Supplementary information

Methods and materials

Mutagenesis of Casp6. ProCasp6(TETD^{179})C163A,D193A was generated using overlapping PCR mutagenic oligonucleotides.

Protein preparation. The caspases constructs were cloned in pET21b vector with a C-terminal $(His)_6$-tag and expressed in *E. coli* Rosetta (DE3) strain at 18 ºC for 20 hrs. Homogeneous proteins were obtained by two-step purification, nickel chelating column (5 ml HisTrap HP column, GE Healthcare) and gel filtration chromatography (120 ml Superdex-75, GE Healthcare), and 10 mM dithiothreitol (DTT) was added to the purified proteins. The active caspases constructs were kindly provided by Dr Guy Salvesen (Burnham Institute, CA).

Proteolytic processing of proCasp6C163A by active caspases.

The substrate, 3 μM (0.1 μg/μL) purified caspases variants (proCasp6C163A, Casp6D193A, proCasp6(TETD^{179})C163A,D193A or Casp3C163A) was incubated with 30 nM (1 ng/μL) active Casp6, Casp7, Casp8 or Casp6D193A (Fig. S2) in 20 mM HEPES, pH 7.4, 50 mM NaCl, 2 mM EDTA, 0.1% CHAPS and 5 mM DTT at 37ºC for 24 hrs. Samples were analyzed by 15% SDS-PAGE.

Fig. S1

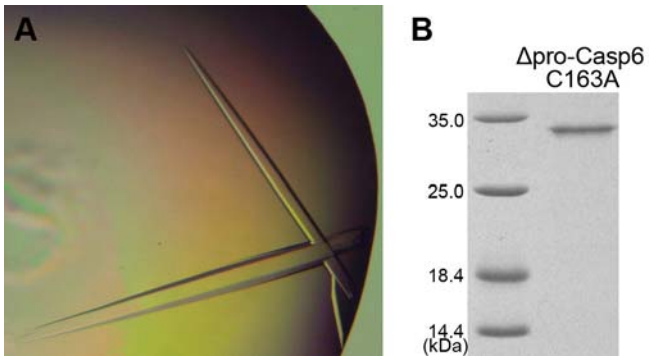

Fig. S1. The Δpro-Casp6C163A was expressed as a single contiguous peptide. (**A**) The crystals of Δpro-Casp6C163A. (**B**) Coomassie-stained SDS-PAGE gel of the crystals of Δpro-Casp6C163A. The crystals in (**A**) were dissolved in water and analyzed by SDS-PAGE.

Fig. S2

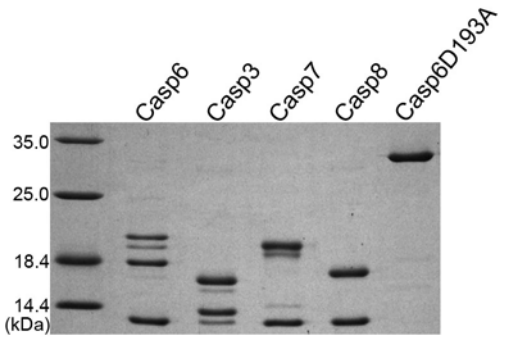

Fig. S2. Coomassie blue-stained SDS-PAGE gel showing all of the proteins used as active enzyme in this study. Each lane contains 1 µl of the proteins at a concentration of 1 µg/µl.

Fig. S3

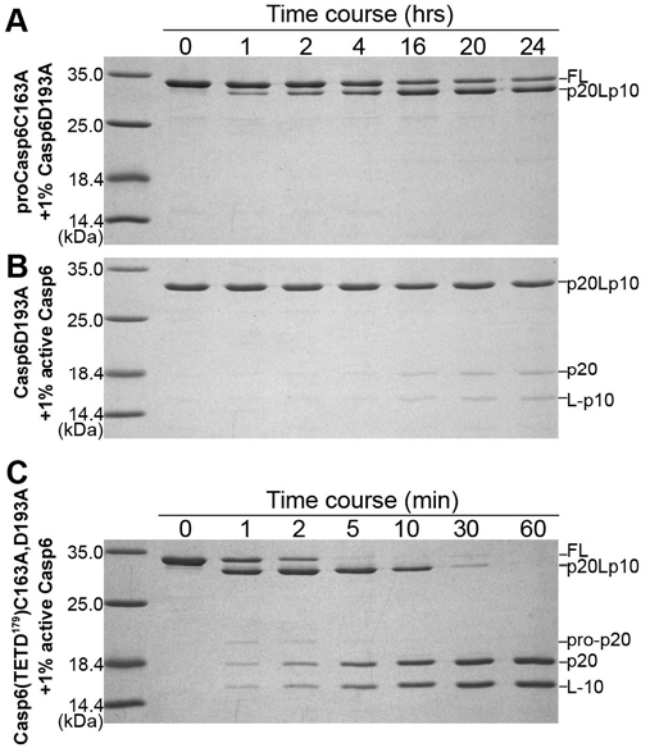

Fig. S3. TETD[23] is a much better substrate for Casp6 than DVVD[179]. Coomassie blue-stained gel of (**A**) proCasp6C163A cleaved by 1% Casp6D193A, showing Casp6D193A has the ability to cleave proCasp6C163A at D23; (**B**) Casp6D193A cleaved by 1% active Casp6, showing active Casp6 only slightly cleaved Casp6D193A at D179; and (**C**) Casp6(TETD[179])C163A,D193A cleaved by 1% active Casp6, showing that when DVVD[179] was mutated to TETD, active Casp6 efficiently cleaved proCasp6(TETD[179])C163A,D193A at the new D179 (TETD) site.

Fig. S4

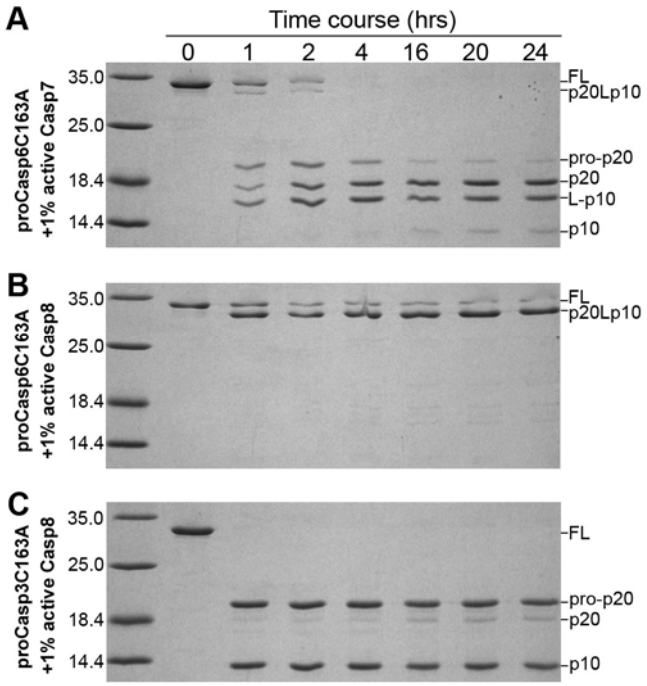

Fig. S4. Casp6 processed by other caspases. (**A**) Pro-Casp6C163A cleaved by 1% active Casp7. (**B**) Pro-Casp6C163A cleaved by 1% active Casp8. (**C**) Pro-Casp3C163A cleaved by 1% active Casp8, as a control to show Casp8 has high activity.

Fig. S5

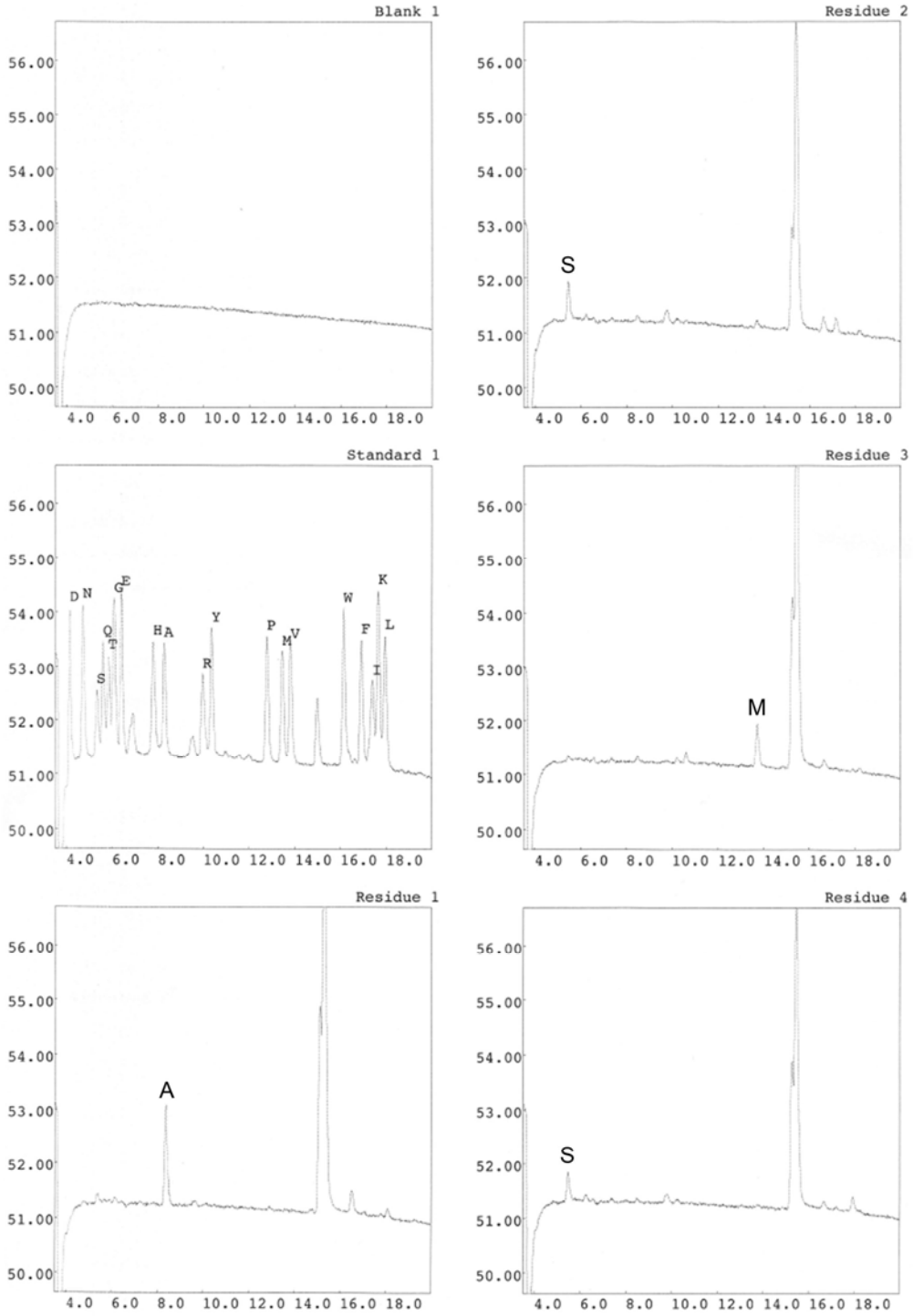

Fig. S5. N-terminal sequencing result of the pro-p20 band of Fig. 2I. The N-terminal four residues were sequenced by Edman degradation, and the sequence of the pro-p20 band was ASMS. The mutant proCasp6D23A was cloned in pET21b vector between *Nhe*I and *Xho*I, and N-terminal sequence of the translated protein was MASMSSAS, and the first methionine was cleaved when expressed in bacteria.

Fig. S6

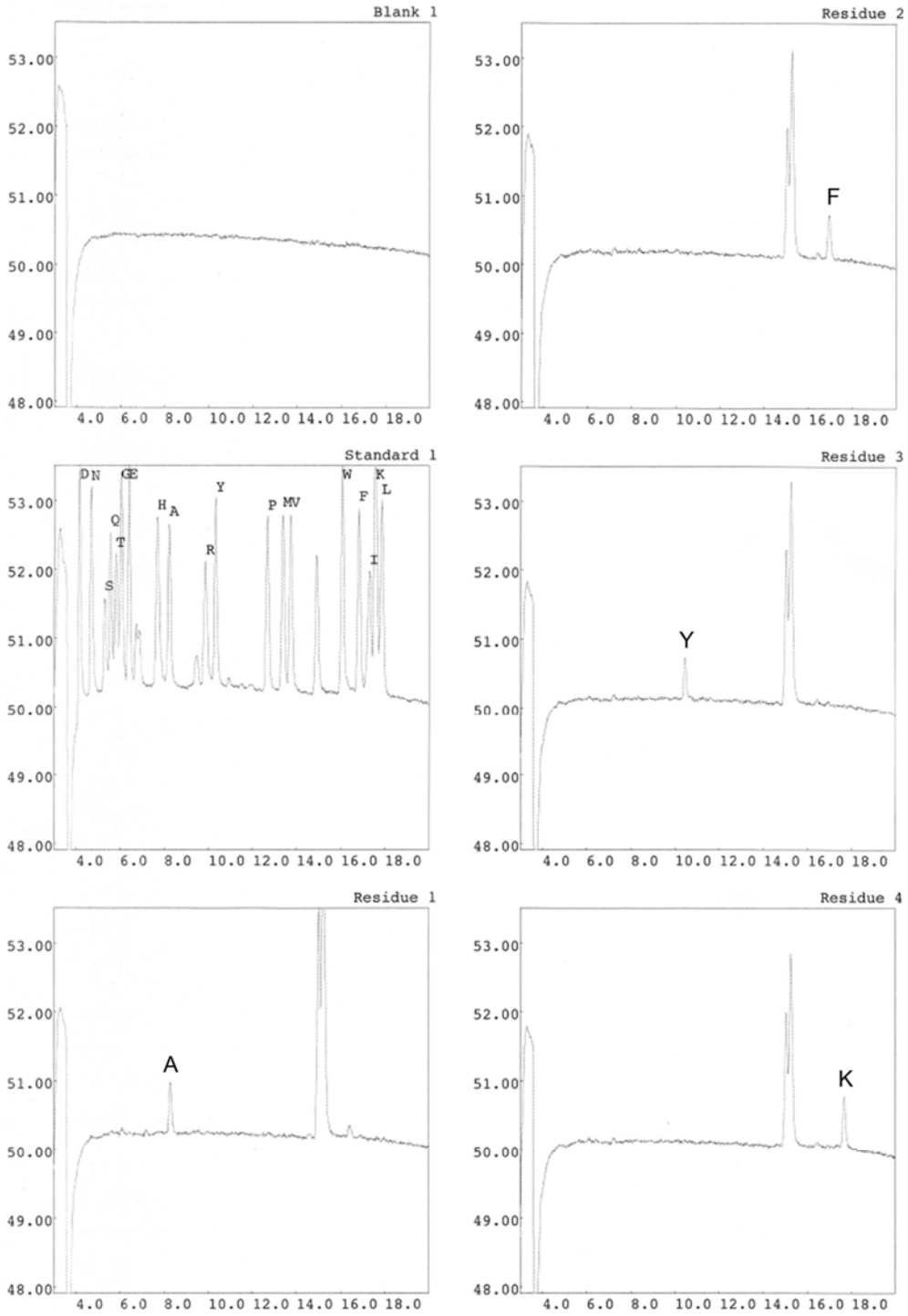

Fig. S6. N-terminal sequencing result of the p20L band of Fig. 2I. The N-terminal four residues were sequenced by Edman degradation, and the sequence was AFYK, which are the 24 to 27 residues of Casp6.