Review

The Interaction Between DAP1 and Autophagy in the Context of Human Carcinogenesis

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Abstract. *Autophagy is an evolutionarily-conserved catabolic process which furthers cell survival, especially in times of nutritional stress. Whilst being by itself a pro-survival mechanism, it has many areas of overlap with apoptosis. Autophagic cell death is recognised as a subset of programmed cell death, with features more typical of autophagy rather than classical apoptosis. The mechanisms preventing autophagy from unravelling into autophagic cell death are still the subject of much controversy. We discuss the current understanding of these mechanisms, including recent research regarding the role of death-associated protein-1 in autophagy and apoptosis.*

Autophagy can refer to several cellular catabolic processes currently under investigation. Conventionally, the term 'autophagy' commonly refers to macro-autophagy, which is an evolutionarily-conserved catabolic process which functions to further cell survival. Whilst being a pro-survival mechanism, it is very similar to a subset of programmed cell death referred to as autophagic cell death. Dysregulation of autophagy and programmed cell death are believed to have a role in carcinogenesis, presumably by furthering tumour cell survival (1).

In the present review, we present an overview of the literature regarding the regulation of autophagy, in particular regarding recent findings pertaining to death-associated protein 1 (DAP1) and its role in the control of autophagy, and its potential clinical implications regarding human carcinogenesis.

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Classification of Autophagy

Microautophagy. Microautophagy is a process not well-known by which the cytoplasm is directly engulfed by the lysosome for degradation. Generally, cytosolic contents are translocated into lysosomes in this manner. Alternatively, this process is also referred to as the 'vacuole import and degradation' (Vid) pathway (2, 3).

Specific forms of microautophagy may target specific organelles, such as the mitochondria (micromitophagy), damaged peroxisomes (micropexophagy), and nucleus (piecemeal microautophagy of the nucleus, or PMN) (2).

Chaperone mediated autophagy. Chaperone-mediated autophagy is a process by which ubiquitin-tagged proteins are translocated into lysosome by a heat-shock protein 70 (HSP-70) -mediated process (4).

Macroautophagy. Macroautophagy (henceforth referred to as autophagy) is an evolutionarily conserved catabolic process which functions to further cell survival. It has been studied as a potential target of therapeutic intervention. It has been shown to occur in response to nutritional stress, DNA damage, growth factors, and chemically induced endoplasmic reticular stress (5, 6).

It can be a generalised bulk process, or a process targeting specific cargo-protein tagged organelles. Examples include autophagic processes targeting mitochondria (mitophagy), nucleus (nucleophagy), peroxisomes (pexophagy), endoplasmic reticulum (reticulophagy), ribosomes (ribophagy), and protein aggregates (aggrephagy). Macroautophagy has also been

implicated in the destruction of foreign microbes within the cell (xenophagy) (5).

This process was initially described by Nobel laureate de Duve (1963), who had earlier identified the lysosome (7, 8). It is regulated by the *atg* genes (also called apg genes) in yeast models, whose mammalian homologues are currently being identified. It has been demonstrated to be cyto-protective (9). Knock-down of the *atg* genes impaired survival of tumour cells (10).

Autophagy involves the budding of an isolation membrane from an intracellular organelle, most likely the endoplasmic reticulum (ER). These membranes separate forming autophagosomes, or a phagophore, which contain organelles or proteins marked for destruction. Autophagosomes are characterised by retention of autophagy 8 protein (atg8) homologue in their membranes, which in mammals is designated LC3. After inclusion of the targeted organelle, the autophagosomes fuse with lysosomes to form autolysosomes in which the engulfed material is hydrolysed. This process is necessary for normal cell functions, and disruption of this process has been shown to lead to cell senescence, and reduced resistance to adverse stimuli (11).

Autophagy, Autophagic Cell death and Apoptosis: Similarities and cross-talk

Evidently, autophagy shares many characteristics with apoptosis. Indeed, a subset of programmed cell death is termed as type II or autophagic cell death, remarkable for microscopic stigmata typical of autophagy. Morphologically, it is defined by a lack of chromatin condensation, with significant cytoplasmic vacuolisation. Specifically, these are double-layer autophagic vacuoles, retaining LC3 proteins in their membranes (12).

Harmol, a β-carboline alkaloid, has been investigated for its anti-neoplastic properties in non-small cell lung carcinoma cell lines. It has been found to induce autophagic cell death, independently of the caspase cascade (13).

There is significant evidence of cross talk between autophagy and apoptosis. Inhibition of caspase-8 using a pan-caspase inhibitor, benzyloxycarbonylvalyl-alanyl–aspartic acid (*O*-methyl)–fluoro-methylketone (zVAD) in murine cells leads to autophagic cell death. Subsequent RNAi of *atg* genes such as beclin-1 and *ATG7* impaired the autophagic cell death inducing properties of zVAD (14).

Studies in MCF7 breast cancer cell lines suggest an inhibitory role for caspase-9 in autophagy, which under certain circumstances did compromise cell survival (15).

Human fibrosarcoma cell lines treated with zVAD underwent autophagic cell death. This was deduced to be dependent on extracellular-signal-regulated kinase (ERK), c-Jun *N*-terminal kinase (JNK) and reactive oxygen species (ROS). These pathways were found to be activated by cytoplasmic sarcoma-related tyrosine kinase (c-SRC). It was observed that inactive c-SRC exists in complex with caspase-8, and is activated by zVAD-induced caspase inhibition (16).

A recent study has shown caspase-8 activated during death-ligand induced apoptosis to have an inhibitory role in autophagy. ATG3 is cleaved by caspase-8. This is believed to be in keeping with the cytoprotective role of autophagy, which needs to be inhibited in the cells undergoing apoptosis (17).

Regulation of Autophagy

The regulation and control of autophagy has been found to be highly conserved across various orders of eukaryotes, and has been closely studied in yeast models. Autophagy in yeast is controlled by 32 genes (all given the designation atg), which give rise to certain pathways and complexes found to be essential for autophagy. These include the atg1-13-17 autophagy induction complex, atg9 (for membrane recycling), the class III phosphatidylinositol 3-kinase (PI3K) complex for vesicle nucleation, the atg18/21-2 complex, and two conjugation systems (atg12-atg5-atg16L and atg8). The yeast model has provided a fairly consistent road map for mammalian pathways, and similar mechanisms with certain variations have been found in human and murine models (18).

Mammalian target of rapamycin complex 1 (mTORC1), AMP activated protein kinase (AMPK) and the UNC-51–like kinase 1 (ULK1) complex. In normal, nutrient-rich conditions, autophagy is inhibited by the influence of mTORC1, which receives upstream signals related to nutritional state, amino acids, and growth factors. In particular, these signals are mediated by protein kinase B (AKT/PKB), which stimulates mTORC1 by inhibiting the tuberous sclerosis protein 1 and 2-rat sarcoma (RAS) homolog enriched in brain (TSC1/2-RHEB) pathway. Alternatively, AKT/PKB can interact directly through the proline-rich AKT substrate of 40 kDa (PRAS40) component of mTORC1. The mTORC1 core protein, raptor, phosphorylates UNC-51–like kinase 1 and 2(ULK1 and 2) proteins in a manner which inhibits the 3 kDa protein complex they reside in (5, 19).

In times of nutritional stress, the inhibitory effect of mTORC1 is lost. In addition, autophagy pathway activation is assisted by AMP-activated protein kinase (AMPK). AMPK is acutely sensitive to the energy state of the cell, and is activated by low ATP to AMP ratios (20). Furthermore, it channels genotoxic signals from p53 (21). It inhibits mTORC1 by activating the TSC1/2-RHEB complex, or by phosphorylating raptor (22). Additionally, AMPK directly activates the ULK1 complex by phosphorylating ULK1 and 2 (23).

ERK and JNK. In addition, the autophagy is also controlled by an mTORC1-independent pathway involving ERK and JNK through the rat sarcoma (RAS)/ v-raf-1 murine

leukemia viral oncogene homolog 1 (RAF-1)/ MAPK and ERK kinase (MEK)/ extracellular signal-regulated kinase (ERK) cascade (24).

Additionally, ERK is also regulated by c-SRC. As mentioned earlier, inhibition of caspases by zVAD in human fibrosarcoma cell lines resulted in autophagic cell death mediated by ERK, which was in turn activated by c-SRC (16).

ULK1 complex. The ULK complex corresponds to the atg1 complex in the yeast model, and receives the majority of the upstream signals controlling the autophagy pathway. It consists of ULK1, and its homologue ULK2, with ATG13, ATG101 and focal adhesion kinase (FAK) family–interacting protein of 200 kDa (FIP200). FIP200 is considered to be the mammalian homologue of atg17 (25, 26).

ULK1 and 2 have been determined to be the mammalian homologues of atg1 (27, 28). ULK3 and ULK 4 are less well-known members of this group (5). ULK3 has been found to have a role in oncogene-induced senescence (29). Knock-down studies have shown ULK1/2 to be essential for autophagosome formation. Knockdown of either ULK 1 or 2 does not result in total blocking of autophagy, indicative of an at least partial redundancy of function (27). In addition, ATG13 and FIP200 depletion have been shown to be phenotypically-similar to a loss of ULK1/2 (5, 30).

ULK1 and 2 function within a much larger complex whose size remains static regardless of nutritional state. When under the inhibitory influence of mTORC1, ULK1/2 and ATG13 are phosphorylated. Loss of mTORC1 activity de-phosphorylates these proteins, allowing ULK1/2 to recruit further components of the autophagy cascade (25, 31).

Most importantly, ULK1/2 phosphorylates FIP200, normally bound to ATG13, which mediates this interaction. As per Gan *et al*., FIP200 has been shown to interact with at least eight effectors. It affects p53 (32). It inhibits tuberous sclerosis 1 (TSC1), which controls mTORC1 (33). It inhibits FAK, which controls cell migration (34). It inhibits proline-rich tyrosine kinase 2 (PYK2), an inducer of apoptosis (35). On the other hand, FIP200 increases cell susceptibility to tumour necrosis factor-α (TNF-α)-mediated apoptosis by providing scaffolding for apoptosis signal-regulating kinase 1 (ASK1) and TNF receptor-associated factor 2 (TRAF2) interaction, as part of the JNK signalling mechanism (36). Furthermore, it has been shown to have a role in cell differentiation and embryogenesis (37).

More recently, ATG101 was identified as a non-conserved component of the ULK1 complex not found in yeast. It has been characterised as an ATP13-binding protein, normally found in the ULK1 complex. Knockdown of *ATG101* impaired autophagy, and specifically affected LC3 translocation from the nucleus, and the conversion of LC3-I to LC3-II. Furthermore, depletion of ATG101 also affected ATG13 expression, and subsequently, overall stability of the ULK1 complex (26).

In human models, there remains significant ambiguity with regards to interaction of the ULK1 complex with autophagosome formation, and this remains an area of intensive research. The ULK1 complex is known to interact with activating molecule in Beclin1-regulated autophagy (AMBRA-1), which is postulated to likely be a component of the Beclin-1 related class III PI3K complex (see below) (38).

Death-associated Protein-1 (DAP1) and the ULK1 Pathway

The *DAP1* gene is localised to chromosome 5 band p15, and encodes a small 15-kDa protein. Human DAP1 is proline-rich, and normally exists as a phosphoprotein *in vivo* (39). The *DAP1* gene was initially identified in HeLa cells subjected to the continuous presence of apoptosis-inducing interferon gamma. Other genes thus identified encoded DAP3, DAP5 and DAP kinase. These genes are thought to have a role in apoptosis (40). A possible role in advanced cancer for *DAP3* has been a recent subject of research (41).

DAP1 lacks any identifiable functional motifs, and its function was little understood until recently. Recent studies in HeLa cells subjected to a continuous apoptotic stimulus (specifically, TNF-γ) have suggested it may have a role in autophagy (42).

Recent studies have shown that *DAP1* is also a substrate for mTOR, and is inactive when phosphorylated. In contrast to ULK1, when activated, it has been found to suppress the process of autophagy. It is thought to serve as a 'brake', preventing autophagy from progressing to cell death (43) (Figures 1 and 2).

A recent study by our group has found evidence suggesting that the mRNA expression of *DAP1* in tissue has associations with clinicopathological parameters of human breast cancer. Specifically, low expression of *DAP1* was significantly associated with adverse clinical outcomes, advanced clinical stage at presentation, and elevated Nottingham Prognostic Index (44).

Furthermore, electrical cell impedance sensing assay on knock-down transgenic sub-lines of MDA-MB-231 and MCF7 cells showed greater migratory potential compared to controls (preliminary results presented at the Scientific Meeting of the British Association of Surgical Oncology held in London, November 2012) (45).

The *in vivo* and *in vitro* findings of our group and others suggest that decreased expression of DAP1 may have significant effects on the behaviour of cancer cells, and may contribute to carcinogenesis in the context of human breast cancer. As yet, the downstream effectors of DAP1 have not been identified. However, extrapolating from those of its co-substrate, FIP200, the pathways downstream of DAP1 are likely to be wide-ranging and varied beyond the direct control of autophagosome formation. These may include

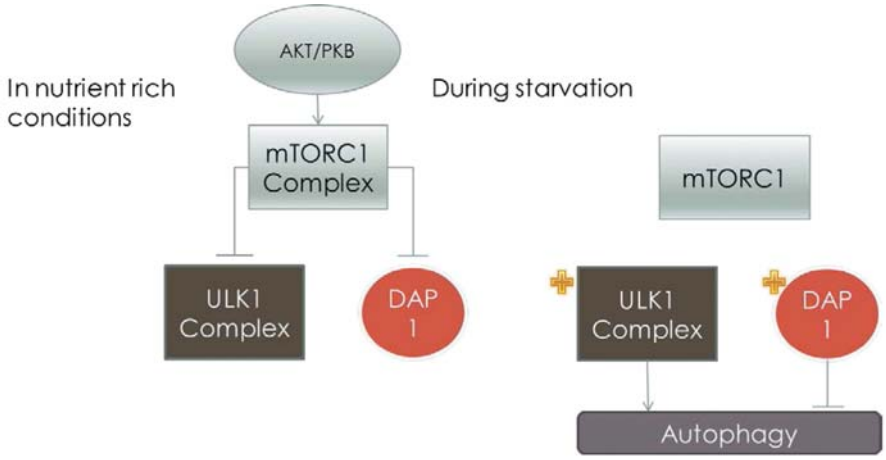

Figure 1. *Unc-51 like protein 1 (ULK1) and death-associated protein 1 (DAP1) acting under the regulatory influence of mammalian target of rapamycin complex 1 (mTORC1) and protein kinase B (AKT/PKB).*

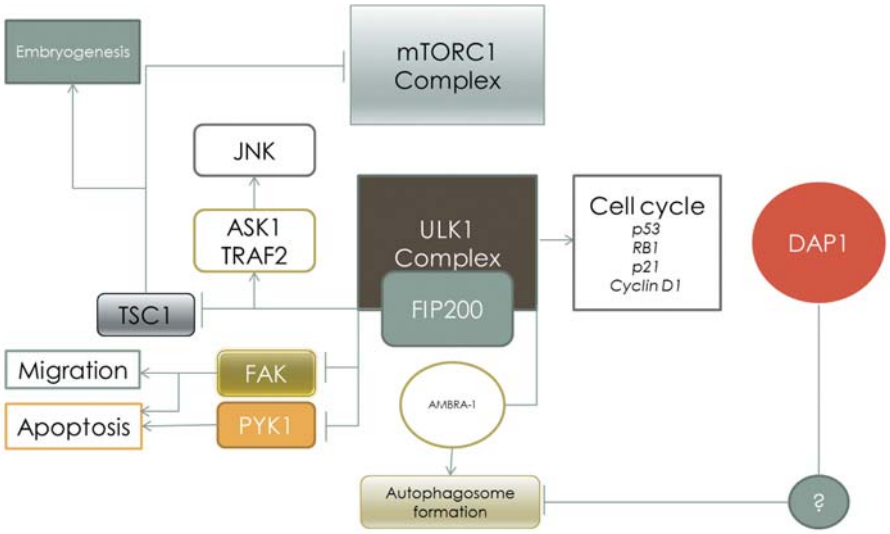

Figure 2. *The downstream pathways affected by the Unc-51 like protein 1 (ULK1) and death-associated protein 1 (DAP1). Abbreviations: Unc-51 like protein 1 (ULK1), death-associated protein 1 (DAP1)ULK1, mammalian target of rapamycin complex 1 (mTORC1), protein 53 (p53), protein 21 (p21), retinoblastoma 1 (RB1), tuberous sclerosis 1 (TSC1), c-Jun N-terminal kinase (JNK), focal adhesion kinase (FAK), focal adhesion kinase family–interacting protein of 200 kDa (FIP200), proline-rich tyrosine kinase 2 (PYK2), apoptosis signal-regulating kinase 1 (ASK1) and TNF receptor-associated factor 2 (TRAF2).*

apoptosis, regulation of the cell cycle, the mTOR pathway, and the pathways controlling cell migration.

BCL2 Family and the Class III PI3K

BCL2, a member of the eponymous family of proteins in the outer membrane of mitochondria, has been demonstrated to have an inhibitory role in autophagy regulation. Canu *et al.* observed early autophagic changes in cerebellar granule cells subjected to serum and potassium deprivation before progressing to apoptosis (46). These changes were inhibited in cell lines overexpressing BCL2. BCL2 overexpression was cytoprotective. This effect of BCL2 on autophagy persisted despite treatment with the pan-caspase inhibitor zVAD, indicating the activation of a BCL2 downstream target other than the canonical caspase pathway (47).

It is posited that BCL2 exerts its effects on autophagy by regulating beclin-1. Beclin-1 associates with intracellular membranes (specifically, the trans-Golgi complex), and has been shown to induce autophagy. Pattingre *et al.* demonstrated a possible role of BCL2-beclin1 complex in the regulation of cytoprotective autophagy (48). It is suggested that during

apoptosis, with depletion of BCL2, beclin-1 is released, and interacts with PI3K vacuole protein sorting-34 (VPS34) and p150 (or VPS15) to form the class III PI3K, an important component of the autophagy pathway, directly responsible for formation of the autophagosomes (49). Other possible components of class III PI3K include AMBRA-1, UV radiation resistance-associated gene (UVRAG), and BAX-interacting factor-1 (BIF-1), among others (50, 51). Takahashi *et al*. suggest that BIF-1 and UVRAG form a complex with beclin-1which contributes to autophagosome formation by mediating curvature formation (52). It is suggested that a variety of combinations may be possible. It is suggested that the inhibition of actions of beclin-1 by BCL2 over-expression promotes oncogenesis. Whilst these conclusions were not reproduced by Zeng (49), they have since been reproduced by Maiuri *et al*. (53), and Priault (54).

The BCL2-beclin1 interaction is mediated by BCL2 homology (BH) region 3 (BH3) on BCL2 and a corresponding motif on beclin1. The BH3 motif is said to mediate antiapoptotic functions of the BCL2 family, and also enables interactions of BCL-xL and BCL2-associated death promoter (BAD) with beclin1 (53, 54). This interaction is prone to disruption by certain mutations in BH3, and specific BH3 mimetic agents, such as ABT737. BAD is a pro-apoptotic member of the BCL2 family with only the BH3 motif. In response to starvation, it is found to associate with both BCL2 and BCL-xL following their dissociation from beclin1. siRNA-mediated depletion studies by Maiuri *et al*. demonstrated that BAD and its interaction with BCL2/BCL-xL facilitates starvation mediated autophagy, but not mTOR-related autophagy, whilst VSP34 is required for both (53). Transfection-induced overexpression of BAD caused autophagy in mouse embryonic fibroblast cells (MEF) (53).

BCL-xL is an antiapoptotic BCL2 protein which, like BCL2 has four BH domains (BH1, BH2, BH3, and BH4). Maiuri *et al*. found BCL-xL to interact with beclin-1 in a manner similar to BCL2 (53). However, Priault *et al*. found BCL-xL to be a stronger regulator of autophagy than BCL2 (54). Furthermore, it was demonstrated that the effect of BCL-xL on autophagosome formation was independent of beclin-1. However, an intact BH3 site was essential for this function. As depletion of Bcl-xL and ATG7 were phenotypically similar, it is suggested that they may constitute a portion of an autophagy pathway which is yet to be delineated (54).

Autophagosome Formation

The pre-autophagosomal structure, or phagophore assembly site (PAS) is the designation given to the assembly point for autophagosome formation. It is by and large believed to originate from the ER. However, some evidence points to mitochondria as the origin of the PAS. Some of the difficulty in ascertaining the origin of the autophagosome is due to the distinct composition of the membrane, which is relatively bereft of proteins or organelle markers clearly denoting origin (55).

Autophagosome formation is believed to be initiated by interaction of the class III PI3K complex (beclin1-VPS30-p150) with ATG14L (homologue of ATG14, also known as Barkor) on ER-derived membrane. This complex generates PI3, which is required for recruitment of further ATG proteins (55). These include the 49 kDa WD-40 repeat containing proteins that interacts with phosphatidylinositol (WIPI49, or WIPI, mammalian orthologue of ATG18). This family of proteins (WIPI-1 to 4), especially WIPI-2, has been found to be essential for formation of punctae which mark the initiation of autophagosome formation. In addition, this family of proteins in involved in recruitment of omegasomes (omega-shaped buds from ER), and initiating LC3 conjugation pathways (56, 57).

Recruitment of lipids for membrane formation involves shuttling of mammalian ATG9 to the PAS from another cell location, most likely the trans-Golgi network. Mammalian ATG9 is a transmembrane protein, believed to be dependent upon ULK1 and VPS 34 for its function in formation of the initial isolation membrane. The only other transmembrane protein involved in autophagy appears to be VMP-1, which may have a role in beclin-1 recruitment to the PAS (58, 59).

Mirroring yeast models, the elongation of the isolation membrane in mammals, including humans, is dependent upon two conjugation systems. ATG12 is a ubiquitin-like molecule. In a manner analogous to ubiquination, ATG12 combines with ATG7 (a E1-like protein), then transfers to ATG10 (E2-like protein). An E3-like protein has not been reliably identified in this process. Finally, ATG12 is covalently conjugated to ATG5. ATG12-ATG5 form a complex with autophagy related 16-like 1 (ATG16L1) (60, 61). The ATG12-ATG5-ATG16L1 complex is essential for isolation membrane elongation, and acts as an E3 ligase of atg8 orthologues, determining the phosphatidylethanolamine (PE) conjugation site (59).

The mammalian orthologue of atg8 consists of three families of proteins. The LC3 is the one best understood, and is a long recognised marker of autophagosome membranes. Other homologues include GABAA receptor-associated protein (GABARAP) and Golgi-associated ATPase enhancer of 16 kDa (GATE-16) (62).

Soluble LC3 is cleaved by human homologue of atg4 (ATG4B) to form LC3-I (63). As was in the case with ATG12, the process of conjugation of LC3-I with PE occurs in a manner similar to analogous to ubiquination (61). LC3-I is conjugated with PE by ATG7 (E1-like), ATG3 (E2-like) and the ATG12-ATG5-ATG16L1 (E3-like) to form LC3-II, which is incorporated into the autophagosome membrane (64, 65). LC3-II disappears from the outer surface of the autophagosome just prior to fusion

with lysosomes. ATG8 proteins are believed to serve as cargo proteins targeting specific cell components, interacting with specific adaptor molecules [*e.g.*, p62, BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like (BNIP3L, or Nix)], and mediating intracellular trafficking to lysosomes in particular (50, 62).

Fusion with lysosome exposes the enclosed cellular organelles and proteins to digestive proteases and hydrolases, making the recycled materials available for further use. The interactions mediating the fusion with the lysosome, and translocation of digested material are poorly understood (18). It has been suggested that a novel protein, tectonin beta-propeller repeat containing 1 (TEPRC1) may play a role in the fusion of the autophagosome with lysosome. Initially, ATG16L1 dissociates from ATG12-ATG5. TEPRC1 then binds ATG12-ATG5 to phosphatidylinositol 3-phosphate, which initiates the autophagosome maturation process (66).

Conclusion

Autophagy is an important pro-survival mechanism which enables cells to endure times of nutritional stress. In addition, it enables clearance of damaged, potentially harmful, organelles and makes their constituent nutrients available for cellular functions. As apparent from the preceding review, disruptions in the regulation of this pathway have been implicated in carcinogenesis. Furthermore, this pathway is known to have significant linkages, overlaps and cross-talk with other cellular pathways known to have significant roles in oncogenesis.

There is still much to be ascertained regarding the control of autophagy. It is suggested that DAP1 provides an inhibitory control to autophagy. However, little is known of its downstream effectors. As mentioned earlier, an extrapolation from the better-understood downstream effectors of FIP200 component of the ULK1 complex would suggest that the pathways downstream from DAP1 may include the mTOR pathway, the cell cycle, and the apoptosis cascade, among others. This may suggest that the normal function of autophagy is predicated on close coordination with other cellular processes, and disruptions in the control of autophagy may have profound effects on other critical cellular pathways.

A more detailed understanding of the downstream substrates of DAP1 requires further research, which may include immunohistochemical, genomic and mechanistic studies. This would hopefully further our understanding of the interactions between several key cellular pathways central to carcinogenesis.

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