Boosting ATM Activity Promotes Longevity in Nematodes and Mice

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Abstract

DNA damage accumulates with age¹. However, whether and how robust DNA repair machinery promotes longevity is elusive. Here, we demonstrate that activation of ataxia-telangiectasia mutated (ATM) via low dose of chloroquine (CQ) promotes DNA damage clearance, rescues age-related metabolic shift, and extends lifespan in nematodes and mice. Molecularly, ATM phosphorylates SIRT6 deacetylase and thus prevents MDM2-mediated ubiquitination and proteasomal degradation. Extra copies of *Sirt6* in *Atm*^{-/-} mice extend lifespan, accompanied with restored metabolic homeostasis. In a progeria mouse model with low ATM protein level and DNA repair capacity, the treatment with CQ ameliorates premature aging features and extends lifespan. Thus, our data highlights a pro-longevity role of ATM, for the first time establishing direct causal links between robust DNA repair machinery and longevity, and providing therapeutic strategy for progeria and age-related metabolic diseases.

Introduction

A variety of metabolic insults frequently generate DNA lesions in mammalian cells, which, if wrongly repaired, may lead to somatic mutations and cell transformation²; if unrepaired, may accumulate and constantly activate DNA damage response (DDR), a unique feature and mechanism of senescence^{3,4}. Ataxia telangiectasia mutated (ATM), a serine/threonine protein kinase, belongs to these key regulators of DDR⁵. Upon DNA damage, self-activated ATM phosphorylates downstream transducers and effectors, thus promoting DNA repair^{6,7}. H2AX is one well-documented phosphorylation target of ATM; phosphorylated H2AX at S139 (γ H2AX) is widely applied as a hallmark of DNA damage⁸. Accompanied with DNA repair function decline, γ H2AX-enriched DNA damage foci accumulate in senescent cells and in tissues from aged animals⁹, supporting causal links between defective DDR and aging. In addition to γ H2AX, CHK2, p53, SMC1, NBS1, BRCA1 and MDC1 are all ATM substrates¹⁰, of which phosphorylated Chk2 and p53 are key players of DDR-induced cell cycle arrest. In human fibroblasts, a dramatic decline of homologous recombination (HR) efficiency, attributable to defective recruitment of Rad51, was observed¹¹. Similar defects in HR were also observed in Hutchinson-Gilford progeria syndrome (HGPS), which is predominantly caused by a *LMNA* G608G mutation¹². In addition to DNA damage accumulation, inherited loss-of-function mutations in essential components of DNA repair machinery also accelerate aging in humans and mice¹³. Patients suffering from ataxia telangiectasia (A-T) develop prominent aging features in their second decades^{14,15}. Werner syndrome, Bloom's syndrome and Rothmund-Thomson syndrome are all progeria syndromes caused by mutations of genes that directly regulate DNA repair¹⁶⁻¹⁹. Homozygous disruption of *Atm* in mice recapitulates many premature aging features of A-T, like growth retardation, infertility, neurodegeneration, immunodeficiency and cancer predisposition²⁰. Mouse models deficient in DNA repair elements, including DNA-PKcs, Ku70, Ku80, DNA ligase IV, Artemis or Ercc1, phenocopy premature aging features^{21,22}. However, though tons of evidences support that defects in DNA repair accelerate ageing, whether and how robust DNA repair machinery promotes longevity is poorly understood.

Metabolic disturbance is another antagonistic hallmark of aging²³. Although DNA repair deficiency is implicated in aging and age-related diseases including metabolic disorders^{24,25}, the mechanistic linker between DNA repair machinery and metabolic reprogramming in aging is poorly understood. Notably, in response to oxidative stress, ATM phosphorylates Hsp27 thus to shift glucose metabolism from glycolysis to the pentose phosphate pathway (PPP)^{26,27}. Inactivating ATM enhances glucose and glutamine consumption by inhibiting P53 and upregulating c-MYC²⁸. The role of ATM in age-onset metabolic disturbances is yet unclear. On the other front, longevity-promoting genes, like NAD⁺-dependent sirtuins, are able to shunt energy metabolism away from anaerobic glycolysis toward TCA cycle^{29,30}. Sirt6 cooperates with Hypoxia inducible factor-1 α (HIF1 α) via deacetylating transcription-active epigenetic marks to regulate glucose homeostasis³¹, and also modulates aerobic glycolysis³². Loss of *Sirt6* accelerates aging, accompanied with hypoglycemia and genomic instability^{33,34}, whereas ectopic *Sirt6* inhibits insulin and insulin-like growth factor 1 (IGF1) signaling and extends lifespan in male mice³⁵. Owing to up-regulated metabolic genes like glucose transporter GLUT-1, lactate dehydrogenase (LDH) and pyruvate dehydrogenase kinase (PDK), the glycolytic activity and lactate production were dramatically increased in *Sirt6*^{-/-} cells and tumors³².

Here, we identified a progressive decline in ATM-centered DNA repair machinery during aging, along with shunted glucose metabolism to glycolysis. DNA damage-free activation of ATM by chloroquine (CQ) promotes DNA damage clearance, rescues the age-related metabolic shift, and extends lifespan in both nematodes and mice. Mechanistically, ATM phosphorylates and stabilizes pro-longevity protein SIRT6. Extra copies of *Sirt6* attenuate metabolic abnormality and extend lifespan in *Atm*^{-/-} mice, and long-term treatment of CQ restores metabolic shift and extends lifespan in a progeria mouse model.

Results

ATM alleviates replicative senescence

In searching for genes/pathways that drive senescence, a gradual decline of ATM-centered DNA repair machinery was identified by RNAseq analysis (Supplementary Fig.

1a-e). Western blotting analysis confirmed progressively downregulated protein levels of ATM and its downstream target NBS1 and RAP80 in senescent human skin fibroblasts (HSFs) (Fig. 1a). Mouse embryonic fibroblasts (MEFs) with a limited growth capacity and senescent phenotypes when cultured *in vitro*³⁶⁻³⁸, and brain tissues from aged mice also showed progressive decline of ATM, NBS1, and PAP80 (Fig. 1b-c). Concomitantly, a reciprocal upregulation of γ H2AX, indicating accumulated DNA damages, and p16^{Ink4a} level was observed in senescent HSFs, MEFs, and aged brain tissues (Fig. 1a-c). Knocking down *ATM* via shRNA accelerated senescence in HSFs, evidenced by increased β -galactosidase activity (Fig. 1d-e), enlarged morphology (Supplementary Fig.2a), accumulated γ H2AX (Fig. 1f) and reduced cell proliferation (Supplementary Fig. 2b). Thus, ATM decline retards DDR and drives senescence.

Other than DNA damage, ATM can be activated by chloroquine (CQ), an antimalarial drug that modulates chromatin confirmation⁶. Indeed, we found that low dose of CQ increased pS1981 auto-phosphorylation of ATM but not γ H2AX (Supplementary Fig. 2c). We then investigated whether activating ATM by CQ ameliorates senescence. As shown, the CQ treatment activated ATM (pS1981), promoted clearance of DNA damage (γ H2AX), and inhibited apoptosis (cleaved Casp3) in HSFs (Fig. 1g). Also, the CQ treatment suppressed β -galactosidase activity, but that was abrogated when *ATM* was knocked down (Fig. 1h-i). Likewise, the CQ treatment activated *Atm*, cleared up accumulated DNA damage (Fig. 1j), suppressed β -galactosidase activity (Fig. 1k and Supplementary Fig. 2d), and prolonged replicative lifespan in MEFs (Fig. 1l). Despite that both doses of 10 μ M and 1 μ M CQ activated ATM, the dose-dependent toxicity assay showed CQ in 1 μ M is suitable for long-term treatment (Supplementary Fig. 2e-f). Of note, *ATM* KD or low dose of CQ applied in this study had little effect on basal autophagic activity (Fig. 1f, j and Supplementary Fig. 2g). Collectively, CQ activates ATM to alleviate replicative senescence.

Figure 1

Figure 1: ATM activation by chloroquine alleviated senescence.

(a) Immunoblots showing protein levels of ATM, NBS1 and RAP80 in human skin fibroblasts (HSFs). Gradually increased level of p16 indicates cellular senescence. Elevated γ H2AX level indicates accumulated DNA damage. (b) Immunoblots showing protein levels of ATM, NBS1 and RAP80 in mouse embryonic fibroblasts (MEFs). (c) Immunoblots showing protein levels of ATM, NBS1 and RAP80 in brain tissues isolated

from 3, 10 and 18-month-old male mice. **(d)** SA- β -Gal staining in HSFs treated with sh-*ATM* or scramble shRNA. Scale bar, 100 μ m. **(e)** Quantification of SA- β -Gal-positive staining of **(d)** from five views randomly captured for each group. Data represent mean $\pm$ SEM. *** $P < 0.001$. **(f)** Immunoblots showing increased γ H2AX and unaffected LC3/II in HSFs treated with sh-*ATM* or scramble shRNA. **(g)** Immunoblots showing protein levels of pS1981 ATM, γ H2AX and cleaved caspase-3 in HSFs treated with 10 μ M of CQ for indicated time. **(h)** SA- β -Gal staining in HSFs expressing either scramble or *ATM* shRNA treated with 1 μ M CQ or DMSO (12 h). Scale bar, 100 μ m. **(i)** Quantification of SA- β -Gal-positive staining of **(h)** from five views randomly captured for each group. Data represent mean $\pm$ SEM. *** $P < 0.001$; 'N.S.' indicates no significant difference. **(j)** Immunoblots showing protein levels of γ H2AX, p62 and LC3 in MEFs treated with 1 μ M CQ or DMSO. Noted that the levels of p62 and LC3 were merely affected. **(k)** SA- β -Gal staining in primary MEFs treated with 1 μ M CQ or DMSO. Scale bar, 100 μ m. **(l)** Cumulative cell numbers of continuously cultured primary MEFs treated with 1 μ M CQ or DMSO. Data represent mean $\pm$ SEM. ** $P < 0.01$.

An ATM-SIRT6 axis underlies age-associated metabolic reprogramming

A-T patients lacking functional ATM display features of premature aging, accompanied with insulin resistance and glucose intolerance^{39,40}. Senescent cells exhibit enhanced glycolysis but impaired mitochondrial respiration, and increased lactate for ATP generation^{41,42}. As such, we must ask whether ATM decline triggers age-associated metabolic shift. Indeed, glycolytic gene *LDHB* and *PDK1* were dramatically increased in senescent MEFs and HSFs (Fig. 2a and Supplementary Fig. 3a), and in liver tissues from *Atm*^{-/-} mice (Supplementary Fig. 3b). Significantly, activating ATM via CQ suppressed senescence-associated glycolysis (Fig. 2a and Supplementary Fig. 3a). The inhibitory effect on glycolysis was diminished when *ATM* was depleted in HepG2 cells (Fig. 2b), suggesting the role of ATM in inhibiting glycolysis.

To address how ATM regulates glycolysis, we performed RNA-Seq in *Atm*^{-/-} MEF cells, and revealed a significant upregulation of glycolytic pathways (Fig. 2c). Specific genes were validated by q-PCR (Supplementary Fig. 3c). As p53 is critical in glycolysis^{43,44}, we

further analyzed metabolomics of *Atm*^{-/-} and control MEF cells in *p53* null background. As shown, the metabolic profile exhibited a clear shift, i.e. mitochondrial electron transport chain and intermediates of TCA cycle were reduced, while intermediates of glycolysis were elevated (Fig. 2d, Supplementary Fig. 3d-e and Table S1). The data suggest *ATM* deficiency enhances anaerobic glycolysis in a *p53*-independent manner.

Sirt6 deacetylase is able to shunt energy metabolism away from anaerobic glycolysis to TCA cycle via H3K9ac-mediated local chromatin remodeling^{31,32}. We noted the level of H3K9ac was enhanced in cells depleted *ATM* (Fig. 2e). Re-expressed *ATM* in A-T cells suppressed H3K9ac level (Fig. 2f). Further ChIP analysis showed that H3K9ac was enriched at the promoter regions of glycolytic genes in *Atm*^{-/-} cells (Fig. 2g), where the relative occupancy of SIRT6 was abolished (Fig. 2h). Consistent with increased H3K9ac level, SIRT6 protein level was dramatically downregulated in *Atm*^{-/-} mouse livers, and *ATM* deficient HepG2, U2OS, and HEK293 cells (Supplementary Fig. 3f-i). In contrast, other sirtuins were merely affected (Fig. 2i), and mRNA levels of all sirtuins remained unchanged (Supplementary Fig. 3j). Moreover, transcriptomic analysis and q-PCR data illustrated that *Sirt6* depletion upregulated a similar cluster of genes essential for glycolysis (Supplementary Fig. 4a-b and Table S2). More importantly, the hyper-activated glycolytic pathway caused by *ATM* deficiency was completely restored by ectopic *SIRT6* in HepG2 cells (Supplementary Fig. 4c). The CQ treatment upregulated SIRT6 level and reduced H3K9ac level, especially at regulatory regions of glycolytic genes (Supplementary Fig. 4d-e). Knocking down *SIRT6* abolished the inhibitory effect of CQ on glycolysis (Fig. 2b). Though levels of SIRT1 and SIRT7 were also declined along with senescence, they were not restored by CQ treatment (Supplementary Fig. 4f). Additionally, *ATM* depletion in HEK293 cells, HSFs and MEFs, significantly downregulated SIRT6 protein level, with no effect on SIRT1 or SIRT7 protein level (Fig. 2i and Supplementary Fig. 4g-h). Thus, these data suggest *ATM* decline triggers age-associated metabolic shift via SIRT6-mediated chromatin remodeling.

Other than metabolic abnormality, depleting *Sirt6* leads to various premature aging features and shortened lifespan⁴⁵, whereas extra copies of *Sirt6* promotes longevity in male mice³⁵. Given that Sirt6 was destabilized in *Atm* null mice, we asked whether *Sirt6*

transgene could rescue premature aging phenotypes and shortened lifespan in *Atm*^{-/-} mice. To this end, we generated *Sirt6* transgenic mice by microinjection, and bred them with *Atm*^{-/-} mice. The overexpression of *Sirt6* was demonstrated by Western blotting (Supplementary Fig. 4i). Significantly, ectopic *Sirt6* restored the elevation of serum lactate and blood glucose levels, and extended lifespan of *Atm*^{-/-} mice in both genders (Fig. 2j and Supplementary Fig. 4j-k). Importantly, *Atm*^{-/-}; *Sirt6*-tg mice exhibited improved glucose tolerance and decreased insulin resistance (Fig. 2k-l). Given little difference was observed in glucose metabolism between young WT and *Sirt6*-transgenic mice³⁵, these data suggest a contributing role of the *Atm*-*Sirt6* axis in the age-associated metabolic reprogramming.

Figure 2: ATM-SIRT6 axis regulates age-related metabolic reprogramming.

(a) Quantitative RT-PCR analysis of mRNA levels of indicated glycolytic genes in different passages of MEFs with or without treatment of CQ. Data represent mean $\pm$ SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (b) Quantitative RT-PCR analysis of mRNA levels of indicated glycolytic genes in Scramble (NC), si-SIRT6 or si-ATM HepG2 cells

incubated with or without CQ (10 μ M, 6 h). Data represent mean $\pm$ SEM. * P < 0.05, ** P < 0.01, *** P < 0.001. (c) Heatmap representation of RNA-Seq data showing relative changes of glycolytic genes in *Atm*^{-/-} MEF cells. Red and green indicate up- and downregulation, respectively. (d) Heatmap showing relative levels of metabolites in *Atm*^{+/+} and *Atm*^{-/-} MEF cells of p53 null background, analyzed by LC-MS. Red and blue indicate up- and downregulation, respectively. (e) Immunoblots showing protein levels of H3K9ac and H3K56ac in *ATM*-deficient HepG2 cells. (f) Immunoblots showing levels of H3K9ac in A-T cells reconstituted with Flag-ATM. (g-h) ChIP analysis showing enrichment of H3K9ac and SIRT6 at the promoter regions of indicated genes in *Atm*^{+/+} and *Atm*^{-/-} MEFs. Data represent mean $\pm$ SEM of 3 independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001. (i) Immunoblots showing protein levels of sirtuins in wild-type (WT) and *ATM* knockout (KO) HEK293 cells. (j) Kaplan-Meier survival of *Atm*^{-/-} and *Atm*^{-/-}; *Sirt6*-Tg male (n = 11 in each group) and female (n = 9 in each group) mice. ** P < 0.01. (k) Results of glucose tolerance tests in *Atm*^{+/+}, *Atm*^{-/-}, and *Atm*^{-/-}; *Sirt6*-Tg mice. Data represent mean $\pm$ SEM, n = 6. ** P < 0.01, *** P < 0.001. (l) Results of insulin tolerance tests in *Atm*^{+/+}, *Atm*^{-/-}, and *Atm*^{-/-}; *Sirt6*-Tg mice. Data represent mean $\pm$ SEM, n = 6. ** P < 0.01.

ATM phosphorylates and stabilizes SIRT6

Next, we examined how ATM regulates SIRT6. Significantly, the overexpression of *ATM* increased SIRT6 level, but that was abolished when ATM was S1981A-mutated (Fig. 3a). Of note, the S1981A mutation blocks dimeric ATM dissociation and therefore the ATM activation⁶. Moreover, in addition to CQ, hypotonic buffer (20 mM NaCl), low glucose (LG), DNA-damaging agent CPT and doxorubicin (Dox) all activated ATM and concomitantly increased SIRT6 protein level (Supplementary Fig. 5a-c), but this effect was abrogated in *ATM*-depleted cells (Supplementary Fig. 5b-c). These data implicate a direct regulation of SIRT6 stability by ATM kinase activity. To confirm it, we first performed co-immunoprecipitation (Co-IP) in cells transfected with various FLAG-sirtuins. Very interestingly, ATM was predominantly associated with SIRT6 among seven sirtuins (Fig. 3b). The interaction was further confirmed at both ectopic and endogenous levels (Fig. 3c and Supplementary Fig. 5d). Immunofluorescence microscopy

showed co-localization of SIRT6 and ATM protein in the nucleus (Fig. 3d). Domain mapping experiment indicated that the C-terminal domain was required for SIRT6 binding to ATM (Supplementary Fig. 5e). To determine whether ATM physically binds to SIRT6, 10 consecutive recombinant GST-ATM proteins were obtained and the binding to purified His-SIRT6 was analyzed. As shown, His-SIRT6 strongly bound to GST-ATM-4 (residues 770-1102) (Fig. 3e), the N-terminal HEAT repeat domain of ATM, which are predicted to bind to phosphorylation substrates, such as NBS1⁴⁶.

We next examined whether ATM phosphorylates SIRT6. Firstly, we found the CQ or CPT treatment significantly enhanced the binding of SIRT6 to ATM (Fig. 3f and Supplementary Fig. 5f), whereas the S1981A mutant blocked such association (Supplementary Fig. 5g). ATM preferentially phosphorylates S/T-Q motif. In the presence of CPT, the increased p-S/TQ level of SIRT6 was identified (Fig. 3g). Of note, lambda protein phosphatase (λ PP) diminished p-S/TQ level of SIRT6 (Supplementary Fig. 5h). Likewise, the p-S/TQ level of SIRT6 was elevated in cells treated with low glucose, which activates ATM by ROS generation^{47,48} (Supplementary Fig. 5i). Moreover, ectopic ATM significantly increased p-S/TQ level of SIRT6, and that was abolished in case of S1981A-mutated (Fig. 3h). Consistently, a pronounced reduction of p-S/TQ level of SIRT6 was observed in cells lacking *ATM* or treated with KU55933, a selective and specific ATM kinase inhibitor^{49,50} (Fig. 3i-j). The decrease in p-S/TQ level was primarily attributable to loss of *ATM*, as it was restored by ectopic FLAG-ATM in a dose-dependent manner (Supplementary Fig. 5j). Indeed, SIRT6 has one evolutionarily conserved S¹¹²Q¹¹³ motif (Fig. 3k). We therefore constructed S112A and S112D mutations, which resembles hypo- and hyper-phosphorylated form respectively. As shown, these mutations almost abolished the pS/T-Q level of FLAG-SIRT6 (Fig. 3l). Further *in vitro* kinase assay showed that ATM could phosphorylate GST-SIRT6, but not S112A mutant (Supplementary Fig. 5k). Collectively, the data suggest that ATM directly phosphorylates SIRT6 at Serine 112.

Figure 3

Figure 3: ATM interacts with and phosphorylates SIRT6.

(a) Immunoblots showing protein levels of SIRT6 in HEK293 cells expressing Flag-ATM or Flag-ATM S1981A. (b) Immunoblots showing endogenous ATM and p-S/TQ motif in anti-Flag immunoprecipitates in HEK293 cells transfected with empty vector or Flag-sirtuins. (c) Immunoblots showing Flag-ATM and HA-SIRT6 in anti-HA (upper) or anti-Flag (lower) immunoprecipitates in HEK293 cells transfected with indicated constructs. (d) Representative photos of immunofluorescence staining of SIRT6 and

ATM in U2OS cells, showing the co-localization in nucleus. Scale bar, 50 μ m. (e) GST pull-down assay showing bacterially expressed His-SIRT6 predominantly bound to GST-ATM fragment 4 (770-1102), the N-terminal HEAT-repeat of ATM. (f) Immunoblots showing the increased binding capacity of ATM and SIRT6 under the treatment of (10 μ M) CQ for the indicated time. (g) Immunoblots showing ATM and p-S/TQ in anti-Flag immunoprecipitates in HEK293 cells expressing Flag-SIRT6 treated with CPT (0.4 μ M) or DMSO. (h) Immunoblots showing level of p-S/TQ SIRT6 in HEK293 cells co-transfected with HA-SIRT6 and Flag-ATM, Flag-ATM S1981A, or empty vector. (i) Immunoblots showing p-S/T Q SIRT6 in WT or ATM KO HEK293 cells. (j) Immunoblots showing p-S/T Q level of SIRT6 in *ATM* WT or KO HEK293 cells treated with DMSO and KU55933 (10 or 20 μ M, 2 h). (k) Alignment of protein sequence of human SIRT6 and orthologues in mouse, rat, fruit fly, *Xenopus*, and *C. elegans*. A conserved S¹¹² Q¹¹³ motif was highlighted. (l) Immunoblots showing p-S/T Q level of Flag-SIRT6, Flag-SIRT6 S112A, or Flag-SIRT6 S112D in HEK293 cells.

We next examined whether ATM is involved in regulating SIRT6 protein stability. Notably, compared to wild-type or vehicle control, the degradation rates of ectopic and endogenous SIRT6 were largely increased in *ATM* KO HEK293 cells, *Atm*^{-/-} MEFs, or cells incubated with ATM specific inhibitor KU55933 in the presence of cycloheximide (CHX) (Fig. 4a-b and Supplementary Fig. 6a-c). Recently MDM2 was demonstrated to ubiquitinate SIRT6 and promote its proteasomal degradation⁵¹. We therefore examined the polyubiquitination level of SIRT6. As shown, the ubiquitination level of FLAG-SIRT6 in *ATM* KO cells was significantly elevated compared with wild-type (Supplementary Fig. 6d). While the S112A mutant markedly enhanced polyubiquitination level of SIRT6, the S112D merely affected it (Supplementary Fig. 6e). Moreover, the S112A accelerated SIRT6 degradation, whereas the S112D retarded it (Fig. 4c-d), indicating that the Ser112 phosphorylation by ATM regulates SIRT6 ubiquitination and thus protein stability. Indeed, ectopic MDM2 enhanced polyubiquitination level of FLAG-SIRT6 (Supplementary Fig. 6f). In case of *ATM* depleted or SIRT6 S112A-mutated, the binding capacity of SIRT6 to MDM2 was

enhanced (Fig. 4e and Supplementary Fig. 6g). In searching for key residues that are poly-ubiquitinated by MDM2, we identified two clusters of lysine residues, i.e. K143/145 and K346/349, which are conserved across species. We then generated KR mutations of these residues, and found K346/349R remarkably reduced the poly-ubiquitination level of SIRT6 (Supplementary Fig. 6h). Individual KR point mutation showed that K346R significantly blocked MDM2-mediated ubiquitination and degradation of SIRT6, whereas K349R hardly affected it (Fig. 4f-g). More importantly, the K346R restored the increased ubiquitination and accelerated protein degradation of SIRT6 S112A (Supplementary Fig. 6i-j). Collectively, these data indicate that K346 is subjected to MDM2-mediated ubiquitination and is responsible for the degradation of SIRT6, which is inhibited by ATM-mediated S112 phosphorylation.

Figure 4

Figure 4: ATM prevents ubiquitination and degradation of SIRT6.

(a) Immunoblots showing protein levels of Flag-SIRT6 in WT and ATM KO HEK293 cells treated with CHX (50 μ g/ml) for indicated periods of time. (b) Quantification of protein levels in (a) by ImageJ[®]. Data represent mean $\pm$ SEM of three independent experiments. ** P < 0.01. (c) Immunoblots showing protein levels of Flag-SIRT6, S112A and S112D in the presence of CHX (50 μ g/ml) for indicated periods of time. (d) Quantification of protein levels in (c) by ImageJ[®]. Data represent mean $\pm$ SEM of three

independent experiments. *** $P < 0.001$. (e) Immunoblots showing increased binding capacity between SIRT6 and MDM2 in *ATM* KO HEK293 cells. (f) Immunoblots showing ubiquitination of Flag-SIRT6, K346R, K349R and K346/349R (2KR) in HEK293 cells. Noted that 2KR and K346R abrogated the ubiquitination of Flag-SIRT6. (g-h) Upper, immunoblots showing protein levels of Flag-SIRT6, K346R and K349R in the presence of CHX (50 μ g/ml) for indicated periods of time. Lower, quantification of protein levels by ImageJ[®]. Data represent mean $\pm$ SEM of three independent experiments. *** $P < 0.001$.

Activating ATM via CQ promotes longevity

The cellular data suggest a pro-longevity function of ATM. We then tested it at organismal level. We employed *Caenorhabditis elegans*, which have a short lifespan of approximate 30 days. Nematodes deficient for *atm-1*, an orthologue of mammalian *ATM*, and wild-types were exposed to various doses of CQ (see Materials and Methods). Significantly, the period treatment with CQ (1.0 μ M) extended the median lifespan (~14%) of *C. elegans* (Fig. 5a). The lifespan-extending effect was abolished in *atm-1* KO (Fig. 5b) or in SIRT6 homolog *sir-2.4* KD nematodes (Supplementary Fig. 7a-b). The data suggest that CQ promotes longevity in an ATM- and SIRT6- dependent manner. We further examined the beneficial effect of CQ in a HGPS model, i.e. *Zmpste24*^{-/-} mice, which has a shortened lifespan of 4-6 months⁵² and impaired ATM-mediated DNA repair signaling⁵³. Here we found, *Atm* level was dramatically reduced in *Zmpste24*^{-/-} cells and tissues (Supplementary Fig. 7c-d). Significantly, the CQ treatment activated *Atm*, stabilized *Sirt6*, decreased the accumulated DNA damage, inhibited glycolysis, and alleviated senescence in *Zmpste24*^{-/-} cells (Fig. 5c-d and Supplementary Fig. 7e-f). The CQ treatment also held off body weight decline, increased running endurance, and prolonged lifespan in *Zmpste24*^{-/-} mice (Fig. 5e-f and Supplementary Fig. 7g). We then extended the study to physiologically aged mice. In this case, 12-month-old “old” male mice were intraperitoneally administrated with lose dose of CQ (3.5mg/kg) twice a week. Remarkably, compared to the saline-treated control group, the CQ treatment inhibited glycolysis, lowered down serum lactate level, attenuated body weight decline (Fig. 5g-h

and Supplementary Fig. 7h). Most remarkably, the CQ treatment enhanced survival rate of physiologically aged mice ($p < 0.05$), but showed no significant effect in *Atm*^{-/-} mice (Fig. 5i). Generally, these data demonstrate a lifespan-extending benefit of ATM activation by CQ.

Figure 5

Figure 5: ATM Activation via CQ promotes longevity

(a) Survival analysis of *C. elegans* treated with the indicated dosage of CQ. ** $P < 0.01$. NS indicates no significant difference. (b) Survival analysis of wild-type and *atm-1* null *C. elegans* cultured in medium with or without 1 μ M CQ. (c) Representative images showing SA- β -Gal staining in *Zmpste24*^{-/-} MEFs with or without CQ treatment. Scale

bar, 100 μ m. (d) Quantitative RT-PCR analysis of mRNA levels of p16^{Ink4a} and indicated glycolytic genes in liver tissues of *Zmpste24*^{+/+}, saline-treated, and CQ-treated *Zmpste24*^{-/-} mice. Mice were treated for 8 weeks with two weekly intraperitoneal injections of CQ at 3.5 mg/kg. Data represent mean $\pm$ SEM. * P < 0.05, ** P < 0.01, *** P < 0.001. (e) Body weight of saline- and CQ-treated male *Zmpste24*^{-/-} mice. Data represent mean $\pm$ SEM. ** P < 0.01. (f) Kaplan-Meier survival curves of saline-treated (n = 12) and CQ-treated (n = 7) *Zmpste24*^{-/-} mice. *** P < 0.001. (g) Quantitative RT-PCR analysis of mRNA levels of indicated glycolytic genes in the liver tissues of 4-month-old, saline-treated 12-month-old (n = 3), and CQ-treated 20-month-old (n = 3) mice. Mice were treated for 8 weeks with two weekly intraperitoneal injections of CQ at 3.5 mg/kg. Data represent mean $\pm$ SEM. * P < 0.05, ** P < 0.01, *** P < 0.001. (h) Body weight of saline- and CQ-treated male aging mice. Data represent mean $\pm$ SEM. *** P < 0.001. (i) Kaplan-Meier survival curves of saline-treated and CQ-treated aging C57BL/6J mice (n = 12 for each group) and *Atm*^{-/-} mice (n = 9 for each group). * P < 0.05.

Discussion

DNA damage accumulates with age and defective DNA repair accelerates aging. However, whether boosting DNA repair machinery promotes healthiness and longevity is still obscure. DNA damage stimulates DDR, but if persisted, it rather leads to senescence. Therefore, if enhancing DDR efficacy possibly promotes longevity, it must be DNA damage free. The antimalarial drug CQ could intercalate into the internucleosomal regions of chromatin, unwind DNA helical twist, and thus activate ATM without causing any DNA damage^{6,54}. We demonstrate that long-term treatment with CQ activated ATM, slowed down senescence, restored age-related metabolic shift, and extended lifespan in both nematodes and mice. Mechanistically, ATM phosphorylated longevity gene SIRT6⁵⁵, and prevented the MDM2-mediated ubiquitination and proteasomal degradation of SIRT6. To our knowledge, it is the first time establishing direct causal links between robust DNA repair machinery and longevity. Supporting this notion, the DNA repair efficacy is found enhanced in long-lived naked mole rat⁵⁶, and human longevity is associated with single nucleotide polymorphisms (SNPs) in DNA repair

genes/pathways^{57,58}. Specifically, an *ATM* SNP that could enhance the transcription of *ATM* is associated with longevity in both Chinese and Italian populations^{59,60}. In future study, it would be worthwhile to evaluate whether extra copies of *Atm* could promote longevity in model organisms.

Accumulation of DNA damages and metabolic disturbance are common denominators of aging^{23,61}. Metabolic reprogramming from TCA cycle to glycolysis is prominent in both physiological and pathological aging^{24,62}. Why senescent cells become glycolytic is poorly understood. Although it is recognized that deficiency in DNA repair machinery like ATM, WRN and Ercc1 accelerates aging and causes severe metabolic disorders^{63,64}, it is unclear whether overexpression or enhancing DNA repair capacity, e.g. activating ATM, could not only enhance genomic stability but also hold off age-onset metabolic reprogramming and even extend lifespan. In our study, depletion of *ATM* induced metabolic reprogramming to glycolysis in senescent cells and premature ageing mouse models. Activation ATM by CQ reduced DNA damages and inhibited glycolysis. Remarkably, the long-term treatment of CQ extended lifespan in nematodes and mice. The data suggest a pro-longevity role of CQ-activated ATM, not only in improvement of DNA repair capacity, but also in the inhibition of glycolysis. Further, SIRT6 with diverge functions in biological process including DNA repair and metabolism, was discovered as the novel and direct target of ATM.

Recently, Bohr's group uncovered an increased consumption of NAD⁺ by an early DDR factor poly (ADP-ribose) polymerase (PARP1), owing to accumulated DNA damages, accelerates aging in *Atm* mutant mice⁶⁵. NAD⁺ serves as a cofactor of sirtuins, including SIRT1 and SIRT6. Therefore this work establishes a linear causal link between deficient DDR, DNA damage accumulation, consumption of NAD⁺, decline in sirtuin activity and aging. Moreover, administration of nicotinamide mononucleotide or nicotinamide riboside ameliorates age-related function decline and extends lifespan in mice^{66,67}. Here, we found that *ATM* decline during aging caused DNA damage accumulation and enhanced glycolysis, both of which consume most of NAD⁺, providing an explanation of low NAD⁺ level in aged and *Atm*^{-/-} mice.

Closely resembling normal ageing, HGPS has attracted numerous efforts in understanding of molecular mechanisms and developing therapeutic strategies^{68,69}. We and others have found HGPS and *Zmpste24* null cells undergo accelerated senescence owing to defective chromatin remodeling^{53,70-72}, delayed DDR and impaired DNA repair^{12,72,73}. Specifically, Atm-Kap-1 signaling is compromised and SIRT6 protein and deacetylase activity are reduced in progeria cells^{70,74}. Here we showed that Atm was significantly downregulated, which well explains the reduced SIRT6, delayed DDR and metabolic shift in progeria cells and mice. It would be interesting to investigate whether ectopic *Atm* or *Sirt6* could rescue progeroid features in these mice. Nevertheless, the pharmaceutical activation of ATM via CQ remarkably improved glucose homeostasis, DNA damage clearance, increased running endurance, and extended lifespan in progeria mice.

CQ is FDA-approved and clinically used medicine for treatment of malaria⁷⁵. Through the activation of ATM, long-term treatment of CQ protects against atherosclerosis, improves insulin sensitivity, and rescues glucose tolerance in type 2 diabetes (T2D) models⁷⁶⁻⁷⁸. Lysosomotropic property of CQ also make it as a potent inhibitor of autophagy⁷⁹. The dose (7.0 mg/kg/week in mice) applied in this study is equivalent to 35 mg/week for humans, much lower than that suggested for antimalarial treatment (500 mg/week, maximum 0.8 μ M in plasma) and that for cancer therapy via autophagic inhibition (100-500 mg/day)⁸⁰. Indeed, no obvious autophagic inhibition was observed in our study. The administration of CQ was conducted by intraperitoneal injection as described⁷⁸. It seems that the frequently induced stress by repeated intraperitoneal injection imposes negative effect on lifespan, as saline-treated animals died earlier than untreated. Nonetheless, we treated the two groups of animals under the same conditions (age, diet, health status), and found that CQ-treated C57BL/6J mice exhibited much longer lifespan than saline-treated. When we summarized the lifespan data, i.e. ~840 days after birth of tested animals, which is equivalent to the median lifespan of C57BL/6J^{35,67,81}, more than 60% CQ-treated mice are still alive. Therefore, we believe that the effect of CQ is not just an adaption to chronic stress induced by intraperitoneal injection but rather reflects a general regulation of murine lifespan.

In conclusion, our data establish direct causal links between robust DNA repair machinery and longevity. Together with DNA damage theory of aging, we propose that DNA damage activates DDR, however its constant activation causes senescence; defective ATM-SIRT6 axis underlies premature ageing, exemplified by HGPS and A-T mouse models, which are rescued by treatment of CQ and *Sirt6* transgene respectively; in physiological ageing, DNA damage-free activation of ATM by CQ stabilizes SIRT6, thus promoting longevity in both nematodes and mice (Supplementary Fig 8). Our findings also provide a novel therapeutic strategy for HGPS, and could facilitate clinical trials of CQ as an effective treatment for aging-related diseases.

Experimental Procedures

Mice

Zmpste24^{-/-} mice and *Atm*^{-/-} mice have been described previously^{20,52}. *Sirt6*-transgenic mice (*Sirt6*-tg) in C57BL/6J background were constructed by injecting cloned mSirt6 cDNA with CAG promoter into fertilized eggs. Primers for genotyping of *Sirt6* transgenic allele were as follows; forward: 5'-CTGGTTATTGTGCTGTCTCATCAT-3' and reverse: 5'-CCGTCTACGTTCTGGCTGAC-3'. *Atm*^{-/-} mice were crossed to *Sirt6*-tg mice to get *Atm*^{-/-}; *Sirt6*-tg mice. Chloroquine (CQ) experiments were conducted as described⁷⁸. Briefly, 12-month-old wild-type C57BL/6J male mice and 2-month-old *Zmpste24*^{-/-} male mice were administered with CQ (Sigma, St. Louis, MO) in 0.9% saline twice per week at 7 mg/kg body weight, and control group was treated with saline alone. 8 weeks after treatment of CQ, mice were subjected for functional tests. Body weight and lifespan was recorded. The survival rate was analyzed by Kaplan–Meier method and statistical comparison was performed by Log-rank Test. Mice were housed and handled in the laboratory animal research center of Shenzhen University. All experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC).

C. elegans survival assay

C. elegans nematode survival assay was performed according to standard protocols⁸². Briefly, wild-type and *atm-1* null nematodes (100 to 150 per group) synchronized to

prefertile young adult stage were exposed to NGM plates containing the indicated dosage of CQ. After 1-day incubation, animals were transferred to fresh incubation plates without CQ for another 2 days. This procedure was repeated every three days. Nematodes that showed no response to gentle stimulation were recorded as dead. The survival data was analyzed by Kaplan–Meier method and statistical comparison was performed by Log-rank Test.

Cell lines

HEK293, immortalized mouse embryonic fibroblasts (MEFs), human skin fibroblasts (HSFs), HepG2 and U2OS cells were cultured in Gibco[®] DMEM (Life Technologies, USA) with 10% fetal bovine serum (FBS), 100 U/ml penicillin and streptomycin (P/S) at 37 °C in a 5% CO₂ humidified atmosphere. Primary MEFs were generated from 13.5-day-old embryos as described¹², and were cultured in DMEM supplemented with 0.1 mM nonessential amino acids (Life Technologies), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich, USA). For CQ experiments, cells were maintained in the medium containing 1μM chloroquine for 12 h, and then grown in new fresh medium for 48 h.

Cell lines	Description and Application in this study
HEK293	Transfection; Immunoprecipitation
ATM KO HEK293	Constructed by CRISPR-cas9 system; Ubiquitination and Degradation; p-S/T Q level of ectopic SIRT6
WT, <i>Atm</i> ^{-/-} MEFs and <i>Zmpste24</i> ^{-/-} MEFs	Prepared from WT, <i>Atm</i> ^{-/-} and <i>Zmpste24</i> ^{-/-} embryonic mice; Senescence assay; Glycolysis
WT and <i>Atm</i> ^{-/-} MEFs	RNA-seq; Metabolome; Degradation assay of endogenous Sirt6 protein
HSF cells	shATM by lentivirus; Senescence assay; Glycolysis
HepG2 cells	siSIRT6/siATM to perform the metabolic assay
U2OS cells	DNA repair; Endogenous SIRT6 protein level

Plasmids

Human Flag-SIRT6, pcDNA3.1 Flag-ATM, Flag-ATM S1981A, and pcDNA3 human MDM2 were all purchased from Addgene (Cambridge, MA). Flag-SIRT6 with amino acid substitution mutations (S112A, S112D, K346R/K349R) were generated by PCR-based mutagenesis using pcDNA3-Flag-SIRT6 as a template and QuikChange II site-directed mutagenesis kit (Agilent Technologies), following the manufacturer's instruction. Primer sequences for amino acid mutations of SIRT6 were as follows: SIRT6 S112A: (forward) 5'-cgtccacgttctggcgaccaggaagcgga-3', (reverse) 5'-tccgcttctgtgctgccagaacgtggacg-3'; SIRT6 S112D: (forward) 5'-ccgtccacgttctgtgctgccagaacgtggacg-3', (reverse) 5'-ctccgcttctgtgctgccagaacgtggacg-3'; SIRT6 K346R: (forward) 5'-ggccttcacccttctgggggtctgtg-3', (reverse) 5'-cacagacccccagaagggtgaaggcc-3'; SIRT6 K349R: (forward) 5'-gccttggccctcaccctttgggggggt-3', (reverse) 5'-acccccaaaagggtgagggccaaggc-3. HA-tagged human SIRT6 plasmid was amplified from their respective cDNAs and constructed into pKH3-HA vector. To express four truncated forms of SIRT6 protein, HA-SIRT6 plasmid as a template was constructed by PCR-based deletion.

Protein extraction and Western blotting

For whole cell protein extraction, cells were suspended in 5 volumes of suspension buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, protease inhibitor cocktail), and then added 5 volumes of 2X SDS loading buffer and incubated at 98 °C for 6 min. Mice tissues were homogenized with 1mL of ice-cold tissue lysis buffer (25 mM TrisHCl, pH 7.5, 10 mM Na₃VO₄, 100 mM NaF, 50 mM Na₄P₂O₇, 5 mM EGTA, 5 mM EDTA, 0.5% SDS, 1% NP-40, protease inhibitor cocktail). After homogenization and sonication, lysates were centrifuged at 16,000 g for 15 min. The clean supernatant was carefully transferred to new tubes. Protein concentrations were determined by using bicinchoninic acid (BCA) assay method (Pierce, Rockford, IL) and were normalized with lysis buffer for each sample. Samples were denatured in 1X SDS loading buffer by boiling at 98 °C for 6 min. Proteins were separated by loading to SDS-polyacrylamide gels, and then were transferred to PVDF membrane (Millipore). The protein levels were determined by immunoblotting using respective antibodies. The ImageJ program was used for densitometric analysis of immunoblotting, and the quantification results were normalized to the loading control.

Antibodies

Rabbit anti-SIRT6 (ab62739), ATM (ab78), SIRT1 (ab12193), γ H2AX (ab81299), RAP80 (ab52893), Kap-1 (ab10484), p-KAP-1 (Ser824, ab70369), PDPK1 (ab52893) antibodies were obtained from Abcam (Cambridge, UK). Anti-lamin A/C (sc-20681), p21 (sc-6246), MDM2 (sc-965), P53 (sc-6243) antibodies were purchased from Santa Cruz Biotechnology. Rabbit anti- γ H2AX (05-636), p-ATM (Ser1981) (05-740), histone H3 (07-690), anti-H3K56ac (07-677) and H3K9ac (07-352) antibodies were sourced from EMD Millipore. Mouse anti-p-ATM (Ser1981) (#5883), p-S/TQ (#9607), Ubiquitin (#3936), AMPK (#5831), rabbit anti-p-Akt (Ser308) (#9275), p-Akt (Ser473) (#9271), total Akt (#9272), p-S6 (#2211), S6 (#2217), p-GSK3 β (Ser9) (#9322), GSK3 β (#5676), p-AMPK (Thr172) (#2535), cleaved caspase-3 (#9661) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Mouse anti- HA, Flag, rabbit anti-LC3B, P62 antibodies were obtained from Sigma-Aldrich. Anti-Nbs1 (NB100-143) antibody was purchased from Novus Biologicals. Mouse anti-actin, tubulin antibodies were obtained from Beyotime. Anti-pS112 SIRT6 monoclonal antibodies were prepared by Abmart generating from a specific phosphorylated peptide (peptide sequence CLRFS_pQNV).

Protein degradation assay

HEK293 cells (WT and ATM-deficient cells) were transfected with Flag-SIRT6 alone or together with Mdm2. 48 h later, the cells were treated with 50 μ g/ml of cycloheximide (CHX, Sigma-Aldrich), a translation inhibitor. For endogenous SIRT6 protein degradation assay, ATM wild-type and null MEFs were grown in 6-cm plates, and were treated with 50 mg/ml CHX for indicated time points. Cells were collected and the protein levels were determined by western blotting, the subsequent quantification was performed with ImageJ[®] software.

In vivo ubiquitination assay

In vivo ubiquitination assay was performed by transfecting HEK293 cells in 6-cm dishes with 1 μ g Myc-ubiquitin, 2 μ g Flag-SIRT6 or its mutations, and/or 1 μ g MDM2 vector. 48 h after transfection, cells were lysed in the buffer (25 mM Tris-HCl pH 8.0, 250 mM NaCl, 10 mM Na₃VO₄, 1mM EDTA, 10% glycerol, protease inhibitor cocktail, and 0.1 mM Phenylmethanesulphonyl fluoride), and then incubated with Flag-M2 beads (Sigma-

Aldrich) overnight at 4 °C. Beads were washed with lysis buffer for three times, bound proteins were eluted by adding 1.5 × SDS loading buffer. The ubiquitin levels were analyzed by immunoblotting.

***In vitro* kinase assay**

HEK293T cells were transfected with 10 µg of FLAG-ATM and then treated with CPT. Activated ATM was immune-purified from the cell extracts with FLAG beads (Sigma, M8823). GST-SIRT6 or the S112A mutant was purified from bacteria. Kinase reactions were initiated by incubating purified ATM with GST-SIRT6 in the kinase buffer with or without 1mM ATP for 120 min at 30°. After reaction, proteins were blocked by SDS loading buffer. The membrane was then subjected to Western blotting with antibodies against p-S/TQ.

Immunoprecipitation

Cells under indicated treatments were totally lysed in lysis buffer containing 20 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM Na₃VO₄, 10% glycerol, 2 mM EDTA, protease inhibitor cocktail, and 0.1 mM Phenylmethylsulphonyl fluoride. After sonication and centrifugation, the supernatant was collected and incubated with H3K9ac (Millipore, 2 µg/sample) overnight at 4 °C with a gentle rotation. Protein A/G agarose (Pierce, 10 µl/sample) were added to the tubes and rotated at 4 °C for 2 h. Beads were precipitated by centrifugation at 1000 g for 15 s and washed three times with cold lysis buffer. The pellet was resuspended in 1.5 × SDS loading buffer and incubated at 98 °C for 6 min. The supernatants were collected and used for western blotting.

GST pull-down assay

A series of GST fusion proteins of truncated ATM, which together spanned the full-length of ATM, were constructed into pGEX4T-3 vector. For GST pull-down, bacterially expressed 6 × His-tagged SIRT6 was separately incubated with various GST-ATM fragments in a buffer of 150 mM NaCl, 20 mM Tris-HCl [pH 7.5], 5 mM MgCl₂, 0.2 mM EDTA, 10% glycerol, 0.2% NP-40 and protease inhibitors (Roche Complete). GST-fusion proteins were then precipitated by adding Glutathione Sepharose® fast flow (GE Healthcare). After washed twice with TEN buffer (0.5% Nonidet P-40, 20 mM Tris-HCl

[pH 7.4], 0.1 mM EDTA, and 300 mM NaCl), glutathione agarose beads were analyzed by Western blotting and coomassie staining.

RNA interference

Briefly, cells were transfected with the indicated small interfering RNAs (siRNAs) for 48 h using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions. The following siRNA oligonucleotides targeting human ATM, SIRT6 and HDM2 were purchased from Shanghai GenePharma, China. Si-*ATM* (AAUGUCUUUGAGUAGUAUGUU⁸³, #2: AAGCACCAGUCCAGUAUUGGC⁸⁴); si-SIRT6 (AAGAAUGUGCCAAGUGUAAGA); si-*HDM2* (#1: AACGCCACAAATCTGATAGTA; #2: AATGCCTCAATTCACATAGAT). A scrambled siRNA sequence was used as control. Lentiviral shRNA constructs were generated in a pGLVH1 backbone (GenePharma, China), and virus was produced in HEK293 cells. To deplete ATM in HSF cells, lentiviral infection was performed in the presence of 5 µg/ml polybrene. 2 days later, the infected HSF cells were selected with 2 µg/ml puromycin. To downregulate sir-2.4 expression, the NL2099 worms were exposed to incubation plates containing HT115 bacteria with sir-2.4 RNAi vector.

CRISPR/Cas9-mediated genome editing

Gene mutagenesis by CRISPR/Cas9 system was conducted as described⁸⁵. The following gRNA oligonucleotides targeting human ATM, SIRT6 were constructed in pX459 vector (Addgene, #48139). sg*ATM* F: 5'- CACCGATATGTGTTACGATGCCTTA -3', R: 5'- AAACCTAAGGCATCGTAACACATATC -3'. HEK293 cells were transfected with pX459 or pX459-gRNA by using Lipofetamine[®] 3000 Transfection Reagent according to manufacturer introductions. After 2-day culture, cells were selected with 2 µg/ml puromycin, six colonies were picked and grown to establish stable cell lines. The targeted mutations were identified by Western blotting, and PCR-based sequencing.

EdU (5-ethynyl-2'-deoxyuridine) incorporation assay

EdU incorporation assays were conducted in HSF cells to estimate cell proliferation using the Click-iT[™] EdU Alexa Fluor[®] 488 Kit (Invitrogen). HSF cells, infected by the respective lentiviruses containing shNC and sh*ATM*, were cultured in a 6-well plates

containing the coverslips in the presence of 10 μ M EdU for 12 hours. Cells were fixed in 3.7 % formaldehyde followed by a 0.5% Triton X-100 permeabilization, and then stained with Alexa Fluor picolyl azide. Five random views were captured to calculate the positive staining rate for each group.

Growth curves and SA- β -gal assays

Cell population doublings were monitored using a Coulter Counter. SA- β -galactosidase assay in primary cells was performed using Senescence beta- galactosidase staining Kit (#9860, CST) according to manufacturer instructions, five views were captured randomly to calculate the positive staining rate for each group.

RNA Preparation and Real-Time qPCR

Total RNA was extracted from cells or the tissues of mice using Trizol[®] reagent RNAiso Plus (TaKaRa, Japan) following the phenol–chloroform extraction method. Purified total RNA was used to obtain cDNA using PrimeScript[™] RT Master Mix (Takara, Japan) following this method: 37 °C for 30 min, and 85 °C for 5 s. The gene expression was analyzed with CFX Connected[™] Real-Time PCR Detection System (BioRad) with SYBR[®] Ex Taq Premixes (Takara, Japan). Gene expression levels were normalized to actin.

Glucose tolerance test

Mice were fasted overnight (6 p.m. to 9 a.m.), and D-glucose (2.5 g/kg body weight) was administrated intraperitoneally. Blood glucose levels were determined from tail vein blood using glucometer (Onetouch[®] ultravue, Johnson) at 0, 30, 60, 90, and 120 min after D-glucose injection.

Insulin tolerance test

Mice were fasted for 6 hours (8 a.m. to 2 p.m.), and recombinant human insulin (0.75 U/kg body weight) was administered intraperitoneally. Blood glucose levels were determined from tail vein blood using glucometer (Onetouch[®] ultravue, Johnson) at 0, 30, 60, 90, and 120 min after insulin injection.

Lactate assay

Mouse serum was five-fold diluted, and lactate concentration was determined with the Lactate Colorimetric Assay Kit (BioVision).

Endurance running test

Zmpste24^{-/-} mice were treated for 8 weeks with chloroquine or saline before running on a Rota-Rod Treadmill (YLS-4C, Jinan Yiyang Scientific Research Company, Shandong, China) to test the effect of chloroquine on fatigue resistance. Mice were placed on the rotating lane, and the speed was gradually increased to 10 r/min. When mice were exhausted and safely dropped from the rotating lane, the time latency to fall were automatically recorded.

Metabolite analysis

Wild-type and *ATM* KO cells were grown in normal medium for 24 hours, and methanol-fixed cell pellets were analyzed by two liquid chromatography-tandem mass spectrometry (LC-MS) method as described ⁸⁶.

Immunofluorescence microscopy

The cells were fixed using 4% paraformaldehyde at room temperature for 15 min, and then permeabilized using 0.5% Triton X-100 at room temperature for 10 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 DAPI in anti-fade mounting medium. Images were captured using Zeiss LSM880 confocal/multiphoton microscope.

ChIP assay

Cells were fixed in 1% formaldehyde for 10 min at room temperature. The crosslinking reaction was quenched with 0.125 M glycine. After washed with PBS, cells were lysed with lysis buffer (50mM Tris-HCl pH 8.0, 2mM EDTA, 15 mM NaCl, 1% SDS, 0.5% deoxycholate, protease inhibitor cocktail, and 1mM PMSF). After sonication and centrifugation, the supernatant was collected and precleared in dilution buffer (50mM Tris-HCl pH 8.0, mM EDTA, 150mM NaCl, 1% Triton X-100) with protein A/G Sepharose and pre-treated salmon DNA. The precleared samples were incubated

overnight with H3K9ac antibody (2 µg/sample, Millipore) or appropriate control IgGs (Santa Cruz), and protein A/G Sepharose (Invitrogen). After washed sequentially with a series of buffers, the beads were heated at 65 °C to reverse the cross-link. DNA fragments were purified and analyzed. Real-time PCR was performed with primers as described³¹:

LDHB-ChIP-5': AGAGAGAGCGCTTCGCATAG

LDHB-ChIP-3': GGCTGGATGAGACAAAGAGC

ALDOC-ChIP-5': AAGTGGGGCACTGTTAGGTG

ALDOC-ChIP-3': GTTGGGGATTAAGCCTGGTT

PFKM-ChIP-5': TTAAGACAAAGCCTGGCACA

PFKM-ChIP-3': CAACCACAGCAATTGACCAC

LDHA-ChIP-5': AGGGGGTGTGTGAAAACAAG

LDHA-ChIP-3': ATGGCTTGCCAGCTTACATC

LDHA-ChIP-1Kb-5': TGCAAGACAAGTGTCCCTGT

LDHA-ChIP-1Kb-3': GAGGGAATGAAGCTCACAGC

Statistical analysis

Statistical analyses were conducted using two-tailed Student's t-test between two groups.

All data are presented as Mean ± S.D. or Mean ± S.E.M. as indicated, and a *P* value < 0.05 was considered statistically significant.

Primers for q-PCR

hBPGM-F	TCACATCTGTCCTTAATCGGTCC
hBPGM-R	TGACGCTCATTTAGACGCCAG
mBPGM-F	GGACCAGAACTTAACAACGACG
mBPGM-R	CAGGCTGTGTGAATGGACCT
hHK1-F	CGGAATGGGGAGCCTTTGG
hHK1-R	GCCTTCCTTATCCGTTTCAATGG

mHK1-F	GCTCTCCGATGAAACTCTCATAG
mHK1-R	GGACCTTACGAATGTTGGCAA
hAldoc-F	AGAAGGAGTTGTCGGATATTGCT
hAldoc-R	TTCTCCACCCCAATTTGGCTC
mAldoc-F	TGCTGATGACCGTGTGAAAAA
mAldoc-R	CGGACGAAGGGAACACCAT
hLDHB-F	TGGTATGGCGTGTGCTATCAG
hLDHB-R	TTGGCGGTCACAGAATAATCTTT
hLDHA-F	TTGACCTACGTGGCTTGGAAG
hLDHA-R	GGTAACGGAATCGGGCTGAAT
hPDK1-F	CTGTGATACGGATCAGAAACCG
hPDK1-F	TCCACCAAACAATAAAGAGTGCT
hPDK4-F	GGAGCATTTCTCGCGCTACA
hPDK4-F	ACAGGCAATTCTTGTCGCAAA
hPFKM-F	GGTGCCCGTGTCTTCTTTGT
hPFKM-F	AAGCATCATCGAAACGCTCTC
hGlut1-F	TCTGGCATCAACGCTGTCTTC
hGlut1-F	CGATACCGGAGCCAATGGT
mGlut1-F	TCTCGGCTTAGGGCATGGAT
mGlut1-R	TCTATGACGCCGTGATAGCAG
mPDK1-F	GGACTTCGGGTCAGTGAATGC
mPDK1-R	TCCTGAGAAGATTGTGCGGGA
mPDK4-F	CCGCTTAGTGAACACTCCTTC
mPDK4-R	TGACCAGCGTGTCTACAACT
mLDHA-F	TGTCTCCAGCAAAGACTACTGT
mLDHA-R	GACTGTACTTGACAATGTTGGGA
mLDHB-F	CATTGCGTCCGTTGCAGATG

mLDHB-R	GGAGGAACAAGCTCCCGTG
mPFKM-F	TGTGGTCCGAGTTGGTATCTT
mPFKM-R	GCACTTCCAATCACTGTGCC
hP16-F	GGGTTTTTCGTGGTTCACATCC
hP16-R	CTAGACGCTGGCTCCTCAGTA
mP16-F	CGCAGGTTCTTGGTCACTGT
mP16-R	TGTTACGAAAGCCAGAGCG
hP21-F	TGTCCGTCAGAACCCATGC
hP21-R	AAAGTCGAAGTTCCATCGCTC
hSIRT1-F	TAGCCTTGTCAGATAAGGAAGGA
hSIRT1-R	ACAGCTTCACAGTCAACTTTGT
hSIRT2-F	ATCCACCGGCCTCTATGACAA
hSIRT2-R	CGCATGAAGTAGTGACAGATGG
hSIRT3-F	GACATTCGGGCTGACGTGAT
hSIRT3-R	ACCACATGCAGCAAGAACCTC
hSIRT4-F	GAATCGGGGATACCAGACTACA
hSIRT4-R	GCCAGCCTACGAAGTTTCTCG
hSIRT5-F	GCCATAGCCGAGTGTGAGAC
hSIRT5-R	CAACTCCACAAGAGGTACATCG
hSIRT6-F	CCCACGGAGTCTGGACCAT
hSIRT6-R	CTCTGCCAGTTTGTCCCTG
mSirt6-F	ATGTCGGTGAATTATGCAGCA
mSirt6-R	GCTGGAGGACTGCCACATTA
hSIRT7-F	AGAAGCGTTAGTGCTGCCG
hSIRT7-R	GAGCCCGTCACAGTTCTGAG
hATM-F	TTGATCTTGTGCCTTGGCTAC
hATM-R	TATGGTGTACGTTCCCCATGT

mAtm-F	GATCTGCTCATTTGCTGCCG
mAtm-R	GTGTGGTGGCTGATACATTTGAT
hActin-F	CCAACCGCGAGAAGATGA
hActin-R	TCCATCACGATGCCAGTG
mActin-F	CATCCGTAAAGACCTCTATGCCAAC
mActin-R	ATGGAGCCACCGATCCACA
c act-4-F	GCCACCGCTGCCTCCTCATC
c act-4-R	CCGGCAGACTCCATACCCAAGAAG
c sir-2.4-F	CTGAGATCCGAGACACTGAAAC
c sir-2.4-R	CCGGCTCCGATAAGAACAAA

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