Regulation of Hypoxia-Inducible Factor 2α Signaling by the Stress-Responsive Deacetylase Sirtuin 1

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To survive in hostile environments, organisms activate stress-responsive transcriptional regulators that coordinately increase production of protective factors. Hypoxia changes cellular metabolism and thus activates redox-sensitive as well as oxygen-dependent signal transducers. We demonstrate that Sirtuin 1 (Sirt1), a redox-sensing deacetylase, selectively stimulates activity of the transcription factor hypoxia-inducible factor 2 alpha (HIF-2α) during hypoxia. The effect of Sirt1 on HIF-2α required direct interaction of the proteins and intact deacetylase activity of Sirt1. Select lysine residues in HIF-2α that are acetylated during hypoxia confer repression of Sirt1 augmentation by small-molecule inhibitors. In cultured cells and mice, decreasing or increasing Sirt1 activity or levels affected expression of the HIF-2α target gene erythropoietin accordingly. Thus, Sirt1 promotes HIF-2 signaling during hypoxia and likely other environmental stresses.

Hypoxia-inducible factors (HIFs) are transcriptional regulators that control genes induced during hypoxia and other stresses (*1*). Activation of the founding HIF member, HIF-1α, is increased when oxygen concentrations are reduced. The second HIF alpha member, endothelial PAS domain protein 1 (EPAS1) (*2*), also known as HIF-2α, is closely related to HIF-1α in structure and is likewise activated during hypoxia (*3*). HIF-2α target genes identified from mouse knockout studies include *Sod2* encoding the mitochondrial-localized major antioxidant enzyme manganese superoxide dismutase (*4*), *VegfA* encoding the proangiogenic regulator vascular endothelial growth factor A (*5*), and *Epo* encoding the cytokine erythropoietin (*6*, *7*).

The activity of HIF members is in part regulated by oxygen concentrations. When oxygen levels are normal, HIF-1α and HIF-2α undergo posttranslational modifications by oxygen-dependent prolyl (*8*, *9*) and asparaginyl (*10*) hydroxylases that decrease HIF-α protein stability and activity, respectively. When oxygen levels are low, oxygen-dependent hydroxylases are inactive and HIF-α signaling increases. Although they are subject to similar oxygen-dependent modifications, HIF-1α and HIF-2α differ in other details of their regulation.

HIF-1α, but not HIF-2α, is transcriptionally regulated during hypoxia, and the activity of HIF-1α is proportional to its abundance (*3*, *11*, *12*). Amounts of HIF-2α protein increase modestly during hypoxia, but HIF-2α–dependent transactivation increases markedly, which suggests that additional posttranslational mechanisms besides oxygen-dependent hydroxylation regulate HIF-2α activity (*13*). Hypoxia alters cellular redox state in vivo (*14*, *15*). Redox-sensitive

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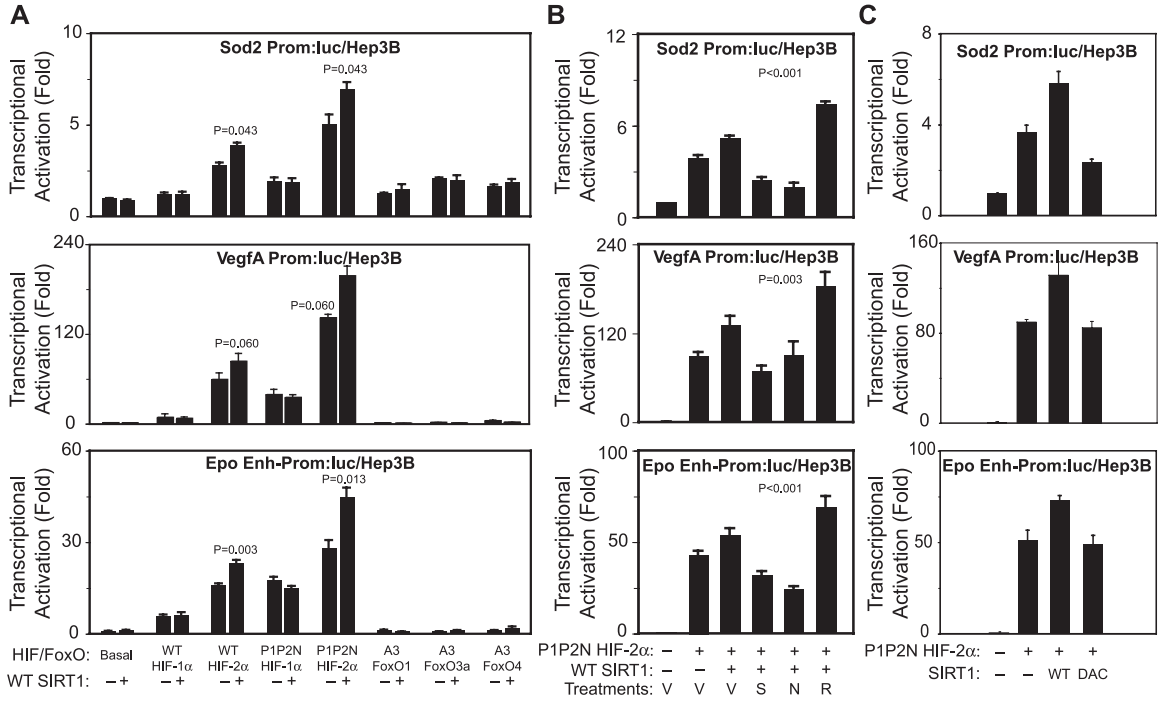

Fig. 1. Augmentation of HIF-2 signaling by Sirt1. (**A**) Activation of transcription from a mouse *Sod2* promoter (Sod2 Prom:luc), *VegfA* promoter (VegfA Prom:luc), or *Epo* enhancer-promoter (Epo Enh-Prom:luc) reporter by exogenous SIRT1 in human hepatoma Hep3B cells expressing WT or constitutively active (P1P2N) HIF-1α or HIF-2α, or constitutively active (A3) FoxO1, FoxO3a, or FoxO4. Statistical significance (Student's *t* test) of transcription factor plus SIRT1, relative to transcription factor alone, is indicated. (**B**) Effects of treatment with vehicle (V), the Sirt1 inhibitor sirtinol (S), the end-product inhibitor nicotinamide (N), or the activator resveratrol (R) on augmentation by exogenous SIRT1 of transcription from the Sod2 Prom:luc, VegfA Prom:luc, or Epo Enh-Prom:luc reporters in Hep3B cells expressing P1P2N HIF-2α. Statistical significance [analysis of variance (ANOVA)] of P1P2N HIF-2α plus SIRT1 with pharmacological treatment relative to P1P2N HIF-2α plus SIRT1 with vehicle is indicated. (**C**) Transcriptional effects of exogenous WT or deacetylase mutant (DAC) SIRT1 expressed with P1P2N HIF-2α on transcription from the Sod2 Prom:luc, VegfA Prom:luc, or Epo Enh-Prom:luc reporters in Hep3B cells. The bars in (A) to (C) represent the mean ± SEM of three independent transfections, with each transfection performed in triplicate.

modifications of HIF-α proteins are one potential mechanism whereby HIF-α activity could be controlled.

Silent information regulator 2 (Sir2) is a redox-sensitive, class III histone deacetylase (HDAC) initially identified from studies of aging in yeast (*16*). Homologs of the Sir2 protein in higher eukaryotic organisms are known as Sirtuin (Sirt) proteins, with Sirt1 being most closely related to Sir2 (*17*). In cultured mammalian cells, Sirt1 is activated in response to growth factor deprivation and increased oxidative stress. Changes in the cellular redox state as reflected by pyridine nucleotide homeostasis, specifically concentrations of oxidized nicotinamide adenine dinucleotide (NAD+) or the ratio of the concentration of NAD+ and its reduced form NADH, control the deacetylase activity of Sir2 and its homologs (*18*).

Sirt1 augments HIF-2 signaling. Because hypoxia affects the cellular redox state, we reasoned that Sirt1 could be activated during hypoxia and could participate in HIF signaling. We investigated whether HIF-responsive regulatory regions responded to Sirt1 overexpression. In human hepatoma Hep3B cells, overexpression of wild-type (WT) HIF-2α activated the isolated mouse *Sod2* promoter reporter, Sod2 Prom:luc, also activated by HIF-2α in human embryonic kidney (HEK) 293 cells (*4*). Overexpression of Sirt1 further augmented transcriptional activity of this reporter induced by WT HIF-2α (Fig. 1A). Sirt1 also augmented WT HIF-2α–activated transcription of isolated mouse *VegfA* and *Epo* regulatory regions (Fig. 1A). In contrast, Sirt1 did not augment transcription of Sod2 Prom:luc, Vegf Prom:luc, or Epo Enh-Prom:luc mediated by WT HIF-1α (Fig. 1A).

We examined whether activation of HIF-2α signaling by Sirt1 was independent of known oxygen-dependent modifications of HIF-α proteins. Alanine substitutions of the proline (P) and asparagine (N) residues that are hydroxylated under normal oxygen conditions result in mutant HIF-α proteins, referred to as P1P2N HIF-1α or P1P2N HIF-2α, that are equally active when oxygen conditions are normal (normoxia) as when they are reduced (hypoxia). Sirt1 augmented transcription of the Sod2 Prom:luc, Vegf Prom:luc, or Epo Enh-Prom:luc reporter by P1P2N HIF-2α, but not by P1P2N HIF-1α (Fig. 1A). Sirt1 augmentation of P1P2N HIF-2α signaling was similar or exceeded that of WT HIF-2α signaling for all three reporters.

Sirt1 deacetylation of FoxO transcription factors results in enhanced transcription of genes encoding prosurvival factors including *Sod2* (*19*), also a target for HIF-2α (*4*). FoxO members also participate in the hypoxia response (*20*). We determined whether Sirt1 augmentation of HIF-2α signaling involves signaling through FoxO proteins. In comparison with HIF-2α, constitutively active FoxO, expressed in the absence or presence of Sirt1, modestly activated transcription of Sod2 Prom:luc and had no effect on transcription of Vegf Prom:luc or Epo Enh-Prom:luc (Fig. 1A). The synthetic HIF-responsive reporter 3xHRE-tk Prom:luc, consisting of three HIF-responsive elements (HREs) upstream of a minimal thymidine kinase (tk) promoter, was activated to a comparable extent by P1P2N HIF-1α or P1P2N HIF-2α, but Sirt1 only augmented HIF-2α–activated transcrip-

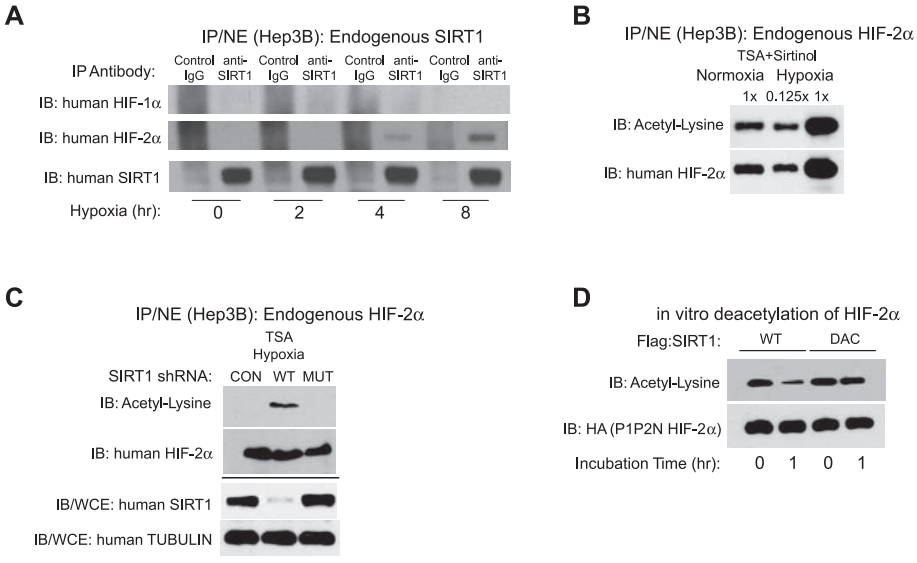

Fig. 2. Regulation of HIF-2α acetylation by Sirt1. (**A**) Association of endogenous SIRT1 and HIF-2α during hypoxia. SIRT1 was immunoprecipitated (IP) from nuclear extracts (NE) of Hep3B cells at the indicated time points. Association of HIF-1α or HIF-2α was detected by immunoblotting (IB). (**B**) Acetylation of endogenous HIF-2α during hypoxia. HIF-2α was immunoprecipitated (IP) from nuclear extracts (NE) prepared from Hep3B cells maintained under normal oxygen conditions (normoxia) or reduced oxygen conditions (hypoxia) and treated with trichostatin A (TSA) plus sirtinol. Equivalent amounts (1x) of the normoxia and hypoxia immunoprecipitated HIF-2α samples, as well as an adjusted amount (0.125x) of the hypoxia HIF-2α sample, were examined for acetylated and total HIF-2α by immunoblotting (IB). (**C**) Depletion of SIRT1 and acetylation of endogenous HIF-2α during hypoxia. Acetylation of endogenous HIF-2α was detected by immunoblotting (IB) nuclear extracts (NE) prepared with TSA from hypoxia-exposed Hep3B cells expressing control (CON), WT, or MUT Sirt1 shRNA. Depletion of SIRT1 was confirmed by immunoblotting of whole-cell extracts (WCE). (**D**) In vitro deacetylation of acetylated P1P2N HIF-2α by WT or DAC SIRT1. After 1 hour, amounts of acetylated and total P1P2N HIF-2α were assessed by IB.

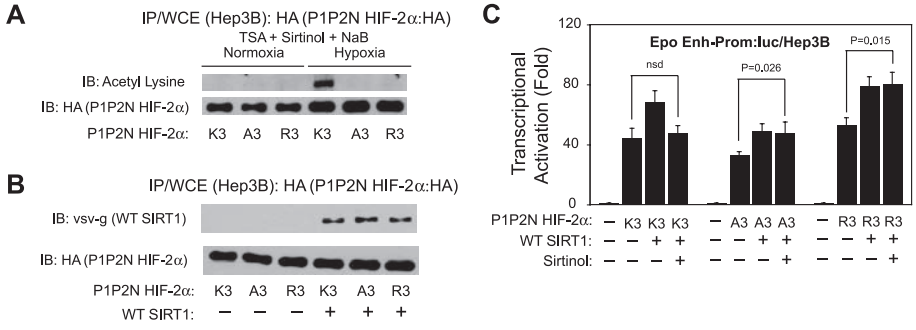

Fig. 3. Regulatory roles of HIF-2α carboxy terminal acetylated lysines. (**A**) Acetylation of P1P2N HIF-2α containing intact lysine (K3), alanine-substituted (A3), or arginine-substituted (R3) amino acids in the carboxy terminus expressed in Hep3B cells and maintained under normal oxygen conditions (normoxia) or under reduced oxygen conditions (hypoxia). P1P2N HIF-2α was immunoprecipitated (IP) from WCE prepared with TSA, sirtinol, and sodium butyrate (NaB). Acetylated or total HIF-2α in immunoprecipitated samples was assessed by IB. (**B**) Association of Sirt1 with HIF-2α lysine substitution mutants. P1P2N HIF-2α with intact carboxy terminal acetylated lysine residues (K3) or with alanine (A3) or arginine (R3) substitutions expressed in Hep3B cells and immunoprecipitated from WCE. Amounts of HIF-2α and associated exogenous SIRT1 were detected by IB. (**C**) Transcriptional activation of HIF-2α lysine substitution mutants by Sirt1. Transcription of the Epo Enh-Prom:luc reporter by exogenous SIRT1 in Hep3B cells in which parental (K3), alanine-substituted (A3), or arginine-substituted (R3) P1P2N HIF-2α was expressed in either the absence or presence of the Sirt1 inhibitor sirtinol. Statistical significance (Student's *t* test) of P1P2N HIF-2α plus SIRT1 in the presence of Sirtinol, relative to P1P2N HIF-2α alone, for each P1P2N HIF-2α form is indicated. The data represent the mean ± SEM of three independent transfections, with each transfection performed in triplicate.

tion (fig. S1A). The synthetic FoxO-responsive reporter 8xFBE-tk Prom:luc, containing eight FoxO binding elements (FBEs) upstream of the minimal tk promoter, responded only to constitutively nuclear FoxO members and not to HIF-α overexpression with or without Sirt1 (fig. S1A).

We examined whether Sirt1 augmentation of HIF-2 signaling was affected by pharmacological modulation of Sirt1 activity. Nicotinamide (NAM), a pyridine nucleotide end product generated after Sir2- or Sirt1-mediated deacetylation, inhibits Sir2 or Sirt1 action (*21*), as does the synthetic compound sirtinol (*22*), whereas the natural polyphenol resveratrol stimulates activity of Sir2 or Sirt1 (*23*). NAM or sirtinol reduced, and resveratrol increased, the ability of Sirt1 to augment HIF-2α transactivation of the *Sod2, VegfA,* and *Epo* reporters (Fig. 1B) (*24*). In comparison, NAM, sirtinol, and resveratrol had no effect on transcription of these reporters by HIF-1α (fig. S1B).

Nuclear-localized Sirt1 modulates the activity of coactivator proteins (*25*, *26*), represses select transcription factors (*27*–*29*), and activates other transcription factors (*19*, *30*–*34*) through its deacetylase activity. We examined whether genetic modulation of Sirt1 deacetylase activity affected augmentation of HIF-2 signaling by Sirt1. A site-directed point mutation that inhibits Sirt1 deacetylase activity (DAC) eliminated the Sirt1 stimulatory effect on expression of all three reporters (Fig. 1C).

HIF-2α acetylation during hypoxia is reversed by Sirt1. Augmentation of HIF-2α signaling by Sirt1 required an intact deacetylase function of Sirt1. We determined whether endogenous HIF-2α can bind to and act as an acetylated substrate for Sirt1 in Hep3B cells. Endogenous HIF-2α, but not HIF-1α, associated with Sirt1 during hypoxia (Fig. 2A). When isolated from hypoxia-exposed Hep3B cells in the presence of the HDAC I/II inhibitor trichostatin A (TSA) and the HDAC III (Sirt1) inhibitor sirtinol, HIF-2α was acetylated (Fig. 2B). After depletion of endogenous Sirt1, acetylated HIF-2α was detected in extracts from hypoxia-exposed Hep3B cells prepared in the absence of pharmacological inhibitors of Sirt1 (Fig. 2C). Acetylated HIF-2α was directly deacetylated by WT Sirt1 but not by DAC Sirt1, as assessed by in vitro deacetylation assays (Fig. 2D).

We next examined whether augmentation of HIF-2α signaling by Sirt1 was restricted to Hep3B cells. Amounts of P1P2N HIF-1α and P1P2N HIF-2α protein in Hep3B and HEK293 cells were similar and were not affected by Sirt1 overexpression (figs. S1C and S3C). Augmentation of HIF-2α signaling by Sirt1 for isolated reporters was observed in HEK293 cells and responded similarly to pharmacological modulation of Sirt1 activity (fig. S2 and S3) (*24*). Sirt1 associated with exogenous P1P2N HIF-2α, but not with exogenous P1P2N HIF-1α, in HEK293 cells maintained under normal atmospheric oxygen concentrations and occurred irrespective of whether class I/II (TSA) or class I/II+III (TSA + NAM) HDAC inhibitors were present (fig. S4A). Acetylation of exogenous P1P2N HIF-2α protein isolated from HEK293 cells exposed to hypoxia was not observed with effective concentrations of TSA (fig. S4B) but was observed when treated with TSA and sirtinol (fig. S4C). Acetylation and amounts of endogenous HIF-2α protein increased during hypoxia in HEK293 cells (fig. S4D). Exogenous as well as endogenous HIF-2α was acetylated during hypoxia when endogenous Sirt1 in HEK293 cells was depleted in the absence of Sirt1 inhibitors (fig. S4, E and F).

Sirt1 augmentation localizes to the HIF-2α carboxy terminus. We tested whether a specific region of HIF-2α binds to and confers augmentation by Sirt1. We expressed truncated forms of P1P2N HIF-2α in HEK293 cells along with Sirt1 and analyzed immunoprecipitated proteins. The carboxy terminus (C terminus) of HIF-2α, encompassing amino acids 350 to 870, was sufficient to form a stable complex with Sirt1 (fig. S5, A and B). In vitro stable and direct binding of WT Sirt1, but not DAC Sirt1, to the HIF-2α C terminus was detected using bacterial-produced HIF-2α (fig. S5C). Sirt1 augmentation of HIF-2α transcription from reporters occurred in both Hep3B and HEK293 cells, with hybrid HIF proteins containing the C terminus of HIF-2α (figs. S6A and S7A). Activation of a mammalian two-hybrid reporter occurred when WT Sirt1 and the C terminus of HIF-2α were coexpressed as hybrid constructs in either Hep3B or HEK293 cells (figs. S6B and S7B).

We asked whether specific acetylated lysine residues in the C terminus of HIF-2α could play a regulatory role in HIF-2 signaling. Mass spectrometry revealed acetylation of exogenous P1P2N HIF-2α during hypoxia at three lysine residues (K385, K685, and K741) within the HIF-2α C terminus (fig. S8, A to C). Substitution mutants of the three acetylated lysines in the C terminus of HIF-2α, with alanine (A3) or arginine (R3) residues, were not acetylated during hypoxia (Fig. 3A and fig. S9A) but retained interaction with Sirt1 (Fig. 3B and fig. S9B).

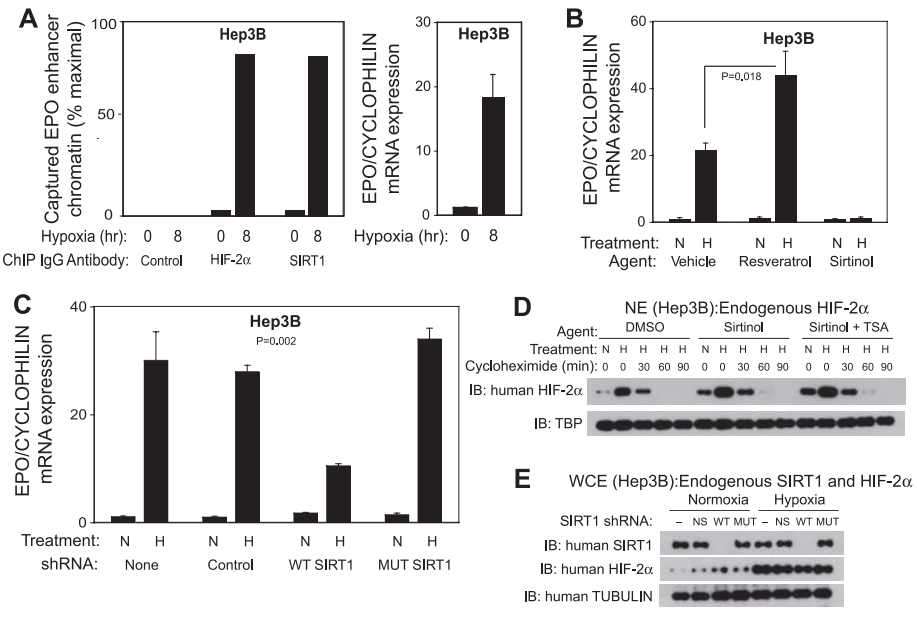

Fig. 4. Modulation of *Epo* gene expression by Sirt1 in cell culture. (**A**) Association of endogenous SIRT1 and HIF-2α to the *EPO* enhancer during hypoxia. Using extracts prepared from Hep3B cells after hypoxia exposure, chromatin immunoprecipitation (ChIP) assays were done with antibodies to SIRT1, HIF-1α, or HIF-2α and with primers encompassing the human *EPO* enhancer. *EPO* mRNA expression was assessed by real-time reverse transcription polymerase chain reaction (rtRT-PCR) for parallel samples and was normalized to expression of the housekeeping gene *cyclophilin B* (CYCLOPHILIN). (**B**) Pharmacological manipulations of Sirt1 activity and *EPO* expression during hypoxia. After vehicle, resveratrol, or sirtinol treatment, *EPO* expression was measured by rtRT-PCR in Hep3B cells maintained under normal (normoxia, N) or reduced (hypoxia, H) oxygen conditions. Statistical significance (Student's *t* test) is indicated. The data represent the mean ± SEM of triplicates for each treatment. (**C**) SIRT1 depletion and *EPO* expression during hypoxia. *EPO* gene expression was measured by rtRT-PCR in Hep3B cells expressing control, WT SIRT1 shRNA, or MUT SIRT1 shRNA and maintained under normoxia (N) or hypoxia (H) conditions. Statistical significance (ANOVA) for hypoxia samples is indicated. The data represent the mean ± SEM of triplicates for each transfection. (**D**) Pharmacological inhibition of Sirt1 activity and endogenous HIF-2α stability. Nuclear extracts (NE) from hypoxia-exposed Hep3B cells treated with dimethyl sulfoxide, sirtinol, or sirtinol plus TSA followed by cycloheximide-mediated protein inhibition were prepared and immunoblotted (IB) with antibodies recognizing human HIF-2α or the normalization control TATA-binding protein (TBP). (**E**) Sirt1 depletion and endogenous HIF-2α amounts. WCE of normoxia- or hypoxia-exposed Hep3B cells treated with control, WT SIRT1 shRNA, or MUT SIRT1 shRNA were immunoblotted (IB) with antibodies recognizing human SIRT1, HIF-2α, or the normalization control α-tubulin (TUBULIN).

Similar to findings observed with lysine substitution mutants of PPAR gamma coactivator 1α (PGC-1α), also a target for Sirt1-mediated deacetylation and augmentation (*35*), transcriptional activities of the HIF-2α lysine substitution mutants were augmented by Sirt1 but were not repressed by a small molecule inhibitor of Sirt1 (Fig. 3C and fig. S9C).

Sirt1 regulates Epo expression in cell culture. We next assessed whether Sirt1 participated in regulation of endogenous HIF-2α target genes in cells. Chromatin immunoprecipitation experiments revealed increased recruitment of Sirt1 and HIF-2α to the *Epo* enhancer region in Hep3B cells during hypoxia (Fig. 4A). Pharmacological manipulations of Sirt1 activity resulted in increased abundance of *Epo* mRNA with agents that stimulate Sirt1 deacetylase activity and decreased *Epo* mRNA concentrations with agents that inhibit Sirt1 deacetylase activity (Fig. 4B). Depletion of Sirt1 resulted in reduced induction of *Epo* mRNA (Fig. 4C). Neither pharmacological manipulations nor genetic inhibition of Sirt1 affected amounts of endogenous HIF-2α protein (Fig. 4, D and E).

Sirt1 contributes to Epo regulation in mice. If inhibiting Sirt1 deacetylase activity blunts HIF-2α signaling in cells, then reduced Sirt1 gene dosage should affect in vivo HIF-2α signaling. We determined whether Sirt1 signaling is physiologically relevant to regulation of the HIF-2α selective target gene *Epo*. During mouse development, the liver is a major source of *Epo* at the mid- to late-gestational stages. At embryonic day 14.5 (E14.5), when HIF-2α deficiency results in lower *Epo* mRNA concentrations in the fetal liver, $Sirt1^{-/-}$ embryos had significantly lower amounts of *Epo* mRNA compared with $Sirt1^{+/+}$ mice (Fig. 5A). In contrast, at E12.5, when HIF-2α deficiency has no effect on *Epo* mRNA concentrations in the fetal liver, $Sirt1^{-/-}$ embryos had similar amounts of *Epo* mRNA as $Sirt1^{+/+}$ mice (fig. S10).

$Sirt1^{-/-}$ mice exhibit substantial prenatal and perinatal lethality as well as marked postnatal pathology (*36*). $Sirt1^{+/-}$ mice, in comparison, lack gross abnormalities or gestational lethality. To assess whether partial Sirt1 deficiency affects in vivo HIF-2α signaling, we tested whether renal *Epo* gene expression differed between $Sirt1^{+/-}$ and $Sirt1^{+/+}$ mice exposed to hypoxia (6% oxygen). $Sirt1^{+/-}$ mice had blunted induction of renal *Epo* mRNA relative to $Sirt1^{+/+}$ mice after hypoxia exposure (Fig. 5B). Both groups of mice had similar amounts of renal *Epo* mRNA under ambient oxygen conditions (21% oxygen, room air).

Congenital Sirt1 deficiency affects fetal and adult *Epo* gene expression in mice. We asked whether acute alterations in Sirt1 could modulate *Epo* gene expression in adult mice. Adenoviruses encoding P1P2N HIF-1α, P1P2N HIF-2α, or WT Sirt1 were injected into tail veins of adult mice; hepatic *Epo* mRNA levels and hematocrits were measured 1 week later. Sirt1 alone had no significant effect on hepatic *Epo* mRNA levels and hematocrits (fig. S11). Ectopic P1P2N HIF-2α, but not P1P2N HIF-1α, expression markedly increased hepatic *Epo* mRNA levels as well as hematocrits; these physiological parameters were further augmented when WT Sirt1 was coexpressed with P1P2N HIF-2α (Fig. 5C).

We determined whether Sirt1 deacetylase activity was required to augment *Epo* gene expression by ectopic P1P2N HIF-2α in adult mice. Adenoviruses encoding P1P2N HIF-1α, P1P2N HIF-2α, WT Sirt1, or DAC Sirt1 were injected into adult mice; hepatic *Epo* mRNA levels and hematocrits were measured 1 week later. Ectopic P1P2N HIF-2α, but not P1P2N HIF-1α, markedly increased hepatic *Epo* mRNA levels (Fig. 5C) and spleen weights, the latter indicative of a dramatic increase in circulating red blood cell mass (fig. S12A). Combined overexpression of P1P2N HIF-2α and WT, but not DAC, Sirt1 further augmented *Epo* mRNA levels (Fig. 5C) and hematocrits (fig. S12B).

Although overexpression of Sirt1 alone does not affect *Epo* gene expression in the liver, endogenous Sirt1 could contribute to HIF-2 signaling in the liver when HIF-2 signaling is active. We examined whether an acute reduction in Sirt1 levels affected hepatic *Epo* gene expression induced by ectopic P1P2N HIF-2α expression. Adenovirus encoding short hairpin RNA (shRNA) against Sirt1 were injected into mice to deplete Sirt1 in livers. The rise in hepatic *Epo* mRNA concentrations (Fig. 5D) and hematocrits (fig. S13) induced by ectopic P1P2N HIF-2α were blunted in mice expressing WT, but not mutant (MUT), Sirt1 shRNA.

Discussion. We reasoned that during hypoxic stress, redox changes would activate Sirt1 and that activated Sirt1 would regulate HIF signaling. Our molecular and biochemical findings support this hypothesis. Furthermore, our cell culture and animal data extend the biological action for Sirt1, beyond its previously defined roles in aging and caloric restriction, to a functional role in hypoxic signaling. Specifically, we demonstrate that Sirt1 augments HIF-2α signaling and consequently participates in regulation of the HIF-2α target gene *Epo*. Erythropoietin, generally considered an erythrogenic growth factor, is also a potent prosurvival factor that protects developing stem cell and progenitor cells in a variety of organs (*37*). Activation of Sirt1 may facilitate HIF-2α–directed production of erythropoietin in cellular niches characterized by hypoxic and possibly other environmental stresses.

In developing mice, hepatic *Epo* gene expression is normally induced by physiological hypoxia induced by growth. Sirt1 deficiency affects embryonic hepatic *Epo* gene expression at the same developmental time point when HIF-2α deficiency exerts its consequence. In adult mice, hypoxia-induced increases in renal *Epo* gene expression are blunted in Sirt1 haploinsufficient mice, similar to that observed with adult HIF-2α haploinsufficient mice (*7*). As further evidence of Sirt1/HIF-2α signaling regulating *Epo* gene expression in vivo, acute Sirt1 knockdown in adult liver

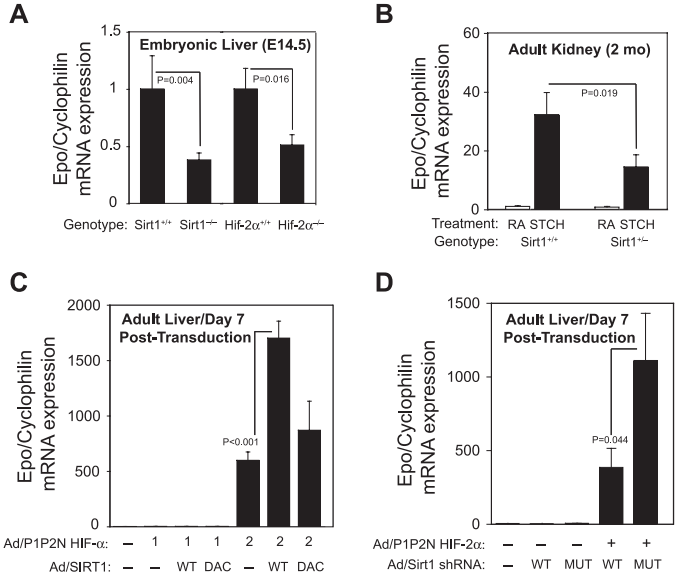

Fig. 5. Modulation of *Epo* gene expression by Sirt1 in animals. (**A**) Hepatic *Epo* expression at E14.5 during development in Sirt1- or HIF-2α–deficient mice. Amounts of *Epo* mRNA expressed in livers of $Sirt1^{+/+}$, $Sirt1^{-/-}$, $HIF\text{-}2\alpha^{+/+}$, and $HIF\text{-}2\alpha^{-/-}$ embryos. Statistical significance (Student's *t* test) is indicated. The bars represent the mean ± SD of five embryos of each genotype. (**B**) Renal *Epo* expression during hypoxia in animals with Sirt1 haploinsufficiency. Amounts of *Epo* mRNA expressed in kidneys of 2-month-old $Sirt1^{+/+}$ or $Sirt1^{+/-}$ mice exposed to short-term continuous hypoxia (STCH). Statistical significance (Z-test) is indicated. The bars represent the mean ± SD of six ($Sirt1^{+/+}$) or eight ($Sirt1^{+/-}$) mice. (**C**) Hepatic *Epo* expression in animals expressing ectopic Sirt1 and HIF-2α. Amounts of *Epo* mRNA expressed in liver at day 7 after injection in adult mice of adenovirus encoding mock, P1P2N HIF-1α, P1P2N HIF-2α, WT Sirt1, or DAC Sirt1, as indicated. Statistical significance (Student's *t* test) is indicated. The bars represent the mean ± SD of five mice per treatment group. (**D**) Hepatic *Epo* expression in animals depleted in Sirt1 and expressing ectopic HIF-2α. Amounts of *Epo* mRNA expressed in liver at day 7 after injection in adult mice of adenovirus encoding mock, Sirt1 WT shRNA, Sirt1 MUT shRNA, or P1P2N HIF-2α, as indicated. Statistical significance (Student's *t* test) is indicated. The bars represent the mean ± SD of five mice per treatment group. For (A) to (D), amounts of *Epo* mRNA were measured by rtRT-PCR and were normalized to amounts of *cyclophilin B* (cyclophilin) mRNA, a housekeeping gene whose expression levels are similar between the different groups.

blunts ectopic HIF-2α–induced increases in hepatic *Epo* gene expression, whereas concomitant Sirt1 and HIF-2α overexpression further augments hepatic *Epo* gene expression in normoxic mice compared with ectopic HIF-2α overexpression alone.

Although Sirt1 overexpression augments HIF-2α–induced *Epo* expression, increased Sirt1 activity alone is not sufficient to induce hepatic *Epo* gene expression in mice. Interestingly, HIF signaling and Sirt1 activity in the liver are inversely regulated during caloric restriction. Whereas caloric restriction of aged rats results in reduced HIF-1 signaling and blunted expression of HIF target genes in the liver, including *Epo* (*38*), caloric restriction is associated with increased hepatic Sirt1 activity (*39*), which indicates that Sirt1 or HIF-2α signaling pathways can be controlled independent of each other in a stress-dependent manner. In addition to repressing HIF-1α signaling, caloric restriction may directly repress HIF signaling induced by HIF-2α. Alternatively, caloric restriction may induce expression of a repressor of *Epo* gene expression that suppresses Sirt1/HIF-2α signaling in a dominant fashion.

Our data integrate Sirt1-HIF-2α signaling with other stress-responsive, prosurvival signal transduction pathways that are modulated by Sirt1 in mammals. HIF-2α is only present in vertebrates and regulates expression of prosurvival factors under hypoxia and other adverse environmental conditions (*2*). HIF-2 signaling, regulated in part by hypoxia-induced acetylation, and Sirt1 augmentation of HIF-2 signaling, conferred through Sirt1/HIF-2α complex formation as well as by Sirt1-mediated deacetylation of acetylated HIF-2α, likely have a specialized role in higher metazoans. Signaling from Sirt1 to HIF-2α could be induced by other environmental stresses besides hypoxia that alter pyridine nucleotide homeostasis and activate HIF-2α signaling. Identifying the relevant environmental stressors that induce Sirt1/HIF-2α signaling and defining the role of Sirt1/HIF-2α signaling in the regulation of protective cellular mechanisms in mammals may provide novel therapeutic opportunities for human disease states.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/324/5932/1289/DC1
Materials and Methods
Figs. S1 to S13
References

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Did Warfare Among Ancestral Hunter-Gatherers Affect the Evolution of Human Social Behaviors?

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Since Darwin, intergroup hostilities have figured prominently in explanations of the evolution of human social behavior. Yet whether ancestral humans were largely "peaceful" or "warlike" remains controversial. I ask a more precise question: If more cooperative groups were more likely to prevail in conflicts with other groups, was the level of intergroup violence sufficient to influence the evolution of human social behavior? Using a model of the evolutionary impact of between-group competition and a new data set that combines archaeological evidence on causes of death during the Late Pleistocene and early Holocene with ethnographic and historical reports on hunter-gatherer populations, I find that the estimated level of mortality in intergroup conflicts would have had substantial effects, allowing the proliferation of group-beneficial behaviors that were quite costly to the individual altruist.

Intergroup hostilities figure prominently in a number of explanations of the evolution of human social behavior, starting with Darwin (*1*). The underlying mechanism is that (as Darwin put it) groups with "a greater number of courageous, sympathetic and faithful members, who were always ready to warn each other of danger, to aid and defend each other…would spread and be victorious over other tribes" [(*1*), p. 156]. An implication is that if intergroup conflict is frequent and lethal, then more altruistic group-beneficial behaviors—those entailing greater costs to the individual altruist—will be able to proliferate.

Notwithstanding a number of insightful recent studies (*2*–*4*), however, lethal intergroup conflict among hunter-gatherers during the Late Pleistocene and early Holocene remains a controversial subject, with little agreement on either its extent or consequences (*5*, *6*). Among the empirical challenges are the lack of written accounts, the difficulty in making inferences from hunter-gatherers in the ethnographic record about conditions before the domestication of plants and animals and the emergence of states, and the fact that most foragers made little use of fortifications and killed each other with the same weapons that they used to hunt other animals, thus leaving few distinctive archaeological traces other than skeletal remains.

In light of the available archaeological and ethnographic evidence, could war among ances-

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Regulation of Hypoxia-Inducible Factor 2α Signaling by the Stress-Responsive Deacetylase Sirtuin 1

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