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SYMPOSIUM REVIEW

Vitamin D binding protein/GC-globulin: a novel regulator of alpha cell function and glucagon secretion

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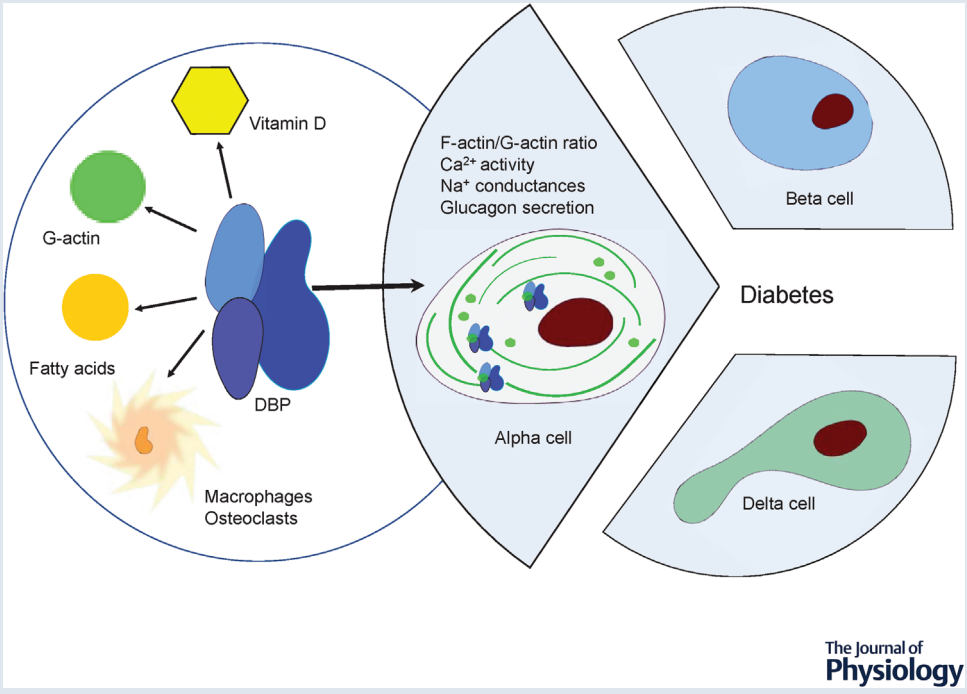

Abstract The contribution of glucagon to type 1 and type 2 diabetes has long been known, but the underlying defects in alpha cell function are not well-described. During both disease states, alpha cells respond inappropriately to stimuli, leading to dysregulated glucagon secretion, impaired

Katrina Viloria conducted her undergraduate degree at the University of Alaska Anchorage, before undertaking PhD studies with Dr Natasha Hill at Kingston University. Her current postdoctoral research focuses on vitamin D-binding protein action in pancreatic alpha cells. **Martin Hewison** is Professor of Molecular Endocrinology at the University of Birmingham, having previously spent 9 years at Cedars-Sinai Medical Center and UCLA. He is noted for his seminal contributions to the field of vitamin D biology, in particular the role of vitamin D metabolism in immune function. **David J. Hodson** qualified as a Veterinary Surgeon, before conducting PhD studies at the University of Bristol and postdoctoral studies at CNRS, France. He is currently Professor of Cellular Metabolism at the University of Birmingham and leads a lab devoted to the understanding of pancreatic islet biology.

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glucose tolerance and hypoglycaemia. The mechanisms involved in this dysfunction are complex, but possibly include changes in alpha cell glucose-sensing, alpha cell de-differentiation, paracrine feedback, as well as alpha cell mass. However, the molecular underpinnings of alpha cell failure are still poorly understood. Recent transcriptomic analyses have identified vitamin D binding protein (DBP), encoded by *GC/Gc*, as an alpha cell signature gene. DBP is highly localized to the liver and alpha cells and is virtually absent from other tissues and cell types under non-pathological conditions. While the vitamin D transportation role of DBP is well characterized in the liver and circulation, its function in alpha cells remains more enigmatic. Recent work reveals that loss of DBP leads to smaller and hyperplastic alpha cells, which secrete less glucagon in response to low glucose concentration, despite vitamin D sufficiency. Alpha cells lacking DBP display impaired Ca^{2+} fluxes and Na^{+} conductance, as well as changes in glucagon granule distribution. Underlying these defects is an increase in the ratio of cytoskeletal F-actin to G-actin, highlighting a novel intracellular actin scavenging role for DBP in islets.

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Abstract figure legend The multifunctional vitamin D binding protein (DBP) has a novel role in alpha cell function. DBP regulates F-actin/G-actin ratios and glucagon granule distribution. Loss of DBP leads to abnormal alpha cell shape, Ca^{2+} activity, Na^{+} conductance and impaired glucagon secretion. Understanding the role of DBP in alpha cells may further reveal the critical role of alpha cell regulation in the development of diabetes.

Introduction

Glucagon is the major counter-regulatory hormone that prevents hypoglycaemia by inhibiting insulin secretion and increasing endogenous glucose production. As the second most abundant cell type in the islet of Langerhans, alpha cells are the main source of (pro)glucagon and work in close cooperation with insulin-secreting beta cells and somatostatin-secreting delta cells to control glucose homeostasis. During type 2 diabetes mellitus (T2DM) (and type 1 diabetes; T1D), alpha cell function becomes dysregulated, leading to inappropriate glucagon secretion and exacerbation of blood glucose levels (D'Alessio, 2011), as well as impaired counter-regulatory responses (McCrimmon & Sherwin, 2010). Indeed, glucagon hypersecretion and impaired glucagon counter-regulation have been proposed to contribute to beta cell failure and T2DM development (Müller *et al.* 1970; Reaven *et al.* 1987; Dinneen *et al.* 1995; Larsson & Ahren, 2000; Shah *et al.* 2000; Unger & Cherrington, 2012).

Recent advances in RNA sequencing of single human islet cells have revealed novel genes that are specifically enriched in alpha, beta, and delta cells. Among the top enriched genes in alpha cells is *GC/Gc*, which encodes vitamin D binding protein (DBP), primarily considered to be the major transporter of vitamin D metabolites in the circulation (also known as GC-globulin or group-specific component) (Daiger *et al.* 1975; Dorrell *et al.* 2011; Ackermann *et al.* 2016; Segerstolpe et al. 2016). However, DBP is a multifunctional and pleiotropic protein, and is also known to bind fatty acids, activate macrophages and potently scavenge actin released into serum (Van Baelen *et al.* 1977; Williams *et al.* 1988; Bouillon *et al.* 1992; Yamamoto & Naraparaju, 1996; Kanda *et al.* 2002). Although well-characterized polymorphic DBP variants have been associated with increased risk of developing diabetes (Malik *et al.* 2013; Bikle & Schwartz, 2019; Bouillon *et al.* 2019), the influence of DBP on alpha (and other islet) cell function has not been considered beyond its classical marker role. Recent studies in mouse and human tissue have demonstrated that DBP/*Gc* contributes to normal alpha cell function (Viloria *et al.* 2020), is upregulated in de-differentiated beta cells in high fat diet-fed mice (Kuo *et al.* 2019; Kuo & Accili, 2020), and as such plays a hitherto under-appreciated role in the regulation of both glucagon and insulin secretion. This symposium review summarises the known functions of DBP that are relevant for alpha cell function, effects of global DBP deletion, and how this information can be potentially leveraged to modify glucagon and insulin secretion in health and disease.

Alpha cell physiology and regulation

Pancreatic islets control glycaemia through a tightly coordinated secretion of endocrine hormones (Islam, 2015; Gilon, 2020). The rodent islet mass comprises

∼60–80% insulin-secreting beta cells, ∼15–20% glucagon-secreting alpha cells with less than ∼1% as somatostatin-secreting delta cells (Brelje *et al.* 1989; Brissova *et al.* 2005). Located at the islet core are beta cells, surrounded by alpha and delta cells at the outer periphery or mantle (Steiner *et al.* 2010). Suggesting a more intimate paracrine regulation in humans, the proportion of alpha cells increases up to ∼30–40% of the total islet mass, and they are more interspersed with beta cells and delta cells due to a tertiary folding-step (Cabrera *et al.* 2006; Bosco *et al.* 2010).

Hyperglycaemia stimulates beta cells to secrete insulin, signalling to muscle tissues for glucose uptake and the liver to inhibit endogenous glucose production, consequently lowering glucose levels to normoglycaemia (Edgerton *et al.* 2006; Quesada *et al.* 2008; Fu *et al.* 2013; Gilon, 2020). As glucose levels continue to decrease and reach hypoglycaemia, alpha cells begin to secrete glucagon, stimulating hepatic glycogenolysis and gluconeogenesis, thus releasing glucose back into the circulation as part of the counter-regulatory response (Band & Jones, 1980; Quesada *et al.* 2008; Unger & Cherrington, 2012; Gilon, 2020). Control of glucagon secretion operates through both glucose-dependent (endogenous) and -independent (exogenous) pathways.

Intrinsic regulation of alpha cell function. Alpha cells express several ion channels that together contribute to membrane depolarisation, ion influx and exocytosis (Fig. 1). At low glucose (1 mM), ATP-sensitive K^+ channels (K_{ATP} channels) are moderately activated (cf. beta cells), leading to a membrane potential of −60 mV. This slight depolarisation is sufficient to open T-type Ca^{2+} channels, further depolarising the membrane to −40 mV, which subsequently activates L-type, N-type, and P/Q-type Ca^{2+} channels as well as Na^+ channels. Opening of these high voltage-activated Ca^{2+} channels allows a large influx of Ca^{2+} into the cytoplasm, generating large amplitude action potentials to trigger glucagon exocytosis (Zhang *et al.* 2013, 2020). By contrast, rising blood glucose levels increase ATP/ADP ratios, causing K_{ATP} channels to close. This further depolarisation leads to partial voltage inactivation of Na^+ channels, depressing action potential peak amplitude, reducing voltage-gated P/Q-Ca^{2+} channel activation and thus inhibiting glucagon secretion (Zhang *et al.* 2013, 2020) (Fig. 1*A*). The K_{ATP} channel model of alpha cell regulation remains debated, however, since opposing effects of K_{ATP} channel blockers (sulfonylureas) on glucagon release have been reported (Cheng-Xue *et al.* 2013; Zhang *et al.* 2013), including a strong glucagonotropic effect in the absence of somatostatin input (Lai *et al.* 2018), amongst other arguments.

A second model has been suggested to operate through K_{ATP} channel-independent mechanisms via store-operated Ca^{2+} channels (SOC) (Liu *et al.* 2004; Vieira *et al.* 2007; Gylfe, 2013) (Fig. 1*B*). At low glucose, SOC are open, maintaining a depolarising potential. As glucose levels rise and ATP/ADP levels increase, Ca^{2+} is sequestered into the endoplasmic reticulum via sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), causing the closure of SOC and re-polarization of the alpha cell membrane. This leads to low frequency action potentials and inhibition of glucagon secretion (Liu *et al.* 2004; Vieira *et al.* 2007; Gylfe, 2013). Glucose concentrations in the hypoglycaemic range have also been shown to increase sub-plasma membrane levels of cAMP (Tengholm & Gylfe, 2017; Yu *et al.* 2019). This nucleotide exerts a number of effects on alpha cell function, including release of Ca^{2+} from intracellular stores, increased Ca^{2+} entry via L-type Ca^{2+} channels and protein kinase A- and Epac2-dependent increases in exocytosis (Gromada *et al.* 1997; De Marinis *et al.* 2010; Tengholm & Gylfe, 2017; Yu *et al.* 2019). Other hypotheses also exist for the intrinsic regulation of alpha cell function and the reader is directed to several excellent reviews for further information (Quesada *et al.* 2008; Rorsman *et al.* 2012; Briant *et al.* 2016; Hughes *et al.* 2018; Gilon, 2020).

Extrinsic regulation of alpha cell function. Paracrine mechanisms activated at high glucose levels contribute to glucagon inhibition. Alpha cells express the insulin and somatostatin receptors, which following activation by neighbouring beta cells and delta cells, suppress glucagon secretion, decreasing blood glucose levels and post-prandial plasma glucagon (Kumar *et al.* 1999; Yoshimoto *et al.* 1999; Gromada *et al.* 2001; Diao *et al.* 2005; Dunning *et al.* 2005). Other beta cell secretagogues including zinc, amylin, GABA and 5-HT have been demonstrated to inhibit glucagon secretion to varying degrees (Rorsman *et al.* 1989; Wendt *et al.* 2004; Diao *et al.* 2005; Gedulin *et al.* 2006; Gyulkhandanyan *et al.* 2008; Quesada *et al.* 2008; Almaca *et al.* 2016; Hughes *et al.* 2018). Conversely, glucagon is a potent stimulator of insulin secretion. Recent studies have shown that intra-islet glucagon levels are sufficient to stimulate insulin secretion from human islets under low (2.7–7 mM) and high (10 mM) glucose conditions (Rodriguez-Diaz *et al.* 2018; Capozzi *et al.* 2019), whereas mouse islets respond only in the presence of high glucose (Capozzi *et al.* 2019; Zhu *et al.* 2019). Nonetheless, these data further indicate that alpha cell regulation during normo- and hyper-glycaemia is also essential and warrants further investigation. Finally, the parasympathetic nervous system is a strong driver of glucagon release, largely via both cholinergic and non-cholinergic mechanisms (Thorens, 2011).

Gut-derived incretins. Intestinal glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) exert glucagonostatic and glucagonotropic effects, respectively

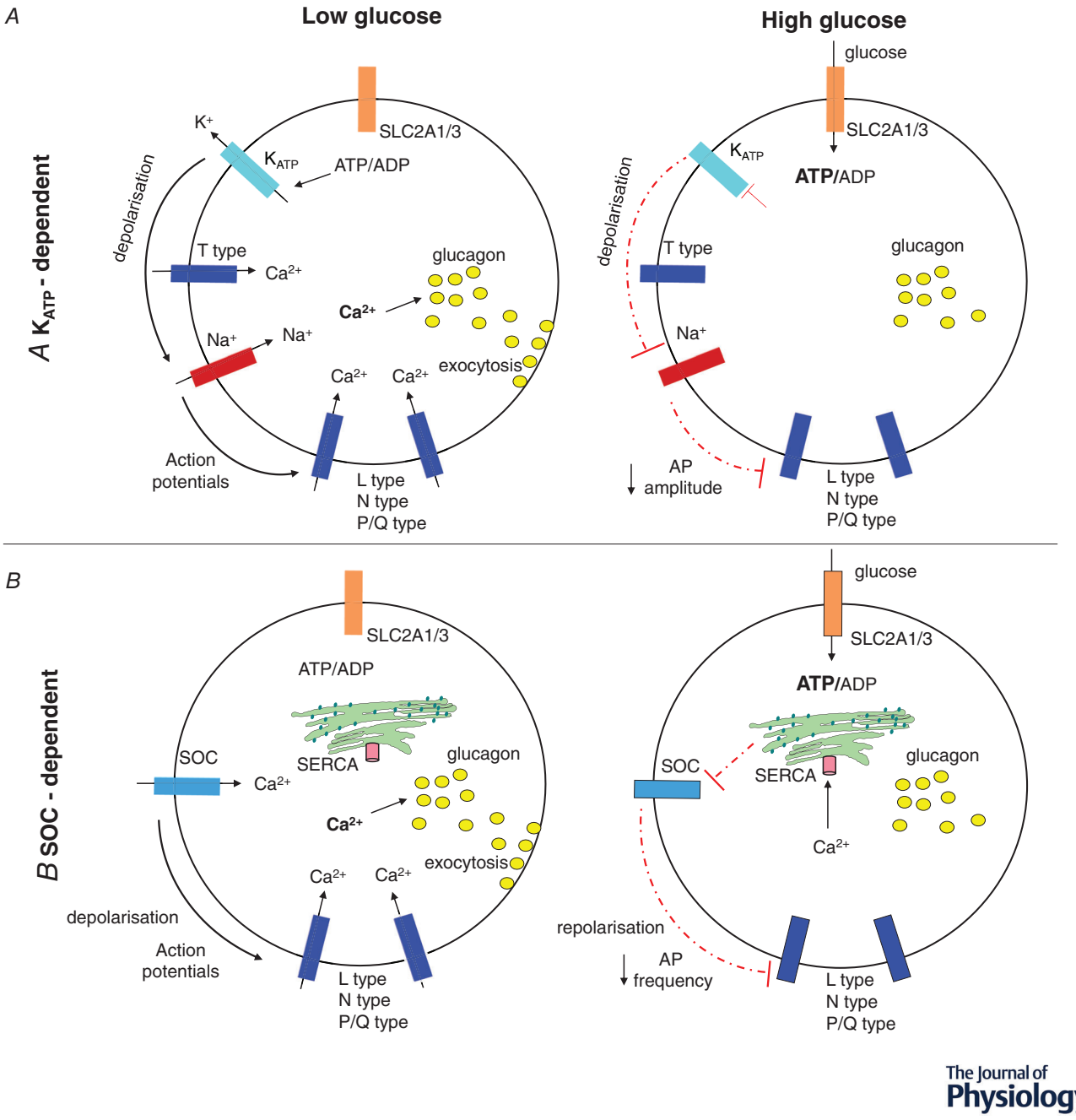

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Figure 1. Major models of alpha cell stimulus–secretion coupling
Glucose enters alpha cells through GLUT1 and GLUT3 (encoded by *Slc2a1/SLC2A1* and *Slc2a3/SLC2A3*, respectively). Two models are proposed to then couple glucose to alpha cell electrical activity and secretion. *A*, in the first model, K_{ATP} -dependent pathways regulate Ca^{2+} influxes. At low glucose, adequate ATP/ADP levels maintain a membrane potential that opens T-type Ca^{2+} channels. Further depolarization opens Na^+ channels and other voltage-dependent Ca^{2+} channels such as L, N and P/Q type. Increased Ca^{2+} influx generates strong action potentials that trigger glucagon exocytosis. At high glucose, the resulting increase in ATP/ADP levels shuts off K_{ATP} channels, leading to the closure of Na^+ channels and partial depolarization. This generates low amplitude action potentials, thereby inactivating high voltage Ca^{2+} channels, preventing large Ca^{2+} influxes and reducing glucagon secretion. *B*, a second model of glucose-dependent alpha cell regulation operates through store operated Ca^{2+} channel (SOC)-dependent pathways. At low glucose, SOC are open, allowing Ca^{2+} entry and glucagon secretion. However, at high glucose, Ca^{2+} is incorporated into the endoplasmic reticulum via sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA). This results in the closure of SOC, thereby generating a repolarizing membrane potential and low frequency action potentials, shutting off Ca^{2+} influxes and glucagon secretion.

(Meier & Nauck, 2005; Parker *et al*. 2009; Hare *et al*. 2010; El & Campbell, 2020). Moreover, clinically-approved GLP-1 receptor (GLP-1R) agonists suppress glucagon secretion whilst augmenting insulin release in a GLP-1R-dependent manner (Juhl *et al*. 2002; Degn *et al*. 2004; Drucker, 2018). Since GLP-1R is largely absent from alpha cells, such effects of GLP-1R agonists are likely to be indirect via other islet cell types, although electrophysiological studies have shown direct effects of GLP-1 itself (De Marinis *et al*. 2010), possibly via degradation products acting via the glucagon receptor (Guida *et al*. 2020).

Alpha cells in diabetes

Loss of glucagon control. Alpha cell glucose-sensing is compromised in T2DM and impaired glucagon secretion is proposed to exacerbate hyperglycaemia (Unger & Cherrington, 2012; Gromada *et al*. 2018; Gilon, 2020), which may then further impair beta cell function. Persistent hyperglucagonaemia in the fasting and postprandial states is commonly observed in diabetes, indicating loss of alpha cell inhibition at hyperglycaemic states (Müller *et al*. 1970). It has been suggested that hyperglucagonaemia is due to alpha cells developing resistance to insulin and hyperglycaemia (Unger & Cherrington, 2012; Yosten, 2018), alongside dysregulated somatostatin, GIP and GLP-1 input/signalling (Lund *et al*. 2014; Yosten, 2018; Kellard *et al*. 2020). Furthermore, the aberrantly high glucose levels may stimulate increased glucagon secretion, exacerbating hyperglycaemia (Salehi *et al*. 2006). Recent studies in human alpha cells show that both glucose (intrinsic) and paracrine (extrinsic) signals interact to regulate exocytosis of glucagon granules, and this interaction becomes dysfunctional during T2DM (Omar-Hmeadi *et al*. 2020). On the other hand, hypoglycaemia unawareness is commonly observed in T1D and T2DM, with impaired alpha cell stimulation at low glucose leading to loss of glucagon counter-regulation and increased risk of hypoglycaemia (Bolli, 2003; UK Hypoglycaemia Study Group, 2007; Yosten, 2018).

Changes in alpha cell mass and morphology. Changes in alpha cell mass have been reported during diabetes, with studies in both T1D and T2DM showing an increase in alpha cell mass (Rahier *et al*. 1983; Clark *et al*. 1988; Plesner *et al*. 2014) while others have reported decreases (Pechhold *et al*. 2009; Bru-Tari *et al*. 2019) or no changes at all (Stefan *et al*. 1982; Sakuraba *et al*. 2002; Henquin & Rahier, 2011; Campbell-Thompson *et al*. 2016). It is important to note, however, that findings may depend on age and disease stage, as well as imaging or quantification techniques used. Nonetheless, changes in alpha cell mass are likely to occur early in disease progression, as shown in mouse experiments where increased alpha cell mass and hypertrophy were observed prior to frank diabetes onset induced by streptozotocin (Plesner *et al*. 2014). Since alpha cells persist during T1D and T2DM, restoration of their function represents a viable therapeutic target.

Vitamin D binding protein

Vitamin D transport. Initially isolated in 1959 from the liver, GC was found to be a polymorphic serum protein (Hirschfeld *et al*. 1960). It was not until 1979 that GC was shown to bind vitamin D and was also referred to as DBP (Daiger *et al*. 1975). Subsequent studies indicated that DBP was structurally related to albumin and α-fetoprotein, with the *GC* gene being a member of the albumin/α-fetoprotein gene family on chromosome 4 (Harper & Dugaiczyk, 1983; Cooke *et al*. 1986). In common with other steroid-like molecules, the active, hormonal form of vitamin D, 1,25-dihydroxyvitamin D (1,25(OH)2D), and its precursor, 25-hydroxyvitamin D (25(OH)D), can circulate through low-affinity binding to common serum proteins such as albumin. Although less abundant than albumin, high affinity binding to DBP means that this is the major serum transporter of vitamin D metabolites (Fig. 2*A*). The major circulating form of vitamin D, 25(OH)D, shows the highest binding affinity for DBP resulting in 85% of 25(OH)D being bound to DBP and only 15% to albumin, leaving less than 1% unbound in circulation (Bikle & Schwartz, 2019; Bouillon *et al*. 2019). Binding of 25(OH)D is fundamental to vitamin D endocrinology with facilitated endocytic uptake of 25(OH)D–DBP via the megalin–cubilin complex being essential for renal synthesis of 1,25(OH)2D in the proximal tubules (Nykjaer *et al*. 1999). Outside the kidneys, a wide range of tissues are known to express megalin–cubilin and are therefore also able to acquire DBP-bound vitamin D metabolites via endocytic uptake (Lundgren *et al*. 1997). Nevertheless, expression of megalin–cubilin is not universal and so other mechanisms are required for uptake of 25(OH)D and 1,25(OH)2D by many target cells. The free hormone hypothesis describes the unbound hormone as the bioavailable fraction for cell uptake (Mendel, 1989; Hammond, 2002; Chun *et al*. 2014). Lipophilic in nature, unbound vitamin D metabolites can freely diffuse through the plasma membrane to reach intracellular targets such as the vitamin D-activating enzyme 25-OHD-1 α-hydroxlyase (CYP27B1) or the nuclear vitamin D receptor (VDR) for 1,25(OH)2D. Hormone carrier proteins such as DBP therefore play a crucial role in controlling the amount of circulating hormone available for cell uptake by either megalin/cubilin-dependent or megalin-independent mechanisms (Bikle & Schwartz, 2019; Bouillon *et al*. 2019).

Other DBP substrates. Though less studied than vitamin D transport, DBP binds to many other substrates such as monomeric G-actin and fatty acids (Van Baelen *et al.* 1977; Williams *et al.* 1988; Bouillon *et al.* 1992), and a deglycosylated form of DBP can act as a macrophage-activator factor (maf) (Yamamoto *et al.* 1991, 1996; Yamamoto & Kumashiro, 1993; Yamamoto & Naraparaju, 1996). Related to the albumin family of proteins, DBP is composed of 460 amino acids in rodents and 458 amino acids in humans, with three main domains consisting of α-helices (Law & Dugaiczyk, 1981; Verboven *et al.* 2002). Domain I contains the vitamin D binding region while G-actin binding occurs between domains II–III, suggesting that actin does not compete with vitamin D binding (Haddad *et al.* 1992; Head *et al.* 2002) (Fig. 2*B*). With higher affinity for G-actin (K_d = 10 nM) than other actin-binding proteins such as gelsolin (K_d = 50 nM), DBP binding blocks the

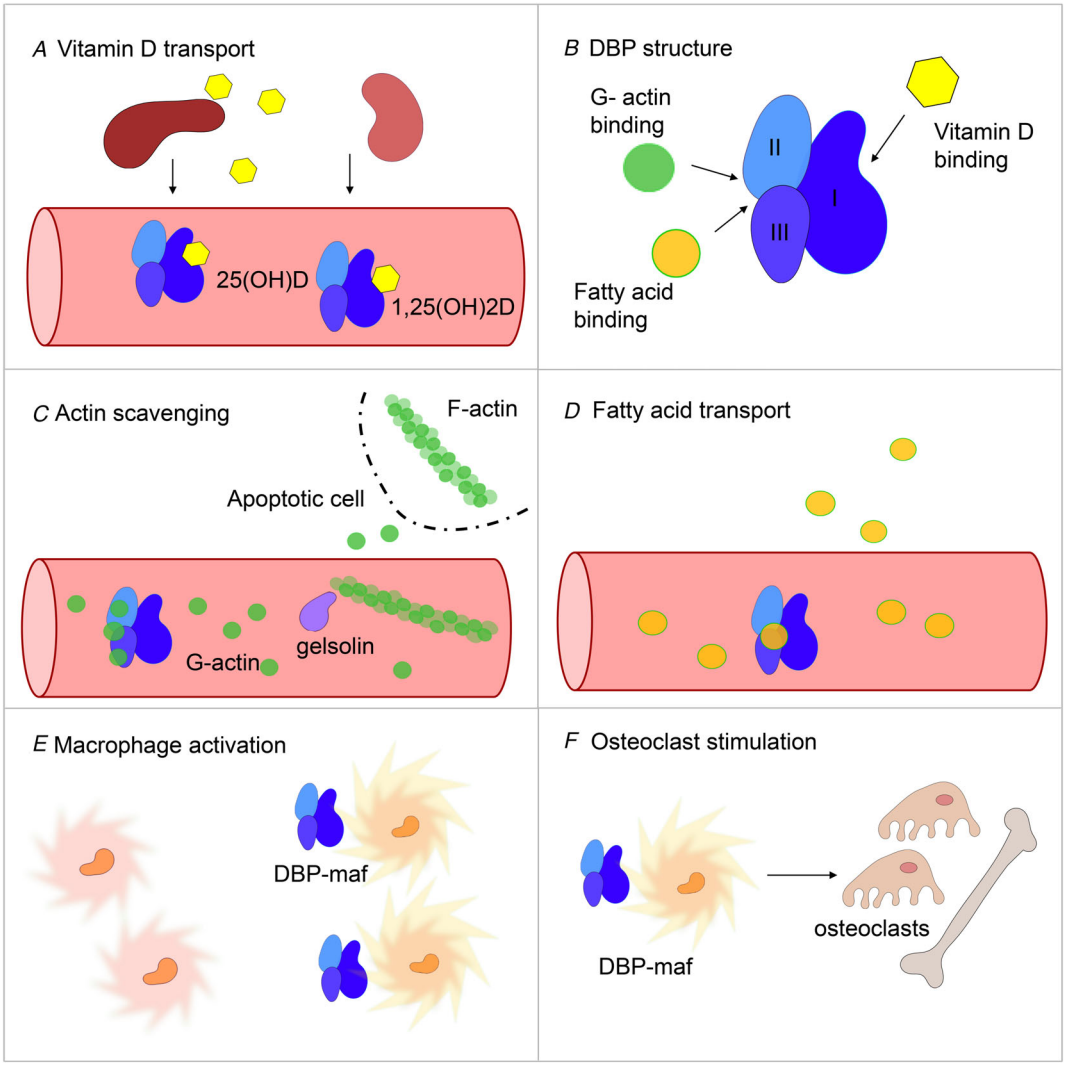

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Figure 2. Vitamin D-binding protein has multiple functions in the circulation and cells
A, vitamin D-binding protein (DBP) binds to vitamin D with high affinity and is the major serum transporter of vitamin D metabolites. As such, DBP plays a central role in regulating circulating free vitamin D levels. *B*, structurally related to the albumin family, DBP has 3 main domains consisting of α-helices. Domain I contains the vitamin D-binding region while domains II–III contains G-actin and fatty acid binding regions. *C*, DBP binds to G-actin, preventing polymerization of F-actin. Operating in concert with gelsolin, DBP plays a role in actin scavenging in serum to prevent fibrosis. *D*, DBP transports fatty acids. Binding to unsaturated fatty acids may alter DBP binding to vitamin D. *E*, DBP activates macrophages (DBP–maf complex) and plays a role in regulating inflammation. *F*, DBP–maf stimulates osteoclasts and regulates bone remodelling.

fast growing end of actin monomers, effectively preventing actin from repolymerizing (Mc Leod *et al.* 1989; Vasconcellos & Lind, 1993) (Fig. 2*C*). For this reason, DBP is amongst the most potent actin scavengers in the body.

DBP also binds to mono-unsaturated, poly-unsaturated and saturated fats (Calvo & Ena, 1989; Ena *et al.* 1989; Bouillon *et al.* 1992; Swamy & Ray, 2008), although with lower affinity ($K_a = 10^5–10^6$ M^{-1}) than albumin ($K_a = 10^7–10^8$ M^{-1}) (van der Vusse, 2009) (Fig. 2*D*). Little is known about the role of DBP in fatty acid transport, but it is suggested that mono and poly-unsaturated fatty acid binding may alter DBP configuration and modify binding to 25(OH)D and 1,25(OH)2D (Williams *et al.* 1988; Ena *et al.* 1989; Bouillon *et al.* 1992). A ∼58 kDa protein, DBP may be deglycosylated to form complexes with macrophages (Fig. 2*E*). The DBP–maf complex activates macrophages and related cells such as osteoclasts (Fig. 2*F*) and thus plays a role in inflammation and bone remodelling (Yamamoto *et al.* 1991, 1994; Schneider *et al.* 1995; Nykjaer *et al.* 1999). Additionally, DBP–maf has been of interest in cancer research and has been shown to inhibit pancreatic tumour growth with antiangiogenic and pro-apoptotic functions (Kisker *et al.* 2003).

DBP polymorphisms

To date, more than 124 DBP variant alleles have been described in humans (Chalk & Kodicek, 1961; Van Baelen *et al.* 1977; Cleve & Constans, 1988; Speeckaert *et al.* 2006; Bikle & Schwartz, 2019; Bouillon *et al.* 2019). DBP variants were first characterised by varying electrophoretic mobility and were therefore initially referred to group-specific component. Three major codominant alleles have been identified, *GC1f* and *GC1s* located at the rs7041 *GC* locus and *GC2* at the rs4588 *GC* locus. The two subtypes of GC1 differ in their charge, with GC1f running electrophoretically faster than GC1s (Speeckaert *et al.* 2006; Bikle & Schwartz, 2019; Bouillon *et al.* 2019). DBP polymorphisms are major determinants of the genetic variability in serum 25(OH)D concentrations (Wang *et al.* 2010), and also show distinct patterns of expression in different racial groups (Bouillon, 2017). Polymorphisms in DBP have been associated with multiple chronic diseases such as cancer, chronic obstructive pulmonary disease, asthma, thyroid autoimmunity, liver and inflammatory bowel diseases, diabetes as well as susceptibility to infectious diseases including HIV, rheumatoid fever and tuberculosis (Speeckaert *et al.* 2006; Malik *et al.* 2013). The exact role of DBP and its variants in the pathophysiology of these diseases has yet to be defined as it is unclear whether genetic variations in DBP impact its ability to bind vitamin D, fatty acids, or G-actin.

DBP variation and diabetes risk. *GC* gene variants may affect circulating DBP serum levels as well as vitamin D binding affinity, thus influencing the risk of developing vitamin D deficiency. Individuals harbouring the GC2 variant, for example, were found to have 5–10% lower serum levels of vitamin D *versus* those with the GC1 variant (Bouillon *et al.* 1980; Lauridsen *et al.* 2001; Bouillon, 2017). Furthermore, the GC2 variant was shown to have the least affinity for 25(OH)D, followed by GC1s, with GC1f showing the highest affinity (Arnaud & Constans, 1993). However, these findings were challenged by other studies showing no such difference in vitamin D affinity between the variants (Bouillon *et al.* 1980; Boutin *et al.* 1989). Several studies have shown differences between the association of DBP polymorphisms with glucose tolerance and diabetes incidence. GC1s-2 and Gc1s-1s were associated with higher fasting plasma insulin compared to Gc1f in a Japanese and Dogrib Indian cohort (Szathmary, 1987; Hirai *et al.* 2000). However, no such association was detected in Hispanic or Caucasian participants (Baier *et al.* 1998; Klupa *et al.* 1999). By contrast, although no association with fasting plasma glucose or insulin was found in Pima Indians, GC1f was found to have the highest postprandial glucose (Baier *et al.* 1998). However, in a study of Japanese individuals, participants with diabetes were more likely to carry the heterozygous GC1s-2 variant (Hirai *et al.* 1998), but no strong differences in variant expression were observed between healthy and T1D or T2DM in Pima Indians or in Caucasians (Baier *et al.* 1998; Klupa *et al.* 1999). Nonetheless, reduced serum DBP levels have been associated to T1D (Blanton *et al.* 2011), and additionally DBP has in fact been classified as an autoantigen, activating T cells in non-obese diabetic mice (Kodama *et al.* 2016). Most recently, large-scale Mendelian randomisation studies of European and Chinese adults have shown an association between *GC* and T2DM. However, the study included other vitamin D-related single nucleotide polymorphisms, which were used to link serum DBP levels with genetically determined variation in 25(OH)D status and T2DM (Lu *et al.* 2018). Thus, *GC* gene variants are present and might be linked to T2DM, but there is no way at present of knowing how this relates to DBP tissue expression and actin binding.

Suggesting that DBP action and variation may have a wider impact than simple vitamin D transport are reports from DBP-null mice. Mice lacking DBP possess markedly decreased serum vitamin D, but do not display any signs of vitamin D-related diseases or vitamin D deficiency. DBP-null mice show normal bone and immune phenotypes, providing evidence that the low levels of 25(OH)D and 1,25(OH)2D that circulate either free or bound to albumin are able to fulfil most of the functions of vitamin D. In support of this, DBP-null mice only show symptoms of vitamin D deficiency when

placed on a diet low in vitamin D (Safadi *et al*. 1999). More recently, the first human with homozygous *GC* deletion was described, also showing reduced serum 25(OH)D and 1,25(OH)2D with no signs of deficiency (Henderson *et al*. 2019). Together, these studies show that deletion of DBP depletes vitamin D levels, but enough bioavailable vitamin D is retained to exert biological effects (Safadi *et al*. 1999). Investigations on the implications of DBP in diabetes should therefore consider non-vitamin D binding roles of DBP. Indeed, following detailed whole body assessment of DBP-null mice (∼500 animals per genotype), significant changes were only detected in metabolic homeostasis, including decreased fed glucose, increased circulating alanine transaminase and decreases in cholesterol, high-density lipoprotein cholesterol and triglyceride (https://www.mousephenotype.org/data/genes/MGI:95669#phenotypesTab). These data point to changes in glucagon release, liver function, adipose function and alpha cell–liver communication.

DBP as an alpha cell regulator

Gene tissue-expression patterns show that *GC* is predominantly expressed in the liver, with pancreatic islets being the only other organ/tissue to have significant expression of *GC*. Subsequent cell type-specific RNA sequencing identified the *GC* transcript among the alpha cell enriched genes expressed in human islet cells (Dorrell *et al*. 2011; Ackermann *et al*. 2016; Segerstolpe et al. 2016). Resembling other known alpha cell markers such as *ARX*, *DPP4*, and *GCG*, the *GC* gene was found to contain cell type-specific open chromatin regions at its promoter, indicating that *GC* is an alpha cell signature gene (Ackermann *et al*. 2016). Despite the known (potent) biological functions of DBP, an effect on alpha cell physiology has only recently been examined. Using DBP-null mice, we were able to show that loss of DBP results in major alpha cell impairments (Viloria *et al*. 2020) (Fig. 3). Mice with DBP deletion displayed reductions in insulin- and low glucose-stimulated glucagon release. Mechanistically, fewer alpha cells responded to low glucose with Ca^{2+} rises, although those that were responsive displayed increased Ca^{2+} amplitude. This compensatory response was reflected at the level of Na^{+} channel function, with DBP-null alpha cells showing increased Na^{+} currents and an increased slope factor for Na^{+} channel inactivation (Fig. 3*A*). However, when recordings were subjected to mathematical prediction models (Briant *et al*. 2017), alpha cells lacking DBP displayed an electrophysiological fingerprint that more closely resembled a delta cell-like signature.

Also suggesting a role for DBP in maintaining alpha cell morphology, deletion of DBP in mice resulted in smaller and hyperplastic alpha cells (Fig. 3*B*). Immunohistochemical analysis of DBP in pancreata from human donors revealed an increase in DBP with age, in parallel with glucagon expression, suggesting that DBP might become relatively more important as alpha cells fully mature. These changes were unlikely to be associated with alpha cell de-differentiation, however, since expression levels of *Arx*, *Pax6*, *Pou3f4*, and *Irx2* were similar in $DBP^{-/-}$ and $DBP^{+/+}$ islets. In pancreata from donors with late-onset T1D, DBP was decreased, and this was associated with decreased glucagon expression and reduced cell size (Viloria *et al*. 2020). Thus, loss of DBP leads to impaired alpha cell morphology, function and glucagon release and may represent a marker of late-onset T1D. It is important, however, to consider these results in light of potential effects of the liver on alpha cells (e.g. via amino acids) (Wewer Albrechtsen *et al*. 2019), as well as the indirect nature of DBP–T1D correlations (i.e. changes in DBP might be a consequence of rather than the cause of T1D). Future studies will be required using both alpha cell- and liver-specific DBP deletion models.

DBP as a novel intracellular (and extracellular) actin regulator

Actin-related functions of DBP have largely been explored in the circulation and in the extracellular space where its primary role is to clear actin monomers released by apoptotic cells. The actin-scavenging system operates with gelsolin as the primary F-actin depolymerising agent. The resulting G-actin monomers are sequestered by DBP with high affinity, inhibiting repolymerisation of fibrils and thus preventing fibrosis and potential obstruction of vasculature (Mc Leod *et al*. 1989; Vasconcellos & Lind, 1993; Speeckaert *et al*. 2006; Bikle & Schwartz, 2019; Bouillon *et al*. 2019). The use of DBP and gelsolin to scavenge actin is currently patented for therapeutic use in respiratory diseases (Stossel *et al*. 1995), but practically nothing is known about whether DBP is able to bind actin within the cell. Due to the endogenous expression of DBP, alpha cells thus provide a unique opportunity to understand the contribution of cytoplasmic actin scavenging to cell function.

Using phalloidin to stain F-actin fibrils, loss of DBP was found to increase the density of polymerised F-actin fibrils, with a concomitant decrease in G-actin monomer abundance (Viloria *et al*. 2020) (Fig. 3*B*). Suggesting that these changes in F-actin and G-actin are associated with changes in actin-dependent processes, distribution and size of glucagon granules were found to be altered in DBP-null alpha cells. Thus, it appears that DBP may assist dynamic actin remodelling in alpha cells, similarly to that described for neural cell adhesion molecule (NCAM) and ephrin type-A receptor 4 (Olofsson *et al*. 2009; Hutchens

& Piston, 2015; Hughes *et al.* 2018). DBP may plausibly sequester G actin monomers near granules, restricting supply of monomers and controlling the F-actin/G-actin ratio for fibril polymerisation and secretory regulation, as well as ion channel function. The fact that alpha cells express their own specialised supply of an actin binding protein, in addition to actin remodelling proteins, further supports the importance of cytoskeletal re-arrangement in alpha cell function (Olofsson *et al.* 2009; Hutchens & Piston, 2015; Hughes *et al.* 2018).

We propose that changes in the F-actin cytoskeleton lead to many of the reported defects in DBP-null alpha cells. Indeed, assembly of polymerised actin filaments is a fundamental process involved in cell morphology (Pollard & Cooper, 2009), and F-actin has been shown to influence the trafficking of various ion channels present in beta cells through the action of actin-binding partners including Rab GTPases, SNARE proteins and tubulin (Sasaki *et al.* 2014). Notably, F-actin has also been shown to directly interact with ion channels, gating their activity (Shin *et al.*

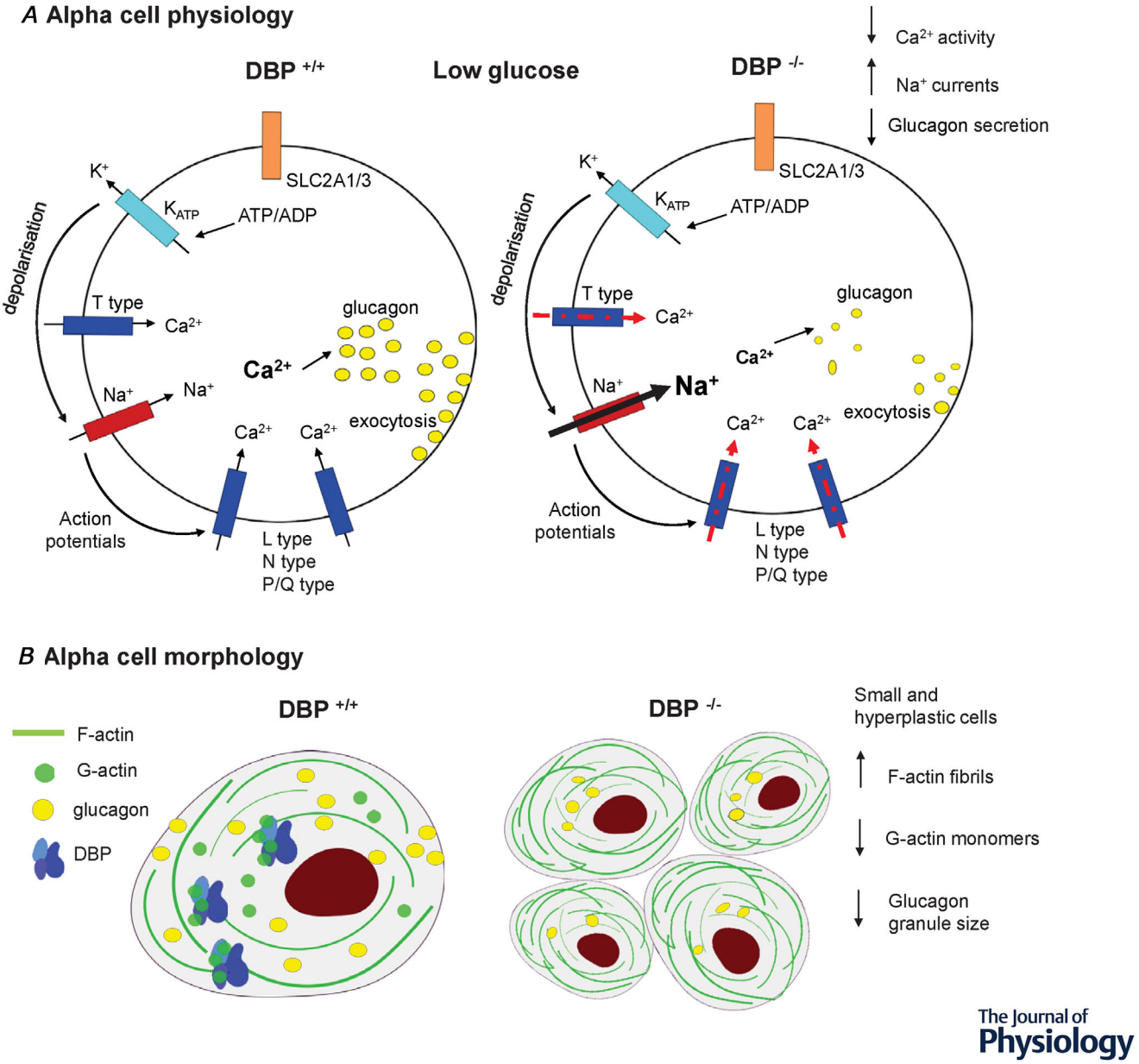

Figure 3. Vitamin D-binding protein regulates alpha cell function
A, global deletion of vitamin D-binding protein (DBP) leads to impairments in alpha cell function, including changes in Ca^{2+} spiking activity and Na^{+} conductance, which result in decreased glucagon secretion. *B*, loss of DBP results in smaller and hyperplastic alpha cells. Changes in cell shape are associated with altered F-actin/G-actin ratios, and decreased size and distribution of glucagon granules.

2012; Sasaki *et al.* 2014). Providing evidence for a role of DBP in ion channel function in alpha cells, treatment with latrunculin to depolymerise F-actin restored Ca^{2+} responses to low glucose in DBP-null islets (Viloria *et al.* 2020). While glucagon was not measured in these specific experiments, inhibition of actin polymerisation restored glucagon secretion in NCAM-null islets in which F-actin distribution is also perturbed (Olofsson *et al.* 2009).

Given that DBP is an important regulator of ion channel activity and exocytosis, why is the gene not expressed in other neuro(endocrine) cell types that also rely on cytoskeletal remodelling for secretion? One explanation is that other neuro(endocrine) cell types might be able to acquire DBP via megalin-mediated endocytic internalisation, as recently shown in trophoblasts (Ganguly *et al.* 2021). Another explanation is that the actin scavenger gelsolin is glucose-dependent, at least in beta cells where its actions are needed for glucose-stimulated insulin secretion (Tomas *et al.* 2006). As such alpha cells might have evolved endogenously expressed actin remodelling mechanisms that respond to low glucose.

Of note, DBP is present in glucagon granules in human alpha cells (Viloria *et al.* 2020), suggesting that it might be released in a regulated manner. As well as acting directly on actin filaments near to the plasma membrane, we speculate that DBP is released into the extracellular space with glucagon in response to low glucose, from where it might exert paracrine effects on neighbouring cell populations, as well as autocrine effects on the alpha cell itself. Further experiments are, however, required to ascertain whether DBP is secreted by islets into culture media and whether DBP can be transported into alpha cells (e.g. via megalin).

Other islet targets for DBP

In healthy rodent islets, *Gc*/DBP gene and protein expression is virtually absent in beta cells, as expected for an alpha cell signature gene. However, recent studies have shown that, during metabolic stress, *Gc* gene expression levels are upregulated in purified beta cells (Kuo *et al.* 2019). Suggesting that *Gc* might be a de-differentiation marker, the gene was upregulated in beta cells from db/db mice. Notably, deletion of *Gc* in high fat diet-fed mice prevented upregulation of Aldh1a3, improved glucose-stimulated insulin secretion and improved glucose tolerance and insulin sensitivity assessed using euglycaemic hyperinsulinaemic clamp (Kuo *et al.* 2019; Kuo & Accili, 2020). Thus, while inhibition of DBP expression is an attractive target to improve glucose tolerance during metabolic stress, it is also important to consider the role of DBP in the maintenance of alpha cell function. Whether these results are associated with the beta cell de-differentiation seen in T2DM is not known, but it will be interesting to confirm findings in human samples. DBP is also expressed in delta cells, confirmed using both RNA-seq (Adriaenssens *et al.* 2016) and immunohistochemistry (Viloria *et al.* 2020), although its downstream functions are unknown. Cell-specific manipulation of DBP in the islet compartment will therefore be integral to any approaches targeting DBP as a diabetes treatment, perhaps using molecular addresses specific to alpha cells. It is also noteworthy that, although beta cells do not normally express *Gc*/*GC*/DBP, they express the 25-OHD-1 α-hydroxlyase (CYP27B1) enzyme and are able to convert 25(OH)D to 1,25(OH)2D (Bland *et al.* 2004), raising the question as to whether exogenous DBP plays a role in the delivery of 25(OH)D to beta cells.

Concluding remarks

Glucagon plays an important role in counteracting insulin action, increasing endogenous glucose production and balancing glucose levels. While growing evidence has shown the benefits of managing glucagon levels in diabetes, there is still much to uncover regarding regulation of alpha cell function. With several suggested models of glucagon control, it is evident that the regulation of alpha cell function is a complex phenomenon. To fully uncover potential targets for maintaining glucagon secretion during metabolic stress, it is thus imperative to study critical alpha cell regulators. Positioning DBP as an important contributor to glucagon release are studies showing expression of this protein localised to alpha cells and the liver in healthy animals/humans, as well as the presence of impaired alpha cell morphology, ionic fluxes, electrical conductance and glucagon secretion in DBP-null animals. While DBP is primarily known for its vitamin D-binding properties, vitamin D metabolites account for only a small amount of DBP binding capacity, indicating that its other substrates such as actin and fatty acids might contribute to its multifunctional role. Further studies are now warranted to understand how DBP levels change in alpha cells during metabolic stress, whether DBP can be supplemented specifically in alpha cells to restore function, and more widely, how the actin cytoskeleton contributes to glucagon secretion. Key to this will be the use of conditional deletion or overexpression models, targeted delivery of DBP and confirmation of DBP function in isolated human islets.

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Additional information

Competing interests

The authors have no interests to declare.

Author contributions

K.V., M.H. and D.J.H. conceived and wrote the review article. K.V., M.H. and D.J.H. approved the final version of the manuscript. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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