Null mutations of **NEUROG3** are associated with delayed-onset diabetes mellitus

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Introduction

**NEUROG3** is a basic helix-loop-helix (bHLH) transcription factor that drives endocrine lineage generation in the gut, pancreas, and hypothalamus (1–3). Human neonates with biallelic dysfunctional **NEUROG3** present clinically with enteric anendocrinosis (MIM: #610370), characterized by generalized malabsorption and an absence of enteroendocrine cells (EECs) (4–6). As these children age, hypogonadotropic hypogonadism and short stature become evident (7), and at a variable age (from 20 days to more than 23 years of age), they develop insulin-dependent diabetes mellitus (IDDM) (8, 9).

An in vitro directed–differentiation protocol fails to generate any significant number of pancreatic endocrine cells from human pluripotent stem cells if **NEUROG3** function is disabled by gene editing (10, 11). **NEUROG3** deletion experiments in pigs (3, 12) and mice (3) have similarly demonstrated failure of endocrine cell generation in the developing pancreas, resulting in a permanent neonatal diabetes mellitus (PNDM) phenotype. Such results have led to the conclusion that **NEUROG3** is essential for human β cell development. Hence, it has also been concluded that the **NEUROG3** mutations affecting patients exhibiting delayed-onset IDDM (e.g., p.R107S) must be hypomorphic, displaying insufficient transactivating activity to enable generation of EECs in the gut, but nonetheless retain sufficient activity to initiate some minimal level of pancreatic endocrine differentiation during development (8, 11).
Standard tests of the functional competence of human NEUROG3 variants have significant background activity, making it difficult to distinguish weak residual hypomorphic activity from effectively null activity (5). Thus far, tests have been limited to in vitro reporter and gel shift assays of mutant NEUROG3 interactions with a well-studied E-box (12) located in the immediate promoter region of neurogenic differentiation factor 1 (NEUROD1) and in vivo assessment of the level of NEUROD1 or glucagon expression driven by mutant NEUROG3 when expressed in Xenopus laevis or chicken embryos, respectively (5, 9).

NEUROG3’s ability to repress the cell cycle offers an alternate assay of its functional competence (13). We recently found that expressing NEUROG3 in a human endocrine cell line induces cellular quiescence in a p21CIP1-dependent fashion, while prolonged expression induces cellular senescence in a p16INK4A-dependent manner (14). Furthermore, early neuroG3-induced cellular quiescence is reversible by inhibition of PTEN, due to a reduction in steady-state NEUROG3 and p21CIP1 levels in BON4 cells and human intestinal enteroids.

Here, we describe and demonstrate the functional incompetence of 2 probands with homozygous severe nonsense mutations of NEUROG3. Importantly, in addition to enteric anendocrinosis, both had delayed-onset permanent IDDM, rather than the PNDM expected to arise if NEUROG3 is indeed essential for human pancreatic endocrine cell generation.

Results

Subjects’ clinical characteristics. The 2 unrelated index cases are a male and female of Mexican-American descent, each the product of consanguineous union. They were both hospitalized within the first weeks after birth with severe diarrhea, weight loss, and dehydration. Both required a combination of parenteral and enteral nutrition for several years, with diarrheal symptoms that improved but persisted throughout their childhoods. Intestinal biopsies revealed that both children lacked EECs, while Na+ glucose/galactose cotransporter (SLC5A1) expression was similar to that of controls (Figure 1E and our unpublished observations). IDDM began by 3 years of age in proband 1 and by 7 years of age in proband 2. The probands are currently 18 and 14 years old, respectively. Detailed clinical information is provided in the Supplemental Clinical Information (supplemental material available online with this article; https://doi.org/10.1172/jci.insight.127657DS1).

Sequencing of the NEUROG3 gene. We sequenced the single coding exon of NEUROG3 from the 2 index cases and their biological parents.

Proband 1 has a homozygous deletion of cytosine at nucleotide 117 that results in a frame shift mutation beginning at amino acid 40 (Figure 1A). This variant, p.P39PfsX38, (hereafter referred to as NEUROG3_NULL) encodes a protein missing the nuclear localization, HLH, and activation (AD) domains, which suggested that it likely has no residual functional activity (Figure 1C).

Proband 2 has a homozygous insertion of cytosine at nucleotide 431 that results in a frame shift mutation beginning at amino acid 145 (Figure 1B). This variant, p.H144Pfs*94 (hereafter referred to as NEUROG3_DN) retains the bHLH domain but lacks the protein’s entire AD, which suggested that it likely exhibits dominant-negative (DN) characteristics without residual functional activity (Figure 1C).

These variants were not previously observed in the Exome Aggregation Consortium (ExAC) database (http://exac.broadinstitute.org) of over 121,000 individuals and are therefore very rare (MAF < 0.0001%) (data not shown).

Reduced pancreatic islets in a patient with NEUROG3 missense mutation. Our original report described a proband with a NEUROG3_R107S missense mutation. This patient succumbed to central catheter sepsis prior to the onset of IDDM (5). Immunostaining of pancreas obtained at autopsy demonstrated both glucagon and insulin+ cells, but the frequency of insulin+ cells was nearly 5-fold less than in age-matched controls (Figure 1D and data not shown).

Intracellular localization of FLAG-tagged recombinant NEUROG3WT, NEUROG3_NULL, and NEUROG3_DW expressed in BON4 cells. To examine the functional consequences of these severe variants, we assessed their behavior in a homogeneous isolate of the endocrine-derived human BON cell line, herein referred to as BON4 cells (14). BON4 cells do not express endogenous NEUROG3 and are therefore entirely responsive to heterologous expression. BON4 cells transduced with lentiviruses that constitutively express a C-terminus FLAG-tagged sequence encoding WT (NEUROG3WT), the 2 previously described missense variants (NEUROG3_R107L and NEUROG3_R107S) (5, 15), or the 2 potentially novel variants (NEUROG3_NULL and NEUROG3_DW) were used to assess the intracellular localization of each variant (Figure 1C). Cells transduced with NEUROG3WT, NEUROG3_R107L, or NEUROG3_R107S appropriately localized exclusively in the nucleus, while the NEUROG3_NULL...
variant was located diffusely across the cytoplasm and nucleus, and the NEUROG3DN mutant appears especially concentrated in some subnuclear structures (Supplemental Figure 1A).

NEUROG3NULL and NEUROG3DN fail to activate the promoter of the NEUROG3 target gene NEUROD1. NEUROG3 activates expression of its downstream target NEUROD1 via interaction with an E-box in the NEUROD1 promoter (5, 16). We assessed the ability of each NEUROG3 variant to activate this promoter. We constructed a separate mammalian expression plasmid for each of NEUROG3 WT, NEUROG3R93L, NEUROG3R107S, NEUROG3NULL, and NEUROG3 DN. Each construct was cotransfected into BON4 cells with a plasmid containing the immediate promoter region of NEUROD1 driving a luciferase reporter (5). The NEUROG3 WT construct induced a pronounced activation of the reporter, the NEUROG3R93L and NEUROG3R107S variants induced only a mild response, and the responses to NEUROG3 NULL and NEUROG3 DN were not significantly different from those induced by an empty expression vector control (Figure 2A). The fidelity of NEUROG3 WT in BON4 cells was confirmed by its failure to upregulate the non-NEUROG3 target — SLC5A1 — via the E-box element in its proximal promoter (ref. 17 and Supplemental Figure 1B). Western blotting confirmed that similar levels of transfected proteins were expressed (Supplemental Figure 1D).

We also tested each NEUROG3 variant’s interaction with the NEUROD1 promoter E-box by performing electrophoretic mobility shift analysis after incubation of the promoter sequence with nuclear extracts isolated from BON4 cells previously transfected with the plasmids containing the various NEUROG3 variants. A prominent gel shift occurred only with extracts isolated from cells transfected with NEUROG3WT (Supplemental Figure 1E).

In summary, the NEUROG3 NULL variant induced only background levels of activation of the NEUROD1 promoter and showed no evidence of direct interaction with its E-box.
NEUROG3\textsuperscript{DN} can function as a DN. NEUROG3\textsuperscript{DN} has intact DNA-recognition and -binding domains but lacks the AD domain. Thus, its failure to activate the NEUROD1 promoter is consistent with the idea that it exhibits DN potential. To assess this possibility, BON4 cells were transduced with a NEUROG3\textsuperscript{WT} lentivirus, generating BON4\textsubscript{NEUROG3\textsuperscript{wt}} cells, which were then cotransfected with a NEUROD1 promoter luciferase reporter construct and increasing amounts of the NEUROG3\textsuperscript{DN} variant. The NEUROG3\textsuperscript{WT}–driven reporter expression decreased in proportion to the amount of transfected NEUROG3\textsuperscript{DN} variant (Figure 2B). Similarly, BON4 cells were transduced with a NEUROG3\textsuperscript{DN}-expressing lentivirus, generating BON4\textsubscript{NEUROG3\textsuperscript{dn}} cells, which were then cotransfected with increasing concentrations of NEUROG3\textsuperscript{WT} plasmid and the NEUROD1 reporter construct. In this case reporter activity remained low and was only mildly enhanced in cells transfected with the largest amount of the NEUROG3\textsuperscript{WT} vector (Supplemental Figure 1C; compare with the ~10-fold increase in Figure 2A).

Next, we compared the DN inhibitory activity of the NEUROG3\textsuperscript{DN} variant with ID1, a well-characterized DN inhibitor of bHLH transcriptional activation (18). BON4\textsubscript{NEUROG3\textsuperscript{wt}} cells were cotransfected with the same amount of the NEUROD1 luciferase reporter and increasing amounts of ID1, NEUROG3\textsuperscript{DN}, or both. The data suggest that the NEUROG3\textsuperscript{DN} variant is more potent than ID1, but the 2 in combination synergistically attenuated NEUROG3\textsuperscript{WT}–induced expression of NEUROD1 promoter activity (Figure 2C).

Overall, these data support the notion that the NEUROG3\textsuperscript{DN} protein can act in a DN fashion to inhibit NEUROG3–induced activity on the NEUROD1 promoter.

The NEUROG3\textsuperscript{NULL} and NEUROG3\textsuperscript{DN} variants fail to repress cellular proliferation. NEUROG3 is known to repress proliferation in BON4 cells (14), offering an independent assay of the functional competence of the NEUROG3 variants. To test for repression, we transduced BON4 cells with lentiviruses encoding each NEUROG3 variant. The corresponding transduced cells were denoted as follows: BON4\textsubscript{NEUROG3\textsuperscript{WT}}, BON4\textsubscript{NEUROG3\textsuperscript{DN}}, BON4\textsubscript{NEUROG3\textsuperscript{NULL}}, BON4\textsubscript{NEUROG3\textsuperscript{R93L}}, and BON4\textsubscript{NEUROG3\textsuperscript{R107S}}. We assessed proliferation in the transduced cells by Ki67 staining as well as the MTT and FACS assays. As expected from previous observations (14), proliferation of BON4\textsubscript{NEUROG3\textsuperscript{WT}} cells was repressed. Thus, Ki67\textsuperscript{+} cells were rarely seen among transduced (GFP\textsuperscript{+}) cells, and there was reduced MTT activity and an increase of cells in the G0/G1 phase of the cell cycle (Figure 3). In contrast, the measures of proliferation in the BON4\textsubscript{NEUROG3\textsuperscript{NULL}} and BON4\textsubscript{NEUROG3\textsuperscript{DN}} cells were indistinguishable from those in GFP controls (Figure 3 and Table 1). Finally, we examined whether NEUROG3\textsuperscript{DN} attenuates the ability of BON4\textsubscript{NEUROG3\textsuperscript{WT}} to induce cell cycle arrest. Indeed, transduction of NEUROG3\textsuperscript{DN} was able to entirely inhibit the NEUROG3\textsuperscript{WT}-induced cell cycle arrest (Figure 4A).

The NEUROG3–induced changes in the cell cycle are mediated in part by binding an E-box element in the immediate promoter region of the p21cip1 gene (19). Therefore, it was important to assess whether NEUROG3\textsuperscript{DN} attenuated NEUROG3\textsuperscript{WT}–mediated induction of the p21cip1 promoter. We transiently cotransfected BON4 cells with a human p21cip1 promoter luciferase reporter vector and a mammalian expression vector containing either a NEUROG3\textsuperscript{WT} or NEUROG3\textsuperscript{DN} insert. Increasing amounts of NEUROG3\textsuperscript{WT} augmented
promoter activity, whereas the NEUROG3DN variant failed to enhance activity (Figure 4B). To assess whether the NEUROG3DN variant could completely attenuate NEUROG3WT-induced expression of the p21CIP1 promoter, we transfected BON4-NEUROG3WT cells with the p21CIP1 promoter luciferase reporter vector and increasing amounts of the NEUROG3DN expression vector. We observed that NEUROG3DN-driven p21CIP1 promoter activity decreased in proportion to the quantity of transfected NEUROG3DN vector (Figure 4C).

Overexpression of NEUROG3DN attenuates the ability of NEUROG3WT to induce cellular senescence in BON4 cells. Another test of the functional competence NEUROG3 variants arises from the observation that overexpression of NEUROG3 in BON4 cells results in a time-dependent increase in p16INK4A expression, with the consequent induction of cellular senescence (14). The NEUROG3 variant transduced BON4 cell lines were examined for evidence of cellular senescence at 5 and 10 days of culture by staining for senescence-associated β-galactosidase (SA β-Gal), a marker of cellular senescence (20). BON4-NEUROG3WT cells exhibited robust SA β-Gal staining by 10 days, while BON4-NEUROG3DN, BON4-NEUROG3NULL, BON4-NEUROG3R93L, and BON4-NEUROG3R107S cell were indistinguishable from control cells (Figure 5A). We quantified SA β-Gal activity by lysing the cells and measuring the lysate’s absorbance at 405 nm. While BON4-NEUROG3WT cells exhibited a 12-fold increase in SA β-Gal levels by 15 days, the levels in BON4-NEUROG3NULL and BON4-NEUROG3DN cells were indistinguishable from those in control cells (Figure 5B). When BON4-NEUROG3WT cells were cotransduced with NEUROG3DN, SA β-Gal activity was strongly repressed, indicating that the cellular senescence induced by NEUROG3WT had been inhibited (Figure 5B). These findings demonstrate that NEUROG3NULL and NEUROG3DN lack the cellular senescence–inducing activity exhibited by NEUROG3WT. Furthermore, NEUROG3DN attenuates the cellular senescence induced by NEUROG3WT, again indicating that NEUROG3DN acts as a DN.

NEUROG3NULL and NEUROG3DN variants fail to induce the endocrine phenotype exhibited by BON4 cells expressing NEUROG3WT. The NEUROG3WT-induced cell cycle arrest in BON4 cells is known to be accompanied by increased expression of various EEC markers, including CHGA and PCSK1 (14). Therefore, we assayed the BON4 cells transduced with NEUROG3WT, NEUROG3NULL, NEUROG3DN, and NEUROG3WT + NEUROG3DN for expression of these EEC markers. CHGA and PCSK1 were detectable by Western blot in NEUROG3WT-transduced cells by 5 days, whereas they remained undetectable in NEUROG3NULL-transduced cells (Figure 6A). The rapid and sustained expression of endocrine markers exhibited by BON4-NEUROG3WT cells was strongly attenuated by cotransducing with NEUROG3DN (Figure 6). Interestingly, faint expression of CHGA and PCSK1 was detectable in NEUROG3DN cells. We confirmed these findings using RT-qPCR for NEUROG3, CHGA, and PCSK1. Expression levels in BON4-NEUROG3NULL cells were always significantly less than those in BON4-NEUROG3DN cells. (Figure 6, B–D).

NEUROG3NULL fails to induce the endocrine phenotype in Neurog3null murine enteroids. Intestinal enteroids grown from 0-day-old WT (Neurog3+) mouse pups exhibited abundant expression of the EEC marker Chga, while similar enteroids grown from Neurog3-null (Neurog3−/−) pups did not (Figure 7, A–C, G, and H). We next tested whether transducing murine enteroids with lentiviruses encoding human NEUROG3WT enforces an EEC program. Neurog3+ and Neurog3−/− enteroids displayed greatly increased Chga expression 7 days after transduction (Figure 7, B, C, G, and H). Thus, the induction of Chga expression in Neurog3−/− enteroids provides a sensitive assay for NEUROG3 functional competence. We next applied this assay to assess the activity of the NEUROG3NULL variant. Transducing Neurog3−/− enteroids with a lentivirus encoding NEUROG3NULL induced no change in Chga expression (Figure 7, C and G), confirming in vivo that the NEUROG3NULL variant identified in proband 1 exhibits no NEUROG3 activity.

NEUROG3DN represses endogenous NEUROG3 activity in human enteroids. We have consistently found that NEUROG3DN represses NEUROG3 activity. We now ask whether it will do so in the context of human intestinal epithelium. Our group and others have described methods to grow human intestinal epithelium that continuously generates each of the differentiated epithelial cell lineages, including the NEUROG3-dependent EEC lineage (21, 22).

Next, WT human intestinal enteroids were transduced with a lentivirus expressing NEUROG3DN. NEUROG3DN expression significantly decreased the expression of the NEUROG3-dependent endocrine markers (CHGA), compared with the expression observed in untransduced enteroids (Figure 7, D, I, and J), confirming that NEUROG3DN acts as a DN factor that impairs NEUROG3-dependent differentiation of EECs. Native enteroids from a DN subject produce NEUROG3 as well as reduced EEC markers grow from proband 2 carrying the NEUROG3DN variant compared with WT enteroids. Control enteroids
expressed abundant CHGA and PCSK1; however, CHGA and PCSK1 were significantly reduced in the NEUROG3DN enteroid sample (Figure 7E). It should be noted that protein levels of the NEUROG3DN variant were comparable to NEUROG3 isolated from control sample. Finally, we assessed relevant transcript levels in control and NEUROG3DN enteroids, and as expected, CHGA and NEUROD1 mRNA levels were reduced in the enteroids from proband 2 when compared with control (Figure 7F). We also found that expression levels of related bHLH transcription factors (i.e., NEUROG1, NEUROG2, and NEUROD2) were not significantly elevated and therefore were not compensating for the loss of NEUROG3 function.

Discussion

Current understanding of the role of NEUROG3 in generating endocrine cells in humans is based on the clinical, pathologic, and metabolic abnormalities observed in children with biallelic mutations impairing NEUROG3 function.

The timing of the onset of diarrheal symptoms appears to be highly similar in children with various forms of enteric endocrinopathy or other enterocyte enteropathies (5, 6). In sharp contrast, the age of onset of IDDM varies widely in children with biallelic NEUROG3 variants (refs. 5, 23 and Supplemental Table 1). When we first described 3 children with missense mutations of NEUROG3 we postulated that unlike in mice, pancreatic endocrine cell fate in humans might be redundant with other transcription factor(s) since the surviving children were older than 8 years of age when they were first diagnosed with persistent IDDM (5). Others subsequently postulated that the failure of these subjects to develop persistent glucose intolerance was secondary to the hypomorphic nature of the variants that were described (24). Since then,
several new cases have been described where the age of onset varies from 20 days (9) to more than 23 years of age (8, 9, 25); some of the younger patients may have developed hyperglycemia that was in part secondary to the use of glucocorticoids and macrolide calcineurin inhibitor (tacrolimus), administered to treat an incorrectly diagnosed autoimmune enteropathy (25). Interestingly, even siblings with identical homozygous \textit{NEUROG3} mutations (R107S) had dramatically disparate age of onset of persistent diabetic symptoms, suggesting that environmental or epigenetic factors may influence the age of onset of IDDM (ref. 8 and Supplemental Table 1). But this could all be attributed to some poorly understood residual \textit{NEUROG3} function present in these potentially hypomorphic variants.

Therefore, the fact that \textit{NEUROG3\textsuperscript{null}} — the entirely nonfunctional nonsense variant of the \textit{NEUROG3} gene described here — results in delayed-onset IDDM, characteristic of the previously described hypomorphic variants, implies that some as-yet undescribed mechanism(s) exists for the generation of insulin-producing cells in the absence of \textit{NEUROG3} function. At least 3 possibly interacting classes of such potential mechanisms seem plausible and should form the basis of future studies:

\textit{NEUROG3}-independent pathways capable of generating some pancreatic endocrine cells. Among the transcription factors known to be important for islet cell development, only \textit{RFX6} is consistently associated with both PNDM and malabsorptive diarrhea in a disorder named Mitchell-Riley syndrome (26). In this disorder, hyperglycemia begins immediately after birth and requires continuous insulin infusion — a phenotype distinct from that seen in most children with \textit{NEUROG3} mutations. Thus, \textit{RFX6} appears to be nonredundant with other factor(s) required for islet cell formation in humans and mice (26, 27). Indeed, genetic complementation studies in a novel murine \textit{Neurog3null} pancreatic organ culture model suggest that \textit{Rfx6} and \textit{NeuroD1} stimulate endocrine cell fate in the absence of \textit{Neurog3} (28). Furthermore, assessment of the \textit{NEUROG3\textsuperscript{null}} variant failed to induce islet cell formation in a murine model system (28). But the role of \textit{NEUROG3} in human islet development remains uncertain. Notably, a human embryonic stem cell line, in which both \textit{NEUROG3} alleles had been disrupted by CRISPR/Cas9 targeting, entirely failed to generate pancreatic endocrine cells (11), seemingly demonstrating that \textit{NEUROG3} is essential for islet development, with the important proviso that the experiment was reliant on induction of differentiation by a standard in vitro differentiation induction protocol that may not enable alternate pathways available in the context of an intact embryo. However, others have been successful in generating a limited number of pancreatic endocrine cells in targeted \textit{NEUROG3} ablation studies that used different differentiation protocol (10). Production of pluripotent stem cell lines from patients with \textit{NEUROG3}-dependent enteric anendocrinosis could help to resolve this. If such \textit{NEUROG3} variant stem cells generated β cells, that would be convincing evidence of hypomorphic \textit{NEUROG3} function. Conversely, if β cells are not generated from stem cells produced from patients displaying clear evidence of β cell function, then the in vitro differentiation protocol would need to be reassessed.

\textit{Ectopic NEUROG3-independent (i.e., extraendocrine pancreas) sources of sufficient insulin to sustain the child}. An example of such a source is the thymus. The neonatal thymus makes proinsulin as part of self-tolerance mechanisms, and this subsides with thymic involution in early childhood (29). However, it seems unlikely that this is the source of insulin that serves to modulate glucose levels in prediabetic \textit{NEUROG3} probands. “In the murine thymus, all pancreatic hormones (i.e., insulin, glucagon, and somatostatin) are expressed (30) at levels that are approximately 10,000-fold lower than in the pancreas (31) and are not modulated by metabolic modifications (32).” Nor is there any evidence that the thymus elaborates the complex cellular machinery necessary to process and secrete functional mature insulin in a glucose-responsive manner. Fur-

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Colors refer to Figure 3. Red represents Ki67, and green represents GFP-positive cells; %KI67 indicates red/green.
thermore, the RFX6-null patients discussed above had diabetes mellitus at birth, arguing against the existence of an independent ectopic source of insulin in the neonate (unless RFX6 has a role in determining ectopic insulin transcription/processing, whereas the lineage factor NEUROG3 does not).

Spontaneous reverting mutations in NEUROG3 that reestablish some degree of NEUROG3 function in a pancreatic progenitor, enabling its resulting NEUROG3+ subclone to generate a limited population of endocrine cells sufficient to sustain the child. This seems improbable at first glance, but perhaps reversion-free NEUROG3 nulls succumb as neonates and hence go undiagnosed. The intestinal epithelium offers a simple test of the idea. If reverting mutations of the NEUROG3 locus occur with sufficient frequency to be a significant factor in pancreatic endocrine development, then this should manifest in the intestine as scattered crypts containing EECs (due to intestinal stem cells incurring reverting mutations, leading to a clone capable of generating EECs). Such EEC-containing crypts have not been seen, suggesting that the rate of reverting mutations is very low.

It is noteworthy that neither diabetes mellitus nor malabsorption have been reported in NEUROG3 heterozygous parents or siblings, including the parents of the index cases reported here. This is an especially interesting aspect of the NEUROG3DN family, because expression of the mother’s DN variant allele would be expected to impair the function of her only normal NEUROG3 allele, implying that her progenitors experienced unusually low levels of NEUROG3 functional activity. This observation supports current understanding that low levels of NEUROG3 expression result in generation of sufficient pancreatic β cells and EECs to avoid disease.

Frameshift mutations can induce nonsense-mediated mRNA decay (NMD) mechanisms, which could reduce the quantity of transcript and protein from the variant allele and, thereby, silence the DN effect in a heterozygote state (33). However, in the case of the NEUROG3DN variant, we show that the native protein in the patient’s own enteroids sample is comparable in quantity in enteroids isolated from a control sample (Figure 7E). These findings and the absence of a concomitant increase in related bHLH transcription factors (Figure 7F) argue against the presence of NMD in our frameshift mutations. Furthermore, since NEUROG3 is encoded by a single exon, our results are consistent with other studies that have shown that NMD requires at least 1 intron with both donor and acceptor splice sites downstream of the nonsense codon (34).

It should also be pointed out that NEUROG3DN induced marginal PCSK1 expression in BON4 cells and in the patient’s native enteroids (despite an absence of frank EECs), the implications of which are unclear.

In mice, Neurog3 is expressed in endocrine pancreas development (30) during 2 fetal transitions that are associated with specific short bursts of Neurog3 expression (35). During the early phase (E8.5–E10.5) Neurog3 induces a cluster of α cells, while the late phase (E12.5–E15) leads to the generation of primarily β and δ cells (35, 36). Postnatally, the endocrine cell mass undergoes a proliferative phase that is independent of Neurog3 expression (37). In the intestine, Neurog3 expression occurs continuously throughout late fetal and postnatal life and is limited to endocrine precursor cells derived from crypt base columnar cells, the intestinal epithelial stem cells (38). Moreover, Neurog3+ progenitor cells are either slowly cycling or postmitotic and express endocrine markers (2). Neurog3DN pups expire during within the first day of life and are
entirely devoid of pancreatic β cells and EECs (38, 39), demonstrating that in mice Neurog3 is essential for generation of both pancreatic and intestinal endocrine cells (30–32).

Thus, it is well established that Neurog3 is required and sufficient for endocrine cell development in the intestines and pancreases of mice and, more recently, pigs (12). Human EEC biology is similarly dependent on NEUROG3; however, our results indicate that it is likely that NEUROG3's role in human pancreatic endocrine development is more complex and may be redundant with other factor(s). Since the age of onset of diabetic symptoms varies so widely, even in patients with identical NEUROG3 genotypes, our findings indicate that the environment, epigenetics, and/or modifier genes influence pancreatic endocrine development in patients with severe NEUROG3 mutations.

Methods

Genomic DNA isolation and sequencing. Genomic DNA was extracted from saliva by standard procedures and measured by Qubit (Invitrogen). The mRNA of NEUROG3 is encoded on a single exon, and the oligonucleotides and PCR conditions used to amplify the coding region were previously described (ref. 5 and Supplemental Table 2).

Histopathology. Endoscopic formalin-fixed, paraffin-embedded biopsies from the small intestine of the index case were processed using standard techniques and stained for hematoxylin and eosin and immunohistochemical assessment with an anti–chromogranin A antibody (Santa Cruz).

Cloning and site-directed mutagenesis. Expression vectors containing the previously described human NEUROG3 expression vector were used for these studies (5). These vectors also served as the template for the various other constructs used in this study (Supplemental Table 2).

Lentiviral production and transduction. Vectors were constructed by insertion into the multiple cloning site of a third-generation, self-inactivating LV lentivirus-based vector (RRL.sin.cPPT.MCS.IRES.GFP) and produced at the UCLA Core Vector for the production of lentivirus (Supplemental Table 2). All the constructs contain a carboxy terminal FLAG tag, as shown in Figure 1B. Virus titers were determined by HIV1 p24 ELISA (Perkin Elmer) and expressed as p24 equivalents units. Transduction was performed on 10⁵ cells with 8 μg/ml polybrene (MilliporeSigma) at 37°C.

Cell proliferation analysis by MTT and FACS. Cell proliferation was assessed by MTT assay per the manufacturer’s instructions (ATCC). BON4 cells were seeded in 12-well flat-bottom well plates (Corning) at a density of 5 × 10⁵ cells per well. Proliferation was also assessed by FACS analysis using 10⁶ cells that were harvested and washed with PBS twice, followed by fixation with 4% PFA for an hour. Fixated cells were washed with 3% BSA/PBS twice and resuspended in PBS containing 50 μg/ml propidium iodine and 10 μg/ml RNaseA for 30 minutes at 25°C in the dark. Cells were assessed for DNA content by FACS and analyzed using Cell Quest software (BD).
TUNEL assay. Apoptosis of BON4 cells transduced with NEUROG3 and NEUROG3 mutants was assessed by TUNEL assay (Roche). BON4 cells growth on glass coverslips were fixed in 4% phosphate-buffered paraformaldehyde for 15 minutes and then washed 3 times with 1× PBS and 0.3% Triton X-100 in PBS for 15 minutes. After additional washes, cells were treated with the TUNEL enzyme solution and incubated for 1 hour in a dark, humidified chamber, and washed with PBS, and converter solution was applied. They were then incubated for 30 minutes in a dark, humidified chamber and washed with PBS. Cells were then stained with a Slowfade gold with DAPI (Invitrogen), and covered with a glass slide. As a negative control, TUNEL enzyme was omitted for some reactions.

Quantitative PCR. Steady-state mRNA levels were assessed by quantitative PCR (qPCR) performed with cDNA synthesized from 5 μg total RNA; this was performed using TaqMan assay (Life Technology) or Perfecta SYBR green fast mix low rox (QUANTA Biosciences) in a real-time thermocycler (Applied Biosystem, 7500). The results obtained for each individual gene were normalized to the β-tubulin. The primers used in this work were designed for Primer3, or as suggested by TaqMan, and sequences are shown in Supplemental Table 3.

Luciferase reporter assay. BON cells were plated in 6-well plates at a density of 5 × 10⁵ cells per well in DMEM-F12 media supplemented with 10% FBS and penicillin/streptomycin. Depending on the experiment, cells were transduced with 1 of several lentiviruses as described earlier. One day later, the cells were transfected with 1 pmol of the pGL3 Basic (Promega) plasmid containing the promoter areas of human NEUROD1 (1 Kb) (5), or p21 (−592/−141), or SLC5A1 (−5370/+22) (ref. 17 and Supplemental Table 2) using the lipofectamine reagent (Invitrogen). Two days later, cell lysates and luciferase levels were measured with the dual luciferase assay kit (Promega). Luciferase levels were normalized by renilla luciferase. Data are showed as mean ± SEM over PGL3-B control.

SA β-Gal staining. SA β-Gal staining was used to determine replicative senescence as previously described (20). Cells plated in 6-well plates were washed twice with PBS and fixed with 4% PFA. They were then washed twice with PBS by gently shaking, and 1 ml SA β-Gal staining solution was added and incubated at 37°C. Cells were observed every 4 hours for 12 hours and then every 12 hours. The optimal incubation period was determined based on the visibility of stained cells in the test sample but not in the control sample. Then, cells were lysed with 200 μl cell culture lysis reagent 5X (Promega), and the absorbance was measured at 405 nm on a VERSAmax microplate reader (Molecular Devices).

Western blot analysis. Cells were lysed with RIPA lysis buffer, scraped into microfuge tubes, incubated 5 minutes at 25°C, and then centrifuged at max speed at 4°C for 10 minutes. Concentration of the proteins

Figure 6. NEUROG3null fails to induce downstream endocrine markers in BON4 cells, and NEUROG3dn attenuates NEUROG3wt-induced changes, while retaining marginal activity. (A) Western blot of cellular extracts transduced several days earlier with lentiviruses expressing NEUROG3wt, NEUROG3dn, or both and assessed with anti-FLAG, CHGA, and PCSK1 antibodies. (B–D) Similarly, mRNA levels of various endocrine markers were assessed at the corresponding times after transduction. *P < 0.05, **P < 0.01, ***P < 0.005, 2-way ANOVA and 2-way Tukey’s multiple comparisons.
was determined using the Qubit protein assay (Life Technologies). Protein (10 μg) was loaded onto a 10% polyacrylamide gel and subjected to SDS-PAGE and blotted onto immune blot Amersham Hybond 0.2 PVDF (GE Healthcare Life Sciences). Membranes were blotted using standard procedures using the primary antibodies shown in Supplemental Table 4. Secondary antibodies used were either an ECL anti-mouse IgG-HRP–linked whole antibody or anti-rabbit IgG-HRP–linked whole antibody. Western blots were visualized using the ECL plus Western blotting kit (GE Healthcare) and scanned on a Typhoon 9410 imager (GE Healthcare) using antibodies shown in Supplemental Table 4.

Figure 7. Assessment of endocrine cell induction in enteroids from Neurog3WT or Neurog3null mice or human intestines from normal and NEUROG3DN proband. (A) Neurog3WT or Neurog3null enteroids transduced with either constitutively active NEUROG3WT- or NEUROG3null-expressing lentivirus and grown in culture for 7 days. (B and C) Western blot of enteroids cell extracts isolated from (B) Neurog3WT or (C) Neurog3null mice. Enteroids were untransduced or transduced with NEUROG3WT or NEUROG3null and assessed for proteins using anti-Neurog3, Chga and tubulin antibodies. (D) Western blot of enteroids cell extracts isolated from a normal human (WT) subject untransduced or transduced with NEUROG3DN and assessed 7 days later for proteins. (E) Human enteroids from WT and NEUROG3DN subjects were examined for specified proteins and (F) mRNA. (G and H) mRNA isolated from murine (G) Neurog3null and (H) Neurog3WT enteroids transduced with NEUROG3WT or NEUROG3null and WT enteroids transduced with NEUROG3WT and assessed 7 days later. (I and J) mRNA isolated from WT human enteroids and transduced with NEUROG3DN and assessed 7 days later. (F–J) *P < 0.05, **P < 0.01, ***P < 0.005, 1-way ANOVA and Dunnett’s multiple comparison. (G–J) *P < 0.05, **P < 0.01, ***P < 0.005, unpaired Student’s t test.

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Microscopy and staining. BON4 cells transduced with NEUROG3 or NEUROG3<sup>DN</sup> were grown on glass cover slips and fixed in 4% PFA for 15 minutes. They were then washed 3 times with 1× PBS and treated with blocking buffer (1× PBS/5% normal serum/0.3% Triton X-100) for 60 minutes and washed 3 times with PBS for 2 minutes, incubated with diluted primary antibody (Supplemental Table 4) for 2 hours at room temperature, and rinsed 3 times in 1× PBS for 5 minutes each. They were then incubated in fluorochrome-conjugated secondary antibody diluted in antibody dilution buffer (1× PBS/1% BSA/0.3% Triton X-100) for 1–2 hours at room temperature in the dark, rinsed 3 times in 1× PBS for 5 minutes each, and mounted using Gold Antifade Reagent with DAPI (Invitrogen).

Generation of BON4 cells. The BON4 cell line used here was generated in-house from the BON cells that were obtained from ATCC. The BON4 cells were generated from the parental BON cell that had a more heterogeneous appearance and growth characteristics. These cells were maintained in DMEM-F12 media supplemented with 10% FBS.

Murine enteroid isolation, propagation, and transduction method. Small intestinal crypts were isolated from Neurog3-null (Neurog3<sup>−/−</sup>) pups immediately after birth, using a previously described protocol (40). Crypts were initially resuspended in 5 mL Basic Crypt Media (BCM) consisting of Advanced DMEM/Ham’s F12 (Invitrogen) with 1× Antibiotic-Antimycotic, 2 mM Glutamax (Invitrogen), and 10 mM HEPES (Invitrogen). Aliquots were microcentrifuged to yield crypts pellets for culture purposes.

Human enteroid isolation, propagation, and transduction method. Duodenal crypts were obtained and grown as enteroids using previously described methods. In general, enteroids were grown in 3D culture with Matrigel (Corning) and 1 mM N-Acetylcysteine (MilliporeSigma), 100 ng/ml recombinant murine Noggin (PeproTech), 50 ng/ml recombinant murine EGF (PeproTech), N2 supplement (Invitrogen), B27 supplement (Invitrogen), and 1 μg/ml recombinant human R-spondin1 (R&D Systems), 10 μM ROCK inhibitor (MilliporeSigma), and 5 μM GSK inhibitor (Stemgent).

Statistics. For multiple comparisons, 1- or 2-way ANOVA was followed by Tukey’s or Dunnett’s multiple-comparisons test. For comparisons involving 1 independent variable with 2 groups, we performed 2-tailed Student’s <i>t</i> tests. <i>P</i> values were considered significant at <i>P</i> < 0.05. Variation is reported as SEM.

Study approval. Discarded human intestinal epithelium was obtained with approval from the UCLA Human Investigational Review Board at the UCLA Department of Pathology, as previously described (41). The index cases and their families were enrolled in the UCLA pediatric diarrhea study that was approved by the UCLA institutional review board. Informed consent was obtained from members of both families. Pertinent medical and pathology records were obtained and summarized for the patients.

Author contributions
RSSV, MGM, MB, and MGGC conceived and designed the experiments. RSSV, JW, MB, HC, and SG performed the experiments. RSSV, MGM, MB, and HC analyzed the data. MGGC and PP contributed reagents, materials, and/or analysis tools. SVW and MSG performed experiments and analyzed the data. MGM, MB, and SG wrote the paper. RSSV, MGGC, MB, HC, and SG critically reviewed the manuscript.

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