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"Mechanism of regulation of Raf-1 by Ca^{2+}/Calmodulin-dependent kinase II"

Coordinator:
Prof. Vittorio Enrico Avvedimento
Tutor: Prof. Mario Vitale

Candidate:
Dr. Marcella Salzano

TABLE OF CONTESTS

ABSTRACT

The calcium-calmodulin dependent kinase II (CaMKII) is an ubiquitous serine/threonine protein kinase involved in multiple signalings and biological functions. It has been demonstrated that in epithelial and mesenchimal cells CaMKII participates with Ras to Raf-1 activation and that it is necessary for ERK activation by diverse factors. Raf-1 activation is complex. Maximal Raf-1 activation is reached by phosphorylation at Y341 by Src and at S338. Although early data proposed the involvement of p21-activated kinase 3 (Pak3), the kinase phosphorylating S338 is not definitively identified.

Aim of my thesis is to go more insight into the molecular mechanisms of CaMKII/Raf-1 interaction and to verify the hypothesis that CaMKII phosphorylates Raf-1 at Ser338. To this purpose, I investigated the role of CaMKII in Raf-1 and ERK activation by oncogenic Ras and other factors, in COS-7 and NIH3T3 cells. Serum, SrcY527 and RasV12 activated CaMKII. CaMKII was necessary for Raf-1 and ERK activation by all these factors. CaMKII was necessary to the phosphorylation of S338 Raf-1 by serum, fibronectin or oncogenic Ras. Conversely, the inhibition of phosphatidylinositol 3-kinase, which in turn activates Pak3, was ineffective. The direct kinase activity of CaMKII on the serine 338 residue, was demonstrated in vitro by interaction of purified kinases.

These results demonstrate that CaMKII phosphorylates Raf-1 at S338 and partecipates to ERK activation upon different physiologic and pathologic stimuli in the MAPK cascade. This kinase, might have a role in cancers harbouring oncogenic Ras and could represent a new therapeutic target for pharmacological intervention in these tumors.

1. INTRODUCTION

1.1 Raf family

The Raf protein, named for Rapidly Accelerated Fibrosarcoma, was discovered over two decades ago as a retroviral oncogene, v-Raf or v-MIL, possessing a serine/threonine kinase activity (Moelling, et al., 1984; Rapp, et al., 1983). v-Raf correlated genes were identified later. In mammalian cells there are three known Raf isoforms: A- Raf, B- Raf and C- Raf, also called Raf-1. Studies in *Drosophila* and in *C. Elegans* have defined D-Raf and lin45 respectively, demonstrating that all these proteins share common architecture and that they are subject to complex and conserved regulation, represented by the presence of a large number of phosphorylation sites distributed along the proteins (Fig. 1). Raf proteins have a key role in the mitogen-activated protein-kinase (MAPK) pathway, a linear and conserved cellular pathway that transduces signals from the membrane to the nucleus. Binding of extracellular ligands such as growth factors, cytokines and hormones to cell-surface receptors activates Ras, a small GTP-binding protein, that initiates Raf activation. This binding leads to activation and phosphorylation of the dual-specificity mitogen activated kinase-1 and -2 (MEK1 and MEK2) which in turn activate and phosphorylate the extracellular signal-regulated kinas-1 and -2 (ERK1 and ERK2) (Wellbrock, et al., 2004). The Ras-ERK pathway can mediate differentiation, proliferation or oncogenic transformation, depending on cellular context (Marshall, 1995). Knock out studies of individual Raf isoforms in mice, seem to demonstrate their essential and non-overlapping roles in embryo- and organogenesis. In general, knockout mice display severe growth retardation in size and weight and abnormal organs development, demonstrating that Raf isoforms are required for normal development beyond the blastocyst stage and for sustaining life (Wojnowski, et al., 1998)

1.1.1 Raf-1 kinase structure

Each of the Raf kinases shares three conserved regions (CR) : CR1 and CR2 in N-terminus and CR3 in the C-terminus (Fig. 1). CR1 contains elements required for Raf membrane recruitment: in particular, it is composed of a Ras-binding domain (RBD) that binds active GTP-Ras, and a cysteine-rich domain (CRD), which can bind two zinc ions and stabilizes the association with Ras. CR2 is a serine/threonine rich domain and it holds a conserved phosphorylation site at S259 for 14-3-3, a regulatory protein. Binding of 14-3-3 to this phosphorylated serine is inhibitory. CR3 contains the catalytic portion holding the Raf kinase domain and a conserved serine at S621 that is a stimulatory 14-3-3 binding site. The Raf protein kinase domain has the characteristic small N-terminal lobe and large C-terminal lobe typical of all protein kinases. The small lobe has a predominantly antiparallel β-sheet structure and anchors and orients ATP. The large lobe is mainly α-helical and binds MEK1/2 . The two lobes move relative to each other and can open or close the cleft. The open form allows access of ATP and represents the active form; in the closed conformation the catalytic domain of Raf is folded and binds the N-terminal regulatory domain (Leicht, et al., 2007; Roskoski).

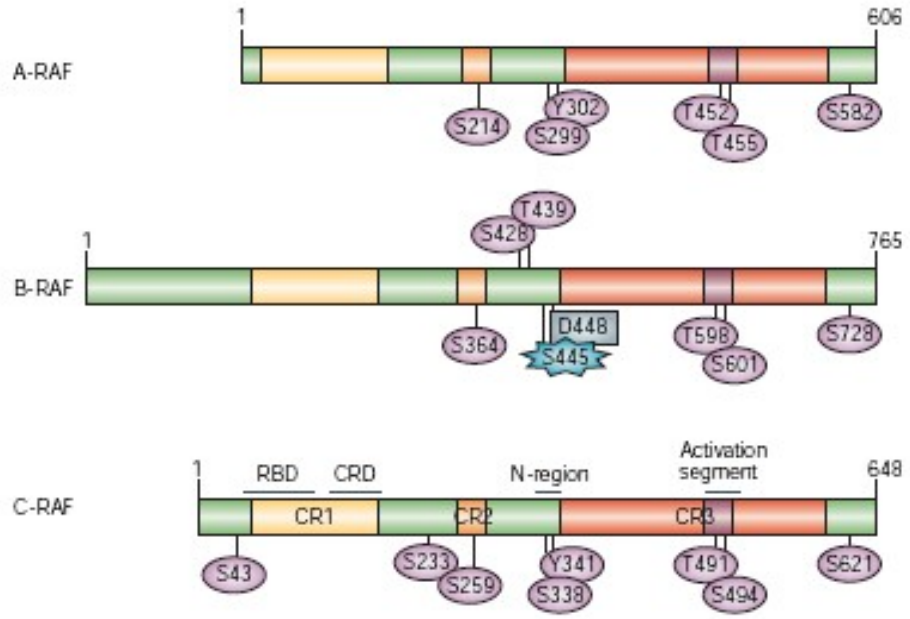

Fig. 1.1 | **Structure of the Raf proteins.** The Raf mammals isoforms are: A-Raf, B-Raf and C-Raf. They share three conserved region: CR1 (yellow), CR2 (orange), CR3 (red) which contains several phosphorylation sites. RBD and CRD, within CR1, are required for membrane recruitment. (Wellbrock, et al., 2004)

1.1.2 Raf kinase regulation

Raf -1 activation is a multistep and highly complex process that involves:

- membrane recruitment
- several phosphorylation sites
- protein-protein interaction (dimerization and oligomerization with B-Raf)
- conformational changes

Most of our understanding of Raf regulation comes from studies using Raf-1; although many of these regulation events seem to be conserved for A-Raf and B-Raf, there are several crucial differences.

The initiating event in Raf-1 activation is Ras-mediated membrane recruitment, due to directly binding of Ras to the N-terminal regulatory domain of Raf-1 (Wellbrock, et al., 2004). Ras-GTP, in its active form and attached to the inner leaflet of the plasma membrane, binds to RBD of Raf-1, but forms also secondary interactions with CRD.

This binding recruits Raf-1 to the cellular membrane, but it is insufficient for the full activation of Raf-1. There are four Ras proteins in humans (H-Ras, N-Ras, K-RasA and K-RasB) and there are clear differences in their binding affinities to the RBDs: transient transfection of oncogenic Ha-Ras leads to a preferential activation of endogenous c-Raf in HEK 293 cells as opposed to A-Raf (Weber, et al., 2000). CR1 and CR2-deleted Raf-1 resulted in a costitutively active form of the kinase, demonstrating that in the absence of stimuli inactive Raf-1 has the catalytic domain folded and bound to the N-terminal half regulatory domain (Stanton, et al., 1989). This interaction is stabilized by the binding of a 14-3-3, a dimeric adaptor/scaffold protein, to CR2 of Raf-1 when S259 and S621 are phosphorylated, interfering with binding to Ras. Following growth-factor stimulation, Ras-GTP interferes with 14-3-3 and enforces conformational changes in Raf-1 necessary for its stable activation and for exposing docking site to MEK (Terai and Matsuda, 2005; Tzivion and Avruch, 2002; Wellbrock, et al., 2004). Although Ras-GTP can distrupt 14-3-3 binding in vitro, 14-3-3 displacement needs dephosphorylation of S259 by protein phosphatase-2A (PP2A) in the cells (Jaumot and Hancock, 2001).

Is a common thinking that Raf-1 undergoes a series of phosphorylation and dephosphorylation events after Ras recruitment that results in an stably active form, in a B-Raf different manner (Wellbrock, et al., 2004). In Raf-1 are known both negative phosphorylation, that suppress Raf-1 activity, and positive phosphorylation that maintain the active Raf-1 conformation. In resting cells, inactive C-Raf is phosphorylated at S43, S259, S621 but several other sites remain to be identified (Leicht, et al., 2007).

As noted above, S259 and S621 phosphorylation serve to bind 14-3-3, stabilizing the basal inactive Raf-1 conformation; S43, S233 and S259 are thought to be a negative regulatory site targeted by PKA, a cyclic-AMP-dependent kinase; S259 is also phosphorylated by AKT/protein kinase B (AKT/PKB) (Dumaz and Marais, 2003; Tzivion, et al., 1998). Accordingly, phosphorylation of S259 by AKT or PKA was shown to negatively regulated Raf-1, whereas its dephosphorylation by protein phosphatase 2A has been reported to be part of the Raf-1 activation mechanism (Balan, et al., 2006). Also S43 phosphorylation seems to sterically hinder binding of the N-terminus of Raf-1 to Ras, but the regulation of Raf-1 by PKA is highly complex and not fully understood: what is sure is that cells are determined to inhibit Raf-1 when cAMP levels are elevated.

Following cell stimulation by growth factors, Raf-1 goes through an activation intermediated state that is stabilized by several positive phosphorylation. To date, the major identified catalytically significant, growth factor-induced C-Raf phosphorylation sites are S338/339 and Y340/341, located at the N-terminal side of CR3 in a subdomain called N-region for several negative charges. SRC and SRC-family kinases seem to phosphorylate Y341 in vitro and in cell culture while there is disagreement about the kinase or kinases that phosphorylate S338 (Marais, et al., 1995). Interestingly, the S338/339 sites are conserved among the Raf proteins (Fig.1), with the exception of A-Raf that has a Glycine at corresponding S339 and Lin-45 that has two Hystidines. In B-Raf the phosphorylation of S445, which corresponds to S338 in Raf-1, is largely constitutive and so does not actively contribute to relief from autoinhibition of the kinase. The YY340/341 are more variable residues among the Raf isoforms: B-Raf, D-Raf and Lin-45, have charged residues as Glu and Asp at their corresponding sites (in B-Raf is D448). For this reason, these kinases require less activation-related modification for full activation respect to Raf-1 and A-Raf (Tran, et al., 2005). It means that B-Raf has strongly elevated basal kinase activity compared to Raf-1 and for its full activation requires only Ras-mediated membrane recruitment.

In literature there is a strongly disagree about the kinase that phosphorylates S338 in Raf-1. One of the suggestions is that it is mediated by Pak3 (King, et al., 1998). Pak proteins are serine/threonine specific kinases that bind and are activated by a membrane-bound Ras-related GTPases Cdc42 and by RAC; has been proposed that Pak3 phosphorylated Raf-1 at S338 and stimulated its activation in a RAC/Cdc42 and phosphatidylinositol-3 kinase (PI3K)-dependent manner (Sun, et al., 2000). Effectively, Pak kinases can phosphorylate Raf-1 at S338 in vitro, but experiments using PI3K inhibitors in vivo (Ly294002 and Wortmannin), did not block S338 phosphorylation in COS-7 cells stimulated by EGF. Importantly, it is well established that S338 phosphorylation Ras- and growth factor-mediated occurs at the plasma membrane in a Ras/Src dependent manner, whereas Pak3 phosphorylates Raf-1 in the cytosol and recruits Raf-1 to the membrane in a Ras independent manner. Furthermore, dominat-negative vectors of Pak3 and Cdc42, that are catalitically inactive, do not block S338 phosphorylation and C-Raf activation EGF-mediated (Chiloeches, et al., 2001).

Taken together, these data strongly oppose the model that Pak kinases can stimulate S338 phosphorylation in a growth factor- or Ras- dependent manner and it is a common view that the Ras-dependent S338 kinase is still to be identified (Wellbrock, et al., 2004).

Importantly, the dephosphorylation of S259 is a prerequisite for the further activation of Raf-1, and the phosphorylation of the key activating residue S338 occurs after S259 dephosphorylation. These two residues are mutually exclusive, as upon stimulation by EGF, S259 phosphorylation decreases and S338 phosphorylation appears (Dhillon, et al., 2007).

Other two phosphorylation are essential for Raf-1 activation: T491 and T494 that are within the kinase domain in a region called *activation segment*. Their mutation blocked Raf-1 activation, but the kinases that mediate their phosphorylation are still to be identified (Wellbrock, et al., 2004). All the principal phosphorylation sites and respectively kinases targeting Raf-1 are schematized in Fig. 1.2.

Interestingly, Raf kinase form both omodimers and heterodimers and it is also regulated through association with chaperones such as HSP-90/p50 and HSP/HSP-70 and with various scaffold proteins such as RKIP (Raf kinases inhibitory protein) (Leicht, et al., 2007). MEK 1/2 is not the only one Raf target: mammalian Raf proteins might have other effectors. One of these is the transcription factor nuclear factor (NF)-kB: C-Raf seems to be able to activate NF-kB but the mechanism is unclear (Wellbrock, et al., 2004).

The mechanism(s) responsible for Raf-1 inactivation is much less understood, although the common view is that Raf-1 dephosphorylation should play a key role in this process (Balan, et al., 2006). The inactivated state of Raf-1 is re-established by the coordinated action of Pin1, a prolyl isomerise that converts pSer and pThr residues from the cis to the trans conformation, which is preferentially recognized and dephosphorylated by PP2A (Baccarini, 2005).

Raf isoforms have also different cellular expression and different sub cellular localization. Morice et al showed that B-Raf and Raf-1 proteins are present in most brain areas, whereas A-Raf is not detected: Raf-1 is localized mainly in the cytosolic fraction around the nucleus, whereas B-Raf is widely distributed in the cell bodies and in the

neuritic processes (Morice, et al., 1999). Finally, Raf-1 translocates to mitochondria and phosphorylates BAD and inhibits BAD-Bcl-2 complex in a Pak1 dependent manner, demonstrating that Raf-1 has also an antiapoptotic role (Jin, et al., 2005).

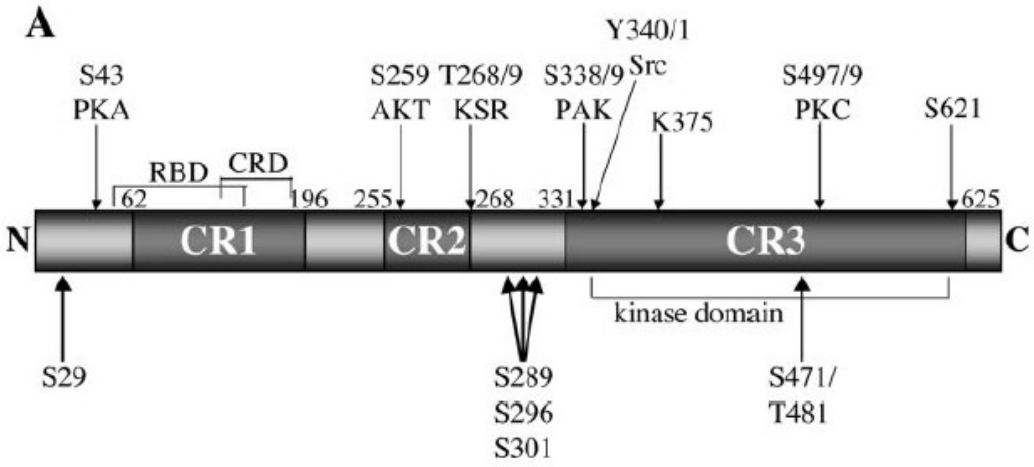

Fig. 1.2 | **Structure and known Raf-1 phosphorylation sites.**

Diagram depicting known Raf-1 phosphorylation sites and potential kinases reported to phosphorylation these sites. RBD: Ras binding domain; CRD: cysteine-rich domain; CR1-3: conserved region 1-3 in Raf family: K375: ATP binding site (Balan, et al., 2006).

1.2 The Ca^{2+}/Calmodulin dependent Kinases (CaMKs)

Calcium (Ca^{2+}) is an important intracellular second messenger in several processes, such as growth factor and hormone signalling, cell cycle regulation, gene expression and apoptosis. Resting cells have an intracellular Ca^{2+} level of 10^{-7} M, which is 10^4 times lower than the level outside the cells. The cells have an intricate network by which control the cytoplasmatic Ca^{2+} levels: ATP-dependent Ca^{2+} pumps, the endoplasmic reticulum (ER) and the extracellular space. Various signals can stimulate Ca^{2+} intracellular increase. Receptors tyrosine kinase and G-protein-coupled receptors can induce Ca^{2+} release from the ER by producing IP3, while ligand gated ion channels and voltage-dependent ion channels in the plasma membrane can initiate Ca^{2+} entry from extracellular stores (Hook and Means, 2001).

One of the key proteins that binds Ca^{2+} in the cells is Calmodulin (CaM). CaM is a small, highly conserved Ca^{2+} sensor ubiquitously expressed in mammalian cells (Bito, 1998). Ca^{2+} ions bind to CaM by each of the 4 helix-loop-helix protein folding motifs called EF hands. When the four binding sites are filled, CaM undergoes to a conformational change that leads to the exposition of a flexible eight-turn α helix, which separates the hydrophobic pockets present at each globural end of the protein. In this way, CaM is activated and capable to interact with one of its many target protein in the cells. CaM-substrate binding is at highly affinity and reversible when Ca^{2+} concentration return to basal level (Means, 2000).

One action of CaM is to activate members of a family of Serine/Threonine protein kinases called Ca^{2+}/CaM-dependent kinases or CaMKs. This family includes kinases having a single substrate, such as myosin-light chain kinase (MLCK), or kinases having multiple substrate. These proteins are: CaMKI, CaMKII and CaMKIV, kinases with similar domain structures and properties (Braun and Schulman, 1995).

The multifunctional CaMKs differ for : tissue distribution, subcellular localization, subunit composition and known requirements for complete activation. Thus, CaMKI and CaMKII are both ubiquitous expressed, while CaMKIV is tissue-specific, expressed mainly in brain, T-cells, timus, testis, ovary, bone marrow and adrenal gland (Wang, et al., 2001). While CaMKI and CaMKII seem to be predominantly cytoplasmic, except some isoforms of CaMKII that can be nuclear, CaMKIV should be nuclear and

cytoplasmic (Hook and Means, 2001). About the structure, CaMKI and CaMKIV are monomeric enzyme, while CaMKII is composed by more subunits and it is the most well characterized and interesting of the multifunctional CaMKs.

1.2.1 CaMKII regulation

CaMKII is encoded by 4 separate genes that for alternate splicing produce 4 isoforms of CaMKII: α, β, γ and δ. Every cell type has at least one isoform of CaMKII. It is a holoenzyme complex composed of 10-12 subunits : every subunit is assembled to each other thanks to a C-terminal association domain forming a pair of hexameric stacked rings. (See Fig. 1.3) (Anderson, et al.). The general structure of every subunit of the CaMKs is a 50/60 KDa polypeptide characterized by: an N-terminal catalytic domain, followed by an autoinhibitory domain containing the binding site for Ca^{2+}/CaM complex and finally, in the case of CaMKII, a C-terminal association region (Fig. 1.3). Studied of mutated or truncated enzymes, indentified the minimum autoinhibitory domain of the CaMKs. The truncation at residue 294 of CaMKI produces a protein that is constitutively active: thus removing 295-299 of CaMKI generates an active enzyme, indicating that these few aminoacids are sufficient for the autoregolation (Hook and Means, 2001). At resting calcium levels, the autoinhibitory portion interact with the catalytic domain keeping the enzyme inactive (Cruzalegui, et al., 1992). CaMKs can rapidly sense elevation of intracellular Ca^{2+} levels binding Ca^{2+}/CaM complex: this binding causes a conformational change that allows access of substrate and ATP to the catalytic domain. CaMKII activation requires that Ca^{2+}/CaM binds to regulatory domains of two neighbour subunits. These interactions, lead to the activation of one catalytic domain and to the phosphorylation of residue Thr 286/287 (the precise numbering varies according to isoform) of the adjacent regulatory domain, until each of 12 subunits are activated (Miller and Kennedy, 1986).

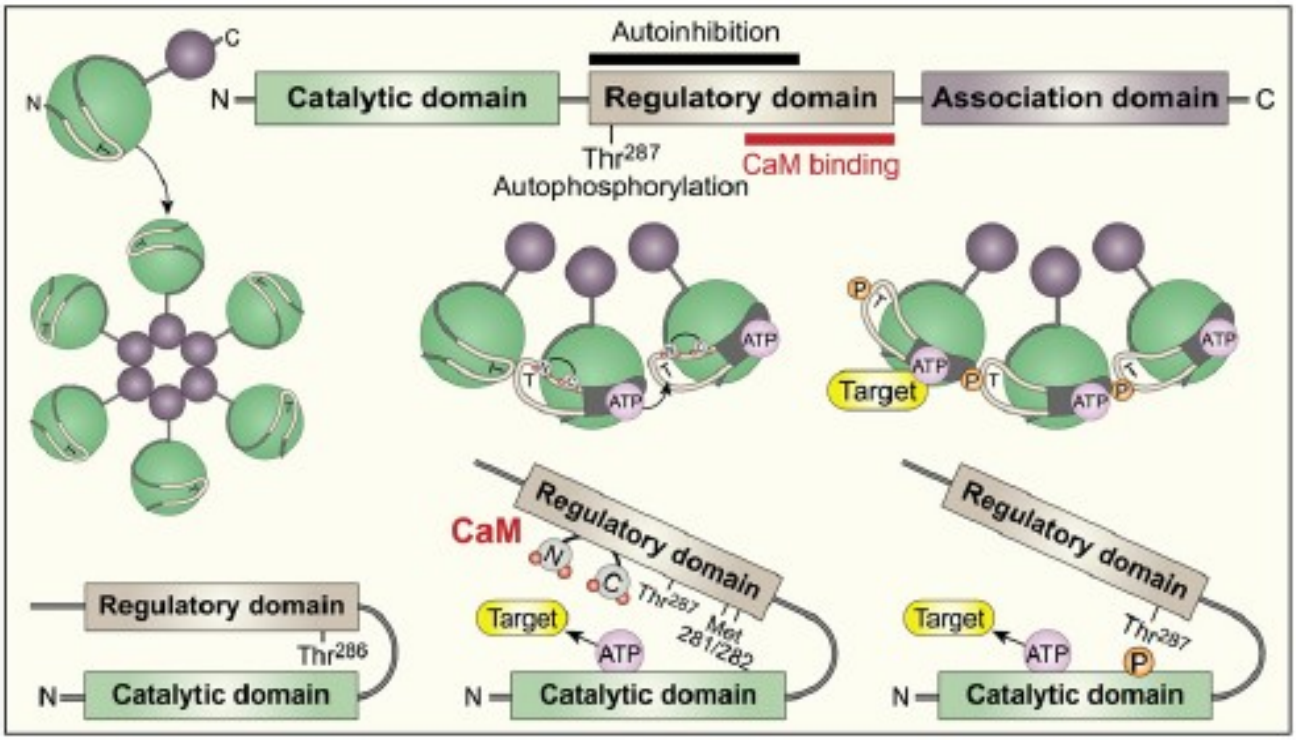

Fig. 1.3 | **Ca^{2+}/CaM-dependent Kinase II structural domains and activation.**

CaMKII monomers consist of an N terminal catalytic domain and a C terminal association domain that bound a regulatory domain (top). The association domains (maroon circles) are required for assembly of the CaMKII monomers into the holoenzyme (middle panels). Under resting conditions the catalytic domain is constrained by the regulatory domain (left middle and bottom panels). After intracellular Ca2+ rises and complexes with calmodulin (CaM) the Ca2+/CaM binds to the C terminal portion of the CaMKII regulatory domain (mid portion of the top, middle and bottom panels) to prevent autoinhibition of the regulatory domain on the catalytic domain, activating CaMKII. With sustained Ca2+/CaM or increased oxidation, CaMKII transitions into a Ca2+/CaM-autonomous active enzyme after autophosphorylation (at Thr 287) or oxidation (at Met281/282) of amino acids in the regulatory domain (Anderson, et al.).

The event of *autotransphosphorylation* has two important consequences: first, the affinity of the enzyme for Ca^{2+}/CaM is increased by a factor of 10^5 in a process called "CaM trapping", because CaM remains "trapped" by the phosphorylation and delays dissociating. Second, the autoinhibitory domain is further disrupted making CaMKII partially independent of Ca^{2+}/CaM and "autonomous" (Hudmon and Schulman, 2002). This means that CaMKII is able to prolong its activity after Ca^{2+} levels have dropped below the activation threshold. To return to an inactive state, dephosphorylation of CaM-KII must occur. Both protein phosphatases 1 and 2A (PP1 and PP2A) can effectively dephosphorylate CaMKII in vitro, and both enzymes appear to play important physiological roles in a subcellular localization of CaMKII-dependent manner (Means, 2000).

The activity of CaMKI and CaMKIV is also modulated by phosphorylation. However, in contrast of CaMKII, the regulatory phosphorylation events are catalysed by a distinct group of kinases called CaM kinases kinases (CaMKKs) (Soderling, 1999). CaMKKs themselves are Ca^{2+}/CaM dependent enzymes. They phosphorylate Ca^{2+}/CaM-bound CaMKI and CaMKIV on a Thr residue located within the activation loop. These phosphorylation events cause the activity of CaMKI and CaMKIV to increase several folds (Chatila, et al., 1996).

CaMKII has a broad range of biological cellular functions: neuronal functions but also regulation of gene expression, regulation of cell cycle and proliferation.

CaMKII is one of the most abundant proteins in the brain, comprising 1% of the total proteins in the forebrain and 2% in the hippocampus, a region associated to memory (Cruzalegui and Bading, 2000). Some studies evidenced that CaMKII autonomous activity is important in such neuronal processes as long-term potentiation (LTP) and long-term depression (LTD). CaMKII seems to be implicated in several pre- and post-synaptic events. Among the presynaptic functions: neurotransmitters synthesis, neurotransmitters secretion, microtubule disassembly; and for the post-synaptic, phosphorylation of a number of proteins and kinases in the post-synaptic density of downstream neurons (Hook and Means, 2001). CaMKIIα, the first isoform identified, is a major component of the postsynaptic membrane (PSD) in pyramidal neurons. In PSD, CaMKII is thought to increase synaptic strength by phosphorylating ion channels and

signalling proteins such as glutamate receptors and N-methyl D-aspartate (NMDA) receptors (Cruzalegui and Bading, 2000).

The literature reveals that CaMKI is predominantly cytoplasmatic while some isoforms of CaMKII and CaMKIV could have also a nuclear localization, owing to a nuclear localization signal (NLS) within their association domain. Because all the CaMKs have quite similar substrate specificity determinants, it is not surprising that they sometimes phosphorylate the same proteins. One of these is the cAMP-response element binding protein, CREB (Sheng, et al., 1991). Thus, CaMKII and CaMKIV can phosphorylate the trascriptor factor CREB in the nucleus and can mediate the expression of genes regulated by CRE such as c-fos. Phosphorylation of CREB on Ser133 is essential for its activation because it is required for binding of CREB binding protein (CBO) and p300, which function is transcriptional integrator (De Cesare, et al., 1999). Protein kinase A (PKA) was originally identified as the kinase that phosphorylates CREB in Ser 133; however also CaMKII can phosphorylate CREB on Ser 133, but it does not induce CRE-mediated transcription. In addiction, CaMKII seems to inhibit CREB in some cell types, phosphorylating the transcriptor factor on a second site, Ser 142. the mechanism by which phosphorylation on Ser 142 inhibits CREB-mediated transcription seems to be by destabilizing the association between CREB and CBP (Parker, et al., 1998). Interestingly, CaMKIV can phosphorylate CREB on Ser 133 and consequently, can markedly stimulate CRE-mediated transcription; while has been demonstrated that also CaMKI is able to phosphorylate Ser 133 in vitro, but its phosphorylation remains controversial in vivo because CaMKI has never be found in the nucleus (Hook and Means, 2001).

The role of CaMKII in the regulation of cell cycle mechanisms and in cell proliferation, has been well demonstrated. In mammalian fibroblast, CaMKII mediates G1 phase progression in a Ca^{2+}/CaM dependent manner, mediating Cyclin D1 levels (Morris, et al., 1998). More recent works, show that CaMKII regulates cell proliferation in different cell types.

1.2.2 CaMKII in the MAPK pathway

Recent works demonstrated that Ras-ERK pathway, activated upon several stimuli, is controlled by CaMKII-mediated Ca^{2+} signalling through the regulation of Raf-1 activity. Illario et al. showed that Fibronectin (FN) binding to integrin in thyroid cells TAD-2, activates the Ras/Raf/MEK/ERK pathway, through the formation of FAK/Grb-2/Sos complex, and also generates an increase of Ca^{2+} intracellular level that leads to a Ca^{2+}/CaMKII signal. Binding to FN induced Raf-1 and CaMKII to form a protein complex, indicating that intersection between Ras/Raf/Mek/Erk and Ca^{2+}/CaMKII signalling pathways occurred at Raf-1 level. Immunoprecipitation experiments demonstrated that Ca^{2+}/CaMKII signal is necessary for ERK activation and interruption of the pathway using pharmacological inhibitors of CaMKII (KN-93 and ant-CaNtide), arrested cell proliferation induced by FN in thyroid cells (Illario, et al., 2003; Illario, et al., 2005). The cross talk between CaMKII and MAPK pathway, has been well demonstrated also in L6 skeletal muscle cells, following insulin stimulation. Insulin-activated CaMKII associates to Raf-1 and it is necessary for DNA synthesis-ERK dependent and for the attenuation of AKT activation, demonstrating the role of CaMKII also in the selective control of insulin signalling (Illario, et al., 2009).

Moreover, CaMKII has been found constitutively activated in absence of any stimulation in primary cultures of papillary thyroid carcinomas (PTC) and in PTC cell lines harboring the oncogenes RET/PTC-1 or BRafV600E. Rusciano et al. showed that the expression of recombinant RET/PTC-3, BRafV600E or RasV12 in COS-7 cells, induced CaMKII activation, in a Phospholipase C/Ca^{2+} dependent manner. In the PTC cell line TPC-1, harboring RET/PTC-1, CaMKII inhibitors attenuated ERK activation and DNA synthesis, suggesting that CaMKII is a component of the ERK signal cascade in this cell line. Taken together, these data demonstrate a new role of CaMKII in the modulation of tumor cell proliferation and that the PLC/CaMKII pathway could therefore provide appropriate targets for therapeutic intervention of tumors harboring RET/PTC.

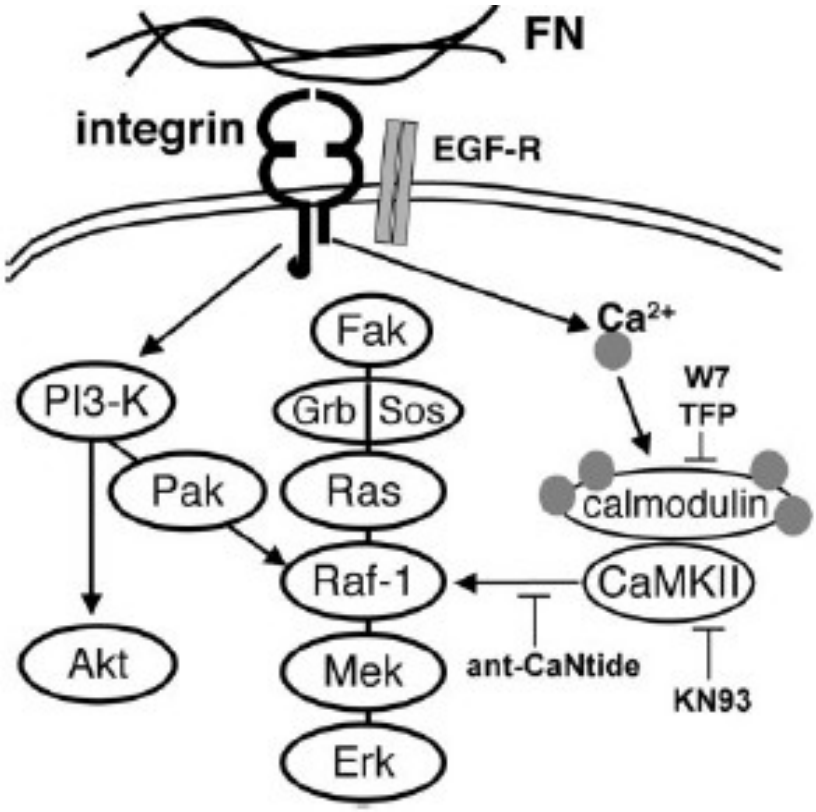

Fig. 1.4 | **Integrin-mediated signalling pathways.**

Integrin activation promotes three signaling pathways: Ras/Raf-1/Mek/Erk, PI3-K/Akt, and Ca^{2+}/CaMKII. The last is necessary to Raf-1 to activate Mek, thus inducing ERK activation. Ca^{2+}/CaMKII signal is necessary but not sufficient for Raf-1 activation (Illario, et al., 2003).

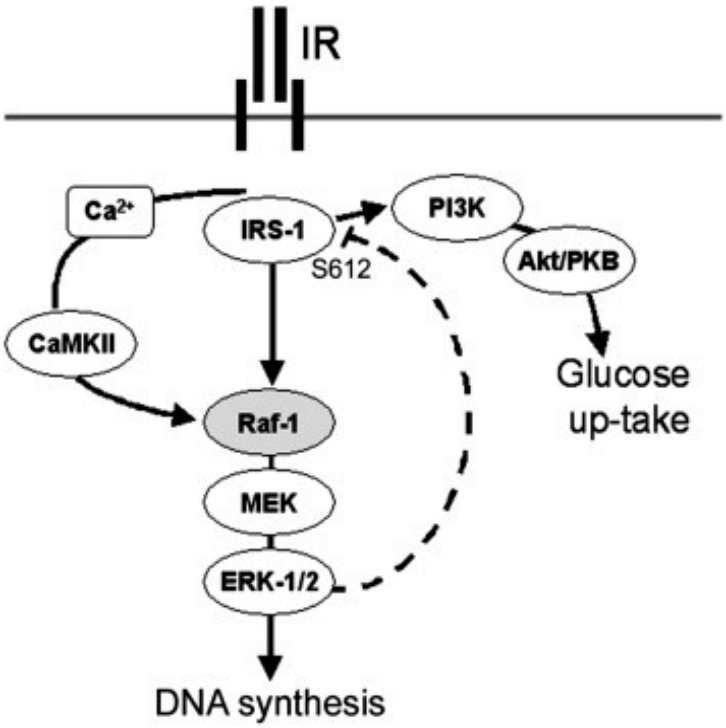

Fig. 1.5 | **Schematic diagram of the insulin receptor signalling in L6 cells**.

Activation of the insulin receptor generates the IRS-1/2→PI3-K→Akt signalling pathway and promotes glucose uptake. Insulin receptor activation generates two other signals: [Ca2+]i→CaMKII and IRS-1/2→Erk-1/2. They both participate to Raf-1 activation, leading to stimulation of cell proliferation (Illario, et al., 2009).

1.3 Phosphorylation of Raf-1 at Serine 338 and Tyrosine 341

As described above, Raf-1 is normally located in the cytosol in an inactive state and its activation counts a complex series of events. Ras-dependent recruitment to the plasma membrane is the first event that leads to Raf activation. EGF treatment or oncogenic Ras expression by plasmid transfection can induce the formation of the Ras/Raf complex in several cellular models. In order to complete Raf-1 activation, following Ras/Raf association, two sequential important modifications have to occur at the plasma membrane. The first modification is the phosphorylation of Tyr341 by membrane-bound Tyr341 kinases, whose activities are induced by EGF and/or Ras. This phosphorylation may relocalize the Ras/Raf complex within specialized plasma membrane microdomains where the second event of phosphorylation, on Ser338, can occur. Ser338 phosphorylation leads Raf-1 competent to phosphorylate downstream effectors like MEK/ERK (Carey, et al., 2003).

Mason et al. demonstrated three topic events for Raf-1 activation: phosphorylation on Ser338 and Tyr341 are both necessary to full Raf-1 activity by EGF stimulation; both phosphorylations require Ras/Raf-1 interaction and its membrane localization; Ras mediates predominantly S338 phosphorylation and Src gives predominantly Tyr341 phosphorylation. Both sites must be phosphorylated for Raf-1 full activity, because they cooperate to activate the kinase. In contrast, the regulation of B-Raf is rather different. S445 (the B-Raf equivalent of Ser338) is constitutively phosphorylated and this phosphorylation with the aspartic acids at 447/448 (the equivalent of Tyr340/341) contributes to the high basal kinase activity of B-Raf (Mason, et al., 1999).

Many studies have demonstrated that members of the Pak family (p21 activated kinase) act as molecular linkers that couple signalling events between Ras and the Rho GTPase family. Pak1, -2 and -3 are cytosolic serine/threonine-specific protein kinases that are activated by direct binding to the small G proteins Cdc42 and Rac. Like Raf-1 activation, Pak activation is very complex and involves membrane recruitment, phosphorylation and dimerization. Paks are implicated in a number of biological processes such as cytosckeletal reorganization, cell cycle progression and apoptosis (Chiloeches, et al., 2001). Evidences suggestes that Pak1 mediates RasV12-induced cell transformation in Rat-1 cell lines (Tang, et al., 1997). King *et al.* first identified Pak2 as

an important kinase responsible for the phosphorylation of Ser338, thus involved in Raf activation. Pak1 and 2 have also been shown to be necessary for the activation of Raf-1 by RasV12 (King, et al., 1998), by an active mutant of phosphatidylinositol 3-kinase (Sun, et al., 2000) and by integrins (Chaudhary, et al., 2000). Nocodazole activates Pak and consequently causes Ser338 phosphorylation and Raf-1 activation, suggesting the existence of a signal generated by changes in microtubule dynamics (Zang, et al., 2001). Another study by Li *et al.* has also positioned Rac/Cdc42 and Pak in the RasV12-induced Raf activation pathway (Li, et al., 2001).

Although an increasing number of studies support that Pak regulates Ser338 phosphorylation in a Rac/Cdc42/PI3K-regulated manner, a dissenting study does not agree with this notion (Zang, et al., 2002). This careful study demonstrated that the PI3K inhibitors LY294002 and Wortmannin, at concentrations that block PI3K activity, do not suppress the EGF-mediated Ser338 phosphorylation (Chiloeches, et al., 2001). In the same study, at higher concentrations, Ser338 phosphorylation was suppressed, but so was also Ras activity. Moreover, Pak3 activated mutant could induce Ser338 phosphorylation but not Raf-1 activity and this phosphorylation occurred in the cytosol and not at the plasma membrane. Thus, taking together these data, the authors conclusion was that the role of PI3K and Pak3 in mediating Ser338 phosphorylation by Ras is not physiological and the kinase that phosphorylates Raf-1 on Ser338 and participates to ERK activation remained to be indentified (Chiloeches, et al., 2001).

Phosphorylation of S338 serves multiple roles. It maintains Raf-1 in its activated state and regulate the translocation of Raf-1 to the mitocondria. In addition has been reported that S338 phosphorylation regulates the binding of two negative regulators as RKIP and protein phosphatise 5 (PP5). Unlike most other serine/threonine phosphatises, PP5 showed remarkable substrate specificity in regard to Raf-1; in fact, both in vivo and in vitro, PP5 only dephosphorylates S338 but not other Raf-1 phosphorylation sites (Dhillon, et al., 2007).

2. AIMS OF THE STUDY

It has been previously demonstrated that several factors, such as integrin activation and insulin stimulation, activate CaMKII in different cell types including thyroid cells, rat mioblasts (L6) or human fibroblasts. In primary PTC cultures and in PTC cell lines harboring the oncogenes RET/PTC-1, CaMKII has been found activated in a PLC/Ca^{2+} dependent manner, also in the absence of external stimuli. In these models, CaMKII binds Raf-1 and this complex is necessary for ERK activation and modulates Ras/Raf/MEK/ERK pathway and cellular proliferation (Illario, et al., 2003; Illario, et al., 2005; Illario, et al., 2009; Rusciano, et al.).

The aim of this doctoral thesis is to go more insight into the molecular mechanisms of CaMKII/Raf-1 interaction and to verify the hypothesis that CaMKII phosphorylates Raf-1 at Ser338.

I asked few specific questions:

1) how the role of CaMKII in Raf-1/ERK signal is extended or is restricted to some cell contexts;

2) which is the role of CaMKII in Raf-1 activation by oncogenic Ras;

3) if CaMKII phosphorylates Raf-1 at Ser338 upon oncogenic Ras and other stimuli.

3. RESULTS

3.1 CaMKII is phosphorylated at Thr286 by RasV12 in a Ca^{2+}/CaM dependent manner

To evaluate whether oncogenic Ras stimulates CaMKII phosphorylation at Thr286, NIH3T3 cells were transiently transfected with 2 μg of vectors encoding oncogenic H-RasV12 and K-RasV12 isoforms. After 24 hours from transfection, the cells were starved from serum for 24 hours and treated for 30 minutes with the calmodulin inhibitors W7 or TFP. CaMKII phosphorylation at Thr286 was visualized by Western Blotting using phospho-specific antibody. Ionomycin, a potent calcium ionofore, and fetal calf serum (FCS) treatment were used as controls for maximal CaMKII activation.
In starved or not transfected NIH3T3, pT286-CaMKII was not visible. Both the oncogenic Ras isoforms induced CaMKII phosphorylation at Thr286 and treatment with W7 or TFP reduced this phosphorylation, demonstrating that CaMKII was phosphorylated by oncogenic Ras by a Ca^{2+}/CaM signal (Fig. 3.1).

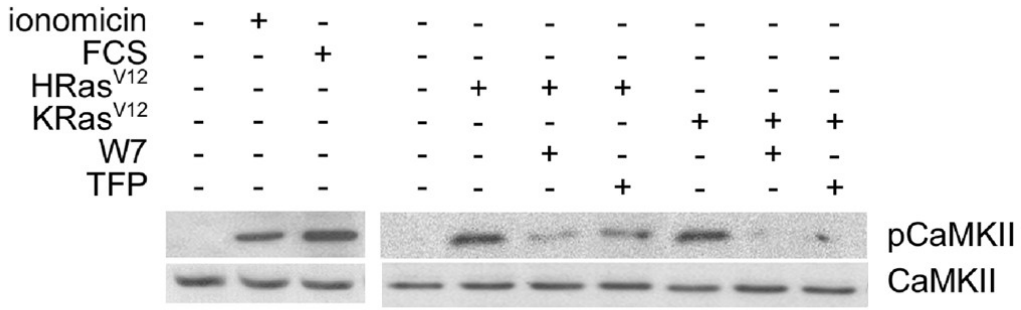

Fig. 3.1 | **CaMKII is phosphorylated by RasV12 in a Ca^{2+}/CaM dependent manner**

NIH3T3 cells were starved from serum for 24 hours and treated with Ionomycin, FCS or transiently transfected with 2 µg of plasmid encoding oncogenic Ras isoforms, HRasV12 and KRasV12. Where indicated the cells were treated for 30 minutes with 30 µM W7 or 50 nM TFP; pT286-CaMKII and total CaMKII were visualized by Western Blotting.

3.2 CaMKII is activated by RasV12

To determine whether oncogenic Ras stimulates CaMKII activation, I performed a CaMKII activity assay in vitro in NIH3T3 stably transfected with KRasV12 vector. In a first reaction step, immunoprecipitated CaMKII was incubated with CaCl2, CaM and cold ATP. In a second reaction step an aliquot from the first reaction was incubated with CaMKII peptidic substrate Autocamtide, EGTA and [γ32P]ATP. The reaction mixture was spotted onto p81 phosphocellulose filters and the level of [32P] incorporation into Autocamtide was determined by liquid scintillation counting.

NIH3T3 were starved and then treated with ionomycin 2 μM for 15 minutes as positive control; stably clones expressing KRasV12, were starved for 24 hours and treated with CaMKs inhibitor (KN93) 10 μM for 30 minutes and with Ras inhibitor (Lovastatin) 5 μM for 24 hours. Stimulation of CaMKII activity by ionomicin was evident in NIH3T3 cells (Fig. 3.2). Stable RasV12 expression induced a comparable CaMKII activity. The specificity of stimulation was demonstrated by the inhibitory effect of both KN93 and lovastatin. This experiment demonstrates that KRasV12 is able to fully activate CaMKII.

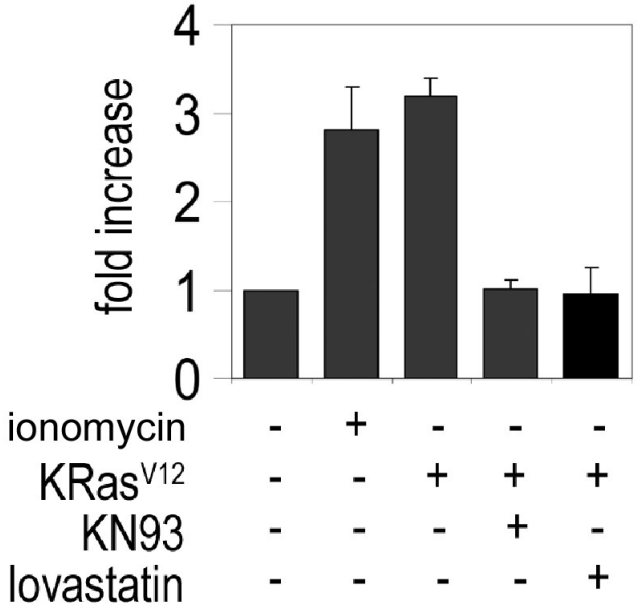

Fig. 3.2 | **RasV12 activates CaMKII.**

NIH3T3 cells and NIH3T3 stably expressing KRasV12 were starved and treated with 2 μM ionomycin, 10 μM KN93 and 5 μM lovastatin as indicated. CaMKII activity were evaluated by *in vitro* kinase activity assay. The reactions were performed as described in *Materials and methods* section. The results are presented as fold increase of incorporated cpm. Data are reported as the mean +/- standard deviation from duplicate experimental points.

3.3 CaMKII is necessary for ERK phosphorylation by RasV12

A cross-talk between the Ca^{2+}/CaMKII and ERK-1/2 pathways was previously demonstrated in epithelial cells (thyroid cells) and in mesenchimal cells (myotubes and fibroblasts) stimulated by diverse factors (integrins and insulin) (Illario, et al., 2003; Illario, et al., 2005; Illario, et al., 2009). In order to investigate the possible existence of a similar cross-talk when the MAPK pathway is triggered by oncogenic Ras, I tested the effects of CaMKII specific inhibitory peptide, AntCaNtide, on ERK-1/2 phosphorylation in NIH3T3 transiently transfected with K-RasV12.

NIH3T3 cells were starved from serum, pretreated with AntCaNtide and then stimulated by FCS. Pretreatment with AntCaNtide was performed at increasing concentration (1-2-5 μM) for 30 minutes, and the levels of ERK-1/2 phosphorylation were evaluated by Western Blotting after additional 30 min of FCS stimulation.

As it shown in Fig. 3.3, AntCaNtide (ant) treatment reduced FCS-induced ERK phosphorylation in a dose dependent manner. In parallel, RasV12 expression was induced by plasmid transfection in NIH3T3 cells, and treated as above. Inhibition of ERK phosphorylation upon AntCaNtide treatment was evident. These data demonstrate that ERK-1/2 activation by FCS and oncogenic Ras, requires active CaMKII in NIH3T3 and that the cross-talk between Ca^{2+}/CaMKII and ERK-1/2 pathway may be considered a general mechanism as it is present in diverse cell types (thyroid cells, fibroblasts, myocites, NIH3T3 cells) stimulated by diverse factors (serum, fibronectin, insulin, RasV12).

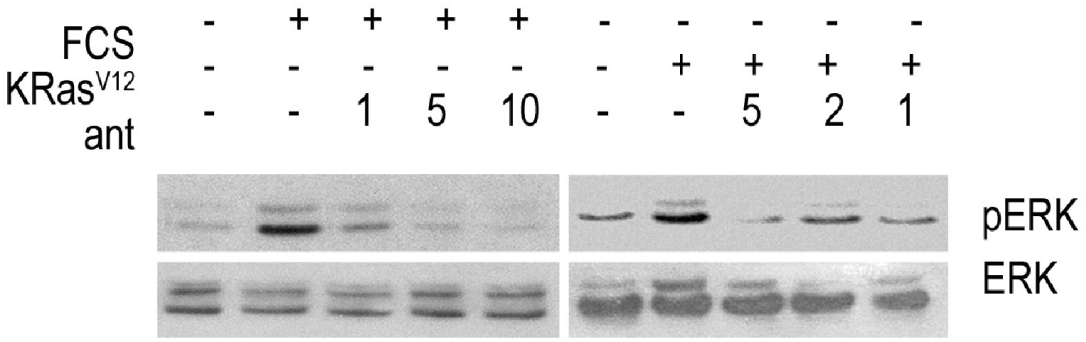

Fig. 3.3 | **CaMKII is necessary for ERK phosphorylation by RasV12**

Serum starved NIH3T3 were pretreated with AntCaNtide at indicated concentration and then stimulated with FCS for 30 min (left). Alternatively, the cells were transiently transfected with a RasV12 expressing plasmid and the treated with AntCaNtide for 30 min. The amount of total ERK1/2 (ERK1/2) and phosphorylated ERK1/2 (p-ERK1/2) was determined by Western Blotting.

3.4 Raf-1 activation by RasV12 is CaMKII mediated

It has previously been demonstrated that Raf-1 activation by fibronectin (FN) and insulin is Ras- and CaMKII- mediated. I ment now to investigate whether CaMKII is necessary for Raf-1 activation by oncogenic Ras. Because most of the studies on Raf-1 activation mechanisms have been performed in COS-7 cells, all experiments have been performed in this cell line. I first evaluated the ability of oncogenic Ras and Src to activate CaMKII in COS-7 cells. The cells were transiently transfected with expression vectors for RasV12 and SrcY527. CaMKII activity was determined by *in vitro* activity assay of immunoprecipitated CaMKII, as described in material and methods. Oncogenic Ras and Src both activate CaMKII, with similar intensity (Fig. 3.4A). Ionomycin 2 μM and KN93 10 μM treatments, were used as positive and negative controls.

In the literature it has been reported that oncogenic Ras and oncogenic Src cooperate for serine and tyrosine phosphoryations of Raf-1 and mediate its full activation (Mason, et al., 1999). I determined whether activated Ras and Src stimulated Raf-1 activation in a CaMKII dependent manner (Fig. 3.4B). COS-7 cells were transiently transfected with expression vectors for RasV12, SrcY527 and CaMKII dominant negative mutant (CaMKIIdn). CaMKIIdn is mutated in K42M that leads to a catalytically inactive kinase that competes with the wild-type kinase for auto-activation and with substrate. Raf-1 activation was determined by immunoprecipation of Raf-1 and *in vitro* kinase assay. A modest Raf-1 activation was induced by SrcY527 alone, while RasV12 alone was more efficient. Maximal Raf-1 activation was achieved by oncogenic cooperation. CaMKII inhibition by CaMKIIdn reduced of about 50% Raf-1 activation by oncogenes, both RasV12 alone and RasV12 plus SrcY527.

These data confirmed that both RasV12 and SrcY527 activate Raf-1 and cooperate to the maximal stimulation. Inhibition experiments demonstrated that Raf-1 activation is CaMKII dependent.

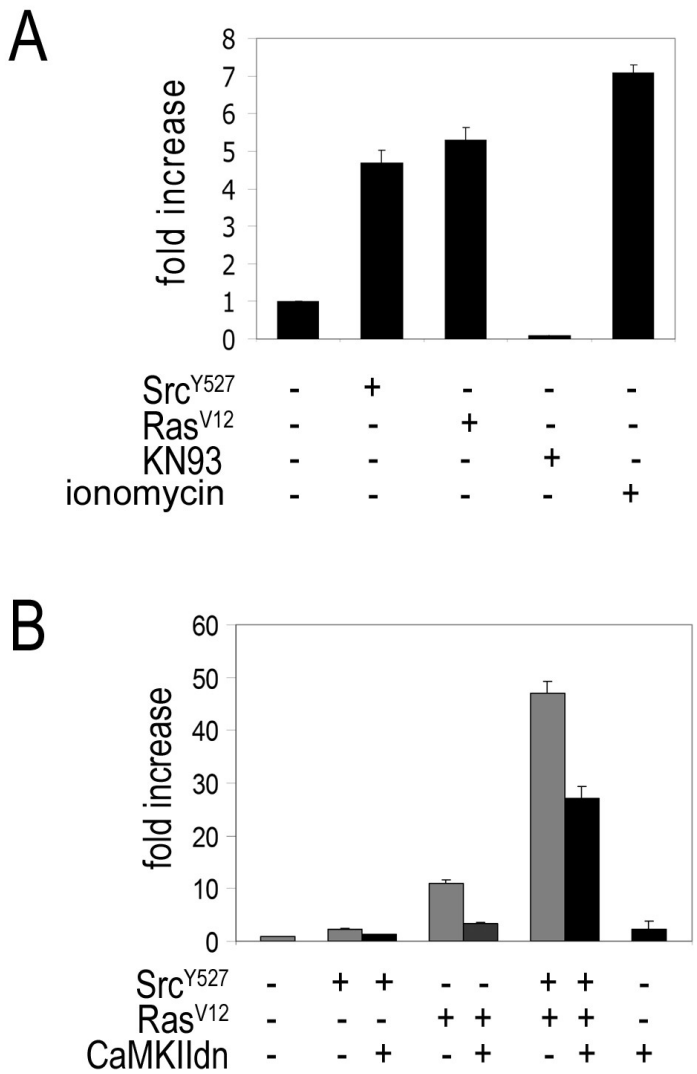

Fig. 3.4 | **Raf-1 activation by RasV12 is CaMKII mediated.**

A) CaMKII activity assay. COS-7 cells were transiently transfected with SrcY527 5 µg or RasV12 2 µg for 48 hours and starved from the serum for 24 hours. CaMKII activity was determined by kinase activity assay, as described in *Material and methods section.* Ionomycin 2 µM 10 minutes and KN93 10 µM 30 minutes, were used as positive and negative control.

B) Raf-1 activity assay. RasV12 2 µg, SrcY527 5 µg and CaMKIIdn 5 µg were transiently expressed for 48 hours in COS-7 cells, as indicated. Raf-1 activity was measured as described in *Material and methods section*. The results are presented as fold increase of incorporated cpm. Data are reported as the mean +/- standard deviation from duplicate experimental point. All differences were significant with the only exception of Src *vs.* Src + CaMKIIdn.

3.5 Phosphorylation of Raf-1 at Ser 338 by FCS and FN is CaMKII mediated

It was previously proposed that RasV12 induces S338 Raf-1 phosphorylation by PAKs protein in a Rac/Cdc42/PI3K-regulated manner. A careful study demonstrated that the PI3K inhibitors do not suppress Ser338 phosphorylation EGF-mediated at concentrations that block PI3K activity (10 μM). The authors conclusion was that the role of PI3K and Pak3 in mediating Ser338 phosphorylation by Ras was not physiological and lacked of direct evidences. In conclusion, the kinase that phosphorylates Raf-1 on Ser338 and participates to ERK activation by growth factors or by oncogenic Ras remained to be indentified (Chiloeches, et al., 2001).

Seven CaMKII consensus sequence (R/KXXS/T) are present along Raf-1 aminoacidic sequence and one of these contains S338 (Fig. 3.5).

According to previous experiments involving PAK, I determined whether phosphorylation of Raf-1 at S338 was CaMKII mediated. To this aim, starved TAD-2 cells were stimulated by serum (FCS) or fibronectin (FN) for 30 minutes upon pretreatment with CaMKII inhibitors (KN93 or AntCaNtide) 5-10 μM or with PI3K inhibitors (Ly-294002) 10 μM. Raf-1 phosphorylation at S338 was visualized by immunoprecipitation of the kinase and Western Blotting. Both FCS and FN stimulated S338 phosphorylation respect to basal condition. The inhibition of CaMKII reduced S338 phosphorylation by FCS and FN, in a dose dependent manner. Conversely, PI3K inhibition was ineffective on S338 phosphorylation (Fig. 3.6A-B).

These data demonstrated that Raf-1 phosphorylation at S338 by FCS or FN is CaMKII dependent and not PI3K/PAK dependent.

Canonical consensus sequence for CaMKII is **R/KXXS/T**:

Raf-1 aa sequence

```
1     MEHIQGAWKT ISNGFGFKDA VFDGSSCISP TIVQQFGYQR RASDDGKLTD PSKTSNTIRV
61    FLPNKQRTVV NVRNGMSLHD CLMKALKVRG LQPECCAVFR LLHEHKGKKA RLDWNTDAAS
121   LIGEELQVDF LDHVPLTTHN FARKTFLKLA FCDICQKFLL NGFRCQTCGY KFHEHCSTKV
181   PTMCVDWSNI RQLLLFPNST IGDSGVPALP SLTMRRMRES VSRMPVSSQH RYSTPHAFTF
241   NTSSPSSEGS LSQRQRSTST PNVHMVSTTL PVDSRMIEDA IRSHSESASP SALSSSPNNL
301   SPTGWSQPKT PVPAQRERAP VSGTQEKNKI RPRGQRDSSY YWEIEASEVM LSTRIGSGSF
361   GTVYKGKWHG DVAVKILKVV DPTPEQFQAF RNEVAVLRKT RHVNILLFMG YMTKDNLAIV
421   TQWCEGSSLY KHLHVQETKF QMFQLIDIAR QTAQGMDYLH AKNIIHRDMK SNNIFLHEGL
481   TVKIGDFGLA TVKSRWSGSQ QVEQPTGSVL WMAPEVIRMQ DNNPFSFQSD VYSYGIVLYE
541   LMTGELPYSH INNRDQIIFM VGRGYASPDL SKLYKNCPKA MKRLVADCVK KVKEERPLFP
601   QILSSIELLQ HSLPKINRSA SEPSLHRAAH TEDINACTLT TSPRLPVF
```

Fig. 3.5 | **Raf-1 aminoacidic sequence and canonical consensus sequence for CaMKII.**

The canonical consensus sequence for CaMKII along Raf-1 aminoacidic sequence are indicated in bold font. The canonical consensus sequence for CaMKII that contain S338 is indicated in red.

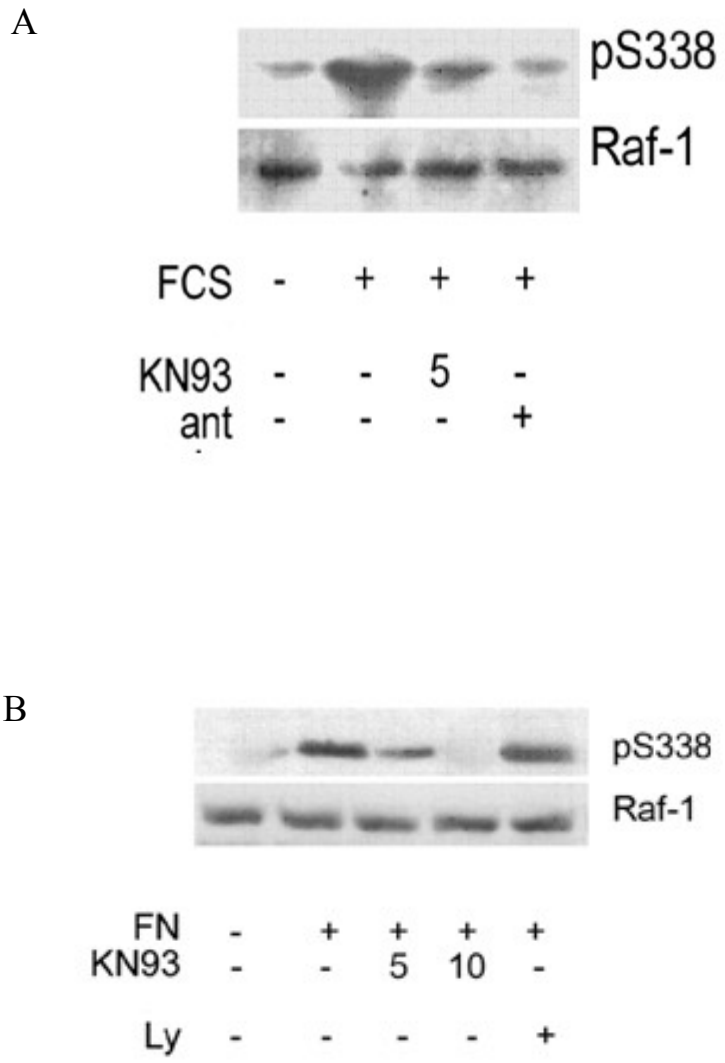

Fig. 3.6 | **Phosphorylation of Raf-1 at Ser 338 by FCS and FN is CaMKII mediated.**

A) TAD-2 cells were starved for 24 hours, stimulated with FCS, upon treatment with CaMKII inhibitors, KN93 or AntCaNtide 5 μM. Raf-1 was immunoprecipitated from the cells and pS338 and total Raf-1 analyzed by Western Blotting using specific antibodies.
B) TAD-2 cells were starved for 24 hours, stimulated with FN for 30 minutes, upon treatment with KN93 5 μM or 10 μM, or PI3K inhibitor (Ly-294002) 10 μM for 30 minutes. Raf-1 was mmunoprecipitated and pS338 and total Raf-1 analyzed by Western Blotting using specific antibodies.

3.6 Phosphorylation of Raf-1 at S338 by RasV12 is CaMKII mediated

To determine whether CaMKII mediated RasV12-dependent Raf-1 phosphorylation at S338, COS-7 cells were transiently transfected with expression vectors for RasV12, SrcY527 with or without CaMKIIdn. Raf-1phosphorylation at S338 was visualized by WB after Raf-1 immunoprecipitation in serum starved cells. EGF stimulation for 10 minutes (used as a positive control of Raf-1 phosphorylation), oncogenic Ras or Src induced S338 phosphorylation. Coexpression of RasV12 and SrcY527 produced a maximal S338 phosphorylation, according to previous study (Mason, et al., 1999).

Cotransfection of CaMKIIdn together with RasV12 and SrcY527 resulted in a 60% reduction of pS338 phosphorylation induced in the absence of CaMKIIdn. Also the expression of the costitutively activated CaMKII mutant (CaMKIIac), induced a striking S338 phosphorylation with respect to control cells.

These data (Fig. 3.7) demonstrated that Ras-dependent Raf-1 phosphorylation at S338 is CaMKII mediated and that CaMKII by itself is able to phosphorylate Raf-1 at the same aminoacidic residue.

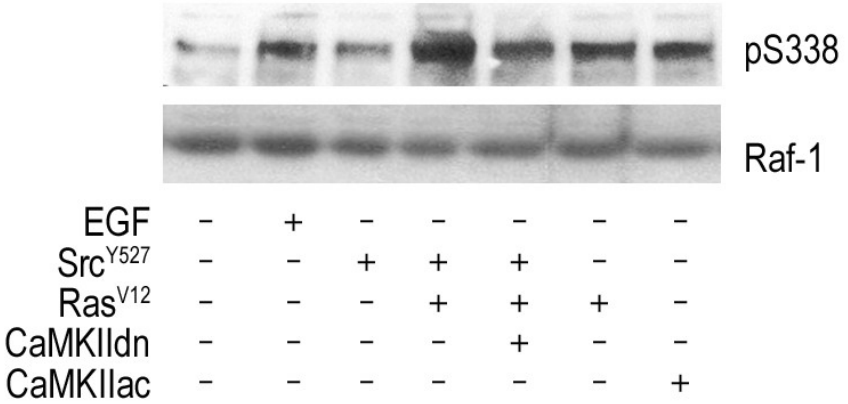

Fig. 3.7 | **Phosphorylation of Raf-1 at S338 by RasV12 is CaMKII mediated.**

COS-7 cells were transiently transfected with RasV12 2 µg, SrcY527 5 µg, CaMKIIdn 5 µg, CaMKIIac 5 µg for 48 hours or treated with EGF 10 ng/mL for 10 minutes. Raf-1 was immunoprecipitated from the cells and pS338 and total Raf-1 analyzed by Western Blotting using specific antibodies.

3.7 CaMKII directly interact with Raf-1 and phosphorylates Raf-1 at S338 in vitro

To determine whether CaMKII and Raf-1 directly interact in vitro and whether CaMKII phosphorylates Raf-1 at S338 in vitro, I performed a Raf-1 activity in vitro as described in *Material and methods*. Raf-1 was immunoprecipitated from unstimulated TAD-2 cells and incubated in vitro for 30 minutes with active recombinant CaMKII. Phosphorylated S338 was visualized by WB with specific antibody. In the absence of calcium (EGTA), CaMKII is inactive, while it is maximally activated in the presence of calcium. The experiment clearly demonstrated that S338 was phosphorylated in the presence of calcium and in the absence of AntCaNtide (Fig. 3.8). As expected, KN93 treatment was ineffective on S338 phosphorylation, because this drug interferes competitively with CaMKII/CaM binding and thus it is ineffective on the autonomous activity of the kinase (Vest, et al., 2007).

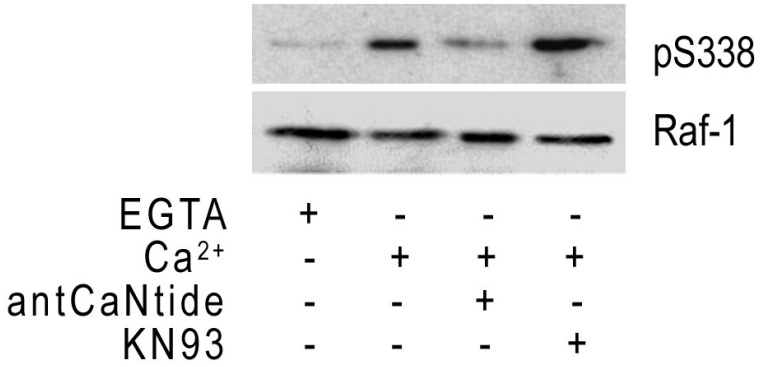

Fig. 3.8 | **CaMKII directly interact with Raf-1 and phosphorylates Raf-1 at S338 in vitro.**

Raf-1 was immunoprecipitated from unstimulated TAD-2 cells, incubated in vitro for 30 minutes with active CaMKII in presence (Ca^{2+}) and absence (EGTA) of calcium and upon antCaNtide 5 μM and KN93 10 μM pre treatment for 30 minutes. pS338 and total Raf-1 visualized by WB.

4. CONCLUSIONS AND DISCUSSION

CaMKII is an ubiquitous kinase with a broad range of biological cellular functions, including regulation of gene expression, cell cycle and proliferation.

This kinase is one of the most abundant protein in the brain, where it is associated to neuronal functions such as memory. The role of CaMKII in the regulation of cell cycle mechanisms and in cell proliferation, has been well demonstrated. In mammalian fibroblast, CaMKII mediates G1 phase progression in a Ca^{2+}/CaM dependent manner, mediating Cyclin D1 levels. More recent works, show that CaMKII regulates cell proliferation in different cell types, including epithelial cells. In the laboratory where I performed my thesis, it has been demonstrated for the first time that the Ras/ERK pathway, activated upon several stimuli, is controlled by CaMKII through the regulation of Raf-1 activity. Illario et al. showed that Fibronectin (FN) binding to integrins in thyroid cells TAD-2, activates the Ras/Raf/MEK/ERK pathway, through the formation of FAK/Grb-2/Sos complex, and also generates an increase of Ca^{2+} intracellular level that leads to a Ca^{2+}/CaMKII signal. Binding to FN induces Raf-1 and CaMKII to form a protein complex, indicating that intersection between Ras/Raf/Mek/Erk and Ca^{2+}/CaMKII signalling pathways occurs at Raf-1 level. Immunoprecipitation experiments demonstrated that Ca^{2+}/CaMKII signal is necessary for ERK activation and interruption of the pathway using pharmacological inhibitors of CaMKII, arrested cell proliferation induced by FN in thyroid cells (Illario, et al., 2003; Illario, et al., 2005). The cross talk between CaMKII and MAPK pathway, has been demonstrated also in L6 skeletal myoblasts and human fibroblasts, following insulin stimulation. Insulin-activated CaMKII associates to Raf-1 and it is necessary for DNA synthesis and for the attenuation of AKT activation, demonstrating the role of CaMKII also in the selective control of insulin signalling (Illario, et al., 2009). A role for CaMKII was demonstrated not only in the physiology of certain cells, but also in cancer. In the study of Rusciano et al., I contributed to demonstrate that CaMKII is activated by the oncogenes RET/PTC-1 and BRafV600E and that it is necessary for ERK activation by RET/PTC (Rusciano, et al.).

These evidences support a pivotal role for CaMKII in the modulation of ERK activation in a number of models. Personal unpublished data are in favour for a constitutive activation of CaMKII in tumors with different oncogenes. In medullary thyroid carcinoma harbouring RET mutations (an oncogene that recognizes Ras as a downstream substrate) and in colorectal carcinoma cell lines harbouring oncogenic Ras, CaMKII resulted constitutively activated, suggesting that Ras is a CaMKII activator.

In the first part of my study, I provide direct evidences that CaMKII is activated by RasV12 in NIH3T3 and that it is necessary for ERK phosphorylation in the Ras-dependent signal. This, together with the other observations obtained in my laboratory, indicate that the role of CaMKII in the MAPK signalling is a general mechanism, hence it is important in the physio-pathology of many cell systems and might represent a target for therapy of proliferating diseases.

Raf-1 activation is a complex process involving multiple converging signalings, protein-protein interactions and Raf-1 phosphorylation at multiple sites. Maximal Raf-1 activation is reached by phosphorylation at Y341 by Src and at S338 (Mason, et al., 1999). Although some studies proposed PAK family proteins as the kinases phosphorylating Raf-1 at S338, which is the kinase involved was not definitively identified. In the second part of my study, I investigated the mechanism by which CaMKII modulates Raf-1. Seven CaMKII consensus sequence (R/KXXS/T) are present along Raf-1 aminoacidic sequence and one of these contains S338 (Fig. 3.5). Previous co-immunoprecipitation experiments, reported that CaMKII associates with Raf-1 in vivo in L6 cells stimulated by insulin (Illario, et al., 2009). Using a phospho-specific antibody against the residue of Ser338, I demonstrated that CaMKII has a role in the phosphorylation at S338 of Raf-1 in thyroid cells by diverse factors (FCS, FN and RasV12). Conversely, the inhibition of phosphatidylinositol 3-kinase, which activates PAK3 was ineffective, demonstrating that, in this cell system, PAK has no role in Raf-1 phosphorylation at S338. The role for this kinase in Raf-1 activation was first sustained and then questioned. My data do not exclude that in other cell types and upon different stimuli, PAK may play the role that was first hypothesized. It is still possible that both PAK and CaMKII compete for Raf-1 and cooperate to its activation. Indeed, in

inhibition experiments in which Raf-1 activation was determined, a residual Raf-1 stimulation was evident, thus leaving the possibility that other kinases than CaMKII participate to the modulation of Raf-1 activation.

My experiments are focused on few aspects of the CaMKII/Raf-1 interaction, while many other are left unsolved. Although constitutively active CaMKII alone was able to phosphorylate Raf-1 at S338, it was not able to activate neither Raf-1 nor ERK (not shown). This observation is in agreement with the large body of data that demonstrate the complex mechanism of events that leads to Raf-1 activation and that require multiple factors. We have no evidence whether CaMKII is involved in the Raf-1 translocation from the cytosol to the plasma membrane, as it is unknown whether CaMKII phosphorylates Raf-1 also in sites other than S338.

Based upon my data, CaMKII can be considered as a new factor in the Ras signaling, involved in the regulation of the important effects of oncogenic Ras action such as aberrant cell proliferation and tumorigenesis. This kinase, by modulating the activated Ras/ERK signal by oncogenes, might have a role in several cancers harbouring oncogenic RasV12 and could represent a new therapeutic target for pharmacological intervention in these kind of tumors.
In conclusion, I gained more insight into the molecular mechanism by which CaMKII modulates Raf-1 activation and I demonstrated that CaMKII is the kinase or is one of the kinases that phosphorylate Raf-1 at S338.

5. MATERIALS AND METHODS

5.1 Cell culture, vectors transfection and fibronectin stimulation

NIH3T3, TAD-2 and COS-7 cells were grown in Dulbecco Modified Eagle Medium (DMEM) (Life Technologies, Inc, Grand Island, NY) supplemented with 10% fetal calf serum (Sigma, St. Louis, MO), 2 mM L-glutamine and 100 units/ml penicillin-streptomycin (GIBCO) and the plates were incubated at 37°C in the presence of 5% CO2.

The cDNAs for oncogenic Ras, H-RasV12 and K-RasV12, were subcloned in expression vector pBABE and pcDNA3 respectively; activated Src, SrcY527, was subcloned in expression vector pEF; catalitically inactive form of CaMKIIα (K42M) was subcloned in pSP72; CaMKIICA is a truncated sequence of CaMKII (from 1 to 290 aminoacids) that leads the enzyme constitutively active (both the vectors CaMKIIDN and CaMKIICA are a generous gift from Dr. A.R.Means, Duke University, Durham, NC). NIH3T3 and COS-7 cells were transiently or stably transfected with Lipofectamine 2000 according to the manufacturer's recommendations (Gibco Invitrogen). Briefly, cells were transfected at 80% confluence with appropriate μg of DNA for each 100mm dish. The Lipofectamine was used 2 μL for each μg of DNA. The mix DNA Lipofectamine was incubated 20 minutes at room temperature to allow the formation of the precipitates. Before adding the mixture, cells were washed with PBS and the growing medium was replaced with medium without antibiotics. For transiently transfection cells were lysate after 48 hours. For stably transfection, after 48 hours from transfection, cells were splitted in appropriate dilutions (1:20, 1:40, 1:80, 1:160, 1:320); after 3 days, G418 or Puromicine were added in the wells (1 μg/mL per plate) and after 3 weeks of continuous selection, single clones were picked, screened for expression of the transgene, and amplified individually in DMEM 10% FCS supplemented with the opportune antibiotic.

The TAD-2 cell line was obtained by simian virus 40 infection of human fetal thyroid cells and was donated by Dr. T. F. Davies, Mount Sinai Hospital (New York, NY). The plates of FN were prepared as described: the wells were filled with PBS, 1% heat-denatured BSA (SIGMA) or 100 g/mL of human FN (Collaborative Research, Bedford,

MA) and after overnight incubation at 4 °C, the plates were washed with PBS and the cells lysated as described in Vitale et al. (Vitale, et al., 1998).

5.2 Western Blotting and immunoprecipitation procedures

Cells were washed in PBS buffer and lysed on ice for 30 min in RIPA buffer (50 mM Tris-HCl, pH 7.4,150 mM NaCl, 1% NP-40,2 mM EDTA,2 mM PMSF, 5 ug/mL leupeptin, 5 ug/mL pepstatin).The lysate were quantified by Biorad DC protein assay. An equal amount of proteins from each sample was loaded with laemly buffer. Protein were resolved by SDS-PAGE and transferred to an Immobilion P membrane (Millipore Corporation, Bedford, MA). Membranes were blocked by incubation with PBS 0,2% tween , 5% nonfat dry milk for one hour at room temperature. The membranes were then incubated overnight with primary antibodies at 4°C (dilution 1:1000), washed for 40 minutes with PBS 0,2% tween and incubated for 1 hour with a horseradish peroxidase - conjugated secondary antibodies (dilution 1:2000). Finally, protein bands were detected by an enhanced chemiluminescence system (ECL, Amersham Bioscience). Computer-acquired images were quantified using ImageQuant software (Amersham Bio-sciences).
For the immunoprecipitation procedures, the cells were lysed in immunoprecipitation buffer (50mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycolate, 0.1% SDS, 10 mM NaF, 5 mM EGTA, 10 mM sodium pyrophosphate, and 1 mM phenylmethylsulfonylfluoride). Primary polyclonal antibody against all CaMKII isoforms (SANTA CRUZ BIOTECHNOLOGY, Santa Cruz, CA) or primary purified mouse antibody against c-Raf (BD Bioscences Pharmingen) was incubated with the lysate for 1 h at 4 °C. Successively, Protein G plus/protein A agarose beads (Oncogene Science, Boston, MA) were incubated with the immunocomplexes for 2 h at 4 °C and used to immunoprecipitate corresponding proteins from 1 mg of total lysate after centrifugations.
Mouse monoclonal antibodies to p44/p42 MAPK, phospho-p44/p42 MAPK , Raf1 and CaMKII were from Santa Cruz Biotechnology. Polyclonal anti-phospho-CaMKII antibody (pT286-CaMKII) was from Promega (Madison, WI). Anti-phospho-Raf-1 (Ser338) rat monoclonal was from UPSTATE.

5.3 CaMKII activity and inhibitors

To inhibit CaMKII activity two different inhibitors have been used: KN93 and AntCaNtide. KN93 is a potent, selective and cell permeant pharmacological inhibitor of the CaMKs (IC50=370 nM). This drug is an isoquinolonesulfonamides and it has the effect of a competitive ATP antagonist (Tokumitsu, et al., 1990). The CaMKII specific peptidic inhibitor Ant-CaNtide is derived from the endogenous CaMKII inhibitor protein CaMKIIN (Chang, et al., 1998) and was made cell permeable by Nterminal addition of an antennapedia-derived sequence (Ant-CaNtide: RQIKIWFQNRRMKWKKR PPKLG QIGRSKRVVIEDDRIDDVLK). Catalitically inactive form of CaMKIIα (K42M) was subcloned in pSP72. Calmodulin inhibitors, trifluoperazine (TFP) e N-(6-123 aminohexyl)-5-chloro-1-nafthalene-sulfonamide (W7), were from Sigma.

CaMKII activity assay was performed as described. In a first reaction step immunoprecipitated CaMKII was incubated for 30 minutes at 30 °C with 5 mM CaCl2 and 5μM CaM in 50 μl of reaction mixture consisting of 50 mM HEPES pH 7.5, 10 mM MgCl2, 0,5 mM dithiothreitol (DTT), 2μM CaM, 100 nM microcystin, 0,5mM cold ATP. A 10 μl aliquot from the first reaction was than incubated with 25mM EGTA, 0,5 mM Autocamtide (Hanson, et al., 1989) and 50 μM ATP (1500 cpm/pmol [γ-32P]ATP) in order to determine CaMKII autonomous activity on its peptide substrate Autocamtide. The reaction was carried out for 30 minutes at 30 °C and 20 μl aliquots of the reaction mixture were spotted on p81 phosphocellulose filters (Upstate Biothechnology, Lake placid, NY). The level of [32P] incorporation into Autocamtide was determined by liquid scintillation counting. Purified CaM and Autocamtide were a kind gift from Dr. AR. Means, (Duke University, Durham, NC). Ionomycin (Sigma) 500 ng/mL was used as positive control of CaMKII activation.

5.4 Raf-1 activity

Raf-1 activity was evaluated by a Raf-1 immunoprecipitation-kinase cascade assay kit (Upstate Biotechnology). Briefly, Raf-1 was immunoprecipitated from 1 mg of cell extracts. The immunocomplexes were washed and incubated in the presence of magnesium/ATP and Mek-1 unactive (0,4 µg/assay) for 30 min at 30 °C in 50 µl of reaction mixture ADBI (consisting of 20 mM MOPS pH 7,2, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol) . An aliquot of the mixture was then incubated with ERK-2 unactive (1 µg/assay), 20 µg of myelin basic protein (MBP) in the presence of [γ-32P]ATP for 30 min at 30 °C and 20 µl aliquots of the reaction mixture were spotted on p81 phosphocellulose filters (Upstate Biothechnology, Lake placid, NY). The level of [32P] incorporation into MBP was determined by liquid scintillation counting.

5.5 Raf-1 activity in vitro

Raf-1 was immunoprecipitated from 1 mg of cells extract. The immunocomplexes and active CaMKII were incubated in the presence of EGTA or Calcium for 30 min at 30 °C in 50 µl of reaction mixture ADBI (consisting of 20 mM MOPS pH 7,2, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol). The reaction was quenched with Laemmli buffer, proteins were separated through a 10% polyacrylamide/tris glycine gel and phosphorylation visualized by phosphospecific antibody.

5.6 Statistical analysis

Results are presented as the mean ± SD. Statistical analysis was performed by using the *t* test. The level of significance was set at *p* less than 0.05.

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8. LIST OF PUBLICATIONS

1) Rusciano MR, **Salzano M**, Monaco S, Sapio MR, Illario M, De Falco V, Santoro M, Campiglia P, Pastore L, Fenzi G, Rossi G, Vitale M.
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