Insm1, Neurod1, and Pax6 promote murine pancreatic endocrine cell development through overlapping yet distinct RNA transcription and splicing programs

Karrie D. Dudek, Anna B. Osipovich, Jean-Philippe Cartailler, Guoquing Gu, and Mark A., Magnuson

Abstract

Insm1, Neurod1, and Pax6 are essential for the formation and function of pancreatic endocrine cells. Here, we report comparative immunohistochemical, transcriptomic, functional enrichment, and RNA splicing analyses of these genes using gene knock-out mice. Quantitative immunohistochemical analysis confirmed that elimination of each of these three factors variably impairs the proliferation, survival, and differentiation of endocrine cells. Transcriptomic analysis revealed that each factor contributes uniquely to the transcriptome although their effects were overlapping. Functional enrichment analysis revealed that genes downregulated by the elimination of Insm1, Neurod1, and Pax6 are commonly involved in mRNA metabolism, chromatin organization, secretion, and cell cycle regulation, and upregulated genes are associated with protein degradation, autophagy, and apoptotic process. Elimination of Insm1, Neurod1, and Pax6 impaired expression of many RNA-binding proteins thereby altering RNA splicing events, including for Syt14 and Snap25, two genes required for insulin secretion. All three factors are necessary for normal splicing of Syt14, and both Insm1 and Pax6 are necessary for the processing of Snap25. Collectively, these data provide new insights into how Insm1, Neurod1, and Pax6 contribute to the formation of functional pancreatic endocrine cells.

Keywords: pancreas; endocrine progenitor cells; gene expression; alternative RNA splicing

Introduction

During pancreas development, the formation of islets of Langerhans requires formation of an endocrine cell-specific gene regulatory network (GRN). Prior studies have identified multiple transcription factors (TFs) that contribute to endocrine cell development. Among these, Neurog3 plays an especially important role. By activating a downstream cascade of pro-endocrine TFs, Neurog3 initiates formation of the endocrine cell-specific GRN. In its absence, the fate of pancreatic pre-endocrine cells is redirected toward the ductal lineage and no endocrine cells are formed. In contrast, when other pro-endocrine TFs downstream of Neurog3 are eliminated, including Insm1, Neurod1, and Pax6, pancreatic endocrine-like cells are formed that exhibit marked defects in proliferation and/or hormone expression, suggesting that each of these factors, in turn, regulates a subnetwork of genes. TFs activate target genes by directly binding DNA in promoter regions, and ChIP-Seq has been widely used to identify genome-wide TF binding sites. Previous ChIP-Seq studies using cell lines have identified many binding sites for Insm1, Neurod1, and Pax6. Analysis of Insm1 and Neurod1 binding sites in a pancreatic β-cell line derived from adult islets has shown that Insm1, Neurod1, and Foxa2 frequently co-bind to the same genomic regions. However, these analyses have also revealed that only 32% of genes in adult β-cells that are regulated by Insm1 have nearby Insm1 binding (Jia et al. 2015). Similarly, while both Pax6 and Neurod1 often bind to enhancers that are active in insulinoma cells, the binding of both factors occurs at only 40% of such binding sites (Lizio et al. 2015). Furthermore, while there is a large degree of overlap in the binding of Neurod1 and Pax6, Pax6 exhibits both activating and repressive functions in mouse and human β-cell lines (Swisa et al. 2017).

RNA processing is also a vitally important process that is regulated during development. There are hundreds of different RNA-binding proteins (RBPs) that affect polyadenylation, stabilization, and localization dynamics of mRNA. Some of these proteins also introduce nucleotide modifications and cause differential
splicing of introns and exons (Licatalosi and Darnell 2010; Wickramasinghe and Venkitaraman 2016; Manning and Cooper 2017; Carazo et al. 2019). Alternative RNA splicing significantly enhances transcriptome diversity, and is crucial for cell differentiation (Fiszbein and Kornblitt 2017). Indeed, Singer et al. (2019) have recently reported that the alternative splicing of Pax6, which requires recruitment of RBPs by the lncRNA Pauper, alters both its transcriptional activity and DNA binding specificity. It has also recently been shown in islets from individuals with type 2 diabetes that many RBPs are dysregulated and that this may impair correct RNA splicing (Jeffery et al. 2019).

In this study, we performed immunohistochemical and bulk transcriptomic analyses to directly compare the effects of eliminating Ins1, Neuro1, and Pax6 during endocrine cell development. Our findings indicate that these three TFs individually and coordinately regulate the expression of common and unique sets of genes necessary for the proliferation and function of pancreatic endocrine cells. Additionally, we find that Ins1, Neuro1, and Pax6 differentially affect the splicing of genes, thereby adding complexity to pancreatic endocrine cell proteomes.

Materials and methods

Mice and genotyping

Ins1tm1.1Mgn (Ins1GFPCre) (Osipovich et al. 2014) and Neuro1tm1.1mle (Neuro1LacZ) (Miya et al. 1999) mice were maintained in a CD-1 background. Pax6tm2Pgr mouse, Pax6lox mouse obtained from Jackson Laboratory and kept in a C57Bl6/J background (Ashery-Padan et al. 2000; Sun et al. 2008). All animal experimentation was performed under the Vanderbilt University Institutional Animal Care and Use Committee’s oversight. The Ins1GFPCre allele expresses a GFP-Cre fusion protein that contains a nuclear localization sequence and were genotyped as previously described (Osipovich et al. 2014). Pax6lox mice containloxP sites flanking the initiation codon in exon 4 and exon 6 (the paired domain), requiring Cre activity for excision and were genotyped using the primer pairs 5′-CTCAACAGAGCCCGTATTC(forward) and 5′-GCCCAACGTCCAGAAAG(reverse). In Neuro1LacZ mice, Neuro1 coding sequences were replaced with a LacZ reporter (Miya et al. 1999) and therefore intrinsically lack Neuro1 expression. To detect wild-type Neuro1 and Neuro1LacIz we used either 5′-ACCATCGCCTGTACCCAGT (forward) or 5′-GAGACTGAGACTCTCTGT (forward) in combination with 5′-AAAGGCCGAGTFAAGCCGTC (reverse), respectively.

Timed matings and tissue collection

Embryos were isolated from timed matings where noon of the day that vaginal plugs were observed was designated E0.5. Embryonic day (E) 15.5 animals were dissected into ice-cold PBS and visually genotyped for the presence of Ins1GFPCre allele based on green fluorescence. For PCR genotyping, embryonic tissues were digested at 55°C in 100 μl of PCR lysis buffer (1 × PCR buffer, 0.1% Triton X-100, 100 μg/ml Proteinase K), inactivated at 90°C for 15 min and used for PCR. Whole pancreas was dissected and either processed for fluorescence-activated cell sorting (FACS) or frozen in OCT for immunostaining analyses.

Immunofluorescence

Pancreata were routinely fixed in 2% paraformaldehyde for 2 h at room temperature, incubated in 30% sucrose in PBS overnight on a shaker, then mounted using Tissue Tek O.C.T. compound and stored at −80°C. Immunofluorescence staining of frozen tissue sections was performed as previously described (Burilson et al. 2008). Ten μm tissue sections were stained using anti-GFP (1:500, ThermoFisher, #A10262), insulin (1:1000, Invitrogen, #PA1-26938), glucagon (1:100, Milipore, #AB932), somatostatin (1:1000, Linco), pancreatic polypeptide (1:1000, Linco), and/or Ki67 (1:100, ThermoFisher, #RM-9106-S1) antibodies, and ProLong Gold anti-fade reagent with DAPI (Life Technologies, #P36941) used to mount coverslips. TUNEL (TMR red) assays were performed following the manufacturer’s protocol (ROCHE). Respective images were obtained via confocal microscopy using an LSM 510 Meta microscope at 20 × magnification. The image processing software, ImageJ (National Institute of Health), was used to manually identify and quantify fluorescently-positive cells (Schneider et al. 2012). The percentage of GFP-positive cells expressing islet hormones were calculated. Three different biological replicates from each genotype and at least 500 cells were analyzed for each measurement.

Fluorescence-activated cell sorting

Pancreatic cells were dispersed into a single-cell suspension by incubation for 5 min at 37°C in Accumax (Sigma) containing 50 μg/ml DNase I. Reactions were quenched by the addition of sorting buffer (FACS staining buffer (R&D Systems), DNase (1:1000)). Cells were then filtered through a 35 μm nylon mesh into a FACS tube (Corning), washed once with FACS staining buffer, centrifuged at 1100 rpm for 3 min, then re-suspended in 500 μl of FACS sorting buffer (Osipovich et al. 2014). 7-aminoactinomycin (7-AAD) was added at a concentration of 1:1000 immediately before sorting using either a Bentley Dickenson FACS Aria-II or Aria-III instrument to distinguish between live and dead cells. GFP-positive, 7-AAD negative cells were collected directly into Trizol LS (Invitrogen) containing 40 μg/ml of mussel glycogen (Roche/Sigma). An average of 6000 cells was obtained per embryo sample.

RNA isolation, library construction, and RNA-Seq

Three replicate samples for each genotype were collected, and total RNA was isolated using Trizol LS then treated with DNase I (Life Technologies). RNA was column-purified using the RNA Clean and Concentrator Kit (Zymo Research) and previously published protocols (Choi et al. 2012). RNA integrity was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA), and samples with an RNA integrity number (RIN) of 7.0 or greater were used for RNA-Seq. All samples were amplified using the SMART-Seq Ultra Low Input RNA Kit (TAKARA/Clontech) at 10 cycles, except for the Neurog3GFP and Neurog3GFP samples, which were prepared using the Ovation RNA-Seq System V2 (NuGEN). cDNA was prepared using the Low Input Library Kit (Clontech) and sequenced using either an Illumina HiSeq3000 genome analyzer to obtain paired-end, 75-bp reads, or Illumina NovaSeq6000 to get paired-end, 100-bp reads. RNA samples were sequenced to an average depth of ~5.0 × 10⁷ reads. RNA-Seq data have been deposited in ArrayExpress (accession no. E-MTAB-10262).

Bioinformatic analysis of RNA-Seq

FastQ files were routinely processed then sanitized using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) (last accessed 2021-08-31) and TrimGalore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) (last accessed 2021-08-31), respectively. Individual reads were aligned to the mouse genome (mm-10) using Spliced Transcripts Alignment to a Reference (STAR) software (Dobin et al. 2013). Pairwise differential gene expression analyses were performed using HTSeq (Anders et al. 2018).
et al. 2015) to obtain read counts, and DESeq2 was used (Love et al. 2014) to quantify and display the differences. Only genes that exceeded an adjusted P-value ≤ 0.05 were included in the analysis. Gene ontology (GO) analyses were performed using the online Database for Annotation, Visualization, and Integrated Discovery (DAVID v6.8) (Huang da et al. 2009a,b). Neurog3<sup>GRP/</sup> and Neurog3<sup>GRP/GFP</sup> samples were separately analyzed as described (Osipovich et al. 2021).

**Bioinformatic analysis of ChIP-Seq**

ChIP-Seq datasets for INSM1 and NEUROD1 binding sites were obtained from ArrayExpress (E-GEOD-54046). Both datasets were obtained using an insulinoma cell line propagated from RIP1-Tag2 transgenic mice (Jia, et al. 2015). Snakemake (5.2.4), a workflow management system, was used to manage the data processing. FastQC (0.11.7) returned a quality score report on each sample’s sequences before and after Trimgalore (0.6.5) discarded sequences with a read length shorter than 20bp were discarded and trimmed read ends that do not meet the threshold of 20. Trimmed sequences were used by the RNA-Seq aligner Bowtie2 (2.3.5) to align against the mouse genome (gencode 17; GRCh38). On average, 95% of reads were mapped to the reference per sample. Samtools (1.9) converted the SAM files into BAM files and filtered out alignments with a lower MAPQ value than 10, and sort alignments by leftmost coordinates and by read name. Macs2 (2.2.6) was used to perform peak calling on the samples using default parameters for bandwidth and effective genome size. The resulting BED files were modified by bedtools (2.26.0) for pile-up visualization via IGV. Unmodified BED files were reported as very high-quality data by phantompeakqualtools (1.2.2).

**Differential splice variant analysis**

DSV analysis was performed using the previously described RNA-Seq datasets from control and knock-out (KO) embryos at E15.5. Snakemake (5.2.4), a workflow management system, was used to call five programs necessary to process the samples. FastQC (0.11.8) returned a quality score report on each sample’s sequences before and after Trimgalore (0.5.0) trimmed the sequences. Trimmed paired-end sequences were passed to STAR (2.6.0), an RNA-seq aligner, to align against the mouse genome (gencode 17; GRCh38), and gene counts for each sample were returned. The average uniquely mapped reads count per sample was 52M (89%). QoRTs (Quality of RNA-Seq toolset) converts the gene counts format for analysis in JunctionSeq. Lastly, Multiqc (1.7) compiled the final summary output from the other jobs into a single report. Gene counts for each sample were passed to R (3.5.3) to do a quality control assessment before analysis. Quality control assessment included analyzing a box plot of normalized sample counts, principal component analysis (PCA) plot, sample to sample heatmap clustering, and a density plot of the normalized counts. All samples passed the quality control assessment. Each sample’s QoRTS formatted gene counts were run through JunctionSeq (1.12.1) to determine any differential gene expression through alternative splicing between the KO and control groups.

**Sashimi plots and exon junction visualization**

Plots of read coverage across exons and corresponding sashimi plots were generated using the Integrative Genomics Viewer (IGV) platform (Robinson et al. 2011). Indexed BAM files from each KO dataset were uploaded directly to IGV for analysis, and mm10 was used as the reference genome. All tracks are set to the same read count scale for a given locus.

**Results**

An integrated analysis of Insm1, Neurod1, and Pax6, three Neurog3-dependent pro-endocrine TFs

Comparison of the gene expression profiles of purified Neurog3<sup>GRPCre</sup> and Neurog3<sup>GRPCre/GFP</sup> (Neurog3 KO) cells from E15.5 embryos by bulk RNA-Seq (Osipovich et al. 2021) revealed a marked reduction in the expression of pro-endocrine TFs Insm1, Neurod1, and Pax6 (Supplementary Figure S1A). The marked dysregulation of these genes provides further evidence that each lies downstream of Neurog3 in a Neurog3-driven endocrine cell-specific gene regulatory network (Supplementary Figure S1B) essential for the formation and function of pancreatic endocrine cells (Naya et al. 1997; St-Onge et al. 1997; Gierl et al. 2006; Mellitzer et al. 2006).

To systematically assess and compare the independent effects of Insm1, Neurod1, and Pax6 in developing endocrine cells, we intercrossed mice containing Insm1<sup>GRPCre</sup>, Neurod1<sup>LacZ</sup>, and Pax6<sup>flux</sup> alleles. From timed matings, we obtained embryos that were heterozygous for Insm1 (Insm1<sup>+/−</sup>) for use as controls, embryos that were globally deficient in Insm1 (Insm1 KO) or Neurod1 (Neurod1 KO), and embryos that lacked Pax6 specifically in Insm1-expressing cells (Pax6 KO) (Figure 1). Our strategy took advantage of the expression profile of Insm1, specifically that it is expressed in developing endocrine cells, that it is maintained in all endocrine cell types through adulthood, and that it is absent in exocrine pancreas. Green fluorescence produced by the Insm1<sup>GRPCre</sup> allele enabled us to purify endocrine cells by FACS from E15.5 embryos for RNA-Seq analysis and to accurately identify pancreatic endocrine cells by immunohistochemistry in tissue samples from E18.5 embryos (Figure 1). The findings were then comparatively analyzed.

Mice lacking Insm1, Neurod1, or Pax6 exhibit defects in endocrine cell differentiation, proliferation, and apoptosis

Immunofluorescence staining and morphometric analysis of pancreatic tissues from Insm1<sup>+/−</sup> (control) and Insm1 KO, Neurod1 KO, and Pax6 KO embryos at E18.5 was done by co-staining and quantifying the percentage of pancreatic hormone-positive cells, cell proliferation marker Ki67-positive, or apoptotic TUNEL-positive cells per total number of GFP-positive endocrine cells.

Similar to prior reports, embryos that lack Insm1 exhibit both a disruption in endocrine cell organization and a reduced number of endocrine cells (Gierl et al. 2006; Osipovich et al. 2014). Insm1 KO pancreata were again seen to have a lower number of insulin-, glucagon-, and somatostatin-expressing endocrine cells, and an increase in pancreatic polypeptide-expressing cells, indicating profound defects in endocrine differentiation and lineage specification (Figure 2, B, F, and I–L). Immunostaining with Ki67 revealed reduced proliferation of the Insm1 KO endocrine cells (3.6% vs. 15% of control cells) (Figure 3, B, F, I, and J), whereas the number of apoptotic cells between the control and KO cells was unchanged.

As in previous studies, Neurod1 KO embryos also exhibited a lower overall number of endocrine cells, disrupted islet organization, and increased percentage of pancreatic polypeptide-expressing endocrine cells increased (Figure 2, C, G, and I–L). The Neurod1 KO embryos had fewer Ki67-positive endocrine cells (2.8% vs. 15% in the controls) and a marked increase in TUNEL-positive cells (4.5% vs. 0.6% in control animals) (Figure 3, C, G, I, and J), which is also similar to prior reports (Naya et al. 1997; Romer et al. 2019).
Similarly, analysis of the Pax6 KO embryos revealed disorganized endocrine islets and a reduced overall number of endocrine cells. However, in these mice, there was a marked decrease in the number of glucagon-positive cells (Figure 2, D and J), although the numbers of other hormone-expressing GFP-positive cells were not significantly affected (Figure 2, H, K, and L). There was also a considerable reduction in the number of Ki67-positive cells in the Pax6 KO animals (4% vs. 15% in the controls) (Figure 3, D and J). Notably, we had difficulty obtaining Insm1GFPCre/+; Pax6KO embryos at E15.5, and only succeeded in obtaining two embryos of the sought-after genotype out of 62 embryos genotyped (4% vs. 15% in the controls) (Supplementary Figure S2, A and B). Among the top DRGs were numerous TFs known to control embryonic survival compared to the effects of a Pax6 KO alone (Figure 3K).

Together, these analyses indicate that mice lacking Insm1, Neurod1, or Pax6 have abnormal islet morphologies, a reduced number of endocrine cells, and varying defects in differentiation toward hormone-expressing cells. Moreover, all three KOs exhibited decreased endocrine cell proliferation rates, with both the Neurod1 and Pax6 KOs also showing increased apoptosis.

Pancreatic pre-endocrine cells lacking Insm1, Neurod1, and Pax6 have distinct transcriptional profiles

Next, to better understand how each factor contributes to the pre-endocrine cell transcriptome, we performed bulk RNA-Seq on FACS-purified cell populations isolated from E15.5 embryos. Cells were isolated at this earlier time point to minimize variance in gene expression caused by maturation toward specific endocrine subtypes. Hierarchical clustering and PCA of the 12 RNA-Seq samples revealed tight clustering by genotype, indicating the low variance between samples and that each condition analyzed has a unique transcriptional profile (Supplementary Figure S2, A and B).

Dysregulated expression of genes involved in hormone secretion, RNA metabolism and processing, and cell development in Insm1 KO endocrine cells

We previously reported the gene expression profile of both Insm1+/− and Insm1 KO mice at E15.5 using a legacy RNA amplification and sequencing method (Osipovich et al. 2014). To accurately compare the transcriptomes of the three TF KOs in this study, we collected new Insm1 KO datasets using the methods, reagents, and instrumentation described herein. A comparison of the two different Insm1+/− and Insm1 KO datasets indicated that they clustered first by amplification method (Nugen vs. Clontech) and secondarily by genotype (Insm1+/− control vs. Insm1 KO), consistent with a method-dependent batch effect. However, a linear regression analysis of the log2-fold changes of differentially expressed genes from the old and new datasets showed strong correlation (R² = 0.85) (Supplementary Figure S3, A–C). Analysis of the new Insm1+/− and Insm1 KO datasets by DESeq revealed a total of 4,694 differentially expressed genes, 2,265 were categorized as downregulated genes (DRGs) and 2429 upregulated genes (URGs) in the KO compared to control embryos (P-value ≤ 0.05) (Supplementary Figures S2C, S4, A and B and Table S1). Among the top DRGs were numerous TFs known to control endocrine cell development, including both Sox9 (McDonald et al. 2012) and Mnx1, which is necessary for endocrine cell fate allocation and in later stages for maintaining the β-cell fate (Sund et al. 2001; Wang et al. 2002; Pan et al. 2015). Also, among the DRGs were the TF Bhlhe23, the histone modifiers Hist1h2bf and Hist2kb, and genes involved in the trafficking and secretion of insulin (Aup, Chgb) (Supplementary Figure S4C). GO and pathway enrichment analysis results from DRGs revealed enrichment in genes involved in cell projection morphogenesis (Bdnf, Brsk2, Camk2b, Brsk1, and Cdc42) and hormone secretion.
Etv4), cellular response to hormone stimulus (Adra2a, Gcgr, Insr, Kcn2), hormone secretion (StxbKp5l, Stx1a, Cacna1d), and mRNA metabolic process (Srsf2, Hnrnpc, Celf4, Celf6) (Supplementary Figure S4, D and F and Table S2).

Among the top URGs, there were many genes necessary for the key functions of mature islet cells. Examples include Kcn4 (Kv3.4), a voltage-gated potassium channel expressed in β- and δ-cells, and Gpr17, an orphan G protein-coupled receptor known to inhibit neural cell maturation (Gopel et al. 2000; Jacobson and Philipson 2007; Chen et al. 2009). GO and pathway enrichment analyses identified an enrichment in genes involved in the regulation of cellular component organization (Adck1, Arap1), Notch and Wnt signaling pathways (Dll1, Notch1/2, Wnt7b, Frzb, Frz8), and the negative regulation of cellular development (Aatk, Dlx1, Rest, Kctd11) (Supplementary Figure S4, E and F and Table S2). These results indicate that Insm1 stimulates many genes involved in hormone secretion and the function of mature endocrine cells, while repressing genes associated with Notch and Wnt signaling.

**Dysregulated expression of genes involved in chromatin organization, cell proliferation, and mitochondrial function in Neurod1 KO endocrine cells**

Comparing the Neurod1 KO and control datasets revealed a different set of 4613 dysregulated genes (2131 DRGs and 2482 URGs) (P-value ≤ 0.05) (Supplementary Figures S2D, S5, A and B and Table S1). The top DRGs included many factors known to impact islet...
cell function, including Prlr, G6pc2, Hspa1a, and Hspa1b (Supplementary Figure S5C). Transcriptional co-regulators (Runx1t1, Myt1l), cell cycle regulation (Cdkn1a, Cdc14b), and chromatin organization (Ctf1, Kat2b) were enriched in DRGs in Neurod1 KO (Supplementary Figure S5D and Table S2).

The top URGs in this dataset included the NF-κB signaling regulators (Tifab, Ccdc3), extracellular matrix factors (Emilin2, Col19a1), and the TRAIL receptor Tnfsrf26 (Supplementary Figure S5C). URGs were enriched in such functional categories as cellular respiration (Coq10a, Uqcc3), mitochondrial organization (Mfn2, Meff1, Meff2, Plekkf1), and autophagy (Atg7, Atg13, Tbc1d17, Vps11) (Supplementary Figure S5D and Table S2). Moreover, genes involved in regulation of cell death were also upregulated (P2rx1, P2rx7, C6). Identification of these enriched GO terms and pathways for the Neurod1 KO mice parallels the changes we and others have observed in islet morphology, including reduced endocrine cell proliferation and increased cell death (Naya et al. 1997; Romer et al. 2019). These findings indicate that the loss of Neurod1 causes a transcriptional response marked by the upregulation of genes involved in NF-κB signaling and energy metabolism, and the downregulation of other pancreatic TFs and genes associated with cell cycle regulation.

Dysregulated expression of genes involved in cell cycle regulation, developmental growth, and apoptosis in Pax6 KO endocrine cells

Comparison of the Pax6 KO embryos and controls revealed a third set of 5770 genes (2756 DRGs and 3014 URGs) that were Pax6 dependent (P-value < 0.05) (Supplementary Figures S2E, S6, A and B and Table S1). Among the top URGs were Prlr, G6pc2, Gcg, Ptgdr,  

![Figure 3](image-url)
and Cdc3 (Supplementary Figure S6C). Inspection of the enriched GO terms and pathways for Pax6-dependent DRGs and URGs suggests a critical role for Pax6 in stimulating cell growth and cell cycle, and regulating endoplasmic reticulum (ER) stress and apoptosis in developing endocrine cells (Supplementary Figures S6, D and E and Table S2). Specifically, many genes involved in chromatin organization (AshH1, p300, Mettl4), cell cycle regulation (Cond1, Cdc27, Cdkn1b, Chek1), and developmental cell growth (Fgf9, Ifg1r, Tgfβ2) were downregulated in Pax6 KO animals (Supplementary Figure S6, D and F). Consistent with the diminished number of α-cells observed at E18.5, there was a marked reduction in Gcg expression, as well as dysregulation of other α-cell-specific genes Bnd4 and Pck2. GO terms and pathways enriched in URGs include cellular component organization (Elmo1/2, Lim1a, Myo7a), response to ER stress (Atf6b, TrAf2), and the apoptotic signaling pathway (Casp9, Casp3, Madd) (Supplementary Figure S6, E and F and TableS2). Analysis of dysregulated genes in Pax6 KO animals shows the downregulation of other TFs, cell cycle regulators and genes important for cellular growth, and the upregulation of apoptotic signaling and genes involved in the response to ER stress.

Comparison of the gene sets dysregulated in the Ins1m1, Neurod1, and Pax6 KO endocrine cells

To further assess the similarities and differences in the genes affected by each gene KO, we started by comparing the gene lists. We found that of the 8729 total dysregulated genes (based on adj. F-value ≤ 0.05) across all three KOs, 4719 (54%) were affected by more than one TF (Figures 4A and 5A). Additionally, 578 (6.6%) of genes were inversely regulated across TFs. For example, Mnx1 is upregulated in Pax6- and Neurod1 KO datasets and downregulated in Ins1m1 KO samples, conversely Rest is downregulated in Pax6- and Neurod1 KO samples and upregulated in Ins1m1 KO data. Similarly, Fev is upregulated in the absence of Pax6, unchanged in Neurod1 KO and downregulated in Ins1m1 KO datasets (Supplementary Table S1). This finding indicates that while Ins1m1, Neurod1, and Pax6 commonly regulate a sizeable fraction of genes, each TF has unique effects on the transcriptome. To quantify these findings and reflect the similarity of the different groups, we determined the percentage of shared genes relative to the total number of dysregulated genes. This analysis revealed a range of 2.6–22.6% similarity between different groups, with the highest similarity between Neurod1 and Pax6 DRGs (22.6%).

Next, to identify commonalities in the functional annotations of genes dysregulated in each gene set, we performed Metascape analyses for both the DRGs and URGs. These analyses revealed many genes and functional GO terms and pathways shared between dysregulated genes from each gene set (Figures 4B and 5B). Of the 620 commonly regulated DRGs (14.07% shared), the enriched GO term and signaling pathway network includes such categories as regulation of mRNA metabolic processes and translation (Akap17b, Cik1, Srf3, Srf6), cell cycle regulation (Bbx8, Cdk6, Cdc14b), and chromatin organization (Kmt2a, Setd5, Kdm7a) (Figure 4C and Supplementary Table S2). Other common DRGs included regulators of insulin secretion and signaling (Ins1, Nnat, Unc3, Insr), transcriptional regulators (St18, Neurod1, Rfx3, Myt11, Runx1,11, Oncut2), kinases (Akt3, Mapk8, Taok1, Frrcd), and anti-apoptotic genes (Xiap, Bcl-W) (Supplementary Table S1). While it is not surprising that these TFs regulate other TFs that are known to be involved in pancreas development and function, it is important to note their role in affecting the expression of cell cycle regulators and chromatin organization. Tight regulation of these processes has long been linked to cellular development, expansion, and differentiation, and their dysregulation likely contributes to the observed developmental delays and perturbed ratios of islet cell types (Dalton 2015; Kim et al. 2015; Boward et al. 2016; Soufi and Dalton 2016; Krentz et al. 2017).

The shared functional network for 863 URGs (17.7% shared) in all three KOs contains GO terms and pathways for ncRNA processing (Aars, Clu1, Lsm6, Tsen2), apoptotic signaling pathway (Casp9, Tradd, Tnfsf21, Ddx47), protein localization (Ipo4, Ipo9, Tmm29), and response to oxidative stress (Pink1, Selonen, Fancc) (Figure 5C and Supplementary Table S2). Genes involved in Wnt-signaling pathways (Fzb, Lts2) and other TFs (Sox10, Tbx2, Foxa2) were also upregulated (Supplementary Table S1). The upregulation of genes associated with apoptosis and oxidative stress response, in combination with the downregulation of anti-apoptotic genes, coincides with the increases in apoptosis in Neurod1 and Pax6 KO pancreata. Likewise, increases in the expression of members of the Wnt signaling pathway and early endocrine TFs are reminiscent of morphological developmental delays (Sharon et al. 2019). This is particularly true of those observed in Ins1m1 KO embryos which are able to generate endocrine cells, but many of those are unable to properly differentiate and mature into hormone expressing endocrine cells (Mellitzer et al. 2006; Osipovich et al. 2014).

Metascape analysis also identified protein–protein interaction (PPI) enrichments common to all three gene sets. Enriched subnetworks of DRGs known to form PPIs included members of the ATF2- (Atf2, Pou2f1, Nfj1, Brcal), cohesin- (Smc1a, Smc3, Atrx, Mepcz), and histone acetylation-complexes (Katu2b, Crebbp, Clock). (Supplementary Figure S7, A, C, and E). The PPI subnetworks of URGs involved in NF-κB signaling (Ikbbk, Ikbbg, Rnf31, Rbbk1) and histone modification (Tubl1a, Hda6c, Kat5b) (Supplementary Figure S8, A and C). Interestingly, NF-κB signaling has been recently shown to regulate β-cell proliferation and apoptosis, and in turn β-cell mass, during development (Sever et al. 2021). Together, these analyses indicate that while there are differences in the genes dysregulated by Ins1m1, Neurod1, and Pax6, there are many shared functionalities, both from a GO and PPI subnetwork perspective.

Prediction of direct targets of Ins1m1 and Neurod1 critical for endocrine cell development

To predict direct targets of Ins1m1 and Neurod1 in developing endocrine cells, we utilized previously reported ChIP-Seq datasets (Jia et al. 2015). Consistent with published data, our analysis showed that Ins1m1 and Neurod1 share a large proportion of binding sites (11,409 sites across the genome, 81.5% of total binding sites) (Supplementary Figure S9A). Integration of ChIP-Seq data with our RNA-Seq data on dysregulated genes suggests that 4180 and 3789 genes, respectively, may be direct targets of Ins1m1 and Neurod1 in endocrine progenitor cells (Supplementary Figure S9, B and C and Table S3). We further compared the lists of direct target genes and found 1,719 (27.5% shared) genes bound and dysregulated by both Ins1m1 and Neurod1. These included genes involved in peptide hormone response and secretion (Gpc2, Gilp1r, Syt4, Stxbp5), zinc finger proteins (Zfp326, Zkbb3, Zbd2f), rhythmic processes (Clock, Dbx9, Kdm2), and mRNA processing (Celf4, Tra2a, Akap17b, Zfp871, Luc7l2, Ddx17) (Supplementary Figure S9D). These findings indicate that of the genes dysregulated in our Ins1m1 and Neurod1 KO endocrine progenitor cells, 89% and 82% are predicted to be direct targets, respectively, based on the presence of binding sites within 5 kb of the transcriptional start site.
Insm1, Neurod1, and Pax6 KO endocrine cells exhibit dysregulated RNA splicing

Among the enriched GO terms and signaling pathways of dysregulated genes found in all three of the KO gene sets was the regulation of RNA metabolic processes, which includes the regulation of alternative splicing. To determine whether mRNA splicing was affected in the Insm1, Neurod1, and Pax6 KO cells, we first examined the expression of several RBPs known to regulate mRNA splicing. We found several RBPs with similarly dysregulated expression patterns, including Elavl3, Elavl4, Tra2A, and Tra2B (Figure 6A). The expression of other differential RNA splicing factors such as serine/arginine-rich proteins that generally act as activators of splicing (Srsf1, Srsf3), heterogeneous nuclear ribonucleoproteins (hnRNPs) with repressive functions (Hnurnpα1, Hnurnpβ, Hnurnpγ), and members of the spliceosome itself (Sf91, Sf92, Sf93, Sf94) are also dysregulated. Importantly, ChIP-Seq data indicate that Insm1 and Neurod1 bind at the promoter regions of many of the same RBPs whose expression levels are dysregulated in our KO data. For example, Elavl3/4, Nova1, Tra2A/B, and Srsf1, in addition to being downregulated, are all bound by both Insm1 and Neurod1 at their promoter regions (Supplementary Figure S10). To determine if the dysregulation of RBP expression in the absence of Insm1, Neurod1, or Pax6 was correlated with differential splicing events, we used our RNA-Seq datasets to perform a computational alternative splicing analysis using JunctionSeq. The results indicated hundreds of differential splicing events in Insm1 KO (356), Neurod1 KO (212), and Pax6 KO (885) datasets (adj. P-value ≤ 0.01, log₂ FC > |1|) (Figure 6B and Supplementary Table S4). Of the 1,262 total differentially spliced genes, 172 were shared between pairs of TFs, with Pax6 itself being alternatively spliced in Insm1 and Pax6 KO datasets (Supplementary Table S4). Additionally, Tcf7l2, which has strong polymorphism associations with T2D and is known to have isoform-specific effects on β-cell function, is also differentially spliced in the Pax6 KO samples (Prokunina-Olsson, 2009; Osmark, 2009; Le Bacquer, 2011; Zhang, 2021). However, only 19 (1.5%) differential splicing events were common to all three KOs (Ncoa4, Syt14, Tnfrsf12a, Muc10) (Supplementary Table S4). Since some genes have several possible transcriptional isoforms, interpreting alternative splicing data is challenging. Therefore, we chose to take a closer look at a few genes that were differentially spliced in our KO datasets, and which have only a few RNA splicing variants.

Syt14 is a Ca²⁺-independent synaptotagmin required for the exocytosis and trafficking of secretory vesicles. Interestingly, differential splicing patterns were revealed for Syt14 across all three
KO datasets, specifically a decrease in expression of the Ensemble splice variant 205. To quantitatively visualize these differences, we generated splice junction plots (Sashimi plots), that tally the exon junction read counts. Sashimi plots of Syt14 reveal a marked decrease of junction reads at exon 4 of the Sty14-205 splice variant (Figure 7A). To determine if dysregulated RBPs show binding preferences near the differentially spliced loci might cause these splicing differences, we used the online oRNAment database to identify local binding motifs (Benoit Bouvette et al. 2020) and found that TRA2A, NOVA1, and ELAVL4 all show binding patterns at exon 4, as well as adjacent exons 3 and 5 (Figure 8A). Importantly, expression of Tra2A and Elavl4 are downregulated in all three KO gene sets, and Nova1 downregulated in Pax6 KO suggesting their potential involvement in differential splicing of Syt14 in developing endocrine cells (Figure 6A).

Similarly, Snap 25 is a critical SNARE protein involved in regulating exocytosis of synaptic vesicles in the brain and hormones in pancreatic islet cells (Kadkova et al. 2019; Liang et al. 2020). Two isoforms, Snap25a and Snap25b, have been identified that differ based on the alternative usage of two different exon 5 sequences (5a and 5b), with corresponding proteins differing in only 9 amino acids (Kadkova et al. 2019). We observe that the Snap25b isoform is increased in the Pax6 KO (P-value < 0.001) and decreased in Insm1 KO (P-value < 0.01) datasets, as indicated in the associated Sashimi plots (Figure 7B). RBP binding site analysis of this locus indicated TRA2A and NOVA1 binding sites at both 5a and 5b exons, and ELAVL4 sites at nearby upstream exons (Figure 8B). Together, these findings indicate that Insm1, Neurod1, and Pax6 are required for the expression of RBPs that bind in the vicinity of exons whose proper splicing may be essential for the function of pancreatic endocrine cells.

**Discussion**

**Insm1, Neurod1, and Pax6 both independently and coordinately govern pre-endocrine cell gene expression**

Insm1, Neurod1, and Pax6 are individually essential for the formation of functional pancreatic endocrine cells (Naya et al. 1997; St-Onge et al. 1997; Gierl et al. 2006; Gu et al. 2010; Osipovich et al. 2014; Jia et al. 2015; Mitchell et al. 2017). Here, we systematically compared the effects of deleting Insm1, Neurod1, and Pax6 in an Insm1GFPCre heterozygous background based on the morphology, gene expression, and differential RNA splicing of nascent pancreatic endocrine cells. By doing so, we obtained new insights into the vital roles that each of these factors has in the development of pancreatic endocrine cells.

**Role of Insm1**

Consistent with previously published results we showed that Insm1 KOs have defects in endocrine cell differentiation that were manifested in decreased numbers of Ins- and Gcg-positive
and increased number of Ppy-positive endocrine cells. This is accompanied by decreased endocrine cell proliferation, as well as changes in expression of genes that affect these processes (Gierl et al. 2006; Osipovich et al. 2014). Our comparative transcriptional analysis revealed both similarities and differences in the genes regulated by Insm1 and Neurod1 and Pax6. For example, Insm1, but not Neurod1 or Pax6, activates the expression of Fev and Mnx1 in endocrine cells, TFs that are known to influence the numbers and types of endocrine cells, as well as islet morphology. Insm1 is specifically important for expression of many genes involved in inducible secretory function of endocrine cells, including protein secretion and Ca²⁺ regulated vesicle exocytosis. This is consistent with the role of Insm1 in the proliferative and secretory functions of mature endocrine cells (Tao et al., 2018).

Our new data, together with re-analysis of previously reported ChIP-Seq data, suggests that 89% of genes dysregulated in endocrine progenitor cells of Insm1 KO mice contain binding sites for Insm1 with 5 kb of the transcriptional start site.

**Role of Neurod1**

Mice that lack Neurod1 exhibit an increased number of Ppy-expressing endocrine cells without significantly altering the proportion of other differentiated endocrine cell types. However, Neurod1 KO mice exhibit both a profound decrease in proliferation and an increase in apoptosis of endocrine cells, thereby causing a marked reduction in overall number of endocrine cells. These findings are consistent with previous studies, supporting the hypothesis that a lack of proliferative cells and increase in apoptosis are primary causes for the reduced total number of endocrine cells observed (Naya et al. 1997; Romer et al. 2019). Likewise, Romer et al. (2019) recently reported a decrease in α- and β-cell numbers only after the time ~P0 stage. Their quantifications at
Figure 7 Identification of differential splicing events in Syt14 and Snap25 genes. The gene structures of (A) Syt14 and (B) Snap25 are illustrated above corresponding Sashimi plots of differentially spliced regions. Sashimi plots show the representative splice junctions as arcs that connect the exons in each of the datasets. The numbers found on each of the corresponding arcs indicate the junction depth, or reads spanning the exon junctions. Dashed boxes highlight the regions of alternative splicing, which are again depicted below the Sashimi plot to provide a visual reference for read alignments. Sashimi plots were generated using the Integrated Genomic Viewer (IGV).

E17 show no differences in the cell numbers or ratios of Ins- or Gcg-expressing cells, indicating that the specific reduction in α- and β-cells occurs during late embryonic or early postnatal stages after islet differentiation has largely concluded, which is consistent with our findings.

Our transcriptional profiling results also suggest that Neurod1 promotes proliferation and maturation through the stimulation of genes important for cell cycle regulation and chromatin modifications, processes that are important for cell differentiation and function (Pauklin and Vallier 2013; Chen and Dent 2014; Dowen et al. 2014; Krentz et al. 2017). Simultaneously, the observed upregulation of apoptotic and autophagy genes likely contributes to an increase in cell death, further decreasing the total number of endocrine cells.

The regulation of endocrine specific TFs (Fov, Mnx1) and other genes important for cellular function further support a role for Neurod1 in mature cells as is suggested both by the expression of Neurod1 in adult β- and α-cells and that the conditional ablation of Neurod1 in β-cells impairs their maturation (Naya et al. 1997; Gu et al. 2010; Mastracci et al. 2013). Additionally, ChiP-Seq data indicates that Neurod1 binds within 5 kb of 82% of the genes dysregulated in this analysis, strongly suggesting that it is the primary effector of the observed changes. Together these findings indicate the critical role of Neurod1 in propagating enough fully functional islet cells during development via the promotion of cellular proliferation and activation of key TFs.

Role of Pax6

It is well established that Pax6 is necessary for the specification of pancreatic α-cells, so the marked reduction of Gcg-positive cells (3.3% vs. 15% in controls) in the Pax6 KO animals is not a surprise (Gosmain et al. 2010). However, our data have revealed that endocrine cells lacking Pax6 not only have a decrease in the expression of Gcg, but also a marked dysregulation of other genes important for α-cell development and function, such as Pou3f4 (or Bmn4), Pcsk2, Slc30a8, Irx2, and Smarca1. Besides its function in α-cells, Pax6 is important for development and function of adult β-cells, which is supported by our findings of decreased expression of Ins1, Ins2, and Insr in Pax6 KOs (Gosmain et al. 2012; Mitchell et al. 2017; Swisa et al. 2017). While the morphological phenotypes of the islets we observed are in line with previously published results, due to the design of our study we cannot exclude a combinatorial effect caused by the haplosufficiency of Insm1 in mice containing the Insm1<sup>GFP<sup>Cre</sup></sup> allele (Sander et al. 1997; St-Onge et al. 1997; Gosmain et al. 2010). Indeed, and as discussed below, the difficulty we had in obtaining Pax6 KO animals at E18.5, which contained only one Insm1<sup>GFP<sup>Cre</sup></sup> allele, could be due defects in the developing central nervous system where both Pax6 and Insm1 are expressed (Farkas et al. 2008; Lan and Breslin 2009; Osipovich et al. 2014). Furthermore, it was previously shown that Pax6 RNA is alternatively spliced, and that β- and α-cells preferentially express different splicing variants (Singer et al. 2019). Thus, our finding that Insm1 affects the processing of Pax6 mRNA also affects the interpretation of our results. Despite this, the downregulation of genes involved in chromatin organization, cell cycle regulation, and cell growth in the Pax6 KOs, similar to Neurod1 KOs, likely contributes to the reduction in endocrine cell expansion, while an increase in apoptotic genes correlates to the increase in cell death.

Regulatory overlap of Insm1, Neurod1, and Pax6

Recently, we have shown that Insm1 and Neurod1 exhibit a high degree of co-expression during endocrine differentiation, and that Pax6 is similar except that it continues to be expressed in adult β-cells (Osipovich et al. 2021). These co-expression relationships are consistent with Insm1, Neurod1, and Pax6 being important components of the GRN in nascent pancreatic endocrine cells. The genes regulated by these three factors also share many functional assignments, such as mRNA processing and alternative splicing and cell cycle regulation. These similarities likely underpin the broadly similar morphological phenotypes when each is knocked-out, which includes a reduced overall number of pro-
endocrine cells, reduced endocrine cell proliferation and increased apoptosis, as well as defects in differentiation toward different kinds of hormone-expressing cells.

Our findings strongly point to a negative epistasis between Insm1 and Pax6, as already noted, due to the fewer than expected numbers of Insm1GFPCre/+; Pax6fl/fl animals at E18.5. Consistent with this, a recent report showed that mice with only a single functional Insm1 allele (Insm1+/−) have impaired β-cell proliferation that results in impaired glucose tolerance in adult animals (Tao et al. 2018). However, the authors observed no impairments in β-cell mass at birth and argued that Insm1 haploinsufficiency has little to no effect on embryonic endocrine development (Tao et al. 2018). Previous studies of global Pax6 KO mice have all observed a marked decrease in hormone-positive endocrine cells, but none quantified apoptosis events (Sander et al. 1997; St-Onge et al. 1997; Ashery-Padan et al. 2004; Heller et al. 2004). Moreover, Ashery-Padan et al. (2004), who utilized a Cre-Lox-based lineage tracing strategy to quantify the proportion of Pax6 expressing cells relative to total pancreatic tissue at E18.5, reported the loss of hormone expression but no difference in total cell number. While apoptosis was not specifically quantified, their study suggests that pro-endocrine cells form and expand but then fail only to express islet hormones (Ashery-Padan et al. 2004). If so, then our observations are consistent with a detrimental synergistic effect, or negative epistasis, between Insm1 and Pax6 that impairs embryo survival.

In addition to the greater lethality of Pax6 KO embryos, our data show an increase in both apoptotic events at E18.5, an increase in expression of pro-apoptotic genes and a decrease in expression of anti-apoptotic genes at E15.5. Previous studies of global Pax6 KO mice have all observed a marked decrease in hormone-positive endocrine cells, but none quantified apoptosis events (Sander et al. 1997; St-Onge et al. 1997; Ashery-Padan et al. 2004; Heller et al. 2004). Moreover, Ashery-Padan et al. (2004), who utilized a Cre-Lox-based lineage tracing strategy to quantify the proportion of Pax6 expressing cells relative to total pancreatic tissue at E18.5, reported the loss of hormone expression but no difference in total cell number. While apoptosis was not specifically quantified, their study suggests that pro-endocrine cells form and expand but then fail only to express islet hormones (Ashery-Padan et al. 2004). If so, then our observations are consistent with

Figure 8 Differentially spliced regions of Syt14 and Snap25 genes are bound by dysregulated RBPs. (A) Schematic of Syt14 gene structure and corresponding binding sites of the RBPs ELAVL4, NOVA1, and TRA2A near the differentially spliced regions. (B) Schematic of Snap25 and corresponding binding sites of ELAVL4, NOVA1, and TRA2A at the differentially spliced site. Colored dashes above the indicated gene region represent the putative binding site of the given RBP. Binding sites were identified via the online oRNAment database.
Insm1 and Pax6 having redundant functions, perhaps in the suppression pro-apoptotic genes. In the future, alternate strategies that do not rely on the Insm1GFP/Cry2 allele and that maintain full expression of Insm1 will need to be utilized to explore how these two genes interact with each other.

**Dysregulation of RBP gene expression and identification of alternative splicing events in Insm1, Neurod1, and Pax6 KO animals**

Alternative RNA splicing enables a single gene to give rise to multiple mRNA products that encode different protein products. The function of these different protein products can vary greatly and impact cellular function (Alvelos et al. 2018). Recent studies have suggested the importance of the unique splicing program innate to pro-endocrine cells, and how the functional outputs help steer the developmental process, yet it remains incompletely understood (Juan-Mateu et al. 2018; Singer et al. 2019; Colli et al. 2020; Alvelos et al. 2021).

Our analyses indicate Insm1, Neurod1, and Pax6 each regulate the expression of RBPs that mediate different RNA splicing events, including expression of Elavl4, Tra2a, Msi1 and Msi2, and Nova1. Consistent with this, we identified multiple global differential slicing events within KO datasets and, specifically, in the Syt14 and Snap25 genes. Synaptotagmin 14 (Syt14) is a member of the SYT family that is necessary for exocytosis of secretory vesicles, and mutations in this gene have been associated with neurodegenerative disorders in humans (Doi et al. 2011). Our data revealed that Syt14 is differentially spliced in all three of the KO mice studied. Similarly, Synaptosome Associated Protein 25, or Snap25, is a member of the SNARE complex. It is necessary for insulin secretion, is known to be downregulated in T2D islet cells, and is differentially spliced in both Insm1 and Pax6 KO endocrine cells (Sadoul et al. 1995; Gonelle-Gispert et al. 1999; Ostenson et al. 2006, Daraio et al. 2017). It is well established that Snap25 has two primary mRNA isoforms that have slightly different localization patterns and functional traits (Nagy et al. 2005; Daraio et al. 2017; Irfan et al. 2019). Considering this information, it is likely that the differential splicing of these both Syt14 and Snap25 contributes to the impaired function of KO islets. Moreover, it also highlights the importance of alternative splicing in endocrine cell development and indicate that Insm1, Neurod1, and Pax6, besides having large effects on the transcriptome, also control differential splicing events through their regulation of RBPs and thereby affect endocrine cell function in this manner. In the future, it would be beneficial to determine the effects of disrupting the TF binding sites at the promoter regions of RBPs on gene expression levels. Likewise, more work needs to be done to determine the role of specific binding activity of RBPs at their target sites within alternatively spliced genes and if those RBPs maintain endocrine-specific functions.

In conclusion, the present study provides additional insights into the roles for Insm1, Neurod1, and Pax6 in driving endocrine development and differentiation. While each of these TFs has its own unique regulatory functions, there is also functional overlap between these factors based on the large number of commonly dysregulated genes represented in our data. Importantly, we show that the differential expression of RBPs correlates with differential splicing events that can either be shared or common to each gene KO, demonstrating a novel role shared by each factor. Finally, our data demonstrate the broad impact of eliminating each TF, further illustrating their individual roles and importance in establishing the GRN within pancreatic endocrine cells.

**Data availability**

RNA sequencing data are available at ArrayExpress with the accession number E-MTAB-10262. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables provided. Supplementary material is available at G3 online.

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**Conflicts of interest**

The authors declare that there is no conflict of interest.

**Author contribution**

K.D.D. and A.B.O. performed experiments and analyzed data. J.P.C. performed RNA-Seq data processing, alignment, and differential expression analyses. K.D.D., A.B.O., and M.A.M. wrote and edited the manuscript. M.A.M. supervised the experiments.

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