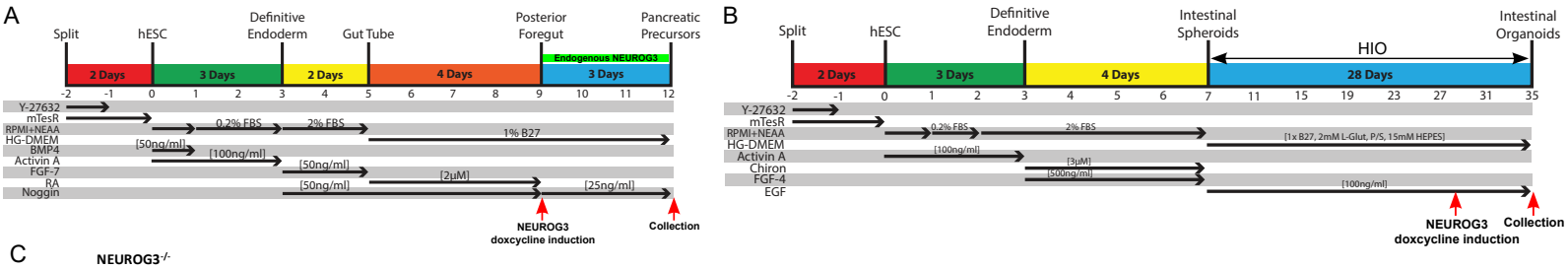
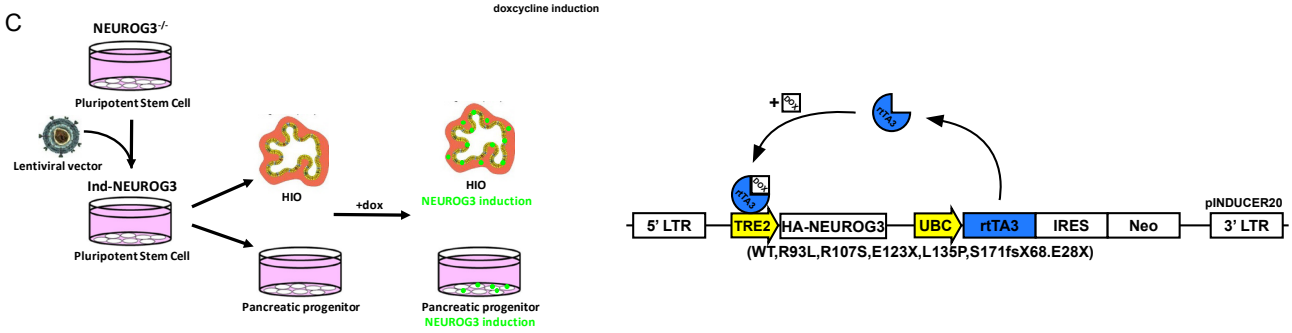
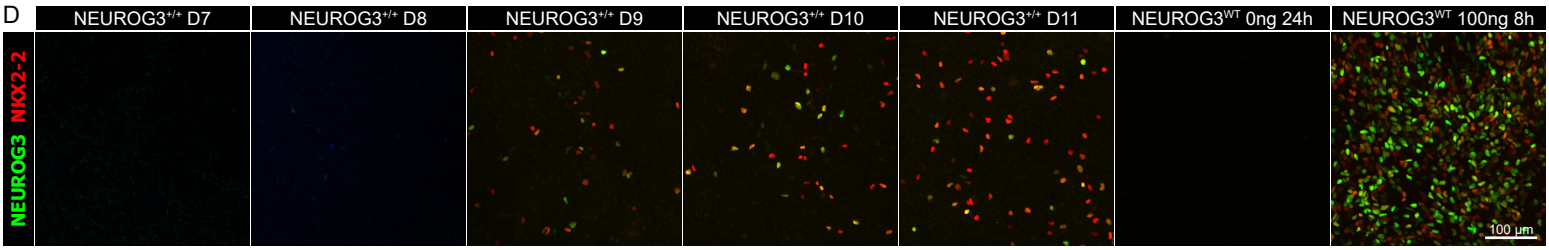
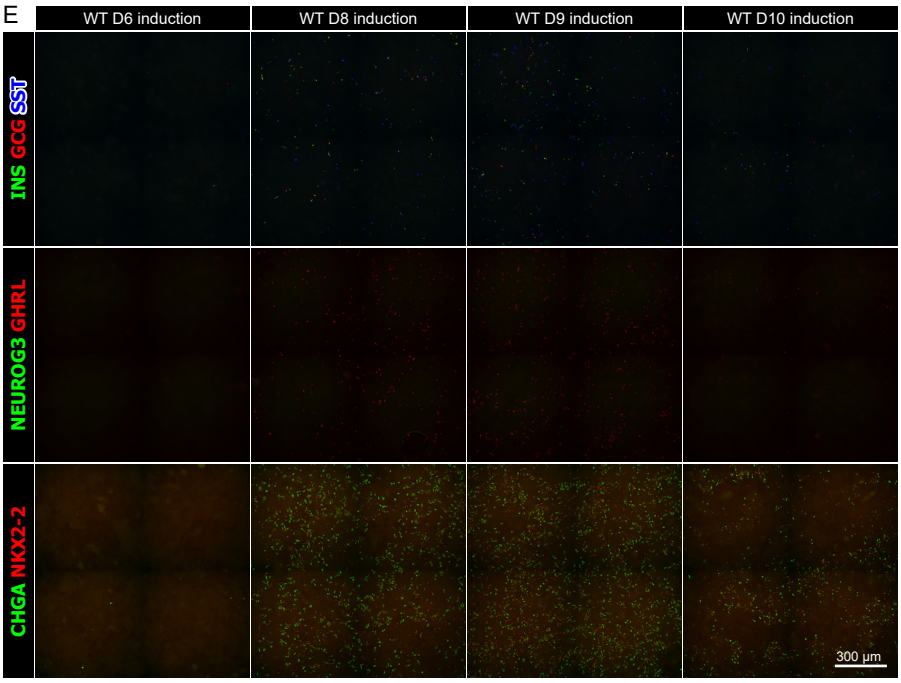
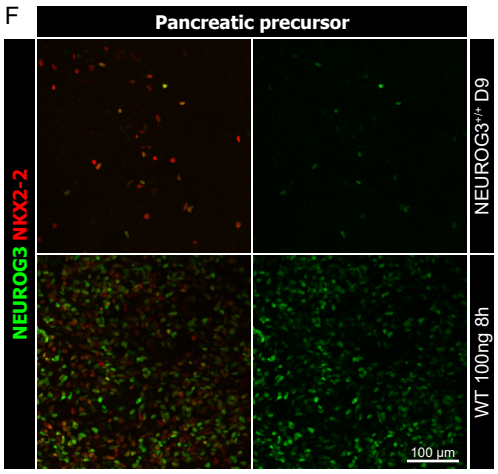
Figure S1

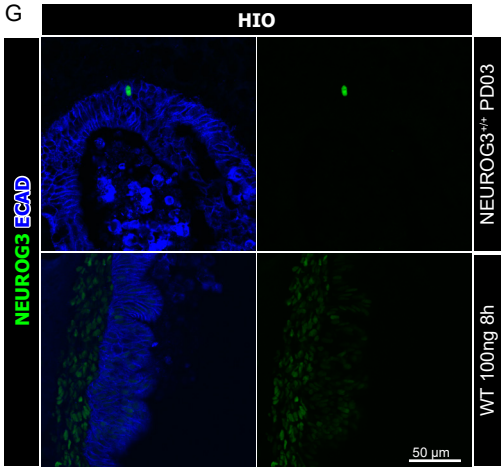

A
Split
hESC
Definitive Endoderm
Gut Tube
Posterior Foregut
Pancreatic Precursors
Endogenous NEUROG3
2 Days
3 Days
2 Days
4 Days
3 Days
Y-27632
mTesR
RPMI+NEAA
HG-DMEM
BMP4
Activin A
FGF-7
RA
Noggin
0.2% FBS
2% FBS
1% B27
[50ng/ml]
[100ng/ml]
[50ng/ml]
[50ng/ml]
[2μM]
[25ng/ml]
NEUROG3 doxcycline induction
Collection
B
Split
hESC
Definitive Endoderm
Intestinal Spheroids
Intestinal Organoids
HIO
2 Days
3 Days
4 Days
28 Days
Y-27632
mTesR
RPMI+NEAA
HG-DMEM
Activin A
Chiron
FGF-4
EGF
0.2% FBS
2% FBS
[1x B27, 2mM L-Glut, P/S, 15mM HEPES]
[100ng/ml]
[3μM]
[500ng/ml]
[100ng/ml]
NEUROG3 doxcycline induction
Collection

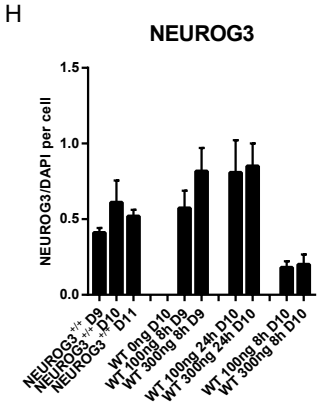

C
NEUROG3-/-
Pluripotent Stem Cell
Lentiviral vector
Ind-NEUROG3
Pluripotent Stem Cell
HIO
+dox
HIO
NEUROG3 induction
Pancreatic progenitor
Pancreatic progenitor
NEUROG3 induction
+Dox
rtTA3
rtTA3
5' LTR
TRE2
HA-NEUROG3
UBC
rtTA3
IRES
Neo
3' LTR
pINDUCER20
(WT,R93L,R107S,E123X,L135P,S171fsX68.E28X)

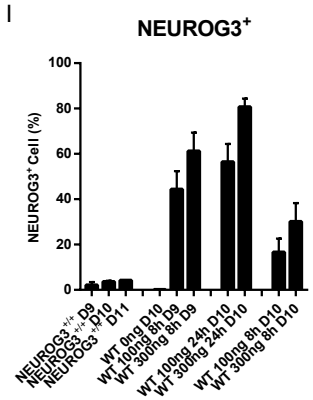

D
NEUROG3+/+ D7
NEUROG3+/+ D8
NEUROG3+/+ D9
NEUROG3+/+ D10
NEUROG3+/+ D11
NEUROG3WT 0ng 24h
NEUROG3WT 100ng 8h
NEUROG3 NKX2-2
100 μm


E
WT D6 induction
WT D8 induction
WT D9 induction
WT D10 induction
INS GCG SST
NEUROG3 GHRL
CHGA NKX2-2
300 μm


F
Pancreatic precursor
NEUROG3 NKX2-2
NEUROG3+/+ D9
WT 100ng 8h
100 μm


G
HIO
NEUROG3 ECAD
NEUROG3+/+ PD03
WT 100ng 8h
50 μm


H
NEUROG3
NEUROG3/DAPI per cell
1.5
1.0
0.5
0.0
NEUROG3+/+ D9
NEUROG3+/+ D10
NEUROG3+/+ D11
WT 0ng D10
WT 100ng 8h D9
WT 300ng 8h D9
WT 100ng 24h D10
WT 300ng 24h D10
WT 100ng 8h D10
WT 300ng 8h D10


I
NEUROG3+
NEUROG3+ Cell (%)
100
80
60
40
20
0
NEUROG3+/+ D9
NEUROG3+/+ D10
NEUROG3+/+ D11
WT 0ng D10
WT 100ng 8h D9
WT 300ng 8h D9
WT 100ng 24h D10
WT 300ng 24h D10
WT 100ng 8h D10
WT 300ng 8h D10

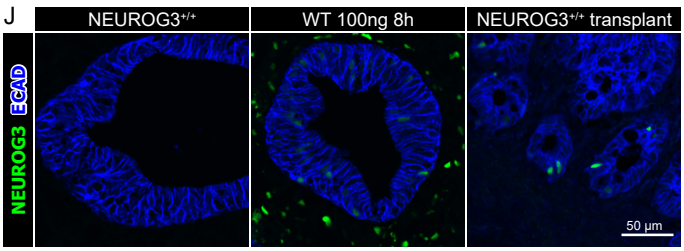
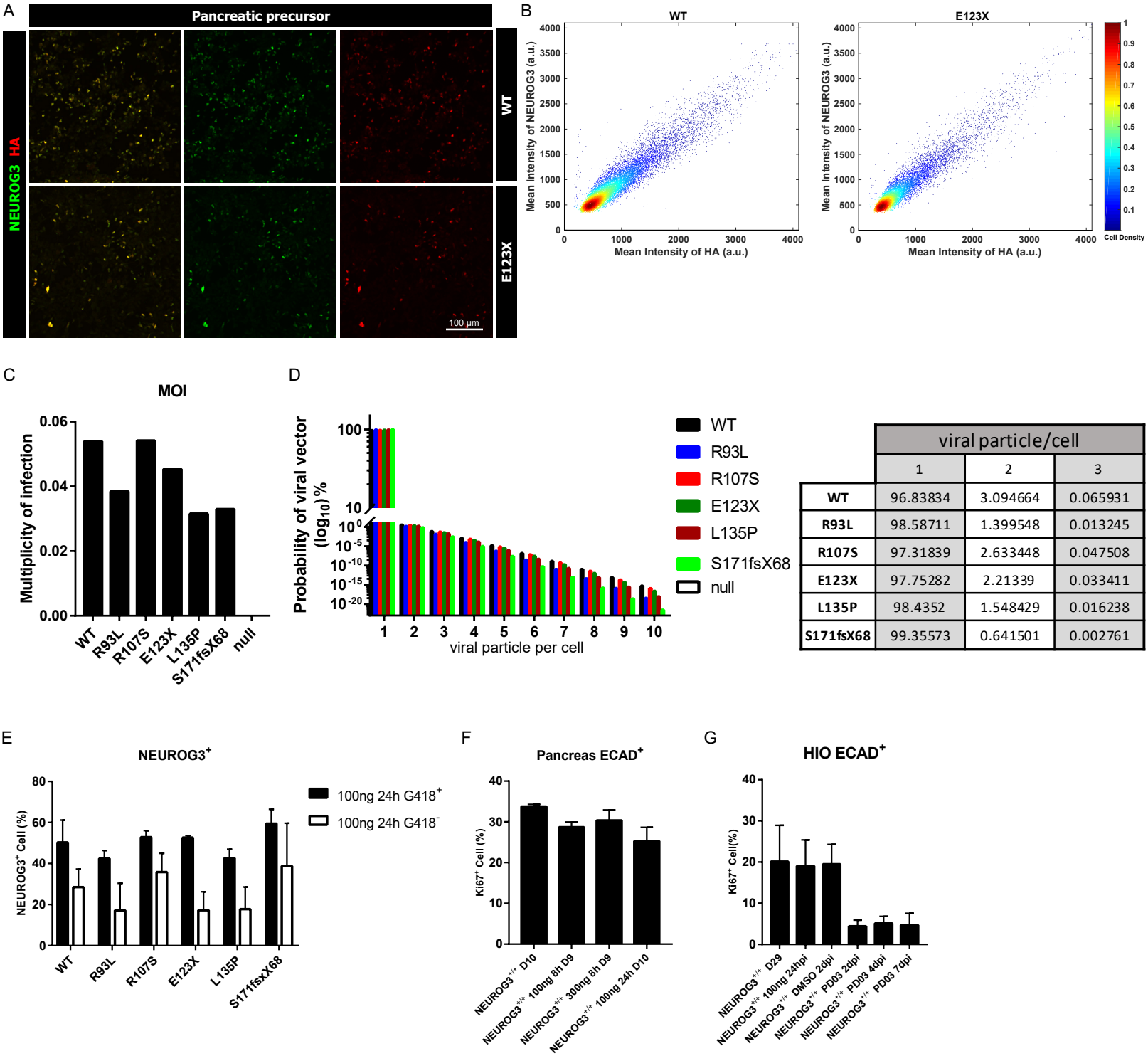

J
NEUROG3+/+
WT 100ng 8h
NEUROG3+/+ transplant
NEUROG3 ECAD
50 μm

Figure S1: Tetracycline inducible NEUROG3 system characterization in pancreatic precursors and HIOs derived from hESCs. Related to Figure 1.
(A) Protocol for pancreatic precursors derived from hESCs. Endogenous NEUROG3 expression starts from D9 throughout D12. To simulate endogenous NEUROG3 expression, NEUROG3 was induced on D9 and cells were collected on D12 for endocrine cell generation (red arrows).
(B) Protocol for HIOs derived from hESCs. Endogenous NEUROG3 expression was not detected. To simulate endogenous NEUROG3 expression, NERUOG3^{WT} was induced on D28 and organoids were collected on D35 for endocrine cell generation (red arrows).
(C) Schematic of tetracycline inducible NEUROG3 regulation with doxycycline treatment in pINDUCER20.
(D) Immunofluorescence analysis of NEUROG3 and NKX2-2 expression in pancreatic precursors derived from NEUROG3$^{+/+}$ (D7-D11), or NEUROG3^{wr} hESCs.
(E) Immunofluorescence analysis of induced NEUROG3 temporal competency for pancreatic precursors. NEUROG3^{WT} were induced on D6, D8, D9, or D10 and cells were collected on D12.
(F) Immunofluorescence analysis of NEUROG3 and NKX2-2 proteins in NEUROG3$^{+/+}$ (D9) and 100ng/ml 8-hour induced NEUROG3^{WT} pancreatic precursors.
(G) Immunofluorescence analysis of NEUROG3and ECAD of NEUROG3$^{+/+}$ (PD03 on D21 through D23) and 100ng/ml 8-hour induced NEUROG3^{WT}35-day HIOs.
(H-I) Quantification of NEUROG3 expression per cell **(H)** and NEUROG3^{+} cell percentage **(I)** in NEUROG3$^{+/+}$ (D9-D11), or NEUROG3^{wr} pancreatic precursors.
(J) Immunofluorescence analysis of NEUROG3 and ECAD expression in NEUROG3$^{+/+}$ (D28), NEUROG3$^{+/+}$ post-transplantation and 100ng/ml 8-hour induced NEUROG3^{WT} HIOs.
For all experiments, the data is representative of a minimum of 2 separate experiments. Scale bar = 50, 100 and 300 μm. Data are represented as mean ± SD.

Figure S2



	viral particle/cell		
	1	2	3
WT	96.83834	3.094664	0.065931
R93L	98.58711	1.399548	0.013245
R107S	97.31839	2.633448	0.047508
E123X	97.75282	2.21339	0.033411
L135P	98.4352	1.548429	0.016238
S171fsX68	99.35573	0.641501	0.002761

Figure S2: Doxycycline induction of NEUROG3 variants viral transfection mimics endogenous NEUROG3 expression level. Related to Figure 1.
(A-B) Immunofluorescence analysis **(A)** and quantification **(B)** of overlapping NEUROG3 and HA-tag of pancreatic precursors with100ng/ml 8-hour and 24-hour induced NEUROG3 variants.
(C-D) Viral transduction multiplicity of infection (MOI) **(C)** and viral particle per cell probability **(D)** based on cell survival analysis post selection for all NEUROG3 variants.
(E) Evaluation of viral silencing effects on pancreatic precursors for all NEUROG3 variants based on NEUROG3^{+} cell percentage post induction.
(F) Quantification of KI67 in NEUROG3$^{+/+}$ pancreatic precursors as evaluation of doxycycline effects on cell proliferation.
(G) Quantification of KI67 in NEUROG3$^{+/+}$ HIOs as evaluation of doxycycline and PD03 effects on cell proliferation.
For all experiments, the data is representative of a minimum of 2 separate experiments. Scale bar = 100 μm. Data are represented as mean ± SD.

Figure S3

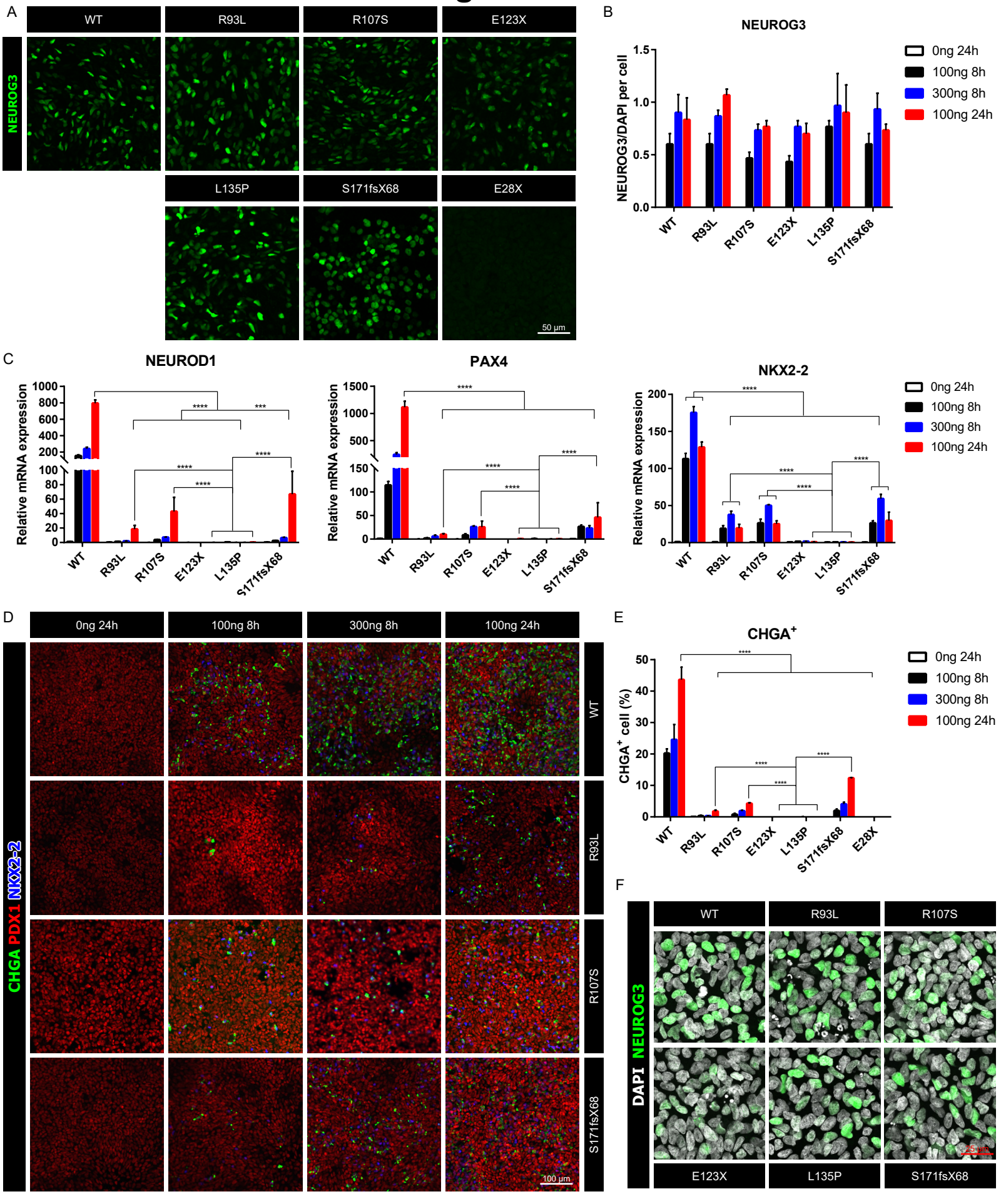

A
WT
R93L
R107S
E123X
NEUROG3
L135P
S171fsX68
E28X
50 μm
B
NEUROG3
NEUROG3/DAPI per cell
0ng 24h
100ng 8h
300ng 8h
100ng 24h
WT
R93L
R107S
E123X
L135P
S171fsX68
C
NEUROD1
PAX4
NKX2-2
Relative mRNA expression
D
0ng 24h
100ng 8h
300ng 8h
100ng 24h
CHGA PDX1 NKX2-2
WT
R93L
R107S
S171fsX68
100 μm
E
CHGA+
CHGA+ cell (%)
E28X
F
DAPI NEUROG3
E123X
L135P
S171fsX68

Figure S3: Overexpression of NEUROG3 variants in pancreatic precursors. Related to Figure 3.
(A-B) Immunofluorescence analysis **(A)** and quantification **(B)** of NEUROG3 with 100ng 8-hour induced NEUROG3 variants in pancreatic precursors.
(C) qPCR analysis of *NEUROD, PAX4,* and *NKX2-2* transcripts with 100, 300ng/ml 8-hour and 100ng/ml 24-hour induced NEUROG3 variants in pancreatic precursors.
(D) Immunofluorescence analysis of CHGA, PDX1 and NKX2-2 with 100, 300ng/ml 8-hour and 100ng/ml 24-hour induced NEUROG3 variants in pancreatic precursors.
(E) Quantification of $CHGA^+$ cell percentage of pancreatic endocrine with 100, 300ng/ml 8-hour and 100ng/ml 24-hour induced NEUROG3 variants.
(F) High magnification immunofluorescence analysis of DAPI NEUROG3 of pancreatic precursors with 100ng/ml 8-hour NEUROG3 variants.
For all experiments, the data is representative of a minimum of 3 separate experiments. Scale bar = 25, 50 and 100 µm. Data are represented as mean ± SD. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, and ****p ≤ 0.0001 for one-way ANOVA with Tukey HSD or Dunnett T3 (unequal variance) post hoc test.

Figure S4

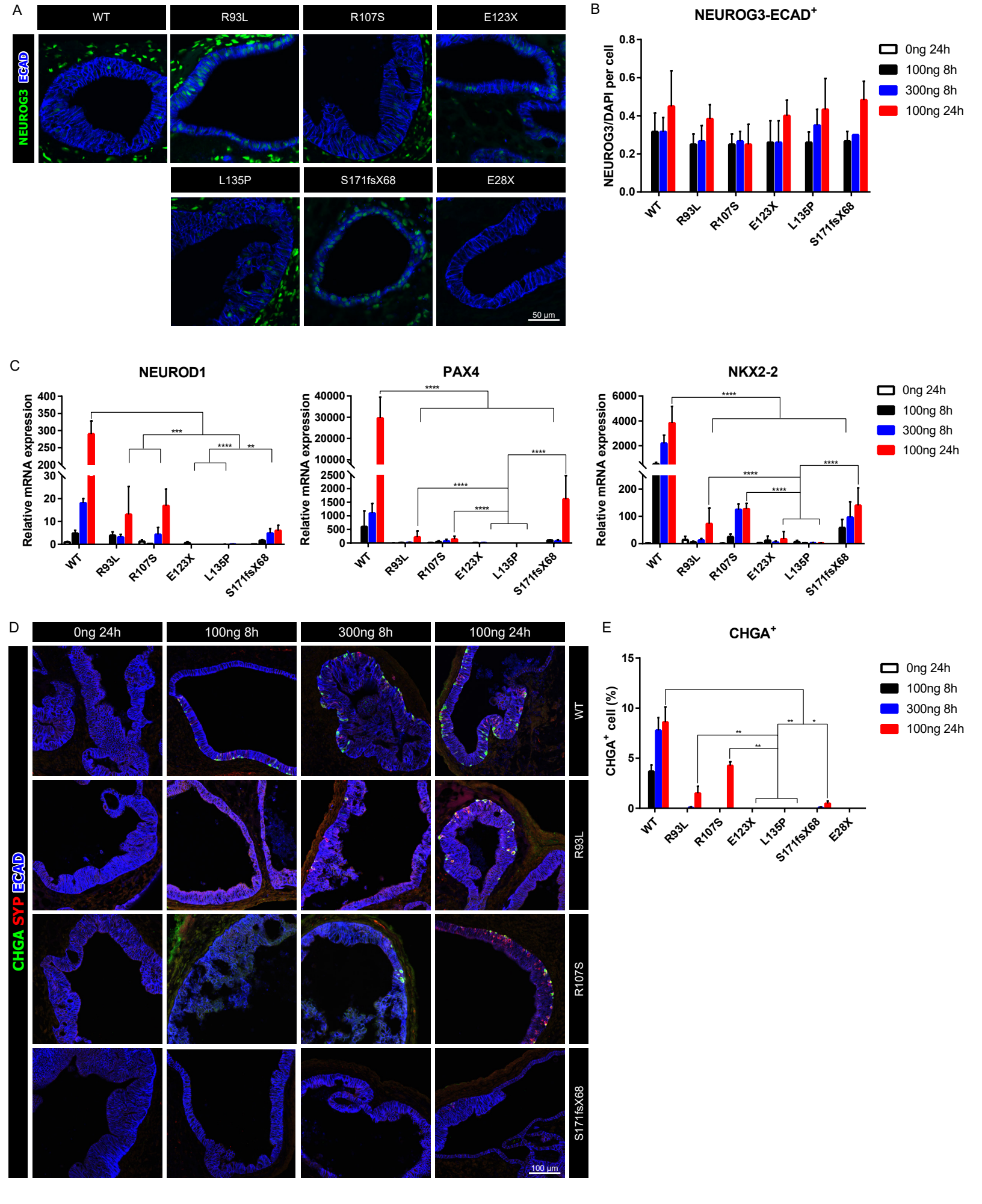
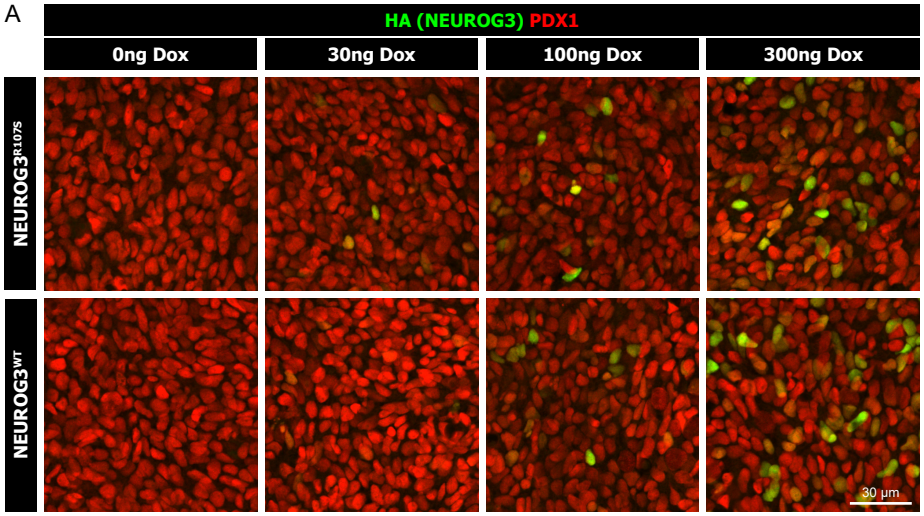
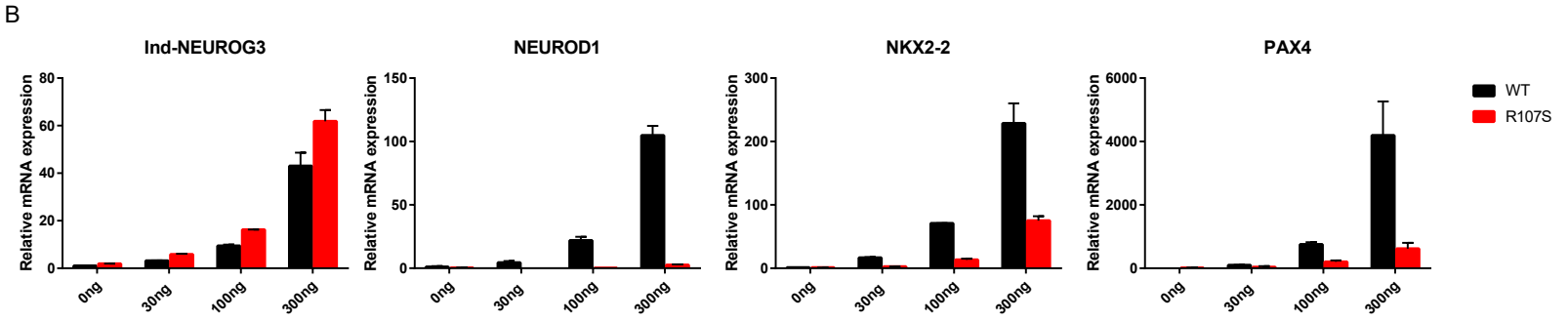
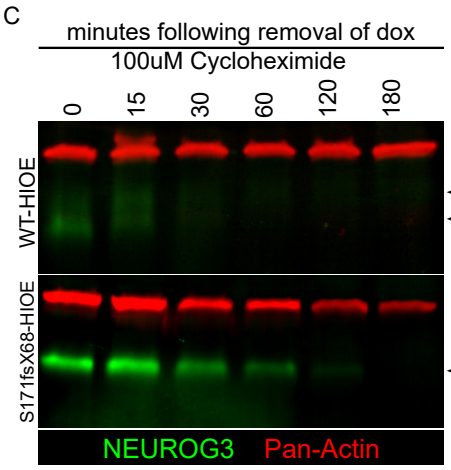

A
WT
R93L
R107S
E123X
L135P
S171fsX68
E28X
NEUROG3 ECAD
50 µm
B
NEUROG3-ECAD+
NEUROG3/DAPI per cell
0ng 24h
100ng 8h
300ng 8h
100ng 24h
C
NEUROD1
PAX4
NKX2-2
Relative mRNA expression
D
0ng 24h
100ng 8h
300ng 8h
100ng 24h
CHGA SYP ECAD
100 µm
E
CHGA+
CHGA+ cell (%)

Figure S4: Overexpression of NEUROG3 variants in HIOs. Related to Figure 3.
(A-B) Immunofluorescence analysis **(A)** and quantification **(B)** of NEUROG3 (green) of HIOs with 100ng/ml 8-hour doxycycline induction of NEUROG3 variants derived from hESCs.
(C) qPCR analysis of *NEUROD, PAX4, NKX2-2* and *CHGA* transcripts in HIOs with 100, 300ng/ml 8-hour and 100ng/ml 24-hour induced NEUROG3 variants.
(D) Immunofluorescence analysis of CHGA (green), PDX1 (red) and ECAD (blue) of HIOs with 100, 300ng/ml 8-hour and 100ng/ml 24-hour induced NEUROG3 variants.
(E) Quantification of $CHGA^+$ cell percentage of HIOs with 100, 300ng/ml 8-hour and 100ng/ml 24-hour induced NEUROG3 derived from NEUROG3 variants.
For all experiments, the data is representative of a minimum of 3 separate experiments. A minimum of 18 organoids were assessed. Scale bar = 50 and 100 µm. Data are represented as mean ± SD. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, and ****p ≤ 0.0001 for one-way ANOVA with Tukey HSD or Dunnett T3 (unequal variance) post hoc test.

Figure S5


A
HA (NEUROG3) PDX1
0ng Dox
30ng Dox
100ng Dox
300ng Dox
NEUROG3R107S
NEUROG3WT
30 µm


B
Ind-NEUROG3
NEUROD1
NKX2-2
PAX4
Relative mRNA expression
0ng
30ng
100ng
300ng
WT
R107S


C
minutes following removal of dox
100uM Cycloheximide
0
15
30
60
120
180
WT-HIOE
S171fsX68-HIOE
NEUROG3
Pan-Actin

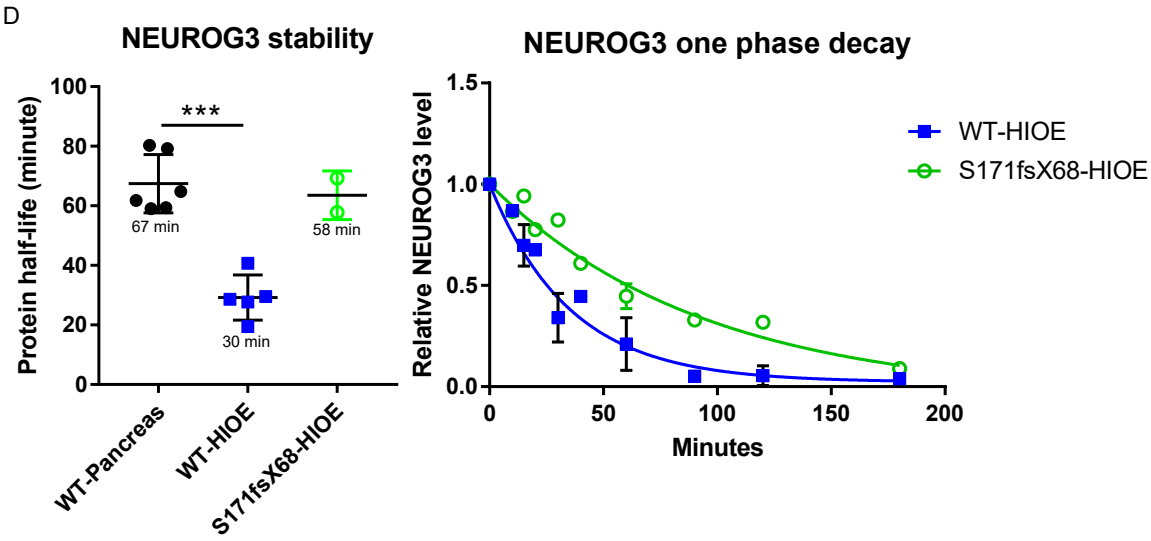
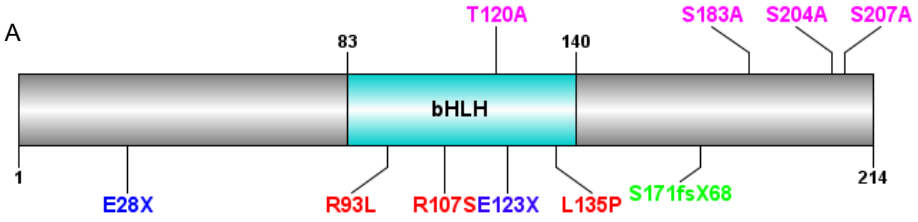
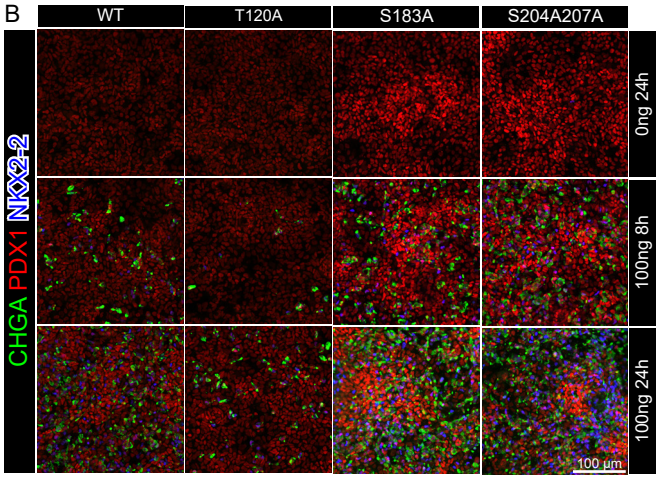
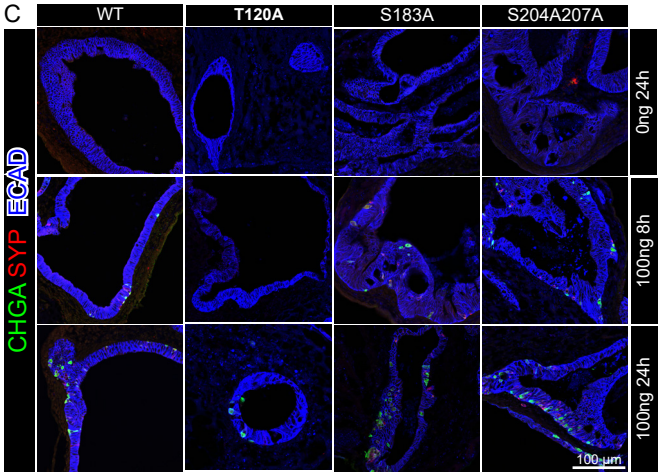

D
NEUROG3 stability
Protein half-life (minute)
***
67 min
30 min
58 min
WT-Pancreas
WT-HIOE
S171fsX68-HIOE
NEUROG3 one phase decay
Relative NEUROG3 level
Minutes
WT-HIOE
S171fsX68-HIOE

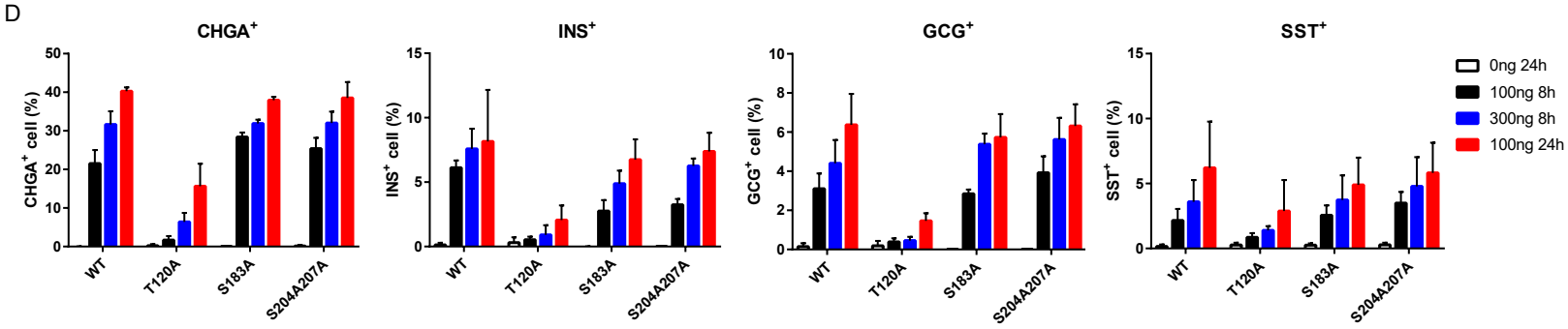
Figure S5: Overexpression of NEUROG3^{R107S} to compensate reduced protein stability and NEUROG3 half-life comparison in pancreas precursors and HIOE. Related to Figure 4.
(A) Immunofluorescence analysis of NEUROG3 and PDX1of pancreatic precursors with 0, 30, 100 or 300ng/ml 8-hour induced NEUROG3 variants.
(B) qPCR analysis of induced *NEUORG3*, *NEUROD, PAX4,* and *NKX2-2* transcripts in pancreatic precursors with 0, 30, 100 or 300ng/ml 8-hour induced NEUROG3 variants.
(C) Western blot analysis of NEUROG3 and ACTIN in human intestinal enteroids with 100ng/ml 24-hour induction of NEUROG3^{WT} and NEUROG3^{S171fsX68}. Arrows indicate multiple bands representing different forms of NEUROG3.
(D) Quantification of induced NEUROG3^{WT} and NEUROG3^{S171fsX68} half-life and corresponding decay curve in pancreatic precursors and human intestinal enteroids over the 180-minute time course using first order decay function. The data from NEUROG3^{WT} in Figure 4G were shown again here for comparison. For stability assays each data point is a separate experiment and for the decay graph one representative experiment is shown. Scale bar = 30 μm. Data are represented as mean ± SD.

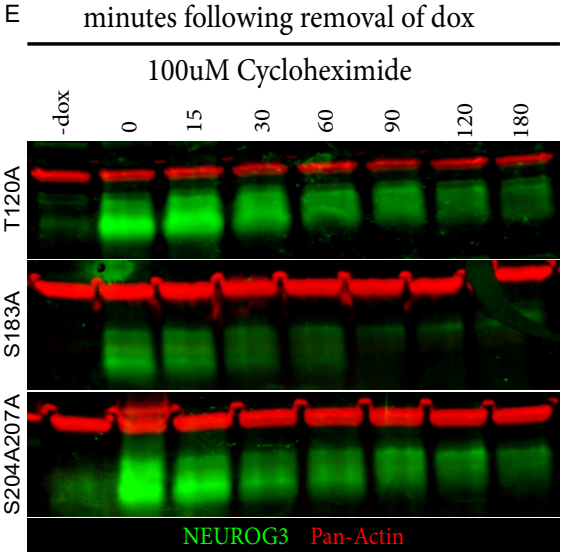
Figure S6

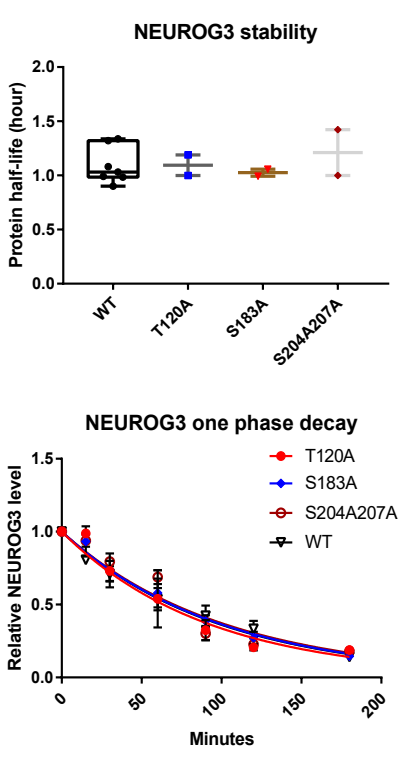

A
T120A
S183A
S204A
S207A
83
140
bHLH
1
214
E28X
R93L
R107S
E123X
L135P
S171fsX68

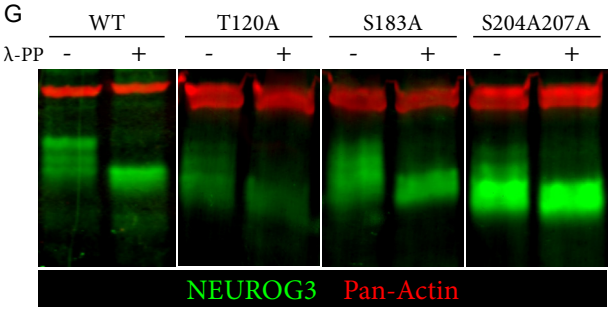

B
WT
T120A
S183A
S204A207A
CHGA PDX1 NKX2-2
0ng 24h
100ng 8h
100ng 24h
100 μm


C
WT
T120A
S183A
S204A207A
CHGA SYP ECAD
0ng 24h
100ng 8h
100ng 24h
100 μm


D
CHGA+
INS+
GCG+
SST+
CHGA+ cell (%)
INS+ cell (%)
GCG+ cell (%)
SST+ cell (%)
WT
T120A
S183A
S204A207A
0ng 24h
100ng 8h
300ng 8h
100ng 24h


E
minutes following removal of dox
100uM Cycloheximide
-dox
0
15
30
60
90
120
180
T120A
S183A
S204A207A
NEUROG3 Pan-Actin


F
NEUROG3 stability
Protein half-life (hour)
WT
T120A
S183A
S204A207A
NEUROG3 one phase decay
Relative NEUROG3 level
Minutes
T120A
S183A
S204A207A
WT


G
WT
T120A
S183A
S204A207A
λ-PP
NEUROG3 Pan-Actin

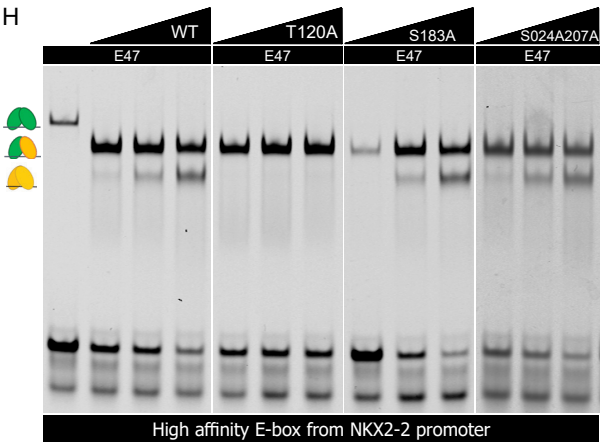

H
WT
T120A
S183A
S024A207A
E47
High affinity E-box from NKX2-2 promoter

Figure S6: NEUROG3 T120, S183, S204, and S207 phosphorylation effects on endocrine development in hESCs derived pancreatic precursors and HIOs. Related to Figure 4.

(A) Schematic of NEUROG3 patient mutations (blue, red and green), predicted C-terminal phosphorylation sites (cyan), and phosphorylation mutations (magenta) mapped onto NEUROG3 structure.

(B) Immunofluorescence analysis of CHGA, PDX1and NKX2-2 of pancreatic endocrine with 100ng/ml 8-hour and 24-hour induced NEUROG3 variants.

(C) Immunofluorescence analysis of CHGA (green), SYP (red) and ECAD (blue) of HIOs with 100ng/ml 8-hour and 24-hour induced NEUROG3 variants.

(D) Quantification of $CHGA^+$, INS^+, GCG^+ and SST^+ cells of pancreatic endocrine with 100ng/ml 8-hour and 24-hour induced NEUROG3 variants.

(E) Western blot analysis of NEUROG3 and ACTIN in pancreatic precursors with 100ng/ml 24-hour induced NEUROG3 variants.

(F) Quantification of induced NEUROG3 variants half-life and corresponding decay curve over the 180-minute time course in pancreatic precursors using first order decay function.

(G) Western blot analysis of NEUROG3 (green) and ACTIN (red) in pancreatic precursors with (+) or without (-) phosphatase λ-PP treatment.

(H) EMSA analysis of purified NEUROG3 variants binding activity to high affinity E-box, in the form of NEUROG3-E47 heterodimer and NEUROG3-NEUROG3 homodimer.

For all experiments, the data is representative of a minimum of 2 separate experiments. A minimum of 10 organoids were assessed. Scale bar = 100 μm. Data are represented as mean ± SD.

Figure S7

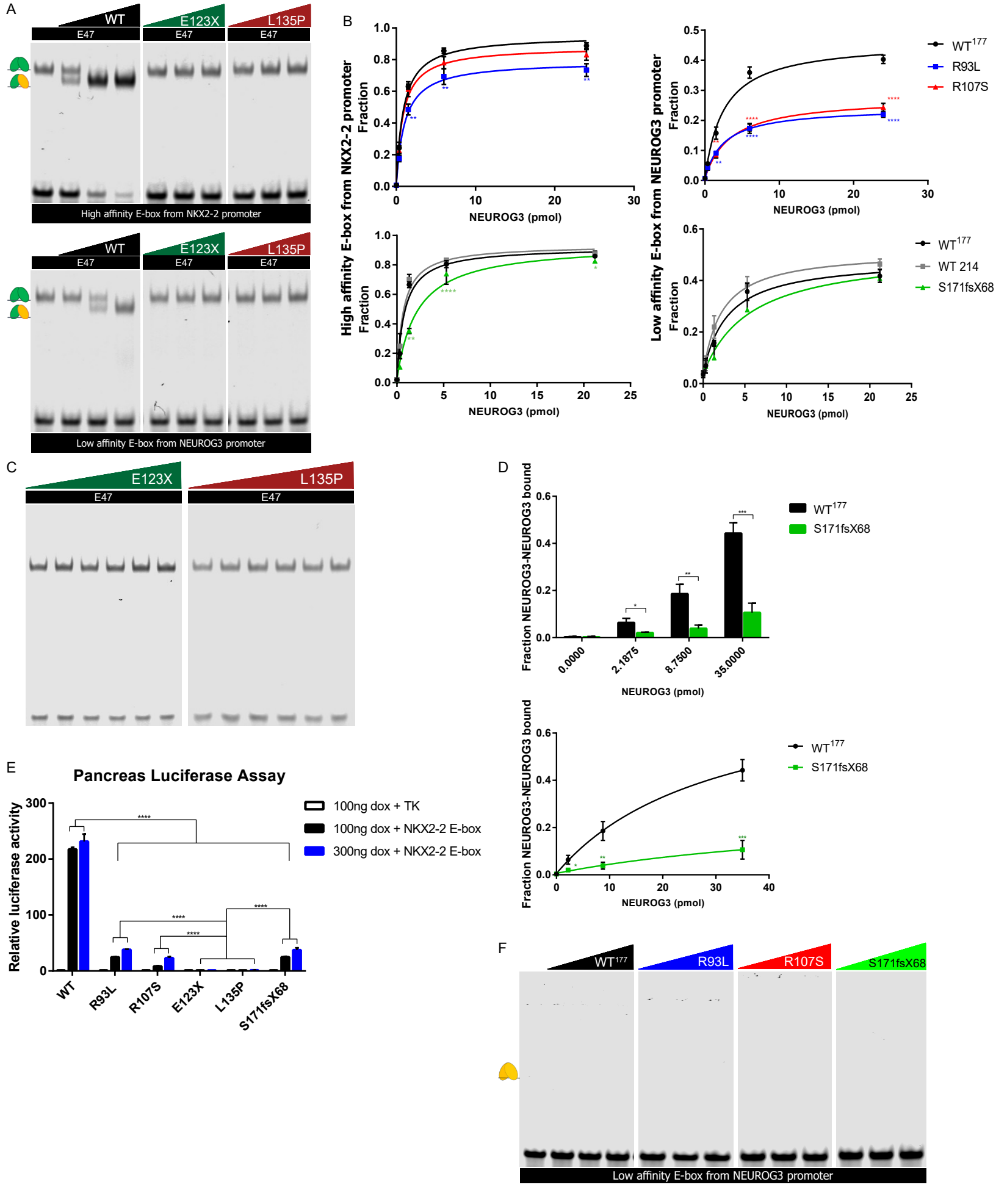

A
WT
E123X
L135P
E47
High affinity E-box from NKX2-2 promoter
Low affinity E-box from NEUROG3 promoter
B
High affinity E-box from NKX2-2 promoter Fraction
Low affinity E-box from NEUROG3 promoter Fraction
NEUROG3 (pmol)
WT177
R93L
R107S
WT 214
S171fsX68
C
D
Fraction NEUROG3-NEUROG3 bound
E
Pancreas Luciferase Assay
Relative luciferase activity
100ng dox + TK
100ng dox + NKX2-2 E-box
300ng dox + NKX2-2 E-box
F

Figure S7: Defective E-box binding affinity of NEUROG3 mutations. Related to Figure 5 and Figure 6.
(A) EMSA analysis of purified NEUROG3 variants heterodimer binding activity to high affinity and low affinity E-box.
(B) Quantification of high affinity and low affinity E-box fraction bound with purified NEUROG3 variants.
(C) EMSA analysis of purified NEUROG3 variants heterodimer binding activity to high affinity E-box.
(D) Quantification of purified NEUROG3 variants homodimer binding activity to high affinity E-box.
(E) Luciferase analysis of *NKX2-2* high affinity E-box promoter activation in pancreatic precursors with 100ng 8-hour, and 300ng-8h doxycycline induction of NEUROG3 variants derived from hESCs.
(F) EMSA analysis of purified NEUROG3 variants homodimer binding activity to low affinity E-box.
For all experiments, the data is representative of a minimum of 3 separate experiments. Data are represented as mean $\pm$ SD. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, and ****p ≤ 0.0001 for two-tailed student's t-test or one-way ANOVA with Tukey HSD or Dunnett T3 (unequal variance) post hoc test.

Table S1

NEUROG3 Mutation	Malabsorptive Diarrhea	Diabetes Onset	Reference
R93L/R93L	Yes	Patient 1: 8 yrs	Wang et al., 2006
		Patient 2: 8 yrs	
E123X/E123X	Yes	Patient 1: 5 mos	Pinney et al, 2011
R107S/R107S	Yes	Patient 1: 12 yrs	Rubio-Cabezas et al., 2014
		Patient 2: >23 yrs	
		Patient 3: >2 yrs	Wang et al., 2006
L135P/L135P	Yes	Patient 1: 3 wks	Rubio-Cabezas et al., 2014
		Patient 2: 13 yrs	
E28X/L135P	Yes	Patient 2: 13 yrs	Rubio-Cabezas et al., 2011
S171fsX68/S171fsX68	Yes	Patient 1: >20 mos	Sayar et al., 2013
Q4X/Q4X	Yes	Patient 1: 2 mos	Hancili et al., 2017
		Patient 2: 13 days	

Table S1: Reported NEUROG3 mutations and related patient phenotypes.

Table S2

NEUROG3 Mutation	Protein Stability		DNA binding		Biological Activity	
	Current	Previous	Current	Previous	Current	Previous
WT	+++	+++	+++	+++	+++	+++
T120A	+++	+	++	++	+	+++
S183A	+++	++++	+++	/	+++	++++
S204A207A	+++	/	+++	/	+++	-

Table S2: Comparison of current NEUROG3 phosphorylation mutation effects with previous reports.