REVIEW

The emerging role of FOXO transcription factors in pancreatic β cells

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Abstract

FOXO transcription factors critically control fundamental cellular processes, including metabolism, cell differentiation, cell cycle arrest, DNA repair, and other reactions to cellular stress. FOXO factors sense the balance between stimuli promoting growth and differentiation versus stress stimuli signaling damage. Integrated through the FOXO system, these divergent stimuli decide on cell fate, a choice between proliferation, differentiation, or apoptosis. In pancreatic β cells, most recent evidence highlights complex FOXO-dependent responses to glucose, insulin, or other growth factors, which include regulatory feedback. In the short term, FOXO-dependent mechanisms help β cells to accomplish their endocrine function, and may increase their resistance to oxidative stress due to transient hyperglycemia. In the long term, FOXO-dependent responses lead to the adaptation of β cell mass, conditioning the future ability of the organism to produce insulin and cope with changes in fuel abundance. FOXO emerges as a key factor for the maintenance of a functional endocrine pancreas and represents an interesting element in the development of therapeutic approaches to treat diabetes. This review on the role of FOXO transcription factors in pancreatic β cells has three parts. In Part I, FOXO transcription factors will be presented in general: structure, molecular mechanisms of regulation, cellular functions, and physiological roles. Part II will focus on specific data about FOXO factors in pancreatic β cells. Lastly in Part III, it will be attempted to combine general and β cell-specific knowledge with the aim to envisage globally the role of FOXO factors in β cell-linked physiology and disease.
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Part I: introduction to FOXO transcription factors

Classification and structure of FOXO proteins

FOXO transcription factors form a subclass of the large family of Forkhead proteins characterized by the presence of a 'winged-helix' DNA-binding domain called Forkhead box, which gave the name FOX proteins to the family (Kaestner *et al.* 2000). FOXO proteins are conserved from worm to human. While only one FOXO species is known in invertebrates (called dauer formation-16 (DAF-16) in *Caenorhabditis elegans* and dFOXO in *Drosophila*), four FOXO species, encoded by four distinct genes, have been identified in mammals: FOXO1 (previously known as FKHR), FOXO3 (previously known as FKHRL1), FOXO4 (previously known as Afx), and FOXO6.

The DNA-binding domain (Forkhead box) is located in the N-terminal portion of these proteins, while the transactivation domain is located in the C-terminal portion. Furthermore, a 'nuclear export sequence' and a 'nuclear localization signal' have been delineated. These motifs allow FOXO factors to shuttle in and out of the nucleus (see below). FOXO proteins may be post-translationally modified by phosphorylation and/or acetylation at differentially conserved serine/threonine and lysine residues respectively. Structural features of FOXO proteins have been detailed in recent reviews (Barthel *et al.* 2005, Greer & Brunet 2005).

Molecular regulation of FOXO transcription factors

As illustrated in Fig. 1, multiple molecular mechanisms regulate FOXO transcription factor functions. Some control subcellular FOXO localization, while others modulate FOXO transactivation properties. Furthermore, FOXO factor abundance is regulated by site-specific protein cleavage or through the control of *foxo* gene expression.

Subcellular localization FOXO factors shuttle between nucleus and cytoplasm. This represents a major event controlling FOXO activity that results from changes in FOXO phosphorylation. Two main classes of stimuli trigger FOXO phosphorylation with opposing effects on FOXO localization.

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On one hand, phosphorylation of FOXO proteins in response to growth factors such as insulin-like growth factor-I (IGF-I), erythropoietin, epidermal growth factor (EGF), or nerve growth factor causes exclusion from the nucleus. For many growth factor-activated protein kinases, the specific phosphorylation sites are known. These include Akt (also known as protein kinase B (PKB)) and serum and glucocorticoid inducible kinase (SGK), which are activated through the PI3K pathway, dual speciticity tyrosine (Y) phosphorylation regulated kinase 1 (DYRK1), and casein kinase 1 (CK1) (Biggs *et al.* 1999, Brunet *et al.* 1999, 2001, Rena *et al.* 2002).

On the other hand, phosphorylation of FOXO proteins in response to oxidative stress, involving, for example, Jun-N-terminal kinase (JNK) or mammalian Ste20-like kinase, results in FOXO import in the nucleus. FOXO residues targeted by these kinases are different from those targeted by growth factor-regulated kinases (Essers *et al.* 2004, Matsumoto & Accili 2005, Lehtinen *et al.* 2006). Interestingly, the effect of oxidative stress appears to prevail on the effect of growth factors (see Fig. 2; Wang *et al.* 2005).

To date, no specific phosphatase dephosphorylating FOXO has been identified.

Transcriptional regulation by FOXO proteins

Nuclear FOXO proteins interacting with DNA and partner proteins regulate the transcription of specific target genes through multiple modes of action as depicted in Fig. 3 (reviewed in Barthel *et al.* 2005). FOXO can recruit transcriptional co-activators (Fig. 3A) or co-operating DNA-binding transcription factors (Fig. 3B). Alternatively, FOXO factors may repress transcription by competing with activatory transcription factors for a common binding site in a gene promoter (Fig. 3C; Kitamura *et al.* 2002). FOXO factors may also act as co-activators or co-repressors (Fig. 3D and E), thereby regulating transcription through promoters which lack FOXO-binding sites.

Modulation of FOXO transcriptional properties

Covalent modifications (phosphorylation and acetylation) as well as protein–protein interactions can modulate the transactivation potential and the DNA binding of FOXO. FOXO-binding partners can modulate FOXO transactivation properties either positively (e.g. β-catenin; Essers *et al.* 2005) or negatively (e.g. PPARγ or the androgen receptor; Dowell *et al.* 2003, Li *et al.* 2003).

In addition to governing nuclear/cytoplasm shuttling, phosphorylation of FOXO can decrease its inherent transactivation potential notably by disrupting the interactions with co-activators (Perrot & Rechler 2003, Puigserver *et al.* 2003, Tsai *et al.* 2003).

FOXO transcription factors are acetylated by p300 and CBP, co-activators displaying histone acetyltransferase activity (Chan & La Thangue 2001), at several conserved lysine residues, many of which are located in the DNA-binding domain (Fukuoka *et al.* 2003, van der Horst *et al.* 2004). Apparently, acetylation of FOXO factors decreases their transactivation potential, possibly by reducing their DNA-binding activity. Given that the requirement for FOXO DNA-binding activity may vary among the different target

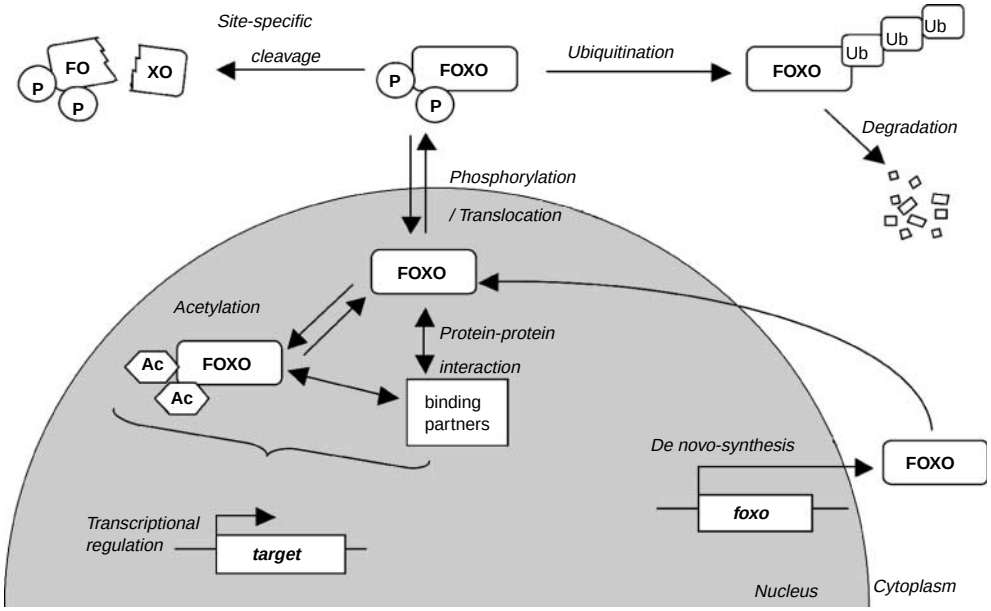

Figure 1 Overview of the main mechanisms regulating FOXO transcription factors. Multiple mechanisms control FOXO transcription factor activity. Some are reversible, such as interaction with other proteins, acetylation, phosphorylation, or translocation, while others are irreversible, such as ubiquitin-dependent degradation or site-specific cleavage by proteases. Ub, ubiquitin; P, phosphate group; Ac, acetyl group.

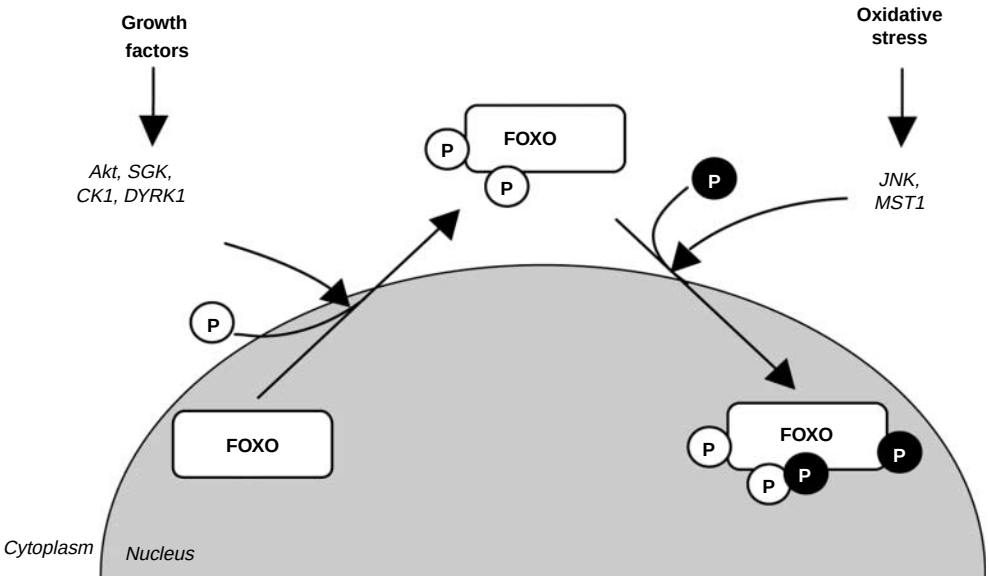

Figure 2 Antagonistic effects of growth factors versus oxidative stress-dependent phosphorylations on the subcellular localization of FOXO.

genes, acetylation may represent a mechanism controlling FOXO factor specificity. Deacetylation of FOXO proteins has been shown to result from the activity of SIRT1, a member of the Sir2 family of NAD-dependent deacetylases (Brunet *et al.* 2004, Motta *et al.* 2004, van der Horst *et al.* 2004). The effects of SIRT1 on FOXO function are complex and vary depending upon the FOXO target genes. SIRT1 appears to promote transcription of FOXO target genes involved in stress resistance, while decreasing transcription of genes involved in apoptosis (Greer & Brunet 2005). This is consistent with the proposal that acetylation/deacetylation of FOXO protein may switch target specificity.

Altogether, interaction with other proteins, phosphorylation, and acetylation offer a large spectrum of options for the fine tuning of FOXO function in complement to the master switch operated by the control of FOXO factors' nuclear localization.

Regulated levels of FOXO proteins mRNA levels of the different *foxo* gene subtypes vary from tissue to tissue in mammals (Furuyama *et al.* 2000) and can be modulated under some circumstances (Furuyama *et al.* 2002, Richards *et al.* 2002). These observations highlight that the transcription of *foxo* genes is subject to regulation.

Probably, a more important determinant of FOXO protein expression is its rate of degradation. After ubiquitination, FOXO proteins are degraded by proteolysis through the proteasome pathway (Vogt *et al.* 2005). This cytosolic process may be favored by FOXO phosphorylation and/or deacetylation (Hu *et al.* 2004, Kitamura *et al.* 2005). A possible role for AMPK in regulating FOXO stability has been suggested in liver (Barthel *et al.* 2002). Recent findings indicate that ubiquitination of the different FOXO proteins may be accomplished by different E3 (ubiquitin ligase) complexes as a consequence of different regulatory processes (Huang *et al.* 2005).

Site-specific cleavage In early stages during Fas-induced apoptosis in lymphoid cells, FOXO3 protein is cleaved by a caspase-3-like protease (Charvet *et al.* 2003). This cleavage separates the N-terminal DNA-binding domain of FOXO3 from its C-terminal transactivation domain and occurs at a caspase-3 consensus cleavage site (amino acid sequence: $DELD^{304}$). Interestingly, similar sequence motifs are conserved in FOXO1 and FOXO4. Site-specific cleavage probably represents more than an inactivation pathway, the N-terminal fragment being able to translocate to the nucleus and having the potential to affect the transcription of FOXO target genes (Charvet *et al.* 2003).

Cell functions regulated by FOXO

FOXO factors contribute to the regulation of various processes such as cell cycle progression, cell size determination, cell death, cell differentiation, resistance to stress, and energetic metabolism (reviewed in Greer & Brunet 2005). In Table 1, we list the best characterized FOXO-regulated target genes. Recent experiments aiming to identify FOXO target genes at a large scale have confirmed the plethora of cellular functions concerned (Modur *et al.* 2002, Ramaswamy *et al.* 2002, Murphy *et al.* 2003).

Role of FOXO in development, integrated physiology, and diseases

Implication of FOXO in development *Foxo1*, *foxo3*, and *foxo4* genes have been knocked out in mice (Castrillon *et al.*

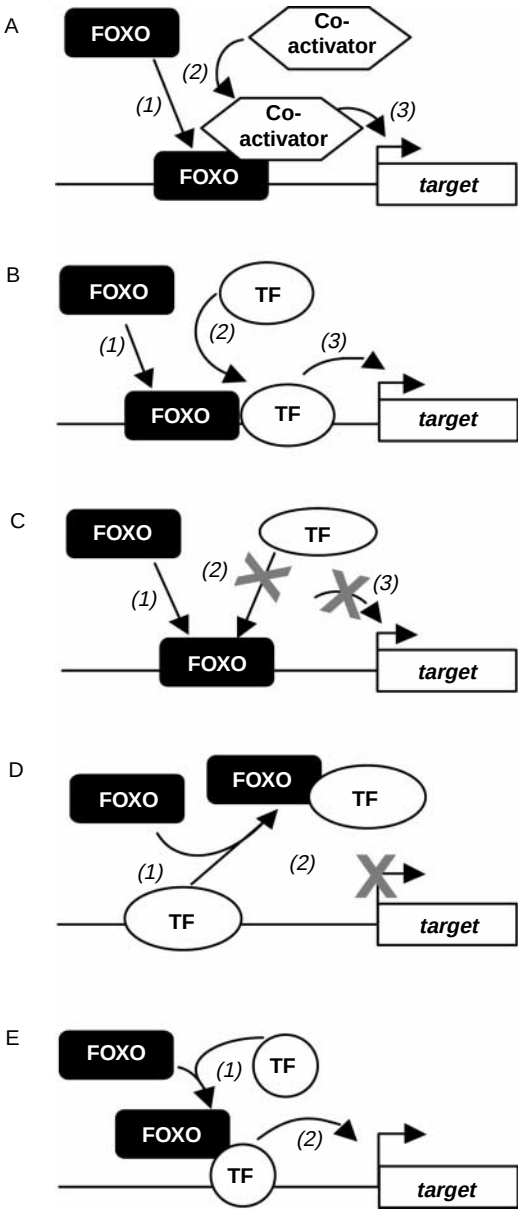

Figure 3 Different FOXO modes of transcriptional regulation. (A) Recruitment of co-activators. (B) Co-operative binding with transcription factors. (C) Competition with transcription factors. (D and E) Co-repression or co-activation (independently of FOXO DNA-binding activity). TF, transcription factor.

2003, Hosaka *et al.* 2004). While heterozygous Foxo1$^{+/-}$ mice are viable, homozygous Foxo1$^{-/-}$ mice are embryonic lethal, emphasizing the importance of FOXO function in embryonic development. Foxo1$^{-/-}$ mice embryos are smaller and die at embryonic day 10·5. The primary defect causing lethality in Foxo1$^{-/-}$ mice is apparently an impaired vascularization. Consistently, experiments in Foxo1$^{+/-}$ mice embryos have shown that FOXO1 is highly expressed in developing vasculature (Furuyama *et al.* 2004, Hosaka *et al.* 2004). Interestingly, FOXO factors continue to contribute to vascularization after birth (Potente *et al.* 2005). In mature endothelial cells, the inhibition of FOXO1 activity by angiopoietin 1 regulates several genes involved in apoptosis and vessel destabilization/remodeling (Daly *et al.* 2004).

In mice, FOXO factors may also have a role in the developing brain. FOXO1, FOXO3, and FOXO6 are expressed in a spatially and temporally restricted manner (Hoekman *et al.* 2006).

Evidence for a role of FOXO factors in embryonic development has also been provided in the frog *Xenopus laevis*, where *xfoxo1* and *xfoxo3* (*foxo* orthologs) present distinct spatial and temporal patterns of expression (Pohl *et al.* 2004).

Implication of FOXO factors in reproductive function Foxo3$^{-/-}$ female mice have a very singular phenotype: being initially fertile, they become infertile after 15 weeks of age (Castrillon *et al.* 2003, Hosaka *et al.* 2004). This secondary infertility appears to be due to premature and global follicular activation leading to oocyte death and subsequent precocious depletion of the follicle population. Accordingly, under normal circumstances, Akt and FOXO3 are expressed in oocytes, mostly in primordial and primary follicles. Communication between oocytes and their surrounding granulosa cells is mediated by stem cell factor (SCF), which regulates primordial to primary follicle transition and subsequent follicle development. In oocytes, SCF activates Akt and suppresses FOXO3 activity through nuclear exclusion. Thus, it has been proposed that granulosa cell-derived SCF will act on the follicular development through activation of an Akt–FOXO3 pathway in oocytes (Reddy *et al.* 2005). There is also evidence for an implication of FOXO1 in ovarian functions, as FOXO1 can regulate follicle-stimulating hormone-dependent proliferation of granulosa cells (Cunningham *et al.* 2003, 2004, Park *et al.* 2005).

Implication of FOXO in cancer The pivotal role of FOXO in cell fate decisions, depending upon the balance between growth factor stimulation versus cellular stress and damage, would predict that FOXOs are tumor suppressors. Indeed, circumstantial and direct evidence suggests the implication of FOXO factors in cancer. *Foxo* genes (*foxo1*, *foxo3*, and *foxo4*) were found at chromosomal breakpoints in human tumors (Galili *et al.* 1993, Parry *et al.* 1994, Borkhardt *et al.* 1997, Hillion *et al.* 1997). Nuclear exclusion of FOXO3 in primary breast tumors correlates with PI3K activation and poor survival of the patients (Hu *et al.* 2004). FOXO factors reduce tumorigenicity in nude mice (Ramaswamy *et al.* 2002, Hu *et al.* 2004, Yang *et al.* 2005). Furthermore, FOXO proteins interact with many oncogenes (i.e. β-catenin; Essers *et al.* 2005) or tumor suppressors such as p53 (Brunet *et al.* 2004).

Implication of FOXO in aging A link between FOXO factors and aging has initially been observed in invertebrates. In the nematode *C. elegans*, DAF-16, the only *foxo* gene ortholog, regulates lifespan (reviewed in Baumeister *et al.*

Table 1 Some of the best characterized FOXO target genes and regulated cellular processes

	Cellular process	Reference
Target gene		
Cyclin-dependent kinase inhibitor 1B (*p27*)	Cell cycle (G1/S transition)	Medema *et al.* (2000)
Cyclin-dependent kinase inhibitor 1A (*p21*)	Cell cycle (G1/S transition)/differentiation (adipocyte)	Seoane *et al.* (2004), Nakae *et al.* (2003)
Cyclin D1	Cell cycle (G1/S transition)	Ramaswamy *et al.* (2002)
Cyclin D2 (*ccnd2*)	Cell cycle (G1/S transition)	Ramaswamy *et al.* (2002)
Cyclin G2	Cell cycle (G0/G1–S transition)	Martinez-Gac *et al.* (2004)
Growth arrest and DNA damage-inducible protein 45	Cell cycle (G2 arrest)/DNA repair	Tran *et al.* (2002)
DNA damage-binding protein 1	DNA repair	Tran *et al.* (2002)
Manganese superoxide dismutase	ROS detoxification	Kops *et al.* (2002)
Catalase	ROS detoxification	Nemoto & Finkel (2002)
Trail	Cell death	Modur *et al.* (2002)
Fas ligand	Cell death	Brunet *et al.* (1999)
Bcl-6	Cell death	Ramaswamy *et al.* (2002)
Bcl-2 interacting mediator of cell death	Cell death	Dijkers *et al.* (2000)
B-cell translocation gene 1	Differentiation (erythrocytes)	Bakker *et al.* (2004)
Atrogenin-1 (MAFbx)	Muscle atrophy	Sandri *et al.* (2004)
Glucose-6-phosphatase	Metabolism	Nakae *et al.* (2001)
Phosphoenolpyruvate carboxykinase	Metabolism	Nakae *et al.* (2001)
Apolipoprotein CIII	Metabolism	Altomonte *et al.* (2004)
Pyruvate dehydrogenase kinase 4	Metabolism	Furuyama *et al.* (2003)

2006). In *Drosophila melanogaster*, dFOXO regulates age-linked decline of cardiac function and longevity (Giannakou *et al.* 2004, Hwangbo *et al.* 2004, Wessells *et al.* 2004).

There is so far no direct evidence which would link FOXO factors to longevity of mammals. FOXO factors are involved in cellular resistance to stress, promoting reactive oxygen species (ROS) detoxification, DNA repair, and cell cycle arrest to allow time for the repair process (Table 1). Therefore, FOXO could reduce detrimental effects of aging. Interestingly, some of the FOXO targets (like MnSOD) are conserved from worm to mammals. Moreover, mice that are deficient in insulin or the IGF-I receptor have an increased lifespan and are more resistant to oxidative stress stimuli (Bluher *et al.* 2003, Holzenberger *et al.* 2003). It is possible that FOXO factors, normally leaving the nucleus after insulin/IGF-I stimulation, mediate in part the longevity of these knockout mice. Furthermore, the effects of SIRT1 on FOXO function may be linked to aging, as Sir2, the SIRT1 orthologs in yeast, worms, and flies, have been linked to the longevity of these organisms (Tissenbaum & Guarente 2001, Rogina & Helfand 2004).

Implication of FOXO factors in energy homeostasis

In mammals, insulin has a key role in regulating glucose homeostasis and co-ordinating body energetic metabolism. Many aspects of the response to insulin require regulation of gene expression. As a major downstream target of insulin-signaling pathway, FOXO transcription factors may mediate adaptive responses of the gene expression program in many insulin target tissues (Barthel *et al.* 2005). There is evidence for this notably in liver (Puigserver *et al.* 2003), muscle (Furuyama *et al.* 2003), adipocytes (Nakae *et al.* 2003), and pancreatic β cells (Kitamura *et al.* 2002). Furthermore, recent findings have indicated that FOXO1 mediates the action of insulin and leptin in the hypothalamus, controlling the production of different neuropeptides and the feeding behavior in mice (Kim *et al.* 2006, Kitamura *et al.* 2006). Thus, FOXO contributes to the orchestration of energy homeostasis by acting in the main energy consuming and storing tissues, as well as in the endocrine and central nervous system.

Part II: FOXO in pancreatic β cells

Pancreatic β cells are located in the islets of Langerhans which constitute the endocrine compartment of the pancreas. β Cells produce insulin and secrete it in response to elevations in circulating blood glucose and to other signaling molecules, providing a key contribution to glucose homeostasis and to the coordination of metabolism within the body. FOXO factors are expressed in β cells, and many pathways that have been shown to regulate FOXO function in other tissues are active in β cells. The following paragraphs will summarize information about these pathways and present recent data pointing to an emerging role of FOXO factors in β cells.

FOXO subtypes expressed in β cells

FOXO1 is the most predominantly expressed FOXO factor in isolated mice islets as well as in the βTC-3 β cell line. In mice islets, FOXO3 is expressed at a lower level, while FOXO4 is apparently undetectable (Kitamura *et al.* 2002). FOXO1 has also been shown to be expressed in isolated human islets (Contreras *et al.* 2002). Interestingly, foxo1 mRNA levels are more elevated in islets of diabetic when

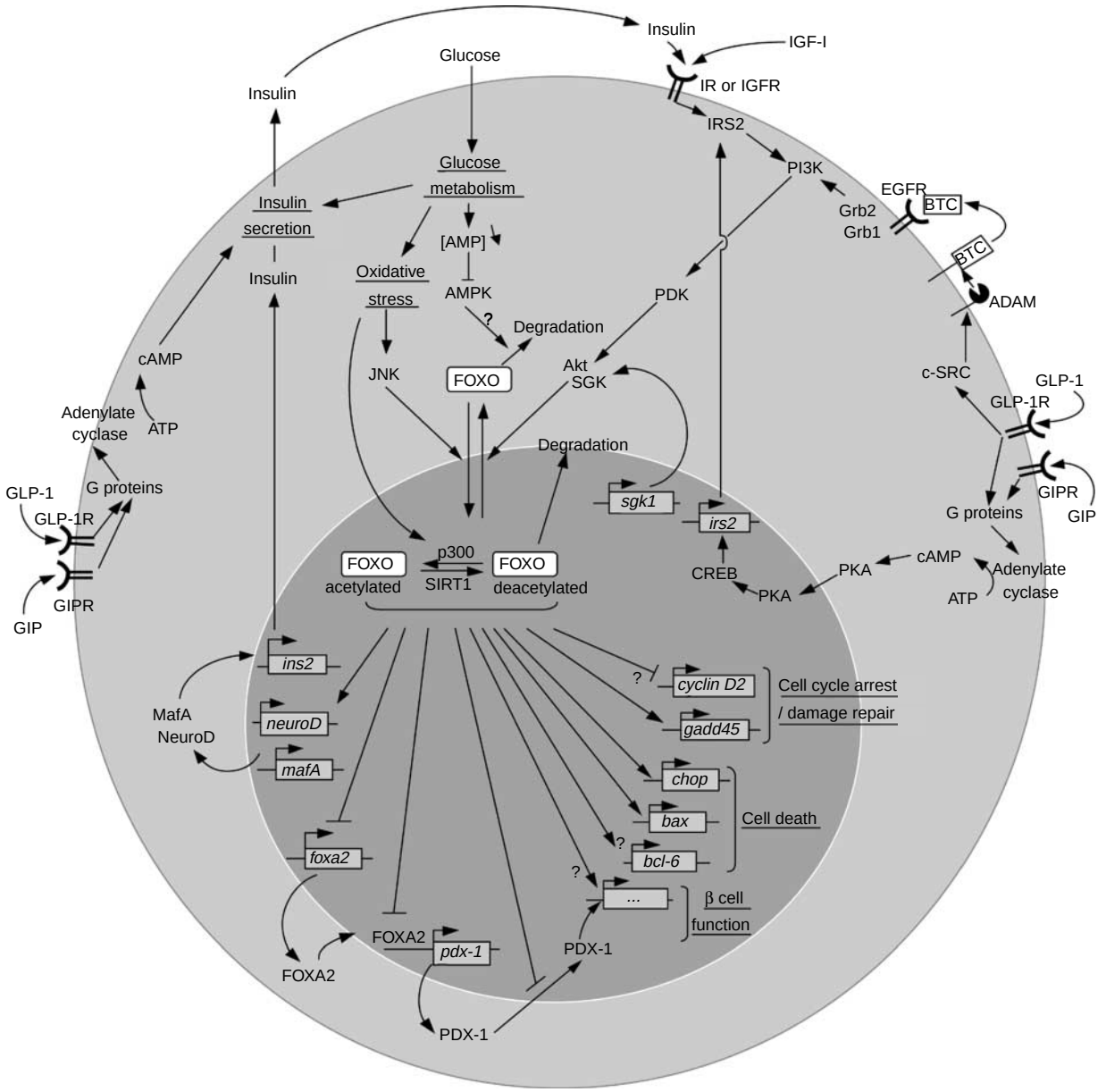

Figure 4 Overview of FOXO-linked signaling pathways and FOXO targets in β cells. See details in the text.

compared with non-diabetic patients, but the reason for this is unknown (Del Guerra *et al.* 2005).

FOXO-linked pathways in β cells

As shown in Fig. 4, several signaling pathways may lead to regulation of FOXO transcription factors in pancreatic β cells.

PI3K–Akt pathway PI3K–Akt pathway is a major upstream signaling module leading to the phosphorylation of FOXO factors and their exclusion from the nucleus. In β cells, FOXO is phosphorylated following the activation of the PI3K–Akt pathway by insulin, IGF-I, glucose, glucagon-like peptide-1 (GLP-1), or glucose-dependent insulinotropic polypeptide (GIP) (Trumper *et al.* 2000, 2001, Wrede *et al.* 2002, Buteau *et al.* 2006).

The binding of IGF-I or insulin to their receptors results in the receptor autophosphorylation on tyrosine residues and in the tyrosine phosphorylation of insulin receptor substrates (IRS) by the receptor tyrosine kinases. IRS may then activate PI3K, leading to 3-phosphoinositide dependent protein kinase-1 (PDK) activation and finally Akt activation.

GLP-1 and GIP are glucoincretin hormones that are released from gut cells upon food intake and potentiate glucose activation of insulin secretion by β cells. A mechanistic explanation for Akt activation by insulin secretagogues would be an autocrine effect of secreted insulin through its binding to the insulin receptor (IR) at the surface of β cells (Ohsugi *et al.* 2005). Consistently, in cultured cells, glucose regulates FOXO1 through the insulin signaling pathway (Martinez *et al.* 2006). However, the ability of insulin to drive activation of the PI3K/Akt pathway in a physiological context, as well as the real physiological significance of any insulin feedback on β cells, is still controversial (Leibiger *et al.* 2002). Alternative mechanisms have been proposed for the activation of PI3K by GLP-1. On the one hand, binding of GLP-1 to its receptor (GLP-1R) can produce c-SRC-mediated activation of a membrane-bound metalloprotease, which would cleave betacellulin membrane integral precursor and release betacellulin soluble ligand (BTC). BTC would in turn activate EGF receptor and PI3K, presumably independently of IRS, but with the help of the structurally related GAB1/GRB2 proteins (Buteau *et al.* 2003). On the other hand, GLP-1 can promote activation of PI3K by increasing expression of IRS-2, through G-protein-dependent activation of adenylyl cyclase, formation of cAMP, activation of PKA, and finally, activation of the CREB transcription factor (Holz & Chepurny 2005).

In spite of distinct receptors for GIP (GIPR) and GLP-1 (GLP-1R), the two glucoincretins share most of their downstream mitogenic signaling pathways (Trumper *et al.* 2001). Thus, it is likely that the mechanisms of activation of the PI3K–Akt–FOXO pathway by GIP are similar to the one triggered by GLP-1.

In summary, the PI3K–Akt–FOXO pathway is regulated by multiple signals in the β cell.

SGK1 In the Min6 β cell line, expression of SGK1 mRNA depends on the glucose concentration (Fig. 5B). Increased expression of SGK1 at low glucose correlates with decrease of FOXO level in the nucleus (Fig. 5A and Martinez *et al.* 2006). This suggests that regulation of SGK1 may also contribute to the glucose responsiveness of FOXO factors in β cells.

JNK pathway Activation of JNK due to cellular stress of various origin (including oxidative stress) may lead to phosphorylation of FOXO factors and drive translocation of FOXO factors into the nucleus. In pancreatic β cells, oxidative stress is induced when glucose levels are high. In diabetes, chronic hyperglycemia inducing oxidative stress is thought to cause β cell dysfunction (Kaneto *et al.* 2005). Recent findings indicate that activation of JNK by oxidative stress can result in FOXO1 translocation to the nucleus in mouse β cells (Kawamori *et al.* 2006).

FOXO acetylation/deacetylation An important molecular mechanism regulating FOXO protein functions consists in acetylation/deacetylation modifications. SIRT1 deacetylase is known to deacetylate FOXO proteins, while CBP and p300 are known to acetylate FOXO proteins. As mentioned earlier in the text, these modifications are likely

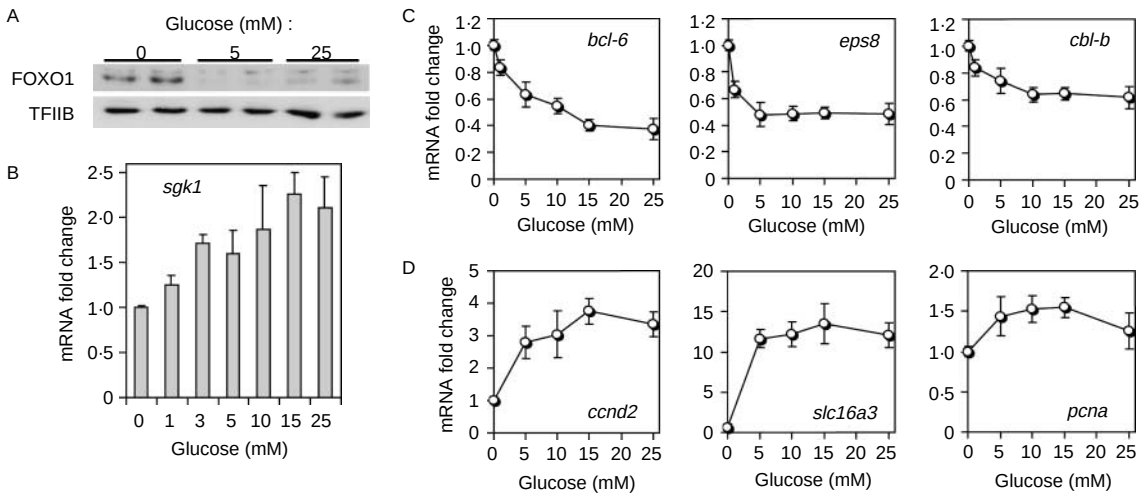

Figure 5 Regulation of SGK and FOXO target gene expression by glucose in Min6 cells. Min6 cells were cultured for 24 h in a medium with 1% serum, at indicated glucose concentration as previously described (Glauser & Schlegel 2006). (A) Nuclear fractions were analyzed by western blot with the antibody raised against FOXO1 (#9462, Cell Signaling) and the general transcription factor TFIIB (loading control; Santa Cruz Biotechnology). FOXO1 is detected in the nucleus at low but not at high glucose concentrations. (B–D) Total RNA was analyzed by quantitative real-time RT-PCR for indicated genes (normalized with 18S rRNA content and depicted as mean ± S.D., $n=3$). Genes known to be up-regulated by FOXO1 in other cell systems (C) are up-regulated at low glucose concentrations (when FOXO1 is present in the nucleus). Inversely, genes known to be down-regulated by FOXO1 in other cell systems (D) are up-regulated at high glucose concentrations (when FOXO1 is absent from the nucleus).

determinants in FOXO transcription factor target specificity and may be key regulatory steps in determining the balance between the resistance to stress effects and the pro-apoptotic effects of FOXO factors.

SIRT1 is expressed in β cells and is involved in β cell physiology. Indeed, increasing the level of SIRT1 in the pancreatic β cells of transgenic mice results in a more efficient glucose handling due to enhanced glucose-stimulated insulin secretion (Moynihan *et al.* 2005). Recent experiments have shown that FOXO1 was subject to acetylation and deacetylation in β cells, and suggested the involvement of SIRT1 and CBP/p300, which interact with FOXO1 in β cells (Kitamura *et al.* 2005).

AMPK AMPK pathway is regulated by the intracellular metabolism, in particular, by the energetic substrates that are available. Under conditions of energy restriction, an increase in AMP levels leads to AMPK activation; reciprocally, under conditions of high energy availability, a decrease in AMP levels inhibits AMPK activation. In the β cell, variations in glucose concentrations over the physiological range efficiently modulate AMPK activity, which in turn regulates insulin secretion and gene expression (Rutter *et al.* 2003). Under conditions of energy restriction, activated AMPK might promote FOXO degradation in the β cells, thereby regulating FOXO activity as proposed for the liver (Barthel *et al.* 2002).

FOXO targets and physiological role of FOXO in β cells

The glucose/IGF-I/GLP-1/GIP-induced activation of Akt in β cells correlates with increased survival. Overexpression of Akt in β cells in transgenic mice promotes β cell survival and increases β cell size (Dickson & Rhodes 2004). Moreover, glucose can promote β cell proliferation in culture through the activation of PI3K–Akt pathway (Srinivasan *et al.* 2002). In contrast, free fatty acids significantly inhibit glucose/IGF-I-induced activation of Akt in β cells, correlating with reduced β cell growth and increased β cell apoptosis (Dickson & Rhodes 2004). GLP-1 also inhibits apoptosis through activation of the PI3K/Akt pathway (Wang *et al.* 2004, Li *et al.* 2005). Thus, Akt appears to be an important positive regulator of β cell mass inhibiting apoptosis and promoting β cell proliferation and expansion.

In the last few years, several studies using transgenic mice have substantiated an important role of FOXO as a downstream effector of insulin/IGF–PI3K–Akt signaling pathway in the regulation of β cell function and growth. In $Irs2^{-/-}$ mice, which lack IRS2 and suffer from β cell failure, ablation of one allele of foxo1 is sufficient to restore β cell proliferation. This indicates that FOXO1 is an important target of insulin/IGF signaling pathway, downstream of IRS2 (Kitamura *et al.* 2002). Similarly, deletion of one allele of foxo1 was found to rescue β cell proliferation in $\beta Pdk1^{-/-}$ mice (β cell-specific knockout for Pdk1), alleviating their diabetic phenotype (Hashimoto *et al.* 2006). Transgenic mice that express insulin receptors (INSR) only in liver, brain, and β cells ($Insr^{-/-}$ rescued by insr transgene under the control of transtyretin promoter) have high levels of circulating insulin compensating for insulin resistance in muscle and fat. Increased β cell mass can explain this compensatory effect, which is reversed by the expression of a constitutively nuclear form of FOXO1 (Okamoto *et al.* 2006). In another transgenic mouse model, pancreas-restricted production of IGF-II acts in a paracrine fashion to promote β cell proliferation. In this model, expression of a constitutively nuclear form of FOXO1 is also able to block the proliferation response (Okamoto *et al.* 2006). Altogether, these findings converge to show that expansion of β cell mass in response to insulin/IGF signaling requires FOXO1 nuclear exclusion.

A probable way through which FOXO1 mediates insulin/IGF effects on β cell proliferation is through down-regulation of PDX1. PDX1 is a major β cell transcription factor that controls β cell growth and function. PDX1 and FOXO1 exhibit mutually exclusive patterns of nuclear localization (Kitamura *et al.* 2002). Two mechanisms by which FOXO may regulate PDX1 have been proposed. First, it was suggested that FOXO1 would control the subcellular localization of PDX1 (Kawamori *et al.* 2006). Secondly, FOXO1 may function as a repressor of FOXA2-dependent expression from the Pdx1 promoter (Kitamura *et al.* 2002).

FOXO1 factor is an important mediator of GLP-1 and GIP effects on β cell proliferation and survival. As mentioned earlier, GLP-1 activates the PI3K–Akt pathway inhibiting FOXO through its exclusion from the nucleus. Expression of constitutively nuclear FOXO1 prevents the proliferative and anti-apoptotic effects of GLP-1 (Buteau *et al.* 2006). GLP-1 up-regulates pdx1 and foxa2 (two FOXO1 target genes), transcription of which is repressed by nuclear FOXO1. Induction of pdx1 and foxa2 may contribute to FOXO exclusion-dependent effect of GLP-1 on proliferation and survival. Similarly, GIP induces FOXO1 nuclear exclusion. This results in the down-regulation of the pro-apoptotic gene *bax*, a FOXO1 target, and promotes β cell survival (Kim *et al.* 2005).

Other transcriptional targets of FOXO1 in β cells include *mafA* and *neuroD* genes, which code for transcription factors that are important for β cell function, notably regulating the expression of the insulin-coding gene Ins2 (Kitamura *et al.* 2005). Upon oxidative stress caused by hyperglycemia, the nuclear localization of FOXO1 is required for the induction of MafA and NeuroD expression. Interestingly, under these circumstances, FOXO1 is initially acetylated and subsequently deacetylated, which not only activates transcription but also promotes ubiquitin-dependent degradation of FOXO1 by the proteasome and reduces the half-life of FOXO1. These findings suggest that FOXO1 orchestrates a compensatory response to transient metabolic stress aiming at maintaining β cell function, but that this response may be inefficient for longer term responses to hyperglycemia since FOXO1 is ultimately degraded. In addition, there is evidence that chop and gadd45 are FOXO targets, up-regulated at low glucose in β cells (Martinez *et al.* 2006). Thus, FOXO may be

involved in β cell responses to stress, including cell cycle arrest, DNA damage repair, or apoptosis.

FOXO most likely regulates the transcription of additional target genes in β cells. A list of FOXO1 target gene candidates was obtained by crossing a list of known FOXO1-regulated genes (Ramaswamy *et al.* 2002) and a list of glucose-responsive genes in Min6 cells (DA Glauser, T Brun, BR Gauthier and W Schlegel, unpublished observations), as glucose regulates FOXO in this cell line (Fig. 5A and Martinez *et al.* 2006). Six probable targets were thus identified (Fig. 5C and D). Among these, cyclin D2, which is known to be down-regulated by FOXO1 (Ramaswamy *et al.* 2002), is down-regulated at low glucose when FOXO1 is abundant in the nucleus. As cyclin D2 represents a major checkpoint in the regulation of β cell cycle progression (Georgia & Bhushan 2004, Kushner *et al.* 2005), its regulated expression possibly mediates the FOXO effect on β cell proliferation. Further investigations are required to elucidate the mechanisms downstream of FOXO which impact on β cell physiology.

Part III: perspectives

Why is FOXO of particular interest to the biology of the endocrine pancreas?

A conserved and fundamental function for FOXO factors In any living organism, nutrient availability and growth are linked, and most organisms deal with an adverse environment by the activation of a repertoire of anti-stress responses. FOXO factors, which decide between proliferation and stress handling, are thus central to very fundamental biology (Fig. 6). Nutrient restriction depleting ATP levels will decrease growth and prompt cells to spend all remaining resources on maintenance. In contrast, when nutrients are very abundant, high rates of oxidative metabolism may produce detrimental reactive oxygen species. Both of these inappropriate energetic situations may thus cause insults to cells, such as DNA damage or protein misfolding resulting from lack of ATP in the endoplasmic reticulum. If such insults are too important or inappropriately controlled, then they may cause apoptosis.

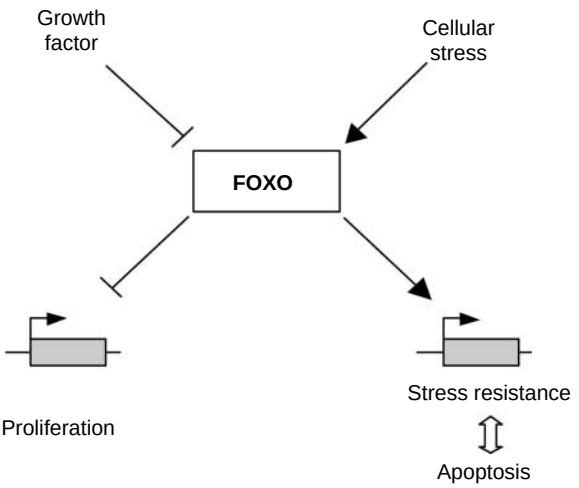

Figure 6 FOXO as integrator of growth and stress signals.

In mammals, the endocrine pancreas sensing the circulating nutrients will produce insulin and glucagon, which in turn handle nutrient homeostasis for the whole organism and regulate growth and differentiation of various organs and tissues. Handled nutrient and hormone levels feed back to the β cells, such that the functionality of the endocrine compartment can be maintained and its performances adapted to varying insulin demand. Indeed, insulin, which likely act in an autocrine manner, as well as glucoincretins are growth/survival factors for β cells. On the other hand, metabolism of nutrients contributes to oxidative stress also in the endocrine pancreas and elicits cellular stress signaling. Thus, FOXO factors, which sense growth signals and cellular stress and relate these to cellular responses, are part of the most important regulatory system governing function, proliferation, differentiation, and survival of the pancreatic β cell (Fig. 7). FOXO-dependent mechanisms function on different time scales. First, FOXO may help β cells cope with acute periods of oxidative stress produced by transient oscillations of glycemia, either by orchestrating the resistance to intracellular stress or by enhancing insulin production and its endocrine action to decrease glycemia. Secondly, in the long term, FOXO factors control the adaptation of β cell mass and the ability of the endocrine pancreas to handle future metabolic loads.

FOXO in diabetes, a possible therapeutic target? These features point to a possible role of FOXO in human diabetes mellitus, especially in type 2 diabetes. Type 2 diabetes is characterized by hyperglycemia that results from a β cell failure to produce sufficient insulin to meet the body's demand. This failure may result from a dysfunction of insulin secretion and/or from a decrease in β cell mass (Rhodes 2005). Obese patients who develop insulin resistance in peripheral tissues and therefore have a high insulin demand reach the limits of their endocrine pancreas earlier in life. This could partially explain the high prevalence of type 2 diabetes in obese patients. Compensatory adaptation of β cell mass, which – in mice models (Okamoto *et al.* 2006) – involves FOXO factors, must have failed in those patients. Furthermore, FOXO-mediated β cell apoptosis may contribute to the long-term deleterious effect of hyperglycemia on β cell mass. Thus, FOXO factors are very likely involved in the progression of type 2 diabetes and may be regarded as possible therapeutic targets.

In addition to controlled diet and exercise, three main types of approaches are used, to date, to treat diabetes: (i) oral drugs, which mostly aim at reducing glucose production in liver, reducing glucose absorption from the digestive tract, or stimulating the secretory capacity of β cells; (ii) insulin injections; and (iii) transplantation of islets from organ donors;

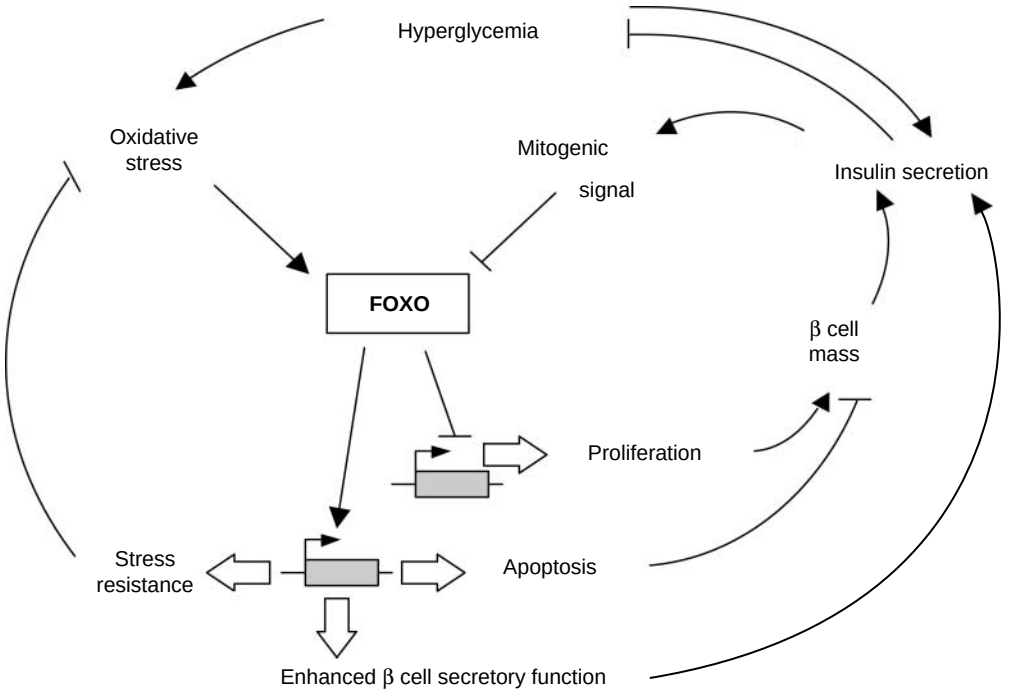

Figure 7 FOXO as an integrator of metabolic and hormonal feedback to β cell function.

the latter two being used when the disease is accompanied by drastic reduction in β cell function and/or mass. An alternative strategy aiming at promoting β cell growth, possibly by acting on the Akt–FOXO pathway, could be envisioned. Note that the systemic administration of drugs addressing the FOXO pathway might favor tumorigenesis and increase the risk of cancer. Therefore, drugs acting on FOXO would be more safely applied *ex vivo*, for example, to improve the success of islet transplantation. In fact, islet transplantation being limited by the availability and viability of isolated islets, promoting β cell survival, or expanding the β cell population in isolated human islets *ex vivo* before transplantation with FOXO-targeting drugs – or even FOXO-based gene therapy – could be an interesting option.

Conclusions

Investigations in model organisms from worm to mice have highlighted the versatility of FOXO factor regulation and the plethora of cellular processes they control. FOXO factors integrate multiple intracellular and extracellular signals. Sensing the balance between growth-promoting extracellular signals and intracellular stress signals, FOXO factors orchestrate the appropriate cellular responses, i.e. either cell growth and resistance to stress or apoptosis. Findings in pancreatic β cells are in line with this general model: FOXO responds to stimuli that are physiologically relevant for the endocrine pancreas, such as insulin, glucose, or GLP-1, and FOXO factors control β cell growth and physiology. As a key 'node' in the regulatory networks controlling the pancreatic β cell, FOXO represents an interesting potential target to develop novel therapeutic approaches for diabetes.

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