Review

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Decoding Skd3 (Human *CLPB*): a Mitochondrial Protein Disaggregase Critical for Human Health

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Abstract: Protein folding is important for all life. Indeed, protein misfolding can result in catastrophic protein aggregation and toxicity. The pathways involved in reversing protein aggregation within human mitochondria had long been unknown. We recently discovered that Skd3 (human *CLPB*) is a potent mitochondrial protein disaggregase, which is regulated by the rhomboid protease PARL, and maintains the solubility of many important mitochondrial proteins. Skd3 variants underlie several debilitating human diseases, including 3-methylglutaconic aciduria, severe congenital neutropenia, and premature ovarian insufficiency. Here, we describe advances in understanding Skd3 function, mechanism, and structure and place these discoveries in the context of physiology and disease.

1. Introduction

Encoded within the primary sequence of all proteins are the instructions to fold into an energetically favorable, functional conformation termed the native state.[1] Chaperones are needed to guide proteins in the complex milieu of the cell to their native fold and away from deleterious monomeric and multimeric conformations.[2] Upon catastrophic misfolding, protein aggregation can occur and some aggregated conformers (particularly, prions and amyloids) can self-template their structure by recruiting and converting natively folded proteins into the aggregated state.[2a,3] Often, protein aggregates are more energetically favorable than the native state and chaperones can have difficulty reversing protein aggregation.[2a] Some protein aggregates can be beneficial;[3–4] however, many are toxic due to the loss of function of the native protein, a gain of function of aggregated conformers, or both.[3,5] To combat these issues, diverse protein-disaggregase machineries exist in various cellular compartments, which disassemble protein aggregates by extracting individual polypeptides and restoring them to soluble, native form and function.[3,6] Indeed, non-metazoan mitochondria contain the AAA+ (ATPases associated with various cellular activities) protein disaggregase, Hsp78, within the mitochondrial matrix, which dissolves and reactivates trapped in protein aggregates (Figure 1).[6h,7] However, metazoa lack Hsp78 and although protein aggregates within mitochondria can be sequestered and eliminated via selective mitophagy, no system for protein disaggregation and reactivation within metazoan mitochondria was known.[8] Recently, we[6b] and others[9] discovered that Skd3 (human *CLPB*) is a potent human mitochondrial protein disaggregase (Figure 2). In this review, we will briefly discuss: (1) the cellular function of Skd3, (2) the mechanism and structure of Skd3, and (3) the relevance of Skd3 to human health and disease.

2. Skd3 Function

Suppressor of potassium (K) transport defect 3 (Skd3; also known as human *CLPB*) was the first human gene discovered with homology to the ClpB/Hsp104 family of protein disaggregases (Figure 1).[10] Although Skd3 appears to be related to the ClpB/Hsp104 family of proteins, some have suggested a closer relationship to the bacterial ClpC family.[8b] Skd3 is conserved in most, but not all metazoan lineages and first appears in choanoflagellates, a group of free-living unicellular and colonial flagellate protozoa considered to be the closest extant relatives of metazoa.[6b,8b] For example, *Monosiga brevicollis* expresses Skd3, Hsp78, and Hsp104, but upon the evolutionary transition to metazoa Hsp78 and Hsp104 are lost, whereas Skd3 is retained, indicating a shift in disaggregase biology in metazoa.[8b] While Hsp104 localizes to the cytosol and Hsp78 localizes to the mitochondrial matrix, intriguingly, Skd3 localizes to the mitochondrial intermembrane space (IMS).[6b,10b,11]

Skd3 is comprised of a N-terminal mitochondrial targeting signal (MTS), a short 35 amino acid hydrophobic region, an ankyrin repeat domain (ANK), a nucleotide-binding domain (NBD), and a C-terminal domain (CTD) (Figure 1).[6b,8b] Although they are sometimes confused for each other within the literature, Skd3 (human *CLPB*) differs appreciably from

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bacterial ClpB (Figure 1). Ankyrin repeats are a flexible structural scaffold that can be readily adapted to specifically bind to a variety of substrates and are often found in association with cellular membranes.[12] No other protein is known to contain both a series of ankyrin repeats and a NBD. The Skd3 NBD is from the HCLR clade of the AAA+ family of proteins.[13] All members of the HCLR clade of AAA+ proteins couple ATP hydrolysis to protein disaggregation or unfolding.[13] Despite the association with HCLR clade AAA+ proteins, the biochemical and cellular function of Skd3 remained unknown long after its initial discovery.[10a]

Challenges with purification of recombinant Skd3 limited the study of its biochemical activity.[14] We discovered that Skd3 has potent protein disaggregase activity *in vitro* against diverse aggregated substrates.[6b] Additionally, another study found that Skd3 had potent refoldase activity against misfolded substrates.[9] It was discovered that the 35 amino acid hydrophobic region in Skd3 is cleaved off by the rhomboid protease, PARL, in human cells and mice; however, the biochemical relevance of this cleavage was not known.[15] We discovered that removal of the hydrophobic region of Skd3 by PARL increased Skd3 disaggregase activity by greater than 10-fold without appreciably altering its ATPase activity (Figure 2).[6b] PARL-cleaved Skd3 was even potent enough to dissolve disease-associated α-synuclein fibrils.[6b,16] These findings indicated that the hydrophobic region of Skd3 acted as an inhibitory peptide to limit Skd3 disaggregase activity.[6b] It is yet unclear how PARL cleavage of Skd3 is physiologically regulated and what factors enable or inhibit PARL-mediated Skd3 cleavage and subsequent activation. For example, it is possible that PARL cleavage of Skd3 increases in response to stress. Alternatively, the inhibitory peptide may limit Skd3 disaggregase activity until it is trafficked to the mitochondria. These possibilities are not mutually exclusive. Other post-translational modifications also likely impact Skd3 function; Skd3 is phosphorylated, acetylated, and potentially ubiquitylated.[17] The consequence of these modifications are not yet understood.

We discovered that Skd3 maintains protein solubility within the mitochondrial intermembrane space.[6b] Indeed, in the absence of Skd3, a large number of mitochondrial proteins become aggregated and mitochondrial proteostasis is perturbed.[6b,18] However, thus far Skd3 has only been shown to have chaperone activity in cells; to demonstrate *bona fide* disaggregase activity, careful experimental design is required. The ANK is required for substrate interaction; therefore, it is likely that the ANK is required for chaperone activity in cells.[19] Indeed, the point mutation Y272C in the ANK reduces Skd3 substrate solubility.[16]

A prominent protein that exhibits reduced solubility in the absence of Skd3 is HAX1.[6b] HAX1 is a highly intrinsically disordered, antiapoptotic protein, which functions via an interaction with HTRA2 and PARL.[6b,20] Skd3 interacts with HAX1.[6b,10b,11d,16,18–19,21] The ANK of Skd3 is required for HAX1 binding; the PEST domain and an adjacent stretch of amino acids in HAX1 are required for binding to Skd3 in cells.[18a] Residue Y272 in Skd3 enables interaction with HAX1 and residue L130 in HAX1 enables interaction with Skd3.[18a] We demonstrated that Skd3 is required to maintain HAX1 solubility in cells (Figure 2).[6b] Indeed, HAX1 forms high molecular weight complexes in the presence of mutant Skd3.[18b] The functional consequence of HAX1 aggregation upon the loss of Skd3 function is not clear. Skd3 mutant cells have increased apoptotic sensitivity, so it is possible that the loss of HAX1 solubility drives increased apoptosis in Skd3 mutant cells.[21–22] However, Skd3 also interacts with and maintains the solubility of other proteins involved in apoptosis such as PARL, OPA1, HTRA2, and SMAC/DIABLO, which may also contribute to the heightened apoptotic sensitivity of Skd3 mutant cells (Figure 2).[6b,11d,19,21] Furthermore, Skd3 mutant cells display aberrant mitochondrial morphology and inner mitochondrial membrane topology, which may also increase apoptotic sensitivity.[21]

Skd3 engages and maintains the solubility of several other important clients, including components of the mitochondrial respiratory chain and its assembly factors, such as complex I proteins NDUFA8, NDUFA10, NDUFA11, NDUFA13, NDUFB7, NDUFB10, ACAD9, and TTC19; complex III proteins CYC1 and COX11; and complex V proteins ATP5F1A, ATP5F1B, ATP5F1C, ATP5PO, ATP5J2, and ATP5MF among others (Figure 2).[6b,11d,19,21] Interestingly, respiratory complex I, III, and TCA cycle proteins were dysregulated in Skd3 knockout cells.[18a] Functionally, Skd3 deficiency results in decreased basal and maximal cellular respiration.[21–22] We suggest that Skd3 regulates mitochondrial respiratory complex assembly and activity by maintaining the solubility of key respiratory chain components and assembly factors.

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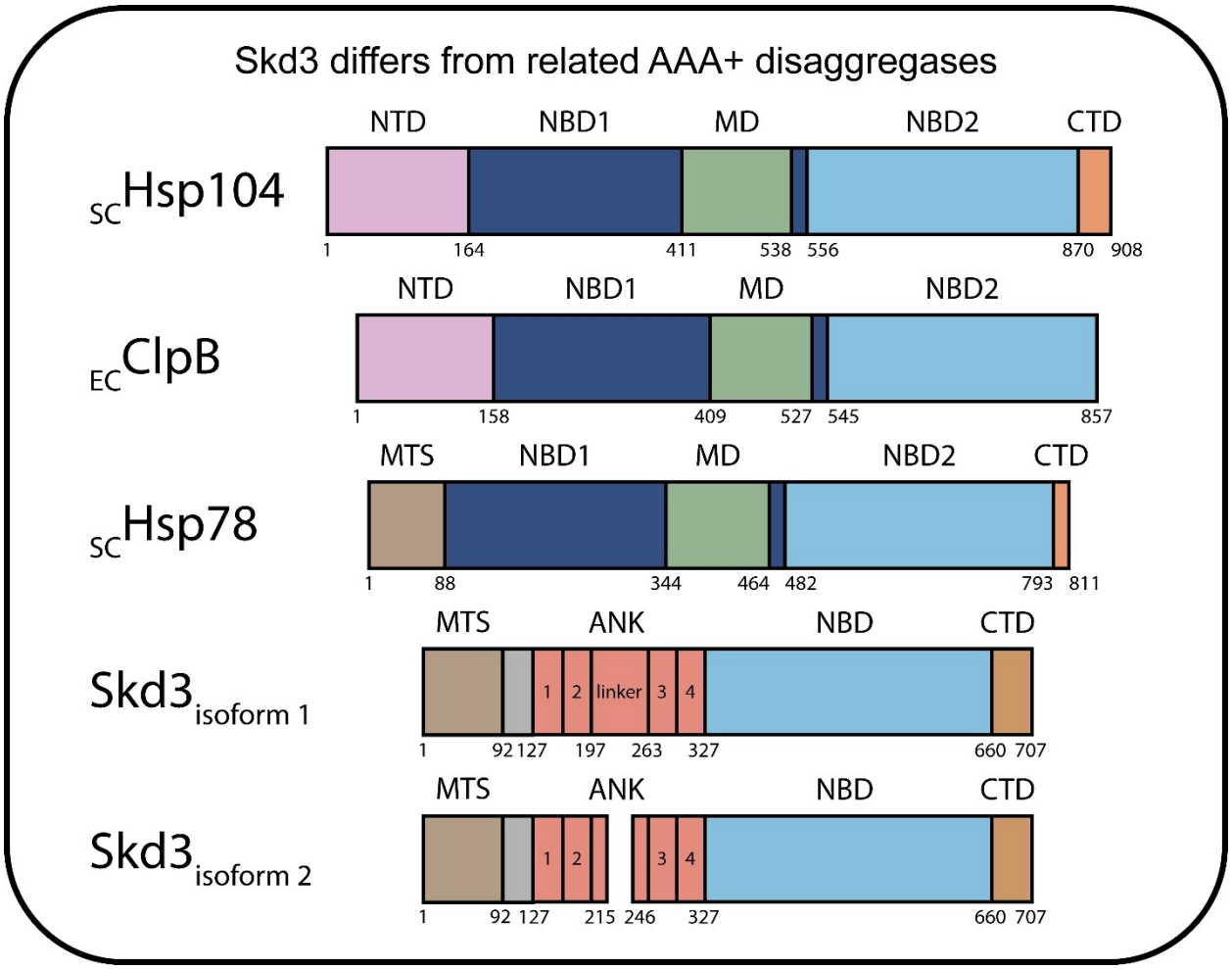

Figure 1. Domain architecture of Skd3 and related AAA+ disaggregases. Domain map depicting *S. cerevisiae* Hsp104, *E. coli* ClpB, *S. cerevisiae* Hsp78, and *H. sapiens* Skd3 (isoforms 1 and 2). Hsp104 is composed of a N-terminal domain (NTD), nucleotide-binding domain 1 (NBD1), middle domain (MD), nucleotide-binding domain 2 (NBD2), and C-terminal domain (CTD). Bacterial ClpB is composed of a NTD, NBD1, MD, and NBD2, but lacks a CTD. Hsp78 is composed of a mitochondrial-targeting signal (MTS), NBD1, MD, NBD2, and CTD. Skd3 is composed of a MTS, a short hydrophobic inhibitory peptide, an ankyrin-repeat domain (ANK) containing four ankyrin repeats, an NBD that is homologous to Hsp104/Hsp78/ClpB NBD2, and a CTD. The Skd3 CTD is not homologous to the Hsp104/Hsp78 CTD. Skd3 isoforms 1 and 2 differ in the loss of 30 amino acids within the linker region of the ANK.

Additionally, several lines of evidence suggest that Skd3 engages mitochondrial calcium uniporter-associated proteins (MCU), members of the mitochondrial carrier family (SLC25 family proteins), translocase of the inner membrane (TIM) components, and Prohibitins (PHB1 and PHB2) (Figure 2).[6b,19] Skd3 interacts with MICU1, MICU2, MCUB, and MCU and we found that Skd3 maintains MICU1 and MICU2 solubility in cells.[6b,19] The functional consequences of MICU1 and MICU2 insolubility are not known. Skd3 binds and solubilizes the mitochondrial carrier family proteins SLC25A13 (ARL2), SLC25A25, SLC25A32 (MTF), SLC25A33, SLC25A36, and SLC25A39.[6b,11d,19] Skd3 also maintains the solubility of 21 additional SLC25 family proteins, including SLC25A4 (ANT1), SLC25A5 (ANT2), SLC25A6 (ANT3), and SLC25A12 (ARL1).[6b,11d,19] Skd3 expression is upregulated in mice lacking the skeletal muscle/heart ANT1 isoform.[23] We speculate that Skd3 may play a role in chaperoning the insertion of SLC25 family members into mitochondrial membranes from the IMS. There is also a decrease in solubility of TIM components in Skd3 knockout cells.[6b] Indeed, we suspect that these proteins might co-aggregate with their substrates such as SLC25A family members. Skd3 binds and solubilizes PHB1 and PHB2 and was found to sub-stoichiometrically migrate with Prohibitin assemblies via complexome profiling.[6b,18b,19] Functionally, Skd3 has been suggested to play a role in RIG-I-MAVS signaling via a PHB1/2, Skd3, and MAVS complex; however, much remains understood about the interaction between Skd3 and PHB1/2.[11d]

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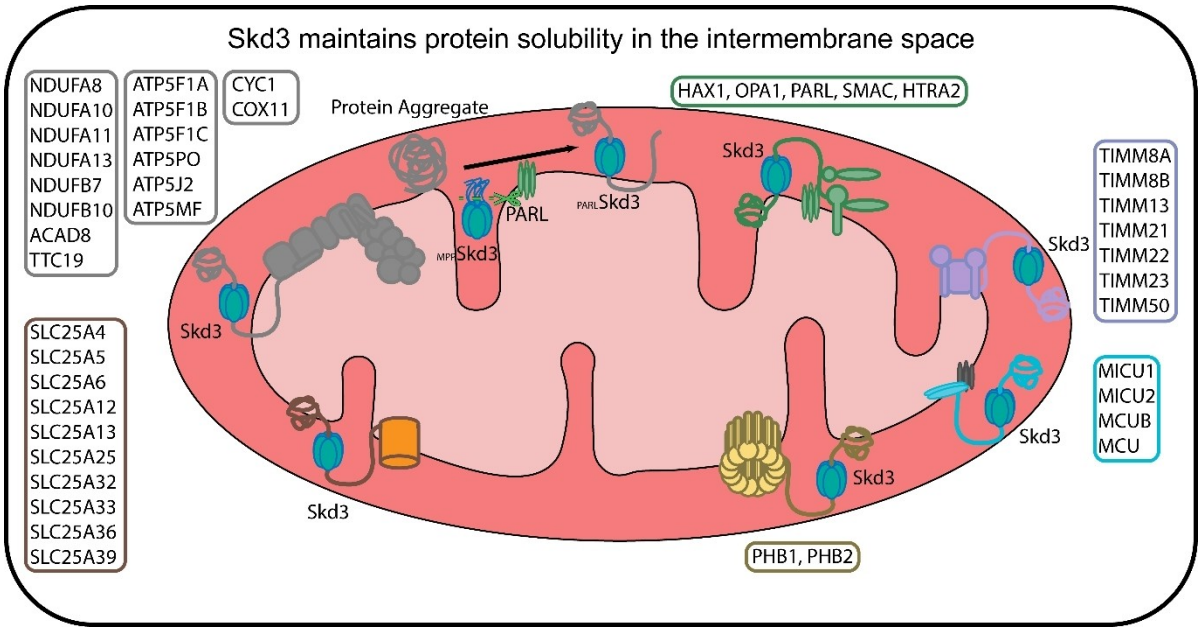

Figure 2. Skd3 is a disaggregase that maintains the solubility of important IMS proteins. The N-terminal inhibitory peptide of $_{MPP}$Skd3 is cleaved by the rhomboid protease PARL to unlock $_{PARL}$Skd3 disaggregase activity (top, center). Skd3 is required to maintain the solubility of key proteins in the IMS involved in apoptosis, mitochondrial protein import, mitochondrial calcium handling, prohibitin complexes, mitochondrial solute transport, and mitochondrial respiration (from top center in clockwise order).

Often disaggregases and chaperones work in concert with cochaperones to assist in protein disaggregation, unfolding, and refolding.[6h,l,24] We found that unlike other AAA+ disaggregases such as Hsp104, Hsp78, and bacterial ClpB, Skd3 does not collaborate with Hsp70 and Hsp40 cochaperones to stimulate disaggregase activity.[6b] Skd3 lacks the requisite middle domain of Hsp104 that enables Hsp70 interaction and the human mitochondrial Hsp70 (HSPA9/Mortalin) and main Hsp40 (DNAJA3/TID1), reside within the mitochondrial matrix and are thus unlikely to physiologically interact with Skd3 to assist in substrate disaggregation or refolding.[25] However, a unique mitochondrial Hsp40, DNAJC11, resides within the IMS and interacts with Skd3.[19] It is unknown whether Skd3 can collaborate with DNAJC11 for protein disaggregation or refolding. Interestingly, in the absence of Skd3 or HAX1 the unphosphorylated (inactive) form of HSP27 accumulates in mitochondria and HSP27 appears in the insoluble fraction of mitochondria.[18a] It is unclear if Skd3 and HSP27 physically interact or can collaborate in protein disaggregation as with Hsp104 and the small heat shock proteins Hsp26 and Hsp42.[26] However, HSP27 was recently discovered to play a role in mitochondrial proteostasis in the IMS.[27]

3. Skd3 Mechanism and Structure

Biochemical studies of the domain architecture of Skd3 have revealed the requirements for activity.[6b] PARL-cleaved Skd3 ATPase activity is stimulated by the presence of a model substrate; however, when the hydrophobic region remains attached to Skd3, ATPase activity is not stimulated by substrate.[6b] We hypothesize that the hydrophobic region of Skd3 may mimic substrate and consequently inhibit substrate binding and disaggregation. We found that both the ANK and NBD are required for Skd3 disaggregase activity and that they cannot cooperate in trans.[6b] The ANK is comprised of two N-terminal ankyrin repeats, a linker that displays some homology to ankyrin repeats, and two C-terminal ankyrin repeats.[6b,28] Unsurprisingly, the isolated ANK does not possess ATPase activity; however, the isolated NBD only preserves ATPase activity when residues 297–326 (corresponding to ankyrin repeat #4 from the ANK) are present.[6b,19] The final helix of ankyrin repeat #4 is contiguous with helix D1 in the NBD of Skd3 and is not conserved in NBD2 of Hsp104 (Figure 3).[6b,28] We hypothesize that this final ankyrin repeat maintains interdomain cooperativity between NBD and the ANK to enable ATP hydrolysis. The Skd3 CTD is patterned by both acidic and basic residues and removal of the CTD mildly increases Skd3 ATPase activity without altering disaggregase activity.[28] This finding suggests a role for the CTD in efficient coupling of ATPase and disaggregase activity.

HCLR clade AAA+ proteins form homo-hexameric rings mediated by their NBDs.[13,29] Skd3 forms higher order oligomers in cells and in solution Skd3 has been observed as both a hexamer and a dodecamer (Figure 4).[9,11a,14,19,28,30] Dodecamer and hexamer formation is observed both with and without substrate in the presence of ATP, ADP, the slowly

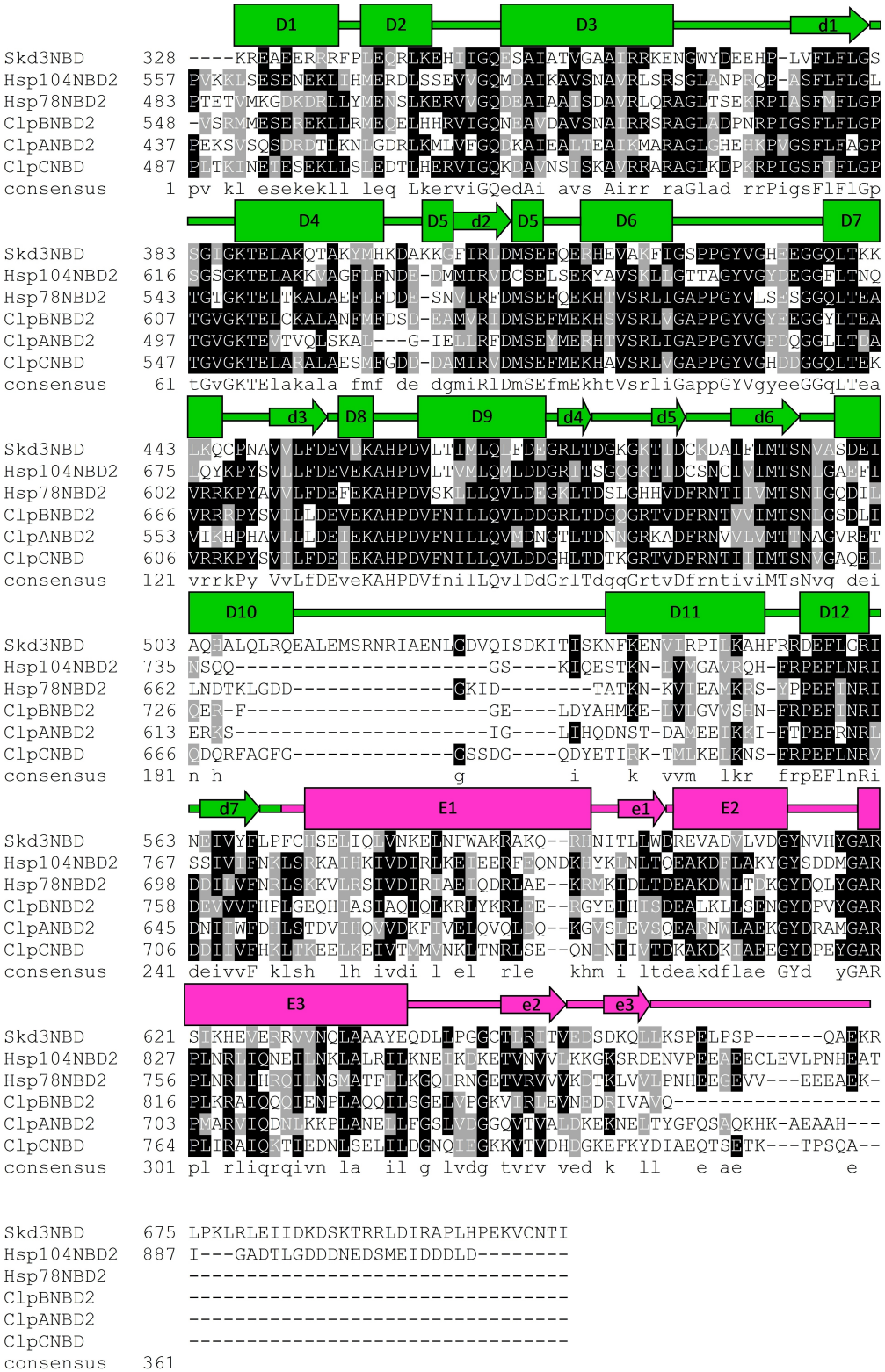

Figure 3. Sequence alignment and secondary structural elements of Skd3. Alignment of the HCLR-clade NBD of *H. sapiens* Skd3, *S. cerevisiae* Hsp104, *S. cerevisiae* Hsp78, *E. coli* ClpB, *E. coli* ClpA, and *S. aureus* ClpC. Sequences are from UniProt and aligned using ClustalOmega. Consensus sequence is listed at the bottom. Secondary structural elements are shown as bars (α-helix) or arrows (β-strand) and are enumerated with upper case (α-helix) or lower case (β-strand) letters. The large sub-domain of the NBDs is labeled in green, and the small sub-domain of the NBDs is labeled in pink. This nomenclature is referenced throughout the manuscript and in Table 1.

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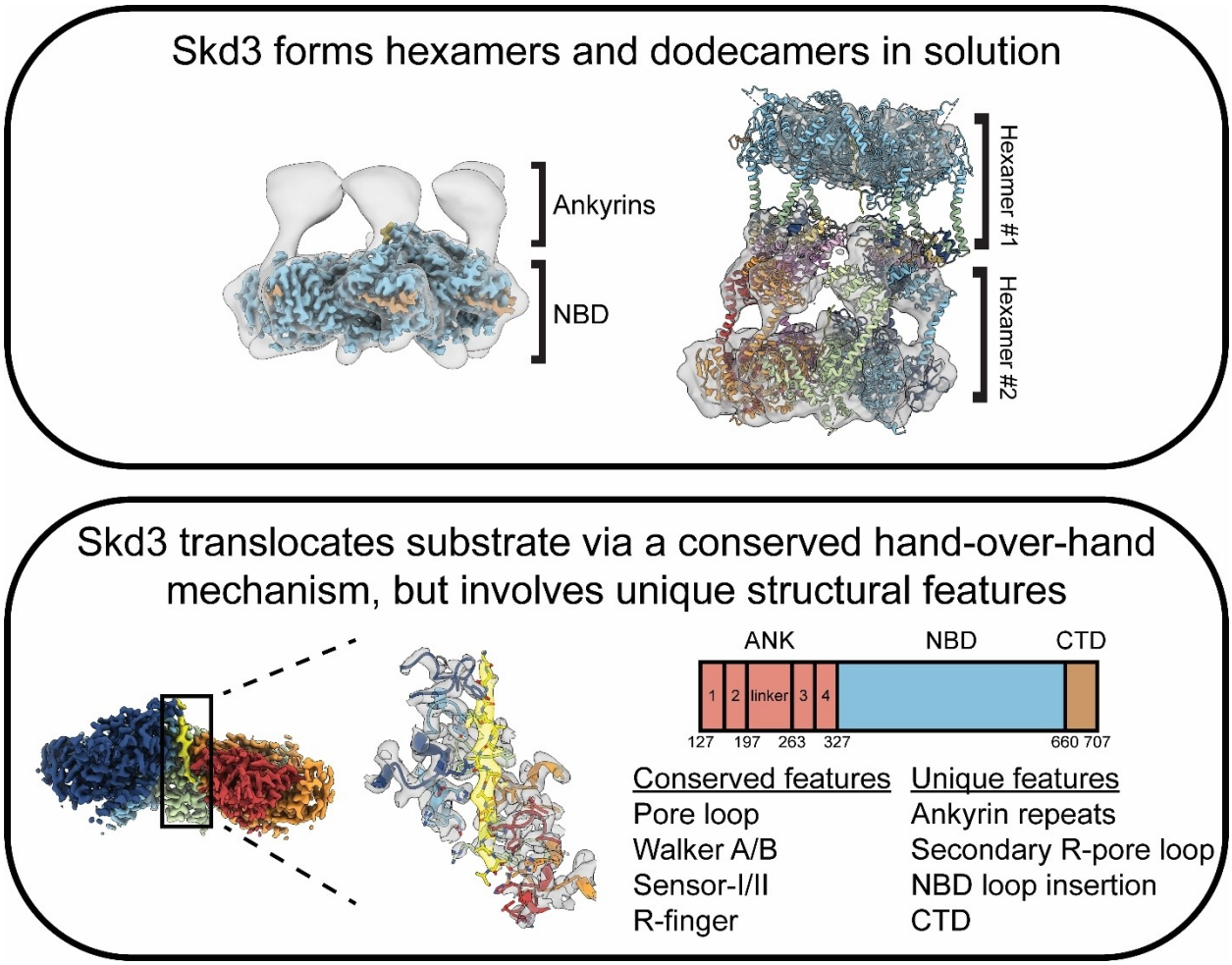

Figure 4. Skd3 forms hexamers and dodecamers and utilizes conserved AAA+ elements as well as unique features to power disaggregase activity. Skd3 forms hexamers and dodecamers in solution. The hexamer is mediated by the NBDs arranged in a spiral staircase around substrate with the ANKs protruding from the top of the hexamer. The dodecamer is comprised of two hexamers binding via head-to-head contacts which are mediated by the ANKs (top). Skd3 utilizes conserved AAA+ elements such as the pore loop, Walker A, Walker B, sensor-I, sensor-II, and R-finger motifs to power disaggregase activity. However, Skd3 also uses the unique ANK, a secondary arginine pore loop, an insertion in the NBD, and a divergent CTD to regulate disaggregase activity (bottom).

hydrolyzable ATP analog ATPγS, and the non-hydrolyzable ATP analog AMP–PNP.[28,30b] Similar to other AAA+ proteins, the formation of the homo-hexamer is mediated by the NBD of Skd3 whereby adjacent Skd3 protomers assemble to form a ring, with the nucleotide binding pocket of each NBD forming at the interface between adjacent protomers (Figure 4).[19,28,30a] In contrast, the dodecamer is mediated via the novel ANK and is comprised of two hexamers forming head-to-head contacts (Figure 4).[19,28,30a] Mutational analysis shows that Skd3 variants lacking the first two ankyrin repeats or the final two ankyrin repeats are all capable of dodecamer formation.[28] However, deletion of the linker region resulted in a defect in dodecamer formation.[28] It is yet unclear what functional role the Skd3 dodecamer plays; mutations that abrogate the dodecamer without altering the substrate-binding function of the ANK will be of key importance. It has been suggested that the Skd3 dodecamer might promote more effective substrate disaggregation, refolding, or both.[28,30a] One study found that Skd3 mutants deficient in dodecamer formation maintain effective disaggregase activity but are defective in client refolding; however, whether this effect is directly from dodecamer formation or some other aspect of Skd3 function is yet to be disambiguated.[30a]

Thus far the ANK has been poorly resolved in full-length structures and the only high-resolution structures have been from isolated ANKs.[16,19,28,30a] Crystal structures of the isolated ANK from Skd3 isoform 1 and 2 have been resolved.[16,19] The first three ankyrin repeats share the canonical helix-turn-helix-hairpin-loop fold; however, the final ankyrin repeat does not have the hairpin and has a 3_{10}-helix substituted for the first helix.[16] Deletion of the first two ankyrin repeats, the linker, or the final two ankyrin repeats decreased disaggregase activity

but had no significant effect on Skd3 ATPase activity.[19,28] Deletion of the first two ankyrin repeats had the least drastic effect; however, all three deletions maintained some level of disaggregase activity.[28] Skd3 isoform 1 contains 30 additional residues in the linker between ankyrin repeats 2 and 3.[16,19] Isoform 1 has a β-hairpin-helix motif in the linker region whereas isoform 2 has two short helices in the linker region.[16,19] One report found that Skd3 isoform 1 and 2 displayed similar ATPase and disaggregase activity.[19] However, another study reported that Skd3 isoform 1 and 2 displayed similar ATPase activity in the presence of substrate, but Skd3 isoform 2 displayed decreased basal ATPase activity.[16] This study also suggested that Skd3 isoform 2 displayed increased disaggregase activity compared to isoform 1.[16] More research is required to determine the precise differences between Skd3 isoforms 1 and 2.

The ANK has a hydrophobic groove, and it is tempting to speculate that this groove acts as a substrate-binding site analogous to the hydrophobic groove in the N-terminal domain of Hsp104.[16,19,28,30a,31] Mutation of residues in this hydrophobic groove ablated disaggregase activity, but it is yet unclear if this effect is due to limiting substrate binding to the ANK.[30a] In support of direct substrate binding by the ANK, the isolated ANK was competent to bind a model substrate and exhibited anti-aggregation activity against the amyloid-β (Aβ42) peptide.[30a] Disease-associated mutations in the ANK also limited Skd3 disaggregase activity.[6b,16,30a] It is suggested that helix α5 of the ANK may compete with substrate interaction.[16] In the full-length Skd3 structure, the ANKs are more poorly resolved possibly due to the flexible or dynamic nature of the domains.[19,28] In the hexamer, the ankyrin repeats are arranged above the NBDs in a spiral around the central pore.[28] Crystal structures and solution measurements of the isolated ANK demonstrate a potential dimerization of the ANKs and electron densities of the ANKs in the full-length hexamer indicate proximity and possible dimerization of adjacent ANKs.[16,28] The function of ANK dimerization is unknown; mutations that ablate ANK dimerization will help to understand the dimer.

The NBD has been well resolved in both full-length structures and in structures of isolated NBD domains (Figure 4).[16,19,28,30a] The Skd3 homohexamer is comprised of adjacent NBDs in a right-handed spiral staircase around a central channel through which substrate is threaded (Figure 4).[19,28,30a] The protomers rise ~6 Å and rotate ~60° along the substrate from protomers P1 to P5 and protomer P6 is at the interface between the lowest (P1) and highest (P5) protomers.[28] This spiral is reminiscent of the NBD2 from ClpA, ClpB, and Hsp104.[28–29] The Skd3 NBD differs in that the dual-ring AAA+ proteins have an additional NBD formed in a spiral on top of the NBD2, whereas Skd3 has the ANKs with flexible density arranged above the NBD.[28–29] The Skd3 tyrosine pore-loops (Y430) are likewise arranged in a spiral staircase around substrate, directly bind substrate backbone, and are the primary points of substrate contact within the NBD.[28] Mutation of the tyrosine pore-loops and adjacent residues (V431 and P427) abolishes disaggregase activity and drastically diminish ATPase activity.[6b,18b,28] The ATPase activity of most other HCLR clade AAA+ proteins is not affected by pore-loop mutations;[13,32] however, pore loop mutations in ClpC similarly abrogate ATPase activity.[33] We speculate that there may be allosteric communication between the tyrosine pore-loops in Skd3 and nucleotide-binding residues required for ATP hydrolysis.

Like other HCLR-clade NBDs, Skd3 contains a secondary pore-loop that is arranged in a spiral staircase around substrate, but unlike other NBDs the identity of the amino acid at this position is an arginine, R417.[28–29] Mutation of this residue does not alter ATPase activity but completely abolishes disaggregase activity.[28] Arginine has a guanidinium group and thus can mimic the unfolding properties of guanidine hydrochloride.[34] The AAA+ proteins p97/VCP and Vps4 have pore-facing arginines that similarly contact substrate.[35] Due to the guanidyl group of these pore-facing arginines, these residues have been proposed to form an 'arginine denaturation collar' that fosters an environment that helps to promote or maintain substrate unfolding within the central channel of p97/VCP and Vps4.[35] We hypothesized that R417 might also act as an arginine denaturation collar within the central pore of Skd3 and thus play an essential role in protein disaggregation. We measured the pore volume that contains all six arginine groups to be ~575 $Å^3$.[28] Assuming one arginine functional group is equivalent to two-thirds of a guanidine molecule, the six arginine residues facing into the central pore of Skd3 promote an effective guanidine concentration of 11.6 M.[28] A guanidine concentration of 11.6 M is more than sufficient to promote the unfolding of most proteins. This property would be beneficial to disaggregases as substrates are in an unfolded state in the central pore of AAA+ proteins and thus have highly hydrophobic sites exposed which could in turn clog the pore.[29] We propose that the Skd3 arginine denaturation collar limits hydrophobic sticking of residues within the central pore.

The nucleotide-binding pockets within the Skd3 hexamer are formed by the interface between two adjacent protomers.[28] ATP occupies the nucleotide-binding pocket of protomers P2–P5, which are also substrate-engaged protomers.[28] Protomer P6 is in an apo (nucleotide unbound) state, whereas P1 likely contains ADP.[28] These data indicate that post-ATP-hydrolysis subunits likely disengage from substrate.[28] Skd3 has a low Michaelis constant (~65 μM) for ATP hydrolysis, which is about two orders of magnitude lower than that of Hsp104.[9,28,36] Skd3 also can efficiently disaggregate at low concentrations of ATP and is not as potently inhibited by ADP as Hsp104.[28,36a,37] We hypothesize that Skd3 is biologically tuned to operate as a disaggregase under conditions of mitochondrial stress where ATP is limited and ADP is elevated. The structure of the NBD and the nucleotide-binding pocket is largely similar to other HCLR clade NBDs; however, there is a unique insertion in the NBD between helix D10 and D11 (residues L507–I534) that is not conserved in other NBDs (Figure 3).[28] Deletion of this insertion results in elevated ATPase and disaggregase activity, suggesting a

potential role as a molecular handbrake for the Skd3 NBD engine.[28]

The residues K387 (Walker A motif), E455 (Walker B motif), N496 (sensor I motif), and R620 (sensor II motif) from one protomer and R561 (arginine-finger motif) from the adjacent protomer form the canonical nucleotide-binding pocket.[28] The Walker A residue contacts the β- and γ-phosphate of ATP and Walker B primes the water molecule adjacent to the β- and γ-phosphate of ATP for nucleophilic attack on the γ-phosphate of ATP.[13,28,38] The R-finger from one protomer contacts the γ-phosphate of ATP in the nucleotide-binding pocket of the adjacent protomer and is key for ATP hydrolysis; whereas Sensor I coordinates the attacking water residue relative to the γ-phosphate of ATP and transmits a conformational change upon nucleotide engagement to displace the R-finger in the adjacent nucleotide-binding pocket.[13,28,38] The sensor II residue contacts both the β- and γ-phosphate of ATP to mediate a conformational change that sequesters the catalytic site from water.[13,28,38] Mutation of the Walker A, Walker B, R-finger, Sensor I, and Sensor II residues in Skd3 abolish both ATPase and disaggregase activity.[6b,22,28]

Skd3 is a highly, but subglobally cooperative enzyme in which the incorporation of just two mutant subunits into a hexamer can poison the disaggregase activity of the hexamer.[22,28] However, subunits with different mutations have different effects on the Skd3 hexamer.[28] Thus, by ranking how resilient the hexamer is against subunits with different AAA+ motif mutations we can draw conclusions about the relative importance of those motifs for (1) ATP hydrolysis and (2) coupling ATP hydrolysis to mechanical work. For ATPase activity in descending order, Skd3 is most affected by sensor I, R-finger, sensor II, Walker A, Walker B, and pore-loop mutations. For disaggregase activity in descending order, Skd3 is most affected by R-finger, sensor I, Walker A, sensor II, Walker B, and pore-loop mutations. We hypothesize that the Skd3 hexamer is most sensitive to R-finger and sensor I residues because they are involved in inter-subunit coordination of ATP hydrolysis.[28] We speculate that the Skd3 hexamer is next most sensitive to Walker A and sensor II mutations because they coordinate both the β- and γ-phosphates for ATP hydrolysis.[28] Finally, we hypothesize that the priming of the water molecule by the Walker B motif for nucleophilic attack is the least important conserved motif within the Skd3 nucleotide binding-pocket as Skd3 hexamers can tolerate incorporation of 2–4 Walker B mutant protomers for ATPase activity and 3–4 mutant protomers for disaggregase activity.[39] Ultimately, much is left to be understood about the cooperativity of Skd3 protomers within the hexamer, and Skd3 hexamers within the dodecamer.

4. Skd3 in Disease

Biallelic mutations in Skd3 (human *CLPB*) are associated with the severe mitochondrial metabolic disorder 3-methylglutaconic aciduria type VII (MGCA7) (Table 1).[10b,40] Patients often present with symptoms that include 3-methylglutaconic aciduria, congenital cataracts, severe encephalopathy, epilepsy, neutropenia with frequent infections that can develop into leukemia, and death in early childhood.[10b,40a–d] Other symptoms can include medullary cysts, microcephaly, small birth weight, severe psychomotor regression during febrile episodes, severe hypertonia, prolonged clonic movements, and nonprogressive intellectual disability.[10b,40a–d] A recent publication has better characterized the neurologic involvement of two MGCA7 patients and found reactive astrogliosis, subependymal white matter loss, and striatothalamic neurodegeneration.[41] Treatment of patients with granulocyte colony stimulating factor (G-CSF) for neutropenia is common, yet there are no cures.[10b,40d]

A Skd3 zebrafish model using a *CLPB* antisense morpholino oligonucleotide had an abnormal touch-evoked response with increased swim velocity and tail beat frequency, reminiscent of touch-evoked responses in patients.[40a] This response is potentially indicative of increased neuronal circuit excitability.[40a] A different *CLPB* zebrafish model had central nervous system defects ranging from depletion of cerebellar midline axonal connections to complete cerebellar atrophy, which recapitulates the most penetrant phenotype in MGCA7 patients.[10b] There are no published *CLPB* mouse models, but Skd3 is present in all mouse tissues and highly expressed in heart, skeletal muscle, kidney, and testis.[10a] A CLPB mouse will be valuable for determining the tissues and cell types important for MGCA7 pathology. The molecular etiology of MGCA7 is yet unclear; however, our data suggest that mutations in Skd3 impair disaggregase (but not ATPase) activity in a manner that correlates with the clinical severity of disease (Figure 5).[6b] Indeed, mutations with a more severe presentation in patients had a more severe defect in disaggregase activity and mutations with more moderate presentation in patients likewise had more moderate impairment of disaggregase activity.[6b] In support of this correlation, a MGCA7-associated mutation in the ANK of Skd3 impairs chaperone activity in cells.[16] Biochemical and cellular evidence is still needed for many of the reported mutations (Table 1). The putative substrates most relevant for MGCA7 pathology are not known; intriguingly, Skd3 interacts with the IMS protease, HTRA2, which is also mutated in 3-methylglutaconic aciduria type VIII (MGCA8).[6b,42] Whether Skd3 and HTRA2 impinge upon the same biochemical pathway in 3-methylglutaconic aciduria is unclear. We find it intriguing that both PARL and HAX1 mutually interact with Skd3/HTRA2 and are likewise implicated in the etiology of mitochondrial disorders.[18a,20,42–43]

Monoallelic mutations in *CLPB* are associated with severe congenital neutropenia (SCN) and cyclic neutropenia.[18,22] SCN is characterized by arrest of granulocyte differentiation, a propensity to develop myeloid malignancies, and frequent infections.[22] As with MGCA7, treatment of SCN patients with G-CSF appears to be beneficial.[18,22] Human hematopoietic progenitor cells with a Skd3 knockout or cells expressing mutant Skd3 had impaired granulocyte differ-

Table 1. Current disease-associated mutations in Skd3/*CLPB*. Table lists the amino-acid identity of all disease-associated mutations in Skd3 using single letter amino-acid abbreviations and referencing Skd3 isoform 1. The subsequent columns list the disease association, the genetic variant of the mutation, how many patients have been described in the literature to bear the mutation, and the clinical references for each patient. The last set of columns list the domain in Skd3 that contains the mutation, whether biochemical and cellular evidence exists for the mutation (and their subsequent references), and finally the proposed mechanism by which each mutation inhibits Skd3. **Partial* indicates either incomplete evidence or a similar but not exact mutation was tested.

Mutation	Clinical Presentation	Variant type	Number of Patients	References	Domain	Biochemical Evidence	References	Cellular Evidence	References	Proposed Mechanism
H164P	MGCA7	Biallelic	1	[40e]	ANK	N/A		N/A		Disrupts helix α3
R250*	MGCA7	Biallelic	2	[10b]	ANK	N/A		N/A		Loss of NBD + partial loss of ANK
T268M	MGCA7	Biallelic	4	[40c]	ANK	Yes	[6b,16]	N/A		Substrate binding
A269T	MGCA7	Biallelic	1	[40d]	ANK	Yes	[16]	N/A		Substrate binding
Y272C	MGCA7	Biallelic	3	[10b,40d]	ANK	Yes	[16]	Yes	[16,18a]	Substrate/HAX1 binding
K321*	MGCA7	Biallelic	1	[40c]	ANK	Partial	[6b]	N/A		Loss of NBD
L339Rfs*5	MGCA7	Biallelic	1	[40j]	NBD	N/A		N/A		Loss of NBD
A359_A360in-sDLVCLAV	MGCA7	Biallelic	1	[40e]	NBD	N/A		N/A		Large insertion likely disrupts helix D3
R362Q	MGCA7	Biallelic	1	[40g,40h]	NBD	N/A		N/A		Replaces highly conserved basic residue with amidic residue in helix D3
R363G	MGCA7	Biallelic	1	[40j]	NBD	N/A		N/A		Replaces highly conserved basic residue with glycine residue in helix D3
R387*	MGCA7	Biallelic	2	[41]	NBD	N/A		N/A		Partial loss of NBD
T388K	SCN and POI	Monoallelic	1	[22,44]	NBD	N/A		N/A		Walker A motif
K404T	SCN	Monoallelic	1	[18b]	NBD	Yes	[18b]	N/A		Replaces basic residue with uncharged polar residue in helix D5
R408G	MGCA7	Biallelic	5	[10b,40i]	NBD	Yes	[10b,22,28]	Yes	[10b]	Disrupts β-strand d2
M411I	MGCA7	Biallelic	2	[10b]	NBD	N/A		Yes	[10b]	Unknown – in helix D5
R417*	MGCA7 and POI	Biallelic	10	[10b,40c,40i,44]	NBD	N/A		N/A		Partial loss of NBD
P427L	SCN	Monoallelic	1	[18b]	NBD	Yes	[18b]	N/A		Pore loop motif adjacent
E435_G436de-linsDP	MGCA7 and POI	Biallelic	1	[10b,44]	NBD	N/A		N/A		Pore loop motif adjacent
D462R*11	MGCA7	Biallelic	1	[40i]	NBD	N/A		N/A		Partial loss of NBD
R475Q	MGCA7	Biallelic	1	[40i]	NBD	Yes	[6b,28]	N/A		Disrupts β-strand d4
C486R	MGCA7	Biallelic	2	[10b]	NBD	N/A		N/A		Adds charged residue between β-strand b5 and b6
N496K	SCN	Monoallelic	1	[22]	NBD	Yes	[22,28]	Yes	[22]	Sensor I motif
E501K	MGCA7	Biallelic	2	[10b]	NBD	N/A		N/A		Reverses residue charge in helix D10
I562Tfs*23	MGCA7	Biallelic	4	[40a]	NBD	N/A		N/A		Partial loss of NBD
Y567C	MGCA7	Biallelic	2	[10b]	NBD	N/A		N/A		Disrupts β-strand d7
Y567S	MGCA7	Biallelic	1	[40g,40h]	NBD	N/A		N/A		Disrupts β-strand d7
E557K	SCN	Monoallelic	1	[22]	NBD	Yes	[22]	Yes	[22]	R-finger motif adjacent
G560R	SCN	Monoallelic	3	[18]	NBD	Yes	[18b]	N/A		R-finger motif adjacent
R561G	SCN	Monoallelic	1	[22]	NBD	Yes	[22,28]	Yes	[22]	R-finger motif
R561Q	SCN and POI	Monoallelic	6	[18a,22,44]	NBD	Partial	[22,28]	Partial	[22]	R-finger motif
R561W	SCN	Monoallelic	2	[18b]	NBD	Partial	[22,28]	Partial	[22]	R-finger motif
A591V	MGCA7	Biallelic	1	[10b]	NBD	Yes	[6b,28]	N/A		Highly conserved residue in helix E1
Y617C	MGCA7	Biallelic	2	[10b]	NBD	N/A		Yes	[10b]	Sensor II motif adjacent
R620C	SCN	Monoallelic	2	[22]	NBD	Yes	[22,28]	Yes	[22]	Sensor II motif
R620H	SCN	Monoallelic	1	[18a]	NBD	Partial	[22,28]	Partial	[22]	Sensor II motif

Table 1. continued

Mutation	Clinical Presentation	Variant type	Number of Patients	References	Domain	Biochemical Evidence	References	Cellular Evidence	References	Proposed Mechanism
R628C	MGCA7, POI, and cyclic neutropenia	Biallelic	5	[22,40b,40f,44]	NBD	N/A		Partial	[40f]	Removes charged residue in helix E3
A635K	MGCA7	Biallelic	1	[40f]	NBD	N/A		Partial	[40f]	Adds charged residue in helix E3
E639K	MGCA7 and POI	Biallelic	2	[40b,44]	NBD	N/A		N/A		Reverses residue charge at end of helix E3
G646V	MGCA7 and POI	Biallelic	1	[10b,44]	NBD	N/A		Yes	[10b]	Highly conserved residue between helix E3 and β-strand e2
C647Lfs 26	MGCA7	Biallelic	1	[10b]	NBD	N/A		N/A		Partial loss of NBD
R650P	MGCA7	Biallelic	1	[40i]	NBD	Yes	[6b]	N/A		Disrupts β-strand e2
I682N	MGCA7	Biallelic	1	[10b]	CTD	N/A		N/A		Disrupts charge patterning in CTD

entiation and increased apoptosis.[22] We found that SCN-associated *de novo* mutations inhibit Skd3 disaggregase activity via a dominant-negative mechanism whereas MGCA7 mutations do not (Figure 5).[22,28] These SCN-linked mutant subunits can oligomerize with wild-type Skd3 subunits into hexamers and for some SCN-linked mutants, the incorporation of only 1–2 mutant subunits is sufficient to inactivate the hexamer (Figure 5).[28] SCN-linked mutations appear to be distinct from MGCA7-linked mutations in that SCN-associated mutations cluster within the nucleotide-binding pocket and affect conserved AAA+ motifs such as Sensor-I, Sensor-II, Walker A, and R-finger (Table 1).[28]

Importantly, MGCA7-linked Skd3 mutant subunits have only minor effects on the disaggregase activity of WT Skd3 subunits within the hexamer.[28] Hence, gene therapy approaches to express WT Skd3 could be beneficial for MGCA7. By contrast, SCN-linked Skd3 mutant subunits can potently poison WT Skd3 subunits within the hexamer.[28] Thus, increasing the expression of WT Skd3 may be less impactful for SCN than MGCA7.[28] For SCN, an allele-specific antisense oligonucleotide or gene-editing based therapy to eliminate expression of the SCN-linked Skd3 variant might be more beneficial.[28]

Interestingly, HAX1 mutations are also associated with SCN.[18a,20b] HAX1 is a substrate of Skd3 and MGCA7-linked mutations inhibit Skd3–HAX1 binding and subsequent chaperone activity.[6b,16,18a] How interplay between HAX1 and Skd3 might contribute to disease is an important area of investigation.

Recently, Skd3 mutations were found to be associated with premature/primary ovarian insufficiency (POI), a disorder of female fertility which is characterized by a loss of ovarian function and elevated levels follicle stimulating hormone.[44] Patients with POI also have an increased risk of diabetes, cardiovascular disease, osteoporosis, and early mortality.[44] Treatment of POI patients using hormone replacement therapy can minimize the risk of comorbidities.[44] Skd3 mutations associated with POI were also associated with SCN, cyclic neutropenia, and MGCA7 (Table 1).[44] Rare coding variants in Skd3 have also been associated with earlier onset menopause, suggesting an important role for Skd3 in female fertility.[45] The variant with the most significant association with earlier onset menopause was R598C; however, the functional importance of this mutation has not been tested and no causative link has yet been established.[45] Given the clinical overlap between MGCA7, SCN, and POI *CLPB* mutations, it is likely that at least some molecular etiology is shared.

A genome-wide screen identified Skd3 as important for Venetoclax (an FDA-approved BCL-2 inhibitor) resistance.[21] Skd3 is upregulated in Venetoclax-resistant cancer cells and in acute myeloid leukemia (AML) patient samples.[21] Skd3 knockout sensitizes cells to apoptotic signaling and consequently Venetoclax treatment.[21–22] Knockdown of Skd3 synergized with Venetoclax treatment to reduce cancer burden in a murine xenograft AML model.[21] The mechanism by which Skd3 reduction enables Venetoclax sensitivity is not

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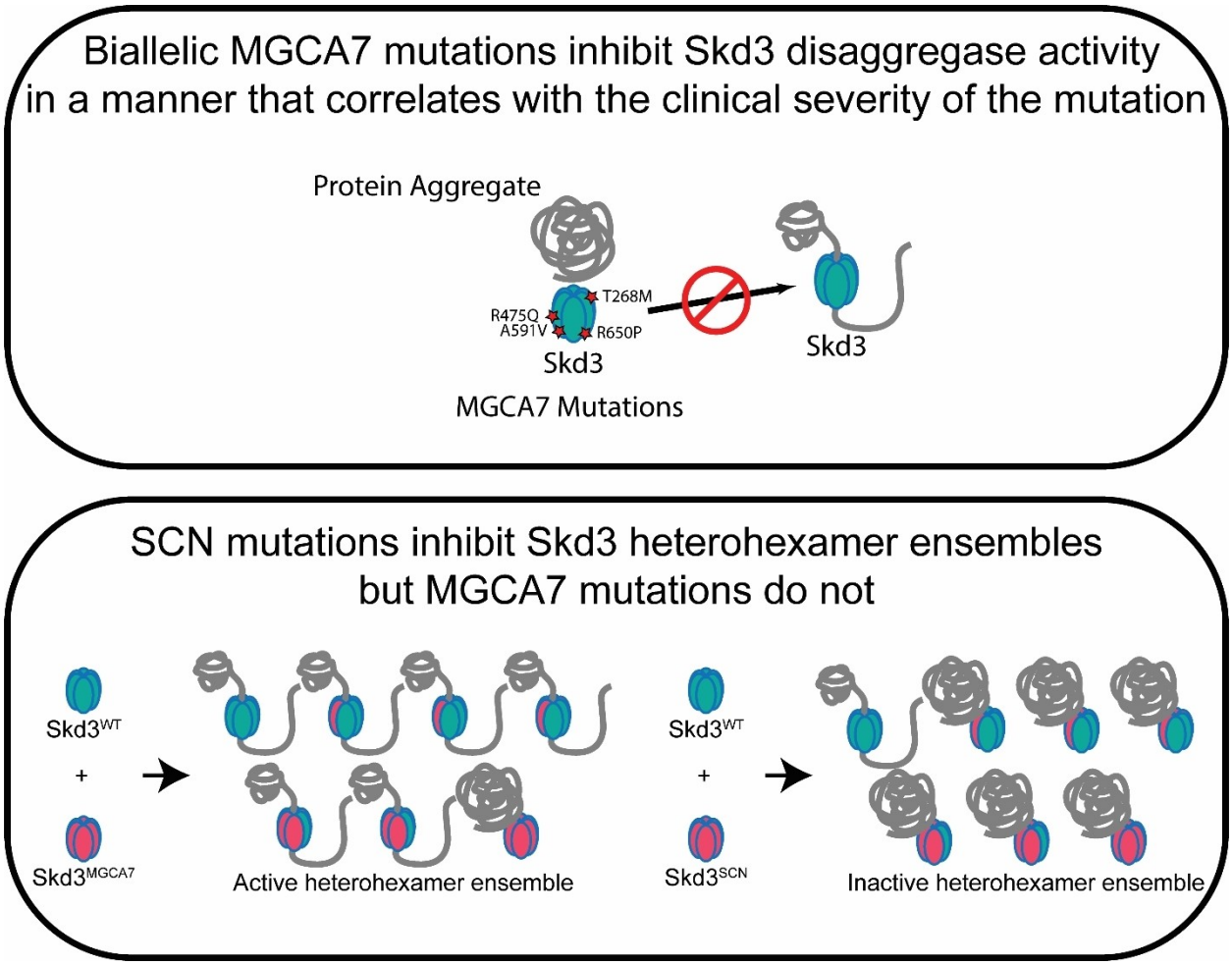

Figure 5. Disease-associated mutations in Skd3 inhibit disaggregase activity. Biallelic MGCA7-linked mutations in Skd3 inhibit Skd3 disaggregase but not ATPase activity in a manner that correlates with the clinical severity of the mutation (top). *De novo*, SCN-associated mutations inhibit Skd3 disaggregase activity in a dominant-negative fashion by potently poisoning the heterohexameric ensemble. MGCA7-linked mutations do not poison the Skd3 hexamer (bottom).

understood, but it is proposed to act via increasing the sensitivity to apoptosis by altering mitochondrial morphology.[21] However, Skd3 could also sensitize cells to apoptotic signaling via (1) altering mitochondrial calcium handling, (2) inhibiting mitochondrial respiration, or (3) its interaction with mitochondrial apoptotic/anti-apoptotic proteins. We propose that Skd3 may be a strong pro-survival signal, which may have relevance for many other cancers. In support of the role of Skd3 as a pro-survival signal for cells, high Skd3 expression is associated with poor prognosis for castration-resistant prostate cancer.[46]

Disease genes are often at the center of key protein–protein interaction hubs, which connect otherwise disparate pathways.[47] Proteomics of putative Skd3 substrates reveal its role at the nexus of mitochondrial biology in the intermembrane space.[6b] Although Skd3 has been implicated in other human health conditions, more research into the associations of *CLPB* with disease is certainly required.[48]

5. Summary and Outlook

Nine years ago, Skd3 was a protein that was often speculated about,[49] but had no ascribed function or role in human disease. In the convening years, mutations in Skd3 associated with MGCA7, SCN, and POI have been discovered.[10b,22,44] Functionally, Skd3 has been discovered to be a human mitochondrial protein disaggregase that chaperones the intermembrane space.[6b] Skd3 has been found to play a role in apoptosis and subsequently cancer resistance to apoptosis-inducing drugs.[21–22] The structure of Skd3 has been revealed and some of the mechanisms that underlie Skd3 function have been elucidated.[28,30a] An important area of research moving forward will be dissecting more of the mechanisms of Skd3 function, especially the role of the intriguing ANK. The discovery of Skd3 as a human mitochondrial protein disaggregase has opened a new field of research. Understanding the molecular and cellular pathways that distinguish MGCA7,

SCN, and POI etiologies is a key area of ongoing interest. Additionally, finding which mitochondrial processes are functionally altered by Skd3 deficiency will be important. Undoubtedly, we still have much to learn about Skd3.

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Data Availability Statement

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

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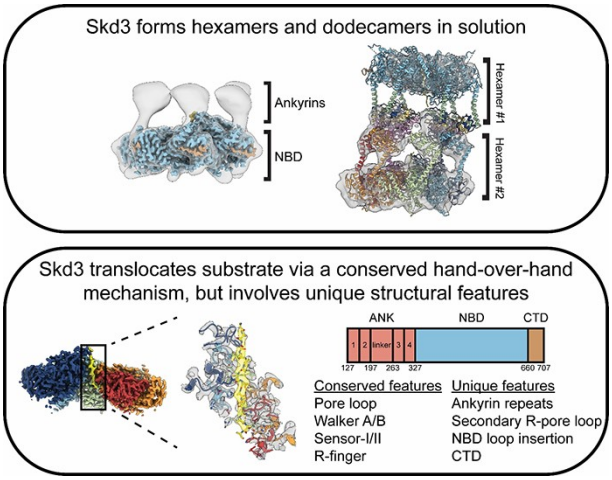

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Decoding Skd3 (Human *CLPB*): a Mitochondrial Protein Disaggregase Critical for Human Health