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FUCCI tracking shows cell-cycle-dependent *Neurog3* variation in pancreatic progenitors

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Abstract

During pancreas organogenesis, *Neurog3*^{HI} endocrine-committing cells are generated from a population of Sox9⁺ mitotic progenitors with only a low level of *Neurog3* transcriptional activity (*Neurog3*^{TA,LO}). Low-level *Neurog3* protein, in *Neurog3*^{TA,LO} cells, is required to maintain their mitotic endocrine-lineage-primed status. Herein, we describe a *Neurog3*-driven FUCCI cell-cycle reporter (*Neurog3*^{P2A.FUCCI}) derived from a *Neurog3* BAC transgenic reporter that functions as a loxed cassette acceptor (LCA). In cycling Sox9⁺ *Neurog3*^{TA,LO} progenitors, the majority of cells in S-G₂-M phases have undetectable levels of *Neurog3* with increased expression of endocrine progenitor markers, while those in G₁ have low *Neurog3* levels with increased expression of endocrine differentiation markers. These findings support a model in which variations in *Neurog3* protein levels are coordinated with cell-cycle phase progression in *Neurog3*^{TA,LO} progenitors with entrance into G₁ triggering a concerted effort, beyond increasing *Neurog3* levels, to maintain an endocrine-lineage-primed state by initiating expression of the downstream endocrine differentiation program prior to endocrine-commitment.

Keywords

endocrine-biased; progenitor; lineage priming

Introduction

Neurogenin3 (*Neurog3*) encodes a bHLH transcription factor essential for endocrine-lineage specification during mouse pancreas organogenesis (Gradwohl et al., 2000). *Neurog3* is also critical to human pancreatic endocrine-cell development, with null mutations causing

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Competing Interests

The authors declare that no competing interests exist.

neonatal diabetes, and blocking β -cell differentiation from hESC (McGrath et al., 2015). During mouse pancreatic development, high-level *Neurog3* expression (*Neurog3^{HI}*) in Sox9⁺ pancreatic epithelial cells causes cell-cycle exit, endocrine commitment and epithelial delamination (Miyatsuka et al., 2011; Wang et al., 2010; Johansson et al., 2007; Bechard et al., 2016). We recently demonstrated, however, that low Neurog3 levels are necessary to allow the maintenance of a population of Sox9⁺ *Neurog3*-transcriptionally-active pancreatic epithelial cells in a mitotic endocrine-biased progenitor state (defined as *Neurog3^{TA,LO}*), which pre-empt the transition to an endocrine-committed *Neurog3^{HI}* state (Bankaitis et al., 2015; Bechard et al., 2016). Our findings presented a significant parallel to how a low level of Neurog2 promotes a neural-progenitor state while high levels cause neural differentiation and cell-cycle exit (Roybon et al., 2009; Shimojo et al., 2011; Ali et al., 2011; Florio et al., 2012). In those studies, higher Cdk activity in rapidly cycling progenitors, which have a relatively short G₁, keeps Neurog2 in a (hyper)-phosphorylated, unstable state that activates neural-progenitor target genes (Hindley et al., 2012; Ali et al., 2011). When the cell cycle of neural progenitors lengthens, however, and G₁ lengthens, Cdk activity decreases, resulting in accumulation of a more stable (hypo)-phosphorylated Neurog2 that preferentially activates neural-differentiation targets (Hindley et al., 2012; Ali et al., 2011). Recently, we demonstrated that keeping Neurog3 levels low leads to an increased mitotic index of *Neurog3^{TA,LO}* progenitors and expands their numbers within the pancreatic epithelium (Bechard et al., 2016). Moreover, time-lapse observations show that the transition from the low level of Neurog3 observed in mitotic *Neurog3^{TA,LO}* progenitors to the high level necessary for endocrine-commitment occurs ~3–6 hours after division of the parental *Neurog3^{TA,LO}* cell, during what is likely the early part of an extended G₁ or long-term cell-cycle exit (Bechard et al., 2016). These findings led to our proposal that the level and stability of Neurog3 in mitotic Sox9⁺ *Neurog3^{TA,LO}* progenitors is regulated by the cell cycle and that G₁ extension promotes Neurog3 stabilization, accumulation, and endocrine commitment (Bechard and Wright, 2017). Two recent reports support this model, demonstrating that Neurog3 is targeted and destabilized by Cdks (Azzarelli et al., 2017) and that G₁ lengthening, by reducing Cdk activity, causes the accumulation of a more stable un(der)phosphorylated form of Neurog3 (Krentz et al., 2017).

We have been independently investigating if Neurog3 protein stability and progenitor maintenance vs. endocrine differentiation decisions are connected to cell-cycle progression in *Neurog3^{TA,LO}* progenitors. To do so, we used recombinase-mediated cassette exchange (RMCE) to replace our previously described *Neurog3^{RG}* BAC transgenic reporter – which was designed as a Loxed Cassette Acceptor (LCA) – with a *Neurog3*-driven single-transgene insert of the FUCCI (Fluorescence Ubiquitin Cell Cycle Indicator) reporter (*Neurog3^{P2A.FUCCI}*). Our analysis of *Neurog3^{P2A.FUCCI}* reporter activity showed that in cycling Sox9⁺ *Neurog3^{TA,LO}* progenitors, Neurog3 protein levels are highest during G₁ and lowest during S-G₂-M. Moreover, Sox9⁺ *Neurog3^{TA,LO}* progenitors in early G₁ show increased expression of downstream Neurog3 targets usually associated with the forward passage into an endocrine commitment and progression program. We propose that these findings support a model in which the endocrine-differentiation program is already accessed, or preformed (albeit at a low or incomplete level), in mitotic *Neurog3^{TA,LO}* progenitors prior to moving into endocrine-commitment. This work provides a new tool for investigating,

under *in vivo* conditions, Neurog3 and cell-cycle connections in lineage-primed progenitors, and new insight on the role of Neurog3 in regulating progenitor maintenance vs endocrine-commitment decisions.

Results and Discussion

Generating a *Neurog3*-driven P2A-fused single transgene FUCCI reporter

The FUCCI reporter relies on cell-cycle-phase-dependent destruction of fluorescent proteins fused to “degradation boxes” from hGeminin and hCdt, specifically the regions hGem^(1/110) and hCdt1^(30/120) (Sakaue-Sawano et al., 2008), allowing cell-cycle phase determination (Figure 1A). To investigate connections between Neurog3 protein levels and cell-cycle progression in *Neurog3*^{TA.LO} progenitors we generated a single mKO2-hCdt1^(30/120)-P2A-mVenus-hGem^(1/110) FUCCI (P2A.FUCCI) cassette, enabling both FUCCI components to be expressed under the control of *Neurog3* (Figure 1B). We selected the pairing of mKO2/mVenus because their fluorophores are spectrally separable from GFP and mCherry, allowing P2A.FUCCI visualization in cells carrying our previously described *Neurog3*-driven H2B^{mCherry}-P2A-GFP^{GPI} (*Neurog3*^{RG1} reporter) (Bechard et al., 2016). As seen with the original FUCCI reporter (Sakaue-Sawano et al., 2008), *CMV*-driven expression of P2A.FUCCI in HeLa cells resulted in mKO2-hCdt1^(30/120) positivity during G₁ and mVenus-hGem^(1/110) positivity during S-G₂-M, with a brief overlap of the two fusion proteins during the G₁/S phase transition (Figure 1B).

To facilitate generating additional *Neurog3* BAC transgenic reporters from the *Neurog3*^{RG} reporter, we had flanked the *Neurog3*^{RG} cassette with tandem *lox71* and *lox2272* sites, making a Loxed Cassette Acceptor (LCA) allele (see Bechard et al., 2016; Figure 2A). This design was to allow *Neurog3*^{RG} to be replaced with any lox66/lox2272-flanked cassette via RMCE in mESCs. To avoid potential issues with performing RMCE in cells carrying multiple LCA alleles, mESCs identified as having stably integrated the *Neurog3*^{RG} BAC LCA transgene were screened for single-copy insertion by a qPCR-based assay (see methods and materials; Figure 2B and C) that accurately estimates transgene copy number (Chandler et al., 2007). Using this assay, two mESC transgenic clones, referred to as *Neurog3*^{RG1} and *Neurog3*^{RG2}, were identified as having copy numbers of 1.25 ± 0.16 and 1.46 ± 0.26 (Figure 2C). Derivation of *Neurog3*^{RG} mESC lines and the subsequent *Neurog3*^{RG1} mouse line are described in (Bechard et al., 2016). Examining various stages of pancreas organogenesis in *Neurog3*^{RG2} mice at the level of gross tissue and islet architecture, ad libitum fed glycemia, and proportion of Sox9⁺ Neurog3 protein-low (Neurog3^{pLO}) versus Sox9⁻ Neurog3 protein-high (Neurog3^{pHI}) cells, revealed no change relative to our previous characterization of the *Neurog3*^{RG1} reporter line (Bechard et al., 2016) (Supplemental Figure 1A–D; data not shown). We next validated the LCA function of the *Neurog3*^{RG} BAC transgene by using the *Neurog3*^{RG2} mESC line to derive a *Neurog3*^{P2A.FUCCI} mESC line. A lox66/lox2272-flanked *Neurog3*^{P2A.FUCCI}-PGK-hygro^R cassette was generated with cassette placement mimicking that of *Neurog3*^{RG} (Supplemental Figure 2A). Following RMCE in *Neurog3*^{RG2} mESCs, PCR was performed to verify replacement of the lox71/lox2272-flanked *Neurog3*^{RG}-PGK-Puro^{TK} cassette with the lox66/lox2272 *Neurog3*^{P2A.FUCCI}-PGK-hygro^R cassette (Supplemental Figure 2B). This

derivative *Neurog3*^{P2A.FUCCI} mESC line was then used to generate *Neurog3*^{P2A.FUCCI} transgenic mice. Given that the genomic integration site is likely different in *Neurog3*^{RG2} vs. *Neurog3*^{RG1} mESC lines, we used *Neurog3*^{RG2} mESCs to derive *Neurog3*^{P2A.FUCCI} mice to allow future breeding of *Neurog3*^{P2A.FUCCI} to *Neurog3*^{RG1} mice to enable four-color reporting of cell-cycle phase and *Neurog3* expression. Such visualization could facilitate future experiments aimed at understanding if, like other progenitor populations, G₁ length or overall cell-cycle length in mitotic *Neurog3*^{TA,LO} progenitors affects progenitor maintenance vs. endocrine-commitment decisions, or even if one endocrine cell-type is produced over another at specific stages or locations within the developing pancreas.

Neurog3 levels and progenitor maintenance vs. endocrine-commitment decisions are coupled to the cell cycle

Recent studies showed that during S-G₂-M, Neurog3 is kept in a hyperphosphorylated unstable state via Cdk phosphorylation, and that decreased Cdk activity associated with entrance into G₁ result in stabilization and accumulation (Azzarelli et al., 2017; Krentz et al., 2017). These findings support our model that, in actively cycling *Neurog3*^{TA,LO} progenitors, Neurog3 protein levels vary according to the cell-cycle phase (Bechard and Wright, 2017). To address this issue, we used *Neurog3*^{P2A.FUCCI} reporter expression in *Neurog3*^{TA,LO} progenitors to track cell-cycle progression in relation to Neurog3 protein levels. Previously, *Neurog3*^{TA,LO} progenitors were defined as a population of Sox9⁺ *Neurog3*-transcriptionally active (low-level *Neurog3*^{RG1} reporter expression) progenitors comprising cells with either low (*Neurog3*^{TA,pLO}) or immunologically undetectable Neurog3 (*Neurog3*^{TA,pUD}) (Bechard et al., 2016). Consistent with this definition, *Neurog3*^{TA,LO} progenitors were herein defined as Sox9-positive and positive for either component of the *Neurog3*^{P2A.FUCCI} reporter, with low or undetectable Neurog3 protein, whereas endocrine-committed *Neurog3*^{TA,HI} cells should be Sox9-negative, Neurog3^{pHI} and positive for mKO2-hCdt1^(30/120) (Figure 3A). Unexpectedly, we detected significant residual cytoplasmic mVenus-hGem^(1/110) fluorescence in post-mitotic, actively delaminating, Muc1⁺ endocrine-committed *Neurog3*^{TA,HI} cells that showed the expected high mKO2-hCdt1^(30/120) signal (Figure 3A). This observation was different from previous reports on the FUCCI reporter, where mVenus-hGem^(1/110) was mostly degraded soon after M-phase, becoming absent by the time of mKO2-hCdt1^(30/120) detection in early G₁ (Abe et al., 2013; Sakaue-Sawano et al., 2008). This unexpected overlap between mVenus-hGem^(1/110) (cytoplasmic) and mKO2-hCdt1^(30/120) (nuclear) signals was essentially completely restricted to the actively delaminating post-mitotic Sox9⁻ *Neurog3*^{TA,HI} population, and >85% of the cells within this pool displayed this co-positivity. Critically, the large majority of *Neurog3*^{P2A-FUCCI} reporter-positive cells in the intraepithelial Sox9⁺ *Neurog3*^{TA,LO} population showed no overlap between mVenus-hGem^(1/110) and mKO2-hCdt1^(30/120) signal. The residual cytoplasmic mVenus-hGem^(1/110) began to disappear in mKO2-hCdt1^(30/120)-positive, fully delaminated Muc1⁻ *Neurog3*^{TA,HI} cells (Figure 3A) and was completely absent in mKO2-hCdt1^(30/120)-positive islet cells that had been incorporated into the nascent islet-cell clusters (data not shown). This finding suggests that high *Neurog3*^{P2A.FUCCI} reporter expression in actively delaminating Muc1⁺ *Neurog3*^{TA,HI} cells overwhelms the ubiquitin-mediated degradation pathway, or these cells behave in a specific manner, prolonging the time to fully degrade mVenus-hGem^(1/110) after entering G₁. We were therefore careful to score

Neurog3^{P2A.FUCCI+} cells as only being in S-G₂-M if definitively nuclear mVenus signal was observed, with no indication of mKO2 (Figure 3A). By these criteria, the majority of Sox9⁺ *Neurog3*^{TA.LO} cells were in S-G₂-M and thus mitotic, while nearly all *Neurog3*^{TA.HI} cells were in G₁ (Figure 3B). To determine if Neurog3 protein levels vary through the cell cycle, we examined the cell-cycle status of Sox9-positive *Neurog3*^{TA.pLO} versus *Neurog3*^{TA.pUD} cells. Quantification revealed that 78% (± 8.1%) of *Neurog3*^{TA.pUD} cells were in S-G₂-M and 22% (± 8.1%) in G₁ (Figure 3B). Although stabilization and accumulation of Neurog3 occurs during G₁, previous work showed that Neurog3 protein is present during S phase, with rapid degradation occurring during G₂-M (Krentz et al., 2017). Corroborating that result, we show that while the majority (55% ± 3.6%) of Sox9⁺ *Neurog3*^{TA.pLO} cells were in G₁, 45% (± 3.6%) were in S-G₂-M (Figure 3B). These findings show that the Neurog3 protein level in cycling Sox9⁺ *Neurog3*^{TA.LO} progenitors is lowest during S-G₂-M and highest during G₁.

Previous work shows that a low Neurog3 protein level is compatible with a mitotic, endocrine lineage-primed progenitor state (Wang et al., 2010; Bechard et al., 2016). Given the cell-cycle-dependent variation of Neurog3 protein level, we hypothesized that the low-level accumulation of Neurog3 in *Neurog3*^{TA.LO} progenitors in G₁ could trigger gene expression changes that were consistent with endocrine lineage-priming, and involving genes other than solely *Neurog3*. Therefore, intraepithelial *Neurog3*^{P2A.FUCCI+} cells (*Neurog3*^{TA.LO} progenitors) were isolated from E14.5 *Neurog3*^{P2A.FUCCI} pancreatic explants by flow sorting of lumen-contacting (Muc1⁺) cells, then sorting cells in S-G₂-M (mKO2⁻ mVenus⁺) or G₁ (mKO2⁺ mVenus⁻) (Figure 4A). As described above, actively delaminating endocrine-committed *Neurog3*^{TA.HI} cells display still-nondegraded cytoplasmic mVenus in addition to the expected high nuclear mKO2 signal associated with G₁ entry and cell-cycle exit (Figure 3A). To exclude this population, the flow-cytometry gating was set very conservatively so that Muc1⁺ mVenus/mKO2 co-positive cells, and therefore Muc1⁺ endocrine-committed *Neurog3*^{TA.HI} cells, were not collected (Figure 4A). Analysis via qRT-PCR showed that while in S-G₂-M, *Neurog3*^{TA.LO} progenitors are enriched for *Sox9* and *Hes1* (mitotic endocrine-progenitor markers) with low expression of *Neurog3* and several markers indicating forward progression towards endocrine commitment and further differentiation (*NeuroD1*, *Insm1*, *Glucagon*, *Insulin*) (Figure 4B). *Neurog3*^{TA.LO} progenitors in G₁, however, showed significantly decreased *Hes1* and increased *Neurog3*, *NeuroD1*, *Insm1*, *Glucagon* and *Insulin* (Figure 4B). Despite the increases in endocrine-commitment markers, Sox9 expression remained unchanged in Muc1⁺ mKO2⁺ G₁ cells, (Figure 4B), confirming that our sorting scheme limited our analysis to intra-epithelial Sox9⁺ *Neurog3*^{TA.LO} progenitors with minimal contamination from actively delaminating, Muc1⁺ *Neurog3*^{TA.HI} endocrine-committed cells (previously shown to be Sox9-negative; Bechard et al., 2016). The data are, therefore, consistent with the idea that mitotic *Neurog3*^{TA.LO} progenitors, when in G₁, initiate the low-level expression of several genes representing the downstream endocrine differentiation program, at a stage prior to commitment. Given the role of Neurog3 in trans-activating *NeuroD1* and *Insm1* (Mellitzer et al., 2004; Huang et al., 2000; Gasa et al., 2008), we speculate that the low-level accumulation of Neurog3 specifically in G₁ could be sufficient to induce low-level *NeuroD1/Insm1* expression in lineage-primed progenitors. It is also possible that signals

initiating the lineage-primed state activate low-level expression of other transcription-factor genes in a *Neurog3*-independent manner. It is plausible that the concerted expression of several trans-acting factors establishes a relatively weak or incomplete form of the GRN that is normally considered to work only in post-mitotic committed cells. There are also several explanations as to why, despite the presence of *Neurog3* protein during S-G₂-M, higher-amplitude expression of downstream *Neurog3* targets (e.g. *NeuroD1* and *Insm1*) only occurs during G₁. Most notable is the possibility that a yet-to-be-defined pathway or mechanism blocks *Neurog3* protein present during S-G₂-M from activating such downstream targets. For example, the unstable phosphorylated form of *Neurog3* (the predominant form during S-G₂-M; Krentz et al., 2017), like *Neurog2* (Ali et al., 2011; Hindley et al., 2012), is unable to activate downstream targets associated with promoting endocrine differentiation. It would be important to discover if entrance into G₁ were also linked to alterations in chromatin architecture and DNA accessibility that allow low-level expression of GRN member genes contributing to lineage priming in *Neurog3*^{TA,LO} progenitors. Understanding how cell-cycle progression regulates such gene expression programs could lead to understanding if the final hormone-secreting cell fate might become preconditioned in the mitotic lineage-biased stage, and possibly how to manipulate cells at this early phase of their lifespan to improve the generation of functional endocrine cells.

Materials and Methods

Mice and transgene copy number analysis

Animal protocols were approved by the Vanderbilt University Institutional Animal Care and Use Committee. All animals were PCR genotyped. Sequences for genotyping primers are listed in Supplemental file 1A. Generation of the *Neurog3*^{RG} BAC transgene and subsequent derivation of the *Neurog3*^{RG2} mESC line and *Neurog3*^{RG2} reporter mice was described previously (Bechard et al., 2016). The analysis of the *Neurog3*^{RG2} line, to ensure that it is a passive and accurate reporter that mimicked normal *Neurog3* expression patterns with no mutant phenotype, was done at the same time as our previous *Neurog3*^{RG1} line (Bechard et al., 2016), but *Neurog3*^{RG2} results were not reported there. Because the *Neurog3*^{P2A.FUCCI} line was derived via RMCE using *Neurog3*^{RG2} ES cells, the analysis of *Neurog3*^{RG2} line are presented here (Supplemental Figure 1) and referenced to our previous work (6). Although not previously reported in (Bechard et al., 2016), mouse ES cells that stably integrated the *Neurog3*^{RG} BAC LCA were analyzed by a qPCR-based assay that accurately estimates transgene copy number (Chandler et al., 2007). Briefly, primers specific for the puromycin-resistance cassette were used in quantitative PCR analysis of 2.5, 10, 20, 40 and 100 ng of genomic DNA from a TL1 mESC knock-in line, known to carry one copy of the *Puro*^R-TK-em7-Neo^R (*Puro*^{TK}) cassette inserted via homologous recombination, to yield a C_T curve reflecting copy number per DNA input (Figure 2B). Using these same primers to target the *Puro*^{TK} cassette in the *Neurog3*^{RG} transgene, triplicate qPCR runs on exactly 20 ng of DNA from 23 candidate *Neurog3*^{RG} mESC lines generated C_T values that defined single-copy insertions.

Generation of P2A.FUCCI transgene and the *Neurog3*^{P2A.FUCCI} reporter mouse line

To generate the mKO2-hCdt1^(30/120)-P2A-mVenus-hGem^(1/110) (P2A.FUCCI) cassette, mKO2-hCdt1^(30/120) and mVenus-hGem^(1/110) were PCR-amplified from plasmids provided by Dr. Atsushi Miyawaki (RIKEN Brain Science Institute) (Sakaue-Sawano et al. 2008). Amplification of mKO2-hCdt1^(30/120) involved attaching a 40 bp *Neurog3* homology region 5' of the mKO2 start codon along with the first 25 base pairs of a P2A sequence 3' of mKO2. Amplification of mVenus-hGem^(1/110) involved attaching a 5' BamHI site and a 3' ApaI site. A third PCR was used to generate a P2A cassette with 25 base pairs of the 3' end of mKO2-hCdt1^(30/120) attached to its 5' end and a BamHI site at its 3' end. The resulting mKO2-hCdt1^(30/120) and P2A amplicons were then fused together by overlap extension PCR (Horton et al., 2013), using a forward primer specific for the mKO2-hCdt1^(30/120) amplicon and a reverse primer specific for the P2A amplicon. The resulting mKO2-hCdt1^(30/120)-P2A amplicon was attached to the mVenus-hGem^(1/110) amplicon via the BamHI site and inserted into a pBS KS(-) vector. The resulting P2A.FUCCI cassette was removed from pBS KS (-) and inserted into a pCMV5 vector with a PGK-neomycin selection cassette for expression in HeLa cells (described below). The P2A.FUCCI cassette was also inserted in place of the RG cassette in the PL451-RG-FRT-Puro^R- TK-em7-Neo^R-FRT-lox2272 vector described previously (Bechard et al., 2016). Using BAC recombineering the resulting P2A.FUCCI-FRT-Puro^R- TK-em7-Neo^R-FRT-lox2272 cassette was inserted immediately upstream of the *Neurog3* start codon in the *Neurog3*-containing RPCI-23-121F10 BAC (Bechard et al., 2016). Using BAC recombineering, the P2A.FUCCI-FRT-Puro^R- TK-em7-Neo^R-FRT-lox2272 cassette was retrieved into a vector containing a lox66 site in a manner that ensured that placement of the lox66 site precisely mimicked that of its lox71 counterpart in the lox71/lox2272 flanked *Neurog3*^{RG} BAC LCA. Subsequently, the FRT-flanked Puro^R- TK-em7-Neo^R cassette was replaced with an FRT-flanked PGK-Hygro^R selection cassette. This final lox66/lox2272 flanked *Neurog3*^{P2A.FUCCI} exchange plasmid was linearized and used to replace, via RMCE, the *Neurog3*^{RG} BAC LCA in the *Neurog3*^{RG2} mESC line. Successful replacement with *Neurog3*^{P2A.FUCCI} was verified by PCR (Figure Supplement 2B). A single, verified, *Neurog3*^{P2A.FUCCI} mES cell line was expanded, karyotyped and injected into blastocyst-stage embryos to derive the *Neurog3*^{P2A.FUCCI} reporter mouse strain. The LCA capability of the *Neurog3*^{RG1} mESC line was also tested and shown to allow efficient RMCE of lox66/lox2272 flanked cassettes (data not shown).

Cell culture

HeLa cells were cultured on tissue culture grade plastic at 37° C in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 100 U/mL penicillin-streptomycin. Cells were passaged by adding 0.05% trypsin-EDTA to a plate of semi-confluent (<90%) cells. To test expression of the P2A.FUCCI reporter, HeLa cells were transiently transfected with the *CMV*^{P2A.FUCCI} expression plasmid using Lipofectamine 2000 (Thermo Fisher) according to manufacturer's instructions. The same conclusions were obtained with stable clonal lines expressing *CMV*^{P2A.FUCCI} selected for neomycin resistance over 14 days (not shown).

Immunodetection

E14.5 dorsal pancreata were fixed in 4% paraformaldehyde (4 hrs, 4°C) then equilibrated in 30% sucrose overnight at 4°C). A Leica CM3050S was used sucrose-equilibrated, OCT-embedded tissue (Tissue-Tek) into 10 µm tissue sections, sequentially placed on three separate sets of slides, each covering ~33% of the dorsal pancreas. Primary and Secondary antibodies are listed in Supplemental file 1B. All images are epifluorescence from a Zeiss ApoTome microscope with Zeiss Axiovision software.

Flow sorting and qRT-PCR analysis

Multiple E14.5 *Neurog3*^{P2A.FUCCI+} dorsal pancreata were pooled and dispersed into a single-cell suspension using Accumax (Sigma) (protocol available on request). Dispersed samples were washed and incubated on ice, first with Muc1 antibody for 1 hr, then anti-hamster Cy5 secondary antibody for an additional hour. DAPI was added to ensure sorting of viable cells. Flow sorting used a BD FACS Aria III. To exclude from the subsequent qRT-PCR analysis the endocrine-committed Sox9⁺ *Neurog3*^{TA.HI} cells, which display cytoplasmic mVenus in addition to nuclear mKO2, the flow-cytometry gating was set conservatively so that mVenus/mKO2 co-positive cells were not collected (Figure 4A). cDNA was generated using iScript cDNA synthesis kit (Bio-Rad) from RNA isolated from flow-sorted cells after TRIzol extraction. qRT-PCR was performed in a Bio-Rad CFX96 with SsoFast EvaGreen Supermix (Bio-Rad) using at least three technical replicates. Relative expression level (normalized to *Gapdh*) was calculated by first assessing the C_T between the gene of interest and *Gapdh* before converting the C_T to relative expression level (2^{-C_T}). The results in Figure 4 were independently repeated (biologically replicated) with similar results. Primer sequences, except for *Insm1* primers (Applied Biosystems), are listed in Supplemental file 1A.

Cell counting, quantification and statistics

Cell counting used NIH ImageJ software with “n” indicating total cells counted, and “N” number of individual dorsal pancreata analyzed (see figure legends for specific numbers). Fluorescence-intensity quantifications for classification of *Neurog3*^{pLO} and *Neurog3*^{pHI} cells were as previously described (Bechard et al., 2016). As previously stated approximately 33% of an entire dorsal pancreas was analyzed for each dorsal pancreas. Previous reports indicate that only 2% of the total pancreas volume needs to be systematically sampled and analyzed to obtain a relative error of ~10% (Chintinne et al., 2010). For all quantifications, error bars were generated using standard error of the mean (SEM), with Student’s t-test (one-tailed) used to calculate *p* values. *p* values were deemed significant when ≤ 0.05 .

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Peptide-2A single-transgene FUCCI transgene. (A) Diagram indicating phases of the cell cycle marked by the components of the FUCCI reporter: *mVenus-hGem*^(1/110) (S-G₂-M) and *mKO2-hCdt1*^(30/120) (G₁) (Sakaue-Sawano et al., 2008). (B) *Top*, Diagram of *CMV*^{P2A}*FUCCI* expression plasmid. *Bottom*, Immunofluorescence images showing *CMV*^{P2A}*FUCCI* reporter expression in HeLa cells at appropriate stages of the cell cycle. Green arrowhead indicates mitotic chromosomes. Scale bars, 20 μ m.

Figure 2. Generation of an LCA-capable BAC transgenic *Neurog3*^{RG} mESC line. (A) Schematic detailing the generation of transgenic mES cell lines carrying a single copy of a *Neurog3*^{RG} BAC transgene designed to serve as an LCA in future RMCE reactions. The *Neurog3*^{RG} BAC transgenic mESCs were previously used to generate *Neurog3*^{RG} reporter mice (Bechard et al., 2016). *Neurog3* 5′ untranslated region (UTR) represents the region 5′ of the start codon containing cis regulatory elements and the *Neurog3* 5′/UTR. (B) Table and graph of a standard curve, generated via a qPCR-based assay (see methods and materials), that relates transgene copy number to a specific C_T value. (C) Table and graph depicting the estimated *Neurog3*^{RG} BAC transgene copy number present in *Neurog3*^{RG1} and *Neurog3*^{RG2} mESC lines. Each data point represents the average of the indicated numbers of qPCR runs ± SEM.

Figure 3.

Neurog3 protein levels vary according to cell-cycle phase. (A) E14.5 pancreatic epithelium showing Sox9, Neurog3, hCdt1^{mKO2} and hGem^{mVenus}. Red arrowheads indicate *Neurog3*^{P2A.FUCCI+} cells that are mKO2⁺ and thus in G₁, green arrowheads indicate *Neurog3*^{P2A.FUCCI+} cells that are mVenus⁺ and thus in S-G₂-M phase. Asterisk indicates Sox9⁺ *Neurog3*^{TA,LO} cells, arrows with no asterisk indicate Sox9⁻ *Neurog3*^{TA,HI} cells. (B) Left, percentage of Sox9⁺ *Neurog3*^{TA,LO} and Sox9⁻ *Neurog3*^{TA,HI} cells in S-G₂-M versus G₁ phase. Right, percentage of Sox9⁺ *Neurog3*^{TA,pLO} and Sox9⁺ *Neurog3*^{TA,pUD} in S-G₂-M versus G₁ phase. ($n = 1600$, $N = 3$). (*) $P = 0.0072$; (**) $P = 4 \times 10^{-6}$; (***) $P = 0.0607$; (****) $P = 0.0039$. Each data point is an average of $N = 3$ with error bars representing $\pm$ SEM. Scale bars, 20 μ m.

Figure 4.

Neurog3 promotes low-level activation of downstream targets during G₁ in the mitotic *Neurog3^{TA.LO}* progenitor state. (A) flow cytometry plot detailing capture of lumen-apposed (Muc1⁺) *Neurog3^{P2A.FUCCI}* cells in S-G₂-M (mKO2⁻ mVenus⁺) (Blue population) or G₁ (mKO2⁺ mVenus⁻) (red population) from E14.5 *Neurog3^{P2A.FUCCI}* pancreata. Flow-sorted cells were collected into TRIzol for RNA isolation and cDNA synthesis. (B) Relative expression level (y-axis), normalized to *Gapdh*, of *Sox9*, *Neurog3*, *Hes1*, *NeuroD1*, *Insm1*, *glucagon*, and *Insulin* for E14.5 flow captured Muc1⁺ *Neurog3^{P2A.FUCCI}* mKO2⁻ mVenus⁺ (blue bars) and Muc1⁺ *Neurog3^{P2A.FUCCI}* mKO2⁺ mVenus⁻ (red bars) cells. Unless where noted differently on the figure, data points are an average of n = 6 technical replicates with error bars representing $\pm$ SEM. See supplemental table 1 for a list of primers used.