SI Appendix

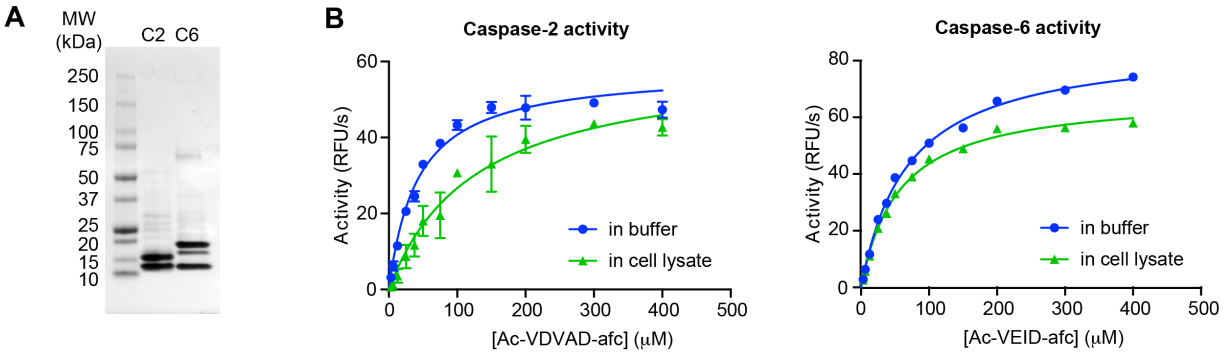

Figure S1. A. Gel electrophoresis of active two-chain recombinant caspase-2 and caspase-6 purified from *E. coli*. **B.** The activity of purified capase-2 and caspase-6 in buffer is comparable to their respective activity in Jurkat cell lysate indicating no significant endogenous inhibitor or activator. No detectable activity was detected by adding zVAD-fmk (100-fold), suggesting that this pan-caspase inhibitor can be use to quench the exogenous enzyme. Ac-VDVAD-afc and Ac-VEID-afc was used to monitor caspase-2 and -6, respectively.

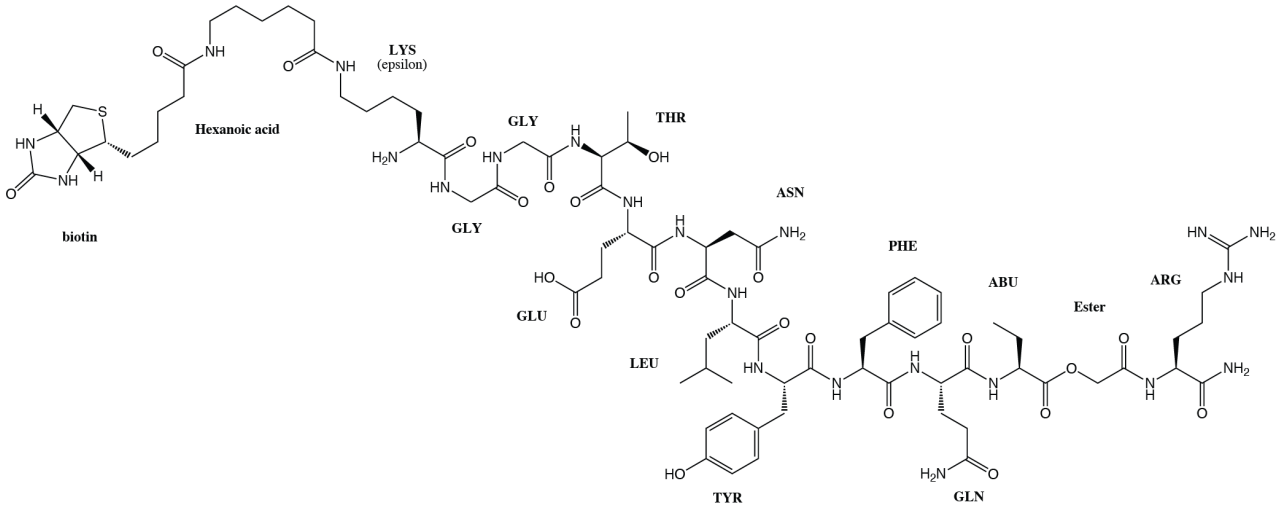

Figure S2. Chemical structure of the ester tag. TEVest4B contains a biotin for avidin capture linked by hexanoic actid to the ε-lysine of KGGT. This is followed by the TEV protease recognition sequence (ENLYFQ↓), then a non-standard amino butyric acid (Abu) for peptide identification, and the ester used for subtiligase tagging which contains a terminal Arg for solubility.

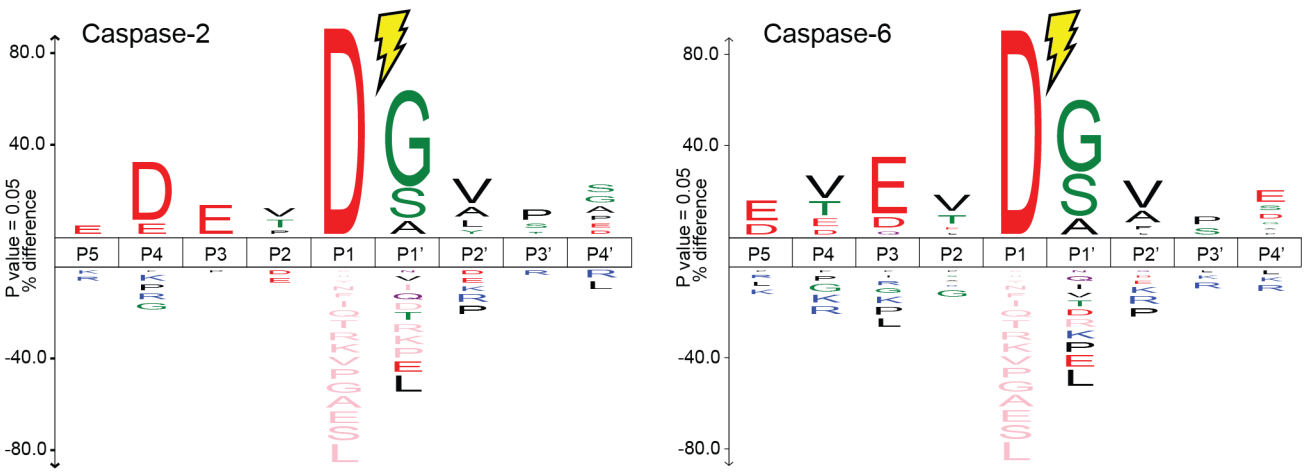

Figure S3. Caspase-2 and -6 specificity. Sequence recognition motifs of caspase cleavages for caspase-2 and caspase-6 shows a consensus DEVD↓(G/S/A) and VEVD↓(G/S/A) motif (P value = 0.05), respectively. Both had a small preference for negatively charged residues at P5 position.

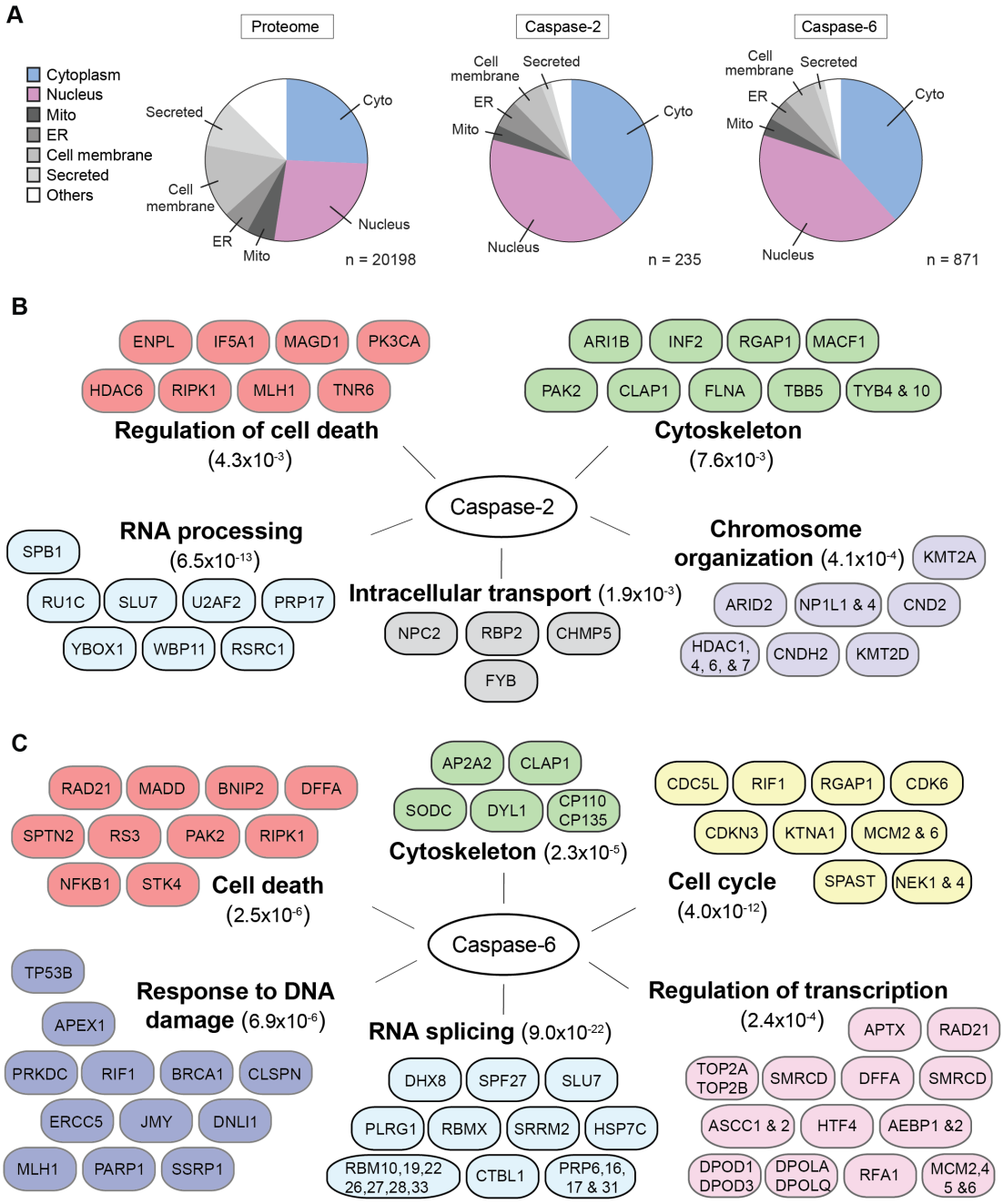

Figure S4. General biological locations, cleavage sites, and functions of caspase-2 and -6 substrates. A. Cellular location of the caspase substrates compared to the full proteome. **B-C**. Gene ontology analysis of caspase-2 and -6 substrates highlights their respective biological roles. A few examples for major groupings are shown, with the p-values per category provided in parentheses. The full list is shown in Datasets S3 and S4.

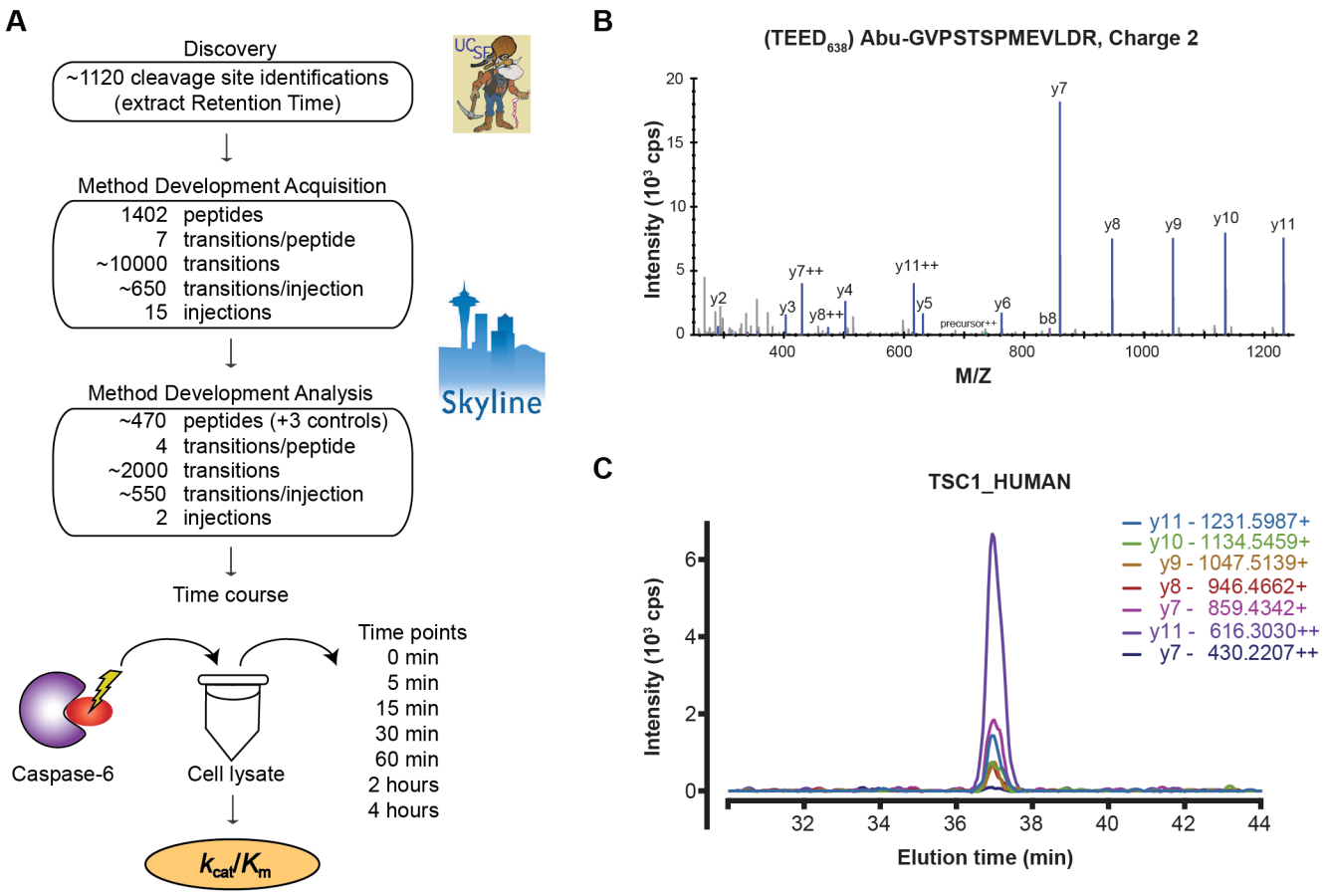

Figure S5. SRM method development for caspase-2 and caspase-6 substrates. A. Schematic of the methodology used to measure kinetics of proteolysis. The peptides corresponding to the caspase cleavage sites were identified using the discovery experiments shown in **Fig. 2**. Using the corresponding retention times, the 7 most intense transitions were selected for each peptide. To monitor the ~8000 transitions, 15 injections of the later time point (4 hour) were used to obtain SRM data for each peptide using a 15 minute retention time window. **B**. The mass spectrum and associated chromatogram of the ($TEED_{638}$) Abu-GVPSTSPMEVLDR peptide from the protein TSC1_HUMAN cleaved by caspase-6 is shown as an example. The 4 most intense transitions were then selected to monitor the appearance of the cleaved peptides as a function of time (see **Fig. 4**) The SRM data were acquired on a QTRAP 5500 LC-MS/MS.

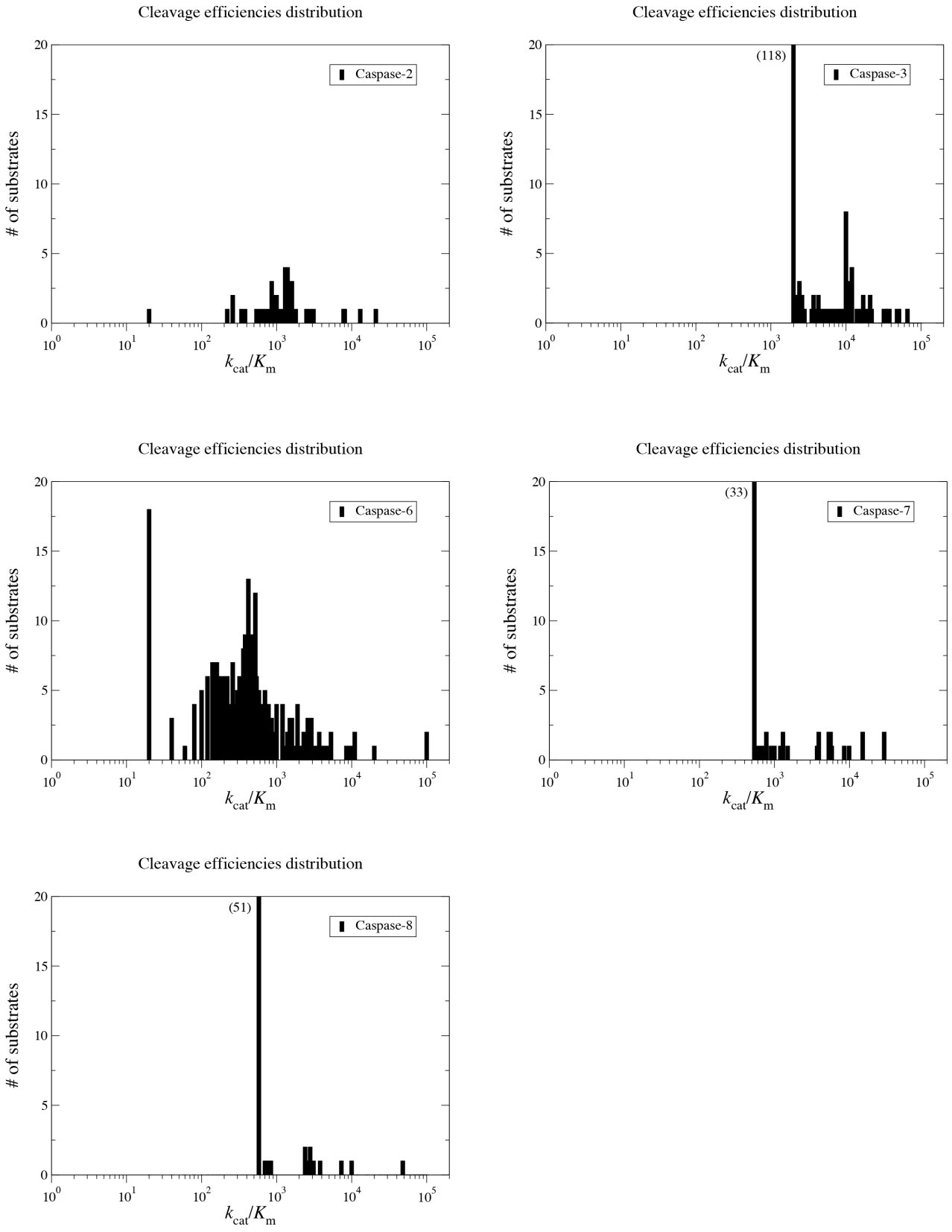

Figure S6. Distribution of catalytic efficiency values for caspase-2, -3, -6, -7, and -8. The broader distribution of k_{cat}/K_M values for caspase-2 and -6 presented in this paper likely reflects the larger data set from improved methods and instrumentation.

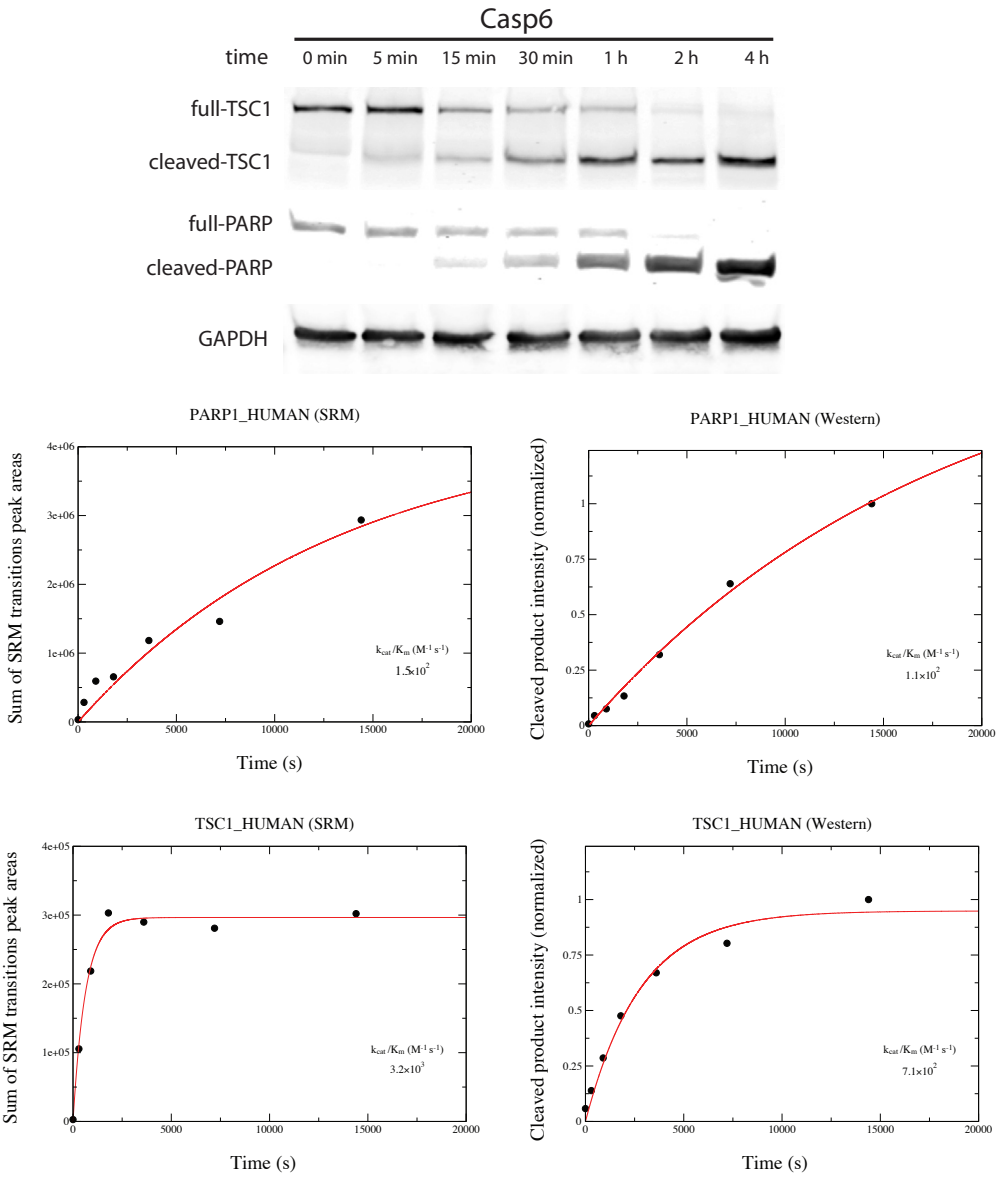

Figure S7. Western blot of caspase-6 substrates. Hamartin (TSC1) and Poly [ADP-ribose] polymerase 1 (PARP-1) antibodies were used to monitor proteolysis of these two substrates by caspase-6 in Jurkat cell lysate. Endogenous proteases were quenched before addition of purified caspase-6 (500 nM) and proteolysis was monitored as a function of time. Gel imaging was performed on a Li-cor (near-infrared instrument). Representative Western blots are shown. The loading control, GAPDH, showed no change over time, while we measured a k_{cat}/K_m of 7.1×10^2 for TSC1 and 1.1×10^2 for PARP-1, compared to 3.2×10^3 and 1.5×10^2 by SRM, respectively. The small discrepancies in rates are probably due to inherent differences in the methods: Western blot values depend on consistent protein transfers, the quality of the antibodies used for a given substrate, the differential specificity of the antibodies for the full length and cleaved fragments, gel migration, gel imager resolution, and software used for analysis. On the other hand, the SRM data depends on the consistency of N-terminal labeling, sample processing, trypsinization, and consistent quantitation LC-MS/MS, and is a lot more sensitive technique in general than WB.

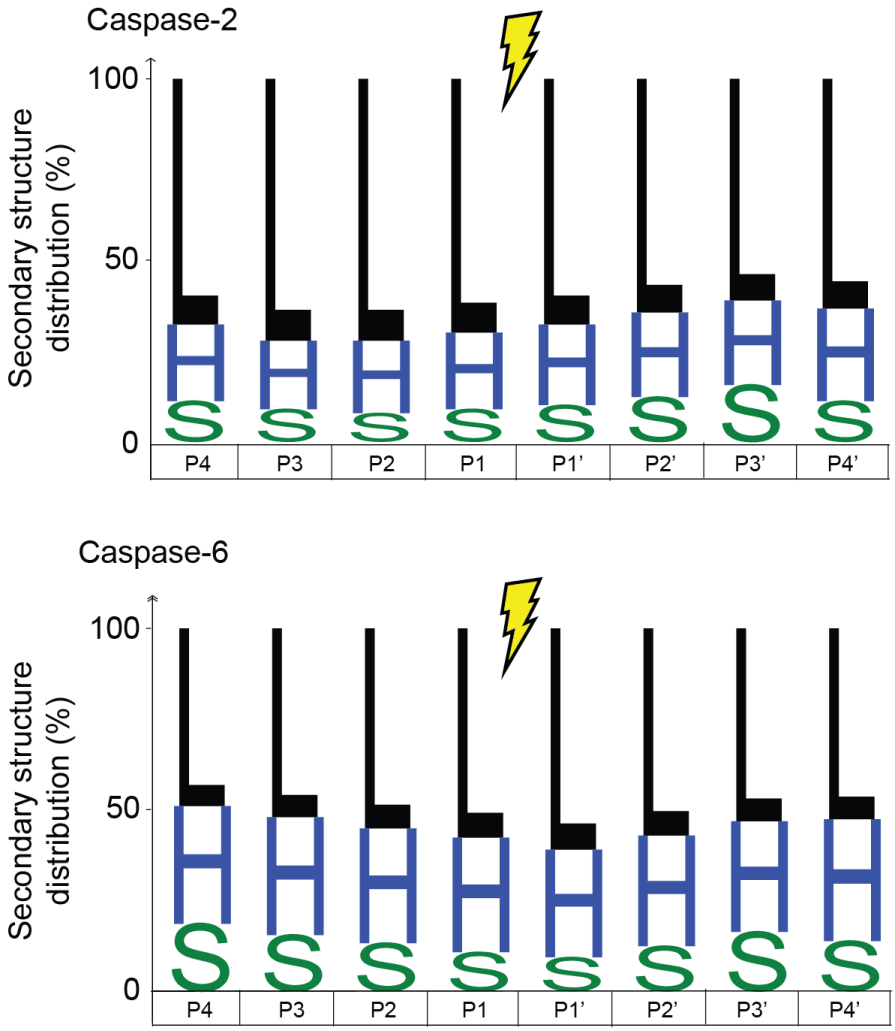

Figure S8. Secondary structure of caspase-2 and -6 cleavage sites from P4 to P4'. The secondary structure distribution of the caspase cleavage sites identified in our discovery experiment showed ~50% of the residues are located in loop (L), ~35% in α-helices, and ~15% in β-sheets. The structural data was extracted from known structures, 90 for caspase-2 and 357 for caspase-6. The secondary structure information was obtained from Barkan et al. (2010), where all possible caspase cleavage sites in the human proteome were reported.

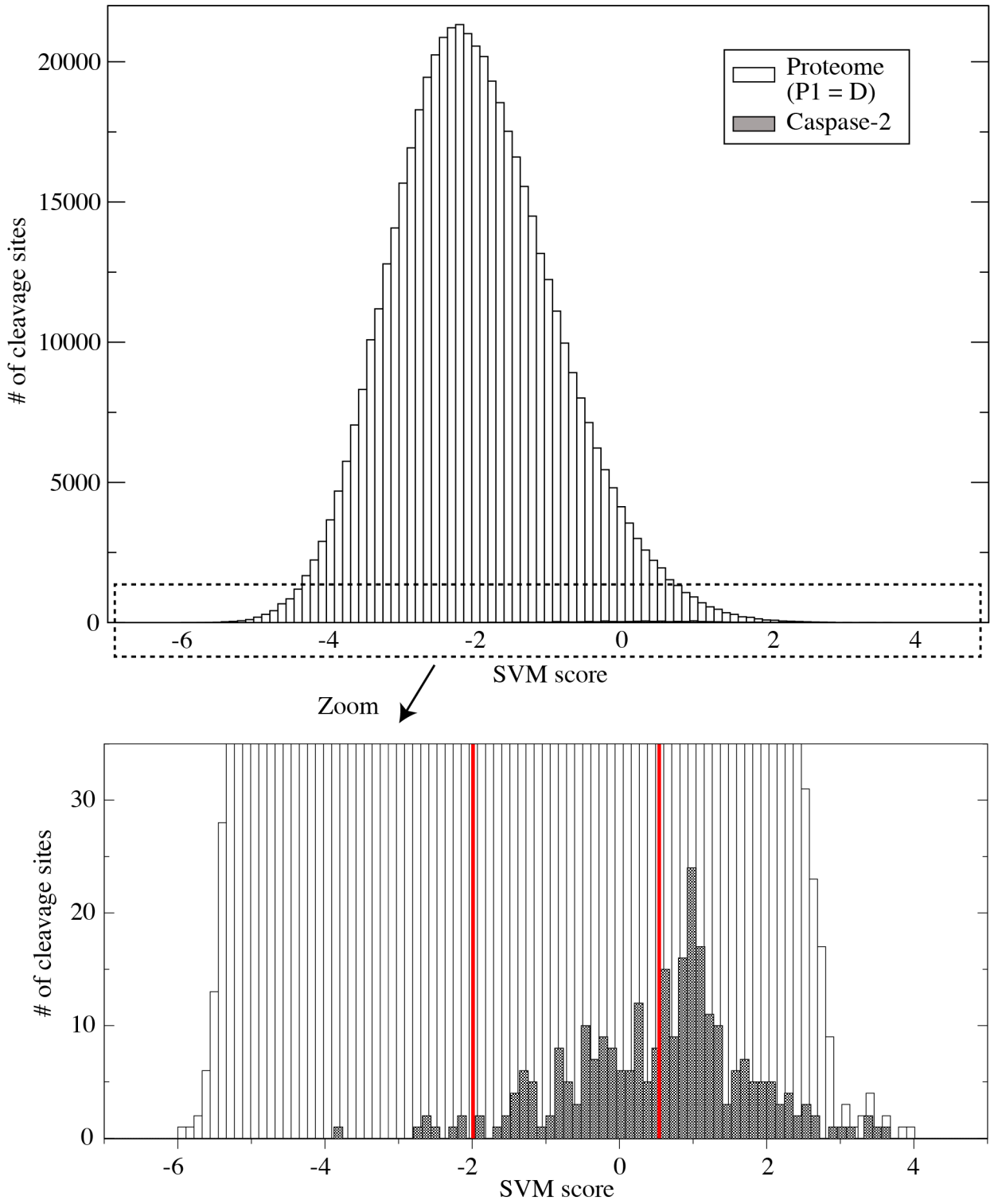

Figure S9. Observed and predicted caspase-2 cleavage sites correlate well. Comparison between the 277 unique N-termini cleavage sites identified in our study with the predictions made by Barkan et al. (2010) for every tetrapeptide in the human proteome with an aspartate at P1 position.

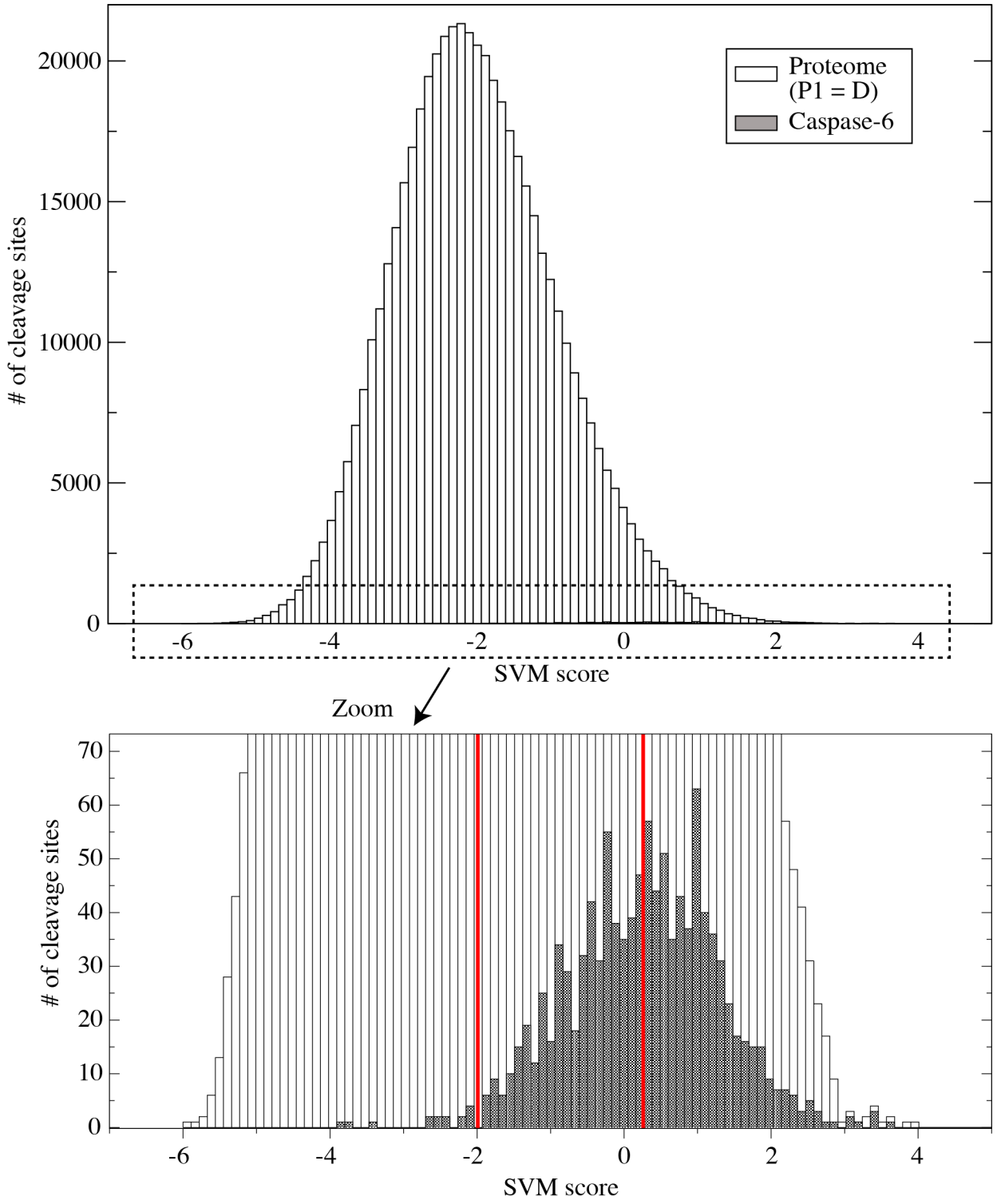

Figure S10. Observed and predicted caspase-6 cleavage sites correlate well. Comparison between the 1120 unique N-termini cleavage sites identified in our study with the predictions made by Barkan et al. (2010) for every tetrapeptide in the human proteome with an aspartate at P1 position.

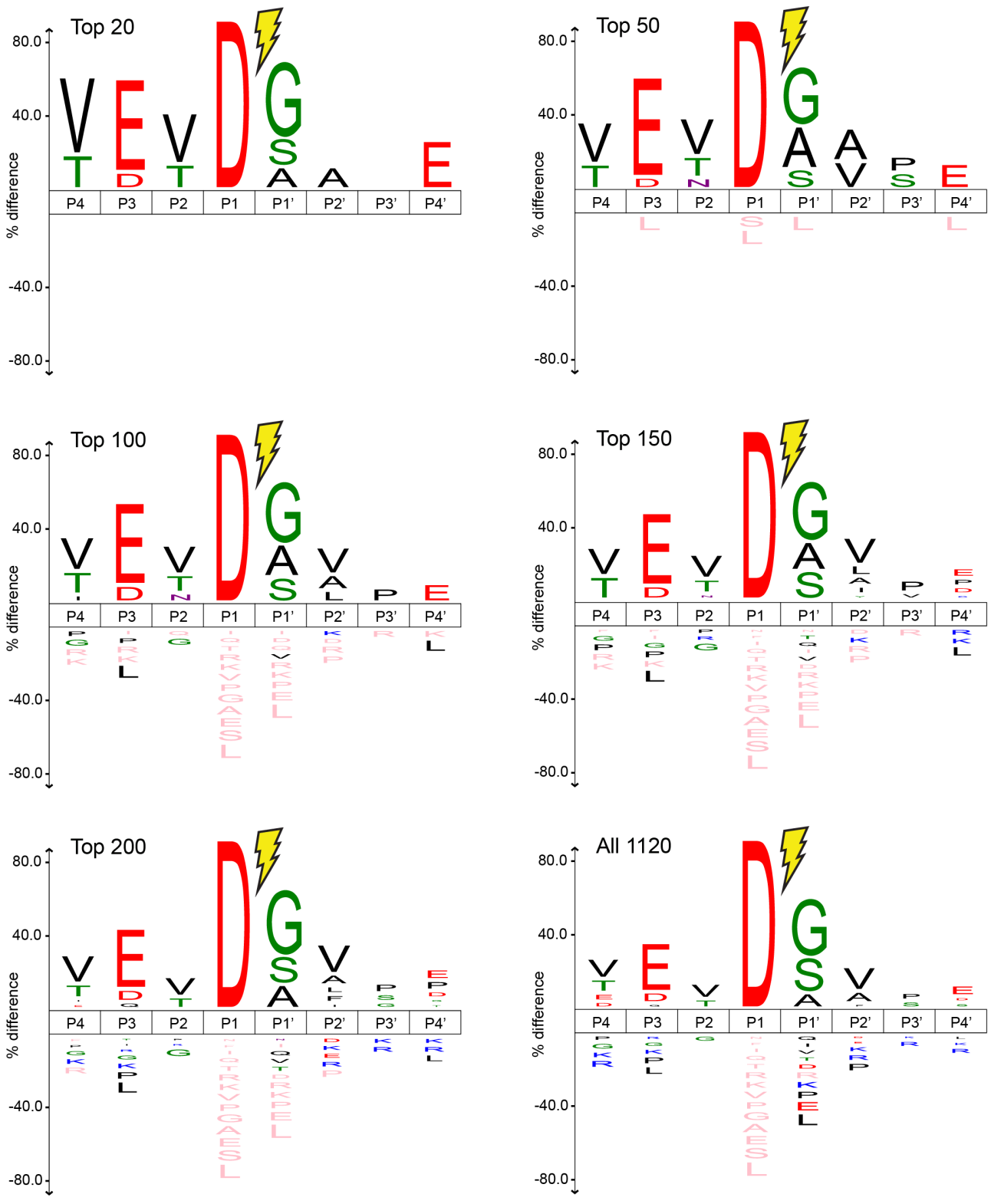

Figure S11. Caspase-6 recognition motifs for fastest cleaved substrates. Top 20, 50, 100, 150, 200, and all 1120 protein cleavage sites show virtually identical sequence specificity.

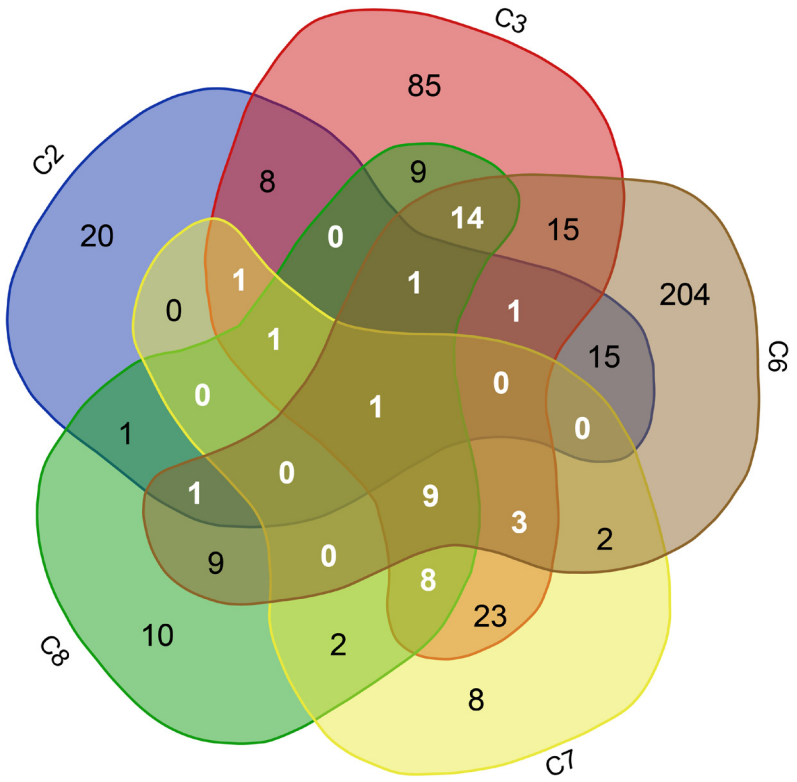

Figure S12. Common substrates between Caspase-2. -3, -6, -7 and -8 for which rate constants were obtained by SRM. Number of common substrates cleaved by at least three different caspases is shown in white. The substrate DCGR8, depicted as “1” in the center of the diagram, was cleaved by all the caspases but at rates that varied over 100-fold (Dataset 7). There were 11 substrates cut by four of the five caspases with rates that varied over 100-fold. The vast majority are unique to individual caspases.

Dataset S1

Caspase-2 discovery MS dataset

Dataset S2

Caspase-6 discovery MS dataset

Dataset S3

Gene Ontology (GO) analysis for caspase-2 substrates

Dataset S4

Gene Ontology (GO) analysis for caspase-6 substrates

Dataset S5

Quantitative MS-based k_{cat}/K_M determination of caspase-2 substrates

Dataset S6

Quantitative MS-based k_{cat}/K_M determination of caspase-6 substrates

Dataset S7

Pair-wise comparisons of rates for common substrates cleaved by caspases-2, -3, -6, -7 and -8

Dataset S8

Rates of proteolysis for protein substrates compared to their corresponding peptide sequences