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Mapping early steps in brain development in Down Syndrome using iPSC differentiation models

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iPSC derivation method

- Induced pluripotent cells are reprogrammed from differentiated fibroblasts via transduction of 4 key transcription factors: Pou5f1 (Oct4), Nanog, Sox-2 and Klf4.(Takahashi, K. and S. Yamanaka (2006). Cell. 126(4):663-76 and modifications thereafter)

- Method of reprogramming matters – previous study rely on retroviral integration, we used episomes.
  - F5 method is (Yu J, Hu K, Smuga-Otto K, et al. Human induced pluripotent stem cells free of vector and transgene sequences. **Science** 2009;324:797-801.).

  Control and DS iPSC were shown to be free of transgene integration or persistence

- iPSC methods provide an opportunity to examine early differentiation events in patient-derived cells
- There are few independent (published) datasets on iPSC-neurons, early human brain development, or Downs Syndrome iPSC: these provide an opportunity to generalise F5 findings from lines obtained from different donors and in different labs

Background to Down Syndrome

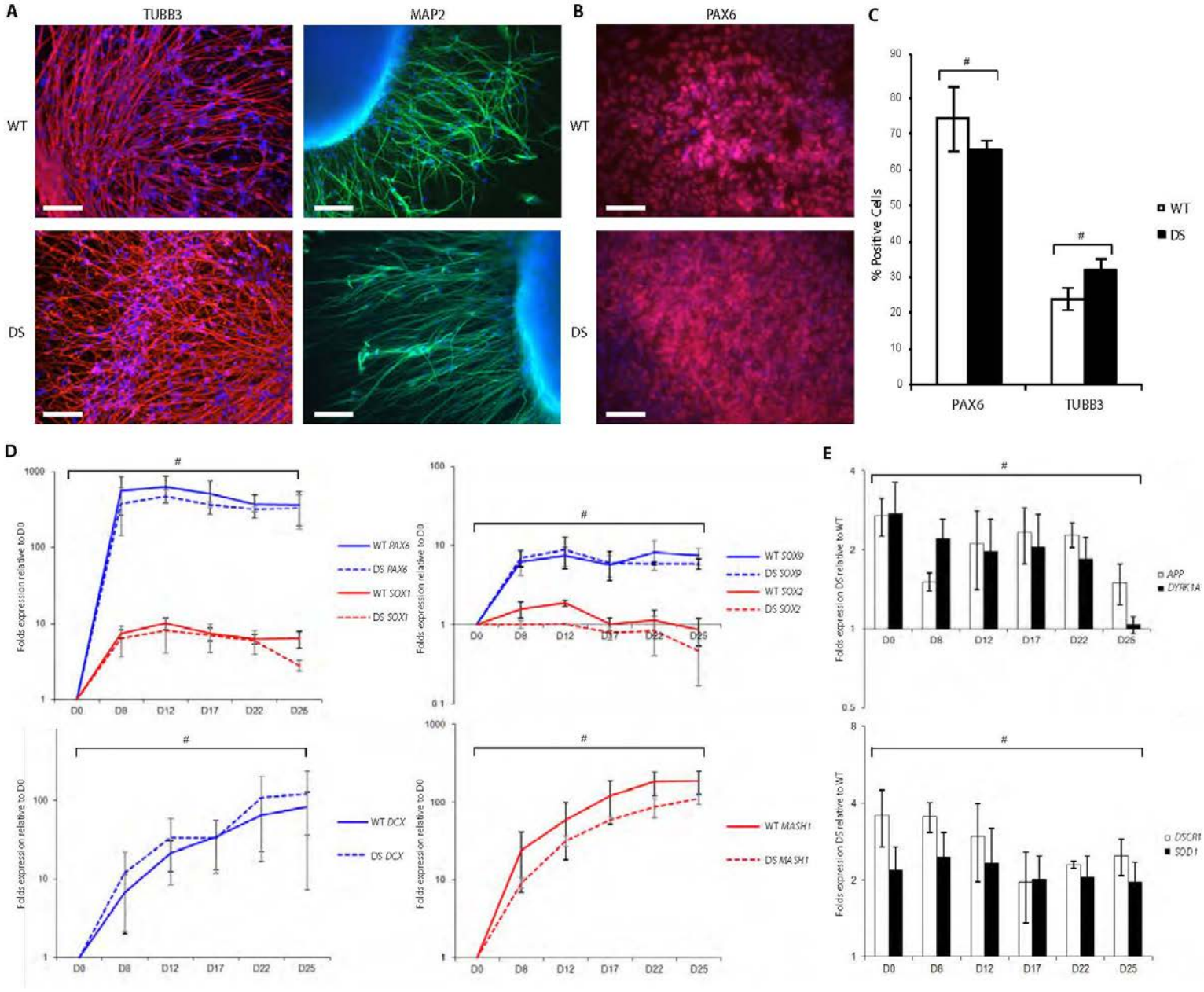
- Trisomy 21 is the most common genetic abnormality in humans (1:700 live births) ~430 genes on chromosome 21

- Highly penetrant phenotypes: mental retardation, early onset Alzheimer disease, motor and hearing impairments.

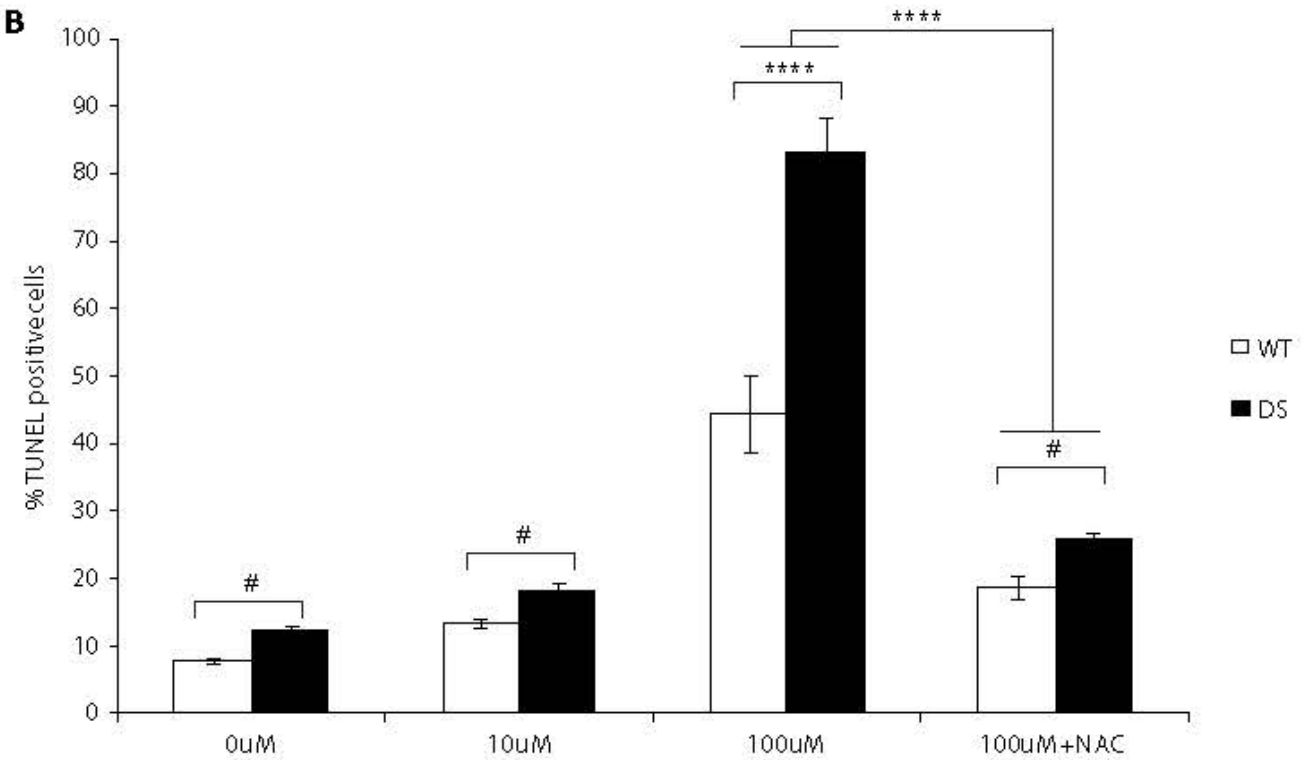
- Postmortem, aborted fetal DS brains and fetal-brain-derived-neurospheres show:
  - neurite and synapse formation defects,
  - over-representation of glial lineages, and
  - reduction in total brain volume, (reduced neurogenesis and increased neuronal apoptosis).

- Neuronally differentiated DS IPSC recapitulate:
  - similar efficiency in forebrain specification and
  - neural stem cell expansion to control iPSC.
  - but have increased (oxidative stress induced) neuronal apoptosis
  - and glial differentiation bias

Despite over-expression of Chr 21 genes previous implicated in neurodegeneration (E) early cortical neurogenesis is not affected in DS iPS cells (A-D).



Increased sensitivity of DS iPSC derived neurons to apoptosis can be rescued by the anti-oxidant NAC.



Motivations for study

- Current choices of prenatal testing for parents of DS foetus is whether or not to abort. Understanding the primary neural defect in DS provides a potential alternative - with the aim to develop in-utero therapeutic strategies.

- Publish a molecular map and GRN of early neural differentiation, which will compare, and extend published neural diff analyses from

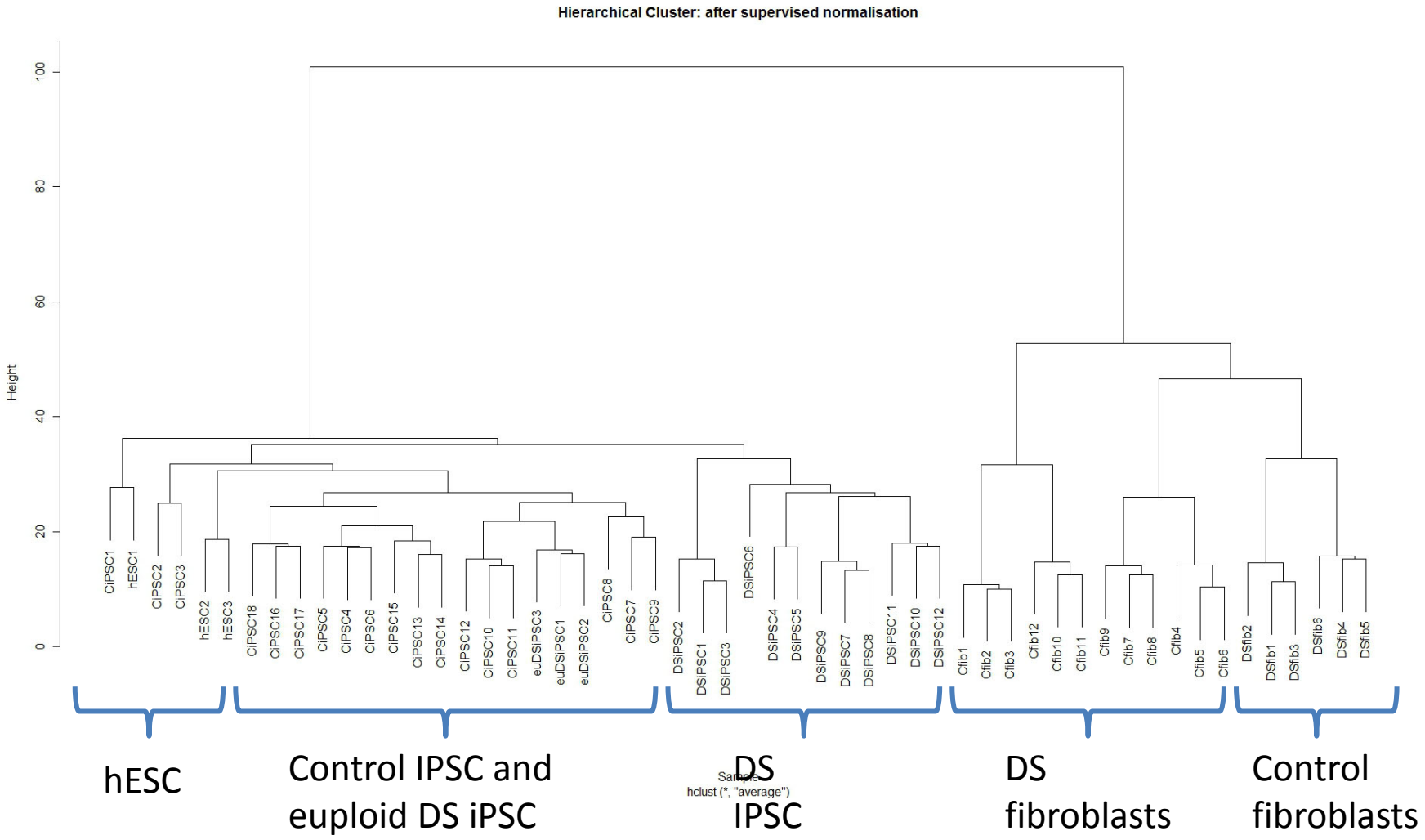
- Wu et al (Snyder lab) Dynamic transcriptomes during neural differentiation of human embryonic stem cells revealed by short, long, and paired-end sequencing Proc Natl Acad Sci U S A. 2010 March 16; 107(11): 5254–5259.

- RNA-Seq of Human Neurons Derived from iPS Cells Reveals Candidate Long Non-Coding RNAs Involved in Neurogenesis and Neuropsychiatric Disorders PLoS ONE 6(9): e23356. doi:10.1371/journal.pone.0023356

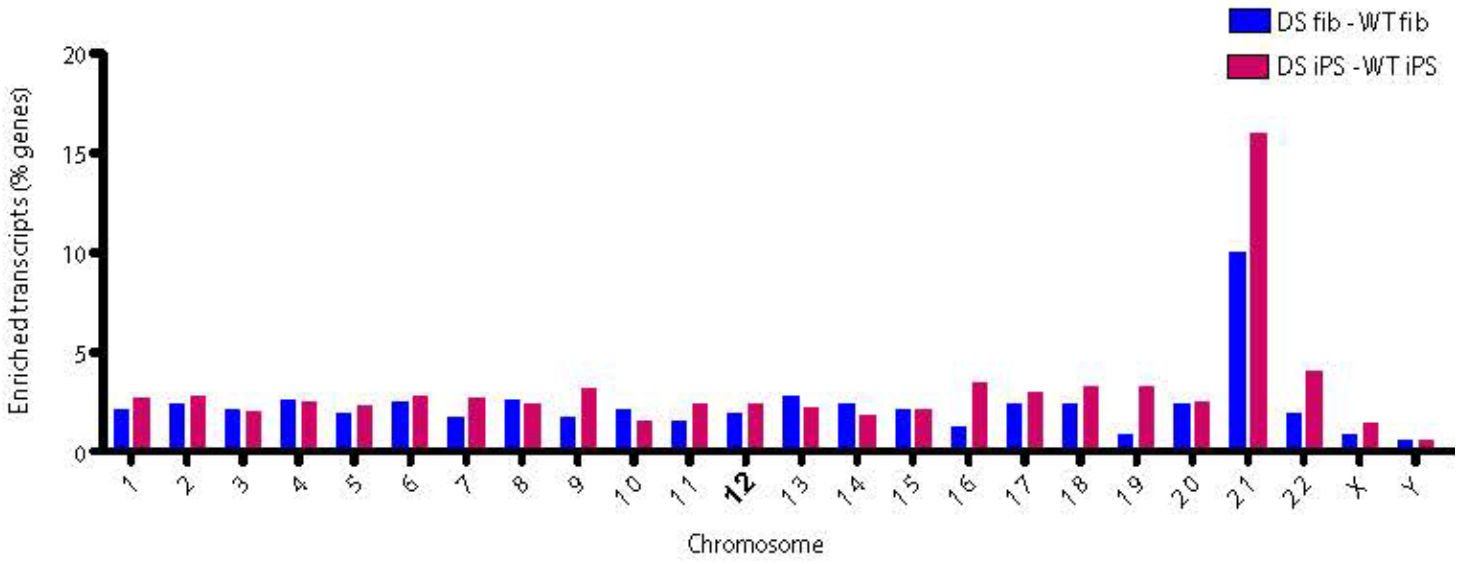
- Predict impact of trisomy21 on this GRN

Gene expression of undifferentiated DS iPS cells is highly similar to, yet distinct from, control pluripotent stem cells.

Illumina HumanHT-12 V4 microarray data and methylation (array) data available:

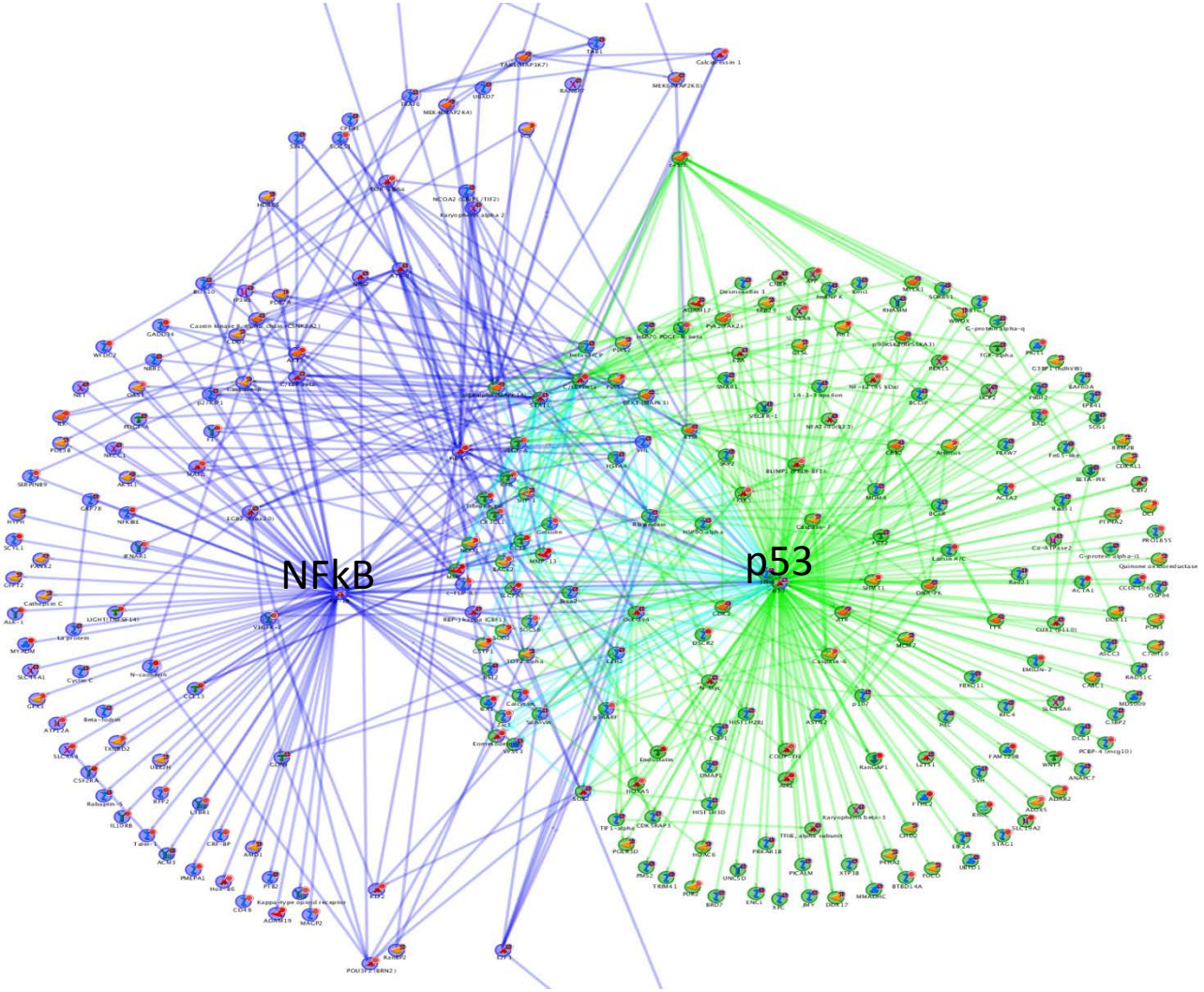


Fibroblasts (DS fib) and iPSC (DS iPS) from Down Syndrome cells have increased expression of Chr21 genes, consistent with increased gene dosage in trisomy21.



Two highly significant pathways distinguish DS and control iPSC

(microarray, B-Stat ranked gene list, enrichment in GENEGO)



Neuronal differentiation

CAGE time points

10uM SB431542

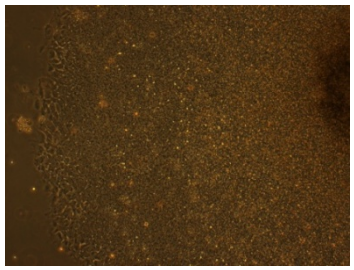
5uM Dorsomorphin

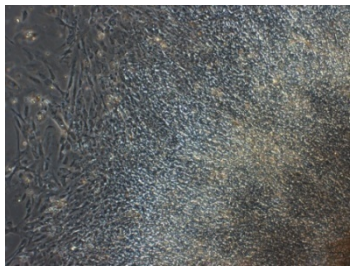
0 2 4 6 8 10 12 14 16 18

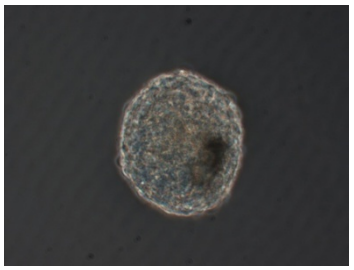
iPS

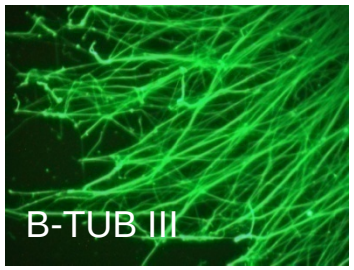
Neurosphere formation

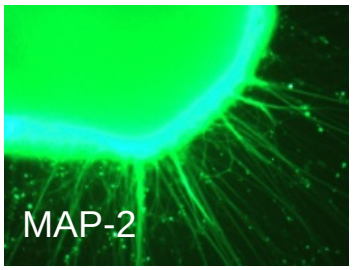
Maturation begins









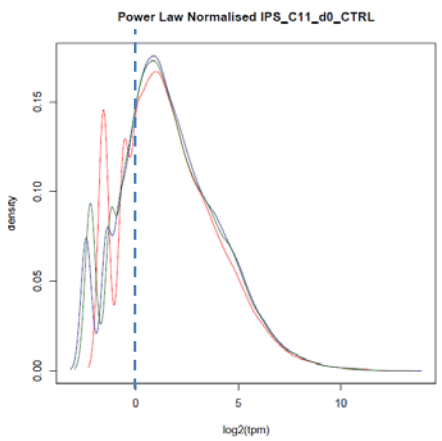


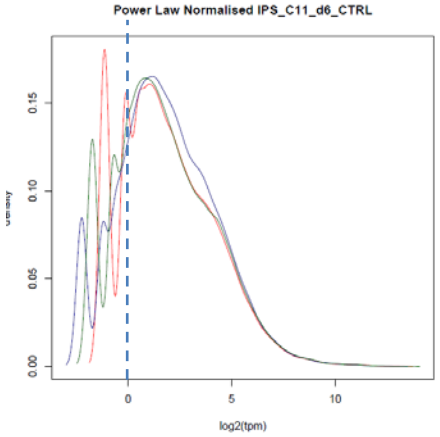
CAGE time course

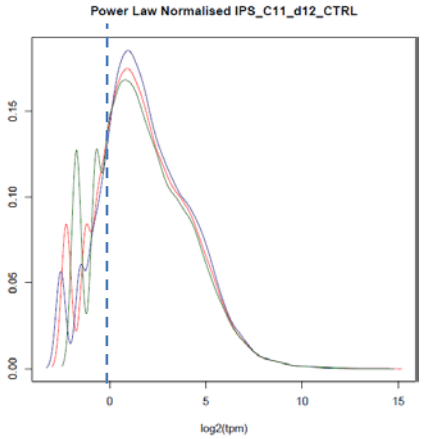
- 2 Donors – control and Down Syndrome
- 2 iPSC clones per donor
  - Control: Colony 11 (C11), Colony 32 (C32)
  - Down Syndrome: Colony 11 (C11), Colony 18 (C18)
- 4 time points per clone
  - Day 0, 6, 12, 18
- 3 replicates per time point
  - (separate culture, separate RNA extraction)

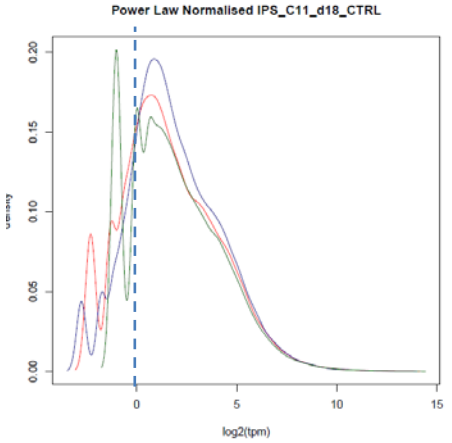
Power-law normalisation (Piotr's code, implemented by Ashley Waardenberg)
Good argument for 1 TPM threshold

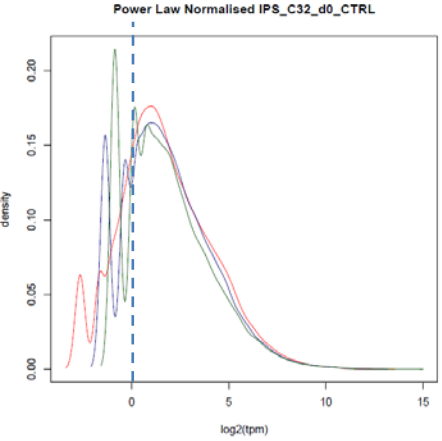
Control CAGE libraries

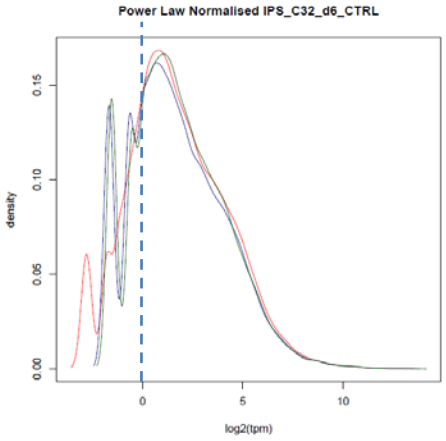


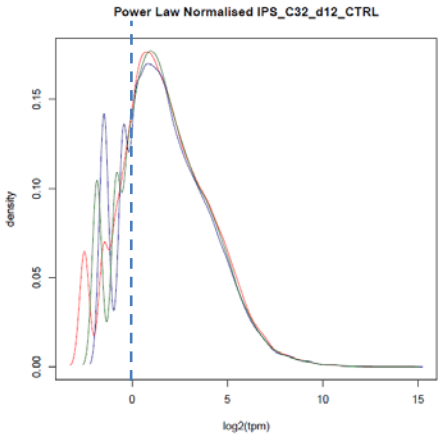


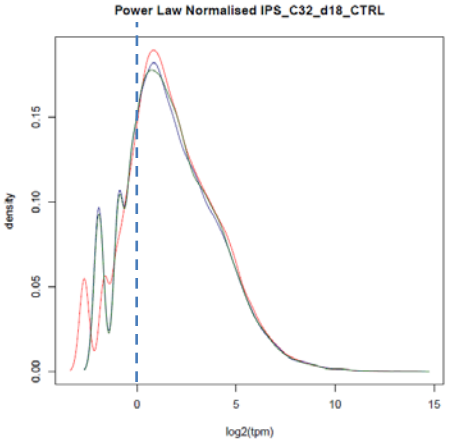




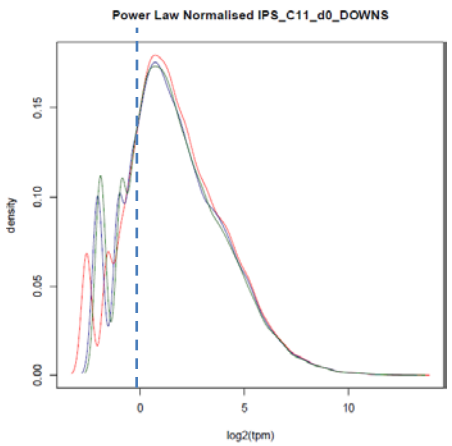


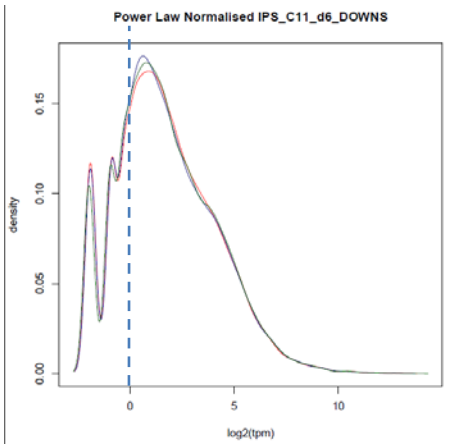


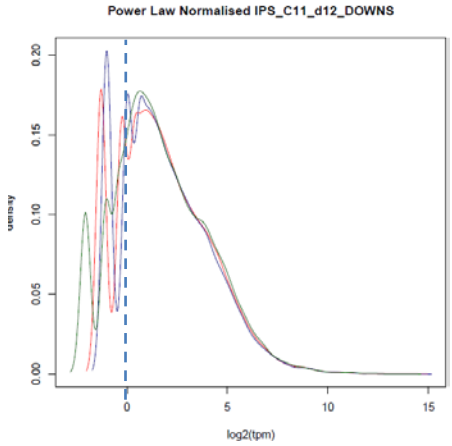


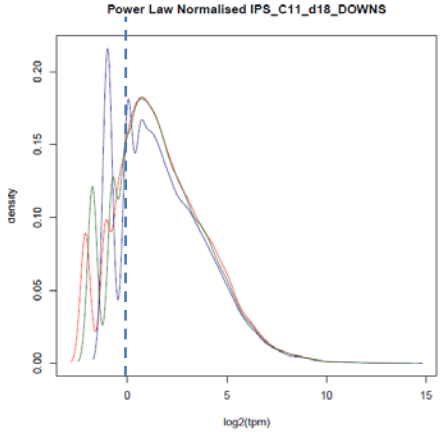


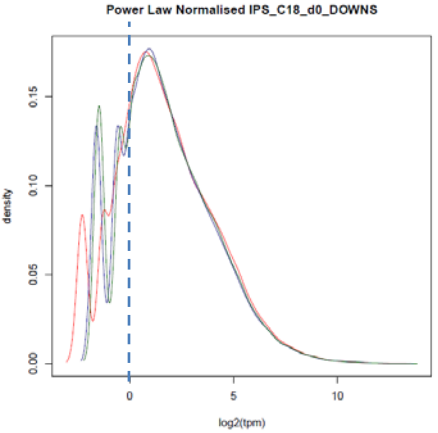
DS CAGE libraries

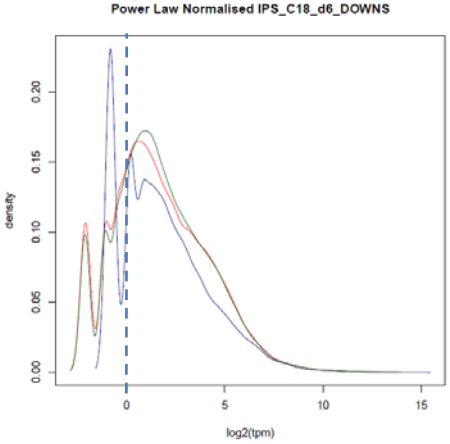


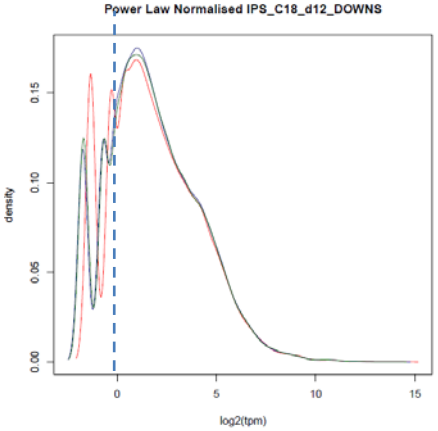


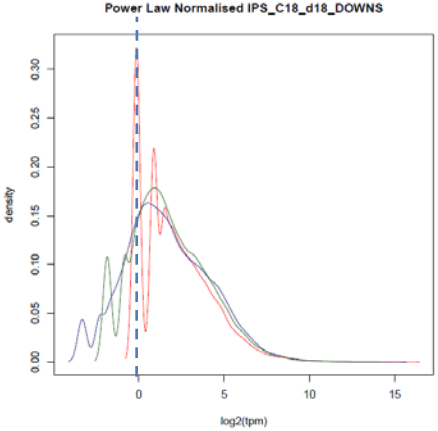




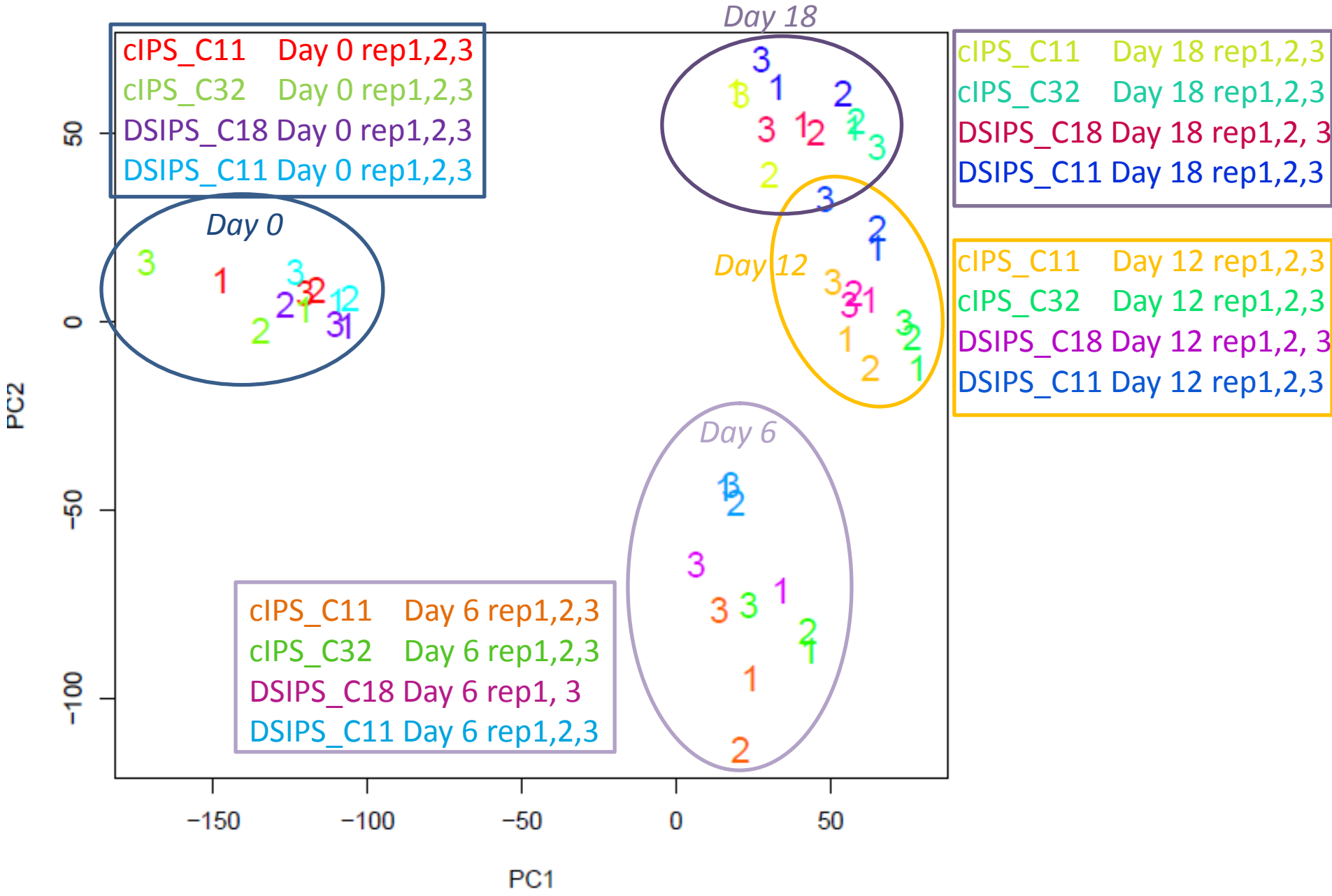








PCA: Erik on FANTOM normalised data



Analysis questions

General neural differentiation questions:

- What is the GRN that drives neural differentiation?
- What are the combinatorial changes in TF activity predicted from CAGE (how much of these are driven by HSA 21 TFs ?)
- Where do we see 'repurposing' of genes in the differentiation process (via alt products)
- Do we see isoform or promoter usage specialisation
- What are the concordant changes in chromatin predicted
    - (e.g. Evidence for hierarchical silencing?)

Down Syndrome questions:

How does gene dosage on Chr21 impact on the GRN?

Do we see phased differences in the GRN

Do we see changes in the combinatorial TF network

Do we see changes in chromatin in DS individual

Can we distinguish *Cis* and *Trans* events?

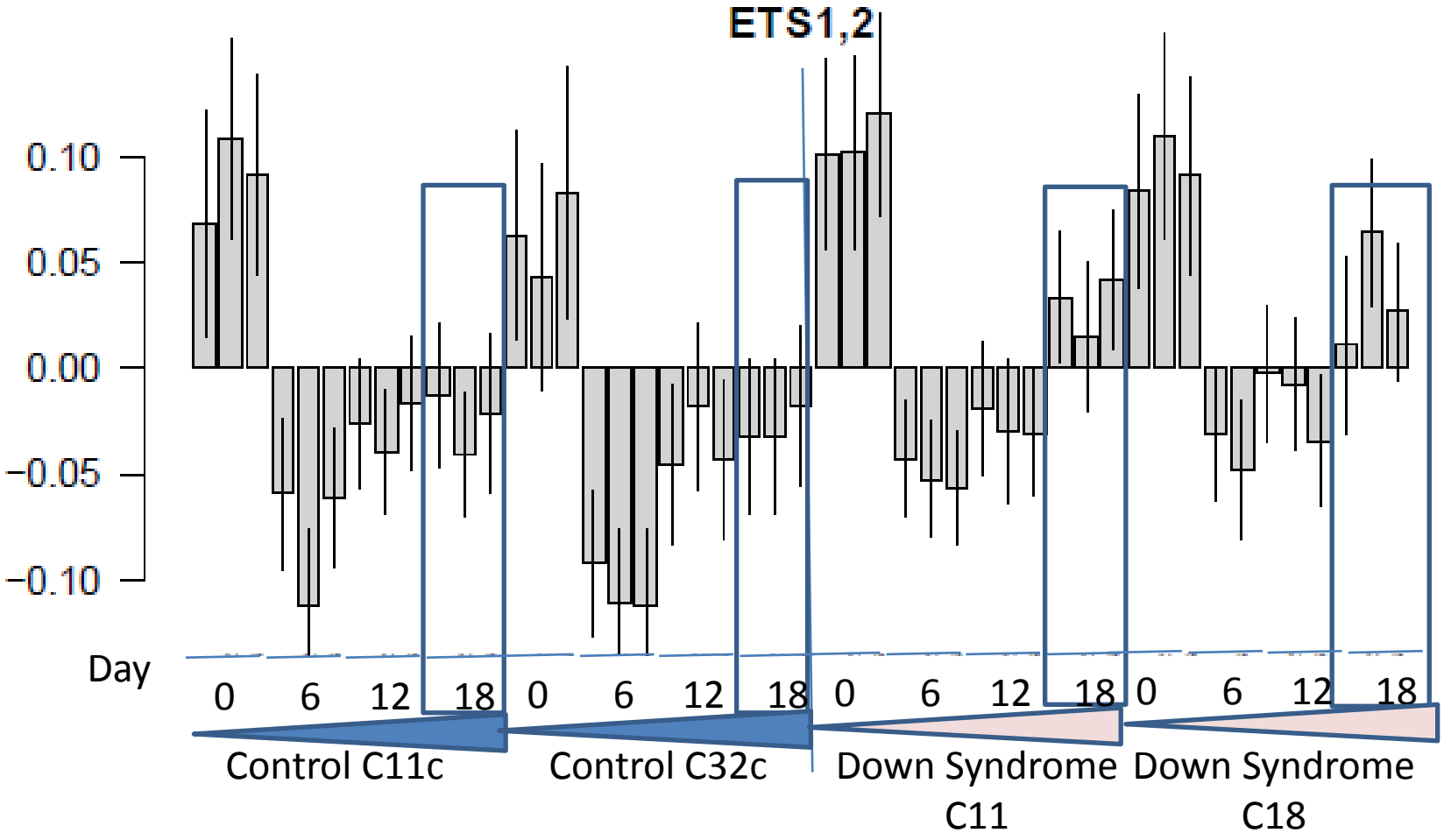
Chr21 candidate 'disruptors' – DNMT3L; ETS2, GABP

Evidence for the molecular basis of DS glial bias, or susceptibility to oxidative stress, and increased neuronal apoptosis?

Pathways most dysregulated in CAGE DS libraries

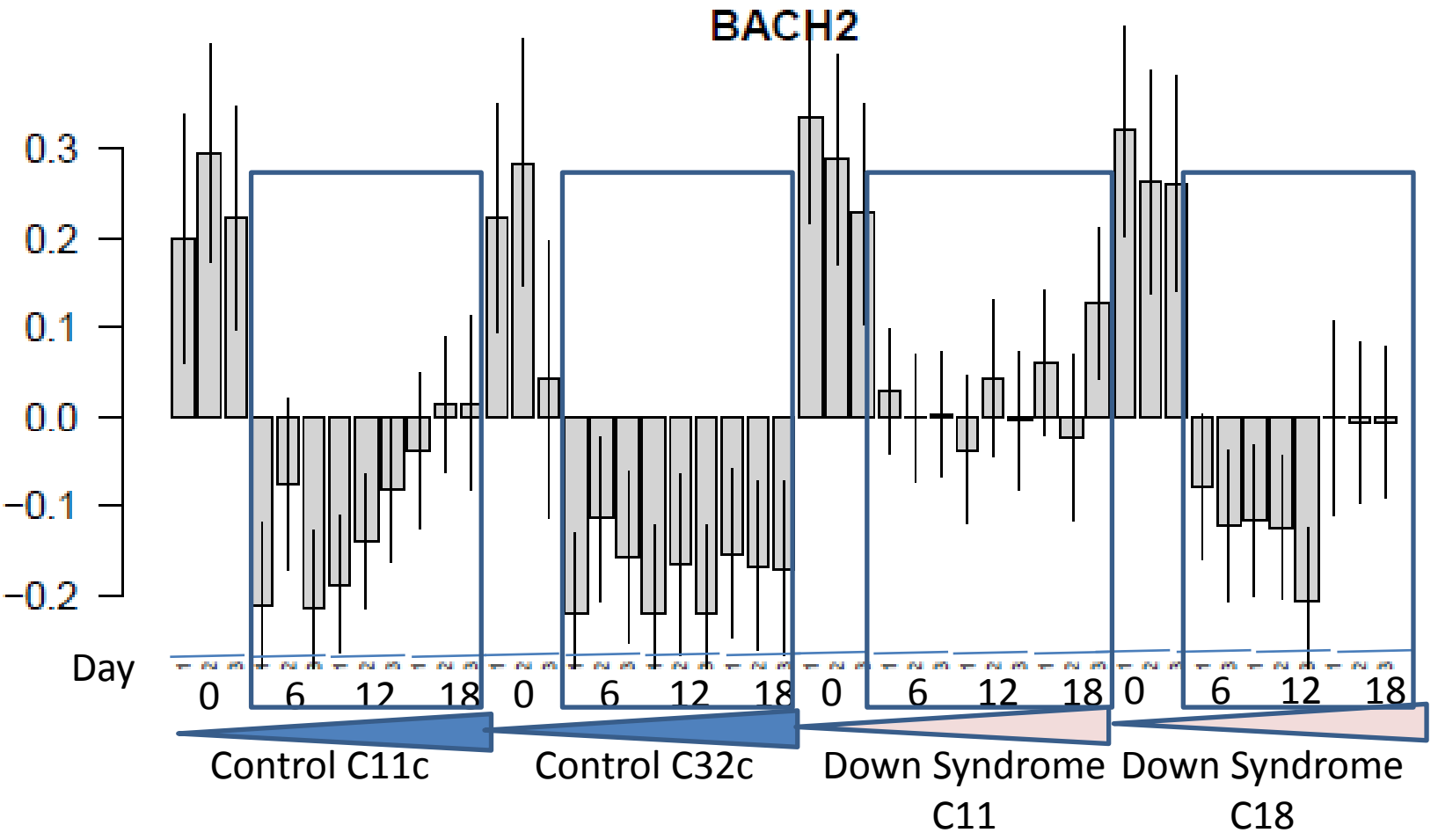
DE TF + MARA analysis

ETS2 p1 (up in DS) p<0.007 genotype; p<0.000 time (ANOVA)
Chr21 gene, known roles in development, apoptosis and telomerase activity



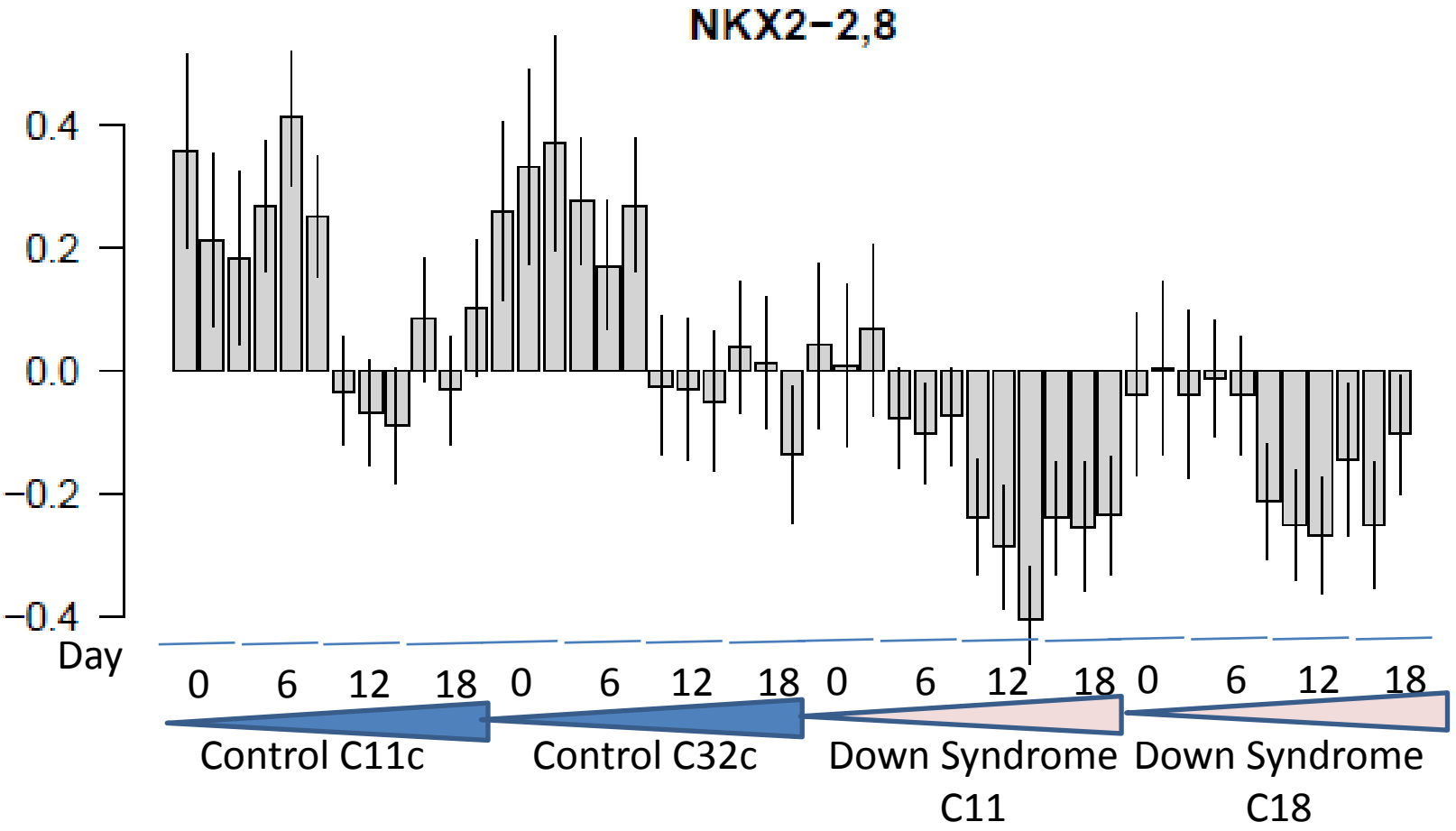
DE TF + MARA analysis

DEG: BACH2 (down in DS) p<0.003 genotype; p<0.000 time (ANOVA)
Chr6 gene, likely to be trans-regulated by Chr21 genes.

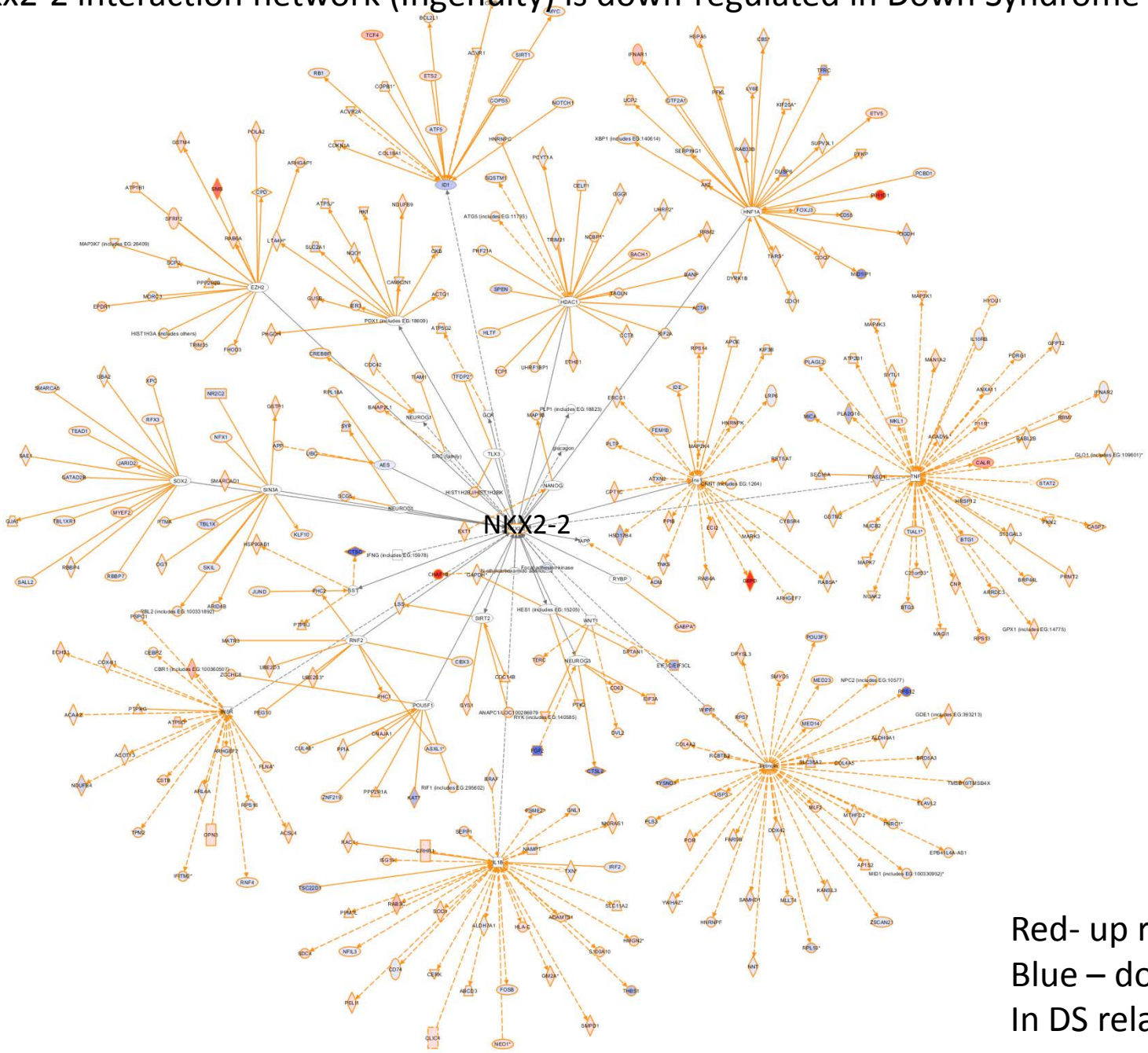


MARA analysis

NKX2-2 is not differentially expressed (Chr20), so next step is to investigate upstream pathway. This homeobox TF is implicated in forebrain development.



The Nkx2-2 interaction network (Ingenuity) is down-regulated in Down Syndrome



Red- up regulated
Blue – down regulated
In DS relative to control

Summary DEG/MARA analysis

- Strong candidate TF from CAGE data:
  - ETS2 is a broadly expressed transcriptional activator: Chr21, gene dosage= upreg in DS
  - BACH2 is part of the NRF-2 network, and is a regulator of cell responses to oxidative stress.
  - Nkx2-2 has been implicated in mouse brain development, but this is the first report of its role in human neural differentiation or DS.

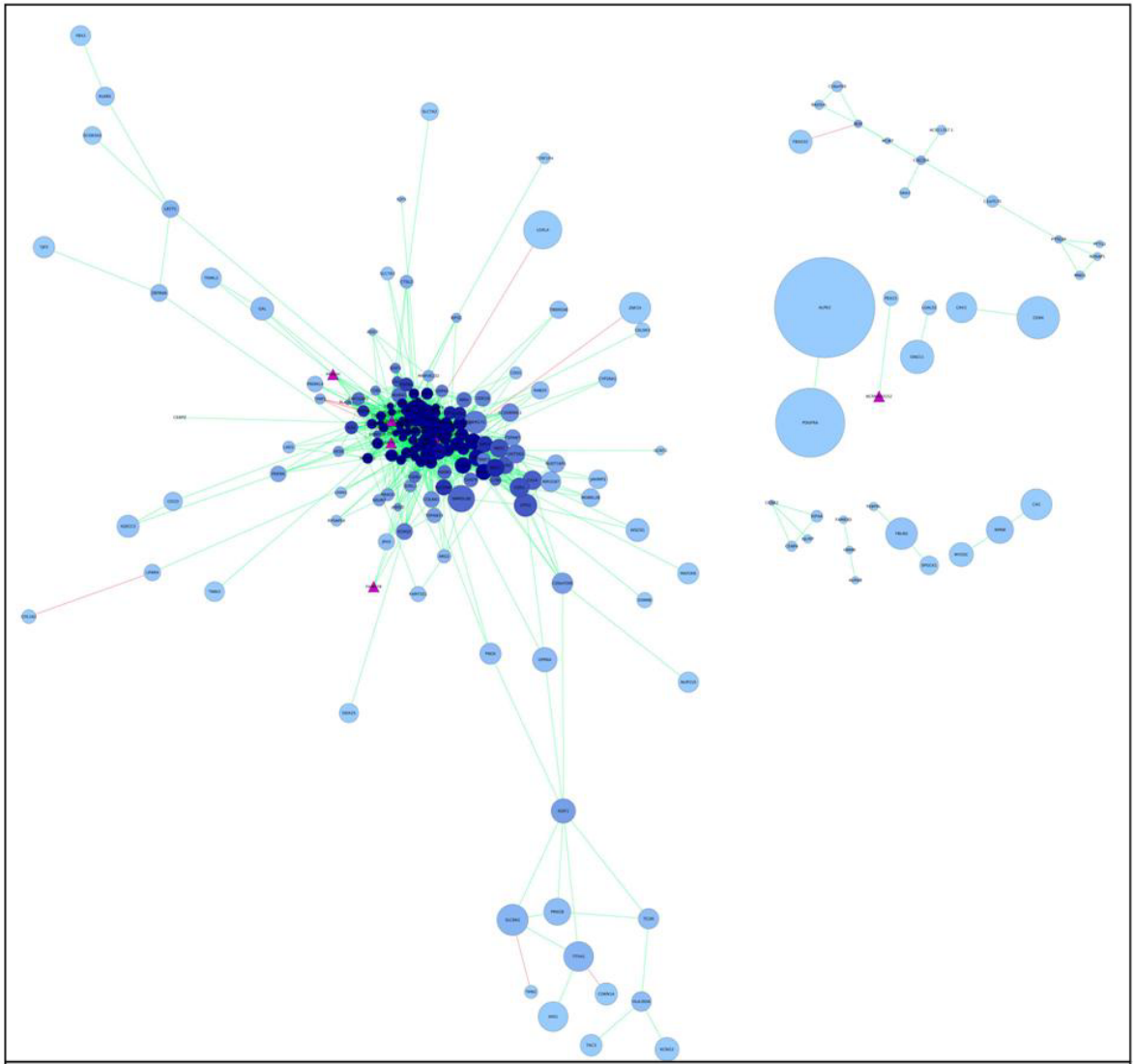
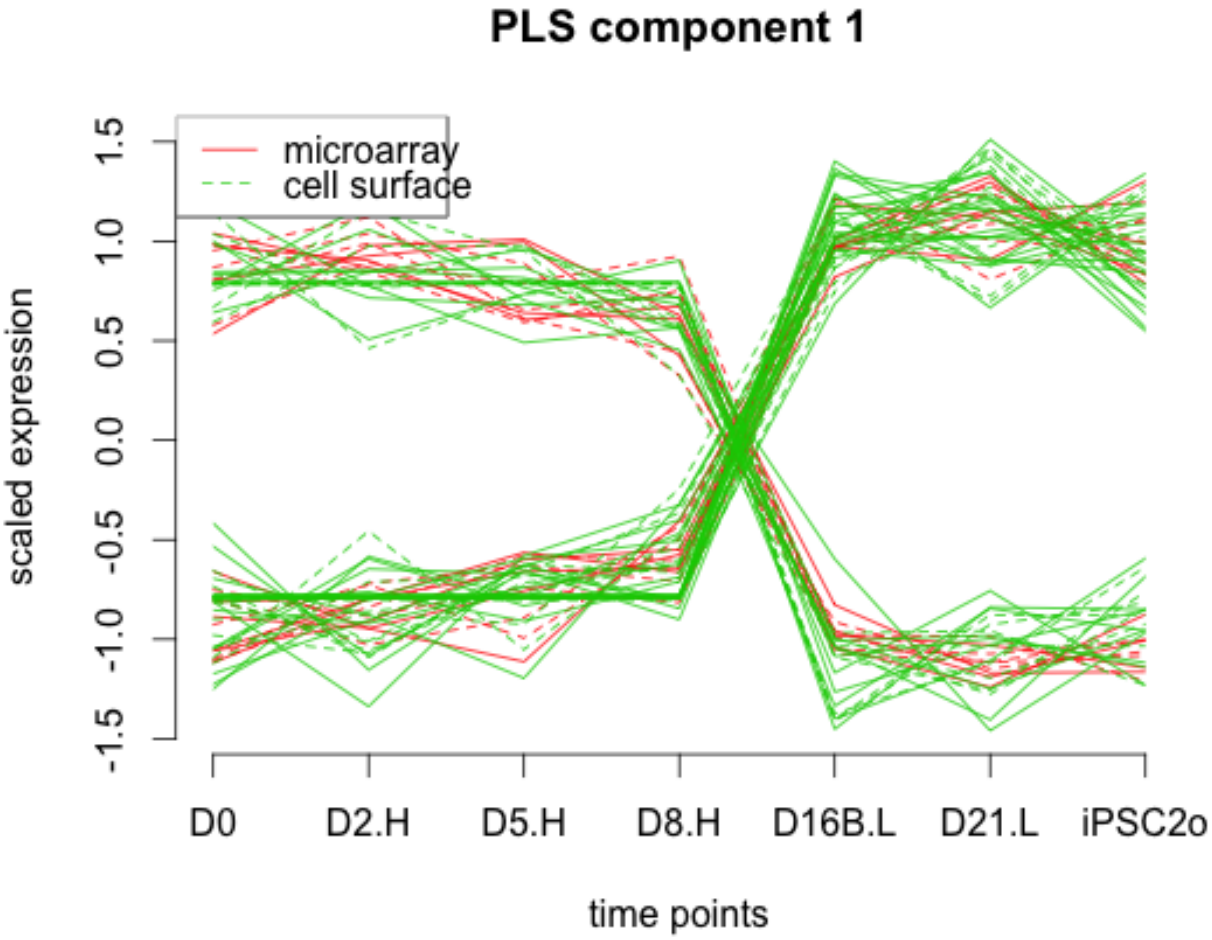


Figure One: *Gene level correlation/co-expression network.*
Nodes represent genes identified belong to the *PluriNet* or Extracellular-Matrix Interaction pathways, or are correlated partners of expression (Pearson R above 0.9 or below Pearson R –0.9). Green edges represent a strong pair-wise positive correlation above 0.995 and red edges represent strong negative correlations of below –0.995. Larger nodes represent high expression variation coefficient in pluripotent iPS cells. Darker coloured nodes have higher degree of connectivity as calculated by the number of edges. Purple nodes are expressed only in fibroblast cells.

Overlapping the CAGE data with our pluripotency network (Lizzi Mason)

Using sPLS for time course analysis



Example from Kim-Anh LeCao, University of QLD.

Validation approaches

Inducible over-expression systems

RU486-GAL4-inducible control, DS and euploid DS iPSC
CHIP-seq

Gene-silencing systems
Lentiviral Dox-inducible shRNA/cDNA

AMAXA Transfection of adherent neurons with shRNA, expression vectors, ASO's.

TALE based editing of HSA21 in DS iPSC