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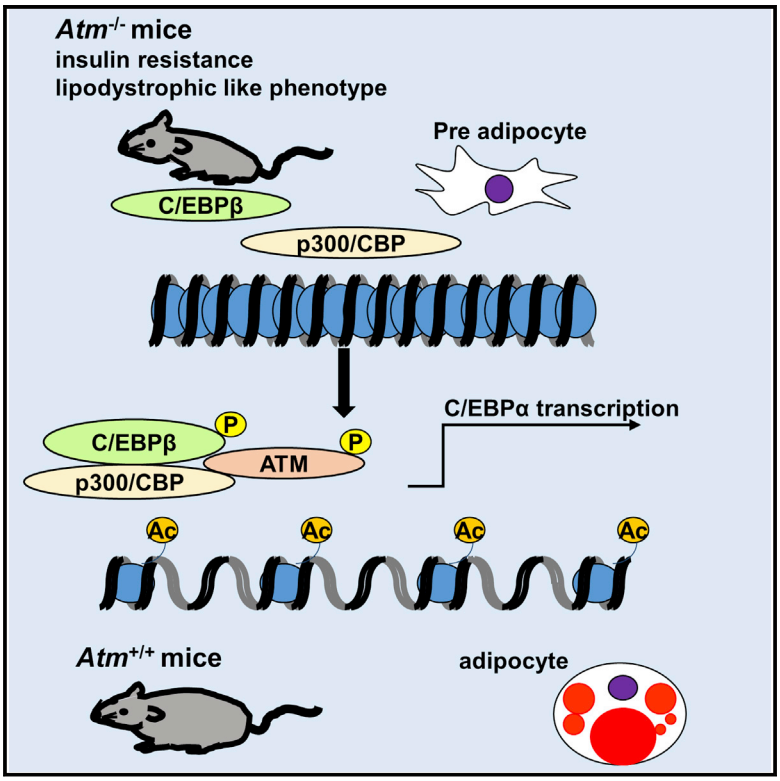
Cell Reports

ATM Regulates Adipocyte Differentiation and Contributes to Glucose Homeostasis

Graphical Abstract



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In Brief

Ataxia telangiectasia (A-T) patients develop diabetes mellitus. ATM, linked to A-T, is known to be involved in the DNA damage checkpoint. Takagi et al. reveal that ATM regulates adipocyte differentiation and attenuates differentiation of adipocytes in A-T patients, contributing to glucose metabolism in vivo.

Highlights

- ATM, linked to ataxia telangiectasia, regulates adipocyte differentiation
- The adipocyte differentiation defect in A-T contributes to type 2 diabetes
- Transcriptional activation of C/EBPα and PPARγ depends on ATM
- Binding of ATM to C/EBPβ and p300 induces transcriptional regulation of C/EBPα

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Article

ATM Regulates Adipocyte Differentiation and Contributes to Glucose Homeostasis

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SUMMARY

Ataxia-telangiectasia (A-T) patients occasionally develop diabetes mellitus. However, only limited attempts have been made to gain insight into the molecular mechanism of diabetes mellitus development in A-T patients. We found that *Atm*$^{-/-}$ mice were insulin resistant and possessed less subcutaneous adipose tissue as well as a lower level of serum adiponectin than *Atm*$^{+/+}$ mice. Furthermore, in vitro studies revealed impaired adipocyte differentiation in *Atm*$^{-/-}$ cells caused by the lack of induction of C/EBPα and PPARγ, crucial transcription factors involved in adipocyte differentiation. Interestingly, ATM was activated by stimuli that induced differentiation, and the binding of ATM to C/EBPβ and p300 was involved in the transcriptional regulation of C/EBPα and adipocyte differentiation. Thus, our study sheds light on the poorly understood role of ATM in the pathogenesis of glucose intolerance in A-T patients and provides insight into the role of ATM in glucose metabolism.

INTRODUCTION

Ataxia-telangiectasia (A-T) is often accompanied by glucose intolerance and insulin resistance (Bar et al., 1978; Blevins and Gebhart, 1996; McFarlin et al., 1972; Morio et al., 2009; Schalch et al., 1970), and our previous study revealed that 17% of A-T patients developed type 2 diabetes mellitus (Morio et al., 2009). A-T patients also exhibit poor weight gain, a progressive decrease in their BMI, and progressive dystrophy (Schubert et al., 2005). In addition to A-T patients, A-T carriers, who comprise an estimated 0.05%–0.1% of the normal population, suffer an increased risk of ischemic heart disease (Su and Swift, 2000) and diabetes (Morrell et al., 1986). As in A-T patients, glucose intolerance has been reported in *Atm*$^{-/-}$, *Atm*$^{+/-}$*ApoE*$^{-/-}$, and *Atm*$^{-/-}$*ApoE*$^{-/-}$ mice (Miles et al., 2007; Schneider et al., 2006); the *Atm*$^{+/-}$*ApoE*$^{-/-}$ mouse model generates a state of insulin resistance similar to that observed in type 2 diabetes. In addition, Miles et al. reported impaired insulin secretion in aged *Atm*$^{-/-}$ mice (Miles et al., 2007). However, the mechanism by which an ATM deficiency affects the development of type 2 diabetes remains unknown.

ATM, the gene responsible for A-T, plays a central role in the DNA damage response. Previous reports have suggested that ATM is activated in response to insulin stimulation and phosphorylates the Cap-dependent translation inhibitor 4E-BP1 (Yang and Kastan, 2000). A recent large-scale proteomic ATM substrate analysis identified several proteins involved in the insulin-signaling pathway, such as AKT and FOXO1 (Matsuoka et al., 2007). Together, these observations strongly support the hypothesis that ATM is involved in the insulin-signaling pathway and modulates glucose homeostasis.

Insulin resistance is a frequent complication of obesity; however, lipoatrophic diabetes is paradoxical because it is

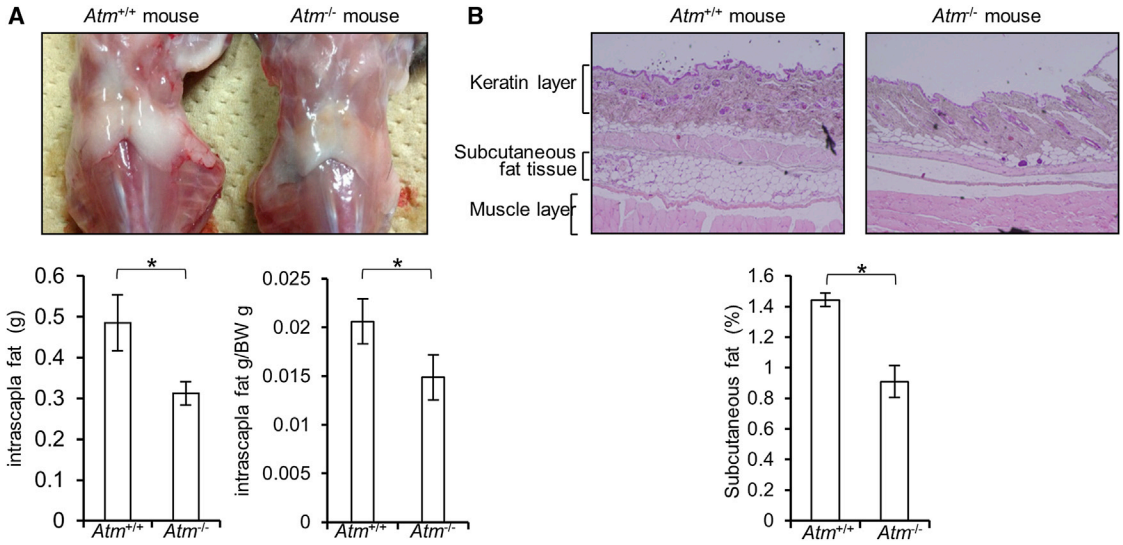

Figure 1. Adipose Tissue Distribution in *Atm*$^{+/+}$ and *Atm*$^{-/-}$ Mice

(A) Intrascapular fat tissue and (B) hematoxylin-eosin staining of the back skin of *Atm*$^{+/+}$ and *Atm*$^{-/-}$ mice. The lower graphs indicate the amount of fat tissue. The *Atm*$^{-/-}$ mice were relatively smaller than the *Atm*$^{+/+}$ mice. The relative amount of fat tissue per body weight is also shown. The mean values from three independent experiments are shown (A and B).

characterized by insulin resistance despite the existence of a low-fat mass. Insulin resistance in lipoatrophic diabetes may be due to the defective development of adipose tissue and the subsequently impaired secretion of adipokines, such as adiponectin or leptin (Rosen and Spiegelman, 2006). Adipocytes secrete several adipokines, such as adiponectin, leptin, visfatin, and omentin, which increase insulin sensitivity. Conversely, adipokines that are secreted by hypertrophic adipocytes, such as resistin and tumor necrosis factor α (TNF-α), act to decrease insulin sensitivity. Alterations in adiposity have profound implications for glucose homeostasis, and an appropriate balance of adiposity is required to maintain adequate glucose homeostasis.

The central engine for adipose differentiation involves CCAAT/enhancer-binding protein α (C/EBPα) and peroxisome proliferator activated receptor γ (PPARγ). When this receptor is activated by an agonistic ligand in fibroblasts, a full program of differentiation is stimulated, including morphological changes, lipid accumulation, and the expression of almost all genes characteristic of fat cells. Multiple C/EBPs, such as C/EBPβ and C/EBPδ, are expressed during the early stages of differentiation; subsequently, C/EBPα and PPARγ expression is driven by C/EBPβ and C/EBPδ. C/EBPs and PPARγ also directly activate many genes in terminally differentiated adipocytes (Rosen and Spiegelman, 2000).

To understand the molecular mechanism of diabetes development in A-T patients, we investigated the adipose tissue distribution and degree of adipocyte maturation in *Atm* knockout mice. Examination of mouse embryonic fibroblasts (MEFs) derived from wild-type (*Atm*$^{+/+}$) or *Atm* knockout (*Atm*$^{-/-}$) mice revealed that adipocytic differentiation did not occur in *Atm*$^{-/-}$ MEFs. The impaired adipocyte differentiation observed in *Atm*$^{-/-}$ MEFs was due to the defective ATM-dependent induction of C/EBPα and PPARγ expression. These observations strongly support the hypothesis that glucose intolerance and insulin resistance in A-T patients are due to attenuated adipocyte functioning.

RESULTS

Atm Knockout Mice Exhibited Glucose Intolerance, Insulin Resistance, and Abnormal Adipose Distribution

As previously suggested, *Atm*$^{-/-}$ mice were glucose intolerant, and their condition mimicked type 2 diabetes mellitus (Figures S1A–S1G). Interestingly, *Atm*$^{+/-}$ mice exhibited neither glucose intolerance nor insulin resistance, although *Atm*$^{+/-}$ male mice fed a high-fat diet exhibited glucose intolerance and insulin resistance (Figures S1H and S1I). A-T patients have lean figures and a reduced level of subcutaneous fat tissue. In contrast, an increased amount of visceral fat tissue was reported in *Atm*$^{+/-}$*ApoE*$^{-/-}$ mice (Schneider et al., 2006). Therefore, the adipose distribution in *Atm*$^{-/-}$ mice was re-evaluated. We detected a decreased amount of intrascapular and subcutaneous fat tissue in *Atm*$^{-/-}$ mice compared to their wild-type littermates (Figures 1A and 1B). Conversely, *Atm*$^{-/-}$ mice showed an increased level of visceral fat tissue, as previously reported, similarly to human metabolic syndrome, which is also associated with a high accumulation of visceral fat (Matsuzawa, 2006) (Figures S1J–S1L). In addition, a reduced serum level of adiponectin was previously observed in *Atm*$^{+/-}$*ApoE*$^{-/-}$ mice (Schneider et al., 2006). As expected, *Atm*$^{-/-}$ mice also showed reduced serum adiponectin and leptin levels, whereas *Atm*$^{+/-}$ mice exhibited levels intermediate between those of *Atm*$^{-/-}$ and *Atm*$^{+/+}$ mice (Figures S1M and S1N). The fat redistribution, increase in visceral fat tissue, and reduction in subcutaneous fat tissue observed in *Atm*$^{-/-}$ mice may be explained by their increased appetite, which is due to their reduced level of leptin (Figure S1O).

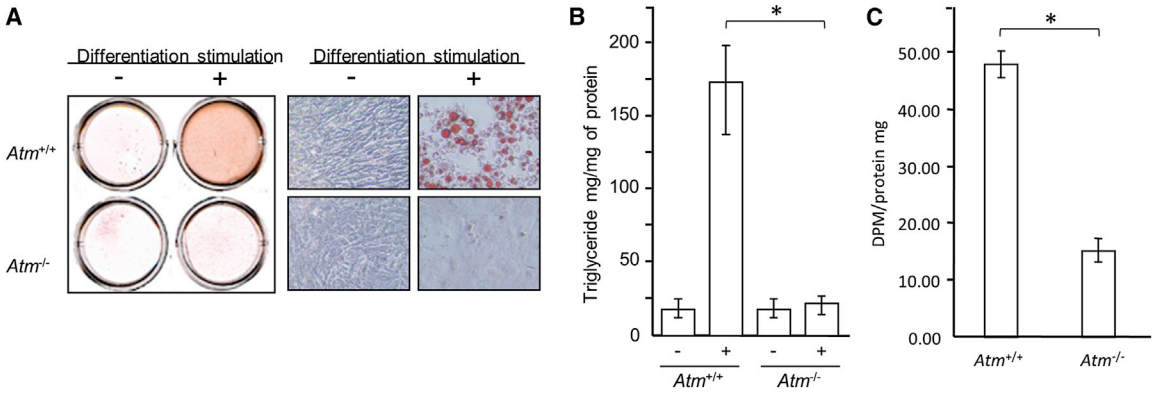

Figure 2. ATM-Null Cells Are Defective in Adipocyte Differentiation

(A) Oil red O staining of in-vitro-differentiated *Atm*$^{+/+}$ and *Atm*$^{-/-}$ MEFs. The right panel shows magnified images of parts of the left panel.

(B) The intracellular triglyceride concentrations of *Atm*$^{+/+}$ and *Atm*$^{-/-}$ MEFs are shown.

(C) In-vitro-differentiated *Atm*$^{+/+}$ and *Atm*$^{-/-}$ MEFs were assayed for radiolabeled 2-deoxyglucose uptake in the presence of 5 μg/ml insulin. The SEs are shown as error bars (*$p < 0.05$).

ATM-Deficient Cells Were Defective in Adipocyte Differentiation

The reduced amount of subcutaneous fat tissue and reduced level of serum adiponectin in *Atm* knockout mice suggested a defect in adipocyte functioning. To evaluate the function of adipose tissue in *Atm* knockout mice, an in vitro adipocyte differentiation model (Tanaka et al., 1997) was employed, using *Atm*$^{-/-}$ and *Atm*$^{+/+}$ MEFs. After the MEFs were stimulated to differentiate into adipocytes, the *Atm*$^{+/+}$ MEFs showed lipid accumulations, as indicated by Oil red O staining, whereas the *Atm*$^{-/-}$ MEFs failed to differentiate into adipocytes (Figure 2A). To evaluate the ability of the cells to differentiate into adipocytes, the intracellular triglyceride levels of these cells after differentiation stimulation were compared. *Atm*$^{-/-}$ MEFs showed an approximately 85% triglyceride level compared with that of *Atm*$^{+/+}$ MEFs (Figure 2B). Glucose uptake was evaluated in cells that were induced to differentiate for 6 days, and the *Atm*$^{-/-}$ MEFs showed approximately 75% less glucose uptake compared to *Atm*$^{+/+}$ MEFs (Figure 2C). Adipocyte differentiation capacity using stromal vascular fractions (SVFs) from *Atm*$^{+/+}$ and *Atm*$^{-/-}$ mice was also examined. As observed in the MEFs, the *Atm*$^{-/-}$ SVFs exhibited a defective adipocyte differentiation capacity (Figure S2A).

Adipocyte differentiation experiments frequently utilize 3T3-L1 preadipocytes. To confirm the results obtained using *Atm*$^{-/-}$ and *Atm*$^{+/+}$ MEFs and SVFs, adipocyte differentiation was also investigated in 3T3-L1 cells treated with the ATM inhibitors caffeine and KU55933. As expected, treatment with caffeine and KU55933 blocked adipocyte differentiation in 3T3-L1 cells (Figure S2B). To confirm that adipocytic differentiation was dependent on ATM function, the genome of *Atm*$^{-/-}$ MEFs was complemented with wild-type or kinase-dead ATM cDNA via expression vectors; only wild-type ATM restored the in vitro ability of the cells to differentiate into adipocytes (Figure S2C). ATM-deficient cells accumulate reactive oxygen species (ROS) (Ito et al., 2004), which may interfere with differentiation in vitro. To investigate whether ROS interfered with adipocyte differentiation, *Atm*$^{-/-}$ MEFs were pretreated with the ROS scavenger N-acetyl cysteine (NAC), and differentiation was induced in the presence of NAC. However, NAC treatment failed to rescue adipocyte differentiation in *Atm*$^{-/-}$ MEFs (Figure S2D). These experiments demonstrated that ATM is required for proper adipocyte differentiation and that ROS are not involved in this process.

ATM-Deficient Cells Showed Defective Induction of Transcriptional Factors Required for Adipocyte Differentiation

To clarify the molecular mechanism of attenuated adipocyte differentiation in *Atm*$^{-/-}$ MEFs, several factors with possible roles in adipocyte differentiation were investigated in *Atm*$^{+/+}$ and *Atm*$^{-/-}$ MEFs upon differentiation induction. C/EBPα and PPARγ expression was observed 4–6 days after the induction of differentiation in *Atm*$^{+/+}$ MEFs, whereas *Atm*$^{-/-}$ MEFs completely lacked C/EBPα and PPARγ expression. In contrast, C/EBPβ and C/EBPδ expression was induced normally upon the differentiation of *Atm*$^{-/-}$ MEFs (Figure 3A). A-T cells have been previously reported to show decreased expression of the IGF1 receptor, which is the primary mediator of the insulin-signaling pathway (Peretz et al., 2001; Shahrabani-Gargir et al., 2004). However, there was no significant difference in the levels of expression of the IGF1 receptor of *Atm*$^{+/+}$ and *Atm*$^{-/-}$ MEFs (Figure 3A). These two observations suggested that signaling pathways upstream of C/EBPβ and C/EBPδ were regulated normally in *Atm*$^{-/-}$ MEFs during differentiation and that the impaired adipocyte differentiation of *Atm*$^{-/-}$ MEFs was caused by defective C/EBPα and PPARγ expression. Real-time qPCR and northern blot revealed that the expression of C/EBPα and PPARγ mRNA was dysregulated at the transcriptional level (Figures 3B, 3C, and S2E). KU55933 blocked adipocyte differentiation in 3T3-L1 cells (Figure S2B), which was accompanied by the aberrant induction of C/EBPα expression (Figure S2F). Adipocyte differentiation via wild-type ATM complementation of *Atm*$^{-/-}$ MEFs restored the induction of C/EBPα expression, whereas kinase-dead ATM complementation failed to restore this process (Figure S2G).

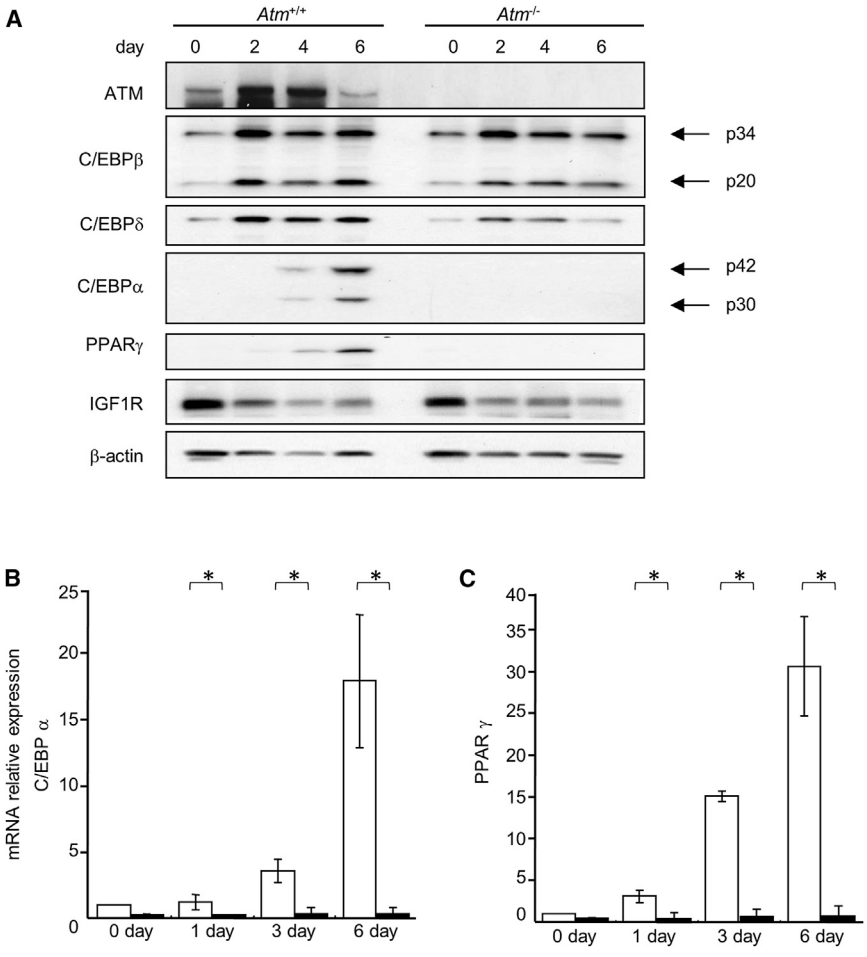

Figure 3. C/EBPα and PPARγ Expression during the Adipocyte Differentiation Process

(A) Western blotting analyses of in-vitro-differentiated $Atm^{+/+}$ and $Atm^{-/-}$ MEFs.

(B and C) The levels of C/EBPα and PPARγ mRNA expression after differentiation were analyzed with qRT-PCR. The mean values from three independent experiments are shown in the bar graphs. The SEs are shown as error bars (*$p < 0.05$).

These results suggested that the impaired adipocyte differentiation observed in $Atm^{-/-}$ cells was caused by a defect in the ATM-dependent transcriptional activation of C/EBPα and PPARγ expression upon differentiation stimulation.

Complementation of C/EBPα or PPARγ Restored the Adipocyte Differentiation Capacity of *Atm*$^{-/-}$ Cells

C/EBPα and PPARγ are necessary for the terminal differentiation of adipocytes, and the aberrant expression of C/EBPα and PPARγ is thought to be the central cause of impaired adipocyte differentiation in $Atm^{-/-}$ MEFs. Thus, we tested whether defective differentiation would be rescued by the overexpression of C/EBPα or PPARγ in $Atm^{-/-}$ MEFs. $Atm^{-/-}$ MEFs were transduced using retroviruses for the expression of HA-tagged C/EBPα or FLAG-tagged PPARγ2 and the internal ribosome entry sequence (IRES)-dependent expression of GFP, and then GFP-positive cells were cultured under conditions that stimulated differentiation. The overexpression of either HA-tagged C/EBPα or FLAG-tagged PPARγ2 restored the ability of $Atm^{-/-}$ MEFs to differentiate into adipocytes (Figures S2H and S2J). Furthermore, the overexpression of HA-tagged C/EBPα or FLAG-tagged PPARγ2 induced the endogenous expression of PPARγ or C/EBPα (Figures S2I and S2K).

ATM-Deficient Cells Were Deficient in Cell-Cycle Regulation after Differentiation Stimulation

In addition to the major adipocyte transcriptional differentiation inducers, such as C/EBPα or PPARγ, several factors that participate in cell-cycle regulation are involved in adipocyte differentiation (Abella et al., 2005; Fajas et al., 2002). Upon differentiation stimulation, the cells that are arrested by contact inhibition re-enter the cell cycle, in a process referred to as clonal expansion (Tang et al., 2003). After two to three rounds of proliferation, the cell cycle is halted in these cells, and they enter the process of terminal differentiation (Rosen and Spiegelman, 2000). Thus, the cell-cycle kinetics of $Atm^{+/+}$ and $Atm^{-/-}$ MEFs undergoing differentiation was investigated. The results of a bromodeoxyuridine (BrdU) pulse-labeling experiment revealed that $Atm^{+/+}$ and $Atm^{-/-}$ MEFs re-entered the cell cycle normally after differentiation stimulation, but the cell cycle was not halted in $Atm^{-/-}$ MEFs at 8 days after differentiation stimulation (Figure S3A). Monitoring the RB phosphorylation status using western blotting analysis showed lack of RB dephosphorylation at the terminal stage of differentiation in $Atm^{-/-}$ MEFs (Figure S3B). The induction of cyclin A expression upon differentiation stimulation was also observed in $Atm^{+/+}$ and $Atm^{-/-}$ MEFs; cyclin A expression declined at 6 days after differentiation stimulation in $Atm^{+/+}$ MEFs, but its expression persisted in $Atm^{-/-}$ MEFs (Figure S3C). The expression of E2F4, which halts the adipogenic differentiation process (Fajas et al., 2002), was gradually upregulated, reaching a maximum level at 6 days after differentiation stimulation in $Atm^{+/+}$ MEFs. Interestingly, the level of expression of E2F4 was constitutively high in $Atm^{-/-}$ MEFs (Figure S3C). These observations demonstrated that, although $Atm^{-/-}$ MEFs could re-enter the clonal expansion phase, they failed to show the cell-cycle arrest necessary for the terminal differentiation process. Interestingly, the overexpression of HA-tagged C/EBPα induced cell-cycle arrest after the clonal expansion of $Atm^{-/-}$ MEFs via RB dephosphorylation (Figures S3D and S3E), suggesting that C/EBPα functions upstream of cell-cycle regulators.

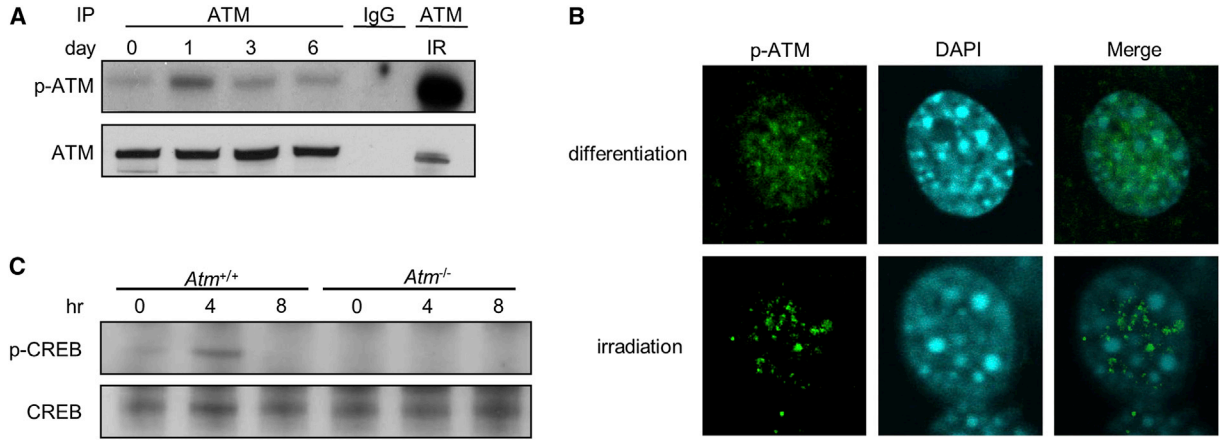

Figure 4. ATM Is Activated during Differentiation

(A) The ATM phosphorylation status after in vitro differentiation was determined by western blotting analysis of immunoprecipitates.

(B) ATM phosphorylation 1 day after in vitro differentiation and 3 hr after 5-Gy irradiation, as detected using immunofluorescence.

(C) CREB phosphorylation upon in vitro differentiation was analyzed using western blotting.

ATM Was Activated by Stimuli that Induce Differentiation

ATM is activated by DNA damage signals as well as insulin stimulation (Yang and Kastan, 2000), and ATM activation can be monitored according to the intermolecular autophosphorylation of its serine-1981 (Bakkenist and Kastan, 2003). Therefore, the autophosphorylation of ATM during adipogenesis was investigated. During adipocyte differentiation, ATM was activated (Figure 4A). Differentiation-stimulated cells showed a diffuse phosphorylated ATM pattern in contrast to the irradiated positive control cells, which showed diffuse and discrete foci of phosphorylated ATM (Figures 4B and S4A). This ATM activation process was not associated with DNA double-strand breakage (Figure S4B). ATM activation upon differentiation stimulation also resulted in the phosphorylation of the ATM downstream target CREB at serine-121 (Shi et al., 2004), which is required for adipocyte differentiation (Zhang et al., 2004) (Figure 4C).

ATM Was Required for the Induction of C/EBPα Transcription by C/EBPβ

The above-described results strongly suggest that ATM activation contributes to adipogenesis via the regulation of C/EBPα and/or PPARγ transcription, although the expression of C/EBPβ and C/EBPδ was induced normally in $Atm^{-/-}$ MEFs. C/EBPβ and C/EBPδ are involved in C/EBPα transcription through binding to the C/EBP-binding sequence in the C/EBPα promoter and acting as upstream transcription factors for C/EBPα. Therefore, C/EBPβ binding to the C/EBPα promoter was investigated. Chromatin immunoprecipitation (ChIP) assays revealed that C/EBPβ bound equally to the C/EBPα promoter in $Atm^{+/+}$ and $Atm^{-/-}$ MEFs upon differentiation stimulation (Figure 5A). However, the activity of the C/EBPα promoter, as determined using a luciferase assay, was completely abolished in $Atm^{-/-}$ MEFs (Figure 5B). Furthermore, this dysregulation of C/EBPα transcription attenuated the histone H3 and H4 acetylation in the C/EBPα promoter region (Figure 5C). Together, these experiments showed that C/EBPβ bound to the C/EBPα promoter but could not transactivate the C/EBPα promoter in the absence of ATM.

ATM Bound to C/EBPβ and p300 Was Recruited to the ATM-C/EBPβ Complex

An electromobility shift assay (EMSA) revealed a protein complex bound to the C/EBPα promoter in $Atm^{+/+}$ and $Atm^{-/-}$ MEFs. However, examination of nuclear extracts of $Atm^{-/-}$ MEFs showed that the mobility of several protein complexes that bind to the C/EBPα promoter was retarded in these cells (Figure S5), suggesting that the composition or modification of protein complexes that bind to the C/EBPα promoter were different in $Atm^{+/+}$ and $Atm^{-/-}$ MEFs. The C/EBPβ binding partners in $Atm^{+/+}$ and $Atm^{-/-}$ MEFs were investigated using an immunoprecipitation assay, which showed that ATM bound to C/EBPβ upon differentiation stimulation (Figure 6A). C/EBPβ is known to bind to CBP/p300, a phosphorylation target of ATM, and modulate its transcriptional activity (Jang et al., 2010; Schwartz et al., 2003; Wang et al., 2007), and the acetylation of histone H3 and H4 near the C/EBPα promoter was attenuated in $Atm^{-/-}$ MEFs. Thus, we hypothesized that histone acetyl transferase is recruited to the ATM-C/EBPβ complex and acetylates histones near the C/EBPα promoter. Therefore, the association of histone acetyl transferase with C/EBPβ was investigated using an immunoprecipitation assay. Screening for several histone acetyl transferases, including GCN5, p300, CBP, and Tip60, demonstrated that ATM and C/EBPβ bound to p300 upon differentiation and that the binding of ATM to C/EBPβ and p300 was augmented upon differentiation stimulation. Furthermore, co-immunoprecipitation assays revealed that ATM, C/EBPβ, and p300 formed a ternary complex upon differentiation stimulation, with C/EBPβ-p300 binding being dependent upon the presence of ATM (Figure 6A). C/EBPβ threonine 188 (threonine 235 in humans) phosphorylation is required for its transcriptional activation; upon differentiation stimulation, $Atm^{+/+}$ MEFs showed phosphorylation of C/EBPβ at threonine 188, whereas this effect was absent in $Atm^{-/-}$ MEFs (Figure 6B).

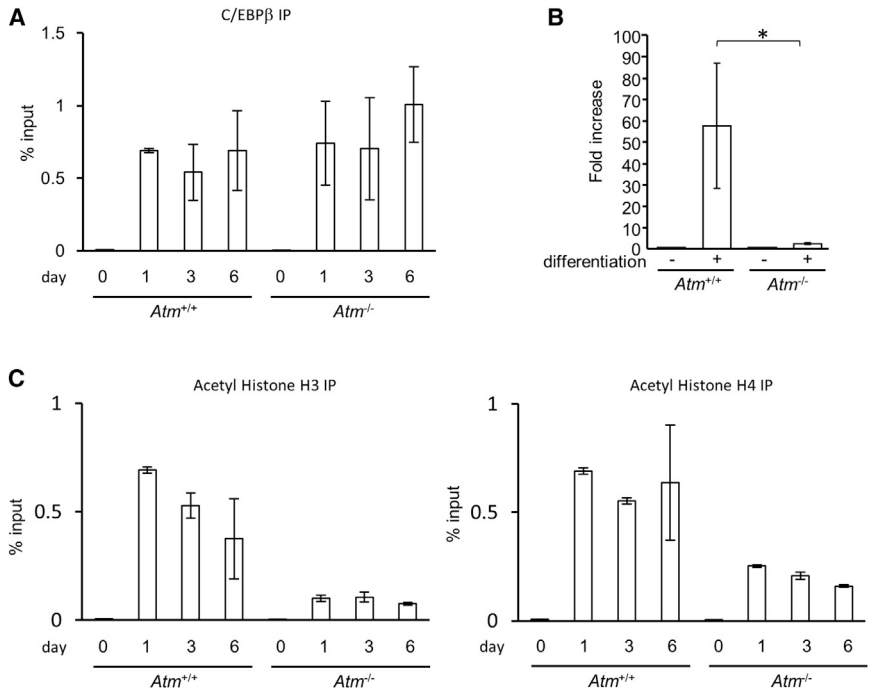

Figure 5. C/EBPβ Transcriptional Activity Depends on ATM

(A) A ChIP assay using an anti-C/EBPβ antibody (H7) showed that C/EBPβ bound to the C/EBPα promoter sequence in $Atm^{+/+}$ and $Atm^{-/-}$ MEFs.

(B) C/EBPα promoter activation in $Atm^{+/+}$ and $Atm^{-/-}$ MEFs before and 3 days after differentiation stimulation, as determined using a luciferase assay.

(C) Analyses of the histone H3 and H4 acetylation status proximal to the C/EBPα promoter using a ChIP assay. The mean values from three independent experiments are shown.

The SEs are shown as error bars (*$p < 0.05$).

It is known that C/EBPβ threonine 188 is directly phosphorylated by ERK, and it is also known that $ATM^{-/-}$ cells exhibit defective MAPK activation (Kim and Wong, 2009; Raman et al., 2007). To link these two observations, we tested whether the activation of the MAPK-signaling pathway was defective in $Atm^{-/-}$ cells after differentiation stimulation. As expected, $Atm^{-/-}$ MEFs exhibited defective MEK1 and ERK activation (Figure S6). Thus, the defect in C/EBPβ phosphorylation in $Atm^{-/-}$ cells may be due to the failure of MAPK-signaling pathway activation.

Restoration of Adipose Functioning in $Atm^{-/-}$ Mice Improved Their Glucose Intolerance

Based on our findings, agents such as the PPARγ ligand thiazolidione, which induces adipocyte differentiation, may be effective candidates for treating glucose intolerance in A-T patients. Indeed, rosiglitazone treatment restored adipocyte differentiation in $Atm^{-/-}$ MEFs (Figures 7A and 7B), and pioglitazone treatment ameliorated glucose intolerance in $Atm^{-/-}$ mice (Figures 7C and 7D) and increased their serum adiponectin concentrations (Figure 7E), suggesting a restoration of fat tissue functioning. Metformin treatment also improved glucose intolerance in these mice (Figures 7C and 7D). The increase in insulin sensitivity with metformin treatment was milder than that with pioglitazone treatment; however, the results were not significantly different. Furthermore, metformin treatment did not affect the serum adiponectin concentration, suggesting that metformin improved the glycemic response through a fat-tissue-independent pathway (Figure 7E). Previously, surgical implantation of normal fat tissue was reported to rescue insulin resistance in lipodystrophic mice (Gavrilova et al., 2000). Therefore, we surgically implanted fat tissue derived from wild-type and *Atm* knockout littermates into $Atm^{-/-}$ mice (Figures S7A and S7B), and only wild-type fat transplantation successfully reversed the glucose intolerance and insulin resistance of $Atm^{-/-}$ mice and increased their levels of serum adiponectin (Figures 7F–7H).

DISCUSSION

A-T patients often exhibit glucose intolerance and insulin resistance and possess less subcutaneous adipose tissue than healthy individuals. Glucose intolerance was reported not only in A-T patients, but also in the *Atm* knockout mouse model (Miles et al., 2007; Schneider et al., 2006). We confirmed that *Atm* knockout mice are insulin resistant and possess less subcutaneous adipose tissue, accompanied by a lower level of serum adiponectin, than their wild-type littermates. Furthermore, in vitro investigations using MEFs revealed that adipocyte differentiation was impaired in $Atm^{-/-}$ cells, which was caused by the lack of induction of C/EBPα and PPARγ, crucial transcription factors involved in adipocyte differentiation. These observations suggest that the glucose intolerance and insulin resistance of A-T patients are due to the improper functioning of their adipose tissue due to the attenuation of adipocyte differentiation.

Recently, a link was established between the DNA damage checkpoint pathway and cellular metabolism. Previous reports have shown that the ATM-p53 pathway participates in glucose metabolism (Armata et al., 2010; Minamino et al., 2009), although the molecular mechanisms involved in this process remain unclear. Our findings demonstrate the importance of the DNA damage checkpoint pathway in the regulation of cellular metabolism and homeostasis in vivo. It is also clear that transcriptional regulation by ATM plays an important role in cellular differentiation. ATM is reportedly required for the retinoic-acid-induced differentiation of SH-SY5Y neuroblastoma cells to neuronal-like cells. Retinoic acid rapidly triggers the activity of ATM kinase, resulting in the ATM-dependent phosphorylation of the transcription factor CREB (Fernandes et al., 2007). In the case of adipocyte differentiation, ATM is also activated by extracellular signals. In our model of adipocyte differentiation, C/EBPα transcription required ternary complex formation by ATM, C/EBPβ, and the histone acetyl transferase p300, which was associated with the phosphorylation of C/EBPβ at threonine 235. The recruitment of p300 to the C/EBPβ complex activated the

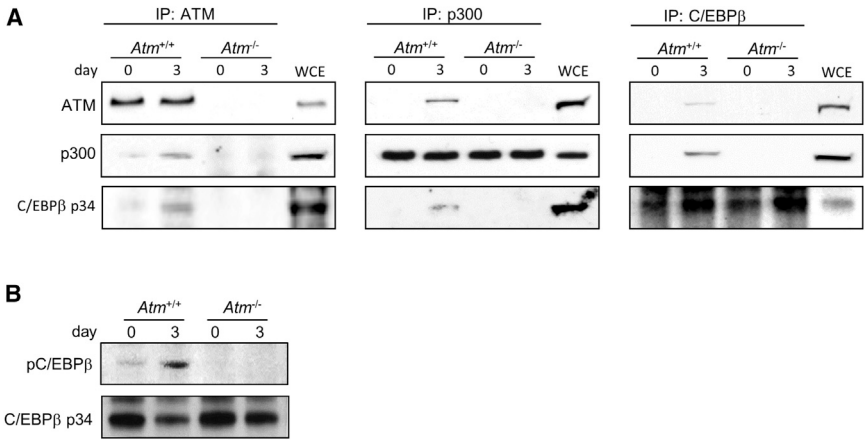

Figure 6. ATM Forms a Ternary Complex with C/EBPβ and p300

(A) ATM bound to p300 and C/EBPβ upon differentiation (left). p300 bound to ATM and C/EBPβ upon differentiation (middle). C/EBPβ bound to ATM and p300 upon differentiation (right). Immunoprecipitation using the indicated antibodies was performed before and after the differentiation of $Atm^{+/+}$ and $Atm^{-/-}$ MEFs. The anti-C/EBPβ (H7) antibody immunoprecipitated only the active p34 LAP form of C/EBPβ.

(B) C/EBPβ was phosphorylated in an ATM-dependent manner. C/EBPβ was immunoprecipitated from $Atm^{+/+}$ and $Atm^{-/-}$ MEF lysates and detected using western blotting with an anti-phospho-C/EBPβ threonine 235 (threonine 188 in mouse) antibody.

C/EBPβ-dependent transcription of C/EBPα through the modification of histone acetylation. The phosphorylation of C/EBPβ at threonine 235, reportedly by ERK and GSK3, is required for its transcriptional activity (Park et al., 2004). Moreover, ERK activation by DNA damage is dependent upon ATM (Tang et al., 2002).

It is possible that other components of the C/EBPβ complex(es) are targets of ATM phosphorylation and that this phosphorylation is a prerequisite for the formation of the complex(es). In fact, C/EBPβ and the C/EBPβ-binding partners p300, CREB, and FOXO1, as well as the negative regulator of adipocyte differentiation Sp1 and the positive regulator of adipocyte differentiation E2F1, carry ATM phosphorylation consensus residues and are phosphorylated by ATM (Iwahori et al., 2008; Jang et al., 2010; Lin et al., 2001). Therefore, the phosphorylation of transcription factors and associated proteins by ATM, including the C/EBPβ-p300 complex, may play a central role in adipocytic differentiation.

The DNA damage response pathway has been linked with the state of cellular glucose metabolism (Armata et al., 2010; Minamino et al., 2009) and fat metabolism (Wong et al., 2009). Furthermore, components of the DNA break/repair machinery, including DNA-PK, Ku70/80, PARP-1, and topoisomerase IIβ, as well as protein phosphatase 1, are recruited to the fatty acid synthase promoter in mice immediately after feeding (Wong et al., 2009). Transient DNA breaks were recently reported to be required for estrogen-receptor-regulated transcription (Ju et al., 2006), and ATM is hypothesized to be required for the DNA-break-dependent transcriptional regulation of C/EBPα, similar to the case for DNA-PK. Indeed, topoisomerase β is phosphorylated in an ATM-dependent manner in response to DNA damage (Bensimon et al., 2010).

The amount of intrascapular and subcutaneous fat tissue in $Atm^{-/-}$ mice was less than that of their wild-type littermates. Conversely, $Atm^{-/-}$ mice showed an increased amount of visceral fat tissue, as previously reported (Schneider et al., 2006), comparable to that observed in lipodystrophy, HIV infection, or human metabolic syndrome, conditions that are associated with a high accumulation of visceral fat (Matsuzawa, 2006; Safrin and Grunfeld, 1999). Although it is not known why subcutaneous fat tissue is preferentially affected in $Atm^{-/-}$ mice, we propose several hypotheses to explain this phenomenon. First, the increased appetite of $Atm^{-/-}$ mice due to their reduced leptin level may preferentially induce fat accumulation in the visceral fat tissue (Figure S1O). Second, the caveolin-1 knockout mouse is one of the best-characterized lipodystrophic mouse models. Although this phenotype is observed late in life, all types of fat tissues in this mouse exhibit the lipodystrophic phenotype. As young animals, the only types of adipose tissue that are affected are the female mammary and subcutaneous white adipose tissue (Razani et al., 2002). As previously reported for caveolin-1 knockout mice, subcutaneous fat tissue of $Atm^{-/-}$ mice may be much more sensitive than other fat tissues to defects in the adipocyte differentiation machinery. Third, the association between age and lipodystrophy may be mediated by the increased number of deletions in the mitochondrial (mt) DNA that naturally occur with aging (Walker and Brinkman, 2001). Because visceral abdominal fat is known to be more metabolically active than other fat tissues, altered adipocyte mitochondrial functioning would affect central adipocytes more than peripheral adipocytes. It is known that ATM is indispensable for mitochondrial metabolism (Valentin-Vega et al., 2012). Based on these observations, one can speculate that loss of ATM would preferentially affect peripheral adipocytes, in which mitochondrial functioning is relatively low.

Our study revealed a previously uncharacterized function for ATM as a regulator of key adipocyte transcription factors, although its role in the DNA damage response pathway remains to be determined. Based on our findings, agents such as the PPARγ ligand thiazolidione, which induces adipocytic differentiation by bypassing the ATM pathway, may be good candidates for treating glucose intolerance in A-T patients.

It is interesting that in $Atm^{+/-}$ mice, a high-fat diet, but not a normal diet, induced glucose intolerance. Schneider et al. reported that $Atm^{+/-}ApoE^{-/-}$ mice fed a normal diet exhibited glucose intolerance (Schneider et al., 2006). However, in our study, $Atm^{+/-}$ mice did not exhibit glucose intolerance, although the genetic background of the $Atm^{+/-}ApoE^{-/-}$ mice or the increased serum lipid level generated by disabling the *ApoE* gene may have affected the state of glucose tolerance. Nevertheless, our observations demonstrated the possibility of an increased risk of metabolic syndrome in A-T carriers, which is consistent with the epidemiological data supporting an

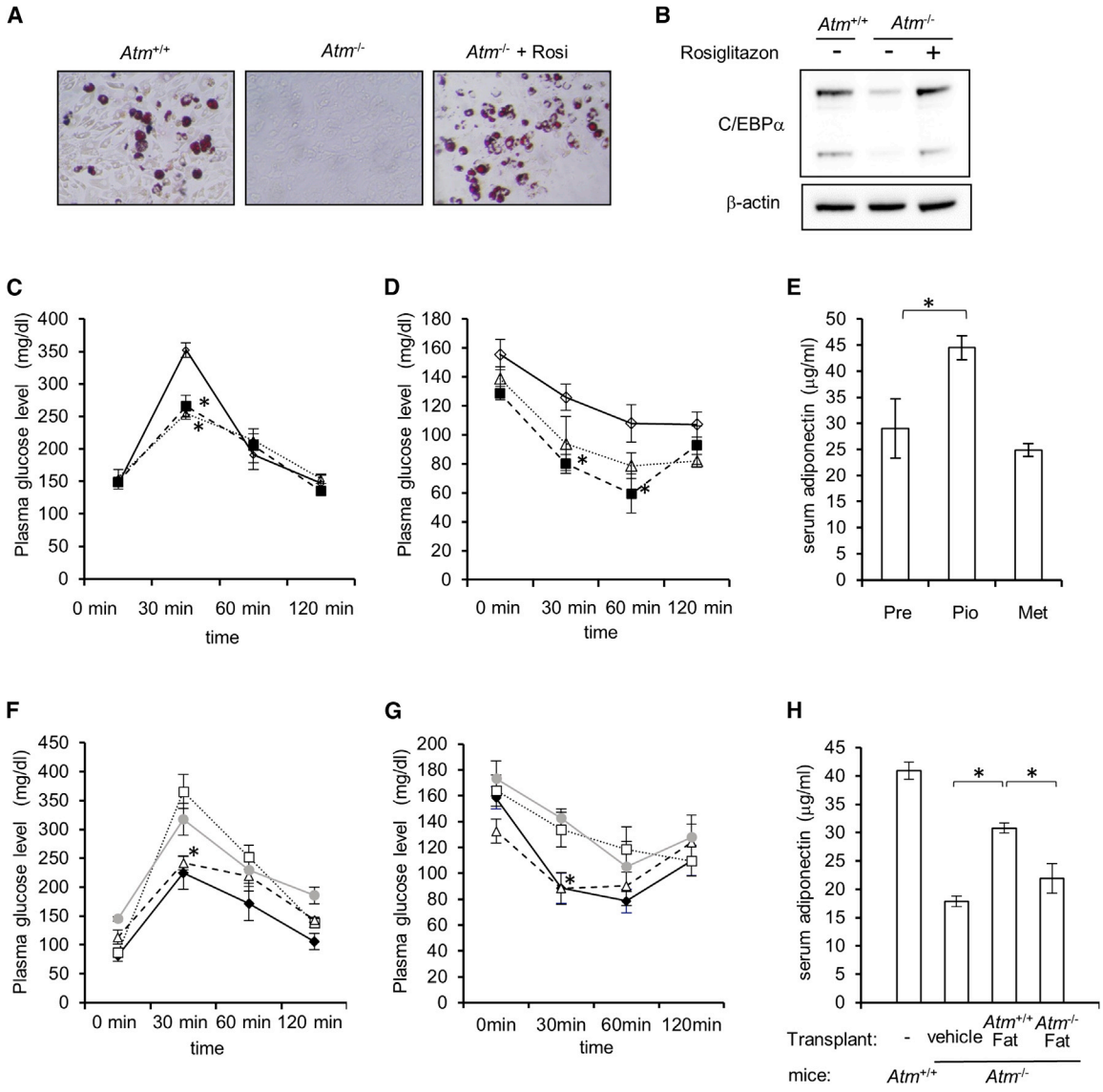

Figure 7. Thiazolidione Treatment and Wild-Type Adipose Tissue Transplantation Rescues the Glucose-Intolerance Phenotype of *Atm* Knockout Mice

(A) Rosiglitazone (Rosi) treatment restored the adipocyte differentiation capacity of $Atm^{-/-}$ MEFs. Oil red O staining (left).

(B) Western blotting analysis. Differentiation was induced using DMSO or 1 μM rosiglitazone.

(C) Results of glucose tolerance tests of $Atm^{-/-}$ mice treated with metformin or pisoglitazone. These tests were performed before treatment (open diamond), after metformin treatment (37.5 mg/kg, open triangle), and after pioglitazone treatment (30 mg/kg, closed square) for 21 consecutive days.

(D) Results of the insulin tolerance tests of mice treated as in (C).

(E) The serum adiponectin concentration of $Atm^{-/-}$ mice before treatment (Pre) with pioglitazone (Pio) or metformin (Met). The mean values from three or four independent experiments are shown.

(F) Results of the glucose tolerance tests performed after fat transplantation; $Atm^{+/+}$ mice (closed diamond), $Atm^{-/-}$ vehicle-transplanted mice (closed square), $Atm^{-/-}$ mice transplanted with $Atm^{+/+}$ fat (open triangle), and $Atm^{-/-}$ mice transplanted with $Atm^{-/-}$ fat (gray circle).

(G) Results of the insulin tolerance tests of mice treated as in (G).

(H) The serum adiponectin concentrations in $Atm^{+/+}$, $Atm^{-/-}$ vehicle-transplanted, and $Atm^{-/-}$ mice that received fat tissue transplants from $Atm^{+/+}$ mice or $Atm^{-/-}$ mice are shown in the bar graph. The mean values from three or four independent experiments are shown.

The SEs are shown as error bars (*$p < 0.05$).

increased risk for ischemic disease in A-T carriers (Su and Swift, 2000).

The *ATM* gene contains a number of SNPs, some of which confer functional deficiencies. The frequency of these SNPs is estimated to be <5%. One report demonstrated that a genetic locus responsible for type 2 diabetes is located on chromosome 11q, where *ATM* is also located (Palmer et al., 2006). A genome-wide association study showed that one SNP,

rs11212617, at the *ATM* locus was associated with the successful treatment of type 2 diabetes using metformin, suggesting that ATM plays a role in the effect of metformin upstream of AMPK (Zhou et al., 2011). Nucleotide variations or the inhibition of ATM using KU-55933 alters the glycemic response to metformin. However, the results of several studies do not support the hypothesis that ATM is involved in the activation of AMPK through metformin (Florez et al., 2012; Woods et al., 2012; Yee et al., 2012). Metformin activates the ATM-dependent pathway and inhibits tumor growth and the sensitivity to irradiation via an AMPK-dependent pathway (Storozhuk et al., 2013; Vazquez-Martin et al., 2011). However, further studies are required to elucidate the relationship between the metformin-dependent glycemic response and ATM-dependent glucose metabolism or the DNA damage response. It is also known that individuals with type 2 diabetes have an increased susceptibility to cancer. Together, these observations raised the question of whether *ATM* SNPs are associated with the concomitant susceptibility to diabetes and malignancy in certain individuals.

Thus, our study revealed a previously uncharacterized function for ATM in the regulation of key adipocyte transcription factors, although its role as a classical DNA damage response molecule remains to be determined. Understanding the functions of ATM that are modulated by the DNA damage response in glucose homeostasis may yield breakthroughs and reconsideration of the current paradigm for general diabetes research.

EXPERIMENTAL PROCEDURES

Animals

The generation of Atm-deficient mice ($Atm^{-/-}$) was previously described (Herzog et al., 1998). These mice have been backcrossed onto the C57BL/6 background for more than 15 generations. The mice were housed in a specific pathogen-free barrier facility and weaned at 3 weeks of age to a standard mouse chow that provided 6% of calories as fat. To produce the high-fat diet group, mice were fed a diet that provided 42% of calories as fat. Animal protocol 010018A was approved by the Animal Study Committee of Tokyo Medical and Dental University.

Glucose Tolerance Test, Insulin Sensitivity Assay, Surgical Implantation of Fat, and Pioglitazone and Metformin Treatment

$Atm^{-/-}$ mice fed normal chow underwent glucose tolerance or insulin resistance tests at 12 weeks of age. The mice were fasted for 12 hr, and then 10% D-glucose (1 g/kg body weight) or human insulin (0.75 U/kg body weight; Sigma-Aldrich) was administered via injection. Tail-vein blood (5 μl) was assayed for glucose at 0, 30, 60, and 120 min after the injection using a glucose meter (Medisense and Precision Xceed, Abbott Laboratories). Surgical implantation of fat was performed as previously reported, with minor modifications (Gavrilova et al., 2000). Intrascapular fat pads lacking brown adipose tissue and inguinal fat pads were used. The homeostasis model assessment (HOMA) index was calculated according to the following formula: insulin (μU/ml) × glucose (mg/dl)/405) (Akagiri et al., 2008). Pioglitazone and metformin were administered as previously described (Kita et al., 2012; Prieur et al., 2013).

Cells and Culture Conditions

3T3-L1 and 293T cells were maintained in DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, and 100 U/ml of penicillin and streptomycin (P/S). Primary MEFs were cultured in medium supplemented with 0.1 mM nonessential amino acids, 55 μM 2-mercaptoethanol, and 100 U/ml P/S. The $Atm^{+/+}$ and $Atm^{-/-}$ MEF cell lines were maintained on an *Arf*-null background as described previously (Kamijo et al., 1999). In terms of adipocyte differentiation, the *Arf*-null background did not affect competency, similar to the case of the 3T3-L1 cell line, which lacks *Arf* expression.

In Vitro Adipocyte Differentiation Assay

The in vitro adipocyte differentiation assay was performed as previously described (Tanaka et al., 1997). To induce adipocytic differentiation, the cells were maintained at confluence for 2 days and were then switched to differentiation medium (DMEM containing 5 μg/ml insulin, 1 μM dexamethasone, and 0.5 mM isobutyl methyl xanthine [Sigma-Aldrich]). The medium was replaced every 2 days until the cells were analyzed. SVF analysis and in vitro differentiation were performed as previously described (Rodeheffer et al., 2008).

Oil Red O Staining and Determination of Triglyceride Content

To stain adipocytes, the cells were washed twice with PBS and then incubated with a filtered solution of 60% Oil red O (0.15 g/50 ml of 2-propanol) for 30 min at 37°C. The cells were washed briefly with 60% 2-propanol and then with water before visualization. To measure their triglyceride content, the cells were suspended in a buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 0.1% Triton X-100, and protease inhibitors (Roche Diagnostics) and were sonicated for 10 s on ice. The lysates were cleared by centrifugation, and the triglyceride content was measured using a serum triglyceride determination kit (Sigma).

Western Blotting and Immunoprecipitation

Western blotting was performed according to routine procedures. The immunoprecipitation experiments were performed as follows. First, the cells were lysed using TGN buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% NP-40, 0.5% Tween 20, and a phosphatase- and protease-inhibitor cocktail), and then the antigens were precipitated using the antibodies described above after precleaning the lysates using a control immunoglobulin G and protein A/G Sepharose beads (Santa Cruz Biotechnology). The precipitants were eluted by boiling the beads in SDS buffer after six thorough washes using TGN buffer containing 0.3 M LiCl. After SDS-PAGE, the blots were incubated with the various antibodies and were visualized using TrueBlot technology (eBioscience).

Immunofluorescence Microscopy

The cells were fixed using 4% paraformaldehyde at room temperature for 15 min, permeabilized using 0.5% Triton X-100/PBS at room temperature for 5 min, blocked using 10% FBS/PBS, and stained using primary antibodies diluted in PBS containing 2% BSA overnight at 4°C. The primary antibodies were detected using an Alexa-488-conjugated anti-mouse secondary antibody (Invitrogen). The nuclei were stained using Vectashield containing DAPI (Vector). Images were captured using an FV10i confocal microscope (Olympus).

ChIP Assay

The ChIP assays were performed as previously described, with minor modifications (Berkovich et al., 2008). Protein-G Dynabeads were used instead of protein-A agarose beads. The antibodies used for the ChIP assays included C/EBPβ (H7) from Santa Cruz Biotechnology and acetyl-histone H3 (06-599) and H4 (06-866) from EMD Millipore. The primers used for the expression analysis of the C/EBPα promoter were previously reported (Tang et al., 2004).

Luciferase Assay

The C/EBPα-promoter luciferase-reporter plasmid was constructed as previously described (Tang et al., 1997). The luciferase activity was determined using the dual-luciferase reporter assay system (Promega).

Real-Time qPCR Assay

After RNA extraction with TRIzol reagent (Life Technologies), cDNAs were synthesized using Superscript III (Life Technologies) and an oligo dT primer. PCR amplification was performed using SYBR GreenER (Life Technologies), and the amplified PCR product was monitored using a Bio-Rad MiniOption cycler.

Additional methods are provided in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.01.027.

AUTHOR CONTRIBUTIONS

M.T. designed and performed experiments, analyzed the data, and wrote the manuscript. H.U. performed majority of experiments and analyzed the data. R.N. performed complementation assay. M.S. performed glucose uptake assay, EMSA assay, and vector construction. S.K. and J.P performed microscopic analysis. N.I. performed micro CT analysis. S.K. (TMDU), S.K. (Juntendo University), Y.T., and Y.K performed hyperinsulinemic-euglycemic clamp experiments. T.S. supported experiment for differentiation assay using stromal vascular fractions. T.S. and A.Y. supervised the experiment performed by H.U. Y.O. and S.M. supervised the project and designed experiments.

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