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Dicer1 Is Required to Repress Neuronal Fate During Endocrine Cell Maturation

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MicroRNAs (miRNAs) are important regulators of gene expression programs in the pancreas; however, little is known about the role of miRNA pathways during endocrine cell specification and maturation during neonatal life. In this study, we deleted *Dicer1*, an essential RNase for active miRNAs biogenesis, specifically from NGN3+ endocrine progenitor cells. We found that deletion of *Dicer1* in endocrine progenitors did not affect the specification of hormone-expressing endocrine cells. However, the islets in the mutant mice in the neonatal period exhibited morphological defects in organization and loss of hormone expression, and the mutant mice subsequently developed diabetes. Dicer1-deficient β-cells lost insulin expression while maintaining the expression of β-cell transcription factors such as Pdx1 and Nkx6.1 early in the postnatal period. Surprisingly, transcriptional profiling showed that that the Dicer1-deficient endocrine cells expressed neuronal genes before the onset of diabetes. The derepression of neuronal genes was associated with a loss in binding of the neuronal transcriptional repressor RE-1-silencing transcription factor to its targets in Dicer1-deficient β-cells. These studies suggest that miRNAs play a critical role in suppressing neuronal genes during the maturation of endocrine cells. ***Diabetes*** **62:1602–1611, 2013**

A potential therapeutic approach to replenish the pancreatic β-cell mass in diabetic patients involves the transplantation of functional, glucose-responsive β-cells differentiated from human pluripotent stem cells. Several attempts have been made at differentiating β-cells in vitro from stem cells, with limited success, (1,2) because the insulin-expressing cells generated lack the characteristic hallmarks of functionally mature β-cells, such as the ability to regulate glucose-stimulated insulin-secretion. Although many transcription factors and signaling pathways underlying the stepwise cell fate acquisition during β-cell development are known (3–6), a complete understanding of the molecular basis of β-cell specification and functional maturation is lacking.

Of significant interest is the role of microRNAs (miRNAs) in regulating the pancreatic developmental program. miRNAs are non–protein-coding small RNAs (~19–25 nucleotides) that negatively regulate gene expression at the post-transcriptional level (7) and have been implicated as important regulators of animal development (8). Newly transcribed miRNAs undergo a series of processing steps that require the RNase III enzymes Drosha and Dicer1 before becoming functional (9,10). Although several miRNAs have been proposed to regulate β-cell transcription factors during development (11), many of these computationally predicted miRNA–mRNA interactions have not been experimentally validated in vivo. The dysregulation of miRNAs through *Dicer1* ablation in the early embryonic pancreatic progenitor cells expressing Pdx-1 resulted in severe deficiencies in the formation of all islet cell lineages (12). More recently, it has been shown that deletion of *Dicer1* in β-cells leads to loss of insulin expression and to development of diabetes in adult mice (13). Although these studies reveal key functions of miRNA-dependent pathways during early pancreatic development and in adult β-cells, they preclude analysis of the role of miRNAs during the specification of endocrine cells and their functional maturation in postnatal life.

In this study, we used a mouse model where expression of Cre recombinase directed by the *Ngn3* promoter conditionally deleted floxed *Dicer1* alleles in endocrine progenitor cells. In addition, by crossing these mice onto the *R26RYFP* reporter line, we were able to trace the lineage of the Dicer1-deficient islet progenitor cells. Our data demonstrate that Dicer1-deficient endocrine progenitors differentiate into hormone-expressing endocrine cells but subsequently lose hormone expression during the neonatal period and develop diabetes. More surprisingly, we found that the Dicer1-deficient islet cells expressed neuronal genes, supporting a model in which miRNA pathways control important transcriptional networks required for suppressing neuronal fate during the maintenance and maturation of newly specified endocrine cells.

RESEARCH DESIGN AND METHODS

Mice and physiology. Mice were maintained in a 12-h light/dark cycle under standard conditions. Studies involving mice were performed in accordance with National Institutes of Health policies on the use of laboratory animals and approved by the University of California, Los Angeles (UCLA) Animal Research Committee. The mice used in this study are the conditional *Dicer1* $^{flox/flox}$ line (14), the *Ngn3-Cre* (15), and the *R26R-YFP* (16) lines. The control mice used throughout were heterozygous for the conditional *Dicer1* allele and the Ngn3-Cre transgene (*NC:Dicer1* $^{fl/+}$). All mice were maintained in the C57BL6 background.

DNA extracted from tails was used for PCR-based genotyping. Blood glucose levels were measured from tail vein blood using a FreeStyle glucometer (Abbot Diabetes Care), and pancreatic insulin content was measured using a mouse insulin ELISA kit (Mercodia) after acid ethanol (0.18 mol/L HCl in 70% ethanol) extraction according to the protocol recommended by the Animal Models of Diabetes Complications Consortium (http://www.amdcc.org/).

Histology and immunohistochemistry. Pancreatic tissue was processed for immunohistochemical analyses as previously described (17). Briefly, the pancreas was dissected and fixed in 4% formaldehyde for 2 h before being embedded in paraffin. Sections (5-μm thick) were deparaffinized, rehydrated,

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subjected to antigen retrieval using Antigen Unmasking Buffer (Vector Laboratories), and permeabilized in 0.4% Triton X-100/TBS. Tissues were subsequently blocked with 3% IgG-free BSA (Jackson ImmunoResearch Laboratories). Incubation with primary antibodies was performed overnight at 4°C in blocking solution at the following dilutions: 1:200 guinea pig anti-insulin (Dako), 1:500 rabbit anti-glucagon (Immunostar), 1:200 rabbit anti-amylase (Sigma-Aldrich), 1:100 mouse anti-Pdx1 (DHSB), 1:200 rabbit anti-Pdx1 (Chemicon), 1:250 chicken anti-green fluorescent protein (GPF; Aves Laboratories Inc.), 1:500 rabbit anti-MafA (18), 1:100 rabbit anti-MafB (Bethyl), 1:1000 rabbit anti-Glut2 (Millipore), 1:250 rabbit anti-tyrosine hydroxylase (Th; Millipore), 1:50 mouse anti-Nkx2.2 (BCBC), 1:50 mouse anti-Nkx6.1, and 1:50 mouse anti-Ki67 (BD Pharmingen). Secondary antibodies (Jackson ImmunoResearch Laboratory) conjugated to fluorescein isothiocyanate (1:200 dilution) or Cy3 (1:1000 dilution) were diluted in blocking buffer. In situ cell death detection assay (TUNEL) was performed according to the manufacturer's instructions (Roche). Slides were mounted with Vectashield with DAPI (Vector Laboratories), and images were obtained using Openlab software (Perkins Elmer) and a Leica DM6000 microscope.

β-Cell mass analysis. β-Cell mass was calculated, as previously described (19), by analyzing pancreata from four mice for each age and genotype.

Islet isolation and fluorescence-activated cell sorter sorting. Islet isolation from mice was performed using a Liberase enzyme blend and purified by centrifugation in a Histopaque gradient, as described previously (17), with slight modifications. Instead of Liberase perfusion through the common bile duct, neonatal pancreata were randomly injected with Liberase blend. To obtain purified pancreatic and endocrine progenitor cells, pancreatic buds from embryonic day (e)12.5 Pdx1-yellow fluorescent protein (YFP) and e14.5 Ngn3-GFP (20) embryos were dissected and dissociated into a single-cell suspension using nonenzymatic cell dissociation buffer (Sigma-Aldrich). GFP+ cells were sorted by fluorescence-activated cell sorter (FACS) (FACSaria BD biosciences). A similar process was followed to obtain β-cells from islets isolated from 2-week-old MIP-GFP (21) mice.

RNA isolation, RT-PCR, and real-time quantitative PCR. Total RNA from islets, dissected pancreata, and Ngn3-GFP and MIP-GFP cells was isolated using an RNeasy RNA extraction Kit (Qiagen). Single-stranded cDNA was prepared using Superscript III Reverse Transcriptase (Invitrogen) with oligodT priming. Real-time quantitaive (q)PCRs were performed using the 7900HT Fast Real-time PCR system (Applied Biosystems). The expression level of each transcript was normalized to the housekeeping gene *Cyclophilin*. Data and standard deviations shown were measured from at least three independent biological replicate experiments. All RT-PCR and real-time qPCR primer sequences used are available upon request.

Chromatin immunoprecipitation assays and chromatin immunoprecipitation-qPCR. To obtain purified β-cells from P7 control and *NC:Dicer1*$^{fl/fl}$ mice for chromatin immunoprecipitation (ChIP) analyses, dissected pancreata were dissociated into a single-cell suspension. The pancreatic cells were immunostained for insulin, after fixation and permeabilization with BD Cytofix reagent (BD PharMingen), using guinea pig anti-insulin antibody, followed by incubation with a Cy3-conjugated secondary antibody, and sorted by FACS (FACSaria BD Bioscience). Pancreatic cells processed without primary antibody were used as a negative control for FACS. ChIP experiments on purified β-cells were performed using the micro-ChIP protocol, as previously described (22). The sequence-specific primers used to amplify the region around the RE1 sites on the *Stmn2*, *Stmn3*, *Th*, and *Syn1* locus are available upon request. The qPCR analysis was performed using the 7900HT Fast Real-time PCR system (Applied Biosystems). The data shown were from independent biological triplicates, and ChIP-qPCR signals are reported as percentage of input.

Microarray gene expression analysis. Two pairs of independent pancreata from control and mutant *NC:Dicer1*$^{fl/fl}$ mice were dissected out at postnatal day 7 (P7), and total RNA was extracted using an RNeasy Plus Mini Kit (Qiagen), according to the manufacturer's protocol. Total RNA quality was assessed using an Agilent 2100 Bioanalyzer and an RNA Integrity Number (RIN) generated using 2100 Expert Software (Agilent Technologies). All RNA samples used had a RIN greater than 7. One microgram of total RNA was processed, labeled, and hybridized to the mouse 1.0ST GeneChip Microarray (Affymetrix), according to manufacturer's recommendations, by the UCLA DNA Microarray Core Facility.

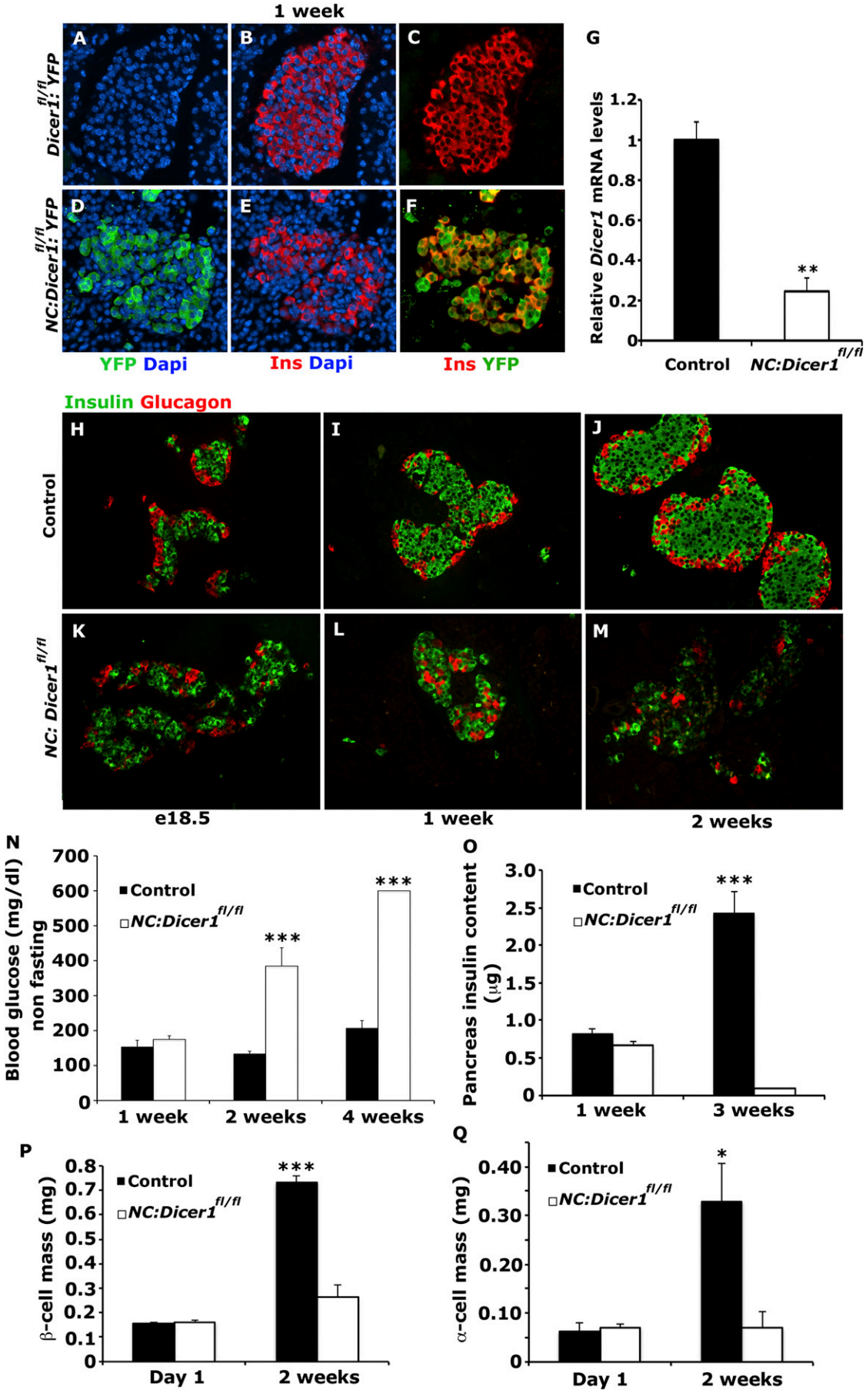
The CEL files (raw expression measurement data) generated from array image analysis were imported into Partek Genomics Suite Software (23), robust multi-array average (RMA) normalized, and converted to $\log_2$ values. The software was further used to perform ANOVA and identify statistically significant differentially expressed genes (P values) between control and *NC:Dicer1*$^{fl/fl}$ pancreata by applying a false discovery threshold of 5% to the P values.

Statistical analyses. All data are expressed as means ± SEM. Statistical significance was determined by an unpaired Student t test. A P value <0.05 was used to reject the null hypothesis.

RESULTS

***Dicer1*-null endocrine precursor cells specify appropriately but display loss of hormone expression during neonatal phase and subsequently develop diabetes.** The catalytic role of *Dicer1* is central to the functional maturation of miRNAs from their precursors (9). To investigate in vivo the role of miRNAs during islet cell specification from endocrine precursor cells expressing *Neurogenin3* (*Ngn3*), the *Dicer1* conditional allele (*Dicer1*$^{fl/fl}$) (14) was deleted specifically from endocrine precursor cells using *NC*-mediated excision (15) thereby preventing the generation of active miRNAs in these cells. In addition, an *R26R-YFP* reporter line (16) was crossed onto the *NC:Dicer1*$^{fl/fl}$ mice to heritably label any cells undergoing recombination and deletion of Dicer1. Co-immunostaining of *NC:Dicer1*$^{fl/fl}$ pancreatic sections for insulin/YFP (Fig. 1*A–F*) and glucagon/YFP (data not shown) showed a high degree of overlap of these markers in the mutant islets, therefore confirming that a high percentage of specified islet cells were derived from endocrine precursor cells that had undergone Cre-mediated recombination. Real-time qPCR analysis revealed that the *Dicer1* transcript was reduced by 80% in the mutant *NC:Dicer1*$^{fl/fl}$ islets at 1 week (Fig. 1*G*), further confirming the deletion of *Dicer1* from a majority of islet cells.

To determine whether loss of *Dicer1* affected the specification of islet cells, pancreatic tissue isolated from control (heterozygous for the *Dicer1* conditional allele *NC:Dicer1*$^{fl/+}$) and mutant *NC:Dicer1*$^{fl/fl}$ mice were co-immunostained for insulin and glucagon at different stages of neonatal development. Immunostaining of pancreata from e18.5 *NC:Dicer1*$^{fl/fl}$ mice for insulin and glucagon revealed that α- and β-cells specified appropriately in the pancreas, and no morphological differences in islet architecture were apparent (Fig. 1*H* and *K*). However, pancreata from 1-week-old *NC:Dicer1*$^{fl/fl}$ mice exhibited altered islet organization, with many instances of α-cells prevalent within the core of the islet rather than at the periphery, an observation also confirmed by Pdx1 and glucagon staining at the same stage (Fig. 1*I* and *L*; Supplementary Fig. 1*A* and *B*). Pancreata from 2-week-old *NC:Dicer1*$^{fl/fl}$ mice displayed severe defects in morphology, and the expression of insulin and glucagon was severely diminished (Fig. 1*J* and *M*). Quantification at 1 day after birth revealed similar β- and α-cell mass (Fig. 1*P* and *Q*). A similar comparison showed a dramatic reduction in endocrine cell mass in the mutant *NC:Dicer1*$^{fl/fl}$ mice at 2 weeks after birth. Consistent with the reduction in β-cell mass by 2 weeks (Fig. 1*P*), an almost total loss of pancreatic insulin content was observed in the mutant *NC:Dicer1*$^{fl/fl}$ animals (shown at 3 weeks in Fig. 1*O*). However, only a very modest decrease in insulin content was noticeable in the mutant *NC:Dicer1*$^{fl/fl}$ animals at 1 week. The mutant *NC:Dicer1*$^{fl/fl}$ animals displayed an inability to metabolize glucose and developed hyperglycemia and frank diabetes within 2 weeks of birth (Fig. 1*N*), consistent with a nearly total loss of β-cells by that age. Taken together, our results suggest that *Dicer1* in the endocrine progenitors was not required for specification of endocrine cells during embryogenesis but was required postnatally to maintain the expression of hormones and the maintenance of endocrine cell mass. These observations therefore underscore the key role miRNAs play during the neonatal period when endocrine cells became functionally mature and capable of maintaining blood glucose levels.


1 week
A
B
C
D
E
F
Dicer1fl/fl:YFP
NC:Dicer1fl/fl:YFP
YFP Dapi
Ins Dapi
Ins YFP
G
Relative Dicer1 mRNA levels
1.2
1
0.8
0.6
0.4
0.2
0
**
Control
NC:Dicer1fl/fl
Insulin Glucagon
H
I
J
K
L
M
Control
NC: Dicer1fl/fl
e18.5
1 week
2 weeks
N
Blood glucose (mg/dl) non fasting
700
600
500
400
300
200
100
0
Control
NC:Dicer1fl/fl
***
***
1 week
2 weeks
4 weeks
O
Pancreas insulin content (μg)
3.0
2.5
2.0
1.5
1.0
0.5
0
Control
NC:Dicer1fl/fl
***
1 week
3 weeks
P
β-cell mass (mg)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
NC:Dicer1fl/fl
***
Day 1
2 weeks
Q
α-cell mass (mg)
0.40
0.30
0.20
0.10
0
Control
NC:Dicer1fl/fl
*
Day 1
2 weeks

Endocrine cells from *Dicer1*-null mice maintain expression of characteristic transcription factors despite losing hormone expression. Next, we asked whether the loss of hormone expression in mutant *NC*:*Dicer1*$^{fl/fl}$ islet cells was due to any potential effect on key islet transcriptional regulators upon ablation of miRNAs. We tested this hypothesis by assessing the expression of characteristic β-cell transcription factors Pdx1, Nkx6.1, and MafA. Immunostaining of pancreata from 2-week-old control mice showed the expected coexpression of all these transcription factors with insulin (Fig. 2*A–C* and data not shown). In contrast, whereas the pancreata from mutant *NC*:*Dicer1*$^{fl/fl}$ littermates showed normal expression levels of Pdx1, Nkx6.1, and MafA in the islets, only a few of these cells were also positive for insulin, with the rest devoid of insulin expression (Fig. 2*D–F*). Similarly, 2-week-old mutant *NC*:*Dicer1*$^{fl/fl}$ pancreatic sections showed a number of cells that expressed MafB but not glucagon (Supplementary Fig. 1*C* and *D*). Furthermore, real-time qPCR analyses of a set of key β-cell genes between control and mutant *NC*:*Dicer1*$^{fl/fl}$ pancreata revealed a significant reduction in the expression of *Insulin1* and *Insulin2* mRNA, whereas no differences in the expression of transcription factors markers was observed (Supplementary Fig. 1*E* and *F* and Supplementary Fig. 2*A*). These results suggested that endocrine hormone expression was lost although the transcriptional regulatory genes that characterize endocrine cells were intact in the absence of *Dicer*1.

Deletion of *Dicer-1* upregulates neuronal genes in islet cells. To investigate the molecular changes in endocrine cells of mutant *NC*:*Dicer1*$^{fl/fl}$ animals that could be responsible for the loss of hormone expression in endocrine cells, we performed genome-wide transcription profiling using microarrays on two independent pairs of control and mutant *NC*:*Dicer1*$^{fl/fl}$ pancreata isolated at P7. The microarray was performed at this stage, which precedes any apparent physiological changes. Transcription profiling analysis revealed 162 differentially expressed genes with at least a twofold change at a false discovery rate of 5%, of which 145 genes were upregulated and 17 were downregulated. A surprisingly disproportionate number of neuronal genes were upregulated in the *NC*:*Dicer1*$^{fl/fl}$ pancreata, whereas the expression of transcription factors and other characteristic islet cell markers did not exceed the twofold change threshold (Fig. 3*A–C*). The upregulation of these neuronal genes in *NC*:*Dicer1*$^{fl/fl}$ pancreata was further validated by performing real-time qPCR analysis from the microarray samples (Fig. 3*E*) as well as on a triplicate of samples independent of those used for the microarray analysis (Supplementary Fig. 2*A*).

Gene ontology analysis for biological processes associated with the dysregulated genes revealed a significant enrichment of genes involved in nervous system development, synaptic transmission, transmission of nerve impulse, and neurogenesis (Fig. 3*D*). The upregulated genes included pan-neuronal markers such as *Scg10*, *Stmn3*, and the neurofilament markers *Nefl* and *Nefm*, as well as molecular markers of noradrenergic neurons, such as the neurotransmitter-synthesizing enzymes *Th* and dopamine β hydroxylase (*Dbh*), the vesicular transport molecule–vesicular monoamine transporter 2 (*Vmat2*), and the plasma membrane transporter–norepinephrine transporter (*Net*). In fact, many components of the noradrenergic program were upregulated. A number of these neuronal genes are expressed to some extent early during endocrine pancreas development, but their expression declines significantly by 3 to 4 weeks of age (24). For example, real-time qPCR analysis indicated that the expression of neuropeptide Y (NPY), which is normally downregulated upon maturation, was expressed at high levels in the mutant *NC*:*Dicer1*$^{fl/fl}$ pancreata.

To test whether the increase in transcript levels of the neuronal genes was associated with a corresponding increase in protein levels, we immunostained 1 week control and *NC*:*Dicer1*$^{fl/fl}$ pancreatic sections for insulin and Th. Very few Th and insulin double-positive cells were observed in control sections (Fig. 4*A–C*). In contrast, the mutant *NC*:*Dicer1*$^{fl/fl}$ sections displayed a larger percentage of cells that coexpressed insulin and Th (Fig. 4*D–F*). Thus, the upregulation of mRNA of neuronal genes due to the absence of *Dicer1* also results in a concomitant increase at the protein level. To assess whether the upregulation of Th was limited to β-cells, we costained Th with Pdx1. All Th-positive cells in the *NC*:*Dicer1*$^{fl/fl}$ islets also coexpressed Pdx1 (Fig. 4*H–M*), suggesting that β-cells upregulated the neuronal marker Th in the absence of *Dicer1*. To verify the endocrine lineage of these Th-positive cells, we performed immunostaining for the lineage trace marker YFP and Th in the mutant *NC*:*Dicer1*$^{fl/fl}$pancreata. All of the Th-positive cells were also positive for YFP staining (Fig. 4*G* and *J*), therefore confirming that the Th-positive cells were derived from endocrine precursor cells that had undergone recombination and lost Dicer1 expression.

We also examined Th expression in the mutant *NC*:*Dicer1*$^{fl/fl}$ islets at 2 weeks. Consistent with observations in islets from 1-week-old control mice, costaining of Th with insulin (Fig. 4*N–S*) revealed that control islets from 2-week-old mice also typically displayed only a few Th-positive cells that also stained for insulin (Fig. 4*N–P*). In contrast, mutant *NC*:*Dicer1*$^{fl/fl}$ islets displayed a massive upregulation of Th staining (Fig. 4*Q–S*). A few insulin and Th-copositive islet cells were evident, but most of the Th-positive cells did not show any insulin staining, suggesting that neuronal gene upregulation persists in mutant islet cells that have already downregulated endocrine hormones. Taken together, our results suggest that miRNAs likely play an important role in suppressing a neuronal gene program during the maturation phase of endocrine islet cells.

FIG. 1. Mutant *NC*:*Dicer1*$^{fl/fl}$ islet cells specify normally but lose hormone expression and develop hyperglycemia during neonatal development. Immunostaining of 1-week-old Cre negative (*A–C*) and mutant *NC*:*Dicer1*$^{fl/fl}$ (*D–F*) pancreatic sections ($n = 3$) for insulin (red) (*B* and *E*), YFP that marks recombined cells (green) (*A* and *D*), and DAPI to visualize the nuclei (blue). *C* and *F*: Most of the insulin$^+$ β-cells colocalize with YFP in the mutant islets but not in the control islets. *G*: Transcript levels of *Dicer1* determined by real-time qPCR using RNA isolated from islets of control and mutant *NC*:*Dicer1*$^{fl/fl}$ mice ($n = 3$) at P7. Expression levels in control were set as one arbitrary unit. Representative pancreatic sections ($n = 3$ for each age and genotype) from e18.5 and 1- and 2-week-old control (*H–J*) and mutant *NC*:*Dicer1*$^{fl/fl}$ (*K–M*) littermates were immunostained for insulin (green) and glucagon (red). *N*: Nonfasting blood glucose levels in neonatal control and mutant *NC*:*Dicer1*$^{fl/fl}$ mice at 1, 2, and 4 weeks ($n = 5$ for each age group). *NC*:*Dicer1*$^{fl/fl}$ mutant mice are hyperglycemic by 2 weeks. *O*: Total pancreatic insulin content in control and *NC*:*Dicer1*$^{fl/fl}$ mice at 1 and 3 weeks reveals minimal pancreatic insulin content remaining in *NC*:*Dicer1*$^{fl/fl}$ mice at 3 weeks ($n \geq 3$). *P–Q*: β- and α-cell mass in control and mutant *NC*:*Dicer1*$^{fl/fl}$ mice at P1 and 2 weeks was assessed as described in RESEARCH DESIGN AND METHODS ($n = 4$). The error bars represent the SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$. In all cases, at least two to three pancreas sections were used for each animal.

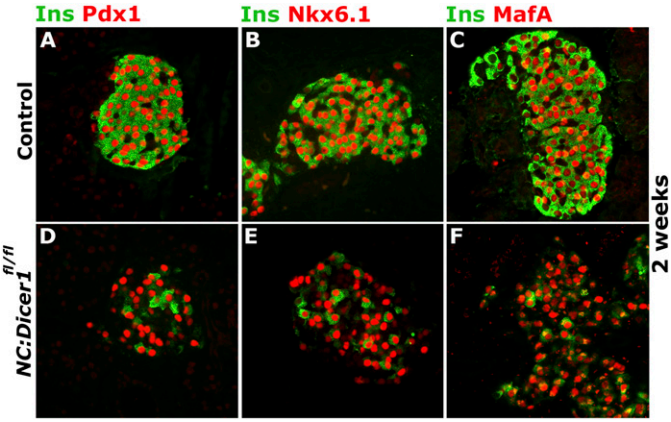


FIG. 2. Loss of insulin expression in mutant *NC:Dicer1*$^{fl/fl}$ β-cells is not preceded by downregulation of characteristic β-cell transcription factors. Representative pancreatic sections from 2-week-old control and mutant *NC:Dicer1*$^{fl/fl}$ mice co-immunostained for insulin (Ins; green) and Pdx1 (red) (*A* and *D*), Nkx6.1 (red) (*B* and *E*), and MafA (red) (*C* and *F*). Many instances of insulin-negative β-cells that still express the β-cell transcription factors are observed in the mutant sections. In control animals, β-cells always coexpress insulin with the transcription factors ($n \geq 3$ for each group of animals). In all cases, at least two to three pancreas sections were used for each animal.

Loss of RE1-silencing transcription factor–mediated repression of neuronal targets in *Dicer1*-null β-cells. We hypothesized that the acquisition of a neuronal transcriptional profile by the mutant *NC:Dicer1*$^{fl/fl}$ islet cells could possibly be attributed to the dysregulation of transcriptional programs dedicated toward the maintenance of islet cell identity. To investigate transcription factors regulating neuronal gene repression in islet cells, we used the MetaCore Interactome characterization tool (GeneGo, Inc., http://www.genego.com) to identify the top transcription factors that exhibited significantly large numbers of interactions with the dysregulated genes in the mutant *NC:Dicer1*$^{fl/fl}$ islet cells. The Interactome analysis revealed that 16% of the dysregulated genes were targets of the neuronal transcriptional repressor RE1-silencing transcription factor (REST), also known as neuron restrictive silencer factor (Fig. 5*A*). REST is a repressor of neuronal genes that binds in a sequence-specific manner to the RE1 elements of its target genes and mediates transcriptional repression (25,26).

The upregulation of REST target genes in *NC:Dicer1*$^{fl/fl}$ islet cells suggested an important role for REST during islet cell development and maturation. We first investigated whether REST was expressed at different stages of islet cell development. Real-time qPCR analysis of REST expression showed that *REST* transcripts were present in FACS-purified Ngn3-positive endocrine precursor cells at e14.5 and in β-cells at P14 at levels comparable to those expressed in e14.5 brain tissue and adult lung tissue where REST is known to be highly expressed (27,28) (Fig. 5*B*). Although microarray and real-time qPCR analysis (data not shown and Fig. 5*D*) did not reveal any changes in REST mRNA levels between control and *NC:Dicer1*$^{fl/fl}$ islets, we hypothesized that changes in miRNAs could potentially be regulating molecular events that may alter recruitment of REST protein and/or members of the REST repressive complex.

To analyze whether REST binding to the RE1 sites of its target genes was lost in the mutant *NC:Dicer1*$^{fl/fl}$ β-cells, ChIP analysis on a subset of the upregulated REST target genes showed that REST was bound to the RE1 sites of the four genes examined in FACS-purified control β-cells at P7. In contrast, REST binding to its target genes was significantly reduced in the mutant *NC:Dicer1*$^{fl/fl}$ β-cells (Fig. 5*C*), and a concomitant increase in mRNA expression of these genes was observed in the mutant *NC:Dicer1*$^{fl/fl}$ pancreata (Fig. 3*E*). This suggests that miRNAs play an important role in regulating the recruitment of the REST repressive complex, which in turn represses the transcription of its target neuronal genes.

In addition to REST repression of the neuronal genes, we also identified other potential candidate transcription factors using the Interactome analysis, including the neuronal homeobox transcription factors Phox2a and Phox2b, which are important regulators of the noradrenergic phenotype in vertebrates (29–31). Real-time qPCR analysis indicated that both transcripts were significantly upregulated in the mutant *NC:Dicer1*$^{fl/fl}$ pancreata (Fig. 5*D*). Taken together, our results reveal an important role of miRNAs in regulating key transcriptional programs required for maintaining islet cell identity during neonatal development.

***Dicer1*-null islet cells die by early adulthood.** To determine the ultimate fate of the *Dicer1*-null islet cells that had acquired an altered transcriptome, we immunostained the islets of control and *NC:Dicer1*$^{fl/fl}$ pancreatic sections for the islet hormone markers at 6 weeks. By 6 weeks, most insulin expression (Fig. 6*A* and *B*, Supplementary Fig. 3*A* and *B*) and other hormone expression in the islet was lost (data not shown). To determine whether the hormone-negative *Dicer1*-null islet cells survived into adulthood, immunostaining of control and *NC:Dicer1*$^{fl/fl}$ pancreatic sections with the lineage trace marker YFP revealed that by 6 weeks, very few YFP-positive cells remained in the mutant *NC:Dicer1*$^{fl/fl}$ pancreatic sections compared with the littermates (Fig. 6*C* and *D*). Because the genetic ablation of *Dicer1* is associated with apoptosis during development (14,32,33), we assessed the apoptosis rate in control and mutant *NC:Dicer1*$^{fl/fl}$ islets using TUNEL immunostaining. A significant increase (11.7-fold) in apoptosis was observed in mutant *NC:Dicer1*$^{fl/fl}$ β-cells at P12 (Fig. 6*E–G*) as well as other islet cells (data not shown), therefore suggesting that *Dicer1* was ultimately responsible for the survival of mature islet cells.

DISCUSSION

Our study illustrates an essential role of Dicer1-dependent miRNA pathways in the suppression of a subset of neuronal markers and the maintenance of endocrine hormone expression during the maturation of newly specified islet cells in neonatal development. Surprisingly, we found that deletion of the miRNA-processing enzyme Dicer1 in islet progenitors cells (*NC:Dicer1*$^{fl/fl}$ mice) did not affect the specification of endocrine cells. However, these newly specified endocrine cells acquired an altered transcriptome in which neuronal genes were upregulated and endocrine hormone expression was lost, resulting in failure to regulate blood glucose and onset of diabetes by age 2 weeks. One possible explanation for this is that the loss of miRNAs early in endocrine development may alter later developmental pathways involved in the maturation of endocrine cells.

Neuronal and β-cells both share the expression of a large number of proteins, and many similarities exist in the transcription factors involved in their differentiation

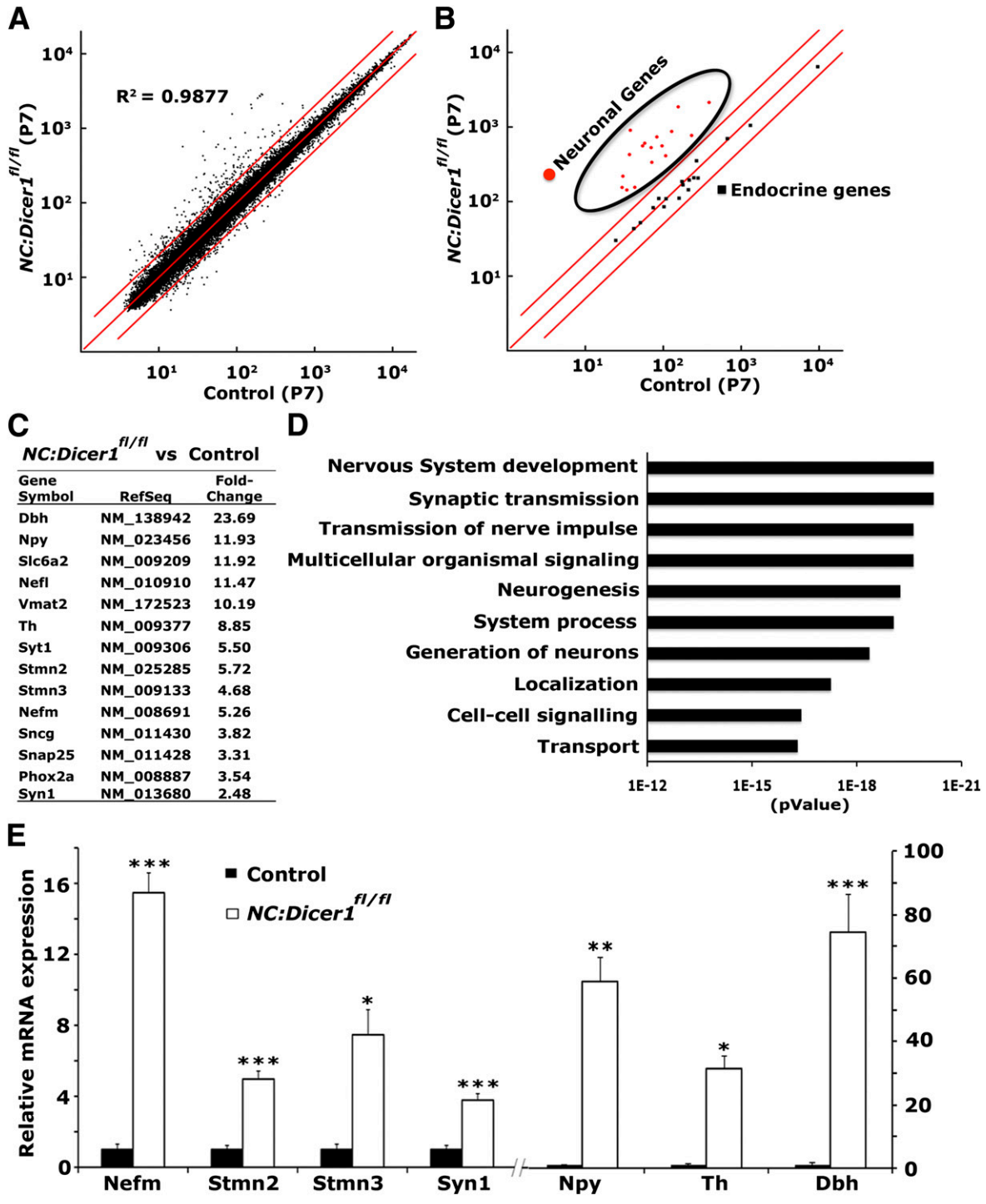


FIG. 3. Unchanged endocrine gene expression but increased neuronal gene expression in mutant *NC:Dicer1*$^{fl/fl}$ islet cells at the presymptomatic stage (P7). *A*: Representative scatter plot of global gene expression obtained from microarray analysis of control and mutant *NC:Dicer1*$^{fl/fl}$ pancreas at P7. A high similarity (R^2) in gene expression is observed between control and mutant *NC:Dicer1*$^{fl/fl}$ samples. Red lines indicate a twofold difference in expression. *B*: The expression levels of many neuronal markers as identified by the Gene Ontology database are upregulated in the mutant *NC:Dicer1*$^{fl/fl}$ samples. Key upregulated neuronal genes (red dots) are circled. The expression levels of most endocrine cell markers are unchanged (black dots). *C*: List of key neuronal genes (circled in *B*) upregulated in microarray ChIP analyses of mutant *NC:Dicer1*$^{fl/fl}$ vs. control samples. *D*: Gene ontology analysis displaying biological processes associated with gene function of the differentially expressed genes with fold-change greater than two. The x-axis values are in logarithmic scale and correspond to raw binomial *P* values. *E*: Real-time qPCR validation of representative upregulated neuronal gene transcripts *Nefm*, *Stmn2*, *Stmn3*, *Syn1*, *Npy*, *Th*, and *Dbh* using RNA isolated from pancreata of control and mutant *NC:Dicer1*$^{fl/fl}$ mice. Expression levels in control pancreata were set as one arbitrary unit. All data points represent means ± SEM of at least three biologically independent experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$. (A high-quality color representation of this figure is available in the online issue.)

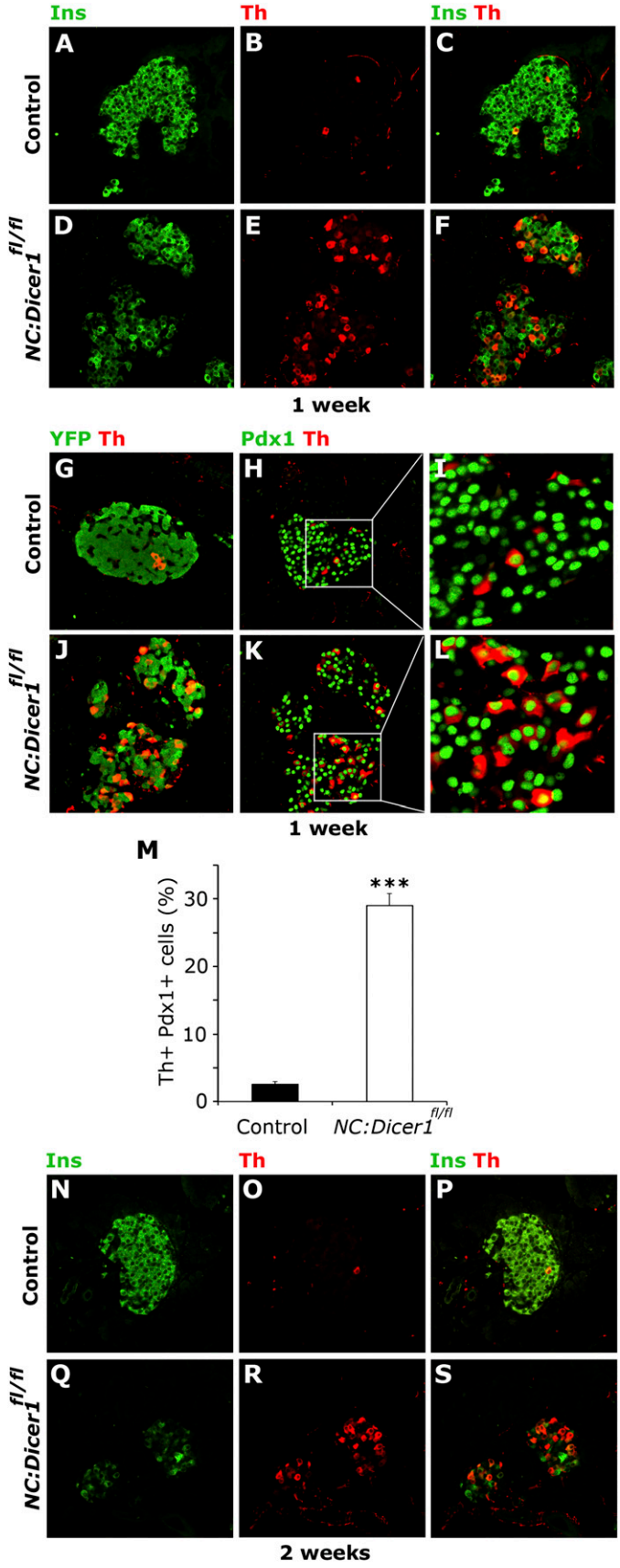


FIG. 4. Expression of Th in mutant *NC:Dicer1*$^{fl/fl}$ pancreas. Pancreatic sections from 1-week-old control and mutant *NC:Dicer1*$^{fl/fl}$ littermates showing immunostaining for insulin (Ins; green) (*A* and *D*), Th (red) (*B* and *E*), and merged view (*C* and *F*) reveals sharp upregulation of Th expression in the mutant β-cells ($n = 3$). Immunostaining of 1-week-old control (*G* and *H*) and *NC:Dicer1*$^{fl/fl}$ mutant (*J* and *K*) pancreatic sections for Th (red)/YFP (green) and Th (red)/Pdx1 (green; $n = 3$). Most Th expression is limited to Pdx1-positive cells. *I* and *L*: Magnified *inset* of an area in *H* and *K* is shown. *M*: Quantification of Th-positive cells expressed as percentage of insulin-positive and Pdx1-positive cells in 1-week-old mice. Data points represent means ± SEM of at least three independent experiments. ***$P < 0.005$. Immunostaining of 2-week-old control and mutant *NC:Dicer1*$^{fl/fl}$ littermates for insulin (Ins; green) (*N* and *Q*), Th (*O* and *R*), and merged view (*P* and *S*) indicates the presence of many insulin-negative β-cells that express Th ($n = 3$). In all cases, at least two to three pancreas sections were used for each animal.

programs (34). The neuronal genes could be direct targets of miRNAs, such that *Dicer1* ablation would result in their derepression in the mutant *NC:Dicer1*$^{fl/fl}$ islet cells. Alternatively, it is a more likely scenario where transcriptional regulators of the neuronal markers that are direct or indirect targets of a combination of miRNAs could be deregulated in the *NC:Dicer1*$^{fl/fl}$ islet cells, resulting in the derepression of neuronal transcripts. The decreased binding of the neuronal transcriptional repressor REST to target neuronal genes, and increased levels of the neuronal transcriptional activators Phox2a and Phox2b in mutant *NC:Dicer1*$^{fl/fl}$ β-cells, suggests that these miRNA-controlled transcriptional regulators are likely part of a broader transcriptional program that actively suppresses neuronal differentiation genes to maintain islet cell identity.

In vivo genome-wide mapping of REST binding sites by ChIPSeq analysis has previously identified the critical islet-cell development transcription factors neurogenic differentiation 1 (NEUROD1), hepatocyte nuclear factor (HNF) 4α, HNF6, Hes1, and Ngn3 as targets of REST (35). Interestingly, genes encoding these endocrine cell regulators were bound relatively poorly by REST compared with terminally differentiated neuronal genes such as *Stmn2*, *Stmn3*, and the neurofilament genes, which bound with greater affinity. This suggests that REST might not play a major role during islet cell differentiation during embryogenesis, but may instead control terminally differentiated neuronal genes that might be involved in maturation of endocrine cells. *Dicer1* deletion results in deregulation of REST recruitment, resulting in the derepression of its target neuronal genes. The eventual loss of islet cells is not surprising given that the loss of Dicer1 expression is associated with massive cell death in a range of different tissues during development (14). Our results are supported by a recent study on the deletion of *Dicer1* in β-cells using the transgenic RIP-Cre line, which showed that the neonatal pancreas contained ultrastucturally normal β-cells, although these mice developed progressive hyperglycemia and full-blown diabetes in adulthood (36). The derepression of neuronal genes in β-cells, however, was not explored.

Given the similarities in the neuronal and islet cell differentiation programs, our studies demonstrate a novel role of miRNA pathways in maintaining islet cell identity through the repression of neuronal markers while maintaining the expression of endocrine hormones. Identifying the individual miRNAs involved in the maturation and maintenance of newly specified islet cells, particularly β-cells, will aid in the development of therapeutic strategies to generate functionally mature replacement β-cells. These results also have important implications for engineering β-cells from embryonic stem cells.

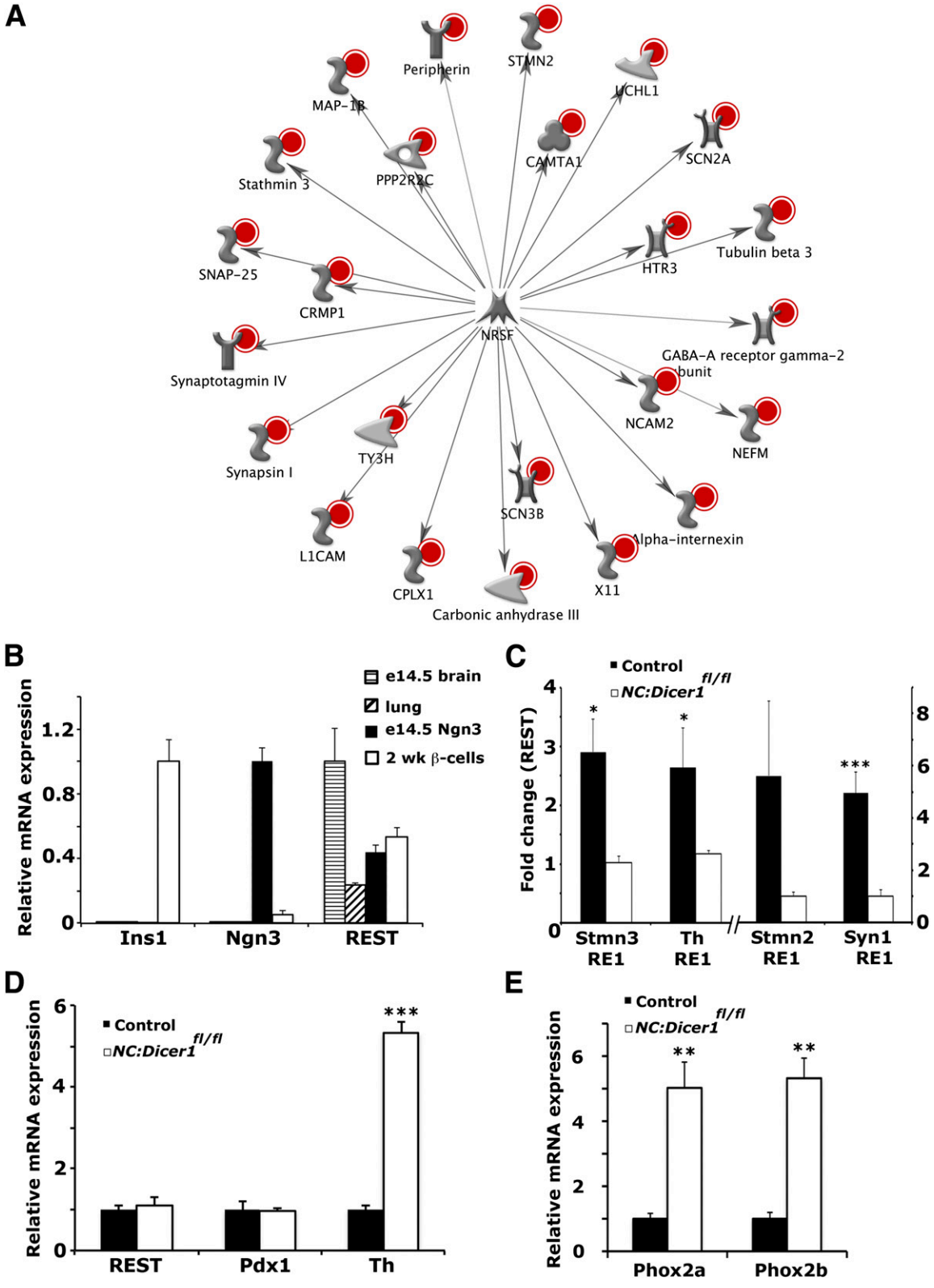


FIG. 5. Loss of REST binding to neuronal targets in mutant *NC:Dicer1*$^{fl/fl}$ β-cells. *A*: MetaCore Interactome analysis (GeneGo, Inc.) revealed that 16% of the dysregulated genes (more than twofold change) are targets of the neuronal transcriptional repressor REST. *B*: Real-time qPCR analysis of *Ins1*, *Ngn3*, and *REST* mRNA transcript levels in adult lung tissue and brain tissue isolated at e14.5, FACS-purified Ngn3-positive pancreatic endocrine progenitor cells at e14.5, and MIP-GFP β-cells at 2 weeks. *C*: ChIP analysis of FACS-purified control and mutant *NC:Dicer1*$^{fl/fl}$ mice β-cells at P7. REST binding to the RE1 regions of *Stmn2*, *Stmn3*, *Th*, and *Syn1* locus was reduced in mutant β-cells. ChIP data are reported as fold-change of REST binding at the RE1 locus of the respective genes relative to a negative control region and represent means ± SEM of at least three biologically independent experiments. *D*: Real-time qPCR analysis of *REST*, *Pdx1*, and *Th* mRNA transcript levels in isolated islets from pancreata of control and mutant *NC:Dicer1*$^{fl/fl}$ mice at P7. *E*: Transcript levels of *Phox2a* and *Phox2b* as determined by real-time qPCR using RNA isolated from pancreata of control and mutant *NC:Dicer1*$^{fl/fl}$ mice at P7. Expression levels in the control were set as one arbitrary unit. All data points represent means ± SEM of at least three biologically independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.005. (A high-quality color representation of this figure is available in the online issue.)

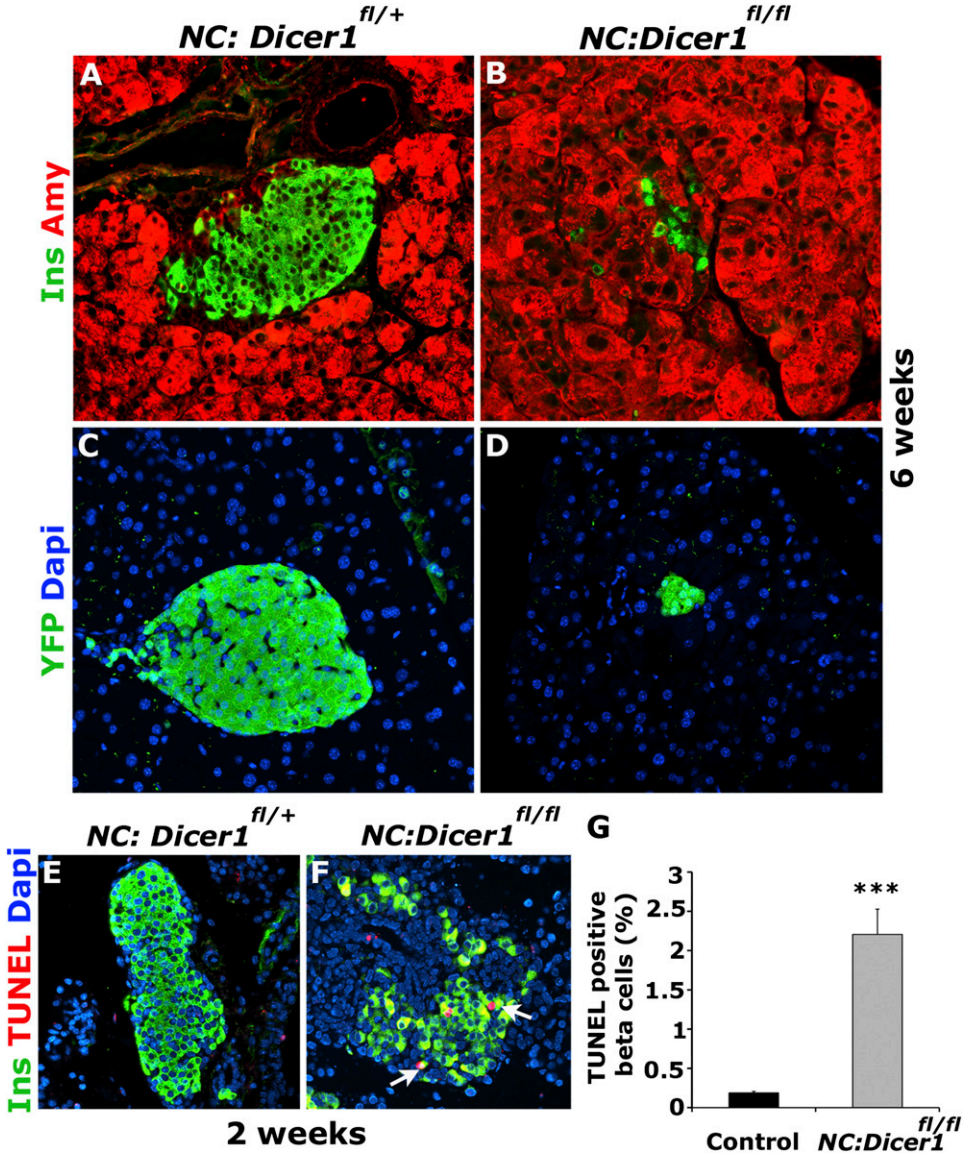


FIG. 6. *Dicer1*-null islet cells undergo apoptosis during neonatal period. *A–D*: Representative pancreatic sections ($n = 3$) from 6-week-old control and mutant *NC:Dicer1*$^{fl/fl}$ mice co-immunostained for insulin (green)/amylase (red) (*A* and *B*) and YFP (green)/DAPI (blue) (*C* and *D*). *E* and *F*: Immunostaining of pancreatic sections ($n = 3$) from 12-day-old control and mutant *NC:Dicer1*$^{fl/fl}$ mice for insulin and TUNEL reveals an increase in TUNEL-positive apoptotic cells in the mutant mice. *G*: Quantification of TUNEL-positive cells expressed as percentage of β-cells in 12-day-old mice. Data points represent means ± SEM of at least three independent experiments. ***$P < 0.005$. In all cases, at least two to three pancreas sections were used for each animal.

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M.K. designed and performed research, analyzed data, and wrote the manuscript. M.G.M. designed research and reviewed and edited the manuscript. A.B. designed research, analyzed data, and wrote the manuscript. A.B. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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