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Role of ATM in Fas-induced apoptosis in lymphoid cells

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ABSTRACT

Ataxia Telangiectasia (A-T) is a rare cancer-predisposing genetic disease, caused by the lack of functional ATM kinase, a major actor of the Double Stand Break (DSB) DNA-damage response. A-T patients show a broad and diverse phenotype, which includes an increased rate of lymphoma and leukemia development. Fas-induced apoptosis plays a fundamental role in the homeostasis of the immune system and its defects have been associated with autoimmunity and lymphoma development.

We therefore investigated the role of ATM kinase in Fas-induced apoptosis. Using A-T lymphoid cells we could show that ATM deficiency causes resistance to Fas-induced apoptosis. A-T cells upregulate FLIP protein levels, a well-known inhibitor of Fas-induced apoptosis. Reconstitution of ATM kinase activity was sufficient to decrease FLIP levels and to restore Fas-sensitivity. Conversely, genetic and pharmacological ATM kinase inactivation resulted in FLIP protein upregulation and Fas resistance.

Both ATM and FLIP are aberrantly regulated in Hodgkin lymphoma. Importantly, we found that reconstitution of ATM kinase activity decreases FLIP protein levels and restores Fas-sensitivity in Hodgkin lymphoma derived cells.

Overall, these data identify a novel molecular mechanism through which ATM kinase may regulate the immune system homeostasis and impair lymphoma development.

ABSTRACT (in Italian)

L'Atassia Telangectasia (A-T) è una rara patologia genetica causata dall'assenza funzionale della chinasi ATM, la quale svolge un ruolo fondamentale nella risposta al danno a doppia elica del DNA. I pazienti A-T manifestano un'ampia gamma di fenotipi, tra i quali una maggior predisposizione allo sviluppo di linfomi e leucemie. L'apoptosi indotta da Fas gioca un ruolo fondamentale nel controllo dell'omeostasi del sistema immunitario e difetti nella via di trasduzione del segnale controllata dal recettore di morte Fas sono stati associati con lo sviluppo di linfomi e malattie autoimmuni.

Abbiamo quindi investigato il ruolo di ATM nell'apoptosi indotta da Fas. Nei nostri studi cellule linfoblastoidi che non hanno una proteina ATM funzionale sono sensibilmente resistenti alla morte indotta dal recettore Fas. Tale resistenza all'apoptosi correla con alti livelli di espressione nelle cellule di una proteina anti-apoptotica chiamata FLIP. La ricostituzione di queste cellule con una forma cataliticamente attiva di ATM è sufficiente per abbassare i livelli di FLIP e ripristinare la sensibilità all'apoptosi indotta da Fas. Invece, l'inibizione dell'attività chinasica di ATM, tramite inibitori farmacologici e/o genetici, porta all'innalzamento dei livelli di FLIP ed alla resistenza all'apoptosi indotta da Fas.

Sia ATM che FLIP sono regolati in modo aberrante nei linfomi di Hodgkins. Noi abbiamo scoperto che la ricostituzione dell'attività chinasica di ATM, in cellule derivate dai linfomi di Hodgkins, fa sì che i livelli di FLIP si abbassino e le cellule diventino sensibili all'apoptosi indotta da FAS.

Nel loro insieme questi dati hanno portato all'identificazione di un nuovo meccanismo molecolare attraverso il quale la chinasi ATM può regolare l'omeostasi del sistema immunitario e lo sviluppo di tumori a carico di questo apparato.

INTRODUCTION

ATM (Ataxia-Telangiectasia Mutated) KINASE

Ataxia Telangiectasia

The ATM (Ataxia-Telangiectasia Mutated) protein was identified as the product of the gene that is mutated (lost or inactivated) in the human genetic disorder Ataxia-Telangiectasia (A-T) (Savitsky et al., 1995). A-T belongs to a group of diseases that are collectively known as 'genomic instability syndromes', each of which results from a defective response to a specific DNA lesion. A-T is characterized by cerebellar degeneration, which leads to severe and progressive neuromotor dysfuction, immunodeficiency, genomic instability, thymic and gonodal atrophy, a striking predisposition to lymphoreticular malignancies and extreme sensitivity to ionizing radiation and Double Stand Break (DSB) DNA damage -inducing agents (Fig.1) (McKinnon, 2004). This human disorder typically combines most of the hallmarks of a defective DNA-damage response, clearly pointing to the DSB as the lesion that elicits this defects. Indeed, cultured cells from A-T patients show a broad defect in responding to DSBs that span almost all of known branches of this response. The identification of ATM, which when mutated is the underlying cause of the disease, supported a rapid progression in the understanding of the molecular basis of this disease. Moreover, the striking clinical and cellular phenotype that is caused by ATM loss, clearly places this protein at the top position in the DSB-response cascade (Shiloh , 2003).

A-T patients suffer as a result of over 400 distinct ATM mutations, among them 85% are accounted for null mutations in the ATM gene (Becker-Catania et al., 2000). Thus approximately 85% of A-T suffers have no detectable ATM protein. There are a few reported genuine A-T cases with normal ATM protein levels; however, in these cases the protein is defective in its enzymatic activity (Stankovic et al., 1998).

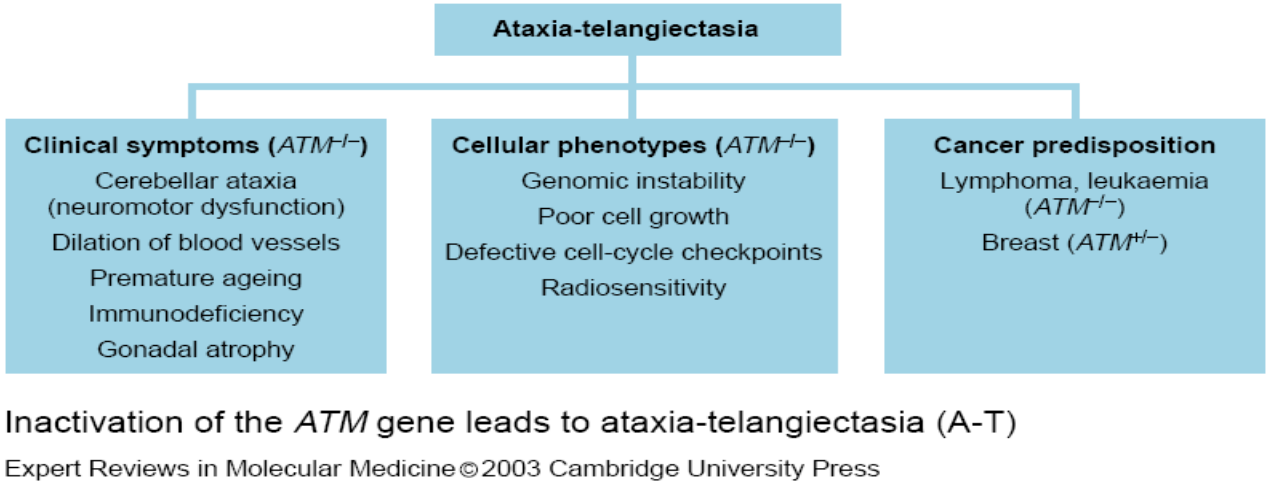

Fig.1. Clinical symptoms, cellular phenotypes and cancer predisposition of individuals with mutations in the ATM (ataxia-telangiectasia mutated) gene.

ATM structure and function

The ATM protein is a large molecule (approximately 350 kDa) which belongs to a conserved family of proteins, most of which possess a serine/threonine kinase activity (Shiloh Y., 2003). All of these proteins contain a domain typical of the lipid kinase phosphatidylinisitol 3-kinase (PI3K) (Fig.2), so they are dubbed 'PI3K-like protein kinases' (PIKKs). The PI3K domain harbours the catalytic site of the active protein kinases of the PIKK family. The ATM protein is characterized by the presence of other structural domains (Fig.2): the FAT domain, located N-terminally to the kinase domain, is conserved among PIKK family of proteins FRAP, ATR and TRRAP; the FATC domain, a 33aa conserved domain in the C-terminal of the protein. Recent works show that these two domains play a critical role in the regulation of ATM protein kinase activity (Bakkenist and Kastan,2003; Jiang X et al., 2006).The FAT domain of ATM contains serine 1981, the site that is autophosphorylated during ATM activation (Bakkenist and Kastan, 2003). The FATC domains binds to histone acetyltransferase TIP60 and this binding is important for ATM activation (Jiang X et al., 2006). Instead the function of the large N-terminal portion of ATM is almost unknown, but this large portion is crucial to form productive complexes with some important ATM substrates proteins as p53 and c-Abl (Khanna et al., 1998; Shafman et al., 1997), supporting the idea that the N-terminal of ATM is also essential for correct ATM function (Fernandes et al., 2005).

The active protein kinases in the family, which are conserved from yeast to mammals, respond to various stress phosphorylating key proteins in the corresponding response pathways (Shiloh Y., 2003). They could therefore simultaneously affect several processes depending on the spectrum of their substrates. Four mammalian PIKKs are known to be involved in the DNA-damage response: the DNA-dependent protein kinase (DNA-PK), ATM, ATR and ATX .Whereas ATM and DNA-PK respond primarily to DSBs, ATR and ATX respond to both ultraviolet (UV) light damage (possibly UV-light-induced replication arrest) and DSBs, and ATR also responds to stalled replication forks. mTOR/FRAP is the only active kinase in this family that is not involved in the DNA damage response.

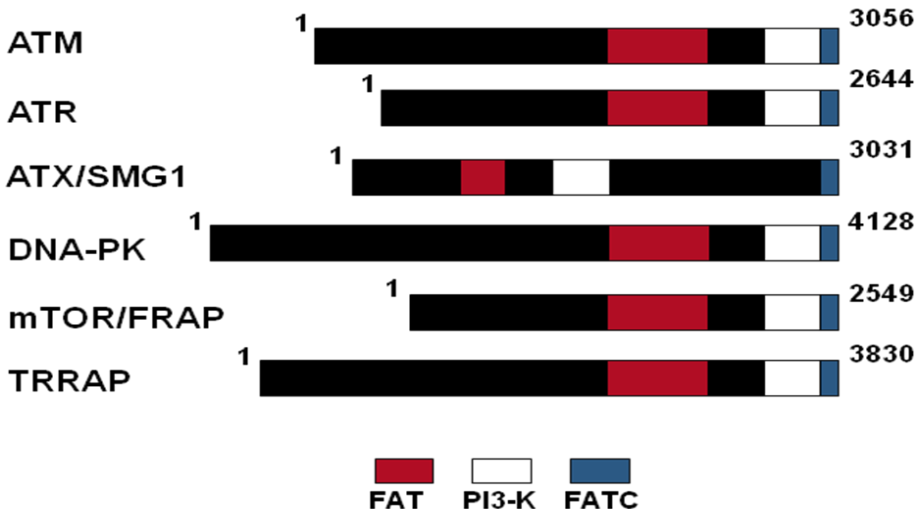

Fig.2. Size and common motifs in the human members of the PIKK family. The number of residues is indicated for each protein. This family comprises six proteins, all of which (except for TRRAP) possess protein kinase activity. These proteins share three motifs: the FAT and FATC domains and the PI3K domain, which contains the phosphatidylinositol 3-kinase motif and harbours the catalytic site in the active kinases of the family (see text).

ATM function in DNA damage signaling

The greatest challenge to genome stability comes from DNA-damaging agents that can be either endogenous (occurring during normal cell metabolism) or exogenous (from the environment). Damaging agents such as radiation and reactive chemicals are capable of inducing a plethora of DNA lesions. Some of them are extremely cytotoxic if not repaired, whereas others are mutagenic and can affect the production, structure and function of cellular proteins, with consequences ranging from malfunction of the cell to malignant transformation. It is not surprising, therefore, that many mutagens are also carcinogens, and that there is a high correlation between their carcinogenic and mutagenic potencies. The basic cellular response is to arrest the cycle to prevent genome instability and to repair the damage, but the type and amount of damage might overwhelm the survival response machinery to the extent that programmed cell death (apoptosis) is initiated instead (Fig.3). The mechanism of this important choice between attempts at survival and programmed death is not entirely clear.

ATM is the prototype transducer of the Double Stand Break DNA damage. DSBs are naturally formed and sealed during processes such as meiotic recombination and the assembly of the T-cell receptor and immunoglobulin genes via V(D)J recombination, in T cells and B cells, respectively. It is safe to assume that cellular DSB repair mechanisms maintain continuous, low-level activity, ensuring that the occurrence and resealing of these breaks leave the cell unharmed. But when DSBs are inflicted on the genome by damaging agents, such as free radicals or ionizing radiation, their threat to cell life is sufficiently serious to set in motion, within minutes, a rapidly mounting, decisive DNA-damage response. Recent models depict the DSB response as developing through a series of steps (Fig. 3) (Shiloh Y., 2003).According to these models, DSBs might first be detected by sensor proteins that recognize the DNA lesion itself or possibly chromatin alterations that follow DNA breakage. The broken ends are then processed — their chemical nature is random, so they cannot serve directly as substrates for repair mechanisms. Then, the transducers are brought into action; these convey the damage signal to downstream effectors. It is this relay system from transducers to effectors that enables a single transducer to quickly affect the operation of many pathways .The transducers might also be involved in the assembly of DNA-repair complexes at the site of the damage , so DSB repair and signaling are probably concomitant and functionally linked . As mentioned above in the case of DSBs, the initial and primary transducer is ATM which transmits the message via a standard signalling mode: protein phosphorylation. In particular ATM kinase activity is induced upon DSB and modulates the cell cycle arrest and repair as well as the apoptotic response in case the damage is very extend preventing DNA replication in the presence of damaged DNA and genomic instability (Fig.3) (Shiloh Y., 2003).

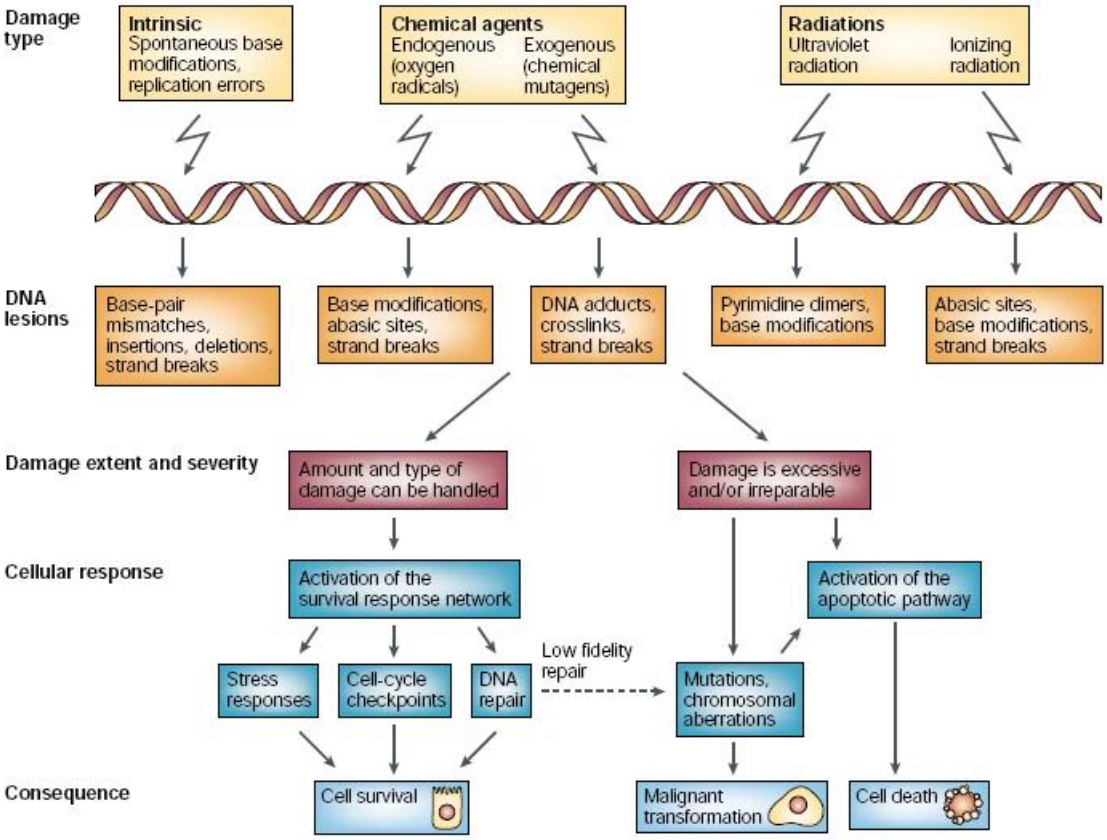

Fig. 3. Cellular responses to DNA damage. Different types of DNA damage cause different types of lesions, and these, in turn, are handled by the cell in different ways. The outcome could be cell survival and resumption of the normal life cycle of the cell, cell death or malignant transformation. The mechanism of choice between attempt at survival and programmed cell death is not completely understood. The survival response is elaborate and encompasses many signalling pathways. (From Shiloh Y. 2003)

ATM function in non-DNA damage signaling

Although the work of several labs supports the idea that ATM is a predominantly nuclear protein, involved in the signaling of DNA damage to the cell cycle checkpoint machinery, some reports demonstrated that ATM is also a cytosolic protein (Watters, D et al. 1997) and that the kinase activity of this protein is also activated by non-DNA damage signaling (Kastan MB & Lim DS., 2000; Lavin MF, 2000). For example Yang and Kastan (Yang DQ & Kastan MB., 2000) provide evidence that the kinase activity of this protein is also activated by insulin through a non-DNA damage signalling pathway to phosphorylate 4E-BP1 (PHAS-I), a regulator of protein synthesis. This report supports a more general signalling function of ATM in cell growth and proliferation .

Moreover, in the cytoplasm, ATM localizes to vesicles and interacts with β-adaptin (Lim et al., 1998) one of the components of the AP-2 adaptor complex, which is involved in clathrin mediated endocytosis of receptors (Robinson, 1994). This interaction between ATM and the vesicle associated proteins may play an important role in regulating vesicle and or protein transport in neurons. Dysfunction in these pathways may contribute to the progressive cerebellar degeneration of AT patients (Lim et al., 1998).

In post-mitotic neuronal tissue samples, it has even been shown that ATM is predominantly cytoplasmic (Oka and Takashima 1998; Barlow et al. 2000), which further indicates a role for cytoplasmic ATM in neuronal cell differentiation and survival. Finally a recent work on Science (Zhao-Hui Wu et al. 2006) demonstrated that ATM can traslocate in the cytosol upon the phosphorylation and the binding to NEMO protein, a regulatory subunit of IKK complex that plays a central role in regulating NFKB transcription factor.

Although the role of ATM kinase in the cytosol remains unclear, understanding this function of ATM might help to explain how mutations in the ATM gene cause the pleiotropic nature of the A-T phenotype. In fact, a role for ATM in intracellular signaling and in the cytosol has been suggested by several of the phenotypic changes observed in AT cells, such as the high level of growth factors required for cell growth (Shiloh, Y et al. 1983), the insulin resistance in AT patients (Bar, R. S et al. 1978), and the altered actin cytoskeleton in AT cells (McKinnon, P. J. & Burgoyne, L. A. 1985). In addition, uncharacterized accumulated cytoplasmic lipid vesicles and an increased number of lysosomes in AT patient samples have been observed in electron microscopic studies (Schoonderwaldt et al, 1977), suggesting altered lipid metabolism or altered lysosomal enzyme activity in AT cells.

So the large size of the ATM protein and its multiple subcellular localizations suggest that ATM may have more than one function.

ATM activation

ATM is activated most efficiently by radiation and radiomimetic agents (Kurz et al. 2004). ATM resides predominantly in the nucleus in dividing cells, and responds swiftly and vigorously to DSBs by phosphorylating numerous substrate. The hallmark of ATM's response to DSBs is a rapid increase in its kinase activity immediately following DSB formation. Researchers have long been impressed by the rapid phosphorylation of the many ATM substrates, which converts them within minutes to phosphorylated derivatives. A marked change in the activity of ATM would account for this massive process. Initial evidence indicated that ATM activation might involve autophosphorylation. A breakthrough in our understanding of this process came in a landmark publication by Bakkenist and Kastan (Bakkenist and Kastan,2003). They reported that ATM molecules are inactive in undamaged cells, being held as dimers or higher-order multimers. In this configuration, the kinase domain of each molecule is blocked by the FAT domain of the other (Fig. 4). Following DNA damage, each ATM molecule phosphorylates the other on a serine residue at position 1981 within the FAT domain , a phosphorylation that releases the two molecules from each other's grip, into fully active monomers (Fig. 4). Within minutes after the infliction of as few as several Double Stand Breaks per turning genome, most ATM molecules become vigorously active.

Mechanism of ATM activation

There are different hypothesis about the molecular mechanism through which ATM is activated upon DSB DNA damage. Bakkenist and Kastan provide evidence that the signal for ATM activation might be chromatin alterations rather than direct contact of ATM with the broken DNA (Bakkenist and Kastan,2003). While it remains unclear whether the DNA double strand break per se or conformational change in chromatin resulting from the break initiates the process of ATM activation, other events associated with the process are better described. NBS1 (a component of the Mre11 complex) is conventionally thought to be a downstream substrate of ATM. However recent studies suggest that NBS1/MRN might function upstream of ATM, by recruiting ATM to the proximity of DNA damage sites and activating its enzimatic function (Uziel et al., 2003). In addition to the requirement for the Mre11 complex for ATM activation a number of post-translational modifications are also required. As mentioned above Bakkenist and Kastan showed that the phosporylation on S1981 on ATM plays an important role in its activation (Bakkenist and Kastan,2003). There is also evidence that dephosphorylation of ATM affects its enzimatic activity. Goodarzi et al. (Goodarzi et al. 2004) showed that the catalytic and scaffolding subunits of PP2A co-immunoprecipitated with ATM, in unirradiated cells, but dissociated from the complex after irradiation. These results suggested that PP2A associates with ATM in unperturbed cells to maintain it in an inactive state.

Moreover Sun Y. et al (Sun Y. et al., 2005) suggest a direct role for the histone acetyltransferase TIP60 in ATM activation in response to DNA damage. ATM in unstressed cells appeared to be associated through the FATC domain with TIP60. Upon DNA damage TIP60 is activated and directly acetylates ATM and this event is causal for kinase activation**.**

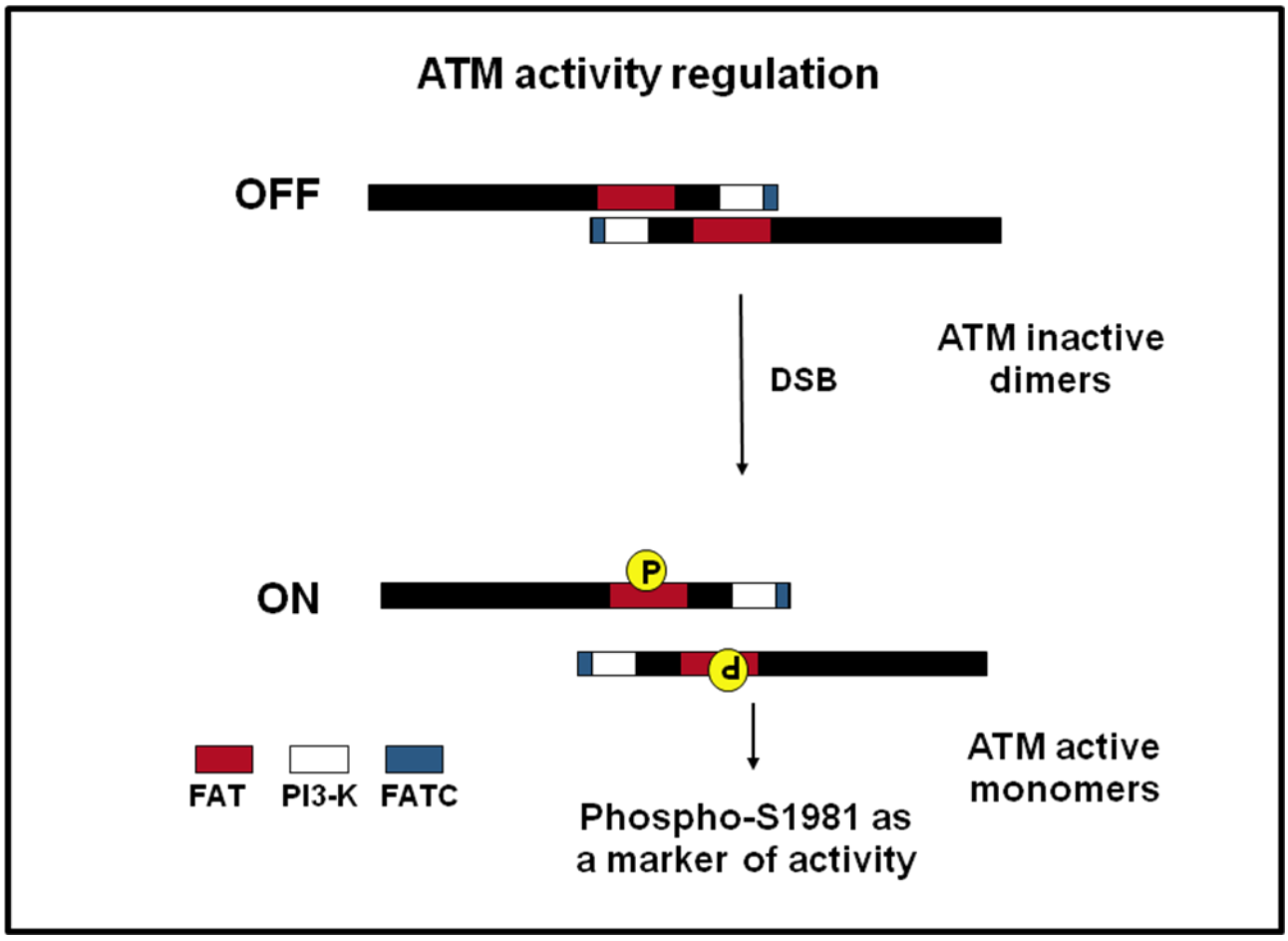

Fig. 4. Model of ATM activation. In unstressed cells, the ATM kinase forms dormant dimers, distributed throughout the nucleus. The 'FAT' domain of one ATM unit interacts with the enzymatic (kinase) domain of the other, and this probably locks ATM into a state in which it cannot interact with and phosphorylate its protein targets. Generation of DNA double-strand breaks (DSBs) by, for instance, ionizing radiation causes intermolecular modifications within ATM dimers that ultimately lead to their activation. The two bound ATM proteins phosphorylate each other on serine residue 1981 to form active monomers, and so this phosphorylation is consider a marker of ATM activity (Bakkenist and Kastan, 2003).

ATM substrates

ATM-mediated phosphorylation either enhances or represses the activity of its targets, thereby affecting specific processes in which these proteins are involved. Similar to other active PIKKs (with the exception of mTOR/FRAP), ATM targets serine or threonine residues followed by glutamine (the 'SQ/TQ'motif) (Traven A. and Heierhorst, 2005). Knowledge of the substrates of a protein kinase is essential to understanding its biological functions. So when ATM was identified as a member of the PIKK family, capable of phosphorylating a variety of substrates involved in Double Stand Break (DSB) DNA damage induced signalling, it was evident that it would play a key role in cell cycle checkpoint control, DNA repair and apoptosis (Fig.5) (Shiloh, 2003).

One of the first processes that is initiated by DSBs is the massive phosphorylation of the tail of a histone protein variant called H2AX (Redon et al., 2002). Foci of phosphorylated H2AX are rapidly formed at the DSB sites and are thought to be essential for further recruitment of repair factors, such as the MRN complex. H2AX phosphorylation , a very early event in the cascade induced by DSBs , was reported to be ATM dependent following DSB induction (Burma S. et al 2001).This process could therefore serve as a rapid and powerful mechanism for amplifying the damage signal via repeated cycles of H2AX phosphorylation, and recruitment of processing factors.

Another important substrate of ATM after DSB DNA damage is p53. A-T cells were defective in all the cell cycle checkpoints (Beamish and Lavin 1994). ATM plays a complex role in regulating the G1/S, S and G2/M checkpoints, directly phosphorylating substrates (Fig.5), activating other kinases to do this and ensuring rigid maintenance of the checkpoints by controlling signalling at multiple levels. This is illustrated for the G1/S checkpoint. When Kastan et al. (Kastan et al., 1992) demonstrated a defect in p53 stabilization in response to radiation exposure, p53 emerged as crucial mediator of G1/S checkpoint. They had previously shown that radiation exerted its influence on the G1/S checkpoint to delay the passage of cells into S-phase in order to facilitate DNA repair (Kastan et al., 1991). It is now history that ATM phosphorylates p53 on S15 and is responsible for other p53 phosphorylations that contribute to the efficiency of the transcriptional activation of p53 (Banin S. et al., 1998), responsible for G1/S checkpoint activation or induction of apoptosis (Lavin, 2006). ATM also phosphorylates and activates CHK2, a checkpoint kinase that phosphorylates p53 on Ser20 (Bartek, J. et al., 2001). This interferes with the p53–MDM2 interaction. The oncogenic protein MDM2 is both a direct and indirect inhibitor of p53, as it serves as a ubiquitin ligase in p53 ubiquitylation, which mediates its proteasome-mediated degradation. ATM also directly phosphorylates MDM2 on Ser395, which interferes with nuclear export of the p53–MDM2 complex, and hence the degradation of p53 (Khosravi et al. 1999). Finally, it has been reported that phosphorylations of p53 on Ser9 and Ser46 , and dephosphorylation of Ser376 , are ATM dependent as well, although the function of these changes is unknown (Saito S. 2002). This series of ATM-dependent modifications that activate and stabilize p53, although perhaps not complete , illustrates the elaborate way in which ATM handles a single effector, and indicates that ATM might regulate several effectors within the same pathway.

Most of these pathways have not been completely characterized, and the involvement of ATM substrates in them has been inferred simply from defective activation of specific checkpoints following abrogation of ATM-mediated phosphorylation of these proteins. It is possible that such proteins have a dual role in processes upstream and downstream of ATM. As mentioned above such is the case with NBS1 as well: on one hand, it is a component of the MRN complex that is thought to be

involved in the initial processing of the DSB, and, on the other hand, it is a downstream effector of ATM in a checkpoint pathway.

So the emerging complex relationships between ATM and its substrates are drawing new flow charts for the DNA-damage signal that deviate from the traditional linear ones and assign to several proteins more than one role ‘upstream’ or ‘downstream’ in this chart (Fig.5).

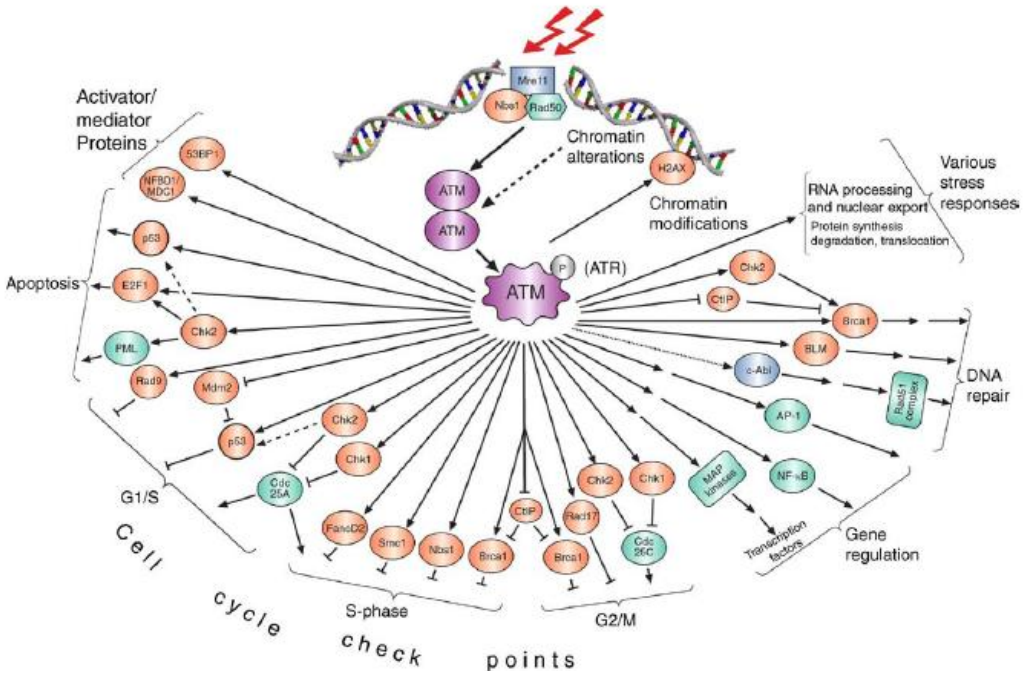

Fig. 5 Current view of the ATM-dependent DSB response network. (From Shiloh et al. 2004)

Proteomic approach for ATM substrates identification

The identification of downstream components of the ATM signaling network provide a necessary starting point for functional studies and will stimulate insights into diseases such as Ataxia Telangiectasia and the avoidance of cell cycle checkpoints in cancer, a critical early event in cancer progression.

The list of published ATM substrates is far from complete, and many ATM-dependent responses are likely to involve ATM targets that are unknown at present. Although a key to understanding any kinase network is the identification of the in vivo substrates, few techniques are available to identify protein kinase substrates so unbiased identification of kinase substrates is a difficult endeavor. Various techniques have emerged to identify phosphoproteins and kinase substrates (Ptacek et al., 2005; Dephoure et al., 2005), but few give in vivo confirmation or the sites of phosphorylation. However recently several groups made large-scale proteomic analysis of proteins phosphorylated by ATM kinase. Mu et al. (Mu et al., 2007) screened for potential ATM/ATR substrates using phospho-specific antibodies, against known ATM/ ATR substrates that recognize pSQ motifs, to immunoprecipitate potential new substrates. The proteins cross-reacting with phospho-specific antibodies, in response to DNA damage, were identified by mass spectrometry and the subset of candidate substrates for ATM/ATR-dependent phosphorylation was valitated *in vivo*. Interestingly, using this approach they identified proteins that belong to the ubiquitin-proteasome system (UPS) to be required in mammalian DNA damage checkpoint control thus revealing protein ubiquitylation as an important regulatory mechanism downstream of ATM/ATR activation for checkpoint control. Moreover recently Matsuoka et al. (Matsuoka et al., 2007) performed a large-scale proteomic analysis of proteins phosphorylated in response to DNA damage on consensus sites recognized by ATM and ATR and identified more than 900 regulated phosphorylation sites encompassing over 700 proteins. The identified proteins were annotated by the authors in gene ontology format (Fig.6). Functional analysis of a subset of this data set indicated that this list is highly enriched for proteins involved in the DNA damage response. More interestingly they identified also a large number of protein modules and networks not previously linked to the DNA damage response. This database paints a much broader landscape for the DNA damage response that was previously appreciated and opens new avenues of investigation into the responses to DNA damage in mammals.

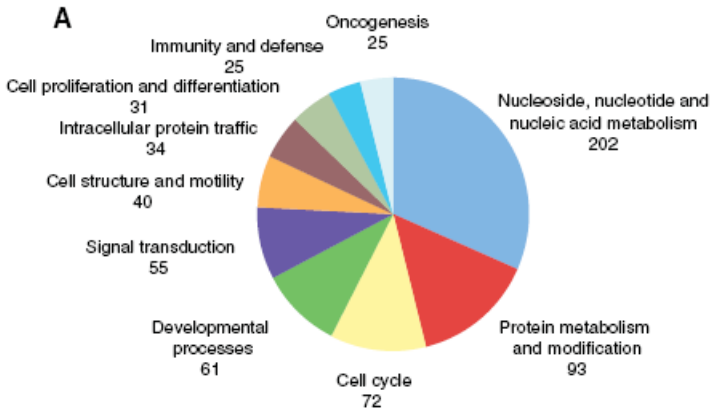

Fig.6 Gene ontology analysis of the candidate ATM and ATR substrates (from Matsuoka et al., 2007). Gene ontology analysis of the candidate substrates was done with the PANTHER program. Of the 700 identified proteins, 421 were assigned with 769 biological processes. Proteins for which no biological process could be assigned were omitted from this display. Categories with more than 20 assigned proteins are shown.

ATM in the immune system and cancer

Like the other genomic instability syndromes, A-T is a cancer-predisposing disorder. The higher cancer predisposition of A-T patients has been associated with the lack of DNA damage response, which results in genomic instability (Shiloh Y. 2003; Khanna KK. & Jackson SP, 2001) However, despite the nervous system being markedly affected in AT, the tumor types occurring in this disease are primarily lymphoma and leukemia (Gumy-Pause F. et al., 2004; Taylor AM, et al. 1996). This clinical feature is consistent with the central role of ATM in the management of the DNA DSBs generated during the immune system development and function in physiological conditions (Matei IR et al. 2006; Starczynski J et al 2003).

As mentioned above DNA damage checkpoints and repair of DNA double strand breaks (DSB) are necessary to preserve genomic integrity during V(D)J recombination, a process required to rearrange B-cell and T-cell receptor (TCR) gene segments. Although responsible for enormous diversity of the immune system, chromosome breakage and rejoining during V(D)J recombination contribute to oncogenic transformation in the context of defective DNA damage checkpoints and/or DNA DSB repair (Matei IR et al. 2006). Thus, impairments in ubiquitous DNA damage detection and/or repair pathway result in genomic instability. Defects in repair protein, as ATM kinase, involved in rejoining V(D)J recombination-induced DSBs preclude the generation of antigen receptor, profoundly compromising T- and B-cell development and causing severe immune deficiencies (Revy P et al. 2005). Moreover *Atm*-deficient mice show a striking predisposition to lymphoid malignancies, particularly thymic lymphomas, to which they succumb before the age of 1 year (Shiloh Y. 2001; Shiloh T. and Kastan MB 2001).

However, much of the literature on *ATM* mutations and cancer is not about A-T patients, but is, instead, on heterozygous carriers of A-T mutations. For more than two decades, *ATM* has been of interest to cancer epidemiologists and the public because of observations of cancer predisposition among A-T carriers. These studies pointed to a high incidence of malignancies, particularly breast cancer, among unaffected members of A-T families (Khanna, K.K., 2000). In view of the estimated 1–2% frequency of A-T carriers in the general population, this observation has important implications for public health. A-T mutations lead, in most cases, to truncated, unstable protein products, and these *ATM* alleles therefore fail to produce any ATM at all. Carriers of such mutations have a reduced amount of otherwise functional ATM. Other A-T mutations lead to amino-acid substitutions (missense mutations) or in-frame deletions that produce a catalytically inactive protein. Cells of carriers of these mutations are expected to contain both functional and inactive ATM molecules in various ratios. A meticulous study of the type of *ATM* mutations in A-T families with high incidence of cancers disclosed a high frequency of missense mutations (Stankovic, T. *et al* 1998*)*. The importance of missense mutations in predisposing carriers to cancer can be explained simply by the dominant-negative effect of the inactive version of the protein leading to a reduction in ATM function .

Further evidence of the importance of *ATM* missense mutations as cancer-causing genomic alterations came from the search for somatic *ATM* mutations in sporadic human tumours. Indeed, *ATM* was found to undergo somatic mutations in sporadic lymphoid tumours, behaving like a tumour-suppressor gene in these malignancies (Stankovic *et al.*2002). In particular ATM gene alterations, mainly missense mutations, have been reported frequently in adult lymphoid malignancies (Gumy-Pause et al. 2004). More recently, childhood acute leukemias and Hodgkin disease were investigated for ATM gene alterations. ATM germline missense variants were frequently reported (Gumy Pause F et al. 2003; Liberzon E et al. 2004) and two studies showed that some of the detected variants were pathogenic, encoding functionally abnormal protein (Takagi M et al. 2004; Oguchi K et al. 2003) These results suggest that ATM is also involved in the pathogenesis of childhood lymphoid malignancies. These observations seem to reconcile the debate on the role of *ATM* mutations in

genetic predisposition to cancer (Khanna, K.K., 2000; Stankovic, T. *et al* 1998; Gatti r. et al, 1999), and place the gene encoding the ATM protein well within the list of genes that are involved in cancer morbidity in the general population.

Thus understanding the contribution of *ATM* gene mutations to cancer predisposition in human populations will keep this gene in the spotlight of cancer epidemiology.

Death receptor induced apoptosis: Fas pathway

Apoptosis overview

Multicellular animals often need to get rid of cells that are in excess, or potentially dangerous. To this end, they use an active dedicated molecular programme, which is as important as cell division and cell migration and allows the organism to tightly control cell and tissue homeostasis. Various researchers during the past two centuries have observed this phenomenon and apoptosis is the term finally adopted, coined by Currie and colleagues in 1972 (Kerr et al., 1972).

Apoptosis is a genetically programmed, morphologically distinct form of cell death that can be triggered by a variety of physiological and pathological stimuli. This evolutionarily conserved form of cell suicide requires specialized machinery and the central components of this machinery is a proteolytic system involving a family of cysteinyl aspartate specific proteinases known as caspases (Earnshaw et al., 1999).

The dying cells shared morphologically features that can be seen under a microscope as this sequence:

1. The cell becomes circular because the cytoskeleton is digested by the caspases.
2. The chomatin undergoes degradation and condensation into compact patches against the nuclear envelope.
3. The nuclear double membrane started to be dismantle. The caspases begun to degrade the lamin that underlies the nuclear envelope.
4. The nuclear envelope becomes discontinous and the DNA inside it is fragmented.
5. The nucleus breaks into several discrete chromatin bodies or nucleosomal units.
6. Plasma membrane blebbings
7. The cell or the apoptotic bodies are phagocytesed.

This readily visible transformation (Fig.7) is accompanied by a number of biochemical changes like the externalization of phosphatidylserine at the cell surface and the activation of the caspase proteolityc cascade.

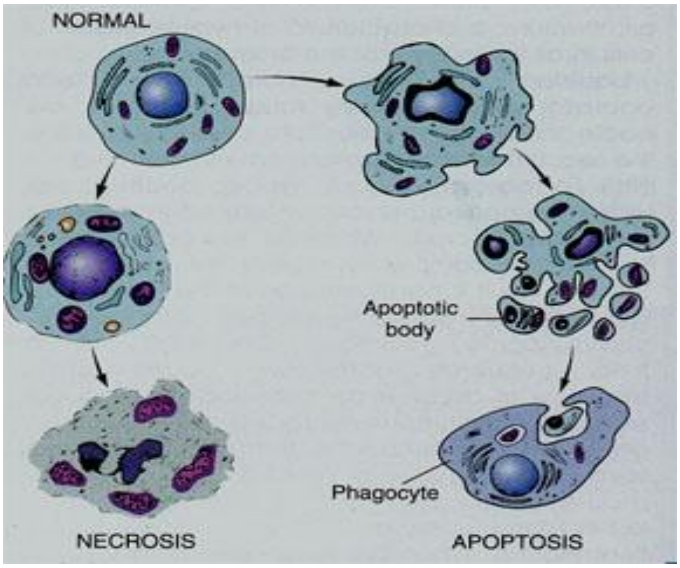

Fig.7. Apoptotic morphology.

Apoptosis can be triggered essentially in two distinct ways: intrinsic and extrinsic . In both cases effector caspases activated downstream are responsible for all the biochemical and morphological changes observed during apoptosis (Fig.8). Irreparable genome damage, caused by mutagens, pharmaceuticals or ionizing radiation, activates the **intrinsic pathway**, in which cytochrome c is released from the mitochondria and starts the assembly of the apoptosome. Apaf-1, an oligomeric cytoplasmic protein, binds the released cytochrome c and then undergoes an ATP-dependent conformational change that allows binding of pro-Caspase-9 through N-terminal CARD domains present in both molecules (Hofmann et al., 1997). This binding increases the local concentration of Caspase-9, leading to dimerization and promoting reorientations of the caspase's activation loop (Renatus et al., 2001). In addition the juxtaposition of pro-Caspase-9 molecules in the complex permits transcatalytic cleavage (Srinivasula et al., 1998).

In the **extrinsic pathway**, the apoptotic signal is initiated by direct ligand-mediated activation of death receptors like CD95 at the cell surface and the apical caspases involved are pro-Caspase-8 and pro-Caspase-10 (Kischkel et al., 2001).

The death-receptor and mitochondrial pathways converge at the level of Caspase-3 activation. Cross-talk and integration between the death-receptor and mitochondrial pathways is provided by Bid, a pro-apoptotic Bcl2 family member. Caspase-8 mediated cleavage of Bid greatly increases its pro-death activity, and results in its translocation to mitochondria, where it promotes cytocrome c exit. Nevertheless the contribution of this cross-talk is minimal and the two pathways operate largely independently each other (Hengartner, 2000) (Fig.8).

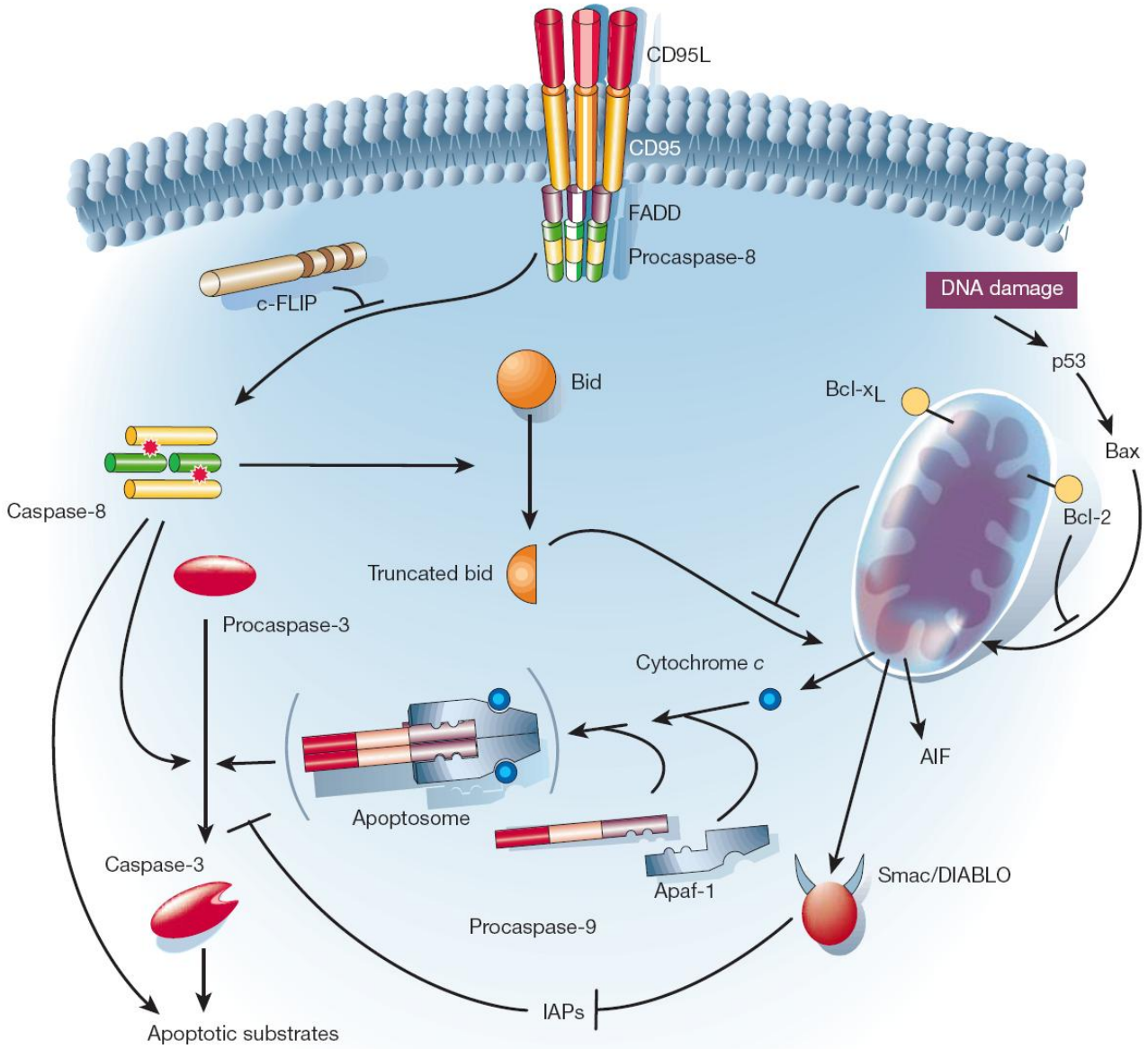

Fig.8. Two major apoptotic pathways in mammalian cells. (Hengartner, 2000). The death-receptor pathway (left pathway in the figure) is triggered by members of the death receptor superfamily (such as CD95 and tumour necrosis factor receptor I). Binding of CD95 ligand to CD95 induces receptor clustering and formation of a death inducing signalling complex (DISC). This complex recruits, via the adaptor molecule FADD (Fas-associated death domain protein), multiple procaspase-8 molecules, resulting in caspase-8 activation. Caspase-8 activation can be blocked by recruitment of the degenerate caspase homologue c-FLIP. The mitochondrial pathway (right) is used extensively in response to extracellular cues and interna insults such as DNA damage These diverse response pathways converge on mitochondria, often hrough the activation of a pro-apoptotic member of the Bcl-2 family.

Fas pathway

CD95 (APO1/Fas) is the best characterized member of the tumor necrosis factor (TNF) superfamily of receptors like TNF and TRAIL receptors (Fig.9). Members of the TNF-R family have pleiotropic action. Depending on the signal and on the cell type these receptors can trigger proliferation, survival, differentiation or death. CD95 has three cysteine-rich extracellular domains and an intracellular death domain (DD) essential for signalling. The receptor mediates apoptosis when triggered by agonistic antibodies or its cognate oligomerizing ligand, CD95L, expressed on cell membranes or in a soluble form. CD95L belongs to a corresponding coevolved family of proteins, the family of TNF and its related cytokines (Peter et al., 1997).

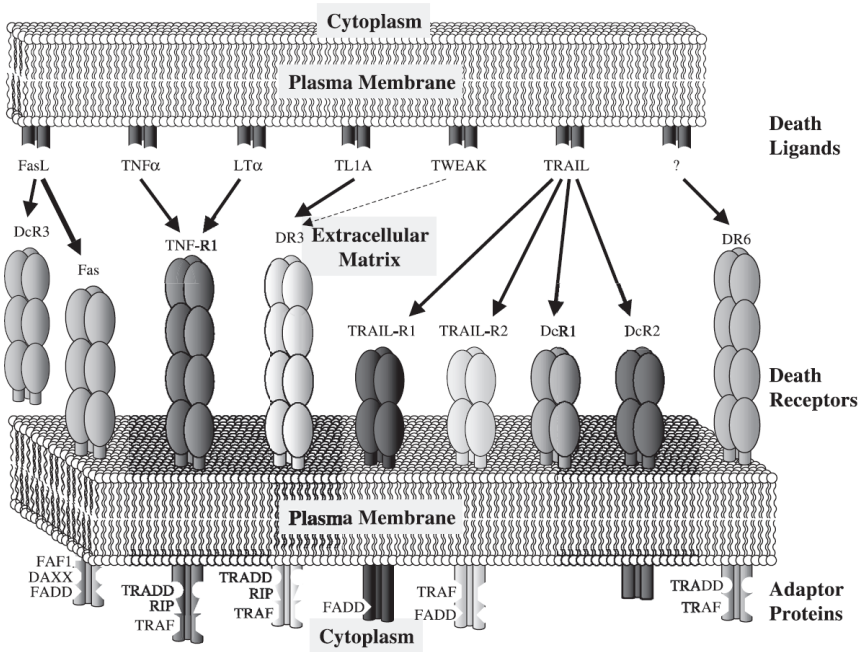

Fig.9. Tumor necrosis factor superfamily (Curtin JF and Cotter TG., 2003).Schematic diagram depicting the death receptors Fas, TNF-R1, DR3, TRAIL-R1, TRAIL-R2 and DR6 and the decoy receptors DcR1, DcR2 and DcR3. Ligands that are known to bind to these receptors are all shown and are predominantly membrane bound. Some death receptors can bind with more than one ligand and some ligands bind to more than one receptor as indicated. Important adaptor proteins that are recruited to each receptor and that are involved in signal transduction are also indicated.

CD95 receptors are expressed on the surface of cells as preassociated homotrimers (Papoff et al., 1999) and mutations on the domain that impairs this association cause the autoimmune disorder called ALPS, 'Autoimmune Lympho Proliferative Syndrome' (Siegel et al., 2000). Upon specific ligand binding (Wallach, 1999) there is assembly of a complex of adaptor proteins that leads to clustering of the two Fas homotrimers (Holler et al., 2003). This enables the adapter molecule FADD/MORT1 to interact with the Death receptor via homophilic interaction of the death domains (DD). FADD is a protein of 22 kDa containing two structurally similar protein motifs, the N-terminal Death-Effector Domain (DED) and the C-terminal Death-Domain (DD).

FADD molecules interact then with the death protease Caspase-8 (FLICE) via homophilic interaction of death effector domains (DED). The assembly of this proteins constitutes the death-inducing signalling complex (DISC) (Fig.10) (Medema et al., 1997).

When pro-Caspase-8 dimerizes, its activity is induced and it acquires the capability to cleave the adjacent dimer (Boatright, 2003) (Chang et al., 2003) (Donepudi, 2003). This starts the processing (Medema et al., 1997) (Muzio, 1996) of pro-Caspase-8 to form the active Caspase-8, and initiate the caspase cascade (figure 5). Upon prolonged triggering of CD95 with agonistic antibodies all cytosolic caspase-8 gets proteolitically activated. Physiological caspase-8 cleavage requires association with the DISC and occurs by a two-step mechanism. Initial cleavage generates a p43 and a p12 fragment further processed to a p10 fragment. Subsequent cleavage of the receptor-bound p43 results in formation of the prodomain p26 and the release of the active site-containing p18 (Medema et al., 1997). Pro-Caspase-8 activation is absolutely required to trigger this apoptotic response (Juo et al., 1998) and its catalytic activity has to be tightly regulated to avoid inappropriate activation and undesired cell death (Peter, 2004). Several mechanisms contribute to the control of the apoptotic response regulating caspase-8 activity. In particular the most important player in the control of caspase-8 activation and Fas signalling is FLIP protein that modulates the caspase-8 recruitment to the DISC (Peter, 2004). Moreover recently we demonstrated (Cursi et al., 2006) that tyrosine phosphorylation plays a central role in the control of caspase-8 processing and activity. We have shown that Src kinase directly phosphorylates Caspase-8 on Tyr 380. Src activity triggers endogenous Caspase-8 tyrosine phosphorylation and protects cells from Fas-induced apoptosis, suggesting that tyrosine phosphorylation directly modulates Caspase-8 activity and function (Cursi et al., 2006).

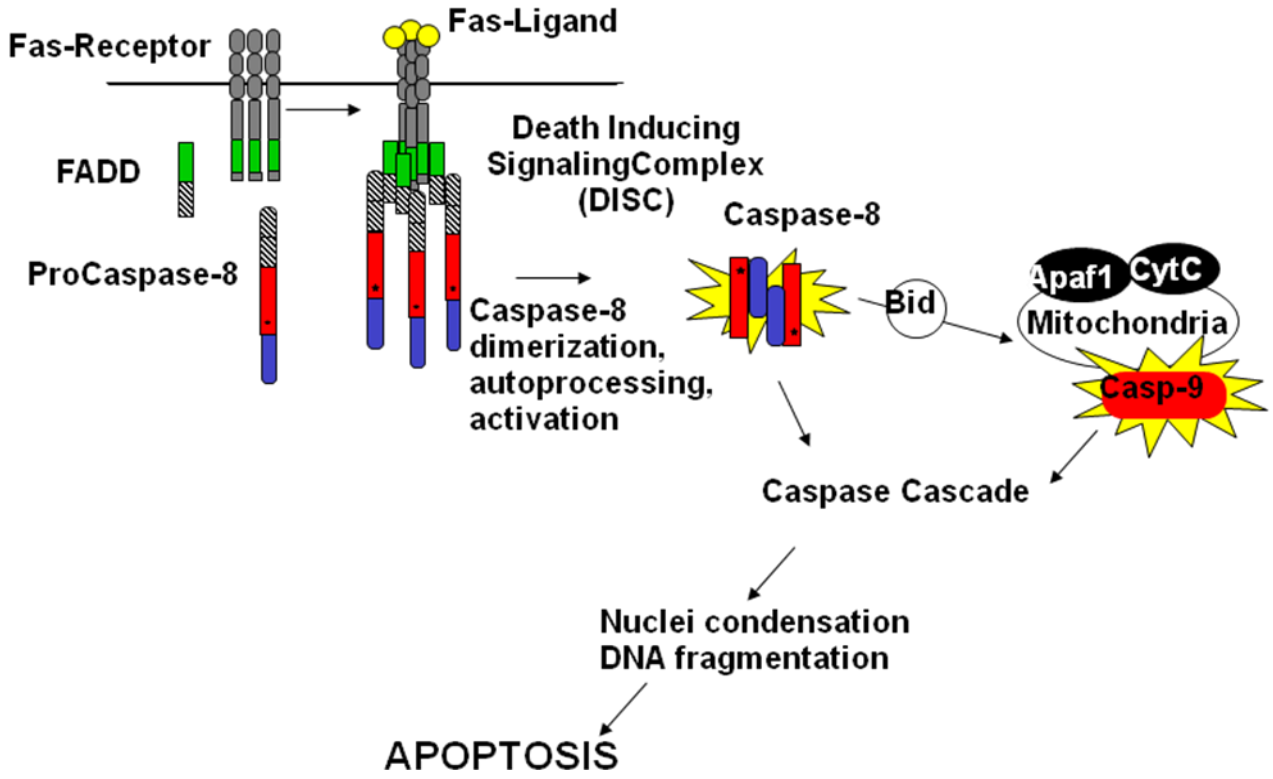

Fig.10. Fas pathway and DISC formation.

Fas pathway regulation: FLIP protein

To avoid uncontrolled cell death or tissue damage, apoptosis is tightly controlled by a collection of inhibitors. Both viral and cellular inhibitors exist which block cell death at different levels. Proteins of the inhibitor of apoptosis (IAP) family directly bind and inhibit caspase-3, -6, -7 and -9 (Deveraux *et al.*, 1999), whereas Bcl-2 family members regulate apoptosis that is induced by the mitochondrial pathway (Adams *et al.*, 2001).

There have been several recent advances in defining the molecular and physiological function of a novel family of inhibitors of death-receptor induced apoptosis, called FLIP, also known as FLICE/caspase-8 inhibitory proteins. FLIP was originally identified as a viral gene product, viral FLIP (vFLIP), while investigators were searching genomes for proteins that contain a DED in an effort to identify molecules that interact with caspases (Thome, M. *et al*.1997; Hu, S. et al. 1997; Bertin, J. *et al*.1997). The vFLIPs each contain two DEDs and are members of a family of DED-containing proteins that includes FADD, caspase-8, caspase-10 and PEA15 (phosphoprotein enriched in astrocytes 15 kDa) (Fig.11).

Following the characterization of vFLIPs, the mammalian cellular homologue was identified and called cFLIP (also known as CASH, Casper, CLARP, FLAME, I-FLICE, MRIT and usurpin)(Irmler, M. *et al*.1997; Inohara, N. et al. 1997). As many as 11 distinct cFLIP splice variants have been reported, two of which are mainly expressed as proteins: the 26 kDa short form (FLIP-S) and the 55 kDa FLIP-L (Fig.11) (Tschopp, J et al. 1998; Golks, A et al. 2005; Krueger, A.et al2001). FLIP-S is similar in structure to the vFLIPs, except that the two DEDs of FLIP-S are followed by ~20 amino acids that seem to be crucial for its ubiquitylation and therefore its targeting for proteasomal degradation (Poukkula, M. et al., 2005). FLIP-L contains a longer C terminus than FLIP-S and this full-length form of cFLIP closely resembles the overall structure of caspase-8 and caspase-10 (Fig.11)

(Tschopp, J et al. 1998). However, the C-terminal portion of FLIP-L lacks caspase enzymatic activity, owing to the substitution of several amino acids, including the crucial cysteine residue in the Gln-Ala-Cys-X-Gly motif (where X denotes any amino acid) and the histidine residue in the His-Gly motif, both of which are necessary for caspase catalytic activity and are conserved in all caspases (Cohen, G. M.1997).

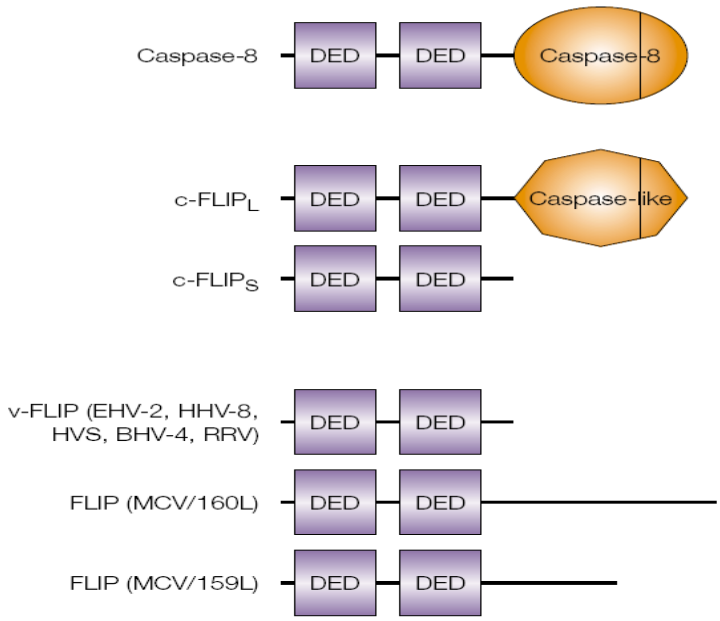

Fig.11. Molecular structure of viral and cellular FLIPs (Thome and Tschopp, 2001). Both herpes viral FLIP and the short form of cellular FLIP (FLIP-S) consist essentially of two repeats of a death effector domain (DED).The long splice variant of c-FLIP (FLIP-L) contains a carboxy terminal inactive caspase-like domain which confers on the molecule an overall structural homology with caspase-8 and caspase-10. (FLIP, FLICE/caspase-8 inhibitory protein.)

FLIP function

The inhibition of Fas signaling is based on the ability of the DED domains of FLIP to compete with the DED domain caspase-8, thus preventing caspase-8 recruitment to the adaptor protein FADD and consequently preventing the binding of caspase-8 to the death-inducing signalling complex (DISC), which is required for caspase-8 activation (Fig.12) (Irmler, M. *et al*.1997). Therefore, vFLIPs and FLIP-S function as dominant-negative inhibitors of caspase-8 by blocking recruitment of caspase-8 to the DISC, and as a result, block its subsequent processing and activation (Irmler, M. *et al*.1997) (Krueger, A.et al. 2001). FLIP-L function is more complex. Although FLIP-L can compete with caspase-8 for recruitment to the DISC, and can therefore inhibit caspase-8 activation downstream of CD95 ligation, it also forms a heterodimer with caspase-8 through interactions between both the DEDs and the caspase-like domains of the two proteins. The C-terminus of FLIP-L contains an activation loop that overlaps and exposes the enzymatic pocket of caspase-8. (Micheau, O. et al.2002). This allows partial auto-processing of caspase-8, which releases the p10 fragment to generate the p43 cleavage product but inhibits further cleavage to fully active caspase-8 (Krueger, A.et al. 2001). In this capacity, FLIP-L functions also as an activator of caspase-8 but then restricts the degree of

caspase-8 activation to a moderate, non-apoptotic range. This is probably crucial for the moderate caspase activation observed in activated T cells (Ralph C et al.2006).

However ligation of CD95 induces the rapid recruitment of FADD, caspase-8 and the various forms of c-FLIP to the DISC. The ratio of total c-FLIP to caspase-8 in the DISC is generally higher than that observed in whole-cell lysates, even in cells that express very low levels of FLIP-L. This indicates that FLIP-L–caspase-8 heterodimers might have a higher affinity for CD95–FADD complexes than do caspase-8 homodimers. This phenomenon might also reflect, at least in part, a greater affinity of caspase-8 for FLIP-L than for other caspase-8 molecules. In agreement with this observation that homodimerization of a caspase-8 mutant that lacks DED domains is less efficient than heterodimerization of FLIP-L and caspase-8 molecules that both lack DEDs (Scaffidi et al., 1999).

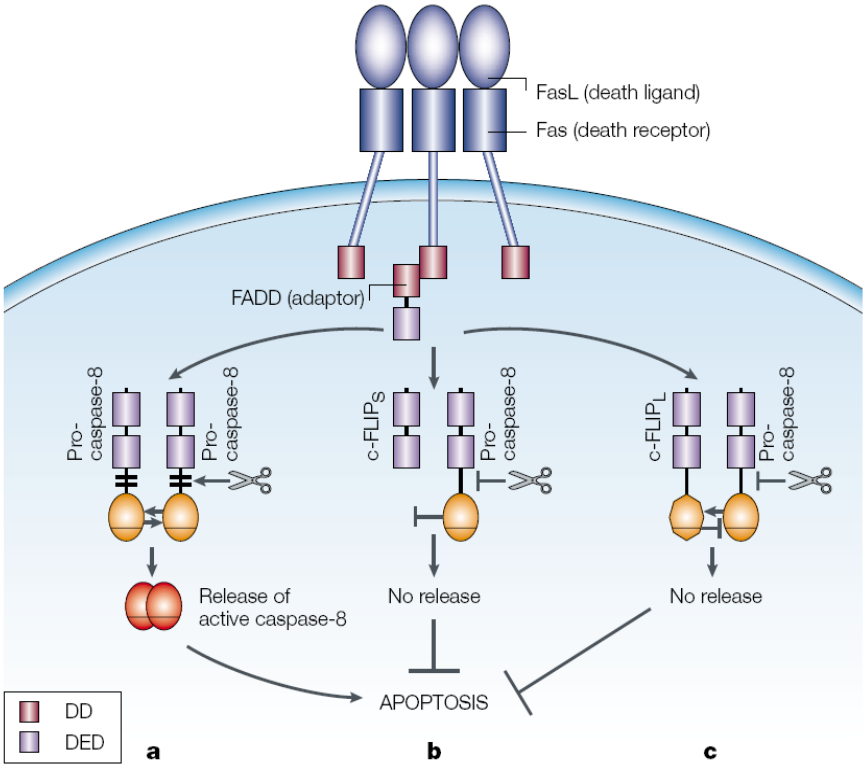

Fig.12. Death-receptor signalling in the absence or presence of FLIP (Thome and Tschopp, 2001). Binding of a trimeric ligand to a death receptor leads to recruitment of the adapter molecule Fas-associated death domain (FADD). In the case of Fas, tumour-necrosis-factor-related apoptosis-inducing ligand receptor 1 (TRAIL-R1) and TRAIL-R2, the binding of FADD is direct, whereas its recruitment to TNF-R1 is mediated by additional adapter molecules. These interactions are due to homophilic interactions between the death domains (DD) of receptors and adapter molecules. FADD has an additional domain, the death effector domain (DED), which serves to recruit caspase-8, again through homophilic interactions. (**a)**In the absence of FLIP, caspase-8 is recruited and initially activated by autocatalytic cleavage, followed by cleavage between the large and small subunit and between the caspase domain and the DED of the neighbouring caspase. As a result, the DED-containing amino-terminal fragment of caspase-8 stays transiently at the death-inducing signalling complex, whereas the active caspase-8 protease dimer is released into the cytoplasm to initiate the apoptotic cascade. (**b)** Viral FLIP (v-FLIP) and the short form of cellular FLIP (FLIP-S) have two DEDs and bind to FADD and caspase-8. v-FLIP and FLIP-S inhibit the processing of caspase-8 at the receptor level and protect the cells from apoptosis. (**c)** Long cellular FLIP (FLIP-L) has two DEDs and a caspase-like domain that lacks catalytic activity due to absence of a cysteine residue. In the presence of FLIP-L, both caspase-8 and FLIP-L are recruited and partially processed at the receptor level. Because no cleavage between the caspase domain and DED occurs, the partially processed proteins stay bound to the receptor and no active caspase-8 can be released into the cytosol .

Regulation of FLIP function and expression

The ability of cells to modulates the apoptotic response is crucial during development and differentiation. In this light, c-FLIP proteins are essential regulator of casapase-8 activity and play a central role in the control of death receptor signaling. Consequently, disturbances in c-FLIP expression have been implicated in several malignancies. For example, an elevated expression of c-FLIP is associated with resistance of many lymphomas tumours to Fas-induced apoptosis (Budd et al., 2006). Moreover an elevated expression of c-FLIP results in the escape of tumors from immune surveillance (Djerbi M. et al., 1999; Medema JP et al. 1999).

c-FLIP expression is carefully regulated at different levels. The transcriptional regulation is linked to a number of growth and survival promoting signaling pathways, including NF-kB (Kreuz s. et al, 2001), MAPK/ERK (Yeh JH et al, 1998) and Akt (Panka et al, 2001).

In addition to gene expression, it has been reported that the turnover of c-FLIP is actively regulated by ubiquitin-mediated degradation (Kim Y, et al, 2002)(Perez D. & White, E. , 2003). Although very little is known about the molecular mechanisms underlying this regulation, recently it has been identified the E3 ubiquitin ligase involved in the ubiquitination of Flip-L, ITCH (Chang et al., 2006). Chang et al. (2006) demonstrated that TNFa-mediated JNK activation accelerates turnover of the anti-apoptotic protein FLIP-L. This is not due to direct FLIP-L phosphorylation but depends on JNK-mediated phosphorylation and activation of the E3-ubiquitin ligase ITCH, whichs specifically ubiquitinates FLIP-L and induces its proteasomal degradation.

Role of Fas pathway in the immune system and in cancer progression

The death receptor system is essential for the regulation of the lymphoid system homeostasis (Krammer PH., 2000**)**. One of the principal roles of Fas receptor is regulating the immune response and this is the most clearly characterized function of Fas receptor. A number of studies have highlighted the multiple modes by which Fas receptor signalling can regulate T cell and B cell development, maturation and deletion (Newton et al., 2000; Rathmell JC ., 1996; Bras A et al.1997). It is assumed that the negative selection process of B as well as T cells in the germinal center (GC) and thymus, respectively, depends on Fas system (Siegel RM. Et al., 2000**)**. Moreover several mouse mutations have been identified that cause complex disorders of the immune system, manifested as lymphadenopathy and autoimmunity. One is the recessive *lpr* (lymphoproliferation) mutation. The symptoms of the disease arising from *lpr* are similar to those in systemic lupus erythematosus. The mutations *lprcg* (allelic to *lpr*) and *gld* (generalized lymphoproliferative disease) cause a very similar disease. In all three cases, aberrant T cells accumulate. In *lpr* mice a splicing defect results in the greatly decreased expression of CD95. In *lprcg* mice a point mutation in the intracellular death domain of CD95 abolishes the transmission of the apoptotic signal. In *gld* mice a point mutation in the carboxy terminus of CD95L impairs its ability to interact successfully with its receptor. Thus, a failure of Fas-induced apoptosis accounts for the complex immune disorder in *lpr* and *gld* mutant mice (Nagata, S., 1998). In humans a similar disease with a dysfunction of the CD95–CD95L system (type Ia 'autoimmune lymphopro liferative syndrome' (ALPS) has been reported. Children with ALPS (or Canale Smith syndrome) show massive, non-malignant lymphadenopathy, an altered and enlarged T-cell population and a severe autoimmunity (Fisher, G. H. *et al*. , 1995). In some cases (type II ALPS), defective CD95-mediated apoptosis is observed without mutations in CD95 or CD95L (Wang, J. *et al 1999*). This suggests the existence of defects that affect CD95 signalling , for example mutations of altered expression of some components of this pathway.

Indeed, resistance to apoptosis is believed to be one of the hallmarks of cancer (Hanahan D & Weinberg RA, 2000). Most cancer cells are relatively resistant to apoptosis mediated through Fas, with molecular defects being identified at several levels of the apoptotic signaling pathway (Fig.13) (Houston A. & O'Connell J, 2004). In particular Fas mutations where have been identified in lymphomas, especially those deriving from GC B cells (Muschen M et al. 2002). A common mechanism employed by cells to decrease sensitivity to Fas-mediated apoptosis is to regulate cell surface expression of Fas (Ivanov et al., 2003). Alternatively, cells may secrete an antagonistic 'decoy' receptor (Pitti et al, 1998). The Fas signal can also be inhibited at the level of the DISC via increased expression of cFLIP (FLICE-inhibitory protein), which can inhibit interaction of caspase-8 and -10 with the DISC (Irmler et al. 1997), or reduced expression of FADD (Tourneur et al., 2003) or caspase-8 (Fulda et al., 2001). Thus, because of their insensitivity to Fas-mediated apoptosis, tumor cells can express FasL without undergoing apoptosis (Houston et al., 2003).

However, the control of Fas pathway imparted by the isoforms of the caspase-8-related FLICE-inhibitory protein (FLIP) is of particular interest for cancer progression. There is growing evidence that FLIP can act as a tumor progression factor (Thome et al., 2001; Igney & Krammer, 2002). For example, FLIP expression correlates with resistance against death receptor-induced apoptosis in a variety of B-cell lymphomas, and FLIP-transfected tumor cell lines develop more aggressive tumors in vivo (Thome et al., 2001; Igney & Krammer, 2002).In particular there are a lot of work that demonstrated that Hodgkin/Reed Sternberg (HRS) cells, the malignant cells of classical Hodgkin's lymphoma (cHL, a common human lymphoma), are resistant to Fas-induced apoptosis (Re et al. 2000) because in these cells there is an aberrant upregulation of FLIP proteins (Dutton et al. 2004)

(Mathas et al., 2004). Moreover Fas resistance has been proposed to play an active role in the development of HRS cells (Re et al., 2000).

The particular relevance of FLIP for apoptosis-resistance has been pointed in recent reports showing that decreased expression of FLIP is sufficient to confer sensitivity against death receptor induced apoptosis (Dutton et al. 2004; Mathas et al., 2004). Future studies will show whether selective decreases in FLIP expression accounts for differential sensitization of tumor cells and normal cells for death receptor-induced apoptosis (Dutton A, Young LS, Murray PG , 2006). Interesting administering chemotherapeutic drugs to sensitize cells that are resistant to death receptor–induced apoptosis often correlates with decreased expression of FLIP (Wajant, et al.,2002).

The identification of defective steps in Fas receptor signalling pathways in tumours, might be useful to design therapies able to overcome the resistance of tumours to Fas ligand and thus improve the clinical outcome of patients when used in combination with standard chemotherapy. Understanding the complex regulation of Fas-mediated apoptosis is crucial to this process.

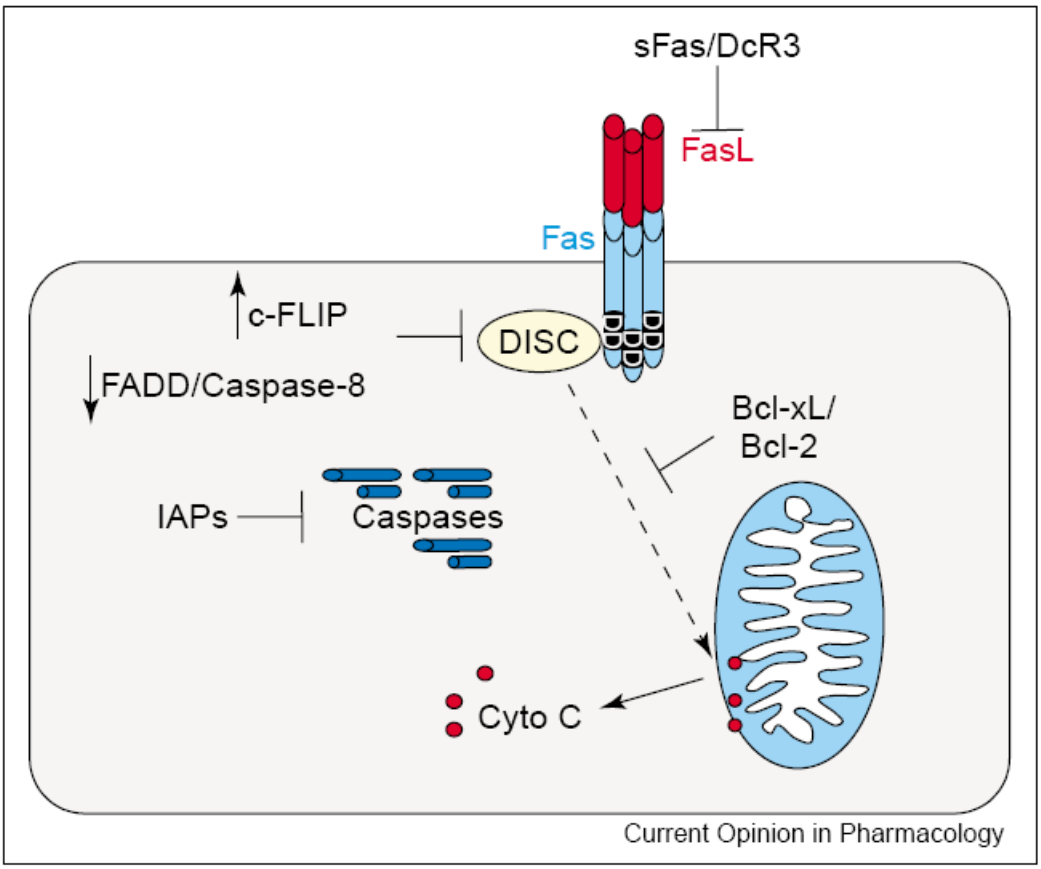

Fig.13. Mechanisms of resistance to Fas-mediated apoptosis (Houston A. & O'Connell J, 2004). Fas-mediated apoptosis can be inhibited at different points in the apoptotic signalling pathway. Cells may secrete soluble 'decoy' receptors, such as sFasL or DcR3, which can bind to FasL and inhibit FasL-induced apoptosis. FADD-like interleukin-1b-converting enzyme inhibitory protein (FLIP) binds to the DISC and prevents the activation of caspase-8; reduced expression of FADD or caspase-8 can also inhibit Fas signalling. IAPs present in the cytosol can bind to and inhibit caspases, whereas upregulation of Bcl-2 or Bcl-xL can render type II cells resistant to Fas-mediated apoptosis. Cyto c, cytochrome c; IAP, inhibitor-of-apoptosis protein.

AIM OF THE PROJECT

Role of ATM in Fas-induced apoptosis

Ataxia Telangiectasia (A-T) is an autosomal recessive disorder characterized by cerebellar progressive neurodegeneration leading to ataxia, dilatation of blood vessels in the eye and facial area (telangiectasia), sensitivity to γ-irradiation, high incidence of tumorigenesis in the lymphoid system and deficiency in immunoresponses. A-T pathology is characterized by the loss of functional ATM protein kinase (Shiloh, 2003). Following DNA damage, ATM is rapidly activated, (auto)phosphorylated (Bakkenist et al., 2003**)** and, in turn, it phosphorylates a number of substrates which all contribute to cell growth arrest or, alternatively, apoptosis (Shiloh, 2003). The higher cancer predisposition of A-T patients has been associated with the lack of DNA damage response, which results in genomic instability (Khanna KK, Jackson SP, 2001). The immune system is the major target of tumor development in these patients, and lymphoma and leukemia are very frequent (Gumy-Pause et al. 2004; Taylor et al., 1996). In particular ATM is an interesting candidate for a tumor suppressor gene. A-T patients show an increased predisposition to develop cancer, in particular, neoplasms of the lymphoid system including both B- and T-cell tumors (Taylor et al.1996). The risk of these patients developing leukemia is approximately 70 times higher than in the normal population (Morrell et al.1986). Moreover mutational inactivation of the ATM gene recently has been demonstrated in T-prolymphocytic leukemia (T-PLL) and a subset of B-cell chronic lymphocytic leukemias (B-CLL) in patients without A-T history, indicating a tumor suppressor function of ATM in both sporadic leukemias (Stilgenbauer et al., 1997; Stoppa-Lyonnet et al., 1998; Stankovic T, et al.1999; Gilad S. et al., 1996). ATM is a key regulator of the cellular response to DNA double-strand breaks induced by irradiation or physiological processes, such as V(D)J recombination (Shiloh, 2003). Indeed most of the lymphoma developed in A-T patients are characterized by aberrant VDJ recombination (Matei et al., 2006). However molecular mechanisms by which ATM inactivation may act as a tumorigenic promoter are not very clear.

The death receptor system, in particular Fas pathway, is essential for the regulation of the lymphoid system homeostasis (Krammer PH, 2000). Resistance to death receptor–mediated apoptosis is supposed to be important for the deregulated growth of B cell lymphoma. Several lines of evidence indicate the importance of this system for the balance between B cell proliferation and apoptosis (Defrance et al. 2002). Indeed, mice lacking functional Fas expression suffer from autoimmunity and increased incidence of B cell lymphomas (Adachi et al. 1995; Davidson et al., 1998). Patients with mutations that impair the function of proteins involved in Fas-dependent apoptosis develop the autoimmune lymphoproliferative syndrome (ALPS), which predisposes them to autoimmune disorders and to lymphoma development (Fleicher et al., 2001; Straus et al., 2001). Finally, Fas mutations where identified in lymphomas, in particular those deriving from GC B cells (Muschen et al., 2002).

However, it is quite astonishing that, despite the fact that ATM and Fas play a central role in the control of the immune system and in lymphoma development, any relationship between these pathways has been reported in literature. Interestingly, there are many type of lymphomas that are resistant to Fas induced apoptosis (Re et al., 2000) and that express aberrantly low level of ATM kinase (Starczynski et al., 2003). Moreover, there is a close linkage between Fas impairment and the development of those tumors that are more frequent in A-T patients.

Thus, taking into account these observations, the aim of this project is to investigate whether any relationship exists between Fas and ATM signalling pathways. In particular, in this work we will

present data supporting the idea that ATM kinase is an essential component to modulate Fas sensitivity through the control of FLIP proteins stability. Furthermore, we test the hypothesis that the ability of ATM kinase to modulate FLIP protein levels plays a central role in the pathogenesis of cancer as Hodgkin's lymphoma.

MATERIAL AND METHODS

DNA constructs

pcDNA3-Flag-ATMWT, pcDNA3-Flag-ATMKD were kindly provided by M. Kastan. shFLIP construct and its control were kindly provided by H. Walczak (Ganten TM et al. 2001**)**.pCR3.V64-Flag-FLIPL was kindly provided by J Tschopp (Irmler et al., 1997).

Antibodies and other reagents

The following antibodies and reagents were used: anti-phosphoSer1981-ATM (Rockland), anti-ATM (MAT3, generously provided by Y.Shiloh), anti-phosphoSer15-p53 (Cell Signaling), anti-p53 (Santa Cruz, Pab240), anti-phosphoThr68-Chk2 (Cell Signaling), anti-Chk2 (kindly provided by D. Delia), anti-pS139 H2A.X (UBI), anti-Fas IgM monoclonal antibody (CH11; UBI), anti-Flag (Sigma), anti-Caspase-8 (clone 5F7, MBL), anti-FLIP(S and L) (H-202 Santa Cruz), anti-active Caspase-3 (Cell Signaling), caspase-inhibitor zVAD (Biomol), NCS (kindly provided by Y.Shiloh), KU-55933 (kindly provided by KUDOS), Cycloheximide (SIGMA) .

Cell culture and transfections

C3ABR, L6 cells **(**kindly provided by M. Lavin and Y. Shiloh) and HL-derived cell line, L428 (kindly provided by Martin Kronke) were cultured in RPMI 1640 medium with 10 mM HEPES, 1.0 mM sodium pyruvate, 10% fetal bovine serum. C3ABR (wild-type) (Lavin et al., 1981) and L6 (derived from A-T patients) (Gilad et al., 1996) cells were Epstein-Barr virus-transformed lymphoblastoid cell lines (EBV-LCL). L428 was derived from plural effusion that was histologically confirmed as HL (Shaadt et al, 1980).

C3ABR and L6 cells were stably transfected by electroporation. C3ABR and L6 cells were diluited with medium to a density of $5X10^5$ cells/ml. After 24 hours $10X10^6$ cells were washed twice with serum-free RPMI1640 medium and electroporeted using 20 μg of the indicated construct. Electroporetion was performed using a Gene-Pulser II (Bio-Rad laboratories) with 950 μF and 0.18 kV; cells were then incubated in culture media. Trasfection-associated cell death was of 30-50% so after 12 hours density gradient separation with Lympholyte-H (Cedarlane cat.no.: CL5010) was used to eliminated cellular debris and dead cells. Trasfection efficiency of C3ABR cells was 5-10% and of L6 cells it was 3-5%. Stably transfected cells were selected in the presence of 500 μg/ml G418**.**

HL-derived cell line, L428 was transiently trasfected using the Nucleofactor unit supplied by AMAXA GmbH . In brief, $4X10^6$ L428 cells were pelleted at 1000 rpm for 10 minutes: following resuspension in 100 μl of freshly prepared Nucleofactor kit L (amaxa cat no:VCA-1005), 1 μg of plasmid DNA pEGFP-C3 and 4 μg of pcDNA3 or pcDNA3-Flag-ATMWT or pcDNA3-Flag-ATMKD were added. Subsequently cells were pulsed using program X-001;cells were then incubated in culture media and after 24 hours were stimulated with anti-Fas antibody. Trasfection efficiency of L428 cells was 50-70% and trasfection-associated cell death was negligible.

Analysis of apoptosis

C3ABR, L6, L6pCDNA, L6-Flag-ATM-wt, L6-Flag-ATM-Kin- and L6-shFLIP cells lines ($5x10^5$ per ml) were treated to undergo apoptosis with 250 ng/ml anti-Fas antibody. Where indicated in western blot and immunofluorescence analysis cells were also treated with NCS (100 ng/ml for 1h) or stimulated in the presence of 40 μM zVAD caspase-inhibitor, which was added 30 min before stimulation with Fas.

Apoptosis was quantificated by propidium iodide (Sigma) nuclear staining or by the analysis of Annexin V (Pharmigen) exposure using a FACScan (Becton Dickinson). Staning with propidium was performed as described by Kalejta et al. (Kalejta et al. 1999).Staining with Annexin V was performed with 1μg AnnV-PE in 100ul AnnexinV binding buffer (10mM Hepes pH 7.5, 150mM NaCl, 5mM KCl, 1mM MgCl2 and 2 mM CaCl2) following incubation for 30' at RT.

Specific apoptosis was determined as follows: (% of apoptotic cells with anti-Fas - % of apoptotic cells without anti -Fas) / (100 - % of apoptotic cells without anti -Fas).

Analysis of Fas-receptor levels

To analyze the expression of Fas protein $1X10^6$ cells per ml were incubated for 30 min RT with mouse anti-human Fas antibody 1:100 (APO1,Transduction Laboratories). Next, cells were reacted with PE-conjugated goat anti-mouse IgG(H+L)1:200 (Pharmigen) for 30 min at RT. Cells were analyzed using a flow-cytometer. For each cell line incubation with PE-conjugated alone served as negative controls. Mean fluorescence intensity of cell stained with anti-Fas used to compare the level of Fas expression.

Flow cytometry of phosho-Ser1981-ATM in apoptotic cells

Our protocol is a variation of a recent method used to evaluate phosphoepitope status by flow cytometry (Perez et al. 2004**)**. 5 x 10^5 cells were fixed in 4% formaldehyde and incubated 15 min at 4 C. They were then permeabilized by resuspending with vigorous vortexing in 1 ml ice-cold MeOH and incubated at -20°C O/N. Cells were washed and resuspended in PBS-Tween 0.5% containing 5% NGS containing anti-phospho-Ser1981-ATM (monoclonal) and anti-active-Caspase-3 (polyclonal) primary antibodies and incubated for 1 h at room temperature. After washing and repeating the process with anti-mouse-AlexaFluor488 and anti-rabbit-AlexaFluor633 conjugated secondary antibodies, flow cytometry was evaluated with FACScan (Beckton Dickinson).

Immunofluorescence analysis

C3ABR, L6-pCDNA, L6-FlagATM-wt and L6-Flag-ATM-Kin- cells line were fixed, permebilized and Immunofluorescence were carried out as previously described (Tritarelli et al., 2004). Flag-ATM protein was visualized with monoclonal anti-Flag (Sigma, 1:500) followed by fluorescein-conjugated anti-mouse antibody (Alexis, 1:200) in blocking buffer. Phospho-S1981ATM was labeled with anti-pS1981 ATM (Rockland, 1:1000) followed by rhodamine-conjugated anti-rabbit diluted (Alexis, 1:600) or by fluorescein-conjugated anti-rabbit antibody diluted 1:200. Nuclei were visualized with Hoechst 33342 (Molecular Probes) diluted 1:20,000 in PBS-0.1% Triton X-100.

Immunoblotting

Cell extracts were prepared in IP buffer (50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 1% NP-40, 5 mM EDTA, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 25 mM NaF, 1 mM orthovanadate, 10 µg/ml TPCK, 5 µg/ml TLCK, 1 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor, 1µg/ml aprotinin)(Barilà D et al. 2003). For immunoblotting, 80-100 µg of protein extract were separated by SDS-PAGE, blotted onto nitrocellulose membrane and detected with specific antibodies. All immunoblots were revealed by ECL (Amersham).

Caspase-8 activity assay

To determine Caspase-8 activity in C3ABR, L6pCDNA, L6-FlagATM-WT and L6-FlagATM-KD cells line, cells were induced to undergo apoptosis with 250 ng/ml of anti-Fas mAb. Protein extracts were assayed for caspase-8 activity using IETD-AMC as a substrate Ac-IETD-AMC at 37 ºC in 200 µl assay buffer (20 mM Tris, pH 7.4, 0.1 M NaCl, 10% sucrose, 0.1% CHAPS, 10 mM DTT) containing 700 µg protein extract. Reaction was started by the addition of 10 µM Ac-IETD-AMC. Cleavage of the substrate as a function of time was monitored reading the absorbance at 460 nm upon excitation at 390 nm. The enzymatic activity was determined from the linear portion of the curve.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNAs were isolated using Trizol reagent (Invitrogen). In all,1 µg of total RNA was subjected to Reverse Transcription in a 20 µl reaction volume using oligo(dT) primer and M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's protocol. RT reaction (3 µl) was then amplified by PCR for 30 cycles using the following conditions: 94°C for 30 s ; 58°C for 1 min and 72°C for 2 min. The primers used were: flip short forward (sense): 50-CGAGGCAAGATAAGCAAGGA-30;flip short reverse (antisense): 50-CACATGGAACAATTTCCAAGAA-30; flip long forward (sense): 50-CTTGGCCAATTTGCCTGTAT-30; flip long reverse (antisense): 50-GGCAGAAACTCTGCTGTTCC-30 (Salon et al., 2006). Amplification of of actin was performed in the same PCR reaction as internal control. PCR products were run on a 2% agarose gel and visualized by ethidium bromide staining.

Statistical methods

All data were analyzed and presented as mean ± SD ($n<10$). The significance of differences between populations of data were assessed according to the Student's two tailed T-test with a level of significance of at least $p < 0.05$ (alpha conventionally equal to 0.05). This analysis arises in the problem of estimating the mean of a normally distributed population when the sample size is small.

RESULTS

ATM deficient cells are resistant to Fas-induced apoptosis

To investigate whether ATM could participate in Fas-mediated apoptosis, we compared the sensitivity to Fas induced apoptosis of two lymphoblastoid cell lines widely used in studies on ATM activity, one established from an AT patient (L6) (Gilad et al., 1996), the other one from a healthy control donor (C3ABR) (Lavin et al., 1981). Fas was stimulated with agonistic anti-Fas antibodies that mimic the binding of Fas-Ligand and triggers the apoptotic response. Cells undergoing apoptosis display typical changes in their morphological and physical properties (cell shrinkage, condensation of chromatin and cytoplasm) which are well measurable by flow cytometry. Toevaluate the apoptotic response we used mainly two methods. In the first method we stained cellular DNA with propidium iodide. This method is based on the reduced DNA stainability following staining with propidium of apoptotic cells due partial loss of DNA after activation of endogenous nucleases and diffusion of low-molecular weight DNA outside apoptotic cells. Therefore, the presence of cells with DNA stainability lower than that of G_1-cells (hypodiploid or sub-G_1 peaks) has been considered a marker of cell death by apoptosis. In the second method we stained the cells with Annexin V. This assay takes advantage of the fact that phosphatidylserine (PS) is translocated from the inner (cytoplasmic) side of the plasma membrane to the outer (cell surface) side soon after the induction of apoptosis, and that the annexin V protein has a strong, specific affinity for PS. Phosphatidylserine on the outer side is available to bind labeled annexin V and provides a simple and effective method to detect apoptosis at a very early stage.

Interestingly L6 cells, which lack the expression of ATM protein, were significantly resistant to Fas-induced apoptosis measured with propidium ioide staining and with AnnexinV staining (Fig. 1A, B). Similar results were obtained also with other A-T lymphoblastoid cell lines, such as GM-03189 and GM-02782, (Fig.1C). Therefore we can conclude that A-T cells are resistant to Fas-induced apoptosis.

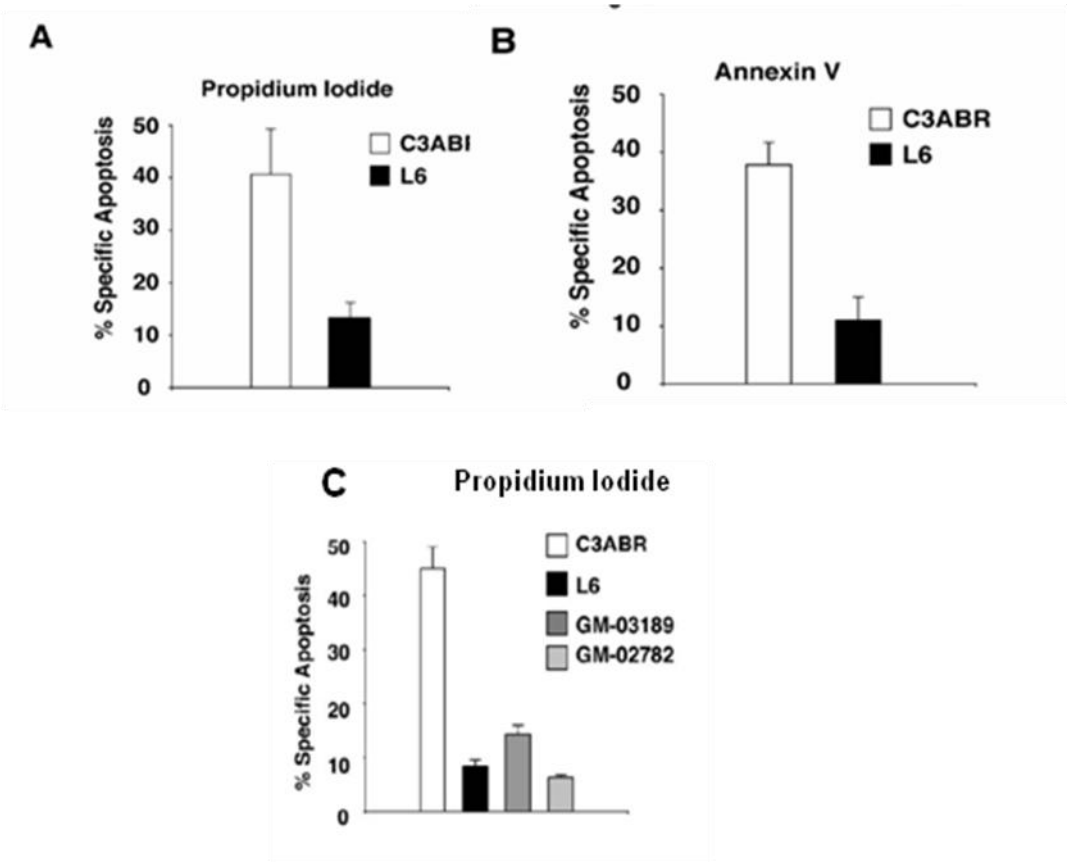

Fig.1. ATM deficient cells are resistant to Fas-induced apoptosis. ATM proficient cells (C3ABR) and ATM deficient cells (L6, A-T-44, A-T-3189 and A-T-2782) were treated with 250 ng/ml of anti-Fas mAb. Apoptosis was determined by the analysis of DNA fragmentation in propidium iodide stained cells (P.I) **(A) (C)** or by the analysis of Annexin V binding **(B)**, 24 hrs after anti-Fas treatment.

ATM kinase activity enhances Fas-induced apoptosis

To be able to work in the same cellular contest and to address the question of whether ATM kinase activity is required for Fas sensitivity, we stably reconstituted ATM expression in L6 cells. For this purpose L6 cells were stably transfected with constructs that allow the expression of either FLAG-ATM-wt protein (L6-ATM-wt) or the kinase inactive FLAG-ATM-Kin- protein (L6-ATM-Kin-), or with the empty vector as control (L6-pCDNA). ATM expression was monitored by immunoblotting with specific antibodies (Fig. 2A). L6-ATM-wt and L6-ATM-Kin- cells expressed same levels of ATM protein. Interestingly, the reconstitution of the expression of ATM in the L6-ATM-wt cells dramatically sensitized these cells to Fas-induced apoptosis (Fig.2B,C). The expression of the ATM-kinase-defective mutant, FLAG-ATM-Kin-, completely failed to restore Fas-sensitivity. Overall these results, suggest that ATM kinase activity enhances Fas-induced apoptosis.

These experiments have been made in collaboration with Dr. Maria Giovanna Di Bari, a phD student at the department of Experimental Medicine headed by Prof. Testi at the Faculty of Medicine, Tor Vergata University and with Dr. Ivano Condo', a post-doc at the laboratory of Signal Transduction and Immunology headed by Prof. Testi at the Faculty of Medicine, Tor Vergata University.

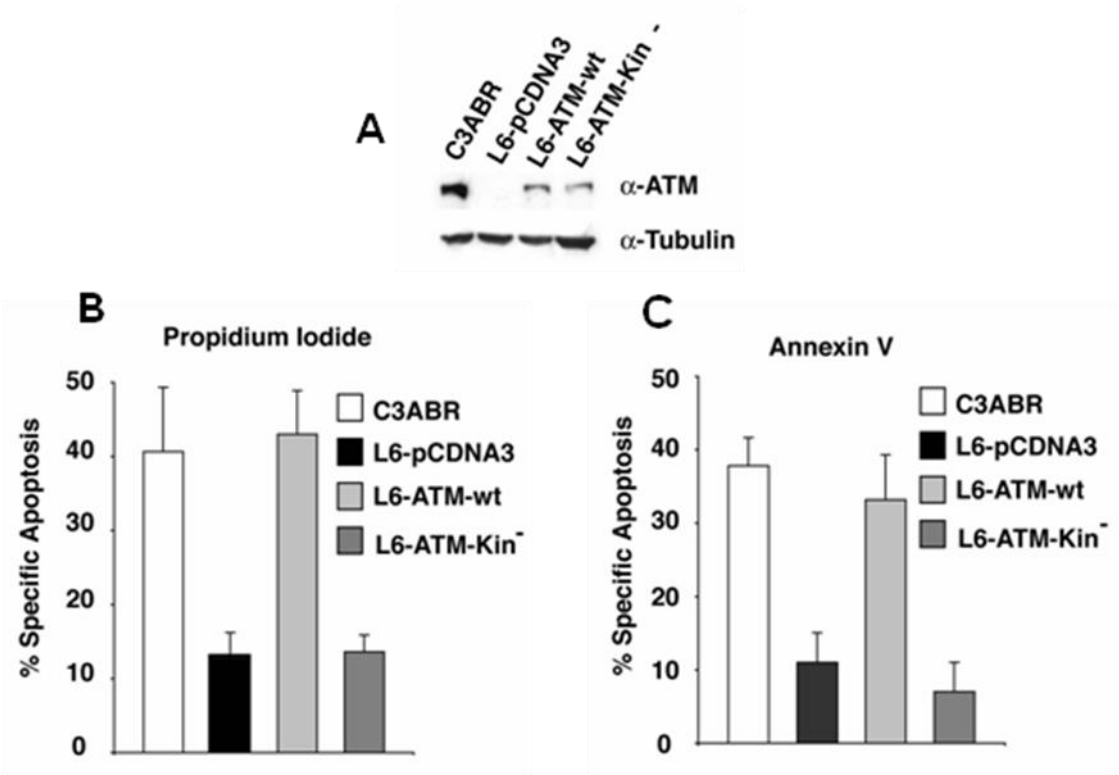

Fig.2. ATM kinase activity enhances Fas-induced apoptosis. (A) ATM deficient cells (L6) were stably transfected with ATM-wt, ATM-Kin- or with empty vector as control using 20 μg of the indicated constructs. For immunoblotting, 80-100 μg of protein extract were separated by SDS-PAGE, and transferred on nitrocellulose. ATM protein was revealed with anti-ATM (MAT3) antibodies. **(B,C)** Cells were treated to undergo apoptosis with 250 ng/ml of anti-Fas mAb. Apoptosis was determined by the analysis of DNA fragmentation upon propidium iodide nuclear staining (P.I) **(B)** or by the analysis of Annexin V exposure **(C)** , at 24 hrs after anti-Fas treatment.

ATM kinase activation upon Fas stimulation does not play a major role in Fas-sensitivity

We therefore asked the question of whether Fas-stimulation triggers ATM kinase activation and if this may contribute to Fas-sensitivity. To evaluate the effect of Fas-stimulation on ATM kinase activity, we analyzed protein extracts at different times of stimulation. Fas stimulation results in ATM phosphorylation on Ser1981, a marker of ATM activation (Bakkenist and Kastan, 2003), and p53, Chk2 and H2AX became phosphorylated respectively at Ser15, Thr68 and Ser139, as described in the DNA damage response (Ahn et al., 2000; Khanna et al., 1998; Burma et al. 2001) (Fig. 3). These data suggest that Fas stimulation results in ATM activation. Importantly, ATM activation is completely prevented by preincubation with the general caspase-inhibitor z-VAD (Fig. 3), thus suggesting that Fas-induced ATM activation occurs downstream caspase activity.

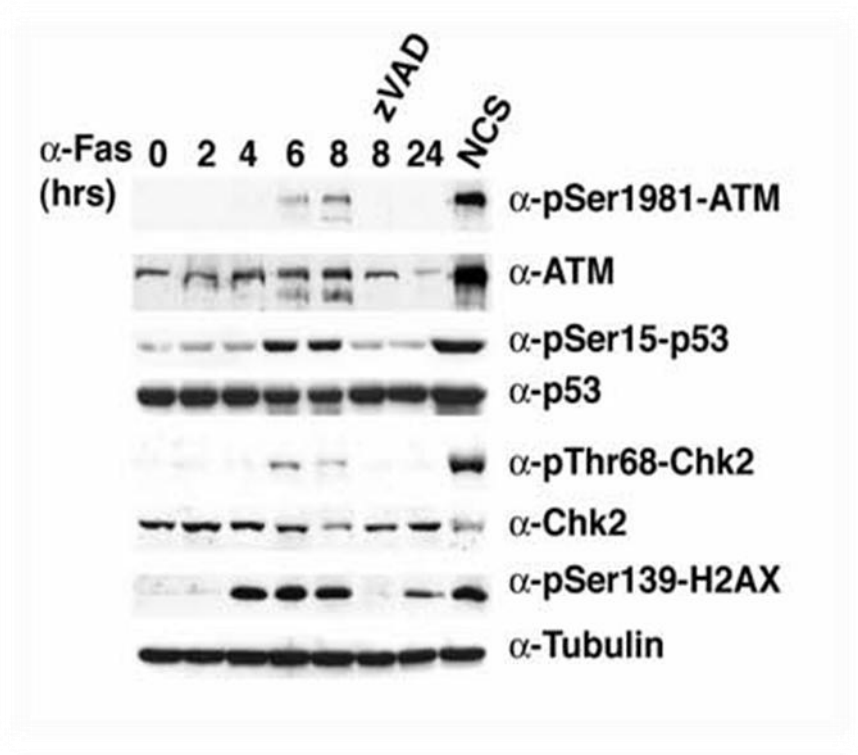

Fig.3. ATM kinase activation following Fas-induced apoptosis is a late passive event. C3ABR cells were induced to apoptosis with 250 ng/ml anti-Fas IgM monoclonal antibody. Untreated and NCS-treated cells that triggers DSB and classically induces ATM activation (Uziel et al. 2003), were used as controls. For immunoblotting, 80-100 µg of protein extract were separated by SDS-PAGE, and transferred on nitrocellulose. The proteins of interest and their phosphosphorylation were revealed by immunoblotting with specific antibodies.

To evaluate this possibility, we established a new flow cytometry–based assay that allow us to analyze the levels of phospho-Ser1981-ATM and of activated Caspase-3, which labels apoptotic cells (Fig. 4A). For this aim, after Fas stimulation, we visualized the activation of ATM kinase with anti-phospho-Ser1981-ATM conjugated with AlexaFluor488 and the activation of caspase-3 with anti-active-caspase3 conjugated with a AlexaFluor633. ATM phosphorylation on Ser1981 occurs mainly on those cells where caspase-3 has been cleaved (activated) as shown by Flow cytometry in Fig. 4A. This approach allowed us to conclude that ATM activation mainly occurs in cells that activate caspase-3, supporting the hypothesis that Fas-dependent ATM activation is downstream caspase-3 activation, and therefore most likely does not play a major role in Fas sensitivity (Fig. 4A). Fas stimulation also resulted in the cleavage of ATM protein (Fig. 3), according to the effect of other apoptotic stimuli, which trigger ATM kinase cleavage most likely through Caspase-3 activity (Smith et al., 1999). Moreover we could show by immunofluorescence that Fas stimulation triggered ATM phosphorylation on Ser1981 only on those cells that show apoptotic morphology characterized by nuclei condensation or fragmentation (Fig. 4B). We failed to detect any active ATM in our cells with intact nuclei. Overall, these findings strongly suggest that ATM activation upon Fas stimulation is a passive event subsequent to DNA fragmentation, and therefore most likely does not contribute significantly to cell fate. These experiments have been made in collaboration with Dr. Silvia Cursi, a post-doc in our laboratory.

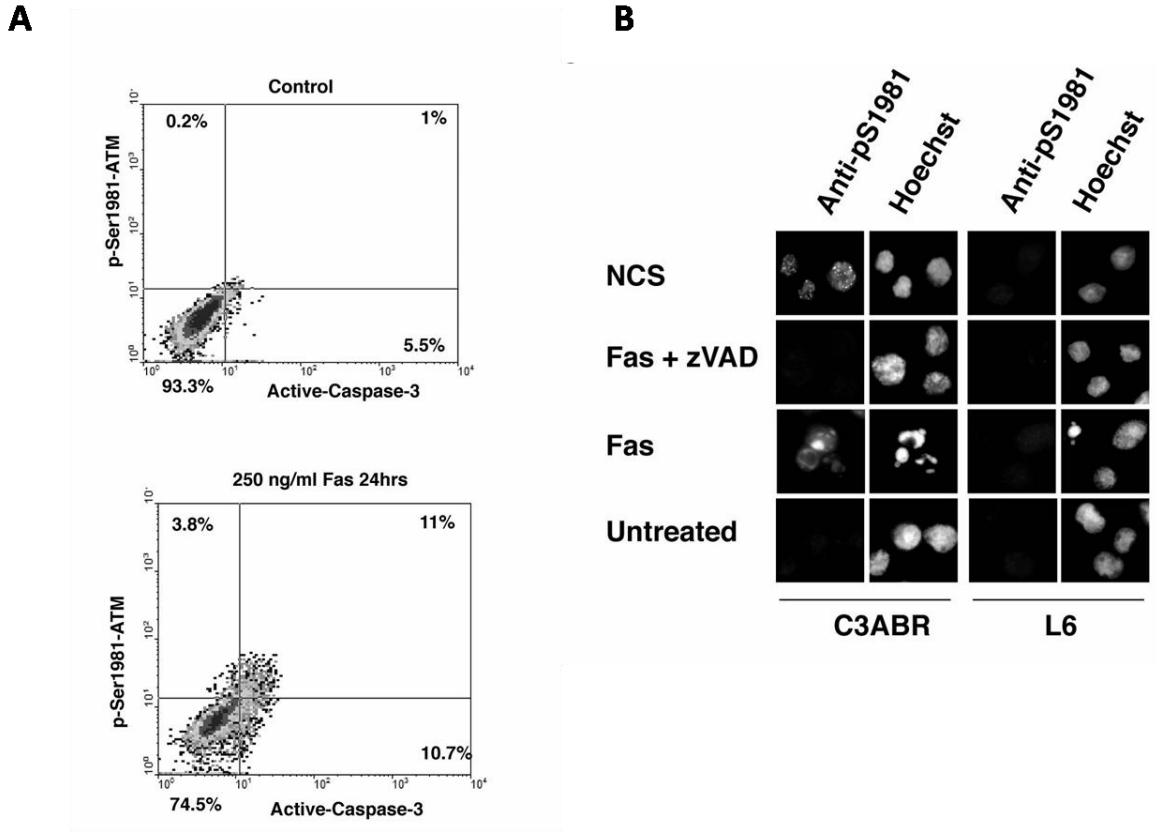

Fig.4. ATM kinase activation following Fas-induced apoptosis is a late passive event. (A) C3ABR cells were treated to undergo apoptosis with 250 ng/ml anti Fas IgM monoclonal antibody (CH11; UBI). Untreated and treated cells were analyzed with multiparameter flow cytometry for active caspase-3 and phospho-Ser1981-ATM. **(B)** C3ABR cells were treated to undergo apoptosis as in B. Untreated and NCS-treated cells were used as controls. Cells were fixed, permeabilized and immunofluorescences were carried out as previously described (Tritarelli et al., 2004). Nuclear condensation and fragmentation has been evaluated by Hoechst staining.

ATM kinase activity promotes Caspase-8 activation

To get more insight in the molecular mechanism by which ATM modulates Fas-induced apoptosis, we analyzed the expression profile of those proteins that are relevant for this signalling. FACS analysis showed that ATM expression and activity do not modulate the levels of Fas (Fig. 5A). Immunoblotting analysis showed that all cell lines express comparable levels of Caspase-8, independently on ATM activity (Fig. 5B). Remarkably, despite the observation that Caspase-8 is equally expressed in all cell lines, its activation following Fas crosslinking is significantly delayed in the ATM deficient cells (L6-pCDNA) as well as in the ATM kinase activity deficient cells (L6-ATM-Kin-) (Fig. 5C, 5D). Full activation of Caspase-8 upon Fas-stimulation requires its processing, essential to get a stable active caspase-8 tetramer and to allow its release from the DISC and subsequent cleavage of cytoplasmic substrates, such as executioner caspases (Wallach et al., 1999). Immunoblotting experiments, using an anti-Caspase-8 antibody raised against the p18 subunit, showed that the lack of ATM results in the delayed accumulation of the intermediate processing product p43 and of the p18 subunit (Fig. 5C). Moreover, ATM deficiency delayed Fas-induced Caspase-8 activation, measured as its ability to cleave its substrate peptide IETD (Fig. 5D). These experiments have been made in collaboration with Dr. Silvia Cursi, a post-doc in our laboratory.

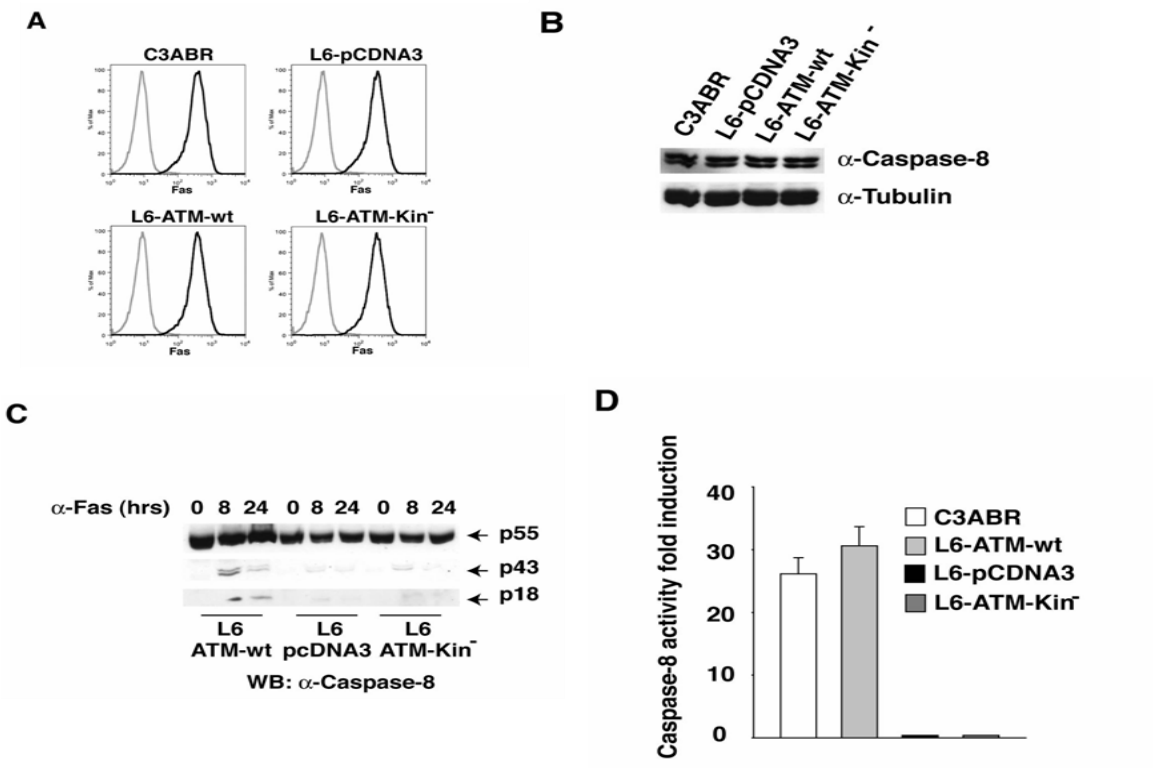

Fig.5. ATM kinase activity promotes Caspase-8 activation. (**A)** Fas receptor levels were detected by flow-cytometry analysis. Cells were incubated with anti-Fas antibodies followed by PE-conjugated secondary antibodies (dark lines). For each cell line an incubation with PE-conjugated alone served as negative controls (light lines). (**B)** Caspase-8 expression was revealed by immunoblotting on extracts obtained by the indicated cell lines. 80-100 µg of protein extract were separated by SDS-PAGE, transferred on nitrocellulose and Caspase-8 expression revealed using specific antibodies. (**C)** Protein extracts from the indicated cell lines stimulated to undergo apoptosis with anti-Fas antibodies, have been separated by SDS-PAGE and Caspase-8 revealed by immunoblotting with specific antibodies. The arrows point to the entire protein, p55, as well as to the processing products p43 and p18. (**D)** Caspase-8 activity from the same extracts was measured by the hydrolysis of the Caspase-8 substrate Ac-IETD-AMC.

ATM kinase activity downregulates c-FLIP protein levels

c-FLIP is a well characterized inhibitor of Fas signaling, so we wanted to investigate the possible relationships between ATM activity and c-FLIP expression. It is important to remind that the lack of ATM expression triggers the upregulation of c-FLIP (Fig. 6A,B), which may account for Fas resistance in A-T cells (Fig. 1). Reconstitution of ATM kinase activity in L6-ATM-wt cells significantly decreased FLIP-L and FLIP-S expression levels (Fig.6A), which may account for the recovery of Fas sensitivity (Fig. 2). Again, the ATM-Kin- mutant completely failed to downregulate FLIP (Fig. 6A).

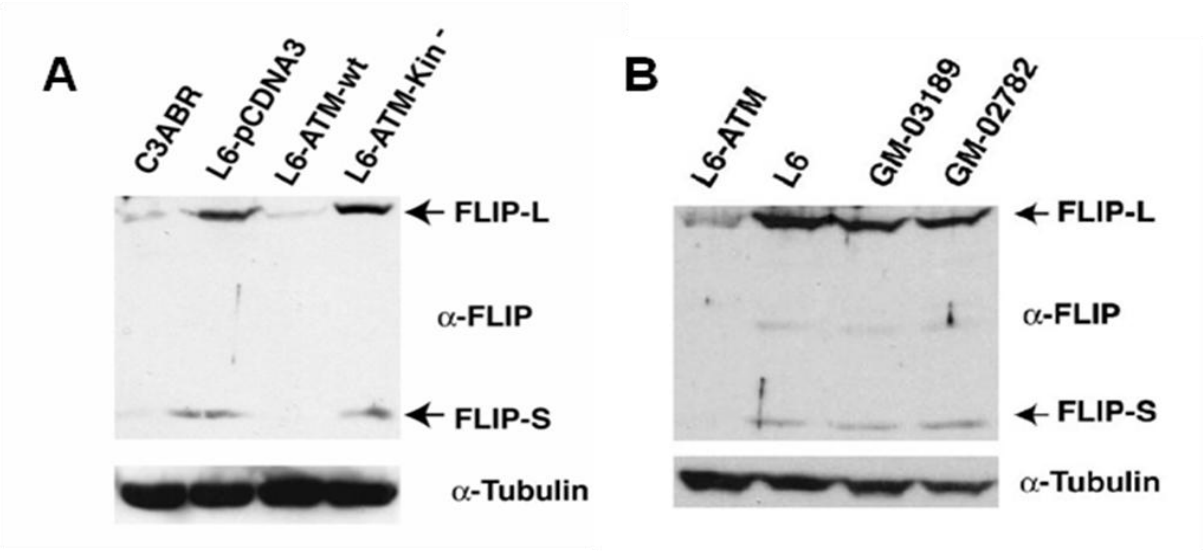

Fig.6. Basal ATM kinase activity regulates FLIP protein levels. (A) (B) FLIP expression was revealed by immunoblotting on extracts obtained from the indicated cell lines. 80-100 µg of protein extract were separated by SDS-PAGE, transferred on nitrocellulose and FLIP expression revealed using specific antibodies. The arrows point to FLIP-L and FLIP-S isoforms.

To test whether ATM activity modulates Fas sensitivity via the regulation of FLIP levels, we generated a stable A-T cell line, L6-shFLIP, where FLIP expression has been genetically reduced through specific shRNA that selectively targets FLIP-L and FLIP-S isoforms. These cells express FLIP proteins at a level comparable to the endogenous level of ATM kinase reconstituted cells (Fig. 7A). By the analysis of DNA fragmentation upon propidium iodide nuclear staining we could show that the reduction of FLIP sensitizes A-T cells to Fas-induced apoptosis (Fig.7B), indicating that the aberrant levels of FLIP proteins may be responsible for Fas resistance in A-T cells. Overall these experiments show that ATM kinase sensitizes cells to Fas-induced apoptosis through the modulation of FLIP levels and suggest that ATM kinase activity may downregulate FLIP.

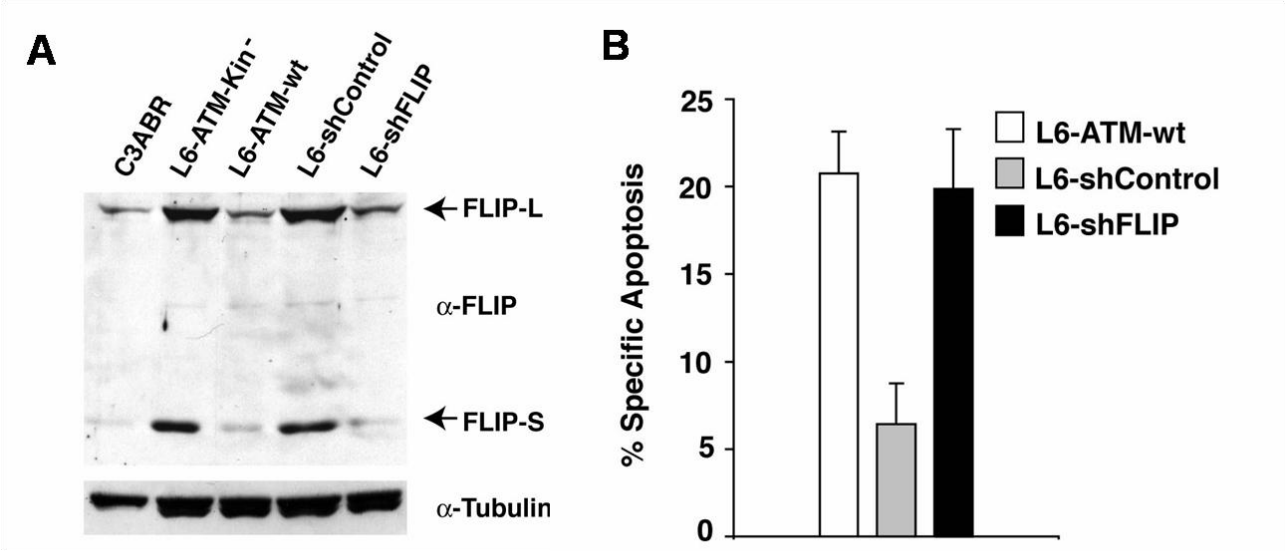

Fig.7. Downregulation of FLIP sensitizes A-T cells to Fas-induced apoptosis. (A) ATM deficient L6 cells were stably transfected with shFLIP or with a scrambled oligo as control. Protein extracts from the indicated cell lines were probed for FLIP expression by immunoblotting as described in Fig.6. The arrows point to endogenous FLIP-L and FLIP-S. (**B)** The indicated cell lines were stimulated to undergo apoptosis with 250ng/ml of anti-Fas mAb. Apoptosis was determined by the analysis of DNA fragmentation upon propidium iodide nuclear staining (P.I) 24 hrs after anti-Fas treatment.

To test the hypothesis that ATM kinase activity downregulates FLIP, we treated the cells with NCS, a radiomimetic drug (kindly provided by Dr. Yossi Shiloh, Tel Aviv University, Israel), that classically triggers ATM kinase activation (Uziel et al. 2003). ATM kinase activation results in a reduction of the levels of FLIP protein (Fig. 8A). This effect is completely abrogated in cells that lack ATM protein or reconstituted with the ATM kinase defective mutant (Fig. 8A). Furthermore, according to downregulation of FLIP levels, NCS treatment significantly sensitized cells to Fas-induced apoptosis (Fig. 8B).

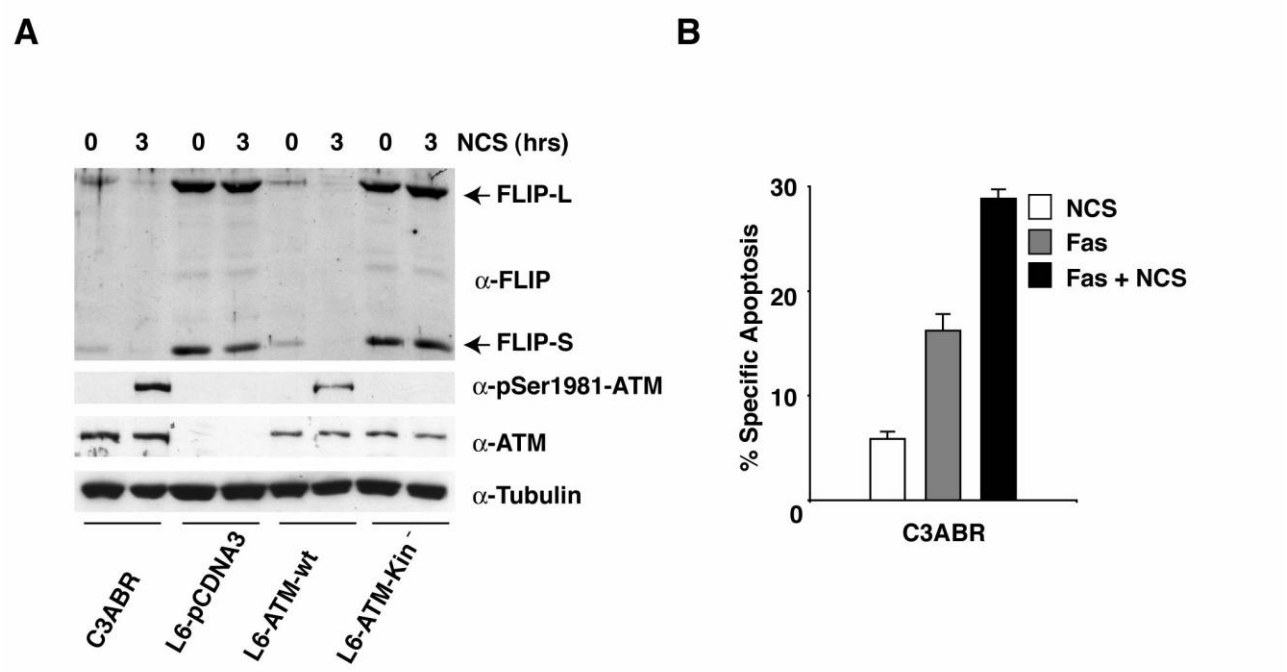

Fig.8. Modulation of ATM kinase activity results in the regulation of FLIP protein levels which in turn determines Fas-sensitivity. (A) Different cell lines were treated with NCS for 3 hrs to trigger ATM kinase activation. 80-100 μg of protein extract were separated by SDS-PAGE, transferred on nitrocellulose and FLIP expression revealed using specific antibodies. (B) C3ABR cells were stimulated to undergo apoptosis with 250ng/ml of anti-Fas mAb in the presence or in the absence of NCS pretreatment for 3 hrs. Apoptosis was determined by the analysis of DNA fragmentation in propidium iodide stained cells (P.I) 8 hrs after anti-Fas treatment.

The observation that FLIP is aberrantly upregulated in A-T cells as well as in A-T cells reconstituted with inactive ATM (Fig. 6A) suggests that the endogenous basal activity of ATM is sufficient to downregulate FLIP protein levels. To unambiguously address this issue, ATM proficient cells were incubated in the presence of the ATM kinase specific inhibitor KU-55933 (Hickson et al., 2004). Indeed, this treatment triggered FLIP upregulation (Fig. 9A). Interestingly, preincubation with KU-55933 for 1 hour is not sufficient to increase FLIP protein levels (Fig. 9A) and consistently fails to protect cells from Fas-induced apoptosis (Fig. 9B). Conversely, preincubation with KU-55933 for 8 hours, which is sufficient to trigger FLIP protein accumulation, dramatically impairs Fas-induced apoptosis to the same extent of A-T cells (Fig. 9B). These data clearly show that ATM kinase activity is required to modulate Fas sensitivity through the control of FLIP protein levels.

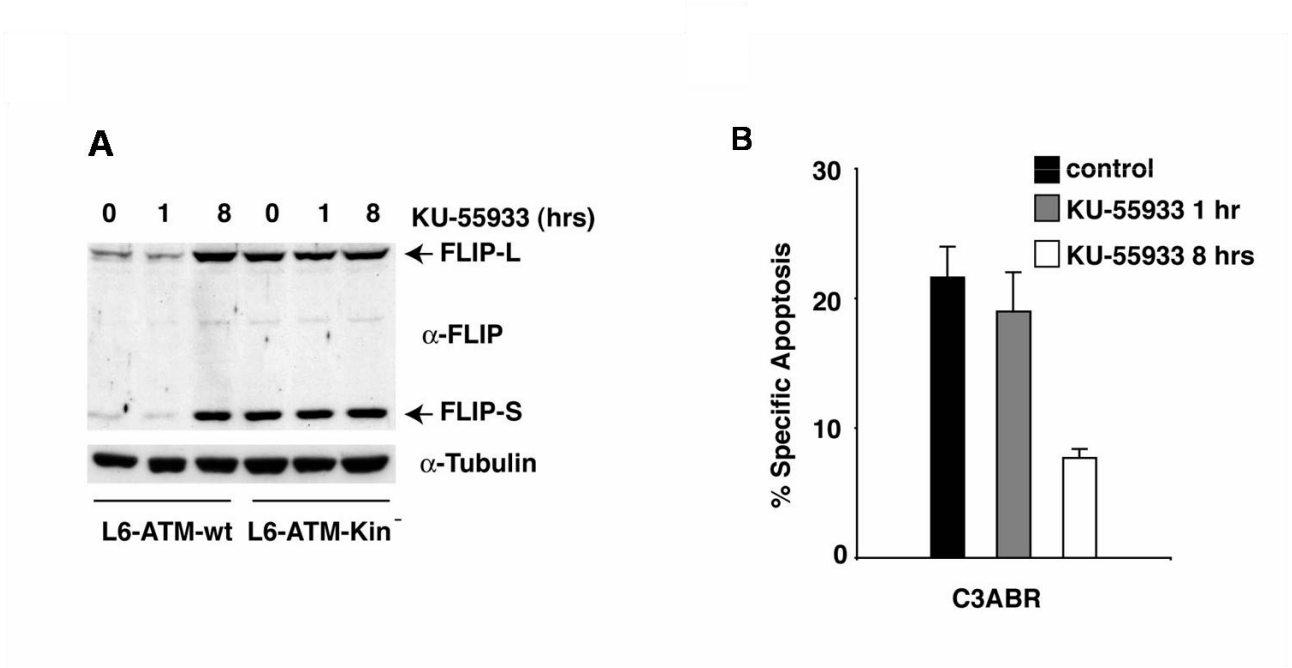

Fig.9. Basal ATM kinase activity regulates FLIP protein levels which in turn determines Fas-sensitivity. (A) The indicated cell lines were incubated in the presence of the specific ATM kinase inhibitor KU-55933 (10 μM) for 1 or 8 hrs. 80-100 μg of protein extract were separated by SDS-PAGE, transferred on nitrocellulose and FLIP expression revealed using specific antibodies. **(B)** C3ABR cells were preincubated for 1 or 8 hrs with the specific ATM kinase inhibitor KU-55933 (10 μM), to allow endogenous ATM kinase inactivation and FLIP levels upregulation and then stimulated to undergo apoptosis with 250ng/ml of anti-Fas mAb. Apoptosis was determined by the analysis of DNA fragmentation in propidium iodide stained cells (P.I) 24 hrs after anti-Fas treatment.

ATM kinase activity does not modulate FLIP mRNA levels

Next we asked the question of how does ATM modulates FLIP levels. To evaluate whether ATM modulates the mRNA levels of FLIP, we analyzed FLIP mRNA levels in A-T proficient and A-T deficient cell lines. RT-PCR experiments allowed us to conclude that the levels of FLIP transcript are comparable in all cell lines independently on ATM expression and activity (Fig. 10), supporting the idea that FLIP levels could be regulated at a non transcriptional level.

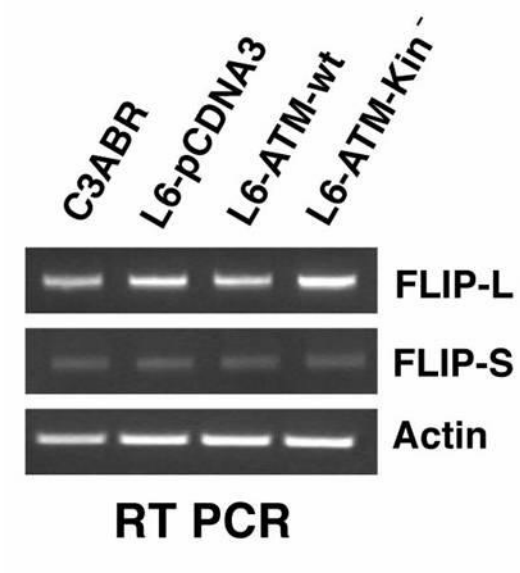

Fig.10. ATM kinase activity does not modulate FLIP mRNA levels. RT-PCR analysis of FLIP-L and FLIP-S RNA expression levels was performed in the indicated cell lines. Amplified actin was used as an internal control.

ATM kinase activity modulates FLIP protein stability

We therefore tested the hypothesis that ATM kinase activity modulates FLIP protein stability. In agreement with this assumption, an exogenous FLAG-tagged FLIP-L, driven by a heterologous promoter, was repressed similarly to the endogenous FLIP-L when stably transfected C3ABR-FLAG-FLIP-L cells where stimulated with NCS to trigger ATM kinase activity (Fig. 11A). So we investigated whether ATM kinase activity accelerates FLIP proteins degradation. The classical approach to evaluate protein stability is the treatment of cells with cycloheximide (CHX), a drug that blocks novel protein synthesis. The amount of the protein of interest may be evaluated by immunoblotting with specific antibodies at different times after CHX incubation. Indeed, we blocked nascent translation of FLIP with CHX and we compared FLIP protein stability in L6-ATM-WT cells and in L6-ATM-Kin- cells. Cells where pretreated with the ATM kinase inhibitor KU-55933 for 8 hrs to obtain the same initial levels of FLIP proteins and after KU-55933 removal, cells were incubated for different times with CHX. This approach allowed us to conclude that FLIP-protein degradation is significantly increased dependently on ATM kinase activation and that ATM kinase downregulates FLIP protein stability (Fig. 11B).

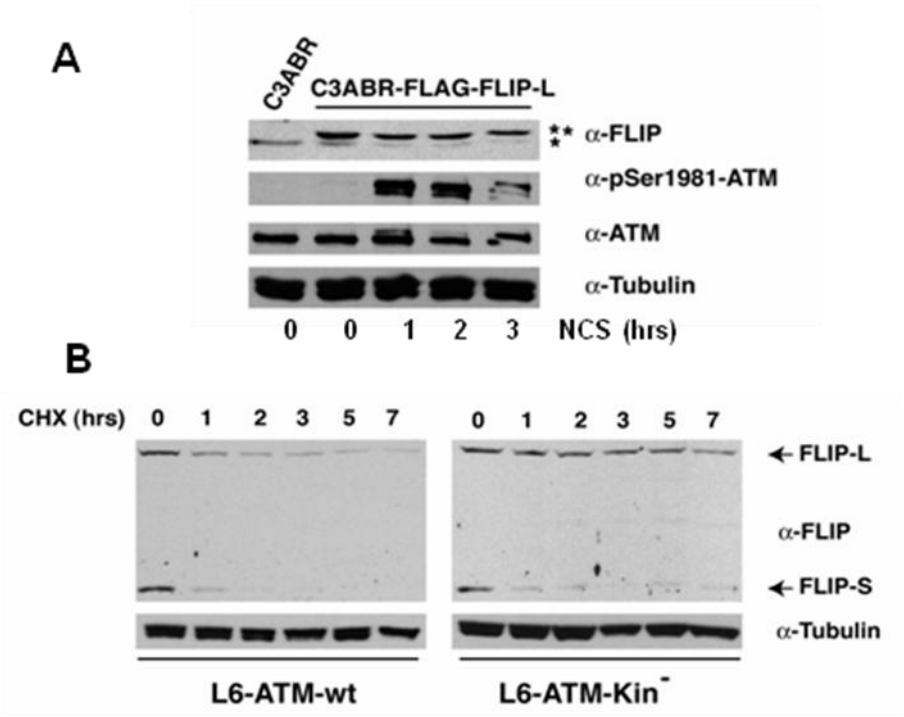

Fig.11. ATM kinase activity modulates FLIP protein stability. (A) C3ABR-FLAG-FLIP-L stably transfected cells were incubated with NCS for different times to trigger ATM kinase activation. 80-100 μg of protein extract were separated by SDS-PAGE, transferred on nitrocellulose and endogenous (*) and transfected FLIP (**) expression revealed using specific anti-FLIP antibodies. **(B)** Cells were pretreated with KU-55933 O/N, washed, and then incubated with CHX for the indicated times.

ATM kinase activity sensitizes Hodgkin Lymphoma cells to Fas-induced apoptosis

Resistance to death-receptor-mediated apoptosis is supposed to be important for the deregulated growth of B cell lymphoma. Hodgkin/Reed Sternberg (HRS) cells, the malignant cells of classical Hodgkin's lymphoma (cHL), resist to Fas induced apoptosis. Fas resistance in this system is due to the aberrant upregulation of FLIP proteins (Dutton et al., 2004; Mathas et al., 2004). Conversely, ATM expression and function is impaired in many HL cases (Starczynski et al., 2003) and in several HL-derived cell lines (Takagi et al., 2004)(Dutton et al., 2006). To test whether ATM loss of function may contribute to Fas resistance through FLIP protein upregulation in a pathological situation, we took advantage of a Hodgkin's lymphoma cell line, L428, that has been previously characterized for the aberrant downregulation of ATM activity (Dutton et al., 2006) and for the aberrant upregulation of FLIP protein levels (Dutton et al., 2004; Mathas et al., 2004). Transient transfection of a functional ATM kinase downregulates FLIP levels (Fig. 12A) and restores Fas sensitivity (Fig. 12B), suggesting that targeting of ATM kinase activity significantly contributes to death receptor resistance of HL cell lines and most likely plays a functional role in this pathology.

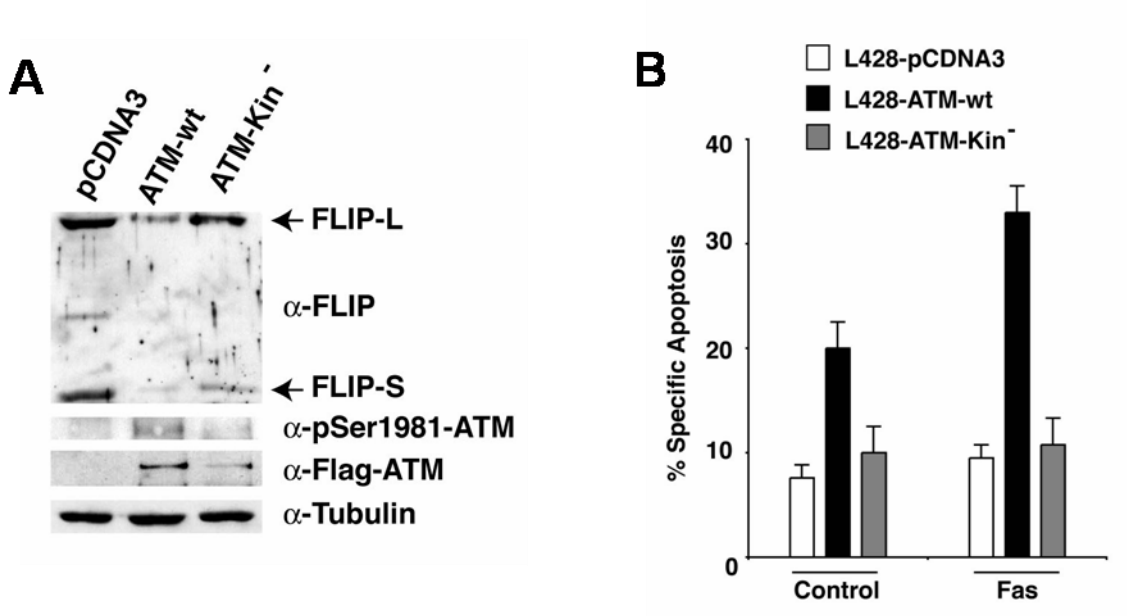

Fig.12. ATM kinase activity downregulates FLIP protein levels and sensitizes Hodgkin Lymphoma cell lines to Fas induced apoptosis. (A) L428 HL cells were transiently transfected with the indicated constructs along with GFP. 24 hrs after transfection GFP positive cells were isolated by FACS sorting, and incubated for additional 12 hrs. For immunoblotting, 80-100 μg of protein extract were separated by SDS-PAGE, and transferred on nitrocellulose. The proteins of interest and their phosphosphorylation were revealed by immunoblotting with specific antibodies. **(B)** L428 HL cells were transiently transfected with the indicated constructs along with GFP. 24 hrs after transfection cells were stimulated to undergo apoptosis with 500ng/ml of anti-Fas mAb. Apoptosis was determined by the analysis of Annexin V binding 24 hrs after anti-Fas treatment, upon FACS sorting of GFP positive cells.

DISCUSSION

The ataxia telangiectasia mutated (ATM) kinase is a key tumor suppressor. A deficiency of ATM results in early onset of lymphomas in mice (Barlow et al., 1996), whereas germline mutations in the ATM gene cause ataxia-telangiectasia (A-T), a multisystem disorder associated with a predisposition to lymphoma and acute leukemia (Shiloh and Kastan, 2001). Moreover, ATM gene alterations, mainly missense mutations, have been reported frequently in adult lymphoid malignancies (Gumy-Pause et al., 2004). ATM kinase plays a central role in the DSB DNA damage response and this response is required in some physiological context such as the immune system homeostasis. The lack of ATM activity has been proposed to be responsible for aberrant chromosomal translocations, originated as a consequence of the failure of the DNA damage response and indeed associated to several lymphomas and leukemias (Matei et al., 2006).

Fas-dependent apoptosis plays a fundamental role in the regulation of the homeostasis of the lymphoid system (Krammer PH., 2000). Failure in the Fas signaling causes, both in mice and in humans, autoimmunity as well as aberrant proliferation and lymphoma development (Adachi et al., 1995; Davidson et al., 1998; Fleisher et al., 2001).

We reasoned that since defects in Fas-induced apoptosis, result in defects in the immune system that partially resemble some of the abnormalities characteristic of the immune system of A-T patients, ATM kinase may play a role in Fas-induced apoptosis. According to our hypothesis, the present work shows that cells that lack ATM kinase are significantly resistant to Fas induced apoptosis (Fig.1). Reconstitution experiments showed that ATM catalytic activity is required to sensitize cells to Fas (Fig.2). We show that Fas stimulation triggers ATM kinase activation. However, our data strongly suggest that ATM activation upon Fas stimulation occurs when the apoptotic signaling is already irreversible, as a consequence of DNA condensation and fragmentation during the apoptotic response. Therefore, ATM activation does not seem to play a major role in the sensitization to Fas-induced apoptosis. This apparent paradox prompted us to investigate whether basal ATM kinase might modulate the level and/or the activity of any central player of Fas signaling. Fas sensitivity mainly relies on Fas-receptor expression on cell surface and on Caspase-8 activity, which is absolutely required to drive the caspase cascade and execute the apoptotic programme. Importantly, ATM protein does not modulate Fas-receptor or Caspase-8 protein levels (Fig. 5A,B). However, A-T cells are impaired in Caspase-8 activation consistently with their resistance to Fas (Fig.5C,D).

It has been clearly established that FLIP proteins may modulate Caspase-8 activation *in vitro* and *in vivo* (Peter ME, 2004). FLIP levels are tightly regulated during T and B cell activation and its decrease parallels the enhancement of Fas sensitivity (Thome M and Tschopp J, 2001). We show that A-T cells significantly accumulate FLIP proteins (Fig. 6). Reconstitution of ATM kinase activity, downregulates FLIP proteins. Conversely, a catalitically inactive ATM fails to downregulate FLIP. Importantly, there is a strict relationship between FLIP levels and the sensitivity of the different cell lines to Fas-induced apoptosis. To further test the hypothesis that ATM kinase sensitizes cells to Fas-induced apoptosis through the downregulation of FLIP proteins, we knocked out FLIP expression in A-T cells, by specific shRNA constructs. Following this approach it was possible to downregulate FLIP to the same levels observed in ATM proficient cells which, in its turn resulted in the restoration of Fas sensitivity in A-T cells (Fig. 7). Therefore we concluded that ATM kinase activity modulates Fas-sensitivity through the regulation of FLIP protein levels. The observation that FLIP levels decrease in A-T cells upon reconstitution with kinase active ATM but not with a kinase defective mutant (Fig. 6A) suggests that a basal ATM kinase activity may be sufficient to downregulate FLIP levels. The presence of an endogenous basal ATM activity, which may be further induced upon DNA damage, is currently a central topic in the ATM field (Banin et al., 1998)(Kozlov et al., 2003). DNA double strand break is an event that occurs constantly and the cells are continuously subjected to basal stress, cells in culture even more. The observation that cells costitutively express activated ATM and the degree of

constitutive ATM activation differs depending on cell type and cell phase has been reported by several groups (Di Tullio et al. 2002; Bartkova et al. 2005; Huang et al. 2003; Tanaka et al. 2006). This observation further supports the idea that immortalized cell lines have a basal constitutive activity of ATM. Therefore we provide novel evidence for a basal endogenous activity of ATM kinase independent of the exogenous DNA damage induction, which probably accounts for differences in the level of expression of FLIP protein. This basal activity of ATM could be relevant also in other cellular processes and contribute, at least in part, to the complexity of A-T phenotype. Investigations comparing different structural and functional features of wt and A-T cells in the absence of DNA damage, may strongly contribute to broaden current knowledge on ATM kinase function and A-T pathology.

The existence of such a basal activity is confirmed by the observation that NCS treatment, which triggers ATM activation, downregulates FLIP in the presence of a kinase competent ATM protein (Fig. 8A). Conversely, the treatment of ATM proficient cells with the ATM kinase inhibitor KU-55933 triggers FLIP upregulation (Fig. 9A). These data allow us to hypnotized that ATM kinase activity might modulate FLIP protein levels. Consistently, while the decrease of FLIP levels following NCS treatment sensitizes cells to Fas induced apoptosis (Fig. 8B), the upregulation of FLIP levels after 8 hours preincubation with KU-55933 protects cells from Fas-induced apoptosis (Fig. 9B). Overall, we provide evidence for A-T cell resistance to Fas-induced apoptosis and we demonstrate that ATM kinase activity may modulate Fas sensitivity through the regulation of FLIP proteins level. Overall, these findings points to the upregulation of FLIP protein levels as a putative novel marker of A-T cell lines. We are currently investigating the levels of FLIP protein in heterozygous derived A-T cell lines. This study along with further experiments on peripheral blood cells from A-T patients will address the question whether FLIP upregulation could be used as a novel A-T prognostic marker.

Importantly, A-T patients have an increased rate of lymphoma and leukemia development, with a frequent occurance of B-cell lymphomas such as Hodgkin Lymphomas (Gumy-Pause et al., 2004; Taylor et al., 1996). Several independent studies on HL reported the aberrant downregulation of ATM activity as a common event, thus suggesting that ATM loss may promote HL development (Starczynski et al., 2003; Greiner et al., 2006). Furthermore it has been clearly shown that HL are very resistant to Fas- and TRAIL-induced apoptosis and this correlates clearly with the aberrant upregulation of FLIP levels. Indeed the downregulation of FLIP is sufficient to sensitize back these cells to death-receptor-induced apoptosis (Dutton et al., 2004; Mathas et al., 2004). We have shown that ATM kinase activity modulates FLIP protein levels. To test whether the lack of ATM kinase activity in Hodgkin/Reed Sternberg (HRS) cells may contribute to FLIP downregulation we restored ATM activity in L428 cells, an HL-derived cell line previously characterized for aberrantly low ATM function (Dutton et al., 2006) and for aberrantly high FLIP levels (Dutton et al., 2004; Mathas et al., 2004). Using this approach we could show that ATM activity is sufficient to decrease FLIP levels and to sensitize L428 cells to Fas-induced apoptosis (Fig.12). It should be noted that the expression of ATM itself induces apoptosis in L428 cells (Fig.12B). Indeed this cell line express Fas Ligand (FasL) but are protected from autonomous cell death because overexpress FLIP protein (Dutton et al., 2004) . The downregulation of FLIP, through the use of specific siRNAs, leads to reduced viability of L428 cells independently of the stimulation with anti-Fas antibody (Dutton et al., 2004) , because of the presence of the endogenous FasL. Therefore it is not so unexpected that the expression of ATM itself in L428 cells induces apoptosis through downregulation of FLIP, aside from Fas stimulation. Consequently Fas stimulation further sensitize L428 cells to apoptosis. However, our data show a new molecular mechanism trough which ATM inactivation may act as a tumorigenic promoter in Hodgkin Lymphomas. Moreover, this finding, along with the data present in literature on ATM deficiency in HL (Starczynski et al., 2003; Greiner et al., 2006), allows us to speculate that AT deficiency could also contribute to lymphoma development via the loss of control on FLIP levels which in turn triggers Fas resistance.

In summary, we identified a novel function for ATM kinase as a regulator of FLIP levels and of Fas sensitivity (Fig.13) and suggested that this signalling may contribute to the homeostasis of the

immune system (Stagni et al., 2008). We could also speculate that failure of the ATM-dependent FLIP regulation, might be at least partially responsible for the increased frequency of lymphomas associated to A-T as well as for the development of lymphoma in those situations where ATM kinase activity is downregulated through alternative mechanisms other than homozygous deletion. Furthermore, the induction of ATM activation may provide a novel tool to downregulate FLIP protein levels and to sensitize those lymphomas where endogenous ATM is still functional to death receptor induced apoptosis, suggesting that this mechanism might be diagnostically and therapeutically relevant.

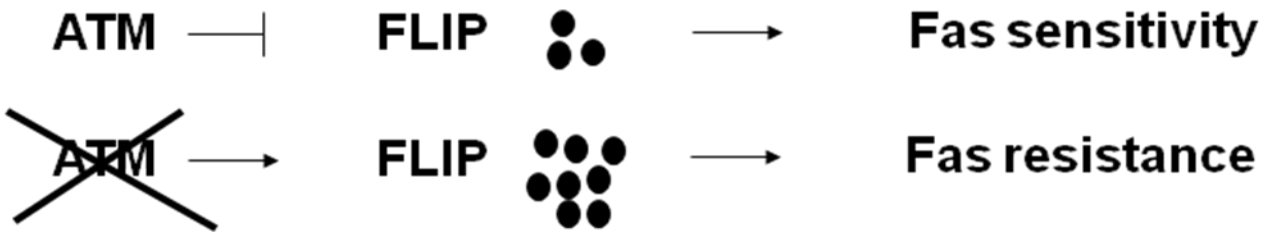

Fig.13. Novel function for ATM kinase as a regulator of FLIP levels and of Fas sensitivity. The lack of ATM expression triggers the upregulation of c-FLIP, which may account for Fas resistance of cells that express no fuctional ATM kinase.

Future directions

In Fig.11 we could show that ATM modulates FLIP protein stability through a post-transcriptional mechanism. Moreover, our preliminary data suggest that ATM kinase activity modulates FLIP protein levels depending on the proteosome-dependent pathway. In particular, we treated the L6, L6-ATM-WT and L6-ATM-KD cells with the proteosoma inhibitor MG132 and then we looked at the levels of FLIP by immunoblotting analysis (data not shown). We observed that MG132 increases FLIP protein levels and stability only in cells that express the catalytically active form of ATM (L6-ATM-WT). These data suggest that ATM kinase activity modulates FLIP levels depending on the proteosoma pathway.

We are currently work on two main hypothesis (Fig.14). One possibility is that ATM regulates FLIP stability through a direct phosphorylation, as some works demonstrated that ATM may modulate the ubiquitination of some substrate proteins (Wu ZH et al., 2006). Our second hypothesis is that ATM can modulates the activity of an E3-ubiquitin ligase which is able to trigger FLIP degradation. Indeed, some E3-ubiquitn ligases, such as COP1 and MDM2, have been identified as novel ATM targets (Dornan D. et al, 2006; Khosravi R. et al. 1999). Recently, the E3-ubiquitin ligase ITCH has been identified as the one responsible for specific ubiquitination of FLIP-L and its induction to proteosomal degradation (Chang et al., 2006). In addition, ITCH ubiquitin ligase regulates also the stability of some proteins involved in DNA damage response as p73 and p63 (Rossi et al., 2005; Melino et al., 2006).

These observations prompted us to investigate whether ITCH could be involved in ATM-dependent degradation of FLIP. Further experiments will clarify the molecular mechanism beyond this regulation.

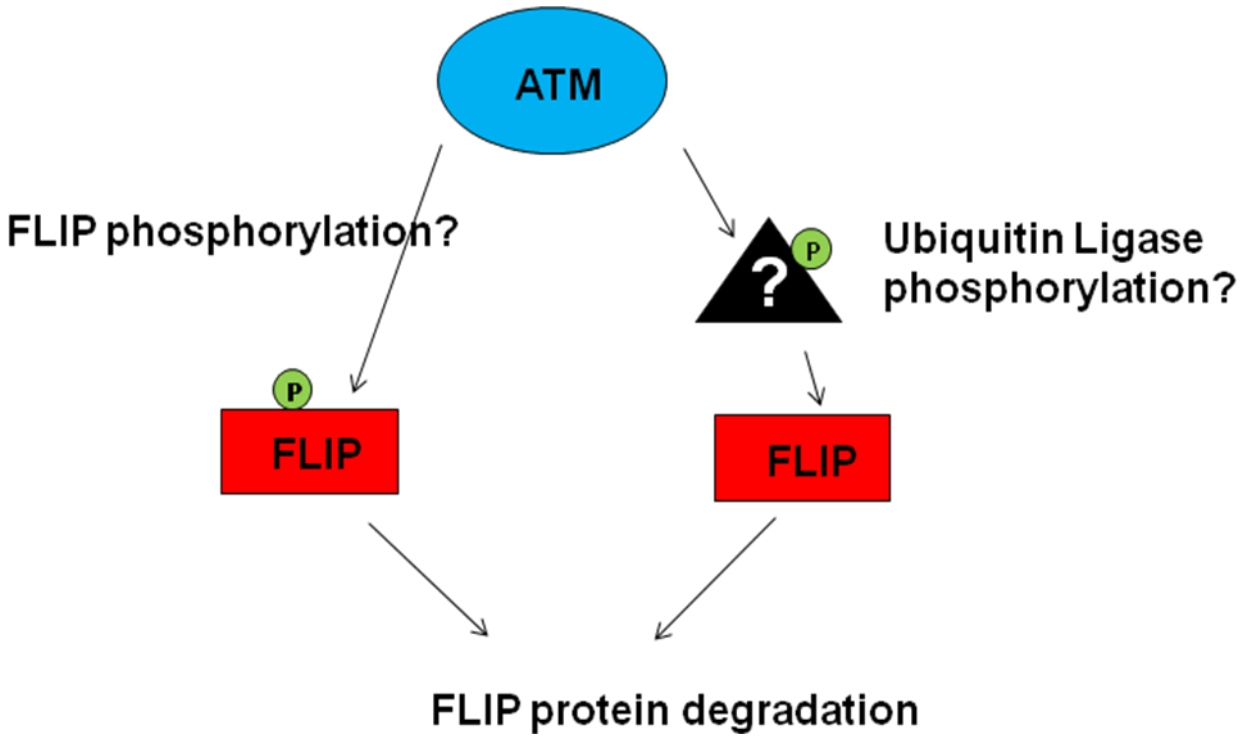

Fig.14. Hypothesis of the molecular mechanism through which ATM kinase activity modulates FLIP levels .

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