Rfx6 directs islet formation and insulin production in mice and humans.
Rfx6 Directs Islet Formation and Insulin Production in Mice and Humans

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A.M.P., J.M, J.D, S.V.E., M.A., N. Kacet, J.W., M.E.R., M.G., I.H., and A. H. recruited the human subjects and provided phenotypic information.

Competing interests
M.S.G. is an inventor on patents held by the University of California covering Neurogenin3 and its use.
Abstract

Insulin from the β-cells of the pancreatic islets of Langerhans controls energy homeostasis in vertebrates, and its deficiency causes diabetes mellitus. During embryonic development, the transcription factor Neurogenin3 initiates the differentiation of the β-cells and other islet cell types from pancreatic endoderm, but the genetic program that subsequently completes this differentiation remains incompletely understood. Here we show that the transcription factor Rfx6 directs islet cell differentiation downstream of Neurogenin3. Mice lacking Rfx6 failed to generate any of the normal islet cell types except for pancreatic-polypeptide-producing cells. In human infants with a similar autosomal recessive syndrome of neonatal diabetes, genetic mapping and subsequent sequencing identified mutations in the human RFX6 gene. These studies demonstrate a unique position for Rfx6 in the hierarchy of factors that coordinate pancreatic islet development in both mice and humans. Rfx6 could prove useful in efforts to generate β-cells for patients with diabetes.

Expression of Rfx6

In independent screens for genes co-expressed with Neurogenin3 in islet progenitor cells, activated by Neurogenin3, and uniquely expressed in islets (M.E.W. and J.D.J., unpublished data), we identified Rfx6, a member of the RFX (Regulatory Factor X-box binding) family of winged-helix transcription factors. Rfx6 transcripts could be detected in mouse and human embryonic pancreas by RT-PCR, but, unlike all other known islet transcription factors, not in brain (Fig. 1a, c), and not in mouse embryonic pancreas lacking Neurogenin3 (Fig. 1a). In contrast, mouse Rfx4, the mammalian RFX gene with the highest homology to Rfx6, was amplified from brain and not from pancreas, and the other RFX genes were more widely expressed (Fig. 1a).

To explore the pattern of Rfx6 protein expression, we used antiserum generated against recombinant Rfx6 protein for immunofluorescence studies. In mice, Rfx6 was detected as...
As early as embryonic day 7.5 (e7.5) throughout the definitive, but not extraembryonic, endoderm and persisted broadly in gut endoderm at e9.0, after which it becomes progressively restricted to the pancreas and scattered cells in the gut (Fig. 2a-c, Supplemental Fig. S1 and data not shown). At e10.0, immunofluorescence staining detected Rfx6 in foregut/midgut epithelium and in scattered cells in the nascent pancreatic buds, as indicated by staining for pancreatic transcription factor Pdx1 (Fig. 2d-f). Most of these scattered Rfx6-expressing cells did not co-express Pdx1, but many co-expressed Nkx2.2 and Neurogenin3 (Supplemental Figs. S1 and S2). By e12.5, the Rfx6-expressing cells were generally distinct from the Pdx1-expressing progenitor cells, but most co-expressed glucagon, demonstrating restricted expression of Rfx6 in the endocrine lineage even at this early stage (Supplemental Fig. S3). In pancreata from Neurog3\(^{−/−}\) embryos, there were no Rfx6-expressing cells (Supplemental Fig. S4).

During the peak of endocrine cell differentiation at e15.5, Rfx6 co-localized with Neurogenin3 in the nuclei of a subset of the endocrine progenitor cells (Fig 2g), and overlapped with the islet transcription factors Nkx2.2, Nkx6.1, and Pdx1 (Supplemental Fig. S5). At e18.5, Rfx6 could be found in the nuclei of cells expressing each of the major pancreatic endocrine hormones (Fig. 2h and Supplemental Fig. S6). In the adult pancreas, Rfx6 expression was restricted to the islets where it could be detected in all endocrine lineages (Fig. 2i and Supplemental Fig. S7).

To generate Rfx6 null mice, we used homologous recombination to replace the first five exons of the Rfx6 gene, including the sequences encoding the DNA-binding domain, with a cassette encoding an eGFP-cre fusion protein (Supplementary Fig. S8). By crossing mice heterozygous for the mutant allele with mice carrying the marker gene ROSA26 loxP-stop-loxP lacZ (R26R)\(^{11}\), we generated Rfx6\(^{+/−}\)eGFPcre/R26R double heterozygous mice in which Rfx6-expressing cells and their descendents are marked by the expression of β-galactosidase and can be visualized with the X-gal substrate (Fig. 3a-d and Supplemental Fig. S9). β-galactosidase expression was detected in all embryonic tissue derived from the endoderm germ layer, but not in other embryonic or in extraembryonic tissues, demonstrating that Rfx6 is broadly expressed in and restricted to the definitive endoderm prior to the formation of the endoderm-derived organs. Taken together, the immunohistochemistry data and lineage tracing demonstrate that Rfx6 is expressed initially broadly in the definitive endoderm after gastrulation, becomes restricted to the gut and pancreatic bud at mid gestation, is reactivated by Neurogenin3 in islet progenitor cells and is ultimately restricted to pancreatic islets in the mature pancreas.

\(\text{Rfx6}^{−/−}\text{eGFPcre/eGFPcre} \text{ mice}\)

From heterozygous crosses, homozygous Rfx6\(^{−/−}\)eGFPcre/eGFPcre mice were born at the expected Mendelian ratio, but failed to feed normally, exhibited gross bowel distension due to small bowel obstruction (Fig. 3e-f) and died within 2 days post partum. Some, but not all, of the Rfx6 null animals also had reduced pancreas size (data not shown).

To test for effects on gene expression prior to birth, we harvested RNA from e17.5 pancreata, and used low density TaqMan arrays\(^6\) to measure the levels of a set of pancreatic
genes (Supplemental Table S1). \( Rfx6^{eGFPcre/eGFPcre} \) pancreata had almost no expression of the islet hormones genes, except for pancreatic polypeptide (\( Ppy \)). Several other islet/β-cell genes, such as the zinc transporter \( Slc30a8 \) and G-protein coupled receptor \( GPR40/Ffar \) genes, were similarly reduced; but other β-cell genes were more modestly reduced, including the glucose sensing genes \( Gck, Slc2a2, \) and \( Kcnj11 \). Immunofluorescence staining with the endocrine markers Chromogranin A or Synaptophysin at e17.5 demonstrated that the \( Rfx6^{eGFPcre/eGFPcre} \) pancreata still contained a large number of endocrine cells, but confirmed that none of these cells expressed insulin, glucagon, somatostatin or ghrelin (Fig. 4). Although the number of Ppy-expressing cells was increased in the \( Rfx6^{eGFPcre/eGFPcre} \) pancreata, they only accounted for a subset of the endocrine cells (Fig. 4i-l), leaving the identity of the remaining endocrine cells unknown.

We also tested whether Rfx6 regulates other transcription factor genes (Supplemental Table S2). The absence of Rfx6 did not affect Neurogenin3 expression, and this was confirmed at the protein level (data not shown). In sharp contrast, expression of genes downstream of Neurogenin3 encoding factors involved in alpha-cell development, including \( Irx2 \) and \( Arx \), was markedly reduced in \( Rfx6^{eGFPcre/eGFPcre} \) pancreata. Interestingly, genes encoding several factors involved in insulin gene transcription (\( Pax6, MafA, NeuroD1 \) and \( Pdx1 \)) also had reduced expression, but some key genes involved in β-cell specification either did not significantly change (\( Nkx2.2 \) and \( Nkx6.1 \)), or increased (\( Pax4 \)). Immunofluorescence staining at e17.5 revealed that the field of Nkx6.1 expression expanded from β-cells alone in wildtype pancreata to include all of the Chromogranin A + endocrine cells, including the PP cells in \( Rfx6^{eGFPcre/eGFPcre} \) pancreata (Fig. 4i-l and Supplementary Table S3). These studies suggest that while Rfx6 regulates the transcription factors involved in β-cell maturation and function, it restricts the expression of the β-cell differentiation and specification genes, and thus the β-cell fate choice.

Mice with a targeted disruption of the \( Rfx3 \) gene have an islet phenotype that is similar to, but less extreme than, the \( Rfx6^{eGFPcre/eGFPcre} \) mice, with reductions in the numbers, but not complete loss, of insulin- and glucagon-expressing cells and an increase in pancreatic polypeptide-expressing cells. In a proteome-wide screen of protein-protein interactions, Rfx6 was found to interact with Rfx2 and 3. Since the RFX transcription factors generally bind to their target DNA sites (the “X box”) as dimers, we tested whether Rfx6 and Rfx3 form a heterodimeric DNA binding complex in an electromobility shift assay (EMSA). We found that both full length Rfx3, and truncations that retain the DNA-binding and dimerization domains, bound to an X box site together with Rfx6 (Fig. 5a and Supplementary Fig. S10), and that the two factors cooperated in activating a promoter containing multimers of this X box site (Fig 5b).

It has been proposed that the islet phenotype of the \( Rfx3^{-/-} \) mice results from defects in primary cilia formation on islet cells, although islets lacking any primary cilia develop fairly normally. Unlike in the \( Rfx3^{-/-} \) mice, we found that primary cilia formation was unaffected in the \( Rfx6^{eGFPcre/eGFPcre} \) islets (Supplementary Fig. 11a-d). In addition, expression of the cilia genes \( Ifit88 \) and \( Dync2li1 \), which are reduced in the pancreas of \( Rfx3^{-/-} \) mice, was not reduced in the pancreas of \( Rfx6^{eGFPcre/eGFPcre} \) mice (Supplementary Fig.
We conclude that Rfx3 and Rfx6 cooperate in regulating a set of genes involved in islet development but not in cilia formation. The more modest islet phenotype in the Rfx3+/− mice may be due to the ability of Rfx2 to compensate partially for the loss of Rfx3 (Fig. 1a), or the ability of Rfx6 to direct gene expression as a homodimer (Fig. 5a, b).

**Human mutations in RFX6**

The phenotype of the Rfx6 eGFPcre/eGFPcre mice is remarkably similar to human patients born with neonatal diabetes and small bowel obstruction due to bowel atresia. Despite some reduction in pancreatic size, these cases were not deficient in enzymes of the exocrine pancreas, and autopsies of two cases (proband #1 and case 3 in Ref 17) revealed normal-appearing exocrine pancreata with clusters of ChromograninA-positive cells but total absence of cells staining for insulin, glucagon, or somatostatin. In addition, the syndrome involves hypoplastic gall bladder, and intractable diarrhea unresponsive to pancreatic enzyme replacement.

The disease locus was mapped using overlapping homozygosity in probands #1 and #2 (see Supplementary material for pedigree information and references to previous clinical case reports) respectively the offspring of first and second cousins. High-resolution homozygosity mapping identified 10 homozygosity-by-descent (HBD) segments >500 kb in proband #1 (after excluding those that overlapped with her unaffected sibling, Supplementary Table S4), and 25 HBD segments >500kb in proband #2 (Supplementary Table S5). Only three HBD regions were common in the two probands, totaling 24 Mb (Table 1). Altogether, 194 RefSeq genes map to these regions. Of these genes, only RFX6, which falls in the largest segment at 6q21-22, had pancreas−enriched expression in the TiGER database (Supplementary Table 6), and also increased in expression in human pancreas between foetal ages 10 and 20 weeks (Fig. 1b and Supplementary Figs. S13 and S14), concordant with the appearance of endocrine cells.

Two parallel, independent approaches unequivocally identified mutations in the RFX6 gene in this human syndrome: direct sequencing of the RFX6 gene and unbiased deep sequencing of all exons within the three overlapping HBD regions.

For deep sequencing, exons were captured from DNA obtained from Proband #2 using a tiled oligonucleotide array covering 1,309 of 1,322 exons mapping within the HBD regions. Amplification and sequencing of the captured fragments generated 40,379 sequences of at least 100 bp that aligned within the target regions. Median target coverage depth was 9.2, with 80% of targets having a depth of at least 4. Given that we were searching for a homozygous mutation, this was sufficient for unequivocal detection of exonic variants. Altogether, 30 novel sequence variants were detected (Supplementary Table S7): 15 in introns, 3 in both introns and untranslated regions (UTRs), 9 in UTRs, and only three in coding sequences, two synonymous. The only non-synonymous variant was 217 Ser>Pro in RFX6, identifying this gene as the most likely candidate.

In parallel, direct sequencing was performed on the 19 exons and the splicing junctions of RFX6 in all probands. Missense, splicing or frameshift mutations in RFX6 were found in five of the six available probands (Fig. 5e) with an interesting genotype-phenotype.
correlation. Proband #1, 4 and 5 all died in the first few months of life and were homozygous for, respectively, a loss of the donor splicing site in intron 2 (IVS2+2 t>c), an out-of-frame deletion in exon 7, and the missense mutation 181 R>Q involving a highly conserved arginine in the DNA-binding domain (Supplementary Fig. S15). Proband #3, still alive at the age of 9 and intermittently off insulin, was a compound heterozygote for donor-site loss in intron 6 (IVS6+2 t>g) and disruption of the acceptor site in intron 1 (IVS1-12 a>g). Proband #2, still alive at age 4.5 years18, had the homozygous missense mutation 217 Ser>Pro, confirming the unbiased exon sequencing described above. All mutations were inherited from carrier parents.

To determine the significance of the homozygous intron 2 splice donor splicing site mutation in proband #1, we amplified RFX6 mRNA by RT-PCR of high-quality RNA from autopsy pancreas and failed to detect the properly spliced transcript, which was easily amplified from normal foetal pancreas as was the reference gene cyclophilin in the proband’s RNA. We also failed to detect any RNA from exons 1+2, upstream of the splicing mutation, probably due to nonsense-mediated decay (Supplementary Fig. S16).

We also tested the two missense mutations for their effect on DNA binding by Rfx6. We found that 181R>Q (proband #5), which alters a conserved amino acid in the DNA binding domain, completely abrogated DNA binding, while 217S>P (proband #2), which lies between the DNA-binding domain and dimerization domain of Rfx6, only modestly reduced DNA binding (Fig. 5d) and did not affect dimer formation (data not shown).

Finally, we failed to identify any mutation in RFX6 in proband #6. In the absence of DNA from the proband, we sequenced both parents and found no point mutation of RFX6 or NEUROG3; and long-range PCR did not reveal any deletions of RFX6 (Supplementary Fig. S17). In the absence of proband DNA we cannot rule out a de novo mutation, but this case is most likely a phenocopy. We also failed to find RFX6 mutations in a case of the Martinez-Frias syndrome27. Finally, a search of the RFX6 linkage disequilibrium block in our genome-wide association data, combined with those of the WTCC30, did not reveal any common variants associated with type 1 or type 2 diabetes (data not shown).

Discussion

In the pancreas, Rfx6 acts downstream of the pro-endocrine factor Neurogenin3 (Fig. 5c), and mutation of the two genes give similar but distinct phenotypes. Like RFX6 mutations, mutation of NEUROG3 in humans also causes intractable diarrhoea and diabetes31, but NEUROG3 mutation does not cause the small bowel atresia or biliary abnormalities seen with RFX6 mutation, which is not surprising since Neurogenin3 is not expressed in the early gut endoderm as is Rfx64,32,33. Despite severe intestinal malabsorption, pancreatic exocrine function is intact in both syndromes, and the only histological abnormality in the gut in the mutant NEUROG3 syndrome is loss of the intestinal endocrine cells34. Comparison of the gut endocrine cells affected by these two related syndromes may provide new insight into mechanisms that regulate nutrient absorption in the small bowel.
In addition, RFX6 mutations cause diabetes at birth, while the reported patients with homozygous mutations in Neurogenin3 did not develop diabetes until several years later, despite evidence that Neurogenin3 is absolutely required for the generation of islet cells and production of insulin in mice. Incomplete loss of function in the reported human NEUROG3 mutations could explain the continued insulin production in these patients. Alternatively, recent evidence that Neurog3-/ mice still generate a small number of islet cells suggests some redundancy of Neurogenin3’s pro-endocrine function, possibly due to the presence of related bHLH proteins in the pancreas. In contrast, our data do not indicate any redundancy of Rfx6 function in endocrine differentiation and demonstrate remarkable conservation of the genetic control of islet development, despite some discrepancy between mouse and human NEUROG3 mutants.

It should be noted that our patients with RFX6 mutations have some similarities with other reported cases, especially those with the Martinez-Frias syndrome (MFS), under which OMIM currently lists our cases. However, since the original MFS patients also had oesophageal atresia and hypospadias, and did not have diabetes, and we did not find any RFX6 mutation in the parents of one case of MFS, our cases are distinct from MFS. We propose to name their condition, now of defined molecular aetiology, the Mitchell-Riley syndrome after the two clinicians who first described it.

In summary, we have identified a novel factor in endoderm and islet development, Rfx6, that is required for the differentiation of 4 of the 5 islet cell types and for the production of insulin in both humans and mice. In the hierarchy of islet developmental factors, it lies downstream of Neurogenin3 and upstream of many of the other islet transcription factors. A full understanding of the role of Rfx6 will help to clarify how islets and β-cells are generated, what goes wrong in this process in diabetes, and how to generate new β-cells for patients with diabetes.

**Methods summary**

All studies involving mice were approved by the UCSF Institutional Animal Care and Use Committee. Timed matings were carried out with embryonic day 0.5 being set as midday of the day of discovery of a vaginal plug. The Rfx6 targeting allele (Supplementary Fig. S8) was generated by recombineering in a modified bacterial artificial chromosome followed by recombination into plasmid DNA by gap-repair. This construct was used by the UCSF DERC Transgenic Core Laboratory to target the Rfx6 allele in 129 (E14) mouse embryonic stem cells, which were injected into mouse blastocysts to generate chimeric Rfx6+/eGFPcre mice. Chimeras and subsequent generations were crossed to C57BL/6 mice. Mouse tissue processing, β-galactosidase detection, immunofluorescence staining, automated cell counting, transfections of mPAC cells, EMSA and mRNA quantification with low density TaqMan arrays were performed as previously described. All antisera used for immunofluorescence studies are listed in Supplementary Table S8. Sequences of oligonucleotides used for RT-PCR, ESC colony screening, mouse genotyping and EMSA are available on request.
The human subjects protocol was approved by the IRB of the Montreal Children’s Hospital and written informed consent was obtained from all participating families. Clinical findings and case-report references are summarized in the web supplement. For homozygosity mapping, results from the Illumina Hap 550 (proband #1, call rate 0.99) or 1M (proband #2, call rate 0.978) microarrays were used to scan autosomes in 300-kb windows and HBD was defined as absence of any heterozygous SNP in the proband and presence of at least one in either parent or the unaffected sibling. Long-oligo Nimblegen capture arrays included around 100 bp extension of intronic sequence coverage from the boundary of the exons. The eluted enriched regions from proband #1 were run on the Roche 454 FLX Genome Sequencer. The 19 exons of RFX6 were PCR-amplified manually for Sanger sequencing in the remaining probands. For the long range PCR, we used the Kit # K0182 from Fermentas for fragments ranging from 6 to 17 Kb (Supplementary Fig S17).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Expression of Rfx6 in mice and human tissues
In a, the mRNA for Rfx genes 1-6 were amplified by RT-PCR from RNA isolated from the pancreas and brain of mouse embryos at e17.5 and from NIH3T3 fibroblasts. In b, levels of RFX6 mRNA were determined by real-time PCR of RNA from whole pancreas of human foetuses at the ages shown. n= 5 samples per foetal age group. *p= 0.017, weeks 8-10 vs. 19-21, by two-tailed Student’s t test. In c, mRNA for RFX6 and control gene Cyclophilin A (PPIA) genes were amplified by RT-PCR from RNA isolated from the human adult tissues shown. In d, levels of Rfx3 and Rfx6 mRNA were determined by real-time RT-PCR (TaqMan) of RNA isolated from the pancreata of wildtype and Neurog3−/− mouse embryos at e17.5 values and expressed relative to the level of Gusb. n= 3 samples per group. **p = 0.0025, wildtype vs. mutant, by two-tailed Student’s t test.
Figure 2. Expression of Rfx6 in mice

Immunofluorescence staining was performed for Rfx6 (red) in mouse embryos. In a - c, at e9, Rfx6 staining overlaps with Foxa2 in the gut epithelium (including foregut, FG) and nascent dorsal pancreatic bud (DP), but Foxa2 is expressed alone in the liver bud (Li) and extraembryonic endoderm (EE). Separate colour channels are shown for red (a and d) and green (b and e). In d - f, costaining was performed with Pdx1 (green) in gut (duodenum, Du), dorsal pancreas (DP) and ventral pancreas (VP) at e10. In g, e15.5 pancreas was costained for Neurogenin3 (green). Costaining nuclei appear yellow. In h, e18.5 pancreas was costained for insulin (green). In i, adult pancreas was costained for insulin (green).

Higher resolution photomicrographs from additional dates with additional markers can be found in Supplementary Figs. 1-7. Scale bars, 25 μm.
Figure 3. Targeting of the Rfx6 gene in mice

In panel a-d, lineage tracing was performed on Rfx6^{+/+}/R26R (left in a and c) or Rfx6^{+/eGFPcre}/R26R (b and d, and right in a and c) mice at e10.5 (a, b) and e12.5 (c, d) by staining for β-galactosidase activity with Xgal (blue). Panel b shows a close-up view of the animal on the right in panel a, and panel d shows a close-up view of the animal on the right in panel c. In panel e, an Rfx6^{eGFPcre/eGFPcre} pup at p2 is shown on the right, with a wildtype litter mate on the left. In panel f, the dissected abdominal viscera are shown for wildtype (left) and Rfx6^{eGFPcre/eGFPcre} (right) pups at p0.5. Additional photographs of the lineage tracing and mutant animals can be found in Supplementary Fig. 10. Li, liver; Du, duodenum; GB, gall bladder; VD, vitelline duct; Th, thymus; Tr, trachea; Oe, oesophagus; Lu, lung; St, stomach; DP, dorsal pancreas; VP, ventral pancreas; Hg, hindgut.
Figure 4. Expression patterns of islet markers in wildtype and Rfx6<sup>−/−</sup> mice at e17.5
On pancreas sections from e17.5 embryos with the genotypes shown at the left, immunofluorescence costaining was performed for ChromograninA (ChromoA, red, a-f, green k, l), Synaptophysin (Syn, red, g, h), insulin (green, a, b), glucagon (green, c, d), somatostatin (Sst, green, e, f), ghrelin (green g, h), Nkx6.1 (red in i-l), and pancreatic polypeptide (Ppy, green, k, l). Quantification of cells expressing Ppy and Nkx6.1 is shown in Supplementary Table S3. Scale bars, 25 μm.
Figure 5. Function of the human Rfx6 protein

In a and c, DNA binding of the human in vitro-translated proteins shown above each lane to the double-stranded, radiolabeled oligonucleotide HBV X-box probe was tested by electromobility shift assay (EMSA). In a, combined proteins were co-translated, and probe bound by the heterodimer partners has a mobility between that of the two homodimers. Truncated proteins Rfx3T1 and Rfx3T2 have the first 119 and 160 amino-terminal amino acids removed respectively, but retain the DNA-binding and dimerization domains. In vitro-translated luciferase is included as a negative control. A close-up view of a longer gel is shown in Supplementary Fig. S10a. In b, mouse pancreatic ductal mPac L20 cells were co-transfected with a DNA plasmid containing the reporter constructs shown and another expressing the RFX cDNAs shown, luciferase reporter expression was assayed for each combination. \(*p = 0.0026 vs. “no cDNA”, 0.0024 vs. Rfx3 alone, and 0.011 vs. Rfx6 alone by two-tailed Student’s t test. In c, a schematic shows the proposed interactions, either direct or indirect, of several transcription factors during pancreas development. In d, increasing amounts of the in vitro-translated human Rfx6 wild type and R181Q and S217P mutant proteins were assayed for binding to the X-box DNA probe. Efficiency of mutant protein production is demonstrated in Supplementary Fig. S10b. In e, mutations found in patients are indicated on a map of the RFX6 gene. All mutations were homozygous except for proband 3.
Table 1

Regions of homozygosity-by-descent common to probands #1 and #2

| Chromosome | start SNP  | start position | end SNP  | end position | Size    |
|------------|------------|----------------|----------|--------------|---------|
| 2p15-16    | rs6754038  | 57,761,104     | rs1426699| 64,418,856   | 6,657,752|
| 6q21-22    | rs6913656  | 112,589,928    | rs10499129| 124,975,906  | 12,385,978|
| 6q23       | rs7744295  | 131,173,627    | rs17065195| 136,360,914  | 5,187,287 |