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The dual role of *RFX6* in directing β cell development and insulin production

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Abstract

RFX6 transcription factor is believed to play a central role in directing cell development of insulin-producing pancreatic islet. *RFX6* homozygous mutations cause syndromic neonatal diabetes with hypoplastic pancreas. However, *RFX6* heterozygous mutations cause maturity-onset diabetes of the young (MODY) with normal pancreas development. Here, we show that *RFX6* may control islet cell development and insulin production in different manners. The *rfx6* knockout zebrafish generated by CRISPR/Cas9 exhibited an overt diabetes phenotype. Pancreatic islet failed to form compact structures in the knockout fish. While endocrine pancreatic islet non- β -cells were absent, insulin-producing β -cells were present in the knockout fish. Although insulin mRNA level was normal in the β -cells of the knockout fish, insulin protein level was decreased. High-throughput RNA sequencing (RNAseq) showed that differentially expressed genes were enriched in the translation term in islet β -cells from the knockout fish. Chromatin immunoprecipitation sequencing (ChIPseq) of normally developed islet β -cells from mice demonstrated that *rfx6* interacted with translation initiation factors and controlled insulin translation. Our data indicate that *Rfx6* may act as a transcription factor regulating the transcription of genes involved in mRNA translation, which may represent a new mechanism and treatment strategy for diseases.

Key Words

- ▶ *Rfx6*
- ▶ Cas9
- ▶ islet development
- ▶ insulin
- ▶ RNA-seq
- ▶ ChIP-seq

*Journal of Molecular
Endocrinology*
(2021) **66**, 129–139

Introduction

Diabetes is a global health problem that is tightly associated with the dysfunction or loss of insulin-producing pancreatic islet β -cells. In this context, seeking the mechanisms underlying β -cell development and function serves a crucial challenge. Specific transcription factors including *PDX1*, *NEUROD*, *NKX2.2*, *NKX6.1* and *PAX6* are involved in the development of pancreatic islet β -cells (Desai *et al.* 2008, Papizan *et al.* 2011, Taylor *et al.* 2013, Pauerstein *et al.* 2015, Zhu *et al.* 2016).

The regulatory factor X6 (*RFX6*) transcription factor is expressed at a high level only in the pancreas (Smith *et al.*

2010). Homozygous mutations in *RFX6* cause Mitchell–Riley syndrome, an autosomal recessive syndrome of severe neonatal diabetes, hypoplastic or annular pancreas and intestinal atresia (Smith *et al.* 2010, Pearl *et al.* 2011, Concepcion *et al.* 2014). It has been proposed that this severe diabetes in patients with homozygous mutations in *RFX6* contributed to the overall deficiency of pancreatic islet development and function including β -cell insulin production (Smith *et al.* 2010, Pearl *et al.* 2011). On the other hand, heterozygous mutations in *RFX6* can cause mild maturity-onset diabetes of the young (MODY) with

reduced penetrance (Patel *et al.* 2017). In these patients with *RFX6*-MODY, defective insulin secretion was attributed to insulin-secreting function failure of the islet β -cells with normal development of the islets (Chandra *et al.* 2014, Piccand *et al.* 2014). Therefore, we propose that *RFX6* controls both pancreatic islet development and insulin production in different manners.

RFX6 knockdown in human EndoC-BH1 cells showed changes in insulin mRNA (Chandra *et al.* 2014). Mice with *Rfx6* knockout die within 2 days after birth, and hence the role of *RFX6* in β -cell function and insulin production is limited (Smith *et al.* 2010, Pearl *et al.* 2011). In this study, we used *rfx6* knockout zebrafish (*Danio rerio*) generated by CRISPR/Cas9 to illustrate the precise role of *rfx6* in controlling insulin production in fetal and postnatal stages (Zhang *et al.* 2016b). To study the role of *rfx6* in controlling insulin production in postnatal stages in animals with normal development, we also performed chromatin immunoprecipitation sequencing (ChIPseq) in mice. Our study shows that *rfx6* is both a transcription factor essential for non- β -cell development and a translation factor or initiator for insulin protein translation.

Materials and methods

Zebrafish strains and maintenance

The endocrine pancreas was isolated from the bacterial artificial chromosome *neurod1:GFP* transgenic fish (Obholzer *et al.* 2008) and *insulin:mCherry* transgenic fish (Pisharath *et al.* 2007). Adult *neurod1:GFP*, *neurod1:GFP/insulin:mCherry* and WT (Tubingen strain) zebrafish (*Danio rerio*) were raised and cared for according to standard protocols in a recirculating system (The Aquatics Habitat, Gloucester, UK). After treatment, the experimental fish were maintained in isolation tanks with daily water changes. All experiments with laboratory animals were complied with the Ethical Review Committee at Beijing Tongren Hospital, Capital Medical University, China (No. TRECKY2018-037).

CRISPR/Cas9 design

The CRISPR/Cas9 gene sequence was designed and ordered commercially (Sangon, Shanghai, China). The full-length products were cloned into pcDNA3.1 (Invitrogen). The human U6 promoter and gRNA scaffold were obtained commercially (Sangon) and cloned into the pUC19 vector. The sequence of Cas9 was designed as 5'-GAAGAAGACCTGGGCCATCTGG-3'.

Blood glucose determination

Blood glucose measurements were obtained as follows: zebrafish were anesthetized in 4°C ice-water for 2 min, and a pulled capillary pipet was inserted into the atrium of the beating heart. Approximately 5 μ L of blood was removed and rapidly transferred to a Freestyle Freedom Lite device. We did not use blood from the tail vein because this technique caused more lasting damage than heart puncture. Using the Freestyle Freedom Lite device, 0.3 μ L of blood was sufficient for glucose measurement.

Immunohistochemistry analysis of zebrafish tissue

Tissues were fixed by immersion in 4% paraformaldehyde for 4 h at room temperature, then embedded in paraffin. Heat-induced antigen retrieval was then performed on the paraffin-embedded sections (5 μ m) in citrate buffer (pH 6.0) at 99°C for 5 min by using a microwave. The slides were incubated in normal serum at room temperature for 1 h, followed by incubation with the primary antibody, guinea pig anti-insulin antibody; diluted 1:100 (Abcam) or *rfx6* antibody PA5-68655; diluted 1:100 (Thermo). The sections were washed three times with PBS and then incubated with Alexa Fluor 555 guinea pig anti-goat; diluted 1:200 (SC-362265, Santa Cruz). These sections were mounted with fluorescent mounting medium containing DAPI (ZSGB-BIO, Beijing, China). Hematoxylin and eosin (H&E) staining was used to observe islet morphology changes. Images were recorded with a laser-scanning confocal microscope, Leica TCSP5 (Leica).

Western blotting

The pancreas were solubilized in radio-immunoprecipitation assay (RIPA) buffer containing 100 mmol/L phenylmethylsulfonyl fluoride (PMSF) and 25 mmol/L proteinase inhibitors (Roche). The supernatant was collected following centrifugation of the lysate at 10,000 *g* for 20 min at 4°C and the enhanced BCA Protein Assay Kit (Beyotime Biotechnology, Beijing, China) was used to determine protein concentration in the samples. Equivalent amounts of protein were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore). The membrane was blocked with 5% non-fat dry milk for 1 h followed by immunoblotting with specific antibodies (1:1000, ab210560, Abcam). Western blotting images were scanned and analyzed with ImageJ software for densitometry measurements.

RT-PCR and real-time RT-PCR

Total RNA was purified using an RNA prepPure Tissue Kit (Tiangen Biotech, Beijing, China). RNA samples were converted to cDNA using FastQuant RT Super Mix (Tiangen Biotech). Real-time RT-PCR was then performed on a LightCycler® 480 Real-Time PCR System (Roche) using SYBR Green I Master Mix reagent (Roche). Three distinct amplifications were performed in duplicate for each transcript, and mean values were normalized to the mean value of the reference mRNA cyclophilin.

RNA probes, whole-mount *in situ* hybridization (WISH) and imaging

Antisense RNA probes were prepared by transcribing linearized cDNA clones with SP6, T7 or T3 polymerase using digoxigenin in or DNP labeling mix (Roche) according to the manufacturer's instructions. The probes were subsequently purified on NucAway spin columns (Thermo) and ethanol-precipitated. The zebrafish *insulin*, *glucagon*, *somatostatin*, *ghrelin* and *trypsin* probes were used. Single whole-mount and double fluorescent *in situ* hybridizations were performed as described (Tong *et al.* 2014). For visible WISH, the numbers of cells were quantified by counting the cells under the microscope by focusing successively on each layer of stained cells.

RNAseq

RNAseq experiment was performed from isolated pancreas of zebrafish. A total of 3 µg of RNA per sample was used as an input material for RNA sample preparation. Sequencing libraries were generated using the NEB Next®Ultra™ RNA Library Prep Kit for Illumina® (New England Biolabs, MA, USA). The index-coded samples were clustered using a cBot Cluster Generation System and a TruSeq SR Cluster Kit v3-cBot-HS (Illumina) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an IlluminaHiSeq 2000/2500 platform, and 150-bp/100-bp/50-bp paired/single-end reads were generated.

Functional analysis of differentially expressed genes

The gene ontology (GO) enrichment analysis and the web-based Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was provided by DAVID Bioinformatics Resources 6.7 (<http://david.abcc.ncifcrf.gov>). Gene functional enrichment was performed using

the default parameters in DAVID to obtain an adjusted *P*-value <0.05 for the test gene group vs the zebrafish COG ontology annotation set.

ChIPseq

For ChIP, tissue preparation and ChIP protocol closely adhered to steps outlined in the SimpleChIP® Plus Sonication Chromatin IP System manual #56383 (Cell Signaling Technology), according to manufacturer's instructions as described previously (Cheng *et al.* 2019). Several antibodies were used to perform ChIP assay. We found that the most efficient antibody was from Millipore (#ABD28). ChIPseq experiment were repeated three times.

ChIP-qPCR

ChIP was performed using a protocol that described previously for ChIPseq. qPCR data were generated using a LightCycler96 thermocycler (Roche) and the qPCR SuperMix (Transgen Biotech, Beijing, China) according to manufacturer's instructions.

Statistics and data analysis

All statistical analyses were conducted with MedCalc version 11.4 (<http://www.medcalc.be>) or GraphPad Prism version 6.0. Data are presented as means ± s.e.m., and statistical comparisons were performed using Student's *t* tests or the Mann–Whitney *U* test as appropriate. Significance was defined as *P* < 0.05. In gene ontology and DAVID analysis, the statistical data were generated by the software.

Results

Rfx6 knockout causes diabetes in zebrafish

We designed a CRISPR/Cas9 short guide RNA (sgRNA) that specifically targets exon 5 of zebrafish *rfx6*. Restriction site disruption assay and PCR sequencing were used to confirm the target site modification. Three different frameshift mutant lines were obtained, which we named *rfx6*⁺¹³, *rfx6*⁺¹⁴ and *rfx6*⁺²². We used both male and female fish in this study and found no gender-specific phenotypes. Western blotting results showed that the protein level of *rfx6* decreased dramatically in KO fish which demonstrated that *rfx6* mutant fish was successfully constructed (Fig. 1A). Each of the line were used to perform the next experiments. These three *rfx6* mutants showed decreased survival from approximately

Figure 1

Diabetes caused by mutation of *rfx6* in zebrafish. (A) The protein level of *rfx6* decreased dramatically in KO fish. (B, C, D and E) The *rfx6* knockout zebrafish model showed diabetic phenotype with smaller body shape at different stages (B), shorter growth curve (C), lower survival rates (D), elevated blood glucose at 60 dpf (left) and 90 dpf (right) (E) and impaired fin regeneration (E) compared with the WT zebrafish. (F) The pancreas of *rfx6* knockout fish were smaller than the WT at 90 dpf (F). H&E staining showed a loose, dispersed and larger shape in *rfx6* knockout fish compared with adult WT (G, Scale Bars, 25 μ m). $n = 10$ fish (A, B, C), $n = 100$ fish (D), $n = 6$ fish (E, F) and $n = 8$ fish (G) per group for the mutant (Mu) and WT. $**P < 0.01$, $***P < 0.001$; Mann-Whitney *U* test (G) Data are mean $\pm$ s.e.m. Mu, mutant. A full color version of this figure is available at <https://doi.org/10.1530/JME-20-0119>.

7 days post fertilization (dpf), with most fish dead by 14 dpf. Surviving mutants were infertile and exhibited a gaunt body type (Fig. 1B and C). Only 5% of mutant embryos survived to adulthood (Fig. 1D).

Previous reports have shown that mutations of human *RFX6* resulted in neonatal diabetes (Smith *et al.* 2010, Pearl *et al.* 2011, Concepcion *et al.* 2014). When examining the glucose metabolism of *rfx6* knockout zebrafish, we found that, compared with their WT siblings, the adult *rfx6* knockout fish exhibited a significantly increased blood glucose levels at both 60 dpf and 90 dpf (Fig. 1E), gaunt body type and diabetic complications including fin regeneration in zebrafish (Intine *et al.* 2013) (Fig. 1F). The *rfx6* knockout fish failed to cope with a cold stimulus, thus implying that *rfx6* may function in maintaining the stress response system of zebrafish. All of which is the characteristics of insulin-dependent diabetes mellitus.

Rfx6 is necessary for compact islet structure

To further validate the morphological changes of islets from the adult *rfx6* knockout fish, we conducted hematoxylin and eosin (H&E) staining histological experiments. According to reference, main pancreas of the adult zebrafish has one large islet and three to six smaller islets (Namba 2019, Toselli *et al.* 2019). The pancreas of the *rfx6* knockout fish was smaller compared with WT. The WT islets presented compact and dense shapes, while the islets of *rfx6* knockout fish were significantly looser and larger. Total number of β -cell numbers and

β -cell proliferation were unchanged both in Wt and Mu fish (Fig. 1G). These data show that *rfx6* is necessary for normal islet morphology.

Rfx6 knockout decreases insulin protein production

In this study, we choose a *neurod1:eGFP* transgene that is broadly expressed in the CNS during embryonic development as the target integration site (Obholzer *et al.* 2008). The *eGFP* transgene allows the direct visualization of target gene disruption and should not compromise survival upon loss of gene function. We used *neurod:eGFP* line in this study. In WT embryos, *neurod:eGFP* transgenic fish showed eGFP expression within the compact islet structure at 3 dpf (Fig. 2A). The numbers of *neurod*⁺ cells were unchanged in *rfx6* mutants, thus demonstrating that *rfx6* mutation does not affect the early stages of pancreatic endocrine development (Fig. 2B). In WT islet cells, which were identified by *neurod:eGFP*, labeling of insulin showed clear insulin protein in the islets of embryo WT fish, while the knockout fish showed decreased insulin protein at the age of 3 dpf (Fig. 2B) and 90 dpf (Fig. 2C).

We further examined whether insulin protein was influenced in the adult *rfx6* knockout fish (90 dpf). Total number of β -cell numbers and β -cell proliferation were also unchanged both in Wt and Mu fish. Western and histochemical staining showed clear insulin protein in the islets of adult WT fish, while dramatically decreased insulin protein was detected in the islets of adult *rfx6* knockout fish (Fig. 2D).

Figure 2

Significantly decreased insulin protein of *rfx6* knockout fish. (A and B) *Neurod:eGFP* transgenic line was used to incross with *rfx6*^{-/-} line. *rfx6*^{-/-}/*Neurod:eGFP* showed green fluorescence (A, Scale Bars, 1 μ m). (B and C) Significantly decreased insulin protein of *rfx6* knockout fish were detected both in *rfx6*^{-/-}/*Neurod:eGFP* in the embryos (B, Scale Bars, 100 μ m) at the age of 3 dpf and *rfx6*^{-/-} adult fish (C, Scale Bars, 10 μ m) at the age of 90 dpf. *n* = 4 (B, C) independent samples per group for the mutant (Mu) and WT. (D) Significantly decreased insulin protein were detected in Mu adult fish compared with Wt fish. $**P < 0.01$, $***P < 0.001$; Mann-Whitney *U* test (B, C, D). Data are mean $\pm$ S.E.M. A full color version of this figure is available at <https://doi.org/10.1530/JME-20-0119>.

These data show that the *rfx6* mutation does not influence the development of β -cells and that the gene is necessary for the production of insulin protein and therefore for regulating glucose metabolism in postnatal stage.

Rfx6 only regulates islet non- β -cells development

Because *rfx6* was found to be necessary for the development of the endocrine pancreas, we next examined whether it affects exocrine pancreas development. At 4 dpf, *rfx6* knockout fish showed normal *trypsin* expression compared with that of WT animals, thus indicating that the development and function of acinar cells were normal (Fig. 3A). Briefly, *rfx6* did not influence the development of the

exocrine pancreas, demonstrating that its effect is specific to the endocrine pancreas.

We next explored the islet cell development through whole-mount *in situ* hybridization (WISH) and RT-PCR. The results showed that mRNA levels of *glucagon* (*gcga*), *somatostatin* (*sst*) and *ghrelin* (*ghrl*) were significantly downregulated in the *rfx6* knockout fish at 2 dpf, thus indicating a failure of α -, δ - and ϵ -cell development (Fig. 3B). However, mRNA levels and sequence of *insulin* (*ins*) appeared normal in the *rfx6* knockout fish, suggesting that β -cell development remained unaffected (Fig. 3C). RNAseq results further confirmed that *glucagon* (*gaga*), *somatostatin* (*sst*) and *ghrelin* (*ghrl*) expression were downregulated in *rfx6* knockout fish, and that *insulin* (*ins*) expression remained unaffected. The results showed that

Figure 3

Failure of non- β endocrine cell differentiation in the *rfx6* knockout fish. (A and B) *In situ* hybridization showed that the expression levels of *glucagon* (*gcga*), *somatostatin* (*sst*) and *ghrelin* (*ghrl*) were decreased in the *rfx6* knockout fish (B, Scale Bars, 20 μ m), whereas *trypsin* (A, Scale Bars, 10 μ m) and *insulin* (*ins*) (B) level was normal. (C, D and E) *Neurod:eGFP/insulin:mCherry* transgenic line was used to incross with *rfx6*^{-/-} line (D, Scale Bars, 100 μ m), *rfx6*^{-/-}/*Neurod:eGFP/insulin:mCherry* showed green and red fluorescence in one cell (E), suggesting insulin secreting β -cell development remained unaffected (E, Scale Bars, 200 μ m). *n* = 10 fish for the mutant (Mu) and *n* = 22 fish for WT (A), *n* = 23, 26 or 23 fish for the mutant (Mu) and *n* = 11, 12 or 23 fish for WT (B), *n* = 8 fish for the mutant (Mu) and WT (C). *n* = 5, Mann-Whitney *U* test (E). Data are mean $\pm$ S.E.M. A full color version of this figure is available at <https://doi.org/10.1530/JME-20-0119>.

the knockout fish specifically regulated the development of non- β endocrine cells.

In a further set of experiments, we used *neurod:eGFP/insulin:mCherry* transgenic fish line to observe the mRNA levels of *neurod* and *insulin* in the same cell. When examining *neurod:eGFP/insulin:mCherry* expression, we found a non-compact islet structure phenotype in the *rfx6* knockout fish, which was the same as the H&E staining results. The islets of *rfx6* mutant embryos were loose and disperse in shape and were larger than those in WT embryos. *Neurod*⁺ and *ins*⁺ cells were both detected in the WT and knockout fish, suggesting that β -cell development remained unaffected (Fig. 3D and E).

These results show that the *rfx6* mutation does not affect the development of endocrine progenitors, but specifically regulate the development of non- β endocrine cells.

Rfx6 is not necessary for insulin gene transcription

To search for genes that might be involved in the *rfx6* mutant phenotype, we performed real-time RT-PCR. In *rfx6* knockout fish, pancreatic duodenal homeobox-1 (*pdx1*) and *neurod* were both upregulated, suggesting that *rfx6* acted downstream of these two genes (Fig. 4A). Remarkably, the expression of *arx* was strongly reduced in *rfx6* knockout fish, thus suggesting that *rfx6* acted upstream of *arx* in the regulatory cascade of α -cell development (Collombat *et al.* 2003, Wang *et al.* 2007, Dorrell *et al.* 2011). While *glucagon*, *somatostatin* and *ghrelin* were downregulated, *insulin* was unchanged in

rfx6 knockout fish (Dryja *et al.* 1993, Støy *et al.* 2007, Edghill *et al.* 2008) (Fig. 4B, C and D). The expression of key regulators of islet β -cell development, including *mafa*, was also normal in the *rfx6* knockout fish (Rual *et al.* 2005, Desai *et al.* 2008, Papizan *et al.* 2011, Taylor *et al.* 2013) (Fig. 4E). As *glut2* is also expressed in zebrafish pancreatic β cells, we detected the mRNA level of *glut2* (Marín-Juez *et al.* 2015). The results showed that reduced expression in the mutant fish (Fig. 4E). These results were consistent with RNAseq of RNA isolated from the adult pancreas of WT fish and *rfx6* knockout fish.

In summary, *rfx6* specifically regulates pancreatic islet non- β -cell development and may decrease insulin synthesis at the translational but not transcriptional level in zebrafish (Fig. 4F).

Rfx6 regulates expressed gene related to protein translation

To better understand the precise function of *rfx6* in the zebrafish, especially the gap between insulin mRNA and protein, high-throughput RNAseq was performed. As it is very hard to dissect pancreas from the adult zebrafish, we isolated pancreas from 5 zebrafish for each group, then mix them for RNAseq. Solexac DNA libraries were constructed from the pancreas of WT and knockout zebrafish. The criteria of threshold ≥ 1.5 or ≤ 0.67 in expression were chosen as significantly upregulated or downregulated genes. Using these criteria, a total of 5821 genes were significantly differentially expressed, including 2734 upregulated genes and 3087 downregulated genes.

Figure 4

The mRNA levels of pancreas transcription factors. The pancreas of the zebrafish were extracted from the mutant (Mu) and WT fish at the stage of 3-month. (A, B, C, D and E) The mRNA levels of *pdx1* and *neurod* were upregulated (A) and *rfx6* (A), *arx*, *glucagon* (*gcga*) (B), *somatostatin* (*sst*) (C) and *ghrelin* (*ghrl*) (D) were downregulated, while the mRNA levels of *insulin* (*ins*) remained unchanged in the adult knockout fish (Mu). (F) Summary of endocrine cell development in the *rfx6* knockout fish. *n* = 6 (A, B, C, D and E) for the mutant (Mu) and WT, three independent experiments (A, B, C, D and E). **P* < 0.05, ***P* < 0.01, ****P* < 0.001; Mann-Whitney *U* test (A, B, C, D and E). Data are mean $\pm$ S.E.M. A full color version of this figure is available at <https://doi.org/10.1530/JME-20-0119>.

The Clusters of orthologous groups (COG) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were provided by DAVID Bioinformatics Resources 6.7 (Huang *et al.* 2008) (<http://david.abcc.ncifcrf.gov>). Among the 5821 differentially expressed genes, DAVID provided functional annotation for 4972 genes (Fig. 5A). KEGG pathway analysis showed that spliceosome pathway was ranked No 1 ($P = 2.40\text{E}-07$). Spliceosome pathway was represented by three upregulated and 65 downregulated genes (data not shown). We performed qPCR of *hhex* and *pax6* in the mutant zebrafish. They were no significant change in the mutant fish compared with WT fish. These data meant that hormone–endocrine+ (NeuroD+) cell population was molecularly considered as α/δ cells, but just not producing hormone. Real-time RT-PCR showed that the expression trends of nine randomly selected genes including *snrnp*, *usp39*, *eftud*, *imp3*, *dhx15*, *cpsf3*, *mill1*, *mcts1* and *rbm25* were consistent with RNAseq results. Differentially expressed genes from the mutant zebrafish were mapped to the spliceosome pathways. However, the mRNA sequence of *insulin* was unchanged in the *rfx6* knockout fish, suggesting that the splicing of *insulin* was normal.

Figure 5

COG ontology enrichment analysis of RNAseq results. (A) RNAseq showed a total of 5821 genes were significantly differentially expressed. (B, C, D and E) COG Ontology enrichment analysis showed that 'Translation, ribosomal structure and biogenesis' may be related with *rfx6* gene (B). Differentially expressed genes related with translation were listed (C), which was selectively confirmed by real-time RT-PCR (C). A full color version of this figure is available at <https://doi.org/10.1530/JME-20-0119>.

COG ontology enrichment analysis showed that the differentially expressed gene may be mainly related to translation, ribosomal structure and biogenesis ($P = 2.70\text{E}-06$) (Fig. 5B). There was also a statistically significant amount of mapped genes for other functions, such as posttranslational modification. Meanwhile, the detection of real-time RT-PCR showed that the expression trends of 12 randomly selected genes including *eif2s1*, *eif2g2*, *eif3hb*, *eif4h*, *magoh*, *sf3a2*, *eif2a*, *snrnp40*, *snrpe*, *yars*, *eif2ba* and *eif1axb*, detected by the two methods were consistent. These results suggested that the RNAseq results were reliable (Fig. 5C and D).

Rfx6 directly binds to promoter and enhancer of *eif2ak1* and *eif5*

To determine whether translation-related genes might be the direct targets of *rfx6*, we performed ChIPseq experiments in the pancreas of C57/BL6 mouse. We used C57/BL6 mouse instead of the zebrafish for several reasons. First, zebrafish is too small to get enough pancreas to perform the experiments. Another reason is antibody. We tried but failed to find a good ChIP-grade antibody for zebrafish. The most important is to avoid the confounding factor caused by abnormal development of *rfx6* knockout fish.

Here, 4146 unique peaks in the genome region of the adult murine pancreas were captured. Most of the binding peaks were located in intron or intergenic regions. Over 1000 genes were captured as *Rfx6*-binding genes in the pancreas. One peak was detected in the *eif5* promoter region. Similarly, a peak was observed in a conserved region of intron 1 and intron 4 of the *eif2ak1* gene. *Eif2ak1* plays an important role at the translation initiation level to downregulate protein synthesis in response to stress (Lu *et al.* 2001, Zismanov *et al.* 2016). *Eif5* is a GTPase-activating protein specific to *Eif2*, which may act as a part of pre-initiation complex assembly and ensure correct AUG selection during eukaryotic translation initiation (Nanda *et al.* 2013). Quantitative ChIP experiments using a *rfx6* antibody, confirmed that endogenous *rfx6* binds to both promoter and enhancer region of *eif2ak1* (Fig. 6A and B). *Rfx6* can also bind to the promoter region of *eif5* (Fig. 6C). These findings were consistent with qRT-PCR results in the zebrafish (Fig. 6D). ChIPseq results also showed that *rfx6* can bind to *tars* and *dus4l*, which were closely related with translation. Taken together, our results suggest that *rfx6* is a direct positive transcriptional regulator of *eif2ak1* and *eif5* and that the downregulation of these genes could impact insulin translation which is consistent with RNAseq results.

Discussion

The pancreas is an important visceral organ that comprises two portions, the hormone-expressing endocrine islets and the digestive enzyme-expressing exocrine tissues. The endocrine islets are clusters of five types of hormone-producing cells, α -, β -, δ -, PP- and ϵ -cells, which produce glucagon, insulin, somatostatin, pancreatic polypeptide and ghrelin, respectively. In this study, we showed that *rfx6* was necessary for the development of non- β endocrine cells in fetal zebrafish. *Rfx6* is also involved in the formation of a compact islet structure. Postnatal *rfx6* knockout fish exhibited a diabetic phenotype resulting from the failure of insulin translation, suggesting that *rfx6* was not only a transcription factor but also a translation initiator (Fig. 4F).

Although much of the current understanding of pancreas organogenesis has relied on mouse and human genetic studies, the use of zebrafish has played an important role in studies of genetic pathways especially the early phases of pancreatic development (Moss *et al.* 2009). Previous studies of zebrafish *rfx6* have used morpholinos to focus on its role in the development of pancreas. The predicted zebrafish *Rfx6* protein displays 60% sequence identity with the mouse *Rfx6*. All mouse *Rfx* proteins could be identified in zebrafish. However, *rfx6* was not present in the gut endoderm, which meant differences in the mechanisms controlling endoderm and/or pancreas specification between the mouse and zebrafish. One study on *rfx6* morphants showed that *rfx6*

was a transcription factor affecting the development of glucagon-, ghrelin- and somatostatin-expressing cells, while insulin-expressing cell remained normal (Soyer *et al.* 2010). Another study showed that knockdown of *RFX6* in xenopus led to a loss of both endocrine and exocrine gene expression, including insulin expression (Pearl *et al.* 2011). These conflicting results, which may be due to the limitations of morpholinos, leave the function of *rfx6* in pancreatic islet cell development unclear. The recently discovered CRISPR/Cas9 system can create insertion and deletion mutations (indels) at specifically targeted sites, thereby causing gene disruption (Soyer *et al.* 2010, Le *et al.* 2013, Mali *et al.* 2013, Qi *et al.* 2013). This system has been used for gene knockout studies in several model species, including nematodes, fruit flies, zebrafish and mice (Dai *et al.* 2012, van Schendel *et al.* 2015, Zhang *et al.* 2016a,b).

Here, we generated zebrafish *rfx6* knockout lines using the CRISPR/Cas9 system. Most homozygous mutants died at approximately 7–14 dpf, with 5% surviving as infertile adults. *In situ* hybridization and qPCR results showed that *rfx6* is necessary for the development of non- β endocrine cells in natal zebrafish. However, β -cell development and insulin transcription was not affected by the *rfx6* knockout fish. Thus, the results mean that *rfx6* is necessary for the development of non- β endocrine cells in fetal zebrafish.

The few surviving *rfx6* knockout fish exhibited a gaunt body type and high blood glucose levels, both of which are characteristic of diabetes. These phenomena contradicted our qPCR results which showed insulin gene expression were not altered. To explain this controversy,

Figure 6

ChIPseq analysis. (A, B and C) ChIPseq and ChIP qPCR results showed that *Rfx6* can directly bind to promoter (A) and enhancer (B) of *eif2ak1* and promoter of *eif5* gene (C). (D) qRT-PCR results showed that *eif2ak1*, *eif5* and other genes were downregulated in *rfx6* knockout zebrafish. A full color version of this figure is available at <https://doi.org/10.1530/JME-20-0119>.

we further detected the level of insulin protein in postnatal *rfx6* knockout fish. Insulin protein was dramatically decreased in the *rfx6* knockout fish compared with that of control, thus indicating that insulin transcripts failed to translate to insulin protein. The decreased level of insulin protein may be the cause of diabetic phenotype in postnatal.

We suspected there may be two reasons to explain the gap between normal insulin mRNA level and decreased insulin protein level in postnatal fish. First is the islet structure. The generation of insulin-producing pancreatic β -cells from human pluripotent stem cells (hPSC) gives the possibility of drug discovery and cell transplantation therapy in diabetes (D'Amour *et al.* 2005, Cheng *et al.* 2012, Hrvatin *et al.* 2014). Stem-cell-derived β -cells secrete human insulin into the serum of mice only shortly after transplantation. This indicates that not only maturation by upregulation of key β cell regulators like MAFA but also the structure of islet may be important for insulin protein secretion. Our study showed that the islets of the *rfx6* mutant embryos were loose and disperses in shape and were larger than those in WT embryos. H&E staining experiments showed that WT islets presented compact and dense shapes, whereas the islets of *rfx6* knockout fish were not compact. We did not find ECM or cell adhesion pathway changing in the RNAseq. While some cell adhesion factors including rap1b, gon4l and crb2a were decreased in the RNAseq. This information may explain the structural changes in KO islets. Our model demonstrates that without integrant islets structure, insulin-producing pancreatic β -cells cannot secret insulin.

Another reason for the gap between normal *insulin* mRNA level and decreased insulin protein level may be the dysfunction of insulin translation. Previous study showed that transcription factor *PAX6* was crucial for β -cell function through controlling some key genes involved in insulin biosynthesis (Gosmain *et al.* 2012). RNAseq technology has been successfully applied to several disease models of zebrafish. However, none has used RNAseq approach to elucidate *rfx6* precise function in zebrafish. *Rfx6* is a member of the *RFX* family of transcription factors, so a better understanding of transcriptome changes in *rfx6* mutant may help to analyze the phenotype and find the potential downstream target genes regulated by *rfx6*. In our study, we performed a high-throughput RNAseq of isolated pancreas from adult *rfx6* mutant and control. Interestingly, DAVID enrichment analysis showed that the differentially expressed genes were enriched in 'Translation, ribosomal structure and biogenesis'. It's interesting why and how *rfx6* defect may

cause the downregulation of a number of translation-related genes. To avoid the confounding factor caused by abnormal development of *rfx6* knockout fish, we performed ChIPseq using normally developed islet β -cells from C57/BL6 mice. Previous study performed ChIP-seq experiments performed in the mouse β cell line Min6 transfected with HA-tagged *Rfx6* (Piccand *et al.* 2014). Here, we performed ChIP-seq in the mouse pancreas directly. Our previous results found that *Rfx6* play an important role in pancreas development in adult mice (Cheng *et al.* 2019). In this study, we found that *rfx6* was a direct positive transcriptional regulator of *eif2ak1* and *eif5*, which played important roles at the translation initiation level. This can explain why the mRNA level of *insulin* remained unchanged, while the protein level of insulin dramatically decreased. To our knowledge, this study is the first report that *rfx6* is involved in controlling insulin protein translation.

Differentially expressed genes from the knockout fish were mapped to the translation signaling pathways (Walsh & Mohr 2011) (Fig. 7). Regulated mRNA translation can be subdivided into three stages including initiation, elongation and termination, each of which requires specific factors. At the initiation stage, *eif1* and *eif1a*, which can bind to 40s ribosome, along with *eif2*, *eif4a*, *eif4e*, *eif4g*, *eif4h*, *eif5* were downregulated in the *rfx6* knockout fish. Importantly, *rfx6* can directly bind to the promoter and enhancer elements of *eif2ak1* or *eif5*. *eef2k*, which has an inhibition role in transitioning from initiation to elongation, was upregulated. Termination and re-initiation may be linked through *pabp* with *erf1*, *pelo*, *gsgt*. The genes were downregulated in the *rfx6* knockout fish. Based on the above data, our results suggest a novel function of transcription factor *rfx6* in protein translation, which may explain the normal *insulin* mRNA level but dramatically decreased insulin protein level in postnatal stage.

Our study is limited due to we only focus on insulin protein translation, while protein translation is a global impairment. We will use *pdx1*-cre mice interlink *Rfx6* conditional knockout mice in the future to detect the detail function of *RFX6*.

In our study, we found that *rfx6* was necessary for the development of non- β endocrine cells in fetal zebrafish, which may be one reason for diabetes. While in postnatal fish, mutation of *rfx6* causes dysfunction of insulin translation, which may be another reason for diabetes. In conclusion, mutation of *rfx6* disrupts islet non- β -cell development and insulin translation. A transcription factor regulating the transcription of genes involved in

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Received in final form 29 November 2020

Accepted 16 December 2020

Accepted Manuscript published online 23 December 2020