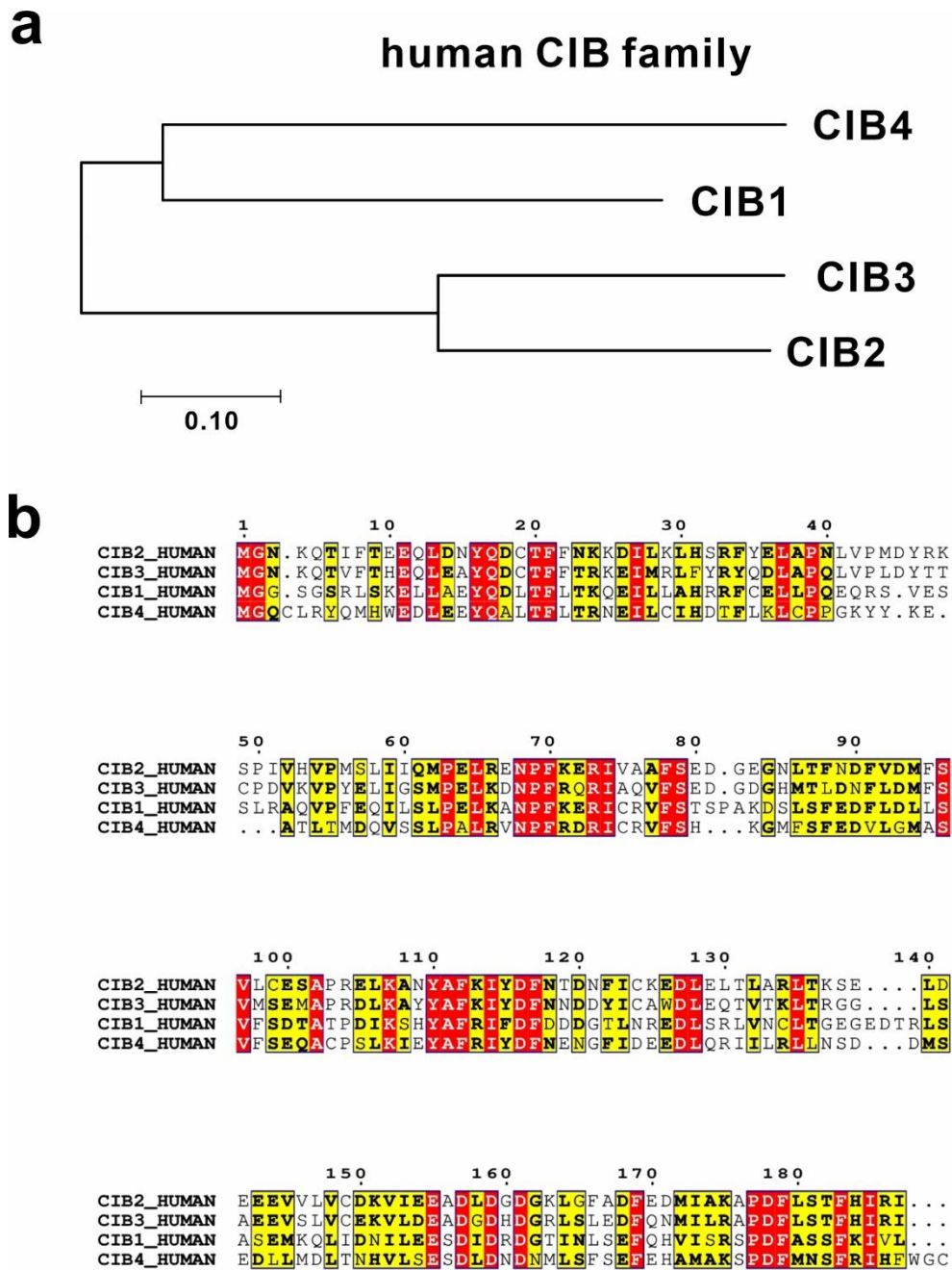
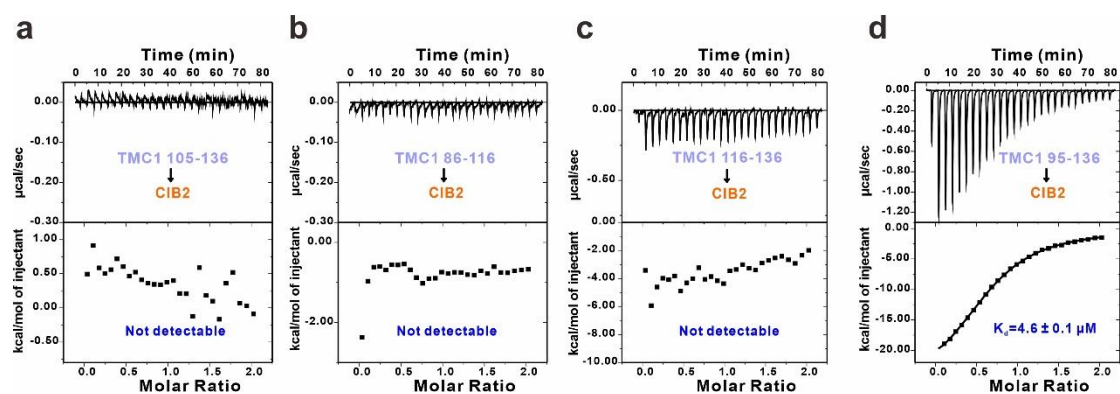




Supplementary Figure 1 CIB family protein evolution tree and conservation

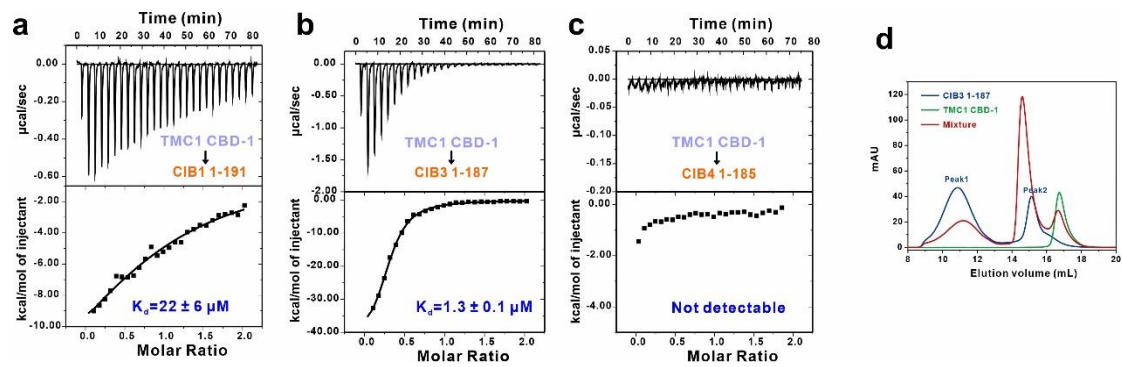
(a) Phylogenetic tree of the human CIB family protein. CIB3 is identified as the closest homolog to CIB2 compared to the larger evolutionary distance with CIB1/4.

(b) Multi-sequence alignment of human CIB family proteins. Residues that are identical and highly similar are indicated in red and yellow boxes, respectively.



Supplementary Figure 2 The binding region mapping between CIB2 and TMC1^{CBD-1}

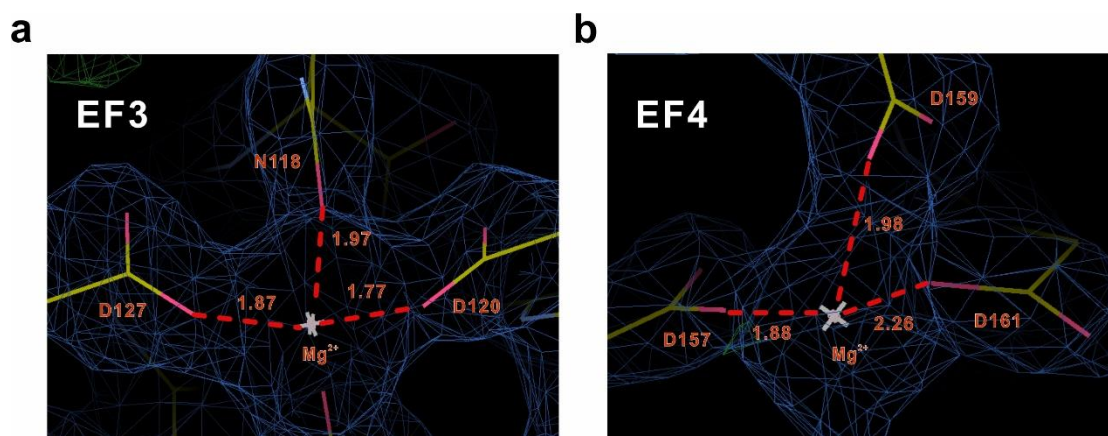
(a-d) ITC-based measurements of the binding affinities of CIB2 1-187 and various TMC1 truncations including 105-136 (a), 86-116 (b), 116-136 (c), and 95-136 (d).



Supplementary Figure 3 Both CIB2 and CIB3 bind to TMC1^{CBD-1}

(a-c) ITC-based measurements of the binding affinities of TMC1^{CBD-1} and CIB1 1-191 (a), CIB3 1-187 (b), and CIB4 1-185 (c).

(d) Size-exclusion chromatograms for CIB3, revealing monomer peak (peak2) and dimer peak (peak1). AGFC showed that CIB3 1-187 interacts with TMC1^{CBD-1}.

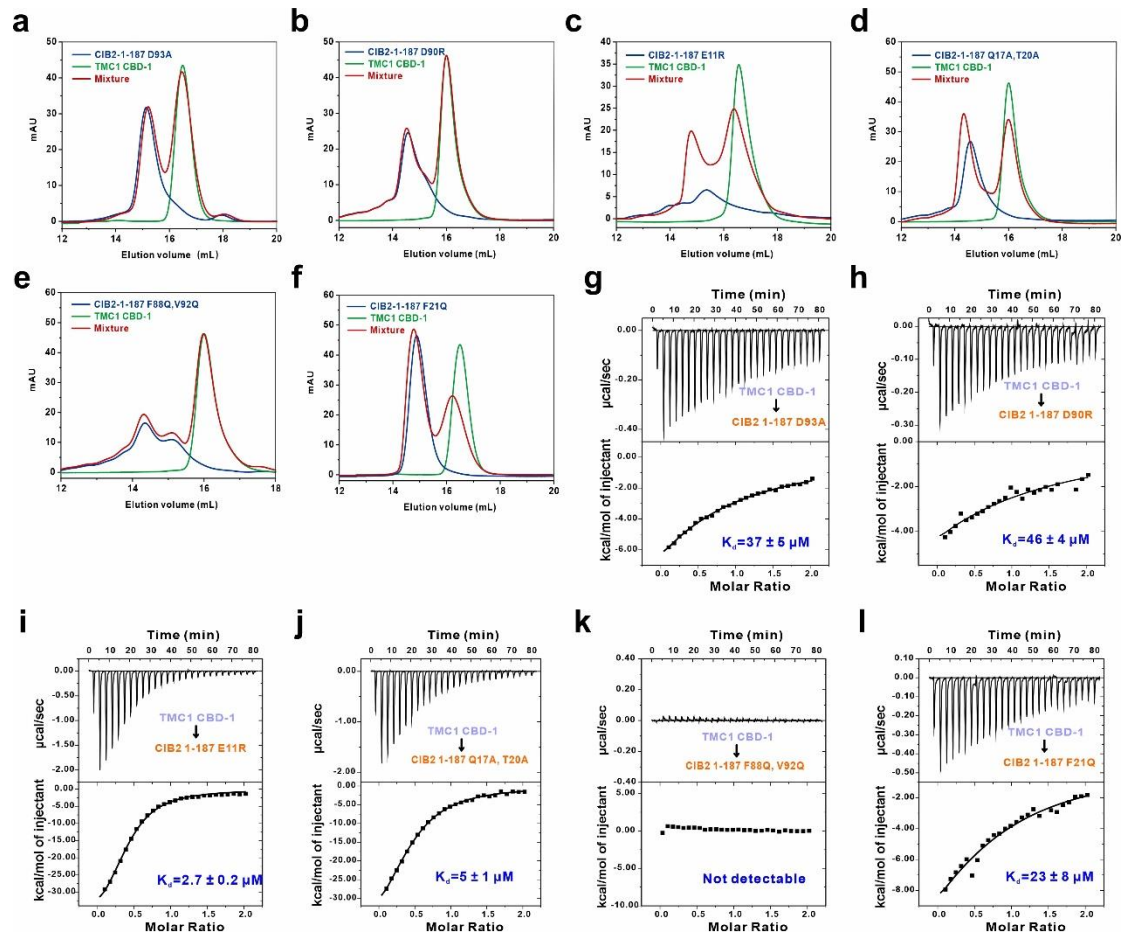


Supplementary Figure 4 Mg^{2+} is found in the complex structure of CIB2-TMC1

Residues and ion density map are displayed in blue and all residues shown as sticks. The central coordinates of magnesium ions are represented by gray cross markers, while the distances from carboxylate oxygen atoms to the central coordinates of the magnesium ions are depicted by red dashed lines.

(a) Magnesium ion within EF3. In EF3, the magnesium ion forms coordination bonds with the carboxylate oxygen atoms of N118, D120, and D127, with bond lengths of 1.97 Å, 1.77 Å, and 1.87 Å, respectively.

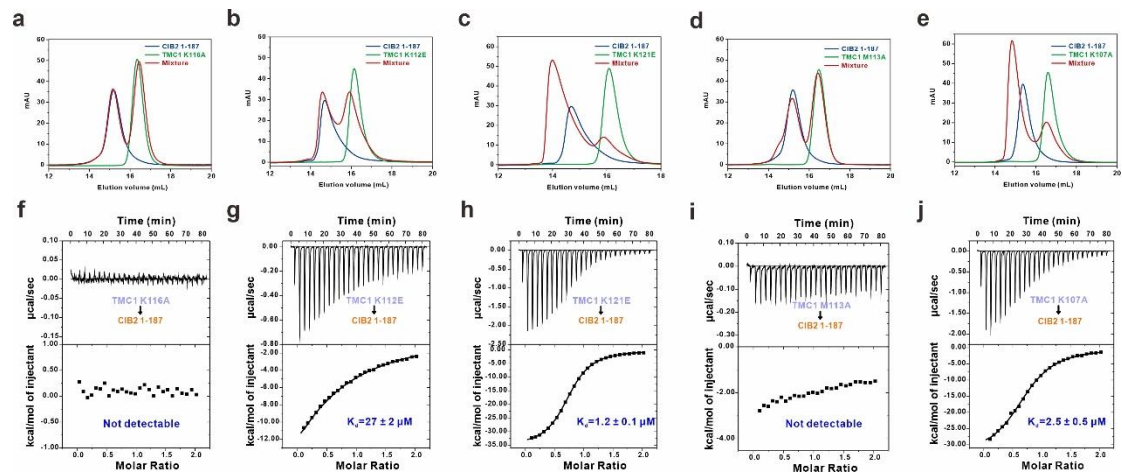
(b) Magnesium ion within EF4. In EF4, the magnesium ion forms coordination bonds with the carboxylate oxygen atoms of D157, D159, and D161, with bond lengths of 1.88 Å, 1.98 Å, and 2.26 Å, respectively.



Supplementary Figure 5 CIB2 mutations linked to interaction interface decrease the binding affinities with TMC1.

(a-f) Analytical gel filtration analysis showing the interactions between TMC1 CBD-1 and CIB2 1-187 D93A (a), D90R (b), E11R (c), Q17A/T20A (d), F88Q/V92Q (e), and F21Q (f).

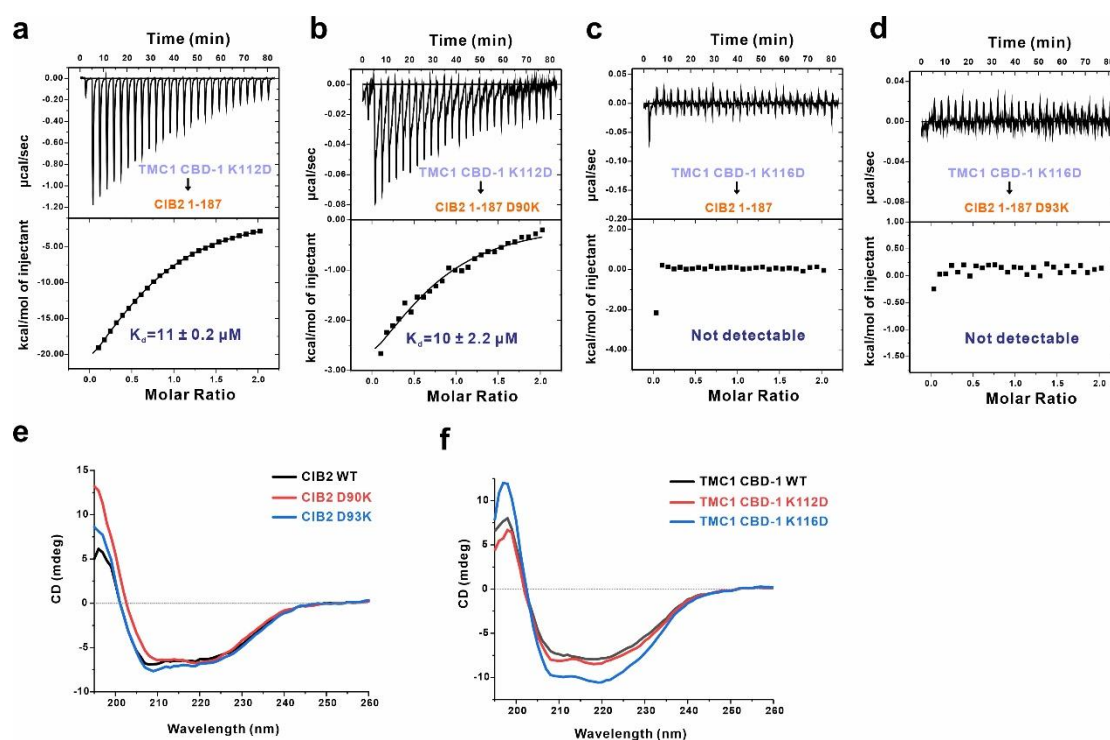
(g-l) ITC-based measurements of the binding affinities of TMC1 CBD-1 and various CIB2 1-187 variants including D93A (g), D90R (h), E11R (i), Q17A/T20A (j), F88Q/V92Q (k), and F21Q (l).



Supplementary Figure 6 TMC1 mutations linked to interaction interface decrease the binding affinities with CIB2

(a-e) Analytical gel filtration analysis showing the interaction of CIB2 1-187 and TMC1 CBD-1 K116A (a), K112E (b), K121E (c), M113A (d), and K107A (e).

(f-j) ITC-based measurements of the binding affinities of CIB2 1-187 and various TMC1 CBD-1 variants including K116A (f), K112E (g), K121E (h), M113A (i), and K107A (j).



Supplementary Figure 7 Reversed mutations of TMC1 and CIB2 cannot restore the bindings.

(a) ITC result of TMC1 CBD-1 K112D to CIB2 1-187. (b) ITC result of TMC1 CBD-1 K112D to CIB2 1-187 D90K. (c) ITC result of TMC1 CBD-1 K116D to CIB2 1-187. (d) ITC result of TMC1 CBD-1 K116D to CIB2 1-187 D93K. (e) CD spectroscopy profiles of WT CIB2 and its two mutants. (f) CD spectroscopy profiles of WT TMC1 and its two mutants.

	1	10	20	30
CIB2_HUMAN	MGN.....KQTIFTEEQLDNYQDCTFFNKKDILKLHSRFYELAP			
CIB2_MOUSE	MGN.....KQTIFTEEQLDNYQDCTFFNKKDILKLHARFYELAP			
CIB2_DOG	MGN.....KQTIFTEEQLDNYQDCTFFNKKDILKLHARFYELAP			
CIB2_ZEBRAFISH	MGN.....KQTIFTDEQLDAYQDCTFFTRKEILRLHGRFYELAP			
CIB2_FROG	MGN.....KQTIFTDEQLDAYQDCTFFTRKEILRLHGRFYELAP			
CIB2_DROSOPHILA	MGN.....KVVTFTEQELDDYQDCTFFTRKEILRVHKKRFRELRP			
CALM-1_C.elegans	MGNASSLSSELNLFSSKGGVFTREQLDEYQDCTFFTRKDIIRLYKRFYALNP			

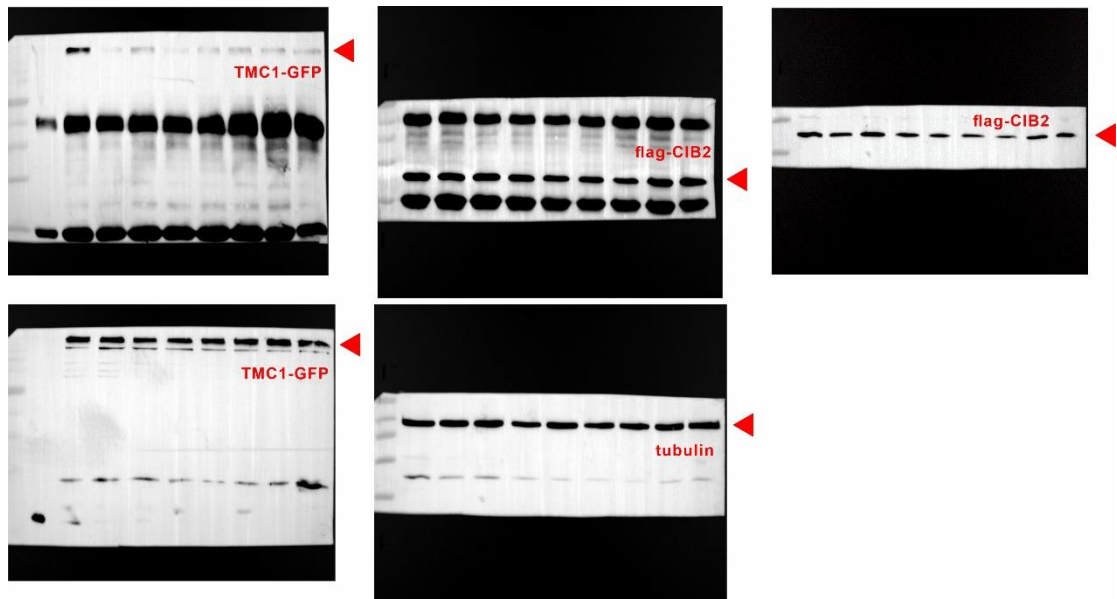
	40	50	60	70	80
CIB2_HUMAN	NLVLP..MDYRKSPIVHVPMSLIIQMPELRENPFKERIVEAFSEEDGEGNLTF				
CIB2_MOUSE	NLVLP..MDYRKSPIVHVPMSLIIQMPELRENPFKERIVEAFSEEDGEGNLTF				
CIB2_DOG	NLVLP..MDYRKSPIVHVPMSLIIQMPELRENPFKERIVEAFSEEDGEGNLTF				
CIB2_ZEBRAFISH	HLVLP..MDYTNDPDKVPLALIVNMPELKENPFRRNRIVESFSEDDGQGNLSF				
CIB2_FROG	NLVLP..MDYTDDPDKVPLMLIINMPELVENPFKERIVQSFSEDGEGNLSF				
CIB2_DROSOPHILA	DLVLPQMTGQASSVKVPCECIEKMPELRENPFRRRICEAFSRDQGNLSF				
CALM-1_C.elegans	HKVP.TNMQGNRPAITITLTFEVEKMPPELKENPFKRRICEVSEDDGRGNLSF				

	90	100	110	120	130
CIB2_HUMAN	NDFVDMFSVLCESAPRELKAN YAFKIYDFNTDNFICKEDLELTLARLTKSE				
CIB2_MOUSE	NDFVDMFSVLCESAPRELKAN YAFKIYDFNTDNFICKEDLEMTLARLTKSE				
CIB2_DOG	NDFVDMFSVLCESAPRELKAN YAFKIYDFNTDNFICKEDLERTLARLTKSE				
CIB2_ZEBRAFISH	NDFVDMFSVLSMAPRELKAIYAFKIYDFNVNYICKEDLEKTLNKLTKEE				
CIB2_FROG	NDFVDMFSVMSMAPRELKAIYAFKIYDFNTDNFICKSDLEKTLNKLTKREE				
CIB2_DROSOPHILA	EDFLDALSVFSQAPRDIKVYAFKIYDFDQDGFIGHADLMSCLTMTKNE				
CALM-1_C.elegans	DDFLDMFSVFSMAPLQLKLKYAFRIYDYDGDDELGHDDLSKMIRSLTRDE				

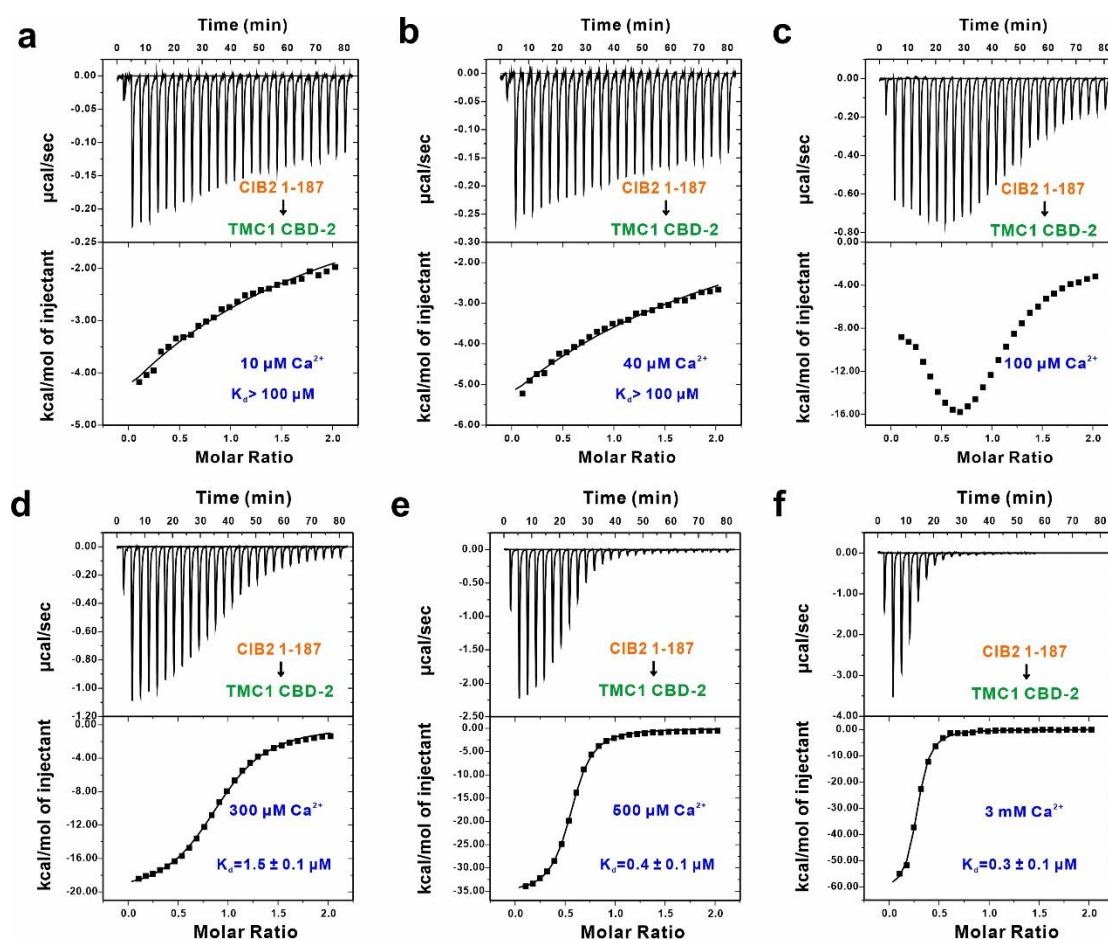
	140	150	160	170	180
CIB2_HUMAN	LDDEEVV LVCDKVIEEADLDGDGKLG FADFEDMIAKAPDFLS TFHIRI				
CIB2_MOUSE	LDDEEVV LVCDKVIEEADLDGDGKLG FADFEDMIAKAPDFLS TFHIRI				
CIB2_DOG	LDDEEVV LVCDKVIEEADLDGDGKLG FADFEDMIAKAPDFLS TFHIRI				
CIB2_ZEBRAFISH	LTPEEVN LVCEKAIIEEADLDGDNKLS FADFENMIS RAPDFLS TFHIRI				
CIB2_FROG	LDDEEVN LVCEKVIEEADM DGDGKLS FADFENMIS KAPDFLS TFHIRI				
CIB2_DROSOPHILA	LSPEEHQ QIADKVIEEADV DGDGKLS ILEFEHVIL RAPDFLS TFHIRI				
CALM-1_C.elegans	LSDVEVEFI IERIIEEADLDGDSSI NF AEF EHVVS RS PDI RTFHIRI				

Supplementary Figure 8 CIB2 family conservation across species.

Sequence alignment of CIB2 in different species showing a high conservation through evolution. Residues that are identical and highly similar are indicated in red and yellow boxes, respectively.

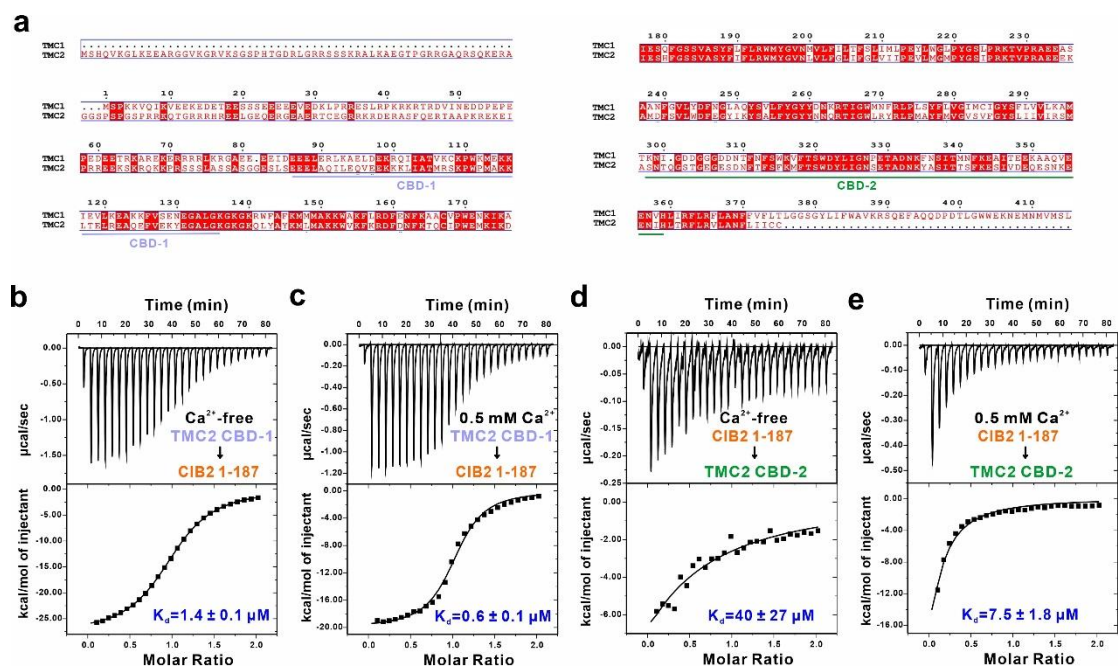


Supplementary Figure 9 Uncropped and unedited blot images present in Figure 4e.



Supplementary Figure 10 Calcium-dependent modulation of CIB2-TMC1 CBD-2 interactions.

(a-f) ITC experiments showing the CIB2-CBD2 interaction with various concentrations of Ca^{2+} , including 10 μM (a), 40 μM (b), 100 μM (c), 300 μM (d), 500 μM (e), and 3 mM (f).

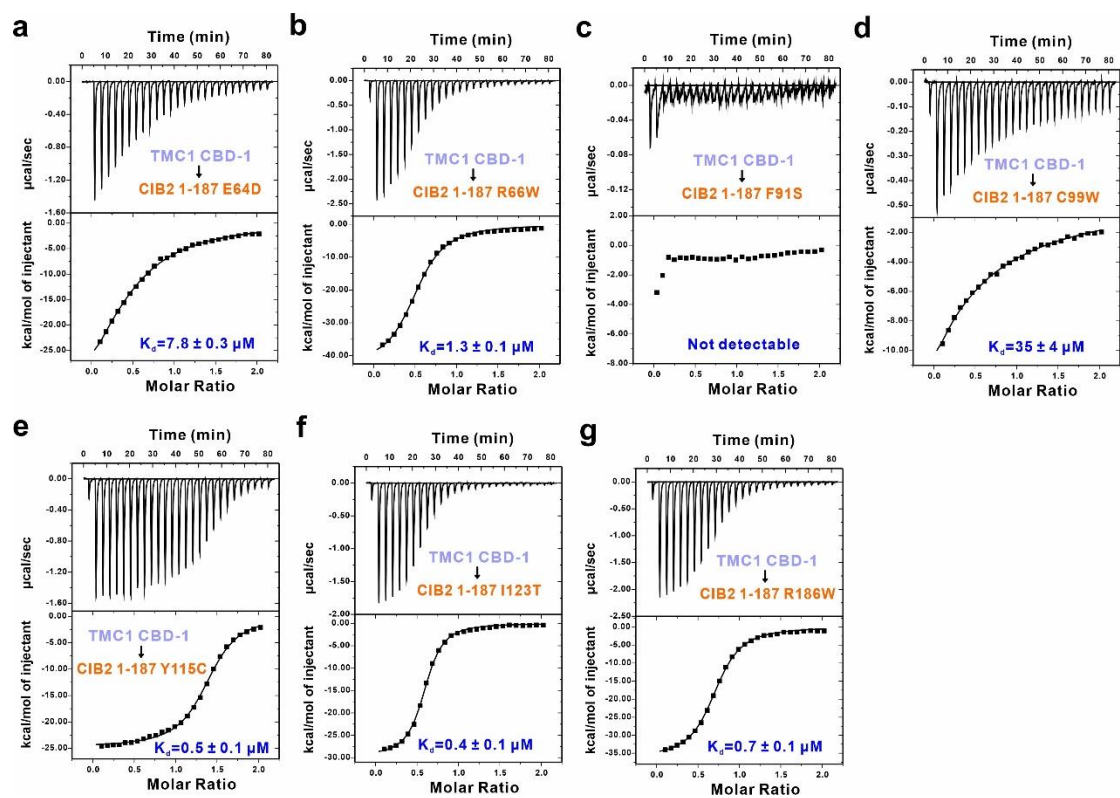


Supplementary Figure 11 Calcium-dependent modulation of CIB2-TMC2 interactions.

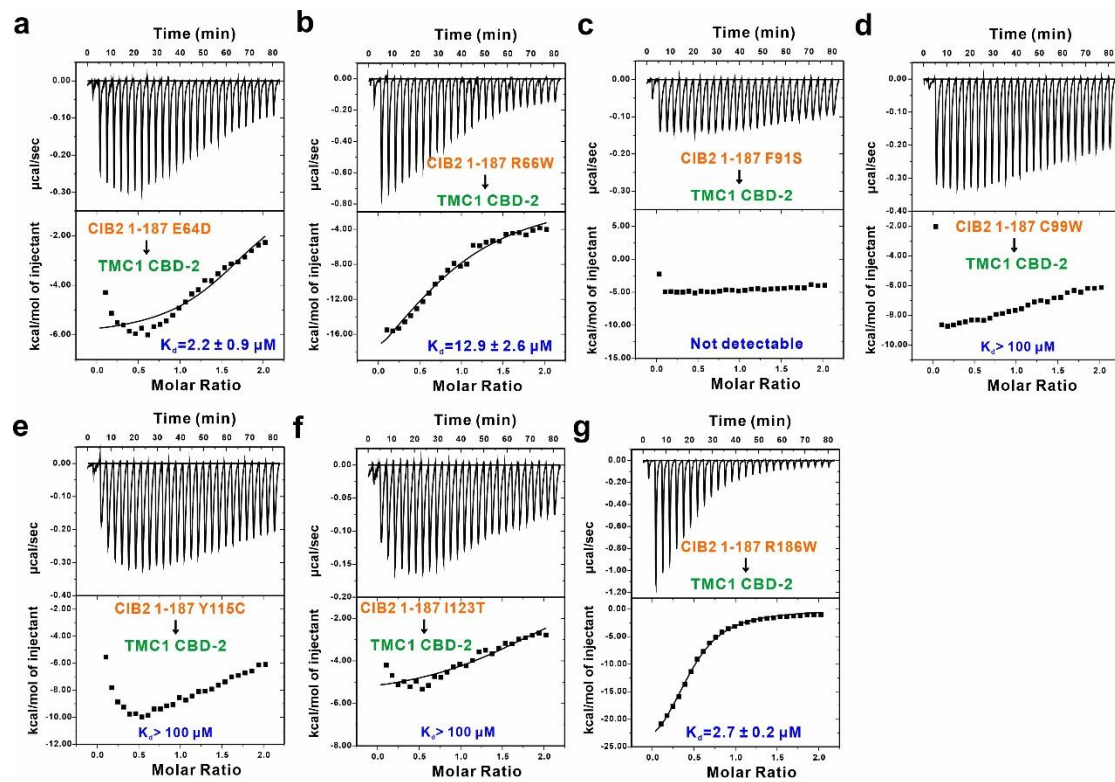
(a) Sequence alignment of human TMC1 and human TMC2. Residues that are identical and highly similar are indicated in red and transparent boxes, respectively. The location of TMC2 CBD1 (light blue line) and TMC2 CBD2 (green line) for CIB2 binding are indicated on the sequence alignment.

(b-c) ITC-based measurements of the binding affinities of CIB2-TMC2 CBD-1 in calcium free (b) and 0.5mM CaCl₂ (c).

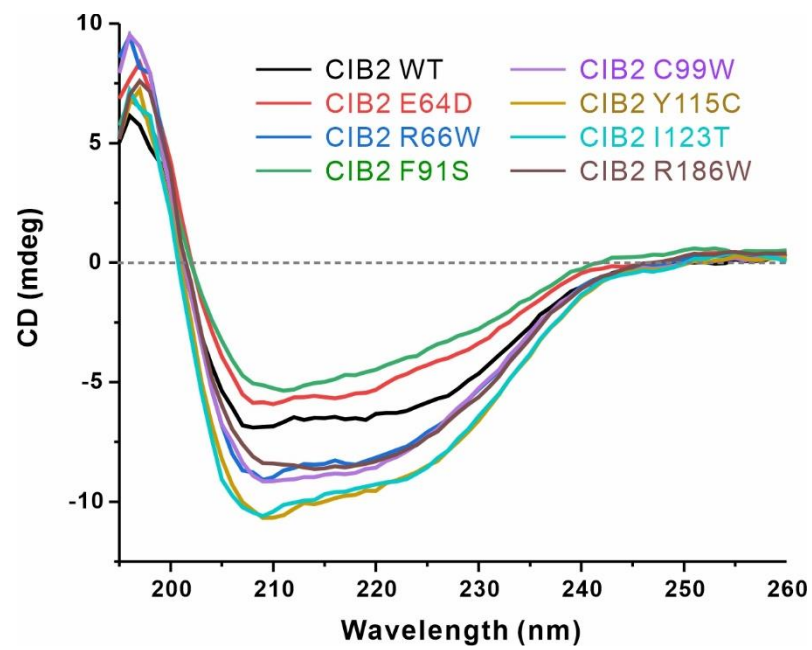
(d-e) ITC-based measurements of the binding affinities of CIB2-TMC2 CBD-2 in calcium free (d) and 0.5mM CaCl₂ (e).



Supplementary Figure 12 ITC-based measurements of the binding affinities of CBD-1 and various CIB2 deafness related mutants, including E64D (a), R66W (b), F91S (c), C99W (d), Y115C (e), I123T (f), and R186W (g).



Supplementary Figure 13 ITC-based measurements of the binding affinities of CBD-2 and various CIB2 deafness related mutants including, E64D (a), R66W (b), F91S (c), C99W (d), Y115C (e), I123T (f), and R186W (g).



Supplementary Figure 14 CD spectroscopy profiles of various CIB2 deafness related mutants.