Convergent biological pathways underlying the Kallmann syndrome-linked genes Hs6st1 and Fgfr1

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Abstract

Kallmann syndrome (KS) is a congenital disorder characterized by idiopathic hypogonadotropic hypogonadism and olfactory dysfunction. KS is linked to variants in >34 genes, which are scattered across the human genome and show disparate biological functions. Although the genetic basis of KS is well studied, the mechanisms by which disruptions of these diverse genes cause the same outcome of KS are not fully understood. Here we show that disruptions of KS-linked genes affect the same biological processes, indicating convergent molecular mechanisms underlying KS. We carried out machine learning-based predictions and found that KS-linked mutations in heparan sulfate 6-O-sulfotransferase 1 (HS6ST1) are likely loss-of-function mutations. We next disrupted Hs6st1 and another KS-linked gene, fibroblast growth factor receptor 1 (Fgfr1), in mouse neuronal cells and measured transcriptome changes using RNA sequencing. We found that disruptions of Hs6st1 and Fgfr1 altered genes in the same biological processes, including the upregulation of genes in extracellular pathways and the downregulation of genes in chromatin pathways. Moreover, we performed genomics and bioinformatics analyses and found that Hs6st1 and Fgfr1 regulate gene transcription likely via the transcription factor Sox9/Sox10 and the chromatin regulator Chd7, which are also associated with KS. Together, our results demonstrate how different KS-linked genes work coordinately in a convergent signaling pathway to regulate the same biological processes, thus providing new insights into KS.

Introduction

Kallmann syndrome (KS) is a congenital disorder characterized by idiopathic hypogonadotropic hypogonadism and olfactory dysfunction (1,2). Individuals with KS show symptoms such as incomplete or absent puberty, infertility, low levels of sex steroids, low levels of gonadotropin and loss of smell. The idiopathic hypogonadotropic hypogonadism is caused by deficits in the production, secretion or action of the gonadotropin-releasing hormone (GnRH), which is mainly because of defects in the development or migration of the GnRH producing neurons (3–5). The olfactory dysfunction, either absence of smell (anosmia) or partial loss of smell (hyposmia), is likely because of defects in the development of the olfactory system, such as impaired axon guidance of the olfactory neurons (6,7). Although some KS individuals can be treated with hormone-replacement therapy (2), many of the KS-associated symptoms are not treatable, which is in part due to our limited understanding of the molecular mechanisms underlying KS.

KS has a strong genetic basis (1,2,8,9). The first KS-linked gene, anosmin 1 (ANOS1, also known as KAL1), was identified in 1991 (10,11). Since then, studies in KS patients have identified KS-linked genetic variants in >34 genes (1,2,4,5,8,12). Although the association between KS and several of these genes, such as fibroblast growth factor 8 (FGF8) (13) and heparan sulfate 6-O-sulfotransferase 1 (HS6ST1) (14), has been validated using animal models, the exact mechanisms by which these genes contribute to KS pathogenesis are not fully understood. Particularly, given that the KS-linked genes show diverse biological functions, how disruptions of these different genes cause the same outcome of KS remains elusive.

Most KS-linked genes show low penetrance and cause variable clinical severity (1,2,4,5,8). These findings lead to a proposed model of oligogenicity (15), in which mutations in multiple genes interact together to manifest a more severe phenotype. Indeed, large cohort studies revealed that >20% of individuals with KS or idiopathic hypogonadotropic hypogonadism are oligogenic (4,15). The oligogenicity of KS indicates that proteins encoded by the KS-linked genes might be involved in a same signaling pathway. For instance, HS6ST1 (14), FGF8 (13), FGF17 (16) and FGF receptor 1 (FGFR1) (17) are genetically linked to KS. HS6ST1 catalyzes the 6-O-sulfation on heparan sulfate (HS), a glycosaminoglycan that presents at the cell surface and extracellular matrix (ECM) of all human cells. Notably, HS 6-O-sulfation directly interacts with FGF and FGFR and enhances the bindings between the two (18–20), suggesting that HS6ST1, FGF8/FGF17 and FGFR1 belong to a same molecular pathway. However, it remains unclear how other KS-linked proteins, such as the transcription factor SOX10 (21) and the chromatin regulator CHD7 (22), contribute to this signaling pathway.

In this study, we disrupted Hs6st1 and Fgfr1 in mouse neuronal cells and carried out RNA sequencing (RNA-seq) to assess the transcriptome changes upon the disruptions. We found that disruptions of Hs6st1 and Fgfr1 altered the expression of genes in the same biological processes, suggesting that the two genes...
belong to a same molecular pathway. We also found that Hs6st1 and Fgfr1 regulate gene transcription likely via Sox9 and Chd7. Taken together, our results indicate a convergent signaling pathway underlying multiple KS-linked genes, thus providing novel insights into KS.

**Results**

**KS-linked mutations in HS6ST1 affect the protein structure**

Mutations in HS6ST1 are genetically linked to KS (14) (Fig. 1A), but the underlying mechanisms are not fully understood. To determine how KS-linked mutations affect HS6ST1 function, we assessed the effects of these point mutations on the protein structure of HS6ST1 using a novel machine learning-based approach. We used the deep learning algorithm in the AlphaFold software (23) to predict the three-dimensional structures of the wild-type (WT) and mutant HS6ST1. We found that the WT HS6ST1 has 11 α-helices and four β-strands (Fig. 1B and Supplementary Material, Fig. S1), which is similar to the crystal structure of the zebrafish Hs6st3 catalytic domain (24). We also used the AlphaFold to predict the structures of the mutant HS6ST1 (Supplementary Material, Fig. S2). We found that the KS-linked mutations affect the local properties of the protein (Fig. 1C and Supplementary Material, Fig. S2), but they do not alter the global protein structures (Supplementary Material, Fig. S2). For example, R306W and R306Q, which localize at the ninth α-helix of the protein, increase the hydrophobicity and negative electrostatic potential of a valley-like structure at the catalytic core of the protein (Fig. 1C and Supplementary Material, Fig. S2). Thus, these two mutations likely affect the enzyme activity. This finding is consistent with previous in vitro biochemical studies that the R306Q, R306W, R382W, M404V and R375H mutations reduce the enzyme activities (14,24,25).

In contrast, no obvious alternation of protein surface properties was observed in the R232Q HS6ST1 (Supplementary Material, Fig. S2). Notably, the R232 localizes in the substrate-binding pocket of the protein and interacts with the cofactor product 3′-phosphoadenosine 5′-phosphate (24). Thus, the R232Q mutation may directly abolish the substrate recognition or binding instead of altering the protein structure, which is also consistent with the in vitro biochemical studies (14,24). Together, these results suggest that KS-linked mutations in HS6ST1 affect the protein structures and are likely loss-of-function mutations.

**Disruption of Hs6st1 in Neuro 2a cells alters the transcriptome**

To illustrate the molecular mechanisms by which HS6ST1 dysfunction contributes to KS, we disrupted Hs6st1 in mouse neuronal cells and assessed the transcriptome changes (Fig. 2A). We used Neuro 2a cells, a widely used mouse neuroblastoma cell line that expresses high levels of Hs6st1 and low levels of the other Hs6st genes (Supplementary Material, Fig. S3A). To disrupt Hs6st1, we transfected Neuro 2a cells with siRNAs that specifically target the messenger RNA (mRNA) of Hs6st1. Neuro 2a cells transfected with control siRNAs against a scrambled sequence were used as the control. We found that siRNA treatment decreased the mRNA levels of Hs6st1 by 78% (Fig. 2B, P-value < 0.001, one-tailed t-test).

To assess the transcriptome changes upon the knockdown of Hs6st1, we performed ribosomal RNA-depleted RNA sequencing (RNA-seq) experiment. We sequenced three biological replicates for the control and Hs6st1 knockdown samples and obtained 517 million high-quality sequencing reads for the six RNA-seq libraries (Supplementary Material, Fig. S3B). We next used a computational pipeline to analyze the RNA-seq data and quantified the expression levels of all genes in the mouse mm10 genome (described in detail in the Materials and Methods section). We found a high correlation among the biological replicates (Pearson’s correlation coefficient r > 0.994). To further assess the six transcriptome profiles, we performed a principal component analysis (PCA). We found a clear separation of the three control samples and the three Hs6st1 knockdown samples (Fig. 2C), suggesting that the siRNA knockdown of Hs6st1 consistently alters the transcriptome.

To identify genes that were affected by the Hs6st1 knockdown, we compared the expression profiles of all genes between the control and the knockdown RNA-seq data using edgeR (26). We identified 1436 genes that were significantly affected by the Hs6st1 knockdown (false discovery rate (FDR) < 0.05) (Fig. 2D, Supplementary Material, Fig. S3C and Supplementary Material, Table S1), including 740 upregulated genes and 696 downregulated genes (Fig. 2D). For example, the cellular communication network factor 1 (Con1) and two Fgf genes (Fgf1 and Fgf2) were upregulated by the knockdown, whereas the leucine rich repeat neuronal 4 (Lrrn4) and the beta-1,3-galactosyltransferase 6 (B3galt6) were downregulated (Supplementary Material, Fig. S3D).

To validate the gene expression changes upon Hs6st1 knockdown, we carried out independent experiments to knock down Hs6st1 using the same siRNAs and measured the expression changes of six genes using quantitative reverse transcription PCR (qPCR). We found a significant decrease of the mRNA levels of Hs6st1 (Fig. 2E), supporting the knockdown efficiencies. For the other five genes, the expression levels of Ccn1, Fgf1 and Fgf2 were increased, and the expression levels of Lrrn4 and B3galt6 were decreased in the knockdown experiments (Fig. 2E). These results are consistent with the results from the RNA-seq (Supplementary Material, Fig. S3D). Notably, we also found the increased expression of Con1 and the decreased expression of Lrrn4 in the brain of the Hs6st1 nervous system-specific knockout mice (data not shown). Together, these results suggest that knockdown of Hs6st1 in Neuro 2a cells alters the transcriptome.

**Hs6st1 knockdown promotes genes in the extracellular pathways and inhibits genes in the chromatin pathways**

To determine the biological pathways affected by the Hs6st1 knockdown, we carried out Gene Ontology enrichment analysis (27) and Gene Set Enrichment Analysis (GSEA) (28). We found that the upregulated and downregulated genes were enriched for distinct gene ontology pathways (FDR < 0.05). The 740 upregulated genes were enriched for extracellular pathways, such as glycolytic process, ECM structural constituent and integrin binding (Fig. 3A). In contrast, the 696 downregulated genes were enriched for chromatin pathways, such as DNA replication, chromosome, DNA repair and nucleosome assembly (Fig. 3B). The GSEA results also showed that extracellular pathways, such as the ECM glycoproteins, were upregulated, whereas chromatin pathways, such as the covalent chromatin modification, were downregulated (Fig. 3C). Together, these results indicate that Hs6st1 knockdown promotes genes in the extracellular pathways and inhibits genes in the chromatin pathways.

**Disruption of Fgfr1 in Neuro 2a cells alters the transcriptome**

To determine the extent to which the biological pathways affected by the Hs6st1 disruption are shared among other KS-linked genes, we next carried out siRNA knockdown and RNA-seq experiments
Figure 1. KS-linked mutations in HS6ST1 affect the protein structure. (A) Diagram of the human HS6ST1 protein and the six KS-linked mutations. (B) Three-dimensional protein structure of human HS6ST1 predicted using the AlphaFold. (C) The hydrophobicity and electrostatic potential of the WT and two mutant HS6ST1 proteins. Yellow arrows indicate the loci of property changes at the protein surface.

Figure 2. Hs6st1 knockdown alters the transcriptome. (A) Diagram of the experiment design. (B) qPCR results showing the relative mRNA levels (2-ΔΔCt values) of Hs6st1 in the control (Ctrl) and Hs6st1 knockdown (KD) samples. Ctrl, n=3; KD, n=3. **P-value < 0.001, one-tailed t-test. (C) Principal component (PC) analysis results. (D) Volcano plot showing the fold change and FDR. Red dots represent the 740 upregulated genes, and green dots represent the 696 downregulated genes. (E) qPCR results showing the relative mRNA levels (2-ΔΔCt values) of six genes in the independent experiments of Hs6st1 knockdown. Ctrl, n=2; KD, n=8. *P-value < 0.05; **P-value < 0.01; ***P-value < 0.001; one-tailed t-test.

For another KS-linked gene, Fgfr1 (Fig. 4A). Neuro 2a cells express high levels of Fgfr1 and low levels of the other Fgfr genes (Supplementary Material, Fig. S3F). To disrupt Fgfr1, we transfected Neuro 2a cells with siRNAs that specifically target Fgfr1 mRNA. We found that siRNA treatment decreased the mRNA levels of Fgfr1 by 83% (Fig. 4B, P-value < 0.001, one-tailed t-test). We then carried out RNA-seq experiment with four biological replicates (Supplementary Material, Fig. S3B). The PCA showed a clear separation of the three control samples and the four Fgfr1 knockdown samples (Fig. 4C), suggesting that siRNA knockdown of Fgfr1 consistently alters the transcriptome.

To identify genes that were affected by the Fgfr1 knockdown, we used our RNA-seq analysis pipeline and identified 2526 upregulated genes and 2278 downregulated genes (FDR < 0.05) for another KS-linked gene, Fgfr1 (Fig. 4A). Neuro 2a cells express high levels of Fgfr1 and low levels of the other Fgfr genes (Supplementary Material, Fig. S3F). To disrupt Fgfr1, we transfected Neuro 2a cells with siRNAs that specifically target Fgfr1 mRNA. We found that siRNA treatment decreased the mRNA levels of Fgfr1 by 83% (Fig. 4B, P-value < 0.001, one-tailed t-test). We then carried out RNA-seq experiment with four biological replicates (Supplementary Material, Fig. S3B). The PCA showed a clear separation of the three control samples and the four Fgfr1 knockdown samples (Fig. 4C), suggesting that siRNA knockdown of Fgfr1 consistently alters the transcriptome.

To identify genes that were affected by the Fgfr1 knockdown, we used our RNA-seq analysis pipeline and identified 2526 upregulated genes and 2278 downregulated genes (FDR < 0.05)
of Fgfr1 (Fig. 4E), supporting the knockdown efficiencies. The qPCR results of the other five genes are consistent with the RNA-seq results, including the upregulation of the Con1, Fgf1 and polypeptide N-acetylgalactosaminyltransferase 15 (Galnt15) and the downregulation of the Lm4 and B3gal16 (Fig. 4E and Supplementary Material, Fig. S3G). Together, these results suggest that knockdown of Fgfr1 in Neuro 2a cells alters the transcriptome.

To determine the biological pathways affected by the Fgfr1 knockdown, we carried out Gene Ontology enrichment analysis and GSEA analysis. We found that the upregulated genes were enriched for extracellular pathways (Fig. 4F), and the downregulated genes were enriched for chromatin pathways (Fig. 4G). The GSEA results also showed that extracellular pathways, such as the ECM binding, were upregulated, whereas chromatin pathways, such as the mRNA splicing, were downregulated (Fig. 4H). Notably, the upregulated pathways and downregulated pathways are in the same biological processes between the Hs6st1 knockdown (Fig. 3A–C) and the Fgfr1 knockdown (Fig. 4F–H). Together, these results indicate that Hs6st1 knockdown and Fgfr1 knockdown affect the same biological processes.

A group of 1052 genes are regulated by both Hs6st1 and Fgfr1

To identify genes that are regulated by both Hs6st1 and Fgfr1, we compared the two lists of differentially expressed genes (DEGs) in the two knockdown experiments. We found that 1052 genes were affected in both knockdown experiments (Fig. 5A and Supplementary Material, Table S3), which we term overlapping DEGs. To determine the expression changes of the 1052 overlapping DEGs in the two knockdown experiments, we analyzed their values of fold changes. We found that 99.7% (1049 out of 1052) of the 1052 overlapping DEGs showed the same directions of the gene expression changes upon Hs6st1 knockdown and Fgfr1 knockdown (Fig. 5B and C). These results suggest that Hs6st1 and Fgfr1 belong to a same signaling pathway to regulate the 1052 genes. To determine the biological pathways of the 1052 overlapping DEGs, we carried out Gene Ontology enrichment analysis. We found that the 492 upregulated genes were enriched for extracellular pathways (Fig. 5D), whereas the 557 downregulated genes were enriched for chromatin pathways (Fig. 5E). Together, these results suggest that a group of 1052 genes are regulated by both Hs6st1 and Fgfr1.

Hs6st1 and Fgfr1 regulate gene transcription likely via the transcription factor Sox9

Although Hs6st1 and Fgfr1 regulate the transcription of the 1052 genes (Fig. 5), the transcription factors involved in this process remain unknown. To identify transcription factors that are downstream of Hs6st1 and Fgfr1, we first carried out a Homer
motif analysis (29) using the promoter regions of the 1052 overlapping DEGs (Fig. 6A). We found that these promoter regions were enriched for 16 de novo motifs (Supplementary Material, Fig. S3H). Figure 6B shows the top five de novo motifs identified using Homer, including the transcription factor Sox9. Sox9 belongs to the Sox-E transcription factor family, which also includes Sox10 (30). Notably, mutations in Sox10 are also genetically linked to KS (21). Furthermore, we found that although Sox10 is lowly expressed in Neuro 2a cells, Sox9 was significantly upregulated upon the Hs6st1 knockdown and the Fgfr1 knockdown (Fig. 6C).

To assess the genomic bindings of Sox9, we analyzed the publicly available genome-wide binding profiles of Sox9 (31), which were generated by chromatin immunoprecipitation followed by sequencing (ChIP-seq). We first analyzed Sox9 ChIP-seq signals at the promoter regions of the 1052 overlapping DEGs and found an enrichment of ChIP-seq signals (Fig. 6D), suggesting that Sox9 directly binds to the promoters of these genes. In addition, to assess the chromatin environment of these promoters, we analyzed our previously generated ChIP-seq profiles of histone H3 trimethylation at lysine 4 (H3K4me3) and histone H3 acetylation at lysine 27 (H3K27ac) (32). We found an enrichment of H3K4me3 and H3K27ac ChIP-seq signals at the promoter regions of the 1052 overlapping DEGs (Fig. 6D), suggesting that these promoters are in active chromatin status. Furthermore, to determine whether the Sox9 bindings are specific to the 1052 overlapping DEGs, we randomly selected 1000 control genes that were expressed in Neuro 2a cells but were not affected by the two knockdown experiments. We found a significant enrichment of Sox9 ChIP-seq signals at the promoters of the 1052 overlapping DEGs compared with the 1000 control genes (Fig. 6F and F P-value = 0.001, one-tailed t-test), indicating that Sox9 bindings are specific to the DEGs. We also showed the ChIP-seq profiles at four DEGs as an example (Fig. 6G). Lastly, although Sox10 was not affected by the two knockdown experiments (Fig. 6C), we analyzed the publicly available Sox10 ChIP-seq data (33) and found an enrichment of Sox10 bindings at the promoters of the 1052 overlapping DEGs (Supplementary Material, Fig. S3J). Together, these results indicate that Sox9 is likely the transcription factor downstream of Hs6st1 and Fgfr1.

A convergent signaling pathway underlying the KS-linked genes

Thirty-four genes are genetically linked to KS (1,2,4,5,8,12) (Supplementary Material, Fig. S3J). Given that the three KS-linked genes, Hs6st1, Fgfr1, and likely Sox10, regulate the same biological processes, we next asked whether other KS-linked genes also play a role in these pathways. To answer this question, we first analyzed the expression changes of the 34 KS-linked genes upon Hs6st1 knockdown and Fgfr1 knockdown. We found that except Hs6st1 and Fgfr1, three other KS-linked genes, chromodomain helicase DNA binding protein 7 (Chd7), dual specificity phosphatase 6 (Dusp6) and coiled-coil domain containing 141 (Ccdc141), were also significantly affected by the knockdown experiments (Supplementary Material, Tables S1 and S2). For instance, Chd7, which encodes a chromatin regulator, was significantly downregulated (Fig. 7A). Given that disruptions of Hs6st1 and Fgfr1 inhibit chromatin processes (Fig. 5E), we next examined whether these inhibitions are mediated by Chd7. Thus, to determine the binding profiles of Chd7, we analyzed the publicly available Chd7 ChIP-seq data (34). We found an enrichment of Chd7 bindings at the promoters of the 1052 overlapping DEGs (Fig. 7B and C), suggesting that Chd7 may modify the chromatin status of these genes to regulate their expression. In addition, we found that genes affected by the knockdown of Hs6st1 and Fgfr1 tend to exhibit the same directions of expression changes in the brain of Chd7 knockout mice (34) (Supplementary Material, Fig. S3K).

Together, our results indicate a model by which multiple KS-linked genes, including Hs6st1, Fgfr1, Sox10 and Chd7, converge on a same signaling pathway (Fig. 7D). In this model, Hs6st1 catalyzes the HS 6-O-sulfation to control the HS/FGF/FGFR interactions to regulate the expression of Sox9 and Chd7. Sox9 upregulation will promote the ECM pathways, which has been reported by a previous study (35). Chd7 downregulation will modify chromatin status to inhibit the chromatin pathways.

Discussion

KS, once considered as a monogenic disorder, has been linked to >34 genes (1,2,4,5,8,12). In this study, we found that multiple
KS-linked genes, including Hs6st1, Fgf, Fgfr1, Sox10 and Chd7, work coordinately in the same signaling pathway. To determine KS-linked mutations in Hs6st1, we carried out machine learning-based predictions and found that KS-linked mutations in Hs6st1 are likely loss-of-function mutations. In addition, by carrying out siRNA knockdown and RNA-seq, we found that disruptions of Hs6st1 and Fgfr1 in mouse neuronal cells affected the same biological processes. Lastly, by performing genomics and bioinformatics analyses, we found that Hs6st1 and Fgfr1 regulate gene transcription likely via the transcription factor Sox9/Sox10 and the chromatin regulator Chd7. Taken together, our study indicates a convergent signaling pathway underlying multiple KS-linked genes.

ECM pathways in the brain play an important role in the regulation of brain development and function, such as neuron migration, axon guidance and synapse maturation (36). Intriguingly, defects in the migration and development of the GnRH neurons and the olfactory neurons are the two hallmarks of KS (3–7). In addition, the first identified KS gene, ANOS1, encodes a glycoprotein that plays an important role in ECM pathways (10,11). In this study, we found that disruptions of Hs6st1 and Fgfr1 in mouse neuronal cells both promote the expression of genes in the ECM pathways. Thus, our findings highlight a role of ECM pathways in the KS pathogenesis. Moreover, we found that disruptions of Hs6st1 and Fgfr1 upregulated the expression of the transcription factor Sox9. Given that Sox9 directly promotes the expression of ECM genes in the brain (35), our findings together suggest a model by which the HS/FGFR signaling regulates Sox9 expression to promote the ECM processes. Further studies are needed to directly dissect the role of Sox9 and the ECM pathways in the pathogenesis of KS.

Sox9 belongs to the E subgroup of the SOX transcription factor family (SOX-E). SOX-E family includes three members, SOX8, SOX9 and SOX10, which exhibit similar DNA binding motifs and function redundancy (30,37,38). SOX-E transcription factors play an important role in development. Notably, mutations in SOX10 have been found in KS individuals (21). In this study, we found a central role of Sox9 in the signaling pathways that are critical to KS. Therefore, it will be intriguing to investigate genetic variants in SOX9 as well as SOX8 in KS individuals.

We identified that disruptions of Hs6st1 and Fgfr1 in mouse neuronal cells both downregulated genes in chromatin pathways, such as chromatin modification, nucleosome assembly, DNA binding and chromatin binding. Given that Hs6st1 and Fgfr1 are localized at the Golgi and the cell surface, they are unable to directly regulate biological processes in the nucleus. One possible mediator is Chd7, which is a chromatin remodeling enzyme that plays an important role in promoting chromatin accessibility, enhancer activation and active histone modifications in the brain (34,39). We found that disruptions of Hs6st1 and Fgfr1 downregulated the expression of Chd7, suggesting a model by which the HS/FGFR signaling regulates Chd7 expression to inhibit the chromatin processes. Notably, a previous study found that Chd7 interacts and cooperates with Sox10 to regulate chromatin status and gene transcription in the brain (39). Thus, further investigations are needed to reveal how the two KS-linked proteins, Chd7 and Sox10, and the chromatin pathways contribute to KS pathogenesis.

Oligogenicity is a key feature of the genetic basis of KS. Genetic interactions among KS-linked genes have been reported by previous studies. Many of these interactions are centering on the FGFR signaling. For instance, ANOS1 functionally interacts with the FGF–FGF–HS complex in human embryonic GnRH olfactory neuroblasts (40). ANOS1 function in Caenorhabditis elegans also requires FGFR and HS 6-O-sulfation (14). In addition, the localization of interleukin 17 receptor D (IL17RD), a KS-linked protein, in the olfactory placode requires FGFR8 and the FGFR signaling (16). Furthermore, Dusp6 is linked to KS and is a negative feedback regulator of the FGF/FGFR signaling in mouse development (41). The β-Klotho (KLB) function also needs the FGF/FGFR signaling (42). Notably, some KS-linked genes can interact independent of the FGFR signaling. For instance, Chd7 and Sox10 work corporately to regulate the myelination in the mouse brain. In this study, we found that multiple KS-linked genes, including Hs6st1, Fgf, Fgfr1, Chd7, Sox10 and likely Dusp6, converge on a same signaling pathway to regulate genes in the ECM processes and chromatin processes. Together, these findings indicate a potential gene interaction network among KS-linked genes. Further studies are needed to determine whether and how other KS-linked genes involve in this network and contribute to the oligogenicity.

In summary, our data demonstrate convergent molecular pathways underlying the KS-linked genes. Moving forward, we propose that these convergent pathways may involve other KS-linked genes and may directly contribute to the etiology of KS. Given that this study is focused on the signaling pathways underlying KS using neuronal cell lines, the role of these signaling pathways in KS pathogenesis, such as deficits in neuron migration and axon guidance, remains elusive. As such, we think further investigations are warranted to reveal the association and the detailed molecular mechanisms of these convergent pathways in KS pathogenesis in animal models and in KS individuals. Furthermore, to validate the transcriptome changes identified in this study, future investigations are needed to assess and integrate the transcriptome of GnRH neurons and olfactory neurons from KS patients and KS animal models, which are still lacking at present. Given that the six KS-linked variants in Hs6st1 may affect different aspects of the Hs6st1 protein function, future investigations, such as variant-specific rescue experiments in Hs6st1 disrupted neurons, are needed to pinpoint the common and variant-specific effects. Lastly, these convergent pathways also could be a potential therapeutic target for KS.
Materials and Methods

Cell cultures

Neuro 2a cells were purchased from the ATCC (CCL-131). The cells were cultured and maintained in Dulbecco’s modified Eagle’s medium (DMEM, Gibco # 11960592) and were supplemented with 10% fetal bovine serum (FBS, Gibco # 26140079), 2 mM L-glutamine (Gibco # 25300081), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco # 15240062). The cells were incubated at 37°C in a humidified atmosphere incubator containing 5% CO2. The cells were sub-cultured in fresh medium after reaching 70–80% confluence (about every 2–3 days).

siRNA knockdown

The siRNA transfection of Neuro 2a cells was performed using siRNA transfection reagent (Santacruz Biotech, sc-29528) according to the manufacturer’s instructions. The siRNAs used in this study include siRNAs for Hs6st1 (Santacruz Biotech, sc-146089), siRNAs for Fgf1r (Santacruz Biotech, sc-29317) and control siRNA (Santacruz Biotech, sc-37007). Briefly, for each transfection, Neuro 2a cells were plated for 1 day and then were incubated with siRNA (60 pmol), the transfection reagent and the transfection medium for 6 h at 37°C incubator. After the incubation, the cells were overlaid with the same volume of fresh growth medium containing 10% FBS and were cultured overnight in 37°C incubator. In the next day, the medium was exchanged to fresh growth medium containing 10% FBS, and the cells were incubated at 37°C, 5% CO2 for an additional 72 h before harvesting for analysis.

RNA isolation and quality assessment

RNA was isolated from Neuro 2a cells, and the total RNA was then extracted using the Trizol reagent (Invitrogen, #15596026) following the manufacturer’s instruction. After the isolation, the quality, concentration and integrity of the isolated RNA were assessed using the Nanodrop spectrometer and the Bioanalyzer (Agilent 2100) using RNA 6000 Nano Chip. RNA samples with a RIN value of > 8 were included for the downstream experiments.

RNA-seq library preparation, quality control and sequencing

RNA-seq libraries were prepared according to the manufacturer instructions with the Stranded Total RNA Library Prep kit (ZYMO, #R3003). The libraries were prepared with 1000 ng of total RNAs. Library quality was confirmed by the Bioanalyzer (Agilent 2100) using DNA 7500 chip. The fragment sizes of all libraries were within the range of 300–500 bp. The libraries were sequenced using the Illumina NovaSeq sequencer.

qPCR

To assess the relative mRNA levels, after RNA isolation, cDNA was synthesized with Prime script reverse transcriptase kit (Takara, RR037A). Real-time PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems, #4309195). The 18S rRNA was used as the endogenous reference. The primers used were listed as follows: B3gal5 forward, 5′-GGCAACTCTGGCACTACACTG-3′; B3gal5 reverse, 5′-CGATACGTCTTCACTGTGC-3′; Ccn1 forward, 5′-ATGAGAAGCATTAGAAGCTC-3′; Ccn1 reverse, 5′-TGAGG TCG AGGGTTGAAAGAAC-3′; Fgfr1 forward, 5′-CACCAGGCCACTTCAAGGA-3′; Fgfr1 reverse, 5′-AGCACCTCCATTCTTGCTGG-3′; Galnt15 forward, 5′-TGGCCAATGCTCTACCCTGAG-3′; Galnt15 reverse, 5′-CCATCCCGC AACCTCCCAT-3′; Hs6st1 forward, 5′-TGGACGGAAACTCACCACACTGTC-3′; Hs6st1 reverse, 5′-CATTCCAAGTACGGTACGGGATAC-3′; Lrn4 forward, 5′-TGGATTTTCTTGTTGTCTTGG-3′; Lrn4 reverse, 5′-GTGTTGACATCCACGAGAAGGAGC-3′; Hs6st2 forward, 5′-ACTCTCCA TCCTCCACAAAGGCC-3′; Hs6st2 reverse, 5′-CCAGTTGCTCCTCCTCT CGACA-3′; Hs6st3 forward, 5′-CTGGACCCGGACTCACCACAC-3′; Hs6st3 reverse, 5′-CGTCCGCACATATGGAGAGGT-3′; 18S rRNA forward, 5′-GAACCGGTTAGACCCACTTCT-3′; 18S rRNA reverse, 5′- CCATCCCAATCGTGATGAGG-3′. The cycling parameters used for qPCR amplification reactions were: AmpliTag activation at 95°C for 10 min, denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min (40 cycles).

RNA-seq alignment and statistical analyses

The cycle threshold (Ct) values were obtained from the StepOne-Plus real-time PCR system (Applied Biosystems) for both the endogenous reference gene (18S rRNA) and target genes. The Microsoft Excel was used for the downstream data analyses. For each sample, we had two to three technical replicates and two to eight biological replicates. First, we calculated the mean Ct value for the 18S rRNA for each sample [ΔCt18S] on the basis of the 18S rRNA Ct values of different technical replicates of that sample. We next calculated the ΔCt values for target genes in the control and knockdown samples by subtracting ΔCt18S of each sample from the Ct values of these target genes. We then calculated the mean ΔCt value of control sample [ΔACtcontrol] using the ΔCt values of the control samples of all biological and technical replicates. To calculate the ΔACt values, we subtracted ΔACtcontrol from the ΔCt values of target genes in the control and knockdown samples. Lastly, we transformed the ΔACt values into 2−ΔΔCt values. We used the 2−ΔΔCt values to calculate the mean and standard error and to plot. We used the ΔCt values and the Excel t test (one-tailed, two-sample unequal variance) to calculate the P-values for the gene expression differences between the control and knockdown samples.

Protein structure prediction and visualization

The AlphaFold v2.0 (23) was used to predict the protein structures. The human H6EST1 protein sequence was obtained from the National Center for Biotechnology Information (NCBI) database by the accession number of NP_004798.3. For mutant proteins, the according amino acid was replaced by the mutant allele. The AlphaFold docker was installed. The AlphaFold was carried out using the default parameters except for the ‘—max_template_date=2020-05-14’. The ‘ranked_0.pdb’ file was selected as the predicted protein structure. The UCSF Chimera (43) was used to visualize the protein structures.

RNA-seq alignment and read counting

The RNA-seq data analyses were performed as previously described (44–48). Briefly, the FASTQ files of RNA-seq were aligned to the mouse mm10 genome using STAR 2.7.7a (49) by the parameters of ‘–runThreadN 40 –outFilterMultimapNmax 1 –outFilterMismatchNmax 3’ followed by the parameters of ‘–runThreadN 40 –outFilterMultimapNmax 1 –outFilterScoreMinOverLread 0.25 –outFilterMatchNminOverLread 0.25’. To increase the speed of the alignment, we used the ‘–runThreadN 40’ parameter to use 40 processors/CPUs. To exclude ambiguously mapped reads, we used the ‘–outFilterMultimapNmax 1’ parameter to include reads that are uniquely mapped in the mouse mm10 genome and to exclude reads that are mapped to more than one locus. To control the mapping specificity and potential SNPs, we used the ‘–outFilterMismatchNmax 3’ parameter to manage that the maximum number of mismatches per read-pair is three. The
samples. The top 1000 highly variable genes were used for the PCA analysis to check for batch effects and the similarity among samples for rows with small counts and normalize to library size. The count data into the log2 scale, minimize differences between the dispersion, fit a Negative Binomial GLM model and perform the DESeqDataSetFromMatrix command. Next, the DESeq command was used to estimate the library size factors, estimate the common dispersion and tagwise dispersions were estimated using the edgeR estimateDisp command. We next used the quasi-likelihood methods with empirical Bayes shrinkage in edgeR to fit the TPM data into a quasi-likelihood negative binomial generalized log-linear model using the glmQLFit command. Lastly, we carried out the quasi-likelihood F-test to compare gene expression using the glmQLFTest command. The cutoff for a significant difference in gene expression was FDR < 0.05. The Gene Ontology enrichment analyses were performed using the DAVID (27), and the genes expressed in Neuro 2a cells were used as the background genes.

RNA-seq PCA
The DESeq2 (50) was used to perform the PCA. Briefly, the table of read counts was converted into a list-based data object by the DGEList command. Lowly expressed genes (average count-per-million < 0.5) were excluded from the downstream analysis. The library size was calculated by the colSums command, and the read counts were normalized to library size using the calcNormFactors command in edgeR to obtain the tag per million (TPM) values. The common dispersion and tagwise dispersions were estimated using the edgeR estimateDisp command. We next used the quasi-likelihood methods with empirical Bayes shrinkage in edgeR to fit the TPM data into a quasi-likelihood negative binomial generalized log-linear model using the glmQLFit command. Lastly, we carried out the quasi-likelihood F-test to compare gene expression using the glmQLFTest command. The cutoff for a significant difference in gene expression was FDR < 0.05. The Gene Ontology enrichment analyses were performed using the DAVID (27), and the genes expressed in Neuro 2a cells were used as the background genes.

Fragments per kilobase of transcript per million mapped read pairs (FPKM) calculation
FPKM value was calculated using the edgeR TPM value to normalize to the length of the exonic region of the gene.

GSEA
The GSEA analyses were performed using the GSEA software (28). The TPM values from edgeR were used as the input files. The msigdb.v7.4.symbols.gmt was used as the gmt file. The Mouse_Gene_Symbol_Remapping_MSigDB.v7.0.chip was used as the chip file. The parameters of ‘1000 permutation’, ‘collapse’ and ‘permutation type: gene_set’ were selected.

Motif enrichment analysis
The motif enrichment analysis was carried out using the ‘findMotifs.pl’ command in Homer (29). The input file contains the gene Ensembl IDs of the 1052 genes. The parameters used were ‘mouse -start -1000 -end 1000 -len 8,10,12 -p 30’. The P-value < 1E-12 was used as the cutoff to identify enriched motifs from the Homer de novo Motif Results.

ChIP-seq data and analysis
The FASTQ files of the ChIP-seq data were downloaded from the EMBL-EBI European Nucleotide Archive database. Bowtie (51) was used to align the FASTQ files to the mouse mm10 genome by the parameters of ‘-v 2 -m 1 -p 40’. The ‘samtools view’ (52) was used to convert the sam files into bam files. The bamCoverage (53) was used to convert the bam files into bw files by the parameters of ‘–binSize 10 -p 40’. Integrative genomics viewer (IGV) (54) was used to visualize the bw files. The 1000 control genes were selected from the genes expressed in Neuro 2a cells, and the ‘int rand’ function in Perl was used.

Data plotting and statistical analysis
Most of the data plotting and statistical analysis were carried out using the R version 4.0.4. The ‘pheatmap’ in R was used to plot the heatmaps. The ‘t.test’ in R was used to carry out the t-test. The ‘cor’ in R was used to calculate the Pearson’s correlation coefficient.

Availability
All computational scripts used in this study are available in the GitHub repository (https://github.com/Jerry-Zhao/KS2022).

Accession numbers
The RNA-seq data of raw and processed files generated in this study have been deposited with the NCBI Gene Expression Omnibus under the accession number of GSE201401. The accession numbers for publicly available ChIP-seq data are GSM1693007 (Sox9), GSE91043 (H3K4me3 and H3K27ac), GSE69949 (Sox10) and GSE164360 (Chd7). The accession number for publicly available RNA-seq data is GSE164360 (Chd7 WT and knockout).

Supplementary Material
Supplementary Material is available at HMG online.

Acknowledgements
We thank Dr Weikang Cai, Dr Raddy Ramos and members of the Zhao Laboratory for helpful discussions and comments on the manuscript. We thank the Center for Biomedical Innovation at the New York Institute of Technology College of Osteopathic Medicine for support.

Conflict of Interest statement. The authors declare that they have no competing interests.

Funding
This work was supported by start-up funds from the New York Institute of Technology College of Osteopathic Medicine.

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