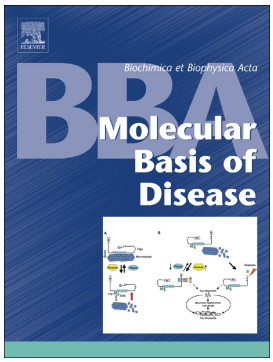
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Metformin regulates mitochondrial biogenesis and senescence through AMPK mediated H3K79 methylation: Relevance in age-associated vascular dysfunction

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Keywords: Metformin, Mitochondrial function, endothelial dysfunction, cardiovascular diseases, atherosclerosis, aging.

ABSTRACT

Endothelial senescence in conjunction with mitochondrial dysfunction orchestrates age-associated cardiovascular disorders. In this study we investigated the causal link between these two processes and studied the molecular mechanisms by which metformin acts to coordinate the delay of endothelial senescence via enhancing mitochondrial biogenesis/function. AMPK activators metformin and AICAR delayed endothelial senescence via SIRT1-mediated upregulation of DOT1L, leading to increased trimethylation of H3K79 (H3K79me3). Treatment of cells with either siAMPK or siSIRT1 repressed DOT1L-mediated enhancement of H3K79me3. Moreover, the increase in SIRT3 expression and mitochondrial biogenesis/function by AMPK activators was H3K79me-dependent as H3K79N mutant or siDOT1L abrogated these effects. This was confirmed by the enrichment of H3K79me3 in the SIRT3 promoter with AMPK activation. Intriguingly, enhanced PGC-1α expression by SIRT3 via AMPK activation was responsible for increased hTERT expression and delayed endothelial senescence. In contrast, SIRT3 knockdown caused increased oxidative stress and premature senescence, possibly by depleting hTERT expression. Furthermore, a chronic low dose administration of metformin significantly attenuated vascular aging and inhibited age-associated atherosclerotic plaque formation in $ApoE^{-/-}$ mice. Overall, the results of this study show a novel regulation of mitochondrial biogenesis/function, and cellular senescence by H3K79me acting through SIRT3, thus providing a molecular basis for metformin-mediated age-delaying effects.

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Introduction

Dysregulated mitochondrial metabolism is a hallmark of aging and a compromised respiratory capacity is associated with age in many tissues. Epidemiological studies have shown that age is the dominant risk factor for atherosclerotic cardiovascular diseases and endothelial cell senescence(1,2). Decrease in the activity of telomerase, a replicative senescence marker, contributes to the pathogenesis of age-associated vascular diseases, including atherosclerosis(3). While longevity extension strategies will not only eliminate aging-related diseases, they are expected to postpone their age of onset, thus contributing to the objective of extending healthspan(4-6). Since the last decade, there is a growing body of evidence showing that the aging process is genetically determined and there is reasonable hope that the function of genes that control lifespan can eventually be therapeutically modulated(7,8). 'Caloric Restriction' (CR) mimetics are shown to delay the aging process due to decrease in the production of free radicals, apart from regulating other metabolic events like glucose homeostasis, adenosine monophosphate-activated protein kinase (AMPK) activation, Nrf2/ARE pathway etc(9,10). One such putative CR-mimic is metformin, a well-known anti-diabetic drug, commonly prescribed to manage type-II diabetes. Metformin exerts pleotropic effects independent of its anti-diabetic properties including the vaunted cardiovascular protective effects(11,12). Also, metformin administration has shown promise in the treatment of polycystic ovarian syndrome(13). However, hyper activation of AMPK in certain situations including stroke has shown to be detrimental(14).

In agreement with the beneficial effects of metformin, recently we showed that metformin inhibits monocyte-to-macrophage differentiation and attenuates angiotensin-II-induced atherosclerosis in $ApoE^{-/-}$ mice(15). Previous studies also indicate that long-term metformin administration improve health and lifespan in mice(4-6,16). While the precise target of metformin is still elusive, it has been shown that apart from acting as a complex-1 inhibitor in some situations, metformin activates AMPK; a cardinal regulator of energy homeostasis, independent of complex-1 inhibition(17). AMPK primarily functions as a fuel gauge, sensing the changes in the energy status of the cell and thus, plays a critical role in regulating systemic energy balance. AMPK activation declines as a function of age(10). Moreover, the responsiveness of AMPK activation by AICAR (a direct activator of AMPK) treatment also declines in muscle tissue of aged rats compared to young rats(18).

Reduced mitochondrial mass with age contributes to increased mitochondrial ROS with a concomitant depletion of ATP production(19,20). Enhanced SIRT3, a major mitochondrial biogenetic marker along with PGC-1α, correlates with longevity in mammals(21,22). SIRT3 guards' cardiomyocytes from aging and oxidative stress and subdues cardiac hypertrophy(23-25). Nevertheless, it is inconclusive in the vascular system about the factors that are involved in regulating the altered mitochondrial biogenesis and the resultant changes that correlates to vascular aging.

AMPK activators like metformin are receiving much attention for their ability to extend healthy lifespan ranging from worms to humans (4,6,26,27). In the present work, we sought to investigate the mechanisms by which metformin and AICAR regulate cellular senescence and mitochondrial biogenesis. The causal relationships between these processes were examined in early and late passage endothelial cells. We present novel data showing that metformin or AICAR dependent-AMPK activation enhances trimethylation of H3K79 (H3K79me3) through

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SIRT1-DOT1L axis. This lead to increased SIRT3 levels resulting in improved mitochondrial biogenesis/function, as well as delay of cellular senescence by enhancing hTERT expression. Furthermore, a chronic low-dose administration of metformin to cohorts of $ApoE^{-/-}$ mice ameliorated age-associated atherosclerosis.

RESULTS

Chronic AMPK activation delays endothelial senescence. To study the role of AMPK activation on hTERT (telomerase reverse transcriptase), the enzyme involved in adding the telomeric repeats to the ends of chromosome, early passage HAEC (p3-p9) were treated time-dependently with either metformin or AICAR, the two well-known activators of AMPK. Metformin and AICAR significantly increased hTERT levels by 8h (Fig. 1a and b). Under these conditions, both these agents also enhanced AMPK activation as measured by phospho active-AMPKα (Thr-172) (Fig. 1a, b and S9). Next, to see if AMPK activation by metformin was indeed responsible for the increase in hTERT, cells were treated with compound C, a known AMPK activation inhibitor. Compound C greatly inhibited the metformin-mediated increase of hTERT (Fig. 1c, d and S9). These results were further confirmed by siAMPKα treatment (Fig. 1e). Thereby indicating that, AMPK activation regulates hTERT expression in endothelial cells. In agreement with this, metformin treatment failed to reverse the siAMPKα -induced senescence in HAEC (Fig. 1f and S14). We then investigated the differential responsiveness of AMPKα activation in early and late passage HAEC for its ability to regulate cellular senescence. Based on the senescence features, we considered HAEC with passages 3-9 as early, and 12-17, as late passage endothelial cells. Metformin treatment resulted in a significant activation of AMPKα in early and late passage endothelial cells, albeit, the responsiveness was marginally more in early passage in comparison to late passage cells (Fig. 1g). Interestingly, AMPKα activation was robustly enhanced when HAEC were cultured in the presence of metformin for several passages (Fig. 1h). Thereby by raising the possibility that chronic metformin treatment may have a role in delaying the endothelial cell senescence. To investigate this, both early and late passage cells were incubated with metformin either at single dose (2 mM) or they were cultured continuously with metformin for several passages (p6-p15) and measured hTERT activity in nuclear and cytosolic fractions along with other known senescence markers like p53, p21, p27, and p16. Single dose administration of metformin in early passage but not in late passage cells resulted in a significant increase of hTERT (Fig. 1i and S9). Similarly, single dose of metformin to late passage cells did not affect the senescence markers that were measured in this study (Fig. 1i, S1 and S9). In contrast, hTERT protein as well as its activity in nuclear and cytosolic fractions was significantly increased along with a concomitant reduction in all the senescence markers in cells that were grown in the presence of metformin continuously from p6-p15 (Fig. 1j-l, S1 and S9). In addition, cell proliferation was 30% slower in chronically metformin treated cells in comparison to untreated cells (Fig. S1b). Taken together these results suggest that, AMPKα activation is involved in the regulation of hTERT expression and moreover, chronic administration of metformin delays endothelial cell senescence.

SIRT1 and DOT-1L are involved in AMPKα-induced hTERT expression. AMPK activation leads to functional activation of SIRT1(28). To explore if AMPKα-mediated hTERT expression involves SIRT1 activation, HAEC were treated either with metformin or AICAR and found that these AMPK activators increased SIRT1 levels (Fig. 2a, b and S10). However, metformin

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treatment failed to upregulate either SIRT1 in the presence of siAMPKα, or hTERT in the presence of siSIRT1 (Fig. 2c, d and S10). Moreover, siSIRT1 treatment alone increased cellular senescence in HAEC (Fig. 2e and S14). Thereby suggesting that, SIRT1 is required for AMPKα-induced hTERT expression. As expected, the basal level of SIRT1 was significantly low in late passage HAEC in comparison to early passage HAEC (Fig. 2f and S10). Furthermore, in accordance with the results shown in Fig. 1, on the relative responsiveness of AMPK activation with metformin in early and late passage HAEC, it was observed that late passage cells were only marginally responsive to metformin treatment in regulating SIRT1 in comparison to early passage HAEC (Fig. 2f and S10). In contrast, chronic metformin treatment resulted in significant increase of SIRT1 levels (Fig. 2g and S10).

Previously it was reported that SIRT1 physically and functionally interacts with DOT1L (Disruptor of Telomeric Silencing-1 Like protein or Kmt4) to enhance the distributive activity of DOT1L on H3K79 methylation(29). So far it has been shown that DOT1L is the only known DNA/histone lysine methyltransferase that specifically methylates H3K79. In this context, we next hypothesized that AMPKα-induced SIRT1 mediates hTERT regulation via involving DOT1L and H3K79 methylation. To test this, cells were treated either with AICAR or metformin. AMPK activators dose- and time-dependently enhanced DOT1L and H3K79 trimethylation (me3) in HAEC (Fig. 3a-d, S2a and S11). Interestingly, siAMPKα or AMI-5 (histone lysine methyl transferase inhibitor) treatments greatly reversed metformin-mediated increase of DOT1L and H3K79me3 levels (Fig. 3e, f and S11). In addition, AMI-5 and siDOT1L treatments significantly inhibited metformin-mediated increase of hTERT levels (Fig. 3g, h and S11). To further prove that H3K79me is indeed involved in hTERT regulation, we made adenoval viral constructs of H3K79 wild type and H3K79N mutant. H3K79N viral transduction impressively hampered hTERT transcripts as well as protein expression (Fig. 3i, j and S11). In tune with these observations, treatment of cells with either siDOT1L or with H3K79N mutant caused premature senescence in HAEC, and metformin treatment failed to regulate senescence under these conditions (Fig. 3k and S14). Also, metformin treatment failed to increase DOT1L and H3K79me3 under SIRT1 depleted conditions (Fig. 3l and S11). Besides, only chronic (from p6-p15) but not single administration of metformin to late passage (p15) endothelial cells increased DOT1L and H3K79me3 levels (Fig. S2a-c). Interestingly, H3K79N mutant transduction caused increased accumulation of subG0/G1 population (49.2%) in comparison to vector control (13.4%) or H3K79 wild type (10.3%) along with increased expression of senescent markers, p16 and p27 (supplementary figure S2d and e). Thus suggesting that, H3K79me regulates apoptotic process. Taken together, AMPKα activation leads to hTERT expression via the enhancement of SIRT1-DOT1L-H3K79me axis, and this pathway is likely responsible for controlling cellular senescence in endothelial cells.

DOT1L and H3K79me facilitate AMPKα-induced increase in mitochondrial biogenesis/function. Earlier we showed that AMPK activation increases mitochondrial biogenesis in endothelial cells(30). To further investigate the mechanisms involved during this process, early passage HAEC were treated with metformin or AICAR and found increased mitochondrial biogenesis by mitotracker staining (Fig. 4a and S15) as well as increased SIRT3 and PGC-1α (mitochondrial biogenetic markers) protein levels (Fig. 4b, c, S3a, S3b and S12). In contrast, treatment of cells with either compound C or siAMPKα or siSIRT1 decreased metformin-mediated increase of SIRT3 and PGC-1α levels (Fig. 4b-d and S12). Treatment of cells with either AMI-5 or siDOT1L greatly decreased metformin-mediated increase of SIRT3

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and PGC-1α levels (Fig. 4e, f and S12). The involvement of DOT1L-H3K79me in regulating mitochondrial biogenesis was further verified in cells transduced with H3K79N mutant construct, wherein, SIRT3 and PGC-1α along with mitochondrial DNA content, as well as the indicated candidate mitochondria-encoded gene expressions that were measured in this study, were significantly decreased with H3K79N transduction (Fig. 4g, h S3c, S3d and S12). Thus indicating that, DOT1L-induced H3K79 methylation possibly regulates mitochondrial biogenesis. Further, the basal levels of SIRT3 and PGC-1α along with total mitochondrial DNA content were low in late passage cells in comparison to early passage endothelial cells (Fig. 4 i, j, l and S12). In tune with the results shown in figures 1-3, only chronic but not single dose administration of metformin to late passage cells increased mitochondrial staining, DNA content, as well as the aforementioned mitochondrial biogenetic marker expressions (Fig 4i-l, S3e and S12). In addition, metformin treatment failed to increase PGC-1α levels in the presence of siSIRT3 (Fig. 4m and S12). Similarly, metformin treatment failed to enhance hTERT levels in the presence of either siSIRT3 or siPGC-1α (Fig. 4m, n, and S12). Further, SIRT3-mediated increase in hTERT expression was compromised in the presence of siPGC-1α (Fig. 4m and 4n). In support of this observation, recently it was reported that PGC-1α regulates TERT expression and activity in high fat diet-induced and age-dependent arteriopathy(31). These results were further confirmed with SA-β-gal staining wherein; SIRT3 depletion enhanced premature senescence (Fig. 4o and S14). It is to be noted that, siSIRT3 treatment caused significant cytotoxicity to HAEC presumably by inducing H_2O_2 production and oxidative stress as measured by Amplex red assay and DCFH oxidation, and metformin treatment failed to rescue these effects under SIRT3 depleted conditions (S5 a-c). Furthermore, to gain more mechanistic insights on the regulation of SIRT3 by H3K79 methylation, we performed ChIP assay to study the occupancy and enrichment of H3K79me3 in the promoter (P) and the actively transcribed region (G) of SIRT3. In agreement with the earlier results, metformin treatment significantly increased the occupancy of H3K79me3 in the promoter region of SIRT3 (Fig. 4p). Thus the results suggest that, SIRT3 plays a critical role in the regulation of hTERT expression and endothelial cell senescence and that; depletion of SIRT3 causes stress-induced premature senescence (SIPS).

On a similar note, the differential responsiveness of metformin in early and late passage cells on mitochondrial biogenesis was even reflected in the function of mitochondria. Single dose of metformin decreased the mitochondrial respiration as measured by Oxygen Consumption Rate (OCR) in early passage (p6) HAEC (Fig. 5a), perhaps attributed to its mild complex-1 inhibitory activity(4,32). However, OCR was significantly increased in cells chronically administered with metformin (from p6-p15) in comparison to either early passage (p6-p8) or late passage (p14-p16) untreated cells (Fig. 5a). Similar results were observed with basal respiration, maximal respiration, spare respiratory capacity, and ATP production (supplementary figure S4, a-d). To account for the net increase in OCR in chronically metformin treated cells, we then studied the involvement of other complex subunits of the electron transport chain using complex I (pyruvate), complex II (succinate), and complex IV (TMPD) substrates in late passage as well as in chronically metformin treated cells. Interestingly, addition of pyruvate did not alter the OCR in chronic metformin treated cells (Fig. 5b and d). While addition of succinate caused marginal, albeit, statistically significant increase in OCR, intriguingly, addition of TMPD, a complex IV substrate, resulted in a drastic increase in OCR in chronically metformin treated late passage endothelial cells in comparison to untreated cells (Fig. 5c and d). The results of Seahorse extracellular flux analyzer were further confirmed by measuring Ox-Phos subunits expression by

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Western blotting. In agreement with OCR results, complex II and IV subunit levels were significantly increased in chronic metformin treated cells in comparison to untreated cells (Fig. 5e and S28). Overall, although metformin is known to inhibit complex I activity, the net increase in OCR observed in chronically metformin treated conditions is presumably because of its ability to increase complex II and IV activities.

Chronic metformin administration delays vascular aging and protects from age-associated atherosclerosis in ApoE$^{-/-}$ mice. It is well known that age is a dominant risk factor for the development of several disorders including atherosclerosis. In this work we have taken four months old ApoE$^{-/-}$ mice and chronically administrated them with a low dose of metformin (50 mg/kg.bd.wt) for fourteen months in drinking water to study the effect of metformin on the age-associated atherosclerotic disease progression. The mean plasma concentration of metformin was found to be 1.03ng/mg protein at the end of the treatment protocol (Fig. S6). ApoE$^{-/-}$ mice are known to spontaneously develop atherosclerosis as well as these mice age faster compared to their C57BL/6 counterparts(33-35). At the end of the treatment protocol, thoracic and abdominal aortic diameters of metformin-administered ApoE$^{-/-}$ mice were significantly decreased in comparison to untreated ApoE$^{-/-}$ mice (Fig. S7a). The results observed with metformin administration were comparable to C57BL/6 mice. Similar results were observed with aortic plaque incidence, lumen diameter, and plaque diameter (Fig. S7b-f). In addition, metformin administration resulted in reduced body weight, blood pressure, plasma insulin and glucose levels and lipid profile, while; serum GSH levels were elevated in comparison to ApoE$^{-/-}$ mice (Fig. S8 a-j).

We further analyzed the vascular remodeling in tissue sections of thoracic aorta. H&E staining of ApoE$^{-/-}$ mice aorta showed severe atherosclerotic lesions with both thick internal and external walls and intimal plaques. These changes were significantly reduced in metformin-administered mice and were comparable to C57BL/6 mice (Fig. 6a and b). These changes were further confirmed with Van Geison staining indicating increased presence of collagen tissue and Masson's trichrome staining indicating accumulation of fibrous connective tissue with ruptured medial layer in ApoE$^{-/-}$ mice. However, these changes were greatly inhibited in metformin-administered ApoE$^{-/-}$ mice (Fig. 6c-f). In tune with this, metformin administration also regressed macrophage accumulation (as measured by Mac-3), and other pro-inflammatory indices like Cd45.2 and Icam-1 by immunofluorescence and flow cytometry in the aorta and blood respectively, in comparison to untreated ApoE$^{-/-}$ mice (Fig. 6g and h). Thereby suggesting the anti-inflammatory role of metformin(15).

Furthermore, chronic metformin administration resulted in a significant reduction of graying of hair and complete absence of visceral fat tissue compared to untreated ApoE$^{-/-}$ mice, as shown in Fig. 7a. In addition, a significant reduction in the SA-β-gal staining of aorta was observed in metformin-administered mice in comparison to untreated ApoE$^{-/-}$ mice (Fig. 7a). These gross observations clearly indicate that metformin administration not only greatly delays the overall aging process, but also significantly attenuate the vascular aging in ApoE$^{-/-}$ mice. To corroborate with the results seen in endothelial cells (as shown in figures 1-4), we next sought to see if chronic metformin administration modulates the markers of mitochondrial biogenesis, and senescence in the aortic tissue. Enhanced levels of activated Ampk, H3K79me3, Dot1L, Pgc-1α, and Sirt3 were observed in endothelial layer of metformin-administered aorta by immunohistochemistry (Fig. 7b). Also, increased transcript levels of Sirt1, Dot1L, Sirt3, and

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Pgc-1α as well as a decrease in the inflammatory markers like Vcam-1 and Icam in the total aorta by qRT-PCR were found with metformin administration in comparison to untreated $ApoE^{-/-}$ mice (Fig. 7c). Similarly, while the transcript and protein levels of Tert were increased with metformin administration, the senescent markers (p16, p21, p27, p53) were decreased in the total aortic homogenate (Fig. 7d, e and S13) in comparison to untreated $ApoE^{-/-}$ mice. Taken together, chronic metformin administration delays vascular aging, in part, by enhancing mitochondrial biogenesis, that perhaps is mediated through increased Sirt1-Dot1L-H3K79me-Sirt3 axis.

DISCUSSION

The findings of the present study indicate that metformin, a widely used anti-diabetic drug and an AMPK activator, and AICAR, a distinct AMPK activator, greatly attenuate endothelial senescence by increasing telomerase expression and activity. Forced expression of telomerase, the enzyme responsible for the addition of telomeric repeats at the ends of the chromosome for its maintenance, thwarts replicative senescence in normal human cell types(36). In contrast, telomerase deficient mice show accelerated aging(37,38). One of the important observations of the current study was that, only chronic administration of metformin significantly enhanced AMPK activation and inhibited senescence markers like p16, p53, p21 and p27 and senescence-associated β-gal staining(3,39). Single dose metformin treatment to late passage endothelial cells failed to enhance AMPK activation as well as senescence-associated markers. This observation is consistent with earlier reports on the marginal responsiveness of AMPK activation in aged muscle tissue compared to young muscle tissue(18,40). Also, we observed depletion of mitochondrial biogenetic markers like SIRT1, SIRT3 and PGC-1α, as a function of endothelial cell aging. Only a chronic administration of metformin significantly increased these marker proteins compared to age-matched untreated cells. It is well established that SIRT1 and SIRT3 proteins play a key role in increasing the longevity and our results show that persistent AMPK activation maintains the level of SIRT1/3, which in turn, enhances mitochondrial biogenesis and function. While exploring the possible molecular and epigenetic events that may be involved during AMPK activation-mediated delay of endothelial cell senescence, we found that DOT1L, a specific H3K79 methyl transferase, whose basal expression was compromised as a function of endothelial senescence, was upregulated with metformin or AICAR treatments. AMPKα activation resulted in increased levels of SIRT1 that led to increase in H3K79me3 via DOT1L upregulation. DOT1L deficient cells undergo irreversible cell cycle arrest in G1 phase leading to premature senescence(41). The increase in the occupancy of H3K79me3 in the promoter region of SIRT3 by AMPKα activation observed in the current study appears to be responsible for the increased expression of SIRT3, which in turn enhanced not only mitochondrial biogenesis, but also increased hTERT expression and activity and thereby delayed the endothelial cell senescence. Experiments with H3K79N mutant further confirmed the regulation of SIRT3 expression and mitochondrial biogenesis by H3K79me. In addition, H3K79N mutant via down regulating SIRT3 expression, inhibited hTERT expression that led to premature senescence in endothelial cells. A recent report showed that PGC-1α deletion reduces expression and activity of hTERT and increases p53 levels(31). Studies in the past showed that methyl marks contribute to transcriptional regulation and serve as a stage for the recruitment of effector proteins(42). In general, methylation at H3K9, H3K27, and H4K20 correlates with transcriptional repression, while methylation at H3K4, H3K36, and H3K79 correlates with gene transcription(43-45).

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Based on the results of the present study, it is conceivable that SIRT1-mediated longevity effects are mediated in part by increasing methylation of H3K79 via DOT1L and that, H3K79me plays a decisive role in the regulation of hTERT expression and senescence through SIRT3. Moreover, SIRT3 depletion resulted in increased oxidative stress and premature senescence in endothelial cells. Thereby suggesting that the increased oxidative stress observed in general, in late passage cells, may in part be due to the loss of SIRT3(21).

Metformin at single dose caused inhibition of mitochondrial oxygen consumption rate (OCR) and decreased ATP levels, perhaps due to its mild complex I inhibitory activity in endothelial cells. In contrast, importantly, chronic metformin treatment resulted in the net increase in OCR and ATP levels due to the enhancement of complex II and IV subunits and thereby bypassing the inhibitory complex I effect. Thus suggesting that, endothelial cell adaptation to chronic metformin administration would be important to mediate its beneficial effects on mitochondrial biogenesis and function, and in turn, for its age-delaying effects. These results are in agreement with a recent report that showed, while short term administration of metformin caused mild inhibition of complex I activity, long term exposure of metformin is associated with benefits, including reduced oxidative stress and increased antioxidant defenses, leading to lower oxidative damage accumulation and inhibition of chronic inflammation(4,15,32,46). In support of the observed favorable effects of metformin on endothelial age-delaying effects, we observed that a low dose chronic metformin administration for fourteen months resulted in a significant attenuation of age-associated atherosclerotic plaque formation in $ApoE^{-/-}$ mice, presumably by delaying the vascular senescence as evidenced by a significant reduction in SA-β-gal staining of the aorta. Metformin attenuates vascular senescence and atherosclerosis in high fat diet fed $ApoE^{-/-}$ mice(47-49) Earlier we showed that metformin treatment regress Ang-II-induced atherosclerosis in $ApoE^{-/-}$ mice, owing to its anti-inflammatory effects(15). Consistent with this, the current study shows a significant inhibition of macrophage recruitment into the sub-endothelial space of aorta accompanied by a reduction in the levels of pro-inflammatory mediators in metformin-administered $ApoE^{-/-}$ mice in comparison to untreated cohorts. Although this study found that a chronic low dose metformin administration significantly delayed vascular senescence, one of the limitations of this study is that we did not investigate the effect of metformin treatment on other organ functions. It known that chronic metformin treatment causes lactic acidosis and the drug is contraindicated in patients with kidney dysfunction(50).

Overall, clinically metformin is a drug of choice to treat insulin resistance and prevent type-2-diabetes mellitus. However, the growing interests on the pleotropic effects of metformin indicates that it may soon find its way in the treatment of other complications including cardiovascular, cancer, polycystic ovarian syndrome, and other neurological diseases. Moreover, two recent clinical studies on metformin namely, 'targeting aging with metformin (TAME)' and 'metformin in longevity study (MILES)', have been initiated in the United States. These trials aim to investigate the usefulness of metformin in the elderly population to see if it can delay the age-associated diseases by increasing the health span(5).

In conclusion, AMPK activation leads to H3K79me3-induced upregulation of SIRT3 levels, that in turn, enhance mitochondrial biogenesis/function as well as delay vascular senescence, and that, metformin attenuates age-associated atherosclerotic plaque formation perhaps by delaying the vascular senescence.

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Methods

Endothelial cell culture: Human aortic endothelial cells (HAECs) were obtained from ATCC (Manassas, VA) and maintained (37 ^{0}C, 5% CO_2) in basal medium supplemented with 10% FBS and growth supplements (EBM-2 bullet kit, Lonza-CC3162, Walkersville, MD). Cells used in this study were between passages 3 and 17. Based on the senescence features, we considered HAECs from passage p3-9 as early passage, and from p12-17 as late passage cells.

Mice experiments: Experiments were conducted in four months old male apolipoprotein E knockout ($ApoE^{-/-}$) mice according to the guidelines formulated for care and use of animals in scientific research (ICMR, India) at a CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) registered animal facility. The experimental protocols were approved by the IAEC at CSIR-IICT (IICT/13/2015). Animals were randomly divided into 3 groups each n=12; 1) C57BL/6, 2) $ApoE^{-/-}$ and 3) $ApoE^{-/-}$ + metformin treatment. C57BL/6 and $ApoE^{-/-}$ groups received normal saline whereas metformin treatment group received the drug at 50 mg/kg/day in normal drinking water for a period of fourteen months. All the animals were fed on normal chow throughout the study.

Senescence-Associated β–Galactosidase (SA-β-gal) Staining: SA-β-gal activity for cellular senescence was examined using Senescence Detection kit (Millipore-Cat No.KAA002).

hTERT assay: At the end of the treatments, nuclear and cytosolic fractions were made according to manufacturer's protocol, NE-PER (Thermo Scientific-78833). hTERT activity in these fractions was measured using hTERT ELISA kit (E3294Hu-Bio Assay Technology Laboratory, Shanghai).

Analysis of mitochondrial respiration. An extracellular flux analyzer was used to analyze the mitochondrial function. A detailed protocol is described in the supplemental material(30).

H_2O_2 levels: Measurement of H_2O_2 levels was carried out as described in the supplemental material(30).

Detection and quantification of metformin in plasma samples by High Resolution Mass Spectrometry: The detailed procedure is provided in the supplemental material.

Generation of recombinant adenoviruses expressing HA-H3 and HA-H3K79N: The detailed protocol is provided in the supplemental material.

ChIP assay: The occupancy and enrichment of H3K79me3 on hTERT and SIRT3 promoters as well as in their actively transcribed regions was performed by ChIP assay as described in the supplemental material(51).

Western blot analysis: The procedure employed and the antibodies with their respective catalogue numbers used in this study are provided in the supplementary material.

Histopathological analysis: Aortic tissues were either fixed in 10% formalin or frozen in liquid nitrogen and immediately stored at -80 ^{o}C until further use as described in supplemental material.

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Statistical Analysis: All experiments were performed in biological triplicates and results represent the mean ± S.D. The results of statistical analyses were subjected to two-tailed, unpaired Student's t-test with a significance level of $p<0.05$ or 0.01.

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Author contributions. S.Ka. contributed to the experimental design, data analysis, and writing of the manuscript. S.Ka., P.D. and S.M. contributed to the animal experiments. S.Ka and P.D. contributed to immuno histopathological studies. S.Ka. and P.D. contributed to qRT-PCR measurement and analysis. M.K.J contributed to provision of animals for *in vivo* experiments and data analysis. N.P.K., and N.J. helped in H3-WT and H3K79N adenoviral construction. S.Ka. and Sa.Ko. contributed to ChIP experiments. Sr.Ko. contributed to the experimental design, provision of reagents and other material required for performing both *in vitro* and *in vivo* experiments, data analysis, and writing of the manuscript.

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Figure legends.

Figure.1 AMPK activation enhances hTERT levels and delays endothelial cell senescence. **(a)** Human Aortic Endothelial Cells (HAEC) were treated with metformin (2 mM) for various time points (8-24 h) and then, phospho-AMPKα, AMPKα and hTERT levels were measured by immunoblot analysis. (**b**) HAEC were treated with AICAR (1 mM) for different time points (8-24 h) and then, phospho-AMPKα, AMPKα and hTERT levels were measured by immuno blot analysis. (**c** and **d**) HAEC were pretreated with compound C (20 μM) for 2 h prior to the addition of metformin (2 mM) for 24 h and then, hTERT mRNA (**c**) and protein (**d**) levels were measured by RT-PCR and Western blot analysis respectively. (**e**) HAEC were transfected with siAMPK (40 nM) for 16 h before the addition of metformin (2 mM) for another 24 h and hTERT levels were measured by Western blot analysis. (**f**) Same as *e*, except that SA-β-gal levels were measured as described in methods. (**g**) HAEC (early *versus* late passage) were treated with metformin (2 mM) for 24 h, p-AMPKα and AMPKα levels were measured by Western blot analysis. (**h**) Same as *g*, except that HAEC were continuously grown in the presence of metformin (2 mM) for at least seven/eight passages (from p6 to p15). (**i**) HAEC (early *versus* late passage) were treated with metformin (2 mM) for 24 h and then, hTERT, p53, p27, p21 and p16 levels were measured by immunoblot analysis. (**j**) Late passage HAEC were treated with AICAR (1 mM) for 24 and then, hTERT levels were measured by immunoblot analysis. (k) Same as *i*, except that HAEC were continuously grown in the presence of metformin for at least seven/eight passages (from p6 to p15). (**l**) early and late passage cells were treated with single dose of metformin (2 mM) for 24 h or cells were continuously grown in the presence of metformin (2 mM) for at least seven/eight passages (from p6 to p15) and hTERT activity was measured by ELISA in cytosolic and nuclear fractions. (**m**) SA-β-gal staining in HAEC, early *versus* late passage controls, as well as in cells continuously grown in the presence of metformin from p5-p16. The data shown represents from three independent experiments.*Significantly different ($p<0.05$). **Significantly different($p<0.01$).

Figure.2 AMPKα activation enhances SIRT1 levels in endothelial cells. (**a** and **b**) HAEC were treated with different concentrations of either metformin (0.25-2 mM) or AICAR (0.5-1 mM) for 24 h and SIRT1 levels were measured by Western blot analysis. (**c**) HAEC were transfected with siAMPKα1 (40 nM) for 16 h before the addition of metformin (2 mM) for another 24 h and phospho-AMPKα, AMPKα and SIRT1 levels were measured by Western blot analysis. (**d**) Cells were transfected with siSIRT1 for 16 h before the addition of metformin (2 mM) for another 24 h and then, SIRT1 and hTERT levels were measured by Western blot analysis. (**e**) Cells were transfected with siSIRT1 (40 nM) for 16 h and then, SA-β-gal activity was measured. (**f**) HAEC (early *versus* late passage) were treated with metformin (2 mM) for 24 h and then, SIRT1 levels were measured by Western blot analysis. (**g**) Same as *f*, except that HAEC were continuously grown in the presence of metformin (2 mM) for seven/eight passages (p5-p16). The data shown represents from three independent experiments. **Significantly different ($p<0.01$).

Figure.3 AMPKα activation mediated enhancement of SIRT1-DOT1L/H3K79me3 axis increases hTERT levels and regulates aortic endothelial cell senescence. (**a**) HAEC were treated with different concentrations of AICAR (0.5-1 mM) for 24 h and DOT1L and H3K79me3 levels were measured by Western blot analysis. (**b**) Same as *a*, except that cells were

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treated with AICAR (1 mM) for different time points (8-24 h). (**c**) HAEC were treated with different concentrations of metformin (0.25-2 mM) for 24 h and then, DOT1L and H3K79me3 levels were measured by Western blot analysis. (**d**) Same as *c,* except that HAEC were treated with metformin (2 mM) for various time points (8-24h). (**e**) HAEC were transfected with siAMPKα1 (40 nM) for 16 h before the addition of metformin (2 mM) for another 24 h and then, DOT1L and H3K79me3 levels were measured by Western blot analysis. (**f**) Cells were pretreated with histone lysine methyltransferase inhibitor, AMI-5 (50 μM) for 2 h prior to the addition of metformin (2 mM) for 24 h and then, H3K79me3 levels were measured by Western blot analysis. (**g**) Same as *f,* except that hTERT levels were measured. (**h**) HAEC were transfected with siDOT1L (40 nM) for 16 h before the addition of metformin (2 mM) for another 24 h and then, hTERT levels were measured with immunoblot analysis. (**i** and **j**) HAEC were transduced with H3-WT and H3K79N mutant viral constructs for 3 h, replaced with complete medium and left for another 36 h and mRNA (**i**) and protein (**j**) levels were measured by qRT-PCR and Western blot analysis respectively. (**k**) Cells were transfected with siDOT1L (40 nM) for 16 h before the addition of metformin (2 mM) for another 24 h and then, SA-β-gal was performed. Simultaneously SA-β-gal staining was also measured in HAEC transduced with H3-WT and H3K79N viral constructs as mentioned in *i,* after 36 h. (**l**) Cells were transfected with siSIRT1 (40 nM) for 16 h before the addition of metformin (2 mM) for another 24 h and then, DOT1L and H3K79me3 levels were measured by immunoblot analysis. The data shown represents from three independent experiments. **significantly different ($p<0.01$)

Figure.4 AMPKα activation improves mitochondrial biogenesis and delays endothelial senescence. (**a**) HAEC (p7) were treated with metformin for 24 h and then, mitochondrial content was measured using MitoTracker Red CMXRos staining. (**b**) HAEC were pretreated with compound C (20 μM) for 2 h prior to the addition of metformin (2 mM) for another 24 h, SIRT3 and PGC-1α levels were measured by Western blot analysis. (**c**) Cells were transfected with siAMPKα1 (40nM) for 16 h before the addition of metformin (2 mM) for another 24 h and then, SIRT3 and PGC-1α levels were measured by Western blot analysis. (**d**) Cells were transfected with siSIRT1 (40 nM) for 16 h before the addition of metformin (2 mM) for another 24 h, PGC-1α and SIRT3 levels were measured with immunoblot analysis. (**e**) HAEC were pretreated with AMI-5 (50 μM) for 2 h prior to the addition of metformin (2 mM) for another 24 h, PGC-1α and SIRT3 levels were measured by Western blot analysis. (**f**) HAEC were transfected with siDOT1L (40 nM) for 16 h before the addition of metformin (2 mM) for another 24 h, PGC-1α and SIRT3 levels were measured by immunoblot analysis. (**g** and **h**) HAEC were transduced with H3-WT and H3K79N viral constructs for 3 h and replaced with complete medium for 36 h, SIRT3 and PGC-1α levels were measured by Western blot analysis (**g**) and mitochondrial DNA content (**h**) was estimated by RT-PCR. (**i**) HAEC (early *versus* late passage) were treated with metformin (2 mM) for 24 h, PGC-1α and SIRT3 levels were measured by immuno blot analysis. (**j**) Same as *i,* except that HAEC were continuously grown (p6-p15) in the presence of metformin. (**k**) Late passage HAEC were treated with single dose of metformin (2mM) *versus* cells grown continuously in the presence of metformin from p6 to p15, and mitochondrial content was measured using MitoTracker Red CMXRos. (**l**) Mitochondrial DNA content was measured in HAEC (early and late passage) treated with single dose of metformin (2 mM) for 24 h *versus* cells grown continuously in the presence of metformin from p6 to p15 by RT-PCR. (**m**) HAEC were transfected with siSIRT3 for 16 h before the addition of metformin (2 mM) for another 24 h, PGC-1α, hTERT and SIRT3 levels were measured by Western blot analysis. (**n**) HAEC were transfected with siPGC-1α for 16 h before the addition of metformin (2

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mM) for another 24 h, PGC-1α and hTERT levels were measured by Western blot analysis. (**o**) Same as *m*, except that SA-β-gal staining was performed. (p) HAEC were treated with metformin for 24h, and ChIP assay was performed to study the occupancy and enrichment of H3K79me3 in the promoter and the actively transcribed region of SIRT3 by qRT-PCR analysis. Primer sequences used for qRT-PCR of SIRT3 are presented in supplemental material. The data shown represents from three independent experiments. *significantly different ($p<0.05$)

Figure.5 Chronic metformin treatment improves mitochondrial function in endothelial cells by increasing complex-II and IV activities. (a) Oxygen Consumption Rate (OCR) was measured in HAEC (early and late passage) treated with single dose of metformin (2 mM) for 24 h *versus* cells grown continuously in the presence of metformin from p6 to p15 by extracellular flux analyzer (detailed protocol is provided in supplemental material). (b) Oxygen Consumption Rate (OCR) was measured in HAEC chronically treated with metformin (2 mM) for 7-8 passages *versus* late passage untreated; pyruvate (10 mM) was preloaded in port-A and rotenone (1 µM) in port-B. (c) Same as b, except that rotenone (1 µM), succinate (10 mM), antimycin-A (1 µM) and TMPD (100 µM) were preloaded in A, B, C and D ports respectively. (d) OCR levels were calculated from figures 5b and c. (e) Ox-phos complex subunits were measured in HAEC (early and late passage) treated with single dose of metformin (2 mM) for 24 h *versus* cells grown continuously in the presence of metformin from p6 to p15 by Western blot analysis.

Figure.6 Chronic metformin administration prevents age-associated atherosclerotic plaque formation, collagen and fibrous tissue formation and macrophage accumulation in the aorta of ApoE$^{-/-}$ mice. (**a**) H & E stained histopathological images of aorta of C57BL/6, ApoE$^{-/-}$ and ApoE$^{-/-}$ + metformin (50 m/kg.bd.wt for fourteen months) groups, (**b**) quantitative analysis of % plaque thickness. (**c-f**) Histopathological images of aorta stained with Van-Geison and quantitation of percent collagen tissue accumulation (**c** and **d**) and Masson trichrome staining and quantitation of percent fibrous tissue content (**e** and **f**). (**g**) Immunofluorescence of Mac-3, Cd45.2, and Icam-1 by confocal microscopy in aortic sections (photographs were taken under rhodamine and FITC filters and represented merged images with yellow fluorescence as positive staining). (**h**) percent Mac-3, Cd4 and Cd45.1 levels in blood measured by flow cytometry (n=9). *Significantly different ($p<0.05$) compared to C57BL/6 mice. $^{\#}$significantly different ($p<0.05$) compared to ApoE$^{-/-}$ mice.

Figure.7 Chronic metformin administration delays senescence, enhances mitochondrial biogenesis, and inhibit inflammatory mediators in the aorta of ApoE$^{-/-}$ mice. (**a**) Photographs showing the grey hair formation, adipose tissue and SA-β-gal staining of aorta in C57BL/6, ApoE$^{-/-}$ and ApoE$^{-/-}$ + metformin groups. (**b**) Shows the p-AMPKα, H3K79me3, Dot1L and Sirt3 levels in the endothelial layer of aortic sections by immunohistochemistry. (**c**) mRNA levels of indicated genes were measured in the aortic tissue by qRT-PCR (n=9). (**d**) Same as *c*, except that Tert, p16, p21, and p27 levels were measured by qRT-PCR (n=9). (**e**) Protein levels of Tert, p53, p21, and p16 in the aortic tissue homogenate by immunoblot analysis. *Significantly different ($p<0.05$) compared to C57BL/6 mice. $^{\#}$significantly different ($p<0.05$) compared to ApoE$^{-/-}$ mice.

Figure.8 Proposed mechanism of action of metformin-mediated AMPK activation in the regulation of mitochondrial biogenesis and cellular senescence.

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Highlights:

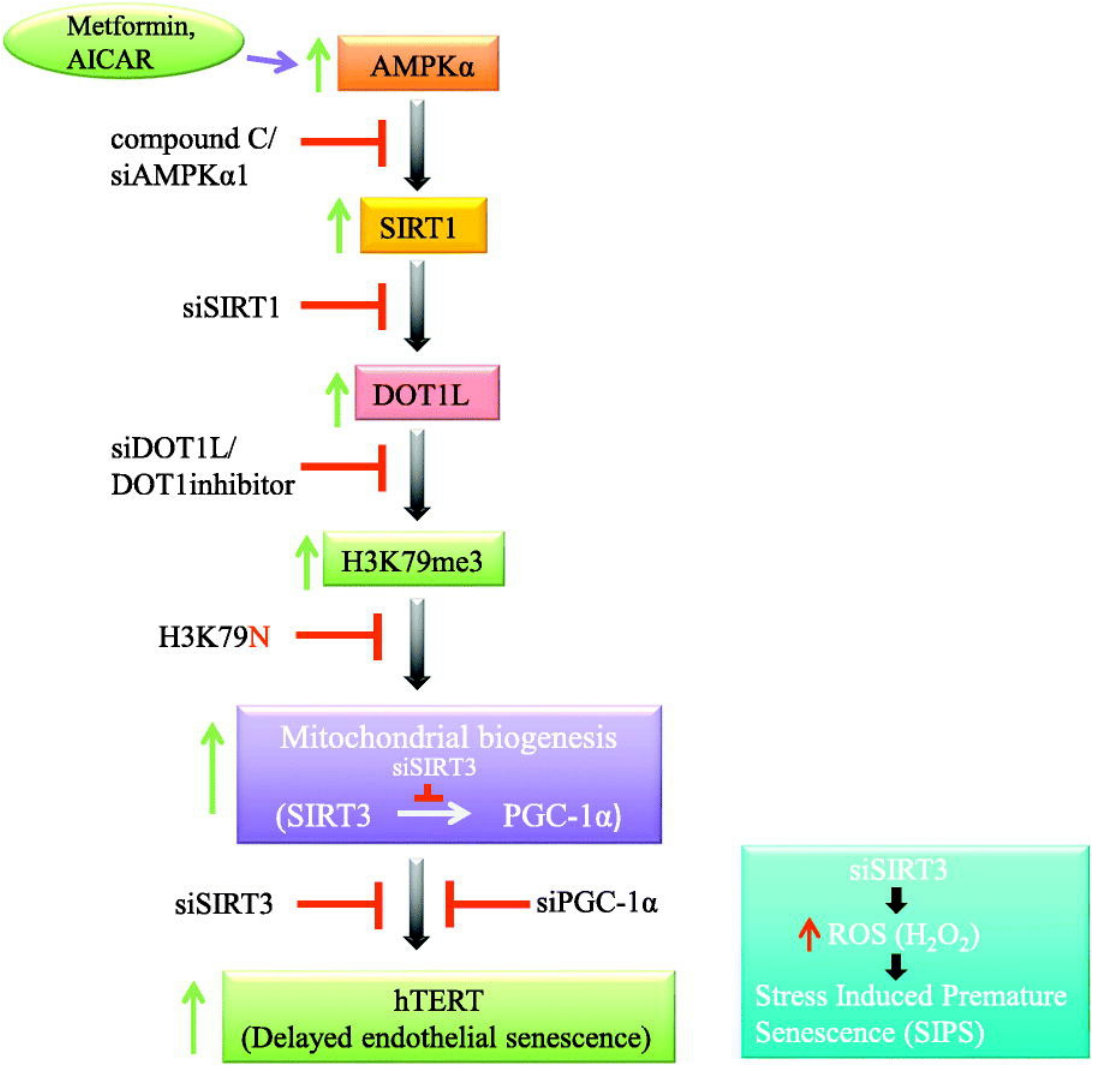
-AMPK activation increases H3K79me3 through SIRT1-DOT1L axis.

-Enhanced SIRT3 by AMPK-mediated H3K79me improves mitochondrial biogenesis/function and delays endothelial senescence.

-Chronic metformin treatment increases mitochondrial oxygen consumption rate by enhancing complex IV.

-Chronic metformin administration delays endothelial senescence and vascular aging in $ApoE^{-/-}$ mice.

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Graphics Abstract

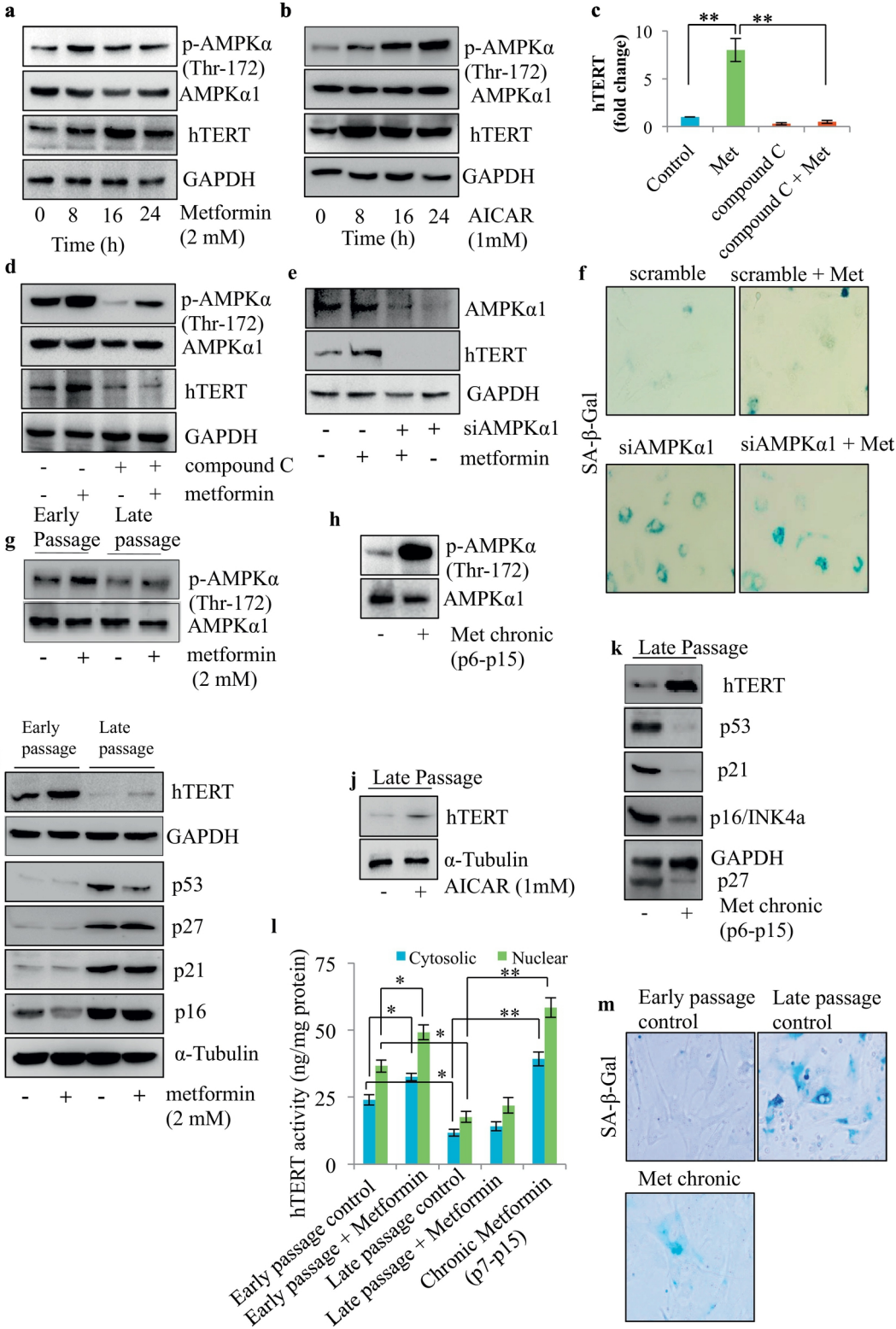

a
p-AMPKα (Thr-172)
AMPKα1
hTERT
GAPDH
0 8 16 24
Time (h)
Metformin (2 mM)
b
p-AMPKα (Thr-172)
AMPKα1
hTERT
GAPDH
0 8 16 24
Time (h)
AICAR (1mM)
c
hTERT (fold change)
10
5
0
**
**
Control
Met
compound C
compound C + Met
d
p-AMPKα (Thr-172)
AMPKα1
hTERT
GAPDH
- - + + compound C
- + - + metformin
e
AMPKα1
hTERT
GAPDH
- - + + siAMPKα1
- + + - metformin
f
scramble
scramble + Met
siAMPKα1
siAMPKα1 + Met
SA-β-Gal
g
Early Passage
Late passage
p-AMPKα (Thr-172)
AMPKα1
- + - + metformin (2 mM)
h
p-AMPKα (Thr-172)
AMPKα1
- + Met chronic (p6-p15)
i
Early passage
Late passage
hTERT
GAPDH
p53
p27
p21
p16
α-Tubulin
- + - + metformin (2 mM)
j
Late Passage
hTERT
α-Tubulin
- + AICAR (1mM)
k
Late Passage
hTERT
p53
p21
p16/INK4a
GAPDH
p27
- + Met chronic (p6-p15)
l
Cytosolic
Nuclear
hTERT activity (ng/mg protein)
75
50
25
0
Early passage control
Early passage + Metformin
Late passage control
Late passage + Metformin
Chronic Metformin (p7-p15)
m
Early passage control
Late passage control
SA-β-Gal
Met chronic

Figure 1

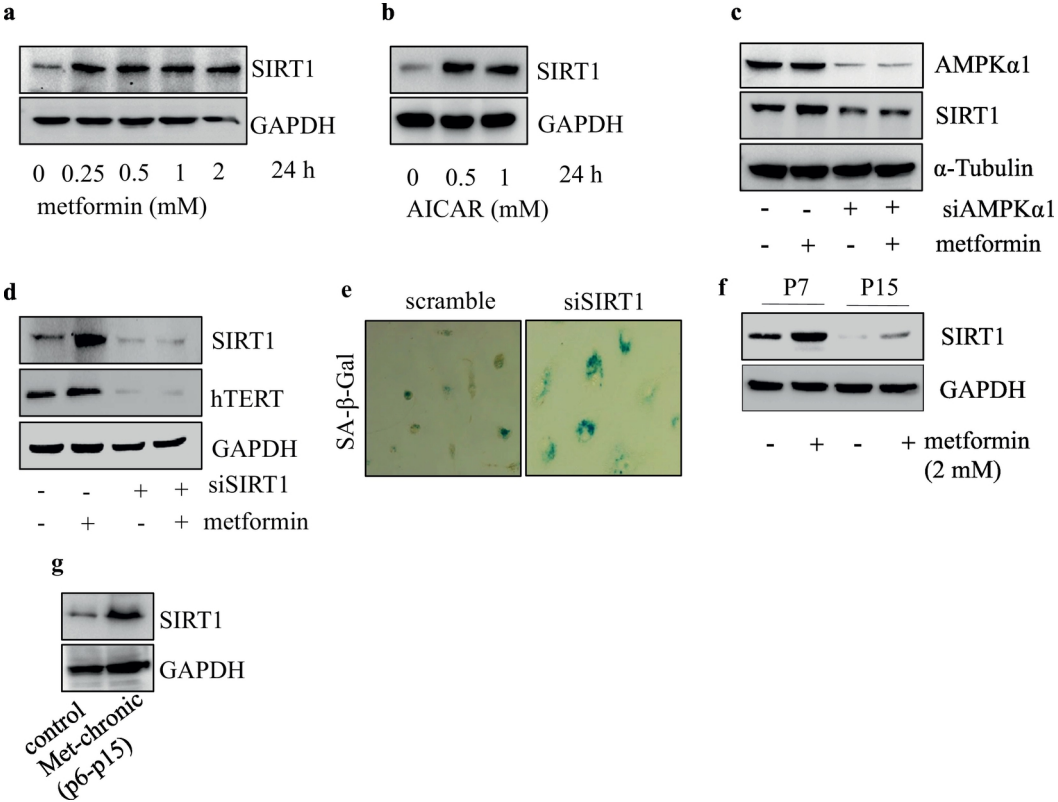

a
SIRT1
GAPDH
0 0.25 0.5 1 2 24 h
metformin (mM)
b
SIRT1
GAPDH
0 0.5 1 24 h
AICAR (mM)
c
AMPKα1
SIRT1
α-Tubulin
- - + + siAMPKα1
- + - + metformin
d
SIRT1
hTERT
GAPDH
- - + + siSIRT1
- + - + metformin
e
scramble
siSIRT1
SA-β-Gal
f
P7
P15
SIRT1
GAPDH
- + - + metformin
(2 mM)
g
SIRT1
GAPDH
control
Met-chronic
(p6-p15)

Figure 2

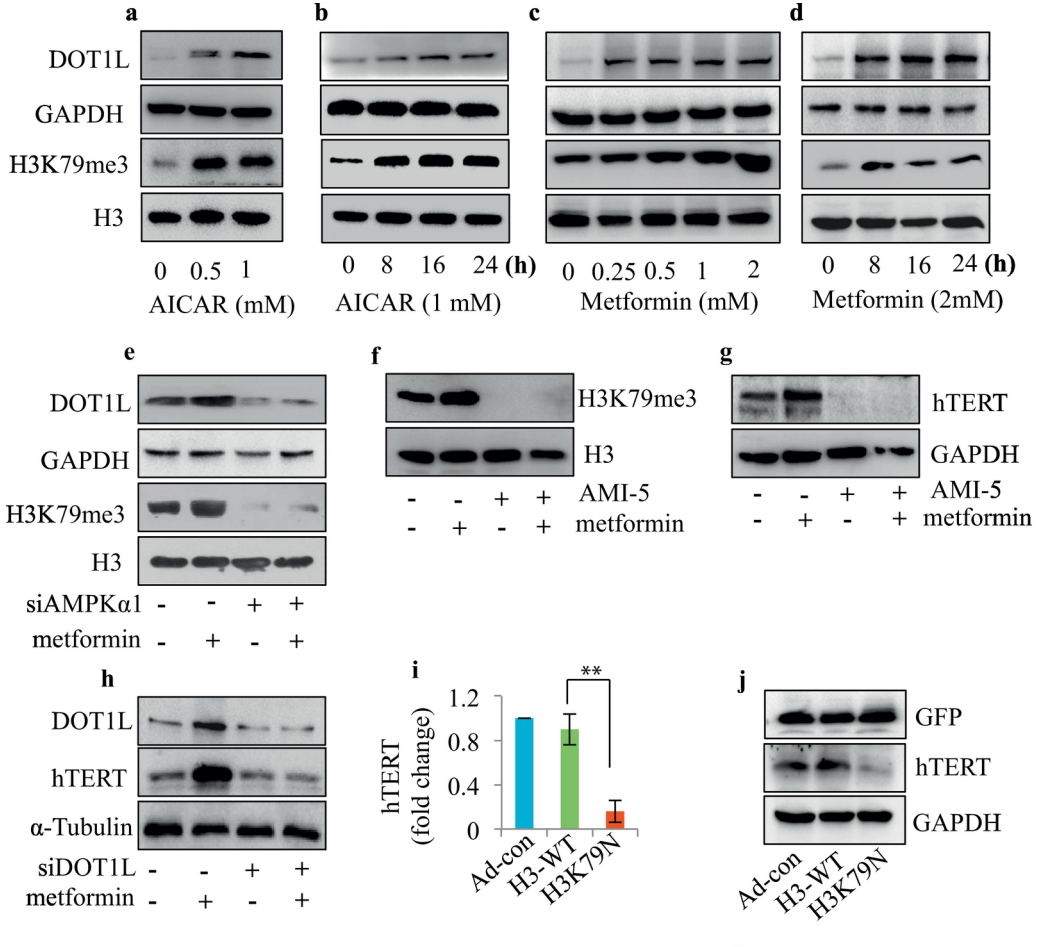

a
DOT1L
GAPDH
H3K79me3
H3
0 0.5 1
AICAR (mM)
b
0 8 16 24 (h)
AICAR (1 mM)
c
0 0.25 0.5 1 2
Metformin (mM)
d
0 8 16 24 (h)
Metformin (2mM)
e
DOT1L
GAPDH
H3K79me3
H3
siAMPKα1 - - + +
metformin - + - +
f
H3K79me3
H3
- - + + AMI-5
- + - + metformin
g
hTERT
GAPDH
- - + + AMI-5
- + - + metformin
h
DOT1L
hTERT
α-Tubulin
siDOT1L - - + +
metformin - + - +
i
hTERT (fold change)
1.2
0.8
0.4
0
**
Ad-con
H3-WT
H3K79N
j
GFP
hTERT
GAPDH
Ad-con
H3-WT
H3K79N
k
control
Met
siDOT1L
siDOT1L+Met
Ad-con
H3-WT
H3K79N
l
SIRT1
DOT1L
H3K79me3
α-Tubulin
- - + + siSIRT1
- + - + metformin

Figure 3

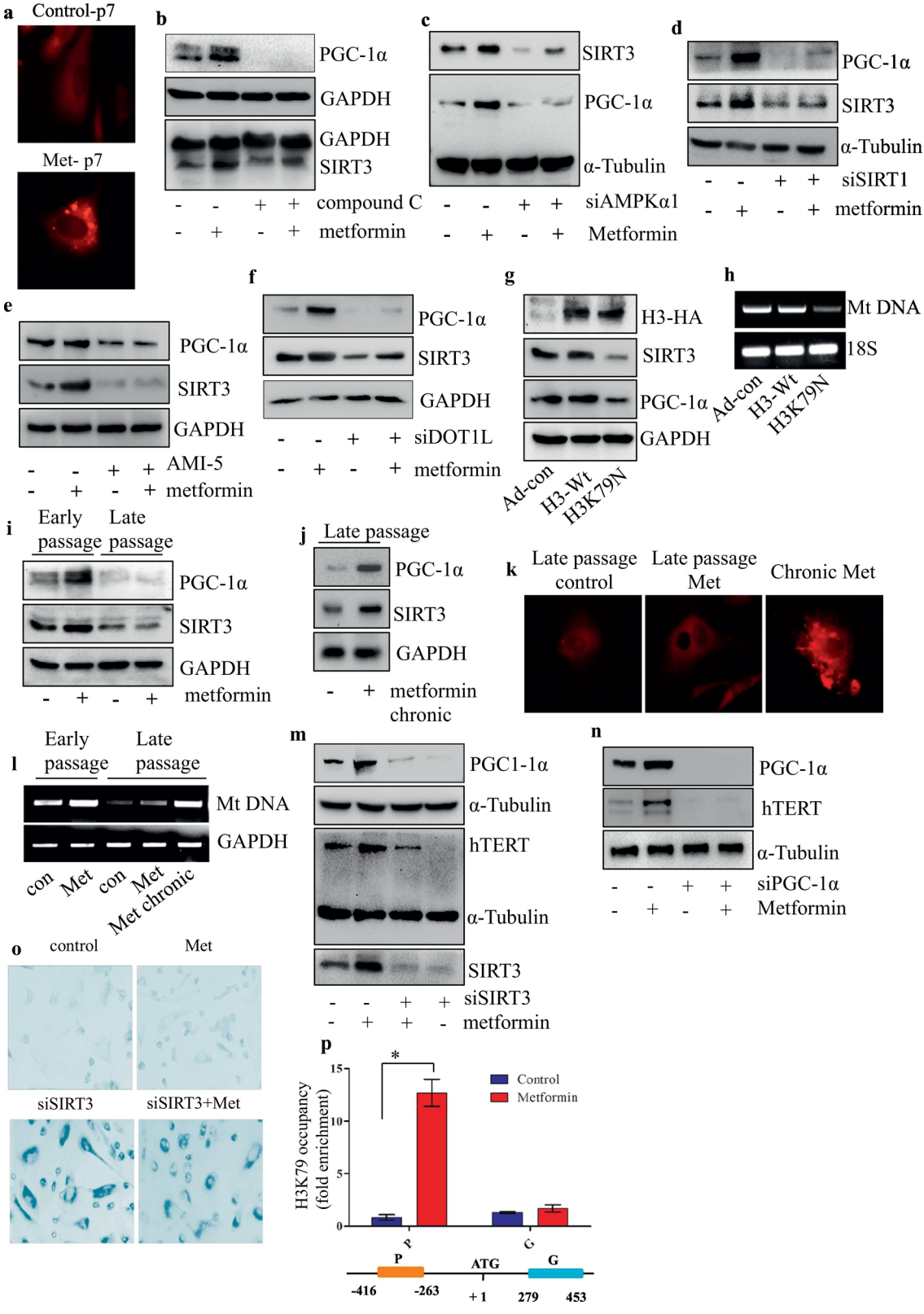

a
Control-p7
Met- p7
b
PGC-1α
GAPDH
GAPDH
SIRT3
- - + + compound C
- + - + metformin
c
SIRT3
PGC-1α
α-Tubulin
- - + + siAMPKα1
- + - + Metformin
d
PGC-1α
SIRT3
α-Tubulin
- - + + siSIRT1
- + - + metformin
e
PGC-1α
SIRT3
GAPDH
- - + + AMI-5
- + - + metformin
f
PGC-1α
SIRT3
GAPDH
- - + + siDOT1L
- + - + metformin
g
H3-HA
SIRT3
PGC-1α
GAPDH
Ad-con
H3-Wt
H3K79N
h
Mt DNA
18S
Ad-con
H3-Wt
H3K79N
i
Early passage
Late passage
PGC-1α
SIRT3
GAPDH
- + - + metformin
j
Late passage
PGC-1α
SIRT3
GAPDH
- + metformin chronic
k
Late passage control
Late passage Met
Chronic Met
l
Early passage
Late passage
Mt DNA
GAPDH
con
Met
con
Met
Met chronic
m
PGC1-1α
α-Tubulin
hTERT
α-Tubulin
SIRT3
- - + + siSIRT3
- + + - metformin
n
PGC-1α
hTERT
α-Tubulin
- - + + siPGC-1α
- + - + Metformin
o
control
Met
siSIRT3
siSIRT3+Met
p
*
Control
Metformin
H3K79 occupancy (fold enrichment)
15
10
5
0
P
G
P
ATG
G
-416
-263
+1
279
453

Figure 4

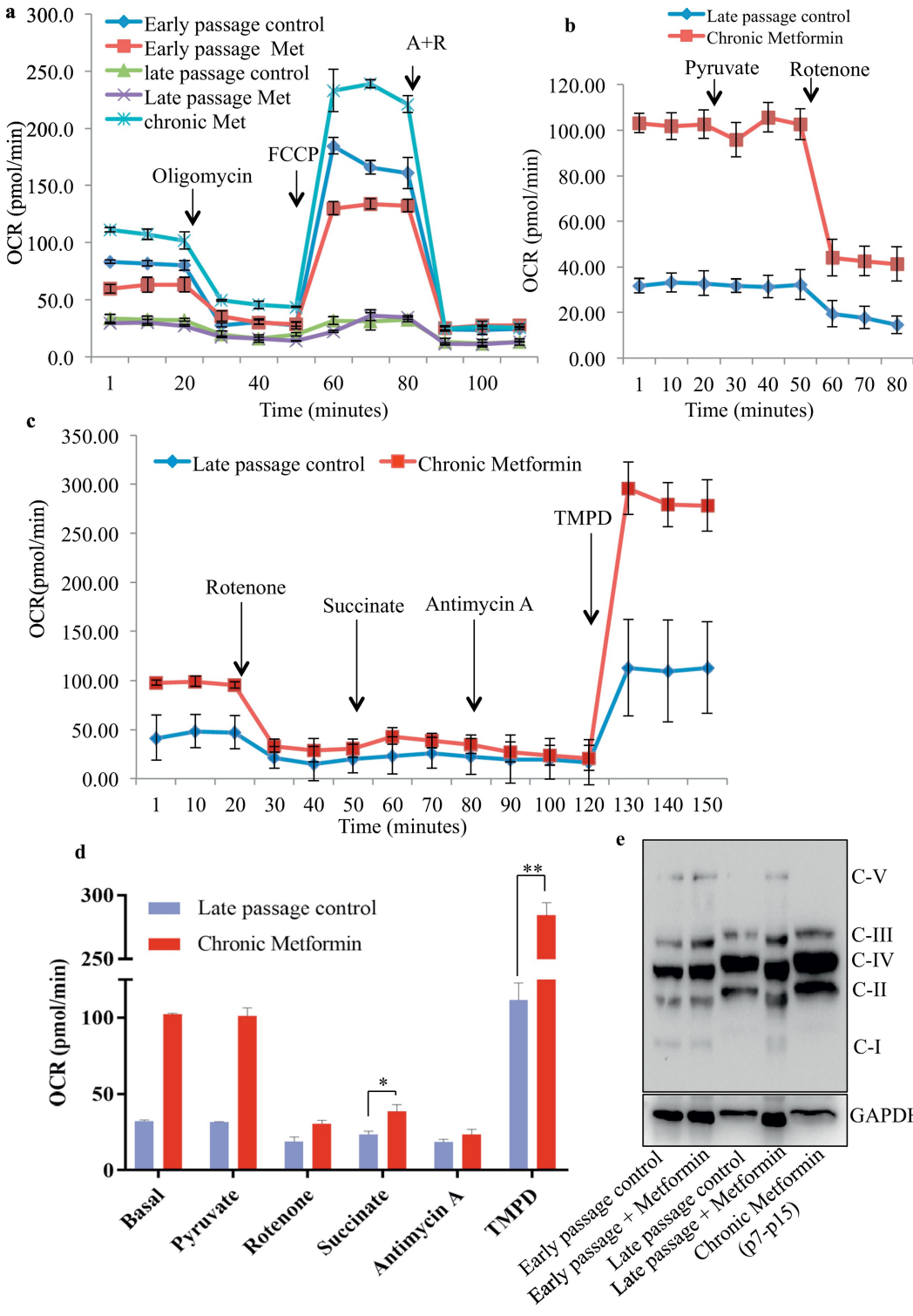

a
Early passage control
Early passage Met
late passage control
Late passage Met
chronic Met
A+R
FCCP
Oligomycin
OCR (pmol/min)
Time (minutes)
b
Late passage control
Chronic Metformin
Pyruvate
Rotenone
OCR (pmol/min)
Time (minutes)
c
Late passage control
Chronic Metformin
TMPD
Rotenone
Succinate
Antimycin A
OCR(pmol/min)
Time (minutes)
d
Late passage control
Chronic Metformin
OCR (pmol/min)
Basal
Pyruvate
Rotenone
Succinate
Antimycin A
TMPD
e
C-V
C-III
C-IV
C-II
C-I
GAPDH
Early passage control
Early passage + Metformin
Late passage control
Late passage + Metformin
Chronic Metformin
(p7-p15)

Figure 5

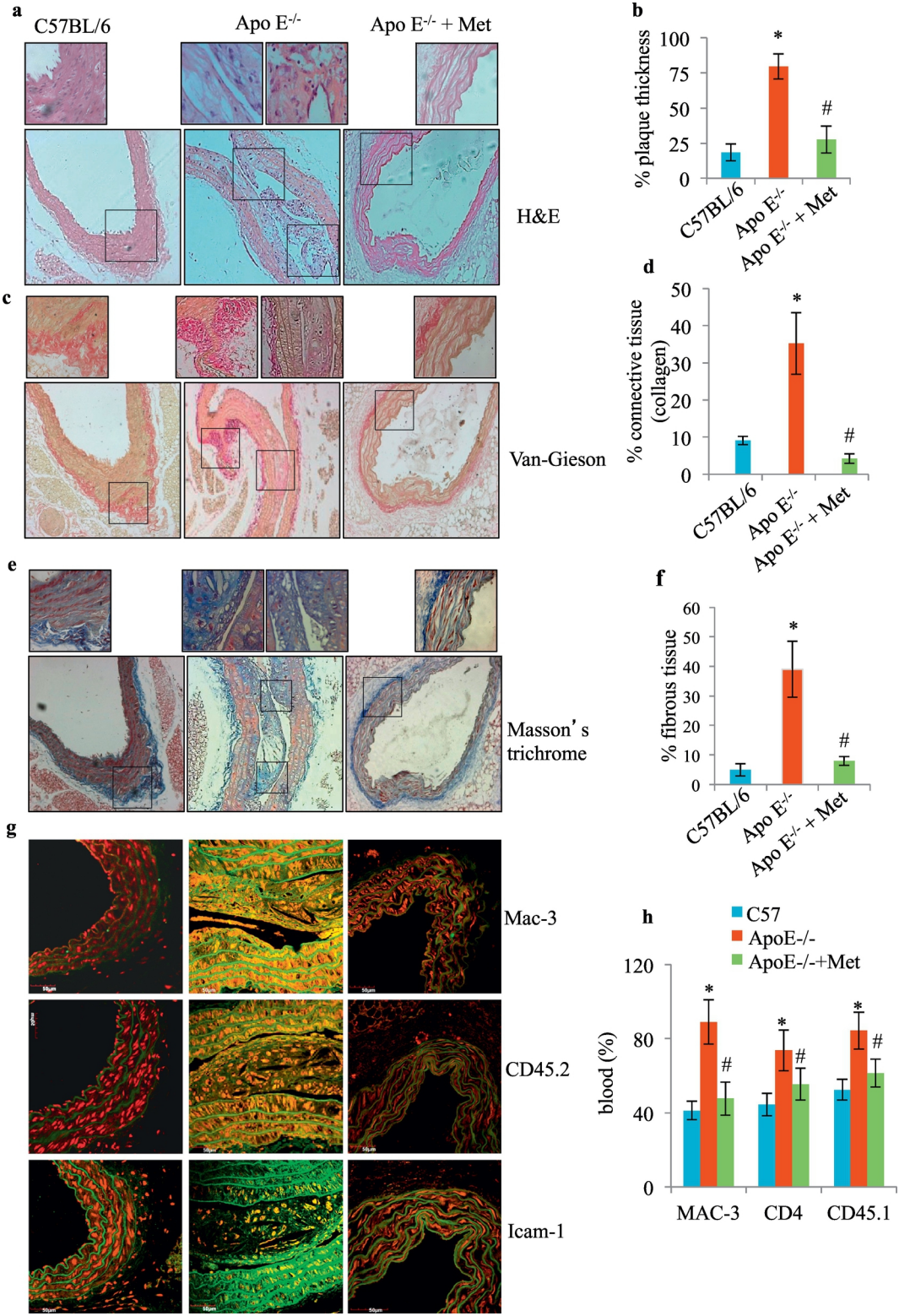

a
C57BL/6
Apo E-/-
Apo E-/- + Met
H&E
b
% plaque thickness
100
75
50
25
0
*
#
C57BL/6
Apo E-/-
Apo E-/- + Met
c
Van-Gieson
d
% connective tissue (collagen)
50
40
30
20
10
0
e
Masson's trichrome
f
% fibrous tissue
60
50
40
30
20
10
0
g
Mac-3
CD45.2
Icam-1
h
C57
ApoE-/-
ApoE-/-+Met
blood (%)
120
80
40
0
MAC-3
CD4
CD45.1

Figure 6

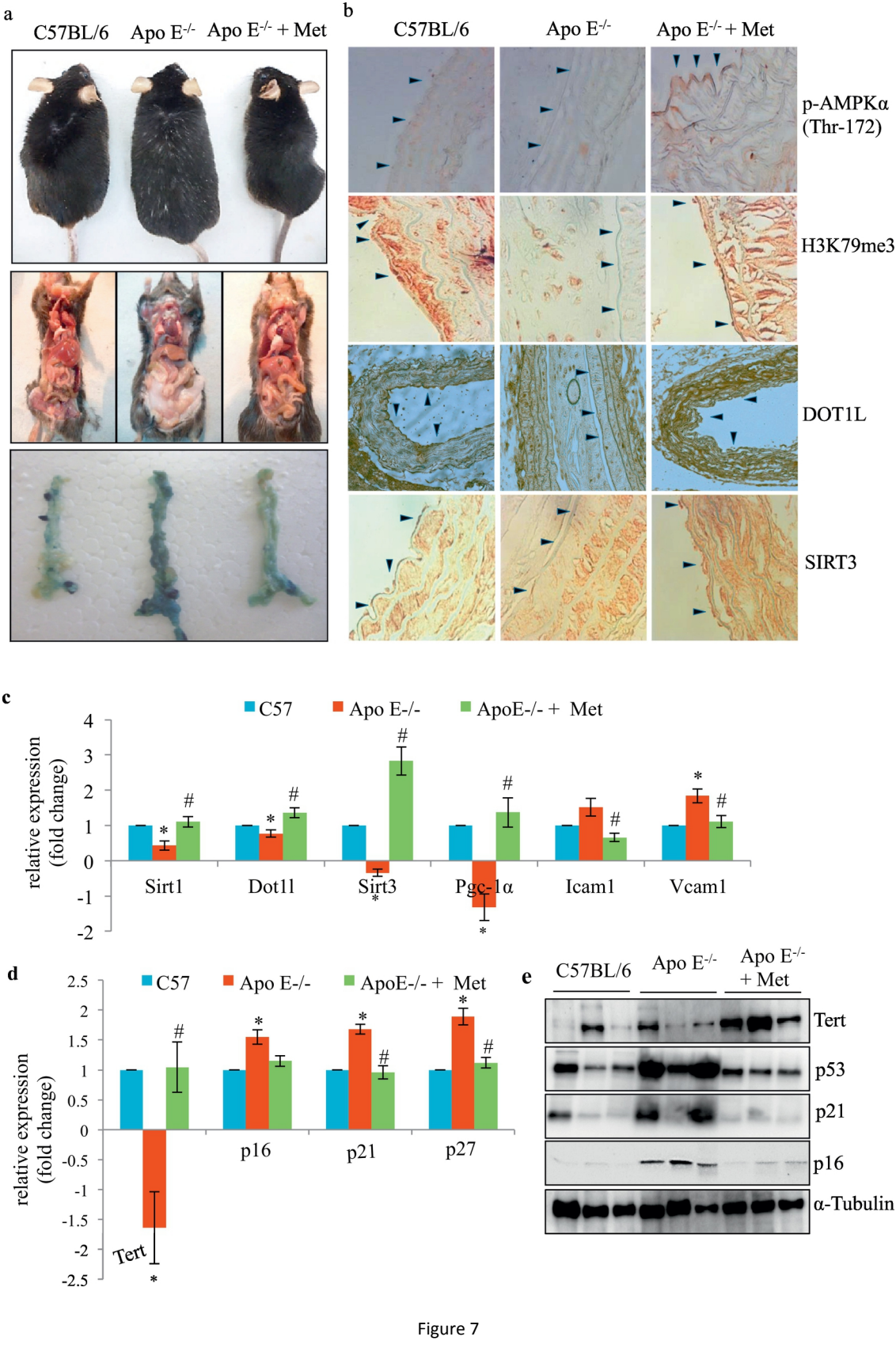

a
C57BL/6
Apo E-/-
Apo E-/- + Met
b
C57BL/6
Apo E-/-
Apo E-/- + Met
p-AMPKα (Thr-172)
H3K79me3
DOT1L
SIRT3
c
C57
Apo E-/-
ApoE-/- + Met
relative expression (fold change)
Sirt1
Dot1l
Sirt3
Pgc-1α
Icam1
Vcam1
d
C57
Apo E-/-
ApoE-/- + Met
relative expression (fold change)
Tert
p16
p21
p27
e
C57BL/6
Apo E-/-
Apo E-/- + Met
Tert
p53
p21
p16
α-Tubulin

Figure 7

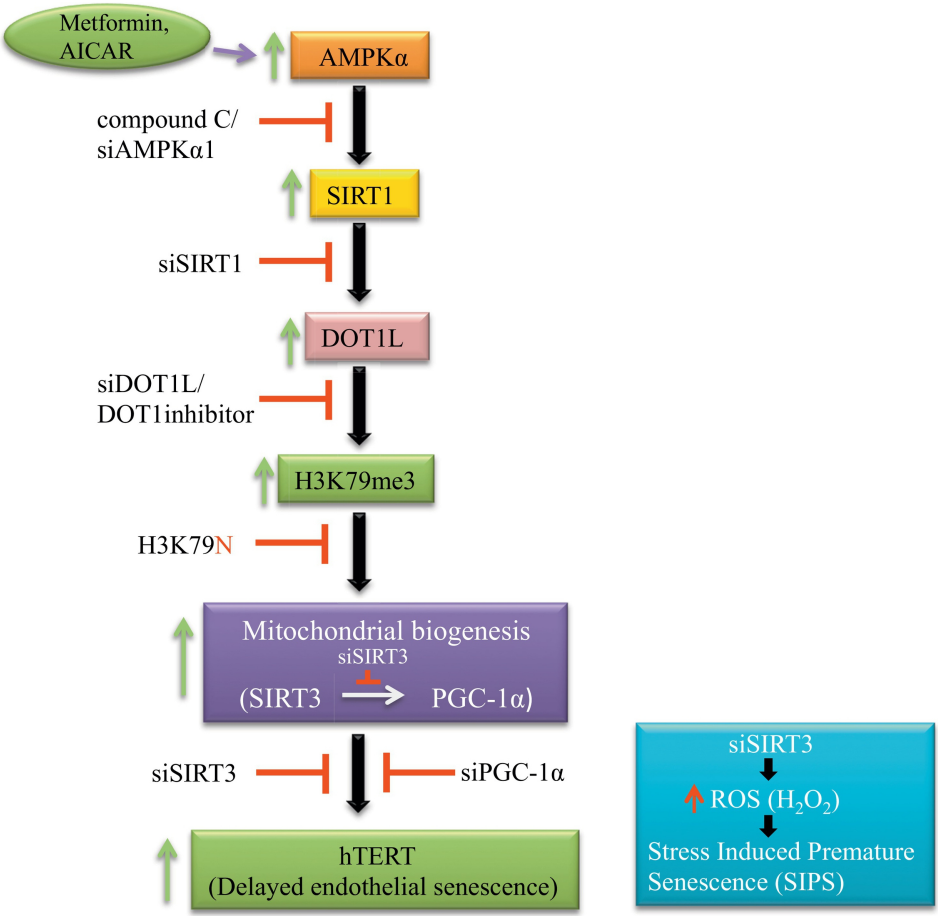

Metformin,
AICAR
AMPKα
compound C/
siAMPKα1
SIRT1
siSIRT1
DOT1L
siDOT1L/
DOT1inhibitor
H3K79me3
H3K79N
Mitochondrial biogenesis
siSIRT3
(SIRT3 → PGC-1α)
siSIRT3
siPGC-1α
hTERT
(Delayed endothelial senescence)
siSIRT3
ROS (H_2O_2)
Stress Induced Premature
Senescence (SIPS)

Figure 8