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ATM kinase activity modulates ITCH E3-ubiquitin ligase activity

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Abstract

Ataxia Telangiectasia Mutated (ATM) kinase, a central regulator of the DNA damage response regulates the activity of several E3-ubiquitin ligases and the ubiquitination-proteasome system is a consistent target of ATM. ITCH is an E3-ubiquitin ligase that modulates the ubiquitination of several targets, therefore participating to the regulation of several cellular responses, among which the DNA damage response, TNFα, Notch and Hedgehog signalling and T cell development.

Here we uncover ATM as a novel positive modulator of ITCH E3-ubiquitin ligase activity. A single residue on ITCH protein, S161, which is part of an ATM SQ consensus motif, is required for ATM-dependent activation of ITCH. ATM activity enhances ITCH enzymatic activity, which in turn drives the ubiquitination and degradation of c-FLIP-L and c-Jun, previously identified as ITCH substrates. Importantly, Atm deficient mice show resistance to hepatocyte cell death, similarly to Itch deficient animals, providing in vivo genetic evidence for this circuit.

Our data identify ITCH as a novel component of the ATM-dependent signaling pathway and suggest that the impairment of the correct functionality of ITCH caused by Atm deficiency may contribute to the complex clinical features linked to Ataxia Telangiectasia.

Keywords

Ataxia Telangiectasia; ATM kinase; ITCH E3-Ubiquitin Ligase; c-FLIP-L; c-Jun; Protein ubiquitination and degradation

INTRODUCTION

Ataxia Telangiectasia (A-T) is an autosomal recessive disorder characterized by neurodegeneration, radiosensitivity and increased cancer predisposition. A-T pathology arises from the loss of functional ATM protein kinase, a central guarantee for the genomic integrity of the cell. ATM is induced by DNA damage and its activity modulates the cellular

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response, mainly through the downstream phosphorylation of target proteins (1, 2). Proteomic studies uncovered more than 700 ATM kinase substrates in response to DNA damage underlying the complexity of the signaling cascade initiated by this kinase. These studies identified the ubiquitin-proteasome system as a relevant target of ATM kinase (3-5). In response to DNA damage ATM phosphorylates and modulates the function of several E3-Ubiquitin ligases, among which the MDM2/MDM4 complex, whose activity and stability is downregulated by ATM, allowing in turn the stabilization of p53 (reviewed in (6)). Similarly, ATM phosphorylates Siah-1 E3-Ubiquitin ligase, impairing its activity and therefore promoting HIPK2 stabilization and p53 pro-apoptotic function (7). Moreover, ATM phosphorylates the RNF20-RNF40 E3 ubiquitin ligase complex recruited to the Double Strand Breakes sites, promoting the monoubiquitination of histone H2B and the recruitment of molecular players in repair pathways (8).

ITCH is an E3-ubiquitin ligase that modulates the ubiquitination of several targets, among which c-FLIP-L, c-Jun and JunB, p73 and p63, Notch and Gli1, therefore participating to the regulation of several cellular responses, such as TNFα, Notch and Hedgehog signalling, differentiation of “naïve” lymphocytes into T helper type 2 (Th2) cells, and DNA damage response (reviewed in (9, 10)). ITCH is a member of the Nedd4 like family of HECT E3 ubiquitin ligases, a family of proteins that participate to several physiological signalling pathways, whose activity is regulated through various molecular mechanisms, including phosphorylation (11). ITCH activity is tightly controlled by intramolecular interactions which block the enzyme in an inactive conformation (12, 13). These inhibitory intramolecular interactions are relieved upon ITCH phosphorylation by JNK1 activity, which triggers ITCH enzymatic activity and in turn promotes the ubiquitination and degradation of ITCH target proteins among which c-Jun and c-FLIP-L (12-14).

The family of c-FLIP proteins plays an essential role in the modulation of death receptor signaling (15). We have recently identified ATM as a modulator of c-FLIP proteins, and subsequently, of death receptor signaling. ATM deficient cells show higher levels of c-FLIP-L and c-FLIP-S proteins and are therefore resistant to death receptor induced apoptosis (16, 17). Furthermore, upon DNA damage, ATM activity promotes c-FLIP-L ubiquitination and degradation (17). Importantly, it has been shown that in response to TNFα, the turnover of c-FLIP-L is enhanced by the activation of the E3 Ubiquitin Ligase ITCH. Consistently, ConcanavalinA (ConA) injection failed to trigger c-FLIP-L down-regulation and hepatocyte cell death in Itch −/− mice (14), pointing to ITCH as an essential modulator of c-FLIP-L protein stability and function.

The observations that ATM modulates c-FLIP-L stability (16, 17) along with the identification of the phosphorylation of ITCH as a molecular mechanism promoting its activity and c-FLIP-L ubiquitination (14)**,** suggest that ATM may promote ITCH enzymatic activity, and in turn, control the ubiquitination and stability of ITCH substrates.

Here we provide evidence that, ATM kinase enhances ITCH ubiquitin ligase activity. Point mutagenesis identified Ser161 as a putative ATM-target site required for the ATM-dependent modulation of ITCH catalytic activity. ATM activity promotes ITCH-dependent ubiquitination of c-FLIP-L and c-Jun, previously identified as ITCH substrate, pointing to ITCH as a novel transducer of ATM signaling. Importantly, similarly to Itch −/− mice, Atm −/− mice are resistant to ConA induced c-FLIP-L down-regulation and induced hepatocyte cell death, providing *in vivo* genetic evidence for our model.

RESULTS

ATM modulates ITCH activity

It has been previously shown that, similarly to other HECT-E3-Ubiquitin ligases, upon activation ITCH can trigger its own auto-ubiquitination, pointing to the auto-ubiquitination level as a valuable marker of enzymatic activation (13, 18-20). Therefore, to ascertain whether ATM may modulate ITCH activity, ITCH-Myc-wt was transfected along with increasing amount of ATM kinase or its catalytically inactive mutant, and Ub-HA. In all the experiments cells were treated with the proteasome inhibitor MG132 for 2 hrs before the preparation of protein extracts to enhance the accumulation of ubiquitinated proteins, and ITCH autoubiquitination was revealed upon immunoprecipitation and immunoblotting. Increasing amounts of ATM kinase but not of its catalytically inactive mutant, significantly augmented ITCH autoubiquitination (Fig. 1A). Interestingly, ATM did not significantly modulate ITCH protein levels suggesting that the enhancement of its autoubiquitination is indeed due to the enhancement of its enzymatic activity (Supplementary Figure S1). As expected, ITCH enzymatic activity is required for its auto-ubiquitination, and ATM failed to promote the autoubiquitination of the catalitically inactive mutant ITCH-CA, (ITCH-C830A) (Fig. 1B). Importantly, ATM expression did not significantly modulate the autoubiquitination of Nedd4, another component of the HECT family of E3 ubiquitin ligases, supporting the idea that ATM activity selectively promotes ITCH activation (Fig. 1C).

ATM enhances the ubiquitination of ITCH Ubiquitin-E3-Ligase target proteins

To further ascertain whether ATM may modulate ITCH activity we asked the question whether ATM could promote the ITCH-dependent ubiquitination of ITCH substrates. c-FLIP-L protein has been identified as ITCH substrate (14), and we have previously shown that ATM activity may modulate c-FLIP-L ubiquitination (16, 17). Therefore, HEK-293T cells were transiently transfected with c-FLIP-L and Ubiquitin-HA, and ITCH-wt or ITCH-CA (ITCH-C830A, defective for its enzymatic activity), in the presence or in the absence of ATM. Cells were treated with MG132 to allow the accumulation of ubiquitinated proteins. As expected, ITCH-wt but not ITCH-CA expression enhanced c-FLIP-L ubiquitination and more importantly ATM significantly increased the ubiquitination of c-FLIP-L. (Fig 2A and Supplementary Figure S2). Conversely, ATM failed to modulate c-FLIP-L ubiquitination in the absence of the enzymatically competent ITCH, suggesting that ITCH activity is required for ATM-dependent c-FLIP-L ubiquitination. Furthermore ATM failed to increase c-FLIP-L ubiquitination in the presence of Nedd4, another component of the HECT family of E3 ubiquitin ligases (9), supporting the idea that ATM enhances c- FLIP-L ubiquitination by the selective modulation of ITCH activity (Fig 2B). To ascertain whether ATM may modulate the ubiquitination of other ITCH targets we tested whether ATM may promote the ITCH-dependent ubiquitination of c-Jun, previously identified as an ITCH target (12). As a control, cells were transfected with ΔMEK kinase, a constitutively active mutant of MEK kinase able to induce JNK1 activity and to promote ITCH activity and c-Jun ubiquitination (12, 13). As expected, ITCH overexpression enhanced the level of c-Jun protein ubiquitination and, more importantly, c-Jun ubiquitination was further increased by ATM expression, similarly to what observed in the presence of ΔMEK kinase, (Fig. 2C) supporting our hypothesis. Conversely, ATM failed to enhance the levels of ITCH-dependent p73 ubiquitination (21), suggesting that although ATM modulates ITCH activity it may not modulate the ubiquitination and therefore the stability of all ITCH substrates (Supplementary Figure S3).

Endogenous ATM activity may transiently promote ITCH activity in response to DNA damage

As ATM plays a central role in the DNA damage response, the observation that ATM kinase activity induction may enhance ITCH activity raised the question on how ATM may contribute to ITCH modulation in response to DNA damage. To this aim, HEK293T cells transiently transfected with ITCH and Ub-HA were treated with Neocarzinostatin (NCS) for different times to induce DNA damage and trigger endogenous ATM activation and then incubated with MG132 to prevent the degradation of ubiquitinated proteins. Importantly, DNA damage triggers the induction of ATM kinase, as expected, which positively correlates with an early and transient induction of ITCH autoubiquitination. Later on ITCH activity is down-regulated (Fig. 3A). Moreover we could show that ITCH autoubiquitination is significantly increased in response to DNA damage, induced by NCS treatment, only in those cells expressing endogenous ATM, while the genetic inhibition of ATM expression strongly impaired ITCH activation (Fig. 3B). Since NCS may trigger ATM activation already upon few minutes of treatment, we performed a shorter kinetic experiment. As expected, ATM activation was induced by NCS already upon 15 min of treatment and this event significantly preceded the induction of ITCH activity, which was detectable and reached its maximum at 1hr and then decreased (Supplementary Figure S4). The observation that in response to DNA damage ATM activity can modulate ITCH activity in a narrow window of time suggest that additional components may contribute to this interplay and will deserve further investigation.

We could show that in response to DNA damage ATM activity may transiently promote ITCH autoubiquitination. Consistently, endogenous ATM expression and activation is also required for the induction of c-FLIP-L ubiquitination triggered by DNA damage (Fig. 3C). Similarly, the treatment of cells with the ATM kinase inhibitor KU-55933 resulted in the downregulation of ITCH-dependent c-FLIP ubiquitination triggered by ATM overexpression as well as by DNA damage (Fig. 3D).

ATM modulates ITCH activity through Ser161

We next sought to investigate the molecular mechanism through which ATM modulates ITCH activity and ITCH-dependent c-FLIP-L ubiquitination.

The analysis of ITCH protein sequence revealed 9 Ser/Thr residues as potential ATM target sites identified because of their localization as part of S/T–Q motifs. Moreover, S161 and S430 received the best NetPhos and ScanSite scores respectively as putative phosphorylated sites. Importantly, immunoblotting experiments using an antibody specifically directed against phosphorylated Ser residues, support the hypothesis that ATM activity may promote ITCH phosphorylation on Ser residues (Fig.4A). To assay whether S161 and S430 residues may be directly targeted by ATM-dependent phosphorylation, we generated unphosphorylatable mutants by substitution of the S residues with A residues. ATM failed to enhance ITCH-S161A autoubiquitination (Fig. 4B) while it promoted the autoubiquitination of ITCH-wt as well as of ITCH-S430A (Fig. 4C). Interestingly, S161 is dispensable for the induction of ITCH autoubiquitination in response to ΔMEK kinase overexpression (Fig. 4D), a constitutively active mutant of MEK kinase which has been previously shown to be able to induce JNK1 and trigger ITCH phosphorylation and activation (12, 13), supporting the conclusion that the S161A mutant can still be activated and that S161 is a major and specific target of ATM kinase activity. Consistently with the auto-ubiquitination experiments, ITCH-S161A mutant, but not ITCH-S430A mutant, failed to enhance c-FLIP-L protein ubiquitination in the presence of ATM (Fig 5 A and B). Moreover, ITCHS161A mutant was unable to augment c-Jun protein ubiquitination in the same conditions (Fig. 5C).

Overall these data suggest that S161 is necessary to enable the ATM-dependent modulation of ITCH enzymatic activity towards its substrates.

We then sought to investigate the molecular mechanism through which phosphorylation on S161 may drive ITCH activation. To this aim we tested the hypothesis that ATM-dependent phosphorylation on S161 may lead, similarly to what reported for JNK-dependent phosphorylations, to the release of the intramolecular interaction between the HECT domain and the WW domains, that has been shown to engage the enzymatic domain in a catalytically inactive conformation. HEK293T cells were transfected with ITCH-wt or ITCHS161A in the presence or in the absence of ATM (Fig. 6A). We could show that as expected, GST-HECT fusion could pull down ITCH from cells transfected with ITCH. Importantly ATM cotransfection, significantly reduced the pull down efficiency, suggesting that S161 phosphorylation may destabilize the binding to the HECT domain (Fig. 6B). Consistently, ATM failed to modulate the interaction of the HECT domain with the ITCHS161A unphosphorylatable mutant (Fig. 6C).

S161 is required for ATM-ITCH-dependent downregulation of FLIP

The observation that ATM activity is required for the enhancement of c-FLIP-L ubiquitination in response to DNA damage (Fig. 3) is in agreement with our previous report showing that ATM activity is required for the DNA damage dependent down regulation of c-FLIP-L protein levels in HepG2 cells (17). Interestingly, the down-regulation of ITCH expression by specific shRNA in the same cellular system resulted in the significant delay of c-FLIP-L down-regulation in response to DNA damage and ATM activation (Figure7A). To evaluate whether AT-dependent phosphorylation on S161 is required for ITCH ability to down-regulate c-FLIP levels in response to DNA damage, HepG2 cells where silenced for ITCH expression were reconstituted either with ITCH-wt or with the ITCHS161A mutant (Fig. 7B). As expected ITCH wt expression rescued the ability of DNA damage to drive endogenous c-FLIP degradation while ITCHS161A mutant failed to recover this phenotype (Fig. 7C). Overall these data unambiguously show that in response to DNA damage, ATM activity transiently instructs ITCH through S161 and this signalling cascade allows c-FLIP-L down-regulation.

To further investigate how the ATM-ITCH-FLIP pathway may modulate the DNA damage response we asked how this signaling may contribute to regulation of cell cycle and apoptotic markers. In response to DNA damage, ITCH expression is required for FLIP protein downregulation and this event correlated with a faster decrease of cyclin D1 expression and with a significant decrement of p53 phosphorylation on S15 and on S46 (Fig. 7D). These data suggest that in response to DNA damage ITCH activity may promote G1-S cell cycle checkpoint and delay the apoptotic response. This is consistent with reports that point to c-FLIP as a promoter of cyclin D1 expression and of cell proliferation (22). Indeed, we could show c-FLIP expression interference significantly reduced the rate of cell proliferation (Fig. 7E),

ATM activity modulates ConA induced hepatocyte cell death

It has been shown that Itch-deficient mice are resistant to ConA-induced acute liver failure, and hepatocytes derived from these mice do not display inducible c-FLIP-L degradation (14). To test the hypothesis that ATM kinase activity may contribute to modulate ITCH activity and c-FLIP-L degradation *in vivo*, the ability of ConA to trigger massive hepatocyte cell death in wt and in *Atm* −/− mice was investigated. As expected, ConA injection triggered FLIP-L down-regulation in wt mice. Conversely, the decrease of c-FLIP-L protein levels is significantly compromised in *Atm* −/− mice (Fig. 6A). The failure to decrease c-FLIP-L protein levels has been linked to a severe impairment of the induction of cell death

in response to ConA injection in Itch −/− mice. Consistently with the observation that ATM is necessary for c-FLIP-L down-regulation, ConA injection triggers Caspase-3 activation and PARP cleavage only in wt mice (Fig. 8A). Importantly, ConA injection significantly induced ATM kinase activity further supporting the hypothesis that ATM activation may be required to trigger a death signaling cascade in this context (Fig. 8B). Indeed, we could show that *Atm* −/− mice are highly resistant to cell death induction in the liver (Fig. 8C and Supplementary Fig. S5A). ATM expression and activity seems to sustain JNK1 activation, as the induction of JNK1 activity is slightly compromised in Atm −/− mice (Supplementary Fig. S5B). We can conclude that ATM and ITCH modulate the balance between death and survival of hepatocytes, as both are required for the down-regulation of c-FLIP-L protein levels. These experiments genetically support *in vivo* the link between ATM and ITCH enzymatic activities.

DISCUSSION

We identify ATM as a novel modulator of ITCH E3-ubiquitin ligase. The molecular mechanisms that ensure ITCH enzymatic activity regulation have only partially been elucidated. It has been proposed that a complex of intramolecular interactions between the WW domain and the HECT domain block the enzyme in an inactive conformation (12, 13). Phosphorylation events ensure the fine modulation of ITCH activity: JNK1 kinase phosphorylates ITCH in S199, S232, and T222 causing the release on the inhibited conformation and therefore allowing ITCH activation. Conversely, tyrosine phosphorylation may negatively modulate the ability of ITCH to selectively bind and ubiquitinate some of its targets such as JunB and c-Jun (23, 24). Our data demonstrate that ATM may directly modulate ITCH. A single point mutation in S161, which is part of an ATM SQ consensus motif, makes ITCH significantly insensitive to ATM activation. Pull down experiments allow to speculate that S161 phosphorylation destabilizes the inhibitory intramolecular interaction mediated by the HECT domain similarly to what described for the S/T residues targeted by JNK activity (13).

We report that in response to DNA damage ATM kinase activation precedes an early and transient induction of ITCH E3-Ub ligase activity, which is then down-regulated, consistently with a reduction of its protein expression levels and with p73 stabilization previously described (21). We provide evidence for the ability of ATM activity as modulator of ITCH-dependent ubiquitination of c-FLIP. This is consistent with our previous reports that identify ATM as a regulator of c-FLIP-L protein stability and describe c-FLIP-L downregulation in response to DNA damage (16, 17).Although the significance of this event has not been completely elucidated yet, evidence obtained in different systems indicate that c-FLIP may not only impair apoptosis but also positively promote cell proliferation (22, 25), suggesting that ATM may promote transiently ITCH activation to allow the down-regulation of c-FLIP and promote cell cycle arrest in response to DNA damage. Indeed, we could show that c-FLIP down-regulation correlates with the decrease of cyclin D1 expression, and with reduced cell proliferation, supporting our hypothesis. Interestingly, we reported that ATM activity enhances also ITCH-dependent ubiquitination of c-Jun, suggesting that indeed ATM may impinge on the protein levels of several ITCH targets. It has been previously shown that the AP1 pathway is constitutively induced in Atm-deficient mouse brain (26). Consistently, preliminary results support the hypothesis ATM activity may downregulate c-Jun protein levels (data not shown). Future experiments will further elucidate the molecular mechanism that link ATM and c-Jun proteins and more importantly the functional significance and this connection. Conversely ATM overexpression does not modulate ITCH ability to trigger p73 ubiquitination. This is in agreement with the observation that drugs that trigger DNA damage did not downregulate p73 protein levels even at early times of treatment (21), when we could detect ITCH activation and suggests that ATM activity at

early time points may direct ITCH activity selectively on a subset of its substrates involved in cell cycle control more than in apoptosis. Future experiment will better elucidate the underneath molecular mechanisms and clarify whether in addition to NCS and Adriamycin, other DNA damaging agents may exert the same effect.

One open question is whether ITCH activation in response to DNA damage may in turn modulate ATM protein expression in response to DNA damage. Preliminary experiments suggest that indeed ITCH overexpression may down-regulate endogenous ATM protein levels independently on proteasome activity (data not shown). Further studies will clarify this issue.

It has been previously shown that ConA failed to trigger c-FLIP downregulation and apoptosis in ITCH deficient mice as well as in Jnk1 KO animals. These observations suggested that Jnk1 may modulate ITCH activity in vivo (14). We could show that similarly to JNK1-ITCH signalling, the ATM-ITCH signalling cascade may impinge on c-FLIP-L protein levels also *in vivo.* Similarly to what reported for ITCH deficient and for JNK1 deficient mice, Atm KO livers, fail to down-regulate c-FLIP-L protein levels following ConA injection and consistently, display resistance to cell death. Interestingly, JNK1 activation is partially compromised in Atm KO mice, suggesting that ATM may impinge on ITCH activity both directly as well as through JNK1. This observation is in agreement with previous studies where the crosstalk between ATM and JNK1 has been reported (27). Further experiments will be necessary to formally address the requirement of ATM and JNK1 for ITCH activity modulation. Conditional ablation of c-FLIP in hepatocytes enhances death receptor mediated apoptosis and toxic liver injury *in vivo* (28), suggesting the idea that ATM and ITCH may exert the same response in this context because of their convergence on c-FLIP protein regulation. Recent evidence identify ATM activity as a central player for the induction of steatoptosis in non-alcoholic fatty liver disease (29) further supporting ATM signaling as an essential promoter of hepatocellular apoptosis.

The identification of ITCH as a novel target of ATM raises the question on the significance of the impairment of the correct functionality of ITCH in the development of the complex clinical features of A-T. As both ITCH and ATM have been implicated in the immune system proficiency (30, 31), it will be important to ascertain whether ATM and ITCH may be functionally linked in this context. To further ascertain the functional significance of the ATM-ITCH signalling cascade it will be intriguing to address whether ATM activity may modulate the stability of a panel of ITCH substrates and to clarify the significance if any, of the loss of their modulation, in A-T pathology. Importantly, ITCH modulates the ubiquitination of several targets, among which c-FLIP-L, c-Jun and JunB, p73 and p63, Notch and Gli1, therefore participating to the regulation of several cellular responses (reviewed in (9, 10)). As cerebellar neurodegeneration is a major feature of A-T, it will be challenging to test the hypothesis that Atm deficiency may result in the aberrant regulation of Gli-1 protein, a central player of Hedgehog signaling, down-regulated during cerebellum differentiation and aberrantly up-regulated in medulloblastoma (32), recently identified as ITCH substrate (20, 33). To summarise, we identify ITCH ubiquitin ligase as a novel target of ATM kinase activity and suggest that the loss of its modulation may contribute to A-T.

METHODS

pcDNA3-Flag-ATM-wt and pcDNA3-Flag-ATM-kin^{-} were kindly provided by M. Kastan. The shATM construct, generously provided by Y. Lerenthal and Y. Shiloh, had the following sequence: 5′ GACTTTGGCTGTCAACTTTCG 3′ (34). Control shRNA, siR5, had the following sequence: 5′ GGGATATCCCTCTAGATTA 3′ (35). The shITCH constructs (shITCH2 seq 5′ AAACATTAAAGTCAAACAATATG 3′) has been described

in (36); shFLIP has been kindly provided by H. Walczak (37); Flag-Ubiquitin (kindly provided by S. Polo), HA-Ubiquitin (kindly provide by D. Bohmann), pcDNA3 Myc-ITCH WT and pcDNA3 Myc ITCH C830A, prK5 ITCH WT, prK5 ITCH C830A and GST-HECT plasmid, were kindly provided by M. Rossi and G. Melino and described in (21). pCR3.V64-Met-Flag FLIP-Long kindly provided by J. Tschopp), ΔMEK kinase (ΔMEKK1, kindly provided by D. Bohmann). ITCH-S161A and ITCH-S430A constructs were generated using the QuickChange site-directed mutagenesis kit (Stratagene) using pCDNA3-Myc-ITCH-wt as template.

Antibodies and other reagents

The following antibodies and reagents were used: anti–phospho-Ser1981-ATM (Cell Signaling, Beverly, MA), anti-ATM (2C1; Santa Cruz Biotechnology), anti-FLIP (S and L) (H-202 rabbit polyclonal; Santa Cruz Biotechnology), anti-FLIP (S and L) (NF6; Alexis Biochemicals, Farmingdale, NY), anti-a-Tubulin (Sigma, St Louis, MO), anti-ITCH (BD Transduction Laboratories), anti-c-Myc (9E10) (sc-40 mouse monoclonal; Santa Cruz Biotechnology), anti-phospho-SAPK/JNK (Thr138/Tyr185; Cell Signaling), anti-SAPK/JNK (Cell Signaling), anti-GST: (GE Antibodies, 274577-50), monoclonal anti-HA peroxidase conjugate clone HA-7 (Sigma, St Louis, MO), anti-PARP (Cell Signaling), anti-cleaved Caspase-3 (Asp175 antibody; Cell Signaling), anti- Phosphoserine (ab9332; Abcam), anti-HA (12CA5; Roche), Anti-Ubiquitin (FK-2; Upstate, Billerica, MA). Neocarzinostatin (NCS), MG132 were commercially available (Sigma, St Louis, MO) as well as KU-55933 (Calbiochem), Adriamycin (Sigma) was kindly provided by S. Soddu.

Cell culture and transfections

Cells were cultured and transfected as previously described (17, 38).

Briefly, HEK-293T and HepG2 cell lines were maintained in DMEM containing 10% fetal bovine. HEK-293T cells were transfected with the calcium phosphate precipitation method as previously described while HCC cell lines were stably transfected with psuper-shControl and psuper-shITCH, with Lipofectamine 2000 (according to Invitrogen protocol) and positive clones were selected in the presence of Puromycin (2 μg/ml).

Immunoblotting and Immunoprecipitation

Cell extracts were prepared in lysis 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, 1 μg/mL aprotinin) (16). Liver extracts were prepared in RIPA Buffer (150 mM NaCl, 1% NP-40, 0.5%DOC-deoxycholic acid- 0.1% SDS, 50mM Tris pH 8.0). For immunoblotting, 50μg protein extract were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto nitrocellulose membrane, and detected with specific antibodies.

For Immunoprecipitation protein extracts prepared in lysis buffer were pre-cleared to reduce non-specific binding of proteins to sepharose beads. The samples were incubated for 2hrs with several specific antibodies against Flag-tag, Myc-tag and HA-tag. Then were added G- or A- coupled sepharose beads to precipitate the antibody/antigen complex (Amersham). Immunocomplex were then resolved and analysed by SDS–PAGE. All immunoblots were revealed by ECL (Amersham).

GST pull down

Lysates of HEK293T transfected cells were incubated with GST and GST-HECT proteins bounded to Gluthatione Sepharose beads for 2 hours in binding buffer (4- (2-

hydroxyethyl)-1-piperazineethanesulfonic acid 20mM, $MgCl_2$ 2mM, KCl 100mM 20% glycerol, EDTA 0.2 mM, 0.05% NP-40 and protease inhibitors DTT, phenylmethylsulfonyl fluoride 2mM, NaF 0.5M, βGlicerophosphate 20mM). Beads were washed 3-4 times with wash buffer (binding buffer described above) and analyzed by immunoblotting.

MTT Cell Proliferation Assay

About 3000 cells per well (HepG2-pSuper and HepG2-shFLIP) were seeded in 96- well plates and cultured 24 hours for re-attachment.Proliferation of cells was analyzed using assessed Cell Titer Glo Luminescent Assay (Promega). Growth index was calculated as optical density of the sample after 24h, 48h and 72h of culture divided by the optical density of cells at 24h of culture.

Mice and liver injury model

Experiments using animals were performed in accordance with the European Community Council Directive of 24.11.1986 on the protection of animals used for experimental purposes (86/609/EEC). All experiments were performed in compliance with the Tor Vergata University Institutional Animal Care.

The Atm −/− strain was developed in the laboratory of Dr. Anthony Wynshaw-Boris at the National Institutes of Health. These mice are now commercially available (Jackson129S6/SvEvTac-Atmtm1Awb/J).

ConA was injected i.v. at 25mg/kg in PBS. Animals were sacrificed and livers were fixed in 10% formaldehyde, dehydrated, embedded in paraffin, and sectioned (5 mm). Sections were stained with TUNEL Assay Cell Death Detection Kit, POD (Roche Applied Science).

For the immunohistochemistry experiments, formalin-fixed paraffin-embedded liver specimens from Atm wt and ATM −/− mice were cut on SuperFrost Plus slides (Menzel-Glaser, Braunschweig, Germany). Two micron-thick sections were stained with a streptavidin-enhanced immunoperoxidase technique (Supersensitive Multilink, Novocastra, Menarini Florence, Italy) in an automated autostainer (Bond Max, Menarini) using the ATM-p monoclonal antibody (clone 7C10D8, Rockland, tebu-bio, Italy), and a pH 6 citrate buffer antigen retrieval protocol.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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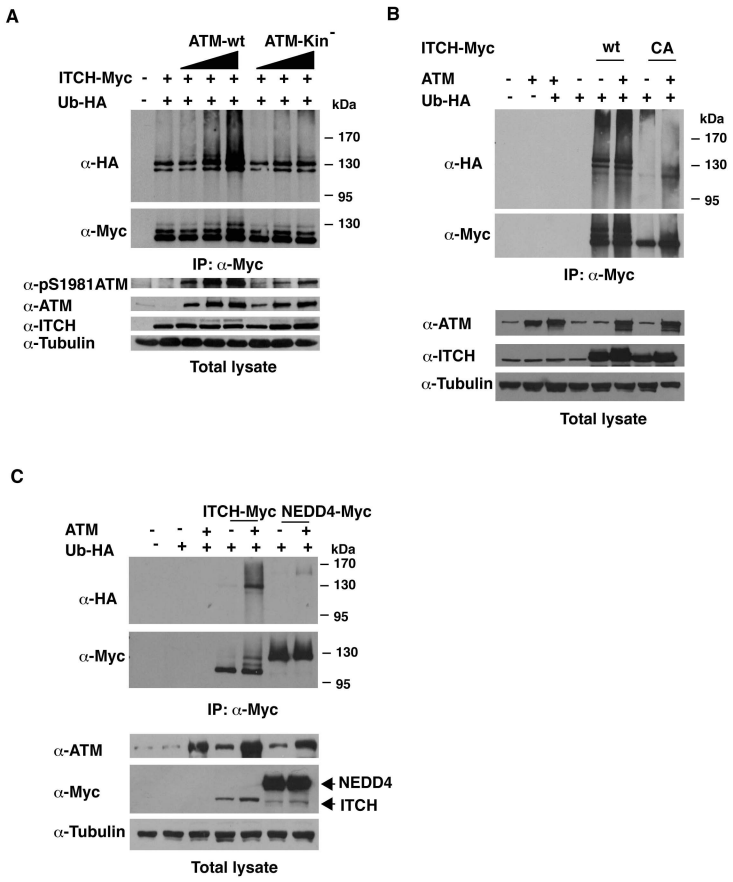

Figure 1. ATM activity specifically enhances ITCH activity

A) ATM activity is required to increase ITCH autoubiquitination. ITCH-Myc was transfected along with increasing amount of ATM-wt or ATM-Kin- in the presence of Ub-HA and its autoubiquitination revealed upon ITCH immunoprecipitation and immmunoblotting. **B)** ITCH autoubiquitination is dependent on ITCH activity. ITCH-wt or the enzymatically inactive ITCH-CA constructs were immunoprecipitated from protein extracts described in Fig. 1A and ITCH proteins autoubiquitination revealed upon immunoprecipitation with anti-Myc antibodies and immunoblotting with anti-HA antibodies. **C)** ATM activity does not modulate NEDD4 autoubiquitination. ITCH or NEDD4 ubiquitin ligases were immunoprecipitated from protein extracts described in Fig. 1B and their autoubiquitination revealed upon immunoprecipitation with anti-Myc antibodies and immunoblotting with anti-HA antibodies.

In all experiments cells were incubated for 2 hrs with MG132 (10μM) to enhance protein ubiquitination..

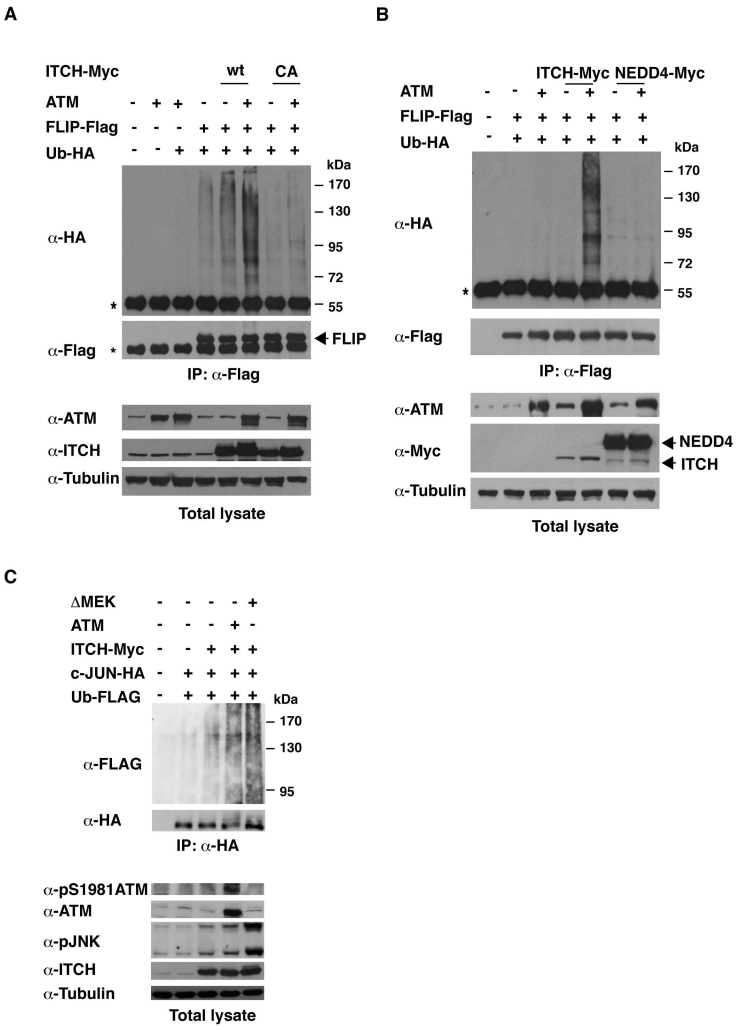

Figure 2. ATM kinase activity promotes ITCH ability to ubiquitinate its substrates

A, B) HEK-293T cells were transfected with the indicated constructs and FLIP-L ubiquitination revealed by immunoprecipitation and immunoblotting. The * points to the cross-reacting heavy chain of the IgG. **C**) HEK-293T were transfected with the indicated constructs and c-Jun-HA ubiquitination revealed upon immunoprecipitation and immunoblotting. ΔMEK kinase transfection was used as a positive control that enhances ITCH-dependent ubiquitination of c-Jun.

In all the experiments cells were incubated for 2 hrs with MG132 (10μM) to enhance protein ubiquitination.

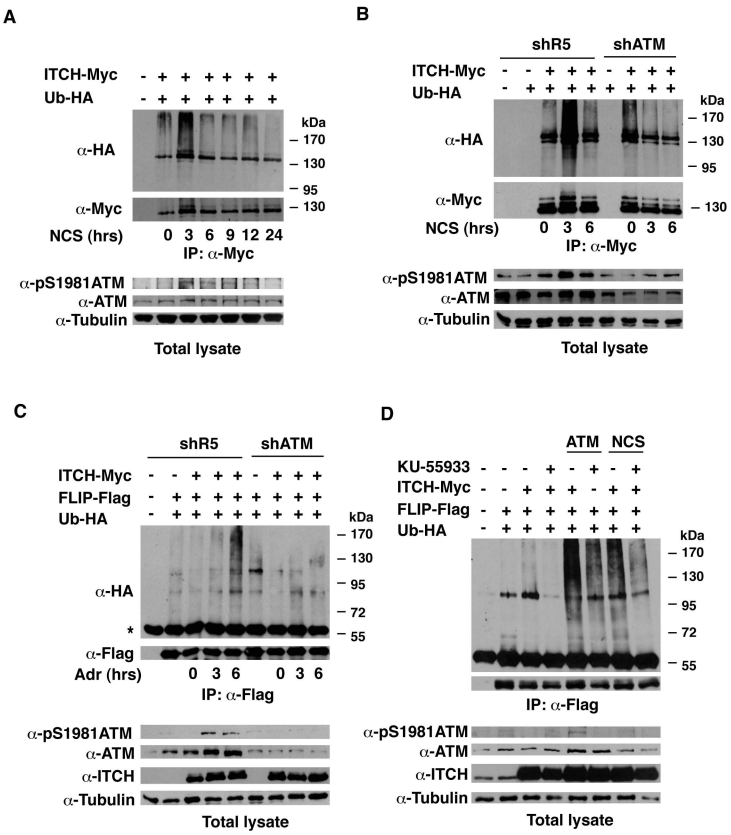

Figure 3. Endogenous ATM activity promotes ITCH activity in response to DNA damage

A) ITCH autoubiquitination is transiently enhanced in response to DNA damage. ITCH-Myc was transfected along with Ub-HA and DNA damage was triggered by NCS (500ng/ml) for the indicated times. ITCH autoubiquitination was revealed as in Fig.1. **B)** ATM activation in response to DNA is required for the transient enhancement of ITCH autoubiquitination levels. HEK-293T cells were transiently transfected with the shATM plasmid or with the shSR5 as a control, along with the indicated constructs. DNA damage was induced by NCS (500ng/ml) for the indicated times. ITCH autoubiquitination was revealed as in A. **C)** ATM activation in response to DNA damage is required for induction of c-FLIP protein ubiquitination. HEK-293T cells were transiently transfected with the shATM plasmid to interfere ATM expression or with the shSR5 as a control, along with the indicated constructs. DNA damage was induced by Adriamycin (Adr, 3μM) treatment for the indicated times. FLIP-L ubiquitination was revealed as described in A. **D)** Inhibition of ATM kinase activity downregulates ITCH dependent ubiquitination of c-FLIP. HEK-293T cells were transiently transfected with the indicated constructs and ITCH activity induced either by ATM overexpression or by NCS treatment. ATM activity was modulated by incubation with the ATM kinase inhibitor KU-55933 (10μM). FLIP ubiquitination was analyzed as in A. In all experiments cells were incubated for 2 hrs with MG132 (10μM) to enhance protein ubiquitination.

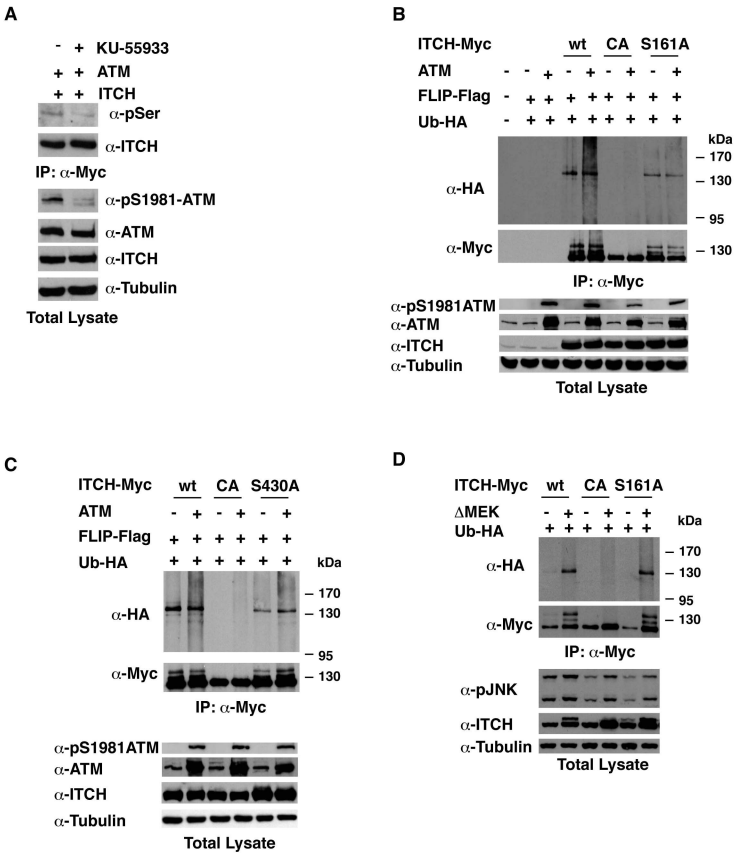

Figure 4. Ser161 of ITCH is required for ATM-dependent ITCH activation

A) ATM kinase activity promotes ITCH phosphorylation on Ser. HEK-293T cells were transfected with ITCH-myc and ATM plasmids, incubated or not with ATM inhibitor KU-55933 (10μM) for 16 hrs. ITCH phosphorylation on Ser was revealed upon immunoprecipitation and immunoblotting. **B, C)** ITCH S161 residue is required for the ATM-dependent enhancement of ITCH activity. HEK-293T cells were transfected with the indicated constructs. ITCH autoubiquitination levels from protein extracts were revealed upon immunoprecipitation and immunoblotting. **D)** ITCH S161 residue is dispensable for ΔMEKK-dependent enhancement of ITCH activity. HEK-293T cells were transfected with the indicated constructs and ITCH autoubiquitination revealed as in B. In all experiments cells were incubated with MG132 as in Fig.1.

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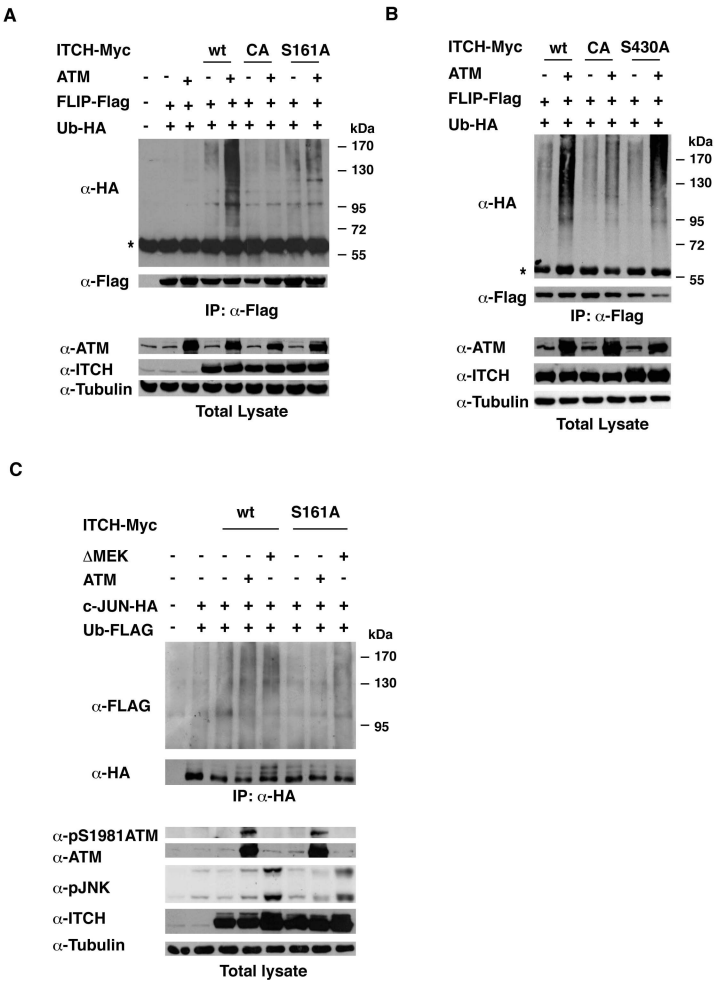

Figure 5. Ser161 of ITCH is required for ATM dependent modulation of ITCH substrates ubiquitination

A, B) ATM modulates c-FLIP-L protein ubiquitination through Ser161. HEK-293T cells were transfected with the indicated constructs. FLIP-L ubiquitination was revealed upon immunoprecipitation and immunoblotting. as described in Fig. 2. **C)** ATM modulates c-Jun protein ubiquitination through Ser161. HEK-293T cells were transfected with the indicated constructs. c-Jun ubiquitination was revealed upon immunoprecipitation and immunoblotting.

In all experiments cells were incubated with MG132 as in Fig.1.

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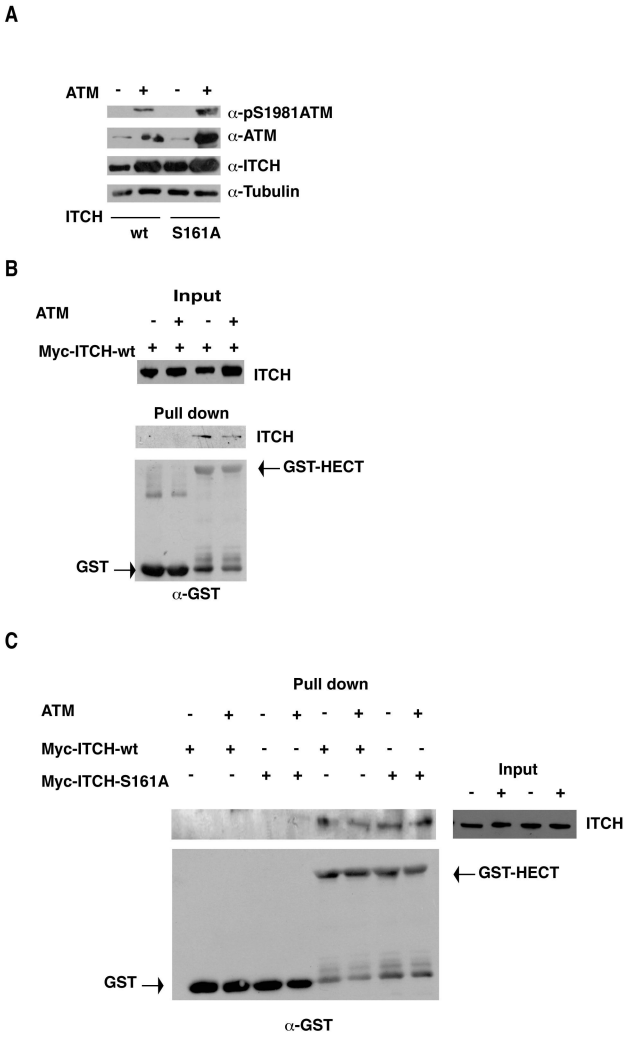

Figure 6. ATM dependent phosphorylation of ITCH on Ser161 impairs ITCH interaction with the HECTdomain

A) HEK 293 T cells were transiently transfected with Myc-ITCH wt, Myc-ITCH S161A in the presence or in the absence of Flag-ATM. Protein expression was revealed by immunoblotting.

B, C) Protein extracts were subjected to pull down assay using GST-HECT fusion protein or GST as control, bound to glutathione sepharose beads. ITCH bound protein was revealed by immunoblotting using anti-ITCH antibodies.

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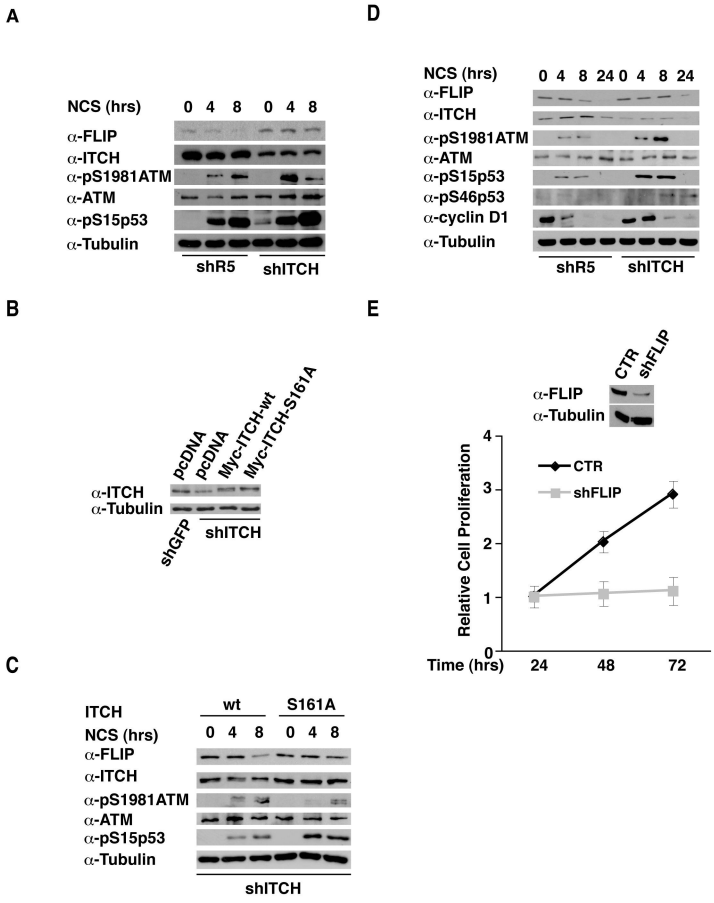

Figure 7. ITCH dependent downregulation of c-FLIP promotes cell cycle arrest in response to DNA damage

A) ITCH Ubiquitin ligase is required for the ATM-dependent downregulation of c-FLIP-L protein levels in response to DNA damage. HepG2 cells were interfered or not for ITCH expression and ATM activation triggered by NCS treatment (500ng/ml for the indicated times. The levels of expression of the proteins of interest were revealed by immunoblotting with specific antibodies. **B)** HepG2 cells stably interfered for ITCH were reconstitute for Myc-ITCH-wt or Myc-ITCH-S161A expression. The levels of expression of the proteins of interest were revealed by immunoblotting with specific antibodies. **C)** S161 is required for ITCH dependent downregulation of c-FLIP in response to DNA damage. HepG2 interfered or not for ITCH expression were reconstituted by transient transfection with ITCH-wt or ITCHS161A mutant. ATM activation was triggered by NCS treatment as in A. The levels of expression of the proteins of interest were revealed as in A. **D)** ITCH expression enhances c-FLIP-L, cyclin D1 and p53 phosphorylation downregulation in response to DNA damage. HepG2 cells were transiently interfered or not for ITCH expression and DNA damage induced by NCS treatment as in A. The levels of expression of the proteins of interest were revealed as in A. **E)** FLIP protein interference results in a reduction of cell proliferation. HepG2 cells were stably interfered for FLIP expression. Interference was verified by immunoblotting with specific antibodies. 3000 cells per well were seeded in 96- well plates and cultured 24 hours for re-attachment. Proliferation of cells was analyzed using assessed Cell Titer Glo Luminescent Assay (Promega). Growth index was calculated as optical

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density of the sample after 24h, 48h and 72h of culture divided by the optical density of cells at 24h of culture.

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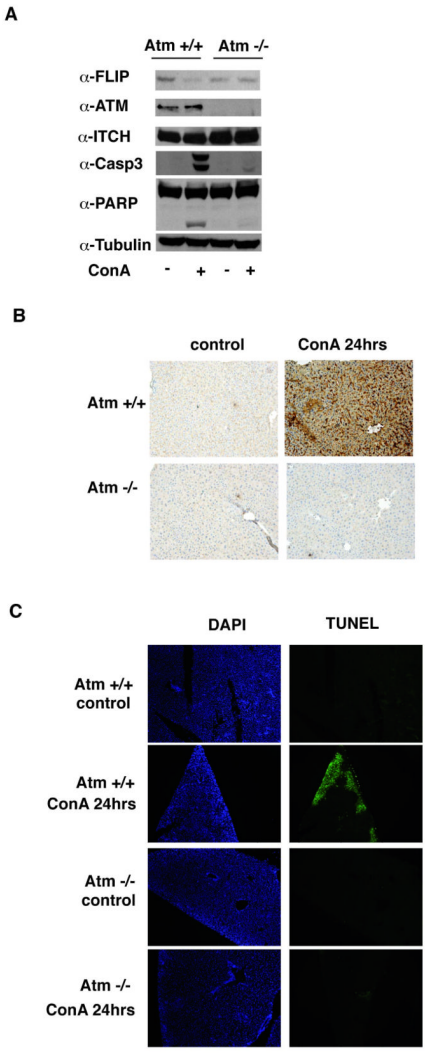

Figure 8. ATM is required for ConA induced hepatic failure

A) Liver extracts were subjected to immunoblotting analysis of the indicated cell death markers. This figure is representative of three different experiments. **B)** Atm+/+ mice (n=3) and Atm −/− mice (n=3) received a ConA injection (25mg/kg) via tail vein. ATM activation in liver tissues was revealed by immunohistochemistry using anti-pS1981-ATM antibody. **C)** Atm+/+ mice (n=3) and Atm −/− mice (n=3) received a ConA injection (25mg/kg) via tail vein as in B. TUNEL assay was performed in liver. *Left panel*: DAPI staining nuclei; *Right panel:* apoptotic/necrotic cells are labelled with green fluorescence. This figure is representative of three different experiments.