Supplementary information

AIFM1 variants associated with auditory neuropathy spectrum disorder cause apoptosis due to impaired apoptosis-inducing factor dimerization

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Materials and methods

Cell culture

HEK 293T cells were cultured in a complete culture medium (DMEM with 2 mmol/L L-glutamine, 4.5 g/L D-glucose, and 110 mg/L sodium pyruvate, supplemented with 10% FBS, Gibco) and maintained at 37 °C in a humidified 5% CO_2 atmosphere. 1.2×10^6 cells were seeded into a 6 cm dish.

PCR amplification and plasmid construction

Total RNA was obtained using Trizol reagent (Invitrogen) from 5×10^6 cells. cDNA was reverse transcripted from one microgram of total RNA using a Prime Script RT reagent Kit (Takara). The *AIFM1* gene was amplified with the corresponding primers (Table 1). Wide-type and mutant *AIFM1* were cloned into several expression vectors (Table 2). These vector constructs were

referred to as pCDH-*AIFM1*-WT/mut, p3×Flag/HA-*AIFM1*-WT/mut, pET28a-*AIFM1*-WT/mut, and pFlag/His- *AIFM1*-WT/ mut. The corresponding primers are listed (Table 1).

Table 1 Primers for vector construction

		Sequence (5'→3')
AIFM1-WT	Forword	GGGGTACCGAAGGAGGAGGTCCCGAATAG
	Reverse	CCTTAAGTGCAGTGGGTTTGCCAATTCC
AIFM1-778A>G[1]	Forword	GGGGTACCGAAGGAGGAGGTCCCGAATAG
	Reverse	GTACCTCCTGCTGCAATCAAGC
AIFM1-778A>G[2]	Forword	GCTTGATTGCAGCAGGAGGTAC
	Reverse	CCTTAAGTGCAGTGGGTTTGCCAATTCC
AIFM1-1264C>T[1]	Forword	GGGGTACCGAAGGAGGAGGTCCCGAATAG
	Reverse	TCTGCATTTACCCAGAAGCCAC
AIFM1-1264C>T[2]	Forword	GTGGCTTCTGGGTAAATGCAGA
	Reverse	CCTTAAGTGCAGTGGGTTTGCCAATTCC
AIFM1-1352G>A[1]	Forword	GGGGTACCGAAGGAGGAGGTCCCGAATAG
	Reverse	ATGGTGCTCTACCTGCCTCCTT
AIFM1-1352G>A[2]	Forword	AAGGAGGCAGGTAGAGCACCAT
	Reverse	CCTTAAGTGCAGTGGGTTTGCCAATTCC
pCDH-*AIFM1*	Forword	TAGAAGATTCTAGAGCTAGCGAATTCATGTTCCGGTGTGGAGGCCTG
	Reverse	AGCGATCGCAGATCCTTCGCGGCCGCTGCAGTGGGTTTGCCAATTCC
p3×Flag-*AIFM1*	Forword	TTAAGCTTGCGGCCGCGAATTCATGTTCCGGTGTGGAGGCCTG
	Reverse	TCCTCTAGAGTCGACTGGTACCGTCTTCATGAATGTTGAATA
p3×HA-*AIFM1*	Forword	ATTGAATTCCCCGGGGATCCATGTTCCGGTGTGGAGGCCTG
	Reverse	GTATGGGTAGTCGACTCTAGAGTCTTCATGAATGTTGAATA
pFlag/His-*AIFM1*[1]	Forword	GCTAGCGCCACCATGGCGGCCGCA ATGTTCCGGTGTGGAGGCCT
	Reverse	TCACCGGTAAGCTTTGCGATCGC GTCTTCATGAATGTTGAATA
pFlag/His-*AIFM1*[2]	Forword	GCTAGCGCCACCATGGCGGCCGCA GGGCTGACACCAGAACAGAA
	Reverse	TCACCGGTAAGCTTTGCGATCGC GTCTTCATGAATGTTGAATA
pET28a-*AIFM1*	Forword	AATGGGTCGCGGATCCGAATTC GGGCTGACACCAGAACAGAA
	Reverse	TCGAGTGCGGCCGCAAGCTT GTCTTCATGAATGTTGAATA

Table 2 Expression plasmid for vector construction

Plasmid	Company	Cleavage sites
pCDH-CMV-MCS-EF1-Puro	Novagen	*Eco*R I/*Not* I
p3xFLAG	Sigma	*Eco*R I/*Kpn* I
p3xHA	Sigma	*Bam*H I/*Xba*l I
pSpCas9(BB)-2A-Puro	Addgene	*Bbs* I
pFlag/His	Addgene	*Not* I/*Sfa*A I
pET28a	Addgene	*Eco*R I/*Hin*d III

Generation of AIF-null, AIF-WT, and AIF-mut cell lines

An AIF-null cell line was generated using the CRISPR/Cas9 system. The gRNA was designed online and was cloned into a pSpCas9(BB)-2A-Puro (PX459) V2.0 vector. The detailed protocols

are referred to in Dr. Zhang's paper (Ran et al., 2013). The primers used for CRISPR/Cas9 are listed in Table 3. Subsequently, AIF-WT and AIF-mut stable transfection cell lines were generated using the lentivirus infection system with pCDH-*AIFM1*-WT/mut, psPAX2, and pMD2G co-transfected into AIF-null cells. These were selected using 4 μg/mL puromycin. At least two monoclonal cell lines of each transfection were singled out for subsequent experiments after gene and protein evaluation.

Table 3 Primers for CRISPR/Cas 9

		Sequence (5'→3')
SgRNA-Cas9	Forword	CACCGCCTCGGGCTTCGGACGCACA
	Reverse	AAACTGTGCGTCCGAAGCCCGAGGC
CRISPR-test	Forword	GAGTCTGCGTAATGTGCG
	Reverse	AGCCAGTTGTTCTGGGAT
Off-Target-1	Forword	CTGGCCTGATGCCTTTCACTG
	Reverse	GGTGCGTCATAGGCTTGCTG
Off-Target-2	Forword	CTGGAACCACGGGTAGTGA
	Reverse	TCTGCAAGCCAAGGATGAA
Off-Target-3	Forword	CGGCTCCGCTCGACTTCCT
	Reverse	GCATTTGCCCCTTTTGTTTCC
Off-Target-4	Forword	GGTGTCCCTTCTCAGTCCC
	Reverse	CCAAGACCCTTTACCTTTGC
Off-Target-5	Forword	GCCTCGAACTGTGACATG
	Reverse	AGGTGGGAGCTGAAACCC
Off-Target-6	Forword	CCCATGTAACCGCCACCT
	Reverse	TCCAGCCTCCTCATAGAGC
Off-Target-7	Forword	GGCTGAGTGTCCATTCCTC
	Reverse	CCATCCAGTGATGCCAGAG
Off-Target-8	Forword	CCCATTATTAACAAGTCCC
	Reverse	TGCTAATCATGTAGGCAGT
Off-Target-9	Forword	AAAGCAATTTCCTTTCCTCTAA
	Reverse	CCTGATGCTGCGGGTTGG
Off-Target-10	Forword	GGCGGAGTAGCCCGTGAA
	Reverse	GCCGCCTGTGGCAGTATCTT

Western blotting analysis

Proteins obtained from cell lines were denatured and loaded on 12% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). The proteins were then electro-transferred to PVDF membrane and blocked with Tris-Buffered Saline and Tween20 (TBST) (150 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 7.5 and 0.1% (v/v) Tween 20) containing 5% (w/v) milk for 1 hour. The membranes were then incubated with corresponding primary and secondary antibodies. The primary antibodies are listed in Table 4. Protein signals were detected using a CLINX chemiscope

and the ECL system (CWBIO) with Peroxidase Affini Pure goat anti-mouse IgG and goat anti-rabbit IgG (BIOKER) used as secondary antibodies.

Table 4 Antibody used for western blot

Antibody	Company	Lot
anti-AIF	santa cruz	ab32516
anti-GAPDH	abcam	ab181602
anti-Caspase3	CST	#9662
anti-Caspase7	CST	#12827
anti-Caspase9	CST	#9502
anti-HA	Sino Biological	100028-MM10
anti-Flag	Protein tech	20543-1-AP

Native-PAGE

Cells were lysed on ice for 20 min with non-denaturing lysis buffer (50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT, and 0.5% NP-40) containing protease inhibitor cocktail (Roche). The proteins were mixed with 5×Native loading buffer (Fdbio science) and heated at 70 ℃ for 5 min. The samples were then loaded on 4-15% Precast-gel Tris-Glycine PAGE (Sangon Biotech) using Tris-Glycine Native PAGE Running Buffer (Sangon Biotech). The following steps were similar to those of western blotting analysis.

Co-IP analysis

Cells were lysed on ice for 20 min with cold lysis buffer (50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT, and 0.5% NP-40) containing protease inhibitor cocktail (Roche). After quantification, 1.5 mg protein was added into 5 μL Flag beads (Sigma) and incubated at 4 °C for 3 h. After incubation, the beads were washed three times with cold lysis buffer and 100 μL 1×loading was added to the beads for further western blotting.

Protein expression and purification in *E. coli*

All $hAIF_{\Delta 1-102}$ constructs were cloned into the pET28a vector with a C-terminal 6X His affinity tag. They were then transformed into Rosetta2 (DE3) cells. At OD600 0.6~0.8, cultures were induced with IPTG at 16 ℃ overnight. Cells were resuspended in PBS (pH 7.4) containing 1 mmol/L phenylmethyl sulfonyl fluoride and subsequently sonicated at 4 ℃ for 10 min. The supernatant was incubated with the Ni-affinity resin (Sangon Biotech) at 4 ℃ overnight. Then, the supernatant was discarded and the Ni-affinity resin was transferred into an Affinity

Chromatography Column (Sangon Biotech). After washing with 40 mmol/L imidazole, AIF was eluted with 150 mmol/L imidazole. The protein solution was concentrated and purified on a UFC5100BK ultrafiltration column (Merck Millipore). The concentration of the purified protein was verified using Bradford assay. Before NADH reduction titration, the purified proteins were oxidized for 2 hours in pure oxygen to completely oxidize the flavin. Protein preparations used in the study had A280/452 ratios≤7.0.

Protein expression and purification in eukaryotic cells

The plasmids of p Flag/His-*AIFM1*-WT/mute were transfected into AIF-null cells. The cell pellets were collected 48 h after transfection. After extraction, 30 mg protein was mixed with 60 μL Flag beads (Sigma) and incubated at 4 °C for 6 h. After incubation, the beads were washed three times with wash buffer (50 mmol/L Tris-HCl (pH 7.4), 500 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L DTT, and 1% NP-40). Then, 60 μL 3×Flag Peptide (Sigma) was added to the beads for competitive elution of the recombinant protein overnight. In eukaryotic cells, the N-terminal of AIF precursor will be cleaved to form $AIF_{\Delta 1\text{-}54}$ proteins. The concentration of the purified protein was verified using Bradford assay. Before NADH reduction titration, the purified proteins were oxidized for 2 h in pure oxygen to fully oxidize the flavin.

Kinetics of AIF reduction with NADH

After purification in *E. coli*, 10 μmol/L AIF was dissolved in PBS (pH 7.4). Absorbance spectra were measured on a Spark 10M spectrophotometer (TECAN). The basal value was measured firstly without NADH. Then, 100 μmol/L NADH was added to prime FAD reduction and CTC formation. Absorbances from 400 to 800 nm were again detected at 20, 40, 60, 120, 240 and 480 s after mixing AIF protein with NADH at room temperature.

Western blotting analysis of AIF reduction with NADH

After purification in eukaryotic cells and in *E. coli*, 1 μmol/L AIF was mixed with various concentrations of NADH (5, 10, and 20 μmol/L) and the reaction was incubated for 15 min at room temperature to reduce AIF. Then, 1 mmol/L disuccinimidyl suberate (DSS) was added to crosslink the AIF dimer via incubating for 30 min at room temperature. Reactions were quenched using 1×SDS loading buffer, followed by boiled and subjected to SDS-PAGE.

Western blotting analysis of AIF dimer in stably transfected cell lines

The stably transfected cell lines of wild type AIF and AIF variants were seeded 24 h before NADH treatment. Then, 200 μmol/L NADH was added and incubated for 24 h. After treatment, 1×10^6 cells were incubated with 4 mmol/L DSS to crosslink the AIF dimer. Reactions were quenched using 20 mmol/L Tris (pH 8.0) for 15 min at room temperature. The proteins were then extracted and subjected to SDS-PAGE.

Apoptosis analysis

The apoptosis of cells was detected using an Annexin V-FITC/PI Apoptosis Detection Kit (YEASEN) through flow cytometry according to the manufacturer's instructions. Blank control: cells were incubated without staurosporine (STS) and Z-VAD-FMK to measure background cell apoptosis. Apoptosis stimulation: cells were incubated only with 1 μmol/L STS (Gene Operation) for 1.5 h. Selectively induced caspase-independent apoptosis: cells were incubated with 1 μmol/L STS for 1.5 h and pre-incubated with 50 μmol/L Z-VAD-FMK (MCE) for 0.5 h.

Models of free AIF and AIF-Ligands starting structure

The starting structures of AIF monomers were generated using the SWISS-MODEL server, according to chain A and chain C of the reduced AIF complexed with NAD (PDB: 4BUR) (Ferreira et al., 2014; Waterhouse et al., 2018). The AIF dimer structure was then created corresponding to the coordinates of AIF-4BUR. This model was subsequently used to generate the AIF-1FAD-2NADH dimer. To simulate the reducing physiological environment, the NAD+ was modeled to its reduced form (NADH) by adding an electron to N1 and a hydrogen atom to C4 in the pyridine ring. Coordinates for the FAD and NADH (A and B) ligands from the reduced AIF complexed with NAD (PDB: 4BUR) were placed into the active site. All AIF variants (p.T260A, p.R422W, p.R451Q) were generated using PyMOL.

Molecular dynamics simulation

To test the stability of the AIF dimer in AIF variants, an all-atom molecular dynamics simulation was performed. The simulations were carried out using the GROMACS software package (version 2020.6) (Hess et al., 2008), together with the CHARMM36 force field set in an explicit TIP3P water solvent. The temperature is 300 K and the pressure is 1 bar. The long-range electrostatic interactions were analyzed via PME method and the van der Waals (vdW) interactions were calculated using a cutoff distance of 1.0 nm. The forcefield in the form of charmm-36 for FAD and NADH was generated from the Ligand Reader & Modeler in the CHARMM-GUI server (Brooks

et al., 2009; Lee et al., 2016; Kim et al., 2017). The AIF dimers with or without ligands were solvated in a 12.5 Å x 12.5 Å x 12.5 Å water box with a 60310 TIP3P water model. The system was then neutralized with 187 sodium and 185 chloride ions in a normal saline concentration. The solvated system was firstly energy-minimized by 10,000 steps, followed by a 2500, 000 step equilibration (2 fs for each step). For each system, 300 ns long simulations were performed in 3 replicates. The backbone atoms of residues from 128 to 611 were used to evaluate the RMSDs. The number of H-bonds between residues at the dimer interface along the trajectory were analyzed.

Statistical analysis

Statistical analysis was carried out using Student's unpaired, two-tailed *t*-tests in the Microsoft-Excel program. All data represent two control AIF-WT cell lines (2 clones) and two AIF-variants cell lines (2 clones) with at least three independent experiments performed. Three replicates for each clone were performed in three independent experiments. Data are represented as mean±SEM. $^{ns}P \geq 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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Supplemental figures

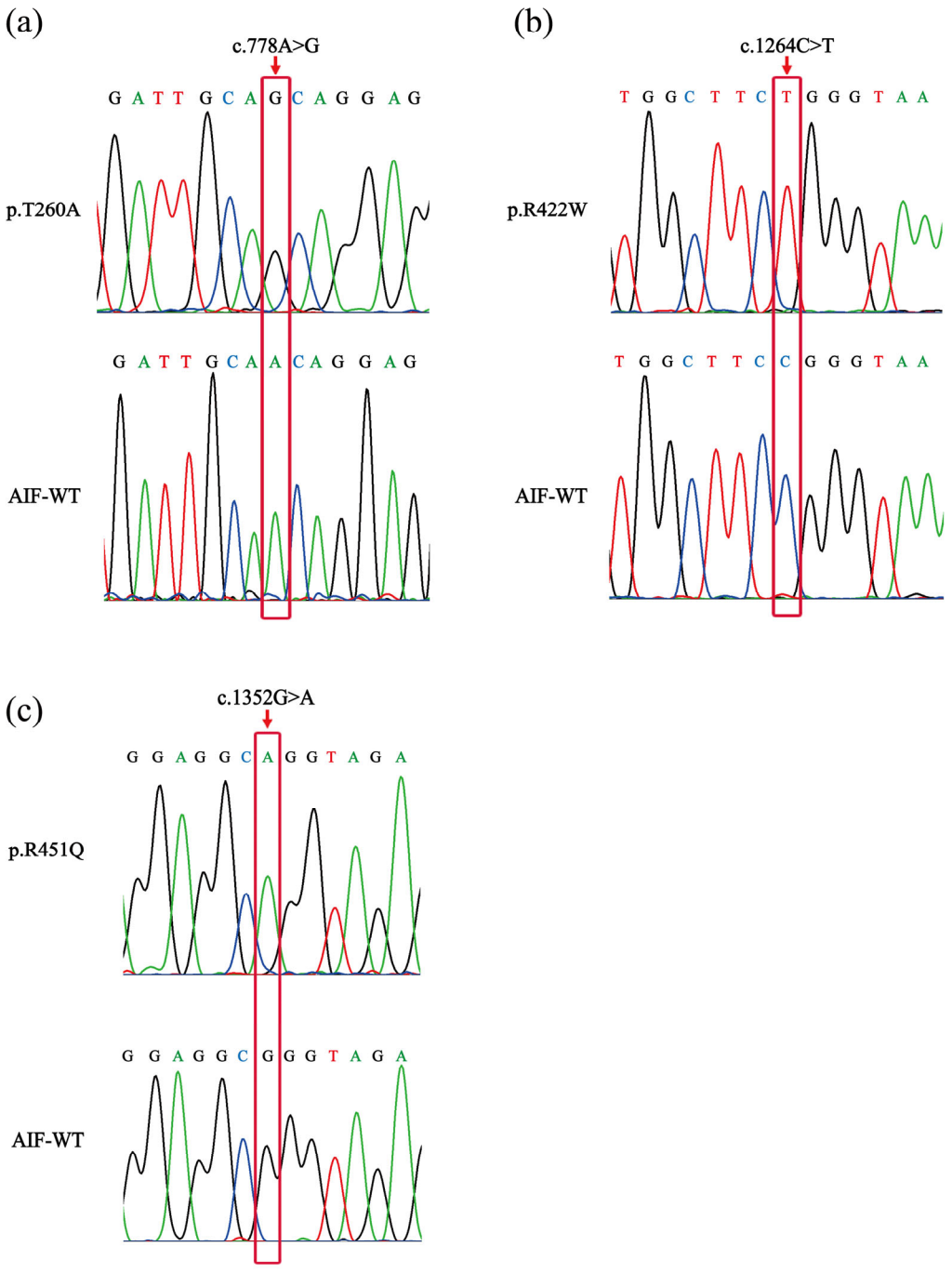

Fig. S1 Identification of *AIFM1* sequence in the AIF-WT and AIF variants cell lines. The *AIFM1* variants (c.778A>G (a), c.1264C>T (b), and c.1352G>A (c)) were present in the AIF variants cell lines, but absent in the AIF-WT cell lines.

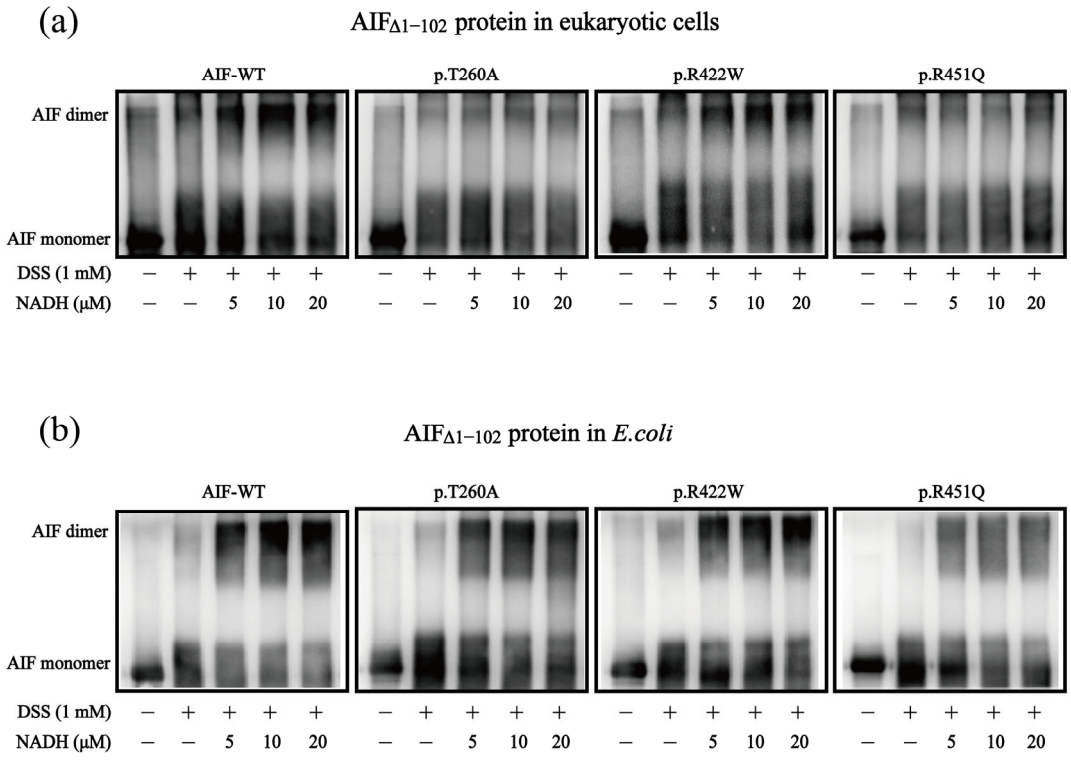

Fig. S2 FAD reduction assays of AIF$_{\Delta 1-102}$ proteins in eukaryotic cells and *E. coli*. Western blotting analysis after mixing 1 μmol/L AIF$_{\Delta 1-102}$ proteins in eukaryotic cells (a) and in *E. coli* (b) with various concentrations of NADH (5, 10, and 20 μmol/L) for 15-min incubation. DSS was added to crosslink the AIF dimer.

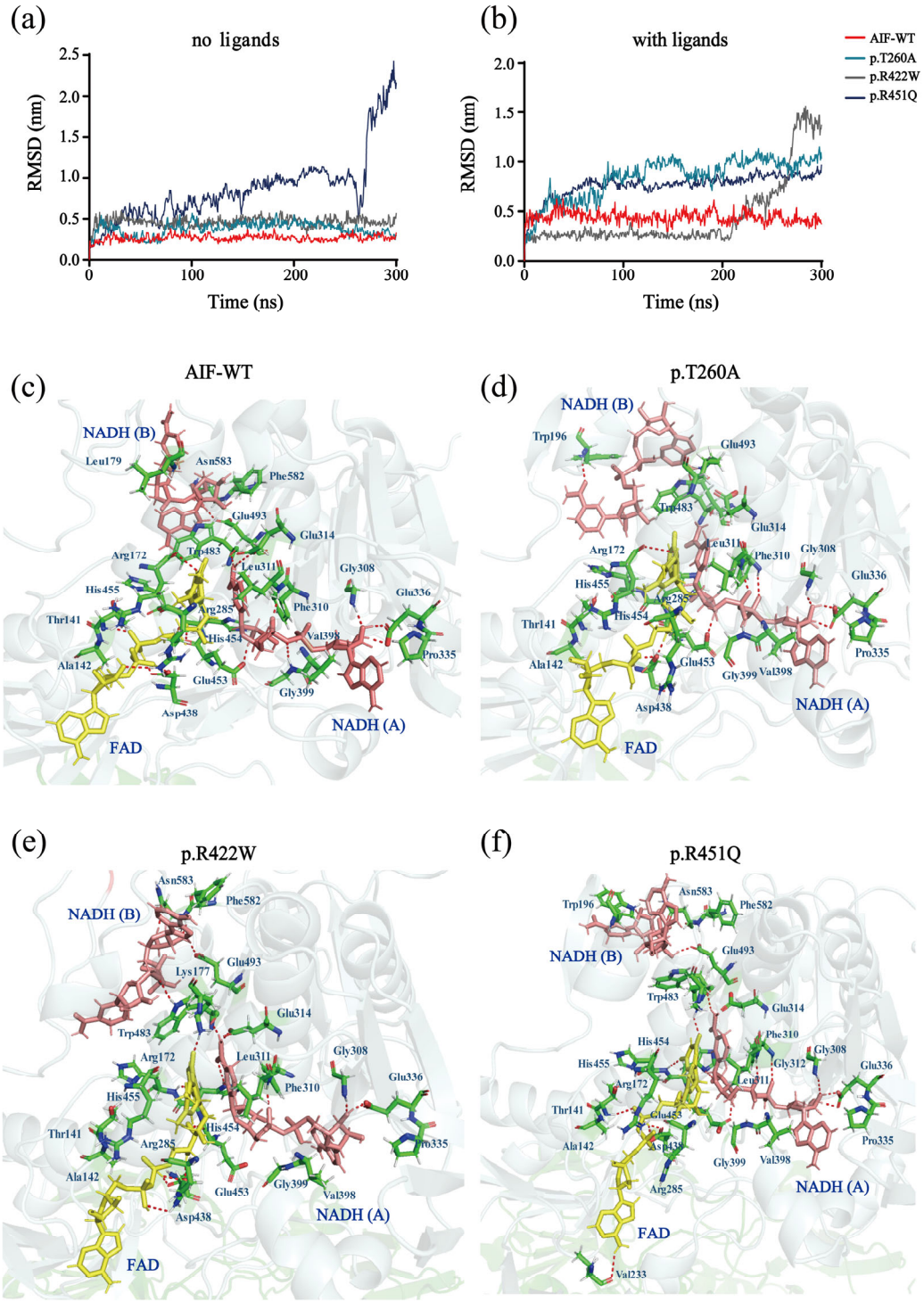

Fig. S3 Unstable AIF dimer structure. (a) Smoothed RMSD values (0.5 ns window) plotted across the trajectory time course without ligand. MD simulations were performed for 300 ns. (b) Smoothed RMSD values (0.5 ns window) were plotted across the trajectory time course with NADH and FAD ligands. MD simulations were performed for 300 ns. (c) Structure of redox active site in AIF-WT, (d) p.T260A, (e) p.R422W, (f) p.R451Q. These selected snapshots were the most stable structures from the trajectories. These conformations were then analyzed, and figures produced using PyMOL. The residues were colored by element, with the C atom in green, the H atom in silver, the N atom in blue, the O atom in red, and the S atom in yellow. The NADH(A/B) is shown in pink and the FAD in yellow. Dashed red lines represent hydrogen bonds.