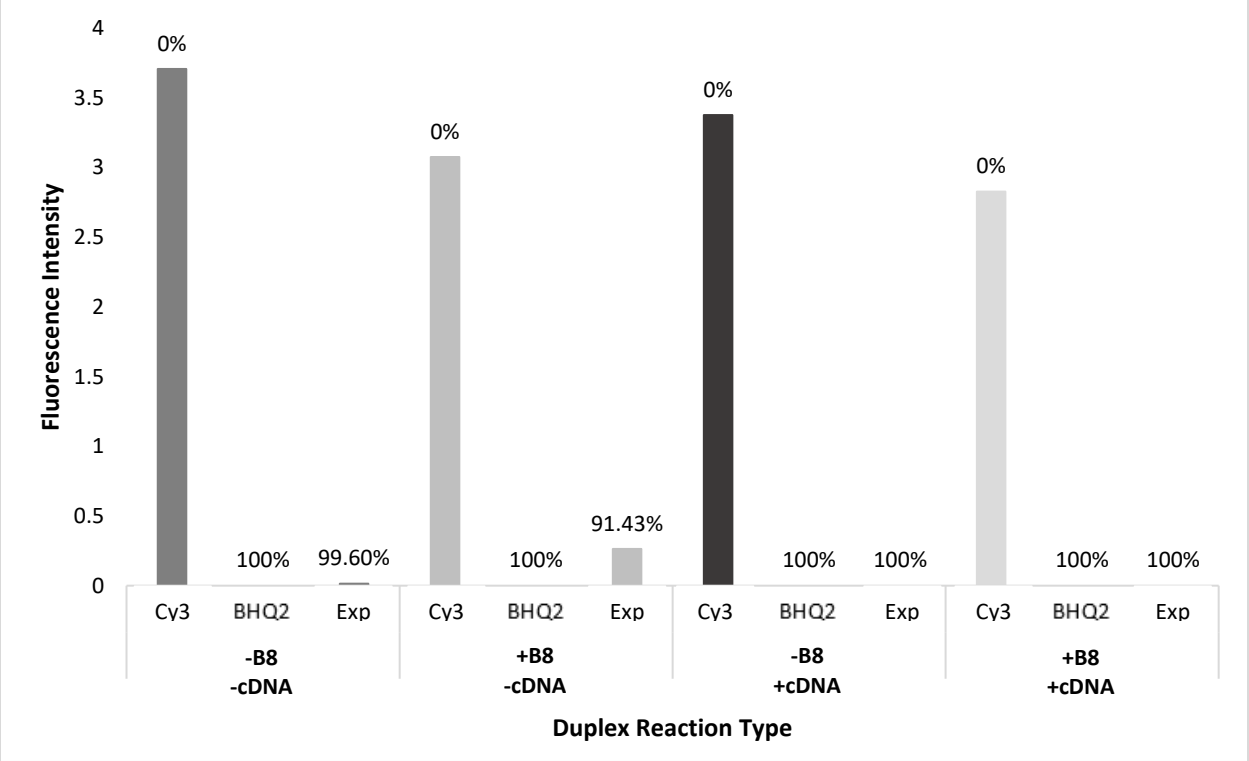


**Figure 6**. Fluorescence intensity when annealing two, 107 nM RNA oligos in the presence (+) or absence (-) of Ded1 storage buffer and competitive DNA. B8 stands for Ded1 Storage Buffer (version 8, Hilliker at al., 2011). cDNA stands for competitive DNA (oAKH838). The conditions of the reactions when fluorescence was tested are under the x-axis. The duplex reaction types are the same as in Figure 5. The fluorescence intensity of these solutions was determined at an excitation wavelength of 540 nm using a Jasco Spectrofluorometer. Percent annealing was calculated via equation 1.1.

## Discussion

Our goal is to compare the helicase activity of methylated vs unmethylated WT Ded1, using a fluorophore-quencher assay, to determine whether the methylation of Ded1 has an effect

on its helicase efficiency. So far, we have found a successful protocol resulting in at least 80% annealing percent of the RNA duplex, which consists of a 92ºC high temperature, an intermediate cooling length of ~1 hour, a magnesium concentration of around 2.0 mM, and a two oligo procedure.

The next step will be to add recombinant, methylated or unmethylated Ded1, purified in lab, to the RNAs annealed under these conditions to determine helicase rate. Ded1's helicase activity requires specific coordination between RNA binding, ATP binding, ATP hydrolysis, and duplex disruption. So, this single helicase assay is sensitive to changes in any of those steps, and future assays can be utilized to dissect a specific step if methylation is found to affect Ded1's overall helicase activity. An ATPase assay could be conducted to monitor how methylation impacts ATP hydrolysis by Ded1, or a fluorescence resonance energy transfer (FRET) assay could be utilized to measure the association between methylated or unmethylated Ded1 and RNA. Future experimentation will pursue analysis of these sub steps of Ded1 helicase activity.

Other RNA helicases have been found to be regulated by PTM methylation. For example, DDX5 is methylated by protein arginine methyltransferase 5 (PRMT5) at its RGG/RG motif. This motif is where DDX5 interacts with XRN2, which is an exonuclease whose association with DDX5 has been found to increase helicase activity (Mersaoui et al., 2019). The methylation of DDX5 at this RGG/RG motif was proposed to serve many functions, including to help recruit XRN2 and coordination with other translational substrates to regulate unwinding of RNA (Zhao et al., 2016). The knowledge that other DEAD-box RNA helicases undergo PTM methylation to regulate their helicase activity was the basis of our hypothesis that Ded1's methylation could serve a similar function.

Also, past research has found that Ded1's helicase ability is impacted by its association with other initiation factors, such as eIF4A and eIF4G (Gao et al., 2016). Translational initiation factors commonly interact with Ded1 at its N- (Gupta & Hinnebusch, 2018) or C-terminus, which is where the five arginine methylation sites lie, so it is possible that the methylation of Ded1 could have some impact on these protein-protein interactions and thus its helicase ability. If we see no change between methylated and unmethylated Ded1, a possible reason for such could be that the difference in helicase activity is not obvious until a combined assay is conducted with eIF4A and eIF4G with Ded1. This would establish that the protein-protein interactions of Ded1 are not only key to its helicase ability but also affected by methylation.

DDX3 is the homolog of Ded1 in humans, which functions as a tumor suppressor and oncogene. However, its mutation has been linked to many diseases, such as different cancers and Hepatitis B and C (Mo et al., 2021). Further understanding of the cellular control mechanisms of Ded1 in yeast could lead to future cancer treatments, or preventative measures, in humans due to the high level of evolutionary conservation between Ded1 and DDX3, and the presence of PTM methylation of both proteins (Hsu et al., 2017).

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