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Commentary

Kinases leave their mark on caspase substrates

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Apoptosis is a cell death program that is executed by the caspases, a family of cysteine proteases that typically cleave after aspartate residues during a proteolytic cascade that systematically dismantles the dying cell. Extensive signaling crosstalk occurs between caspase-mediated proteolysis and kinase-mediated phosphorylation, enabling integration of signals from multiple pathways into the decision to commit to apoptosis. A new study from Maluch et al. examines how phosphorylation within caspase cleavage sites impacts the efficiency of substrate cleavage. The results demonstrate that while phosphorylation in close proximity to the scissile bond is generally inhibitory, it does not necessarily abrogate substrate cleavage, but instead attenuates the rate. In some cases, this inhibition can be overcome by additional favorable substrate features. These findings suggest potential nuanced physiological roles for phosphorylation of caspase substrates with exciting implications for targeting caspases with chemical probes and therapeutics.

Apoptosis is a form of programmed cell death that involves a series of morphological changes to the cell, including membrane blebbing, cytoskeletal rearrangement, chromatin condensation, and DNA fragmentation [1]. These changes facilitate orderly destruction of the dying cell, enabling it to be phagocytosed and minimizing the impact of its demise on neighboring cells. The cellular remodeling observed during apoptosis is orchestrated by the caspases, a family of cysteine proteases named for their ability to cleave after aspartate residues [2]. Caspases are synthesized as inactive zymogens whose activity can be triggered by a variety of cell-intrinsic and cell-extrinsic stimuli [3]. When an apoptotic signal is received, initiator caspases (caspases-2, -8, and -9) are activated by recruitment to activating protein complexes. The activated initiator caspases subsequently proteolytically cleave and activate the executioner caspases (caspases-3, -6, and -7). Once active, the executioner caspases cleave numerous substrates to systematically dismantle the dying cell (Figure 1A). This proteolytic cascade results in irreversible caspase activation, tilting the cell's fate toward apoptosis. However, the activity of caspases can be fine-tuned by other mechanisms including protein–protein interactions and posttranslational modifications [4], enabling the integration of other signals into the cellular decision to commit to apoptosis (Figure 1A,B).

Phosphorylation is a posttranslational modification that plays a key role in regulating apoptosis [5]. Kinases directly phosphorylate caspases to promote or inhibit their activation (Figure 1B), while caspases cleave kinases to impact their activity, localization, substrate repertoire, and lifetime to propagate the apoptotic signal (Figure 1C). While this bidirectional communication between kinases and caspases is well-documented, the ability of kinases to regulate caspase cleavage of individual substrates by phosphorylation within the cleavage site has remained less explored. In particular, it has remained unclear to what extent natural phosphorylation events regulate caspase substrate cleavage and how well phosphoresidues at different positions close to the cleavage site are tolerated by different caspases. In a recent issue of *Biochemical Journal*, Maluch et al. report a systematic investigation of the ability of caspases-3, -6, -7, and -8 to cleave phosphorylated peptides derived from natural substrates [6], shedding light on factors that influence caspase tolerance of phosphoresidues.
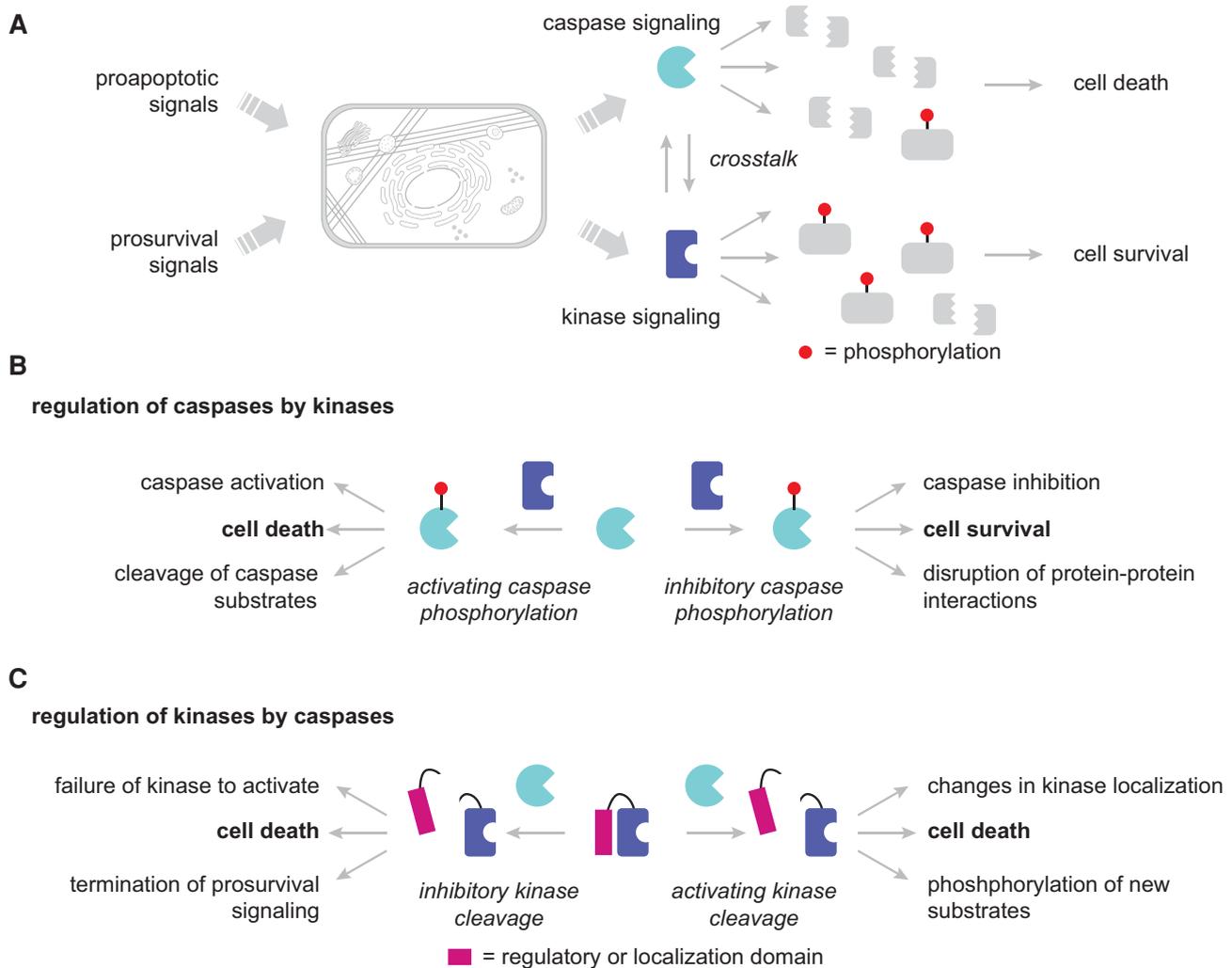
Figure 1. Crosstalk between caspase signaling and kinase signaling.

(**A**) Proapoptotic signals lead to caspase activation and proteolytic cleavage of substrates, while prosurvival signals often lead to kinase activation and phosphorylation of substrates. (**B**) Caspases are regulated by kinase phosphorylation, which is usually inhibitory and more rarely activating. (**C**) Kinases are regulated by caspases in a manner that promotes cell death by either activating or inhibiting kinase activity.

All of the apoptotic caspases are subject to regulation by phosphorylation, which may be inhibitory or, more rarely, activating (Figure 1B) [5,7–11]. Direct phosphorylation of caspases serves to tune the threshold between cell survival and cell death, enabling information from other signaling pathways to be factored into the apoptotic decision. For example, inhibitory phosphorylation of caspases ties initiation of apoptotic signaling to metabolic conditions in the cell [12] and to growth factor signaling [13,14]. Caspase phosphorylation has also been suggested to provide a means for cells to discriminate situational differences in signals that sometimes trigger apoptosis but that are also associated with other biological pathways. Loss of attachment to neighboring cells is associated with the apoptotic pathway known as anoikis but is also characteristic of cells undergoing mitosis. However, during mitosis, caspase-9 is phosphorylated by the cell cycle regulator cyclin-dependent kinase 1 (Cdk1), preventing loss of contact from triggering cell death [15,16]. Caspase inhibition by phosphorylation occurs at a basal level in some cell types, setting a threshold for initiation of apoptosis that is important during development [17]. Phosphorylation of caspases has also been proposed to regulate the activity of specific caspases to allow them to play nonapoptotic roles. Caspase-8 phosphorylation at Tyr 380 is implicated in targeting to membrane ruffles, modulating the nonapoptotic functions of caspase-8 in cell migration and adhesion [18,19]. Although kinase regulation of caspases generally inhibits apoptosis, phosphorylation of caspase-3 by

PKCδ has been reported to promote its activation [20], although the exact site of phosphorylation and activation mechanism have not been elucidated.

While phosphorylation of caspases typically opposes commitment to apoptosis, proteolytic cleavage of kinases by caspases can be a mechanism of amplifying the apoptotic signal (Figure 1C). Caspase cleavage of kinases often serves to rewire the cellular phosphorylation network by activating or inhibiting kinase activity or by triggering changes in subcellular localization that influence kinase access to substrates [5]. In one paradigm of caspase modulation of phosphorylation signaling, a caspase cleaves a kinase, separating it from its autoinhibitory domain to produce a propapoptotic, constitutively active kinase fragment. For example, cleavage of Rho associated kinase-1 (ROCK1) by caspase-3 at the DETD^{1113}↓G cleavage site separates the N-terminal kinase domain from the C-terminal autoinhibitory domain, producing a constitutively active ROCK1 fragment. This fragment hyper-phosphorylates myosin light chain (MLC), resulting in generation of an actin-myosin contractile force that produces the membrane blebs characteristic of apoptotic cells [21,22]. Similarly, p21-activated kinase 2 (PAK2) is cleaved during apoptosis between its catalytic and regulatory domains (SHVD^{212}↓G) to produce a constitutively active fragment [23,24]. In addition to releasing its regulatory domain, this cleavage event also removes a nuclear export sequence, resulting in translocation of PAK2 to the nucleus, where it has been suggested to play a role in chromatin condensation [25]. Caspase cleavage of PAK2 has also been reported to unmask a myristoylation site, resulting in localization of the catalytic domain to the plasma membrane and increasing signaling through the c-Jun N-terminal kinase (JNK) pathway [26]. Caspase cleavage of numerous other kinases has been reported to produce active fragments that contribute to both apoptotic progression and non-apoptotic caspase-mediated pathways such as differentiation and the cell cycle [5,27]. In another paradigm of caspase modulation of phosphorylation signaling, caspase cleavage can inactivate kinases, supporting execution of the apoptotic program by terminating pro-survival signals. Examples of kinases that are inactivated by caspase cleavage include focal adhesion kinase (FAK) [28,29], Akt [30], and NF-κB [31], among many others [5].

In addition to the bidirectional regulation of caspase and kinase activity, phosphorylation of specific caspase substrates has been previously reported to both enhance and inhibit their cleavage (Figure 2A). In one mechanism, phosphorylation of the substrate at a site distant from the caspase cleavage site allosterically enhances caspase cleavage [32]. In another mechanism, phosphorylation within the caspase cleavage site directly enhances or attenuates caspase cleavage. Caspases generally recognize four to five residues on the N-terminal side (or non-prime side, P4–P1) of the scissile bond and two residues on the C-terminal side (or prime side, P1′–P2′) of the scissile bond [3]. Proteomic studies of the apoptotic phosphoproteome have identified numerous phosphorylation events that occur within this cleavage site [33,34]. For caspase-8, phosphorylation at the P3 position has been demonstrated to accelerate cleavage of a handful of substrates by up to 20-fold [33]. P3 phosphorylation of these same substrates was tolerated by caspase-3, but neither enhanced nor inhibited the
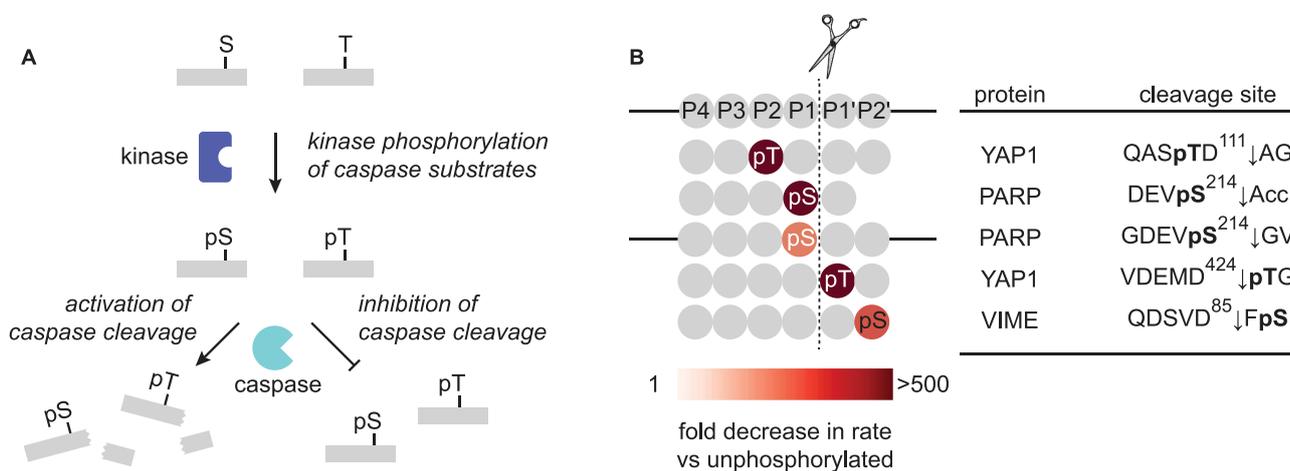

Figure 2. Substrate-level regulation of caspase cleavage by phosphorylation.
(**A**) Kinases act on caspase substrates to phosphorylate them, which made stimulate or inhibit caspase cleavage. (**B**) Maluch et al. systematically explored the role of substrate phosphorylation at the P2–P2′ positions of caspase substrate peptides designed based on natural caspase substrates that are subject to phosphorylation.

rate of cleavage. Another study of caspase-3 and caspase-7 cleavage of the apoptotic phosphoproteome identified several substrates whose cleavage was inhibited by phosphorylation, but also found a substrate, mammalian STE20-like protein kinase 3 (MST3), whose cleavage appeared to be enhanced by phosphorylation at P2 and P4′ [34]. However, these results could not be recapitulated *in vitro* using internally quenched fluorescent peptide substrates, suggesting that factors beyond the cleavage site sequence, such as the structure of the folded protein, presentation of the cleavage site in the cellular context, or the presences of an exosite, could also be important for the effect [35–38]. In another study, a survey of apoptosis-specific proteolytic events in the Degrabase suggested that phosphoserine (pSer) and phosphothreonine (pThr) sites are enriched at the P1 position and may be accepted by caspases in place of the canonical P1 aspartate residue based on their negative charge [39]. However, the consensus motif of these substrates in the P4–P2 positions did not match that of any human caspase. The authors nonetheless showed that internally quenched fluorescent substrates with phosphoserine at P1 were cleaved by caspase-3 with only a threefold reduction in catalytic efficiency [39]. It has thus remained an open question whether caspases-3 and -7 can accommodate phosphorylated residues in their active sites, what additional substrate features impact their ability to do so, and what the quantitative effect of phosphorylation on substrate cleavage is.

In new work, Maluch et al. use a series of internally quenched fluorescent peptides to systematically investigate the impact of natural phosphorylation events at the residues most proximal to the cleavage site (P2–P2′) on proteolysis by caspases-3, -6, -7, and -8 (Figure 2B) [6]. The series of peptides are derived from the bona fide caspase substrates yes-associated protein 1 (YAP1) and vimentin (VIME), both of which contain experimentally validated phosphorylation sites that overlap with their cleavage sites. By testing the ability of the phosphorylated and unphosphorylated forms of these substrates to be cleaved by various caspases, the authors show that Thr phosphorylation at P1′, Thr phosphorylation at P2, and Ser phosphorylation at P2′ are generally inhibitory toward caspase cleavage. Measurement of the Michaelis–Menten kinetic parameters for cleavage of these six substrates by caspase-3 showed that phosphorylation does not lead to complete inhibition of cleavage in all cases. Rather, the catalytic efficiency is decreased by >500-fold by Thr phosphorylation at P1′ and by >50-fold for Ser phosphorylation at P2′, while detectable cleavage is completely abolished by Thr phosphorylation at P2 (Figure 2B). Interestingly, although the P2′-phosphorylated vimentin-derived substrate is cleaved much less efficiently than its unphosphorylated counterpart, it is cleaved with similar efficiency to the unphosphorylated YAP1 substrate. An intriguing possibility raised by this finding is that phosphorylation could tune cleavage efficiencies of specific substrates within the physiological range of observed rates [40], with potential impacts on the order of events during apoptosis. The Michaelis–Menten kinetics data further reveal that the inhibitory effect on phosphorylation is almost completely attributable to a decrease in k_{cat} rather than an increase in K_M, suggesting that phosphorylated substrates maintain the ability to bind to the caspase-3 substrate-binding cleft but are not well positioned for proteolytic cleavage. An interesting hypothesis raised by this result is that phosphorylated caspase substrates may act as competitive inhibitors that disrupt cleavage of other substrates in a physiological context. It should be noted that in order for this type of inhibition to occur, phosphorylated substrates would have to be present at high concentrations that would enable them to compete with other substrates, and would have to be turned over with very low k_{cat}.

At present, there are no well validated physiological caspase substrates in which the P1 position of the cleavage site contains pSer rather than Asp or Glu. However, hints that this may be possible exist in proteomics datasets [39], and there have been conflicting reports suggesting that replacement of P1 Asp by pSer in the caspase cleavage site of poly(ADP-ribose) polymerase (PARP) (DEVD^{214}↓GV) either abolishes substrate hydrolysis completely [41], or that it has only a modest threefold effect on the catalytic efficiency of hydrolysis [39]. Maluch et al. addressed these conflicting results by generating synthetic peptides of different lengths derived from the PARP cleavage site in which the P1 position was either Asp, Glu, Ser, or pSer (Figure 2B) [6]. While the natural acidic residues Asp and Glu were cleaved efficiently in all cases, only the longer peptides containing pSer could be cleaved by caspases-3 and -7. These results suggest that weak recognition of P1 pSer can be overcome by interactions with a longer peptide or protein, consistent with the subsite cooperativity that is observed in many proteases [42]. This leaves open the possibility that P1 pSer could be found in yet-undiscovered physiological caspase substrates.

The results of the new work from Maluch et al. open several exciting avenues of future research. The *in vitro* results suggest that phosphorylation within caspase cleavage sites may have implications for caspase competitive inhibition and could tune cleavage rates within a physiological range to impact the ordering of substrate cleavage events. Application of proteomics approaches designed to unravel the crosstalk between proteolysis and

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phosphorylation [33,43] in combination with candidate-based studies will be important to address these issues going forward. The ability of specific caspases to recognize and cleave different phosphorylated positions within the cleavage site also provides a new opportunity to design activity-based probes with increased power to discriminate between caspases of overlapping specificity using approaches that have been used to target numerous other proteases [44,45]. Application of the internally quenched fluorescent peptide approach to probe an even larger cohort of substrates is likely to provide further insights into the effect of sequence composition and substrate length, making it possible to predict kinase regulation of caspase substrates from sequence alone. Building on the work of Maluch et al. future studies in this area will provide fundamental insights into caspase biology and will open up new opportunities to target caspases with chemical probes and therapeutics.

Competing Interests

The author declares that there are no competing interests associated with this manuscript.

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Abbreviations

FAK, focal adhesion kinase; JNK, c-Jun N-terminal kinase; MLC, myosin light chain; MST3, mammalian STE20-like protein kinase 3; PAK2, p21-activated kinase 2; PARP, poly(ADP-ribose) polymerase; ROCK1, Rho associated kinase-1; VIME, vimentin; YAP1, yes-associated protein 1.

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