Normal and defective pathways in biogenesis and maintenance of the insulin storage pool

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Both basal and glucose-stimulated insulin release occur primarily by insulin secretory granule exocytosis from pancreatic β cells, and both are needed to maintain normoglycemia. Loss of insulin-secreting β cells, accompanied by abnormal glucose tolerance, may involve simple exhaustion of insulin reserves (which, by immunostaining, appears as a loss of β cell identity), or β cell dedifferentiation, or β cell death. While various sensing and signaling defects can result in diminished insulin secretion, somewhat less attention has been paid to diabetes risk caused by insufficiency in the biosynthetic generation and maintenance of the total insulin granule storage pool. This Review offers an overview of insulin biosynthesis, beginning with the preproinsulin mRNA (translation and translocation into the ER), proinsulin folding and export from the ER, and delivery via the Golgi complex to secretory granules for conversion to insulin and ultimate hormone storage. All of these steps are needed for generation and maintenance of the total insulin granule pool, and defects in any of these steps may, weakly or strongly, perturb glycemic control. The foregoing considerations have obvious potential relevance to the pathogenesis of type 2 diabetes and some forms of monogenic diabetes; conceivably, several of these concepts might also have implications for β cell failure in type 1 diabetes.

Brief description of insulin mRNA quantity and quality

Many factors may contribute to the control of insulin mRNA levels (1, 2), including transcriptional networks that control β cell function (3) as well as mRNA stability (4). While a detailed analysis of the transcriptional regulation of β cell development, differentiation, and dedifferentiation is beyond the scope of this Review, it is certainly clear that PDX1 and NEUROD1 can act together to directly stimulate transcription from the *INS* promoter (5). Moreover, PDX1, in combination with NKX2.2 (downstream of NEUROG3; ref. 6) and FOXA2, stimulates the expression of the β cell–specific activator MafA (7); in turn, MafA homodimers (or MafA/MafB heterodimers) help support β cell *INS* gene transcription (8) (two alleles in humans, four alleles in mice and rats), along with additional upstream factors (9). A number of human diabetes–related genes associated with *INS* mRNA expression are summarized in Table 1.

Classic studies of the islets of insulin-resistant C57BL/KsJ *db/db* mice, a genetic model of type 2 diabetes (T2D), have shown that at 5 weeks of age (when glycemia is not yet significantly perturbed and pancreatic insulin content is still similar to that in *+/db* controls), there is an elevation of *Ins* mRNA that is likely

to be disproportional to β cell mass, which helps to provide the insulin needed to mount a hyperinsulinemic response in these animals (10). The *db/db* mice then progress to diabetes, and with this, the abundance of *Ins* mRNA is not sustained (although interventions to limit obesity and diabetes with diet or medications that provide β cell rest tend to preserve some of the elevation of *Ins* mRNA levels, as well as insulin secretory capacity; refs. 10, 11). Thus, regulating *INS* mRNA level is at least part of the response by which β cells compensate for insulin resistance, although this may not be maintained as hyperglycemia progresses. Accumulated clinical and basic research data suggest that declining *INS* mRNA levels may be just one feature of a larger phenomenon of β cell dedifferentiation, and this may be a relatively early signature of β cell failure in the natural progression of T2D in humans and animal models (12–15). Not surprisingly, many genes associated with β cell differentiation (including *INS* mRNA expression) also appear as (loss-of-function) GWAS candidates for T2D in humans (Tables 1, 2, 3, and 4).

There are also murine models in which hyperglycemia (from experimentally induced partial insulin deficiency) appears to lead directly to decreased β cell transcription factors (PDX1, Nkx6.1, HNF1A, and MafA — all of which contribute to differentiated function) in pancreatic islets, along with downregulation of β cell–enriched genes (*Glut2* and *Gck*) and accompanied by increased endocrine progenitor markers (*Ngn3*) and β cell–disallowed genes (*Ldha* and *G6pc*). The effects of hyperglycemia have also been observed in residual islets after incomplete pancreatectomy (16),

Table 1. Human diabetes–related genes associated with *INS* gene transcription[A]

Gene	Function	Diabetes phenotypes and other symptoms	References
INS	Encodes preproinsulin	MODY; PNDM; T2D; T1D	60, 201
PDX1	Transcription factor; stimulates transcription from the *INS* promoter; involved in early pancreas development	MODY4 or PNDM, usually with pancreatic agenesis; T2D	5, 202
NEUROD1	Transcription factor; stimulates transcription from the *INS* promoter; involved in pancreatic and neuronal development	MODY6; PNDM with neurological abnormalities; T2D	203, 204
MAFA	Transcription factor; binds to insulin enhancer elements and stimulates *INS* gene transcription	Insulinomatosis; diabetes; T1D	205–207
PAX6	Transcription factor; involved in islet, eye, brain, and pituitary development	PNDM with brain anomaly; diabetes and aniridia	208, 209
GLIS3	Positively regulates *INS* gene transcription; expressed early in embryogenesis	PNDM with congenital hypothyroidism; T2D; T1D	210, 211

[A]Primarily focused on genes mentioned in the main text. MODY, maturity-onset diabetes of the young; PNDM, permanent neonatal diabetes mellitus.

as well as in transgenic mice expressing a mutated form of the ATP channel (Kir6.2-V59M, which impairs insulin secretion in β cells, causing hyperglycemia; ref. 17). Additionally, even without known genetically predisposing factors, *INS* mRNA has been shown to be dramatically decreased in human islet grafts exposed in vivo to hyperglycemic conditions (i.e., after transplantation into NSG mice), again suggesting that chronic hyperglycemia — along with other factors (18) — is detrimental to the maintenance of β cell *INS* mRNA as well as other key transcriptional machinery needed for the differentiated β cell phenotype (19, 20).

The *INS* mRNA may also be subject to abnormal splicing, including formation of the *INS-IGF2* chimera, which — although it may occur at only a low level (21) — could offer an *INS*-specific sequence contribution limited to the signal peptide, B chain, cleavage site, and a short portion of the C-peptide (22). Another errant splice donor-acceptor combination occurs in patients with early-onset autosomal dominant diabetes (MIDY, discussed in greater detail below) caused by a mutation within the *INS* gene intron 2, creating a preferential splice acceptor site, thus terminating the normal preproinsulin sequence in the same place as in *INS-IGF2*, but thereafter leading to a frameshifted protein replacing the remaining C-peptide and A chain with a novel peptide sequence (23).

Preproinsulin translation and translocation into the ER

Insulin biogenesis (Figure 1) begins with preproinsulin translation and translocation across the endoplasmic reticulum (ER) membrane, to synthesize proinsulin. Translation from the *INS* mRNA is acutely regulated by multiple drugs, hormones, and nutrients, especially glucose and its metabolites (24–27). Glucose-dependent preproinsulin translation is, to a considerable extent, dependent on factors that associate with untranslated regions (UTRs) of the *INS* mRNA (4, 28, 29). In isolated rat pancreatic islets, *total* islet protein synthesis increases about 2-fold within ≤1 hour of high glucose exposure, but much of this overall increase comprises the biosynthesis of preproinsulin along with copackaged secretory granule content proteins (30), with a much higher fold change during the same period (31, 32). Most *Ins* mRNA is associated with the ER, and a key step of glucose-dependent preproinsulin biosynthesis includes ribosome recruitment to initiate translation (33).

The preproinsulin mRNA yields a polypeptide that can be synthesized via cap-dependent or -independent translation (4), encoding a protein from N- to C-terminus comprising the signal peptide to direct translocation into the ER, the B chain, the connecting (C)-peptide (that is removed within immature secretory granules), and concluding with the A chain. Mature, optimally bioactive insulin requires a two-chain structure with two disulfide bonds that link the B and A chains plus one internal disulfide bond within the A chain.

Problems with preproinsulin translation may affect either the quantity or the quality of the translation product. Interestingly, based on rodent studies (that will require further validation in humans as well as animal models), it has been suggested that there is a steady decline of preproinsulin translation as a function of age (34); this might correlate with the age-related increase in the incidence of T2D (35). As for quality, aberrant preproinsulin translation has been linked to defects in *Cdkal1* (a tRNA methylthiotransferase required for the fidelity of reading AAA and AAG), and its deficiency can result in the misreading of Lys codons (36). Thus, in addition to effects on insulin target tissues, *Cdkal1* defi-

Table 2. Human diabetes–related genes linked to preproinsulin translation and/or translocation[A]

Gene	Function	Diabetes phenotypes and other symptoms	References
CDKAL1	tRNA methylthiotransferase; required for the fidelity of AAA and AAG codon translation	T2D	37
EIF2B1	Guanine nucleotide exchange factor for eukaryotic initiation factor 2; regulates translation initiation	PNDM with transient hepatic dysfunction	212
EIF2S3	Involved in the translation of protein synthesis and regulation of the integrated stress response	MEHMO syndrome	213
SSR1	Involved in preproinsulin translocation and proinsulin processing	T2D; GDM	71, 214, 215

[A]Primarily focused on genes mentioned in the main text. GDM, gestational diabetes mellitus; PNDM, permanent neonatal diabetes mellitus.

Table 3. Human diabetes–related genes involved in proinsulin folding or ER homeostasis[A]

Gene	Function	Diabetes phenotypes and other symptoms	References
HYOU1	HSP70 family member; participates in misfolded proinsulin degradation	Immunodeficiency 59 and hypoglycemia (IMD59)	96
SPRY2	A target and a negative regulator of the PERK arm of the UPR	T2D	9
SEL1L	Plays a role in ERAD system; involved in ubiquitin-dependent degradation of misfolded ER proteins	T1D	216
P4HB	Participates in the oxidative folding of proinsulin to the native state in β cells	As a carbonylated autoantigen in T1D	217
EIF2AK3	Induced by ER stress and rapid decrease of translational initiation and repression of global protein synthesis to regulate ER stress	Wolcott-Rallison syndrome	218

[A]Primarily focused on genes mentioned in the main text.

ciency can adversely affect the quality of the proinsulin translation product (triggering ER stress; ref. 37) as well as subsequent proinsulin-to-insulin conversion (38). Further, it was recently demonstrated that proper β cell iron status, mediated by iron-responsive element binding protein 2 (IRP2), is required for the ongoing function of *Cdkal1*, and IRP2 deficiency also results in Lys codon misreading that leads to impaired proinsulin folding with diminished insulin biosynthesis (39). (Alternatively, diabetogenic SNPs in the *CDKAL1* locus may reflect alteration in the expression of SRY-box transcription factor 4 [SOX4], as reviewed in ref. 40.) Moreover, translational initiation at an improper AUG start site could potentially lead either to the production of a preproinsulin bearing an N-terminally foreshortened signal peptide, or to a novel defective ribosomal initiation product that shares no sequence identity

whatsoever with preproinsulin (41). Along with aberrant splicing noted above, these mistranslated products are fascinating proteins, potentially generating neoantigens that may be recognized by the immune system to trigger autoimmune response (42, 43).

A critical step for preproinsulin entry into the secretory pathway involves its translocation across the ER membrane. There are at least two modes of entry: cotranslational translocation that is signal recognition particle–dependent (SRP-dependent), and posttranslational translocation that does not require SRP (44–46). Cotranslational translocation is the main ER entry pathway for most secretory proteins; however, posttranslational translocation may be important especially for small secretory proteins and those with suboptimal SRP recognition/binding sequences (47, 48). Preproinsulin had long been thought to enter the ER exclusively

Table 4. Human diabetes–related genes associated with insulin granule packaging, processing, or secretion[A]

Gene	Function	Diabetes phenotypes and other symptoms	References
PTPRN	Plays a role in vesicle-mediated secretory processes; helps to support luminal protein condensation in young granules of β cells	Associated with presence of islet autoantibody (IA-2) and a high risk of T1D	219
GCK	Acts as "glucose sensor" in glucose-stimulated insulin secretion	MODY; T2D; hypoglycemia	220
SLC2A2	Facilitates bidirectional glucose transport and triggers adequate insulin secretion in β cells	Fanconi-Bickel syndrome	221
SLC30A8	Encodes zinc transporter 8, which provides zinc for insulin maturation and/or storage	T2D	222
KCNJ11	ATP-sensitive potassium channel subunit; involved in regulating insulin secretion of β cells	MODY; NDM; DEND syndrome; hyperinsulinemic hypoglycemia	223
ABCC8	ATP-sensitive potassium channel subunit; involved in regulating insulin secretion of β cells	MODY; NDM; DEND syndrome; hyperinsulinemic hypoglycemia	223
KCNQ1	Adjusts insulin secretion through regulating the potassium channel current	T2D	224
KCNK16	Pancreatic β cell potassium channel that modulates β cell excitability, controlling Ca^{2+} influx and insulin secretion	T2D	225
KCNH6	Stimulates insulin secretion through regulating intracellular calcium concentration	Hypoglycemia; MODY	226
CAMKK2	Contributes to the regulation of insulin production in pancreatic β cells though Ca^{2+} signaling	T2D	227
BCL11A	Transcription factor; regulates multiple genes involved in insulin exocytosis in β cells	T2D	228
PAM	Has effects on insulin availability and the dynamics of granule exocytosis	T2D	229
PIM3	Negatively regulates insulin secretion by inhibiting the activation of MAPK1/3 (ERK1/2), through SOCS6	T2D	230
WFS1	Participates in the regulation of cellular Ca^{2+} homeostasis; involved in insulin secretion accompanied by regulation of cellular calcium responses to the secretagogue	Wolfram syndrome	231
ADRA2A	Overexpression impaired insulin granule docking at the plasma membrane and reduced exocytosis in β cell	T2D	232
ZMIZ1	Involved in exocytosis and insulin secretion	T2D	233, 234
MADD	Participates in glucose-induced insulin release	T2D	235
STARD10	Involved in glucose-stimulated Ca^{2+} dynamics and insulin secretion and proinsulin processing	T2D	236
SLC9B2	Critical for insulin secretion and clathrin-mediated endocytosis in β cells	T2D	237

[A]Primarily focused on genes mentioned in the main text. MODY, maturity-onset diabetes of the young; NDM, neonatal diabetes mellitus.
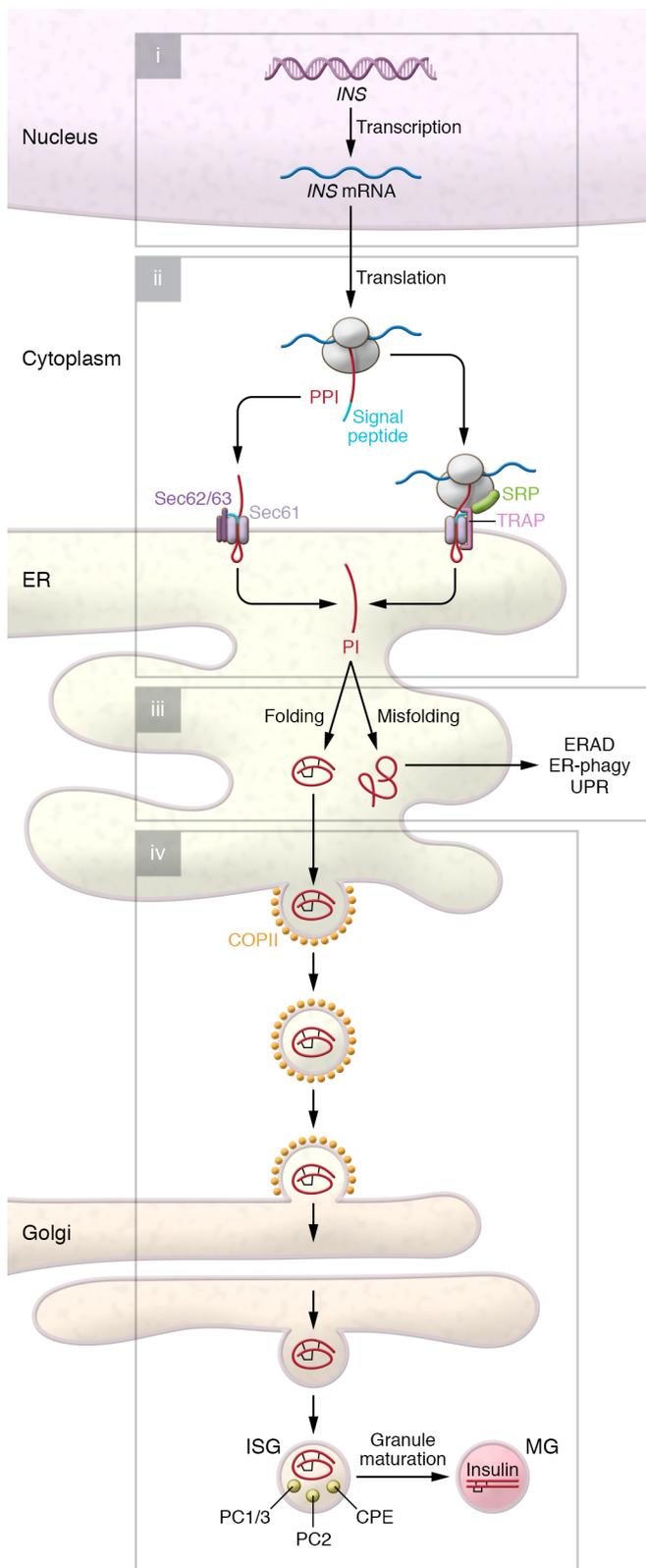
Figure 1. An overview of insulin biosynthesis in β cells. (i) Expression of *INS* gene is controlled quantitatively and qualitatively by transcriptional networks including PDX1, NGN3, NEUROD1, NKX2.2, FOXA2, and MAFA. (ii) Newly synthesized preproinsulin (PPI) undergoes cotranslational (SRP- and Sec61-dependent) and posttranslational (Sec62/63-dependent) translocation across the ER membrane, in which TRAP plays an important yet poorly defined role. (iii) Upon delivery to the ER lumen, the preproinsulin signal peptide is rapidly excised, forming proinsulin (PI) that undergoes rapid oxidative folding — ultimately forming three highly conserved disulfide bonds. ER folding machinery (BiP, ERO1, PDI, ERp46, GRP94, etc.) acts in concert to promote folding, and may recognize unfolded/misfolded proinsulin for degradation through ERAD and ER-phagy. Misfolded proinsulin can also activate the unfolded protein response (UPR; including three classic arms involving PERK, IRE1, and ATF6). (iv) Well-folded proinsulin is exported from the ER via COPII vesicles for delivery to the Golgi complex, where proinsulin forms hexamers in the presence of Zn^{2+}. Upon proinsulin delivery to (more acidic, Ca^{2+}-rich) immature secretory granules (ISGs), PC1/3, PC2, and CPE act to convert proinsulin to insulin, which is ultimately stored in mature granules (MGs).

sulin) indicates that up to 15%–20% of full-length preproinsulin (110 residues) can be detected immediately after its biosynthesis. As cleavage of the signal peptide is not itself thought to be rate-limiting, these data suggest that completion of the full-length translation product is too rapid to fully engage SRP-dependent cotranslational translocation (50, 51). This subset of fully translated molecules can nevertheless undergo subsequent conversion to proinsulin, strongly suggesting posttranslational translocation into the ER (47, 48). This may be an important "backup" mechanism to enhance newly synthesized preproinsulin translocation efficiency (44, 47). A positive charge within the N-terminal portion of the preproinsulin signal peptide appears to be an important factor in determining translocation efficiency for preproinsulin and other small secretory proteins (47, 50, 52).

Importantly, recent studies suggest that translocation into the ER is regulated, and decreased translocation efficiency may be a cellular response to overload of the ER (53, 54). This has been termed "preemptive quality control" accompanied by proteasomal disposal of untranslocated proteins, with the notion that decreased ER protein loading can alleviate ER stress (53, 55, 56). This preemptive quality control has been observed primarily for small secretory proteins and/or those with a suboptimal SRP recognition sequence (44, 57, 58). However, it is worth noting that although this mechanism may reduce ER stress, it may also result in cytosolic accumulation of untranslocated preproteins. Indeed, failure to translocate prion protein has been linked to neurodegenerative diseases (56). Thus, in order to avoid toxic accumulation in cells, untranslocated preproteins need to be efficiently degraded. Components of the ER-associated protein degradation (ERAD) machinery, including Derlins, p97 (valosin-containing protein), Bag6, and AIRAPL (zinc finger AN1-type containing 2B), have all been suggested to play a role in the preemptive proteasomal clearance of untranslocated preproteins (54, 59). Given the fact that preproinsulin is a small protein that may be predisposed to inefficient translocation (44, 50), it will be important to know whether it is also subject to preemptive regulation under ER stress, and whether inefficiently translocated preproinsulin can contribute to β cell failure during the ER stress of type 1 diabetes (T1D), T2D, or selective forms of monogenic diabetes (60–65). Certainly, the

through cotranslational translocation (i.e., formation of the ribosome-preproinsulin-SRP complex for delivery to the Sec61 translocon via binding to the SRP receptor on the ER membrane; ref. 49). However, recent experimental evidence from human islets (as well as non-β cells transfected to express wild-type preproin-

pathophysiological significance of inefficient translocation for preproinsulin is highlighted by the recent discovery of *INS* gene–encoded signal peptide mutations that cause early-onset diabetes in humans (66–69). Further, even in the absence of such signal peptide mutations, defective IRE1α-mediated ER stress signaling is known to result in the accumulation of unprocessed preproinsulin (70), which normally is virtually undetectable in healthy β cells (71). Recently, translocon-associated protein-α (TRAPα), encoded by the T2D-associated gene *SSR1* (signal sequence receptor 1), has also been shown to play an important role in the efficiency of preproinsulin translocation (71). These realizations open new avenues for further studies to evaluate the potential pathophysiological importance of defective preproinsulin translation and/or translocation during the development of diabetes, and the gene variants that may contribute to these defects (Table 2).

ER quality control in proinsulin folding/ misfolding

In general, proteotoxic forms of proinsulin are selected against in evolution (72), as they seem to impair β cell mass in addition to their impairment of insulin biogenesis (73, 74) as well as general dysfunction of the secretory pathway (75). Nevertheless, misfolding of the *INS* gene product is not incompatible with life, as a large family of *INS* gene mutations underlie the autosomal dominant disorder known as MIDY (mutant *INS* gene–induced diabetes of youth) (76). MIDY does not represent all *INS* gene mutations, as some mutant alleles may merely decrease preproinsulin expression (77), or alter aspects of the biology of the *INS* gene product other than proinsulin folding (60). All MIDY patients thus far described are heterozygotes, still bearing one wild-type (WT) *INS* allele (78). It is clear that misfolded MIDY proinsulins are synthesized yet entrapped within the ER lumen (79), and this is the location where the mutant protein first engages (and blocks the trafficking of) WT bystander proinsulin (80). Indeed, MIDY mutant proinsulins can form aberrant, intermolecular disulfide bonds with WT proinsulin, propagating the defects of impaired folding and ER export onto the WT bystanders (51, 76, 81). These abnormal interactions appear to be an initiating event leading to insulin-deficient diabetes, well before decreased β cell mass (82–84).

How these aberrant interactions are initiated has not been clear, but recent evidence suggests that initial contact between mutant and WT molecules involves one or more residues that would normally be used for noncovalent proinsulin dimerization (85), which engages a relatively hydrophobic helical region spanning residues 8–29 of the insulin B chain from two apposed partners. Structural analysis indicates that Tyr-B16 contributes more surface area to the dimerization contact surface than any other single residue (85). Whereas substitution of Tyr-B16 with either Ala or Asp does not affect the folding and trafficking of WT proinsulin, introduction of these substitutions into a MIDY proinsulin (such as the *Akita* mutant) functions as an intragenic suppressor, substantially limiting cross-dimerization, decreasing abnormal intermolecular disulfide bonding between mutant and WT proinsulin, and increasing WT insulin production. These data support the idea that dominant-negative propagation of misfolding from mutant proinsulin onto bystander proinsulin is initiated by intermolecular contact involving residues of the natural proinsulin

dimerization surface. Independently, misfolded proinsulin may also impair, in trans, other exportable proteins in the β cell secretory pathway, such as the precursor insulin receptor (ProIR), which can inhibit ProIR maturation and insulin signaling in β cells (86).

In addition to the foregoing interactions, proinsulin has the potential to interact with multiple resident proteins of the ER, and these interactions are likely to be altered under pathological conditions. Proinsulin has been reported to coprecipitate with ERp46, but the expression level of this ER oxidoreductase (and thus its probable interaction with proinsulin) decreases upon onset of diabetes (at least in C57BL/KsJ *db/db* mice) — although this may be partially rescued upon treatment with GLP-1 agonists (87). BiP is a well-established ER chaperone with preferential binding to misfolded proinsulin (88, 89). BiP function is potentially supported by the Sil1 nucleotide exchange factor (90). Additionally, GRP94 function is needed for proper post-ER processing of proinsulin, and deficiency of GRP94 increases the numbers of abnormally enlarged secretory granules bearing diminished mature insulin (91).

Further, proinsulin that is misfolded under altered redox conditions may present itself for interactions with ER-resident proteins that perhaps do not participate as importantly in the normal proinsulin folding pathway. New evidence indicates that PRDX4 may be an important proinsulin interactor in normal human islets — particularly under altered redox conditions (92). Further, the lectin-like chaperone calreticulin appears to interact with reduced proinsulin and facilitates its lysosomal degradation (93). Misfolded mutant proinsulin is also known to interact with PDIA1 (also known as PDI; encoded by *P4HB*) (94), which assists proinsulin under conditions of oxidative stress (95); misfolded proinsulin also interacts with GRP170 (encoded by *HYOU1*) (96). ERO1 is thought to function upstream of PDIA1 and other isomerases/oxidoreductases. Deletion or downregulation of ERO1 impairs proinsulin oxidative folding and decreases insulin production (97, 98). Conversely, overexpression of ERO1 in β cells appears to accelerate oxidative folding and enhances the ER export of WT proinsulin in the presence of MIDY mutants (51, 99), although excessive β cell–specific expression of ERO1β does not improve insulin production (100), suggesting that a fine-tuned oxidative state of the ER is needed to optimize insulin biosynthesis.

Misfolded proinsulin, at least in part, undergoes intracellular disposal (101) by ERAD and ER macroautophagy. ERAD has an important role to play in normal β cell ER homeostasis (102). The evolutionarily conserved ERAD core machinery includes Hrd1 (involved in retrotranslocation of the target protein) and Sel1L (the cofactor essential for Hrd1 protein stability; ref. 103); additional factors may contribute to the expression and/or stability of these core components (104). The basic steps involved in ERAD of proinsulin (105) and other secretory proteins involve (a) recognition of the (presumably misfolded) protein target via binding to various resident proteins within the ER that either positively or negatively facilitate the efficiency of the degradation process (96, 106); (b) retrotranslocation from the luminal side of the ER membrane to the cytosolic side (94); (c) ubiquitylation of the target protein (107) via Hrd1; (d) extraction of the ubiquitylated target protein via the p97 ATPase for the delivery to proteasomes (108); and (e) proteasomal degradation, which may involve the stress-inducible proteasome subunit β5i (109).
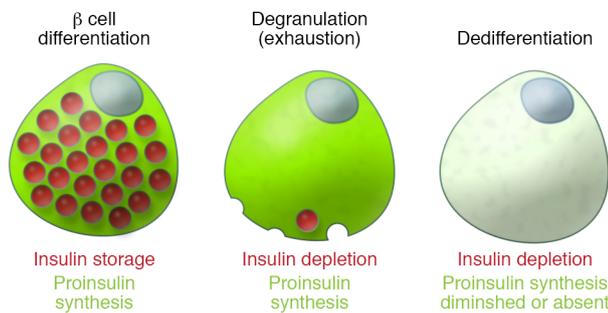
Figure 2. β Cell phenotypes of defective insulin production and storage. β Cell dysfunction may begin many years before diagnosis of diabetes, and progressive deterioration of β cell function represents the natural history of T2D. In healthy, well-differentiated and well-compensated β cells (left), the synthesis of proinsulin (green) and its folding and processing are homeostatically regulated to meet metabolic demand in order to maintain the biogenesis of insulin (red), which is stored in granules for insulin secretion. Well before end-stage β cell failure, proinsulin biosynthesis may be actively ongoing even in β cells that no longer maintain an insulin storage pool (middle). β Cell degranulation appears (by immunofluorescence analysis of insulin content) as a loss of β cell identity. As the disease progresses further (right), there is also a diminution of proinsulin biosynthesis in addition to the loss of insulin storage. This schematic figure shows individual β cells for clarity, but given the heterogeneity of islet β cells, these representations should be viewed not as a fixed stage for all β cells in the population, but rather as the phenotype for a prevailing portion of islet β cells across the pancreas during disease progression. β Cell death is not shown in the figure, but this is also a relevant consideration in many forms of diabetes.

Deficiency of Sel1L expression (which causes instability of Hrd1 protein; ref. 110) has long been known to block glucose-stimulated insulin secretion from islet β cells (111). Hrd1 may act on proinsulin but also on other β cell degradation substrates (112). Indeed, new work has highlighted that Sel1L-Hrd1 activity in β cells is needed for the maintenance of full functionality and identity of β cells — especially including insulin biosynthesis — in particular by limiting the activity of the TGF-β signaling pathway (113). Thus, loss of β cell Sel1L appears to trigger hyperactivation of TGF-β signaling, which accompanies a dedifferentiated β cell phenotype.

Beyond ERAD, quality control of misfolded proinsulin results in active clearance by ER autophagy (ER-phagy); this occurs under all conditions but is especially true when ERAD of proinsulin is impaired (114), or in β cells bearing *Ins* mutations that cause proinsulin misfolding (115). In the latter case, mTOR activity appears diminished (73) and AMP kinase activity appears stimulated. While ER-phagy is likely to be beneficial in clearing misfolded proinsulin and thereby enhancing efficiency of the remaining proinsulin to be properly folded and to undergo successful intracellular transport (114), generalized hyperactivation of autophagy is not a cure-all for β cells, as this can stimulate enhanced autophagic turnover of transport-competent proinsulin (116) as well as turnover of mature insulin secretory granules, leading to a diminution of the insulin storage pool (117, 118). The regulation of lysosome-mediated proinsulin turnover is also not yet understood (119); however, if therapies can be designed that selectively activate β cell ER macroautophagy, such therapies may hold promise as potential treatments for diabetes linked to proinsulin misfolding (114).

ER stress and ER stress response signaling pathways in pancreatic β cells have been extensively reviewed elsewhere (120–123), including heterogeneity in responses that accompany the heterogeneity of proinsulin biosynthetic/folding activity among islet β cells (124). The biosynthesis and folding of proinsulin itself controls a set-point of ER stress activation within β cells (125). Additionally, β cell ER stress appears to be a hallmark of T2D in both humans and rodent models (126) — and failure to mount adaptive ER stress responses is known to make matters worse (127, 128).

The Ire1 limb of the ER stress response is of acute and profound benefit to insulin biosynthesis (129). In particular, Ire1-Xbp1 upregulates multiple ER factors promoting proinsulin biosynthesis, including preproinsulin translocation across the ER membrane (70, 130) (see previous section). Moreover, Ire1-Xbp1 upregulation of various ER oxidoreductases is thought to participate in the oxidative folding of proinsulin to the native state (including PDIA1, PDIA5 [also known as PdiR], PDIA6 [P5], ERp44 [PDIA10], and TXNDC5 [PDIA15, or ERp46]) — of these, PDIA1 has been demonstrated to contribute to the reduction of aberrant intermolecular proinsulin disulfide bonds that are formed upon redox stress (94, 95). Nevertheless, Ire1 activation must be fine-tuned, as its hyperactivation appears clearly detrimental to insulin biosynthesis and β cell survival (131–133).

The role of PERK in regulating insulin biogenesis is firmly established, but the proposed mechanism(s) have varied considerably between different groups and systems. Classic work described PERK as one of the four kinases involved in the phosphorylation of eIF2α (134), negatively regulating pancreatic (exocrine and endocrine) translation (135, 136). Consistent with this, conditions of insufficient PERK activity have been reported to result in hypersynthesis of proinsulin, albeit with an increase in the abundance of non-native folded forms (88, 89, 137) that may contribute to the β cell failure of Wolcott-Rallison syndrome (138, 139). However, other studies have not corroborated that PERK activity negatively regulates proinsulin biosynthesis (140), concluding instead that PERK regulates multiple other functions (141) including β cell proliferation (142, 143) — although this too has been challenged (144). Further, there is reason to suspect that PERK expression is needed for the maintenance of β cell differentiation as a result of its role in regulating multiple aspects of ER homeostasis (82, 145). Like the findings regarding Ire1, these findings may not necessarily be internally contradictory, as they may reflect the homeostatic importance of PERK in β cells in which both too little and too much activity can be detrimental (146). However, in studies of proinsulin translation and its regulation by PERK, we do wish to sound a strong note of caution about conclusions based largely on the INS-832/13 rat β cell line. These cells may indeed be useful for studies of proinsulin trafficking, conversion to insulin, and glucose-stimulated insulin secretion, as well as sensitivity to ROS and other β cell toxins (147). Nevertheless, this clone of INS-1 cells (expressing endogenous rat proinsulin-1 and -2) is uniquely complicated for studies of proinsulin biosynthesis, because the INS-832/13 cells also synthesize transfected human proinsulin expressed from a nonphysiological CMV promoter and bearing nonphysiological 5′- and 3′-UTR sequences (148) — thus its translation cannot be regulated in a physiological way.

PERK activity is also thought to support the expression of another ER stress sensor, ATF6 (149). As with Ire1 and PERK, dif-
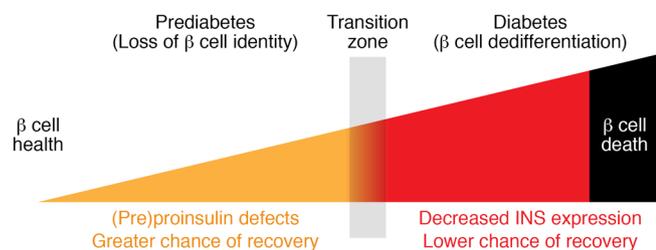
Figure 3. Proinsulin and insulin during progression to β cell failure.
Prediabetes: β Cell function has a greater chance of recovery. β Cell insulin synthesis increases and insulin content is maintained, although intracellular proinsulin/Cpep or proinsulin/insulin ratio begins to rise, accompanied by defective GSIS and elevated proinsulin/Cpep or proinsulin/insulin ratio in the secretion. *Early T2D:* β Cell function still has a good chance of recovery. Insulin content declines and progresses toward depletion as demonstrated by immunostaining, making it more difficult to identify β cells ("loss of identity"), but (pre)proinsulin biosynthesis is still actively ongoing. Proinsulin/Cpep or proinsulin/insulin ratio in the islets and secretion is more markedly elevated. *Later T2D:* β Cell function may still be recoverable, but the chance of recovery is lower at this stage. Evidence of β cell dedifferentiation is detectable and (pre)proinsulin biosynthesis drops (though still detectable) with a decreased expression of pancreatic proinsulin. β Cell–specific transcription factors exhibit diminished expression; endocrine progenitor cell markers increase. *End-stage T2D:* β Cell function appears unrecoverable and β cell mass is diminished, consistent with some level of β cell demise. Cpep, C-peptide; GSIS, glucose-stimulated insulin secretion; PI, proinsulin.

ferent studies of ATF6 in β cells have reached somewhat differing conclusions regarding its impact on insulin production and release (150). In brief, physiological (i.e., mild) ER stress is thought to promote ATF6 expression in a manner that is favorable to β cell growth and survival (128, 151); on the other hand, transcription activated by ATF6 stimulates production of the proapoptotic transcription factor CHOP (152, 153). There is reason to think that inadequate ATF6 and Xbp1 expression may be linked to inadequate insulin production during the progression of T2D (154). In summary, each of the ER stress response signaling limbs requires fine tuning and has the potential to lead to both helpful and harmful effects on insulin biogenesis and β cell viability. A number of genes involved in ER homeostasis whose variants may be linked to human diabetes are listed in Table 3.

Delivery to granules with processing and insulin storage

A list of human diabetes–related genes associated with insulin granule packaging, processing, or secretion is presented in Table 4. Formation of insulin secretory granules, and maintenance of the granule compartment, is a broad topic that will be only briefly summarized here. It is important to note, however, that morphometric studies of human T2D islets have shown that the volume density of total mature insulin granules in β cells is significantly diminished, and this pool size correlates with the diminished magnitude of the glucose-stimulated insulin secretion response (155).

Recent proteomics studies have suggested that to support the upregulation and maintenance of insulin secretory granule biogenesis (in mice; ref. 156), there are differential posttranslational modifications of ER molecular chaperones and proteins involved in

redox homeostasis (see previous section), as well as those involved in protein trafficking. This may be important, because ongoing ER-to-Golgi transport itself helps to maintain the ER environment and limit ER stress (157). Indeed, augmented proinsulin export is thought to be linked to enhanced insulin secretion (158).

Upon transport through the Golgi complex, proinsulin and its processing enzymes emerge in immature secretory granules (ISGs) budding from the trans-Golgi network (a process that appears to be microtubule-dependent; ref. 159). The formation and maturation of insulin granules involve a complex set of processes that coordinate ionic control of the organelle interior (160, 161), acquisition of competence for stimulus-dependent secretion (162, 163), enzymatic processing of proinsulin (164, 165), packaging (protein condensation) (166), and membrane trafficking and lipid modification (167–170), as well as stability of the organelle during its intracellular transport through the cytoplasm (171–173); many of these steps are interconnected, and they are ultimately transcriptionally orchestrated (174). For example, the peripheral membrane protein HID-1 is thought to support proinsulin-to-insulin conversion that is synchronized with other steps of insulin secretory granule maturation (175).

As for processing enzymes, ISG acidification and high Ca^{2+} are both required for optimal proinsulin conversion to insulin (176). Prohormone convertase-1 (PC1/3) is the dominant endoproteolytic activity required for proinsulin-to-insulin conversion, with a lesser contribution from prohormone convertase-2 (177). Additionally, the activity of carboxypeptidase E (CPE) is strongly linked to β cell insulin production and storage (178). These enzymes depend on the dibasic endoproteolytic cleavage sites of proinsulin; diabetes-related insulin deficiency is observed either when these cleavage sequences are not present, or when they are present but inefficiently acted upon. Indeed, deficiency of proinsulin conversion appears to correlate with insulin deficiency in diabetes (179–183).

With regard to intragranular packaging, chromogranins A and B have both been long suspected to play an important role (184), but their precise molecular role has remained somewhat elusive (185, 186), in part because it is difficult to distinguish direct and indirect effects of the expression of these molecules. Additionally, other proteins such as VGF or IA-2/ICA512 (islet cell autoantigen 512, an integral membrane protein with either a detached or an intact luminal domain) may also help to support luminal protein condensation in young granules (187, 188). While luminal condensation is occurring, there appears to be active modeling/remodeling of the ISG membrane, controlled in large part by the recruitment of cytosolic factors that interact with ISG membrane proteins to drive the remodeling (189), as well as dynamic membrane interchange with the endosomal system — in part regulated by the endosome-associated recycling protein (EARP) complex and the EARP-interacting protein EIPR1 (190).

Diabetes caused by defective β cell insulin storage

Absolute/relative insulin deficiency is a hallmark of both T1D and T2D. However, beginning with relatively healthy compensation including robust (or even increased) insulin biosynthesis, the onset and subsequent development of T2D leads β cells through a gradual progression of different phenotypes, from diminished

insulin storage, to functionally exhausted cells that can no longer be identified as β cells owing to loss of immunostaining with anti-insulin antibodies, and ultimately to the loss of islet β cells that may result from dedifferentiation and eventual β cell death (Figure 2) (15, 36, 191–193). Importantly, accumulating evidence from both mouse (17) and human (14, 194, 195) studies supports the idea that at some points during the progression of T2D, β cell function can be partially or even fully restored in those favorably responding to weight loss; however, the ability to regain β cell function appears to be time-dependent (196). Although the rate of remission after extensive intervention can be as high as 50%–90% for patients with T2D of ≤1.5 years' duration (194, 195), reversal essentially cannot be achieved in patients who have had T2D for more than 11 years (197). These data suggest progression beyond one or more transition points that determine recoverability across the β cell population (Figure 3).

Unfortunately, to date, there are no reliable biomarkers to identify/distinguish these stages in a living individual, or even in histological analysis of the pancreas from deceased individuals. Although absence of islet insulin immunostaining is perhaps the most widely used marker to define loss of β cell identity, depletion of insulin storage does not necessarily mean dedifferentiation or β cell death. For example, it has been shown that depleted insulin storage in the islets of C57BL/KsJ *db/db* diabetic mice can still be significantly restored when the islets are cultured ex vivo in 5.6 mmol/L glucose (198). It has also been found that in islets from *Akita* diabetic mice with profound insulin deficiency, (pre) proinsulin biosynthesis is nevertheless still ongoing, although this fails to result in the efficient formation of mature insulin (84). A generally similar phenotype can be discerned in the islets of young C57BL/KsJ *db/db* mice, in which many insulin-depleted β cells continue to abundantly express proinsulin (199). Such data indicate early depletion of the insulin storage pool prior to any diminution of translation of the *INS* mRNA.

Concluding questions and comments

Recent genetic and biological evidence suggests that early steps of insulin biosynthesis, including preproinsulin translocation into the ER, proinsulin folding, and ER export, are not as efficient as was once thought. Indeed, key questions in these areas remain to be addressed:

(a) Does the efficiency of co- and posttranslational translocation vary between healthy β cells and the islets of individuals with diabetes? To what extent do signaling pathways regulate the efficiency of preproinsulin translocation under different physiological and/or pathological conditions?

(b) Proinsulin dimerizes in the ER, and cross-dimerization between misfolded proinsulin and bystander WT proinsulin contributes to dominant-negative effects. If proinsulin dimerization

in the ER is nonessential, could the dimerization contact surface be a druggable target to promote increased insulin biogenesis, comparable to pharmacochaperones being developed for other degenerative diseases caused by protein misfolding (200)?

(c) Correct disulfide pairing is a critical event both for proinsulin folding in the ER, and for the ultimate bioactivity of insulin. Does the oxidative environment of the β cell ER change in T2D, and if so, is this too a druggable target?

(d) Proinsulin folding is a kinetic process, during which we hypothesize that there are discrete folding intermediates. How do β cells differentiate "on-pathway" folding intermediates from "dead-end" misfolded proinsulin in order to distinguish the fates of not-yet-folded versus misfolded proinsulin molecules?

(e) If there really are transition zones during the development and progression of T2D at which β cell function can be fully (or even partially) restored, can β cell rest promote recovery of β cell function even after dedifferentiation has occurred, and could proinsulin status be used as a biomarker to determine this?

Accumulating evidence indicates that genetic or acquired defects in the Ins gene translation product are central to virtually all of these key questions. In the coming years, we would hope to see development of therapies designed to attenuate the detrimental effects of improperly translocated preproinsulin or misfolded proinsulin. These strategies may span approaches designed to increase the biological yield of properly folded proinsulin (and thus insulin) in β cells, or to enhance the degradative clearance of improperly folded products. Such approaches may well have broad translational significance for future developments in the management of β cells in diabetes.

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