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# Molecular Bases of Ataxia Telangiectasia: One Kinase Multiple Functions

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Additional information is available at the end of the chapter

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## 1. Introduction

Ataxia Telangiectasia (A-T) is an autosomal recessive hereditary progressive neurodegenerative and multisystem disease characterized by cerebellar ataxia, telangiectasia, recurrent sinopulmonary infections, variable immunologic defects among which a significantly higher incidence of leukaemia and lymphoma and type 2 diabete. This disorder has been clearly linked to the loss of expression of the serine/threonine kinase ATM (Ataxia Telangiectasia Mutated), a central player of the DNA Damage Response (DDR). Several clinical features of A-T patients, as well as the pleiotropic phenotypes observed in *Atm* deficient mice, can be associated to a defective DDR. Moreover, ATM deficient cellular models display radiosensitivity and failure to activate cell cycle checkpoints in response to DNA damaging agents. Emerging evidences indicate that ATM kinase may be involved in several additional signalling pathways, among which the signalling cascades triggered by oxidative stress, hypoxia, autophagy, metabolic changes, growth factors and death receptors, suggesting that the endangerment of these functions in the absence of ATM activity may importantly contribute to the development of A-T pathology.

The aim of this chapter is to provide a schematic, although exhaustive, description of the signalling cascades that may modulate and may be modulated by ATM kinase activity. Data obtained from different model systems, including *in vitro* and *in vivo* studies, human and mouse models will be integrated. The chapter will be subdivided in paragraphs to improve the readability and to emphasize different aspects of the genetic, molecular and functional features of this pathology. The structure of the protein as well as its primary function in the DDR will be discussed. However, particular emphasis will be given to the enrolment of ATM to other signalling pathways and to the molecular mechanisms that ensure ATM kin-

ase activity regulation. Finally, we will deeply discuss the functional links between ATM kinase and cancer, immune system defects and neurodegeneration.

## 2. Ataxia telangiectasia pathology

Ataxia-telangiectasia (A-T) [1], is a multisystem neurodegenerative syndrome that occurs early in childhood. A-T is an autosomal recessive disease with an estimated frequency in the range of one per 40000 to one per 300 000 births. At birth, infants generally appear normal and begin walking at a normal age (approximately age 1 year); however, by age 2–3 ataxia (loss of muscle co-ordination) becomes visible and generally by age 10 patients are confined to a wheelchair (for a recent review [2]). Clinically, A-T presents with uncoordinated or ataxic movements that are often associated with ocular telangiectasia (dilated blood vessels of the eye). Ataxia generally precedes telangiectasia, which is described as the chronic dilation of a group of capillaries causing elevated, dark red blotches on the skin or eyes. The prominent neurological sign of A-T is an inexorable loss of cerebellar function, and progressive dysarthria (speech defects) and choreoathetosis (abnormal body movements). Studies have shown a gradual decrease in granular and Purkinje cells, which are large branching cells of the nervous system and are located in the cerebellum. Characteristic eye movement abnormalities also feature strongly in A-T, and these might be related to cerebellar dysfunction. Although, the hallmark of clinical presentation is a debilitating progressive neurodegeneration, other characteristics are associated with A-T, such as extreme radiosensitivity, immunodeficiency (frequently manifested as decreased or absent IgA, IgE and IgG2), cancer predisposition (particularly lymphoma and leukaemia), insulin-resistant diabetes and premature aging.

Clinical diagnosis of A-T is relatively easy once the characteristic neurodegeneration and ocular telangiectasia have developed. In these cases the diagnosis can usually be confirmed by finding an elevated serum  $\alpha$ -fetoprotein level, although so far it is not clear why  $\alpha$ -fetoprotein remains high in A-T patients since there is no obvious liver damage. In young children where ataxia and/or telangiectasia did not occur yet, the diagnosis is still challenging. A-T pathology has been clearly linked to the loss of function of the product of the ATM gene. Most patients are compound heterozygotes with different mutations in the two ATM alleles. The identification of the ATM gene has facilitated diagnosis, although the large dimensions of the gene and the lack of mutational hot spots, prevent mutational analysis for clinical screening. Additional confirmatory laboratory test results include absence of the ATM protein on immunoblots, lack of ATM protein kinase activity, increased frequency of chromosomal breaks after exposure to  $\gamma$ -radiation, radioresistant DNA synthesis and decreased colony survival after  $\gamma$ -radiation. None of these methods is 100% specific or 100% sensitive, and clinical correlation is essential. The two most common causes of death are chronic lung disease (about one-third of cases) and cancer (about one-third of cases) (reviewed in [2]).

### 3. Genetic and molecular bases of A-T: From ATM gene to ATM protein

Ataxia–telangiectasia (A-T) is a rare autosomal recessive disorder caused by deficiency of the Ataxia–Telangiectasia Mutated (ATM) protein kinase. A-T patients generally lack functional ATM protein due to missense or nonsense mutations in the ATM gene, which has been identified by positional cloning strategy in 1995 [3].

These mutations occur throughout the entire coding sequence of the gene and overall lead to the production of truncated or unstable ATM variants [4]. The human ATM gene is located at 11q22-23 and covers 160 kb of genomic DNA; the gene product, ATM protein, is produced from a 13 kb transcript that codes for a 350 kDa protein.

ATM protein belongs to the phosphatidylinositol 3 kinase-like kinase (PIKK) family of Ser/Thr-protein kinases, which includes ATR (ataxia–telangiectasia and RAD3-related), DNA-PKcs (DNA-dependent protein kinase catalytic sub-unit) and mTOR (mammalian target of rapamycin), and many others. The proteins that are members of this family show a conserved domain organization in their C-terminal portion, which includes the presence of a kinase domain, flanked by a FAT domain (conserved sequence in FRAP, ATM and TRAPP proteins), that precedes the catalytic domain, and a FATC domain (FAT-C-terminal) that is located at the very C-terminal of the protein. Interestingly the FAT and the FATC domain have been proposed to play a role in the conformational maintenance of the catalytic domain and therefore in the control of the functionality of these kinases [5]. This model is also supported by the identification in these domains of several post-translational modifications that play a role in the modulation of ATM activity. Among these, S1981 in the FAT domain, a major autophosphorylation site that modulates the assembly of the inactive dimeric conformation [6], and C2991 in the FATC, whose acetylation contributes to the modulation of S1981 phosphorylation and to ATM kinase activation [7].

The N terminus of ATM is composed of HEAT (Huntingtin, Elongation factor 1A, protein phosphatase 2A A-subunit, TOR) repeats [8]. Moreover, several motifs that allow ATM interaction with other proteins have been mapped in the N-terminal region of ATM. Among these, sequences for the interaction with NBS1, c-Abl, p53, which play an essential role in the regulation and in the execution of ATM function, as well as with chromatin. The protein is heavily subjected to several post-translational modifications, among which phosphorylations and acetylation, that overall play a role in the modulation of ATM kinase activity as described in the next paragraph (for recent reviews on ATM [9] [10]).

### 4. ATM function: The DNA damage response

ATM function in the DNA damage response has been deeply investigated by several groups. Several excellent reviews in this topic are currently available [9, 11-13]. For this reason this paragraph will only briefly summarize the state of the art on this issue.

ATM has been first identified as an essential component of the DNA damage response, a complex of signalling cascades that ensures the maintenance of genomic stability. The oc-

currence of a DNA damage triggers cell cycle arrest and the initiation of the repair process. Alternatively, the cell that contains the damaged DNA may undergo apoptosis or senescence. The molecular mechanisms that allow the choice among these responses have not been clearly elucidated yet, although the common idea is that apoptosis or senescence are initiated in case the damage is not repairable. Genetic defects that perturb these mechanisms almost invariably cause severe syndromes that are characterized by the degeneration of specific tissues (especially the nervous and immune system), sensitivity to specific DNA-damaging agents and predisposition to cancer (reviewed in [11], see next paragraph on “DNA damage response and other genomic instability syndromes”). Different types of DNA damage may trigger different types of DNA repair responses (for a recent review see [13]).

In particular, ATM is a major player of the cellular response to Double Strand Breaks which represent the most toxic type of DNA lesion, elicited mainly by ionizing radiation (IR or  $\gamma$ -radiation) and other genotoxic stresses. DSBs occur also during physiological 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. The central role of ATM in the DDR to DSBs has been strongly suggested by some observations derived from A-T patients or *Atm* KO mice as well as from *Atm* deficient cells. Indeed A-T cells are highly sensitive to IR, and are characterized by failure of checkpoint induction in response to IR. As already pointed out, ATM deficiency leads both in human and mice to immune system defects (see also next paragraph on “Functional links between ATM kinase and the immune system defects”) as well as to genomic instability and to higher incidence of cancer development (see also next paragraph on “Functional links between ATM kinase and cancer”).

Within minutes after the induction of DSBs, most ATM molecules become vigorously active and participate to checkpoints activation, as well as to DNA repair and to the induction of senescence or apoptosis [14]. Overall, the signalling cascades that allow ATM to participate and modulate all these responses have been only partially elucidated and rely on the ability of ATM to trigger the phosphorylation of a large number of substrates, among which several kinases, that amplify the signal, and transcription factors, DNA repair components and other that execute the different responses (see also next paragraph on “Identification of ATM substrates: proteomic studies”). The activation process includes ATM recruitment to the DSBs, which is mediated by the MRN complex and by the damaged DNA (see next paragraph on “Molecular mechanisms that ensure the modulation of ATM kinase activity”). Activation of this pathway includes a plethora of phosphorylation and ubiquitination events triggered by ATM kinase, among which the activation of c-Abl kinase which in turn modulates the activity of Rad51 and Rad52 proteins and the phosphorylation of histone H2AX which marks the DSBs. Therefore activated ATM participates directly to the repair of the DNA lesion. In particular, ATM modulates Homologous Recombination (HR) and contributes to repair also through its interplay with other PI3K-like kinases such as ATR and DNA-PK.

Activated ATM is also released from the damaged DNA and may therefore trigger the cell cycle checkpoints activation. ATM may modulate cell cycle arrest as well as the apoptotic or

senescence induction. For these responses, among the others, a crucial effector of ATM signalling is p53 transcription factor. ATM may modulate p53 functionality by directly phosphorylating p53 on S15 (promoting p53 transcriptional activity) as well as via the activation of Chk2 kinase, which in turn triggers p53 phosphorylation on S20 (impairing p53 interaction with Hdm2 ubiquitin ligase, therefore promoting its stabilization). Moreover ATM may directly phosphorylate and modulate Hdm2 and may also modulate HIPK2 and therefore p53 phosphorylation on S46, which enhances p53 apoptotic activity. To summarize, the interplay between ATM and p53 is a good example of the complexity of the signalling cascades that modulate the balance and the integration of the different checkpoints with the apoptotic response.

An exhaustive picture of the signalling cascades through which ATM modulates cell cycle, cell death and DNA repair is beyond the scope of this chapter and it is available in these suggested reviews [9, 11-13].

#### **4.1. DNA damage response and other genomic instability syndromes**

A-T belongs to a group of human diseases that are collectively known as “genomic instability syndromes”, each of which results from a defective response to a specific DNA lesion [11]. Genetic defects that affect specific DNA damage response pathways lead to syndromes that combine various degrees of tissue degeneration, growth and developmental retardation, premature signs of ageing, chromosomal instability, sensitivity to the corresponding DNA-damaging agents and cancer predisposition. The prominent genomic instability syndromes – Xeroderma pigmentosum (XP), Cockayne’s syndrome, Trichothiodystrophy (TTD), Bloom’s syndrome (BS), Werner’s syndrome (WS), Rothmund–Thompson syndrome (RTS), Fanconi’s anemia (FA), Nijmegen breakage syndrome (NBS), ataxia-telangiectasia-like disease (ATLD) - are all autosomal recessive and display defects in the main damage-response pathways, each of which is activated by a different class of damaging agent (see for an exhaustive review [11])

#### **4.2. Identification of ATM substrates: Proteomic studies**

It has been shown that ATM and ATR kinases mainly phosphorylate a subset of Serine and Threonine residues located inside the S/T-Q motif [15]. Antibodies that allow the identification of proteins phosphorylated on S/T-Q residues have been generated and are commercially available. These tools allowed therefore the development of several proteomic studies aimed to an exhaustive identification of ATM/ATR substrates in cells treated with IR. Matsuo and colleagues 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. Functional analysis of a subset of this data set indicated that this list is highly enriched for proteins involved in the DDR [16]. Mu and colleagues performed a similar study and identified proteins that belong to the ubiquitin-proteasome system (UPS) to be required in mammalian DNA damage checkpoint control, particularly the G1 cell cycle checkpoint, thus revealing protein ubiquitylation as an important regulatory mechanism downstream of

ATM/ATR activation for checkpoint control [17]. Other proteomic studies were aimed to the analysis of the phosphoproteome dynamic changes, dependent or independent on ATM, involved in the DNA damage response [18, 19]. Recently, a comparative analysis of ATM deficient and proficient lymphoblastoid cells by label-free shotgun proteomic experiments has been conducted. This study provided an insight on the functional role of ATM deficiency in cellular carbohydrate metabolism's regulation [20].

Overall proteomic approaches, identified several changes dependent on ATM expression and activity increasing the comprehension of the DNA damage response. More intriguingly, these studies identified as possible substrates or effectors of ATM several proteins whose known functions are not linked to the DDR, supporting the idea that ATM may perform several other functions in addition of its well known role in the DDR.

## 5. ATM kinase: Other functions

First evidences that induce scientists to postulate a more general signaling role for ATM, independent from DNA damage, rise from several observations made on A-T patients as well as from a series of abnormalities of ATM-deficient cells.

First of all, cerebellar degeneration, ataxia and telangiectasia in A-T patients fit less well with the assumption that exclusive function of ATM is in DDR. It is evident, for example, that post-mitotic neurons of the cerebellum are less dependent on the DNA damage response-associated cell-cycle regulation mediated by ATM [21]. Furthermore, some A-T patients develop insulin-resistant diabetes [22, 23]. This feature is also part of the clinical profile of the metabolic syndrome and cannot be explained by the aberrant DNA damage signaling activation.

Importantly, ATM-deficient cells exhibit some abnormalities, which are difficult to ascribe only to the role of ATM in DNA damage response [12]. These alterations include:

-reduced internalization of phytohaemagglutinin (PHA), defective  $\text{Ca}^{2+}$  mobilization, depolarization in response to extracellular  $\text{K}^+$ ;

- decreased duration of  $\text{Ca}^{2+}$  and  $\text{Na}^+$  firing;
- increased growth factor demand and defective signalling through the epidermal growth factor (EGF) receptor;
- occurrence of markers for oxidative stress such as protein nitrosylation, increased thiol conjugates, and lipid peroxidation.

Finally, although ATM is primarily localized to the nucleus, it has been shown that a minor fraction of the protein is present in the cytoplasm of various cell types [24]. First evidence for a cytoplasmic form is the discovery of ATM association with both peroxisomes and endosomes [25, 26]. In neurons of the cerebellum, ATM is equally distributed between the nucleus and the cytoplasm [27, 28], which further suggests additional roles for ATM also

outside the nucleus. ATM activation in the cytosol has been described upon insulin treatment [29] or, more recently, upon oxidative stress induction [30].

All of these observations provide evidence for additional and unexplored ATM functions mainly linked to its extranuclear localization. However, only in the last years different groups start to investigate more deeply and systematically the molecular mechanism and the biological significance of these new functions. In this section a summary of these studies will be provided.

### 5.1. ATM and oxidative stress

Cells living in an oxygen-rich environment are constantly challenged by oxidative stress. Oxidative stress is defined as an imbalance between cellular oxidants and antioxidants in which the production of Reactive Oxygen Species (ROS) exceeds the anti-oxidative capacity. ROS include the superoxide anion radical ( $\bullet\text{O}^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), and the hydroxyl radical ( $\bullet\text{OH}$ ). Together with other reactive nitrogen species (RNS), these ROS are the major mediators of oxidative stress. Numerous exogenous and endogenous stress stimulators can disrupt cellular homeostasis and evoke oxidative stress. Physical factors (ultraviolet light and ionizing radiation), oxygen level changers (hypoxia and subsequent reoxygenation) and chemical factors (hydrogen peroxide and chemotherapeutic reagents) are some of the possible exogenous sources of ROS. Several potential endogenous sources of ROS exist within the cell. ROS generated as byproducts and normal metabolites during aerobic metabolism in mitochondria are the primary sources of ROS. In conclusion ROS produced by both endogenous and exogenous sources either directly or indirectly activate antioxidant machinery and physiological stress signaling pathways.

Over the past two decades, evidence has accumulated that links ATM deficiency to increased oxidative stress in cells, which is thought to play a key role in neurodegeneration, metabolic dysregulation and oncogenesis [31].

Cells from individuals affected by A-T syndrome have constitutive oxidative stress and this altered oxidative stress has long been linked to A-T as both a cause and a consequence of the disease [10].

Because treatment of ATM-null mice with antioxidants can ameliorate intrinsic defects in stem cell renewal [32, 33] and delay their tumor onset [34, 35], it has been suggested that increased accumulation of intracellular ROS associated with ATM dysfunction contributes to the clinical features of this pathology [31, 36, 37].

Although, the increased oxidative damage associated with ATM deficiency has largely been attributed in the past to the defects in the DDR pathway, the basis for this phenomenon remains unclear, and recent data have provided some possible new clues that are independent of DDR. For example, a potential role for ATM in the control of an antioxidant response via the pentose phosphate pathway (PPP) has been reported and may be relevant in ATM null tissues showing increased oxidative stress [38]. The authors demonstrated that ATM regulates the PPP by inducing glucose-6-phosphate dehydrogenase (G6PD) activity, which in turn promotes NADPH (Nicotinamide Adenine Dinucleotide Phosphate) production and



nucleotide synthesis. Consistently, ATM might contribute to maintain the reducing power of the cellular environment by promoting NADPH production. Moreover ATM can modulate ROS also through modulation of mitochondria activity (see next paragraph “ATM and Mitochondria”).

Although, it has been recognized since several years that ATM is activated also by oxidative stress (as  $H_2O_2$ ), the biochemical mechanisms underlying the response of ATM to oxidative stress were described only recently. Guo and colleagues proposed that ATM functions as a redox sensor in the cytoplasm and, as such, may regulate global cellular responses to oxidative stress [39]. ATM activation by oxidative stress involves the formation of a disulfide bridge between cysteine (C2991) residues to form a ATM dimer [39] (described in the following paragraph “Molecular mechanisms that ensure the modulation of ATM kinase activity”). Importantly, this mode of ATM activation can occur independently of the MRN complex, suggesting a role for ATM in signaling other than direct DNA damage.

Notably, ionizing radiations that generate DNA DSBs can also produce ROS that inactivate key DNA repair. Hence, ATM oxidative activation may allow cells to respond to DNA DSBs and maintain genetic integrity under these toxic conditions. ATM appears to function as a key nodal point, bringing together DNA damage response and also the response to oxidative stress [40]. Clearly, further analysis of the importance of oxidative stress-induced activation of ATM will illuminate the possible contribution of this feature toward specific aspects of the A-T phenotype.

A possible role of ATM in vascular stability has long been suspected because of the manifestation of telangiectasia (dilated blood vessels) and vascular leakiness in both patients with ataxia telangiectasia and aged ATM-deficient mice. Recently, Okuno and colleagues demonstrated a very interestingly correlation between oxidative stress-induced activation of ATM and the occurrence of telangiectasia [41]. In this papers the authors demonstrated that ROS substantially accumulates in newly formed immature vessels and activates ATM. Loss of ATM in endothelial cells minimizes pathological ocular and tumor neoangiogenesis as a consequence of defective oxidative defense rather than an impaired DDR [41]. This paper provides the first evidence for a link between a clinical feature of A-T and DNA damage independent function of ATM kinase.

## 5.2. ATM and mitochondria

Mitochondria play an important role in ATP synthesis and apoptosis and are also the major source of intracellular ROS. A number of human diseases are linked to mutations of the mitochondrial genome. Among these are premature ageing, cancer, diabetes mellitus, and a variety of syndromes involving the muscles and the central nervous system [42]. A-T is similar to other progressive neurological disorders that are characterized by oxidative stress and intrinsic mitochondrial dysfunction [43].

Different studies, using immortalized cell lines established from patients with A-T, have reported that cells lacking ATM function exhibit alterations in mitochondrial homeostasis, in-

cluding defects in mitochondrial structure, decreased membrane potential, and respiratory activity [44, 45].

An important step in the control of mitochondrial function is the biogenesis of these organelles, which involves mitochondrial DNA (mtDNA) replication and mitochondrial mass increase. Due to limited coding capacity of mtDNA, mitochondria rely largely on nuclear genes (over 1000 genes) for their proliferation. Mitochondrial biogenesis therefore requires complex coordination between the nuclear and mitochondrial genomes [46]. Upon energy depletion, activated AMPK turns off ATP-consuming processes such as synthesis of lipids, carbohydrates, and proteins, and turns on ATP-generating pathways including mitochondrial biogenesis [47].

Interestingly ATM has been implicated in the mitochondrial biogenesis pathway mediated through AMPK activation [48, 49] supporting a role of ATM in mitochondrial functions.

It is of interest also that several ATM substrates show mitochondrial translocation (CREB, p53) and affect mitochondrial functions (HMGA1) [10]. Moreover A-T cells have lower cytochrome c oxidase activity than normal cells, which could explain their reduced respiratory activity; interestingly, treatment of normal cells with an ATM inhibitor also results in reduced cytochrome c oxidase activity [50]. While there are numerous external sources of ROS, the great majority of ROS within eukaryotic cells derives from the mitochondrion as by-products during the generation of adenosine triphosphate (ATP), through the process of oxidative phosphorylation. These evidence lead to postulate that mitochondrial dysfunction may be responsible for elevated ROS production and oxidative stress of A-T cells [10, 45].

However, there are some inconsistencies between these studies, such as discrepancies in the nature of mitochondrial DNA content abnormalities. Recently Valentin-Vega and colleagues clarify this point and report that *in vivo* loss of *ATM* results in striking mitochondrial dysfunction in thymocytes, leading to elevated mitochondrial number and increased mitochondrial ROS production. The increase in mitochondrial content is associated with defects in the intracellular destruction of abnormal mitochondria (mitophagy). Therefore, they conclude that ATM has major role in modulating mitochondrial function and ROS generation *in vivo* and *in vitro* and suggest that decreased mitophagy, rather than increased mitochondrial biogenesis, is associated with the increased mitochondrial content [51].

### 5.3. ATM, hypoxia and autophagy

Although maintenance of oxygen homeostasis is an essential cellular and systemic function, it is only within the past few years that the molecular mechanisms underlying this fundamental aspect of cell biology started to be elucidated and their connections to development, physiology and pathophysiology have been established. HIF-1 (hypoxia-inducible factor 1) is the transcriptional activator that functions as a master regulator of oxygen homeostasis [52]. Recently, advances in delineating upstream signal transduction pathways leading to the induction of HIF-1 activity, and expression of downstream target genes, have been made and these lead to significant contribution to the understanding of oxygen homeostasis regulation [52]. Interestingly low oxygen tension or hypoxia is a common feature of all solid tu-

mors [53]. It is strongly associated with tumor development, malignant progression, metastatic outgrowth, and resistance to therapy and is considered an independent prognostic indicator for poor patient prognosis in various tumor types [53].

In this context it is well established that ATM is activated under hypoxic conditions not only in a DNA damage dependent way [54] but also through an MRN-independent mechanism in the absence of DNA damage. Phosphorylated ATM is found in a diffuse pattern in the nucleus [55]. The mechanism of ATM activation is not clear: although acute hypoxia induces release of ROS from mitochondria [56], this is not essential for ATM activation under these conditions [55]. Recently Mongiardi and colleagues have demonstrated that ATM may function as an oxygen sensing protein. In particular they demonstrated that A-T cells exhibit a blunted response to mild hypoxia, being defective in upregulating HIF-1 $\alpha$ . The disability of ATM-negative cells to upregulate HIF-1 $\alpha$  is a consequence of an impaired sensing of oxygen variations [57]. In addition, ATM is a direct regulator of the transcription factor complex HIF-1, a heterodimer of HIF-1 $\alpha$  and HIF-1 $\beta$  subunits that regulates metabolism, mitochondrial function and angiogenesis under hypoxic conditions [58]. ATM phosphorylation of HIF1 $\alpha$  on Ser696 stabilizes the protein under hypoxic conditions, which promotes mTORC1 inhibition and growth suppression. Moreover authors suggest that suppression of ATM may significantly contribute to the signalling through which TORC1 activity can remain elevated in hypoxic tumor [58].

Interestingly mTORC1 negatively regulates autophagy a catabolic process in which cells deliver cytoplasmic components for degradation to the lysosome [59]. Concomitant with mTORC1 repression by ROS, autophagy increased in cells treated with H<sub>2</sub>O<sub>2</sub>. Consistently Alexander and colleagues demonstrated that ATM signaling in response to ROS also leads to mTORC1 inhibition and is involved in the consequential induction of autophagy[60]. Whether autophagy is activated as a survival mechanism in response to ROS or functions in an ATM-driven programmed cell death pathway remains to be explored.

#### **5.4. ATM and metabolic syndrome**

Metabolic syndrome is a cluster of metabolic abnormalities and related clinical syndromes among which the most relevant ones are insulin resistance and atherosclerosis [61]. Insulin resistance along with visceral adiposity, dyslipidemia and chronic subclinical proinflammatory state are the main characteristic features of metabolic syndrome. The role of ATM in the regulation of metabolism is emerging as a very interesting topic. A-T patients have an increased risk of developing type 2 diabetes and display growth impairments associated with insulin resistance and glucose intolerance [22, 23]. Diabetic complications are not considered a primary characteristic associated with A-T owing to their late onset and the fact that most A-T patients succumb to the disease early in life. However, several studies have demonstrated a relationship between ATM and metabolic signaling pathways. For example, there is a close interplay between ATM and insulin pathway (reviewed in [10]). The identification of cytoplasmic ATM as an insulin-responsive protein provides the first indication that a defective response to insulin could be related to the development of insulin resistance and type 2 diabetes in A-T patients [29]. Moreover ATM is required for AKT phosphorylation at Ser473

and for translocation of the cell's surface glucose transporter 4 (GLUT4) in response to insulin stimulation [62]. These results suggest that reduced expression of ATM may trigger the development of insulin resistance because of the consequent downregulation of AKT activity. Recent evidence indicates that the effects of ATM on insulin function and glucose metabolism may be mediated through p53 phosphorylation [63]. Deletion of the p53- encoding gene, or its mutation to generate p53 variants that lack the primary ATM phosphorylation site, results in elevated ROS levels, glucose intolerance, insulin resistance, reduced AKT phosphorylation and reduced expression of sestrin proteins, which are involved in the regulation of intracellular antioxidants [63]. These effects can be rescued by the addition of dietary antioxidants, suggesting that ATM affects insulin function and glucose metabolism by regulating intracellular ROS levels through p53 phosphorylation. Moreover Schneider and colleagues discovered a new relationship between ATM deficiency and metabolism in mice, looking specifically at aspects of the metabolic syndrome such as insulin resistance, adiposity, blood pressure, circulating cholesterol and lipid levels, and atherosclerosis. They have shown that transplantation of bone marrow with ATM<sup>-/-</sup>ApoE<sup>-/-</sup> mice increases atherosclerosis, whereas activation of ATM in ATM<sup>+/+</sup>ApoE<sup>-/-</sup> mice alleviates the vascular disease. The results indicate that ATM deficiency causes insulin resistance, resembles the metabolic syndrome, and increases vascular disease [64]. Interestingly, ATM has recently been identified as a functional target of metformin, a drug widely used in the treatment of type 2 diabetes [65]. Metformin is a very interesting drug because reduces insulin resistance and increases glucose uptake in skeletal muscle, but the mechanism of its action is not fully understood. ATM seems to function upstream of metformin- induced AMPK activation, because treatment of rat hepatoma cells with an ATM inhibitor reduced AMPK activation and phosphorylation following metformin treatment [65]. However the role of ATM downstream metformin treatment is very controversial and several research groups are currently trying to clarify this point [66].

### 5.5. ATM and growth factors

Growth factors regulate essential processes in cells as cell proliferation, motility, survival and morphogenesis. Several growth factors activate receptor tyrosine kinases (RTK), leading to activation of cellular signaling pathways as PI3K/AKT signalling, which promotes cell survival, and the mitogen-activated protein (MAP) kinase cascade [67].

First evidences of functional interactions between ATM and growth factor-mediated signaling are:

- the observation that cultured A-T cells display an increased demand for growth factors in the media compared to wt cells;
- the identification of ability ATM as a mediator of the insulin-mediated signaling, which in turn regulates AKT signaling [29, 62].

Moreover, it has been recently shown that also MEK/ERK signaling is modulated by ATM [68] and that inhibition of ATM activity inhibits cell proliferation and induces apoptosis in cancer cell lines with overactive AKT [69]. Collectively, these studies suggest that ATM may

modulate prosurvival signaling downstream growth factor stimulation. Recently, ATM activation has been identified also downstream the growth factor receptor HER2 in a breast cancer mouse model [70]. Thus, it is clear that the ATM is activated upon growth factor stimulation, but the mechanism of this activation is still unknown. It is tempting to speculate that ROS production induced by growth factor stimulation could be responsible for activation of cytoplasmic ATM. Another hypothesis is that ATM could be indirectly activated by hyperproliferation induced by growth factor signaling.

ATM can also regulate the expression of some growth factor receptors and in particular of some Receptor Tyrosine Kinases (RTKs). For example, the expression of the insulin-like growth factor 1 receptor (IGF1R) is reduced in ATM-deficient cells, and the radiosensitivity of A-T cells, following IR treatment is affected by IGF1R expression levels; both effects can be rescued by ATM cDNA expression [71]. More recently, De Bacco and colleagues demonstrated that DNA damage induces ATM dependent transcription of the growth factor receptor MET and this regulation contributes to radioresistance of MET-dependent tumors [72].

Interestingly RTKs signaling is often aberrantly regulated in different type of tumors, so overall these data suggest also a functional role for ATM in RTKs-dependent tumor progression. In this regard, a possible role of ATM inhibition in cancer therapy will be discussed in the paragraph "Functional links between ATM kinase and cancer".

### 5.6. ATM and death receptors

The observation that A-T patients display an increased rate of lymphoma and leukaemia onset, has been largely explained by the identification of ATM as a major modulator of the DNA damage response and by the central role that physiological DNA damage plays in the development of the immune system [73-75] (see next paragraph "Functional links between ATM kinase and the immune system defects").

Other important modulators of the immune system development and function are death receptors such as Fas (CD95/APO-1) and Tumour necrosis factor (TNF)-Related Apoptosis-Inducing Ligand (TRAIL). The death receptor system is essential for the regulation of the lymphoid system homeostasis [76]. 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 [77, 78]. Mice lacking functional Fas expression suffer from autoimmunity and increased incidence of B cell lymphomas [79, 80]. 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 [81, 82]. Finally, Fas mutations were identified in lymphomas, in particular those deriving from GC B cells (reviewed in [83]).

Fas (CD95/APO-1) is a transmembrane protein belonging to the tumor necrosis factor superfamily. Upon binding of Fas ligand or agonistic antibodies, the Fas receptor recruits several cytosolic proteins to form the death-inducing signalling complex (DISC). This is necessary to catalyze Caspase-8 activation, which triggers the caspase cascade [84]. Caspase-8 activation is absolutely required to trigger receptor-activated apoptotic response and its catalytic activ-

ity has to be tightly regulated to avoid inappropriate activation and undesired cell death. This regulation is ensured by FLIP proteins, which are structurally similar to Procaspase-8 and can therefore compete with Procaspase-8 for binding to DISC, thus preventing Caspase-8 activation and the following apoptotic cascade [85].

Taking into account the linkage between Fas impairment and the development of immune system tumors that are more also frequent in A-T patients, we asked whether any relationship exists between Fas and ATM signaling pathways. We could show that ATM deficiency results in a significant resistance of lymphoid cells derived from A-T patients to Fas-induced apoptosis. Interestingly, loss of endogenous ATM kinase activity results in the aberrant up-regulation of FLIP protein levels. Consistently, ATM kinase activation downregulates FLIP protein levels providing a novel mechanism to modulate Fas sensitivity. Interestingly, Hodgkin Lymphoma cells that are characterized by Fas-resistance and by FLIP overexpression, may be sensitized to Fas upon ATM kinase expression, which triggers FLIP downregulation. These data point to ATM as a novel player in Fas-induced apoptosis and suggest a novel molecular mechanism for the increased lymphoma susceptibility of A-T patients and for the development of B cell lymphoma [86]. These observations have been further extended also to TRAIL receptor. ATM modulates TRAIL sensitivity similarly to what described for Fas [87]. This observation provides a rationale for the employment of several DNA damaging agents largely used in chemotherapy, to enhance TRAIL sensitivity, by triggering ATM activation which in turn drives FLIP protein downregulation [87]. This is consistent with the identification of ATM and Chk2 activation downstream death receptor stimulation [88]. ATM would therefore represent a crucial interplay between the modulation of DNA damage response and death receptor induced apoptosis (reviewed in [89]).

## 6. Molecular mechanisms that ensure the modulation of ATM kinase activity

ATM activation has been at first identified as a response to DSBs DNA damage. ATM is recruited to DSBs and activated by DNA damage through interactions with the MRE11–RAD50–NBS1 (MRN) complex, which is bound to DNA ends at the site of the break. The activated ATM is important for the initiation of DNA end resection that is an essential step to initiate DNA repair via the homologous recombination pathway (reviewed in [11]). *In vitro* experiments have shown that ATM activation in response to DSBs requires the interaction with free ends DNA and with the MRN complex [90]. In this context, it has been proposed that ATM protein exists as an inactive dimer in which the catalytic domain of one molecule is engaged in an intermolecular interaction with the FAT domain of the other molecule. DSBs triggers autophosphorylation on S1981, a serine residue located within the FAT domain, and this phosphorylation causes the release of the intermolecular interaction leading to release of an active monomer [6]. The mutation of the autophosphorylation site disrupts ATM signaling in human cells [91, 92]. Nevertheless, the functional significance of ATM autophosphorylation is still debated as mouse models and *in vitro* studies have shown that ATM autophosphorylation is not required for ATM activation by DNA damage or oxi-

dation [30, 90, 93, 94]. However, ATM autophosphorylation at Ser1981 is at present considered a hallmark of ATM activation and several antibodies that specifically recognize ATM when phosphorylated on S1981 are commercially available and commonly employed to detect the occurrence of ATM activation. Moreover, additional DSBs-induced ATM autophosphorylation sites, Ser367, Ser1893 and Ser2996, have been identified and have been shown to be required for ATM signaling in human cells [91, 92]. Global phosphoproteomic screens of the DNA damage signalling network independently confirmed the identification of these autophosphorylation sites. Furthermore these studies also revealed additional ATM phosphorylation sites [16-19]. An updated list of ATM phosphorylation sites is available at PhosphoSite database <http://www.phosphosite.org/proteinAction.do?id=1393&showAllSites=true>.

Several phosphatases have been identified as important modulators of ATM activity, consistently with the central role that the regulation of phosphorylation plays in the modulation of ATM kinase activation in response to DNA damage. PP2A, PP5 and WIP1 phosphatases regulate ATM activity [95-97]. PP2A directly modulates the state of phosphorylation of S1981 [95]. Conversely PP5 interacts with ATM in a DNA-damage-inducible manner and its activity sustains ATM activation [96]. Wip1 phosphatase has been identified as a novel player of the ATM-dependent signaling pathway, as it directly dephosphorylates Ser1981. Deficiency of Wip1 resulted in activation of ATM kinase, while its overexpression triggers the downregulation of the ATM-dependent signaling cascade after DNA damage [97].

The activity of other kinases may also contribute to the modulation of ATM kinase activity. It has been shown that Cdk5 (cyclin-dependent kinase 5), activated by DNA damage, directly phosphorylates ATM at S794 in postmitotic neurons. This phosphorylation precedes and is required for ATM autophosphorylation at S1981, and sustains ATM kinase activation and signaling. The downregulation of Cdk5-ATM interplay attenuates DNA damage-induced neuronal cell cycle reentry and expression of p53 targets PUMA and Bax, protecting neurons from DNA damage-induced cell death [98]. Similarly, c-Abl, a non receptor tyrosine kinase previously identified as a target and effector of ATM kinase activity in the DNA damage response [99, 100], has been recently identified as important modulator of ATM kinase activation. DNA damage triggers ATM kinase dependent induction of c-Abl activity, which in turn triggers ATM tyrosine phosphorylation. This phosphorylation is required to enhance ATM autophosphorylation on S1981 and ultimately to sustain ATM activity, allowing the apoptotic response [101].

It has also been shown that DNA damage induces the rapid acetylation of ATM. This acetylation depends on the Tip60 histone acetyltransferase (HAT). Suppression of Tip60 blocks ATM kinase activation and prevents the ATM-dependent phosphorylation of p53 and Chk2. [7]. The systematic mutagenesis of lysine residues identified a single acetylation site at K3016, which is located in the highly conserved C-terminal FATC domain [102]. K3016 acetylation is required for the DNA damage induced autophosphorylation on S1981. The acetylation of ATM on lysine 3016 by Tip60 is therefore a key step linking the detection of DNA damage and the activation of ATM kinase activity.

Recent studies have identified a completely different mechanism for ATM activation in response to oxidative stress. According to the proposed models ATM would be present as inactive monomers (reviewed in [10]). Oxidation triggers the assembly of an active dimer in which the two monomers are covalently linked by intermolecular disulfide bonds [30, 39]. C2991, located in the C-terminal FATC domain, has been identified as a crucial residue for ATM activation by oxidation, as a C2991L mutant cannot be activated by H<sub>2</sub>O<sub>2</sub>. The interplay between the molecular mechanisms that trigger ATM activation in response either to DSBs or to oxidative stress has not been clearly investigated yet. It has been shown that the S1981A mutant is still competent for activation in response to oxidation. Similarly, the C2991L mutant is competent for DNA damage induction. ATM activation in response to these two different stresses triggers the ATM-dependent phosphorylation of an overlapping subset of substrates although significant differences have also been identified. As an example, although low levels of H<sub>2</sub>O<sub>2</sub>, which specifically trigger oxidative stress, drive the phosphorylation of p53 and Chk2 proteins, similarly to what observed in response to DNA damage, they fail to mediate histone H2AX and KAP1 phosphorylation which seem to be peculiar for the DSBs response [30]. Importantly, the distinction between ATM activation mediated through oxidation and that mediated by DNA damage is difficult, because oxidative stress and ROS production may usually induce DNA damage, and indeed ATM is often exposed to both these stresses simultaneously. IR treatment is able to trigger both DNA damage and oxidative stress suggesting that the large number of substrates identified by proteomic approaches aimed to characterize the global pattern of ATM substrates in this context probably represent targets from both the DNA repair and oxidation pathways. This observation may provide an explanation for the identification, among more than 700 substrates of ATM, of proteins clearly involved not only in the control and execution of cell cycle checkpoints and DNA repair, but also in many other pathways such as the insulin signaling [16].

A-T has a pleiotropic phenotype that affects multiple systems, and most likely the complex clinical features arise from the synergistic effects of a defective DNA damage response and oxidative stress in the absence of ATM. It is intriguing to speculate that a subset of clinical features associated with A-T might be mainly due to defects in ROS response by ATM, whereas other features might primarily result from the impairment of a functional DNA damage response. Interestingly, some A-T patients present a form of ATM with a truncated C-terminal region (R3047X), that therefore lacks C2991 ([10] and references therein). Although these patients develop ataxia similarly to the others, their cells are less sensitive to IR compared to other A-T cells. Moreover, one patient that expresses the R3047X variant does not display immunodeficiency ([10] and references therein). Future studies will clarify whether the variability of the clinical features displayed by A-T patients may arise from different mutations which impact differently on the different functions performed by ATM.

At present the occurrence of other post-translational modification that may modulate ATM activation by oxidative stress has not been investigated. Future systematic proteomic experiments will also define the complete profile of the oxidative stress dependent ATM sub-



strates. These studies will clarify the differences and the similarities among the different mechanisms of activation of ATM kinase and their potential cross-regulation.

## 7. Functional links between ATM kinase and cancer

### 7.1. ATM expression and cancer

ATM is considered one of the principle guardians of the genome as a consequence of its principle role in the coordination and execution of the DNA damage response. According to this function, ATM is generally defined as a tumor suppressor gene. Several *in vitro* and *in vivo* evidences support this idea. First of all ATM deficiency is clearly associated with an increased onset of tumorigenesis both in human and in mouse models. Indeed, about 20% of A-T patients display a significantly higher incidence in the development of leukaemia and lymphoma, according to the decreased ability of A-T cells to correctly handle the physiological double strand breaks occurring during the maturation of the immune system (reviewed in [73, 75]). Consistently, *Atm* <sup>-/-</sup> mice develop lymphoma and leukaemia within the first three months of life and die of malignant thymic lymphoma by 4–5 months of age [103-105]. Several reports also describe an increased rate in the development of solid tumors for the heterozygous relatives of A-T patients compared to the whole population. In particular a link between ATM heterozygosity and a higher predisposition to breast cancer onset has been well established (reviewed in [106]). In 1987 it has been proposed that relatives of A-T patients might be at increased risk of cancer and in particular breast cancer [107]. Later on, several other epidemiologic studies support the same conclusion [108]. A large study conducted on 1160 relatives of 169 A-T patients, estimated the overall relative risk of breast cancer in carriers to be 2.23 (95% CI = 1.16-4.28) compared to the general population [109]. Although this observation was of importance to A-T families, it was immediately clear that it might have a much wider significance, as it has been estimated that about 1% of the whole population might be carriers of an *ATM* gene mutation.

The frequency of ATM variants in human breast cancer has been largely investigated by several laboratories. Unfortunately, the results of these studies were often inconclusive mainly because of the low number of cases included in each study as well as for the technical difficulties linked to the sequencing of *ATM* gene (reviewed in [75]). More recently, 76 rare sequence variants in the ATM gene have been analyzed in a case-control family study of 2,570 cases of breast cancer and 1,448 controls. The risk estimates from this study suggest that women carrying the pathogenic variant, ATM c.7271T > G, or truncating mutations have a significantly increased risk of breast cancer with a penetrance similar to that conferred by germline mutations in BRCA2 [110].

Consistently with the observation that loss of ATM expression or heterozygosity may enhance cancer predisposition, some reports also describe loss of ATM expression in some tumor samples in the whole population. In particular ATM expression is strongly reduced or lost in some leukaemia and lymphoma [73, 111-113]. The modulation of ATM expression levels in breast cancer has been largely investigated. Several reports, demonstrate the occur-

rence of low levels of ATM expression in breast cancer. In particular, it has been proposed that ATM may be aberrantly reduced or lost in BRCA1/BRCA2-deficient and ER/PR/ERBB2-triple-negative breast cancer [114, 115].

Several molecular mechanisms, alternative to the occurrence of genetic mutations that lead to loss or reduction of ATM expression, have been identified. ATM is down-regulated by N-Myc-regulated microRNA-421 and this may play a role in neuroblastoma [116]. Over-expression of miR-100 is responsible for the low-expression of ATM in the human glioma cell line M059J [117]. Furthermore, it has been shown that miR-18a is upregulated in cell lines as well as in patients' tissue samples of breast cancer. miR-18a triggers the downregulation of ATM expression by directly targeting the ATM-3'-UTR and abrogated the IR-induced cell cycle arrest [118].

Alternatively, ATM promoter methylation has been shown to epigenetically trigger ATM expression downregulation in cancer. The ATM gene is aberrantly methylated and silenced in locally advanced breast cancer [119] and aberrant methylation has been correlated with low levels of ATM expression and increased radiosensitivity in colorectal cancer and glioblastoma cell lines [120, 121].

## 7.2. ATM and the DDR as barrier to tumor development

Recent evidence from both cell culture, animal models and analysis of clinical specimens show a correlation between oncogene activation or loss of tumor suppressor expression and the occurrence of DNA replication stress and induction of the DNA Damage Response (DDR) (reviewed in [122]). The key initial observations that inspired the hypothesis that tumorigenic insults may drive DDR activation as a sort of barrier that delays or prevent cancer progression *in vivo*, were:

1. The occurrence of activated DNA damage signalling in a subset of human cancer cell lines, especially those defective for p53 function.
2. The occurrence of activated DNA damage response, exemplified by Thr68 phosphorylation on Chk2 protein, in clinical specimens of large subsets of human and lung carcinomas [123].

These results suggested that some disease-associated event (not occurring in the adjacent normal tissue) led to activation of the DDR. Therefore, it has been postulated that oncogenic events may trigger DNA damage and the consequently activation of ATM-Chk2 signaling cascade. To test whether this activation may represent a barrier to the transformation process two sets of experimental approaches have been conducted. The first one, was to develop cell culture models of conditional oncogene activation, while the second was to extend the analysis of the occurrence of the DDR to a large panel of human tumor biopsies derived from various type of cancers at various stages, especially from premalignant and pre-invasive lesions which represent very early stages of cancer progression. Two studies jointly provided evidence for a role of the DDR machinery as an inducible barrier against cancer in clinical specimens from various tissues [124, 125]. The authors found that according to their hypothesis, tumor cells in clinical specimens from various tissue (and not cells located in the

adjacent normal tissue) show a constitutive activation of checkpoints kinases such as ATM and Chk2, phosphorylated H2AX and p53 and foci formation by the DDR proteins such as 53BP1. Importantly, in the early pre invasive lesions, the DDR activation preceded occurrence of mutations or loss of expression of DDR component, consistently with the idea that the DDR barrier generates a sort of selective pressure for these mutations that would allow the escape from the barrier and consequently drive cancer progression. The DDR activation was also well recapitulated in human cell culture models following oncogene expression, as well as in xenograft models [124, 125]. One key question related to the induction of the DDR as a barrier to tumor development is the mechanistic basis on the induction of DNA damage in cancer. It has been postulated that oncogene expression triggers DNA replication stress, including replication forks collapse and subsequent formation of DSBs [124-127]. Additional events that may contribute to the induction of DDR in this context are telomere erosion and ROS generation (reviewed in [122]). It has been shown that, the activation of DDR is required for the oncogene-mediated induction of senescence [126, 127], a state of permanent growth arrest refractory to physiological proliferation stimuli, that would counteract tumor progression (reviewed in [128]).

### 7.3. ATM kinase inhibition and cancer therapy

The central function of ATM in the DNA damage response and in the modulation of IR-sensitivity, suggested that the modulation of its activity may be exploited for cancer therapy. For this reason a great effort is still ongoing to develop and improve ATM kinase inhibitors and to define the conditions in which their employment could be beneficial for the cancer therapy. A major obstacle in the development of a specific inhibitor of ATM catalytic activity is linked to the high similarity among the kinase domains of the PI3K-like family proteins. For a long time caffeine has been largely employed to modulate ATM/ATR kinase activity. It has been shown that depending on its concentration caffeine may be able to equally block ATM and ATR activities (10  $\mu$ M) or, alternatively, to selectively interfere with ATM activity (5  $\mu$ M) [129, 130]. Later on, screening a small molecule compound library developed for the phosphatidylinositol 3'-kinase-like kinase family, an ATP-competitive inhibitor, 2-morpholin-4-yl-6-thianthren-1-yl-pyran-4-one (KU-55933), that inhibits ATM with an  $IC_{50}$  of 13 nmol/L and a  $K_i$  of 2.2 nmol/L. KU-55933 has been identified. KU-55933 shows specificity with respect to inhibition of other phosphatidylinositol 3'-kinase-like kinases. Inhibition of ATM by KU-55933 resulted in the ablation of IR-dependent phosphorylation of several ATM targets, including p53, H2AX, NBS1, and SMC1 and sensitize cells to the cytotoxic effects of ionizing radiation and to the DNA double-strand break-inducing chemotherapeutic agents, etoposide, doxorubicin, and camptothecin. Inhibition of ATM by KU-55933 also caused a loss of ionizing radiation-induced cell cycle arrest. By contrast, KU-55933 did not potentiate the cytotoxic effects of ionizing radiation on ataxia-telangiectasia cells, nor did it affect their cell cycle profile after DNA damage [131]. More recently, it has been developed an improved analogue of KU-55933, named KU-60019, with  $K_i$  and  $IC_{50}$  values half of those of KU-55933 [68]. KU-60019 is 10-fold more effective than KU-55933 at blocking radiation-induced phosphorylation of several ATM targets in human glioma cells. KU-60019 inhibits the DNA damage response, reduces AKT phosphorylation and prosurvival signaling, inhibits

migration and invasion, and effectively radiosensitizes human glioma cells [68]. A library of 1500 compounds was selected based on known kinase inhibitor templates and calculated kinase pharmacophores from the Pfizer proprietary chemical file. These compounds were screened with potential inhibitors being identified by a decreased ability of purified ATM kinase to phosphorylate GST-p53[1–101] substrate. This screening approach identified the compound CP466722 as a potential novel ATM inhibitor. The ATM-related kinase, ATR, was not inhibited by CP466722 *in vitro*, although inhibitory activities against Abl and Src kinases were reported [132]. CP466722 was not toxic and importantly, inhibition of cellular ATM kinase activity was rapid and completely reversed by removing CP466722. Interestingly, clonogenic survival assays demonstrated that transient inhibition of ATM is sufficient to sensitize cells to ionizing radiation and suggests that therapeutic radiosensitization may require ATM inhibition for short periods of time [132]. Despite the large effort made so far to improve the specificity and the efficacy of these type of inhibitors, these compounds still deserve further investigation as none of them can be used in *in vivo* studies in animal models because of their elevated toxicity. The identification of ATM as a novel player of other signalling cascades, not related to the DNA damage response, raised also the question that its ability to prevent tumorigenicity may be strictly dependent on the specific tumor context. In particular several studies identified ATM activity as a promoter of AKT phosphorylation and activity, which in turn sustains proliferation and cellular transformation [133] [62] [69]. These reports are consistent with the observation that ATM may be activated downstream Receptor Tyrosine Kinases [29, 134], as well with the observation that AKT may be activated in response to DNA damage [68, 135, 136]. Furthermore, ATM activation in response to IR has been shown to promote the expression of MET receptor tyrosine kinase [72]. MET, in turn, promotes cell invasion and protects cells from apoptosis, thus supporting radioresistance. Drugs targeting MET increase tumor cell radiosensitivity and prevent radiation-induced invasiveness. Overall, these observations suggest that in some conditions, ATM activity may sustain tumorigenicity by modulating the levels of the receptor as well as by sustaining AKT activation. Therefore its inhibition may be enhance cancer therapy efficacy. However, the inhibition of ATM may be beneficial or conversely detrimental to cancer therapy, depending on the specific context [137], suggesting that extreme caution should be taken in this regard. Further investigation will clarify this issue.

## 8. Functional links between ATM kinase and the immune system defects

A-T patients display several immunological dysfunctions (reviewed in [75]). The central role of ATM kinase in the control and execution of the DNA Damage Response (DDR), along with the observation that DNA damage occurs physiologically to ensure the development and the functionality of the immune system, strongly suggest that most of the immune defects linked to A-T may arise from defects in the DDR. The activation of the DDR is an important component of the V(D)J recombination, a genetically programmed DNA rearrangement process occurring during the early development of lymphocytes that results in assembly of highly diversified antigen receptors essential to functional lymphocytes. De-

fects in repair proteins involved in rejoining V(D)J recombination-induced DSBs preclude the generation of antigen receptors, profoundly compromising T- and B-cell development and causing severe immune deficiencies. The role of ATM in V(D)J recombination has been largely investigated. Immunoglobulin class switch recombination has been shown to be impaired in *Atm*-deficient mice [138]. Moreover, during the V(D)J recombination ATM participates to the stabilization of DNA double-strand-break complexes [139]. ATM may also function directly in end joining, end processing or end protection [140]. A recent study reported also the persistence of chromosomal breaks in actively dividing ATM-deficient peripheral lymphocytes [141] suggesting a role for ATM in cell-cycle control in addition to facilitating DNA repair. However, the mechanism behind the involvement of ATM in the cell-cycle checkpoint during V(D)J recombination, along with the functions of ATM downstream targets responsible for cell-cycle control, has yet to be determined.

AT patients exhibit a wide range of cellular and humoral immune system abnormalities, resulting in variable lymphopenia [142]. The most common abnormalities are the absence or marked reduction of IgA, IgG subclasses and IgE [75, 142]. Moreover, the peripheral T-cell population of both AT patients and *Atm*<sup>-/-</sup> mice is characterized by a bias toward terminally differentiated effector cells, reflected by an extremely low ratio of naive to memory T cells (reviewed in [75]).

As a consequence immunodeficiency is very frequent in A-T and A-T patients display a high predisposition to sinopulmonary infections and bacterial pneumonia and chronic lung disease are a major cause of mortality in these patients. (reviewed in [75]).

Another important abnormality of the immune system linked to A-T pathology is the higher incidence of leukaemia and lymphoma development observed both in mice and in humans deficient for ATM gene expression. The risk of developing a lymphoid neoplasm is increased approximately 200-fold in AT patients compared with the normal population. The vast majority of lymphoid tumors that develop in A-T children are T-cell ALL/lymphoma (reviewed in [75]). Consistently, *Atm*<sup>-/-</sup> mice generally succumb to pre-T-ALL between 3 and 6 months of age [103-105]. A-T patients display characteristic cytogenetic abnormalities involving chromosomes 7 and 14 that result in disruption of antigen receptor loci [74]. Some of these chromosomal translocations may lead to the juxtaposition of a TCR locus and a proto-oncogene like *TCL1* or *MTCPI* (chromosomes 14 and X, respectively). T cells harboring these translocations clonally expand, accumulate additional cytogenetic abnormalities, and eventually develop into leukemias/lymphomas. In addition, ATM is frequently inactivated in sporadic cancers, particularly lymphoid malignancies. Loss of 11q22-23 (the location of the human ATM gene) is often observed in leukemias/lymphomas [73, 111].

A recent study found a surprising role for ATM in promoting the self-renewal capacity of hematopoietic stem cells (HSCs) [108]. The mechanism for HSC depletion in the absence of ATM appears to be increased oxidative stress, suggesting that indeed abnormalities in the oxidative stress response may also contribute to the immune system phenotype in addition to the DDR deficiency [32, 33].

## 9. Functional links between ATM kinase, neurodegeneration and other neurological features

The neurodegenerative phenotype of A-T is the cardinal aspect of the disease. In A-T, the neurodegeneration is progressive and spinocerebellar in nature, and it usually becomes apparent between 6 and 18 months of age. Patients with A-T manifest hallmarks of cerebellar dysfunction such as dyssynergia, muscle hypotonia, truncal swaying while sitting or standing, and sudden falls [1, 21]. Atrophy of the cerebellum, particularly is a key feature of A-T and is evident upon magnetic resonance imaging and computed tomography imaging [21]. Purkinje cell loss is a hallmark feature of A-T and Purkinje cells have less complex arborizations and are often localized ectopically in the molecular layer of the cerebellum [143].

Although extensive effort has been made to understand how ATM deficiency could result in neuronal degeneration, the mechanisms behind neuronal degeneration of A-T are still poorly understood. It has been speculated that defective responses of ATM to DNA damage could be the cause of neuronal degeneration in A-T. However, *Atm*<sup>-/-</sup> mice show compromised function in DNA repair but fail to develop significant neuronal degeneration or exhibit symptoms of ataxia, suggesting a lack of correlation between dysfunction in DNA repair and neuronal degeneration of the A-T disease [103].

Importantly, a substantial amount of ATM resides in the cytoplasm in human and mouse brain, a sub-cellular localization incongruous for a mediator of DDR [Barlow, 2000 #283; Boehrs, 2007 #798; Li, 2012 #820]. In the cytoplasm ATM appears to be involved in the homeostasis of lysosomes [144], in the spontaneous release of synaptic vesicles and in establishment and maintenance of long-term potentiation (LTP) [145].

In contrast with these data, Biton and colleagues showed that ATM is predominantly nuclear in human neuronal-like cells, and that the ATM-mediated response is as robust as in proliferating cells. Knockdown of ATM abolished that response [146]. Similar observations have been obtained from studies in murine cerebellar neurons, in which ATM seems to localize essentially in the nucleus and ATM activation measured by autophosphorylation and downstream signaling is comparable with that in other cell lines. This is supported by genetic evidence showing that MRE11 facilitates the activation of ATM at DNA DSBs, and that patients that are hypomorphic for mutations in *MRE11* (patients with ATLD) have a neuronal phenotype that is similar to that in A-T [12].

Recently, the finding that oxidation can directly activate ATM [30], strongly suggests that the enrolment of ATM in the oxidative stress response may provide a molecular base for some features of neurodegeneration observed in A-T patients, which cannot be explained by the classical ATM function in the DDR. Neurons are cells particularly vulnerable to oxidative stress as shown by the fact that oxidative injury is a key feature of both acute brain pathologies such as stroke and traumatic brain injury and neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease and amyotrophic lateral sclerosis [147]. Neurons have a high energy demand, principally to maintain ion gradients necessary for neuronal signal transmission as well as for the synthesis, the uptake and the

recycling of neurotransmitters. To fulfil this requirement neurons depend principally on oxidative phosphorylation, a process that necessarily generates a certain amount of ROS as a byproduct. At the same time neurons are relatively poor in antioxidant defenses as compared, for instance, to astrocytes [147]. Indeed, loss of ATM enhances intracellular ROS levels, and their aberrant excess, may contribute to the neurodegeneration described in A-T [148]. Although mouse mutants of ATM do not recapitulate the cerebellar degeneration, ATM deficiency has been shown to be associated with increased levels of reactive oxygen species in Purkinje cells [27, 149]. Furthermore, an age-dependent reduction in the number of dopaminergic neurons present in the substantia nigra and striatum has been observed in ATM<sup>-/-</sup> mice, which was accompanied by severe gliosis [150] suggesting that ATM-deficient mice may model some of the neurological defects observed in A-T. Persistent oxidative stress in the ATM-deficient brain disturbs intracellular antioxidant defence systems and redox homeostasis, thereby activating downstream signaling pathways, including those involving p38 and ERK1/2 [149]. Overall these observations suggest that, in A-T, neuronal degeneration and ataxia do not only depend from a defective DDR but are also a consequence of the inability to mount an efficient antioxidant response because of a defective ATM signaling. In light of this information, it is not surprising that treatment with antioxidants prevents Purkinje cell loss [151] and partially corrects neurobehavioral deficits of Atm<sup>-/-</sup> mice [152].

In conclusion, defective DNA damage response associated with ATM deficiency might be sufficient to induce the neurological pathology associated with A-T, but the compounded oxidative stress and DNA repair defects in A-T patients would potentially increase the rate and severity of neurodegeneration.

### 9.1. ATM and neuronal stem cells

In the normal brain, the number of neuronal stem cells (NSCs) is the result of a tightly controlled balance between self-renewal, differentiation, and death [153]. This means that control of proliferation of the neural stem cells/precursor cells plays a critical role in determining the number of neurons, astrocytes, and oligodendrocytes in the brain. Importantly, ATM expression is abundant in neural stem cells (NSCs), but it is gradually reduced as the cell differentiates [152], suggesting that ATM may play an essential role in NSC survival and function. Paul K. Wang's laboratory reported that ATM is required to maintain normal self-renewal and proliferation of NSCs, due to its role in controlling the redox status. Loss of ATM impairs proliferation of neural stem cells through oxidative stress-mediated p38 MAPK signaling [149, 154].

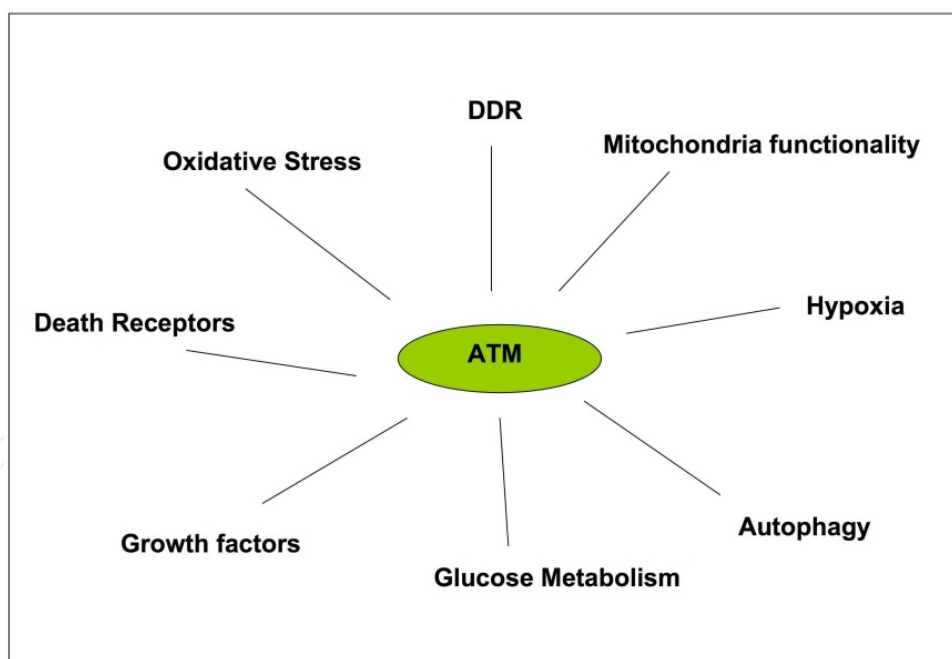
In addition, it is increasingly apparent that stem cell proliferation and maturation require supportive microenvironment including astrocytes. Astrocytes have well-established roles in regulating the microenvironment in the central nervous system, including redox homeostasis. Astrocytes also support stem cell proliferation and maintenance [155-157]. Abnormal neuronal and astrocytic development was reported in ATM knockout mice [151, 152], which could be the result of abnormal differentiation of NSCs. Interestingly, ATM is also required

to maintain survival and proliferation of astrocytes by controlling the redox status of these cells [149].

Recently, Carlessi and colleagues, used a human neural stem cell line model (ihNSCs) to get more insight into the mechanisms of neuronal degeneration in A-T. They could show that ATM plays a central role in terminal differentiation of ihNSCs through its function in DDR [158]. All these data support a role of ATM in the control of neuronal differentiation through its DDR dependent functions and oxidative stress dependent functions and suggest that defective proliferation of NSC could be in part responsible of the neurodegenerative phenotype in *Atm*<sup>-/-</sup> mice and A-T patients.

## 10. General conclusions, remarks and future perspectives

The loss of ATM kinase function leads to A-T, a multisystemic disorder. Contribution from several laboratories allowed, in the recent years, to significantly improve the knowledge on the signalling networks involving ATM kinase. The emerging picture clearly points to ATM as a central player of several cellular functions in addition to the well-established role as master regulator of the DDR.



**Figure 1. Schematic summary of the signalling pathways in which ATM kinase activity has been described as an important player.** ATM activity is modulated by the indicated factors. The molecular mechanisms underneath its modulation and its function deserve further elucidation, as well as the contribution of the individual loss of each function to the development of A-T pathology.

At present there is no therapy to prevent or cure the progression of A-T. It is possible to alleviate some of the symptoms linked to immunodeficiency and deficient lung function, but



neither the cancer predisposition, nor the progressive neurodegeneration, can be prevented. In this regard, the identification of cytoplasmic functions of ATM, and in particular its connection with glucose metabolism and with oxidative stress, provided novel hints for the comprehension of the development of this disorder and suggest possible alternative therapeutic strategies. Treatments with antioxidants and phytonutrients have been suggested as potential treatment strategies. Additional approaches include the employment of read-through drugs to allow the production of ATM kinase in those patients with truncating mutations, and the development of stem cell based therapies (reviewed in [2]). The large amount of information produced by high throughput approaches such as the proteomic studies will deserve further attention and implementation to allow a further step into the elucidation of the networks in which ATM is implicated and of the contribution of each interactor, modulator and substrate of ATM to their functionality.

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