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Supplemental information

Limitations of fluorescent timer protein maturation kinetics to isolate transcriptionally synchronized human neural progenitor cells

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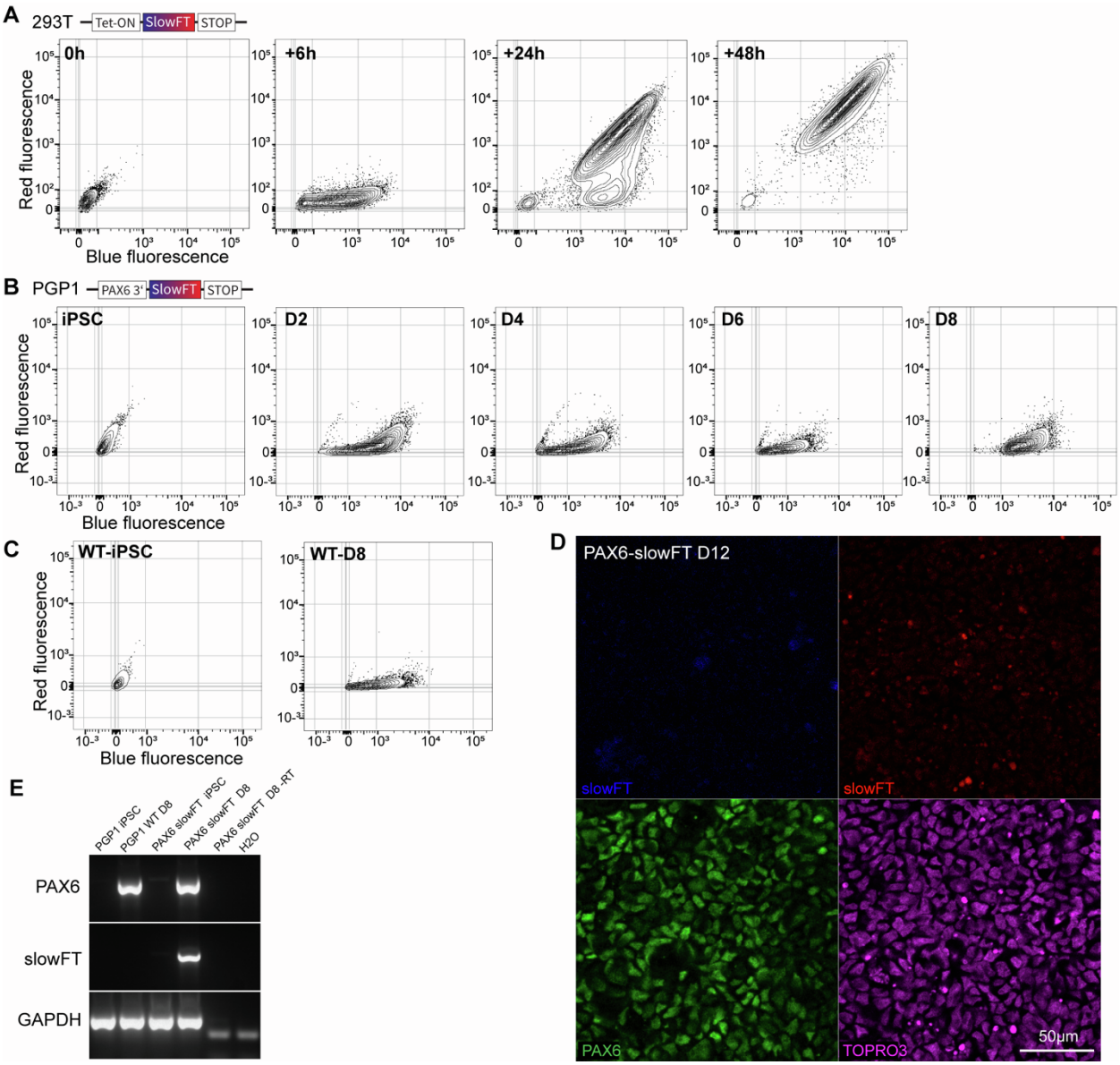
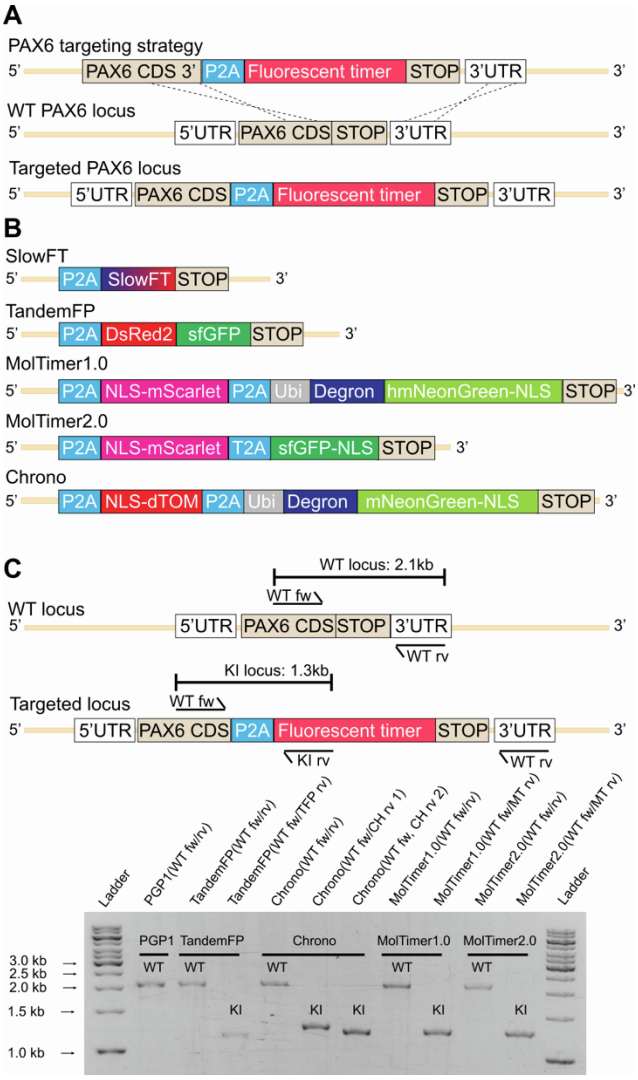
Figure S1: SlowFT expression has distinct dynamics in 293T cells vs. neural progenitor cells, related to Figure 1



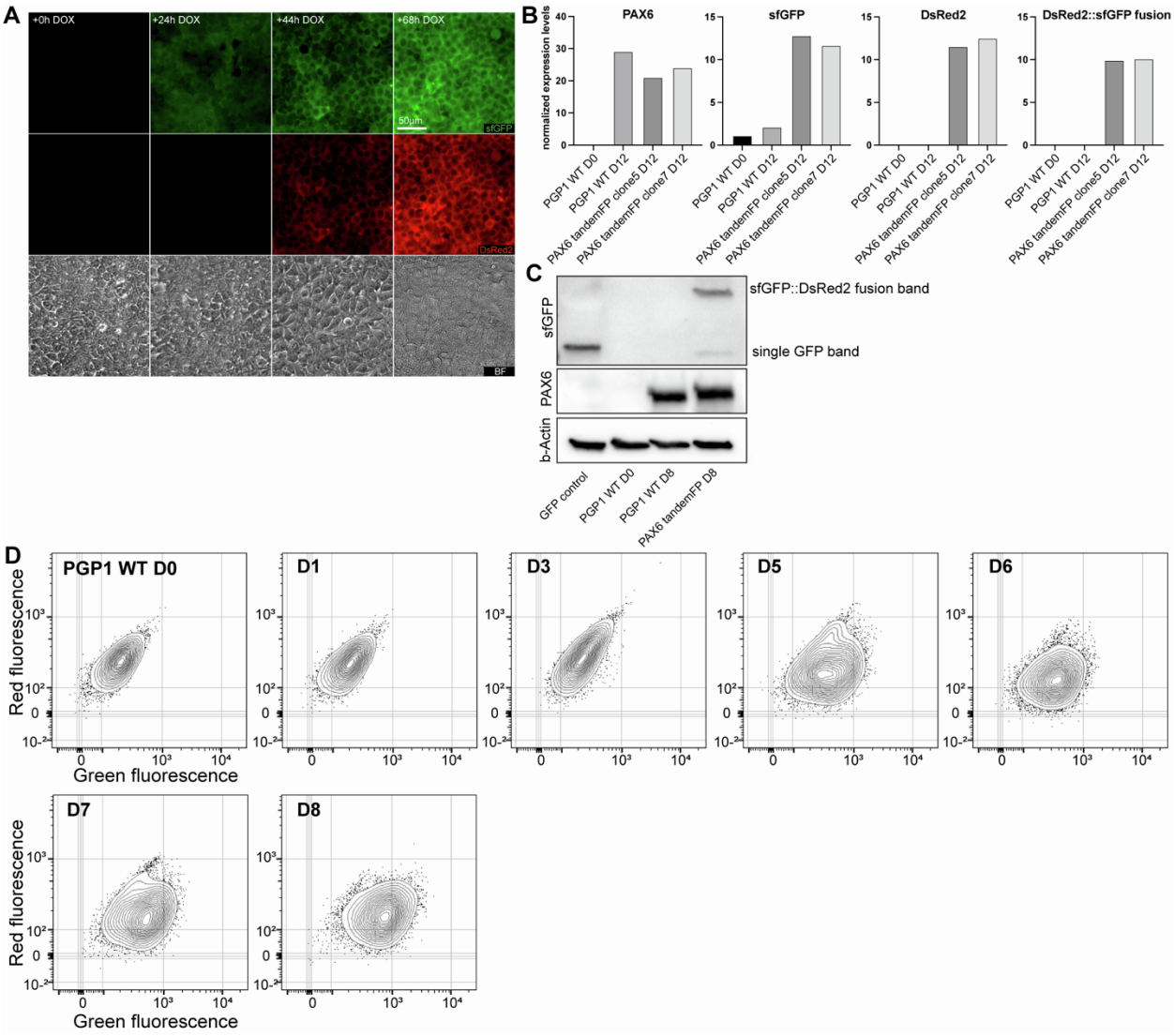
A: FACS plots of 293T cell expressing SlowFT from the Tet-ON promoter. Expression was induced by adding Dox to the cell culture medium. Cells were harvested and analyzed 6h, 24h, and 48h later. Blue fluorescence strongly increases by 6h after induction, followed by most cells displaying both blue and red fluorescence at 24h and 48h. B: FACS plots of PGP-1 iPSC-derived neural progenitor cells expressing SlowFT from the endogenous PAX6 promoter. PGP1-PAX6-SlowFT iPSCs were induced to undergo neural differentiation at D0. Neural progenitors were harvested and analyzed daily for eight days. Blue fluorescence strongly increased by D2, without similar increase of red fluorescence through D8. C: FACS plots of wild type (WT) PGP1 iPSCs and WT D8 neural progenitor cells. Note the strong increase of blue fluorescence in WT cells that do not express SlowFT. D: Confocal imaging stack of PAX6-slowFT neural progenitor cells at day 12. No blue or red cellular fluorescence is detected. Bright red signal is fluorescent debris outside of cell bodies. E: RT-PCR of WT and PAX6-SlowFT iPSCs and neural progenitor cells. PAX6 is highly expressed by both WT and PAX6-SlowFT neural progenitor cells. PAX6-SlowFT mRNA is only detected in PAX6-SlowFT neural progenitor cells.

Figure S2: Targeting strategy enables expression of fluorescent timer constructs under control of the human PAX6 promotor, related to Figures 1, 2, 3, 4, S1, and S6



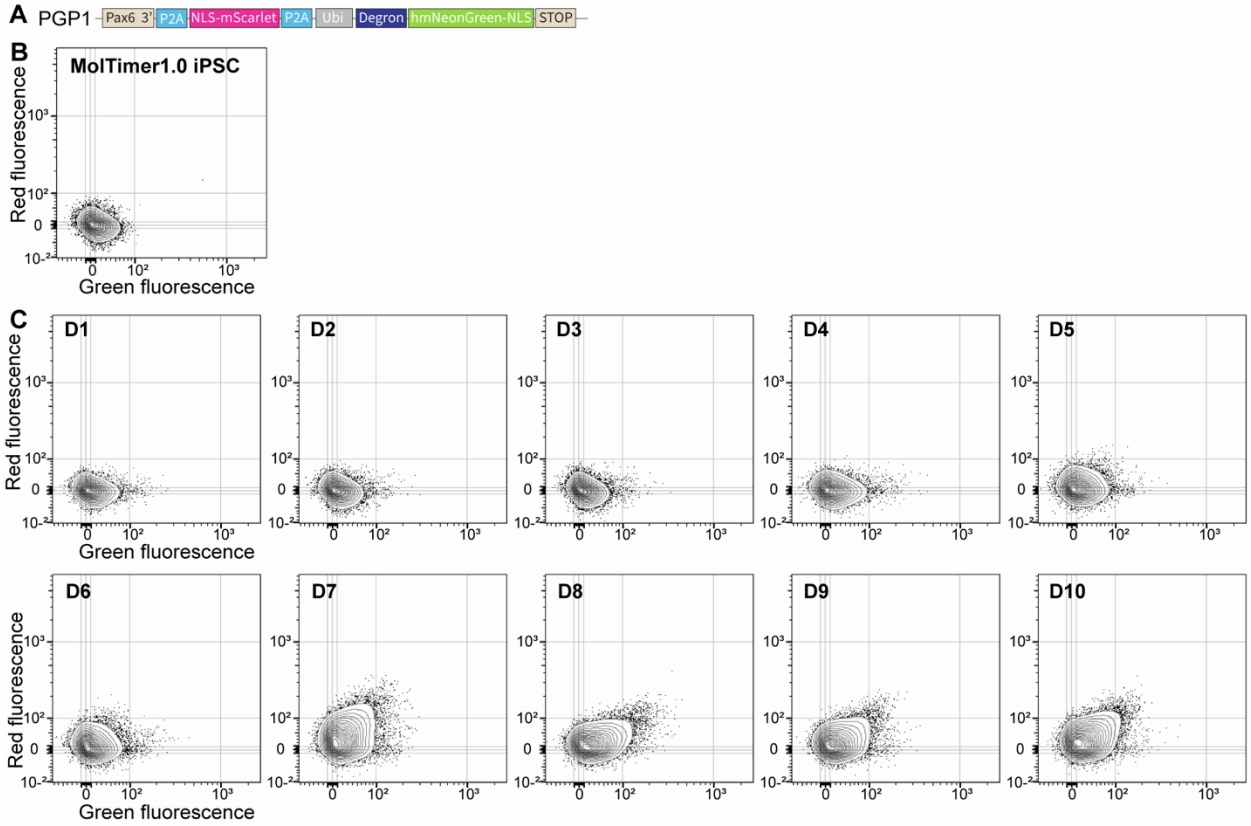
A: Targeting strategy for the integration of fluorescent timer constructs and their expression from the endogenous PAX6 promoter. The 3' stop codon of the PAX6 gene was removed, and each fluorescent timer construct together with a P2A site was integrated in frame with the PAX6 gene. B: Fluorescent timer constructs used in this study. SlowFT consists of a single fluorescent timer molecule that matures and changes color from blue to red. TandemFP is a fusion of the red FP DsRed2 and the green FP sfGFP. MolTimer1.0 consists of a nuclear localized (NLS) mScarlet and NLS humanized hmNeonGreen separated by a P2A site. hmNeonGreen is destabilized with a Degron. MolTimer2.0 consists of NLS mScarlet and NLS sfGFP separated by a T2A site. Chrono[1] consists of NLS dTom and a destabilized NLS mNeonGreen separated with a P2A site; it was used as a comparison strategy published following development and application in mice. C: Genotyping strategy for FT clones (top): Correct PAX6 integration was assayed using two or three primer pairs targeting the WT and knock-in (KI) loci. The WT locus yields a 2.1kb PCR product, and the KI locus yields either a 1.2kb (TandemFP, MolTimer1.0, MolTimer2.0, Chrono pair two) or 1.3kb (Chrono pair one) PCR product. Bottom: Agarose gel of genotyping PCR products showing that all PAX6-FT iPSCs are heterozygous.

Figure S3: TandemFP is expressed appropriately by both 293T cells and human iPSC-derived neural progenitor cells at both RNA and protein levels, related to Figure 1



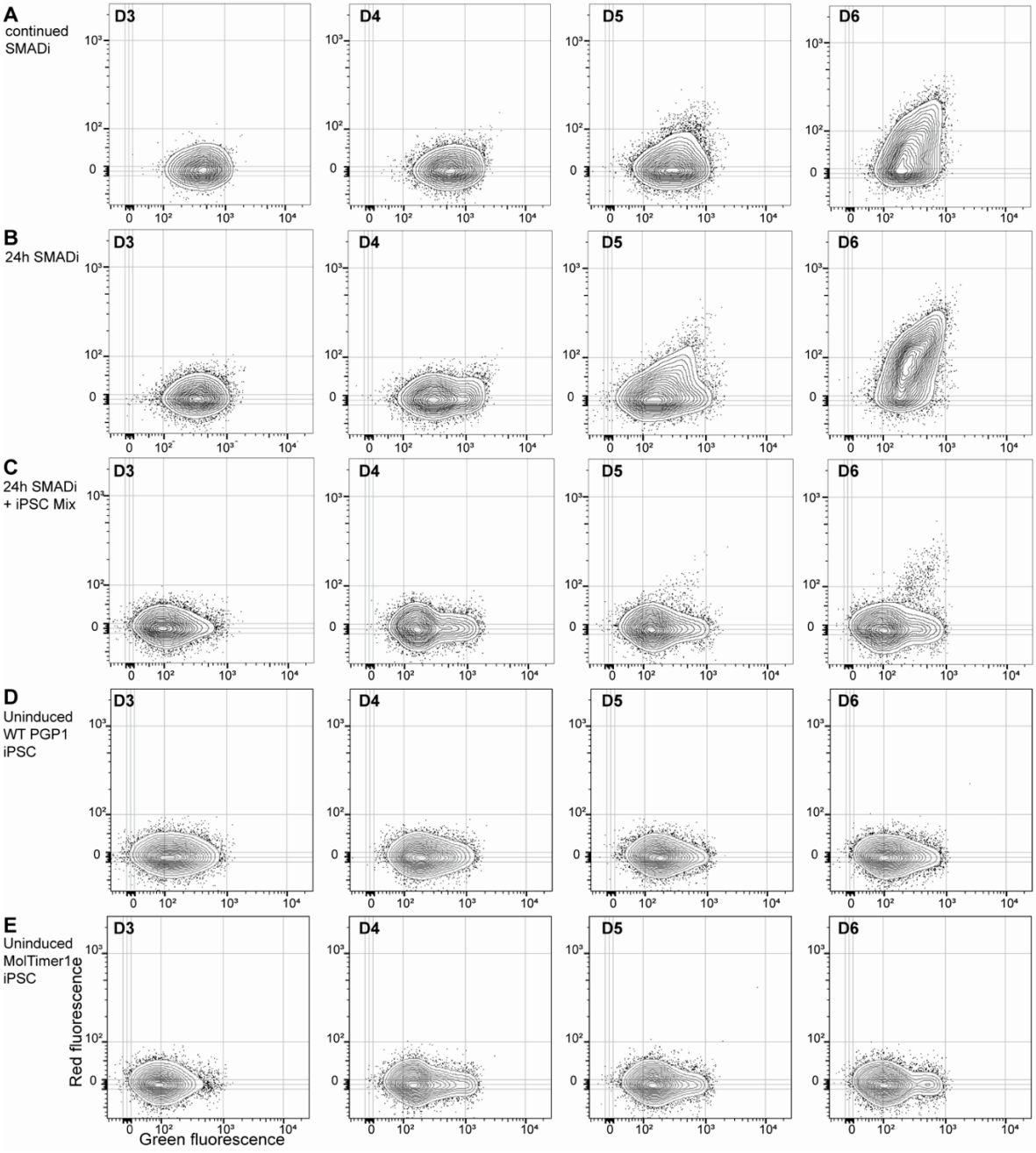
A: Fluorescence and brightfield images of TandemFP-expressing 293T cells after Dox induction. Green fluorescence is detected by 24h after induction. Green and red double-positive cells appear by 24h and increase in number and fluorescence intensity thereafter. B: qPCR of WT PGP1 hiPSCs and PGP1-PAX6-tandemFP expressing hiPSC and neural progenitor cells. The sfGFP, DsRed, and sfGFP::DsRed2 fusion mRNAs increase along with PAX6 in PGP1-PAX6-tandemFP neural progenitor cells. C: WB for PAX6 and sfGFP of WT PGP1 and PGP1-PAX6-tandemFP progenitor cells. The sfGFP::DsRed2 fusion band is detected in PGP1-PAX6-tandemFP neural progenitor cells, indicating both expression and translation of the TandemFP fusion protein. D: FACS plot of PFA-fixed, uninduced WT PGP1 iPSC and PGP1-PAX6-TandemFP neural progenitor cells. PGP1-PAX6-TandemFP iPSC were induced at day (D) 0, and neural progenitors were harvested and analyzed from D1 – D8. Green fluorescence is detected by D5, but no red fluorescence is detected by D8.

Figure S4: Fluorescence time course of MolTimer1.0 expressed by human iPSC-derived neural progenitor cells, related to Figure 2



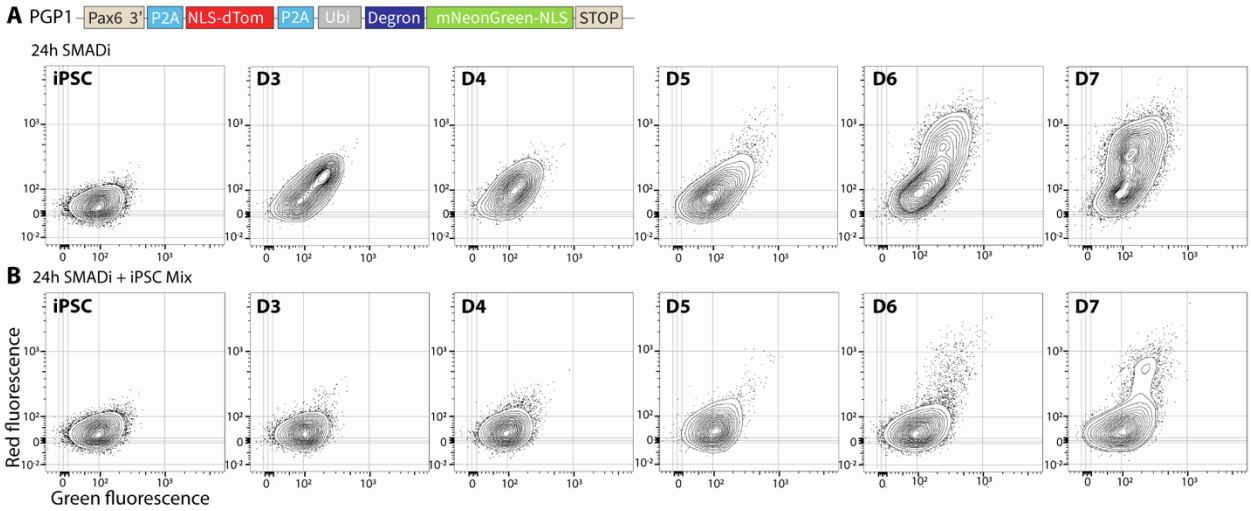
A: Schematic of MolTimer1.0 expression construct. B: FACS plot of uninduced, PFA-fixed PGP1-PAX6-MolTimer1.0 iPSCs C: FACS plots of PFA fixed, human iPSC-derived neural progenitor cells expressing MolTimer1.0 from the endogenous PAX6 promoter. PGP1-PAX6-MolTimer1.0 iPSCs were induced at day (D) 0, and neural progenitors were harvested and analyzed daily from D1 – D10. Green and red double-positive cells start to appear from D6 onward, with increasing fluorescence intensity until D10.

Figure S5: Dynamic live cell FACS analysis of MolTimer1.0 expression by human iPSC-derived neural progenitor cells, related to Figure 2



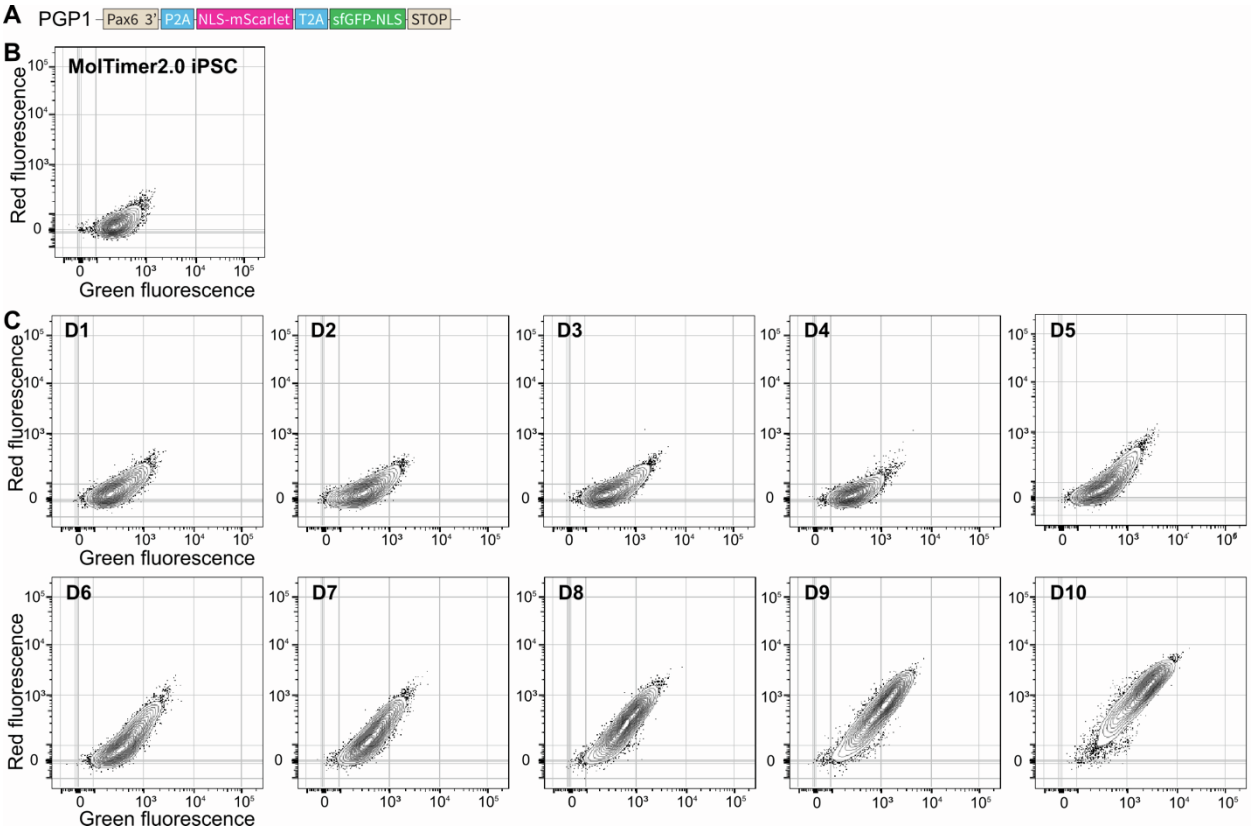
A: FACS plots of live PGP1-PAX6-MolTimer1.0 neural progenitor cells. Neural induction was induced with continuous dual SMAD inhibition (SMADi). Red fluorescent cells appear by D4, and increase in number and intensity through D6. Weakly green fluorescent cells appear by D5. B: FACS plots of PGP1-PAX6-MolTimer1.0 neural progenitor cells that were induced by a pulse of SMADi for 24h from D0 to D1, yielding very similar results as continuous SMADi induction. C: FACS plots of MolTimer1.0expressing neural progenitors that were mixed at a 1:20 ratio with uninduced PGP1-PAX6-MolTimer1.0 iPSC prior to analysis. Red fluorescent cells can be detected by D5 against a background of >95% non-fluorescent iPSCs. D: FACS plots of uninduced WT PGP1 iPSC on D3 – D6. E: FACS plots of uninduced PGP1-PAX6-MolTimer1.0 iPSC on D3 – D6.

Figure S6: Dynamic live cell FACS analysis of Chrono[1] expression by human iPSC-derived neural progenitor cells, related to Figure 2



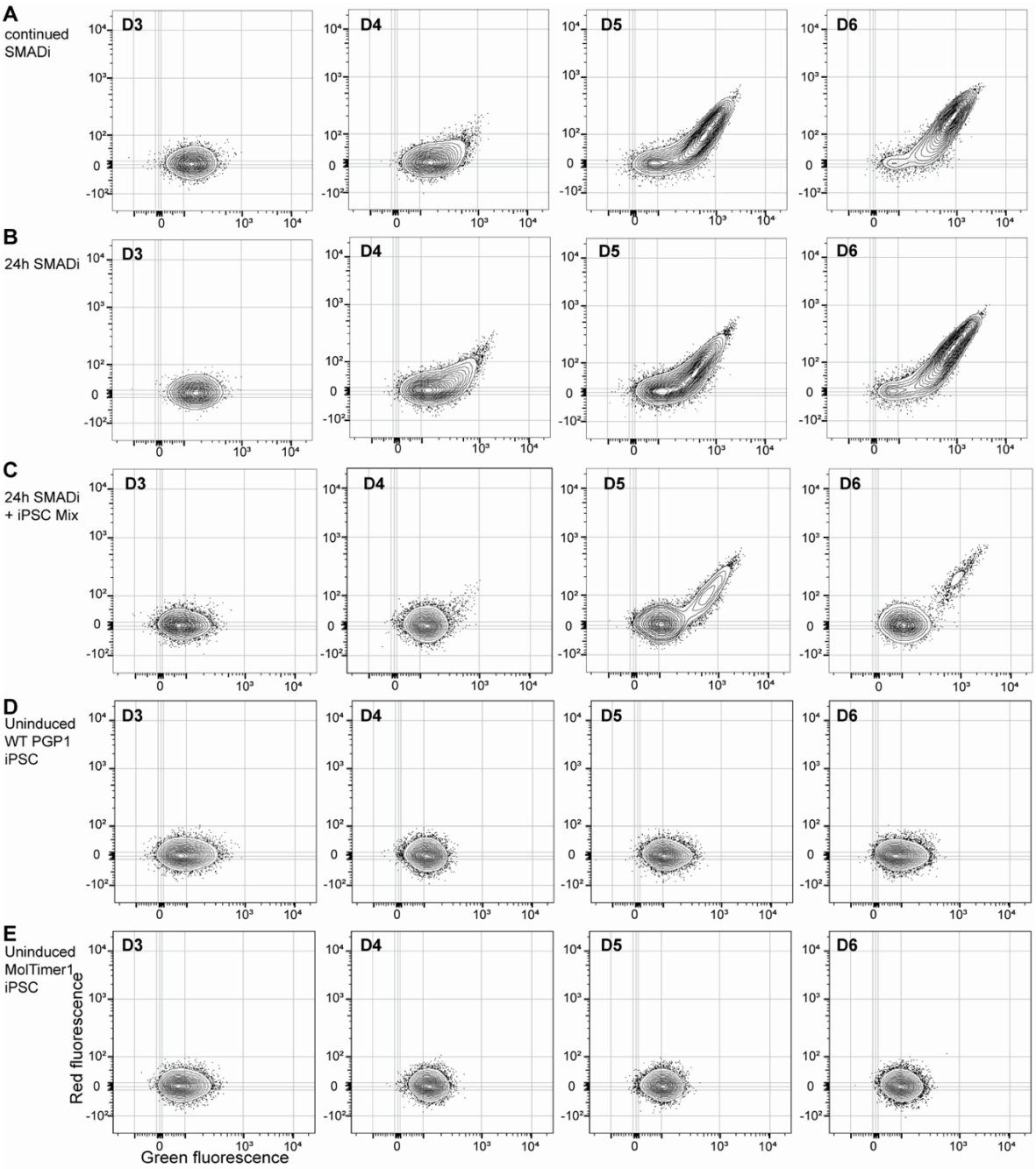
A: FACS plots of live PGP1-PAX6-Chrono neural progenitor cells. Neural differentiation was induced for 24h with SMADi. Red and green double-positive cells can be detected by D3, with increased numbers of cells and fluorescence intensity through D6-7. B: FACS plots of undifferentiated PGP1-PAX6-Chrono iPSCs and Chrono-expressing neural progenitors mixed at a 1:20 ratio with undifferentiated PGP1-PAX6-Chrono iPSC prior to analysis, indicating that red and green double-positive cells can be detected by D3 against a background of >95% non-fluorescent hiPSCs. No green or red signal is detected in undifferentiated PGP1-PAX6-Chrono iPSCs.

Figure S7: Fluorescence time course of MolTimer2.0 expressed by human iPSC-derived neural progenitor cells, related to Figures 3 and 4



A: Schematic of MolTimer2.0 expression construct. B: FACS plot of PFA-fixed, uninduced PGP1-PAX6-MolTimer2.0 iPSCs C: FACS plots of PFA-fixed human iPSC-derived neural progenitor cells expressing MolTimer1.0 from the endogenous PAX6 promoter. PGP1-PAX6-MolTimer1.0 iPSCs were induced at day (D) 0, and neural progenitors were harvested and analyzed daily from D1 – D10. Green and red double-positive cells start to appear from D5 onward, with increasing fluorescence intensity until D10.

Figure S8: Dynamic live cell FACS analysis of MolTimer2.0 expression by human iPSC-derived neural progenitor cells, related to Figures 3 and 4



A: FACS plots of live PGP1-PAX6-MolTimer2.0 hiPSC-derived neural progenitor cells. Neural differentiation was induced with continuous SMADi. Red and green double-positive cells can be detected by D4, with increasing number and fluorescence intensity through D6. B: FACS plots of MolTimer2.0 neural progenitor cells that were induced by a 24h pulse of SMADi from D0 to D1. C: FACS plots of MolTimer2.0-expressing neural progenitors that were mixed at a 1:20 ratio with undifferentiated PGP1-PAX6-MolTimer2.0 hiPSCs prior to analysis. Red and green double-positive fluorescent cells can be detected by D4 against a background of >95% non-fluorescent hiPSCs. D: FACS plots of uninduced WT PGP1 iPSC on D3 – D6. E: FACS plots of uninduced PGP1-PAX6-MolTimer2.0 iPSC on D3 – D6.

Table S1: Primers used in this study, related to STAR methods

Primer name	Sequence 5' - 3'
NeonGreen_FW	GCAAAGGCGAAGAAGACAAC
NeonGreen_RV	CAGTACCCTGTCCAACCATATC
sfGFP_FW	GCTGAAGGGCATCGACTT
sfGFP_RV	CTTGTCGGCGGTGATATAGAC
mScarlet_FW	GTGCTGAAGGGCGACATTA
mScarlet_RV	CATCTGCACGGGCTTCTT
HPRT1_FW	ACCCTTTCCAAATCCTCAGC
HPRT1_RV	GTTATGGCGACCCGCAG
PAX6_FW	GTGTCCAACGGATGTGTGAG
PAX6_RV	CTAGCCAGGTTGCGAAGAAC
sfGFP_tandem_FW	GAACCGCATCGAGCTGAA
sfGFP_tandem_RV	CTTGTCGGCGGTGATATAGAC
DsRed2_tandem_FW	AGTTCATGCGCTTCAAGGT
DsRed2_tandem_RV	TTCAGCTTCACGGTGTTGT
DsRED_sfGFP_fusion_FW	TACACCATCGTGGAGCAGTA
DsRED_sfGFP_fusion_RV	GTTTACGTCGCCGTCCAG
SlowFT_RT_FW	CAGTCAGCCAATGGGCACCTC
SlowFT_RT_RV	CCTCGCCCTTGCTCACCATG
GAPDH_FW	AATGAAGGGGTCATTGATGG
GAPDH_RV	AATGAAGGGGTCATTGATGG
WT fw	GGAATTCCAGTACTTCACGTGAAGGCATC
WT rv	GTGAAAGTAACCATTGGTTTAGAATGTTG
TandemFP rv	CCATGCGCACCTTGAAGCGC
CH rv1 (Chrono)	CTTCAGCTTGGCGGTCTGGGTGC
CH rv2 (Chrono)	CGAACTCGTGGCCGTTCATGG
MT rv (MolTimer)	CATGGAGCCCTCCATGTGCACCTTG

Supplemental reference list

1. Gehart, H., van Es, J.H., Hamer, K., Beumer, J., Kretzschmar, K., Dekkers, J.F., Rios, A., and Clevers, H. (2019). Identification of Enteroendocrine Regulators by Real-Time Single-Cell Differentiation Mapping. Cell *176*, 1158-1173 e1116. 10.1016/j.cell.2018.12.029.