Role of IAPs in modulating C-RAF stability and cell motility

Dissertation

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SUMMARY

Inhibitor of apoptosis proteins (IAP) are evolutionarily conserved anti-apoptotic regulators. C-RAF protein kinase is a direct RAS effector protein, which initiates the classical mitogen-activated protein kinase (MAPK) cascade. This signaling cascade mediates diverse biological functions, such as cell growth, proliferation, migration, differentiation and survival.

In this thesis, it is demonstrated that XIAP and c-IAPs bind directly to C-RAF kinase and that siRNA-mediated silencing of XIAP and c-IAPs leads to stabilization of C-RAF in human cells. XIAP binds strongly to C-RAF and promotes the ubiquitylation of C-RAF *in vivo* through the Hsp90-mediated quality control system, independently of its E3 ligase activity.

In addition, XIAP or c-IAP-1/2 knockdown cells showed enhanced cell migration in a C-RAF-dependent manner. XIAP promotes binding of CHIP (carboxy terminal Hsc70-interacting protein), a chaperone-associated ubiquitin ligase, to the C-RAF-Hsp90 complex *in vivo*. Interfering with CHIP expression resulted in stabilization of C-RAF and enhanced cell migration, as observed in XIAP knockdown cells.

The data of this thesis show an unexpected role of XIAP and c-IAPs in the turnover of C-RAF protein, thereby modulating the MAPK signaling pathway and cell migration.

ZUSAMMENFASSUNG

Apoptose Inhibitoren (IAP) sind eine evolutionär konservierte Proteinfamilie, die als anti-apoptotische Regulatoren wirken. Die Serin-Threonin Kinase C-RAF ist ein unmittelbares RAS-Effektorprotein, das die klassische MAP-Kinase (MAPK)-Kaskade auslöst. Diese Signalkaskade vermittelt unterschiedliche biologische Funktionen wie Zellwachstum, Proliferation, Migration, Differenzierung und Überleben.

In dieser Doktorarbeit wird gezeigt, dass XIAP und c-IAPs an C-RAF-Kinase binden und siRNA-vermittelte Hemmung von XIAP und c-IAPs zur Stabilisierung von C-RAF in humanen Zellen führt. XIAP bindet fest an C-RAF und fördert mittels des Hsp90-vermittelten Qualität-Kontrol-Systems die Ubiquitinierung von C-RAF *in vivo*. Dies geschieht unabhängig von der E3-Ligase-Aktivität XIAPs.

Darüber hinaus konnte gezeigt werden, dass sowohl XIAP als auch c-IAP-1/2 knock-down Zellen eine erhöhte C-RAF-abhängige Zellwanderung aufweisen. XIAP fördert die Bindung von CHIP (carboxy terminal Hsc70-interacting protein), einer Chaperon-assoziierten Ubiquitin-Ligase, an den C-RAF-Hsp90-Komplex *in vivo*. Wird die Expression von CHIP verhindert, führt dies zur Stabilisierung von C-RAF und eine hohe Migrationsrate, wie in XIAP knock-down Zellen konnte beobachtet werden.

Bisher waren IAPs überwiegend in ihrer Rolle als Unterdrücker von Apoptose bekannt. Die Daten dieser Doktorarbeit zeigen nun eine unerwartete Rolle für XIAP und c-IAPs. Durch ihren Einfluß auf den Proteinumsatz von C-RAF modulieren sie die MAPK-Kaskade und somit die Wanderung von Zellen.

1. INTRODUCTION

1.1 Programmed cell death

Programmed Cell Death (PCD) and necrosis have been identified as two major forms of cell death. Necrosis is a localized, unnatural death of living tissue or cells characterized by cytoplasm swelling, destruction of organelles, and disruption of plasma membrane. Conversely, PCD is a normal physiological phenomena carried out in a regulated manner during an organism's life-cycle[1]. There are various types of PCDs such as apoptosis, autophagic cell death, anoikis, cornification, and Wallerian degeneration. The most well characterized forms of PCD are apoptosis and autophagic cell death[1-4]. The word "Apoptosis" means the "falling of leaves from a tree in autumn" in the ancient Greek[5, 6]. This form of cell death plays an important role in terms of homeostasis in multicellular organisms both during embryonic development as well as in adult life[3]. The plethora of diseases associated to apoptosis regulation such as neuro-degenerative diseases, cancer, autoimmune diseases, stress related disorders etc., led to an explosion of research in this field[3]. Unlike necrosis, where there is spillage and inflammation, apoptosis is an energy dependent physiological phenomenon, a distinct set of biochemical and physical changes involving the cytoplasm, nucleus and plasma membrane, that is genetically programmed with the involvement of regulatory genes, stimulatory events and signaling pathways. Currently, two different apoptotic pathways have been proposed depending on the death signal: the **extrinsic (recep tor-mediated) pathw ay** starting with aggregation of death receptors and the **intrinsic (cellular/mitochondrial) pathw ay** initiating with the disruption of intracellular homeostasis such as release of mitochondrial factors and DNA degradation[7] (Fig.1.1). Of course, these signaling pathways do not always proceed independently of each other since interaction at diverse stages has become obvious in numerous models. And triggering of these pathways results in activation of a family of cysteine aspartate specific proteases known caspases. The death signal is transmitted through the cell in the form of a proteolytic caspase cascade, whereby the executioner caspases inactivate or activate their specific cellular targets by proteolysis. However, there are several caspase independent apoptosis like PCDs. For example, some proteases like lysosomal cathepsins, calcium dependent calpains and granzymes, are also involved in alternative pathways leading to cell death[2]. Thus defects in apoptosis can disrupt many of the cell's homeostasis and repair mechanisms leading to dire consequences ranging from cancer to neurodegenerative disorders. Apoptosis and the major components of the apoptotic machinery like the caspases are very much conserved during the course of evolution[8].

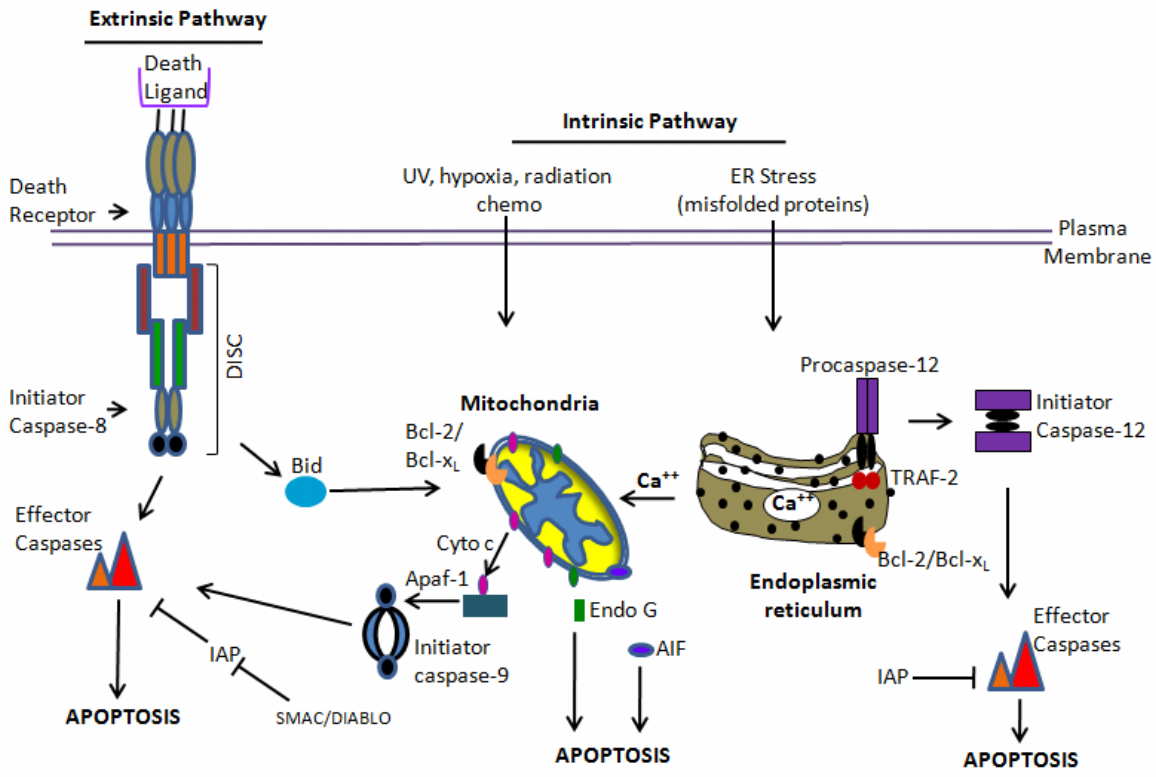

Fig.1.1 The two main pathways for the initiation of apoptosis. Binding of ligand to specific death receptors initiates the extrinsic pathway. On the other hand, various stimuli such as UV, hypoxia, misfolded proteins, etc. initiate the intrinsic pathway via both mitochondria and endoplasmic reticulum. Initiator caspase-8, -9, and -10 then activate executioner caspases such as caspase-3, -6, and -7. Executioner caspases cleave death substrates and, finally, apoptosis occurs (modified from Ref.7).

1.1.1 The extrinsic pathway

One major pathway for the induction of apoptosis is the receptor-mediated or extrinsic pathway. The extrinsic pathway is triggered by the death receptors, which are activated by the extracellular ligands. TNF-R1 (tumor necrosis factor alpha receptor 1), DR4 (death receptor 4), DR5 (death receptor 5), and Fas/Apo/CD95 (Fas receptor) are the best described surface receptors which trigger death signals when activated by their natural ligands TNF-alpha (tumor necrosis factor alpha), TRAIL (TNF-alpha-related apoptosis inducing ligand), or FasL (Fas ligand), respectively[9]. These receptors belong to TNF-R super family, which regulates a large number of biological functions, such as growth and differentiation, in addition to apoptosis. The death receptors of this family contain variable numbers of cysteine rich repeats in their extracellular domain (extracellular ligand-binding region), and an intracellular death domain (DD), which distinguishes these from other TNFR members e.g. TNF-R2 and CD40[9-11] (Fig.1.2).

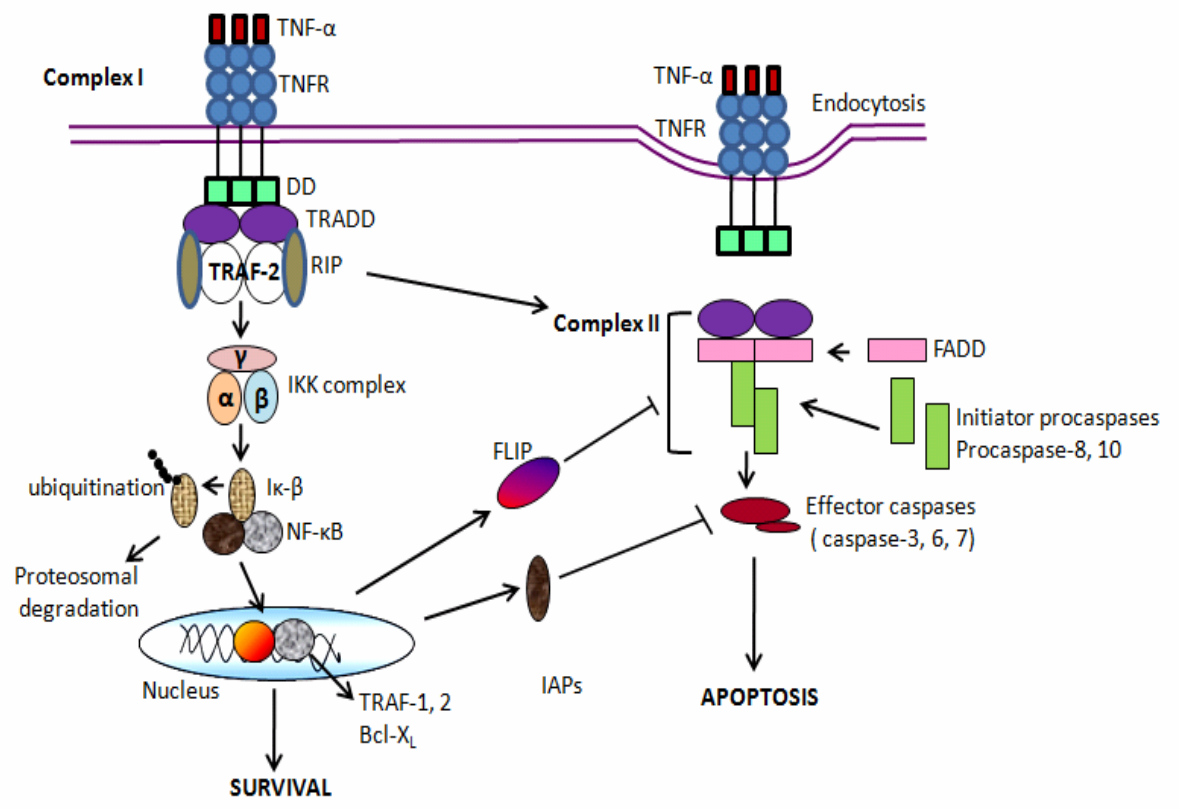

Fig.1.2 TNF receptor (TNFR) pathw ay signaling. After stimulation with TNF-α, TNFRI undergo trimerization. This recruits various adapter molecules leading to NF-κB activation (Complex I formation) and cell survival signaling. As a consequence, endocytosis of receptor complex occurs, resulting in dissociation of certain adaptor proteins (TRAF-2, RIP) as well as recruitment of fas-associated death domain (FADD) and procaspase-8 to form death-inducing signaling complex (DISC). DISC activates caspase-8, which is then released in the cytoplasm and activates effector caspases leading to apoptotic induction (modified from Ref.7).

Beside DD, death receptors contain death effector domain (DED) and caspase recruitment domain (CARD), which also play very important role in death signaling and show very similar 3D structures. The DDs are present in the intracellular parts of death receptors, whereas the DEDs and CARDs are found in the adapter molecules, which mediate receptor or mitochondrial death signals[11]. TNF-alpha (TNF-α) is produced by T-cells and activated macrophages in response to infection. Binding of TNF-α to TNFR1 results in receptor trimerisation, which in turn leads to clustering of intracellular death domains, but it does not result in cell death in every cell type, unlike FasL-receptor binding. The binding of TNF ligand to its receptor results in the binding of an intracellular adaptor molecule called TRADD (TNFR-associated death domain) through the death domains. TRADD in turn recruits additional proteins to this complex: The recruitment of TRAF2 (TNFR-associated factor 2) and RIP (receptor-interacting protein) can lead to activation of NF-κB (Nuclear factor κB) and the

JNK (c-Jun N-terminal kinase) pathway[11, 12]. TRADD can also associate with FADD and this binding results in the formation of a death inducing signaling complex (DISC), a complex constituting the activated receptors, adaptor proteins, and initiator protease Caspase-8 and leads to activation and cleavage of pro-caspase-8. Activated caspase-8 can in turn cleave and activate effector caspases (Caspase-3 and -7), which leads to the cleavage of various cellular proteins[11, 13]. In some cell types, active caspase-8 can also cleave BID (BH3 interacting domain death agonist) protein to tBID, which leads to the mitochondrial outer membrane permeabilization leading to release of cytochrome c in the intrinsic pathway[14] (Fig.1.1 and 1.2). Deng *et al* revealed an unexpected role of JNK in the cleavage of BID in a caspase-8-independent manner during TNF-induced apoptosis[15].

In case of FasL, ligation leads to the activation of apoptosis in a similar way to the TNF receptor. Ligand binding promotes receptor clustering, DISC formation and the activation of the caspase cascade. However, Fas receptor signaling is slightly different than TNF receptor as the adaptor protein FADD can be recruited directly to the death domain on the Fas receptor, which is not the case in TNF receptor signaling. TNF receptor requires the prior recruitment of TRADD. Also, the Fas receptor is generally thought to activate apoptosis[11], but its role in non-apoptotic signaling pathway including proliferation, differentiation, cytokine production, and axonal regeneration in neurons has also been shown[16, 17]. Induction of apoptosis by TRAIL is similar in action to FasL. Binding of TRAIL to its receptors DR4 or DR5 triggers rapid apoptosis in many tumor cells. Interestingly, tumor cells express decoy receptors (DcR1 and DcR2) that compete for binding of TRAIL. While DcR1 does not possess a cytoplasmic domain, DcR2 has a truncated death domain lacking 4 out of 6 amino acids essential for recruiting adapter proteins. So, binding of TRAIL to DcR1 and DcR2 will not result in the activation of caspase-8 and apoptosis[5, 18].

1.1.2 The intrinsic pathway

The intrinsic pathway is triggered by cellular stress, specifically mitochondrial stress caused by factors such as DNA damage by mutagens and ionising radiation, heat shock, oxidative stress, treatment with cytotoxic drugs, or by the deprivation of essential growth factors. This stress triggers a series of events leading to the release of a range of pro-apoptotic molecules from the intermembrane space of the mitochondria: cytochrome c, apoptosis-inducing factor (AIF), second mitochondrial activator of caspases/direct IAP binding protein with low pI (Smac/DIABLO), endonuclease G (EndoG), Omi/HtrA2 (high temperature requirement protein A2), and some caspases[19-21]. Mitochondrial membrane permeabilization (MMP) is one of the crucial events that happen during the intrinsic pathway of apoptosis induction. This includes

mostly permeabilization of the outer mitochondrial membrane (OMM) and sometimes the inner mitochondrial membrane (IMM). When outer membrane integrity is lost, proapoptotic proteins from the mitochondrial inter-membrane space are leaked into cytosol and either activate caspases or act in a caspase-independent manner leading to cell death[19, 21]. In case of the cytochrome c release, this protein forms a complex in the cytoplasm with adenosine triphosphate (ATP), and the adapter molecule Apaf-1 (apoptotic protease activating factor-1). Apaf-1 contains at least three functional domains including a CARD, a CED-4 domain required for self-oligomerisation, and a series of WD40 repeats which possibly mediate protein-protein interactions[22]. This complex will activate an initiator caspase, caspase-9. In return, the activated caspase-9 works together with the complex of cytochrome c, ATP and Apaf-1 to form the apoptosome, an ~1MDa oligomeric complex, which in turn activates caspase-3. Caspase-9 in the apoptosome is more active than processed caspase-9[21-24]. Also it has been shown that caspase-2 activation is required during stress induced apoptosis in several cell types upstream of Bax (Bcl-2-associated X protein) translocation and mitochondrial membrane permeabilization[22]. Like caspase-9, caspase-2 is also recruited to a multimeric protein complex called PIDDosome during apoptosis[25].

Besides cytochrome c, AIF translocates from the mitochondrial intermembrane space via the cytosol to nuclei to facilitate DNA fragmentation and induce an apoptosis-like cell death, that is independent of caspases[13]. EndoG also translocates to the nucleus and mediates internucleosomal DNA-fragmentation[23]. Other intermembrane proteins, Smac/DIABLO and Omi/HtrA2 facilitate activation of caspases indirectly, by inactivating inhibitor of apoptosis proteins (IAPs). Like other intermembrane proteins, Smac/DIABLO is synthesized in the cytoplasm as a precursor carrying an N-terminal mitochondrial localization sequence (MLS). Upon import into mitochondria, this MLS is cleaved off and Smac/DIABLO become mature and apoptogenic[26] (Fig.1.1 and 1.3).

1.1.3 ER-mediated Pathway

The endoplasmic reticulum (ER) is an intracellular organelle serving many vital functions as intracellular Ca^{2+} store and also acts as a site for protein folding and modification (e.g. glycosylation). It consists of a membranous network, which extends throughout the cytoplasm of the cell and is contiguous with the nuclear envelope. The ER is the major site of protein quality control systems, which ensures that only correctly folded proteins transit to the Golgi and unfolded or misfolded proteins are recognized and ultimately degraded. When the load of unfolded proteins rises above the capacity of the ER, a stress response named UPR (unfolded

protein response) is triggered resulting in an upregulation of regulatory factors (e.g. chaperones) and downregulation of protein sythesis. If these repair measures do not take hold, apoptosis is induced as final response[27-30] (Fig.1.1). The proapoptotic pathways emanating

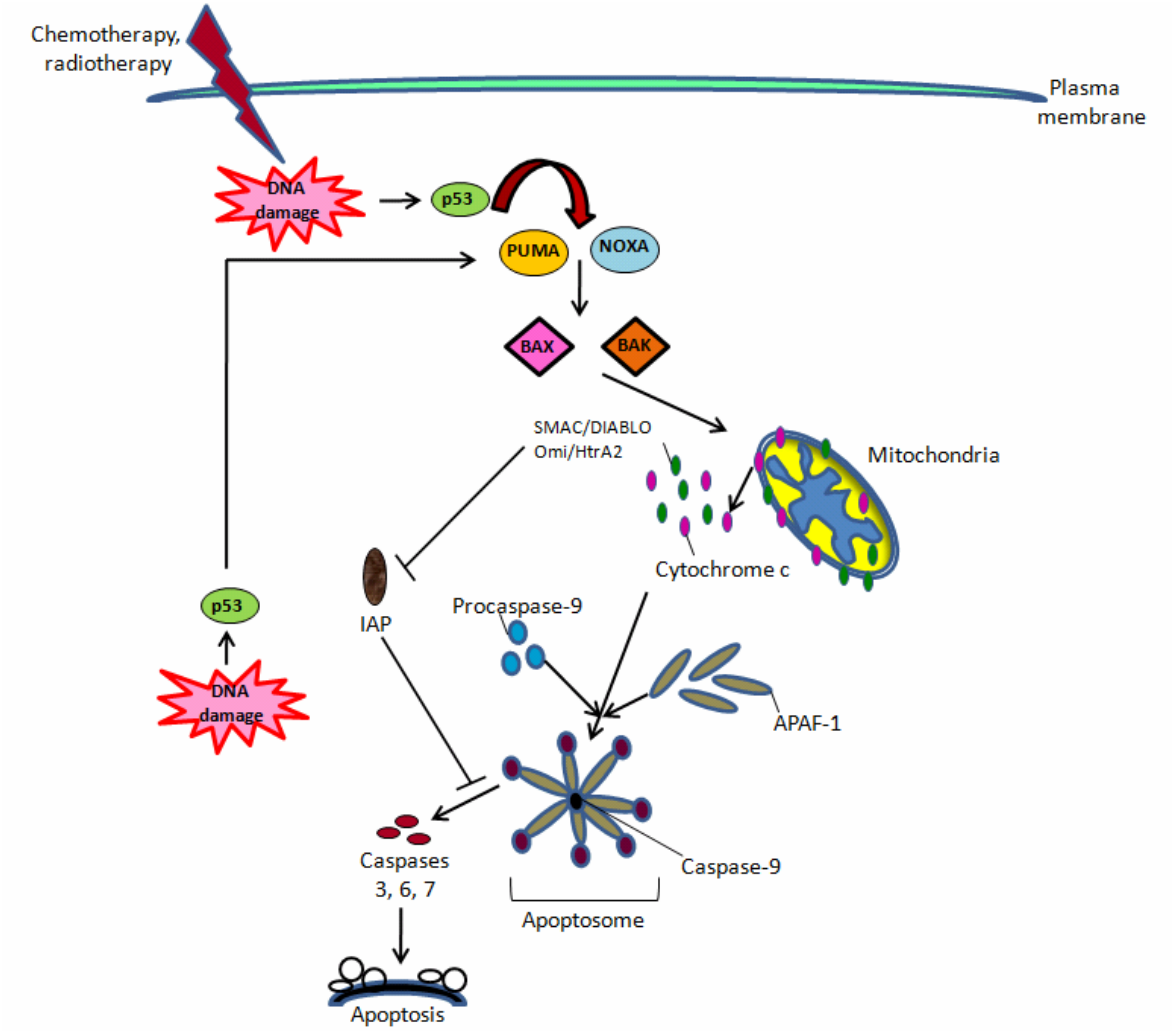

Fig.1.3 The intrinsic apoptosis p athway. Intracellular stress signals like DNA damage, defective cell cycle, hypoxia, detachment from extracellular matrix, loss of cell survival factor, etc., result in activation of the intrinsic apoptotic pathway. This signaling cascade involves the release of pro-apoptotic proteins from the mitochondria that activate caspases, ultimately culminating in apoptosis.
(modified from http://www.researchapoptosis.com/apoptosis/index.jsp).

from the ER are mediated by IRE1 (ER transmembrane bifunctional serine/threonine protein kinase/endoribonuclease), caspase-12, and PERK/CHOP (protein kinase-like endoplasmic reticulum kinase/ C/EBP homologous protein), respectively[29]. Caspase-12 was first detected in murine fibroblasts[31]. It is located exclusively on the cytoplasmic side of the ER membrane, and MEFs defective in this enzyme are partially resistant to ER stress-induced apoptosis. Upon ER stress, activated caspase-12 activates caspase-9, which in turn activates caspase-3, leading to

apoptosis[32]. An isoform of caspase-8, caspase-8L associates with ER membrane and is recruited to another ER surface protein called BAP31 (B-cell antigen receptor-associated protein) allowing self-activation[33]. It has been recently shown that neuronal apoptosis in INCL (Infantile neuronal ceroid lipofuscinosis) is caused by ER stress-mediated caspase-4 activation leading to caspase-3 activation and apoptosis[34]. Apart from unleashing active caspases, ER stress signals attract Bax and Bak (Bcl-2 homologous antagonist killer) to the ER membrane which also induce Ca^{2+} release[35].

1.2 Apoptosis regulation by the BCL-2 protein family

The best characterized regulators of apoptosis are the Bcl-2 (B cell lymphoma) family of proteins, which directly modulate outer mitochondrial membrane permeability during apoptosis. This family of proteins can be categorized into **anti-apoptotic members** and **pro-apoptotic members** (Fig.1.4). The Anti-apoptotic members such as Bcl-2, Bcl-xL, and Mcl-1 prevent the release of apoptogenic factors from mitochondria and therefore protect outer membrane permeabilization by a not fully understood mechanism[36]. The pro-apoptotic members consist of multidomain proteins (such as Bax and Bak) and BH3-only proteins (including Bid, Bim, Bik, Bad, Noxa, and Puma)[36, 37]. Many molecules are suggested to be involved for the formation of pores that releases Cytochrome c, including Bax, VDAC (voltage-dependent anion channel), the mitochondrial permeability transition pore PTP or lipids, through the involvement of Bak, Bax and/or VDAC is mostly suggested for the same[38]. It has been shown that Bak and Bax are essential for the release of Cytochrome c and apoptosis in response to various apoptosis inducers[39]. Bax is loosely attached to the outer membrane or sequestered in cytosol, whereas Bak has an anchor that attaches it to the mitochondrial outer membrane in a complex with the VDAC2[40]. Upon a death stimulus Bak and Bax undergo conformational changes, oligomerize and induce the formation of a pore in the OMM through which e.g. Cytochrome c and Smac are released. Another model proposes that Bax targets one or more components of the permeability transition in the IMM[41]. However, it has been recently shown that there is also another mechanism, which is Bax/Bak-independent, but serine protease(s)-dependent[42]. Bid is thought to induce the conformational change in Bax/Bak; by active caspase-8 cleaved BID protein, tBID, can oligomerise with Bax and they can form supra molecular pores in the OMM, which are large enough to release cytochrome c in the intrinsic pathway[43] (Fig.1.3 and 1.4).

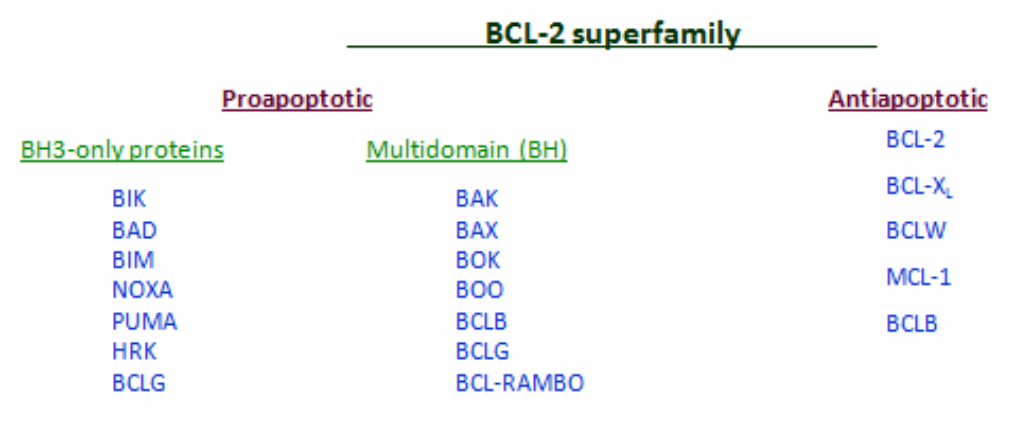

Fig.1.4 Pro- and anti-apoptotic members of the BCL-2 superfamily of proteins. BCL-2 protein superfamily plays a pivotal role in regulation of the intrinsic apoptotic pathway. Activation of its pro-and anti-apoptotic members modulate the permeability of the mitochondrial outer membrane.
(modified from http://www.researchapoptosis.com/apoptosis/index.jsp)

1.3 Caspases

The name "caspase" was derived from their cysteine protease mechanism "c" and the term "asp-ase", referring to their cleavage ability after an aspartic acid[44]. Caspases and their homologues are evolutionary wide spread and found in yeast, worms up to humans. However, meta-and paracaspases are found even in some bacteria, plants, fungi, protists, and animals[45, 46]. There are at least 14 distinct mammalian caspases, 12 of which are human[47]. When the caspases are classified into groups according to their structures, two main categories (caspase with long or short prodomain) are formed. Long prodomains comprise caspase-recruitment domain (CARD) and death-effector domain (DED), which are the responsive elements for the recruitment of initiator caspases into death- or inflammation-inducing signaling complexes, where caspases (caspases 1, 2, 4, 5, 8, 9, 10, 11, 12) are activated. Caspases 3, 6, 7 and 14 have short prodomains and are activated by other caspases upon proteolytic cleavage[47, 48] (Fig.5). On the other hand, classification of caspases by their primary function leads to two groups comprised of inflammatory and apoptotic caspases, whereas the latter can be divided into initiator (caspases 2, 8, 9, 10, 12) and effector (caspases 3, 6, 7, 14) caspases[49]. Caspases are expressed as inactive zymogens, the pro-caspases, which share a similar structure: A N-terminal prodomain followed by a large (~ 20 kDa) subunit, which contains the catalytic cysteine residue within a conserved QACXG motif, and a small subunit (~ 10 kDa). To

become catalytically active the zymogen is first cleaved between the large and the small subunit, and the prodomain is removed. Mature caspases are formed by association of two processed zymogens, with each zymogen comprising the large (p20) and the small (p10) subunits. Each tetramer contains two active sites formed by residues of both the large and small subunit. Active effector caspases are responsible for the regulated disassembly of the apoptotic cell by cleaving the death substrates[47, 50].

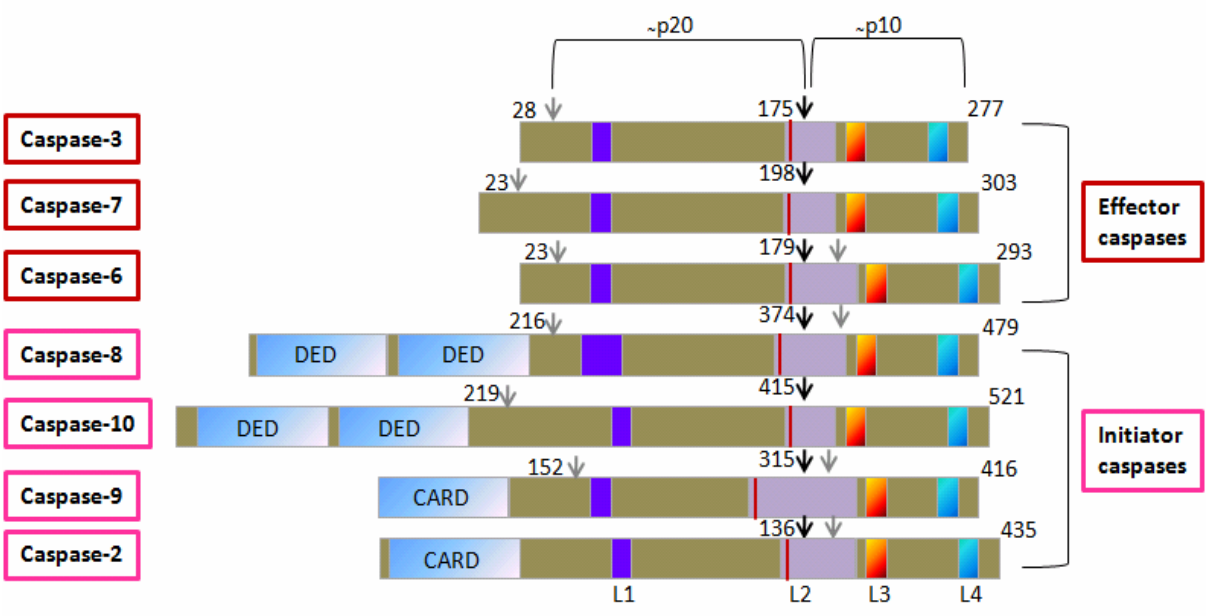

Fig.1.5 Apoptotic caspases in mammals. The effector and initiator caspases are shown in red and purple, respectively. The black arrows highlight the positions of the first intra-chain activation cleavage, and the grey arrows represent the other cleavage sites, which are thought to modulate caspase activity by inhibitor of apoptosis (IAP) proteins and by other proteins. The N-terminal pro-domain of the initiator caspases contains homotypic interaction motifs, such as CARD and DED, which are necessary for catalytic activity of the caspases. The p20 and p10 subunits together form a caspase monomer. The catalytic groove is shaped by the four surface loops (L1–L4), and the catalytic cysteine is indicated by a red line at the beginning of L2. The caspases and the location of functional segments are drawn to scale (modified from Ref.47).

1.4 The Inhibitor of apoptosis proteins

Inhibitor of apoptosis (IAP) proteins were first identified in baculoviruses 15 years ago by Lois Miller and colleagues as they protected infected cells from death[51]. IAPs are expressed in organisms ranging from yeast to mammals. There are eight mammalian IAPs, which include XIAP (X-linked IAP), c-IAP-1 and c-IAP-2 (cellular IAPs), ML-IAP (melanoma IAP)/Livin, ILP2 (IAP-like protein-2), NAIP (neuronal apoptosis-inhibitory protein), Bruce/Apollon and survivin (Fig.1.6), and two fruit fly IAPs, which include Diap 1 (also known as Thread) and

Diap2[52-54]. Even though the IAPs were identified as antiapoptotic proteins, which inhibit caspases, they are now known as a family of multifunctional proteins that also play crucial roles in receptor signaling, cell division, copper metabolism, and ubiquitylation of proteins for proteosomal degradation[53, 55, 56]. All the IAPs contain one to three BIR (baculorovirus IAP repeats) domains, although several IAPs contain as many as three. Characteristic BIR domain consists of about 80 amino acids with a conserved spacing of cysteine and histidine residues ($Cx_2Cx_6Wx_3Dx_5Hx_6C$) at the N-terminus, which suggests this structure represents a novel zinc-binding fold[52, 53]. But some BIR domain-containing proteins, such as mammalian survivin/BIRC5 and its homologues in yeast and invertebrates, are suggested not to inhibit apoptosis. A significant amount of *in vivo* data now shows that these IAPs regulate chromosome segregation during mitosis. Because of that, it can be misleading when these proteins have been defined as a member of the IAP family[57]. Therefore, proteins with BIRs can also be referred to as BIRc (BIR-containing) proteins. Except for survivin, all other IAPs/BIRCs with clearly defined roles in apoptosis also contain a highly conserved zinc ring finger (RING) at their C terminus, which has been implicated in E3 ligase activity to drive the proteasome-mediated degradation[56]. The human c-IAP-1 and c-IAP-2 are unique members, which contain a caspase recruitment domain (CARD), also present in many of the adaptor molecules controlling apoptosis signaling[58].

1.4.1 IAPs and caspase inhibition

In principle, to terminate the activity of a caspase two ways are suggested: a) removing them from the cell by using the ubiquitin-targeted proteasome degradation machinery, b) direct inhibition of its enzymatic activity. Evidently, some members of IAP family are capable of both functions[53]. Earlier studies concluded that all caspase-regulatory IAPs function as direct enzyme inibitors and block effector caspases by binding to their catalytically active pockets. While many IAP proteins can attenuate cell death, XIAP appears to be the only IAP family member to directly inhibit caspases (caspase-3, -7 and -9) with high affinity [47]. It has been shown that BIR domains of IAPs are responsible for the caspase-inhibition [53, 59]. In case of XIAP, the conserved linker region preceeding BIR2 seems to be the binding site of caspase-3 and -7, while the BIR2 domain itself serves as a regulatory element for caspase binding. However, caspase-9 inhibition takes place via BIR3 domain[60]. Both BIR domains use a two-site binding mechanism for potent caspase inhibition. One of these sites is a conserved surface groove found in BIR2 and BIR3 of XIAP, and in other BIR domains. Scott *et al* defined this

site as the IAP-binding motif (IBM)-interacting groove, because of its specificity for proteins containing an IBM at their immediate amino terminus[61]. These include Smac/DIABLO,

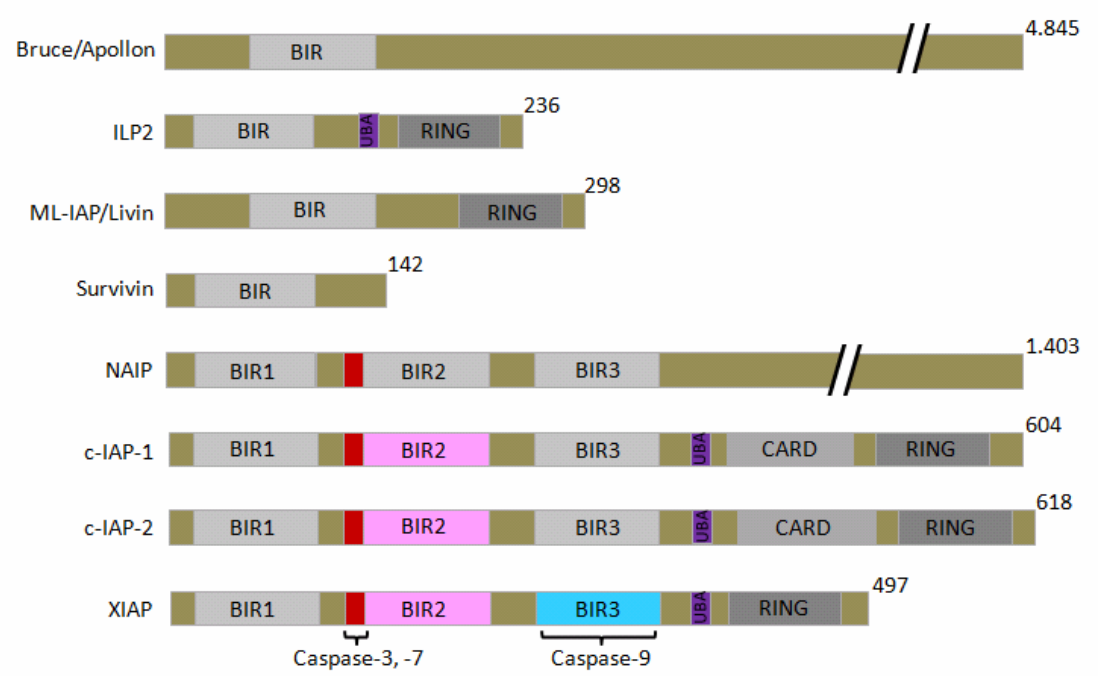

Fig.1.6 IAPs in mammals. Mammalian IAP family consists of XIAP (X-linked IAP), c-IAP-1, c-IAP-2, ILP2 (IAP-like protein-2), ML-IAP (melanoma IAP)/Livin, NAIP (neuronal apoptosis-inhibitory protein) and survivin, characterized by the presence of one to three BIR domains. A conserved linker region preceding BIR2 domain of XIAP, c-IAP-1 or c-IAP-2 (shown in red) is responsible for inhibiting caspases-3 and -7. In NAIP the same function is predicted to be carried out by residues 126–143, as shown in red. Only the BIR3 domain of XIAP can potently inhibit caspase-9. The BIR domains that are biochemically characterized to have known functions are highlighted in colour, whereas other domains in the various IAPs are shown in grey. (RING, really interesting new gene; UBA, Ubiquitin-associated domain) (modified from Ref.47).

Omi/HtrA2, and the Drosophila proteins Hid, Grim and Reaper[62]. BIR1 domain does not contain the IBM-interacting groove[53]. Similar to DIAPs, the mammalian IAP c-IAP1 interacts with caspase-7 in an exclusively IBM-dependent, but active site pocket-independent, manner[63]. The BIR3 domain of XIAP forms a heterodimer with a caspase-9 monomer, thus preventing the formation of the catalytic active dimer form of caspase-9. The specific recognition of the caspase-9 dimerization interface requires four amino acids in the BIR3 domain of XIAP, which are not conserved in c-IAP-1 or c-IAP-2. Because of that, c-IAP-1 and c-IAP-2 can bind to, but do not inhibit, caspase-9. It should be noted that c-IAPs can bind caspases *in vitro*, in an active-site-independent manner, although the significance of this has yet to be established[63, 64]. Unlike XIAP and IAP-like protein 2 (ILP-2), c-IAPs lack the crucial residues required for the direct inhibition of caspases[65]. Interestingly, ILP-2 is highly unstable as the expression of this IAP in cells needs to be established. Survivin, the smallest member of the IAP family, has a single

BIR domain, which is closely related to the BIR3 of XIAP and several studies showed that survivin is capable of binding caspase-3, -7 and -9 but this is still controversially discussed[66]. However, together with hepatitis B X-interacting protein (HBXIP), survivin might prevent recruitment of caspase-9 to Apaf-1 and its activation at the apoptosome[53]. c-IAP-1 and c-IAP-2 have been also shown to prevent binding and activation of caspase-8 to the TNF receptor[67].
It is far from clear which mammalian IAP could perform targeting of activated caspases for proteasomal degradation. For example, XIAP might participate in caspase polyubiquitylation and monoubiquitylation *in vitro*, but this ubiquitylation might not result in degradation by the proteasome. There is also some evidence that c-IAP-2 can also monoubiquitinate executioner caspases *in vitro*, but this modification is not normally associated with proteasome targeting and degradation. Bruce/Apollon can bind caspase-3, -7 and -9, and can ubiquitinate caspase-9 *in vitro*, but the mechanism is unclear[53]. Vucic *et al* showed that ML-IP is not a strong inhibitor of caspase-9, and the mutation of three residues in its BIR3 domain endowed ML-IAP with potent caspase-9-inhibitory function[68].

1.4.2 How are the IAPs regulated?

IAPs can be regulated by transcriptional/post-transcriptional and post translational levels or by binding of regulatory proteins[69].

1.4.2.1 Regulation of IAP expression

Expression levels of certain IAPs are subjected to tight transcriptional control. For example, TNF-R1 triggers NF-κB activation upon TNF ligand binding, leading to nuclear translocation and transcription of important cell survival molecules, and c-IAP-1, c-IAP-2 and XIAP are regulated via this TNF-R1-NF-κB pathway[67, 69]. Morover, it has been reported that JNK1 and JNK2 double knockout MEFs show increased sensitivity to TNFα-induced cell death, that this increased sensitivity is due to defective JNK-mediated upregulation of c-IAP-2[70]. Synthesis of XIAP protein is also regulated at the translational level by a cap-independent mechanism of translation initiation which is mediated by a 162-nucleotide unique internal ribosome entry site (IRES) sequence located in the 5' untranslated region (UTR) of XIAP mRNA. Furthermore, this sequence allows XIAP mRNA to be actively translated during conditions of cellular stress when the majority of cellular protein synthesis is inhibited[71]. The heterogenous nuclear ribonucleoprotein A1 (hnRNPA1) can interact with XIAP IRES RNA both *in vitro* and *in vivo* and negatively regulates XIAP IRES activity. Morover, XIAP IRES-dependent translation is

significantly reduced when hnRNP A1 accumulates in the cytoplasm under cellular stress like osmotic shock[72].

XIAP-deficient mice showed no changes in development, histology, and not even in the induction of caspase-dependent and –independent apoptosis. This is probably due to the compensation of the loss by elevation of c-IAPs levels in XIAP-deficient mice[73]. However, another independent study showed that XIAP deficiency in mice exhibits delays in the development of the mammary gland in a manner that correlates with delayed NF-κB activation[74]. Considering the binding affinities of c-IAPs to the caspases and that of XIAP to adaptor molecules like TRAF it is tempting to propose that IAPs are functionally non redundant[69]. Because XIAP-deficient mice display no overt apoptotic phenotype, the primary function(s) of XIAP in most normal tissues may be to regulate pathways, which are distinct from those involving apoptotic caspase cascades. This has similarity to many apoptotic regulators like cytochrome c and Bad, which under normal conditions participate also in other processes than cell death regulation[75, 76]. Another study showed that the survivin protein is upregulated in G2/M phase and is transcriptionally regulated by the oncogenic transcription factor STAT3[77].

1.4.2.2 Post-translational control of IAPs

The RING domain had been identified in several cellular proteins with a range of functions, and the RING finger proteins play also critical roles in the ubiquitin-proteasome system. They transfer Ubiquitin (a 76-residue protein) onto specific lysine residues in the target protein[56]. In the case of XIAP, the E3 activity of XIAP mediates also auto-ubiquitylation, which occurs at the residues lysine-322 and lysine-328 within the BIR3 domain[78]. Ubiquitylation of these residues and subsequent degradation may play an important role in self-regulation of their abundance and activity, which may be a mechanism to facilitate apoptosis[78-80]. Mutations of the RING domain reduce but do not abolish the antiapoptotic effects of XIAP[81]. Intriguingly, degradation of XIAP can be markedly reduced by phosphorylation of Serine-87 by AKT1 and AKT2[80, 82]. The transactivation domain of Notch, a novel binding partner of XIAP, interacts directly with the RING region of XIAP to block the binding of E2 and prevent the *in vivo* and *in vitro* ubiquitylation and degradation of XIAP[83]. It has been recently shown that X-linked inhibitor of apoptosis-associated factor 1 (XAF1) mediates Survivin down-regulation through a XIAP-XIAF1-Survivin complex, in which XAF1 activates E3 activity of XIAP and targets Survivin by direct ubiquitylation[84].

1.4.2.3 Inhibitors of IAPs

Apart from the inhibition of XIAP through autoubiquitylation, other, direct inhibitors are known. Smac/DIABLO, Omi/HtrA2 and XAF1 are well-studied inhibitors of XIAP, which belong to a family of proteins that contain an IAP-binding motif[67, 85]. However, there are other inhibitors, which are not so well understood, e.g. the mitochondrial ARTS protein[86].

Once released into the cytosol during apoptosis, Smac/DIABLO binds directly to XIAP via its own N-terminal IAP binding domain that interacts with BIR2 and BIR3 of XIAP, and thereby disrupts its function to block caspase-3, -7, and-9[69]. Smac/DIABLO has also been described to inhibit several other members of the IAP family including c-IAP1, c-IAP2, ML-IAP and survivin but not NAIP[87-89]. Smac/DIABLO-XIAP-binding can reduce the E3 ubiquitin ligase activity of XIAP which may interfere with the inhibitory capacities of XIAP but does not result in ubiquitylation of XIAP. Stressing that, rapid degradation upon massive ubiquitylation mediated by Smac/DIABLO is reported for c-IAPs but not for XIAP. On the other hand, IAPs are able to regulate the effect of Smac/DIABLO. Thus, rapid degradation of Smac/DIABLO after its release has been reported as well as the ability of XIAP to ubiquitinate Smac/DIABLO *in vitro*[56, 85].

The serine protease Omi/HtrA2 is a mitochondrial protein, which upon apoptotic induction is released to the cytosol to bind IAPs. This binding disrupts the inhibitory interaction of IAPs with caspases, inactivates XIAP by cleavage and results in caspase activation. However, Omi/HtrA2 is able to induce apoptosis in a caspase-independent manner too. These effects seem to be linked to its serine protease activity[85].

1.4.3 IAPs – More than caspase inhibitors

IAPs are known to have crucial roles in caspase inhibition. But IAPs also have activities that are unrelated to caspase inhibition. They play critical roles in receptor signaling, cell division, copper metabolism, and ubiquitylation of proteins for proteasomal degradation[53, 55, 90].

Evidence for linking IAPs to NF-κB came with the observation that XIAP also activates NF-κB by promoting its translocation to the nucleus, and by promoting the degradation of the NF-κB inhibitor IκB. The E3 ubiquitin ligase activity of the RING domain of XIAP is required for activation of NF-κB and JNK by XIAP, and this effect is dissociated from the caspase inhibitory effects of XIAP. Thus, inhibitors of XIAP that target its RING domain, could also induce apoptosis by blocking NF-κB activation[91]. The signaling function of XIAP in NF-κB and MAP kinase activation is conserved in at least two other IAP members, NAIP and ML-IAP, while the caspase inhibitory function of XIAP does not appear to be conserved in other

IAP family members. In addition, association of c-IAP-1 and c-IAP-2 with TRAFs in the TNF signaling pathway may facilitate or regulate TRAF-mediated NF-κB and MAP kinase activation[67].

XIAP also inhibits apoptosis through mechanisms unrelated to its ability to bind caspases. For example, during TGF-b and BMP signaling or upon XIAP upregulation, XIAP forms a complex with a MAP kinase kinase kinase (MAP3K), TAK1, and its cofactor TAB1 that activates JNK signal transduction pathways. Activated JNK initiates the MAP kinase phosphorylation cascade, which leads to activation of NF-κB[91]. It has been recently shown that the BIR1 domain of XIAP, which has no previously ascribed function, directly interacts with TAB1 to induce NF-κB activation. TAB1 is an upstream adaptor for the activation of TAK1, which in turn couples to the NF-kB pathway[92]. Previous studies have suggested that XIAP RING domain involves in XIAP oligomerization[93, 94]. This oligomerization ability of the RING domain of XIAP could be the reason for its requirement in some situations[95]. Therefore, it appears that while the RING domain may make XIAP a constitutive oligomer, the BIR1 domain mediates a specific and dynamic dimerization for TAK1 activation[92]. BIR1 domain is highly conserved across species. One surface of BIR1 is responsible for TAB1 interaction while the opposite surface is used for BIR1 dimerization, which is similar to the Smac binding surface of BIR2 and BIR3[92]. In this context, it is interesting to note that the BIR1 domain of two other IAP family members, c-IAP1 and c-IAP2, has also been shown to mediate signaling and dimerization. The BIR1 domain of these two cellular IAPs leads to the interaction with TRAF-1 and TRAF-2[96-98]. Such as XIAP and c-IAPs, NAIP and ML-IAP may also induce TAK1 activation via a similar TAB1-mediated mechanism[92].

Kaur *et al* illustrate a novel role of XIAP during protection from TGF-β1-induced apoptosis and showed that NF-κB plays a role in the up-regulation of *XIAP* gene expression in response to TGF-β1 treatment and forms an -inducible complex with TAK1. Furthermore, the RING domain of XIAP mediates TAK1 polyubiquitylation for proteosomal degradation. This degradation of TAK1 inhibits TGF-β1-mediated activation of JNK and apoptosis. Conversely, transient knock down of XIAP leads to persistent JNK activation and potentiates TGF-β1-induced apoptosis[99]. But the results of Kaur *et al* contradict other findings explained above.

Evidence also implicates XIAP as a regulator of the cell cycle. XIAP binds to the cell cycle regulator MAGE-D1, but the significance of this interaction is unclear[100].

The recent reports have shown that XIAP also controls intracellular copper levels, both through ubiquitin ligase-dependent regulation of the copper binding protein COMMD1[101] and by binding copper directly[102].

In muscle of insulin-deficient mice, XIAP was decreased while total muscle protein degradation, caspase-3 activity, and myofibril protein destruction were increased, and overexpression of XIAP by a recombinant lentivirus attenuated the excessive muscle protein degradation, reduced proteasome activity, caspase-3 activity and myofibril protein breakdown[103].

A transient interaction of XIAP via its BIR3 domain with checkpoint kinase 1 (Chk1) during mitosis may imply a mechanism of coupling between the regulatory networks that control cell cycle progression and apoptosis[104].

In the nucleus, survivin binds to microtubules and assists in chromosomal segregation and cytokinesis during mitosis. In the cytoplasm, survivin inhibits apoptosis as described in (**IAPs and caspase inhibition).** Survivin is also expressed in normal proliferating hematopoietic cells as well as in terminally differentiated neutrophils, where following upregulation by hematopoietic growth factors, it inhibits apoptosis independent of the cell cycle[105]. Down-regulation of XIAP and Survivin after treatment of A172 human glioma cells with ciglitazone (a synthetic peroxisome proliferator-activated receptor-gamma (PPARgamma) agonist) is responsible for the ciglitazone induced caspase-independent cell death and these findings suggest that XIAP and Survivin may play an active role in mediating a caspase-independent and –PPARgamma-dependent cell death[106].

Very recently Aird *et al* has identified a novel functional link between epidermal growth factor receptor-2 (ErB2) signaling and antiapoptotic pathway mediated by XIAP[107].

1.5 MAP kinase pathways

Mitogen-activated protein (MAP) kinase pathways are signal-transduction protein phosphorylation cascades, which consist of three well characterized major groups, including extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinases (JNK), and p38. In response to diverse range of stimuli containing growth factors, cytokines, irradiation and osmotic stress they can be activated[108].

1.5.1 The RAS/RAF/MAPK Pathway

The best characterised MAP kinase signaling pathway is the RAS/RAF/MAPK signal transduction pathway. The function of this pathway is to transduce signals from the extracellular milieu to the cell nucleus where specific genes are activated for cell growth, division and differentiation. However, cell cycle regulation, wound healing and tissue repair, integrin signaling, cell migration and angiogenesis are also other cellular processes, in which

the RAS/RAF/MAPK play a remarkable role[109-111]. Thus, signaling through this pathway is also important for tumorigenesis.

Activation of the pathway begins when a signal binds to a protein tyrosine kinase receptor. The epidermic growth factor receptor (EGFR) and the platelet-derived growth factor receptor (PDGFR) are the best-known receptors in the pathway. Binding of ligand to one of these receptors induces oligomerization of the receptor, a process that results in juxtaposition of the cytoplasmic, catalytic domains in a manner that allows activation of the kinase activity and transphosphorylation[112]. The ligand binding leads to the activation of the small GTP-binding protein RAS. RAS is activated by nucleotide exchange factors that stimulate the release of RAS-bound GDP and thereby promote its exchange for GTP. RAS in its GTP-bound form binds to a range of effector molecules including PI3-kinase, Ral-GDS and RAF kinases[113, 114]. Once activated, all RAF family members activates MEK and MEK in turn activates ERK, which then can phosphorylate numerous substrates to relay the signals initiated by the growth factor receptors[115].

1.5.2 RAF kinases

The receptor tyrosine kinase effector, RAF (Rapidly Accelerated Fibrosarcoma), was discovered by two groups independently as a retroviral oncogene, v-Raf or v-MIL[116, 117]. RAF is the best characterized RAS effector and is a member of a family of serine/threonine kinases. Whereas mammals express three isoforms of RAF, invertebrates have only one of the RAF isoforms resembling the mammalian B-RAF isoform; D-RAF in *Drosophila*, and Lin-45 in *Caenorhabditis elegans* were identified. While A- and B-RAF display a tissue-specific pattern of expression, C-RAF (Raf-1 or c-Raf-1) is widely expressed[118].

1.5.2.1 RAF regulation

All three mammalian RAF kinases (A-, B, and C-RAF) share a common structure comprising three conserved regions (CR): CR1 contains two RAS-binding sites required for RAF membrane recruitment; the RAS binding domain (RBD), which binds to the active RAS-GTP, and the cysteine rich domain (CRD) that has a zinc finger structure homologous to the zinc fingers of PKCs and stabilizes the association with RAS through interaction with a lipid moiety present on processed RAS. CR2 of C-RAF has a conserved phosphorylation site at S259, which is a known regulatory binding site for 14-3-3 proteins. CR3 contains the catalytic portion having the RAF kinase domain and a conserved serine at S621 in C-RAF, which is another second binding site for 14-3-3[118] (Fig.1.7). The N-terminal half of RAF has been

shown as a negative regulatory domain and in the absence of stimulation this domain helps to maintain RAF in an inactive state[119]. The catalytic domain of RAF can be folded and binds to the N-terminal regulatory domain, and the binding of a 14-3-3 dimer at two C-RAF phosphorylates sites, S259 and S621 (and the corresponding sites on the other RAF isoforms, Fig.1.7) stabilizes this interaction. On the other hand, binding of 14-3-3 to the S259 site is disrupted by the binding of RAS-GTP to the RBD and CRD domains and this binding brings about conformational changes in C-RAF necessary for its stable activation[118]. It also suggested that RAS-C-RAF interaction can expose a docking site for MEK, which is downstream target of C-RAF[120].

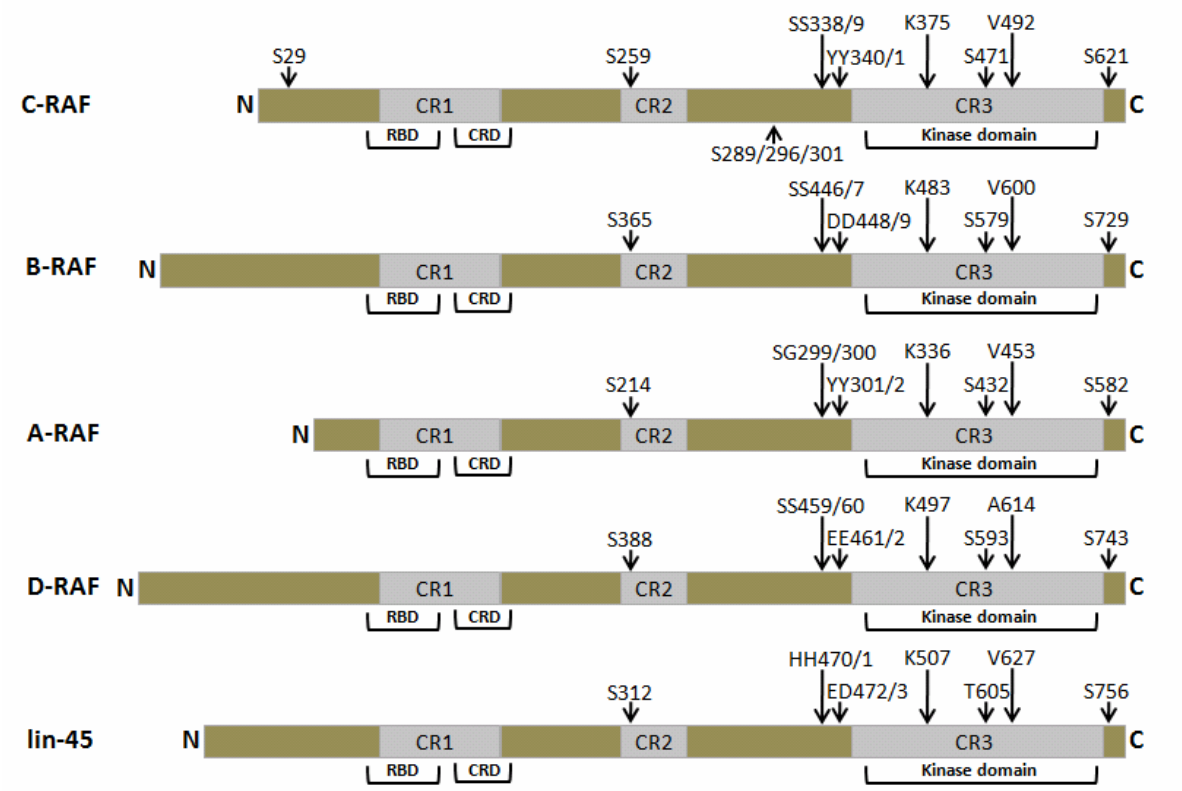

Fig.1.7 Structural align ment of R AF isoforms. The human RAF isoforms (A-, B- and C-RAF) are aligned with the *Drosophila* RAF (D-RAF) and *C. elegans* RAF (lin-45). The positions of conserved regions (CR1, 2 and 3) are indicated. The CR1 consists of the RAS binding domain (RBD) and the zinc finger/cysteine rich domain (CRD), and CR3 consists of the kinase domain. The known phosphorylation sites on C-RAF and their corresponding sites in the other RAF isoforms, position of the ATP binding site (K375) and V600 of B-RAF found mutated in cancers are also indicated (modified from Ref.118).

1.5.2.1.1 Regulation of RAF by phosphorylation

The events leading to activation of RAF is not fully understood which encompass multiple phosphorylation and dephosphorylation events coupled to conformation changes assisted by the binding of multiple proteins. There are at least thirteen regulatory phosphorylation sites on C-RAF[121]. When C-RAF is inactive some of these sites (e.g., S43, S259 and S621) are

phosphorylated. As explained above, S259 and S621 phoshorylations allow 14-3-3 to bind C-RAF and then C-RAF changes its own configuration and becomes inactive. Phosphatases such as *p*rotein *p*hosphatase 2 *A* (PP2A) dephosphorylate S259 leading to disassociation of 14-3-3 from C-RAF. This allows C-RAF to be phosphorylated at S338/9 and Y340/1, rendering C-RAF active[122]. Prohibitin (PHB) was also shown having important role for this displacement to facilitate plasma membrane localization and phosphorylation of C-RAF at Serine 338[123]. Both the *p*21 Rac-*a*ctivated *k*inase family (PAK) and the Src family of tyrosine kinases can mediate phosphorylations at these sites. The phosphorylations at S338/9 and Y340/1 release the inhibitory effect of the N-terminal part on the catalytic domain and stabilize the active form of C-RAF. On the other hand, it has been recently shown that protein phosphatase 5 (PP5) is required for dephosphorylation of C-RAF Ser338 in cells and inactivation of C-RAF-MEK-ERK signaling[118].

The phosphorylation sites Y340 and Y341 for Src family kinases are conserved in A-RAF (Y299 and Y300), but are replaced with aspartic acid (D) at the corresponding positions in B-RAF (D492 and D493)[122]. The RAS-dependent phosphorylation of T491 and S494 in the kinase domain activation loop of C-RAF is indispensable for its activation[110]. As other autophosphorylation site and a target site for a ceramide activated kinase or KSR (kinase suppressor of Ras-1) S268 and T269 were suggested[118]. It has been recently shown that while phosphorylation of C-RAF at 471 promotes its kinase activity and MEK binding, S43, S233, and S259 are thought to be a negative regulatory site targeted by PKA or PKB (S259), which creates additional binding sites for the 14-3-3 proteins[124-126]. Maximal activation of C-RAF and A-RAF requires both RAS and SRC activity while B-RAF activation is SRC-independent[122]. Interestingly, with the exception of A-RAF and Lin-45, the S338/339 sites are conserved among most of the known RAF proteins[118]. However, in B-RAF (S445) this corresponding site is found constitutively phosphorylated in resting cells, where the kinase is inactive, suggesting that phosphorylation at these sites, though may be an essential step in the activation process, does not activate RAF per se[127]. Thus, the potential activating RAF phosphorylation site(s) remains to be examined. Recent studies showed a couple of novel activating C-RAF phosphorylation sites: S29, S296, S289, S301 and S642. They all have a
proline-directed phosphorylation motif and their phosohorylations are largely mediated by a proline directed kinase family member, ERK[125, 128, 129].

In case of B-RAF, the V600E substitution leads a more open configuration of B-RAF, which may recruit its substrate better. This suggests that in response to growth factor stimulation

under normal conditions phosphorylations at proximal sites to V600 may be responsible for B-RAF activation[110, 130].

1.5.2.1.2 Regulation of RAF by its binding partners

Not only phosphorylation of RAF kinases controls their regulation, various protein-protein interactions also play key roles in RAF regulation including RAF-RAS and RAF-14-3-3 interactions, RAF homo-and heterodimerization, and association with chaperones such as Hsp-90, and Hsp/Hsc-70[118]. For example, binding of Hsp90 to C-RAF may serve to stabilize activated C-RAF, while Geldanamycin, an inhibitor of Hsp90, inhibits this binding and result in the rapid degradation of C-RAF[131].

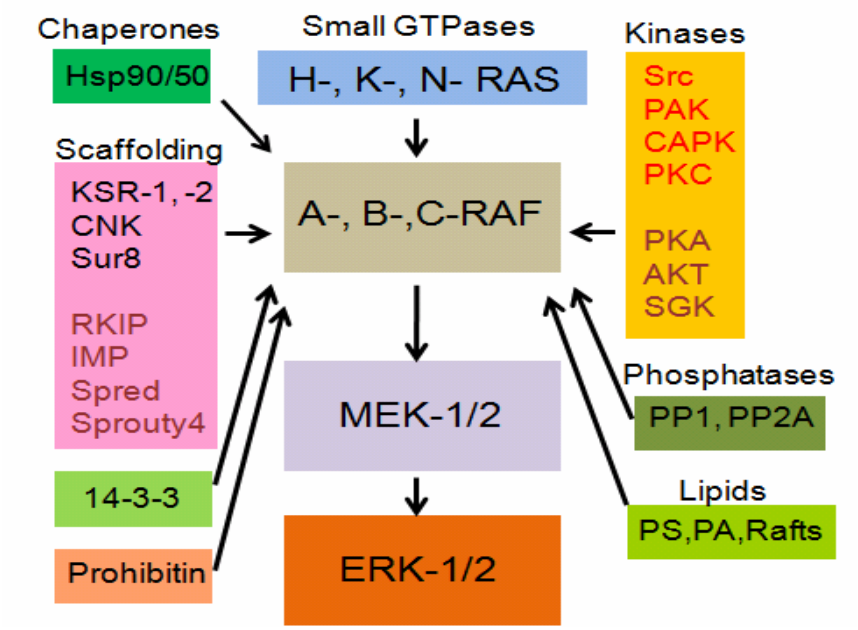

Fig.1.8 RAF kinase signaling and its regulation by interacting molecules. RAF kinases have a plethora of interacting molecules regulating their signaling at multiple levels. Shown are the proteins modulating RAF activation and intracellular localization like kinases, phosphatases, scaffolding proteins and chaperones (modified from Ref.132).

RAF is also regulated through association with various scaffold proteins such as KSR, RKIP, CNK and others[115, 132] (Fig.1.8). For example, KSR1 (kinase suppressor of Ras-1) can bind all kinase members of the ERK/MAPK pathway and is necessary for functional RAF/MEK complex formation, which is limited by a RAS effector, IMP (impedes mitogenic signal propagation)[133, 134]. RKIP-1 (RAF kinase inhibitory protein 1) has been shown to inhibit C-RAF activation and downstream MEK is attenuated when RKIP-1 blocks the interaction between C-RAF and MEK[135].

Another evolutionary conserved protein named prohibitin (PHB), which can directly interact with C-RAF, is required for the displacement of 14-3-3 from C-RAF and indispensable for the activation of the RAF-MEK-ERK pathway by RAS[123].

1.5.3 The RAS/RAF/MAPK pathway and cell motility/migration

Cell migration is a complex and highly coordinated process which plays a vital role in many physiological and pathological situations such as angiogenesis, wound healing, embryonic development and tumor metastasis[136]. The MAPK family proteins including JNK, ERK and p38 play crucial roles in cell migration, and the ERK MAPKs are the most extensively studied subfamily of MAPKs. While ERK translocates into the nucleus and phosphorylates transcription factor complexes, ERKs also have a number of cytoplasmic substrates that can influence cell motility. There are some substrates identified for ERK, which involve in ERK-mediated cell migration: such as MLCK (Myosin light chain kinase), calpain and FAK (a non-receptor protein tyrosine kinase). MLCK is phosphorylated by active MEK and also by both ERK1 and ERK2, and critical in MLC mediated myosin function. ERK phosphorylates m-calpain protease, and this activation is required for adhesion, turnover and cell migration. It is known that the RAS/RAF/MEK/ERK pathway regulates integrin activation. ERK might participate in cell migration by suppressing the binding of integrin to their ligands. Integrin activation of MAPK requires c-Src and FAK, and ERK activity is required for FAK stimulated focal adhesion disassembly to promote cell migration[137, 138]. The small GTPases of the Rho family are key regulators of cytoskeletal dynamics and of migration. The activities of Rho and its effector, ROCK, can be suppressed by FAK or v-Src, which need ERK signaling to modulate actin and focal adhesion dynamics[139]. It has been shown that in response to cell matrix proteins, such as fibronectin, vitronectin and collagen, the best known MEK/ERK inhibitors PD98059 and UO126 inhibit the migration of diverse cell types. Also, growth factor (such as EGF, FGF, VEGF, and insulin) -mediated cell migration is blocked by these inhibitors. Morover, a dominant negative ERK mutant or inhibition of ERK by siRNA also inhibits cell migration[136, 138]. It is known that the SDF-1α and CXCR4 play a crucial role in adhesion and transendothelium migration (TEM) of prostate cancer cells[140] and a recent report showed that the chemokine stromal-derived factor-1α (SDF-1α/CXCL-12) –induced expression of CXCR4, (receptor of SDF-1α), in PC3 cells is dependent on MEK/ERK signaling cascade and NF-κB activation[141]. CDC42 is a well-known regulator of cell chemotaxis, and activation of CDC42 controls the formation of filopodia and downregulates MMP-1 (matrix metalloproteinase-1) expression by inhibiting the ERK1/2 pathway[142, 143]. Szczur K *et al*

showed that CDC42 activity appears to mediate neutrophil chemokinesis via ERK-dependent podosome-like structures[144]. On the other hand, Rac induces membrane protrusion via lamellipodia, and ERK is also implicated in the co-ordinate control of Rho and Rac activities in fibroblast models of transformation and human tumor cells[136]. A very recent study reported that activation of LMP1 (latent membrane protein 1), which is the major transforming protein of Epstein-Barr virus (EBV), by the ERK/MAPK pathway is responsible for LMP1-induced cell motility and haptotactic migration[145]. Thus, ERK is an important factor in the regulation of cell migration.

The direct role of RAF kinases in cell migration is not well understood. It has been suggested that Rac can potentiate C-RAF's ability to activate MAPK through p21-activated kinases, one of the downstream targets of Rac[146]. However, Rac is able to synergize with C-RAF and this synergy increases the chemotactic sensitivity of COS cells to epidermal growth factor by 1000-fold[147]. After observation of inhibitory effect of overexpressed wild type C-RAF or the catalytic domain of C-RAF on the EGF-stimulated migration, it has been suggested that, wheras RAS activity is necessary to promote EGF-stimulated migration, sustained activation of C-RAF may be important in down-regulation of migratory signaling pathways triggered by EGF-R activation[148]. More recently, RKIP (RAF kinase inhibitor ptotein) has emerged as an important suppressor of metastasis[149]. Another observation in this topic is that C-RAF is required for normal wound healing *in vivo* and for the migration of keratinocytes and fibroblasts *in vitro*. The target of C-RAF in motility is the Rho effector Rok-α, which is hyperactive and mislocalizes to the membrane of C-RAF-deficient cells that showed a symmetric, contracted appearance, characterized by cortical actin bundles and by a disordered vimentin cytoskeleton. For efficient migration, kinase activity of C-RAF is not required in fibroblasts[150].

1.6 Molecular chaperones

Proteins must fold into their native three dimensional conformations after being synthesized and maintain this state throughout their lifetime. Molecular chaperones help in initial folding of proteins to their native form, and also the assembly of multi-protein complexes. Also proteins that are translocated into mitochondria or endoplasmic reticulum, need molecular chaperones associated with these cellular compartments for their folding[151]. Molecular chaperones are involved not only in the folding of proteins but also in their quality control including recognition of misfolding, prevention of protein aggregation and facilitation of refolding of partially unfolded proteins. Failure of correct folding in polypeptides leads to their aggregation

in the aqueous cellular environment. This aggregation can result in the formation of toxic protein precipitates, which can further cause severe disease conditions such as Alzheimer's disease, Parkinson-disease or Creutzfeldt-Jakob-disease in humans or bovine spongiform encephalopathy (BSE) in cattle[151, 152] (Fig.1.9). This whole process is essential to all cells.

A variety of molecular chaperones have been found and characterized upto date. Based on differences regarding size, structure, function, mechanism or cellular compartmentalization, different families of chaperones can be classified: Hsp100/Clp, Hsp90, Hsp70, chaperonins (Hsp60), small Hsps and calnexin/calreticulin[151, 153].

In cooperation with co-chaperones, molecular chaperones refold misfolded or unfolded proteins. However, these proteins are subjected to proteasomal degradation when the capacity for refolding is inadequate. E3 ubiquitin ligases play crucial role in protein quality control and could prevent the accumulation of such abnormal proteins[154].

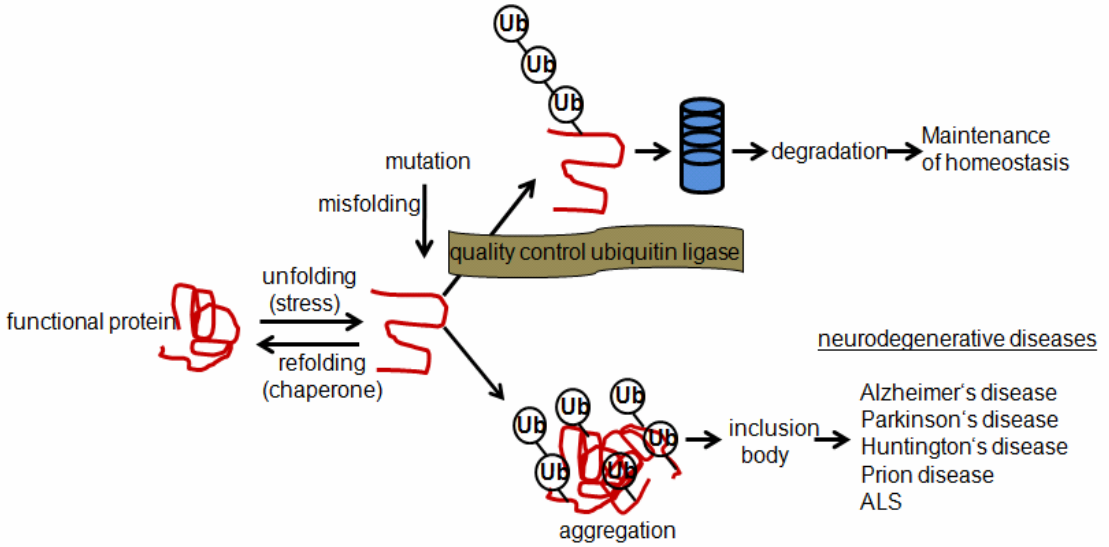

Fig.1.9 Concept of p rotein quality control ubiquitin ligase in the pathogenesis of neurodegenerative disease. Environmental stresses such as heat and oxidation, and genetic mutations (heredity diseases) result in protein misfolding. These misfolded proteins are rescued by molecular chaperones that refold them in cooperation with co-chaperones. Nonetheless, excessive protein misfolding, exceeding the refolding capacity of chaperones, results in activation of protein quality control system. Ubiquitin ligases have a pivotal role in protein quality control system. Proteins evading refolding as well as proteosomal degradation form aggregates and toxic inclusion bodies that cause neurodegeneration. Accumulation of such abnormal gene products can be obviated by E3 ligases of proteosomal degradation machinery (modified from Ref.154).

1.6.1 Hsp70 and Hsp90

The Hsp70 proteins are the most abundant class of molecular chaperones and are found in a rich diversity. They are involved in nearly every protein folding process and constitute a central component of the cellular chaperone network[155]. Hsp90 is one of the most abundant

cytosolic proteins in the cell and not a single player, its function is rather highly dependent on specific cofactors that recruit client substrates, regulate its ATPase cycle, and capture the substrate[156]. Hsp90 often works together with Hsp70, because of that the two chaperones could be considered as parts of a single multichaperone machinery, whose activities are modulated by a wide range of cofactor proteins. These cofactor proteins can interact directly and specifically with either, or in some cases, both of the chaperones. Both Hsp90 and Hsp70 contain a similar cofactor binding motif that is unique for the eukaryotic chaperone system: Separate TPR (tetratricopeptide repeat) domains of Hop/Sti1 (Hsp-organizing protein), a cochaperone, specifically interact with the distinct C-terminal motifs of Hsp70 (IEEVD) or Hsp90 (MEEVD) and bridges both chaperones, and it is thought to promote the substrate transfer from Hsp70 to Hsp90[154, 156, 157] (Fig.10).

Hsp90 ensures the correct conformation, intracellular localization, activity and proteolytic turnover of various proteins, which play crucial roles in cell growth, differentiation and survival[158]. Another importance of Hsp90 is its essential place for the stability and the function of many oncogenic client proteins include ERBB2, BCR-ABL, AKT/PKB, C-RAF, B-RAF(V600E), CDK4, PLK-1, MET, mutant p53, HIF-1α, streoid hormone receptors, survivin and telomerase hTERT[159].

Hsp90 has been reported to regulate RAF in the RAS-RAF-MEK-ERK pathway. For example, disruption of the C-RAF-Hsp90 molecular complex leads to destabilization of C-RAF and loss of C-RAF-RAS association[160]. Dou *et al* showed that Hsp90, Hsp70 and RAF exist in the same complex, which indicates that RAF interacts directly with molecular chaperones and they found that the Hsp90 inhibitor regulates tau phosphorylation through the RAF-MEK-ERK pathway by releasing RAF from the Hsp90 complex. Once it is released from the complex, RAF will be more vulnerable to the proteasome[161]. In contrast to C-RAF and A-RAF, which require Hsp90 for stability, B-RAF does not need it, whereas mutationally activated B-RAF (V600E) binds to an Hsp90-Cdc37 complex, which is required for its stability and function[162, 163].Very recently, regulatory role of extracellular Hsp90 in cell motility of human dermal fibroblast cells was reported, and also this report showed that treatment of mice with cream that contained recombinant Hsp90 protein enhanced wound healing *in vivo*[164].

Inhibition of Hsp90 function results in the proteasome-dependent degradation of associated client proteins. This can occur by the recruitment of the E3-ubiquitin ligase, CHIP, which is a TPR protein that is able to interact with both Hsp70 and Hsp90. Another co-chaperone, which helps Hsp70-Hsp90 machinery to interact with the ubiquitin-mediated proteasome-degradation system, is Bcl-2-associated athanogene-1 (Bag1). The anti-apoptotic protein Bag1 regulates the

ATPase cycle of Hsp70, and also contains a ubiquitin-like domain that mediates contacts with the proteasome[154, 156, 157] (Fig.1.10). It has been shown also that Bag1 promotes the activation of C-RAF kinase and the disruption of Bag1-C-RAF-kinase complexes during heat shock reduces kinase activity, which contributes to cellular growth arrest[165].

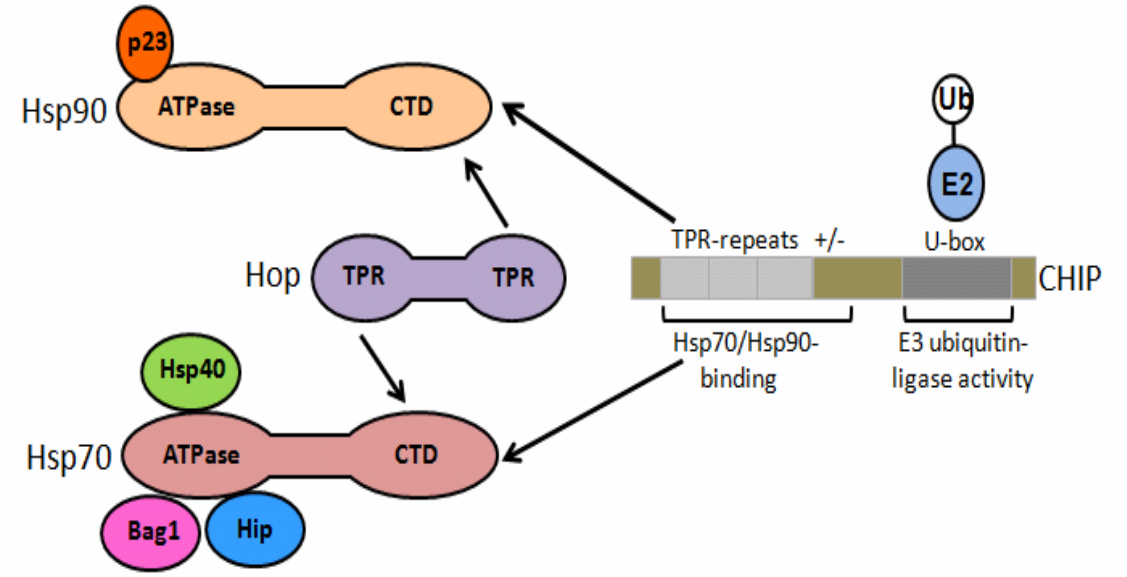

Fig.1.10 Hsp70/Hsp90 chaperones and their interaction w ith CHIP. Hsp70 functions in cooperation with Hsp40 protein family members as well as cofactors Hip and BAG-1. These cofactors compete for binding to the ATPase domain of Hsc70. On the other hand, Hop and CHIP associate through their TPR motif with C-terminal domain of both Hsc70 and Hsp90 in a competitive manner. CHIP binding to Hsc70 leads to attenuation of Hsp40 effect on ATPase activation of Hsc70. Likewise, Hsp90-CHIP interaction results in p23 detachment from the ATPase domain of chaperone (modified from Ref.154, 157).

1.6.2 Co-chaperone CHIP

The TPR-containing co-chaperone, C-terminus of Hsc70-interacting protein (CHIP), has been identified first from heart tissue[166]. In humans, CHIP is expressed ubiquitously, but most highly expressed in striated muscle tissue and, to a lesser extent, in the brain and pancreas. CHIP constitutively localizes to the cytoplasm, but translocates into the nucleus during stress[166-168]. CHIP consists of a TPR domain and a U-box domain. The TPR-domain at its amino terminus serves as the protein-protein interaction domain. CHIP is associated with the carboxyl-terminus of Hsc70 and Hsp90 through its TPR and adjacent charged domain[169] (Fig.1.10). CHIP attenuated the Hsp40-stimulated ATPase activity of Hsc70 and then diminished the refolding activity of the Hsc70-Hsp40 complex for denatured substrates[166, 169].

The U-box domain at the carboxyl-terminal region is unique and it is not found in other TPR containing proteins. The U-box domain of the yeast Ufd2 (ubiquitin fusion degradation) protein performs "E4" activities, i.e. recognizes oligoubiquitin chains and elongates them further, producing polyubiquitin chains[170]. Such factors that can catalyse an extension of the multiubiquitin chain in collaboration with E1, E2, and E3 have been termed E4 enzymes. CHIP is one of these U-box-containing proteins that can function as E4. At present, it is unclear whether all U-box containing proteins can show E4 activity[171]. Furthermore, the tertiary structure of the U-box resembles that of the RING-finger domain, which is responsible for E3 activities of many ubiquitin ligases[172]. CHIP displays E3 activity and mediates the ubiquitylation of a variety of chaperone-bound substrates, which are then recognized by molecular chaperones that leads to their degradation by the 26S proteasome. Thus, CHIP maintains a fine balance between folding and degradation of proteins. For example, it has been shown that CHIP multiubiquitylates the unfolded Pael receptor (Paerl-R) in collaboration with Parkin[173]. Chaperone associated C-RAF can be also diverted to degradation pathway through co-chaperone/ubiquitin ligase CHIP[169, 174]. Further, previous studies have revealed that CHIP can ubiquitinate C-RAF directly[175]. CHIP has been demonstrated to enhance the clearance of expanded polyglutamine proteins and protects the expanded polyglutamine protein-induced cell death[176, 177]. It has also been shown that CHIP induces the Hsp70 levels during acute stress whereas enhances its degradation during stress recovery[168, 178]. Rosser *et al* reported that CHIP possesses an intrinsic chaperone activity, which enables it to selectively recognize and bind proteins in non native states and then suppress protein aggregation. In addition, the chaperone function of CHIP is temperature-sensitive and is enhanced by heat-stress[179]. All these data suggest that CHIP is a dual function protein that acts as both co-chaperone and E3 ubiquitin ligase. The ability of CHIP to serve as a molecular link between protein folding and degradation is an important feature of its action in protein triage and cell stress protection[179, 180]

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2. RESULTS

2.1 Interaction of C-RAF with XIAP and c-IAPs

XIAP has been identified as an interacting partner of C-RAF[181, 182]. To confirm these observations, *in vitro* and *in vivo* binding experiments were performed and a direct interaction between XIAP and C-RAF was detected (Fig.2.1). In addition, HeLa and neuroblastoma cell line, SH-SY5Y, were stimulated with EGF or NGF to check whether activation of MAPK pathway has any influence on this interaction. It was observed that stimulation with the growth factors had no effect on the interaction between C-RAF and XIAP (Fig.2.1A).

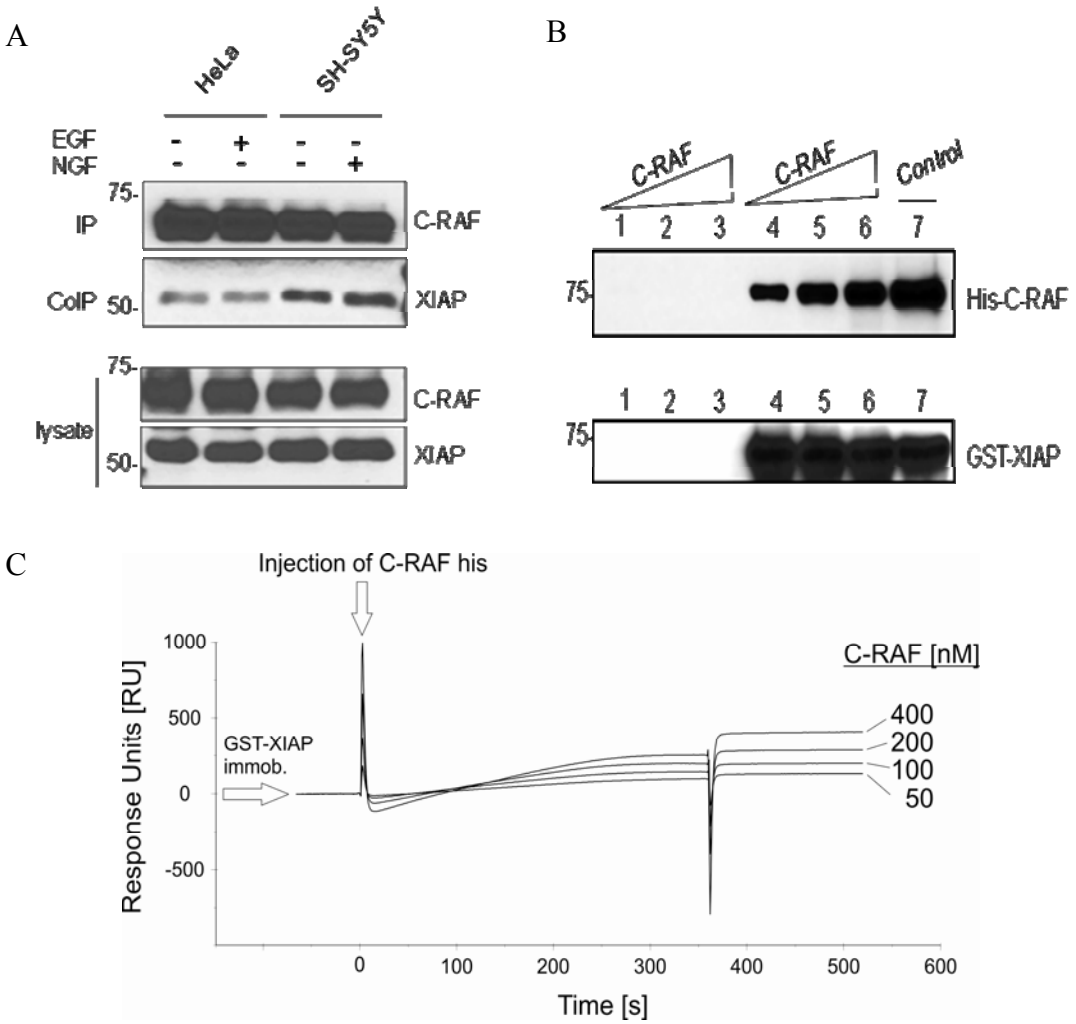


Fig.2.1 XIAP interacts w ith CRAF. (A) Endogenous C-RAF was immunoprecipitated from HeLa and SHSY-5Y cells and the co-precipitating XIAP was detected by immunoblot analysis. The cells were induced with EGF (20 ng/ml) and NGF (20 ng/ml) respectively for 5 min. (B) Interaction between His-tagged C-RAF and GST tagged XIAP recombinant proteins was detected by *in vitro* pull down experiments. Lane 1-3: increasing amounts of C-RAF incubated with GST-sepharose beads alone. Lane 4-6: increasing amounts of C-RAF incubated with GST-XIAP (10 µl) bound to sepharose. C-RAF bound to XIAP was then detected by immunoblots. Lane7: purified proteins were loaded as a control. (C) The kinetics of His-C-RAF binding to GST-XIAP was measured with Biacore system.

In addition, as XIAP and c-IAPs are structurally homologues, a possible direct interaction between C-RAF and c-IAPs was analyzed both *in vitro* and *in vivo* (Fig.2.2 and data not shown).

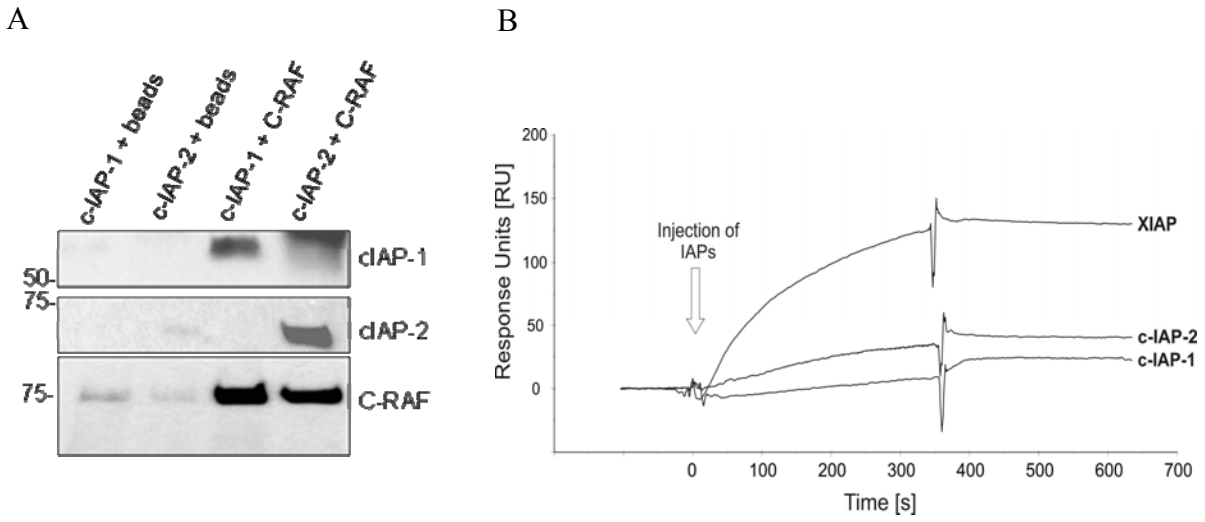


Fig.2.2 c-IAPs interact w ith C- RAF. (A)The interaction between c-IAPs and C-RAF was monitored by GST pulldown assays. (B) Biacore analysis of interaction between IAPs and C-RAF. XIAP binds strongly with C-RAF when compared to c-IAPs.

Mammalian RAF proteins make up a family of three closely related kinases (A-, B- and C-RAF) characterized by the presence of three conserved domains[110]. *In vitro* binding experiments with a Biacore system revealed that C-RAF binds to XIAP with high affinity (K_D=1.2 nM) in comparison to B- and A-RAF (Fig.2.3).

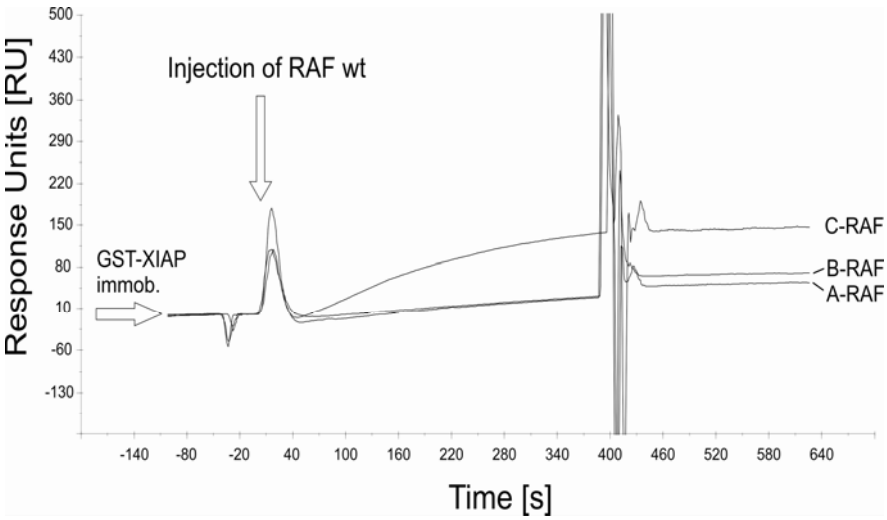
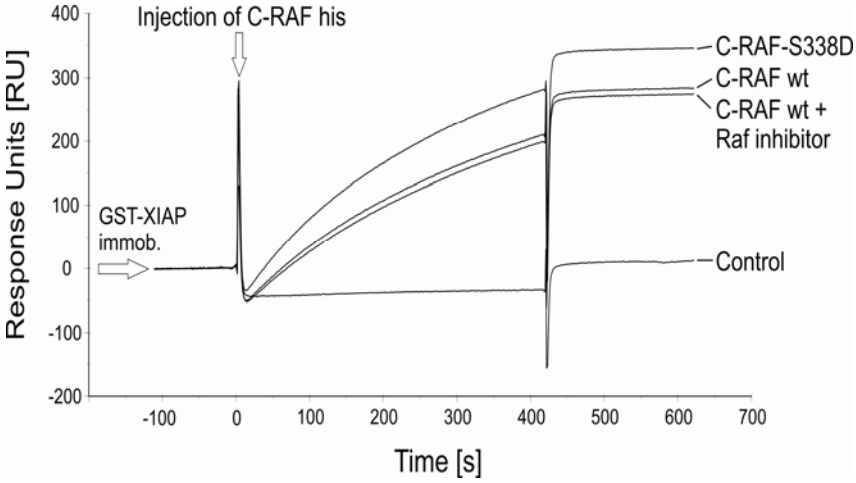


Fig.2.3 C-RAF is the best binding partner of XIAP among RAF family memb ers. The binding affinity of His-A-, B- and C-RAF proteins to GST-XIAP was measured with Biacore system. Compared to A- and B-RAF, C-RAF is the best binder for XIAP.

2.2 Interaction of C-RAF with XIAP does not depend on its kinase activity

As C-RAF is a Serine/Threonine kinase, the requirement of kinase activity of C-RAF for its interaction with XIAP was analyzed. To answer this question, interaction studies with purified proteins in the presence of RAF inhibitor as well as co-immunoprecipitation of mutant proteins were carried out. The results of both experiments showed that the kinase activity of C-RAF is not required but may facilitate interaction with XIAP (Fig.2.4).

A



B

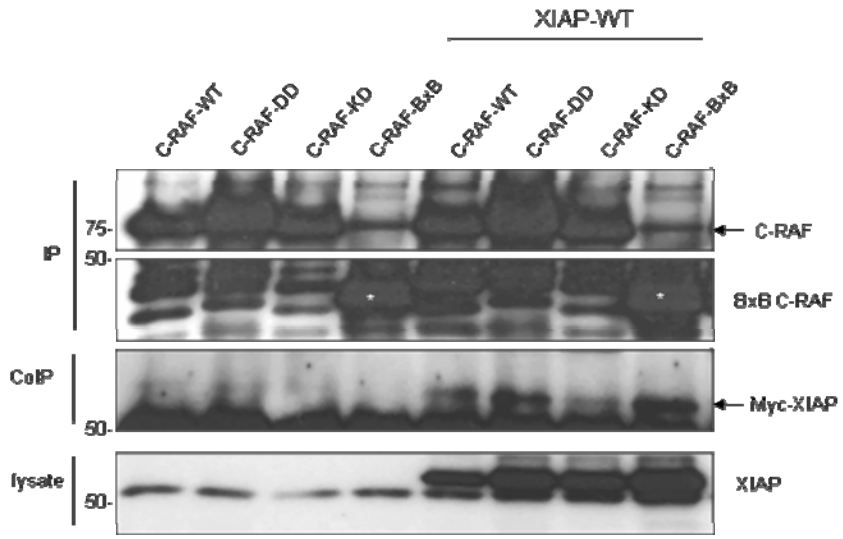


Fig.2.4 Interaction of C-RAF w ith XIAP does not dependent on its kinase activity. (A) The kinase dependency of C-RAF in modulating the interaction with XIAP was analysed with Biacore system by co-incubating C-RAF kinase with BAY43-9006 (10 µM). C-RAF-S338D is the active form of C-RAF. (B) 293T cells were co transfected with XIAP and various mutants of C-RAF. C-RAF was then immunoprecipitated and the co-precipitating XIAP was detected by immunoblot analysis. Note that C-RAF BxB and C-RAF-DD were highly expressed despite that equal amounts of plasmids were used for transfection. The * indicates the overexpressed C-RAF BxB.

2.3 XIAP is not phosphorylated by either C- or B-RAF kinase

To decipher the mode of interaction between XIAP and C-RAF, we analysed if C-RAF phosphorylates XIAP as previously suggested[182]. In contrast to the published observation, no phosphorylation of XIAP by either C- or B-RAF was detected. Phosphorylation of XIAP and MEK by RAF kinases was monitored by an *in vitro* phosphorylation assay (Fig.2.5)

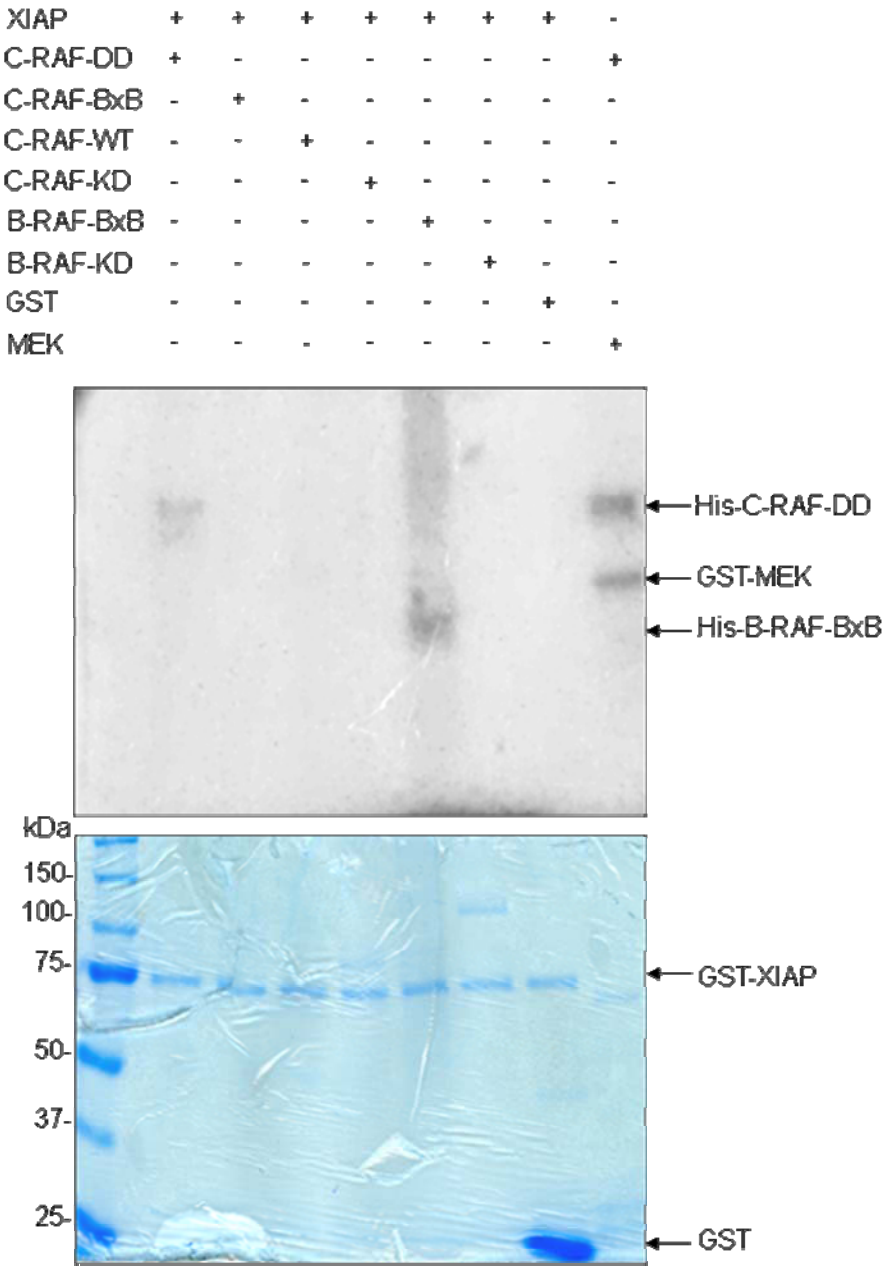


Fig.2.5 XIAP is not phosphorylated by either C- or B-RAF kinase. MEK-1 was used as a positive control of *in vitro* phosphorylation by C-RAF (last lane). Autophosphorylation of C-RAF-DD and B-RAF-BxB were also seen (lanes 1 and 5). But no phosphorylation of XIAP could be detected on the (lanes 1, 2, 3 and 5). Upper panel is from autoradiogarphy. Lower panel is the sample-loaded gel stained by coomassie brilliant blue as a loading control.

2.4 Down-regulation of C-RAF does not affect the protein levels of XIAP

As XIAP binds to C-RAF, the influence of C-RAF on the protein level of XIAP was examined. Depletion of C-RAF using siRNAs did not alter the protein levels of XIAP in HeLa and CAPAN-1 cells (Fig.2.6).

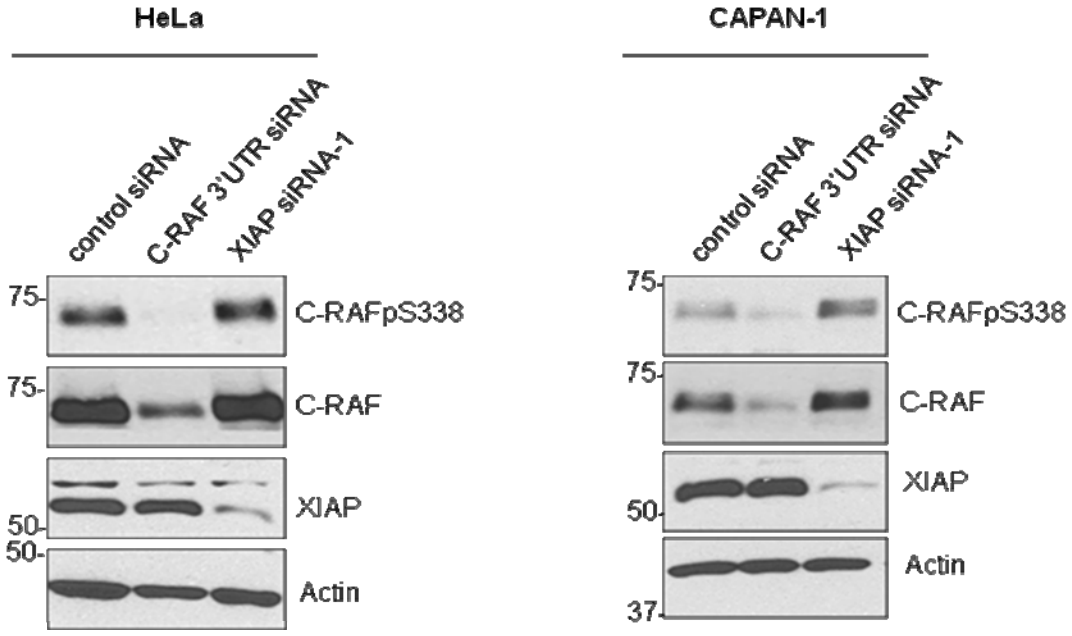


Fig.2.6 Effect of transient suppression of endogenous C-RAF in HeLa and CAPAN-1 cells. Subconfluent cultures of HeLa and CAPAN-1 cells were transfected with siRNAs directed against C-RAF and XIAP, respectively and the alteration in C-RAF, C-RAFpS338 and XIAP levels at 48 h after transfection was monitored by immunoblot.

2.5 Silencing of XIAP leads to stabilization of C-RAF

As XIAP has been shown to have E3 ubiquitin ligase activity, it was tested if XIAP plays any role in modulating C-RAF stability. Surprisingly, an increase in protein but not mRNA levels of C-RAF was detected in HeLa cells when XIAP expression was specifically reduced by siRNAs (Fig.2.6 and 2.7).

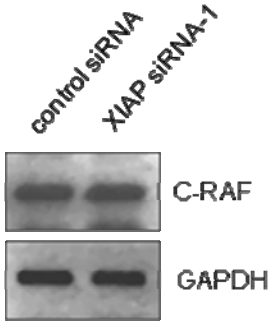


Fig.2.7 XIAP depletion failed to alter C-RAF mRNA levels. RT-PCR analysis revealed no alteration in the mRNA levels of C-RAF in HeLa cells transfected with XIAP siRNAs for 48 h. GAPDH was used as a control.

Moreover, these observations were not confined to HeLa and CAPAN-1 cells as silencing of XIAP expression led to stabilisation of C-RAF in several additional cell lines derived from various human tumours (A549, HEp-2, SHSY-5Y and WM852) (Fig.2.8).

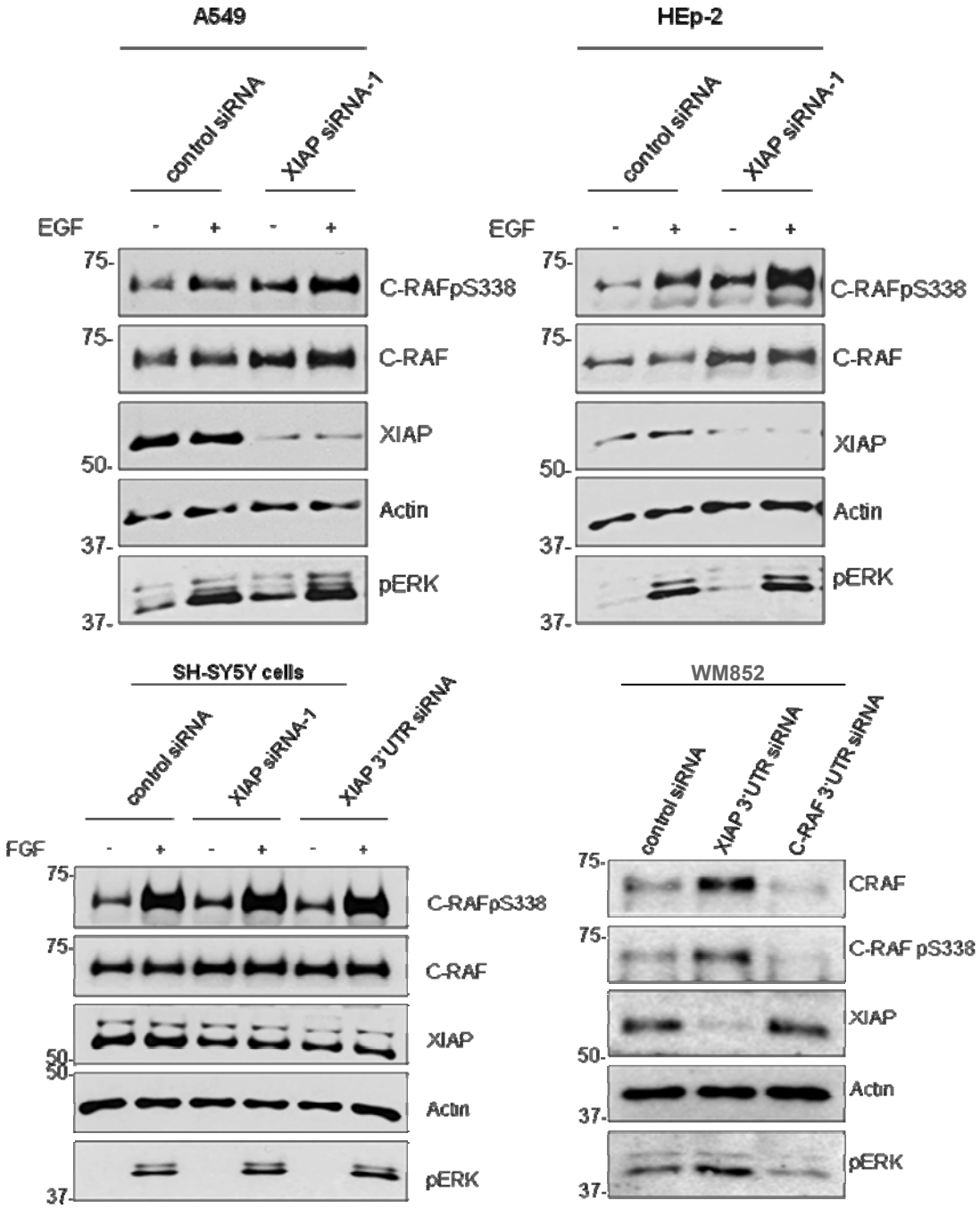


Fig.2.8 Loss of XIAP leads to stab ilization of C-RAF. XIAP expression was interfered with siRNAs in A549, HEp-2, SH-SY5Y and WM852 cells. The protein levels of RAF, XIAP, pERK, C-RAFpS338 and actin were detected by immunoblot analysis.

C-RAF is normally localised to cytosol and activation of RAS leads to relocation of C-RAF at the membranes followed by crucial phosphorylation (Ser338 and/or Tyr341) and dephosphorlyation (Ser259) events leading to its full activation[110]. Therefore, we assessed whether phosphorylation of C-RAF at Ser338 and levels of phospho-ERK were increased concomitantly. Basal as well as EGF stimulated C-RAFpS338, p-ERK and total C-RAF levels were all found to be increased in HeLa and other cell lines transfected with XIAP siRNAs (Fig.5, 7-9).

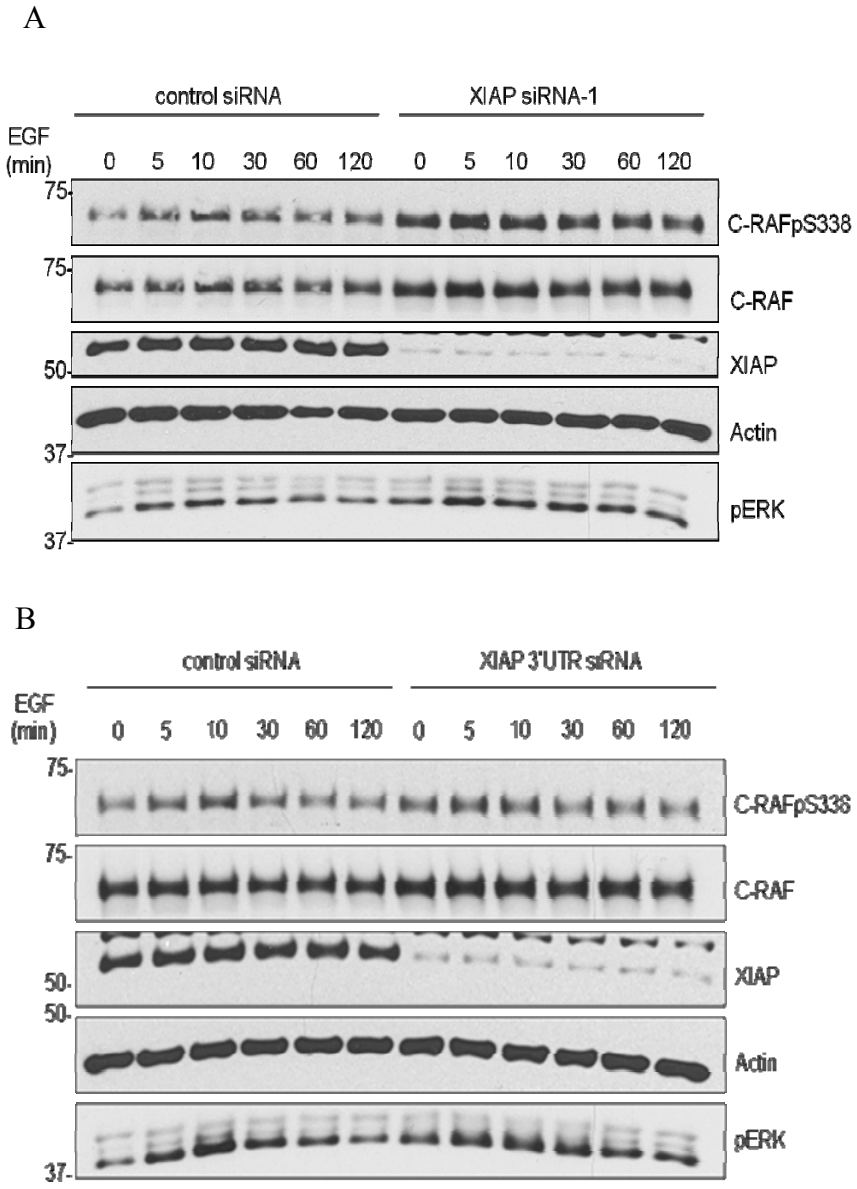


Fig.2.9 XIAP modulates C-RAF stability. HeLa cells were transfected with control and XIAP siRNA (A), and XIAP3'UTR- siRNA (directed towards the 3'UTR region) (B), respectively, and the protein levels of C-RAFpS338, pERK, XIAP and C-RAF were monitored by immunoblot analysis after stimulation with EGF (20 ng/ml) for various time points. Actin was used a loading control.

In order to exclude any unspecific effects of the siRNAs employed, three more sets of siRNAs have been obtained. All siRNAs silenced expression of XIAP efficiently and high levels of C-RAF protein were detected in XIAP silenced HeLa cells (Fig.2.10).

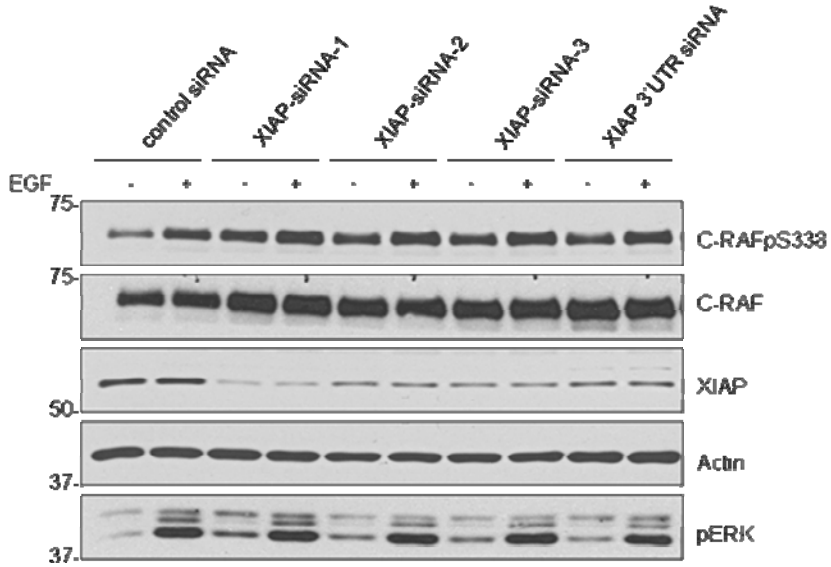


Fig.2.10 Silencing of XIAP expr ession le ads to stabiliz ation of C-RAF. HeLa cells were transfected with four sets of XIAP siRNAs and the levels of various proteins were monitored by immunoblot analysis after stimulation with EGF (20 ng/ml).

Finally, complementation experiments by expressing a myc-tagged version of *XIAP* cDNA were performed, in cells, where the endogenous XIAP levels were reduced with a 3'UTR siRNA. Exogenously expressed XIAP reduced the high C-RAF level in siXIAP cells confirming the role of XIAP in modulating C-RAF stability in these cells (Fig.2.11).

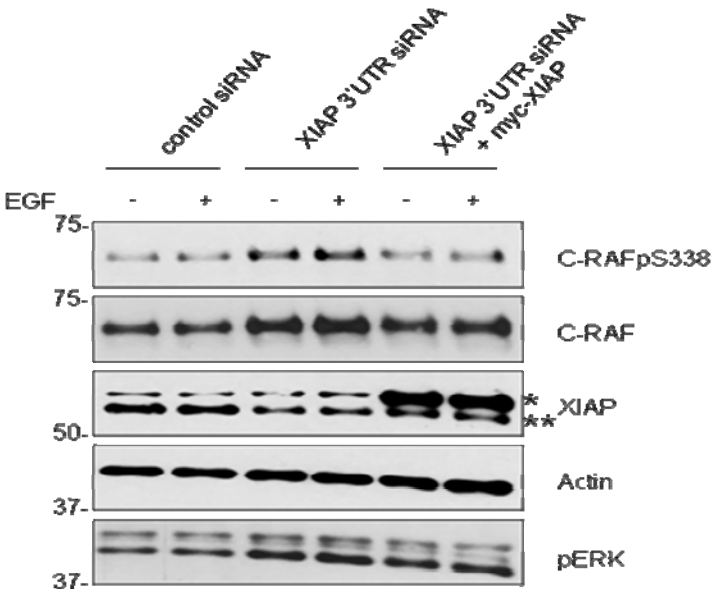


Fig.2.11 Complemen tation of XIAP in siXIAP cells. HeLa cells were co-transfected with XIAP 3'UTR siRNA and pcDNA3-XIAP-myc plasmids to rescue the expression of XIAP. Exogenous and endogenous XIAP are labelled with * and **, respectively.

To further confirm these observations, XIAP was transiently expressed in HeLa cells. As shown in Fig.2.12, C-RAF levels were reduced in cells expressing high levels of XIAP-GFP.

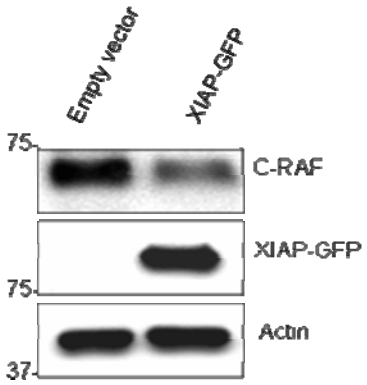


Fig.2.12 Over expression of XIAP caus es degradation of C-RAF pr otein. HeLa cells were transfected with either pEGFP-MCS or with wild-type pEGFP-XIAP. GFP-positive cells were sorted using a FACS sorter and the changes in C-RAF protein levels were determined by immunoblot analysis.

The stabilisation of C-RAF in the absence of XIAP was further confirmed by a kinetics experiment, in which protein synthesis was blocked by the addition of cycloheximide (Fig.2.13).

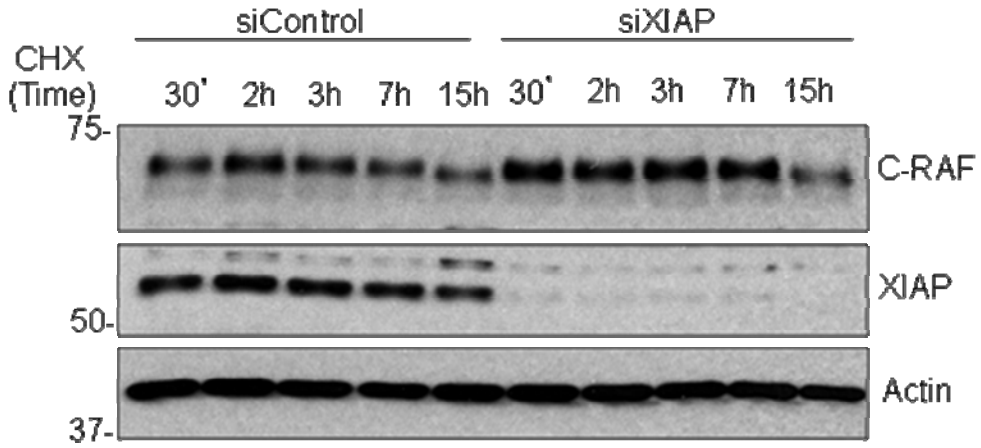


Fig.2.13 Stability of C-RAF in siXIAP cells treated w ith cycloheximide. HeLa cells were transfected with XIAP siRNAs and the protein level of C-RAF was monitored after treating the cells with 10 µg/ml of cycloheximide for various time points.

2.5.1 c-IAPs modulate C-RAF stability

As c-IAPs also bind to C-RAF, it was analyzed, whether ablation of c-IAP-1 and/or c-IAP-2 has any effect on C-RAF stability. As expected, silencing of c-IAP-1 and c-IAP-2 also led to increase in total C-RAF levels in HeLa cells (Fig.2.14).

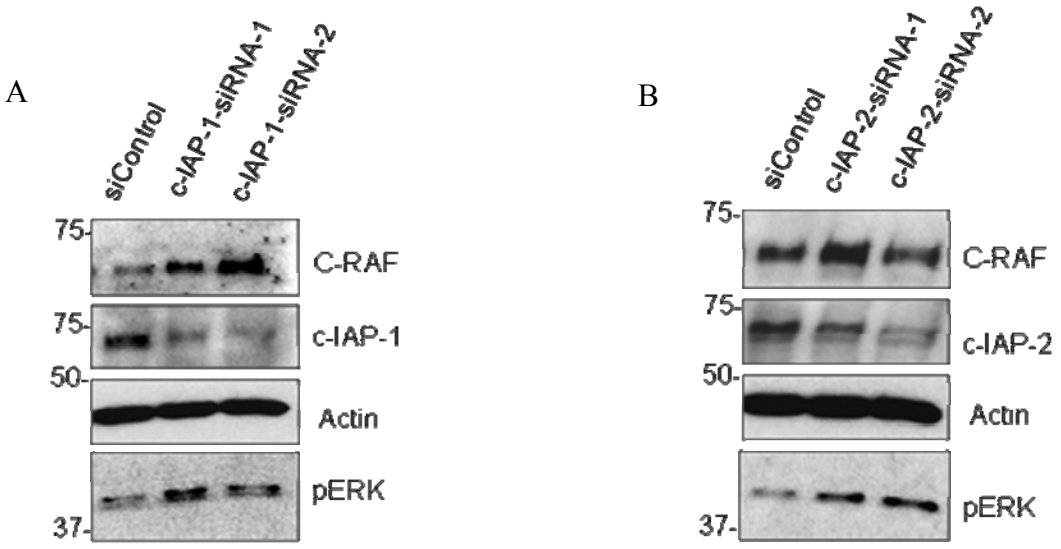


Fig.2.14 c-IAPs modulate C-RAF stability . HeLa cells were transfected with control or two sets of *c-IAP-1* (A) or *c-IAP-2* (B) siRNAs and the alterations in the levels of various proteins were assessed by immunoblot analysis.

2.5.2 Depletion of XIAP stabilizes C-RAF both in cytosol and membranes

To investigate if C-RAF is stabilized both in the cytosol and membranes, cell fractionation experiments were carried out. As expected, we detected increased C-RAF levels in both cytosol and membrane fractions in siXIAP cells (Fig.2.15A). Consistent with this, phosphorylation of C-RAF at Ser 259 was also increased (Fig.2.15B), which along with phosphorylated S621 residue, leads to the interaction of C-RAF with 14-3-3 and in turn C-RAF is localised in the cytosol.

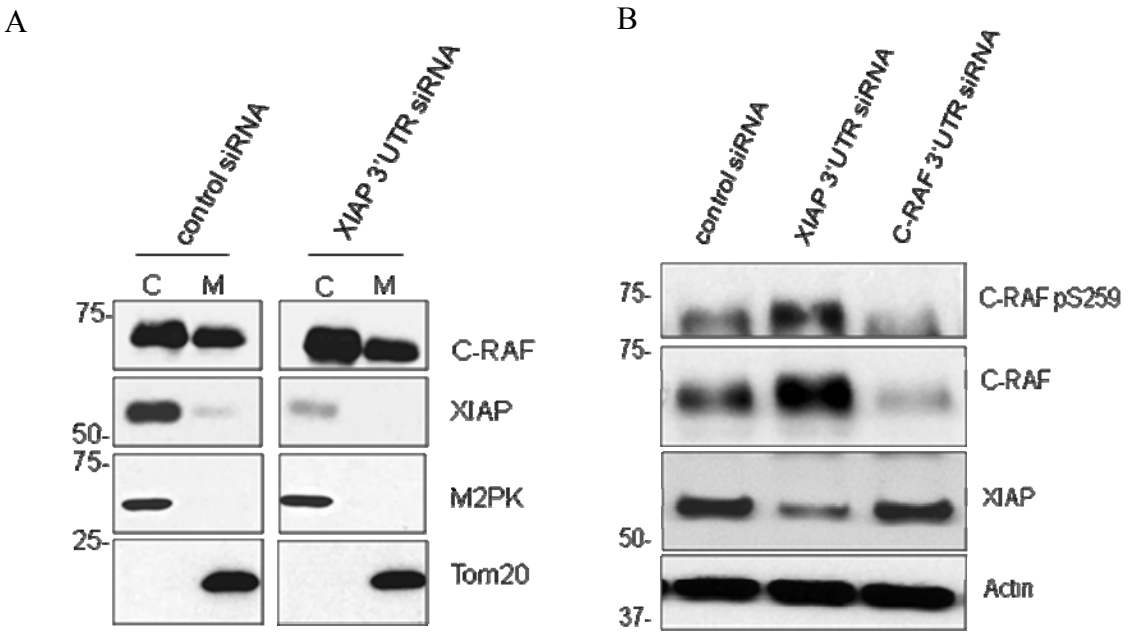


Fig.2.15 CRAF is stabiliz ed in both cytosol and membrane fractions. (A) HeLa cells were transfected with control and XIAP siRNAs and various sub cellular fractions were isolated and the proteins of interest were detected by immunoblots as indicated. C: Cytosol fraction, M: Membrane fraction. Presence of M2PK and Tom20 was checked to evaluate the purity of the fractions. (B) HeLa cells were transfected with control or XIAP or C-RAF siRNAs and the alterations in C-RAF, C-RAFpS259, XIAP and actin levels were monitored by western blot analysis.

2.5.3 XIAP controls C-RAF levels in primary cells

As XIAP is highly expressed in tumour cells, next question was whether XIAP controls C-RAF levels in primary cells. Consistent with our previous observations, an increase in total C-RAF protein levels in the absence of XIAP was detected in human primary lung fibroblasts (Fig.2.16). When stimulated with growth factors (EGF, NGF and FGF), there was a concomitant increase in C-RAFpS338, p-ERK levels as observed in tumour cells. No significant changes in B-RAF were observed suggesting that the effect was specific to C-RAF (Fig.2.16).

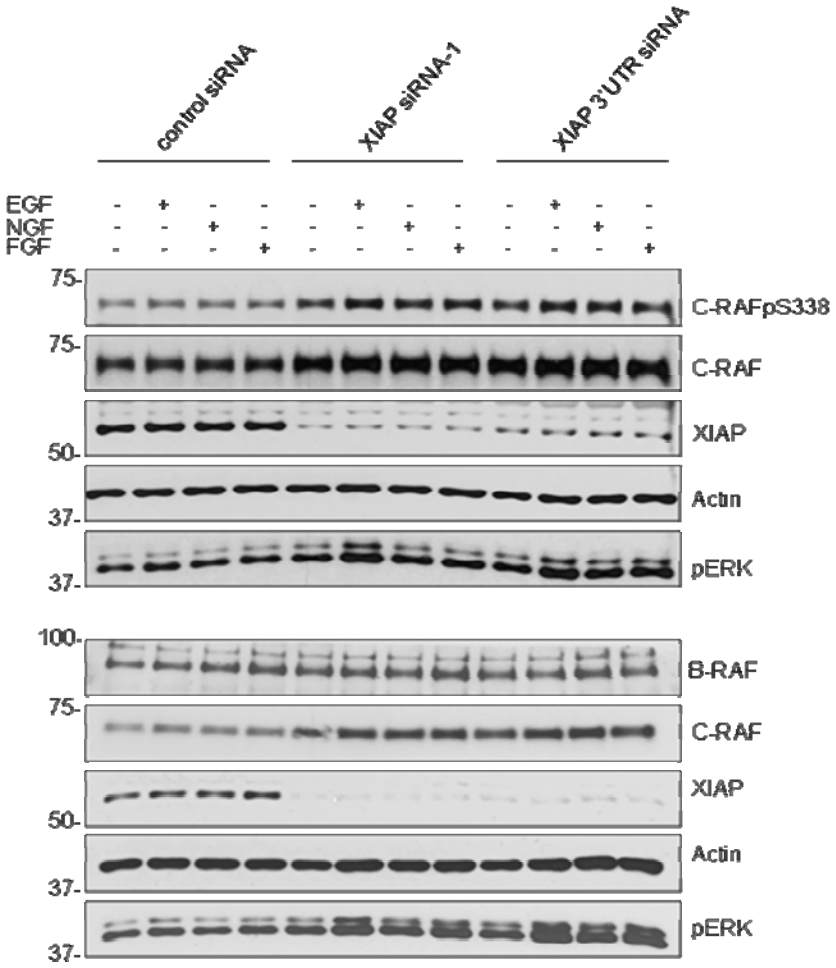


Fig.2.16 IMR90 human primary fibroblasts (passage-2) were transfected with control and two sets of XIAP siRNAs and stimulated with EGF, NGF and FGF. The protein levels were monitored by immunoblot analysis as indicated (upper panel). The same lysates were loaded again to detect B-RAF (lower panel).

2.5.4 RAF kinase inhibitor does not influence the stabilization of C-RAF in XIAP knock down cells

Finally, to analyze whether the kinase activity of C-RAF is required for its stability, BAY43-9006, a RAF kinase inhibitor, was used. Treatment with this RAF inhibitor led to mild reduction in total RAF levels in E1A immortalized IMR90 cells or HeLa cells (Fig.2.17). However, silencing of XIAP led to relatively high levels of C-RAF protein suggesting that the kinase activity of C-RAF was not required for stabilisation under these conditions. Taken together, these results confirmed the role of XIAP in modulating C-RAF homeostasis in human cells.

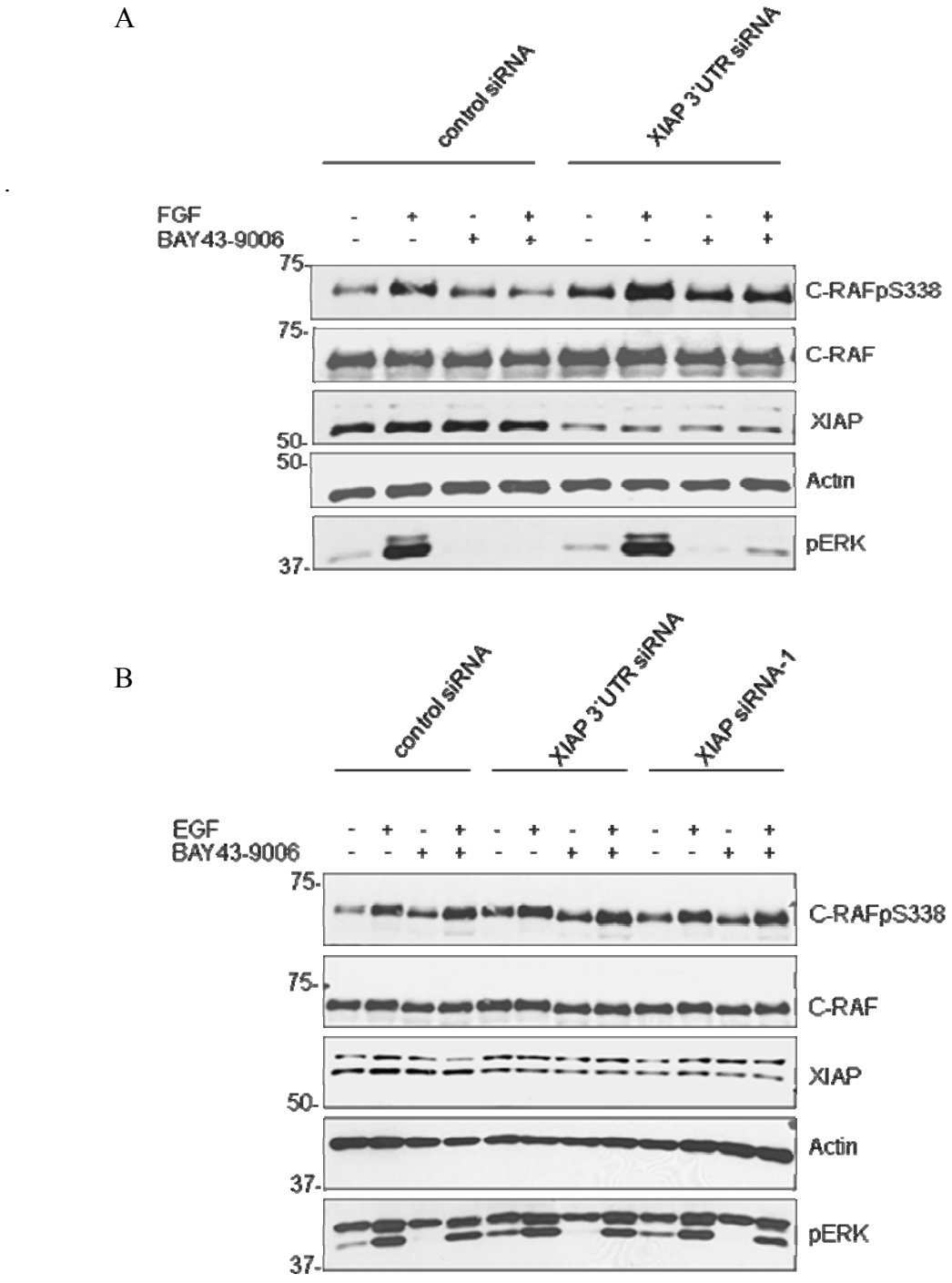


Fig.2.17 Kinase activity of C-RAF is not requi red for its stabiliz ation in XIAP knock down cells. (A) Control and XIAP siRNAs were transfected to E1A immortalized IMR90 cells and the levels pERK, XIAP and C-RAF were assessed by immunoblot analysis after stimulation with FGF or pre-treatment with RAF inhibitor BAY43-9006 (10 μM). (B) Control and XIAP siRNAs were transfected to HeLa cells treated with EGF and BAY43-9006 (10 μM) as indicated. C-RAFpS338, C-RAF, XIAP and pERK levels were detected by immunoblots. Actin was used a loading control.

2.6 XIAP modulates cell motility

As high levels of active C-RAF and p-ERK were detected in XIAP depleted cells, morphological changes in siXIAP cells were next examined. HeLa cells transfected with XIAP siRNAs exhibited an altered morphology as revealed by their elongated fibroblast like structures (Fig.2.18).

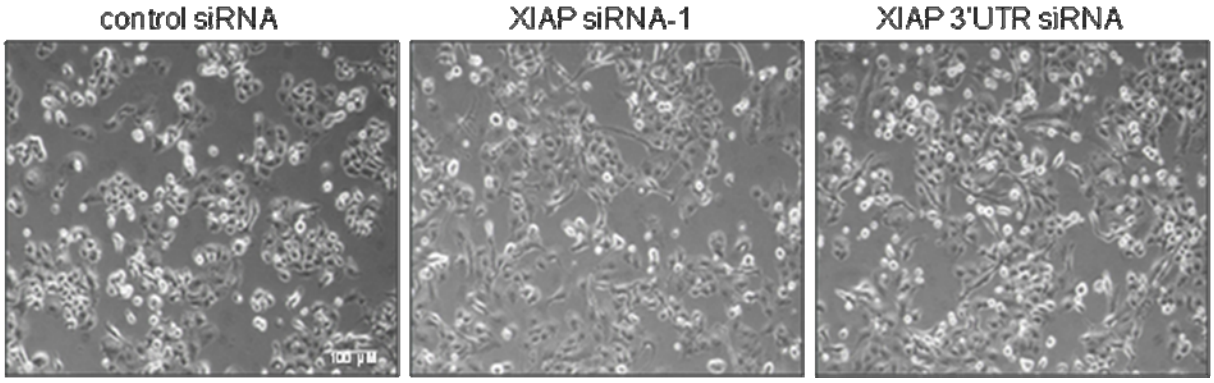
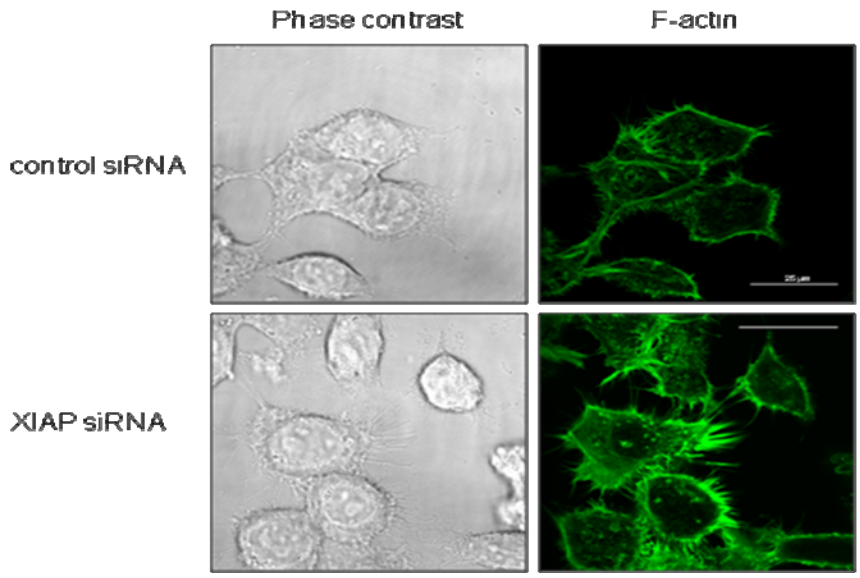


Fig.2.18 Morphology of XIAP depleted cells. HeLa cells were transfected with control and two sets of XIAP siRNAs and cell morphology was monitored at 48 h post transfection. Shown are images from a representative experiment under 10X magnification.

To demonstrate whether the alterations in cell morphology were due to enhanced lamellipodia and filopodia formation, the signatures of cell migration, staining for filamentous actin was performed. As shown in Fig.2.19, siXIAP cells had profuse lamellipodia and filopodia accompanied by enhanced spreading even without EGF stimulation.

A



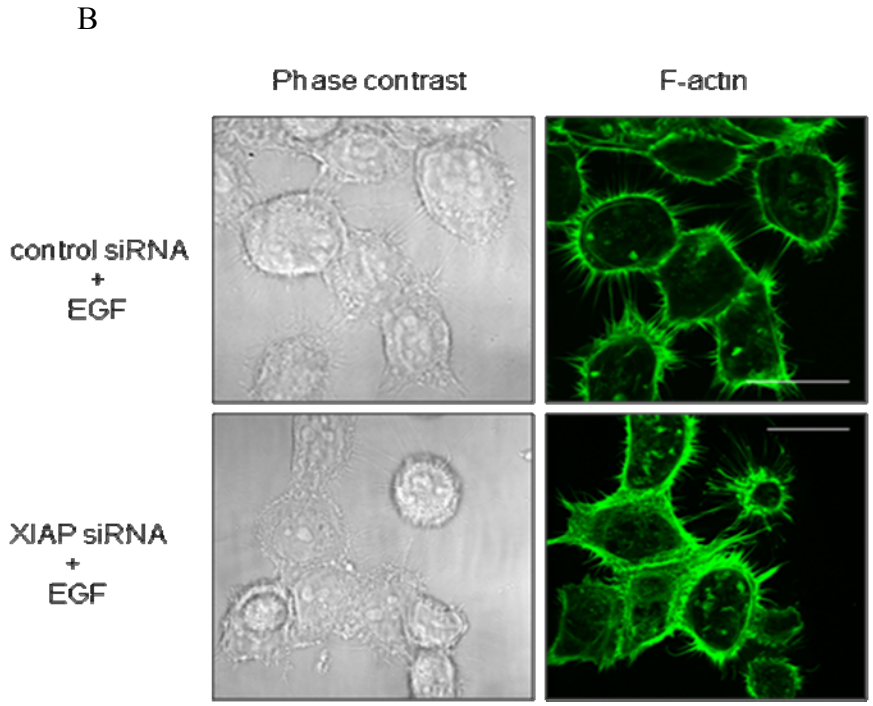


Fig.2.19 Staining of siXIAP cells. The formation of lamellipodia and filopodia was analysed in control and XIAP siRNAs transfected cells by Phalloidin staining. Cells were grown in the presence of serum (A) or stimulated with EGF for 5 min (B).

2.6.1 Activation of Small GTPases like Cdc42 and Rac1 was enhanced in siXIAP cells

GTPase Rac1 plays an important role in the organization of actin filament networks and in membrane ruffling. Rac1 has been implicated in cell transformation, apoptosis and migration. Another GTPase Cdc42 regulates the organization of the actin cytoskeleton and gene transcription. Activation of Cdc42 promotes actin polymerization to form filopodia or microspikes and is associated with integrin complexes. Furthermore, Cdc42 has been shown to activate Rac, such that filopodial extensions are usually seen associated with lamellipodial protusions[183]. As expected, an enhancement in the basal activity of Cdc42 and Rac1 in siXIAP cells was detected (Fig.2.20).

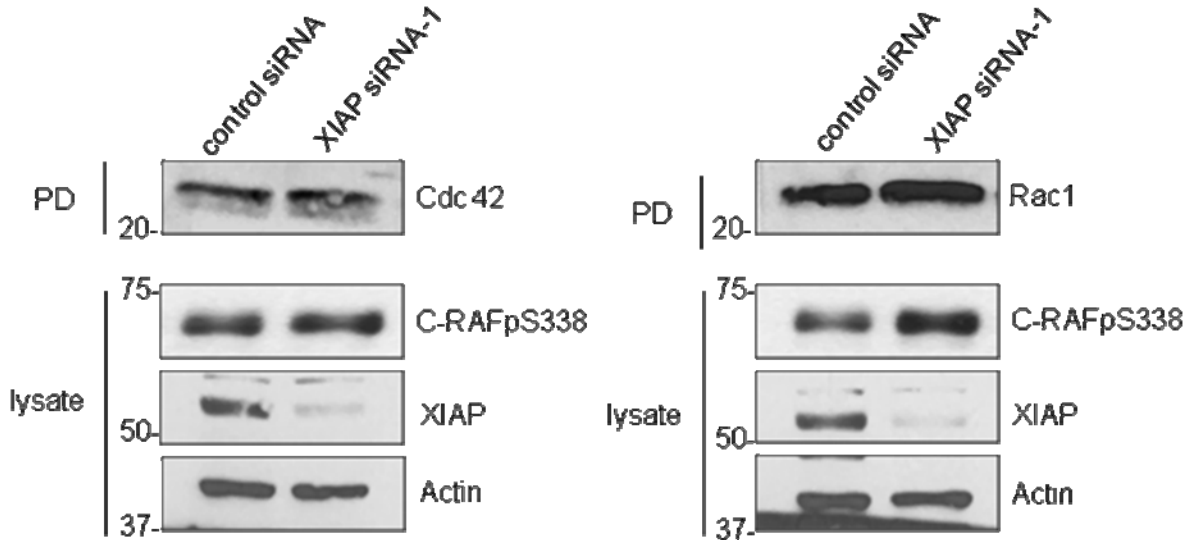
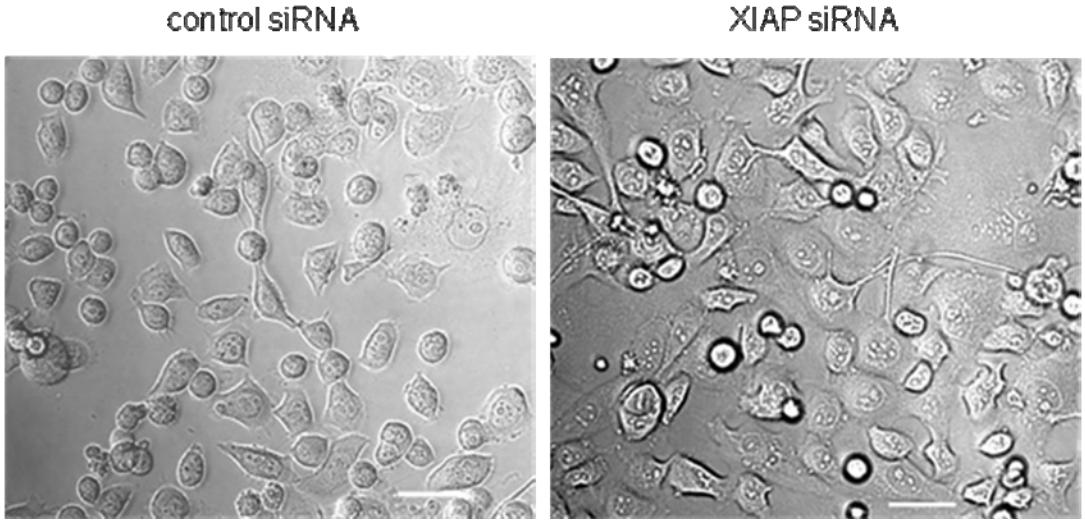


Fig.2.20 Cdc42 and Rac1 are more active in siXIAP cells. Active Cdc42 and active Rac1 was precipitated from control and XIAP knock down cells. PD: Pull down, lysate: total cell lysate

2.6.2 Knock down of XIAP enhances the spreading and the motility of HeLa cells

All the morphological alterations mentioned above in siXIAP cells could point to the fact that these cells might exhibit enhanced migration. To test this possibility, the ability of HeLa cells (with or without XIAP) to spread and migrate on collagen was tested. Most of the control cells remained rounded at the time point where siXIAP cells have spreaded well on collagen, and XIAP depleted cells exhibited enhanced motility as analyzed by time lapse microscopy (Fig.2.21 and 2.22).

A



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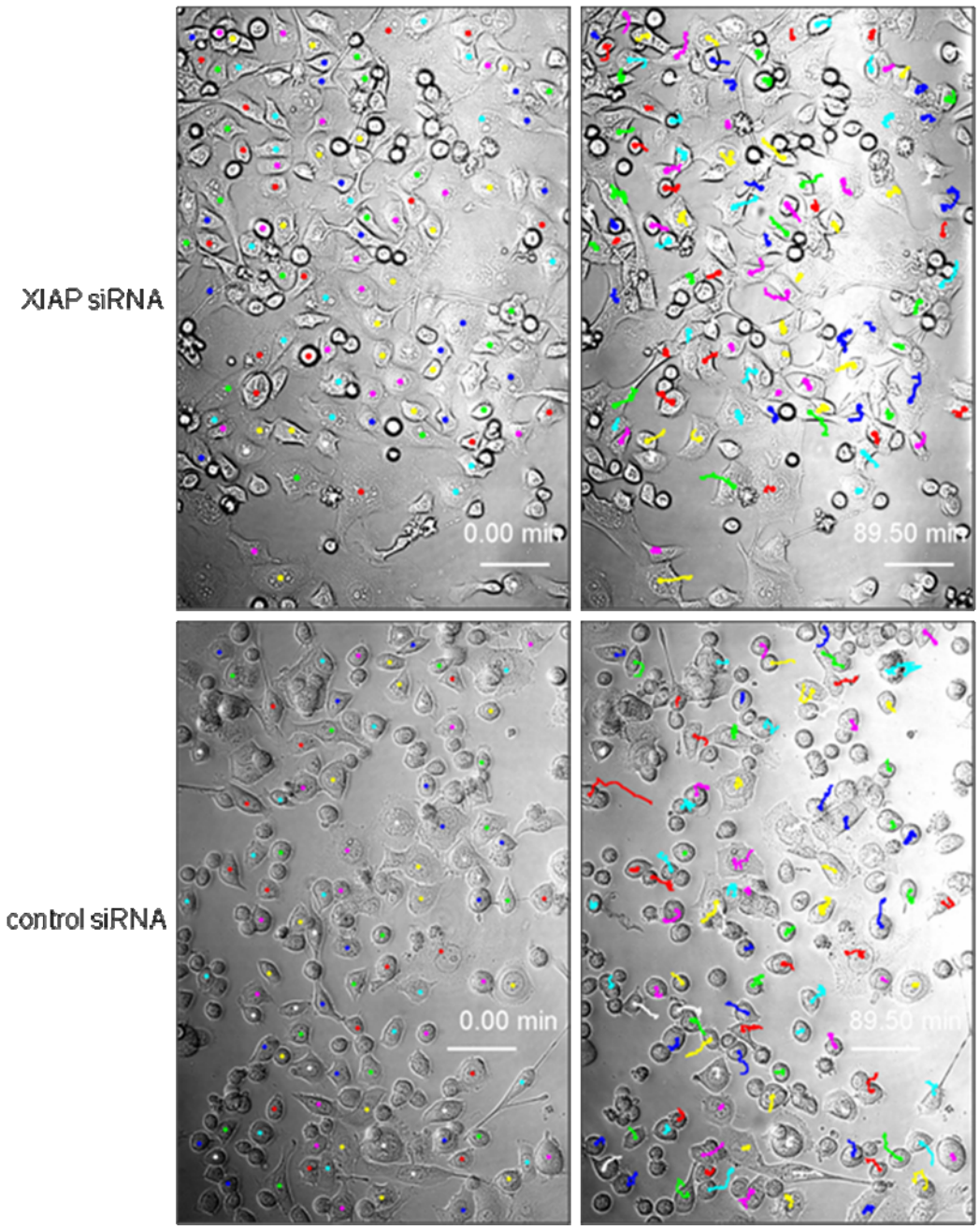


Fig.2.21 XIAP silenced cells exhibited enhanced random cell motility. (A) Spreading of control and XIAP siRNAs transfected cells. The cells were seeded on special tissue culture chamber slides coated with collagen. Shown are images from 0 min of time lapse analysis in B. (B) Analysis of motility of control and XIAP knock down HeLa cells by time lapse microscopy. The cells were seeded on ibidi chambers coated with collagen and their motility was observed for 90 min under a confocal microscope. Shown are representative frames from 0 min and 89.5 min from the movies. The motility of cells was tracked using cell tracking software.

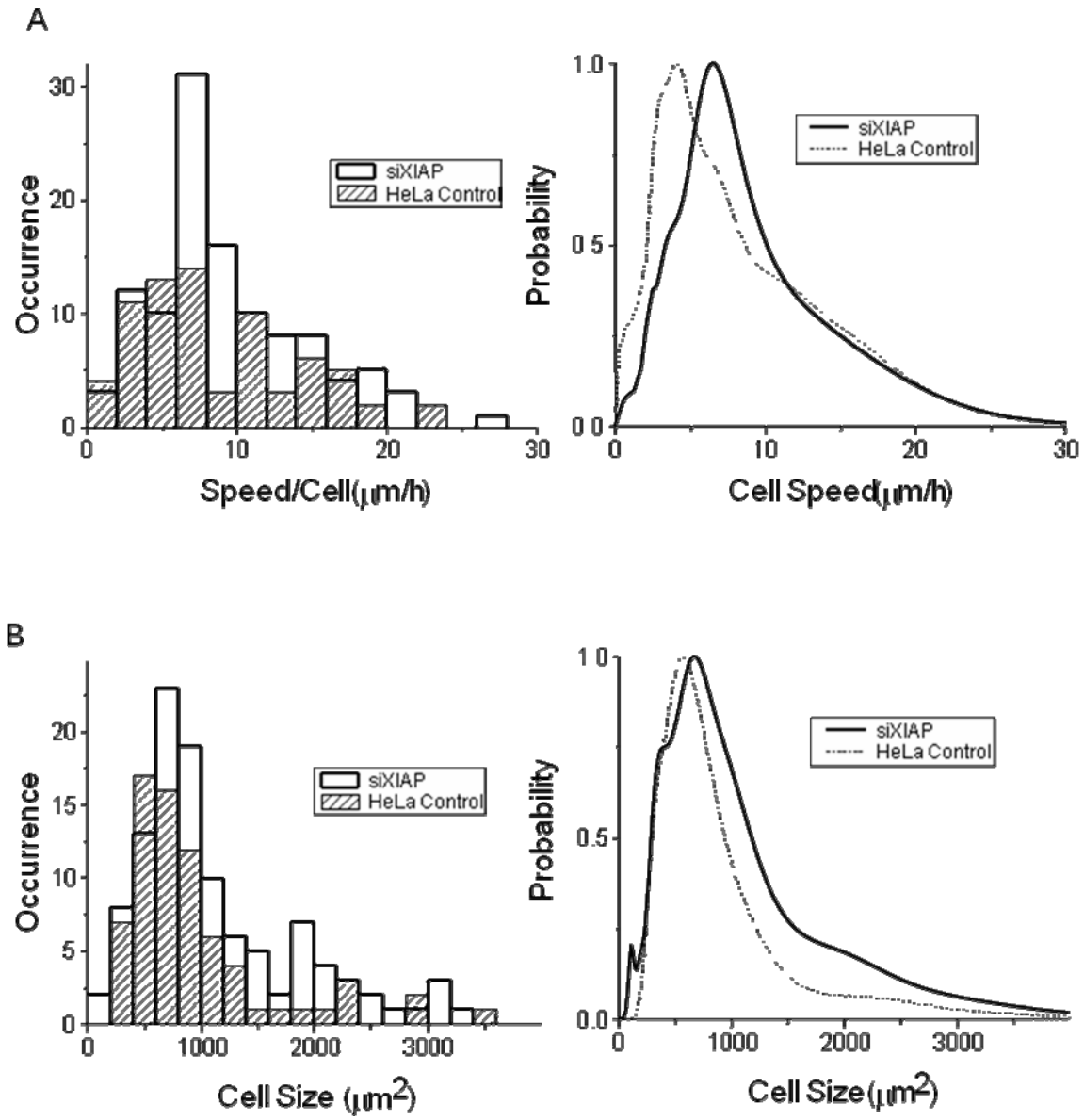
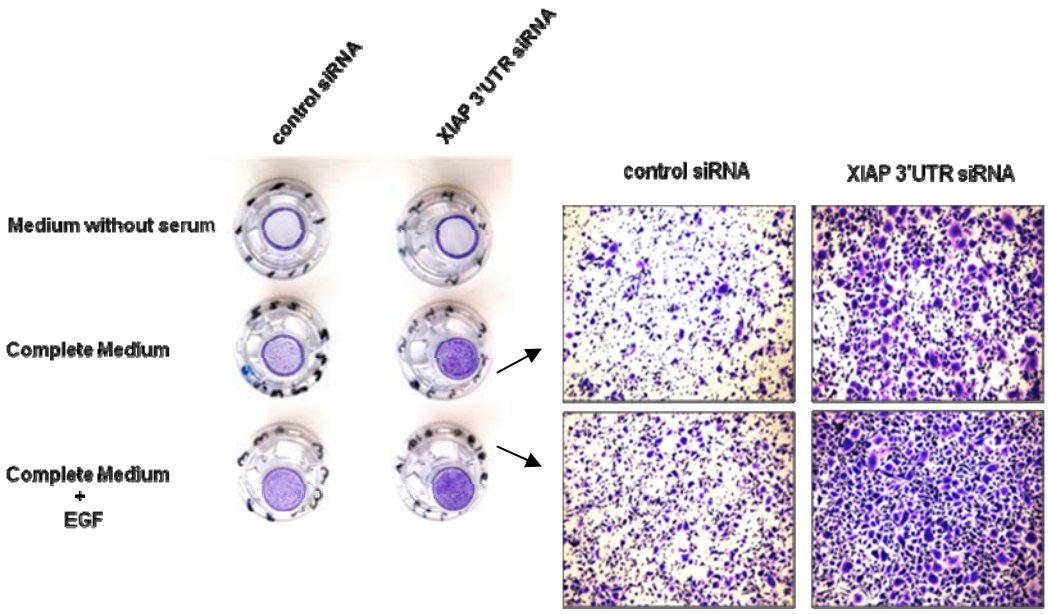


Fig.2.22 Measurement of the cell speed and size. The cell speed and size were calculated for cells indicated by tracks in Fig.2.21B. Only flattened cells have been considered for analysis. The read out was made with histograms and probability density curves for more clarity.

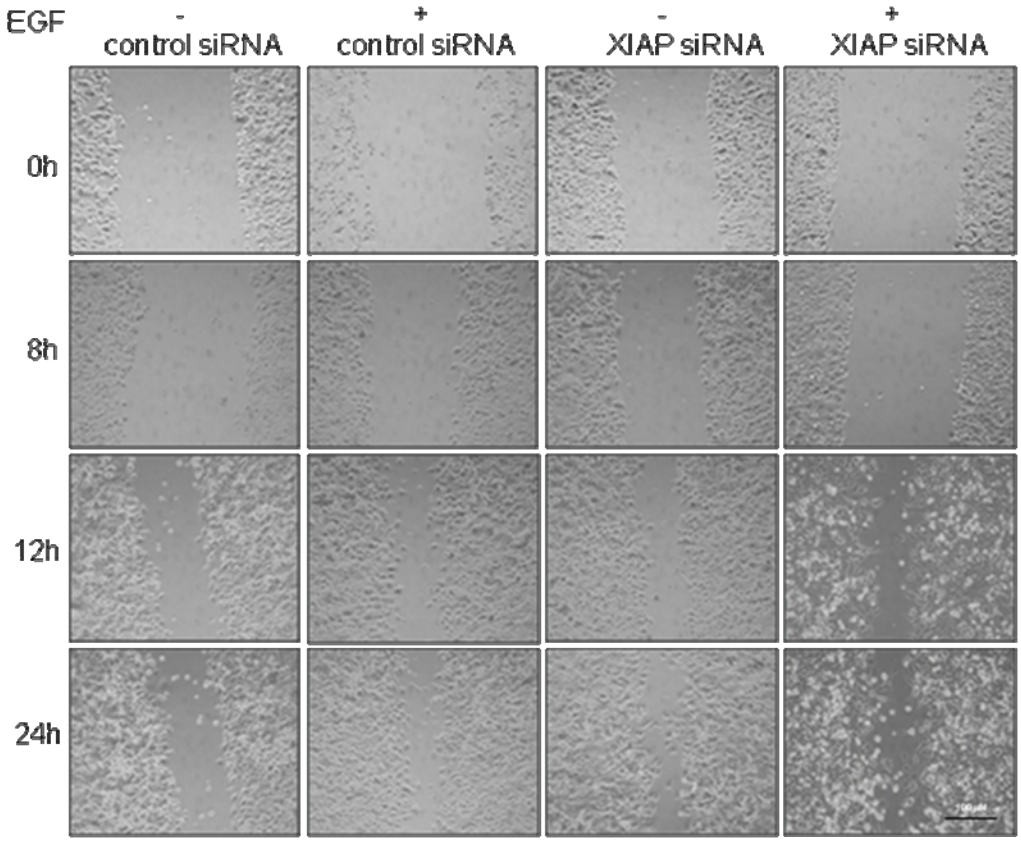
2.6.3 Loss of XIAP increases the migration of HeLa cells

As XIAP knock down cells exhibited enhanced spreading, it was then tested if these cells exhibit enhanced migration. Transwell migration and wound healing experiments revealed a significant increase in the migration of HeLa cells transfected with XIAP siRNAs (Fig.2.23).

A


control siRNA
XIAP 3'UTR siRNA
Medium without serum
Complete Medium
Complete Medium
+
EGF
control siRNA
XIAP 3'UTR siRNA

B


EGF
-
control siRNA
+
control siRNA
-
XIAP siRNA
+
XIAP siRNA
0h
8h
12h
24h

C

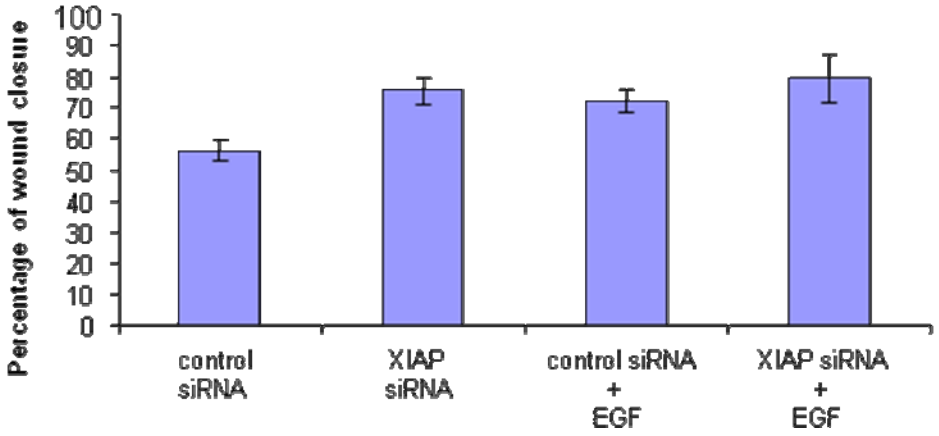
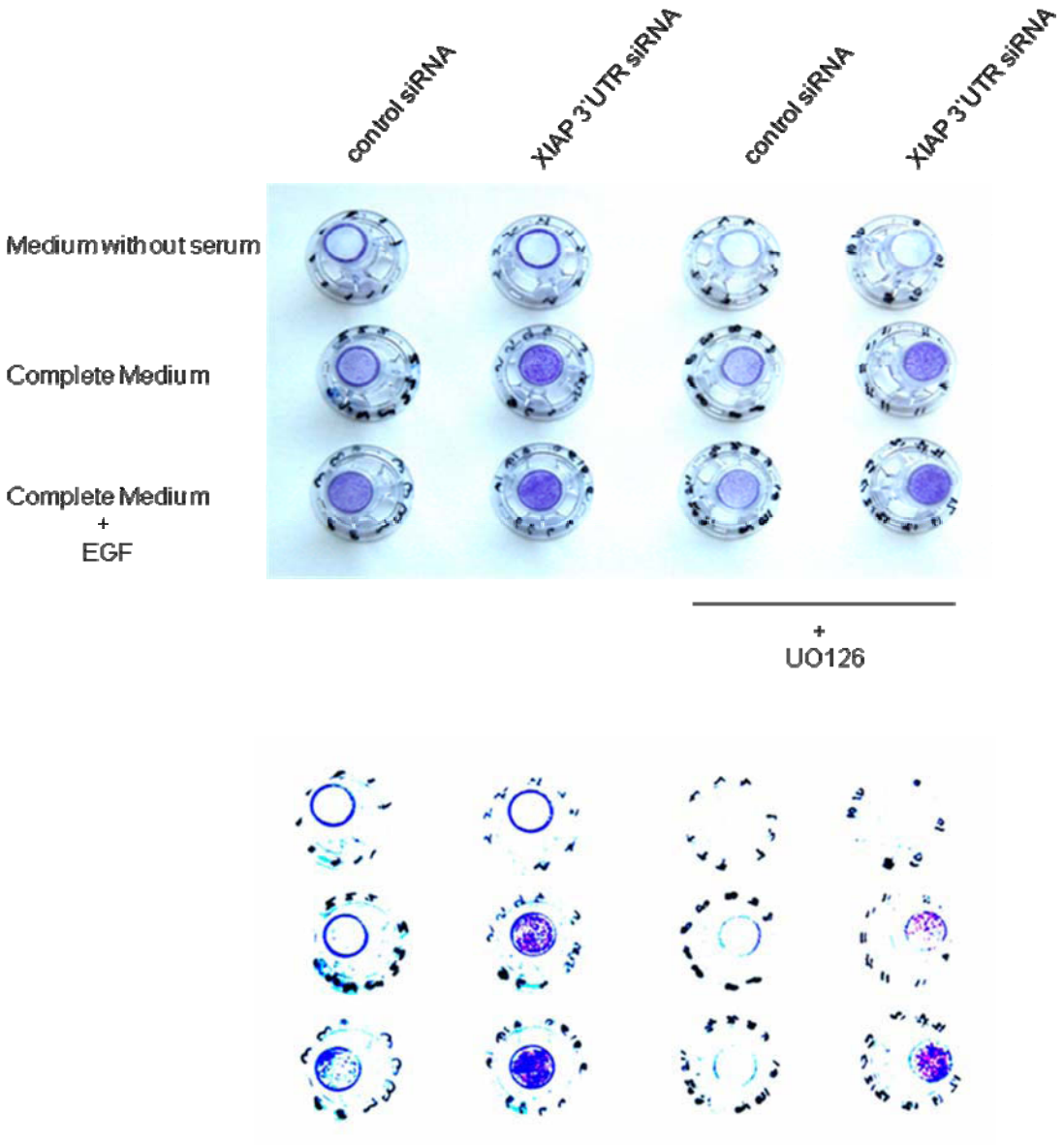


Fig.2.23 Reduction of XIAP levels leads to enhanced migration and w ound healing. (A) HeLa cells transfected with control and XIAP siRNAs were allowed to migrate in a transwell migration chamber coated with collagen for 15 h. The migrated cells were fixed with PFA and stained with crystal violet. Shown are the images made under a phase contrast microscope at 10 X magnification from one representative experiment. (B) HeLa cells were transfected with control and siXIAP siRNAs for 48 h. Scratches were made on the confluent monolayers using a pipette tip and the extent of wound closure was monitored under a phase contrast microscope at 10X magnification. Shown are the images from a representative experiment. (C) The percentage of wound closure was calculated using ImageJ software tools as mentioned in materials and methods. Shown are the data from three independent experiments. The error bars represent ±SD of the mean.

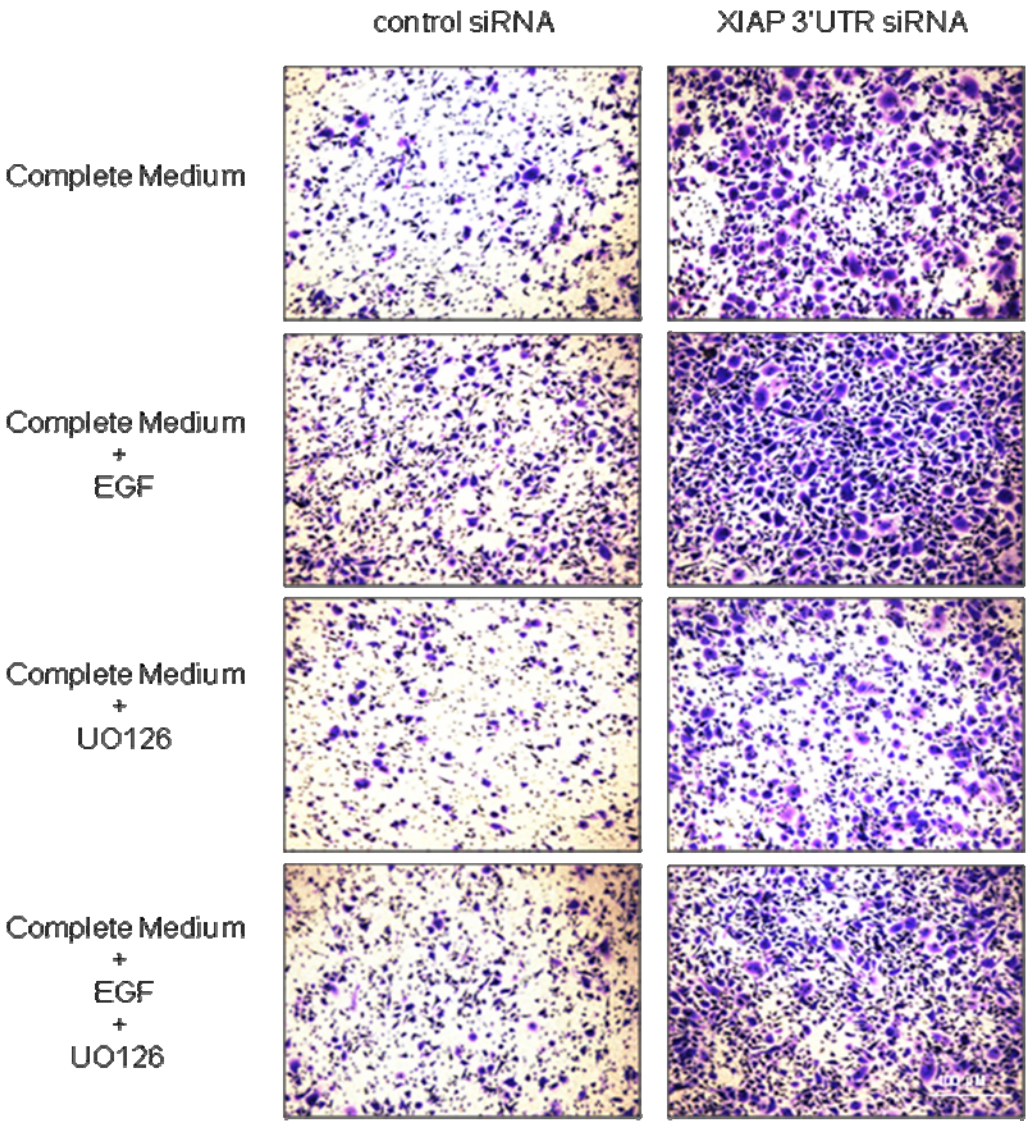
2.6.4 RAF/MAPK pathway is important for th e modulation of cell motility by XIAP

Previous studies have well-established a role for the RAF-MEK-ERK cascade in modulating cell motility[123, 184]. To investigate if the enhanced cell motility observed in siXIAP is due to the high MAPK activity observed in these cells, MEK-1 inhibitor U0126 was employed. As expected, treatment with this inhibitor led to a significant reduction in the transwell invasion observed in control and siXIAP cells suggesting a major contribution of the RAF-MEK-ERK pathway to the observed phenotype (Fig.2.24). These results confirmed a crucial role for XIAP in modulating MAPK pathway and cell motility in human cells.

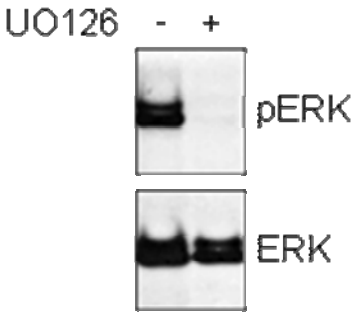
A


control siRNA
XIAP 3'UTR siRNA
control siRNA
XIAP 3'UTR siRNA
Medium without serum
Complete Medium
Complete Medium
+
EGF
+
UO126

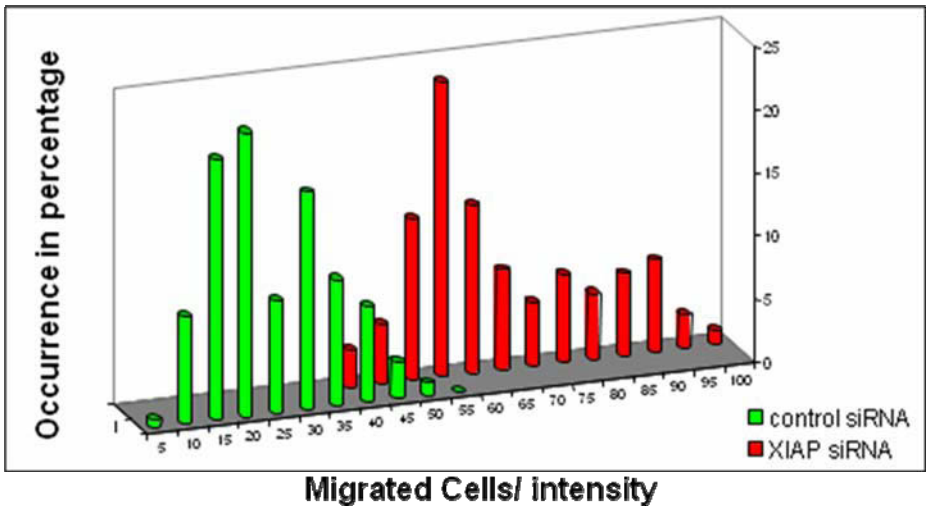
B


control siRNA
XIAP 3'UTR siRNA
Complete Medium
Complete Medium + EGF
Complete Medium + UO126
Complete Medium + EGF + UO126

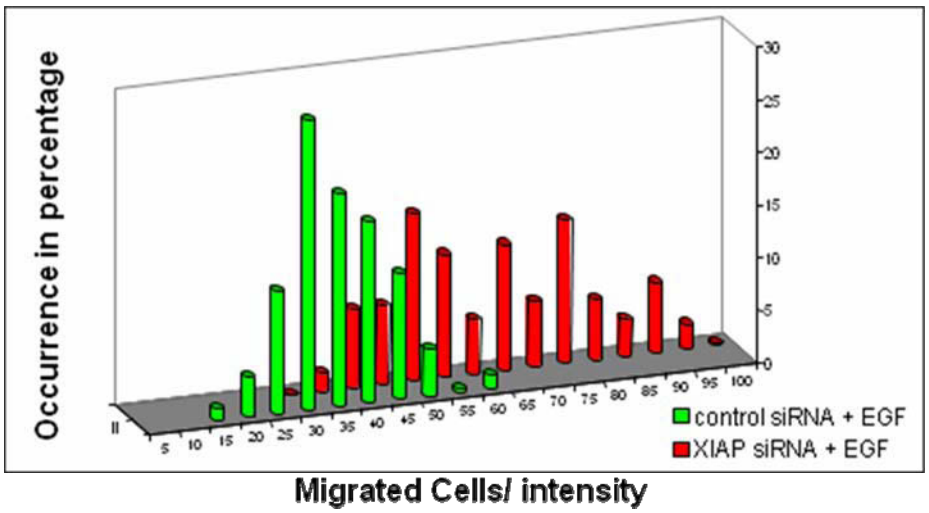
C


UO126 - +
pERK
ERK

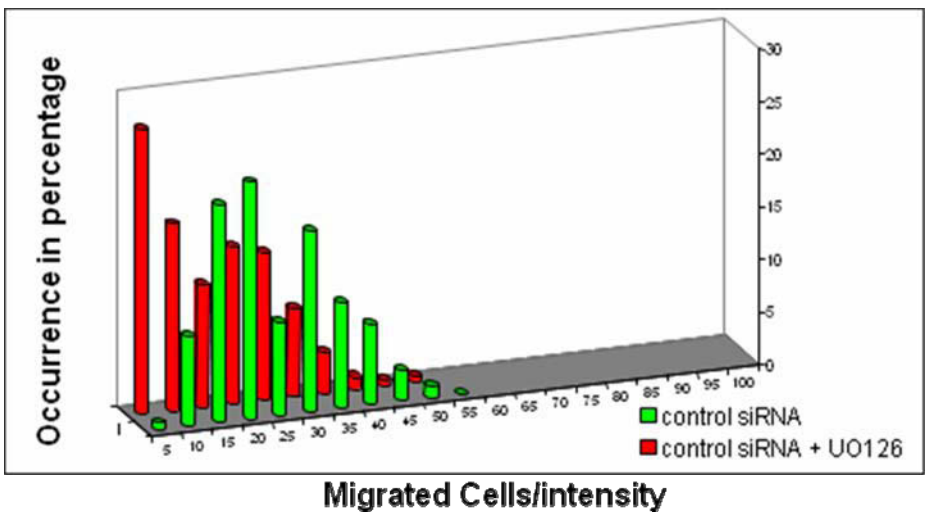
D


Occurrence in percentage
control siRNA
XIAP siRNA
Migrated Cells/ intensity

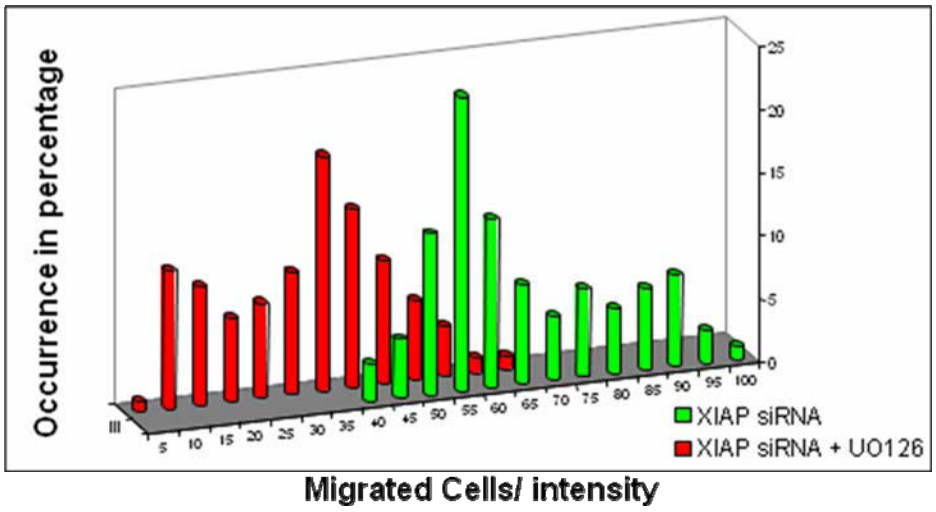
E


Occurrence in percentage
control siRNA + EGF
XIAP siRNA + EGF
Migrated Cells/ intensity

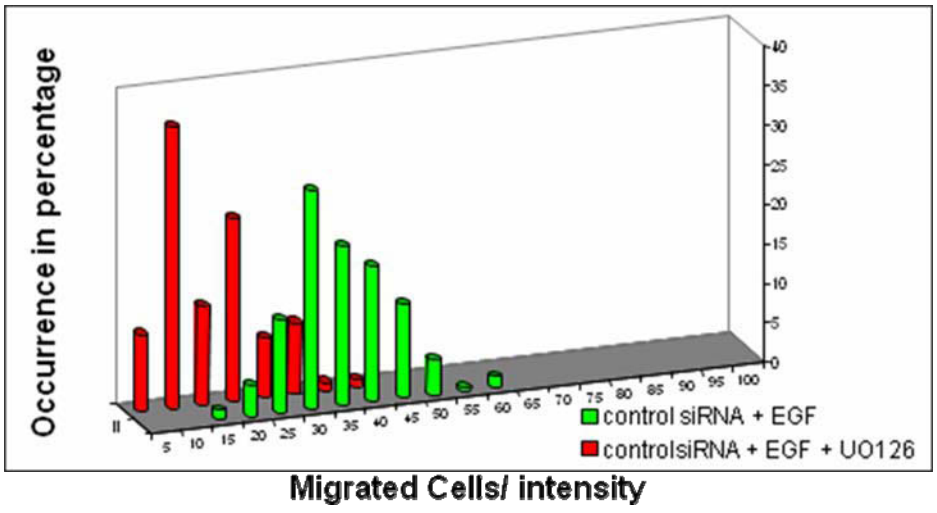
F


Occurrence in percentage
control siRNA
control siRNA + UO126
Migrated Cells/intensity

G



H



I

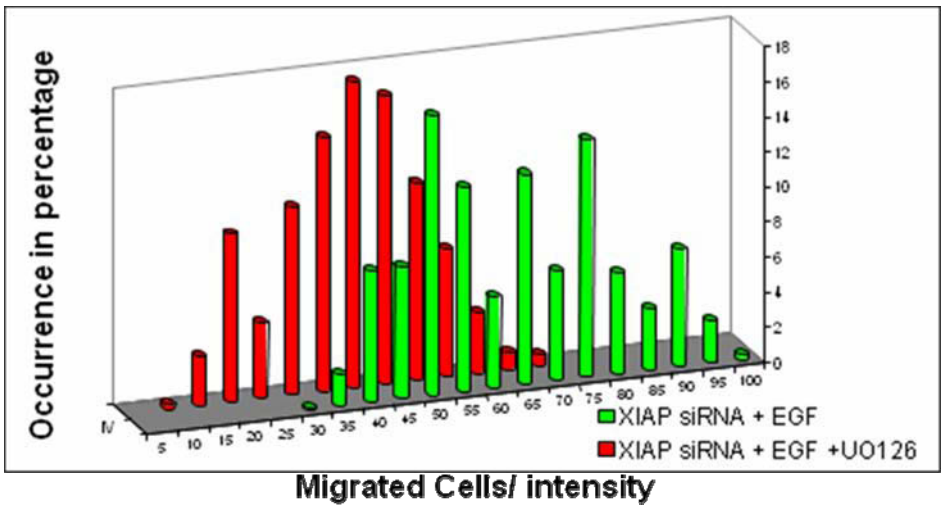


Fig.2.24 MAPK pathw ay is required for cell migration . (A) Transwell migration experiments with control and siXIAP cells were performed as mentioned in the text and the influence of MAPK was analysed by pretreatment of cells with MEK-1 inhibitor U0126 (10

μM). Shown are images of the fixed cells on the transwell chambers and stained with crystal violet. The enhanced brightness/contrast of the image in the upper panel is shown below for better clarity and comparison. (B) The fixed cells were stained and visualized under a phase contrast microscope at 10X magnification. Shown are representative fields from one experiment. (C) Treatment with U0126 (10 μM) significantly reduced pERK levels in HeLa cells. (D-I) The quantification of invaded cells was performed using computer algorithms. Shown are the data from three independent experiments. The bars represent occurrence in percentage.

2.6.5 XIAP and c-IAPs modulate cell motility in a C-RAF dependent manner

To check if the altered morphology and increased motility seen upon XIAP ablation is explicitly linked to C-RAF, double knock down experiments were performed. As expected, enhanced ERK activity and cell migration observed in XIAP siRNA cells is rescued with the reduction in C-RAF levels (Fig.2.25)

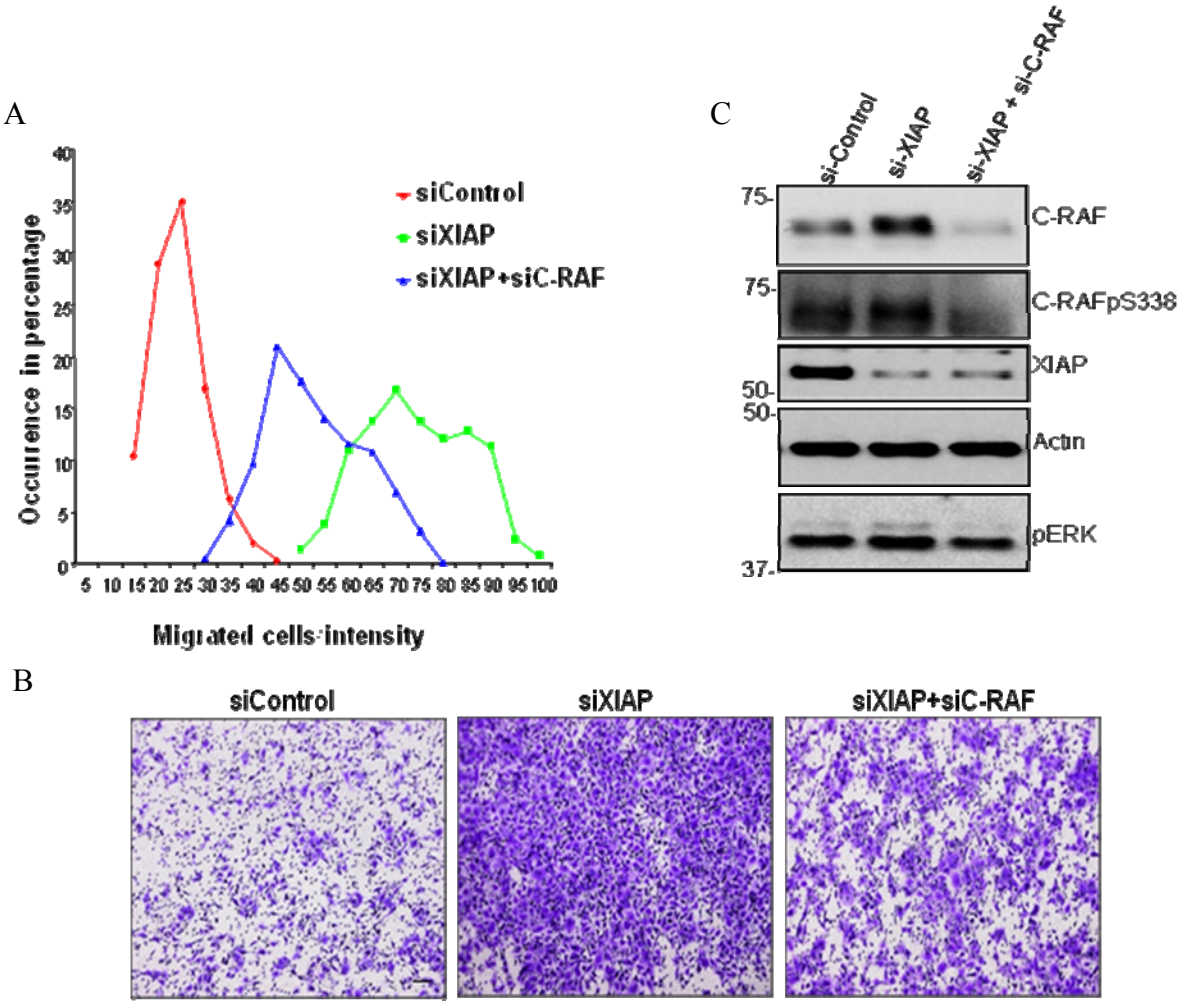


Fig.2.25 XIAP modulates cell motili ty in a C-RAF dependent manner. (B) The transwell migration assays shown in A were quantified with computer algorithms. Data from three independent experiments are shown. (C) HeLa cells were transfected with various siRNAs and the levels of various proteins were monitored.

As silencing of c-IAPs also led to an increase in C-RAF levels (Fig.2.14), it was examined whether silencing of c-IAPs contributes to alterations in cell migration. siRNA-mediated reduction of c-IAPs led to an increase in transwell migration of HeLa cell in a C-RAF-dependent manner (Fig.2.26).

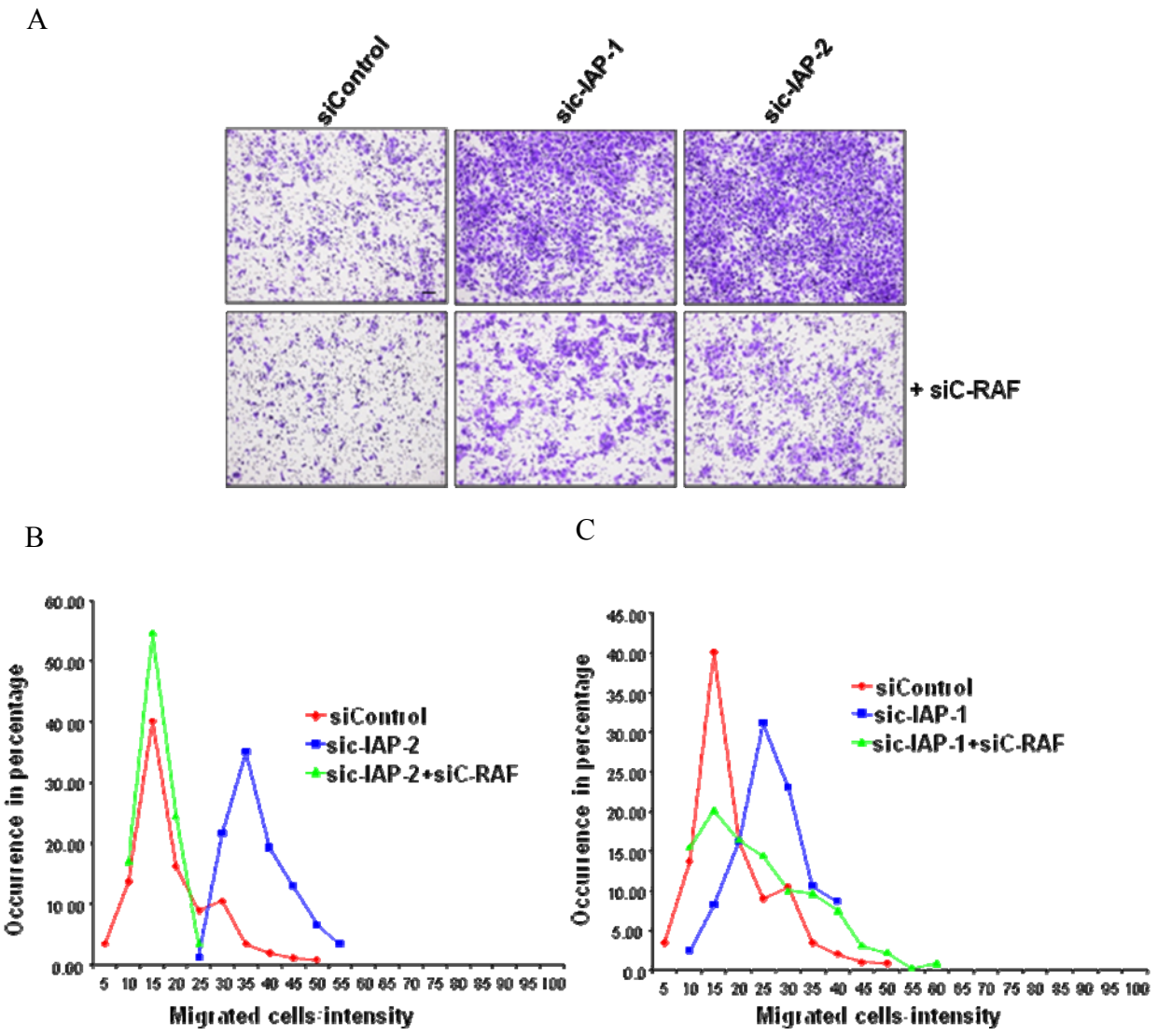


Fig.2.26 c-IAPs modulate cell motility in a C-RAF dependent manner. (A) HeLa cells were tranfected with control and c-IAP siRNAs either alone or in combination with C-RAF siRNAs. The transfected cells were allowed to migrate in a transwell migration chamber. Data from one representative experiment are shown. (B, C) The migrated cells were fixed, stained and quantified with computer-assisted algorithms. Quantification of six fields from a single representive experiment is shown for c-IAP-2 (B) and c-IAP-1 (C).

2.7 BIR1 and BIR2 domains of XIAP are sufficient to modulate C-RAF levels *in vivo*

To investigate an interaction domain(s) of C-RAF for the binding to XIAP the *in vitro* translated C-RAF mutants were used, but they failed to identify any specific domain(s) suggesting that the entire protein is probably required for driving interaction with XIAP (unpublished data). Next, it was attempted to identify the domain of XIAP responsible for driving its interaction with C-RAF. *In vitro* binding experiments with purified proteins encompassing various domains of XIAP suggested a role for the BIR3 domain (Fig.2.27).

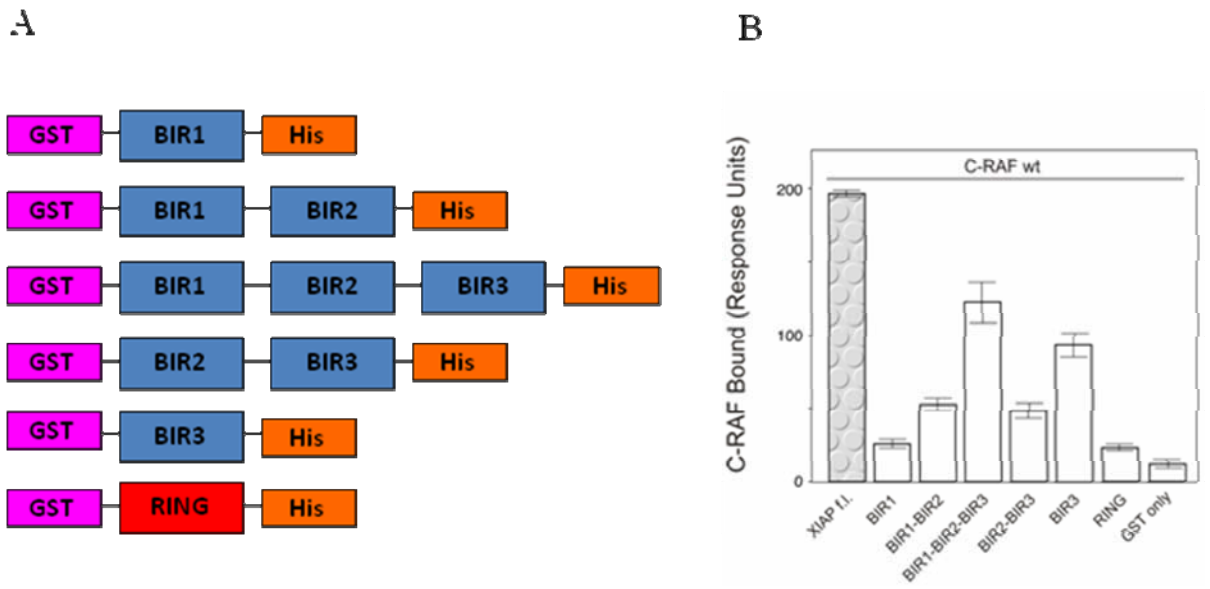


Fig.2.27 BIR3 domain of XIAP shows enhanced binding to C-RAF *in vitro*. (A) Schematic representation of various recombinant XIAP mutant proteins. (B) The interaction of these mutant proteins with C-RAF was measured by Biacore system. Shown are the data representing three independent experiments. The error bars represent ± SD of the mean.

However, complementation experiments with a mutant XIAP lacking C-terminus including BIR3 domain (1-240) failed to stabilise C-RAF in HeLa cells transfected with 3'UTR siRNAs suggesting that BIR1 and BIR2 domains were sufficient to modulate C-RAF levels *in vivo* (Fig.2.28). Furthermore, Smac, as an IAP antagonist, binds to the BIR domains of IAPs[185, 186] and as expected, addition of Smac peptides disrupted the interaction between XIAP and C-RAF *in vitro* in a concentration-dependent manner (Fig.2.29A). In addition, when Smac peptides were added onto the cells, an increase in C-RAF levels was detected (Fig.2.29B).

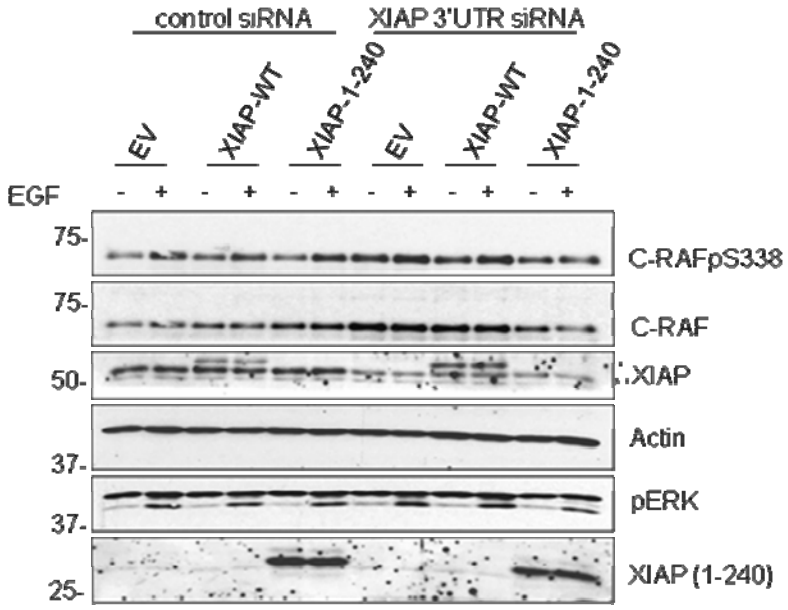


Fig.2.28 BIR3 domain of XIAP is not required for modulating RAF stability *in vivo*. HeLa cells were co-transfected with siRNAs and plasmids encoding XIAP wt and XIAP (1-240) deletion mutants. The cells were stimulated with 20 ng/ml of EGF and the C-RAFpS338, C-RAF, XIAP and pERK levels were detected by immunoblots. Actin was used a loading control. Exogenous and endogenous XIAP were labeled with * and **, respectively.

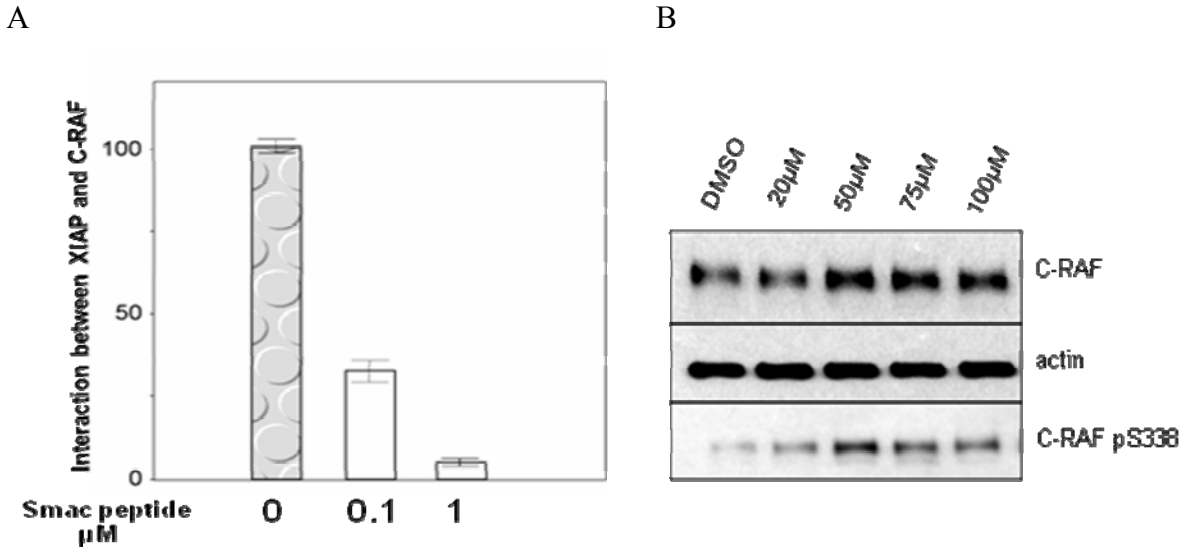
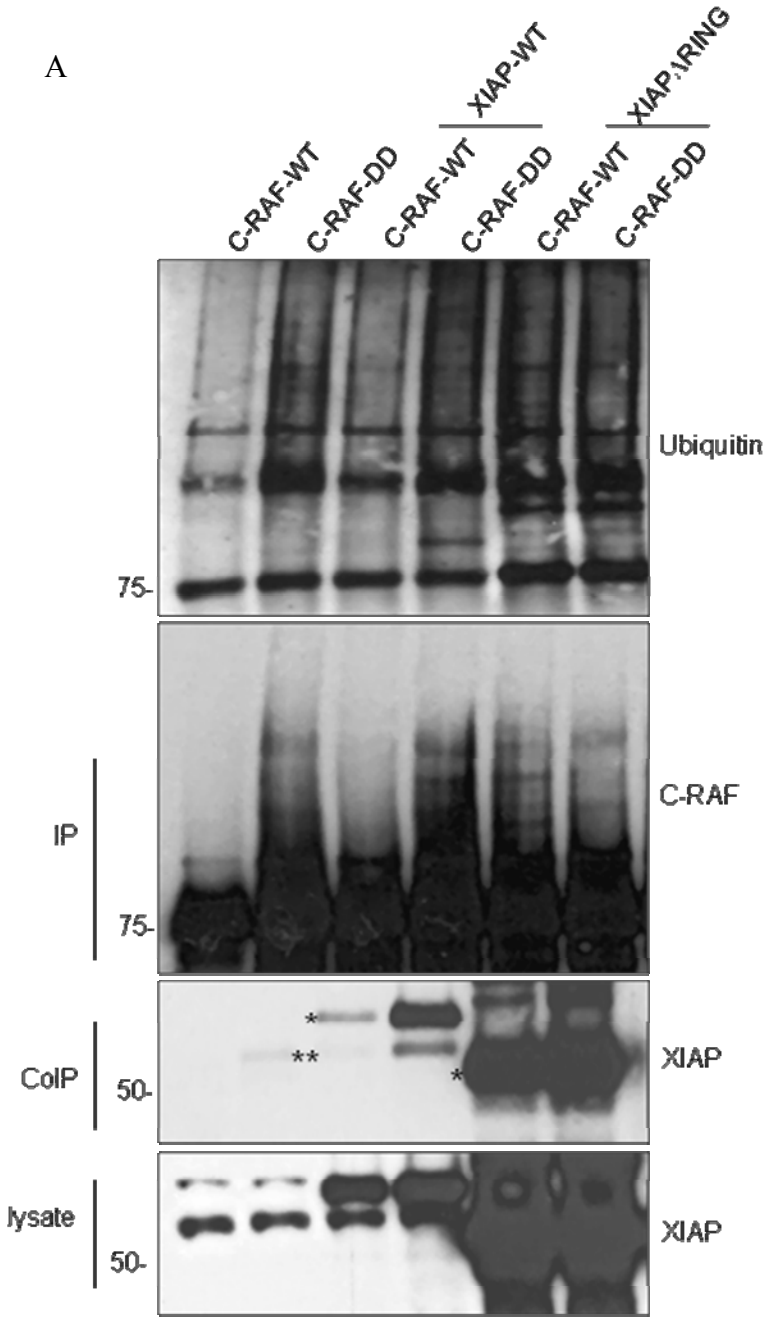


Fig.2.29 BIR-mediated interaction of XIAP w ith C-RAF. (A) The interaction between XIAP and C-RAF is disrupted by Smac AVPI peptide in a concentration dependent manner. Shown are the data from three independent experiments performed with purified proteins in a Biacore system. (B) HeLa cells were loaded with various concentrations of cell permeable Smac-AVPI peptides for 7 hours and the levels of various proteins were monitored by immunoblot analysis.

2.8. RING domain of XIAP is not required for C-RAF stability

As XIAP carries a RING domain, it was hypothesized that XIAP might ubiquitylate C-RAF directly. However, co-immunoprecipitation experiments revealed that XIAP promoted the ubiquitylation of C-RAF in a RING independent manner (Fig.2.30A). Further, complementation experiments with a RING deletion mutant of XIAP (1-336) as well as XIAP (1-240) confirmed that XIAP is probably not the E3 ubiquitin ligase of C-RAF *in vivo* (Fig.2.30B and Fig.2.28).



B

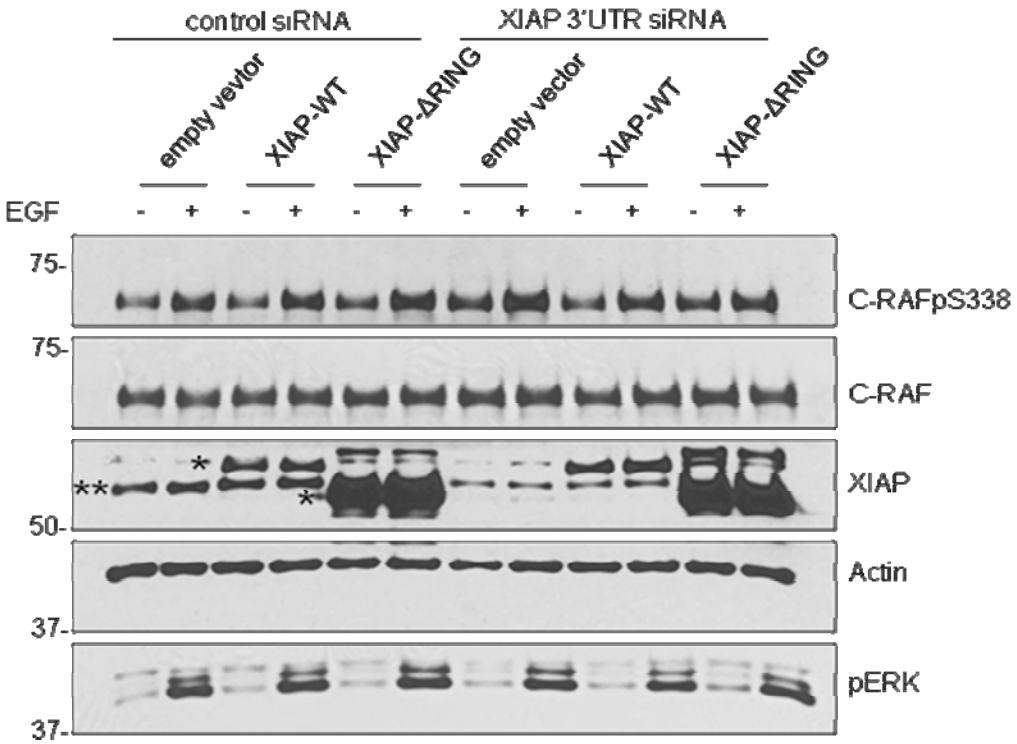


Fig.2.30 XIAP is not the E3 ubiquitin ligase of C-RAF. (A) 293T cells were co-transfected with various plasmids and the ubiquitylation of immunoprecipitated C-RAF was monitored by immunoblots. All samples were pretreated with proteasome inhibitor MG132 (10 μM). (B) HeLa cells were co-transfected with XIAP3'UTR siRNA and XIAP wild type or RING deletion mutant plasmids and the protein levels of RAF and XIAP were monitored by immunoblots. The * indicates the overexpressed XIAP and its ΔRING mutant, ** indicates endogenous XIAP.

2.9. XIAP modulates RAF stability via Hsp90 quality control system

C-RAF is a well-established Hsp90 client protein. Recently, activated B-RAF has also been demonstrated to be a chaperone client and that treatment with Hsp90 inhibitor leads to degradation of mutationally active but not wild type B-RAF[162, 163]. Molecular chaperones are recognized by their ability to bind to newly translated polypeptides and assist them to attain their native conformation. Disruption of Hsp90 activity with benzoquinone ansamycin 17-allylamino-17-demethoxygeldanamycin (17-AAG) leads to ubiquitylation and degradation of C-RAF via proteasomes[187, 188]. However, depletion of XIAP led to an increase in C-RAF protein levels even in the presence of 17-AAG (Fig.2.31).

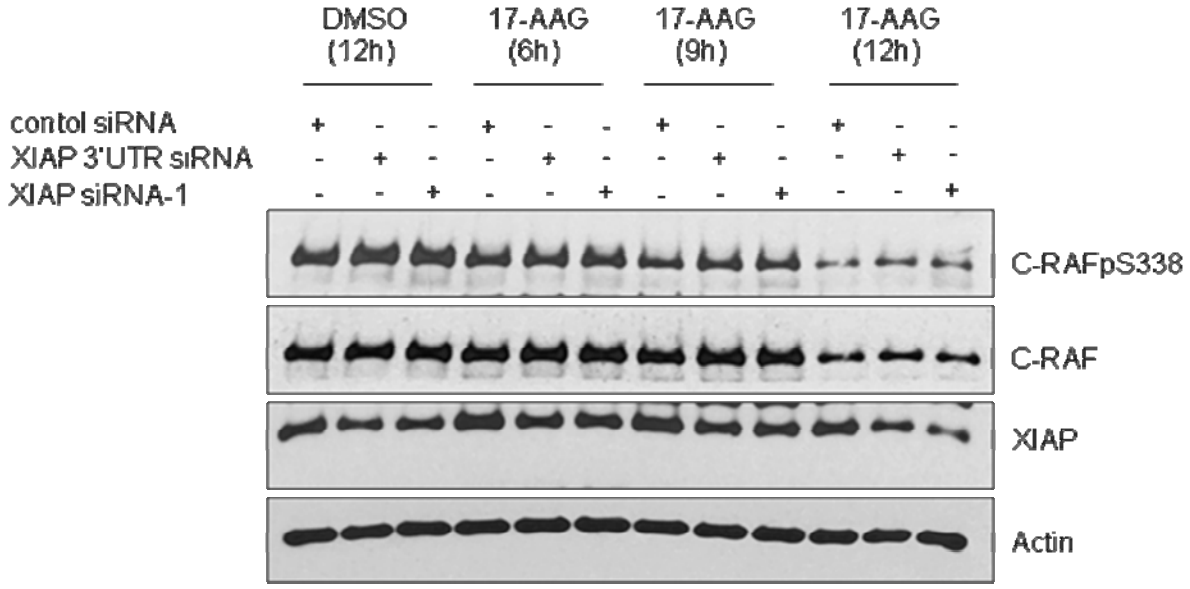


Fig.2.31 Degradation of C-RAF in siXI AP cells treated w ith 17-AAG. Control and XIAP siRNA transfected HeLa cells were treated with 17-AAG (10 µg/ml) for various time points as mentioned in the figure and protein level of RAF and XIAP was detected by immunoblots.

Intriguingly, increasing amounts of co-precipitating Hsp90 with C-RAF in the presence of XIAP in HeLa cells was detected (Fig.2.32).

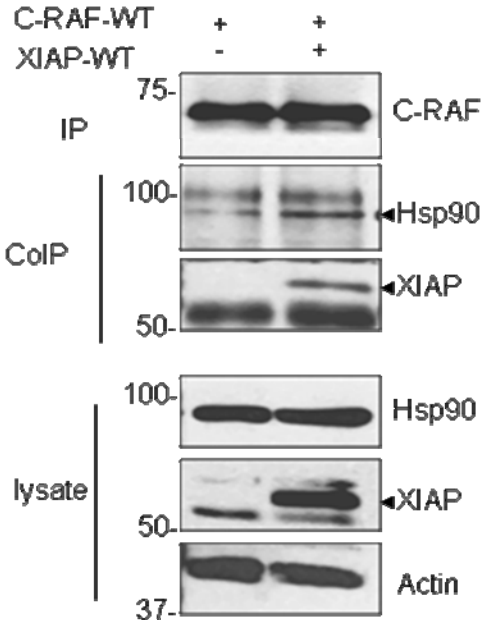


Fig.2.32 C-RAF-XIAP complex encompass Hsp90. HeLa cells were transfected with XIAP expression plasmids and total C-RAF was immunoprecipitated. Co-precipitating Hsp90 and XIAP were monitored by immunoblots.

2.9.1 XIAP promotes the binding of CHIP to the Hsp90-C-RAF complex

Recent evidence suggested that chaperones also direct non native proteins to degradation machinery by recruitment of various co-chaperones and CHIP has been shown to be responsible for the polyubiquitylation and degradation of various chaperone substrates[169]. Furthermore, C-RAF is a known chaperone Hsp90 client protein, and co-chaperone CHIP can ubiquitylate C-RAF directly and leads to its degradation[169, 174, 175].

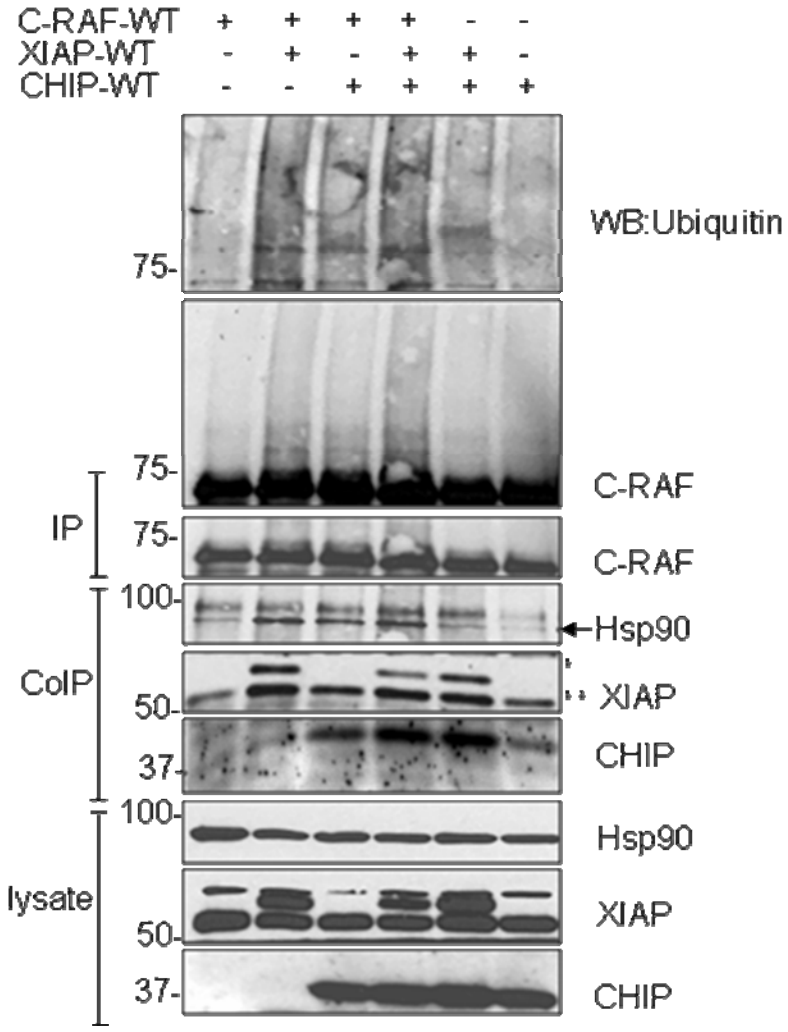


Fig.2.33 XIAP promotes binding of CHIP to the Hsp90-C-RAF complex. The interaction of CHIP with C-RAF in the presence of XIAP was analysed by immunoprecipitation of C-RAF from 293T cells after transfecting with various combinations of plasmids for 48 hours as indicated. All samples were treated with proteasome inhibitor MG132. Exogenous and endogenous XIAP were labeled with * and ** respectively.

To check if XIAP promotes the binding of CHIP to Hsp-90/C-RAF complex, 293T cells were co-transfected with various combinations of plasmids expressing C-RAF, XIAP and CHIP. Immunoprecipitation of C-RAF revealed that XIAP promoted the binding of CHIP to the Hsp90-C-RAF complex and enhanced C-RAF ubiquitylation *in vivo* (Fig.2.33). As expected, co-transfection of XIAP and CHIP led to significant reduction in the total protein levels of C-RAF (Fig. 2.34).

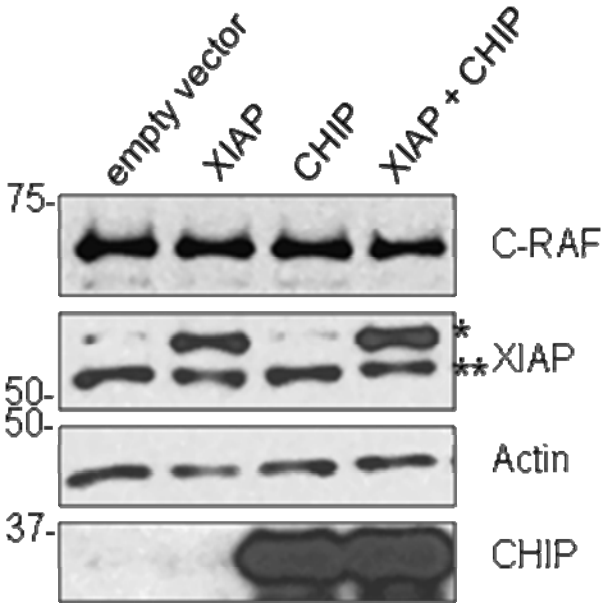


Fig.2.34 Degradation of endogenous C-RAF after co-transfection of XIAP and CHIP. 293 T cells were transfected with various combinations of plasmids as indicated and the alterations in C-RAF levels were monitored by immunoblots as indicated. Exogenous and endogenous XIAP were labeled with * and ** respectively.

2.9.2 RING and BIR3 domains of XIAP are not required for the recruitment of CHIP to C-RAF complex

The contribution of the various domain of XIAP in modulating the interaction between CHIP and C-RAF was then characterized. Consistent with the previous observations (Fig.2.28 and 2.30), neither the RING nor the BIR-3 domains of XIAP are required for the recruitment of CHIP or polyubiquitylation of C-RAF, irrespective of C-RAF activation status *in vivo* (Fig.2.35).

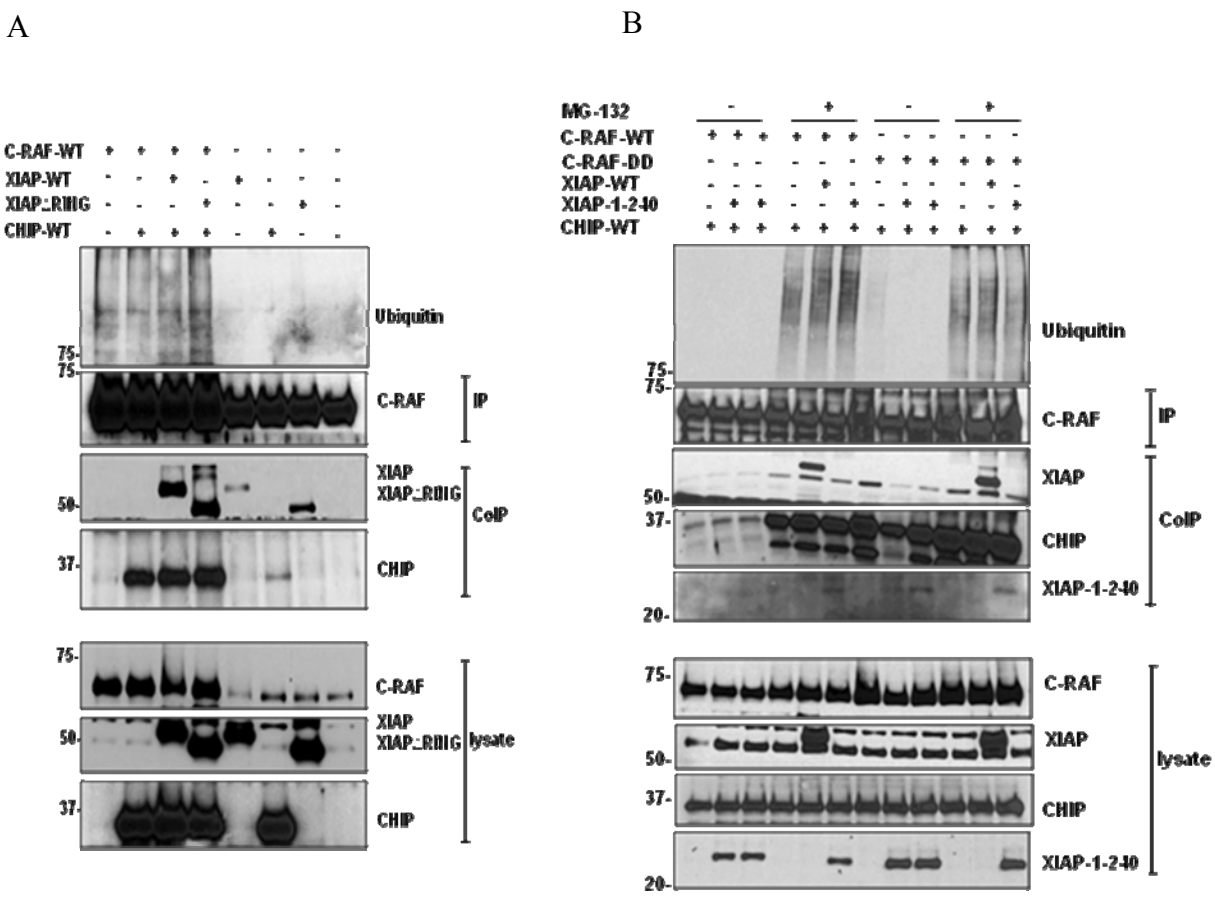
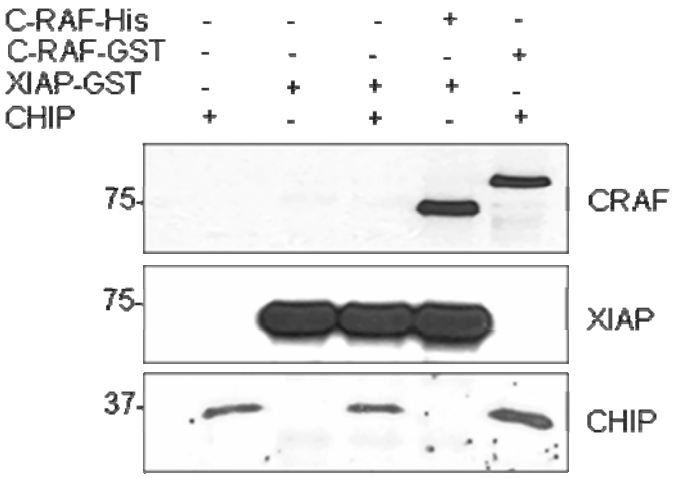


Fig.2.35 RING and BIR-3 domains of XIAP are not required for the recruitment of CHIP to C-RAF complex and poly ubiquitylation of C-RAF. (A) 293T cells were co-transfected with various combinations of plasmids encoding (A) XIAP wild type, XIAP (1-336), C-RAF wild type and CHIP or (B) XIAP wild type, XIAP (1-240), C-RAF wild type, C-RAFDD (active form) and CHIP. C-RAF was immunoprecipitated at 48 h post transfection and the co-precipitating proteins were detected by immunoblot analysis. All samples were treated with proteasome inhibitor MG-132 (10 μM) for 6 h before lysis.

2.9.3 Direct interaction of CHIP with C-RAF and XIAP

Next, it was explored whether there is any direct interaction between XIAP and CHIP. *In vitro* binding assays using purified proteins revealed that CHIP binds directly to C-RAF, but not to XIAP (Fig.2.36A). A direct interaction between CHIP and C-RAF was also detected by Biacore analysis (Fig.2.36B).

A



B

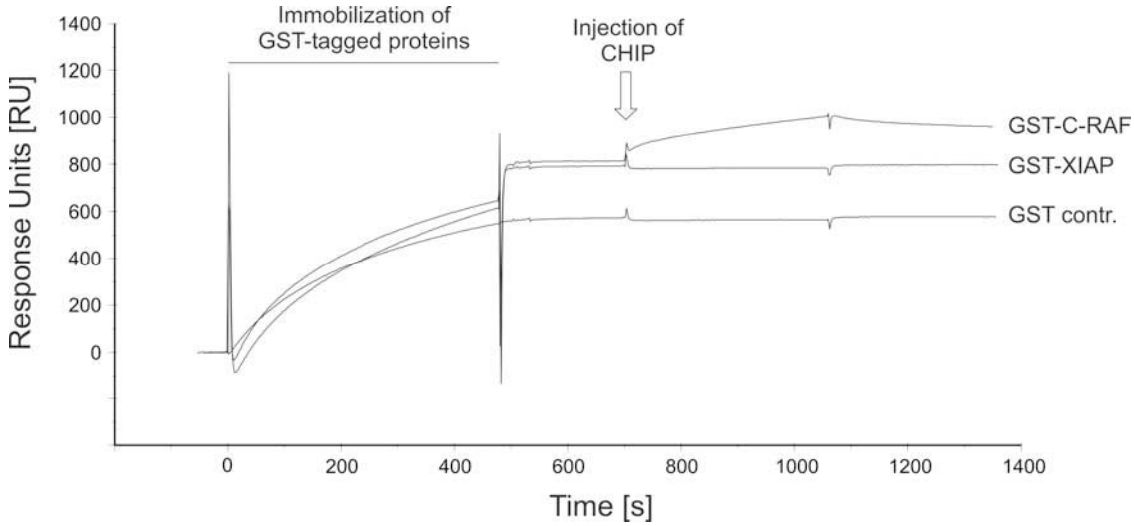


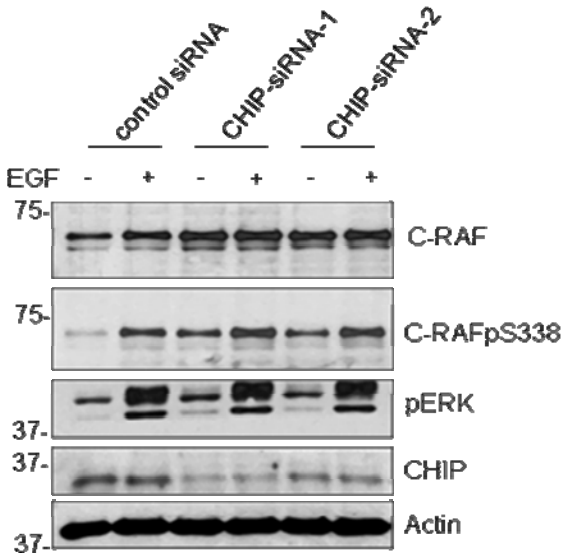
Fig.2.36 CHIP binds directly to C-RAF but not to XIAP *in vitro*. (A) Binding of CHIP with GST-C-RAF and GST-XIAP was checked by an *in vitro* binding assay using purified proteins. (B) The interaction of CHIP with XIAP or C-RAF was measured by Biacore analysis using purified proteins.

These observations suggested that XIAP promoted binding of CHIP to Hsp90/C-RAF complex indirectly by interfering with the folding/conformation of C-RAF in a RING independent manner.

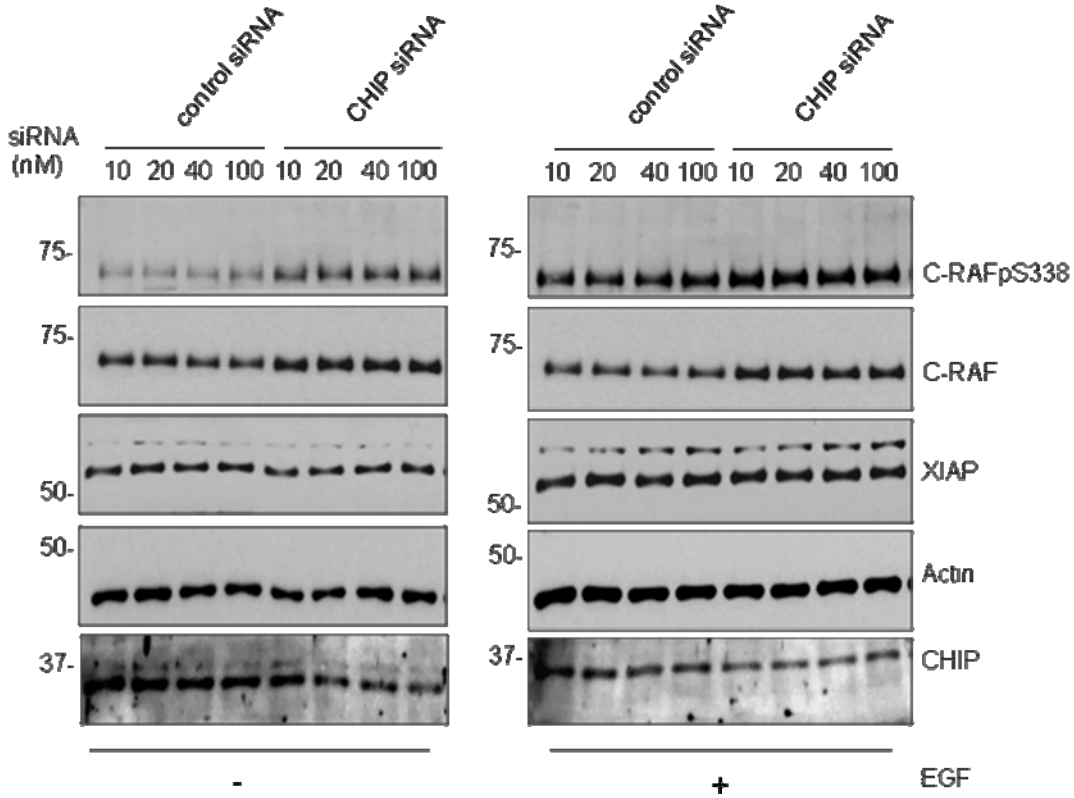
2.9.4 Silencing CHIP expression stabilizes C-RAF

To investigate whether CHIP modulates stabilization of C-RAF *in vivo*, the expression of CHIP was silenced in HeLa cells using siRNAs. An increase in C-RAF protein was readily observed in siCHIP cells without altering XIAP protein levels (Fig.2.37A,B), and on the other hand, depletion of XIAP did not alter CHIP protein levels (Fig.2.37C), suggesting that both XIAP and CHIP were required for modulating the stability of C-RAF *in vivo*.

A



B



C

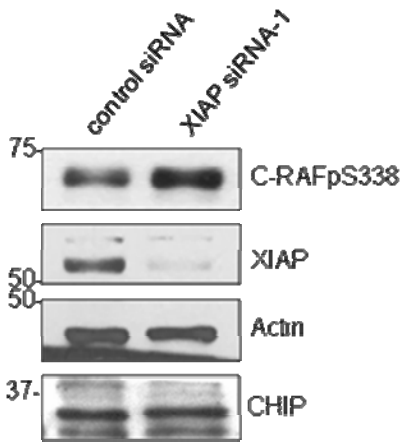
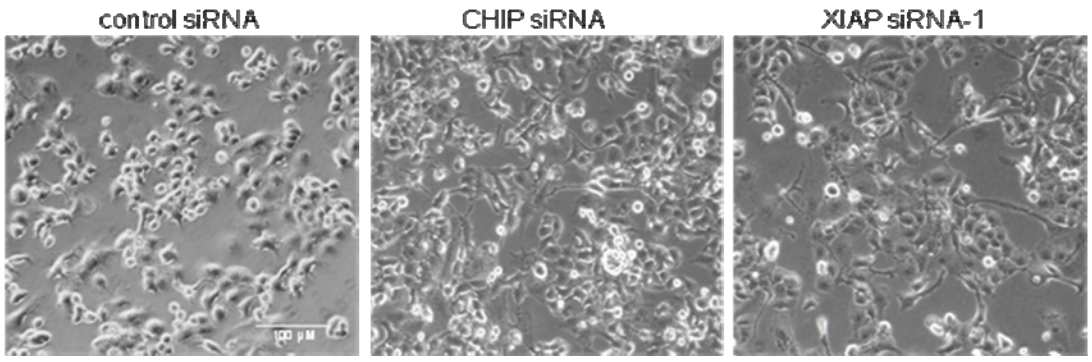


Fig.2.37 Silencing CHIP expression stabilises C-RAF without influencing of XIAP protein levels. (A) HeLa cells were transfected with two sets of siRNAs directed against CHIP for 48 h and the protein levels of C-RAF, C-RAFpS338, pERK, CHIP and actin were detected by immunoblot analysis. (B) Increasing concentrations of control and CHIP siRNAs were transfected in HeLa cells for 48 h and protein levels of XIAP, C-RAF, C-RAFpS338, CHIP and actin were detected by immunoblots. (C) CHIP levels are not altered in XIAP knock down cells. HeLa cells were transfected with control and XIAP siRNAs and the endogenous levels of CHIP and C-RAFpS338 were monitored by immunoblots. Actin was used as loading control.

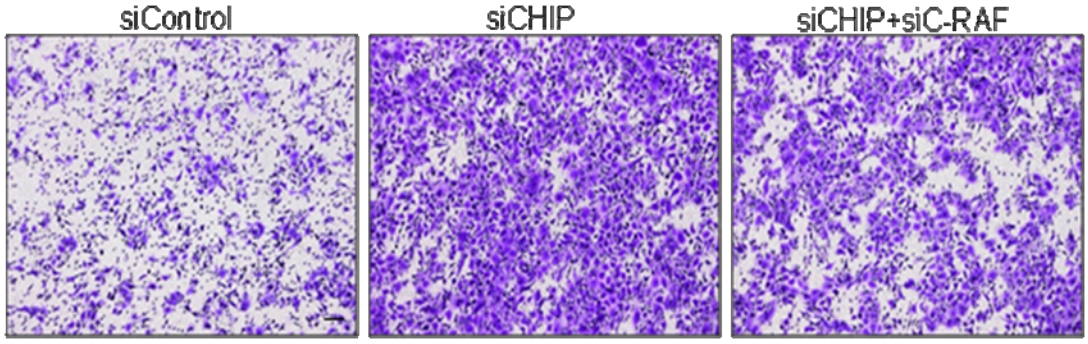
2.9.5 Silencing CHIP induces morphological changes and cell migration

As C-RAF is stabilized in CHIP knock down cells (Fig.2.37A,B), it was tested whether these cells also exhibit morphological changes and enhanced cell migration as observed in siXIAP cells. As expected, siCHIP cells also showed both elongated fibroblast like structures and enhanced transwell migration in a C-RAF-dependent manner (Fig.2.38).

A



B



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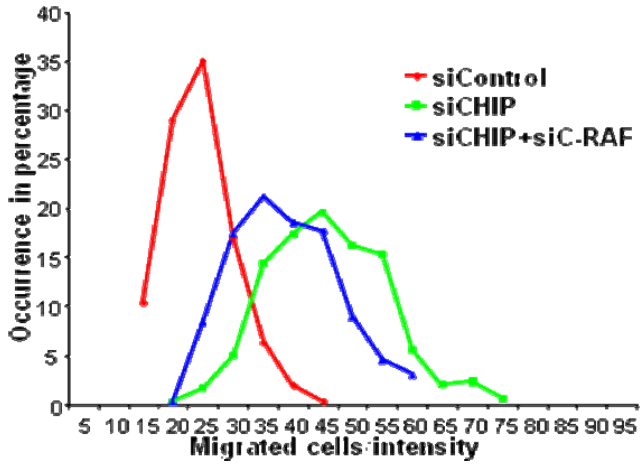

Fig.2.38 Silencing CHIP enhances cell migr ation in a C-RAF dependent manner. (A) Three days after transfection with control, XIAP and CHIP siRNAs, the morphology of HeLa cells on the tissue culture plates was monitored under a phase-contrast microscope. Medium-resolution phase-contrast cell images from one representative experiment are shown. (B) Transwell migration assays were performed with HeLa cells transfected with control and CHIP siRNAs either alone or in combination with C-RAF. Data from one representative experiment are shown.

3. DISCUSSION

3.1 XIAP and c-IAPs interact with C-RAF

Inhibitor of apoptosis proteins (IAPs), originally identified in baculoviruses, have been conserved during the course of evolution and have a crucial role in regulating apoptosis[189]. In human, eight IAPs have been recognized so far. Although IAPs are known as caspase inhibitors, recent studies have shown that apart from their roles in the regulation of caspases, IAPs have also been implicated in activation of several signaling cascades, including the JNK, TGFβ, NF-κB pathways, and in mammary gland development and copper homeostasis[74, 101, 190]. Furthermore, XIAP has been identified as an interacting partner of C-RAF[181, 182]. In this study the interaction between XIAP and C-RAF has been characterized in more detail. A direct interaction between these two proteins was affirmed with purified proteins using both *in vitro* binding assay and Biacore system (Fig.2.1B-C, 2.2B, 2.3A, 2.34A). The interaction between endogenous XIAP and C-RAF was detected in tumor cells irrespective of stimulation status (Fig.2.1A). BAY43-9006 is a small-molecule inhibitor, which was designed specifically to target RAF kinases and is used as a potent inhibitor of RAF kinases *in vitro* and *in vivo*, with significant dose-dependent antitumor activity[191]. Interaction studies with purified proteins in the presence of the RAF inhibitor BAY43-9006, as well as co-immunoprecipitation of mutant proteins, revealed that the kinase activity of C-RAF is not required but may facilitate interaction with XIAP (Fig.2.4). This observation was confirmed also with overexpressed eukaryotic cDNA-constructs transfected in HEK293 cells (Fig.2.4B, 2.28A, 2.30, 2.31 and 2.33).

All known IAP proteins share a characteristic sequence motif, the Baculovirus IAP Repeat (BIR), which contains a number of highly conserved residues[69]. As BIR domains are structurally homologues, the ability of c-IAPs in binding to C-RAF has been tested. These experiments revealed a direct interaction between C-RAF and c-IAPs both *in vitro* and *in vivo* (Fig.2.2 and Ref.[192]). However, c-IAPs bind with relatively less affinity to C-RAF as compared to XIAP. For example, XIAP binds to C-RAF with higher affinity (K_D=1.2 nM) when compared to c-IAP-2 (K_D=29.5 nM). This difference could be attributed to the subtle differences in the BIR domains of these IAPs. Despite weak binding of c-IAPs with C-RAF, these IAPs still modulate the stability of C-RAF. It would be interesting to know whether other IAP members can also bind to C-RAF. For example, one of the human IAPs family members, a melanoma specific IAP, ML-IAP (Livin) shows high-affinity interaction with SMAC similar to XIAP and this interaction will effectively compete with the XIAP-SMAC interaction and regulate apoptosis by sequestering SMAC and preventing it from antagonizing XIAP-mediated

inhibition of caspases[68]. Therefore, Livin acts as a Smac inhibitor, rather than a direct suppressor of caspases and appears to be involved in tumor cell resistance to chemotherapeutic agents. The possible interaction between ML-IAP and RAF kinases and role of these interactions especially in human tumour cells expressing high levels of Livin should be tested.

This study also analyzed the possible interaction between other RAF family members and XIAP. Because of high homology of the RAF family members[110], it could be surmised that XIAP can also interact with both A-RAF and B-RAF kinases. *In vitro* binding assays revealed that C-RAF binds to XIAP with high affinity when compared to B-and A-RAF kinases (Fig.2.3). The observed difference could be attributed to the regions other than those conserved between RAF kinases. Immunoprecipitation assays between RAF chimeras (B- and C-RAF) and XIAP showed that the chimeras containing more N-terminal part of C-RAF protein interact stronger with XIAP, compared with other chimeras (data not shown). Also experiments with *in vitro* translated C-RAF mutants failed to identify any specific domain(s) supporting the idea that the entire protein is probably required for leading interaction with XIAP (unpublished observation; A. Galmiche).

To obtain a complete scenario of IAP-RAF interaction, it is vital to explore the possible binding of various IAPs to RAF isoforms.

3.1.1 XIAP is not phoshorylated by C-and B-RAF

C-RAF is a Serine/Threonine kinase and its specific phosphorylation sites are responsible for kinase activity. Active C-RAF gains another conformation which leads to the binding of its substrates or several other proteins[118]. XIAP is thought to be another substrate of C-RAF and kinase activity of C-RAF may be required for their interaction with XIAP. Tian *et al* showed that C-RAF binds and phosphorylates XIAP *in vitro*[182]. In contrast to this publication, no phosphorylation of XIAP by C-RAF could be detected (Fig.2.5). In this study, beside C-RAF-wt, other active forms of C-RAF purified from SF9 insect cells were employed. Tian *et al* have used active C-RAF purified from bacteria. The observed discrepancy may be attributed to the difference in the sources from which C-RAF kinase is purified.

As B-RAF can also bind to XIAP *in vitro*, it was analyzed if B-RAF can phosphorylate XIAP. No phosphorylation of XIAP by B-RAF was detected (Fig.2.5). Another interesting question remained open in this part is whether other member(s) of IAP family can be phosphorylated by any of RAF kinases and if this phosphorylation contributes to stability of these IAPs.

3.2 XIAP and c-IAPs modulate C-RAF stability

Tian *et al* showed that constitutively active C-RAF inhibits XIAP degradation in response to apoptotic stimuli suggesting a role of C-RAF in modulating the stability of XIAP[182]. Depletion of C-RAF using siRNAs did not alter the protein levels of XIAP in HeLa, Capan I and WM852 cells under normal cell culture conditions, and to exclude eventual unspecific effect of siRNA, two different C-RAF-siRNAs were used (Fig.2.6 and data not shown). Surprisingly, loss of XIAP by siRNA lead to an increase in protein levels of C-RAF (about 2-fold) in HeLa and CAPAN-1 cells (Fig.2.6, 2.9 and 2.10), but no significant changes in the C-RAF mRNA levels after silencing XIAP were detected (Fig.2.7). In addition, no significant changes in the protein levels of B-or A-RAF were observed (Fig.2.16 and data not shown). These observations support that this effect could be very C-RAF specific, which could be attributed to the strong interaction between XIAP and C-RAF.

The effect on C-RAF upon XIAP depletion was not restricted only to HeLa and CAPAN-1 cells, but also detected in various other human tumor cell lines (A549, Hep-2, T24, SH-SY5Y, WM852 and Colo829) and in IMR90 human primary lung fibroblasts (Fig.2.8, 2.13 and data not shown). This suggests that the effect is not confined to tumour cells alone.

It has been shown that crucial phosphorylation (Ser 338 and/or Tyr 341) and dephosphorylation (Ser259) events lead to full activation of C-RAF and its relocation at the membranes, which is normally localized in the cytosol in a multimeric protein complex encompassing 14-3-3 and Hsp90[110]. Both basal and EGF-stimulated C-RAF pSer 338, as well as total C-RAF levels, were found to be increased in HeLa and other cell lines transfected with XIAP siRNAs (Fig.2.6,2.8-10). These results suggested that C-RAF could be stabilized both in cytosol and in membrane. To verify this suggestion, cell fractionation experiments were performed and as expected, an increase in C-RAF levels in both cytosol and membrane fractions in XIAP siRNA cells were detected (Fig.2.15A). On the other hand, as XIAP is primarily cytosolic, it is tempting to propose that XIAP may influence 14-3-3-C-RAF interaction in the cytosol. Also, it is known that phosphorylation of Ser 259 and S621 residues leads to the interaction of C-RAF with 14-3-3 and in turn C-RAF is localised in the cytosol[118]. Consistent with these, phosphorylation of C-RAF at Ser 259 was also increased in siXIAP cells (Fig.2.15B). Furthermore, pull down assay with overexpressed XIAP and C-RAF showed that less 14-3-3 is associated with XIAP-bound C-RAF (data not shown). Taken together, these results confirm the role of XIAP and c-IAPs in modulating the homeostasis of C-RAF in these cells.

3.2.1 Stability of C-RAF in XIAP null MEFs

Despite the important role of IAPs in apoptosis, no significant abnormalities were found initially in IAP-deficient mice[73, 189]. Later studies revealed that XIAP mutant mice have alterations in intracellular copper levels and mammary development[74, 101]. XIAP levels were found to be elevated during late stages of pregnancy and deletion of XIAP leads to aberrant mammary differentiation with elevated levels of active but not total ERK at day 16 of pregnancy in mice[74]. Throughout this study, siRNA-based approaches were used to modulate XIAP levels and their effects on C-RAF stability were explored. Although several different siRNAs were used, and as XIAP and C-RAF are highly conserved, it would be reassuring to know, whether the effects of XIAP on C-RAF stability can be reproduced using XIAP null MEFs. In this work the possible up-regulation of C-RAF in MEFs derived from both the strain-matched wild type and the XIAP knock out mice was analyzed, which were generated by two different groups[73, 74]. In the primary MEFs of first group, no significant changes in the C-RAF levels, but a slight reduction in the C-RAF levels was detected in the transformed XIAP deficient MEFs (Fig.3.1), which may be due to the compensatory increase in the protein levels of c-IAPs[193].

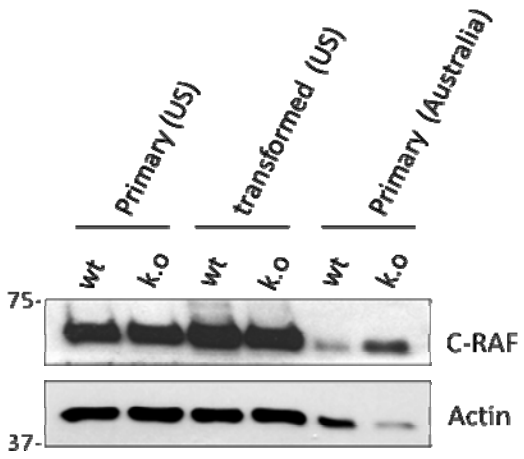

Fig.3.1 Levels of C-RAF in XIAP knock out MEFs. Mouse embryonic fibroblasts derived from wild type and XIAP deficient mice were lysed in sample buffer and the levels of total C-RAF were monitored by immunoblot analysis.

Compared to the MEFs of first group, a significant increase in the C-RAF levels was detected in the XIAP deficient MEFs of second group (Fig.36). These XIAP deficient mice have been demonstrated to have an increase in the phospho- but not total ERK levels in their mammary glands[74]. It should be noticed that, while the US MEFs were transformed with RAS and E1A, the Australian MEFs were transformed with SV40. This could be one reason for the observed discrepancy between these MEFs. In addition, it should not be forgotten that XIAP has also

been previously shown to have species and cell type specific functions. For example, while the XIAP knock-out mice are almost normal, XLP (X-linked Lymphoproliferative Syndrome) patients, which carry mutations in XIAP, have lymphoproliferative disorders and low NKT cells[194].

As c-IAPs also influence C-RAF stability, attempts should be made to test for C-RAF protein levels in cells from c-IAP knock-out mice.

3.3 XIAP and c-IAPs regulate cell motility in a C-RAF-dependent manner

It is known that Rho GTPases interact with and activate downstream effector proteins when bound to GTP, thereby stimulating a variety of processes, including migration, cell division, morphogenesis, neuronal development and adhesion. In addition, they regulate microtubule dynamics, vesicle transport, cell-cycle progression and gene expression[195]. DIAP1 has been reported to interact directly with Rac and regulates migration in an apoptosis-independent manner in border follicle cells from drosophila ovary, which failed to migrate if DIAP1 is lacking[196]. This finding demonstrated a new and unexpected role of DIAP1 since there had been no previous indication till then that IAP proteins contribute to cell migration. On the other hand, it is known well that the RAF-MEK-ERK cascade plays role in modulating cell migration[123, 184]. ERK, JNK and p38 as MAPK family members have the most important role in cell migration[197]. For example, ERK could be activated through Ras-Raf-MEK cascade to mediate cell migration triggered by growth factors, integrin engagement and neurotrophic factor[123, 198, 199]. Beside changes in the levels of C-RAF and p-ERK1/2 in XIAP-depleted cells (see section 3.2), morphological changes were also observed in these cells; e.g. enhanced lamellipodia and filopodia formation (Fig.2.18,19). Consistently, a subtle enhancement in the basal activity of Rac1 and Cdc42 in XIAP siRNA cells was detected (Fig.2.20), which may suggest that active C-RAF could target additional factors to stimulate migration. However, C-RAF is not the only one component of MEK/ERK signaling. To clarify whether altered morphology and increased motility seen upon XIAP ablation is explicitly linked with C-RAF, double knock down experiments were performed. The enhanced ERK activity and cell migration observed in XIAP siRNA cells were rescued with the reduction in C-RAF levels (Fig.2.25). The participation of RAF-MEK-ERK cascade in the siXIAP cell migration was also confirmed by treatment of cells with the MEK inhibitor U0126, which is one of the best known MEK inhibitors[200, 201] and this treatment caused a significant reduction in the transwell invasion observed in control and XIAP siRNA cells, suggesting a major contribution of the RAF-MEK-ERK pathway to the observed phenotype (Fig.2.24B-I). But it is difficult to tell if

the U0126 is acting on XIAP-dependent or independent processes since the motility of control RNAi-treated cells is also reduced with the drug.

Attempts to perform cell migration assays on transwell migration chamber with XIAP knock out MEFs failed as the cells died after seeding on chambers. The option of wound healing experiment could not be explored as well as they never attained confluency under the recommended culture conditions. In this case, it could be helpful if MEFs are isolated fresh from mice and all these assays would be performed with freshly isolated/cultured MEFs. Also, it would be very interesting to know whether c-IAP-1/-2 knock out MEFs would show the same morphological changes and increased motility like sic-IAPs cells.

It would be also interesting to know whether ablation of other RAF isoforms (A-and B-RAF) could reverse the increased cell motility in XIAP siRNA cells. As no significant changes in the protein levels of A-or B-RAF in XIAP depleted cells were detected, it would not be expected that A-and/or B-RAF could modulate the increased cell migration in these cells. Indeed, knock down of XIAP leads to increase in C-RAF protein levels in both RAS mutant and B-RAF mutant (V600E) cells. However, pERK levels are influenced only in RAS mutant cells but not in B-RAF-V600E cells (Fig.2.8 and Ref.[192]). On the other hand, it has been shown that C-RAF and B-RAF can form heterodimer[202], and oncogenic BRAF mutations containing many cancer samples have deregulated C-RAF activity[130], suggesting a possible cross-regulation of RAF isoforms.

Also, it would be interesting to know, whether overexpression of XIAP reduces cell motility, and if a mutant XIAP incapable of binding C-RAF fails to reduce motility. But, the establishment of a stable HeLa cell line expressing high levels of XIAP to perform these experiments failed. However, transwell migration assays with GFP-XIAP-expressed cells showed reduced levels of C-RAF (Fig.2.12), also failed, as the cells couldn't survive long after the sorting procedure. Finally, with a transfection efficiency of 25-30% transwell migration assay was performed and compared to the control cells a significant decrease in the transwell invasion of XIAP-overexpressed HeLa was observed (Fig.3.2)

These results confirmed and extended the role of IAPs in modulating C-RAF and cell motility in human cells. As there are several evidences demonstrating the links between cell migration and survival[203-206] and as Drosophila IAP1 shows a new and unexpected molecular link between survival and migration[196], it could be conceivable that XIAP and c-IAPs may participate with RAF kinases together in one of these biochemical pathways that possibly modulates cell survival and cell motility.

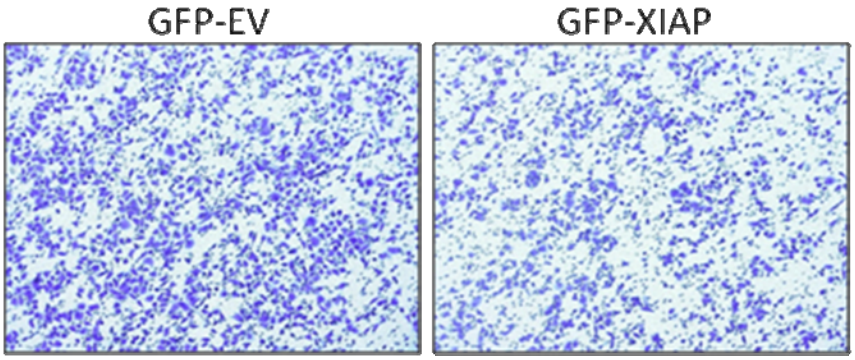

Fig.3.2 XIAP reduces cell motility. HeLa cells were transfected with GFP-EV (empty vector) or GFP-XIAP constructs for 36h. Equal number of cells were allowed to migrate in a transwell migration chamber. The migrated cells were fixed and stained with crystal violet.

3.4 Molecular mechanism by which XIAP modulates C-RAF stability

First, *in vitro* binding assays with purified proteins containing various domains of XIAP showed that BIR3 domain of XIAP could be responsible for the binding to C-RAF (Fig.2.27). Then, the indispensability of BIR3 domain for modulating C-RAF levels was examined using complementation assays with a mutant XIAP (1-240) lacking the BIR3 domain *in vivo*. It was clear that BIR1 and BIR2 domains are enough to modulate C-RAF levels *in vivo* (Fig.2.28, 2.30). Smac/DIABLO is an IAP antagonist normally localized to the mitochondria and released into the cytosol during apoptosis. Smac binds to the BIR domains of IAPs through its four amino acids at the N-terminus (AVPI)[185, 186]. As expected, Smac peptides disrupted the interaction between XIAP and C-RAF *in vitro* in a concentration-dependent manner (Fig.2.29A). In addition, when Smac peptides were added onto the cells, an increase in C-RAF levels was detected (Fig.2.29B). However, no alterations in transwell migration assays in Smac-peptide-treated cells were observed (data not shown). This could be attributed to the instability of the peptide over longer durations, leading to a transient increase in C-RAF levels, compared with the levels seen in XIAP or c-IAPs siRNA cells. Furthermore, expression of a cytosolic form of Smac such as a Ubiquitin-Smac fusion protein, which is processed by Ubiquitin-specific proteases to reveal the IAP-binding motif, would be predicted to produce the same phenotypic effects of XIAP and c-IAPs knock down and increase cell motility by neutralizing these IAPs. As expected, transfection of Ub-coupled AVPI-Smac failed to induce cell death. However, expression of ΔAVPI-Smac induced c-IAP degradation as did AVPI-Smac in these cells (data not shown). In addition, the transfection efficiency of these constructs was too low to perform any motility experiments.

In particular, a number of Smac-mimetic compounds and anti-sense oligonucleotides directed against IAPs are currently undergoing clinical trials[186]. Although Smac-mimetic compounds kill tumour cells through TNF-α signaling, the effects on C-RAF stability and cell migration needs to be tested to adroitly administer IAP antagonist-based anti-tumour therapeutics.

3.4.1 XIAP promotes CHIP-mediated C-RAF degradation

C-RAF is a well-established Hsp90 client protein and ubiquitylation of C-RAF leads to its degradation by the proteasome[161, 187, 207]. Also, as XIAP has E3 ligase activity, it can be surmised that the enhanced stability of C-RAF in XIAP siRNA cells could be due to the fact that XIAP might ubiquitylate C-RAF and lead to its degradation via the proteasome. But several experiments with regard to this aspect showed that XIAP is probably not the E3 ubiquitin ligase of C-RAF (Fig.2.28,2.30). As known, co-chaperones can perform various roles ranging from ubiquitylating the substrate proteins to the delivery of polyubiquitylated proteins to the proteasome degradation machinery. One of the key co-chaperones is CHIP which binds to both Hsp70 and Hsp90[188]. The binding of CHIP to chaperones is mediated by the N-terminal tetratricopeptide repeat (TPR-) domain in conjunction with a highly charged region. CHIP possesses a U-box motif which facilitates the interaction with E2 ubiquitin conjugation-enzymes of Ubc4/5 family[169]. Chaperone associated C-RAF can be diverted to degradation pathway through co-chaperone/ubiquitin ligase CHIP[174, 188, 208]. Further, previous studies have revealed that CHIP can ubiquitylate C-RAF directly[175]. Disruption of the C-RAF-Hsp90 interaction with geldanamycin (17-AAG) leads to C-RAF misfolding and its consequent degradation via the proteasome[175, 187]. However, depletion of XIAP led to an increase in C-RAF protein levels even in the presence of 17-AAG (Fig.2.31). Further, analyses were performed to determine the possible role of XIAP in CHIP-mediated degradation of C-RAF. These results suggested that XIAP promotes the binding of CHIP to the Hsp90-C-RAF complex and enhances C-RAF ubiquitylation and degradation in a RING independent manner (Fig.2.33-35). In addition, XIAP does not bind to CHIP directly (Fig.2.36). To investigate whether CHIP modulates stabilization of C-RAF *in vivo*, siRNAs for depletion of CHIP were used and an increase in C-RAF protein (~2-fold) was readily observed in CHIP siRNA cells, with no significant change in XIAP protein levels, suggesting that both XIAP and CHIP are required for modulating the stability of C-RAF *in vivo* (Fig.2.37A,B). On the other hand, depletion of XIAP did not alter CHIP protein levels (Fig.2.37C). These observations differ from those of a recent study, which suggested that CHIP may not be involved in modulating C-RAF levels in cells derived from genetically modified mice[209]. Apart from species and cell-

type-specific differences, the observed discrepancy could also be attributed to the transformation status of the cells. Further, morphological changes similar to siXIAP cells were observed in CHIP-silenced HeLa cells (Fig.2.38A). As expected, CHIP-silenced HeLa cells also showed enhanced transwell migration in a C-RAF-dependent manner (Fig.2.38B,C). In summary, these results suggest that interaction with XIAP and c-IAPs destabilizes C-RAF and shift the "molecular triage" controlling C-RAF toward the degradation machinery (Fig.3.3).

As c-IAPs also control C-RAF levels, it would be interesting to check if c-IAPs could ubiquitylate C-RAF in a CHIP-dependent or independent manner.

C-RAF contains a PEST sequence, which within proteins mostly acts to promote degradation[210], and a lysine at residue 309 in its PEST domain could serve as a ubiquitin binding site[209]. Ubiquitin is amongst the most versatile proteins in the cell because of its ability to modify substrate proteins by its monomeric form (monoubiquitylation) or by formation of conjugated Ub chain (polyubiquitylation)[211, 212]. Monoubiquitylation can be a signal for several functional consequences for the targeted protein, including changes in binding properties, subcellular localization and activity, viral budding and DNA repair[211, 213, 214], whereas polyubiquitylations occur at lysine residues at position 48 (Lys48) and regulate mostly protein stability by proteosomal degradation[215]. But polyubiquitin chains that polymerize through lysine 63 of ubiquitin seem to be involved in proteasome-independent function, such as signal transduction of the inflammatory cytokine interleukin-1-β (IL1β), protein-protein interaction and subcellular localization[215, 216]. Therefore, it would be very interesting to know which lysine residue(s) of C-RAF play(s) crucial role for its mono-/polyubiquitylation. Additionally, the requirements of IAPs and CHIP as E3 ligases in this process should be explored, and since C-RAF degradation by CHIP is facilitated by the ubiquitin-binding protein BAG-1, which targets ubiquitylated proteins for the proteasome machinery[165, 175], it could be expected that silencing of BAG-1 may show the same effect on C-RAF stability like XIAP and CHIP depletion. Also, it should be tested whether E3 ligase deficient and/or C-RAF-binding deficient CHIP mutants can fail to ubiquitylate C-RAF, confirming that CHIP is indeed the E3 ligase of C-RAF.

Apart from ubiquitin, as a ubiquitin-related modifier, SUMO (small ubiquitin-related modifier) could also be considered to affect the posttranslational modification of C-RAF, and indeed, the SUMOplot™Predictum program from ABGENT predicted a motif with high probability within C-RAF for SUMOylation (http://www.abgent.com.cn/doc/sumoplot/, data not shown). Like ubiquitin, SUMO proteins covalently and reversibly bound to specific lysine residues in the eukaryotic target proteins. In contrast to ubiquitylation, SUMOylation does not target proteins for proteosomal degradation[217]. SUMOylation is involved in diverse biological

pathways, such as genome integrity, chromosome packing and dynamics, meiosis, mitosis and especially in various aspects of signal transduction[218-222]. The SUMO-conjugated lysine residues can also play a role for other post-translational modifications, including ubiquitylation and acetylation. For example, SUMOylation can stabilize the target protein by competition with ubiquitylation of the same lysine residue, as has been shown for IκBα [218 223 224]. Further, phosphorylation may regulate the SUMOylation of a substrate through a highly conserved motif named phosphorylation-dependent sumoylation motif (PDSM) and the majority of the PDSM-containing proteins are transcriptional regulators[225]. Many SUMOylation sites lie in a consensus motif ΦKXE/D, where Φ is a large hydrophobic amino acid, K is lysine, X is any amino acid, and E/D is glutamate or aspartate. As a motif within C-RAF for SUMOylation was predicted, and as C-RAF has various phosphorlyation sites and is ubiquitylated, it would be very interesting to know whether C-RAF is SUMOylated, and if it does, a cross-talk between its SUMOylation and other post-translational modifications like ubiquitylation and phosphorylation should be analyzed. On the other hand, there is also another cross-talk between phosphorylation, ubiquitylation, and proteosomal degradation, and many kinases are now known to be involved in regulatory steps like phosphorylation that generally promote proteasome-mediated degradation of their target proteins[226]. Very recent finding showed that autophosphorlyation of C-RAF at serine 621 prevents the proteasome- mediated degradation of C-RAF[209].

3.5 Impact of C-RAF stabilization by IAPs on Tumorigenesis

As discussed earlier, IAP deficient mice do not have any significant abnormalities except that in XIAP knock-out mice some alterations in intracellular copper levels and mammary development have been detected[73, 74, 101, 189]. Also, XIAP levels were found to be elevated during late stages of pregnancy and deletion of XIAP leads to aberrant mammary differentiation with higher levels of active but not total ERK at day 16 of pregnancy in mice[74]. The observations in this thesis may have relevance in this process of terminal differentiation. In tumours where XIAP and c-IAPs are frequently overexpressed, C-RAF may not necessarily be degraded. This process depends on the availability of CHIP and other components of the Hsp90 quality control system. In addition, IAPs are localized in several multimeric protein complexes, cross-regulate each other and can undergo post-translational modifications[69, 227].

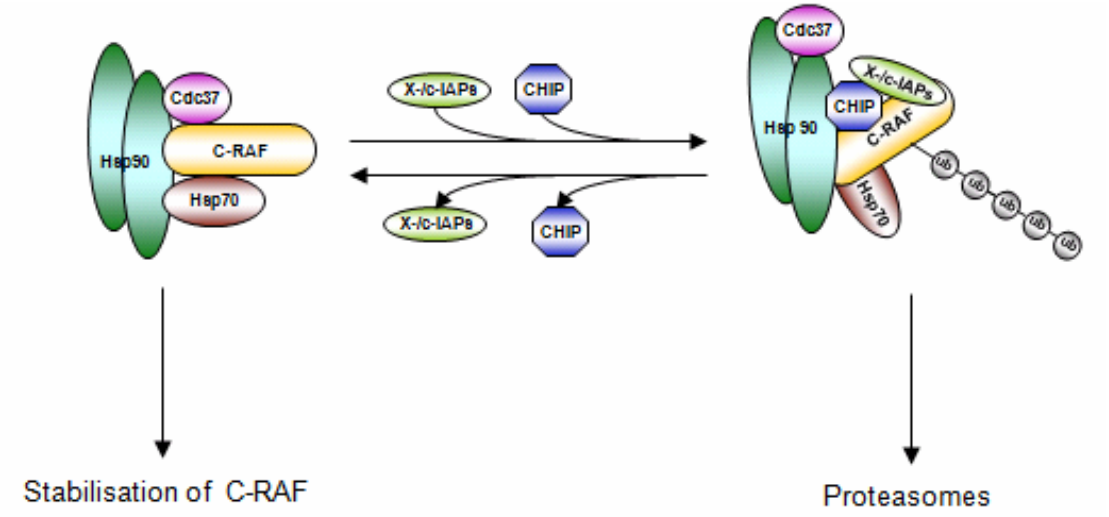

Fig.3.3 XIAP favours the association of CHIP to Hsp90-CRAF com plex. Simplified model demonstrating the suggested role of XIAP and c-IAPs in modulating C-RAF stability. Binding of XIAP alters the conformation of C-RAF which leads to the recruitment of co-chaperone/Ubiquitin ligase CHIP and promotes polyubiquitylation of C-RAF, which is then targeted to proteasomes for degradation.

The ratio of XIAP to other components of the complex may also influence its function. Deciphering the composition, dynamics and function of IAP-IAP-CRAF complexes will further strengthen our understanding of these molecules which are extensively targeted by cancer therapeutics[186, 228].

4. MATERIALS AND METHODS

The methods described in this section are all based on standard molecular and cellular biology techniques.

4.1 Materials

4.1.1 Instruments

Hardware	*Manufacturer*
Autoclave	Webeco
Blotting chamber	Bio-Rad
Bacterial incubator	Heraeus B 6200
Bacterial shaker	New Brunswick Scientific innova 4330
Cell culture hood	Heraeus Instrument
Cell culture incubator	Heraeus Instrument
Dissection microscope	Leica
DNA Sequencer	ABI PRISM 373, ABI
Electrophoresis power supply	Bio-Rad
Electrophoresis unit, small	Bio-Rad Mini-Protean II
Exposure cassettes	Dr. Goss-Suprema
FACS Calibur	Becton Dickinson
Filter paper	Schleicher & Schuell
Fluroscence Microscope (CCD)	Leica (Hamamatsu Orca)
Freezer-20°C	Liebherr
Freezer-80°C	Nunc
Fridge	Liebherr
Gene pulser	Bio-Rad
Heating block	Gebr. Liebisch
Ice machine	Scotsman
Inverted microscope	Olympus
Mega centrifuge	J-6B, Beckman; Megafuge 1.0 R, Heraeus; RC 5B plus, Sorval
Microlumat	EG&G, Berthold
Microscope Slides, Super Frost® Plus	Menzel-Gläzer
Microvave	Severin
Mini centrifuge	5417R, Eppendorf; Biofuge 15, Heraeus
PCR machine	Eppendorf
pH meter	Microprocessor, WTW, Hartenstein
Phosphoimager	Fujix BAS-2000 III, Fuji, with plates BAS-MP 2040P, Fuji

Photometer	Hitachi
Pipettes	Gilson
Pipette boy	Eppendorf
Power supply	Bio-Rad
Scale	BP2100S, BP310S, Sartorius
Shakers	BellcoBiot, Heidolph, Unimax 2010
Spectrophotometer	Ultraspec 3000, Pharmacia Biotech
Thermocycler	PE9600, Perkin Elmer; T3, Biometra®
Timer	Roth
Ultraviolet Crosslinker	UVC500 Hoefer Scientific Instruments, Sony
Vortex	Scientific Industries Genie-2
Water bath	GFL 1083, Amersham-Buchler

4.1.2 Chemical reagents and general materials

Reagent	***Purchased from***
17-AAG	Calbiochem
[α-^{32}P]dATP (370MBq/ml)	Amersham
1 kb DNA ladder	Invitrogen, MBI
Acrylamide /Bisacrylamide (40%)	Bio-Rad
Adenosin-5'Triphosphate (ATP)	Sigma
Agarose, ultra pure	Invitrogen
Ammonium Acetate	Sigma
Ammonium peroxydisulfate (APS)	Sigma
Ampicillin	Sigma
AntifoamA	Sigma
Aprotinin	Roth
Bacto-Agar	Roth
Bovine serum albumin (BSA)	Sigma
Bradford-reagent	Bio-Rad
Bromodeoxyuridine (BrdU)	Sigma
Bromphenolblue	Sigma
β-Mercaptoethanol	Roth
Calciumchloride ($CaCl_2$)	Sigma
Chloroform	Roth
Chloralhydrate	Roth
Cisplatin	Sigma
Collagen type I	Sigma
Crystal violet	Sigma
Cyclohexamide	Sigma
DAPI	Sigma
Deoxycholate (DOC)	Sigma
Diethyl pyrocarbonate (DEPC)	Merck
Dimethylsulfoxide (DMSO)	Sigma
Dithiothreitol (DTT)	Sigma
dNTPs	MBI
ECL western blotting detection reagents	Amersham
Ethylenediaminetetraacetic acid-disodium salt (EDTA)	Sigma

EGTA	Sigma
Ethanol	Roth
Ethidiumbromide	Invitrogen
Formaldehyde	Roth
Formamide	Roth
Glutathione-sepharose	Pharmacia
Glycerol	Sigma
Glycine	Sigma
Guanidine thiocyanate	Roth
HEPES	Roth
Hydrochloride (HCl)	Roth
Hybond-N membrane	Amersham
IGEPAL (NP-40)	Sigma
Isoropyl-1-thio-β-D-thiogalactopyranoside (IPTG)	Roth
Isopropanol	Merck
LB-Broth Base	Sigma
Leupeptin	Sigma
Magnesiumchloride	Sigma
MG-132	Calbiochem
Mitomycin C	Sigma
Mowiol® 4-88 Reagent	Calbiochem
NaF	Sigma
Natrium Chloride	Roth
3-(N-morpholino)propanesulfonic acid (MOPS)	Sigma
Non fat dried milk powder	AppliChem
Oregon Green® 488 phalloidin	Invitrogen
Paraformaldehyde (PFA)	Sigma
PBS	Gibco-BRL
PD 98059	Calbiochem
Pefablock	Roth
Phenol	Roth
Phenol:Chloroform:Isoamylacohol	Roth
Phenol/Chloroform (TE saturated)	Roth
PMA	Sigma
Polyethylenimine (PEI)	PolyScience
Ponceau S	Sigma
Potassium acetate (KAc)	Sigma
Potassiumchloride (KCl)	Sigma
Potassiumdihydrophosphate (KH_2PO4)	Merck
Precision Plus Protein ™ Dual Color Standars	Bio-Rad
Protein A-Agarose	Boehringer
Protein G-Agarose	Boehringer
Protein ladder, BenchMark™	Invitrogen, MBI
Protease Inhibitor Coctail Set I	Calbiochem
PROTRAN membrance	Schleicher & Schüll
Recombinant human EGF	Labgen
Recombinant human NGF-beta	Labgen
Recombinant human FGF	Labgen
SDS ultra pure	Roth
Sodium citrate	Merck
Sodiumdihydrophosphate (NaH_2PO4)	Merck
Sodiumhydrophosphate (NaHPO4)	Merck
Sodiumhydroxide (NaOH)	Sigma
sodium morpholineethanesulfonate (Na-MES)	Sigma
Sodium orthovanadate	Sigma
Staurosporine	Sigma
TEMED	Roth
Tris-(hydroxymethyl)-aminomethane (Tris)	Roth
Triton-X100	Sigma

Tween 20	Sigma
UO126	Calbiochem
Uracil	Sigma
Whatman 3MM Paper	Schleicher & Schüll
X-gal	Sigma
X-ray film	Amersham
Xylencyanol	Roth
Yeast extract	Invitrogen

4.1.3 Cell culture supplies

Reagent	***Source***
µ-Slide VI flow	Ibidi
Cell culture flask and tissue culture dishes	Greiner
DMEM	Gibco-BRL
Fetal calf serum (FCS)	PAN Systems
G418 sulfate	Calbiochem
Horse serum	Invitrogen
Lipofectamine2000	Invitrogen
L-Glutamine	Invitrogen
McCoy's 5A Medium	Gibco-BRL
Non-essential amino acids	Invitrogen
Poly-DL-ornithine	Sigma
Phosphate buffered saline (PBS)	Invitrogen
Penicillin/Streptomycin	Gibco-BRL
Poly-D-Lysine	Sigma
RPMI	Gibco-BRL
Sorafenib (BAY 9006)	MSZ (Prof.Rapp)
TC-plate 6/12 well with lid	Greiner
Transwell plate (8 µm chambers)	Corning Life Sciences
Trypsin-EDTA	Gibco-BRL
Trypanblue	Sigma

4.1.4 Antibodies

Primary antibody	***Species***	***Source***
anti-14-3-3 β (K-19)	rabbit, polyclonal	Santa Cruz
anti-Actin	rabbit, polyclonal	Sigma
anti-A-RAF	rabbit, polyclonal	Santa Cruz
anti-B-RAF (C-19)	rabbit, polyclonal	Santa Cruz
anti-C-RAF (C-12)	rabbit, polyclonal	Santa Cruz
anti-Bad	rabbit, polyclonal	Cell Signaling
anti Bag1 (Y166)(ab32109)	rabbit, monoclonal	Abcam
anti-Bag1 (ab54817)	mouse, monoclonal	Abcam
anti-Cdc37 (C1)	mouse, monoclonal	Alexis
anti-Cdc42	rabbit, polyclonal	PIERCE
anti-CHIP	mouse monoclonal	Calbiochem
anti-ERK2	rabbit, polyclonal	Santa Cruz
anti-FLAG®M2	mouse, monoclonal	Sigma

anti-His Tag, clone 4D11	mouse, monoclonal	Upstate
anti-N-terminal human XIAP	rabbit, polyclonal	Alexis
anti-HSC 70(B-6)	mouse, monoclonal	Santa Cruz
anti-Hsp70	mouse, monoclonal	Stressgen
anti-Hsp90	mouse, monoclonal	Stressgen
anti-Hsp90	rabbit, polyclonal	Santa Cruz
anti-c-IAP-1	goat, polyclonal	R&D Systems
anti-c-IAP-2	mouse, monoclonal	BD Pharmingen
anti-M2PK	mouse monoclonal	ScheBo® Biotech
anti-Myc (9E10)	mouse monoclonal	Santa Cruz
anti-p44/42 MAP Kinase	rabbit, polyclonal	Cell Signaling
anti-Phospho-p44/42 Map Kinase (Thr202/Tyr204)	rabbit polyclonal	Cell Signaling
anti-Phospho-C-Raf (Ser338)(56A6)	rabbit, monoclonal	Cell Signaling
anti-Rac1	mouse monoclonal	PIERCE
anti-TOM20 (FL-145)	rabbit polyclonal	Santa Cruz
anti-Ubiquitin	mouse, monoclonal	Zymed® Laboratories
anti-Ubiquitin Clone 6C1	mouse, monoclonal	Sigma
anti-XIAP	mouse, monoclonal	BD Transduction Laboratories
normal IgG_1	mouse	Santa Cruz
normal IgG	rabbit	Santa Cruz

secondary antibody	***Species***	***Source***
anti-Mouse IgG conjugated peroxidase	sheep	Amersham
anti-Rabbit IgG conjugated peroxidise	donkey	Amersham

4.1.5 Enzymes

Enzymes	***Source***
BioTherm DNA polymerase	Gene Craft
Calf Intestinal Phosphatase (CIP)	New England Biolabs (NEB)
DNase I, PCR grade	MBI
Klenow Fragment	MBI
M-MuLV-RT (reverse transcriptase)	MBI
Pfu polymerase	Stratagene
Proteinase K	Roth
Restriction Endonucleases	MBI, NEB, Amersham
RNaseA	Roche
T4 Ligase	NEB

4.1.6 Recombinant Proteins

Items	*Source*
A-RAF His-Tagged	MSZ
B-RAF His Tagged	MSZ
B-RAF-BxB His-Tagged	MSZ
B-RAF-KD His-Tagged	MSZ
C-RAF GST-Tagged	MSZ
C-RAF His-Tagged	MSZ
C-RAF-S338D His-Tagged	MSZ
C-RAF BxB His-Tagged	MSZ
CRAF-KD His-Tagged	MSZ
CHIP human recombinant	Alexis
c-IAP-1 human recombinant	R&D Sytems
c-IAP-2 human recombinant	R&D Sytems
GST	MSZ
MEK GST-Tagged	MSZ
UbcH5a, human recombinant	BostonBiochem
UbcH5b, human recombinant	BostonBiochem
UbcH5b, GST-Tagged	Calbiochem
UbcH7, human recombinant	BostonBiochem
Ubiquitin, His-Tagged	Calbiochem
Ubiquitin, FLAG-Tagged, human recombinant	BostonBiochem
XIAP human recombinant	R&D Systems
XIAP GST-Tagged	MSZ
XIAPΔRING GST-Tagged	MSZ
XIAPΔBIR2-BIR3 GST-Tagged	MSZ
XIAPΔBIR3 GST-Tagged	MSZ
XIAPΔBIR1	MSZ
XIAPΔBIR1-BIR2-BIR3	MSZ

4.1.7 Plasmid DNA

Plasmids	*Source*
pEGZ-Flag-XIAP	Taner Dogan
pEGZ-Flag-XIAPΔRING	Taner Dogan
pcDNA3-Myc-XIAP	gift from Prof. Dr. Harald Wajant
pEGFP-C1-XIAP	gift from Prof. Dr. Harald Wajant
pEGFP-C1-MCS	MSZ
pcDNA3-XIAP	gift from Prof. Dr. Emad S. Alnemri
pcDNA3-XIAP (1-336)	gift from Prof. Dr. Emad S. Alnemri
pcDNA3-XIAP (1-240)	gift from Prof. Dr. Emad S. Alnemri
pcDNA3-MCS	MSZ
pcDNA3-CHIP	gift from Dr. Len Neckers
pcDNA3-CHIPK30A	gift from Dr. Len Neckers
pcDNA3-CHIPH260Q	gift from Dr. Len Neckers
pcDNA3-Flag-C-RAF-DD	MSZ
pcDNA3-C-RAF-KD	MSZ
pKRSPA-HA-CRAF	MSZ
pGEX-6P-1-GST-XIAP-His	Taner Dogan
pGEX-6P-1-GST-XIAPΔRING-His	Taner Dogan
pGEX-6P-1-GST-XIAPΔBIR2-BIR3-His	Taner Dogan
pGEX-6P-1-GST-XIAPΔBIR3-His	Taner Dogan
pGEX-6P-1-GST-XIAPΔBIR1-His	Taner Dogan
pGEX-6P-1-GST-XIAPΔBIR1-BIR2-BIR3-His	Taner Dogan

4.1.8 Oligonucleotides

siRNA	***Sequence***
XIAP siRNA-1	Validated siRNA, Catalog No: SI00299446 (OIAGEN)
XIAP siRNA-2	GUAGAUAGAUGGCAAUAUGUU (Dharmacon)
XIAP siRNA-3	GAACUGGGCAGGUUGUAGAUU (Dharmacon)
XIAP 3'UTR siRNA	Target Sequence: CTGACTGATCTAATTGTATTA (QIAGEN)
C-RAF siRNA	Validated siRNA, Catalog No: SI00301623 (QIAGEN)
C-RAF 3'UTR siRNA	Target Sequence: GTGGATGTTGATGGTAGTACA(QIAGEN)
CHIP siRNA-1	CGCUGGUGGCCGUGUAUUAUU (Dharmacon)
CHIP siRNA-2	CGCUGGUGGCCGUGUAUUA (QIAGEN)
Control siRNA	Catalog No: D-001210-01-20 (Dharmacon)
c-IAP-1 siRNA-1	Validated siRNA, Catalog No: SI02654435 (QIAGEN)
c-IAP-1 siRNA-2	Validated siRNA, Catalog No: SI02654442 (QIAGEN)
c-IAP-2 siRNA-1	Validated siRNA, Catalog No: SI00299439
c-IAP-2 siRNA-2	Validated siRNA, Catalog No: SI02654449

Primer name	***Sequence 5'→3'***
<u>*Cloning Primer*</u>	
Flag-AsuII-EcoRI-for	AATTATGCCACCATGGACTACAAGGACGATGATGACAAA CTGGAATTCCGTACGTTCGAAAT
Flag-AsuII-EcoRI-rev	AATTATTTCGAACGTACGGAATTCCAGTTTGTCATCATCG TCCTTGTAGTCCATGGTGGCAT
XIAP-BIR1-EcoRI-for	GTAGAAGAATTCAATAGATTAAAAACTTTTGC
XIAP-BIR1-His-XhoI-rev	AAAGCCCTCGAGTTAGTGGTGGTGGTGGTGGTGGTTGAT AAATCTGCAATTTGG
XIAP-BIR1-BIR2-His-XhoI-rev	CCGGCCCTCGAGTTAGTGGTGGTGGTGGTGGTGCAAAAC AAAGAAGCAATTAGG
XIAP-BIR2-BIR3-EcoRI-for	GTATAGTGAATTCGCTAGATTAAAGTCCTTTCAG
XIAP-BIR2-BIR3-His-XhoI-rev	CTAACAGCTCGAGTTAGTGGTGGTGGTGGTGGTGATATT TGCACCCTGGATACC
XIAP-RING-EcoRI-for	CGCCTGGAATTCGAGAAGCTTTGCAAAATCTG
XIAP-RING-His-XhoI-rev	GAAAGTAATCTCGAGTTAGTGGTGGTGGTGGTGGTGGAC TGTGTAGCACATGGG
XIAP-BIR3-EcoRI-for	CCATGGCAGAATTCGAAGCACGGATCTTTACTTTTGGG
pcDNA3-XIAP-EcoRI-for	GGATCCGAATTCACTTTTAACAGTTTTG

pcDNA3-XIAP-His-XhoI-rev	GCATGCTCGAGTTAGTGGTGGTGGTGGTGGTGAGACATA AAAATTTTTTGCTT
XIAP-full-AsuII-rev	GCATGTTCGAATTAAGACATAAAAATTTTTTGC
XIAP-Ring-del-AsuII-rev	CAAAGCTTCGAATTATCTTGCAGGCGCC
XIAP-BIR-del-EcoRI-for	CTGTTAGAATTCAAGGGACAAGAATATATA
XIAP-Ring-del-XhoI-rev	CAAAGCCTCGAGTTATCTTGCAGGCGCC
XIAP-full-XhoI-rev	GCATGCTCGAGTTAAGACATAAAAATTTTTTGC
Sequencing primer	
pEGZ-Flag-Seq-for	CCGCCCTCAAAGTAGACG
pEGZ-Flag-Seq-rev	CCAAGCGGCTTCGGCCAG
pGEX-6P-1-Seq-for	GGGCTGGCAAGCCACGTTTGGTG
pGEX-6P-1-Seq-rev	CCGGGAGCTGCATGTGTCAGAGG
H1-Seq Primer	GCAGGAAGATGGCTGTGAG
pLVTHM-ClaI-LoxP-Seq-rev	ACGAAGTTATTCCCATGCGA
Primer for RT-PCR	
C-RAF-Hu-RT-for	TGAAAGCACTCAAGGTGAGG
C-RAF-Hu-RT-rev	CTTCTCCAATCAAAGACGCA
GAPDH-Hu-RT-for	GGTATCGTGGAAGGACTCATGAC
GAPDH-Hu-RT-rev	ATGCCAGTGAGCTTC CCGTTCAG
XIAP-Hu-RT-for-1	TCACTTGAGGTTCTGGTTGC
XIAP-Hu-RT-for-2	GGGGTTCAGTTTCAAGGACA
XIAP-Hu-RT-rev	CGCCTTAGCTGCTCTTCAGT

4.1.9 Cell lines and bacterial strains

Cell lines	*Source*
A549	human carcinomic alveolar basal epithelial cells
CAPAN-1	human caucasian pancreatic adenocarcinoma cells
HEK293	human embryonic kidney cells (epithelial),transformed with adenovirus
293T	HEK293 cells transformed with the SV40 Large T Antigen
HeLa	human cervical epithelial (carcinoma) cells
HEp-2	human epithelial type-2 cells (laryngeal carcinoma cells)
IMR90	human fetal lung fibroblast, primary cells, passage 2-4
IMR90 E1A	human fetal lung fibroblast, transformed with adenovirus
MEF	mouse embryonic fibroblast
PC12	rat pheochromocytomic adrenal medulla cells
SH-SY5Y	human neuroblastoma cells
WM852	human melanoma cells

Bacterial strains	*Source*
DH5α	Bethesda Research Laboratories. Optimized for DNA transformation and replication.
BL21	Stratagene. Optimized for DNA transformation and protein translation.

4.2 Solutions and buffers

4.2.1 Bacterial medium

LB (Luria-Bertani) medium

10 g/L Bacto-tryptone

10 g/L NaCl

5 g/L yeast extract

Adjust pH to 7.5 with NaOH

For plates, add 15 g Bacto-agar per liter

SOB medium (1 L)

20 g Bacto-tryptone

0.5 g NaCl, 5g yeast extract

10 ml 250 mM KCl

Adjust pH to 7.5 with NaOH

(Before use add 5 ml 2 M $MgCl_2$)

TB- buffer

For 250 ml final volume add 0.756 g 10 mM PIPES; 0.55 g 15 mM $CaCl_2$; 4.66 g 250 mM KCl and fill up with dd.H_2O; all components were mixed; KOH was used for pH 6.7 and 2.72 g 55 mM $MnCl_2$ were added at last. Tris- Borate solution was sterile filtrated (0.2 µm Corning) and kept at 4 ˚ C.

X-Gal stock solution

20 mg/ml X-gal (5-Bromo-4-Chloro-3-Indolyl-β-D-Galactopyranoside) dissolved in DMF and aliquoted at -20°C in darkness.

Ampicillin stock solution

100 mg/ ml in ddH_2O, kept at - 20˚ C

Kanamycin stock solution

25 mg/ ml in dd. H_2O, kept at - 20˚ C

Antibiotics that are mainly dissolved in water were sterile filtrated and kept as mentioned above.

4.2.2 DNA buffers

10X TE

0.1 M Tris-HCl

10 mM EDTA; pH 7.5

6X DNA Gel Loading Buffer

60 % (w/v) Glycerol

20 mM EDTA pH 8

60 mM Tris pH 7.5

0.05 % Bromphenolblue

1X Tris-Acetate-EDTA (TAE)

40 mM Tris-HCl

40 mM Acetic acid

2 mM EDTA; pH 7.8

4.2.3 Protein analysis buffers

NP40 lysis buffer

10 mM HEPES, pH 7.4

145 mM KCl

5 mM $MgCl_2$

1 mM EGTA

0.2 % IGEPAL

1 mM pefablock

5 mM benzamidine

5 µg/ml aprotinin

5 µg/ml leupeptin

1 mM Na_3VO_4

1:100 Protease Inhibitor Coctail (w/w)

Phosphate Lysis Buffer

10 mM NaCl

10 mM NaH_2PO_4 + Na_2HPO_4

2 mM EGTA

1 mM EDTA

10 mM NaF

10 mM $Na_4P_2O_7$

0.5 % Sodium Deoxycholate

0.05 % SDS

0.5 % Glycerol

1 % NP40

0.1 mM Na_3VO_4

1:100 Protease Inhibitor Coctail (w/w)

Lysis Buffer (for cells used for Ubiquitylation)

50 mM Tris/HCl pH 7.5

250 mM NaCl

10 % Glycerol

1 % Triton-X

1 mM PMSF

1 mM Natriumorthovanadate

1.5 mM $MgCl_2$

25 mM NaF

0.01 % β-Mercapthoethanol

1:100 Protease Inhibitor Coctail (w/w)

5X SDS-loading Buffer (for SDS-PAGE)

70 mM Tris-HCl, pH6.8

3 % SDS

40 % Glycerin

5 % β-Mercaptoethanol

0.05 % Bromphenoblue; use as 1x solution

10X Running Buffer (for SDS-PAGE)

143.75 g Glycine

30 g Tris

10 g SDS

add 1 L dH_2O

10X Blotting Buffer (1 L)
29 g Glycine

58 g Tris

18.5 ml of 20% SDS

(or 3.7 g SDS)

add 1 L dH_2O

10X Immunoblot Stripping Buffer

1 M Glycine/HCl pH 2.5

1% SDS

10X Tris-Buffered Saline (TBS)

500 mM Tris-HCl, pH 7.4

1.5 M NaCl

TBST
1x TBS + 0.05 % Tween 20

Immunoblot Blocking Buffer

5% (w/v) of non-fat dry milk in TBST

10X Uiquitination Reaction Buffer

50 mM Tris/HCl pH 7.5

100 mM NaCl

2.5 mM $MgCl_2$

1 mM DTT

10X Kinase Buffer

100 mM $MgCl_2$,

250 mM β-Glycerophosphate

250 mM HEPES pH 7.5

50 mM Bezamidine

5 mM DTT

10 mM Sodiumorthovanadate

TCA stock solution

100 % (w/v) Trichloroacetic acid

500 g TCA

350 ml dH_2O, store at RT.

4.3 Methods

4.3.1 Bacterial manipulation

Since DNA is a very hydrophilic molecule, it won't normally pass through a bacterial cell's membrane. In order to incorporate plasmids into bacteria, the latter must first be made "competent" to take up DNA. This is accomplished by creating small holes in the bacterial cells by suspending them in a solution with a high concentration of calcium. DNA can then be forced into the cells by incubating the cells and the DNA together on ice, placing them briefly at 42^{o}C (heat shock), and then putting them back on ice. This causes the bacteria to take in the DNA. The cells are then plated out on antibiotic containing media.

Plasmid transformed bacteria were selected on LB or BHI plates with the corresponding antibiotics at 37°C for 24 h. For overnight cultures, single colonies were picked and inoculated in LB medium with respective antibiotic and shaken overnight at 37°C. These precultures were then used for preparing frozen glycerin cultures as well as for purification of protein or plasmid DNA. For storage of bacteria, a glycerol stock culture was prepared by growing bacteria to an OD of 0.8 at a wavelength of 600 nm in culture medium. 300 ml bacterial culture was taken and added to 900 ml of 80% glycerine and then mixed thoroughly in a small 1.5 ml tube. This stock solution was subsequently frozen at –80°C. To inoculate an overnight culture again, bacteria was taken out and held at RT until surface was thawed. Pick a small amount of cells and mix into 2-5 ml culture medium and allow to grow for several hours at 37°C in a bacterial culture shaker. The frozen stock is immediately returned to the -80°C.

4.3.1.1 Establishment of chemical competent cells

Sterile SOB medium (250 ml) in a 2 liter flask was inoculated with 10 - 12 large single colonies picked from source plate. The culture was grown to an OD of 0.6 with vigorous shaking (200-250 rpm) at 18°C approximately for 35-40h and then placed on ice for 10 min. The cells were harvested in a centrifuge at 2500xg for 10 min at 4°C and the supernatant was drained. The pellets were resuspended first in 5 ml of ice cold TB buffer and an additional 75 ml of cold TB buffer was added. After incubation of suspension on ice for 10 min the cells were centrifuged as above and then resuspended in 20 ml of ice cold TB-DMSO mixture (1.4 ml of DMSO to 18.6 ml of TB). The suspension was aliquoted in 200 µl aliquots. The aliquots were frozen in liquid nitrogen and then stored at -70°C.

4.3.1.2 Transformation of chemical competent cells

The competent cells were thawed on ice. Generally around 20 ng of plasmid or 15 µl of ligation product was added onto 200 µl of cells. The tubes were swirled gently for a few seconds by finger flicking and incubated on ice for 30 min. After incubation the tubes were placed in 42°C heat block for 45 sec without shaking and replaced on ice for 2-5 min. 750µl of SOB medium was added onto cells and the cells were incubated by shaking at 200 rpm for 60 min at 37°C. The transformed cells were spreaded on LB agar plates containing appropriate antibiotic (e.g., 100 µg/ml ampicillin for pcDNA3). The plates were incubated at 37°C for overnight.

4.3.2 DNA/RNA Methods

4.3.2.1 Electrophoresis of DNA on agarose gel

The DNA fragments were separated on agarose gels. To make an agarose gel agarose was added to 0.5X TBE buffer to obtain a final concentration between 0.8 and 2%, cooked in a microwave and 12 µl of 0.5 % ethidium bromide solution was added. DNA samples were mixed with 3 µl of loading buffer. Electrophoretic separation was dependent on fragment size and performed in 0.5X TBE buffer at 180 volts for 30-60 min. The DNA can be visualized under UV-light. Standard size of markers was compared with separated DNA fragments.

4.3.2.2 Amplification of DNA fragments (PCR)

Polymerase chain reaction is the process used to amplify specific parts of a DNA molecule, via the temperature-mediated enzyme DNA polymerase. PCR usually consists of a series of 20 to 35 repeated temperature changes called cycles to duplicate specific DNA strands. As PCR progresses, the DNA thus generated is itself used as template for replication.

A basic PCR set up requires several components and reagents:

A DNA template that contains the DNA region to be amplified which is flanked by two sets of oligonucleotides (primer) that each bind to one of the two DNA strands. For this reaction a heat stable DNA- Polymerase, usually *Taq*- Polymerase is used working at a temperature optimum around 70 ºC. To synthesize new DNA strands Deoxynucleotide triphosphates (dNTPs) are necessary as they function as building blocks. A suitable chemical environment for optimum activity and stability of the DNA polymerase should be applied.

The following steps are repeated cyclically during PCR:

Denaturation step: It causes melting of DNA template and primers by disrupting the hydrogen bonds between complementary bases of the DNA strands, yielding single strands of DNA (ssDNA).

Annealing step: The reaction temperature is lowered allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5 degrees below the melting temperature (Tm) of the primers used. The polymerase binds to the primer-template hybrid and begins DNA synthesis.

Elongation step: At this step, the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. At its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute.

To avoid unspecific PCR- products the samples should be applied into the thermocycler only when 80ºC are established.

Standard PCR- conditions: „Hot Start PCR“

	3’ 94°C	Starting denaturation
35 cycles:	30’’ 94°C	Denaturation, 45’’ 55°C Annealing, 2’ 72°C Elongation
	8’ 72°C	Final elongation → hold on 4°C

One aliquot of amplified DNA was loaded on a 1 % agarose gel and electrophoresis was employed for size separation of the PCR products. A photo under UV- light was taken to check whether the PCR generated a DNA fragment of anticipated size.

4.3.2.3 Reverse Transcription-PCR (RT-PCR)

Total RNA was extracted from cells using RNAeasy Kit (Qiagen) and treated with DNase using DNase treatment Kit (Ambion) according to the manufacturers’ instructions. To synthesize first-strand cDNA First Strand cDNA Synthesis Kit (Fermentas) was used: 1 µg of total RNA was mixed with 0.5 µg of oligo$(dT)_{18}$ primer and incubated in DEPC treated water in a total volume of 11 µl at 70°C for 5 min to resolve all secondary RNA structures. Then 4 µl of 5X reaction buffer, 20 U of RiboLock Ribonuclease Inhibitor, 2 µl of 10 mM dNTPs mix and 40 U of M-MuLV Reverse Transcriptase were added to make a final 20 µl mixture and incubated at 37°C for 1 h. The reaction was stopped by heating at 70°C for 10 min. The first strand cDNA can be used directly for amplification by PCR or stored at -20°C.

4.3.2.4 Quantitative real-time PCR

Quantitative real-time PCR was performed using DyNAmo™ HS SYBR Green qPCR Kit (Finnzyme). PCR reactions were performed in a total volume of 20 µl (10µl of 2X DyNAmo HS Master Mix, 2 µl of cDNA produced as described above, 1 µl of each primer (20 pmol) and 6 µl of sterile dH_2O) in Rotor-Gene RG-300 (Corbett Research). The PCR programme was as follows: 1 cycle for 5 min at 94°C; 45 cycles for 10s at 94°C, 20s at 55-60°C (depend on primer), 30s at 72°C. The specificity and identity of the PCR product was checked by performing a melting curve test. After quantified with real-time PCR, quantification was performed using the comparative CT method. The target transcript was normalized to an endogenous reference (simultaneous *β*-actin or GAPDH reactions). All samples were analyzed in triplicate.

4.3.3 Working with protein

4.3.3.1 SDS-Polyacrylamide gel electrophoresis and immunoblotting

Cell lysates were heat denatured with laemmli buffer (5X). The denatured samples were resolved on polyacrylamide gels. The samples were electrophoresed at 30-60 mA for 1-1.5 h at room temperature with electrophoresis buffer in miniapparatus, Mini Protean II from BioRad. SDS-PAGE gels are electroblotted at 400 mA in transfer buffer for 45 min. The electroblotted nitrocellulose membranes were incubated with 1X PBST with 5 % milk (blocking solution) for 1 h at room temperature. The membranes were then incubated with the primary antibody appropriately diluted in blocking for 2 h at room temperature, or overnight at 4^oC. After incubation, the membranes were washed thrice with PBST for 30 min each at room temperature, followed by incubation with diluted secondary antibody for 1 h at room temperature. Appropriate secondary antibodies tagged with horse radish peroxidase (HRP) were diluted in blocking solution (1:5000). The membranes were washed thrice with PBST for 30 min at room temperature. This step is followed by the standard enhanced chemiluminescence reaction (ECL-system): incubating the membranes in a 1.1 mix of ECL solutions 1 and 2 (Amersham). The signals were then detected with "Lumi-film Chemiluminiscent" detection film.

4.3.3.2 Coomasie staining

The SDS-PAGE gels were washed thrice in 100 ml of ddH_2O for 10 min and stained with 20 ml (or enough to completely cover the gel) of PageBlue™ Protein Staining Solution

(Fermentas) with gentle agitation for 60 min and then, the gels were washed thrice in 100 ml of ddH_2O for 10 min again.

4.3.3.3 Stripping of immunoblots

The membranes were washed twice with stripping buffer for 15 min and then once with 1X PBST at room temperature. The membranes were then reprobed or stained directly for the presence of proteins.

4.3.3.4 Co-immunoprecipitation

A purification procedure to determine if two different proteins interact. An antibody specific to the protein of interest is added to a cell lysates. Then the antibody-protein complexes are pelleted usually using protein-G sepharose which binds most antibodies. If there are any proteins that bind to the first protein, they will also be pelleted. Identification of proteins in the pellet can be determined by western blot (if an antibody exists) or by sequencing a purified protein band.

Two days post transfection the cells were washed twice with 1x PBS. Washed cells were resuspended in 500 µl of ice-cold 1X lysis buffer without EDTA or phosphate lysis buffer contained fresh added 1:100 Protease Inhibitor Coctail Set I and 100 µM Sodium Orthovanadate, and incubated on ice for 10 min. After sonication, cells were pelleted at 15,000 rpm for 15 min. Supernatant were transferred into fresh eppendorf tubes. Depending on the proteins, the lysates were incubated with corresponding antibody over night at 4°C on a rotator. On the following, day 50µl of a 50% slurry of protein A -, or G- agarose beads were added and then the samples were rotated for additional 2 h at 4°C. Beads were washed thrice in 500µl of lysis buffer without protease inhibitors and pelleted everytime at 2600 rpm for 2 min, and then boiled in 50 µl of sample buffer for 5 min. The samples can be directly used for Western Blot or they can be frozen at -20°C.

4.3.3.5 Purification of GST-XIAP

XIAP was expressed in *E. coli* as glutathione S-transferase (GST) fusion protein using pGEX-4T2 vector (Pharmacia) and purified by glutathione-Sepharose affinity chromatography using standard protocols. Elution of XIAP was performed by 20 mM glutathione in 50 mM Tris-HCl buffer (pH 8.0). The purity of the proteins was assessed by SDS polyacrylamide gel electrophoresis and staining with Coomassie Blue.

4.3.3.6 *In vitro* binding assay (GST pull down assay)

Purified GST fusion proteins (5µg) were incubated with 50 µl of Glutatione Sepharose Beads in 500 µl of 1X Phosphate Lysis Buffer for 1h at 4°C on a rotator. Beads were washed thrice with 500 µl of lysis buffer and pelleted at 2600 rpm for 2 min. His fusion proteins, which should be tested as binding partners of protein of interest, and 500µl of lysis buffer were added on the beads. The samples were incubated for 1h at 4°C on a rotator. Again the beads were washed thrice with lysis buffer and boiled at 100^{o}C for 5 min in 50 µl of sample buffer. The possible interaction between the proteins of interest was analyzed by SDS-PAGE.

4.3.3.7 Biosensor measurement

The Biosensor measurements were carried out either on BIAcore-X or BIAcore-J system (Biacore AB, Uppsala, Sweden) at 25°C. The biosensor chip CM5 was loaded with anti-GST antibody using covalent derivatization according to the manufacturer's instructions. Purified and GST-tagged XIAP was immobilized in Biosensor buffer (10 mM Hepes, pH 7.4, 150 mM NaCl and 0.01% NP-40) at a flow rate of 10 µl/min which resulted in a deposition of approximately 900-1200 response units (RU). Next the proteins (A- , B-, and C-RAF and activated C-RAF-S338D mutant proteins) were injected at indicated concentrations. The values for unspecific binding measured in the reference cell were subtracted. The evaluation of kinetic parameters was performed by non-linear fitting of binding data using the BiaEvaluation 2.1 analysis software. The apparent association (k_a) and dissociation rates constant (k_d) were evaluated from the differential binding curves (Fc2-Fc1) assuming a A+B=AB association type for the protein-protein interaction. The dissociation constant K_D was calculated from the equation $K_D = k_d/k_a$. For measuring the interaction of CHIP with XIAP and C-RAF, GST-tagged XIAP or C-RAF was immobilised and purified CHIP (Alexis) was injected.

4.3.3.8 *In vivo* ubiquitylation assay

The cells were transfected with correspond constructs by one of transfection protocols as described in 4.3.4.2. At 36 h post-transfection, cells were treated with 10µM MG132 for 6h, and then immunoprecipitation assay has been carried out. The ubiquitylation of protein of interest was tested by western blot.

4.3.3.9 *In vitro* Ubiquitylation assay

Reaction mixture contained 2.5 µg substrate, 5 µM ubiquitin, 100 nM E1, 100-500 nM E2, 100 nM E3, 4 nM ATP and 1X Reaction Buffer (10X) in a total volume of 20 µl was incubated at 37°C for 2h. After the reaction, the samples were resuspended in SDS sample buffer, heated at 100°C for 5 min, and resolved on SDS–PAGE. The ubiquitylation of proteins was determined by blotting with anti-ubiquitin antibody or with appropriate antibody.

4.3.3.10 *In vitro* kinase assay

The purified kinase and its substrates (2-5µg) were mixed in 20 µl 1X Kinase Buffer. Add 5µCi [γ-^{32}P] and 0.1 mM ATP, incubate the mixture at 30°C for 30 min. The reaction was then stopped by adding SDS-loading Buffer and the mixture was incubated at 100°C for 2 min. After separation of proteins by SDS-PAGE the gel was placed in a lightproof cassette. The γ-radiation was detected with X-ray detection film. Loading of proteins was controlled by coomassie staining of gel.

4.3.3.11 Trichloroacetic acid (TCA) protein precipitation

After adding of TCA (1:4) into the protein samples the mixtures were incubated for 1h or overnight at 4°C and centrifuged at 14.000 rpm for 5 min. The pellets were washed twice with 200 µl of cold acetone at 14.000 rpm for 5 min and then dried by placing the tube in 95°C heat block for 5-10 min to get rid of acetone. For SDS-PAGE 2X or 4X sample buffer was added onto each pellet and the western blot described in 4.3.3.1 has been carried out.

4.3.4 Cell culture techniques

4.3.4.1 Maintenance of eukaryotic cell lines in culture

All cell culture work was carried out under sterile conditions using autoclaved and sterile filtered solutions, glassware and plastics. All cell lines were cultured at 37°C, 5% CO_2 and appropriate humidity ensured. Adherent cells were- when confluent- split into 1:2- 1:10 depending on the requirements of specific experiments. The cells were washed with PBS and trypsinized for 3-5 min in order to be detached from culture flask. After resuspending the cells in fresh media they were either seeded for experiments or passaged into a new flask.

For long term storage cells were pelleted and approximately 5x 10^6 cells were taken up in culture media that contained 20% FCS and 10% DMSO. Aliquots of 1 ml were first kept at -80°C before being transferred to liquid nitrogen.

To re- cultivate cells they were thawed, transferred into 10 ml medium and pelleted by centrifugation. The DMSO containing supernatant was removed and cells were taken up in fresh culture media.

4.3.4.2 Transient transfection of cells

The cells were transfected by several transfection tecniques for DNA and siRNA, respectively:

Lipofectamine™ Reagent Transfection (Invitrogen)

Polyethylenimine (PEI) Transfection Reagent (PolyScience Inc)

DharmaFECT®Duo Transfection Reagent (Dharmacon)

HiPerFect Transfection Reagent (QIAGEN)

Magnet Assisted Transfection (MATra)

Nucleofection Transfection (Amaxa)

All of kits and Reagents were used according to the manufacturers' instructions.

4.3.4.2.1 Calcium phosphate transfection (also used to generate lentivirus)

2 x 10^6 293T cells were seeded one day before transfection on 10 cm culture dishes with 10 ml DMEM growth medium without antibiotics. For each 10 cm plate, 20 µg recombinant vector, 15 µg psPAX2 and 6 µg pMD2.G (VSVG) were made up to 250 µl with sterile filtered water, and mixed with 250 µl 1M $CaCl_2$. Then 500 µl of 2X HBS (pH 7.0) was added dropwise while vortexing onto the mixture and then incubated at RT for 30 min (max. 40 min). The transfection mix was then added onto the cells. 6-8 h post-transfection, the cells were washed with 1X PBS prewarmed to 37°C and 10 ml DMEM medium was added to each dishes. Transfection efficiencies were monitored via GFP expression from recombinant vector by fluorescence microscopy the very next day.

4.3.4.3 Immunofluorescent staining

The cells were seeded on cover slips in 12-well plate and transfected the next day using various reagents according to manufactures' instructions. Two days post-transfection, the cells were washed thrice with 1X PBS for 10 min, and fixed in 4 % PFA in 1X PBS for 10 min. The cells were washed again thrice with 1X PBS for 10 min, and permeabilised and blocked in the same time with blocking buffer (0.1 % BSA, 0.3 % Triton-X-100 in PBS) for 30 min. 0.1 % BSA in blocking buffer is added to prevent non-specific antibody binding. The first antibodies were diluted according to the manufacturers' recommendations in blocking buffer and incubated for 2h. The unbound antibodies were washed away by incubating thrice with 1X PBS for 10 min. The fluorescence-conjugated secondary antibodies were then diluted appropriately in blocking

buffer and incubated for 90 min protected from light. The cells were then washed thrice with 1X PBS for 10 min to remove the excess antibodies. All steps till here were carried out at RT. To stain the cell nuclei the cells were incubated with Hoechst 33342 (diluted 1:250 in Methanol) at 37°C for 15 min. The cells were then washed twice with 1X PBS for 10 min and finally once with dH_2O for 10 min and carefully mounted on a glass slide using Mowiol.

4.3.4.4 Time lapse microscopy

Cells transfected with XIAP-3'UTR siRNA or XIAP siRNA-1 were seeded onto chamber slides (Ibidi, Germany, Cat#80602) coated with collagen type IV about 15 h in advance and were incubated in complete RPMI medium (RPMI+10%FCS+1% antibiotics). Cells were analyzed before and after treatment with 20ng/ml of EGF. Live cell imaging was performed at 37°C via an objective heater (PeCon, Germany). The fluorescence and transmission images were recorded on a modified confocal microscope (Leica SP5 confocal microscope with a 20X 0.7 NA oil immersion objective, Leica Wetzlar and Mannheim). The fluorescence emission resulted from excitation with 488 (for Oregon Green) and occasionally, 405 nm (for DAPI fluorescence and transmission images) laser lines from an argon ion laser (Lasos, Germany) and a 405 nm diode laser (Cube, Coherent, Germany) with the Leica SP5 LSM confocal with internal spectral parameter settings of 488 nm for GFP and 405 nm for DAPI/transmission. The fluorescence was detected with the SP5 spectral detectors using a 500 - 560 nm spectral band width setting for GFP. The live images of XIAP knock down cells and control HeLa cells were obtained at the same time using the "tile scan" option of the Leica confocal SP5. The images were processed with standard routines ("plugins") on Image J (Scion Image, NIH, USA), and the cells were tracked with the imaging and analysis software on Meta Morph (Universal Imaging, USA). The cell speed and size were calculated for cells indicated by tracks in the supporting movies 1-4. HeLa control (n=145, number of flattened cells= 73 and number of rounded cells=72; mean speed=8.5±0.6µm/h, mean area=1014±105 μm^2) XIAP control (n=150, Number of flat cells=112 and number of rounded cells =38; mean speed=12.2±2.7µm/h, mean area=1218±96 μm^2).

4.3.4.5 Transwell migration experiments

HeLa cells were seeded on 12 well plates and the siRNAs directed against XIAP (XIAP3'UTR siRNA or XIAP siRNA-1) and control (scrambled) sequences were transfected as described above. At 36h post transfection, 2 x 10^5 cells were seeded on to 8 µm transwell migration chambers (Corning Cat# 3422) and the membranes were pre-coated with 0.1 % Collagen type I

derived from Calf skin (Sigma Cat# C8919). The cells were stimulated with serum-free RPMI, or RPMI with 10 % FCS, or RPMI with 10 % FCS and 20 ng/ml EGF, added to the lower chamber. The cells were left to migrate for 12 h. Cells on the upper part of the membrane were scraped using a cotton swab and the migrated cells were fixed in 3.7 % (v/v) paraformaldehyde and stained with 0.4 % Crystal Violet in 10 % ethanol. The experiment was performed in triplicates for all conditions described. From every transwell, several images were taken under a microspcope at 10X magnification and two broad fields were considered for quantification. Analysis was performed using two special algorithms. One determines the value of every pixel in the images and added all values above the background. This gives a good estimate of the fraction of the transwell covered with cells. Secondly, all possible squares with 200 pixels on a side were examined for the ratio of dark/bright areas the results of which were depicted in the form of histograms.

4.3.4.6 Wound healing assay

Cells transfected with control and XIAP3'UTR siRNAs were seeded on to tissue culture plates and scratches (4-5) were made on confluent monolayers with a pipette tip. The extent of wound closure was monitored for 0, 8, 12 and 24 h with or without EGF stimulation. The images were acquired using a Leica fluorescent microscope (DMIRE2) fitted with a digital camera at 10X magnification. The percentage of wound closure was calculated as follows: The width of the wound formed was measured using IMAGEJ software tools from the acquired images and the percentage of wound closure (comparing 0 and 24 h) was calculated.

4.3.4.7 TUNEL (Apoptosis) assay

The cells were plated into 12 well plates to yield 80% confluence the following day. After incubation with apoptosis inducers for appropriate time cell death was observed under the microscope and characterized by cell rounding up and detaching from the plates. For quantification of cell death, DeadEnd Fluorometric TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) System Kit (Promega) was used according to the manufacturer's instructions. TUNEL-positive cells were viewed by fluorescence microscopy.

4.3.5 KITs

All the kits were used according to the manufacturers' instructions;

The QIAGEN Plasmid Purification Kits (Mini-Midi-Maxi) for the purification of plasmids,

The QIAquick PCR Purification Kit for the purification of PCR products,

The QIAquick Gel Extraction Kit for the purification of DNA fragments,

The QIAEX II DNA Extraction Kit,

The RNAeasy Mini Kit (QIAGEN) for the isolation of RNA from mammalian cells,

The TURBO DNA-free Kit (Ambion) for the removing of contaminating DNA from RNA preparations,

The QIAGEN OneStep RT-PCR Kit (QIAGEN) and the First Strand cDNA Synthesis Kit (Fermentas) for the reverse transcription,

The DeadEnd Fluorometric TUNEL System Kit (Promega) for the staining of apoptotic cells,

The ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem) for the fractionation of eukaryotic cells.

The EZ-Detect^{TM} Cdc42 Activation Kit (PIERCE),

The EZ-Detect^{TM} Rac1 Activation Kit (PIERCE),

The EZ-Detect^{TM} Rho Activation Kit (PIERCE) for the detection of Cdc42-, Rac1- and Rho-activation in cells.

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6. APPENDIX

Abbreviations

17-AAG	17-allylamino-17-demethoxygeldanamycin
ABL	V-abl Abelson murine leukemia viral oncogene homolog 1
AIF	Apoptosis-inducing factor
AKT/PKB	Protein kinase B
Apaf-1	Apoptotic protease activating factor-1
APS	Ammonium peroxydisulfate
ATP	Adenosine triphosphate
BAD	Bcl-2-antagonist of cell death
BAG-1	Bcl-2-associated athanogene-1
BAK	Bcl-2 homologous antagonist killer
BAP31	B-cell antigen receptor-associated protein
BAX	Bcl-2-associated X protein
BCL-2	B cell lymphoma 2
BHI	Brain-Heart Infusion Agar
BID	BH3 interacting domain death agonist
BIR	Baculovirus IAP repeat
BMP	Bone morphogenetic protein
BSA	Bovine serum albumin
BSE	Bovine spongiform encephalopathy
C-terminal	Carboxy-terminal
ca.	Circa
$CaCl_2$	Calciumchloride
cAMP	Cyclic adenosine monophosphate
CARD	Caspase recruitment domain
cDNA	Complementary DNA
CDC37	Cell division cycle 37
CDC42	Cell division cycle 42
CDK4	Cyclin-dependent kinase 4
CHIP	C-terminus of Hsc70-interacting protein
Chk1	Checkpoint kinase 1
CHOP	C/EBP homologous protein
CHX	Cycloheximide
Ci	Curie
c-IAP-1	Cellular IAP 1
c-IAP-2	Cellular IAP 2
CNK	Connector enhancer of KSR
COMMD1	Copper metabolism (Murr1) domain containing 1
CoIP	Co-immunoprecipitation
CR	Conserved regions
CRD	Cysteine rich domain
C-RAF	RAF kinase C
CXCR4	Chemokine (CXC motif) receptor 4
Cyto c	Cytochrome c
Da	Dalton
DAPI	4'-6-Diamidino-2-phenylindole
D-RAF	Drosophila RAF
DcR1	Decoy receptor1

DcR2	Decoy receptor2
ddH_2O	Double distilled water
DD	Death domain
DED	Death effector domain
DIABLO	Direct IAP binding protein with low pI
DIAP1	Drosophila IAP protein 1
DIAP2	Drosophila IAP protein 2
DISC	Death-inducing signaling complex
DMEM	Dulbecco's Modification of Eagle's Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DR4	Death receptor 4
DR5	Death receptor 5
DTT	Dithiothreitol
E1A	Early Region 1A
E.coli	*Escherichia coli*
EBV	Epstein-Barr virus
ECL	Enhanced chemiluminescence reaction
EDTA	Ethylenediaminetetraacetic acid
e. g.	*Exempli gratia*
EGF	Epidermic growth factor
EGFR	Epidermic growth factor receptor
EGTA	Ethylene glycol tetraacetic acid
EndoG	Endonuclease G
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase 1/2
ErB2	Epidermal growth factor receptor-2
EV	Empty vector
FADD	Fas-associated death domain
FAK	Focal adhesion kinase
FasL	Fas ligand
FCS	Fetal calf serum
FGF	Fibroblast growth factor
Fig	Figure
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
h	Hour
HA	Hemagglutinin
HBS	HEPES buffered saline
HBXIP	Hepatitis B X-interacting protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF-1α	Hypoxia-inducible factor-1α
His	Histidine
hnRNPA1	heterogenous nuclear ribonucleoprotein A1
HRP	Horse radish peroxidase
Hsp70	Heat shock protein 70 (Hsc70)
Hsp90	Heat shock protein 90

hTERT	Human telomerase reverse transcriptase
IAP	Inhibitor of apoptosis
IBM	IAP-binding motif
IgG	Immunoglobulin G
IL1β	Interleukin-1-β
ILP-2	IAP-like protein 2
IMM	Inner mitochondrial membrane
IMP	Impedes mitogenic signal propagation
INCL	Infantile neuronal ceroid lipofuscinosis
IB	Immunoblot
IP	immunoprecipitation
IRE1	ER transmembrane bifunctional serine/threonine protein kinase/endoribonuclease
IRES	internal ribosome entry site
JNK1	c-Jun N-terminal kinase 1
JNK2	c-Jun N-terminal kinases 2
K_a	apparent association
kb	Kilobase
K_D	dissociation rates constant
kDa	KiloDalton
KSR	Kinase suppressor of Ras
L	Liter
LB	Luria-Bertani
LMP1	Latent membrane protein 1
M2PK	M2 Pyruvate Kinase
MAGE-D1	Melanoma antigen family D, 1
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MAP3K	MAP kinase kinase kinase
MCLK	Myosin light chain kinase
MCS	Multiple cloning site
MDa	MegaDalton
MEF	Mause embryonic fibroblasts
min	minutes
ML-IAP	Melanoma IAP (Livin)
MLS	Mitochondrial localization sequence
MMP	Mitochondrial membrane permeabilization
M-MuLV	Moloney Murine Leukemia Virus
MMP-1	Matrix metalloproteinase-1
mRNA	Messenger RNA
MSZ	Medizinische Strahlenkunde und Zellforschung
MW	Molecular weight
Na_3VO_4	Sodium orthovanadate
NaCl	Sodium chloride
NaF	Sodium fluoride
NAIP	Neuronal apoptosis-inhibitory protein
NF-κB	Nuclear factor κB
NGF	Nerve growth factor
NKT	Natural killer T
NOXA	Phorbol-12-myristate-13-acetate-induced protein 1
NP-40	Nonidet P40

OD	Optical density
Omi/HtrA2	High temperature requirement protein A2
OMM	Outer mitochondrial membrane
PAK	*p*21 Rac-*a*ctivated *k*inase
PAX2	Paired box gene 2
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline tween
PCD	Programmed cell death
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PERK	Protein kinase-like endoplasmic reticulum kinase
PDSM	Phosphorylation-dependent sumoylation motif
PEST	proline (P), glutamic acid (E), serine (S), and threonine (T)
PFA	Paraformaldehyde
PHB	Prohibitin
PI3-kinase	Phosphoinositide 3 kinase
PIDD	p53-induced protein with death domain
PKA	Protein kinase A
PKB	Protein kinase B (AKT)
PLK-1	Polo-like kinase 1
PMA	Phorbol myristate acetate
PMSF	Phenylmethanesulphonylfluoride
PP2A	Protein phosphatase 2 A
PP5	Protein phosphatase 5
PPARgama	Peroxisome proliferator-activated receptor-gamma
PTP	Permeability transition pore
RAC1	Ras-related C3 botulinum toxin substrate 1
RAF	Rapidly Accelerated Fibrosarcoma (v-RAF, v-MIL)
Ral-GDS	Ral guanine nucleotide dissociation stimulator
RBD	RAS binding domain
RING	Really interesting new gene
RIP	Receptor-interacting protein
RKIP1	RAF kinase inhibitory protein 1
ROCK	Rho-associated, coiled-coil containing protein kinase
Rok- α	Rho-binding kinase *α*
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RU	Response units
SD	Standard deviation
SDF-1α	Stromal-derived factor-1α
SDS	Sodium lauryl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	Seconds
Sf9 cells	Insect cell line derived from pupal ovarian tissue of *S. frugiperda*
SMAC	Second mitochondrial activator of caspases
SOB	Super Optimal Broth
Src	v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
STAT3	Signal transducer and activator of transcription 3
SUMO	Small ubiquitin-related modifier
Survivin	BIRC5

TAB1	TAK1 binding protein 1
TAK1	TGF beta-activated kinase 1
TB Buffer	Tris-borate buffer
TBE	Tris-borate-EDTA
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TE Buffer	Tris-EDTA buffer
TEM	Transendothelium migration
TEMED	Tetramethylethylenediamine
TGF-b	Transforming growth factor beta
TGF-β1	Transforming growth factor beta 1
TNF	Tumor necrosis factor
TNF-R1	Tumor necrosis factor alpha receptor 1
TOM 20	Translocase of outer membrane
TPR	Tetratricopeptide repeat
TRAF	TNFR-associated factor
TRAF2	TNFR-associated factor 2
TRADD	TNFR-associated death domain
TRAIL	TNF-alpha-related apoptosis inducing ligand
Tris	Tris-(hydroxymethyl)-aminomethane
U	Unit
UBA	Ubiquitin-associated domain
Ub	Ubiquitin
UPR	Unfolded protein response
UTR	untranslated region
US	United States
UV	Ultra violet
VDAC	Voltage-dependent anion channel
VEGF	Vascular endothelial growth factor
VSVG	Vesicular stomatitis virus G-protein
v/v	Volume/Volume
v	Viral
WT	Wild type
w/v	Weight/Volume
XAIF1	X-linked inhibitor of apoptosis-associated factor 1
XIAP	X-linked IAP protein (BIRC4)
XLP	X-linked Lymphoproliferative Syndrome

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Publications

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Erklärung gemäß § 4 Absatz 3 der Promotionsordnung der Fakultät für Biologie der Bayerischen Julius-Maximilians-Universität Würzburg vom 15. März 1999.

Hiermit erkläre ich ehrenwörtlich, dass ich die vorliegende Dissertation selbständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

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Außer den in dem Zulassungsgesuch urkundlich vorgelegten Graden habe ich keine weiteren Grade erworben oder zu erwerben versucht.

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