The Localization and Functional Characterization of a DNAJC5-like Protein in *Dictyostelium discoideum*

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Abstract

The Localization and Functional Characterization of a DNAJC5-like Protein in *Dictyostelium discoideum*

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DNAJC5, an HSP40 member, supports synaptic vesicle release and protein folding by activating HSP70 ATPase activity. In humans, it localizes to presynaptic terminals and endomembrane compartments that are involved in protein trafficking. Mutations in *DNAJC5* cause CLN4 disease, a rare adult-onset Batten disease. *Dictyostelium discoideum*, a model for neurodegenerative research, encodes a putative homolog of DNAJC5, Dnajc5 (DDB0306688), which remains uncharacterized. This study examined Dnajc5 localization and function in *D. discodieum*. Dnajc5 localized to the endoplasmic reticulum, cytoplasm and nucleolus under both growth and starvation conditions, suggesting a role in proteostasis. Unlike human DNAJC5, Dnajc5 was absent from endomembrane compartments and extracellularly during starvation. Protein quantification revealed increased levels during early development, peaking at the mound stage, and declining thereafter—paralleling gene expression. Immunoprecipitation of Dnajc5 showed no serine phosphorylation or ubiquitination, unlike human DNAJC5. These findings suggest functional differences despite a possible common role in proteostasis.

Keywords: DNAJC5, heat shock proteins, chaperones, Batten disease, CLN4 disease, *Dictyostelium discoideum*, multicellular development, endoplasmic reticulum, plasma membrane, endosomes, cytoplasm, nucleolus, secretion, immunofluorescence, actinomycin- D, western blotting, Grp78, immunoprecipitation, serine/threonine phosphorylation, ubiquitination.

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List of Abbreviations

Actinomycin D	AM-D
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
cAMP	3′,5′-cyclic Adenosine Monophosphate
CB	Conditioned Buffer
CLN4	Neuronal Ceroid Lipofuscinosis type 4
CM	Conditioned Media
Countin	CtnA
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CUPS	Compartment for Unconventional Protein Secretion
eMI	Endosomal Microautophagy
ER	Endoplasmic Reticulum
ERAD	ER-Associated Degradation
ESCRT	Endosomal Sorting Complex Required for Transport
Glucose-Regulated Protein 78	Grp78
HPD	Histidine-Proline-Aspartic acid
HRP	Horseradish Peroxidase
HSP	Heat Shock Protein
ILV	Intraluminal Vesicles

IP	Immunoprecipitation
LC-MS	Liquid Chromatography-Mass Spectrometry
MAPS	Misfolded-Associated Protein Secretion
MVB	Multi Vesicular Bodies
NCL	Neuronal Ceroid Lipofuscinosis
NoLS	Nucleolar Localization Signal
PD	Protein Depleted
pI	Isoelectric Point
PVDF	Poly-Vinylidene DiFluoride
qRT-PCR	Quantitative Reverse Transcriptase Polymerase Chain Reaction
REMI-Seq	Restriction Enzyme-Mediated Integration Sequencing
rRNA	ribosomal RNA
SBDS	Shwachman-Bodian-Diamond Syndrome
SDS	Sodium Dodecyl Sulfate
TBST	Tris-Buffered Saline with 1% Tween-20
UPS	Ubiquitin-Proteasome System
WC	Whole Cell lysate

Chapter 1

1. Introduction

1.1 Proteostasis and Its Regulation

Proteostasis, or protein homeostasis, is crucial for cellular function and overall organismal health, ensuring the proper synthesis, folding, trafficking, and degradation of proteins (Díaz-Villanueva et al., 2015). Proteins must fold correctly to function. Disruptions in proteostasis can lead to severe diseases, including neurodegenerative, metabolic, and cardiovascular disorders, as well as cancers. These conditions often result from misfolded or aggregated proteins that overwhelm cellular maintenance and cause irreversible damage (Balch et al., 2008; Hipp et al., 2014). To maintain proteostasis, cells employ interconnected mechanisms that involve the endoplasmic reticulum (ER), which plays a key role in protein synthesis and folding. When proteins misfold, the unfolded protein response alleviates this stress by increasing molecular chaperones and reducing overall protein synthesis (Braakman et al., 2013; Cybulsky, 2017). Additionally, the ubiquitin-proteasome system tags defective proteins for degradation via the proteasome (Hershko, 1998), while autophagy eliminates damaged organelles and large protein aggregates through lysosomal degradation (Kelekar, 2006). These systems collectively ensure protein quality control and prevent toxic protein accumulation, preserving cellular health and function.

1.1.1 Molecular Chaperones and Heat Shock Proteins

Molecular chaperones are proteins that help other proteins fold, assemble, and stabilize, without becoming incorporated into their final structure (Hartl, 1996). Although many proteins can fold into their native state independently, chaperones are essential in preventing misfolding and aggregation. Most molecular chaperones depend on ATP to drive the conformational changes required for binding, folding, and releasing proteins. They assist by attaching to polypeptides,

helping them fold correctly, and then letting them go to prevent interference from other cellular components (Clare and Saibil, 2013; Hartl, 1996). During this process, ATP enables chaperones to alternate between a low-affinity state—where they bind unfolded proteins loosely when ATP is attached—and a high-affinity state—where they bind tightly after ATP is hydrolyzed to ADP, stabilizing the protein for proper folding (Farr et al., 1998). If a protein fails to fold correctly despite chaperone assistance, the chaperone remains bound to the protein, triggering the activation of second messengers or molecular inducers. These inducers either stimulate the production of additional chaperones or promote the degradation of the misfolded protein (Cybulsky, 2017). Chaperones can independently assist with protein folding; however, to finely regulate protein substrate interactions, folding, disaggregation, degradation, and trafficking within the cell, they often work in combination with various cochaperones (Care and Sabil, 2013). A specific group of molecular chaperones, known as Heat Shock Proteins (HSPs), forms an intricate, cooperative network within the cell. HSPs are upregulated during stressful conditions when levels of aggregation-sensitive unfolded proteins rise (Hu et al., 2022). These chaperones are categorized by their molecular weight (HSP20, HSP40, HSP60, HSP70, HSP90, and HSP100) and are well conserved across species (Feder et al., 1999; Hu et al., 2022). They play a significant role in preventing misfolding and aggregation, ensuring the integrity of the cellular proteome. When proteins cannot be repaired, chaperones help direct them to degradation pathways for removal. One such chaperone, HSP40, is essential in this process. As a co-chaperone, HSP40 aids other chaperones, particularly HSP70, by stabilizing unfolded proteins and facilitating their proper folding (Wyttenbach et al., 2000; Alderson et al., 2016). This helps prevent the accumulation of misfolded proteins like alpha-synuclein, amyloid-beta peptides, and huntingtin, thereby maintaining overall proteostasis within the cell (Witt et al., 2013; Ring et al.,

2022; Lotz et al., 2010) Integral to this quality control system are several organelles and compartments within mammalian cells:

- **Endoplasmic Reticulum (ER):** The ER is a membrane-bound organelle central to protein synthesis, folding, and quality control (Palade, 1956). It hosts molecular chaperones such as GRP78/BiP (Hsp70 family) that assist in protein folding and regulate calcium homeostasis (Lee, 1987; Pobre et al., 2019). Misfolded proteins in the ER are identified and removed via the ER-associated degradation (ERAD) pathway, targeting them for destruction by the ubiquitin-proteasome system (Ruggiano et al., 2014; Pukatzki et al., 1998).

- **Golgi Apparatus:** After initial folding in the ER, proteins are trafficked to the Golgi apparatus—a stack of membrane-bound sacs responsible for post-translational modification, sorting, and transport (Rios & Bornes, 2003). Modifications like glycosylation enhance protein stability and function (Schneider et al., 2010). The Golgi also acts as a checkpoint, ensuring that only correctly folded and processed proteins proceed to their final destinations (Alberts et al., 2002).

- **Proteasome:** The proteasome is a multi-subunit complex that degrades misfolded, damaged, or unnecessary proteins. Proteins tagged with ubiquitin are directed to the proteasome for breakdown into peptides, a vital process for maintaining cellular homeostasis, especially during stress conditions (Pukatzki et al., 1998; Ciechanover & Schwartz, 2018; Smalle, 2006)

- **Nucleolus:** Traditionally known for its role in ribosome biogenesis (Boisvert et al., 2007), the nucleolus is also involved in protein quality control and stress responses

(Frottin et al., 2019). In mammalian cells, nucleolar proteins such as nucleophosmin and HSPs contribute to folding and stability under stress (Frottin et al., 2019). Disruption of transcription with agents such as Actinomycin D (AMD) causes nucleolar fragmentation and alters the localization of nucleolar proteins, highlighting the nucleolus's sensitivity to cellular stress (Yung et al., 1985).

Altogether, these organelles and the intricate network of molecular chaperones coordinate with each other to ensure protein homeostasis, supporting cell survival and function during both normal and stressful conditions.

1.1.2 HSP40 Protein Family

HSP40, also known as the DnaJ family, is a crucial co-chaperone that assists HSP70 in protein quality control by regulating its ATPase activity. The defining feature of HSP40 is the J-domain, a conserved region of approximately 70 amino acids containing the HPD (Histidine-Proline-Aspartic acid) motif, which is essential for stimulating HSP70's ATPase function (Kityk et al., 2018). Hsp40 first binds to Hsp70 while it is in its ATP-bound state. The ATP binding site is located in the N-terminal nucleotide-binding domain (NBD) of Hsp70, where ATP binding and hydrolysis regulate its conformational changes and substrate interaction. In this conformation, HSP70's substrate-binding domain remains open, allowing unfolded or misfolded proteins to interact efficiently (Figure 1) (Hasegawa et al., 2018). HSP40 plays a key role in delivering these misfolded or unfolded proteins to HSP70, ensuring they are properly engaged for folding or refolding. Once the substrate is positioned, Hsp40 enhances Hsp70's ATPase activity, leading to ATP hydrolysis. This reaction converts ATP to ADP and releases an inorganic phosphate (Pi), which is freed into the cytoplasm to be recycled or used in other cellular processes (Muller et al., 2019). The energy released drives Hsp70's conformational change into a high-affinity, closed

state, allowing it to tightly hold the substrate protein (Figure 1) (Hasegawa et al., 2018). The loss of Hsp40 binding occurs immediately after ATP hydrolysis, as this conformational change reduces Hsp40's affinity for Hsp70. Although these steps are tightly coupled and often described together, ATP hydrolysis precedes or coincides with Hsp40 dissociation, making them sequential but rapid events. This step is crucial for preventing protein aggregation and promoting proper folding (Hasegawa et al., 2018) (Figure 1). After the folding process, a nucleotide exchange factor (NEF) facilitates the release of ADP from HSP70, allowing a new ATP molecule to bind (Kityk et al., 2018). This ATP binding resets HSP70 into its open conformation, leading to either the release of the properly folded protein or another cycle of chaperone activity if needed (Figure 1) (Hasegawa et al., 2018). This cycle ensures protein homeostasis and prevents the accumulation of misfolded or aggregated proteins, which could be detrimental to cellular function (Wyttenbach et al., 2000; Alderson et al., 2016). HSP40 proteins are classified into three types. Type I (e.g., DnaJA1) possesses a J-domain, G/F-rich region, and a zinc-finger-like domain for direct substrate binding. Type II lacks the zinc-finger domain but retains chaperone function. Type III contains only the J-domain, modulating HSP70 without direct substrate interaction (Li et al., 2009). This HSP70-HSP40 system maintains proteostasis, aiding protein folding, transport, and stress responses. Dysregulation is implicated in neurodegeneration, cancer, and protein misfolding disorders. A notable Type III member, DNAJC5 (CSPα), is highly expressed in neurons, regulating synaptic vesicle function and neuroprotection via membrane-associated palmitoylation (Fernández-Chacón et al., 2023).

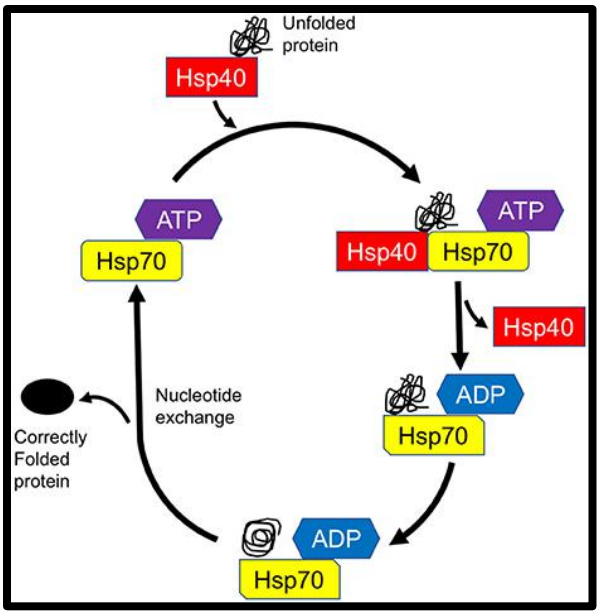

Figure 1. Model of Protein Folding Mediated by the Hsp70-Hsp40/DnaJ Chaperone System. Hsp40/DnaJ initially recognizes and transiently binds to unfolded or misfolded proteins, facilitating their delivery to Hsp70. Through its conserved J-domain, Hsp40 interacts directly with Hsp70 and stimulates its intrinsic ATPase activity. This stimulation accelerates the hydrolysis of ATP bound to Hsp70's nucleotide-binding domain, converting it into the ADP-bound state. In this ADP-bound conformation, Hsp70 undergoes a structural change that allows it to bind tightly and stabilize the unfolded protein, preventing aggregation. Subsequently, nucleotide exchange factors promote the release of ADP and the binding of a new ATP molecule, triggering Hsp70 to adopt a low-affinity conformation that releases the now properly folded protein. This cyclical process ensures efficient folding and quality control of proteins within the cell (Hasegawa et al., 2018).

1.2 DNAJC5 (Cysteine String Protein, CSPα)

DNAJC5, also known as CSPα (cysteine string protein alpha), is a 198-amino-acid-long type III HSP40 that contains a single J-domain (Musskopf et al., 2018) (Figure 2). Despite its simple structure, DNAJC5 plays a crucial role in neurotransmitter release and neuronal proteostasis at synaptic terminals (Fernández-Chacón et al., 2018). The human *DNAJC5* gene, located on chromosome 20q13.33, encodes this protein, which is essential for nervous system function (Deloukas et al., 2001). Mutations in DNAJC5 affect its palmitoylation, causing the protein to mislocalize and aggregate, which contributes to the development of neurodegenerative diseases (Naseri et al., 2020

DNAJC5 consists of three main domains:

- N-terminal and J-domain (~70 amino acids): This domain interacts with Hsp70, activating its ATPase function to facilitate protein folding and degradation. It is highly conserved across species and contains the essential HPD (histidine-proline-aspartic acid) motif required for Hsc70 binding (Figure 2) (Hennessy et al., 2000; Tsai and Douglas, 1996). Additionally, it features a ubiquitination site at lysine-58, enabling DNAJC5 to participate in the ubiquitin degradation pathway (Wang et al., 2021; Patel et al., 2016). Phosphorylation at serine-10 via serine/threonine specific protein kinasecan inhibit lysine-58 interaction, thereby modulating DNAJC5's interactome and function (Patel et al., 2016). While Ser10 is the most well-characterized phosphorylation site, phosphoproteomic studies suggest that DNAJC5 contains multiple additional serine phosphorylation sites that may regulate its activity, interactions, and stability in various cellular contexts. Of the 41 DNAJ proteins, 36 are known to undergo serine/threonine phosphorylation (Hornbeck et al., 2015). While DNAJC5 is known to be phosphorylated at serine 10 by a serine/threonine kinase, it is possible that specific threonine residues are also phosphorylated, although this has not been experimentally demonstrated (Patel et al., 2016).
- Adjacent to the J-domain, a 30-amino-acid linker region plays a crucial role in synaptic vesicle exocytosis by maintaining normal resting calcium ion (Ca^{2+}) levels in synaptic terminals, which are essential for regulating neurotransmitter release (Boal et al., 2011).
- Cysteine-string region: This domain contains 13–15 cysteine residues that undergo palmitoylation, enabling DNAJC5 to associate with membranes, particularly synaptic

vesicles and the presynaptic membrane, facilitating synaptic vesicle exocytosis (Noskova et al., 2011) (Figure 2). A similar mechanism is observed in non-neuronal cells, where DNAJC5 mediates the export of misfolded proteins through the misfolded-associated protein secretion pathway (Wu et al., 2023). This region is essential for vesicle fusion and efficient neurotransmitter release. Mutations or disruptions within this domain can impair synaptic transmission and contribute to neurodegenerative disorders (Noskova et al., 2011).

- C-terminal domain: This domain is subject to alternative splicing, generating different isoforms that may influence DNAJC5's function in protein interactions and vesicle trafficking (Boal et al., 2004; Boal et al., 2007). This variability allows DNAJC5 to adapt to neuronal activity and synaptic demands, ensuring proper synaptic function (Boal et al., 2004).

DNAJC5 is uniquely characterized by its membrane association, which is regulated by palmitoylation (Fernández-Chacón et al., 2023). This modification enables DNAJC5 to localize predominantly to synaptic vesicles in presynaptic nerve terminals, distinguishing it from many other chaperones that function freely in the cytosol. In addition to synaptic vesicles, DNAJC5 is also found on intracellular membranes such as ER, endosomes, lysosomes, and the plasma membrane, with its localization dynamically shifting in response to neuronal activity (Wu et al., 2023; Xu et al., 2018). This activity-dependent redistribution allows DNAJC5 to efficiently regulate synaptic vesicle recycling, neurotransmitter release, and vesicle trafficking. Functionally, DNAJC5 plays a crucial role in maintaining neuronal proteostasis by ensuring proper protein folding, trafficking, and degradation at synapses. Working in conjunction with HSP70, it prevents protein misfolding and aggregation, particularly in the highly active

environment of neuronal synapses (Piette et al., 2021). Beyond its role in proteostasis, DNAJC5 is also essential for synaptic vesicle exocytosis and endocytosis, maintaining the balance between vesicle fusion and retrieval—processes that are vital for sustained neurotransmission (Nyaka et al., 2021). Disruptions in DNAJC5 function can impair synaptic transmission, ultimately contributing to neurodegenerative diseases such as neuronal ceroid lipofuscinosis or Batten disease (Henderson et al., 2016).

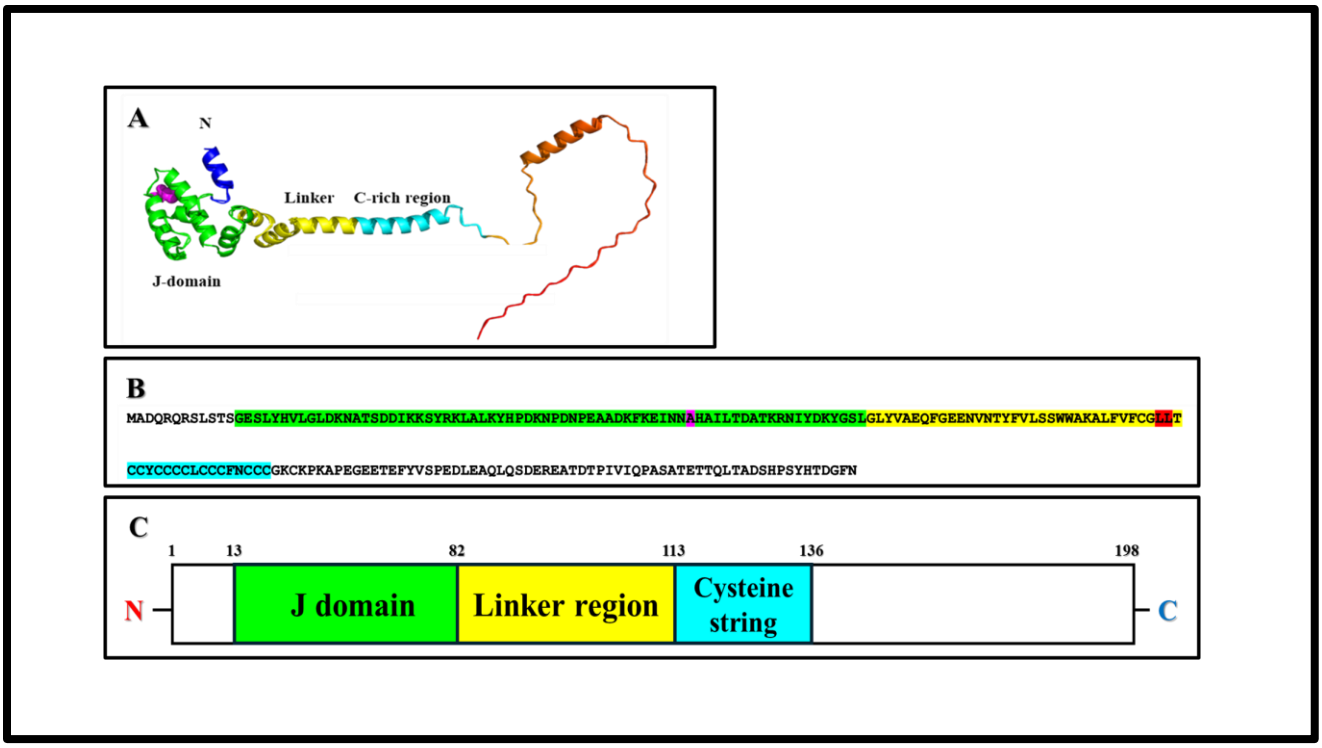

Figure 2. Structural and domain organization of the human DNAJC5 protein. A) Predicted 3D structure of DNAJC5 generated using PyMOL, with domain-specific color coding: J-domain (green), linker region (yellow), and cysteine-string domain (cyan). **B)** Amino acid sequence of DNAJC5 with functional domains highlighted using the same color scheme. Uncommon mutation site (Ala63) and common mutation (Leu115 and Leu116) sites are marked in pink and red, respectively. **C)** Schematic representation of the DNAJC5 domain architecture, indicating the N-terminal (N) and C-terminal (C) ends. DNAJC5 is a type III J-protein that plays key roles in molecular chaperone activity and synaptic vesicle function.

1.2.1 Batten Disease Overview

Batten disease, or Neuronal Ceroid Lipofuscinosis (NCL), is a group of genetically inherited neurodegenerative disorders that primarily affect children (Cooper et al., 2022). Characterized by a progressive loss of motor control, seizures, vision impairment, and dementia, Batten disease ultimately leads to death (Schulz et al., 2013). There are 13 known subtypes of NCL, each named according to the mutated gene. The severity of symptoms, age of onset, and progression rate vary across these subtypes, but they all share a common pathological hallmark: the accumulation of toxic substances in the brain (Zaib et al., 2023). At the cellular level, NCL is classified as a lysosomal storage disorder. In affected individuals, lysosomes fail to properly degrade and remove waste products from cells (Simonati, and Williams, 2022). One such waste product, ceroid lipofuscin, is a fluorescent material that builds up in lysosomes, causing cellular toxicity and neurodegeneration (Rodríguez, 2017). The inability of lysosomes to clear waste in a timely manner results in the malfunction of numerous cell processes, ultimately causing neuronal death and the clinical symptoms seen in Batten disease (Simonati & Williams, 2022; Rodríguez, 2017). Ceroid lipofuscin is a product of lysosomal digestion, consisting of oxidized lipids and proteins. Its accumulation in cells is associated with aging and lysosomal dysfunction, which impairs cellular waste removal, leading to cellular degeneration, including brain degeneration (Seehafer and Pearce, 2006).

1.2.2 CLN4 Disease

Mutations in *DNAJC5* cause neuronal ceroid lipofuscinosis type 4 (CLN4) disease, a rare adult-onset form of Batten disease (Mink et al., 2013). Autosomal dominant mutations, primarily in the cysteine-string domain, disrupt palmitoylation, leading to protein misfolding, defective membrane localization, and synaptic dysfunction (Nosková et al., 2011; Mink et al., 2013; Jarrett

et al., 2018; Valenzuela-Villatoro et al., 2018). Common mutations include Leu115Arg and Leu116Δ, while Ala63Val is rarer (Nosková et al., 2011; Faruq et al., 2021). These alterations impair synaptic vesicle dynamics and increase neuronal toxicity. Symptoms of CLN4 disease typically appear between ages 25 and 46, but cases can emerge during puberty. Common symptoms include speech difficulties, involuntary movements, and seizures (Anderson et al., 2013). Neuropathologically, autofluorescent ceroid lipofuscin accumulates in neurons, visible under UV light, alongside dark granular osmiophilic deposits seen via electron microscopy (Anderson et al., 2013). The dysfunction of DNAJC5 in CLN4 disease highlights its crucial role in synaptic function and neuronal health, linking it to broader protein misfolding disorders such as Alzheimer's, Parkinson's, and Huntington's diseases (Hasegawa et al., 2018). Given its essential function in proteostasis, DNAJC5 represents a key target for research into neurodegenerative diseases and the development of therapeutic strategies.

1.3 Molecular Pathways Involving DNAJC5 and Its Mechanistic Role

Understanding the pathogenesis of CLN4 disease requires insight into the diverse functions of DNAJC5 in neuronal proteostasis. As a key molecular chaperone, DNAJC5 regulates neurotransmitter release, the ubiquitin-proteasome system (UPS), endosomal microautophagy (eMI), misfolded-associated protein secretion (MAPS), and cellular stress responses (Sharma et al., 2011; Wang et al., 2021; Lee et al., 2022; Xu et al., 2018; Hasegawa et al., 2018). These pathways coordinate protein folding, trafficking, and degradation, maintain neuronal homeostasis. DNAJC5 dysfunction disrupts proteostasis, leading to synaptic impairment, lysosomal dysfunction, and neurodegeneration (Mink et al., 2013; Jarrett et al., 2018; Valenzuela-Villatoro et al., 2018). The molecular mechanisms of DNAJC5 aid in understanding CLN4 disease pathology and in the development of therapeutic strategies.

1.3.1 Exocytosis of Synaptic Vesicles via DNAJC5

DNAJC5 plays a vital role in nerve cell function, particularly in the communication between neurons, as it is essential for neurotransmitter release from nerve cell terminals, facilitating synaptic transmission (Fernández-Chacón et al., 2018). At pre-synaptic vesicles, DNAJC5 forms a chaperone complex with HSP70 (Heat Shock Protein 70) and small glutamine-rich tetratricopeptide (SGT) proteins, which helps maintain the proper conformation of Synaptosome-Associated Protein 25 (SNAP25) at the nerve terminals. By stabilizing SNAP25, DNAJC5 ensures the proper formation of a membrane fusion synaptic complex, essential for synaptic vesicle binding to the membrane, leading to neurotransmitter release into the synaptic cleft and enabling neuron-to-neuron communication (Kashyap et al., 2016; Jarrett et al., 2018; Sharma et al., 2011) (Figure 3). In DNAJC5 knockout mice and Drosophila, SNAP25 misfolding disrupts SNARE complex formation, impairing synaptic vesicle fusion, neurotransmitter release, and recycling, leading to severe neurological issues and premature death (Huang et al., 2022; Imler et al., 2019). In CLN4 disease, DNAJC5 mutations prevent palmitoylation, hindering neurotransmitter release and driving neurodegeneration (Noskova et al., 2011). Proper DNAJC5 function is essential for synaptic health, with dysfunction causing severe neurological consequences.

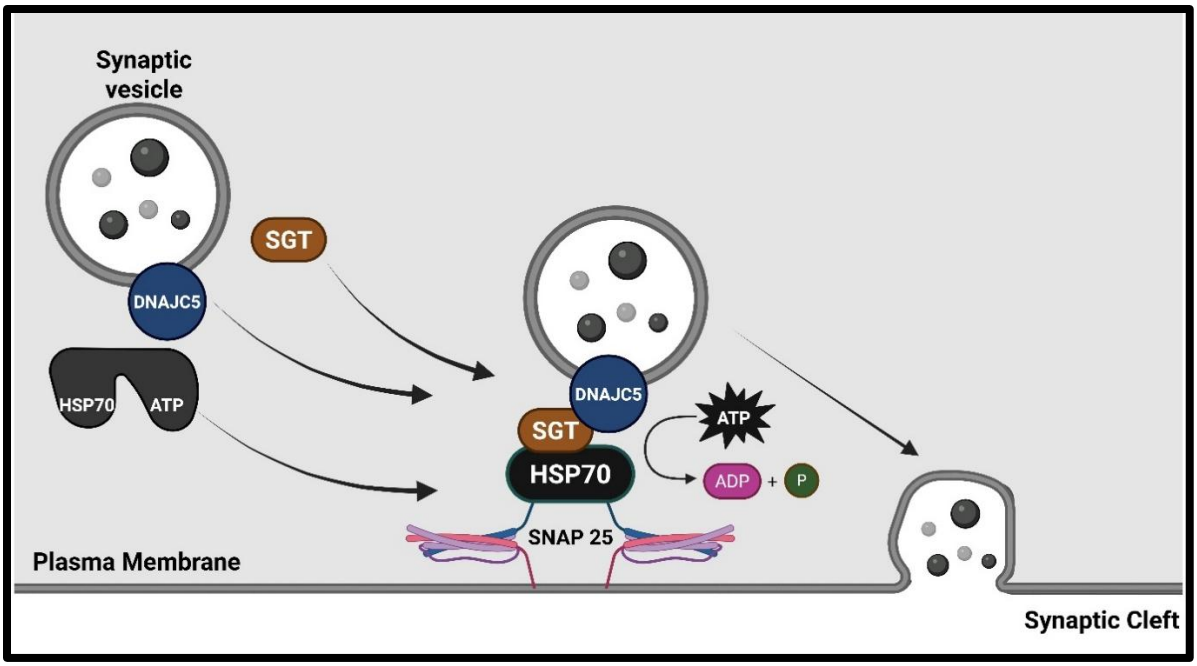

Figure 3. CSPα-HSP70-SGT Chaperone Complex Regulates Synaptic Vesicle Dynamics. The role of CSPα, HSP70, and SGT in synaptic vesicle dynamics and neurotransmitter release. CSPα, in complex with HSP70 and SGT, facilitates proper protein folding and prevents protein aggregation at synaptic terminals. This chaperone complex interacts with SNAP-25, utilizing ATP hydrolysis to regulate synaptic vesicle exocytosis.

1.3.2 Cellular Stress Response and Neuroprotection by DNAJC5

Neurons are particularly susceptible to oxidative stress, heat shock, and proteotoxic stress, which can cause protein misfolding and aggregation. The molecular chaperone system, involving DNAJC5 and HSP70, serves as the defense mechanism against these stressors. When cells experience stress, DNAJC5 upregulates chaperone activity, stabilizing misfolded proteins and preventing them from forming aggregates (Figure 1) (Haswega et al., 2018). If a protein is irreversibly damaged, DNAJC5 facilitates its degradation through various protein quality control mechanisms.

1.3.3 DNAJC5 Role in UPS

UPS plays a crucial role in cellular homeostasis by degrading damaged or excess proteins. This process involves ubiquitin tagging and the 26S proteasome, with E1, E2, and E3 enzymes mediating the pathway, where E3 ligases are essential for substrate recognition and polyubiquitination (Ciechanover & Schwartz, 2018; Smalle, 2006). DNAJ proteins, such as DNAJC5, regulate E3 ligase activity by stabilizing ligases and enhancing substrate interactions. DNAJC5 supports the SCF (Skp1-Cul1-F-box) E3 ligase complex by stabilizing SKP2, an F-box protein responsible for ubiquitinating p27, a key regulator of the cell cycle (Wang et al., 2021) (Figure 4). Elevated DNAJC5 expression increases SKP2 stability, promoting p27 degradation and advancing cell cycle progression (Wang et al., 2021; Kossatz et al., 2004). Conversely, DNAJC5 knockdown reduces SKP2 stability, restoring p27 levels and inhibiting proliferation. Dysregulation of this pathway is implicated in hepatocarcinoma (Wang et al., 2021). Phosphorylation at serine-10 (Ser-10) plays a regulatory role in modulating DNAJC5 function by influencing the accessibility of lysine-58 (Lys-58), a known site for ubiquitination. Patel et al. (2016) demonstrated that phosphorylation at Ser-10 blocks access to Lys-58, likely through conformational or electrostatic changes that prevent E3 ubiquitin ligases from recognizing and binding to this site. This prevents DNAJC5 from being tagged for degradation via the ubiquitin-proteasome system. While this finding suggests that Ser-10 phosphorylation may alter the fate of DNAJC5—potentially redirecting it toward alternative protein quality control pathways such as chaperone-mediated refolding or autophagy—these downstream effects have not been experimentally confirmed. Further studies are needed to determine whether phosphorylation at Ser-10 actively reroutes DNAJC5's function or simply prevents its degradation. Nonetheless, this modification likely serves as a regulatory switch that helps fine-tune DNAJC5's role in

proteostasis, especially under changing physiological or stress conditions in neurons. Mutations in *DNAJC5* may disrupt SKP2 stability, impair p27 degradation, and lead to protein accumulation. In CLN4 disease, defective DNAJC5 function disrupts protein homeostasis, contributing to neurodegeneration. These findings highlight the essential role of DNAJC5 in proteostasis and disease prevention.

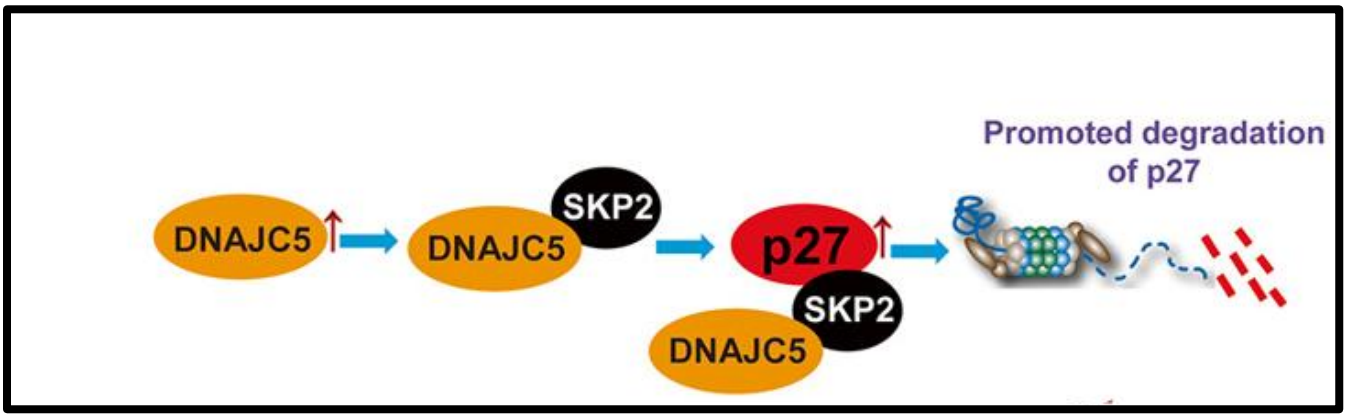

Figure 4. DNAJC5-Mediated Ubiquitin-Proteasome Degradation of p27.
The role of DNAJC5 in promoting p27 degradation through the ubiquitin-proteasome pathway. DNAJC5 interacts with SKP2, an E3 ubiquitin ligase component, to facilitate the ubiquitination and subsequent degradation of p27. The reduction of p27, a key cell cycle regulator, leads to aberrant cell cycle progression and increased cellular proliferation (Wang et al., 2021).

1.3.4 The Role of DNAJC5 in MAPS

MAPS pathway is a compensatory mechanism that allows neurons to export misfolded and aggregation-prone proteins into the extracellular space instead of degrading them intracellularly (Lee et al., 2018). DNAJC5 plays a key role in recognizing and directing misfolded proteins toward secretion pathways. Under normal conditions, misfolded proteins are identified by USP19 and HSP70 at the ER and trafficked to a perinuclear compartment, known as the Compartment for Unconventional Protein Secretion (CUPS), a Golgi-proximal structure that serves as a sorting hub for MAPS (Lee et al., 2022) (Figure 5). Within CUPS, DNAJC5, in association with CD98hc (SLC3A2), facilitates the retrieval and sorting of these proteins for

secretion (Lee et al., 2022) (Figure 5). A crucial aspect of DNAJC5's function in MAPS is its cysteine string domain, which undergoes palmitoylation, a modification necessary for its localization to CUPS and its ability to form large oligomeric assemblies that enhance misfolded protein secretion (Xu et al., 2018; Lee et al., 2022; Wu et al., 2022). This modification involves the covalent attachment of palmitic acid to the cysteine residues via thioester bonds, catalyzed by DHHC-type palmitoyl acyltransferases (Tabaczar et al., 2017). Palmitoylation increases the hydrophobicity of DNAJC5, promoting its stable association with intracellular membranes, including those that form CUPS (Longo et al., 2014). This membrane anchoring and oligomerization are necessary for DNAJC5 to participate effectively in the misfolding-associated protein secretion pathway. In the absence of palmitoylation, DNAJC5 becomes mislocalized and loses its ability to support secretion of misfolded cytosolic proteins, thereby disrupting proteostasis (Wu et al., 2022). While DNAJC5 and USP19 significantly contribute to MAPS, their absence only partially impairs the process, suggesting functional redundancy with other membrane-associated chaperones or the existence of alternative secretion mechanisms (Xu et al., 2018). This model highlights how MAPS serves as an essential pathway for maintaining proteostasis, with DNAJC5 and CUPS acting as key regulators in the extracellular release of misfolded proteins, providing an alternative to degradation-based quality control mechanisms. Therefore, mutations in the *DNAJC5* gene could lead to the accumulation of misfolded proteins, resulting in cellular toxicity and neurodegeneration (Wu et al., 2018).

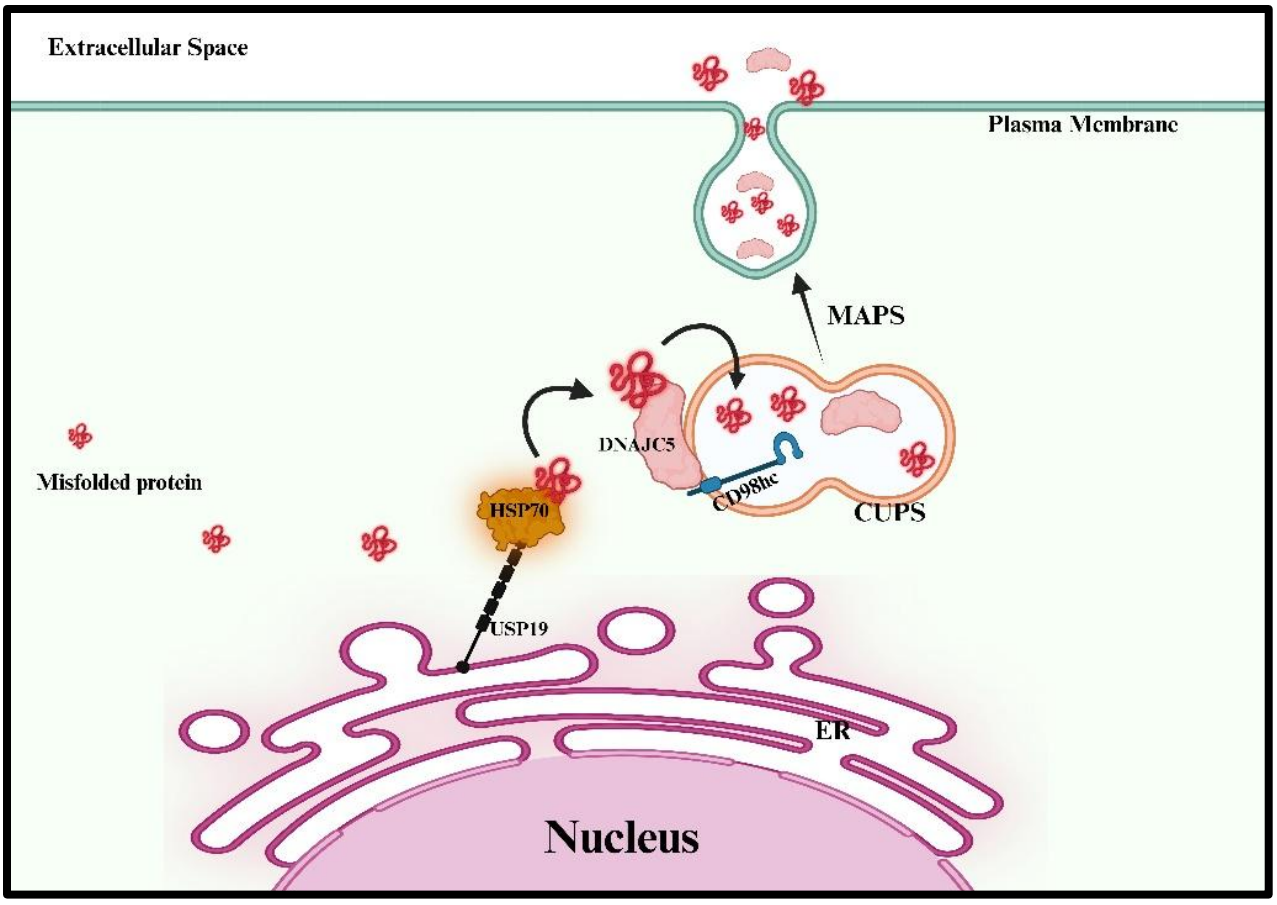

Figure 5. DNAJC5-mediated Misfolded Associated Protein Secretion (MAPS). Misfolded proteins are identified by USP19 and HSP70 at the ER and transported to the Compartment for Unconventional Protein Secretion (CUPS), a perinuclear compartment, where DNAJC5 facilitates their sorting. CD98hc plays a role in processing these proteins for secretion. Through MAPS, misfolded proteins, along with DNAJC5, are trafficked to the plasma membrane and released into the extracellular space**.**

1.3.5 DNAJC5 Facilitates eMI

Endosomal microautophagy (eMI) is a specialized autophagic pathway in which cytoplasmic proteins and organelles are sequestered within intraluminal vesicles (ILVs) of multivesicular bodies (MVBs) via late endosomal membrane invagination (Marzella et al., 1981; Oku and Sakai, 2018). Unlike macroautophagy, which relies on autophagosome formation, eMI enables direct substrate uptake into late endosomes prior to lysosomal degradation, playing a crucial role in proteostasis across species (Klionsky and Codogno, 2013; Mukherjee et al., 2016;

Lee et al., 2022). The endosomal sorting complex required for transport (ESCRT), a membrane-remodeling machinery, mediates ILV formation and cargo sorting during eMI (Boura et al., 2012). Protein substrates can be targeted through ubiquitination or chaperone recognition, with HSP70 aiding in selective translocation (Shields et al., 2009; Tekirdag and Cuvero, 2018). DNAJC5, a key regulator of selective eMI, localizes to late endosomal/lysosomal membranes and directs misfolded proteins into MVBs via an ESCRT-dependent mechanism (Lee et al., 2022; Sahu et al., 2011) (Figure 6). Unlike chaperone-mediated autophagy, which relies on the lysosomal receptor LAMP2A for substrate translocation and requires KFERQ-like motifs for HSP70 recognition, DNAJC5 functions independently of these elements (Kacal et al., 2021). Instead, DNAJC5 directly binds misfolded proteins and directs them into intraluminal vesicles (ILVs) for either lysosomal degradation or extracellular release via exosomes (Sahu et al., 2011) (Figure 6). DNAJC5 dysfunction in neurodegenerative diseases such as CLN4 and Alzheimer's disrupts misfolded protein processing, impairing eMI and leading to the accumulation of toxic protein aggregates (Lee et al., 2022). This dysregulation alters amyloidogenic signaling and neuronal proteostasis, ultimately contributing to synaptic dysfunction and disease progression.

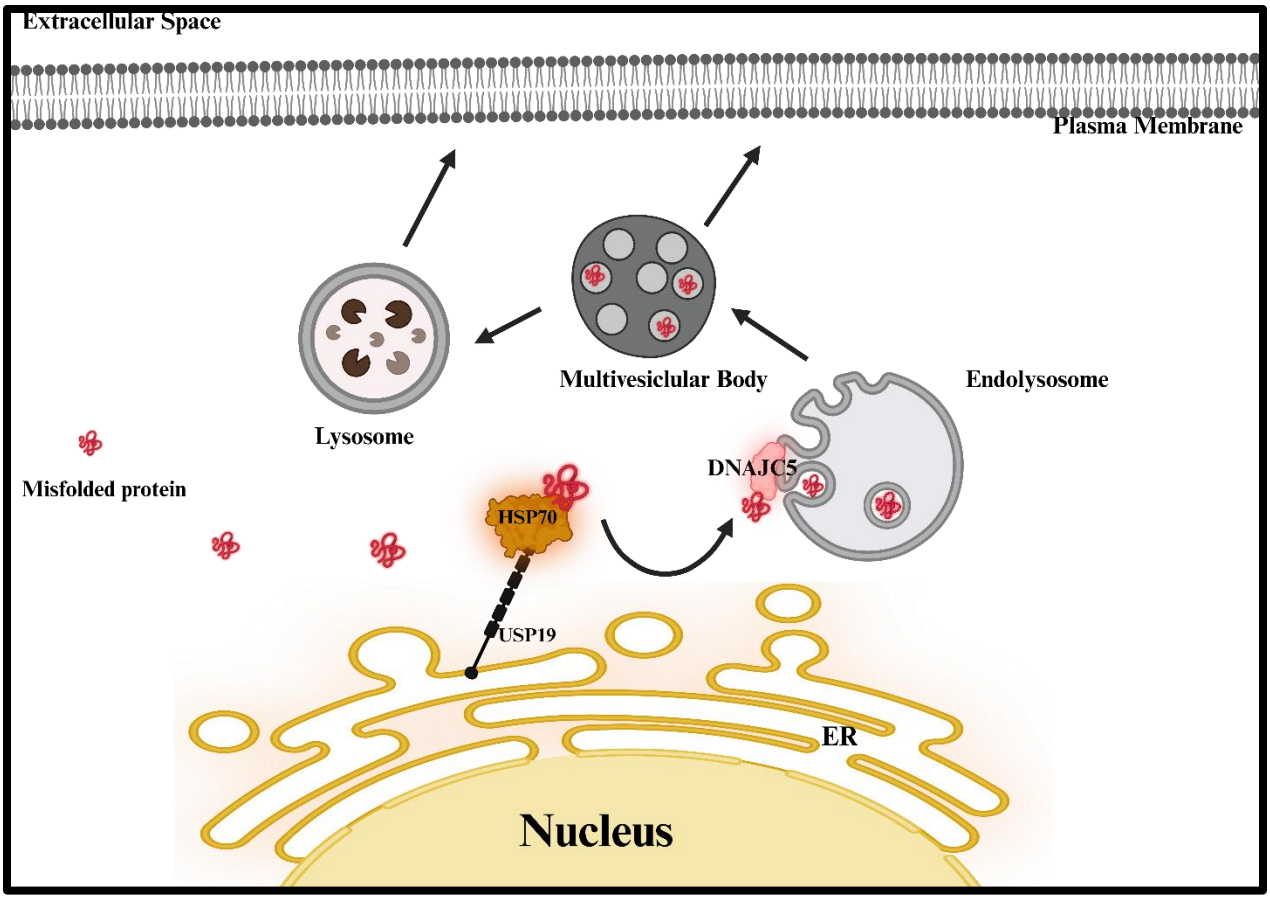

Figure 6. Endosomal microautophagy facilitated by DNAJC5. Misfolded proteins are recognized by the ER-associated chaperone HSP70, recruited by USP19, and directed towards endosomal microautophagy. DNAJC5 mediates the uptake of misfolded proteins into endolysosomes, where they can be processed and degraded. Alternatively, misfolded proteins may be sorted into multivesicular bodies (MVBs) or targeted to lysosomes for degradation.

1.4 Research gap in DNAJC5 Function and Its Role in CLN4 Disease

Despite advancements in understanding DNAJC5 in CLN4 disease, key mechanistic questions remain. While DNAJC5 dysfunction causes misfolding, defective palmitoylation, and impaired membrane localization, its precise impact on neuronal function, synaptic vesicle dynamics, and the accumulation of toxic storage material is unclear (Nosková et al., 2011; Mink et al., 2013; Jarrett et al., 2018; Valenzuela-Villatoro et al., 2018). Additionally, the role of DNAJC5 in endolysosomal function remains poorly defined. It is uncertain whether it directly mediates cargo selection for degradation or primarily influences lysosomal trafficking. Its

interplay with other proteostasis pathways, including the ubiquitin-proteasome system and chaperone-mediated autophagy, also requires further investigation to explain the neuron-specific vulnerability in CLN4 disease. The variability in disease onset and severity suggests the influence of genetic and environmental modifiers. Despite its autosomal dominant inheritance, CLN4 disease exhibits diverse clinical presentations, and it remains unknown whether genetic factors or external stressors like neuronal activity levels contribute to disease progression (Mink et al., 2013). Beyond CLN4 disease, DNAJC5's role in neurodegeneration remains an open question. Given its function in proteostasis and synaptic regulation, its dysfunction can contribute to diseases such as Alzheimer's and Parkinson's (Huang and Zhang et al., 2022). However, whether *DNAJC5* mutations directly drive these disorders or if its dysregulation is secondary to broader proteostasis failure remains unresolved. Addressing these gaps will be crucial for advancing targeted therapies for CLN4 disease and related neurodegenerative diseases. Various models, including mammalian cell lines, mice, *Drosophila*, and *C. elegans*, have provided insights into DNAJC5's role in synaptic vesicle trafficking, endolysosomal function, and protein quality control, particularly in neurodegenerative diseases such as CLN4 (Wu et al., 2023; Fernández-Chacón et al., 2004; Imler et al., 2019; Kashyap et al., 2014). However, their genetic complexity, compensatory mechanisms, and evolutionary differences limit precise dissection of DNAJC5-specific functions. *Dictyostelium discoideum*, a genetically tractable eukaryote with conserved cellular processes, offers a powerful alternative. Its rapid life cycle and ability to be genetically modified enable detailed studies of DNAJC5 in protein degradation, vesicle recycling, and disease mechanisms.

1.5 Background on *Dictyostelium discoideum*

Dictyostelia, or social amoebae, are unicellular eukaryotes that transition to a multicellular state via aggregation under nutrient scarcity, forming fruiting bodies for spore dispersal (Baldauf & Strassmann, 2017). Taxonomically classified within Amoebozoa, the sister group of Obazoa (which includes animals and fungi), they share conserved cellular pathways with more complex eukaryotes (Cavalier-Smith et al., 2015). Among ~150 identified species, *D. discoideum* is a widely studied model for cellular processes such as chemotaxis, autophagy, and signal transduction (Bozzaro, 2013; Müller-Taubenberger et al., 2013). Its relatively simple 34 Mb haploid genome, encoding ~12,500 proteins, can be genetically manipulated which facilitates research on fundamental eukaryotic processes, including cell motility, phagocytosis, and stress responses, with relevance to mammalian systems including human (Cox et al., 1990; Kuspa & Loomis, 1996).

1.5.1 Life cycle of *D. discoideum*

In a nutrient-rich environment, *D. discoideum* grow as individual cells, proliferating through mitosis and absorbing nutrients via endocytosis. Under conditions of nutrient deprivation, *D. discoideum* initiates a well-defined 24-hour developmental cycle that involves a series of highly regulated events (Wilson, 1953). The cycle begins when individual amoebae, which have been proliferating as single cells while feeding on bacteria, enter a state of starvation (Wilson, 1953). In response to this environmental stress, the cells secrete 3′,5′-cyclic adenosine monophosphate (cAMP) as a signaling molecule, which triggers a process known as aggregation (Shaffer, 1975; Wilson, 1953). During the initial phase of this cycle, approximately 10^5 – 10^6 amoebae come together to form a massive, multicellular aggregate (Figure 7). The cells move toward each other through chemotaxis, directed by the cAMP gradient, which causes the

amoebae to align and form a structure known as the pseudoplasmodium, or slug (Tyson, 1989). The aggregation and differentiation of *D. discoideum* is a dynamic process, where cells exchange signals and work in coordination to migrate as a unit (Figure 7). The slug is a highly organized, motile structure that can travel toward light or heat sources, a behavior known as phototaxis or thermotaxis (Marée et al., 1999). During this process, countin (CtnA), a component of the counting factor complex, plays a crucial role in regulating group size during early aggregation by controlling the number of cells that aggregate together (Rosin et al., 2000). CtnA is secreted by the cells and contributes to maintaining the stability and motility of the slug, ensuring the coordinated migration of cells for efficient fruiting body formation (Rosin et al., 2000; Brock et al., 1999; Smith et al., 2008). Over the next several hours, the slug undergoes a process of cellular differentiation. Cells at the front of the slug begin to differentiate into stalk cells, while cells at the rear differentiate into spore cells (Figure 7) (Wilson, 1953). These cells undergo changes in gene expression and alter their morphology to facilitate fruiting body formation (Cardelli et al., 1985). The stalk cells elongate (~80% of total cell population) and form the structure that supports the fruiting body, while the remaining ~20% of cells become spores that are dormant and resilient to environmental stress. After 24 hours, the fruiting body is fully formed. This dispersal of spores into the environment, allow them to survive until conditions improve, at which point they will germinate and restart life cycle (Figure 7) (Cotter et al., 1966).

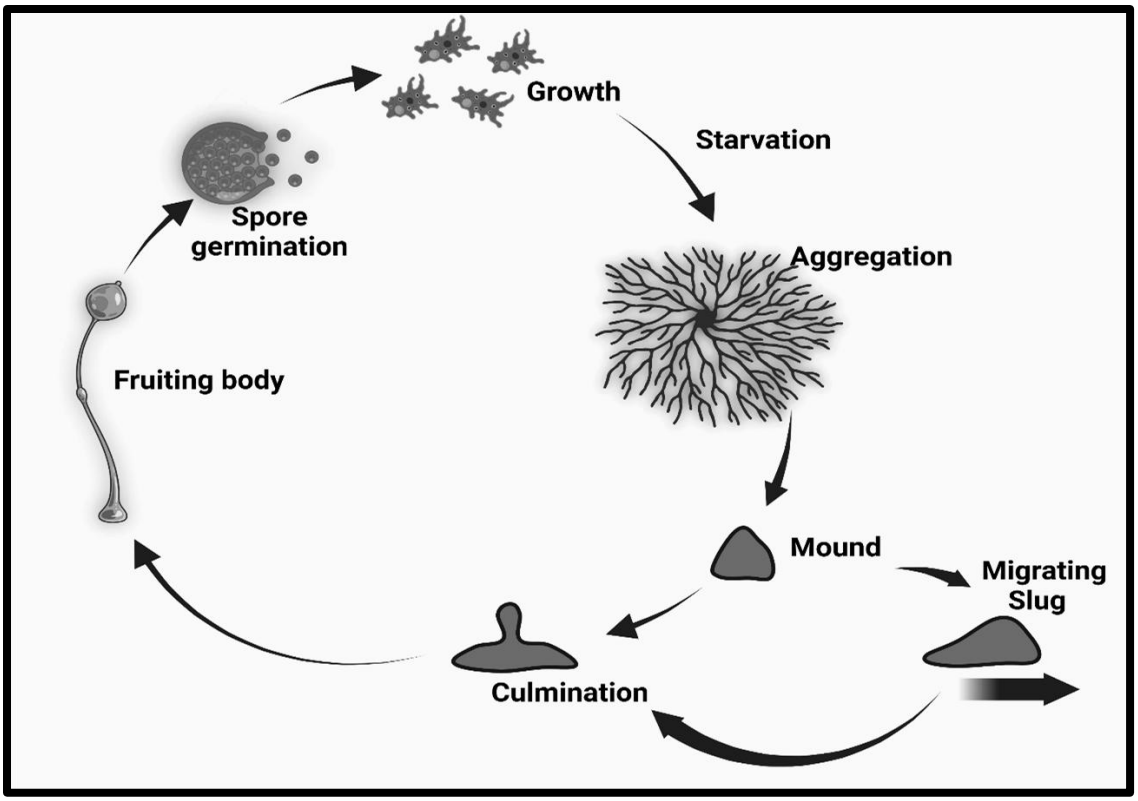

Figure 7. The life cycle of *D. discoideum*. The life cycle of *D. discoideum*, from solitary amoebae to a multicellular fruiting body. The cycle begins with individual amoeboid cells that consume bacteria and divide by binary fission. Under nutrient depletion, the amoebae aggregate to form a migrating slug, which subsequently forms a fruiting body. The fruiting body consists of a stalk and spore cells, which are released to germinate and restart the cycle as new amoebae.

1.5.2 Protein Folding and Stress Response in *D. discoideum*

During the unicellular to multicellular transition in *D. discoideum*, proper protein folding and cellular stress management play crucial roles in ensuring the survival and function of the cells involved (Czarna et al., 2010). Protein folding is required for cellular homeostasis, particularly under stress. In *D. discoideum*, the aggregation and differentiation processes associated with nutrient deprivation increase the metabolic burden on cells, raising the risk of protein misfolding (Domínguez-Martín et al., 2018). To mitigate this, heat shock proteins such as Hsp70 and Hsp90 are upregulated. These chaperones facilitate the proper folding of nascent proteins, prevent misfolded protein aggregation, and promote the degradation of irreparably

damaged proteins via autophagy or the proteasomal pathway (Czarna et al., 2010; Otto et al., 2003; Calvo et al., 2010). Proper protein folding is essential for the coordinated differentiation of stalk and spore cells, as it ensures the functionality of regulatory proteins and transcription factors necessary for fruiting body formation (Czarna et al., 2010). Additionally, during environmental stress, HSPs help protect cellular structures from oxidative damage, enabling the transition to the dormant spore state (Domínguez-Martín et al., 2018). The survival in extreme conditions such as desiccation, ultraviolet radiation, and heat relies on this stress response (Garcia et al., 2010; Taminato et al., 2002). There are multiple molecular systems that assist in *D. discoideum* survival.

In *D. discoideum*, molecular chaperones such as Glucose-Regulated Protein 78 (Grp78), the sole ER-resident member of the Hsp70 family, facilitate protein folding and help maintain proteostasis during tunicamycin-induced ER stress (Domínguez-Martín et al., 2018). In humans, GRP78 also plays a role in regulating calcium homeostasis while functioning as a molecular chaperone (Lee, 1987; Pobre et al., 2019). Under stress conditions, misfolded proteins accumulate within the cell and are targeted for degradation via autophagy, UPS, and ERAD (Glick et al., 2010; Müller & Hoppe, 2024; Nishikawa et al., 2005). Post-translational modifications, many of which occur in the Golgi apparatus — such as glycosylation and ubiquitination — play an important role in determining protein fate; for example, ubiquitinated proteins are directed toward degradation via UPS (Rios & Bornes, 2003; Schneider et al., 2010; Ciechanover & Schwartz, 2018; Smalle, 2006). Later, these ubiquitinated proteins can be degraded through the proteasome. Previously, the 20S proteasome was isolated from *D. discoideum* and found to localize predominantly in the cytoplasm and nucleus, where it carries out proteolysis (Schauer et al., 1993). These cellular mechanisms function in a coordinated

manner to ensure proper protein folding, post-translational modifications, and the degradation of misfolded proteins, thereby maintaining cellular homeostasis in *D. discoideum* during development and environmental stress (Palade, 1956; Schneider et al., 2010; Pergolizzi, et al., 2007). Disruptions in any of these processes can lead to protein aggregation and cellular dysfunction, underscoring the importance of molecular chaperones such as heat shock proteins in stress adaptation.

As discussed earlier, heat shock proteins are highly conserved and play an important role in the stress response and protein quality control. In *D. discoideum*, major heat shock proteins such as Hsp70, Hsp60, Hsp20 and Hsp32 contribute to cellular protection during stress and development (Czarna et al., 2010). Hsp32 is an intriguing protein as it was the first member of the HSP family shown to localize in the nucleolus of *D. discoideum*, where it is predominantly concentrated around the periphery of the nucleolus. Its nucleolar localization was further confirmed by treating *D. discoideum* cells with AM-D which inhibits transcription, resulting in nucleolar disintegration and reduced staining for Hsp32 (Moerman, & Klein, 1998).

HSP homologs in other species perform analogous functions, emphasizing their evolutionary significance in protein folding, oxidative stress regulation, and cellular organization (Feder et al., 1999; Hu et al., 2022). Given this high degree of conservation, it is reasonable to infer that HSP40 proteins, including DNAJC5, are also preserved in *D. discoideum*, potentially fulfilling similar roles in chaperone-mediated protein folding and proteostasis (Nydam et al., 2013).

1.6 *D. discoideum* as a Model for Neurological Disease Research

D. discoideum is widely recognized as a valuable model organism for biomedical and neurological disease research (Martin et al., 2021). This social amoeba has provided significant

insights into the functions of proteins linked to disorders such as Alzheimer's disease, Parkinson's disease, Huntington's disease, and NCL (McMains et al., 2010; Fernando et al., 2020; Myre et al., 2012; McLaren et al., 2019). Notably, *D. discoideum* contains several proteins that are similar to CLN proteins in humans surpassing other model organisms such as yeast, *C. elegans*, and *D. melanogaster* (Huber, 2016). Among these conserved genes, DNAJC5 is one of the NCL-associated genes present in *D. discoideum*.

1.6.1 Ddj1 and Its Limited Homology to DNAJC5

The *D. discoideum* genome encodes two proteins similar to human DNAJC5. The first is Ddj1 (DnaJ homolog 1, DDB0215016), which is a 411-amino-acid, 46 kDa protein localized to the cytoplasm and cell cortex. The *ddj1* gene consists of two exons and exhibits highly dynamic expression, peaking during growth, early development (0–4 hours), and fruiting body formation, while reaching its lowest level at 8 hours before rising again to peak at 20 hours, suggesting roles in growth and terminal differentiation (Stajdohar et al., 2017). Proteomic analyses have identified Ddj1 in the centrosome and macropinocytic pathway, linking it to chromosome segregation, membrane dynamics, protein trafficking, and cellular differentiation (Journet et al., 2012; Reinders et al., 2006; Silkworth et al., 2012; Cardelli, 2001). The centrosome organizes microtubules to ensure proper chromosome segregation during mitosis and meiosis (Bornens, 2012). The macropinocytic pathway enables cells to engulf extracellular fluid via macropinosomes, supporting nutrient uptake, environmental sensing, and migration (Swanson and Colin, 1995). Despite some functional overlap with human DNAJC5, Ddj1 is significantly larger (411 amino acids) and shares only a 69-amino acid conserved region, making it an unlikely homolog.

1.6.2 Dnajc5: a potential DNAJC5 homolog

Another potential DNAJC5 homolog is a protein encoded by the uncharacterized gene DDB_G0290017 (DDB0306688), which consists of 176 amino acids and has a molecular weight of 20 kDa. Sequence analysis reveals that 44% of its amino acids are conserved with those in DNAJC5. The protein likely shares a similar fold and key functional domains with human DNAJC5, such as the J-domain, which is essential for Hsp70 interaction. While this suggests some functional overlap, differences outside conserved regions may affect activity, so experimental validation is needed to confirm functional equivalence. Given its comparable size (176 amino acids) to human DNAJC5 (198 amino acids), DDB0306688 is considered the most likely candidate and will be referred to as Dnajc5 in this study. During *D. discoideum* growth, Dnajc5 localizes to the macropinocytic pathway, facilitating nutrient uptake through macropinosomes, which are essential for cell growth and survival (Journet et al., 2012). Also, the mRNA expression of *dnajc5* increases during early development, peaking during tight mound formation before declining in later stages (Stajdohar et al., 2017) (Figure 8). Beyond these observations, current knowledge about Dnajc5 in *D. discodieum* remains limited. Sequence alignment indicates that Dnajc5 has a well-conserved N-terminal region, including the J-domain, suggesting potential interactions with Hsp70 proteins in *D. discoideum* (Hennessy et al., 2000; Tsai and Douglas, 1996). Additionally, two of the three CLN4-associated mutation sites identified in human DNAJC5—alanine 63 and leucine 116—are conserved in *D. discoideum* Dnajc5, along with serine 10, a known phosphorylation site (Nosková et al., 2011; Faruq et al., 2021; Patel et al., 2016) (Figure 9). Although lysine 58, the only experimentally confirmed ubiquitination site in human DNAJC5, is not conserved in *D. discoideum* Dnajc5, other lysine residues such as lysine 56 are conserved and may serve as alternative ubiquitination sites (Patel

et al., 2016). Phosphorylation of serine 10 in human DNAJC5 is mediated by a serine/threonine-specific kinase, which can also phosphorylate threonine residues (Hornbeck et al., 2015). Since threonine residues are present in *D. discoideum* Dnajc5, it is plausible that this protein may also be phosphorylated at threonine sites, although this has not been experimentally confirmed either in human or *D. discoideum*. In the C-terminal region, conservation between human DNAJC5 and *D. discoideum* Dnajc5 is limited, especially in the cysteine-rich domain, which in human DNAJC5 undergoes S-palmitoylation, a lipid modification dependent on the presence of cysteine residues (Nosková et al., 2011). Notably, *D. discoideum* Dnajc5 lacks cysteine residues in this region, suggesting that it does not undergo S-palmitoylation, highlighting a possible functional divergence between these two proteins (Figure 9). While the sequence conservation and domain architecture suggest potential structural and functional similarities, key aspects of Dnajc5's localization, post-translational modifications, protein-protein interactions, and physiological roles in *D. discoideum* remain unclear and require further investigation. Dnajc5 may provide important information about DNAJC5's roles in non-neuronal cells, especially regarding lysosomal regulation and protein folding in the context of CLN4 disease.

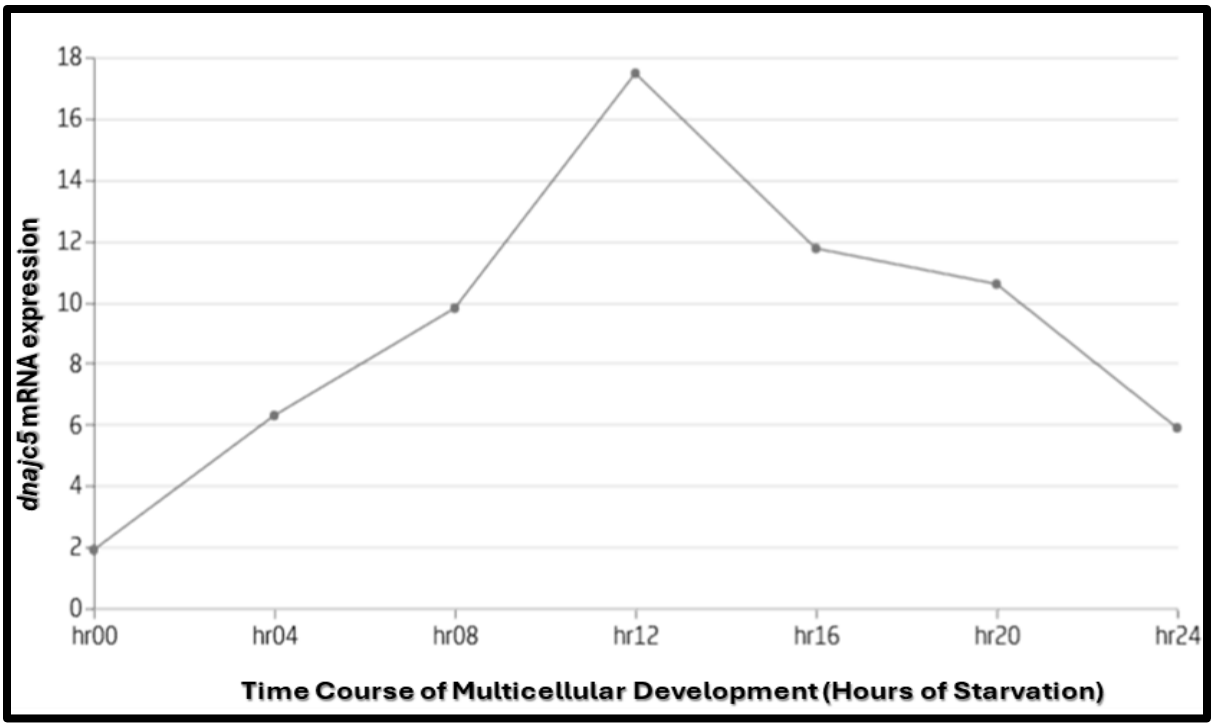

Figure 8. The mRNA expression of dnajc5 during multicellular life cycle of *D. discoideum*. The y-axis represents the RPKM (Reads Per Kilobase of transcript per Million mapped reads) values, indicating the relative abundance of *dnajc5* mRNA. The x-axis shows the hours of starvation, reflecting the temporal changes in expression during the transition from the vegetative stage to the multicellular stages of the life cycle (Stajdohar et al., 2017).

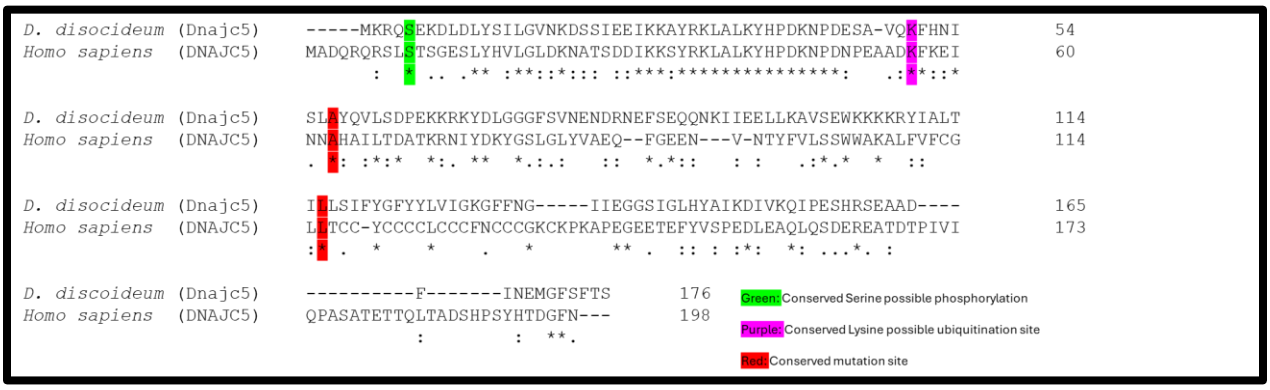

Figure 9. Sequence alignment of *D. discoideum* Dnajc5 and Human DNAJC5 highlighting conserved residues. Alignment of *D. discoideum* Dnajc5 and human DNAJC5 showing conserved amino acids. Conserved serine (S) residues are marked in green, lysine (K) residues in purple, and alanine (A) and leucine (L) residues in red. These residues correspond to functionally important sites, including phosphorylation (serine) and ubiquitination (lysine) sites.

1.7 Purpose and Objectives

1.7.1 Aims

This study aims to investigate the function and localization of Dnajc5 in *D. discoideum* to better understand its role in cellular processes, particularly those linked to lysosomal function, protein homeostasis, and neurodegeneration. By analyzing its localization, post-translational modifications, and secretion dynamics, this research seeks to determine whether Dnajc5 exhibits conserved features with its human homolog, DNAJC5, and its potential involvement in the ubiquitin degradation pathway and misfolded protein secretion.

1.7.2 Hypotheses and predictions:

1. **Localization Conservation** – The localization of Dnajc5 is conserved, meaning it should be primarily found in the cytosol, plasma membrane and perinuclear region, particularly in association with the ER as human DNAJC5 is known to localize to these regions.

2. **Starvation-Induced Secretion** – Similar to human DNAJC5, Dnajc5 is expected to be secreted upon starvation in *D. discoideum*, indicating a potential conserved function in extracellular signaling or proteostasis. If Dnajc5 is secreted under these conditions, it may play a role in the secretion of misfolded proteins, such as the misfolding-associated protein secretion (MAPS) pathway observed in mammalian cells.

3. **Post-Translational Modifications** – Key post-translational modifications, such as serine phosphorylation and potential ubiquitination sites, are conserved, suggesting that Dnajc5 is involved in the ubiquitin degradation pathway (Figure 9). These modifications might be crucial for regulating protein stability, function, and degradation, indicating that

Dnajc5 is likely involved in the ubiquitin-proteasome pathway and might play a role in the clearance of misfolded proteins, similar to DNAJC5.

1.7.3 Rationale

DNAJC5 is implicated in neurodegeneration, lysosomal function, and the clearance of misfolded proteins. Investigating its homolog, Dnajc5, in *D. discoideum* provides a simplified model to study its fundamental cellular roles. Using immunofluorescence microscopy, western blotting, and immunoprecipitation, this study will explore Dnajc5's localization, expression dynamics, and post-translational modifications throughout the *D. discoideum* life cycle. Furthermore, by examining its secretion during starvation, this research will assess whether Dnajc5 contributes to misfolded protein clearance through a MAPS-like mechanism. These findings may provide insights into the molecular mechanisms underlying DNAJC5-related neurodegenerative diseases, such as CLN4 disease.

Chapter 2

2.1 Abstract

Mutations in *DNAJC5* cause CLN4 disease, a rare adult-onset form of neuronal ceroid lipofuscinosis (NCL), a group of fatal neurodegenerative disorders collectively known as Batten disease. Although DNAJC5 has been studied in various model organisms, the lack of a well-characterized homolog in less complex eukaryotes has limited functional analysis in simpler systems. In this study, the DNAJC5 homolog in *D. discoideum*, Dnajc5, was characterized to investigate its cellular localization, expression dynamics, and post-translational modifications. Using a custom antibody, Dnajc5 was detected in the cytoplasm, ER, and nucleolus during growth and 4-hour starvation. Dnajc5 protein levels closely mirrored dnajc5 mRNA expression, indicating coordinated regulation throughout the organism's multicellular life cycle. Unlike human DNAJC5, the *D. discoiduem* homolog is not secreted and lacks key post-translational modifications such as serine/threonine phosphorylation and ubiquitination. While Dnajc5 exhibits partial localization overlap with its human counterpart, it does not replicate the features seen in human DNAJC5. These findings suggest that although Dnajc5 may share some structural features with DNAJC5, it may not serve as a true functional homolog. This work provides the first in-depth characterization of Dnajc5 in *D. discoideum*, offering a foundation for exploring conserved and divergent aspects of DNAJC5-related biology.

2.2 Introduction

Batten disease, or NCL, is a group of inherited neurodegenerative disorders that affect individuals of all ages and ethnicities (Cooper et al., 2022). Most forms of NCL are juvenile in onset and result from autosomal recessive mutations in one of 13 known NCL genes, leading to the accumulation of autofluorescent material in cells causing progressive motor decline, seizures, vision loss, dementia, and premature death (Schulz et al., 2013; Nosková et al., 2011). In

contrast, CLN4 disease is a rare adult-onset form caused by mutations in the *DNAJC5* gene (Nosková et al., 2011). While NCL proteins are thought to function within a common biological pathway, the precise mechanisms remain poorly understood. Studying one form of NCL may offer valuable insights into the underlying processes of neurodegeneration and inform our understanding of other NCL subtypes.

Autosomal dominant mutations in the *CLN4* gene, also known as *DNAJC5*, are linked to CLN4 disease (Nosková et al., 2011; Faruq et al., 2021; Mink et al., 2013). *DNAJC5* encodes CSPα or DNAJC5, a presynaptic co-chaperone that enhances the ATPase activity of HSP70 proteins (Musskopf et al., 2018; Hennessy et al., 2000; Tsai and Douglas, 1996). While predominantly localized at presynaptic terminals in neurons, DNAJC5 is also found in cells that exist outside the nervous system, where it localizes to various subcellular compartments such as the cytoplasm, endoplasmic reticulum, endosomes, lysosomes, melanosomes, the plasma membrane, and extracellularly (Fernández-Chacón et al., 2018; Wu et al., 2023; Xu et al., 2018). It plays roles in neurotransmitter release, synaptic vesicle recycling, protein folding, eMI, UPS and the secretion of misfolded proteins via MAPS (Sharma et al., 2011; Wang et al., 2021; Lee et al., 2022; Xu et al., 2018; Hasegawa et al., 2018). Despite extensive research using model organisms such as mice, fruit flies, zebrafish, and nematodes, the full cellular functions of DNAJC5 remain poorly understood (Wu et al., 2023; Fernández-Chacón et al., 2004; Imler et al., 2019; Kashyap et al., 2014). Post-translationally, DNAJC5 is phosphorylated at a serine residue in its N-terminal region, which inhibits ubiquitination at lysine 58, potentially diverting it from the UPS to an alternative quality control mechanism (Patel et al., 2016). One proposed route is the MAPS pathway, since DNAJC5 interacts with USP19, a deubiquitinase involved in secreting

misfolded proteins such as alpha-synuclein (Lee et al., 2022). However, this mechanistic possibility requires experimental validation.

D. discoideum has proven to be a valuable and cost-effective model organism for studying fundamental cellular and developmental processes such as autophagy, adhesion, cell movement, and cell differentiation (Bozzaro, 2013; Müller-Taubenberger et al., 2013). This organism has a fascinating life cycle, alternating between unicellular and multicellular stages (Wilson, 1953). Under nutrient-rich conditions, *D. discoideum* exists as amoeboid cells, feeding on bacteria. During starvation, these cells transition into a multicellular phase, beginning with the aggregation of individual cells (Shaffer, 1975; Wilson, 1953). The aggregated cells then form slug, which eventually form a fruiting body. The fruiting body consists of a cluster of viable spores supported by a slender stalk (Wilson, 1953). *D. discodieum* is a valuable model for studying neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's, as well as NCLs such as CLN1, CLN2, CLN3, and CLN5 (McMains et al., 2010; Fernando et al., 2020; Myre et al., 2012; McLaren et al., 2019). It expresses several CLN-like proteins, many of which are involved in the macropinocytic pathway—aligning with findings in mammalian models. The macropinocytic pathway of *D. discoideum* also includes a DNAJC5-like (DDB0306688) protein, highlighting *D. discoideum*'s relevance for investigating CLN4 disease and its associated mechanisms (Journet et al., 2012).

The *D. discoideum* homolog of human DNAJC5 is encoded by the uncharacterized gene *DDB_G0290017* (*dnajc5*), which produces a 176-amino acid, 20 kDa protein (DDB0306688 – Dnajc5). Based on sequence alignment, Dnajc5 has been identified as a potential homolog of DNAJC5 in *D. discoideum*, detailed characterization of its functional role remains limited. The conserved J-domain indicates potential interactions with Hsp70 chaperones, yet the effects of

conserved disease-related mutations on Dnajc5's activity are unknown. Given the importance of post-translational modifications such as phosphorylation and ubiquitination in regulating DNAJC5 function, Dnajc5 may undergo similar modifications that influence its stability and interactions. Notably, Dnajc5 has been found to localize within the macropinocytic pathway, a key route for nutrient uptake and membrane trafficking in *D. discoideum* (Stajdohar et al., 2017). The dynamic expression pattern of *dnajc5* during early development suggests it may have critical roles in cellular homeostasis under stress or nutrient-limiting conditions (Journet et al., 2012). However, its exact subcellular localization and specific function remain unclear

In the current study, an antibody against Dnajc5 was used to reveal its localization in the ER and cytoplasm. Actinomycin D (AM-D) was applied to confirm its nucleolar localization. It was also demonstrated that Dnajc5 was not secreted during the early development of *D. discoideum*. The findings revealed that Dnajc5 protein levels paralleled *dnajc5* mRNA expression, suggesting tight regulation of Dnajc5 during its multicellular life cycle. Additionally, after immunoprecipitating Dnajc5, it was found that, unlike human DNAJC5, Dnajc5 lacked post-translational modifications such as serine phosphorylation, threonine phosphorylation, or ubiquitination. While the localization of Dnajc5 is somewhat conserved in *D. discoideum*, it does not appear to replicate many of the functions of DNAJC5. Therefore, Dnajc5 may not be a true homolog of DNAJC5.

2.3 Methods

2.3.1 Cells, chemicals and antibodies

The *D. discoideum* Ax3 parental strain was obtained from the Dicty Stock Center and maintained at room temperature on SM (Sussman Maurice) agar plates seeded with *Klebsiella*

aerogenes (Fey et al., 2007). Axenic growth was conducted in HL5 medium (Formedium, Hunstanton, Norfolk, UK) at room temperature with continuous shaking at 150 rpm on a rotary shaker (Bioshop, Burlington, ON, CA) (Fey et al., 2007). Cultures were supplemented with ampicillin (100 µg/mL) and streptomycin sulfate (300 µg/mL). KK2 buffer (2.2 g/L KH_2PO_4, 0.7 g/L K_2HPO_4, pH 6.5) was used for cell starvation and washing during experiments. Unless otherwise specified, all experimental cells were harvested during the mid-log phase of growth ($1–5 \times 10^6$ cells/mL) (Fey et al., 2007).

A polyclonal antibody against *D. discoideum* Dnajc5 was raised in rabbits using a synthetic peptide corresponding to the N-terminal epitope shown in Figure 10 and was produced by GenScript (Piscataway, New Jersey, USA). Grp78 was also sourced from GenScript. HL5 and low-fluorescence HL5 media were obtained from Formedium (Hunstanton, Norfolk, UK). Alexa Fluor-conjugated secondary antibodies and ProLong Gold Antifade Reagent with DAPI were purchased from Fisher Scientific (Ottawa, Ontario, Canada). The mouse monoclonal anti-calreticulin antibody (clone 252-234-2) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, Iowa, USA). AM-D was purchased from New England Biolabs Ltd. (Whitby, Ontario, Canada). The mouse monoclonal anti-serine antibody was sourced from Sigma-Aldrich (St. Louis, Missouri, USA), while the mouse monoclonal anti-threonine and anti-ubiquitin antibodies, protein A magnetic beads, and Horseradish peroxidase (HRP) were obtained from New England Biolabs Ltd. (Whitby, Ontario, Canada). Precision plus protein ladder (Bio-Rad, Mississauga, ON, Canada) was used for western blots.

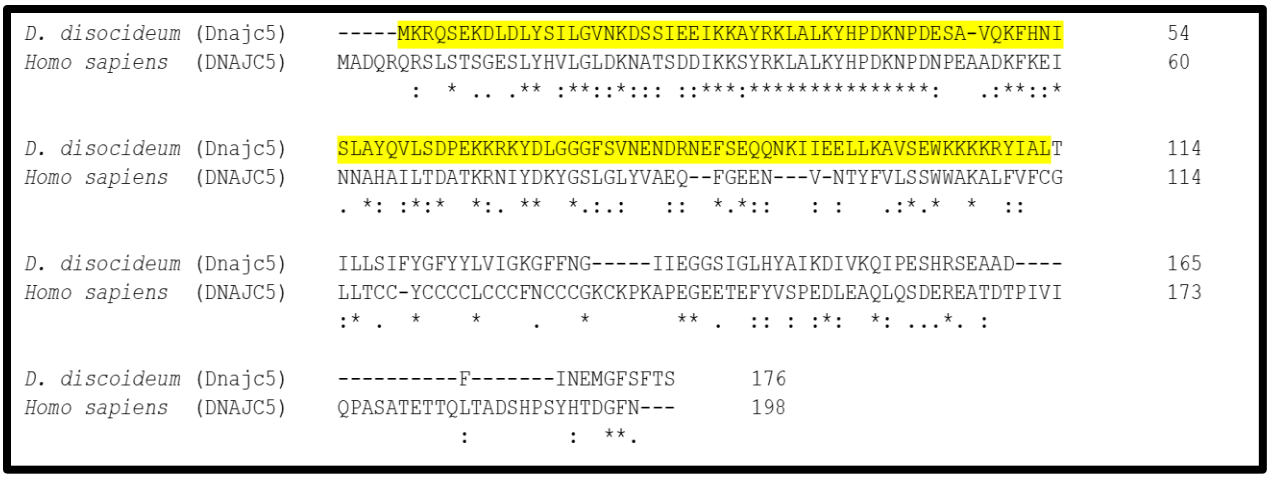

Figure 10. Sequence Alignment Between *D. discoideum* Dnajc5 and Human DNAJC5 with highlighting epitope of the antibody generated against Dnajc5. Amino acid sequence alignment of *D. discoideum* Dnajc5 (top) and human DNAJC5 (bottom). Yellow highlights indicate the conserved epitope regions recognized by antibodies, which is about 2/3 of the protein.

2.3.2 Immunofluorescence Assay

Ax3 cells (5×10^5) were seeded onto sterilized circular coverslips in a 12-well dish and incubated overnight at room temperature in low-fluorescence HL5 medium. The coverslips were sterilized by briefly flaming them to ensure a contaminant-free surface (Eichinger and Rivero, 2006). The following day, cells were gently washed with KK2 buffer to remove residual medium and non-adherent cells. For the starvation condition, cells were incubated in 1x KK2 buffer for 4 hours to induce nutrient deprivation. After the starvation period, cells were fixed by immersing the coverslips in ultracold methanol (−80°C) for 45 minutes to preserve cellular structures and proteins (Hagedorn et al., 2006; Huber et al., 2024). Following fixation, the samples were incubated in blocking buffer (0.2% gelatin and 0.01% Triton X-100 in 1× KK2 buffer) for 30 minutes at room temperature to reduce non-specific antibody binding. The cells were then incubated with primary antibodies, diluted 1:50 in blocking buffer, followed by incubation with secondary antibodies, diluted 1:100 in blocking buffer (Huber et al., 2024). The following

antibodies were used for immunolocalization: rabbit anti-Dnajc5 (1:50), mouse anti-calreticulin (1:50), anti-rabbit Alexa Fluor 488 (1:100), and anti-mouse Alexa Fluor 555 (1:100). After antibody staining, coverslips were mounted onto microscope slides using ProLong Gold Antifade Reagent with DAPI to preserve fluorescence and counterstain the nuclei. The slides were sealed with nail polish to prevent drying and enable long-term imaging.

In a separate experiment, Ax3 cells were cultured in low-fluorescence HL5 medium and supplemented with AM-D at a final concentration of 0.05 mg/mL for 4 hours to disrupt the nucleolus (Catalano et al. 2011). After treatment, the cells were fixed using the same ultracold methanol protocol and processed for immunostaining as described. Fixed cells were imaged using a Nikon Ts2R-FL inverted microscope equipped with a Nikon Digital Sight Qi2 monochrome camera (Nikon Canada Instruments Division, Mississauga, ON, Canada). Image acquisition and analysis were performed using NIS Elements BR version 5.02, and image merging was carried out with ImageJ/Fiji.

2.3.3 Secretion analysis

Ax3 cells (5×10^6) were seeded into Petri dishes and incubated overnight in HL5 medium at room temperature to allow for proper adhesion and growth. The following day, cells were washed twice with KK2 buffer to remove residual nutrients and subsequently starved in fresh KK2 buffer for 12 hours to induce multicellular development. Three independent biological replicates were prepared to ensure reproducibility. In this assay, samples were collected every 4 hours at three key developmental stages: 4-hour starvation, 8-hour aggregation, and 12-hour mound formation. Throughout the starvation period, cell morphology and behavior were monitored and imaged every 4 hours using a Nikon Ts2R-FL inverted microscope equipped with a Nikon Digital Sight Qi2 monochrome camera (Nikon Canada Incorporated Instruments

Division, Mississauga, Ontario, Canada). During each time point, conditioned buffer (CB) was collected to assess secreted factors. The CB was first centrifuged at 2000 × g for 5 minutes to separate the supernatant from cellular debris. The supernatant was then processed using Amicon Ultra-4 10 K centrifugal filter units (Fisher Scientific Company, Ottawa, Ontario, Canada) and centrifuged at 4200 × g for 25 minutes at 4 °C to concentrate secreted proteins while eliminating smaller contaminants (Huber & Mathavarajah, 2018). Additionally, to analyze intracellular proteins during growth, one of the plates was maintained in HL5 medium without starvation. From this plate, conditioned media (CM) was collected, and the cells adhered to the dish were lysed using 0.5% Nonidet P-40 lysis buffer (150 mM NaCl, 50 mM Tris, 0.5% NP-40, pH 8.3) to collect whole cell (WC) lysate. Unlike the KK2-CB, HL5 medium was not concentrated using centrifugal filters, as the presence of rich nutrients and serum components in HL5 could interfere with the filtration process and affect downstream analyses. At the end of the starvation period, cells were lysed using NP-40 lysis buffer to extract total cellular proteins. To prevent protein degradation, the lysis buffer was supplemented with a protease inhibitor tablet (Fisher Scientific Company, Ottawa, Ontario, Canada). The protein concentration of the samples was quantified using Qubit Protein Assay Kit and Qubit 2.0 Fluorometer (Fisher Scientific, Whitby, ON, CA). Protein samples from each replicate were subsequently analyzed by SDS-PAGE, followed by Western blotting to assess Dnajc5 protein amounts.

2.3.4 Multicellular development assay

Ax3 cells (8×10^6) were seeded into Petri dishes containing 8 mL of 1% KK2 agar and incubated at room temperature for 24 hours to initiate the multicellular development. This incubation allowed the cells to undergo the necessary starvation and aggregation processes to form multicellular structures. In parallel, the night before the assay, Ax3 cells (5×10^6) were

incubated in a separate Petri dish with HL5 medium under standard growth conditions at room temperature to maintain cells in the vegetative phase for comparison. Cell morphology and behavior during the development cycle were carefully monitored every 4 hours using a Nikon Ts2R-FL inverted microscope (Nikon Canada Incorporated Instruments Division, Mississauga, Ontario, Canada). Images were captured at each time point to document the transition from the individual amoeboid stage (0 h growth) to fruiting body state (24 hours). Samples were collected at the following developmental stages from the 1% KK2 agar plates: 4 hours (starvation stage), when cells were in the early stages of starvation before aggregation; 8 hours (aggregation stage), when cells began to aggregate into early clusters; 12 hours (mound formation), when aggregated cells formed distinct mounds; 16 hours (tipped mound), when cells had formed tipped mounds and started to elongate; 20 hours (slugs), when multicellular structures progressed into elongated slug-like forms; and 24 hours (fruiting body), when cells had formed mature fruiting bodies, completing the developmental cycle. For each replicate, two 12-hour plates were created at the 12-hour mound formation stage to ensure consistency. For sample harvesting, after every 4 hour the cells were gently scraped from the 1% KK2 agar plates. The areas of the agar plates covered by the cells were washed with 1x KK2 buffer to collect the cells, ensuring the removal of any residual agar or debris. The KK2 buffer containing the cells was then carefully transferred to microcentrifuge tubes and centrifuged to pellet the cells. The supernatant was discarded, and the cell pellet was lysed using NP40 lysis buffer supplemented with a protease inhibitor tablet to prevent protein degradation. The collected sample was analysed by SDS-PAGE, and Western blotting.

2.3.5 Immunoprecipitation Assay

Ax3 cells (15×10^6) were seeded into Petri dishes and incubated overnight at room temperature in HL5 medium to ensure optimal growth conditions. Following incubation, the cells were gently washed with KK2 buffer to remove residual nutrients and any non-adherent cells. Cellular lysis was performed using 0.1% NP40 lysis buffer (150mM NaCl, 50 mM Tris, 0.1% NP-40, pH 7.5) to facilitate the extraction of proteins. Also, a separate set of Ax3 cells was washed with KK2 buffer and incubated in the same buffer for four hours to induce nutrient deprivation. Following this period, WC were collected from both the nutrient-rich condition (HL5 medium) and the 4-hour starvation condition (KK2 buffer) for further analysis. For immunoprecipitation (IP), approximately 1 mg of total protein from the collected WC was incubated with 5 µL of DNAJC5 antibody overnight on a tube rotator at 4°C to allow for specific antigen-antibody binding. The total reaction volume was adjusted to 750 µL to maintain an optimal antibody concentration. The following day, 50 µL of protein A magnetic beads were thoroughly washed with lysis buffer to remove any residual storage solution and equilibrated for efficient binding (Kim et al., 2021). The pre-incubated protein-antibody complexes were then mixed with the washed protein A magnetic beads and incubated for four hours on a tube rotator at 4°C to facilitate the capture of antibody-bound protein complexes via protein A interactions. Following incubation, the beads were separated from the supernatant using a magnetic rack. The supernatant or protein depleted (PD) sample was collected for further analysis, while the beads were subjected to two washes with lysis buffer to remove unbound proteins. To elute the bound proteins, 2× Laemmli buffer (120 mM Tris-HCl pH 6.8, 4% sodium dodecyl sulfate [SDS], 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue) was added to the beads, and the mixture was heated at 95°C for 5 minutes. The supernatant containing the eluted proteins was

then collected, and the beads were discarded. The collected protein samples were subsequently analyzed using SDS-PAGE, followed by western blotting to assess protein expression and interaction.

2.3.6 SDS-PAGE and Western Blotting

WC and CM or CB were each mixed 1:1 with 2× Laemmli buffer and heated at 95°C for 5 minutes to denature proteins and reduce disulfide bonds. Proteins were separated using SDS-PAGE based on molecular weight and transferred to polyvinylidene difluoride (PVDF) membranes (Immun-Blot®, Bio-Rad, Mississauga, ON, Canada) via wet electroblotting at 90 volts for 90 minutes. PVDF Membranes were then blocked for 1 hour at room temperature using 5% non-fat dry milk in TBST (tris-buffered saline with 1% Tween-20) to minimize nonspecific antibody binding, followed by three washes with TBST. Subsequently, membranes were incubated for 2 hours at room temperature with the following primary antibodies: anti-Dnajc5 (rabbit, 1:500), anti-tubulin (mouse, 1:1000), anti-countin (rabbit, 1:1000), anti-Grp78 (rabbit, 1:500), anti-ubiquitin (mouse, 1:1000), anti-phosphoserine (mouse, 1:1000), and anti-phosphothreonine (mouse, 1:1000). After incubation with primary antibodies, membranes were washed three times with TBST and incubated with HRP-conjugated secondary antibodies (anti-rabbit IgG-HRP or anti-mouse IgG-HRP, diluted 1:2000 in blocking buffer). Following secondary incubation, membranes were washed three times with TBST. The chemiluminescent signal was developed using Clarity™ or Clarity Max™ ECL substrates (Bio-Rad, Mississauga, ON, Canada) and imaged using the ChemiDoc™ Imaging System (Bio-Rad, Mississauga, ON, Canada). To verify equal protein loading, replicate gels not used for transfer were stained with the Pierce™ Silver Stain Kit (Fisher Scientific Company, Ottawa, Ontario, Canada) and imaged using the Invitrogen™ iBright™ Imaging System (Bio-Rad, Mississauga, ON, Canada).

Also, a peptide competition assay was carried out to verify the specificity of the anti-Dnajc5 antibody. The antibody was incubated overnight at 4 °C in TBST with its corresponding peptide—specifically, the same synthetic peptide that was originally used to generate the antibody—to allow binding. The next day, this antibody-peptide mixture was diluted in blocking buffer and used to incubate the membrane. To serve as a negative control, a separate blot was incubated with the anti-Dnajc5 antibody diluted in blocking buffer alone, without the peptide.

2.4 Results

2.4.1 Antibody specificity and validation:

To assess the specificity of the antibody, a western blot was performed to determine the size of the bands. WC from *D. discoideum* cells during growth was loaded at 10 µg of protein. A band around 20 kDa was observed, which corresponds to the expected size of Dnajc5 (Figure 11a). A peptide competition assay was performed by incubating the recombinant peptide and antibody together overnight before probing the blot. No bands were observed on the blot incubated with the peptide-blocked antibody, confirming that the peptide effectively inhibited the antibody's binding to its target. Conversely, the blot treated with the antibody alone showed the expected banding pattern, demonstrating specific binding of the antibody to the target protein (Figure 11b).

However, some non-specific binding appeared in the peptide-blocked blot around 20 kDa as a smear rather than a distinct band. Additionally, a band near 15 kDa was observed both in the protein sample lane and the empty lane of the control blot, suggesting that this band is likely non-specific binding. These issues may be due to using only a limited amount of synthetic peptide for blocking, which prevented using a higher concentration, along with suboptimal

incubation conditions—specifically, incubating the membrane in a Falcon tube that likely limited proper antibody solution contact. Combined with the higher antibody dilution (1:1000 vs. 1:500) and lower volume (5 mL vs. 10 mL), these factors likely reduced blocking efficiency and caused uneven binding.

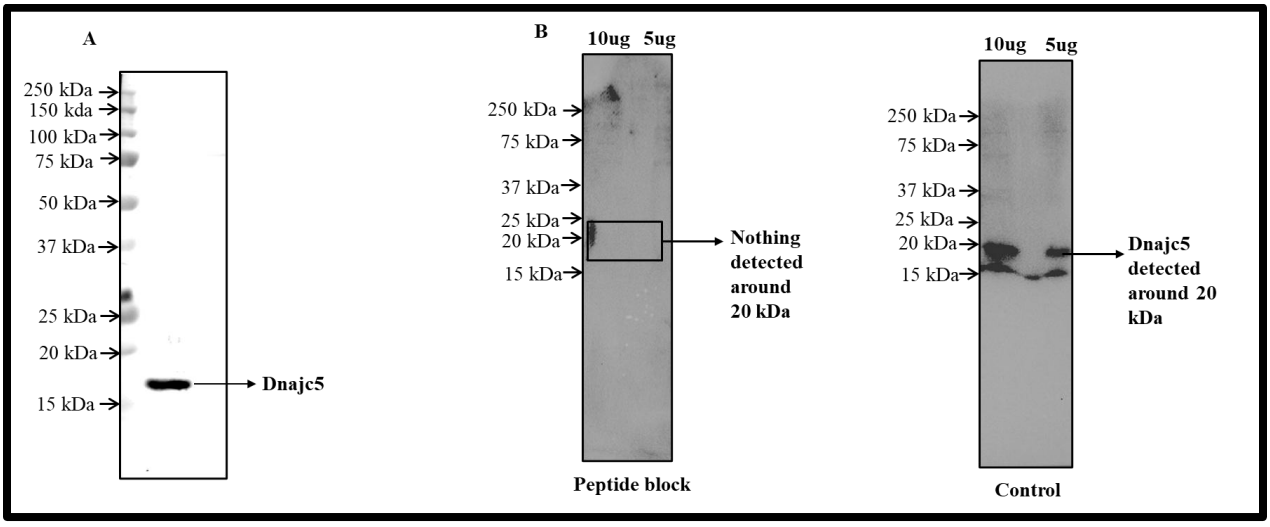

Figure 11. Antibody validation by Western blot analysis of Dnajc5. A) The blot shows a band around 20 kDa, confirming the specificity of the anti-Dnajc5 antibody at a 1:500 dilution, with 16 µg of WC loaded. **B)** Peptide competition assay (left) and control blot (right) demonstrating the specificity of the anti-Dnajc5 antibody, with WC loaded with 5 µg and 10 µg of protein and probed at a 1:1000 dilution.

2.4.2 Dnajc5 localizes to the nucleolus

Initial immunofluorescence analysis revealed that Dnajc5 localized strongly to two or three compact, oval structures situated either adjacent to or within the nuclear region. These structures were not visible in DAPI-stained images, as DAPI selectively binds to A-T rich regions of DNA and does not stain nucleoli, leading to the initial assumption that the observed ovoid structures represented the nucleoli of *D. discoideum* (Tarnowski et al., 1991). To verify this localization, cells were treated with 0.05 mg/mL AM-D for 4 hours or left untreated as controls, followed by fixation and immunofluorescent staining. DAPI was used to label nuclear DNA, and Dnajc5 was detected using an anti-Dnajc5 antibody. In untreated cells (Figure 12, top row),

Dnajc5 was highly enriched in the oval structures within the nucleus, suggesting a specific subnuclear localization. In contrast, AM-D–treated cells (Figure 12, bottom row) showed a marked loss of this concentrated signal, with Dnajc5 appearing more diffusely distributed throughout the nucleus. AM-D is known to specifically disrupt nucleolar structure and function, the sensitivity of Dnajc5 localization to AM-D treatment provides strong evidence that the oval structures represent nucleoli. Thus, these findings confirm that Dnajc5 localizes to the nucleoli of *D. discoideum* under normal conditions, and that this localization is dependent on intact nucleolar integrity.

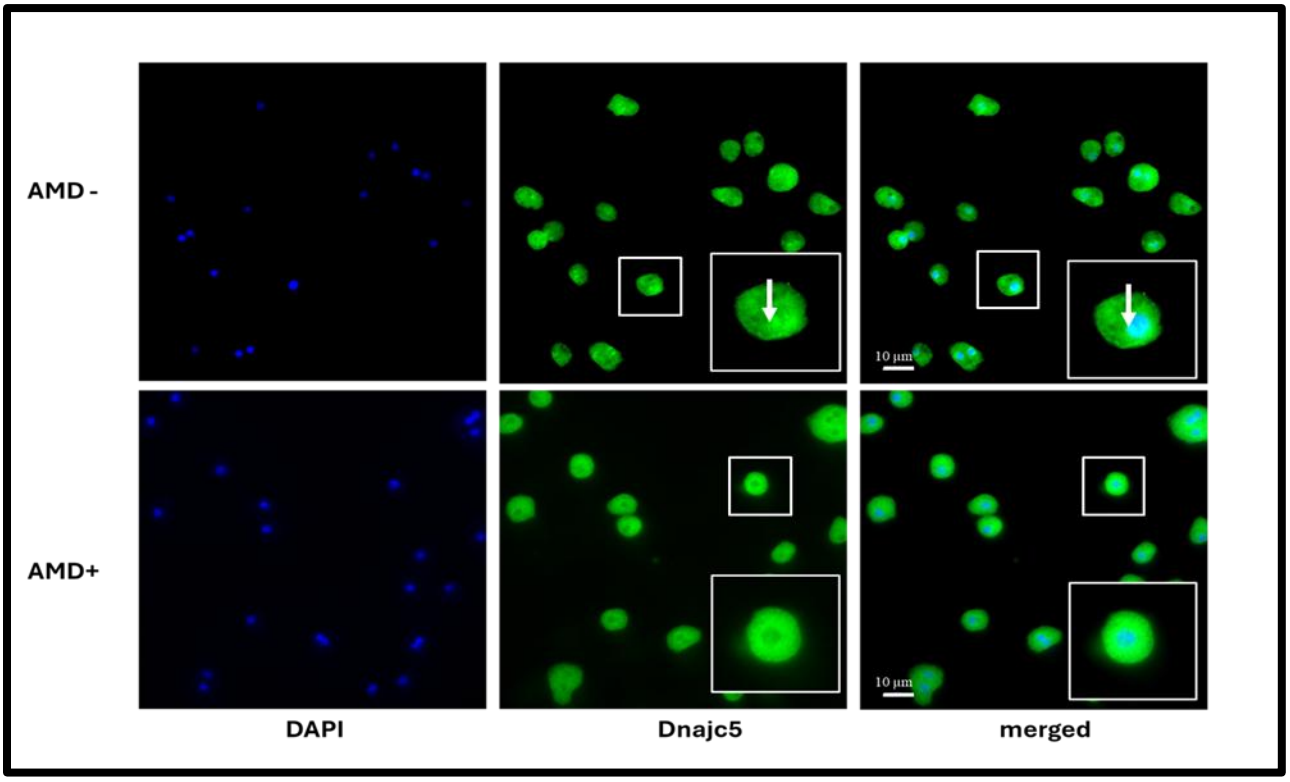

Figure 12. Dnajc5 localizes to nucleoli and cytoplasm in *D. discoideum*, and the nucleolar localization is disrupted by AM-D treatment. *D. discoideum* cells were either left untreated (control; top row) or treated with 0.05 mg/mL AM-D for 4 hours (bottom row), followed by fixation and immunofluorescence staining. Nuclei were labeled with DAPI (left), and Dnajc5 was detected using a primary anti-Dnajc5 antibody (middle). Merged images are shown in the right panels. In control cells, Dnajc5 displayed strong nuclear localization with pronounced enrichment in discrete ovoid structures consistent with nucleoli. AM-D treatment resulted in a marked reduction of this nucleolar enrichment, yielding a more diffuse nuclear distribution.

These findings indicate that Dnajc5 localizes to nucleoli under normal conditions and that this localization is dependent on intact nucleolar architecture. White arrows pointing at the nucleolar structure, Scale bar: 10 μm.

2.4.3 Dnajc5 localization limited to ER and cytoplasm during growth and after 4 hours of starvation

Immunofluorescence analysis of *D. discoideum* revealed that Dnajc5 is localized to both the ER and the cytoplasm under both growth and starvation conditions. During the growth phase, Dnajc5 was observed in both the perinuclear region and the cytoplasm, with a notable colocalization with the ER marker calreticulin (Figure 13). Merged images indicated significant overlap of Dnajc5 and calreticulin, especially in the perinuclear area, suggesting that Dnajc5 localizes to the ER. After a 4-hour starvation in 1x KK2 buffer, the localization of Dnajc5 remained unchanged, continuing to appear in both the ER and cytoplasm (Figure 14). The pattern of colocalization with calreticulin was the same that observed during growth, with strong perinuclear overlap of the two signals. These findings indicate that Dnajc5 consistently localizes to the ER and cytoplasm, regardless of the nutritional state of the cells. We also used anti-p80, anti-VatC and anti-cortexillin antibodies as markers to label the plasma membrane, acidic vesicles, and secretory vesicles, respectively (Figure 15; Figure 16; Figure 17; Figure 18; Figure 19; Figure 20). Dnajc5 did not co-localize with any of these markers, suggesting that its localization is restricted to the endoplasmic reticulum, cytoplasm, and nucleolus.

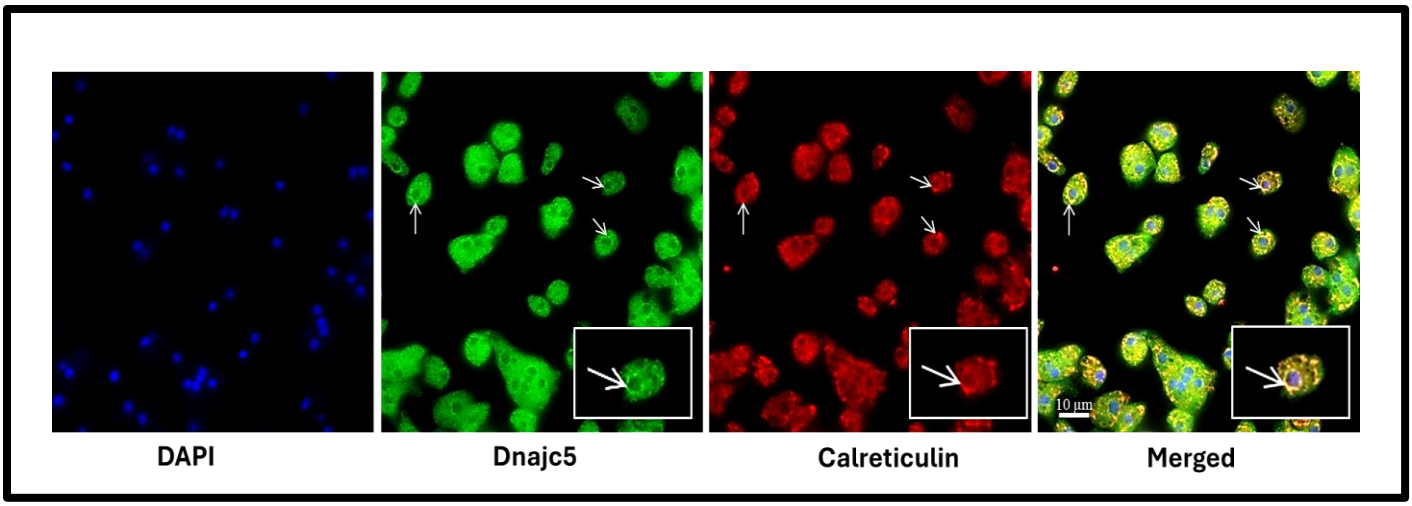

Figure 13. Immunofluorescence staining of *D. discoideum* during growth reveals the localization of Dnajc5 in both the ER and the cytoplasm. From left to right: DAPI staining (blue) marks the nuclei, the green channel shows the localization of Dnajc5 (in both the perinuclear region and cytoplasm), the red channel indicates the localization of calreticulin (an ER marker), and the merged image highlights the colocalization (yellow/orange) of the proteins. The boxed regions emphasize cells with strong perinuclear overlap of green and red signals, suggesting that Dnajc5 is localized to the ER. White arrows point to areas of clear colocalization. Scale bar: 10 µm.

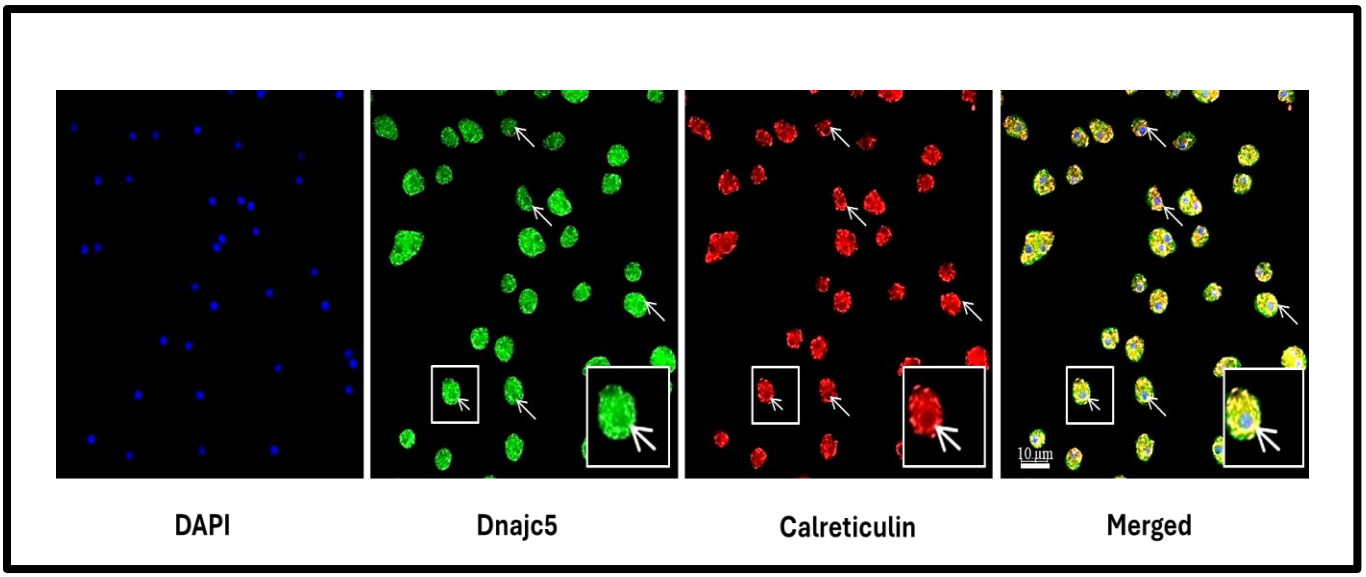

Figure 14. Immunofluorescence staining of *D. discoideum* after 4-hour starvation in 1x KK2 buffer reveals the localization of Dnajc5 in both the ER and the cytoplasm From left to right: DAPI staining (blue) marks the nuclei, the green channel shows Dnajc5 localization (in both the perinuclear region and cytoplasm), the red channel highlights the localization of calreticulin (an ER marker), and the merged image displays the colocalization (yellow/orange) of the proteins. The boxed regions highlight cells with strong perinuclear overlap of green and red signals, suggesting that Dnajc5 is localized to the ER. White arrows point to areas of clear colocalization. Scale bar: 10 µm.

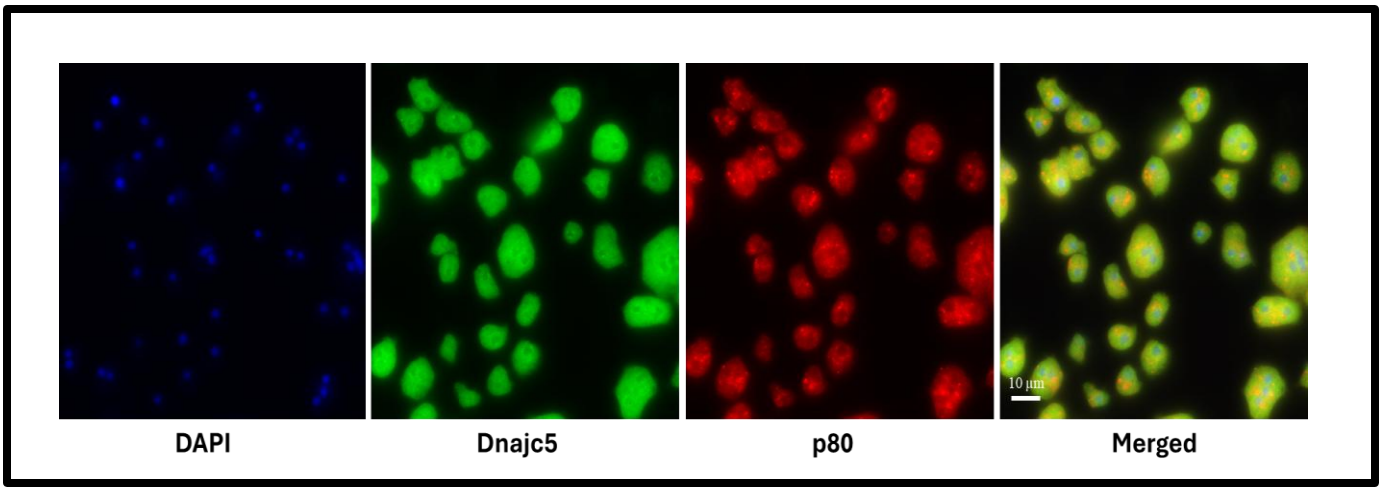

Figure 15. Immunofluorescence staining of *D. discoideum* during growth reveals the localization of Dnajc5 relative to p80, a marker for secretory vesicles. From left to right: DAPI staining (blue) marks the nuclei, the green channel shows Dnajc5 localization, the red channel highlights p80 distribution, and the merged image shows no significant overlap between the two signals. The lack of colocalization suggests that Dnajc5 is not associated with p80-positive secretory vesicles under growth conditions. Scale bar: 10 µm.

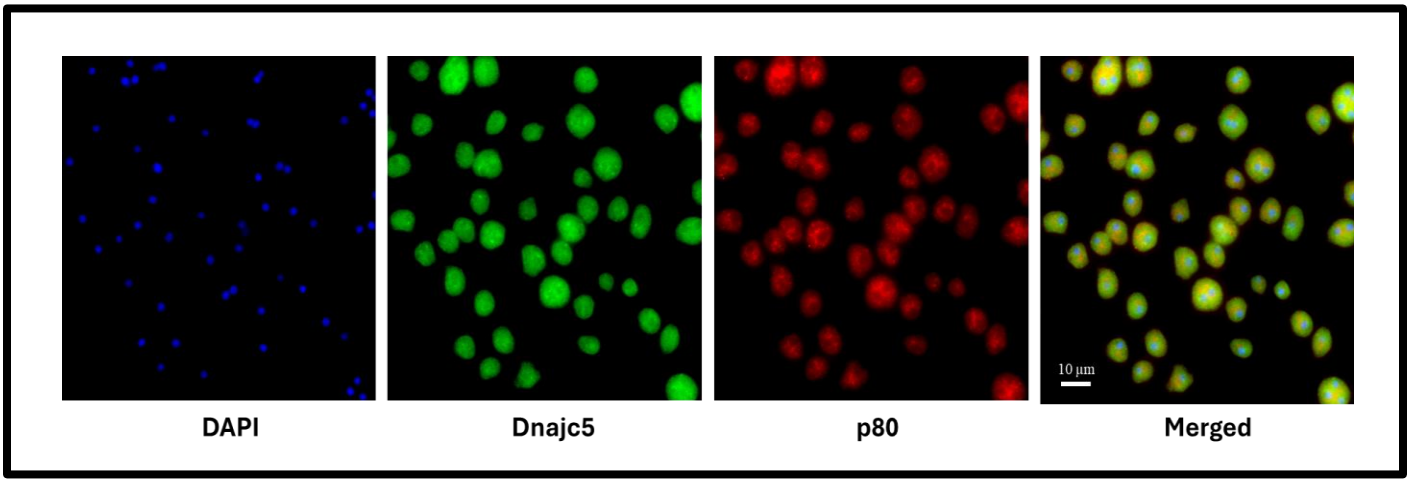

Figure 16. Immunofluorescence staining of *D. discoideum* after 4-hour of starvation in 1x KK2 reveals the localization of Dnajc5 relative to p80, a marker for secretory vesicles. From left to right: DAPI staining (blue) marks the nuclei, the green channel shows Dnajc5 localization, the red channel highlights p80 distribution, and the merged image shows no significant overlap between the two signals. The lack of colocalization suggests that Dnajc5 is not associated with p80-positive secretory vesicles under starved conditions. Scale bar: 10 µm.

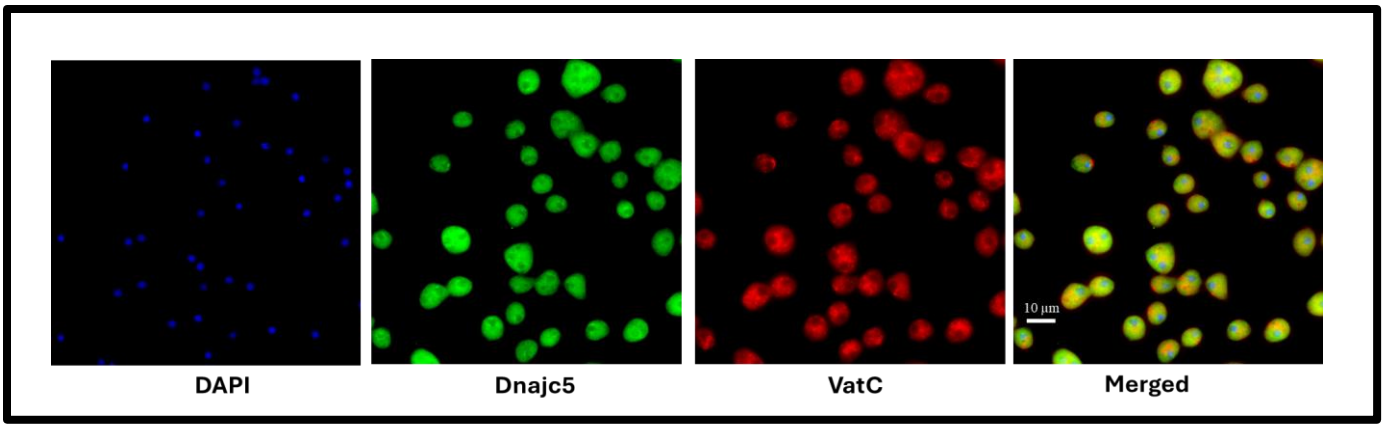

Figure 17. Immunofluorescence staining of *D. discoideum* during growth reveals localization patterns of Dnajc5 and VatC. DAPI staining (blue) marks the nuclei, the green channel shows Dnajc5 localization, and the red channel highlights VatC, a marker for the vacuolar-type H^+-ATPase associated with acidic compartments such as lysosomes and post-lysosomes. The merged image shows no significant overlap between Dnajc5 and VatC, indicating that Dnajc5 is not localized to acidic organelles during growth. Scale bar: 10 µm.

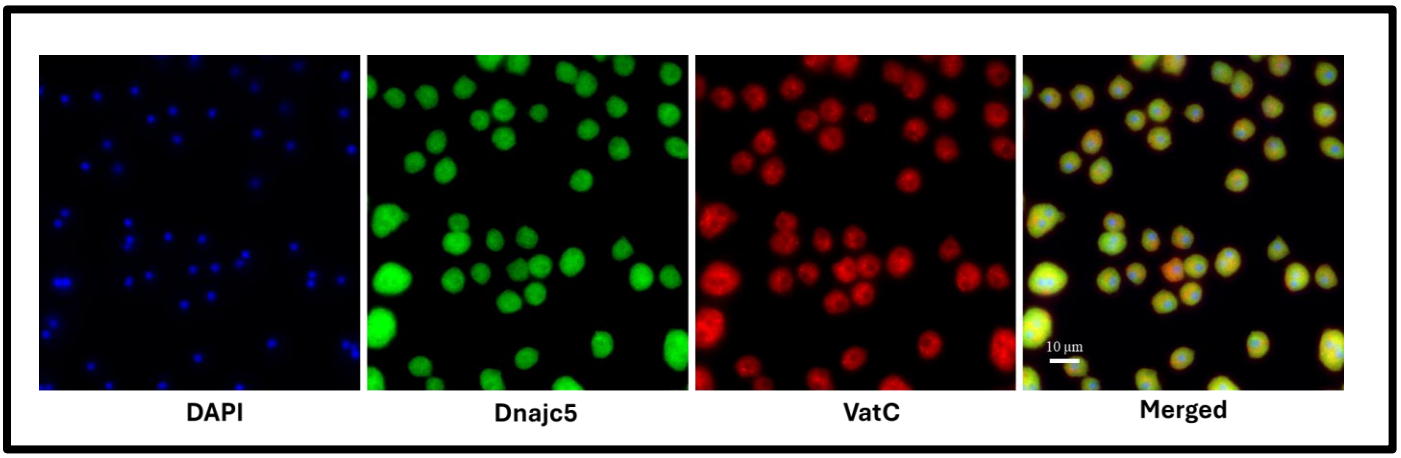

Figure 18. Immunofluorescence staining of *D. discoideum* after 4-hours of starvation in 1x KK2 reveals localization patterns of Dnajc5 and VatC. DAPI staining (blue) marks the nuclei, the green channel shows Dnajc5 localization, and the red channel highlights VatC, a marker for the vacuolar-type H^+-ATPase associated with acidic compartments such as lysosomes and post-lysosomes. The merged image shows no significant overlap between Dnajc5 and VatC, indicating that Dnajc5 is not localized to acidic organelles after 4 hours of starvation. Scale bar: 10 µm.

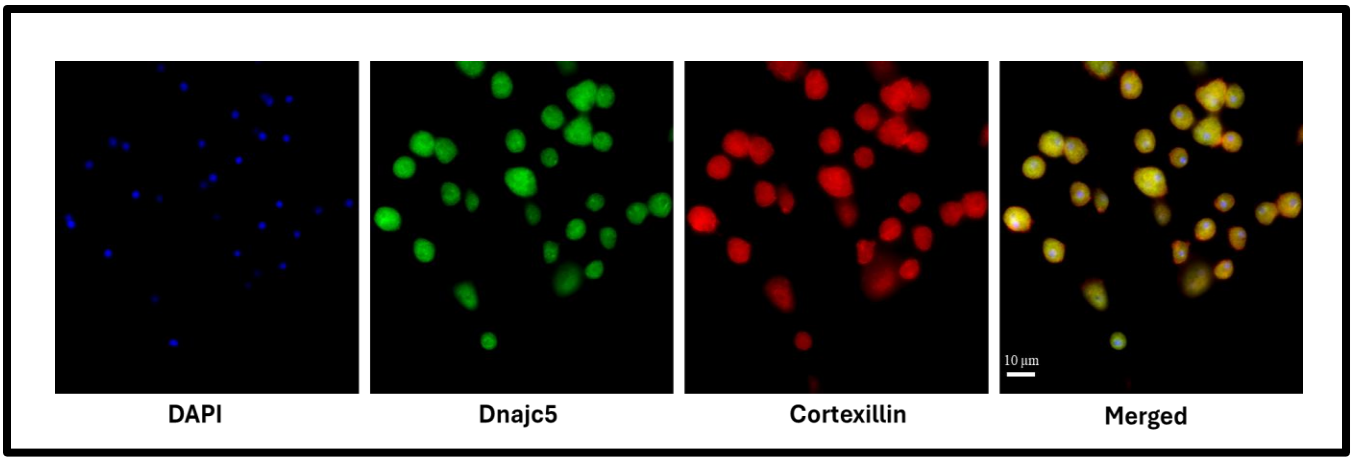

Figure 19. Immunofluorescence staining of *D. discoideum* during growth reveals localization patterns of Dnajc5 and Cortexillin. DAPI staining (blue) marks the nuclei, the green channel shows Dnajc5 localization, and the red channel highlights cortexillin, a marker for the plasma membrane. The merged image shows no significant overlap between Dnajc5 and VatC, indicating that Dnajc5 is not localized to plasma membrane during growth. Scale bar: 10 µm.

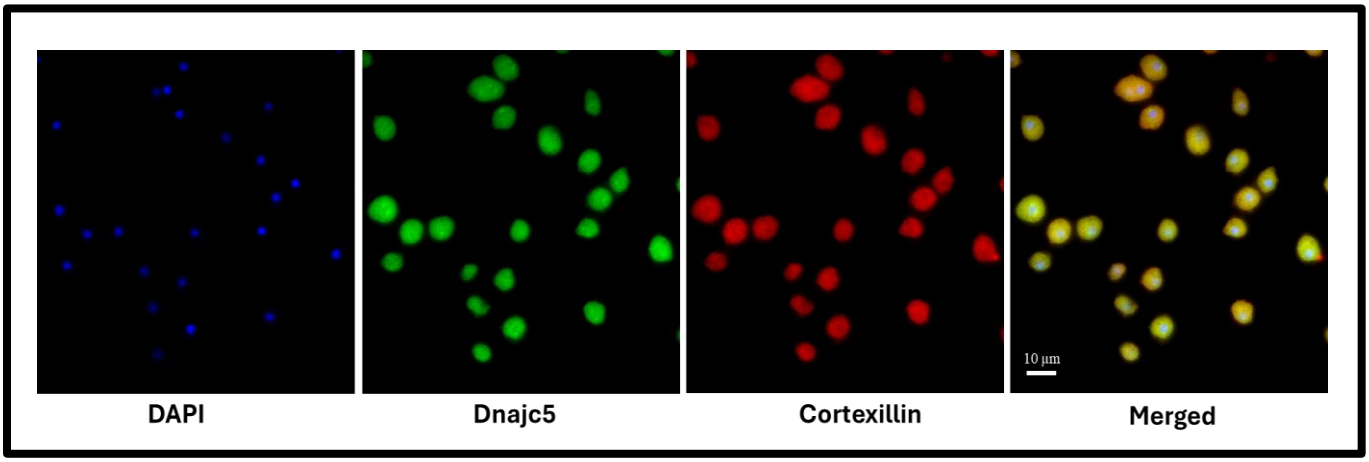

Figure 20. Immunofluorescence staining of *D. discoideum* after 4-hours of starvation in 1x KK2 reveals localization patterns of Dnajc5 and Cortexillin. DAPI staining (blue) marks the nuclei, the green channel shows Dnajc5 localization, and the red channel highlights cortexillin, a marker for the plasma membrane. The merged image shows no significant overlap between Dnajc5 and VatC, indicating that Dnajc5 is not localized to plasma membrane after *D. discoideum* undergoes starvation for 4 hours. Scale bar: 10 µm.

2.4.4 Dnajc5 is not secreted during early developmental stages of *D. discoideum*

To determine whether Dnajc5 is secreted during the early developmental transition in *D. discoideum*, western blot analysis was performed on WC and CM/CB collected at defined time points corresponding to vegetative growth, and 4, 8, and 12 hours of starvation. Dnajc5 was consistently detected in WC across all time points, indicating stable intracellular expression during early development (Figure 15A). However, no Dnajc5 signal was observed in the CB at any stage, suggesting an absence of secretion during this period (Figure 15A). To validate sample integrity, tubulin was used to confirm the absence of cell lysis in CM/CB samples, while countin served as a positive control for secretion, with expression beginning at the 4-hour starvation time point. No secretion control was available for the growth condition, silver staining confirmed the presence of total protein in both WC and CM/CB, excluding the possibility of technical artifacts or insufficient protein recovery (Figure 15B). Collectively, these results demonstrate that Dnajc5 is not secreted during the transition from growth to early starvation in *D. discoideum*, suggesting that Dnajc5 functions intracellularly during the initial stages of development rather than participating in early extracellular signalling events.

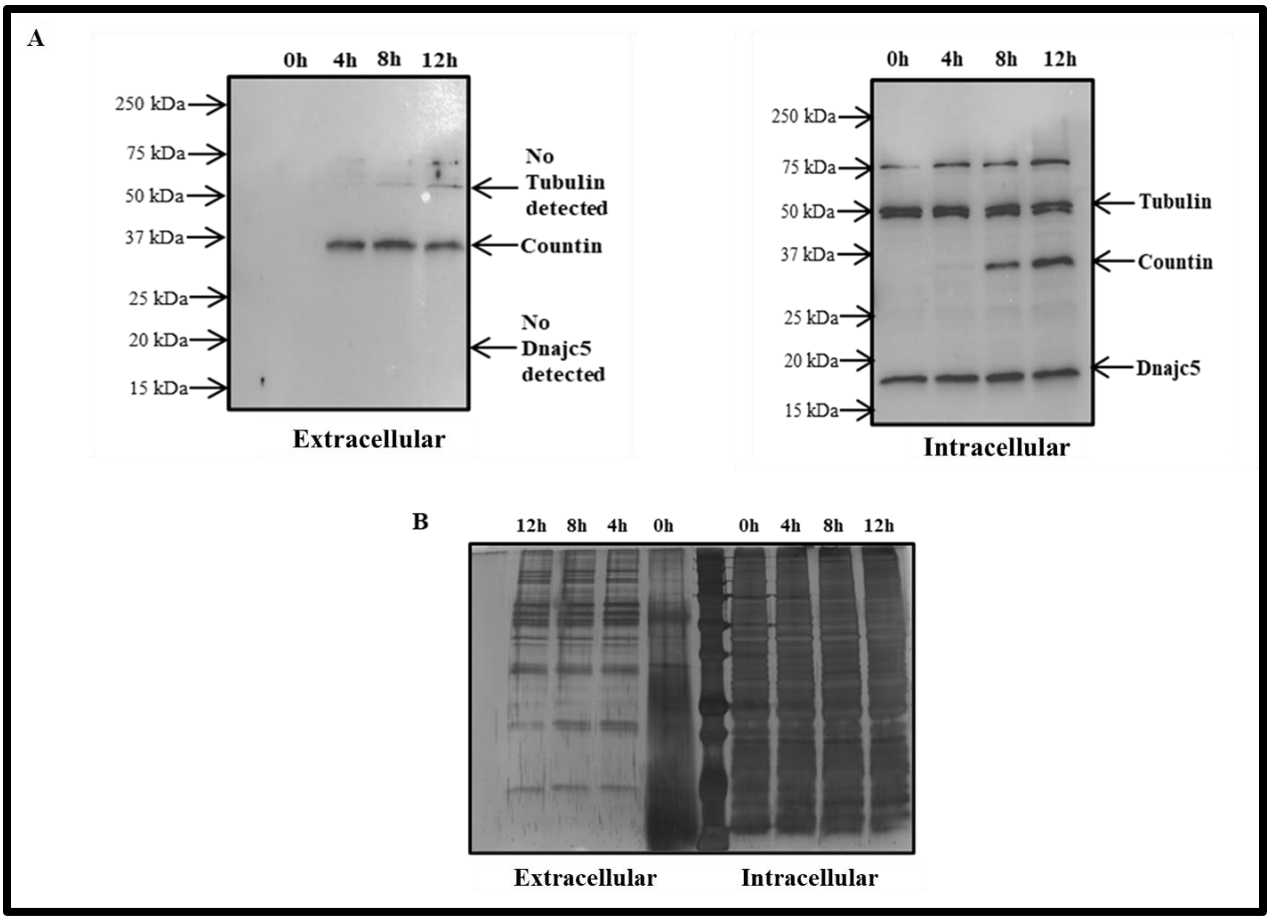

Figure 21. Western blot and silver staining analysis of Dnajc5 secretion in *D. discoideum* during the transition from growth to early development. A) Western blot analysis of Dnajc5 in extracellular material (conditioned media/buffer) and intracellular fractions (whole cell lysates) collected during growth and at 4-, 8-, and 12-hour starvation time points. Dnajc5 was not detected in any extracellular samples, indicating it is not secreted during these stages. Intracellular (WC) samples were loaded at 16 µg per lane, while extracellular (CM/CB) samples were loaded at 1 µg per lane, except for the growth condition, where 15 µL of CM mixed with 2× Laemmli buffer was used. Tubulin (1:1000) was used to confirm the absence of cell lysis in extracellular samples, and countin (1:1000) was used as a positive control for secretion. **(B)** Silver staining of total protein in extracellular (CM/CB, left) and intracellular (WC, right) samples was performed to assess overall protein content and verify consistent sample loading. The experiment was conducted in biological triplicates for reproducibility.

2.4.5 Dnajc5 levels are upregulated during early development of *D. discoideum*

To investigate the temporal regulation of Dnajc5 during multicellular development in *D. discoideum*, WC were collected at defined developmental stages: vegetative growth and at 4, 8, 12, 16, and 24 hours of starvation on 1xKK2 agar plates. Western blot analysis was performed

using an anti-Dnajc5 antibody (1:500), with 15 µg of total protein loaded per lane (Figure 16A). Silver staining was used to verify equal protein loading across all samples by comparing the overall intensity and pattern of total protein bands in each lane. Consistent banding patterns and similar total staining intensity across lanes indicated that comparable amounts of protein were loaded for each sample, confirming loading uniformity (Figure 16B). Dnajc5 protein was detected throughout the developmental time course, with a progressive increase in expression from the growth phase through 4 and 8 hours of starvation, reaching a peak at 12 hours—a stage corresponding to early mound formation and active cell signaling (Figure 16A; Figure 17). Following this peak, Dnajc5 levels declined markedly at 16 and 24 hours, consistent with the transition to later stages of morphogenesis and terminal differentiation. Quantitative analysis of Dnajc5 signal intensity confirmed this expression pattern, demonstrating a gradual increase in protein levels through 12 hours of development, followed by a pronounced decrease at later time points (Figure 17). Notably, this protein expression trend closely mirrors the mRNA expression profile of the *dnajc5* observed during development (Figure 8; Figure 17).

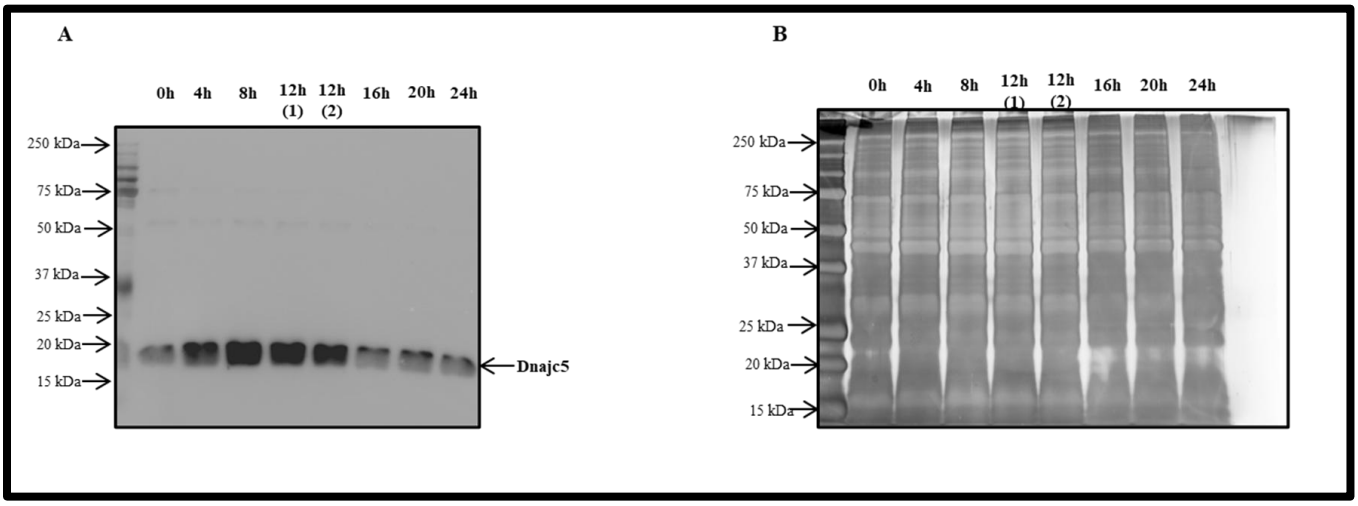

Figure 22. Western blot and silver staining analysis of Dnajc5 expression during multicellular development in *D. discoideum.* A) Western blot analysis of Dnajc5 expression in WC collected at growth and at 4-hour intervals from 4 to 24 hours of starvation (4 h, 8 h, 12 h, 16 h, 20 h, and 24 h). Each lane was loaded with 15 µg of total protein. An anti-Dnajc5 antibody

(1:500) was used to monitor changes in protein expression during development. **(B)** Silver staining of the same samples was performed to verify equal loading and assess total protein content across all time point. This experiment was performed in biological triplicates to ensure reproducibility.

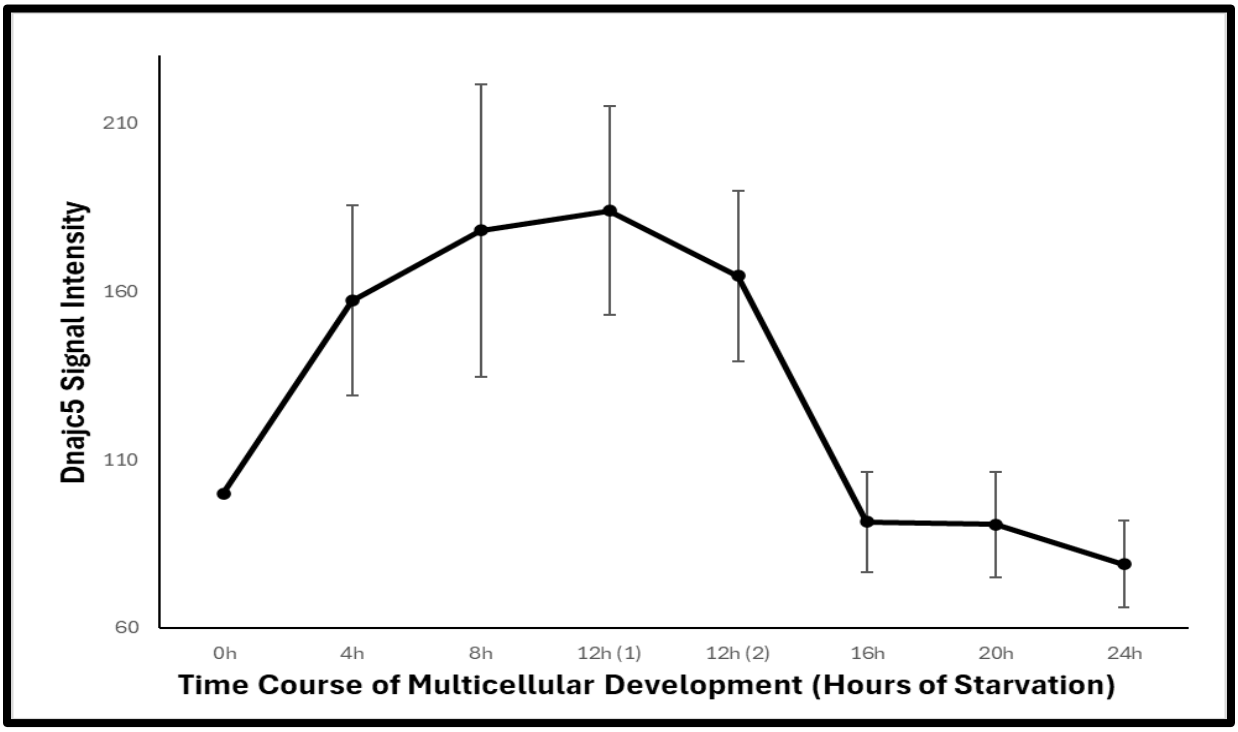

Figure 23. Quantification of Dnajc5 signal intensity during multicellular development in *D. discoideum*. Dnajc5 protein levels were measured by western blot at multiple time points: growth (0 hour) and after 4, 8, 12, 16, 20, and 24 hours of starvation. Signal intensities were quantified, normalized to the growth (0 hour) time point, and plotted to show changes in Dnajc5 levels over time. Error bars represent the standard error of the mean from three biological replicates per time point.

2.4.6 Dnajc5 is neither ubiquitinated nor phosphorylated and does not interact with Grp78.

IP of Dnajc5 was successfully validated, as evidenced by a strong band in the IP lane under both growth and starvation conditions (Figure 18 and Figure 19), confirming efficient pulldown of the target protein. Immunofluorescence analysis revealed that Dnajc5 localizes to the ER and the cytoplasm (Figure 13 and Figure 14). Since, DNAJC5 is known to interact with cytosolic HSP70 family proteins in humans (Hennessy et al., 2000). We suspected that Dnajc5 might also interact with an ER-resident Hsp70 protein. However, the lack of a commercially

available antibody for cytosolic Hsp70 in *D. discoideum*, necessitated that we use the only known ER-resident Hsp70 in *D. discoideum,* Grp78 (Domínguez-Martín et al., 2018). We investigated whether Dnajc5 interacts with Grp78 using a polyclonal custom-made antibody against Grp78. Grp78, which has an expected molecular weight of approximately 75 kDa, was not detected in the Dnajc5 IP lanes. This indicates that under growth conditions or after 4 hours of starvation, Dnajc5 does not form a stable or detectable complex with Grp78 in *D. discoideum* (Figures 18 and 19).

Similarly, the absence of a ubiquitin signal suggests that Dnajc5 is not ubiquitinated during either growth or starvation. Interestingly, under growth conditions, a high molecular weight smear ranging from ~250 kDa to 75 kDa was observed in the IP lane, which disappeared following a 4-hour starvation. No bands were observed at ~20 kDa—the expected molecular weight of Dnajc5—when probing for phosphoserine or phosphothreonine, suggesting that Dnajc5 is not phosphorylated on these residues under the tested conditions (Figure 18). However, during growth, both phosphoserine and phosphothreonine probing revealed multiple bands at approximately 75 kDa and 50 kDa across the IP, PD, and WC lanes. Notably, the 50 kDa band became more intense following starvation. In addition, phosphoserine probing also showed an intense band at 50 kDa during starvation (Figure 18; Figure 19), while phosphothreonine probing revealed bands above 50 kDa, including a prominent band at 250 kDa during growth. Although the 50 kDa band observed in the phosphoserine and phosphothreonine-containing protein blots could correspond to the antibody heavy chain, its absence under growth conditions—despite identical probing conditions—suggests that it may represent a phosphothreonine- and phosphoserine-containing proteins that interacts with Dnajc5 under nutrient-depleted conditions (Figure 19; Figure 18). These findings collectively indicate that while Dnajc5 itself is neither

phosphorylated nor ubiquitinated, its interaction with a phosphothreonine- and phosphoserine-containing proteins appears to be modulated by starvation. This suggests a potential shift in Dnajc5's functional role in response to changes in nutritional status.

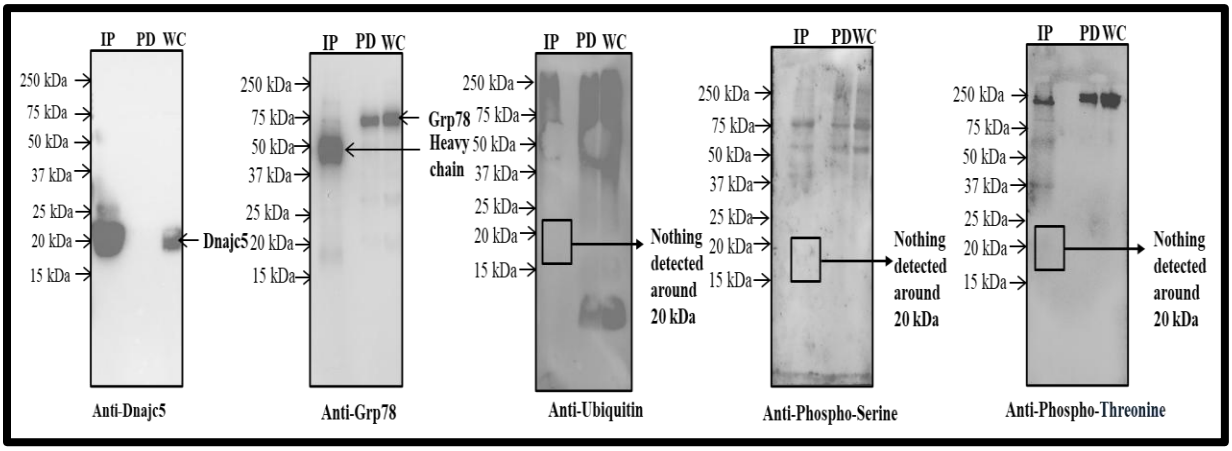

Figure 24. IP and Western blot analysis of Dnajc5 in *D. discoideum* during growth. The protein Dnajc5 was immunoprecipitated, and the blots were probed with antibodies against Dnajc5 (1:500), Grp78 (1:500), ubiquitin (1:1000), phospho-serine (1:1000), and phospho-threonine (1:1000) to assess potential interactions and post-translational modifications of Dnajc5. IP represents the immunoprecipitated sample, PD (protein-depleted) refers to the supernatant collected after immunoprecipitation, and WC indicates the whole cell lysate without any treatment. Blots are displayed in a single row with labels indicating the specific antibody used for detection. The blots are arranged in a single row with appropriate labels indicating the specific antibody used for detection.

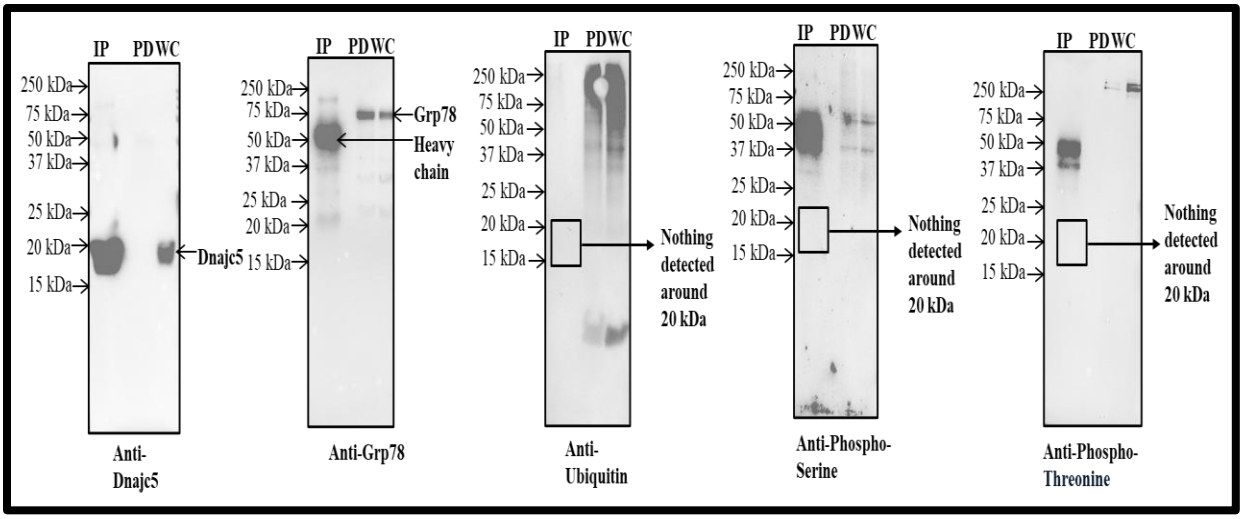

Figure 25. IP and Western blot analysis of Dnajc5 in *D. discoideum* after starvation for 4 hours. The protein Dnajc5 was immunoprecipitated, and the blots were probed with antibodies against Dnajc5 (1:500), Grp78 (1:500), ubiquitin (1:1000), phospho-serine (1:1000), and

phospho-threonine (1:1000) to assess potential interactions and post-translational modifications. IP represents the immunoprecipitated sample, PD (protein-depleted) refers to the supernatant collected after immunoprecipitation, and WC indicates the whole cell lysate without any treatment. Blots are displayed in a single row with labels indicating the specific antibody used for detection.

2.4 Discussion

This study provides the first detailed characterization of Dnajc5 in *D. discoideum*, offering new insights into its developmental regulation, subcellular localization, and expression throughout the life cycle. Using a custom-generated antibody specific to *D. discoideum* Dnajc5, we demonstrated that the protein localizes to both the ER and the nucleolus. Its protein levels pattern parallels reported mRNA expression, suggesting coordinated transcriptional regulation. Although ubiquitination or serine phosphorylation was not detected under our conditions, such modifications may occur under specific or untested stimuli.

Immunofluorescence analysis revealed that Dnajc5 localizes to the nucleolus in *D. discoideum*, with strong enrichment in oval subnuclear structures consistent with nucleoli, as indicated by their sensitivity to AM-D treatment. Sequence analysis of Dnajc5 further identified a lysine-rich region (amino acids 104–107) resembling a nucleolar localization signal (NoLS), suggesting that Dnajc5 may play a role in nucleolar functions such as protein quality control or ribosome biogenesis (Lu et al., 2021; Nielsen et al., 2014; Leary et al., 2001). Human DNAJC5 is an acidic protein with an isoelectric point (pI) of approximately 4.5, in contrast to the predicted pI of ~8.54 for *D. discoideum* Dnajc5, suggesting a more basic character in line with nucleolar localization (Gasteiger et al., 2005; Martin et al., 2015). While nucleolar localization of human DNAJC5 has not been reported, other DNAJ family members, such as DNAJB1 and DNAJA1, are known to localize to the nucleolus during cellular stress (Jung et al., 2023). Given the nucleolus's role in sequestering misfolded proteins, Dnajc5 may contribute to the prevention of

protein aggregation during stress (Frottin et al., 2019). Additionally, Dnajc5 contains a conserved HPD motif, a hallmark of Hsp70 co-chaperones, suggesting potential interaction with the Hsp70 machinery (Piette et al., 2021). Although Hsp70 proteins have not yet been observed in the nucleolus of *D. discoideum*, they are known to localize from the cytoplasm to the nucleolus under stress in other systems, such as HeLa cells (Kotoglou et al., 2009). Alternatively, Dnajc5 may function independently of Hsp70, as some DNAJ proteins can refold misfolded proteins without requiring Hsp70 or even a J-domain (Jana et al., 2011). DNAJ proteins and their homologs have also been implicated in ribosome biogenesis. For instance, in *Escherichia coli*, the DnaJ/Hsp70 chaperone system facilitates the assembly of the 30S ribosomal subunit, which is essential for translation initiation (Maki et al., 2002). This suggests that DNAJ proteins, including Dnajc5, localized to the nucleolus may play a conserved role in promoting cellular growth and survival by maintaining protein homeostasis and facilitating ribosome assembly.

In contrast to more complex eukaryotes such as human, where DNAJC5 localizes to lysosomes, late endosomes, the ER, cytoplasm, and is secreted extracellularly via MAPS-mediated secretion, Dnajc5 in *D. discoideum* was not observed in lysosomal or endosomal compartments (Sharma et al., 2011; Wang et al., 2021; Lee et al., 2022; Xu et al., 2018; Hasegawa et al., 2018). Instead, it localized to the ER and cytoplasm, with no secretion observed during vegetative growth or early starvation. This suggests that Dnajc5 may primarily serve an intracellular role, potentially as a chaperone in proteostasis. Its presence in the ER, where protein folding is essential, aligns with its possible involvement in maintaining protein quality within the cell. Although no direct interaction with the ER-resident Hsp70, Grp78, was detected, studies have shown that ER-localized human DNAJC5 can interact with cytosolic Hsp70 proteins (Sharma et al., 2011; Wang et al., 2021; Lee et al., 2022; Xu et al., 2018; Hasegawa et al., 2018).

Therefore, Dnajc5 in *D. discoideum* may function independently of Grp78, interacting instead with cytosolic Hsp70s to facilitate protein folding and stability.

Post-translational modifications such as phosphorylation and ubiquitination play important roles in regulating protein activity, stability, and interactions (Lee et al., 2023). In humans, DNAJC5 undergoes serine phosphorylation and ubiquitination, processes known to regulate its function (Patel et al., 2016). In this study, immunoprecipitation assays were performed to examine whether Dnajc5 in *D. discoideum* undergoes similar post-translational modifications under growth and starvation conditions, given that serine phosphorylation and potential ubiquitination sites are conserved in Dnajc5. However, no serine/threonine phosphorylation or ubiquitination of Dnajc5 was detected under the conditions tested. This absence suggests that, unlike human DNAJC5, Dnajc5 regulation may occur through alternative mechanisms, although the possibility remains that specific environmental or developmental cues may induce these modifications.

This is further complemented by the temporal regulation of Dnajc5 during the multicellular development of *D. discoideum*. Although transcriptional activity was not directly assessed in this study, the observed protein expression pattern closely mirrors the developmental mRNA expression profile of *dnajc5* reported in publicly available datasets (Figure 8; Figure 17). This correlation suggests that Dnajc5 expression is likely regulated, at least in part, at the transcriptional level during development (Stajdohar et al., 2017). During the first 12 hours of development—when cells transition from unicellular growth to multicellular development—*dnajc5* mRNA expression and Dnajc5 levels are significantly elevated (Wilson, 1953). This upregulation suggests that Dnajc5 might be involved in maintaining protein homeostasis, adapting to stress, and potentially coordinating signaling events during early starvation and

aggregation. After 12 hours, however, as cells progress into tipped mounds and slugs, Dnajc5 expression sharply declines (Wilson, 1953). This decline implies that its function is crucial during the early stages of development but becomes less prominent as later morphogenesis progresses. The importance of Dnajc5 is underscored by the challenges encountered in creating a knockout strain; efforts to disrupt the gene have been unsuccessful, suggesting that Dnajc5 is essential for cellular viability or for key early-stage processes (Gruenheit et al., 2021; Kuspa & Loomis, 1992). Further supporting this, Dnajc5's nucleolar localization suggests it may play a role in ribosome biogenesis, an essential cellular process, as its loss leads to cellular lethality (Leary et al., 2001).

In summary, this study provides an initial characterization of Dnajc5 in *D. discoideum* by examining its localization and expression during the life cycle. While Dnajc5 differs from human DNAJC5 in its observed localization and lack of detectable post-translational modifications under the conditions tested, it retains key features of the DNAJ protein family, including the conserved HPD motif. This confirms its identity as a DnaJ protein and suggests that studying Dnajc5 can provide broader insights into the function and regulation of DnaJ proteins beyond just Dnajc5 itself. These findings indicate that Dnajc5 plays a pivotal intracellular role, particularly during early development, in maintaining proteostasis and potentially contributing to nucleolar functions. Future studies will be essential to explore the wider regulatory mechanisms and functional roles of Dnajc5, including its potential for secretion or modification under alternative conditions. Understanding these conserved mechanisms may offer valuable perspectives on DNAJ protein biology more generally, with possible implications for diseases involving protein misfolding and cellular stress responses.

Chapter 3

3.1 General Discussion

This study presents the first characterization of Dnajc5, an uncharacterized protein in *D. discoideum* and a putative homolog of human DNAJC5. While human DNAJC5 is well-studied for its roles in neuronal physiology, MAPS, UPS-mediated degradation, and cellular stress responses, much less is known about its counterparts in simpler eukaryotes. These simpler organisms provide more accessible systems to dissect the core roles and regulatory mechanisms of protein like DNAJC5, potentially revealing novel functions and pathways. Such insights enhance our understanding of protein homeostasis and cellular stress responses and offer valuable models for studying disease processes associated with DNAJC5 dysfunction in humans.

By examining the developmental regulation, subcellular localization, and conserved motifs of Dnajc5, this work offers preliminary insights into its potential involvement in protein homeostasis, stress response, and nucleolar processes. We observed that Dnajc5 localizes predominantly to the ER, cytoplasm, and nucleolus, with no detectable secretion or post-translational modifications—such as ubiquitination or serine/threonine phosphorylation—under the tested conditions. These findings suggest that Dnajc5 expression and localization are regulated through mechanisms distinct from those seen in its human counterpart. These characteristics differ from those reported for human DNAJC5; however, the presence of a conserved HPD motif suggests that Dnajc5 may function as a member of the DNAJ family in *D. discoideum*. While functional divergence cannot be definitively concluded from our data, these observations provide a foundation for further studies into the specific roles and regulatory mechanisms of Dnajc5 in this model organism.

Dnajc5 was observed in the nucleolus, which is consistent with the effects seen after AM-D treatment. This is further supported by sequence analysis revelated a NoLS motif, which is

generally enriched in positively charged residues. The nucleolus is central to ribosomal RNA (rRNA) synthesis and the assembly of ribosomal subunits (Nielsen et al., 2014; Leary et al., 2001). Defects in ribosome assembly can result in diseases such as bone marrow failure syndromes and various cancers. In *D. discoideum*, the nucleolus carries out similar functions, and proteins involved in nucleolar processes are important for the organism's growth and development. DNAJ family proteins, like DNAJC21, assist in ribosome maturation by acting as chaperones during pre-rRNA processing (Batra et al., 2016). In humans, mutations in DNAJC21 are linked to Shwachman-Bodian-Diamond Syndrome (SBDS), a bone marrow failure disorder (Tummala et al., 2016). Although *D. discoideum* lacks a DNAJC21 homolog, it contains an SBDS homolog, essential for ribosome maturation (Wong et al., 2011). This suggests that Dnajc5 may also be involved in ribosome biogenesis or other nucleolar processes, making it an essential protein. This is further supported by findings from the Restriction Enzyme-Mediated Integration Sequencing (REMI-Seq) project—a large-scale initiative that combined restriction enzyme-mediated integration with high-throughput sequencing to generate and map thousands of insertional mutants in *D. discoideum*. Notably, this project failed to produce viable Dnajc5 knockout mutants, suggesting that Dnajc5 may be essential for cell viability (Gruenheit et al., 2021; Kuspa & Loomis, 1992). The inability to generate Dnajc5 knockout mutants implies that it is indispensable for cell survival, likely due to its involvement in ribosome biogenesis or other fundamental processes. This finding is consistent with studies in other organisms, where defects in ribosome-associated proteins lead to growth defects (Tiller et al., 2013; Moss et al., 2007). Dnajc5's essential role in *D. discoideum* suggests it helps maintain ribosome integrity and overall cellular function, reflecting the conserved nature of these processes across eukaryotes. Additionally, Dnajc5's role in cell survival underscores the importance of DNAJ family proteins

in maintaining cellular homeostasis and responding to stress. DNAJ proteins are known for their involvement in protein quality control, including the prevention of protein aggregation and misfolding. This function is crucial not only during stress responses but also under normal growth conditions (Trotz et al., 2001). In *D. discoideum*, Dnajc5 may perform similar roles in maintaining proteostasis during cellular differentiation, growth, and response to environmental changes. The evolutionary conservation of DNAJ proteins across species highlights their importance in maintaining cellular processes that are fundamental to life.

In addition to its role in ribosomal biogenesis, the nucleolus also contributes significantly to protein quality control (Nielsen et al., 2014; Leary et al., 2001). Misfolded or aggregated proteins within the nucleus can compromise cellular homeostasis and interfere with normal cellular functions (Selkoe, 2003). Although molecular chaperone systems, such as the HSP70 family, often facilitate the refolding of these proteins, some misfolding events are irreversible, leading to the formation of persistent aggregates (Reynaud et al., 2010). Under conditions of cellular stress, the nucleolus can act as a transient sequestration site for misfolded proteins, preventing their aggregation and potential toxicity (Frottin et al., 2019). The HSP70 chaperone system plays a central role in this process by refolding misfolded proteins, which are subsequently released back into the nucleoplasm once properly folded (Frottin et al., 2019). Given that Dnajc5 contains a conserved HPD motif known to mediate interaction with HSP70, it is plausible that Dnajc5 collaborates with Hsp70 within the nucleolus of *D. discoideum* to assist in the refolding or sequestration of misfolded proteins (Hennessy et al., 2000; Tsai and Douglas, 1996; Frottin et al., 2019). However, there are no known Hsp70 proteins that exist within the nucleolus. It is possible that Dnajc5 works independently of Hsp70 to fold proteins and precursors of ribosomal subunit. DNAJB1 can retain its chaperone activity and prevent the

aggregation of misfolded proteins even when its interaction with HSP70 is disrupted (Jung et al., 2023; Jana et al., 2011). This suggests that DNAJ proteins can function independently of HSP70 under certain conditions. Similarly, the *D. discoideum* Dnajc5 may be contributing to ribosomal subunit assembly and assisting in protein folding without relying on HSP70 interaction.

The differences in the localization and potential secretion pathways of DNAJC5 between *D. discoideum* and humans underscore the diversity of cellular mechanisms across species, particularly in the context of protein trafficking and secretion. In humans, DNAJC5 is involved in unconventional secretion pathways, utilizing the MAPS and eMI pathways to facilitate protein secretion through secretory vesicles in neurons and through non-neuronal cells via the endosomal-lysosomal system (Sharma et al., 2011; Wang et al., 2021). This secretion process is essential for cellular communication, particularly in the nervous system, where DNAJC5 plays a role in clearing toxic aggregates such as alpha-synuclein, which is associated with neurodegenerative diseases such as Parkinson's disease and CLN4 disease (Wu et al., 2023; Benitez et al., 2017). The ability of DNAJC5 to undergo S-palmitoylation, a lipid modification that enables membrane association, is a key factor that supports its localization to endosomal and lysosomal compartments, where it is involved in protein sorting and secretion (Naseri et al., 2020).

In contrast, *D. discoideum* appears to lack the ability to execute these unconventional secretion pathways. The absence of a cysteine-rich domain in Dnajc5 prevents palmitoylation, a modification that is important for membrane association and trafficking within the endolysosomal compartments (Wu et al., 2023; Ilmer et al., 2019). As a result, Dnajc5 in *D. discoideum* is localized primarily to the cytoplasm and ER, without involvement in secretory vesicles, the endosomal-lysosomal pathway, or extracellularly. This highlights the functional

divergence between species in their cellular trafficking mechanisms. While *D. discoideum* has evolved a simpler protein trafficking system suited to its unicellular lifestyle, humans rely on more complex protein sorting mechanisms to manage the secretion of various molecules, particularly in specialized cells like neurons. Understanding these species-specific differences in protein secretion pathways not only provides insight into the functional versatility of molecular chaperones but also offers a broader view of how cellular processes evolve to meet the demands of different organisms.

Dnacj5 may fulfill an intracellular chaperone role in *D. discoideum* by localizing to the cytoplasm and ER, which are central sites for protein synthesis, folding, and quality control (Schwarz & Blower, 2016). As a potential co-chaperone of HSP70 family proteins, Dnajc5 may contributes to maintaining proteostasis under both normal and stress conditions. In *D. discoideum*, Grp78 is the only ER-localized Hsp70, while human GRP78 has been shown to associate with other DNAJ proteins, such as DNAJC3, particularly during ER stress (Van Domínguez-Martín et al., 2018; Krieken et al., 2021). These interactions can promote chaperone translocation and broader stress responses, including surface signaling roles for GRP78 that affect immunity and cell survival (Farshbaf et al., 2020). Although DNAJC5 is not known to associate with GRP78 in humans, its interaction with cytoplasmic HSP70s suggests it plays a broader role in chaperone-mediated protein quality control (Sharma et al., 2011; Wang et al., 2021; Lee et al., 2022; Xu et al., 2018; Hasegawa et al., 2018). In *D. discoideum*, Dnajc5 may similarly act in the cytoplasm, assisting cytosolic Hsp70s rather than engaging directly with Grp78. This distinction reflects the potential for functional divergence among DNAJ proteins across species, despite conserved structural domains. Unlike mammals, *D. discoideum* lacks certain components of the unconventional secretion pathways linked to DNAJC5, such as

palmitoylation-dependent endolysosomal targeting. Thus, the chaperone system in *D. discoideum* may rely on simplified or alternative interactions to support cellular homeostasis. Understanding these differences enhances our view of how conserved molecular machinery can be adapted for species-specific roles in development, stress response, and protein management.

Post translational modifications are key modulators of protein function, influencing everything from stability and localization to interaction networks and degradation. In humans, DNAJC5 is regulated by modifications such as ubiquitination and phosphorylation, which allow for fine-tuned control of its activity in response to cellular needs (Patel et al., 2016; Wang et al., 2021). Although Dnajc5 in *D. discoideum* lacks the cysteine-rich domain necessary for S-palmitoylation, it may still be subject to other conserved regulatory mechanisms. Indeed, many DNAJ family proteins—36 of the 41 encoded in the human genome—undergo serine and/or threonine phosphorylation, highlighting the evolutionary conservation and functional importance of such modifications (Hornbeck et al., 2015). This evolutionary perspective suggests that Dnajc5 may engage in stress-responsive regulatory circuits that do not rely on direct modification of the protein itself but rather on its association with modified protein complexes. Such interactions can dynamically shift in response to environmental cues like nutrient availability, potentially altering chaperone functions, protein sorting, or stress granule dynamics (Anderson & Kedersha, 2008; Kampinga & Craig, 2010; Shaid et al., 2013). The divergence of regulatory mechanisms between human DNAJC5 and *D. discoideum* Dnajc5 may reflect adaptations to organism-specific cellular contexts, while still preserving a shared reliance on post translational modification-mediated signaling to coordinate protein homeostasis.

The regulation of gene expression in *D. discoideum* is intricately tied to the organism's developmental stages, with proteins like Dnajc5 playing pivotal roles in these processes. The

dnajc5 mRNA expression and its corresponding protein levels exhibit a clear temporal pattern throughout the life cycle of *D. discoideum* (Stajdohar et al., 2017). From a broader perspective, the close match between mRNA and protein levels may suggest tight regulation with possibly limited influence from later steps such as mRNA processing, translation efficiency, or protein modification. This could indicate that protein production is generally aligned with cellular needs during the dynamic transitions of *D. discoideum* development, though further studies are needed to confirm the exact regulatory mechanisms.

The direct correlation between mRNA and protein levels also suggests that Dnajc5's function is likely governed by its interactions with other proteins, rather than by modifications such as phosphorylation or ubiquitination. This insight points to an alternative form of regulation: one in which Dnajc5's activity is modulated through transient or condition-dependent interactions, rather than by covalent changes in its structure. Such regulation may be important during developmental stages when Dnajc5 is upregulated, particularly at the mound stage, to contribute to processes such as cellular differentiation and stress response, which are vital during early development.

Furthermore, the consistent localization of Dnajc5 to the cytoplasm, ER, and nucleolus, without any significant changes during growth and 4-hour starvation, suggests that its function is not dependent on dynamic shifts in cellular localization. Instead, the stability in localization, coupled with condition-specific interactions, reinforces the idea that the protein's function is primarily regulated by its binding partners, rather than by substantial changes in its subcellular distribution. This indicates a sophisticated layer of regulation, where the protein's functional role in *D. discoideum* development is governed by its interaction network rather than its physical localization. Thus, Dnajc5 appears to play a crucial role during early developmental stages,

suggesting its involvement in initiating or sustaining key cellular processes during this important phase (Yousefi et al., 2021).

This study provides the first detailed characterization of Dnajc5 in *D. discoideum*, revealing a protein with broader functional potential than human DNAJC5. Unlike human DNAJC5, which is primarily associated with cytoplasmic and endolysosomal roles in MAPS and stress responses, Dnajc5 in *D. discoideum* localizes not only to the cytoplasm and ER but also prominently to the nucleolus, suggesting a wider range of cellular functions. Its likely involvement in ribosome biogenesis, protein folding within the nucleolus, and possibly nucleolar quality control highlights roles not described for human DNAJC5. Moreover, Dnajc5 appears to function without classical post-translational modifications such as ubiquitination or phosphorylation, relying instead on dynamic protein-protein interactions. These findings suggest that Dnajc5 has evolved a more diverse functional repertoire in *D. discoideum*, adapting to the organism's unique cellular demands. This highlights the functional plasticity of DNAJ family proteins and underscores the importance of studying them across a broad range of species to fully understand their evolutionary and cellular diversity.

3.2 Broader Implications

The findings presented in this study have broader implications for the understanding of molecular chaperone evolution, nucleolar biology, and cellular stress responses. By characterizing Dnajc5 in *D. discoideum*, this work highlights how highly conserved protein families, such as the DNAJ family, can undergo significant functional diversification across different evolutionary lineages (Craig and Marszalek, 2017). In humans, DNAJC5 is primarily involved in membrane-associated protein secretion and cytoplasmic stress responses, but in *D. discoideum*, Dnajc5 has acquired additional roles within the nucleolus, likely contributing to

ribosome biogenesis and nucleolar quality control. This divergence emphasizes that homologous proteins should not always be assumed to perform identical functions across species, even when important domains such as the HPD motif are conserved. Instead, protein functions must be interpreted within the broader context of an organism's specific cellular architecture and developmental needs. Furthermore, the discovery of a nucleolar-localized DNAJ protein in *D. discoideum* expands the current view of nucleolar biology. Traditionally regarded mainly as a site for ribosome production, the nucleolus is increasingly recognized as a hub for stress sensing and quality control (Boisvert et al., 2007; Frottin et al., 2019). The involvement of Dnajc5 in nucleolar functions suggests that lineage-specific chaperone systems may have evolved to protect essential cellular processes such as ribosome assembly under conditions of stress or rapid growth. Finally, this study underlines the importance of using diverse model organisms to uncover novel aspects of protein function that are not apparent in well-studied systems like mammals. Investigations into proteins like Dnajc5 in amoebozoans not only provide insights into the fundamental principles governing cellular organization and proteostasis but also help trace the evolutionary trajectories that gave rise to specialized roles in more complex organisms (O'Malley et al., 2016). Thus, understanding how proteins such as Dnajc5 operate in different biological contexts can ultimately inform broader fields, including evolutionary cell biology, molecular medicine, and the development of therapeutic strategies targeting chaperone systems in human disease.

This study presents the first characterization of Dnajc5 in *D. discoideum*, identifying its distinct localization, regulation, and function. Unlike its human counterpart, Dnajc5 is not secreted, lacks a cysteine-rich palmitoylation domain, and does not undergo ubiquitination or serine/threonine phosphorylation under growth or starvation conditions. It localizes stably to the

cytoplasm, ER, and nucleolus, where a lysine-rich NoLS suggests a role in ribosome biogenesis or nucleolar protein quality control. Notably, Dnajc5 contains a conserved HPD motif within its J-domain, essential for interaction with Hsp70 chaperones, although no such interaction was detected under tested conditions. Its expression appears to be closely controlled, with both protein and mRNA levels rising during early development and decreasing as terminal differentiation progresses. The inability to generate knockout mutants suggests Dnajc5 is essential for viability, highlighting its potential role in fundamental cellular processes and illustrating the functional divergence and plasticity of DNAJ family proteins across species. As such, while Dnajc5 is classified as a DNAJ protein and shares conserved domains, its role and regulation in *D. discoideum* represent an initial step in understanding its diverse functions across species. These findings underscore the complexity and adaptability of DNAJ proteins and suggest that further studies under different conditions may reveal additional aspects of Dnajc5's function in *D. discoideum* (Yousefi et al., 2021).

3.3 Limitations

While this study provides valuable insights into the function and regulation of Dnajc5 in *D. discoideum*, several limitations should be acknowledged. A major limitation is the absence of a knockout model of *dnajc5*, which would have enabled a more direct investigation of its functional role. Although this study did not attempt gene disruption, data from the REMI-seq project indicate that attempts to generate a mutant were unsuccessful, likely due to the gene being essential for cell growth. Additionally, while the presence of a conserved HPD motif supports the classification of Dnajc5 as a member of the DNAJ family and implies potential interaction with cytosolic Hsp70 chaperones, this interaction could not be experimentally verified. This is due to the unavailability of suitable commercial antibodies against *D.*

discoideum Hsp70 proteins, which prevented co-immunoprecipitation and immunoblotting analyses. Other limitations include the restricted range of experimental conditions tested. Most analyses were conducted under growth and starvation conditions, which may not fully capture the functional dynamics of Dnajc5. Exploring its behavior under additional physiological or stress conditions, as well as in available knockout lines of potential interacting proteins such as Hsp70, and mitochondrial Hsp70 could provide deeper insight into its roles and regulatory mechanisms.

3.4 Future prospectives

Given the possibility that Dnajc5 is an essential protein that localizes to the nucleolus, conventional knockout strategies may not be practical. In a previous study involving the essential *sbds* gene in *D. discoideum,* researchers developed a method to generate conditional mutants by inserting temperature-sensitive, self-splicing inteins into the genomic sequence through homologous recombination (Wong et al., 2011). At a restrictive temperature, inteins fail to splice correctly, resulting in the rapid loss of functional protein (Wong et al., 2011; Tan et al., 2009). A comparable approach could be applied to *dnajc5 gene*, allowing for controlled, conditional inactivation and detailed investigation of its protein product function under various experimental conditions. Also, previous studies have shown that the J-domain of other DNAJ proteins can be deleted using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology, suggesting a similar strategy could be applied to Dnajc5 (Jana et al., 2011). In these mutant lines, one could investigate whether Dnajc5 plays a role in ribosomal biogenesis. Utilizing Northern blotting or quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) to examine pre-rRNA processing is an effective approach to determine if Dnajc5 affects ribosome biogenesis (Wang et al., 2016; Qi et al., 2020). Alterations in the balance of precursor and mature rRNA

levels could reveal defects in processing, providing insights into the functional involvement of Dnajc5 in the nucleolus (Liang et al., 2009). Additionally, to explore potential protein interactions, a custom antibody against Hsp70 proteins could be developed to examine how Hsp70 interacts with Dnajc5 mutants.

Additionally, two CLN4 disease-associated mutations identified in humans are conserved in Dnajc5, making them promising targets for site-directed mutagenesis (Bachman, 2013). One feasible modification would be substituting alanine at position 63 with valine, particularly if Dnajc5 is confirmed to be a true homolog of human DNAJC5 (Faruq et al., 2021). In contrast, deleting leucine at position 115 is likely to be uninformative in *D. discoideum*. In humans, this deletion affects the cysteine-rich domain necessary for S-palmitoylation—a modification essential for secretion of DNJAC5 (Nosková et al., 2011). However, since Dnajc5 in *D. discoideum* lacks this domain and is not secreted under starvation stress, it is unlikely to undergo palmitoylation, rendering the Leu115 deletion irrelevant. Furthermore, samples obtained through IP could be analyzed using liquid chromatography-mass spectrometry (LC-MS) to examine the Dnajc5 interactome under different conditions, including growth, starvation, and other stress treatments (Lin et al., 2003). LC-MS analysis of Dnajc5 interactors under growth, starvation, and ER stress may reveal associations with misfolded ER proteins such as membrane receptors, glycoproteins, and proteases, along with ER chaperones (Rutkowski & Kaufman, 2004; Lee, 2005). It may also identify components of the macropinocytic machinery, including actin regulators, Rab GTPases, and endosomal sorting complexes highlighting Dnajc5's potential chaperone function in proteostasis, vesicle trafficking, and membrane dynamics (Journet et al., 2012; Swanson, 2008; Donaldson, 2019; Hacker et al., 1997;).

While this study was limited to growth and 4-hour starvation conditions, Dnajc5's localization to ER presents an opportunity to investigate its role in ER function. ER stress in *D. discoideum* can be experimentally induced using tunicamycin, which inhibits N-linked glycosylation, thereby impairing the proper folding of glycoproteins (Yoon et al., 2023). This leads to the accumulation of misfolded proteins within the ER, including membrane-bound receptors, surface glycoproteins, and secretory enzymes such as proteases and acid hydrolases (Feasely et al., 2010). In *D. discoideum*, these include surface glycoproteins like gp130, as well as secretory enzymes such as α-mannosidase, all of which rely on proper glycosylation and ER quality control for maturation and function (Barent et al., 2001; Feasely et al., 2010). Investigating Dnajc5's behavior under tunicamycin-induced ER stress could reveal new insights into its potential role in mitigating protein misfolding and maintaining ER homeostasis in *D. discoideum.* Cells treated with tunicamycin for a specific duration could then be analyzed via immunofluorescence to observe changes in Dnajc5 localization. In parallel, western blotting could be used to assess changes in Dnajc5 protein levels and to determine whether it is secreted under ER stress conditions. If secretion or change in Dnajc5 level is observed, further investigation into Dnajc5's stress-induced interactome could be conducted through IP followed by LC-MS analysis.

In summary, this study provides the first in-depth characterization of Dnajc5 in *D. discoideum*, highlighting its distinct localization, regulation, and potential functional roles. The close correlation between mRNA and protein levels throughout the life cycle suggests that Dnajc5 expression is tightly controlled, with its activity likely modulated more by protein interactions than by post-translational modifications. Despite the absence of a knockout model, these findings open several avenues for future research, including the development of conditional

mutant lines to better understand the precise functional role of Dnajc5 in ribosome biogenesis, nucleolar function, and early development. Further investigations into its interactions and the mechanisms underlying its regulation will provide valuable insights into the diverse roles of DNAJ proteins across different species.

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