Evolutionary History of GLIS Genes Illuminates Their Roles in Cell Reprograming and Ciliogenesis

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Abstract

The GLIS family transcription factors, GLIS1 and GLIS3, potentiate generation of induced pluripotent stem cells (iPSCs). In contrast, another GLIS family member, GLIS2, suppresses cell reprogramming. To understand how these disparate roles arose, we examined evolutionary origins and genomic organization of GLIS genes. Comprehensive phylogenetic analysis shows that GLIS1 and GLIS3 originated during vertebrate whole genome duplication, whereas GLIS2 is a sister group to the GLIS1/3 and GLI families. This result is consistent with their opposing functions in cell reprogramming. Glis1 evolved faster than Glis3, losing many protein-interacting motifs. This suggests that Glis1 acquired new functions under weakened evolutionary constraints. In fact, GLIS1 induces induced pluripotent stem cells more strongly. Transcriptomic data from various animal embryos demonstrate that glis1 is maternally expressed in some tetrapods, whereas vertebrate glis3 and invertebrate glis1/3 genes are rarely expressed in oocytes, suggesting that vertebrate (or tetrapod) Glis1 acquired a new expression domain and function as a maternal factor. Furthermore, comparative genomic analysis reveals that glis1/3 is part of a bilaterian-specific gene cluster, together with rfx3, ndc1, hspb11, and lrc46. Because known functions of these genes are related to cilia formation and function, the last common ancestor of bilaterians may have acquired this cluster by shuffling gene order to establish more sophisticated epithelial tissues involving cilia. This evolutionary study highlights the significance of GLIS1/3 for cell reprogramming, development, and diseases in ciliated organs such as lung, kidney, and pancreas.

Key words: ortholog group, gene duplication, neofunctionalization, comparative transcriptomics, microsynteny, ciliogenic gene cluster.

Introduction

Among Krüppel-like zinc-finger transcription factors, GLI-similar transcription factors (GLIS) constitute a large family, together with GLI and ZIC (Hatayama and Aruga 2010; Kang et al. 2010; Scoville et al. 2017; Aruga and Hatayama 2018). GLI, GLIS, and ZIC share a DNA-binding domain consisting of five C2H2 zinc-finger domains, two of which, near the N-terminus, are characterized as a tandem pair of CWCH2 motifs (Hatayama and Aruga 2010; Aruga and Hatayama 2018). It has been proposed that GLI, GLIS, and ZIC originated from a common ancestral gene and that ZIC is an early branching gene group, relative to GLI and GLIS (Layden et al. 2010; Aruga and Hatayama 2018). However, phylogenetic relationships between GLI and GLIS have never been determined, possibly because zinc-finger domains from only a small number of taxa have been used for phylogenetic analysis (Kim et al. 2003; Kang et al. 2010; Layden et al. 2010; Aruga and Hatayama 2018).

In mammals, three GLIS genes (GLIS1-3) have been identified and their embryonic expression patterns in mice and adult organ expression levels in mice and/or humans have been determined (reviewed in Kang et al. [2010]). All three genes are most abundantly expressed in kidney, with moderate expression in various other organs: GLIS1 is expressed in brain, thymus, adipose tissue, colon, testis, and placenta (Kim et al. 2002; Nakashima et al. 2002); GLIS2 is in brain, lung, heart, esophagus, intestine, colon, thyroid, liver, and prostate (Zhang and Jetten 2001; Zhang et al. 2002); and GLIS3 is in brain, lung, thymus, thyroid, liver, pancreas, spleen, testis, ovary, and uterus (Kim et al. 2003; Senee et al. 2006; Beak et al. 2008). GLIS3 is also expressed in a number of human cancers, suggesting that elevated GLIS3 expression leads to cancer progression (Kang et al. 2010). Remarkably, only Glis1 is significantly expressed in early mouse embryos (oocyte to two-cell stage), possibly related to its proreprogramming functions, discussed below (Maekawa et al. 2011).

Knock-out mouse studies have revealed that Glis2 is essential for normal renal functions (Attanasio et al. 2007; Kim et al. 2008), and that Glis3 is required for kidney development, pancreatic β-cell development, and spermatogenesis.
GLIS1 and GLIS3 evolved as ohnologs in vertebrates. (A) A schematic phylogenetic tree of GLI/GLIS/ZIC genes supported by ORTHOSCOPE analysis and ML trees (see supplementary figs. S1–S4, Supplementary Material online, for more details). (B) Presumed evolution of protein–protein interaction motifs of GLIS1/3 after WGD in vertebrates. GLIS3 retains all conservative motifs in chordates, whereas GLIS1 lost many of them. Ubiquitination/SUMOylation motifs and an ITCH-binding motif are strongly conserved among GLIS1/3 in vertebrates. See supplementary figures S5 and S6, Supplementary Material online, for phylogenetic analysis and sequence alignment, respectively.

(Kang, Beak, et al. 2009; Kang, Kim, et al. 2009; Kang et al. 2016). In studies of kidney disease, Glis2 and Glis3 were localized to primary cilia (Attanasio et al. 2007; Kang, Beak, et al. 2009). GLIS2 dysfunction was responsible for nephronophthisis (NPHP), and loss of GLIS3-function leads to neonatal diabetes and hypothyroidism (NDH), polycystic kidneys, and other abnormalities (Jetten 2018). Notably, in generation of induced pluripotent stem cells (iPSCs) from human and mouse fibroblasts by so-called Yamanaka factors (Oct4, Sox2, Klf4, and c-Myc), replacement of c-Myc with GLIS1 mouse fibroblasts by so-called Yamanaka factors (Oct4, Sox2, Klf4, and c-Myc), replacement of c-Myc with GLIS1 decreases tumorigenicity (Maekawa et al. 2011).

In other vertebrates, Glis2 was first identified as a neuronal Krüppel-like protein (Nkl) in Xenopus, and misexpression of Glis2 induced extra primary neurons (Zhang and Jetten 2001). In zebrafish, Glis2 was described as NPHP7 and loss of function analysis showed that Glis2 is required for cilium motility (Kim et al. 2013). Loss of function experiments involving Glis3 led to a significant decrease in β-cell mass in zebrafish (O’Hare et al. 2016). Glis3 deficiency also resulted in a medaka mutant with shortened renal cilia and caused polycystic kidney disease (Hashimoto et al. 2009). These studies imply conserved roles of Glis2 and Glis3 for primary cilium function and kidney development, and Glis3 for pancreatic development in vertebrates. However, little is known about Glis1 functions in other vertebrates.

**Results and Discussion**

Phylogenetic Relationships of GLIS Genes Are Consistent with Differences in Gene Functions

To infer GLIS gene ancestry, we first performed comprehensive phylogenetic analysis using a species tree-based ortholog group identification tool, ORTHOSCOPE (Inoue and Satoh 2018). The result demonstrates that GLIS1 and GLIS3 are “ohnologs” derived from a single ancestral gene (GLIS1/3) via two-rounds of whole genome duplication (WGD) in vertebrates (supplementary figs. S1 and S2, Supplementary Material online). In addition, the orthologous gene group GLIS1/3 is a sister group to the GLI subfamily. On the other hand, GLIS2 and its invertebrate orthologs form an outgroup relative to GLI and GLIS1/3 (fig. 1A). These results were further validated by maximum likelihood (ML) trees constructed using the same sequence set as used with ORTHOSCOPE (supplementary figs. S3 and S4, Supplementary Material online). Therefore, these data clarify relationships between GLI, GLIS1, GLIS2, and GLIS3 with higher reliability than previous studies (Materna et al. 2006; Shimeld 2008; Hatayama and Aruga 2010; Layden et al. 2010).

The deep evolutionary origins of Glis1/3 and Glis2 are reconciled with their structural and functional differences. First, a nuclear localization signal is located at the fourth zinc-finger domain (ZF4) of Glis3, but at ZF3 of Glis2 (Beak et al. 2008; Vasanth et al. 2011; Hatayama and Aruga 2012). Second, Glis3 contains a ciliary localization signal in the N-terminal region that is conserved among Glis transcription factors and presumably binds to Transportin1 (TNPO1) (Han et al. 2017; Jetten 2018). However, the ciliary localization signal motif is not present in Glis2, although Glis2 is reportedly localized in the primary cilium (Attanasio et al. 2007; Jetten 2018). Third, Glis1 and Glis3 contain transactivation domains in the C-terminal region, and self-repressive domains in the N-terminal region (Kim et al. 2002, 2003). In contrast, Glis2 contains transactivation domains in the N-terminal region, and self-repressive domains in the C-terminal region (Zhang et al. 2002). Fourth, both GLIS1 and GLIS3 have comparable activity in reprogramming of human adipose-derived stromal cells, together with Yamanaka factors, whereas GLIS2 suppresses reprogramming (Lee et al. 2017).

The phylogenetic tree of Glis1/3 in chordates further demonstrates that Glis1 evolved faster than Glis3 after WGD in vertebrates (supplementary fig. S5, Supplementary Material online). This implies that Glis1 experienced additional evolutionary events such as acquisition of new functions, loss of ancestral functions, and adaptive evolution, whereas Glis3 retained ancestral form and functions. Actually, GLIS1 can replace Klf4 as a transcription factor for iPSC generation, but other GLI/GLIS/ZIC factors, GLI4, GLIS2, GLIS3, and ZIC4, cannot (Maekawa et al. 2011). Hereafter, we focus on similarities and differences between the ohnologs, Glis1 and Glis3.

Glis1 Lacks Several Conserved Motifs

The Cullin3 complex binds Glis3 for degradation via polyubiquitination, whereas Suppressor of Fused (Sufu) inhibits its degradation by binding to Glis3 via a YGH motif (ZeRuth et al. 2011). Similarly, an E3 ubiquitin ligase, Itch, reportedly binds to Glis3 via a PPPY motif for degradation.
(ZeRuth et al. 2015). For transactivation activity, a Hippo signal pathway regulator Wwtr1/TAZ binds to a P/LPXY motif in the C-terminal region of Glis3 but does not bind to Glis1 or Glis2 (Kang, Beak, et al. 2009). Glis3 also interacts with CBP/p300 through its C-terminal transactivation domain (ZeRuth et al. 2013). Interacting partner proteins of Glis1 and Glis2 have been little studied compared with those of Glis3.

Because of the deep origins of Glis1/3 and Glis2, sequence homology between Glis1 and Glis3 helps us to understand evolution of vertebrate ohnologs. As a result of its faster evolutionary rate (supplementary fig. S5, Supplementary Material online), Glis1 lost several protein motifs that are conserved in Glis3 and invertebrate Glis1/3 (supplementary fig. S6, Supplementary Material online). For example, Glis1 lacks a binding motif for Sufu, which is conserved in Glis3 and Glis3 (ZeRuth et al. 2011). Glis1 also lacks all putative phosphorylation sites identified in Glis3 (ZeRuth et al. 2015). On the other hand, a binding motif for Itch is widely conserved among chordate Glis1/3 genes. In addition, ubiquitination/sumoylation motifs are also conserved, suggesting that Itch is heavily involved in degradation of Glis1 and Glis3. Because invertebrate Glis1/3 does not have a Wwtr1-interacting motif, Glis3 may have acquired that motif for transactivation. Thus, Glis1 lost many ancestral features and may have changed its functions, whereas Glis3 is highly conserved and probably retains ancestral functions (fig. 1B). It is worth investigating whether Glis1 acquired new binding partners for cell reprogramming.

**Faster Evolving glis1/3 Genes Are Prone to Elimination from Genomes after Extra WGD**

We next performed synteny analysis to examine genomic organization of glis1 and glis3 (fig. 2A and B). When the human genome is compared with sarcopterygian and shark genomes, both loci are well conserved, whereas in actinopterygians, both loci are highly rearranged, with a few exceptions such as ndc1, lrrc42, and rfx3. Those will be further discussed below.

Among teleosts, medaka and zebrafish retain two glis1 genes (glis1a and glis1b) that originated from teleost-specific WGD, whereas fugu lost glis1a (fig. 2A). Because Glis1a sequences are more derived than Glis1b (supplementary fig. S5, Supplementary Material online), Glis1a must be less constrained and has been more easily eliminated during teleost evolution. By contrast, extant teleosts only retain a single copy of glis3 (fig. 2B), possibly due to rapid gene loss in an early stage of teleost evolution (Inoue et al. 2015). Because teleost glis3 genes have longer branch lengths in phylogenetic trees than other vertebrate glis3 genes (supplementary fig. S5, Supplementary Material online), teleost Glis3 possibly evolved under weakened functional constraints.

Another case of additional WGD in vertebrates is the African clawed frog, Xenopus laevis, which has an allotetraploid genome composed of two subgenomes, denoted L and S (Session et al. 2016). Surveying glis1/3 genes in X. laevis, we found that glis1 and surrounding genes have been eliminated from the S subgenome (fig. 2C), whereas the glis3 locus is conserved, except for copy number variations of crystallin gamma (cryg) genes (fig. 2D). As with Glis1a and Glis3 in teleosts, Xenopus Glis1 is more divergent among vertebrate Glis1 (supplementary fig. S5, Supplementary Material online). Taken together, loss of faster evolving genes in vertebrate genomes may reflect differences of evolutionary constraints on Glis1/3 functions.

**Expression Profiles Suggest Neofunctionalization of Glis1 in Vertebrate Oocytes**

In mice, Glis1 is enriched in unfertilized eggs and one-cell stage embryos (Maekawa et al. 2011). Transcriptomic data of mouse early embryos (Tang et al. 2011; Xue et al. 2013) further demonstrate that Glis1, but not Glis3, is maternally expressed (fig. 3A and supplementary fig. S7B, Supplementary Material online). However, in humans, GLIS1 and GLIS3 are not expressed in oocytes (supplementary fig. S7A, Supplementary Material online), suggesting gain or loss of maternal expression of Glis1 in mice or humans, respectively. To examine these possibilities, we next examined transcriptomic data from bovine preimplantation embryos (Jiang et al. 2014). The data showed that Glis1, but not Glis3, is expressed in bovine oocytes (supplementary fig. S7C, Supplementary Material online), supporting the possibility that the mammalian ancestor possessed Glis1 expression in oocytes, but that humans lost it.

To infer the origin of maternal expression of glis1, we further surveyed transcriptomic data of other vertebrates. Interestingly, transcriptomic data of chicken early embryos (Hwang et al. 2018) demonstrated that both glis1 and glis3 are expressed in oocytes (fig. 3B). High temporal-resolution transcriptomic data from Xenopus tropicalis embryos (Owens et al. 2016) showed that glis1, but not glis3, is expressed as a maternal factor (fig. 3C). In teleosts, both medaka and zebrafish transcriptomic data during developmental stages (Ichikawa et al. 2017; White et al. 2017) indicate that glis1/3 genes are not expressed in oocytes, except for medaka glis3, which exhibits fairly low-level expression (fig. 3D and supplementary fig. S7D, Supplementary Material online). These results indicate that glis1 may have been expressed maternally, at least in the tetrapod ancestor, and that lineage-specific gain and loss of maternal expression occurred for glis1/3 genes in several lineages. To determine the origin of maternal expression of glis1 in vertebrates, we need to examine more expression profiles, especially from basal vertebrates such as chondrichthyans and cyclostomes. Because the elephant shark genome retains more conserved synteny around glis1 and glis3 with tetrapods than those of actinopterygians (fig. 2A and B), it is possible that chondrichthys retain maternal expression of glis1 as an ancestral feature of gnathostomes.

Given the likely tetrapod origin of glis1 maternal expression, it is interesting that dmrtb1 (also called dmrt6) adjoins glis1 in tetrapod genomes (fig. 3A). DMRTB1 was also screened as a candidate proreprograming transcription factor, although DMRTB1 could not be replaced with c-MYC as GLIS1 could (Maekawa et al. 2011). In addition, transcriptomic data showed that dmrtb1 is also maternally expressed.
Evolutionary History of GLIS Genes

in human, mouse, and frog oocytes, but not in bovine or chicken oocytes (supplementary fig. S8, Supplementary Material online). Thus, it is worth examining the possibility that GLIS1 and DMRTB1 work collaboratively as maternal factors. It should be noted that dmrtb1 was eliminated from both subgenomes of *X. laevis* (fig. 2C), as previously described (Watanabe et al. 2017), although it is maternally expressed in *X. tropicalis* (supplementary fig. S8, Supplementary Material online). This fact implies that maternal gene regulatory networks vary considerably between *X. laevis* and *X. tropicalis*.

To reveal the ancestral expression pattern of glis1/3 before WGD, we further examined transcriptomic data of bilaterian embryos. Recently published transcriptomic data of the cephalochordate, *Branchiostoma lanceolatum* (Marletaz et al. 2018), showed that glis1/3 is not expressed in eggs and early stage embryos (fig. 3F) just as glis3 is not in many vertebrates (fig. 3A, C, and D and supplementary fig. S7A–D, Supplementary Material online). Among nonchordate deuterostomes, glis1/3 is maternally expressed in the sea urchin *Strongylocentrotus purpuratus*, as shown by QPCR (quantitative polymerase chain reaction) (Materna et al. 2006) and RNA-seq (Tu et al. 2012) (fig. 3F). However, transcriptomic data of another sea urchin, *Paracentrotus lividus* (Gildor et al. 2016), showed that glis1/3 is not expressed in eggs (supplementary fig. S7F, Supplementary Material online), indicating that glis1/3 is not necessarily a maternal factor in deuterostomes. In protostomes, glis1/3 is rarely expressed in eggs and early embryos, as shown in transcriptomic data from brachiopods (Luo et al. 2015) and scallops (Wang et al. 2017) (fig. 3G and supplementary fig. S7F, Supplementary Material online). These data suggest that, after WGD, glis3 retained ancestral expression patterns, whereas glis1 may have acquired new expression domains and functions in vertebrate (or tetrapod) oocytes.

**glis1/3 Retains Evolutionarily Conserved Microsynteny in Bilaterians**

To further examine conserved microsynteny around the glis1/3 locus in animals, we surveyed invertebrate genomic data. We found that glis1/3, rfx3, ndc1, hspb11, and lrrc42 are clustered in 100–600-kb regions of most bilaterian genomes (fig. 4A and supplementary table S1, Supplementary Material online). *Drosophila* lost two genes and the remaining three
**FIG. 3.** *glis1* is maternally expressed in tetrapods, but vertebrate *glis3* and invertebrate *glis1/3* are rarely expressed as maternal factors. Expression levels of *glis1/3* genes in various animal embryos are shown in graphs. Individual data from biological replicates are indicated in orange circles for *glis1* (or *glis1a* in teleosts), blue squares for *glis3* (or *glis1/3* in invertebrates), and green triangles for *glis1b* in teleosts. Lines represent the average of biological replicates. ZGA represents the period of zygotic gene activation, proposed for each species or closely related species (Tadros and Lipshitz 2009; Yang et al. 2016; Jukam et al. 2017). (A) Early mouse embryos (Xue et al. 2013). *Glis1* but not *Glis3* is expressed before ZGA and immediately degraded after ZGA in mouse. RPKM, reads per kilobase of exon per million mapped reads. See supplementary figure S7A–C, Supplementary Material online, for more data sets from mammals (human, mouse, and bovine). (B) Early chicken embryos (Hwang et al. 2018). Both *glis1* and *glis3* are expressed in chicken oocytes but greatly reduced in zygotes afterward. (C) Frog (*X. tropicalis*) embryos (Owens et al. 2016). *glis1* is expressed maternally at levels of >10,000 transcripts per egg (0hpf) or embryo (others), whereas *glis3* is only expressed zygotically in *Xenopus*. (D) Medaka embryos (Ichikawa et al. 2017). *glis3* is weakly expressed before ZGA but *glis1a* and *glis1b* are not in medaka. TPM, transcripts per million. See supplementary figure S7D, Supplementary Material online, for zebrafish data. (E) Amphioxus embryos (Marletaz et al. 2018). *glis1/3* is hardly expressed before 18 hpf (neurula) in amphioxus. cRPKM, corrected (per mappability) reads per kb of mappable positions and million reads. (F) Sea urchin (*S. purpuratus*) embryos (Tu et al. 2012). *glis1/3* is maternally expressed but is greatly reduced at ZGA in *S. purpuratus*. See supplementary figure S7F, Supplementary Material online, for data from another sea urchin (*P. lividus*). (G) Brachiopod embryos (Luo et al. 2015). *glis1/3* is rarely
genes are distantly located on the same chromosome, whereas interestingly, octopus retains the intact gene cluster. We also examined synteny conservation of genes neighboring the cluster in humans, amphioxus, octopuses, and brachiopods, genomes of which contain clusters that are almost intact (supplementary fig. S9, Supplementary Material online). Results showed that synteny of genes other than these five genes is not conserved among the four genomes, emphasizing remarkable conservation of these five genes as a cluster.

Among nonbilaterians, only the sea anemone (Nematostella vectensis) possesses glis1/3 and lrrc42 in the same vicinity, but others do not have a putative gene cluster. In the choanoflagellate, Monosiga brevicollis, a unicellular organism closely related to animals, genes other than rfx3 are missing in its genome. These facts suggest that the common ancestor of bilaterians acquired this cluster by shuffling gene order. A characteristic feature of this highly conserved gene cluster is that all five genes belong to different gene families, in contrast to clusters of duplicated copies of the same gene family, such as Hox, ParaHox, and Wnt gene clusters (Takeuchi et al. 2016).

What then is the role of this cluster? Strikingly, the known functions of these genes are related to cilia (fig. 4B). Glis3 is localized in primary cilium and is associated with cystic renal diseases (Kang, Beak, et al. 2009). In amphioxus, glis1/3 is highly expressed in gill bars, which are densely populated with ciliated cells, compared with other tissues (supplementary fig. S10A, Supplementary Material online). In brachiopods, glis1/3 is enriched in lophophores, which contain ciliated tentacles (supplementary fig. S10B, Supplementary Material online). Among scallop adult organs, glis1/3 is strongly expressed in the ciliated gill (supplementary fig. S10C, Supplementary Material online). Even in ctenophores, a basal metazoan lineage, glis1/3 is expressed in ciliated cells (Layden et al. 2010), implying that Glis1/3 had an ancient role in ciliogenesis. Remarkably, glis1/3 is also expressed at...
moderate levels in guts of amphioxus and brachiopods, and in digestive glands of brachiopods and scallops. These expression data imply that functions of Glis3 in pancreatic β-cell differentiation (Kang, Beak et al. 2009; Kang, Kim et al. 2009; O’Hare et al. 2016) originated the digestive system of the bilaterian ancestor.

Rfx3 positively regulates ciliary genes in most animals, and perhaps in choanoflagellates (Piasecki et al. 2010). Importantly, Rfx3 also regulates pancreatic β-cell differentiation (Ait-Lounis et al. 2010), suggesting cooperative functions with Glis3 in islets. Ndc1 is a component of the nuclear pore complex and also of the ciliary pore complex, which mediate protein transport to nuclei and cilia, respectively (Mansfeld et al. 2006; Ounjai et al. 2013). Hspb11 is an ortholog of intraflagellar transport 25 (Ift25) that participates in transport of Hedgehog signaling molecules, including Gli in primary cilium (Keady et al. 2012). Lrrc42 has been reported as a nuclear protein expressed in lung cancer (Fujitomo et al. 2014), suggesting that Lrrc42 interacts with transcription factors that may include Glis1/3 and Rfx3, to regulate ciliogenesis. Taken together, this “ciliogenic gene cluster” may serve to establish ciliated tissues in organs such as gill, gastrointestinal epithelium, lung, kidney, and pancreas, since the origin of bilaterians. In other words, formation of this cluster with other ciliogenic genes further suggests that ciliogenesis was the original function of Glis1/3, and that Glis1 has been coopted for cell reprogramming in vertebrates.

Conclusions and Perspectives

In this study, we first clarified relationships between GLI and GLIS genes by comprehensive phylogenetic analysis. The similar gene names are confusing, but the first emergence of GLIS2 by duplication of the ancestral GLI/GLIS/ZIC gene in metazoans greatly predates the appearance of GLIS1 and GLIS3 by WGD in vertebrates. Amino acid sequences of Glis1 and Glis3 were compared to identify conserved and diversified protein–protein interaction motifs. Surveys of transcriptomic data emphasized that maternal expression of glis1 is characteristic of tetrapods. Glis1 appears to have been released from evolutionary constraints for conventional roles and has acquired new functions in oocytes. Together with preprogramming activity of GLIS1, we hypothesize that Glis1 was neofunctionalized for cell reprogramming in vertebrates (or tetrapods). The cell reprogramming activity of Glis1/3 from various animals should be examined using iPSC or other reprogramming assays. Then, Glis1-specific transcriptional machinery for cell reprogramming should be determined.

Comparative genomic analysis revealed a highly conserved gene cluster containing glis1/3 and other ciliogenic genes. Transcriptomic data also support ancestral roles of Glis1/3 in ciliated tissues. The next question is how these clustered genes are regulated for ciliogenesis. To answer this question, we surveyed previously identified, conserved noncoding sequences for the human genome using UCNEbase (Dimitrieva and Bucher 2013). We found two candidate cis-regulatory modules around the cluster (UCNE34150 and UCNE3883), but these are not conserved among bilaterians.

To identify “the cluster controlling region,” more comprehensive analysis for noncoding sequences should be performed. This study highlights the importance of carefully considering orthologous relationships between homologs without preconceptions stemming from classical gene names, in order to better understand and predict gene functions. Expression profiles and comparative genomics provide us with many clues to unravel how genes evolved. The evolutionary history of GLIS genes illuminates potential functions of GLIS1/3 genes for cell reprogramming and ciliogenesis. Taken together with previous studies on GLIS1 for iPSC technologies and those on GLIS3 for development and disease in kidney and pancreas, our study will facilitate applications of GLIS1/3 to stem cell biology and medical sciences.

Materials and Methods

Phylogenetic Analysis

To identify ortholog groups of GLIS genes, protein-coding DNA sequences of human GLIS1, GLIS2, GLIS3, GLI2, and ZIC1 were submitted as queries to ORTHOSCOPE, a species tree-based ortholog identification tool (Inoue and Satoh 2018), with the following settings: analysis group, vertebrata; E-value threshold for reported sequences, 1e-5; number of hits to report per genome, 3; aligned site rate threshold within unambiguously aligned sites, 0; data set, DNA (Exclude 3rd); rearrangement BS (bootstrap) value threshold, 60%. To produce NJ and ML trees of Glis1/3, amino acid sequences of Glis1/3 were aligned with MAFFT (v7.221) (Katoh et al. 2002) using the –auto strategy. Unaligned regions were trimmed with TrimAl (v1.2rev59) (Capella-Gutierrez et al. 2009) using the --gappyout option.

To generate nucleotide alignments, corresponding cDNA sequences were forced onto the amino acid alignment using PAL2NAL (Suyama et al. 2006). The maximum likelihood method with PROTGAMMAAUTO (amino acid sequences) or GTRGAMMA (nucleotide sequences) was used to construct phylogenetic trees with RAxML (v8.2.70) (Stamatakis 2014). For the nucleotide tree, we used codon partitions.

Synteny Analysis

Genomic synteny of GLIS genes in vertebrates was analyzed using genome assemblies of Homo sapiens, GRCCh38.p12 (human), Gallus gallus, GRCg6a (chicken), X. laevis, xenLae2 (African clawed frog), X. tropicalis, xenTro9 (tropical clawed frog), Latimeria chalumnae, LatCh1 (coelacanth), Lepisosteus oculatus, LepOcu1 (spotted gar), Danio rerio, GRCe10 (zebrafish), Oryzias latipes, ASM223467v1 (Japanese medaka), Takifugu rubripes, FUGUS (pufferfish), and Callorhinchus mili, ESHARK1 (elephant shark). For the synteny search for invertebrates, genome versions are listed in supplementary table S1, Supplementary Material online. The ciliogenic gene cluster (fig. 4A) fulfills a pipeline used to identify conserved microsynteny blocks in previous studies (Simakov et al. 2013, 2015; Albertin et al. 2015); Nmax 10 (maximum of 10 intervening genes) and Nmin 3 (minimum of 3 genes in a syntenic block). Unfortunately, this cluster was not detected in those studies, possibly because they used a limited number of gene families to simplify gene family assignments. The false-positive rate for
this cluster falls to <0.1%, because random genome reshuffling produces ~10% false positives in pairwise genome comparisons (Simakov et al. 2015), but the cluster was observed in more than three species across bilaterian phyla.

Transcriptomic Data from Embryos and Adult Tissues of Various Animals
Publically available transcriptomic data of human early embryos (Xue et al. 2013), mouse early embryos (Tang et al. 2011; Xue et al. 2013), bovine early embryos (Jiang et al. 2014), chicken early embryos (Hwang et al. 2018), X. tropicalis embryos (Owens et al. 2016), medaka embryos (Ichikawa et al. 2017), zebrafish embryos (White et al. 2017), amphioxus embryos, and adult tissues (Marletaz et al. 2018), sea urchin embryos (S. purpuratus [Tu et al. 2012] and P. lividus [Gildor et al. 2016]), brachiopod embryos and adult tissues (Luo et al. 2015), and scallop embryos and adult tissues (Wang et al. 2017) were used to examine expression levels of gls genes and dmr1b. Data for X. tropicalis, zebrafish, amphioxus, and sea urchins (S. purpuratus) were collected from Xenbase (http://www.xenbase.org/entry/; last accessed September 11, 2019), Expression Atlas (https://www.ebi.ac.uk/gxa/experiments/E-ERAD-475-Results; last accessed September 11, 2019), Amphioncode (http://amphioncodegithubio/; last accessed September 11, 2019), and EchinoBase (http://www.echinobase.org/EchinoBase/; last accessed September 11, 2019), respectively.

Supplementary Material
Supplementary data are available at Molecular Biology and Evolution online.

Acknowledgments
We thank Jun Inoue for conducting ORTHOSCOPE analysis and critiquing the manuscript, Naoki Irie for sharing medaka transcriptomic data, Steven D. Aird for technical editing of the manuscript, and Atsushi Hijiikata and Tsuyoshi Shirai for their initial analyses of protein-interacting motifs based on the Basis for Supporting Innovative Drug Discovery and Life Science Research (BINDS) from the AMED, Japan (Grant No. JP17am0101069). We thank all members of Laboratory for Comprehensive Genomic Analysis, especially Shohei Noma, Tomoko Hirata, and Yuki Yasuoka, for technical support. This work was supported in part by a Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) (Grant Nos. 16K21559, 17KT0114, 18K14745, and 18H04825 to Y.Y.; and No. 18K19591 to M.M.), the Translational Research Network Program from the AMED, Japan (Grant No. JP18lm0203004 to M.M.), Kawano masanori memorial public interest incorporated foundation for promotion of Pediatrics (M.M.), and research grants for the RIKEN Center for Integrative Medical Sciences from MEXT, Japan.

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