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Transcription factor GLIS3: critical roles in thyroid hormone biosynthesis, hypothyroidism, pancreatic beta cells and diabetes

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Abstract

GLI-Similar 3 (GLIS3) is a member of the GLIS subfamily of Krüppel-like zinc finger transcription factors that functions as an activator or repressor of gene expression. Study of GLIS3-deficiency in mice and humans revealed that GLIS3 plays a critical role in the regulation of several biological processes and is implicated in the development of various diseases, including hypothyroidism and diabetes. This was supported by genome-wide association studies that identified significant associations of common variants in *GLIS3* with increased risk of these pathologies. To obtain insights into the causal mechanisms underlying these diseases, it is imperative to understand the mechanisms by which this protein regulates the development of these pathologies. Recent studies of genes regulated by GLIS3 led to the identification of a number of target genes and have provided important molecular insights by which GLIS3 controls cellular processes. These studies revealed that GLIS3 is essential for thyroid hormone biosynthesis and identified a critical function for GLIS3 in the generation of pancreatic β cells and insulin gene transcription. These observations raised the possibility that the GLIS3 signaling pathway might provide a potential therapeutic target in the management of diabetes, hypothyroidism, and other diseases. To develop such strategies, it will be critical to understand the upstream signaling pathways that regulate the activity, expression and function of GLIS3. Here, we review the recent progress on the molecular mechanisms by which GLIS3 controls key functions in thyroid follicular and pancreatic β cells and how this causally relates to the development of hypothyroidism and diabetes.

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Keywords

Gene transcription; Hypothyroidism; Thyroid hormone biosynthesis; Diabetes; Pancreatic β cells; Insulin

1. Introduction

Krüppel-like zinc finger proteins form a large family of Cys_2His_2 (C_2H_2)-type zinc finger (ZF)-containing transcription factors that exhibit many critical roles in the control of various biological processes, including embryonic development, cell proliferation, and differentiation. Genetic alterations and common gene variants that alter the expression, activity, and function of these proteins have been associated with a wide range of pathologies, including developmental abnormalities and cancer. The GLI-Similar (GLIS) proteins constitute a subfamily of Krüppel-like zinc finger transcription factors comprised of three members, GLIS1–3 (Jetten, 2018; Kim, et al., 2002; Kim, Nakanishi, Lewandoski, & Jetten, 2003; Lamar, Kintner, & Goulding, 2001; Nakashima, et al., 2002; Zhang & Jetten, 2001; Zhang, et al., 2002). GLIS1–3 are most closely related to members of GLI and ZIC subfamilies of Krüppel-like zinc finger proteins. Beyond their DNA binding domain (DBD), GLIS1–3 exhibit little homology with each other or the GLI and ZIC family members.

Genetic alterations in *GLIS* genes have been implicated in many pathologies. Chromosomal translocations between paired box 8 (*PAX8*) and *GLIS1* are associated with hyalinizing trabecular tumors (HTT) and common genetic variants in *GLIS1* have been reported to be associated with autism spectrum disorder and Alzheimer's disease (Deming, et al., 2017; Jetten, 2019; Kuo, et al., 2015; Nikiforova, et al., 2019). Mutations in *GLIS2* that abolish GLIS2 function cause nephronophthisis, a cystic renal disease that is the most common genetic cause of end-stage renal disease in young adults (Attanasio, et al., 2007; Kim, et al., 2008; Lu, et al., 2016). It is characterized by renal atrophy, inflammation and fibrosis. Intrachromosomal translocations between the CBFA2/RUNX1 partner transcriptional co-repressor 3 (*CBFA2T3*, also referred to as *ETO2*) and *GLIS2* genes are associated with non-Down's syndrome acute megakaryoblastic leukemia (Gruber, et al., 2012; Jetten, 2019; Masetti, et al., 2013; Thiollier, et al., 2012). GLIS3 is critical in the regulation of many physiological processes and has been implicated in a number of pathologies, including neonatal diabetes, glaucoma, polycystic kidney disease, neurological disorders, congenital hypothyroidism, and cancer (Dimitri, 2017; Jetten, 2018, 2019; Kang, et al., 2010; Lichti-Kaiser, ZeRuth, Kang, Vasanth, & Jetten, 2012; Scoville, Kang, & Jetten, 2017). In this review, we provide an overview of our current knowledge of the function of GLIS3 in the thyroid gland and the pancreas, particularly pancreatic β cells, and its role in the development of hypothyroidism and diabetes. Considering the important role of GLIS3 in these processes, a better understanding of the molecular mechanisms underlying the regulation of GLIS3 action and activity might lead to the development of new strategies in the management of these pathologies.

2. GLIS3 functional domains and transcriptional regulation

2.1. DNA binding domain (DBD)

The human *GLIS3* gene is located on chromosome 9p24.2 and encompasses more than 540 kb. Its 11 exons generate a 7.7 kb transcript encoding a protein of 930 amino acids that contains several functional domains (Fig. 1)(Jetten, 2018; Kim, et al., 2003; Senee, et al., 2006). GLIS proteins share their highly conserved DBD with members of the GLI and ZIC subfamilies. The DBD consists of a tandem repeat of 5 ZFs, in which zinc is in a tetrahedral configuration with the two cysteine and histidine residues. Analysis of the 3D structures of GLI1 and ZIC3 revealed that ZF1 and ZF2 form a heterodimer that is mediated via interactions between their hydrophobic amino acids, and that ZF2-5 bind the major groove of the DNA (Hatayama & Aruga, 2010; Pavletich & Pabo, 1993). Based on the high similarities between GLIS1-3 and GLI1 DBDs, one might hypothesize that the ZFs of GLIS proteins have a comparable 3D structure. The DBDs also contain a Lys/Arg-rich sequence at their C-terminal end that mediates the nuclear localization of these proteins.

GLIS3 regulates gene transcription by binding specific DNA sequences that are recognized by its DBD (Beak, Kang, Kim, & Jetten, 2008; Jetten, 2018; Kim, et al., 2003). Motif analysis of the GLIS3-bound DNA (ChIP-Seq analysis) identified a G-rich sequence as the consensus GLIS binding site (GLISBS) (Jeon, et al., 2019; Kang, et al., 2017; Scoville, Lichti-Kaiser, Grimm, & Jetten, 2019). This consensus sequence is very similar to that of other GLIS, GLI, and ZIC proteins, as well as other Krüppel-like zinc finger transcription factors. Therefore, these proteins might compete with each other for GLISBS binding, as for example has been reported for the regulation of *Wnt4* by GLIS2 and GLI1 (Li, et al., 2011). Although some overlap in the regulation of the same target genes likely occurs, their functions appear to be very different from each other based on their regulation of distinct physiological functions and roles in disease. This suggests that they largely regulate the transcription of distinct sets of target genes. How these different proteins regulate distinct target genes is not well understood. In addition to differences in their cell type-specific expression patterns, the specificity might be determined by differences in their affinity for the binding site, the promoter context, interaction with other transcriptional mediators, as well as in the manner by which upstream signaling pathways might regulate their binding affinity.

2.2. Transcriptional activation and interacting proteins

GLIS3 functions as strong activator of gene transcription that is mediated through the transactivation domain (TAD) at its C-terminus (Beak, et al., 2008; Jetten, 2018)(Fig. 1). The TAD facilitates the recruitment of co-activators that includes the C-terminal binding protein (CBP) (ZeRuth, Takeda, & Jetten, 2013). The WW domain containing transcription regulator 1 (WWTR1, also referred to as TAZ) is another protein reported to interact with GLIS3 and enhance GLIS3 transcriptional activity by binding to a P/LPXY motif in the C-terminus (Kang, Kim, et al., 2009; Meng, Moroishi, & Guan, 2016). WWTR1 is also part of the Hippo signaling pathway where it functions as a regulator of gene transcription through its interaction with TEA domain transcription factors (TEAD) (Hillmer & Link, 2019). Interestingly, similar to GLIS3, the Hippo pathway has an important role in polycystic

kidney disease and pancreatic β cells (Ardestani & Maedler, 2018; Ma & Guan, 2018). These observations suggest that there might be a connection between the GLIS3 and Hippo signaling pathways.

Several studies demonstrated that GLIS3 protein is phosphorylated and methylated at multiple sites and can be ubiquitinated and sumoylated (ZeRuth, Williams, Cole, & Jetten, 2015; ZeRuth, Yang, & Jetten, 2011). Although their functional significance is still largely unknown, some of these posttranslational modifications likely play a role in the regulation of GLIS3 activity or protein stability. A number of GLIS3 interacting proteins have been identified that mediate posttranslational modification of GLIS3. The observed interaction of GLIS3 with the arginine methyltransferase PRMT5 and the lysine demethylase KDM4C might have a dual function in the (de)methylation of GLIS3 as well as histones and be part of the regulatory mechanism by which GLIS3 modulates gene transcription. Members of the HECT E3-ubiquitin ligase family interact with a PPxY motif in GLIS3 through their WW-domains and subsequently stimulate GLIS3 ubiquitination and promote its proteosomal degradation (ZeRuth, et al., 2015). The latter may be responsible for the observed reduced transcriptional activity of GLIS3. Sumoylation of GLIS3 mediated by Protein Inhibitor of Activated STAT 4 (PIAS4) and Ubiquitin conjugating enzyme E2 I (UBE2I, also named UBC9) has also been reported to regulate GLIS3 activity (Hoard, Yang, Jetten, & ZeRuth, 2018).

2.3. Highly conserved region (HCR)

In addition to its nuclear localization, proteomic analysis of primary cilium-associated proteins as well as immunohistochemical analysis detected GLIS3 protein in the primary cilium (Hashimoto, et al., 2009; Kang, Beak, Kim, Herbert, & Jetten, 2009; Mick, et al., 2015). The primary cilium is a long extension of the plasma membrane that functions as an important signaling hub (Nachury & Mick, 2019). Immunofluorescent staining of cultured cells and mouse kidney sections localized GLIS3 to the tip of the primary cilium. Although in mice GLIS3 deficiency does not affect the length of the primary cilium, mutation of *glis3* in Medaka (*Oryzias latipes*) results in shorter cilia in renal tubules (Hashimoto, et al., 2009; Kang, Beak, Kim, Herbert, & Jetten, 2009). In the case of members of the GLI family, the translocation into the primary cilium is mediated by a primary cilium localization signal (CLS) consisting of the consensus sequence SSXR-X6-R/KKR-X5-PY/L (Han, et al., 2017). The CLS is localized within a 60 aa highly conserved region (HCR) at their N-terminus. This sequence binds transportin 1 (TNPO1) which facilitates the entry of these proteins into the primary cilium. Interestingly, this HCR is also shared with GLIS3, suggesting that GLIS3 enters the primary cilium by a similar mechanism (Jetten, 2018; ZeRuth, et al., 2011) (Fig. 1). The primary cilium functions as an important signaling hub for many external signals, including the hedgehog signaling pathway, which is essential in the regulation of GLI protein activity (Bangs & Anderson, 2017). We hypothesize that GLIS3 activity is controlled by multiple upstream pathways that include primary cilium-associated signaling pathways and pathways that are independent of the primary cilium (Fig. 1, Pathways 1 and 2) (Jetten, 2018). This might involve a yet unknown external signals that acts on a G-protein coupled receptors, resulting in the activation of a protein kinase pathway and subsequent modulation of GLIS3 activity and function.

3. Role of GLIS3 in the thyroid gland and hypothyroidism

3.1. GLIS3 mutations and polymorphisms in hypothyroidism

The first indications of an association between GLIS3-deficiency and hypothyroidism came from clinical studies of patients with a rare syndrome referred to as Neonatal Diabetes and congenital Hypothyroidism (NDH)(Dimitri, 2017; Dimitri, et al., 2016; Dimitri, et al., 2015; Dimitri, et al., 2011; Jetten, 2018; Senee, et al., 2006; Taha, Barbar, Kanaan, & Williamson Balfe, 2003). These patients displayed a wide range of pathologies that, in addition to congenital hypothyroidism (CH) and neonatal non-autoimmune diabetes, can include elevated intraocular pressure, cholestasis, polycystic kidney disease, facial dysmorphisms, and mild mental retardation. Mutations among these patients include frameshift mutations that cause deletion of the GLIS3 TAD, point mutations in the DBD that abolish the GLIS3 ability to bind GLISBS, deletion of exons, and several large deletions removing major parts of the gene (Alghamdi, Alsaedi, Aljasser, Altawil, & Kamal, 2017; Dimitri, 2017; Dimitri, et al., 2016; Dimitri, et al., 2015; Dimitri, et al., 2011; Jetten, 2018; Senee, et al., 2006).

Characteristic for CH, blood levels of free T4 (FT4) are greatly reduced in these patients, while TSH levels are substantially elevated. NDH patients show a high degree of variability in thyroid gland anatomy that ranges from an almost normal morphology to thyroid aplasia (Dimitri, 2017; Dimitri, et al., 2016). The cause underlying this variability is not yet understood. Since NDH patients acquire a wide range of pathologies, including hyperglycemia and hypoinsulinemia, these phenotypes may also affect the development and function of the thyroid gland. This hypothesis is supported by studies showing that the insulin and insulin-like growth factor-1 receptors (INNSR and IGF1R) play a critical role in thyroid folliculogenesis (Ock, et al., 2018) and a GWAS showing the importance of the *insulin receptor* (*INSR*) locus in the regulation of thyroid function (Porcu, et al., 2013). The latter study also identified an association between the *GLIS3* variant, rs1571583, and the regulation of thyroid dysfunction (Porcu, et al., 2013). Recent studies identified an association of several missense mutations in *GLIS3* with thyroid dysgenesis and dyshormonogenesis (de Filippis, et al., 2017; Teumer, et al, 2018; Yamaguchi, et al., 2020); however, their significance to congenital hypothyroidism was found to be uncertain.

3.2. Thyroid gland development and thyroid hormone biosynthesis

Thyroid organogenesis involves two distinct progenitors: thyroid progenitors in the pharyngeal floor of the endoderm, which generate the thyroid follicular cells, and neural crest cells that give rise to the parafollicular C cells (De Felice, Postiglione, & Di Lauro, 2004; Fernandez, Lopez-Marquez, & Santisteban, 2015; Mio, Grani, Durante, & Damante, 2020; Nilsson & Fagman, 2017). In mouse development, thyroid organogenesis, from the assembly of thyroid progenitors and the formation of the thyroid bud to the generation of the follicles (folliculogenesis), takes place between E8.5 and E15.5. Thyroid progenitors express the transcription factors paired box 8 (PAX8), thyroid transcription factor 1 and 2 (TTF1 and TTF2, also referred to as NKX2.1 and FOXE1), and hematopoietically expressed homeobox (HHEX), which play key roles in committing the cells to differentiate along the thyroid lineage (Fernandez, et al., 2015; Mio, et al., 2020; Persani, et al., 2018). Deficiency in any of these transcription factors leads to impaired thyroid development and thyroid hormone (TH)

biosynthesis. Folliculogenesis is characterized by the formation of the thyroid follicles and the expression of genes required for TH biosynthesis. Thyroglobulin is one of the first genes induced, while several other TH biosynthetic genes, including the iodide transporter *Nis,* become expressed and TH synthesis is initiated by the end of folliculogenesis (embryonic day E15.5).

After their synthesis by thyroid follicular cells, triiodothyronine (T3) and thyroxine (T4) are released into the blood stream and delivered to target cells where they regulate gene expression in various organs, including several neural, endocrine, and metabolic tissues, as well as heart (Mullur, Liu, & Brent, 2014; Rutigliano & Zucchi, 2017; Sinha, Singh, & Yen, 2018; Stepien & Huttner, 2019). The transcriptional regulation of target genes is mediated largely through the binding of T3 to thyroid hormone receptors (TRs). THs are critical in the regulation a wide range of biological processes, including cardiovascular functions, growth, thermogenesis, and neural development. Loss of TH production leads to hypothyroidism. CH is the most common neonatal endocrine disorder that, without proper treatment, causes severe neurological abnormalities in infants and children (Peters, van Trotsenburg, & Schoenmakers, 2018; Wassner, 2017). Defects in thyroid gland development (thyroid dysgenesis) are the major cause of CH and are due to mutations in transcription factor genes critical for thyroid development, including *PAX8, NKX2.1, FOXE1*, and *HHEX* (Fernandez, et al., 2015; Mio, et al., 2020; Nilsson & Fagman, 2017; Persani, et al., 2018). About 15% of CH cases are related to impairments in TH production (dyshormonogenesis)(Grasberger & Refetoff, 2011).

TH biosynthesis consists of a multistep process in which iodide, an essential element provided by the diet, is first taken up by thyroid follicular cells from the blood stream via the Sodium/Iodide Symporter (NIS, encoded by *SLC5A5*) located on the basolateral membrane. Iodine is then transported into the lumen of thyroid follicles by Pendrin (PDS, encoded by *SLC26A4*) and Anoctamin 1 (*ANO1*), which are located on the apical membrane (Braun & Schweizer, 2018; Ravera, Reyna-Neyra, Ferrandino, Amzel, & Carrasco, 2017)(Fig. 2). Subsequent oxidation of iodide is catalyzed by thyroid peroxidase (TPO) using H_2O_2 produced by the dual oxidase (DUOX) enzymes, DUOX2, DUOX1, DUOXA1, and DUOXA2 (Citterio, Targovnik, & Arvan, 2019; Grasberger & Refetoff, 2011). TH synthesis starts in the lumen of thyroid follicles with the iodination of tyrosine residues in thyroglobulin (TG). Iodination of multiple tyrosine residues in TG leads to the formation of mono-iodotyrosine (MIT) and di-iodotyrosine (DIT), which then undergo coupling reactions required for T3/T4 synthesis. The iodinated thyroglobulin is then internalized by the follicular cells via endocytosis and T3/T4 dissociated from thyroglobulin by lysosomal degradation. The free T3 and T4 are then released into the blood stream through the monocarboxylate transporters 8 (MCT8) and 10 (MCT10). Iodide is released from uncoupled MIT and DIT by iodotyrosine dehalogenase (IYD1) and recycled. Mutations in genes related to TSH signaling and TH biosynthesis, including *TSHR, NIS, PDS, TPO, TG*, and *DUOXA2*, are associated with thyroid dyshormonogenesis and congenital hypothyroidism in humans (Grasberger & Refetoff, 2011; Persani, et al., 2018; Targovnik, Citterio, & Rivolta, 2017; Kus, Chaker, Teumer, Peeters, & Medici, 2020). Defects in iodide transport resulting from inactivating mutations in *NIS* or *PDS* cause deficient iodide accumulation in the lumen of the follicles leading to dyshormonogenesis and congenital

hypothyroidism (Banghova, et al., 2008; Ishii, et al., 2019; Levy, Ginter, De la Vieja, Levy, & Carrasco, 1998; Martin, et al., 2019; Pohlenz, et al., 1998).

Circulating TH levels are under the control of a negative feedback loop referred to as the hypothalamus-pituitary-thyroid (HPT) axis (Joseph-Bravo, Jaimes-Hoy, & Charli, 2016). Insufficient TH levels induce the production of thyrotropin releasing hormone (TRH) in the hypothalamus and thyroid stimulating hormone (TSH) in the pituitary gland. TSH then binds the TSH receptor (TSHR), a guanine nucleotide-binding (G) protein-coupled receptor on thyroid follicular cells, that subsequently activates different G protein subtypes and signaling pathways leading to increased expression of several TH biosynthetic genes (Citterio, et al., 2019; Kleinau, Neumann, Gruters, Krude, & Biebermann, 2013). Chronic hypothyroidism, as caused by low iodide diet (LID) for example, is associated with a greatly elevated blood TSH level that significantly enhances the expression of TH biosynthetic and cell cycle genes, leading to increased thyroid follicular cell proliferation and ultimately the development of goiter (Zimmermann & Boelaert, 2015).

3.3. Thyroid development, hypothyroidism and Glis3 expression in mice

Study of *Glis3* knockout mice (*Glis3KO*) showed that they exhibit phenotypes very similar to those observed in human NDH patients, including congenital hypothyroidism (Kang, Beak, Kim, Herbert, & Jetten, 2009; Kang, Kim, et al., 2009; Kang, et al., 2017; Watanabe, et al., 2009; Yang, et al., 2011). Levels of T3 and T4 in the thyroid and blood are greatly reduced in both one-month old male and female *Glis3KO* mice compared to wild type mice, while blood levels of TSH are greatly increased. However, at postnatal day 7 (PND7) circulating T4 was not significantly different between WT and *Glis3KO* mice. This provided the first indication that *Glis3* plays an important role in postnatal but not embryonic thyroid development. Although little morphological difference was observed between thyroid glands of wild type and *Glis3KO* mice at postnatal day 1 (PND1), at PND7 and beyond the size of the thyroid and follicles in *Glis3KO* mice was significantly smaller than in wild type mice (Kang, et al., 2017; Watanabe, et al., 2009). These observations support the hypothesis that GLIS3 does not play a major role in the regulation of embryonic thyroid development, including formation of the thyroid primordium, migration, bifurcation of the thyroid primordium, and folliculogenesis (Kang, et al., 2017). This conclusion is consistent with findings showing that unlike the transcription factors PAX8, FOXE1, and NKX2.1, which are critical for thyroid development, GLIS3 protein was not expressed during early thyroid gland development, but was first observed in follicles at E17.5 (unpublished). Moreover, at E15.5 the expression of NKX2.1 and PAX8 was not affected in *Glis3KO* thyroids, although at PND28 *Pax8* expression was significantly higher in *Glis3KO* mice (Kang, et al., 2017). Together these observations suggested that the development of hypothyroidism in *Glis3*-deficient mice is not due to thyroid dysgenesis, but to thyroid dyshormonogenesis. The latter was supported by histochemical analysis showing that the expression of several proteins related to TH biosynthesis, including NIS, was significantly reduced in *Glis3KO* thyroid (Kang, et al., 2017).

3.4. Thyroid development, hypothyroidism and glis3 expression in zebrafish

The observed thyroid phenotype in mice is very different from that in *glis3*-deficient zebrafish (Kang, et al., 2017; Rurale, Marelli, Duminuco, & Persani, 2020). *glis3* morphants, in which *glis3* was knocked down by microinjection of *glis3* morpholinos in one- or two-cell stage embryos, exhibited reduced expression of *nkx2.4* and *pax2a* in the thyroid primordium. The reduced expression of *nxk2.4* and *pax2a* led to thyroid dysgenesis in *glis3* morphants. The number of T4-positive cells was also significantly decreased in *glis3* morphants at 5 days post fertilization (dpf) compared to control embryos. This appears to be a consequence of impairments in early thyroid development as well as a reduction in the expression of thyroid functional genes, such as *tg* and *nis,* in *glis3* morphants. The discrepancy in thyroid phenotype between murine and zebrafish models could be due to the differences in the temporal expression of *Glis3* during embryonic development. In zebrafish, *glis3* is expressed in the pharyngeal endoderm earlier than *nxk2.4* and *pax2a* (Rurale, et al., 2020; Rurale, Persani, & Marelli, 2018), whereas during murine thyroid development GLIS3 protein was detectable in thyroid follicular cells no earlier than E17.5. Another apparent difference in *Glis3* mRNA expression between mouse and zebrafish thyroid glands is in adults. In zebrafish, *glis3* mRNA is no longer expressed in mature thyroid gland, while GLIS3 mRNA remains highly expressed in mature murine thyroid (Kang, et al., 2017; Rurale, et al., 2020). These observations indicate that *Glis3* expression in mouse and zebrafish thyroid is regulated very differently.

3.5. Regulation of TH biosynthesis by GLIS3

Analysis of the gene expression profiles of thyroids from *Glis3KO* and wild type mice revealed that several TH biosynthesis associated genes were expressed at a significantly reduced level in knockout mice, suggesting a role for GLIS3 in the regulation of TH biosynthesis (Fig. 2) (Kang, et al., 2017). Differences in gene expression between *Glis3KO* and WT thyroids were much more pronounced in mice fed low iodide diet (LID) than a normal diet (ND). In wild type mice fed a LID, the highly elevated level of TSH greatly induced the expression of a number of TH biosynthesis-related genes, including *Pds*, *Nis*, *Duoxa2*, *Mtc8,* and *Tpo.* In contrast, although blood TSH levels were equally elevated in *Glis3KO* mice fed LID, the expression of several TH biosynthesis associated genes, including the iodide transporters *Nis* and *Pds*, were not induced, while the induction of some TH biosynthetic genes was moderately inhibited or not affected. These observations indicate that GLIS3 selectively regulates a subset of TH biosynthetic genes, particularly *Nis* and *Pds*.

These studies further indicate that gene regulation downstream of TSH/TSHR is impaired in *Glis3KO* thyroid follicular cells, suggesting that GLIS3 functions as a downstream mediator of TSH/TSHR signaling. The regulation of TH biosynthetic genes by TSH is believed to be mediated through a TSHR-coupled $G_{\alpha s}$ protein that catalyzes the formation of cAMP by adenyl cyclase leading to the subsequent activation of several kinase pathways (Fig. 2). Activation of cAMP-dependent protein kinase A (PKA) and possibly other kinases may then promote the transcriptional activity of transcription factors that play a critical role in the regulation of TH biosynthesis-related genes, such as GLIS3. This activation might involve phosphorylation of these factors and promote the recruitment of coactivators and/or increase protein stability.

Cistrome analysis revealed that GLIS3 was bound to several TH biosynthesis associated genes that were down-regulated in *Glis3KO* thyroids, including *Nis*, *Pds*, *Duoxa2*, and *Mct8* (Kang, et al., 2017). These findings are consistent with the hypothesis that GLIS3 controls TH biosynthesis by directly regulating the transcription of several TH biosynthetic genes (Fig. 2). *De novo* motif analysis identified CCTGGGA/GGG as the *in vivo* GLISBS consensus, a sequence very similar to GLISBS motifs found in other cell types (Beak, et al., 2008; Jeon, et al., 2019; Scoville, et al., 2019). Motif analysis identified, in addition to the GLISBS consensus, consensus binding motifs for PAX8 and NKX2.1, transcription factors with critical roles in the control of thyroid development and TH biosynthesis (Fernandez, et al., 2015; Mio, et al., 2020; Persani, et al., 2018). Cistrome analysis of PAX8 and NKX2.1 binding sites showed a substantial overlap between the binding of these transcription factors and that of GLIS3, particularly at regulatory regions of TH biosynthesis associated genes, such as *Nis*, *Pds*, *Tpo*, *Tg*, and *Duoxa2* (Kang, et al., 2017)(unpublished). And although PAX8 and NKX2.1 still associate with these target genes in the absence of GLIS3, they are insufficient to strongly activate TSH-induced transcription of these genes without GLIS3 (Kang, et al., 2017). Together, these observations suggest that GLIS3 regulates TH biosynthesis in coordination with other thyroid transcription factors, including PAX8 and NKX2.1 (Fig. 2).

3.6. Regulation of thyroid follicular cell proliferation

The size of the thyroid follicles and the thyroid gland was significantly smaller in *Glis3KO* mice that could be due to increased apoptosis and/or reduced cell proliferation (Kang, et al., 2017; Watanabe, et al., 2009). However, no increase in apoptosis was observed, suggesting that GLIS3 regulates the proliferation of thyroid follicular cells. The latter is consistent with the lower percentage of $EdU^{+}PAX8^{+}$ thyroid follicular cells in *Glis3KO* mice. These differences were particularly apparent in mice fed a LID. Under this condition, the highly elevated level of TSH greatly stimulates thyroid follicular cell proliferation in wild type mice, but not in *Glis3KO* thyroid. In wild type thyroid, this is accompanied by increased expression of several cell cycle genes, including cyclin B1 (*Ccnb1*), cyclin B2 (*Ccnb2*), centromere protein F (*CenpF*), cyclin A2 *(Ccna2*), cell division cycle associated 2 (*Cdca2*), and Baculoviral IAP repeat-containing 5 (*Birc5*) (Kang, et al., 2017). In contrast, many of these genes were not induced in the thyroid of *Glis3KO* mice fed a LID. Cistrome analysis indicated that several cell cycle genes, such as *Cdca2, Cdc6*, and *Ccnd2,* are direct transcriptional targets of GLIS3. These data support the conclusion that in addition to regulating TH biosynthesis, GLIS3 plays a role in the control of thyroid follicular cell proliferation.

Thyroid follicular cell proliferation is controlled by TSH and a number of growth factors. The stimulation of thyroid follicular cell proliferation by TSH/TSHR is believed to involve activation of $G_{\alpha q/11}$, which stimulates phospholipase C-mediated generation of diacylglycerol and inositol-1,4,5-trisphosphate (PI3) leading to the subsequent activation of protein kinase C (PKC)/MAPK and PI3 kinase (PI3K)/mTOR pathways (Fig. 2) (Brewer, Yeager, & Di Cristofano, 2007; Citterio, et al., 2019; Kero, et al., 2007; Vella & Malaguarnera, 2018). TSH and various growth factors stimulate thyroid follicular cell proliferation mainly through activation of the mTOR signaling pathway and the subsequent

increased expression of cell cycle genes (Blenis, 2017; Brewer, et al., 2007). Elevated TSH levels activated the mTOR signaling pathway in the thyroid gland of wild type mice, as indicated by the phosphorylation of ribosomal protein S6 (RPS6), but not in *Glis3KO* mice (Kang, et al., 2017). Thus, the suppression of cell proliferation in *Glis3KO* thyroid is at least in part due to an impairment in mTOR pathway activation.

3.7. GLIS3 rearrangements in Hyalinizing Trabecular Tumor (HTT)

Recently, two independent studies implicated interchromosomal rearrangements between *GLIS3* and *PAX8* in a rare thyroid neoplasm referred to as hyalinizing trabecular tumor (HTT), a tumor type that originates from thyroid follicular cells (Jetten, 2019; Marchio, et al., 2019; Nikiforova, et al., 2019). It involves rearrangement between exon 1–2 of *PAX8* located on chromosome 2q14.1 and exon 3–11 of *GLIS3* located on chromosome 9q24.2, that generates a transcript encoding a fusion protein consisting of the first 9 N-terminal amino acids of PAX8 and 800 amino acids at the C-terminus of GLIS3. Although the first 130 amino acids of GLIS3 are deleted, this fusion protein retains the HCR, DBD, and TAD of GLIS3 and therefore remains transcriptionally active. The expression of the fusion transcript is under the control of the *PAX8* regulatory region and causes a 6.8-fold higher expression of GLIS3 mRNA compared to normal tissue (Nikiforova, et al., 2019). The elevated GLIS3 expression stimulates cell proliferation and significantly increases the expression of several TH biosynthesis-related genes, including *NIS* and *MCT8*, as well as several extracellular matrix genes, such as *COL1A2*, *COL4A1*, and *COL5A2*. The latter is supported by histological analysis of HTT sections showing increased deposition of extracellular collagen (Marchio, et al., 2019; Nikiforova, et al., 2019). These observation are consistent with *Glis3KO* studies demonstrating that GLIS3 directly regulates the expression of a subset of cell cycle, extracellular matrix, and TH synthetic genes (Kang, et al., 2017). The role of the fusion protein in oncogenesis was supported by a recent study demonstrating that exogenous expression of the PAX8-GLIS3 fusion protein increases proliferation, migration, and clonogenic potential of the nonmalignant human thyroid cell line, Nthy-ori 3–1 (Basili, et al., 2020). In addition, these cells generated larger tumors xenografts. These effects appear to be mediated in part via the activation of the sonic hedgehog (SHH) pathway. Together, these studies suggest that the PAX8-GLIS3 fusion protein promotes oncogenesis by directly stimulating the transcription of cell cycle and extracellular matrix (ECM) genes.

4. Role of GLIS3 in pancreatic β cells and diabetes

4.1. GLIS3 plays a critical role in the development of diabetes in humans

Diabetes mellitus is a growing health problem, with the global incidence tripling from 151 million cases in 2000 to 463 million cases in 2019 (IDF, 2019). Diabetes is broadly classified into two types: Type 1 Diabetes (T1D) is characterized by autoimmune destruction of pancreatic β-cells, whereas Type 2 Diabetes (T2D) is characterized by peripheral insulin resistance and a subsequent inability of islet β-cells to secrete adequate insulin, and accounts for ~90% of diabetes cases ((ADA), 2015). In addition, impaired β-cell function can occur during pregnancy, termed gestational diabetes, which correlates with poorer health outcome for both mother and child. Diabetes is thought to be the result of both genetic and

environmental factors, with some genetic factors so critical that they can lead to neonatal diabetes or Maturity Onset Diabetes of the Young (MODY) ((ADA), 2015). Regardless of the exact factors involved, diabetes centrally revolves around an inability of the pancreas to secrete adequate insulin to regulate glycemia.

The pancreas itself is a multifunctional organ with both endocrine and exocrine functions (Gittes, 2009; Guney & Gannon, 2009; Murtaugh, 2007; Pan & Wright, 2011). The acinar cells form the exocrine compartment of the pancreas that produces digestive enzymes, which are secreted through the pancreatic ducts into the duodenum to aid in digestion. The endocrine function of the pancreas is carried out by the islets of Langerhans, which account for only ~5% of the pancreas and includes alpha, beta, delta, epsilon, and pp-cells. Pancreatic β cells, through their production and secretion of insulin, play a critical role in controlling blood glucose levels and the development of diabetes. In response to increased blood glucose levels, β cells secrete insulin, which signals to peripheral tissues to store glucose and prevent hyperglycemia. Loss or dysfunction of β-cells leads to insufficient secretion of insulin, and results in hyperglycemia and eventual diabetes (DiMeglio, Evans-Molina, & Oram, 2018; Letourneau & Greeley, 2018; Prentki & Nolan, 2006; Roden & Shulman, 2019). GLIS3 has been shown to play a central role in the development of islets, as well as the generation of functional β-cells and maintenance of their cellular identity, and is therefore a prime candidate gene for understanding the development and possible treatment of diabetes (Jetten, 2018; Kang, Kim, et al., 2009; Scoville, et al., 2019; Watanabe, et al., 2009; Yang, Bush, Wen, Cao, & Chan, 2017; Yang, Chang, & Chan, 2013).

As mentioned above, initial studies in humans linked deletions in *GLIS3* to a syndrome of neonatal diabetes and congenital hypothyroidism (NDH) (Senee, et al., 2006), with subsequent studies expanding on the number of observed deletions and reinforcing this association (Alghamdi, et al., 2017; Dimitri, et al., 2016; Dimitri, et al., 2015; Dimitri, et al., 2011; Fu, et al., 2017; Habeb, et al., 2012). The resulting phenotype was severe hyperglycemia observed within the first month of life, requiring treatment with exogenous insulin. GWAS in humans have found an association between common variants in *GLIS3* and T1D, T2D, and gestational diabetes, as well as various measures of β-cell function, such as fasting blood glucose or Homeostatic Model Assessment of β-cell function (HOMA-β, see Table 1). It is important to note that, while these GWAS span different global populations and individual Single Nucleotide Polymorphisms (SNPs), all of these SNPs reside within intronic regions of *GLIS3*. Observations showing that several islet transcription factors bind in close proximity to these intronic SNPs in both humans and mice (Fig. 3) are consistent with a potential cis-regulatory role for these intronic regions (Aylward, Chiou, Okino, Kadakia, & Gaulton, 2018; Ediger, et al., 2017). Combined, these studies indicate that GLIS3 expression and regulation is essential for proper β-cell development and postnatal function.

Not all GWAS have been able to establish a statistically significant link between individual SNPs in *GLIS3* and diabetes. For example, several groups reported that individual SNPs in *GLIS3* (such as rs7034200 or rs7041847 for T2D, and rs7020673 or rs10758593 for T1D) lack statistical association with T1D or T2D alone, but were associated with T1D or T2D when grouped with up to 9 other SNPs (Duarte, Assmann, Dieter, de Souza, & Crispim,

2017; Frohnert, et al., 2018; Fujita, et al., 2012; Sakai, et al., 2013; Winkler, et al., 2014). Other studies found an association with fasting insulin levels (rs7034200) or production of islet autoantibodies (rs7020673) (Florez, et al., 2012; Steck, et al., 2014). Two additional studies focused on rare missense variants of *GLIS3* in patients with MODY and T2D (Shakhtshneider, et al., 2018; Sun, et al., 2019). Shakhtshneider *et al.* lacked a sufficient sample size to draw firm conclusions, but Sun *et al.* found that patients with T2D are more likely to have at least one of 79 rare variants than non-diabetic individuals. While these genetic studies have yielded some knowledge about the association of GLIS3 variants and T2D in humans, it also shows the shortcomings of studying rare variants and further highlights the importance of developing more targeted model systems, such as mice.

4.2. Human GLIS3 likely has one functional isoform

It should be noted here that when *GLIS3* was initially studied in humans, at least three isoforms were detected via northern blot analysis of multiple tissues (Senee, et al., 2006). These included a ~7.5kb long isoform in kidney, pancreas, and thyroid, as well as shorter 0.8 and 2.0kb isoforms observed in heart, liver, skeletal muscle, and adrenal gland. Separately, *in silico* analysis identified 2 isoforms, referenced by Wen and Yang as isoforms A and B, with the long isoform (A) corresponding to the ~7.5kb mRNA observed by Senee *et al.* However, it is important to note that isoform B is encoded by a ~6.7kb mRNA, and thus does not correspond to the ~0.8–2.0kb bands seen in Senee *et al.* We feel this is an especially important point to raise as several publications have conflated the isoforms identified in these publications. The existence of isoform B is not strongly supported by the Northern blot analysis from Senee *et al*, and while ENSEMBL does annotate several transcripts <2KB in length, none of them contain the DNA binding domain of GLIS3. While it is possible that these shorter isoforms could play some dominant-negative role, we consider it much more likely that only one functional isoform of GLIS3 exists in humans (at least in kidney, pancreas, and thyroid), which produces a 930 amino acid protein.

4.3. Pancreas development and Glis3 expression in mice

The mouse model provides an excellent system to dissect the molecular relationships between GLIS3, regulation of pancreatic β cell functions, and diabetes. In mice, after initial formation of dorsal and ventral pancreatic buds, the pancreas undergoes branching morphogenesis, producing “tip” and “trunk” domains (Pan & Wright, 2011; Villasenor, Chong, Henkemeyer, & Cleaver, 2010). The tip domains contain both multipotent progenitor cells, which express low levels of PTF1A, PDX1, SOX9, and HNF1β, as well as NKX6.1, GATA4, and NR5A2, and cells committed to the acinar cell lineage, in which the expression of PDX1, SOX9, HNF1β, and NKX6.1 becomes repressed and expression of PTF1A is highly induced. In contrast, the trunk domains consist of bipotent progenitors, which are capable of differentiating along the endocrine or ductal lineages and activate high expression of SOX9 and HNF1β. *Glis3* mRNA is first detected in the pancreas starting at around E11.5–12.5 (Kang, Kim, et al., 2009), but protein is not detectable until E13.5 (Kang, Takeda, Jeon, & Jetten, 2016), or roughly the time period where branching domains become well established. GLIS3 expression is restricted to the bipotent progenitor cells, as established by the absence of $GLIS3^{+}PTF1A^{+}$ cells and the presence of $GLIS3^{+}SOX9^{+}$ cells (Kang, et al., 2016).

Bipotent cell commitment to either a ductal or endocrine lineage appears to be largely driven by Notch signaling (Shih, et al., 2012). High levels of Notch signaling activate SOX9 and HES1, both markers of the ductal lineage. However, lower levels of Notch signaling appear to only activate SOX9, which in turn activates the expression of neurogenin 3 (NGN3), a critical component of endocrine lineage determination. Mice lacking *Ngn3* fail to develop functional endocrine cells (Gradwohl, Dierich, LeMeur, & Guillemot, 2000). In order to commit to the endocrine lineage, cells must subsequently turn off SOX9 expression. GLIS3 expression is maintained when bipotent progenitors differentiate into either the ductal lineage or endocrine progenitor cells. GLIS3, presumably together with SOX9, is thought to promote the differentiation of bipotent progenitors into the proendocrine lineage by directly regulating *Ngn3* expression (Kim, et al., 2012). The fact that GLIS3 expression is maintained during this differentiation likely indicates that GLIS3 is not a target of Notch signaling, although it cannot be ruled out that Notch regulates GLIS3 activity through posttranslational modifications. The differentiation of proendocrine cells into the various hormone-positive lineages (α-, β-, δ-, ε-, and pp-cells) is under the control of distinct transcription factors that are induced, repressed, or remain expressed. During endocrine lineage determination GLIS3 expression becomes restricted to the β- and pp-cells (Kang, et al., 2016).

This expression pattern correlates well with the observed phenotypes of various mouse models deficient in *Glis3* function. Mice in which *Glis3* is globally deleted have reduced numbers of all hormone-positive endocrine cells (Kang, Kim, et al., 2009; Watanabe, et al., 2009; Yang, et al., 2011), likely due to reduced *Ngn3* expression (Kim, et al., 2012) that subsequently leads to the generation of fewer proendocrine cells. In contrast, postnatal deletion of *Glis3* results in a reduction in insulin expression, but does not appear to affect glucagon expression (Yang, et al., 2017), reflecting the selective expression of GLIS3 in β-cells, but not α-cells. Unlike its role in endocrine cells, loss of GLIS3 in pancreatic ducts causes dilation of the ducts similar to the polycystic phenotype observed in the kidneys of these mice (Kang, Beak, Kim, Herbert, & Jetten, 2009; Kang, Kim, et al., 2009; Scoville, et al., 2019). This suggests that in the pancreas, GLIS3 exhibits multiple, cell type-specific functions, including a critical role in the development and maintenance of β-cells. In order to understand the relationship between GLIS3 and diabetes established in the GWAS studies and mouse models, it is essential to obtain a deeper understanding of the mechanisms by which GLIS3 regulates β-cell functions.

4.4. GLIS3 role in β cell dysfunction versus apoptosis

Initial studies in ubiquitous *Glis3* knockout mice established that key markers of endocrine cells, such as *Ngn3* and *Pax4*, or the hormones themselves (*Ins2*, *Gcg*, *Sst*), were greatly reduced in the knockout mice compared to control littermates (Kang, et al., 2009; Watanabe, et al., 2009; Yang, et al., 2011). This was hypothesized to be the result of a failure of proper differentiation, as cell death was not observed (Kang, Kim, et al., 2009; Yang, et al., 2011). This observation appears to be consistent with studies of the thyroid gland and kidney, which did not observe increased cell death in thyroid follicular cells or renal tubules in *Glis3*-deficient mouse or zebrafish models (Kang, et al., 2017; Rurale, et al., 2020). Pancreas-specific deletion of *Glis3* using *Pdx1-Cre* mice further confirmed downregulation of a

variety of genes involved in β-cell function and identity as well as absence of increased cell death in pancreatic islets (Scoville, et al., 2019). It is therefore likely that a lack of GLIS3 during development prevents endocrine progenitor cells from differentiating into functional β-cells, leaving behind improperly differentiated cells, halting differentiation and growth entirely, or potentially shifting endocrine development towards other cell fates.

Interestingly, postnatal deletion of *Glis3* in adult β-cells using a *Pdx1-Cre*$^{ERT+}$ mouse line did lead to increased cell death in islet β-cells (Y. Yang, et al., 2013); however, this cell death only occurs following hyperglycemia, a phenotype which causes apoptosis via glucotoxicity (Poitout & Robertson, 2008). This is similar to the enhanced apoptosis observed in models of islet stress in response to either high fat diet (Yang, et al., 2013) or unfolded protein response (Dooley, et al., 2016) within β-cells in a *Glis3* heterozygous background. The relationship between GLIS3 and apoptosis is further complicated by reports in rat β-cells and cultured human islets, which showed an increase in cell death in response to siRNA-mediated *GLIS3* knockdown (Calderari, et al., 2018; Juan-Mateu, et al., 2018; Nogueira, et al., 2013). One proposed mechanism for this increased cell death is impaired autophagy, through GLIS3 regulation of a variety of autophagy related genes (Calderari, et al., 2018). However, the evidence provided to support this is not convincing, as the changes in LC-I and LC-II protein levels are very modest, and much of the gene regulation mentioned is not observed in mouse models (Scoville, et al., 2019). A more likely mechanism involves upregulation of the short isoform of BCL-2 interacting protein (BIM, also named BCL2L11)(Nogueira, et al., 2013), which drives the mitochondrial-associated apoptotic pathway in response to cytokines and glucotoxicity (Barthson, et al., 2011; McKenzie, et al., 2010; Santin, et al., 2011). Nogueira et al. proposed that the lower levels of SRSF6 (also known as SRP55), observed in cells in which GLIS3 is downregulated, leads to an increase in the BIM short isoform. This proposed mechanism is different than what has been reported previously, where an increase in SRSF6 levels correlated with an increase in production of the more pro-apoptotic BIM short isoform (Jiang, et al., 2010; Lai, Jiang, Farrelly, Zhang, & Hersey, 2012).

An additional study in human embryonic stem cells (hESCs), utilizing a CRISPR knockout strategy to produce *GLIS3*$^{-/-}$ cells (Zhu, et al., 2016), also observed increased apoptosis upon terminal differentiation into monohormonal β-cells in the absence of GLIS3 (Amin, et al., 2018). However, the authors did not observe a decrease in *SRSF6* expression in RNA-seq data from *GLIS3*$^{-/-}$ cells. Instead, they observed an increase in transforming growth factor β (TGFβ) signaling pathway components, such as *TGFB2*, *TGFB3*, and *TGFBR2*, that led to caspase 3 cleavage and SMAD2 phosphorylation (Amin, et al., 2018). Taken together, this indicates that in certain contexts, the absence of GLIS3 might promote apoptosis through the TGFβ pathway, which leads to phosphorylation of SMAD2 and increased production of BIM.

It should be noted here that neither effects on *Srsf6* or *Bcl2l11* (i.e. BIM) expression, nor TGFβ signaling pathways, were observed in RNA-seq data from pancreas-specific deletions of *Glis3* (Scoville, et al., 2019). These islets were collected at a timepoint prior to the onset of severe hyperglycemia, highlighting a role of possible additional stressors in the link between GLIS3 deficiency and apoptosis. These findings are consistent with earlier

mentioned studies of *Glis3* heterozygous mice exposed to high fat diet or unfolded protein stress (through impaired *Manf* induction) within the islet (Yang, et al., 2013, Dooley, 2016 #1327). Taken together, these studies provide evidence that external stressors, such as glucolipotoxicity or cytokine signaling, may stimulate cell death through SRP55 or TGFβ-mediated stimulation of BIM production, and that GLIS3 may act as a repressor of these pathways. However, further research is needed to clarify what role reduced or lost GLIS3 expression plays in cell death/dysfunction, and how this connects to the pathophysiology of T1D and T2D.

4.5. GLIS3 connection with WNT signaling and primary cilia

GLIS3 is expressed not only in the pancreatic endocrine progenitor cells and β-cells, but also in both pancreatic ductal progenitors and mature pancreatic ductal cells (Kang, et al., 2016). Consequently, GLIS3 is also present in pancreatic colony forming units (PCFUs), colonies formed by $CD133^{high}/CD71^{low}$ pancreatic ductal cells isolated from adult mice (Tremblay, Lopez, & Ku, 2019). PCFUs have self-renewal capacity and the capability to differentiate into endocrine- and acinar-like cells in culture, and appear to function as potential pancreatic progenitor-like cells, at least *in vitro* (Jin, et al., 2016). In order to promote their expansion, PCFUs are treated with a potent WNT agonist, R- spondin-1 (RSPO1). Interestingly, in cells where *Glis3* expression has been depleted via shRNA, self-renewal is inhibited in spite of the presence of RPSO1. Furthermore, GLIS3 does not appear to affect the activation of the WNT receptor LRP6, but controls expression of *Lrp6* and other WNT pathway components (Tremblay, et al., 2019). In mouse islets, GLIS3 appears to directly control expression of *Wnt4*, indicating a potential role in controlling WNT signaling within islet β-cells. We obtained similar results in neural cell differentiation of hESCs, with GLIS3 directly regulating the expression of *WNT3A* and other WNT pathway genes, leading to a stimulation of the WNT signaling pathway (Jeon, et al., 2019). This, together with the reported functions of WNT signaling pathways in the control of different stages of pancreatic development (Scheibner, Bakhti, Bastidas-Ponce, & Lickert, 2019), raises the possibility of a connection between GLIS3 and WNT signaling in the regulation of endocrine and ductal lineages.

As mentioned in the introduction, GLIS3 has been detected in the primary cilium, which functions as critical signaling hub (Kang, Beak, Kim, Herbert, & Jetten, 2009; Mick, et al., 2015) and is present in most cell types in which GLIS3 is expressed. For example, pancreatic ductal and endocrine cells contain a primary cilium, whereas acinar cells, which lack GLIS3 expression, do not (Lodh, O'Hare, & Zaghloul, 2014). This observation raises the question of whether there is a relationship between regulation of GLIS3 activity and function in these cells and its ciliary localization, and whether GLIS3 plays an important role in conveying primary ciliary signaling to the nucleus for transcriptional regulation (Jetten, 2018). Several studies have revealed a function for ciliary signaling in the regulation of pancreatic endocrine cell functions and a connection with diabetes (Lodh, 2019). Primary cilia are required for the formation of functional β- cells and loss of cilia postnatally leads to β-cell dysfunction (Gerdes, Davis, & Katsanis, 2009; Volta, et al., 2019). Additionally, many ciliary genes are reduced in T2D, which may inhibit the compensatory proliferation of β-cells in response to insulin resistance (Kluth, et al., 2019). However, the specific signaling

pathways involved in these processes remain mostly undetermined. TGFβ signaling through SMAD phosphorylation is thought to take place in the primary cilia (Clement, et al., 2013), and pancreatic cells that lack cilia have increased WNT signaling (Cano, Murcia, Pazour, & Hebrok, 2004), indicating that these pathways previously identified as targets of GLIS3 are also connected to the primary cilia. This highlights the critical importance of obtaining a better understanding of the upstream signaling pathways that regulate GLIS3 activation as well their connections to primary ciliary signaling.

4.6. GLIS3 regulates genes critical to β-cell function

Within the pancreatic β-cell, GLIS3 acts as a transcriptional activator and repressor to regulate a variety of genes and pathways, many with a direct role in insulin secretion. Indeed, the *Ins2* gene (one of the two genes that encode insulin protein in mice) was the first direct target of GLIS3 identified in β-cells (Kang, Kim, et al., 2009; Yang, Chang, Samson, Li, & Chan, 2009; ZeRuth, et al., 2013). Although loss of *Glis3* does not appear to induce an inactive chromatin conformation at the *Ins2* locus in postnatal mouse pancreas, a recent unpublished study showed that GLIS3 may play a pioneering role in binding the *INS* promoter in human pancreatic β cells as well as non-pancreatic cells (Akerman, et al., 2020). Subsequent studies also identified *Ngn3*, a key marker of progenitor endocrine cells, as a direct target of GLIS3 (Kim, et al., 2012; Yang, et al., 2011). More recently, RNA-seq analysis of islets from a pancreas-wide deletion of *Glis3* combined with ChIP-seq analysis of GLIS3 binding in islets has identified ~1,500 potential direct target genes (Scoville, et al., 2019). Among these targets were several genes with critical functions in β-cell development (*Nkx6.1*, *Mafa*) and function (*Ins1/2*, *Slc2a2*, *Glp1r*, *Chga*). Other gene pathways regulated included various metabolic pathways, regulation of lipolysis, and other signaling pathways.

Interestingly, the regulatory regions of many of these genes with a direct role in β-cell development, maintenance, and function are bound not only by GLIS3, but by other islet-enriched transcription factors (Fig. 4)(Khoo, et al., 2012; Raum, et al., 2010; Pasquali et al 2014; Ediger, et al., 2017; Miguel-Escalada, et al., 2018; Scoville, et al., 2019). Further evidence suggests that GLIS3 interacts with some of these transcription factors, such as PDX1, NEUROD1, and MAFA (Yang, et al., 2009). Together, these findings indicate that there is some level of coordination between various transcription factors to properly regulate critical β-cell genes via their binding within close proximity to each other (Fig. 4) (Scoville, et al., 2019). Whether GLIS3 is required for or stabilizes the binding of these factors, or is necessary for the recruitment of co-regulators, remains to be determined. However, considering the significant impact GLIS3 deficiency has on β-cell generation and function, it is likely that GLIS3 plays some non-redundant role in transcriptional activation. This is supported by a recent study (Akerman, et al., 2020) showing that a GLIS3-bound regulatory region of the *INS* promoter was essential for transitioning from a poised chromatin state to an active chromatin state. Future studies should seek to address the relationship between loss of GLIS3 and chromatin architecture, both in mature and developing β-cells, on *INS* and other β cell genes.

It should be noted that several additional genes have been proposed to be regulated by GLIS3. For example, some authors report regulation of *Srp55*, *Bcl2l11*, or TGFβ pathway

components, while others do not (Amin, et al., 2018; Calderari, et al., 2018; Juan-Mateu, et al., 2018; Nogueira, et al., 2013; Scoville, et al., 2019). We believe that cellular stress is a key requirement for such observations. Therefore, findings with regard to GLIS3 function and gene regulation should be carefully considered in the context of the model system, and what type of stress that system is under. This also highlights how the mouse model remains a critical system for testing *in vivo* function in the absence of many of the exogenous stressors inherent to cell line systems. In future studies, both approaches will likely yield useful information, with studies of GLIS3 deficiency in the absence of cell stress giving important mechanistic information on β- cell development and function, while studies in the presence of cell stress represent a model of pathophysiology with potential similarities to the complex balance of genetic and environmental factors leading to T1D/T2D.

5. Conclusions and potential therapeutic strategies

GLIS3 is specifically expressed in thyroid follicular cells and pancreatic β-cells, where it regulates TH biosynthesis and cell proliferation or β-cell identity, respectively. Deficiency in *GLIS3* in humans causes congenital hypothyroidism and neonatal diabetes, while GLIS3 overexpression is associated with the development of a thyroid follicular cell-related cancer, HTT (Jetten, 2018; Nikiforova, et al., 2019). Additionally, GWAS have linked *GLIS3* to both Type 1 and Type 2 diabetes (Table 1), as well as the regulation of thyroid dysfunction (Porcu, et al., 2013). Moreover, studies with *Glis3*$^{+/-}$ mice revealed that these mice are more sensitive to developing diabetes in response to high fat diet and endoplasmic reticulum stress (Dooley, et al., 2016; Yang, et al., 2013). This sensitization to stress is likely related to reduced *Glis3* expression, which might also pertain to the association of certain variants within *GLIS3* introns (therefore likely affecting GLIS3 expression levels) with increased risk of diabetes (Fig. 3). Stress response-induced apoptosis was also observed in a differentiation model of *GLIS3*$^{-/-}$ hESCs into β-cells (Amin, et al., 2018), in which treatment with the TGFβR1 kinase inhibitor galunisertib significantly reduced apoptosis. Although these studies suggest that GLIS3 has a role in regulating stress, the mechanisms underlying the relationship between GLIS3, cellular stress, apoptosis, and diabetes remain largely unknown. Thus, enhancing GLIS3 activity may assist in restoring β-cell function in diabetic patients and may help patients with other GLIS3-related pathologies. To promote the development of new therapeutic approaches, it will therefore be of vital importance to identify GLIS3 regulatory pathways and obtain insights particularly into the upstream signaling pathways that regulate GLIS3 expression and transcriptional activity.

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Abbreviations:

GLIS	GLI-Similar
ZF	zinc finger
DBD	DNA binding domain

TAD	transactivation domain
TH	thyroid hormone
GLISBS	GLIS binding site
TSH	thyroid stimulating hormone
TSHR	TSH receptor
T2D	type 2 diabetes
NIS	sodium iodide symporter
SNP	single nucleotide polymorphism
GWAS	genome wide association studies

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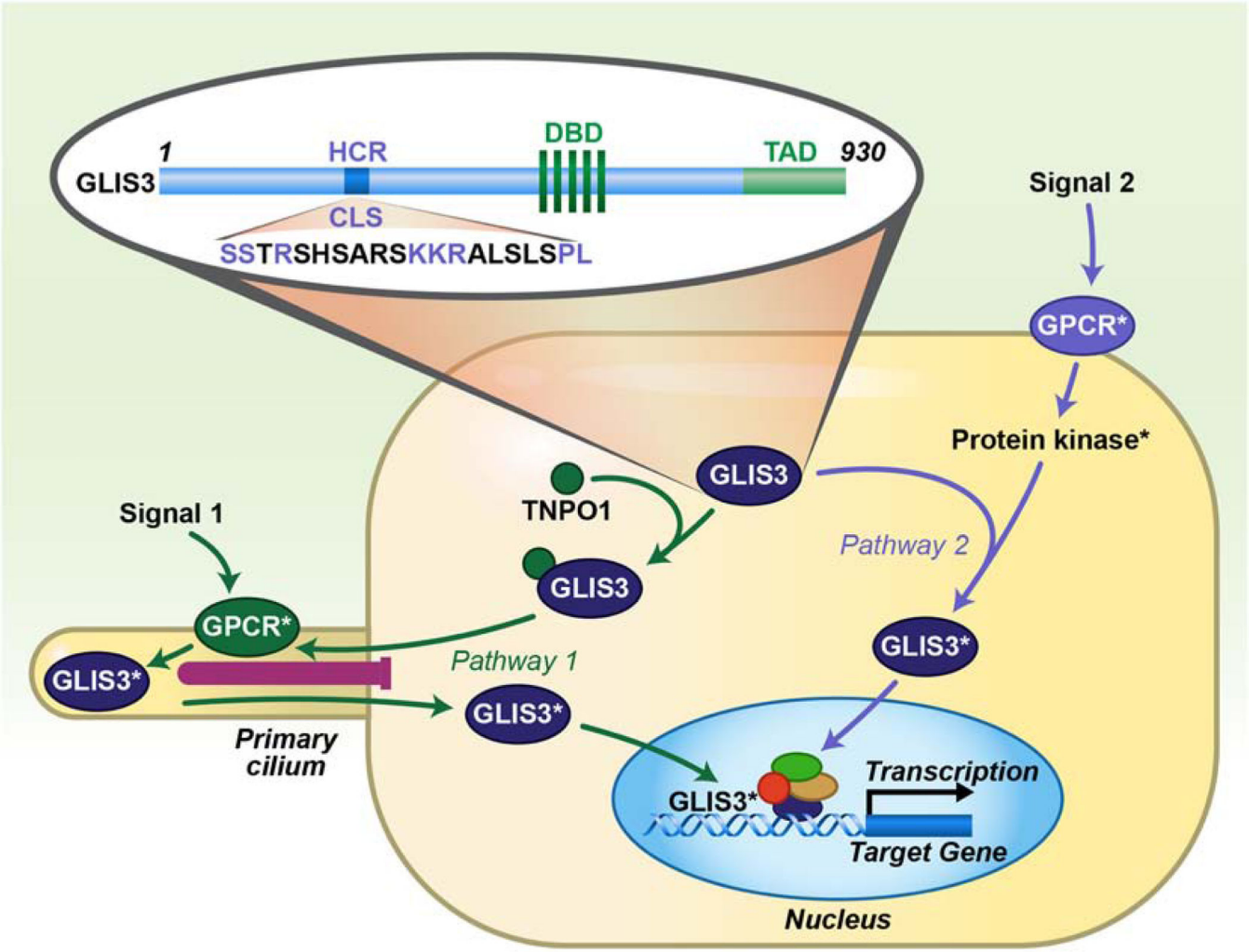

Fig. 1.

Structure and function of human GLIS3 protein. The DNA binding domain (DBD) containing five C_2H_2 zinc finger motifs, the transactivation domain (TAD), and the highly conserved region (HCR) are indicated. The HCR contains a ciliary localization signal (CLS) that interacts with TNPO1 and is believed to facilitate GLIS3 entry into the primary cilium. GLIS3 transcriptional activity is regulated by multiple upstream pathways, including primary cilium-associated and primary cilium-independent pathways (Pathways 1 and 2, respectively), through the activation of different G protein-coupled receptors (GPCRs) by their respective external signals and downstream protein kinases.

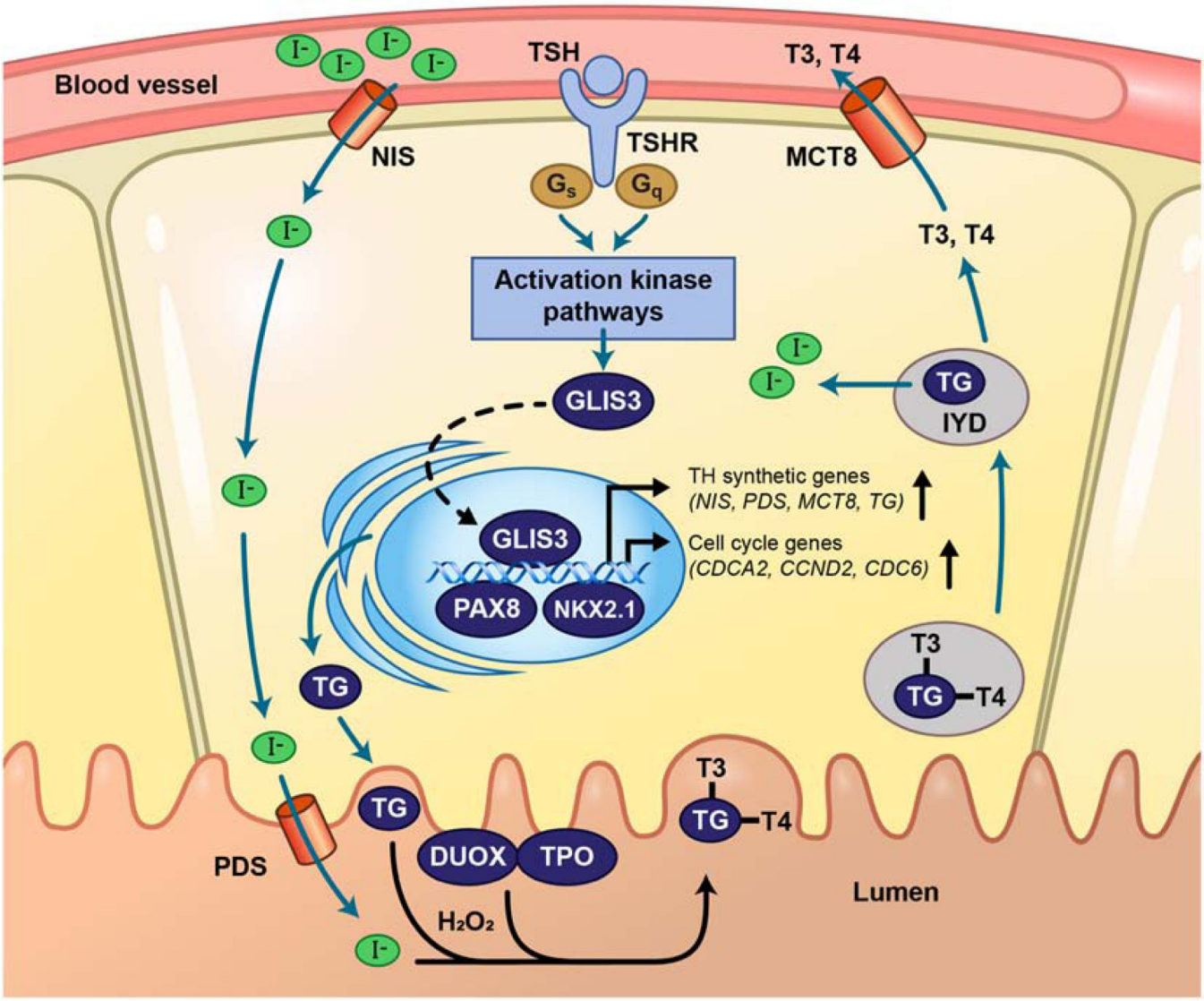

Fig. 2.

GLIS3 plays a critical role in thyroid hormone biosynthesis. GLIS3 is expressed specifically in thyroid follicular cells where it directly regulates the expression of several thyroid hormone biosynthetic genes, including *Nis*, *Pds*, *Mct8*, and *Tg*. It also has a role in thyroid follicular cell proliferation and regulates several cell cycle genes, such as *Cdca2*, *Ccnd2*, and *Cdc6*. Low TH levels cause an increase in blood TSH, which by its interaction with TSHR leads to the subsequent activation of several kinase pathways through the TSHR coupled G proteins, G_s and G_q. It is believed that these kinases posttranslationally modify GLIS3 thereby significantly enhancing its transcriptional activity that results in strong induction of thyroid hormone biosynthetic genes. GLIS3 regulates transcription of these genes in coordination with other thyroid transcription factors, including PAX8 and NKX2.1.

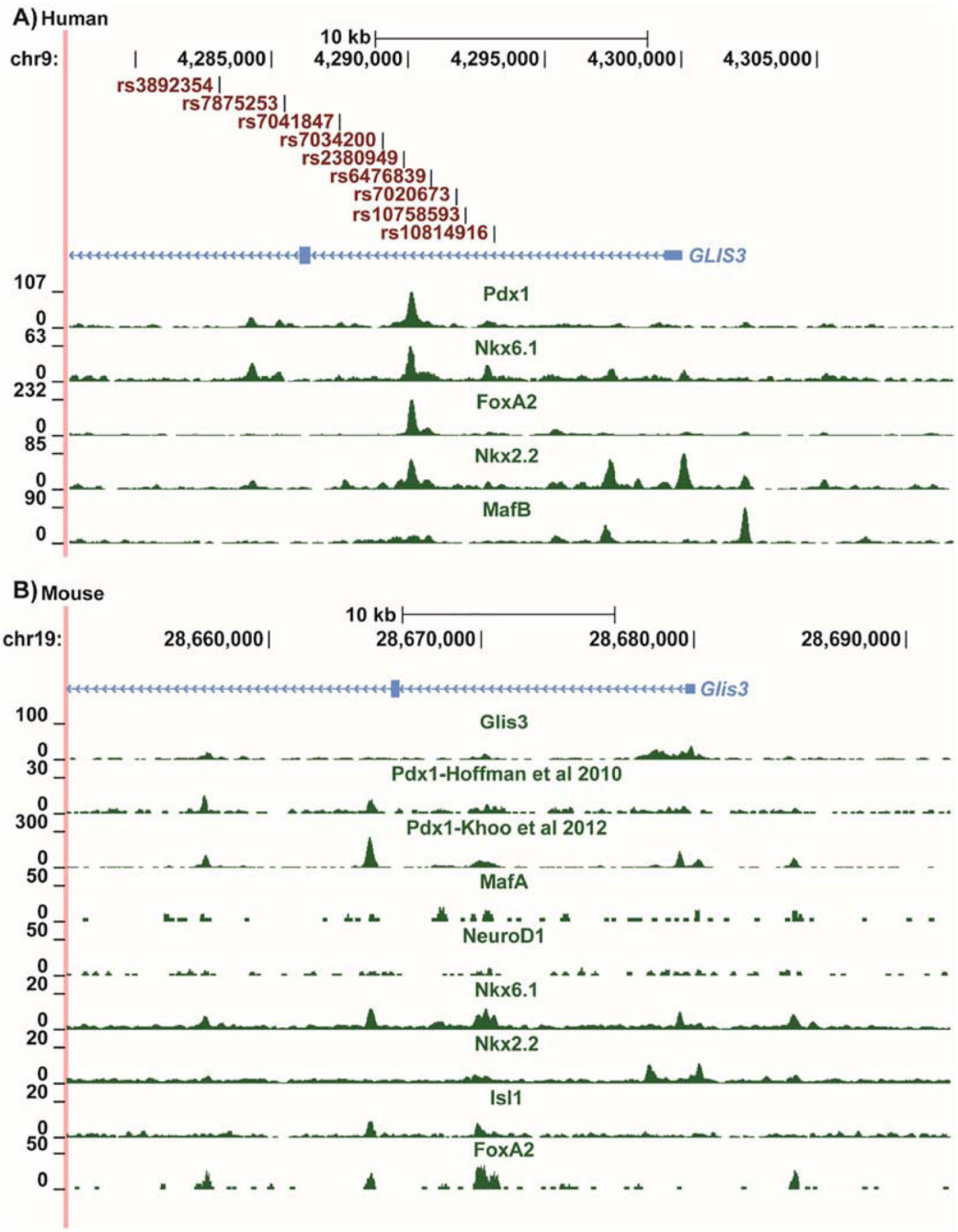

Fig. 3.

Many of the *GLIS3*-associated SNPs related to diabetes and β cell dysfunction identified by GWAS located in intron 1 neighbor a region of β cell transcription factor binding. (A) Top panel shows the localization of several *GLIS3*-associated SNPs to intron 1 and 2. Below it are UCSC genome browser tracks of this region with the binding peaks of the indicated transcription factors as identified by ChIP-Seq with human pancreatic islets (Pasquali, et al., 2014). The y-axis indicates normalized read counts. (B) UCSC genome browser tracks of intron 1 and 2 of *Glis3* showing the binding peaks of the indicated transcription factors as

identified by ChIP-Seq with mouse pancreatic islets or β-cell lines (Ediger, et al., 2017; Gutierrez, et al., 2017; Hoffman, et al., 2010; Khoo, et al., 2012; Scoville, Lichti-Kaiser, Grimm, & Jetten, 2019; Taylor, Liu, & Sander, 2013; Tennant, et al., 2013). Details on data analysis can be found in Scoville et al. 2019. This comparison suggests a potential cis-acting regulatory role of this intronic region in the control of *GLIS3* expression.

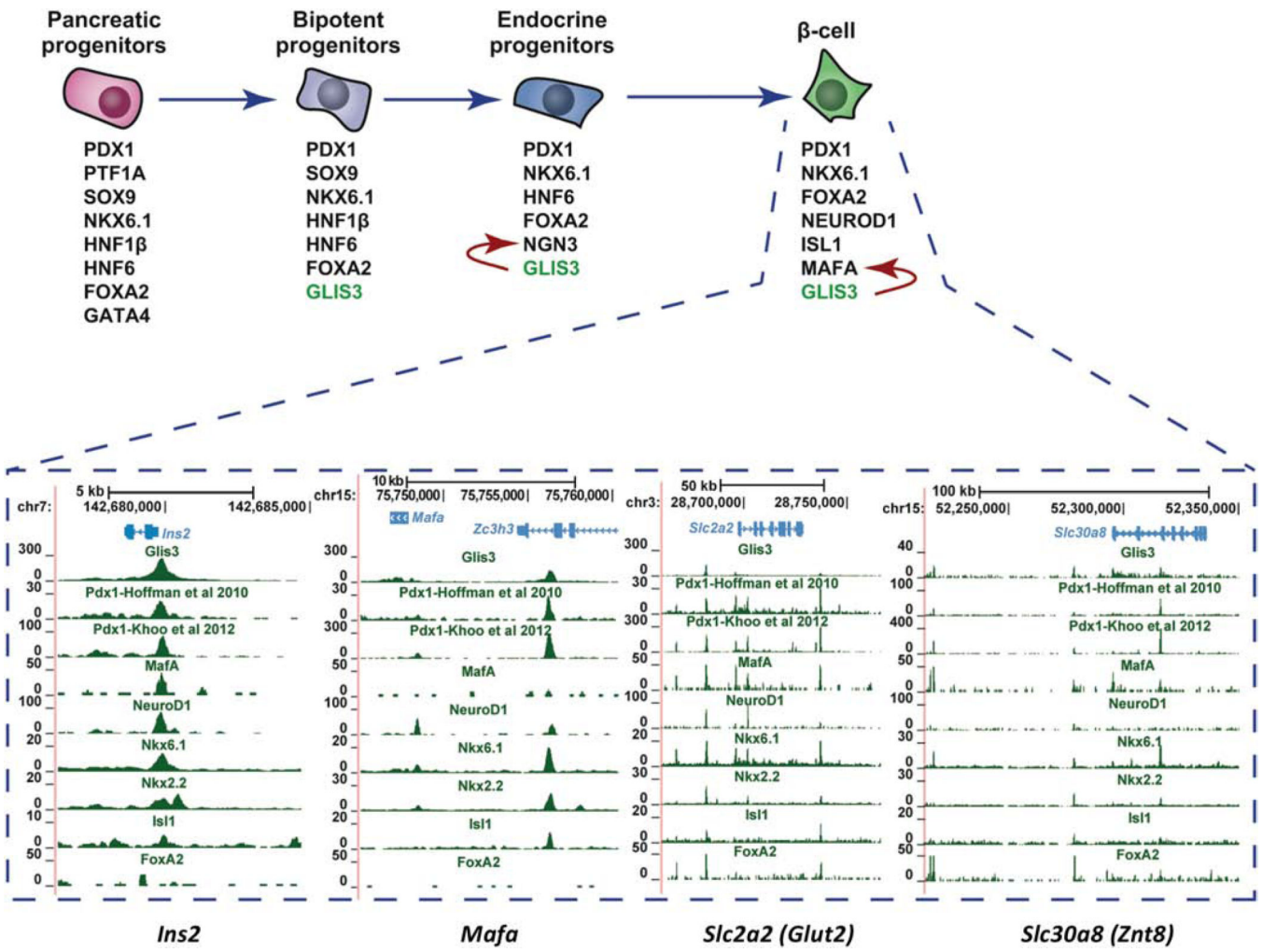

Fig. 4.

GLIS3 regulates pancreatic β cell gene transcription in coordination with other transcription factors. Top: Schematic of the expression of GLIS3 and other transcription factors during pancreas development. GLIS3 protein is first detectable in bipotent cells and remains expressed in endocrine progenitors and pancreatic β cells where it regulates the transcription of several genes. Lower panel: Comparison of genome browser tracks showing the binding peaks of the indicated transcription factor in the *Ins2*, *Mafa*, *Slc2a2*, and *Slc30a8* genes as identified by ChIP-Seq of mouse pancreatic islets or β-cell lines (Ediger, et al., 2017; Gutierrez, et al., 2017; Hoffman, et al., 2010; Khoo, et al., 2012; Scoville, Lichti-Kaiser, Grimm, & Jetten, 2019; Taylor, Liu, & Sander, 2013; Tennant, et al., 2013). Many of the β cell transcription factors are bound in close proximity to one another on multiple critical genes suggesting they coordinate the transcriptional control of these genes. *Mafa* transcription is controlled by a previously identified upstream regulatory region within the *Zc3h3* gene (Raum, et al., 2010). Details on data analysis can be found in Scoville et al. 2019. The y-axis indicates normalized read counts.

Table 1.

Association of various *GLIS3* variants with β cell dysfunction and different types of diabetes.

Phenotype	SNP	References
Fasting glucose/β-cell function	rs7034200	(Barker et al., 2011; Boesgaard et al., 2010; Dupuis et al., 2010; Hong. Chung. & Cho. 2014; Liu et al., 2011; Rees et al., 2011; Wagner et al., 2011)
	rs2380949	(Goodarzi et al., 2013)
Type 1 diabetes	rs7020673	(Barrett et al., 2009; Kiani et al., 2015; Steck et al., 2017)
	rs6476839	(Inshaw, Cutler, Crouch, Wicker, & Todd, 2020; Steck et al., 2017)
	rs10758591	(Fortune et al., 2015)
Type 2 diabetes	rs7034200	(Dou et al., 2017; Dupuis et al., 2010; Hu et al., 2010; Liu et al., 2011; Miranda-Lora et al., 2019; Rees et al., 2011)
	rs7041847	(Cho et al., 2012; Morris et al., 2012; Muller et al., 2017; Zhang et al., 2019)
	rs10758593	(Mahajan et al., 2014; Morris et al., 2012)
	rs10814916	(Li et al., 2013)
	rs7875253	(Muller et al., 2017)
	rs180867004	(Muller et al., 2017)
	rs3892354	(Hong, Kim, Zhang, & Park, 2018)
	rs2027393	(Hong et al., 2018)
	rs486163	(Hong et al., 2018)
	rs10758591	(Fortune et al., 2015)
Gestational diabetes	rs10814916	(Ding et al., 2018)
	rs7041847	(Ding et al., 2018)