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RESEARCH ARTICLE

Novel GATA6 Mutations in Patients with Pancreatic Agenesis and Congenital Heart Malformations

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Abstract

Patients with pancreatic agenesis are born without a pancreas, causing permanent neonatal diabetes and pancreatic enzyme insufficiency. These patients require insulin and enzyme replacement therapy to survive, grow, and maintain normal blood glucose levels. Pancreatic agenesis is an uncommon condition but high-throughput sequencing methods provide a rare opportunity to identify critical genes that are necessary for human pancreas development. Here we present the clinical history, evaluation, and the genetic and molecular analysis from two patients with pancreatic agenesis. Both patients were born with intra-uterine growth restriction, minor heart defects and neonatal diabetes. In both cases, pancreatic agenesis was confirmed by imaging studies. The patients are clinically stable with pancreatic enzymes and insulin therapy. In order to identify the etiology for their disease, we performed whole exome sequencing on both patients. For each proband we identified a de novo heterozygous mutation in the GATA6 gene. GATA6 is a homeobox containing transcription factor involved in both early development of the pancreas and heart. In vitro functional analysis of one of the variants revealed that the mutation creates a premature stop codon in the coding sequence resulting in the production of a truncated protein with loss of activity. These results show how genetic mutations in GATA6 may lead to functional inactivity and pancreatic agenesis in humans.

Introduction

Pancreatic agenesis is a rare disease with individual and compiled case reports totaling approximately 77 cases with the largest contribution from Allen et al. in 2012 [1–4]. However, this number is likely an underestimate of the total incidence, as many cases are likely unrecognized

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either due to rapid perinatal mortality, or phenotypic variability [4–6]. To survive, patients with complete pancreatic agenesis require early identification and treatment of neonatal diabetes with insulin and pancreatic enzyme replacement therapy shortly after birth. Even with therapy, Baumeister *et al.* originally reported only 31% (4/13) infant survival in the neonatal period, with deaths attributed to bronchopneumonia or complications from other congenital anomalies [1]. Most patients (92%, 38/41 total cases) with pancreatic agenesis are born with intrauterine growth restriction (IUGR), reflecting a requirement for fetal insulin to support in utero growth [2,5,7–22]. In addition, pancreatic agenesis has been associated with malformations of the biliary, cardiac, intestinal, and nervous systems, with gall bladder agenesis as the most common biliary abnormality [1–3,8,13,16,18,23–25]. Congenital heart defects associated with pancreatic agenesis include atrial septal defect, ventricular septal defect, tetralogy of fallot, pulmonary stenosis, transposition of great vessels, tricuspid atresia, and double outlet right ventricle (S1 Table) [1,2,8]. Elucidating novel mutations that cause pancreatic agenesis are needed to further our understanding of the relationship between genotypes and these associated anomalies, and to provide insight into the cause of the variable clinical courses between patients.

Mutations affecting transcription factors critical for pancreas development have been implicated as the basis of human pancreas agenesis. This includes mutations in the coding region of pancreatic duodenal homeobox 1 (Pdx1; also known as IPF1), and mutations in the locus encoding Pancreatic transcription factor 1A (PTF1A) [14,15,25–27]. In mice, two GATA Binding proteins, Gata4 and Gata6, have been shown to regulate pancreatic and cardiac development [31–34]. Shaw-Smith *et al.* (2014) showed that a heterozygous mutation of GATA4 was linked to a case of complete pancreatic agenesis [28]. Recently, Allen *et al.* used next generation exome sequencing and discovered that 15/27 (56%) in one series of patients with pancreatic agenesis had *de novo* heterozygous mutations in the GATA6 gene [3]. In these patients with GATA6 haploinsufficiency, 92% (23/25) also had congenital heart defects (S1 Table) [3,4,6,9,16,21]. Thus, Pdx1, Ptf1a, Gata4 and Gata6 are crucial early regulators of pancreas development in mice, and the discovery of loss-of-function mutations affecting these genes in patients with pancreatic agenesis indicates that the developmental functions of these transcription factors may be conserved in humans [29–32].

In humans, heterozygous loss-of-function GATA6 mutations have been linked to pancreatic agenesis, but in mice, Gata6 heterozygous null mutant mice appear phenotypically normal and do not have abnormal pancreas development. In mice, prior studies show that Gata6 and the paralogue Gata4 are expressed and crucial for heart and pancreas development [33–36]. Simultaneous conditional inactivation of Cre-recombinase sensitive alleles of Gata6 and Gata4 resulted in mice with pancreatic agenesis [34,37]. Thus, the requirement for GATA6 function may be distinct in human and rodent pancreatic development, emphasizing the need for functional studies of GATA6 genetics in human pancreas development.

Here we describe the clinical history, evaluation and molecular studies of two patients with pancreatic agenesis. We performed whole exome sequencing and identified two previously unreported variants in the GATA6 gene. We discovered that one patient has a single base pair change that alters the splice site at exon 4 to 5 intron/exon boundary and the other has a seven base pair nucleotide deletion that results in a frame shift and premature stop codon. We demonstrate that the premature stop results in a truncated GATA6 protein, leading to loss-of-function in transcription assays. In both probands, the novel GATA6 variants are *de novo* and heterozygous, similar to previous GATA6 variants found in pancreatic agenesis, supporting a mechanism of haploinsufficiency in humans that is distinct from rodent models.

Methods

Human subject research and ethics

Skin biopsies and/or whole blood were obtained from proband (n = 2), siblings (n = 2) and parents (n = 4) using an approved IRB protocol from Stanford University. Written informed consent was obtained from each participant at the time of skin and/or blood specimen collection. Both probands were male and one proband had one sister and one brother, which totaled to three female and five male participants. All participants were of Eastern European decent.

Whole exome sequencing

Skin biopsies were obtained from all participants. Fibroblast cell lines were established from patient skin biopsies and grown in sterile culture media (DMEM, 10% FBS, 1x Penicillin/Streptomycin, and 1ug/uL Fungizone). Whole genomic DNA was isolated from fibroblast cells (Gentra Puregene Cell Kit; Qiagen) and whole blood (PAXgene Blood DNA Kit; Qiagen). Exome sequencing was performed using Agilent SureSelect Human All Exon v4–51Mb kit and HiSeq2000 and bioinformatics was done using raw sequence alignment with human genome build 19 using BWA software to analyze for SNPs and In/Dels (Centrillion Biosciences). Ingenuity Variant Analysis was also used to analyze variant calls. Variants were confirmed using direct DNA Sanger Sequencing. Using previously published primers from Allen et al. in exon 2 and exon 4 PCR products of genomic DNA of probands, siblings and parents were sequenced [3]. In order to Sanger Sequence both alleles PCR products of the probands were subcloned into a TOPO-Blunt II vector (Invitrogen) and colonies were picked and sequenced. The exome sequences will be deposited into dbGaP.

GATA6 site directed mutagenesis

The variant identified in proband 2 was generated *in vitro* using a wild-type GATA6 expression plasmid (GATA6 myc-DDK tagged ORF clone; Origene), and Stratagene site-directed mutagenesis (Agilent Technologies). The sequence of the forward primer was ccgctgaacgggaccaccac cacc and the reverse primer was ggtggtggtgtccggttcagcgg. The wild-type human GATA6 myc-DDK tagged ORF clone was used as the template. Sanger sequencing confirmed the deletion of the ACGT bases resulting in a frameshift mutation and a premature stop codon mimicking proband 2 deletion.

Luciferase reporter assays

Luciferase assays were performed using the insulinoma cell line INS-1 (gift from Dr. Justin Annes, Stanford University School of Medicine). Cells were cultured in RPMI 1640 with 2mM glutamine and 5% fetal calf serum, 1mM sodium pyruvate, 50 uM 2-mercaptoethanol, 10 mM HEPES, 100 U/ml penicillin and 100 ug/ml streptomycin. Transfections were performed with technical triplicates that were averaged and compared across biological replicates of the experiment, which was repeated six times. Plasmids were transfected into cells using Lipofectamine LTX Plus (Life Technologies) according to the manufacturer's protocol (4uL per sample reaction). Co-transfections included Hnf4 α _P2–2200 promoter (Addgene Plasmid 31062, [38]) (0.1ug) and vector only (pCMV-Tag3), 'GATA6 wildtype', 'GATA6 variant' (0.5ug) or combined GATA6 wild-type (0.5ug) and GATA6 variant (0.5ug) in 24 well plates. 'Vector only' is an empty pCMV plasmid. 'GATA6 variant' refers to GATA6 with the Proband 2 mutation, GATA6^{Y323fsX21}. After 48 hours of incubation, Dual Luciferase Assays (Promega) were performed to measure relative luciferase activity. Relative luciferase activity was normalized to renilla (5ng per transfection) and compared to vector only. We compared firefly/renilla

luciferase values for GATA6 wild-type, GATA6 variant, and GATA6 wild-type with GATA6 variant to firefly/renilla luciferase values with vector alone, and calculated statistical significance using a two sided t-test.

Western blot analysis

Transfections of HEK 293T cells were performed using Polyfect (10 ul per reaction) according to manufacturer's protocol. GATA6 wild-type or GATA6 variant plasmids (1ug) were transfected in 12 well plates. Cells were lysed in modified RIPA buffer. Protein lysates were run on a 10% SDS-PAGE gel, transferred to PVDF membrane, and probed with mouse anti beta actin (Sigma 1:5000) and goat anti GATA6 antibody (Santa Cruz N-19 sc-7245 1:500). GATA6 antibody required pre-clearing, in which the antibody was initially pre-cleared with protein lysates to eliminate non-specific binding.

Results and Discussion

Clinical history

Proband 1. Proband 1 is a male born at term gestational age with intrauterine growth restriction via cesarean section due to maternal fibroids. Although his delivery was uneventful, at one hour of life he developed severe respiratory distress requiring intubation and transfer to the neonatal intensive care unit. He developed diabetic ketoacidosis and hyperglycemia, which improved with insulin therapy. Other health conditions in proband 1 include mild anemia, an atrial septal defect (ASD) and Patent Ductus Arteriosus (PDA). On initiation of feeding, exocrine pancreatic dysfunction was discovered, and a subsequent CT scan confirmed pancreatic agenesis. His current medications are insulin and pancreatic enzyme replacement. He is reported to have mild developmental delay. Proband 1 has no siblings. Parental history includes impaired glucose tolerance in the pre-diabetic range with fasting blood sugars between 100–120 mg/dL and post prandial blood sugars above 200 mg/dL in the mother. The father of the proband does not have diabetes, known congenital anomalies or autoimmunity. There is a paternal cousin with biliary atresia who required a liver transplant at the age of five years old.

Proband 2. Proband 2 is a male who was born at term gestational age with intrauterine growth restriction. At birth neonatal diabetes was discovered, requiring intensive care for several weeks. His condition improved with insulin therapy. At three months of age, he was noted to have a poor growth velocity and was referred to gastroenterology where he was diagnosed with pancreatic exocrine insufficiency. Ultrasounds performed at 6 months and 13 months of age, both failed to identify any pancreatic tissue but visualization was limited due to bowel gas. At 14 months of age an endoscopic retrograde cholangiopancreatography confirmed that the cystic duct, gallbladder and pancreas were all absent. There were short ductal structures, with two very small branches emanating medially that were thought to be tiny main pancreatic ducts. He was found to have mitral valve stenosis with mild regurgitation and a PDA. The PDA was subsequently ligated. His current medications are insulin, pancreatic enzymes and fat-soluble vitamins. He has an older brother and younger sister. There is no family history of diabetes, autoimmune disease or congenital abnormalities.

Proband 1 and 2 have pancreatic agenesis and congenital heart defects similar to other patients with pancreatic agenesis (summarized in [S1 Table](#)) [14,15,25,26]. Both had neonatal diabetes at birth and required ligation of a PDA. Consistent with other patients with pancreatic agenesis, proband 2 also has gall bladder agenesis [3,16,19]. Our report provides additional evidence showing patients born with neonatal diabetes as well as congenital heart defects may require imaging for pancreatic agenesis, and pancreatic exocrine sufficiency testing to exclude pancreatic agenesis as a cause of neonatal diabetes.

Identification of novel GATA6 variants

To determine genetic variants associated with pancreatic agenesis, high-throughput whole exome sequencing was performed on DNA isolated from probands 1 and 2. Sequencing coverage of the exomes resulted in a total yield of 27.17 megareads and 5.43 gigabases for proband 1, and 39.98 megareads and 7.88 gigabases for proband 2. We performed bioinformatics analysis comparing the variant calls from the probands' exome sequences to the genome reference consortium human build 19 also known as hg19 (GenBank Assembly ID GCA_000001405.1 http://www.ncbi.nlm.nih.gov/assembly/GCF_000001405.13/ on May 26th 2014).

Analysis of the exome sequence of proband 1 identified a novel heterozygous G to T transition in GATA6 at position c.1428+1, which was confirmed by Sanger sequencing (Fig. 1A). This nucleotide change in proband 1 is located in the exon 4 splice donor site on GATA6 c.1428+1 G>T. The location of this variant likely abrogates proper splicing, resulting in a run-on transcript with downstream exons out of frame or could possibly result in skipping exon 4, which would truncate part of a zinc-finger DNA binding domain (Fig. 1B). Interestingly, nucleotide changes in GATA6 splice regions have recently been reported in 2 other patients with pancreatic agenesis (De Franco et al (S1 Table, cases 19 and 20) [4]. These types of splice sites disruptions can lead to anomalous alternative splicing and rapid degradation of the transcripts or the resulting truncated protein [39–41].

In proband 2 the exome sequencing identified a de novo heterozygous mutation in GATA6 located at c.964_970delACGTACC. Our sequencing confirmed that proband 2 has a deletion of seven nucleotides (c.964_970delACGTACC) in the GATA6 gene (Fig. 2). Direct sequencing of the PCR amplicons of genomic DNA for proband 2 showed a mixed population, suggesting a heterozygous state. To confirm this, the amplicons were subcloned into a plasmid vector and 8 independent clones were sequenced. We found that four of the clones were the wild-type allele with an intact ACGTACC sequences and the other four were a variant: GATA6 with c.964_970delACGTACC DNA sequences. These results are most consistent with a heterozygous variant allele. In addition, we sequenced both parents and siblings and found that the sequences of the GATA6 gene matched the normal reference sequence in each of these cases (data not shown), indicating that the variant in proband 2 occurred de novo.

Translation of the c.964_970delACGTACC GATA6 sequence we identified in proband 2 is predicted to produce a frame shift mutation in exon 2 at tyrosine 323 changing it to a threonine

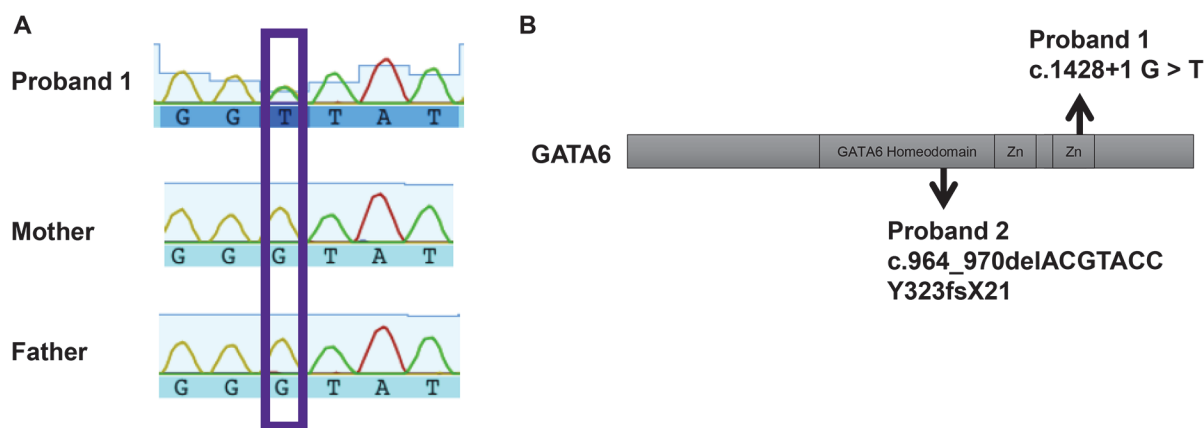


Fig 1. GATA6 variant proband 1. A) DNA sequence of proband and parents showing a single base pair change from G in the parents to a T in the proband at nucleotide position c.1428+1 G>T. B) Diagram of the GATA6 protein containing a GATA family homeodomain and two DNA binding zinc fingers. Arrows indicate locations of GATA6 variants discovered in proband 1 and 2.

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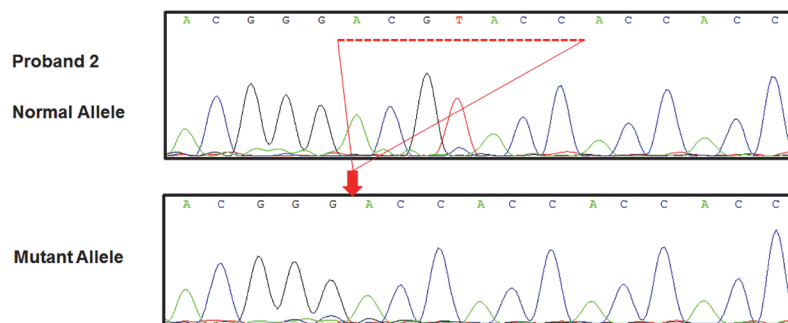


Fig 2. GATA6 variant proband 2. DNA Sanger sequencing of proband 2 demonstrates he has a normal allele (upper) and a mutant allele (lower). The wild-type GATA6 sequence c964_970 (underlined region) is deleted in the mutant allele (arrow).

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(Y323T) and to introduce a premature stop codon 21 amino acids downstream of the frame shift (Y323fsX21) (Fig. 1B). The predicted protein for this variant Y323fsX21 is a truncated GATA6 protein that terminates at amino acid 343, compared to the full-length 595 residue wild-type protein. The deleted C-terminal region of GATA6 contains both zinc finger DNA binding domains and the nuclear localization sequence (Fig. 1B). To test the prediction that Y323fsX21 will produce a truncated protein product, we re-created this variant using site directed mutagenesis in a GATA6 mammalian expression vector. Western blotting revealed that cells transfected with mutant GATA6 generate a truncated protein (Fig. 3B). Thus, the $GATA6^{Y323fsX21}$ allele encodes for a truncated protein.

Protein produced from $GATA6^{Y323fsX21}$ fails to activate transcription

GATA6 is a zinc finger transcription factor that is important for the development of the hematopoietic, cardiac and gastrointestinal systems. Mouse knockout studies demonstrated that

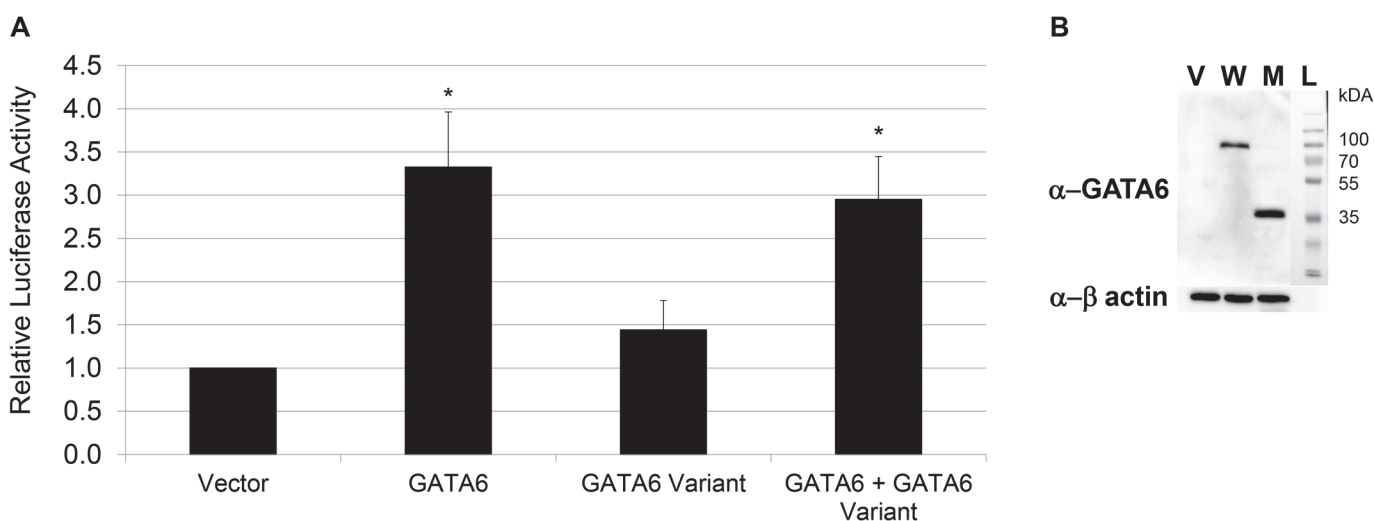


Fig 3. Loss of Hnf4 α promoter activation by GATA6 with $GATA6^{Y323fsX21}$. A) Human wild-type GATA6 and/or $GATA6^{Y323fsX21}$ labeled 'GATA6 variant' were co-expressed with Hnf4 α promoter in INS-1 cells. Hnf4 α promoter luciferase activity was measured and levels normalized to vector alone. *P = 0.00001. B) Western blot analysis of human GATA6 protein (W lane) and $GATA6^{Y323fsX21}$ (M lane) expressed in HEK 293T cells. Vector (V lane) only was a negative control and to approximate the size a protein ladder (L lane) was utilized. GATA6 was detected with a GATA6 antibody against the N-terminal of the protein and beta actin was measured as a loading control.

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Gata6 directly regulates expression of *hepatocyte nuclear factor 4 alpha* (*HNF4α*), a crucial regulator of pancreas development and beta-cell function by binding to an enhancer element in *HNF4α* [3,38,42,43]. To test the impact of the *GATA6*^{Y323fsX21} variant on functional activity, we measured the ability of this variant to transactivate the *HNF4α* promoter using a reporter construct that contains this *GATA6* enhancer element and the endogenous *HNF4α* promoter, preceding a luciferase reporter gene (*HNF4α*-Luc) [3,38,42]. INS-1 cells were transfected with *HNF4α*-Luc and wild-type or *GATA6*^{Y323fsX21} or both and luciferase activity was quantified. We found that wild-type *GATA6* transactivates the *HNF4α* promoter as expected (Fig. 3A). Conversely, *GATA6*^{Y323fsX21} failed to transactivate *HNF4α* promoter (Fig. 3A). In addition, co-transfection of *GATA6*^{Y323fsX21} with wild-type *GATA6* did not inhibit *HNF4α*-Luc activation (Fig. 3A). Thus, in this setting, we did not detect dominant negative activity of the *GATA6*^{Y323fsX21} variant. In sum, our results confirm that the novel *GATA6*^{Y323fsX21} variant is likely a loss-of-function mutation that could be caused by either loss of the critical zinc finger domain or from nonsense mediated mRNA decay of the truncated transcript. Further, these results support a mechanism of haploinsufficiency causing disease in humans that is distinct from the rodent *GATA6* models [34,37].

Our studies are consistent with previous results that found point mutations in the zinc fingers of *GATA6* result in loss of *Hnf4α*-Luc activation [3]. Here we show the *GATA6*^{Y323fsX21} variant also results in loss of *Hnf4α*-Luc activation. There are two previous reports on patients with pancreatic agenesis and *GATA6* mutations where single base pair insertions were predicted to produce a frameshift and premature stop codon resulting in a truncated protein (S1 Table, case 4 and 5) [4,21]. Thus, with our current study, there are three reported patients with pancreatic agenesis who have mutations producing a truncation of *GATA6* protein after the Tyrosine 323 residue. Since it is unlikely that all variants in *GATA6* will result in functional changes in protein activity, we believe it will be important to assess protein function resulting from nucleotide changes in this gene in order to classify a specific mutation as disease-causing.

Supporting Information

S1 Table. Summary of *GATA6* mutations in patients with pancreatic agenesis.
(PDF)

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Author Contributions

Conceived and designed the experiments: BJF SKK. Performed the experiments: CSC KDM. Analyzed the data: BJF SKK CSC KDM. Contributed reagents/materials/analysis tools: ALC KLC. Wrote the paper: BJF SKK CSC KDM ALC KLC.

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