Suppl. Fig. S1. **Cloning scheme for the generation of bacterial polycistronic TAK1 complex expression vectors.** *A,* cDNAs encoding for TAK1, TAB1 or TAB2 were individually subcloned into pOLY3.TEV and pOLY1 bacterial expression vectors, respectively. All vectors carry an ampicillin resistance gene (ampR). *B,* Following enzymatic restriction reaction, single cistrons from pTAB1 and pTAB2 were successively ligated into pHis-TAK1 using the compatible SpeI and XbaI as well as the BlpI restriction sites, which results in the tricistronic pHis-TAK1-TAB1-TAB2 construct. *C,* The resulting polycistronic gene consists of a promoter, ribosome-binding sites (RBS), the open reading frames of the according TAK1 complex proteins and a transcriptional terminator. Modified after Neumann et al., (2003) *Protein Expr Purif* **30**(2), 230-237.

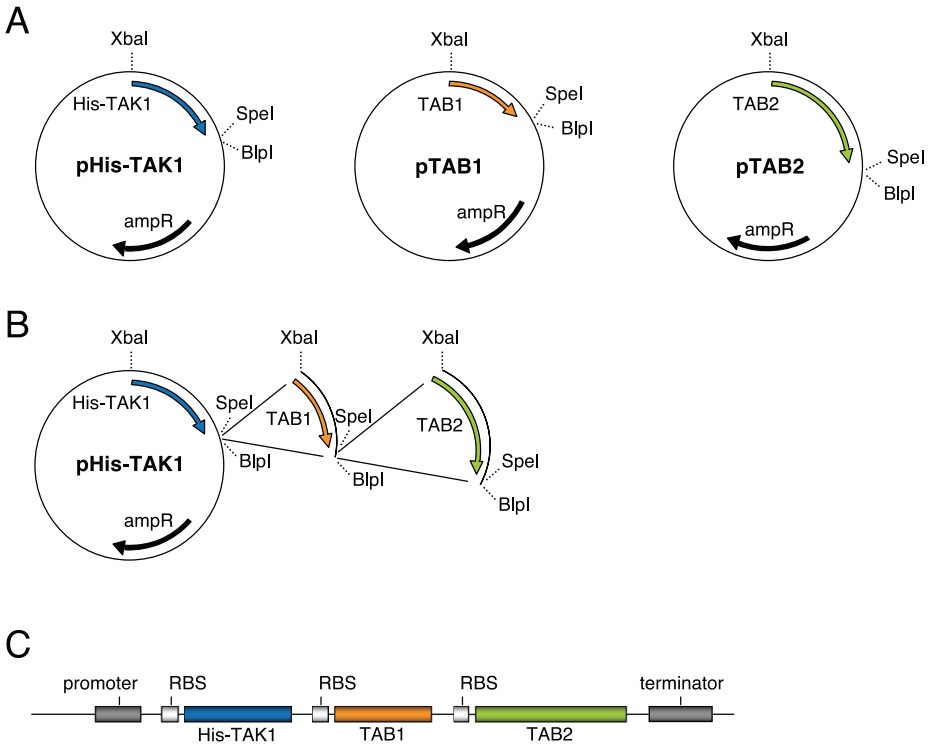
Suppl. Fig. S2. **Bacterial expression and Nickel affinity purification of TAK1 and TAK1 containing complexes.** *A,* Western blot analysis of different purification steps of bacterially expressed His-TAK1 using anti-Penta-His and anti-TAK1 antibodies. *B, C,* and D, as in *A* but describing the purification of bacterially expressed His-TAK1-TAB1, His-TAK1-TAB2 and His-TAK1-TAB1-TAB2, respectively. Additionally, anti-TAB1 and anti-TAB2 antibodies were used for Western blot analysis. Whole bacterial cell lysate (lysate, 2.5 µg/lane), pellet of insoluble protein after centrifugation of cell lysate (P, 2.5 µg/lane), corresponding supernatant containing all soluble proteins (SN, 2.5 µg/lane), flow through fraction (FT, 2.5 µg/lane), first washing step (W1, 2.5 µg/lane), second washing step (W2, 7.5 µl/ lane) and elution (Elu, 2.5 µg/lane).

Suppl. Fig. S3. **Overexpression of HA-TAK1 and FLAG-TAK1 in AMPKα subunit double knockout (α1-/-, α2-/-) MEFs.** AMPKα subunit double knockout (α1-/-, α2-/-) MEFs were transfected with plasmids encoding HA-TAK1 and FLAG- TAK1 and expression of respective proteins was confirmed by Western blot analysis of the total cell lysates.

Suppl. Fig. S4. **Recovery of intermolecular TAK1 αG-helix mutant phosphorylation by potential TAK1 rescue mutants.** *A,* Western blot analysis of bacterially expressed TAK1 (T178E, T184E) αG-helix mutants TAK1(A236K, M240K)-TAB1 (AM), TAK1(F237K, W241K)-TAB1 (FW), TAK1(W241K, V243K)-TAB1 (WV), and TAK1(I239K, V243K)-TAB1 (IV). Note, all αG-helix mutants additionally carried mutations replacing Thr178 and Thr184 by glutamate thus potentially mimicking phosphorylation of the corresponding activation segment sites. WT-TAK1-TAB1 and KW-TAK1-TAB1 served as a control. Western blotting utilized anti-TAK1, anti-TAB1 and phospho-specific anti-TAK1 Thr184/Thr187 (α-P184/187) antibodies. *B,* upper left panel, TAK1(A236E, M240E)-TAB1 (AEME) was incubated with TAK1(T178E, T184, A236K, M240K)-TAB1 for 20 min at 30 °C to analyze the potential of restoring activation segment phosphorylation by electrostatic attraction of corresponding αG-helix residues. 150 µg/ml of each TAK1 complex was applied. In lane 5, 75 µg/ml of each TAK1 complex was used resulting to a total of 150 µg/ml in the assay mix. Other panels, incubation of TAK1(F237E, W241E)-TAB1 (FEWE) with TAK1(T178E, T184E, F237K, W241K)-TAB1, TAK1(W241E, V243E)-TAB1 (WEVE) with TAK1(T178E, T184E, W241K, V243K)-TAB1 and TAK1(I239E, V243E)-TAB1 (IEVE) with TAK1(T178E, T184E, I239K, V243K)-TAB1, respectively.

Suppl. Fig. S5. **αG-helix-mediated crystal contacts between symmetry-related monomers of the TAK1 kinase domain.** *Upper panel:* Two symmetry-related monomers A (dark grey) and B (light grey) of human TAK1 kinase domain (Protein Data Bank code 2eva). The αG-helices (residues 235 to 245) are highlighted in green and blue, respectively. *Lower panel:* Magnified front and top view of crystal contact site between both monomers A and B involving hydrophobic TAK1 kinase domain αG-helix residues Phe-237, Met-240 and Trp-241 (residues shown: 231 to 248).

Suppl. Fig. S1


A
XbaI
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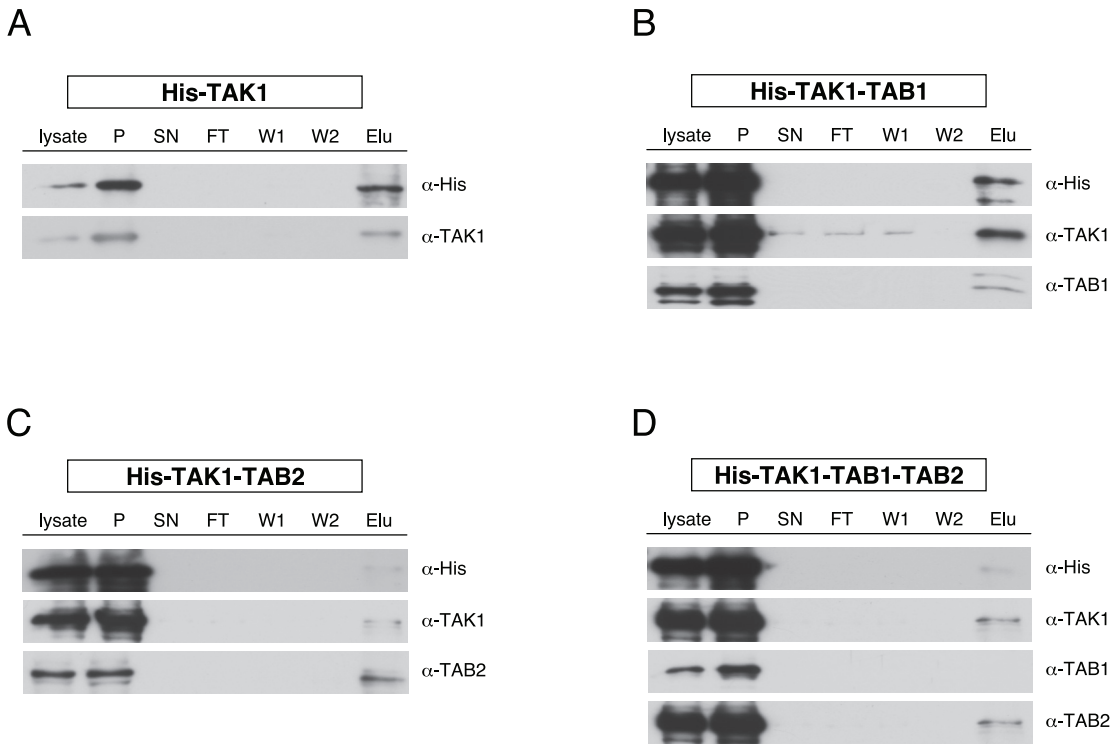
SpeI
BlpI
pHis-TAK1
ampR
XbaI
TAB1
SpeI
BlpI
pTAB1
ampR
XbaI
TAB2
SpeI
BlpI
pTAB2
ampR
B
XbaI
His-TAK1
SpeI
BlpI
pHis-TAK1
ampR
XbaI
TAB1
SpeI
BlpI
XbaI
TAB2
SpeI
BlpI
C
promoter
RBS
His-TAK1
RBS
TAB1
RBS
TAB2
terminator

Suppl. Fig. S2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α-His
α-TAK1
B
His-TAK1-TAB1
lysate P SN FT W1 W2 Elu
α-His
α-TAK1
α-TAB1
C
His-TAK1-TAB2
lysate P SN FT W1 W2 Elu
α-His
α-TAK1
α-TAB2
D
His-TAK1-TAB1-TAB2
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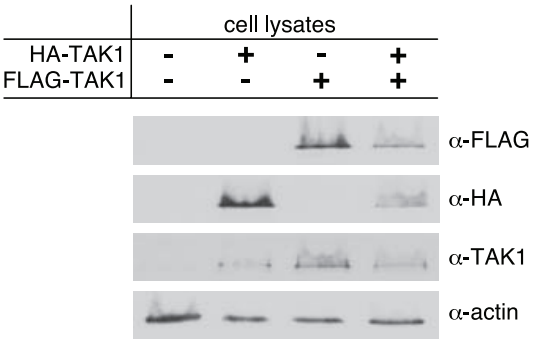
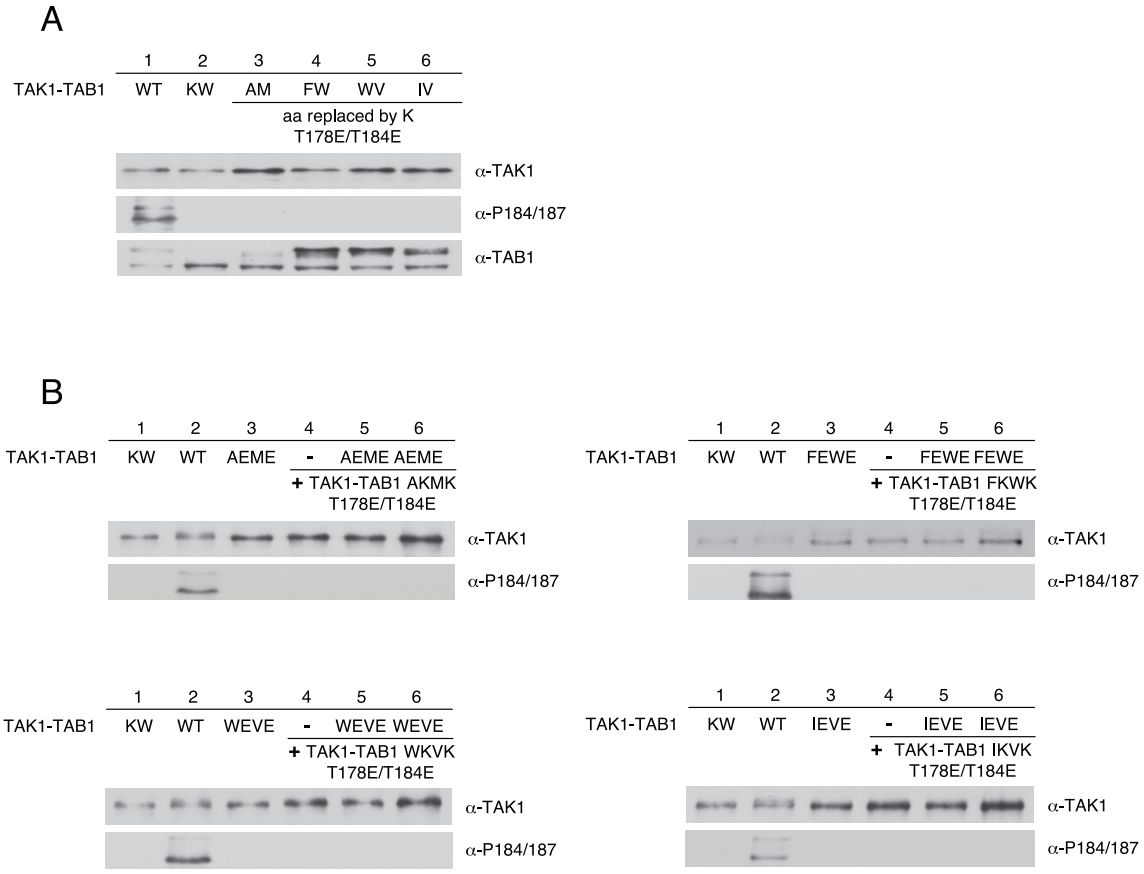
α-His
α-TAK1
α-TAB1
α-TAB2

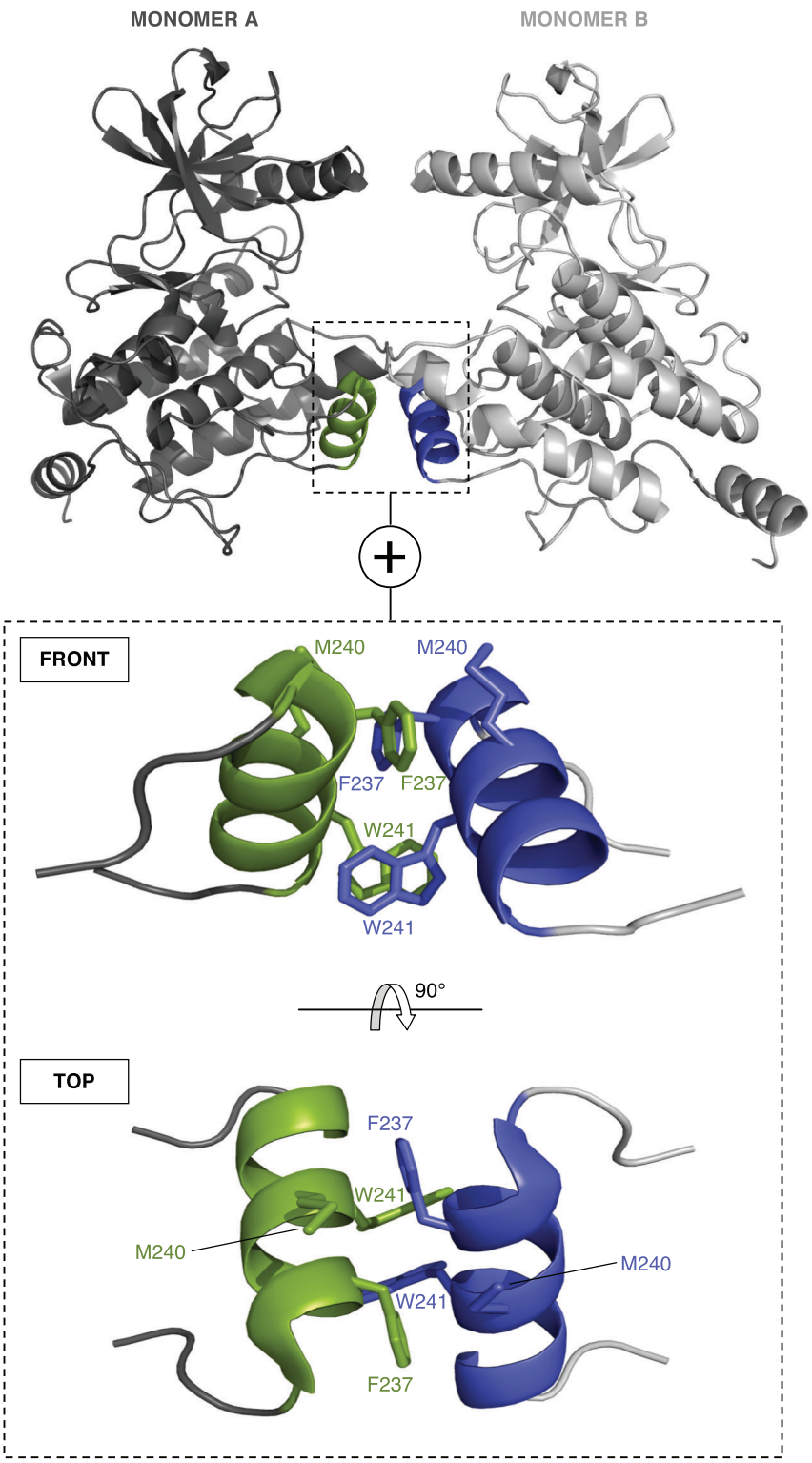
Suppl. Fig. S3



Suppl. Fig. S4


A
1 2 3 4 5 6
TAK1-TAB1 WT KW AM FW WV IV
aa replaced by K
T178E/T184E
α-TAK1
α-P184/187
α-TAB1
B
1 2 3 4 5 6
TAK1-TAB1 KW WT AEME - AEME AEME
+ TAK1-TAB1 AKMK
T178E/T184E
α-TAK1
α-P184/187
1 2 3 4 5 6
TAK1-TAB1 KW WT FEWE - FEWE FEWE
+ TAK1-TAB1 FKWK
T178E/T184E
α-TAK1
α-P184/187
1 2 3 4 5 6
TAK1-TAB1 KW WT WEVE - WEVE WEVE
+ TAK1-TAB1 WKVK
T178E/T184E
α-TAK1
α-P184/187
1 2 3 4 5 6
TAK1-TAB1 KW WT IEVE - IEVE IEVE
+ TAK1-TAB1 IKVK
T178E/T184E
α-TAK1
α-P184/187

Suppl. Fig. S5


MONOMER A
MONOMER B
FRONT
M240
M240
F237
F237
W241
W241
90°
TOP
F237
W241
M240
M240
W241
F237