Metformin-mediated increase in DICER1 regulates microRNA expression and cellular senescence

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Summary

Metformin, an oral hypoglycemic agent, has been used for decades to treat type 2 diabetes mellitus. Recent studies indicate that mice treated with metformin live longer and have fewer manifestations of age-related chronic disease. However, the molecular mechanisms underlying this phenotype are unknown. Here, we show that metformin treatment increases the levels of the microRNA-processing protein DICER1 in mice and in humans with diabetes mellitus. Our results indicate that metformin upregulates DICER1 through a post-transcriptional mechanism involving the RNA-binding protein AUFI. Treatment with metformin altered the subcellular localization of AUFI, disrupting its interaction with DICER1 mRNA and rendering DICER1 mRNA stable, allowing DICER1 to accumulate. Consistent with the role of DICER1 in the biogenesis of microRNAs, we found differential patterns of microRNA expression in mice treated with metformin or caloric restriction, two proven life-extending interventions. Interestingly, several microRNAs previously associated with senescence and aging, including miR-20a, miR-34a, miR-130a, miR-106b, miR-125, and let-7c, were found elevated. In agreement with these findings, treatment with metformin decreased cellular senescence in several senescence models in a DICER1-dependent manner. Metformin lowered p16 and p21 protein levels and the abundance of inflammatory cytokines and oncoproteins that are hallmarks of the senescence-associated secretory phenotype (SASP). These data lead us to hypothesize that changes in DICER1 levels may be important for organismal aging and to propose that interventions that upregulate DICER1 expression (e.g., metformin) may offer new pharmacotherapeutic approaches for age-related disease.

Key words: aging; AUFI; caloric restriction; diabetes mellitus; microRNA; RNA-binding proteins.

Introduction

Metformin has been used to treat type 2 diabetes mellitus since the 1950s. Metformin enhances insulin sensitivity and lowers blood glucose levels by inhibiting gluconeogenesis in the liver. Metformin elicits these effects by inhibiting the mitochondrial respiratory-chain complex I and glycolytic phosphofructokinase (PFK) and also by activating adenosine monophosphate-activated protein kinase (AMPK) (Foretz et al., 2014; Madiraju et al., 2014). Activation of AMPK, in turn, leads to a plethora of signaling cascades that regulate energy homeostasis and metabolism (Hardie et al., 2012). However, AMPK-independent pathways activated by metformin have also been described (Pollak, 2012; Foretz et al., 2014) and recent data also suggest that systemic effects of metformin may have different mechanisms of action in different tissues (Duc et al., 2015). Gaps in our knowledge about the mechanisms that underlie its therapeutic effects persist.

Numerous epidemiologic studies show that in addition to its role in modulating metabolic pathways, metformin also influences factors associated with cancer incidence and cancer mortality among diabetics treated with the drug (Pollak, 2012). These retrospective epidemiologic studies have led to intensive focus on the potential use of metformin as an anticancer therapeutic agent. Although many studies have focused on uncovering how metformin elicits anticancer effects, the exact mechanism of tumor inhibition remains unknown (Foretz et al., 2014). Studies using cancer cells in culture and murine tumor xenografts have found that metformin inhibits tumorigenesis by upregulating DICER1 (Blandino et al., 2012), a key enzyme that processes microRNAs (miRNAs). Therefore, it is possible that upregulation of DICER1 may in part mediate metformin’s antitumorigenic effects. In general with few exceptions, cancer cells have decreased global miRNA expression (Lu et al., 2005; Kumar et al., 2007), leading to the hypothesis that upregulation of DICER1 may lead to increased miRNA expression, which would then contribute to lowering tumorigenic activity. Consistent with this idea, DICER1 expression is decreased in several types of human cancers (Babu et al., 2011; Fouiloux et al., 2014).

Conditional loss of mouse Dicer in skin or adipocytes induces DNA damage and activates a p19ARF-p53 signaling pathway leading to increased cellular senescence (Mudhasani et al., 2008; Mori et al., 2012), indicating that modulation of DICER1 levels may influence aging-related pathways. Additionally, dicer loss-of-function mutations in Caenorhabditis elegans reduce lifespan and homeostatic stress responses (Mori et al., 2012). We reported that miRNA levels decrease with advancing age in human peripheral blood mononuclear cells (PBMCs) and primate skeletal muscle, while others have found decreased levels of miRNAs with age in mouse brain and in C. elegans (Noren Hooten et al., 2010, 2013; Smith-Vikos & Slack, 2012; Mercken et al., 2013). Given the conservation of miRNAs across species, and that a single miRNA can regulate C. elegans lifespan and predict the mortality of individual C. elegans (Smith-Vikos & Slack, 2012), it is likely that miRNAs play an important role in regulating lifespan in humans and other species.

The fact that modulating DICER1 and miRNAs may be important for regulating lower organismal aging and our recent findings that metformin significantly increases the longevity of mice (Martin-Montalvo et al., 2013) led us to examine mechanisms by which DICER1 expression...
is altered by metformin and the possible role of DICER1 in aging. As gene expression is affected post-transcriptionally by noncoding RNAs and RNA-binding proteins (RBPs), we hypothesized that post-transcriptional mechanisms may play a role in modulating levels of DICER1 in response to metformin. Previous evidence indicated that the RBP AU-rich element-binding factor 1 (AUF1), also known as hnRNPd (heterogeneous nuclear ribonucleoprotein D), lowers the stability of DICER1 mRNA through binding to multiple regions in the coding region and 3’-untranslated region (UTR) of DICER1 mRNA (Abdelmohsen et al., 2012). AUF1 comprises 4 isoforms (p37, p40, p42, and p45) all of which contain two RNA-recognition motifs (RRMs) (White et al., 2013; Yoon et al., 2014). In general, cytoplasmic AUF1 promotes the decay of target mRNAs (White et al., 2013). However, new data using the PAR-CLIP (photoactivatable ribonucleoside-enhanced cross-linking and immunoprecipitation) method indicate that AUF1 also affects mRNA translation and enhances the stability of a subset of transcripts (Yoon et al., 2014). Through these activities, AUF1 regulates cellular processes such as senescence, proliferation, and stress and immune responses that impact upon aging and age-related diseases.

Here, we explored the role of DICER1 in mice chronically treated with metformin or dietary caloric restriction, two interventions that extend mouse lifespan. We found higher levels of DICER1 in mice treated with metformin or caloric restriction as well as in humans with diabetes mellitus on metformin. In contrast, DICER1 mRNA levels decreased with age in untreated human subjects. We present evidence that metformin treatment prevents the interaction of the decay-promoting RBP AUF1 with DICER1 mRNA, resulting in increased expression of DICER1. Given that metformin inhibits cellular senescence only in the presence of DICER1, our results highlight a possible mechanism that may explain in part the anti-aging effects of metformin and identify relevant pathways and targets for further investigation.

**Results**

**Age and metformin treatment alter DICER1 levels**

We hypothesized that changes in DICER1 levels may contribute to the age-associated decline in miRNAs (Noren Hooten et al., 2010). Therefore, we examined DICER1 mRNA levels by reverse transcription (RT) followed by real-time quantitative (q)PCR analysis from PBMCs from young (~30 years) and old (~64 years) individuals and found a significant decrease in DICER1 mRNA levels with human age (Fig. 1A; Table 1A). In contrast, the levels of DROSHA mRNA, encoding another key protein in miRNA biosynthesis, were not significantly different with human age (Fig. 1A). Therefore, we focused on investigating whether changes in DICER1 levels may explain alterations in miRNA expression with age.

Previously, metformin was found to increase the healthspan and longevity of mice (Martin-Montalvo et al., 2013). Given that metformin elevates DICER1 in cultured cancer cells (Blandino et al., 2012), we wanted to examine whether changes in DICER1 expression occur in mice chronically treated with metformin and to compare these findings with those of mice on dietary caloric restriction, an intervention that extends lifespan in mice. In response to metformin and dietary caloric restriction, there is an upregulation in Dicer1 mRNA levels in the livers of treated mice compared with control mice on standard diet (Fig. 1B), and livers of metformin-treated mice had higher DICER1 protein levels than control mice (Fig. 1C).

Given that DICER1 mRNA levels decreased in human PBMCs from older individuals compared with younger individuals (Fig. 1A, first panel) and that DICER1 levels increased by metformin treatment in mice, we investigated whether individuals being treated for diabetes with metformin also had higher levels of DICER1. Individuals in the Healthy Aging in Neighborhoods of Diversity across the Lifespan (HANDLS) study...
Metformin modulates DICER and senescence, N. Noren Hooten et al.

Fig. 2 Metformin stabilizes DICER1 expression through modulating the binding of the RNA-binding protein AUF1. (A) Serum-starved HepG2 cells were treated with 500 μM metformin or PBS for 1 h and ribonucleoprotein (RNP) immunoprecipitation (RIP) assays followed by RT-qPCR analysis was used to measure the enrichment of DICER1 mRNA in AUF1 immunoprecipitates. Each IP was compared to control IgG IP and normalized to GAPDH mRNA levels. (B) HeLa cells were transfected with either AUF1 or Control (Con) siRNA and 24 h later, cells were treated with metformin for 24 h. The levels of HNRNPD mRNA (which encodes AUF1) and DICER1 mRNA were measured by RT-qPCR analysis and normalized to GAPDH mRNA levels. (C–E) Cells were transfected with either AUF1 siRNA-1 (Qiagen), AUF1 siRNA-2 (Santa Cruz) or the indicated FLAG-tagged plasmids were treated as in (B), and lysates were analyzed by immunoblotting to assess protein levels of DICER1, AUF1 and actin using specific antibodies. Histograms represent the mean ± SEM from three independent experiments. ***p < 0.001 or *p < 0.05 by Student’s t-test.

(Evans et al., 2010) were chosen based on the following criteria. The participants were either euglycemic normal controls (i), diabetics treated with sulfonylurea (ii), or diabetics treated with metformin (iii) (Table 1B). The sulfonylurea group was added in the study because diabetic individuals treated with metformin have increased survival compared with nondiabetics and with diabetics on sulfonylurea (Bannister et al., 2014). All groups were matched based on age, sex, race and body mass index (BMI) (n = 20 per group; Table 1B). After isolation of RNA from participant PBMCs and RT-qPCR analysis, diabetics treated with metformin were found to have significantly higher levels of DICER1 mRNA compared with nondiabetics; diabetics taking sulfonylureas also had higher levels of DICER1 mRNA, but this difference was not significant (Fig. 1D).

Metformin regulates the RBP AUF1 localization and binding to DICER1 mRNA

Given that chronic metformin treatment in mice increased DICER1 levels, we hypothesized that metformin may alter post-transcriptional processes that would affect the stability and/or turnover of DICER1 mRNA. In response to changes in the environment, RNA-binding proteins (RBP) regulate gene expression by affecting the turnover and/or translation of bound mRNAs (Moore, 2005). Previous studies showed that the RBP AUF1 binds DICER1 mRNA and negatively regulates DICER1 protein levels by lowering the stability of DICER1 mRNA (Abdelmohsen et al., 2012). Therefore, we hypothesized that metformin may enhance DICER1 expression by altering the binding of AUF1 to DICER1 mRNA. To test this possibility, we performed a ribonucleoprotein immunoprecipitation (RIP) assay to examine the effect of metformin on the AUF1-DICER1 mRNA complex. While AUF1 was found to bind DICER1 mRNA under basal conditions, metformin treatment dramatically reduced this interaction (Fig. 2A). As AUF1 binding degrades DICER1 mRNA, these data suggest that by triggering the dissociation of AUF1-DICER1 mRNA complexes, metformin treatment may stabilize DICER1 mRNA. To further test this possibility, small interfering RNAs were used to downregulate AUF1 levels in cells. Lowering AUF1 increased DICER1 mRNA and protein levels (Fig. 2B–D). DICER1 mRNA levels further increased by metformin treatment, which may reflect an effect on DICER1 at the transcriptional level (Fig. 2B). Conversely, AUF1 overexpression decreased DICER1 protein levels (Fig. 2E). These findings suggest that metformin treatment enhances DICER1 expression at least in part by dissociating AUF1 from DICER1 mRNA.

The abundance of AUF1 was not altered by metformin treatment of mice or cells in tissue culture (Fig. S1A,B); however, metformin affected AUF1 subcellular localization. Although AUF1 was found in both the cytoplasm and nucleus under basal conditions, by 1 h after addition of metformin, AUF1 had redistributed to a predominantly nuclear localization (Fig. 3A,B). This redistribution to the nucleus in response to metformin was confirmed by fractionating cells into the nuclear and cytoplasmic compartments in both HeLa and WI-38 cells (Figs 3C, S1D). Given that AMPK is activated in response to metformin, we tested whether AMPK activation is important for the effects of metformin on AUF1 subcellular localization. Treatment with the AMPK inhibitor Compound C blocked AUF1 redistribution to the nucleus in response to metformin (Figs 3A, S1D), suggesting that the effects of metformin on AUF1 localization are influenced by AMPK. Furthermore, lowering AMPK levels using siRNA inhibited AUF1 redistribution from the cytoplasm to the nucleus in response to metformin (Fig. 3D). This translocation of AUF1 from the cytoplasm to the nucleus was associated with the release of AUF1-DICER1 mRNA complex, facilitating DICER1 mRNA stabilization and translation.
The stoichiometry of the various AUF1 isoforms differed in their subcellular localization in response to metformin. Specifically, the localization of p45 was most affected by metformin, although p42/p40 isoforms (typically found together as a single band) were moderately affected as well. PAR-CLIP data showed that the p40, p42, and p45 isoforms bind to DICER1 mRNA; p45 in particular bound to multiple regions within DICER1 mRNA (Table S1). Given this evidence, we tested whether metformin affected AUF1 isoform binding to DICER1 mRNA. Each FLAG-tagged AUF1 isoform was individually transfected into cells, and RIP assays were performed in control and metformin-treated cells.

As shown (Fig. 3E), metformin treatment did not affect p37 or p40 binding to DICER1 mRNA, but it dramatically decreased DICER1 mRNA bound to the p42 and p45 isoforms, supporting the notion that AUF1 translocation to the nucleus is associated with the dissociation of DICER1 mRNA from AUF1.

We next investigated whether AUF1 is phosphorylated in response to metformin. Metformin treatment increased AUF1 phosphorylation as detected using antibodies that recognize the phosphorylated AMPK substrate motif and with phospho-serine/threonine antibodies (Fig. 3F). Interestingly, several other proteins were prominent in AUF1

![Image](https://example.com/image.png)
immunoprecipitates, among them a ~70-kDa band. It was previously reported that AUF1 binds to HSP70 and that HSP70 shuttles AUF1 to the nucleus in response to heat shock (Laroia et al., 1999). Western blot analysis of HSP70 in the AUF1 immunoprecipitated material revealed that the AUF1-HSP70 interaction was enhanced in the presence of metformin (Fig. 3F). Finally, HSP70 increased in the nucleus after metformin treatment (Fig. 3C). These data suggest that AUF1 translocation to the nucleus may be modulated by HSP70 binding in response to metformin.

**Metformin inhibits cellular senescence through DICER1**

As the functional role of DICER1 in cells is to process miRNAs from precursors to mature miRNAs, it is likely that changes in DICER1 expression would ultimately alter miRNA expression. To test this idea, we analyzed global miRNA profiles using microarrays. Many miRNAs were significantly altered after metformin treatment or calorie restriction (Fig. 4A; Table S2). Consistent with the higher DICER1 levels in the liver of metformin-treated mice, many miRNAs were found to be upregulated by metformin (Figs 4A,B, S2). To assess whether the increases in mature miRNAs were due to elevated transcription, we quantified the primary miRNA (pri-miRNA, the initial unprocessed transcript of miRNAs) transcripts of two miRNAs observed to be differentially expressed in the presence of metformin, miR-130a-3p and miR-92a-3p (Fig. 4B,C). As shown, neither pri-miR-130a nor pri-miR-92a levels were significantly increased by metformin or CR (Fig. 4C). These data suggest that the increase in mature miRNAs in mice treated with metformin was not primarily a result of increased transcriptional activity.

Several of the miRNAs upregulated by metformin (miR-20a, miR-30, miR-34a, miR-106b, miR-130a/b, let-7, miR-125) are important for regulating cellular senescence or organismal aging (Fig. 4B). Furthermore, many of the targets of these miRNAs are important for regulating senescence (Table S3), and some have opposite expression patterns in response to metformin compared with senescence, suggesting that metformin may affect lifespan by inhibiting cellular senescence. To investigate this possibility, we examined whether metformin affects cellular senescence in culture using several different senescence models: WI-38 human fetal lung diploid fibroblasts, IMR-90 human skin fibroblasts, and IDH4 fibroblasts. We chose doses of metformin that were comparable to circulating drug levels of mice treated with 0.1% metformin (~0.45 mM; 74.3 mg L⁻¹) and the therapeutic range in humans (1.64 ± 0.13 mg L⁻¹ to 6.57 ± 0.61 mg L⁻¹) (Sum et al., 1992). Metformin inhibited replicative senescence in WI-38 and IMR-90 cells (Fig. 5A,B,F). In IDH4 cells, senescence is induced by removal of dexamethasone (dex) (Wright et al., 1989). Dex was removed, and cells were cultured in the presence of metformin for 7 days; metformin treatment reduced the number of SA-β-gal-positive IDH4 cells (Fig. 5C). Additionally, we induced senescence by exposing WI-38 and IMR-90 cells to ionizing radiation (IR). Treatment of cells with metformin delayed IR-induced senescence, as assessed initially by SA-β-gal-positive cells.
Fig. 5  Inhibition of cellular senescence by metformin requires DICER1. Presenescent WI-38 or IMR-90 cells were transfected with either the indicated siRNAs or transfected with pDEST-DICER1 and then either treated with PBS or with 500 μM metformin (Met) for 48 h and stained for SA-β-gal activity (A,B,D). (C) IDH4 cells cultured without dex were incubated with metformin. (D) WI-38 and IMR-90 cells exposed to ionizing radiation (IR) were treated with metformin. SA-β-gal-positive cells were counted and normalized to the total number of cells. Representative phase-contrast images from WI-38 cells from (B) are shown, and the histograms represent the mean ± SEM from 3 independent experiments. *P < 0.05, **P < 0.01 and ***P < 0.001 by Student’s t-test. (F) Lysates from the indicated cell lines and treatments were analyzed for protein levels of senescence markers and actin for a protein loading control.

(Fig. 5D) and subsequently confirmed by examination of two well-established protein markers of senescence, p16 and p21. All of these markers of senescence were decreased by metformin treatment (Fig. 5F).

A further hallmark trait of senescent cells is the secretion of inflammatory cytokines, chemokines, and oncogenes (the senescence-associated secretory phenotype or SASP) (Coppe et al., 2008). We quantified the levels of several mRNAs encoding SASP factors in WI-38 cells, in IDH4 cells grown without dex, and WI-38 and IMR-90 cells exposed to IR. The levels of IL6, IL8, CXCL1 (which encodes GRO-a), and CXCL2 (which encodes GRO-b) mRNAs decreased by metformin treatment (Fig. 6).
We next addressed whether the effects of metformin on senescence were dependent on DICER1. SiRNA-mediated downregulation of DICER1 levels increased cellular senescence and blocked the ability of metformin to lower the number of SA-β-gal-positive cells in both WI-38 and IMR-90 cells (Fig. 5A,B). Conversely, overexpression of DICER1 decreased cellular senescence (Fig. 5E). To determine whether AMPK is required for the senescence-inducing effects of metformin, we silenced AMPK levels. This intervention blocked the metformin-mediated decrease in SA-β-gal-positive cells, and this effect was reversed with DICER1 overexpression. Collectively, these data indicate that metformin may reduce cellular senescence by promoting DICER1 expression and that AMPK is important for these effects.

Discussion

Previously, we reported that chronic metformin treatment increases both healthspan and lifespan in mice (Martin-Montalvo et al., 2013). Here, we have found that DICER1 levels are upregulated by metformin treatment in both mice and humans in part through a post-transcriptional mechanism involving the RBP AUF1. Upregulation of DICER1 by metformin leads to higher levels of a subset of miRNAs important for regulating senescence and aging-associated pathways. Furthermore, metformin treatment inhibits cellular senescence through a DICER1-dependent mechanism.

Our data suggest that DICER1 levels may contribute to aging. Consistent results have been observed in a limited number of studies that have examined the role of DICER1 in aging. A study in primary rat cerebrovascular endothelial cells (CMVECs) found a decrease in DICER1 levels with age, with concomitant reduction in miRNA expression (Ungvari et al., 2013). Furthermore, aged mouse adipocytes also have lower DICER1 levels (Mori et al., 2012). Here, we found that DICER1 mRNA levels decreased with age in human PBMCs, which may provide a mechanism for lower miRNA levels we previously observed in this cell population in the same cohort of individuals (Noren Hooten et al., 2010).

Compelling evidence suggests that alterations in DICER1 levels or activity may be important for other age-associated diseases, especially cancer. In general, DICER1 levels are decreased in different types of human tumors and germline mutations in DICER1 are associated with a rare type of childhood cancer called pleuropulmonary blastoma (PPB) (Bahubeshi et al., 2011; Foulkes et al., 2014). Point mutations in DICER1 have also been described leading to tumor predisposition and referred to as DICER1 syndrome (Bahubeshi et al., 2011; Foulkes et al., 2014). However, the role of DICER1 in cancer is complicated as mouse cancer models suggest that loss of one copy of the Dicer1 gene increases tumorigenesis, whereas loss of both copies inhibits it, leading to the idea that DICER1 functions as a haploinsufficient tumor suppressor (Bahubeshi et al., 2011; Foulkes et al., 2014). These pieces of evidence may reflect the importance of DICER1 on cell survival and/or indicate that a subset of miRNAs is important for cancer progression. Nevertheless, these studies point to the importance of DICER1 and the need to fully understand the consequences of altering DICER1 levels and/or activity.

We found increased levels of miRNAs in livers of metformin-treated mice, consistent with the fact that DICER1 processes precursor miRNAs to mature miRNAs. As DICER1 also plays other important roles in the RNA-induced silencing complex (RISC) and in RNA metabolism (Burger & Gullerova, 2015), we cannot exclude the possibility that these other functions of DICER1 may be altered by metformin and contribute to metformin signaling. In addition, upregulation of DICER1 by metformin may also be regulated, in part, at the transcriptional level (Blandino et al., 2012); a comprehensive view of the spectrum of DICER1 functions influenced by metformin is beyond the scope of this investigation.

By examining the miRNAs that were upregulated by metformin treatment, we observed that several of these miRNAs have been
implicated in regulating cellular senescence or aging (Table S3). Notably, we found that metformin treatment upregulated miR-125, a putative homolog of the C. elegans miRNA lin-4, which is a well-established regulator of lifespan in worms (Boehm & Slack, 2005; Ibáñez-Ventoso et al., 2008). Mutation of dicer decreases C. elegans longevity in response to heat stress, an effect that can be rescued by overexpression of lin-4 (Mori et al., 2012). Here, the increase in miR-125-5p levels coincided with lifespan extension in mice in response to metformin (Boehm & Slack, 2005; Martin-Montalvo et al., 2013). It will be interesting in future studies to examine whether miR-125 has life-extending properties, similar to those conferred to C. elegans by its homolog lin-4.

Other miRNAs upregulated by metformin have also been linked to aging in model systems, including miR-34. An age-associated alteration in miR-34 occurs in the mouse and overexpression of miR-34 in Drosophila extends lifespan (Li et al., 2011; Liu et al., 2012). miR-34 may play an important role in responding to DNA damage and also in protecting against osteoporosis and neurodegeneration (Kato et al., 2009; Liu et al., 2012; Krzeszinski et al., 2014). Metformin has been shown to upregulate miR-34a in cancer cells in culture (Do et al., 2014). In addition to miR-125 and miR-34a, compelling evidence also suggests that other miRNAs including miR-20a, miR-30, miR-106b, miR-130a/b, and let-7 family members are important regulators of cellular senescence (Table S3) (Abdelmohsen & Gorospe, 2015). With advancing age, the number of senescent cells increases and contributes to age-related physiology and pathologies (van Deursen, 2014). We propose that one potential mechanism by which metformin increases lifespan is through inhibition of cellular senescence. Metformin decreases senescence in several different cell lines and senescence model systems. We provide evidence that metformin lowers protein levels of p16 and/or p21 and also decreases levels of IL6, IL8, CXCL1, and CXCL2 mRNAs, all encoding critical SASP factors. A previous report found that metformin enhances cellular senescence using different cell model systems and high doses of metformin (1–5 mM) with possible value in cancer therapy (Cuﬁ et al., 2012). Here, we chose a lower range of concentrations (100–500 μM) that reflect physiological circulating levels of metformin in mice and in diabetic patients treated with metformin (Sum et al., 1992).

We describe a post-transcriptional mechanism whereby metformin treatment causes the redistribution of the RBP AUFI from the cytoplasm to the nucleus, associated with a disruption of its interaction with DICER1 mRNA that causes increased DICER1 mRNA and protein levels. Cellular glucose levels also regulate AUFI shuttling to the nucleus and AMPK is important for this effect (Gao et al., 2014). Here, we found that AMPK is also important in mediating the effects of metformin on AUFI redistribution to the nucleus. Furthermore, metformin increased the phosphorylation of AUFI and enhanced AUFI binding to HSP70, an interaction that shuttles AUFI to the nucleus in response to heat shock (Larioa et al., 1999). We found evidence that metformin enhanced AUFI binding to HSP70 and increased HSP70 localization in the nucleus. Interestingly, a ~70 kDa band that was serine/threonine-phosphorylated in AUFI immunoprecipitates might represent HSP70, recently identiﬁed as a substrate of AMPK (Schaffer et al., 2015). It has been observed that phosphorylation of AUFI has been reported to regulate AUFI protein stability and binding to target miRNAs (Wilson et al., 2003; Li et al., 2013). AUFI has also been implicated in regulating cellular senescence and also targets p16ink4a, a potent regulator of senescence (Guo et al., 2010; Pont et al., 2012). It will be interesting to determine whether other AUFI targets are also affected by metformin.

Although the actions of metformin have been extensively studied for decades, the underlying mechanisms remain unclear. Here, we have extended our mouse lifespan studies by showing that chronic metformin treatment affects DICER1 levels both in mice and humans. This new insight will hopefully aid both in identifying novel targets for development of agents that could be important in the primary or secondary prevention of cancer and increasing healthspan in older individuals. Currently, the FDA is considering a clinical trial called Targeting Aging with Metformin (TAME), which aims to assess whether metformin can delay aging and age-related diseases. Our studies provide timely insight into the molecular mechanisms through which metformin elicits anti-aging effects in mice and humans.

**Experimental procedures**

**Human study participants and mouse models**

For Fig. 1A, a subcohort of young (30 years) and older (64 years) participants from the Healthy Aging in Neighborhoods of Diversity across the Life Span study (HANDLS) (Evans et al., 2010) were chosen for examination of DICER1 and DROSHA miRNA levels with age. Clinical information on this subcohort has been described previously and is also listed in Table 1A (n = 14/group) (Noren Hooten et al., 2010) and explained in the Supporting Information. DICER1 levels were quantified from PBMCs by RT-qPCR analysis as described below and in Supporting Information.

Frozen tissue from Martin-Montalvo et al. was used for the current study (Animal protocol #: 352-TGB-2015). Livers from C57BL/6 mice on metformin (0.1% w/v in diet), calorie restriction (60% daily food, AIN-93G, allotment compared with ad lib animals) or standard diet (AIN-93G diet) used for these studies were described previously (Martin-Montalvo et al., 2013).

Global microRNA expression from livers of the same cohort of mice (n = 5 per group) whose global gene expression proﬁle was previously reported (Accession Number: GSE40936) (Martin-Montalvo et al., 2013). Total RNA including miRNAs was isolated using the Absolutely RNA miRNA Kit (Agilent) and analyzed using the Agilent Mouse miRNA Microarray 15.0. Microarray was performed and analyzed as previously described (Noren Hooten et al., 2013). Individual miRNAs with pairwise z-test P value ≤ 0.05, absolute value of Z ratio ≥ 1.5, with fdr < 0.3 were considered signiﬁcantly changed. The microRNA microarray data can be accessed at GEO (Accession Number: GSE73393). miRNA expression information from heat map is in Table S2.

**Cell lines, reagents, and transfections**

HepG2 cells were maintained in Minimal Essential Media containing 10% fetal bovine serum (FBS) and HeLa, IMR-90 and IDH4 cells were grown in Dulbecco’s modiﬁed Eagle’s medium (DMEM) supplemented with 10% FBS. IDH4 cells were further supplemented with 1 μg mL⁻¹ dexamethasone (Wright et al., 1989). WI-38 human fetal lung diploid ﬁbroblasts (HDFs, Coriell Cell Repositories) were grown in DMEM supplemented with 10% FBS and 1% nonessential amino acids. All media contained penicillin/streptomycin. Metformin (Farmhispania S.A., Barcelona, Spain) was made fresh as a 100 mM stock solution in PBS. PBS was used as a vehicle control in all experiments. Compound C (Dorsomorphin) was purchased from Sigma-Aldrich, St. Louis, MO, USA.

Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) with siRNAs directed to AUFI (Qiagen, Hilden, Germany; AAGATCCTATCACAGGGCGAT), AUFI (Santa Cruz;
reverse transcription (RT) followed by real-time, quantitative (q)PCR analysis

RNA was reverse-transcribed using the QuantitiMIR cDNA kit (System Biosciences, Mountain View, CA, USA) according to the manufacturer’s instructions and real-time, quantitative (q)PCR amplification of miRNA, mRNA, pri-microRNA, and microRNA was carried out as described in the Supporting Information.

Immunoprecipitation, immunoblotting, and immunofluorescence

Mouse livers were homogenized in RIPA buffer containing protease and phosphatase inhibitors. Lysates were centrifuged, and equal protein concentrations of the supernatant were analyzed using SDS-PAGE. For immunoprecipitation experiments, HeLa cells were serum-starved in 0.1% FBS-containing media for 18 h then treated for the indicated times with 500 μM metformin or PBS. Cells were lysed in a buffer containing PBS, 1% Triton-X100, 2 mM EDTA, and phosphatase and protease inhibitors, clarified, and incubated with Gamma bind beads precoated with anti-AUF1 antibodies (Cell Signaling, Danvers, MA, USA) for 1.5 h. After extensive washing, immunoprecipitations were analyzed by SDS-PAGE. The antibodies used for immunoblotting are described in the Supporting Information.

AUF1 immunofluorescence in HeLa cells was analyzed as described in Supporting Information, using a Zeiss Observer D1 microscope with an AxioCam1Cc1 camera at a set exposure time.

Immunoprecipitation of ribonucleoprotein (RNP) complexes

HepG2 cells were starved for 18 h and then treated in serum-free media for 1 h with 500 μM metformin or PBS, and ribonucleoprotein (RNP) immunoprecipitation (RIP) assays were carried out as described previously (Yoon et al., 2014). To test AUF1 isoform binding to DICER1 mRNA, HeLa cells were transfected with individual plasmids encoding FLAG-tagged p37, p40, p42, or p45 AUF isoforms. RIP analysis is described in Supporting Information.

Acknowledgments

This study was supported by the Intramural Research Program of the National Institutes of Health, National Institute on Aging. The authors wish to thank Kób Abelhomsen for valuable discussions and critical reading of the manuscript, the HANDLS staff for care of participants and sample acquisition, and William H. Wood III, Yoonseo Kim, and Althaf Lohani for technical assistance.

Funding info

No funding information provided.

Author contributions

NNH and MKE designed the study. NNH performed the experiments. AM-M, MB, and RdC performed the metformin mouse study. MG provided reagents, PAR-CLIP, and advice. ABZ and MKE are coprincipal investigators of HANDLS. DFD isolated RNA and performed primary miRNA RT–qPCR and target prediction analysis. YZ and KGB performed microarray analysis. NNH wrote the paper with input from all authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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