

## 1 **Supplementary information**

### 2 3 ***AIFM1* variants associated with auditory neuropathy** 4 **spectrum disorder cause apoptosis due to impaired** 5 **apoptosis-inducing factor dimerization**

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## 18 19 20 **Materials and methods**

### 21 22 **Cell culture**

23 HEK 293T cells were cultured in a complete culture medium (DMEM with 2 mmol/L L-glutamine,  
24 4.5 g/L D-glucose, and 110 mg/L sodium pyruvate, supplemented with 10% FBS, Gibco) and  
25 maintained at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere. 1.2×10<sup>6</sup> cells were seeded into a 6 cm  
26 dish.

### 27 28 **PCR amplification and plasmid construction**

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

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

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**Table 1 Primers for vector construction**

|                                      |         | Sequence (5'→3')                                |
|--------------------------------------|---------|---|
| <i>AIFM1</i> -WT                     | Forward | GGGGTACCGAAGGAGGAGGTCCCGAATAG                   |
|                                      | Reverse | CCTTAAGTGCAGTGGGTTTGCCAATTCC                    |
| <i>AIFM1</i> -778A>G <sup>1</sup>    | Forward | GGGGTACCGAAGGAGGAGGTCCCGAATAG                   |
|                                      | Reverse | GTACCTCCTGCTGCAATCAAGC                          |
| <i>AIFM1</i> -778A>G <sup>2</sup>    | Forward | GCTTGATTGCAGCAGGAGGTAC                          |
|                                      | Reverse | CCTTAAGTGCAGTGGGTTTGCCAATTCC                    |
| <i>AIFM1</i> -1264C>T <sup>1</sup>   | Forward | GGGGTACCGAAGGAGGAGGTCCCGAATAG                   |
|                                      | Reverse | TCTGCATTTACCCAGAAGCCAC                          |
| <i>AIFM1</i> -1264C>T <sup>2</sup>   | Forward | GTGGCTTCTGGGTAAATGCAGA                          |
|                                      | Reverse | CCTTAAGTGCAGTGGGTTTGCCAATTCC                    |
| <i>AIFM1</i> -1352G>A <sup>1</sup>   | Forward | GGGGTACCGAAGGAGGAGGTCCCGAATAG                   |
|                                      | Reverse | ATGGTGCTCTACCTGCCTCCTT                          |
| <i>AIFM1</i> -1352G>A <sup>2</sup>   | Forward | AAGGAGGCAGGTAGAGCACCAT                          |
|                                      | Reverse | CCTTAAGTGCAGTGGGTTTGCCAATTCC                    |
| pCDH- <i>AIFM1</i>                   | Forward | TAGAAGATTCTAGAGCTAGCGAATTCATGTTCCGGTGTGGAGGCCTG |
|                                      | Reverse | AGCGATCGCAGATCCTTCGCGGCCGCTGCAGTGGGTTTGCCAATTCC |
| p3×Flag- <i>AIFM1</i>                | Forward | TTAAGCTTGC GGCCGCGAATTCATGTTCCGGTGTGGAGGCCTG    |
|                                      | Reverse | TCCTCTAGAGTCGACTGGTACCGTCTTCATGAATGTTGAATA      |
| p3×HA- <i>AIFM1</i>                  | Forward | ATTGAATTCGGGGATCCATGTTCCGGTGTGGAGGCCTG          |
|                                      | Reverse | GTATGGGTAGTCGACTCTAGAGTCTTCATGAATGTTGAATA       |
| pFlag/His- <i>AIFM1</i> <sup>1</sup> | Forward | GCTAGCGCCACCATGGCGGCCGCA ATGTTCCGGTGTGGAGGCCT   |
|                                      | Reverse | TCACCGGTAAGCTTTGCGATCGC GTCTTCATGAATGTTGAATA    |
| pFlag/His- <i>AIFM1</i> <sup>2</sup> | Forward | GCTAGCGCCACCATGGCGGCCGCA GGGCTGACACCAGAACAGAA   |
|                                      | Reverse | TCACCGGTAAGCTTTGCGATCGC GTCTTCATGAATGTTGAATA    |
| pET28a- <i>AIFM1</i>                 | Forward | AATGGGTCGCGGATCCGAATTC GGGCTGACACCAGAACAGAA     |
|                                      | Reverse | TCGAGTGC GGCCGCAAGCTT GTCTTCATGAATGTTGAATA      |

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**Table 2 Expression plasmid for vector construction**

| Plasmid               | Company | Cleavage sites                 |
|-----------------------|---------|--------------------------------|
| pCDH-CMV-MCS-EF1-Puro | Novagen | <i>EcoR</i> I/ <i>Not</i> I    |
| p3xFLAG               | Sigma   | <i>EcoR</i> I/ <i>Kpn</i> I    |
| p3xHA                 | Sigma   | <i>Bam</i> H I/ <i>Xba</i> I   |
| pSpCas9(BB)-2A-Puro   | Addgene | <i>Bbs</i> I                   |
| pFlag/His             | Addgene | <i>Not</i> I/ <i>Sfa</i> I     |
| pET28a                | Addgene | <i>EcoR</i> I/ <i>Hind</i> III |

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

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

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

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**Table 3 Primers for CRISPR/Cas 9**

|               |         | Sequence (5'→3')          |
|---------------|---------|---------------------------|
| SgRNA-Cas9    | Forward | CACCGCCTCGGGCTTCGGACGCACA |
|               | Reverse | AAACTGTGCGTCCGAAGCCCGAGGC |
| CRISPR-test   | Forward | GAGTCTGCGTAATGTGCG        |
|               | Reverse | AGCCAGTTGTTCTGGGAT        |
| Off-Target-1  | Forward | CTGGCCTGATGCCTTTCACCTG    |
|               | Reverse | GGTGCATCATAGGCTTGCTG      |
| Off-Target-2  | Forward | CTGGAACCACGGGTAGTGA       |
|               | Reverse | TCTGCAAGCCAAGGATGAA       |
| Off-Target-3  | Forward | CGGCTCCGCTCGACTTCCT       |
|               | Reverse | GCATTTGCCCTTTTGTTC        |
| Off-Target-4  | Forward | GGTGTCCCTTCTCAGTCCC       |
|               | Reverse | CCAAGACCCTTACCTTTC        |
| Off-Target-5  | Forward | GCCTCGAACTGTGACATG        |
|               | Reverse | AGGTGGGAGCTGAAACCC        |
| Off-Target-6  | Forward | CCCATGTAACCGCCACCT        |
|               | Reverse | TCCAGCCTCCTCATAGAGC       |
| Off-Target-7  | Forward | GGCTGAGTGTCCATTCTC        |
|               | Reverse | CCATCCAGTGATGCCAGAG       |
| Off-Target-8  | Forward | CCCATTATTAACAAGTCCC       |
|               | Reverse | TGCTAATCATGTAGGCAGT       |
| Off-Target-9  | Forward | AAAGCAATTTCTTTCTCTAA      |
|               | Reverse | CCTGATGCTGCGGGTTGG        |
| Off-Target-10 | Forward | GGCGGAGTAGCCCGTGAA        |
|               | Reverse | GCCGCCTGTGGCAGTATCTT      |

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## 52 Western blotting analysis

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

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

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62 **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  |

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#### 64 **Native-PAGE**

65 Cells were lysed on ice for 20 min with non-denaturing lysis buffer (50 mmol/L Tris-HCl (pH 7.4),  
66 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT, and 0.5% NP-40) containing protease  
67 inhibitor cocktail (Roche). The proteins were mixed with 5×Native loading buffer (Fdbio science)  
68 and heated at 70 °C for 5 min. The samples were then loaded on 4-15% Precast-gel Tris-Glycine  
69 PAGE (Sangon Biotech) using Tris-Glycine Native PAGE Running Buffer (Sangon Biotech). The  
70 following steps were similar to those of western blotting analysis.

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#### 72 **Co-IP analysis**

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

78

#### 79 **Protein expression and purification in *E. coli***

80 All hAIF<sub>Δ1-102</sub> constructs were cloned into the pET28a vector with a C-terminal 6X His affinity  
81 tag. They were then transformed into Rosetta2 (DE3) cells. At OD<sub>600</sub> 0.6~0.8, cultures were  
82 induced with IPTG at 16 °C overnight. Cells were resuspended in PBS (pH 7.4) containing 1  
83 mmol/L phenylmethyl sulfonyl fluoride and subsequently sonicated at 4 °C for 10 min. The  
84 supernatant was incubated with the Ni-affinity resin (Sangon Biotech) at 4 °C overnight. Then, the  
85 supernatant was discarded and the Ni-affinity resin was transferred into an Affinity

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

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### 93 **Protein expression and purification in eukaryotic cells**

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

103

### 104 **Kinetics of AIF reduction with NADH**

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

110

### 111 **Western blotting analysis of AIF reduction with NADH**

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

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## 119 **Western blotting analysis of AIF dimer in stably transfected cell lines**

120 The stably transfected cell lines of wild type AIF and AIF variants were seeded 24 h before  
121 NADH treatment. Then, 200  $\mu\text{mol/L}$  NADH was added and incubated for 24 h. After treatment,  
122  $1 \times 10^6$  cells were incubated with 4  $\text{mmol/L}$  DSS to crosslink the AIF dimer. Reactions were  
123 quenched using 20  $\text{mmol/L}$  Tris (pH 8.0) for 15 min at room temperature. The proteins were then  
124 extracted and subjected to SDS-PAGE.

125

## 126 **Apoptosis analysis**

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

133

## 134 **Models of free AIF and AIF-Ligands starting structure**

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

144

## 145 **Molecular dynamics simulation**

146 To test the stability of the AIF dimer in AIF variants, an all-atom molecular dynamics simulation  
147 was performed. The simulations were carried out using the GROMACS software package (version  
148 2020.6) (Hess et al., 2008), together with the CHARMM36 force field set in an explicit TIP3P  
149 water solvent. The temperature is 300 K and the pressure is 1 bar. The long-range electrostatic  
150 interactions were analyzed via PME method and the van der Waals (vdW) interactions were  
151 calculated using a cutoff distance of 1.0 nm. The forcefield in the form of charmm-36 for FAD and  
152 NADH was generated from the Ligand Reader & Modeler in the CHARMM-GUI server (Brooks

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

161

## 162 **Statistical analysis**

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

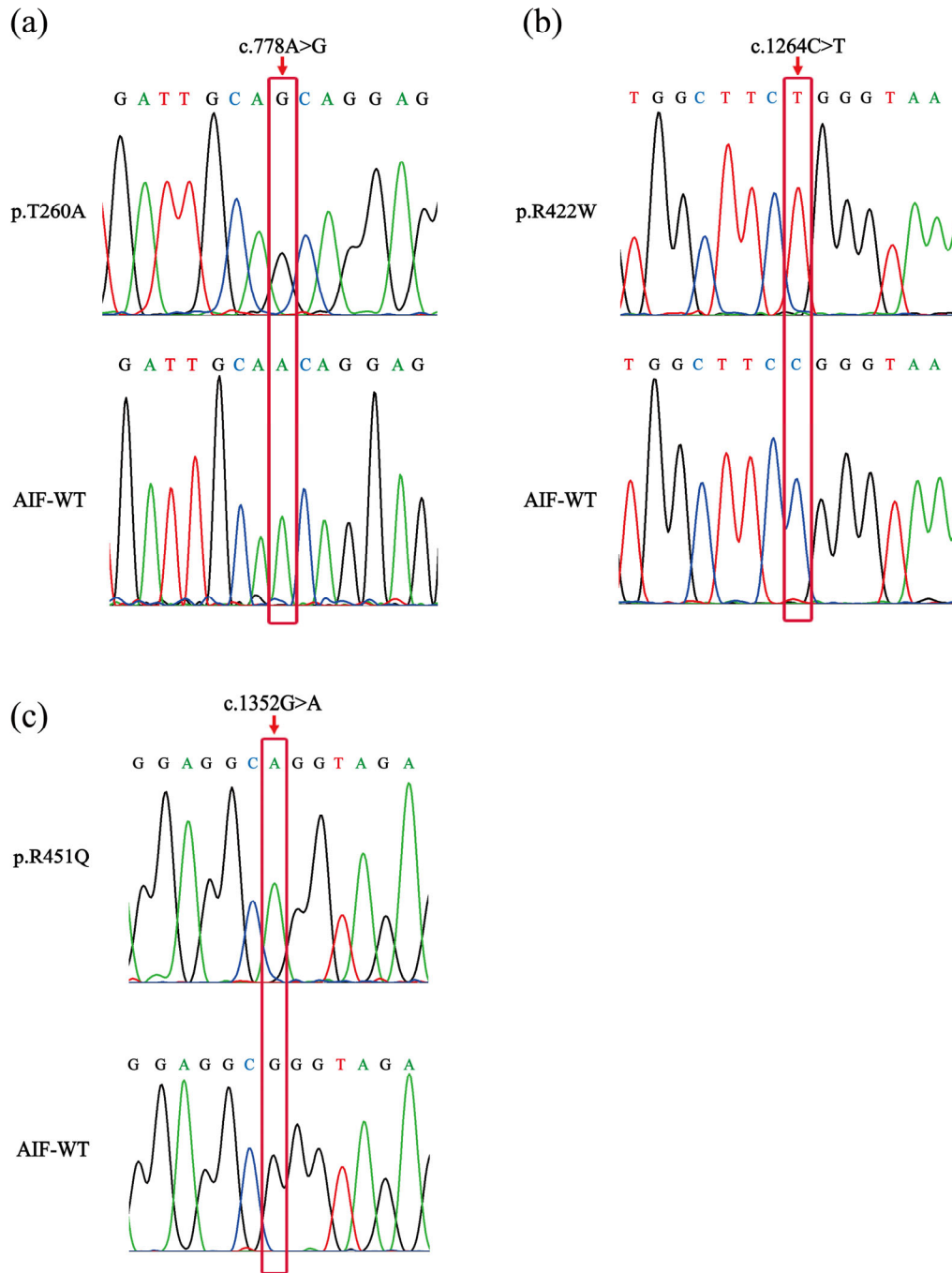
168

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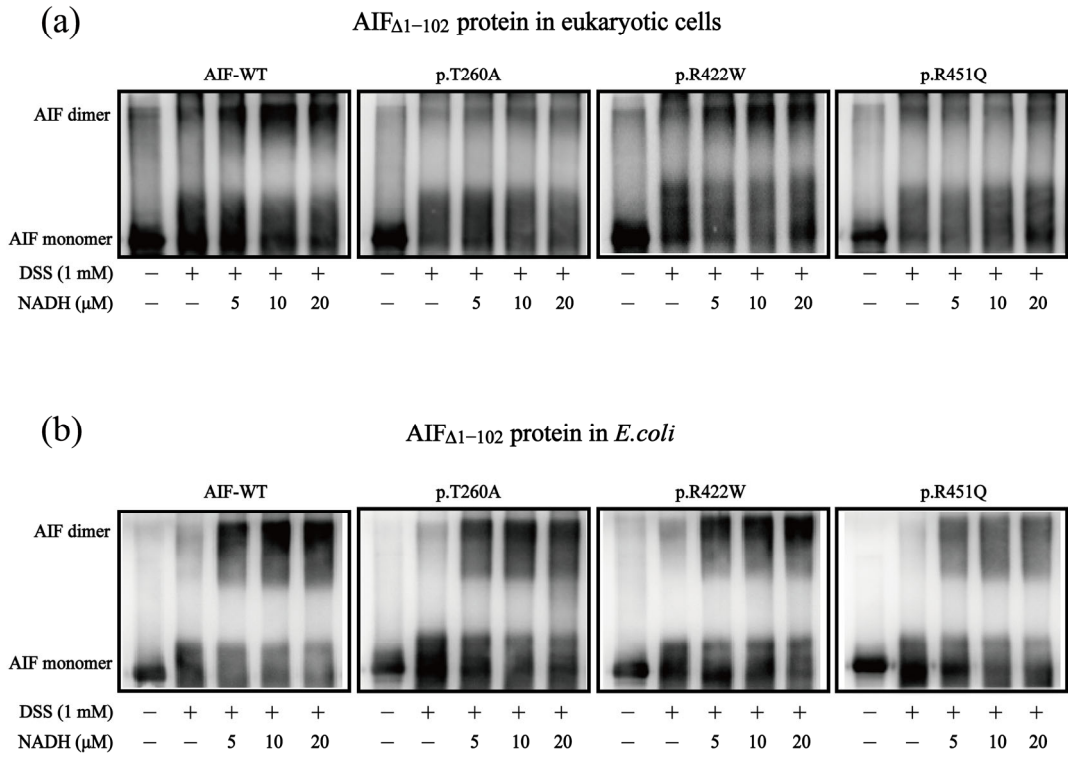
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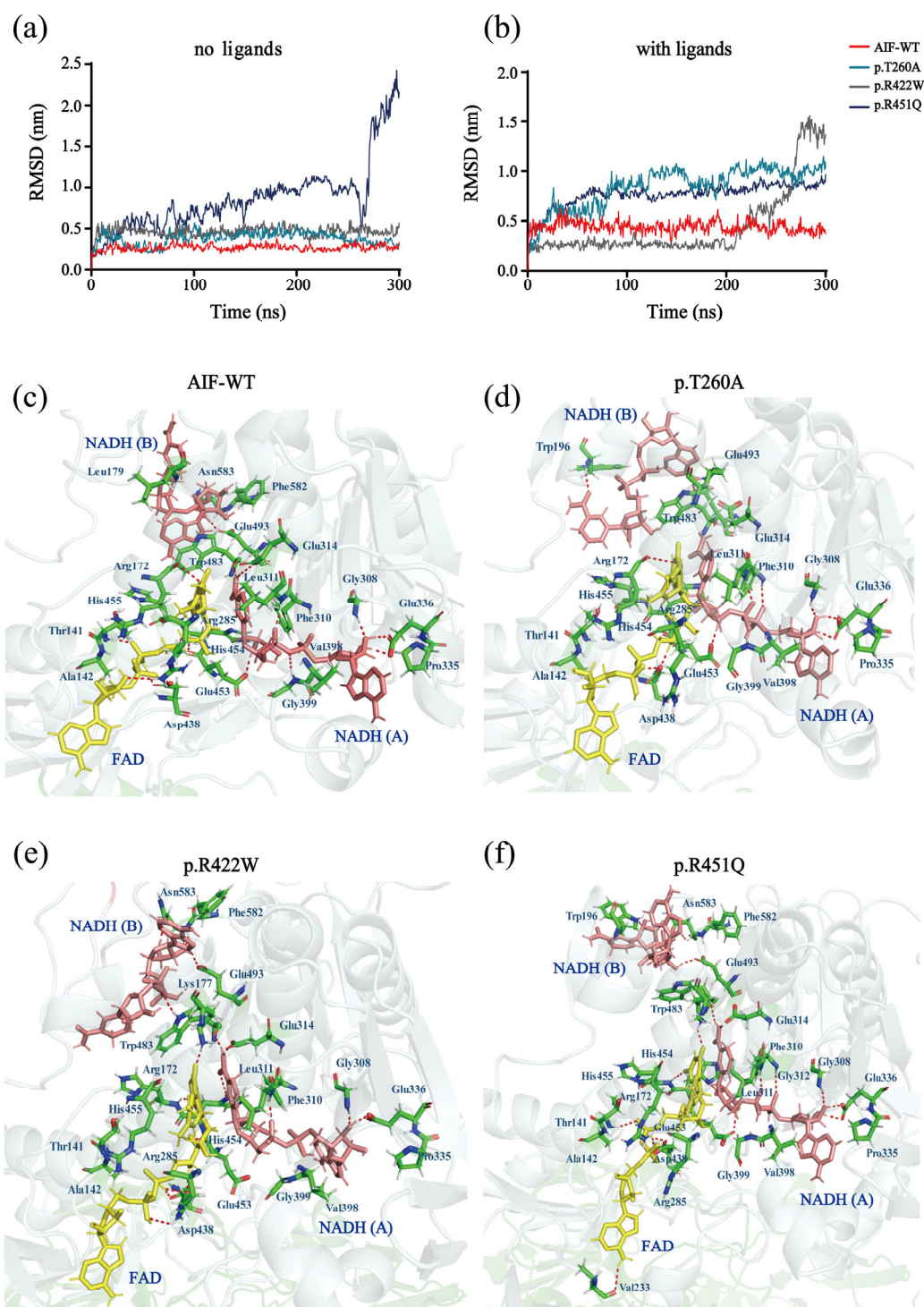
Fig. S1 Identification of *AIFM1* sequence in the AIF-WT and AIF variants cell lines. The *AIFM1* variants (c.778A>G (a), c.1264C>T (b), and c.1352G>A (c)) were present in the AIF variants cell lines, but absent in the AIF-WT cell lines.





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**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.**



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**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.