# UNIVERSITÀ DEGLI STUDI DI NAPOLI

# "FEDERICO II"

## Scuola di Dottorato in Medicina Molecolare

### Dottorato di Ricerca in Genetica e Medicina Molecolare


*"Akt and Erk 8 activity regulation in cellular proliferation control"*

**Coordinatore:**
**Prof. Carmelo Bruno Bruni**

**Candidato:**
**Dott. Mario Acunzo**

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**Dipartimento di Biologia e Patologia Cellulare e Molecolare "Luigi Califano"**

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**Sede amministrativa:**
**Dipartimento di Biologia e Patologia Cellulare e Molecolare "Luigi Califano"**

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**Dipartimento di Biologia e Patologia Cellulare e Molecolare "Luigi Califano"**

**Tesi di Dottorato di Ricerca in Genetica e Medicina Molecolare**
**XX ciclo**

" ***Akt and Erk 8 activity regulation in cellular proliferation control "***

**Canidato: Mario Acunzo**

**Docente guida: Prof.ssa Gerolama Condorelli**
**Dott. Mario Chiariello**

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## Introduction

A key question in developmental biology is how cells perceive and respond properly to their enviroment. Cells must not only sense and distinguish between stimuli, but also transduce the signal accurately, to activate the appropiate responses. Signal transduction is the process by which extracellular signals are detected and converted into intracellular signals, which, in turn, generate specific cellular responses. Signal transduction systems are typically arranged as networks of sequential protein kinases.

## Protein kinases

The eukaryotic protein kinases make up a large superfamily of homologous proteins. They are related by virtue of their kinase domains (also known as catalytic domains), which consist of 250-300 amino acid residues. The kinase domains that define this group of enzymes contain 12 conserved subdomains that fold into a common catalytic core structure, as revealed by the 3-dimensional structures of several protein-serine kinases (Hanks et al 1995).

There are many types of protein kinases involved in signal transduction. They all have same basic catalytic activity: they add a phosphate group to an amino acid in a target protein. The phosphate is provided by hydrolyzing ATP to ADP. A protein kinase has an ATP-binding site and a catalytic center that can bind to the target amino acids. The phosphorylation of the target protein changes its proprieties so that it turn acts to carry the signal transduction pathway to the next stage.

Protein kinases can be classified both by the types of amino acids that they phosphorylate in the protein target and their location in the cell.

Three groups of protein kinases are distinguished by the types of amino acid targets:

- *Protein serine/threonine kinases* are responsible for the vast majority of phosphorylation vents in the cell. As their name indicates, they phosphorylate either serine or threonine in the target protein.

- Protein tyrosine kinases phosphorylate tyrosine in the target protein.
- Dual specificity kinases are less common and can phosphorilate target proteins on either tyrosine or serine/threonine.

Protein kinases are found in two types of location:

- *Cytosolic protein kinases* are most often Ser/Thr protein kinases. They are responsible for the vast majority of phosphorylation events in the cells.
- *Receptor protein kinase* are found in the plasma membrane. They have a domain on the exterior of the cell that binds a ligand, and a catalytic domain within the cell that can act on a target protein. Most receptors with protein kinase activity are protein tyrosine kinases (RTK), although there are also some receptors of Ser/Thr kinase class. ( Hunter T., et al. 1987).

**Akt a serine/threonine kinase**

Akt protein was isolated as a gene product of the Akt gene. It's a serine/threonine kinase with the plackstrin homology domain (PH) in its $NH_2$ terminal region and catalytic domain closely related to protein kinase C (PKC) and protein kinase A (PKA) family members (Staal 1987).
Mammalian genomes contain three widely expressed isoforms of Akt kinase. Tissue and organ expression of individual isoforms is shown in Fig 1.

| | High expression levels | Low / moderate expression level |
|---|---|---|
| *Akt1* | Brain, heart, testis, thimus | Kidney, liver, spleen |
| *Akt2* | Brown fat, cerebellum, heart | Brain, kidney, lung spleen, testis |
| *Akt3* | Brain, testis | Heart, kidney, liver lung, spleen |

**Figure 1:** Akt expression profile

Akt can be activated by a wide variety of growth stimuli such as growth factors and cytokines. The pathway of Akt activation is a process involving membrane traslocation and phosphorilation (Bellacosa et al, 1998). The typical route of Akt activation is mediated *via* tyrosine kinase receptors such as a receptors for insulin or certain growth factors. Upon stimulation, agonists activate receptor kinase function resulting in stimulation of tyrosyl phosphorilation of IRSs provides binding sites for specific proteins containing $SH_2$ domains including the 85 kDa regulatory subunit of phospatidylinositol 3-kinase (PI3K), a crucial molecule in this signaling pathway. (Burgering and Coffer 1995). The plecstrin homology (PH) domain of PI3P triggers Akt traslocation to the plasma membrane (Bellacosa et al 1998). Furthermore, the increasing levels of phosphoinositides function as intracellular second-messenger molecules leading to activation of PI-dependent kinases (PDK1 and PDK2). $PDK_s$ activate Akt upon its membrane traslocation on threonine 308 (Thr 308) (Alessi et al 1997) and serine 473 (Ser 473) (Bellacosa et al. 1998). Thr 308 phosphorilation is necessary for Akt activation, whereas Ser 473 phosphorilation is required as well as for maximal activity. (Downward 1998). Activated Akt becomes avaiable for phosphorilation of its downstream targets in various subcellular localizations and nucleus. Inactivation of Akt is accomplished by dephosphorylation by protein

phosphatases such as protein phosphatase 2A, or by Akt antagonist (ceramides and PTEN) which blok an activation of this enzyme in certain steps of its activation (Ozes et al. 2001).

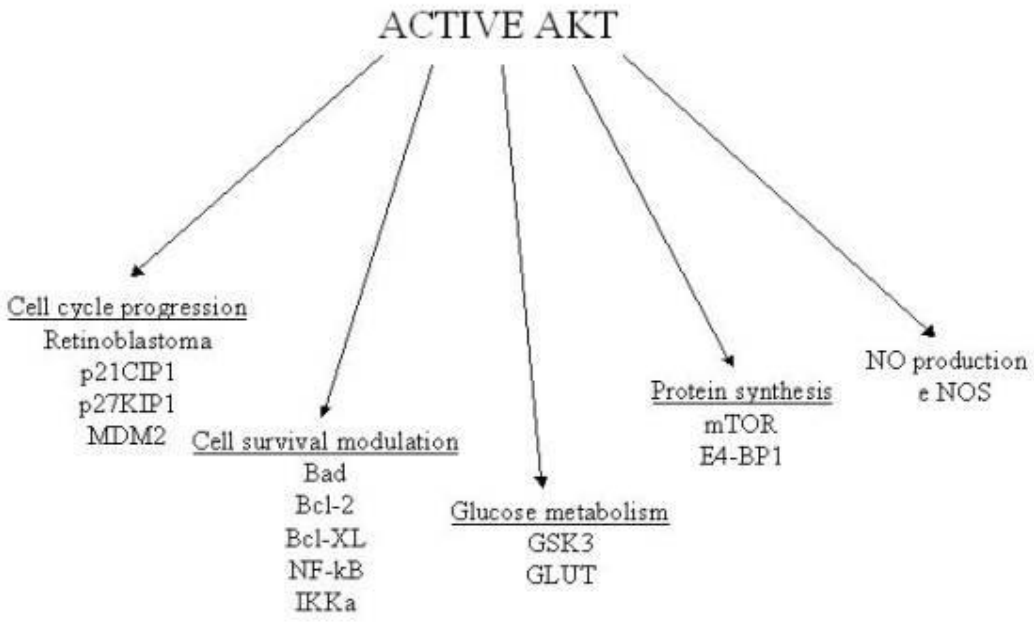



**Figure 2:** Akt downstream target molecules. Downstream target Akt substrates are grouped according to their function.

*Involvment of Akt in prosurvival and antiapoptotic mechanism*

This aspect of Akt physiology and pathophysiology is one of the most intensively studied with respect to its role in a number of areas of biomedical research spanning from oncology to cardiovascular medicine. Akt is critical for cell survival triggered by growth factors, extracellular matrix, and other stimuli. For example, the overexpression of wild type or activated Akt can rescue cells from apoptosis induced by various stress signals (Kauffmann-Zeh et al. 1997).

Promotion of cell survival by Akt is accomplished on two levels.

First, Akt is involved by trascriptional regulation of prosurvival and antiapoptotic genes. Akt promotes cell survival by directly phosphorylating trascription factors that

control the expression of pro-and antiapoptotic genes. Akt appears to both negatively regulate factors that induce survival genes (Biggs et al 1999).

Second, Akt promotes survival by direct phosphorilation of key regulators of the apoptotic cascade. The most widely studied example of this type of regulation involves BAD, a membre of Bcl-2 family, whish promotes apoptosis by binding to and antagonizin the actions of prosurvival members of the family such as Bcl-2 and Bcl-XL. Akt can phosphorilate BAD and this modification promotes the sequestration of BAD in the cytosol, thus preventing BAD from interacting with Bcl-2 or Bcl-XL (del Peso ey al. 1997). Akt has been shown to phosphorilate several trascription factors (CREB, NF-kB, Forkhead) many of which have been implicated in the expression of genes whose protein products regulate susceptibility to apoptosis (Barton et al. 1996).

It appears that one or more factors may be involved in the regulation of FLIP, and studies to determine the role played by these factors in FLIP expression are currently under way. However, FLIP expression is critically dependent upon PI 3-kinase and Akt activity. These data add FLIP to the growing list of survival-related proteins reglated by the PI 3-kinase pathway (David J. Panka et al. 2000).

**C-FLIP$_L$**

Cellular FLICE inhibitory protein (c-FLIP) is an endogenous inhibitor of death receptor-induced apoptosis throught the caspase 8 pathway, and is widely expressed in various tumors (Lee et al. 2003). C-FLIP acts as an inhibitor of apoptosis induced by various agents, including tumor necrosis factor (TNF), TNF-related apoptosis inducing ligand (TRAIL), T cell receptor (TCR), death receptor (DR) and Fas (Rippo et al. 2004). c-FLIP comes in two variants, c-FLIP$_S$ only contains two N-terminal DEDs very similar to the prodomains of caspase-8/-10. In contrast, the long splice form of c-FLIP (c-FLIP$_L$) is identical in lenght with caspase-8, also contains N-terminal tandem DED$_S$, but its caspase domain is altered, rendering it enzymically inactive. The 55 kDa FLIP (c-FLIP$_L$) is expressed in many tissues. The most

abundant expression of the protein is found in the heart, keletal muscle and kidney (Rasper D. Et al. 1998). The 25 kDa FLIP (c-$FLIP_S$) is expressed in lymphatic tissues and its expression is dependent on the degree of cell activation (Imler M. Et al. 1997). c-$FLIP_L$ therefore has the classical structure of dominant-negative inhibitor: an intact upstream protein-protein interaction domain linked to an inactive downstream enzymic domain (Yeh, W.C. et al. 2000). Recently two reports demonstrated that c-$FLIP_L$ is not only an inhibitor of apoptosis, but also an activator of procaspase-8, infact the caspase like-domain of c-FLIP, can activate the caspase-8 through heterodimerization (Chang et al. 2002).

In addiction to the apoptosis inhibition, c-$FLIP_L$ mediate sthe activation of NF-kB and Erk by virtue of its capacity to recuirt the adaptor proteins involved in each signaling pathway, such as TRAF-1, TRAF-2, RIP and Raf-1 (Fang et al. 2004). The inhibition of JNK pathway by direct binding to MKK7 was also reported (Nakajima et al 2006). Homozygous distruption of c-FLIP gene in mice results in a failure of heart development at embryonic day 11.5, suggesting an essential role of cFLIP in cardiac development during embriogenesis (Yeh et al. 2000).

**GSK3β**

GSK3 was discovered 20 years ago as one of several protein kinases that phosphorylated and incativated glycogen synthase, the final enzyme in glycogen biosyntesis (Embi, N. et al . 1980).

It was subsequently purified to homogeneity from skeletal muscle and molecular cloning revealed that there where two closely related isoforms, GSK3α and GSK3β, which are expressed ubiquitously in mammarian tissues ( Woodgett et al. 1990). The proteins share 97% sequence similarity within their kinase catalytic domains, but differ from one another outside this region, with GSK3α possessing an extended N-terminal glycine-rich tail (Woodgett et al. 1991).

The inhibition of GSK3 might underlie the insulin-induced dephosphorilation and activation of glycogen synthase (Cohen, P. 1978). We know that the inhibition of GSK3 by insulin results from its phosphorilation at an N-terminal serine residue

(Ser$^{21}$ in GSK3α and Ser$^{9}$ in GSK3β) and it's catalysed by protein kinase B (PKB; also called Akt)( Cross et al. 1995). In recent years, GSK3 has been implicated in the phosphorylation of many proteins and in the regulation os several cellular events (Cohen et. al 2001). In addiction, the same serine residue that is targeted by PKB/Akt is now known to be phosphorylated by other protein kinases in response to different signals. For example growth factor can inhibit GSK3 activity by means of the classical MAPK cascade (Parker et al. 1983).

In 1992, many years after its initial identification, geneticist studing Drosophila melanogaster "rediscovered" GSK3 in a signalling pathway that is turned on by the secreted glycoprotein Wingless (Wnt). These findings implied that Wingless suppress GSK3 in this pathway (Siegfried et al. 1992). In particular, the pool of GSK3 that partecipates in WNT signalling is not free, but is present in a multiprotein complex that includes axin, β-catenin and the adenomatous polyposis coli (APC) protein ( Zeng et al.1997). In absence of WNT signal, GSK3 is active and phosphorylates axin, β-catenin and APC. This stabilizes axin and facilitates the interaction of APC with β-catenin, but targets β-catenin for ubiquitin-mediated proteolytic destruction (Li. L. Et al. 1999).

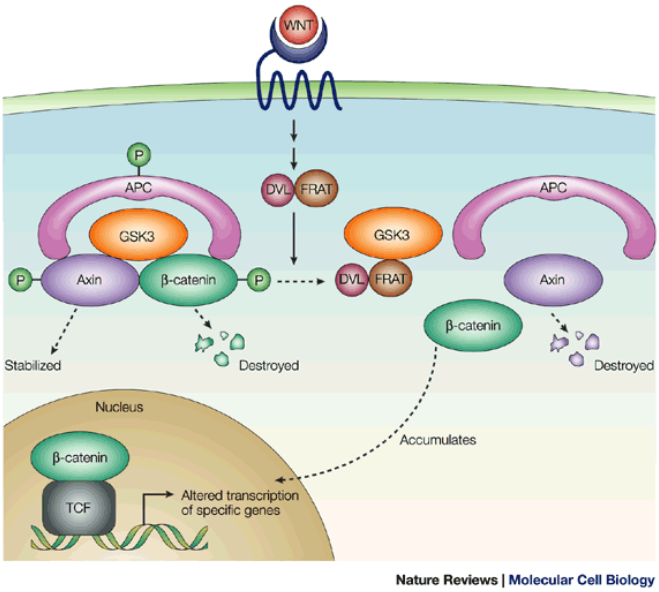



**Figure 3:** A proportion of GSK3 in cells is present in a multiprotein complex together with axin, the adenomatous polyposis coli (APC) protein and β-catenin. In the absence of secreted glycoproteins, called WNTs, the GSK3 in this complex is active and phosphorylates axin, APC and β-catenin. Axin is stabilized by phosphorylation, but phosphorylation of β-catenin targets it for ubiquitylation and subsequent proteolytic destruction. The role of APC phosphorylation is less clear, but it seems to enhance its interaction with β-catenin. After the binding of WNTs to their receptors, a signal-transduction pathway is triggered that has yet to be fully elucidated, but which seems to involve a protein, termed dishevelled (DVL), which, together with FRAT (frequently rearranged in advanced T-cell lymphomas), results in the displacement of axin (and hence APC and β-catenin). This leads to the dephosphorylation of axin, APC and β-catenin. The dephosphorylation of β-catenin leads to its accumulation in cells and translocation to the nucleus, where it binds to members of the T-cell factor (TCF) family of transcription factors (also called LEF, for lymphoid-enhancer factor), and stimulates the transcription of genes that are required for embryogenesis. The same pathway is likely to be involved in regulating the expression of other genes in adult tissues.

GSK3β downregulation induced by the canonical WNT signal pathway and by the FGF-dependent PI3K-AKT signaling pathway is independent of and dependent on Ser 9 phosphorilation, respectively (Frame et al. 2001). Because the mechanisms of GSK3β downregulation by WNT and FGF signaling are not the same, distinct GSK3β pools might be implicated in WNT and FGF signaling. Simultaneous activation of WNT and FGF signaling pathway leads to the potentiation of the canonical WNT signaling pathway trought the promotion of the nuclear traslocation of β-catenin by the FGF-dependent PI3K-AKT signaling pathway. Therefore, coactivation of WNT and FGF signaling pathways during carcinogenesis leads to

more malignant phenotypes due to the potentiation of β-catenin/TCF signaling cascades.

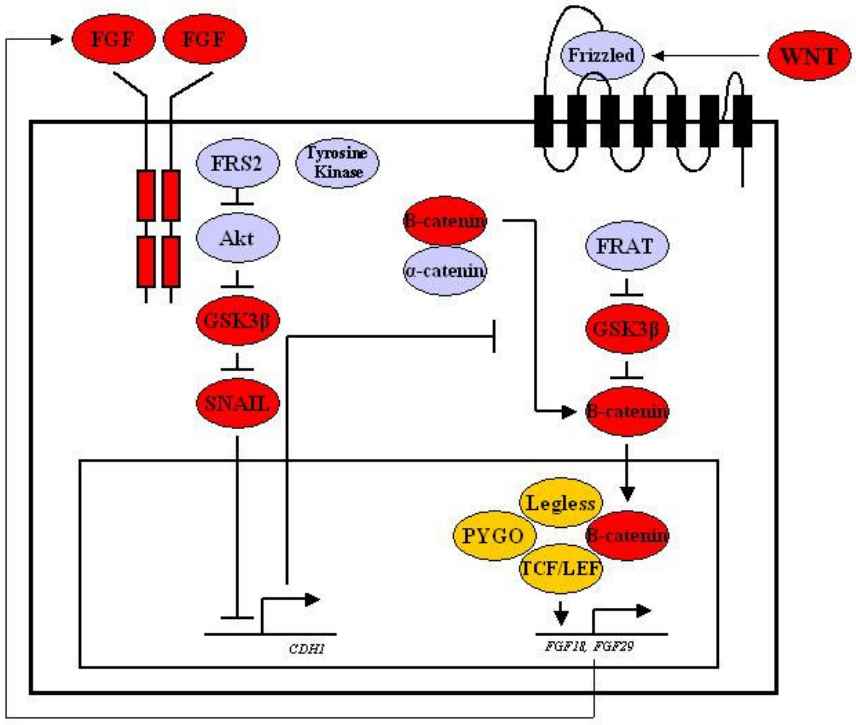



**Figure 4:** Cross-talk of WNT and FGF signaling pathways. Schematic representation of the crosstalk of WNT and FGF signaling pathways during carcinogenesis.

## Receptor tyrosine kinases.

Growth factors control cell growth, proliferation, differentiation, survival and migration by activating receptor tyrosine kinase (RTK) family members (Blume-Jensen and Hunter, 2001). Signalling by RTKs requires ligand-induced receptor oligomerization, but evidences indicate that RTKs oligomerization per se is not always sufficient for kinase activation. There seems to be an additional requirement for ligand-induced conformational switches, ensuring that the catalytic domains are juxtaposed in a proper configuration to enable phosphorylation (Schlessinger, 2000; Jiang and Hunter, 1999). Anyway, upon ligand binding, cytoplasmic tyrosine residues of RTKs becomes autophosphorylated and thus provide docking sites for a variety of

phosphotyrosine-binding proteins. The specific recruitment of these proteins, which harbour various, catalytic and scaffolding domains, determines the signalling output (Blume-Jensen and Hunter, 2001).

Many RTKs, among which epidermial growth factor (EGFR) (Liebman, 2001), platelet-derived growth factor (PDGF) (Satoh et al., 1993; Nanberg and Westmark, 1993) and RET (Chiariello e al., 1998) stimulate, through the small GTP-binding Ras, different MAP kinase pathways.

**MAP Kinases.**

Pathways involving MAP kinases are activated in response to an extraordinary diverse array of stimuli. These stimuli vary from growth factors and cytokines to irradiation, osmolarity, and shear stress of fluid flowing over a cell. These stimuli induce a specific dual phosphorylation on a conserved motif, Thr-Xaa-Tyr, present in all MAP kinases (**Fig. 2**). The best characterized substrates for MAP kinases are transcription factors. However, MAP kinases have the ability to phosphorylate many other proteins including other kinases, phospholipases, and cytoskeleton-associated proteins.

In mammals, there are many MAP kinases with different biological functions, grouped in distinctly regulated groups, of which the best known are ERK1/2 (extracellular signal related kinase, ERK), JNKs (jun amino terminal kinase, JNK) and p38, which are involved in many cellular events such as proliferation, differentiation, apoptosis and stress (Chang and Karin, 2001) (Fig. 3). All MAP kinases recognize similiar phosphoacceptor sites composed of serine or threonine followed by a proline, and the amino acids that surround these sites further increase the specifity of recognition by the catalitic pocket of the enzyme. Full specificity is ensured through the interaction mediated by another site on the kinase that recognizes a distinct site on the substrate (docking site). Moerover, spatial localization of signalling molecules further auguments specificity in signal transduction (Roux and

Bleins, 2004). Finally, cross-talk by scaffolding proteins regulate MAP kinase signaling beyond simple tethering (Chang and Karin, 2001; Qi and Elion, 2005).

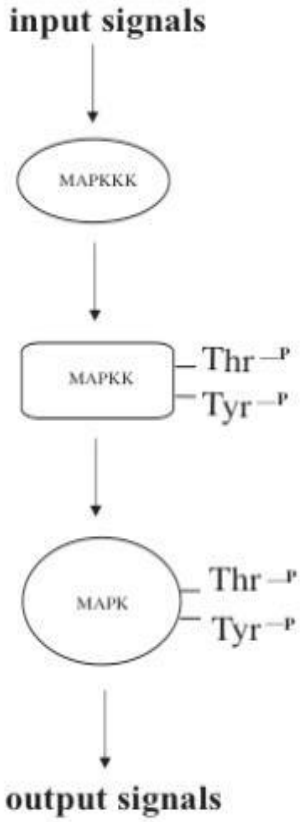



**Figure 5 :** MAPK pathway module. Core module of mitogen-activated protein kinase (MAPK) pathway is composed of three kinases. MAP kinase kinase kinase (MKKK), MAP kinase kinase (MKK), MAP kinase (MAPK), that are sequentially activated by phosphorylation. $MAPK_s$ are actibvated by dual phosphorylation of conserved threonine and tyrosine residues within the activation loop (TXY).

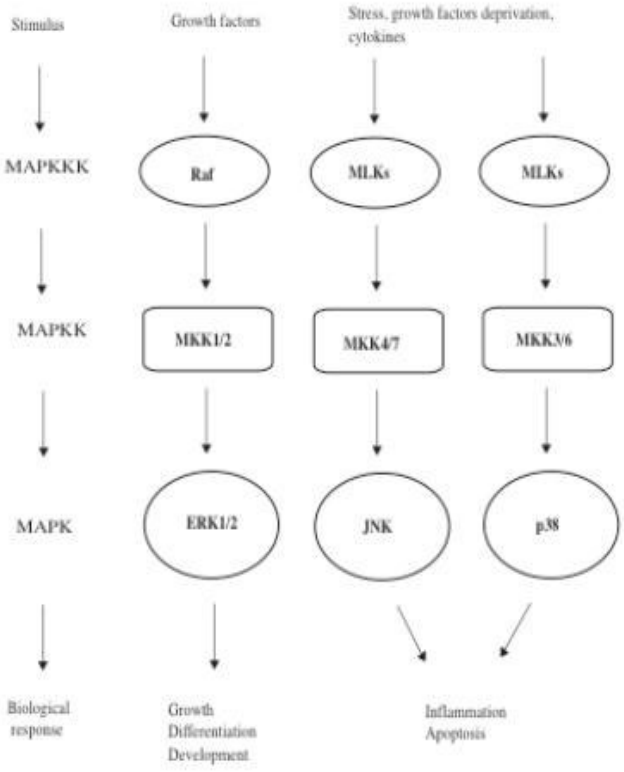



**Fig 6:** Signaling cascade of the MAPK pathways. Schematic representation of signaling cascades leading to activation os MAP kinase to respective biological function

## Big "atypical" MAP kinases.

The recently identified ERK5, ERK7 and ERK8 are significantly larger than the originally identified ERK1 and ERK2 due to an extended C-terminal domain. ERK5, also known as big mitogen-activated kinase 1 (BMK1) (Lee et al., 1995), is a 110 kDa protein, while ERK7 is 61 kDa protein and ERK8 is 60 kDa protein. All these MAP kinases are activated by dual phosphorylation on Thr-Xaa-Tyr motif. Recent information indicates that the C-terminal regions of ERK5 and ERK7 have important regulatory functions. The C-terminal region of ERK5 appears to regulate negatively its kinase activity (Zhou et al., 1995) and contains a putative bipartite nuclear

translocation signal for ERK5 that functions *in vivo* following activation (Yan et al., 2001). The C-terminal region of ERK5 also contains a myocyte enancher-binding factor 2-interacting region and a potent transcriptional activation domain (Kasler et al., 2000). Disruption of the gene encoding ERK5 led to angiogenic defects and embryonic lethality in mice (Yan et al., 2003)

ERK7 is activated by autophosphorylation, which is regulated through its C-terminal domain (Abe et al., 2001). Moreover, the C-terminal region is required for the ability of ERK7 to localize to the nucleus and inhibit growth (Abe et al., 1999).

ERK8 is the last identified member of the MAP kinase family (Abe et al., 2002). ERK8 represents the human orthologue of the rat ERK7 and is present in brain, kidney and lung. The overall amino acid identity of the human ERK8 and rat ERK7 sequences is 69%. Comparison of the kinase domains reveals a sequence identity of about 82%, whereas the amino acid sequence identity of the C-terminal regions is only 53% (Abe et al., 2002). By contrast, sequence identity between other ERK orthologues is significantly higher.

The possible physiological roles of ERK8 remain the less studied. The failure of ERK8 to phosphorylate many of tested substrates, c-jun, c-myc, histone H1, Ets-1, Elk-1 and paxillin has not elucitated its function. Its activation following stimulation by c-Src or cell exposure to serum hints at a function in response to mitogenic factors (Abe et al., 2002). Obviously, many possibilities remain to be explored when describing the function of ERK8.

The *objective* of the present work is to determine the relevant MAP kinase family members involved in the signals from tyrosine kinase receptors to the nucleus. In particular, we examined the role of JNK in *c-myc* expression induced by PDGF and the activation c-Abl mediates RET/PTC3-dependent of the novel ERK8 MAP kinase.

## Materials and Methods

### Expression vectors.

The expression vectors pCEFLP-SrcYF (constitutively active) and pCEFLP-SrcYF KM (dominant negative) were obtained by sub-cloning the corresponding cDNA obtained from pSM-SrcYF and pSM-SrcYF KM, kindly provided by H. Varmus (Chiariello et al., 2001). The HA-tagged form of Erk8 was generated by cloning the corresponding cDNA, kindly provided by M. Abe (Abe et al., 2002), in the pCEFL-HA vector. The expression vector for the dominant negative Erk8 KR molecule was also provided by M. Abe (Abe et al., 2002). To generate the pCEFL-HA-Erk8□ expression vector, we amplified by PCR the corresponding cDNA using an "expressed sequence tag" (est) obtained from ResGen (Clone ID 5742965). This sequence data has been submitted to the GenBank database under accession number AY994058. The pCDNA3-Ptc3 expression plasmid has been previously described (Melillo et al., 2001). The $Ptc3^{Y981}$, $Ptc3^{Y1015}$, $Ptc3^{Y1062}$, Ptc3 $Kin^{dead}$ and $Ptc3^{V804}$ expression plasmid were generated by the QuikChangeTM Site-Directed Mutagenesis Kit (Stratagene), using pCDNA3-PTC3 as a template. Expression vectors for c-Abl and its oncogenic form, Bcr/Abl p210 (Bcr/Abl), have been previously described (Lobo et al., 2005; Sanchez-Prieto et al., 2002). The dominant negative c-Abl (Abl-KD) expression vector was obtained by mutating a critical lysine in the kinase domain of c-Abl, contained in the pCEFL-AU5 vector. The c-myc and c-jun promoter reporter plasmids, pMyc-Luc and pJun-Luc, respectively, and the pCDNAIII-β-galactosidase (β-gal) expression vector have been previously described (Chiariello et al., 2000; Chiariello et al., 2001).

### Reagents.

The PP1 inhibitor was purchased from Biomol. All other chemicals were purchased from Sigma.

**Cell culture and transfections.**

293T cells and thyroid ARO cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 2mM L-glutamine, and 100U/ml penicillin-streptomycin (Invitrogen). NIH3T3 fibroblasts were maintained in DMEM supplemented with 10% calf bovine serum (Bio Whittaker), 2mM L-glutamine, and 100U/ml penicillin-streptomycin (Invitrogen). 293T and NIH3T3 cells were transfected by the LipofectAMINE reagent (Invitrogen), while ARO cells were transfected by the Lipofectamine 2000 reagent (Invitrogen), respectively, in accordance with the manufacturer's instructions. For transfections, 200 ng of HA-Erk8 and HA-Erk8δ and 100 ng of SrcYF, Abl Act., Bcr/Abl, 500 ng, 1 μg and 2μg of $FLIP_L$ and of the different Ptc3 expression vectors were used, unless otherwise indicated.

**Antibodies.**

As primary antibodies rabbit polyclonal antibodies against Erk2 (C-14) and c-Src (N-16) (Santa Cruz), phospho-MAPK (p42/p44) (Cell Signaling), GSK3β and phospho-GSK3β (Cell Signaling), c-FLIP (Abcam Corp.), β-catenin (Cell signaling), RET and phospho-RET (phospho-Tyr905) (Carlomagno et al., 2004); mouse monoclonal antibodies against haemagglutinin (HA) epitopes (HA.11; Berkley Antibody Company, CA); c-Abl (BD Pharmingen) and to phospho-tyrosine; PY (Santa Cruz and Upstate Biotechnology); FLAG (Sigma); actine (Sigma), Akt (cell Signaling).

**Western blot analysis.**

Lysates of total cellular proteins or immunoprecipitates were analyzed by protein immunoblotting after SDS-PAGE with specific rabbit antisera or mouse monoclonal antibodies. Immunocomplexes were visualized by enhanced chemiluminescence

detection (ECL or ECL Plus, Amersham-Pharmacia) with the use of goat antiserum to rabbit or mouse immunoglobulin G, coupled to horseradish peroxidase (Amersham-Pharmacia).

**Reporter gene assays.**

ARO cells were transfected with different expression plasmids together with 20 ng of the pJLuc reporter plasmid. After 24 h incubation in serum-free media, the cells were lysed using reporter lysis buffer (Promega). Luciferase activity present in cellular lysates was assayed using D-luciferin and ATP as substrates, and light emission was quantitated using the $20^n/20^n$ luminometer as specified by the manufacturer (Turner BioSystems).

***In vitro*** **kinase assay.**

Confluent plates of transfected NIH3T3 were kept two hours (JNK assay) or overnight (MAPK assay) in serum-free medium. Cells were then washed with cold phosphate-buffered saline, and lysed at 4° C in a buffer containg 20 mM Hepes, pH 7.5, 10 mM EGTA, 40 mM β-glycerophosphate, 1% IGEPAL, 2.5 mM $MgCl_2$, 1mM dithiothreitol, 2 mM sodium vanadate, 1mM phenylmathylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin. Lysates were clarified by centrifugation at 12,000 x g for 20 min at 4° C, and supernatants were incubated with 1 μg monoclonal antibody against JNK (PharMingen) or with 1 μg polyclonal antibody against Erk2 (C-14) (Santa Cruz), for 1 h at 4° C. Immunocomplexes were recovered with the aid of protein A/G PLUS-Agarose (Santa Cruz Biotechnology). Pecipitates were washed three times with phosphate-buffered saline which contained 1% IGEPAL and 1mM vanadate, once with 100 mM Tris pH 7.5, 0.5 M LiCl, and once in kinase reaction buffer (12.5 mM MOPS, pH 7.5, 12.5 mM β-glycerophosphate, 7.5 mM $MgCl_2$,

0.5mM EGTA, 0.5 mM sodium fluoride, 0.5 mM vanadate).Assays were performed in a reaction buffer containing 1 μCi of [γ-$^{32}$P]ATP, 20 μM ATP, 3mM dithiothreitol and 1 μg GST-ATF2 and myelin basic protein (MBP, Sigma). After 30 min at 30°C, reactions were terminated by addition of 5X Laemli buffer. Samplers were heated at 95°C for 5 min and analyzed by SDS-gel electrophoresis on 12% acrylamide gels. Autoradiography was performed with the aid of an intensifying screen.

## Study I

### Activation of the ERK8 MAP kinase by RET/PTC3, a constitutively active form of the RET proto-oncogene.

RET is a typical trans-membrane receptor tyrosine-kinase (RTK), essential for the development of the sympathetic, parasympathetic and enteric nervous system and of the kidney (Schuchardt et al., 1994). In complex with four glycosylphosphatidylinositol (GPI)-anchored coreceptors, GFR-α 1–4, the RET protein binds growth factors of the glial-derived neurotrophic factor (GDNF) family, mediating their intracellular signaling (Airaksinen & Saarma, 2002). As for other RTKs, ligand interaction triggers autophosphorylation of different RET intracellular tyrosine residues that work as docking sites for several adaptor and effector signaling molecules (Santoro et al., 2004). Among such tyrosines, while $Tyr^{981}$ is a binding site for c-Src, $Tyr^{1062}$ has been shown to mediate the interactions with most of RET effectors and to be responsible for activation of the Ras/Erk, PI3K/Akt, Jnk, p38 and Erk5 signaling pathways (Kurokawa et al., 2003). Finally, $Tyr^{1015}$ is a recognized docking site for PLCγ (Borrello et al., 1996).

Gain-of-function mutations of *RET* have been repeatedly described in several human tumors (Pasini et al., 1996). *RET* germline point mutations are in fact responsible for the three clinical subtypes of the Multiple Endocrine Neoplasias type 2 (MEN2) syndrome, MEN2A, MEN2B and Familial Medullary Thyroid Carcinoma (FMTC) (Santoro et al., 2004). In addition, fusion of the intracellular kinase domain of *RET* with heterologous genes, caused by chromosomal inversions or translocations, generates the *RET/PTC* oncogenes, which represent the genetic hallmark of papillary thyroid carcinomas (PTC), accounting for more than 80-90% of all thyroid carcinomas (Sherman, 2003). Among the at least ten different *RET/PTC* rearrangements, *RET/PTC1* and *RET/PTC3*, generated by the fusion with the *H4* and *RFG* genes, respectively, are the most common types, accounting for more than 90% of all rearrangements (Nikiforov, 2002).

**Erk8 is activated by RET-dependent signaling pathway.**

We performed an *in silico* analysis of *Erk8* gene expression in mouse tissues, through the public Mouse Gene Prediction Database resource (http://mgpd.med.utoronto.ca/) (Zhang et al., 2004). Among other tissues, *Erk8* was expressed at very high levels in the thyroid, therefore suggesting a role for this kinase in signaling pathways involved in the homeostasis and/or pathology of this organ. As the RET/PTC oncogenes are frequently involved in human papillary thyroid carcinomas (Santoro et al., 2004), we decided to investigate their ability to modulate Erk8 activation. In particular, we investigated the role of RET/PTC3, a chimeric oncogene generated by the fusion of *RET* with the *RFG* gene (Fig. 7A) (Santoro et al., 1994).

As an approach to score Erk8 activation, we used an anti-phospho-MAPK (Erk2) antibody that recognizes phosphorylation in the conserved MAP kinase TEY motif. We performed western blot analysis of 293T cells transfected with an HA epitope-tagged form of the Erk8 kinase, as previously described (Abe et al., 2002), and then distinguished the transfected HA-Erk8 and the endogenous Erk2 by their different molecular weights, ~60 kDa and ~45 kDa, respectively. As shown in figure 8B, RET/PTC3 overexpression readily induced Erk8 activation, at a level comparable to an activated form of c-Src (Src YF), used as a positive control (Abe et al., 2002). Of note, no signal in the ~60 kDa range was detected in the absence of HA-Erk8 transfection (Fig. 7B), indicating that the anti-phospho-MAPK antisera specifically recognized the Erk8 protein. As an additional control for the activity of RET/PTC3 and Src YF, both proteins activated the Erk2 MAP kinase (Fig. 7B), also scored by anti-phospho-MAPK western blot. Altogether, these results indicate that RET/PTC3 stimulates Erk8 activity.

**The Erk8 carboxy-terminal modulates activation of the MAP kinsase by RET/PTC3.**

While classical MAP kinases such as Erks, Jnks and p38s are only slightly larger than their minimum Ser/Thr kinase core, the atypical Erk5, Erk7 and Erk8 MAP kinases all

contain long C-terminal domains whose functions are largely unknown. Yet, recent experiments performed on Erk5 (Buschbeck & Ullrich, 2005) and Erk7 (Abe et al., 2001) have demonstrated a role for their C-terminal tail in the regulation of kinase intracellular localization and activity. Thus, we set up to investigate a role for the Erk8 C-terminal domain in RET/PTC3-dependent activation of the kinase.

The genomic organization of the *Erk8* gene has been previously described (Abe et al., 2002). By *in silico* analysis of available "expressed sequence tags" (est) clones we identified an *Erk8* cDNA whose corresponding protein, when expressed, presented a molecular weight shorter (~35 kDa) than the described Erk8 protein (~60 kDa) (Fig. 8A). We named this protein Erk8δ (accession # AY994058). Comparative analysis of the sequences for *Erk8*, *Erk8δ* and the *Erk8* gene (http://www.ncbi.nlm.nih.gov/genome/guide/human/) revealed that *Erk8δ* corresponded to an alternatively spliced form of *Erk8* in which an alternative exon 8 (exon 8a) contained a "stop" codon (Fig. 8B), therefore determining a 254-aminoacid long protein, lacking the Erk8 C-terminal domain (Fig. 8C). Thus, we took advantage of the availability of this naturally occurring C-terminally truncated protein, to evaluate the role of this domain in RET/PTC3-dependent Erk8 activation. As shown in figure 8D, RET/PTC3 was not able to induce Erk8δ activation while, as a control, it strongly activated Erk8. In the same experimental condition, Src YF, a described activator of Erk8 (Abe et al., 2002), also failed to stimulate Erk8δ activation (Fig. 8D), therefore establishing a key role for the C-terminal domain of Erk8 in the activation of this MAP kinase by various upstream stimuli.

**Tyrosine 981 of RET/PTC3 is necessary for Erk8 activation.**

Tyrosine phosphorylated residues in the kinase domain of RET, as well as of its derivate oncogenes, usually represent docking sites for adaptor proteins and enzymes that are able to propagate the signal to the intracellular environment (Santoro et al., 2004). We therefore used RET/PTC3 molecules in which different tyrosine

phosphorylation sites have been inactivated by mutating them to phenylalanines, to ascertain the dependency of RET/PTC3-induced Erk8 activation on the presence of these specific residues. Also, as these tyrosines have already been linked to the activation of different specific signaling pathways (Santoro et al., 2004), this approach could grant us the possibility to suggest the participation of some of these effectors in the modulation of Erk8 activity. In particular, tyrosine$^{981}$ binds c-Src (Encinas et al., 2004), tyrosine$^{1015}$ is a docking site for PLCγ (Borrello et al., 1996) and tyrosine$^{1062}$ is a multiple docking site that mediates most of RET signaling pathways (Kurokawa et al., 2003), including Erk2 activation (Chiariello et al., 1998). 293T cells were transiently transfected with the HA-Erk8 molecule, together with RET/PTC3, RET/PTC3$^{Y981}$, RET/PTC3$^{Y1015}$ or RET/PTC3$^{Y1062}$, respectively (numbers indicating RET/PTC3 tyrosine residues correspond to their position in the wild-type RET receptor). Surprisingly, based on the observation that tyrosine$^{1062}$ mediates most of RET signaling pathways (Kurokawa et al., 2003), the RET/PTC3$^{Y1062}$ mutant activated Erk8 at an extent comparable to the RET/PTC3 molecule while, as expected (Chiariello et al., 1998), this mutation strongly affected Erk2 activation (Fig. 9). The tyrosine$^{1015}$ mutation, involving a known binding site for PLCγ (Borrello et al., 1996), also did not affect Erk8 activation by RET/PTC3 (Fig. 9). Conversely, tyrosine$^{981}$ mutation determined a dramatic reduction in RET/PTC3-dependent Erk8 activation, although resulting irrelevant to Erk2 activation (Fig. 10). As a control, RET/PTC3 Kin$^{dead}$, a kinase-inactive form of RET/PTC3 containing a mutation in the ATP-binding catalytic lysine (Lys$^{758}$), was unable to activate both Erk8 and Erk2 (Fig. 9). These results therefore imply tyrosine$^{981}$ of RET/PTC3 as a major site recognized by signaling molecules mediating RET/PTC3-dependent Erk8 activation. In addition, as tyrosine$^{981}$ has been previously recognized as a key residue for the binding of c-Src to RET (Encinas et al., 2004), they also suggest a role for c-Src in mediating RET/PTC3-initiated signals impinging on Erk8 activation.

**Src activity is dispensable for RET/PTC3-dependent Erk8 activation**

Based on the above information and on the observation that c-Src activates Erk8 (Abe et al., 2002), we next sought to investigate if c-Src was able to mediate RET/PTC3-dependent Erk8 activation. A classical approach to establish a role for Src kinases in cellular processes takes advantage of a pyrazolo-pyrimidine compound, PP1, which binds the ATP-binding pocket of these kinases therefore blocking their enzymatic activity (Hanke et al., 1996) and biological functions (Chiariello et al., 2001). Although PP1 has been described to affect RET kinase activity (*in vitro* $IC_{50}$=100 nM) (Carlomagno et al., 2002), a specific mutation in valine$^{804}$ in the RET kinase domain confers resistance (>50-fold increase of the $IC_{50}$) to the compound (Carlomagno et al., 2004). We, therefore, introduced such mutation in the RET/PTC3 kinase domain (RET/PTC3$^{V804}$) rendering its activity significantly resistant to PP1, as scored by RET/PTC3$^{V804}$ auto-phosphorylation and activation of Erk2 (Fig. 10A). As expected, kinase activity of the parental RET/PTC3 molecule was completely abolished at comparable concentrations (compare the 5-10 μM PP1 lanes) as evidenced by both RET/PTC3 auto-phosphorylation and activation of Erk2 (Fig. 10B). Surprisingly, while strongly inhibiting Src (data not shown), PP1 treatment of RET/PTC3$^{V804}$-transfected cells only slightly affected Erk8 activity even at the highest doses tested (10 μM) (Fig. 10C) and after extensive times of treatment (up to 10 hrs treatment, at 5 μM concentration) (Fig. 4D), thus excluding a role for c-Src and its related kinases (Hanke et al., 1996) in the control of RET/PTC3-induced Erk8 activation. As a complementary approach to ascertain the role of Src kinases in RET/PTC3 activation of Erk8, we also used a dominant negative form of c-Src, Src YF KM (Chiariello et al., 2001). As shown in figure 10E, overexpression of the dominant negative molecule did not affect Erk8 activation while it effectively inhibited PDGF-induced activation of the c-*myc* promoter (Fig. 10F) (Chiariello et al., 2001). Altogether, these data clearly indicate that RET/PTC3 can use a Src-independent pathway to activate the Erk8 MAP kinase.

**c-Abl mediates RET/PTC3-dependent Erk8 activation.**

c-Abl, the cellular homologue of the Abelson murine leukemia virus, has been implicated in different cellular processes ranging from cell growth to survival, cellular stress, DNA-damage response and cell migration (Hantschel & Superti-Furga, 2004). From the structural point of view, c-Abl contains SH3, SH2 and tyrosine kinase domains whose arrangement and sequence very much resemble that of c-Src (Hantschel & Superti-Furga, 2004). These observations prompted us to investigate whether, similarly to Src, an activated form of c-Abl could induce Erk8 activation and, in turn, whether c-Abl could mediate RET/PTC3 activation of Erk8. As shown in figure 11A, an oncogenic, activated form of c-Abl, the Bcr/Abl fusion protein, readily induced Erk8 activation, at a level comparable to an activated form of c-Src (Src YF), used as a positive control (Abe et al., 2002). Thus, we decided to investigate whether c-Abl is able to act as a link between RET/PTC3 and the stimulation of Erk8. As an approach, we used a kinase-defective, dominant negative form of c-Abl, Abl-KD. This dominant negative molecule strongly inhibited the RET/PTC3-dependent activation of Erk8 (Fig. 11B), thus suggesting that c-Abl is a likely mediator in the pathway connecting RET/PTC3 to the activation of the Erk8 MAP kinase.

To control a vast range of cellular processes, c-Abl interacts with a large variety of cellular proteins, including phosphatases, kinases, signaling adaptors, transcription factors, cytoskeletal proteins and cell cycle regulators (Hantschel & Superti-Furga, 2004). To determine whether c-Abl can interact with Erk8 *in vivo*, 293T cells were transfected with HA-Erk8 and either wild-type c-Abl or the control vector, immunoprecipitated with an anti-HA antibody and then analized by western blot with an anti-abl antisera. As shown in figure 11C, c-Abl clearly co-immunoprecipitated with Erk8, therefore suggesting a role for physical interaction in the control of Erk8 activation by c-Abl.

We have previously shown that tyrosine$^{981}$ in RET/PTC3 mediates RET/PTC3-dependent Erk8 activation (Fig. 9), representing a major site recognized by signaling molecules intervening in such process. We therefore investigated whether the

tyrosine$^{981}$ residue was also able to mediate RET/PTC3 activation of c-Abl. Taking advantage of the observation that tyrosine phosphorylation of c-Abl correlates with its activation (Plattner et al., 1999), we cotransfected an autophosphorylation-impaired, AU5-tagged, c-Abl molecule, together with RET/PTC3, RET/PTC3$^{Y981}$, RET/PTC3$^{Y1015}$ or RET/PTC3$^{Y1062}$, respectively, immunoprecipitated these samples by anti-AU5 antibodies and analyzed them by anti-phospho-tyrosine western blot. As show in figure 11D, RET/PTC3 clearly induced Abl phosphorylation. Importantly, RET/PTC3$^{Y981}$ was strongly impaired in its ability to induce phosphorylation of the c-Abl protein, as compared to RET/PTC3 (Fig. 11D). In the same experiment, RET/PTC3$^{Y1062}$ and RET/PTC3$^{Y1015}$ exerted more limited or no effects, as compared to RET/PTC3 (Fig. 11D). Ultimately, the RET/PTC3 Kin$^{dead}$ was unable to induce c-Abl phosphorylation, establishing a requirement for RET/PTC3 kinase activity in c-Abl activation (Fig. 11D). Together, these results clearly indicate that RET/PTC3, through its tyrosine$^{981}$, can utilize an Abl-dependent pathway to stimulate Erk8 activation.

**A kinase-defective mutant for Erk8 interferes with RET/PTC3 signaling.**

The expression of the c-*jun* proto-oncogene is rapidly and transiently induced by different growth factors and cellular oncogenes (Marinissen et al., 1999). Among them, an oncogenic rearrangement of the RET proto-oncogene is able to strongly induce c-*jun* expression (Ishizaka et al., 1991), therefore establishing this gene as part of RET signaling pathway. To investigate whether the RET/PTC3 oncogene was able to stimulate the activity of the c-*jun* promoter, we took advantage of the availability of a reporter plasmid carrying the *luciferase* gene under the control of the murine c-*jun* promoter (Chiariello et al., 2000; Marinissen et al., 1999). Cotransfection of thyroid ARO cells with this reporter plasmid and increasing concentrations of the RET/PTC3 cDNA revealed that this oncogene could strongly induce the activity of the c-*jun* promoter (Fig. 12A). To evaluate whether Erk8 activation is involved in RET/PTC3 signaling to the c-*jun* promoter, we next used a dominant negative, kinase defective (data not shown) Erk8 molecule. For these experiments, we therefore cotransfected

RET/PTC3 with the c-*jun* reporter plasmid and increasing amounts of the Erk8 KR expression vector. As shown in figure 12B, the dominant negative Erk8 molecule caused a strong, although incomplete inhibition of RET/PTC3-dependent c-*jun* promoter stimulation, suggesting the existence of both Erk8-dependent and -independent pathways linking RET/PTC3 to the expression of the c-*jun* proto-oncogene.

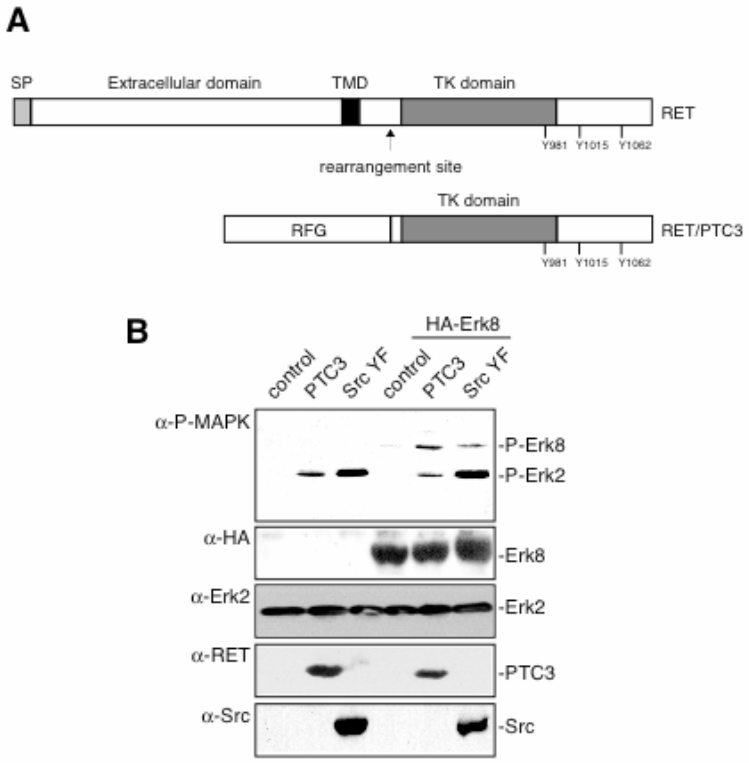



**Fig. 7 RET/PTC3 induces ERK8 activation.**
(A) Schematic representation of the wild-type RET protein and of its activated form, the RET/PTC3 oncogene. The position of three tyrosine autophosphorylation sites and of important protein domains are also showed. SP, signal peptide; TMD, trans-membrane domain; TK, tyrosine kinase; Y, tyrosine; RFG, RET Fused Gene. Numbers indicating RET/PTC3 tyrosine residues correspond to their position in the wild-type RET receptor. (B) RET/PTC3 induces ERK8 activation. Analysis of HA-Erk8 activation in 293T cells cotransfected with the RET/PTC3 (PTC3) and Src YF expression vectors and analyzed by western blot with anti-phospho MAPK antisera. Activation of endogenous Erk2 by RET/PTC3 and Src YF was used as an additional control for the activity of the two oncogenes. The expression of HA-Erk8 (a-HA panel), Erk2(α-Erk2 panel), Src YF (α-Src panel) and RET/PTC3 (α-RET panel) was also confirmed.
Control: cells transfected with β -galatosidase; α: anti.

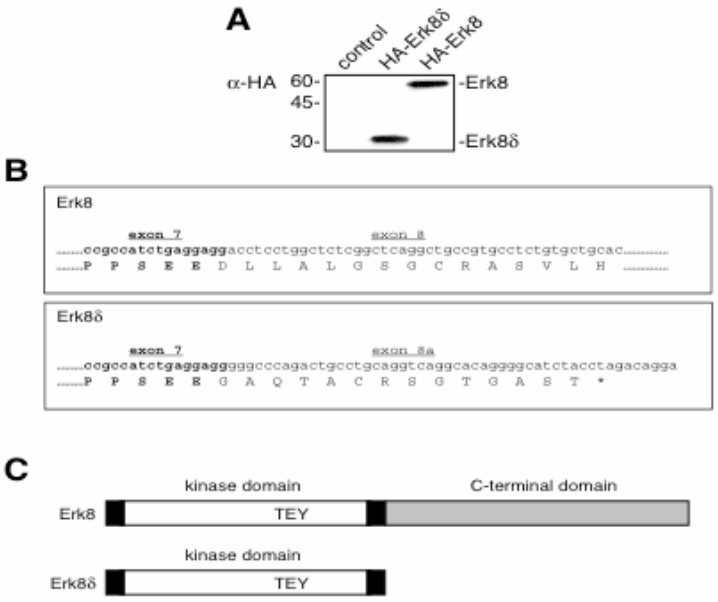
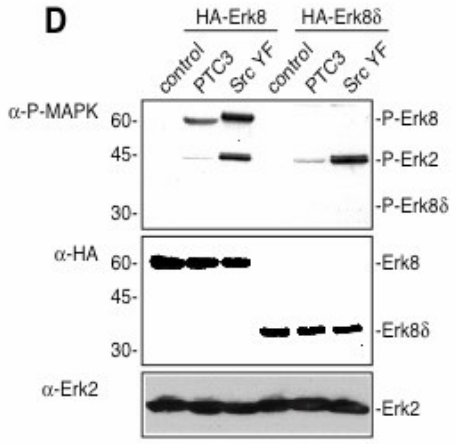



**Fig. 8 The Erk8 carboxy-terminal domain mediates the acivation of the MAP kinase by RET/PTC3.** (A), Erk8 and Erk8δ expression in transiently transfected 293T cells. (B), Comparison of Erk8 and Erk8δ nucleotide and protein sequences in the region of the alternative splicing. (C), Schematic representation of Erk8 and Erk8δ protein structures. The relative position of important residues and protein domains is indicated.




(D), Analysis of Erk8 and Erk8δ activation in 293T cells cotransfected with the RET/PTC3 (PTC3) and Src YF expression vectors. Activation of endogenous Erk2 by RET/PTC3 and Src YF was used as an additional control for the activity of the two oncogenes. The expression of HA-Erk8, HA-Erk8δ (α-HA panel) and Erk2 (α-Erk2 panel) was also confirmed.
Control: cells transfected with b-galatosidase; α: anti.

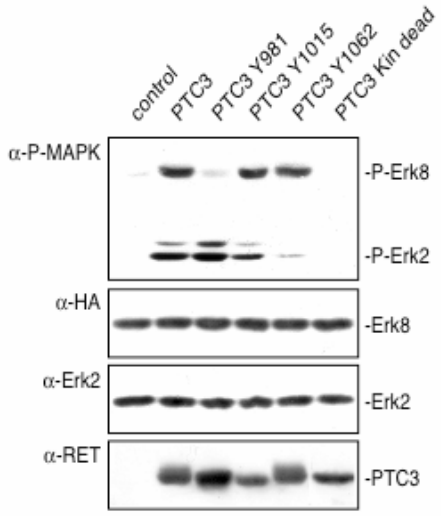
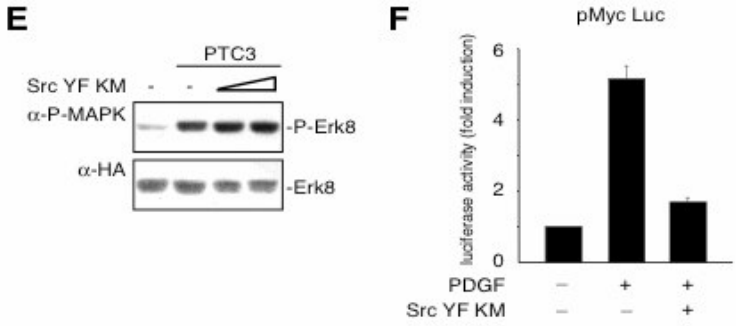



**Fig.9 RET/PTC3 requires tyrosine 981 to activate ERK8.**
Stimulation of Erk8 activation by RET/PTC3 (PTC3) and by tyrosine mutated forms of the oncogene, in 293T cells co-transfected with an expression vector for HA-Erk8 (α-P-MAPK panel). Activation of endogenous Erk2 by RET/PTC3 and its different mutated forms wa s used as a parallel control for the activity of the oncogenes (α-P-MAPK panel). The expression of HA-Erk8 (α-HA panel), Erk2 (α-Erk2 panel) and RET/PTC3 wild-type and mutants (α-RET panel) was also confirmed.
Control: cells transfected with β-galatosidase; α: anti.




(E), Analysis of Erk8 activation (upper α-P-MAPK panel) in 293T cells transfected with HA-Erk8, RET/PTC3 and increasing concentrations (250 and 500 ng) of the dominant negative Src YF KM. The expression of HA-Erk8 (α-HA panel) was also confirmed. Control: cells transfected with b-galatosidase; a: anti. (F), Inhibition of PDGF-induced c-myc promoter activity by Src YF KM. Stimulation of c-myc promoter transcriptional activity in NIH 3T3 ells transfected with the c-myc promoter reporter plasmid (pMyc-Luc) and a Src dominant negative expressing vectors (Src YFKM) and then stimulated for 4 h with PDGF (12.5 ng ml-1). Results represent luciferase activity in each sample, normalized for the corresponding efficiency of transfection and they are the average ± standard errors of triplicate samples from a typical experiment.

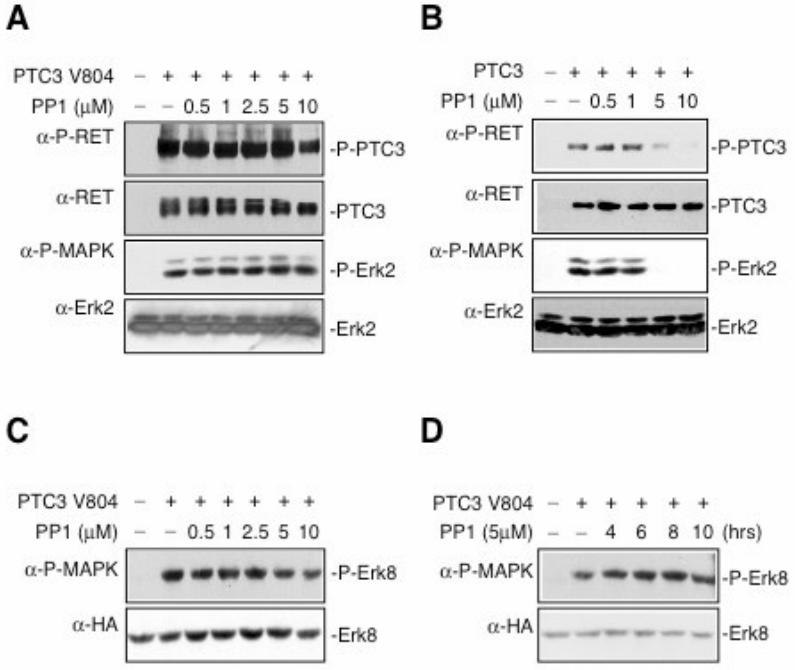
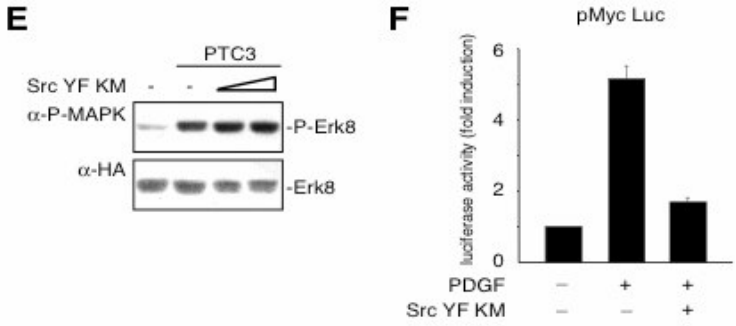



**Fig.10 Erk8 activation by RET/PTC3 does not depend on c-Src.**
(A), Effect of PP1 on RET/PTC3V804 auto-phosphorylation (α-P-RET panel), in 293T cells transfected as indicated, pre-treated three hours with different concentrations of the inhibitor and then analyzed by an anti-phospho RET specific antisera. As a control, activation of endogenous Erk2 was also scored by western blot with anti-phospho MAPK antisera (α-P-MAPK panel). Expression of RET/PTC3V804 and Erk2 was confirmed by western blot performed with anti-RET and anti-Erk2 antisera, respectively (α-RET and a-Erk2 panels). (B), Same as in A, using RET/PTC3 to activate Erk8. (C), Same as in A, analyzing RET/PTC3V804-dependent Erk8 activation. (D), Effect of PP1 on RET/PTC3V804-dependent Erk8 activation (α-P-MAPK panel), in 293T cells transfected as indicated and pre-treated with PP1 (5 μM) for increasing times. Erk8 expression was also confirmed (α-HA panel).




(E), Analysis of Erk8 activation (upper α-P-MAPK panel) in 293T cells transfected with HA-Erk8, RET/PTC3 and increasing concentrations (250 and 500 ng) of the dominant negative Src YF KM. The expression of HA-Erk8 (α-HA panel) was also confirmed. Control: cells transfected with b-galatosidase; a: anti. (F), Inhibition of PDGF-induced c-myc promoter activity by Src YF KM. Stimulation of c-myc promoter transcriptional activity in NIH 3T3 ells transfected with the c-myc promoter reporter plasmid (pMyc-Luc) and a Src dominant negative expressing vectors (Src YFKM) and then stimulated for 4 h with PDGF (12.5 ng ml-1). Results represent luciferase activity in each sample, normalized for the corresponding efficiency of transfection and they are the average ± standard errors of triplicate samples from a typical experiment.

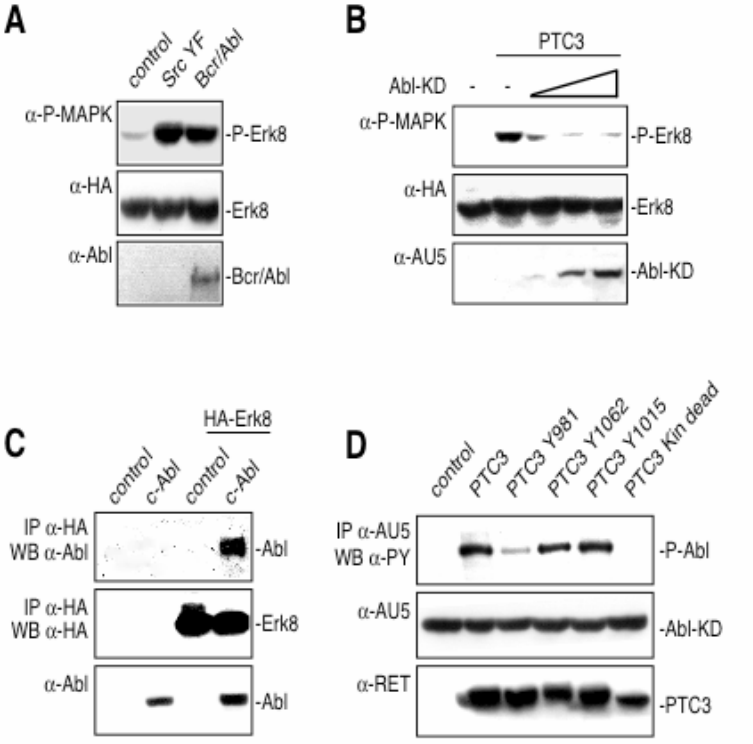



**Fig. 11 c-Abl mediates RET/PTC3-dependent ERK8 activation.**
(A), Analysis of HA-Erk8 activation in 293T cells cotransfected with Src YF and the oncogenic form of c-Abl, Bcr/Abl. Samples were analyzed by western blot with anti-phospho MAPK antisera. The expression of HA-Erk8 (α-HA panel) and Bcr/Abl (α-Abl panel) were confirmed. (B), Analysis of Erk8 activation (α-P-MAPK panel) in 293T cells transfected with HA-Erk8, RET/PTC3 and increasing concentrations (100, 250 and 500 ng) of the dominant negative Abl-KD. The expression of HA-Erk8 (α-HA panel) and of Abl-KD (α-AU5 panel) were also confirmed. (C), In vivo interaction of Erk8 with c-Abl. 293T cells were co-transfected with an expression vector for c-Abl (500 ng) together with plasmids for HA-Erk8 (500 ng) or control vector (β-galactosidase). Samples were next immunoprecipitated by anti-HA antibodies and then analyzed by anti-Abl antibodies. The expression of HA-Erk8 (middle panel) and RET/PTC3 wild-type and mutants (lower panel) was also confirmed. (D), Stimulation of c-Abl phosphorylation by RET/PTC3 (PTC3) and by tyrosine mutated forms of the oncogene. 293T cells were co-transfected with an expression vector for AU5-Abl-KD together with plasmids for RET/PTC3 and its tyrosine mutants. Samples were next immunoprecipitated by anti-AU5 antibodies and then analyzed by anti-phospho-tyrosine antibodies (α-PY). The expression of AU5-Abl-KD (α-AU5 panel) and RET/PTC3 wild-type and mutants (α-RET panel) was also confirmed.
Control: cells transfected with β-galatosidase; α: anti.

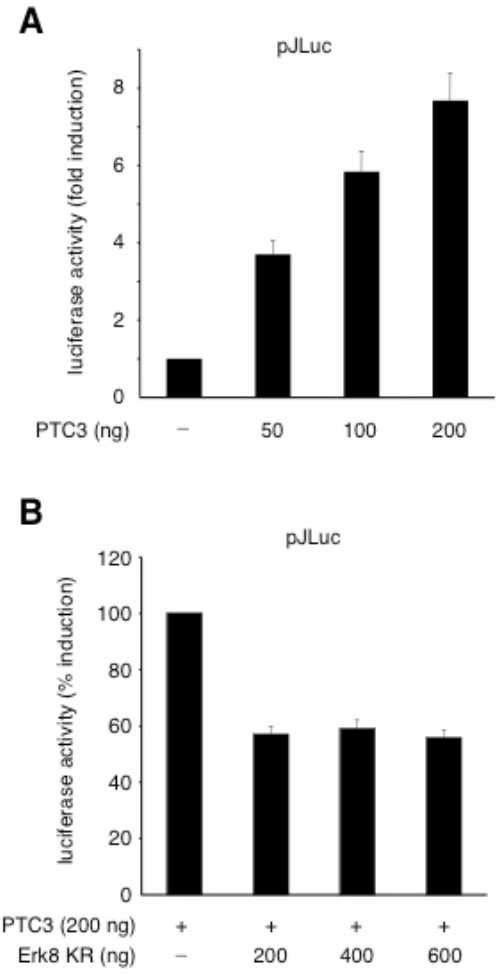



**Fig.12 Inibition of c-jun promoter activation by a dominant negative Erk8 mutant.** (A), Stimulation of c-jun promoter transcriptional activity in ARO cells transfected with the c-jun promoter reporter plasmid (pJLuc) and increasing concentrations of a RET/PTC3 expressing vectors. (B), Inhibition of RET/PTC3-induced c-jun promoter activity by Src YF KM. Stimulation of c-myc promoter transcriptional activity in NIH 3T3 cells co-transfected with pJLuc, RET/PTC3 and a Erk8 dominant negative expressing vector (Erk8 KR). Results represent luciferase activity in each sample, normalized for the corresponding efficiency of transfection and they are the average ± standard errors of triplicate samples from a typical experiment.

## Study 2

### Regulation of Akt activity by c-FLIP$_L$ .

### FLIP$_L$ interacts with Akt.

Using a yeast two-hybrid screening, we identified new unreported Akt interactors. Dual-reporter gene activity identified 82 positive clones. A clone, the number 8, was been choose as a positive interactor with Akt and this clone was identified as the cellular FLIP$_L$. All screenings were repeated in triplicate.

To confirm the interaction between Akt and FLIP$_L$ we performed a coimmunoprecipitation in vivo, between these two proteins. To this aim, Hela cells were transiently trasfected with two plasmids, Akt-HA and c-FLIP$_L$-Flag. We trasfected Hela cells with Akt-HA only like a negative control. An anti-Flag antibody was used to immunoprecipitate Flag-FLIP$_L$ and than we blotted with an anti-HA antibody. The results confirmed the interaction between Akt and Flip; in the negative control we were not able to see any bands (Fig. 13).

### c-FLIP overexpression does not interfere with the expressions levels and phosphorylation of Akt.

In order to understand the interaction between FLIP$_L$ and Akt, we tried to realize if the overexpression of FLIP$_L$, in 293-cells, could in some way modify the expression or activity of Akt. The 293-cells were trasfected with c-FLIP$_L$ and stimulated for 15 min.with insulin ($10^{-6}$M)(infact insuline stimulates Akt activity).

As a control, non trasfected 293-cells were utilized and stimulated with insulin for the same time and at the same concentrations.

The results confirmed that the expression levels and Akt phosphorilation, after c-FLIP$_L$ overexpression, in 293-cells, are unmodified. (Fig. 14)

**c-Flip$_L$ overexpression interferes with the levels of phosphorilation of GSK3B.**

From the data that we obtained by c-Flip overexpresion, we did not find any interference either with the endogenous levels of expression, or with the levels of phosphorylation of Akt, so we focused our studies on the possibility that c-FLIP could have an effect on the expression or on the activity of some Akt substrates. In order to do that, we trasfected, in transient, c-FLIP$_L$ in 293-cells and analyzed the total lysates by western blots for GSK3β (metabolic substrate of Akt) and Bad (involved in the intrinsic pathway of apoptosis).
Astonishingly, the levels of phosphorylation of GSK3β in cells expressing high levels of c-FLIP$_L$ were clearly reduced if compared to the control, whereas Bad expression levels remained unchanged. c-FLIP$_L$ overexpression in 293-cells influences the phosphorylation but not the expression levels of endogenous GSK3β. (Fig 15-16)

**The overexpression of c-FLIP$_L$ influences the cellular localization of GSK3β.**

Besides having a role in the metabolism of glucose, GSk3β is also important in cellular proliferation. Infact, once migrated into the nucleus, it phosphorilates many transcriptional factors such as cyclin D1 and c-Jun (Diehl,J.A.et al. 1998). At this point of our study, we investigated if the c-FLIP overexpression, besides influencing the activity of GSK3, could also regulate its cellular localization. We made a separation between nucleus and cytoplasm in 293-cells trasfected in transient with c-FLIP$_L$. Cytoplasmatic and nuclear extracts were analyzed by Western Blot with an anti-GSK3β antibody. Interestingly the result showed that the overexpression of c-FLIP$_L$ in 293-cells reduces the GSK3β levels in the nucleus preventing, the phosphorilation of some transcriptional factors.(Fig 17)

**c-FLIP$_L$ overexpression is inversely related with β-catenin expression levels.**

As well as in the glucose metabolism, GSK3β is also responsible for the regulation of β-catenin degradation. The active form of GSK3β (dephosphorilated) links the β-catenin with the axin protein in a compound, favouring the degradation by ubiquitination. The β-catenin if not degraded, moves into the nucleus and activates some transcriptional factors important for the proliferation and differentiation (Cohen et al. 2001).

Considering these informations and that the c-FLIP$_L$ overexpression seems to reduce clearly the phosphorilation levels of GSK3β, we wondered if the levels of endogenous β-catenin were also reduced as a consequence of the activation of GSK3β. The results showed that in 293-cells with c-FLIP$_L$ overexpession β-catenin levels were clearly reduced if compared to the control. (Fig 18)

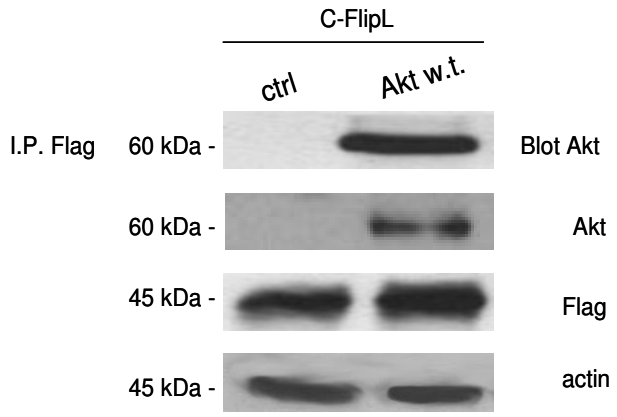
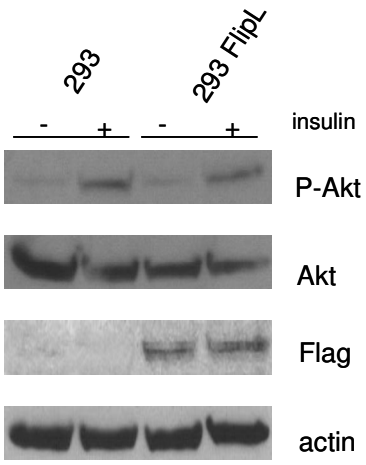



**Figure 13:** The amount of transfectd plasmids (pcDNA3-HA-Akt wt; D +, D-) as (CMV3xFlag -FlipL) is 5 μ g. The total lysates were immunoprecipitati ON A 4 °C using an antibody anti-Flag (Sigma) and then were added to the samples letting to hibridate the wheel at 4 °C for 1h with 20 μl of protein A/G plus (Santa Cruz). The immunoprecipitati were loaded on acrylamide gel to 10% and subjected to SDS page and then transferred to nitrocellulose filter. A quantity of lisati total (50 μ g) was analyzed to normalize the experiment.




**Picture 14:** 293-cells have been trasfected with CMV3xFlag-FlipL and treated with insulin (10-6 M). An amount of total lysates (5μg) have been loaded upon acrylamide-gel at 10% , exposed to SDS page,and later moved on a nitrocellulose-filter. The filter has been hybridized with anti-Akt and anti-P-Akt antibodies in order to evaluate the levels of expression and activities of Akt.

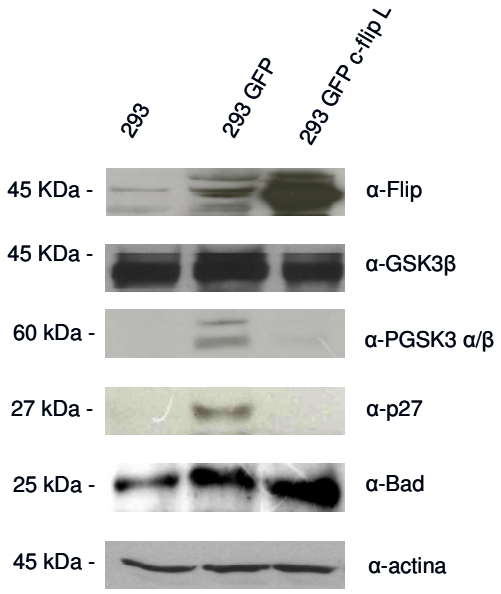



**Fig. 15:** Overexpression of c-FLIP$_L$ in 293 cells induces a reduction of GSK3β in 293 cells, but not on the levels of expression of BAD.

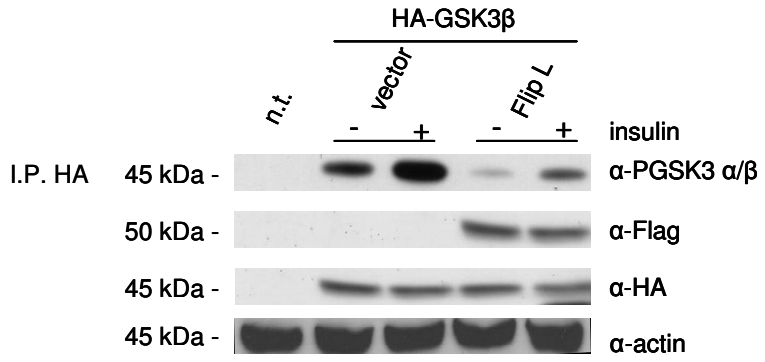



**Fig. 16:** 293-cells have been cootrasfected with GSK3β and c-FLIPL and after 48 hours have been stimulated for 15 min with insulin (10¯6M) so as to start the pathway of Akt. The total extracts have been immunoprecipitated with an anti-Akt antibody and later analyzed via Western Blot with an anti-PGSK3β antibody. Also in this case the overexpression of c-FLIP induces to a remarkable reduction of both basal phosphorilation of GSK3β and of the phosphorilation induced by the insulinic stimulus, confirming the datum we previously obtained that is:the overexpression of c-FLIPL in 293-cells influences the phosphorolation of c-FLIPL in a negative way.

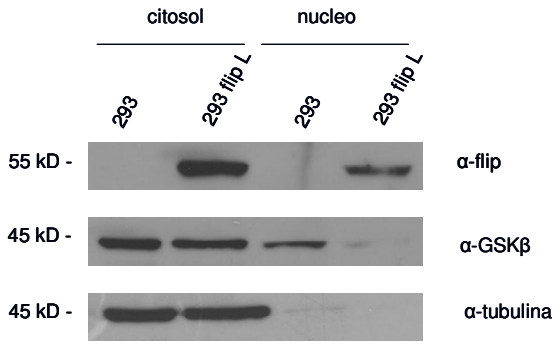
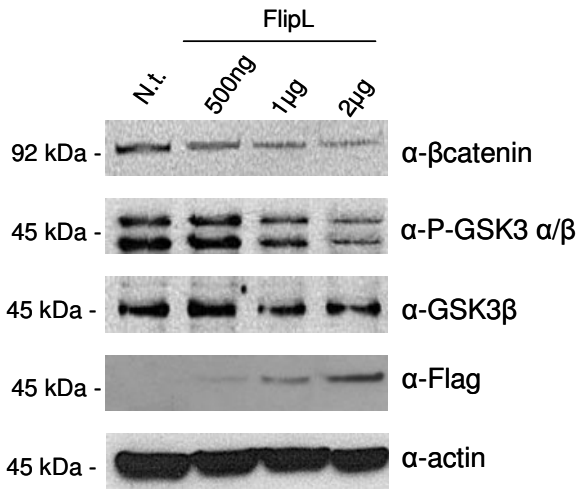



**Fig. 17:** C-FLIPL regulates the cellular compartimentation of GSK3β.




**Picture 18:** 293-cells trasfected with encreasing quantities of c-FLIPL. The bigger is the expression of c-FLIP,the smaller is the quantity of phosphorilated GSK3β; in this active form GSK3β regulates the ubiquitination of β-catenin in a positive way.

## Discussion

The complexity of the mechanisms mediating intracellular signaling by RET and its activated forms, the RET/PTC and MEN2 oncogenes, has just begun to be appreciated. Indeed, the biological functions of these proteins result from the coordinated activity of multiple kinase cascades, whose integrated signals control renal development, histogenesis of the enteric nervous system and, possibly, tumor formation (Nikiforov, 2002; Pasini et al., 1996; Santoro et al., 2004). In the first study, finding that RET/PTC3 activates Erk8 raises the possibility of a novel Erk8-dependent signaling pathway controlling RET biological functions. Interestingly, we have shown that Erk8 activation depends on the integrity of tyrosine$^{981}$, while tyrosine$^{1062}$ mutation does not affect RET/PTC3-dependent activation of the kinase. This result clearly differentiates Erk8 from other MAP kinases already involved in RET signaling whose activation, on the contrary, strictly depends on RET tyrosine$^{1062}$ (Hayashi et al., 2000).

Although RET tyrosine$^{981}$ has been previously recognized as a docking site for c-Src (Encinas et al., 2004) and this kinase modulates Erk8 activation (Abe et al., 2002), surprisingly, RET/PTC3 activation of Erk8 does not depend on c-Src. This result therefore suggests that additional molecules interact with tyrosine$^{981}$ of RET/PTC3 and are responsible for the control of Erk8 activity. Indeed, in this report we present evidences that c-Abl controls RET/PTC3-dependent Erk8 activating phosphorylation (Fig. 14). As a corollary to this finding, for the first time we show that c-Abl is able to mediate RET-dependent signaling pathways. Not only RET/PTC3 induces c-Abl phosphorylation but such phenomenon also seems to be mediated by tyrosine$^{981}$, in line with our observation that this tyrosine mediates Erk8 activation. These findings strongly support each other, especially considering that, up to now, the only known signaling molecule downstream of this tyrosine was c-Src, while most of the other RET effectors depended on the integrity of tyrosine$^{1062}$.

c-Abl as well as c-Src contain well characterized SH3 domains, with an high degree of conservation in terms of sequence identity and structure (Hantschel & Superti-Furga, 2004). In c-Abl, this domain is important both for interaction with different proteins and for participation to an intramolecular regulatory mechanism (Wang, 2004). On the

other hand, Erk8 contains two putative SH3-binding sites in its C-terminal tail (Abe et al., 2002). As the c-Src SH3 domain interacts *in vitro* with Erk8 (Abe et al., 2002) and we have demonstrated that c-Abl interacts *in vivo* with this MAP kinase, it is possible that this interaction is mediated by the c-Abl SH3 domain. This hypothesis is currently under investigation.

The more recently identified Erk5, Erk7 and Erk8 molecules differentiate from classical MAP kinases (Erks, Jnks and p38s) in that they present long carboxy-terminal domains with no strong homology to other mammalian proteins. By using a naturally occurring Erk8 splice variant, Erk8δ, lacking the long carboxy-terminal domain, we show a key role for this domain in RET/PTC3-dependent activation. It is intriguing the possibility that distinct stimuli differently activate the Erk8 and Erk8δ proteins and, conversely, that Erk8δ may represent a modulator of Erk8 activation.

Upon activation of different MAP kinases, a large number of transcription factors appears to control the expression of several growth promoting genes, such as c-*jun* and c-*fos*, and, through these, control a vast variety of cellular functions. Specifically, the c-*jun* promoter has already been shown to represent a key site for the integration of signals coming from both cellular oncogenes (Chiariello et al., 2000) and extracellular ligands (Marinissen et al., 1999). It is therefore not surprising our observation that a dominant negative Erk8 molecule only partially inhibits the activation of the c-*jun* promoter. Indeed, we have previously demonstrated that signaling from RET impinges on the activation of at least another MAP kinase, Jnk (Chiariello et al., 1998), which is able to control the activity of the c-*jun* promoter (Marinissen et al., 1999). We can therefore expect Erk8 to be part of the complex network of kinases, whose activation ultimately determines the specific biological response to the activation of RET and its related oncogenes in different cellular environments.

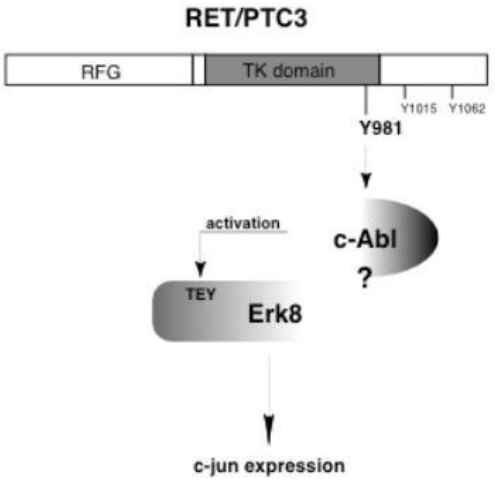



**Figura 19 :** Schematic rappresentation of the pathway connecting RET/PTC3 to the stimulation of ERK8 activation, throught tyrosine 981-dependent activation of c-Abl.
TK, tyrosine kinase; RFG, RET Fused Gene. Numbers indicating RET/PTC3 tyrosine residues corrisponde to their position in the wild-type RET receptor.

Cellular FLICE inhibitory protein (c-FLIP) is an endogenous inhibitor of death receptor-induced apoptosis throught the caspase 8 pathway, and is widely expressed in various tumors (Lee et al. 2003).

Akt is a serine/threonine kinase with the plackstrin homology domain (PH) in its $NH_2$ terminal region and catalytic domain closely related to protein kinase C (PKC) and protein kinase A (PKA) family members (Staal 1987). However, FLIP expression is critically dependent upon PI3-kinase and Akt activity. (David J. Panka et al. 2000). The two-hybrid screening suggested that Akt and $FLIP_L$ interact. We confirmed this interaction by coimmunoprecipitation in vivo of these two proteins. The interaction between $FLIP_L$ and Akt does not interfere either with the expression, or with the phosphorilation of Akt, but the overexpression of c-$FLIP_L$ interferes, instead, on the levels of phosphorilation of GSK3β, a substrate of Akt.

GSK3β is one of several protein kinases that phosphorylated and inactivated glycogen synthase, the final enzyme in glycogen biosyntesis (Embi, N. et al . 1980).
We know that the inhibition of GSK3 by insulin is dependent from its phosphorilation at an N-terminal serine residue ($Ser^{21}$ in GSK3α and $Ser^{9}$ in GSK3β) and this phosphorilation is catalysed by protein kinase B (PKB; also called Akt)(Cross et al. 1995). In recent years, GSK3β has been implicated in the phosphorylation of many proteins and in the regulation of several cellular events (Cohen et. al 2001). The activation of GSk3β activates the degradation-process of β-catenin (Fig.2) (Frame et al. 2001). We proved that the overexpression of c-FlipL in Hela-cells determines a reduction of GSK3β- phosphorilation even when this phosphorilation is induced by the insulinic stimulus through PI3K pathway. Thus, through the activation of GSK3β, $c\text{-}FLIP_L$ expression levels regulate β-catenin degradation.
$c\text{-}FLIP_L$ and Akt interaction leads to a reduced phosphorilation of GSK3β (Akt substrate involved in the metabolism of glucose and cellular proliferation) by Akt and c-FLIPL overexpression in Hela-cells has also an effect on the cellular localization of GSK3β. Infact, the nuclear levels of this protein are drastically reduced when $c\text{-}Flip_L$ is overexpressed.In conclusion c-FLIPL protein is an Akt interactor and this interaction is responsible of the reduction of GSK3β phosphorilation levels and β-catenin cytoplamic levels.

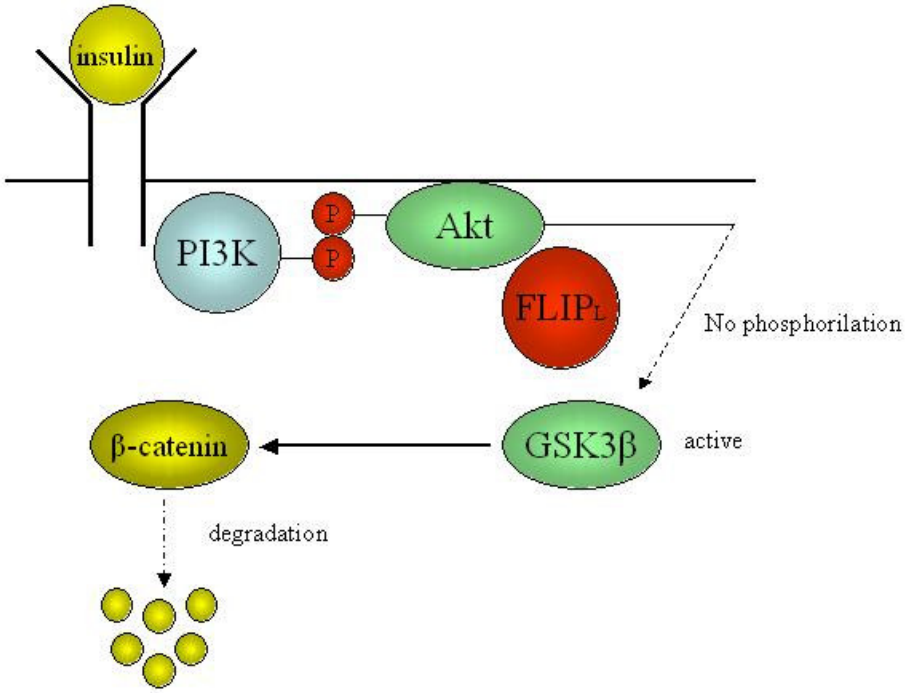



**Figure 20:** When c-FLIPL and Akt interact, the phosphorilation of GSK3β decreases and, as a consequence, its activation takes place. The activation of GSK3β causes an increase of β-catenin degradation.

## References


Abe, M.K., Kahle, K.T., Saelzler, M.P., Orth, K., Dixon, J.E., and Rosner, M.R. ERK7 is an autoactivated member of the MAPK family. 2001. J. Biol Chem., **276**: 21272-21279.

Abe, M.K., Kuo, W.L., Hershenson, M.B., and Rosner, M.R. 1999. Extracellular signal-regulated kinase 7 (ERK7), a novel ERK with a C-terminal domain that regulates its activity, its cellular localization, and cell growth. Mol. Cell. Biol., **19**: 1301-1312.

Abe, M.K., Saelzler, M.P., Espinosa, R. 3rd, Kahle, K.T., Hershenson, M.B., Le Beau M.M., and Rosner, M.R. 2002. ERK8, a new member of the mitogen-activated protein kinase family. J. Biol. Chem., **277**: 16733-16743.

Airaksinen M.S., and Saarma M. 2002. The GDNF family: signalling, biological functions and therapeutic value. Nat. Rev. Neurosci., **3**: 383-394.

Blume-Jensen, P., and Hunter, T. 2001. Oncogenic kinase signalling. Nature, **411**: 355-365.

Borrello, M.G., Alberti, L., Arighi, E., Bongarzone, I., Battistini, C., Bardelli, A., Pasini, B., Piutti, C., Rizzetti, M.G., Mondellini, P., Radice, M.T., and Pierotti, M.A. The full oncogenic activity of Ret/ptc2 depends on tyrosine 539, a docking site for phospholipase Cgamma. 1996. Mol Cell Biol. **16**: 2151-2163.

Buschbeck, M, and Ullrich, A. The unique C-terminal tail of the mitogen-activated protein kinase ERK5 regulates its activation and nuclear shuttling. 2005. J. Biol. Chem. **280**: 2659-2667.

Carlomagno, F., Guida, T., Anaganti, S., Vecchio, G., Fusco, A., Ryan, A.J., Billaud, M., and Santoro, M.. 2004. Disease associated mutations at valine 804 in the RET receptor tyrosine kinase confer resistance to selective kinase inhibitors. Oncogene, **23**: 6056-6063.

Carlomagno, F., Vitagliano, D., Guida, T., Napolitano, M., Vecchio, G., Fusco, A., Gazit, A., Levitzki, A., and Santoro, M. 2002. The kinase inhibitor PP1 blocks tumorigenesis induced by RET oncogenes. Cancer Res., **62**: 1077-10882.

Chang, L., and Karin, M. 2001. Mammalian MAP kinase signalling cascades. Nature, **410**, 37-40.

Chiariello M, Visconti R, Carlomagno F, Melillo, R.M., Bucci, C., De Franciscis, V., Fox, G.M., Jing, S., Coso, O.A., Gutkind, J.S., Fusco A, Santoro M. 1998. Signalling of the Ret receptor tyrosine kinase through the c-Jun NH2-terminal protein kinases (JNKS): evidence for a divergence of the ERKs and JNKs pathways induced by Ret. Oncogene, **16**: 2435-2445

Chiariello, M., Marinissen, M.J., and Gutkind, J.S. 2000. Multiple mitogen-activated protein kinase signaling pathways connect the cot oncoprotein to the c-jun promoter and to cellular transformation. Mol. Cell. Biol. 2000. **20**: 1747-1758.

Chiariello, M., Marinissen, M.J., and Gutkind, J.S.. 2001. Regulation of *c-myc* expression by PDGF through Rho GTPases. Nat. Cell. Biol., **3**: 580-586.

Encinas, M., Crowder, R.J., Milbrandt, J., Johnson, E.M. Jr. 2004. Tyrosine 981, a novel ret autophosphorylation site, binds c-Src to mediate neuronal survival. J Biol Chem., **279**: 18262-18269.

Hanke, J.H., Gardner, J.P., Dow, R.L., Changelian, P.S., Brissette, W.H., Weringer, E.J., Pollok, B.A., Connelly, P.A. 1996. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. J Biol Chem., **271**: 695-701.

Hantschel, O, and Superti-Furga, G. 2004. Regulation of the c-Abl and Bcr-Abl tyrosine kinases. Nat. Rev. Mol. Cell. Biol., **5**: 33-44.

Ishizaka, Y., Takahashi, M., Ushijima, T., Sugimura, T., and Nagao, M. 1991.A high phosphorylation state and increased activity of the TRE motif in the NIH3T3 cell transformant induced by retTPC. Biochem Biophys Res Commun., **179**:1331-1336.

Jiang, G., and Hunter, T. 1999. Receptor signalling: when dimarization is not enough. Curr. Biol., **9**: 568-571.

Kasler, H.G., Victoria, J., Duramad, O., and Winoto, A. 2000. ERK5 is a novel type of mitogen-activated protein kinase containing a transcriptional activation domain. Mol. Cell. Biol., **20**: 8382-8389.

Kurokawa, K., Kawai, K., Hashimoto, M., Ito, Y., Takahashi, M. Cell signalling and gene expression mediated by RET tyrosine kinase. 2003. J. Intern. Med.,**253**: 627-633.

Lee, J.D., Ulevitch, R.J., and Han, J. 1995. Primary structure of BMK1: a new mammalian map kinase. Biochem. Biophys. Res. Commun., **213**: 715-724.

Marinissen, M.J., Chiariello, M., Pallante, M., and Gutkind, J.S. 1999. A network of mitogen-activated protein kinases links G protein-coupled receptors to the c-jun

promoter: a role for c-Jun NH2-terminal kinase, p38s, and extracellular signal-regulated kinase 5. Mol. Cell. Biol., **19**: 4289-4301.

Nanberg, E., and Westmark, B. 1993. Platelet-derived growth factor increases the turnover of GTP/GDP on Ras in permeablized fibroblasts. J. Biol. Chem. **268**: 18187–18194.

Nikiforov, Y.E. RET/PTC rearrangement in thyroid tumors. 2002. Endocr. Pathol., **13**: 3-16.

Pasini B, Ceccherini I, and Romeo G. 1996. RET mutations in human disease. Trends Genet., **12**:138-144.

Plattner, R., Kadlec, L., DeMali, K.A., Kazlauskas, A., and Pendergast, A.M. 1999. c-Abl is activated by growth factors and Src family kinases and has a role in the cellular response to PDGF. Genes Dev., **13**:2400-2411

Qi, M., Elion, E.A. 2005. MAP kinase pathways. J. Cell Science, **118**: 3569-3572.

Santoro M, Melillo RM, Carlomagno F, Vecchio G, Fusco A. Minireview: RET: normal and abnormal functions. 2004. Endocrinology, **145**: 5448-5451.

Santoro, M, Dathan, NA, Berlingieri, MT, Bongarzone, I, Paulin, C, Grieco, M, Pierotti, MA, Vecchio, G, and Fusco, A. 1994. Molecular characterization of RET/PTC3; a novel rearranged version of the RET proto-oncogene in a human thyroid papillary carcinoma.1994. Oncogene, **9**: 509-516.

Satoh T., Fantl, W.J., Escobedo, J.A., Williams, L.T., and Kaziro, Y. 1993. Platelet-derived growth factor receptor mediates activation of ras through different signaling pathways in different cell types. Mol Cell Biol., **13**:3706-3713.

Schlessinger, J. 2000. Cell signalling by receptor tyrosine kinases. Cell, **103**: 211-225.

Schuchardt, A., D'Agati, V., Larsson-Blomberg, L., Costantini, F., and Pachnis, V. 1994. Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. Nature, **367**: 380-383.

Sherman, S.I. Review of undifferentiated thyroglobulin-positive thyroid cancer 45 years after treatment for papillary primary. 2003. Clin. Adv. Hematol. Oncol. **1**: 243.

Yan, C., Luo, H., Lee, J.D., Abe, J., and Berk, B.C. 2001 Molecular cloning of mouse ERK5/BMK1 splice variants and characterization of ERK5 functional domains. J. Biol. Chem., **276**: 10870-10878.

Yan, L., Carr, J., Ashby, P.R., Murry-Tait, V., Thompson, C., and Arthur J.S. 2003. Knockout of ERK5 causes multiple defects in placental and embryonic development. BMC Dev. Biol., **3**: 1-21.

Zhang, W., Morris, Q.D., Chang, R., Shai, O., Bakowski, M.A., Mitsakakis, N., Mohammad, N., Robinson, M.D., Zirngibl, R., Somogyi, E., Laurin, N., Eftekharpour, E., Sat, E., Grigull, J., Pan, Q., Peng, W.T., Krogan, N., Greenblatt, J., Fehlings, M., van der Kooy, D., Aubin, J., Bruneau, B.G., Rossant, J., Blencowe, B.J., Frey, B.J., and Hughes, T.R. 2004. The functional landscape of mouse gene expression. J. Biol., **3**: 21.

Zhou, G., Bao, Z.Q., and Dixon, J.E. 1995. Components of a new human protein kinase signal transduction pathway. J. Biol. Chem., **270**: 12665-12669.

Hanks SK, Hunter T. Protein kinases 6. The eukaryotic protein kinase superfamily: kinase (catalytic) domain structure and classification. FASEB J. 1995 May;9(8):576-96.

Hunter T. A thousand and one protein kinases.
Cell. 1987 Sep 11;50(6):823-9. Review.

Stall SP. Molecular cloning of the akt oncogene and its human homologues AKT1 and AKT2: amplification of AKT1 in a primary human gastric adenocarcinoma. Proc Natl Acad Sci U S A. 1987 Jul;84(14):5034-7.

Bellacosa A, Chan TO, Ahmed NN, Datta K, Malstrom S, Stokoe D, McCormick F, Feng J, Tsichlis P. Akt activation by growth factors is a multiple-step process: the role of the PH domain. Oncogene. 1998 Jul 23;17(3):313-25.

Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PR, Reese CB, Cohen P. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. Curr Biol. 1997 Apr 1;7(4):261-9.

Downward J. Mechanisms and consequences of activation of protein kinase B/Akt.
Curr Opin Cell Biol. 1998 Apr;10(2):262-7. Review.

Ozes ON, Akca H, Mayo LD, Gustin JA, Maehama T, Dixon JE, Donner DB. A phosphatidylinositol 3-kinase/Akt/mTOR pathway mediates and PTEN antagonizes tumor necrosis factor inhibition of insulin signaling through insulin receptor substrate-1. Proc Natl Acad Sci U S A. 2001 Apr 10;98(8):4640-5. Epub 2001 Apr 3.

Kauffmann-Zeh A, Rodriguez-Viciana P, Ulrich E, Gilbert C, Coffer P, Downward J, Evan G. Suppression of c-Myc-induced apoptosis by Ras signalling through PI(3)K and PKB. Nature. 1997 Feb 6;385(6616):544-8.

Biggs WH 3rd, Meisenhelder J, Hunter T, Cavenee WK, Arden KC. Protein kinase B/Akt-mediated phosphorylation promotes nuclear exclusion of the winged helix transcription factor FKHR1. Proc Natl Acad Sci U S A. 1999 Jun 22;96(13):7421-6.

del Peso L, González-García M, Page C, Herrera R, Nuñez G. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science. 1997 Oct 24;278(5338):687-9.

Lee YJ, Song YK, Song JJ, Siervo-Sassi RR, Kim HR, Li L, Spitz DR, Lokshin A, Kim JH. Reconstitution of galectin-3 alters glutathione content and potentiates TRAIL-induced cytotoxicity by dephosphorylation of Akt. Exp Cell Res. 2003 Aug 1;288(1):21-34.

Rippo MR, Moretti S, Vescovi S, Tomasetti M, Orecchia S, Amici G, Catalano A, Procopio A. FLIP overexpression inhibits death receptor-induced apoptosis in malignant mesothelial cells. Oncogene. 2004 Oct 14;23(47):7753-60.

Rasper DM, Vaillancourt JP, Hadano S, Houtzager VM, Seiden I, Keen SL, Tawa P, Xanthoudakis S, Nasir J, Martindale D, Koop BF, Peterson EP, Thornberry NA, Huang J, MacPherson DP, Black SC, Hornung F, Lenardo MJ, Hayden MR, Roy S, Nicholson DW. Cell death attenuation by 'Usurpin', a mammalian DED-caspase homologue that precludes caspase-8 recruitment and activation by the CD-95 (Fas, APO-1) receptor complex. Cell Death Differ. 1998 Apr;5(4):271-88.

Yeh WC, Itie A, Elia AJ, Ng M, Shu HB, Wakeham A, Mirtsos C, Suzuki N, Bonnard M, Goeddel DV, Mak TW. Requirement for Casper (c-FLIP) in regulation

of death receptor-induced apoptosis and embryonic development. Immunity. 2000 Jun;12(6):633-42.

Chang DW, Xing Z, Pan Y, Algeciras-Schimnich A, Barnhart BC, Yaish-Ohad S, Peter ME, Yang X. c-FLIP(L) is a dual function regulator for caspase-8 activation and CD95-mediated apoptosis. EMBO J. 2002 Jul 15;21(14):3704-14.

Fang LW, Tai TS, Yu WN, Liao F, Lai MZ. Phosphatidylinositide 3-kinase priming couples c-FLIP to T cell activation. J Biol Chem. 2004 Jan 2;279(1):13-8. Epub 2003 Oct 24.

Nakajima A, Komazawa-Sakon S, Takekawa M, Sasazuki T, Yeh WC, Yagita H, Okumura K, Nakano H. An antiapoptotic protein, c-FLIPL, directly binds to MKK7 and inhibits the JNK pathway. EMBO J. 2006 Nov 29;25(23):5549-59. Epub 2006 Nov 16.

Embi N, Rylatt DB, Cohen P. Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. Eur J Biochem. 1980 Jun;107(2):519-27. No abstract available.

Woodgett JR. cDNA cloning and properties of glycogen synthase kinase-3. Methods Enzymol. 1991;200:564-77.

Woodgett JR. Molecular cloning and expression of glycogen synthase kinase-3/factor A. EMBO J. 1990 Aug;9(8):2431-8.

Cohen P, Nimmo HG, Proud CG. How does insulin stimulate glycogen synthesis? Biochem Soc Symp. 1978;(43):69-95. Review.

Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature. 1995 Dec 21-28;378(6559):785-9.

Parker PJ, Embi N, Caudwell FB, Cohen P. Glycogen synthase from rabbit skeletal muscle. State of phosphorylation of the seven phosphoserine residues in vivo in the presence and absence of adrenaline. Eur J Biochem. 1982 May;124(1):47-55.

Zeng L, Fagotto F, Zhang T, Hsu W, Vasicek TJ, Perry WL 3rd, Lee JJ, Tilghman SM, Gumbiner BM, Costantini F. The mouse Fused locus encodes Axin, an inhibitor of the Wnt signaling pathway that regulates embryonic axis formation. Cell. 1997 Jul 11;90(1):181-92.

Li L, Yuan H, Weaver CD, Mao J, Farr GH 3rd, Sussman DJ, Jonkers J, Kimelman D, Wu D. Axin and Frat1 interact with dvl and GSK, bridging Dvl to GSK in Wnt-mediated regulation of LEF-1. EMBO J. 1999 Aug 2;18(15):4233-40.

Frame S, Cohen P, Biondi RM. A common phosphate binding site explains the unique substrate specificity of GSK3 and its inactivation by phosphorylation. Mol Cell. 2001 Jun;7(6):1321-7.