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Biophysical Consequences of EVEN-PLUS Syndrome Mutations for the Function of Mortalin

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Abstract

HSPA9, the gene coding for the mitochondrial chaperone mortalin, is involved in various cellular roles such as mitochondrial protein import, folding, degradation, Fe-S cluster biogenesis, mitochondrial homeostasis, and regulation of the anti-apoptotic protein p53. Mutations in the HSPA9 gene, particularly within the region coding for the nucleotide-binding domain (NBD), cause the autosomal disorder known as EVEN-PLUS syndrome. The resulting mutants R126W and Y128C are located on the surface of the mortalin-NBD near the binding interface with the interdomain linker (IDL). We used differential scanning fluorimetry (DSF), biolayer interferometry, X-ray crystallography, ATP hydrolysis assays, and Rosetta docking simulations to study the structural and functional consequences of the EVEN-PLUS syndrome-associated R126W and Y128C mutations within the mortalin-NBD. These results indicate that the surface mutations R126W and Y128C have far-reaching effects that disrupt ATP hydrolysis, interdomain linker binding, and thermostability, and increase propensity for aggregation. The structural differences observed provide insight into how the conformations of mortalin differ from other heat shock protein 70 (Hsp70) homologs. Combined, our biophysical and structural studies contribute to the understanding of the molecular basis for how disease-associated mortalin mutations affect mortalin functionality and the pathogenesis of EVEN-PLUS syndrome.

Abstract Graphic

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Supporting information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

CD spectra, DSF-derived Gibbs free energy of unfolding for wild type and mutant mortalin-NBD, Hsc70, and Hsp70, data fitting to determine ΔG° values for mortalin, thermal reversibility by CD, and enzyme activity assays.

The authors declare no competing financial interest.

Introduction

Hsp70 protein family members are ubiquitous and found throughout all cellular compartments. Hsp70 and its homologs regulate homeostasis through a wide range of functions, such as vesicular trafficking, iron-sulfur cluster biogenesis, stress responses, protein folding, and protein translocation.^{1–4} All Hsp70s undergo a cycle of conformational changes driven by the reversible binding of ATP and subsequent hydrolysis to modulate substrate affinity.⁵ ATP binding in the nucleotide-binding pocket (NBP) leads to a conformational change in the NBD that is transferred through the interdomain linker (IDL) to the substrate-binding domain (SBD) eliciting reduced substrate affinity and a faster substrate exchange rate.⁶ In this state the NBD and SBD, tethered by the short IDL, act as independent beads on a string.⁷ The hydrolysis of ATP to produce ADP and inorganic phosphate promotes the conformational change to a state with stronger substrate affinity and slower substrate exchange.^{8–11} Concomitant conformational changes in the NBD are accompanied by the docking of the interdomain linker and SBD to the NBD.¹² Thus, for the Hsp70 family of proteins, nucleotide driven conformational changes allosterically regulate the affinity of the misfolded protein client for the C-terminal substrate-binding domain contingent on the state of the nucleotide bound to the NBD.¹³

The nuclear gene HSPA9 encodes for the human mitochondrial Hsp70, mortalin.¹⁴ Mortalin is a 679 amino acid protein and has a molecular weight of 73.913 kDa after the 46-residue long signal peptide that targets mortalin to mitochondria is cleaved upon import into the mitochondria.^{15,16} Mortalin is similar to other homologs of Hsp70 in overall structure in that it consists of a ~42 kDa NBD and a ~25 kDa SBD. The SBD is further split into a twisted β -sandwich domain (SBD β) and a ~12 kDa α -helical lid domain (SBD α). The NBD consists of four subdomains IA, IB, IIA, and IIB that fold into a pair of lobes forming a nucleotide-binding pocket located at the central cleft of the domain.¹⁷ The NBD and SBD are connected by a short flexible sequence, the IDL, which is necessary to communicate conformational changes between the two domains upon hydrolysis of ATP by the NBD.⁶ Both the mortalin SBD and the apo state of the NBD have been separately crystallized (PDB ID 3N8E) and (PDB ID 4KBO).¹⁸ Unfortunately, the expression of human full-length mortalin has proven to be difficult without co-expression with the co-chaperone HEP,¹⁹ and due to this limitation most structural studies are still performed on isolated domains of mortalin.

Mutations leading to malfunction of the mortalin-NBD are implicated in cancers, neurological, and many other diseases making the NBD a potential target for therapeutic compounds.^{20,21} Chaperones function in cells in collaboration with co-chaperones, for example, mortalin functions with a specialized Hsp70 escort protein, HEP, to maintain solubility and regulate ATP hydrolysis.²² The mortalin-NBD is also a prospective target for modulation by nucleotide competitive or allosteric compounds such as MKT-077.²³ The key role of the NBD in the conformational changes within the chaperone cycle of mortalin set the stage for further structural and biophysical studies which may be useful in modulating the functions of mortalin.²⁴

Mutations in mortalin are also implicated in the autosomal disease EVEN-PLUS (epiphyseal, vertebral, ear, nose, plus associated findings) syndrome.²⁵ Initially EVEN-PLUS syndrome was misdiagnosed as CODAS (cerebral, ocular, dental, auricular and skeletal) syndrome that is associated with mutations in the LONP1 gene coding mitochondrial AAA+ lon protease.²⁶ Mortalin functions in conjunction with many proteins to maintain mitochondrial homeostasis, one of which is the proteolytic function of lon protease, an enzyme which has been shown to require interaction with mortalin to perform protease functions.^{27,28} Exome sequencing of three patients with similar symptoms to CODAS revealed multiple mutations in the HSPA9 gene.²⁵ One patient was heterozygous for c.383A>G and c.882_883delAG coding for the Y128C mutation in the mortalin-NBD and a deletion of the protein at residue 296 truncating a portion of the NBD and entire SBD of mortalin.²⁵ Two patients were found to be homozygous for variant c.376C>T coding for the R126W mutation in the mortalin-NBD.²⁵ These findings suggest that mutations in the HSPA9 gene coding for mortalin alter the function of mortalin with lon and produces similar symptoms of CODAS, thereby masking the symptoms of EVEN-PLUS syndrome.²⁹ Both variants are colocalized on the NBD near to the IDL interaction site NBD suggesting a potential negative effect on inter-domain communication with the SBD.^{25,30} The R126W variant was also found in Parkinson's disease patients and determined to have a detrimental effect on mitochondrial homeostasis.^{31,32} The presence of the R126W mutation in both Parkinson's disease and EVEN-PLUS syndrome suggests that mortalin plays important roles in both early and late stages of life. Prior to the current study, the biochemical and biophysical mechanisms driving the action of R126W and Y128C mutants were not known. To develop an understanding of these mechanisms we examined the thermostability of mortalin wild type and mutant constructs and compared findings with mortalin to the corresponding mutations in the cytosolic homologs Hsc70 and Hsp70. Thermostability studies included determination of melting temperatures and calculation of the Gibbs free energy of unfolding. Additional experiments to further identify the effects of the R126W and Y128C mutants included binding studies to determine the affinity for the interdomain linker, docking simulations to assess structural perturbations, and ATP hydrolysis assays to measure the effect of R126W and Y128C mutations on enzymatic activity of the NBD, and dynamic light scattering experiments to probe propensity for aggregation. Our results suggest that the R126W and Y128C mutants exert a combinatorial effect through destabilization and aggregation, impairment of interdomain linker binding, and a reduction in NBD-catalyzed ATP hydrolysis.

Methods

Cloning and mutagenesis

A pHis//2-Mortalin-NBD (52–431) plasmid containing the coding sequence for a N-terminal His₆-tag, with a tobacco etch virus (TEV) protease cleavage site before human mortalin residues 52–431 was used as the expression vector for wild type mortalin-NBD. The pET15b-Hsp70-NBD (1–385) and pHis//2-Hsc70-NBD (1–381) plasmids contained the coding sequence for the NBD of cytosolic heat shock protein 70 (Hsp70) and heat shock cognate 70 (Hsc70), respectively. The R126W and Y128C mutations were generated in the pHis//2-Mortalin-NBD (52–431) plasmid using a QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) and sequencing was used to confirm the mutations (Eurofins Scientific). The corresponding mutations R76W and F78C were generated in both the pHis//2-Hsc70-NBD (1–381) and pET15b-Hsp70-NBD (1–385) plasmids by site-directed mutagenesis using the QuikChange kit and was confirmed by sequencing. All plasmids contained an N-terminal His₆-tag, with a TEV protease cleavage site prior to the corresponding coding sequence.

Protein expression

All constructs were sequence-verified before being transformed into *Escherichia coli* Rosetta2 (DE3) cells (EMD Chemicals) for expression. Lysogenic broth (LB) media starter cultures were inoculated with multiple colonies of transformed Rosetta2 (DE3) cells and grown at 37 °C overnight with the addition of 100 µg/ml carbenicillin and 34 µg/ml chloramphenicol. The subsequent morning, LB starter cultures were transferred to Terrific Broth (Research Products International) expression culture (80 mL per one liter), which grew at 37 °C until OD₆₀₀ reached 1.0. Protein expression was then induced by incorporating isopropyl β-D-1-thiogalactopyranoside (IPTG) to a concentration of 400 µM into the culture flask. Harvesting of the induced cell cultures was performed by centrifugation at 4,000 rpm at 4 °C for 20 min and resuspended in a buffer containing 180 mM Tris (pH 8.0), 450 mM NaCl, 10% (v/v) Glycerol, 20 µg/ml DNaseI (MP Biomedicals), 1 mg/ml lysozyme (Sigma), and 100 µg/ml 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF; Gold Biotechnology) were added prior to freezing. The resuspended cells were then flash-frozen in liquid nitrogen and stored at –80 °C until purification.

Protein purification

Frozen cells stocks were thawed overnight at 4 °C on a slow rotator. The cell lysate was then pelleted by centrifugation at 12,500 rpm for 45 min at 4 °C, subsequently filtered through a 0.45 µm (VWR) syringe filter and transferred to a new 50 mL centrifuge tube. The supernatant containing the His₆-tagged mortalin-NBD was loaded onto a 5 mL HisTrap HP column (GE Healthcare) on a fast protein liquid chromatography (FPLC) system (GE Healthcare) equilibrated with buffer A (25 mM HEPES pH 7.8, 150 mM NaCl). The column was subsequently washed with 15 column volumes (CV) of buffer A to remove non-specifically bound proteins and elution of the protein of interest was executed with 5 CVs of 100% buffer B (25 mM HEPES, pH 7.8, 150 mM NaCl, 500 mM imidazole). The fractions containing the His₆-tagged mortalin-NBD were pooled and cleavage of the His₆-tag was then carried out by adding 1 mg of His₆-tagged tobacco etch virus (TEV) protease. To

remove excess imidazole, the pooled samples were then dialyzed into buffer A with 5 mM β -mercaptoethanol and stirred overnight at 4 °C. The cleaved samples were then loaded onto a 5 mL HisTrap HP column and flow through containing the cleaved mortalin-NBD was collected and the column was washed with 5 CV of buffer A. The HisTrap column was then washed with 5 CV of buffer B to elute TEV and any uncleaved His₆-tagged mortalin-NBD. The mortalin-NBD samples were concentrated by centrifugation and applied to a HiLoad 16/60 Superdex 75 (S75) size exclusion column (GE Healthcare) equilibrated with buffer A. Elution fractions from the size exclusion column were then loaded onto an SDS-PAGE gel and stained with coomassie blue reagent. The fractions containing mortalin-NBD were concentrated and applied to 5 mL HiTrap Desalting columns (GE Healthcare) and washed with 5 CV of 25 mM HEPES pH 7.8. The mortalin-NBD sample was then applied to HiTrap Blue HP column (GE Healthcare) and washed with 5 CV of 25 mM HEPES pH 7.8. The protein was eluted with 10 CV gradient of 0.2 M NaCl, 25 mM HEPES pH 7.8. The peak fractions containing the desired eluate were pooled and dialyzed into 25 mM HEPES, pH 7.8, 50 mM NaCl. Identical purification schemes were carried out for Hsc70 and Hsp70 samples.

Crystallization, structure determination and refinement

Crystallization was performed using the sitting drop vapor diffusion method with freshly purified mortalin-NBD concentrated to 20 mg/mL. Crystallization of the ADP bound state of the mortalin-NBD was carried out with the use of sparse matrix crystallization screens JCSG Core I-IV (Qiagen) and MCSG 1–4 (Microlytic). Optimizations of initial hits from the sparse matrix screens identified an optimal reservoir solution of 0.17 M ammonium acetate, 0.085 M sodium citrate: HCl, pH 5.6, 25.5 % (w/v) PEG 4000, and 15 % (v/v) glycerol. In order to determine the post-hydrolysis state ADP, Na₂HPO₄, and MgCl₂ were added the mortalin-NBD sample to a final concentration of 20 mM prior to crystallization. The ADP-bound mortalin-NBD sample was then hand pipetted into matrix crystallization screens MCSG 1–4. During harvesting the selected crystals were transferred through LV CryoOil (MiTeGen) as a cryoprotectant and immediately frozen in liquid nitrogen prior to transport to the synchrotron and subsequent X-ray diffraction. Collection of X-ray diffraction data was performed at 1.00 Å on beamline 4.2.2 at the Advanced Light Source in Lawrence Berkeley National Laboratory followed by processing using XDS.³³ PHASER was used to perform molecular replacement and calculate the phases.³⁴ Molecular replacement was done using structure human Hsp70-NBD in the ADP- and Mg-bound state (PDB ID 3ATU) as the search model in PHASER.³⁵ Structure refinement and model building were carried out using PHENIX and Coot.^{36,37} The structure of the ADP-bound mortalin-NBD was deposited in the PDB with accession code 6NHK. MolProbity was used to analyze the stereochemical and geometric properties of the ADP-bound mortalin-NBD structure.³⁸ PyMOL was utilized to create all molecular structure figures.³⁹

Differential Scanning Fluorimetry

In order to determine the melting temperature (T_m) of the mortalin-NBD, Hsc70-NBD, and Hsp70-NBD variants the fluorescence of SYPRO Orange dye (Thermo Fisher Scientific) was monitored as a function of temperature. The 5,000x SYPRO Orange stock was diluted to a 10x stock in 5 mL of buffer A (20 mM HEPES, 150 mM NaCl, pH 7.8). All NBD

protein samples were dialyzed into buffer A and diluted to 5 μM final concentrations in 1.5ml Eppendorf tubes. NBD proteins were combined with and/or without a combination of magnesium, ADP, ATP, and/or IDL peptides to a final concentration of 1 mM. IDL peptides were purchased as custom synthetic peptides (Lifetein) and concentrations of IDL peptide stocks were determined by UV absorbance. The NBD protein samples were distributed into 96 well Frame Star PCR plates in 50 μL aliquots and covered with clear thermal seal film to prevent evaporation during heating in the instrument. Differential scanning fluorimetry (DSF) data was collected using a BioRad CFX96 RT-PCR. Fluorescence intensity data was collected using the preset “HEX” channel for fluorescence excitation and emission. Generation of the DSF curves was performed by measuring the fluorescence of each sample at 570 nm with temperature increasing in 0.5 $^{\circ}\text{C}$ increments from 25 to 95 $^{\circ}\text{C}$ with 5 second equilibrium at each temperature. T_m values were defined by calculating by fitting the fluorescence intensity data to Boltzmann Sigmoidal curves using GraphPad Prism (GraphPad, La Jolla, CA).

Extraction of the thermodynamic parameters of protein unfolding

The thermodynamic parameters of protein unfolding were extracted with the method for parallelized differential scanning fluorimetry we previously outlined in the literature.⁴⁰ The thermodynamic parameters of protein stability calculated in this method are the standard Gibbs free energy of unfolding, ΔG° , enthalpy of unfolding, ΔH° , and entropy of unfolding, ΔS° . The raw DSF signal is fit to a Boltzmann sigmoid in Prism (GraphPad, La Jolla, CA) to extract the minimal fluorescence, $F_{\min}$, and maximal fluorescence, $F_{\max}$, values. The Boltzmann sigmoid is then normalized so that the minimal fluorescence corresponded to a value of 0 and the maximal signal corresponded to a value of 1. The normalized fluorescence, F , is then assumed to correspond to the proportion of unfolded NBD protein (P_u) and was determined from equation 1.

$$P_u = \frac{F - F_{\min}}{F_{\max} - F_{\min}} \quad (1)$$

In order to determine the proportion of unfolded NBD protein (P_u) as a function of temperature, the T_m was defined as the temperature at which 50% of the protein was denatured, or $P_u = 0.5$. This assumes a two-state model of reversible unfolding for the NBD proteins; the fraction of folded protein is given by equation 2.

$$P_f = 1 - P_u \quad (2)$$

The equilibrium constant for unfolding (K_u) was determined using equation 3.

$$K_u = \frac{P_u}{P_f} \quad (3)$$

The Gibbs free energy of unfolding ($\Delta_u G$) is then determined via equation 4 in which R is defined as the universal gas constant and T represents the temperature.

$$\Delta_u G^\circ = -RT \ln K_u \quad (4)$$

$\Delta_u G^\circ$ is related to the standard enthalpy ($\Delta_u H^\circ$) and entropy ($\Delta_u S^\circ$) of unfolding through the following relationship:

$$\Delta_u G^\circ = \Delta_u H^\circ - T \Delta_u S^\circ \quad (5)$$

This implies that the plot of $\Delta_u G^\circ$ versus temperature should be linear with a slope of $\Delta_u S^\circ$ and an intercept at 0 K of $\Delta_u H^\circ$. Values for $\Delta_u G^\circ$ and temperature from equation 4, corresponding to a range of P_u values between 0.1 and 0.5, were plotted according to equation 5 and fit with a linear regression. The standard Gibbs free-energy of unfolding ($\Delta_u G^\circ$) at 25 °C was determined by extrapolating the linear regression back to a temperature of 298.15 K.

Circular Dichroism Spectroscopy

An AVIV 435 spectropolarimeter was used to obtain circular dichroism (CD) data for the mortalin-NBD, Hsc70-NBD, and Hsp70-NBD wild type and mutant proteins. All NBD proteins were analyzed at 10 μ M protein concentration in 10 mM Na₂HPO₄ pH 7.8 in a 0.1 cm quartz cuvette. CD spectra of the NBD proteins were collected from 190–260 nm at 25 °C for 3 scans with a scan rate of 10 nm/min and a 1 nm bandwidth. The resulting spectra were converted to mean residue ellipticity and the secondary structure content was estimated using the BeStSel analysis program.^{41,42}

Thermal reversibility of the NBD proteins folding transitions were assessed by monitoring the CD signal at 222 nm with the temperature increasing in 2.5 °C increments from 25 °C to the corresponding T_m of each NBD protein, as determined by DSF, and returning to 25 °C in 2.5 °C increments. A comparison spectrum to determine CD signal for folded and unfolded NBD protein was obtained by thermal-induced unfolding by ramping the temperature from 25 °C to 100 °C in 2.5 °C increments with a 30 second hold for equilibration at each temperature increment.

Dynamic Light Scattering

Dynamic light scattering (DLS) signal intensities were measured for all NBD proteins at 10 μ M protein concentration in 10 mM HEPES pH 7.8 using a Zetasizer Nano S instrument (Malvern Instruments). Prior to analysis, the NBD proteins were incubated for 20 min at 25,

31, or 37 °C and subsequently centrifuged at 12,000 rpm for 5 min to remove precipitate. The samples were then transferred to ZEN plastic micro cuvettes and analyzed in triplicate for size measurements with a 173° scattering angle.

Biolayer Interferometry

A BLItz system (ForteBio) at 25 °C was utilized to measure the dissociation constant, K_d , of the NBD proteins for the corresponding N-terminal biotinylated interdomain linker peptides (LifeTein) reconstituted with binding buffer (20 mM HEPES, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM KCl, 5 mM Na_2HPO_4 pH 7.8) to a final concentration of 10 mM IDL peptides. N-terminal biotinylated interdomain linker peptides were immobilized onto streptavidin (SA) biosensors by inserting binding buffer equilibrated biosensors into 5 μ l droplets containing 1 mM IDL peptide in binding buffer. For all binding experiments mortalin-NBD, Hsc70-NBD, and Hsp70-NBD variant proteins were tested for binding affinity to the corresponding biotinDVLLLDVTPLS_{C-term} mortalin, biotinQDLLLDVTPLS_{C-term} Hsc70, and biotinQDLLLDVAPLS_{C-term} Hsp70 IDL peptides, respectively. SA biosensors were soaked for 10 minutes in binding buffer in preparation for IDL peptide binding. All Mortalin-NBD, Hsc70-NBD, and Hsp70-NBD variant protein binding measurements were executed at the specified concentrations over the designated time period. The control for each binding experiment was the response of the biosensor in only binding buffer and a single biosensor was used per binding measurement at each protein concentration. In additional experiments, ADP was added the IDL peptide and NBD samples to a final concentration of 1mM to test for the effects of ADP on binding affinity. The data analysis software provided by ForteBio was used to determine the dissociation constant for each protein at the indicated concentrations. Binding affinity values were then imported into GraphPad Prism to generate a comparison to each variant and to assess statistical significance of differences in binding affinity.

Docking of the Interdomain Linker

Modeling of the IDL peptides onto the ADP bound mortalin-NBD crystal structure was performed using Rosetta FlexPepDock.⁴³ The previously obtained ADP bound mortalin-NBD crystal structure and IDL peptide (₄₄₄SLPTVDLLLV₄₃₄) served as input models for the docking of IDL to the wild type mortalin-NBD. Residues were mutated *in silico* to either R126W or Y128C in the ADP bound mortalin-NBD structure to generate IDL docking models for each of the disease-associated mutations. The resulting docked family of structures are the 5 lowest Rosetta energy score output models from a family of 5000 models (decoys). The decoys with the largest deviation from the wild type mortalin-NBD/IDL model were used for comparing the docked structures of IDL onto the mutant R126W and Y128C mortalin-NBD models.

ATPase Kinetics

ATPase activity for all mortalin-NBD, Hsc70-NBD, and Hsp70-NBD variants were measured spectrophotometrically using the EnzChek phosphate assay kit (Invitrogen). This method allows for quantification over time of inorganic phosphate (Pi) release due to ATP hydrolysis by the enzyme. ATP hydrolysis activity was measured in triplicate using 50 μ l reaction mixtures in 384 well optical bottom plates (Thermo Scientific Nunc™) prepared to

read absorbance at 360 nm with a BioTek Synergy II plate reader. A standard curve using inorganic phosphate was utilized to phosphate concentrations and reaction buffer without enzyme served as the negative control. Mortalin-NBD (2.00 μ M), Hsc70-NBD (2.50 μ M), and Hsp70-NBD variants (2.00 μ M) were all prepared in binding buffer and mixed with ATP (Sigma) with concentrations ranging from 0 to 2.4 mM. The chromogenic substrate, MESG, was added to the samples to a final concentration of 0.2 μ M along with 0.2 U of purine nucleoside phosphorylase (PNP) and left to incubate for 10 min at 25 °C. After incubation the absorbance at 360 nm was immediately measured for a 90 min time period at 25 °C. For data analysis, the values were determined from the experimental reaction values minus the corresponding zero-substrate control values in Excel. In order to obtain the kinetic parameters (k_{cat} and K_M), the amount of P_i released per minute (V_0 , μ M/min) was then plotted against the ATP concentration (mM) using Prism 6 and fit to a Michaelis-Menten curve. The turnover number, k_{cat} , is represented by the equation ($k_{cat} = V_{max}/[enzyme]$), where V_{max} is defined as the maximum amount of P_i released per minute over the NBD protein concentration in each experiment. The K_M is defined as the ATP concentration at which the amount of P_i released per minute is 50% of the V_{max} ($K_M = 1/2 V_{max}$).

Results and Discussion

Structural changes for mortalin-NBD upon ADP-Pi binding

We crystallized human mortalin-NBD in the ADP-bound state and solved the structure to 2.78 Å resolution by molecular replacement. The ADP bound mortalin-NBD refinement and processing statistics are compiled in Table 1. The entire globular NBD containing residues 52–431 were resolved in this structure (Figure 1A). The overall conformations of the apo (PDB ID 4KBO) and ADP bound mortalin-NBD structures are similar with most of the differences occurring in the NBP. Upon ADP binding the C317 side chain rotates to accommodate the adenosine moiety of ADP and participate in a sulfur- π stacking interaction (Figure 1C). In Hsc70 and Hsp70, the corresponding residue in this position is R391. The sulfur- π stacking interaction facilitated by mortalin C317 is analogous the Hsc70/Hsp70 R391 side chain which forms cation- π stacking interaction with the adenosine.^{44,45} The side chains of mortalin E313 and K316 shift closer to form hydrogen bonds to the C2 carboxyl of the ribose moiety of ADP. This shift tugs subdomain IIB to move closer to the NBP and subdomain IB (Figure 1B,C). The backbone amides of G388 and M389 shift closer to the α -phosphate and methoxy group of ribose, respectively. This causes a slight shift of portions of subdomain IIA closer to IA indicating communication between the domains upon ADP accommodation (Figure 1A and 1B). The backbone amide of N64 forms a hydrogen bond to the β -phosphate. E222, T62, and T249 form hydrogen bonds to the inorganic phosphate liberated by hydrolysis of ATP. The hydroxyl of T249 is conserved among Hsp70s and homologous residues with mutations in this residue impair ATP hydrolysis.⁴⁶ Additionally, the Mg^{2+} ion is fixed between the β -phosphate and inorganic phosphate and D244 shifts to form interactions with the Mg^{2+} ion. The presence and position of the Mg^{2+} ion is important for the hydrolysis of ATP and disruption of this alignment has a significant effect on the activity of Hsp70s.^{47,48} The S320 side chain hydroxyl shifts closer to form a hydrogen bond to the N1 of adenosine shifting subdomain IIB closer to the nucleotide. In the ADP bound state, R309 near the subdomain IB and IIB

cleft rotates to form a hydrogen bond with E313, which shifts to hydrogen bond to the ribose of the nucleotide (Figure 1B). This shift of R309 could further tighten the interactions between subdomain IB and IIB relative to the apo structure. In summary, the addition of ADP-Pi causes the residues of the NBP to shift around the nucleotide, eliciting structural changes in the corresponding subdomains. This causes the overall conformation of the mortalin-NBD to contract and rotate about the nucleotide relative to the apo conformation. Although both the apo and ADP-Pi bound mortalin-NBD structures are in the closed conformation, this comparison of the structures shows how ADP-Pi binding causes changes to the active site, interdomain clefts, and to the overall structure of the mortalin-NBD. These changes, relative to the previously solved apo mortalin-NBD structure, could be useful for further elucidation of the mechanisms of the mortalin chaperone cycle. The crystal structure of the mortalin-NBD with ADP-Pi bound also sets the stage for the design of more specific targeting compounds to modulate the activity of mortalin that take advantage of an active site primed for interactions with ADP-Pi and the resulting structural changes in surrounding subdomains. Furthermore, our mortalin-NBD/ADP-Pi structure provides a basis for our strategy to determine the mechanism of action of the EVEN-PLUS syndrome mutations, R126W and Y128C (Figure 1A). Although R126W and Y128C are not located near known co-chaperone binding sites, these residues are positioned near the binding cleft for the mortalin IDL. Introduction of the R126W or Y128C mutations could therefore influence binding of the IDL or induce structural changes that impact solution behavior or activity of the NBD. Thus, building upon our crystal structure we have explored a range of possible effects of the R126W and Y128C mutations including perturbation of the mortalin-NBD structure, thermostability, aggregation propensity, IDL binding, and ATP hydrolysis.

We assessed the secondary structure content for wild type and mutant NBD proteins using CD spectroscopy (Figure S1, S2). For mortalin, Hsp70, and Hsc70 NBD proteins both the R176W/R76W and Y128C/F78C mutants exhibit CD spectra that are highly similar to the wild type constructs (Figure S1). Using the BeStSel program we determined secondary structure content for each construct (Table 2). For all three proteins, similar values are observed for BeStSel-derived secondary structure contents for wild type and mutant constructs. The circular dichroism data (Figure S1) and BeStSel analysis suggest that the mutations, which are located on a surface loop near the end of an α -helix, do not appear to significantly perturb the secondary structure content of mortalin, Hsp70, or Hsc70.

Thermostability of NBD constructs

We sought to address if binding of nucleotides, magnesium, or the IDL peptide impacted the thermostability of the wild type and mutant proteins. Using an established protocol for thermal denaturation using differential scanning fluorimetry we measured the thermostability of Hsc70-NBD, Hsp70-NBD, and wild type mortalin-NBD (Figure 2A). We observed that the addition of nucleotide, with or without magnesium, causes an increase in the melting point (T_m) of wild type mortalin-NBD (Figure 2B), similar to the increase in T_m of wild type Hsc70-NBD and Hsp70-NBD relative to the apo proteins (Figure 2A,B). These results are consistent with prior studies of Mortalin and Hsc70 which found that ATP, ADP, and magnesium stabilize the wild type proteins.^{49,50} This increase in stability suggests that nucleotide binding results in a transition to a conformation with stabilizing interactions, such

as NBP residues that facilitate bridging interactions through the nucleotide between subdomains (Figure 1). The addition of ATP, which is subsequently hydrolyzed to ADP-Pi, is likely slightly higher due to residues of the NBP binding to the P_i and possible further interaction between the adjacent subdomains. Magnesium in combination with ADP or ATP causes a further increase in T_m for mortalin NBD, Hsc70-NBD, and Hsp70-NBD. This data is consistent with the location of the Mg²⁺ ion within the active site, the bridging of the ADP β-phosphate, free phosphate, and D414. In comparison, without nucleotide present, addition of magnesium to apo protein causes only a small change in T_m for mortalin-NBD (Figure 2), Hsc70-NBD, and Hsp70-NBD (Figure 2). In comparison to Hsc70-NBD and Hsp70-NBD, mortalin-NBD exhibits smaller increases in T_m upon ADP binding relative to the cytosolic Hsp70s, which suggests weaker interactions with the nucleotide and corresponds to data indicating mortalin-NBD has a weaker binding affinity for ADP compared to cytosolic Hsp70-NBD. Interestingly, compared to the cytosolic homologs, mortalin-NBD has the largest observed increase in T_m upon addition of ATP:Mg. This may be due to an ATP:Mg bound conformation with more extensive interactions between subdomains compared to the cytosolic Hsp70 homologs. Although our crystallization trials with non-hydrolyzable ATP analogs did not yield an ATP-bound mortalin-NBD structure, future crystallographic efforts may identify these putative interactions.

The disease-associated R126W and Y128C mutants have an overall decrease in thermostability relative to the wild type mortalin-NBD. We observed that the mutations resulted in a decrease in the T_m of about 9 °C for the Y128C mutant and 11 °C for the R126W mutant (Figure 2A). This data indicates a slightly more detrimental effect by the R126W mutation upon the stability of mortalin-NBD compared to the Y128C mutation. We posit that loss of the positive charge of R126, when replaced with tryptophan, results in a hydrophobic surface patch between the IB and IA subdomain which may increase aggregation.¹⁸ This is consistent with our experience that R126W mortalin-NBD exhibits poorer solubility during purification (data not shown). The R126W and Y128C mutants exhibit similar stabilization upon binding nucleotide with or without the addition of magnesium (Figure 2B) and both mutants are most thermostable when ATP and magnesium are present. Despite increases in T_m values for R126W and Y128C mutants that are larger than the corresponding increases observed for wild type protein for each set of ligands tested, when comparing identical conditions, the R126W and Y128C mutants display a lower T_m value than the wild type protein. Interestingly, the increases in T_m values for mortalin-NBD (Figure 2B) upon addition of any of the examined ligands are similar for both R126W and Y128C suggesting that packing defects or structural perturbations that result from each mutation are nearly equally detrimental. Similarly, not only are the corresponding R76W and F78C mutations in both Hsc70-NBD and Hsp70-NBD each destabilizing (Figure 2A), these corresponding mutants also exhibit similar stabilizing effects when binding nucleotides (Figure 2B). In the cytosolic variants, the changes in T_m values for both the R76W and F78C mutations are lower for Hsp70 than for Hsc70, and each are lower than the increases in T_m values observed for mortalin-NBD. The overall behavior across all three homologs indicates that the arginine to tryptophan and tyrosine to cysteine mutations reduce thermostability of each homolog and that binding of nucleotides leads to partial recovery of thermostability.

In addition to the effect of ligand binding at the NBP, we sought to measure the additional contribution to thermostability elicited by interdomain linker peptide (IDL) binding to mortalin, Hsc70, and Hsp70 NBD constructs. Compared to the apo wild type sample for each homolog, substantial additional increases in T_m values are observed for mortalin-NBD variants with bound nucleotides upon addition of the interdomain linker peptide (Figure 2B). The additive contribution of IDL binding is more pronounced for mortalin-NBD, compared to Hsc70 and Hsp70, with exception of wild type Hsp70 in the presence of ADP or ATP. Across all three homologs, the additional stabilization afforded by IDL binding is diminished for the R126W and Y128C mutants. While the additional stabilization observed for mortalin-NBD mutants is similar in magnitude to the stabilization observed for wild type mortalin-NBD, the F76W and F76C Hsc70 and Hsp70 mutants exhibit little to no additional stabilization. The reduced stabilization for all homologs suggests the mutations examined may cause structural changes that alter how the NBD interacts with the IDL. For a more in-depth examination of changes in interactions with the IDL, we extended thermostability studies by calculating thermodynamic parameters of unfolding, pursued binding studies to determine the affinity of the IDL for NBD constructs, and docking simulations of wild type, R126W, and Y128C mortalin-NBD constructs.

R126W and Y128C mutations disrupt thermodynamic parameters of unfolding

We utilized our previously published method to determine the Gibbs free energy of unfolding (ΔG°) from the thermal denaturation curves.⁴⁰ In general, a more-stable protein will have a large positive value for ΔG° at 25 °C indicating that spontaneous unfolding at 25 °C is unfavorable. We herein refer to constructs with larger, positive ΔG° values as more stable, while constructs with smaller ΔG° values are referred to as less stable. A comparison of ΔG° values provides for a more nuanced evaluation of stability compared to comparisons of T_m values.⁴⁰ The thermodynamic formalism required for extracting ΔG° from the thermal denaturation curves requires that the proteins undergo reversible folding transitions in the region analyzed. For our analysis this is the temperature range from 25 °C to the T_m of the corresponding protein. Efforts to directly demonstrate reversibility by DSF were unsuccessful due to the presence of the SYPRO Orange dye which binds to hydrophobic regions of the protein during initial phases of thermal unfolding and interferes with or prevents refolding upon cooling. Therefore, we sought to demonstrate reversibility using CD spectroscopy, a label-free technique. We monitored CD signal at 222 nm by heating each wild type and mutant mortalin-NBD, Hsc70-NBD, and Hsp70-NBD construct in 0.5 °C increments from 25 °C up to the T_m identified for that construct by differential scanning fluorimetry (Figure 2), followed by cooling in 0.5 °C increments back to 25 °C (Figure S3). For a comparison, a full thermal melt was conducted in 0.5 °C increments from 25 °C to 95 °C was also conducted for each construct (Figure S2). The CD reversible unfolding experiments (Figure S3) demonstrate that all wild type and mutant mortalin-NBD, Hsc70-NBD, and Hsp70-NBD constructs maintain a nearly constant CD signal at 222 nm throughout the reversible unfolding experiment and that the CD signal at 222 nm is identical at the beginning and end of the experiment indicating no loss of secondary structure upon heating to the T_m and returning to 25 °C. This data demonstrates that the thermodynamic parameters of unfolding presented here for all wild type and mutant mortalin-NBD, Hsc70-NBD, and Hsp70-NBD constructs were calculated for reversible transitions. Apo Mortalin-

NBD, Hsc70-NBD, and Hsp70-NBD exhibit similar reductions in the Gibbs free energy of unfolding upon introduction of the R126W/R76W and Y128C/F78C mutations (Figure S4). While all three homologs exhibit positive ΔG° values for wild type and mutant constructs at 25 °C, indicating that none of the constructs spontaneously unfold at 25 °C, the decrease in ΔG° upon introduction of the mutations are consistent with the trends observed in T_m values. The relative stabilities of each NBD construct under different conditions are more readily apparent when examining ΔG° values by comparing each construct under different conditions against the ΔG° for that construct in the apo state (Figure 3). Positive ΔG° values indicate that a construct is more stable under the tested condition than the corresponding apo construct. Conversely, negative ΔG° values indicate a reduction in stability compared to the apo construct. For both Hsc70-NBD and Hsp70-NBD constructs, wild type constructs are more stable than the corresponding R76W and F78C mutants under all conditions tested. It is noteworthy that these mutants are substantially less stable than wild type in the apo and magnesium-bound states, with exception of F78C Hsp70-NBD in the presence of magnesium which shows only a small decrease in stability. Similarly, the mortalin-NBD R126W and Y128C mutants in the apo or magnesium-bound states were considerably less stable than apo mortalin-NBD (Figures S5,S6). The primary difference in stability of R126W/R76W and Y128C/F78C mutants with respect to the wild type proteins occurs in the ADP, ADP:Mg, ATP, and ATP:Mg conditions. Under each of these conditions, the wild type NBD constructs are uniformly more stable than the apo state. For both Hsc70 and Hsp70-NBD R76W and F78C mutants, increases in stability relative to the apo state are observed, with the exception of small decreases in stability for R76W Hsp70-NBD. However, the small reduction in stability for R76W Hsp70-NBD is reversed in the presence of the IDL. The mortalin-NBD mutants are, in comparison to Hsc70 and Hsp70 mutants, substantially less stable with only small increases in stability over apo wild type. For mortalin-NBD mutants, stabilization on the order of those seen for Hsc70 and Hsp70-NBD R76W and F78C mutants is only observed for Y128C mortalin-NBD in the presence of ADP, Mg, and the IDL. For nearly every condition tested, the mortalin-NBD mutants are less stable than the corresponding Hsc70-NBD and Hsp70-NBD constructs. Taken together, the R126W/R76W and Y128C/F78C mutants have a clear detrimental impact on the stability of mortalin-NBD, Hsc70-NBD, and Hsp70-NBD constructs. Additionally, mortalin does not exhibit the level of ligand-induced recovery of stability that is observed for Hsc70 and Hsp70, consistent with previous studies indicating that mortalin has a greater tendency for aggregation compared to the cytosolic homologs Hsc70 and Hsp70.^{51,52}

R126W and Y128C mutations increase aggregation propensity

We assessed the effect of the R126W and Y128C mutations upon aggregation propensity of wild type or mutant mortalin-NBD, Hsc70-NBD, and Hsp70-NBD constructs using dynamic light scattering. NBD constructs were incubated at 25, 31, and 37 °C and subsequently analyzed with DLS (Figure 4). The wild-type and mutant mortalin-NBD constructs each exhibit an initial peak with approximate radius of 5 nm consistent with a monomeric NBD (Figure 4A). However, the constructs differ on the intensity of a second peak. Both the R126W and Y128C mutants display a second peak with an approximate radius of 150 nm at a significantly higher intensity than wild type mortalin. This peak at 150 nm is indicative of the increased level of aggregate formation from the caused by the R126W and Y128C

mutations. When incubated at 31 °C the intensity of this large aggregate peak further increases for both R126W and Y128C mutants while wild type mortalin exhibits only a slight increase in intensity for the second peak. Upon incubation at 37 °C the mutant mortalin-NBD constructs exhibit a dramatic decrease in the initial monomer peak with a large shift to peaks corresponding to large aggregates. In contrast, wild type mortalin at 37 °C has a further increase in the intensity of the second peak, however the intensity for wild type mortalin at 37 °C is similar to that of the mutants at 25 °C and is significantly lower than the intensity of the large diameter mutant peaks at 37 °C. While the apparent size of the large diameter R126W and Y128C mutant peaks at 37 °C is smaller than the second peaks at 31 °C, this is likely due to the sample preparation process. After incubation at the indicated temperature, samples were centrifuged to remove precipitate. Both the R126W and Y128C exhibited substantial precipitation at 37 °C and the removal of the precipitate by centrifugation may have removed a significant portion of the large diameter aggregates leading to an apparent decrease in the size of aggregates for R126W and Y128C at 37 °C. Despite the decrease in apparent size, both the R126W and Y128C mutations exhibit substantially higher levels of aggregation compared to wild type. These trends observed for increased aggregate formation for the R126W and Y128C mutants are also shared by the Hsc70 and Hsp70-NBD R76W and F78C mutants (Figure 4 B,C) suggesting that the damaging effects of the R126W and Y128C mutations are conserved between the mitochondrial and cytosolic Hsp70s. Additionally, these shifts in intensities to this second peak indicates a possible common mechanism of aggregation for the NBD proteins and that the EVEN-PLUS syndrome mutations increase aggregation of the mortalin-NBD.

R126W and Y128C mutations disrupt affinity for IDL binding

To determine the effect of R126W and Y128C mutations upon interaction with the IDL we immobilized biotinylated IDL peptide derived from mortalin, Hsc70, or Hsp70 onto streptavidin biosensors. The IDL biosensors were then probed with wild type or mutant mortalin-NBD, Hsc70-NBD, or Hsp70-NBD constructs to determine the dissociation constant (Figure 5). Across the twelve experiments, that addition of ADP to the sample dramatically increased the affinity of IDL, consistent with prior findings that the ADP-bound closed conformation orients NBD subdomains and participating residues to form a tighter interaction with the IDL relative to the open state of apo NBD. Comparing the affinities of wild type, R126W, and Y128C mortalin-NBD in the presence of ADP, the R126W and Y128C mutants exhibit reductions in affinity deemed significant by unpaired t tests. Similarly, Y78C Hsc70-NBD and R76W Hsp70-NBD exhibit significant reductions in affinity compared to the corresponding wild type NBD. Although the affinities of R76W Hsc70-NBD and F78C Hsp70-NBD were not found to be significantly different from wild type NBD in the ADP-bound state, substantial reductions in affinity were seen in the apo state. The similar performance of Hsc70-NBD and Hsp70-NBD in IDL binding assays, compared to mortalin-NBD, suggests that the damaging effects of the disease-associated mutations to IDL peptide binding are at least partially conserved between the mitochondrial and cytosolic Hsp70s. For mortalin-NBD, the R126W mutation has a more deleterious effect on IDL binding, compared to the Y128C mutation. This may be due to the proximity of the R126 to the IDL binding site and the severity of mutating a polar positively charged arginine to an aromatic nonpolar tryptophan, compared to the more distally located Y128.

Docking of the IDL is altered by the disease-associated mutations

Interactions between the IDL and the NBD play an important role in allosterically communicating between the domains of Hsp70s and activity of the NBD.^{53,54} The impaired binding of the IDL to the R126W and Y128C mutant mortalin-NBD constructs (Figure 5) suggests that the R126W and Y128C mutations in the mortalin-NBD disrupt or alter the conformation of the IDL peptide with respect to mortalin-NBD. To examine the effect of the R126W and Y128C mutations on IDL docking to the mortalin-NBD we ran simulations to dock a flexible mortalin IDL peptide (₄₄₄SPLTVDLLLV₄₃₄) onto wild type, R126W, or Y128C mortalin-NBD (Figure 6). Our mortalin-NBD crystal structure with ADP, Pi, and Mg²⁺ bound was used as the starting point for simulations and was mutated *in silico* to produce R126W or Y128C mortalin-NBD. In all three docking simulations the IDL binds between the subdomains IA and IIA with the N-terminal region of the IDL located near Y196, a residue which interacts with the P_i within the NBP of mortalin in the post-hydrolysis closed conformation. In each simulation, the IDL forms at least three hydrogen bonds to the mortalin-NBD and bridges the IA and IIA subdomains (Figure 6B). Hydrophobic packing between the mortalin-NBD and IDL is facilitated by IDL residues V435, L436, L437, V440, and P442. For both wild type and Y128C docking results (Figure 6B,D) four hydrogen bonds form between the IDL and mortalin-NBD, the additional hydrogen bond not present in R126W docking is between the IDL L435 backbone carbonyl and F262 backbone amide from the NBD. In comparison to wild type and Y128C docking conformations, the W126 side chain indole is positioned to buttress the isobutyl of L443 on the IDL, twisting the IDL. This twisting of the IDL peptide ripples through the peptide and ultimately prevents formation of the L435/F262 hydrogen bond. These altered interactions are likely responsible for the diminished affinity of R126W mortalin-NBD for the IDL relative to affinities observed for wild type and Y128C constructs. The R126W simulation also exhibits higher variation in conformations among the lowest Rosetta energy score poses compared to wild type and Y128C simulations. This increased variation is indicative of lower favorability for the binding pose observed in wild type and Y128C simulations and is consistent with a lower affinity for the interaction between R126W mortalin-NBD and the IDL. In comparison, the wild type and Y128C docking poses are similar, with exception of variability in conformation near L443 observed in the Y128C simulation. Changes in hydrophobic packing due to the Y128C mutation could yield small alterations in conformation of the R126-Y128 loop, causing minor changes to the conformation of IDL bound to Y128C mortalin-NBD. These small changes in binding are consistent with the concomitantly small decrease in affinity for the IDL exhibited by the Y128C compared to wild type. Taken in light of the thermostabilities and IDL binding affinities, the IDL docking simulations suggest that the detrimental effects of R126W occur due to a combination of poorer NBD stability and altered IDL binding whereas poorer stability, but not significantly altered IDL binding, appear to determine the effect of the Y128C mutation.

Impaired ATP hydrolysis for EVEN-PLUS syndrome mortalin mutants

Given the detrimental effects of EVEN-PLUS syndrome mutations on the thermostability, aggregation, and IDL binding for each of the NBD constructs we examined phosphate release kinetics for the human mortalin-NBD, Hsc70-NBD, and Hsp70-NBD variants to assess whether these mutations impact ATP hydrolysis (Figure S7–S9). The k_{cat} values

determined for each of the wild type NBD constructs in this study (Table 3) are lower than those measured by Sevier and colleagues,⁴⁸ however the k_{cat} and K_M values we found are consistent with the range of previously reported k_{cat} ($0.83 - 0.031 \text{ min}^{-1}$) and K_M ($270 - 0.11 \text{ }\mu\text{M}$) values for either isolated NBDs or full-length Hsp70s.^{49,55–61} The disease-associated mutations severely impaired catalytic activity of mortalin-NBD (Figure 7). R126W mortalin-NBD exhibited a ~65% decrease in the catalytic activity and 30% weaker K_M of $230 \pm 50 \text{ }\mu\text{M}$ compared to the wild type mortalin-NBD. The Y128C mutation in mortalin-NBD yielded similarly low catalytic activity and weaker K_M compared to wild type. A similar detrimental effect on the catalytic activity is observed in the corresponding mutations in Hsc70-NBD and Hsp70-NBD (Table 3 and Figure 6). R76W Hsc70-NBD exhibited a ~67% decrease in the catalytic activity and a 5.4-fold reduction in K_M . F78C Hsc70-NBD exhibited a ~64% decrease in the catalytic activity and a 4.8-fold weaker K_M compared to wild type Hsc70-NBD.

ATP hydrolysis has been linked to residues from subdomains IA and IB and rearrangement of these subdomains upon ATP and ADP states (Figure 8). For example, in subdomain IA residue T13 in Hsc70, conserved in mortalin as T62, is coupled to conformational change in the subdomain and loss of the threonine hydroxyl hinders ATP hydrolysis and conformational change in the NBD subdomains.⁶² In subdomain IB residue K71 in Hsc70, conserved in mortalin as K121, is essential for ATP hydrolysis and reorients about subdomain IA upon ATP binding and hydrolysis to ADP.^{63,64} The structural changes along the catalytic cycle of mortalin are modeled in Figure 8 with a progression of structures from apo NBD, ATP-bound NBD, and ADP-bound NBD. Taken in light of published mechanisms for ATP hydrolysis by Hsp70 homologs, the substantial hits in catalytic activity for both mutations across all three homologs we studied implies that the disease-associated mutations disturb the allosteric coupling between the two subdomains of mortalin, in which the reorientation of subdomains is communicated through the NBP and defects in this mechanism result in impaired ATP hydrolysis and binding. The location of R126W and Y128C at or near the interface between mortalin-NBD subdomains suggests that the disruptions in charge, hydrophobic packing, or conformation and the proximity of the mutations to the subdomain interface each influence the severity of the effects of the EVEN-PLUS syndrome mutations.

Conclusions

The ADP-bound structure of mortalin-NBD provides a snapshot for a second conformation within the chaperone cycle of mortalin, building upon our earlier structure of apo mortalin-NBD. Using this structure as a basis for understanding the effect of the EVEN-PLUS syndrome mutations R126W and Y128C, we modeled the binding of IDL peptides and conducted CD spectroscopy, thermostability assays, DLS aggregation studies, IDL docking assays, and ATPase activity assays. We found the thermostability of mortalin-NBD was severely diminished by the EVEN-PLUS syndrome mutations R126W and Y128C. Significantly, determination of the Gibbs free energy of unfolding indicates that neither the R126W nor Y128C mutants exhibit significant capability of stabilization by binding nucleotides, magnesium, or the IDL. Additionally, the mutations were determined to increase the propensity of the NBD to form large aggregates. Decreases in stability of each

mutant coincide with reduced affinity for the IDL peptide. Since only the R176W mutation displayed substantial changes in conformation of the IDL in docking simulations, we suggest that while the effects of these mutations may not necessarily be manifested in significant changes in conformation of a bound IDL, small changes in the conformational cycle for mortalin appear to elicit significant decreases in ATPase activity. Given the close ties between ATP hydrolysis and chaperone function, diminished or altered chaperone functions will most likely affect many of the proteins regulated by mortalin creating a cascade of aberrantly functioning proteins, such as for LONP1 protease.²⁵ In light of the data we collected, EVEN-PLUS syndrome mutations likely impair the function of mortalin through a combination of factors including reduced stability and diminished allosteric communication between subdomains. These alterations in the transmission of conformational changes between the two subdomains could be one of the causes of disease symptoms by disrupting the chaperone function of mortalin. We recommend that future efforts to identify therapeutics for EVEN-PLUS syndrome should focus on the dynamics of wild type and mutant constructs to restore or maintain allosteric communication within the mortalin-NBD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Overlay of human mortalin-NBD in apo and ADP-Pi bound conformations. Throughout Figure 1, ADP-Pi bound mortalin (*blue*) is shown in a single color, apo mortalin is shown with each subdomain colored individually (subdomain IA *pink*; subdomain IB *magenta*; subdomain IIA *white*; subdomain IIB *cyan*), ADP-Pi (*yellow*) is shown as sticks, and the Mg^{2+} ion (*green*) is represented as a sphere. The inset region, viewed from the opposite face of subdomain IB, highlights the position of R126 (*green*) and Y128 (*grey*). (B) Zoomed view of interactions between subdomains IB and IIB showing the shifts in residue

orientation upon ADP-Pi binding. (C) Zoomed in view of the mortalin nucleotide binding pocket with the ADP-Pi bound mortalin (*blue*) backbone shown as cartoons. Important residues from the ADP-Pi bound mortalin and apo mortalin structures are colored as in (A) and (C).

Figure 2.

(A) DSF curves for apo Mortalin-NBD, Hsc70-NBD, and Hsp70-NBD constructs. Throughout (A) and (B) wild type (*grey*), R126W or R76W mutants (*red*), and Y128C and F78C mutants (*blue*) are shown as solid bars or lines in the absence of the IDL, or as striped bars in the presence of the IDL peptide. (B) The change in T_m (ΔT_m), calculated relative to the apo wild type sample for the corresponding NBD, determined by DSF for each construct measured in the apo form (*Apo*), in the presence of magnesium (*Mg*), ADP (*ADP*), ADP and magnesium (*ADP:Mg*), ATP (*ATP*), or ATP and magnesium (*ATP:Mg*). The additional change in T_m (ΔT_m) for each sample upon IDL peptide binding is signified by striped stacked bars.

Figure 3.

The change in Gibbs free energy of unfolding (ΔG_u^o) for the mortalin-NBD, Hsc70-NBD, and Hsp70-NBD in the apo form (*Apo*), or in the presence of magnesium (*Mg*), ADP (*ADP*), ADP and magnesium (*ADP:Mg*), ATP (*ATP*), or ATP and magnesium (*ATP:Mg*) is plotted for wild type proteins (*grey bars*), R126W or R76W mutants (*red bars*), and Y128C and F78C mutants (*blue bars*). Solid bars indicate data collected without the IDL peptide, striped bars indicate data collected in the presence of the IDL peptide. Within each set the ΔG_u^o values are calculated relative to the apo wild type sample for the corresponding NBD.

Figure 4.

Aggregation propensity of wild type and mutant NBD constructs as measured by dynamic light scattering for samples incubated at 25 °C, 31 °C, and 37 °C. DLS curves are shown for mortalin-NBD (A), Hsc70-NBD (B), and Hsp70-NBD (C). Constructs are colored according to the legend shown above (A). Error bars indicate the standard deviation from three replicates.

Figure 5.

Dissociation constants (K_d) determined by biolayer interferometry of the mortalin-NBD (circles), Hsc70-NBD (squares), and Hsp70-NBD (triangles) for the IDL peptide. Wild type constructs (grey), R126W or R76W mutants (red), and Y128C and F78C mutants (blue) are shown as filled symbols for experiments in the absence of ADP and outlined symbols in the presence of ADP. Results deemed significantly different by an unpaired t test are denoted as ** for $P < 0.01$, and *** for $P < 0.001$.

(A) The five lowest Rosetta energy scoring poses of the IDL peptide backbone onto wild type mortalin-NBD in magenta, the IDL peptide onto a R126W mortalin-NBD model in orange, and the IDL peptide onto a Y128C mortalin-NBD model in marine. The mortalin-NBD is with the subdomains IA, IIA, IB, IIB shown in pink, pale cyan, dark red, and cyan, respectively. (B) A pose of the IDL peptide in magenta onto wild type mortalin-NBD. (C) A pose of the IDL peptide in orange modeled onto R126W mortalin-NBD. (D) A pose of the IDL peptide in marine docked onto Y128C mortalin-NBD. Throughout panels B-D the IDL

peptide residues are shown in bold, interacting residues are shown in italics, and hydrogen bonds are drawn as yellow dashed lines.

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Figure 7. Phosphate release assays for ATP hydrolysis by mortalin-NBD (circles), Hsc70-NBD (squares), and Hsp70-NBD (triangles). Throughout wild type constructs (*grey*), R126W or R76W mutants (*red*), and Y128C and F78C mutants (*blue*) are shown with nonlinear regression fits and error bars indicating the standard deviation of data collected in triplicate. Error bars for some data points are smaller than the symbol drawn.

Figure 8.

Progression of the structure of the mortalin-NBD throughout the catalytic cycle. Models within the progression include apo mortalin (PDB ID 4kbo), ATP-bound DnaK-NBD (4b9q), and ADP-bound mortalin-NBD (this study). Throughout Figure 7, each NBD is shown with each subdomain colored individually (subdomain IA *pink*; subdomain IB *magenta*; subdomain IIA *white*; subdomain IIB *cyan*). The R126W mutation is colored red and the Y128C mutation is colored yellow.

Table 1.

Data collection and refinement statistics for ADP bound Mortalin-NBD

Data collection	
X-ray source	Beamline ALS 4.2.2
Wavelength (Å)	1.00
Resolution range (Å)	56.870 – 2.777 (2.877 – 2.777)
Space group	C121
<i>a</i> , <i>b</i> , <i>c</i> (Å)	177.3, 60.1, 91.7
α , β , γ (°)	90, 97.6, 90
Reflections	90,280 (7,867)
Unique reflections	24,320 (2,310)
Multiplicity	3.7 (3.4)
Completeness (%)	0.99 (0.94)
Mean <i>I</i> /sigma (<i>I</i>)	13.6 (1.1)
Wilson B-factor (Å ²)	73.5
CC _{1/2}	0.998 (0.54)
Refinement	
PDB ID	6NHK
Number of reflections	24,273 (2,292)
Reflections used for R-free	1,989 (185)
<i>R</i> _{work} / <i>R</i> _{free}	0.255 / 0.296
Number of atoms Protein/water/ligands)	5,290 / 8 / 72
Average <i>B</i> factors (protein/water/ligands)	81.0 / 58.8 / 66.4
Model Quality	
Bond length rmsd (Å)	0.003
Bond angle rmsd (°)	0.610
Ramachandran favored (%)	96
Ramachandran allowed (%)	3.8
Ramachandran outliers (%)	0
Poor rotamers (%) ^b	0
Clashscore ^b	5.74
Clash percentile ^b	100th percentile (N=143; 2.78 Å 0.25 Å)
MolProbity score ^b	1.57
MolProbity score percentile ^b	100th percentile (N=4,489; 2.78 Å 0.25 Å)

^aData for the highest resolution shell is represented by the values in parentheses within the table.^bValues calculated using MolProbity v4.4.³⁸

Table 2.

BeStSel-derived secondary structure content for wild type and mutant mortalin-NBD, Hsc70-NBD, and Hsp70-NBD constructs

Construct	Secondary Structure Content ^a					
	α -Helices	β -Sheets (anti-parallel)	β -Sheets (parallel)	Turns	Unordered Coils	Spectral deviation (NRMSD) ^b
MortalinNBD WT	31.0%	17.3%	6.5%	12.1%	33.2%	0.02496
MortalinNBD R126W	32.9%	17.7%	5.9%	11.2%	32.4%	0.02385
MortalinNBD Y128C	31.0%	16.4%	7.4%	11.8%	33.5%	0.02328
Hsc70NBD WT	37.3%	16.8%	4.3%	7.3%	34.3%	0.03691
Hsc70NBD R76W	39.4%	18.6%	3.7%	6.7%	31.5%	0.04327
Hsc70NBD F78C	38.0%	17.5%	4.2%	7.0%	33.2%	0.04289
Hsp70NBD WT	35.8%	16.8%	4.1%	7.9%	35.4%	0.03370
Hsp70NBD R76W	37.4%	17.4%	4.1%	7.5%	33.5%	0.03495
Hsp70NBD F78C	36.5%	16.9%	4.4%	7.6%	34.6%	0.03464

^aSecondary structure content calculated from best fits performed by BeStSel. Fits and residuals are shown with the corresponding data in Figure S2.

^bNormalized root mean square deviation (NRMSD) as calculated by BeStSel.

Table 3.

Turnover number and Michaelis constant for wild type and mutant mortalin-NBD, Hsc70-NBD, and Hsp70-NBD constructs

	Mortalin-NBD		Hsc70-NBD		Hsp70-NBD	
	K_{cat} (min^{-1})	K_{M} (μM)	K_{cat} (min^{-1})	K_{M} (μM)	K_{cat} (min^{-1})	K_{M} (μM)
wild type	0.069 ± 0.002	160 ± 20	0.087 ± 0.001	25 ± 3	0.079 ± 0.004	280 ± 60
R126W/R76W	0.024 ± 0.01	230 ± 50	0.029 ± 0.001	130 ± 20	0.044 ± 0.001	290 ± 10
Y128C/F78C	0.029 ± 0.002	250 ± 60	0.031 ± 0.001	120 ± 30	0.045 ± 0.001	340 ± 30