SUPPORTING INFORMATION

Text S1: EvoDesign profile construction

Table S1 lists the pair-wise structural alignments generated by the TM-align program [1], between the XIAP scaffold protein (PDB ID: 2OPY) and the 10 non-homologous templates that has a TM-score higher than 0.5 and sequence identity below 80% to the scaffold. These alignments have been used to construct the multiple structural alignment matrix shown in Figure S1A and the profile to guide the EvoDesign simulation search. The pair-wise sequence alignments created by NW-align [2], a program implementing the standard Needleman-Wunsch dynamic programming algorithm [3], are listed in Table S2 as a control.

The average sequence identities in the alignments by TM-align and NW-align are 32.7% and 37.1%, respectively. Despite the lower sequence identity, the structure-based alignments have a slightly lower number of internal gaps than that by the sequence-based alignments. The average number of internal gaps and insertions with size ≥2 is 2.1 and 3.1, respectively, in the alignments by TM-align and NW-align. The difference in the structure and sequence-based alignments are mainly located outside the binding sites regions, where in the binding-site involved regions these two alignments are almost identical (see Figures S1A and S1B).

Text S2: Sequence sample preparation and protein purification

DNA and protein sequences preparations. Dynamic-Interface XIAP (DI-XIAP) and Fixed-Interface XIAP (FI-XIAP) sequences were optimized based on frequent codon usage in *E. coli* (K-12 strain) and listed in Table S2, where ligation independent cloning (LIC) handles are in bold. Protein sequence length is 101 residues and based on residues 250-350 from the 2OPZ X-ray crystal structure. The N-terminal cloning residues “SNA” (lower case in sequence) remain after rTEV protease cleavage during purification extending the length of the purified proteins to 104 amino acids. The XIAP/caspase-9 complex (PDB ID: 1NW9) which has a length of 91 residues (from 256 to 346), was used as the scaffold for conservation score analysis of the interface residues.

Protein expression and purification. Native XIAP and design constructs were transformed into a Rosetta 2 *E. coli* expression cell strain (EMDmillipore). Cells were grown in LB media with ampicillin at 0.1 g/L at 310 K until mid-log phase. At a cell density of 0.6 OD (600 nm wavelength), protein expression was induced by the addition of 0.2 mM IPTG for 4 hours at 303 K. Cells were harvested by centrifugation and frozen. Unless noted all temperatures were at 277 K during purification and biophysical characterization. The cells were thawed and resuspended in 50 mM Tris pH 7.5, 150 mM NaCl, 5 mM imidazole, lysed by sonication (Fisher model 705 series), and the sample was subsequently centrifuged (30,000 g x 30 min Beckman J26-XP (JA25.50 rotor)) to pellet cell debris. The remaining supernatant was mixed with Ni-NTA resin (Qiagen) via batch binding, and washed with 30 column volumes of resuspension buffer. The protein was subsequently eluted with resuspension buffer plus 200 mM imidazole, and concentrated using a 3K MWCO concentrator (Pall). The protein sample was dialized in 50 mM Tris pH 7.5 150 mM NaCl 0.2 mM DTT and the N-terminal affinity tag was removed by rTEV protease digestion. A final purification polishing step by size-exclusion gel filtration using a (GE) AKTA chromatographic work station and an S-100 column, yielded a single elution species at a retention time indicative of a monomer for each design protein. This is in contrast to the native protein, a known dimer (via a C-terminal intermolecular disulfide bridge, this is not required for function regarding peptide-XIAP interactions), which eluted at a retention time corresponding to a dimer (Figure S7).

References

[1] Y. Zhang, J. Skolnick, TM-align: a protein structure alignment algorithm based on the TM-score, Nucleic Acids Res. 33 (2005) 2302-2309.

[2] R. Yan, D. Xu, J. Yang, S. Walker, Y. Zhang, A comparative assessment and analysis of 20 representative sequence alignment methods for protein structure prediction, Sci. Rep. 3 (2013) 2619.

[3] S.B. Needleman, C.D. Wunsch, A general method applicable to the search for similarities in the amino acid sequence of two proteins, J. Mol. Biol. 48 (1970) 443-453.

Table S1. Pair-wise structure and sequence alignment between templates and XIAP-WT protein. ":" indicates the residue pairs with a distance <5 Å in the structural alignments or the pair of same amino acid identity in the sequence alignments. "." indicates the pairs aligned but with a distance >5 Å in the structural alignments.

Template ID: 1C9Q

```
Structural alignment, sequence identity=31.7%, TM-score=0.73
1C9Q: RDHFALDRPSETHADYLLRTGQVVDISDTI-YPRNPAMYSEEARLKSFQNWPDYAHLTPRELASAGLYYTGIGDQVQCFACGGKLKNWEPGDRAWSEHRR
                              : :::: :::::::::::::::::::::  ::::::::::::::::::::::::::::::::::::::::::::::
2OPY: ------------------------F-PNSTNLPRNPSMADYEARIFTFGTWI--YSVNKEQLARAGFYALGEGDKVKCFHCGGGLTDWKPSEDPWEQHAK

1C9Q: HFPNCFFVLGRNLNIRSE----------------
      :::::::::::.
2OPY: WYPGCKYLLEQK------GQEYINNIHLTHSLEE

Sequence alignment, sequence identity=35.6%
1C9Q: -RDHFALDRPSETHADYLLRTGQVVDISDTIYPRNPAMYSEEARLKSFQNWPDYAHLTPRELASAGLYYTGIGDQVQCFACGGKLKNWEPGDRAWSEHRR
               :  :                   :::: :   :::   :  :          :: :: :  : :: : :: ::: :  : :    :  :
2OPY: F--------PNSTNL-----------------PRNPSMADYEARIFTFGTW--IYSVNKEQLARAGFYALGEGDKVKCFHCGGGLTDWKPSEDPWEQHAK

1C9Q: HFPNCFFVLGR-------NLNIR---SE
        : :   :         :        :
2OPY: WYPGCKYLLEQKGQEYINNIHLTHSLEE
```

Template ID: 1E31

```
Structural alignment, sequence identity=24.7%, TM-score=0.66
1E31: TLPP---------AWQPFLKDHRISTFKNWPFLEGCACTPERMAEAGFIHCPTENEPDLAQCFFCFKELEGWEPDDDPIEEHKKHSSGCAFLSVKKQFEE
                   .::::::::::::::::::   . ::::::::::::::::   :::::::::::::::::::::::::::::::::::::::
2OPY: ----FPNSTNLPRNPSMADYEARIFTFGTWIY---S-VNKEQLARAGFYALGE---GDKVKCFHCGGGLTDWKPSEDPWEQHAKWYPGCKYLLEQ-----

1E31: LTLGEFLKLDRERAKNKIAKETNNKKKEFEETAKKVRRAIEQLAAMD-----------------

2OPY: -----------------------------------------------KGQEYINNIHLTHSLEE

Sequence alignment, sequence identity=34.7%
1E31: -----TLPPAWQPFLKDH--RISTFKNWPFLEGCACTPERMAEAGFIHCPTENEPDLAQCFFCFKELEGWEPDDDPIEEHKKHSSGCAFLSVKKQFEELT
            ::    :   :   :: ::  :          :  : :::       : :   :: :   :  : :  :: : : :   ::  :   :  :
2OPY: FPNSTNLPR--NPSMADYEARIFTFGTWIY----SVNKEQLARAGFYAL---GEGDKVKCFHCGGGLTDWKPSEDPWEQHAKWYPGCKYLLEQKGQEYI-

1E31: LGEFLKLDRERAKNKIAKETNNKKKEFEETAKKVRRAIEQLAAMD
                   : :                      :
2OPY: -------------NNI----------------HLTHSLEE-----
```

Template ID: 1JD5

```
Structural alignment, sequence identity=42.6%, TM-score=0.89
1JD5: -GN--YFPQYPEYAIETARLRTFEAWPRNLKQKPHQLAEAGFFYTGVGDRVRCFSCGGGLMDWNDNDEPWEQHALWLSQCRFVKLMKGQLYIDTVAAKPV
       .:  ::::::::::::::::::::::  ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
2OPY: FPNSTNLPRNPSMADYEARIFTFGTWI--YSVNKEQLARAGFYALGEGDKVKCFHCGGGLTDWKPSEDPWEQHAKWYPGCKYLLEQKGQEYINNIHLTH-

1JD5: LAEEKEES
      ...  .
2OPY: SLE--E--

Sequence alignment, sequence identity=41.6%
1JD5: ---GNYFPQYPEYAIETARLRTFEAWPRNLKQKPHQLAEAGFFYTGVGDRVRCFSCGGGLMDWNDNDEPWEQHALWLSQCRFVKLMKGQLYIDTVAAKPV
             :  :  :   ::  ::  :         ::: :::   : :: : :: ::::: ::     :::::: :   :      ::: ::
2OPY: FPNSTNLPRNPSMADYEARIFTFGTWIYSVNKE--QLARAGFYALGEGDKVKCFHCGGGLTDWKPSEDPWEQHAKWYPGCKYLLEQKGQEYINNIHLTHS

1JD5: LAEEKEES
      :     :
2OPY: L-----EE
```

Template ID: 1OXQ

```
Structural alignment, sequence identity=41.6%, TM-score=0.89
1OXQ: AG-ATLSRGPAFPGMGSEELRLASFYDWPLTAEVPPELLAAAGFFHTGHQDKVRCFFCYGGLQSWKRGDDPWTEHAKWFPSCQFLLRSKGRDFVHSVQET
         ...:::::::::::::::::::::::  :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
2OPY: --FPNSTNLPRNPSMADYEARIFTFGTWI--YSVNKEQLARAGFYALGEGDKVKCFHCGGGLTDWKPSEDPWEQHAKWYPGCKYLLEQKGQEYINNIHLT

1OXQ: HS---
      ::
2OPY: HSLEE
```

Sequence alignment, sequence identity=41.6%

```
1OXQ: AGATLSRGPAFPGMGSEELRLASFYDWPLTAEVPPELLAAAGFFHTGHQDKVRCFFCYGGLQSWKRGDDPWTEHAKWFPSCQFLLRSKGRDFVHSVQETH
              :  : :   : :   :  :     :  : :: :::   :  ::: :: : :::  ::   :::  :::: : :  ::  ::         ::
2OPY: FPNSTNL-PRNPSMADYEARIFTFGTWIYS--VNKEQLARAGFYALGEGDKVKCFHCGGGLTDWKPSEDPWEQHAKWYPGCKYLLEQKGQEYINNIHLTH

1OXQ: S---
      :
2OPY: SLEE
```

Template ID: 1QBH

Structural alignment, sequence identity=35.7%, TM-score=0.60

```
1QBH: ------GS--HMQ-TH-AARMRTFMYWPSSVPVQPEQLASAGFYYVGRND---DVKCFCCDGGLRCWESGDDPWVEHAKWFPRCEFLIRMKGQEFVDEIQ
            ::  ::: .  ..:..::::  :::::::::::::::   ::::   :::::::::::::::::::::::::::::::::::::::::::::::
2OPY: FPNSTNLPRNPSMAD-YEARIFTFGT--WIYSVNKEQLARAGF---YALGEGDKVKCFHCGGGLTDWKPSEDPWEQHAKWYPGCKYLLEQKGQEYINNIH

1QBH: GRYPHLLEQLLSTS--
      ::::.
2OPY: LTHSL---------EE
```

Sequence alignment, sequence identity=44.6%

```
1QBH: ---------GSHMQTHAARMRTFMYWPSSVPVQPEQLASAGFYYVGRNDDVKCFCCDGGLRCWESGDDPWVEHAKWFPRCEFLIRMKGQEFVDEIQGRYP
                  :    ::  ::  :  ::    :::: ::::  :  : :::: : :::  :    :::  :::: : :  :   ::::    :
2OPY: FPNSTNLPRNPSMADYEARIFTFGTWIYSV--NKEQLARAGFYALGEGDKVKCFHCGGGLTDWKPSEDPWEQHAKWYPGCKYLLEQKGQEYINNIH--LT

1QBH: HLLEQLLSTS
      : ::
2OPY: HSLEE-----
```

Template ID: 1SE0

Structural alignment, sequence identity=26.8%, TM-score=0.70

```
1SE0: ----------NDLNREETRLKTFTDWPLDWLDKRQLAQTGMYFTHAGDKVKCFFCGVEIGSWEQEDQPVPEHQRWSPNCPLLRRRTTNNVPINAEALDRI
                .::::::::::::::::: :::::::::::::::::::::::::::::::::::::::::::::::::::::::
2OPY: FPNSTNLPRNPSMADYEARIFTFGTWIY-SVNKEQLARAGFYALGEGDKVKCFHCGGGLTDWKPSEDPWEQHAKWYPGCKYLLE----------------

1SE0: LPPIS-YD----------
      ::::: .:
2OPY: QKGQEYINNIHLTHSLEE
```

Sequence alignment, sequence identity=32.7%

```
1SE0: --NDLNRE--------ETRLKTFTDWPLDWLDKRQLAQTGMYFTHAGDKVKCFFCGVEIGSWEQEDQPVPEHQRWSPNCPLLRRRTTNNVPINAEALDRI
        :  :          : :  ::  :      : :::  : :    ::::::: ::     :     :   :  : : :  :         ::   :
2OPY: FPNSTNLPRNPSMADYEARIFTFGTWIYS-VNKEQLARAGFYALGEGDKVKCFHCGGGLTDWKPSEDPWEQHAKWYPGCKYLLEQKGQEY-INNIHLTHS

1SE0: LPPISYD
      :
2OPY: LE----E
```

Template ID: 2QRA

Structural alignment, sequence identity=22.8%, TM-score=0.67

```
2QRA: ---------EEEFVEEFNRLKTFANFPSGSPVSASTLARAGFLYTGEGDTVRCFSCHAAVDRWQYGDSAVGRHRKVSPNCRFINGFYLE-----------
               ..::::::::::::::: : ::::::::::::::::::::::::::::::::::::::::::::::::::::::::.
2OPY: FPNSTNLPRNPSMADYEARIFTFGTW-I-YSVNKEQLARAGFYALGEGDKVKCFHCGGGLTDWKPSEDPWEQHAKWYPGCKYLLEQ---KGQEYINNIHL

2QRA: ------

2OPY: THSLEE
```

Sequence alignment, sequence identity=26.7%

```
2QRA: ---------EEEFVEEFNRLKTFANFPSGSPVSASTLARAGFLYTGEGDTVRCFSCHAAVDRWQYGDSAVGRHRKVSPNCRF---------INGFYL---
                        :  ::        :    ::::::   :::: : :: :      :         : :  : :           ::   :
2OPY: FPNSTNLPRNPSMADYEARIFTFGTWIYS--VNKEQLARAGFYALGEGDKVKCFHCGGGLTDWKPSEDPWEQHAKWYPGCKYLLEQKGQEYINNIHLTHS

2QRA: --E
        :
2OPY: LEE
```

Template ID: 2VM5

Structural alignment, sequence identity=29.7%, TM-score=0.71

```
2VM5: SMRVKNLKS--------RLRMRYQEEEARLASFRNWPFYVQGISPCVLSEAGFVFTGKQDTVQCFSCGGCLGNWEEGDDPWKEHAKWFPKCEFLRSK-KS
                       .. :::::::::::::::: : : :::::::::::::::::::::::::::::::::::::::::::::::::::::::: :.
2OPY: ---------FPNSTNLPRN-PSMADYEARIFTFGTW-I-Y-SVNKEQLARAGFYALGEGDKVKCFHCGGGLTDWKPSEDPWEQHAKWYPGCKYLLEQKGQ

2VM5: SEEITQYIQSYK--------------

2OPY: ------------EYINNIHLTHSLEE
```

Sequence alignment, sequence identity=34.7%

```
2VM5: SMRVKNLKSRLRMRYQEEEARLASFRNWPFYVQGISPCVLSEAGFVFTGKQDTVQCFSCGGCLGNWEEGDDPWKEHAKWFPKCEFLRSKKSSEEITQYIQ
           ::     :     :::   :  :           :  :::   :  : : :: ::: :  :    :::  :::: : :   :   :  : :
2OPY: FPNSTNLPRNPSMA--DYEARIFTFGTW---IYSVNKEQLARAGFYALGEGDKVKCFHCGGGLTDWKPSEDPWEQHAKWYPGCKYLLEQKGQEYINNIHL

2VM5: SY---K

2OPY: THSLEE
```

Template ID: 3M0A

Structural alignment, sequence identity=29.7%, TM-score=0.65

```
3M0A: ML-------------SCELYRMSTYSTFPAGVPVSERSLARAGFYYTGVNDKVKCFCCGLMLDNWKRGDSPTEKHKKLYPSCRFVQ-S------------
                     .:::::::::::: : ::::::::::::::::::::::::::::::::::::::::::::::::::::::: :
2OPY: --FPNSTNLPRNPSMADYEARIFTFGTW-I-YSVNKEQLARAGFYALGEGDKVKCFHCGGGLTDWKPSEDPWEQHAKWYPGCKYLLEQKGQEYINNIHLT

3M0A: -----

2OPY: HSLEE
```

Sequence alignment, sequence identity=30.7%

```
3M0A: ML-SCELYRMSTYSTFPAGV--------PVSERSLARAGFYYTGVNDKVKCFCCGLMLDNWKRGDSPTEKHKKLYPSCRFV------------------Q
         :  : :        :           :    ::::::::  :  :::::: ::  :  ::    : : : : :: :
2OPY: FPNSTNLPRNPSMADYEARIFTFGTWIYSVNKEQLARAGFYALGEGDKVKCFHCGGGLTDWKPSEDPWEQHAKWYPGCKYLLEQKGQEYINNIHLTHSLE

3M0A: S

2OPY: E
```

Template ID: 3T6P

Structural alignment, sequence identity=41.6%, TM-score=0.84

```
3T6P: ----------GSMQTHAARMRTFMYWPSSVPVQPEQLASAGFYYVGRNDDVKCFSCDGGLRCWESGDDPWVEHAKWFPRCEFLIRMKGQEFVDEIQGRYP
                :::::::::::::::::  :::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
2OPY: FPNSTNLPRNPSMADYEARIFTFGTWI--YSVNKEQLARAGFYALGEGDKVKCFHCGGGLTDWKPSEDPWEQHAKWYPGCKYLLEQKGQEYINNIHLTHS

3T6P: HLLEQLLSTEDAVMMNTPVVKSALEMGFNRDLVKQTVQSKILTTGENYKTVNDIVSALLNAEDEKREEEKEKQAEEMASDDLSLIRKNRMALFQQLTCVL
      .
2OPY: LEE-------------------------------------------------------------------------------------------------
3T6P: PILDNLLKANVINKQEHDIIKQKTQIPLQARELIDTILVKGNAAANIFKNSLKEIDSTLYKNLFVDKNMKYIPTEDVSGLSLEEQLRRLQEERTCKVCMD

2OPY: ----------------------------------------------------------------------------------------------------

3T6P: KEVSVVFIPCGHLVVCQECAPSLRKCPICRGIIKGTVRTF

2OPY: ----------------------------------------
```

Sequence alignment, sequence identity=48.5%

```
3T6P: -----------GSMQTHAARMRTFMYWPSSVPVQPEQLASAGFYYVGRNDDVKCFSCDGGLRCWESGDDPWVEHAKWFPRCEFLIRMKGQEFVDEIQGRY
                  ::    ::  ::  :  ::    :::: ::::  :  : :::: : :::  :    :::  :::: : :  :   ::::    :
2OPY: FPNSTNLPRNP-SMADYEARIFTFGTWIYSV--NKEQLARAGFYALGEGDKVKCFHCGGGLTDWKPSEDPWEQHAKWYPGCKYLLEQKGQEYINNI----

3T6P: PHLLEQLLSTEDAVMMNTPVVKSALEMGFNRDLVKQTVQSKILTTGENYKTVNDIVSALLNAEDEKREEEKEKQAEEMASDDLSLIRKNRMALFQQLTCV
       ::
2OPY: -HLTH-----------------------------------------------------------------------------------------------

3T6P: LPILDNLLKANVINKQEHDIIKQKTQIPLQARELIDTILVKGNAAANIFKNSLKEIDSTLYKNLFVDKNMKYIPTEDVSGLSLEEQLRRLQEERTCKVCM
                                                                                       ::::
2OPY: ---------------------------------------------------------------------------------SLEE---------------

3T6P: DKEVSVVFIPCGHLVVCQECAPSLRKCPICRGIIKGTVRTF

2OPY: -----------------------------------------
```

Table S2. DNA and protein sequences of the designed proteins.

(1) FI-XIAP DNA Sequence:

TACTTCCAATCCAATGCAACCGGTAACCTGTCTTACTTCCCGGGTTACCCGGACATGGGTATGGA
AACCGCGCGTATGCGTACCTTCGAAGACTGGCAGGTTGAAGTTCCGCCGGAACAGCTGGCGTCTG
CGGGTTTCTTCTACCTGGGTCGTAACGACAAAGTTAAATGCTTCTCTTGCGGTGGTGGTCTGACCG
ACTGGAAATCTGGTGAAGACCCGTGGGTTCAGCACGCGAAATGGTACCCGCAGTGCCAGTTCGTT
GTTCGTATGAAAGGTCAGGACTTCATCGACTCTGTTCAGGGTCGTCACGCGCTGCAGGAA**TAACA
TTGGAAGTGGATAA**

(2) FI-XIAP protein sequence:

snaTGNLSYFPGYPDMGMETARMRTFEDWQVEVPPEQLASAGFFYLGRNDKVKCFSCGGGLTDWKSG
EDPWVQHAKWYPQCQFVVRMKGQDFIDSVQGRHALQE

(3) DI-XIAP DNA sequence:

TACTTCCAATCCAATGCATCTGGTGGTCCGACCGCGTGGCCGGAACACCCGCAGTACTCTACCGA
AGCGGCGCGTATCAAAACCTTCCAGAACTGGCCGATCGCGGTTTCTCCGGAACAGCTGGCGGAAG
CGGGTTTCTACTACACCGGTCGTGGTGACAAAGTTAAATGCTTCTCTTGCGGTGGTGGTCTGGCGT
CTTGGGAACCGGGTGACGACCCGTGGTCTGAACACCAGAAATGGTTCCCGAACTGCAAATTCATG
CAGGCGATGAAAGGTCAGGACTACGTTGACGTTGAAAAAGCGATGCACGTTGAAGACGAC**TAAC
ATTGGAAGTGGATAA**

(4) DI-XIAP protein sequence:

snaSGGPTAWPEHPQYSTEAARIKTFQNWPIAVSPEQLAEAGFYYTGRGDKVKCFSCGGGLASWEPGD
DPWSEHQKWFPNCKFMQAMKGQDYVDVEKAMHVEDD

Table S3. Pair-wise TM-score between models predicted by different methods for designed sequences.

Designs	Structure prediction tools	I-TASSER	QUARK	Rosetta	RaptorX	Phyre2
DI-XIAP	I-TASSER	1.00	0.83	0.93	0.94	0.91
	QUARK		1.00	0.84	0.83	0.82
	Rosetta			1.00	0.95	0.94
	RaptorX				1.00	0.93
	Phyre2					1.00
FI-XIAP	I-TASSER	1.00	0.83	0.94	0.95	0.90
	QUARK		1.00	0.84	0.83	0.80
	Rosetta			1.00	0.98	0.94
	RaptorX				1.00	0.93
	Phyre2					1.00

Table S4. Comparison of WT-XIAP and models built by different methods for designed sequences.

Structure prediction tools	DI-XIAP		FI-XIAP	
	TM-score	RMSD (Å)	TM-score	RMSD (Å)
I-TASSER	0.92	1.01	0.92	1.12
QUARK	0.84	2.55	0.82	3.84
Rosetta	0.93	2.44	0.94	2.23
RaptorX	0.92	2.34	0.93	2.43
Phyre2	0.91	3.29	0.91	2.62

Table S5. Conservation score for all residues on the BIR3 domain of XIAP calculated based on EvoDesign structure profile.

#	#Residue position	Sequence (WT-XIAP)	Sequence (DI-XIAP)	Interface[a]	Mutation[b]	Conservation score
1	250	F	S		M	-3.00
2	251	P	G		M	-1.33
3	252	N	G		M	2.33
4	253	S	P		M	-0.50
5	254	T	T			0.00
6	255	N	A		M	-1.00
7	256	L	W		M	-2.25
8	257	P	P			5.00
9	258	R	E		M	2.50
10	259	N	H		M	-0.67
11	260	P	P			0.89
12	261	S	Q		M	0.33
13	262	M	Y		M	1.44
14	263	A	S		M	-0.33
15	264	D	T		M	-1.00
16	265	Y	E		M	-1.56
17	266	E	A		M	1.30
18	267	A	A			1.30
19	268	R	R			5.00
20	269	I	I			1.90
21	270	F	K		M	-2.60
22	271	T	T			3.80
23	272	F	F			5.70
24	273	G	Q		M	-1.90
25	274	T	N		M	-0.10
26	275	W	W			7.60
27	276	I	P		M	-2.30
28	277	Y	I		M	-0.90
29	278	S	A		M	-0.90
30	279	V	V			2.20
31	280	N	S		M	0.20
32	281	K	P		M	-0.20
33	282	E	E			1.60
34	283	Q	Q			1.80
35	284	L	L			3.80
36	285	A	A			3.70
37	286	R	E		M	0.70

38	287	A	A			3.60
39	288	G	G			6.00
40	289	F	F			4.80
41	290	Y	Y			2.80
42	291	A	Y		M	-1.90
43	292	L	T	S	M	-1.00
44	293	G	G			3.70
45	294	E	R		M	-0.33
46	295	G	G			2.00
47	296	D	D			6.00
48	297	K	K	SC		1.50
49	298	V	V	SC		3.60
50	299	K	K	SC		2.90
51	300	C	C			9.00
52	301	F	F			6.00
53	302	H	S		M	-1.50
54	303	C	C	C		9.00
55	304	G	G	C		2.00
56	305	G	G	C		2.70
57	306	G	G	SC		1.20
58	307	L	L	SC		3.50
59	308	T	A	SC	M	-1.20
60	309	D	S	SC	M	0.00
61	310	W	W	SC		11.00
62	311	K	E	SC	M	1.70
63	312	P	P			0.20
64	313	S	G		M	0.10
65	314	E	D	SC	M	2.00
66	315	D	D			3.00
67	316	P	P			5.40
68	317	W	W			5.50
69	318	E	S		M	0.80
70	319	Q	E	SC	M	2.10
71	320	H	H			8.00
72	321	A	Q		M	1.50
73	322	K	K	SC		3.70
74	323	W	W	SC		5.70
75	324	Y	F	SC	M	1.50
76	325	P	P	C		5.40
77	326	G	N	C	M	-0.20
78	327	C	C	C		9.00

79	328	K	K	C		2.50
80	329	Y	F		M	2.60
81	330	L	M		M	2.90
82	331	L	Q		M	-0.10
83	332	E	A		M	-1.00
84	333	Q	M		M	-0.20
85	334	K	K			3.17
86	335	G	G			3.33
87	336	Q	Q	C		2.17
88	337	E	D	C	M	1.80
89	338	Y	Y			4.00
90	339	I	V	C	M	2.10
91	340	N	D	C	M	1.00
92	341	N	V	C	M	0.25
93	342	I	E		M	3.50
94	343	H	K	C	M	-0.50
95	344	L	A	C	M	-3.00
96	345	T	M	C	M	0.50
97	346	H	H	C		2.50
98	347	S	V		M	0.00
99	348	L	E		M	-2.00
100	349	E	D		M	1.50
101	350	E	D		M	5.00

[a]'S' indicates that the residue is bound to Smac peptide, and 'C' indicates that the residue is bound with caspase-9.
[b]'M' indicates that the residue is mutated in DI-XIAP.

Table S6. Solvation categorization of the interface residues with or without mutations in DI-XIAP.

	#	Residue	AA	rASAm[a]	rASAc[b]	Category[c]
Interface residues mutated in DI-XIAP	1	292	L	0.406	0.353	Rim
	2	308	T	0.436	0.143	Core
	3	309	D	0.847	0.680	Rim
	4	311	K	0.635	0.630	Rim
	5	314	E	0.211	0.126	Support
	6	319	Q	0.150	0.106	Support
	7	324	Y	0.339	0.226	Core
Interface residues without mutation in DI-XIAP	1	297	K	0.410	0.290	Rim
	2	298	V	0.013	0.000	Support
	3	299	K	0.365	0.280	Rim
	4	306	G	0.400	0.000	Core
	5	307	L	0.088	0.000	Support
	6	310	W	0.059	0.051	Support
	7	322	K	0.390	0.385	Rim
	8	323	W	0.545	0.357	Rim

[a]Relative accessible surface area (rASA) on monomer XIAP structure.
[b]Relative accessible surface area on XIAP/Smac complex structure.
[c]Categorization of interface residues based on Levy (J Mol Biol, 403: 660-670, 2010).

Table S7. Conservation score for the interface residues in the XIAP/caspase-9 complex.

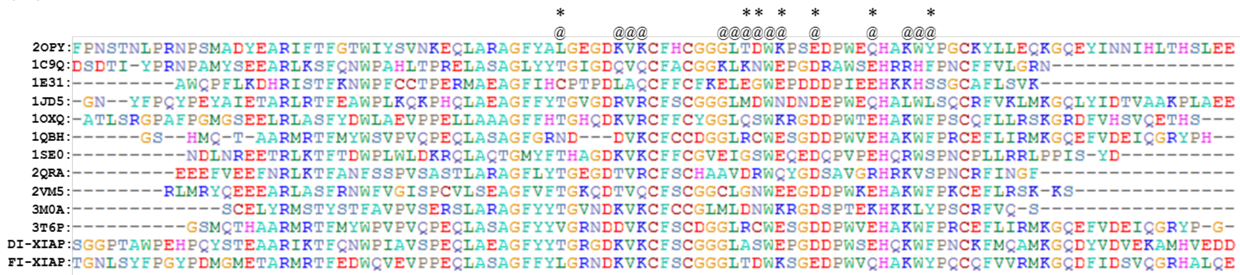
#	#Residue position	Sequence (WT-XIAP)	Sequence (DI-XIAP)	Interface[a]	Mutation[b]	Conservation score
1	297	K	K	SC		1.50
2	298	V	V	SC		3.60
3	299	K	K	SC		2.90
4	303	C	C	C		9.00
5	304	G	G	C		2.00
6	305	G	G	C		2.70
7	306	G	G	SC		1.20
8	307	L	L	SC		3.50
9	308	T	A	SC	#	-1.20
10	309	D	S	SC	#	0.00
11	310	W	W	SC		11.00
12	311	K	E	SC	#	1.70
13	314	E	D	SC	#	2.00
14	319	Q	E	SC	#	2.10
15	322	K	K	SC		3.70
16	323	W	W	SC		5.70
17	324	Y	F	SC	#	1.50
18	325	P	P	C		5.40
19	326	G	N	C	#	-0.20
20	327	C	C	C		9.00
21	328	K	K	C		2.50
22	336	Q	Q	C		2.17
23	337	E	D	C	#	1.80
24	339	I	V	C	#	2.10
25	340	N	D	C	#	1.00
26	341	N	V	C	#	0.25
27	343	H	K	C	#	-0.50
28	344	L	A	C	#	-3.00
29	345	T	M	C	#	0.50
30	346	H	H	C		2.50

[a]'S' indicates that the residue is bound to Smac peptide, and 'C' indicates that the residue is bound with caspase-9.
[b]'#' indicates that the residue is mutated in DI-XIAP.

Table S8. Standard deviations of FoldX physical terms from four independent initialization runs. Four independent 1000-step initialization runs are performed with the same XIAP/'AVPF' protein-peptide complex, each starting with different random numbers. Columns 2-4 are from the new EvoDesign protocol with binding and monomer folding separately counted, where Column 5 is for the old EvoDesign protocol with a united FoldX energy term.

Index of runs	$\delta E_{foldx}(XIAP_{apo})$	δE_{foldx}**(interface)**	$\delta E_{evolution}$	δE_{foldx}**(complex)**
1	55.99	3.85	13.23	57.23
2	55.56	3.71	12.32	58.57
3	56.24	3.74	12.74	57.49
4	56.87	3.90	12.41	56.79
Average	56.17	3.80	12.68	57.52

(A)



(B)

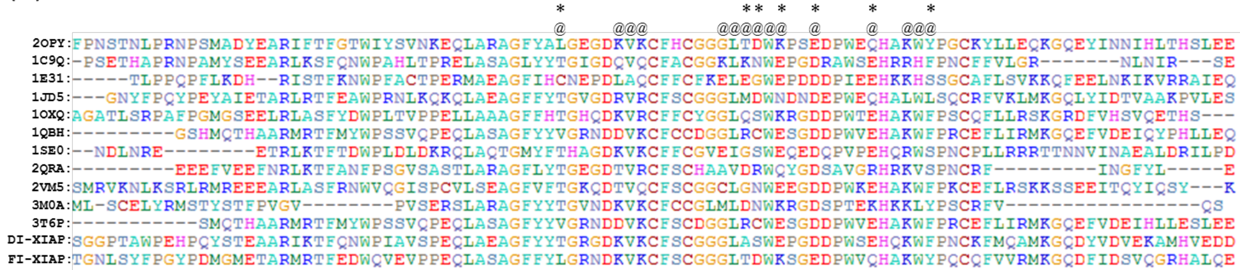


Figure S1. Multiple sequence alignments generated based on (A) pair-wise structure alignments by TM-align and (B) pair-wise sequence alignments by Needleman-Wunsch dynamic programming. The designed DI-XIAP and FI-XIAP sequences are listed at the bottom for comparison. On the top of each matrix, '@' refers to the sites bound with Smac peptide in the WT-XIAP structure and '*' labels the binding sites that are mutated in DI-XIAP. Amino acids are colored using BioEdit.

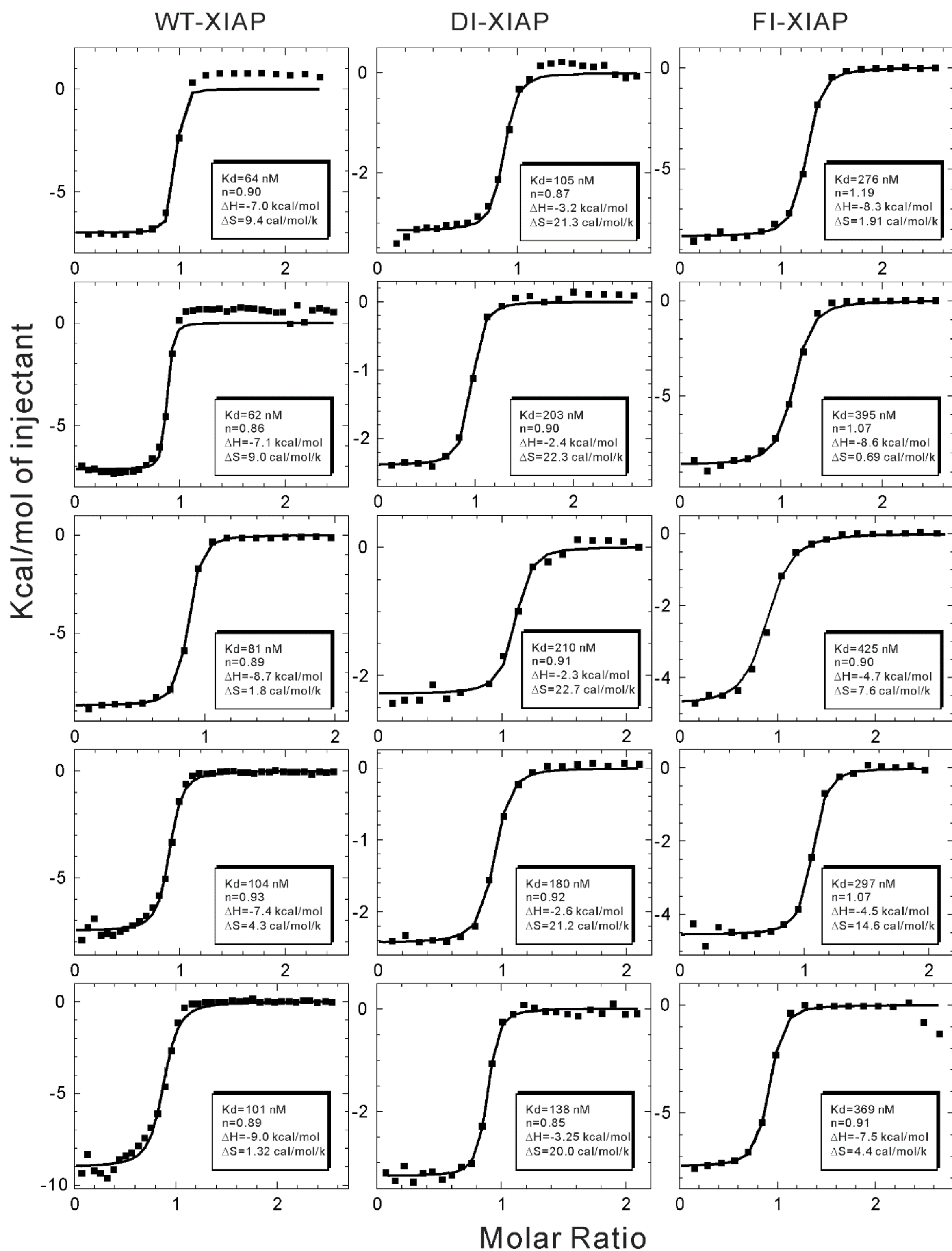


Figure S2. Summary of the isothermal calorimetry (ITC) experiment repeated for the XIAP proteins with the "AVPF" peptide. Left panel: WT-XIAP; Middle panel: DI-XIAP; Right panel: FI-XIAP. Insets show the parameters in each ITC experiment.

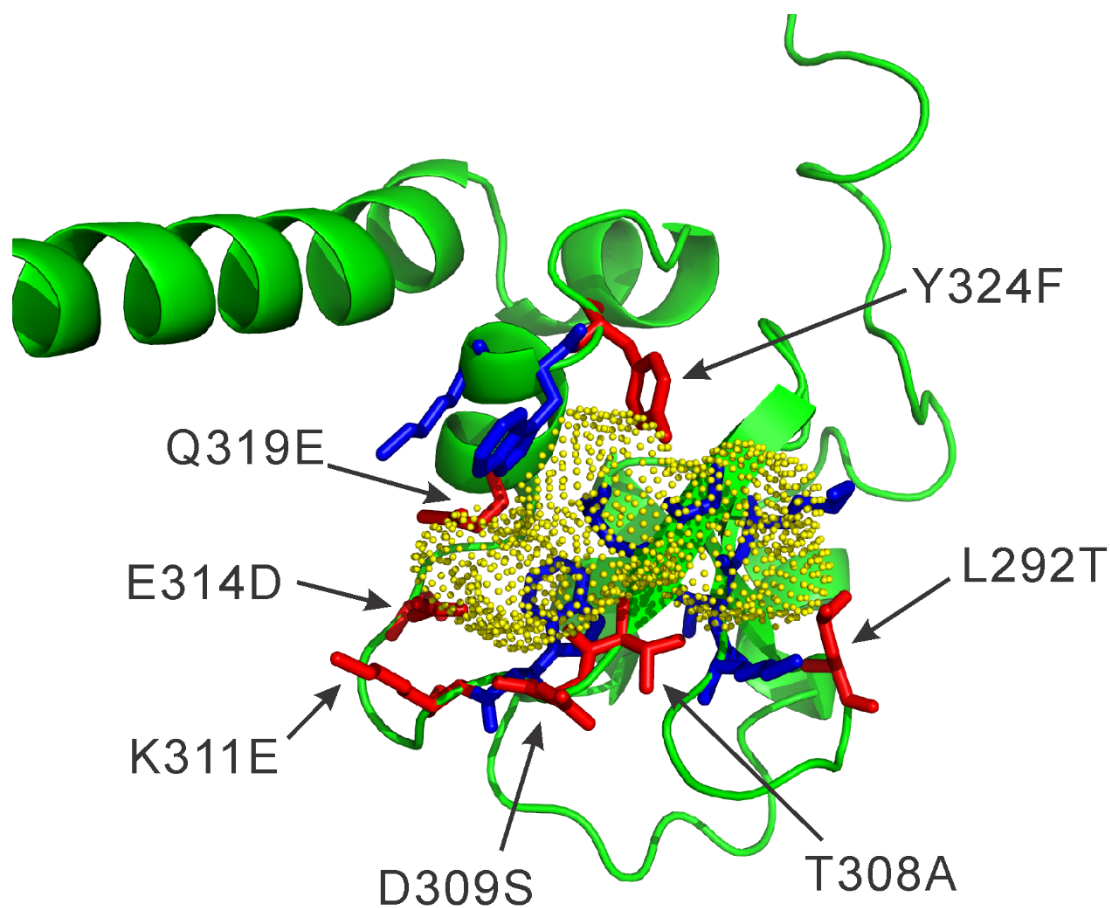


Figure S3. Binding pocket of DI-XIAP with Smac peptide. The XIAP structure is shown in green cartoon and Smac peptide in dots. Sticks are residues in the interface where red and blue are mutated or un-mutated residues in the DI-XIAP sequence.

(A) Sequence alignment to calculate evolution score

```
Position:  123456789abcdefghijk
Target:    SISSRVKSKRIQLGLNQAEL-
            ||||||||||||||||||||
Decoy:     -ISSRVKSKRIQLGLNQAELS
Position:   123456789abcdefghijk
```

(B) Generate structure decoy to calculate physical score

```
Position:  123456789abcdefghijk
Target:    SISSRVKSKRIQLGLNQAEL
           ||||||||||||||||||||
Decoy:     ISSRVKSKRIQLGLNQAELS
Position:  123456789abcdefghijk
```

Figure S4. Illustration of sequence alignment and side-chain reconstruction processes. Here, the target template consists of a sequence of 'SISSRVKSKRIQLGLNQAEL' (red color) indexed from '1' to 'k', where the decoy sequence 'ISSRVKSKRIQLGLNQAELS' (blue color) shifts the first amino acid to the last position. The best alignment pattern should align the 1st residue of decoy sequence with the 2nd residue of target, the 2nd residue of decoy with the 3rd of target and so on (Figure S4A), which was generated for evolutionary score calculation. In the structure decoy generation, however, there is no dynamic sequence re-alignment, and thus the 1st residue (Isoleucine) of decoy sequence will be always anchored to the 1st position of target template, the 2nd residue (Serine) of decoy will be anchored to the 2nd position of target and so on (Figure S4B). Thus, although the evolutionary score is calculated based on dynamic programming, there is no indel in side-chain structure reconstruction and final designs.

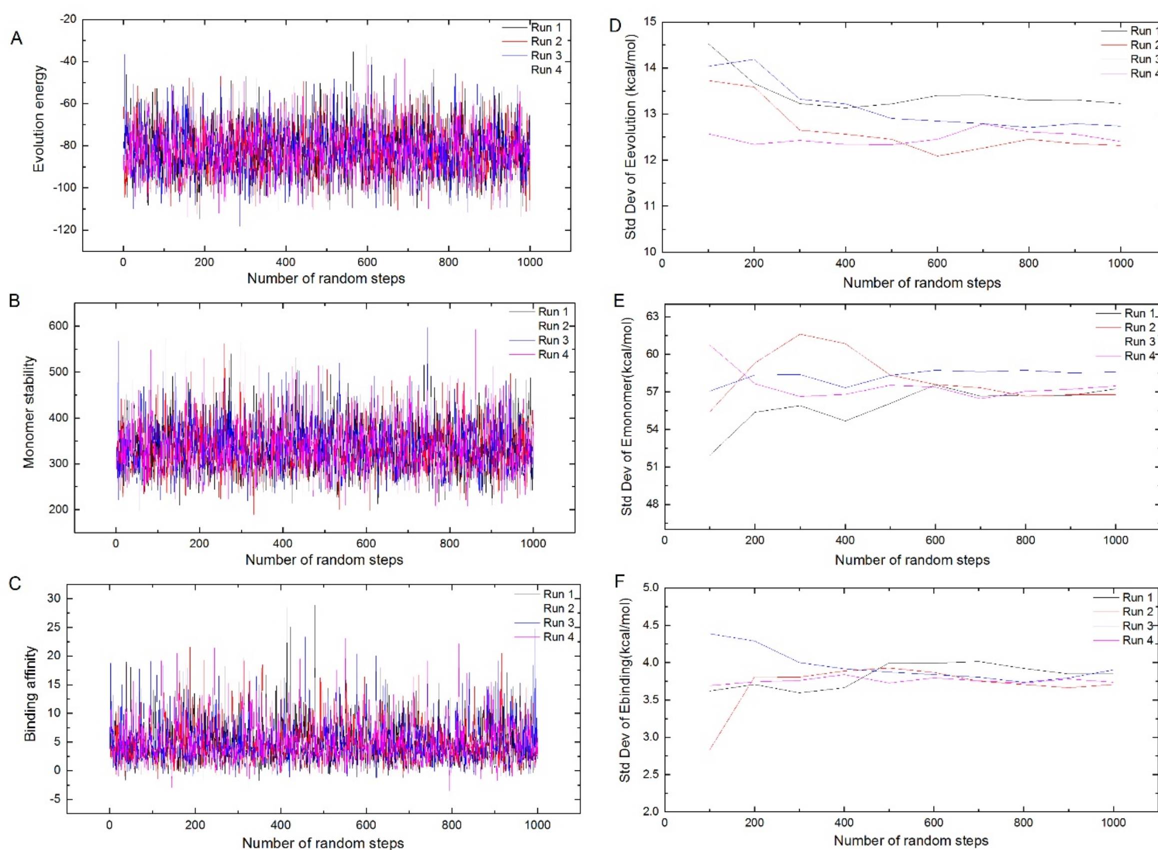


Figure S5. Fluctuations and standard deviations of different energy terms in random sequence decoys. A series of random initialization steps are performed to calculate the standard deviation of evolution, monomer, and interface energy terms separately, where SCWRL4 was used to repack the side chains and FoldX4 was used to calculate the monomer and interface energy terms. Four independent initialization experiments are run, each generating 1000 random sequence and structure decoys, where the standard deviations are calculated with N=100, 200, 300, …, 1000 decoy structures. (A-C). Fluctuation of the evolution, monomer and binding energy terms in (4×) 1000 random sequences; (D-F). Standard deviations calculated at different cutoffs of decoy numbers.

```
                                                 @    @@@      @@@@@@  @    @  @@@
WT:    FPNSTNLPRNPSMADYEARIFTFGTWIYSVNKEQLARAGFYALGEGDKVKCFHCGGGLTDWKPSEDPWEQHAKWYPGCKYLLEQKGQEYINNIHLTHSLEE
New-1: NANTLHYPRAPEMSSEEHRLKTFSEWPLPMSPEQLAEAGFYMSGAGDMVRCFCCGGALMRWEPGEDPWSTHAEFYPQCQFLLRMQGQEFVDEVMGRHAHES
New-2: DGNLLKFPRYPEMQSEEAKLKTFKNWPAPVPPQQLAQAGFYHTGAGDMVKCFACGGGLMKWEPGDDPWEEHAKWFPNCQFLLRMKGQEFIDTIRERYAAES
New-3: DGNLLKFPRYPEMQSEEAKLKTFKNWPAPVPPQQLAQAGFYHTGAGDMVKCFACGGGLMKWEPGDDPWEEHAKWFPNCQFLLRMKGQEFIDTIRERYAAES
New-4: EGNASYWPRFPEMQAEEARLKSFANWPAPVTPEQLAEAGFYHTGKGDMVRCFCCGGGLMKWEPGDDPWESHAKFYPNCEFLMRMRGQEFVDSIQGRYAADT
Old-1: DGNLMWYPRYPQYQTEEARIKTFTNWPIPISPEQMAEAGFFHVGAGDTVKCFCCGGALKNWEPGDDPWTEQAKWFPNCQYLKRKQGQEFMDEILGKYAHEE
Old-2: DGNLLYYPRFPEMQTEEARLKTFANWPAPVPPEQLAEAGFYATGEGDKVKCFCCGGALQNWEPGDDPWEEHAKWFPNCEFLMRMKGQEFVDEVQGRHAAEE
Old-3: DGNLFYYPRFPQYATEEARMKTFQNWPAPVPPEQLASAGMYSTGAGDKVKCFCCGGGLRNWEPGDDPWTEHAKWFPNCRFLMRKRGQEFIDEIQAKYSAQR
Old-4: DGNLFYYPRYPDFATEEARLKTFANWPLPVAPEQLASAGFYATGAGDKVKCFCCGGGLRNWEPGDEPWEEHAKWFPNCEFLKRMRGQEFVDTIQARYTAES
```

Figure S6. Comparison of final designed sequences with two EvoDesign protocols. Here, four independent runs are performed for each of the new (using separate binding and monomer terms as Eq. 7) and old (using uniform binding and monomer terms from FoldX) EvoDesign protocols. 'WT' refers to the wild-type XIAP, and 'New-[1-4]' and 'Old-[1-4]' are the sequences designed using the new and old EvoDesign protocols. On the top of the matrix, '@' indicates the binding sites of Smac peptide with the WT-XIAP structure, where the residues at the 2nd, 7th and 8th interface positions are completely different in new and old designs although they tend to converge within each protocol of independent runs.

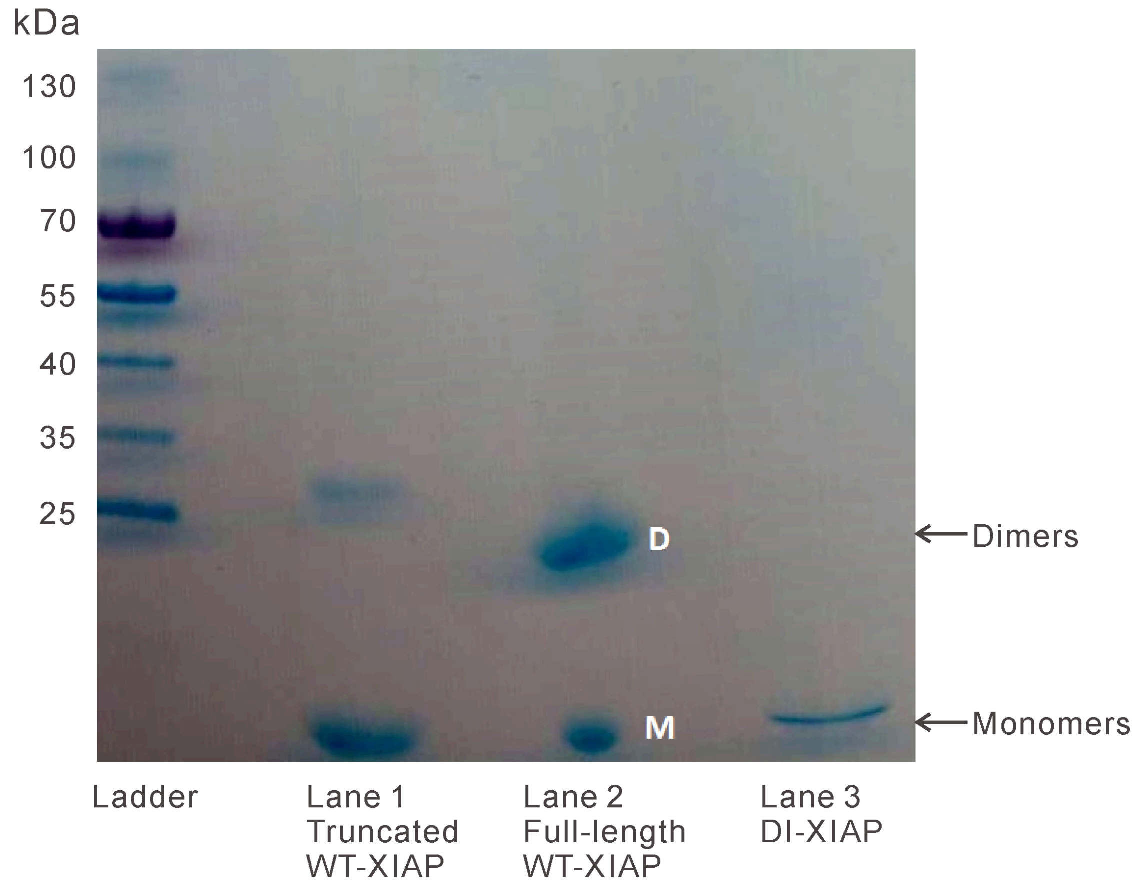


Figure S7. SDS-PAGE gel analysis of wild type (WT) and designed XIAPs. WT-XIAP and DI-XIAP were prepared via Nickel-NTA affinity purification and subjected to SDS-PAGE. Lane-1: Truncated WT-XIAP (~12.8 kDa); Lane-2: Full-length WT-XIAP in which both dimer (D~27.2 kDa) and monomer (M~13.6 kDa) form of full-length wild-type XIAPs are present; Lane-3: DI-XIAP (~13.4 kDa).