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Generation of β -like cell subtypes from differentiated human induced pluripotent stem cells in 3D spheroids

Lisa Morisseau^{1*}, Fumiya Tokito^{2*}, Stephane Poulain^{3*}, Valerie Plaisance⁴, Valerie Pawlowski⁴, Seo Hyeon Kim³, Cecile Legallais¹, Rachid Jellali¹, Yasuyuki Sakai², Amar Abderahmani⁴⁺, Eric Leclerc⁵⁺

¹ CNRS UMR 7338, Laboratoire de Biomécanique et Bioingénierie, Sorbonne universités, Université de Technologies de Compiègne, France.

² Department of Chemical System Engineering, graduate school of Engineering, the University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo, 113-8656, Japan.

³ Institute of Industrial Science, University of Tokyo; 4-6-1 Komaba; Meguro-ku; Tokyo, 153-8505, Japan.

⁴ Univ. Lille, CNRS, Centrale Lille, Univ. Polytechnique Hauts-de-France, UMR 8520, IEMN, F-59000 Lille, France.

⁵ CNRS IRL 2820; Laboratory for Integrated Micro Mechatronic Systems, Institute of Industrial Science, University of Tokyo; 4-6-1 Komaba; Meguro-ku; Tokyo, 153-8505, Japan.

+ corresponding author: Eric Leclerc

*authors with similar contribution

Abstract

Since the identification of four different pancreatic β -cell subtypes and bi-hormonal cells playing a role in the diabetes pathogenesis, the search for *in vitro* models that mimics such cells heterogeneity became a key priority in experimental and clinical diabetology. We investigated the potential of human induced pluripotent stem cells to lead to the development of the different β -cells subtypes in honeycomb microwell-based 3D spheroids. The glucose stimulated insulin secretion confirmed the spheroids functionality. Then, we performed a single RNA sequencing of the cells of the spheroids. Using a knowledge-based analysis with a stringency on the pancreatic markers, we extracted the β -cells INS+/UCN3+ subtype (11%; β 1-like cells), the INS+/ST8SIA1+/CD9- subtype (3%, β 3-like cells) and INS+/CD9+/ST8SIA1- subtype (1%; β 2-like cells) consistently with literature findings. We did not detect the INS+/ST8SIA1+/CD9+ cells (β 4-like cells). Then, we also identified four bi-hormonal cells subpopulations including δ -like cells (INS+/SST+, 6%), of γ -like cells (INS+/PPY+, 3%), of α -like-cells (INS+/GCG+, 6%) and of ϵ -like-cells (INS+/GHRL+, 2%). Using data driven clustering, we extracted four progenitors' subpopulations (with the lower level of INS gene) that included one population highly expressing inhibin genes (INHBA+/INHBB+), one population highly expressing KCNJ3+/TPH1+, one population expressing hepatocyte-like lineage markers (HNF1A+/AFP+), and one population expressing stem like cell pancreatic progenitor markers (SOX2+/NEUROG3+). Furthermore, among the cycling population we found a large number of REST+ cells and CD9+ cells (CD9+/SPARC+/REST+). Our data confirm that our differentiation leads to large β -cell heterogeneity, which can be used for investigating β -cells plasticity under physiological and pathophysiological conditions.

Keywords: pancreas, beta cells, human induced pluripotent stem cells, single cell sequencing

1. Introduction

The pancreatic β -cells are the prominent cell type within islets of Langerhans that release insulin, the only hypoglycemic hormone of the body (Honzawa et al., 2021). The number and function of these cells are critical for maintaining blood glucose, as the reduction in their number and function can lead to relative or absolute insulin deficiency and ultimately thereby to diabetes, one of the deadliest diseases worldwide. β -cell population is heterogenous including four distinct subtypes (Miranda et al., 2021). These subtypes are classified according to the mRNA and protein levels of ST8SIA1 and CD9 genes (Dorrel et al., 2016), INS, GLUT2, GCK (Miranda et al., 2021), cell adhesion markers (Miranda et al., 2021; Ensi et al., 1999) and their position within the islets (peripheral vs central, Miranda et al., 2021). It has been observed that the β -cell subtypes that express the lowest level of ST8SIA1 represent approximately 80% of the cell population (Dorrel et al., 2016). These cells that do express very low level of CD9 are referred as β 1-cells, whereas β 2-cells harbor abundant CD9 (Dorrel et al., 2016). The remaining cells express high levels of ST8SIA1 together with either low (β 3) or high expression of CD9 (β 4) (Dorrel et al., 2016). Glucose competence of β 3 and β 4-cells is lower than this of β 1 and β 2-cells (Dorrel et al., 2016). It has been shown that the β 3/ β 4 subpopulation is increased in diabetes, possibly contributing to the drop of insulin secretion in diabetes (Dorrel et al., 2016). Beside of the four β -cell types, other possible β -cells expressing other hormone exist as exemplified by the detection of bihormonal cells expressing INS and Glucagon (GCG) (Chakravarthy H., 2017) or Somatostatin (SST) (Chera S., 2014) under some circumstances.

The discovery of these new β -cell subtypes and bihormonal cells very likely involved in diabetes is now challenging the field in diabetes research. With the lack of available human islets, the number of the cell model available for investigating their physiology and development under normal and diabetes condition is very limited. Therefore, it is required to develop cell models representing these new β -cell subtypes. Toward this objective, human induced pluripotent stem cells (hiPSCs) are very promising for mimicking β -cell plasticity and heterogeneity. In fact, pancreas *in vitro* lineage can be differentiated from hiPSCs into pancreatic β -like cells (Hosoya, 2012; Zhu et al., 2016; Kahraman et al., 2016; Hohwieler et al., 2019, Millmann et al., 2016).

Although, the maturation and the functionality, similar to those of the primary human β -cells, is still challenging, the hiPSCs sources would provide great insight for investigating the pathogenic mechanisms of pancreas diseases (Balboa et al., 2019; Amirruddin et al., 2020) and for contributing to cell therapies and drug development (Boettler et al., 2016; Kahraman et al., 2016, Millman et al., 2017).

In our previous work, we have proposed a protocol to generate C-peptide and insulin secreting β -like cells in spheroids derived from hiPSCs (Essaouiba et al., 2021, Essaouiba et al., 2022). We noticed that our spheroids contained also cells positive to glucagon. A bulk transcriptomic investigation revealed that the spheroids co-expressed mRNA related to several hormones' activities (such as IAPP, INS, INHBB, GCG, Essaouiba et al., 2022, and SST, Essaouiba et al., 2021) when compared to the control of undifferentiated cells. Those results illustrated a potential cell heterogeneity. The cells also over expressed several genes of the lipids' metabolism (LDLR, SCD, HMGCR, HMGCS1) and various specific transcription factors (HIC2, WRNIP1, SOX8, FOX genes, Essaouiba et al., 2022). In order to clarify the composition of our pancreatic model, we proposed in this work an additional analysis consisting of single cell sequencing analysis to extract the β -cell subpopulations.

2. Materials and methods

2.1 Pancreas iPSC protocol

The pancreatic tissues of the human pancreas monoculture were based on the differentiation protocol from iPSC proposed by Essaouiba et al. 2021 (Supp. Fig. 1 in Supp. File 1). In this protocol, the Cellartis hiPSCs derived β -cells were provided by Takara Bio (Japan). Cellartis hiPSC beta cells were differentiated from ChiPSC12 lines and provided in stage 1 of maturation by the manufacturer (and presented in Essaouiba et al. 2021). The hiPSCs derived β -cells were differentiated using the hiPSCs β -cell media kit (cat. N° Y10108, Takara Bio, Japan). We created spheroids using the PDMS based honeycombs microwell technology developed by Shinohara et al., 2014, 2017. The array of honeycombs, with a 126 μ m diameter, are located at the bottom of a 24 well plate format. The honeycombs were precoated with Pluronic F-127-coated (0.01 g/ml, Wako). After washing with PBS, the cells were inoculated at a

density of 6×10^5 cells per well (corresponding to 3×10^5 cells/cm²). The culture medium changed was performed following the cell supplier (Takara Bio, Japan). The set of experiments was performed three times independently leading to 12 independent honeycombs well cultures.

2.2 Biological assays

Following the manufacturer's recommendations (Mercodia), we measured the level of production of insulin *via* a glucose stimulated insulin secretion. For that purpose, at the end of the end experiments, we first washed 3 times the cells using a low glucose concentration medium (DMEM, 0 mM, Gibco) and exposed them to the low glucose medium (DMEM, 0 mM, Gibco) for 2 hours. Then, we exposed the cells to high glucose medium stimulation (DMEM, 25 mM, Gibco) for 2 hours. Finally, the cells were wash 3 times and exposed once more to the low glucose concentration medium (DMEM, 0 mM, Gibco) for 2h20 min. The culture medium were samples and frozen until measurement.

2.3 Single RNA sequencing

The spheroids from several PDMS-bottom 24-well plate were collected by pipetting and dissociated into single cells by Accumax (Inovative Cell technologies, USA) treatment. After being washed 3 times in total with DMEM media containing 10% FBS, cells were filtrated with a 40 μ m strainer and then resuspended in an appropriate volume of PBS (Mg/Ca-free) containing 0.04% BSA. We recovered prior analysis $4 \pm 0,3 \times 10^6$ cells. Approximately 8,000 cells with 75% cell survival rate were used for GEM (Gel Bead in emulsion) generation using Chromium Controller (10x Genomics), where each RNA molecules contained in each cell are tagged by specific barcode/UMI combinations. The molecular-tagged RNAs were converted into double-stranded cDNA followed by library preparation for next generation sequencing using the Chromium Next GEM Single Cell 3' Reagent Kit v3.1 (10x Genomics). The resulting libraries were quantified by Qubit dsDNA Assay (ThermoFisher Scientific) and TapeStation D1000 (Agilent). The libraries were sequenced on an Illumina HiSeq platform with the following configuration: Read 1: 28 cycles, Read 2: 91 cycles and i7 index: 8 cycles, yielding approximately 400 M paired-end reads per sample. Image analysis and base calling were conducted by Illumina software on the HiSeq instrument.

Raw FASTQ reads were imported into a Cell Ranger pipeline for mapping and gene expression count analyses (10x Genomics, cell ranger 6-1-2; include introns: false, chemistry: single cell 3' v3; transcriptome GRCH38-3.0.0). Additional details are given in Supp. File 2. The single cell gene expression study mentioned above were conducted by Azenta Life Sciences (formerly Genewiz), according to the manufactures' instructions. Samples analysis and comparisons were performed using Loupe Browser 6.0.0 (10x Genomics, statistics methods are given in Supp. File 2) applying a features per barcode filtration threshold set to 9000 to remove potential multiplets. The percentage of UMIs assigned to mitochondrial RNAs were limited to 20% per barcode to remove cells with high mitochondrial gene expression levels from the analysis. Gene expression tables for each cell cluster of interest were exported from Loupe Browser and then processed with Shinygo (<http://bioinformatics.sdstate.edu/go/>) for subsequent pathway analysis (the enrichment analysis is calculated based on hypergeometric distribution followed by false discovery rate, FDR, correction, Ge et al., 2020. The FDR was set at 0.05 in our study) and by iDEP96 (<http://bioinformatics.sdstate.edu/idep96/>) for heatmap clustering and gene representation (Ge et al., 2018). Sequencing data supporting the findings presented in this study, and all supplementary files and figures, were deposited at Zenodo (<https://zenodo.org>) with the following Digital Object Identifier: 10.5281/zenodo.7960673.

3. Results

3.1 Morphology and basal functional analysis

We presented the spheroids' morphologies 24h after seeding (Fig. 1A) and at the end of the culture (16 days, Fig. 1B). The cell suspension aggregated into spheroids within the first 24h of culture. The spheroids were formed inside the honeycomb and their size was controlled by the micro geometry. They growth up to reach the border the honeycomb. This led to spheroids with a controlled size driven by the honeycomb geometry of about 130 μm in diameter. The spheroids remained at the bottom of the honeycomb during the cultures and inside the honeycomb. We did not observe particular over growth above or outside of the honeycombs. We did not detect differences on the spheroids' morphologies when we compared the honeycombs from the different dishes and experimental campaigns. We also confirmed that the insulin

secretion by glucose stimulation was functional as shown in Fig. 1C. The results demonstrated a $3,3 \pm 1,2$ induction in high glucose conditions ($n=3$, $p_value < 0,05$).

3.2 Single sequencing results confirmed the pancreatic differentiation

A knowledge data-based analysis using the pancreatic lineage hallmarks allowed to extract the specific endocrine differentiation patterns. We confirmed the successful differentiation by checking that the cell population largely did not express pluripotent markers (NANOG, POU5F1, Figs. 2A and 2B) neither mesoderm nor definitive endoderm makers (such as FGF4, SOX17, Figs. 2C and 2D). The pancreatic progenitor markers NEUROG3, PAX4, NKX6-1, NKX2-2 were expressed in 20%, 13%, 35% and 60% of the populations respectively (Figs. 2E to 2H). We also confirmed that the cell population expressed the endocrine specification markers (NEUROD1 is expressed in 65% of the population, MAFB is expressed in 73% of the population, Figs. 2I and 2J). However, the most advanced β -cells markers, such as MAFA, UCN3, were weakly expressed (only in 2% and 11% of the population respectively) illustrating partial β -cells maturation (Figs 2K and 2L). Nevertheless, the predominance of the β -cells lineage was illustrated by the overall levels of INS that was expressed in 100% of the sequenced population (Fig. 2M).

3.3 Knowledge based analysis confirmed the presence of seven typical β -cells subtypes.

3.3.1 Identification of the typical β -like cells subpopulation based on literature markers

By addressing the knowledge on specific pancreatic markers, we extracted, from the insulin positive cells, seven β -cells subtypes representing 32% of the overall cells' population based on literature data and markers. The plume plots of mRNA levels of the markers used to discriminate the population, GCG, SST, PPY, CD9, ST8SIA1, GHRL and UCN3 (INS), compared to the overall population, is presented in Fig 3A (Supp File 3). The heatmap of the top 10 discriminating genes is presented in Fig 3B. The INS+/UCN3+ population showed the highest level of INS and UCN3 (mature β -like cells, 11% of the overall population, upregulating also INS-IGF2 as reported by van Gurp et al., 2021). This population was CD9- and ST8SIA1- (representing potentially

a β 1-cells like subtype as identified by Dorrel et al., 2016). The top upregulated genes of this population enriched the GO_biological process related to regulation of peptide hormone secretion, neuropeptide pathway, hormone secretion (Fig. 4A). We also identified an INS+/ST8SIA1+/CD9- population (β 3-like cells subpopulation, 3%, Dorrel et al., 2016). This population expressed cell cycle genes (CDK1, CCNB1, CCNB2) and REST. The top up regulated gene of this population enriched the GO_biological processes related to digestive development and extra cellular matrix organization (Fig. 4B). Then, we detected the INS+/CD9+/ST8SIA1- population (β 2-like-cell subpopulation, 1 %, Dorrel et al., 2016, this population also over expressed SLC2A5). However, the specificity of the INS+/ST8SIA1+/CD9- cells were very low as far as this population was characterized by only 8 genes differentially expressed (Supp. File 3). Using this knowledge-based analysis, we did not extract significant INS+/CD9+/ST8SIA1+ (β 4-cells subpopulation, *nb: in fact there is just one cell in our dataset with this feature*). The specific stratification of the INS+/CD9-/ST8SIA1- (β 1-cells), INS+/CD9+/ST8SIA1- (β 2-cells) and INS+/CD9-/ST8SIA1+ (β 3-cells) populations is given in Fig. 3C (additional stratifications are given in Supp. Fig. 2 in Supp. File 1).

The other β -cells subtypes selectively co-expressed high levels of INS+/ARX+/GCG+ (β - α -like cells, 6%), INS+/SST+ (β - δ -like cells, 6%, population with also high level of HHEX, ISL1, LEPR, ETV1 consistently with literature Zhang et al., 2014, van Gurp et al., 2021), INS+/PPY+ (β - γ -like cells, 3%, population with also high level of IAPP, TPH2 and PYY) and INS+/GHRL+ (β - ϵ -like cells, 2%), illustrating the large pancreatic heterogeneity and the β -cells plasticity of our spheroids. The top upregulated genes of the INS+/GHRL+ subpopulation highly enriched the cholesterol and lipid biological processes (Fig. 4C); the top genes of INS+/SST+ cells enriched nervous system development related biological processes (Fig. 4D); the top genes of INS+/PPY+ cells enriched the angiotensin related processes, the peptide hormone processing, the feeding behavior, endocrine process, and the several inflammation related processes (Fig. 4E); finally the INS+/ARX+/GCG+ up regulated genes enriched the histamine response and heparan sulfate synthesis, the cell mobility and cell migration processes (Fig. 4F).

3.4 Data driven analysis revealed the heterogeneity and new types of β -like cell subpopulations in our spheroids

Then, to identify new potential cell subpopulations, we performed a data driven analysis on our dataset. The analysis contributed to extract 10 typical cell subpopulations (Fig. 5A, Supp. File 4). Using a selected genes list of pancreatic genes including mature, immature, and functional markers (Supp. File 4), we observed that the 10 cell subpopulations were classified under three distinct branches according to the hierarchical clustering presented on the heatmap of gene expression shown in Fig. 5B (a second heatmap showing the expression levels of the top 10 genes in each cluster is given in Supp. Fig. 3 in Supp. File 1)

3.4.1 A first branch grouped the most mature like β -cells subtypes

The first branch regrouped three cell subpopulations. Among them, we distinguished a subpopulation characterized by the highest level of PPY, IAPP, CGA, NTS genes (PPY+/IAPP+ in Fig 5A, 9% of the overall population). This subpopulation also co-expressed high levels of GLP1R, GCGR, WNT4 concomitant with low FZD6, INSR. The top 200 genes expressed by this subpopulation enriched the neuropeptide molecular functions and various neuro/synaptic biological functions (Fig. 6A, KEGG enrichment is given in Supp. Fig. 4 in Supp. File 1).

The second population of this branch was characterized by the high expressions of DPP4, KDR, SPON1, PDK4, CTNND2 genes while co expressing INS, SST and GCG (DPP4+/KDR+ in Fig. 5A; 18%). The top 200 highly expressed genes enriched the reactome of the sodium/calcium exchangers, the reactomes of the synthesis secretion and inactivation (i) of glucose dependent insulinotropic polypeptide, (ii) of incretin, (iii) of GLP1 (Fig. 6B, KEGG enrichment is given in Supp. Fig. 5 in Supp. File 1).

The third subpopulation was characterized by increased levels of TTR, SST, GCG, UCN, CRH and APOA1 genes expressions (TTR/GCG/SST in Fig 5A; 14%) It also expressed low level of ST8SIA1 and CD9 genes. The top 200 genes in this subpopulation enriched the biological processes related to hormones transport, lipid

transport, ions and cations homeostasis (Fig. 6C), the reactome of incretin, ghrelin, peptide hormones, fatty acids, insulin like growth factors (Fig. 6D). KEGG enrichment is given in Supp. Fig. 6 in Supp. File 1.

Those three populations superimposed with most of the cells of the INS+/ARX+/GCG+, INS+/SST+ and INS+/PPY+ subtypes described in section 3.3.

3.4.2 Identification of three type of cycling cells among the β -cells

The second branch of the heatmap of Fig. 5B grouped three subpopulations of cells highly expressing cell cycle markers such as CDK1, CCNA2, CCNB1, CCNB2.

The “pure cycling” population expressed the highest levels of CDK1, CCNA2, CCNB1, CCNB2 and LDHA (Cycling cells in Fig. 5A) and represented 3% of the overall population. As those cells also presented low level of CD9 and moderate expression of ST8SIA1, it could reflect a proliferative portion of a β 3-like cells following the classification of Dorrel et al., 2016 (*nb this population may reflect a potential source of β 3 like-proliferative cells observed in T2DM by Dorrel et al., 2016*). The top genes hallmark the mitotic, microtubule organization as top biological processes (Supp. Fig. 7 in Supp. File 1).

The second subpopulation of this branch was characterized by the high levels of SLC2A2, SCL2A4, GCGR, GCKR and low INSR (6%). Interestingly, this subpopulation also co-expressed ST8SIA1 while moderately expressing the PPY, SST, and GCG genes and with a low level of INS (SLC2A4+/GCKR+ in Fig. 5A). Nevertheless, this population reflected a very poor specificity when compared to other 9 populations (Supp File 4). and no significant enrichment could be detected.

Then, we distinguished in this branch a particular subpopulation expressing immature markers such as REST, PDGFRA, ESRRB, LAMA1, SOX9, SLC2A1 and SPARC genes, but also the highest levels of CD9 (CD9+/SPARC+/REST+ in Fig 5A, 8%). Furthermore, this population presented low level of ST8SIA1. As such it appeared to include a part of the β 2-like-cells described in section 3.3. This subtype expressed various genes involved in TGF β and NOTCH signaling pathways. This population also expressed the lowest levels of INS gene and of pancreatic mature markers such as MAFB, NEUROD1 and NEUROG3, genes when compared to other populations. The top 200 genes enriched extracellular matrix biological process, mesenchyme

development, the tube and blood vessel formation (Supp Fig. 8A and Supp Fig. 8B in Supp File 1; KEGG enrichment is given in Supp. Fig. 9 in Supp. File 1).

Then, we refined the analysis of this CD9+/SPARC+/REST+ subpopulation. We re-clustered those CD9+/SPARC+/REST+ cells, and we extracted four typical subtypes, Fig. 7A (Supp. File 5). More particularly, we found a CD9+/BMP4+/SPARC+ positive subpopulation (1% of the overall population and representing about 16% of cells within the CD9+/SPARC+/REST+ cells) expressing the highest levels of TGF β and NOTCH genes (such as BMP4, SMAD3, TGFB2, and NOTCH3) but also a large panel of ECM genes including LUM, COL6A3, EGFLAM, COL3A1, endothelial membrane markers (VWF, PECAM1) and HGF. The top 200 genes expressed in this subpopulation highlighted the reactome of carbohydrates sulfotransferase (CHST3, CHST4), MET receptor, collagen process and NCAM-1 interactions as well as the biological processes related to the mesenchymal development (Fig. 7B). A second subpopulation co-expressed GHRL+/UCN3+/INS+/SST+, and was positive to KRT20, ISL1, ACSL1, ARX, PAX6, PAX4, FFAR3, ARX, NEUROG3, NKX6-1, NKX2-2 (2% of the overall population and representing about 28% of cells within the CD9+/SPARC+/REST+ cells). The top 200 genes enriched the reactome related to NEUROG3 progenitor, Ghrelin synthesis, serotonin and melatonin biosynthesis and of free fatty acids receptors (Fig. 7C). Altogether, the data converge toward a common root of the endocrine progenitor population expressing typical α , β , γ , δ , ϵ -like cells marker genes in this subpopulation. This population represented about 2% of the overall population. The third one (2% of the overall population and representing about 28% of cells within the CD9+/SPARC+/REST+ cells) was positive to SOX3, NOG and CTGF (involved in connective tissue in pancreas development), various ECM markers (COL2A1, FN1), cell cycle genes (CDK1, CCNB1, CCNB2, CCNE2) but also REST. The top upregulated genes enriched cell cycle processes (Fig 7D). Finally, the last one (2% of the overall population and representing about 28% of cells within the CD9+/SPARC+/REST+ cells) not only expressed CDLN4, CDLN6, SCL2A2, IGF2, and more particularly the genes GP2, LYZ, ANXA4, SPINK1 and SDC4 that are typical pancreatic acinar markers (Kurashima et al., 2021; Segerstolpe et al., 2016), but also the CST4 and SLC4A4 and SPP1 genes (that are pancreas ductal markers, Segerstolpe et al., 2016; Yang et al., 2019; Wu et al., 2021). This subpopulation was also characterized by low expression levels of INS, GCG, TTR, SQLE and SCD when compared to other four populations. The top uregulated genes of this fourth

subpopulation enriched bicarbonate transport, excretion/secretion biological processes (Fig 7E; nb pancreas ductal cells control bicarbonate secretion, Heygi et al., 2011)

3.4.2.2 Identification of four endocrine progenitors of β -cells subtypes

Finally, the third clustering branch extracted from the heatmap presented in Fig. 5B grouped the four populations co expressing the typical pancreatic transcription factors representative of immature endocrine cells such as NEUROG3, NKX6-1, PAX4 (endocrine progenitor markers), and NEUROD1, NKX2-2 (more advanced endocrine maturation) but a moderate level of INS.

Among those four subpopulations, one population co-expressed more specifically the SOX2, NEUROG3, CFAP126, MAPK10 genes, reflecting an on-going differentiation, in parallel to the low levels of GLP1R and GCGR (6%, SOX2+/NEUROG3+). The top 200 genes expressed in this population enriched the endocrine system development and MAPK cascade (Fig. 8A; KEGG enrichment is given in Supp. Fig. 10 in Supp. File 1).

A second subpopulation presented an upregulation of various liver like markers such as HNF1A, FOXA2, APOA1, APOA2, AFP, ALB, NR1H4 and CYP27A1 when compared to other populations (10%, HNF1A+/APOA2+). Most of the GRHL+ cells presented in section 3.3 were associated with this subpopulation. Consistently, the hallmark of this HNF1A+/APOA2+ population enriched the lipids, sterol, cholesterol metabolic pathways (Fig. 8B; KEGG enrichment is given in Supp. Fig. 11 in Supp. File 1).

Finally, the two last subpopulations displayed very closed signatures, as far as both expressed INHBA, INHBB, PDX1, MAPK10, MAPK12, NKX6-1, NKX2-2, GCK, GLP1R. Firstly, the labeled INHBA+/INHBB+ expressed more largely those markers (11% of the over cell population). The top 200 genes of this population enriched amylin, calcitonin receptors activities, hormones activity (Fig. 8C). The biological processes were related to Mast cells degranulation, leucocytes and immune response, angiogenesis (Fig. 8D; *nb*: the top genes did not contribute to extract significant KEGG pathway enrichment). Secondly, the KCNJ3+/MYH4+ subpopulation (16%) was showing high levels of glycolytic genes (HIF1A, GCK, PCK2, SLC2A2, GLP1R, INSR, PPARA), potassium ion channels (KCNJ3) and serotonin biosynthesis (TPH1). The

200 top expressed genes were related to ions and cations transports (Fig.8E; KEGG enrichment is given in Supp. Fig. 12 in Supp. File 1).

4. Discussion

We have performed a characterization of the β -like cell subpopulation derived from induced human pluripotent stem cells in 3D spheroids. The analysis confirmed the endocrine differentiation. We did not achieve a mature β -cells specific tissue as far as we detected immature endocrine like progenitors, endocrine like progenitors and trans differentiated like β -like cells. Although all cell population expressed INS gene, we observed a graduation of the level of expression of this gene. Furthermore, our previous studies revealed that not all the islets expressed the INS protein (Essaouiba et al., 2021). Nevertheless, we confirmed the advanced maturation by the GSIS assays illustrating functional spheroids.

There are several classifications of the β -cell subtypes in the literatures, including from primary islets or from hiPSCs derived tissues (Veres et al., 2019; Dominguez-Gutierrez et al., 2019). In our study, we detected three types of β -like cells, the INS+/UNC3+/ST8SIA-/CD9-, INS+/CD9+/ST8SIA1-, INS+/ST8SIA1+/CD9-, consistently with the β 1, β 2, β 3 cells classification established by Dorrell et al., 2016. We did not find the β 4-like cells (INS+/ST8SIA1+/CD9+ subpopulation) conversely to literature (Dorrell et al., 2016; Liu et al., 2017). Dorrell et al., noticed that the proportion of ST8SIA1 cells (β 3 and β 4 cells) can differ from donors and from healthy to disease patients. They are also less responsive to glucose. Our protocol may also lead to promote a preferential differentiation route which reduced the occurrence of β 4 cells. Furthermore, in our analysis, the INS+/ST8SIA1+ cells present a poor transcriptomic specificity as far as they over express only 5 genes (ASB5, CSMD3, CXCL5, DUSP2) and weakly expressed UCN3 when we compared them to other β -like cells subtypes. As the literature displayed more complex ST8SIA1+ profile with its expression in up to 20% of β -cells profile in human primary islets (Liu et al., 2017), our results illustrate that our spheroids still not reflect a complete adult pancreatic profile.

Then, the pancreatic markers including somatostatin, ghrelin and pancreatic polypeptide genes were largely expressed in our cells. The graduation of the levels of

expression of those pancreatic markers led to extract a large heterogenic cell population. The panel of bi hormonal profile was previously detected in hiPSCs *in vitro* β -cells differentiation (Veres et al., 2019). In addition, several poly-hormonal cells in human islets were classified based on their expression of calcium regulated genes (Yoon et al., 2022). Interestingly, our data driven identified TTR+/GCG+/STT+ cells subpopulation have shown that the top genes enriched the calcium signalling pathway (Supp. Fig. 6 in Supp. File 1). In parallel, the observation of trans differentiation of β -cells in other hormones secreting cells is largely observed in the literature (Weinberg et al, 2007; Spikjer et al, 2013; Gershengorn, et al, 2014; Diedisheim et al, 2018). We confirmed the presence in our spheroids of a β -like cells (high INS+/UCN3+ 11%), β - δ -like cells (INS+/SST+, 6%), β - γ -like cells (INS+/PPY+, 3%), β - α -cells (INS+/GCG+, 6%) β - ϵ - like cells (INS+/GHRL+, 2%). The β -cells plasticity is now largely accepted and is illustrated by their capability to de differentiate. The dedifferentiation is observed under various metabolic stimulations (such as hyperglycemia) and inflammation process (through cytokines for instance) leading to reactive oxygen species accumulation which in turn conduct to β -cells dysfunction (Honzawa et al, 2021). Dedifferentiation often results either to the conversion from mature phenotype to endocrine progenitors' phenotypes, or to cell apoptosis. The consequences are the β -cells dysfunction and the progression of pathology in the pancreas. Dedifferentiation is largely observed in diabetic patients (Wang et al, 2021). The plasticity of the β -cells is also illustrated by their ability to trans-differentiate to other hormones secreting cells (Weinberg et al, 2007; Spikjer et al, 2013; Gershengorn, et al, 2014; Diedisheim et al, 2018). It is suspected that this process occurred naturally with aging patients, diabetic patients. *In vitro*, the trans-differentiation was observed in insulin positive cells changing for glucagon positive cells under free serum cultures (Honzawa et al., 2021; Gershengorn et al., 2004).

In addition to bi-hormonal' profiles and multi lineage like subtypes, several types, mature like cells (α - and β -like cells) of progenitors (NKX6.1+; PDX1+), and several rare cell subtypes (including ONECUT3+; SST+/HHEX+ cells similar to our INS+/SST+ subpopulation) were reported from *in vitro* hiPSC derived tissue (Veres et al., 2019), Our data driven analysis also revealed several types of advanced pancreatic profiles and progenitors. We found an advanced endocrine profile with 18% of the cells co-

expressing INS and GCG and highly co-expressing KDR and DPP4. KDR (VEGFR2) is an endothelial marker found during islet vascularization (Staels et al., 2019; Toselli et al., 2019). DPP4 is also involved in GLP1 response and glucose homeostasis (Deacon et al., 2018). DPP4 is expressed in human pancreatic islets and in both beta cells and alpha cells (Bugliani et al., 2018). Proportion of DPP4 of α and β -cells is reduced in type 2 diabetes. Furthermore, its inhibition improves β -cells function and survival in type 2 diabetes (Bugliani et al., 2018) which is consistent with its lower expression of this marker in our most advanced endocrine profiles extracted by the data driven analysis (PPY/IAPP and TTR/SST/GCG profiles in Fig. 5).

Then, we identified four typical endocrine progenitors' populations. Among them, the first one expressed SOX2+/NEUROG3+ that are typical endocrine progenitor markers (D'Amour et al., 2006). NEUROG3+ positive cells were also classified as pancreatic progenitor in Veres et al., 2019. Secondly, one population present a liver like signature with highly expressing HNF1A transcription factor. This result is coherent with the fact that liver and pancreas arise from common multipotent population (Zaret, 2008; Cerda-Esteban et al., 2017). Furthermore, our results show that this liver like progenitor population superimposed with the Ghrelin expressing like population (INS+/GHRL+). Interesting, literature report that GHRL stimulates the hepatocyte proliferation (Wang et al., 2018) and a key regulator liver steatosis (*via* the regulation of lipid metabolism, Quinones et al., 2020).

Then, another progenitors' population was characterized by high levels of INHBA and INHBB (activin A and B ligands). In fact, endocrine differentiation is reported to interact with activins signals (Zhang et al., 2002). Furthermore, other single cell sequencing identified endocrine maturation via INHBA routes from fetal pancreas population in a mesenchymal compartment (de la O et al., 2022). Thus, it was predicted that the pancreas endocrine development in humans was dependent on signal input from the mesenchymal niche environment (de la O et al., 2022). This population also over expressed NKX6-3 gene and co-expressed high levels of NKX6-1 and PAX4 genes which are two genes appearing in one progenitor like population and one rare premature cell population in Veres et al., clustering (Veres et al., 2019). Then, the targets genes of this population enriched the processes of "the mast cells". Mast cells are rich in heparan and histamine, that can bridge this population with some of the cells of the INS+/ARX+/GCG+ subtype identified by the knowledge-based-analysis in section 3.3 (as this population also enriched histamine and heparan processes). As

INHBA and INHBB are both expressed in normal α -cells, whereas only INHBA is expressed in β -cells (Braun et al., 2014), we suspect that INHBA+/INHBB+ is one potential α -cell progenitor source. Finally, we extracted a subpopulation highly expressing TPH1 gene, co-expressing FEV, LMX1A, SCL18A1, with low ISL1 level, which could be consistent with Veres et al., analysis that identified among a stem cell like enterochromaffin cells (TPH1+/FEV+/LMX1A+/SCL18A1+/ISL1-) during β -cell differentiation.

Finally, we identify four INS+/REST+ subpopulations that displayed a potential pancreatic niche profile with an immature progenitor signature prior the endocrine specification. In fact, those cells expressed the lowest level of INS gene in our overall dataset, which is consistent with the role of REST as a repressor of β -cell identity (Martin et al., 2017). A first CD9+ subtype co expressed TGF β , SPARC, NOTCH3 and ECM genes. The co expression of SPARC, TGF β and NOTCH3 genes in this population could reflect a pancreatic like tumoral population (Liu et al., 2016; Hartley et al., 2020). However, the TGF β signaling is also a key mechanism in β -cell differentiation, in inhibiting the exocrine cell differentiation (Sanvito et al., 1994) and repressing pancreatic ductal cell proliferation (Shin et al., 2011). TGF β also promotes p16 cell cycle regulator which controls self-renewal of adult endocrine stem cells (Dhawan et al., 2016; *nb*: which appeared consistent with the cell cycle genes of this population heatmap of Fig 5B). This subtype was also characterized by the enrichment of biological process related to the pancreatic mesenchyme. Consistently with our result, several evidence shows that pancreatic mesenchyme is supporting pancreatic epithelial cell growth and differentiation during organogenesis (Hibsher et al., 2016). Furthermore, NOTCH plays an important role in the development of pancreas from immature progenitors (Li et al., 2015). Positive NOTCH blocked endocrine differentiation and led to mesenchymal routes to ductal and acinar cells (Li et al., 2015). More particularly, NOTCH 3 is expressed in pancreas mesenchyme (Lammert et al., 2000). The second subtype of CD9+ cells co-expressed SOX9+/PDX1+/NEUROG3+, the markers of endocrine progenitors such as PAX4 and ARX, the NOTCH1 and NOTCH2 genes. The pancreatic ductal niche is characterized by SOX9+/PDX1+/NEUROG3+ positive cells committed to generate endocrine progenitors. 10% of the pancreatic ductal cells are also reported to produce glucagon

and insulin (Carpino et al., 2016). NOTCH1 and NOTCH2 are the first NOTCH genes expressed in the pancreatic epithelium development (Lammert et al., 2000), and need to be repress for endocrine specification (Li et al., 2015). Therefore, the profile of this subtype is consistent with a profile of a common root of the immature pancreatic progenitor as the one observed in developmental embryology (Li et al. 2015). Our CD9+ like-niche profile was completed by a sub population co expressing CLDN6+ (CLND6 is a tumoral marker, Qu et al., 2021; but is also an early marker of the epithelialization during embryogenesis, Turksen et al., 2001), GP2 (acinar marker, Scheele et al., 1994 and progenitor pancreatic marker, Cogger et al., 2017), CST4 and SLC4A4 (pancreas ductal marker) and moderately NOTCH1 and NOTCH2 genes. Finally, the last subtype co-expressed NOTCH1, NOTCH2, NOG (a BMP and TGF β signaling inhibitor promoting pancreatic development, Nostro et al., 2011) and CTGF (involved in the pancreatic β -cells replication during embryogenesis, Charrier et al., 2012) which appeared as another immature progenitor lineage before the endocrine specification.

Conclusions

We performed a β -cells like differentiation into pancreas like spheroids. The islets were responsible to the GSIS simulations. The 3D differentiation did not lead to fully mature specific pancreatic lineage. We observed an important heterogeneity of the β -like cell populations. The subpopulation included hormone secreting like cells with α , β , δ , γ , ϵ profiles. We also extracted four endocrine progenitors' populations. The immature endocrine progenitors were characterized by the expression of CD9. Even if we did not reach fully mature profile, we believed that our model would be a suitable tool to investigate pancreatic development and the pancreatic cells plasticity.

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Conflict of interest statement

The authors declare no conflict of interest.

Authors contributions

EL performed the biological experiments, designed the experiments and participated to data analysis and in the writing of the manuscript, LM, FT, VP, VP were involved in biological experiments, AA contributed to the experimental design and participated to the discussion and in the writing of the manuscript, SP and SHK were involved in the sequencing and bioinformatics treatment, processed transcriptomics data and participated in the writing of the manuscript, FT made the honeycomb plates, CL, RJ and YS were involved in the experimental design and LM supervision, EL is the project supervisor, designed the experiments and participated to data analysis and the writing of the manuscript. All authors are involved in the manuscript revision and approval.

Figures captions

Figure 1: (A) Morphologies of the spheroids after 24h of culture; (B) Morphologies of the spheroids at the end of the culture (day 16); (C) Insulin secretion in glucose stimulation assays: ratio of insulin between low and high glucose (n=3, p_value < 0,05).

Figure 2: UMAP projection of the mRNA expression levels of selected genes in the sequenced cell populations illustrating the levels of maturation of the pancreatic spheroids: (A, B) pluripotent, (C) mesoderm, (D) definitive endoderm, (E, F, G, H)

pancreatic progenitor; (I, J) endocrine specification, (K, L) advanced β -cells, (M) insulin markers.

Figure 3: (A) Plume plots of selected genes used to sort and extract main β -like cells subpopulations: GCG+, SST+, PPY+, CD9+, ST8SIA1+, PPY+, GHRL+ and UCN3+ (INS+); (B) heatmap of expression of the top 10 genes representative of each subpopulation; (C) heatmap of expression of specific genes involved in the stratification of the INS+/CD9-/ST8SIA1- (β 1-like-cells), INS+/CD9+/ST8SIA1- (β 2-like-cells) and INS+/CD9-/ST8SIA1+ (β 3-like-cells) subpopulations.

Figure 4: Enriched GO_biological processes extracted from Shinygo and highlighted from top expressed genes in (A) INS+/UCN3+ subpopulation, (B) in INS+/ST8SIA1+/CD9- subpopulation, (C) in INS+/GHRL+ subpopulation, (D) in INS+/STT+ subpopulation, (E) in subpopulation INS+/PPY+, (F) in INS+/ARX+/GCG+ subpopulation.

Figure 5: (A) UMAP plot presenting the 10 cell subpopulations identified by K-means clustering; (B) heatmap of expression for selected genes specific of each subpopulation.

Figure 6: (A) Enriched biological processes in PPY+/IAPP+ cell subpopulation; (B) Enriched reactomes in DPP4+/KDR+ cell subpopulation; (C) Enriched biological processes in TTR+/GCG+/SST + cell subpopulation; (D) Enriched reactomes in TTR+/GCG+/SST + cell subpopulation. Data are extracted from Shinygo.

Figure 7: (A) We reclustered the CD9+/SPARC+/REST+ subpopulation to extract the various subtypes of this subpopulation. We presented the Heatmap of the expression levels for selected genes illustrating the expression profiles of the different subtypes of cells identified upon the reclustering of the CD9+/SPARC+/REST+ cell population; Enriched pathways identified by the top highly expressed genes in the four subtypes of cells extracting from the reclustering of the CD9+/REST+/SPARC+ population.

Figure 8: (A) Enriched biological processes in SOX2+/NEUROG3+ cell subpopulation, (B) Enriched biological processes HNF1A+/APOA2+ cell subpopulation, (C) Enriched molecular functions in INHBA+/INHBB+ cell subpopulation, (D) Enriched biological processes in INHBA+/INHBB+ cell subpopulation, (E) Enriched biological processes KCN3+/MYH4+ cell subpopulation. Data are extracted from Shinygo.

Supplementary Figure 1: Experimental protocol

Supplementary Figure 2: Heatmap showing the expression level of selected genes in the INS+/UCN3+ (β 1-cells), INS+/CD9+ (β 2-cells), INS+/ST8SIA1+ (β 3-cells), INS+/GRHL+, INS+/ARX/GCG+, INS+/PPY+, INS+/STT+ populations extracted from loop browser analysis.

Supplementary Figure 3: Heatmap showing the expression levels of top 10 highly expressed genes in the 10 clusters identified from the data driven analysis.

Supplementary Figure 4: Enriched KEGG pathways by the top 200 genes of the PPY+/IAPP+ subpopulation

Supplementary Figure 5: Enriched KEGG pathways by the top 200 genes of the DPP4+/KDR+ subpopulation

Supplementary Figure 6: Enriched KEGG pathways by the top 200 genes of the TTR+/GCG+/STT+ subpopulation

Supplementary Figure 7: Enriched biological processes by the top 200 genes of the cycling cell subpopulation

Supplementary Figure 8: (A) Top 200 genes enriched biological processes in CD9+/SPARC+/REST+ subpopulation. (B) Network of enriched biological processes in the CD9+/SPARC+/REST+ subpopulation.

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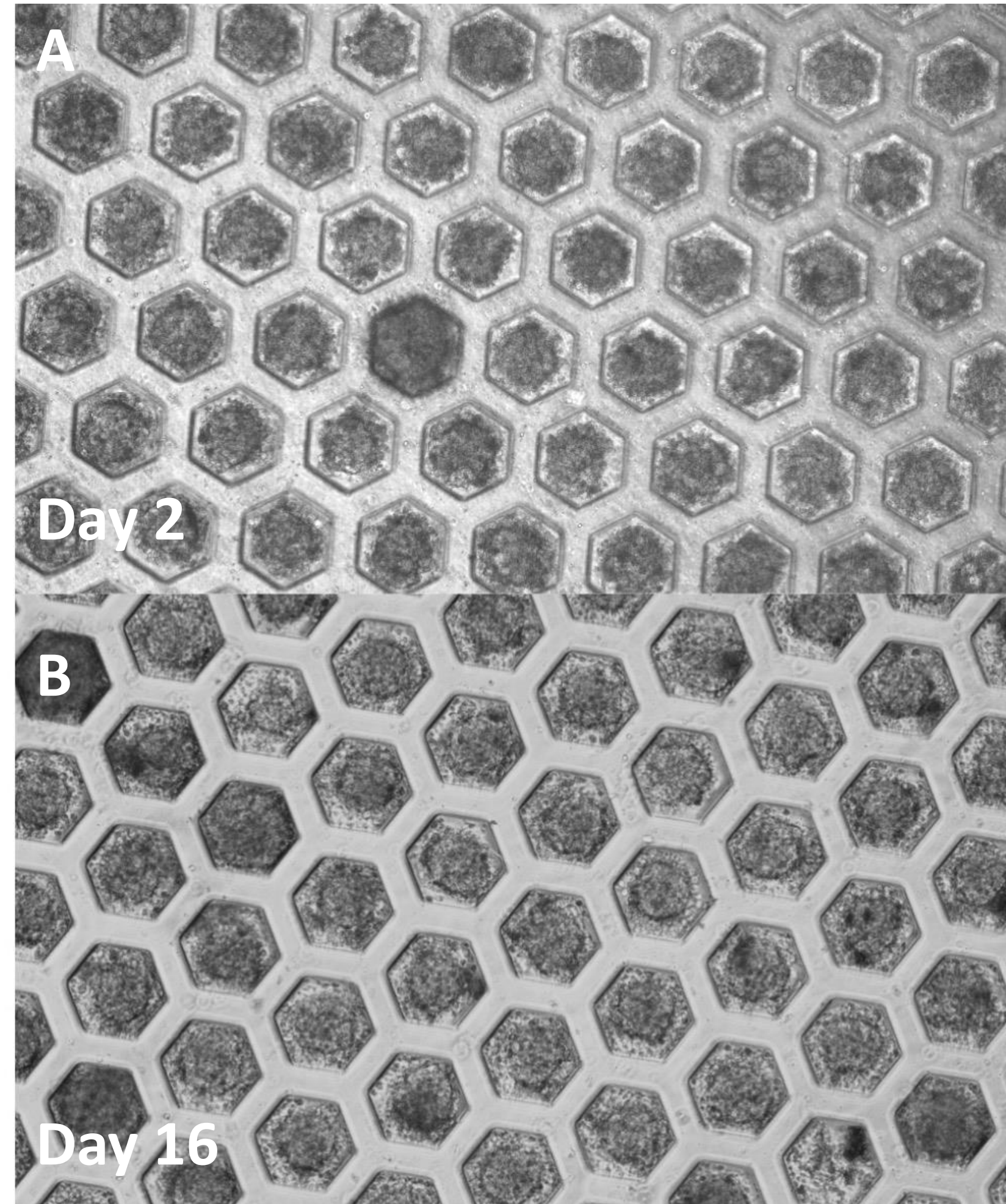
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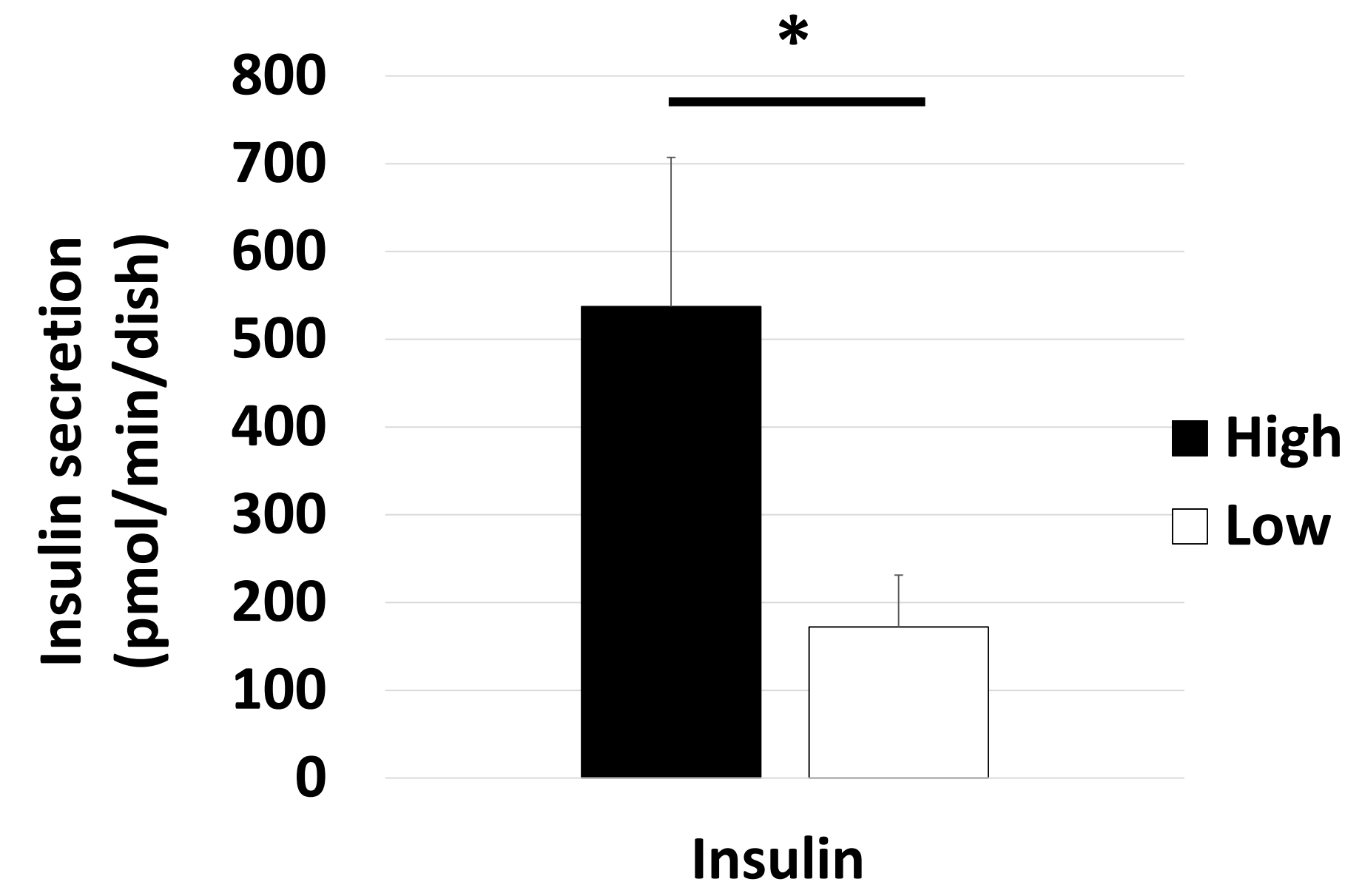
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120 μm

Figure 1

C



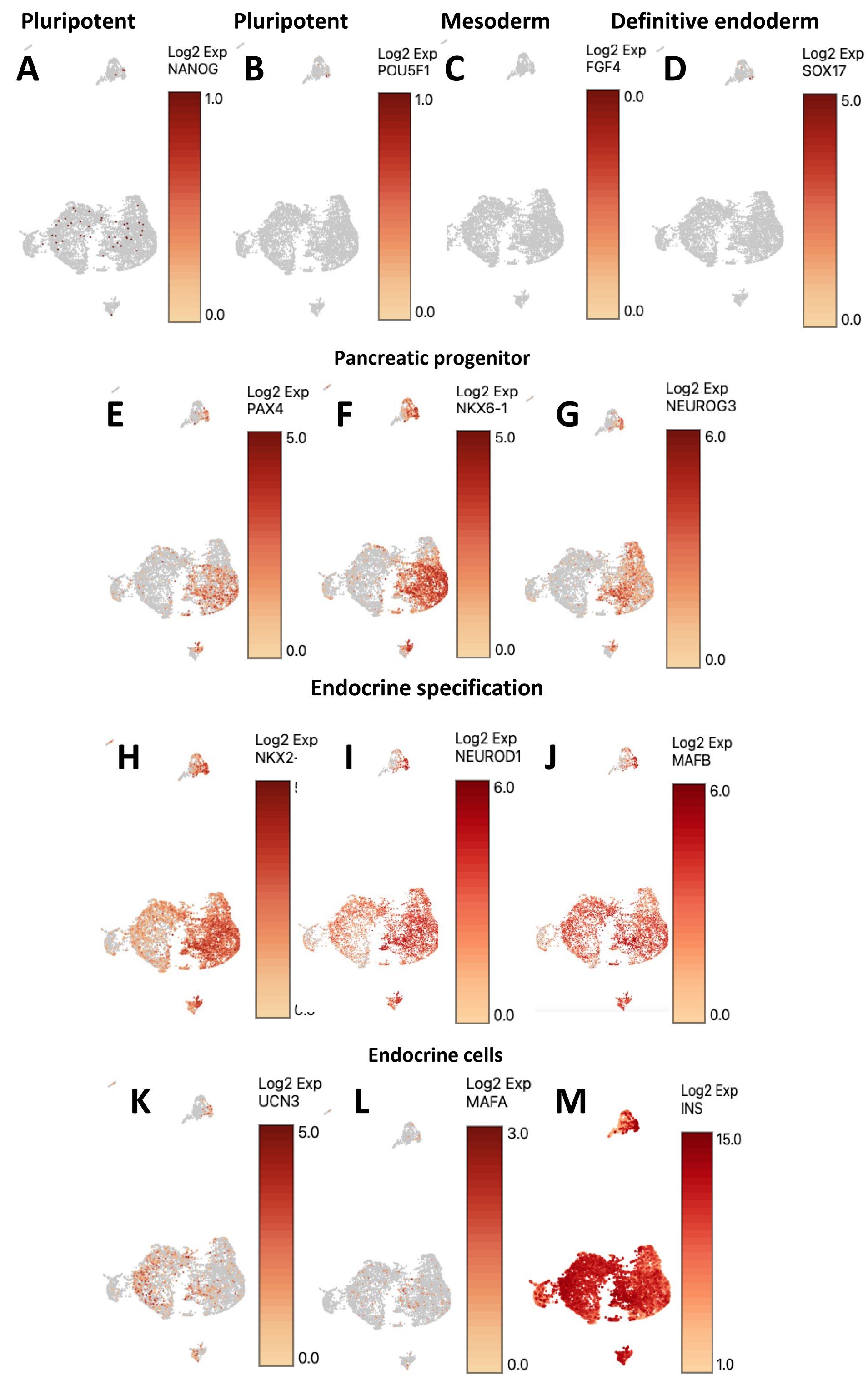


Figure 2

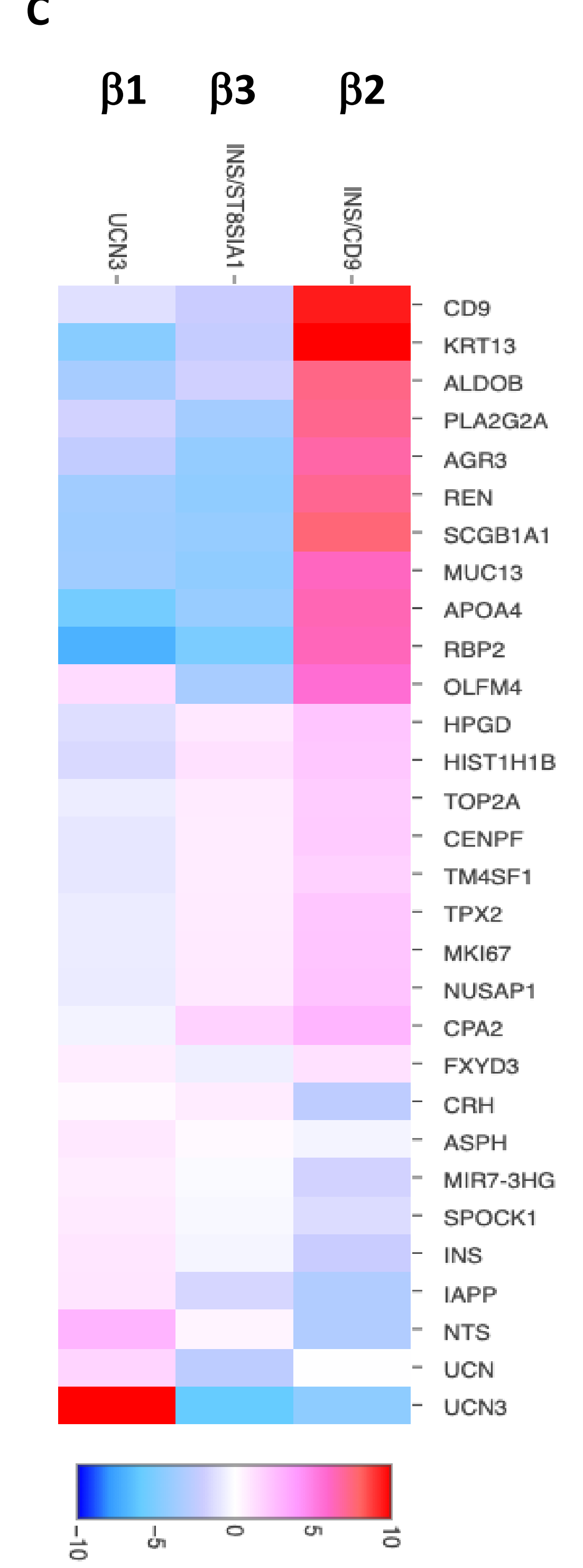
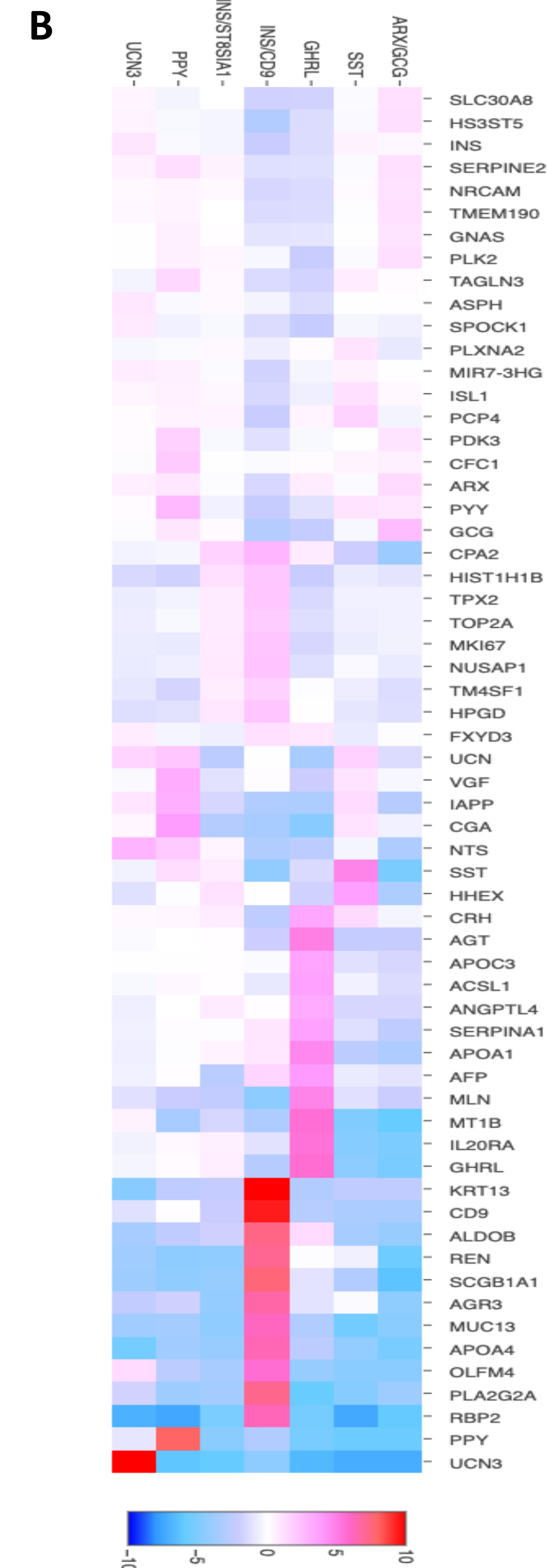
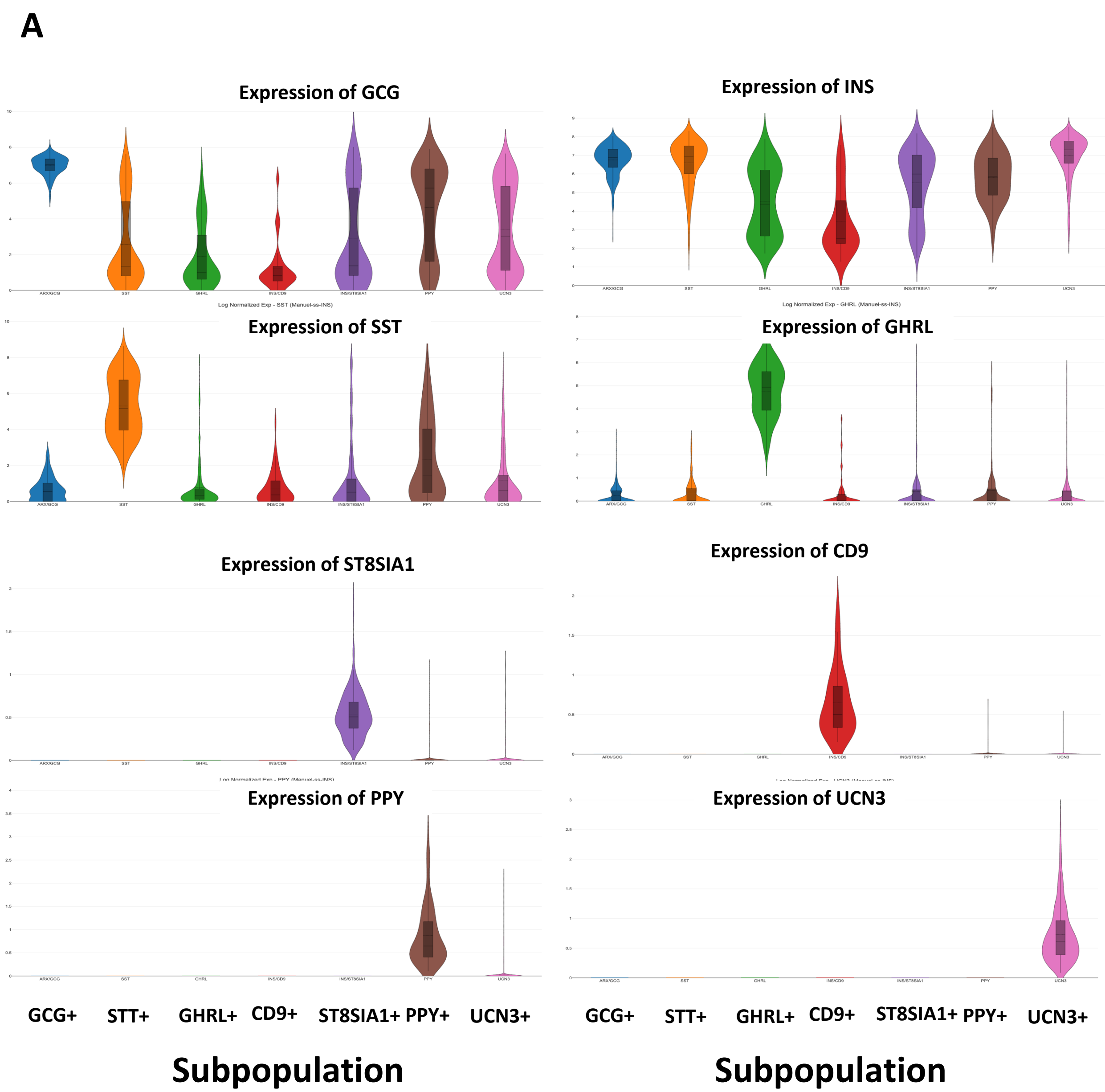


Figure 3

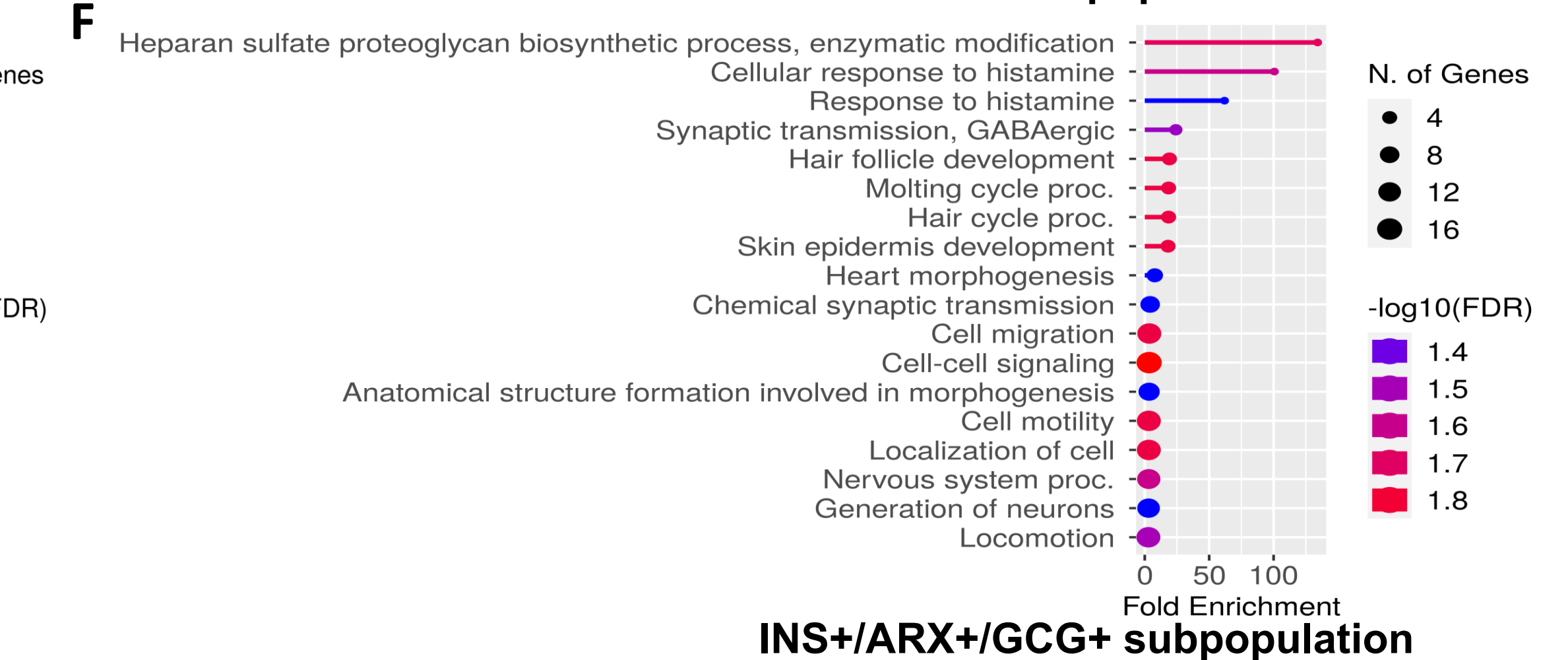
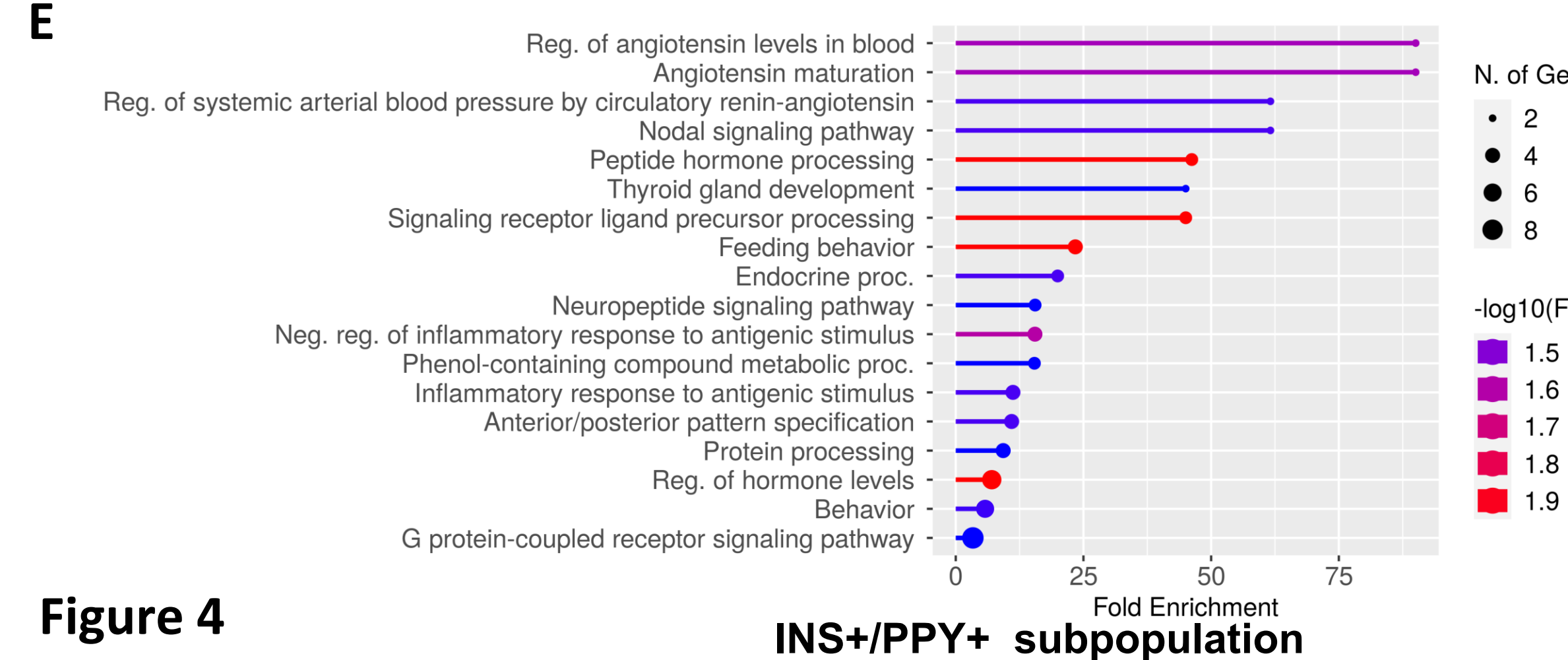
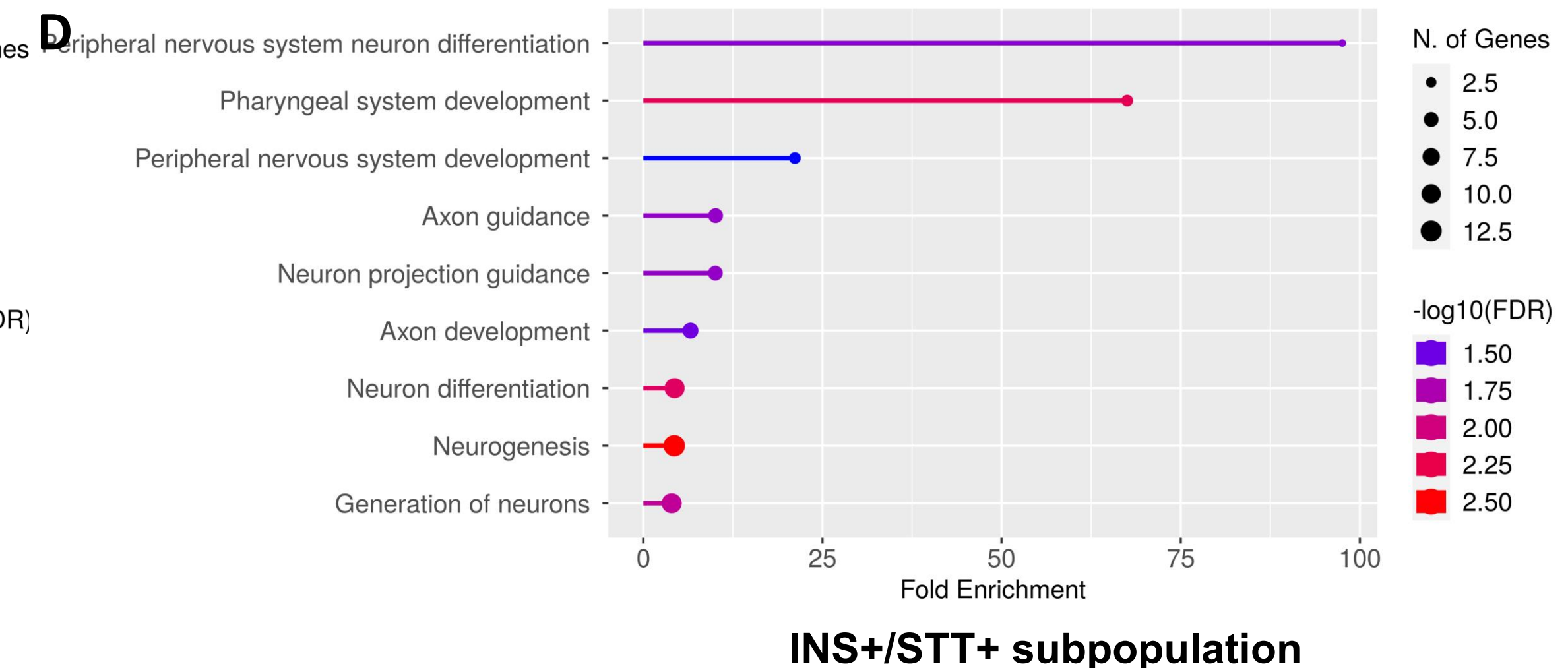
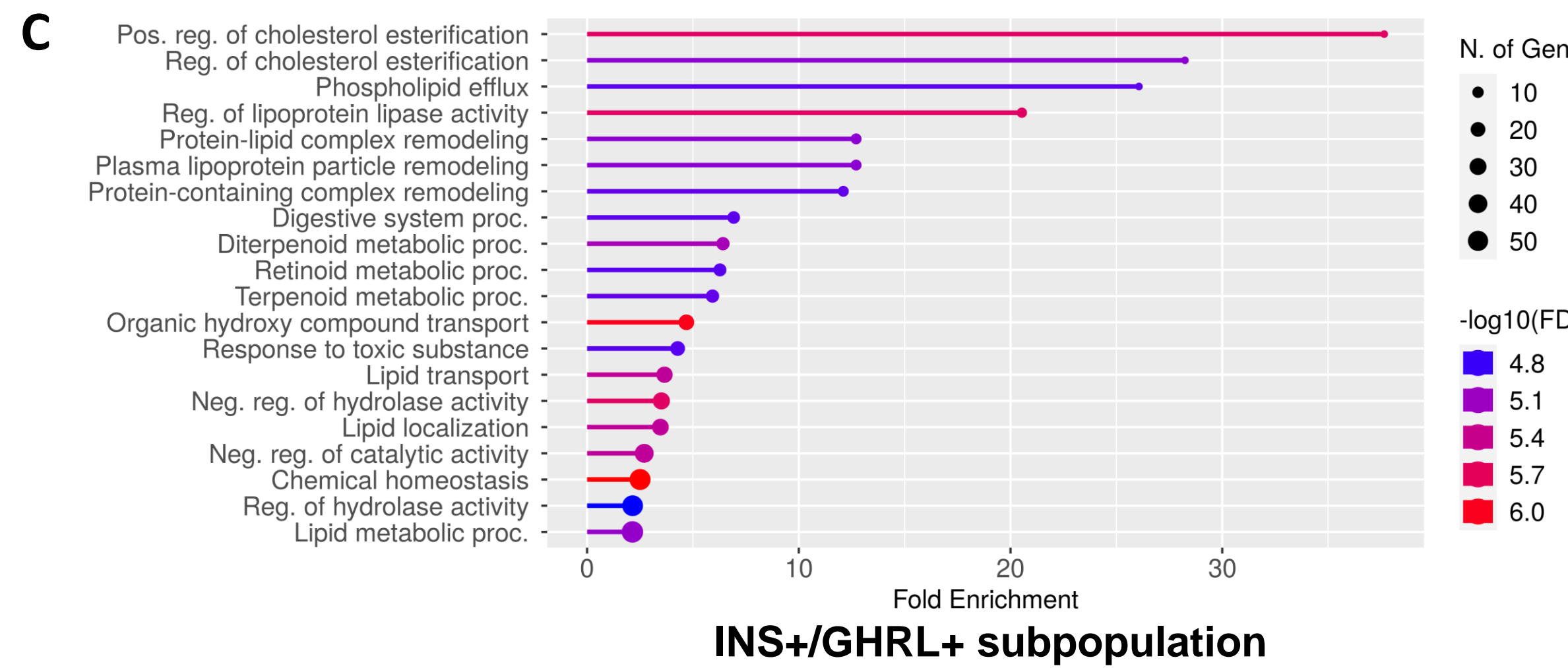
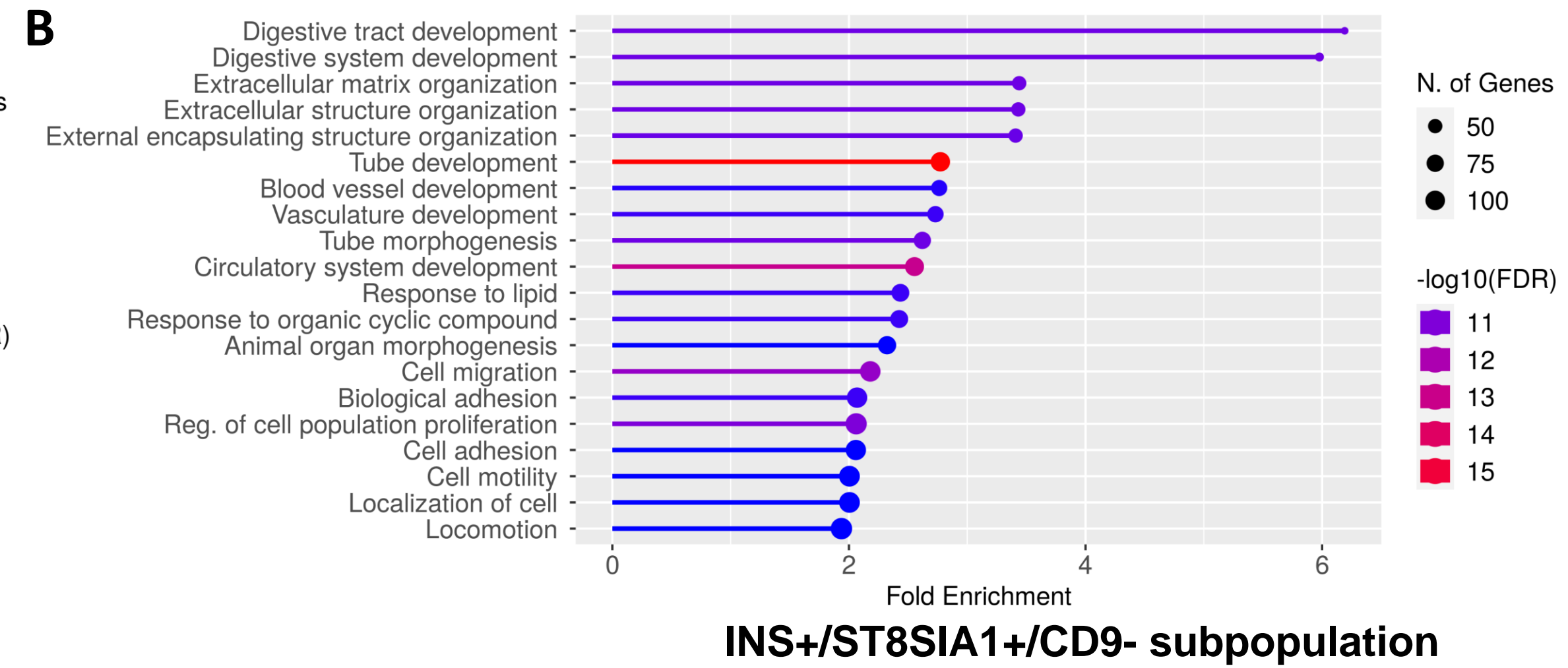
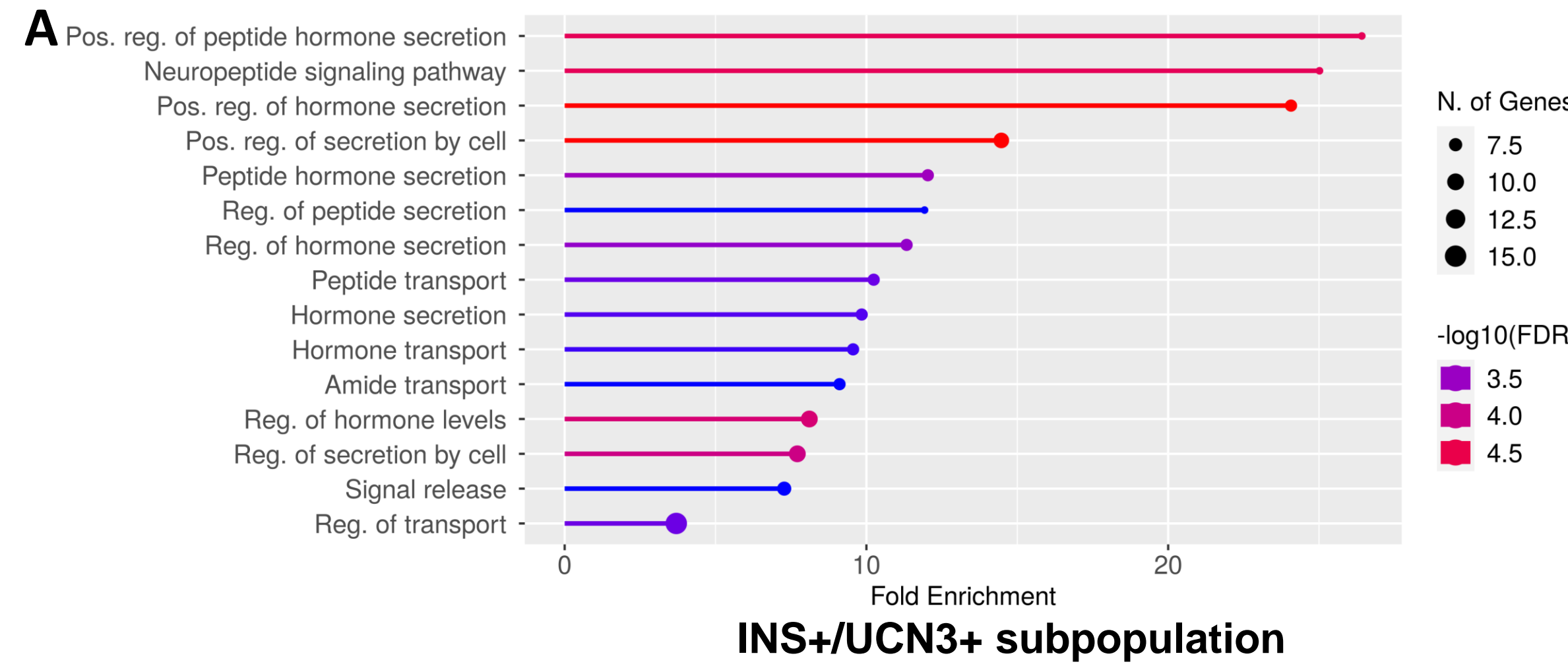


Figure 4

A

- DPP4+/KDR+
- KCNJ3+/TPH1+
- TTR+/GCG+/SST+
- INHBA+/INHBB+
- HNF1A+/APOA2+
- PPY+/IAPP+
- CD9+/SPARC+/REST+
- SLC2A4+/GCKR+
- SOX2+/NEUROG3+
- Cycling-Cells



B

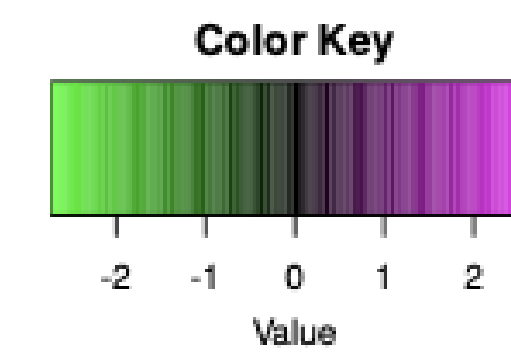
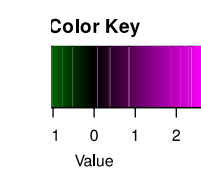
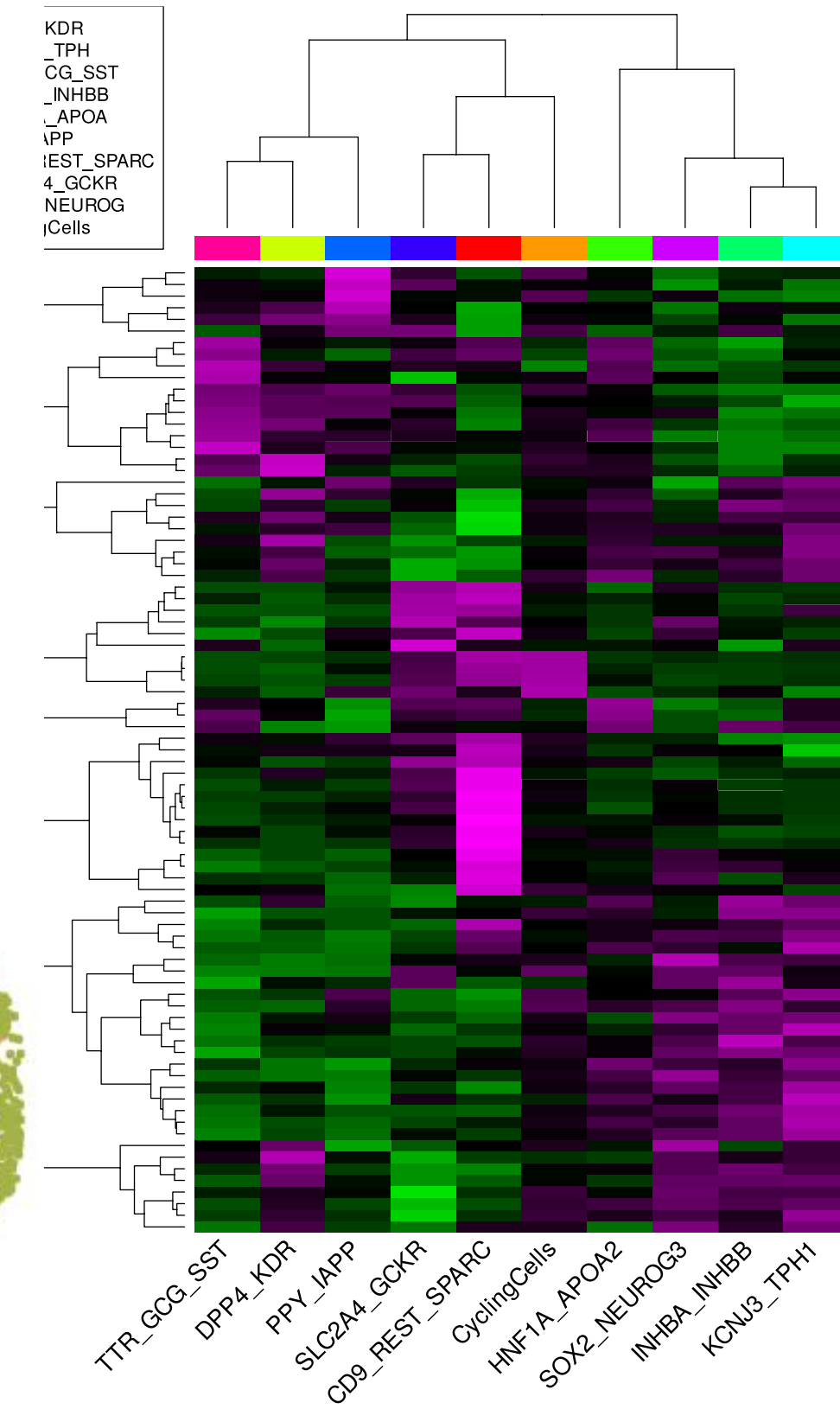
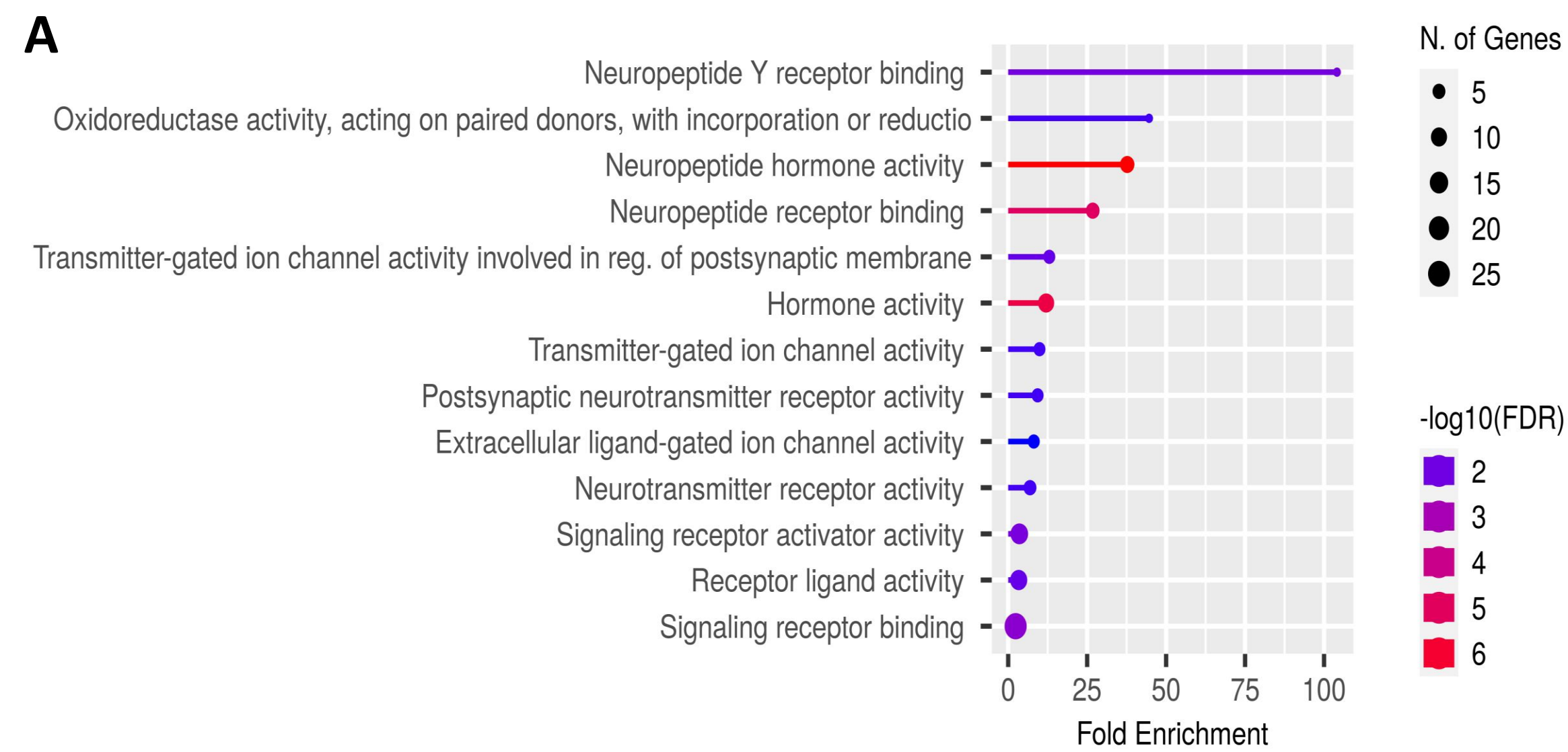
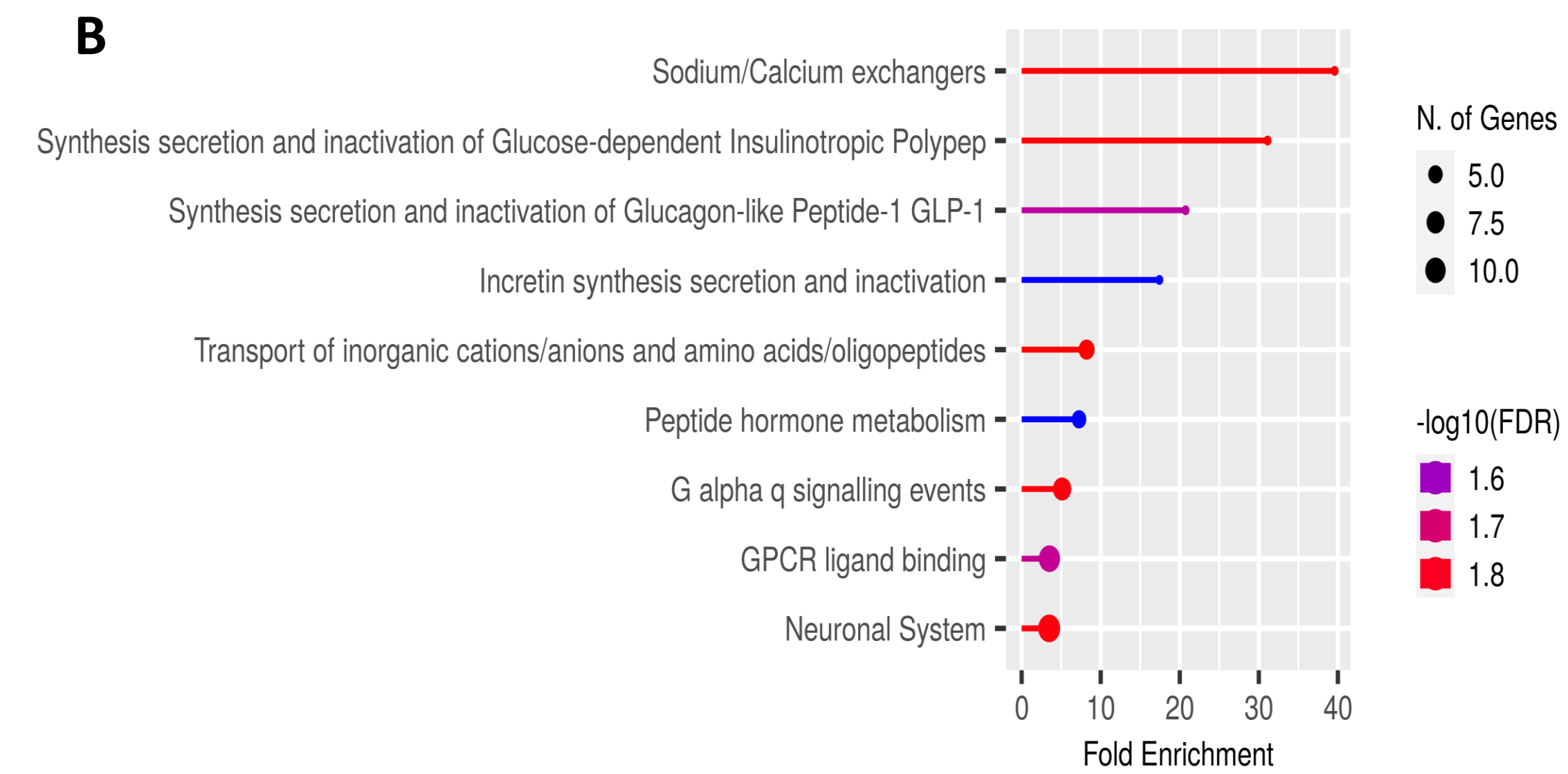


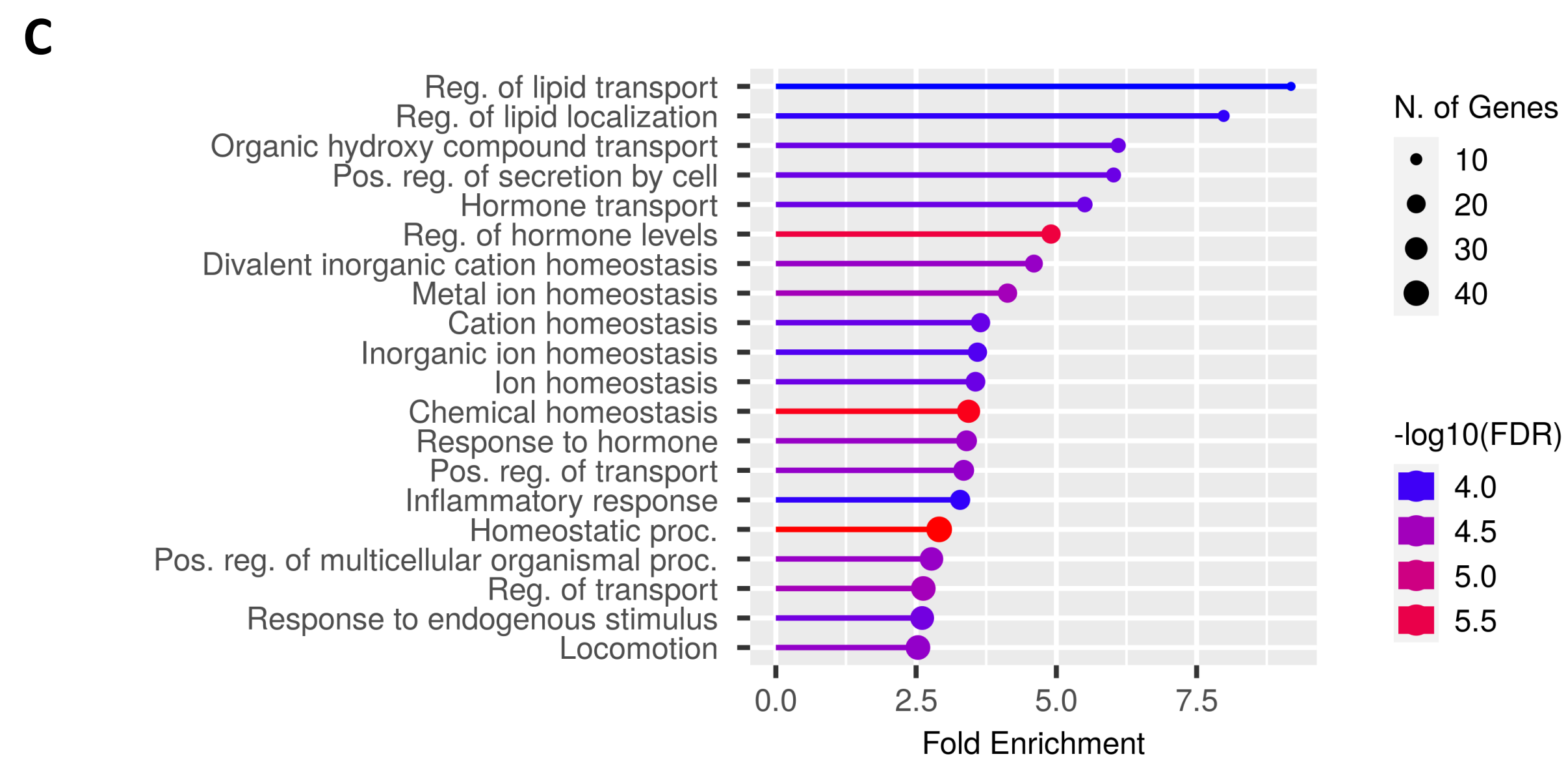
Figure 5



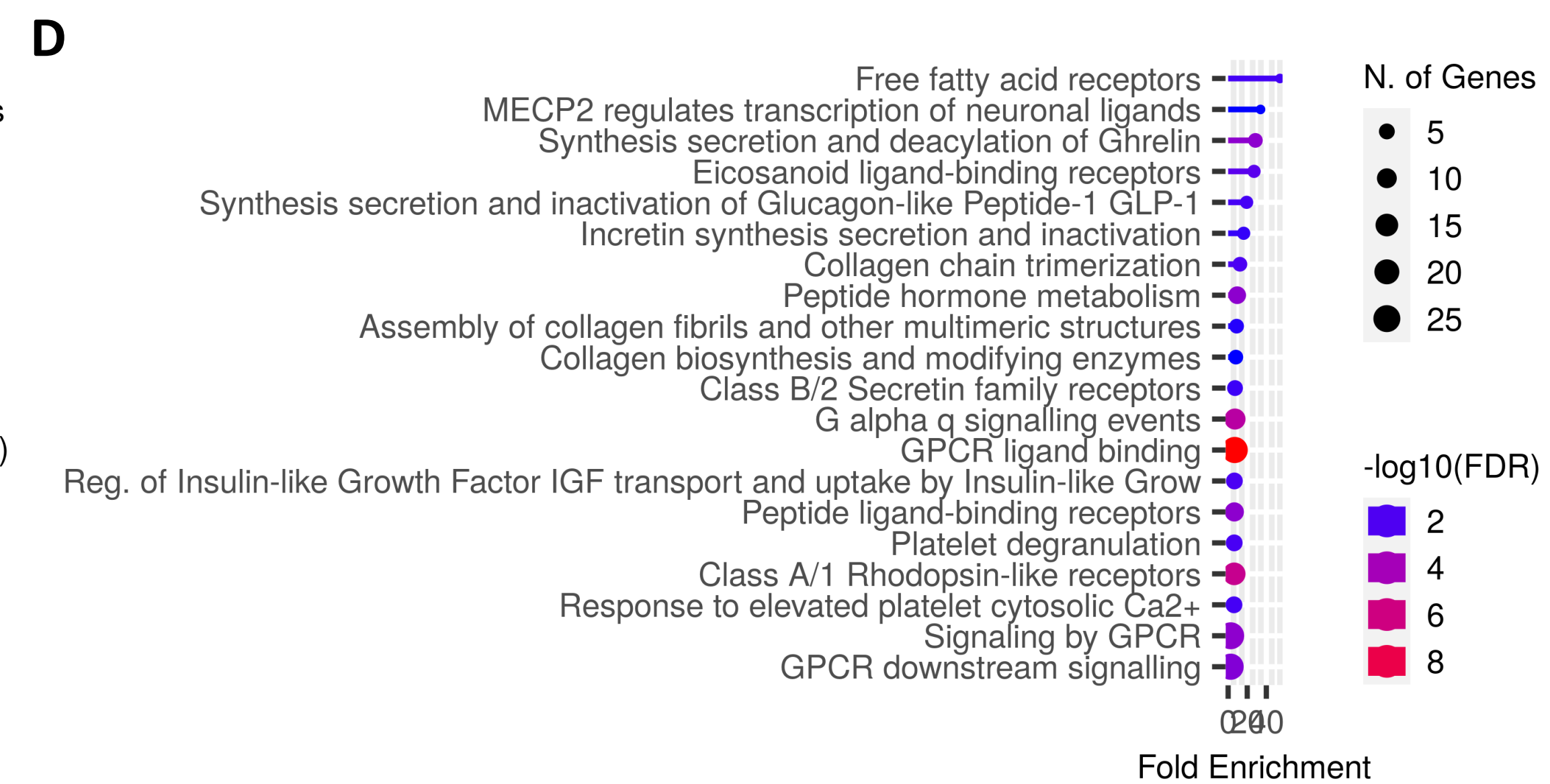
Top 200 genes of PPY+ cell population molecular function



Top 200 genes of DPP4+/KDR+ cell population reactome

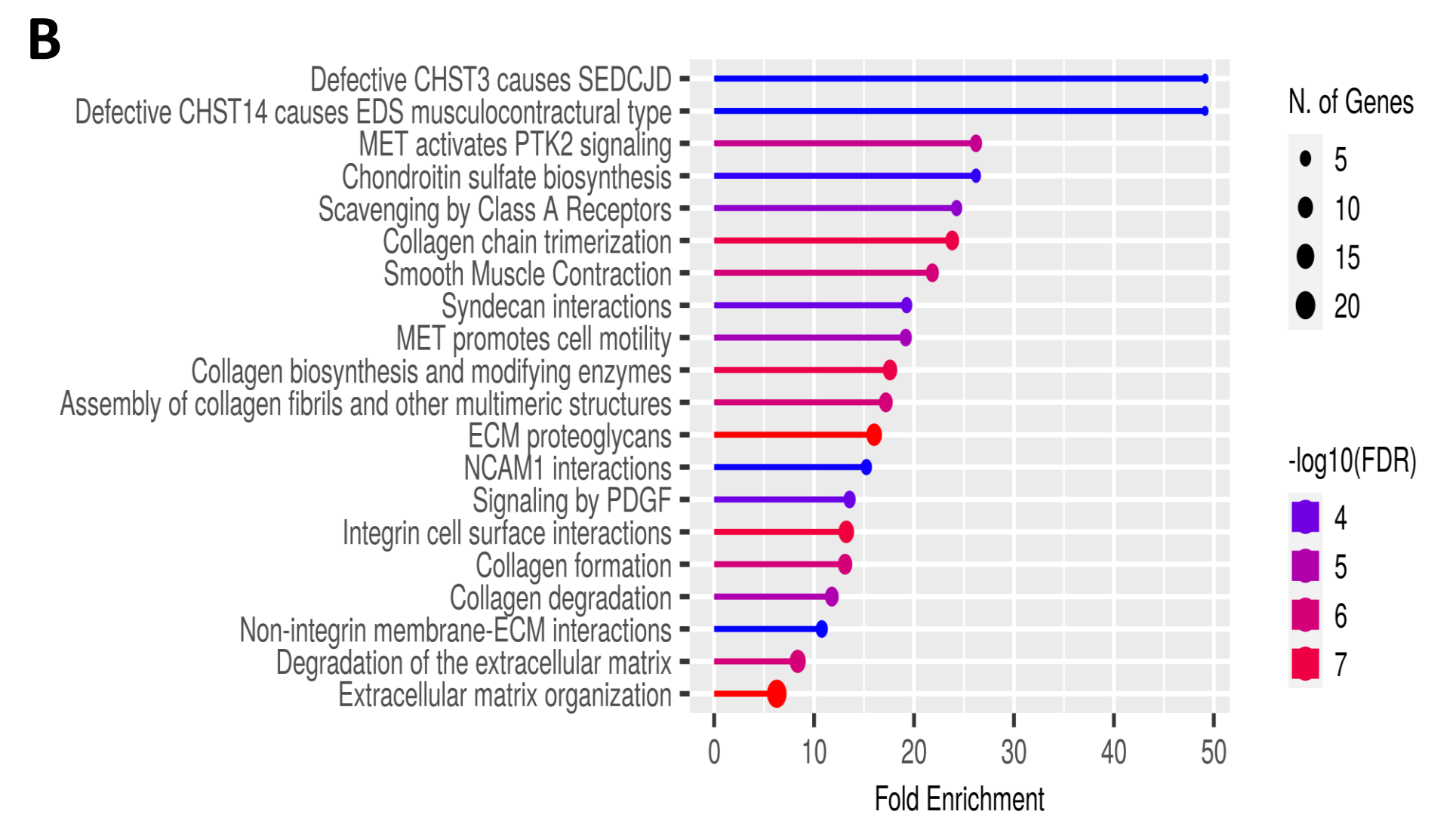
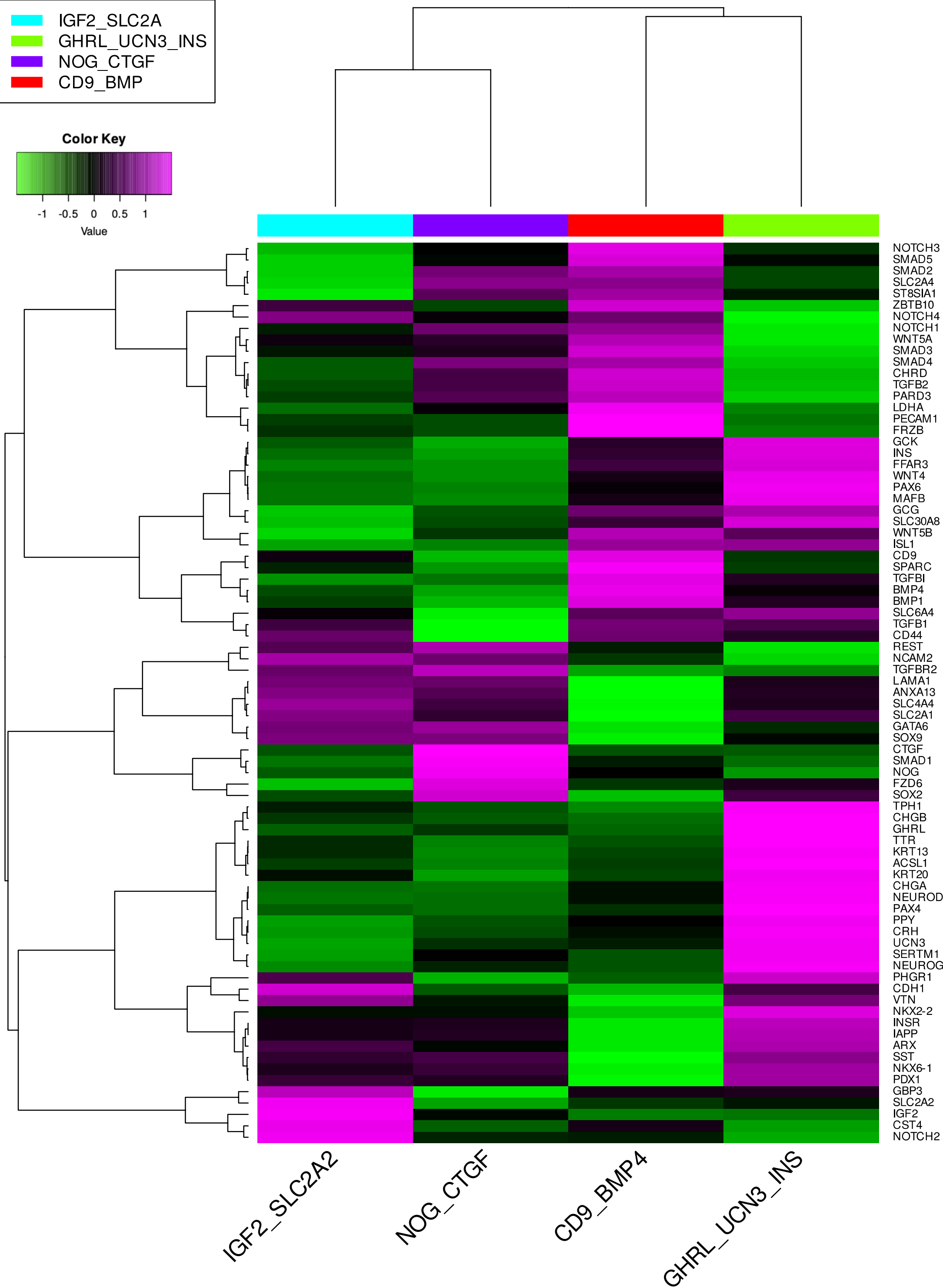


Top 200 genes of the TTR+/CGC+/SST+ cell population biological process

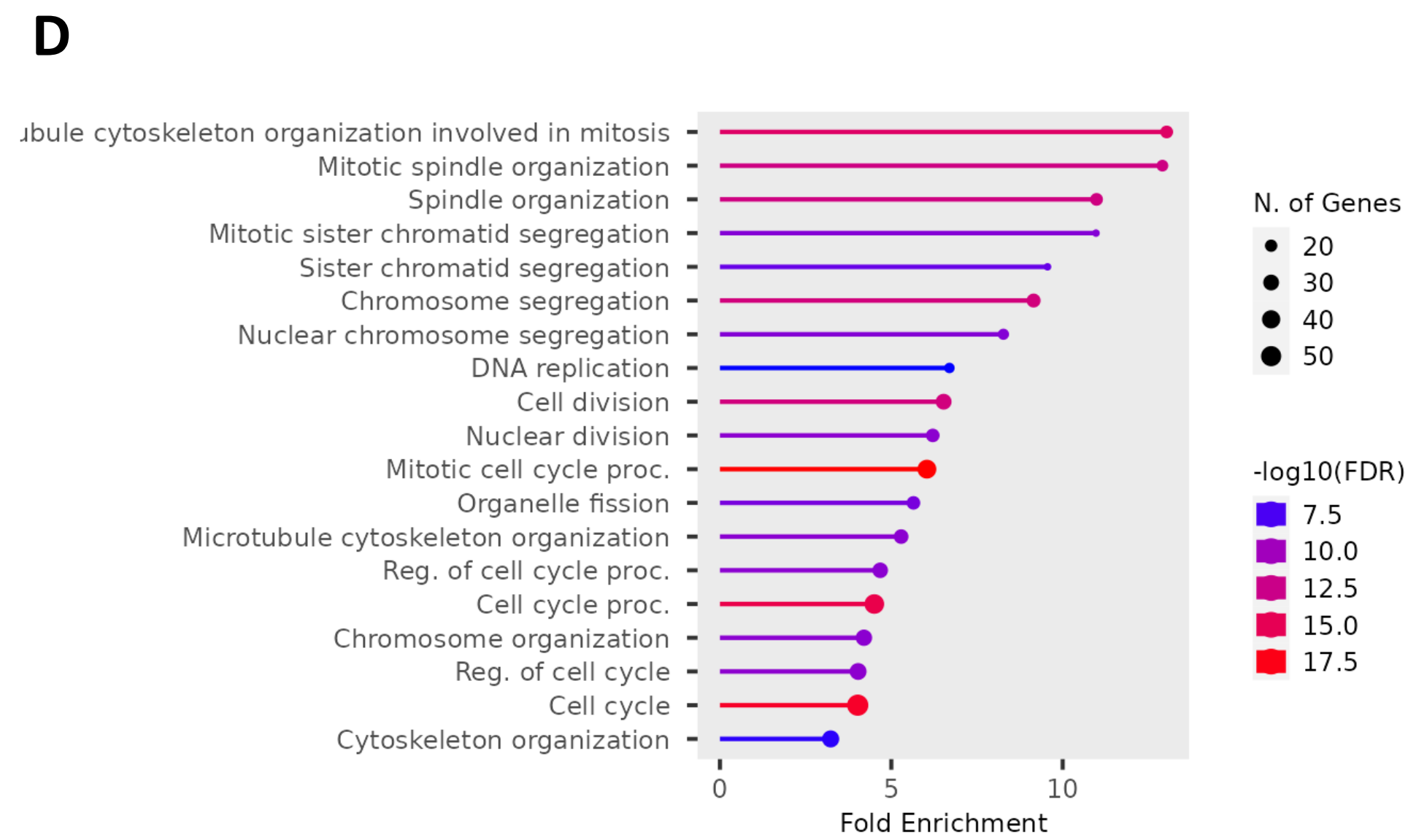


Top 200 genes of the TTR+/CGC+/SST+ cell population reactome

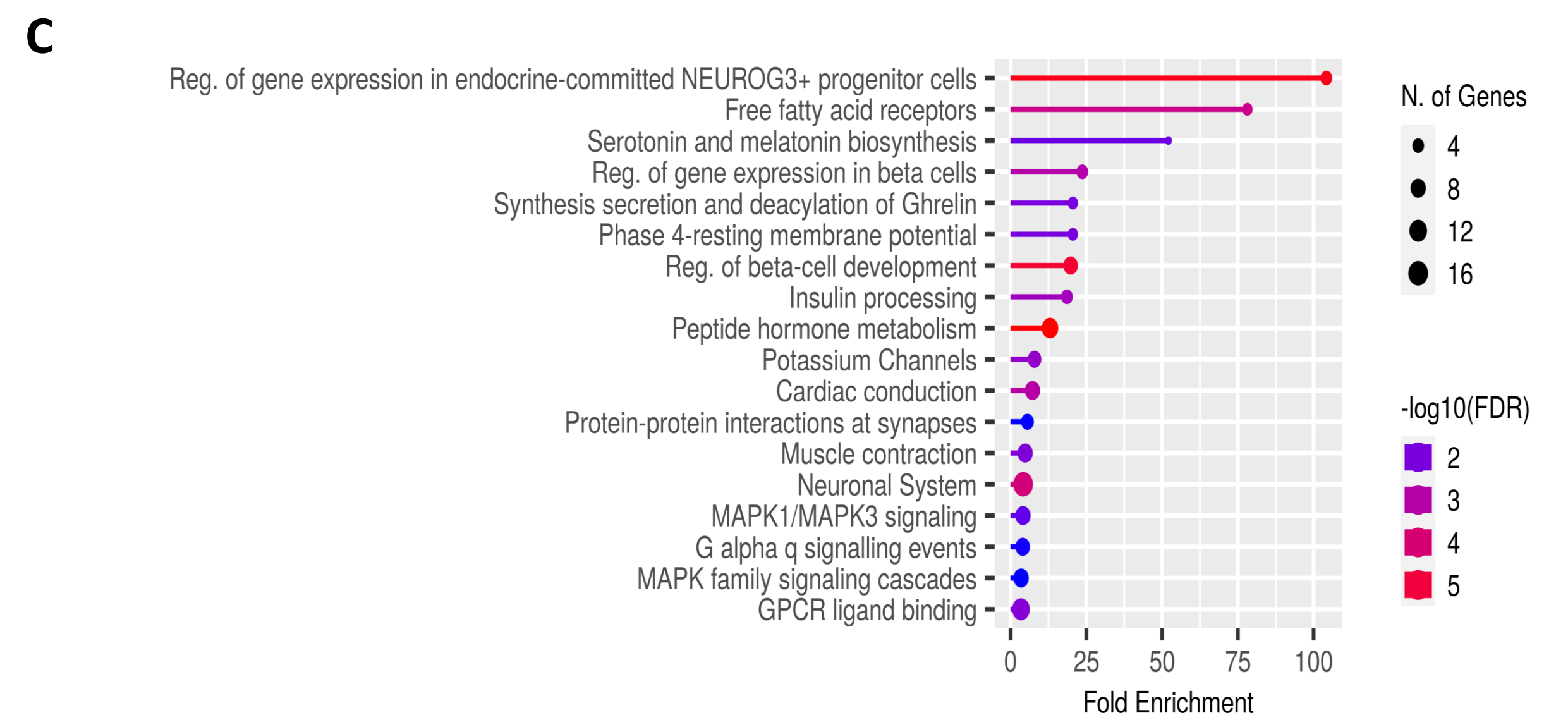
Figure 6



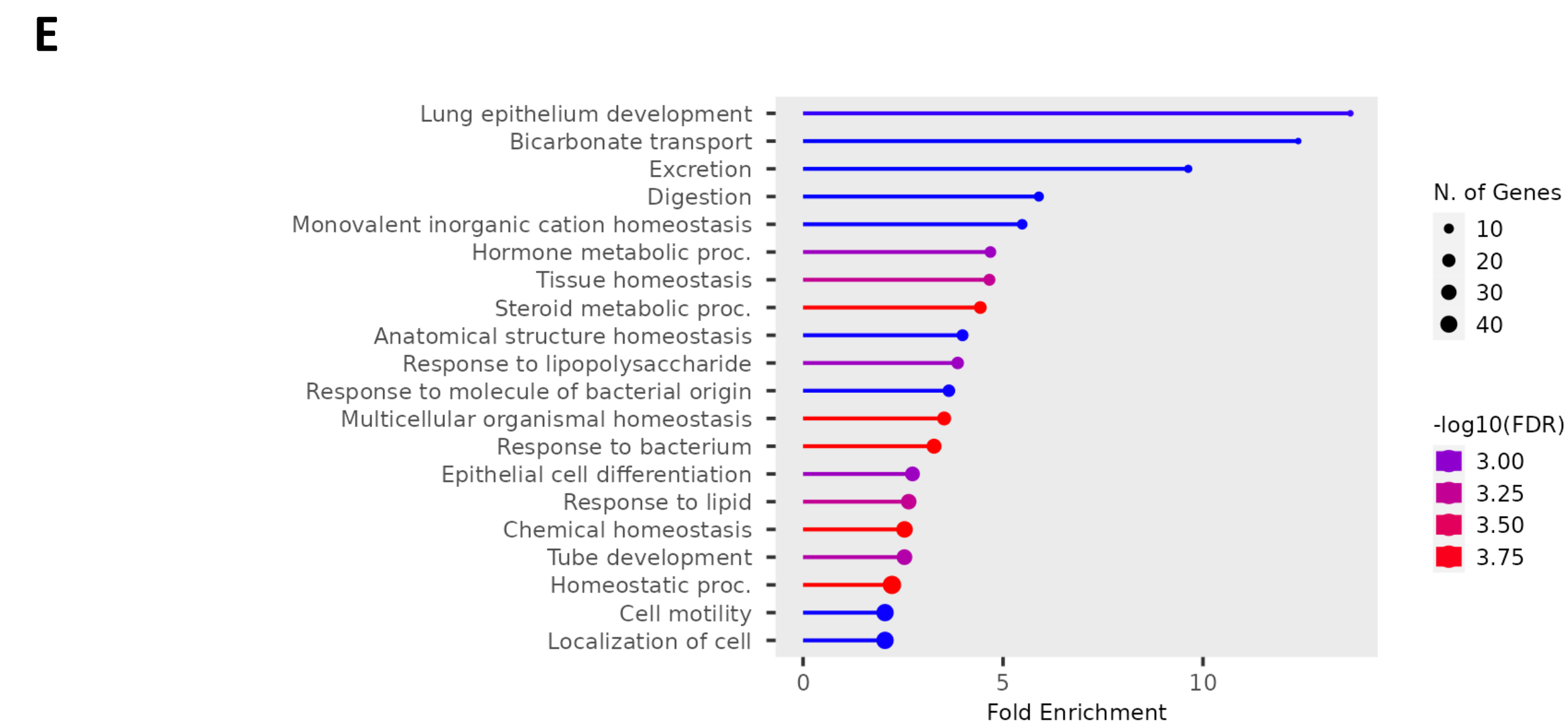
Top enrichment of the CD9+/BMP4+ cell population reactome



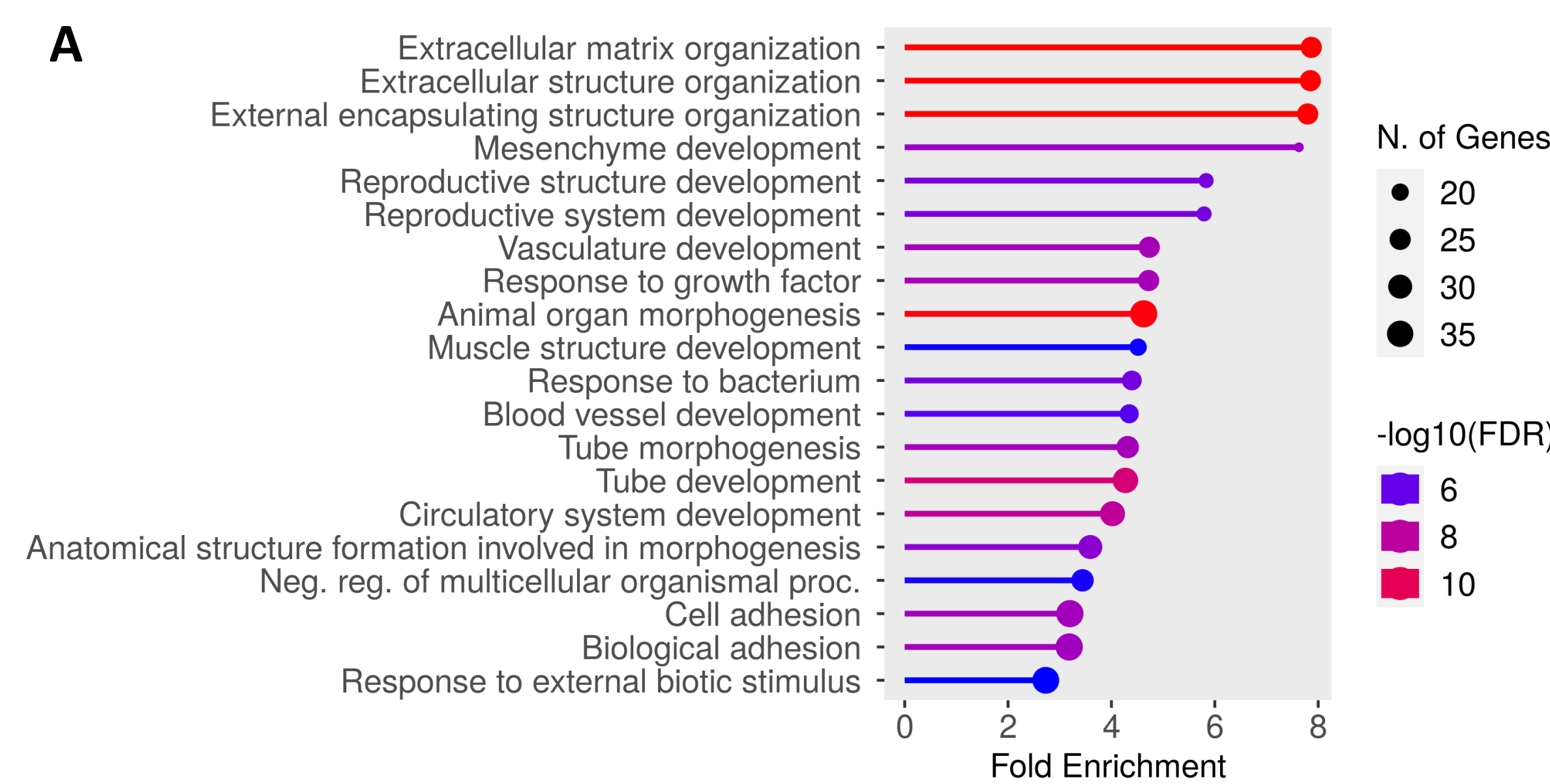
Top enrichment of the NOG+/CTGF+ cell population biological process



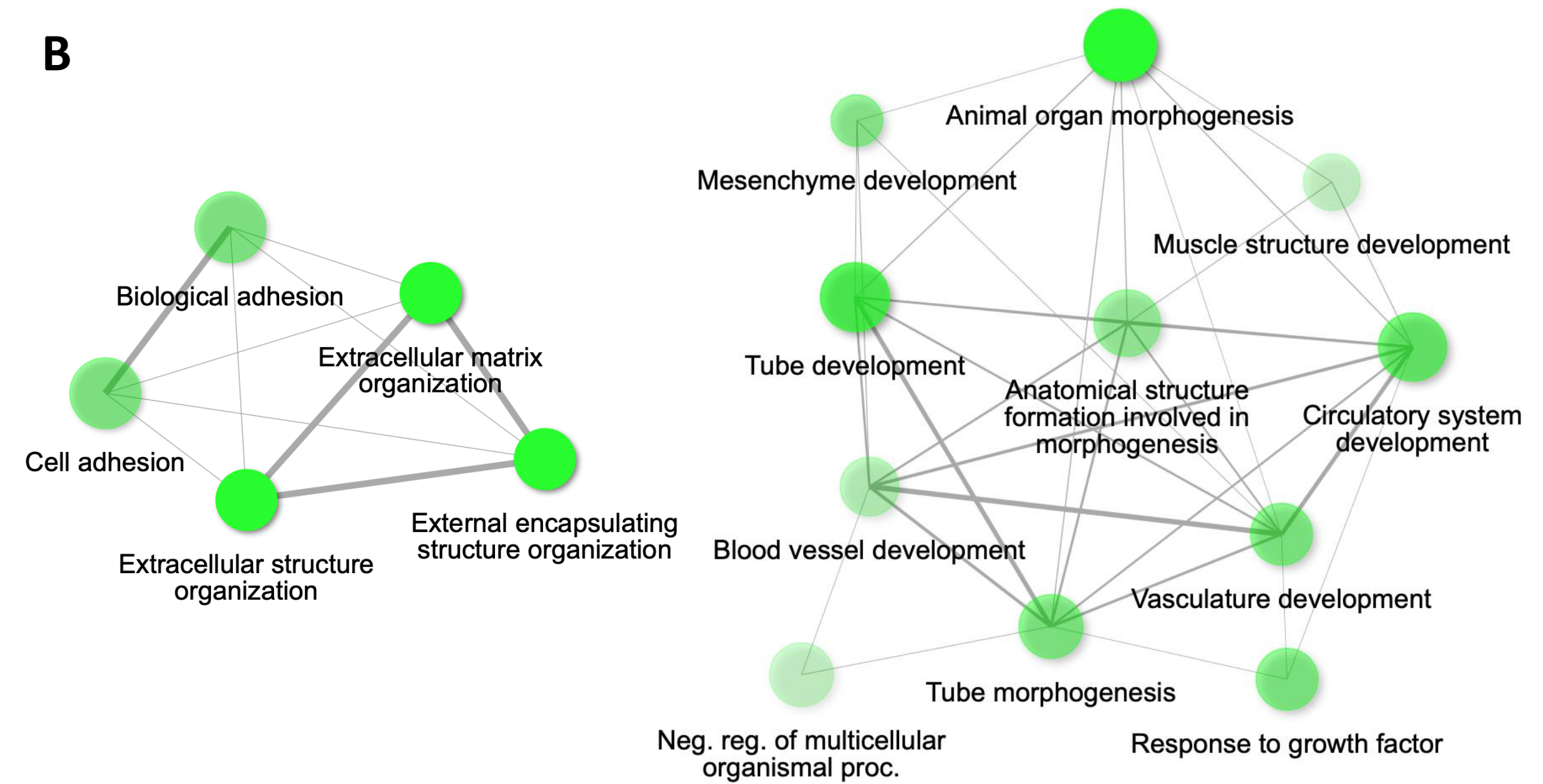
Top enrichment of the GHRL+/UCN3+/INS+ cell population reactome



Top enrichment of the IGF2+/SLC2A2+ cell population biological process



Top 200 genes of CD9+/SPARC+/REST+ cell population enriched biological process



CD9+/SPARC+/REST+ cell population network of enriched biological process

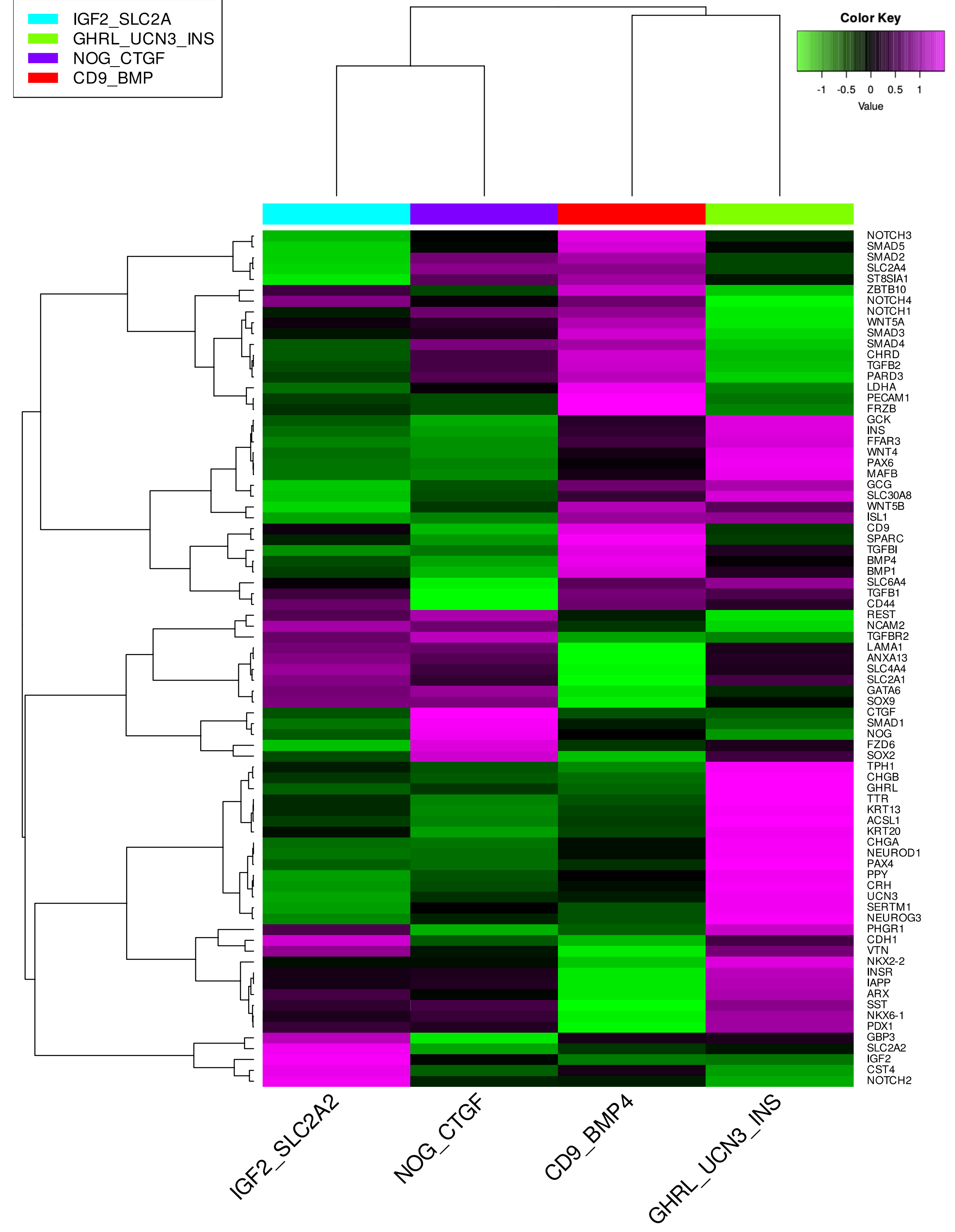
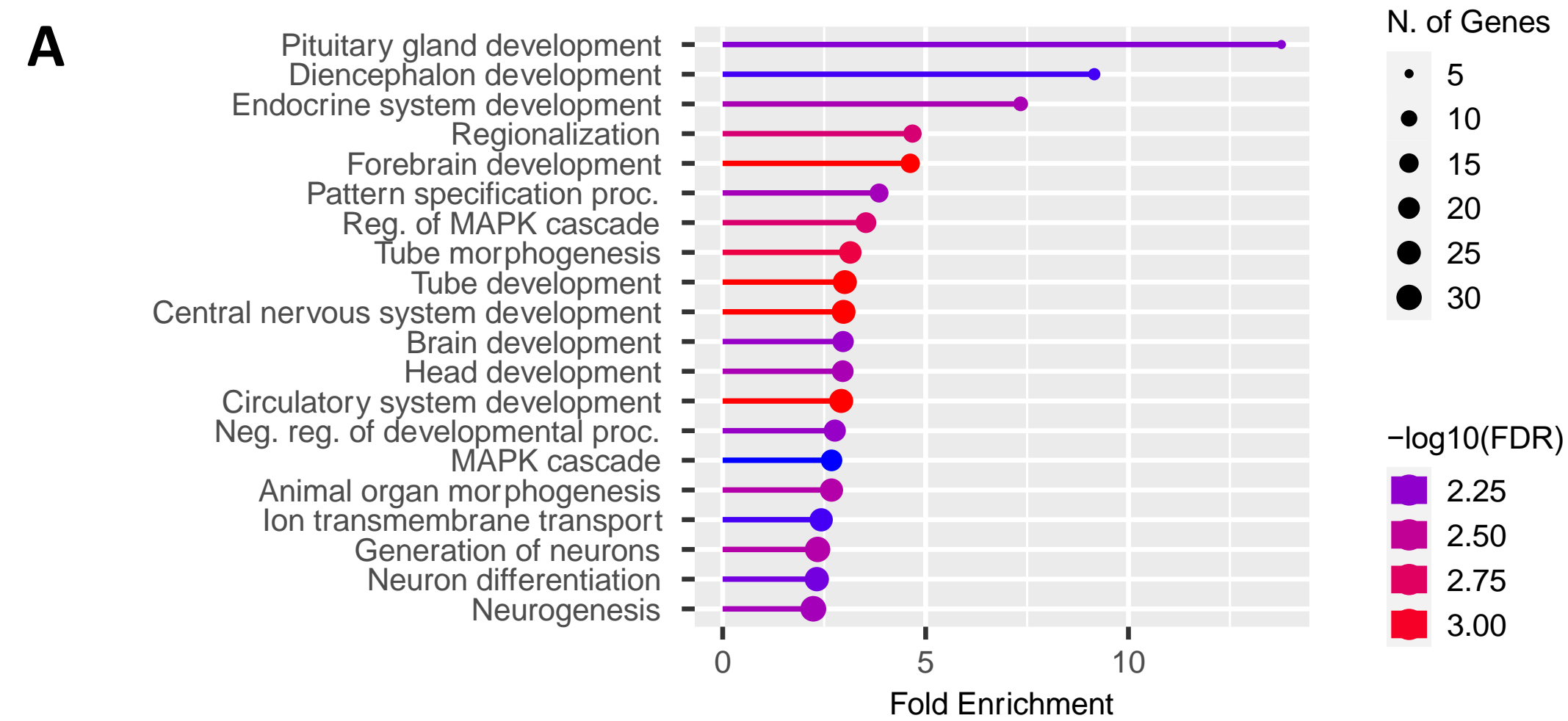
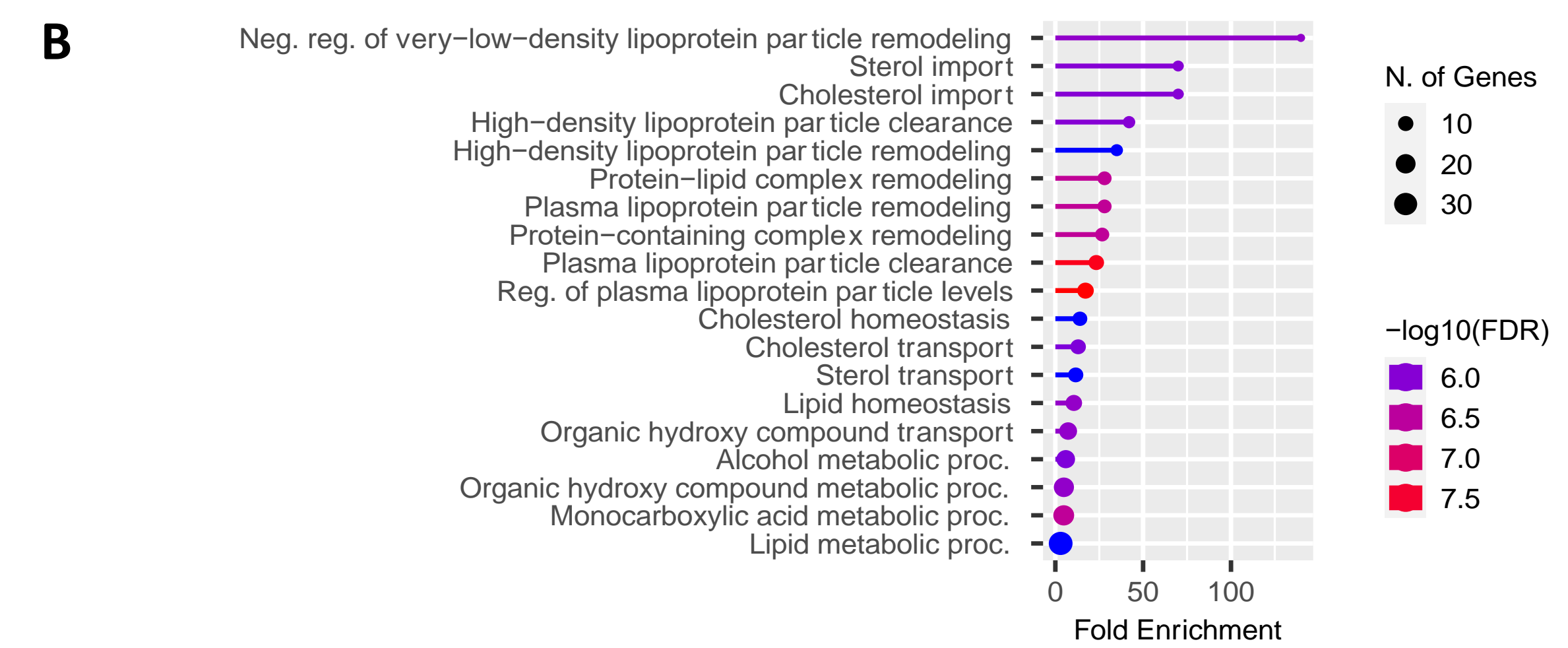


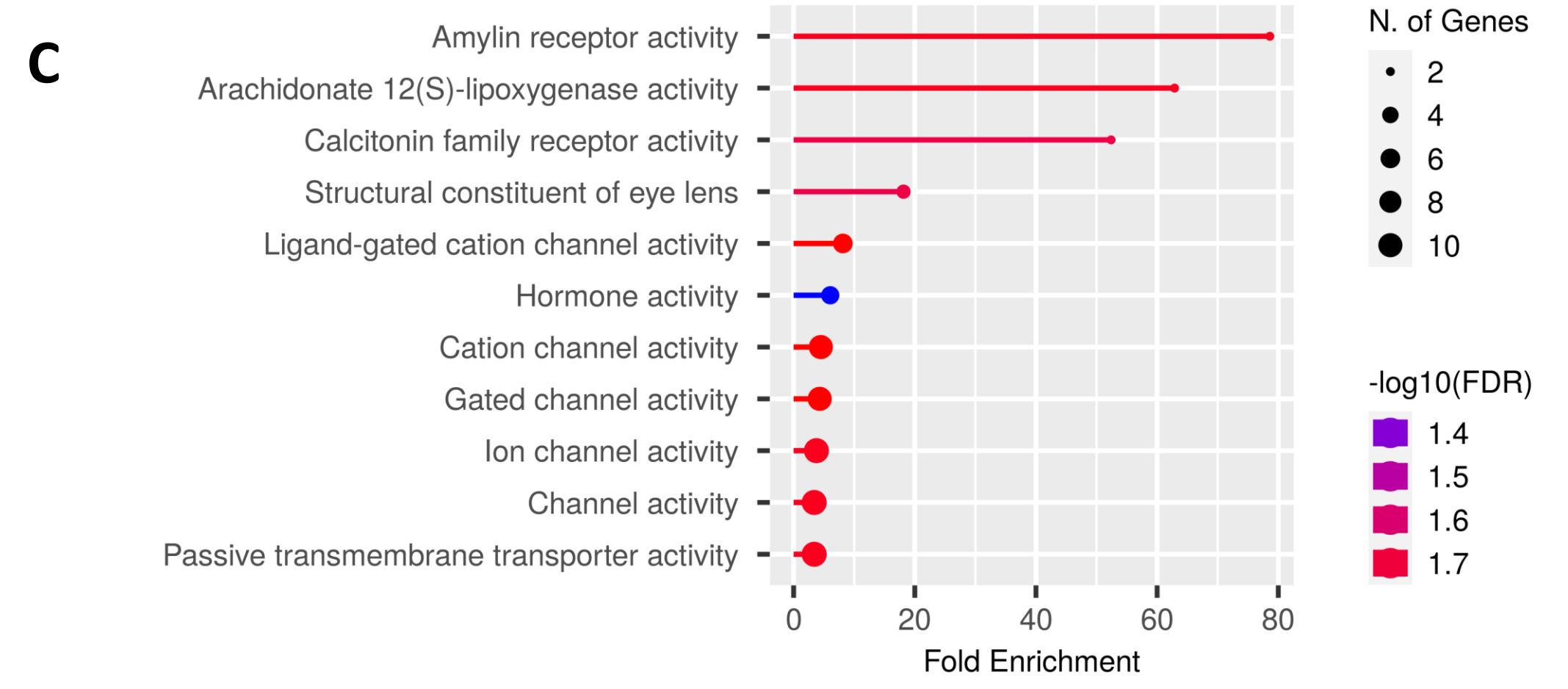
Figure 7



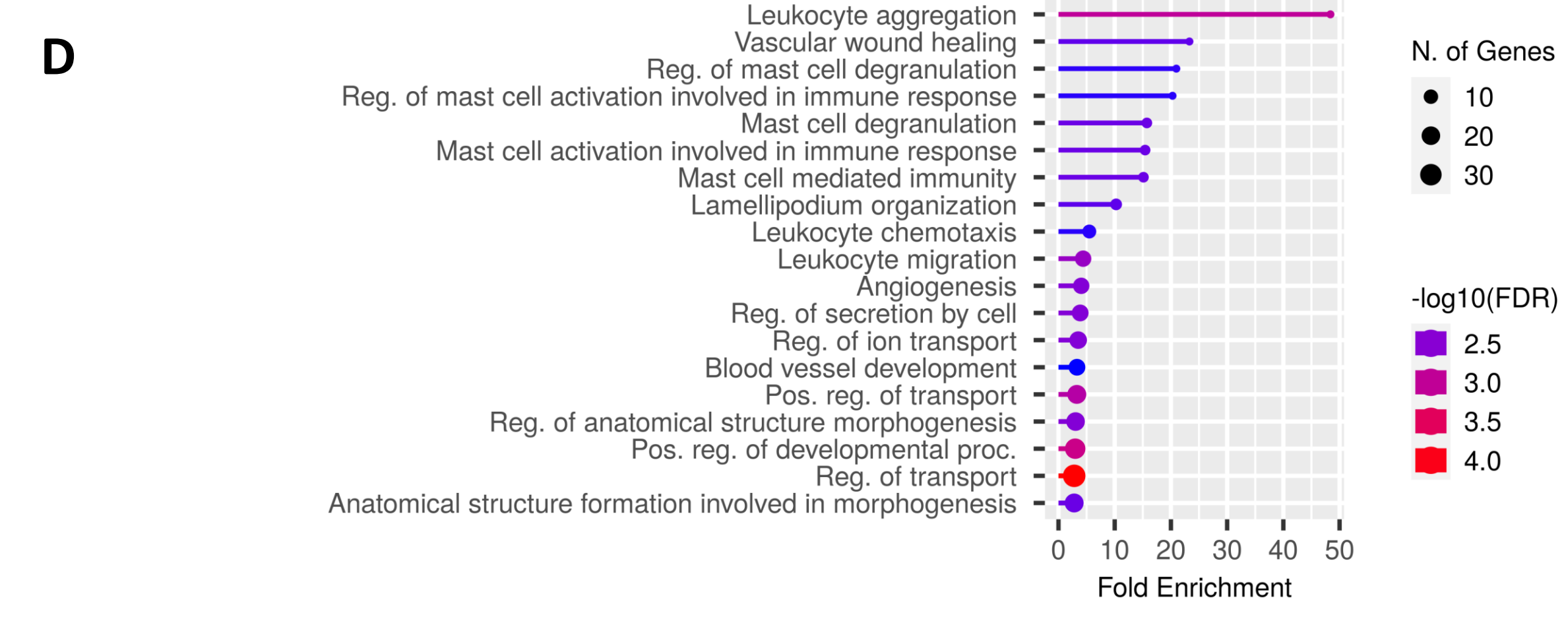
Top 200 genes of SOX2+/NEUROG3+ cell population enriched biological process



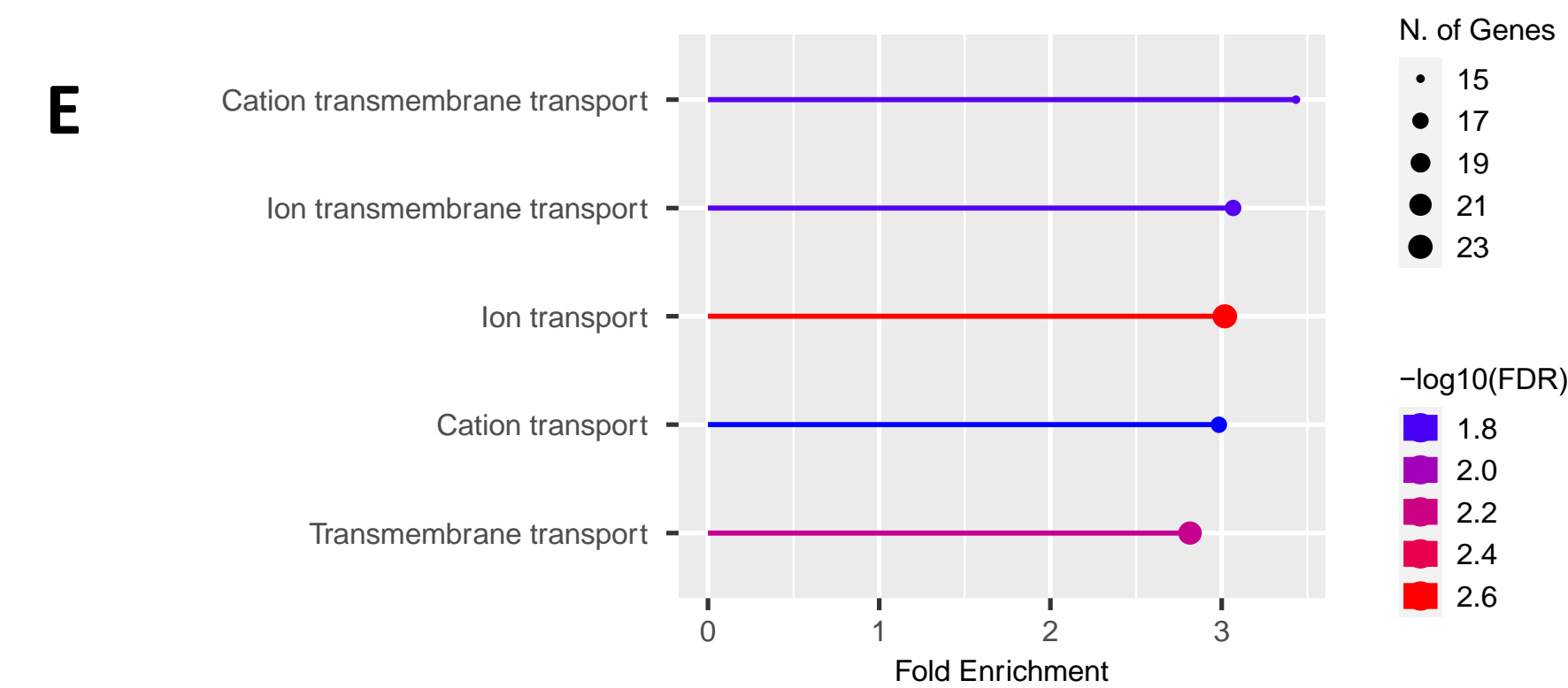
Top 200 genes of HNF1A+/APOA2+ cell population enriched biological process



Top 200 genes of INHBA+/INHBB+ cell population enriched molecular function



Top 200 genes of INHBA+/INHBB+ cell population enriched biological process



Top 200 genes of KCNJ3+/MYH4+ cell population enriched biological process

Figure 8