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Review

Salt-Inducible Kinases: Physiology, Regulation by cAMP, and Therapeutic Potential

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Salt-inducible kinases (SIKs) represent a subfamily of AMP-activated protein kinase (AMPK) family kinases. Initially named because SIK1 (the founding member of this kinase family) expression is regulated by dietary salt intake in the adrenal gland, it is now apparent that a major biological role of these kinases is to control gene expression in response to extracellular cues that increase intracellular levels of cAMP. Here, we review four physiologically relevant examples of how cAMP signaling impinges upon SIK cellular function. By focusing on examples of cAMP-mediated SIK regulation in gut myeloid cells, bone, liver, and skin, we highlight recent advances in G protein-coupled receptor (GPCR) signal transduction. New knowledge regarding the role of SIKs in GPCR signaling has led to therapeutic applications of novel small molecule SIK inhibitors.

Highlights

Salt-inducible kinases (SIKs) control the phosphorylation and subcellular localization of two key classes of transcriptional regulatory factors: class IIa histone deacetylases (HDACs) and cAMP-regulated transcriptional coactivators (CRTCs).

SIK activity is inhibited by upstream signals that increase intracellular cAMP levels.

cAMP-regulated SIK inhibition is a key component in the cellular effects of multiple hormones and paracrine-acting factors.

Small molecule SIK inhibitors represent a novel therapeutic strategy to mimic cAMP-inducing signals in the settings of inflammatory bowel disease, osteoporosis, and skin pigmentation.

Background on SIKs and Their Regulation by cAMP Signaling

The AMPK family contains 14 members that play broad roles in cellular metabolism [1]. Activation of AMPK family members occurs via phosphorylation of the activation loop kinase domain by liver kinase B1 (LKB1) [2]. Within the AMPK family, the subfamily of SIKs contains three kinases (SIK1, SIK2, and SIK3) whose physiologic roles are the subject of this review article. SIK1, the founding member of this subfamily, was identified and named as a kinase whose expression is induced in the adrenal gland of rats fed a high-salt diet [3]. Subsequent homology searches led to the identification of SIK2 and SIK3. All three SIK family members share a characteristic structure, including an N-terminal kinase domain bearing an LKB1 phosphorylation site, a sucrose non-fermenting-1 homology domain, and a long C-terminal extension containing multiple potential protein kinase A phosphorylation sites [4].

All three SIK family kinases are expressed broadly. SIK1 mRNA expression is regulated by multiple stimuli, including high dietary salt intake, adrenocorticotropin hormone signaling [5], glucagon signaling [6], excitable cell depolarization [7], and circadian rhythms [8]. In contrast, SIK2 and SIK3 expression is constitutive in tissues in which these kinases are expressed. In humans, SIK2 and SIK3 are expressed ubiquitously, with the highest SIK2 levels in adipose tissue and the highest SIK3 expression in brain. Interestingly, SIK2 and SIK3 are closely linked on human chromosome 11 and mouse chromosome 9.

Initial studies to define the substrate specificity of SIKs were performed *in vitro* using recombinant SIK1 kinase domain fragments. Through this approach, a canonical substrate phosphorylation motif of LxB(S/T)xS*xxxL (B, basic amino acid; X, any amino acid) was defined [4]. In subsequent years, multiple physiologically important SIK substrates have been identified. Currently, the best-studied SIK substrates are class IIa histone deacetylases (HDACs 4, 5,

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7, and 9) [9] and cAMP-regulated transcriptional coactivators (CRTCs 1, 2, and 3) [10]. Phosphorylation by SIKs plays a crucial role in regulating subcellular localization and biologic activity of class IIa HDACs and CRTC proteins. When phosphorylated, these SIK substrates are retained in the cytoplasm due to association with cytoplasmic 14-3-3 chaperones. When dephosphorylated, these SIK substrates are able to translocate into the nucleus, where they regulate gene expression. In the nucleus, class IIa HDACs function as potent inhibitors of myocyte enhancer factor-2 (MEF2)-driven gene expression [9] and can activate forkhead family transcription factors [11,12], while CRTC factors potentiate the activity of cAMP-responsive element (CRE) binding protein (CREB) and related basic leucine zipper (bZIP) family transcription factors [10]. Beyond class IIa HDACs and CRTC proteins, additional tissue-specific SIK substrates have been suggested [13–15] and are discussed below.

A key role of SIKs is to control dynamic changes in phosphorylation and subcellular localization of class IIa HDACs and CRTC factors. Therefore, upstream control of SIK activity provides an opportunity to integrate diverse extracellular cues into changes in MEF2- and CREB-driven gene expression. In general, SIK cellular activity is tonically in the 'on' state due to constitutive LKB1-mediated phosphorylation [2,16,17]. SIK-mediated phosphorylation of class IIa HDAC and CRTC proteins leads to their cytoplasmic retention and latent inactivation [9,10,18]. Signals that increase intracellular cAMP levels lead to protein kinase A (PKA)-mediated SIK family member phosphorylation [19,20]. PKA-mediated phosphorylation does not alter SIK intrinsic kinase activity [21,22]. However, mutation of PKA phosphoacceptor sites leads to SIK variants whose cellular activity cannot be inhibited by cAMP-inducing signals [23]. PKA-mediated SIK phosphorylation promotes interaction between SIK and 14-3-3 proteins [18,24]. This PKA-inducible SIK/14-3-3 association leads to conformational changes and/or shifts in SIK cytoplasmic distribution that block the ability of these kinases to access and phosphorylate their substrates. As discussed below, reducing CRTC phosphorylation via small molecule SIK inhibitors appears to be sufficient to stimulate CREB-dependent gene expression, even in the absence of boosting cellular cAMP levels. Therefore, the relative importance of PKA-dependent CREB versus SIK phosphorylation in stimulating CREB/CRTC-mediated transcriptional output remains to be determined.

Recent work demonstrated that, of the three SIK isoforms, SIK2 is unique in that it bears four separate PKA phosphorylation sites (SIK1 and SIK3 each have two PKA sites) that, when phosphorylated, serve as 14-3-3 docking sites [24]. Therefore, the cellular activity of all SIK family members can be inhibited by upstream cAMP-inducing signals, with SIK2 perhaps best poised to be blocked by PKA-activating agents. While the role of PKA-mediated SIK1 and SIK2 phosphorylation *in vivo* remains to be explored, a SIK3 mutant allele lacking these PKA phosphorylation sites was identified during a screen for randomly mutagenized mice with disrupted sleep patterns [25]. Of the three SIK isoforms, SIK3 expression is highest in brain. Interestingly, brain phosphoproteomic analysis of these SIK3 gain-of-function mice versus littermate controls revealed increased phosphorylation of synaptic regulatory proteins, indicating a novel role for SIK3 in sleep-related neurotransmission [26]. Although cAMP-activated PKA is a well-accepted mechanism to reduce cellular SIK activity, less is known about the upstream signals that stimulate basal SIK function. Since LKB1 is the best-known SIK activator [2], it is possible that signals that induce LKB1 function [17] may also increase SIK activity.

To highlight the physiologic significance of these signaling events, selected examples of GPCR-linked cAMP-PKA-SIK signaling pathways are now discussed. Although each example reviewed participates very different cellular physiology ranging from cytokine production to bone remodeling to skin pigmentation, the general theme that SIK inhibition is a key

downstream step in cAMP signaling events clearly emerges. Moreover, in each instance, key aspects of hormonal signaling action are mimicked using small molecule SIK inhibitors, hinting at possible new therapeutic strategies.

The Role of SIKs Downstream of Prostaglandins in Gut Myeloid Cells

Crohn's disease and ulcerative colitis are the most common forms of inflammatory bowel disease (IBD), a chronic disorder arising, in part, from impaired anti-inflammatory immune mechanisms that result in an imbalance between pro- and anti-inflammatory cytokines [27]. Multiple lines of evidence from human and mouse genetics have highlighted a central role for the anti-inflammatory cytokine interleukin (IL)-10 in IBD. Impaired IL-10 production by gut-resident myeloid cells drives intestinal inflammation; therefore, boosting IL-10 levels could yield therapeutic anti-inflammatory effects in the appropriate setting.

Relative levels of nuclear factor-κB (NF-κB)- and CREB-target expression programs represent key regulatory nodes governing IL-10 production by myeloid cells [28]. Disruption of signaling pathways activating NF-κB downstream of microbial recognition with small molecule inhibitors of protein kinase C (PKC) or glycogen synthase kinase 3β (GSK3β) upregulates IL-10 production by stimulated macrophages [29,30]. CREB activation by EP2/EP4 prostanoid receptor agonists such as prostaglandin E_2 (PGE_2) promotes IL-10 production by myeloid cells [31]. Recently, SIKs have been identified as key components of the CREB activation cascade downstream of PGE_2 [32,33]. In quiescent myeloid cells, SIKs phosphorylate the CREB transcriptional co-activator CRTC3, resulting in its sequestration by cytoplasmic 14-3-3 proteins. PKA activation following EP2/EP4 stimulation inhibits SIK activity and activates CREB, enabling CRTC3 to enhance transcription of IL-10.

Several of these IL-10 pathway regulators are encoded by genes that are also associated with increased risk for IBD, strengthening the link between defective IL-10 signaling and intestinal inflammation (Figure 1). SNPs near the genetic loci encoding the EP4 prostanoid receptor and CRTC3 both confer increased risk for IBD [34]. Furthermore, an intronic SNP in *SIK2* itself confers susceptibility to primary sclerosing cholangitis, an inflammatory liver disease that is strongly associated with IBD [35]. The central role of PKC, GSK3β, and SIKs in IL-10

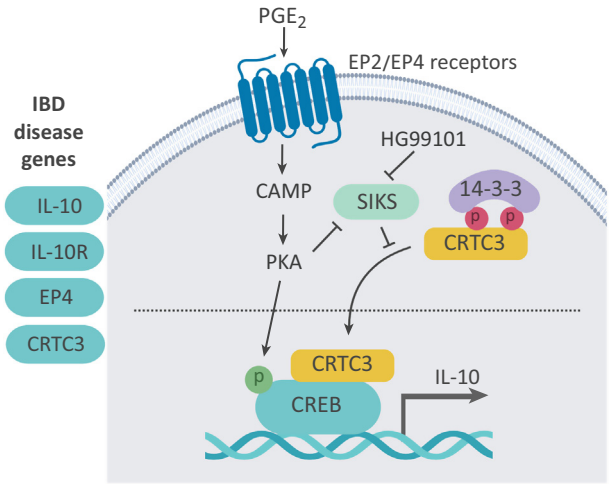

Figure 1. Human Disease Genetics Implicates EP4-SIK2-CRTC3-CREB Pathway in the Regulation of IL-10 Levels and IBD Susceptibility. Prostaglandin E2 (PGE_2) signaling, through protein kinase A (PKA), leads to salt-inducible kinase (SIK) inhibition and therefore reduced phosphorylation of the SIK substrate cAMP-regulated transcriptional coactivator 3 (CRTC3). Un-phosphorylated CRTC3 translocates to the nucleus where it drives interleukin (IL)-10 expression. Small molecule SIK inhibitors such as HG99101 show PGE_2-like effects and boost IL-10 production. CREB, cAMP-responsive element binding protein; EP4, E-type prostanoid receptor 4; IBD, irritable bowel disease; IL10R, interleukin-10 receptor.

production suggests that kinase inhibitors are likely to modulate IL-10 production by dendritic cells (DCs) and macrophages.

To identify compounds that enhance anti-inflammatory IL-10 and suppress proinflammatory IL-12 production, more than 150 kinase inhibitors were recently screened in bone marrow-derived DCs [36]. SIK inhibition was identified as a common feature of several kinase inhibitors identified that enhanced IL-10 production. HG99101 analogs with altered selectivities for SIKs 1, 2, and 3 were used to determine the relationship between SIK isoform inhibition and IL-10 induction. The strong positive correlation between the potencies of SIK2 inhibition and IL-10 upregulation suggested that SIK2 is the key family member regulating IL-10 induction. Consistent with this observation, expression of an HG99101-resistant SIK2 'gatekeeper' mutant is reported to suppress IL-10 potentiation by HG99101 in myeloid cells [23].

Next, selective SIK inhibitors for *in vivo* use were developed [37]. Molecular modeling was used to guide structure–activity relationship optimization of HG99101. Analysis of derivatives synthesized based on modeling predictions identified the analog YKL05099. This compound showed biochemical and cell-based activities consistent with SIK inhibition and improved pharmacokinetic properties suitable for use *in vivo*. Kinome-wide profiling of YKL05099 revealed that this compound inhibits several protein kinases in addition to SIK1, SIK2, and SIK3, including Brk, Lck, Eph-A4, and p38a. YKL05099 modulates cytokine responses *in vivo*. Mice treated with YKL05099 displayed reduced SIK substrate phosphorylation, increased IL-10 production, and reduced tumor necrosis factor (TNF)-α levels in serum and colonic epithelium. Importantly, daily dosing with YKL05099 for 1 week did not yield metabolic defects observed with chronic SIK2 inhibition by genetic deletion. The improved pan-SIK inhibitor YKL05099 was also utilized in mechanistic studies of SIK signaling events in bone and skin, as discussed below.

Notably, the effect of small molecule SIK inhibitors on cytokine production is distinct from that of activation by PGE_2 [36]. While both enhance IL-10 production, SIK inhibitors suppress proinflammatory cytokines including IL-12 and TNF-α to a greater extent than PGE_2, suggesting that SIK inhibition converts gut-derived myeloid cells to an anti-inflammatory phenotype marked by enhancing IL-10 production and reducing production of inflammatory cytokines. These observations support the pursuit of SIK inhibitors as potential therapies to regionally modulate cytokines. Moreover, SIK inhibitors represent a unique class of small molecules for the treatment of IBD because of their dual effect of enhancing IL-10 and dampening IL-12 production. Current therapies such as anti-TNF agents target only one side of this equilibrium, are broadly immunosuppressive, and can cause severe side effects. There is an urgent need to discover new therapies for these disorders that restore cytokine balance within local compartments of the gut. While protein-based therapies are used to effectively treat a wide variety of complex immune-mediated disorders, they have several limitations, notably an inability to regulate intracellular proteins identified as targets by genetic and functional studies. Small molecules constitute a complementary approach to protein-based immunomodulatory drug development by enabling modulation of intracellular networks that regulate cytokine signaling [38]. The recent successful example of Janus kinase inhibitors for rheumatoid arthritis highlights how small molecule kinase inhibitors can be used to target redundancies within cytokine signaling networks for chronic diseases.

The Role of SIKs Downstream of Parathyroid Hormone Action in Bone

Parathyroid hormone (PTH) is a peptide hormone that plays a crucial role in maintaining blood calcium levels [39]. In response to hypocalcemia, PTH is released and acts to maintain

normocalcemia through effects in bone and kidney. Since 99% of total body calcium is stored in bone, skeletal PTH action is key in maintaining calcium homeostasis. Chronic hyperparathyroidism is a disease in which overproduction of PTH from the parathyroid glands causes release of calcium from bone and increases risk of fractures [40]. However, PTH also stimulates bone formation and concomitant slow entry of calcium into bone, perhaps in an attempt to protect from excessive bone loss in response to intermittent hypocalcemia. This property of PTH to alter bone formation is the basis for its pharmacologic use as treatment for osteoporosis in the form of intermittent (once daily) injection of PTH analogs teriparatide or abaloparatide [41,42].

In bone, PTH targets multiple cell types to promote bone resorption and to stimulate bone formation. Osteocytes, terminally differentiated osteoblasts embedded within mineralized matrix, are the most abundant cell type in bone and the cells expressing the highest numbers of PTH receptors [43]. In response to PTH, osteocytes regulate the expression of paracrine-acting factors, such as sclerostin and receptor activator of NF-κB ligand (RANKL), that, in turn, modulate the activity of osteoblasts and osteoclasts on bone surfaces. Sclerostin is a secreted WNT inhibitor that blocks osteoblast activity [44]; reducing its expression is one mechanism used by PTH to stimulate bone formation [45]. RANKL (TNFSF11) is the major osteoclastogenic cytokine made by osteocytes and osteoblasts [46]; increasing its expression represents the major pathway through which PTH stimulates bone resorption [47].

Recently, a key role for SIKs has been described in PTH signaling in osteocytes [48]. MEF2 family transcription factors control sclerostin expression [49,50], and class IIa HDACs block MEF2C action in osteocytes [51]. CREB and related bZIP factors drive RANKL gene expression [52,53]. PTH signaling in a conditionally immortalized osteocyte cell line [54] leads to rapid SIK2 phosphorylation at multiple PKA sites. Subsequent to this, PTH signaling leads to reduced phosphorylation of HDAC4, HDAC5, and CRTC2 and the nuclear translocation of these key SIK substrates. HDAC4 and HDAC5 are required for PTH to suppress sclerostin expression both *in vitro* and *in vivo*. In contrast, CRTC2 is required for PTH-regulated RANKL upregulation *in vitro* [48].

Since SIK2 suppression leads to regulation of key PTH target genes in osteocytes, the effect of the small molecule SIK inhibitor YKL05099 [55] (discussed above in the setting of its initial development as a compound that induces IL-10 in myeloid lineage cells in the gut) on gene expression in osteocytes was tested. As predicted, this compound led to PTH-like changes, including reduced HDAC4 and HDAC5 phosphorylation, CRTC2 nuclear translocation, increased RANKL, and reduced sclerostin expression. Remarkably, RNA sequencing profiling revealed substantial transcriptomic overlap of PTH and small molecule SIK inhibitors. Furthermore, 2-week *in vivo* treatment with YKL05099 led to increased bone formation and increased bone mass in young, eugonadal mice [48]. A working model for the central role of SIK inhibition in skeletal PTH action is shown in Figure 2.

While these PTH-like effects of YKL05099 were predicted, it was surprising that the SIK inhibitor also reduced osteoclast numbers and activity. Indeed, these inhibitory effects on osteoclast numbers and activity occurred despite clear increases in bone RANKL gene expression after *in vivo* SIK inhibitor treatment, similar to the effects of SIK inhibitors on cultured osteocytes *in vitro*. Therefore, while the possibility remains that the effects of YKL05099 on bone are different *in vitro* and *in vivo*, these results suggest that SIKs may directly regulate osteoclast function. Future studies are needed to clarify the mechanism through which SIK inhibitors boost bone formation and reduce bone resorption, including investigation as to whether there might be a distinct cell-autonomous role for SIKs in osteoclasts [56]. Since

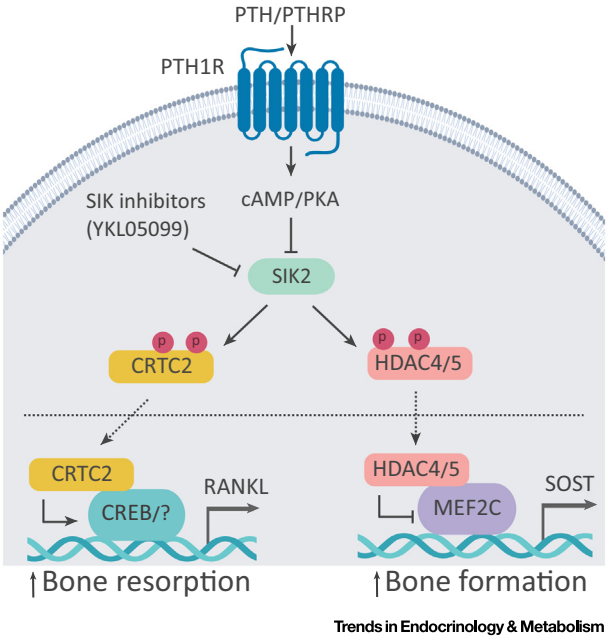

Figure 2. PTH Signaling in Osteocytes Inhibits SIK2 Activity via Protein Kinase A-Mediated Phosphorylation. By reducing salt-inducible kinase (SIK) activity, parathyroid hormone (PTH) reduces phosphorylation levels of SIK substrates including class IIa histone deacetylases (HDACs) and cAMP-regulated transcriptional coactivator (CRTC) family members. Upon dephosphorylation, these factors translocate to the nucleus. Class IIa HDACs block myocyte enhancer factor-2C (MEF2C)-driven sclerostin (SOST) expression. CRTC2 stimulates receptor activator of nuclear factor-κB ligand (RANKL) expression driven by cAMP-responsive element binding protein (CREB) and other basic leucine zipper family transcription factors. Small molecule SIK inhibitors such as YKL05099 mimic the actions of PTH, both *in vitro* and *in vivo*. As schematized here, SIK inhibition represents an intracellular mechanism to ensure that PTH signaling stimulates both bone formation and bone resorption. The small p circles indicate phosphorylation. PTHrP, parathyroid hormone-related peptide; PTH1R, parathyroid hormone 1 receptor.

YKL05099 can potentially inhibit multiple kinases in addition to SIK2, future studies using genetic approaches and more specific small molecule inhibitors are necessary to address this important question. Furthermore, future studies are needed to address the questions of functional redundancy between SIK isoforms in bone, as discussed below in the setting of hepatic glucagon action. Nonetheless, these observations indicate that SIK inhibition is a key signaling mechanism used by PTH to accomplish its physiologic effects in osteocytes. Future work is needed to determine whether the actions of PTH its other cellular targets in bone and kidney utilize an analogous mechanism.

The Role of SIKs Downstream of Hepatic Glucagon Action

Hepatic gluconeogenesis is controlled by the pancreatic hormones insulin and glucagon that regulate transcription of the rate-controlling enzymes glucose-6-phosphatase (encoded by the *G6pc* gene) and phosphoenolpyruvate carboxykinase (encoded by the *Pck1* gene) in hepatocytes. In the fed state, insulin inhibits hepatic glucose production, whereas glucagon upregulates gluconeogenesis during prolonged fasting. Dysregulation of these processes contributes to the development of type 2 diabetes [57]. Over recent years, SIKs have emerged as major downstream effectors of glucagon action in the regulation of hepatic gluconeogenesis.

In the fed state, hepatic insulin signaling reduces the expression of *G6pc* and *Pck1* through Akt-mediated phosphorylation of forkhead box protein-O (FoxO) transcription factors [58]. FoxO phosphorylation by Akt results in binding to 14-3-3 proteins, sequestering FoxO in the cytoplasm and thereby inhibiting gluconeogenic gene expression (Figure 3). Under fasting conditions, glucagon upregulates gluconeogenic gene expression through the PKA-mediated phosphorylation and activation of the transcription factor CREB, which binds to the *G6pc* and *Pck*1 gene promoters [59,60]. PKA-mediated CREB phosphorylation (on Ser133) induces

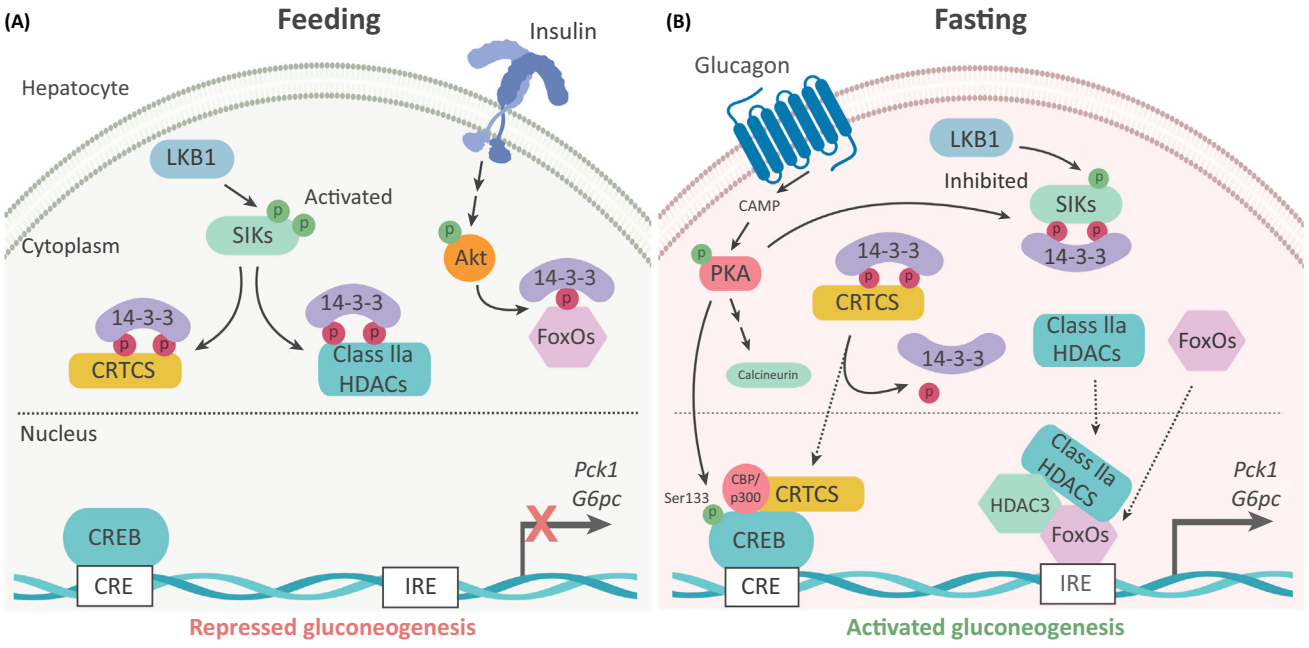

Figure 3. Model of SIK-Dependent Gluconeogenesis Regulation in Liver. (A) Under feeding conditions, Akt-dependent phosphorylation of the forkhead box protein-Os (FoxOs) transcription factor results in binding to 14-3-3 proteins, sequestering FoxOs into the cytoplasm. In parallel, the liver kinase B1 (LKB1)-dependent salt-inducible kinases (SIKs) activation induces the phosphorylation of the cofactors cAMP-regulated transcriptional coactivators (CRTCs) and class IIa histone deacetylase (HDAC) and their binding to the 14-3-3 proteins and retention into the cytoplasm, leading to the repression of gluconeogenic gene expression (*G6pc* and *Pck1)*. (B) During fasting, glucagon/cAMP-activated protein kinase A (PKA) phosphorylates the transcription factor cAMP-responsive element binding protein (CREB) on Ser133, promoting the recruitment of the coactivator proteins CREB binding protein (CBP)/p300. In parallel, PKA inhibits SIKs through phosphorylation-dependent interactions with 14-3-3 proteins, resulting in loss of CRTCs and HDACs phosphorylation. The glucagon-PKA pathway also activates the phosphatase calcineurin that contributes to CRTCs dephosphorylation. Dephosphoryled CRTCs and class IIa HDACs then translocate to the nucleus where CRTCs cooperate with CREB and CBP/p300, while class IIa HDACs recruit HDAC3, causing deacetylation of FoxOs to gluconeogenic gene promoters to activate their transcription. The small p circles indicate phosphorylation; green p circles indicate activating phosphorylation; and pink p circles indicate inhibitory phosphorylation. CRE, cAMP-responsive element; IRE, insulin-responsive element.

recruitment of the histone acetyltransferases p300 and CREB binding protein (CBP) [61]. In concert, the activation of the cAMP-PKA pathway also triggers the dephosphorylation and nuclear translocation of the CRTCs (CRTCs 1, 2, and 3) [6] that then bind to CREB to stimulate gluconeogenic gene transcription (Figure 3).

CRTC coactivators play a key role in the regulation of hepatic gluconeogenesis [6]. During feeding, CRTC2 and CRTC3 are sequestered in the cytoplasm through phosphorylation-dependent interactions with 14-3-3 proteins. Hepatic CRTCs are regulated through phosphorylation at multiple sites by SIKs (SIKs 1, 2, and 3) [6,18,62]. During fasting the activity of all three SIK isoforms is modulated by multiple phosphorylation events outside of the T-loop/kinase domain by the cAMP-PKA pathway. Indeed, as noted earlier, PKA-mediated phosphorylation of the SIKs inhibits their catalytic activity by inducing 14-3-3 protein associations [24], leading to CRTC dephosphorylation and translocation to the nucleus, where they induce the transcription of gluconeogenic genes. The dephosphorylation of CRTCs seems to be, in part, mediated by the cAMP-PKA-dependent activation of the phosphatase calcineurin [63] (Figure 3).

Similar to CRTCs, under fed conditions the localization of class IIa HDACs (HDACs 4, 5, 7, and 9) to the nucleus is inhibited through SIKs-mediated phosphorylation and subsequent 14-3-3

protein interactions, leading to cytoplasmic sequestration. In response to glucagon, PKA-dependent inactivation of SIKs promotes dephosphorylation of class IIa HDACs and their translocation to the nucleus, where they recruit HDAC3 to *G6pc* and *Pck1* promoters and regulate FoxO acetylation, resulting in transcriptional activation of gluconeogenic genes [11] (Figure 3).

AMPK can also phosphorylate CRTCs and class IIa HDACs [6,11]. However, although AMPK was considered as a key regulator of hepatic gluconeogenesis, liver-specific AMPK-deficient mice are normoglycemic and their hepatic gluconeogenesis is unchanged [64–66]. In addition, the use of a small molecule (A769662) that specifically activates AMPK without modifying the cellular AMP/ATP ratio had no effect on hepatic glucose production measured in isolated hepatocytes or *in vivo* [65,67]. In contrast, liver-specific ablation of upstream kinase LKB1 causes increased glucose production in hepatocytes *in vitro* and fasting hyperglycemia *in vivo* [64,65,68]. Although the SIK2 isoform was also proposed to be critical in controlling the gluconeogenic gene program [6,69], liver-specific ablation of SIK2 alone has no effect on glycemia and hepatic gluconeogenesis, and insulin does not modulate SIK2 phosphorylation/activity [23]. Similarly, liver-specific deletion of SIK1 has no impact on hepatic gluconeogenesis [70]. Unexpectedly, global SIK3-deficient mice are hypoglycemic [71]. Since SIK3 is deleted in all tissues in these mice, it is difficult to appreciate the role of hepatic SIK3 in this mouse model, especially since most SIK3 knockout mice die shortly after birth due to abnormal chondrocyte hypertrophy [72]. Finally, pan-SIK inhibitors recapitulated the entire phenotype observed in LKB1-deficient hepatocytes, including dephosphorylation of CRTC2 and CRTC3 and HDAC4 and HDAC5, associated to an increase in gluconeogenic gene expression and glucose production [23,65]. These observations suggest that SIK isoforms play a redundant role in the control of hepatic gluconeogenesis. Furthermore, de-repression of gluconeogenesis observed in response to LKB1 deletion or SIKs inhibition indicates that the LKB1-SIK pathway functions as a key gluconeogenic gatekeeper for hepatic glucose production by keeping the gluconeogenic program repressed, and this can be released by fasting/glucagon signals [23].

With regard of their critical role in the suppression of hepatic glucose production, SIKs may serve as therapeutic targets to treat hyperglycemia in diabetic patients. Indeed, SIK inhibition is sufficient to induce gluconeogenesis [23]. Thus, dysregulation of SIK activity may be involved in the impairment of glycemic control in diabetes. Consistent with this hypothesis, SIK1 and SIK2 activities are decreased concomitantly with CRTC2 dephosphorylation/activation in livers of diabetic models [69,73,74]. In contrast, overexpression of SIKs in liver is sufficient to normalize blood glucose levels in diabetic mice [6]. Therefore, therapeutic strategies that specifically activate hepatic SIK activity may curb excessive hepatic glucose output and thus improve glycemic control in patients with type 2 diabetes.

The Role of SIKs Downstream of MSH Effects in Melanocytes

Mammalian pigmentation is typically described as either constitutive or adaptive. Constitutive pigmentation is genetically determined, and highly polymorphic in many species, whereas adaptive pigmentation responds to environmental signals and is best characterized for UV irradiation/sunlight. Numerous signaling events have been demonstrated to impact control of melanin synthesis [75]. However a central and rate-limiting pathway regulating pigmentation is triggered by the GPCR melanocortin 1 receptor (MC1R) and its ligand α-melanocyte-stimulating hormone (MSH). Non-functional variants of MC1R are present in individuals with the redhair-lightskin phenotype [76,77]. The recognition that redhaired individuals have poor or non-existent tanning responses led to the observation that UV produces strong (>30-fold) p53-mediated induction of pro-opiomelanocortin (POMC)/α-MSH peptide within epidermal

keratinocytes [78,79]. UV's direct effect on melanocytes produced only weak (~50%) upregulation of POMC/α-MSH. MC1R activity triggers cAMP surges via adenylate cyclase and was shown to upregulate expression of the melanocyte master transcription factor MITF, via a conserved CRE motif in its (melanocyte-specific) promoter. The red/blond pigment within hair of non-functional MC1R ('redhair') variants is called pheomelanin and is produced via cysteine- or glutathione-mediated reduction of oxidized tyrosine metabolites. The 'switch' between pheomelanin and brown/black eumelanin species occurs when activity of CREB and expression of MITF are strongly induced downstream of cAMP, thereby stimulating tyrosinase expression (the tyrosine-oxidizing enzyme), consuming and depleting intracellular thiols, and resulting in an alternative thiol-free pathway of melanin biosynthesis. Thus, the control over red/blond versus brown/black pigmentation is traceable to the magnitude of MC1R/cAMP signals.

The SIK-CRTC pathway has been demonstrated to potently modulate melanin synthesis within the melanocyte lineage. A landmark study by Horike *et al.* [80] studied this signaling response within the B16 murine melanoma cell line. The investigators observed that all three family isoforms of both SIK and CRTC were expressed in the melanocyte lineage. They found that overexpression of the CRTCs induced cellular hyperpigmentation as well as induction of CREB activity, MITF, and tyrosinase, all of which were suppressed by simultaneous overexpression of SIK2. Importantly, the authors crossed mice exhibiting red hair (due to overexpression of a natural antagonist of Mc1r) together with germline $SIK2^{-/-}$ mice and observed dose-dependent 'rescue' of dark fur pigmentation [80]. Collectively, these data established a key role for the SIK-CRTC-CREB/MITF pathway in control of pigmentation.

A more recent study [81] asked whether the eumelanin repressive activity of SIK could be targeted using skin-permeable small molecule antagonists. This concept was an extension of a prior study [79] in which topical forskolin (adenylate cyclase agonist) was shown to induce dark pigmentation in redhaired mice lacking functional Mc1r. The darkening effect of forskolin in redhaired mice suggested that cAMP induction was sufficient to stimulate the CREB/MITF axis and had previously been assumed to reflect direct phosphorylation/activation of CREB by PKA. This 'sunless tanning' was further shown to have produced chemically indistinguishable eumelanin compared to genetically black mice and to protect from UV-induced DNA damage, sunburn cell formation, and skin carcinogenesis. While topical forskolin produced impressive murine skin melanization, it was subsequently observed to be impermeable to human skin, which is approximately 5 times as thick as mouse skin. The study of Mujahid *et al.* examined a series of small molecule SIK inhibitors, iteratively modifying their chemical features including lipophilicity and size, aiming to enhance human skin penetration. Several chemical species were ultimately identified that retained cell-based SIK inhibitory activity, induction of MITF, and hyperpigmentation, while also being capable of triggering significant darkening of discarded human skin specimens. Melanization was also accompanied by trafficking of melanin-containing melanosomes from melanocytes into epidermal keratinocytes, where they are maintained in an 'umbrella'-like configuration over the superficial ('sun-exposed') side of the keratinocyte nucleus, a pattern of intracellular trafficking phenomenon that is known to occur after UV-induced tanning/pigmentation. Topical SIK inhibitor was also seen to potently darken the skin of redhaired mice lacking wild-type Mc1r. Collectively, these studies demonstrated that either forskolin/cAMP or SIK inhibition could strongly induce dark eumelanin pigmentation in the redhair/lightskin genetic background and that small molecule SIK inhibitors can be designed that are capable of penetrating human skin. Important remaining questions include a better understanding of the precise role of PKA in either suppressing SIK2, stimulating CREB, or both. Do these activities vary by genetic background among humans with different pigmentation

features? By what pathway is SIK activity regulated constitutively in human skin? Is SIK activity diminished within skin of darkly pigmented humans? If so, via what mechanism(s)?

The Therapeutic Potential of Small Molecule SIK Inhibitors

Kinases have been intensively studied as drug targets, with 38 kinase inhibitors currently FDA approved. While most of these agents are used for oncologic indications, success stories of kinase inhibitors for non-oncologic indications exist [82]. As discussed above, recent evidence indicates that beneficial effects of SIK inhibition might include increased bone mass (by mimicking PTH action) [48], modulation of inflammatory cytokine production (by mimicking PGE_2 action) [32,36,83], and increased skin pigmentation (by mimicking MSH action) [81]. Moreover, liver-specific SIK activation may be of benefit for treatment of type 2 diabetes. Finally, recent evidence indicates that catecholamine- and subsequent PKA-induced SIK inhibition participates in adaptive thermogenesis in brown adipose tissue [84].

In addition to these physiology-based mechanisms, it is clear that SIK inhibition may be of benefit in certain malignancies. For example, SIK2 is overexpressed in certain high-grade serous ovarian cancers in which it functions as a centrosome kinase in cell cycle progression [14]. Moreover, SIK2 may promote omental ovarian cancer metastasis by activating the phosphatidylinositol 3-kinase pathway [15]. The small molecule SIK inhibitor ARN3236 shows promising efficacy in ovarian cancer xenograft models [85]. Other malignancies in which SIK inhibition may be of benefit include triple negative breast cancer [86], prostate cancer [87], and certain subtypes of acute myelogenous leukemia (AML) [88,89]. AML is a particularly interesting example in which a 'rational' therapeutic role for SIK inhibitors may emerge: specific mixed lineage leukemia-fusion AML subtypes harbor high MEF2 activity and are sensitive to genetic and pharmacologic manipulations that target LKB1/SIK3 [89].

Along these lines, a major focus for future efforts will be to identify and optimize potency and specificity of small molecule SIK inhibitors, especially if these agents are to be used for non-oncologic indications. Compounds such as HG99101 [32], MRT67307 [32], and YKL05099 [55] represent invaluable starting points in these efforts. Moreover, recent unbiased profiling of 243 clinical kinase inhibitors revealed that SIK2 is a relatively common target amongst inhibitors designed to block other kinases [90]. Efforts for structure-based drug design are currently limited by the lack of SIK crystal structures.

Concluding Remarks and Future Perspectives

As discussed above, recent evidence points towards a central role for SIK inhibition in the physiologic intracellular actions of several cAMP-linked signals. Despite these advances, major outstanding questions (see Outstanding Questions) remain. First, do important intracellular SIK substrates exist beyond class IIa HDACs and CRTC proteins? Second, kinase inhibition alone is insufficient to explain the rapid reductions in SIK substrate phosphorylation seen after cAMP signaling or treatment of cells with small molecule SIK inhibitors. Therefore, future studies are needed to identify the role of phosphatases in SIK-mediated class IIa HDAC- and CRTC-regulated nuclear translocation. Third, as discussed above, discordant phenotypes are observed comparing the effects of small molecule SIK inhibitors and *in vivo* deletion of individual SIK isoforms. For this reason, *in vivo* mouse genetics studies are needed to address the potential for functional redundancy among the members of this family. Finally, it is possible that HG99101, MRT67307, ARN3236, and YKL05099 represent relatively 'early' efforts in the development of small molecule SIK inhibitors for therapeutic use. Future development of more

Outstanding Questions

Do cell-type-specific SIK substrates exist to account for unique transcriptional outputs downstream of a shared cAMP-regulated signaling mechanism?

Do cAMP-regulated phosphatases participate in rapid signal-dependent reductions in phosphorylation of SIK substrates?

Can cellular SIK activity be increased by signals that stimulate LKB1 kinase activity?

By what pathway is SIK activity regulated constitutively in human skin? Is SIK activity diminished within skin of darkly pigmented humans?

What are the functional similarities and differences between the three distinct members of the SIK family of kinases?

What is the safety profile associated with long-term pharmacologic SIK inhibition?

potent and specific agents will only be boosted by enhanced knowledge of the role of these kinases in physiology and pathophysiology.

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