Abstract

Caspase-3 activation and function has been well defined during programmed cell death, but caspase activity, at low levels, is also required for developmental processes such as lymphoid proliferation and erythroid differentiation. Post-translational modification of caspase-3 is one method used by cells to fine-tune activity below the threshold required for apoptosis, but the allosteric mechanism that reduces activity is unknown. Phosphorylation of caspase-3 at a conserved allosteric site by p38-MAPK promotes survival in human neutrophils, and the modification of the loop is thought to be a key regulator in many developmental processes. We utilized phylogenetic, structural, and biophysical studies to define the interaction networks that facilitate the allosteric mechanism in caspase-3. We show that, within the modified loop, S150 evolved with the apoptotic caspases, while T152 is a more recent evolutionary event in mammalian caspase-3. Substitutions at S150 result in a pH-dependent decrease in dimer stability, and localized changes in the modified loop propagate to the active site of the same protomer through a connecting surface helix. Likewise, a cluster of hydrophobic amino acids connects the conserved loop to the active site of the second protomer. The presence of T152 in the conserved loop introduces a “kill switch” in mammalian caspase-3 while the more ancient S150 reduces without abolishing enzyme activity. These data reveal how evolutionary changes in a conserved allosteric site result in both a common pathway for lowering activity during development as well as introducing a more recent cluster-specific switch to abolish activity.
Caspases (cysteiny1-aspartate specific proteases) are an ancient class of cysteiny1 proteases that maintain cellular homeostasis by controlling the cell death program, apoptosis, when activated at high levels, and they are critical to cell development when activated at low levels (1, 2). Caspases most likely evolved in metazoans from ancestral functions used in immune responses, and due to gene duplication events followed by neofunctionalization, human cells contain eleven caspases that are critical to the immune response or to apoptosis (3). Within the apoptotic caspases, two subclasses are further described based on entry into the apoptotic cascade. Initiator caspases-8, -9, and -10 are responsive to external or internal signals and initiate apoptosis by activating the downstream effector caspases-3, -6, -7, where caspase-3 is the primary executioner of the cell death program (4). Activation of the pre-synthesized zymogen results in an overwhelming response to stimuli, leading to the dismantling of the cell without eliciting an immune response. Although the caspase degradome is a current topic of study, the role of caspases in apoptosis is reasonably well understood (3, 5). Hallmarks of apoptosis are largely observed through the caspase-3 cleavage of Rho effector protein (ROCK 1), apoptotic chromatin condensation inducer in the nucleus (acinus), nuclear lamin proteins, and caspase-activated DNase (CAD) as well as many others, which leads to membrane blebbing, nuclear condensation, and DNA fragmentation (6, 7).

While caspase-3 is a well-known executioner of apoptosis, it is also a key protease in many non-apoptotic processes (8). For example, caspase-3 activity is important for remodeling the cytoplasm and in the development of eye lens and inner ear (9, 10). Caspase-3 is also involved in differentiation of many cell types, such as erythroblasts, keratinocytes, macrophages, lens epithelial cells, sperm cells, skeletal muscles, embryonic stem cells (ESCs), osteoblasts, and placental trophoblasts (8, 11–13). Importantly, caspase-3 is required for terminal erythroid differentiation, starting from the mature BFU-E stage onward, and either inhibition or knockout (KO) of caspase-3 causes a decrease in proliferation due to halted cell cycle progression through the G2/M phase and prevention of terminal erythroid maturation (14, 15). Substrate cleavage data suggest that cells contain regulatory mechanisms to direct caspase-3 activity, although, at present, it is not clear how cells obtain sufficient activity for developmental responses yet maintain activity levels below the threshold required for apoptosis (8).

There are three general post-translational mechanisms that affect the population of active caspase-3 in cells: changes in zymogen maturation, active site-directed inhibitors, and allosteric inhibition through post-translational modifications or metal binding (Figure 1A). Maturation of the caspase-3 zymogen, for example, is inhibited by protein kinase CK2, which phosphorylates T174 and S176 in the intersubunit-linker (IL) (see Figure 1). Phosphorylation of the IL prevents initiator caspases from cleaving caspase-3 at D175, thus inhibiting maturation (16). In the mature caspase-3, X-linked inhibitor of apoptosis (XIAP) competitively inhibits the enzyme by binding to the active site and targeting the caspase for proteasomal degradation (17). In addition, the catalytic cysteine is sensitive to nitrosylation, although the modification may affect only a subset of caspase-3 localized to the mitochondria (18).

Caspase-3 is also allosterically regulated through post-translational modifications, including glutathionylation and phosphorylation (19–21). While systematic
global studies of caspase phosphorylation have not been done, several phosphorylation sites have been mapped on individual caspases (20, 22, 23). Based on the current data, phosphorylation sites of mature caspases can be characterized in two categories: those located near the active site that likely prevent substrate binding, and those located some distance away from the active site that affect caspase activity allosterically. One allosteric site of interest, S150, is located in a loop at the C-terminal end of helix-3 near the dimer interface, and this serine residue (or threonine in caspase-7) is conserved in all human caspases except caspases-10 and -14 (4). Helix-3 is an important regulator of effector caspase activity because fluctuations in the N-terminal region of the helix disrupt conserved water networks in caspase-3 and reposition the catalytic cysteine and histidine (24, 25). In addition, the same region of helix-3 and the adjoining β-strands undergo a coil-to-helix transition in caspase-6, which also disrupts the catalytic residues by extending helix-3 (22).

Phosphorylation of S150 by p38-MAPK was reported to promote survival in human neutrophils (26). Introducing a phospho-null mutant, S150A, into cells rendered Fas-induced apoptosis in neutrophils insensitive to p38-MAPK inhibition, suggesting that phosphorylation of S150 inhibits catalytic activity (26). In addition to effector caspases, the initiator caspase-8 is phosphorylated by p38-MAPK at the same site (S347), suggesting a common mechanism for controlling caspases by allosterically inhibiting the enzyme. The S150 allosteric loop is approximately 33 Å from either active site of the caspase-3 dimer (intra-protomer or inter-protomer) (Figure 2), so it is not clear how the signal from phosphorylation of S150 would propagate to either active site. Although the prevailing view is that phosphorylation of the loop is a common mechanism for inhibiting caspase activity, the previous in cellulo data for caspase-3 also suggested that phosphorylation at S150 may decrease caspase stability, resulting in increased turnover in the cell (26).

We investigated the allosteric mechanism conferred by modifying the loop containing S150 in caspase-3, and we show that, while there is no change in activity against small peptide substrates, the substitutions affect catalytic efficiency against a protein substrate. The effect is due to a pH-dependent decrease in dimer stability. Structural studies and molecular dynamics simulations show that localized changes in the loop propagate through helix-3 and affect the connected β-strand containing the catalytic histidine. Likewise, the modifications disrupt the nearby “loop bundle” that stabilizes the active site of the second protomer (Figure 2), so modifications of the loop affect both active sites. We show that evolutionary changes within the loop of mammalian caspase-3 resulted in the introduction of a “kill switch” in the enzyme where loop modifications abolish, rather than diminish, activity. The evolutionary changes add an additional level of control to the allosteric mechanism in mammalian caspase-3 such that modifications in the common phosphorylation network propagate to both active sites. We note that, in terms of defining the caspase structural units, both the zymogen and the mature caspase-3 are considered a dimer of protomers. Each caspase protomer contains one large (LS) and one small (SS) subunit (Figure 1A) that fold into a single domain containing a six-stranded β-sheet core with five α-helices on the surface (Figure 2). In the zymogen, the intersubunit linker (IL) covalently connects the subunits in the protomer (Figures 1 and 2), while the IL is cleaved in the mature enzyme. Cleavage of the IL (Figure 1A,
maturation) results in active site loop rearrangements and formation of the mature enzyme active site (27, 28).

Results

Evolutionary conservation of helix-3 C-terminal loop

The level of conservation of the loop containing S150, herein called the helix-3 C-terminal loop (H3CL), is not known, aside from its conservation in human caspases. Since current models suggest that modification of S150 represents a common mechanism for inhibiting caspases, we first examined the conservation of the H3CL in order to discern whether the studies of human caspases are representative of other species. To this end, we conducted a comprehensive phylogenetic analysis to probe the level of conservation and functional importance of the H3CL. Our analysis used 1,325 caspase sequences in the NCBI Protein Databank; including caspases-1, -2, -3, -4, -6, -7, -8, -9, -10, and -14. The incorporated species ranged from fish to mammals and span ~500 million years of evolution (29). We calculated the amino acids usage frequency for residues 149-153, which contains S150 and T152 (caspase-3 numbering) (Figure 3 and Supplemental Table 1). For position 150, the data show that a Ser/Thr was observed in >92% of effector caspases (-3, -6, and -7) and in initiator caspases-8 and -9. In contrast, a lower frequency of Ser/Thr was observed in inflammatory caspases - caspase-4 (63.3%), caspases-1 and -2 (40-44%, respectively). Very low frequencies (<5%) were observed in caspases-10 and -14, demonstrating that Ser/Thr is not conserved in the H3CL of those caspases (Supplemental Table 1). We also examined the neighboring T152 because, from previous reports, T152 may also be phosphorylated along with S150 (26). For position 152, the data show that hydrophobic amino acids (Ala, Val, Ile, Leu, Gly) are present in >90% of caspases-3, -6, -7, -8, and -10, while lower frequencies are observed in caspase-9 (51%), and charged amino acids are common in caspases-1, -2, -4, and -14 (Figure 3, Supplemental Figure 3A). Together, the data suggest that position 150 likely evolved as a phosphorylation site after the apoptotic caspases diverged from the inflammatory caspases. The data also show that position 152 is evolutionarily constrained in the apoptotic caspases to require a hydrophobic amino acid.

The analysis of amino acid frequency for the H3CL does not reveal when in evolutionary time the extant residues arose, so we generated a phylogenetic tree for each family member using the maximum likelihood method (30). Neofunctionalization is associated with site-specific rate variation over time and is more readily observed when a phylogenetic tree is parsed into clusters, as shown in Figure 3 (31). For residue 150, the phylogenetic trees support the amino acid frequency data and show that the Ser/Thr residue evolved early in apoptotic caspases and only later in mammals of the inflammatory caspases (caspases-1 and -2 in particular) (Figure 3A and Supplemental Figure 3A). In contrast, T152 evolved in early mammals, while prior evolutionary species utilized alanine or valine (~98%) (Supplemental Table 1). We note that mammalian caspase-3 is the only caspase to utilize a residue at this position with the potential to be phosphorylated while maintaining hydrophobicity in the native state, due to the presence of both the hydroxyl and methyl groups in the side chain. Together, the data suggest that phosphorylation of S150 is an evolutionarily conserved mechanism in apoptotic caspases, while modification of T152 may represent
an allosteric control mechanism that only recently evolved in caspase-3 of mammals.

**Phosphorylation of S150 is predicted to modulate activity while modification of T152 is predicted to abolish activity**

In order to examine the effects of phosphorylating the H3CL, we made several point mutations to mimic phosphorylation at S150 and T152 (Table 1). We introduced phospho-mimetics (Asp and Glu) or phospho-null side-chains (Ala). We also introduced a large polar side-chain (Tyr) at position 150, and we increased hydrophobicity at position 152 by introducing valine. For each of the variants, we determined the steady-state parameters, $k_{\text{cat}}$ and $K_M$, as well as the specificity constant, $k_{\text{cat}}/K_M$, and the results are shown in Table 1. Unlike the previous suggestions, our data show that modifications of S150 (Asp, Glu, Ala, or Tyr) have no effect on enzyme activity, even when a large amino acid (Tyr) is introduced at the site (Table 1) (26). In all cases, the specificity constant was within about three-fold that of wild-type caspase-3. We note, however, that in contrast to previous reports, our data were measured against tetra-peptide substrates using *in vitro* enzymatic assays rather than *in cellulo* apoptosis assays (26).

In contrast to the S150 variants, the T152D substitution abolished activity (Table 1). To determine if the decreased activity was due to the loss of the hydrophobic pocket or to the loss of H-bonds, we made two point mutants (T152A and T152V) to restore local hydrophobicity. In these mutants, the substitution of T152 with alanine removes the hydrogen bonds observed with the hydroxy group and decreases the number of hydrophobic groups in the side-chain, while the isosteric valine simply replaces the hydroxyl group with a methyl group. The results show that both variants (T152A and T152V) retain wild-type levels of activity (Table 1).

In addition to the modification of the mature caspase-3, phosphorylation of S150 was previously shown to occur in the zymogen prior to maturation (26). Procaspase-3 is not fully active because of the covalent connection between active site loops L2 and L2’ (the intersubunit linker), so we wanted to determine if the phosphorylation of the H3CL affected activity of the zymogen. To this aim, we introduced the same mutations within the background of the full-length uncleavable zymogen (D9A,D28A,D275A, called D3A) and determined changes in enzymatic activity. As with the mature caspase-3, only the substitution of T152 with aspartate abolished activity (Table 1), so the effects on the zymogen appear to be the same as those on the mature caspase-3.

We showed previously that the pro-domain increases the stability of caspase-3 (32). Amino acids 1-28 of the pro-domain are intrinsically disordered, but they bind to the protease domain in an extended conformation (33). In order to determine whether the pro-domain modulates the effects due to phosphorylation of S150 or T152, we introduced each of the mutations into the background of mature caspase-3 (cleaved at D175) with an uncleavable pro-domain (D9A,D28A, called D2A) (32). The data show that the presence of the pro-domain does not change the effects of phosphorylation at either S150 or T152. Like the fully mature enzyme, only the substitution of T152 with aspartate resulted in a decrease in activity (Table 1). Overall, the data show that phosphorylation of S150 has no effect on caspase-3 activity when measured against peptide substrates. In contrast, the phosphorylation of T152 is sufficient to abolish activity. The results were not dependent on whether the
intersubunit linker was uncleaved (zymogen) or cleaved (mature) or whether the pro-domain was present.

Our phylogenetic analysis, described above, revealed that the hydrophobic pocket of the H3CL is highly conserved and that T152 is a more recent evolutionary event in mammalian caspase-3 (Figure 3). Based on the consensus sequences generated from the phylogenetic analysis, one observes that the effector caspases contain a polar group at position 149, which, in caspases-3, -6, and -7, H-bonds with the backbone of active site loop 2 across the dimer interface (Figure 2). All caspases contain leucine at position 151, and the hydrophobic side-chain anchors the loop to the protein core (Figure 3C). Caspase-7 contains a charged group at position 153 and is more similar to the inflammatory caspases than to the effector or initiator caspases, which contain glycine at that position. In caspase-7, E176 (equivalent to G153 in caspase-3) forms a salt bridge with R271’ (equivalent to T245 in caspase-3), which is located at the C-terminus of helix 5 and at the base of active site loop 4 (Figures 1B, 3C, 3D). Thus, in caspase-7, the E176-R271’ interaction provides a direct connection between the H3CL of one protomer and the active site of the second protomer.

Because S150 is conserved in caspases-6 and -7, we made point mutations in caspase-6 (S150D) and caspase-7 (T173D) to determine the effects, if any, of phospho-mimetics on enzyme activity. The data reveal that, like caspase-3, the mutations at this conserved site in caspases-6 and -7 also had no significant effect on the activity of these enzymes (Table 1). We note that a recent study by Hardy and coworkers showed similar results for a comparable variant of caspase-7, that of T173E (34). In addition to introducing phospho-mimetic mutations, we removed the salt-bridge between E176 and R271’ in caspase-7 by replacing E176 with glycine, either individually or in combination with the T173D phospho-mimetic. Both variants showed specificity constants similar to wild-type caspase-7 (Table 1), so removing the salt-bridge connecting the two protomers had no effect on activity. Overall, the results for caspases-3, -6, and -7 show that phosphorylation of Ser/Thr150, as assessed through phospho-mimetic substitutions, had no effect on enzyme activity in our in vitro assays. Likewise, variations of the consensus sequence for the H3CL observed in caspase-7, which contains an additional salt-bridge across the dimer interface, does not affect activity. Therefore, phosphorylation of the highly conserved Ser/Thr150 in the H3CL does not decrease activity in vitro, but disrupting the hydrophobic cluster of the H3CL with the loop bundle abolishes activity.

Finally, in order to examine putative interaction networks that propagate allosteric signals from the conserved loop to one or both active sites, we used NetPhos 3.1 and identified two possible sites: T245, which is located at the C-terminus of helix-5; and S249, which is located in active site loop 4 (Figure 2C) (35). This region is also part of the loop bundle, on the side opposite from the H3CL (Figure 2A). Interestingly, T245 is in the same position as R271’ in caspase-7, but the side-chain, while solvent-exposed, is too short to interact across the dimer interface with the H3CL. In contrast, S249 is located in active site loop 4 and forms several H-bonds with the C-terminal end of L4 to stabilize the loop conformation. The N-terminal region of L4, specifically the amide nitrogen of F250, H-bonds with the P4 side-chain of the substrate, and the C-terminal end of L4 forms part of the hydrophobic S2 binding pocket (Figure 2C). In order to examine the effects of phosphorylation of either site, we substituted
each amino acid with aspartate (Table 1). Consistent with the loss of the salt-bridge in caspase-7, the substitution of T245 with aspartate had no effect on enzymatic activity. In contrast, substitution of S249 with aspartate abolished activity, and this was true also for the double mutant, T245D,S249D (Table 1). Overall, the data show that phosphorylation of sites that flank the loop bundle (T152 or Ser249) abolish activity and suggest that changes in the H3CL propagate across the dimer interface, through the loop bundle, which consists of active site loops of the second protomer.

**Modifications of the H3CL affect dimer stability**

In order to examine whether modifications of the H3CL affect activity for a larger substrate, we examined the cleavage of caspase-7 by the caspase-3 variants. As part of the maturation process for caspase-7, the pro-domain is removed by caspase-3 at \( \text{DSVD}^{23} \), and the protomer is cleaved in the intersubunit linker at \( \text{NDTD}^{206} \) to generate the large and small subunits. Cleavage of larger protein substrates can distinguish effects that may not be observed with small peptides that bind only to the active site (36, 37). We also examined cleavage at two pHs (7.5 and 6) because the pH of the cytoplasm is known to decrease during apoptosis (38). The data show that the pro-peptide of caspase-7 is efficiently removed by wild-type caspase-3 and that the intersubunit linker is readily cleaved, at pH 7.5 and pH 6 (Figure 4A, lanes 4-5). In contrast, the variants showed comparable cleavage of the pro-domain but not of the intersubunit linker. For example, the S150A variant cleaved the intersubunit linker comparably to wild-type at pH 7.5 but showed reduced cleavage at pH 6 (Figure 4A, lanes 6-7). The S150D variant showed reduced cleavage at both pHs (Figure 4A, lanes 8-9). Consistent with the activity assays described above for small peptides, the T152V variant efficiently cleaved both the pro-peptide and the intersubunit linker of caspase-7 (Figure 4A, lanes 10-11), while the T152D variant showed greatly diminished activity (Figure 4A, lanes 12-13).

Previous studies in cellulo suggested that phosphorylation of S150 either reduced enzyme activity or decreased protein stability, and either mechanism could result in lower apoptosis (26). Our data show that changes in activity are observed in large protein substrates but not in tetra-peptide substrates, when S150 is modified. In order to further examine the differential effects of pH on the variants, we measured the pH-dependent assembly of the caspase dimer. Our previous studies showed that the caspase-3 dimer dissociates to the two protomers below pH \( \sim 5.5 \), and the dimer reversibly assembles above pH \( \sim 4 \) (Figure 4B) (32, 39-41). In those studies, we utilized several biochemical and biophysical assays to monitor caspase-3 dimer dissociation versus pH. Changes in fluorescence emission, as monitored by the average emission wavelength (AEW), and size exclusion chromatography showed a blue-shift in fluorescence emission between pH 5.5 and pH 4 that corresponds to dissociation of the dimer to the two protomers (40, 41). Below pH 4, the subunits within the protomer unfold, which is accompanied by a red-shift in fluorescence emission. In our previous studies, we confirmed that the dimer of wild-type caspase-3 dissociates between pH 5.5 and pH 4 by extensive pH experiments in which we monitored changes in fluorescence emission and circular dichroism as a function of urea over a broad pH range (41), as well as protein concentration and salt dependence (32). Finally, using a combination of fluorescence emission and circular dichroism combined...
with quenching by iodide, cesium, and acrylamide, and limited proteolysis by trypsin and V8 proteases, we showed that the protomer remains well-folded at pH 4 and that the tryptophan residues in the protomer are not exposed to solvent (39, 40, 42). Together, our previous studies establish that the caspase-3 dimer dissociates between pH 5.5 and pH 4 and that dimer dissociation is accompanied by a blue-shift in fluorescence emission (Figure 4B). The minimum in AEW at pH~4 and the red-shift in AEW at pH<4 reports on the unfolding of the protomer.

In order to examine changes in the dimer of the H3CL variants, we first characterized the fluorescence emission of each protein. Caspase-3 has two tryptophan residues, and both are located in the active site near the surface of the substrate-binding loop (L3). The tryptophan fluorescence emission, therefore, reports on possible conformational changes to the active site. The results show that, at pH 7.5, the average emission wavelength (AEW) of wild-type caspase-3 is ~350 nm, as expected for tryptophans that are solvent accessible (Figure 4C). There was no significant difference in the AEW for the S150A/D/E or T152A/V variants, all of which showed AEW of ~350 nm (Figure 4C). In contrast, the T152D variant demonstrated a significant blue-shift in AEW to 346 nm (Figure 4C). When unfolded in 8 M urea-containing buffer, the AEW of all proteins was ~350 nm, so the blue-shift observed for the T152D variant shows that the active-site tryptophans are in a less solvent-exposed environment compared to those of the wild-type enzyme at pH 7.5 (Figure 4D). As noted below, all proteins were dimeric at pH 7.5, so the altered fluorescence emission for the T152D variant suggests that the mutation affects the conformation of the active site tryptophans.

The activity observed for a caspase is dependent on dimerization because loops from each protomer comprise the loop bundle (see Figure 2A), and one measure of dimer stability in caspases is the pH-dependent dissociation of the dimer (Figure 4B) (32, 41). In order to examine the effects of mutations in the H3CL, we incubated the phospho-mutants in buffer between pH 3 and pH 7.5, as described previously, and we monitored enzyme activity after returning the protein to pH 7.5 (Figure 4E) and allowing the sample to equilibrate (14 hour minimum) (32). Relative to controls for each protein, which were incubated for equivalent times at pH 7.5, the major loss of activity occurred when protein was incubated between pH 6 and pH 4 prior to returning the protein to pH 7.5. Furthermore, the wild-type caspase-3 dimer reassembled reversibly when incubated at pH 6, as shown by the complete recovery of activity upon return to pH 7.5. However, in the S150 and T152 variants, only 65-85% activity was recovered in protein incubated at pH 6.

To further examine dissociation of the dimer and changes resulting from mutations in the H3CL, we determined the AEW for each protein over the pH range of 3 to 7.5. For wild-type caspase-3 (Figure 4F), the data show one primary transition where the AEW for tryptophan fluorescence decreases between pH 6 and 4, with a mid-point for the transition of ~5. As described above, the fluorescence emission of the protomer is blue-shifted compared to the dimer, so the major transition in AEW between pH 6 and pH 4 is due to dimer dissociation. The results were independent of whether we monitored only the tryptophans (Figure 4F) or all aromatic residues (Supplemental Figure 1). The variants (S150A and S150D) demonstrated an increase in the transition mid-point to pH ~5.2-5.4. Below pH 4, the protomers dissociate to the large and small subunits, which is accompanied by an
increase in AEW due to exposure of the tryptophan residues. For the S150A and T152V variants, the pH-dependent minimum in AEW increased when compared to that of wild-type caspase-3 from pH 4 to pH >4.5. Together, the fluorescence data are consistent with a destabilized dimer and protomer in the H3CL variants (with the exception of T152V). Both the mid-point for dimer dissociation and the minimum in AEW are shifted to higher pH values. The lower recovery of activity versus pH for the mutants (Figure 4E) may be due to the destabilized protomer, since protomer unfolding is not reversible. Finally, we note that all proteins were dimers at pH 7.5, as determined by size exclusion chromatography (Supplemental Figure 2), so the blue-shift in fluorescence emission and loss of activity observed for the T152D variant at pH 7.5 were not due to dimer dissociation.

**High-resolution structures of H3CL variants reveal localized changes in hydrogen bonding and hydrophobic interactions.**

We examined changes in the H3CL due to mutations at S150 or T152 by X-ray crystallography, and each mutant was solved to 2.1 Å or higher resolution (Supplemental Table 2). We note that the presence of the pro-domain (in the D2A variants) did not affect the changes caused by mutations at S150 or T152. As expected, the pro-domain was disordered in the crystal. In general, the presence of the pro-domain appeared to increase the flexibility of the N-terminus because several residues were disordered in the D2A variants that were ordered in the fully mature variants, namely residues 29-34. As N35 is the first residue to contact the dimer, the effects of the pro-domain are considered inconsequential to the conclusions described below.

The data show no gross structural changes in the protein resulting from the mutations at S150 or T152, as the RMSD compared to wild-type caspase-3 was generally <0.3 Å (Figure 5). Not surprisingly, the two H-bonds contributed by the S150 side-chain were lost in the S150 variants (Figure 5A-D). Although the hydroxyl of S150 is partially buried, replacement with larger side-chains (aspartate, glutamate, tyrosine) is accommodated by rotation of the side-chain toward solvent. The S150D variant shows a net increase in H-bonds with helices-2 and -3, while S150A has a net loss of three H-bonds (Figure 5A,B). In addition, the larger side-chains caused a displacement of 1-1.5 Å between the H3CL and the neighboring loop connecting helix-2 to the protein core (Figure 5C,D).

In the T152 variants, removal of the hydroxyl group of T152 and replacement of the side-chain with alanine or valine results in disruption of the H-bond between R149 and E173’ in the loop bundle as well as loss of the H-bond with the amide nitrogen of G145 (Figure 5E-G). In the case of R149, the side-chain is observed in two rotamers, where one rotamer is rotated toward solvent. In addition, the shorter alanine side-chain results in changes to the hydrophobic cluster of A152, I172’ and I187’, where the distance of the A152 methyl group to that of I187 increases by ~2 Å compared to that of T152. The changes in the hydrophobic cluster result in a partially disordered side-chain for E173’ in the loop bundle. The hydrophobic pocket is restored in the T152V mutant, although as noted above, the H-bonds from the T152 hydroxyl are lost. Notably, L2’ of the loop bundle is disordered beyond H185. In wild-type caspase-3, residues 179-184 of one protomer interact with active site loop L4 and the S4 substrate-binding pocket of the second protomer, and those interactions are
disrupted in the H3CL variants (43). We note that the residues of L2 and L2' that form the hydrophobic pocket between the H3CL and the loop bundle (I172'/I187') are also highly conserved in effector caspases (Supplemental Figure 3B). Thus, changes at T152 affect both the hydrophobic interactions and the H-bonding in the loop bundle as well as the flexibility of the termini of loops L2 and L2'.

We next characterized the structures of variants in the two sites that are thought to be part of the H3CL interaction network: T245 and S249 near active site loop L4. The T245D variant, with or without the pro-domain (D2A,T245D), had no significant structural changes. Indeed, the H3CL contacts were the same as for wild-type caspase-3 at S150 and T152. Closer to the mutation site, the D245 side-chain forms a new salt-bridge with R241 in helix 5, causing the R241 side-chain to adopt another rotamer in order to form the interaction (Supplemental Figure 4). In wild-type caspase-3, R241 H-bonds with the backbone carbonyl of T270’ and with the side-chain of N35’ across the dimer interface, so these interactions are lost in the mutant, but the repositioned R241 forms new interactions with the side-chain of D34’.

We were unable to crystallize the S249D single mutant, but we were able to determine the structure of the double mutant, T245D,S249D. In this case, the changes noted above for T245D were also observed in the double mutant. Contrary to our expectation that active site loop L4 would be disordered in the variant, the D249 residue forms new H-bonds near the active site (Supplemental Figure 4). For example, the D249 carbonyl H-bonds to the amide backbone of L157 of active site loop L2, and the side-chain H-bonds with the backbone amide of K259. Thus, based on our structural studies, it is not clear why the S249D variant is not active, unless the new H-bonds with loop L2 affect the catalytic efficiency of C163 or the dynamics of active site loop L4.

**Molecular dynamics simulations show increased fluctuations in active site loops**

We conducted MD simulations for 50 ns on each variant to determine whether changes to the H3CL affected fluctuations in the active site loops. In order to set a baseline with which to compare the H3CL variants, we performed three independent MD simulations on wild-type caspase-3 (dimer of PDB entry 2J30) and used the average data in further analyses (Supplemental Figures 5 and 6). A comparison of the average structures from the three MD simulations of wild-type caspase-3 to that of the crystal structure is shown in Supplemental Figure 5 (panels A,B). For wild-type caspase-3, the MD simulations show very little change in the substrate-binding pocket (active site loop L3) or in L2. In contrast, active site loops L1 and L4 show the most flexibility, with RMSF for each loop of ~3 Å (Supplemental Figure 6). In the crystal structure, L2’ is bound near the S4 binding pocket and forms through-water H-bonds with L4 and the substrate. The simulations, however, show that the loop does not remain bound near the S4 pocket, but rather it rotates toward the H3CL and L2 (Supplemental Figure 5B). As expected, the N-terminus is flexible from S29-D34 but has lower fluctuations at N35, where the N-terminus contacts the protein core. In protomer B, the elbow loop (A200-Y203) and turn above (called the 124-loop) are displaced compared to the crystal structure and show fluctuations of ~1.5-2 Å (Supplemental Figures 5 and 6). Together, the simulations show that there are little to no changes in the H3CL over the course of the simulations. In the three simulations of...
wild-type caspase-3, the protein core shows very similar fluctuations, and the largest variations among the three simulations occur at the chain termini (Supplemental Figure 6).

Representative data for the H3CL variants are shown in Supplemental Figures 5C and 5D as the average structures compared to that of wild-type caspase-3. The root mean square fluctuation (RMSF) for each amino acid is presented in Supplemental Figures 7-13 for all variants. The RMSF data are shown as the difference between the mutant and wild-type caspase-3 (ΔRMSF). In this case, values above zero indicate increased fluctuations in the mutant, while values below zero indicate decreased fluctuations in the mutant compared to wild-type caspase-3. The average structures for the variants were similar overall to that of wild-type caspase-3 in that the active site loops L1 and L4 showed the largest fluctuations, as did the terminus of L2’. There were small fluctuations in the H3CL, but the mutations resulted in repositioning helix-3 as well as the loop connecting the surface strands β1 and β2 (the 124-loop), which showed increased fluctuations of ~2 Å in some cases (Supplemental Figure 5C,D). The increased motion in the surface loop was also observed in the ΔRMSF analysis (Supplemental Figures 7-13). In the S150 and T152 variants, there were little additional fluctuations in active site loops L1 or L4 compared to wild-type caspase-3. That is, the ΔRMSF for those regions were generally <1 Å. The loop 4 mutants (T245D; T245D,S249D) showed increased fluctuations in the 124–loop, similar to the H3CL variants, but the double mutant also resulted in increased fluctuations in active site loop 4 compared to wild-type caspase-3 (Supplemental Figure 13). In some cases, S150E for example, the displacement of 1-1.5 Å observed in the crystal structure between the H3CL and the neighboring helix-2 loop, was observed to propagate along helices-2 and -3 to the short surface β-strand, called β1-β3 (Figure 6A). The short surface strands not only contain the catalytic histidine (H121), but they also contribute to the oxyanion hole as well as stabilize active site residues (28). The increased fluctuations in the 124-loop (connecting β1 and β2) resulted in transient interactions between E123 and H121, where E123 was observed to move to within ~3 Å of H121, from ~11 Å in wild-type caspase-3 (Figure 6B). A similar displacement of helices-2, -3, and β1 is observed in the T152D mutant (Supplemental Figure 5D). Our phylogenetic analysis of the 124-loop shows that E123 is highly conserved in apoptotic caspases (Supplemental Figure 14). We note that changes to fluctuations in the hydrophobic cluster of the H3CL and active site loop 2 were not observed in the MD simulations, but the chain termini of L2 and L2‘ are among the most mobile in the protein, so it is difficult to determine whether the mutations further increased mobility of the termini.

Discussion

The regulation of caspase activity is an essential determinant to cell survival, and post-translational modifications provide reversible mechanisms to control caspase activity below the threshold of activity required for cell death (28). Although poorly understood, the phosphorylation of caspase-3 in the C-terminal loop of helix-3 is of particular interest because it is thought to represent a common allosteric mechanism in all caspases (25, 26). In addition, T152 is part of a hydrophobic cluster of amino acids that stabilize the active site loops across the dimer interface.

Our data show that there are two allosteric networks in caspase-3 that
facilitate signal propagation following phosphorylation of the H3CL. On one side of the loop, S150 is conserved in most vertebrate species, and the residue evolved after the apoptotic caspases split from inflammatory caspases. The phylogenetic data support the hypothesis that phosphorylation at S150 represents an allosteric mechanism that has been conserved in vertebrates. For T152, however, small hydrophobic residues are common, which likely stabilize the hydrophobic cluster in the loop bundle. The use of threonine at position 152 arose in mammalian caspase-3, and as our results show, T152 provides a unique mechanism to abolish activity of the enzyme. The backbone carbonyl of T152 also makes through-water H-bonds with the N-terminus of the protein, so the H3CL not only bridges the dimer interface, but it also bridges the N- and C-termini of the protein. Importantly, this region of the N-terminus has been shown to be phosphorylated in other caspases (44). Thus, interactions in the H3CL form extensive H-bonding and hydrophobic contacts that contribute to the stability of helix-2, helix-3, the loop bundle of L2 and L2', and possibly the N- and C-termini of the protein.

We show that the allosteric mechanism of the H3CL is propagated through both intra- and inter-protemer contacts. In the intra-protemer allosteric network, small changes in positioning the neighboring loop near helix-3 propagate along helix-3 and affect the adjoining surface β-sheet of β1-β3. The surface β-sheet not only contains the catalytic H121 but it also forms stabilizing interactions with active site loop L2 in the dimer interface (28). Molecular dynamics simulations also demonstrated a repositioning of helix-3 and increased fluctuations in the β1-β3 sheet. At the end of the allosteric network, the conserved E123 transiently interacts with the catalytic H121, and the interactions likely decrease the rate of proton transfer during the catalytic reaction. The conservation of S150 in the H3CL as well as a charged residue at position 123 suggests a common allosteric mechanism in the apoptotic caspases (-3, -6, -7, -8, -10). Interestingly, the surface β-sheet undergoes a coil-to-helix transition in caspase-6, so fluctuations in helix-3:β1-β3 may reflect an ancient allosteric mechanism coupled to S150 (22). The allosteric network in caspase-6 may have further evolved to allow the additional coil-to-helix transition, providing a unique control mechanism from the common allosteric network.

The inclusion of T152 is a more recent evolutionary event in mammalian caspase-3 and provides an added control mechanism by engaging an inter-protemer allosteric network that connects the H3CL to the second active site via the loop bundle. The loss of activity due to modifications of the H3CL may be due to the increased flexibility of active site loop L4, which is stabilized through interactions with loops L2 and L2', and the hydrophobic cluster that connects L4 to the H3CL is less ordered in the T152D variant. Our results identify T152 as an allosteric kill switch that evolved in mammalian caspase-3. While phosphorylation of S150 decreases enzyme activity, the phosphorylation of T152 is sufficient to abolish activity.

The pH of the cell decreases during early apoptosis from ~7.4 to ~6.8, so phosphorylation of the H3CL may couple changes in cellular pH with caspase dimer stability (38). The wild-type caspase-3 dimer dissociates to the inactive protomer in a pH-dependent manner, and the S150 variants appear to affect the mid-point of the transition, resulting in transition to the inactive state at higher pH. The mid-point for the pH-dependent transition of ~5-5.5 is consistent with the titration of one or more
histidine residues. Including the catalytic H121, there are eight histidine residues in the caspase-3 protomer (see Figure 1B and Figure 7A). Close to the site of modification, H108 is located in a turn between helix 2 and β-strand 3, and the side-chain of S150 H-bonds with the backbone amide of H108 (Figure 7B). In addition, active site loops 1, 4, and L2’ each contain one histidine. Although it is not clear at present how modifications in the H3CL may affect the pKa of the histidine residues, the most likely candidate for the pH-dependent effect on dimer stability is H234 (Figure 7C). Helix 5 makes several charge-charge interactions across the dimer interface with helix 5’ of the second protomer, and H234, E231, and E272 form salt bridges with E231’, E272’ and H234’ in the interface (Figure 7C). The H3CL is ~27 Å from the salt-bridges, but the changes may propagate through interactions with the N-terminus. Because the N-terminus is phosphorylated in other caspases, the H3CL-N-terminus-Helix 5 interactions appear to represent a common network for signal propagation due to phosphorylation.

Targeting the dimerization of caspase-3 via phosphorylation is a novel allosteric mechanism and explains the previous suggestion that modification of the H3CL destabilizes the enzyme and leads to survival of the cell (26). Because apoptotic cells initially undergo an acidification process, the H3CL modification might help cells maintain a level of activity that allows for continued progression to cell death or toward “incomplete apoptosis” for processes such as erythroid or monocyte differentiation (45, 46). Taken together, our results reveal the structural interaction network of allostery that connects evolutionarily conserved and novel phosphorylation sites directly to the active site.

**Experimental Procedures**

**Cloning, protein expression and purification**

All mutants were generated by PCR site-directed mutagenesis using primers containing the mutation (see Supplemental Data) and pET21b expression plasmid that contained either wild-type caspase-3, caspase-3 (D9A,D29A: called D2A), or caspase-3 (D9A,D29A,D175A: called D3A) with a C-terminal 6X-His-tag (33, 42). Mutants of caspase-6 and caspase-7 were generated by PCR site-directed mutagenesis using primers containing mutant of interest (see Supplemental Data) in pET21b and pET23b expression plasmid that contained wild-type caspase-6 or caspase-7 with a C-terminal 6X-His-tag (47, 48). Mutations were confirmed by sequencing both DNA strands. Plasmids were transformed into E. coli BL21(DE3) pLysS, and proteins were expressed as described (33, 49).

**Phylogenetic analysis**

A fasta file for each caspase family member was compiled, and a preliminary alignment of the sequences was performed with MUSCLE (50). Sequences that opened gaps or had large deletions were removed from the analysis to avoid noise or bias. We used 1,325 sequences for the analysis, and the number of sequences for each individual caspase is shown in the left-most column of Supplementary Table 1. A multiple sequence alignment (MSA) was generated for each family member with PROMALS3D (51). The data in Supplementary Table 1 were generated by analyzing the amino acid frequency at each position with ProtParam on the ExPaSy server (52). The phylogenetic tree for each caspase family member was created by using the Maximum Likelihood method in MEGA 7 (30, 31). One hundred bootstraps were performed as a test of confidence for the tree topology (53).
Each MSA was analyzed on the ProtTest3 server, and the Jones-Taylor Thornton (JTT) substitution model of protein evolution plus gamma (+G) distribution was selected to construct the trees (54, 55). The trees are drawn to scale, with the branch lengths measured in the number of substitutions per site. Positions in the MSA with less than 80% site coverage were eliminated. Fewer than 20% alignment gaps, missing data, and ambiguous bases were allowed at any position. In each figure, the tree with the highest log likelihood from each family member is shown. The positions corresponding to S150 and T152 in caspase-3 are displayed on the branches of currently existing species, and the most likely ancestors (MLA) are displayed at the nodes for each caspase family member. The bootstrap results are shown next to the branches and represent the percentage of trees in which the associated taxa are clustered together.

**Enzyme activity assays**

Enzyme activity was determined in a buffer of 150 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM DTT, 1% sucrose, 0.1% CHAPS (assay buffer) at 25 °C, as previously described (56). The total reaction volume was 200 µL and the final enzyme concentration was 10 nM. Following the addition of substrate, the samples were excited at 400 nm, and emission was monitored at 505 nm for 60 seconds. The steady-state parameters, $K_M$ and $k_{cat}$, were determined from plots of initial velocity versus substrate concentration and are presented in Table 1.

**Crystallization and data collection**

Caspase-3 variants were crystallized as described previously for wild-type caspase-3 (43). Briefly, each protein was dialyzed in a buffer of 10 mM Tris-HCl, pH 8.5, and 1 mM DTT, and concentrated to ~7 mg/mL. Inhibitor, Ac-DEVD-CMK (reconstituted in DMSO), was added at a 5:1 (w/w) inhibitor/protein ratio, and DTT and NaN$_3$ were added to final concentrations of 10 mM and 100 mM, respectively. Samples were incubated for one hour in the dark on ice. Crystals were obtained at 18 °C by the hanging drop vapor diffusion method using 4 µL drops that contained equal volumes of protein and reservoir solutions over a 0.5 mL solution of 100 mM sodium citrate, pH 4.9-5.2, 8-18 % PEG 6000 (w/v), 10 mM DTT, and 3 mM Na$_3$N. Crystals appeared within 3-5 days and were briefly immersed in a cryogenic solution containing 20 % MPD (2-methylpentane-2,4-diol) and 90 % reservoir solution. Crystals were stored in liquid nitrogen. Data sets were collected at 100 K at the SER-CAT synchrotron beamline (Advance Photon Source, Argonne National Laboratory, Argonne, IL, U.S.A.). Each data set contained 180 frames at 1° rotation. The proteins crystallized in either the monoclinic space group C2 or the orthorhombic space group I222 and were phased with a previously published human CASP3 structure (PDB entry 2J30) using Phaser-MR in the Phenix software (57). Data reduction and model refinements were done using HKL2000, COOT, and Phenix, and a summary of the data collection and refinement statistics is shown in Supplemental Table 2 (57–60).

**Molecular dynamics simulations**

Molecular dynamics (MD) simulations were performed as described previously with GROMACS 2016, using the Amber99 force field and the TIP3P water model (61–65). All simulations started with the structure obtained from X-ray crystallography, as described above, and the inhibitor was removed from the structure file. As described previously for human caspase-3, the proteins were solvated in a periodic box of 62 Å x 48 Å x 66 Å, with approximately 13,500 water molecules (65). Sodium or chloride ions were added as
required to neutralize the charge on the system. The system was first minimized using steepest descent, and then the waters were relaxed during a 20 ps MD simulation with positional restraints on the protein. Simulations of 50 ns were then run for each protein under constant pressure and temperature (300 K). A time step of 2 fs was used, and coordinates were saved every 5 ps. In each simulation, the protein was equilibrated within 500 ps.

**pH studies and fluorescence emission**

Proteins were incubated in a buffer of 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM DTT or in 8 M urea-containing buffer. Samples (1 µM) were excited either at 280 nm or at 295 nm, and emission scans were collected between 300 and 400 nm (PTI C61 spectrofluorometer). The average emission wavelength (AEW or <λ>) was determined for each protein as

\[<\lambda> = \frac{\sum_{i=1}^{N}(l_i \lambda_i)}{\sum_{i=1}^{N}(l_i)},\]

where \(l_i\) refers to fluorescence intensity and \(\lambda_i\) refers to wavelength, as described previously (66). We performed two experiments to examine pH-induced dimer dissociation and reassembly. First, proteins were incubated over a pH range of 3.0-7.5, and the return of enzyme activity was determined after the proteins were re-equilibrated at pH 7.5. In those experiments, proteins were incubated for 4 hours at 25 °C over a pH range of 3.0-7.5 using either 50 mM citrate buffer (pH 3-6), or 50 mM Tris-HCl, pH 7.5, containing 2 mM DTT, as described previously (32, 40). Following the initial incubation, samples were then dialyzed in a buffer of 50 mM Tris-HCl, pH 7.5, 2 mM DTT for 14 hours at 25 °C. Reassembly of the active dimer was monitored by measuring enzyme activity after incubation at pH 7.5 for a minimum of 14 hours. The data were compared to samples that were incubated at pH 7.5 for 18 hours (25 °C). Second, we monitored the intrinsic fluorescence emission of each protein (in the absence of substrate) as a function of pH. In those experiments, samples were incubated for a minimum of 12 hours at 25 °C over a pH range of 3.0-7.5 using either 50 mM citrate buffer (pH 3-6), or 50 mM Tris-HCl, pH 7.5, containing 2 mM DTT. Samples (at each pH) were then excited at 280 nm or 295 nm, and the fluorescence emission was measured from 300 to 400 nm. All data were corrected for background signal, and the average emission wavelength (AEW or <λ>) was calculated as described above (66).

**Whole protein digest**

The caspase-7(C186S) inactive variant was used as a substrate for caspase-3 cleavage assays, and the protein was diluted into activity buffer (pH 7.5 or pH 6) to a final concentration of 75 µM. Caspase-3 (wild-type or variant) was then added to a final concentration of 10 µM, and the samples were incubated for 20 minutes at 37 °C. The cleavage reaction was stopped by adding 6X SDS (1X final) followed by boiling. Cleavage products were analyzed by 12.5% SDS-PAGE gel.

**Size exclusion chromatography**

Protein oligomeric state was examined using a Superdex75 Increase 10/300GL column (AKTA-FPLC). Proteins were dialyzed in a buffer of 10 mM phosphate, pH 7.5, and the column was equilibrated with the same buffer. Protein (100 µL) at a concentration of 1-5 mg/mL was loaded onto the column at a flow rate of 0.8 mL/min. The column was calibrated using the gel filtration LMW calibration kit (GE Health Sciences- 28-4038-41) following the manufacturer instructions.

**Accession numbers**

Protein data bank accession number for caspase-3 variants: S150A (PDB ID 6BDV), S150D (PDB ID 6BGQ), S150E (PDB ID 6BGR), T152A (PDB ID 6BH9), T152D (PDB ID 6BG4), T152V (PDB ID 6BHA),
S150Y (PDB ID 6BGS), S150D,D2A (PDB ID 6BG0), S150E,D2A (PDB ID 6BG1), T152D,D2A (PDB ID 6BGK), T245A (PDB ID 6BFK), T245D (PDB ID 6BFO), T245D,D2A (PDB ID 6BFL), T245D,S249D (PDB ID 6BFJ).
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Author contributions. ACC conceived and coordinated the study and performed experiments shown in Figure 5. RG and MET designed the experiments shown in Figure 1. MET designed, performed, and analyzed the experiments shown in Figures 1-4. MET and PD crystallized proteins and determined structures shown in Figure 4. ACC and MET wrote the paper.
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Footnotes:

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Table 1. Catalytic parameters for caspase-3, -6, and -7 and H3CL phosphorylation mutants.

|        | $K_M$ ($\mu$M) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_M$ (M$^{-1}$s$^{-1}$) |
|--------|----------------|----------------------|----------------------------------|
| WT     | 8.0 ± 2.7      | 0.91 ± 0.001         | 1.14*10^5                       |
| S150A  | 16.3 ± 0.8     | 5.70 ± 0.001         | 3.50*10^5                       |
| S150D  | 20.2 ± 1.5     | 4.40 ± 0.001         | 2.20*10^5                       |
| S150E  | 19.8 ± 2.3     | 3.50 ± 0.001         | 1.77*10^5                       |
| S150Y  | 23.3 ± 1.5     | 8.78 ± 0.002         | 3.76*10^5                       |
| T152A  | 18.7 ± 1.0     | 2.90 ± 0.001         | 1.55*10^5                       |
| T152D  | 0              | 0                    | 0                                |
| T152V  | 17.3 ± 0.7     | 4.34 ± 0.01          | 2.50*10^5                       |
| S249D  | 0              | 0                    | 0                                |
| T245A  | 31.9 ± 3.01    | 3.51 ± 0.01          | 1.10*10^5                       |
| T245D  | 29.3 ± 3.27    | 3.30 ± 0.02          | 1.13*10^5                       |
| S150D(D$_2$A) | 27.2 ± 1.35 | 3.00 ± 0.006        | 1.10*10^5                       |
| S150A(D$_2$A) | 28.8 ± 2.3 | 5.57 ± 0.018        | 1.93*10^5                       |
| T152A(D$_2$A) | 30.9 ± 1.1  | 5.59 ± 0.008        | 1.82*10^5                       |
| T152A(D$_3$A) | 28.3 ± 4.99 | 0.01 ± 0.0001      | 4.45*10^2                       |
| T152D(D$_2$A) | 0           | 0                    | 0                                |
| T152D(D$_3$A) | 0           | 0                    | 0                                |
| T245A(D$_2$A) | 28.6 ± 3.6  | 2.91 ± 0.014        | 1.02*10^5                       |
| S150E(D$_2$A) | 23.6 ± 2.6  | 4.80 ± 0.02         | 2.09*10^5                       |
| S150E(D$_3$A) | 19.0 ± 7.1  | 0.002 ± 0.00002     | 1.05*10^2                       |
| T245D+S249D | 0          | 0                    | 0                                |
| Cp6 WT | 43.2 ± 3.34    | 0.67 ± 0.0002       | 1.55*10^4                       |
| Cp6 S150D | 86.7 ± 4.6 | 2.60 ± 0.008       | 3.00*10^4                       |
| Cp7 WT | 69.5 ± 3.3    | 11.30 ± 0.003       | 1.63*10^5                       |
| Cp7 T173D | 61.9 ± 2.8  | 12.90 ± 0.003       | 2.08*10^5                       |
| Cp7 E176G | 62.5 ± 3.6  | 5.40 ± 0.002        | 8.64*10^4                       |
| Cp7 T173D+E176G | 79.1 ± 4.3 | 13.70 ± 0.004      | 1.74*10^5                       |

Cp6 = Caspase-6, Cp7 = Caspase-7; All other proteins refer to caspase-3 variants; WT refers to wild-type enzyme; D$_2$A refers to D9A,D28A uncleavable pro-domain; D$_3$A refers to D9A,D28A,D175A uncleavable procaspase-3.
Figure 1. A, Model of caspase-3 activation, regulation by XIAP binding, phosphorylation, and degradation. The caspase protomer is represented by one large (LS) and one small (SS) subunit. In the zymogen, the subunits in the protomer are covalently connected by the intersubunit linker (IL). In the mature caspase, the IL is cleaved to yield the mature caspase protomer. Following cleavage, the IL provides two of the five active site loops, called L2 and L2’ (see panel B and Figure 2). Both the zymogen and the mature caspase-3 are considered a dimer of protomers, but the IL is cleaved in the mature enzyme. Phosphorylation of the zymogen inhibits maturation, while phosphorylation of the mature caspase inhibits enzyme activity. Binding of XIAP inhibits the mature caspase and leads to proteasomal degradation. B, Secondary structural elements mapped onto the caspase-3 sequence. This figure was generated using Polyview-2D (67) and the structure of human caspase-3 (PDB ID 2J30). β-strands 1-6 (green), α-helices 1-5 (red, H1-H5), and active site loops (blue) L1-L4 and L2’ are indicated (see also Figure 2). H3CL refers to the Helix-3 C-terminal Loop, and the arrow indicates site of cleavage (D175) of the IL to yield loops L2 and L2’ of the mature protomer.
Figure 2. A, Structure of caspase-3 (PDB ID 2J30). The large (LS) and small (SS) subunits (see Figure 1) fold into a single domain with a central six-stranded β-sheet core (β1–β4 contributed by the LS and β5–β6 contributed by the SS; labeled in protomer 1) and five external α-helices (H1–H3 contributed by the LS and H4–H5 contributed by the SS). Five loops comprise the active site of each protomer, where L1, L2, L3, and L4 are contributed by one protomer, and L2′ is contributed by the second protomer of the dimer (see Figure 1). The loop bundle refers to interactions between L4, L2, and L2′, which stabilize the active site. B, Interactions in the H3CL (helix-3 C-terminal loop, green) with helix-2 (H2, blue/brown) and the loop bundle (L2, red; L2′, cyan). Hydrogen bonds from S150 and T152 are shown as dashed lines. C, Active site loop L4 (brown) is stabilized, in part, by hydrogen bonds from S249, which contributes to substrate binding through interactions with F250 and P4 of the substrate. Inhibitor refers to DEVD-chloromethylketone (CMK) used during crystallization of the wild-type enzyme. Note that, for clarity, the active site in panel C is rotated 180° relative to the orientation in panel A.
Figure 3. A-B, collapsed phylogenetic trees of 175 caspase-3 sequences, bootstrapped one hundred times, with chondrichthyes (cartilaginous fish) as the out group. Groups are labeled according to their phylogenetic class or subclass. A, The most probable ancestor of position S150 at the nodes shows that S150 is highly conserved from chondrichthyes to human. B, The most probable ancestor of position T152. Metatherian mammals are not collapsed to show that threonine at position 152 evolved early in the mammalian lineage; between Ornithorhynchus anatinus (platypus) and Monodelphis domestica (opossum). Panels A and B are expanded in Supplemental Figure 3B. C, Multiple sequence alignment for each caspase using every known caspase sequence from NCBI using WebLogo (68). Numbers below each position refer to caspase-3 numbering. D, Salt-bridge between E176 and R271 in caspase-7 that spans across the dimer interface.
**Figure 4.** A, Whole protein digest, Lane 1=Protein MW standards, Lane 2=10 µM WT caspase-3 control, Lane 3=75 µM caspase-7(C186S) control, Lane 4=WT+CP7 (pH 7.5), Lane 5=WT+CP7 (pH 6), Lane 6=S150A+CP7 (pH 7.5), Lane 7=S150A+CP7 (pH 6), Lane 8=S150D+CP7 (pH 7.5), Lane 9=S150D+CP7 (pH 6), Lane 10=T152V+CP7 (pH 7.5), Lane 11=T152V+CP7 (pH 6), Lane 12=T152D+CP7 (pH 7.5), Lane 13=T152D+CP7 (pH 6), Lane 14= Protein MW standards. In panel A, CP7 refers to caspase-7. B, Model of pH-dependent oligomeric states of caspase-3 from dimer to unfolded protomer. LS = Large Subunit; SS = small subunit. C-D, Average emission wavelength (AEW) following excitation at 295 nm in the native state (C) and unfolded in 8M urea (D). E, pH gradient vs relative activity for WT and caspase-3(S150 or T152). Error bars show standard deviation from three experiments. F, Average emission wavelength (AEW) following excitation at 295nm vs pH for WT, S150, and T152 caspase-3 proteins. The following symbols were used: WT (♦), S150A (○--), S150D (--□--), T152V (—□—), and T152D (—●—).
Figure 5. A-G, Hydrogen bonds and electrostatic interactions in the H3CL of WT (grey) superimposed to each caspase-3 mutant (green). Blue spheres represent conserved waters as described in the text (24). Panel A= S150A, panel B= S150D, panel C= S150E, panel D= S150Y, panel E= T152V, panel F= T152A, panel G= T152D. For each panel, black dashes represent hydrogen bonds in WT caspase-3, and red dashes represent hydrogen bonds in the mutant.
Figure 6. A, Representative frame of a molecular dynamics simulation of S150E (green) compared to the X-ray crystal structure of wild-type caspase-3 (PDB code 2J30) showing movement of surface strands $\beta^1$ and $\beta^2$ toward the catalytic residue H121. Black dashes represent initial distance between the side chain of E123 and the catalytic histidine ($\sim$11 Å), and the red dashes represent the distance between E123 and H121 in the mutant ($\sim$3.5 Å). B, Positions of the catalytic dyad (H121 and C163) and of E123 shown as 200 frames from the 50 ns MD simulation.
Figure 7. A, Location of histidine residues in caspase-3. Active site loops L1, L4, and L2' each contain one histidine (H56, H257, H185, respectively). Two histidine residues at the C-terminus (H277 and H278) are not labeled. B, Near the H3CL (helix-3 C-terminal loop), S150 hydrogen bonds to H108. C, In wild-type caspase-3, the dimer is stabilized by electrostatic interactions between helix-5 and helix-5’ across the dimer interface, facilitated by E231, H234, and E272 of each protomer. The prime (’) indicated amino acids of the second protomer.
Modifications to a common phosphorylation network provide individualized control in caspases

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