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Deregulation of mTOR signaling is involved in thymic lymphoma development in *Atm*−/− mice

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ABSTRACT

Abnormal thymocyte development with thymic lymphomagenesis inevitably occurs in *Atm*−/− mice, indicating that ATM plays a pivotal role in regulating postnatal thymocyte development and preventing thymic lymphomagenesis. The mechanism for ATM controls these processes is unclear. We have shown previously that c-Myc, an oncoprotein regulated by the mammalian target of rapamycin (mTOR), is overexpressed in *Atm*−/− thymocytes. Here, we show that inhibition of mTOR signaling with its specific inhibitor, rapamycin, suppresses normal thymocyte DNA synthesis by downregulating 4EBP1, but not S6K, and that 4EBP1 phosphorylation and cyclin D1 expression are coordinately increased in *Atm*−/− thymocytes. Administration of rapamycin to *Atm*−/− mice attenuates elevated phospho-4EBP1, c-Myc and cyclin D1 in their thymocytes, and delays thymic lymphoma development. These results indicate that mTOR downstream effector 4EBP1 is essential for normal thymocyte proliferation, but deregulation of 4EBP1 in *Atm* deficiency is a major factor driving thymic lymphomagenesis in the animals.

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Introduction

Ataxia-telangiectasia (A-T) is an autosomal recessive disease. Most A-T patients die of lymphoid tumors or immunodeficiency infection either before or during their teenage years [1]. The *Atm* gene product, ataxia-telangiectasia mutated (ATM), belongs to the phosphoinositide 3-kinase like kinase (PIKK) superfamily of serine/threonine protein kinases [2]. ATM is activated by intramolecular autophosphorylation at serine 1981 following oxidative DNA damage [3]. ATM activation results in phosphorylation of many downstream substrates, triggering cell cycle arrest or apoptosis, both of which prevent the damaged DNA from being replicated and transferred to daughter cells [4]. The *Atm*−/− mice used in this study recapitulate some of the features of human A-T. By 3–5 months of age, all *Atm*−/− mice die of thymic lymphomas [5,6]. These findings suggest that ATM is important in the prevention of tumorigenesis during postnatal thymocyte development, but the mechanisms of its action remain to be elucidated.

It is known that c-Myc is required for thymocyte proliferation during development [7]. As an oncogenic transcription factor, c-Myc regulates cell proliferation, differentiation and apoptosis in many cell types [8,9]. An essential role of c-Myc in prenatal thymocyte development is indicated by the death of *c-myc*−/− mouse embryos at embryonic day 10 with small thymi [10]. During postnatal thymocyte development, c-Myc is expressed at high levels in proliferating $CD4^-/CD8^-$ double negative (DN) thymocytes, at lower levels in $CD4^+/CD8^+$ double positive (DP) thymocytes, and at intermediate levels in mature $CD4^+$ or $CD8^+$ single positive (SP) thymocytes [11].

We have shown that c-Myc levels are progressively increased with age in primary thymocytes from *Atm*−/− mice, and that the upregulation of c-Myc parallels the steady elevation of spontaneous DNA synthesis in these cells, suggesting that deregulation of c-Myc may drive the uncontrolled thymocyte proliferation in *Atm*−/− mice [12]. It appears that deregulation of c-Myc in *Atm*−/− thymocytes is associated with thymic lymphomagenesis, since administration of dexamethasone to *Atm*−/− mice suppresses c-Myc expression [13], and prevents thymic lymphoma development [14].

There are many factors that control c-Myc levels, one of which is the mammalian target of rapamycin (mTOR). This evolutionarily conserved checkpoint protein kinase controls proliferation, differentiation, migration, and survival of cells by regulating protein synthesis [15]. mTOR is known to bind to at least two molecules, raptor (regulatory-associated protein of mTOR) and rictor (rapamycin-insensitive companion of mTOR), forming the mTOR complex1 (mTORC1) and the mTOR complex2 (mTORC2), respectively [16]. When mTOR is autophosphorylated via its intrinsic serine/thronine kinase activity, it phosphorylates the S6 kinase (S6K) and the eukaryotic initiation factor 4E (eIF4E)-binding protein (4EBP1). Phosphorylation of 4EBP1, in turn, results in release of functional eIF4E from the eIF4E/4EBP1 complex, allowing it to become active [17]. Activated eIF4E functions as an mRNA

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cap-binding protein that regulates the synthesis of essential proteins involved in cell cycle progression, such as cyclin D1 and c-Myc [18,19].

The serine/threonine protein kinase AKT, a downstream target of PI3K, is a positive regulator of mTOR activity [20]. PI3K regulates phosphatidylinositol (3,4,5)-trisphosphate (PIP_3). PIP_3, in turn, activates the 3-phosphoinositide-dependent protein kinase 1 (PDK1), which then activates AKT. The phosphatase and tensin homolog deleted on chromosome 10 gene (PTEN) counteracts PI3K in this process by dephosphorylating PIP_3, thereby preventing activation of AKT [21]. Phosphorylations at both sites of T308 and S473 are necessary for full AKT activation [22]. It is now clear that AKT upregulates mTOR by phosphorylating and inhibiting the activity of the tuberous sclerosis complex-2 (TSC2) [23–25], thereby reducing GAP (GTPase-activating protein) activity of TSC2 towards Rheb (Ras homolog enriched in brain), a positive regulator of mTOR [26–28].

In this study, we demonstrate that the mTOR signaling component 4EBP1, but not S6K, is participates in normal thymocyte development, and that deregulation of 4EBP1 and its targeting proteins c-Myc and cyclin D1 in *Atm*−/− thymocyte is critically involved in thymic lymphomagenesis in *Atm* deficient mice. Thus, our findings provide new clues linking ATM and mTOR to thymocyte development and to thymocyte tumorigenesis. These findings may lead to exploration of innovative strategies, such as targeting mTOR with rapamycin, to prevent lymphoid tumors in A-T patients.

Materials and methods

Mice. The *Atm*−/− mice used in this study were created by Barlow et al. [5]. Heterozygous *Atm*+/− mice of this line were purchased from the Jackson Laboratory, mated, and kept in the Animal Center at The University of Texas MD Anderson Cancer Center, Science Park-Research Division. Genotyping was carried out by polymerase chain reaction, as described by Liao et al. [29]. The mice were bred in the institution-accredited specific pathogen-free animal facility under standard conditions with a 14:10 light/dark cycle and were given standard diet and water ad libitum. All studies were reviewed and approved by the Institutional Animal Care and Use Committee of MD Anderson Cancer Center.

Animal treatments. Atm+/+ and *Atm*−/− mice were treated with rapamycin (Calbiochem) in normal saline with 5.2% Tween 80 (Sigma) and 5.2% polyethylene glycol-400 (Sigma) or with the same volume of vehicle, by intraperitoneal (ip) injection every three days or twice a week for indicated period of times.

[^{3}H] thymidine incorporation into DNA. Thymocytes were isolated as described previously [30]. Freshly isolated thymocytes were cultured in 96-well plastic tissue culture plates in 5×10^5 cells/well in the presence or absence of various concentrations of rapamycin (Calbiochem) or of FK 506 (Sigma) at 37 °C for 8 h with RPMI-1640 medium (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco B RL). For the last 4 h of culture, 0.5 μCi of [^{3}H] thymidine (Amersham) was added into each well. By the end of the culture, the cells were harvested and [^{3}H] thymidine incorporation into DNA was measured in a scintillation counter (Packard). Results were expressed as mean counts per min (cpm) ± SD in triplicate cultures.

Western blot analysis. Total cellular extracts were prepared in lysis buffer (50 mM Tris–HCl, pH 7.5, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.25 mM PMSF, 1 mM sodium orthovanadate, 1 mM sodium fluoride, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin). Proteins (30 μg) from each sample were separated on sodium dodecyl sulfate–polyacrylamide gels, transferred to a polyvinylidene fluoride membrane, and immunoblotted with primary antibodies (Cell Signaling) to mTOR, p-mTOR at S2448, 4EBP1, p-4EBP1 at T37/46, cyclin D1, c-Myc, S6K, p-S6K at T389, p-S6K at S371, PTEN, p-PTEN at S380/T382/T383, AKT, p-AKT at T308, p-AKT at S473. The blots were stripped and immunoblotted with a monoclonal anti-β-actin antibody (Sigma), used as a control for protein loading. Since *Atm*−/− thymi are atrophied, thymocytes from 4 to 6 *Atm*−/− mice at the same age were pooled to obtain sufficient proteins for Western blot analysis.

Statistical analysis. The results were expressed as means ± SD, and the statistical significance of the results was determined by analysis with Student's two-tailed, unpaired *t* test. For the experiments testing for rapamycin effects on thymic lymphoma development in *Atm*−/− mice, the statistical significance of the results was determined by the Kaplan–Meier survival/log-rank significance test (Chicago). p values of <0.05 were considered statistically significant.

Results and discussion

Inhibition of mTOR signaling with rapamycin suppresses DNA synthesis and 4EBP1 phosphorylation in normal thymocyte

It is known that the oncoprotein c-Myc is essential for thymocyte proliferation [11]. Since mTOR closely links PI3K/AKT and c-Myc [31], we reasoned that it might play an important role in regulating normal thymocyte development. To confirm that this is so, primary thymocytes from 4-week-old *Atm*+/+ mice were cultured with various concentrations of rapamycin, or with FK 506, a calcineurin inhibitor, for 6 h, and the DNA synthesis of the cells was measured. Fig. 1A shows that, as expected, rapamycin suppresses thymocyte DNA synthesis in a dose-dependent manner, while FK 506 does not. The Western blots in Fig. 1B show that rapamycin (2 μM), but not FK 506 (2 μM), suppresses levels of 4EBP1 at T37/46 (p-4EBP1) in thymocytes treated with rapamycin for 1 h.

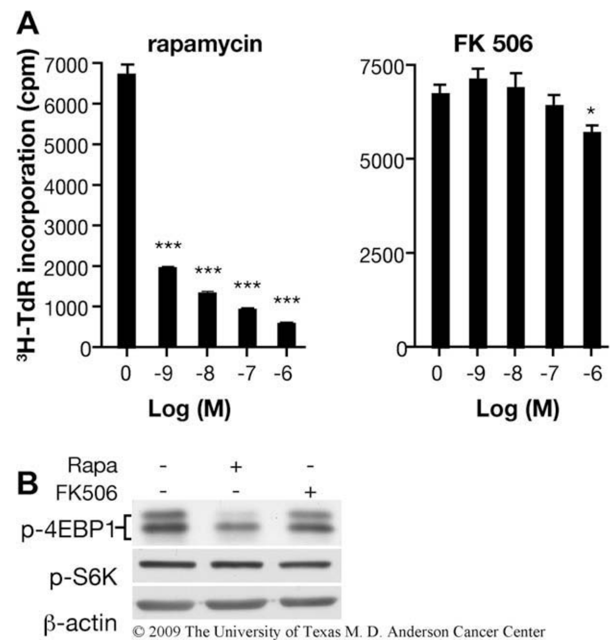

Fig. 1. Effects of rapamycin on thymocyte DNA synthesis and 4EBP1 phosphorylation. (A) Freshly isolated normal thymocytes from 4-week-old *Atm*+/+ mice were cultured with various concentrations of rapamycin, or with FK 506, for 6 h. DNA synthesis was measured by [^{3}H] thymidine incorporation into DNA. $^{***}p < 0.001$; $^{*}p < 0.05$ for drug-treated group vs. untreated controls. (B) Freshly isolated normal thymocytes from 4-week-old *Atm*+/+ mice were cultured with rapamycin (2 μM), or with FK 506 (2 μM) for 1 h. Protein samples were extracted from the cultured thymocytes and screened for p-4EBP1 at T37/46, and p-S6K at T 389, by Western blotting.

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