Spatiotemporal gating of SIRT1 functions by O-GlcNAcylation is essential for liver metabolic switching and prevents hyperglycemia

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Inefficient physiological transitions are known to cause metabolic disorders. Therefore, investigating mechanisms that constitute molecular switches in a central metabolic organ like the liver becomes crucial. Specifically, upstream mechanisms that control temporal engagement of transcription factors, which are essential to mediate physiological fed–fast–refed transitions are less understood. SIRT1, a NAD^+-dependent deacetylase, is pivotal in regulating hepatic gene expression and has emerged as a key therapeutic target. Despite this, if/how nutrient inputs regulate SIRT1 interactions, stability, and therefore downstream functions are still unknown. Here, we establish nutrient-dependent O-GlcNAcylation of SIRT1, within its N-terminal domain, as a crucial determinant of hepatic functions. Our findings demonstrate that during a fasted-to-refed transition, glycosylation of SIRT1 modulates its interactions with various transcription factors and a nodal cytosolic kinase involved in insulin signaling. Moreover, sustained glycosylation in the fed state causes nuclear exclusion and cytosolic ubiquitin-mediated degradation of SIRT1. This mechanism exerts spatiotemporal control over SIRT1 functions by constituting a previously unknown molecular relay. Of note, loss of SIRT1 glycosylation discomposed these interactions resulting in aberrant gene expression, mitochondrial dysfunctions, and enhanced hepatic gluconeogenesis. Expression of nonglycosylatable SIRT1 in the liver abrogated metabolic flexibility, resulting in systemic insulin resistance, hyperglycemia, and hepatic inflammation, highlighting the physiological costs associated with its overactivation. Conversely, our study also reveals that hyperglycosylation of SIRT1 is associated with aging and high-fat-induced obesity. Thus, we establish that nutrient-dependent glycosylation of SIRT1 is essential to gate its functions and maintain physiological fitness.

fed–fast cycle | gluconeogenesis | PGC1α | insulin signaling | ubiquitinylation

Metabolic states, such as fed/fasted, are a continuum and an inability to mediate physiological transitions is often associated with diseases and aging (1–6). Therefore, it is critical to identify processes that establish a relay of molecular interactions during such physiological transitions. Decades of work have revealed that mechanisms governing gene expression during fed and fasted states are necessary to regulate physiological anabolic and catabolic processes (4, 7–9). While protein lysine acetyltransferases (KATs) and lysine deacetylases (KDACs) regulate most transcription factors (TFs), including those involved in metabolic homeostasis (10), mechanisms that define context-specific interactions between them are poorly understood. For example, the temporal resolution of TFs engaging with KATs/KDACs, during fed–fast–refed cycles is still unclear. Moreover, given that futile and aberrant expression of the fed–fasted genes is detrimental, investigating mechanisms that constrain functions of master transcriptional regulators becomes fundamentally relevant and important. Therefore, in addition to revealing protein–protein interaction dynamics that tune transcription bidirectionally, such efforts would greatly enable our understanding of age-associated decline in physiological fitness and metabolic diseases.

Abnormal activation or inhibition of hepatic fed- or fasting-responsive genes are known to cause metabolic diseases (5–7, 11). Fasting-induced NAD^+-dependent deacetylase SIRT1 plays a central role in regulating hepatic functions and is also essential for bringing about metabolic homeostasis (1, 2, 7, 12–17). Hepatic SIRT1 exerts a tight control over transcription of genes and maintains organism-wide physiology by its ability to interact with, and deacetylate, several transcription factors and coactivators (18–20). While SIRT1-dependent deacetylation and activation of factors, such as PGC1α, PPARα, and FOXO1 is required for fatty acid oxidation and gluconeogenesis (19, 21), inhibition of HIF1α and SREBP1 is known to repress expression of fed-responsive genes (22, 23). While optimal SIRT1 function is necessary to counter age-related diseases, its sustained overactivation is often detrimental (24–26). Furthermore, pharmacological approaches to activate SIRT1 functions have gained significant traction and clinical attention (27, 28). Hence, it is even more necessary now to investigate mechanisms that control SIRT1 turnover and homeostasis under normal and diseased states.

Significance

Emerging literature indicates that, besides circadian inputs, fed–fast cycles are vital for maintaining physiological homeostasis. While several studies have highlighted the importance of feeding and state-specific molecular signatures, information regarding mechanisms that bring about efficient fed–fast–refed transitions is sparse. Here we show how a glucose-derived modification (glycosylation) of SIRT1, a master regulator of transcription and insulin signaling, regulates this metabolic switch in the liver by restricting SIRT1 functions during fasted-to-refed transition. SIRT1 glycosylation orchestrates molecular interactions to suppress fasting genes, derepress fed genes, and activate insulin signaling upon refeeding. We show that aberrant SIRT1 glycosylation causes hepatic dysfunctions resulting in a diabetes-like state and is also associated with aging and obesity.

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In addition to regulation of transcription, metabolic switching to a refed state is intrinsically dependent upon insulin signaling (IS). It should be noted that both precocious or abnormal activation, and an inability to induce IS following a refed transition, lead to hypo- or hyperglycemia and metabolic dysfunction (29). Interestingly, SIRT1 is also necessary to activate IS and its loss-of-function is associated with insulin resistance (30, 31). Specifically, SIRT1 deacetylates and activates AKT, a cytosolic nodal kinase in IS (32). Therefore, given that SIRT1 is largely nuclear, temporal regulation of SIRT1–AKT interactions during such state transitions are yet to be unraveled. IS is also known to inhibit the expression of fasting genes and activate transcription downstream to SREBP1 and HIF1α, which are necessary for anabolism (29). Paradoxically, even though SIRT1 activates IS, it counters the induction of lipogenic and glycolytic genes by inhibiting the aforementioned factors (18, 22, 33). Taken together, these data posit upstream mechanisms that enable metabolic changeover during fasted–refed transitions by exerting an as yet unknown spatiotemporal control over SIRT1 interactions and function.

While SIRT1 is known to interact with several nuclear and cytosolic factors (19, 20, 30), it is only recently that mechanisms, which determine specificity of interactions, have begun to be understood. In this regard, we and others have recently highlighted the importance of the N-terminal domain of SIRT1 (34–37). Specifically, we have shown that a domain encoded by exon-2 in SIRT1, which forms an intrinsically disordered region (IDR), acts to tether substrate proteins and is specifically required for interaction with PGC1α, PPARα, and FOXO1 (34). Given that modifications on IDRs are known to determine protein–protein interactions (38), it is enticing to hypothesize SIRT1-IDR modification as a means to regulate interactions, especially during metabolic transitions that we refer to here.

Dynamic and reversible O-GlcNAcylation (glycosylation) on protein serine and threonine residues has also emerged as a major regulator of nuclear and cytosolic proteins involved in diverse cellular functions (39–41). The donor metabolite for glycosylation, UDP-GlcNAc, is derived primarily from the hexosamine biosynthetic pathway, with contributions from various anabolic inputs, and is therefore considered to act as a signal of a nutrient-rich state (42). O-GlcNAc-transferase (OGT) mediates glycosylation across cellular compartments, and O-GlcNAcase (OGA) catalyzes de-glycosylation (43, 44). Furthermore, OGT-dependent glycosylation is enhanced following insulin stimulation and glucose uptake (45). In the context of fed–fast cycles, factors such as FOXO and PGC1α have been shown to be glycosylated by OGT (41, 44). However, given that these are also modulated by metabolic status-dependent de-/acetylation (12, 46), if and how such diverse signals impinge on downstream transcription and thus contribute to physiological homeostasis is currently unknown.

In the present study, we show that SIRT1 is posttranslationally modified under nutrient-replete conditions via O-GlcNAcylation and we propose that aberrant SIRT1 glycosylation could potentially be a major factor contributing to metabolic diseases and aging. Interestingly, upon refeeding nutrient-dependent glycosylation occurs within a domain of SIRT1 that encodes substrate specificity, and thus switches its interaction from transcriptional factors/coregulators to a nodal kinase in insulin signaling. Molecular and physiological assays clearly establish that glycosylation of SIRT1 is critical to spatiotemporally resolve its nuclear and cytosolic functions, which is required for efficient fed–fast–refed transitions. Furthermore, glycosylation also causes SIRT1 degradation via ubiquitin-proteasomal pathway and limits its function in a fed state. Besides identifying a molecular mechanism that constrains SIRT1 functions, we also establish that active glycosylation and de-glycosylation cycles in SIRT1 are essential for liver functions and maintenance of metabolic homeostasis.

Results

Nutrient-Dependent Glycosylation of SIRT1. Although SIRT1 levels are known to decrease during a fed state (46, 47), nutrient inputs regulating SIRT1 homeostasis are still unknown. As shown in Fig. 1 *A* and *B* and *SI Appendix*, Fig. S1 *A* and *B*, and as previously reported (11, 46), total SIRT1 levels decreased following a normal physiological transition to a fed state, which was both glucose- and insulin-dependent. We hypothesized that a nutrient-derived post-translational modification (PTM), sensitive to cellular glucose levels, could potentially affect SIRT1 protein levels under fed conditions.

Since O-GlcNAcylation or glycosylation has been proposed to link nutrient-rich states to molecular functions of a diverse set of intracellular proteins, we investigated if altered SIRT1 levels during fed–fast cycles could be mediated by glycosylation. Interestingly, we found that SIRT1 was glycosylated in response to both high-glucose (HG) conditions (Fig. 1*C*) and insulin treatment (Fig. 1*D*). We confirmed SIRT1 glycosylation by reciprocal pull-downs, namely by assaying for glycosylation on immunoprecipitated SIRT1 (Fig. 1 *C* and *D*) and by checking for SIRT1 enrichment on succinylated wheat germ agglutinin (sWGA)-beads that enrich most O-GlcNAcylated proteins (*SI Appendix*, Fig. S1*C*). Importantly, insulin-induced glycosylation of SIRT1 was starkly reduced when cells were simultaneously treated with 2-deoxy-glucose (2-DG), a competitive inhibitor of glycolysis (*SI Appendix*, Fig. S1*D*), suggesting that glucose metabolism is essential for SIRT1 glycosylation under fed conditions.

OGT-mediated addition of O-linked β-*N*-acetylglucosamine (GlcNAcylation) is dependent upon intracellular UDP-GlcNAc concentrations (44). Interestingly, estimating UDP-GlcNAc levels in primary hepatocytes and HEK293 cells, demonstrated a significant increase under fed conditions (Fig. 1*E*). Enhanced SIRT1 glycosylation and increased UDP-GlcNAc levels under a glucose-replete condition prompted us to probe for OGT–SIRT1 interactions under altered metabolic states. We found that interaction of SIRT1 with OGT was higher under fed conditions (Fig. 1*F* and *SI Appendix*, Fig. S1*E*) and substantially decreased upon 2-DG treatment (*SI Appendix*, Fig. S1*F*). These results suggested that a fed response likely led to an OGT-mediated glucose-dependent enhanced glycosylation of SIRT1. Inhibiting the de-glycosylase enzyme, OGA (using PUGNAc) or OGT (using BZX) led to a significant increase and decrease in SIRT1 glycosylation, respectively, indicating this to be an active process (Fig. 1 *G* and *H*). Our findings on enhanced SIRT1 glycosylation during a fed state are also consistent with earlier reports on the interplay between IS and OGT, which results in increased OGT activity and thus global induction of protein glycosylation (39, 45).

Since nutrient-dependent changes in both total SIRT1 and its glycosylation status were observed, we wanted to investigate if the two processes were linked. Treating cells with the translational inhibitor cycloheximide (CHX) revealed increased degradation of SIRT1 in the presence of PUGNAc (Fig. 1*I* and *SI Appendix*, Fig. S1*G*). As SIRT1 mRNA levels did not significantly change under these conditions (*SI Appendix*, Fig. S1*H*), our results suggest that SIRT1 glycosylation decreased its stability. Corroborating this, we also found that levels of SIRT1 reduced upon CHX treatment in the presence of insulin (Fig. 1*J*). Interestingly, while inhibiting OGA led to enhanced degradation of SIRT1, inhibiting OGT (using BZX) stabilized SIRT1 even under fed conditions (*SI Appendix*, Fig. S1*I*), indicating that SIRT1 stability and glycosylation were correlated with a metabolic shift. Therefore, we surmised that this nutrient-dependent PTM likely modulates SIRT1 stability and in turn its downstream molecular functions. Moreover, given that SIRT1 plays a pivotal role in maintaining metabolic homeostasis, next we systematically tested if SIRT1 glycosylation was critical for mediating physiological transitions.

PHYSIOLOGY

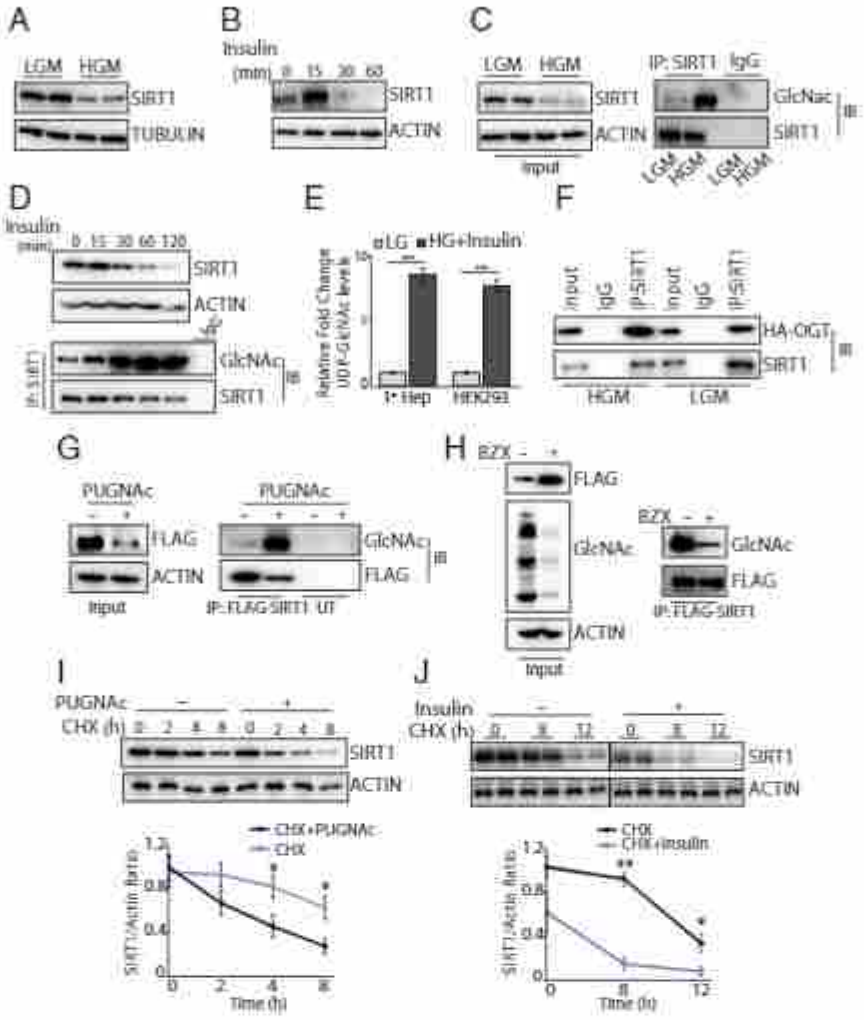

Fig. 1. Nutrient dependent stability and glycosylation of SIRT1. (*A* and *B*) Immunoblot for endogenous SIRT1 levels in primary hepatocytes treated with (*A*) LG and HG-media for 12 h and (*B*) mouse liver tissue, harvested at indicated time points in response to insulin (0.75 IU/kg body weight) administered intraperitoneally. (*C* and *D*) Immunoblot for glycosylated SIRT1 in Primary hepatocytes treated with (*C*) 12 h of LG and HG media and (*D*) mouse liver tissue harvested at indicated time points upon insulin (0.75 IU/kg body weight) administration. (*E*) UDP-GlcNAc level in Primary hepatocytes and HEK293 cells. (*F*) Immunoblot for endogenous SIRT1 in HA immunoprecipitates from HEK293T cells expressing HA-OGT treated with LG and HG media. (*G* and *H*) Immunoblot for glycosylated SIRT1 in FLAG-M2 immunoprecipitates from HEK293T cells expressing FLAG-SIRT1^{WT} treated with (*G*) 100 μM PUGNAc and (*H*) 100 μM BZX for 16 h. (*I* and *J*) Immunoblot for endogenous SIRT1 levels in HepG2 cells treated with (*I*) PUGNAc (100 μM) and CHX (100 μg/mL) for indicated time points ($n = 4$), and (*J*) insulin (100 nM, pretreated for 12 h) and CHX (100 μg/mL) with insulin for indicated time points ($n = 4$). Data are represented as mean ± SEM and analyzed by Student's *t* test. A value of *P* less than 0.05 was considered statistically significant. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$.

SIRT1 Is Glycosylated at Its N-Terminal Specificity Domain. In silico prediction of potential glycosylation sites within SIRT1 indicated that Thr^{160}/Ser^{161} (T^{160}/S^{161}) within the exon-2 domain could be modified (*SI Appendix*, Fig. S2*A*). Although other putative sites were also predicted to be glycosylated, we focused on characterizing the modification at the N-terminal T^{160}/S^{161} residues, as the exon-2 domain that harbors these residues confers binding specificities of SIRT1 to various TFs (34, 36). Moreover, this region is intrinsically disordered and raises the intriguing possibility of a PTM-based mechanism of regulating such protein–protein interactions (35, 36).

We therefore hypothesized that glycosylation at T^{160}/S^{161} in mouse SIRT1 (mSIRT1) (Fig. 2*A*) could play a critical role in regulating SIRT1 functions. Importantly, the predicted glycosylation site (Ser^{161}/Ser^{169} in mouse/human, respectively) and the exon-2 domain are highly conserved in mammals, with 84% sequence identity between humans and mice (Fig. 2*A*). We generated single mutants (Thr-160-Ala and Ser-161-Ala) and assessed their glycosylation under HG conditions. Relative to WT controls, both single mutants demonstrated decreased glycosylation (*SI Appendix*, Fig. S2 *B* and *C*), suggesting that both of these residues were likely glycosylated under nutrient-replete conditions. Hence, we generated the double-site mutants (T^{160}/S^{161}-A) of SIRT1 (SIRT1-E2$^{mut\text{-}AA}$), and found that glycosylation of SIRT1-E2$^{mut\text{-}AA}$ was starkly reduced and its levels were insensitive to nutrient alterations, unlike the WT (Fig. 2 *B*–*D*). Despite this drastic decrease, we do not rule out glycosylation at other putative sites, which may contribute toward SIRT1 functions. Given a recent report on C terminus SIRT1 glycosylation (Ser-549) under starvation and stress (48), we mutated this site and assayed for changes in modification under nutrient-replete conditions. We found that SIRT1-S^{549}-A displayed a similar increase in glycosylation as in the WT under fed conditions (*SI Appendix*, Fig. S2*D*). Our data thus show that the N-terminal T^{160}/S^{161} residues contribute the most toward a fed-dependent SIRT1 hyperglycosylation. Interestingly, we found that the exon-2 domain was necessary to mediate SIRT1 interaction with OGT, and SIRT1 glycosylation was markedly reduced in its absence (*SI Appendix*, Fig. S2 *E* and *F*).

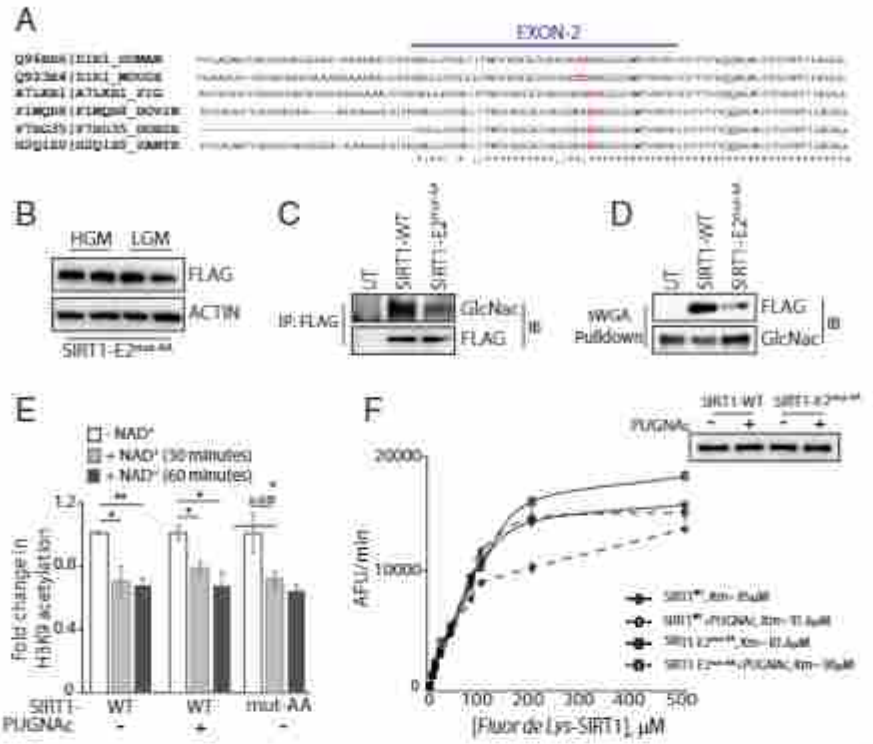

Fig. 2. Identification of glycosylation sites in the exon-2 region of SIRT1. (*A*) ClustalW alignment of amino acid sequence of SIRT1^{WT} from human, mouse, pig, bovine, horse, and panther, respectively. Serine at position-161 of SIRT1 in the exon-2 region, which is evolutionarily conserved, is indicated in red. (*B*) Immunoblot for FLAG-SIRT1-E2^{mut-AA} in HEK2931 cells grown in LG and HG culture media. (*C* and *D*) Immunoblot for glycosylated SIRT1 from HEK2931 cells expressing FLAG-SIRT1^{WT} and FLAG-SIRT1-E2^{mut-AA} in (*C*) FLAG-M2 immunoprecipitates and (*D*) sWGA pull-down. (*E*) Comparison of SIRT1 activity on acetylated histones along with saturating levels of NAD$^+$ for 30 and 60 min from FLAG-M2 immunoprecipitates in HEK2931 cells overexpressing FLAG-SIRT1^{WT} (±PUGNAc) and -E2^{mut-AA} ($n = 3$). (*F*) Substrate saturation curves of fluoregenic peptide using FLAG-SIRT1^{WT} and -E2^{mut-AA} (± PUGNAc). Values for K_m were calculated from a Lineweaver–Burke plot derived from these data. Data are represented as mean ± SEM and analyzed by the Student's *t* test. A value of $P \leq 0.05$ was considered statistically significant. *$P \leq 0.05$; **$P \leq 0.01$.

However, it is important to note that the decreased glycosylation in SIRT1-E2^{mut-AA} was not due to reduced interaction with OGT but a resultant loss of residues T^{160}/S^{161} that are glycosylated within the exon-2 domain (*SI Appendix*, Fig. S2*E*).

PTMs (e.g., phosphorylation and SUMOylation) on SIRT1 are known to modulate its activity (49–52). Notably, some of these modifications have opposing effects on SIRT1 activity (52). However, a possible interplay between any of these PTMs in fine-tuning SIRT1 functions remains poorly understood. Hence, to investigate if glycosylation resulted in altered SIRT1 activity, we carried out biochemical assays using immunoprecipitated SIRT1^{WT} and SIRT1-E2^{mut-AA} (Fig. 2*E*). In vitro NAD$^+$-dependent SIRT1 deacetylation assay revealed comparable activities for both forms of SIRT1 on lysine-9 acetylation of histone H3 (H3K9Ac) (Fig. 2*F* and *SI Appendix*, Fig. S2 *G*–*I*). Furthermore, kinetic analysis of SIRT1^{WT} and SIRT1-E2^{mut-AA} under saturating NAD$^+$ concentrations also showed comparable K_m values for the fluorogenic p53 peptide (Fig. 2*F* and *SI Appendix*, Fig. S2*J*). This was not surprising, as the loss of the exon-2 domain, which although was necessary for modification of endogenous proteins by differential tethering, did not affect catalytic activity of SIRT1 on accessible acetylated peptides (34). This suggested that glycosylation or its loss do not significantly impact the intrinsic activity of SIRT1 on peptide substrates. Taken together, our results establish that T^{160}/S^{161} within the exon-2 domain were nutrient-sensitive glycosylation sites. Importantly, since we have previously established the exon-2 domain as being crucial for determining substrate tethering/interaction, we next wanted to study the effect of glycosylation at T^{160}/S^{161} on SIRT1 functions at molecular, cellular, and organismal levels.

SIRT1 Glycosylation Determines a Spatial and Temporal Shift in Molecular Interactions. SIRT1 deacetylates several TFs, exerting a master control over gene expression (19, 21, 33). To ascertain if metabolic inputs orchestrate SIRT1 interactions and functions in response to changes in nutrient availability, we examined the interaction of SIRT1 with TFs (FOXO1 and PPARα) and coactivators (PGC1α), which are essential to mediate a fasting response (19, 21, 33, 46). We found that compared to low-glucose (LG) conditions, SIRT1's interaction with these transcriptional factors and coactivators were significantly reduced under HG conditions (Fig. 3*A*). Intriguingly, we also found a similar decrease in interactions with SREBP1, a well-known mediator of fed response (Fig. 3*B*). These indicated that fed inputs led to a disruption of SIRT1 interactions with nuclear TFs and coactivators, and pointed toward a nutrient (glucose) -dependent control of SIRT1 dynamics, and perhaps localization.

To check if glycosylation was involved in integrating nutrient inputs with SIRT1 functions, we assayed for the ability of WT SIRT1 to interact with TFs and coactivators under conditions that lead to its hyperglycosylation. Interestingly, we found that SIRT1 glycosylation, upon PUGNAc treatment, significantly reduced its interaction with transcriptional regulators that operate during both starved and fed conditions (Fig. 3 *C* and *D*). In contrast, the nonglycosylated form of SIRT1, SIRT1-E2^{mut-AA}, had enhanced interactions with these factors (Fig. 3 *E* and *F*). These striking findings indicate that hypoglycosylation of SIRT1 at T^{160}/S^{161}, under starved or nutrient-deprived conditions, most likely increases its association with PGC1α, FOXO1, PPARα, and SREBP1. Furthermore, these results suggested that an absence of SIRT1 glycosylation can both up-regulate and

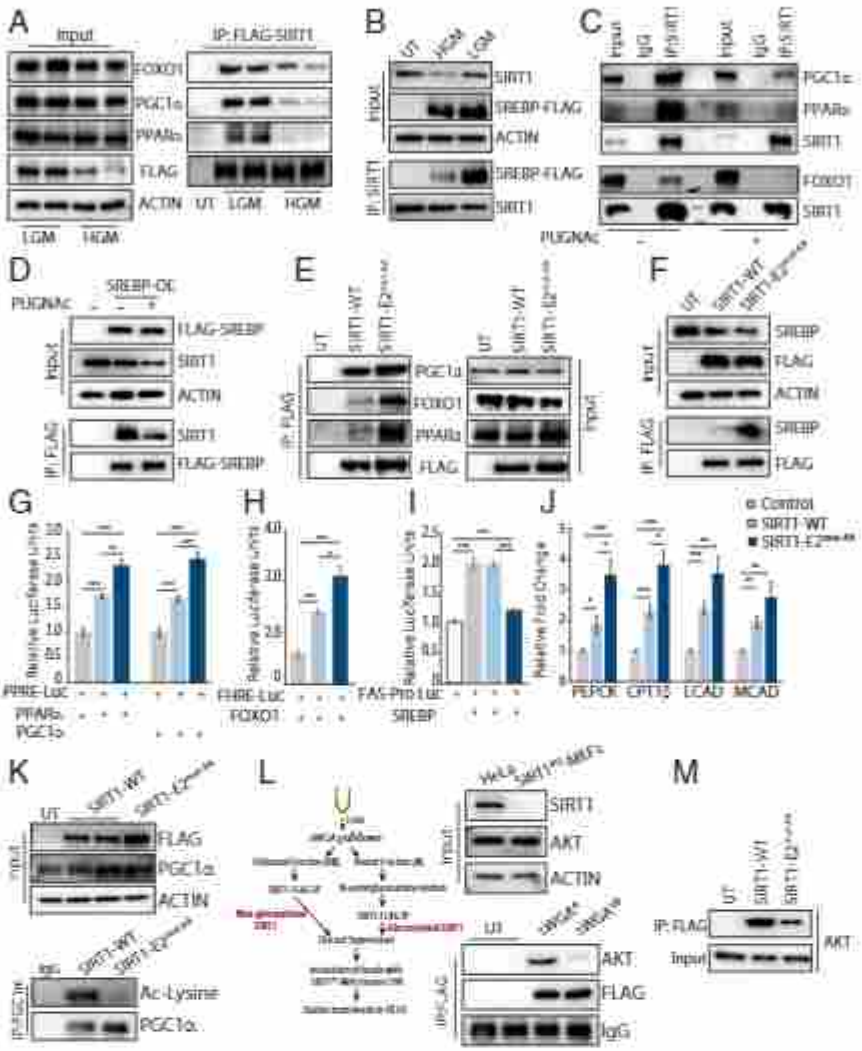

Fig. 3. Nutrient-dependent SIRT1 glycosylation modulates interactions and affects transcription. (*A* and *B*) Coimmunoprecipitation from HEK293T expressing either FLAG-SIRT1^{WT} or FLAG-SREBP1 using FLAG-M2 beads and immunoblotting for (*A*) interactions and (*B*) SIRT1 in response to 12 h of LG and HG culture conditions. (*C*–*F*) Immunoblot for interactions in *C* and *D* HEK293T cells treated with 100 μM PUGNAc for 12 to 16 h from (*C*) SIRT1 immunoprecipitates and (*D*) FLAG-M2 immunoprecipitates overexpressing FLAG-SREBP1. (*E* and *F*) Immunoblot of FLAG-M2 immunoprecipitates from HEK293T cells expressing either FLAG-SIRT1^{WT} or SIRT1-E2^{mut-AA} 36 to 40 h posttransfection without nutrient replenishment. (*G*–*I*) Luciferase assay in HEK293T cells expressing either FLAG-SIRT1^{WT} or SIRT1-E2^{mut-AA} and (*G*) quantifying PGC1α- or PPARα-dependent transcription as measured by PPRE-luciferase assay ($n = 3$–4, $n = 3$). (*H*) FOXO1-dependent transcription as measured by FHRE-promoter luciferase assay ($n = 3$, $n = 2$) and (*I*) SREBP1-dependent transcription as measured by FAS-Promoter luciferase assay ($n = 3$ to 4, $n = 2$). (*J*) Gluconeogenic and β-oxidation gene-expression analysis from HepG2 cells expressing either FLAG-SIRT1^{WT} or SIRT1-E2^{mut-AA} ($n = 3$, $n = 2$). (*K*) Immunoblot for acetylated-lysine in PGC1α immunoprecipitates, following nutrient replenishment, from HEK293T cells overexpressing either FLAG-SIRT1^{WT} or -E2^{mut-AA}. (*L*) Immunoblot for AKT interaction with sWGA^{B} and sWGA^{UB} fractions from HeLa cells overexpressing FLAG-SIRT1^{WT} incubated with SIRT1^{KO}-MEF lysates. (*M*) Immunoblot for AKT interaction in FLAG-M2 immunoprecipitates from HEK293T cells expressing either FLAG-SIRT1^{WT} or -E2^{mut-AA}. Data are represented as mean ± SEM and analyzed by the Student's *t* test. A value of $P \leq 0.05$ was considered statistically significant. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

suppress expression of genes required for starvation and fed response, respectively.

To investigate this, we assayed for the expression of genes downstream of these TFs, using both luciferase-based reporter constructs and by measuring endogenous mRNA levels of fasting-/fed-responsive genes. We found that nonglycosylated SIRT1 enhanced transcription downstream to PGC1α, PPARα, and FOXO1, including genes involved in oxidative phosphorylation, gluconeogenesis, and β-oxidation, and inhibited expression of lipogenic genes downstream to SREBP1 (Fig. 3 *G*–*J* and *SI Appendix*, Fig. S3*A*). Overexpression of SIRT1-E2^{mut-AA} resulted in increased deacetylation of PGC1α compared to SIRT1^{WT} under HG conditions (Fig. 3*K*), further strengthening the mechanistic underpinnings. Moreover, at a functional level, increased palmitate-induced fatty acid oxidation and ATP production rate were observed in hepatocytes expressing SIRT1-E2^{mut-AA} compared to SIRT1^{WT} (*SI Appendix*, Fig. S3 *B* and *C*). In addition to corroborating the differential SIRT1 interactions, these results demonstrate that nutrient-dependent SIRT1 glycosylation is necessary to switch the transcriptional program for an efficient transition from a starved to fed state.

Besides its role in regulating transcription, SIRT1 is well documented to be a key factor that determines insulin sensitivity (30, 53), and a loss of SIRT1 induces insulin resistance (30–32, 54). Even though SIRT1-mediated deacetylation of AKT is important for its membrane localization and activity in response to insulin stimulation (32), spatiotemporal regulation of SIRT1–AKT interactions during starved to refed transition are unclear.

Therefore, we asked if glucose and insulin affected the ability of SIRT1 to interact with AKT. Addition of insulin to cells led to a temporal regulation of SIRT1–AKT interactions with a significant increase at 15 min (*SI Appendix*, Fig. S3*D*), which thereafter decreased drastically by 30 min, coinciding with increased SIRT1 glycosylation (Fig. 1*D*).

In vitro pull-down assays using purified glycosylated or nonglycosylated forms of SIRT1 indicated that AKT preferentially interacted with glycosylated SIRT1 (Fig. 3*L*). On assaying for SIRT1–AKT interactions using SIRT1-E2$^{mut\text{-}AA}$, absence of glycosylation at T^{160}/S^{161} diminished AKT binding significantly (Fig. 3*M*). Moreover, overexpression of this nonglycosylated form of SIRT1 resulted in dampened IS compared to SIRT1^{WT} (*SI Appendix*, Fig. S3*E*), corroborating the differential interaction observed in Fig. 3*M*. Interestingly, cells expressing SIRT1-E2$^{mut\text{-}AA}$ also displayed reduced glycolytic-flux under both basal and insulin-stimulated conditions, and clearly highlighted the metabolic consequence of reduced IS upon loss of glycosylation at T^{160}/S^{161} (*SI Appendix*, Fig. S3*F*). This illustrates that nutrient-driven SIRT1 glycosylation, a fasting factor, during the initial phase of a refed response is required for activation of IS. Taken together with reduced SREBP1 interaction (Fig. 3*D*), this shows how SIRT1 glycosylation, while necessary for IS, also ensures that a fed-responsive transcriptional program is not inhibited upon transition to a fed state. These significant findings provide a molecular basis to the paradoxical role of SIRT1 in activating IS and inhibiting downstream fed-responsive transcriptional programs.

Glycosylation and Phosphorylation Interplay Posits a Complex Regulatory PTM Code for SIRT1 at Its N-Terminal Domain. Phosphorylation and O-GlcNAcylation are known to regulate protein functions by creating a "PTM code" (44). Although not widely investigated, competing or proximal site phosphorylation is known to impact O-GlcNAcylation and such an interplay likely affects protein function. Recent in silico approaches have tried to propose sequence or motif-based mechanisms that might bring about such cross-talks (55). SIRT1 is phosphorylated at multiple sites with divergent effects on its functions (52, 56). Based on the recently reported SIRT1 phosphoproteome analyses and functionally validated phosphorylation sites in the exon-2 domain (57–59), we chose Ser-154 and Thr/Ser-160/161 to study the potential interplay with glycosylation. Although no known phosphorylations have been reported at T^{160}/S^{161} sites, we wanted to check the effect of phosphor-mimic mutations (T^{160}/S^{161} replaced by glutamate; SIRT1-E2$^{mut\text{-}EE}$) on the ability of SIRT1 to activate transcription downstream to PPRE-Luc. SIRT1-E2$^{mut\text{-}EE}$ did not show any nutrient dependent loss in SIRT1 functions unlike the WT, which was consistent with SIRT1-E2$^{mut\text{-}EE}$–PGC1α interactions (*SI Appendix*, Fig. S3 *G*–*I*). Our results thus indicate that phosphorylation and glycosylation at T^{160}/S^{161} likely marks distinct functional states of SIRT1.

CK2-dependent phosphorylation of Ser-154, which is proximal to T^{160}/S^{161} in mouse SIRT1, has been reported to affect downstream functions, including fatty acid oxidation and cell survival (58, 59). To check if a de-phosphomimic or a phosphomimic mutation at this site impinged on glycosylation at T^{160}/S^{161}, we generated SIRT1$^{S154\text{-}A}$ and SIRT1$^{S154\text{-}E}$ mutants. Interestingly, we found that while SIRT1$^{S154\text{-}A}$ did not affect glycosylation, SIRT1$^{S154\text{-}E}$ seemed to reduce glycosylation of SIRT1 (*SI Appendix*, Fig. S3*J*). Therefore, in addition to establishing T^{160}/S^{161} glycosylation-dependent molecular dynamics of SIRT1 functions, our results also suggest a complex interplay with phosphorylation both at the same site and in its proximal vicinity. It will be exciting to unravel, in the future, the impact of diverse cellular cues that impinge on T^{160}/S^{161} or other neighboring sites by way of PTMs.

Overexpression of a Nonglycosylatable Form of SIRT1 Leads to Hyperglycemia and Hepatic Inflammation. To unravel the physiological significance of SIRT1 glycosylation, we overexpressed both SIRT1^{WT} and SIRT1-E2$^{mut\text{-}AA}$ in the liver of C57Bl6 mice using adenovirus (Fig. 4 *A* and *B*). We found that mice with ectopic expression of SIRT1-E2$^{mut\text{-}AA}$ led to disrupted glucose homeostasis, as evident from glucose-, insulin-, and pyruvate-tolerance tests (GTT, ITT, and PTT respectively) (Fig. 4 *C*–*E*), along with a significant increase in fasting blood glucose (Fig. 4*F*). Together, these indicated increased gluconeogenesis in the presence of SIRT1-E2$^{mut\text{-}AA}$, which was further confirmed by measuring glucose output in primary hepatocytes (*SI Appendix*, Fig. S4*A*). Higher expression of PEPCK and G6Pase (Fig. 4*G*) substantiated the above findings of increased glucose output from livers of SIRT1-E2$^{mut\text{-}AA}$–expressing mice. Furthermore, reduced oil-red staining along with elevated transcripts of genes involved in fatty acid oxidation upon fatty acid feeding and ectopic SIRT1 expression indicated altered fat metabolism in the absence of SIRT1 glycosylation (Fig. 4*G* and *SI Appendix*, Fig. S4 *B*–*D*). SIRT1-E2$^{mut\text{-}AA}$ mice also had decreased lipogenic and fatty acid transporter gene expression even upon 6 h of refeeding (Fig. 4*H*). Highlighting gross perturbation at the level of metabolic signaling, ectopic expression of SIRT1-E2$^{mut\text{-}AA}$ resulted in heightened pAMPK and dampened pAKT (Fig. 4*I*) even in a fed state. Emerging literature indicates that a perturbation in liver metabolism is associated with hepatic inflammation (1, 5, 6). In this context, SIRT1-E2$^{mut\text{-}AA}$–overexpressing mice had significantly higher expression of inflammatory genes in their livers (*SI Appendix*, Fig. S4*E*). Taken together, these results demonstrate that altered molecular interactions of SIRT1-E2$^{mut\text{-}AA}$ with PGC1α-PPARα/FOXO and AKT caused physiological hepatic deficits.

Glycosylation Results in Nuclear Export of SIRT1. Results presented above showed glycosylation-dependent differential interactions of SIRT1 with nuclear and cytosolic factors were necessary for maintaining hepatic physiology. This prompted us to check if glycosylation was associated with altered cellular localization of SIRT1. Although SIRT1 has been shown to shuttle between the nucleus and cytoplasm (60–62), a nutrient-dependent control of SIRT1 localization is unknown. Consistent with earlier reports, we found SIRT1 to be predominantly nuclear under LG conditions (Fig. 5*A*). It relocalized to the cytoplasm in PUGNAc or insulin-treated cells under HG (Fig. 5*A* and *SI Appendix*, Fig. S5*A*). Furthermore, glycosylated SIRT1 was enriched in the cytoplasmic fraction of sWGA pull-downs (*SI Appendix*, Fig. S5*B*). Moreover, inhibiting glycosylation enhanced nuclear retention of SIRT1^{WT} even under nutrient excess conditions (Fig. 5*B*). Notably, there was a stark contrast between the localization patterns of SIRT1^{WT} and SIRT1-E2$^{mut\text{-}AA}$ (Fig. 5*C*), with predominant nuclear localization for the latter. These results established that nutrient-dependent SIRT1 glycosylation determined its nuclear-cytosolic pools.

Next, we addressed if SIRT1 was glycosylated within the nucleus and if the relocalization was an active mechanism. Cells treated with Leptomycin B (LMB), a known inhibitor of exportin protein (CRM-1), resulted in the loss of nuclear extrusion of SIRT1 and indicated it to be an active fed-state mechanism (Fig. 5*D* and *SI Appendix*, Fig. S5*C*). OGT is known to localize to the nucleus, apart from cytoplasm and mitochondria, and glycosylate various nuclear proteins (44, 63, 64). On assaying for glycosylation of SIRT1 immunoprecipitated from LMB-treated cells under HG, we found that SIRT1 was indeed modified within the nuclear compartment (*SI Appendix*, Fig. S5*D*). Thus, our results demonstrate that nutrient-dependent glycosylation of SIRT1 at its N-terminal domain resulted in its cytoplasmic export (Fig. 5 *E* and *F*).

Proteasome-Mediated Degradation of SIRT1 during a Refed State Is Governed by Glycosylation. While mechanisms that induce SIRT1 during a fasting phase are well known (52, 65), relatively little is known about those that lead to its degradation during a fed state. Importantly, if/how nutrient status of the cells control

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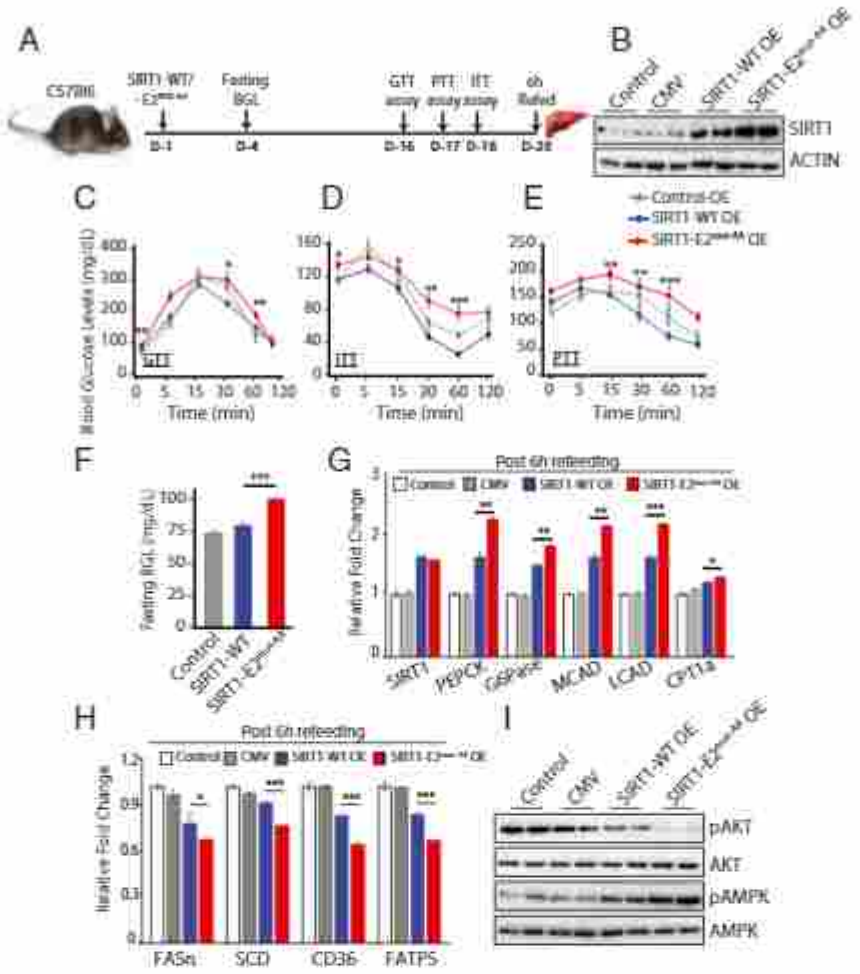

Fig. 4. SIRT1 glycosylation regulates liver physiology. (*A*) Schematic representation of experimental paradigm used for hepatic overexpression of FLAG-SIRT1^{WT} and SIRT1-E2^{mut-AA} in C57BL6 mice. (*B–F*) Physiological assays following overexpressing of either SIRT1^{WT} or SIRT1-E2^{mut-AA}, along with control injected and uninjected mice and (*B*) immunoblotted for SIRT1 in the liver. (*C*) GTT, (*D*) ITT, and (*E*) PTT ($n = 6$, $n = 3$). (*F*) Blood glucose levels upon 12 h of fasting ($n = 6$, $n = 3$). (*G–I*) Liver tissues harvested from mice with hepatic overexpression of either SIRT1^{WT} or SIRT1-E2^{mut-AA}, along with relevant controls, and analyzed for (*G*) gluconeogenic and β-oxidation gene expression ($n = 3$, $n = 2$), (*H*) lipogenic and fatty acid transporter genes ($n = 3$, $n = 3$), and (*I*) immunoblot of pAMPK and pAKT for fasted and fed signaling, with total AMPK and total AKT. Data are represented as mean ± SEM and analyzed by the Student's *t* test. A value of $P \leq 0.05$ was considered statistically significant. *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$.

SIRT1 turnover remains to be unraveled. Since nutrient-dependent SIRT1 glycosylation dictated its interactions and cellular localization, we wondered whether glycosylation determined its protein levels in the fed state. PUGNAc treatment led to decreased SIRT1 levels (Fig. 1*F*), indicating that increased glycosylation resulted in its degradation. Inhibiting glycosylation stabilized SIRT1, even under glucose-replete conditions (*SI Appendix*, Fig. S1*F*). Together, these results illustrate that nutrient-dependent glycosylation was involved in regulating SIRT1 levels.

To further identify whether SIRT1 degradation was proteasome mediated, cells were treated with proteasomal inhibitor MG132 in the presence or absence of insulin. As shown in Fig. 6*A*, SIRT1 degradation was abrogated in the presence of MG132. Immunoblotting for ubiquitination of immunoprecipitated SIRT1-WT and -E2^{mut-AA} under these conditions showed that sustained glycosylation of WT caused increased ubiquitination in cells treated with PUGNAc and MG132 (Fig. 6*B*). However, it has been difficult to tease out the temporal control of glycosylation dependent ubiquitination during the continued fed condition. Moreover, whether nutrient-dependent inputs activate specific E3-ligases needs to be investigated in the future. Importantly, we found that the stability of SIRT1-E2^{mut-AA} was significantly enhanced as compared to the WT SIRT1, even in the presence of PUGNAc (*SI Appendix*, Fig. S6*A*). Stability of SIRT1-E2^{mut-AA} protein remained unchanged following PUGNAc (Fig. 6*C*), and in cells that were treated with insulin and MG132 (*SI Appendix*, Fig. S6*B*), and possibly ruled out other mechanisms that contributed to differential degradation kinetics. These clearly demonstrated that the observed effect on protein turnover was primarily driven by glycosylation at T^{160}/S^{161}. It was also interesting to note that nuclear retention of SIRT1^{WT}, in LMB- and PUGNAc-treated cells, did not lead to increased destabilization and suggested that cytoplasmic export was necessary for its degradation (Fig. 6 *D* and *E*). Together, these data conclusively demonstrate that glycosylation leads to ubiquitin-mediated degradation of SIRT1 under nutrient-rich conditions, which was hitherto unknown.

Reduced SIRT1 Expression during Aging and Obesity Is Associated with Glycosylation. Reduced SIRT1 expression has been associated with aging and metabolic diseases, both causally and consequentially (56, 65, 66). However, little is known about nutrient- and age-dependent mechanisms that control SIRT1 levels. We found that total cellular protein glycosylation was enhanced in

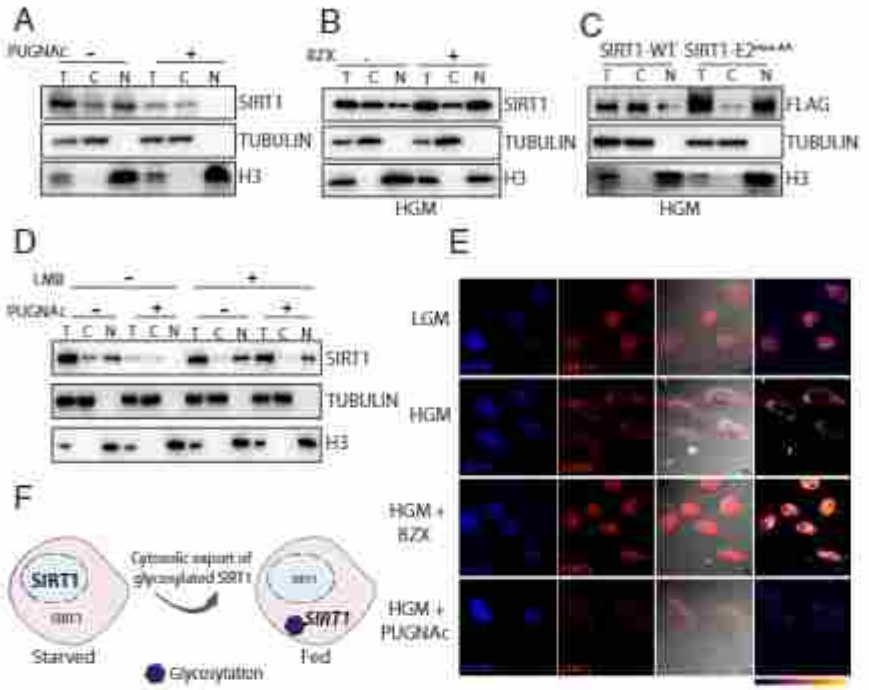

Fig. 5. *N*-terminal glycosylation dependent cytoplasmic export of SIRT1. (*A*–*D*) Nucleo-cytoplasmic distribution of SIRT1 in HeLa cells treated with (*A*) 100 μM PUGNAc for 12 h and (*B*) 100 μM BZX for 12 h under HG conditions; (*C*) expressing either FLAG-SIRT1^{WT} or -E2^{mut-AA} under HG conditions, and (*D*) treated with 100 μM PUGNAc and 10 ng/mL LMB for 12 h. (*E*) Representative immunofluorescence image for endogenous SIRT1 localization upon treatment (16 h) with either OGT inhibitor (BZX) or OGA inhibitor (PUGNAc) in HeLa cells grown in LG or HG media, as indicated ($n = 3$ to 4). (*F*) Schematic representation of cytosolic export of SIRT1 upon nutrient dependent glycosylation at the N terminus. C, cytoplasmic fraction; N, nuclear fraction; T, total cell lysate.

the livers isolated from refed, high-fat–induced obese and aged mice (*SI Appendix*, Fig. S7*A*). Taking together the enhanced UDP-GlcNAc levels (Fig. 1*E*), which is indicative of high nutrient flux, and the elevated total protein glycosylation demonstrated increased cellular O-GlcNAcylation in a nutrient excess state. Probing for SIRT1 expression in this context, we found that there was a drastic reduction under these conditions, consistent with our earlier results and previous reports (*SI Appendix*, Fig. S7 *B*–*D*) (11, 47, 50). Notably, we found a significant increase in glycosylation as compared to their respective controls in immunoprecipitated SIRT1 from these mice livers (Fig. 7 *A*–*C*). Although correlative, these data suggest that glycosylation of SIRT1 could be one of the major underlying causes for its reduced expression during aging or in obesity.

Glycosylation-Mediated SIRT1 Oscillation Is Essential for Fasted-To-Refed Transition. Recent reports have highlighted small-molecule activators of SIRT1, which enhance its activity by either stabilizing or decreasing the K_m for its substrate (27, 28, 52). While literature clearly highlights the beneficial effects of activating SIRT1, potential drawbacks of over-/sustained activation of SIRT1 have been largely ignored. As shown in Fig. 4, hepatic overexpression of SIRT1-E2^{mut-AA}, which abrogated typical oscillation of SIRT1 protein in response to the fed–fast transition, resulted in hepatic defects and inflammation. Thus, it can be surmised that rampant and nonspecific activation of SIRT1 may be deleterious to organismal physiology, contrary to the current assumption of SIRT1 being beneficial irrespective of the metabolic state. To rule out any possible artifact due to overexpression, we knocked out endogenous SIRT1 in the livers of SIRT1^{liver} mice and restored it with SIRT1^{WT} or SIRT1-E2^{mut-AA} forms using adenoviral systems (*SI Appendix*, Fig. S7*E*). Since degradation of SIRT1 in the fed state enabled the expression of fed-responsive genes, we were curious to check if glycosylation or its absence had any effect on fasted-to-refed transitions. Both the SIRT1 forms were expressed to amounts that were comparable to the endogenous SIRT1 in the liver (Fig. 7*D*). Overexpression of SIRT1-E2^{mut-AA} in SIRT1-floxed mice increased gluconeogenesis (PTT assay) (Fig. 7*E*) and modestly elevated fasting blood glucose levels (Fig. 7*F*) when compared to control and SIRT1^{WT}-rescued mice. These mirrored the changes seen in mice with ectopic overexpression of mutant SIRT1 (Fig. 4 *C*–*F*).

Next, we wanted to assess the importance of SIRT1 glycosylation in mediating efficient physiological fasted–refed transitions. Toward this, mice expressing SIRT1-E2^{mut-AA} were starved for 24 h followed by 6 h of refeeding. It should be noted that unlike when SIRT1^{WT} was restored in SIRT1-floxed mice, in cohorts expressing SIRT1-E2^{mut-AA} the levels of mutant SIRT1 did not decrease upon a refed transition (Fig. 7*G*). Moreover, decreased protein levels of SIRT^{WT} were associated with increased glycosylation (Fig. 7*G*). As expected, we observed reduced FOXO1 and pAMPK levels upon refeeding and a concomitant increase in pAKT (Fig. 7*H*). However, upon hepatic SIRT1-E2^{mut-AA} expression in SIRT1-floxed mice there was no change in FOXO1, pAKT, or pAMPK levels between the starved and the refed states (Fig. 7*H*). Additionally, gluconeogenic and β-oxidation genes were elevated in the SIRT1-E2^{mut-AA} mice livers and did not decrease upon refeeding when compared to SIRT1^{WT} (*SI Appendix*, Fig. S7*F*). Together, these results establish that loss/abrogation of SIRT1 glycosylation led to continued expression of fasting genes and elevated blood glucose levels, resulting in a failure to transit to a refed state, even upon nutrient availability (Fig. 7*I*).

Discussion

Temporal control of protein–protein interactions is vital for mediating physiological transitions, specifically in the context of dynamic toggling between fed–fast–refed states. Here, we have discovered a nutrient-dependent control of SIRT1 functions, which is manifested through a temporal switch in its interactions

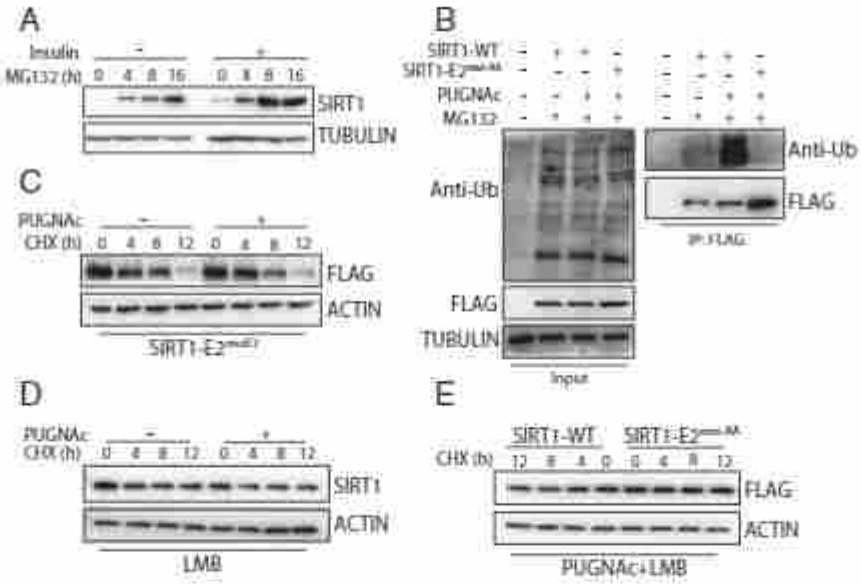

Fig. 6. Glycosylation-dependent proteasomal degradation of SIRT1. (*A*) Immunoblot for endogenous SIRT1 levels in HepG2 cells treated with insulin (100 nM, 16 h) and proteasomal inhibitor MG132 (20 μM) for indicated time points. (*B*) Immunoblot of ubiquitinated $SIRT1^{WT}$ and $SIRT1\text{-}E2^{mut\text{-}AA}$ from SIRT1 immunoprecipitates in HEK293 cells treated with PUGNAc and MG132 for 16 h, as indicated. (*C*) Immunoblot for SIRT1 levels in HepG2 cells expressing FLAG-$SIRT1\text{-}E2^{mut\text{-}AA}$ treated with PUGNAc (100 μM, 16 h) and CHX (100 μg/mL) for indicated time points. (*D*) Immunoblot for endogenous SIRT1 in HeLa cells treated with LMB (10 ng/mL, 12 h), CHX (20 μM, indicated time points), and ± PUGNAc (100 μM, 16 h). (*E*) Immunoblot for SIRT1 in HeLa cells overexpressing FLAG-$SIRT1^{WT\text{-}mutAA}$ treated with and PUGNAc (100 μM, 16 h), LMB (10 ng/mL, 12 h), and CHX (20 μM) for indicated time points.

to regulate hepatic glucose and fat metabolism. Specifically, we found a regulatory PTM on SIRT1 (i.e., glycosylation), which determines interactions with its target transcription factors/coregulators. Several studies have identified positive and negative regulators of SIRT1, including proteins like DBC1 (36, 67) and modifications, such as SUMOylation and phosphorylation (52). However, mechanistic insights into their spatial and temporal regulation of substrate selectivity of SIRT1, during any physiological state transitions, remain unknown. In this regard, our results demonstrate that SIRT1 glycosylation during the initial phases of refed state, reduces its interaction with nuclear transcription factors/coregulators (PPARα, PGC1α, FOXO1, and SREBP1), and is also coupled with nuclear exclusion of SIRT1. Furthermore, we show that this cytoplasmic export of glycosylated SIRT1 is necessary for enhanced interaction with AKT, a key component of insulin signaling, and subsequently mediates SIRT1 degradation through the proteosomal machinery during fed–fast cycles.

Similar or distinct PTMs at overlapping or separate sites are known to affect protein functions variably. In this context, we found that mutating C-terminal S^{549}, which was recently reported to be glycosylated in response to stress (48), did not abrogate fed-dependent N-terminal glycosylation (at T^{160}/S^{161}) in SIRT1. Unlike this, comparing molecular interactions of $SIRT1^{WT}$, $SIRT1\text{-}E2^{mut\text{-}AA}$, and $SIRT1\text{-}E2^{mut\text{-}III}$ unveiled the significance of PTMs in the N-terminal domain during fed–fast cycles. Our results suggest that in a WT condition glycosylation at T^{160}/S^{161} residues, which are either 1) unmodified in a fasted-state or 2) dephosphorylated upon a fasted-to-refed transition, is necessary for the molecular switch that enables physiological transition. Hence, projecting ahead, it will be interesting to investigate the interplay between such similar/dissimilar modifications on same and proximal sites, and how diverse extra- and intracellular signals orchestrate SIRT1 functions. It should be noted that our results are also broadly consistent with the roles played by N and C termini vis-à-vis SIRT1 activity and functions, with the N terminus having a deterministic role in substrate interaction/selection.

Modifications on IDRs have been thought to differentially modulate protein–protein interactions (38) and it should be noted that the specificity domain in SIRT1 (encoded by exon-2) is part of the N-terminal IDR (34, 36). In this context, the results presented here, along with literature precedence (34), indicate that metabolite-driven modification of the exon-2 domain acts to regulate the functions of this master transcriptional regulator. In a recent paper, Krzysiak et al. (36) demonstrated that in response to IS, interaction of SIRT1 with DBC1-/PACS2 (via the N-terminal IDR) inhibits its activity and thus attenuates starvation-responsive gene transcription. However, if/how a fed response leads to gating or attenuation of SIRT1 functions by altering its interactions and stability has not been addressed, to the best of our knowledge. Our findings highlight a direct interplay between glucose inputs and SIRT1 regulation, which seems to be upstream and is required to sensitize IS itself. Importantly, mutating glycosylation sites on the SIRT1 exon-2 domain stabilized its interactions with TFs and also caused reduced binding to AKT, which together affected both gene transcription and IS. Thus, our results clearly show that glycosylation of SIRT1 in the exon-2 domain is an important determinant of temporal interaction specificities with transcription factors and signaling proteins.

In addition to drawing attention to the benefits associated with restricted feeding regimes, recent literature has emphasized the importance of fed–fast cycles (68–70). Some of these reports have highlighted the fact that aberrant fed–fast cycles lead to metabolic diseases and specifically compromises hepatic functions (71–73). However, the mechanistic basis for nutrient- and endocrine-dependent inputs that encode temporal molecular switches and enable physiological transitions are poorly understood. Here we show that, while SIRT1 glycosylation is necessary for efficient transition from a fasted to a refed state, its abrogation caused metabolic inflexibility and contributed to perturbed glucose homeostasis both in fasted and refed conditions. Moreover, hepatic expression of nonglycosylated SIRT1 displayed signs of hyperinflammation.

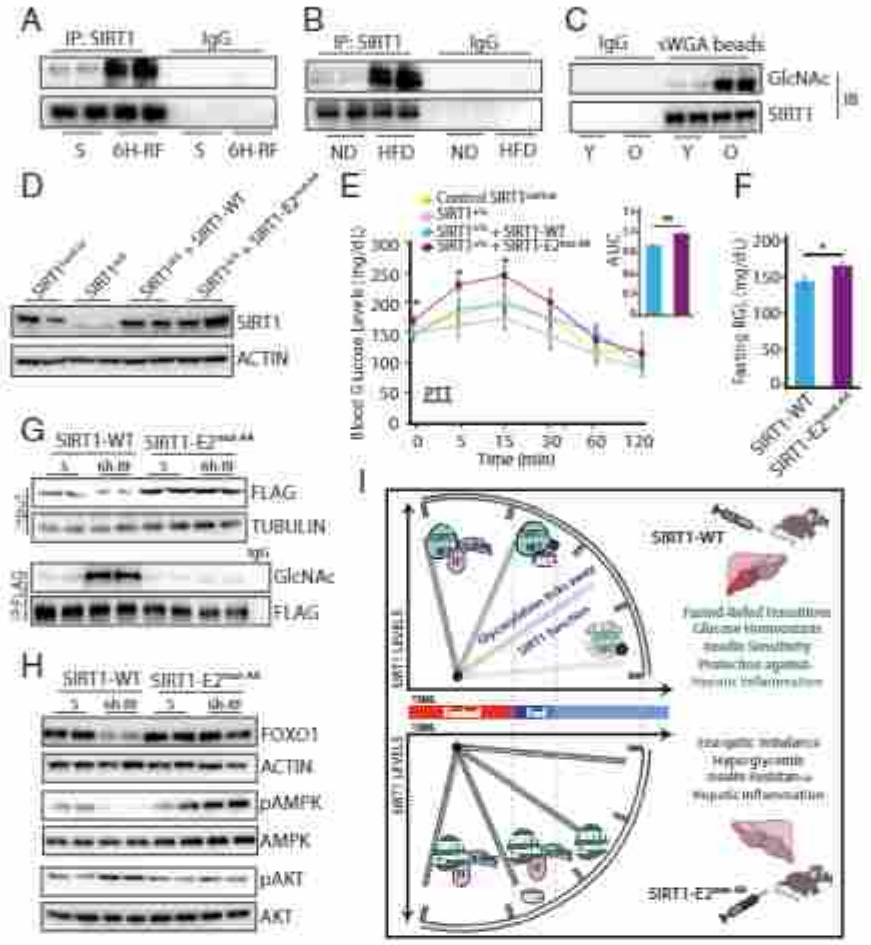

Fig. 7. Hepatic SIRT1 glycosylation is essential to mediate starved–refed transition. (*A–C*) Immunoblot of glycosylated SIRT1 from SIRT1 immunoprecipitates from livers of (*A*) 24-h starved and 6-h refed, (*B*) normal fed chow diet (ND) and 3-mo fed high-fat diet (HFD), and (*C*) young (3-mo) and old (20-mo) mice. (*D–F*) Physiological assays in SIRT1^{hepKO} mice rescued with either SIRT1^{WT} or SIRT1-E2$^{mut\text{-}AA}$ after flox-ing out endogenous SIRT1 along with uninjected control and (*D*) immunoblotted for SIRT1 in liver tissues. (*E*) PTT and area under the curve (AUC) ($n = 6$) and (*F*) blood glucose levels upon 12 h of fasting ($n = 6$). (*G* and *H*) Twenty-four-hour starved and 6-h refed liver tissues harvested from SIRT1^{hepKO} mice rescued with either SIRT1^{WT} or SIRT1-E2$^{mut\text{-}AA}$ after flox-ing out endogenous SIRT1 and immunoblotted for (*G*) glycosylated FLAG-SIRT1 and (*H*) FOXO1, pAMPK, and pAKT. (*I*) Schematic representation of nutrient-dependent glycosylation-mediated switch in SIRT1 functions during fed–fast cycles, which is necessary for physiological state transition, and whose absence causes metabolic inflexibility and diabetes-like state. Data are represented as mean ± SEM and analyzed by the Student's *t* test. A value of $P \leq 0.05$ was considered statistically significant. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$.

In conclusion, our study brings to the fore the importance of SIRT1 oscillation in terms of interactions and expression during fed–fast cycles. Given that there have been many attempts to create gain-of-function models of SIRT1 (15, 24, 27, 28, 74, 75), our study points out that uncontrolled (over)-activation of SIRT1, as in the case of hypoglycosylation, can have detrimental pathological consequences. In contrast, we have found that glycosylation is also positively correlated with reduced SIRT1 levels during aging and obesity. This suggests that, although glycosylation is necessary to restrict SIRT1 activity upon refeeding, excess glycosylation might eventually lead to a loss of SIRT1 function. Therefore, we propose that developing interventions, which regulate SIRT1 glycosylation and hence bias its interactions or maintain its homeostatic expression, will have therapeutic potential.

Methods

Animals. C57BL/6 and SIRT1^{hepKO} mice were housed under standard animal house conditions. Three- to 4-mo-old and 20- to 22-mo-old male mice were considered as young and aged cohorts, respectively. The procedures and the project were approved and were in accordance with the Institutional Animal Ethics Guidelines of Tata Institute of Fundamental Research.

Immunoprecipitation/Purification of FLAG-mSIRT1$^{WT/mut\text{-}AA}$ for Activity-Assays. HEK293T cells were transfected in 150-mm^2 culture dishes with either FLAG-SIRT1^{WT} or FLAG-SIRT1-E2$^{mut\text{-}AA}$ constructs. After indicated treatments, cells were washed and lysed in TNN-buffer and immunoprecipitation was done using anti-FLAG-M2 beads. Elution was performed with 100 μL of 10 mM Tris pH 8.0, 150 mM NaCl, 150 μg/mL FLAG-peptide on a thermomixer (16 °C, 3 h, 1,000 rpm). To determine relative expression of the eluted fusion proteins, sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) Western blotting was done using diluted eluate, anti-Flag antibody and anti-mouse-HRP secondary antibody, chemiluminescent-detection, and imaging on GE-Amersham Imager-600. Expression ratio of SIRT1$^{WT/mut\text{-}AA}$ was estimated via densitometry in ImageJ (NIH) and loads in subsequent assays were adjusted proportionally.

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1. C. E. Geisler, C. Hepler, M. R. Higgins, B. J. Renquist, Hepatic adaptations to maintain metabolic homeostasis in response to fasting and refeeding in mice. *Nutr. Metab. (Lond.)* **13**, 62 (2016).
2. F. Dang *et al.*, Fasting and feeding signals control the oscillatory expression of Angptl8 to modulate lipid metabolism. *Sci. Rep.* **6**, 36926 (2016).
3. M. R. Soeters, P. B. Soeters, M. G. Schooneman, S. M. Houten, J. A. Romijn, Adaptive reciprocity of lipid and glucose metabolism in human short-term starvation. *Am. J. Physiol. Endocrinol. Metab.* **303**, E1397–E1407 (2012).
4. C. Chen, P. F. Williams, G. J. Cooney, I. D. Caterson, J. R. Turtle, The effects of fasting and refeeding on liver glycogen synthase and phosphorylase in obese and lean mice. *Horm. Metab. Res.* **24**, 161–166 (1992).
5. E. Mezey, Liver disease and nutrition. *Gastroenterology* **74**, 770–783 (1978).
6. M. C. Petersen, D. F. Vatner, G. I. Shulman, Regulation of hepatic glucose metabolism in health and disease. *Nat. Rev. Endocrinol.* **13**, 572–587 (2017).
7. J. D. Horton, Y. Bashmakov, I. Shimomura, H. Shimano, Regulation of sterol regulatory element binding proteins in livers of fasted and refed mice. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 5987–5992 (1998).
8. J. Y. Altarejos, M. Montminy, CREB and the CRTC co-activators: Sensors for hormonal and metabolic signals. *Nat. Rev. Mol. Cell Biol.* **12**, 141–151 (2011).
9. M. Naims, C. Arous, E. Van Obberghen, Energetic cell sensors: A key to metabolic homeostasis. *Trends Endocrinol. Metab.* **21**, 75–82 (2010).
10. T. Narita, B. T. Weinert, C. Choudhary, Functions and mechanisms of non-histone protein acetylation. *Nat. Rev. Mol. Cell Biol.* **20**, 156–174 (2019).
11. B. Manjyadath *et al.*, Loss of hepatic oscillatory fed microRNAs abrogates refed transition and causes liver dysfunction. *Cell Rep.* **26**, 2212–2226.e7 (2019).
12. S. Jitrapakdee, Transcription factors and coactivators controlling nutrient and hormonal regulation of hepatic gluconeogenesis. *Int. J. Biochem. Cell Biol.* **44**, 33–45 (2012).
13. L. Rui, Energy metabolism in the liver. *Compr. Physiol.* **4**, 177–197 (2014).
14. A. Purushotham *et al.*, Hepatocyte-specific deletion of SIRT1 alters fatty acid metabolism and results in hepatic steatosis and inflammation. *Cell Metab.* **9**, 327–338 (2009).
15. A. S. Banks *et al.*, SirT1 gain of function increases energy efficiency and prevents diabetes in mice. *Cell Metab.* **8**, 333–341 (2008).
16. F. Xu *et al.*, Lack of SIRT1 (Mammalian Sirtuin 1) activity leads to liver steatosis in the SIRT1−/− mice: A role of lipid mobilization and inflammation. *Endocrinology* **151**, 2504–2514 (2010).
17. P. T. Pfluger, D. Herranz, S. Velasco-Miguel, M. Serrano, M. H. Tschöp, Sirt1 protects against high-fat diet-induced metabolic damage. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 9793–9798 (2008).
18. R. B. Ding, J. Bao, C. X. Deng, Emerging roles of SIRT1 in fatty liver diseases. *Int. J. Biol. Sci.* **13**, 852–867 (2017).
19. X. Li, SIRT1 and energy metabolism. *Acta Biochim. Biophys. Sin. (Shanghai)* **45**, 51–60 (2013).
20. T. Yang, M. Fu, R. Pestell, A. A. Sauve, SIRT1 and endocrine signaling. *Trends Endocrinol. Metab.* **17**, 186–191 (2006).
21. M. C. Sugden, P. W. Caton, M. J. Holness, PPAR control: It's SIRTainly as easy as PGC. *J. Endocrinol.* **204**, 93–104 (2010).
22. R. Ponugoti *et al.*, SIRT1 deacetylates and inhibits SREBP-1C activity in regulation of hepatic lipid metabolism. *J. Biol. Chem.* **285**, 33959–33970 (2010).
23. J. H. Lim *et al.*, Sirtuin 1 modulates cellular responses to hypoxia by deacetylating hypoxia-inducible factor 1alpha. *Mol. Cell* **38**, 864–878 (2010).
24. T. Kawahara *et al.*, Constitutive SIRT1 overexpression impairs mitochondria and reduces cardiac function in mice. *J. Mol. Cell. Cardiol.* **51**, 1026–1036 (2011).
25. D. Wu, Y. Qiu, X. Gao, X. B. Yuan, Q. Zhai, Overexpression of SIRT1 in mouse forebrain impairs lipid/glucose metabolism and motor function. *PLoS One* **6**, e21759 (2011).
26. L. Vilà *et al.*, AAV-mediated *Sirt1* overexpression in skeletal muscle activates oxidative capacity but does not prevent insulin resistance. *Mol. Ther. Methods Clin. Dev.* **5**, 16072 (2016).
27. B. P. Hubbard, D. A. Sinclair, Small molecule SIRT1 activators for the treatment of aging and age-related diseases. *Trends Pharmacol. Sci.* **35**, 146–154 (2014).
28. J. C. Milne *et al.*, Small molecule activators of SIRT1 as therapeutics for the treatment of type 2 diabetes. *Nature* **450**, 712–716 (2007).
29. S. Guo, Insulin signaling, resistance, and the metabolic syndrome: Insights from mouse models into disease mechanisms. *J. Endocrinol.* **220**, T1–T23 (2014).
30. F. Liang, S. Kume, D. Koya, SIRT1 and insulin resistance. *Nat. Rev. Endocrinol.* **5**, 367–373 (2009).
31. R. H. Wang *et al.*, Hepatic Sirt1 deficiency in mice impairs mTorc2/Akt signaling and results in hyperglycemia, oxidative damage, and insulin resistance. *J. Clin. Invest.* **121**, 4477–4490 (2011).
32. N. R. Sundaresan *et al.*, The deacetylase SIRT1 promotes membrane localization and activation of Akt and PDK1 during tumorigenesis and cardiac hypertrophy. *Sci. Signal.* **4**, ra46 (2011).
33. J. T. Rodgers, P. Puigserver, Fasting-dependent glucose and lipid metabolic response through hepatic sirtuin 1. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 12861–12866 (2007).
34. S. Deota *et al.*, Identification of a tissue-restricted isoform of SIRT1 defines a regulatory domain that encodes specificity. *Cell Rep.* **18**, 3069–3077 (2017).
35. F. Ghiwya *et al.*, The N-terminal domain of SIRT1 is a positive regulator of endogenous SIRT1-dependent deacetylation and transcriptional outputs. *Cell Rep.* **10**, 1665–1672 (2015).
36. T. C. Krzysiak *et al.*, An insulin-responsive sensor in the SIRT1 disordered region binds DBC1 and PACS-2 to control enzyme activity. *Mol. Cell* **72**, 985–998.e7 (2018).
37. M. Pan, H. Yuan, M. Brent, E. C. Ding, R. Marmorstein, SIRT1 contains N- and C-terminal regions that potentiate deacetylase activity. *J. Biol. Chem.* **287**, 2468–2476 (2012).
38. M. M. Babu, The contribution of intrinsically disordered regions to protein function, cellular complexity, and human disease. *Biochem. Soc. Trans.* **44**, 1185–1200 (2016).
39. G. W. Hart, Dynamic O-linked glycosylation of nuclear and cytoskeletal proteins. *Annu. Rev. Biochem.* **66**, 315–335 (1997).
40. L. Wells, K. A. Whelan, G. W. Hart, O-GlcNAc: A regulatory post-translational modification. *Biochem. Biophys. Res. Commun.* **302**, 435–441 (2003).
41. M. R. Bond, J. A. Hanover, O-GlcNAc cycling: A link between metabolism and chronic disease. *Annu. Rev. Nutr.* **33**, 205–229 (2013).
42. N. E. Zachara, G. W. Hart, Cell signaling, the essential role of O-GlcNAc! *Biochim. Biophys. Acta* **1761**, 599–617 (2006).
43. C. Slawson, M. P. Housley, G. W. Hart, O-GlcNAc cycling: How a single sugar post-translational modification is changing the way we think about signaling networks. *J. Cell. Biochem.* **97**, 71–83 (2006).
44. C. Butkinaree, K. Park, G. W. Hart, O-linked beta-N-acetylglucosamine (O-GlcNAc): Extensive crosstalk with phosphorylation to regulate signaling and transcription in response to nutrients and stress. *Biochim. Biophys. Acta* **1800**, 96–106 (2010).
45. S. A. Whelan, M. D. Lane, G. W. Hart, Regulation of the O-linked beta-N-acetylglucosamine transferase by insulin signaling. *J. Biol. Chem.* **283**, 21411–21417 (2008).
46. J. T. Rodgers *et al.*, Nutrient control of glucose homeostasis through a complex of PGC-1alpha and SIRT1. *Nature* **434**, 113–118 (2005).
47. Y. Kanfi *et al.*, Regulation of SIRT1 protein levels by nutrient availability. *FEBS Lett.* **582**, 2417–2423 (2008).
48. C. Han *et al.*, O-GlcNAcylation of SIRT1 enhances its deacetylase activity and promotes cytoprotection under stress. *Nat. Commun.* **8**, 1491 (2017).
49. J. Ford, S. Ahmed, S. Allison, M. Jiang, J. Milner, JNK2-dependent regulation of SIRT1 protein stability. *Cell Cycle* **7**, 3091–3097 (2008).
50. Z. Gao *et al.*, Sirtuin 1 (SIRT1) protein degradation in response to persistent c-Jun N-terminal kinase 1 (JNK1) activation contributes to hepatic steatosis in obesity. *J. Biol. Chem.* **286**, 22227–22234 (2011).
51. L. Peng *et al.*, Ubiquitinated sirtuin 1 (SIRT1) function is modulated during DNA damage-induced cell death and survival. *J. Biol. Chem.* **290**, 8904–8912 (2015).
52. J. R. Revollo, X. Li, The ways and means that fine tune Sirt1 activity. *Trends Biochem. Sci.* **38**, 160–167 (2013).
53. D. Ramachandran *et al.*, Sirt1 and mir-9 expression is regulated during glucose-stimulated insulin secretion in pancreatic β-islets. *FEBS J.* **278**, 1167–1174 (2011).
54. S. Schenk *et al.*, Sirt1 enhances skeletal muscle insulin sensitivity in mice during caloric restriction. *J. Clin. Invest.* **121**, 4781–4788 (2011).
55. A. C. Leney, D. El Atmioui, W. Wu, H. Ovaa, A. J. R. Heck, Elucidating crosstalk mechanisms between phosphorylation and O-GlcNAcylation. *Proc. Natl. Acad. Sci. U.S.A.* **114**, E7255–E7261 (2017).
56. B. Zschoernig, U. Mahlknecht, SIRTUIN 1: Regulating the regulator. *Biochem. Biophys. Res. Commun.* **376**, 251–255 (2008).
57. T. Sasaki *et al.*, Phosphorylation regulates SIRT1 function. *PLoS One* **3**, e4020 (2008).
58. H. Kang, J. W. Jung, M. K. Kim, J. H. Chung, CK2 is the regulator of SIRT1 substrate-binding affinity, deacetylase activity and cellular response to DNA-damage. *PLoS One* **4**, e6611 (2009).
59. D. Kong *et al.*, SIRT1-mediated ERβ suppression in the endothelium contributes to vascular aging. *Aging Cell* **15**, 1092–1102 (2016).
60. C. Tong *et al.*, Impaired SIRT1 nucleocytoplasmic shuttling in the senescent heart during ischemic stress. *FASEB J.* **27**, 4332–4340 (2013).
61. M. Tanno, J. Sakamoto, T. Miura, K. Shimamoto, Y. Horio, Nucleocytoplasmic shuttling of the NAD+-dependent histone deacetylase SIRT1. *J. Biol. Chem.* **282**, 6823–6832 (2007).
62. N. Nasrin *et al.*, JNK1 phosphorylates SIRT1 and promotes its enzymatic activity. *PLoS One* **4**, e8414 (2009).
63. X. Yang, K. Qian, Protein O-GlcNAcylation: Emerging mechanisms and functions. *Nat. Rev. Mol. Cell Biol.* **18**, 452–465 (2017).
64. T. Insel, M. Kuo, O-GlcNAc modification of transcription factors, glucose sensing and glucotoxicity. *Trends Endocrinol. Metab.* **19**, 380–389 (2008).
65. H. S. Kwon, M. Ott, The ups and downs of SIRT1. *Trends Biochem. Sci.* **33**, 517–525 (2008).
66. V. D. Longo, B. K. Kennedy, Sirtuins in aging and age-related disease. *Cell* **126**, 257–268 (2006).
67. J. E. Kim, J. Chen, Z. Lou, DBC1 is a negative regulator of SIRT1. *Nature* **451**, 583–586 (2008).
68. M. Hatori *et al.*, Time-restricted feeding without reducing caloric intake prevents metabolic diseases in mice fed a high-fat diet. *Cell Metab.* **15**, 848–860 (2012).
69. A. Chaix, A. Zarrinpar, P. Miu, S. Panda, Time-restricted feeding is a preventative and therapeutic intervention against diverse nutritional challenges. *Cell Metab.* **20**, 991–1005 (2014).
70. A. Chaix, T. Lin, H. D. Le, M. W. Chang, S. Panda, Time-restricted feeding prevents obesity and metabolic syndrome in mice lacking a circadian clock. *Cell Metab.* **29**, 303–319.e4 (2019).
71. G. Sulli, E. N. C. Manoogian, P. B. Taub, S. Panda, Training the circadian clock, clocking the drugs, and drugging the clock to prevent, manage, and treat chronic diseases. *Trends Pharmacol. Sci.* **39**, 812–827 (2018).
72. V. D. Longo, S. Panda, Fasting, circadian rhythms, and time-restricted feeding in healthy lifespan. *Cell Metab.* **23**, 1048–1059 (2016).
73. K. Kinouchi *et al.*, Fasting imparts a switch to alternative daily pathways in liver and muscle. *Cell Rep.* **25**, 3299–3314.e6 (2018).
74. L. Bordone *et al.*, SIRT1 transgenic mice show phenotypes resembling calorie restriction. *Aging Cell* **6**, 759–767 (2007).
75. D. Herranz, M. Serrano, SIRT1: Recent lessons from mouse models. *Nat. Rev. Cancer* **10**, 819–823 (2010).