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Extra-view

ALK IS A NOVEL DEPENDENCE RECEPTOR : POTENTIAL IMPLICATIONS IN DEVELOPMENT AND CANCER

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ABSTRACT

ALK (anaplastic lymphoma kinase) is a transmembrane receptor tyrosine kinase, initially discovered as part of the NPM-ALK fusion protein, resulting from a chromosomal rearrangement frequently associated with anaplastic large cell lymphomas. The native ALK protein is normally expressed in the developing and, at a weaker level, adult nervous system. We recently demonstrated that ALK is a novel dependence receptor. As such, in the absence of ligand, the ALK receptor is kinase inactive and its expression results in enhanced apoptosis, whereas kinase activation, due to a ligand or constitutive as in NPM-ALK, decreases apoptosis. Unligated/kinase unactivated ALK receptor facilitates apoptosis via its own cleavage by caspases, a phenomenon allowing the exposure of a proapoptotic juxta-membrane intra-cellular domain. This review summarizes the biological significance of the ALK receptor in cancer and development, in perspective with its dependence receptor function. The dual function of ALK in the physiology of development is illustrated in the visual system of *Drosophila*. In this part of the nervous system, ALK in the presence of ligand appears essential for axonal guidance, whereas in the absence of ligand, ALK expression can lead to developmental neuronal apoptosis. ALK is also found expressed in neural crest-derived tumors such as human neuroblastomas or glioblastomas but its role is not fully elucidated. However, an excessive or constitutive ALK tyrosine kinase activation can lead to deregulation of cell proliferation and survival, therefore to human cancers such as lymphomas and inflammatory myofibroblastic tumors. Our observations could have important implications in the therapy of ALK-positive tumors harboring the chimeric or wild type ALK protein.

Increasing evidence indicates that a single molecular actor can lead to opposite cellular effects depending on the environmental conditions. Indeed oncogenes, receptors, or apoptosis modulators (for example of the Bcl-2 family) can drive cells either to proliferate (or differentiate or migrate) and survive, or to die in a programmed fashion.[1,2] The balance between these positive and negative signals in individual cells determines their fate, and the resulting number and nature of proliferating/surviving and dying cells subsequently determines the fate of the tissue or organ. Such phenomena are pivotal in physiology, *e.g.*, in development, and are deregulated in pathology leading to cancer.

The recently described "dependence receptor" family seems to fit the above criteria. These receptors, defined by their functional rather than structural similarity, create cellular states of dependence on their respective ligands by inducing or favoring apoptosis when unoccupied by ligand, but inhibiting apoptosis in the presence of ligand. All these receptors appear to be cleaved during apoptosis, most of them by caspases (reviewed by Bredesen et al[3]), leading to the release or the exposure of a proapoptotic peptide and creating a positive feedback loop for apoptosis enhancement. Several members are involved in neuronal development and migration or axonal guidance. They include $p75^{NTR}$, a low affinity receptor for nerve growth factor,[3] the netrin receptors DCC (deleted in colorectal cancer) and UNC5H,[4-7] the integrin $\alpha v\beta 3$,[8] Patch (a receptor for the morphogen Sonic hedgehog),[9] and neogenin.[10] An interesting example of a dependence receptor is given by RET (rearranged during transfection), a receptor tyrosine kinase acting as a coreceptor for the glial cell-derived neurotrophic factor (GDNF). RET induces apoptosis in the absence of ligand, and the addition of GDNF is then sufficient to block RET pro-apoptotic activity. Moreover RET is cleaved *in vitro* by caspase 3 and the caspase cleavage sites are crucial for RET-induced cell death, probably by allowing the release of a pro-apoptotic fragment.[11] In humans, mutations that inactivate RET lead to

Hirschprung's disease (congenital aganglionic megacolon), a developmental defect due to the lack of enteric neurons.[12,13] In contrast, mutations activating the receptor tyrosine kinase RET lead to multiple endocrine neoplasia type 2, an inherited cancer syndrome.[14] Thus this dependence receptor can play a role as an oncogene as a result of constitutive activation of its kinase activity.[15] Alternatively, some dependence receptors can play a role in cancer as conditional tumor suppressors. By functioning as a dependence receptor, DCC induces apoptosis unless DCC is engaged by its ligand, netrin-1. Mehlen's group showed that inhibition of cell death by enforced expression of netrin-1 in mouse gastrointestinal tract leads to the spontaneous formation of hyperplastic and neoplastic lesions.[16,17] Thus when their expression is ectopic or inappropriately regulated, dependence receptors can lead to cancer (as they become oncogenes or conditional tumor suppressors) or to severe developmental defects.

ALK is a dependence receptor

We recently demonstrated that ALK (anaplastic lymphoma kinase), a receptor tyrosine kinase of the insulin receptor superfamily, is a dependence receptor.[18] ALK was initially discovered as part of the NPM-ALK fusion protein, resulting from the t(2 ;5) translocation that is frequently associated with anaplastic large cell lymphomas (ALCL).[19,20] It has been demonstrated that the NPM portion is responsible for the dimerization of the fusion protein leading to constitutive activation of the ALK kinase and to oncogenicity,[21] as shown for other fusion proteins involving tyrosine kinases such as BCR-ABL.[22] The native ALK protein, a type I transmembrane receptor, is mainly expressed in discrete regions of the developing central and peripheral nervous system, but is not present in hematopoietic cells.[23, 24] We had previously demonstrated that the oncogenic, constitutively kinase activated NPM-ALK protein was antiapoptotic when expressed in Jurkat lymphoblastic cells treated with cytotoxic drugs.[25] In order to approach the function of the wild type ALK receptor, which is still

elusive, we expressed ALK in cell lines of either lymphoid or neuronal origin as models. Apoptosis was induced by doxorubicin in Jurkat human T lymphoblastic cells stably expressing ALK, or triggered by serum deprivation in transiently ALK-transfected 13.S.1.24 murine immortalized neuroblasts. The expression of ALK clearly enhanced apoptosis in both models. Furthermore, *ALK* transfection into primary cortical rat neurons (which do not express endogenous ALK) significantly decreased their survival. Our results showed for the first time that ALK expression could enhance and/or trigger apoptosis in lymphoid and neuronal cells. Apoptosis enhancement could be prevented in the presence of ALK-specific ligands such as agonist monoclonal antibodies. We also demonstrated that the ALK protein was cleaved during apoptosis in a caspase-dependent manner, and mapped the caspase cleavage site at the aspartic residue 1160 located in the juxta-membrane region of ALK. Mutation at this site abolished both caspase cleavage and apoptosis enhancement.[18]

Thus in the absence of ligand, the ALK receptor kinase is inactive and enhances apoptosis, whereas kinase activation, due to a ligand or constitutive as in NPM-ALK, decreases apoptosis. Unligated/kinase unactivated ALK receptor facilitates apoptosis via its own cleavage by caspases, a phenomenon allowing the exposure of a proapoptotic juxta-membrane intra-cellular domain (Figure 1).

It has been shown that lymphocytes can survive limited caspase activation,[26] and that active caspase 3 was detected in ALK-positive ALCL tumors.[27] In the lymphoid Jurkat cells, we have observed that a certain degree of caspase activation was present both in parental and transfected cells (unpublished results). It is possible that ALK does not itself trigger apoptosis but acts as a sensitizer to whichever trigger is existant. Two signals would thus be necessary to enhance apoptosis. If the level of caspase activation reaches a certain threshold, depending on the cell itself and its environment, then we propose a model in which ALK is cleaved by caspase 3 (or possibly a related subfamily caspase member), exposing a proapoptotic region

and creating a positive feedback loop for apoptosis enhancement. This model could also apply to neuronal cells, where serum deprivation, a stimulus which *per se* does not induce cell death in murine immortalized neuroblasts, "pushes" ALK to induce cell death (Figure 1).[18]

ALK is involved in development

Like several dependence receptors,[28] ALK is involved in neural development[23,24] (Table 1) and possibly morphogenesis, in spite of the lack of a lethal or abnormal phenotype in ALK-knock-out mice (Morris, personal communication, and Duyster et al[29]). *In situ* hybridization analysis performed in rodents showed that the *ALK* mRNA is essentially and transiently expressed in specific regions of the central and peripheral nervous systems such as the thalamus, mid-brain, olfactory bulb and peripheral ganglia and that it is mainly localized in neuronal cells.[23,24] Since ALK expression is maintained, albeit at a lower level, in the adult brain, it might play an important role both in the normal development and function of the nervous system. Interestingly, two recent papers analyzed the temporal spatial expression of the ALK receptor during the development of mice[30] and chicken,[31] showing that ALK is dynamically expressed on subsets of neurons of both the central and peripheral nervous system. It is noteworthy that the expression of the *ALK* transcript in spinal motor neurons overlaps temporally with the period of programmed cell death (which affects 50% of these neurons) in the chick embryo, suggesting that ALK could play a role in this phenomenon.[31] Our results showing a pro-death effect of ALK in both murine immortalized neuroblasts and in *ex vivo* primary neurons support the potential proapoptotic role of ALK under certain physiological conditions that remain to be determined.

A current difficulty encountered in elucidating the physiological role of ALK is that the ligands of ALK are still a matter of controversy, at least in mammals/vertebrates. Pleiotrophin (PTN) and midkine, two heparin-binding growth factors with pleiotrophic activities involved

in normal development and tumor growth,[32,33] have been proposed as potential ligands of ALK, based on a genetic screen by peptide phage display. The expression pattern of these growth factors partially overlaps that of the ALK receptor in the rodent developing nervous system.[34,35] However, recent studies performed by different groups including ours argue against PTN as a specific ALK ligand.[18,29,36-38] In contrast, a ligand named jelly belly (jeb) has been clearly identified in *Drosophila melanogaster*, based on the similar phenotypes displayed by fly mutants lacking either the *Drosophila* homologue of *ALK*, *DALK* , or the *jeb* gene. Both defects caused an abnormal development of the visceral mesoderm, due to a lack of visceral muscle founder cells.[39-42] DALK is also expressed within the nervous system of *Drosophila* at later developmental stages,[43] but its role in this tissue is only starting to be elucidated. A very elegant study from Palmer's group recently found complementary patterns of expression for DALK and its ligand jeb in the fly developing visual system. These authors demonstrate that the ALK/jeb couple plays a central role as an anterograde signaling pathway mediating neuronal circuit assembly in the *Drosophila* visual system. ALK is expressed and required in target neurons in the optic lobe, whereas jeb is primarily generated by photoreceptor axons and functions in the eye to control target selection of specific photoreceptor cell axons.[44] Interestingly, the authors pointed out that the level of neuronal cell death (measured by active caspase 3 expression) in the optic lobe medulla, an area in which ALK is expressed, increases in mutants lacking expression of jeb. Moreover, caspase-dependent neuronal apoptosis dramatically decreases in mutants overexpressing jeb.[44] These results suggest ALK could play a role in the physiological negative selection of neurons shaping the optic lobe in the *Drosophila* nervous system, by favoring apoptosis in the absence of the ALK ligand. These *in vivo* observations provide a strong support to our classification of ALK as a dependence receptor.[18] Together, these findings suggest that jeb/ALK signaling helps photoreceptor cell axons to shape their environment for target neuron recognition. [44] It

should also be noted that an *ALK* homologue has been identified in *C. elegans*. It is expressed at low levels in the nervous system and is proposed to regulate synapse differentiation at neuromuscular junctions.[45]

Taken together, these studies (summarized in Table 1) show that in vertebrates, ALK is expressed early in development in the ectoderm that gives rise to the neural crest and its derivatives. Expression of ALK in the mesoderm is substantiated by studies showing its role in *Drosophila* gut development.[39-41] The observation that in chick, a distinct set of skeletal muscles of the limbs, together with motor neurons innervating these regions, are ALK-positive during embryo development,[31] suggests a possible role in neuromuscular synapse formation.

Inverse correlation between ALK kinase activation and ALK-mediated proapoptotic effect

The activation of receptor tyrosine kinases typically requires ligand-induced receptor oligomerization, which results in tyrosine autophosphorylation of the receptors.[46] We have shown that in the absence of ligand, ALK expressed by transfection in lymphoid or neuronal cells was not tyrosine phosphorylated (therefore the kinase was inactive) and exerted a proapoptotic effect. In the presence of a specific ligand (an agonist antibody) however, ALK became phosphorylated on tyrosine and the cells were partially rescued.[18] Thus there is an inverse correlation between the kinase activation and the proapoptotic activity of ALK. Accordingly, the antiapoptotic activity of NPM-ALK in doxorubicin-treated Jurkat cells depended on the constitutive activation of the kinase.[25] Mutation of the ATP-binding site ("killing" the kinase) in NPM-ALK not only abrogated this antiapoptotic effect, but resulted in enhanced apoptosis, as efficiently as wild type ALK expression.[18] We noticed that the constitutively kinase-activated NPM-ALK chimeric protein, which contains the entire intra-

cellular domain of ALK, was cleavable by caspase 3 *in vitro,* but was relatively resistant to cleavage after *in vivo* doxorubicin treatment of cells. The kinase-dead NPM-ALK mutant was more readily cleaved *in vivo* than its kinase-active counterpart, indicating that ALK phosphorylation partially prevents caspase cleavage, probably through a conformation change, and protects cells from apoptosis.[18]

Expression of a transmembrane ALK receptor truncated at the site of caspase cleavage led to significant enhancement of apoptosis in both the lymphoid and neuronal cell models, whereas the expression of the cleaved C-terminal fragment of ALK did not have any significant effect on apoptosis. These findings strongly suggest ALK cleavage exposes a juxta-membrane intra-cellular region that favors apoptotic signaling (Figure 1). Caspases have been shown to associate to death receptors such as Fas/CD95, TNFR1 or TRAIL via intracellular adaptor proteins through different motifs within the death-like domain superfamily: death domain (DD), death-effector domain (DED) or caspase-recruitment domain (CARD).[47, 48] In order to see whether ALK could associate with proapoptotic effectors, we searched databases but were unable to find any death-like domain within the juxta-membrane intra-cytoplasmic sequence of ALK. The direct or indirect interaction of ALK with proapoptotic effector molecules will be the object of further studies.

In summary, we propose the following model for ALK positive and negative signaling (Figure 1). In the presence of ligand, the ALK receptor homodimerizes, activating its tyrosine kinase domain by transphosphorylation and mediating a decrease of apoptosis through various signaling pathways. In the absence of ligand, the ALK receptor is likely monomeric, the kinase is inactive and it enhances mitochondrial-dependent apoptosis : release of cytochrome c by the mitochondria, formation of the apoptosome associating cytochrome c with apoptotic protease activating factor 1 and procaspase 9, leading to caspase 9 and caspase 3 activation and eventually to apoptosis.[49] In conditions generating a sufficient level of activated caspases,

ALK is cleaved by caspase 3 (or possibly a related subfamily caspase member), exposing a proapoptotic region within the juxta-membrane intra-cellular domain and creating a positive feedback loop for apoptosis enhancement.

ALK in cancer

ALK is involved in several chromosomal translocations or inversions, apart from the most common t(2;5) giving rise to the NPM-ALK fusion. Most of these X-ALK fusions are also found in ALCL, while a few can affect (though rarely) diffuse large B-cell lymphomas.[50] ALK represents one of few examples of a receptor tyrosine kinase implicated in oncogenesis in both hematopoietic and non-hematopoietic tumors, given that ALK fusions also occur in the mesenchymal tumor known as inflammatory myofibroblastic tumor (reviewed by Pulford et al[51]) (Table 2). The oncogenic properties of some X-ALK proteins have been demonstrated,[52] sharing the same molecular mechanism, i.e., homodimerization of the chimera followed by constitutive kinase activation.

The wild type full size ALK protein has also been found expressed in human tumors, notably in neuroblastomas, in which ALK was generally not found tyrosine phosphorylated.[36,53] These tumors, thought to originate from neural crest cells during embryonic development of the peripheral nervous system, occur in infants and young children, and often show spontaneous differentiation and regression.[54] It is noteworthy that spontaneous caspase activation observed in neuroblastomas correlates with a good prognosis.[55] Our observation of an apparent cleavage of ALK in two cell lines by western blotting (unpublished results) suggests that *in vivo* caspase activation could occur in neuroblastoma cells and favor ALK-dependent proapoptotic processes. The full length ALK is also found expressed in glioblastomas and melanomas,[36] that may originate from neural crest-derived cells. Whenever it has been studied, ALK tyrosine phosphorylation was not detected in primary tumors, but has been

reported in rare cases for neuroblastoma[56] and possibly glioblastoma[57] cell lines. One may wonder whether it is a coincidence that ALK is involved in visceral and skeletal muscle development in *Drosophila*[42,43] and chicken,[31] respectively, as well as in a subset of human rhabdomyosarcomas (skeletal muscle malignant tumors),[23] all these tissues having a mesodermal origin (Tables 1 and 2).

In summary, we have shown that inappropriate expression of ALK, in the absence of ligand and/or in the wrong cell setting, triggers or facilitates apoptosis.[18] On the other hand, mechanisms which inhibit apoptosis should enhance or promote tumorigenesis. Indeed when ALK is under its oncogenic form NPM-ALK, the proapoptotic effect of ALK due to its cleavage by caspases is balanced by the proliferative and pro-survival effect of the constitutively activated tyrosine kinase. In studies aimed at decreasing ALK expression in tumor cells by specific ribozymes[57] or siRNA,[56,58] or inactivating ALK kinase using selective tyrosine kinase inhibitors,[59-61] it is interesting to note that the tumor cells rapidly die by apoptosis. We propose that apoptosis associated with inappropriately regulated ALK expression or ALK kinase inactivation could limit the tumorigenic effect of the ALK proto-oncogene. Our findings could have important implications in the therapy of ALK-positive tumors harboring the chimeric or wild type ALK protein.

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FIGURE LEGEND

Figure 1 : Model for ALK positive and negative signaling. In the presence of ligand (left), the ALK receptor homodimerizes, activating its tyrosine kinase domain by transphosphorylation. The main signaling pathways triggered by activated ALK are the phospholipase C gamma (PLCγ), STAT3 and STAT5 (STAT), the protooncogene c-src, the mitogen-activated protein kinase (MAPK) and the PI3-kinase (PI3K) pathways (reviewed by Pulford et al[51]), leading to proliferation, differentiation or/and survival. Kinase-activated ALK mediates a decrease of apoptosis, partially (but not exclusively) through the PI3K pathway. In the absence of ligand (right), the ALK receptor is likely monomeric, the kinase is inactive and it enhances mitochondrial-dependent apoptosis : release of cytochrome c by the mitochondria (mito), formation of the apoptosome associating cytochrome c with apoptotic protease activating factor 1 (APAF 1) and procaspase 9, leading to caspase 9 and caspase 3 activation and eventually to apoptosis. We propose a model in which unligated/kinase unactivated ALK is cleaved by caspase 3 (or possibly a related subfamily caspase member), exposing a proapoptotic region within the juxta-membrane intra-cellular domain and creating a positive feedback loop for apoptosis enhancement. P-Y, Y-P : phosphorylation on tyrosine residues.

Table 1 : ALK in development

Organism (stage of development)	Tissue expression	Putative function*	References
Mouse (embryonic d10.5-16.5 and neonatal mice)	- CNS : thalamus, hypo-thalamus, mid-brain, olfactory bulb, dorsal root ganglia - PNS : enteric ganglia - testis, ovary - head: skin, cartilage,…	- neuronal and glial differentiation - neuronal guidance	Iwahara et al[24] Morris et al[23] Vernersson et al[30]
Chick (Embryonic d3-15)	- CNS : dorsal root ganglia, spinal cord motor neurons innervating the limbs - PNS : sympathetic ganglia - limb skeletal muscles	- motor neuron programmed cell death - limb innervation - synaptogenesis	Hurley et al[31]
Drosophila (pup and adult)	- visceral mesoderm : gut visceral muscle founder cells - CNS - optic lobe : target neurons of retinal photoreceptors (in lamina and medulla)	- gut development - CNS development - visual system development : anterograde retinal axon targeting	Englund et al[39] Lee et al[40] Stute et al[41] Loren et al[42, 43] Bazigou et al[44]
C. elegans	- nervous system : motor neurons	- synaptogenesis	Liao et al[45]

* Except for development in *Drosophila*, the functions assigned to ALK in this table have not been formally demonstrated yet.
CNS: central nervous system; PNS : peripheral nervous system.

Table 2 : ALK in cancer

Tumor	Genetic rearrangements	Protein*	Activated kinase	References
Anaplastic large cell lymphoma	t(2;5)(p23;q35)	NPM-ALK	Yes	Pulford et al[51]
	t(1;2)(p25;p23)	TPM3-ALK	Yes	Armstrong et al[52]
	t(2;3)(p23;q21)	TFG-ALK	Yes	
	inv(2)(p23;q35)	ATIC-ALK	Yes	
	t(2;17)(p23;q23)	CLTC-ALK	Yes	
Diffuse large B-cell lymphoma	t(2;17)(p23;q23)	CLTC-ALK	Yes	Gascoyne et al[50]
Inflammatory myofibroblastic tumor	t(1;2)(p25;p23)	TPM3-ALK	Yes	Pulford et al[51]
	t(2;19)(p23;p13.1)	TPM4-ALK	Yes	
Neuroblastoma	No	ALKwt	No	Lamant et al[53]
	No	ALKwt	Yes	Osajima-Hakomori et al[56]
Glioblastoma	No	ALKwt	No	Dirks et al[36]
	No	ALKwt	Yes?[+]	Powers et al[57]
Melanoma	No	ALKwt	No	Dirks et al[36]
Rhabdomyosarcoma	No	ALKwt	No	Morris et al[23]

*The list of X-ALK chimeric proteins is not exhaustive. For a review, see Pulford et al.[51]

[+] Powers et al indirectly suggest ALK is activated in a glioblastoma cell line, but do not show ALK phosphorylation.[57]

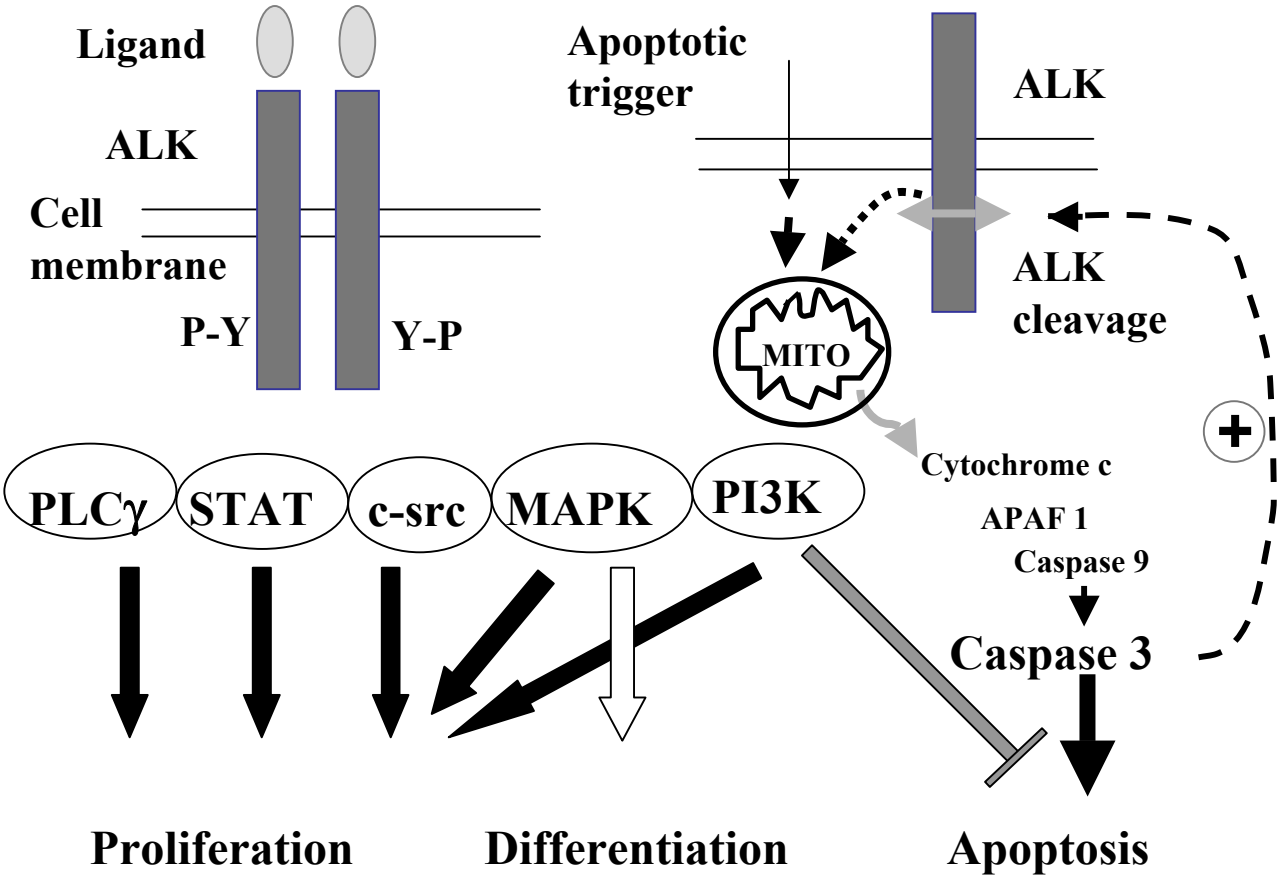

Figure 1

(Allouche)