A novel inverse relationship between metformin-triggered AMPK-SIRT1 signaling and p53 protein abundance in high glucose-exposed HepG2 cells

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IN RESPONSE TO A DECREASE in cellular energy state, activation of AMP-activated protein kinase (AMPK) and the NAD+−dependent histone/protein deacetylase sirtuin 1 (SIRT1) are metabolic sensors that can increase each other’s activity. They are also both activated by the antidiabetic drug metformin and downregulated in the liver under conditions of nutrient excess (e.g., hyperglycemia, high-fat diet, obesity). In these situations, the abundance of the tumor suppressor p53 is increased; however, the relevance of this to the changes in AMPK and SIRT1 is not known. In the present study we investigated this question in HepG2 cells under high glucose conditions. Metformin induced activation of AMPK and SIRT1 and decreased p53 protein abundance. It also decreased triglyceride accumulation and cytosolic oxidative stress (a trigger for p53 accumulation) and increased the deacetylation of p53 at a SIRT1-targeted site. The decrease in p53 abundance caused by metformin was abolished by inhibition of murine double minute 2 (MDM2), a ubiquitin ligase that mediates p53 degradation, as well as by overexpression of a dominant-negative AMPK or a shRNA-mediated knockdown of SIRT1. In addition, overexpression of p53 decreased SIRT1 gene expression and protein abundance, as well as AMPK activity in metformin-treated cells. It also diminished the triglyceride-lowering action of metformin, an effect that was rescued by incubation with the SIRT1 activator SRT2183. Collectively, these findings suggest the existence of a novel reciprocal interaction between AMPK/SIRT1 and p53 that may have implications for the pathogenesis and treatment of metabolic diseases.

The tumor suppressor p53 is a stress-responsive transcription factor that has been studied extensively with regard to its role in DNA-damage response pathways and apoptosis (2). More recently it has also been examined in the context of metabolic dysfunction and cellular senescence. In rodents fed a high-fat, high-sucrose diet (39) or with obesity (57), the protein abundance of p53 in the liver is elevated, whereas AMPK and SIRT1 are downregulated (10, 15, 18, 59). Whether a linkage exists between diminished hepatic AMPK and SIRT1 and increased p53 protein under conditions of energy excess has yet to be examined.

In this study we investigated the relationship between AMPK-SIRT1 signaling and p53 abundance.1 Human hepatoma (HepG2) cells were exposed to glucose concentrations that have been shown to inhibit AMPK and SIRT1, induce lipid accumulation, and cause insulin resistance (49, 60). It is important to note that although HepG2 cells exhibit chromosomal (29) and some oncogenic gene abnormalities (23, 24, 61), they also express wild-type p53 (3, 21, 23) and show many of the metabolic characteristics of primary hepatocytes (46, 60, 62). Our data demonstrate that, in high glucose-exposed HepG2 cells, activation of AMPK and SIRT1 by metformin, a widely used antidiabetic drug and activator of AMPK (62), results in decreased p53 protein abundance. They also reveal that overexpression of p53 decreases SIRT1 abundance and diminishes the ability of metformin both to activate AMPK and to decrease cellular triglycerides. Overall, these findings suggest the existence of a reciprocal relationship between hepatic AMPK-SIRT1 signaling and p53 protein under conditions of nutrient excess and in response to metformin.

MATERIALS AND METHODS

Materials. HepG2 cells were purchased from American Type Culture Collection (Manassas, VA). DMEM and penicillin-streptomycin (PS) were from Gibco (Grand Island, NY). FBS was from Thermo Scientific (Hyclone, Logan, UT). (−)−Glucose solution, 45%, and metformin (1,1-bimethylbiguanide hydrochloride) were purchased from Sigma-Aldrich (St. Louis, MO). (±)−Nutlin-3 was from Cayman Chemical (Ann Arbor, MI). SRT2183 was provided by Sirtris Pharmaceuticals (Cambridge, MA). Lys382 acetyl-p53, acetyl-CoA carboxylase (ACC), AMPK, and Thr172 phospho-AMPKα (40H9) antibodies as well as secondary horseradish peroxidase-linked antibodies were purchased from Cell Signaling Technology (Danvers, MA). Ser79 phospho-ACC, acetyl-histone H3, and histone H3 (CT, pan, clone A3C) antibodies were from Upstate/Millipore (Temecula, CA). SIRT1 (H-300) and p53 (DO-1) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody-β-actin was from Sigma-Aldrich.

1 This article is the topic of an Editorial Focus by Mary C. Sugden and Mark J. Holness (49a).
Cell culture and treatments. HepG2 cells were cultured in low-glucose (5.5 mM) DMEM supplemented with 10% FBS and 1% PS. Media were replaced every 24 h and cells were passaged upon reaching 80–90% confluency. Glucose-, pyruvate-, and FBS-free DMEM supplemented with 1% PS and glucose to a final concentration of 25 mM was used for all experimental incubations. Metformin was dissolved in H₂O. Nutlin-3 and SRT2183 were dissolved in DMSO.

Adenoviruses and cell infection. A replication-defective adenoviral vector expressing β-galactosidase (pCMV-β-gal) was used as a control (4). The recombinant adenoviral vector expressing a dominant negative mutant of AMPKα2 (DN-AMPK) was constructed from AMPKα2 bearing a mutation of lysine 45 to arginine (K45R) as described previously (40, 41, 63). Wild-type p53 was subcloned from a pCMV-p53 vector purchased from Clontech (Mountain View, CA), and the short hairpin RNA sequence used to knock down SIRT1 (shSIRT1) was generated as previously described (32). These two sequences were incorporated into an adenovirus vector as described by Cacicedo et al. (4). Cells were infected with approximately 50–200 plaque-forming units per cell for 24–48 h before the start of the experimental incubations.

Glucose assays. Residual glucose concentration in the media was measured using an enzymatic glucose assay kit (GAHK-20) from Sigma. The assay was adapted for use in a 96-well plate by loading 2 μl sample or standard and 200 μl reagent per well, incubating for 15 min at room temperature, and reading at 340 nm in a spectrophotometer. Glucose concentrations were calculated from the standard curve linear regression.

SDS-PAGE and Western blot analysis. Analyses were carried out as previously described (49, 60) with the following modifications: cells were washed once on ice with Dulbecco’s PBS + 10 mM nicotinamide, lysed in buffer containing 20 mM Tris-HCl pH 8.0, 1% IGEPAL, 1 mM EGTA, 10 mM nicotinamide, 1 μM trichostatin A, 10 mM sodium butyrate, 1 mM PMSF, 1× phosphatase inhibitor cocktail (Sigma), and 1× protease inhibitor cocktail containing 1 mM EDTA (Complete Mini, Roche, Basel, Switzerland). Nicotinamide was added to inhibit sirtuin deacetylase activity, and sodium butyrate and trichostatin A were added to inhibit other histone deacetylases (HDACs).

Detection of cytosolic oxidative stress. Cells were incubated for 24 h in DMEM containing 25 mM glucose ± the indicated treatments. They were loaded with 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate dye (Invitrogen, Carlsbad, CA) during the last 30 min of this incubation, and analyses of DCF fluorescence were carried out as previously described (17).

Measurement of cellular triglyceride content. Cellular triglyceride was determined in whole cell lysates (prepared as described above) using Infinity Triglycerides reagent (Thermo Fisher Scientific, Middletown, VA) as previously described (22, 60).

Real-time quantitative PCR. Cells were washed once on ice with Dulbecco’s PBS + 10 mM nicotinamide and immediately placed in TRIzol. RNA was extracted, 700 ng of total RNA were reverse transcribed using Moloney murine leukemia virus (MMLV; Invitrogen), and real-time RT-PCR was performed using SYBR Premix Extaq in a Cepheid Smart Cycler (Cepheid, Sunnyvale, CA). SIRT1 primers were as follows: forward, CACTGTGGTAGAGCTTGCAT, reverse, GGGGGGTGGGATATTGGTTC. Actin primers were as follows: forward, CAGGATCAAGGGGAGGTGAAG; reverse, CTTCGCTCCAGGATGCAGGG.

**Fig. 1.** AMP-activated protein kinase (AMPK) and sirtuin 1 (SIRT1) activation by metformin. HepG2 cells were incubated in 25 mM glucose DMEM for 24 h with or without 2 mM metformin followed by whole cell lysis and Western blot analysis. A and B: representative blot from experiments repeated at least 3 times showing activation of AMPK evidenced by increased phosphorylated acetyl-CoA carboxylase (p-ACC) (Ser79) and p-AMPK (Thr172) and decreased NH₂-terminal histone H3 acetylation (Ac-H3). C: densitometric analysis represented as fold change demonstrating increased p-ACC, increased p-AMPK, and reduced Ac-H3 in response to metformin, consistent with an increase in AMPK and SIRT1 activity. Results are means ± SE (n = 6); *P < 0.05.
and reverse, ACACCTCCCCAGTAGAAGT. Primers for the housekeeping gene RPS18 were as follows: forward, TCCAGCATATTTCGGAGTACT, and reverse, CCACATGAGCATATCTTCGG. Data are expressed relative to the housekeeping gene and were calculated using the ΔΔC_T method and are presented as fold change from control, within each time point.

Statistical analysis. Results are reported as means ± SE. Statistical significance was determined by a two-tailed unpaired Student’s t-test or ANOVA with Tukey’s post hoc test. A level of P < 0.05 was considered statistically significant.

RESULTS

AMPK and SIRT1 are activated by metformin in high glucose-exposed HepG2 cells. We first set out to confirm that metformin increases the activity of both AMPK and SIRT1 under conditions of nutrient excess. In initial studies, we measured the remaining glucose concentration in the media at 24 h. In cells incubated with a starting glucose concentration of 5.5 mM, glucose was completely depleted by 24 h. In contrast, at least 10 mM glucose remained at 24 h when the starting glucose concentration was 25 mM (data not shown). Under the high glucose conditions, the addition of 2 mM metformin increased the activities of AMPK, as assessed by phosphorylation of AMPK (Thr172) and its downstream target ACC (Ser79), and of SIRT1, as reflected by deacetylation of histone H3 (Fig. 1). Metformin increased AMPK activity (p-ACC and p-AMPK) under conditions of low glucose as well, but had no effect on SIRT1 activity, as evidenced by unchanged histone H3 acetylation (data not shown).
Metformin triggers a decrease in p53 protein abundance that is dependent on AMPK and SIRT1. We next determined the effect of metformin on p53 abundance. Western blot analysis showed a dose-dependent decrease in p53 protein in response to metformin under high glucose conditions (Fig. 2A). Overexpression of a DN-AMPK increased p53 abundance under basal conditions and prevented the metformin-induced decrease in p53 (Fig. 2, B and C), supporting a loss of SIRT1 activity in the absence of AMPK activation. Similarly, knockdown of SIRT1 by adenovirus-mediated expression of shRNA interference (shSIRT1) blunted the ability of metformin to decrease p53 protein abundance, although it did not significantly alter the acetylation status of p53 (Fig. 3, A and B). Importantly, knockdown of SIRT1 had no effect on metformin-induced AMPK activity (Fig. 3C), suggesting that metformin acts independently of SIRT1 to activate AMPK.

Metformin-induced decreases in p53 are associated with reduced oxidative stress, a decrease in its acetylation, and are attenuated by murine double minute 2 inhibition. Various cellular stressors including oxidative stress can trigger p53 accumulation (2). To determine whether a decrease in oxidative stress occurs in response to metformin treatment under high glucose conditions, we assessed the production of cytotoxic reactive oxygen species (ROS) using DCF fluorescence. Consistent with its observed effect on p53 abundance, metformin diminished cytotoxic ROS production under high glucose (Fig. 4A). To determine whether the ability of metformin to decrease ROS production under high glucose conditions was dependent on AMPK, cells were infected with adenovirus (Ad)-DN-AMPK prior to the DCF experiment. The knockdown of AMPK resulted in increased ROS production, which was partially blunted by metformin treatment (Fig. 4B).

In conjunction with the degree of cellular stress, the abundance of p53 is regulated by the rate of its degradation by the
To determine whether MDM2-mediated p53 degradation contributes to the observed effect of metformin, we incubated the cells with nutlin-3, a pharmacological inhibitor of MDM2 (50). As shown in Fig. 4C, the decrease in p53 abundance that occurs in response to metformin treatment was abolished in the presence of nutlin-3. Overexpression of p53 with nutlin-3 treatment had no significant effect on ROS production (Fig. 4D).
It has been reported that acetylation makes p53 more resistant to ubiquitination by MDM2 and increases its half-life in vivo (33). SIRT1 has been shown to deacetylate p53 at lysine 382 (36, 51), a known ubiquitination site (42, 47). By overexpressing wild-type p53 in the HepG2 cells, we were able to detect a decrease in lysine 382 acetylation of p53 in response to metformin treatment, consistent with an increase in SIRT1-mediated deacetylation (Fig. 4E). Overall, these results suggest that activation of AMPK and SIRT1 by metformin decreases p53 abundance by reducing oxidative stress, decreasing lysine 382 acetylation, and increasing MDM2-mediated degradation.

Overexpression of p53 decreases SIRT1 abundance and attenuates the effects of metformin on AMPK activation and cellular triglycerides. Overexpression of p53 decreased SIRT1 protein abundance and attenuated metformin-induced AMPK and ACC phosphorylation (Fig. 5A and B). In addition, treatment with nutlin-3, which increased the abundance of p53 (Fig. 4C), reduced the transcription of SIRT1 as evidenced by reduced SIRT gene expression at both 12 and 16 h (Fig. 5C). Since both AMPK and SIRT1 have lipid-lowering effects in vitro and in vivo (8, 12, 22, 35, 60, 62), we hypothesized that p53 overexpression would attenuate the lipid-lowering effect of metformin. As shown in Fig. 6, the ability of metformin to inhibit triglyceride accumulation was blunted in HepG2 cells in which p53 was overexpressed.

Metformin effects are partially restored by the SIRT1 activator SRT2183 in cells overexpressing p53. We next tested whether SIRT1 activation in cells overexpressing p53 would restore the effects of metformin on AMPK activity and triglyceride accumulation. Coincubation with the SIRT1 activator SRT2183 restored some, but not all, of the effects of metformin in these cells, as evidenced by their decreased triglyceride content and increased AMPK phosphorylation (Fig. 7). Although no effect on ACC phosphorylation was observed (Fig. 7A), these results support the notion that p53 overexpression attenuates the effects of metformin in part by decreasing SIRT1 activity.

**DISCUSSION**

In this study, we investigated the interactions between AMPK/SIRT1 signaling and p53 protein abundance in HepG2 cells

![Image](https://example.com/image.png)

Fig. 5. Overexpression of p53 decreases SIRT1 abundance and diminishes AMPK activation by metformin. Cells infected with adenovirus Ad-β-gal (control) or Ad-p53 for 24 h in 25 mM glucose, followed by 24 h incubation in 25 mM glucose with or without 2 mM metformin. A: representative Western blot demonstrating the inhibitory effects of Ad-p53 on SIRT1 abundance and phosphorylation of AMPK (Thr172) and ACC (Ser79) in response to metformin. B: densitometric analysis of SIRT1, β-actin, p-AMPK/AMPK, and p-ACC/ACC. C: RT-PCR analysis of SIRT1 gene expression in response to p53 overexpression with nutlin-3. Results are means ± SE (n = 3–10); *P < 0.05 and ***P < 0.001.
cells under conditions of nutrient excess. The results demonstrate that activation of AMPK and SIRT1 in response to metformin treatment decreases p53 abundance. They also reveal that metformin diminishes oxidative stress and enhances p53 deacetylation, likely contributing to the MDM2-mediated increase in p53 degradation. Conversely, we showed that overexpression of p53 diminishes SIRT1 abundance and prevents metformin-induced activation of AMPK and reductions in triglyceride content.

The findings that AMPK activation by metformin diminishes p53 abundance and oxidative stress are in agreement with those of Eid et al. (11), who reported that AMPK activation by AICAR (5-aminoimidazole-4-carboxamide-1-β-d-ribofuranoside) and by overexpression of wild-type AMPKα2 decreased p53 abundance in glomerular epithelial cells under high glucose conditions. They also showed that this effect was mediated by an AMPK-induced decrease in Nox4 expression that inhibited ROS production by NADPH oxidase. In addition, several other groups have reported an inhibitory effect of AMPK on NADPH oxidase activity (11, 45, 54). To our knowledge, this report and that of Eid et al. (11) are the only studies to date in which the effect of AMPK activation on p53 abundance was examined under high glucose conditions. In contrast, in response to extreme glucose deprivation, it has been reported that AMPK activation promotes cellular survival by phosphorylation and activation of p53 (27, 28).

We found that inhibition of the ubiquitin ligase MDM2 with nutlin-3 abolished the p53-lowering effect of metformin. Although many factors may contribute to the stability of the p53-MDM2 interaction, including posttranslational modifications of p53, some studies suggest that modifications of MDM2 may play a role (37, 55). In particular, it was recently reported that the mammalian target of rapamycin (mTOR)-ribosomal protein S6 kinase 70-kDa polypeptide 1 (S6K1) pathway (mTORC1) contributes to serine 163 phosphorylation of MDM2, resulting in inhibition of MDM2-mediated p53 ubiquitination and degradation (31). Although the effect of AMPK on mTOR/S6K1 was not examined in this study, it is known that AMPK phosphorylates and activates tuberous sclerosis 2 (TSC2), which in turn leads to inhibition of mTOR-S6K1 signaling (26). Thus it is plausible that AMPK activation could increase MDM2-mediated degradation of p53 by decreasing S6K1 activity, although this remains to be proven.

We also investigated whether metformin increased the deacetylation of p53, perhaps making it more susceptible to MDM2 degradation. In control cells, metformin caused a robust reduction in p53 abundance but had no measureable effect on p53 acetylation (Fig. 3). It is plausible that metformin caused the deacetylation of p53 in these cells, contributing to p53 degradation. Importantly, in cells overexpressing wild-type p53 and resistant to degradation of p53, metformin decreased lysine 382-acetylated p53 (Fig. 4E). This experimental model provides evidence for a role of metformin in decreasing p53. Furthermore, the striking reduction in p53 protein abundance by metformin was abolished by knockdown of SIRT1. These findings support the notion that SIRT1 activity is enhanced and contributes to metformin-induced decreases in p53 stability. However, we cannot entirely rule out effects of other HDACs, histone acetyltransferases (HATs), or other sirtuins.

The increase in ROS production caused by AMPK knockdown with Ad-DN-AMPK is in agreement with previous reports that AMPK activation can reduce ROS generation (16). In DN-AMPK cells, metformin was effective in reducing ROS, but not to the level of control cells; thus metformin may act independently of, as well as through AMPK to attenuate ROS production. Pharmacologic overexpression of p53 by nutlin-3 had no effect on ROS (Fig. 4D), indicating that, although oxidative stress increases p53 (2), p53 does not affect ROS generation. Nutlin-3 treatment had no effect on ROS production (Fig. 4D).

Numerous studies have shown that AMPK activation can lead to increased SIRT1 activity (5–7, 9, 14), with two of them demonstrating that metformin increases the NAD+/NADH ratio and SIRT1 abundance and activity (5, 7). Under control conditions, we did not observe an increase in SIRT1 protein; however, metformin did decrease histone H3 acetylation, consistent with an increase in SIRT1 activity. On the other hand, in cells incubated with Ad-DN-AMPK it no longer had this effect (Fig. 2, B and C). In contrast, metformin-induced increases in p-AMPK and p-ACC (indicators of AMPK activity) were unchanged when SIRT1 was knocked down (Fig. 3C). These findings suggest both that metformin can activate AMPK independently of SIRT1 and that it acts primarily through AMPK when it increases SIRT1 activity. These data corroborate the findings of Caton et al. (7), which demonstrated that compound C blunted the metformin-induced increase in SIRT1 activity. Although metformin appears to be acting through AMPK, it is also noteworthy that several reports have demonstrated that SIRT1 activation or overexpression can also activate AMPK (22, 32, 53).

The findings discussed thus far support the notion of an inhibitory effect of AMPK/SIRT1 activation on the abundance of p53 protein in HepG2 cells under high glucose conditions. We also investigated the effect of p53 overexpression and found that it decreased SIRT1 abundance and AMPK activity in metformin-treated cells. Since SIRT1 has been shown to regulate LKB1 (32), an upstream kinase for AMPK, it is likely that the observed decrease in SIRT1 protein caused by p53

![Graph](http://ajpcell.physiology.org/)

**Fig. 6.** Increasing p53 abundance attenuates the effect of metformin on cellular triglycerides. HepG2 cells infected with adenovirus Ad-β-gal (control) or Ad-p53 for 24 h in 25 mM glucose media, followed by 24 h incubation in 25 mM glucose with or without 2 mM metformin. Triglyceride levels from a representative experiment are shown. Results are means ± SE (n = 4–6); *P < 0.05 and ***P < 0.001.
could have contributed to the diminished AMPK activation. In further studies, we found an attenuation of the triglyceride-lowering effect of metformin in cells overexpressing p53 that was reversed by coincubation with the SIRT1 activator SRT2183. Acetylation status of p53 and histone H3 did not reflect SIRT1 activation; however, there is debate as to the effectiveness of this compound to directly activate SIRT1 (38, 44). There was, however, a modest but significant increase in AMPK phosphorylation with SRT2183 (Fig. 7B), possibly contributing to the reduction in triglyceride. These results suggest that p53 interferes with the triglyceride-lowering effect of metformin by decreasing AMPK activation and SIRT1 protein. Although not directly tested, the latter effect could have been mediated by p53-induced transcription of miR-34a, a microRNA that inhibits SIRT1 expression by binding to the 3’-untranslated region (UTR) of the SIRT1 transcript (58), or by direct p53-induced repression of the SIRT1 promoter that occurs under conditions of nutrient abundance (43).

Taken together, these results suggest the existence of a novel reciprocal relationship between p53 and the AMPK-SIRT1 signaling cycle. Increasing AMPK and SIRT1 activity under conditions of nutrient excess diminishes p53 abundance and cellular triglycerides, whereas increasing p53 dampens AMPK-SIRT1 signaling and blunts the triglyceride-lowering effect of metformin. In response to metformin, AMPK is the primary target, leading to increased activation of SIRT1. However, both molecules are necessary for the metformin-induced reduction in p53 protein abundance. The experiments presented here were limited to HepG2 cells; however, our novel data are consistent with a growing body of literature regarding AMPK, SIRT1, p53, and metabolic abnormalities. In accordance with this model, the phosphorylation of AMPK and the abundance of SIRT1 are diminished in the liver following high-fat feeding (1, 10, 18), whereas p53 protein abundance is increased (39). Hepatic p53 abundance is also elevated in two distinct models of hepatosteatosis, ob/ob mice and transgenic...
mice overexpressing sterol regulatory element-binding protein-1 (SREBP-1) (57). Furthermore, the transcription of miR-34a, which is regulated by p53 and results in decreased SIRT1 protein, is elevated in the livers of ob/ob and streptozotocin (STZ)-induced diabetic mice (34, 58).

On the basis of our findings, one could hypothesize that when hepatic p53 abundance is increased in conditions of obesity or fatty liver, it could play a role in diminished SIRT1 and AMPK activity, which would further propagate the diseased state. Thus the ability of metformin to lower p53 abundance could represent a novel additional therapeutic pathway that contributes to its beneficial metabolic effects. Whether metformin lowers p53 abundance in in vivo models of hepatosteatosis remains to be determined. Importantly, metformin has been shown to inhibit cellular proliferation and decrease cancer risk in diabetic patients (30); thus any decrease in p53 abundance that it produces in vivo would not likely increase the propensity towards tumorigenesis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

L.E.N., J.M.C., Y.I., and N.B.R. conception and design of the research; L.E.N., R.J.V., J.M.C., M.-S.G., Y.I., and N.B.R. edited and revised the manuscript. L.E.N., R.J.V., J.M.C., M.-S.G., Y.I., and N.B.R. approved the final version of the manuscript; R.J.V., J.M.C., L.E.N. drafted the manuscript; L.E.N., R.J.V., J.M.C., M.-S.G., Y.I. performed the experiments; L.E.N. and J.M.C. analyzed the data; L.E.N., R.J.V., J.M.C., Y.I., and N.B.R. interpreted the results of the experiments; L.E.N., R.J.V., and M.-S.G. prepared the figures; L.E.N. drafted the manuscript; L.E.N., R.J.V., J.M.C., M.-S.G., Y.I., and N.B.R. approved the final version of the manuscript; R.J.V., J.M.C., M.-S.G., Y.I., and N.B.R. edited and revised the manuscript.

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