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# Lethal weapons: DAP-kinase, autophagy and cell death

## DAP-kinase regulates autophagy

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Recently, DAP-kinase was identified as one of the essential regulators of autophagy, activated by signals such as cytokines and ER stress. DAP-kinase is a tumor suppressor that mediates several cell death pathways, such as apoptosis and programmed necrosis. Likewise, functional studies suggest that DAP-kinase may direct autophagy specifically towards autophagic cell death. Several recent studies have mapped DAP-kinase function to distinct stages in autophagy signaling. These include the Beclin-1/phosphatidylinositol 3-kinase (PI(3)K) complex, which is necessary for autophagosome formation, and an interaction with the LC3 binding protein, MAP1B, which may regulate vesicle trafficking. This review will summarize the functional and mechanistic studies that have linked DAP-kinase to the regulation of autophagy in general, and autophagic cell death, in particular.

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### Introduction

Autophagy is the process by which intracellular contents are engulfed and self-consumed by *de novo* formed autophagosomes (reviewed in Ref. [1]). It is a mechanism by which the cell recycles cellular building blocks and nutrients, scavenges damaged organelles and aggregated, misfolded proteins, and disposes of intracellular pathogens. As such, the autophagic process plays a crucial role in maintaining cellular homeostasis, and especially contributes to cell survival during times of stress. Autophagy is often observed during programmed cell death, where it may serve to either prevent further damage and counter the death stimulus, or may actually facilitate cell death by excessive self-consumption (see Ref. [2] for a recent review of this issue). At the molecular level, autophagy is mediated by a group of Atg genes, originally identified

in yeast [3,4]. While the basic machinery is conserved between yeast and mammals, several crucial regulators are present in higher organisms, including those that lie at signaling junctions between apoptosis and autophagy, governing a cell's decision to activate one or both pathways, and ultimately affecting overall cell survival or death. DAP-kinase (DAPk) is one such gene and will be the focus of this review.

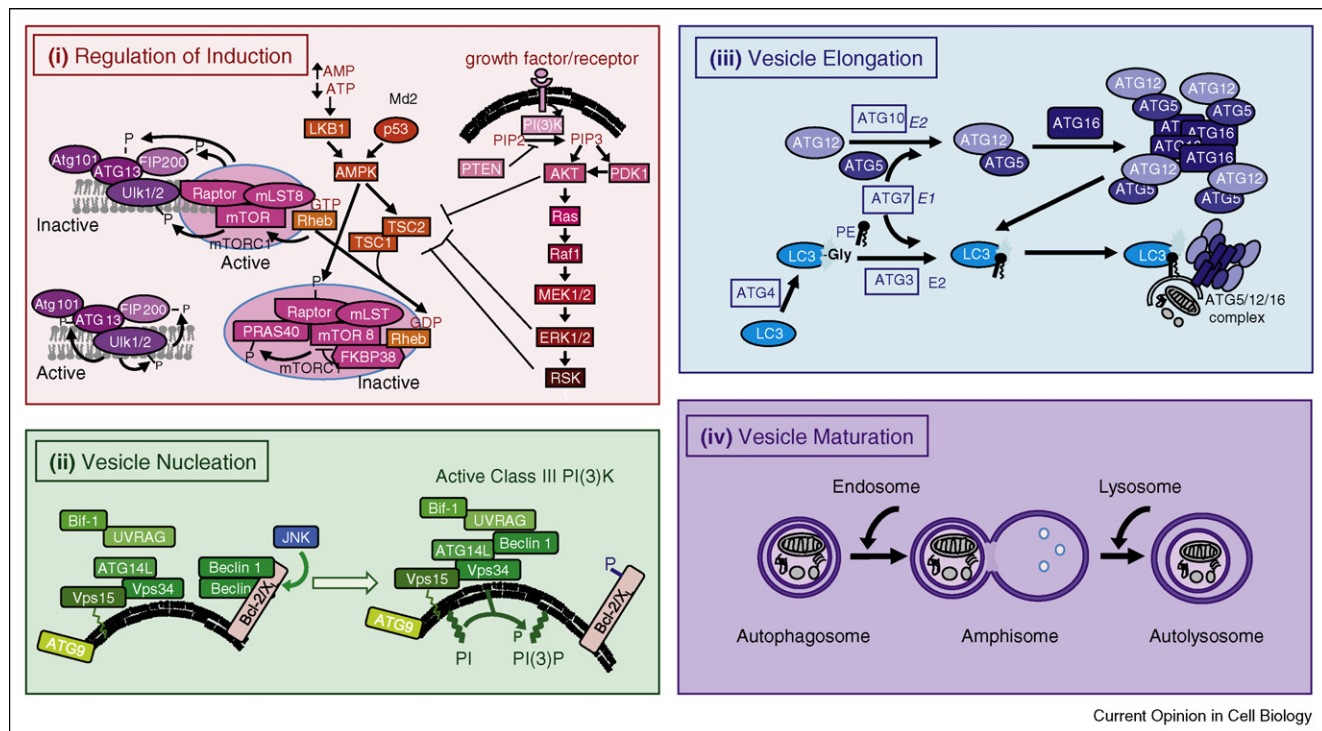
DAPk, a  $\text{Ca}^{2+}$ /calmodulin regulated Ser/Thr kinase, is a tumor suppressor that functions as a regulator of cell death. It has been shown necessary for apoptosis induced by several stimuli (see Ref. [5] and references therein). It has also been recently implicated in the regulation of caspase-independent cell death with necrotic hallmarks through a PKD–JNK signaling pathway [6]. Furthermore, DAPk has been linked to the activation of autophagy and is one of the few autophagic genes associated specifically with autophagic cell death [7,8]. Thus, not only is it an example of cross-talk and joint control of several death pathways, but it may represent a molecular switch that determines whether autophagy has a pro-survival or pro-death role. The molecular regulation of mammalian autophagy is constantly being updated and refined, and this has enabled a more precise analysis of DAPk-induced autophagy and its mechanism of action. Several papers have emerged in the past 2–3 years that map DAPk to different stages of autophagy regulation. This review will present the data showing DAPk's involvement in autophagy and will also discuss these recent papers.

### Regulation of autophagy

The basic autophagy machinery, comprised of the Atg genes, can be grouped into modules that function at various stages of the autophagic process (Figure 1). The first stage of autophagosome generation is dependent on a kinase complex containing Ulk1 (yeast Atg1) and several recently identified interacting proteins, which localizes to the isolation membrane [4,9]. Except for members of its own complex, the substrates of Ulk1 have not yet been identified [10]. A Class III phosphatidylinositol 3-kinase (PI(3)K) complex that includes the PI(3)K Vps34, Beclin-1 (Atg6), and others produces PI3-phosphate, a lipid signaling molecule that is crucial in the early stages of autophagosome nucleation. Beclin-1 is essential for PI(3)K activity and autophagy, and is in turn negatively regulated by Bcl-2/Bcl-X<sub>L</sub>, an interaction that involves binding of Beclin-1's BH3 domain to the BH3-binding groove of the Bcl-2 proteins [11–15].

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Figure 1



Schematic diagram of the various stages of autophagy. Stage I involves the regulation of autophagy induction, in which mTOR, a sensor of energy and nutrients, is inactivated, allowing for the activation of the Ulk1 kinase complex. In Stage II, nucleation, the Class III PI(3)K complex, composed of Vps34 and its interacting partners, forms PI(3)P, which is necessary for formation of the isolation membrane. For simplicity, UVRAG and Atg14L are shown in the same complex, although they define separate Vps34 complexes, the functional significance of which is still not clear. The membrane expands to engulf cytosolic contents in Stage III, vesicle elongation, a process that requires the two ubiquitin-like conjugation steps of Atg5-Atg12 and LC3-PE. Stage IV, vesicle maturation, involves trafficking and fusion of the fully enclosed double-membrane autophagosome to various endosomal compartments to form the amphisome, which finally fuses with the lysosome to form the autolysosome. In the final stage, Stage V, degradation (not shown), the contents of the autolysosome are degraded by resident lysosomal enzymes.

Finally, two ubiquitin-like pathways are required for vesicle membrane recruitment and elongation [16]: Atg5 is conjugated to Atg12, which then binds Atg16L, forming a large multi-protein complex that is recruited to the forming autophagosome's isolation membrane, and Atg8 (mammalian LC3) is conjugated to the lipid phosphatidylethanolamine (PE). Lipidation of LC3 converts it from its soluble, cytoplasmic form (LC3-I) to the membrane-bound, autophagosome-associated, form (LC3-II), which is required for membrane expansion. Additional factors regulate autophagosome trafficking and fusion with endosomal compartments and the lysosome, to form the amphisome and finally the autolysosome, wherein its contents are degraded by lysosomal proteases [4].

Autophagy is negatively regulated by mTOR (mammalian target of rapamycin) kinase, a sensor of growth factor, nutrient and energy availability [17]. mTOR is activated by the small GTP binding protein, Rheb, whose GTPase activity is under negative control by the TSC1/2 dimer. Various signaling pathways modulate this regulatory step by phosphorylation of TSC2, including Akt/PKB, ERK,

and RSK1, which inactivate TSC1/2, and AMP kinase (AMPK), which stimulates TSC1/2, thereby activating or inhibiting mTOR, respectively. mTOR's substrates include those that regulate cell growth and protein translation, such as 4E-BP1 and p70S6K, and members of the Ulk1 complex, including Atg13 and Ulk1, which are inactivated by phosphorylation (reviewed in Ref. [9]). Thus signals that suppress mTOR, such as starvation, lead to initiation of autophagy via the Ulk1 signaling module.

### DAPk and autophagy: a functional connection

DAPk was originally isolated in a functional anti-sense based screen for genes that were necessary for IFN $\gamma$ -induced cell death in HeLa cells [18]. Further characterization of this system indicated that cell death was caspase-independent and accompanied by the appearance of autophagosomes [7]. Expression of the DAPk anti-sense isolated in the original screen reduced the extent of autophagosome formation, implying that DAPk was necessary for autophagy in this system [7]. Likewise, when expressed in 293T cells, active DAPk induced a

caspace-independent cell death that was characterized by membrane blebbing and the formation of autophagosomes, as seen by EM [7] and the accumulation of GFP-LC3 in intracellular puncta that represent autophagic vesicles [19<sup>••</sup>,20].

DAPk was also shown to have a contributing role in cell death by autophagy during ER stress. DAPk catalytic activity was stimulated by tunicamycin-induced ER stress, through the dephosphorylation of an inhibitory auto-phosphorylation site, Ser308 [8<sup>••</sup>]. Systemic administration of tunicamycin to mice leads to ER stress-induced cell death of kidney tubular cells, a process involving apoptosis and autophagy. DAPk knockout mice, however, were protected from cell death and showed greatly reduced pathology. Further analysis of tunicamycin-induced death of primary mouse embryonic fibroblasts (MEFs) indicated that cell death could only be attenuated when both apoptosis and autophagy were suppressed either by small molecule inhibitors or specific gene knockout/down. Thus, unlike other systems wherein autophagy serves to limit cell death during ER stress [21–24], here, autophagy contributed to cell death. Significantly, both apoptotic and autophagic pathways were reduced in DAPk<sup>-/-</sup> MEFs, and cell death triggered by tunicamycin was attenuated [8<sup>••</sup>]. Thus in this particular setting, DAPk lies at a junction connecting two death pathways: a caspase-dependent one and an autophagic one.

DAPk is but one of a family of highly related death-associated kinases, all of which share significant homology within their common catalytic domains [5]. The closest members, DRP-1 and ZIPk, have also been shown to regulate autophagy. DAPk and ZIPk form a kinase cascade, whereby DAPk phosphorylates and activates ZIPk. Expression of the phosphomimetic, activated form of ZIPk led to autophagy induction [20]. Likewise, expression of active DRP-1 in 293T cells induced autophagosome formation ([7], and unpublished data, Ber, Y. and Kimchi, A.), while expression of a dominant negative form blocked autophagy by amino acid starvation or anti-estrogens in MCF7 cells [7]. Furthermore, ectopic DRP-1 has been localized by immuno-EM to the interior of the autophagosome [7].

The *Caenorhabditis elegans* DAPk ortholog has been shown necessary for autophagy during starvation [25]. Overactivation of muscarinic acetylcholine receptors during starvation leads to excessive autophagy in the pharyngeal muscle and eventual death of the organism. It should be noted that in this case, autophagy did not cause death of pharyngeal muscle cells; organismal lethality was due to malfunction of the pharynx feeding organ. Deletion or knockdown of DAPk, however, suppressed starvation-induced autophagy and partially rescued the death phenotype. Thus DAPk is involved in the

signaling pathway that regulates autophagy in a physiological setting.

### DAPk and autophagy: molecular mechanisms

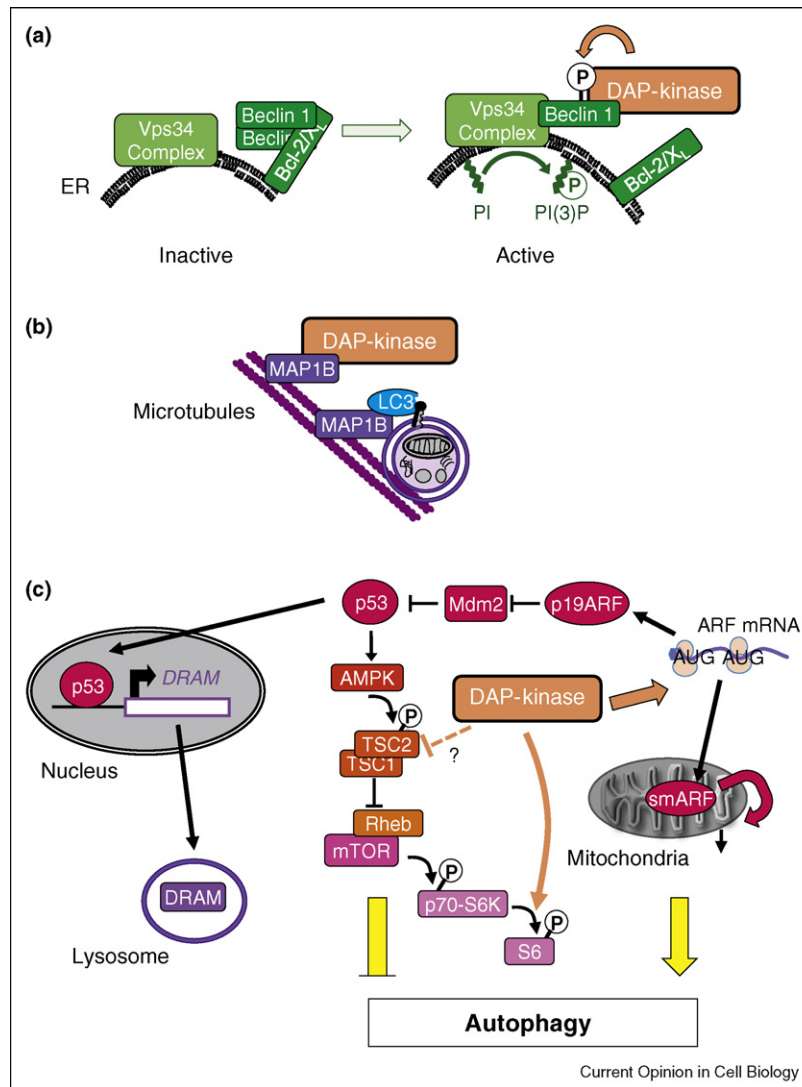
Several recent studies have shown DAPK to functionally interact with several of the autophagic modules described above (Figure 2). While some of these mechanisms are still hypothetical, positioning DAPk within the autophagic signaling pathway has substantiated its role as an autophagy regulator.

Knockdown of Beclin-1 suppresses DAPk-induced autophagy, implying that DAPk functions upstream to the Beclin-1/Vps34 complex [19<sup>••</sup>]. In fact, DAPk interacts with Beclin-1 through its BH3 domain, and significantly, phosphorylates it both *in vitro* and *in vivo* on Thr119 within Beclin-1's BH3 domain [19<sup>••</sup>]. Co-expression of DAPk with Beclin-1 led to reduced co-immunoprecipitation of Bcl-2/X<sub>L</sub> with wild type Beclin-1, but not a mutant in which the phosphorylation site was changed to Ala. Moreover, this non-phosphorylated mutant showed a greater affinity for Bcl-X<sub>L</sub> than the corresponding phosphomimetic mutant (Thr119Glu). Thus phosphorylation within the BH3 domain disrupts Beclin-1's interaction with Bcl-2 and Bcl-X<sub>L</sub>, thereby promoting Beclin-1 autophagic activity [19<sup>••</sup>,26<sup>•</sup>] (Figure 2A). Consistent with this, expression of the phosphomimetic mutant led to increased autophagosome induction, indicating the significance of this phosphorylation site for the regulation of Beclin-1. DAPk is not the only regulator of the Beclin-1/Bcl-2 complex. Phosphorylation of Bcl-2 by JNK also serves to release Beclin-1 from Bcl-2's inhibition. This latter mode of regulation has been observed in cells following starvation or ceramide treatment [27,28]. It is not yet known what signals activate the DAPk-Beclin-1 axis, and whether the DAPk and JNK regulatory events are complementary, or represent different modes of activating Beclin-1 in response to different signals.

A second molecule potentially linking DAPk to autophagy is MAP1B, a member of the MAP1 family of microtubule lattice binding proteins [29]. MAP1B can interact with both LC3-I and LC3-II in brain tissue, and a phosphorylated MAP1B specifically associates with autophagosome-localized LC3-II [30]. In degenerating Purkinje cells of Lurcher mice, autophagosomes and phospho-MAP1B accumulate in the axonal dystrophic swellings. This study suggested that MAP1B may be involved in autophagosome trafficking within the axon terminals, or may facilitate retrograde transport of autophagosomes to the cell body and delivery to the lysosome [31]. DAPk interacts with MAP1B, and in fact, can be co-localized to microtubules [32<sup>•</sup>]. At the endogenous level, this interaction is induced by amino acid starvation. Furthermore, DAPk is highly expressed in specific adult brain regions, including cerebellar Purkinje cells [33],

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Figure 2



Proposed models by which DAPk regulates autophagy. (a) DAPk interacts with and phosphorylates Beclin-1, which disrupts the inhibitory Beclin-1–Bcl-2/X<sub>L</sub> interaction. (b) DAPk interacts with MAP1B, which may be involved in autophagosome trafficking along microtubules. (c) DAPk modulates upstream signaling events that regulate autophagy induction. DAPk activates p53 in a p19-dependent manner, leading to upregulation of both proteins. p53 in turn activates AMPk, which suppresses mTOR signaling through TSC2, and upregulates the lysosomal protein DRAM. DAPk may also induce smARF levels, which triggers autophagy through damage to the mitochondrial membrane potential ( $\Delta\psi$ ). A third potential pathway, which needs further validation, may involve an interaction with TSC2, which leads to its phosphorylation and inactivation. This activates mTOR, and has been shown to lead to ribosomal protein S6 phosphorylation, also a direct substrate of DAPk.

similar to MAP1B [29]. Expression of MAP1B synergized with DAPk to suppress growth and cell viability, and conversely, partial depletion of MAP1B attenuated the growth suppression activity of overexpressed DAPk [32<sup>\*</sup>]. The DAPk/MAP1B synergy affected both the ability of DAPk to induce autophagosomes and membrane blebbing [32<sup>\*</sup>]. Whether DAPk is involved in the proposed role that MAP1B plays in autophagosome activity is unknown, but it is tempting to speculate that the synergistic effects of DAPk and MAP1B on autophagy stem

from the MAP1B/LC3 interaction (Figure 2B). Further experimentation, including whether DAPk can phosphorylate MAP1B, and whether DAPk can associate with MAP1B and LC3 on the autophagosome, are necessary to validate the mechanistic significance of this intriguing data.

Preliminary evidence suggests that the mTOR complex may be another autophagic module regulated by DAPk, although this reported connection stimulates rather than

inhibits mTOR activity (Figure 2C). It is mainly based on the finding that DAPk interacts with TSC2, the negative regulator of mTOR [34]. Overexpression of DAPk led to increased phosphorylation of TSC2 *in vivo* and to partial disruption of the TSC1/TSC2 dimer in serum starved cells. The predicted functional effect of this regulation is to activate mTOR, and in fact, expression of DAPk enhanced rapamycin-sensitive phosphorylation of the mTOR substrate p70S6K and its substrate S6. Crucial data to establish this model are still missing, such as convincing experiments that prove that TSC2 is indeed a direct substrate of DAPk, and assessment of whether the DAPk-dependent increase in mTOR activity is in fact accompanied by suppression of autophagy in this system. Significantly, a study in *Drosophila* suggested that mTOR's effects on S6K and autophagy may represent independent functional arms, and furthermore, mutation of S6K led to reductions in starvation-induced autophagy [35]. Thus activation of S6K downstream of mTOR does not necessarily equate with suppression of autophagy. Interestingly, DAPk can phosphorylate S6 directly [36], so that activation of this particular arm downstream of mTOR may have significance to DAPk mode of action. In conclusion, further research is required to determine whether TSC2 connects DAPk to the regulation of autophagy.

DAPk may also be linked to autophagy through more indirect connections, as depicted in Figure 2C. DAPk is an upstream regulator of p53, necessary for p19ARF-dependent p53 induction by oncogenes in MEFs. Furthermore, DAPk expression results in elevations in both p19ARF and p53 [37]. p53, in addition to its role in apoptosis, can regulate autophagy by several mechanisms. In response to etoposide, p53 can activate AMPK to phosphorylate TSC2, thereby suppressing mTOR [38]. p53-dependent autophagy requires DRAM, a p53 target gene that is induced in response to DNA damage [39]. The role of DRAM in autophagy is unknown, although it has been shown to localize to the lysosome. p19ARF also may link DAPk to autophagy. The p19ARF mRNA can produce a second shorter protein by internal translation, known as smARF, which lacks the nucleolar targeting signals [40]. smARF localizes to the mitochondria and its overexpression induces depolarization of the mitochondrial membrane and autophagy, leading ultimately to cell death [40]. Endogenous smARF levels are extremely low in normal cells, owing to rapid proteasome-mediated turn-over. However, like the full length p19ARF, smARF can be upregulated by oncogene expression [40]. DAPk's ability to induce p19ARF [37] presumably enables it to induce smARF expression as well, although this has not been shown directly. Thus DAPk may regulate autophagy through p53 and/or ARF-dependent pathways (Figure 2C), which differ from the previously described ones that take place in p53 deficient cells and do not involve mitochondrial dysfunction [7].

## Conclusions and future prospects

It has become increasingly clear over the past several years that DAPk is a crucial regulator of autophagy. The recent studies reported here have implicated several steps along the autophagic process that are targets for DAPk regulation. Yet, these mechanisms have only begun to be delineated and still require more research. DAPk's function in autophagy may contribute to its anti-tumor capacity, in the specific settings where autophagy has been shown to block tumorigenesis [41]. It will be interesting to discover specific mutations in DAPk that abrogate either its apoptotic or autophagic functions, to determine their individual contribution to tumor suppression. Furthermore, it remains to be discovered whether DAPk's family members function in redundant roles to induce autophagy, or whether they map to different regulatory stages in the autophagy signaling pathway. Last but not least, DAPk, as a death-associated protein, may provide a key to understanding the controversial switch in which autophagy converts from a survival mechanism to a pro-death process. This is especially relevant to cellular contexts in which the caspase-dependent pathways of DAPk are not operative and cells die without displaying apoptotic features. While the functional studies indicate that DAPk activates autophagy as a death pathway, the mechanistic studies have so far linked it to the basic autophagy regulators, without suggesting how this specifically directs autophagy towards cell death. One possibility is that other lethal caspase-independent pathways of DAPk (e.g., JNK activation) may act in concert with the direct effects on the autophagic machinery. Alternatively, DAPk's ability to activate the autophagic machinery may differ from pro-survival autophagy signals not in signaling mechanisms but rather in degree; DAPk may overactivate the basic machinery, perhaps at more than one stage, resulting in excessive autophagy that leads to death. Clarifying these aspects will require future investigation.

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