

## **Supplementary Information for “Rapid neurogenesis through transcriptional activation in human stem cells”**

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### ***Table of contents***

- **Supplementary Text**
- **Supplementary Materials and Methods**
- **Supplementary Video S1** (as a separate file)
- **Supplementary References**
- **Supplementary Figures and Legends: S1 – S13** (Figures included in Supplementary Information file)
- **Supplementary Tables S1 – S8** (combined in one separate excel file, one spreadsheet per table)

### **Supplementary Text**

#### **Neuronal content cannot explain higher similarity to specific brain regions**

To test if some brain regions show higher correlation to our iNGN cells solely for having a higher proportion of neurons in the tissue, we looked deeper into the comparison of brain regions to the 500 most highly differentially expressed gene in our iNGN cells, as reported in the main text. To test this idea, we took GFAP expression levels (a proxy for

glial cell abundance in each tissue sample) and looked to see if the expression is anti-correlated with the Z-scores for each brain region at each time point. If brain region association was the result of higher neuron populations, then one would expect that regions with a higher Z-score would have lower glial cell count (or lower GFAP expression in our analysis). However, only two time points showed anti-correlation: 9 post conception weeks and 37 years, but the p-values were not significant ( $p = 0.45$  and  $p = 0.2$ , respective). This suggests that higher Z-scores in our comparison to the various brain regions analysis do not result from the brain regions having higher neuron populations.

### **Some details on transcription factors that could help drive the rapid neurogenesis**

The rapid acquisition of homogeneous bipolar neurons following Neurogenin induction suggests that there exists a robust gene regulatory network underlying the response. To find transcription factors that contribute to this regulatory network, we analyzed the time-course of mRNA and miRNA expression data in the context of known transcriptional regulatory network processes using Ingenuity's IPA database.

Over the course of differentiation, iNGN cells lost their initial pluripotency. By day 1, common stem cell markers were repressed, as shown in Figure 2A in the main text. By day 1, POU5F1 (OCT4) regulatory targets were differentially expressed ( $p = 7.8 \times 10^{-5}$ ), and changed consistent with decreased POU5F1 activity (Figure 5A). Similarly, expression of targets for NANOG and SOX2 were significantly downregulated ( $p < 7.2 \times 10^{-4}$ ), a finding consistent with the inhibition of their regulatory activities (Figure 5A). Thus, the down regulation of NANOG, POU5F1, and SOX2 is accompanied with the

inhibition of their activities. Our data suggest that by day 1 (Figure 5A), the Neurogenins likely inhibit SOX2, NANOG and POU5F1. In accordance with published work (Evsen et al, 2013; Li et al, 2012; Sun et al, 2001) that suggests that NEUROG2 could inhibit stem cell factors by directly activating NEUROD1 or activating it after binding a p300/CREBBP complex (Sun et al, 2001), we found that downstream targets of these factors were all significantly changing consistent with the possibility of them contributing to the differentiation process. In addition, NEUROD1 activation could also be inhibiting SOX2 (Evsen et al, 2013). Additional transcriptional regulators may reinforce the inhibition of stem cell factors based on our analysis, centered on STAT3 repression. It has been shown that STAT3 phosphorylation maintains pluripotency by enabling the binding to distal enhancers of POU5F1 and NANOG (Do et al, 2013). After day 1, our analysis suggests that STAT3 activity drops considerably in iNGN cells. This might occur as p300/CREBBP is sequestered by NEUROG1 (Sun et al, 2001), which, based on previous literature could indirectly suppress STAT3 activity (Nakashima et al, 1999). This suppression of STAT3 is reinforced as the STAT3 repression of SNAI1 is relieved, leading to the activation of SPARC, which further decreases STAT3 phosphorylation (Bhoopathi et al, 2011). The sequestered p300/CREBBP complex could also fail to activate FOXO1 (Yamagata et al, 2004), which would subsequently be unable to activate POU5F1 and SOX2 (Zhang et al, 2011). In summary, our pathway analysis revealed potential connections of Neurogenins with repression of stem cell factors enabling the destabilization of the stem cell state.

Alongside the increased inhibition of stem cell factors, several neuronal transcription factors are predicted to be activated in our data (Figure 5B). NEUROG1 and NEUROG2

likely activate NEUROD1 (Roybon et al, 2010), a key factor in adult neurogenesis (Gao et al, 2009), and our data suggests that its regulatory functions are strongly activated on day 1 and fortified each day thereafter (Figure 5B). NEUROD1 could subsequently activate other neuronal transcription factors including NEUROD2. The Neurogenin expression also induces neuronal transcription factors, such as ISL1, PAX6, POU3F2, POU4F1, TLX3 and ZEB1. In addition to the activation of many transcription factors, inhibitors of neurogenesis showed suppression of their activity over time, including HES1 and REST. Our transcriptomic data shows increasing inhibition of REST regulation each subsequent day ( $p < 0.003$ ; Figure 5B) that was accompanied by the upregulation of many of its targets. While there are not any known direct connections between the Neurogenins and REST, it is known that REST is activated by POU5F1 and NANOG (Campbell et al, 2007; Soldati et al, 2012) and repressed by NEUROD2 via ZEB1 (Ravanpay et al, 2010). REST suppression would enable activation of a few dozen neuronal genes. Thus, as the Neurogenins activate several core neuronal transcription factors, many downstream neuronal genes were expressed in the iNGN cells resulting in a concerted activation of neuronal fate commitment.

### **Comparison of singly expressed Neurogenin constructs and the iNGN construct in human iPS cells**

In this work, murine Neurog1 and Neurog2 were expressed together from the same construct (iNGN). Previous studies demonstrated that the individual Neurogenins could contribute to neuron differentiation, albeit with lower efficiency (Farah et al, 2000; Stein et al, 2014) or by using a selection marker (Zhang et al, 2013). Here we compared the

differences in gene expression resulting from our iNGN construct and doxycycline-induced constructs with human NEUROG1 or NEUROG2 alone. When this induction was implemented, neurons were obtained from each experimental setup (Figure S13A). While there are considerable similarities in the genes these transcription factors regulate, both had unique genes they regulated (Figure S13 B-D). Indeed, GO term enrichment (Table S9) showed some differences in the classes of genes that were differentially regulated between the two Neurogenins when compared to the construct used in the iNGN cells, suggesting that both transcription factors complement each other in the iNGN cell line.

## **Supplementary Materials and Methods**

### **DNA constructs**

NEUROG1 was PCR-amplified from a plasmid containing the cDNA (MHS1010-57998, Thermo Scientific, Hudson NH). NEUROG2 was PCR-amplified from a plasmid containing the cDNA (MHS1010-9204603, Thermo Scientific, Hudson NH). The respective PCR products were recombined into pDORN221 using BP clonase, yielding pENTR\_L1\_hNgn1\_L2 and pENTR\_L1\_hNgn2\_L2. Sequencing confirmed the correct DNA sequence.

### **Microarray analysis**

PGP1 iPS cells were transfected with lentiviral particles delivering constructs containing NEUROG1 or NEUROG2. These were induced using doxycycline, and induced neurons differentiated over the course of 4 days in mTeSR media. Subsequently, cells were

harvested, and expression profiles were assayed using Agilent microarrays. Arrays were normalized using quantile normalization and differentially expressed genes were determined using the two sample t-test ( $FDR < 0.05$ , with a fold-change greater than a magnitude of  $\log_2(1.5)$ ).

**Supplementary Video S1:** Representative time-lapse recording of differentiating iNGN cells from day 2 to day 4.

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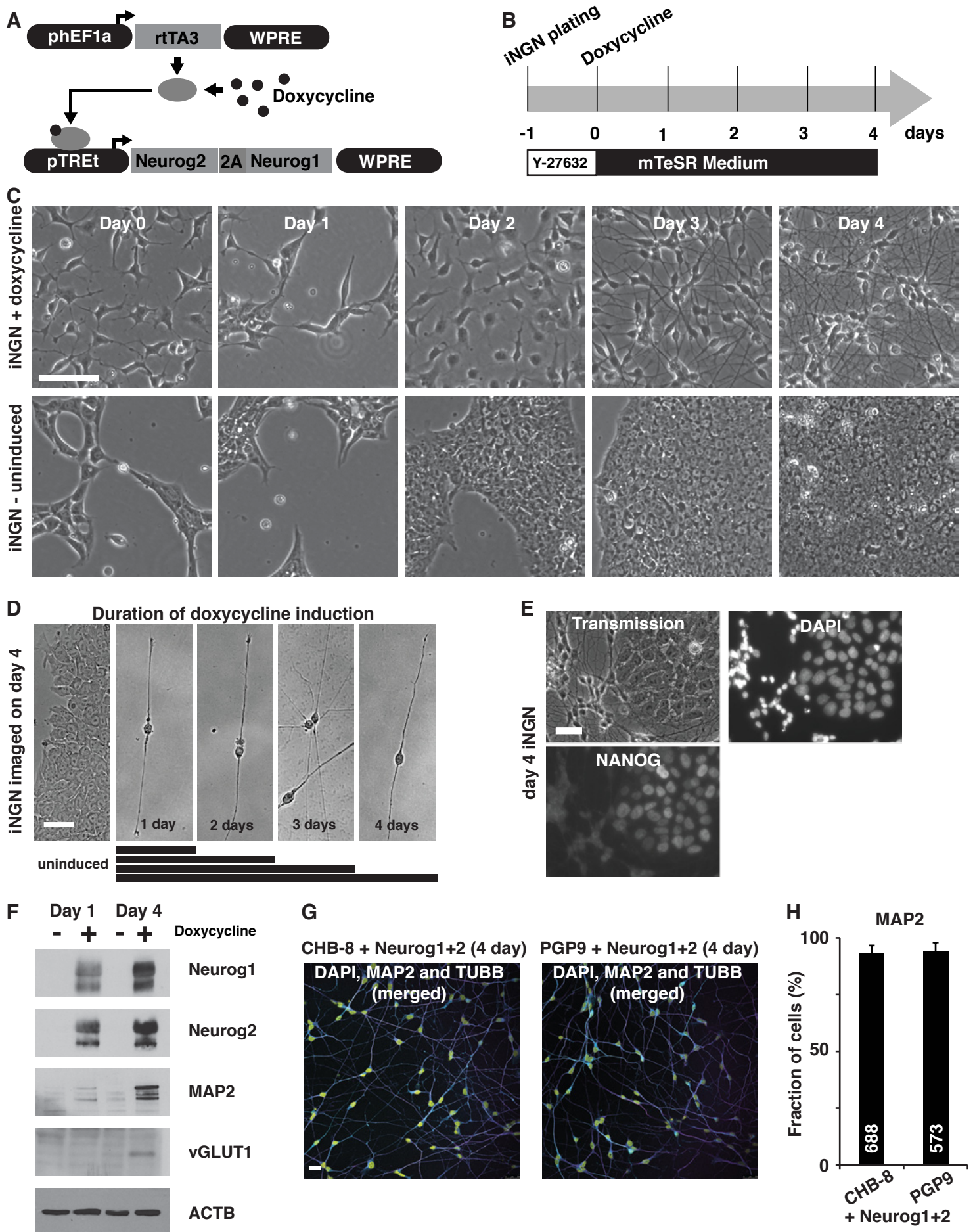
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### **Supplementary Figures and Legends**



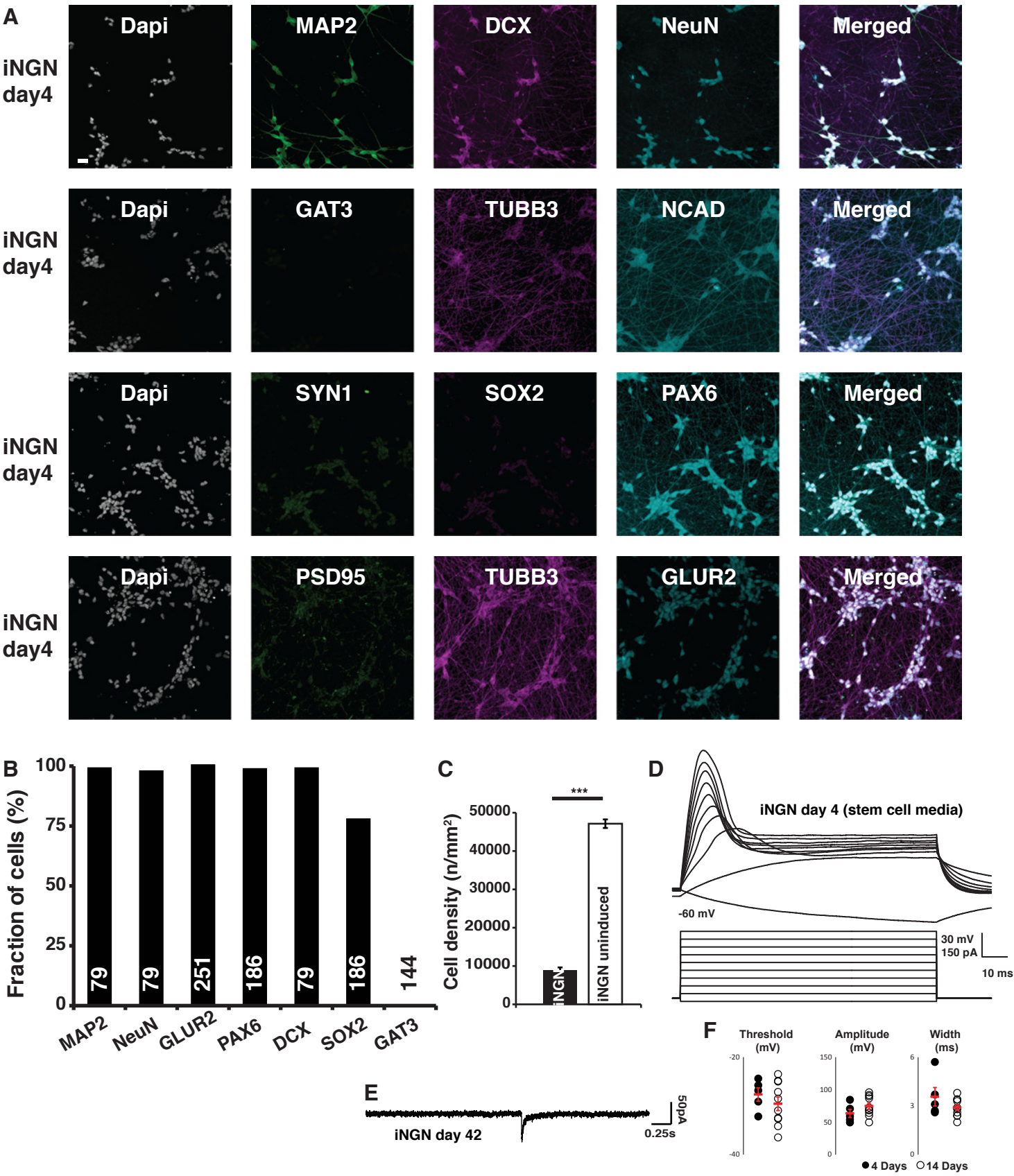
**Figure S1 - Extended characterization of the iNGN cell line.** (A) Schematic outline of the lentiviral constructs used to generate iNGN cells. One construct contains the transcription activator rTA3 that when bound to doxycycline, induces expression of the bicistronic Neurogenin cassette, contained in the second construct. (B) Experimental outline: the cells were maintained in defined iPS media (mTeSR1) and doxycycline was applied at day 0. The Rho Kinase inhibitor Y27632 was applied for 24h after plating. (C) Contrast images illustrating the induction time course, with the doxycycline induced cells in the upper row and uninduced cells in the lower row. (D) Representative transmission light microscopic images of iNGN cells with different lengths of doxycycline treatment, demonstrating that neuron morphology appears to be unaffected by the length of doxycycline treatment; all cells imaged on day 4. (E) Representative NANOG immunostaining of induced neurons next to an iPS colony. Transmission light (TM), DAPI and NANOG channels are shown. (F) Western Blot analyses for Neurog1, Neurog2, MAP2 and vGLUT1 of induced (+doxycycline) or uninduced (-doxycycline) iNGN samples at day 1 and day 4. ACTB served as a loading control. (G) The bicistronic Neurog1+2 construct also rapidly differentiated human ES cells (CHB-8 line, left panel) and another human iPS cell line (PGP9) within four days. Merged channels for DAPI (yellow), MAP2 (cyan) and TUBB3 (magenta) are shown. (H) Quantification of MAP2 positive Neurog1+2-induced CHB-8 and PGP9 cells on day 4. The number indicates the number of analyzed cells of three independent biological samples. Scale bars, 20  $\mu$ m.

Figure S1



**Figure S2 - Neuronal features of iNGN cells.** (A) Representative immunostainings of iNGN cells after 4 days of induction, for MAP2, DCX, NeuN, GAT3, TUBB3, NCAD, SYN1, SOX2, PAX6, PSD95, GLUR2 and nuclear DAPI dye. The cells are positive for the neuronal markers MAP2, TUBB3, NCAD and NeuN as well as for the neural progenitor markers DCX, SOX2 and PAX6. Synaptic markers such as SYN1, PSD95 and GLUR2 were also detected whereas iNGN cells were immuno-negative for the GABA transporter GAT3. (B) Quantification of MAP2, NeuN, GLUR2, PAX6, DCX, SOX2 and GAT3 immuno-positive cells. The number refers to the number of analyzed cells. (C) Cell density of induced and uninduced iNGN cells measured by quantifying DAPI signals in biological triplicates at day 4. (D) Representative whole-cell voltage recording of an immature iNGN cell cultured entirely in stem cell media on day 4 in response to the injection of current as indicated. (E) Representative spontaneous excitatory postsynaptic current recording of a day 42 iNGN cell under neuronal culturing conditions. (F) Action potential threshold (in mV), amplitude (in mV), and width (in ms) at 4 days (filled circles) and 14 days (open circles) post-induction. Circles represent individual recordings. Error bars, standard error of the mean (SEM). p-value, \*\*\* $\leq$  0.001. Scale bars, 20  $\mu$ m.

Figure S2

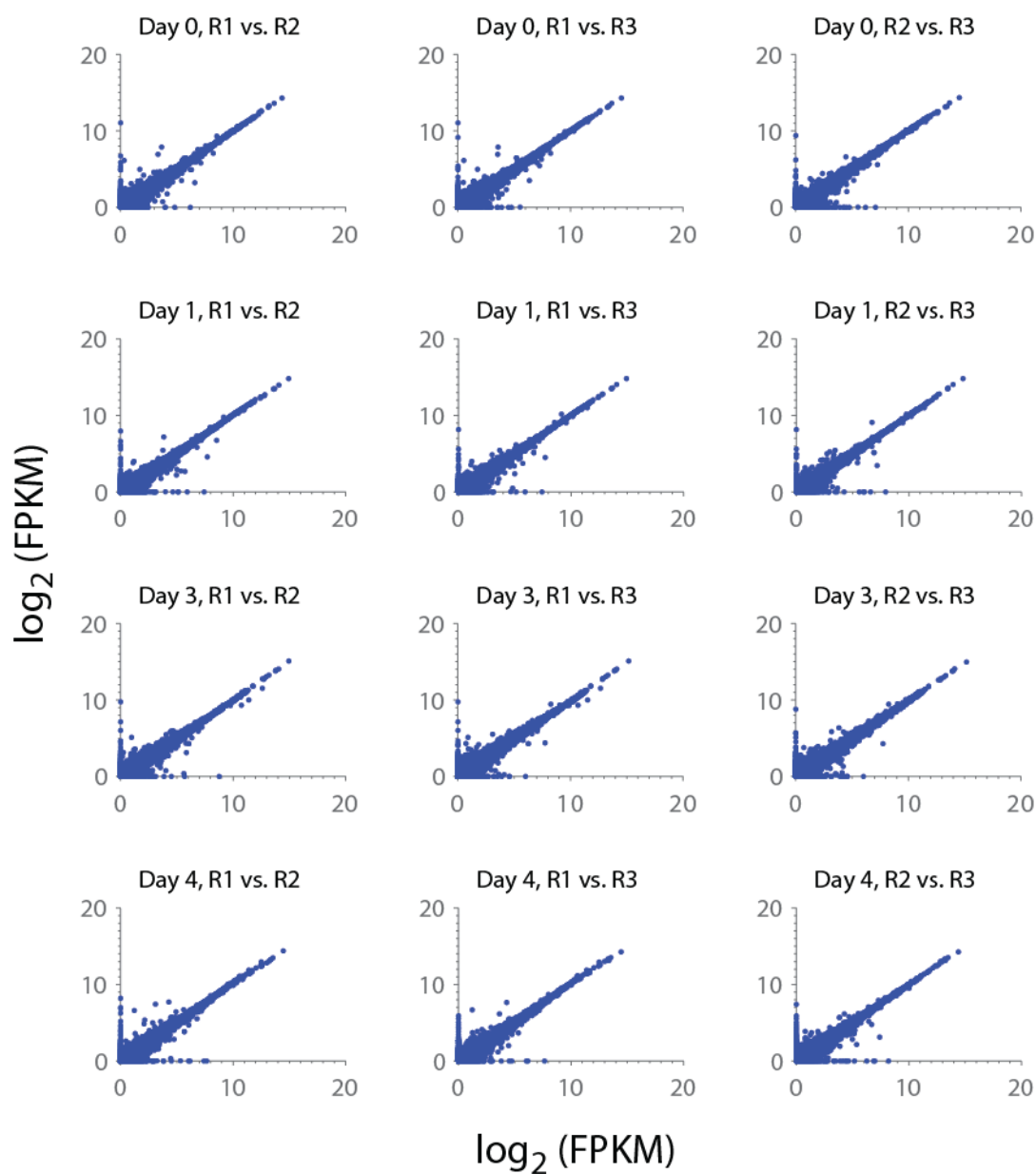


**Figure S3 - RNA-Seq statistics.** (A) Scatter plots comparing biological replicates (R) at each time point. (B) Table showing the number of reads per sample.



**Figure S3**

**A**

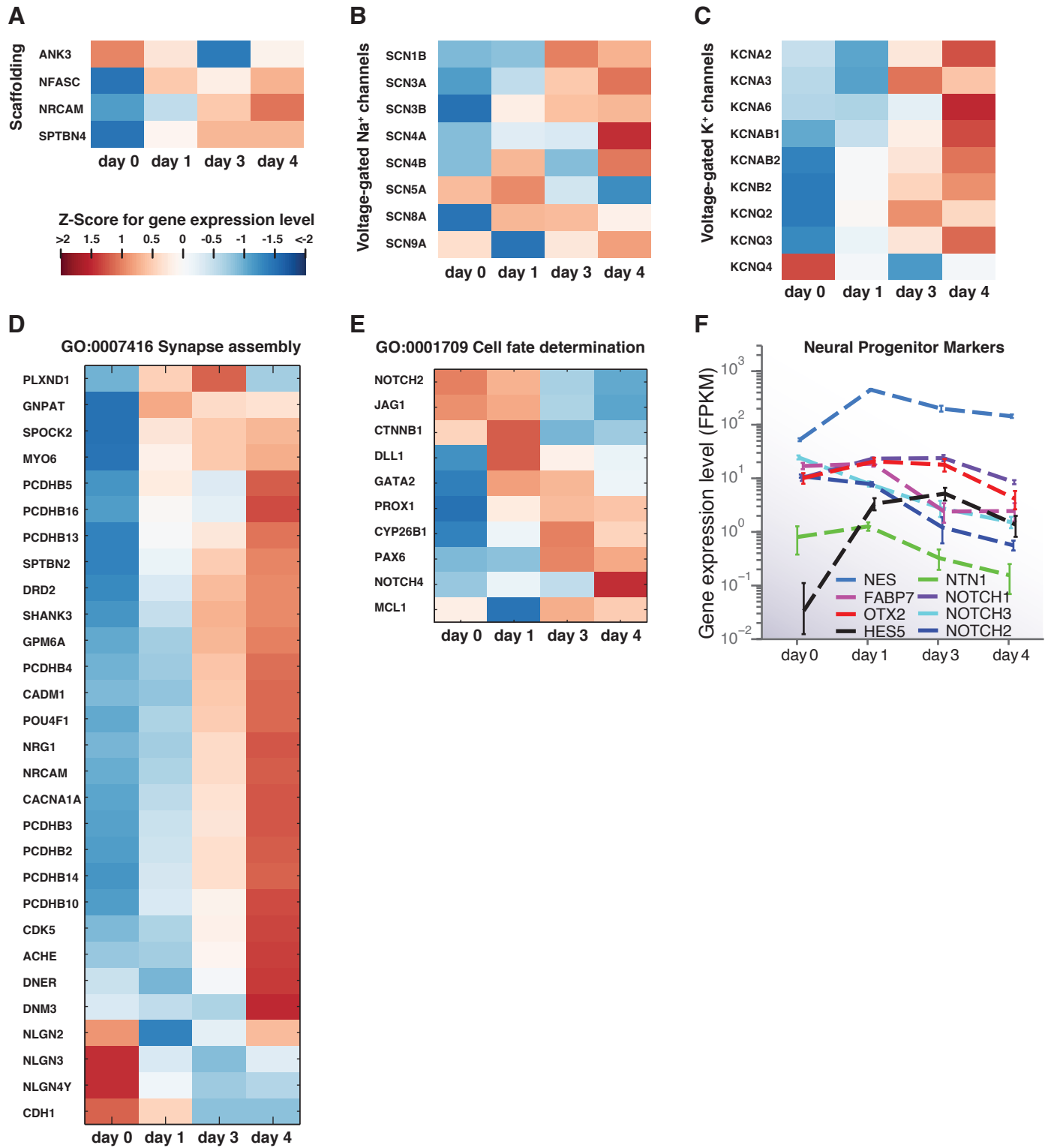


**B**

| Sample:  | Total Reads: | Sample:  | Total Reads: | Sample:  | Total Reads: | Sample:  | Total Reads: |
|----------|--------------|----------|--------------|----------|--------------|----------|--------------|
| Day 0-R1 | 44,476,956   | Day 1-R1 | 39,152,322   | Day 3-R1 | 34,838,784   | Day 4-R1 | 44,028,220   |
| Day 0-R2 | 35,754,396   | Day 1-R2 | 47,420,988   | Day 3-R2 | 39,168,634   | Day 4-R2 | 40,587,180   |
| Day 0-R3 | 40,372,848   | Day 1-R3 | 42,688,716   | Day 3-R3 | 35,508,036   | Day 4-R3 | 39,403,714   |

**Figure S4 - Extended expression analyses of neuronal features.** (A-C) Expression levels of gene categories over days 1-4. Genes associated with the axon hillock and initial segment are significantly upregulated, including transcripts for cell adhesion and ion channel scaffolding proteins (A), voltage-gated sodium channels (B), and voltage-gated potassium channels (C). Most genes in the Gene Ontology classification of “Synapse assembly” (D) are significantly upregulated on day 4 whereas genes of “Cell fate determination” (E) pulse in activation between day 1 and 3. Heat maps represent the Z-score for average expression levels for all isoforms over time. (F) Gene expression levels of neural progenitor markers measured by RNA-Seq in Fragments Per Kilobase of transcript per Million mapped reads (FPKM). Error bars represent the 95% confidence interval of the abundance of the gene.

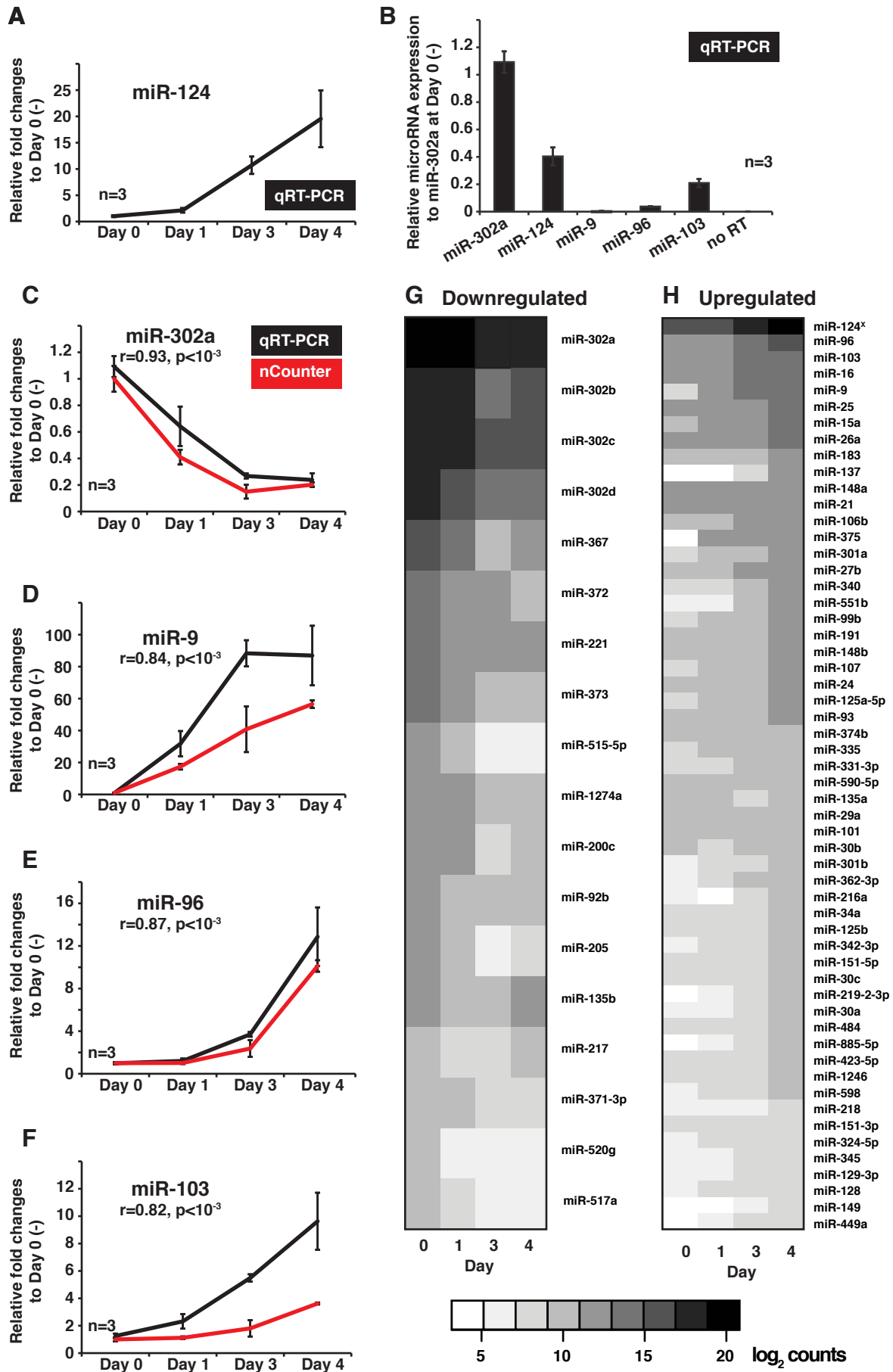
### Figure S4





**Figure S5 - Quantitative RT-PCR (qRT-PCR) for selected miRNAs in biological triplicates.** (A) Relative fold-changes of miR-124 over the course of iNGN differentiation. (B) miRNA levels normalized to miR-302a expression levels at day 0. (C-F) Relative fold-changes of mir-302a (C), miR-9 (D), miR-96 (E) and miR-103 (F) over the course of iNGN differentiation. qRT-PCR (black) and nCounter fold-changes (red) are shown; Pearson correlation coefficients  $r$  and  $p$ -values are indicated, demonstrating good correlations between nCounter and qPCR expression levels. (G, H) Heatmaps of  $\log_2$  miRNA counts assayed by nCounter technology of downregulated (G) and upregulated (H) miRNAs (miR-124<sup>x</sup> refers to expression estimated from qPCR). Only miRNAs that are significantly differentially expressed between day 0 to day 4 and that reach a threshold of at least 500 counts on the nCounter platform at one given time point are shown,  $q$ -values  $< 0.05$ . qRT-PCR data was normalized to 5S rRNA.

Figure S5

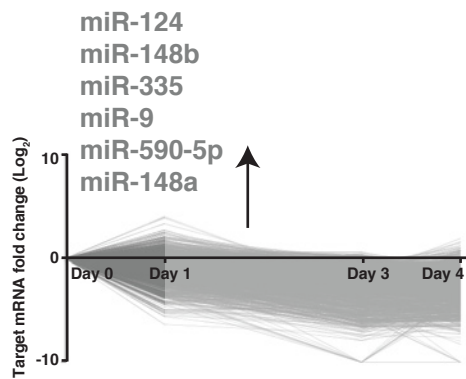
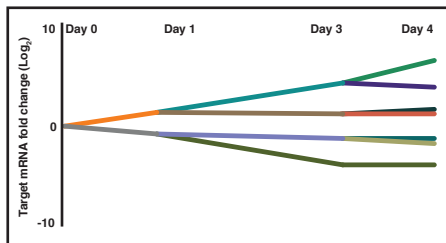
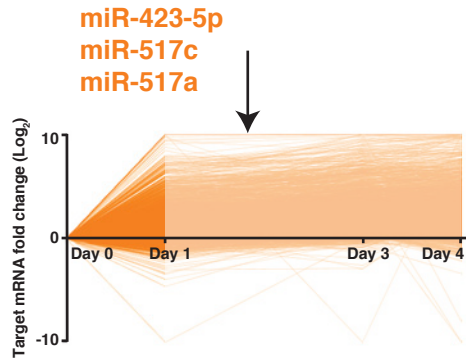


***Figure S6 - The dynamics of gene expression, analyzed in an effort to identify miRNAs that could contribute to the regulation of gene expression over time, using mirDREM***

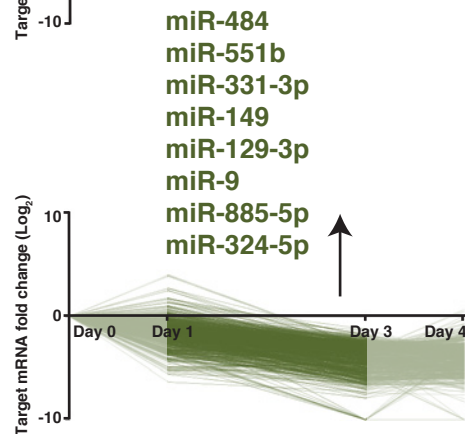
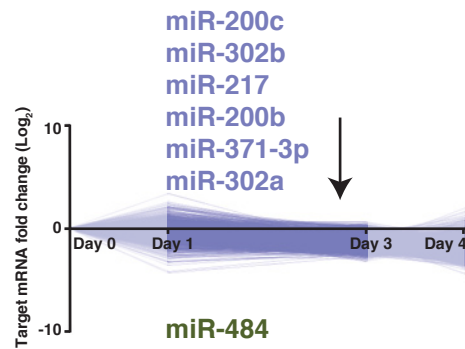
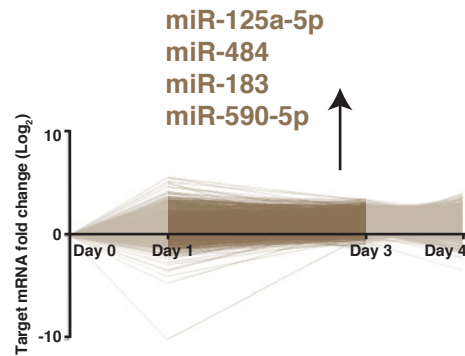
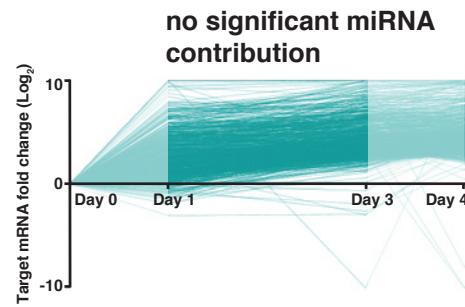
(Schulz et al, 2013). In this method, gene expression profiles with an average FPKM > 0.5 were obtained for all genes that are known to be regulated by a transcriptional regulator with known regulation. Regulated genes included those provided in the mirDREM package and transcriptional regulatory interactions from Ingenuity IPA. These gene expression profiles were clustered such that significant bifurcations (splits in up/down expression) can be found at each day. Then, predicted and known targets (included in mirDREM and obtained from Ingenuity IPA) are used to identify miRNAs that could explain the bifurcation of gene expression changes. For each bifurcation, miRNAs with a p-value for split contribution < 0.1 are shown.

Figure S6

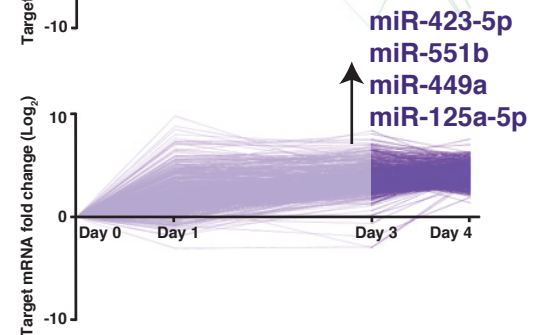
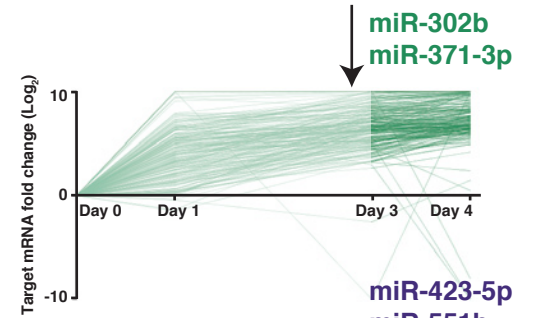
Day 0 to Day 1 changes



Day 1 to Day 3 changes



Day 3 to Day 4 changes



**Figure S7 - Expanded transcription regulatory network during iNGN cell differentiation.** Transcription factors were identified that best matched the daily transcriptomic changes. These were used to construct a transcription regulatory network that best explained the mechanisms underlying Neurogenin-mediated neurogenesis (see Materials and methods for details). **(A)** For each day, each transcription factor received an activation Z-score, which describes how consistent the downstream activation or repression was with the experimentally derived knowledge of downstream activation or repression (Ingenuity IPA). For each day, the computed score represents how strongly the downstream genes expression changes support increased activation (positive) or repression (negative) of the upstream transcription factor for that day. **(B)** In addition, transcription factor targets in our regulatory network were associated with miRNAs that were upregulated (red text) or downregulated (blue text). Interactions were based on experimentally validated interactions, as reported in miRTarBase 4.4. The majority of upregulated miRNAs target repressed transcription factors, and most downregulated miRNAs are targeting activated transcription factors. This suggests that the miRNAs are likely contributing to the activity of the regulatory network.



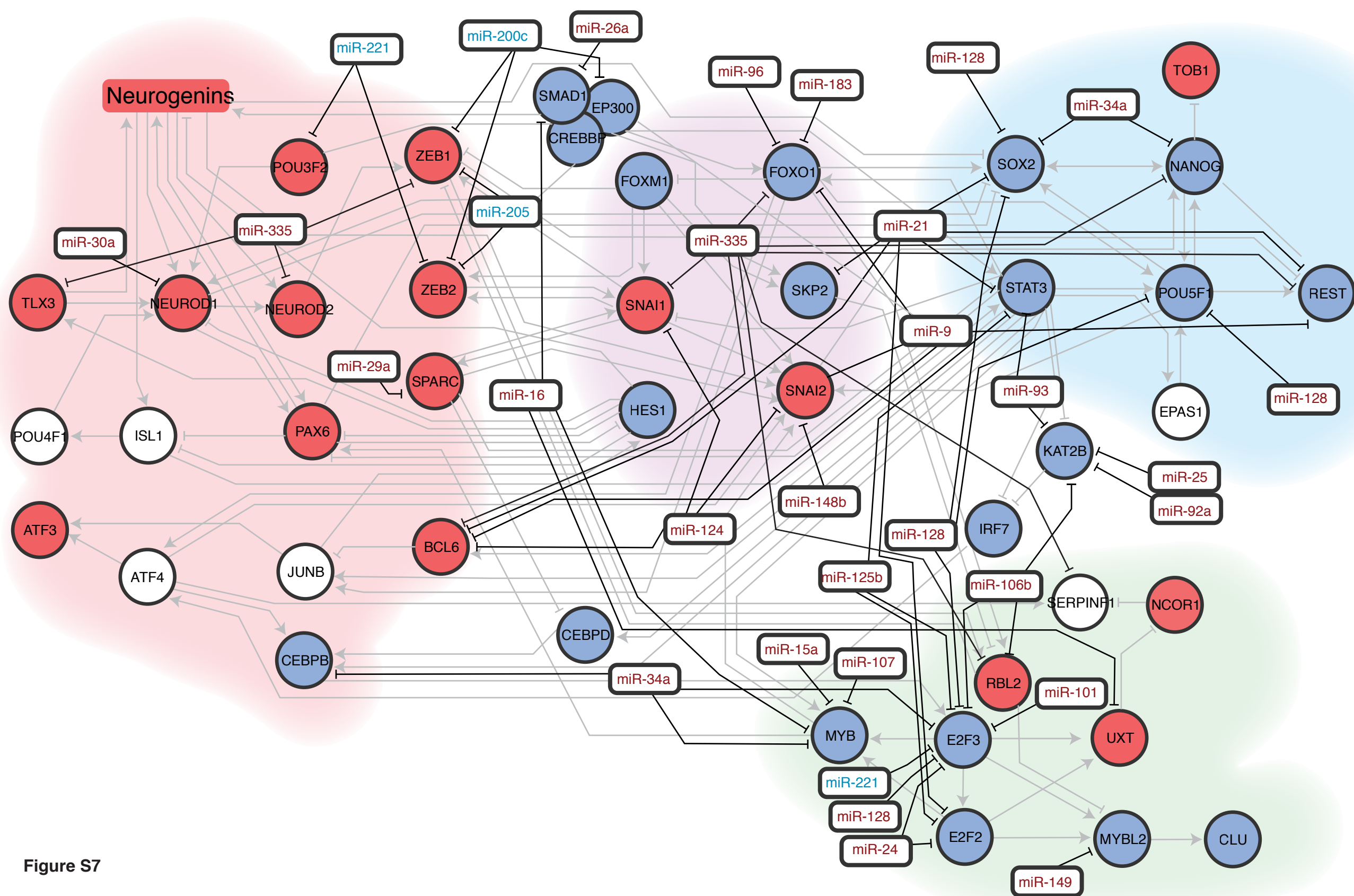
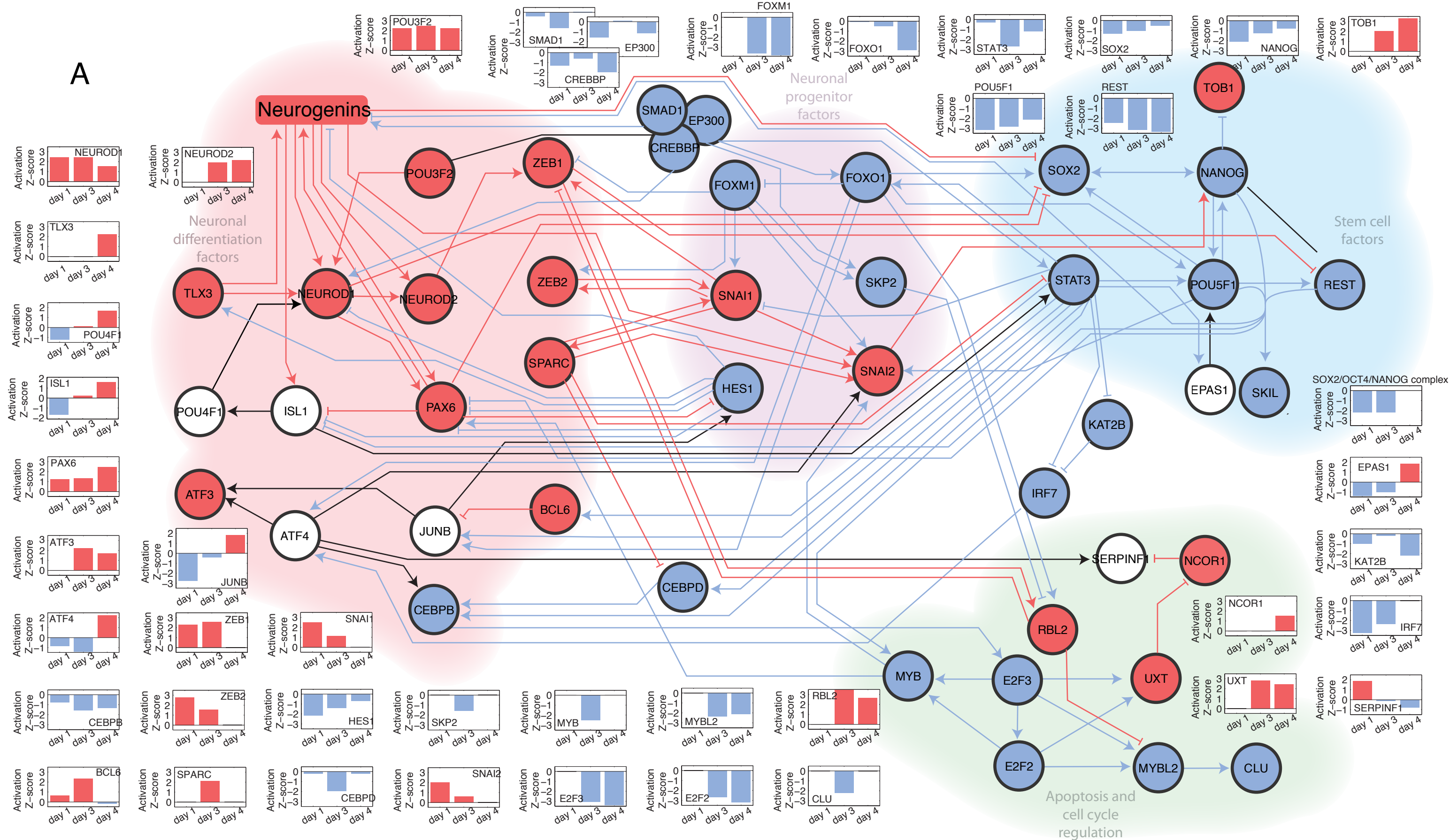
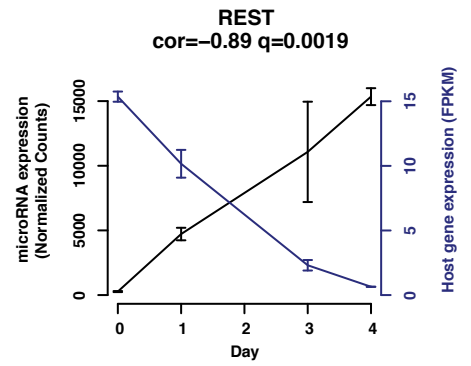
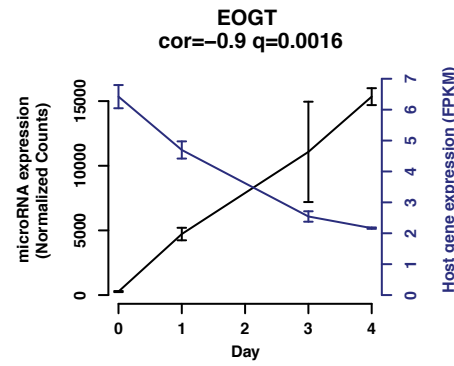
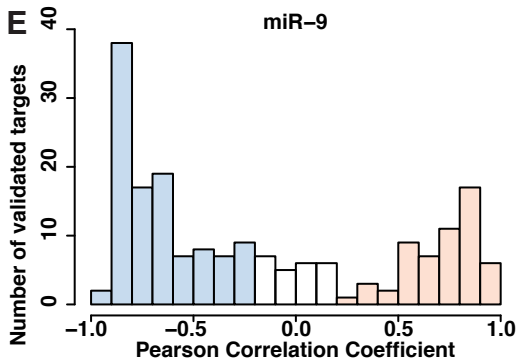
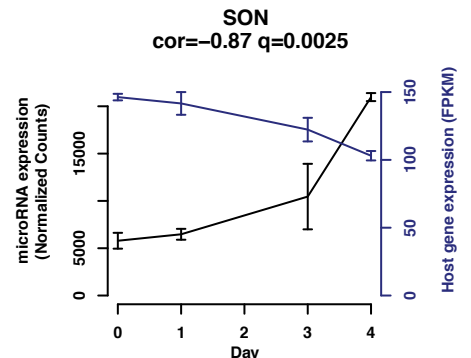
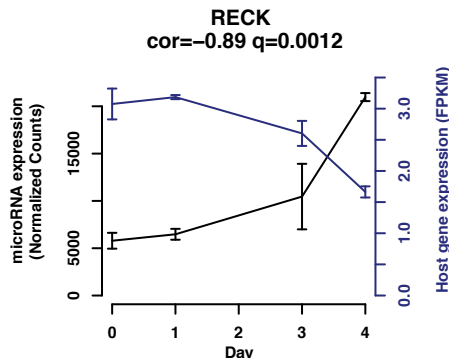
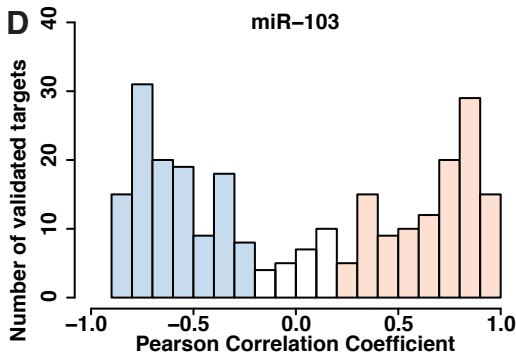
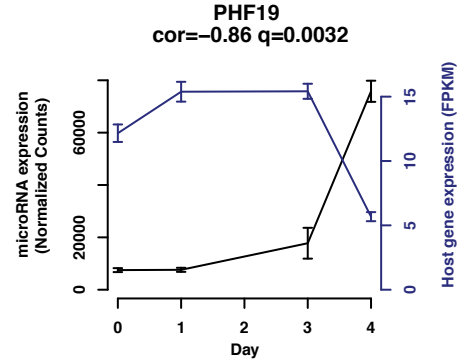
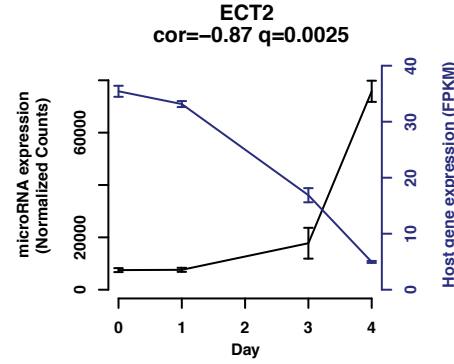
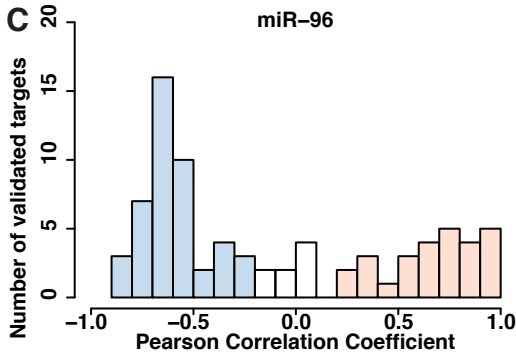
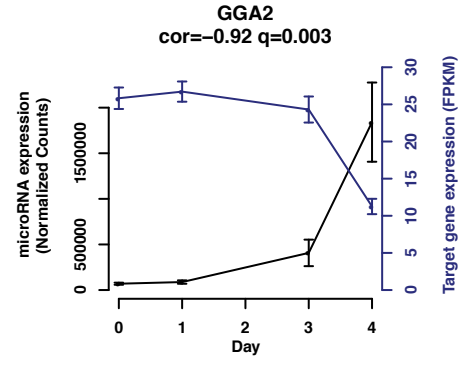
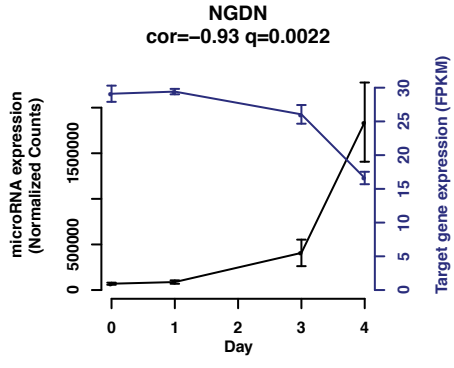
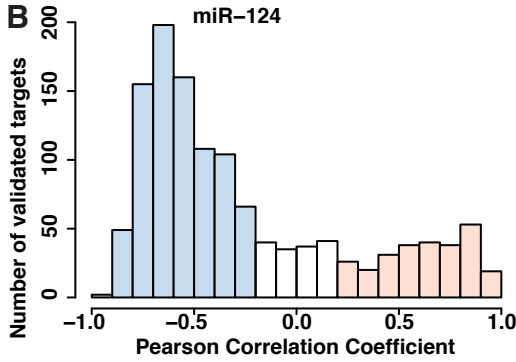
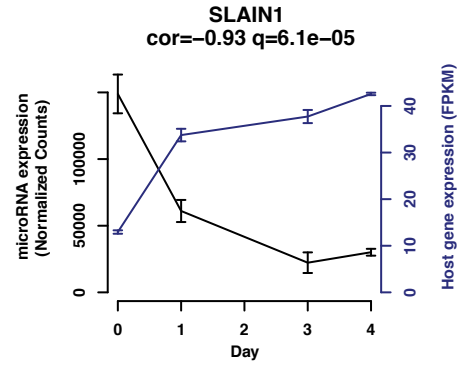
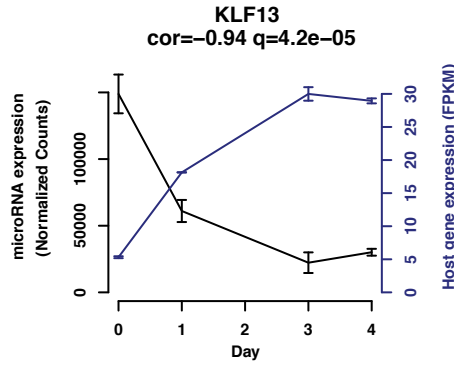
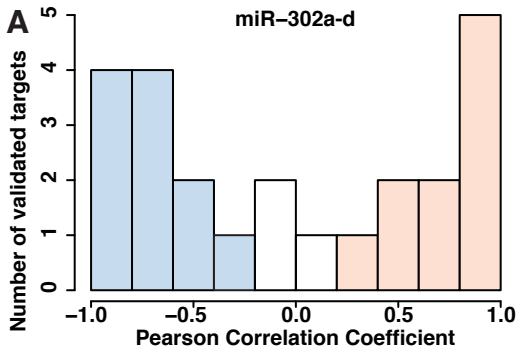


Figure S7

***Figure S8 - Correlation analysis of selected miRNAs and their validated targets.***

Histograms of correlation coefficients of miR-302abcd (A), miR-124 (B) miR-96 (C), miR-103 (D) and miR-9 (E) with their validated mRNA targets. miRNAs are repressors of gene expression, therefore one would expect less mRNA expression if a regulating miRNA is upregulated (miR-124, -9, -96 and -103) and increased gene expression if a miRNA is downregulated (miR-302abcd). Negative Pearson correlation coefficients are indicated in blue, positive ones in red. Next to histograms, two examples of each indicated miRNA/validated target pair are shown, miRNA counts (black) and mRNA levels in FPKM (blue) over time.

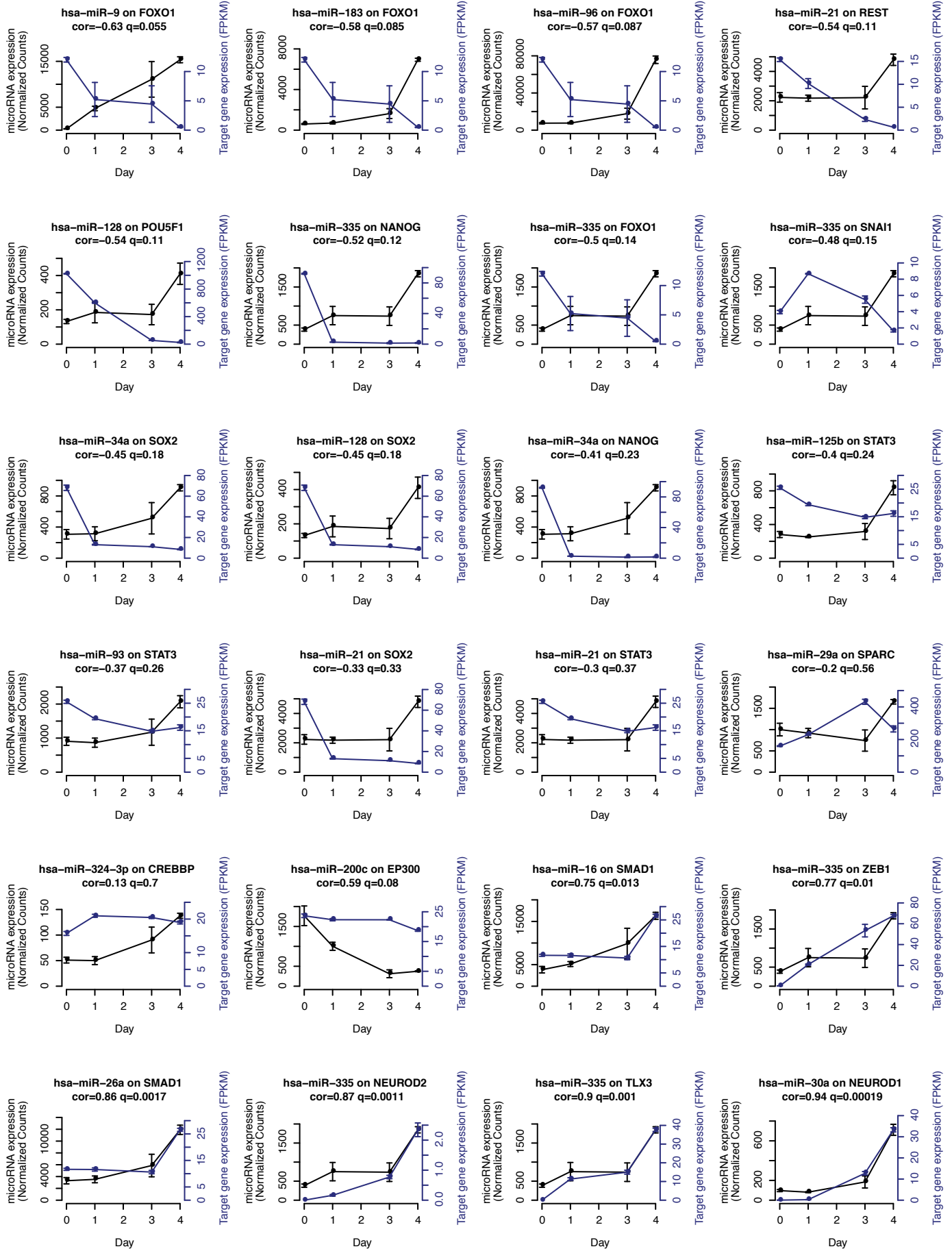
Figure S8





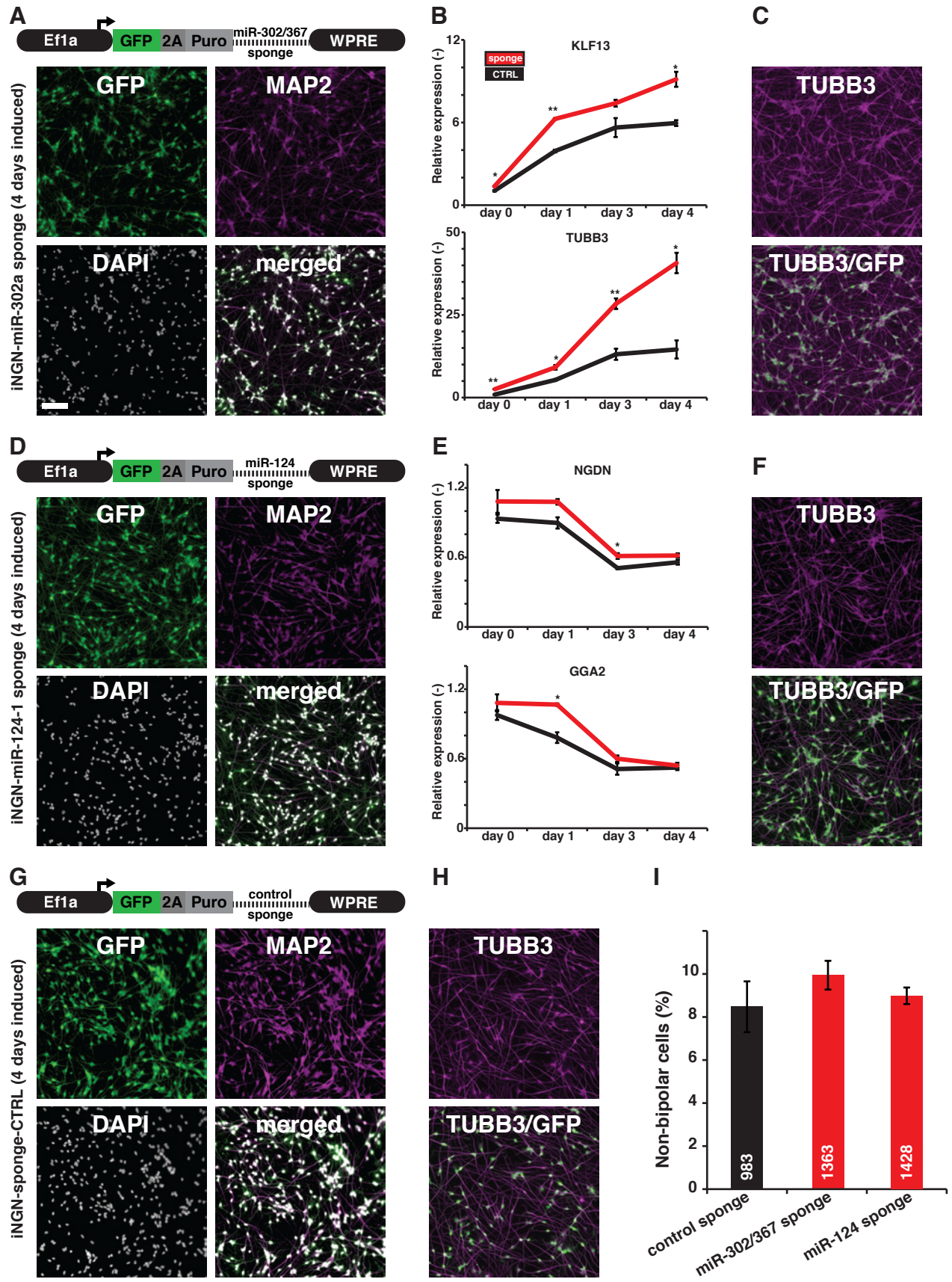
***Figure S9 - Correlation analysis of miRNA interactions with gene regulatory network members.*** Pairs of experimentally validated interactions between differentially expressed miRNAs and target transcription factors in our core gene regulatory network were identified, and their expression dynamics were compared. The significance of anti-correlation in expression levels for each miRNA-transcription factor pair was computed. Those showing significant correlation ( $\text{FDR} < 0.05$ ) are plotted in Figure 6, while the remaining pairs are presented here.

Figure S9



**Figure S10 - Perturbations of miRNA expression by miRNA sponges.** (A-C) Sponges against the miR-302/367 cluster, miR-124 (D-F) and control (G, H). The sponge sequences were placed downstream of a GFP-2A-Puromycin cassette that was driven by the EF1 $\alpha$  promoter and stably introduced into iNGN cells by lentiviral gene transfer. Untransfected iNGN cells were removed by puromycin selection. (A, D, G) Representative corresponding iNGN cells were stained at day 4 for GFP (green), MAP2 (magenta) and DAPI (white). (B, E) qPCR analysis over time of KLF13 and TUBB3, two validated miR-302 targets (B), as well as NGDN and GGA2, two validated miR-124 targets (E), demonstrated increased expression levels in sponged cells (red) versus control samples (black). (C, F, H) Additional representative immunostainings for TUBB3 as well as merged channels for GFP and TUBB3. (I) Quantification of non-bipolar control (black) and miRNA-sponged (red) iNGN cells, in biological triplicates. The number of analyzed cells is indicated in the columns. \*\*, p-value  $\leq 0.01$ , \*, p-value  $\leq 0.05$ , error bars, SEM, scale bar, 100 $\mu$ m.

Figure S10



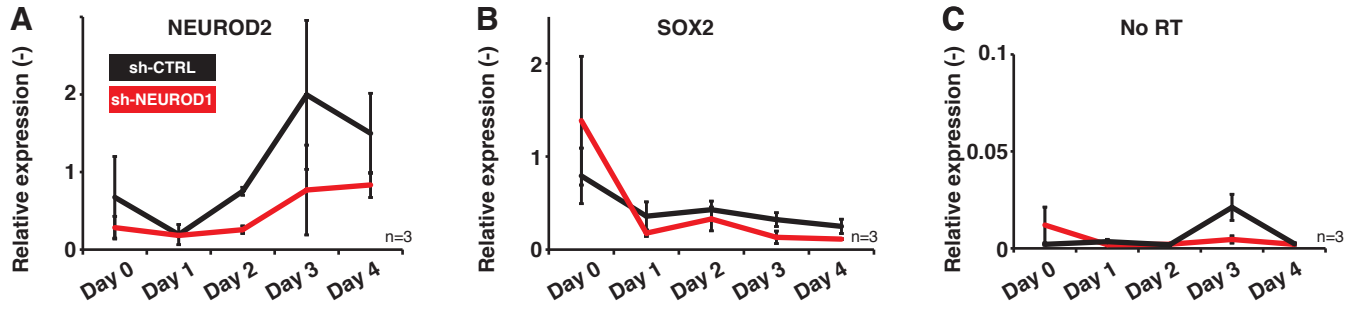
***Figure S11 - Extended analyses of perturbations of the gene regulatory network.***

Knockdown experiments using shRNAs (**A-C**) and siRNAs (**D, E**) against NEUROD1 as well as upregulation of the transcription factor REST (**G-I**). qRT-PCR analysis of the NEUROD1 regulated genes NEUROD2 (**A**) and SOX2 (**B**) including a no RT control (**C**). Corresponding gene expression levels over time of NEUROD1-shRNA (red) and sh-CTRL (black) iNGN samples are shown, normalized to ACTB in biological triplicates. (**D, E**) Immunohistochemical analysis for TUBB3 and NeuN and nuclear DAPI staining of NEUROD1-siRNA treated iNGN cells (**D**) and control-siRNA treated iNGN cells (**E**) imaged at day 4 of induction. Merged channels are indicated. Scale bar, 50  $\mu$ m. (**G**) Transmission light images of day 4 REST-overexpressing and control iNGN cells (left) and corresponding uninduced cells (right). The lentiviral doxycycline inducible REST-overexpression construct is shown on the top. (**H**) Quantitative RT-PCR for REST of day 1 induced and uninduced iNGN + REST (black) and iNGN (grey) cells, normalized to ACTB; n refers to the number of biological replicates. (**I**) Day 4 iNGN + REST cells had significantly enlarged soma diameters; n, number of analyzed cells from three independent samples. Scale bar, 20  $\mu$ m. \*\*\*, p-value  $\leq 0.001$ . \*\*, p-value  $\leq 0.01$ . (**J**) Proportion of neuronal genes (members of neuronal GO terms) under unique NEUROD1 and REST regulation taken from Ingenuity IPA. Error bars,  $\pm$  SEM.

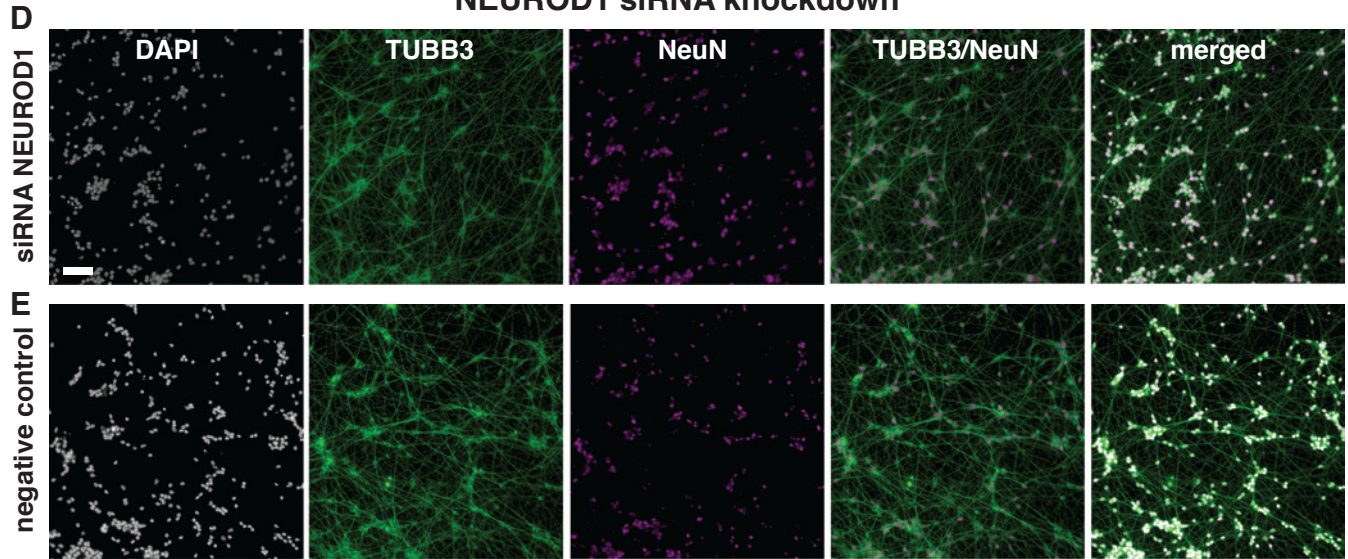


Figure S11

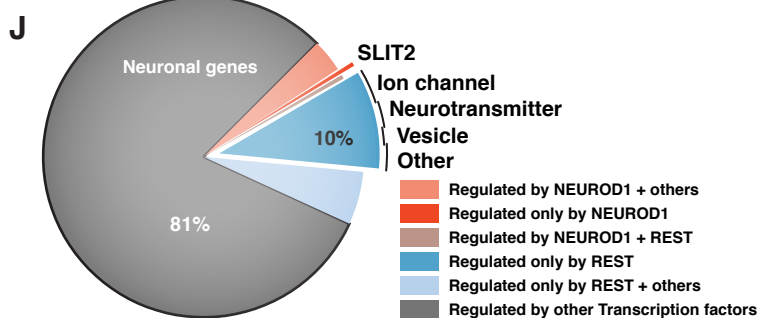
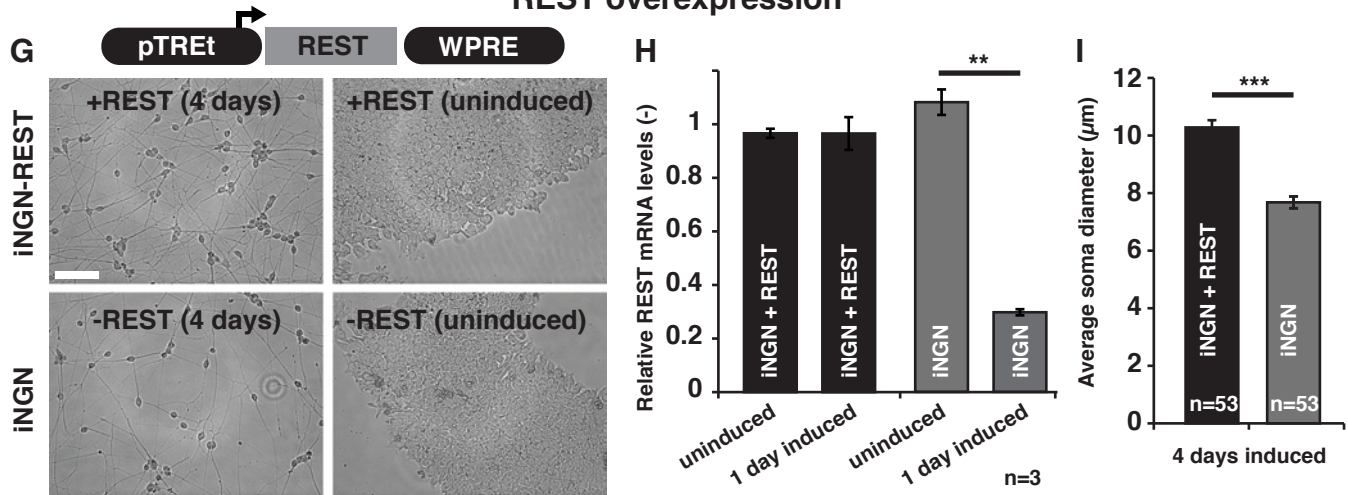
### NEUROD1 shRNA knockdown



### NEUROD1 siRNA knockdown

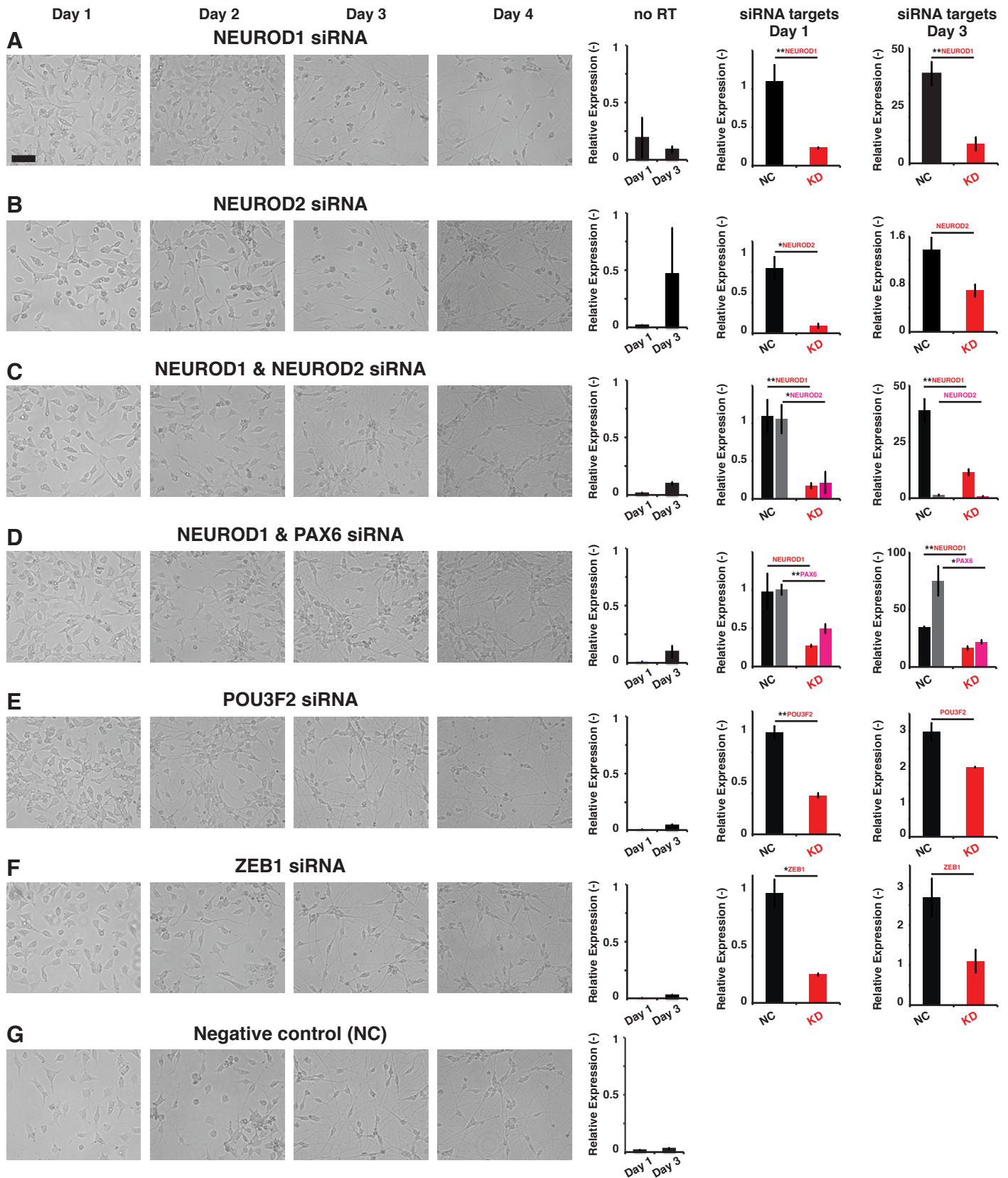


### REST overexpression



**Figure S12 - Transient perturbations of core regulatory members.** siRNA-knockdown experiments against NEUROD1 (A), NEUROD2 (B), NEUROD1/NEUROD2 (C), NEUROD1/PAX6 (D), POU3F2 (E) and ZEB1 (F) during iNGN differentiation. A scrambled siRNA served as a negative control (NC) shown in (G). Uninduced iNGN cells were transfected with siRNAs one day prior doxycycline induction. Contrast images from day 1 to day 4 of induction for each siRNA treatment are shown. Total RNA for qRT-PCR analysis was harvested at day 1 and day 3. The no RT controls served as a measure of DNA contamination. Corresponding gene expression levels at day 1 and day 3 of siRNA knockdown samples (KD) (red, magenta) and NC (black, grey) iNGN samples are shown, normalized to ACTB in biological triplicates. \*\*, p-value  $\leq 0.01$ , \*, p-value  $\leq 0.05$ , scale bar, 20 $\mu$ m.

### Figure S12





***Figure S13 - Comparison of individual Neurogenins and the iNGN cassette.***

Differences in gene expression resulted when comparing cells derived using by the iNGN construct (Neurog1+Neurog2) and doxycycline-induced constructs with NEUROG1 or NEUROG2. **(A)** NEUROG1 (upper panel) and NEUROG2 (lower panel) both individually differentiated PGP1 iPS cells into MAP2-positive (green) neurons after four days of induction. Scale bar, 100µm. **(B-D)** Microarray gene expression analysis of neurons (day 4) generated by the iNGN or individual Neurogenin constructs. **(B)** After correction for multiple hypotheses, 1732 and 305 genes were differentially expressed for the NEUROG1 and NEUROG2 cells, respectively, when compared to the iNGN cells. Significantly differentially expressed genes are plotted in red, and GO terms that were significantly enriched in differentially expressed genes are shown to the right (Fisher's exact test,  $p < 0.001$ ).

Figure S13

