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online data

Original Paper

SUPPLEMENTARY ONLINE DATA

Allosteric modulation of caspase 3 through mutagenesis

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MATERIALS AND METHODS

Site-directed mutagenesis of caspase 3

The single mutants V266H, Y197C and E124A were made in the background of wild-type caspase 3 using site-directed mutagenesis and plasmid pHC332 as a template [1]. All mutations were confirmed by sequencing both DNA strands, and the mutated bases are shown in bold. For V266H, primers 1 and 2 were used: 5′- CAGATTCCATGTATT**CA**-TAGCATGCTCACAAAAGAACTC-3′ and 5′-GAGTTCTTT-TGTGAGCATGCTA**TG**AATACA-TGGAATCTG-3′, respectively. For Y197C, primers 3 and 4 were used: 5′-GGCCGAC-TTCTT-GTATGCAT**GC**A**GT**AC**T**GCACCTGG-3′ and 5′-CCAGGTGCAGTACTGCATGCATACAAG-AAGTCGGCC-3′ respectively. For E124A, primers 5 and 6 were used: 5′-CTG-AGCCATGGTGAAG**CC**GGCATAATTTTTGGAAC-3′ and 5′-GTTCCAAAAATTATGCCGGCTTCACCAT-GGCTCAG-3′ respectively. Primers 1, 2, 3 and 4 introduced a unique SphI site. Primers 5 and 6 introduced a unique NaeI site (underlined). The resulting plasmids (in pET21b) are referred to as pHC33203, pHC33226 and pHC33247 for the V266H, Y197C and E124A single mutants respectively.

The double mutants Y197C,V266H and E124A,V266H mutations were made in the background of V266H using plasmid pHC33203 as a template. For Y197C,V266H, primers 7 and 8 were used: 5′-GGCCGACTTCTTGTATGCAT**GC**A**GT**A**T**G-CACCTGG-3′ and 5′-CCAGGTGCAGTA-CTGCATGCATA-CAAGAAGTCGGCC-3′ respectively. For E124A,V266H, primers 9 and 10 were used: 5′-CTGAGCCATGGTGAA-G**CC**GGCATAATTTTTGGAAC-3′ and 5′-GTTCCA-AAAAT-TATGCCGGCTTCACCATGG CTCAG -3′ respectively. Primers 7 and 8 introduced a SphI site, while primers 9 and 10 introduced a NaeI site (underlined). The resulting plasmids are referred to as pHC33227 and pHC33271 for the Y197C,V266H and E124A,V266H double mutants respectively.

The double mutant E124A,Y197C was made in the background of Y197C using plasmid pHC33226 as a template. For E124A,Y197C, primers 11 and 12 were used: 5′-CTG-AGCCATGGTGAAG**CC**GGCATAATTTTTGGAAC-3′ and –GTTCCAAAAATTATGC-CGGCTTCACCATGGCTCAG-3′ respectively. Primers 11 and 12 introduce a unique NaeI site. The resulting plasmid for E124A,Y197C is referred to as pHC33270.

The triple mutant, E124A,Y197C,V266H was made in the background of Y197C,V266H using plasmid pHC33227 as a template and primers 13 and 14: 5′-CT-GAGCCATGGTGAAG**CC**GG**C**ATAATTTTTGGAAC-3′ and 5′-GTTCCAA-AAATTATGCCGGCTTCACCATGGCTCAG-3′. Primers 13 and 14 introduce a unique NaeI site. The resulting plasmid for E124A,Y197C,V266H is referred to as pHC33272.

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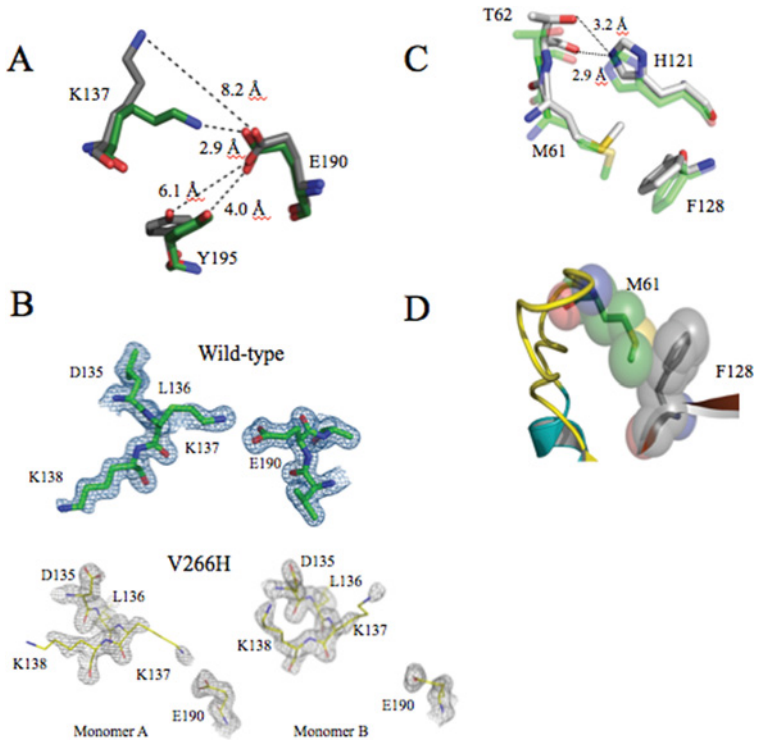

Figure S1 **Changes in caspase 3 resulting from V266 to histidine mutation**
(**A**) Interactions among Lys[137], Glu[190] and Tyr[195] showing increases in distances for V266H variant (grey) versus wild-type (green). (**B**) Electron density maps for Lys[137] and Glu[190] for wild-type (upper panel) or V266H variant (lower panel). (**C**) Comparison of active site for wild-type (green) versus V266H variant (grey) demonstrating different rotamer for Met[61] and increased H-bonding distance for the catalytic residue His[121]. (**D**) Steric clashes between Phe[128] and Met[61] result in the different rotamer observed in (**C**).

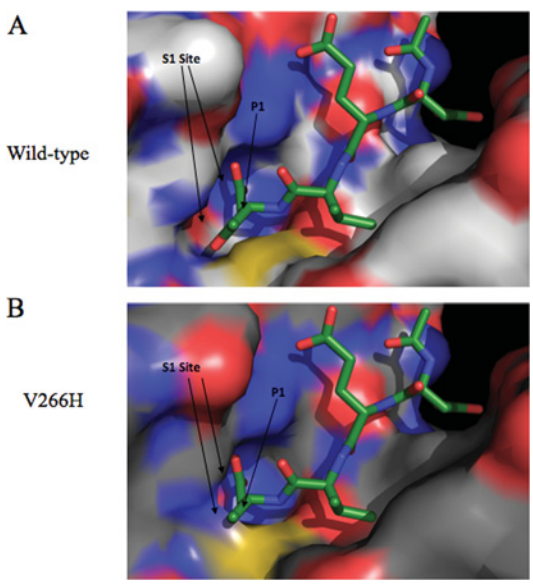

Figure S2 The S1 and S1′ sites are wider in wild-type caspase 3
(**A**) Compared with the V266H variant (**B**).

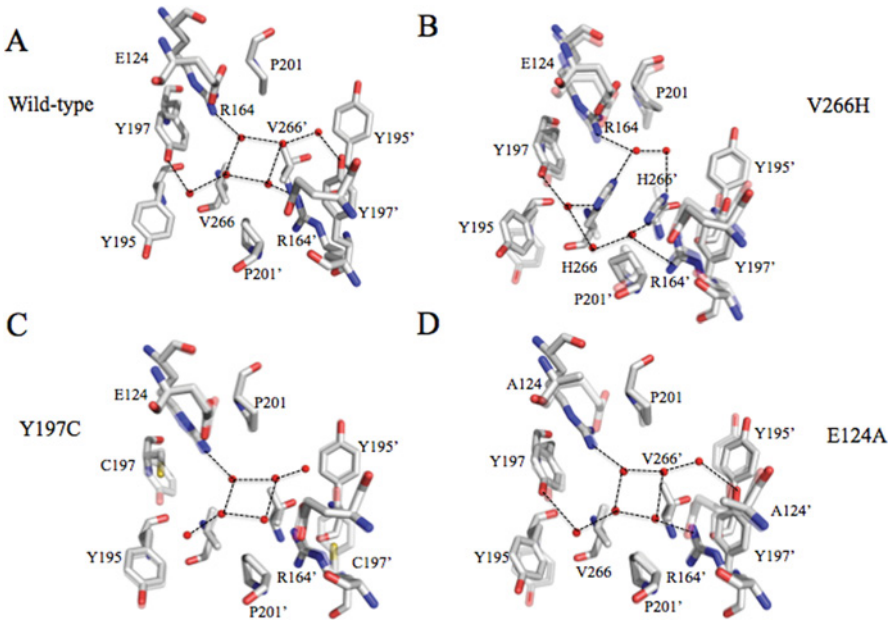

Figure S3 **Comparison of H-bonding and water molecules in the dimer interface of wild-type caspase 3 (A) and single mutants V266H (B), Y197C (C) or E124A (D)**
(**A**–**D**) Red spheres indicate water molecules, and the prime (′) indicates amino acids from the second monomer. For (**B**–**D**), Wild-type caspase 3 is shown as partially transparent.

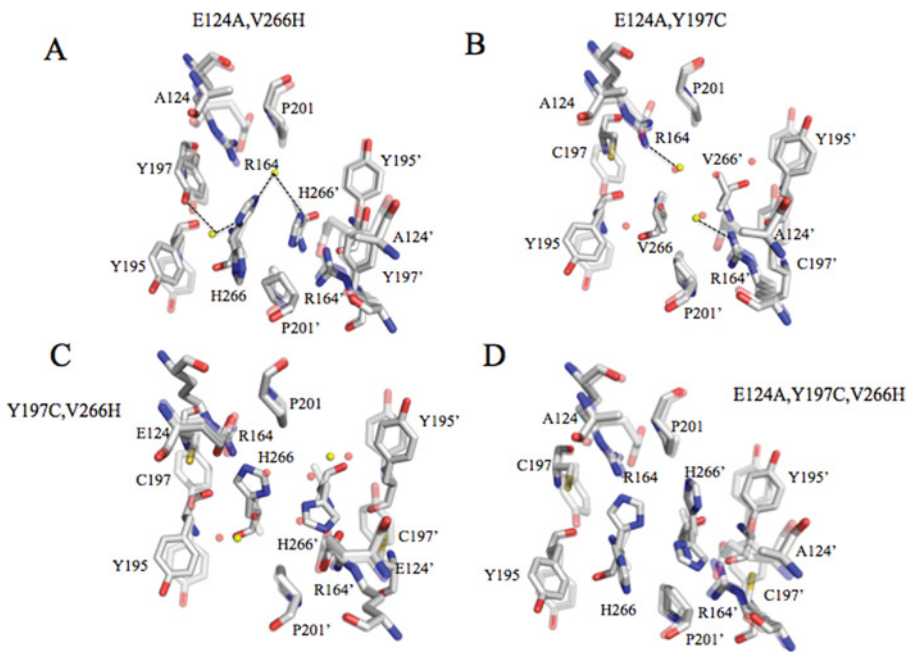

Figure S4 **Comparison of H-bonding and water molecules in the dimer interface of wild-type caspase 3 and double mutants E124A,V266H (A) E124A,Y197C (B), Y197C,V266H (C) and triple mutant E124A,Y197C,V266H (D)**
(**A**–**D**) Red spheres indicate water molecules in wild-type caspase 3 and yellow spheres indicate water molecules in the mutant. The prime (′) indicates amino acids from the second monomer, and wild-type caspase 3 is shown as partially transparent.

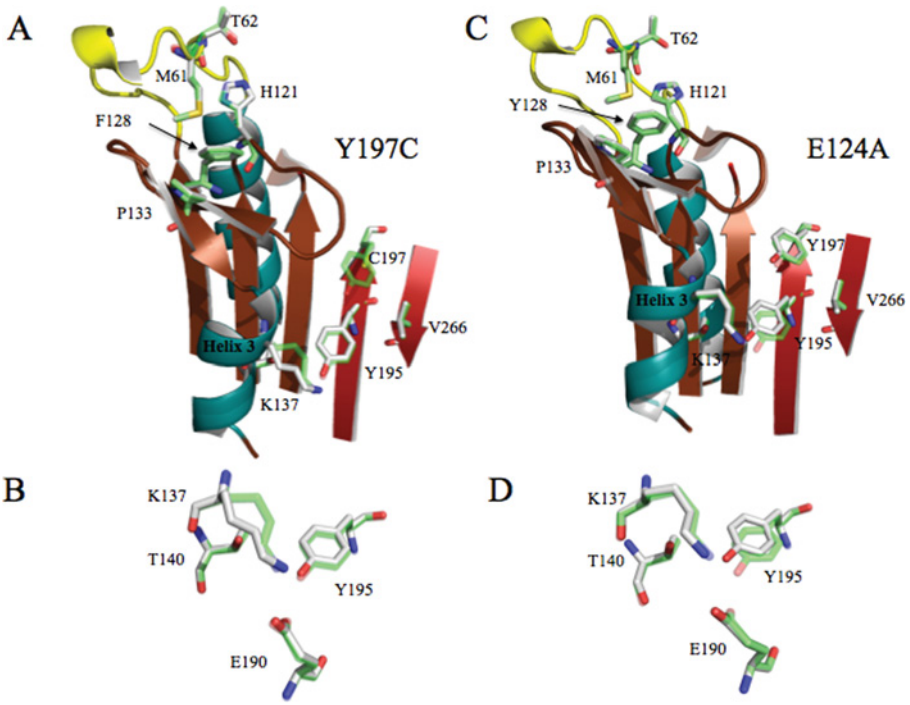

Figure S5 **Comparison of single mutants Y197C (A, B) or E124A (C, D) with wild-type caspase 3**
(**A**–**D**) Wild-type caspase 3 is shown as partially transparent and amino acid side chains are coloured green. Amino acid side-chains for the mutants are coloured grey.

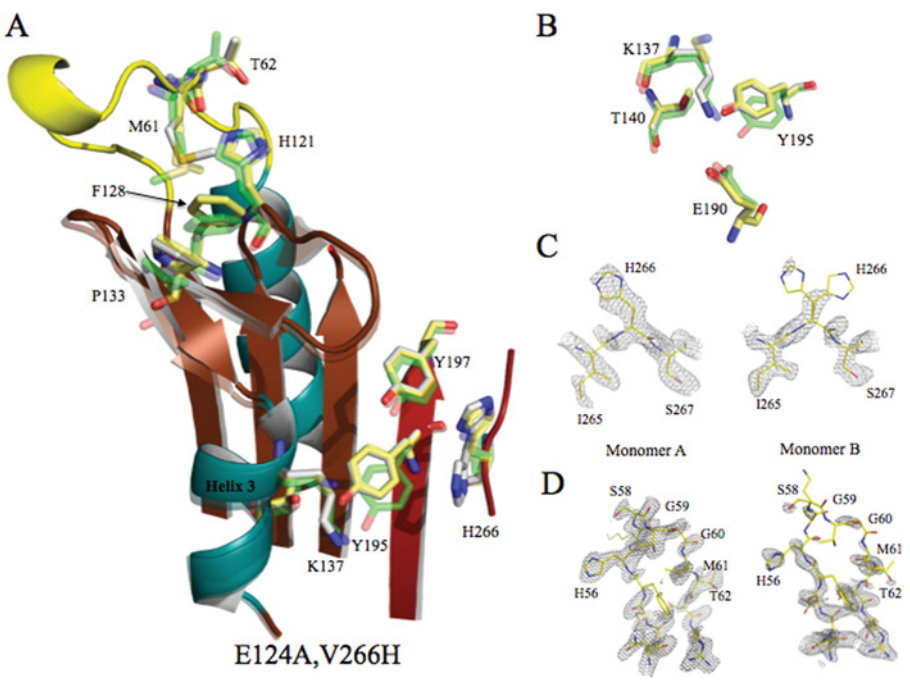

Figure S6 **Comparison of the double mutant E124A,V266H (grey) with V266H (yellow) and wild-type (green) caspase 3**
(**A**) Changes in helix 3 and active site regions due to the mutations. (**B**) Comparison of interactions among Lys^{137}, Thr^{140}, Glu^{190} and Tyr^{195}. (**C**) Electron density maps of His^{266} in monomer A (left panel) or monomer B (right panel) demonstrating evidence for two conformations of His^{266} in monomer B. (**D**) Electron density maps of L1 of monomer A (left panel) or monomer **B** (right panel) demonstrating disorder in L1 of monomer B. For (**C**, **D**), the mesh is drawn at $\sigma = 2.0$.

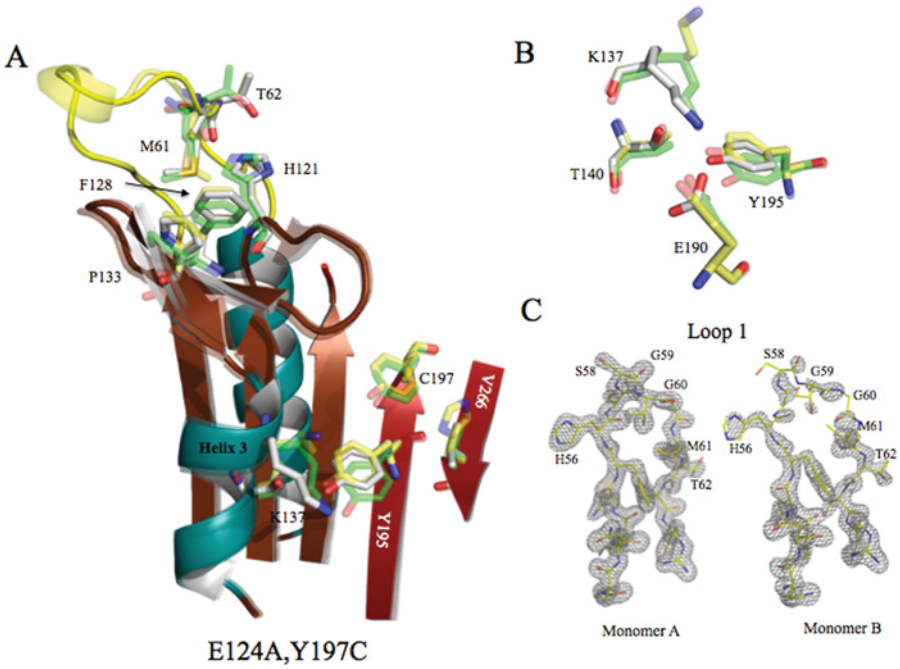

Figure S7 **Comparison of the double mutant E124A,Y197C (grey) with V266H (yellow) and wild-type (green) caspase 3**
(**A**) Changes in helix 3 and active site regions due to the mutations. (**B**) Comparison of interactions among Lys^{137}, Thr^{140}, Glu^{190} and Tyr^{195}. (**C**) Electron density maps of L1 of monomer A (left panel) or monomer B (right panel) demonstrating disorder in L1 of monomer B. For (**C**) the mesh is drawn at $\sigma = 2.0$.

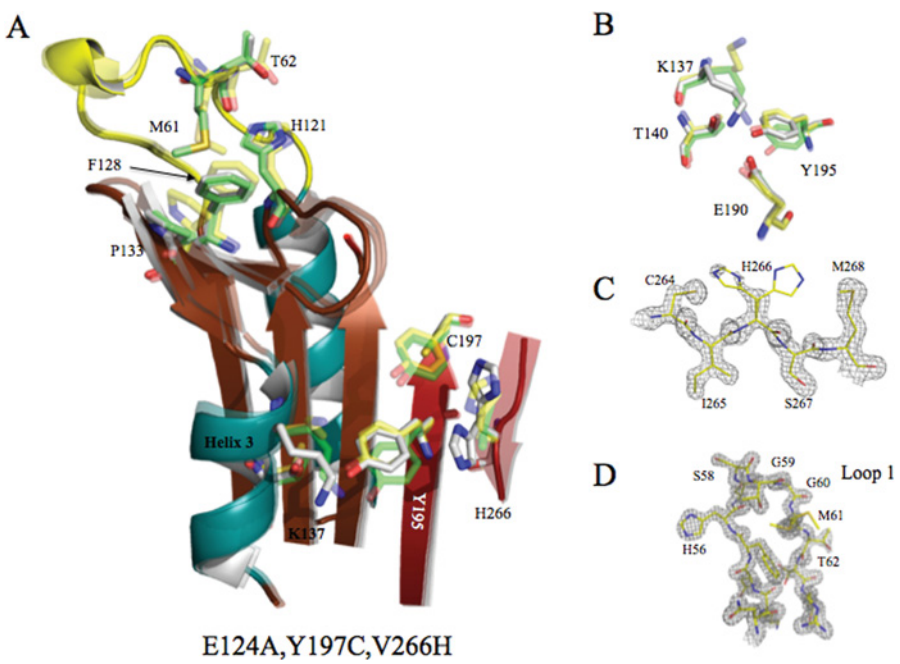

Figure S8 **Comparison of the triple mutant E124A,Y197C,V266H (grey) with V266H (yellow) and wild-type (green) caspase 3**
(**A**) Changes in helix 3 and active site regions due to the mutations. (**B**) Comparison of interactions among Lys^{137}, Thr^{140}, Glu^{190} and Tyr^{195}. (**C**) Electron density map of His^{266} demonstrating evidence for two conformations of His^{266}. (**D**) Electron density map of L1 demonstrating order in L1. For (**C**, **D**), the mesh is drawn at $\sigma = 1.5$.

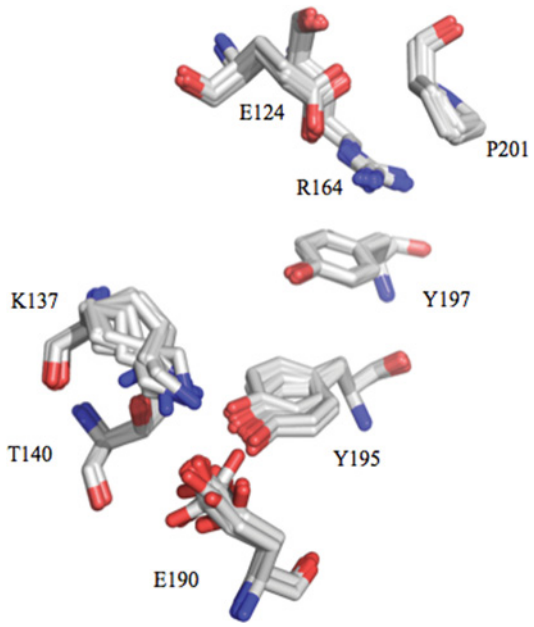

Figure S9 Comparison of indicated amino acid side chains in 23 structures of caspase 3 from the protein data bank, as shown in Table S2

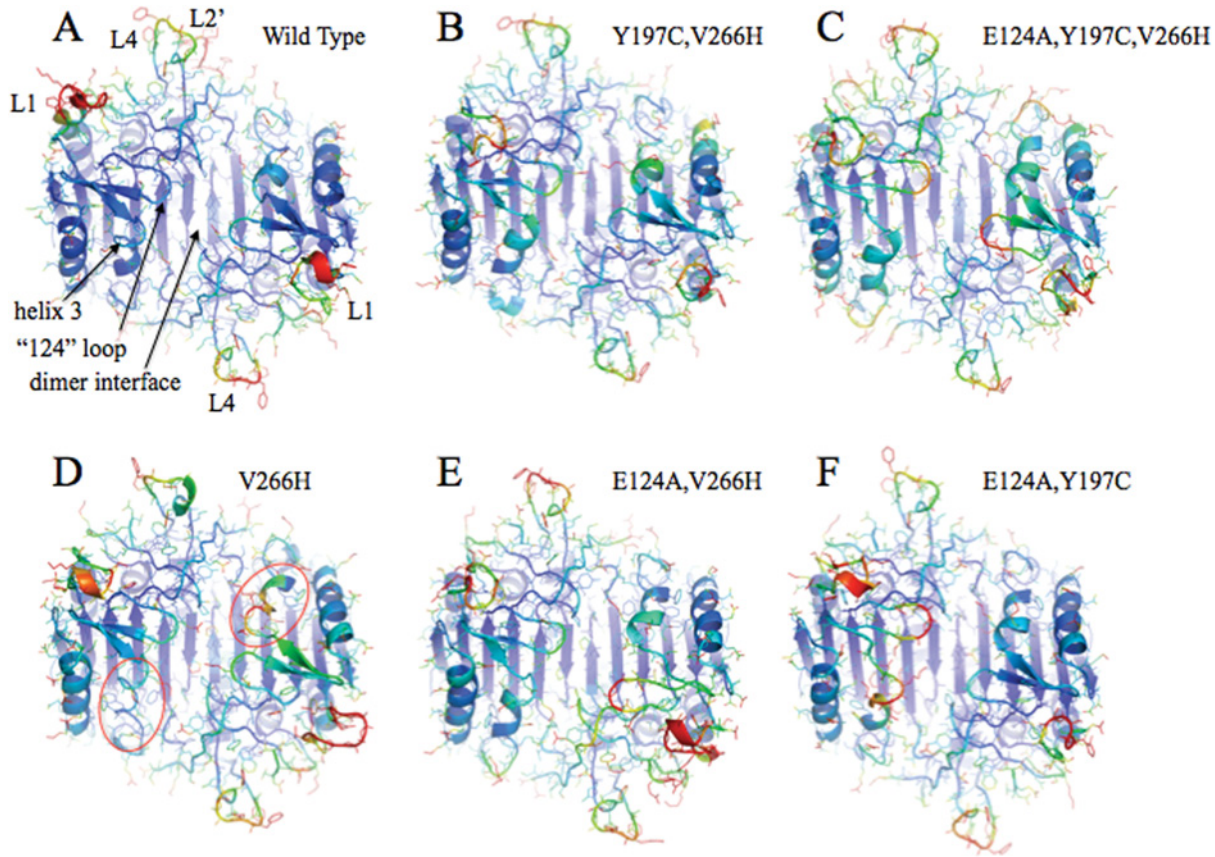

Figure S10 **Structures representing the final frame of the simulation (50 ns) with b-factors indicated by colour**
A blue to red spectrum indicates low to high b-factors respectively, with values greater than 100 set as red. Active site loops 1, 4 and 2′ and regions discussed in the text (dimer interface, '124' loop, helix 3) are indicated in (**A**). Helix 3 is indicated by the red ovals for the V266H variant (**D**). Enzymatically active mutants are shown in the top row (**A**–**C**), and inactive mutants are shown in the bottom row (**D**–**F**).

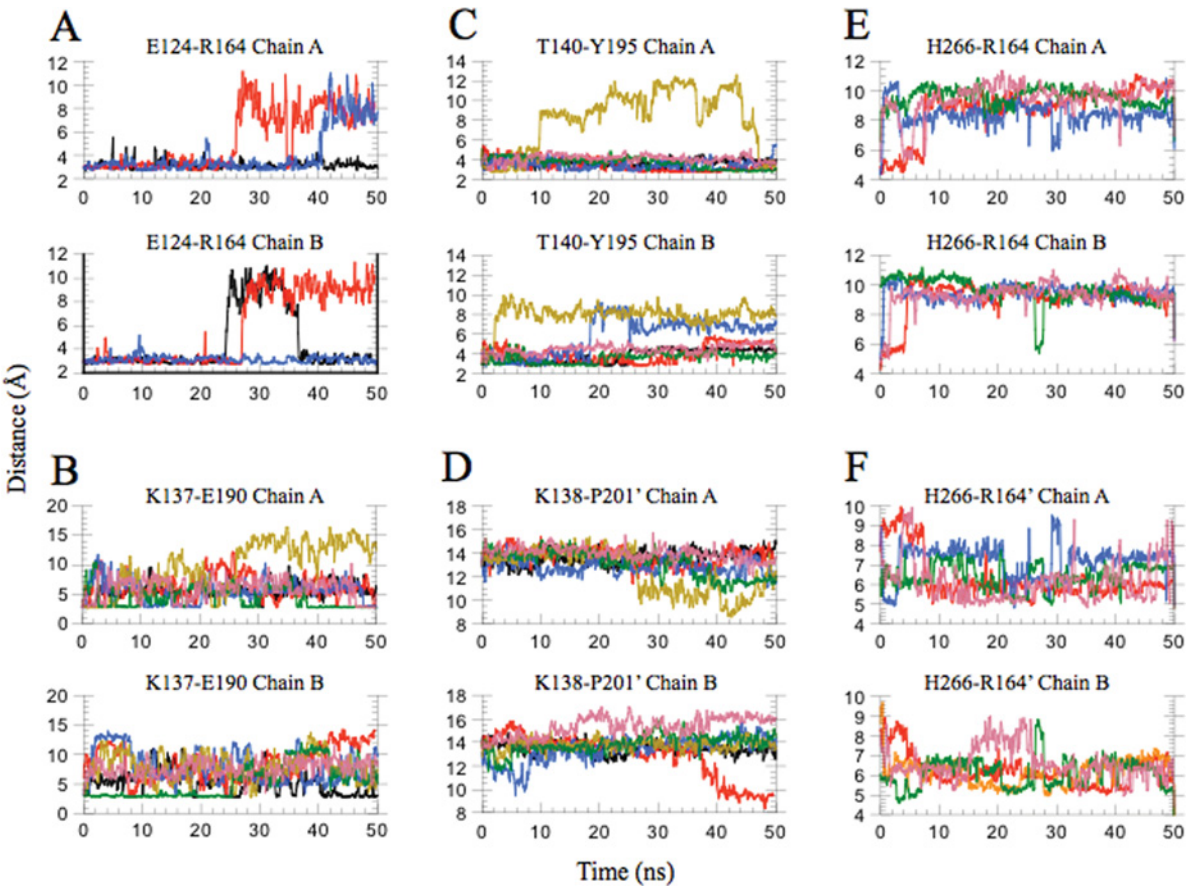

Figure S11 **Distances were calculated for atom pairs over the course of the molecular dynamics simulation**
Plots indicate distance in Å on the *y*-axis, and simulation time in ns on the *x*-axis. Atom pairs are indicated on each plot. The following colour scheme is used for each protein: wild-type (black), V266H (red), Y197C,V266H (blue), E124A,Y197C (olive), E124A,V266H (green) and E124A,Y197C,V266H (purple).

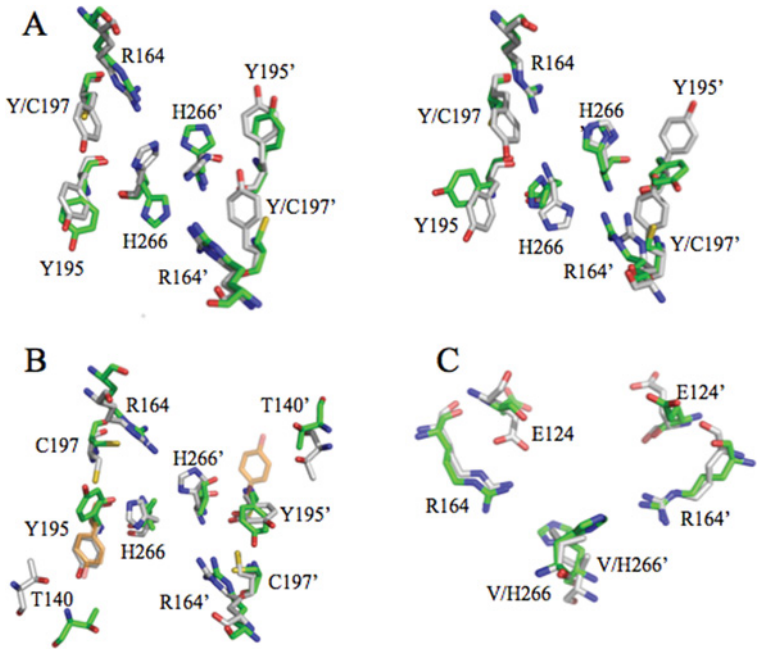

Figure S12 **Comparison of conformations of key residues in the dimer interface from molecular dynamics simulations** (**A**) Movement of His^{266} for the V266H (grey) and Y197C,V266H (green) variants at 2 ns (left) and 50 ns (right). (**B**) In monomer B of Y197C,V266H (grey), at 30 ns Tyr^{195} moves from the native orientation (wild-type conformation shown in orange) and points towards the cavity introduced by Cys^{197}. This movement occurs in both monomers of the E124A,Y197C variant (green). (**C**) At 30 ns, Glu^{124} in monomer B of wild-type caspase 3 (grey) transiently moves to a solvent-exposed position. In the V266H variant (green), the same movement for Glu^{124} is observed in both monomers, and the side-chain remains in the solvent-exposed position for the duration of the simulation. For (**A**–**C**), monomer B is indicated by the prime (′) notation.

Table S1 Summary of data collection and refinement statistics

Parameter	Wild-type*	V266H	Y197C	E124A	Y197C, V266H	E124A, V266H	E124A, Y197C	E124A, Y197C,V266H
Space group	I222	C2	I222	I222	I222	C2	C2	I222
Unit cell								
a (Å)	68.73	109.91	69.33	69.02	68.55	109.36	109.82	68.89
b (Å)	84.40	96.77	84.49	84.75	84.60	96.73	96.78	85.52
c (Å)	96.35	69.78	95.92	96.26	96.48	69.21	69.18	96.34
α (°)	90	90	90	90	90	90	90	90
β (°)	90	127.19	90	90	90	127.11	127.39	90
γ (°)	90	90	90	90	90	90	90	90
Temperature (K)	100	100	100	100	100	100	100	100
Resolution (Å)	1.40	1.70	1.64	1.78	1.72	1.86	1.68	1.69
Number of reflections	54 279	54 485	36 196	25 638	33 032	33 894	64 820	31 715
Completeness (%)	97.9	84.7	93.2	93.5	97.7	63.7	97.1	98.5
Redundancy	5.8	3.5	4.8	6.4	4.8	1.7	3.5	4.6
Average I/σ	46.2 (1.34)	36.7 (11.6)	15.1 (5.9)	20.2 (1.99)	28.4 (11.9)	40.4 (14.6)	49.2 (16.7)	20.0 (6.3)
R_{work} (%)	19.6	19.8	18.0	23.8	16.2	16.0	19.6	16.0
R_{free} (%)	20.7	23.3	19.9	28.1	18.5	21.0	23.0	19.2
R_{merge} (%)†	5.9	6.5	13.2	17.3	11.1	6.6	5.1	6.6
RMSD for bond lengths (Å)	0.005	0.007	0.007	0.007	0.007	0.007	0.007	0.007
RMSD for bond angles (°)	1.30	1.15	1.14	1.10	1.19	1.06	1.15	1.12
No. of protein atoms	1974	3905	2060	1996	2086	3848	3824	1989
No. of water molecules	290	386	263	239	207	369	401	261

*PDB code 2J30.
†$R_{merge} = \Sigma_h \Sigma_i |I(h,i) - I(h)| / \Sigma_h \Sigma_i I(h,i)$, where $I(h,i)$ values are symmetry-related intensities and $I(h)$ is the mean intensity of the reflection with unique index h. $R_{work} = \Sigma_h |F_{obs}| - |F_{calc}| / \Sigma |F_{obs}|$,where F_{obs} and F_{calc} are observed and calculated structure factors respectively. $R_{free} = \Sigma_T |F_{obs}| - |F_{calc}| / \Sigma_T |F_{obs}|$,where T is a test dataset of 10% of the total reflections randomly chosen and set aside prior to refinement.

Table S2 Structure files used to compare the position of Tyr^{195}

PDB code	Resolution (Å)	Space group
1QX3	1.9	$P2_12_12$
1NME	1.6	I222
2CJY	1.67	I222
2CJX	1.7	I222
2DKO	1.06	I222
2H5I	1.69	I222
2C2M	1.94	I222
2C2K	1.87	I222
2C1E	1.77	I222
2J30	1.4	I222
2J31	1.5	I222
2J32	1.3	I222
2J33	2.0	I222
2CDR	1.7	I222
2CNO	1.95	I222
2CNN	1.7	I222
2CNL	1.67	I222
2CNK	1.75	I222
3ITN	1.63	I222
3KJF	2.0	$P2_12_12$
3PDO	2.0	I222
3PD1	1.62	I222
3PCX	1.5	I222

REFERENCE

1 Pop, C., Chen, Y.-R., Smith, B., Bose, K., Bobay, B., Tripathy, A., Franzen, S. and Clark, A. C. (2001) Removal of the pro-domain does not affect the conformation of the procaspase-3 dimer. Biochemistry **40**, 14224–14235

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