Boosting ATM Activity alleviates ageing and extends lifespan in a mouse model of progeria

Minxian Qian,1,2,3,9 Zuojun Liu,1,2,3,9 Linyuan Peng,1,2,3 Xiaolong Tang,1,2,3 Fanbiao Meng,1,2,3 Ying Ao,1,3 Mingyan Zhou,1,2,3 Ming Wang,1,2,4 Xinyue Cao,1,2,3 Baoming Qin,4 Zimei Wang,1,3 Zhongjun Zhou,5 Guangming Wang,6,7 Zhengliang Gao,7,8 Jun Xu,6 Baohua Liu1,2,3,*

1Guangdong Key Laboratory of Genome Stability and Human Disease Prevention, 2Medical Research Center, 3Department of Biochemistry & Molecular Biology, Shenzhen University Health Science Center, Shenzhen 518060, China 4South China Institute for Stem Cell Biology and Regenerative Medicine, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou 510530, China 5School of Biomedical Sciences, Li Ka Shing Faculty of Medicine, The University of Hong Kong, 21 Sassoon Road, Hong Kong 6East Hospital, Tongji University School of Medicine, Shanghai 200120, China. 7Shanghai Tenth People's Hospital, Tongji University School of Medicine, Shanghai 200072, China. 8Advanced Institute of Translational Medicine, Tongji University, Shanghai 200092, China. 9Minxian Qian and Zuojun Liu contributed equally to this work. *Correspondence should be addressed to Dr Baohua Liu (ppliew@szu.edu.cn).

Abstract

DNA damage accumulates with age (Lombard et al., 2005). However, whether and how robust DNA repair machinery promotes longevity is elusive. Here, we demonstrate that ATM-centered DNA damage response (DDR) progressively declines with senescence and age, while low dose of chloroquine (CQ) activates ATM, promotes DNA damage clearance, rescues age-related metabolic shift, and prolongs replicative lifespan. Molecularly, ATM phosphorylates SIRT6 deacetylase and thus prevents MDM2-mediated ubiquitination and proteasomal degradation. Extra copies of Sirt6 extend lifespan in Atm−/− mice, with restored metabolic homeostasis. Moreover, the treatment with CQ remarkably extends lifespan of Caenorhabditis elegans, but not the ATM-1 mutants. In a progeria mouse model with low DNA repair capacity, long-term
administration of CQ ameliorates premature ageing 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 transformation (Vijg, 2014), if unrepaided, may accumulate and constantly activate DNA damage response (DDR), a unique feature and mechanism of senescence (Halliwell and Whiteman, 2004; Tanaka et al., 2006). Ataxia telangiectasia mutated (ATM), a serine/threonine protein kinase, belongs to these key regulators of DDR (Guleria and Chandna, 2016). Upon DNA damage, self-activated ATM phosphorylates downstream transducers and effectors, thus promoting DNA repair (Bakkenist and Kastan, 2003; Paull, 2015). H2AX is one well-documented phosphorylation target of ATM; phosphorylated H2AX at S139 (γH2AX) is widely applied as a hallmark of DNA damage (Burma et al., 2001). Accompanied with decline of DNA repair function, γH2AX-enriched DNA damage foci accumulate in senescent cells and in tissues from aged animals (Maslov and Vijg, 2009), supporting causal links between defective DDR and ageing. In human fibroblasts, a dramatic decline of homologous recombination (HR) efficiency, attributable to defective recruitment of Rad51, was observed (Li et al., 2016). Similar defects in HR were also observed in Hutchinson-Gilford progeria syndrome (HGPS), which is predominantly caused by a \textit{LMNA} C1024T mutation (Liu et al., 2005).

In addition to DNA damage accumulation, inherited loss-of-function mutations in essential components of DNA repair machinery accelerate ageing in humans and mice (Hoeijmakers, 2009b). Patients suffering from ataxia telangiectasia (A-T) develop prominent ageing features in their second decades (Boder and Sedgwick, 1958; Shiloh and Lederman, 2016). Werner syndrome, Bloom’s syndrome and Rothmund-Thomson syndrome are all progeria syndromes caused by mutations of genes that directly regulate DNA repair (Balajee et al., 1999; Cooper et al., 2000; Lebel et al., 1999; Li and Comai, 2000). Homozygous disruption of \textit{Atm} in mice recapitulates many premature ageing features of A-T like growth retardation, infertility, neurodegeneration, immunodeficiency and cancer predisposition (Barlow et al., 1996). Mouse models deficient in DNA repair factors, including DNA-PKcs, Ku70, Ku80, DNA ligase IV, Artemis or Ercc1 etc., phenocopy premature ageing features (Hasty, 2005; Hoeijmakers, 2009a), supporting that
defects in DNA repair accelerate ageing. However, whether and how robust DNA repair machinery promotes longevity is poorly understood.

Metabolic disturbance is another antagonistic hallmark of ageing (Lopez-Otin et al., 2013). Although DNA repair deficiency is implicated in ageing and age-related diseases including metabolic disorders (Lopez-Otin et al., 2016; Shimizu et al., 2014), the mechanistic linker between declined DNA repair machinery and metabolic reprogramming during ageing 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) (Cosentino et al., 2011; Kruger and Ralser, 2011). Inactivating ATM enhances glucose and glutamine consumption by inhibiting P53 and upregulating c-MYC (Aird et al., 2015). The role of ATM in age-onset metabolic disturbances is yet unclear.

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

Results

ATM activation alleviates replicative senescence

In searching for genes/pathways that drive senescence, we employed human primary endothelial cells, which underwent replicative senescence at passage 21, with increased p21 expression and β-galactosidase activity (Figure 1—figure supplement 1a-b). By RNAseq analysis, a gradual decline of ATM-centered DNA repair machinery was identified (Figure 1—figure supplement 1c-e). Western blotting analysis confirmed the progressively downregulated protein levels of ATM and its downstream target NBS1 and
RAP80 in senescent human skin fibroblasts (HSFs) (Figure 1a). Mouse embryonic fibroblasts (MEFs) with a limited growth capacity and senescent phenotypes when cultured in vitro (Parrinello et al., 2003; Samper et al., 2003; Sherr and DePinho, 2000), and brain tissues from aged mice also showed progressive decline of ATM, NBS1, and RAP80 (Figure 1b-c). Concomitantly, an upregulation of γH2AX, indicating accumulated DNA damage, and an increase of p16\textsuperscript{Ink4a} were observed in senescent HSFs, MEFs, and aged brain tissues (Figure 1a-c). Knocking down ATM via shRNA accelerated senescence in HSFs, evidenced by increased β-galactosidase activity (Figure 1d-e), enlarged morphology (Figure 1—figure supplement 2a), accumulated γH2AX (Figure 1f) and reduced cell proliferation (Figure 1—figure supplement 2b). These data indicate that ATM decline retards DDR and drives senescence.

Other than DNA damage, ATM is activated by chloroquine (CQ), an antimalarial drug that modulates chromatin confirmation (Bakkenist and Kastan, 2003). Indeed, we confirmed that low dose of CQ increased the level of pS1981 auto-phosphorylation of ATM but not γH2AX (Figure 1—figure supplement 2c). We then investigated whether activating ATM by CQ can ameliorate senescence. As shown, the CQ treatment activated ATM (pS1981), promoted clearance of DNA damage (γH2AX), and inhibited apoptosis (cleaved Casp3) in HSFs (Figure 1g). Also, the CQ treatment suppressed β-galactosidase activity, which was abrogated if ATM was knocked down (Figure 1h-i). Importantly, CQ treatment extended the replicative lifespan of HSFs (Figure 1j). Likewise, CQ treatment activated Atm, cleared up accumulated DNA damage, suppressed β-galactosidase activity (Figure 1k and Figure 1—figure supplement 2d-e), and prolonged replicative lifespan in MEFs (Figure 1l). Despite that both 10 μM and 1 μM of CQ activated ATM, dose-dependent toxicity assay showed that CQ in 1 μM is suitable for long-term treatment (Figure 1—figure supplement 2f-g). Of note, ATM KD or low dose of CQ applied in this study had little effect on basal autophagic activity (Figure 1f, j and Figure 1—figure supplement 2g). Collectively, CQ activates ATM to alleviate replicative senescence.

An ATM-SIRT6 axis underlies age-associated metabolic reprogramming
A-T patients lacking functional ATM display features of premature ageing, accompanied with insulin resistance and glucose intolerance (Bar et al., 1978; Espach et al., 2015). Senescent cells exhibit impaired mitochondrial respiration, but enhanced glycolysis producing more lactate (Hagen et al., 1997; Lenaz et al., 2000). As such, we ask whether ATM decline triggers age-associated metabolic shift. Indeed, the levels of glycolytic gene \textit{LDHB} and \textit{PDK1} were dramatically increased in senescent MEFs and HSFs (Figure 2a and Figure 2—figure supplement 1a), and in liver tissues from \textit{Atm-/-} mice (Figure 2—figure supplement 1b). Significantly, activating ATM via CQ suppressed senescence-associated glycolysis (Figure 2a and Figure 2—figure supplement 1a). Similarly, the inhibitory effect on glycolysis was diminished when ATM was depleted in HepG2 cells (Figure 2b). These data suggest a role of ATM in inhibiting glycolysis.

To address how ATM regulates glycolysis, we performed RNA-Seq in \textit{Atm-/-} MEF cells, and revealed a significant upregulation of glycolytic pathways (Figure 2c, and Figure 2—source data 1). Specific genes were validated by q-PCR (Figure 2—figure supplement 1c). As p53 is critical in glycolysis (Kruiswijk et al., 2015; Schwartzemberg-Bar-Yoseph et al., 2004), we further analyzed metabolomics of \textit{Atm-/-} and control MEFs in \textit{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 (Figure 2d, Figure 2—figure supplement 1d-e and Figure 2—source data 2). The data suggest that ATM deficiency enhances anaerobic glycolysis in a p53-independent manner.

Sirt6 deacylase is able to shunt energy metabolism away from anaerobic glycolysis to TCA cycle via H3K9ac-mediated local chromatin remodeling (Sebastian et al., 2012; Zhong et al., 2010). We noted that the level of H3K9ac was enhanced in cells depleted ATM (Figure 2e). Re-expressed ATM in A-T cells suppressed H3K9ac level (Figure 2f). ChIP analysis showed that H3K9ac was enriched at the promoter regions of glycolytic genes in \textit{Atm-/-} cells (Figure 2g), where the relative occupancy of SIRT6 was abolished (Figure 2h). Consistent with increased H3K9ac, SIRT6 protein level was dramatically downregulated in \textit{Atm-/-} mouse livers, and ATM deficient HepG2, U2OS and HEK293 cells (Figure 2—figure supplement 1f-i). In contrast, protein levels of other sirtuins were
not much affected in ATM KO HEK293 cells (Figure 2i), and mRNA levels of all sirtuins remained unchanged (Figure 2—figure supplement 1j). Moreover, transcriptomic analysis and q-PCR data illustrated that Sirt6 depletion upregulated a similar cluster of genes essential for glycolysis (Figure 2—figure supplement 2a-b and Figure 2—source data 3). More importantly, the hyper-activated glycolytic pathway caused by ATM deficiency was completely restored by ectopic SIRT6 in HepG2 cells (Figure 2—figure supplement 2c). The CQ treatment upregulated SIRT6 level and reduced H3K9ac level, especially at the regulatory regions of glycolytic genes (Figure 2—figure supplement 2d-e). Knocking down SIRT6 abolished the inhibitory effect of CQ on glycolysis (Figure 2b).

Additionally, ATM depletion in HEK293 cells, HSFs and MEFs, significantly downregulated SIRT6 protein level, with little effect on SIRT1 or SIRT7 (Figure 2i and Figure 2—figure supplement 2f-g). Thus, these data suggest that ATM decline triggers age-associated metabolic shift via SIRT6-mediated chromatin remodeling.

Other than metabolic abnormality, depleting Sirt6 leads to premature ageing features and shortened lifespan (Mostoslavsky et al., 2006a), whereas extra copies of Sirt6 promote longevity in male mice (Kanfi et al., 2012). Given that Sirt6 was destabilized in Atm null mice, we asked whether Sirt6 transgene could rescue premature ageing 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 (Figure 2—figure supplement 2h). Significantly, ectopic Sirt6 restored the elevation of serum lactate, and extended lifespan of Atm-/- mice in both genders (Figure 2j and Figure 2—figure supplement 2i). Importantly, Atm-/-; Sirt6-tg mice exhibited improved glucose tolerance and decreased insulin resistance (Figure 2k-l). Given that little difference was observed in glucose metabolism between young wild-type (WT) and Sirt6-transgenic mice (Kanfi et al., 2012), these data suggest a contributing role of the Atm-Sirt6 axis in the age-associated metabolic reprogramming.

**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 to block dimeric ATM dissociation (Bakkenist and Kastan, 2003; Berkovich et al., 2007) (Figure
Moreover, in addition to CQ, hypotonic buffer (20 mM NaCl), low glucose (LG), DNA-damaging agent camptothecin (CPT) and doxorubicin (Dox) all activated ATM and concomitantly increased SIRT6 protein level (Figure 3—figure supplement 1a-c), which was abrogated in ATM-depleted cells (Figure 3—figure supplement 1b-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 (Figure 3b). The interaction was further confirmed at both ectopic and endogenous levels (Figure 3c and Figure 3—figure supplement 1d). Immunofluorescence microscopy showed co-localization of SIRT6 and ATM protein in the nucleus (Figure 3d). Domain mapping experiment indicated that the C-terminal domain was required for SIRT6 binding to ATM (Figure 3—figure supplement 1e). 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 (Figure 3e), His-SIRT6 bond predominantly to GST-ATM-4 (residues 770-1102) and relatively weakly to GST-ATM-1 (residues 1-250); both belong to the N-terminal HEAT repeat domain of ATM.

We next examined whether ATM phosphorylates SIRT6. Firstly, we found that CQ or CPT treatment significantly enhanced the binding of SIRT6 to ATM (Figure 3f and Figure 3—figure supplement 1f), whereas the S1981A mutant blocked such association (Figure 3—figure supplement 1g). ATM preferentially phosphorylates S/T-Q motif. In the presence of CPT, increased p-S/TQ level of SIRT6 was identified (Figure 3g). Of note, lambda protein phosphatase (λPP) diminished p-S/TQ level of SIRT6 (Figure 3—figure supplement 1h). Likewise, the p-S/TQ level of SIRT6 was elevated in cells treated with low glucose, which activates ATM by ROS generation (Assaily et al., 2011; Sarre et al., 2012) (Figure 3—figure supplement 1i). Moreover, ectopic ATM significantly increased the p-S/TQ level of SIRT6, and that was abolished in case of S1981A-mutated (Figure 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 (Berkovich et al., 2007; Hickson et al., 2004) (Figure 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 (Figure 3—figure supplement 1j). Indeed, SIRT6 has one evolutionarily conserved S\textsuperscript{112}Q\textsuperscript{113} motif (Figure 3k). We therefore constructed S112A and S112D mutants, which resembles hypophosphorylated and hyperphosphorylated SIRT6 respectively. As shown, these mutations almost abolished the pS/T-Q level of FLAG-SIRT6 (Figure 3l). The \textit{in vitro} kinase assay showed that ATM could phosphorylate GST-SIRT6, but not S112A (Figure 3—figure supplement 1k). Further, compared with SIRT6 S112A, ectopic S112D exhibited much higher inhibitory effect on glycolytic gene expression in sh-SIRT6 HepG2 cells (Figure 3m and Figure 3—figure supplement 1l), and enhanced chromatin association of SIRT6 (Figure 3n).

Collectively, the data suggest that ATM directly phosphorylates SIRT6 at Serine 112.

We next examined whether ATM is involved in regulating SIRT6 protein stability. Notably, compared to WT or vehicle control, the degradation rate of ectopic and endogenous SIRT6 was largely increased in ATM KO HEK293 cells, Atm\textsuperscript{-/-} MEFs and cells incubated with KU55933 in the presence of cycloheximide (CHX) (Figure 4a-b and Figure 4—figure supplement 1a-c). Recently MDM2 was demonstrated to ubiquitinate SIRT6 and promote its proteasomal degradation (Thirumurthi et al., 2014). 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 WT (Figure 4—figure supplement 1d). While S112A mutant markedly enhanced the polyubiquitination level of SIRT6, S112D had little effect (Figure 4—figure supplement 1e). Moreover, S112A accelerated SIRT6 degradation, whereas S112D retarded it (Figure 4c-d), indicating that the Ser112 phosphorylation by ATM regulates SIRT6 ubiquitination and thus protein stability. Indeed, ectopic MDM2 enhanced the polyubiquitination level of FLAG-SIRT6 (Figure 4—figure supplement 1f). In case of ATM depleted or SIRT6 S112A-mutated, the binding capacity of SIRT6 to MDM2 was enhanced (Figure 4e and Figure 4—figure supplement 1g). In searching for key residues that are polyubiquitinated 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 (Figure 4—figure supplement 1h). Individual KR mutation showed that K346R significantly blocked MDM2-mediated ubiquitination and degradation of SIRT6, whereas
K349R hardly affected it (Figure 4f-g). More importantly, K346R restored the increased ubiquitination and accelerated protein degradation of SIRT6 S112A (Figure 4—figure supplement 1i-j). Collectively, these data indicate that K346 is subjected to MDM2-mediated ubiquitination, which is inhibited by ATM-mediated S112 phosphorylation.

**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 WTs were exposed to various doses of CQ (see Materials and Methods). Significantly, the period treatment with CQ (1.0 µM) extended the median lifespan (~14%) of *C. elegans* (Figure 5a). The lifespan-extending effect was abolished in *atm-1* KO (Figure 5b) or in SIRT6 homolog *sir-2.4* KD nematodes (Figure 5—figure supplement 1a-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 months (Pendas et al., 2002) and impaired ATM-mediated DNA repair signaling (Liu et al., 2013a). We found that the level of Atm was dramatically reduced in *Zmpste24*-/— MEFs and tissues (Figure 5c and Figure 5—figure supplement 1c). Significantly, CQ treatment activated Atm, stabilized Sirt6, decreased the accumulated DNA damage, inhibited glycolysis, and alleviated senescence in *Zmpste24*-/— cells (Figure 5d-e and Figure 5—figure supplement 1d-e). The CQ treatment also held off body weight decline, increased running endurance, and prolonged lifespan in *Zmpste24*-/— mice (Figure 5f-h), whereas showed no significant effect on the lifespan of *Atm*-/— mice (Figure 5- figure supplement 1f).

Physiologically aged mice frequently develop ageing-associated metabolic disorders, with high glucose and lactate (Houtkooper et al., 2011). Given that ATM declines with age, and activation of ATM by CQ inhibits glycolysis in senescent cells and *Zmpste24*-/— mice, we intraperitoneally administrated 12-month-old “old” male mice with low dose of CQ (3.5mg/kg) twice a week. Remarkably, compared to saline-treated group, CQ treatment inhibited glycolysis, lowered down serum lactate level, and attenuated body weight decline (Figure 5i and Figure 5—figure supplement 1g-h), implicating potential
benefits of CQ in physiological aged mice. Generally, these data demonstrate a lifespan-extending benefit of ATM activation by CQ.

Discussion

DNA damage accumulates with age and defective DDR and DNA repair accelerates ageing. 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 (Bakkenist and Kastan, 2003; Krajewski, 1995). We demonstrate that long-term treatment with CQ activates ATM, improves DNA repair, restores age-related metabolic shift, alleviates cellular senescence, and extends lifespan of nematodes and Zmpste24 null mice. Mechanistically, ATM phosphorylates longevity gene SIRT6 (Tasselli et al., 2016), and prevents 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 is that the DNA repair efficacy is enhanced in long-lived naked mole rat (MacRae et al., 2015), and that human longevity is associated with single nucleotide polymorphisms (SNPs) in DNA repair genes/pathways (Debrabant et al., 2014; Soerensen et al., 2012). Interestingly, the heterozygous instead of homozygous status of a SNP, albeit both enhance the transcription of ATM, is associated with longevity in Chinese and Italian populations (Chen et al., 2010; Piaceri et al., 2013). Therefore, in future study, it would be worthwhile to evaluate whether and how many extra copies of Atm could promote longevity in model organisms.

Accumulation of DNA damage and metabolic disturbance are common denominators of ageing (Lopez-Otin et al., 2013; Moskalev et al., 2013). Metabolic reprogramming from TCA cycle to glycolysis is prominent in both physiological and pathological ageing (Feng et al., 2016; Shimizu et al., 2014). Why senescent cells become glycolytic is poorly understood. The crosstalk between cellular metabolism and DDR is not well elucidated.
Upon genotoxic stress, ATM represses the rapamycin-sensitive mammalian target of
rapamycin (mTORC1) pathway (Alexander et al., 2010), but rather activates the pentose
phosphate pathway (PPP) (Cosentino et al., 2011), suggesting that cell metabolism may
serve as a key downstream of DDR signaling. Moreover, it is recognized that deficiency
in DNA repair machinery like ATM, WRN and Ercc1 accelerates ageing and causes
severe metabolic disorders (Garinis et al., 2008; White and Vijg, 2016). In this study, we
showed that boosting ATM activity by low dose of CQ enhances genomic stability, holds
off age-onset metabolic reprogramming, alleviates senescence, and extends lifespan in
mice. The data demonstrate for the first time that enhanced DNA repair machinery
(ATM-SIRT6 axis) promotes longevity. Considering that ATM and SIRT6 function not
only in maintenance of genome integrity but also as homeostatic protein modifiers, the
pro-longevity role of ATM is most likely benefited from enhanced DNA repair and
metabolic homeostasis, but it is hard to determine which one is more important.

Recently, Bohr’s group uncovered an increased consumption of NAD+ by an early DDR
factor poly (ADP-ribose) polymerase (PARP1), owing to accumulated DNA damage,
accelerates ageing in Atm mutant mice (Fang et al., 2016). 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 ageing. Moreover, administration of nicotinamide mononucleotide or
nicotinamide riboside ameliorates age-related function decline and extends lifespan in
mice (Mills et al., 2016; Zhang et al., 2016). Here, we found that ATM decline during
ageing causes DNA damage accumulation and enhances glycolysis, both of which
consume most of NAD+, providing an explanation of low NAD+ level in Atm-/− mice and
physiological aged mice.

Closely resembling normal ageing, HGPS has attracted numerous efforts in
understanding of molecular mechanisms and developing therapeutic strategies (De
Sandre-Giovannoli et al., 2003; Eriksson et al., 2003). We and others have found that
HGPS and Zmpste24 null cells undergo premature senescence owing to defective
chromatin remodeling (Ghosh et al., 2015; Krishnan et al., 2011; Liu et al., 2013a; Liu et
al., 2013b), delayed DDR and impaired DNA repair (Liu et al., 2005; Liu et al., 2013b;
Varela et al., 2005). Specifically, Atm-Kap-1 signaling is compromised (Liu et al.,
2013a), and SIRT6 protein level and deacetylase activity are reduced in progeria cells (Endisha et al., 2015; Ghosh et al., 2015). Here we showed that Atm is 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 activation of ATM via CQ remarkably improves glucose homeostasis, DNA damage clearance and running endurance, and extends lifespan in progeria mice. It would be worthwhile to evaluate the pro-longevity benefits of CQ in physiological ageing.

CQ is FDA-approved and clinically used medicine for treatment of malaria (2015). Via activating ATM, long-term treatment of CQ protects against atherosclerosis, improves insulin sensitivity, and rescues glucose tolerance in type 2 diabetes (T2D) (Emami et al., 1999; Razani et al., 2010; Schneider et al., 2006). Lysosomotropic property of CQ also make it as a potent inhibitor of autophagy (Yang et al., 2013). The application of CQ for antimalarial treatment (500 mg/week, maximum 0.8 µM in plasma) and for cancer therapy (100-500 mg/day) (Kimura et al., 2013) is attributed to its inhibitory action on autophagy. Of note, CQ also attenuates inflammatory response by inhibiting autophagy (Szatmari-Toth et al., 2016; Whelan et al., 2017; Wu et al., 2018), requiring a high dosage of 50 mg/kg for mice. In current study, we utilized low dose of CQ to activate ATM, i.e. 1-10 µM for cell line and 3.5 mg/kg twice a week for mice (Schneider et al., 2006). The results showed that low dose of CQ has no toxicity and little effect on basal autophagic activity. Moreover, low dose of CQ prolongs lifespan in progeroid mice, but exhibits little effect at Atm KO background, supporting an ATM-dependent pro-longevity function of CQ. Unfortunately, we could not test the CQ effect in Sirt6-/- mice with only 1-month lifespan (Mostoslavsky et al., 2006b). Here, we addressed the pro-longevity benefits of CQ-activated ATM, most likely attributable to improved DNA repair and glucose metabolism. Given that ATM also displays anti-inflammatory function (Erttmann et al., 2017; Shoelson, 2006), we couldn’t rule out the anti-inflammatory effect in lifespan extension observed in CQ-treated mice.

In conclusion, our data establish direct causal links between robust DNA repair machinery and longevity. In line with DNA damage theory of ageing, 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 nematodes and most likely also in mice (Figure 5—figure supplement 2). Our findings provide a novel therapeutic strategy for HGPS, and could facilitate clinical trials of CQ as an effective treatment for age-related diseases.

Figure Legend

Figure 1: ATM activation by chloroquine alleviates 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 γH2AX 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 µm. (e) Quantification of SA-β-Gal-positive staining of (d) from five views randomly captured for each group. Data represent means ± SEM. ***P < 0.001. (f) Immunoblots showing increased γH2AX and unaffected LC3I/II in HSFs treated with sh-ATM or scramble shRNA. (g) Immunoblots showing protein levels of pS1981 ATM, γH2AX and cleaved caspase-3 in HSFs treated with 10 µM of CQ for indicated time. (h) SA-β-Gal staining in HSFs expressing either scramble or AT M shRNA treated with 1 µM CQ or DMSO (12 h). Scale bar, 100 µm. (i) Quantification of SA-β-Gal-positive staining of (h) from five views randomly captured for each group. Data represent means ± SEM. ***P < 0.001; ‘N.S.’ indicates no significant difference. (j) HSFs at passage 20 were continuously cultured with 1 µM CQ or DMSO, and cell number was calculated at each passage. Data represent means ± SEM. ***P < 0.01. (k) Immunoblots showing protein levels of γH2AX, p62 and LC3 in MEFs treated with 1 µM CQ or DMSO. Noted that CQ had little effect on the expression levels of p62 and LC3. (l) MEFs at passage 1 were continuously cultured in 20% O2 with 1 µM CQ or DMSO, and cell number was determined at each passage. Data represent means ± SEM. ***P < 0.01.
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 means ± 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 (10µM, 6 h). Data represent means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

(c) Heatmap representation of RNA-Seq data (GSE109280) showing relative changes of glycolytic genes in Atm-/− MEF cells. The transcript levels are qualified in reads per kilobase of exon per million mapped sequence reads (RPKM), which is a normalized measure of exonic read density. 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) ChIP analysis showing enrichment of H3K9ac at the promoter regions of indicated genes in Atm+/+ and Atm-/− MEFs. Data represent means ± SEM of 3 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

(h) ChIP analysis showing enrichment of Sirt6 at the promoter regions of indicated genes in Atm+/+ and Atm-/− MEFs. Data represent means ± SEM of 6 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 means ± 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 means ± SEM, n = 6. **P < 0.01. ‘ns’ indicates no significant difference.

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 µm. (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 µM) 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 µM) 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 µM, 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. (m) Quantitative RT-PCR analysis of mRNA levels of indicated glycolytic genes in sh-SIRT6 HepG2 cells re-expressing SIRT6, SIRT6 S112A or 112D mutation. Data represent means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001. ‘ns’ indicates no significant difference. (n) Immunoblots showing SIRT6 protein level in total cell extract (TCE) and chromatin-enriched fractions (P2). Densitometry analysis was performed to determine the relative ratio of SIRT6/H2B within chromatin fractions.

**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 µg/ml) for indicated periods of time. (b) Quantification of protein levels in (a) by ImageJ®. Data represent means ± 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 µg/ml) for indicated periods of time. (d) Quantification of protein levels in (c) by ImageJ®. Data represent means ± 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 μg/ml) for indicated periods of time. Lower, quantification of protein levels by ImageJ®. Data represent means ± SEM of three independent experiments. ***P < 0.001.

Figure 5: CQ extends lifespan in an ATM-dependent manner.
(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 1μM CQ. (c) Immunoblots showing protein levels of Atm and γH2AX in brain tissues of Zmpste24+/+ (2 months), Zmpste24+/− (2 months), and Zmpste24+/− (4 months) mice. (d) Representative images showing SA-β-Gal staining in Zmpste24+/− MEFs with or without CQ treatment. Scale bar, 100 μm. (e) Quantitative RT-PCR analysis of mRNA levels of p16Ink4a 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 means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001. (f) Maximum running duration in saline- and CQ-treated Zmpste24−/− mice. Data represent means ± SEM. ***P < 0.001. (g) Body weight of saline- and CQ-treated male Zmpste24−/− mice. Data represent means ± SEM. **P < 0.01. (h) Kaplan-Meier survival curves of saline-treated (n = 10) and CQ-treated (n = 8) Zmpste24−/− mice. ***P < 0.001. (i) 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. Data represent means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure Supplement Legend
Figure 1-figure supplement 1: Decline of ATM-centered DNA repair machinery during senescence.
(a) Real-time PCR analysis showing progressively elevated mRNA level of p21 in continuously cultured human endothelial cells (HUVEC). **P < 0.01. (b) SA-β-Gal staining of HUVEC cells at indicated passages. Scale bar, 100 µm. (c) HUVEC cells at P21, P18, P12 and P7 were subjected for transcriptome analysis. A minimum average rpkm value of 1.0 and maximum 10% fluctuation in young cells (P7 Vs P12) was set as threshold. Genes were downregulated by more than 20% in pre-senescent and senescent cells compared with young cells (P21/P18 Vs P12/P7) were selected. (d) Pathway analysis of genes identified in (c) by STRING v10. (e) Downregulation of ATM-related DNA repair genes during senescence.

**Figure 1-figure supplement 2: ATM regulates replicative senescence.**

(a) Representative images showing cells treated with Scramble (sh-NC) or sh-ATM. (b) Percent EdU-positive cells in sh-NC or sh-ATM treated HSFs. Views were randomly captured and at least 100 cells were included for each group. Data represent mean ± SEM. ***P < 0.001. (c) Immunoblots showing protein levels of pS1981 ATM and γH2AX in HSFs treated with 10 µM chloroquine (CQ) or 0.4 µM CPT (4 h). Noted that CQ activated ATM (pS1981) without increasing γH2AX, while CPT activated ATM accompanied with increased γH2AX. (d) SA-β-Gal staining in primary MEFs treated with 1 µM CQ or DMSO. Scale bar, 100 µm. (e) Quantification of SA-β-Gal-positive staining of (d) from five views randomly captured for each group. Data represent means ± SEM. ***P < 0.001. (f) Percent EdU-positive cells in HSFs treated with DMSO, 1 µM or 10 µM CQ. Views were randomly captured and at least 100 cells were included for each group. Data represent mean ± SEM. ***P < 0.001. (g) Representative images showing proliferative HSFs treated with different doses of CQ for the indicated time points. (h) Immunoblots showing LC3B levels in HSFs treated with indicated dose of CQ for indicated period of time.

**Figure 2-figure supplement 1: Atn deficiency promotes glycolysis.**

(a) Quantitative RT-PCR analysis of mRNA levels of indicated glycolytic genes in different passages of HSFs with or without treatment of CQ. Data represent mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001. (b) Quantitative RT-PCR analysis of mRNA levels of glycolytic genes in liver tissues from Atm+/+, Atm+/- and Atm-/- mice. Data represent
mean ± SEM. *P < 0.05, **P < 0.001. (c) Quantitative RT-PCR analysis of mRNA levels of glycolytic genes to validate the RNA-seq data set of Atm+/+ and Atm-/− MEFs. Data represent means ± SEM of 6 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001. (d) Metabolic pathway enrichment in Atm null MEFs compared to wild-types. (e) AMP/ATP ratio and NAD+ levels in wild-type and Atm null MEFs. (f) Immunoblots showing ATM and SIRT6 protein levels in indicated clones of ATM KO HEK293 cells generated by the CRISPR/Cas9 system. (g) Immunoblots showing protein levels of Sirt6 and H3K9ac in liver tissues from 4-month-old Atm+/+, Atm+/− and Atm−/− mice. (h–i) Immunoblots showing SIRT6 levels in si-NC and si-ATM treated HepG2 and U2OS cells. (j) Quantitative RT-PCR analysis of sirtuin mRNA levels in wild-type and ATM KO HEK293 cells. Data represent mean ± SEM.

**Figure 2-figure supplement 2: SIRT6 reduction underlies age-related metabolic reprogramming triggered by ATM decline**

(a) Heatmap representation of RNA-Seq data showing relative changes of glycolytic genes in Sirt6−/− MEF cells. Red and green indicate up- and downregulation, respectively. (b) Quantitative RT-PCR analysis of mRNA levels of glycolytic genes to validate the RNA-seq data set of Sirt6+/+ and Sirt6−/− MEFs. Data represent means ± SEM of 6 independent experiments. **P < 0.01, ***P < 0.001. (c) Quantitative RT-PCR analysis of indicated glycolytic genes in HepG2 cells transfected with Scramble, si-Atm, si-SIRT6, or si-ATM plus Flag-SIRT6. Data represent mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001. (d) Immunoblots showing protein levels of SIRT6 and H3K9ac in HepG2 cells treated with 10 μM CQ for indicated periods of time. (e) ChIP analysis showing enrichment of H3K9ac at promoter regions of glycolytic genes in HepG2 cells treated with 10 μM CQ for indicated periods of time. Data represent mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001. (f) Immunoblots showing SIRT1, SIRT6 and SIRT7 protein levels in ATM knockdown (KD) or control HSFs. (g) Immunoblots showing protein levels of Atm, Sirt1, Sirt6 and γH2AX levels in Atm+/+ and Atm−/− primary MEF cells. (h) Immunoblots showing Sirt6 expression in liver tissues of Sirt6-tg mice. (i) Serum lactate levels in 3-month-old Atm+/+, Atm−/− and Atm−/−; Sirt6-tg male mice. Data represent mean ± SEM of 5–6 animals/group. *P < 0.05.

**Figure 3-figure supplement 1: ATM directly phosphorylates SIRT6**
(a) Immunoblots showing a significant increase in SIRT6 protein level, pS1981ATM and pS824KAP-1 in the presence of DNA damage reagents-CPT and Dox in U2OS cells. (b) Immunoblots showing Sirt6 protein level in immortalized wild-type and Atm null MEFs in response to 0.4 μM CPT treatment. (c) Immunoblots showing pS1981-ATM and SIRT6 levels in wild-type and ATM KO HEK293 cells treated with CQ, low glucose (LG), and hypotonic swelling (20 mM NaCl). (d) Immunoblots of indicated immunoprecipitates showing the interaction between endogenous ATM and SIRT6 in HepG2 cells. (e) Immunoblots showing interaction between ATM and truncated fragments of SIRT6 in HEK293 cells. F-L, full-length of SIRT6, ΔC, C-terminal deletion, ΔN, N-terminal deletion, ΔN-C, N-and C-terminal truncation. (f) Immunoblots showing ATM protein in anti-Flag immunoprecipitates in HEK293 cells expressing Flag-SIRT6, treated with 0 μM, 4 μM or 8 μM of CPT for 1 h. (g) Immunoblots showing ATM protein in anti-HA immunoprecipitates in HEK293 cells expressing Flag-ATM or Flag-ATM S1981A, as well as empty vector or HA-SIRT6. (h) Immunoblots showing p-S/T Q of Flag-SIRT6 in HepG2 cells treated with 5 mM of glucose for indicated time. (i) Immunoblots showing p-S/T Q of Flag-SIRT6 in HEK293 cells, treated with or without λPP (30 min). (j) Immunoblots showing p-S/T Q of SIRT6 in WT and ATM KO HEK293 cells, transfected with empty vector, 2 μg Flag-ATM, or 6 μg Flag-ATM. (k) Immunoblots showing p-S/T Q of GST-SIRT6 after incubation with Flag-ATM purified from CPT-treated HEK293 cells. (l) Immunoblots showing SIRT6 protein levels in HepG2 cells with lentiviral infection containing sh-NC or sh-SIRT6.

Figure 4-figure supplement 1: ATM-mediated phosphorylation of SIRT6 prevents its ubiquitination and degradation.

(a) Immunoblots showing Sirt6 levels in wild-type and Atm null MEFs in the presence of CHX (50 μg/ml). (b-c) Immunoblots showing SIRT6 protein level in HEK293 cells in the presence of CHX (50 μg/ml) and/or KU55933 (10 or 20 μM). Densitometry analysis was performed to determine the SIRT6/Actin ratio. (d) Immunoblots showing increased ubiquitination of SIRT6 in ATM KO HEK293 cells. (e) Immunoblots showing ubiquitination of Flag-SIRT6, Flag-SIRT6 S112A and Flag-SIRT6 S112D in HEK293 cells co-transfected Myc-Ub. (f) Immunoblots showing increased ubiquitination of Flag-SIRT6 in HEK293 cells over-expressing MDM2. (g) Immunoblots showing MDM2 in
anti-Flag immunoprecipitates in HEK293 cells expressing Flag-SIRT6 or Flag-SIRT6 S112A. (h) Immunoblots showing ubiquitination of Flag-SIRT6, K143/145R and K346/349R in HEK293 cells. Noted that K364/349R abrogated the ubiquitination of Flag-SIRT6. (i) Immunoblots showing the ubiquitination of Flag-SIRT6, Flag-SIRT6 S112A, and Flag-SIRT6 S112A/K346R. (j) Immunoblots showing protein levels of Flag-SIRT6, Flag-SIRT6 S112A and Flag-SIRT6 S112A/K346R in the presence of CHX (50 μg/ml). Noted that K346R rescued the accelerated degradation of S112A.

Figure 5-figure supplement 1: ATM activation ameliorates aging-associated features
(a) Survival analysis of sir-2.4 downregulated C. elegans exposed in the medium with or without 1μM CQ. ‘N.S.’ indicates no significant difference. (b) Quantitative RT-PCR analysis of mRNA levels of sir-2.4 in vehicle and sir-2.4 RNAi-treated C.elegans. (c) Immunoblots showing protein levels of Atm in wild-type and Zmpste24 null MEFs. (d) Immunoblots showing levels of proteins involved in pS1981-ATM, γH2AX, Sirt6 and p16 in liver tissues of saline-treated, and CQ-treated Zmpste24-/ mice. (e) Quantification of SA-β-Gal staining in (Figure 5d) from five views randomly captured for each group. Data represent mean ± SEM. *P < 0.05. Scale bar, 100 μm. (f) Kaplan-Meier survival curves of saline-treated and CQ-treated Atm-/ mice (n = 9 for each group). ‘ns’ indicates no significant difference. (g) Body weight of saline- and CQ-treated male aging mice. Data represent means ± SEM. ***P < 0.001. (h) Serum lactate levels in saline- and CQ-treated 20-month-old mice. Data represent means ± SEM. **P < 0.01.

Figure 5-figure supplement 2: Schematic model of ATM-SIRT6 axis in regulating aging and longevity
Left, DNA damage activates DDR cascade, and its constant activation leads to permanent cell cycle exit and senescence. Right, defective ATM-SIRT6 axis underlies premature ageing in mouse models resembling HGPS and A-T, which is rescued by treatment of CQ and Sirt6 transgene respectively. Middle, during physiological aging, DNA damage-free activation of ATM by CQ promotes longevity at organismal levels.

Materials and Methods
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### Mice

Zmpste24-/ mouse and Atm/- mice have been described previously (Barlow et al., 1996; Pendas et al., 2002). 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’; reverse: 5’-CCGTCTACGTCTGCTGAC-3’. Atm/- mice were crossed to Sirt6-tg mice to get Atm-/-; Sirt6-tg mice. Chloroquine (CQ) experiments were conducted as described (Schneider et al., 2006). Briefly, 12-month-old wild-type C57BL/6J male mice, 2-month-old Zmpste24-/ and Atm/- male mice and 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. At least 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). The protocols were approved by the Animal Welfare and Research Ethics Committee of Shenzhen University (Approval ID: 201412023).

### C. elegans survival assay

C. elegans nematode survival assay was performed according to standard protocols (Kenyon et al., 1993). 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

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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 (CRL-1573), HepG2 (HB-8065) and U2OS (HTB-96) cells have been purchased from ATCC. Human skin fibroblasts HSFs (F2-S) and primary MEFs were prepared as described previously (Liu et al., 2005). Immortalized \textit{Atm-/-; p53-/-} and \textit{Sirt6-/-} MEFs were provided as a kind gift from Dr. Yosef Shiloh (Tel Aviv University, Israel) and Dr. Raul Mostoslavsky (Massachusetts General Hospital Cancer center, USA), respectively. These cell lines were authenticated by short tandem repeat (STR) profile analysis and genotyping, and were mycoplasma free. Cells were cultured in Gibco\textsuperscript{®} DMEM (Life Technologies, USA) with 10% fetal bovine serum (FBS), 100 U/ml penicillin and streptomycin (P/S) at 37 °C in a 5% CO\textsubscript{2} and atmospheric oxygen conditions. For CQ experiments, cells were maintained in the medium containing 1µM chloroquine for 1β h, and then grown in new fresh medium for 48 h.

**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'-cgctcagcttgctgcaacagcggaacgg-3', (reverse) 5'-tcgctcagcttgctgcaacagcggaacgg-3'; SIRT6 S112D: (forward) 5'-tcgctcagcttgctgcaacagcggaacgg-3', (reverse) 5'-tcgctcagcttgctgcaacagcggaacgg-3'; SIRT6 K346R: (forward) 5'-ggcctttccctttctgtt-ggtgtgtgt-3', (reverse) 5'-ggcctttccctttctgtt-ggtgtgtgt-3'; SIRT6 K349R: (forward) 5'-ggcctttccctttctgtt-ggtgtgtgt-3', (reverse) 5'-ggcctttccctttctgtt-ggtgtgtgt-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 Na3P2O7, 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), γH2AX (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-γH2AX (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), 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 CLRFVSPQNV).

**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 µg/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 µg Myc-ubiquitin, 2 µg Flag-SIRT6 or its mutations, and/or 1 µg 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 Phenylmethylsulphonyl 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°C. 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 Na$_3$VO$_4$, 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 MgCl$_2$, 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 and shRNA lentiviral infection**

Briefly, cells were transfected with small interfering RNAs (siRNAs) for 48 h using Lipofectamine® 3000 (Invitrogen, USA) according to the manufacturer’s instruction. The siRNAs targeting human ATM, SIRT6 and HDM2 were purchased (GenePharma, China) and sequences are as follow, si-ATM#1: 5’-AAUGUCUUUGAGUAGUAUGUU-3’ (Zhou et al., 2003); Si-ATM#2: 5’-AAGCACCAGUCCAGUUAUGGC-3’ (Zhang et al., 2005); si-SIRT6#1: 5’-CCGGCTCTGACCAGCTGGCTAA-3’; si-SIRT6#2: 5’-CCGGCTCTGACCAGCTGGCTAA-3’; si-HDM2#1: 5’-AACGCCACCAAATCTGATAGTA-3’; si-HDM2#2: 5’-AATGCCTCAATTCATAGAT-3’. 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 and SIRT6 in HepG2 cells, lentiviral infection was performed in the presence of 5 μg/ml polybrene. 2 days later, the infected HSF cells or HepG2 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 described (Ran et al., 2013). The following gRNAs targeting human ATM, SIRT6 were constructed in pX459 vector (Addgene, #48139). sgATM F: 5’-CACCGATATGTGTTACGATGCCTTA-3’, R: 5’- AAACTAAGGCATCGTAACACATATC-3’. HEK293 cells were transfected with pX459 or pX459-gRNA 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, USA). HSF cells, infected by the respective lentiviruses containing shNC and shATM, were cultured in a 6-well plate containing the coverslips in the presence of 10 μM 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 mouse tissues 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, USA) 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 in 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 Yiyan 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 (Luo et al., 2007).

**Immunofluorescence microscopy**

The cells were fixed by 4% paraformaldehyde at room temperature for 15 min, permeabilized by 0.5% Triton X-100 at room temperature for 10 min, blocked using 10% FBS/PBS, and then incubated with 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 (50 mM Tris·HCl pH 8.0, 2 mM EDTA, 15 mM NaCl, 1% SDS, 0.5% deoxycholate, protease inhibitor cocktail, 1 mM PMSF), followed by sonication and centrifugation. The supernatant was collected and precleared in dilution buffer (50 mM Tris-HCl pH 8.0, 2 mM EDTA, 150 mM 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 described (Zhong et al., 2010):

- LDHB-ChIP-5': AGAGAGAGCGCTTCGCATAG
- LDHB-ChIP-3': GGCTGGATGAGACAAAGGC
- ALDOC-ChIP-5': AAGTGGGGCACTGTTAGGTG
- ALDOC-ChIP-3': GTTGGGGATTAAGCCTGGTT
- PFKM-ChIP-5': TTAAGACAAAGCCTGGCACA
- PFKM-ChIP-3': CAACCACAGCAATTGACCAC
LDHA-ChIP-5’: AGGGGTGTGTGAAAAACAG
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 $\pm$ S.D. or Mean $\pm$ S.E.M. as indicated, and a $P$ value $< 0.05$ was considered statistically significant.

Acknowledgements

We thank Dr. Yosef Shiloh (Tel Aviv University, Israel) for Atm-/-; p53-/- MEF cells and Dr. Raul Mostoslavsky (Massachusetts General Hospital Cancer center, USA) for Sirt6-/- MEFs. This project is supported by research grants from National Natural Science Foundation of China (81422016, 91439133, 81571374, 81501206, 81501210), National Key R&D Program of China (2017YFA0503900, 2016YFC0904600), Research Grant Council of Hong Kong (773313, HKU2/CRF/13G), Natural Science Foundation of Guangdong Province (2014A030308011, 2015A030308007, 2016A030310064) and Shenzhen Science and Technology Innovation Commission (CXZZ20140903103747568 and JCYJ20160226191451487) and Discipline Construction Funding of Shenzhen.

Source Data Files

Figure 1-source data 1
Figure 1-source data 2
Figure 2-source data 1
Figure 2-source data 2
Figure 2-source data 3
Figure 5-source data 1
Figure 5-source data 2

Reference


Figure 1

(a) HSFs
(b) MEFs
(c) Brain

(d) Scramble, shATM #1, shATM #2

(e) Percent SA-β-Gal positive cells

(f) Scr, shATM #1, shATM #2

(g) CQ 0 3 6 9 Hr

(h) Veh, CQ

(i) Percent SA-β-Gal positive cells

(j) Log cell number vs. Days in culture (HSFs)

(k) Veh, CQ

(l) Log cell number vs. Days in culture (MEFs)
Figure 5

a) Percent survival over days for different concentrations of an inhibitor.
b) Percent survival over days for different genotypes and treatments.
c) Western blot images showing ATM, (Pre)-lamin A/C, γH2AX, and Actin in brain tissues.
d) Image of Zmp-/- MEFs treated with Veh or CQ.
e) Bar graph showing relative mRNA levels of various genes in different genotypes.
f) Dot plot showing running duration (s) for Zmp-/- and Zmp-/-+CQ.
g) Graph showing body weight change over weeks for different genotypes.
h) Graph showing percent survival over days for different genotypes.
i) Bar graph showing relative mRNA levels of various genes in different genotypes.
Figure 1-figure supplement 1

(a) Relative mRNA levels bar chart:

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(b) Images of cell culture:
P7, P12, P21

(c) Graph showing gene expression changes across stages:
P7-Young-P12, Pre-sen, Sen, P18, P21

(d) Network diagram highlighting genome maintenance genes:
- BRCC3
- DDX1
- UIMC1
- ATM
- NBN
- ATR

(e) Table of RPKM values:

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RPKM values in parentheses:
- ATM: (99.2%) (100.8%) (78.5%) (74.0%)
- ATR: (99.8%) (100.2%) (63.1%) (36.5%)
- NBS1: (97.6%) (102.4%) (58.6%) (62.4%)
- DDX1: (103.1%) (96.9%) (76.7%) (63.7%)
- UIMC1: (101.6%) (98.4%) (64.0%) (67.4%)
- BRCC3: (104.4%) (95.6%) (69.7%) (70.6%)
Figure 4-figure supplement 1

(a) 

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CHX
ATM
SIRT6
Actin

(b) 

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(c) 

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(d) 

Vector
Flag-SIRT6
Input

ATM KO
ATM

(e) 

WT
S112A
S112D

IP FLAG
Ub
Flag

Input
Flag

(f) 

FI-SIRT6
FI-Mdm2

Ub
Flag

Input
Flag

(g) 

MDM2

Vector
Flag-SIRT6
Flag-SIRT6

MDM2
Flag

(h) 

WT
S112A
K143,145R
K346,349R

IP FLAG
Ub
Flag

Input
Flag

(i) 

WT
S112A
S112A/K346R

Ub
Flag-SIRT6

Input
Flag-SIRT6

(j) 

CHX
0 4 8

FLAG-SIRT6
Tubulin

FLAG-SIRT6
S112A
Tubulin

FLAG-SIRT6
S112A/K346R
Tubulin
Figure 5-figure supplement 2

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- **DNA damage**
  - DDR
  - ATM
  - SIRT6
  - Ageing

- **Longevity Genes**
  - ATM
  - ATM-1
  - SIRT6
  - Longevity

- **Inherited Mutations**
  - Progeria
  - Ataxia telangiectasia
  - HGPS
  - A-T
  - Genomic Instability

**Legend**
- ATM
- SIRT6
- Ageing
- Longevity
- Premature ageing