



GLIS1–3 transcription factors: critical roles in the regulation of multiple physiological processes and diseases

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Abstract

Krüppel-like zinc finger proteins form one of the largest families of transcription factors. They function as key regulators of embryonic development and a wide range of other physiological processes, and are implicated in a variety of pathologies. GLI-similar 1–3 (GLIS1–3) constitute a subfamily of Krüppel-like zinc finger proteins that act either as activators or repressors of gene transcription. GLIS3 plays a critical role in the regulation of multiple biological processes and is a key regulator of pancreatic β cell generation and maturation, insulin gene expression, thyroid hormone biosynthesis, spermatogenesis, and the maintenance of normal kidney functions. Loss of GLIS3 function in humans and mice leads to the development of several pathologies, including neonatal diabetes and congenital hypothyroidism, polycystic kidney disease, and infertility. Single nucleotide polymorphisms in *GLIS3* genes have been associated with increased risk of several diseases, including type 1 and type 2 diabetes, glaucoma, and neurological disorders. GLIS2 plays a critical role in the kidney and GLIS2 dysfunction leads to nephronophthisis, an end-stage, cystic renal disease. In addition, GLIS1–3 have regulatory functions in several stem/progenitor cell populations. GLIS1 and GLIS3 greatly enhance reprogramming efficiency of somatic cells into induced embryonic stem cells, while GLIS2 inhibits reprogramming. Recent studies have obtained substantial mechanistic insights into several physiological processes regulated by GLIS2 and GLIS3, while a little is still known about the physiological functions of GLIS1. The localization of some GLIS proteins to the primary cilium suggests that their activity may be regulated by a downstream primary cilium-associated signaling pathway. Insights into the upstream GLIS signaling pathway may provide opportunities for the development of new therapeutic strategies for diabetes, hypothyroidism, and other diseases.

Keywords GLIS1 · GLIS2 · GLIS3 · Krüppel-like zinc finger protein · Pancreatic beta cells · Diabetes · Insulin · Polycystic kidney disease · Nephronophthisis · Primary cilium · Reprogramming · Stem cells · Hypothyroidism · Spermatogenesis · Thyroid hormone biosynthesis · NIS · Development · HECT E3 ubiquitin ligase · TAZ · SUFU · ITCH · GWAS · Mutations

Introduction

GLI-Similar 1–3 (GLIS1–3) constitute a subfamily of Krüppel-like zinc finger transcription factors that contain a zinc finger domain (ZFD) that is highly conserved among GLIS1–3 as well members of the closely related GLI1–3 protein subfamily [1–8] (Fig. 1). The ZFDs of GLIS1 and GLIS3 exhibit the highest homology suggesting that they are evolutionary the most closely related. Outside their ZFD,

GLIS1–3 exhibit little homology with each other or GLI proteins with the exception of an N-terminal, conserved region (NCR) of about 60 aa that GLIS3 has in common with GLI1–3 [9].

Although several alternatively spliced GLIS transcripts and variants have been described, their physiological relevance has yet to be determined. GLIS homologs have been identified in all mammalian species, as well as *Zebrafish*, *Medaka*, *Xenopus* and *Drosophila* [10–12]. The GLIS homolog in *Drosophila* is referred to as gleeful (*gfl*) or lame duck (*lmd*).

GLIS1–3 play a critical role in the regulation of many physiological processes and are been implicated in a variety of pathologies, including neonatal diabetes, glaucoma, cystic kidney disease, neurological disorders, congenital hypothyroidism, and cancer [1, 4, 5, 13, 14]. Study of this

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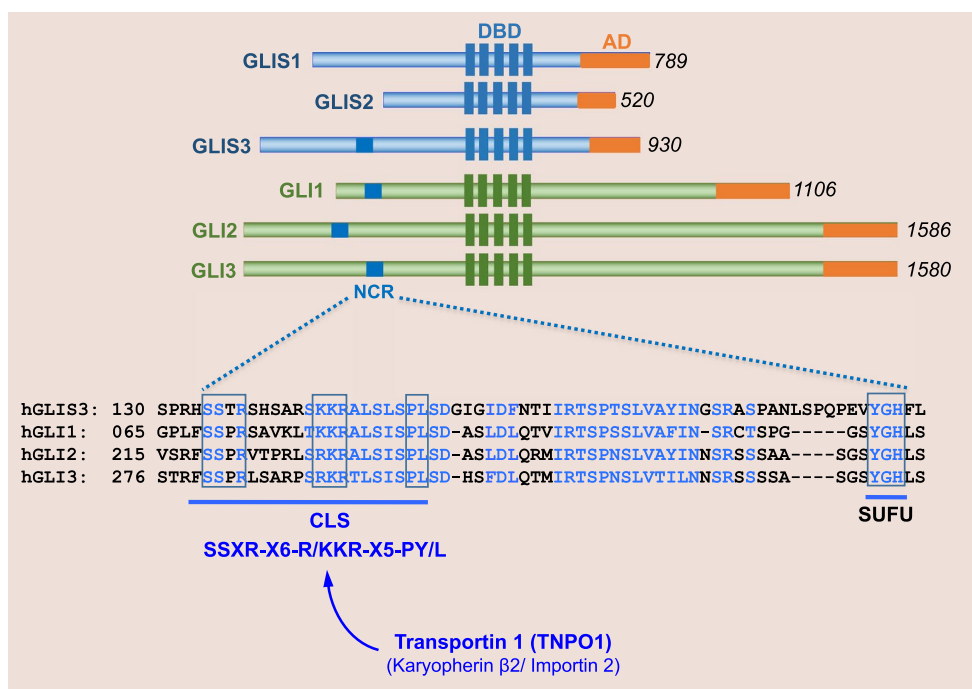


Fig. 1 Schematic presentation of human GLIS1–3 proteins. The DNA binding domain (DBD) containing five zinc finger motifs, the activation domain (AD), and the N-terminal conserved region (NCR) are indicated. Numbers on the right indicate the size of the GLIS1–3 and GLI proteins. Lower panel: comparison of the amino acid sequence

between the NCRs of GLIS3 and GLI1–3. Homologous amino acids are shown in blue. The ciliary localization signal consensus (CLS) and the SUFU binding domain are indicated. The CLS binds TNPO1, which guides transport into the primary cilium

subfamily of transcription regulators has provided greater mechanistic insights into the regulation of several physiological processes and the development of various pathologies, and might eventually lead to the discovery of novel therapeutic approaches for these diseases.

Transcriptional activity and protein interactions

Activator and repressor functions

GLIS1 and GLIS3 function primarily as transcriptional activators, whereas GLIS2 can activate transcription, but appears to function mainly as a transcriptional repressor [8, 15–18]. Deletion analysis revealed that GLIS1 and GLIS3 contain a strong activation domain at their C terminus that mediates their interaction with co-activators. GLIS proteins regulate gene transcription by interacting with GLIS-binding sites (GLISBS) in the regulatory region of target genes. Insulin (*Ins2*), cyclin D2 (*Cdnd2*), fibroblast growth factor 18 (*Fgf18*), neurogenin 3 (*Ngn3*), pendrin (*Pds*), and Na⁺/I⁻ symporter (*Nis*) are among the genes directly regulated by GLIS3, while *GLI1*, the Wnt family member 4 (*Wnt4*) and *Snail1* (*Snai1*) are target genes of GLIS2 [17–22]. The

interaction of GLIS proteins with GLISBS is mediated by their ZFD, which consists of a tandem repeat of five C2H2 zinc finger motifs [1, 5, 8, 16, 19]. All five zinc finger motifs are required for optimal binding of GLIS proteins to GLISBS; loss of the first zinc finger has the least impact on this interaction. The *in vivo* consensus sequence of GLISBS derived from ChIP-Seq analysis exhibits high similarity with the GLISBS consensus sequence, (G/C)TGGGGG(A/C), identified by an *in vitro* screen [17, 23, 24]. Members of the closely related GLI and ZIC families also bind G-rich DNA response elements similar to GLISBS and therefore might compete for the same binding site and interfere with each other's action in cells in which they are co-expressed. This is supported by a report showing that GLIS2 can inhibit GLISBS-dependent activation of a reporter gene by GLI1 and repress GLI1-induced transcriptional activation of *Wnt4* by competing for the same binding site [17, 18]. In addition, different members of these protein families may interact by forming heterodimers as have been reported for other Krüppel-like zinc finger transcription factors, including members of the ZIC and GLI subfamilies [25].

GLIS proteins activate or repress transcription through the recruitment of co-activators or co-repressors, respectively. The co-activator, C-terminal binding protein (CBP), was shown to interact with GLIS3 and be part of a

multiprotein co-activator complex [19]. Mass spectrometric analysis of GLIS3 protein complexes identified several phosphorylation and arginine methylation sites, and a number of GLIS3-interacting partners [9, 26]. Still very little is known about the role of GLIS3 phosphorylation and the upstream kinase signaling pathways that might regulate GLIS3 activity and function. The arginine methyltransferase PRMT5 and the lysine demethylase KDM4C were among the GLIS3 interacting proteins identified. PRMT5 catalyzes the formation of H3K4me₃, which is associated with active chromatin, while KDM4C demethylates histone H3K9me₃, which is associated with the repressed state of a gene. Thus, these (de)methylation activities correlate with transcriptionally active genes and are consistent with GLIS3 acting as an activator of gene transcription. Since GLIS3 was found to be methylated, PRMT5 might also be involved in mediating its methylation and as such modulate GLIS3 transcriptional activity or protein stability or alter its interactions with other proteins.

C-terminal binding protein 1 (CtBP1) functions as a co-repressor for a number of transcription factors, including GLIS2, by interacting with PXDLS consensus motifs, in which X is any amino acid [27]. CtBPs mediate transcriptional repression by recruiting histone deacetylases (HDACs) and histone methyltransferases. HDAC3 was found to be part of the GLIS2-CtBP1 transcription silencing complex. The latter is consistent with the concept that GLIS2 functions as a transcriptional repressor as indicated by data showing that GLIS2 represses the expression of several genes, including *Gli1* and *Wnt4* [18].

TAZ

GLIS3, but not GLIS1 or GLIS2, was found to interact with TAZ (WWTR1), a PDZ binding motif-containing transcriptional co-activator [28, 29]. TAZ is part of the Hippo signaling pathway that regulates its nuclear localization and activity [30]. The Hippo pathway plays a role in the regulation of many biological functions, including cell migration, differentiation, proliferation, and cell polarity. The WW-domain of TAZ recognizes a P/LPXY motif in the C terminus of GLIS3 [28]. Co-expression with GLIS3 promotes the translocation of TAZ from the cytoplasm to the nucleus where it co-localizes with GLIS3. TAZ enhances the transcriptional activity of GLIS3 and appears to function as a co-activator of GLIS3-mediated transcriptional activation. Whether GLIS3 activity *in vivo* is regulated by the Hippo signaling pathway is an attractive hypothesis that needs further study. Moreover, it is worthwhile noting that GLIS3-deficiency as well as loss of TAZ function leads to the development of polycystic kidney disease [28, 31]. These observations are consistent with a functional link between these two proteins and the development of polycystic kidney disease.

β-Catenin and p120 catenin

Yeast two-hybrid analysis identified β-catenin as a GLIS2-interacting partner [32]. The tetrahedral configuration of the first zinc finger of GLIS2 and the armadillo repeats of β-catenin are required for this interaction. GLIS2 inhibits β-catenin/T cell factor (TCF)-mediated transcription as well as the expression of the TCF target gene, cyclin D1 (*Ccnd1*) suggesting that it functions as a negative modulator of the Wnt/β-catenin-TCF signaling pathway consistent with GLIS2 repressor function.

GLIS2 also interacts with p120 catenin (CTNND1) and induces its translocation to the nucleus [33]. This interaction involved the 140 aa, N-terminal region of GLIS2 and the C terminus of p120 and was enhanced by the tyrosine kinase SRC. Interaction with p120 induces proteolytic cleavage of GLIS2 between ZF4 and ZD5 that results in the generation of a C-terminal-truncated GLIS2-lacking ZF5 (GLIS2ΔC). Since ZF5 plays a critical role in the recognition of GLISBS by GLIS2, one would predict that GLIS2 is unable to bind this site and regulate GLISBS-dependent transcription. However, GLIS2ΔC was still able to bind DNA possibly by recognizing a DNA sequence different from that of GLISBS or via interaction with another transcription factor. Moreover, GLIS2ΔC was capable of inhibiting neural tube differentiation suggesting that it still has a function. Co-expression with E-cadherin, which sequesters p120, inhibited the p120–GLIS2 interaction.

Ubiquitin and SUMO ligases

Both GLIS2 and GLIS3 have been reported to be ubiquitinated and sumoylated [9, 34, 35]. Yeast two-hybrid and mass spectrometric analyses revealed that a number of WW-domain containing HECT E3 ubiquitin ligases, including ITCH, NEDD4, and SMURF1/2, interact with GLIS3 [9, 26]. These interactions with GLIS3 are mediated through their WW-domain, which recognizes a PPYP⁴⁶¹ motif in GLIS3. ITCH was shown to enhance polyubiquitination of GLIS3 and promote its proteolytic degradation by proteasomes, and consequently reduce GLIS3 protein stability. ITCH inhibits the transcriptional activity of wild-type GLIS3, but not that of a PPYP mutant that is unable to interact with ITCH. This inhibition of GLIS3-mediated transactivation might be related to decreased GLIS3 protein levels. This study suggests that ubiquitination plays an important role in regulating GLIS3 protein stability and as a consequence its activity and function.

GLIS2 was found to interact with the E3 ubiquitin ligase, tripartite motif containing 32 (TRIM32, also named Bardet–Biedl syndrome 11 or BBS11), and BBS1 [35, 36], proteins that are part of the Bardet–Biedl syndrome (BSS) multiprotein complex, which plays a role in ciliogenesis and cilia

maintenance. In zebrafish, deficiency in either TRIM32 or GLIS2 leads to the development of renal cysts. The 141–359 region of GLIS2 that includes the ZFD, interacts with the TRIM32 N-terminus containing the Ring and B-box domains. TRIM32 enhances K63-dependent polyubiquitination but inhibits K48-linked polyubiquitination of GLIS2, thereby inhibiting its proteasomal degradation. Unexpectedly, the catalytic activity of TRIM32 was not required for this increase in K63 ubiquitination suggesting the involvement of another ubiquitin ligase. TRIM32 also modifies GLIS2 transcriptional activity; it reversed the inhibition of β -catenin-mediated transcriptional activation by GLIS2 and inhibited the GLIS2-induced activation of the *Ins2* promoter. In addition, TRIM32 changes the subnuclear localization of GLIS2 and instead of a diffuse pattern of expression it becomes localized to nuclear bodies [35], including PML bodies, which are involved in the regulation of multiple cellular functions, such as apoptosis, DNA repair, and cell cycle [37]. This co-localization and interaction with promyelocytic leukemia protein, PML (also named TRIM19) suggest that GLIS2 might have a role in these processes as well.

E3 SUMO ligase, PIAS4, was reported to also interact with GLIS2 and catalyze sumoylation of GLIS2 at multiple sites, including K195 [34]. This sumoylation was specific for SUMO3. The PIAS4-mediated sumoylation was shown to interfere with GLIS2 ubiquitination, thereby inhibiting its degradation by the proteasome and extending its half-life. Although mutation of K195R prevents ubiquitination and sumoylation at this site, the overall GLIS2 ubiquitination was increased and GLIS2 protein stability decreased. Increased sumoylation did not affect the inhibitory effect of GLIS2 on β -catenin-mediated gene activation, but inhibited its activation of the *Ins2* promoter. These results suggest that sumoylation selectively influences GLIS2 transcriptional activity and function.

SUFU

As has been reported for GLI proteins [38], both GLIS2 and GLIS3 have been shown to interact with suppressor of fused (SUFU) [9, 18, 39]. GLIS3 interacts with SUFU through a VYGHF motif located within the NCR as well as with an undefined site at the C terminus of GLIS3. SUFU protects GLIS3 from proteasomal degradation, thereby stabilizing GLIS3 protein. The cullin-RING ubiquitin ligase cullin 3 (CUL3) also interacts with GLIS3 and promotes the ubiquitination and degradation of GLIS3. CUL3 interacts with the N terminus of GLIS3, a region that includes the SUFU binding motif. This raised the possibility that the stabilization of GLIS3 by SUFU may be due to its inhibition of GLIS3 interaction with CUL3. SUFU plays also an important role in the control of hedgehog/GLI signaling and regulates this pathway at multiple levels [40, 41]. In the case of GLIS3,

co-expression of GLIS3 and SUFU causes translocation of SUFU to the nucleus, suggesting that GLIS3 and SUFU are part of a nuclear protein complex. Although SUFU was shown to moderately reduce GLIS3-mediated transcriptional activation, the precise function of SUFU in GLIS3-mediated transcriptional activation is not fully understood [9]. In contrast to GLIS3, the GLIS2–SUFU complex appears more susceptible to ubiquitination and degradation by the proteasome suggesting a different mode of regulation [18].

Genetic alterations in human GLIS1–3

GLIS3-associated mutations and variants

Genetic variations and mutations in the human *GLIS3* gene, which maps to chromosome 9p24.2 (Fig. 2a), have been linked to a wide range of pathologies. Patients with loss-of-GLIS3-function mutations most consistently develop a syndrome referred to as neonatal diabetes and congenital hypothyroidism (NDH) and have a greatly reduced life span of a few days to several years [13, 42–48]. Abnormalities associated with *GLIS3* mutations can extend to intrauterine growth retardation (IUGR), developmental delay, development of polycystic kidneys, congenital glaucoma, hepatic cholestasis, osteopenia, atrial septal defects, and minor facial dysmorphisms. Facial anomalies associated with *GLIS3* mutations typically include depressed nasal bridge, bilateral low-set ears, long philtrum, large anterior fontanelle, and elongated, upslanted palpebral fissures. GLIS3-deficiency shows some phenotypic variability among patients; some patients do not develop cystic kidneys or skeletal abnormalities or show abnormal thyroid gland development. The phenotypic abnormalities observed in GLIS3-deficient mice largely mimic those observed in human patients [1, 4, 5, 22, 28, 49–51].

Mutations in human *GLIS3* that cause loss of function, are extremely rare and were first described in several NDH patients from Saudi Arabia and France and subsequently in a few other patients [13, 42–47]. Genetic mutations in *GLIS3* include deletions encompassing exons 5–9, 3–4, 9–11, 10–11, and several larger deletions covering regions > 100 kb that include exons 1–2 and part of intron 2 or exons 1–4 and part of intron 4 (Fig. 2a). Homozygous frameshift mutations, p.Arg780Profs*79, p.Gly311Alafs*15, and p.Pro772Leufs*35 resulting in an early termination codon and the loss of the GLIS3 transactivation domain, were identified in several NDH patients [43, 45, 46, 48]. In addition, several homozygous missense mutations, p.Arg589Trp, p.Cys536Trp and p.His561Tyr, within the DNA-binding domain of GLIS3, were found to be associated with NDH [43]. p.Cys536Trp and p.His561Tyr within the second ZF motif cause the collapse of the tetrahedral

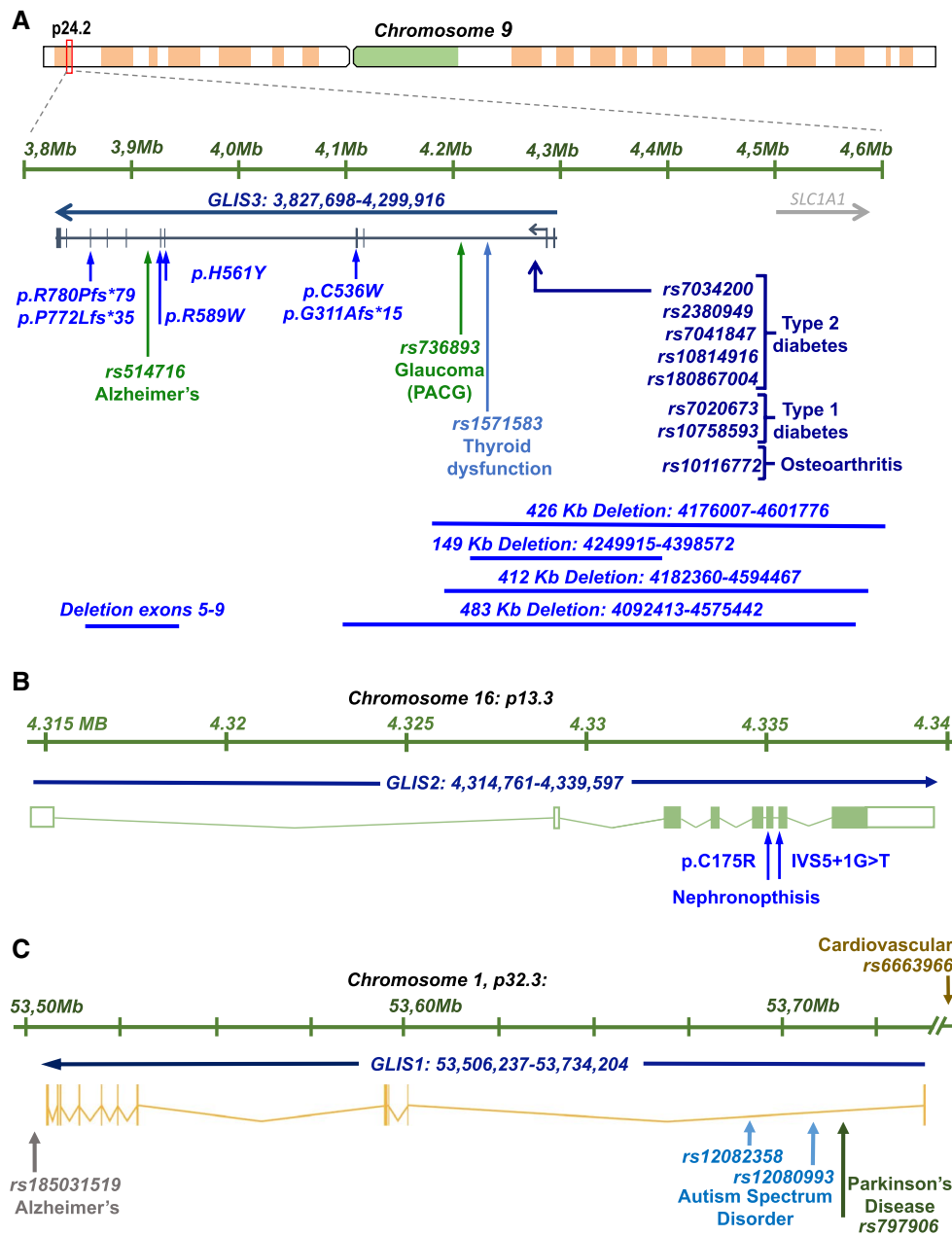


Fig. 2 Schematic presentation of the genomic maps of the human *GLIS1–3* genes. Missense and frame shift mutations, and deletions implicated in disease are indicated. SNPs associated with increased risk for several pathologies are also shown. **a** *GLIS3*. **b** *GLIS2*. **c** *GLIS1*

configuration of ZF2 and greatly diminishes the ability of *GLIS3* to bind *GLISBS*. A patient with a p.Phe857Tyr missense mutation in *GLIS3* was reported to develop neonatal diabetes and liver dysfunction and died of liver and kidney failure at 1.5 years of age [52]. A combination of heterozygous variants in *GLIS3* (p.R720Q) and *DUOX2* (p.R683L/p.L1343F) was found to be associated with congenital hypothyroidism [47].

Genome wide association studies (GWAS) have uncovered associations between single nucleotide polymorphisms

(SNPs) in *GLIS3* and several pathologies. The *GLIS3*-associated SNPs, rs7034200, rs7875253, rs7041847, and rs10814916, have been linked to elevated fasting glucose levels, altered β cell function, and increased risk for type 2 diabetes (T2D) [53–71] (Fig. 2a). The *GLIS3* SNP rs2380949 was found to be associated with reduced insulin clearance, a predictor of T2D incidence [72], while the *GLIS3* SNP, rs180867004, exhibited a sex-specific association with a higher risk for early onset of T2D in male American Pima Indians [68].

More than 40 genes have been identified as a susceptibility locus for type 1 diabetes (T1D); however, *GLIS3* is one of a handful of genes that have also been linked to both T1D and T2D. The *GLIS3* SNPs, rs7020673 and rs10758593, have been reported to be significantly associated with increased risk for T1D [54, 63, 73–76]. However, in a study of diabetic patients in Brazil no significant association was observed between these SNPs and T1D [77]. The low-frequency p.Ala908Val variant of *GLIS3* was found to be strongly associated with resistance to T1D [78].

GLIS3 variations have been linked to a number of additional pathologies. Certain susceptibility loci are shared between different autoimmune disorders. For the T1D-associated *GLIS3* SNP rs7020673, a significant association was found with rheumatoid arthritis risk [79]. Rs10116772 was found to be associated with osteoarthritis [80], while rs736893, was identified as a risk factor for primary angle closure glaucoma (PACG) [81, 82]. A rare *GLIS3* duplication was reported to be associated with congenital heart defects [83]. Rs476155 was identified as a susceptibility locus for low HDL-cholesterol levels, a risk factor for coronary artery disease in ethnic Arabs [84]. A significant association was also found between the *GLIS3* SNP, rs514716, and elevated levels of cerebrospinal fluid (CSF) tau and phosphorylated tau (ptau₁₈₁), established biomarkers for AD [85, 86]. No significant correlation was found between T2D-associated *GLIS3* SNP, rs10814916, and risk of Parkinson's or Alzheimer's disease (AD) [87].

GLIS2-associated mutations and variants

GLIS2 maps to chromosome 16p13.3 (Fig. 2b). Loss of *GLIS2* function in humans as well as in mice lead to the development of type 7 nephronophthisis (NPHP7) [2, 88–91]. Nephronophthisis is a rare autosomal recessive cystic kidney disease characterized by renal atrophy, fibrosis, and interstitial infiltration of inflammatory cells. It is the most common genetic cause of end-stage renal disease in children and young adults. A homozygous transversion at the acceptor splice site of intron 6 (IVS5 + 1G > T) in *GLIS2* was found to lead to loss of *GLIS2* function and the development nephronophthisis [88]. A homozygous missense mutation, p.Cys175Arg (rs587777353), in exon 6 of *GLIS2* was identified in a patient with nephronophthisis [89, 91]. This mutation, which is within the first ZF, destroys its tetrahedral configuration and thereby the ability of *GLIS2* to optimally bind *GLISBS* and regulate *GLISBS*-dependent transcription. This mutation also changed the subcellular localization of *GLIS2* from nucleus to cytoplasm, but had no effect on *GLIS2* protein stability or its interaction with CTBP1, HDAC3 or β -catenin [89].

GLIS1-associated mutations and variants

The human *GLIS1* gene maps to chromosome 1p32.3 (Fig. 2c). *GLIS1* variants have been reported to be a risk factor for several neural pathologies. The intergenic SNP, rs185031519, near the *GLIS1* locus was found to be associated with lower cerebrospinal fluid (CSF) amyloid-beta1-42 (A β ₄₂) levels and higher tau and phosphorylated tau (ptau₁₈₁) levels [86]. Lower CSF A β ₄₂ levels and higher tau and ptau₁₈₁ correlate with the number of neurofibrillary tangles and plaque load and are well-established AD endophenotypes. Rs185031519 was found to be associated with faster disease progression and increased AD risk.

A different GWAS study found an association between two *GLIS1* SNPs, rs12082358 and rs12080993, and increased risk of autism spectrum disorder in a Taiwanese Han population [92]. An additional SNP in *GLIS1* (rs797906) was identified as a susceptibility locus for late-onset Parkinson's disease (PD) in a Han Chinese population [93]. And although individual studies did not find a significant correlation between this SNP and PD in Caucasian populations, pooled data appear to support an association between PD and rs797906 [93–97]. Another *GLIS1*-associated SNP (rs6663966) was found to be linked to increased severity of coronary artery calcified plaques and risk for cardiovascular disease in African Americans with T2D [98].

Molecular and physiological functions of GLIS1–3

GLIS as reprogramming factors

Several studies have established roles for *GLIS* proteins in reprogramming. Somatic cells can be reprogrammed into induced pluripotent stem cells (iPSCs) by ectopic expression of several reprogramming factors, OCT3/4 (POU5F1), SOX2, KLF4, and c-MYC (OSKM) [99]. *GLIS1* significantly promotes the reprogramming of both human and mouse fibroblasts into iPSCs when co-expressed with OSK (referred to as OSKG), whereas down-regulation of *GLIS1* by shRNAs reduced reprogramming efficiency [14, 100–102]. iPSCs generated by OSKG were shown to be fully able to produce germline competent chimaeras.

The mechanism by which *GLIS1* enhances reprogramming efficiency is not fully understood. Gene profiling analysis demonstrated that *GLIS1* enhanced the expression of several pluripotent genes, including *NANOG*, *ESSRB*, *LIN28A*, *MYCN*, *MYCL1*, and several WNT ligands, such as *WNT3* and *WNT8A*. Most of these genes appeared to be indirectly regulated by *GLIS1*. Mesenchymal–epithelial transition (MET) is a critical element in the reprogramming of somatic cells. *GLIS1* regulates

several genes implicated in MET, including increased expression of the transcription factor FOXA2, a repressor of epithelial–mesenchymal transition (EMT), which as a consequence would promote MET and iPSC generation. GLIS1 was found to be in a protein complex with OCT4, KLF4, and SOX2. The N terminus and ZFD of GLIS1 are required for the interaction with KLF4. Whether this interaction has a role in mediating the increase in programming efficiency by GLIS1 needs further study [100].

The ability of GLIS1 to function as an efficient reprogramming factor was also demonstrated by transfecting human primary foreskin fibroblasts with a modified version of a non-infectious Venezuelan equine encephalitis (VEE) virus RNA replicon expressing OCT4, KLF4, SOX2, and GLIS1 [102]. Moreover, OCT4, KLF4, SOX2, GLIS1 and c-MYC, robustly generated iPSCs from human fibroblasts from older adults, age 54–77 [103]. A different study showed efficient reprogramming of human urine-derived cells using a high-efficiency episomal system containing *OCT4*, *GLIS1*, *KLF4*, *SOX2*, *L-MYC*, and the miR-302 cluster [104].

GLIS1 also enhances reprogramming induced by an alternative pathway. C-JUN functions as a powerful inhibitor of somatic cell reprogramming by inducing the expression of EMT genes, including *TGF β* and *SNAIL*, and inhibiting the expression of pluripotent genes, such as *SOX2* and *NANOG* [105]. In contrast, JUN dimerization protein 2 (JDP2), which represses JUN-mediated transactivation, promotes reprogramming in conjunction with KLF4, SOX2 and MYC. JDP2 also induces reprogramming of MEFs into iPSCs via an alternative pathway in combination with the expression of GLIS1, lysine demethylase KDM2B, and the transcription factors ID1/3, SALL4, and LRH1 (NR5A2) [105].

A recent study demonstrated that GLIS3 can promote reprogramming in human somatic cells as efficiently as GLIS1 [106]. In contrast to GLIS1 and -3, GLIS2 had a negative effect on reprogramming [106, 107]. However, knockdown of GLIS2 expression in human ESCs by siRNA abolished the expression of OCT4, SOX2, and NANOG and loss of stem cell phenotype [107]. This was accompanied by differentiation and expression of multiple genes associated with endodermal and extraembryonic lineages. Knockdown of OCT4 also induced differentiation that was associated with decreased expression of GLIS2. OCT4 was found to regulate *GLIS2* transcription directly by binding to its promoter region. Thus, these observations suggest that GLIS2 may have a role in maintaining the pluripotent state of hESCs. GLIS3, together with several other transcription factors, also promotes the reprogramming of human fibroblasts into retinal pigmented epithelial cells [108].

GLIS3 and thyroid hormone biosynthesis

Loss of GLIS3 function in humans and mice causes congenital or neonatal hypothyroidism [4, 43–46, 50, 109]. Congenital hypothyroidism is the most common inborn endocrine disorder and is caused by abnormalities in either thyroid development (thyroid dysgenesis) or thyroid hormone (T3/T4) biosynthesis (dysmorphogenesis) [110, 111]. The thyroid phenotype in GLIS3-deficient patients is quite variable ranging from aplasia/dysplasia to dysmorphogenesis [13] suggesting that GLIS3 might have a role in both thyroid gland development and thyroid hormone biosynthesis.

The development of neonatal hypothyroidism in GLIS3-deficient mice has been serving as a model to study the mechanisms underlying this pathology. GLIS3 expression in the thyroid gland is restricted to the thyroid follicular cells [24]. The basic morphology of the thyroid gland and the expression of several genes critical for thyroid development, such as *Pax8*, *Tf1* (*Nkx2.1*), and *Tf2* (*FoxE1*), are not greatly affected in PND7 Glis3-KO mice, suggesting that abnormal thyroid gland development does not play a major role in the development of hypothyroidism in these Glis3-KO mice, but has a major function in regulating thyroid hormone biosynthesis and homeostasis (Fig. 3).

Thyroid hormone biosynthesis is controlled by the hypothalamic–pituitary–thyroid axis, in which low T3/T4 leads to increased production of thyrotropin-releasing hormone (TRH) by the hypothalamus and thyroid-stimulating hormone (TSH) by the pituitary, which subsequently increases thyroid hormone production and blood levels [112] (Fig. 3). Increasing blood levels of thyroid hormone subsequently act as a negative feedback loop. Human and mice deficient in GLIS3 function exhibit low blood levels of T3 and T4, and highly elevated levels of thyroid-stimulating hormone (TSH). These observations indicate that the development of hypothyroidism is not due to the inability of the pituitary to respond to low thyroid hormone levels or to produce TSH.

Study of gene expression profiles of thyroid glands from wild-type and GLIS3-deficient mice revealed that GLIS3 regulates the expression of a selective set of genes critical for thyroid hormone biosynthesis. The expression of particularly the iodide transporters, *Nis* (*Slc5a5*) and pendrin (*Pds*, *Slc26a4*), was greatly repressed in the thyroid gland of GLIS3-deficient mice (Fig. 3). Previous studies reported that mutations in *NIS* that result in a nonfunctional NIS protein [113], as well as mutations in *PDS* [114], cause congenital hypothyroidism in humans. Thus, the suppression of these genes appears to be a major contributory factor to the impaired T3/T4 biosynthesis and the development of neonatal hypothyroidism in GLIS3-deficiency.

In addition to controlling T3/T4 biosynthesis, TSH regulates thyroid follicular proliferation. These actions are mediated through the interaction of TSH with the TSH-receptor

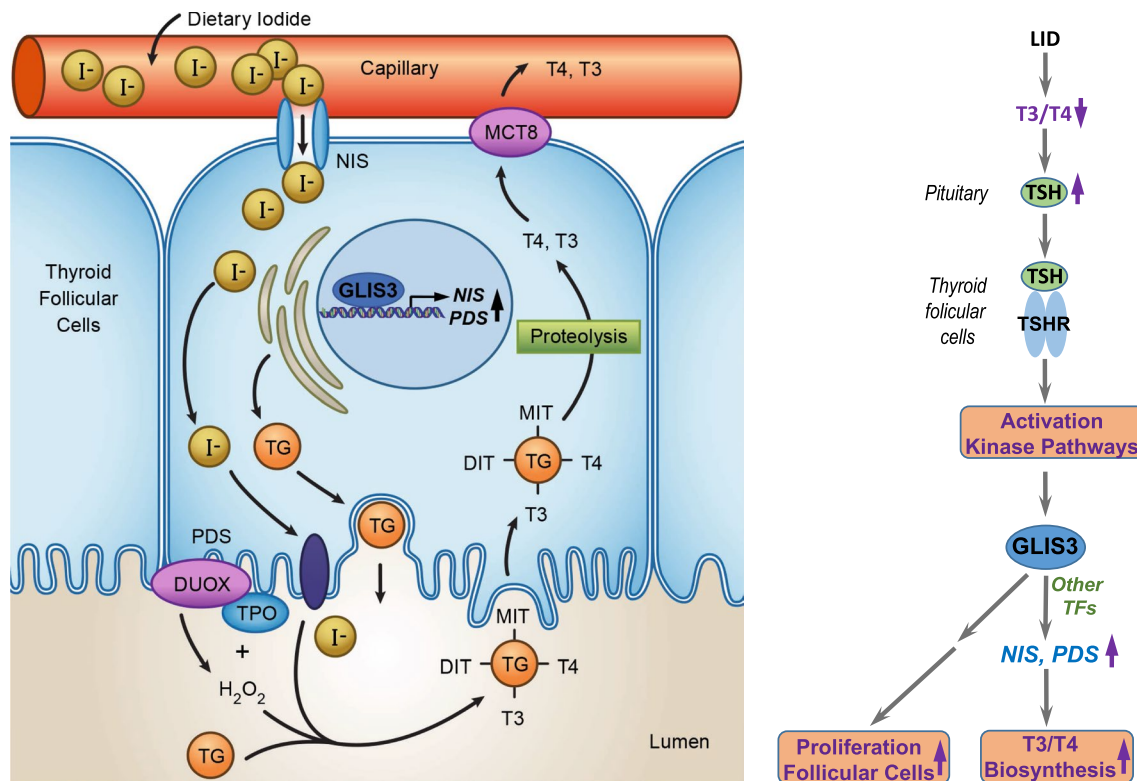


Fig. 3 GLIS3 is essential for thyroid hormone biosynthesis. **a** GLIS3 expression is restricted to thyroid follicular cells where it directly regulates the transcription of several genes required for thyroid hormone biosynthesis, particularly the two iodide transporter genes, *NIS* and *PDS*. Loss of GLIS3 causes a dramatic decrease in *NIS* and *PDS* expression and iodide transport, and consequently reduced thyroid hormone biosynthesis and hypothyroidism. **b** Low iodide diet

(LID) greatly elevates blood TSH levels. Interaction of TSH with the TSHR in thyroid follicular cells activates several kinase pathways and expression of the thyroid biosynthetic genes, including *NIS* and *PDS*, and cell cycle genes. However, these genes are not induced by TSH in GLIS3-deficient thyroid follicular cells suggesting that GLIS3 mediates the downstream effects of TSH/TSHR

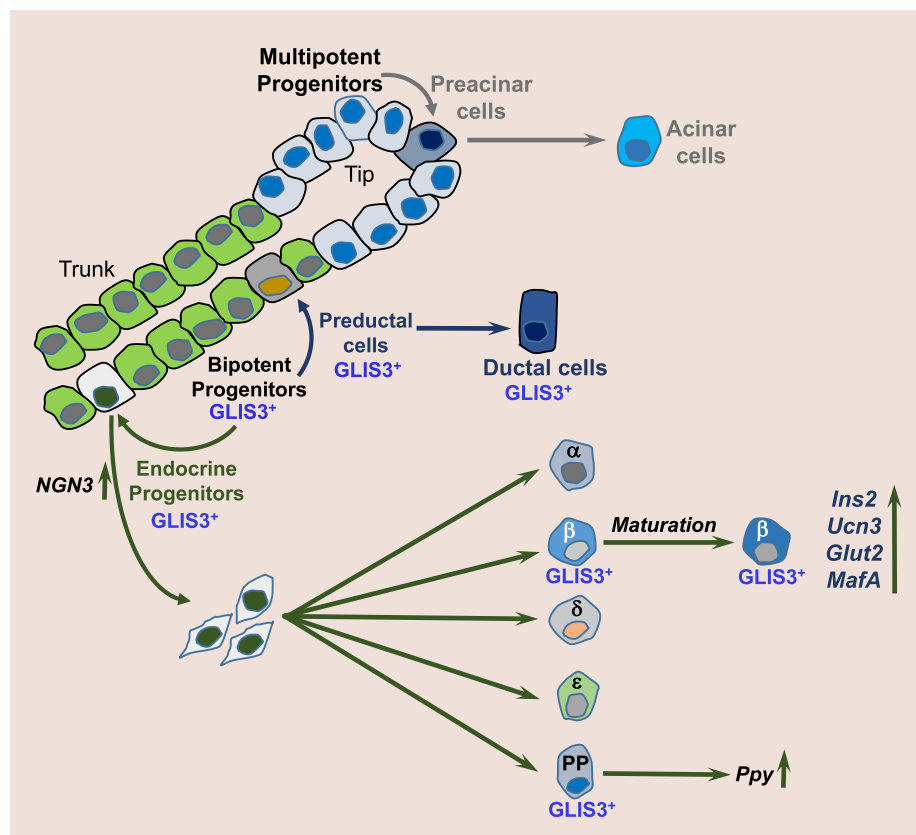
(TSHR), a G-protein coupled receptor consisting of G α and G β , and the subsequent activation of several kinase pathways, including cAMP- and phosphoinositol-dependent kinase, and the mTORC1/RPS6 pathway [115, 116]. In addition to the activation of several thyroid hormone biosynthetic genes, including *Nis* and *Pds*, persistent high TSH levels induce activation of many cell proliferation regulatory genes, including cyclins A2, B1 and B2 (*Ccna2*, *Ccnb1*, *Ccnb2*), and cell division cycle-associated protein 2 (*Cdca2*). Blood TSH levels are persistently elevated in low iodide diet resulting in increased proliferation of thyroid follicular cells and a significantly enlarged thyroid gland (goiter) (Fig. 3). In contrast to wild-type mice fed a low iodide diet, elevated TSH levels in GLIS3-deficient mice fed either a normal or low iodide diet do not induce expression of *Nis* and *Pds* or cell proliferation regulatory genes, such as *Ccna2*, *Ccnb1*, and *Cdca2*, and mice do not develop an enlarged thyroid gland. The lack of a TSH response is not due a loss of TSHR since *Tshr* expression was not altered in GLIS3-deficient mice. Together, these observations suggest that GLIS3 acts downstream of TSH/TSHR and mediates the transcriptional

activation of *Nis* and *Pds* by TSH (Fig. 3). The TSH-dependent induction of cell cycle genes involves activation of the mTORC1/RPS6 pathway. The repression of most cell cycle genes in GLIS3-deficient mice does not involve direct transcriptional regulation by GLIS3, but appears to involve inhibition of RPS6 kinase activation.

GLIS3 and pancreas development

Studies demonstrating that loss of GLIS3 function in humans and mice causes neonatal diabetes indicated a regulatory role for GLIS3 in pancreatic β cells [1, 4, 5, 13, 22, 28, 42–50]. The development of hyperglycemia and hypoinsulinemia in GLIS3 deficiency was found to be related to aberrant pancreatic β cell generation and insulin production, suggesting that GLIS3 may have a role in the regulation of pancreas development. In the mouse, pancreas organogenesis starts at about E9.5 when multipotential pancreatic cells (MPCs) emerge from the foregut endoderm and form the dorsal and ventral pancreatic buds, which subsequently undergo extensive expansion and branching (Fig. 4) [117,

Fig. 4 Schematic of the multiple functions of GLIS3 in the pancreas. GLIS3 protein is first detectable in bipotent cells (BPCs) and is not expressed in multipotent progenitor cells (MPCs) or acinar cells. GLIS3 protein remains expressed in preductal and ductal cells and in endocrine progenitors. When the endocrine progenitors differentiate into the five endocrine cell types, it remains expressed in β and PP cells, but is repressed in α , γ , and δ cells. GLIS3 deficiency inhibits differentiation of BPCs into NGN3⁺ endocrine progenitors and as a consequence the generation of endocrine cells. In addition, loss of GLIS3 function inhibits β cell maturation and greatly reduces insulin gene expression. GLIS3 deficiency also causes dilation of the pancreatic ducts and repression of *Ppy* expression in PP cells



118]. By E12.5, the MPCs expressing PDX1, PTF1A, NKX6.1, HNF1 β , and SOX9 become restricted to the branch tips, where they give rise to preacinar cells and subsequently mature acinar cells, while the trunk regions differentiate into PDX1⁺PTF1A⁻NKX6.1⁺HNF1 β ⁺SOX9⁺ bipotent progenitors (BPs). GLIS3 protein is first observed in BPs and is not detectable in MPCs or acinar cells [119]. The BPs subsequently give rise to neurogenin 3⁺ (NGN3⁺) endocrine progenitors and preductal cells, which then mature into ductal cells. The endocrine progenitors delaminate from the trunk via a still poorly understood process that involves EMT, and differentiate along the five endocrine cell lineages, α , β , γ , δ , and PP cells that produce glucagon, insulin, somatostatin, ghrelin, and pancreatic polypeptide (PP), respectively, thereby leading to the formation of pancreatic islets. GLIS3 protein remains expressed in (pre)ductal cells, NGN3⁺ endocrine progenitors, and in insulin⁺ and PP⁺ cells, but is repressed in α , γ , and δ cells (Fig. 4). This differential pattern of GLIS3 expression raises the question whether GLIS3 might have a role in endocrine lineage determination.

In human patients with GLIS3 dysfunction, as well as in Glis3-deficient mice, the size of pancreatic islets and the number of β cells are greatly reduced leading to the development of neonatal diabetes [28, 45, 50]. This was found to be at least in part related to a reduction in the generation of NGN3⁺ endocrine progenitors during the secondary

transition stage (E13.5–E15.5) of pancreas development [5, 14, 28]. The decrease in endocrine progenitors appears to be in part due to suppression of NGN3 expression, a key transcription factor that is required for the commitment of BPs to endocrine progenitors [117, 118, 120]. GLIS3 was shown to regulate *Ngn3* transcription directly by binding to GLISBS in its promoter region [21, 22], likely in collaboration with other transcription factors, such as PDX1, SOX9, and HNF1 β [121, 122]. The inhibition of NGN3⁺ endocrine progenitors is at least in part responsible for the observed decrease in pancreatic β cell generation, smaller islet size, and the development of neonatal diabetes in GLIS3-deficient mice. In addition to endocrine progenitor and pancreatic β cell generation, evidence is accumulating that in postnatal mouse pancreas GLIS3 regulates the maintenance and maturation of β cells [14, 19, 20, 28, 119]. GLIS3 transcriptionally regulates the expression of *Ins1*, *Ins2*, and *Glut2* (*Slc2a2*), and the maturation markers, urocortin 3 (*Ucn3*) and *MafA* [123, 124], all of which are significantly down-regulated in postnatal GLIS3-deficient pancreas (Fig. 4). In addition to its role in β cells, GLIS3 regulates duct morphogenesis as well as the expression pancreatic polypeptide (*Ppy*) in PP cells [28]. GLIS3 deficiency causes significant dilation of pancreatic ducts and greatly reduces *Ppy* expression. Together these studies indicate that GLIS3 has multiple critical regulatory functions during pancreas development

and in the adult pancreas: regulating the development of endocrine progenitors, the generation and maturation of pancreatic β cells, regulation of insulin and Ppy expression, as well as in maintaining normal duct morphology (Fig. 4).

Although global knockout of *Glis3* results in neonatal diabetes, a pancreas-specific knockout of *Glis3* in *Glis3^{fl/fl}/RipCre* mice, expressing Cre under control of the rat insulin promoter, does not cause a diabetic phenotype [125, 126]. However, *Pdx1Cre*-mediated knockout of *Glis3* yielded a mixed phenotype depending on the efficiency of the *Pdx1Cre* with 30% of the mice developing severe diabetes around PND21, 55% became mildly diabetic with aging, while 15% did not develop diabetes. The diabetic phenotype correlated with the degree by which GLIS3 was knocked out indicating that it is dose dependent. These observations are consistent with the conclusion that GLIS3 is required for the maintenance of postnatal pancreatic beta cells.

Several studies have provided evidence for a possible role of GLIS3 in the control of pancreatic β cell proliferation and apoptosis. This is indicated by studies showing that *Glis3* heterozygous mice exhibit increased susceptibility to high fat diet-induced hyperglycemia and age-induced diabetes that appears to be associated with impaired proliferation and expansion of pancreatic β cells [126]. Tamoxifen-inducible, β cell-specific knockout of GLIS3 in *Glis3^{fl/fl}/Pdx1Cre^{ERT2}* mice led to hypoinsulinemia and hyperglycemia and was associated with decreased insulin expression and an increase in apoptotic β cells [125–127]. Using a model of pancreatic β cell-selective endoplasmic reticulum (ER) stress in NOD mice, an association was found between increased apoptosis and the down-regulation of *Glis3* and the anti-apoptotic gene, *Manf* [127]. This study further showed that GLIS3 heterozygous mice exhibit increased sensitivity to ER-stress induced apoptosis and diabetes. A different study reported that down-regulation of GLIS3 expression by siRNAs in rat pancreatic β cells INS-1E enhanced cell death via the intrinsic apoptotic pathway that involved modulation of the alternative splicing of the pro-apoptotic favoring the expression of BIM_s, its pro-apoptotic form BIM [128]. Further studies are needed to understand the molecular mechanisms by which GLIS3 regulates various pancreatic β cell functions and how this relates to the development of diabetes in GLIS3-deficiency.

GLIS3 and regulation of insulin transcription

In addition to regulating pancreatic β cell generation, GLIS3 plays a critical role in the regulation of mouse insulin (*mIns2*) gene expression [1, 19, 20, 28, 45, 119]. The β cells in postnatal pancreas from GLIS3-deficient mice contain fewer and smaller insulin-containing granules than WT cells and exhibit a greatly reduced expression of *mIns2* mRNA [119]. This is further supported by data showing that ectopic

expression of GLIS3 can induce *Ins2* expression in β cell lines. *Ins2* expression is under complex control and a number of transcription factors, including Pdx1, MafA, and NeuroD1, which bind, respectively, A-, C-, and E-boxes in the proximal *Ins2* promoter, have been implicated in the regulation of *Ins2* transcription [123, 129]. *Ins2* promoter analysis showed that GLIS3 can efficiently activate the *Ins2* promoter by binding two GLISBS in its proximal promoter region. Mutation of both GLISBS sites abolishes the GLIS3-mediated activation [19]. The transcriptional activation by GLIS3 was mediated through its interaction with the co-activator CBP/p300. ChIP-Seq analysis supported the conclusion that GLIS3 regulates *Ins2* transcription directly. Moreover, GLIS3 binding is required for optimal activation of the *Ins2* promoter by PDX1, MAFA, and NEUROD1 [19, 20]. We proposed that GLIS3–CBP/p300 coactivator complex might help recruiting PDX1, MAFA, and NEUROD1 or stabilize their interaction with their respective DNA binding sites. GLIS3 was also shown to directly regulate *MafA* expression [20, 28]. Thus, the down-regulation of MAFA in GLIS3-KO islets likely contributes to the repression of *Ins2* activation. Together, these data suggest that GLIS3 appears to regulate *Ins2* transcription through several different mechanisms.

GLIS3 is essential for spermatogenesis

Glis3 is highly expressed in the testis where it plays a critical role in the regulation of early postnatal spermatogenesis [16, 130]. In the mouse testis at PND1, nondividing gonocytes migrate to the basal compartment of the seminiferous tubule where they differentiate into self-renewing spermatogonial stem cells (SSCs), which are distinguished by the high expression of inhibitor of DNA binding 4 (*Id4*) [131–134]. The SSCs sequentially differentiate into several subsets of spermatogonial progenitor cells (SPCs) that are classified as A-single (A_s), A-paired (A_{pr}), and A-aligned spermatogonia (A_{al4} , A_{al8} , A_{al16}) consisting of 1, 2, 4, 8 and 16 cell(s), respectively (Fig. 5). The SSCs and SPCs together are referred to as undifferentiated spermatogonia and share the expression of a number of genes implicated in SSC maintenance and self-renewal, and PGC development, including *Pax7*, *Etv5*, *Gfra1*, *Zbtb16* (*Plzf*), *Lhx1*, *Sall4*, *Lin28*, and *Nanos2*. The A_{pr} and A_{al} SPCs are transient amplifying cells that become irreversibly committed to differentiation into type B spermatogonia [131–134]. This is accompanied by down-regulation of the expression of various genes, including *Gfra1*, *Zbtb16*, E-cadherin (*Cdh1*), *Nanos2*, and *FoxO1* and induction of *cKit*. The type B spermatogonia then give rise to meiotic spermatocytes, which after meiosis is completed, differentiate into spermatids and ultimately mature spermatozoa. During postnatal spermatogenesis, GLIS3 protein is detectable in gonocytes, and most GFRA1⁺ and PLZF⁺ SSCs and SPCs, but not in differentiated c-Kit⁺

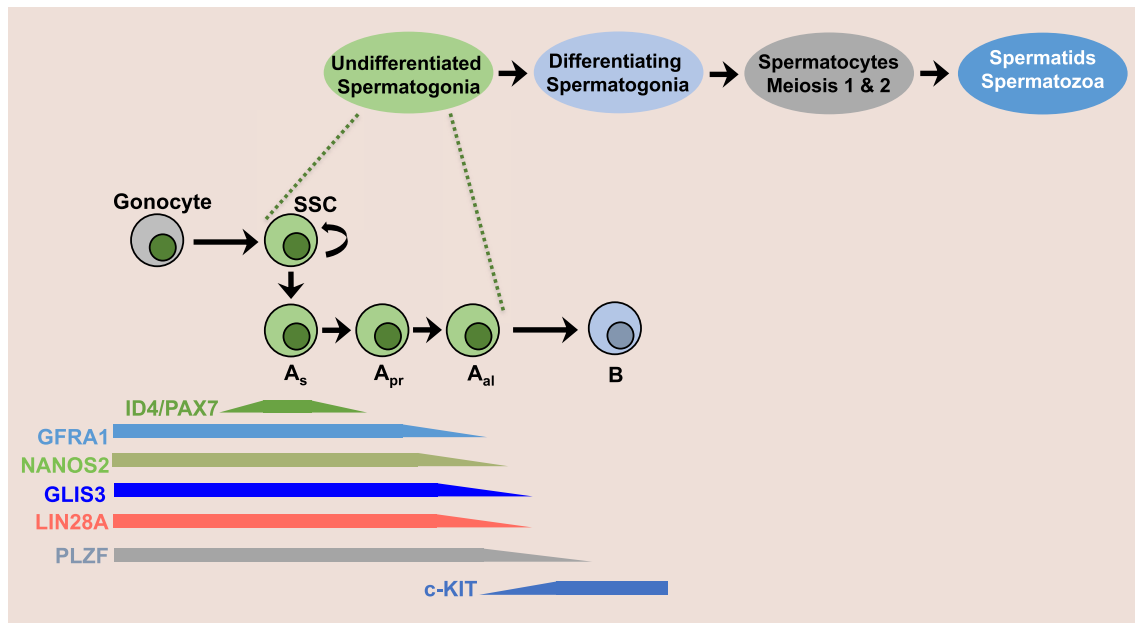


Fig. 5 GLIS3 is required for early postnatal spermatogenesis. At PND1, non-mitotic gonocytes differentiate into spermatogonial stem cells (SSCs), which give rise to several spermatogonial progenitor (SPCs) subpopulations (A_s , A_{pr} , A_{al}) with decreasing self-renewal capacity. These cells subsequently give rise to c-KIT⁺ differentiated

spermatogonia and eventually mature spermatozoa. GLIS3 expression is restricted to gonocytes, SSCs, and SPCs. Loss of GLIS3 expression affects self-renewal of SSCs and early differentiation and impairs the generation of c-KIT⁺ differentiated spermatogonia leading to infertility

spermatogonia and germ cells at later stages [130] (Fig. 5). GLIS3 expression is lost during the A_{al} stages, after the down-regulation of GFRA1, but before the suppression of PLZF. GLIS3 is not detectable in Sertoli or Leydig cells. The number of undifferentiated PLZF⁺ spermatogonia is significantly decreased in testes from PND1 and PND7 GLIS3-deficient mice [130]. This decrease appeared not to be due to increased apoptosis, but to inhibition of differentiation and/or cell proliferation of SSCs and SPCs. As a consequence, the number of differentiated c-Kit⁺ spermatocytes, as well as the number of HSPA2⁺ cells, representing meiotic and post-meiotic cells, was greatly diminished in testes of Glis3-KO mice. Histological observations showed that spermatids and spermatozoa were largely absent in 3–5 weeks old Glis3-KO testes. This was accompanied by a dramatically reduced expression of genes associated with meiosis, spermatids and spermatozoa, including aurora kinase C (*Aurkc*), protamines 1 and 2 (*Prm1*, *Prm2*), sperm motility kinase 2a (*Smok2a*), and spermatogenesis associated 3 (*Spata3*) [130]. Together, these studies identify GLIS3 as a key regulator of early stages of postnatal spermatogenesis and demonstrate that loss of GLIS3 function greatly impairs the generation of spermatozoa, thereby rendering the Glis3-KO males infertile.

GDNF produced by Sertoli cells plays a critical role in regulating the proliferation and differentiation of SSCs and SPCs through action of its receptor, the GFRA1–RET

complex [131, 133]. The expression of *GFRA1* and *RET* was significantly decreased in Glis3-KO testis as was the expression of several GDNF-dependent genes that are important in SCC maintenance, including *Etv5*, *Lhx1*, and Brachyury homolog (*T*) [130]. The expression of the transcription factors, *Cxcr4*, *Zbtb16*, *Ngn3* and *Dmrt1*, and the RNA binding proteins, *Nanos2*, *Nanos3* and *Lin28a*, which are involved in the regulation of the maintenance proliferation, and differentiation of undifferentiated spermatogonia was also diminished in Glis3-KO testis. NANOS2 and LIN28A have been reported to be important in regulating self-renewal of SSCs and proliferation of SPCs, respectively [135, 136]. The expression of Piwi Like RNA-Mediated Gene Silencing 4 (*Piwil4*), which is expressed in gonocytes and SSCs [137], as well as several other genes involved in retrotransposon silencing suggest that GLIS3 might be involved in regulating the repression of transposable elements [130]. These studies suggest that GLIS3 may play a critical role in regulating the dynamics of the gonocytes–SSC–SPC transition and the proliferation and differentiation of these cells. The observation that the translocation of FoxO1 to the nucleus, which marks the gonocyte-to-SSC transition and is required for SSC proliferation [138], was significantly inhibited in Glis3-KO testes consistent with the hypothesis that GLIS3 might positively regulate the gonocyte-to-SSC transition and SCC self-renewal [130]. This is supported by data showing that the expression of FoxO1 target genes, *Lhx1* and *Sall4*, and

that of *Id4*, a specific marker of SSCs, were greatly reduced in Glis3-KO testes.

GLIS1 functions

Relatively little is known about the physiological functions of GLIS1. In postnatal mouse testis, *Glis1* is expressed in spermatogonia, but not in pachytene spermatocytes and spermatids [15]. *Glis1* null mice are fertile and display abnormalities in spermatogenesis upon aging. *Glis1* is also highly expressed in mouse and bovine oocytes and 1-cell embryos. Glis1 mRNA expression is significantly reduced in 2-cell embryos and is no longer detectable in 4-cell mouse embryos and blastocysts or 8-cell bovine embryos [100, 139]. Down-regulation of GLIS1 in 4-cell bovine embryos by siRNAs resulted in defective development of embryos beyond the 16-cell stage, a time at which the developmental control by maternal transcription factors is shifted to embryonic control, a process referred to as zygotic gene activation (ZGA). The expression of PDHA1 and HSPA8, which are induced after ZGA, was significantly decreased in Glis1-downregulated bovine embryos suggesting that ZGA is not proceeding [139]. Together these observations suggest that GLIS1 is important for preimplantation development; however, whether GLIS1 is required for ZGA or whether it affects development at a preceding stage needs further study.

A recent study reported a positive role for GLIS1 in brown adipocyte differentiation [140]. This was indicated by data showing that exogenous expression of GLIS1 in mouse myoblast C2C12 cells decreases the expression of myogenic markers and enhances adipogenic marker expression, whereas down-regulation of GLIS1 by siRNAs has the inverse effects. This association was supported by the observed increase in Glis1 expression during brown adipocyte differentiation in C2C12 cells in which Kdm1a (Lsd1) expression was down-regulated.

Functions of GLIS2 in the kidney

During embryonic kidney development, *Glis2* is well expressed in the ureteric bud, which subsequently forms the collecting ducts, but at low levels in S-shaped bodies that will form the nephrons [2, 88]. However, at later stages of development *Glis2* is also detectable in renal tubules. *Glis2* is highly expressed in the adult kidney [8]. As discussed above, loss of GLIS2 function causes nephronophthisis, a cystic kidney disease characterized by cyst formation, renal atrophy, inflammation, and fibrosis [2, 88–91]. GLIS2 deficiency in mice is also associated with an increase in apoptotic tubular cells and interstitial infiltration of inflammatory cells [2]. Progression of the disease leads to the development of proteinuria, elevated blood levels of urea nitrogen and creatine, and ultimately end-stage renal disease. Gene

expression profiling showed that kidneys from Glis2-null mice exhibited increased expression of several inflammatory genes (e.g., *Cxcl10*, *Ccl2*, *Cx3cl1*) and genes related to fibrosis (e.g., *Col1a1*, *Col3a1*, *Ltbp1*), apoptosis (e.g., *Casp4*), and EMT (e.g., *Snai1*, *Fsp1/S100A4*, *Ctgf*, *Tgfb1*) [2, 88]. The increased expression of these genes at least partially explains the development of inflammation, fibrosis, and renal atrophy in Glis2-deficient kidneys. The increased inflammatory response likely contributes significantly to the progression of nephronophthisis.

Observations showing that kidney tubule cells in Glis2-knockout mice express a number of mesenchymal markers is consistent with the concept that they undergo EMT [2, 88]. This is further supported by studies showing that down-regulation of GLIS2 expression in murine inner medullary collecting duct IMCD3 cells, in which GLIS2 is abundantly expressed, leads to increased cell migration and changes in gene expression that are characteristic of EMT [18]. This includes suppression of E-cadherin and induction of FSP1. In addition, down-regulation of GLIS2 significantly increased the expression of *Gli1* as well as two GLI1 target genes, *Snai1* and *Wnt4*. The transcription factor SNAI1 promotes EMT by inhibiting E-cadherin (*Cdh1*) transcription and inducing the expression of mesenchymal markers, such as vimentin and fibroblast-specific protein 1 (FSP1 or S100A4). ChIP analysis demonstrated that GLIS2 was associated with the upstream promoter regulatory region of *Snai1* and *Wnt4* and repressed the activation of the *Snai1* and *Wnt4* promoter. Together, these findings led to the hypothesis that GLIS2 negatively regulates *Snai1* and *Wnt4* transcription through both an indirect and direct mechanism by, respectively, repressing the expression of the positive regulator *Gli1* and by directly binding to GLISBS in the *Snai1* and *Wnt4* promoters thereby competing with GLI1 for the same sites [18]. Thus, GLIS2 acts as a suppressor of the SHH/GLI signaling pathway and thereby helping to maintain the epithelioid phenotype of renal tubule cells.

A recent study showed that deficiency in GLIS2 function suppresses the growth of cysts in Kif3a knockout kidneys [141]. Kidney-specific knockout of Kif3a (^{Ksp-Cre}Kif3a^{fl/-}), one of the kinesins required for primary cilium formation, causes loss of primary cilia and development of polycystic kidneys [142]. This leads to increased blood urea nitrogen and serum creatinine levels, renal failure and death around the age of 6 weeks. Crossing these mice with Glis2-deficient mice suppressed proliferation of renal tubule cells, decreased DNA damage and apoptosis, and led to reduced growth of renal cysts and improved kidney function. Thus, GLIS2 deficiency was able to partially rescue the polycystic phenotype in ^{Ksp-Cre}Kif3a^{fl/-} mice [141]. Immortalized tubular epithelial cells derived from these Kif3a null mice also exhibited accelerated cell cycle progression, decreased p53 stability, and increased DNA damage and apoptosis, while

down-regulation of GLIS2 by shRNA reversed this phenotype. In addition, loss of GLIS2 function in Kif3a null as well as wild-type kidney tubule cells induced senescence as indicated by the positive staining for the senescence markers, H3K9me3 and β -galactosidase.

Unilateral ureteral obstruction, a mouse model of kidney injury, caused more severe injury and dilated tubules in kidneys of heterozygous *Glis2* null mice than WT mice [143]. *Glis2* mutant tubular epithelial cells appeared less differentiated and interstitial collagen depositions were increased, which was at least in part related to enhanced proliferation of pericytes and interstitial fibroblasts due to increased expression of SHH in heterozygous *Glis2*-deficient kidneys.

Functions of GLIS3 in the kidney

Glis3 is highly expressed in the kidney, where it localizes to the epithelial cells of renal tubules, collecting ducts, and Bowman's capsule [2]. Although not in all human patients, *GLIS3* deficiency in humans and mice leads to the development of polycystic kidney disease [5, 13, 45, 46, 49]. *Glis3*-KO mice develop prominent glomerulocysts as well as dilated distal tubules and collecting ducts. Deficiency in Zinc Finger E-Box Binding Homeobox 2 (ZEB2), a SMAD-interacting transcription factor, also causes glomerulocystic kidney disease [144]. This is associated with decreased *GLIS3* expression suggesting that it might be part of the mechanism by which ZEB2 deficiency causes glomerulocyst formation. Similarly, knockout of hepatocyte nuclear factor 1b (HNF1b) in mice causes polycystic kidney disease as well as cyst formation in pancreatic ducts [121, 145]. The down-regulation of *Glis3* expression in HNF1b-KO pancreas may be causally related to the development of dilated ducts.

MRI studies of polycystic kidneys from *GLIS3*-deficient mice showed that kidney volume, cyst volume and cyst-to-kidney volume ratio, increased significantly with age and progression of the disease [51]. Treatment of *Glis3* null mice with rapamycin inhibited the progression of polycystic kidney disease, but did not improve renal functions significantly. The molecular mechanism(s) involved in polycystic kidney disease are still poorly understood. Changes in cell proliferation, planar cell polarity, and defects in primary cilium structure or signaling have been implicated in polycystic kidney disease [146–150]. Inflammation and fibrosis also play an important role in the pathogenesis of cystic renal disease [151].

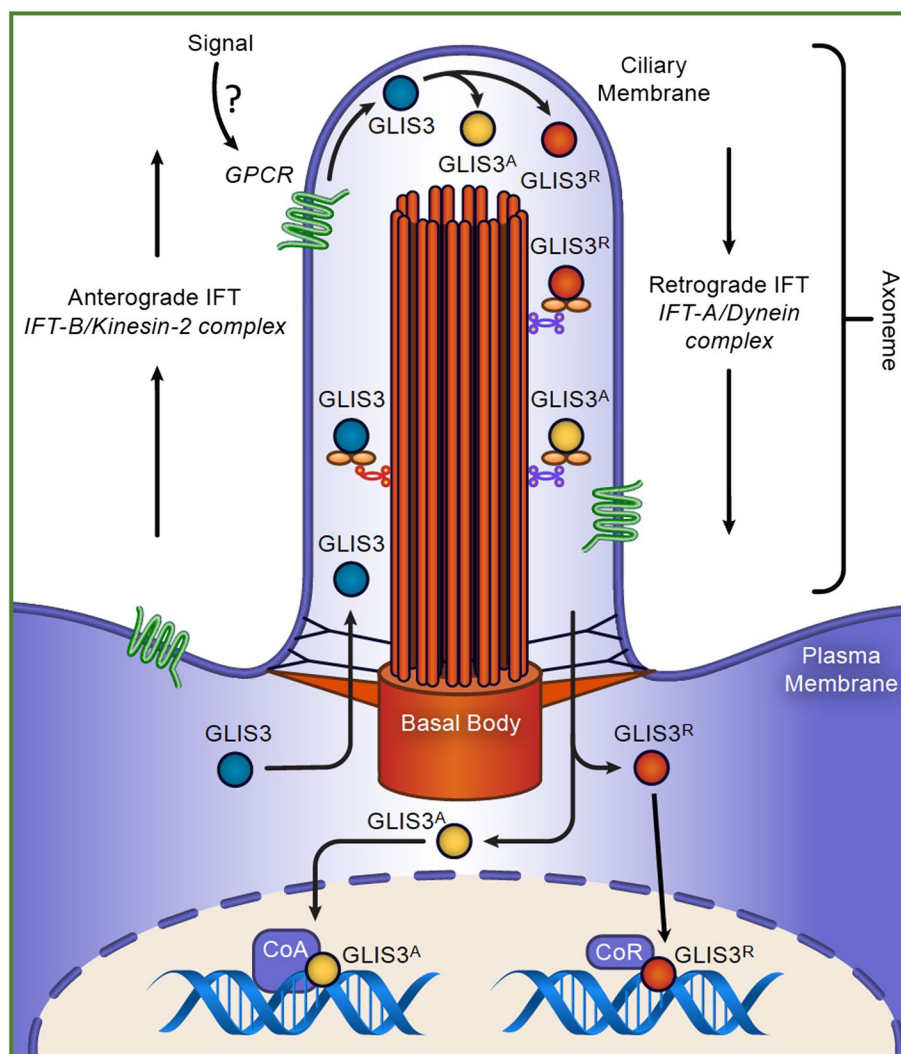
GLIS proteins, the primary cilium, and cystic kidney disease

The primary cilium is a non-motile, microtubule-based organelle that protrudes from the surface of many eukaryotic cells [150, 152–155] (Fig. 6). It constitutes a subcellular

compartment that allows enrichment of membrane receptors and their downstream signaling molecules, thereby serving as an important sensory organelle and signaling hub. An increasing number of membrane receptors for a wide variety of signals as diverse as peptide hormones, neurotransmitters, odorants, lipids, and light, have been associated with the primary cilium. These include many G protein-coupled receptors (GPCRs), including receptors for Sonic Hedgehog (SHH), WNT, and PDGFA, somatostatin, dopamine, and serotonin [152, 156, 157]. In addition to GPCRs, several Ca^{2+} channels have been reported to localize to the primary cilium, including the transient receptor potential (TRP) channels, TRPM4 and TRPV4, and the calcium-activated chloride channel ANO1 [158–160]. The primary cilium plays a key role in the regulation of embryonic development, proliferation, and autophagy. Disruption of the primary cilium or associated signaling pathways has been implicated in a broad range of genetic disorders, collectively referred to as ciliopathies. This includes autosomal recessive and autosomal dominant polycystic kidney disease (ARPKD and ADPKD, respectively), nephronophthisis (NPHP), Bardet–Biedl syndrome, Joubert syndrome and Meckel–Gruber syndrome, cancer, and several neuropsychiatric disorders [152, 153, 161–164]. Polycystin PC2, a member of the TRP family, forms with polycystin PC1 a receptor-channel complex that is localized to the primary cilium [147, 165]. Mutations in PC1 and PC2 are implicated in the majority of autosomal dominant polycystic kidney disease PKD1 and PKD2, respectively, while PKHD1, encoding fibrocystin, is associated with autosomal recessive polycystic kidney disease.

Study of the subcellular localization of *GLIS3* protein has indicated that it largely localizes to the nucleus in cells *in vivo* and when it is ectopically expressed in cultured cells [16, 23, 24, 119]. Immunohistochemistry using proximal tubule TKPTS cells ectopically expressing *GLIS3*-EGFP and kidney sections from transgenic mice expressing an *GLIS3*-EGFP fusion protein, showed that in addition to its nuclear localization, *GLIS3* is also detectable in the tip of the primary cilium [49]. This is supported by proteomics identifying *GLIS3* as a primary cilium-associated protein [166]. A recent study showed that the localization of *GLI1–3* proteins to the primary cilium is mediated by transportin 1 (TNPO1, also named importin 1 or karyopherin beta-2), which recognizes a primary cilium localization signal (CLS) in the NCR of *GLI1–3* proteins [167]. Interestingly, *GLIS3* also contains this CLS consensus motif within its NCR (Fig. 1) suggesting that its primary cilium localization might be mediated by a similar mechanism. It is further interesting to note that *GLIS3* interacts with *SUFU*, a protein that has been demonstrated to interact and co-localize with *GLI* proteins to the tip of the primary cilium [9, 10, 28, 166]. The ciliary localization of *GLIS3* raises the question whether

Fig. 6 Regulation of GLIS3 activity and function by an upstream primary cilium-associated signaling pathway. Several studies have provided evidence for the localization of GLIS3 to the primary cilium. TNPO1-GLIS3 interaction allows GLIS3 to enter the primary cilium, where it is transported to the tip of the primary cilium by the anterograde intraflagellar transport (IFT) system. We hypothesize that activation of a GPCR localized to the ciliary membrane by an external signal modulates GLIS3 activity possibly through posttranslational modification or proteolytic cleavage. This may lead to the generation of either a GLIS3 activator (GLIS3^A) or GLIS3 repressor (GLIS3^R). The retrograde IFT mediates the transportation of GLIS3 out of the primary cilium. GLIS3 then regulates target gene transcription after its translocation to the nucleus



GLIS3 activity is under the control of an external signal that acts through a specific primary cilium-associated GPCR, as has been demonstrated for the hedgehog-GLI pathway [4, 155, 168]. We hypothesize that GLIS3 activity is regulated by an as-yet unknown external signal that activates a distinct GPCR signaling pathway that subsequently modulates GLIS3 activity and promotes GLIS3 nuclear localization [4] (Fig. 6). This modulation of GLIS3 activity might involve posttranslational modification (e.g., phosphorylation) and/or proteolytic cleavage of GLIS3. Further studies are needed to identify such upstream signal(s) to understand the role of the primary cilium in the control of GLIS3 activity. The identification of such a signal might open possibilities to regulate GLIS3 activity in vivo and as such might provide opportunities for the development of new therapeutic strategies for diabetes, hypothyroidism, and other diseases.

Deficiency in GLIS2 function leads to the development of nephronophthisis, a different type of renal ciliopathy [49, 88]. Evidence has been provided indicating that GLIS2

interacts with SUFU and might localize to the primary cilium [18]; however, GLIS2 does not contain a CLS consensus sequence similar to that of GLIS3. Whether GLIS2 localizes to the primary cilium in vivo and whether this is part of a physiologically relevant signaling pathway needs further study. Nothing is known about the potential ciliary localization of GLIS1.

Summary and conclusions/concluding remarks

Recent studies revealed that the GLIS1–3 transcription factors function as critical regulators of embryonic development and many biological processes. Significant molecular insights have been obtained about the mechanisms by which GLIS3 regulates thyroid hormone biosynthesis, pancreatic β cell generation and insulin expression, and spermatogenesis, as well as the role of GLIS2 in the maintenance of

normal kidney functions. These studies have provided not only a better understanding of these biological processes, but also provided insights into the mechanisms by which GLIS3 dysfunction leads to hypothyroidism, diabetes, and infertility and GLIS2-deficiency results in nephronophthisis. Future studies are needed to elucidate the roles of GLIS1–3 in other biological processes and pathologies, such as the role of GLIS3 in the development cystic kidney disease, as well as their emerging regulatory functions of GLIS1–3 in different stem/progenitor populations. Progress would highly benefit from the availability of good GLIS antibodies, which have been a limiting factor in these studies. In addition, future studies have to determine the roles of different posttranslational modifications and GLIS-interacting proteins in the modulation of GLIS1–3 activity and function. Increasing evidence showing that GLIS2 and GLIS3 localize to the primary cilium and might be part of a primary cilium-associated GPCR signaling pathway is very exciting. Further insights into the nature of this upstream signaling pathway may allow regulation of GLIS activity by small molecules and provide opportunities for the development of new therapeutic strategies for diabetes, hypothyroidism, cystic kidney disease and other pathologies.

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