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To initiate or not to initiate: A critical assessment of eIF2A, eIF2D, and MCT-1·DENR to deliver initiator tRNA to ribosomes

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Abstract

Selection of the correct start codon is critical for high-fidelity protein synthesis. In eukaryotes, this is typically governed by a multitude of initiation factors (eIFs), including eIF2·GTP that directly delivers the initiator tRNA (Met-tRNA$_i^{Met}$) to the P site of the ribosome. However, numerous reports, some dating back to the early 1970s, have described other initiation factors having high affinity for the initiator tRNA and the ability of delivering it to the ribosome, which has provided a foundation for further work demonstrating non-canonical initiation mechanisms using alternative initiation factors. Here we provide a critical analysis of current understanding of eIF2A, eIF2D, and the MCT-1·DENR dimer, the evidence surrounding their ability to initiate translation, their implications in human disease, and lay out important key questions for the field.

Keywords

eIF; translation initiation; translational control

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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1 | INTRODUCTION

Messenger RNA (mRNA) translation is a highly orchestrated process composed of four main steps: initiation, elongation, termination, and ribosome recycling (Jackson et al., 2010; Ramakrishnan, 2002; Schmeing & Ramakrishnan, 2009). Most mechanistic details have been elucidated with biochemical, genetic, and structural approaches using *Escherichia coli*, yeast (*Saccharomyces cerevisiae*), rabbit reticulocyte lysates, and common mammalian cell lines. Comparing the three domains of life, the molecular mechanisms of elongation are remarkably analogous, and a few distinct differences are seen in termination and ribosome recycling (Dever & Green, 2012; Schmeing & Ramakrishnan, 2009). However, initiation is recognized as vastly distinct (Jackson et al., 2010; Schmeing & Ramakrishnan, 2009; Voigts-Hoffmann et al., 2012). For example, in prokaryotes, initiation uses three initiation factors (IFs) to ensure the ribosome locates the correct start codon; whereas in eukaryotes, a minimum of 12 eukaryotic IFs (eIFs), many of which are multi-subunit or multi-factor complexes, is required for high fidelity initiation. Since protein synthesis is very energetically costly, initiation is highly regulated, deeming it the rate-limiting step for most mRNAs and open reading frames (ORFs). It is thought that the increase in molecular complexity and the number of required eIFs (relative to prokaryotic initiation) provides eukaryotes with more regulatory targets to ensure proteins are synthesized with the spatiotemporal accuracy necessary for physiology or biological fitness.

The canonical eukaryotic translation initiation pathway is well characterized and can be separated into four main stages: pre-initiation complex (PIC) recruitment to the 5′ 7-methylguanosine cap of mRNA, PIC mRNA scanning, start codon recognition, and subunit joining. Heterotrimeric eIF2 delivers the initiator tRNA in the form of the ternary complex (TC; eIF2·GTP·Met-tRNA$_i^{Met}$) to the 40S subunit. The TC along with eIF1, eIF1A, eIF3, and eIF5 binds the 40S subunit to form the 43S PIC. The PIC is recruited to the 5′ cap by the eIF4F complex (comprised of eIF4A, eIF4E, and eIF4G), where it begins to scan the mRNA for the AUG start codon. Once the anticodon loop of the initiator tRNA base pairs with the AUG start codon, eIF2 hydrolyzes GTP with aid from the GTP-activating protein, eIF5, and releases Pi (Algire et al., 2005). eIF2·GDP then dissociates from Met-tRNA$_i^{Met}$ and the 40S ribosome. eIF1A and eIF5B interact with the ribosome and reorient the initiator tRNA to allow for subsequent 60S subunit joining, 80S ribosome formation, and dissociation of the remaining eIFs (Lapointe et al., 2022).

The eIFs were first identified and characterized from crude preps from high salt-washed ribosomes, often from rabbit reticulocyte lysate (RRL) (Adams et al., 1975; Benne & Hershey, 1978; Schreier et al., 1977). An early focus was to identify factor(s) responsible for initiator tRNA binding and delivery to the ribosomal P site. In the early 1970s, Anderson et al. first identified eIF2A (originally named IF-M1) from RRL to have the ability to deliver initiator tRNA to the 40S ribosomal subunit. However, soon after, between 1972 to 1975, the Acs, Staehelin, Gupta, Bose, Stanley, Henshaw, and Robinson laboratories found that eIF2 (referred to as IF-L3, Factor C, IF1, and IF-E2 in these papers) also had the ability to bind and deliver initiator tRNA to the ribosome (Cashion & Stanley, 1974; Chen et al., 1972; Dettman & Stanley, 1972; Gupta et al., 1973; Levin et al., 1973a, 1973b; Schreier & Staehelin, 1973; Smith & Henshaw, 1975; Treadwell & Robinson, 1975). It was not until

1975 that a single group purified and compared the function of eIF2A and eIF2 (named IF-MP) (Adams et al., 1975). Adams et al. found that while both factors had initiator tRNA carrier capabilities, only eIF2 was able to direct translation of natural globin mRNA (Adams et al., 1975), and it is now widely understood that eIF2 is the primary initiator tRNA carrier in eukaryotes.

However, several reports have provided evidence that other initiation factors have high affinity for initiator tRNA and ability to bind the 40S ribosomal subunit, namely eIF2D and the MCT-1·DENR dimer, in addition to the aforementioned eIF2A. As such, these three factors are sometimes referred to as alternative or non-canonical initiation factors as they clearly do not function in canonical eukaryotic initiation but are thought to function in a transcript- or condition-specific manner. eIF5B functions in canonical initiation but others have raised the possibility that it may also deliver the initiator tRNA in a selective manner; however, recent single-molecule FRET and structural work has shed light on how eIF5B directly interacts with Met-tRNA$_i^{Met}$ during the late states of initiation and 80S formation (Chukka et al., 2021; Huang & Fernandez, 2020; Lapointe et al., 2022; Wang et al., 2019; Wang et al., 2020), most likely contributing to previous observations of its affinity for Met-tRNA$_i^{Met}$ and its ability to direct initiation on the Hepatitis C virus (HCV) internal ribosome entry site (IRES) (Pestova et al., 2008; Yamamoto et al., 2014). Other alternative initiation factors such as DAP5 (eIF4G2, p97, Nat1) (de la Parra et al., 2018; Henis-Korenblit et al., 2000; Henis-Korenblit et al., 2002; Marash et al., 2008; Shestakova et al., 2023; Weber et al., 2022), eIF3d (Lamper et al., 2020; Lee et al., 2015; Lee et al., 2016; Ma et al., 2023), and paralogs of eIF4G (eIF4GI and eIF4GII) (Hundsdoerfer et al., 2005; Kaiser et al., 2008; Zhao et al., 2017) have been reported in facilitating cap-dependent and -independent initiation on subsets of endogenous mRNAs and viral IRESs. However, eIF2A, eIF2D, and the MCT-1·DENR dimer are the only factors reported to have a propensity to bind and deliver the initiator tRNA to the 40S ribosomal subunit. In this review, we will focus on the current understanding of eIF2A, eIF2D, and the MCT-1·DENR dimer, and the evidence supporting their ability to stimulate initiation through their interaction with initiator tRNA and/or affect ribosome recycling. Additionally, we will highlight their potential impact as regulators of aberrant translation that cause human disease and lay out important outstanding questions that would push the field forward.

2 | eIF2A

2.1 | Initial discovery of eIF2A and new insights

Early experiments focusing on identifying the factor(s) that deliver Met-tRNA$_i^{Met}$ were conducted using high salt-washed ribosome fractions and partially purified initiation factors. Individual proteins were then purified to further study their respective functions. eIF2A is a 65 kDa monomer (for a comprehensive review on eIF2A see (Komar & Merrick, 2020)) that was purified from RRL using an eight-step purification scheme: (1) 0.5 M KCl wash, (2) ammonium sulfate precipitation, (3) DEAE-cellulose chromatography, (4) ammonium sulfate precipitation, (5) Sephadex G-200 chromatography, (6) CM-cellulose chromatography, (7) phosphocellulose chromatography, and (8) Sephadex G-100 chromatography (Merrick & Anderson, 1975; Shafritz & Anderson, 1970). This purification

scheme produced 2.1 mg of seemingly homogenous eIF2A as analyzed via SDS-PAGE, which maintained ~15% yield of the original eIF2A activity (Merrick & Anderson, 1975). eIF2A activity was assessed using poly(U)-directed phenylalanine synthesis at 4 mM Mg^{2+} levels and the ability of eIF2A to deliver labeled Met-$tRNA_i^{Met}$ to ribosomes programmed with an AUG trinucleotide was tested using nitrocellulose filter binding assays at 5 mM Mg^{2+}. Low Mg^{2+} levels are required for N-formylmethionine initiator tRNA (fMet-$tRNA_F$) binding in bacterial systems (Nakamoto & Kalokofsky, 1966), which supported eIF2A as a plausible candidate for being the primary Met-$tRNA_i^{Met}$ carrier in eukaryotes. The activity and binding assays made it clear that the purified protein had the same function as the factor in the high salt-washed ribosome fraction; however, the partially purified protein from an earlier purification fraction (step 5) was able to deliver 2-fold more initiator tRNA to ribosomes than the final purified product (step 8) (Merrick & Anderson, 1975). Soon thereafter, it was found that eIF2, and not eIF2A, was the primary initiator tRNA carrier in eukaryotes (Adams et al., 1975).

In 2010, Dmitriev et al. identified that eIF2D (also a 65 kDa monomer) co-purifies with eIF2A from RRL due to its similar chromatographic properties (Dmitriev et al., 2010). Further investigation of completely homogenous eIF2A (verified by mass spectrometry) indicated a loss of initiator tRNA binding and carrying activity as demonstrated by ribosome toe printing and filter binding assays (Dmitriev et al., 2010). eIF2D purified from RRL, HeLa cells, and recombinantly expressed and purified from *E. coli* was able to deliver initiator tRNA to the 40S ribosomal subunit (Dmitriev et al., 2010) (see Section 3 below for a more in-depth discussion of eIF2D function). This raises the question: Could the loss of eIF2A activity be indicative of an incorrectly attributed function that actually belonged to contaminating eIF2D? A bottleneck for the field to answer this question has been obtaining highly pure and soluble recombinant eIF2A (Grove et al., 2023). A recent report has described an expression and purification scheme that produces highly pure recombinant eIF2A with yields ~360- to 6000-fold higher yield than previous reports depending on the expression system used (bacteria or insect cells). Inconsistent with having a stimulating role in initiation, titration of tagged or tagless recombinant eIF2A into in vitro RRL translation reactions inhibited translation initiation prior to start codon recognition, regardless of cap dependency, 5′ UTR, start codon, and coding sequence (Grove et al., 2023).

Furthermore, recent work demonstrated that eIF2A is able to directly bind the 40S ribosomal subunit without additional eIFs, initiator tRNA, or mRNA, and, in doing so, actually inhibits cap-dependent and -independent translation at physiological and higher concentrations (Grove et al., 2023). Notably, eIF2A inhibited translation of reporters under control of the HCV IRES and cricket paralysis virus intergenic region (CrPV IGR) IRES. Since the HCV and CrPV IGR IRESs recruit the 40S ribosomal subunit in distinct manners, Grove et al. separated translation reactions on sucrose gradients to gain insight if eIF2A prevented ribosome recruitment by one or both IRESs. However, there was no robust change in either IRES binding to the 40S ribosomal subunit when eIF2A was present. The addition of eIF2A also did not lead to an accumulation of reporter mRNA at the top of the gradient. Thus, eIF2A most likely does not interfere with IRES·40S binding within the E, P, and A sites. Nevertheless, for all IRESs, mRNA must still be inserted into the mRNA channel for subsequent elongation to occur. These data may suggest that

eIF2A is preventing mRNA insertion into the mRNA channel. Possibly supporting this idea, Wei et al. immunoprecipitated GFP tagged eIF2A (eIF2A-GFP) from UV-crosslinked HEK293T cells and observed over 50% of crosslinks mapped to the 18S rRNA, which accounted for ~20% of the input RNAs (Wei et al., 2023). Further analysis showed that these crosslinks were enriched near the mRNA entry site, specifically between two rRNA expansion segments, which are evolutionarily divergent parts of rRNA found in eukaryotes that vary in biological function, in the V4 variable region of 18S rRNA (Wei et al., 2023). Structural characterization of eIF2A bound to the 40S subunit will be required to confidently determine the inhibitory mechanism of eIF2A.

2.2 | Lessons learned from yeast eIF2A

Yeast has proven to be a powerful model system in providing evidence that eIF2A, somehow, functions in translation. In addition to *Schizosaccharomyces pombe* and human eIF2A sharing a similar β-propeller N-terminus (Kashiwagi et al., 2014) and overall similar predicted structure (Figure 1) (Jumper et al., 2021; Varadi et al., 2022), *Saccharomyces cerevisiae* and human eIF2A primary sequences are 28% identical and 58% similar. Deletion of eIF2A (eIF2AΔ) from *S. cerevisiae* did not result in a slow or fatal growth phenotype, but the sporulation count was one-third of wildtype cells (Komar et al., 2005). Since *S. cerevisiae* sporulation results from nutrient deficiencies (usually from nitrogen and/or carbon starvation) (Neiman, 2011), this phenotype indicates loss of eIF2A impacts the ability of this yeast strain to have a standard response to and reproduce during cell stress. Polysome profiling analysis of eIF2AΔ strains were no different from wildtype yeast strains; however, HA-tagged yeast eIF2A did co-sediment with 40S ribosomal subunits and 80S ribosomes (Zoll et al., 2002). eIF2AΔ strains also had equal levels of protein synthesized from the *GCN4* re-initiation reporter mRNA when compared with WT strains. Gaikwad et al. recently concluded using ribosome profiling and eIF2AΔ strains in normal and cell stress conditions (when functional eIF2 levels are low) that eIF2A has little to no role on mRNA translation in yeast (Gaikwad et al., 2024).

Since eIF2A does not hydrolyze GTP, it has been speculated that release of the initiator tRNA after start codon recognition could be regulated by a binding partner, namely eIF5B. In canonical initiation, eIF5B directly interacts with Met-$tRNA_i^{Met}$ during the late stages of initiation and 80S formation in a GTP-dependent manner (Chukka et al., 2021; Huang & Fernandez, 2020; Lapointe et al., 2022; Wang et al., 2019; Wang et al., 2020). Unlike mammalian eIF5B, yeast eIF5B is non-essential. However, eIF5B null stains do have a severe growth phenotype (Choi et al., 1998). While eIF2A null and eIF5B null strains are viable, the double knockout (KO) results in a synthetically lethal phenotype, providing evidence that the two gene products function in the same pathway (Zoll et al., 2002). Subsequently, a direct interaction between recombinant human eIF2A and recombinant human eIF5B was verified (Kim et al., 2018) (see below), which is consistent with this model. However, translation initiation of mRNAs that require the interaction between eIF2A and eIF5B still needs to be determined.

Komar et al. deleted eIF2A from temperature-sensitive eIF4E mutant strains to determine if eIF2A had any apparent genetic interactions with initiation factors other than eIF5B (Komar

et al., 2005). Under non-permissive temperatures, the eIF4E-ts/eIF2AΔ mutant grew 1.68 times slower than the parental strains, indicating eIF2A and eIF4E also function in the same pathway, but whether eIF2A directly interacted with eIF4E was not tested. Although many eukaryotic genomes do not encode IRESs, the atypical yeast URE2 IRES was sensitive to eIF2A levels and, to a lesser extent, to eIF4E levels when grown at non-permissive temperatures. Expression of the URE2 IRES reporter was upregulated in the eIF2AΔ strain 13-fold relative to WT; conversely, URE2 IRES reporter expression was down-regulated 9-fold in the eIF2AΔ strain complemented with HA-eIF2A (both yeast and human) relative to the eIF2AΔ strain alone. Interestingly, the eIF4E-ts/eIF2AΔ strain had no increase in URE2 levels, which indicates that at least one of the factors needs to be present for URE2 IRES expression. Together, these data suggest that eIF2A levels do affect translation and this factor probably physically interacts with some members of the translation machinery but does so in a more transcript-specific manner.

Immunoprecipitation of HA tagged eIF2A (HA-eIF2A) from yeast cells followed by mass spectrometry analysis has identified new putative interacting partners for eIF2A. The most confident hits included various large and small subunit ribosomal proteins, eEF1A, and ribosome chaperone protein SSB2 (Reineke et al., 2011), which supports that eIF2A has some role in translation. RNA-independent association between eEF1A or SSB2 and eIF2A was confirmed through GST pull down assays with recombinant GST-eEF1A and recombinant GST-SSB2 with RNase-treated lysates from cells that expressed HA-eIF2A (Reineke et al., 2011). Endogenous eEF1A also co-immunoprecipitated with HA-eIF2A from cell lysates. The co-immunoprecipitation experiments were repeated with various HA-eIF2A deletion mutants and the C-terminus of eIF2A was identified to be important for this interaction. The same group found that URE2 IRES expression in yeast was inversely correlated with eIF2A levels (Komar et al., 2005). Complementation of an eIF2A C-terminal deletion into an eIF2AΔ strain no longer increased URE2 IRES expression and the authors contributed this to its inability to bind eEF1A without its C-terminus (Reineke et al., 2011).

2.3 | Structural basis of eIF2A

Studies in yeast have also provided early insights into eIF2A structure; however, full-length crystal or cryo-EM structures are still lacking for any orthologue. Full-length recombinant *S. pombe* eIF2A (residues 1–585) was not able to form crystals, possibly due to a highly dynamic C-terminus (Kashiwagi et al., 2014). The C-terminally truncated *S. pombe* eIF2A (residues 1–424) crystal structure was solved at 2.5 Å (Figure 1a) and was the first nine-bladed β-propeller identified, as most β-propellers contain six to eight blades (Chen et al., 2011). In addition to the unconventional nature of the nine-bladed β-propeller, the funnel formed was inverted (wider at top rather than the bottom) (Kashiwagi et al., 2014). The top of the β-propeller contained conserved and positively charged residues (Kashiwagi et al., 2014), which would support potential interactions with RNA or other translation initiation factors. Coincidentally, the structure of eIF3b was solved by Liu et al. within months of the *S. pombe* eIF2A structure and also shows a nine-bladed β-propeller that has high structural homology (Liu et al., 2014), but it remains unknown whether one protein evolved from the other or if the two share ribosome binding sites due to this structural homology.

The crystal structures of eIF2A β-propellers from *S. pombe* (residues 1–424; Figure 1a) (Kashiwagi et al., 2014) and *Homo sapiens* (residues 4–427; Figure 1b) indicate a conserved structure between yeast and humans. Since full-length empirically determined structures for eIF2A are unavailable, we have also included the AlphaFold predicted structure (Figure 1c, left) (Jumper et al., 2021; Varadi et al., 2022) which has high prediction confidence in the β-propeller but much lower confidence in the flexible linkers. Nevertheless, an overlay of the β-propellers from the two crystal structures and the AlphaFold structure (Figure 1d) show high structural homology. Using numerous recombinant eIF2A truncations, Kim et al. determined eIF2A contains three domains (Figure 1c, right) that bind initiator tRNA (residues 1–461), eIF5B (residues 462–501), and mRNA (residues 502–585) (Kim et al., 2018). However, the properties of these three domains have yet to be subsequently validated. The binding affinities (apparent K_d) between eIF2A and either uncharged or charged radio-labeled initiator tRNA were found to be 16.5 nM and 12.4 nM (Kim et al., 2011), respectively, which are similar to the 15 nM (apparent K_d) binding affinity eIF2 has for charged initiator tRNA (Erickson & Hannig, 1996). It should also be noted that the affinities between initiator tRNA and eIF2A or eIF2 were measured years apart by different groups. The mRNA binding domain has only been shown to interact with HCV IRES reporter mRNA (Kim et al., 2018) and whether other mRNAs share this binding site remains unknown. Additionally, the inhibitory phenotype of recombinant eIF2A in vitro does not seem to rely on its interaction with eIF5B since eIF2A lacking the helix that was mapped to interact with eIF5B still inhibited translation (Grove et al., 2023).

2.4 | Connections of eIF2A to pathology and human disease

While characterization of the yeast orthologue and identification of genetic interactions provide some evidence that eIF2A functions in translation, the in vivo function in mammalian systems remains enigmatic. To provide new insight, Golovko et al. generated an eIF2A KO mouse and observed no phenotype in the first 3–5 months after birth (Golovko et al., 2016); however, once the mice reached ~1 year of age, they developed a metabolic syndrome, were pre-diabetic, and had decreased life spans, all of which were more prominent in females (Anderson et al., 2021). Most notably, eIF2A KO mice had fat deposits with increased adipocyte levels and displayed signs of fatty liver disease. The increase in fatty tissues also provides a connection with the observed glucose tolerance and pre-diabetic state in both male and female eIF2A KO mice. However, to the best of our knowledge, diabetes or lipid metabolism phenotypes have not been genetically linked to eIF2A levels, gene copy number, or mutations in humans. As eIF2A had previously been implicated in the integrated stress response (ISR) (Kim et al., 2011; Kim et al., 2018; Sonobe et al., 2018; Starck et al., 2012; Starck et al., 2016; Ventoso et al., 2006), Anderson et al. sought to measure potential changes in the levels of major ISR proteins in the KO mice (Anderson et al., 2021). Levels of these proteins (e.g., BiP, CHOP, phosphorylated PERK, p-eIF2α)—which are typically up-regulated, resistant to translation inhibition, or modified during cell stress due to their roles in re-establishing homeostasis—during normal conditions and under cell stress (ER stress induced by tunicamycin treatment) were measured by Western blot and showed no significant differences.

However, Sendoel et al. have provided a strong link to eIF2A and squamous cell carcinoma (SCC) (Sendoel et al., 2017), with the latter relying on the SOX2 transcription factor (Boumahdi et al., 2014; Sarkar & Hochedlinger, 2013). Using an inducible $SOX2^+$ epidermis model, Sendoel et al. performed a lentiviral-delivered shRNA screen for components of the translation machinery that were not necessary for $SOX2^+$ epidermal growth but were required for normal epidermal growth. Among the highest hits were the α and β subunits of eIF2 (Sendoel et al., 2017). With ribosome profiling, a shift toward translating more uORFs was noticed during SOX2 expression. These data, and the observation of higher p-eIF2α levels in the $SOX2^+$ keratinocytes and SCC suggested to Sendoel et al. that the shift in translation toward uORFs may be independent of eIF2. Since eIF2A has been implicated in near-cognate start codon-mediated initiation (described in more detail in Section 2.5), Sendoel et al. then tested and demonstrated that delivery of shRNAs targeting eIF2A did not allow $SOX2^+$ epidermis to develop. The authors concluded that these data indicated that translation in $SOX2^+$ epidermis is more dependent on eIF2A than eIF2. eIF2A deletion in SCC keratinocytes did not prevent proliferation in serum rich media, nor was a change in global protein synthesis observed by either O-propargyl-puromycin incorporation or ribosome profiling. However, Sendoel et al. did report with ribosome profiling data that the ratio of uORF to ORF translation was decreased in eIF2A KO SCC keratinocytes compared with WT controls. Whether these uORFs were enriched in near-cognate start codons was not clearly addressed. Additionally, eIF2A KO SCC keratinocytes stimulated little to no tumor growth 2 months after tumor engraftment, while WT SCC keratinocytes developed tumors within 1–2 weeks. However, eIF2A complementation only partially rescued tumor formation to ~40% compared with WT SCC keratinocyte controls. It is possible that the lack of full rescue could be attributed to the low eIF2A rescue expression as Western blotting showed low amounts of eIF2A protein in the rescue compared with the control. While eIF2A does not function in a global initiation pathway, these phenotypes indicate it has an important role in tumor biology and uORF translation in mammals.

Finally, eIF2A has also been implicated in neurological disorders, specifically with two repeat expansion disorders that cause neurodegeneration through repeat-associated non-AUG (RAN) translation. First, with the expanded *GGGGCC* repeats contributed to amyotrophic lateral sclerosis frontotemporal dementia (ALS-FTD), Sonobe et al. transiently transfected HEK293 WT or eIF2A KO cells with reporter mRNA containing the expanded *GGGGCC* repeat and found that reporter protein levels decreased in the eIF2A KO cells, albeit the effect was <2-fold (Sonobe et al., 2018). However, this effect was not seen by Green et al. with similar reporters with eIF2A knockdown in HEK293 cells (Green et al., 2022). Second, Tusi et al. provided evidence that RAN translation of expanded *CCUG·CAGG* repeats that causes myotonic dystrophy 2 is sensitive to eIF2A levels, as well as levels of functional eIF2 (Tusi et al., 2021). By generating stable HEK293T cell lines expressing either *CCUG* (encodes QAGR repeat protein) or *CAGG* (encodes LPAC repeat protein) repeat reporter mRNAs, they found that protein levels of both reporters increased during viral and ER cell stress. Knockout of PKR and PERK decreased transient expression of the QAGR product to <1% and 28%, respectively, when compared with WT cells (Figure 2a–c). LPAC protein expression was similarly reduced in PKR and PERK KO cell lines

to ~10% (Figure 2a–c). Protein levels of QAGR robustly decreased to ~2% in an eIF2α S51A mutant cell line, while LPAC expression only decreased to 56% compared with WT (Figure 2a,d), suggesting QAGR protein synthesis heavily relies on eIF2α phosphorylation. The ability of eIF2A to function in RAN translation of these two mRNAs was assessed by generating HEK293T eIF2A KO cells. Similar reduced expression of QAGR and LPAC protein levels was observed in the eIF2A KO as in the eIF2α S51A mutant cells (Figure 2a,d,e) and overexpression of eIF2A in eIF2A KO cells rescued expression of both RAN translation reporters. The authors went on to overexpress eIF2A in the eIF2α S51A mutant cells and observed no rescue of QAGR levels (Figure 2a,f), indicating eIF2A requires eIF2α phosphorylation for its role in QAGR protein synthesis. Together these data suggest that the observed eIF2A dependence in RAN translation of the *CCUG* reporter mRNA is downstream of eIF2α S51 phosphorylation. Multiple reports have shown that expanded repeats themselves and the repeat proteins encoded by them can induce a stress response that induced eIF2α S51 phosphorylation (Cheng et al., 2018; Green et al., 2017; Tian et al., 2000; Westergard et al., 2019; Zu et al., 2020). Whether this was happening within the stable cell lines or during transient eIF2A overexpression was not addressed. Additionally, Tusi et al. did not provide clear evidence of a negative control reporter that did not respond to alteration of eIF2A levels.

2.5 | Evidence of eIF2A directing initiation at near-cognate start codons

The recent advancement of ribosome footprint mapping (i.e., ribosome profiling) has expanded our view on the use of near-cognate start codons (CUG, GUG, UUG, etc.) in mammalian cells (Ingolia et al., 2009; Ingolia et al., 2011). While only a few mammalian mRNAs are solely encoded by a near-cognate start codon (e.g., *DAP5* mRNA using a GUG start codon), many mRNAs can use upstream near-cognate start codons to encode an N-terminal extension or a regulatory uORF (Arnaud et al., 1999; Brar et al., 2012; Chew et al., 2016; Ingolia et al., 2011; Johnstone et al., 2016; Liang et al., 2017; Renko et al., 1990). Near-cognate start codons in eukaryotic cells are 50- to 100-fold less efficient (Clements et al., 1988; Hann et al., 1988; Peabody, 1987, 1989; Zitomer et al., 1984); however, cell stress can sometimes increase the efficiency to some degree (Green et al., 2022). eIF2A levels have also been shown to affect the translation of several mRNAs that harbor functional near-cognate start codons. For example, Starck et al. concluded that proper translation of *binding immunoglobin protein* (*BiP*) mRNA during cell stress is dependent on the translation of the UUG-encoded uORF1 and eIF2A (Figure 3a) (Starck et al., 2016). BiP is a chaperone protein that is resistant to ER stress induced by the unfolded protein response to aid in re-establishing homeostasis. Using innovative small tracer peptides to assess the translation of uORF1 from a *BiP-FLAG* reporter mRNA, levels of the peptide encoded by uORF1 remain unchanged during oxidative cell stress induced by sodium arsenite in WT cells. When eIF2A was depleted by RNAi, uORF1 expression diminished and BiP-FLAG protein levels decreased by ~30% (Figure 3b). Mutation of the UUG start codon in uORF1 in cells depleted of eIF2A and treated with thapsigargin (Tg) decreased BiP-FLAG protein levels by ~60% when compared with WT cells, suggesting the importance of both uORF1 and eIF2A in translation of *BiP-FLAG* mRNA during cell stress (Figure 3b). However, the same eIF2A knockdown did not alter endogenous BiP levels during the same stress

condition, and eIF2A KO mice showed no difference in BiP protein levels during ER stress when assayed by Western blot (Anderson et al., 2021).

The effect of eIF2A levels on *phosphatase and tensin homolog* (*PTEN*) mRNA translation also provides evidence for a connection between eIF2A and near-cognate start codons. *PTEN* is a tumor suppressor gene that is commonly mutated in cancer and contains a functional in-frame CUG start codon upstream of the AUG-encoded main *PTEN* ORF (Liang et al., 2014). Liang et al. inserted a FLAG-tag fused to the C-terminus of the endogenous mouse PTEN gene to immunoprecipitate and confirm the production of both the CUG-encoded extension isoform (PTEN-α) and the canonical AUG-encoded isoform (PTEN) from the same locus. eIF2A knockdown in HeLa cells reduced the abundance of the N-terminally extended PTEN product (PTEN-α) but did not affect the canonical AUG-encoded isoform (PTEN). Importantly, the reciprocal experiment was performed and, in agreement with the authors' conclusions, overexpression of eIF2A resulted in an increase in PTEN-α protein levels. Nevertheless, how eIF2A preferentially initiates at the nearcognate start codon in either example (uORF1 in *BiP-FLAG* or main *PTEN* ORF) is unclear and is not yet supported by direct biochemical evidence.

One mechanism proposed that allows eIF2A to initiate selectively at near-cognate start codons centers around the use of tRNA other than Met-tRNA$_i^{Met}$ for initiation. Starck et al. suggested that eIF2A uses Leu-tRNA^{Leu} to initiate translation at CUG start codons (Starck et al., 2012). They first showed in both cells and RRL that translation of a small peptide reporter mRNA encoded by an AUG start codon (*AUG-YL8*) but not a CUG start codon (*CUG-YL8*) was inhibited by the small molecule NSC119893 that dissociates the eIF2·GTP·Met-tRNA$_i^{Met}$ ternary complex. Conversely, eIF2A knockdown resulted in a decrease in expression of only the CUG-YL8 reporter. These data suggested that translation of *CUG-YL8* in cells does not require eIF2 and uses an eIF2A-based initiation mechanism. Upon assessing which tRNAs co-purify with the initiation complexes of each small peptide reporter isolated from rabbit reticulocyte lysates by tRNA microarrays, Starck et al. found Leu-tRNA^{Leu} to associate with only the *CUG-YL8* reporter mRNA initiation complex. While a direct interaction between eIF2A and Leu-tRNA^{Leu} (with or without the 40S ribosomal subunit and mRNA) has not been reported, these data together led Starck et al. to speculate that eIF2A could be responsible for delivering tRNAs other than Met-tRNA$_i^{Met}$ for initiation at near-cognate start codons.

Ichihara et al. developed a strategy to more consistently identify translation initiation start sites in HEK293T cells referred to as Translation Initiation Site detection by translation Complex Analysis sequencing (TISCA-seq) (Ichihara et al., 2021). They, like others, confirmed that near-cognate start codons were much less efficiently translated than their AUG counterparts. Through proteomics analysis and identified N-terminal methionine residues, they also found that Met-tRNA$_i^{Met}$ was used for the majority of translation events even at these near-cognate start sites and that eIF2 (not eIF2A or eIF2D) was the primary initiator tRNA carrier for these sites, even during cell stress. eIF2A knockout only changed translation efficiency of one near-cognate start codon encoded ORF during normal growth conditions and this was less than a two-fold decrease compared with WT cells. The authors used sodium arsenite to induce oxidative stress in eIF2A knockout cells and identified three

ORFs encoded by near-cognate start codons that were translated 0- to 1-fold less efficiently, and one near-cognate encoded ORF that was translated 1- to 2-fold less efficiently compared with WT cells treated with sodium arsenite. In the same study, Ichihara et al. identified nine ORFs with near-cognate start sites that increased in translation efficiency. Among these, four ORFs demonstrated a 0- to 1-fold increase in translation efficiency, while the other five exhibited more than a 2-fold increase in translation efficiency during oxidative stress in eIF2A KO cells compared with WT cells. The latter is consistent with newer data showing eIF2A inhibits translation in vitro (Grove et al., 2023). Nevertheless, these identified ORFs should be useful substrates to mechanistically determine how eIF2A stimulates and inhibits translation, at least during oxidative stress in HEK293T cells.

To our knowledge, the only direct biochemical data providing evidence that eIF2A can form an initiation complex with an mRNA is with the HCV IRES; however, the HCV IRES initiation mechanism is not found in endogenous eukaryotic mRNAs. Using biotinylated HCV IRES mRNA incubated with a series of eIF2A-FLAG deletion mutants expressed in HEK293FT cells, Kim et al. demonstrated that eIF2A residues 502–585 are required for HCV IRES binding (Kim et al., 2018). Subsequent filter binding assays showed that eIF5B increases the ability of eIF2A to deliver labeled initiator tRNA to purified 40S ribosomal subunits via the HCV IRES by 1.77-fold. Kim et al. demonstrated that eIF2A knockdown in Huh7 cells abrogated HCV IRES directed translation of *Firefly luciferase* (*FFLuc*) reporter mRNA during cell stress but did not affect cap-dependent translation of the control *Renilla luciferase* reporter (Kim et al., 2011). Importantly, complementation of eIF2A into the knockdown cells rescued expression of *FFLuc* mRNA directed by the HCV IRES. However, these results were not replicated independently by Gonzalez-Almela et al. when testing HCV IRES translation in WT and eIF2A KO HAP1 cells (Gonzalez-Almela et al., 2018), suggesting the role of eIF2A in translation directed by the HCV IRES could be cell type specific. Experiments in a variety of other cell types would be beneficial for further characterizing the role of eIF2A in IRES-mediated translation.

2.6 | eIF2A localization

Uncharacteristic of a translation initiation factor, several studies have provided evidence that eIF2A is non-cytosolic (Kim et al., 2011; Panzhinskiy et al., 2021; Thul et al., 2017; Uhlen et al., 2010); however, no two reports are in agreement. Unfortunately, eIF2A is not included in the Yeast GFP Fusion Localization Database (Huh et al., 2003). First, Kim et al. reported that eIF2A is shuttled from the nucleus to the cytoplasm during viral infection to participate in HCV IRES reporter mRNA translation in Huh7 cells (Kim et al., 2011). This shuttling was not specific to viral infection as oxidative stress by sodium arsenite resulted in the same phenotype, suggesting that eIF2A localization was sensitive to cell stress in general. There are other instances of nuclear to cytoplasmic re-localization that occur during cell stress that are often regulated by reversible post-translational modifications such as phosphorylation (Averna et al., 2001; Sakurai et al., 2017; van der Sluijs et al., 1992). It would be of interest to determine whether eIF2A is differentially modified during cell stress in Huh7 cells and if this modification determines eIF2A localization. To our knowledge, whether this localization pattern is due to an alternate function of eIF2A, such as ribosome assembly, is not reported. Second, Panzhinskiy et al. demonstrated that eIF2A co-localizes

with the endoplasmic reticulum (ER) in MIN6 cells during normal physiological conditions (Panzhinskiy et al., 2021). ER localization during normal conditions is uncharacteristic of a translation initiation factor since translation initiation occurs in the cytosol. Whether eIF2A localization changes upon cell stress in MIN6 cells was not reported at the time of this review, but determining whether this occurs would be informative to elucidate if eIF2A is able to participate in translation initiation in this cell type. Finally, the Human Protein Atlas reports that eIF2A has a localization pattern consistent with mitochondria in both A-431 and U2OS cells; however, only one out of two antibodies showed this pattern and a control co-staining for mitochondria was lacking (Thul et al., 2017; Uhlen et al., 2010). Investigating eIF2A localization during both normal conditions and cell stress is vital to determine the mechanism and function of eIF2A in cells. A recent model suggests that eIF2A at levels similar or above other eIFs inhibit translation in vitro by directly binding and sequestering 40S ribosomal subunits (Grove et al., 2023). Non-cytosolic localization of eIF2A could be a method used by cells to prevent translational repression by keeping eIF2A away from the translational machinery.

3 | eIF2D AND MCT-1·DENR

3.1 | MCT-1·DENR resembles eIF2D

As described above, eIF2D (Tma64 in yeast) was initially identified in an intermediate purification fraction of eIF2A from rabbit reticulocyte lysate and was observed to possess GTP-independent initiator tRNA binding activity (Dmitriev et al., 2010). eIF2D is a 65 kDa monomer with N-terminal DUF 1947 and PUA domains, a central winged helix (WH) domain, and C-terminal SWIB/MDM2 and SUI domains (Figure 4) (Hellen, 2018; Lomakin et al., 2017; Tempel et al., 2013; Vaidya et al., 2017; Weisser et al., 2017). Uniquely, eukaryotes also contain genes that encode proteins that have homology to separate parts of eIF2D. Multiple copies in T-cell lymphoma-1 (MCT-1; Tma20 in yeast; 48% sequence identity) (Young et al., 2021) and density-regulated protein (DENR; Tma22 in yeast; 34% sequence identity) are homologous to the N- and C-terminal portions of eIF2D, respectively (Figure 4a) (Skabkin et al., 2010). Recent work, discussed in greater detail below, has provided evidence that MCT-1·DENR function as a dimer and has similar functions to eIF2D.

Structural studies of eIF2D in complex with initiator tRNA, an HCV IRES lacking domain II ΔdII HCV IRES), and the 40S ribosomal subunit have provided potential mechanistic insights (Weisser et al., 2017). The ΔdII HCV IRES was used since other HCV-like IRESs lacking this domain were previously shown to be more efficient in promoting 48S complex formation mediated by eIF2D or MCT-1·DENR than the wildtype versions of the IRESs (Skabkin et al., 2010). eIF2D makes several contacts with initiator tRNA through both the PUA and SWIB/MDM2 domains. Interactions between eIF2D and the methionine group of initiator tRNA were not detected, suggesting that eIF2D interacts indiscriminately with acylated and deacylated initiator tRNA in vitro, at least once the initiation complex is formed. In addition to resembling eIF1, structural data also showed that the SUI domain interacts similarly with the ribosome based upon its location near the P site and that it makes contacts with the codon-anticodon duplex. eIF2D overlaps with locations that would

be occupied by eIF1, eIF2, eIF5B, portions of eIF3, and possibly eIF1A on the 48S initiation complex (Weisser et al., 2017). This has led to implications of eIF2D in initiator tRNA recruitment, start codon recognition, subunit joining, and ribosome recycling.

Similar structural studies were performed for MCT-1·DENR in complex with the 40S ribosomal subunit alone and with initiator tRNA, ΔdII HCV IRES, and the 40S ribosomal subunit (Lomakin et al., 2017; Weisser et al., 2017). In these structures, the MCT-1·DENR dimer was shown to occupy similar locations on the ribosome and make similar contacts as eIF2D. To date, no structure has been obtained for eIF2D or MCT-1·DENR in complex with initiator tRNA alone (Weisser et al., 2017). Furthermore, efforts to show direct binding to initiator tRNA although UV crosslinking have also yielded unsatisfactory results (Skabkin et al., 2010). Therefore, direct evidence of eIF2D being able to bind and subsequently deliver initiator tRNA to the ribosome with endogenous mRNA substrates are lacking, although eIF2D is able to do so with an artificial AUG triplet. Consequently, more biochemical studies aimed at supporting the observations from these structural-based studies would be beneficial to strengthening the mechanistic details surrounding eIF2D and MCT-1·DENR involvement in initiation (Dmitriev et al., 2010; Skabkin et al., 2010).

3.2 | Evidence for a role in non-canonical initiation

Early work aimed at deciphering the mechanism of eIF2D in translation initiation used different in vitro reconstitution approaches. Using toe printing assays or RelE-cleavage assays, it was shown that recombinant eIF2D and MCT-1·DENR could successfully deliver initiator tRNA to the P site of 40S ribosomal subunits with HCV IRES and leaderless mRNA substates (Dmitriev et al., 2010; Skabkin et al., 2010). With eIF2D or MCT-1·DENR, both substrates allow for the direct placement of the start codon in the P site of the recruited 40S ribosomal subunit. Nevertheless, as observed by ribosome toe printing, the levels of 48S complex formation were still low in comparison to that of canonical initiation by eIF2. Interestingly, 48S complex formation via eIF2D could be accomplished without the requirement of GTP hydrolysis (Dmitriev et al., 2010). Remarkably, it was also observed that eIF2D could deliver other aminoacyl-tRNAs to non-canonical start codons, at least for the HCV IRES substrate. For example, when the AUG start codon of the HCV IRES was substituted to a UUU codon and the system was programmed with Phe-tRNA$^{Phe/UUU}$, 48S complex formation was still observed (Dmitriev et al., 2010). Efficient initiation was also observed for CUG and GUG codons in the presence of their respective cognate aminoacyl-tRNAs, Leu-tRNA$^{Leu/CUG}$ and Val-tRNA$^{Val/GUG}$ (Kearse & Wilusz, 2017; Skabkin et al., 2010; Starck et al., 2012).

Before obtaining structural information, how eIF2D and MCT-1·DENR were interacting with the ribosome or tRNA to promote formation of 48S complexes independent of the HCV IRES was initially addressed using sucrose gradient density centrifugation. This was accomplished by incubating both native and recombinant eIF2D or MCT-1 with purified 40S ribosomal subunits, 60S ribosomal subunits, and 80S ribosomes, performing centrifugation through a 10%–30% sucrose gradient, and collecting the ribosomal peak fractions and analyzing factor association through SYPRO staining and Western blotting (Skabkin et al., 2010). It was shown that eIF2D and MCT-1 could stoichiometrically bind to the

40S ribosomal subunit and the 80S ribosome but less efficiently to the 60S ribosomal subunit (Skabkin et al., 2010). The observation that these factors have potentially similar affinities to 40S ribosomal subunits and 80S ribosomes is interesting considering that eIF2 is displaced during subunit joining and does not have similar affinity for 80S ribosomes (Lapointe et al., 2022; Unbehaun et al., 2004). However, as explained in Section 3.3 below, these factors have been implicated in ribosome recycling and being able to recognize a terminating ribosome and recycle ribosomes would provide a logical reason for why these factors recognize both ribosome species with equal affinities. The association of eIF2D and MCT-1 with the different ribosome species can be inhibited by preincubation with eIF1 and to a greater extent when preincubation occurred with eIF1 and eIF1A (Skabkin et al., 2010), suggesting they share the same space on the ribosome. However, this did not impact association with 60S ribosomal subunit. Additionally, at least in the context of initiator tRNA recruitment with the HCV IRES-like Classical Swine Fever Virus (CSFV) IRES that lacks the non-essential domain II and the 40S ribosome by MCT-1·DENR, eIF1 could substitute for DENR. Whether or not eIF2D recruits initiator tRNA to the 40S ribosomal subunit through a direct interaction or indirectly by allosteric changes to the 40S subunit-IRES complex remains unclear. Attempts to show direct binding of eIF2D to initiator tRNA using UV-crosslinking resulted in weak detection of eIF2D bound to initiator tRNA, suggesting the mechanism of recruitment may be more indirect (Skabkin et al., 2010).

Despite several lines of evidence indicating that eIF2D, MCT-1, and DENR can interact with the translation machinery and form initiation complexes with the HCV IRES, most work to date has been performed in vitro. It remains to be determined whether these observations and principles occur within cellular environments when eIF2 is naturally competing for initiator tRNA and ribosomes. However, at least in HAP1 cells, HCV IRES translation has been shown to be resistant upon eIF2D knockdown under normal and cell stress conditions (Gonzalez-Almela et al., 2018). Single knockout of eIF2D, MCT-1, and DENR in yeast does not result in appreciable defects to translation across the transcriptome (Dmitriev et al., 2010). Thus, if eIF2D and MCT-1·DENR do function in initiation in cells, it may do so in a transcript-selective manner or in response to a specific environmental or developmental cue.

3.3 | Evidence for a role in ribosome recycling

Skabkin et al. noticed that the ribosomal salt wash fraction obtained from the purification of the 60S ribosome recycling factor, ABCE1, possessed the ability to dissociate deacylated tRNA from 40S ribosomal subunits that had been recycled off the mRNA (Skabkin et al., 2010). By mass spectrometry, the factor from the ribosomal salt wash fraction was identified as eIF2D. Using an in vitro reconstitution approach, it was discovered that both recombinant and native eIF2D could promote the release of deacylated tRNA and mRNA from unrecycled 40S ribosomes at stop codons. It was also shown that when recombinant MCT-1 and DENR were individually added to the reconstituted system, neither could produce efficient release of deacylated tRNA and mRNA from the recycled 40S ribosomal subunits. However, MCT-1·DENR could but at much lower efficiency than eIF2D (Skabkin et al., 2010).

Profoundly, Young et al. demonstrated in vivo, using 80S ribosome profiling in yeast, that double knockout of eIF2D (Tma64) and MCT-1 (Tma20) or DENR (Tma22) resulted in accumulation of 80S ribosomes upstream of stop codons (Young et al., 2018). Signal for 80S ribosomes was not detected at the stop codon, suggesting that the 40S ribosomal subunits were not recycled off the mRNA (Young et al., 2018). A similar approach using DENR knockout cells resulted in the same observation in HeLa cells (Bohlen, Harbrecht, et al., 2020). To provide more direct evidence, Young et al. subsequently used 40S ribosome profiling and confirmed the presence of unrecycled 40S ribosomal subunits at the stop codon (Young et al., 2021). More specifically, in yeast, MCT-1 (Tma20)·DENR (Tma22) contributed to most of the recycling activity; whereas eIF2D (Tma64) only minorly contributed when these factors were examined individually. Additionally, whether or not the identity of the penultimate codon to the termination codon influences the ability of eIF2D (Tma64), MCT-1 (Tma20), or DENR (Tma22) to recycle the 40S ribosome was also explored. It was shown in the double deletion yeast strains that codons that generally have slower tRNA dissociation rates (such as AAN codons) were more dependent on these recycling factors as evidenced by an increase in the average ratio of 40S termination codon peaks between the mutant strain and the wildtype strain. In contrast, codons that had generally fast tRNA dissociation rates (such as the UGU codon) were found to be less dependent on these factors (Gunisova et al., 2018; Young et al., 2021). The dependency on the penultimate codon for recycling by DENR was further corroborated in mammalian cells (Bohlen, Harbrecht, et al., 2020). Together, these data suggest that eIF2D (Tma64), MCT-1 (Tma20), or DENR (Tma22) might have a preference to recognizing ribosomes stalled at termination codons and that their role(s) are to primarily function as recycling factors, not as initiation factors.

3.4 | Evidence for a role in translation re-initiation

Given the observed ability of eIF2D and MCT-1·DENR to deliver aminoacyl-tRNAs to 40S ribosomal subunits and promote initiation in vitro, these factors have also been implicated in translation re-initiation (Bohlen, Harbrecht, et al., 2020; Castelo-Szekely et al., 2019; Schleich et al., 2014, 2017; Sherlock et al., 2023; Zinoviev et al., 2015). However, most instances of re-initiation governed by eIF2D and MCT-1·DENR could most likely be explained by their strong role in ribosome recycling. At least in yeast, most data shows that re-initiation occurs when eIF2D and MCT-1·DENR are depleted, suggesting they are both inhibitors of re-initiation.

In yeast, double knockout of eIF2D (Tma64) and MCT-1 (Tma20), or DENR (Tma22) resulted in increased reinitiation as evidenced by elevated ribosome occupancy at start codons in the 3’ untranslated region (UTR) for several different transcripts (Young et al., 2018). Further analysis showed that this phenotype is most likely due to MCT-1·DENR activity, as single MCT-1 (Tma20) and DENR (Tma22) deletion strains had increased re-initiation in the 3’ UTR but was only minorly present in the eIF2D (Tma64) deletion strains (Young et al., 2021). Thus, these factors play more of an inhibitory role toward translation re-initiation. It is important to note that the faulty ribosome recycling phenotypes seen in eIF2D and MCT-1·DENR deletion strains are highly consistent with subsequent re-initiation (Young et al., 2018; Young et al., 2021; Young & Guydosh, 2022). In fact,

a similar phenotype is seen when the bona fide recycling factor Rli1/ABCE1 is depleted (Young et al., 2015). The unrecycled ribosomes will have a greater opportunity to re-initiate downstream since they are kept on the mRNA (Hellen, 2018; Hronova et al., 2017; Vattem & Wek, 2004; Young & Guydosh, 2022; Young & Wek, 2016).

In mammalian cells, eIF2D, MCT-1, and DENR depletion causes an apparent decrease in re-initiation downstream of uORFs at the major or primary ORF (Figure 5) (Bohlen, Harbrecht, et al., 2020; Schleich et al., 2014; Schleich et al., 2017). This is best exemplified by Bohlen et al. with endogenous ATF4 protein levels in the presence and absence of these factors under normal conditions and in response to cellular stress (Bohlen, Harbrecht, et al., 2020). *ATF4* mRNA, which contains at least two uORFs in the 5' UTR (Lu et al., 2004; Pavitt & Ron, 2012; Vattem & Wek, 2004), is a classically studied eukaryotic transcript that is often used as a tool to derive principles of translation re-initiation. Under normal conditions, uORF1 is thought to be translated and that translation re-initiation favors uORF2, which overlaps with the main coding sequence and prevents ATF4 from being translated (Figure 5a, top). However, under stress conditions, uORF1 is still translated and translation re-initiation at the main ORF becomes favored (Figure 5a, bottom) (Vattem & Wek, 2004; Wek, 2018; Young & Wek, 2016). eIF2D KO cells, DENR KO cells, and MCT-1 KO cells showed similarly low levels of ATF4 protein expression under both normal growth and stress conditions (Figure 5b), and ATF4 protein levels could be rescued when KO cells were transfected with a plasmid expressing eIF2D, MCT-1, or DENR into their respective KO cells (Bohlen, Harbrecht, et al., 2020) (Figure 5c). The low ATF4 protein levels were further reduced under both conditions when eIF2D was knocked down in DENR KO cells. It was also observed, in corroboration with a role in ribosome recycling, that the identity of the penultimate codon resulted in uORFs being more dependent on eIF2D, MCT-1, or DENR for translation re-initiation. Specifically, uORFs that encoded AUG, GCG, or CTG as their penultimate codon were found to be more DENR-dependent (Bohlen, Harbrecht, et al., 2020). Nevertheless, upon eIF2D, MCT-1, or DENR deletion, re-initiation at uORF2 is most likely increased and further decreases ATF4 levels due to impaired ribosome recycling, in both normal and cell stress conditions (Figure 5b). However, direct measurement of uORF2 translation in this study was not reported.

3.5 | Connections to human disease

Considering the potential role eIF2D, DENR, and MCT-1 play in translation initiation, ribosome recycling, and translation re-initiation, it is not surprising that their dysregulation has been linked to several different pathologies. Tissue from patients with pancreatic ductal adenocarcinoma have been found to have lower levels of eIF2D (Golob-Schwarzl et al., 2020), whereas overexpression of MCT-1 has been linked to B- and T-cell lymphoma (Nandi et al., 2007; Prosniak et al., 1998; Shi et al., 2003). In the case of diffuse large B-cell lymphomas, it was discovered that the elevated MCT-1 protein levels were the result of increased stabilization of the protein. Specifically, following DNA damage via irradiation, MCT-1 is phosphorylated on residue T81 by p44/p42 MAPK in a lymphoma cell line and this phosphorylation results in enhanced protein stability (Nandi et al., 2007). When mutational analysis studies were performed by substituting the threonine residue for alanine, which prevents the residue from being phosphorylated by p44/p42 MAPK, MCT-1 protein

levels in NIH 3T3 cells were shown to gradually decay following DNA damage. The importance of the stabilization of MCT-1 to the progression of cancer was highlighted by growth curves in human dermal fibroblasts and NIH 3T3 cells that showed that cells ectopically expressing mutant MCT-1 had reduced cell proliferation in comparison to wildtype MCT-1 (Nandi et al., 2007). Moreover, eIF2D and MCT-1·DENR have been shown to regulate translation re-initiation of several oncogenes including *ATF4, a-Raf, c-Raf*, and *CDK4* mRNAs (Bohlen, Harbrecht, et al., 2020). How these factors are being regulated and what drives changes in their expression levels in cells is still largely unknown. Future studies aimed at addressing these gaps in knowledge could provide potential therapeutic insights for different types of cancers.

In addition to alterations in expression levels of eIF2D, MCT-1, and DENR correlating to different diseases, it has also been shown that mutations to the proteins themselves have been found in patients with specific diseases. For example, mutations in DENR have also been observed in autism spectrum disorder patients (Haas et al., 2016; Neale et al., 2012). The C37Y mutation (Figure 4a) disrupts the zinc-binding domain of DENR that is required for interaction with MCT-1, and the P121L mutation (Figure 4a) is found within the β1 loop of the SUI domain (Lomakin et al., 2017; Weisser et al., 2017). Young et al. tested these different mutations in yeast to determine their effects on ribosome recycling using ribosome profiling. It was observed that for the orthologous mutation of C37Y in yeast (C11Y) that ribosome recycling was impaired; whereas, for the orthologous mutation of P121L (A105L), no defects in recycling were detected (Young et al., 2021). It was speculated that these differences in observations were perhaps due to the C11Y mutation disrupting the association for DENR with MCT-1 and that this lack of association leads to degradation of DENR; however, no data for this was shown. A more recent example has demonstrated that mutations in MCT-1 from patients with Mendelian susceptibility to mycobacterial disease (MSMD) also result in defective recycling and consequently reduction in translation re-initiation (Bohlen et al., 2023). In all cases, it was shown that the different mutations in MCT-1 result in lower expression levels of MCT-1. Specifically, it was shown using 40S and 80S ribosome foot printing that one of the patient mutations that introduces a premature stop codon (R72X) (Figure 4a) into the coding sequences results in accumulation of 40S ribosomes on the stop codons of both the main ORF and uORFs in SV40-fibroblasts from that patient (Bohlen et al., 2023). The impaired recycling phenotype could then be rescued by transduction with a lentiviral vector that expressed the wildtype version of MCT-1. It was also observed that accumulation in 40S ribosomal subunits were more pronounced on coding sequences that had AUG and GCG codons as their penultimate codon, which further corroborates previous observations in mammalian cells depleted of DENR (Bohlen, Harbrecht, et al., 2020). It was also shown using 80S ribosome foot printing that, in addition to the R72X mutation, patients that expressed MCT-1 lacking residues 133–181 (Figure 4a) had lower levels of translation re-initiation across the genome. One of the genes found to have 3- to 4-fold lower expression levels than control cells was Janus kinase 2 (JAK2). It was concluded that loss of JAK2 results in decreased synthesis of interferon-gamma (IFN-γ) in lymphocytes, which drives the disease phenotype (Bohlen et al., 2023). Together, these data provide further support for the role of MCT-1 and DENR in ribosome recycling and highlight the importance of ribosome recycling in preventing disease phenotypes.

eIF2D, MCT-1, and DENR have also been tested as genetic modifiers or modulators of neurological disorders caused by non-canonical translation of expanded repeats (similar to the discussion in Section 2.4 for eIF2A). For example, Green et al. showed using expanded GGGGCC and CGG repeat reporters in HEK293 cells that DENR knockdown resulted in a 50% reduction in RAN translation (Green et al., 2022). This phenotype was also observed when MCT-1 was knocked down. Interestingly, knockdown of eIF2D did not result in a similar inhibitory effect, but other reporter-based studies in HEK293 cells by Sonobe et al. showed that knockdown of eIF2D results in >50% reduction in RAN translation of an expanded GGGGCC repeat (Sonobe et al., 2021). Consistent with these data from mammalian cells, it was shown in vivo using a fly model of CGG RAN translation, that knockdown of DENR results in reduction of RAN translation (Green et al., 2022). Survival of flies that ubiquitously expressed an expanded GGGGCC repeat only showed prolonged survival rates when DENR was depleted (Green et al., 2022). Although eIF2D appears to be unable to ameliorate disease conditions from RAN translation in flies, others have demonstrated that the introduction of loss-of-function mutations in eIF2D using CRISPR/Cas9 genome editing in *Caenorhabditis elegans* models of RAN translation have increased survival rates (Sonobe et al., 2021). The rationale behind these contrasting results is still unclear and more studies are needed to clarify these observations. Furthermore, more complementary biochemical experiments would be beneficial to deciphering if these observations are due to defects in initiation, re-initiation, or recycling.

4 | CRITICAL QUESTIONS MOVING FORWARD

The GTP-independent nature of eIF2A, eIF2D, and MCT-1·DENR provides a challenge to efficiently deliver initiator tRNA to the P site of the 40S ribosomal subunit. In canonical eukaryotic translation, once the scanning 43S PIC recognizes a start codon and forms the 48S initiation complex, eIF2 loses affinity for the initiator tRNA upon GTP hydrolysis and release of Pi (Algire et al., 2005; Kapp & Lorsch, 2004). eIF2 then dissociates upon eIF5B association and 60S ribosomal subunit joining. An analogous regulated affinity for initiator tRNA has not been described for eIF2A, eIF2D, or MCT-1·DENR, but one must exist for efficient initiation. Steady association with initiator tRNA throughout initiation and the first two cycles of elongation until the initiator is released from the E site could bypass this requirement; however, such a mechanism has not been described for any eIF or tRNA binding factor in translation, nor is their sufficient space within the P and E sites to accommodate a relatively large (~65 kDa) protein. Whether dissociation of eIF2A, eIF2D, or MCT-1·DENR from initiation complexes is driven by conformational changes during subunit joining or elongation has also not been reported. Recent single molecule FRET experimental paradigms have been extremely valuable in deciphering the exact order and kinetics of eIF association and dissociation during canonical initiation (Lapointe et al., 2022). Adopting a similar strategy for eIF2A-, eIF2D-, and MCT-1·DENR-dependent translation initiation would be highly informative.

Identifying which open reading frames are regulated by eIF2A, eIF2D, and MCT-1·DENR is crucial and most likely a key to understanding true function. Multiple reports using ribosome profiling demonstrate that nearly all ORFs are not regulated by eIF2A and support that eIF2D and MCT1·DENR primarily function in ribosome recycling (Gaikwad et al., 2024;

Ichihara et al., 2021; Sendoel et al., 2017; Young et al., 2018; Young et al., 2021; Young & Guydosh, 2022). Despite having a reported equal high affinity for initiator tRNA, deletion of eIF2A in multiple systems does not affecting global translation, and eIF2A null yeast strains and mice are completely viable (Gaikwad et al., 2024; Ichihara et al., 2021; Komar et al., 2005; Sendoel et al., 2017; Starck et al., 2016; Zoll et al., 2002). In mice, a phenotype is only seen after 1 year of age (Anderson et al., 2021). For eIF2D and MCT-1·DENR, strong data supports a function in ribosome recycling rather than initiation. Specifically, ribosome profiling studies using eIF2D and MCT-1·DENR deletion yeast strains have demonstrated a shared role in ribosome recycling after termination on most ORFs and mRNAs (Young et al., 2018, 2021), but stronger phenotypes were seen with MCT-1·DENR deletion. Similar studies in mammalian cells have shown that depletion of DENR results in defects to 40S ribosomal subunit recycling on mRNAs with specific penultimate codons (Bohlen, Harbrecht, et al., 2020). With having a role in ribosome recycling, eIF2D and MCT-1·DENR must have the ability to interact with tRNA and ribosomes. Thus, this raises the important question—are the observations that eIF2D and MCT-1·DENR can form complexes with initiator tRNA and 40S subunits in vitro merely because of the biochemical properties they have to truly function in ribosome recycling? More evidence is needed for eIF2A, eIF2D, and MCT-1·DENR to show direct selectivity of specific mRNAs, if any. UV CLIP-seq-based experiments could be informative (as shown for DAP5 (David et al., 2022; de la Parra et al., 2018)) if specific contacts are made with a subset or class of mRNAs. However, if eIF2A, eIF2D, and MCT-1·DENR do not contact mRNAs in a selective manner, selective 40S profiling (e.g., combining formaldehyde crosslinking, immunoprecipitation, and ribosome profiling) (Bohlen, Fenzl, et al., 2020; Wagner et al., 2020) may provide to be a more fruitful strategy.

eIF2D was shown to occupy the P site and make direct contact with the HCV IRES and initiator tRNA when in complex with the 40S ribosomal subunit in vitro (Weisser et al., 2017). During normal growth conditions, when ternary complex (eIF2·GTP·initiator tRNA) levels are high, most free 40S ribosomal subunits are bound by ternary complexes, most likely preventing eIF2A, eIF2D, and MCT-1·DENR from occupying the P site. If this was not so, one would expect an increase in translation upon eIF2A, eIF2D, and MCT-1·DENR depletion; yet translation levels are reported to be stable in such depletion strains and lines. How do eIF2A, eIF2D, and MCT-1·DENR successfully outcompete eIF2·GTP·initiator tRNA for the 40S ribosomal subunit to initiate translation? In the case of HCV IRES, eIF2D has been reported to directly interact with the IRES in vitro, at least when the 40S ribosomal subunit and initiator tRNA are present. To our knowledge and at the time of this review, no evidence has been published showing direct specific interaction of eIF2D and cellular mRNAs. Furthermore, translation of the HCV IRES in HAP1 cells has been reported to be unaffected upon eIF2D depletion (Gonzalez-Almela et al., 2018).

Cell stress is commonly associated with reports demonstrating the effect of increasing or decreasing levels of eIF2A, eIF2D, and MCT-1·DENR on specific mRNA reporters. In many cases, negative control reporters that do not respond are lacking, but are critical to demonstrate a specific effect. Nevertheless, how could cell stress stimulate translation initiation by eIF2A, eIF2D, and MCT-1·DENR? At least two non-mutually exclusive pathways may exist. First, free 40S subunit concentrations rise during cell stress (due

to low levels of TCs) and could provide more opportunity for other initiation factors to associate. During cell stress in mammalian cells, one of the four mammalian ISR kinases (GCN2, HRI, PERK, PKR; yeast only encode GCN2) phosphorylate eIF2± at residue S51, resulting in the sequestration of eIF2B—the guanine exchange factor for eIF2. Without free eIF2B, eIF2·GDP is not able to form eIF2·GTP·initiator tRNA, severely limiting canonical translation initiation. Since eIF2 outnumbers eIF2B 10:1 (Bogorad et al., 2018), even 10% of p-eIF2α would result in near complete eIF2B sequestration and significantly decrease TC levels after free eIF2 goes through one round of initiation. In such conditions, free 40S ribosomal subunit concentrations would increase rather dramatically and be available for eIF2A, eIF2D, and MCT-1·DENR. However, Gaikwad et al. recently tested this scenario with WT and eIF2A null yeast strains and found eIF2A had little to no function in translation (Gaikwad et al., 2024). Second, eIF2A, eIF2D, and MCT-1·DENR could preferentially associate with the 40S ribosomal subunit during cell stress if the affinity between the two increased during cell stress. Do these factors somehow become “activated” by the ISR kinases or another stress-induced pathway in parallel or downstream? It is not commonly believed that any of the four ISR kinases have targets other than eIF2α, at least none that regulate translation initiation since cells that encode the eIF2α-S51A mutation are completely resistant to stress-induced translation inhibition (Baird et al., 2014; Scheuner et al., 2001). However, at least one report has shown that eIF2A shuttles from the nucleus to the cytoplasm in Huh7 cells during cell stress and viral infection. Whether eIF2A shuttling occurs in other cell types and the mechanism was not reported. Enigmatically, eIF2A has been reported to also co-localize with the ER in MIN6 cells (Panzhinskiy et al., 2021) and have localization pattern resembling mitochondria in U2OS and A-431 cells. It is not clear how eIF2A could function in translation initiation if localized or sequestered away from the translation machinery in the cytosol.

5 | CONCLUSION

Many exciting and mechanistic questions remain for eIF2A, eIF2D, and MCT-1·DENR. With the advancements in gene editing, selective ribosome profiling, and cyro-EM, the field is on the brink of finding important insights into their exact function during translation. At least for eIF2D, and MCT-1·DENR, most data from genetic deletion studies suggests their primary role is to aid in ribosome recycling. Nevertheless, direct in vivo evidence of eIF2A, eIF2D, and MCT-1·DENR to form initiation complexes on cellular mRNAs—either in natural, disease, developmental contexts, upon environmental cues—is a critical next step.

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DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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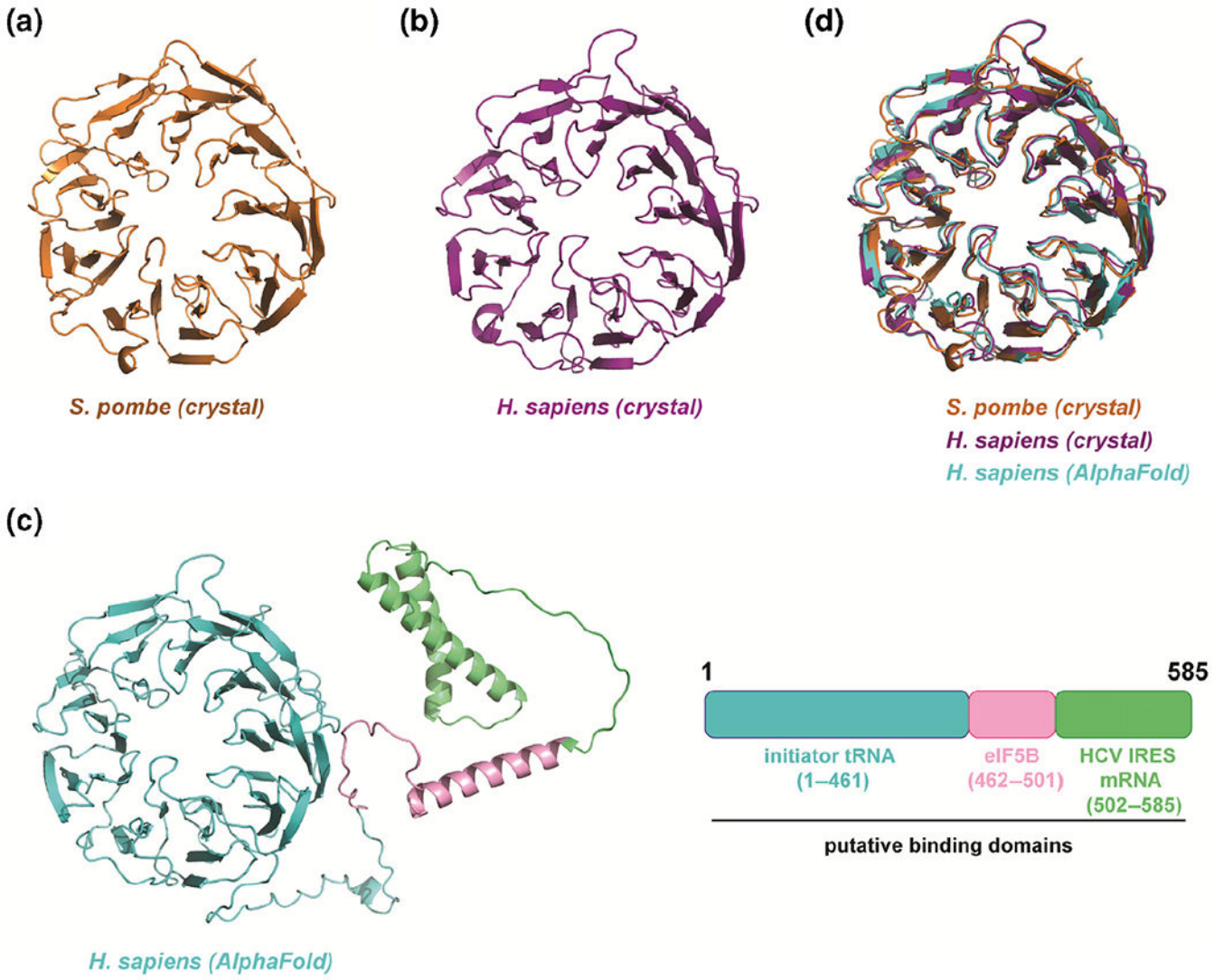

FIGURE 1.

Crystal structures of *S. pombe* and *H. sapiens* eIF2A N-terminus are homologous. (a) Crystal structure of C-terminally truncated *S. pombe* eIF2A (residues 1–424) solved at 2.5 Å. PDB: 3WJ9. (b) Crystal structure of C-terminally truncated *H. sapiens* eIF2A (residues 4–427) solved at 1.8 Å. PDB: 8DYS. (c) AlphaFold predicted structure of full-length *H. sapiens* eIF2A with specific putative binding domains colored (left). Schematic of labeled putative binding domains (right). (d) Overlay of the N-terminal β-propeller in eIF2A from panels a–c.

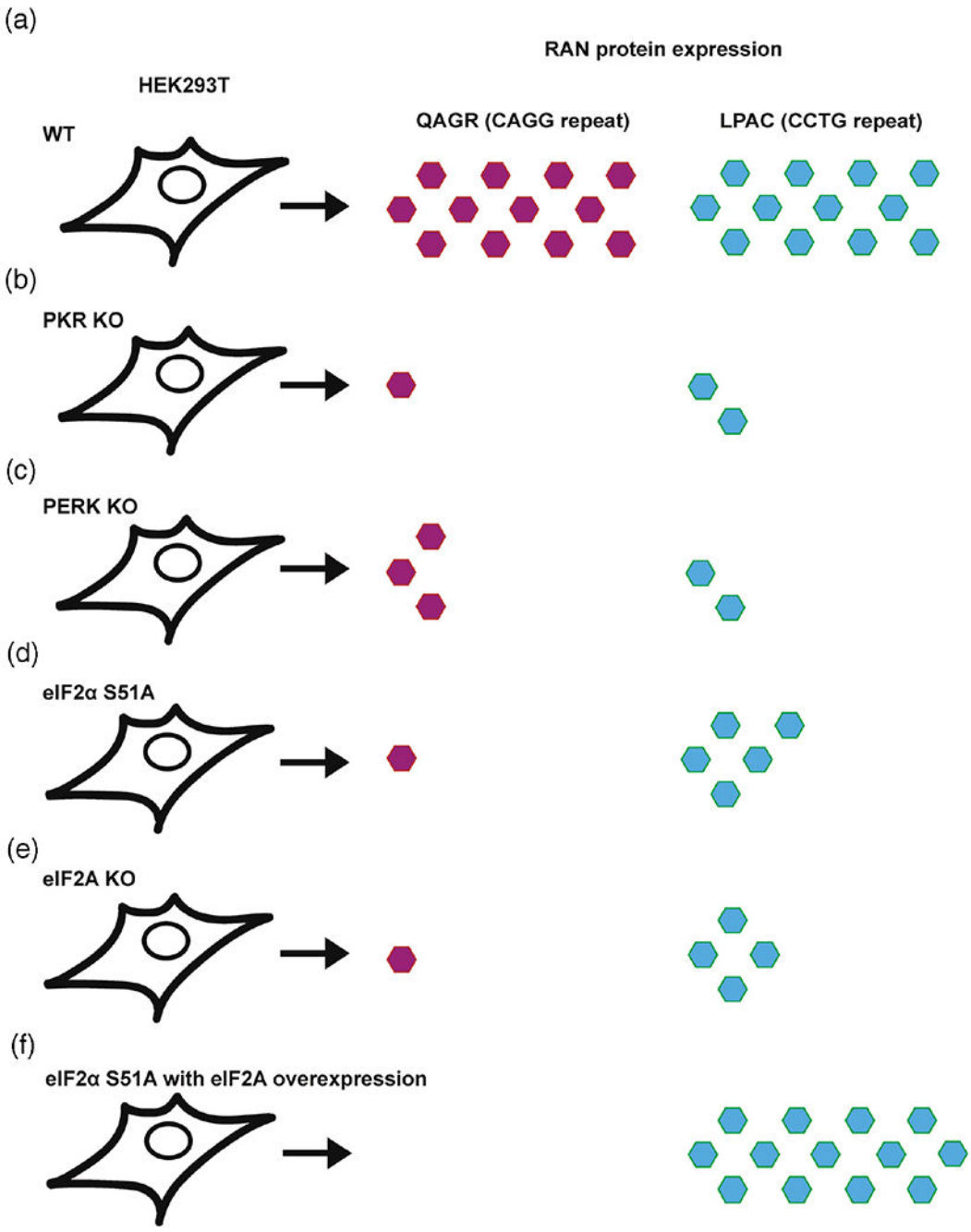

FIGURE 2.

(a–f) eIF2A-dependent RAN translation of the *CCUG* repeat is downstream of eIF2α S51 phosphorylation. RAN translation of expanded *CCUG* repeats (encodes QAGR repeat protein) or expanded *CAGG* repeats (encodes LPAC repeat protein) in WT HEK293T cells, PKR KO, PERK KO, eIF2 S51A mutants, eIF2A KO, and eIF2 S51A mutant with eIF2A overexpression. Protein levels were respective to those measured in WT cells for each reporter.

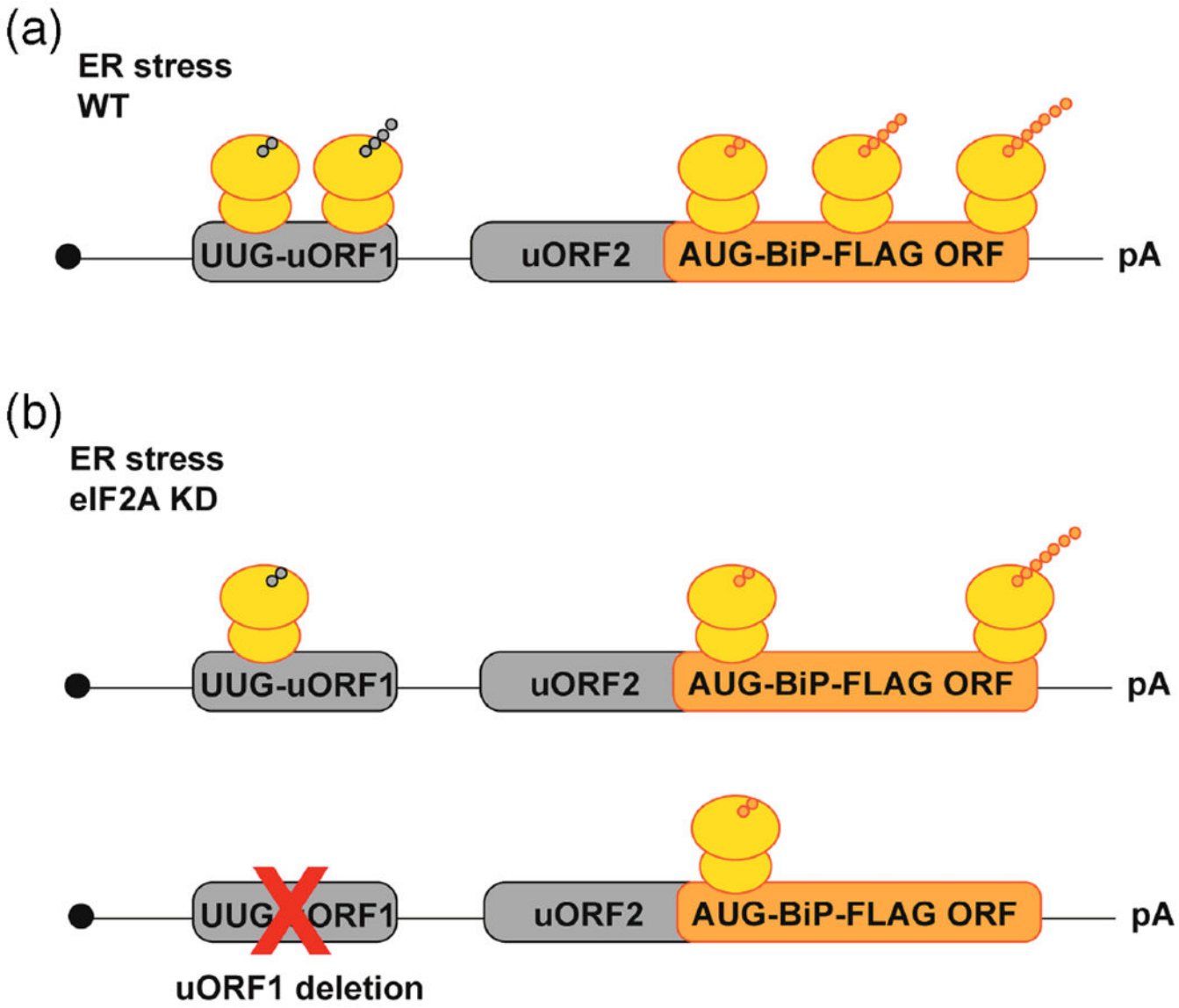

FIGURE 3.

BiP-FLAG reporter expression is sensitive to eIF2A protein levels. (a) UUG-encoded uORF1 and BiP-FLAG protein levels are resistant to translational repression during ER stress. (b) Upon eIF2A depletion, both uORF1 and BiP-FLAG levels decrease during ER stress (top). This decrease is exacerbated when uORF1 is mutated and cannot be translated (bottom).

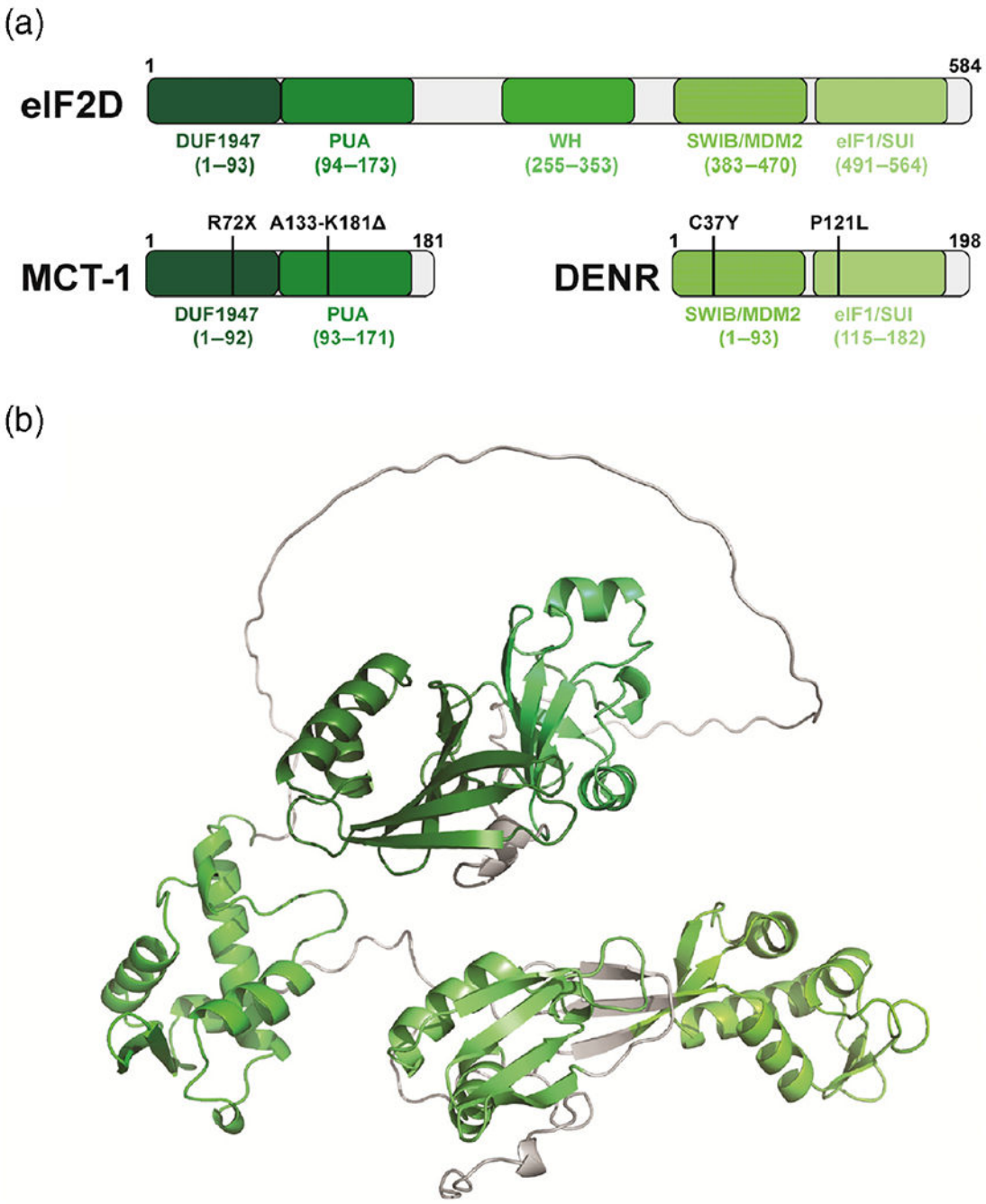

FIGURE 4.

MCT-1·DENR resembles eIF2D. (a) Schematic of *H. sapiens* eIF2D, MCT-1, and DENR showing homologous domains and location of patient-associated Mendelian susceptibility to mycobacterial disease (MSMD) mutations in MCT-1 and autism spectrum disorder mutations in DENR. DUF1947, domain of unknown function 1947; MDM2, mouse double minute 2 homolog; PUA, pseudouridine synthase and archaeosine transglycosylase; SUI, suppressor of initiator codon mutations; SWIB, SWI/SNF complex including complex B;

WH, central winged helix. (b) AlphaFold predicted structure of *H. sapiens* eIF2D structure with domains colored as described in panel a.

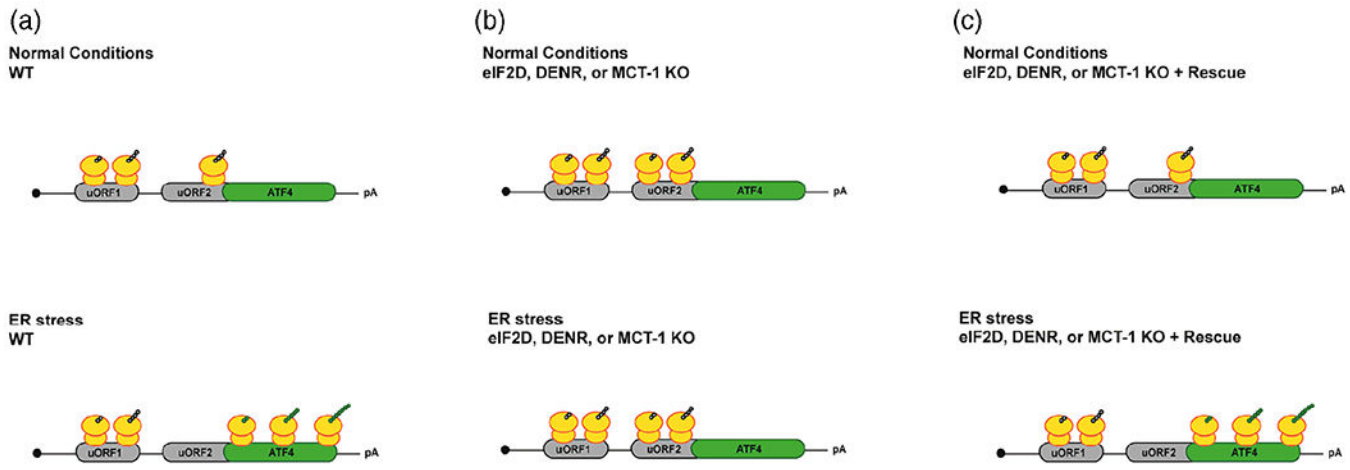

FIGURE 5.

ATF4 expression is sensitive to eIF2D, MCT-1, and DENR protein levels under normal growth and ER stress conditions. (a) In the presence of eIF2D, MCT-1, and DENR, expression of ATF4 by re-initiation is favored under tunicamycin-induced ER stress in HeLa cells. (b) When eIF2D, MCT-1, and DENR are deleted, re-initiation is decreased under both conditions, which results in lower levels of ATF4 protein. (c) Rescue of eIF2D, MCT-1, and DENR in their knockout cells returns ATF4 expression to being favored under tunicamycin-induced ER stress.