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## Targeting hepatic glucose output in the treatment of type 2 diabetes

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### Abstract

Type 2 diabetes mellitus is characterized by the dysregulation of glucose homeostasis resulting in hyperglycemia. Although current diabetes treatments have exhibited some success in lowering blood glucose, their effect is not always sustained and their use may be associated with undesirable side effects, such as hypoglycemia. Novel diabetic drugs, which may be used in combination with existing therapies, are therefore needed. The potential of specifically targeting the liver in order to normalize blood glucose levels has not been fully exploited. Here, we review the molecular mechanisms controlling hepatic gluconeogenesis and glycogen storage, and assess the prospect of therapeutically targeting associated pathways to treat type 2 diabetes.

### Introduction

Type 2 diabetes is the seventh leading cause of death in the United States and affects more than an estimated 29.1 million Americans, with 1.7 million new diagnoses per year<sup>1</sup>. Additionally, more than 86 million Americans are considered pre-diabetic. Worldwide, this disease is found in 9% of the adult population and directly causes at least 1.5 million deaths annually. Moreover, diabetes significantly increases comorbidities of several other chronic health problems, including cardiovascular disease, stroke, and kidney disease, which contribute heavily to the diabetes health and cost burden<sup>2</sup>. The debilitating and chronic nature of type 2 diabetes requires effective and long-lasting drug treatments.

Therapies for type 2 diabetes must ameliorate its pathophysiology, the hallmark of which is decreased insulin secretion and/or insulin insensitivity<sup>3</sup>. In normal individuals, insulin is secreted by the pancreas to decrease glucose production and increase uptake of blood glucose into peripheral tissues such as skeletal muscle and adipose tissue (Figure 1). In diabetes, decreased insulin release and/or suppressed insulin action leads to increased glucose production and decreased glucose uptake by peripheral tissues, resulting in elevated blood glucose levels.

Although several existing type 2 diabetes drugs lower blood glucose levels - including metformin<sup>4</sup>, sulfonylureas<sup>5</sup>, glucagon-like peptide 1 (GLP-1) agonists<sup>6</sup>, glitazones/

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#### Competing Interests Statement

The authors declare no competing financial interests.

thiazolidinediones (TZDs)<sup>7</sup>, alpha-glucosidase inhibitors<sup>8</sup>, sodium-glucose co-transporter 2 (SGLT2) inhibitors<sup>9</sup>, and bile acid sequestrants<sup>10</sup> (Box 1) - these therapies each have their limitations and drawbacks. In particular, the most widely used drug, metformin, although known to decrease hepatic gluconeogenesis, does not have a well-defined molecular target<sup>4</sup>, and is associated with gastrointestinal side effects<sup>11</sup>. Other classes of drugs are also accompanied by side effects, and may cause hyperinsulinemia, sometimes resulting in hypoglycemia<sup>12-14</sup>. Novel therapeutic approaches are therefore warranted.

Existing diabetes drugs target only a subset of the potentially druggable pathways that regulate glucose homeostasis, particularly in the liver<sup>15</sup>. As the chief producer of glucose that is released to the circulation, the liver is an especially important organ in controlling blood sugar<sup>16</sup>. Moreover, in addition to its ability to produce glucose, the liver can also store glucose as glycogen or utilize glucose as a carbohydrate fuel. Leveraging novel targets for decreasing liver glucose production or promoting liver glucose storage may enable more efficient suppression of diabetic symptoms. Such agents would likely be used combinatorially with existing drug therapies when enhanced suppression of blood glucose levels is necessary, and may enable the use of lower concentrations of individual drugs, thereby limiting side effects.

A small number of liver-targeted approaches, which aim to reduce gluconeogenesis or promote glycogen storage, are already being investigated in the clinic. These include glucokinase activators<sup>17-24</sup>, glycogen phosphorylase inhibitors<sup>25-34</sup>, glucagon receptor antagonists<sup>35-41</sup>, fructose 1,6-bisphosphatase inhibitors<sup>42-44</sup>, as well as protein tyrosine phosphatase 1B inhibitors<sup>45-54</sup>, with new potential targets continuing to emerge. In this review, we will provide an overview of the physiology and molecular pathways of liver glucose homeostasis, gluconeogenesis and glycogenolysis, assessing current and novel type 2 diabetes drug targets.

## Glucose homeostasis and type 2 diabetes

Circulating blood glucose is derived from intestinal absorption from food, glucose production (or gluconeogenesis), and glycogen breakdown (or glycogenolysis)<sup>55</sup>. The liver is a major metabolic organ, and among its many functions, it is chiefly responsible for gluconeogenesis (with the kidneys contributing an average 20% to glucose release<sup>56</sup>, and the gut supplying up to 15–20%<sup>57</sup>) and glycogenolysis<sup>58</sup>. Gluconeogenesis and glycogenolysis by the liver lead to glucose release into the blood and a rise in blood glucose levels, with subsequent uptake of glucose by peripheral tissues including the kidney, skeletal muscle, splanchnic organs, and adipose tissue<sup>59</sup> (Figure 1). Following feeding, postprandial insulin is secreted from pancreatic beta cells to directly inhibit glycogenolysis and increase glycogen synthesis<sup>60,61</sup>, and to indirectly inhibit gluconeogenesis through suppression of glucagon and inhibition of adipose free fatty acid release<sup>62</sup>. Insulin also preferentially increases uptake of glucose by peripheral tissues<sup>63</sup>. Conversely, during times of fasting, glucagon is secreted from pancreatic alpha cells to increase gluconeogenesis and glycogenolysis in the liver<sup>64</sup>. Insulin levels also fall, and peripheral tissues utilize proportionately less glucose as fuel relative to fatty acids. In the 24 hours following absorption of a meal, glycogenolysis in the liver substantially contributes to glucose release into the circulation<sup>65,66</sup>.

The nexus of gluconeogenesis and glycogenolysis lies at glucose-6-phosphate, which is a phosphorylated form of glucose that cannot be exported by the cell until it is dephosphorylated by glucose-6-phosphatase (G-6-Pase, Figure 2). In gluconeogenesis, the substrates lactate, amino acids, and glycerol are used by the liver to generate glucose-6-phosphate<sup>67</sup>. During glycogenolysis, glycogen is broken down and converted to glucose-6-phosphate. Glycogen, which consists of polymerized glucose, serves as a readily mobilized storage pool of carbohydrate fuel which can be phosphorylated and quickly used to generate glucose when needed.

Normally, glucose production and uptake are kept in balance to maintain glucose homeostasis and a fasting blood glucose range of 70 to 90 mg/dL in humans<sup>68</sup>. Type 2 diabetes develops when insufficient insulin is produced to maintain this level of blood glucose, generally as a combined result of insulin resistance in peripheral tissues and beta cell dysfunction, leading to persistently elevated blood glucose, or hyperglycemia<sup>68</sup>. As diabetes is a chronic condition, its progression is characterized by advanced beta cell failure<sup>69</sup>. Additionally, aberrantly high levels of glucagon can persist in diabetic patients, leading to elevated rates of gluconeogenesis and glycogenolysis in the liver that contribute to hyperglycemia<sup>70,71</sup>. In diabetic patients, gluconeogenesis also has a proportionately larger contribution to glucose production relative to normal individuals<sup>72</sup>. Moreover, the ability of diabetic patients to store glycogen may be decreased compared to healthy individuals, which also enhances postprandial hyperglycemia<sup>73</sup>. Prolonged hyperglycemia during diabetes causes vascular dysfunction that damages major organs, including the kidney, brain, heart, blood vessels, and eyes<sup>74</sup>. Thus, treating the hyperglycemia of diabetes is of the utmost importance in these patients.

## Strategies to modulate liver glucose and glycogen metabolism

Overall, an imbalance in glucose release from the liver and uptake from peripheral tissues can lead to the persistent hyperglycemia that is a major contributing factor to diabetes development. Therefore, drugs to treat type 2 diabetes can be aimed at maintaining normal blood glucose levels through inhibition of gluconeogenesis and/or glycogenolysis in the liver, or through stimulation of glucose uptake into tissues. The most commonly used type 2 diabetes therapy, metformin, is known to decrease hepatic gluconeogenesis, but the precise molecular mechanisms of this agent have not been well defined<sup>4</sup> (see Box 1). Moreover, metformin can cause various side effects and it typically must be used in combination with other drugs in order to achieve long term suppression of hyperglycemia<sup>11,75</sup>. Other liver-targeted agents which have been or are currently being investigated in clinical trials include: activators of glucokinase (which converts glucose to glucose-6-phosphate in the first step of glycolysis)<sup>17-23</sup>, inhibitors of fructose 1,6-bisphosphatase (which converts fructose-1,6-bisphosphate to fructose 6-phosphate in gluconeogenesis)<sup>42-44</sup>, inhibitors of protein tyrosine phosphatase 1B (which is a negative regulator of the insulin signaling pathway)<sup>45-54</sup>, inhibitors of glycogen phosphorylase (which catalyzes the rate-limiting step in glycogenolysis)<sup>25-34</sup>, as well as antagonists of the glucagon receptor<sup>35-41</sup>. However, so far, no agents in these categories have received approval. The development of novel agents aimed at targeting gluconeogenesis and glycogen breakdown in the liver may provide effective alternative diabetic treatment options. Potential therapeutic strategies include the

direct targeting of specific metabolic enzymes of gluconeogenesis and glycogenolysis, or of regulators of these processes.

### Glucokinase activation

Glucokinase (GK), an enzyme predominately expressed in the liver and pancreas which phosphorylates glucose to glucose-6-phosphate in the first step of glycolysis, may be activated to inhibit gluconeogenesis. Unfortunately, several early studies with direct allosteric GK activators, such as AZD1656 (AstraZeneca)<sup>17</sup>, were abandoned during the clinical phases of research due to a decline in efficacy of the compounds over time, as well as hypoglycemic effects<sup>18</sup>. However, several GK activators remain in clinical development<sup>18</sup>. The liver-selective GK inhibitor PF-04991532 (Pfizer) exhibited favorable glycemic effects in diabetic rats<sup>19</sup>, but newer findings indicated the presence of oxidative metabolites of the compound in human plasma<sup>20</sup>, which will require further careful examination if development is continued. More recent clinical studies employing the GK activators piragliatin (Roche) and AMG 151 (Amgen; previously Array BioPharma (ARRY-403)) demonstrated that both agents resulted in hypoglycemia, with a slight increase in circulating triglycerides for the latter<sup>21,22</sup>. The outcomes of a 6-month phase II clinical trial with a liver-selective GK activator TTP399 (Trans Tech Pharm) are pending following promising short-term effects, including glucose lowering after 6 weeks without hypoglycemia or increased plasma lipids<sup>24</sup>.

Another approach to activating GK is to inhibit its binding to GK regulatory protein (GKRP). GKRP binds cytoplasmic GK when glucose levels decrease, sequestering GK in the nucleus in its inactive form. The benefit of this approach is that liver-specific GKRP inhibition decreases blood glucose without the risk of hypoglycemia that can result from activating GK or inhibiting pancreatic GKRP<sup>76</sup>. The piperazine compound AMG-3969 dissociated GK and GKRP in rat livers, and decreased blood glucose in diabetic but not normoglycemic mice<sup>23</sup>. These findings suggest that GKRP inhibitors may represent promising candidates for clinical investigation.

### PTP-1B inhibition

Insulin exerts suppressive effects on glucose production through the promotion of insulin receptor phosphorylation, which triggers downstream signaling<sup>77</sup>. Protein tyrosine phosphatase 1B (PTP-1B) dephosphorylates the insulin receptor, opposing the action of insulin. Since phosphorylation of the insulin receptor is decreased in type 2 diabetes<sup>78</sup>, PTP-1B is an attractive diabetes drug target. Mice deficient in PTP-1B display enhanced hepatic insulin sensitivity, as indicated by an increase in phosphorylation of the insulin receptor in liver; furthermore, these mice are resistant to gaining weight on a high fat diet<sup>79</sup>. Mice with PTP-1B specifically deleted in the liver display enhanced hepatic insulin signaling, improved glucose tolerance and homeostasis, and increased suppression of hepatic glucose production in insulin-resistant and high fat diet-induced obesity models<sup>80,81</sup>. These and other studies further justify targeting hepatic PTP-1B for diabetes treatment<sup>82</sup>. Studies in monkeys and mice have reported that ISIS 113715 (ISIS Pharmaceuticals), an antisense oligonucleotide targeting PTP-1B that is not liver-specific, reduces fasting concentrations of glucose, while improving insulin sensitivity and decreasing hepatic triglyceride

accumulation<sup>46,47</sup>. ISIS 113715 has shown promising effects in decreasing fasting blood glucose levels in combination with sulfonylureas in a phase II clinical trial involving type 2 diabetes patients<sup>45</sup>.

Targeting PTP-1B with small molecule inhibitors has also been attempted. Ertiprotafib (Wyeth Pharmaceuticals; acquired by Pfizer) showed initial promise by lowering blood glucose levels when orally administered to mice<sup>48</sup> and progressed to a phase II clinical trial. Further studies also indicated decreased insulin levels, along with lowered triglyceride and free fatty acid levels; however, these outcomes were reported to be likely due to off-target effects of ertiprotafib, which include activation of peroxisome proliferator-activated receptor  $\alpha$  (PPAR $\alpha$ ) and PPAR $\gamma$ <sup>49</sup>, and potent inhibition of I $\kappa$ B kinase  $\beta$  (IKK- $\beta$ )<sup>50</sup>. Phase II trials with ertiprotafib were discontinued, likely due to its lack of *in vivo* efficacy, coupled with unwanted side effects<sup>49</sup>. Trodusquemine (MSI-1436), developed by Genaera Corporation (formerly Magainin Pharmaceuticals), is a spermine analog that was initially observed to modulate body weight, glucose homeostasis and serum cholesterol levels in *ob/ob* mice<sup>51</sup>, and was later determined to selectively inhibit PTP-1B<sup>52</sup>. As of 2013, trodusquemine was in phase II clinical trials for treatment of obesity and diabetes<sup>53</sup>. More recently, a chemical biology approach has uncovered an allosteric mechanism of PTP-1B inhibition by trodusquemine<sup>54</sup>. Carefully designed small molecules allosterically targeting PTP-1B could enhance specificity, which could improve *in vivo* efficacy and reduce unwanted off-target effects.

### Glycogen synthesis stimulation

**Glycogen phosphorylase inhibition**—One method to inhibit glucose release by the liver is to increase its storage as glycogen. In diabetic patients, hepatic glycogen synthesis is impaired<sup>83</sup> and the stimulation of glycogen synthesis in skeletal muscle by insulin is stunted, contributing to insulin resistance<sup>84</sup>. Ectopic lipid accumulation in the liver and skeletal muscle leads to diminished insulin signaling and decreased hepatic glycogen synthesis<sup>85</sup>. Human liver glycogen phosphorylase inhibitors can increase glycogen synthesis by interfering with glucose-6-phosphate generation during glycogenolysis<sup>26</sup>. A major appeal of these compounds is that they inhibit the enzyme at elevated blood glucose levels, but their potency diminishes when blood glucose is lowered, decreasing the likelihood of developing hypoglycemia<sup>27</sup>. Indeed, treatment of *db/db* mice with the dihydropyridine diacid glycogen phosphorylase inhibitor U6751 or *ob/ob* mice with CP-91149 lowered blood glucose without producing hypoglycemia, but had no effect on blood glucose levels in normal mice<sup>28,29</sup>. On a molecular level, indole-2-carboxamides act as allosteric inhibitors that bind to the indole site at the dimer interface of glycogen phosphorylase in order to stabilize its inactive conformation, and which also synergize with other inhibitors such as glucose<sup>30</sup>. Notably, the IC<sub>50</sub> of a compound designed to fit into both chloroindole binding pockets of glycogen phosphorylase was only 6 nM<sup>30</sup>.

The glycogen phosphorylase inhibitor FR258900 was discovered in a screen for increased glycogen synthesis in rat primary hepatocytes<sup>86</sup>. FR258900 is a 23-carbon pentanedioic acid that binds to the AMP site of glycogen phosphorylase and stabilizes its inactive conformation<sup>87</sup>. This compound significantly decreased plasma glucose levels and increased

hepatic glycogen levels in *db/db* and streptozocin-induced diabetic mouse models<sup>31</sup>, but long-term effects or actions in other tissues were not studied. CP-316,819, is an indole carboxamide compound<sup>32</sup> that decreased hepatic glucose output in fasted dogs with basal or elevated glucagon levels<sup>33</sup>, although thorough analysis of potential effects on other tissues was not performed. Another commercially available glycogen phosphorylase inhibitor, BAY R 3401, which acts by a similar mechanism as indole carboxamides, also successfully decreased hepatic glucose output and plasma glucose levels in fasted dogs<sup>34</sup>.

Limited clinical studies were performed with CP-316,819, which showed lowering of peak hyperglycemia after a glucagon challenge in normal subjects<sup>25</sup>. A related compound, CP-368,962, was able to dose-dependently lower blood glucose in type 2 diabetic human subjects, but the effect was lost after 4 weeks of treatment, demonstrating a lack of durability<sup>25</sup>. However, glycogen phosphorylase has five different ligand binding sites including its catalytic and AMP-binding sites, revealing several possible methods for targeting its enzyme activity<sup>88</sup>. Thus, although glycogen phosphorylase inhibitors may have thus far failed due to lack of durability, alternative approaches may enable the development of an inhibitor that is able to overcome this barrier.

A potential problem with targeting glycogen phosphorylase is that it is also present in the skeletal muscle, where it is needed to support energetic deficits<sup>89</sup>. The issue of tissue selectivity could be abated by selecting an inhibitor that is more potent against the liver isoform<sup>90</sup>. However, tissue selectivity may not be a substantial confounding issue, as a study in rat gastrocnemius-plantaris-soleus muscle suggested that treatment with glycogen phosphorylase inhibitors did not deplete skeletal muscle function<sup>89</sup>. Additionally, glycogen phosphorylase inhibitors may be beneficial to cardiac muscle, where they can reduce glycolysis and proton production which are harmful during myocardial ischemia<sup>91</sup>.

**Glycogen synthase activation**—Glycogen synthesis can also be targeted through activation of glycogen synthase<sup>92</sup>. Although glycogen phosphorylase inhibition can decrease inhibition of glycogen synthase<sup>93</sup>, this enzyme could also be targeted independently. A major regulatory kinase for glycogen synthase is glycogen synthase kinase 3 (GSK-3), which phosphorylates and inactivates glycogen synthase<sup>94</sup>. GSK-3 levels and activity are increased in skeletal muscle of patients with type 2 diabetes<sup>95</sup>, and in the adipose tissue of obese diabetic mice<sup>96</sup>, suggesting that GSK-3 contributes to insulin resistance.

Two maleimide compounds, SB-216763 and SB-415286, were discovered to be GSK-3 inhibitors within the nanomolar range with considerable specificity over other kinases<sup>97</sup>. These compounds were effective in activating glycogen synthase in human liver cells<sup>97</sup>, and suppressed PEPCCK and G-6-Pase in hepatoma cells<sup>98</sup>. Other specific and potent GSK-3 inhibitors have also been used *in vivo*<sup>99</sup>. The aminopyrimidine derivatives CHIR-99021 and CHIR-98023 significantly increased glucose disposal in Zucker diabetic rats, which was accompanied by an increase in hepatic glycogen synthesis with no change in skeletal muscle glycogen synthesis<sup>100</sup>. These inhibitors also lowered blood glucose in *db/db* mice without causing hypoglycemia<sup>101</sup>. In addition, the GSK-3 peptide inhibitor L803-mts decreased blood glucose and PEPCCK expression, and increased hepatic glycogen in *ob/ob* mice<sup>102</sup>.

While the effects of GSK3 inhibitors on blood glucose are promising, hesitation to move these inhibitors to the clinic may stem from concerns over their potential activation of the oncogene  $\beta$ -catenin, although no studies have shown increased tumorigenicity with GSK-3 inhibitor use<sup>103</sup>. Moreover, potent GSK-3 inhibition can lead to toxicity (as occurs with lithium treatment in the brain, causing motor deficits<sup>104</sup>), so care must be taken to only mildly inhibit the enzyme. AZD1080, a GSK-3 inhibitor from AstraZeneca, exhibited some success in limited phase I clinical trials for Alzheimer's disease, but blood glucose was not measured<sup>105</sup>. Another GSK-3 inhibitor, tideglusib (Noscria), was used in a phase II clinical trial for progressive supranuclear palsy<sup>106</sup>. Although tideglusib was generally found to be safe, it was not effective in treating progressive supranuclear palsy and the effects on blood glucose were not evaluated. Concerns were raised surrounding the irreversible nature of this inhibitor<sup>107</sup>, which may make toxicity more likely and its clinical use difficult.

### Glucagon receptor antagonism

In patients with type 2 diabetes, increased secretion of glucagon from pancreatic  $\alpha$ -cells, in addition to heightened hepatic sensitivity towards glucagon, contributes to the elevated hepatic glucose production observed in these individuals<sup>108,109</sup>. Targeting the glucagon receptor may therefore be a promising approach for decreasing hepatic glucose production and reducing fasting plasma glucose levels<sup>110</sup>. In support of this approach, two glucagon receptor peptide antagonists decreased blood glucose and improved insulin sensitivity in *ob/ob* and diet-induced obese mice<sup>111</sup>. In mice expressing human glucagon receptor, the glucagon antagonist compound 1 decreased blood glucose in response to glucagon administration<sup>112</sup>. In addition, NNC 25-0926, a potent glucagon antagonist with an  $IC_{50}$  of 12 nm, blunted the glucagon-induced increase in blood glucose in fasted dogs<sup>113</sup>. Additionally, the selective and reversible competitive glucagon receptor antagonist compound 9m (also known as MK-0893 (Merck)), lowered glucagon-induced blood glucose in rhesus monkeys, and in humanized *ob/ob* mice fed a high fat diet<sup>39</sup>.

Glucagon receptor antagonists have been used in several studies in humans. The orally available Bay 27-9955 (Bayer) decreased short-term fasting glucose levels without exhibiting any prominent side effects<sup>37</sup>, but was no longer pursued for clinical development for undisclosed reasons. LGD-6972 (Ligand Pharmaceuticals) similarly demonstrated a dose-dependent decrease in fasting glucose in normal and diabetic subjects and favorable safety profiles in a phase 1b clinical study, and a phase II trial is currently underway to better assess its safety and efficacy<sup>36</sup>. In addition, once daily dosing with PF-06291874 (Pfizer) decreased fasting and postprandial glucose levels in patients on metformin, with or without combined treatment with a sulfonylurea, for up to 28 days<sup>35</sup>. However, although this drug exhibited little risk of hypoglycemia, it was associated with a small increase in LDL cholesterol and aminotransferase levels. A similar increase in LDL cholesterol levels was observed in clinical trials of MK-0893, resulting in discontinuation of this agent<sup>38,39</sup>. While an initial short-term clinical study with LY2409021 (Eli Lilly) showed encouraging decreases in fasting and postprandial glucose levels, they also raised concerns due to a rise in aminotransferases<sup>40</sup>. However, a more recent longer duration phase II study indicated that doses of LY2409021 which are sufficient to lower fasting glucose levels, only modestly increased aminotransferase levels, and clinical development is ongoing<sup>41</sup>. These results

suggest that glucagon antagonists may be titrated for therapeutic use with minimal side effects.

### Modulating pyruvate flux

**Pyruvate carboxylase inhibition**—Insulin has been found to decrease hepatic glucose production by suppressing pyruvate flux through inhibition of adipose lipolysis, which decreases hepatic acetyl-CoA, a potent activator of pyruvate carboxylase<sup>114</sup>. Therefore, inhibiting pyruvate carboxylase may be a strategy for diabetes treatment. Indeed, in a study of human liver biopsies, pyruvate carboxylase levels correlated significantly with hyperglycemia<sup>115</sup>. Furthermore, inhibition of pyruvate carboxylase through antisense oligonucleotide targeting in the liver and adipose tissue of high fat diet-fed and diabetic mice lowered blood glucose and rates of gluconeogenesis<sup>115</sup>. Additionally, adiposity and hepatic steatosis were decreased owing to reduced glycerol synthesis. Although phenylalkanoic acids can inhibit pyruvate carboxylase, and phenylpropionic acid decreased gluconeogenesis at least acutely in normal and diabetic rats<sup>116</sup>, these compounds lack tissue specificity. Additionally, phenylalkanoic acids may inhibit insulin secretion, as phenylacetic acid treatment of cultured beta cells and rat islets decreased glucose-stimulated insulin release<sup>117</sup>. Therefore, although this is a promising antidiabetic strategy, improved pharmacological or non-pharmacological approaches would need to be developed in order to selectively inhibit this enzyme in a clinical setting.

**Inhibition of mitochondrial pyruvate import**—Another method to inhibit pyruvate flux into gluconeogenesis is to block pyruvate transport across the inner mitochondrial membrane into the mitochondrial matrix, where it generates oxaloacetate for use in glucose production<sup>118</sup>. A heterologous complex of mitochondrial pyruvate carrier 1 (MPC1) and MPC2 is necessary and sufficient for this transport of pyruvate<sup>119,120</sup>. Independent studies assessed the role of these proteins in the control of gluconeogenesis. Liver MPC1 KO mice exhibited compensatory increased glutamine usage and urea cycle activity, which also produce oxaloacetate, preventing a decrease in basal gluconeogenesis<sup>121</sup>. However, despite this compensation, hyperglycemia was decreased and glucose tolerance was improved in the MPC1 KO mice fed a high fat diet. Similarly, knockout of hepatic MPC2 induced compensatory amino acid metabolism and decreased hyperglycemia in *db/db* and streptozotocin-induced models of diabetes<sup>122</sup>.

The effects of MPC1 and MPC2 on blood glucose in diabetes models suggest that targeting of these proteins by drugs may represent a promising diabetes therapy. The established MPC inhibitor UK-5099 suppressed glucose production from primary hepatocytes<sup>121</sup> and increased glucose uptake into myocytes<sup>123</sup>. Intriguingly, TZD compounds (Box 1) can also directly inhibit the MPC and improve glucose handling in a PPAR $\gamma$ -independent manner<sup>123,124</sup>. Specific targeting of MPC in the liver may therefore improve blood glucose levels without leading to undesirable side effects due to effects on other tissues, as can occur with TZDs. In addition, such liver-specific MPC inhibition would ensure that pyruvate uptake into tissues that are critically dependent on glucose as a fuel source is not inhibited. However, the potential for compensatory effects would need to be carefully evaluated in a clinical setting, particularly with regard to the increase in glutamine metabolism, as



glutamine anaplerosis and oxidation can help fuel the TCA cycle and fatty acid synthesis which drive tumorigenesis<sup>121,125</sup>.

### Targeting gluconeogenesis enzymes

**Inhibition of PEPCK and G-6-Pase**—Two gluconeogenic enzymes with the potential to be targeted for diabetic treatment are PEPCK, which converts oxaloacetate to PEP, and G-6-Pase, which catalyzes the conversion of glucose-6-phosphate to glucose. The expression of these enzymes are highly regulated by glucagon and insulin in correlation with gluconeogenic flux<sup>126–129</sup>. In addition, PEPCK expression is dysregulated in diabetes, and a 7-fold increase in the expression of PEPCK results in hyperglycemia in mice<sup>130</sup>. PEPCK has therefore been thought to be a rate-limiting enzyme for gluconeogenesis and has been implicated as a potential target to reduce hepatic glucose production and blood glucose. Indeed, inhibition of PEPCK by 3-mercaptopicolinic acid results in hypoglycemia<sup>131</sup>. However, the notion that PEPCK is rate-limiting in gluconeogenesis has recently been challenged, particularly since neither PEPCK nor G-6-Pase levels are elevated in livers from patients with type 2 diabetes<sup>132</sup>. Specifically, liver-specific PEPCK knockout mice maintain normal blood glucose levels even after 24 hours of fasting<sup>133,134</sup>, due to increased extrahepatic gluconeogenesis and reduced whole-body glucose turnover<sup>134</sup>. In addition, the amount of PEPCK in the liver exhibits poor control strength on hepatic glucose production, but very strong control of flux through the TCA cycle, the inhibition of which ultimately leads to triglyceride accumulation in liver<sup>133,135,136</sup>. These studies highlight the importance of PEPCK in cataplerosis (i.e. the removal of TCA cycle intermediates), and suggest that it might also support the energetic demands of gluconeogenesis via maintenance of TCA cycle flux. Although the control strength of PEPCK on gluconeogenic flux is less than is expected of a rate-limiting enzyme, small changes in PEPCK can still lead to decreased gluconeogenic flux<sup>136</sup>.

Interestingly, mice lacking G-6-Pase in the liver also maintain normal blood glucose levels during prolonged fasting<sup>137</sup>. As noted for PEPCK knockout mice, this is due to increased levels of extrahepatic gluconeogenesis<sup>137</sup>. In addition, G-6-Pase knockout mice are protected from diet-induced obesity, due to increased energy expenditure in peripheral tissues<sup>138</sup>.

The studies with PEPCK and G6Pase genetic models imply that complete inhibition of one of these enzymes singularly in the liver might not be sufficient to reduce blood glucose *in vivo* in the nondiabetic state. However, these studies do not preclude the possibility that decreases in the activity of these genes may improve hyperglycemia, particularly in the diabetic state. To this end, partial silencing of PEPCK by RNAi decreased blood glucose levels and free fatty acids in diabetic mice while avoiding hepatic steatosis and lactic acidosis<sup>104</sup>. Moreover, PEPCK and G-6-Pase are part of a coordinated response to the fasted state, which involves many cellular signaling changes including upregulation of gluconeogenic pathways, a switch from glucose to fatty acid oxidation, and increased autophagy, among others<sup>139,140</sup>. Therefore, targeting this response (for example, by targeting post-translational modifications of PGC-1 $\alpha$  – see below), rather than direct inhibition of either enzyme alone, might prove to be a more effective strategy to control hyperglycemia.

Targeting PEPCK and G-6-Pase indirectly through modulation of its regulators may also allow for additional metabolic alterations, including redirection of carbon intermediates, which can circumvent potential problems of targeting these enzymes directly.

**FBPase inhibitors**—FBPase, which converts fructose-1,6-bisphosphate to fructose-6-phosphate, is another gluconeogenic enzyme with the potential to be inhibited for diabetes treatment. Treatment of diabetic rats with the direct FBPase inhibitor and phosphanate prodrug MB06322 (Metabasis Therapeutics, also known as CS-917) led to decreased hyperglycemia, without causing aberrant metabolic alterations<sup>42</sup>. This inhibitor was an 8,9-disubstituted purine which acts as an AMP mimetic to bind to the allosteric site of FBPase. Benzimidazole analogs with IC<sub>50</sub> values of less than 100 nM were developed which lowered glucose in rats and inhibited human liver FBPase<sup>43</sup>. Further steps have been taken to determine whether this class of compounds could be developed for the clinic. Specifically, MB07803 is a second generation FBPase inhibitor based on CS-917 which was used in a phase Ib clinical trial in type 2 diabetes patients<sup>44</sup>. In this short-term study, blood glucose levels were lowered in the 6 hours following a 12 hour fast. However, some patients experienced nausea and vomiting at higher doses. Additional studies will need to be conducted regarding the long term effects of this drug class, as well as the potential development of an FBPase inhibitor that does not induce gastrointestinal side effects.

**Activation of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase**—A potent regulator of glycolytic and gluconeogenic flux is fructose-2,6-bisphosphate (F-2,6-P<sub>2</sub>), which is a product of the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (6PFK2/FBP2). Insulin stimulates the kinase activity of this enzyme, resulting in increased levels of F-2,6-P<sub>2</sub> that allosterically activates phosphofructokinase-1 (PFK-1), the committed step in glycolysis which has the opposite effect of FBPase and converts fructose-6-phosphate to fructose 1,6-bisphosphate<sup>141</sup>. In contrast, glucagon stimulates the BPase activity of the enzyme, resulting in reduced levels of F-2,6-P<sub>2</sub> and elevated gluconeogenesis<sup>141</sup>. In accordance to its importance in controlling glucose flux, increasing the levels of F-2,6-P<sub>2</sub> in the liver improves insulin sensitivity and lowers blood glucose in mice<sup>142</sup>, making the bifunctional enzyme a potential target to reduce hepatic glucose production. In addition, overexpression of the bifunctional enzyme in mouse liver lowers blood glucose through suppression of hepatic glucose production<sup>143</sup>, further supporting the concept that activation of this enzyme by small molecules might have beneficial effects on glucose homeostasis.

### Modulating gluconeogenic transcription factor and coactivator activity

Targeting transcription factors and coactivators could be a potentially effective method for developing treatments for type 2 diabetes, as these regulators can affect entire pathways and responses that are physiologically regulated by fasting and feeding. However, transcription factors are frequently found in multi-protein complexes, and designing small molecules that will potentially change complex activity can be difficult<sup>144</sup>. Various confounding factors include the large molecular area involved in protein-protein interactions, which may not contain ideal pockets for small molecules to bind; the large and flexible structure of coactivators which can be accompanied by an unknown crystal structure; a lack of ligand-

binding sites; and limited small molecule libraries with properties ideal for targeting transcription<sup>145,146</sup>. However, small molecules may allosterically affect protein-protein interactions to ultimately affect multi-protein complex activity either by disrupting the integrity of the complex or by changing post-translational modifications (PTMs) of specific subunits<sup>147</sup>. Indeed, successful approaches have been employed to target transcriptional proteins, including the identification of a direct inhibitor of steroid receptor coactivator 3 (Src-3) through high-throughput screening of small molecules that alter Src-3-dependent transcription<sup>145</sup>. Moreover, modulating transcription factor activity – by intervening with the interaction of transcription factors with their cofactors - has been a major focus in drug development for the treatment of cancer<sup>148,149</sup>.

**PGC-1 $\alpha$** —Several regulators of liver metabolic transcription have the potential for diabetes drug targeting. PPAR $\gamma$  coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) is a transcriptional coactivator that is activated in response to nutrient signals<sup>150</sup>. Initially, PGC-1 $\alpha$  was identified in brown adipose tissue as a cold-inducible coactivator of nuclear receptors<sup>151</sup>, with high expression observed in other metabolic tissues<sup>151,152</sup>. PGC-1 $\alpha$  is a chief regulator of mitochondrial biogenesis and function, and consistent with its cold-inducible status, is an activator of mitochondrial uncoupling<sup>153</sup>.

In the liver, PGC-1 $\alpha$  expression is modulated acutely by nutritional status, as it acts as a primary inducer of gluconeogenesis<sup>154</sup>. In the fed state, hepatic PGC-1 $\alpha$  levels are low, and gluconeogenesis is suppressed<sup>154</sup>. Conversely, under fasting conditions, PGC-1 $\alpha$  levels are markedly enhanced to stimulate gluconeogenesis and fatty acid oxidative metabolism<sup>154</sup>. The induction of PGC-1 $\alpha$  under fasting is achieved through the action of glucagon<sup>155</sup> (Figure 3a). When induced, PGC-1 $\alpha$  coactivates the glucocorticoid receptor (GR), hepatic nuclear factor 4 $\alpha$  (HNF4 $\alpha$ ), and forkhead box O1 (FoxO1), leading to PEPCK and G-6-Pase transcription<sup>139,154,155</sup>. In addition, PGC-1 $\alpha$  increases hepatic insulin resistance and fatty acid oxidation through coactivation and induction of PPAR $\alpha$ <sup>156</sup>. Insulin resistance is increased through PPAR $\alpha$ -dependent upregulation of Tribbles homolog 3 (Trb3), a negative regulator of Akt, while fatty acid oxidation is enhanced by PPAR $\alpha$ -driven transcription of fatty acid oxidation genes.<sup>156</sup> This increase in fatty acid oxidation provides carbon substrates to the TCA cycle, which in turn contributes to increased gluconeogenic flux.

Several studies have investigated the role of PGC-1 $\alpha$  in regulating blood glucose levels in knockout or knockdown mouse models. While one study found no change in gluconeogenesis or PEPCK and G-6-Pase gene expression in liver-specific knockout mice<sup>157</sup>, others determined that fasting hypoglycemia occurs with homozygous knockout, heterozygous knockout, and/or with RNAi-mediated PGC-1 $\alpha$  knockdown<sup>156,158,159</sup>. Another study found that PGC-1 $\alpha$  knockout specifically decreases glucose production due to reduced gluconeogenic flux from PEP, secondary to reduced fatty acid oxidation and TCA cycle flux<sup>160</sup>. The discrepancy likely results from differences in the mouse models and strains used, but the majority of the evidence is in support of PGC-1 $\alpha$  as a regulator of blood glucose levels. Complete and chronic ablation of PGC-1 $\alpha$  may result in activation of compensatory mechanisms that mask the effect of PGC-1 $\alpha$  inactivation; however, suppressing PGC-1 $\alpha$  in the liver has the potential to reduce glucose production.

In addition its transcriptional regulation, PGC-1 $\alpha$  activity is also regulated by PTMs, including phosphorylation<sup>161,162</sup>, acetylation<sup>163–167</sup>, and methylation<sup>168</sup> (Figure 3a). Insulin suppresses PGC-1 $\alpha$  activity by increasing its phosphorylation at serine 570 by Akt<sup>161</sup>, and through Akt-induced Cdc2-like kinase 2 (Cdk2), which phosphorylates the PGC-1 $\alpha$  serine-arginine (SR) domain<sup>162</sup>. SR domain phosphorylation is also increased by insulin-induced S6 kinase (S6K), enabling decreased gluconeogenic activity of PGC-1 $\alpha$  specifically by interfering with its interaction with HNF4<sup>169</sup>. PGC-1 $\alpha$  activity has been found to be increased by deacetylation and decreased by acetylation<sup>163</sup>. The deacetylation of PGC-1 $\alpha$  is regulated by fasting and feeding, as the activity of the deacetylase sirtuin 1 (Sirt1) and its interaction with PGC-1 $\alpha$  are enhanced during fasting<sup>164</sup>. Additionally, Sirt6 has been found to deacetylate general control of amino acid synthesis protein 5 (GCN5), increasing its acetyltransferase activity toward PGC-1 $\alpha$ <sup>165,166</sup>. PGC-1 $\alpha$  can also be acetylated through cyclin D1-Cdk4, whose activity is increased by feeding and in *db/db* mice, and which activates GCN5<sup>167</sup>. A protein discovered to deacetylate PGC-1 $\alpha$ , named CBP- and p300-interacting transactivator with glutamic acid- and aspartic acid-rich COOH-terminal domain 2 (CITED2), is a glucagon-stimulated inhibitor of GCN5, and knockdown of hepatic CITED2 decreased gluconeogenesis in mice<sup>170</sup>. Additionally, PGC-1 $\alpha$  activity can be modified through methylation, with protein arginine methyltransferase 1 (PRMT1) stimulating the activity of PGC-1 $\alpha$ <sup>168</sup>. The regulation of PGC-1 $\alpha$  methylation during fasting and feeding has not yet been determined.

Due to its robust effect on gluconeogenesis, drug targeting of PGC-1 $\alpha$  in the liver is a potentially appealing strategy for the treatment of type 2 diabetes. The most significant challenge with targeting PGC-1 $\alpha$  is achieving selective inhibition of its gluconeogenic function in the liver, without inhibiting its effect on mitochondrial function in the liver and other metabolic tissues. However, potential strategies to possibly achieve this goal are multifold. One such strategy is to target specific PTMs of PGC-1 $\alpha$  that only affect its activity toward gluconeogenic targets, perhaps by targeting the interaction between PGC-1 $\alpha$  and HNF4 $\alpha$  or FoxO1. This strategy is supported by the finding that S6K can specifically target the interaction of PGC-1 $\alpha$  with HNF4 $\alpha$ <sup>169</sup>. Although manipulating specific protein-protein interactions or PTMs within a multi-protein complex is not a simple task, lessons learned from the regulation of other transcriptional complexes, such as the NF- $\kappa$ B pathway, suggest that it may be achievable<sup>171</sup>.

Another strategy is to alter PGC-1 $\alpha$  stability or activity by targeting upstream regulators that are expressed relatively specifically in the liver. Utilizing existing inhibitors of PGC-1 $\alpha$  activators such as Sirt1 and AMPK is of limited usefulness for decreasing gluconeogenesis, as these proteins have a broad arsenal of targets in addition to PGC-1 $\alpha$ <sup>172,173</sup>. Furthermore, global inhibition of Sirt1 and/or AMPK would be detrimental, as these proteins have been found to beneficially alter metabolism to improve cardiac and skeletal muscle function, aging, and brain function, among other effects<sup>174,175</sup>. However, other PGC-1 $\alpha$  regulators discussed above have the potential to be targeted in order to alter hepatic gluconeogenesis, without aberrantly affecting other metabolic pathways. Additionally, several compounds were found to increase PGC-1 $\alpha$  expression through high-throughput screening in skeletal muscle cells, suggesting that chemical modifiers of PGC-1 $\alpha$  expression could also be probed in the liver<sup>176</sup>.

A small molecule named ZLN005 was discovered in a screen for compounds that increased PGC-1 $\alpha$  transcription in HEK293 cells, and was confirmed to activate its transcription in L6 myotubes<sup>177</sup>. When used *in vivo* in *db/db* mice, ZLN005 activated transcription in skeletal muscle as expected, but inhibited PGC-1 $\alpha$  transcription in the liver. The expression of G-6-Pase and PEPCK were decreased, while pyruvate tolerance was improved. There was also no effect on mitochondrial gene targets in the liver. The mechanism of action of this drug is unclear, as it had no cell autonomous effect on primary hepatocytes. However, these results suggest that it may be possible to therapeutically target PGC-1 $\alpha$  in specific tissues, with desired inhibitory effects in liver but stimulatory effects in skeletal muscle or adipose tissue. Further understanding of the precise mechanisms regulating PGC-1 $\alpha$  activity will aid in the development of potential liver-specific therapeutics.

**FoxO proteins**—As indicated above, FoxO1 is a transcription factor that is coactivated by PGC-1 $\alpha$  in response to insulin in order to regulate hepatic gluconeogenesis<sup>155</sup>. Mammals express four evolutionally conserved FoxO proteins, named FoxO1, FoxO3a, FoxO4, and FoxO6<sup>178</sup>. While the role of FoxO1 in gluconeogenesis is well-established<sup>155</sup>, there is evidence that other FoxO proteins also contribute to this process. Mice with triple ablation of FoxO1, FoxO3, and FoxO4 have increased fasting hypoglycemia and insulin sensitivity compared to mice with FoxO1 knockout alone, suggesting that FoxO3 and FoxO4 contribute to the control of gluconeogenesis<sup>179</sup>. Additionally, hepatic glucose output is increased in FoxO6 transgenic mice, while depletion of hepatic FoxO6 causes fasting hypoglycemia, demonstrating that FoxO6 also takes part in regulating gluconeogenesis<sup>180</sup>. In addition to controlling gluconeogenesis, FoxO proteins have also been shown to regulate hepatic lipid metabolism<sup>181</sup>. FoxO1 knockout alone does not alter lipid metabolism<sup>182</sup>, but hepatic steatosis and lipid secretion are increased in FoxO1 and FoxO3 double knockout mice<sup>182</sup>, while FoxO1, FoxO3, and FoxO4 knockout mice exhibit hepatic lipid accumulation<sup>183</sup>.

FoxO activity is distinctly controlled by PTMs, which has implications for hepatic glucose output. In particular, FoxO1, FoxO3a and FoxO4 are phosphorylated by Akt in response to insulin at three consensus sites<sup>184</sup> (Figure 3b). Phosphorylation at these sites enables binding by the 14-3-3 protein and subsequent FoxO polyubiquitination and degradation. This phosphorylation by Akt is also dependent on binding to the scaffold protein WD40/ProF, and this interaction is inhibited by atypical Protein Kinase C (aPKC)<sup>185</sup>. Ubiquitination of FoxO1 can also be modulated by ubiquitin-specific protease 7 (USP7)<sup>186</sup>, which decreases monoubiquitination of FoxO1 to deplete its transcriptional activity, and COP1, which acts as an E3 ubiquitin ligase to polyubiquitinate FoxO1 and increase its degradation<sup>187</sup>. Moreover, FoxO proteins are regulated by acetylation, whereby histone deacetylases (HDACs) 4 and 5 are phosphorylated by AMPK in response to insulin, leading to recruitment of HDAC3 and deacetylation and activation of FoxO transcription<sup>188</sup>.

Targeting of FoxO proteins, either dependent on or independent of PGC-1 $\alpha$  activity, may be a potential avenue for developing drugs for type 2 diabetes. Indeed, a direct FoxO1 inhibitor, named AS1842856, was discovered to bind and block FoxO1 transactivation, and was successful in decreasing fasting blood glucose in *db/db* mice without affecting wild type mice<sup>189</sup>. Another compound found to inhibit FoxO1, named AS1708727, similarly decreased blood glucose and triglyceride levels when administered for four days to *db/db*

mice<sup>190</sup>. Further investigation into the precise mechanism of action of these or similar compounds may yield insights into how to specifically target FoxO proteins in the liver. Additional studies regarding the nature of PTMs of FoxOs will likely help in this endeavor. The design and development of drugs that would target multiple FoxOs would also need to consider the potential hepatic steatosis that can result from inhibition of FoxO proteins<sup>182,183</sup>.

**CREB**—cAMP response element binding protein (CREB) is a transcription factor that binds to CRE-response elements and alters gene transcription in various tissues, with known effects on neuronal plasticity, memory, inflammation, and hepatic gluconeogenesis<sup>191</sup>. In the liver, glucagon activates PKA through cAMP to phosphorylate CREB at serine 133<sup>192</sup>, which enables it to interact with the coactivators p300 and CREB-binding protein (CBP)<sup>193</sup> (Figure 3c). Glucagon also stimulates dephosphorylation of CREB-regulated transcription coactivator 2 (CRTC2, also known as TORC2), which then coactivates CREB on gluconeogenic genes<sup>194</sup>. The involvement of CREB in controlling glycemia was demonstrated in CREB knockout mice, which had fasting hypoglycemia<sup>195</sup>. PGC-1 $\alpha$  was also found to be a direct transcriptional target of CREB, and its expression is increased with chronic CREB activation during prolonged fasting<sup>195</sup>. In addition to its effects on gluconeogenesis and fatty acid oxidation through PGC-1 $\alpha$ , CREB also increases the flux of fatty acids towards the TCA cycle by decreasing lipogenesis through inhibition of PPAR $\gamma$ <sup>196</sup>.

Several other proteins have been shown to modulate CREB activity. Small heterodimer partner (SHP) is a transcriptional repressor that binds to CREB to inhibit its interaction with CRTC2<sup>197</sup>. Insulin also inhibits CRTC2 through salt inducible kinase 2 (Sik2)-mediated phosphorylation, leading to CRTC2 degradation and reduced CREB-mediated transcription<sup>198</sup>. TCF7L2 is a transcription factor that binds to CRE to block their occupation by CREB<sup>199</sup>. Circadian proteins have also been recognized in the regulatory control of CREB and gluconeogenesis. Specifically, cryptochrome 1 and 2 (Cry1 and 2) decrease the gluconeogenic gene program by blocking glucagon-induced increases in cAMP and PKA-mediated phosphorylation of CREB<sup>200</sup>.

As CREB exerts effects on other tissues, particularly the brain, and affects PGC-1 $\alpha$ , it is important to specifically target CREB in the liver. Targeting coactivators or inhibitors of CREB, or the interaction of CREB with these proteins, is likely a more feasible approach than directly inhibiting CREB activity. In this regard, a bromodomain inhibitor named CBP30 has been developed which selectively targets CBP and P300<sup>201</sup> and exerts anti-inflammatory effects. Additionally, the protein-protein interaction between CREB and CBP was successfully inhibited by the small molecule KG-501 (2-naphthol-AS-E-phosphate), which decreased cAMP-responsive gene induction<sup>202</sup>. The potential of these agents to decrease gluconeogenesis could be explored.

Although CRTC2 has not been pharmacologically targeted, CRTC2 knockout mice exhibited decreased hepatic glucose production with improved insulin sensitivity, and without apparent neurological deficits<sup>203</sup>. In addition, selective drug targeting of Cry proteins has been achieved by the carbazole derivative KL001, which interacts with Cry proteins and

prevents their ubiquitin-dependent degradation<sup>204</sup>. This compound effectively decreased gluconeogenic gene expression and glucose production after glucagon treatment in primary hepatocytes, although it was not utilized in a mouse model.

Together, these observations suggest that it may be feasible to target the CREB pathway, through its coactivators or inhibitors, in order to selectively target gluconeogenesis in the liver.

**C/EBP $\alpha$  and  $\beta$** —CCAAT/enhancer-binding protein alpha (C/EBP $\alpha$ ) is a transcription factor that increases gluconeogenic gene expression, and the loss of which results in neonatal death within 8 hours of birth due to hypoglycemia<sup>205</sup>. This regulation of gluconeogenesis is PGC-1 $\alpha$ -independent, but dependent on particular C/EBP $\alpha$  residues to control subsets of target genes<sup>206</sup>. Specifically, three consensus residues for glycogen synthase kinase 3 (GSK3) phosphorylation (T222, T226, and S230) were shown to be insulin-responsive, and knock-in mouse models were generated in which these residues were mutated to alanines<sup>206</sup>. These knock-in mice exhibited elevated hepatic expression of PEPCK and G-6-Pase in conjunction with glucose intolerance, demonstrating that these residues are responsible for regulating gluconeogenic genes and glucose tolerance without affecting lipid metabolism. Another study demonstrated that phosphorylation of C/EBP $\alpha$  at S21 by p38 MAPK led to increased PEPCK expression in hepatoma cells<sup>207</sup>, suggesting that liver-specific inhibition of this phosphorylation site may decrease gluconeogenesis. The related family member C/EBP $\beta$  also modulates gluconeogenic gene expression, although it is not necessary for basal regulation<sup>208</sup>. Instead, C/EBP $\beta$  decreases hyperglycemia and gluconeogenesis in streptozotocin-induced diabetes, with decreases in expression of gluconeogenic genes<sup>208</sup>.

The partial redundancy in action of C/EBP $\alpha$  and  $\beta$  makes them potentially appealing drug targets for type 2 diabetes, particularly C/EBP $\beta$  which has not been found to affect basal gluconeogenesis, but instead partially reverses hyperglycemia<sup>208</sup>. Therefore, theoretically, a drug that specifically inhibits C/EBP $\beta$  may be able to reduce blood glucose levels without causing hypoglycemia. Additionally, drug targeting of specific phosphorylation sites on C/EBP $\alpha$  may have the ability to fine-tune the control of gluconeogenesis by C/EBP $\alpha$  without affecting other metabolic pathways, as modulation of GSK3 phosphorylation sites affects gluconeogenic gene expression without altering lipid metabolism<sup>206</sup>. Although technically challenging, identifying small molecules that inhibit specific phosphorylation sites of target proteins has been accomplished for other pathways through screening methods including phosphopeptide binding to fusion proteins<sup>209</sup>.

**REV-ERBs and RORs**—REV-ERBs and retinoic acid receptor-related orphan receptors (RORs) are nuclear receptors involved in several cellular processes, notably metabolism and circadian rhythm regulation<sup>210</sup>. REV-ERBs constitutively repress transcription at ROR response elements through binding of co-repressors. In response to binding by heme, REV-ERB $\alpha$  suppresses gluconeogenic genes and glucose production in HepG2 cells through increased recruitment of the nuclear receptor corepressor–histone deacetylase 3 (NCoR-HDAC3) complex<sup>211</sup>. The ROR family, which includes ROR $\alpha$ ,  $\beta$ , and  $\gamma$ , activates transcription at ROR response elements through recruitment of co-activators such as

PGC-1 $\alpha$ <sup>212</sup> and steroid receptor co-activator 2 (SRC2)<sup>213</sup>. Loss of ROR $\alpha$  leads to fasting hypoglycemia and decreased expression of gluconeogenic genes in the liver<sup>214</sup>.

Manipulation of REV-ERBs and RORs may be of therapeutic benefit for type 2 diabetes. Activation of REV-ERBs with agonists or inhibition of ROR $\alpha$  with inverse agonists has the potential to inhibit hepatic gluconeogenesis<sup>215</sup>. The REV-ERB agonist GSK4112, a tertiary amine, was found to decrease gluconeogenic genes and glucose output in hepatocytes, but has been reported to lack plasma exposure<sup>216</sup>. Two additional compounds, SR9011 and SR9009, were discovered through high-throughput screening as synthetic activators of REV-ERB repressor activity<sup>217</sup>. Administration of these drugs to wild type mice for 6 days significantly altered metabolic gene expression in the liver. Although gluconeogenic gene expression was not measured in this study, hepatic PGC-1 $\alpha$  mRNA expression was repressed, suggesting gluconeogenesis inhibition. Moreover, treatment of diet-induced obese mice with SR9009 caused weight loss, with lowered plasma glucose and lipids. The selective ROR $\alpha$  inverse agonist and benzenesulfonamide SR3335 suppressed gluconeogenic gene expression in HepG2 cells, and decreased glucose production and plasma glucose levels in a mouse model of diet-induced obesity<sup>218</sup>.

**Src1 and 2**—Src-1 and -2 are members of the p160 family of coactivators that control transcription of metabolic gene networks<sup>219</sup>. Src-1 is needed to induce the gluconeogenic program during the transition from the fed to fasting state, and Src-1 KO mice develop hypoglycemia without exhibiting decreases in insulin secretion or sensitivity<sup>220</sup>. The effect on gluconeogenesis is dependent on increased expression and transcriptional activity of C/EBP $\alpha$ <sup>220</sup>. Src-2 also regulates gluconeogenesis, specifically through coactivation of ROR $\alpha$  leading to increased expression of G-6-Pase<sup>213</sup>. Emphasizing the importance of Src-2 in controlling liver glucose metabolism, knockout of Src-2 in mice leads to a Von Gierke's disease phenotype, characterized by fasting hypoglycemia and increased glycogen storage<sup>213</sup>.

Drug targeting of Src-1 and Src-2 may be feasible, as the drug gossypol was discovered to specifically inhibit Src-1 and Src-3, resulting in cancer cell toxicity<sup>221</sup>. Additionally, a direct inhibitor of Src-3 has been identified<sup>145</sup>. However, potent inhibition of these coactivators would be undesirable due to the development of fasting hypoglycemia, and the potential toxicity to non-cancer cell types is unknown. Instead, a drug exhibiting partial and specific inhibition of Src1 and/or Src2 may be beneficial for treating type 2 diabetes. Such inhibition may potentially be accomplished by targeting PTMs of Src-1 and Src-2, as the stability, intracellular localization, and transcription factor specificity and activation of these proteins has been found to be regulated by PTMs including phosphorylation, ubiquitination, acetylation, and methylation<sup>219</sup>. This approach may enable fine-tuning of the activity of these proteins to decrease blood glucose levels to desirable levels, without inducing hypoglycemia or other toxic side effects.

### Mitochondrial uncoupling

Another strategy for targeting hepatic glucose output is to enhance glucose utilization in the liver by altering mitochondrial uncoupling. Uncoupling of the mitochondria diffuses the



proton gradient across the inner mitochondrial membrane that is used to drive ATP synthesis, leading to the release of heat and the increased consumption of metabolic substrates used by the TCA cycle to fuel oxidative phosphorylation<sup>222</sup>. Both fatty acid and glucose oxidation are thus increased by mitochondrial uncoupling. Although the mitochondrial protonophore 2,4-dinitrophenol (DNP) has been known for many years to cause weight loss, it was found to be toxic and even lethal when used clinically due to hyperthermia<sup>223</sup>. To address this, a DNP derivative (DNP-methyl ester, or DNP-ME) that can only be metabolized by the liver was developed, and its administration to diabetic rats decreased fasting plasma glucose, triglycerides, insulin, and glucose production, while improving glucose tolerance<sup>224</sup>. The direct effects of DNP-ME on the liver also indirectly reduced muscle fat content and improved muscle insulin sensitivity<sup>224</sup>. Furthermore, a controlled-release orally available version of DNP - controlled-release mitochondrial protonophore (CRMP) - was developed, which induces mild hepatic uncoupling over a much longer time-frame<sup>225</sup>. Administration of CRMP to high fat diet-fed wild type or Zucker Diabetic Fatty rats lowered plasma glucose, triglycerides, insulin, and glucose production, while improving glucose tolerance. These compounds highlight the potential of utilizing mild oral hepatic uncouplers as an antidiabetic therapeutic strategy to increase substrate oxidation by the liver to decrease gluconeogenesis and blood glucose levels.

## Considerations and challenges in targeting liver glucose homeostasis

It is generally accepted that increased hepatic glucose production, due to enhanced gluconeogenesis, is the major contributor to the increased blood glucose levels observed in diabetic patients<sup>71,226–229</sup>. Directly targeting glucose production or storage may therefore represent an effective approach to regulating blood glucose levels. Inhibiting gluconeogenic and glycogenolytic enzymes would provide the most direct route of decreasing glucose production by the liver, and indeed there are drugs under investigation that target these enzymes (Table 1). Metformin, the first-line drug for the treatment of diabetes (despite its disputable mechanism of action), is believed to reduce blood glucose by decreasing gluconeogenesis and hepatic glucose production<sup>4</sup>, further supporting the notion that targeting liver glucose metabolism is a useful approach to control hyperglycemia. Novel diabetes drugs targeting glucose homeostasis in the liver would most likely be administered in combination with existing type 2 diabetes drugs, such as metformin and SGLT2 inhibitors, to achieve sustained normal blood glucose levels, and to allow for the use of lower drug concentrations in order to avoid side effects (Box 2).

Modulating glucose homeostasis in the liver presents a myriad of challenges and potential advantages when considering novel treatments for type 2 diabetes. Decreasing hepatic glucose production could strongly inhibit blood glucose levels, but hypoglycemia must be avoided, and designing a drug that specifically targets the liver without affecting other tissues can be challenging. Therefore, potency and specificity are two key concerns when developing these drugs for *in vivo* administration. The potency of drugs that target hepatic metabolic enzymes must be carefully considered, as hypoglycemia or aberrant perturbations in liver fat metabolism can result from high levels of enzyme inhibition, as occurs with PEPCK and G-6-Pase knockout<sup>134,230,231</sup>. Potent targeting of these enzymes in extrahepatic tissues can also result in metabolic dysfunction in other tissues, as occurs in kidney-specific

G-6-Pase KO mice which have renal lipid accumulation and glycogen overload<sup>232</sup>. One potential avenue to avoid hypoglycemia is to target proteins that decrease hyperglycemia without affecting basal blood glucose levels, such as glycogen phosphorylase or C/EBP $\beta$ <sup>27,208</sup>. Targeting enzymes that are expressed predominately in the liver, such as G-6-Pase, is one obvious approach to increasing specificity<sup>233</sup>.

Another strategy to increase tissue specificity would be to modify drugs so that they can be metabolized only by the liver, as was performed in the development of the DNP derivative discussed above<sup>224</sup>. The development of this liver-specific DNP mitochondrial uncoupler also revealed a more indirect method of decreasing glucose production in the liver by increasing hepatic metabolic substrate oxidation. This drug targets the liver specifically, but also has beneficial extrahepatic secondary effects such as decreased fat and improved insulin sensitivity of muscle. Titrating a therapeutic to generate mild uncoupling without inducing toxicity will be a particular challenge for translating this class of drug to the clinic.

Modulation of coactivators and transcriptional regulators of metabolic enzymes, such as PGC-1 $\alpha$ , C/EBPs, and others discussed above, could represent an effective method of lowering blood glucose. However, achieving liver specificity when modifying these proteins, potentially through liver-specific PTM regulation or tissue-specific regulation of downstream effectors, would be extremely important given the effects of these proteins on metabolism and other processes in other tissues.

In addition to the potential for hypoglycemia, other possibly detrimental effects of targeting hepatic glucose production should also be considered, including the risk of redirecting carbons to triglyceride or cholesterol synthesis. This redirection in anaplerotic flux could potentially lead to aberrant increases in lipogenesis and hepatic steatosis, or increased cholesterol. Despite its known effects on inhibiting gluconeogenesis, metformin does not increase triglycerides or blood glucose levels<sup>234</sup>, suggesting that gluconeogenesis inhibition does not necessarily lead to aberrations in these parameters. However, novel drug targets would need to be carefully evaluated for their potential effects on triglycerides and cholesterol. Recent technical advances using <sup>13</sup>C labeling combined with <sup>13</sup>C magnetic resonance spectroscopy could be useful in detecting hepatic fatty acid oxidation in human livers *in vivo* to determine the effects of candidate drugs on TCA cycle and anaplerotic flux<sup>235</sup>.

Another potential complication of gluconeogenesis inhibition is lactic acidosis, since anaerobic respiration increases and may lead to lactate accumulation. The incidence of lactic acidosis has been explored with metformin. Since metformin is renally cleared, the drug may accumulate in the kidneys of patients with renal disease, leading to lactic acidosis. However, metformin is not elevated above the therapeutic range in patients with renal failure<sup>236</sup>. Only a slight increase in lactic acidosis was found to occur with metformin treatment in these patients, with no substantial risk demonstrated in patients with mild to moderate kidney disease. Lactic acidosis is likely not a substantial risk of using drugs to inhibit gluconeogenesis, unless pharmacokinetic data reveals that a particular drug accumulates in tissues above the therapeutic range, which must be tested systematically.

## Conclusions and future directions

Despite the existence of several anti-diabetic drugs, type 2 diabetes remains a widespread medical burden. Targeting gluconeogenesis or glucose homeostasis in the liver is an appealing strategy for developing new diabetes therapeutics. Although metformin is currently the most widely used diabetes drug and works at least primarily through inhibition of gluconeogenesis, its mechanism of action has not been fully clarified and it can cause gastrointestinal side effects and typically must be used in combination therapy for long-term sustainability<sup>4,11,75</sup>. Therefore, there is room for novel therapeutic agents that specifically inhibit hepatic glucose output.

Numerous molecular pathways in the liver contributing to glucose production have the potential to be targeted pharmacologically in order to decrease blood glucose levels. Efforts to generate drugs that directly inhibit the metabolic enzymes of gluconeogenesis and glycogenolysis are most advanced. There is also a wealth of targets within additional regulatory pathways, including PGC-1 $\alpha$ , FoxOs, CREB, C/EBPs, RERs, RORs, and Srcs, that have the potential to be therapeutically modulated in order to regulate gluconeogenesis. Hepatic glucose output could also be inhibited by administering a liver-specific mitochondrial uncoupler. Although concerns about potency, tissue selectivity, and undesirable metabolic effects such as lactic acidosis must be addressed, further investigation into specific methods for pharmacologically targeting these pathways in the liver will likely be fruitful in the search for new type 2 diabetes drugs.

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## Glossary Terms

<b>Type 2 diabetes</b>	Chronic disease of aberrant glucose homeostasis that is characterized by elevated blood glucose and insulin resistance
<b>Hyperglycemia</b>	Elevated blood glucose above normal levels
<b>Hypoglycemia</b>	Suppressed blood glucose below normal levels
<b>Gluconeogenesis</b>	Metabolic pathway utilizing carbon substrates to generate glucose
<b>Glycogenolysis</b>	Metabolic pathway that breaks down glycogen into glucose
<b>Hepatic steatosis</b>	Fatty liver disease
<b>Hyperinsulinemia</b>	Excess insulin levels in the blood, often caused by insulin resistance

<b>Lactic acidosis</b>	Increased acidity in the body due to a buildup of lactate, a potential concern with inhibiting hepatic gluconeogenesis
<b>Sulfonylureas</b>	Type 2 diabetes drugs that stimulate insulin secretion from the pancreas through inhibition of $K_{ATP}$ channels in beta cells
<b>Thiazolidinediones</b>	Insulin-sensitizing type 2 diabetes drugs that act as PPAR $\gamma$ agonists, also known as glitazones
<b>Insulin secretagogue</b>	Any agent that increases insulin release from the pancreas

## Biographies

Amy K. Rines, Ph.D., earned her B.A. in Integrated Science and Biology and Ph.D. in Cell Biology from Northwestern University, where her doctoral research focused on kinase regulation of cell proliferation and metabolism. She joined Pere Puigserver's group at Dana-Farber Cancer Institute and Harvard Medical School as a postdoctoral fellow to pursue her research interests of discovering cellular signaling mechanisms and therapeutics for type 2 diabetes and obesity.

Kfir Sharabi, Ph.D., completed his graduate studies at The Hebrew University of Jerusalem in genetics studying the molecular response of worms to environmental changes in CO<sub>2</sub> levels. He is currently pursuing postdoctoral training where he uses a chemical approach to study how glucose homeostasis is regulated in diabetic states. His main research interests include studying how nutrients affect specific metabolic pathways to control energy homeostasis.

Clint D. J. Tavares, Ph.D., is currently a postdoctoral research fellow in the laboratory of Pere Puigserver at the Dana-Farber Cancer Institute and Harvard Medical School. Supported by a postdoctoral fellowship from The American Diabetes Association, his research is aimed at investigating certain molecular components, mechanisms, and transduction pathways involved in nutrient-regulated metabolic responses in the context of diabetes, with the objective of chemically targeting specific components for the therapeutic purpose of treating type 2 diabetes and obesity. He previously received his Ph.D. in Cell and Molecular Biology from The University of Texas at Austin, under the guidance of Kevin N. Dalby.

Pere Puigserver, Ph.D., received his B.S. in Biological Sciences and Ph.D. in Biochemistry at the University of Illes Balears, Spain. His graduate work focused on mitochondrial energetics using *in vitro* and *in vivo* approaches, and included research at the University of Stockholm. He pursued postdoctoral training in molecular and cellular biology at Dana-Farber Cancer Institute and Harvard Medical School. In 2002, he was appointed Assistant Professor of Cell Biology at Johns Hopkins University School of Medicine. In 2006, he was recruited back to the Dana-Farber Cancer Institute and Harvard Medical School to continue his research program in cell metabolism.

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### Box 1: Current type 2 diabetes drugs

The most commonly used diabetes therapy is metformin (N,N-dimethylbiguanide), a biguanide compound that decreases gluconeogenesis<sup>237</sup>. Its popularity stems from its ability to lower blood glucose without inducing hypoglycemia or weight gain, while maintaining an excellent safety profile<sup>4</sup>. However, the molecular mechanism of metformin has not been well defined. A generally acknowledged site of action of metformin is the mitochondria, where it partially inhibits complex I<sup>238,239</sup> to decrease cellular energy and gluconeogenesis<sup>240</sup>. How a decrease in cellular energy (as represented by an increase in the AMP:ATP ratio) causes a change in gluconeogenesis is unclear. Some reports have indicated that activation of AMP-activated protein kinase (AMPK) is necessary<sup>241</sup>. Others have found that AMPK is not needed, but rather that accumulation of AMP:ATP directly inhibits gluconeogenic flux<sup>240</sup> and inhibits adenyl cyclase to decrease cAMP and activation of protein kinase A (PKA)<sup>242</sup>. Metformin has also been reported to inhibit mitochondrial glycerophosphate dehydrogenase (mGPD), which blocks the glycerophosphate shuttle and alters the hepatic redox state to decrease the conversion of lactate and pyruvate to glucose and therefore decrease gluconeogenesis<sup>243</sup>. In addition to affecting gluconeogenesis, metformin also decreases tissue lipid storage through AMPK phosphorylation and inactivation of acetyl-coA carboxylase (ACC), which then can improve insulin sensitivity and decrease blood glucose levels<sup>244</sup>. Although considered safe, metformin is accompanied by gastrointestinal side effects including nausea, which may result from its effects on multiple tissues<sup>11</sup>. Additionally, the effect of metformin on glycemic control is typically reduced over time, requiring combined treatment with other drugs<sup>75</sup>.

Several other classes of drugs affect insulin secretion from the pancreas or uptake of glucose into tissues. Sulfonylureas and meglitinides/D-phenylalanine increase insulin secretion by closing  $K_{ATP}$  channels in pancreatic beta cells<sup>5,245</sup>. While effective at lowering blood glucose, these agents can cause hypoglycemia, epithelial damage, or beta cell exhaustion or apoptosis<sup>12</sup>. Glucagon-like peptide 1 (GLP-1) is a gut-secreted hormone that stimulates insulin and impairs glucagon secretion, and its action can be increased through direct agonism or by inhibition of dipeptidyl peptidase-4 (DPP-4), which leads to enhanced GLP-1 secretion<sup>6</sup>. Side effects of GLP-1 agonists include nausea, diarrhea, and headaches, while DPP-4 inhibitors may cause upper respiratory tract infections and headaches<sup>246</sup>. Aside from the potential side effects, the insulin-stimulating action of sulfonylureas and GLP-1 agonists may not be the best course of treatment for diabetes, as this can result in weight gain, and hyperinsulinemia has been associated with comorbidities including cardiovascular disease and cancer<sup>13,14</sup>. However, no substantial link has yet been found between the use of these drugs and the incidence of these comorbidities.

The glitazone and thiazolidinedione (TZD) class of drugs act as agonists for PPAR $\gamma$  in order to increase glucose uptake into peripheral tissues, thereby increasing insulin sensitivity<sup>7</sup>. While often effective, this drug class has potential complications including increased risks of myocardial infarction, skeletal fractures, and bladder cancer<sup>7</sup>. Alpha-glucosidase inhibitors lower blood glucose by a different method, blocking the digestion

of carbohydrates through inhibition of enterocyte enzymes that cleave oligosaccharides to monosaccharides<sup>8</sup>. These drugs can be useful particularly if postprandial glucose absorption is a concern, although they are often associated with undesirable gastrointestinal effects.

Another approach to lowering blood glucose is through sodium-glucose co-transporter 2 (SGLT2) inhibition, which blocks reabsorption of glucose by the kidney and increases its secretion into the urine<sup>9</sup>. These inhibitors have been recently FDA-approved, are effective at decreasing hyperglycemia, and may decrease adverse cardiovascular outcomes in type 2 diabetes patients<sup>247</sup>. However, they lack long-term safety data and may also cause urinary tract infections. Finally, bile acid sequestrants which lower cholesterol by binding to bile acids, also lower blood glucose by an unknown mechanism<sup>10</sup>. However, as such agents bind bile acids in the intestine, they are also associated with adverse gastrointestinal effects.

**Box 2: Combination strategies to lower blood glucose**

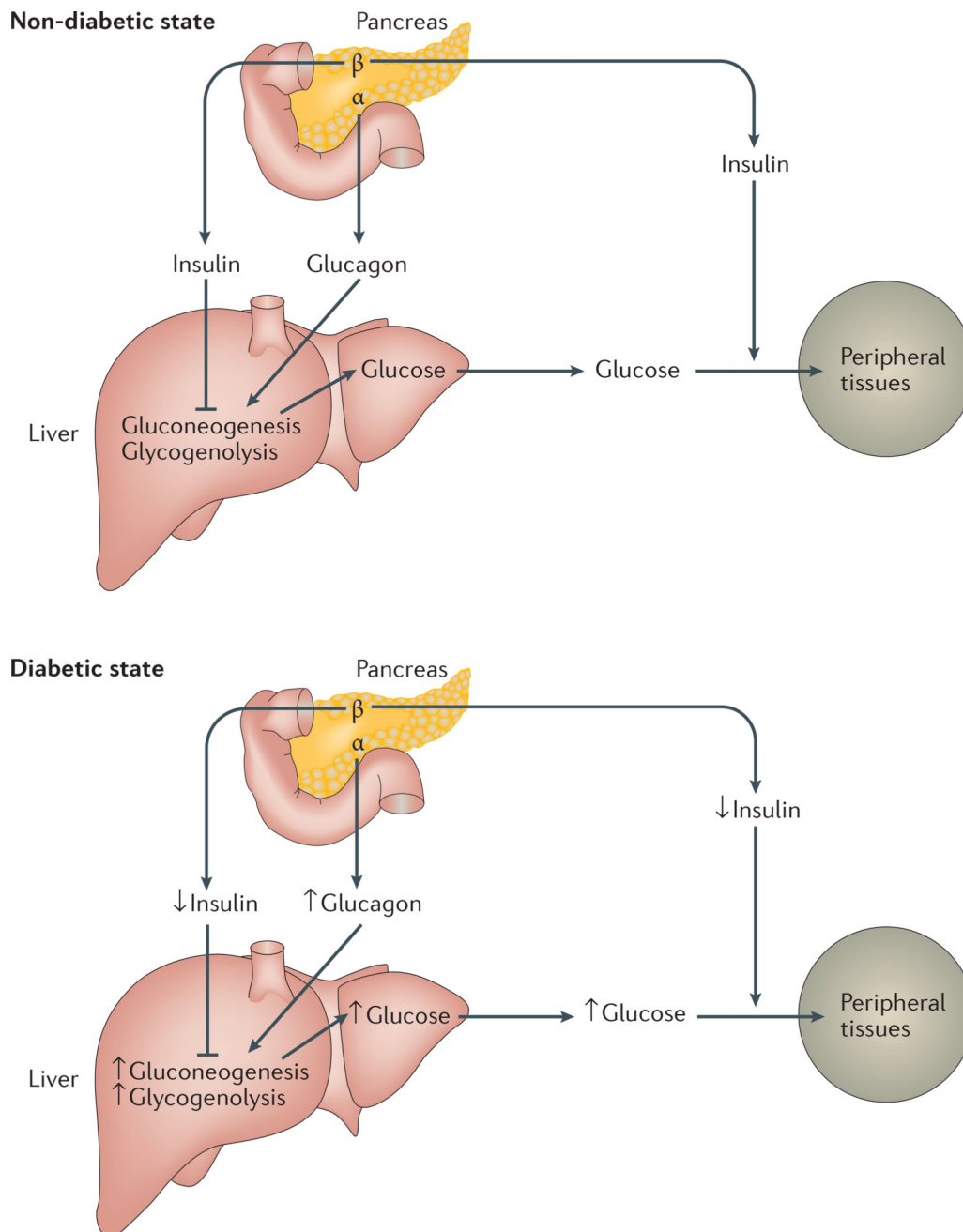
Combining novel and existing diabetes therapies, targeting different mechanisms of glucose homeostasis control, could lead to enhanced effects on blood glucose. Indeed, combinations of existing diabetes drugs are already used in the clinic to achieve blood glucose level goals. Often, metformin is used first; however, in most patients, glycemic control declines over time, making combination therapy an essential approach<sup>75</sup>. Metformin is usually combined with one or two drugs from different classes including insulin, sulfonylureas, TZDs, SGLT2 inhibitors, DPP-4 inhibitors, and GLP-1 agonists<sup>248,249</sup>. Clinically, considerations for what combinations to use involve assessment of efficacy, side effects, contraindications, cost, the complexity of dosing if multiple daily injections are necessary, and patient aversion to using injectable medications, as is needed for insulin and GLP-1 agonists<sup>250</sup>.

Novel diabetes drugs targeting glucose homeostasis in the liver would most likely be used in combination with existing type 2 diabetes drugs, such as metformin, to allow the use of lower drug concentrations to avoid side effects, or to prolong the anti-diabetic effect. However, as metformin also targets glucose homeostasis in the liver, such novel agents may be particularly beneficial in decreasing hyperglycemia when used in combination with drugs that target other mechanisms.

Combining novel drugs targeting hepatic glucose production with insulin sensitizers, like TZDs, which cannot result in uncontrolled insulin secretion, may impose lower risks of hypoglycemia compared with insulin secretagogues such as GLP-1 agonists. However, water retention, heart failure, and weight gain are all known possible side effects of TZDs, which must be carefully considered. While increased lactic acidosis is a potential concern when inhibiting hepatic glucose output, combination with TZDs, which increase the activity of pyruvate dehydrogenase, can ameliorate lactic acidosis by increasing lactate entry into the TCA cycle<sup>251</sup>. This highlights an additional beneficial outcome of combination therapy, which is a more balanced effect on total energy metabolism that might improve overall glycemic control outcomes. SGLT2 inhibitors that reduce glucose reabsorption by the kidney and increase its secretion, like TZDs, do not result in increased insulin secretion, and thus also impose a lower risk of hypoglycemia. Combined treatment with SGLT2 inhibitors may therefore efficiently complement the action of drugs targeting hepatic glucose output.

### Key Points

- Type 2 diabetes is characterized by elevated blood glucose and insulin resistance. Current diabetes drugs can lower blood glucose, but often have side effects, and the most widely used drug metformin does not have a clear mechanism of action.
- Targeting glucose and glycogen metabolism in the liver is a strategy for type 2 diabetes treatment which can decrease hepatic glucose output, but this approach has not been fully explored.
- Targeting gluconeogenic and glycogenolytic enzymes or their regulators presents numerous drug targets that are currently in development or have the potential to be developed.
- Transcriptional coactivators and transcription factors are emerging diabetes drug targets with the ability to control entire gene programs involved in glucose and glycogen metabolism. These transcriptional regulators may be able to be targeted more specifically by modulating their protein-protein interactions or post-translational modifications.
- Novel diabetes drugs would most likely be used in combination with existing therapies to enable sustained blood glucose suppression, and so that each drug could be used at a lower concentration in order to reduce side effects. Drugs decreasing hepatic glucose output may be most effectively used with drugs that work by other mechanisms, such as thiazolidinediones or SGLT2 inhibitors.
- Challenges of inhibiting hepatic glucose output include preventing hypoglycemia, enabling tissue-specific targeting, analyzing the possible effects of redirecting carbons to triglyceride or cholesterol synthesis, and avoiding lactic acidosis.



**Figure 1. Schematic of glucose homeostasis in non-diabetic and diabetic states**

After feeding, pancreatic beta cells release insulin to inhibit gluconeogenesis and glycogenolysis in the liver, decreasing glucose output to the circulation. Insulin also acts at peripheral tissues to increase glucose uptake, resulting in decreased blood glucose. During fasting, pancreatic alpha cells release glucagon to increase gluconeogenesis and glycogenolysis in the liver, increasing circulating blood glucose. In the diabetic state, insulin action is decreased at the liver and/or peripheral tissues and glucagon action is enhanced, leading to increased hepatic gluconeogenesis and glycogenolysis, increased glucose release

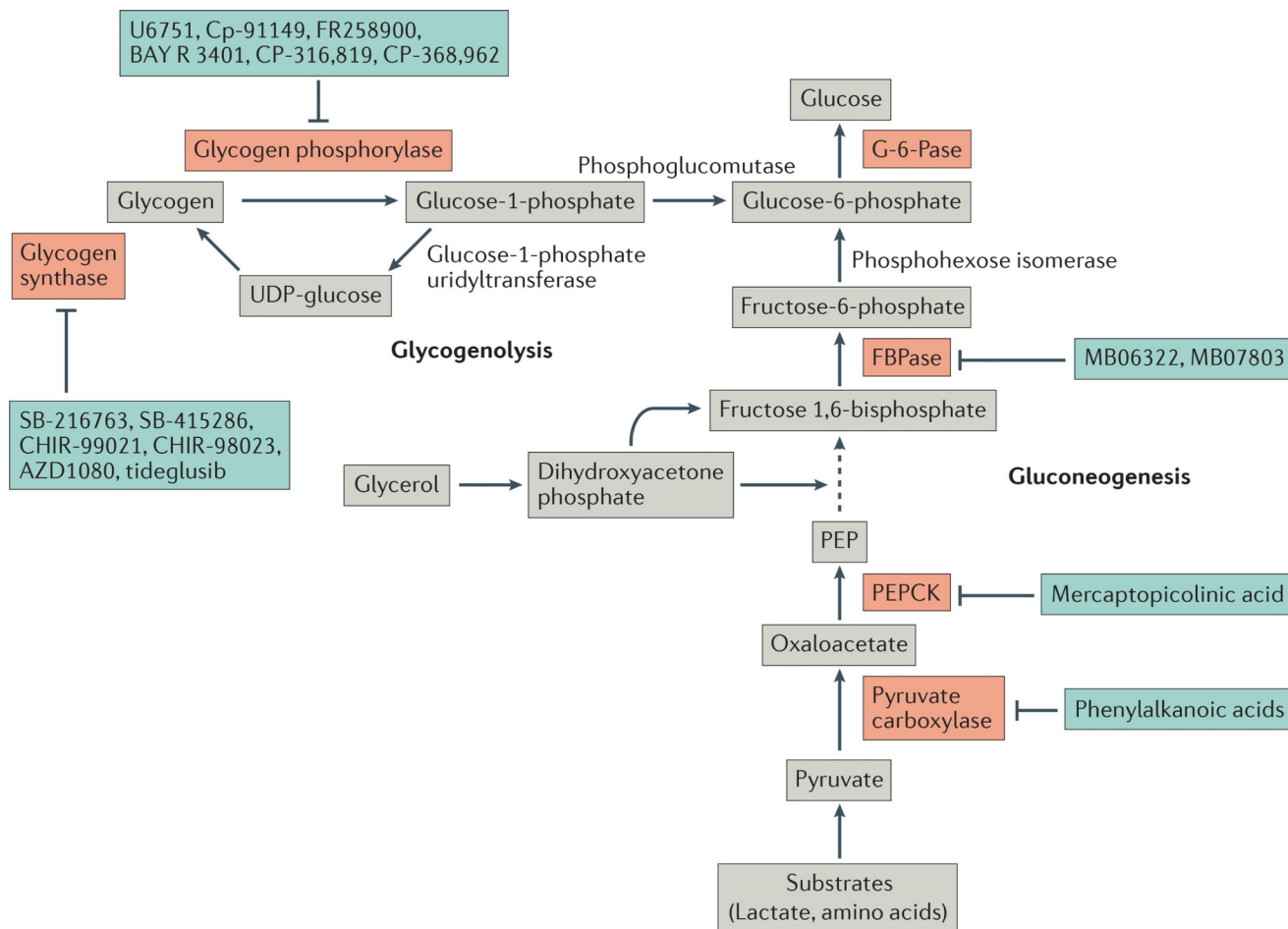
to the circulation, repressed glucose uptake into peripheral tissues, and increased blood glucose levels.

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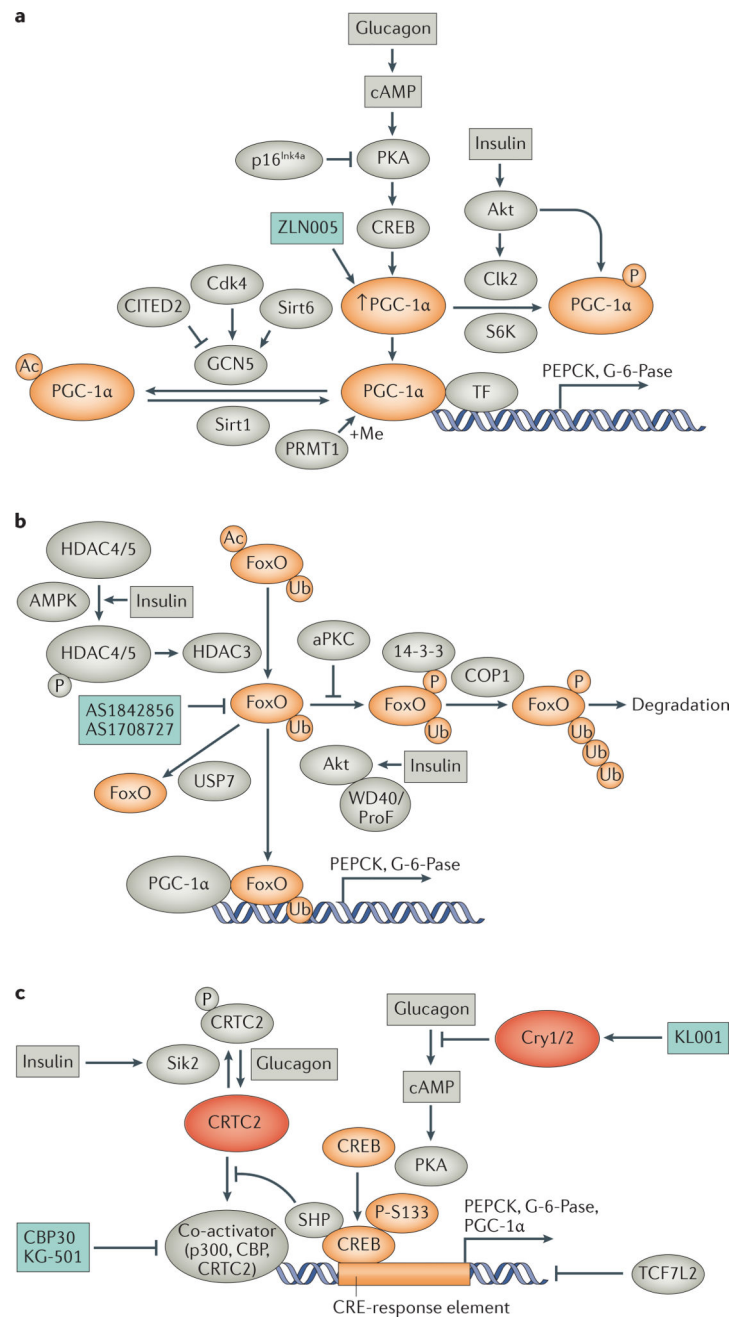
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**Figure 2. Key steps of gluconeogenesis and glycogenolysis**

In gluconeogenesis, lactate and amino acids are first converted to pyruvate, either directly or indirectly through tricarboxylic acid (TCA) cycle intermediates. Pyruvate is then shuttled from the cytosol into the mitochondria where it is used to generate oxaloacetate by pyruvate carboxylase. Oxaloacetate is converted to aspartate, exported from the mitochondria and re-converted to oxaloacetate, and then is made into phosphoenolpyruvate (PEP) by PEP carboxylase (PEPCK). PEP is catalytically altered to fructose 1,6-bisphosphate, and glycerol can also enter gluconeogenesis through conversion to fructose 1,6-bisphosphate. Fructose 1,6-bisphosphate is then altered to fructose-6-phosphate by fructose 1,6-bisphosphatase (FBPase), then to glucose-6-phosphate by phosphohexose isomerase. Finally, the phosphate on glucose-6-phosphate is removed by the liver-specific enzyme glucose 6-phosphatase (G-6-Pase) to generate glucose, which can be exported to the circulation. During glycogenolysis, the glucose residues in glycogen are phosphorylated by glycogen phosphorylase to produce glucose-1-phosphate, then glucose-6-phosphate through phosphoglucomutase<sup>92</sup>. The reverse reaction is catalyzed by glycogen synthase, which generates glycogen from UDP-glucose that is converted from glucose-1-phosphate by glucose-1-phosphate uridylyltransferase. As in gluconeogenesis, the glucose-6-phosphate produced through glycogenolysis is converted to glucose by G-6-Pase, and released to the circulation. Enzymes that have been investigated for drug targeting are highlighted in red.





**Figure 3. Regulation of gluconeogenic transcription factor and coactivator activity**

**a.** Regulation of PGC-1 $\alpha$ . During fasting, induction of PGC-1 $\alpha$  in the liver is achieved through the action of glucagon, which increases cyclic AMP (cAMP), PKA activity, and CREB to increase PGC-1 $\alpha$  transcription<sup>155</sup>. Enhanced PGC-1 $\alpha$  expression is inhibited by the Cdk inhibitor p16<sup>Ink4a</sup>, which decreases gluconeogenic gene expression through suppression of PKA activity<sup>252</sup>. Activated PGC-1 $\alpha$  coactivates transcription of several transcription factor (TF) binding partners, including HNF4, FoxO1, and GR, to increase transcription of target genes such as PEPCK and G-6-Pase. PGC-1 $\alpha$  activity can be altered by PTMs, including acetylation and phosphorylation which decrease its activity, and

methylation which increases its activity. Insulin decreases PGC-1 $\alpha$  activity by increasing its phosphorylation by Akt and Clk2, and S6K also inhibits PGC-1 $\alpha$  through phosphorylation. PGC-1 $\alpha$  acetylation is decreased by Sirt1 and increased by GCN5, whose activity is increased by Cdk4 and Sirt1 and inhibited by CITED2. PGC-1 $\alpha$  is methylated by PRMT1.

**b. Regulation of FoxO activity.** The transcription factor FoxO is co-activated by PGC-1 $\alpha$  to increase transcription of gluconeogenic genes. FoxO activity is modulated by PTMs, including phosphorylation by Akt which leads to its polyubiquitination and degradation, and removal of monoubiquitination by USP7 which decreases its transcriptional activity. FoxO can also be activated through deacetylation by HDAC3 in response to insulin signaling.

**c. Regulation of CREB activity.** CREB is a transcription factor that increases gluconeogenic gene expression from CRE-response elements when activated by a co-activator such as p300, CBP, or CRTC2. CREB activity is increased by phosphorylation at S133, which is enhanced by glucagon-stimulated PKA activity, which is inhibited by Cry1/2. CRE-response element transcription can also be increased by glucagon through dephosphorylation of CRTC2, which increases its activity as a CREB co-activator, or inhibited by insulin-dependent Sik2-mediated phosphorylation of CRTC2, decreasing its activity as a co-activator. CREB activity is inhibited by binding of the transcription factor TCF7L2 to CRE-response elements, which blocks binding of CREB.

Potential therapeutic strategies and agents targeting the activity of PGC-1 $\alpha$ , FOXO and CREB are shown.

P, phosphorylation; Ac, acetylation; Me, methylation; Ub, ubiquitination.

**Table 1**

Pharmacological compounds targeting liver glucose metabolism that have been shown to inhibit hepatic glucose production *in vivo*.

Drug	Target	<i>In vivo</i> and clinical effects	Potential Limitations	Refs.
Metformin	Unclear, involves complex I and mGPD	Extensively used in the clinic to lower blood glucose	Gastrointestinal side effects, precise target unknown, long-term durability as monotherapy lacking	4, 11, 75
GK activators, GKRP inhibitors	GK, GKRP	Reduced blood glucose in diabetic rat and mouse models, early clinical trials demonstrated hyperglycemia normalization	Hypoglycemia and potential toxic metabolites with GK activators, unclear durability	17–24
PTP-1B antisense oligonucleotides	PTP-1B	Decreased fasting glucose in monkey and mouse models, decreased fasting glucose in phase II clinical trial with sulfonylureas	Chronic effects unknown, no small molecule inhibitor available	45–47
PTP-1B small molecule inhibitors (ertiprotafib, trodusquemine)	PTP-1B	Lowered blood glucose in phase II trials	Sub-optimal efficacy, off-target effects on PPAR $\alpha$ , PPARY, IKK- $\beta$	48–53
Glycogen phosphorylase inhibitors (U6751, CP-91149, FR258900, CP-316,819, CP-368,962)	Glycogen phosphorylase	Reduction of blood glucose in <i>db/db</i> and <i>ob/ob</i> mice treated with U6751, CP-91149, or FR258900; decreased hepatic glucose output in fasted dogs treated with CP-316,819; lowered peak hyperglycemia in clinical trial of CP-316,819; short-term blood glucose lowering in clinical trial of CP-368,962	Chronic and extrahepatic effects not well-characterized, ability to lower blood glucose chronically in the clinic is unproven	25, 28–29, 31–34
GSK-3 inhibitors (CHIR-99021, CHIR-98023, L803-mts)	GSK-3	Decreased blood glucose in <i>db/db</i> and <i>ob/ob</i> mice	Possible inhibition of oncogene $\beta$ -catenin	99–102
Glucagon receptor antagonists (Bay 27–9955, LGD-6972, MK-0893, PF-06291874, LY2409021)	Glucagon receptor	Decreased fasting and postprandial glucose levels in phase Ib and II clinical trials	Elevated LDL cholesterol, increased aminotransferases	35–41, 112–113
Phenylpropionic acid	Pyruvate carboxylase	Acute inhibition of gluconeogenesis in normal and diabetic rats	No tissue specificity, may inhibit insulin release, chronic effects unknown	116
FBPase inhibitors (MB06322 and MB07803)	FBPase	Acute and chronic decreased hyperglycemia in diabetic rats treated with MB06322; lowered blood glucose for 6 hours in clinical trial of MB07803	Ability to inhibit FBPase chronically in the clinic is unknown, may cause gastrointestinal side effects	42–44

Drug	Target	<i>In vivo</i> and clinical effects	Potential Limitations	Refs.
FoxO1 inhibitors (AS1842856, AS1708727)	FoxO1	Decreased blood glucose and triglycerides in <i>db/db</i> mice	No tissue specificity, chronic effects unknown	189–190
SR3335	ROR $\alpha$	Decreased plasma glucose in high fat-diet fed mice	No tissue specificity, chronic effects unknown	218
DNP-ME and CRMP	Mitochondrial uncoupling	Lowered blood glucose and triglycerides in high fat diet-fed and diabetic rats	Possible toxicity from mitochondrial uncoupling	224–225

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