Transcriptional regulation of neurodevelopmental and metabolic pathways by the psychiatric illness candidate gene NPAS3

Li Sha

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Declaration

I hereby declare this thesis has been composed by myself, and the work presented within is my own and has not been accepted for any previous application for a degree. Information obtained from sources other than this study is acknowledged in the text or included in the references.

Li Sha

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Abstract

The basic helix-loop-helix PAS domain transcription factor gene *NPAS3* is a risk factor for psychiatric disorders. A knockout mouse model also exhibits behavioural and adult neurogenesis deficits consistent with human illness. To define the location and mechanism of NPAS3 aetiopathology immunofluorescent and transcriptomic approaches were used.

Npas3 was co-localised with Dcx, but not other neurogenesis markers, in the hippocampal subgranular zone - the site of adult neurogenesis. This implied that NPAS3 might be involved in maturing, rather than proliferating, neuronal precursor cells. Microarray analysis revealed that the transcriptional activities of NPAS3 and its truncated form (C-terminal deletion) in the HEK293 cell line are sensitive to circadian rhythm context. The most highly up-regulated NPAS3 target gene, *VGF*, encodes secretory peptides with established roles in neurogenesis, depression and schizophrenia. *VGF* was one of many NPAS3 target genes also shown to be regulated by the SOX family of transcription factors, suggesting an overlap in neurodevelopmental pathways. The transcriptional repression of multiple glycolytic genes indicated that NPAS3 has a second role in metabolic regulation. This finding was also confirmed by collaboration with a metabolomics research group at the University of Strathclyde.

SOX11, a transcription factor known to play a role in neuronal and glial cell differentiation, was shown to be down-regulated by NPAS3. The set of genes targeted by SOX11 and their ontologies were deduced by a microarray analysis in a

SOX11 overexpressing HEK293 cell line. Regulated genes include a previously established SOX11 target, known markers of neurogenesis as well as genes implicated in neuropsychiatric disorders. Multiple histone and zinc finger genes are regulated by SOX11, many of which were located in two clusters on chromosomes 6 and 19. The chromosome 6 cluster lies within a region of the genome showing the strongest genetic association with schizophrenia. SOX11 may alter localised expression competence and its targets induce a complex programme of chromatin remodelling and downstream gene expression changes to achieve the mature neuronal phenotype.

This thesis details how transcription factors are involved in biological processes linked to psychiatric illness. The dual neurodevelopmental and metabolic aspects of NPAS3 activity described here increase our understanding of aspects of neurogenesis relevant to mental illness and may explain the innate and medication-induced susceptibility to diabetes reported in psychiatric patients.

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ABBREVIATIONS

α	Alpha
aa	Amino acid
ATP	Adenosine triphosphate
BAC	Bacterial artificial chromosome
BDNF	Brain derived neurotrophic factor
BLAST	Basic local alignment search tool
bp	Base pair
BP	Bipolar disorder
BSA	Bovine serum albumin
°C	Degrees centigrade
cDNA	Complementary DNA
CACNA1C	Calcium channel, voltage-dependent, L type
CACNG2	Voltage-dependent calcium channel gamma-2
CAD	Coronary artery disease
cAMP	Cyclic adenosine monophosphate
cM	centiMorgans
CNS	Central Nervous System
CNTNAP2	Contactin-associated protein-like 2 precursor
CNV	Copy Number Variant
CT	Computed tomography
DAB	Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DA	Dopamine
DAAO	D-amino acid oxidase
DAG	Diacylglycerol
D_2	Dopamine type-2 receptors
Δ	Delta
Del	Deletion
DG	Dentate gyrus
DGKH	Diacyl glycerol kinase
dH_2O	Distilled water
DISC1	Disrupted-In-Schizophrenia-1
DMD	Duchenne Muscular Dystrophy
DZ	Dizygotic
DNA	Deoxyribonucleic acid
DNase	A deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
DSM	Diagnostic and Statistical Manual of Mental Disorders
DTNBP1	Dysbindin
DYNC1I1	Dynein, cytoplasmic 1, intermediate chain 1
E-Coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
EEG	Electroencephalographic
EFAs	Essential fatty acids

EGFR	Epidermal growth factor receptor isoform b
EPSCs	Excitatory postsynaptic currents
EPSC$_{KA}$	Excitatory postsynaptic currents kainate receptor
ER	Endoplasmic Reticulum
fMRI	Functional Magnetic Resonance Imaging
g	Grams
GABA	Gamma-aminobutyric acid
GAD$_{67}$	Glutamate decarboxylase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
G6PD	Glucose-6-phosphate dehydrogenase
GPR24	G protein-coupled receptor 24
GRIA1	Glutamate ionotrophic receptor, AMPA 1
GRIA3	Glutamate ionotrophic receptor, AMPA 3
GRID2	Ionotropic glutamate receptor, delta subunit 2
GRIK4	Ionotropic glutamate receptor, kainate 4 (protein KA1)
GRIN1	NMDA receptor 1
GRIN2B	NMDA receptor subunit 2B
GWAS	Genome-Wide Association Study
HD	Huntington's disease
HGU	Human Genetics Unit
HPA	Hypothalamic-pituitary-adrenal axis
5-HT	5-hydroxytryptamine /serotonin
IHC	Immunohistochemistry
IP3	Inositol-1, 4,5-triphosphate
IPSC	Inhibitory post synaptic currents
IPTG	Isopropyl β-D-thiogalactopyranoside
kb	Kilobase
KCNB1	Potassium voltage-gated channel, subfamily G
KCND3	Potassium voltage-gated channel, Shal-related
KCNF1	Potassium voltage-gated channel, subfamily F
kDA	Kilo Daltons
KO	Knock out
LSD	Lysergic acid diethylamide
μg	Micrograms
μl	Microlitre
μM	Microns
m	Stratum lucunosum moleculare
M	Molar
Mb	Megabase
MDD	Major Depressive Disorder
MeOH	Methanol
mg	Milligrams
MgCl$_2$	Magnesium chloride
mGluR	Metobotropic glutamate receptor
min	Minutes

ml	Millilitres
mM	Millimolar
MR	Mental retardation
MRC	Medical Research Council
mRNA	Messenger RNA
MYO5B	Myosin VB
MZ	Monozygotic
nACh	Nicotinic acetylcholine
NaCl	Sodium chloride
NAD	Non affected with psychiatric illness
NaOH	Sodium hydroxide
NBD	Nucleotide binding domain
NCBI	National Center for Biotechnology Information
ng	Nanograms
NMDA	N-methyl-D-aspartic acid
NMR	Nuclear magnetic resonance
NPL	Non parametric linkage analysis
NPAS3	Neuronal PAS domain-containing protein 3
NRG1	Neuregulin
OD	Optical density
Oml	Outer molecular layer
OR	Odds ratio
PBS	Phosphate buffered saline
PCM1	Pericentriolar material 1 protein
PCP	Phencyclidine
PCR	Polymerase chain reaction
PDE4D	Phosphodiesterase 4d
PDE4	Phosphodiesterase 4
PET	Positron emission tomography
pH	Power of Hydrogen
PI	Phosphoinositide
PIP$_2$	Phosphatidylinositol 4,5-bisphosphate
qs	*quantum sufficiat* /quantity sufficient
QTL	Quantitative trait loci
r	Stratum radiatum
RGS4	Regulator of G-protein signaling 4
RIN	RNA Integrity Number
RNA	Ribonucleic acid
RNaseA	Ribonuclease A
rpm	Revolutions per minute of rotor
RTA	Road traffic accident
RT-PCR	Reverse transcription – PCR
s	Subiculum
SADS-L	Schedule for Affective Disorders and Schizophrenia-Lifetime
SCZ	Schizophrenia
SDS	Sodium dodecyl sulphate
SDSC	San Diego Super Computer
SDS-PAGE	SDS-polyacrylamide gel electrophoresis

SEM	Standard error of the mean
SEMA3A	Semaphorin 3E
SGZ	Subgranular zone
SHANK3	Proline-rich synapse-associated protein 2
SLC26A4	Pendrin
SLC26A5	Prestin isoform d
SLC6A4	Solute carrier family 6 member 4 / serotonin transporter gene
SORCS2	Sortilin-related VPS10 domain containing receptor 2
SNHL	Sensori-neural hearing loss
SNP	Single nucleotide polymorphism
SOX2	Sry-related HMG box transcription factor
SSC	Standard saline citrate
SSRI	Specific serotonin re-uptake inhibitor
STAR*D	Sequenced Treatment Alternatives to Relieve Depression
STGD	Stargardt disease
TAC1	Tachykinin 1 isoform
Taq	*thermus aquaticus*
TBE	Tris-Borate-EDTA
TBlast	Translated sequence database
TD	Tangier disease
TE	Tris-EDTA
THEX1	Three prime histone mRNA exonuclease 1
TMDs	Transmembrane cluster domains
TPH$_2$	Tryptophan hydroxylase 2
TRIOBP	TRIO and F-actin binding protein
TSPAN8	Transmembrane 4 superfamily member 3
Tween 20	Poly(oxyethylene) sorbitan monolaurate
TX-100	Triton-X-100
U	Units
UCSC	University of California Santa Cruz
UPD	Uniparental disomy
USS	Upstream sequence
dUTP	2'-Deoxyuridine 5'-Triphosphate
UTR	Untranslated region
3'UTR	3 prime untranslated region
5'UTR	5 prime untranslated region
UV	Ultraviolet
VGCNL1	Voltage gated channel like 1
VEGF	Vascular endothelial growth factor
WFS1	Wolfram syndrome
WGH	Western General Hospital, Edinburgh
WHO	World Health Organization
WS	Wolfram syndrome
WTCCC	Welcome Trust Case Control Consortium
WT-CRF	Welcome Trust Clinical Research Facility
xg	Measure of relative centrifugal force
YAC	Yeast artificial chromosome

CHAPTER ONE

Introduction

1.1 Psychiatric illnesses

1.1.1 Schizophrenia

Schizophrenia, which affects about 4-6 in a thousand individuals (Bhugra, 2005; Goldner et al., 2002), is a neuropsychiatric disorder characterised by abnormalities in a person's thinking, mood, movement, and perceptions. Schizophrenia is described in terms of positive and negative symptoms. In the acute phases of illness, a person often presents with positive symptoms. Typically a person may become very distressed and agitated, express delusional beliefs and may experience hallucinations in the form of voices, smells or experience of touch and usually require treatment in hospital. Anti-psychotic medication is very effective at controlling these positive symptoms. To prevent relapse, long-term medication may be required and patients rarely return to their previous level of function. Negative symptoms include apathy, poverty of speech, inability to experience pleasure, lack of desire to form relationships and psychological deficits in executive function and episodic memory. A person with negative symptoms is often withdrawn without drive, ambition and goal-directed behaviour. They may appear and sound emotionally 'flat'. Speech may be slowed and show evidence of unusual words and grammar. Repetitive body movements are common. These symptoms do not respond well to medication. Thus a person with chronic schizophrenia has many difficulties affecting all aspects of life.

The cognitive deficits in schizophrenia include excessive attention to potential threats, jumping to conclusions, impaired reasoning about social situation and mental states etc. (Broome et al., 2005; Brune et al., 2007; Lewis, 2004; Sitskoorn et al.,

2004). Some neurocognitive deficits are related to issues in memory, attention, problem-solving, executive function and social cognition (Kurtz, 2005).

The peak years for the onset of symptoms are typically late adolescence and early adulthood in males and females (the mean age of onset is 20–28 years for males and 26–32 years for females). Increased physical health problems and higher self-harming and suicide rate are the main reasons which reduce the life expectancy of schizophrenia patients by 10-12 years compared to the healthy population (Brown et al., 2000; Palmer et al., 2005). It incurs high public health cost and results in a poor quality of life and has a devastating impact on societies across the whole world.

1.1.1.1 Factors which can contribute to schizophrenia

It is clear that a full explanation of schizophrenia must take account of both genetic and environmental influences. (Corcoran et al., 2003; Harrison and Owen, 2003). It is very difficult to separate the effects of genetic and environmental factors in the heritability of schizophrenia, although twin and adoption studies have suggested a high level of heritability.

The environmental factors which contribute the disease include: hypoxia at birth, migration, living in a city, season of birth (winter has a higher risk for schizophrenia), cannabis, starvation etc. Fetal Hypoxia has been supported as an important external factor that influences the susceptibility of schizophrenia (Handford, 1975). In numerous studies, fetal hypoxia has been correlated with neuronal dysfunction and subtle damage (Rosso et al., 2000; Van Erp et al., 2002). Normal embryonic and fetal hypoxia or pathological hypoxia may affect neurodevelopment by regulating several genes. Schmidt-Kastner *et al.* reviewed that more than 50% of the schizophrenia candidate genes met criteria for ischemia-hypoxia regulation (Schmidt-Kastner et al., 2006). Cannon *et al.* found that obstetric complications involving hypoxia were associated with neurodevelopmental impairments in childhood and with the later development of schizophreniform disorders through a longitudinal study (Cannon et al., 2002). A study of Japanese monozygotic twins discordant for schizophrenia (one has the diagnosis while the other does not) suggests that hypoxic brain damage maybe a differentiation factor for the abnormalities in psychosocial development and subsequent schizophrenia (Kunugi et al., 2003). Although hypoxic impairment does not account for all schizophrenia cases, it is very possibly that prenatal and perinatal

hypoxia play an important role in the neurodevelopment and subsequent development of schizophrenia, which suggests that some forms of schizophrenia may be preventable (Marin et al., 1991).

Schizophrenia is a condition of complex inheritance with many different major or minor genes increasing risk. Adoption studies showed increased risk in those raised in normal environment but with schizophrenic biological parents. These studies suggest that schizophrenic phenotype is genetically influenced. Individual twin studies and meta-analyses of twin studies estimate that schizophrenia is a heterogeneous syndrome with a heritability score of approximately 0.8 (Huntley et al., 2003). (This refers to the proportion of variation between individuals in a population that is influenced by genetic factors, not the degree of genetic determination of individual risk). Concordance rates of schizophrenia are about 50% in monozygotic twins and 17% between dizygotic twins. There is still limitation in the methodology of the twin studies. Although the concordance of schizophrenia in monozygotic twins has traditionally been used to estimate a genetic component, the result could be skewed due to the environmental factors (including shared placenta etc.) (Davis et al., 1995).

1.1.1.2 Traditional hypotheses based on neuropharmacology

1.1.1.2.1   Dopamine hypothesis of schizophrenia

Much evidence supports the hypothesis that a malfunction in the dopamine pathway is involved in the pathology of schizophrenia.

Post-mortem studies have revealed elevated D2 receptors are present in untreated schizophrenia patients (Abi-Dargham et al., 2000; Abi-Dargham et al., 2009; Seeman and Kapur, 2000; Wong et al., 1986). After large and prolonged use of amphetamine and cocaine, which increase levels of dopamine in the brain, these drugs can produce symptoms indistinguishable from those present in psychosis. The majority of schizophrenia patients (up to 75%) also have increased symptoms after treatment with moderate doses of methylphenidate or amphetamine or other dopamine-like compounds, while healthy controls do not have any psychologically disturbing effects (Curran et al., 2004; Lieberman et al., 1987). Several studies also show that schizophrenia patients have greater levels of dopamine release than normal individuals after amphetamine treatment.

Another important piece of evidence is the observation that several antipsychotic drugs, including chlorpromazine and haloperidol, can antagonize dopamine binding to the D_2 dopamine receptors and thus reduce positive symptoms (Creese et al., 1976; Seeman et al., 1976). There is also genetic evidence which supports the dopamine hypothesis of schizophrenia. Several genes or specific variants of genes, which relate to dopamine function, are more prevalent in schizophrenia patients than normal

population. These include *COMT, DRD4* and *AKT1* (Arguello and Gogos, 2008). However, the dopamine hypothesis can not be posited as a complete explanation for schizophrenia.

1.1.1.2.2   Glutamate hypothesis of schizophrenia

This hypothesis has also been supported by several lines of evidence. The first one is that abnormally low levels of NMDA glutamate receptors are found in post-mortem brains of schizophrenia patients (Halene et al., 2009; Konradi and Heckers, 2003). Additional evidence is that some mind-altering drugs such as phencyclidine and ketamine, which are glutamate blocking drugs, can produce the symptoms and cognitive problems similar to the condition of schizophrenia (Lahti et al., 2001). Finally, glutamatergic drugs, which can coagonistically act at the NMDA or non-NMDA receptors, can reduce some of the positive symptoms of schizophrenia (Tuominen et al., 2005). NMDA receptor *Grin1* (-/-) mice have a disinhibition in sensory processing and reduced behavioral inhibition and impaired social interactions. Impaired NMDAR function is possibly relevant to the negative symptoms in schizophrenia (Boulay et al., 2010; Halene et al., 2009; Ramsey, 2009). This hypothesis does not negate the dopamine hypothesis. These two hypotheses possibly function together by circuit-based models (Lisman et al., 2008).

1.1.1.2.3   Serotonin and schizophrenia

Serotonin, also called 5-Hydroxytryptamine (5-HT), is a monoamine neurotransmitter, which is active in the gastrointestinal tract, platelets, and central nervous system (CNS) of humans and animals. The serotonin synthesized in serotonergic neurons in the CNS can regulate various types of processes, including mood, appetite, sleep, muscle contraction, and has cognitive functions, including memory and learning. Individuals with depression usually have lower levels of dopamine and serotonin in the brain (Meltzer and Sumiyoshi, 2008; Slowik, 1967). Several types of antidepressant medicines act through modulation of serotonin at synapses (Marino and Caballero, 2010; Wong et al., 2010). For instance, selective serotonin reuptake inhibitors (SSRIs) are typical antidepressants to treat depression, anxiety disorders and some personality disorders. SSRIs could inhibit the reuptake of serotonin into the presynaptic cell, increasing the extracellular level of serotonin. SSRI was reported to increase the level of brain derived neurotrophic factor (BDNF) through cAMP signal pathways on the postpsynaptic neuronal cell. Therefore, the growth and survival of cortical neurons and synapses are enhanced (Rumajogee et al., 2004)

1.1.1.3 Abnormal brain structure in schizophrenia patients

Brain imaging technologies and neuropsychological tests (including CT, MRI and PET) have produced a lot of extensive and precise findings on the deviation of brain function and structure in schizophrenia patients. Magnetic resonance imaging (MRI) can measure the volumes of anatomical structure and distinguish gray and white matter abnormalities. It was identified that functional differences in brain activity are usually occur in the frontal lobes, hippocampus and temporal lobes (Kircher and Thienel, 2005), which are linked to the neurocognitive deficits in schizophrenia (Green, 2006).

The size and structure of certain brain areas in schizophrenia patients are also found different from healthy individuals, including lateral ventricular enlargement, gray and white matter reduction (Colter et al., 1987), loss of normal asymmetries, frontal, volume reduction in temporal total and superior temporal gyrus etc (Schlaepfer et al., 1994). The reduced whole brain and hippocampal volume and increased ventricular volume in patients with a first psychotic episode have been found in a meta-analysis of MRI studies (Steen et al., 2006).

It is possible that the brain volume change is due to weight gain and changes in the physiological balance and general hydration of an individual (Wenz et al., 1994). But it can not be ruled out that some of the brain volume changes, particularly in the early stage of illness, may be epiphenomena.

1.1.1.4 Medication treatment for Schizophrenia

Medication treatment is used to control acute psychotic symptoms and to prevent relapse in the longer-term. Antipsychotic drugs are popularly used in the treatment of schizophrenia and bipolar disorders.

There are older (typical) and newer (atypical) antipsychotics. Typical antipsychotics, including chlorpromazine, thioridazine, fluphenazine, haloperidol, flupenthioxl and pimozide are classified according to their different chemical structures and were designed to work by blocking dopamine at synapses in the brain. The typical antipsychotics tend to be associated with many unwanted side-effects such as trembling, rigidity, abnormal face and body movements, restlessness and abnormal insulin absorption. Many of these side-effects are dose related. Newer (atypical) psychotics tend to act in a different way (affecting different chemical messengers, such as serotonin) and are less likely to cause side-effects and can help negative symptoms.

1.1.2 Bipolar disorders

Bipolar disorder or manic-depression is one of the most highly heritable psychiatric diagnoses and describes a category of mood disorders with abnormally elevated (manic and hypomanic) and abnormally depressed states or mixed episodes for a period of time. Bipolar disorder is sometimes accompanied by hallucinations, delusions and cognitive changes.

Bipolar disorder is equally prevalent in males and females, and across all cultures and ethnic groups (Jamison, 1990). It is often difficult to diagnose as not everyone's symptoms are the same and no blood test can confirm this disorder. In some cases, bipolar disorder is a devasting and long-lasting illness, especially during depressive episodes, as its episodes of abnormality are associated with distress and disruption and a higher risk of suicide than normal population (Goldberg and Harrow, 2004; Kessler et al., 2006a). Because relapse of bipolar disorder is very common, and this disorder can be profoundly disabling, the personal and societal cost of bipolar disorder are enormous (Kessler et al., 2006b). The onset of bipolar disorder generally occurs in late adolescence or early adulthood.

1.1.2.1 The genetics of Bipolar disorder

The overall heritability of the bipolar spectrum is estimated at 0.71 (Edvardsen et al., 2008). It was reported that the risk of bipolar disorder in first-degree relatives of bipolar disorder patients is approximately 9%, nearly 10 times that of the general population. The genetic causes are complex and multifactorial, due to many genes with individually small effect (Serretti and Mandelli, 2008). Twin studies have indicated that both genetic and environmental factors influence bipolar disorders. For bipolar I, the concordance rates are around 40% in monozygotic twins compared to 4.5% to 5.6% in dizygotic twins (Kieseppa et al., 2004). The concordance rates of combination of bipolar I, II and cyclothymia is 42% in monozygotic twins, while 11% in dizygotic twins. Since the concordance in monozygotic twins is not 100%, genetic factors are very important but can not be sufficient for bipolar disorder.

A large number of candidate genes have been evaluated by gene-finding research, especially association studies. Until recently, efforts to identify specific susceptibility variants have been restricted to studies of biological and positional candidate genes.

1.1.2.2 Medication treatment for Bipolar disorders

For bipolar disorders, Lithium carbonate, sodium valproate or lamotrigine are widely used mood stabilizer medications (Bauer and Mitchner, 2004; Geddes et al., 2004). Lithium is the only known mood stabilizer which can reduce suicide in bipolar patients. Lamotrigine is shown to be of benefit to prevent depression (Calabrese et al., 1999). Carbamazepine is also widely used to treat rapid cycling bipolar disorder. However, the mode of action is not well understood.

1.1.2.3 Abnormal brain structure in bipolar disorder patients

Anatomical differences in amygdala, prefrontal cortex and hippocampus are found in the brains of bipolar and other mood disorders patients. A meta-analysis based on 98 MRI or CT imaging studies found that the lateral ventricles in bipolar disorder patients were on average 17% larger than healthy controls, and the ratios of having deep white matter hyperintensities were 2.5 times of controls.

1.1.2.4 Circadian rhythm and bipolar disorder

The human circadian rhythm is a roughly 24-hour endogenous cycle in biochemical, physiological or behavioural processes. The primary circadian clock in human is found in the suprachiasmatic nucleus (SCN) in the hypothalamus. SCN receives daylight information through the eyes and passes it on to the pineal gland. This process results in the hormone melatonin secretion. Dysfunction of SCN will result in an irregular wake-sleep rhythm. Other organs and cells, such as lungs, liver, pancreas, spleen, thymus and skin, also have circadian rhythms (Zanello et al., 2000).

Disruption of circadian rhythm in humans is usually associated with a number of disorders such as bipolar disorder and sleep disorder (Harvey, 2008; McClung, 2007). It was reported that sleep deprivation can alleviate depression and regulate mood in bipolar disorders (Fahndrich, 1981; Larsen et al., 1976; Wehr et al., 1982). Circadian rhythm disturbances of bipolar disorder patients can be positively affected by lithium through an effect on clock genes (Martino et al., 2008). Lithium and valproate, which are effective medicine for bipolar disorders, also alter circadian rhythm in human and other species (Dokucu et al., 2005; Hafen and Wollnik, 1994; Johnsson et al., 1983). Dopaminergic (Ashby et al., 1999; Barbano and Cador, 2007; Lima et al., 2008; Yuferov et al., 2005) and serotonergic pathways (Adrien, 2002; Brummett et al., 2007; Sprouse et al., 2006; Yuan et al., 2005) are critical in the link between circadian rhythm and emotion. But, unfortunately, there is little research on the integration of the circadian rhythm and neurotransmitter systems to psychiatric disorders. Several bipolar disorder candidate genes are involved in circadian rhythms, including the *CLOCK* and *BMAL1* genes (Benedetti et al., 2003; Mansour et al., 2006;

Nievergelt et al., 2006; Serretti et al., 2003; Shi et al., 2008). Decreased levels of CRY2 were found in bipolar patients in a depressive state (Sjoholm et al., 2010). CRY2 participates in the core clock to regulate circadian rhythm and responds to total sleep deprivation (Takahashi et al., 2008). Taken together, circadian rhythm regulation may be an important process in stabilizing mood.

1.2 Molecular approaches to the study of psychiatric disorders

As schizophrenia and bipolar disorder are highly heritable diseases, identification of the genetic factors which affect the risk of psychiatric disorders are very significant and necessary. These genetic factors have been partially revealed by new technological developments. These technological changes include linkage studies, association methodologies, genome-wide approaches (a form of association), copy number variation studies, and gene expression microarray studies. The knowledge derived from each of these approaches is summarized here.

1.2.1 Linkage studies

Linkage studies are suitable for detecting markers spread across the genome of family members who are affected and unaffected with the disorder. The linkage studies can be applied to determine the chromosomal regions where susceptibility genes are located by examining a few hundred or thousand markers spread across the genome through detecting which markers (or regions) appear to be coinherited with disease within the family (Badner and Gershon 2002; Segurado, Detera-Wadleigh et al. 2003; McQueen, Devlin et al. 2005) Linkage studies are performed with single large families, collections of many smaller families and with two or more affected members. Many linkage studies were done with large numbers of affected sibling pairs. An advantage of linkage is that it can detect rare and common alleles. A disadvantage is that the region of linkage is large covering several mega bases and containing many genes. It does not allow the detection of individual candidate genes. The success of linkage studies in psychiatric disorders has been limited as linkage studies only work well in determining the single-gene Mendelian disorders (such as Huntington disease and Cystic fibrosis) or disorders in which genetic risk is conferred by a relatively small number of genes. These further support the hypotheses that psychiatric disorders are genetically complex diseases which are determined by a large number of genes, each of the susceptibility genes has a relatively small effect on disease risk. The linkage study works well on a large family with a major effect gene, but when comparing linkage data between families, it is not accurate due to heterogeneity.

1.2.2 Association studies

Association studies typically examine susceptibility variants that are relatively common in the population. The specific variants can include a number of different types of variation, including single nucleotide polymorphisms (SNPs) and repeat polymorphisms. Over the past 10 years, genetic association studies have found many specific alleles which are more common in affected individuals than in matched controls (case-control studies) and specific variants which are transmitted from parents to affected individuals more often than expected by chance (family-based studies). The markers are selected based on positional evidence from linkage studies and/or from hypotheses about the underlying neurobiology of the disorder.

1.2.3 Genome-wide association studies (GWAS)

Common genetic variation across the entire genome can be surveyed using microarray chips that simultaneously assay more than 1 million SNPs which match the complement of common variants. Thus, GWAS is a new and better approach to identify susceptibility genes without any prior hypothesis. This approach has been successfully used in the research of a broad range of complex disorders, including diabetes, breast cancer, cardiovascular disease, inflammatory bowel disease etc. A few groups carried out smaller GWAS studies, but in 2009, in order to fully take advantage of the technique, the psychiatric GWAS Consortium, a collaborative consortium, was been formed to look at many samples over many disorders (including bipolar disorder).

GWAS is suited to a particular type of genetic effect, which is modest in size but with a risk allele frequency that is relatively common in the general population (Newton-Cheh and Hirschhorn, 2005). GWAS can also detect copy number variants, duplications and deletions, which have been reported for psychiatric disorders. The exceptional success of GWAS for complex disease has produced numerous associations with a *P*-value $< 5 \times 10^{-8}$ (a recognised threshold for genome-wide significance).

1.2.4 Copy-number variations

A copy-number variant (CNV) is a DNA segment, usually 1 kilobase to several megabases, with different copy numbers in comparison the normal diploid state of the genome. In humans, CNVs are a widespread and common phenomenon which can be caused by genomic rearrangements such as deletions, duplications, inversions and translocations. For example, for normal DNA sequence (parental copies), the copy number is 2, but recently it was found that a proportion of our DNA was 1 (deletion) or 3 (duplication). This seems to be another form of genetic variation alongside SNPs. Although much copy number variation is common, the rare ones that alter gene dosage seem to be linked with illness. CNVs may be inherited or due to *de novo* mutation. Several techniques have been used to discover CNVs, including fluorescent in situ hybridization, array comparative genomic hybridization and GWAS data.

1.2.5 Gene expression Microarray analysis—High-throughput approaches to study gene expression

Numerous susceptibility genes for psychiatric disorders have been discovered by the approaches discussed above, including novel genes for which a contribution to disease was unknown previously. Microarray analysis is very good at discovering the roles and regulatory profiles of a gene defect or a condition. A microarray contains tens of thousands of gene probes and can measure their expression simultaneously. As advances in gene annotations progress, standardization of microarray platforms and data analysis have been introduced. Microarrays are now an ideal approach to determine the changes in transcription of the genome in response to disease status (e.g., schizophrenia brain vs. healthy control brain), specific drug treatments, or as a consequence of mutations of psychiatric illnesses candidate genes in cell or animal models. These approaches can discover relevant target genes, canonical pathways and further generate new hypotheses. It is very necessary to confirm the key data by independent technologies, such as real-time quantitative PCR, in situ hybridization, immunoblots and other appropriate methods. It must be remembered that microarrays detect changes in the mRNA level which might not necessarily be translated into changes at the protein level.

1.2.6 Candidate genes and chromosome regions associated with schizophrenia identified by these approaches

Several detailed mapping studies based on positive linkage findings have identified several putative susceptibility genes for schizophrenia, such as genes encoding dysbindin (*DTNBP1*) (Schwab et al., 2003; Straub et al., 2002; van den Oord et al., 2003; Williams et al., 2004), neuregulin 1 (*NRG1*) (Corvin et al., 2004; Stefansson et al., 2003; Yang et al., 2003), D-aminao-acide oxidase (*DAO*), D-amino-acid oxidase activator (*DAOA* or *G72*) and regulator of G-protein signalling 4 (*RGS4*) (Kirov et al., 2004). The U2AF homology motif (*UHM*) kinase 1 (*UHMK1*) on the chromosome 1q23.3 was previous reported as a schizophrenia susceptibility gene (16978587) and the genetic association between this gene and schizophrenia has been further confirmed in a fine-mapping study (Puri et al., 2008).

Many hundreds of genes have been identified by association analysis. These genes include disrupted in schizophrenia *(DISC1)*, the dopamine receptor (*SLC6A3*), brain-derived neurotrophic factor (*BDNF*), the NMDA glutamate receptor submit 2B (*GRIN2B*), d-amino-acid oxidase activator (*DAOA*), peroxisome proliferators-activated receptor delta (*PPARD*), neurogulin 1 (*NRG1*), the 5-HT transporter *(SLC6A4)*, tryptophan hygroxylase-2 (*TPH2*), pericentriolar material 1 (*PCM1*) and catechol-o-methyl transferase (*COMT*) (Cardno and McGuffin, 2006; Craddock and Forty, 2006; De Luca et al., 2005; Fallin et al., 2005; Fan and Sklar, 2008); (Bass et al., 2009); (Datta et al., 2010; Gurling et al., 2006).

A susceptibility locus in an intron of *ZNF804A* (zinc finer protein 804A) is with a genome-wide significance (p=9.96 x10^{-9}) (O'Donovan et al., 2009; Williams et al., 2010). This gene encodes a transcription factor, which is associated with the activities of hippocampus and dorso-lateral prefrontal cortex (Esslinger et al., 2009). Shifman and colleagues found a strong evidence for a female-specific association between *reelin* and schizophrenia (Birkhofer et al., 2007; Shifman et al., 2008). Reelin has been reported having a reduced expression in the brain of schizophrenia patients (Knable et al., 2001) and being involved in corticogenesis and implicated in an autosomal recessive form of lissencephaly (Hong et al., 2000).

Like other type of genetic variations, CNVs have been associated with autism, schizophrenia and idiopathic learning disability. 22q11DEL is reported to be associated with a 25-fold increase in the risk of schizophrenia (Bassett et al., 2008; Murphy et al., 1999). Deletions mapping to 1q21.1 and 15q13.3, which are associated with schizophrenia, were discovered in two CNV studies: the ISC one (ISC, 2008) and the one lead by decode genetics (Stefansson et al., 2008). Deletions at 1q21 not only increase risk of schizophrenia but were also associated with mental retardation, autism and ADHD (Mefford et al., 2008). Deletions at 15q13.3 are also involved in Idiopathic Generalized Epilepsy (Helbig et al., 2009). There are other CNV loci that are with strong statistical evidence for association such as 2p16.3 (Del), 15q11.2 (Del), 16p13.1 (Dup), 16p11.2 (Dup)(McCarthy et al., 2009) and 17p12 (del) etc. Although it cannot be denied that CNVs are involved in schizophrenia, the individual genes relevant to disease pathophysiology are still uncertain, as the implicated CNVs span multiple genes.

There are numerous findings in schizophrenia using gene expression microarray technology. Genes involved in the regulation of presynaptic function are found decreased in the prefrontal cortex of schizophrenia patients (Mirnics et al., 2000), which can explain the brain imaging observation that reduced activation of the prefrontal cortex during tasks engage working memory (Carter et al., 1998; Heckers et al., 1999; Petronis, 2003). Reduction in gene expression involved in metabolic pathways and ubiquitin degradation are found in prefrontal cortex in schizophrenia (Middleton et al., 2002; Vawter et al., 2002). Decreased ionotropic glutamate receptor (AMPA) (Vawter et al., 2002), oligodendrocyte specific transcripts (Hakak et al., 2001; Tkachev et al., 2003) and ubiquitin conjugating enzyme E2N were also found in schizophrenia using expression microarrays. Increased transcripts of the apolipoprotein L family genes, which are located close to a genetic high-susceptibility locus for schizophrenia and velocardiofacial syndrome (an illness with schizophrenia-like features) on chromosome 22, were found in the brains of schizophrenia patients (Coon et al., 1994; Karayiorgou et al., 1995; Mimmack et al., 2002; Pulver et al., 1994).

1.2.7 Candidate genes and chromosome regions associated with bipolar disorders identified by these approaches

McQueen et al. combined 11 of the largest linkage studies for bipolar disorder and found that chromosome 6q and 8q met statistical criteria for genome-wide significance (McQueen et al., 2005). *TRPM2* and C*21ORF29* (*TSPEAR*) on chromosome 21q22.3 were found associated with bipolar and unipolar affective disorder by a fine mapping study (McQuillin et al., 2006; Xu et al., 2009; Xu et al., 2006).

There have been several independent published GWAS of bipolar disorder based on individual genotyping or DNA pooling. A significant locus in *DGKH (diacylglycerol kinase eta)* was detected in a sample of 1,233 cases and 1,439 controls (Baum et al., 2008). Diacylglycerol kinases are very important in a series of signalling pathways including a phosphatidyl insositol pathway, which is sensitive to lithium. A genome wide significant association ($P = 6.3 \times 10^{-8}$) for a locus on chromosome 16p2 was obtained in a GWAS based on 1,868 cases and 2,938 control (WTCCC 2007). This region spans several genes including *PALB2 (partner and localizer of the breast cancer gene, BRCA2), NDUFAB1 (NADH dehydrogenase (ubiquinone) 1, alpha/beta)* and *DCTN5 (dynactin 5)*. The strongest finding ($P = 1.7 \times 10^{-7}$) in *MYO5B (myosin 5B)* was observed in another GWAS study, known as STEP-UCL, based on 1,461 cases and 2,008 controls, although it did not pass the threshold of the genome wide significance (Sklar et al., 2008). A meta-analysis comprising the WTCCC, STEP-UCL GWAS datasets and a supplemental GWAS data identified two associations with genome-wide significance: *ANK3* ($P = 9.1 \times 10^{-9}$) and *CACNA1C (calcium*

channel, voltage-dependent, L type, alpha 1 C subunit) ($P = 7.0 \times 10^{-8}$) (Ferreira et al., 2008). ANK3 links integral membrane proteins to the underlying spectrin-actin cytoskeleton and is involved in cell motility, activation, proliferation, contact, and modulation of the activity of neuronal sodium channels. CACNA1C is a subunit of brain L-type voltage gated calcium channels and is responsive to synaptic activity (Vacher et al., 2008).

For bipolar disorders, gene expression microarray studies found that reduced oligodendrocytes in the cortex (Tkachev et al., 2003) and reduced expression of the mitochondrial respiratory chain, which suggests abnormal energy regulation in the brain of bipolar patients (Kato and Kato, 2000; Kato et al., 1992). Down-regulation of proteasomal subunits in the hippocampus was also identified in bipolar disorder using gene expression microarray studies (Konradi et al., 2004). McQuillin and colleagues performed a microarray study the mRNA change in the mice brain after treated by lithium, a widely used mood stabilizer for bipolar. They found that period gene 2 (*Per2*), metabotropic glutamate receptor (*Grm3*) and secretogranin II (*Scg2*) as well as several myelin-related genes and protein phosphatases show significant change in expression (McQuillin et al., 2007).

1.2.8 Overlapping findings associated with both schizophrenia and bipolar disorders

Although bipolar disorder and schizophrenia were assumed to be separate disease entities, genetic linkage studies have identified some chromosome overlapping regions associated with both disorders. These include regions in 13q, 22q, 6p, 4p and chromosome 18 (Badner and Gershon, 2002; Berrettini, 2003). These findings are also enhanced by association studies, CNVs studies and epidemiological studies (Christoforou et al., 2007).

Many susceptibility genes are also associated both disorders. These include *NPAS3, DISC1, ZNF804A, BRD1, PDE4B, SLC6A4 and G72/G30(DAOA)* etc (Gomez et al., 2009; Kahler et al., 2010; Nyegaard et al., 2010; Schosser et al., 2010).

1.3 Adult neurogenesis and psychiatric illnesses

1.3.1 The relation of neurogenesis and psychiatric disorders

Adult neurogenesis, the process of generation of new neurons from adult neural stem cells, is sustained throughout adulthood in the mammalian brain (Gage, 2000). Adult neurogenesis might be involved in hippocampal aspects of neurodegenerative disorders or psychiatric disorders (Kempermann et al., 2008). Adult neurogenesis in SGZ can be activated by several physical and chronic antidepressant treatments (Warner-Schmidt and Duman, 2006). Cell proliferation is deregulated in several neurodegenerative diseases, such as Alzheimer's disease (Verret et al., 2007), Parkinson's disease (Nuber et al., 2008) and Huntington's patients (Kohl et al., 2007).

Although schizophrenia is not specifically a hippocampal disorder, failing adult neurogenesis may reflect the latest stages of a subtle misregulation of brain development and result in a particular set of hippocampal symptoms. Reif et al. (Reif et al., 2006) found a reduction in putative precursor cell proliferation in adult dentate gyrus by 63% in schizophrenia by investigating the brains of 15 patients with depression, schizophrenia or bipolar disease and controls with immunohistochemistry.

1.3.2 Adult Neurogenesis

Two main locations of adult neurogenesis in the mammalian brain have been demonstrated under normal conditions: the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus (Zhao, 2008). Adult hippocampal neurogenesis can be regulated by genetic background at levels of cell proliferation, differentiation, and survival (Kempermann, 2002).
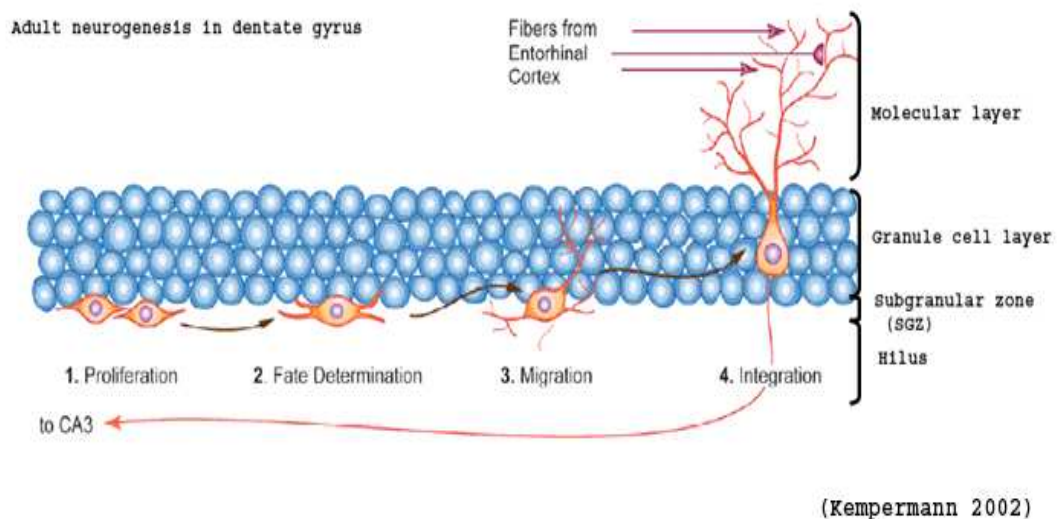
Figure 1.1 Adult neurogenesis in dentate gyrus

Neurogenesis involves cellular processes at several levels, including the proliferation of adult neural stem cells (NSCs) or progenitors, differentiation and fate determination of progenitor cells, and the survival, maturation, and integration of newborn neurons (Grote and Hannan, 2007).

1.3.3 A number of candidate genes for susceptibility to psychiatric disorders are involved in neuronal development.

Reduced expression of reelin in the cortex and the hippocampus in schizophrenia patients (Toro and Deakin, 2007) may result in disturbed adult neurogenesis (Forster et al., 2002; Heinrich et al., 2006) by a disturbance of neuronal migration and glial scaffolding (Won et al., 2006; Zhao et al., 2007).

Disrupted in schizophrenia 1 (*DISC1*) is a schizophrenia susceptibility gene (Blackwood and Muir, 2004; Millar et al., 2000). It is expressed in the hippocampus and has diverse functions in neuronal migration, differentiation, neurite outgrowth and synaptic plasticity (Ross et al., 2006). A study on mutant mice for *Disc1* shows that DISC1 and its co-operator, NDEL1, can regulate adult neurogenesis (Dranovsky and Hen, 2007; Duan et al., 2007). DISC1 is highly expressed in the embryonic ventricular/subventricular zones of the cortex, where neural progenitor cells reside, and plays important roles in the growth of the embryonic and postnatal brain (Mao et al., 2009). When DISC1 RNAi was introduced into neural progenitors in the developing neocortex of mouse brains, the proliferation of progenitor cells was significantly reduced and the premature neuronal differentiation and cell cycle exit were increased. The same group also found that knockdown of DISC1 resulted in a decrease in the proliferation of adult progenitor cells in the dentate gyrus.

Neuronal basic helix-loop-helix (bHLH) PAS domain transcription factor 3 (NPAS3) is a member of basic bHLH PAS domain transcription factors, which is enriched in the inter-neurons in brain. NPAS3 is mutated in a family affected by schizophrenia

(Kamnasaran et al., 2003; Pickard et al., 2008; Pickard et al., 2005). Also Pickard et al. showed that interactions between variants across the NPAS3 gene might contribute to susceptibility to schizophrenia and bipolar disorders (Pickard et al., 2008). A very significant reduction in hippocampal neurogenesis was found in *Npas3* knock-out mice (Pieper et al., 2005). These mice exhibited abnormal performance, such as reduced memory function, disturbed social behaviour and had reduced paired pulse inhibition (Erbel-Sieler et al., 2004).

1.4   NPAS3 is a strong candidate gene for psychiatric illness.

Disruption of the *NPAS3* (Neuronal basic helix-loop-helix (bHLH) PAS domain transcription factor 3) gene as a result of a chromosomal abnormality [t(9;14)(q34;q13)] carried by a mother and daughter diagnosed with schizophrenia and mild learning disability provided the first indication of a role for this gene in psychiatric illness (Kamnasaran et al., 2003; Pickard et al., 2005; Pickard et al., 2006). This disruption lies within the intron 3 of *NPAS3* gene. It results in the bHLH and PAS domains are being seperated from the transactivation domain, therefore, the functions of DNA binding and dimerisation may have been destroyed (Kamnasaran et al., 2003). Subsequent gene-specific and genome-wide case-control association studies have linked single nucleotide polymorphisms in the *NPAS3* gene with increased risk of schizophrenia (Pickard et al., 2009) major depression disorder and bipolar disorder (Ferreira et al., 2008; Huang et al., 2010; Pickard et al., 2009). A recent report detailed the association of three common *NPAS3* exonic variants with increased risk of schizophrenia (Macintyre et al., 2010). Additionally, genetic variation at the *NPAS3* locus has been provisionally associated with risk of multiple sclerosis (Comabella et al., 2008), response to interferon beta treatment of multiple sclerosis (Byun et al., 2008), predisposition to addiction (Liu et al., 2006) and response to treatment with the antipsychotic drug, iloperidone (Lavedan et al., 2009).

1.4.1 The structure and function of NPAS3

NPAS3 is expressed in 13 adult brain tissues including the hippocampus, thalamus, and cortex, especially in developing centre nervous system (Brunskill et al., 1999). *NPAS3* encodes a member of the basic helix-loop-helix PAS domain transcription factor family that typically integrate environmental signals and heterodimeric binding partner availability to generate a transcriptional response (Brunskill et al., 1999; Gilles-Gonzalez and Gonzalez, 2004; Zhou et al., 1997). In humans, the *NPAS3* gene has 11 exons spanning 791 kb of 14q12-q13.

Figure 1.2. The structure of NPAS3. NPAS3 protein is composed a bHLH domain at the amino terminus, followed by two PAS domains. The carboxyl-terminal end contains transactivation domain.

1.4.2 The biology of the psychiatric disorder candidate gene-NPAS3

NPAS3 is a brain-enriched transcription factor containing basic-helix-loop-helix motif and PAS domain. The bHLH-PAS superfamily has diverse functions in development or physiological events, including circadian rhythms, neurogenesis, toxin metabolism, response to hypoxia and tracheal development.

The structures of bHLH-PAS proteins are highly conserved. The bHLH domain is located the amino terminus. Then it is followed by the PAS domain. The carboxyl-terminal end contains transcriptional activation domains (Franks and Crews, 1994; Jain et al., 1994) or repression domains (Moffett et al., 1997).

1.4.3 The basis bHLH domain transcription factors

A basic helix-loop-helix domain is composed of 2 α-helices connected by a loop. Generally, the smaller helix can bind to another protein by folding and packing against another helix. The larger helix, which contains the DNA binding region, typically binds to a consensus DNA sequence (E-box, CANNTG) (Chaudhary and Skinner, 1999). Some bHLH transcription factors can also bind to non-palindromic sequence.
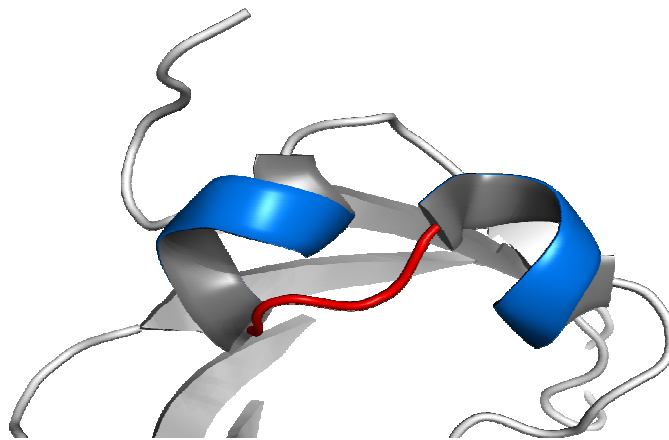
Figure 1.3. basic helix-loop-helix DNA binding domain (Massari and Murre, 2000). Two alpha-helices (blue) are connected by a short loop (red)

The bHLH-PAS proteins share a number of similarities with other members of bHLH proteins (Littlewood and Evan, 1995). For example, bHLH proteins are important in regulation of cell lineage (Isaac and Andrew, 1996; Thomas et al., 1988) as well as other bHLH proteins (Jan and Jan, 1993; Weintraub et al., 1991).

1.4.4 The functions of bHLH superfamily

There are many transcription factors containing a bHLH domain, such as:

- AhR
- Beta2/ NeuroD1
- BMAL-1-CLOCK
- C-Myc, N-Myc
- MyoD
- Pho4
- HIF
- NPAS3, NPAS1, NPAS2, MOP5
- Scl
- Neurogenins
- BHLHB2, BHLHB3, BHLHB4, BHLHB5, BHLHB8

Usually, transcription factors with this domain exert their function with another subunit through heterodimerization, including BMAL1-NPAS3 complex and BMAL1-CLOCK complex. The activity of the heterodimeric complex is often determined by the dimerization of the subunits. The level or availability of one subunit is restricted, whereas the other subunit is broadly expressed. The combinatorial properties of bHLH members explain the possibility that bHLH proteins play important roles in a variety of complex biological events. For example, BMAL1-CLOCK is a very important complex in regulating circadian rhythm, and c-Myc and HIF-1 are linked to cancer through their effect on cell growth and metabolism.

1.4.5 The structure and function PAS domain

The PAS domain is a signal sensor in many signalling proteins (Dunham et al., 2003; Hefti et al., 2004; Ponting and Aravind, 1997). This domain was named after three proteins which contain it:

- Per- period circadian protein

- Arnt- Ary hydrococarbon receptor nuclear translocator protein

- Sim- single-minded protein

The PAS domain includes two degenerate direct repeats (around 50 amino acids) (Crews, 1998). The PAS domain in bHLH-PAS proteins is about 260-310 amino acids. It includes two conserved regions (PAS-A and PAS-B) linked by a spacer (Crews et al., 1988). Many PAS-domain proteins exert their function by dimerization between PAS proteins (Huang et al., 1993), interaction with non-PAS proteins (Dolwick et al., 1993; Gekakis et al., 1995) and binding small moleculars, such as the heme ligand (Gilles-Gonzalez and Gonzalez, 2004)

1.4.6 Npas3 deficient animal model

A mouse *Npas3* knockout displays a range of behavioural phenotypes consistent with it being a representative model of human psychiatric disorders, including increased locomotor activity, stereotypic darting behaviour at weaning, subtle gait defects, impairment of prepulse inhibition of acoustic startle, deficit in recognition memory and altered anxiety-related responses. (Brunskill et al., 2005; Erbel-Sieler et al., 2004). It also displays an additional deficit in adult hippocampal neurogenesis (Pieper et al., 2005).

Both *Npas1* and *Npas3* knockout mouse strains also show developmental lung phenotypes impacting neonatal survival rates but how this pathology relates to central nervous system observations is currently unknown (Levesque et al., 2007; Zhou et al., 2009).

1.5   Summary and hypothesis

Schizophrenia and bipolar disorder are common, lifelong psychiatric illnesses affecting mood, perception and cognition. Family and epidemiological studies have indicated that these conditions are strongly influenced by genetic facors. Therefore, the functional study of candidate genes will aid the description of underlying pathologies and guide the search for new therapeutic approaches. *NPAS3* is a strong risk factor for schizophrenia and bipolar disorder. *Npas3* knockout mice also exhibit behavioural and adult neurogenesis deficits consistent with human illness. A set of questions about NPAS3 were addressed in my PhD project.

1.5.1 What is the expression pattern of NPAS3 in adult hippocampus?

It was reported that Npas3 (-/-) mice are associated with hippocampal neurogenesis deficit. This result suggested NPAS3 might directly regulate the developmental pathway associated with new neuron production. Investigation of the expression pattern of NPAS3 in adult mice hippocampus will be necessary to reveal its role during adult neurogenesis.

1.5.2 What is the regulatory profile of NPAS3?

As a transcription factor, the investigation of NPAS3's transcriptional regulatory profile is particularly significant because observed expression changes are likely to represent direct activity rather than secondary or homeostatic reactions. Full-length (FLNPAS3) and artificially truncated (ΔNPAS3) forms of NPAS3 were over-expressed in the human embryonic kidney cell line, HEK293, followed by microarray analysis to identify target genes.

1.5.3 How does the regulation profile of NPAS3 compare with SOX transcription factors?

Many SOX members are known neurodevelopmental regulators and SOXD/E members are particularly important during the process of converting neural progenitors into immature neurons. Sox11 was found strictly colocalized with Dcx – expressing precursors and immature neurons, but not with Sox2-expressiong non-committed precursors and immature cells (Haslinger et al., 2009). Npas3 was found only colocalized with Dcx in adult mouse hippocampus in our study, furthermore. There are also association evidence supporting roles for *SOX5* in metabolic side-effects of antipsychotic treatment and *SOX10* in increased risk of psychiatric illness (Maeno et al., 2007). We were really interested to investigate whether SOX members and NPAS3, as transcription factors might have some interaction. The target genes of NPAS3 were compared with the SOX family of transcription factors (Azim et al., 2009; Bergsland et al., 2006; Cheung and Briscoe, 2003; Hamada-Kanazawa et al., 2004; Haslinger et al., 2009; Kim et al., 2003; Kwan et al., 2008; Lefebvre, 2009; Prior and Walter, 1996).

1.5.4 How does circadian rhythm affect NPAS3?

NPAS2 is one of the components of the circadian clock oscillator. The other components include the CRY proteins, CLOCK, BMAL1, BMAL2, CSNK1D, CSNK1E, TIMELESS and the PER proteins. BMAL1 is able to heterodimerise with NPAS3 to regulate transcription of genes. As NPAS3 closely interacts with circadian regulators, we wished to know whether NPAS3 activity was sensitive to circadian context or if, indeed, it could directly regulate it. An additional microarray study was

carried out in which cells over-expressing NPAS3 were stimulated to commence synchronous circadian cycling.

In order to answer these questions, several projects were carried out. The detail will be introduced in the later chapters

CHAPTER TWO

2   Materials and Methods

2.1   Materials

2.1.1 Suppliers

Sigma-Aldrich Limited, Poole, Dorset, UK

Sigma-Genosys, Ltd, London Road, Pampisford, Cambridge, UK

Invitrogen Ltd, Paisley, UK

Promega, Ltd, Southhampton, UK

Qiagen, Ltd, West Sussex, UK

GRI, Essex, UK

New England Biolab (UK) Ltd, Hitchin, Hertfordshire, UK

Stratagene, Amsterdam, Netherlands

Cambrex BioScience Rockland Inc, Rckland, USA

Roche Diagnostics Ltd, Lewes, East Sussex, UK

Hybaid, Teddington, UK

ABgene, Surrey, UK

Anachem Ltd, Bedfordshire, UK

Sarstedt, Leicester, UK

Corning Ltd, Buckinghamshire, UK

Cheshire Scientific Ltd, South Wirral, UK

Fisher Scientific Ltd, Leicestershire, UK

Sciquip Ltd, Shropshire, UK

Applied Biosystems, CA, USA

USB Corporation, Cleveland Ohio, USA

Amersham Pharmacia Biotech UK Ltd, Buckinghamshire, UK

2.1.1.1 General Plastic ware

Micro tubes 0.5ml, 1.5ml, and 2ml	Sarstedt
Tissue culture plasticware	Corning
0.2ml Flat and Domed cap tub	Abgene
Thermo-Fast 96, skirted Plate	Abgene
Flat cap strip	Abgene
Culture Loops	Cheshire
Microbiological spreader	Cheshire

2.1.1.2 Apparatus

Horizontal electrophoresis tank	Hybaid
MJ Research Thermocycler	GRI
Balance Fisherbrand PS-200	Fisher
Microcentrifuge eppendorf 5415D	Fisher
IKA Minishaker MS 2	Fisher
Syngene Bio Imaging system	Fisher
Sigma Laboratory centrifuge 4-15, 4K15	Fisher
Micro Pipette (2µl, 10µl, 20µl, 200µl, 1000µl)	Gilson

2.1.1.3 Chemicals

Isopropyl alcohol	Sigma-Aldrich
Ethanol	Sigma-Aldrich
Formamide	Sigma-Aldrich

2.1.1.4 Media and Media Components

Alpha MEM	Invitrogen
PBS	Invitrogen
Glutamine	Invitrogen
Penicillin	Invitrogen
Trypsin/EDTA	Invitrogen
Yeast extract	Invitrogen

2.1.1.5 Molecular Biology products

Dual Luciferase Reagent	Promega
pGL-3 Luciferase vector (control& basic)	Promega
pRL-TK vector	Promega
Taq PCR core kit	Qiagen
PRISM BigDye Terminator cycle sequencing kit	Applied Biosystems
NuPAGE® Novex® Tris-Acetate gels	Invitrogen
SYBR Green^{TM} qPCR SuperMix Universal	Invitrogen
ECL^{TM} Western Blotting Detection Reagents	Amersham^{TM}
QIAquick gel extraction kit	Qiagen
Qiagen PCR purification kit	Qiagen
Qiagen plasmid mini kit	Qiagen
Subcloning Efficiency TM DH5α competent cells	Invitrogen
T4 DNA Ligase and buffer	Roche
Shrimp Alkaline Phosphatase	USB Corporation
PCR primers and oligos	Sigma-Genenosys
Orange G loading dye	Sigma-Aldrich
Ethidium Bromide	Sigma-Aldrich
Restriction endonucleases and buffer	New England Biolabs
T4 DNA polymerase, buffer and EDTA	NEB
Agarose Powder	Bio-Rad
0.1M DTT	Invitrogen
DNase I	Invitrogen
dNTPs	Invitrogen
Bovine serum albumin	New England Biolabs
1 kb DNA Ladder	Promega
Hinc II DNA ladder	Abgene

2.1.2 Solutions, buffers and gel loading dyes

Ampicillin stock solution (10mg/ml)

Ampicillin sodium salt   5g

Qs (Quantity sufficient) 50ml dsH2O

 Filter-sterilized and store at -20°

Blocking buffer (western blot)

Marvel Skimmed milk powder   2.5g

PBS   50ml

TWEEN   100µl

Blocking buffer (immunofluorescence)

donkey serum   100µl

PBS/1% TWEEN 20   10ml

1M DDT

Dithiothreitol   0.7g

Qs 5ml dsH2O

EDTA solution 0.5M pH8.0

Disodium ethylenediaminetetra acetate   1.861g

dsH2O   800ml

pH8.0

30% Ethanol

30ml ethanol (99.7-100% v/v) to every 100ml dsH2O

70% Ethanol

70ml ethanol (99.7-100% v/v) to every 100ml dsH2O

90% Ethanol

90ml ethanol (99.7-100% v/v) to every 100ml dsH2O

50% Formamide Solution

Formamide (99%)	500ml
2×SSC	100ml
dsH2O	400ml

Luria-Bertani broth (LB)

1% (w/v) Bacto-Tryptone (Difco)

0.5% (w/v) Bacto-Yeast extracts (Difco)

0.1% (w/v) NaCl

pH 7.0 autoclave

LB-agar

LB broth with 15g Bacto-Agar (Difco) per litre

Autoclave

Orange G loading buffer

Glycerol	30ml
dsH2O	70ml
Orange G	1g

1×PBS

KH_2PO_4	1.157g
Na_2HPO_4	6.971g
NaCl	43.83g
Qs 500ml dsH_2O	

PBS/1%TX100

PBS	495ml
TX100	5ml

Primer mix

Glycerol	6ml
TE	7ml
DMSO	1ml

Running Buffer (for Tris-acetate gels)

20x Nupage Tris-acetate SDS running buffer	25ml
dsH_2O	475ml

RIPA buffer

Tris (hydroxymethyl)-methylamine (pH7.5)	0.61g
NaCl (150mM)	0.9g
dsH_2O	80ml
1% Triton X-100	1ml
Sodium deoxycholate (10% solution)	2.5ml
Sodium deoxycholate (20% solution)	250μl
1mM EGTA (100mM solution)	1ml

Add 100μl protease inhibitors to 5ml RIPA lysis buffer before use

20x TBE

Tris	242.0g
20mM EDTA	14.88g
Boric acid	123.4g

Qs 1L dsH_2O autoclave

TE pH 8.0

10mM Tris Cl pH 7.4

1mM EDTA   pH 8.0

Transfer Buffer

20x NuPage Transfer buffer	25ml
Methanol	100ml
dsH20	375ml

0.05 Tris-HCL pH 6.7

Trizma hydrochloride   7.88g

Qs dsH$_2$O   1L

pH 6.7

Wash Buffer (western blot)

10x PBS   100ml

dsH2O   900ml

Tween   2ml

2.1.3 Primers

Genomic sequence information for genes was obtained from NCBI. Ologonucleotide primers were designed using PRIMER3 [http://workbench.sdsc.edu] (Rozen and Skaletsky, 2000). Specificity of all primers was checked by Blast and BLAT analysis http://www.ncbi.nlm.nih.gov/Blast.cgi, http://genome.ucsc.edu/cgi-bin/hgBlat. Primer sequences are listed in (table 2.1 and 2.2) PCR Primers were re-suspended at a concentration of 100μg/μl in primer mix and stored at -20° C.

Table 2.1 PCR primers used in validation of SOX11 microarray and timecourse QPCR assay.

Primer Name	Sequence
SOX11 QPCR forward	GACTTCCCCGACTACTGCAC
SOX11 QPCR reverse	TAGGTG AACACCAGGTCGGA
SEMA3B QPCR forward	GACCTCATGGGACGAGACTT
SEMA3B QPCR reverse	ACCTTGACAAACTTGGGCTC
NEDD9 QPCR forward	ACACCATCTACCAAGTGCCA
NEDD9 QPCR reverse	ATGCTTCTCTGCACTGATGG
GSTA2 QPCR forward	AACCTGAGGAACAAGATGCC
GSTA2 QPCR reverse	AAGGTAGTCTTGTCCGTGGC
GPC2 QPCR forward	GCTCCTTTCTGGTTCACACA
GPC2 QPCR reverse	CGTAGGAGTGGGAGAAGAGC
BAG1 QPCR forward	ACAGCAATGAGAAGCACGAC
BAG1 QPCR reverse	CTGTGGAACCCCTATGACCT
HIST1H2BE QPCR forward	ACACCGGCATCTCCTCTAAA
HIST1H2BE QPCR reverse	GTCGAGCGCTTGTTGTAATG
HIST1H2AE QPCR forward	CTAAAACGCGTTCTTCCAGG
HIST1H2AE QPCR reverse	TCGTTCGGACTAGTTGCCTT
SCG2 QPCR forward	GAAGCGAGTTCCTGGTCAAG
SCG2 QPCR reverse	TCCTGAGAGCTGCCTTGATT
CD24 QPCR forward	TAGCTGGGATTACAGGCACC
CD24 QPCR reverse	GTCAGGAGTTCGAAACCAGC
FILIP1 QPCR forward	TCAGTTTAAACGGTCCCCTG
FILIP1 QPCR reverse	TTCGGCTGTCACGTTTACTG
TUBB3 QPCR forward	AGGCCTGAAGAGATGTCCAA
TUBB3 QPCR reverse	AACGAGGCCTCTTCTCACAA
*CYP39A1*QPCR forward	CGAAGGAGAGCTGCAAAAGT
CYP39A1 QPCR reverse	CTTGCTTCTTTTCTGTGGGC
NDP QPCR forward	TGTCGTTCAGCACTGTCGCG
NDP QPCR reverse	CTTCAGCTTGGAAGTCTGGG
GAPDH QPCR forward	ACAGTCAGCCCGCATCTTCTT
GAPDH QPCR reverse	ACGACCAAATCCGTTGACTC
SOX11 attb forward primer	GGGGACAAGTTTGTACAAAAAAGCAGGCTGATCACGGTT CAACACACGGAAC
SOX11 attb reverse primer	GGGGACAAGTTTGTACAAAAAAGCAGGCTACCGCTTCCC GAGCCGTTTAGAG

Table 2.2 PCR primers used in validation QPCR for NPAS3 microarray, circadian gene detection and VGF promoter assay.

Primer name	Sequence
SOX11 QPCR FORWARD	GACTTCCCCCACTACTGCAC
SOX11 QPCR REVERSE	TAGGTGAACACACCAGGTCGGA
SOX3 QPCR FORWARD	ATGAACGGCTGGACTAATGG
SOX3 QPCR REVERSE	GGAGCTCTGCTGGTTGTAGG
SOX4 QPCR FORWARD	GTGAGCGAGATGATCTCGGG
SOX4 QPCR REVERSE	CAGGTTGGAGATGCTGGACTC
HK2 QPCR FORWARD	ATACGGTTGCTTCACCTTGG
HK2 QPCR REVERSE	TCAGGCTCACATCTCAGTG
ENO2 QPCR FORWARD	TGTCTCATCCTCCTGGAACC
ENO2 QPCR REVERSE	TCAATCAGGGAAGTTCTGGG
NPAS3 QPCR FORWARD	TCGGCATTTTCGTTTAGACC
NPAS3 QPCR REVERSE	AAGAAAGAGGGGGTGGAATG
VGF QPCR FORWARD	GAGCATAAAGAGCCGGT
VGF QPCR REVERSE	GAAAGCTCTCCCTCGTCCT
18s RNA QPCR FORWARD	GGGAGGTAGTGACGAAAAATAACAAT
18s RNA QPCR REVERSE	TTGCCCTCCAATGGATCCT
VGF PROMOTER FORWARD	GGCCCTCGAGGGAGGTTAGAAGGAGGGTCAGT
VGF PROMOTER REVERSE	GGCCCCATGGCTACCGGCTCTTTATGCTCAGA
M13 FORWAR SEQUENCING PRIMER	GTAAAACGACGGCCAG
M13 REVERSE SEQUENCING PRIMER	CAGGAAACAGCTATGAC
VGF PROMOTER SEQUENCING PRIMER FORWARD 787	GGTGGAGAGAGCTGGAGTTG
VGF PROMOTER SEQUENCING PRIMER FORWARD 1325	ACGCTGGGACTACCCTTTTT
VGF PROMOTER SEQUENCING PRIMER REVERSE 806	CAACTCCAGCTCTCTCTCCACC
VGF PROMOTER SEQUENCING PRIMER REVERSE 1344	AAAAAGGGTAGTCCCAGCGT
VGF PROMOTER SEQUENCING PRIMER REVERSE 1945	GAGAGGTGGAGAGGAGGGTC
PER1 FORWARD	AGGTACCTGGAGAGCTGCAA
PER1 REVERSE	TTCTTGGTCCCCACAGAGAC
PER2 FORWARD	AAATGGATCCCCCTTGAATC
PER2 REVERSE	AGCACCACCTGGTGTACCTC
PER3 FORWARD	TCCTGGCGTCTTCTCACTTT
PER3 REVERSE	TCATACCGTGCAGCTCTTTG

2.1.4 Cell lines

In-house cell lines HEK293 (human embryonic kidney), SH-SY5Y (human neuroblastoma derived) and U373 were cultured using standard laboratory practice.

2.2   General methods

Molecular biology techniques were developed from (Sambrook et al., 1989), unless otherwise stated.

2.2.1   Miniprep preparation of Bacterial DNA

A single bacterial colony was used to inoculate 5ml of LB-Broth with the appropriate antibiotic added. The cultures was grown overnight at 37°C in a shaker incubator. 2ml of bacterial culture was centrifuged for 5 minutes at 3000rpm. DNA was isolated from the pellets using the Qiagen plasmid mini kit. In brief, the pellet was resuspended in 0.3ml of P1 solution before adding 0.3ml P2 and incubated for 5 minutes at room temperature. Next, 0.3ml of P3 was added and the lysate incubated for 10 minutes on ice before centrifugation at 13000rpm for 10 minutes.

The supernatant was transferred to a new micro-tube, 0.8ml of isopropanol added and left on ice for 15 minutes followed by centrifugation at 13000rpm for 15 minutes. The supernatant was subsequently removed and 0.5ml of 70% alcohol added to wash the pellets before centrifugation at 13000rpm for 5 minutes. The supernatant was again removed and the pellets air dried at room temperature. Once the DNA pellets had become translucent they were re-suspended in 40µl TE (pH 8.0) and left to sit for one hour at room temperature before storage at -20°C. The quality of the DNA was determined by agarose gel electrophoresis.

2.2.2 Sequencing

2.2.2.1 Polymerase Chain Reaction

Standard PCR amplification reactions were performed using Invitrogen reagents. However, when amplifying long sequences, or if the sequence had a high GC content, the Expand long-range PCR kit (Roche) was used. Using Invitrogen reagents, PCR was performed with a final volume of 25ɥl comprised of:

25-50ng	template DNA
2.5ɥl of 2mM	dNTPs
5μmol	primer
2.5μl	10xPCR Invitrogen amplification Buffer;
7μl	dsH$_2$O;
0.25U	*Taq* DNA polymerase (Invitrogen)
10μl	1x enhancer solution.

The PCR reactions were carried out on a Gene Engine thermocycler (MJ Research Inc, USA).

2.2.2.2 Agarose gel electrophoresis

DNA/RNA integrity and PCR reaction efficacy were determined by Agarose gel electrophoresis. In general, agarose gels of 2% and 0.8% were used for resolution of small (~ 0.2kb-1kb) and large (>1kb) fragments respectively. Agarose gels were prepared by heating agarose in 1xTBE buffer. After dissolution of the agarose, SYBR Safe^{TM} was added to the gel at a final concentration of 1μl/100ml. Sample and 1kb DNA ladder were loaded using orange G loading buffer. Electrophoresis was performed using Hybaid Electro-4 gel tanks. The gel was submerged in 1xTBE buffer and a current of 80-100V was passed through the buffer to allow migration of the DNA through the gel. Fragments were visualized using by UV transillumination (Thistle Scientific) and images captured using a digital imager, Uvidoc (Uvitec).

2.2.3 Cloning PCR products

The cloning of PCR products was used to create a SOX11 expression plasmid contruction. Following purification, PCR products were cloned using the Qiagen PCR cloning kit (Qiagen). In brief, 1μl of pDrive cloning vector and 5μl ligation master mix was added to 4μl of purified PCR DNA gently mixed and incubated at 16°C overnight.

Transformation of PCR DNA was executed using a heat shock method as follows. 2μl of ligation reaction mix was added to 25μl of E-coli competent cells, incubated for 30 minutes, heat shocked at 42°C for 30 seconds and left on ice for one minute. Subsequently the ligation reaction/competent cell mix was added to 250μl of L-broth growth media already warmed to 37°C and left on a shaker to incubate for one hour.

The L-broth medium mix was plated on agar plates treated with chromogenic substrates IPTG and X-Gal to determine the efficacy of transformation - a technique termed 'blue/white screening'. Agar plates were made by heating agar to 50°C, adding appropriate antibiotics (1μl/ml of ampicillin [100mg/ml stock]), poured into Petri dishes and briefly dried in an oven. 40μl of IPTG (100mM) and X/Gal (40 mg/ml) was spread over each plate before the L-broth medium transformation mix was plated and left to incubate in an inverted position at 37°C overnight. As approximately half of the cloned PCR product colonies should contain the mutant allele, DNA from 10 colonies of each variant was purified. The white colonies which indicated no production of the enzyme β-galactosidase and hence successful incorporation of plasmid DNA were subsequently cultured in L-broth, pelleted and

DNA isolated using the GenElute^{TM} Plasmid Purification kit (Sigma-Aldrich) according to manufacturer's instructions.

The cloned PCR product DNA was directly sequenced using the Qiagen PCR cloning kit T7 promoter primer. The sequencing reaction was performed using the BigDye$^®$ Terminator Ready Reaction Mix v3.1 with a final volume of 10ul comprising of 1μl BDv3.1, 1μl primer 3.2μM, 2μl cloned DNA and 6μl dsH$_2$O. Primer details are presented in table2.2. Sequencing reactions were performed on a MJ Research Peltier Thermal Cycler using following cycling conditions: 96°C -1min, 96°C -10s, 50°C -5s 60°C -4 min times 25 cycles; 4°C -10min. Following the sequencing reaction, products were precipitated in an Ethanol/EDTA solution and run on an Applied Biosystems 3730 DNA analyser.

2.2.4 Reverse-transcriptase PCR

2.2.4.1 RNA preparation

RNA was extracted using RNeasy Mini Kit (Qiagen) as per manufacturer's instructions. In brief, cell samples were thawed before adding 600µl RLT buffer (containing guanidine-thiocyanate to inactivate RNases hence ensuring purification of intact RNA) and homogenized in a QIA shredder spin column (Qiagen) spun for 2 minutes at 13000 rpm. An equal amount of 70% EtOH was added to the lysate, mixed well and applied to an RNeasy mini spin column spun at 10000 rpm for 15 seconds. The sample was then washed in 350µl RW1 buffer, spun at 10000 rpm for 15 seconds before 80µl of DNase1 incubation mix was dispensed directly onto the spin column membrane and left at room temperature for 15 minutes. The column was then again washed in RW1 buffer and 500µl RPE buffer added, spun for two minutes at 10000 rpm. RNA was then eluted in 30µl RNase-free water and stored at -70°C.

5 µl of the solution was lorded onto a standard (non-denaturing) 1.5 % agarose gel with 0.5xTBE buffer to check the amount and integrity of the RNA. Ethidium bromide (EtBr) is added to the gel to avoid the additional (potentially RNAse-prone) step of gel staining. Load a known amount of DNA in a neighboring lane to use as standard for determining the RNA concentration. Intact RNA should exhibit sharp band(s) of ribosomal RNA.

2.2.4.2 cDNA synthesis

RNA was reverse transcribed to cDNA with First Strand cDNA Kit (Roche) using oligo-p(dT)$_{15}$ primer. This technique produces single stranded RNA using AMV reverse transcriptase. The synthesis reactions were performed with a final volume of 20µl master mix comprised of:

2µl	10 x reaction buffer
4µl	25mM mgCl$_2$
2µl	10mM dNTPs
2µl	oligo-p(dT)$_{15}$ primer
1µl	RNase inhibitor
0.8µl	AMV reverse transcriptase
8.2µl	dsH$_2$O

The reactions were cycled on a thermal cycler (Pelter PTC-225) as follows: 25°C - 10min, 42°C -10min, 99°C -5min, 4°C -5 min. cDNA was stored at -20°C .

2.3 Immunofluorescence

2.3.1 Fixation of Brain Tissue

Adult mouse brains were submerged in a solution of 4% paraformaldehyde in 1×
PBS for 8-16 hours at 4°C on a rocking platform. The brains were then washed with
1×PBS and placed in a 30% sucrose:PBS solution for cryoprotection and left
overnight at 4°C with agitation until the brains sank. Brains were placed in OCT
(Lamb), positioned in the horizontal, coronal or sagittal plane, frozen on dry ice and
then stored at -80°C.

2.3.2 Sectioning of frozen brain tissue

Brains for cryostat sectioning were removed from storage at -80°C and equilibriated
at -20°C to -26°C for one hour in the cryostat. 10µm-thick sections were cut on the
cryostat and collected onto Superfrost Plus (BDH) slides. All sections were air-dried
prior to storage at -80°C with a dessicant until use. Prior to immunofluorescence,
slides were taken straight into ice cold actone for 7 minutes fixation.

2.3.3 Fluorescent endpoint

The sections were fixed in ice cold acetone for 5 minutes and air dried for 30 minutes, then washed in 0.1% Triton in phosphate-buffered saline (pH 7.4). Unless stated otherwise, all steps were carried out at room temperature. The sections were first incubated for 1 h in 2% donkey serum to reduce background staining. The sections were then placed in single/double antibody solutions in 2% donkey serum, and incubated in these solutions for overnight at 4 °C. The following antibody solutions and dilutions were used: 1/500 Sox11 rabbit anti mouse, 1/400 Npas3 goat anti mouse, 1/400 Dcx rabbit anti mouse, 1/400 Gfap rabbit anti mouse, 1/500 Nestin rabbit anti mouse, 1/400 Gpc2 goat anti mouse (Santa Cruz Biotechnology). Following several washes over the period of 1 h, the sections were incubated for 1h in appropriate combinations of 1:400 donkey anti-goat or -rabbit IgG secondary antibodiest, conjugated to Alexa Fluor 594 for red fluorescence, or to Alexa Fluor 488 or FITC for green fluorescence (the Alexa Fluor secondary antibodies were purchased from Invitrogen Life Technologies, Paisley, UK). Finally, after several washes, the sections were mounted on slides with Prolong antifade reagent with 4'6-diamidino-2-phenylindole (DAPI) (Invitrogen) and the coverslips were then sealed in nail varnish.

2.4 Luciferase Reporter Assay

2.4.1 An overview of the technique

Luciferase reporter assay systems are currently one of the best non-toxic, rapid and sensitive methods to measure gene expression. The assay is based on the detection of luciferase activity driven by a promoter/enhancer cloned upstream of the luciferase gene. Luciferase activity correlates with the transcription regulated by the cloned DNA regulatory elements as well as response to extracellular and intracellular signals. In the quantification of gene expression using firefly luciferase (pGL3-control/Basic vector), a second Rennila luciferase vector (pRL-TK vector) is commonly used as internal control co-transfected into mammalian cells. The Dual-Luciferase ® Reporter (DLR) Assay system integrates the assays of both Firefly and Renilla luciferase from the same sample by the sequential measurement of their cognate substrate luciferins respectively.

2.4.2 Construction of VGF promoter-Luciferase Reporter Vectors

2.4.2.1 Generating 5' VGF promoter fragment by Long-PCR

To create the reporter vector, PCR was carried out to amplify 2029kb 5' human VGF sequence containing promoter, exon1, intron1 and part of exon2 with the following primers. *HindIII* forward: GGCCCTCGAGGGAGGTTAGAAGGAGGGTCAGT and *XhoI* reverse: GGCCCCATGGCTACCGGCTCTTTATGCTCAGA. Using the Expand long-range PCR kit (Roche), a total volume of 16ųl comprised; 25ng template DNA; 25mM of

each dNTP; 5μmol primer; 1.7μl expand long-range Buffers 2 &3; 13.3μl dsH$_2$O and, 0.25U Invitrogen *Taq* DNA polymerase.

The cycling conditions for this PCR reaction consisted of an initial incubation for 5 mins at 94°C, followed by 9 cycles of 15 sec at 94°C, 1 min at 56°C and 4 min at 68°C followed by 20 cycles of 94°C for 1 min, 56 for 1 min and 68°C for 4 min with 10 sec increase per cycle. The PCR product was then digested by *HindIII and XhoI* restriction enzymes and purified with Qiagen PCR purification Kit. 1 μl of the purified digested product was then loaded on 1% agarose gel along with 1Kb DNA ladder (NEB, UK) for an approximate quantification.

2.4.2.2 Generating VGF-luciferase Reporter Vector

The pGL3-basic vector was kindly provided by Professor Rob van t' Hoff. 3µg of the modified pGL-3 basic vector was digested by 30 units of HindIII and 30 units of XhoI restriction enzymes (NEB, UK) with 3µl 10×NEbufferB, 1.5µl 20×BSA and deionized water up to 30µl at 37°C for 2 hours. The digestion mix was purified with Qiagen PCR purification kit and then dephosphorylated with shrimp Alkaline Phosphatase (SAP, USB Corporation, UK)

The SAP treatment was set up as following:

3µl   Shrimp Alkaline Phosphatase (1U/µl)

4µl   10 × Shrimp Alkaline Phosphatase reaction buffer

30µl   purified digested DNA (<3µg)

3µl   ddH2O

The reaction was incubated at 37°C for 30 minutes and inactive at 65°C for 15 minutes. The DNA from the SAP treatment was used directly as vector in the ligation reaction. The following formula was used to calculate the amount of insert and vector in the ligation reaction:

[{ Amount of vector(ng)* length of insert (kb)}/length of vector(kb)]* A

The molecular ratio of vector DNA to insert ('A' in the formula) is 1:3 for sticky ends when insert DNA and vector were approximately similar in length, otherwise ratio 1:1 or 1:2 was used.

In this study, the molar ratio of insert to vector was 1:2. Therefore, the reaction mix contained 100ng of 4.8kb vector, 300ng 2.0kb insert, 1µl to T4 DNA ligase (1U/µl,

Roche, UK), 1µl of 10× ligation buffer and water up to 10 µl. The ligation mix was incubated at 16°C for 16 hours. 2 µl of the ligation mix was added to 50 µl of Subcloning Efficiency TM DH5α competent cells (Invitrogen, UK). The transformation and the incubation of the agar plate were carried out as described above. Five clones were picked out to incubate in 5 mls LB broth at 37 °C overnight with constant shaking at 225rpm. After the same procedures for miniprep and quantification, the correct integration of promoter insert into plasmid DNA was confirmed by HindIII and XhoI double digestion. The plasmid with correct digestion products was sequenced with primers listed in table 2.2. The sequence reactions with these primers covered all the 4kb insertion and 100bp of luciferase gene.

2.4.3 Quantification of reporter vectors by Fluorimetry

DNA concentration was measured using Quant-iTTM PicoGreen® dsDNA Assay Kit on a Bio-TEK Synergy HT multiwell plate reader. First a standard curve was constructed using double-stranded λ DNA at known concentrations. DNA samples were analyzed in duplicate and their average concentrations calculated from the standard curve regression equation. Pico green assays (100μl at 20μg/ml) was added to each well of a black 96 well microtitre plate with clear bottom along 100μl of either DNA samples or DNA standards of known concentrations. The plate was read after incubation at room temperature for 5 minutes. The fluorimeter measured the relative absorption ratio of each sample at 360nm excitation and 460 emission wavelength to create a concentration value. Each well was read twice and a mean value caculated.

2.4.4 Transfection of the vectors into human cells.

All transient transfection experiments were carried out using the human HEK293 and SHSY-5Y cell lines. Cells were cultured in 75 cm^2 tissue culture flasks in alpha MEM medium (Invitrogen) supplemented with 10% fetal calf serum (FCS, Invitrogen), 50 IU/ml penicillin (Invitrogen) and 2 mM glutamine (Invitrogen). Medium was renewed every 3 days. Cells were incubated at 37°C in a humidified atmosphere of 5%CO_2.

Twenty-four hours prior to the transfection, cells were cultured in 24-well plates with the density of 4×10^4. On the day of transfection, cells with less than 50% confluence

were transfected using lipofectamine 2000 (invitrogen) with these sets of plasmids respectively:

1) VGF luciferase reporter vector, pRL-TK and pDEST40-FLNPAS3

2) VGF luciferase reporter vector, pRL-TK and pDEST40- ΔNPAS3

3) VGF luciferase reporter vector, pRL-TK and pDEST40-SOX5

4) VGF luciferase reporter vector, pRL-TK and pDEST40-SOX6

5) VGF luciferase reporter vector, pRL-TK and pDEST40-SOX5+SOX6

6) VGF luciferase reporter vector, pRL-TK and pDEST40-SOX9

7) VGF luciferase reporter vector, pRL-TK and pDEST40-SOX10

8) VGF luciferase reporter vector, pRL-TK and pDEST40-SOX9+10

9) VGF luciferase reporter vector, pRL-TK and pDEST40.
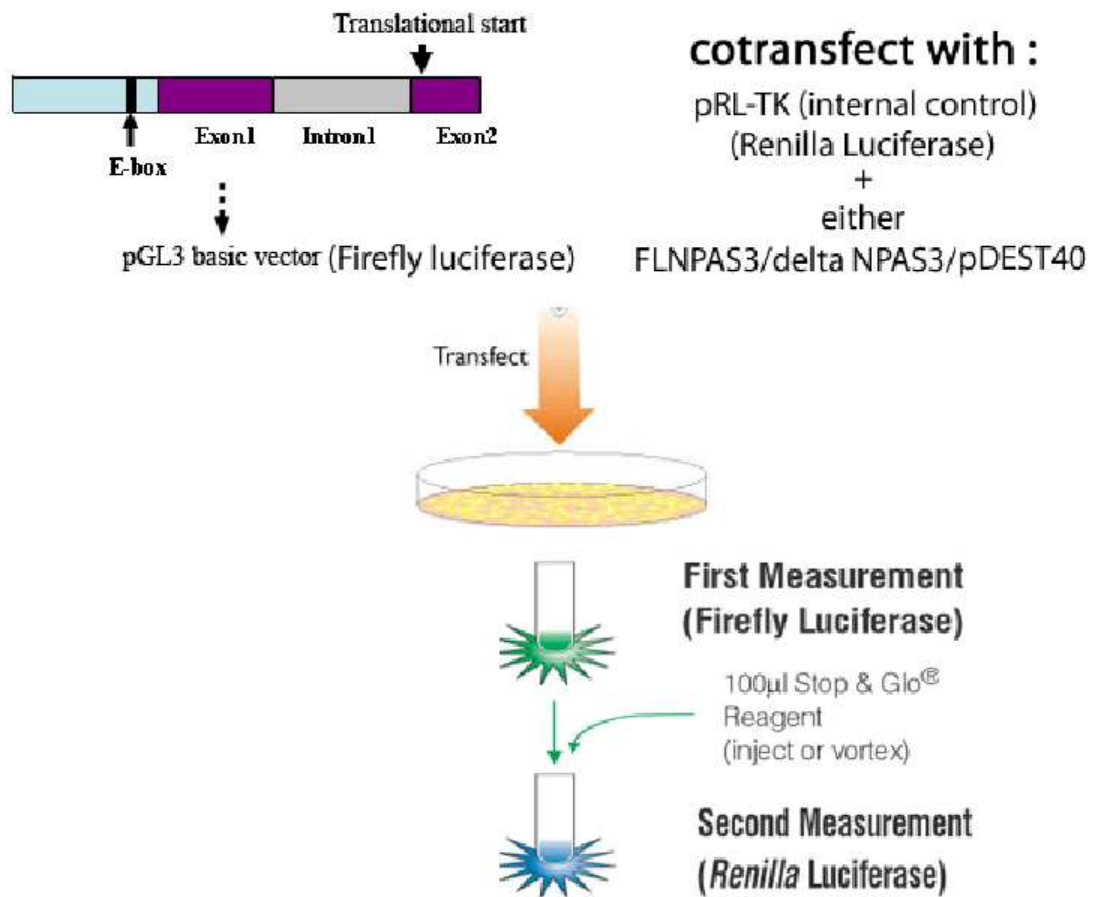
Figure 2.1 The procedure of a dual luciferase assay

2.4.5 Measurement of the luciferase activity

Twenty–four hours after transfection, cells were checked to be no more than 95% confluent. The growth medium was removed from the cultured cells and 2ml of phosphate buffered saline (PBS) was added to wash the surface of 24-well plate. The plate was swirled briefly to remove detached cells and residual growth medium. The rinse solution was completely removed before applying passive lysis buffer (PLB, Promega, UK). 80µl of 1×PLB was added into each well in the 24-well plate to completely cover the cell monolayer. The plate was wrapped with foil paper and stored at -80°C for at least 24 hours prior to performing the DLRTM Assay.

Luciferase assay regeant II (LAR II, Promega, UK) was prepared by resuspending the provided lyophilized Luciferase Assay Substrate in the 10ml of the supplied Luciferase Assay buffer II. Once the substrate and buffer had been mixed, LAR II was aliquoted and stored at -80°C. Frozen aliquot (80µl/sample, 2.5ml) of LAR II were thawed at room temperature before use. Stop & Glo$^®$ reagent was prepared by adding 1 volume of 50× Stop & Glo$^®$ substrate (Promega, UK) to 50 volumes of Stop & Glo$^®$ buffer. For each measurement (80µl/sample) 50 µl of 50× Stop & Glo$^®$ substrate was added to 2450 µl of Stop & Glo$^®$ buffer just before use**.** 40 µl of cell lysate from each sample was carefully transferred into 96-well clear bottom Castor plate without producing bubbles. 40 µl of LAR II and Stop & Glo$^®$ reagents were added sequentially into the same sample and the each luciferase acitivity was read by BIO-TEK Synergy HT multiwell plate reader.

To confirm the expression of NPAS3, ΔNPAS3, SOX5, SOX6, SOX9 and SOX10, total RNA of the cells were extracted and reverse transcribed into cDNA. QPCR

was carried out as decribed above. 18sRNA was used for the reference gene in the QPCR.

2.5   Illumina microarray analysis

2.5.1   SOX11 cloning

The SOX11 gene was cloned using the Invitrogen Gateway system. The human *SOX11* open reading frame was amplified from SH-SY5Y cell lines using SOX11 primers (table 2.1) containing attB sites and then cloned into the intermediate vector pDONR and then, subsequently, into the pDEST40 mammalian expression vector. Sequence verification of the clone was performed using a BigDye Terminator Cycle Sequencing Kit.

2.5.2   Transient transfection of SOX11 gene into HEK293 cells

HEK293 cells were cultured in DMEM (Invitrogen, Carsbad, CA) supplemented with 10% fetal bovine serum (Lab Tech Int.) at 37 ^{0}C in an atmosphere of 5% CO_2 in humidified air. The culture medium was replaced by OPTI-MEM (OMEM) and then the HEK293 cells were transiently transfected by pDEST40-SOX11 or control plasmid (pDEST40) using lipofectamine^{TM} 2000 transfection kit (Invitrogen) according to the manufacturer's instructions. After 4-6 hours, the transfection medium was replaced with standard culture medium. Cells were removed using trypsin/versene at 24 hours post-transfection, washed in cold PBS and then frozen at -80°C.

SH-SY5Y cells were also cultured and transiently transfected in the same manner for the western blot experiments.

2.5.3 Transient tranfection SOXD/E into HEK293 cells

SOXD/E expression constructs were generously gifted by Chuanju Liu (SOX5/SOX6/SOX9) and Fabien Murisier/Friedrich Beerman (SOX10). All SOXD/E microarray experiments were carried out as transient transfections of HEK293 cells using Optimem/Lipofectamine 2000/plasmid DNA (Invitrogen) incubation for 6 hours followed by 24 hours in standard culture conditions. HEK293 cells were maintained in DMEM supplemented with 10% Foetal Bovine Serum.

2.5.4 Preparation of stable FLNPAS3/ΔNPAS3 overexpression sample

The full-length NPAS3 open reading frame (acc. NM_001164749) cloned into the pCDNA expression plasmid was a gift from Dr. Nicholas Brandon, Merck, Sharpe and Dohme, now at Wyeth. This was transferred into a similar TET-inducible expression plasmid (pT-REx-DEST30, Invitrogen, UK) using a restriction digest, Gateway (Invitrogen) cloning linker ligation and the BP/LR reactions (Invitrogen). The truncated form, ΔNPAS3, was generated by cleavage and removal of sequence between internal and multiple cloning site XhoI sites thus deleting the second PAS domain and the putative transactivation domain. After linearisation and transfection of FLNPAS3 and ΔNPAS3 plasmids into HEK293 [T-REx-293] cells (Invitrogen), selection for stable integration was achieved with Geneticin and Blasticidin; the latter to maintain the activity of the TET repressor-expressing pcDNA-6/TR plasmid already present within the cell line.

2.5.5 In vitro circadian induction by serum shock method

For circadian induction, cells were washed in DMEM alone and then maintained in the same for 36 hours. In order to confirm the success of circadian rhythm induction, at zero hour time-point, HEK293 cells were shifted into a DMEM with 50% horse serum for 2 hours incubation. At +2 hours, HEK293 cells were washed with serum free DMEM and cultured with the same medium for the remaining experiment. Cell samples were collected every 4 hours. Gene expression levels of several circadian genes were determined by RT-PCR and Agarose gel electrophoresis. DNA binds were scaned by BIO-TEK Synergy HT soft ware.

For microarray analysis, at the zero hour time-point, the cell medium was replaced with DMEM supplemented with 50% horse serum plus tetracycline in order to induce circadian cycling and NPAS3 over-expression (Balsalobre et al., 1998; Huang et al., 2009). At +2 hours, cells were washed with DMEM and then incubated with the same plus tetracycline for the remaining period of the experiment. Cells were collected at either +12 or +24 hours and frozen as above. In order

5% Fetal Calf Serum
6 days

50% Horse Serum

Time= 0

Incubated for 2 h

Serum Free Medium

Samples are collected at
different time points
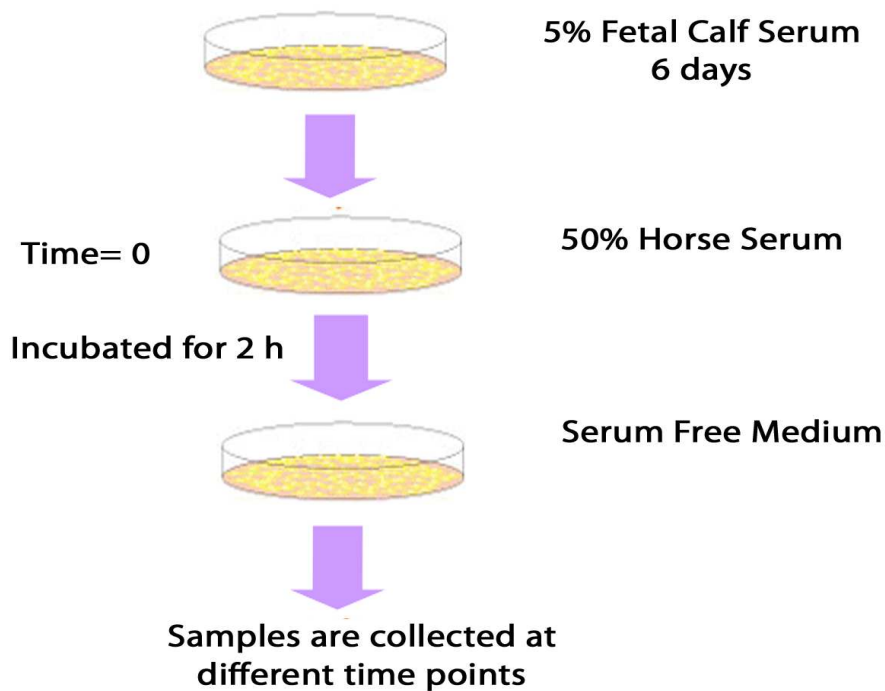
Figure 2.2 The induction of circadian rhythm in cultured cells by serum shock method.

All experimental cells were removed from flasks by incubation with trypsin/versene, washed in cold phosphate buffered saline and then immediately frozen at -80°C.

2.5.6 Total RNA extraction

Extraction of total RNA from transfected and control cells was performed with the mini RNeasy Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The purity and integrity of each total RNA sample was monitored using the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). The ratio of A_{260} to A_{280} values of each sample, an effective measure of RNA purity, fell in the range of 1.8-2.1 indicating high purity. The RNA integrity number (RIN) of each

sample was also evaluated using the Agilent 2100 bioanalyzer. Only RNA samples which demonstrated RIN values above 9.5 were used for probe synthesis purposes.

2.5.7 cRNA probe preparation

Biotinylated cRNA was prepared using the Illumina total Prep RNA application Kits (Ambion. Inc., Austin, TX) according to the manufacturer's direction starting with 100ng total RNA. The size of cRNA was evaluated using Agilent 2100 bioanalyzer. The cRNA profile of each sample was distributed from 250-5500 bp with most of the cRNA at 1000-1500 bp. All cRNA samples passed the quality filter and met our criteria for inclusion into further Illumina Beadarray analysis.

2.5.8 Illumina microarray

For NPAS3 experiments parental HEK293 [T-REx-293] cells were used as negative controls because stably integrated FLNPAS3/ΔNPAS3 cell lines showed a low level of leaky transcription in the absence of tetracycline. In the SOX microarray studies, Quadruplicate transient transfections of pDEST40 plasmid were used as controls were used as shared negative controls compared to triplicate transfections with each of the SOX expression constructs. For all standard cell culture experiments with FLNPAS3/ΔNPAS3/parental negative control cell lines, duplicate biological samples were assessed.

An Illumina Beadstation platform was used in conjunction with Sentrix® HumanRef-8 v1 and v2 chips (Illumina, Inc., San Diego, CA) capable of examining expression of over 24,500 gene transcripts. Hybridization, washing and scanning were performed according to the Illumina BeadStation 500* manual (revision C) by experienced staff located within the Genetics Core of the Wellcome Trust Clinical Research Facility at the Western General Hospital, Edinburgh. Three control replicates and 3 *SOX11* transient transfection replicates were carried out on one Illumina BeadChip.

2.5.9 Analysis of microarray data

For the investigation of the SOX11 regulatory profile, expression differences between the cell lines were assessed using two Bioconductor (Gentleman et al., 2004) algorithms implemented in the statistical programming language, R. Genes differentially expressed between cell-line samples were identified using *limma* (this part of work was performed by Robert Kitchen) and *SAM (Signifacance analysis of Microarray)* (Tusher et al., 2001). The latter was implemented as part of the *BRB-ArrayTools (3.70)* freeware developed by the Biometric Research branch of the US National Cancer Institute (http://linus.nci.nih.gov/BRB-Arraytools.html). In both analyses, the Illumina probe profile expression data were log-transformed and normalised using quantile normalisation. For the analysis using the *limma* package, genes were defined as being differentially expressed after satisfying a minimum fold-change of ±1.5 and a maximum, Benjamini-Hochberg adjusted, p-value of 0.01. For the *SAM* analysis, the differentially expressed genes were selected at a maximum predicted false discovery rate of 0.01.

For NPAS3 and SOXD/E microarray analysis, microarray data analysis was carried out using BRB-ArrayTools 3.8.0 freeware and included normalisation and log transformation of raw data followed by hierarchical clustering of samples, identification of statistically significantly up- or down-regulated genes through Statistical Analysis of Microarrays (SAM).

Regulated genes were further categorised by bioinformatics tools such as *Ingenuity Pathway Analysis* (Ingenuity systems

) and *GeneCodis2* for particular gene ontologies, biological processes, and associated canonical pathways.

2.5.10 Confirmation by Q-RTPCR

Quantitative reverse transcriptase PCR (QPCR) was used to validate mircroarray results using the same set of RNA and also measure time-dependent changes in gene expression levels due to Sox11 over-expression at 0h, 3h, 6h, 12h and 24h and 48h of transient transfection. Briefly, the total RNA of HEK293 cells were purified with RNeasy columns (Qiagen). This RNA served as template for oligo-dT-primed cDNA synthesis with reverse Transcriptase (Roche). QPCR was performed with SYBR green QPCR Master Mix (Invitrogen) and a Real-Time QPCR machine (BIO-RAD). For relative quantification of mRNA expression, geometric means were calculated using the comparative double delta method described previously (Dracheva et al, 2005). Primers used in QPCR were designed using Primer3 Software and sequences are included in table 2,1. The gene expression levels of housekeeping gene (*GAPDH*) in SOX11 overexpressing cells and control cells are similar, so this gene was selected as the endogenous references in this QPCR study. Sample concentrations were normalized to *GAPDH* according to the respective ratios of *GAPDH* levels per HEK293 cell sample, with 3 replicates per time point. Student's T-test was used to determine significant at each time point and changes of $P<0.05$ were considered significant.

In the FL/ΔNPAS3 microarray studies, as GAPDH was found upregulated by FL/ΔNPAS3 when comparing its expression in control cells, it could not be used as a reference gene. The expression levels of 18sRNA, one housekeeping gene, are similar in FL/ΔNPAS3 overexpressing and control cells, so it was selected as the endogenous references in the confirmation of genes targeted by FL/ΔNPAS3 by QPCR assay. Sample concentrations were normalized. Three experimental replicates were used. Student's T-test was used to determine significant at each time point and changes of $P<0.05$ were considered significant.

2.5.11 Confirmation by Western

Samples prepared from HEK293 cells and SH-SY5Y cells were subjected to SDS-PAGE gel electrophoresis (NuPAGE® Novex® Tris-Acetate gels). Protein was transferred to 0.2 μm Polyvinylidene Difluoride (PVDF) membrane (Invitrogen), blocked in 5% dried milk powder in 50 mM Tris-HCl, pH 7.5, 15 mM NaCl, 0.5% Tween-20 (TBST), washed briefly in TBST several times for 1 hour and incubated in primary antibody solutions in 2% donkey serum, and incubated in these solutions for overnight at 4 °C. The following antibody solutions and dilutions were used: 1/1000 SOX11 rabbit anti human, 1/1000 YWHAZ rabbit anti human, 1/800 SCG2 goat anti human (Santa Cruz Biotechnology) and 1/800 TUBB3 rabbit anti human (Abcam®) Blots were then washed 3 times in TBST, incubated for 1 hour in anti-rabbit horse radish peroxidase-conjugated secondary antibody (1:1000 in TBST), washed 3 times in TBST and incubated in ECL plus Western blotting detection reagent for 1-5 minutes based on the manufacturer's instructions (GE Healthcare, Buckinghamshire, UK).

2.6   Bioinformatics

Blast: http://ncbi,nlm.nih.gov/BLAST/

BLAT: http://genome.ucsc.edu/cgi-bin/hgBlat

ClustalX: http://www.clustal.org/

Decipher: https://decipher.sanger.ac.uk/

Ensemble: http://www.ensembl.org/index.html

FinchTV : http://www.geospiza.com

Gen Bank: http://www.ncbi.nlm.nih.gov

GraphPad: http://www.graphpad.com/quickcalcs/contingency

HapMap: http://www.hapmap.org

Haploview: http://www.broad.mit.edu/mpg/haploview/

HWE calculator: http://www.changbioscience.com/genetics/hardy.html

Image J: http://rsb.info.nih.gov/ij/

PubMed: http://www.ncbi.nlm.nih.gov/Literature/

Illumina: http://www.illumina.com/.

Plink: http://pnug.mgh.harvard.edu/~purcell/plink

Repeat Masker: http://repeatmasker.genome.washington.edu/

OR calculator: http://www.hutchon.net/ConfidOR.htm

SDSC Biology workbench: http://workbench.sdsc.edu/

Splice Site Predication http://www.fruitfly.org/seq_tools/splice.html

SwissProt: http://www.expasy.ch/sprot/

UCSC: http://genome.ucsc.edu/

SigmaPlot: http://www.sigmaplot.com/

CHAPTER THREE

3 Transcriptional regulation by a psychiatric candidate gene- NPAS3

3.1 Introduction

In this chapter, I will use immunofluorescence and gene microarrays to explore the function of NPAS3. As the *Npas3* deficient animal model shows a deficit in neurogenesis, NPAS3 was suggested to be involved in the regulation of new neuron production. In order to determine the spatio-temporal contribution of NPAS3 to adult neurogenesis, co-staining with several known neurogenesis markers in the adult mouse hippocampus was carried out using immunofluorescence.

Based on the role of NPAS3 in adult hippocampus neurogenesis and its contribution to schizophrenia, bipolar disorder and major depression, we supposed that the regulatory profile of NPAS3 might provide valuable clues to better understand the underlying pathological mechanisms of psychiatric illness.

The regulatory profiles of full-length (FLNPAS3) and artificially truncated (ΔNPAS3) forms of NPAS3, were indentified by Illumina microarray analysis in an *in vitro* system, the human embryonic kidney cell line (HEK293). ΔNPAS3 was generated by cleavage at an XhoI site which removes the second PAS domain and the putative transactivation domain. Thus, the properties of the bHLH domain and transactivation domain can be identified by comparing of the regulatory profiles of FLNPAS3 and ΔNPAS3. Biostatistical software (BRB), QPCR validation and gene ontological analysis (IPA and GeneCodis2) were carried out in this project.

3.2   Results

3.2.1 Npas3 expression in mouse brain supports a direct role in neurogenesis

The expression pattern of Npas3 in the adult mouse hippocampus was investigated using immunofluorescence microscopy. The sections from frozen mouse brain were cut using a Thermo cryostat. The following antibody solutions and dilutions were used: 1/400 Npas3 goat anti mouse, 1/400 Dcx rabbit anti mouse, 1/400 Gfap rabbit anti mouse, 1/500 Nestin rabbit anti mouse, (Santa Cruz Biotechnology). Npas3 is strongly expressed in the sub-granular zone of the dentate gyrus with processes radiating into the granule cell layer proper (Figure 3.1). Npas3 has a lower expression level in other brain regions, including ependymal cells and axo-dendritic regions adjacent to the cell soma in many cortical neurons (data are not shown).

To define the specific stage at which Npas3 exerts its function during neurogenesis, several neurogenesis markers, including Dcx, Gfap and Nestin, were co-stained with Npas3 in the hippocampus in the adult mouse brain. Npas3 was found to colocalise with Dcx, but not other markers of neurogenesis in the adult mouse hippocampus. This suggests that NPAS3 might exert its function at the same stage in development as Dcx, which has a role in converting progenitors into mature granule cells. This finding may suggest a possible site of action for NPAS3 in psychiatric illness.
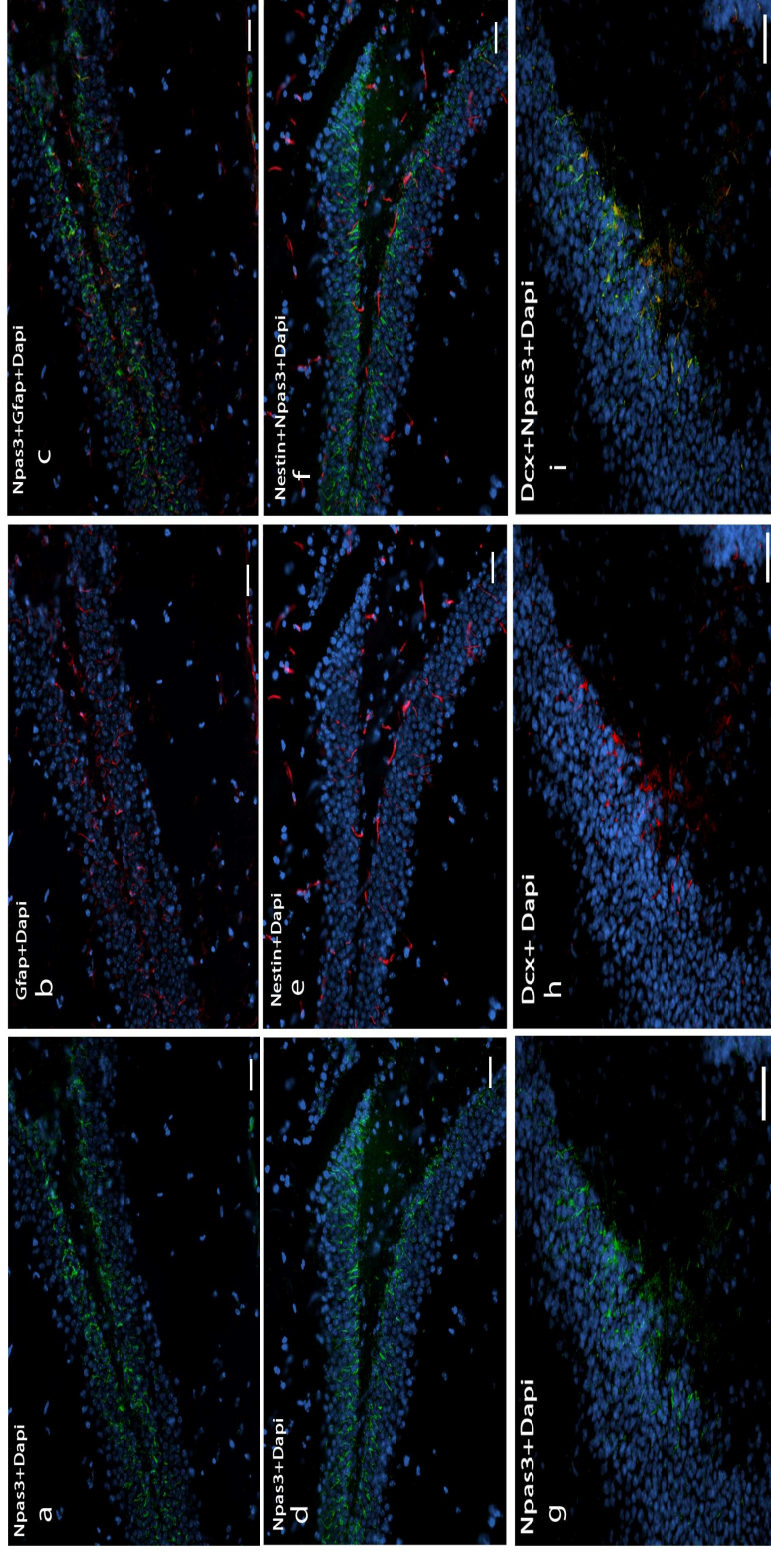
Figure 3.1 Co-immunofluorescence of Npas3 and neurogenesis stage markers in the dentate gyrus of mouse hippocampus

Npas3, Gfap, Nestin and Dcx protein expression was examined using immunofluorescence of frozen mouse brain sections. Anti-Npas3 was labeled with a FITC-conjugated secondary antibody (green). Npas3 (a, d and g) expression was mainly localized to the inner face/subgranular zone of the dentate gyrus. Compared to other sites of Npas3 expression (e.g., cortical neurons and ependymal cells), Npas3 shows no nuclear localisation in the subgranular zone but was distributed within projections that permeate through the granule cell layer. Anti-Gfap, -Nestin and -Dcx/Doublecortin antibodies labeled with a Texas-red conjugated secondary antibody (b,e,h) were used in co-immunofluorescence studies (c,f,i). No overlap was identified between Npas3 and Gfap/Nestin whereas Dcx colocalised with Npas3 in the subgranular zone (yellow, i), scale bar=80μm.

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3.2.2 NPAS3 target genes in standard culture conditions

Illumina microarray analysis was carried out to investigate the gene expression changes in HEK293 cells after over-expressing FLNPAS3 and ΔNPAS3. The fragments of FLNPAS3 and ΔNPAS3 were transferred into a similar TET-inducible expression plasmid and transfected into HEK293 [T-REx-293] cells (Invitrogen). Selection for stable integration was achieved with Geneticin and Blasticidin. Parental HEK293 [T-REx-293] cells were used as negative controls because stably integrated FLNPAS3/ΔNPAS3 cell lines showed a low level of leaky transcription in the absence of tetracycline. Duplicate biological samples were assessed for all standard cell culture experiments with FLNPAS3, ΔNPAS3 or parental negative control cell lines. Microarray probes synthesised from RNA extraction products were quantified using an Agilent Bioanalyzer to ensure high quality probes of equal quantity between experiments. Sentrix® HumanRef-8 v2 chips (capable of examining expression of over 24,500 gene transcripts) were used to detect gene expression profiles.

BRB analysis software was used to statistically analyse gene expression among the 22,177 well annotated RefSeq transcripts present in each array. Unsupervised hierarchical clustering of microarray gene expression profiles using centred correlation and average linkage revealed related transcriptional profiles for FLNPAS3 and ΔNPAS3 over-expression samples but distinct from control samples (Figure 3.2)

**Dendrogram for clustering experiments,
using uncentered correlation and complete linkage.**
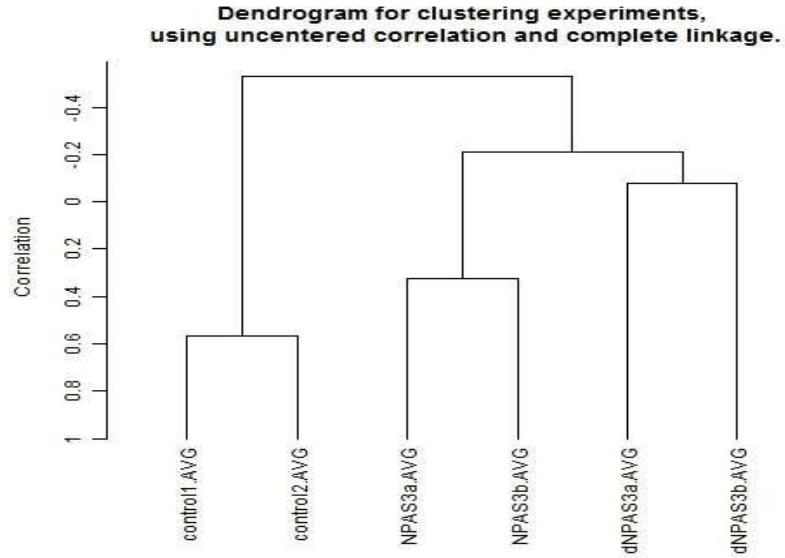
Figure 3.2 Hierarchical clustering dendrogram illustrating relationships between FLNPAS3, ΔNPAS3 and control HEK293 cell microarray data. Using centered correlation and average linkage, two distinct clusters corresponding to FL/ΔNPAS3 over-expressed and control cells were revealed. Thus FL/ΔNPAS3 over-expression induces a reproducible and global change in gene expression. Control1-2:AVG, HEK293 parental cell line treated with tetracycline; NPAS3a/b:AVG, Tetracycline-induced FLNPAS3 over-expression in a HEK293 cell line, ΔNPAS3a/b:AVG, Tetracycline-induced ΔNPAS3 over-expression in a HEK293 cell line.

3.2.3   Genes regulated by FLNPAS3 in standard conditions

Microarray data was filtered using the SAM algorithm (Significance Analysis of Microarrays) with a 0.01 target proportion of false discoveries and 100 permutations. 3476 genes were found to discriminate between FLNPAS3 and control. In a univariate comparison test (applying a random variance model) between control and FLNPAS3 microarray experiments, 282 genes showed >=1.5-fold up-regulation by FLNPAS3 and 359 genes were similarly down-regulated.

As the oligonucleotide probe for NPAS3 is complementary to 3'sequence, which is deleted in ΔNPAS3, NPAS3 was only found in the FLNPAS3 data list with the top up-regulated gene (69-fold) (Table 3.1). This result further confirms the over-expression of NPAS3 in the transfected cell samples.

Table 3.1 The 51 genes most up-regulated by FLNPAS3 (*NPAS3*) overexpression is shown for comparison) in standard cell culture conditions. Enrichment for glycolysis and hypoxia gene targets is evident in the latter group.

Illumina ID	Accession	Symbol	Description	Foldiff	Glycolysis	Hypoxia	Circadian
ILMN_1752550	NM_022123.1	NPAS3	neuronal PAS domain protein 3	62.49			
ILMN_1757497	NM_003378.2	VGF	VGF nerve growth factor inducible	2.92			
ILMN_1705153	NM_021076.2	NEFH	neurofilament, heavy polypeptide 200kDa	2.58			
ILMN_1716264	NM_014391.2	ANKRD1	ankyrin repeat domain 1 (cardiac muscle)	2.40			
ILMN_1660841	NM_001001870.1	MGC14376	hypothetical protein MGC14376	2.36			
ILMN_1730818	NM_174947.2	C19orf30	chromosome 19 open reading frame 30	2.33			
ILMN_1655741	NM_181617.1	KRTAP21-2	keratin associated protein 21-2	2.31			
ILMN_1789096	NM_152672.4	OSTalpha	organic solute transporter alpha	2.23			
ILMN_1734011	NM_001010866.1	RP13-15M17.2	hypothetical protein LOC199953	2.20			
ILMN_1725524	NM_003727.1	DNAH17	dynein, axonemal, heavy chain 17	2.15			
ILMN_1680085	NM_004881.2	TP53I3	tumor protein p53 inducible protein 3	2.12			
ILMN_1657148	NM_152480.1	C19orf23	chromosome 19 open reading frame 23	2.08			
ILMN_1684886	NM_013452.2	VCX	variable charge, X-linked	2.07			
ILMN_1669410	NM_001275.3	CHGA	chromogranin A (parathyroid secretory protein 1)	2.07			
ILMN_1695199	NM_003543.3	HIST1H4H	histone cluster 1, H4h	2.05			
ILMN_1667295	NM_138440.2	VASN	Vasorin	2.01			
ILMN_1810191	NM_003706.1	PLA2G4C	phospholipase A2, group IVC (cytosolic, calcium-independent)	1.98			
ILMN_1747506	NM_014681.4	DHX34	DEAH (Asp-Glu-Ala-His) box polypeptide 34	1.92			
ILMN_1754894	NM_174896.2	C1orf162	chromosome 1 open reading frame 162	1.92			
ILMN_1810514	NM_014655.1	SLC25A44	solute carrier family 25, member 44	1.92			
ILMN_1800425	NM_003047.2	SLC9A1	solute carrier family 9 (sodium/hydrogen exchanger), member 1	1.92			
ILMN_1754061	NM_022917.4	NOL6	nucleolar protein family 6 (RNA-associated)	1.92			
ILMN_1667194	NM_144684.1	ZNF480	zinc finger protein 480	1.89			
ILMN_1658800	NM_015695.2	BRPF3	bromodomain and PHD finger containing, 3	1.88			
ILMN_1666690	NM_052957.3	ACRC	acidic repeat containing	1.83			

Probe ID	Accession	Symbol	Description	Value	
ILMN_1669113	NM_012068.3	ATF5	activating transcription factor 5	1.83	
ILMN_1769931	NM_005066.1	SFPQ	splicing factor proline/glutamine-rich	1.82	
ILMN_1710544	NM_002589.2	PCDH7	protocadherin 7	1.80	
ILMN_1786024	NM_001018052.1	POLR3H	polymerase (RNA) III (DNA directed) polypeptide H (22.9kD)	1.80	
ILMN_1718977	NM_015675.2	GADD45B	growth arrest and DNA-damage-inducible, beta	1.80	yes
ILMN_1694731	NM_001287.3	CLCN7	chloride channel 7	1.79	
ILMN_1697268	NM_032048.2	EMILIN2	elastin microfibril interfacer 2	1.79	yes
ILMN_1757406	NM_005319.3	HIST1H1C	histone cluster 1, H1c	1.77	
ILMN_1725510	NM_014762.3	DHCR24	24-dehydrocholesterol reductase	1.75	
ILMN_1807719	NM_004937.2	CTNS	cystinosis, nephropathic	1.74	
ILMN_1784037	NM_001083621.1	ZBTB40	zinc finger and BTB domain containing 40	1.74	
ILMN_1761411	NM_024834.2	C10orf119	chromosome 10 open reading frame 119	1.73	
ILMN_1795671	NM_018269.1	ADI1	acireductone dioxygenase 1	1.72	
ILMN_1787826	NM_078483.2	SLC36A1	solute carrier family 36 (proton/amino acid symporter), member 1	1.72	
ILMN_1760650	NM_001384.4	DPH2	DPH2 homolog (S. cerevisiae)	1.72	
ILMN_1722059	NM_002967.2	SAFB	scaffold attachment factor B	1.71	
ILMN_1689959	NM_020170.3	NCLN	nicalin homolog (zebrafish)	1.70	
ILMN_1733847	NM_003857.2	GALR2	galanin receptor 2	1.70	
ILMN_1762037	NM_144582.2	TEX261	testis expressed 261	1.70	yes
ILMN_1665212	NM_014329.3	EDC4	enhancer of mRNA decapping 4	1.69	
ILMN_1754643	NM_022719.2	DGCR14	DiGeorge syndrome critical region gene 14	1.69	
ILMN_1797903	NM_014480.1	ZNF544	zinc finger protein 544	1.69	
ILMN_1743397	NM_178517.3	PIGW	phosphatidylinositol glycan anchor biosynthesis, class W	1.69	
ILMN_1737298	NM_005911.4	MAT2A	methionine adenosyltransferase II, alpha	1.67	
ILMN_1668012	NM_014251.1	SLC25A13	solute carrier family 25, member 13 (citrin)	1.67	
ILMN_1694837	NM_015590.2	GPATCH4	G patch domain containing 4	1.67	

Table 3.2 The 50 genes most down-regulated by FLNPAS3 in standard cell culture conditions. The enrichment for glycolysis and hypoxia genes is evident in the latter group.

Illumina id	Accession	Symbol	Description	Fold down-regulation	Metabolic	Hypoxia	Circadian
ILMN_1756417	NM_181726.2	ANKRD37	ankyrin repeat domain 37	-4.49		yes	
ILMN_1758164	NM_003155.2	STC1	stanniocalcin 1	-4.05		yes	
ILMN_1661599	NM_019058.2	DDIT4	DNA-damage-inducible transcript 4	-3.75		yes	
ILMN_1695880	NM_002317.3	LOX	lysyl oxidase	-3.53		yes	
ILMN_1659990	NM_013332.3	HIG2	hypoxia-inducible protein 2	-3.27		yes	
ILMN_1755974	NM_005165.2	ALDOC	aldolase C, fructose-bisphosphate	-3.21	glycolysis	yes	
ILMN_1728057	NM_145267.2	C6orf57	chromosome 6 open reading frame 57	-2.85			
ILMN_1748124	NM_198057.2	TSC22D3	TSC22 domain family, member 3	-2.77			yes
ILMN_1785703	NM_198271.2	LMOD3	leiomodin 3 (fetal)	-2.64			
ILMN_1809931	NM_006096.2	NDRG1	N-myc downstream regulated gene 1	-2.63		yes	
ILMN_1759092	NM_178124.3	CXorf40A	chromosome X open reading frame 40A	-2.60			yes
ILMN_1697448	NM_006472.2	TXNIP	thioredoxin interacting protein	-2.57			
ILMN_1658289	NM_032118.2	WDR54	WD repeat domain 54	-2.55			
ILMN_1715324	NM_014234.3	HSD17B8	hydroxysteroid (17-beta) dehydrogenase 8	-2.48			
ILMN_1724658	NM_004052.2	BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	-2.44		yes	
ILMN_1776602	NM_194431.1	RNASE4	ribonuclease, RNase A family, 4	-2.40		yes	
ILMN_1671791	NM_004563.2	PCK2	phosphoenolpyruvate carboxykinase 2	-2.39	glyc/TCA		
ILMN_1719476	XR_015982.1	LOC731786	similar to 60S ribosomal protein L32	-2.38			
ILMN_1693789	NM_001632.3	ALPP	alkaline phosphatase, placental (Regan isozyme)	-2.38			
ILMN_1723486	NM_000189.4	HK2	hexokinase 2	-2.36	glycolysis	yes	
ILMN_1666750	NM_145111.2	C7orf38	chromosome 7 open reading frame 38	-2.36			
ILMN_1809292	NM_032549.2	IMMP2L	IMP2 inner mitochondrial membrane peptidase-like	-2.34			
ILMN_1800796	NM_000997.3	RPL37	ribosomal protein L37	-2.33			
ILMN_1756338	NM_030971.3	SFXN3	sideroflexin 3	-2.32			

ILMN ID	Accession	Gene	Description	Value	Pathway	
ILMN_1696183	NM_005331.3	HBQ1	hemoglobin, theta 1	-2.31		
ILMN_1651610	XM_001126202.1	LOC730525	hypothetical protein LOC730525	-2.30		
ILMN_1780825	NM_006270.3	RRAS	related RAS viral (r-ras) oncogene homolog	-2.30		
ILMN_1693334	NM_000917.2	P4HA1	proline 4-hydroxylase, alpha polypeptide I	-2.29		yes
ILMN_1798581	NM_032485.4	MCM8	minichromosome maintenance complex component 8	-2.29		
ILMN_1671661	NM_016371.2	HSD17B7	hydroxysteroid (17-beta) dehydrogenase 7	-2.28		
ILMN_1753342	NM_002970.1	SAT1	spermidine/spermine N1-acetyltransferase 1	-2.27		
ILMN_1708778	NM_000050.4	ASS1	argininosuccinate synthetase 1	-2.25	urea	
ILMN_1679093	NM_016535.3	ZNF581	zinc finger protein 581	-2.24		
ILMN_1726460	NM_001034996.1	RPL14	ribosomal protein L14	-2.23		
ILMN_1692145	NM_021030.2	ZNF14	zinc finger protein 14	-2.23		
ILMN_1797668	NM_002196.2	INSM1	insulinoma-associated 1	-2.22		
ILMN_1787885	NM_024815.3	NUDT18	nudix (nucleoside diphosphate linked moiety X)-type motif 18	-2.22		
ILMN_1772492	NM_033412.1	MCART1	mitochondrial carrier triple repeat 1	-2.21		
ILMN_1809750	NM_198795.1	TDRD1	tudor domain containing 1	-2.20		
ILMN_1801584	NM_003467.2	CXCR4	chemokine (C-X-C motif) receptor 4	-2.17		yes
ILMN_1781388	NM_021965.3	PGM5	phosphoglucomutase 5	-2.16	glycolysis	
ILMN_1760727	NM_0010097577.1	ANG	angiogenin, ribonuclease, RNase A family, 5	-2.16		
ILMN_1765796	NM_001975.2	ENO2	enolase 2 (gamma, neuronal)	-2.15	glycolysis	yes
ILMN_1671766	NM_000505.3	F12	coagulation factor XII (Hageman factor)	-2.12		
ILMN_1809291	NM_004615.2	TSPAN7	tetraspanin 7	-2.12		
ILMN_1755075	NM_004508.2	IDI1	isopentenyl-diphosphate delta isomerase 1	-2.12		yes
ILMN_1680279	NM_018561.3	USP49	ubiquitin specific peptidase 49	-2.10		
ILMN_1770922	NM_018004.1	TMEM45A	transmembrane protein 45A	-2.10		yes
ILMN_1733559	NR_003287.1	LOC100008589	28S ribosomal RNA	-2.10		
ILMN_1782743	NM_015416.3	LETMD1	LETM1 domain containing 1	-2.10		

3.2.4 Comparison of FLNPAS3 and ΔNPAS3 regulation

In order to compare the regulatory activities of FLNPAS3 and ΔNPAS3, the fold change of genes regulated by both FLNPAS3 and ΔNPAS3 were plotted against each other in Excel. There was substantial correlation of target gene fold-change between FLNPAS3 and ΔNPAS3 cells in standard culture conditions, with linear regression r^2 values of 0.59 (up-regulated genes) and 0.78 (down-regulated genes). These results suggest that FLNPAS3 and ΔNPAS3 have the same transcription activity in the standard conditions. This result is very surprising because the ΔNPAS3 does not retain the PASB and transcriptional activation/ depression domain.

A

Inverse fold-change for shared up-regulated genes

$R^2 = 0.5899$

$y = 0.6028x + 0.2622$

Truncated (Delta) NPAS3

Full-length NPAS3

B

Fold-change for shared down-regulated genes

$R^2 = 0.781$

$y = 0.5996x + 0.5553$

Truncated (Delta) NPAS3

Full-length NPAS3
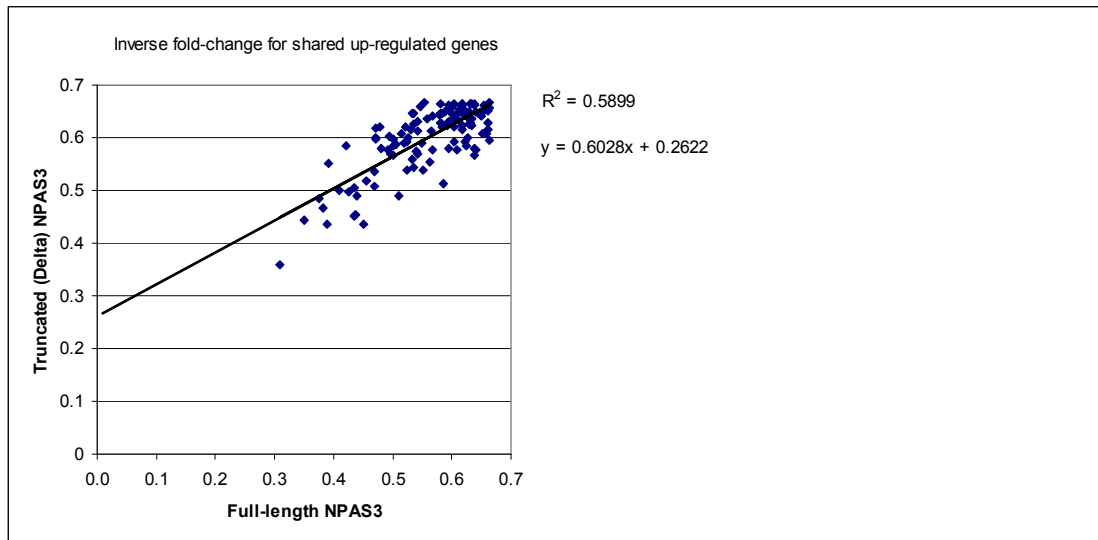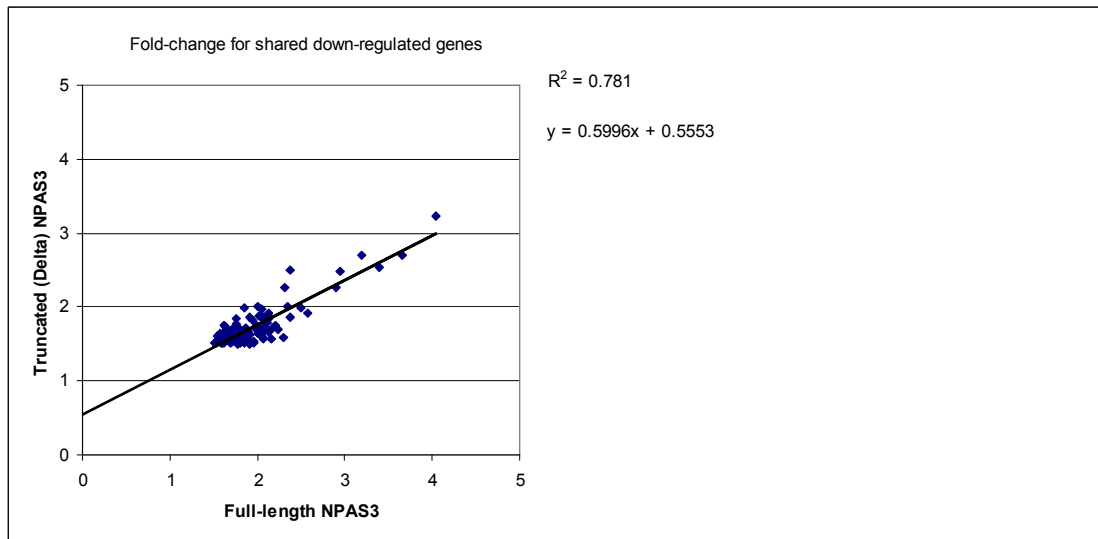
Figure 3.3 FLNPAS3 and ΔNPAS3 shared target gene regulation is highly correlated under standard cell culture conditions indicating minimal effect of truncation. A) up-regulated genes. B) down-regulated genes. All expression data is log transformed and normalised as part of the microarray analysis.

3.2.5 Confirmation of our FLNPAS3/ ΔNPAS3 microarray findings

In order to validate our microarray findings, QPCR analysis was carried out on several selected genes using the same RNA used in our microarray experiment. The QPCR reactions were performed in triplicate and Double delta method was used to calculate the gene expression level. *VGF*, *HK2*, *ENO2*, and *SOX11* showed similar fold changes in QPCR and microarray assays (Figure 3.4). These target genes were chosen because: a) they are in the top list of regulated genes, (VGF, HK2 and ENO2); b) they are relevant to the function of glycolysis and hypoxia (ENO2, HK2); c) they are associated with neurogenesis or psychiatric illness (VGF and SOX11).
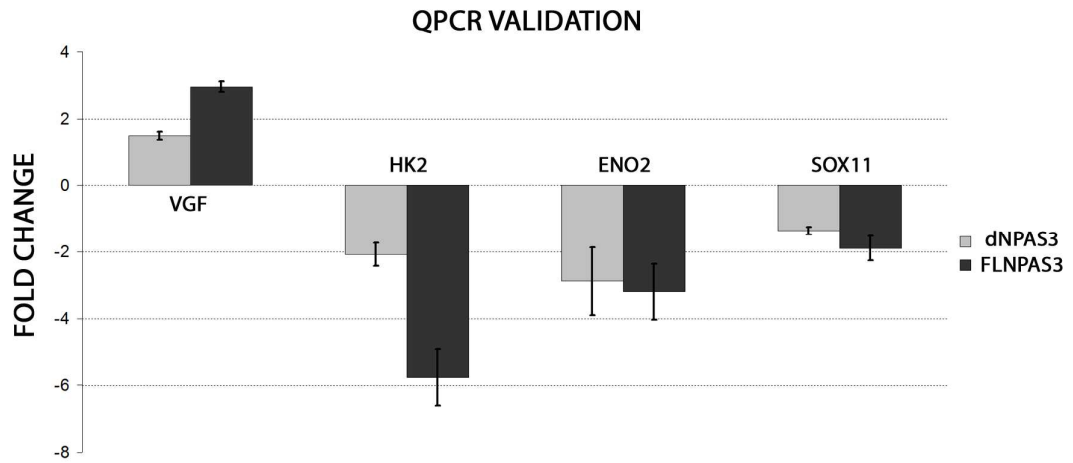
Figure 3.4 QPCR verification of microarray findings.

Expression of *VGF*, *HK2, ENO2* and *SOX11* was determined by triplicated QPCR analysis of the RNA used in the microarray assays. Gene 18sRNA was used to normalise data from FLNPAS3 and ∆NPAS3 (dNPAS3) over-expression samples and values are expressed as fold-change in gene expression in comparison to the control samples (Parental HEK293 [T-REx-293] cells were used as negative controls).

3.2.6 The relevant functions of up-regulated genes

The up-regulated set of genes were examined using the online tool GeneCodis2 (Nogales-Cadenas et al., 2009) to investigate relevant biological processes. Thirty-six of the 282 up-regulated genes (corrected hypergeometric p-value = 2.07 X 10^{-8}) were categorised by the gene ontology term 'transcription' (GO:0006350) being either transcription factors or DNA-binding proteins with regulatory function. An interacting network of transcription factors is a common feature of developmental biological programmes including neurogenesis.

3.2.7 The function analysis of down-regulated genes

3.2.7.1 Several SOX family genes are negatively regulated by NPAS3

In our microarray data list, three members of the SOX family of transcription factors, *SOX3*, *SOX11* and *SOX12,* were down-regulated by NPAS3. The expression levels of these genes were further analysed using QPCR on FLNPAS3 over-expressed and control HEK293 cells. Triplicated reaction and double delta method were performed (Figure 3.5). SOX3 is a marker of neural progenitor proliferation (Axell et al., 2009) and SOX11 is a master-regulator of neuronal differentiation (Azuma et al., 1999; Bergsland et al., 2006; Dy et al., 2008; Hargrave et al., 1997; Haslinger et al., 2009). Both the microarray and QPCR results provide evidence for a direct interaction of NPAS3 with SOX transcriptional networks.
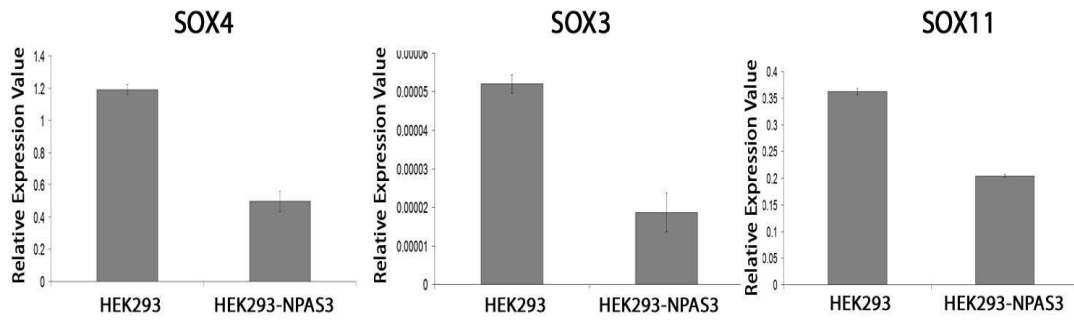
Figure 3.5   Three SOX family genes, SOX4, SOX3 and SOX11, were down-regulated in FLNPAS3 over-expressing HEK293 cells. Expression of these genes was verified by triplicated QPCR analysis of the same RNA used in the microarray assays.

3.2.7.2 Functional analysis of down-regulated genes by IPA and Genecodis2

In order to assess the principal biological functions or gene classes over-represented in the down-regulated genes, Ingenuity Pathway Analysis (IPA) and Genecodis2 were selected to run the microarray data.

The networks illustrate functional relationships between gene products based on published interactions in the literature. The scores for the networks were calculated based on the number of network eligible genes and the size of the network. This provides an estimate of the relevance of any particular network was to the total list of eligible genes. IPA identified five significant pathways (Table.3.7): glycolysis/gluconeogenesis (16 genes, p=6.14 X 10^{-6}), ERK/MAPK signalling (16 genes, p=8.67 X 10^{-3}), Neuregulin signalling (9 genes, p=2.35 X 10^{-2}), Notch signalling (6 genes, p=2.37 X 10^{-2}), and Circadian rhythm signalling (4 genes, p=4.16 X 10^{-2}). Full IPA analysis is presented in the Tables 3.3-3.7.

Using GeneCodis2, 8 genes of the glycolysis pathway (GO:0006096) were of greatest significance (corrected hypergeometric p-value = 5.21 X 10^{-7}) Figure 3.6 Additionally, 21 genes participating in the 'oxidation:reduction' biological process (GO:0055114) were also significantly down-regulated (p = 6.30 X 10^{-7}). Together, these findings strongly suggest that NPAS3 is involved in the regulation of anaerobic catabolism of glucose into pyruvate and, more generally, enzymatic redox processes.

Table 3.3 Ingenuity pathway analysis (IPA) identifies canonical pathway and networks regulated by over-expression of NPAS3.

ID	Associated network functions	Score
1	Carbohydrate Metabolism, Amino Acid Metabolism, Post-translational Modification	51
2	Protein Synthesis, Carbohydrate Metabolism, Small Molecular Biochemistry	46
3	Cellular Development, Nervous System Development and Function, Embryonic Development	40
4	Amino Acid metabolism, Small Molecular Biochemistry, Cellular Function and Maintaenance	39
5	Developmental Disorder, Organ Morphology, Reproductive System disease	39

Table 3.4 Top molecular and cellular functions (IPA)

Name	p-value	Molecules
Cell Death	7.00E-06 – 3.70E-02	168
Cellular Development	1.26E-05 – 3.84E-02	103
Cellular Growth and Proliferation	5.22E-06 – 4.08E-02	194
Protein Synthesis	1.84E-04 – 3.54E-02	49
Carbohydrate Metabolism	3.09E-04 – 3.96E-02	44

Table 3.5 Physiological System Development and Functions (IPA)

Name	p-value	Molecules
Nervous system development and function	7.36E-05 – 3.76E-02	77
Hair and skin development and function	7.21E-04 – 2.04E-02	17
Lymphoid tissure structure and development	9.20E-04 – 3.99E-02	91
Reproductive system development and function	9.20E-04 – 4.03E-02	25
Tissue Morphology	9.90E-04 – 3.76E-02	25

Table 3.6 Disease and disorders (IPA)

Name	p-value	Molecules
Genetics disorders	7.50E-06 – 4.03E-02	105
Neurological disease	7.50E-06 – 3.16E-02	226
Skeletal and muscular disorders	7.50E-06 – 4.03E-02	200
Cancer	4.90E-05 – 4.03E-02	235
Respiratory disease	1.53E-04 – 2.13E-02	51

Table3.7 Canonical pathways

Canonical pathway	Relevant genes	p-value	ratio
Glycolysis /Gluconeogenesis	ACYP1, AKR1A1, ALDH2, ALDOA, ALDOC, ENO2, GALK1,GAPDH, HK2, LDHA, PFKP, PGK1, PGM1, PGM5, PTGR1	6.14E-06	16/144
ERK/MAPK Signaling	ATF4, BAD, CREB5, EIF4EBP1, FOS, HSPB1, MAPK12, MYC, PLA2G4C, PPP1R3C, PPP2R3B, PRKCB, RAC3, RAPGEF1, RRAS	8.67E-03	16/192
Neuregulin Signaling	BAD, EGFR, ERBB3, MAPK12, MATK, MYC, NRG4, PRKCB, RRAS	2.35E-02	9/100
Notch Signaling	DLL3, HES1, HES7, NOTCH1, RBPJ, RFNG	2.37E-02	6/43
Circadian Rhythm Signaling	ATF, BHLHB2, CREB5, GRIN2C	4.16E-02	4/32

Glycolysis
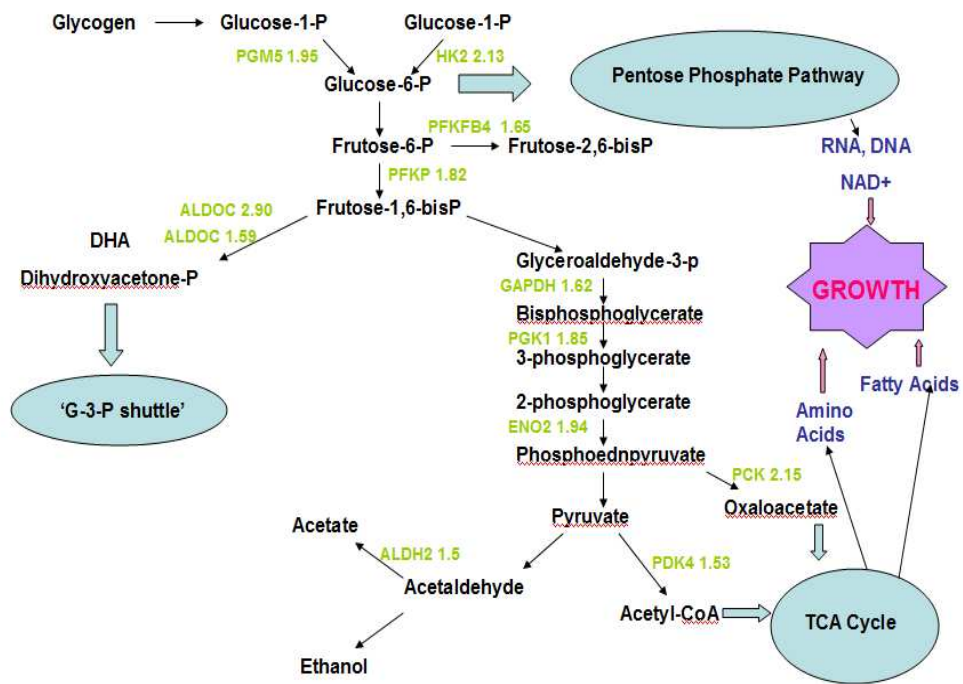
Figure 3.6 Most glycolytic enzyme genes are down-regulated in the presence of NPAS3. Fold change in expression is shown in green next to each gene. The up-regulation of Lactate Dehydrogenase (LDH) by NPAS2 is included for comparison.

3.2.8 Histone and zinc finger genes and chromosomal domain regulation by NPAS3

In order to detect whether some NPAS3 regulated genes lie in clusters on chromosomes, the microarray data were plotted according to chromosome order. Strikingly, seven histone genes were down-regulated by overexpression of NPAS3: *HIST2H2BF*, *HIST2H4A*, *HIST2H3C*, *HIST2H2AA3*, *HIST2H2BE*, *HIST2H2AC*, and *HIST2H2AB*. Closer inspection showed that many were present within a cluster on chromosome 1. This suggests that the transcriptional effects of NPAS3 might be indirect in some circumstances: mediated through the alteration of regional chromatin state rather than by direct promoter effects.
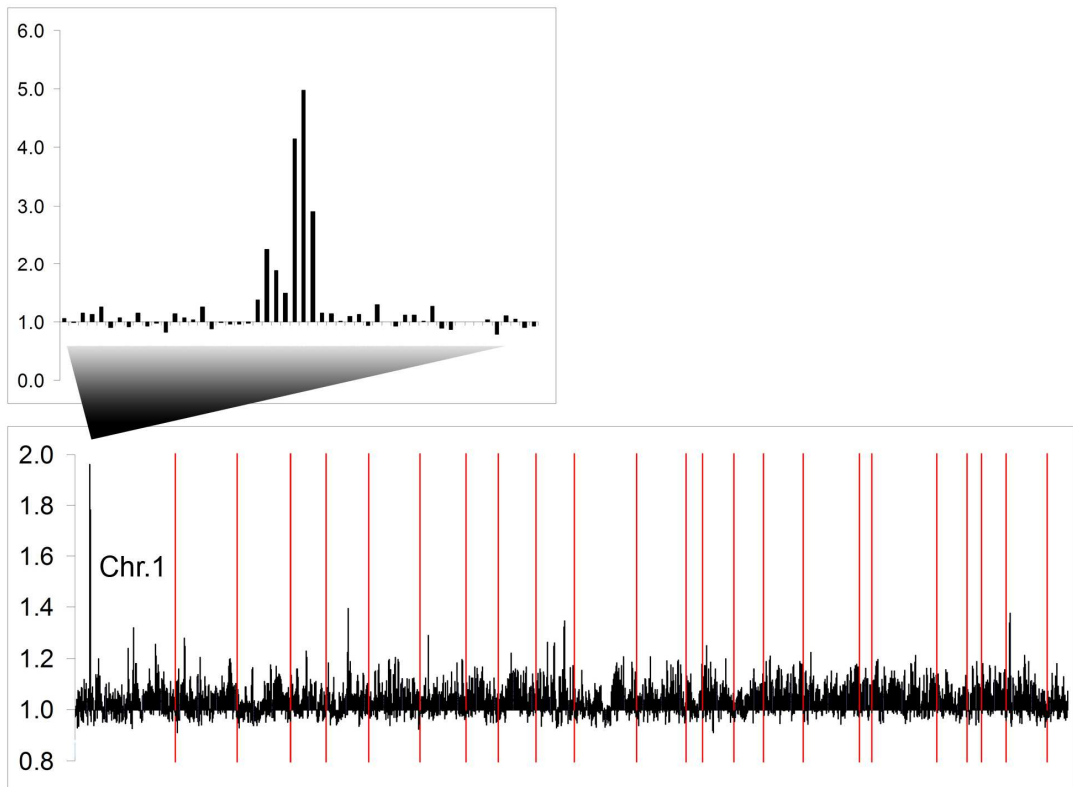
Figure 3.7 NPAS3 regulates the expression of a cluster of histone genes located on chromosome 1. A sliding window analysis of average expression change of genes ordered along each chromosome (Chromosome 1 to the extreme left, X then Y to the right. Red lines represent chromosome boundaries). Expression is displayed as a positive fold-change here but represents down-regulation of the genes in reality. The peak on chromosome 1 corresponds to a cluster of histone genes. This region is expanded above where fold-change down-regulation of individual genes is plotted. The down-regulated cluster consists of: *HIST2H2BF*, *LOC653604*, *HIST2H4A*, *HIST2H3C*, *HIST2H2AA3*, *HIST2H2BE*, *HIST2H2AC*, and *HIST2H2AB*.

3.3   Discussion

Previously, Erbel-Sieler and colleagues had reported that Npas3 was expressed in brain inhibitory interneurons (Erbel-Sieler et al., 2004). In our results, Npas3 was expressed in the inner site of dentate gyrus, where neurogenesis takes place. Strikingly, Npas3 is co-expressed with Doublecortin (Dcx) (not other neurogeneis markers) in the dentate gyrus (Figure 3.1 and 3.8), which supports an intermediate/maturation role in the neurogenic process (Kerjan et al., 2009).   This finding identifies the specific stages of adult neurogenesis where Npas3 may exert its function.
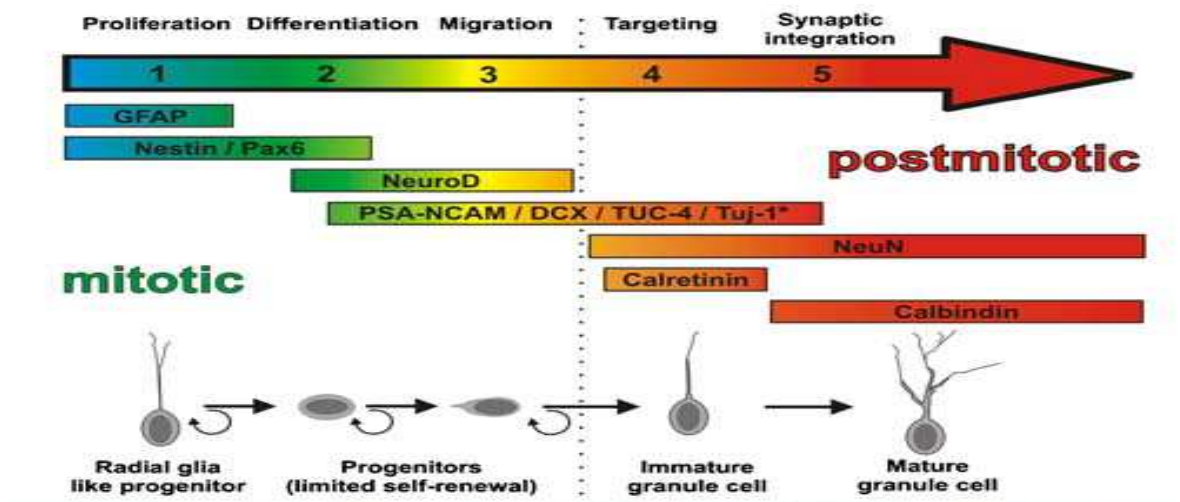
Figure 3.8 Dcx is a neurogenetic marker during the process of converting progenitors into immature granule cell. (Adapted from Halbach, 2007)

The regulatory profiles of FLNPAS3 and ΔNPAS3 are highly similar. Only one gene, *OST-alpha*, encoding an organic solute transporter, displayed down-regulation specific to FLNPAS3. These observations match those from a study of the related

bHLH transcription factor, ARNT, which indicated that dimerisation and transcriptional regulation functions were largely preserved in a C-terminal deletion form analogous to ΔNPAS3 - lacking the second PAS and transactivation domains (Reisz-Porszasz et al., 1994).

Glycolysis is the metabolic pathway that converts glucose into pyruvate thus releasing energy to form ATP and NADH. Glucose is the primary source of energy for the brain. Hence, glucose metabolism, or glycolysis, influences biological process in the brain. Dysfunction of glysolysis results in an inability for the cell to respire and therefore cause the apoptosis of the cell at an early stage. Glycolysis is deranged in a variety of disorders of the nervous system, including Huntington's disease (Oliveira, 2010), Alzheimer's disease (Brinton, 2008; Gibson et al., 1998) and depression (Talisman and Marzabadi, 2008). Our microarray findings show that many genes in the glycolysis pathway are negatively regulated by NPAS3. This may provide the underlying mechanism for the abnormalities in brain structure and adult neurogenesis found in psychiatric illness.

The microarray data also revealed another role for NPAS3 in the regulation of hypoxic response. Brain hypoxia has been proposed as a risk factor for the development of schizophrenia (Handford, 1975; Prabakaran et al., 2004; Van Erp et al., 2002). Under hypoxia conditions, energy metabolism is changed (Webster, 2003). In our microarray data, NPAS3 was shown to be involved in the reduced expression of many hypoxia responsive genes (Table 3.2). I hypothese that although the concentration of oxygen in the brain may remain normal, the expression of hypoxia

responsive genes can still be increased in the brain of individuals with schizophrenia, who have a deletion of *NPAS3* gene.

More details about the role of NPAS3 in glycolysis, circadian rhythm and hypoxia will be further discussed in discussion chapter (Chapter 7).

CHAPTER FOUR

4 Comparison of NPAS3 with SOX transcription factors

4.1 Introduction

The molecular processes regulating the proliferation of progenitor cells followed by their differentiation into functional neurons has been the subject of much study – particularly in relation to adult neurogenesis. As discussed in the chapter 3, NPAS3 colocalised with Dcx (doublecortin) in the dentate gyrus of adult mouse brain. This supports the hypothesis that NPAS3 might exert its function during the later stages of neurogeneis. If this is true then the original interpretation of the knockout model that Npas3 deletion causes a failure in proliferation is incorrect, instead it is possible that NPAS3 deletion causes a loss of neurogenesis (through apoptosis) during the differentiation phase. The aim of this section is to identify the genes regulated by NPAS3 for clues about its role in neurogenesis. To place NPAS3 in the context of known regulators of neuronal differentiation, the regulatory profiles of NPAS3 and the SOX family of transcription factors were compared.

4.1.1 Sox family genes

4.1.1.1 The structure of Sox family gene

Sry box-containing (Sox) family of transcription factors bind sequence-specifically to the minor groove in DNA by means of a conserved high-mobility group (HMG) domain. Sox genes are defined as containing the HMG box of a gene, *Sry*. *Sry* (Sex-determining Region Y) is a sex determining gene containing HMG-box on the Y chromosome in the placental mammals and marsupials (Wallis et al., 2008). The HMG box domain contains three alpha helices separated by loops (Thomas, 2001).

Proteins containing HMG domain are involved in the regulation of DNA-dependent processes, including transcription, replication and DNA repair through changing the conformation of chromatin (Thomas, 2001).

4.1.1.2 The classification of the Sox genes

Based on phylogenetic analysis of this HMG domain, Sox factors can be separated into several subgroups; A-H subgroup are represented in mouse and human (Bowles et al., 2000). Members of the Sox family of genes have diverse functions during the development and differentiation of multiple organ systems (Pevny and Placzek, 2005).

- SoxA: *SRY*
- SoxB1: *SOX1, SOX2, SOX3*
- SoxB2: *SOX14, SOX21*
- SoxC: *SOX4, SOX11, SOX12*
- SoxD: *SOX5, SOX6, SOX13*
- SoxE: *SOX8, SOX9, SOX10*
- SoxF: *SOX7, SOX17, SOX18*
- SoxG: *SOX15*
- SoxH: *SOX30*

4.1.1.3 The location of Sox genes

Several Sox genes are expressed in adult human brain (Table 4.1) and appear to regulate differentiation and cell fate in a cell type specific manner (Cahoy et al., 2008). Sox proteins are found to form stable transcription complexes with a wide range of transcription factor proteins, either to activate or repress transcription (Wilson and Koopman, 2002).

Table 4.1 Sox proteins and their locations in human brain

Sox proteins	Locations in brain	Partners
Sox2	Developing neurons, oligodendrocytes, astrocytes and high in adult astrocytes	
Sox5	Very high in developing oligodendrocytes and high in adult astrocytes	Sox6
Sox6	Very high in developing oligodendrocytes and high in adult astrocytes	CtBP1
Sox7	Adult neurons	
Sox9	High in astrocytes	SF1, HSP70
Sox10	High in oligodendrocytes	Pax3, EGR2, Krox-20
Sox11	Developing neurons/oligodendrocytes/astrocytes	Brn-1
Sox17	Adult neurons	
Sox21	Developing neurons/oligodendrocytes/astrocytes and high in adult astrocytes	

4.1.1.4 The functions of different Sox groups.

SRY (A-group Sox factor) initiates male sex determination. Mutations in this gene cause XY female (Swyer syndrome) (Shahid et al., 2008), while translocation of this gene to the X chromosome results in XX male syndrome (Wallis et al., 2008). SRY proteins are reported to link to dopamine-related diseases, including schizophrenia and Parkinson's disease through control of the concentrations of dopamine (Dewing et al., 2006).

B-group Sox factors play an important role in the processing of progenitors in neurogenesis (Figure 4.1). Sox B1 members, Sox1, Sox2 and Sox3 are expressed in most neuronal progenitors to maintain the pluripotent identity of progenitor cells and thus prevent cells from entering a differentiated state (Bylund et al., 2003). Sox21, a member of SoxB2 family, inhibits the function of SoxB1 members and initiates a programme of differentiation in cells (Sandberg et al., 2005).

Sox C members, including Sox4, Sox11 and Sox12, possibly play a key role during the regulation of later aspects of neurogenesis (Figure 4.1). They are mainly expressed in neuronal cells which have been committed to neuronal differentiation (Cheung et al., 2000). Sox4 and Sox11 regulate the establishment of pan-neuronal protein expression by inducing precocious expression of neuronal markers in self-renewing precursors (Bergsland et al., 2006).

SoxD and SoxE proteins regulate multiple stages of oligodendrocyte specification and differentiation (Stolt et al., 2006). Members of group E (Sox9 and Sox10) are required during specification and terminal differentiation in the oligodendrocyte precursors (Finzsch et al., 2008). Whereas, two D-group genes (Sox 5 and Sox6) negatively regulate Sox E genes, and so maintain the pluripotent identity of oligodendrocyte progenitors (Stolt et al., 2006) (Figure 4.1).
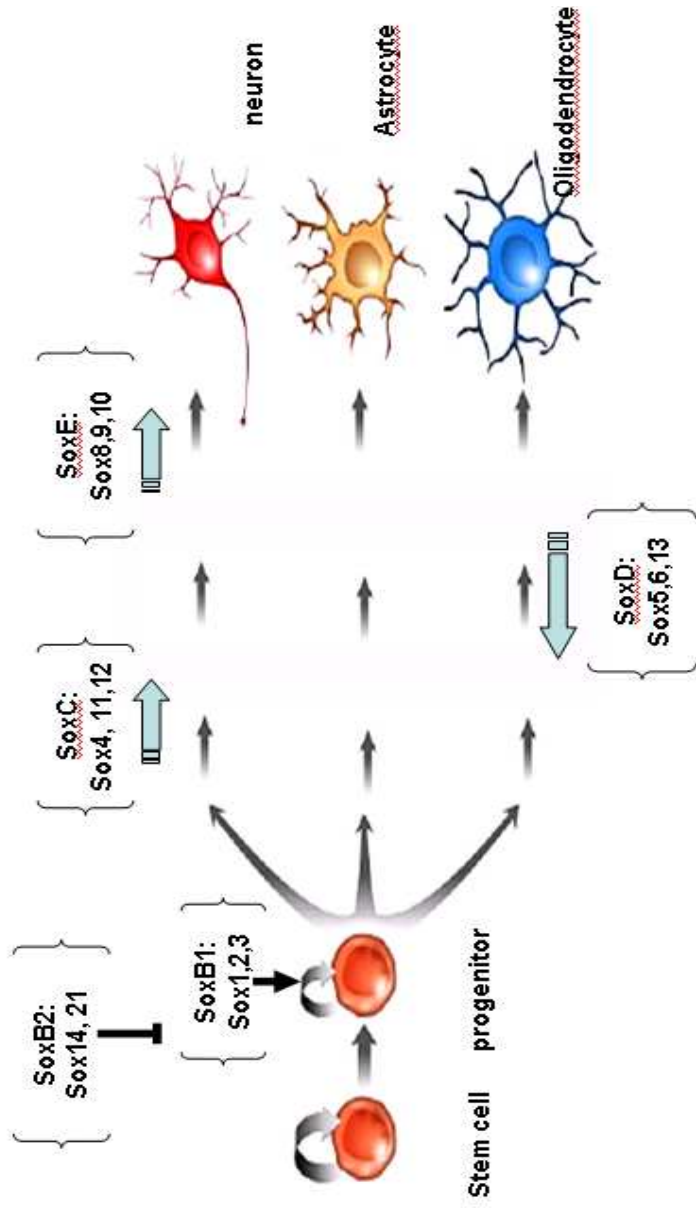
Figure 4.1 SOX memebers are involved in the process of neurogenesis Modified from Kiefer (2007).

4.1.1.5 The SOXD and SOXE memebers

The SOXD group is composed of 3 members, SOX5, SOX6 and SOX13. They contain two highly conserved functional domains: the HMG box in the C-terminal and a group-specific coiled-coil in the N-terminal ends, which mediates the dimerization of the SOXD proteins with other (Lefebvre et al., 1998). Both SOX5 and SOX6 are highly expressed in spermatids, neurons, oligodendrocytes and chondrocyte (Connor et al., 1995; Denny et al., 1992; Lefebvre et al., 1998; Stolt et al., 2006). SOX5 and SOX6 are co-express with SOXE proteins in chondrocytes, oligodendrocytes and melanocytes. The SOXD proteins have no known transactivation or transrepression domain, but they are involved in transcriptional regulation. The human *SOX5* is expressed as long transcripts (6 kb) in the tissues except testis. SOX5 and SOX6 not only can facilitate SOX9 in activating many genes' expression (Han and Lefebvre, 2008), but also can block the transactivation ablility of SOX9 and SOX10 by competing with these proteins for binding to the promoters of different markers (Stolt et al., 2006).
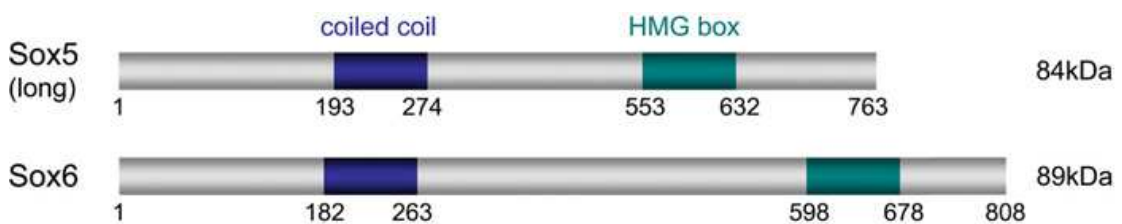
Figure 4.2 The structure of Sox5 and Sox6 proteins

Mutations in *SOX9* and *SOX10* genes result in the neural defects and neurological symptoms of Campomelic Dysplasis, Waardenburg-Hirschsprung and PCWH syndromes. In schizophrenia patients, increased DNA methylation of SOX10 was linked to oligodendrocyte dysfunction (Schlierf et al., 2006). It was also reported that association evidence provisionally supports roles for *SOX5* in metabolic side-effects of antipsychotic treatment (Adkins et al., ; Adkins et al., 2010) and *SOX10* in increased risk of psychiatric illness (Maeno et al., 2007).

SOXD and SOXE proteins regulate multiple stages of oligodendrocyte specification and differentiatiom. Members of group E (Sox9 and Sox10) are required during specification and terminal differentiation in the oligodendrocyte precursors. Whereas, two D-group genes (Sox 5 and Sox6) negatively regulate Sox E genes, and so maintain the pluripotent identity of oligodendrocyte progenitors. (Azim et al., 2009; Bergsland et al., 2006; Cheung and Briscoe, 2003; Hamada-Kanazawa et al., 2004; Haslinger et al., 2009; Kim et al., 2003; Kwan et al., 2008; Lefebvre, 2009; Prior and Walter, 1996). It was also reported that SOX5 is involved in the metabolic side-effects of antipsychiotic treatment (Adkins et al., ; Adkins et al., 2010) and SOX10 is associated with the increased risk of psychiatric illness (Maeno et al., 2007). All of these evidences support the possibility that NPAS3 might be relevant to the network composed by SOXD, SOXE and SOX11.

4.2　Aims of this study

We investigated the regulatory profile of SOX5 and SOX6 (SOX D group), SOX9 and SOX10 (SOX E group), and SOX11 by Illumina microarray. SOX factors were inserted into pDEST40 plasmids and transiently transfected into triplicate HEK293 cells using Optimem/Lipofectamine 2000. After 24 hours, (6 hour incubation with transfection mix followed 24 hrs in standard culture condition), the cells were collected and used for the probe synthesis. Quadruplicate transient transfections of pDEST40 plasmid were used as controls. Sentrix® HumanRef-8 v2 chips (capable of examining expression of over 24,500 gene transcripts) were used to detect gene expression profiles.

4.3   Result

4.3.1 Genes regulated by SOX5 in HEK293 cells

Illumina microarray analysis was carried out to investigate the consequences of SOX5 overexpression in HEK293 cells. BRB analysis software was used to analyse the expression of the 22,177 well annotated RefSeq transcripts present in each array. Microarray data was also filtered using the SAM algorithm (Significance Analysis of Microarrays). 688 genes were found to discriminate between cells overexpressing SOX5 and controls. The estimated false discovery rate among the 688 genes was 0.09. In a univariate comparison test (applying a random variance model) between control and SOX5 microarray experiments, 44 genes showed >=1.5-fold up-regulation by SOX5 and 359 genes were similarly down-regulated (Table 4.2)

Table 4.2: SOX5 top up-/down-regulated genes

Unique id	Clone	GB acc	Gene symbol	Description	Fold change
ILMN_9112	1940528	NM_003378	VGF	VGF nerve growth factor inducible (VGF), mRNA.	6.67
ILMN_22076	5290215	NM_014365	HSPB8	heat shock 22kDa protein 8 (HSPB8), mRNA.	3.57
ILMN_138334	2810270	NM_015675	GADD45B	growth arrest and DNA-damage-inducible, beta (GADD45B), mRNA.	3.45
ILMN_23390	4060209	NM_001275	CHGA	chromogranin A (parathyroid secretory protein 1) (CHGA), mRNA.	3.33
ILMN_13561	6180692	NM_016084	RASD1	RAS, dexamethasone-induced 1 (RASD1), mRNA.	3.13
ILMN_9306	6650520	NM_021076	NEFH	neurofilament, heavy polypeptide 200kDa (NEFH), mRNA.	3.13
ILMN_28586	5810687	NM_015193	ARC	activity-regulated cytoskeleton-associated protein (ARC), mRNA.	3.13
ILMN_13776	7510195	NM_016358	IRX4	iroquois homeobox protein 4 (IRX4), mRNA.	2.94
ILMN_16399	2850156	NM_006086	TUBB3	tubulin, beta 3 (TUBB3), mRNA.	2.94
ILMN_884	3400767	NM_032551	KISS1R	KISS1 receptor (KISS1R), mRNA.	2.86
ILMN_4188	160739	NM_003857	GALR2	galanin receptor 2 (GALR2), mRNA.	2.86
ILMN_16252	3170497	NM_001878	CRABP2	cellular retinoic acid binding protein 2 (CRABP2), mRNA.	2.78
ILMN_1557	6900204	NM_003407	ZFP36	zinc finger protein 36, C3H type, homolog (mouse) (ZFP36), mRNA.	2.56
ILMN_25193	4570427	NM_000735	CGA	Glycoprotein hormones, alpha polypeptide (CGA), mRNA.	2.50
ILMN_11654	2190008	NM_016639	TNFRSF12A	tumor necrosis factor receptor superfamily, member 12A (TNFRSF12A), mRNA.	2.44
ILMN_3329	3190551	NM_003820	TNFRSF14	tumor necrosis factor receptor superfamily, member 14 (herpesvirus entry mediator) (TNFRSF14), mRNA.	2.44
ILMN_7067	7160398	NM_031479	INHBE	inhibin, beta E (INHBE), mRNA.	2.38
ILMN_3304	6520500	NM_0010109985	MYBPHL	myosin binding protein H-like (MYBPHL), mRNA.	2.33
ILMN_26323	6840609	NM_001004354	MGC61598	similar to ankyrin-repeat protein Nrarp (MGC61598), mRNA.	2.27
ILMN_20689	3610601	NM_000076	CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2) (CDKN1C), mRNA.	2.22
ILMN_2040	4920634	NM_012109	C19orf4	chromosome 19 open reading frame 4 (C19orf4), mRNA.	2.22
ILMN_27341	4670025	NM_014279	OLFM1	olfactomedin 1 (OLFM1), transcript variant 1, mRNA.	2.22
ILMN_22135	940142	NM_003900	SQSTM1	sequestosome 1 (SQSTM1), mRNA.	2.17
ILMN_138248	160377	NM_002305	LGALS1	lectin, galactoside-binding, soluble, 1 (galectin 1) (LGALS1), mRNA.	2.17
ILMN_14210	630471	NM_002773	PRSS8	protease, serine, 8 (prostasin) (PRSS8), mRNA.	2.17
ILMN_22033	4570408	NM_001009820	SNRP70	small nuclear ribonucleoprotein 70kDa polypeptide (RNP antigen) (SNRP70), transcript variant 2, mRNA.	2.17

ILMN_29328	2940255	NM_003706	PLA2G4C	phospholipase A2, group IVC (cytosolic, calcium-independent) (PLA2G4C), mRNA.	2.13
ILMN_28176	2640170	NM_004352	CBLN1	cerebellin 1 precursor (CBLN1), mRNA.	2.13
ILMN_17452	6860041	NM_000160	GCGR	glucagon receptor (GCGR), mRNA.	2.08
ILMN_5188	5090044	NM_001251	CD68	CD68 antigen (CD68), mRNA.	2.08
ILMN_30180	6270241	NM_014587	SOX8	SRY (sex determining region Y)-box 8 (SOX8), mRNA.	2.04
ILMN_23858	5560446	NM_201525	GPR56	G protein-coupled receptor 56 (GPR56), transcript variant 3, mRNA.	2.04
ILMN_29799	4180201	NM_004821	HAND1	heart and neural crest derivatives expressed 1 (HAND1), mRNA.	2.04
ILMN_4647	1780358	NM_001029885	MGC10334	Hypothetical protein MGC10334 (MGC10334), mRNA.	2.04
ILMN_11525	4150338	NM_000041	APOE	apolipoprotein E (APOE), mRNA.	2.04
ILMN_3867	2190653	NM_173842	IL1RN	interleukin 1 receptor antagonist (IL1RN), transcript variant 1, mRNA.	2.04
ILMN_2789	360554	NM_000558	HBA1	hemoglobin, alpha 1 (HBA1), mRNA.	2.00
ILMN_17660	7210543	NM_032895	MGC14376	Hypothetical protein MGC14376 (MGC14376), transcript variant 1, mRNA.	2.00
ILMN_18321	2690326	NM_080618	CTCFL	CCCTC-binding factor (zinc finger protein)-like (CTCFL), mRNA.	2.00
ILMN_16763	1450132	NM_003727	DNAH17	dynein, axonemal, heavy polypeptide 17 (DNAH17), mRNA.	2.00
ILMN_20363	380731	NM_006000	TUBA1	tubulin, alpha 1 (testis specific) (TUBA1), mRNA.	2.00
ILMN_4021	4730768	NM_002290	LAMA4	laminin, alpha 4 (LAMA4), mRNA.	-2.07
ILMN_13166	1850132	NM_005407	SALL2	sal-like 2 (Drosophila) (SALL2), mRNA.	-2.35
ILMN_137098	2480497	NM_005868	BET1	BET1 homolog (S. cerevisiae) (BET1), mRNA.	-3.09

4.3.2 Genes regulated by SOX6 in HEK293 cells

The gene expression of SOX6 overexpressed and control HEK293 cells were identified by Illumina microarray and further used BRB analysis software to statistically analyse gene expression among the 22,177 well annotated RefSeq transcripts present in each array. 2085 genes were identified using SAM, the estimated false discovery rate among the 2085 genes was 0.01. 46 genes regulated by SOX6 with a fold change >=±1.5 are listed here. (Table 4.3)

Table 4.3: SOX6 top up-/down-regulated genes

Unique id	Clone	GB acc	Gene symbol	Description	Fold-change
ILMN_9112	1940528	NM_003378	VGF	VGF nerve growth factor inducible (VGF), mRNA.	5.88
ILMN_138334	2810270	NM_015675	GADD45B	growth arrest and DNA-damage-inducible, beta (GADD45B), mRNA.	3.85
ILMN_22076	5290215	NM_014365	HSPB8	heat shock 22kDa protein 8 (HSPB8), mRNA.	3.70
ILMN_13561	6180692	NM_016084	RASD1	RAS, dexamethasone-induced 1 (RASD1), mRNA.	3.12
ILMN_26323	6840609	NM_001004354	MGC61598	similar to ankyrin-repeat protein Nrarp (MGC61598), mRNA.	3.03
ILMN_28586	5810687	NM_015193	ARC	activity-regulated cytoskeleton-associated protein (ARC), mRNA.	2.86
ILMN_9306	6650520	NM_021076	NEFH	neurofilament, heavy polypeptide 200kDa (NEFH), mRNA.	2.86
ILMN_13776	7510195	NM_016358	IRX4	iroquois homeobox protein 4 (IRX4), mRNA.	2.86
ILMN_27341	4670025	NM_014279	OLFM1	olfactomedin 1 (OLFM1), transcript variant 1, mRNA.	2.86
ILMN_3329	3190551	NM_003820	TNFRSF14	tumor necrosis factor receptor superfamily, member 14 (herpesvirus entry mediator) (TNFRSF14), mRNA.	2.86
ILMN_23651	7160743	NM_000641	IL11	interleukin 11 (IL11), mRNA.	2.78
ILMN_11525	4150338	NM_000041	APOE	apolipoprotein E (APOE), mRNA.	2.78
ILMN_23858	5560446	NM_201525	GPR56	G protein-coupled receptor 56 (GPR56), transcript variant 3, mRNA.	2.70
ILMN_25193	4570427	NM_000735	CGA	Glycoprotein hormones, alpha polypeptide (CGA), mRNA.	2.56
ILMN_16252	3170497	NM_001878	CRABP2	cellular retinoic acid binding protein 2 (CRABP2), mRNA.	2.5
ILMN_4891	1010358	NM_014446	ITGB1BP3	integrin beta 1 binding protein 3 (ITGB1BP3), transcript variant 1, mRNA.	2.44
ILMN_4898	2100446	NM_004750	CRLF1	cytokine receptor-like factor 1 (CRLF1), mRNA.	2.44
ILMN_27030	4250379	NM_005252	FOS	Homo sapiens v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS), mRNA.	2.44
ILMN_3304	6520500	NM_001010985	MYBPHL	Homo sapiens myosin binding protein H-like (MYBPHL), mRNA.	2.44
ILMN_137300	6580575	NM_004917	KLK4	kallikrein 4 (prostase, enamel matrix, prostate) (KLK4), mRNA.	2.38
ILMN_884	3400767	NM_032551	KISS1R	KISS1 receptor (KISS1R), mRNA.	2.38
ILMN_2040	4920634	NM_012109	C19orf4	chromosome 19 open reading frame 4 (C19orf4), mRNA.	2.33
ILMN_16399	2850156	NM_006086	TUBB3	tubulin, beta 3 (TUBB3), mRNA.	2.33
ILMN_23390	4060209	NM_001275	CHGA	chromogranin A (parathyroid secretory protein 1) (CHGA), mRNA.	2.27
ILMN_30180	6270241	NM_014587	SOX8	SRY (sex determining region Y)-box 8 (SOX8), mRNA.	2.22
ILMN_11654	2190008	NM_016639	TNFRSF12A	tumor necrosis factor receptor superfamily, member 12A (TNFRSF12A), mRNA.	2.17

ILMN_24980	5050088	NM_005211	CSF1R	colony stimulating factor 1 receptor, formerly McDonough feline sarcoma viral (v-fms) oncogene homolog (CSF1R), mRNA.	2.17
ILMN_5188	5090044	NM_001251	CD68	CD68 antigen (CD68), mRNA.	2.17
ILMN_17452	6860041	NM_000160	GCGR	glucagon receptor (GCGR), mRNA.	2.17
ILMN_4188	160739	NM_003857	GALR2	Galanin receptor 2 (GALR2), mRNA.	2.17
ILMN_6548	6060431	NM_024027	COLEC11	collectin sub-family member 11 (COLEC11), transcript variant 1, mRNA.	2.13
ILMN_13603	6040086	NM_006732	FOSB	FBJ murine osteosarcoma viral oncogene homolog B (FOSB), mRNA.	2.13
ILMN_138248	160377	NM_002305	LGALS1	lectin, galactoside-binding, soluble, 1 (galectin 1) (LGALS1), mRNA.	2.13
ILMN_2789	360554	NM_000558	HBA1	hemoglobin, alpha 1 (HBA1), mRNA.	2.08
ILMN_20689	3610601	NM_000076	CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2) (CDKN1C), mRNA.	2.08
ILMN_20406	6510202	NM_004669	CLIC3	chloride intracellular channel 3 (CLIC3), mRNA.	2.08
ILMN_1557	6900204	NM_003407	ZFP36	zinc finger protein 36, C3H type, homolog (mouse) (ZFP36), mRNA.	2.08
ILMN_19535	4250735	NM_001001852	PIM3	pim-3 oncogene (PIM3), mRNA.	2.08
ILMN_12941	2900128	NM_001033026	C19orf6	chromosome 19 open reading frame 6 (C19orf6), transcript variant 1, mRNA.	2.00
ILMN_13166	1850132	NM_005407	SALL2	sal-like 2 (Drosophila) (SALL2), mRNA.	-2.01
ILMN_29428	4590242	NM_006755	TALDO1	transaldolase 1 (TALDO1), mRNA.	-2.10
ILMN_30176	2510296	NM_004257	TGFBRAP1	transforming growth factor, beta receptor associated protein 1 (TGFBRAP1), mRNA.	-2.21
ILMN_8399	1450703	NM_006934	SLC6A9	solute carrier family 6 (neurotransmitter transporter, glycine), member 9 (SLC6A9), transcript variant 1, mRNA.	-2.27
ILMN_24486	2810075	NM_006105	RAPGEF3	Rap guanine nucleotide exchange factor (GEF) 3 (RAPGEF3), mRNA.	-2.43
ILMN_21258	7380059	NM_033528	CDC2L2	cell division cycle 2-like 2 (PITSLRE proteins) (CDC2L2), transcript variant 3, mRNA.	-2.47
ILMN_20013	5550735	NM_017544	NKRF	NF-kappaB repressing factor (NKRF), mRNA.	-2.72

4.3.3 Gene regulated by SOX9 in HEK293 cells

To investigate the gene expression of SOX10 overexpressed and control HEK293 cells, Illumina microarray analysis was carried out. BRB analysis software was used to analyse the expression of the 22,177 well annotated RefSeq transcripts present in each array. Microarray data was also filtered using the SAM algorithm (Significance Analysis of Microarrays). 311 genes were identified using the software SAM, the estimated false discovery rate among the 311 genes was 0.001. 89 genes regulated by SOX9 with a fold change $>=\pm1.5$ are listed here.

Table 4.4: SOX9 top up-/down-regulated genes

Unique id	Clone	GB acc	Gene symbol	Description	Fold-change
ILMN_1096	6130181	NM_181611	KRTAP19-5	keratin associated protein 19-5 (KRTAP19-5), mRNA.	3.7
ILMN_17827	2350333	NM_003469	SCG2	secretogranin II (chromogranin C) (SCG2), mRNA.	2.94
ILMN_19455	4010301	NM_174896	C1orf162	chromosome 1 open reading frame 162 (C1orf162), mRNA.	2.78
ILMN_7829	5690131	NM_002557	OVGP1	oviductal glycoprotein 1, 120kDa (mucin 9, oviductin) (OVGP1), mRNA.	2.56
ILMN_26722	3520148	NM_006209	ENPP2	ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin) (ENPP2), mRNA.	2.27
ILMN_22033	4570408	NM_001009820	SNRP70	small nuclear ribonucleoprotein 70kDa polypeptide (RNP antigen) (SNRP70), transcript variant 2, mRNA.	2.13
ILMN_27652	5860301	NM_004071	CLK1	CDC-like kinase 1 (CLK1), transcript variant 1, mRNA.	2
ILMN_1498	7210504	NM_198538	SBSN	suprabasin (SBSN), mRNA.	2
ILMN_29328	2940255	NM_003706	PLA2G4C	phospholipase A2, group IVC (cytosolic, calcium-independent) (PLA2G4C), mRNA.	1.96
ILMN_2831	510176	NM_052872	IL17F	interleukin 17F (IL17F), mRNA.	1.96
ILMN_746	380754	NM_207014	WDR78	WD repeat domain 78 (WDR78), transcript variant 2, mRNA.	1.92
ILMN_19918	7100630	NM_182975	ZNF326	zinc finger protein 326 (ZNF326), transcript variant 3, mRNA.	1.89
ILMN_30163	4730195	NM_003543	HIST1H4H	histone 1, H4h (HIST1H4H), mRNA.	1.82
ILMN_12021	670474	NM_001407	CELSR3	cadherin, EGF LAG seven-pass G-type receptor 3 (flamingo homolog, Drosophila) (CELSR3), mRNA.	1.82
ILMN_4188	160739	NM_003857	GALR2	galanin receptor 2 (GALR2), mRNA.	1.79
ILMN_25508	1400731	NM_206919	ARL9	ADP-ribosylation factor-like 9 (ARL9), mRNA.	1.79
ILMN_20358	110411	NM_014270	SLC7A9	solute carrier family 7 (cationic amino acid transporter, y+ system), member 9 (SLC7A9), mRNA.	1.79
ILMN_22987	6200315	NM_014174	THYN1	thymocyte nuclear protein 1 (THYN1), transcript variant 1, mRNA.	1.75
ILMN_17622	3390075	NM_138720	HIST1H2BD	histone 1, H2bd (HIST1H2BD), transcript variant 2, mRNA.	1.75
ILMN_9895	4040767	NM_130897	DYNLRB2	dynein, light chain, roadblock-type 2 (DYNLRB2), mRNA.	1.72
ILMN_9116	60500	NM_017578	ROPN1	Ropporin, rhophilin associated protein 1 (ROPN1), mRNA.	1.72
ILMN_4387	7210035	NM_001008226	DKFZp666G057	hypothetical protein DKFZp666G057 (DKFZp666G057), mRNA.	1.72
ILMN_21875	50424	NM_020783	SYT4	synaptotagmin IV (SYT4), mRNA.	1.72
ILMN_137281	4540575	NM_005738	ARL4	ADP-ribosylation factor-like 4 (ARL4), transcript variant 1, mRNA.	1.72

ILMN ID	ID	Accession	Symbol	Description	Value
ILMN_7731	2570703	NM_0010013672	LOC400566	hypothetical gene supported by AK128660 (LOC400566), mRNA.	1.69
ILMN_7731	2570703	NM_0010013672	LOC400566	hypothetical gene supported by AK128660 (LOC400566), mRNA.	1.69
ILMN_27286	2140253	NM_001024646	CLK1	CDC-like kinase 1 (CLK1), transcript variant 2, mRNA.	1.69
ILMN_25028	5080474	NM_001063	TF	transferrin (TF), mRNA.	1.69
ILMN_14262	2480619	NM_170713	RASSF1	Ras association (RalGDS/AF-6) domain family 1 (RASSF1), transcript variant C, mRNA.	1.69
ILMN_14013	3310484	NM_032681	TRIM51	Tripartite motif-containing 51 (TRIM51), mRNA.	1.69
ILMN_5526	1340193	NM_001029869	PLAC8L1	PLAC8-like 1 (PLAC8L1), mRNA.	1.67
ILMN_28170	4290376	NM_004040	RHOB	ras homolog gene family, member B (RHOB), mRNA.	1.67
ILMN_8691	1740601	NM_001001342	BLOC1S2	biogenesis of lysosome-related organelles complex-1, subunit 2 (BLOC1S2), transcript variant 2, mRNA.	1.64
ILMN_27627	6280164	NM_181610	KRTAP19-4	keratin associated protein 19-4 (KRTAP19-4), mRNA.	1.64
ILMN_15226	6250327	NM_000785	CYP27B1	cytochrome P450, family 27, subfamily B, polypeptide 1 (CYP27B1), nuclear gene encoding mitochondrial protein, mRNA.	1.64
ILMN_1241	5900201	NM_207392	UNQ467	KIPV467 (UNQ467), mRNA.	1.64
ILMN_7366	3990091	NM_030580	ZNF34	zinc finger protein 34 (KOX 32) (ZNF34), mRNA.	1.61
ILMN_28424	6130328	NM_004861	GAL3ST1	galactose-3-O-sulfotransferase 1 (GAL3ST1), mRNA.	1.61
ILMN_24419	1300241	NM_025145	C10orf79	chromosome 10 open reading frame 79 (C10orf79), mRNA.	1.61
ILMN_22247	6590753	NM_001001661	ZNF425	zinc finger protein 425 (ZNF425), mRNA.	1.61
ILMN_20449	3130735	NM_001819	CHGB	chromogranin B (secretogranin 1) (CHGB), mRNA.	1.61
ILMN_11057	620722	NM_002896	RBM4	RNA binding motif protein 4 (RBM4), mRNA.	1.61
ILMN_8523	1010739	NM_013243	SCG3	secretogranin III (SCG3), mRNA.	1.59
ILMN_8297	3850242	NM_007182	RASSF1	Ras association (RalGDS/AF-6) domain family 1 (RASSF1), transcript variant A, mRNA.	1.59
ILMN_24927	2070309	NM_005968	HNRPM	heterogeneous nuclear ribonucleoprotein M (HNRPM), transcript variant 1, mRNA.	1.59
ILMN_23731	7000619	NM_002201	ISG20	interferon stimulated exonuclease gene 20kDa (ISG20), mRNA.	1.59
ILMN_14828	3190164	NM_031421	TTC25	tetratricopeptide repeat domain 25 (TTC25), mRNA.	1.59
ILMN_11266	870592	NM_182597	FLJ39575	hypothetical protein FLJ39575 (FLJ39575), mRNA.	1.59
ILMN_7061	1660653	NM_013376	SERTAD1	SERTA domain containing 1 (SERTAD1), mRNA.	1.56
ILMN_28352	4290433	NM_021107	MRPS12	mitochondrial ribosomal protein S12 (MRPS12), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA.	1.56
ILMN_23331	6370392	NM_152474	C19orf18	chromosome 19 open reading frame 18 (C19orf18), mRNA.	1.56

ILMN ID		NM Accession	Gene	Description	Value
ILMN_18732	2640497	NM_182541	TMEM31	transmembrane protein 31 (TMEM31), mRNA.	1.56
ILMN_23076	1110437	NM_138998	DDX39	DEAD (Asp-Glu-Ala-Asp) box polypeptide 39 (DDX39), transcript variant 2, mRNA.	1.54
ILMN_22365	5870093	NM_052813	CARD9	caspase recruitment domain family, member 9 (CARD9), mRNA.	1.54
ILMN_16021	1770538	NM_006727	CDH10	Cadherin 10, type 2 (T2-cadherin) (CDH10), mRNA.	1.54
ILMN_13703	5360520	NM_174898	LOC129530	hypothetical protein LOC129530 (LOC129530), mRNA.	1.54
ILMN_11269	2680161	NM_006584	CCT6B	chaperonin containing TCP1, subunit 6B (zeta 2) (CCT6B), mRNA.	1.54
ILMN_9700	130441	NM_032561	C22orf23	chromosome 22 open reading frame 23 (C22orf23), mRNA.	1.52
ILMN_26085	6650142	NM_003467	CXCR4	chemokine (C-X-C motif) receptor 4 (CXCR4), transcript variant 2, mRNA.	1.52
ILMN_24020	6370307	NM_025057	C14orf45	chromosome 14 open reading frame 45 (C14orf45), mRNA.	1.52
ILMN_22180	360114	NM_198175	NME1	non-metastatic cells 1, protein (NM23A) expressed in (NME1), transcript variant 1, mRNA.	1.52
ILMN_16651	2030746	NM_001976	ENO3	enolase 3 (beta, muscle) (ENO3), transcript variant 1, mRNA.	1.52
ILMN_26937	7650433	NM_005756	GPR64	G protein-coupled receptor 64 (GPR64), mRNA.	-1.5
ILMN_14229	540128	NM_005630	SLCO2A1	solute carrier organic anion transporter family, member 2A1 (SLCO2A1), mRNA.	-1.5
ILMN_4674	3190400	NM_005194	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta (CEBPB), mRNA.	-1.51
ILMN_24466	6220692	NM_003641	IFITM1	interferon induced transmembrane protein 1 (9-27) (IFITM1), mRNA.	-1.51
ILMN_1673	3290195	NM_025218	ULBP1	UL16 binding protein 1 (ULBP1), mRNA.	-1.51
ILMN_12740	670189	NM_001793	CDH3	Cadherin 3, type 1, P-cadherin (placental) (CDH3), mRNA.	-1.51
ILMN_8472	4180195	NM_001001520	HDGF2	hepatoma-derived growth factor-related protein 2 (HDGF2), transcript variant 1, mRNA.	-1.52
ILMN_5029	5810164	NM_000071	CBS	cystathionine-beta-synthase (CBS), mRNA.	-1.52
ILMN_28130	7320056	NM_002467	MYC	v-myc myelocytomatosis viral oncogene homolog (avian) (MYC), mRNA.	-1.52
ILMN_506	3450280	NM_152322	BTBD11	BTB (POZ) domain containing 11 (BTBD11), transcript variant 1, mRNA.	-1.53
ILMN_3351	1500110	NM_021020	LZTS1	leucine zipper, putative tumor suppressor 1 (LZTS1), mRNA.	-1.53
ILMN_23782	6770095	NM_006636	MTHFD2	methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase (MTHFD2), nuclear gene encoding mitochondrial protein, mRNA.	-1.54
ILMN_22054	4830551	NM_006158	NEFL	neurofilament, light polypeptide 68kDa (NEFL), mRNA.	-1.54
ILMN_29852	6940725	NM_001008218	AMY1B	Amylase, alpha 1B; salivary (AMY1B), mRNA.	-1.55
ILMN_24176	4390500	NM_001495	GFRA2	GDNF family receptor alpha 2 (GFRA2), mRNA.	-1.55

ILMN_16225	2230215	NM_003155	STC1	stanniocalcin 1 (STC1), mRNA.	-1.56
ILMN_3066	1050070	NM_000599	IGFBP5	insulin-like growth factor binding protein 5 (IGFBP5), mRNA.	-1.61
ILMN_26428	5340685	NM_019064	SDK2	Sidekick homolog 2 (chicken) (SDK2), mRNA.	-1.62
ILMN_17425	3800095	NM_030948	PHACTR1	phosphatase and actin regulator 1 (PHACTR1), mRNA.	-1.62
ILMN_18789	5260026	NM_000050	ASS	argininosuccinate synthetase (ASS), transcript variant 1, mRNA.	-1.63
ILMN_19523	5360075	NM_003638	ITGA8	integrin, alpha 8 (ITGA8), mRNA.	-1.66
ILMN_24095	5810392	NM_003670	BHLHB2	basic helix-loop-helix domain containing, class B, 2 (BHLHB2), mRNA.	-1.69
ILMN_13176	1190193	NM_019058	DDIT4	DNA-damage-inducible transcript 4 (DDIT4), mRNA.	-1.7
ILMN_13166	1850132	NM_005407	SALL2	sal-like 2 (Drosophila) (SALL2), mRNA.	-1.93
ILMN_8399	1450703	NM_006934	SLC6A9	solute carrier family 6 (neurotransmitter transporter, glycine), member 9 (SLC6A9), transcript variant 1, mRNA.	-1.94
ILMN_2489	650446	NM_024111	CHAC1	ChaC, cation transport regulator-like 1 (E. coli) (CHAC1), mRNA.	-1.94
ILMN_9893	1300743	NM_004089	TSC22D3	TSC22 domain family, member 3 (TSC22D3), transcript variant 2, mRNA.	-1.99

4.3.4 Gene regulated by SOX10 in HEK293 cells

Illumina microarray analysis and BRB analysis software were used to investigate the gene expression of SOX10 overexpressed and control HEK293 cells. Microarray data was also filtered using the SAM algorithm (Significance Analysis of Microarrays). There are 111 genes significant by SAM, the estimated false discovery rate among the 111 genes is 0.01. 37 SOX10 regulated genes with a fold change >=±1.5 are listed here.

Table 4.5: SOX10 top up-/down-regulated genes

Unique id	Clone	GB acc	Gene symbol	Description	Fold-change
ILMN_1096	6130181	NM_181611	KRTAP19-5	keratin associated protein 19-5 (KRTAP19-5), mRNA.	5.88
ILMN_26722	3520148	NM_006209	ENPP2	ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin) (ENPP2), mRNA.	2.13
ILMN_27627	6280164	NM_181610	KRTAP19-4	keratin associated protein 19-4 (KRTAP19-4), mRNA.	2
ILMN_19455	4010301	NM_174896	C1orf162	chromosome 1 open reading frame 162 (C1orf162), mRNA.	2
ILMN_27652	5860301	NM_004071	CLK1	CDC-like kinase 1 (CLK1), transcript variant 1, mRNA.	1.92
ILMN_9116	60500	NM_017578	ROPN1	ropporin, rhophilin associated protein 1 (ROPN1), mRNA.	1.75
ILMN_19011	2100575	NM_006501	MOBP	myelin-associated oligodendrocyte basic protein (MOBP), transcript variant 2, mRNA.	1.72
ILMN_26085	6650142	NM_003467	CXCR4	chemokine (C-X-C motif) receptor 4 (CXCR4), transcript variant 2, mRNA.	1.72
ILMN_7829	5690131	NM_002557	OVGP1	oviductal glycoprotein 1, 120kDa (mucin 9, oviductin) (OVGP1), mRNA.	1.69
ILMN_17827	2350333	NM_003469	SCG2	Secretogranin II (chromogranin C) (SCG2), mRNA.	1.64
ILMN_1498	7210504	NM_198538	SBSN	suprabasin (SBSN), mRNA.	1.59
ILMN_22987	6200315	NM_014174	THYN1	thymocyte nuclear protein 1 (THYN1), transcript variant 1, mRNA.	1.59
ILMN_25028	5080474	NM_001063	TF	Transferring (TF), mRNA.	1.56
ILMN_4387	7210035	NM_001008226	DKFZp666G057	hypothetical protein DKFZp666G057 (DKFZp666G057), mRNA.	1.56
ILMN_30273	4860327	NM_006786	UTS2	urotensin 2 (UTS2), transcript variant 2, mRNA.	1.54
ILMN_29328	2940255	NM_003706	PLA2G4C	phospholipase A2, group IVC (cytosolic, calcium-independent) (PLA2G4C), mRNA.	1.54
ILMN_2831	510176	NM_052872	IL17F	interleukin 17F (IL17F), mRNA.	1.54
ILMN_22365	5870093	NM_052813	CARD9	caspase recruitment domain family, member 9 (CARD9), mRNA.	1.52
ILMN_26937	7650433	NM_005756	GPR64	G protein-coupled receptor 64 (GPR64), mRNA.	-1.5
ILMN_9515	6980458	NM_005460	SNCAIP	synuclein, alpha interacting protein (synphilin) (SNCAIP), mRNA.	-1.5
ILMN_22531	6580152	NM_004735	LRRFIP1	leucine rich repeat (in FLII) interacting protein 1 (LRRFIP1), mRNA.	-1.5
ILMN_13176	1190193	NM_019058	DDIT4	DNA-damage-inducible transcript 4 (DDIT4), mRNA.	-1.53
ILMN_7290	6110678	NM_031459	SESN2	sestrin 2 (SESN2), mRNA.	-1.53
ILMN_28794	4590468	NM_018379	FAM63A	family with sequence similarity 63, member A (FAM63A), mRNA.	-1.53
ILMN_20580	1050762	NM_212503	PCTK3	PCTAIRE protein kinase 3 (PCTK3), transcript variant 1, mRNA.	-1.54
ILMN_12839	2100301	NM_198336	INSIG1	insulin induced gene 1 (INSIG1), transcript variant 2, mRNA.	-1.55

ILMN	ID	Accession	Gene	Description	Value
ILMN_5029	5810164	NM_000071	CBS	cystathionine-beta-synthase (CBS), mRNA.	-1.57
ILMN_19523	5360075	NM_003638	ITGA8	integrin, alpha 8 (ITGA8), mRNA.	-1.61
ILMN_18789	5260026	NM_000050	ASS	argininosuccinate synthetase (ASS), transcript variant 1, mRNA.	-1.61
ILMN_26790	5270433	NM_003761	VAMP8	vesicle-associated membrane protein 8 (endobrevin) (VAMP8), mRNA.	-1.65
ILMN_9587	4760767	NM_013361	ZNF223	zinc finger protein 223 (ZNF223), mRNA.	-1.67
ILMN_3851	4250609	NM_198271	LMOD3	Leiomodin 3 (fetal) (LMOD3), mRNA.	-1.67
ILMN_3066	1050070	NM_000599	IGFBP5	insulin-like growth factor binding protein 5 (IGFBP5), mRNA.	-1.69
ILMN_2603	5700110	NM_004563	PCK2	phosphoenolpyruvate carboxykinase 2 (mitochondrial) (PCK2), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA.	-1.7
ILMN_8399	1450703	NM_006934	SLC6A9	solute carrier family 6 (neurotransmitter transporter, glycine), member 9 (SLC6A9), transcript variant 1, mRNA.	-1.8
ILMN_2489	650446	NM_024111	CHAC1	ChaC, cation transport regulator-like 1 (E. coli) (CHAC1), mRNA.	-1.96
ILMN_9893	1300743	NM_004089	TSC22D3	TSC22 domain family, member 3 (TSC22D3), transcript variant 2, mRNA.	-2.01

4.3.5 Shared targets of NPAS3 and SOX factors

In order to identify the overlap of transcriptional targets between the NPAS3 and SOX factors, the up-regulated genes of NPAS3 were compared with those from SOX5, SOX6, SOX9 and SOX10. Two observations were made from these data. Firstly, the related pairs of SOX genes showed a greater overlap with each other than with NPAS3, confirming functional conservation and established redundancy. Secondly, SOX5 and SOX6 have a greater numerical overlap with NPAS3 than SOX9/SOX10 but a similar proportion. Genes regulated by SOX5/6 and by NPAS3 included *VGF*, *NEFH*, *MGC14376*, *C19orf30*, *DNAH17*, *CHGA*, *HIST1H4H*, *GADD45B*, *DPH2*, and *GALR2*. Genes regulated by SOX9/10 and by NPAS3 included *PLA2G4C*, *C1orf162*, *GALR2*, *CARD9*, *OVGP1*, *SBSN*, *CELSR3*, and *CLK1*.

The most significantly up-regulated gene by NPAS3, SOX5 and SOX6 was *VGF* encoding a precursor for a number of processed and secreted signalling peptides such as TLQP-21. Vgf studies in the mouse have produced compelling evidence that it mediates the connection between physical activity, increased adult neurogenesis and attenuation of depression-like phenotypes. VGF peptides have also been associated with the regulation of metabolic rate matching the metabolic aspect of NPAS3's function. VGF and other neuropeptides such as secretogranin II and NPY are regulated in the hippocampus when voluntary exercise was employed as a mood stimulator. It was also reported that VGF peptide can produce a robust antidepressant response in a dose dependent manner in FST and tail suspension tests (Hunsberger et al., 2007). VGF was reported as an important factor in the central nervous system in

a peptidomic analysis on normal cerebrospinal fluid (CSF) (Yuan and Desiderio, 2005).

The highly conserved *VGF* gene encodes a 617 amino acids precursor polypeptide, which is then processed into more than 10 different mature peptides by neuroendocrine-specific prohormone convertases PC1/3 and PC2 (Possenti et al., 1999; Trani et al., 2002). The most prominent mature VGF peptides are 20 (NAPP-129) and 10kDa (TLQP-62). VGF is expressed exclusively in neurons of several areas, such as the olfactory system, cerebral cortex, hypothalamus, hippocampus, the adrenal medulla and motor neurons of the spinal cord. *VGF* is a highly relevant target gene as it shares a number of key features with NPAS3; regulation by circadian rhythm (Cirelli and Tononi, 2000), involvement in metabolic control (Altshuler and Hirschhorn, 1999; Bartolomucci et al., 2006; Bartolomucci et al., 2007; Jethwa and Ebling, 2008; Jethwa et al., 2007; Salton et al., 2000), contribution to activity-related adult neurogenesis (Thakker-Varia et al., 2007), and association with neurological diseases (Altar et al., 2009; Pasinetti et al., 2006; Ruetschi et al., 2005; Selle et al., 2005) including depression (Hunsberger et al., 2007; Malberg and Monteggia, 2008; Thakker-Varia and Alder, 2009; Thakker-Varia et al., 2007) and psychosis (Huang et al., 2006).

We also observed a substantial overlap in target genes down-regulated both by NPAS3 and SOX11 (data is also included on SOX11. the background to SOX11 is presented in Chapter 6). The most significant down-regulation with NPAS3 and SOx11 was *ANKRD37*, *STC1*, and *DDIT4* (Figure 4.3).
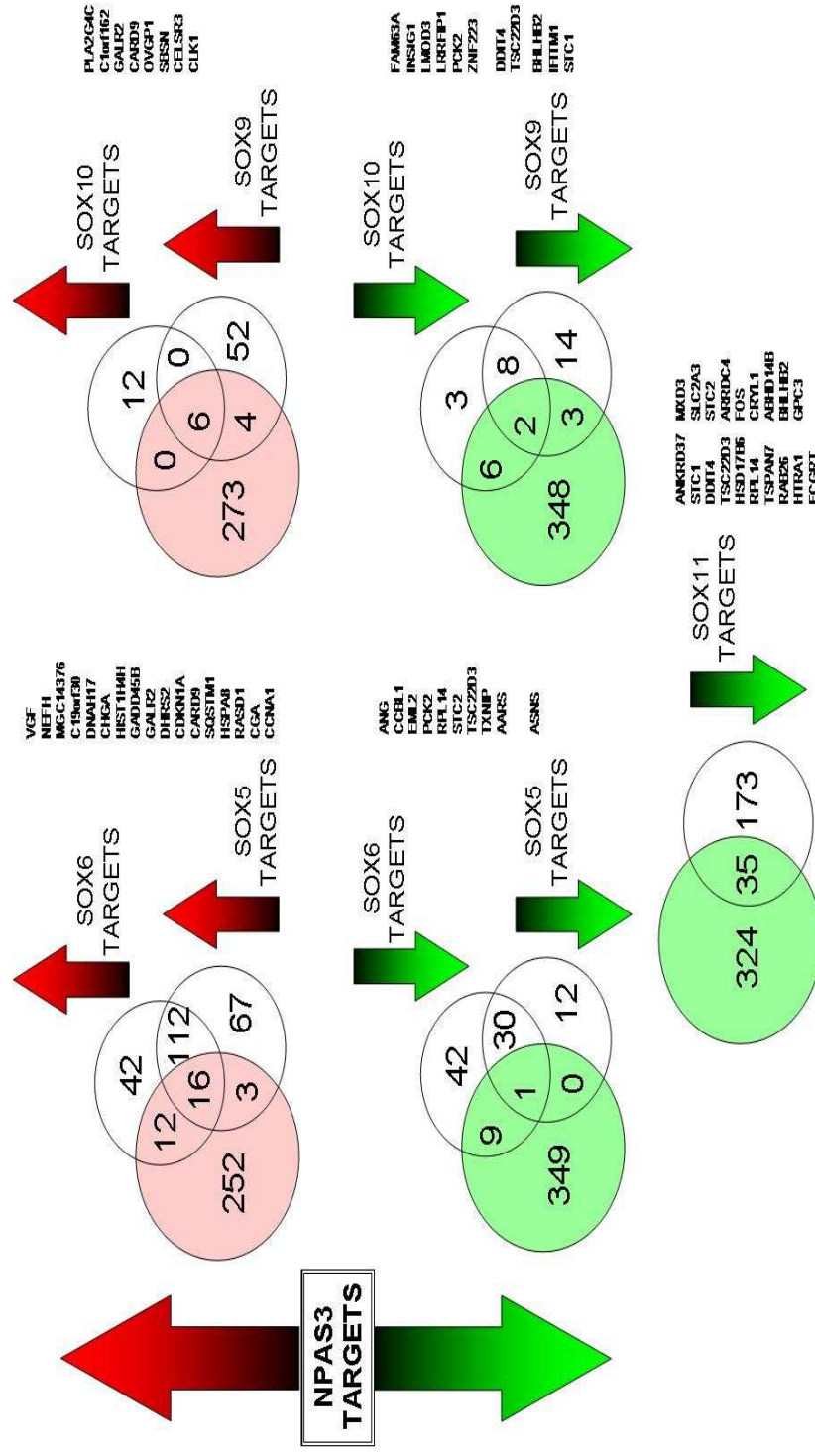
Figure 4.3 Venn diagrams illustrating the overlap between up- (red) and down- (green) regulated targets of NPAS3 and the target genes regulated by the SOX family of transcription factors. The accompanying gene lists detail selected shared targets of the SOX factors and NPAS3. No substantial overlap between SOX11 up-regulated genes and NPAS3 up-regulated genes was observed.

4.3.6 Testing transcriptional control at the VGF promoter

The *VGF* gene promoter contains several specific regions which are thought to be critical for the neuronal expression of VGF. These include a CREB binding site (Bonni et al., 1995), which is critical for BDNF-induced VGF expression, a CCAAT box, a G(S)G element and an E-box near the transcription start sites (D'Arcangelo et al., 1996). As it is known that basic helix-loop-helix domain transcription factors (such as NPAS3) bind specifically to E-box and VGF is a top upregulated gene of NPAS3 in our microarray data set, we can hypothesise that NPAS3 might activate the promoter of VGF.

A human VGF promoter fragment, containing promoter, exon1, intron1 and part of exon2, was cloned into a pGL3 basic vector (Figure 4.4). The activity of the VGF promoter was analysed using a dual luciferase assay in FLNPAS3/ΔNPAS3 overexpression and control cells. The values of VGF promoter (Firefly luciferase) in each sample represent the percentage of the relative activity of the empty pRL-TK vector (Renilla luciferase). Data were analysed by one way ANOVA. In HEK293 cells, the luciferase activities of VGF promoter are significantly different from each other in the NPAS3/ΔNPAS3 over-expressed cells and control vector HEK293 cells (Figure 4.5 A). While, in SHSY-5Y cells, luciferase expression in the FLNPAS3 over-expressed cells was significantly higher than ΔNPAS3 and control vector SH-SY5Y cells (Figure 4.5 B).

5' Human VGF promoter sequence
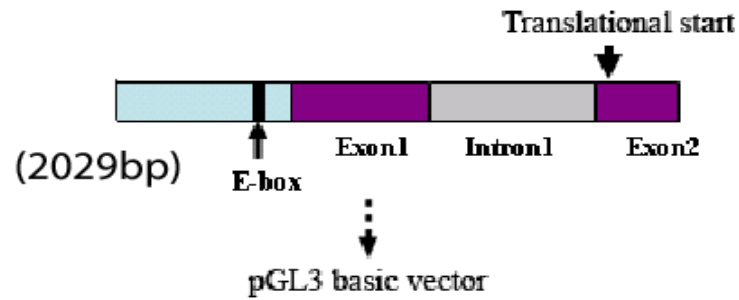
Figure 4.4 The structure of human VGF promoter fragment.
This fragment is a 2029bp sequence and includes 5' untranslated part, exon1, intron1 and part of exon2. E-box, which is specially recognised by NPAS3, is within the 5' untranslated region. The translational start of *VGF* gene is localised in exon2.
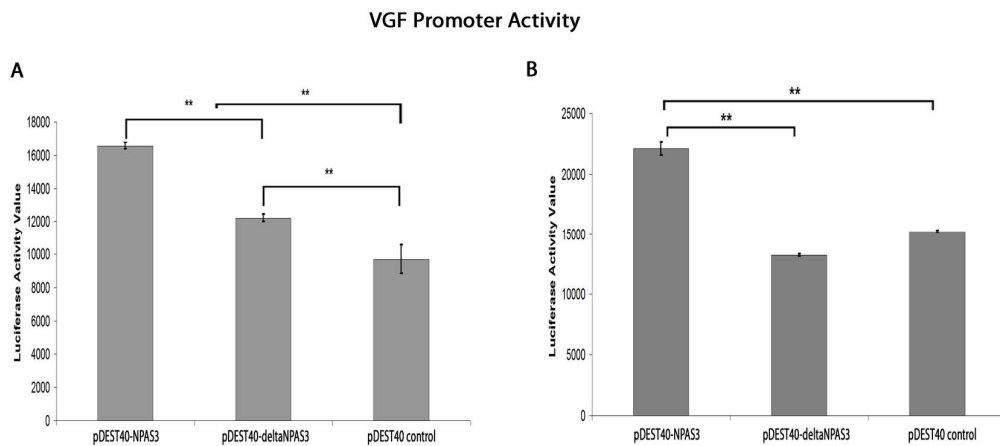
Figure 4.5 Luciferase expressions in the NPAS3 /ΔNPAS3/control vector over-expressed cells. A: Luciferase expression in the NPAS3 /ΔNPAS3/control vector over-expressed HEK293 cells.
B: Luciferase expression in the NPAS3 /ΔNPAS3/control vector over-expressed SH-SY5Y cells. The values represent the percentage of the relative activities to the pRL-TK vector (mean±SEM).
Asterisks mean significantly different VGF promoter activities at $P < 0.05$ (*) or $P < 0.01$ (**) (Analysed by RM-ANOVA).

4.4 Discussion

This study strongly suggests that NPAS3 participates in a neurodevelopmental regulatory network overlapping and interacting with the SOX transcription factor family. We propose that the overlap between NPAS3 and SOX target genes may reflect the intermediate position of NPAS3 in the neurogenic transcriptional programme dictating progression from neural progenitor, through fate-committed cell, to differentiated neuron.

4.4.1 Genes with overlapping regulatory functions

SCG2 protein is strongly up-regulated in our studies by SOX9, SOX10 and SOX11. *SCG2* (Secretogranin II/Chromogranin-C) is a cell surface sialoglycoprotein. Among many functions it has been shown that SCG2 promotes the differentiation of neuroblastoma cells into neurons (Li et al., 2008). Similarly, secretoneurin II, a brain peptide derived from endoproteolytic processing of SCG2, can promote the outgrowth of immature cerebellar granule cells (Shyu et al., 2008). It has been reported that Schizophrenia in humans is associated with upregulation of human *SCG2* mRNA in dorsolateral prefrontal cortex (Hakak et al., 2001).

Importantly, in a published proteomic study of cerebrospinal fluid, VGF and the related SCG2 protein were identified as the two biomarkers most able to discriminate between depression and healthy control samples, whereas VGF alone was critical in distinguishing schizophrenia from control samples (Huang et al., 2006). Their regulation by the NPAS3/SOX developmental cascade suggests a unifying

hypothesis: these two secreted biomarkers report a neurogenic pathology associated with psychiatric disorders.

GADD45B, another highly up-regulated target by NPAS3, SOX5 and SOX6, plays a similar role in adult activity-dependent neurogenesis by stimulating BDNF/FGF signalling and promoter demethylation (Ma et al., 2009b). GALR2, encoding a galanin receptor, was the only gene to be up-regulated by all 5 transcription factors. Galanin and its receptors have established roles in neurogenesis, mood, depression and anxiety (Karlsson and Holmes, 2006; Kuteeva et al., 2008; Lu et al., 2008).

The neurofilament heavy polypeptide (NEFH) is one of the major components of neuronal cytoskeleton neurofilaments (Lee and Cleveland, 1996). NEFH was found to mediate the interaction between neurofilaments and brain mitochondria (Wagner et al., 2003). Absent or diminished NEFH expression has been reported in human automic neuronal tumours or central neurocytomas, human prostate cancer, clear-cell epithelioid tumor and small lung carcinoma. (Bobos et al., 2006; Mena et al., 2001; Schleicher et al., 1997; Segal et al., 1994; You et al., 2005). Variants of NEFH are associated with the development of amyotrophic lateral sclerosis, which is a neurodegenerative disorder primarily affecting motor neurons (Figlewicz et al., 1994).

4.4.2 Evidence that SOX genes are involved in neurogenesis

Sox11 was found prominently expressed in the neurogenic area of adult brain where it is strictly expressed with Dcx –expressing precursors and immature neurons, but not with Sox2-expressing non-committed precursors and immature cells (Haslinger et al., 2009). As Npas3 is also found colocalised with Dcx in the adult mouse hippocampus and they are both transcriptional regulators, we hypothesise that NPAS3 and SOX11 are both involved in the regulation of specific genes at the stage of the immature neuron. In particular, it will be important to further characterise the roles of NPAS3 and SOX target genes in embryonic and adult neurogenesis-processes that seem to correlate with the risk of depression and psychosis (Kempermann et al., 2008).

4.4.3 Npas3 down-regulates SOX11, why do these genes both act as down-regulators of several genes?

A surprising observation was that many genes were down-regulated both by NPAS3 and by SOX11. This is puzzling because SOX11 is down-regulated by NPAS3 (Figure 3.4 and Figure 3.5). Although SOX11 and NPAS3 are very powerful transcription factors, the number of genes directly regulated by them is very limited. It is very possible that a small change in the activity of particular genes causes a similar change nearby, which then causes another similar change and so on in liner sequence. Thus, these genes may not be directly regulated by NPAS3 and SOX11, but may show altered expression due to the interactions with other genes in the pathway.

There is also another possibility that SOX11 is not directly regulated by NPAS3, but is regulated by downstream genes.

CHAPTER FIVE

5   An interaction between NPAS3 and the circadian rhythm

5.1   Introduction

NPAS2 (Neuronal PAS domain protein 2) is another member of the bHLH-PAS superfamily of transcription factors, which is highly expressed in the CNS. NPAS2 contains a basic-helix-loop-helix structure motif and PAS domain, like NPAS3. It is one of the components of the circadian clock oscillator. The other components include the CRY proteins, CLOCK, BMAL1, BMAL2, CSNK1D, CSNK1E, TIMELESS and the PER proteins. NPAS2 polypeptides are highly similar in primary amino acid sequence to CLOCK and have been shown to behave similarly in biochemical and cell based experiments (Hogenesch et al., 1998; McNamara et al., 2001; Reick et al., 2001; Rutter et al., 2001). In mammals, a heterodimer composed of CLOCK and BMAL1 is the positive transcription factor in the negative feedback cycle of the regulation of circadian rhythm. This regulation is inhibited in a feedback loop by CRY and PER. NPAS2 has also been implicated in the redox-sensitive control of gene transcription (Rutter et al., 2002; Rutter et al., 2001). In particular, NPAS2 was shown to up-regulate the expression of one unit of lactate dehydrogenase (LDH), an enzyme that inter-converts lactate and pyruvate generated by glycolysis (Rutter et al., 2001). NPAS2 was shown to be associated with seasonal affective disorder (SAD), which describes depression made worse by the absence of sunlight during winter months (Partonen et al., 2007).

NPAS3 has similar function domains to NPAS2, and we can speculate that NPAS3 might also participate in circadian signalling. Furthermore, NPAS3 (like all basic

helix-loop-helix transcription factors) functions as a heterodimer with partners such as the BMAL (ARNTL) proteins. BMAL1 is also involved in circadian time-keeping. Additionally, altered circadian rhythmicity, producing symptoms of sleep disturbance or metabolic dysfunction, is found in individuals diagnosed with depression and other mood disorders, although it is probably unrelated to the speed of 'cycling' between the manic and depressed states (Kato, 2007; Lamont et al., 2007). Based on this background information, the question was addressed whether constitutive NPAS3 overexpression actively alters circadian rhythm in these cells or is, alternatively, passively influenced by it.

5.2   Aims of this study

To test the hypothesis that NPAS3 is involved in the circadian rhythm, Illumina microarray analysis was carried out to investigate the gene expression changes in circadian induced HEK293 cells after over-expressing FLNPAS3 or ΔNPAS3.

5.3 Results

5.3.1 Confirmation of in vitro circadian induction by serum shock approach

In order to induce the circadian rhythmicity in an *in vitro* system, HEK293 cells were treated using a serum shock approach. HEK293 cells were cultured close to confluence and at the zero hour time-point, the cells were shifted into a serum-rich medium to induce circadian cycling (Balsalobre et al., 1998; Huang et al., 2009). At +2 hours, cells were washed with DMEM and then incubated with the same for the remaining period of the experiment. Cells were collected at different time points (0hr, 4hr, 8hr, 12hr, 16hr, 20hr, 24hr…….72hr). Expression of *period* (*PER*) regulator genes was analysed at different time points over 72hrs using RT-PCR and agarose gel electrophoresis (Fig 5.1). DNA bands were scaned and intensity values were normalised to control genes. PER1, PER2 and PER3 show expression changes with a 18-28 h cycle. Per1 and Per2 are out of phase with each other as previously described. This confirms the success of my methodology for circadian induction in the HEK293 cells.
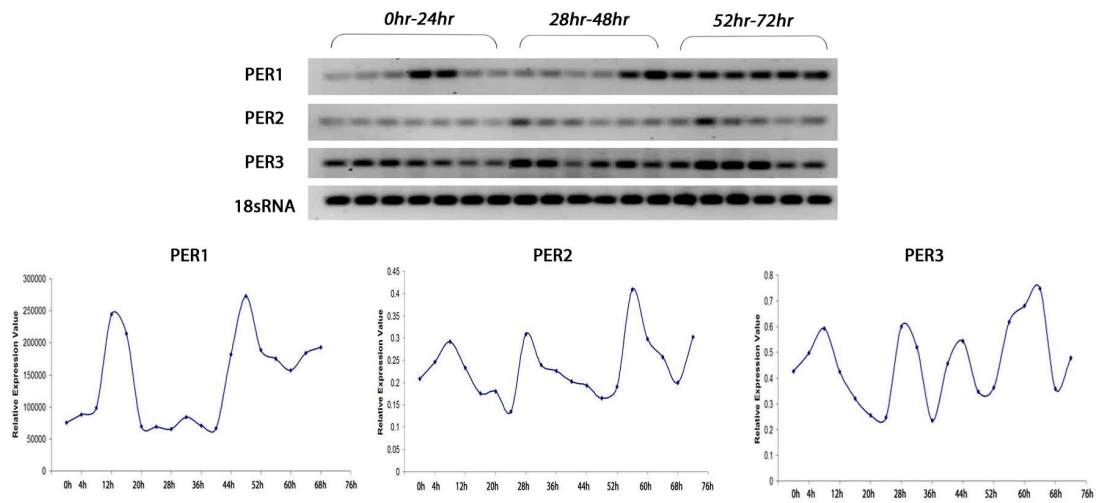
Figure 5.1 RT-PCR analysis of a serum shock induces circadian gene expression in HEK293 cells.

HEK293 cells were grown close to confluence and shifted to a serum-rich medium, as described in the methods. The levels of the cDNA indicated at the left side of the figures were determined at different at different times (top of the figure) after serum shock. 18sRNA was used as a reference gene which indicated the total cDNA of different samples.

5.3.2 NPAS3-specific changes in gene expression in HEK293 cells after circadian induction

The purpose of this experiment was to see the effect of NPAS3 on gene expression at +12 and +24 hours after circadian induction in HEK293 cells using Illumina microarray. (Appendix Table S3-4) The fragments of FLNPAS3 and ΔNPAS3 open reading frames were transferred into a similar TET-inducible expression plasmid and transfected into HEK293 [T-REx-293] cells (Invitrogen). At the zero hour time-point, cells were shifted into a serum-rich medium plus tetracycline in order to induce both circadian cycling and NPAS3 overexpression (Balsalobre et al., 1998; Huang et al., 2009). Parental HEK293 [T-REx-293] cells without NPAS3 constructs were treated in the same way to act as negative controls. Ideally, unactivated (tetracycline) FLNPAS3/ΔNPAS3 cells would have bee used as controls but these cell lines showed a low level of leaky transcription in the absence of tetracycline. At +2 hours, cells were washed with DMEM and then incubated with the same plus tetracycline for the remaining period of the experiment. Cell samples from the three experimental groups were collected at +12hrs and +24hrs respectively. For all circadian cell culture experiments with FLNPAS3/ΔNPAS3/parental negative control cell lines, duplicate biological samples were assessed. Microarray probes synthesised from RNA extraction products were quantified using an Agilent Bioanalyzer to ensure high quality probes of equal quantity between experiments. Sentrix® HumanRef-8 v2 chips (capable of examining expression of over 24,500 gene transcripts) were used to detect gene expression profiles.

As a first test of the data, changes in gene expression in control cells were studied (Appendix table S1). Many genes were showed higher expression at +24 hrs compared to +12 hrs, including *FOS*, *FOSB*, *EGR1*, *EGR2*, *EGR3* and *PER2*, all involved in transducing light entrainment during the dark phase of the circadian rhythm. A search for significant gene functions in the top 100 circadian genes with lower expression at +24 hrs compared to +12 hrs identified a group of 10 genes involved in the regulation of cell cycle progression (GO:0007049, p=1.31546 $\times 10^{-6}$). These were *FBXO5*, *SPC25*, *CDKN1B*, *RAD21*, *CKAP2*, *MAD2L1*, *GAS1*, *ZWILCH*, *CDC2* and *RBBP4*. Cell cycle genes were also reported to be deregulated in a cell model of NPAS2 dysfunction (Hoffman et al., 2008).

5.3.3 The regulatory activities of FL/ΔNPAS3 have been changed by circadian environment

In this culture system FL/ΔNPAS3 activity was strongly modified by circadian cycling in two principal ways. Firstly, NPAS3 activity on target gene expression was almost universally inhibitory at +12 hours. Secondly, NPAS3 down-regulated target genes differed considerably between +12 hours and +24 hours (Appendix table S3-4). The detail of modification by circadian cycling on FL/ΔNPAS3 activity will be further investigated in the later part of this chapter.

5.3.4 Many standard culture down-regulated genes were also down-regulated in the circadian experiments: a link to hypoxia signalling

Only seven genes up-regulated in the standard culture conditions NPAS3 over-expression study were also up-regulated in the circadian experiment: *CDKN1A*, *CENPF*, *GALR2*, *HIST1H1C*, *HIST1H2BD*, *PCDH7*, and *SPHK2*. By contrast, a larger overlap existed between standard and circadian down-regulated genes. While the majority of the overlapping genes were specific to +12 hours, 21 were down-regulated by FLNPAS3 at both time-points. The over-represented ontologies were identified by a GeneCodis2 search. 4 genes (*PLOD2, ALDOC*, *BNIP3*, *DDIT4*) were found to be associated with a response to hypoxia (GO:0001666) with a significant corrected hypergeometric p-value of 4.86×10^{-6}. Further manual annotation showed that all of the 20 genes, except *HOXA13* have published evidence for up-regulation in conditions of hypoxia or nutrient stress.
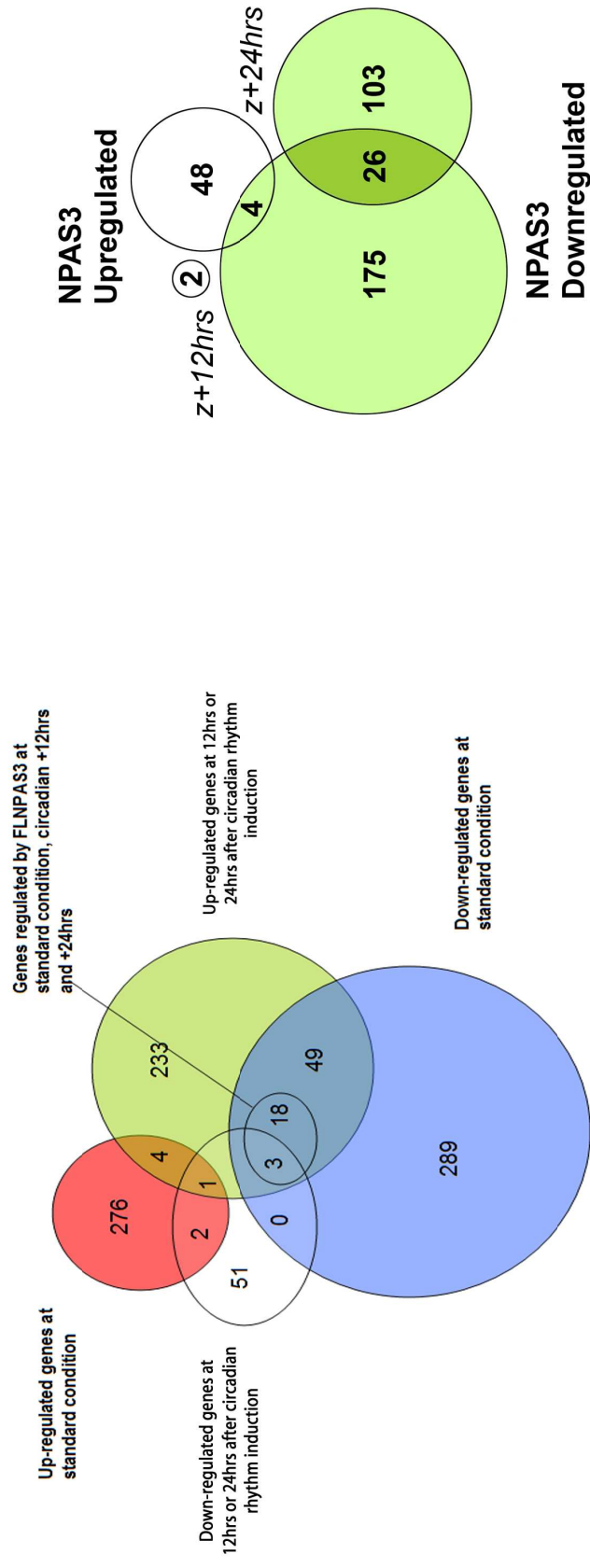
Figure 5.2 Relationships between target gene profiles of standard and circadian microarray experiments. Down-regulated but not up-regulated target genes show overlap between standard and circadian conditions (left). Only a proportion of down-regulate genes are shared between the circadian +12hr and +24hr time-points indicating that NPAS3 target gene regulation is circadian time-point sensitive.

5.3.5 ΔNPAS3 and FLNPAS3 differences exposed by circadian stimulation

In order to compare the difference of FLNPAS3 and ΔNPAS3 in the circadian environment, the gene regulation of these two factors was analysed by scatter plots (Figure 5.3). In contrast to standard culturing conditions, the circadian microarray exposed significant functional differences between ΔNPAS3 and FLNPAS3 at +12 hours after induction of circadian rhythm (Appendix table S2). This was generally evident as reduced ΔNPAS3 potency specifically at +12 hours.
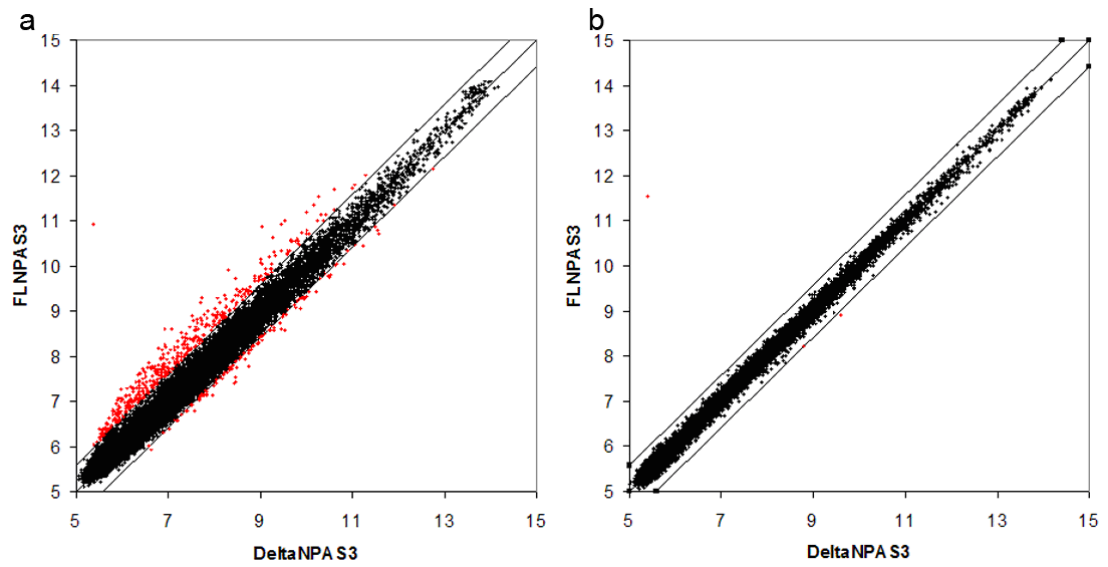
Figure 5.3 Normalised and log-transformed expression data for over 24,000 probes (18,000 genes). The diagonal represents equal expression in both conditions. Genes (shown in red) outside of the two parallel diagonal lines show at least 1.5-fold difference between the two conditions. Regulatory differences between FLNPAS3 and ΔNPAS3 at the +12 (a) and +24 hour (b) time-points after circadian induction indicate that ΔNPAS3 acts differentially only at +12hrs.

5.3.6 An interaction between NPAS3 activity and the circadian rhythm

To further explore the relationship between circadian time-point, FL/ΔNPAS3 activity on target gene regulation, we visualised microarray data using ternary plots. Briefly, a set of functional related genes were chosen for different comparision. Then, the expression values of these genes were looked up in different cell samples in our circadian microarray data file. Finally, the gene expression values were input into ProSim, to generate the ternary plot results. Ternary plots indicate the relative influence of each of the three experimental conditions - FLNPAS3, ΔNPAS3 and control – on gene expression. Any deviation from the centre of the plot indicates that either one or two conditions is/are disproportionally influencing expression. Gene expression at +12hr is indicated by green and +24hr is indicated by red.

As a first test, we input 109 randomly selected genes into ternary plots to look for

biases in the data (Figure 5.4 a). The randomly selected genes show no deviation

from the centre (Figure 5.4 a) discounting systemic microarray biases, and give an

example of a group of genes which are not affected by the overexpression of
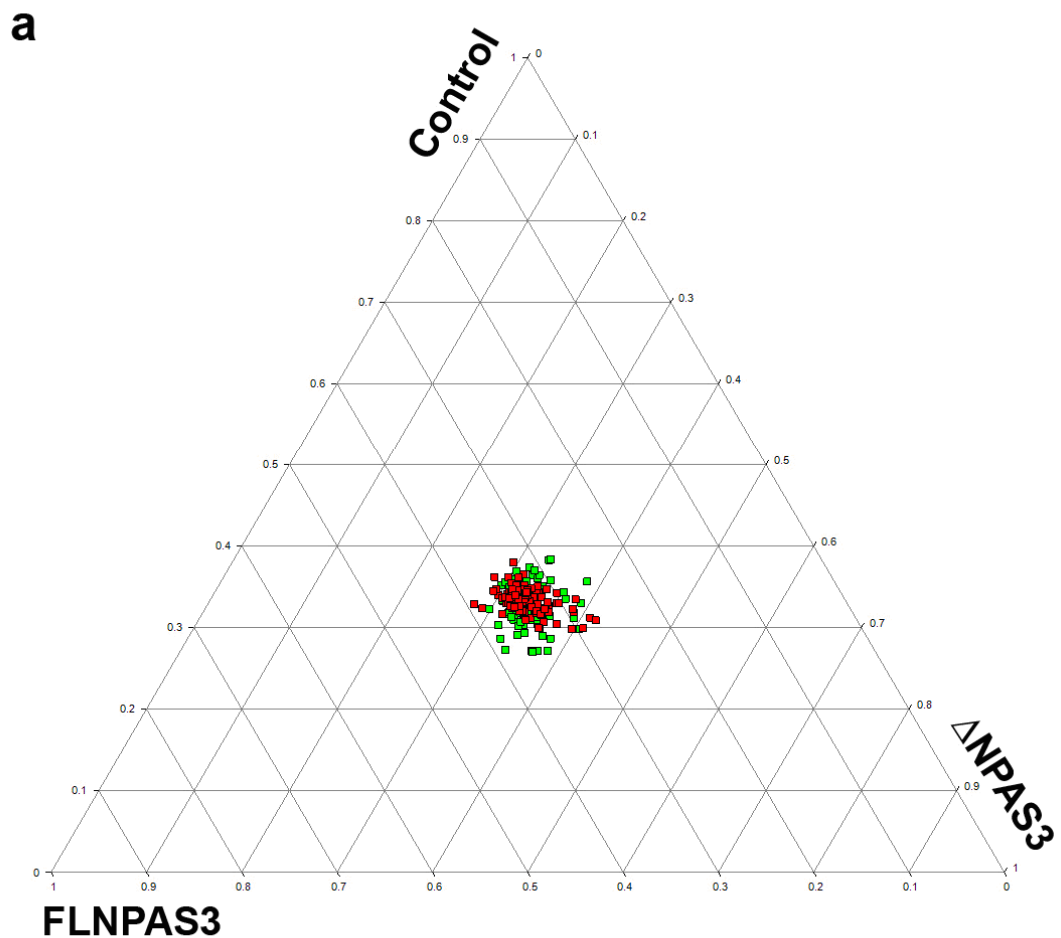
FLNPAS3/ΔNPAS3.

Figure 5.4 a Ternary plot illustrates the relative effects of FLNPAS3 over-expression, ΔNPAS3 over-expression and control conditions on target gene expression. The relative expression levels of 109 randomly selected genes in the three conditions are plotted for +12hrs (green) and +24hrs (red) time-points. The absence of substantial deviation from the centre indicates the overall similarity between the conditions.

The next set of genes analysed were two sets of control cell line (circadian induced HEK293 cells without overexpression of FLNPAS3/ΔNPAS3) genes (200 genes) appearing to show circadian regulation: 100 up-regulated genes between +12 and +24 hrs (circles) and 100 down-regulated genes between +12 and +24 hrs (squares). These were plotted to test the influence of FLNPAS3 or ΔNPAS3 over-expression on circadian processes. (Figure 5.4 b). This result suggests that at +24hr time point (red squares and circles), FL/ΔNPAS3 effects on gene expression are small and equivalent (along the vertical 'control' axis distribution), whereas a pronounced effect was seen at +12 hours (green squares and circles). The substantially deviating group (green squares) indicates that ΔNPAS3 strongly inhibits (movement away from the ΔNPAS3 triangle vertex) the expression of the group of genes at +12 hrs that will be further down-regulated by +24hrs.
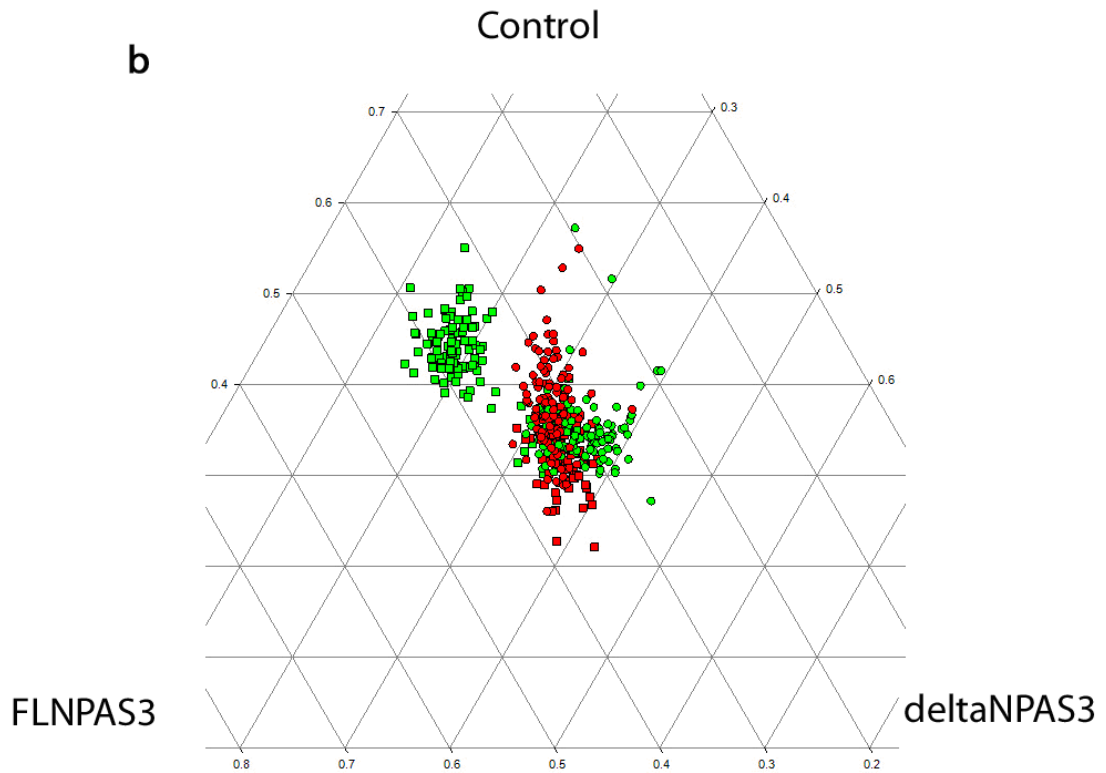
Figure 5.4 b Ternary plots illustrate the relative effects of FLNPAS3 over-expression, ΔNPAS3 over-expression and control conditions on target gene expression. Two sets of control cell line genes appearing to show circadian regulation: 100 up-regulated genes between +12 and +24 hrs (circles) and 100 down-regulated genes between +12 and +24 hrs (squares). Again, +12hr expression is indicated by green and +24hr by red. The substantially deviating group (green squares) indicates that ΔNPAS3 strongly inhibits (movement away from the ΔNPAS3 triangle vertex) the expression of the group of genes at +12 hrs that will be further down-regulated by +24hrs. Both FLNPAS3 and ΔNPAS3 alter the expression of a minority of circadian genes at +24 hrs (red squares and circles).

The third set of genes studied were a panel of 139 published genes known to be up-regulated in response to hypoxia-stimulated HIF1A (Benita et al., 2009; Chi et al., 2006; Greijer et al., 2005; Mense et al., 2006) (Figure 5.4 c), Many hypoxia pathway genes were inhibited equally by ΔNPAS3 and FLNPAS3, particularly at +12hrs (Figure 5.4c).
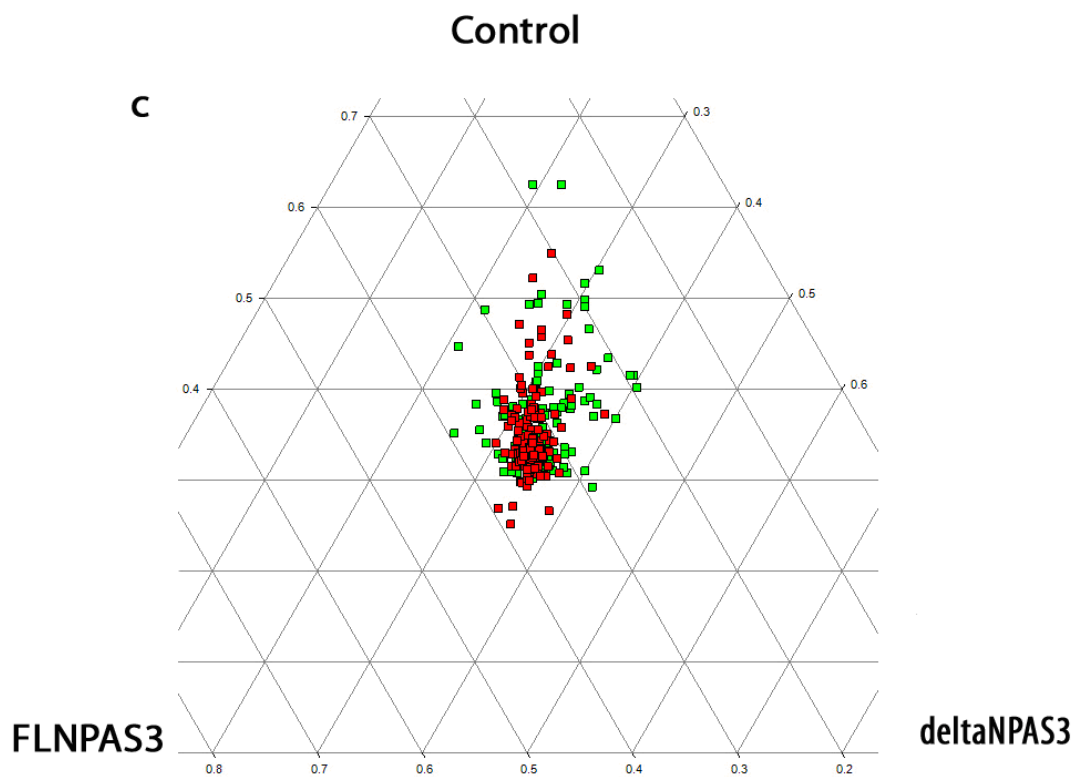
Figure 5.4 c Ternary plots illustrate the relative effects of FLNPAS3 over-expression, ΔNPAS3 over-expression and control conditions on target gene expression. Both FLNPAS3 and ΔNPAS3 inhibit the expression of many hypoxia genes at +12 (green) and +24 hrs (red)

Fourthly, 15 microarray probes corresponding to 14 NPAS3-regulated glycolysis genes were also analysed by ternary plots, these genes include *PFKFB4*, *PCK2*, *PKM2*, *ALDOA*, *ALDOC*, *ENO2*, *PKM2*, *GAPDH*, *PFKP*, *HK2*, *PFKFB3*, *PGK1*, *PKM2*, and *PGM1*. (Figure 5.4 d). The set of glycolysis genes showed deviations from the centre consistent with both NPAS3 forms inhibiting expression at +24hrs, but with a stronger FLNPAS3 inhibitory effect at +12hrs (Figure 5.4 d).
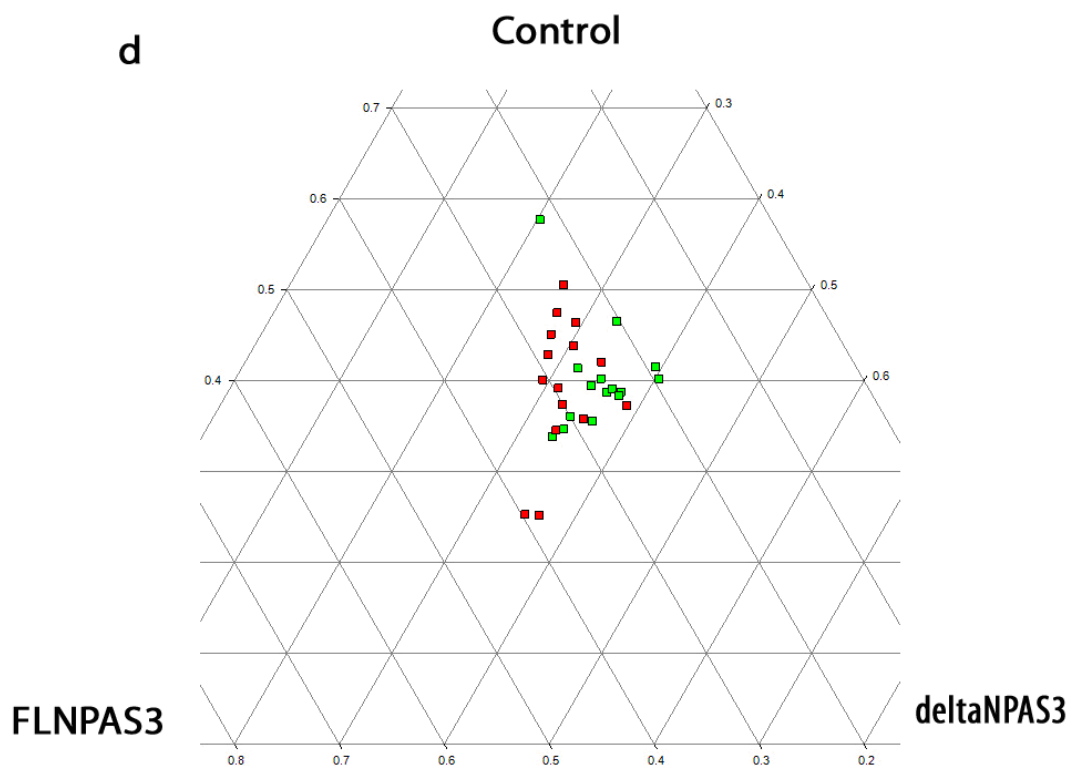
Figure 5.4 d Ternary plots illustrate the relative effects of FLNPAS3 over-expression, ΔNPAS3 over-expression and control conditions on target gene expression. 15 glycolysis genes are inhibited by FLNPAS3 and ΔNPAS3 although FLNPAS3 activity is much stronger than ΔNPAS3 at +12 hrs (green) and PCK2 is up-regulated by both at +24hrs (red).

Microarray data arranged by chromosomal gene position revealed NPAS3 regulation of clustered histone target genes (chapter3). In the fifth ternary plot histone genes on chromosome 6 regulated by SOX11 (circles) and on chromosome 1 regulated by NPAS3 (squares) were assessed .This result shows a striking inhibition of chromosome 1 histone expression by FLNPAS3 and ΔNPAS3 at +12hrs only (Figure 5.4 e). Furthermore, the inhibition capabilities of FLNPAS3 and ΔNPAS3 are equal.
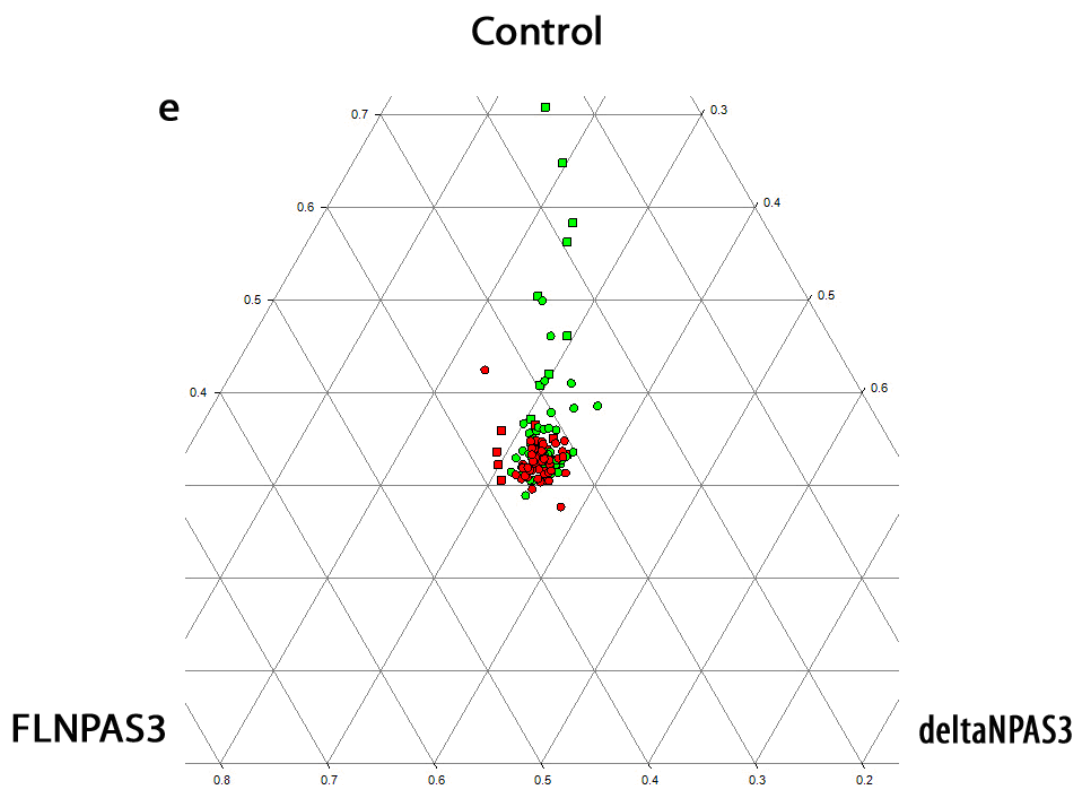
Figure 5.4 e Ternary plots illustrate the relative effects of FLNPAS3 over-expression, ΔNPAS3 over-expression and control conditions on target gene expression. Chromosome 1 histones (squares) are substantially inhibited by both FLNPAS3 and ΔNPAS3 at +12 hrs (green) in comparison to +24hrs (red) and chromosome 6 histones (circles)

Finally, there were a large number of small (RPS) and large (RPL) ribosomal protein genes regulated in the microarray datasets. Ribosomal proteins (small subunits: squares, large subunits: circles) were input into ternary plot. This result provides evidence for FL/ΔNPAS3 inhibitory activity at +12hrs and some stimulatory activity at +24hrs (Figure 5.4 f)
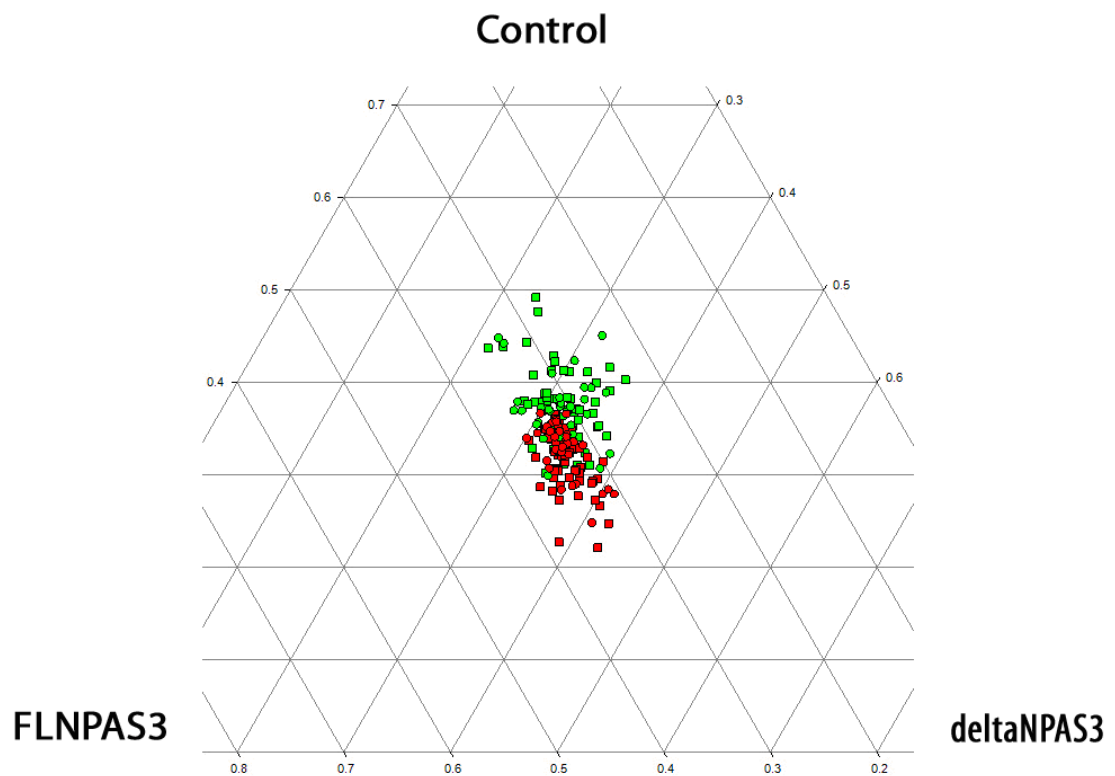
Figure 5.4 f Ternary plots illustrate the relative effects of FLNPAS3 over-expression, ΔNPAS3 over-expression and control conditions on target gene expression. Ribosomal proteins (small subunits: squares, large subunits: circles) show up-regulation at +12hrs (green) and down-regulation at +24 hrs (red).

5.4 Discussion

Although not presented here, a previous RT-PCR experiment did not show circadian changes in the expression of the *NPAS3* gene itself suggesting that the gene is not a central part of the circadian control pathway. But the microarray results show that the regulatory activity of FLNPAS3 was strongly modified by circadian cycling. These might be due to the fact that many co-functioning partners of NPAS3 (such as BMAL) are bHLH family member and controlled by circadian rhythms.

Although the second PAS domain and the putative transactivation domain are absent in ΔNPAS3 the microarray data show that the regulatory actions of FLNPAS3 and ΔNPAS3, as in the standard culture conditions, are largely similar at 24hrs time point (Figure 5.3 b and Figure 5.4 b-e red). As ΔNPAS3 has a bHLH domain, it still keeps the ability to form heterodimerized complex.

In our microarray results, the regulatory profiles of these two proteins are different at 12 hrs. At 12 hr, the dominant function of NPAS3 is due to the transactivation/transdepression domain, and the actions of bHLH domain and PAS-A are limited, therefore, the transcriptional regulatory activities of FLNPAS3 and ΔNPAS3 are significantly different. While at 24 hrs, it is possible that the bHLH domain, which has the property to form a heterodimerized complex, has the dominant role in the transcriptional regulatory of NPAS3. Because both ΔNPAS3 and NPAS3 keep the bHLH domain, there is no big difference between NPAS3 and the truncated form, ΔNPAS3.

This hypothesis also explains why NPAS3 activity is sensitive to circadian time-point. FL/ΔNPAS3 activity was strongly modified by circadian cycling in two main ways. This possibly due to the expression levels of several potential NPAS3 partners are affected by circadian rhythm.

Based on the ternary plot results, there are several interesting findings:

Firstly, the circadian microarray result revealed a direct modulatory interaction between NPAS3 activity and circadian rhythmicity. This interaction includes: 1), ΔNPAS3, but not FLNPAS3, inhibited the expression levels of a set of potential circadian genes which showed a decrease between +12 and +24 hours in the control group (Figure 5.3.b). 2), both FLNPAS3 and ΔNPAS3 inhibited the expression of multiple target gene groups such as glycolysis (Figure 5.3.d), hypoxia response genes (Figure 5.3.c) and chromosome 1 histone cluster (Figure 5.3.e).

Secondly, in both instances these effects were either exclusive to, or amplified at the +12 hour time-point. It is intrinsically difficult to separate cause from effect from the limited time-points studied here. However, only the +12hr ΔNPAS3 effect suggests a direct modulation of circadian master regulator gene activity resulting in circadian phase (wavelength shift). ΔNPAS3 is generated by cleavage at an XhoI site which removes the second PAS domain and the putative transactivation domain. So it only has the properties to heterodimerize with other partners and bind to specific DNA sequence. Therefore, one hypothesis to explain this observation could be that the truncation of NPAS3 overcomes restrictions on the normal range of transcriptional binding partners– permitting abnormal interaction with circadian bHLH transcription

factors active at +12hrs. It can be appreciated that gene mutations with similar effects to the truncation might also upset circadian rhythms.

Thirdly, our ternary plot analysis on hypoxia genes revealed that both FLNPAS3 and ΔNPAS3 can inhibit many hypoxia genes at +12hr and +24hr (Figure 5.4.c). The underlying mechanism of this phenomenon might due to 'binding partner choice'. In fact, after NPAS1, SIM1 and SIM2, HIF1A possesses the most homologous bHLH domain to NPAS3. Because the inhibitory capabilities of FLNPAS3 and ΔNPAS3 are different at +12hrs and +24hrs in our cell models, I further propose that this NPAS3/hypoxia pathway functions is sensitive to circadian state. Reports that hypoxia can affect the amplitude of the circadian output also strengthen the argument that HIF1A participates in cross-talk with circadian pacemaker factors (Bosco et al., 2003; Ghorbel et al., 2003; Mortola, 2007; Vargas et al., 2001). As NPAS3 did not show a circadian expression pattern in our cell models (both in the normal PCR result and in the microarray result), I do not hypothesise that NPAS3 has the capacity to sense its environment directly, although it is highly related to transcription factors such as NPAS2 and HIF1A.

CHAPTER SIX

6 SOX11 target genes: implications for neurogenesis and neuropsychiatric illness

6.1 Introduction

The proliferation of progenitor cells and their differentiation into functional neurons are critical processes during developmental and adult neurogenesis. As growing evidence suggests that neurogenesis deficits play a role in neurological and psychiatric disorders, such as schizophrenia and bipolar disorder (more information is available in 1.3.1), it is important to understand the underlying molecular mechanisms. SOX family members play an important role in adult neurogenesis and SOX11 is one of the negatively regulated genes of NPAS3. This chapter describes experiments to define the regulatory profile of SOX11.

Sox C members, including Sox4, Sox11 and Sox12, possibly play a key role during the regulation of later aspects of neurogenesis. They are mainly expressed in neuronal cells which have been committed to neuronal differentiation (Cheung et al., 2000). Sox4 and Sox11 regulate the establishment of pan-neuronal protein expression by inducing precocious expression of neuronal markers in self-renewing precursors. SOX11 is the most powerful transcription factor among these three factors (Bergsland et al., 2006). SOX11 is critical during the later stages of neurogenesis. However, the regulatory targets and downstream pathways at the molecular level are yet to be fully demonstrated.

Sox11 null mice die during the neonatal period due to malformations of heart and internal organs including dysgenesis of the anterior eye segment, hypoplastic lungs, undermineralized bones and lack of a spleen (Bergsland et al., 2006). SoxC triple-deleted mouse embryos die at midgestation and are tiny. Although the patterning and lineage specification are normal, there is massive apoptotic loss of neural and mesenchymal progenitor cells (Bhattaram et al., 2010).

The 'neurodevelopmental' hypothesis of neuropsychiatric disorders such as schizophrenia, bipolar disorder and autism suggests that deficits in the proliferation, differentiation and connectivity of neurons during the formation of the brain might contribute towards increased risk of illness. Supporting a role for SOX11 in this context is a case report recently describing a 7-year-old patient with autism, moderate mental retardation, secondary microcephaly, agenesis of right opti nerve, and dysmorphic features who carried a deletion of the *SOX11* gene (Lo-Castro et al, 2009).

6.2 Aims of this study

Here we describe the over-expression of SOX11 in the HEK293 cell line followed by microarray analysis to deduce the set of target genes and their ontologies. Briefly, HEK293 cells were transiently transfected by pDEST40-SOX11 or control plasmid (pDEST40) using lipofectamine[TM] 2000 transfection kit (Invitrogen). After 4-6 hours, the transfection medium was replaced with the standard culture medium for 24 hours. Microarray probes synthesised from RNA extraction products was quantified using an Agilent Bioanalyzer to ensure high quality probes of equal quantity between experiments. Sentrix® HumanRef-8 v2 chips (capable of examining expression of over 24,500 gene transcripts) were used to detect gene expression profiles.

In addition, to investigate the distribution of SOX11 in the adult mouse hippocampus, immunofluorescence was used to localise SOX11 and several of its targets within the dentate gyrus of the hippocampus.

6.3   Result

6.3.1 Transient overexpression of SOX11 in HEK293 cells results in specific gene expression changes as assessed by microarray

Illumina microarray analysis was carried out to investigate the gene expression changes in HEK293 cells after over-expressing SOX11. For all standard cell culture experiments with SOX11/ parental negative control cell lines, triplicate biological samples were assessed. BRB analysis software was used to analyse the expression of the 22,177 well annotated RefSeq transcripts present in each array. Unsupervised hierarchical clustering of gene expression profiles using centred correlation and average linkage revealed that human *SOX11* and control transfection samples have distinct profiles (Figure 6.1). This was an indication of both the consistency of the experimental samples used and also the quality of the array hybridization and detection procedures.
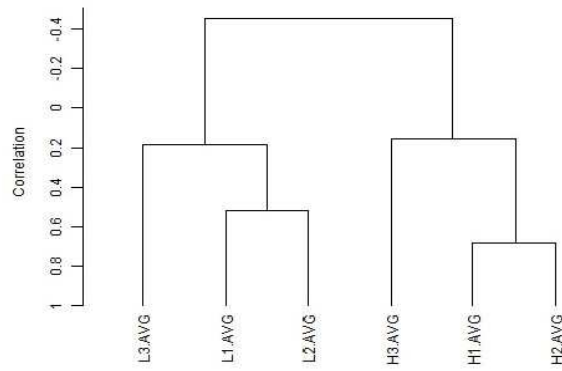
Figure 6.1 Dendrogram of *SOX11* transfected and control HEK293 cell samples. Hierarchical clustering based on microarray data from each sample reveals two distinct clusters corresponding to *SOX11* transfected (H1-3AVG) and control cells (L1-3AVG), using centered correlation and average linkage. Thus *SOX11* transfection induces a reproducible and global change in gene expression.

6.3.2 Identification and validation of SOX11 regulated genes

This section of the thesis was a collaboration with Rob Kitchen, a PhD student working in the bioinformatics department of University of Edinburgh.

Limma (designed by Rob Kitchen) and *BRB-Array Tools* approaches used in the microarray data analysis generated highly similar results at the levels of rank, fold-change and P-value. 932 genes were significant by SAM analysis. The estimated false discovery rate among the 932 significant genes was set at 0.01. Supervised analyses were next performed to identify gene expression differences between human *SOX11* transfected and untransfected HEK293 cells, at a significance level of P<0.01. 251 genes with expression levels altered by at least 1.5-fold were selected for further analysis. 63 of these genes changed by at least 2-fold are shown in Table 6.1.

Table 6. 1 SOX11 upregulated genes with fold-change more than 2

Unique id	GB acc	Gene symbol	Description	Fold-change
ILMN_17533	NM_015687	FILIP1	filamin A interacting protein 1 (FILIP1), mRNA.	6.25
ILMN_30163	NM_003543	HIST1H4H	histone 1, H4h (HIST1H4H), mRNA.	5.26
ILMN_6771	NM_152742	GPC2	glypican 2 (cerebroglycan) (GPC2), mRNA.	4.17
ILMN_22946	NM_000266	NDP	Norrie disease (pseudoglioma) (NDP), mRNA.	4.00
ILMN_28723	NM_013230	CD24	CD24 antigen (small cell lung carcinoma cluster 4 antigen) (CD24), mRNA.	4.00
ILMN_17622	NM_138720	HIST1H2BD	histone 1, H2bd (HIST1H2BD), transcript variant 2, mRNA.	3.70
ILMN_19059	NM_052913	KIAA1913	KIAA1913 (KIAA1913), mRNA.	2.94
ILMN_1003	NM_005767	P2RY5	purinergic receptor P2Y, G-protein coupled, 5 (P2RY5), mRNA.	2.94
ILMN_22069	NM_003548	HIST2H4	histone 2, H4 (HIST2H4), mRNA.	2.86
ILMN_24012	NM_016593	CYP39A1	cytochrome P450, family 39, subfamily A, polypeptide 1 (CYP39A1), mRNA.	2.86
ILMN_7731	NM_001013672	LOC400566	Hypothetical gene supported by AK128660 (LOC400566), mRNA.	2.86
ILMN_5522	NM_001031692	LRRC17	leucine rich repeat containing 17 (LRRC17), transcript variant 1, mRNA.	2.70
ILMN_650	NM_182492	LRP5L	low density lipoprotein receptor-related protein 5-like (LRP5L), mRNA.	2.70
ILMN_16399	NM_006086	TUBB3	tubulin, beta 3 (TUBB3), mRNA.	2.70
ILMN_18421	NM_182909	DOC1	downregulated in ovarian cancer 1 (DOC1), transcript variant 1, mRNA.	2.63
ILMN_28977	NM_014890	DOC1	downregulated in ovarian cancer 1 (DOC1), transcript variant 2, mRNA.	2.56
ILMN_19455	NM_174896	C1orfl62	Chromosome 1 open reading frame 162 (C1orf162), mRNA.	2.50
ILMN_692	NM_003545	HIST1H4E	histone 1, H4e (HIST1H4E), mRNA.	2.50
ILMN_27652	NM_004071	CLK1	CDC-like kinase 1 (CLK1), transcript variant 1, mRNA.	2.50
ILMN_12847	NM_025231	ZNF435	zinc finger protein 435 (ZNF435), mRNA.	2.44
ILMN_4727	NM_018351	FGD6	FYVE, RhoGEF and PH domain containing 6 (FGD6), mRNA.	2.33
ILMN_7731	NM_001013672	LOC400566	Hypothetical gene supported by AK128660 (LOC400566), mRNA.	2.33
ILMN_22440	NM_152490	B3GALNT2	UDP-GalNAc:betaGlcNAc beta 1,3-galactosaminyltransferase, polypeptide 2 (B3GALNT2), mRNA.	2.33
ILMN_10151	NM_006299	ZNF193	zinc finger protein 193 (ZNF193), mRNA.	2.33
ILMN_588	NM_006550	FSBP	fibrinogen silencer binding protein (FSBP), mRNA.	2.33
ILMN_24579	NM_144661	C10orf82	Chromosome 10 open reading frame 82 (C10orf82), mRNA.	2.27
ILMN_18282	NM_005319	HIST1H1C	histone 1, H1c (HIST1H1C), mRNA.	2.27

ILMN_11057	NM_002896	RBM4	RNA binding motif protein 4 (RBM4), mRNA.	2.22
ILMN_15870	NM_021908	ST7	suppression of tumorigenicity 7 (ST7), transcript variant b, mRNA.	2.22
ILMN_17949	NM_181427	GABPB2	GA binding protein transcription factor, beta subunit 2 (GABPB2), transcript variant gamma-3, mRNA.	2.22
ILMN_21089	NM_003518	HIST1H2BG	histone 1, H2bg (HIST1H2BG), mRNA.	2.22
ILMN_22535	NM_007149	ZNF184	zinc finger protein 184 (Kruppel-like) (ZNF184), mRNA.	2.22
ILMN_25666	NM_001005914	SEMA3B	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B (SEMA3B), transcript variant 2, mRNA.	2.17
ILMN_28905	NM_006417	IFI44	interferon-induced protein 44 (IFI44), mRNA.	2.17
ILMN_22538	NM_020801	ARRDC3	arrestin domain containing 3 (ARRDC3), mRNA.	2.17
ILMN_19687	NM_198329	UBE1DC1	ubiquitin-activating enzyme E1-domain containing 1 (UBE1DC1), transcript variant 2, mRNA.	2.17
ILMN_11266	NM_182597	FLJ39575	Hypothetical protein FLJ39575 (FLJ39575), mRNA.	2.17
ILMN_16129	NM_018153	ANTXR1	anthrax toxin receptor 1 (ANTXR1), transcript variant 3, mRNA.	2.17
ILMN_25145	NM_014969	WDR47	WD repeat domain 47 (WDR47), mRNA.	2.17
ILMN_10688	NM_014037	SLC6A16	solute carrier family 6, member 16 (SLC6A16), mRNA.	2.13
ILMN_1395	NM_002849	PTPRR	protein tyrosine phosphatase, receptor type, R (PTPRR), transcript variant 1, mRNA.	2.13
ILMN_11563	NM_032875	FBXL20	F-box and leucine-rich repeat protein 20 (FBXL20), mRNA.	2.13
ILMN_17827	NM_003469	SCG2	secretogranin II (chromogranin C) (SCG2), mRNA.	2.13
ILMN_18286	NM_170662	CBLB	Cas-Br-M (murine) ecotropic retroviral transforming sequence b (CBLB), mRNA.	2.13
ILMN_21620	NM_134428	RFX3	egulatory factor X, 3 (influences HLA class II expression) (RFX3), transcript variant 2, mRNA.	2.13
ILMN_7089	NM_005025	SERPINI1	serpin peptidase inhibitor, clade I (neuroserpin), member 1 (SERPINI1), mRNA.	2.08
ILMN_8765	NM_177422	EIF2C3	eukaryotic translation initiation factor 2C, 3 (EIF2C3), transcript variant 2, mRNA.	2.08
ILMN_29976	NM_006286	TFDP2	Transcription factor Dp-2 (E2F dimerization partner 2) (TFDP2), mRNA.	2.08
ILMN_21620	NM_134428	RFX3	regulatory factor X, 3 (influences HLA class II expression) (RFX3), transcript variant 2, mRNA.	2.08
ILMN_14828	NM_031421	TTC25	tetratricopeptide repeat domain 25 (TTC25), mRNA.	2.08
ILMN_7829	NM_002557	OVGP1	Homo sapiens oviductal glycoprotein 1, 120kDa (mucin 9, oviductin) (OVGP1), mRNA.	2.08
ILMN_15139	NM_000846	GSTA2	glutathione S-transferase A2 (GSTA2), mRNA.	2.04
ILMN_16141	NM_152909	ZNF548	zinc finger protein 548 (ZNF548), mRNA.	2.04
ILMN_25508	NM_206919	ARL9	ADP-ribosylation factor-like 9 (ARL9), mRNA.	2.04

6.3.3 Confirmation of SOX11 microarray findings at the transcriptional and protein levels and in a second cell line

In order to validate our microarray findings, QPCR analysis was carried out on several selected genes using the same RNA used in our microarray experiment. The QPCR reactions were performed in triplicate and the double delta method was used to calculate the gene expression level.

A panel of eight representative genes (*NDP, NEDD9, HIST1H2BE, HIST1H2AE, SEMA3, BAG1, TUBB3, FILIP1, CYP39A, SCG2, CD24*) with a robust fold change in microarray data (Figure 6.2 A) were chosen for validation using QPCR on the same RNA as used in the microarray experiment. Gene 18sRNA was used to normalise data from SOX11 over-expression and control samples and values are expressed as fold-change in gene expression in comparison to the control samples (HEK293 cells tansiently transfected with pDEST40 plasmid). Results of this analysis are shown in (Figure 6.2B).

To determine if SOX11 regulates similar targets in an alternative cell line, SH-SY5Y cells were transiently transfected with the SOX11-expressing construct. Western blot analysis was carried out on two proteins, SCG2 and TUBB3, which displayed up-regulation in the microarray analysis. Both showed clear up-regulation of expression in comparison to 'empty vector' or 'no DNA' control transfections when normalised to YWHAZ protein loading control (Figure 6.2C).
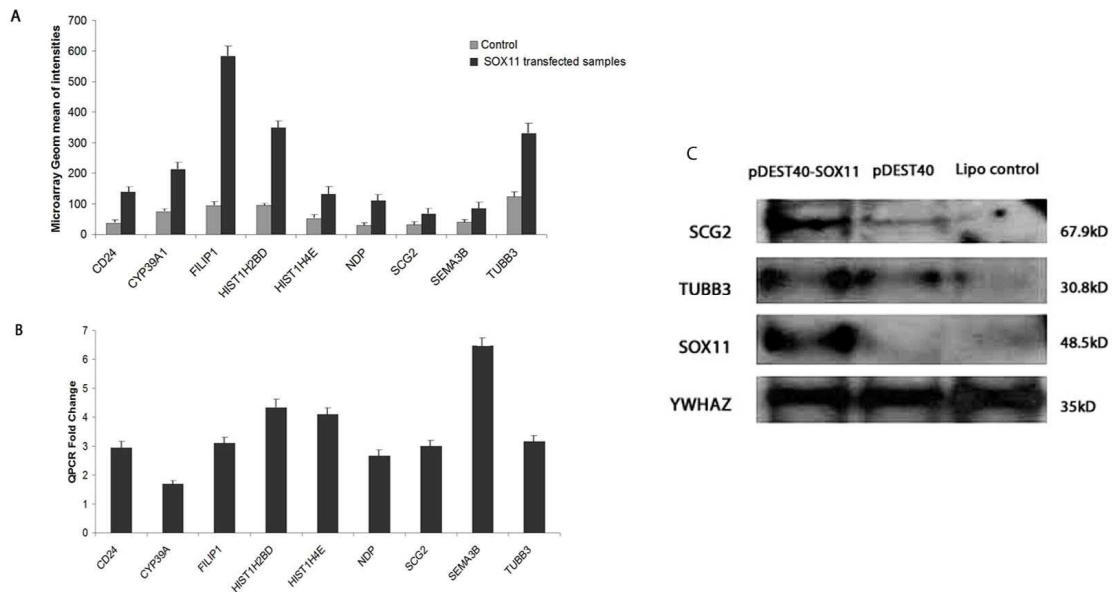
Figure 6.2. QPCR and Western blotting verify microarray findings.

Expression of CD24, CYP39A1, FILIP1, HIST14E, HIST1H2BD, NDP, SCG2, SEMA3B and TUBB3 determined by microarray (A) and QPCR (B). (A) Microarray signal intensities are expressed as an average of cDNA triplicates from HEK293 cells treated lipofactemine (as control) and cDNA triplicates from SOX11-overexpressed HEK293 cells. (B) fold-change values in the QPCR figure were calculated from triplicated analysis of the same RNA used in the microarray assays. (C) SH-SY5Y cells were transiently transfected with plasmids pDEST40-*SOX11* or empty pDEST40, or a Lipofectamine alone control and cultured for 48h. YWHAZ was used as a reference protein. The protein levels of TUBB3 and SCG2 were significantly up-regulated in the cell that over-expressed SOX11 protein.

6.3.4 Kinetics of SOX11 activity: time-course Q-PCR

To assess the kinetics of up-regulation of selected genes from the microarray, a time-course QPCR experiment was carried out. This would provide information on how quickly SOX11 acts on target gene promoters (or perhaps if some of the targets were indirectly/secondarily regulated). Transient transfection of SOX11 was followed by harvesting of RNA at 0h, 3h, 6h, 12h, 24h and 48h (the time-point at which microarray analysis was performed). We compared the temporal changes in expression of several upregulated genes *FILIP1, CYP39A1, SEMA3B, SCG2, GSTA2, TUBB3,* relative to control genes (Figure 6.3). These genes were chosen based on the following criteria: 1) these genes were identified as candidate molecules participating in the process of early neuron development, and 2) these genes can show fairly robust changes in our microarray list (fold change more than 2).

Comparing the fold changes of these genes revealed a dynamic set of expression profiles that fell into 3 sequentially and transiently upregulated groups: 1*) CYP39A1* and *TUBB3* were increased at 6h, reached their peaks by 24h, 2) *GSTA2, SCG2* and *SEMA3B* were up-regulated by 12h, 3) *FILIP1* was up-regulated and reached its peak at 24h. This result suggests that regulation mechanisms by SOX11 on these genes might be different. The detail of the possible mechanisms will be discussed in the discussion section.
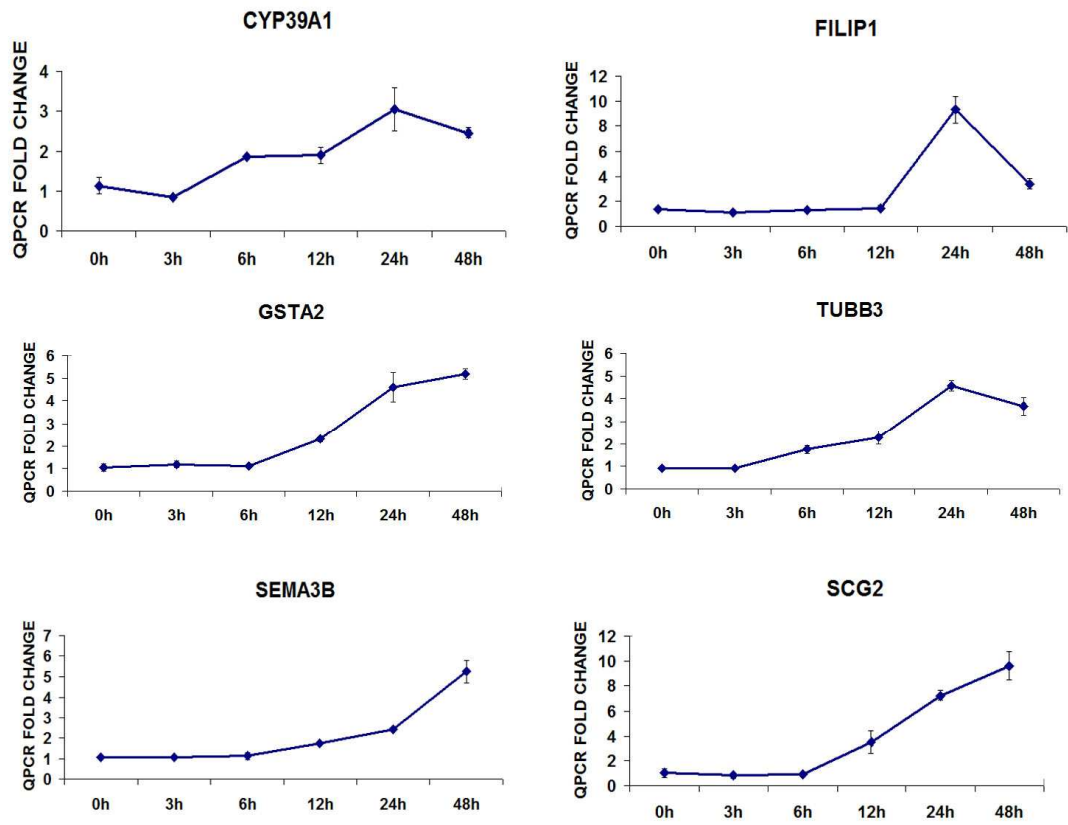
Figure 6.3 Timecourse QPCR of SOX11 transiently transfected HEK293 cells. RNA obtained at different time point (from 0h-48h) after HEK293 cells were transiently transfected with SOX11. Each time point represents fold change (mean +/- s.d. of assay triplicates) in comparison to gene expression in HEK293 cells transfected by control plasmid (pDEST40).

6.3.5 IPA analysis of SOX11 microarray data

Ingenuity Pathway Analysis (IPA) was used to identify canonical networks and biological functions/ gene ontology over-represented within the list of 251 SOX11 up-regulated genes with a fold change more than 1.5. IPA functional analysis produces three primary categories of output: molecular and cellular functions; physiological system development and functions; diseases and disorders. Several neurogenesis relevant functions and neurological diseases were over-represented in the dataset (Table 6.2) including cell cycle, cell death, cell signalling, cell-to-cell signalling and interaction, cell growth and proliferation, gene expression (regulation), and lipid metabolism. 18 SOX11 up-regulated genes were also shown to be involved in nervous system development and function with roles in chemotaxis, growth cone collapse, and familial encephalopathy with neuroserpin inclusion bodies, transformation, senescence, cell death, and leakage.

Table 6.2 Functions and Diseases

RELEVANT FUNCTIONS & DISEASES	RELEVANT GENE SYMBOL	MOLECULES
Cell Cycle	*CDKN2B, CDKN2C, COMMD5, HOXA10, IL8, IL1A, ING4, POLB, PPM1D, PPP1R15A (includes EG:23645), PPP2R2A, SKIL, TFDP2, CXCL12,IVNS1ABP*	16
Cell Death	*CXCL12, IL8, IL1A, MCL1, TUBB3, CDKN2C, ANTXR1, POLB*	9
Cell Signaling	*CBLB, IL8*	2
Cell-To-Cell Signaling and Interaction	*AKT2, CBLB, CD24, CXCL12, IL8, IL1A, NME1, PPP1R15A 9includes EG:23645), PLXNB1, NEDD9, GJC2, SEMA3B*	12
Cellular Growth and Proliferation	*CXCL12, IL8, PBX1, MSI2, CBLB,*	6
Developmental Disorder	*PHF6, PBX1, HOXA10, TGIF1,*	5
Gene Expression	*AKT2, IHPK2, IL8, IL1A, SOCS4, GABRB2, EIF5, HOXA10, NME1,*	10
Lipid Metabolism	*CYP39A1, PROX1, AKR1C3, PLA2G4C (includes EG:8605), IL1A, CXCL12, ETNK1, IL8*	8
Nervous System Development and Function	*CD24, CXCL12, DPF1, DPYSL4, MAP2, NDP, PCDHB12 (includes EG:56124), POLB, ST8SIA4, TNRC4, GJC2, CHN1(includes EG:1123), PLXNB1, SEMA3B, DLG2*	18
Neurological Disease	*CXCL12, IL8, SERPINI1, HOXA10, CDKN2B,*	5
Visual System Development and Function	*CXCL12*	2

6.3.6 Several neurogenesis and psychiatric illness candidate genes are regulated by SOX11

SOX11 is enriched in developing neurons, oligodendrocytes and astrocytes in human brain (Cahoy et al., 2008). Our hypothesis is that it is involved in the processes involved in CNS development and disease was supported by the microarray data as they revealed several genes with known involvement in adult and developmental neurogenesis as well as genes with established links to neuropsychiatric illness. These genes include:

1) Glypican-2, a heparan sulphate proteoglycan. It is significantly up-regulated by SOX11 in our microarray list (Fold change:4.225). It was reported associated with developing nervous system in axons growth cones (Kurosawa et al., 2001). In our study, Gpc2 is found expressed in the inner side of subgranular zone of adult mouse hippocampus, with very low expression in other parts of the brain. This expression pattern is quite similar to those markers for early axon growth from new neurons. These findings suggest that SOX11 maybe involved in the process of axon growth through its transcriptional activity on other genes.

2) *Scg2* (Secretogranin II/Chromogranin-C) is a cell surface sialoglycoprotein. Among many functions it has been shown that SCG2 promotes the differentiation of neuoblastoma cells into neurons. It was reported that Schizophrenia in humans is associated with upregulation of human SCG2 mRNA in dorsolateral prefrontal cortex (Hakak et al., 2001). And also Secretoneurin II, a peptide derived by endoproteolytic processing from SCG2 in brain, can promote the outgrowth of

immature cerebellar granule cells (Shyu et al., 2008). Also SCG2 is a marker for schizophrenia in cerebrospinal fluid (Bartolomucci et al., 2010).

3) Our microarray results show that FILIP1 is the top unregulated gene by SOX11. It interacts with Filamin A and plays a regulatory role in neocortical cell migration from ventricular zone (Nagano et al., 2002).

4) NDP is another SOX11 up-regulated gene with robust expression change. NDP is a secreted protein with a cystein-knot motif that activates the Wnt/beta-catenin pathway. It forms disulfide-linked oligomers in the extracellular matrix. *NDP* was isolated as a candidate gene for the Norrie Disease gene which is associated with congenital blindness sometimes associated with mental retardation and hearing loss (Chen et al., 1993). The characterization of deletions of this gene in Norrie disease and function analysis was carried out by Chen et al (Chen et al., 1993). NDP protein acts as an alternative to wnt ligand on the FZD4 receptor and has been implicated in angiogenesis.

5) TUBB3 is on the list of significantly up-regulated genes and also shows significant upregulation in SOX11-overexpressed SHSY5Y cells (Figure 6.2, western result). TUBB3 is abundant in the brain and widely regarded as a marker for differentiating neurons (Katsetos et al., 2003). Tubb3 has three potential binding sites for Sox4 and Sox11, which are located upstream of the Tubb3 transcriptional start site and has therefore been previously suggested as a target gene (Bergsland et al., 2006; van Beest et al., 2000). A recent paper highlights the role of TUBB3 in axon

guidance in a human CNS syndrome with behavioural phenotypes (Tischfield et al., ; Tischfield and Engle, 2010).

6) CD24 is in the top 10 of the genes up-regulated by SOX11. It is a small, glycosylphosphatidylinositol-anchored membrane protein, plays a role in B-cell development and neurogenesis (Calaora et al., 1996; Poncet et al., 1996). In adult mouse CNS, the expression of Cd24 is restricted to differentiating neurons in the subventricular zone and dentate gyrus and is a frequently used biomarker for neurogenesis in the brain (Belvindrah et al., 2002; Nieoullon et al., 2005).

7) *ST7*, also known as *RAY1 or FAM4A1*, is disrupted by a translocation breakpoint in a patient diagnosed with autism (Vincent et al., 2000; Vincent et al., 2002)

6.3.7 Histone and zinc finger genes and chromosomal domain regulation by SOX11

Thirteen histone genes were up-regulated by overexpression of SOX11. Closer inspection showed that many were present within a cluster on chromosome 6. We therefore reassessed the microarray fold-change data in relation to gene chromosomal coordinates and observed apparent clustering of up-regulated genes in several areas, with chromosomes 6p22.2 and 19q13.43 showing the most robust findings (Figure 6.4 a, b and c). This suggested that the transcriptional effects of SOX11 might be indirect in some circumstances: mediated through the alteration of regional chromatin state rather than by direct promoter effects. Some circumstantial evidence for this is provided by the bell-shaped distribution of SOX11 histone gene up-regulation in the chromosome 6 cluster suggesting a spreading and attenuating influence from a central regulatory point. The subsets of genes that are present within these two clusters are listed in Table 6.3. Most of them are members of the histone or zinc finger families. The chromosome 6 region is the site of a very promising genetic finding relating to increased risk of schizophrenia. A genome-wide association study found multiple single nucleotide polymorphism (SNP) associations. Four of the most significant markers, SNPs rs6913660, rs13219354, rs6932590, and rs13211507, are found within our SOX11-regulated cluster (Purcell et al., 2009; Stefansson et al., 2009). This association has been previously interpreted to indicate a role for the nearby HLA genes in disease risk (Figure 6.4b). However, we now suggest that SOX11 exerts its transcriptional control by altering chromatin conformation
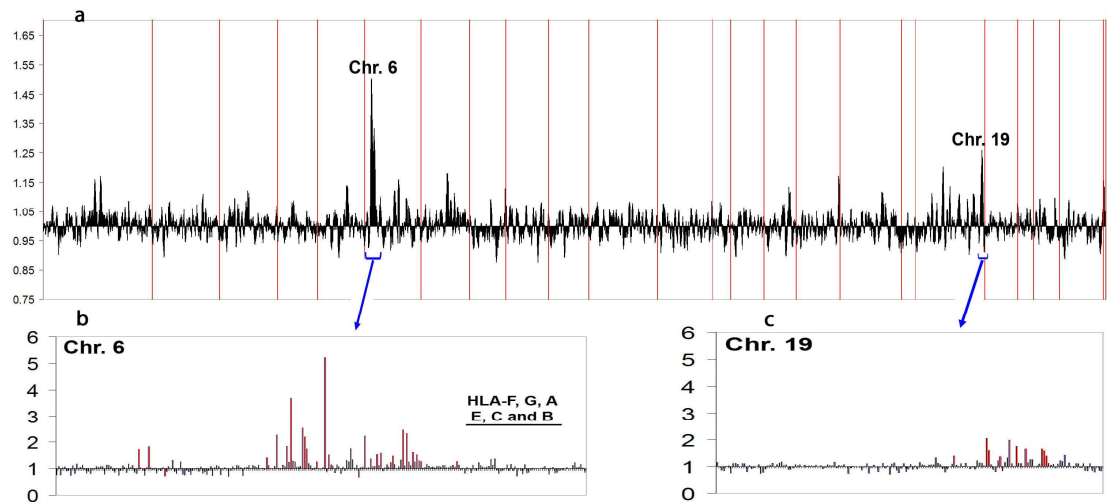
Figure 6.4 Clustering of SOX11-regulated genes.

Average fold-changes in a sliding window of 30 genes along the length of all chromosomes were calculated and plotted (top). Strong peaks indicating clustered up-regulation of genes were observed on chromosomes 6 and 19 (below). In the lower figures, red bars indicate statistically significant gene up-regulation (predominantly histone/zinc finger genes) as determined in the SAM analysis. The horizontal black bar indicates the approximate location of the HLA gene cluster

Table 6.3 Significantly (SAM) regulated genes, shown in order, that are found within clusters on chromosomes 6 and 19. The two genes highlighted in bold show down-regulation, the rest are up-regulated. Histones and zinc finger proteins comprise the majority of the list.

Cluster	Gene symbol	Acc. No.	Fold-change
Chr.6	*MAK*	NM_005906	1.73
Chr.6	*NEDD9*	NM_182966	1.82
Chr.6	*HIST1H4B*	NM_003544	1.41
Chr.6	*HIST1H1C*	NM_005319	2.27
Chr.6	*HIST1H2AC*	NM_003512	1.84
Chr.6	*HIST1H2BD*	NM_138720	3.69
Chr.6	*HIST1H2BE*	NM_003523	1.28
Chr.6	*HIST1H4E*	NM_003545	2.53
Chr.6	*HIST1H2BG*	NM_003518	2.21
Chr.6	*HIST1H2AE*	NM_021052	1.75
Chr.6	*HIST1H3F*	NM_021018	1.25
Chr.6	*HIST1H4H*	NM_003543	5.21
Chr.6	*BTN2A2*	NM_181531	1.53
Chr.6	*BTN3A1*	NM_007048	1.16
Chr.6	*ZNF184*	NM_007149	2.23
Chr.6	*HIST1H3H*	NM_003536	1.36
Chr.6	*HIST1H4K*	NM_003541	1.54
Chr.6	*HIST1H2BN*	NM_003520	1.60
Chr.6	*HIST1H2AM*	NM_003514	1.48
Chr.6	*ZNF435*	NM_025231	2.47
Chr.6	*ZNF193*	NM_006299	2.32
Chr.6	*ZNF187*	NM_001023560	1.62
Chr.6	*ZNF323*	NM_030899	1.53
Chr.6	*ZNF96*	NM_014724	1.27
Chr.6	*UBD*	NM_006398	1.28
Chr.19	*ZNF71*	NM_021216	1.39
Chr.19	*ZNF548*	NM_152909	2.05
Chr.19	*ZNF17*	NM_006959	1.60
Chr.19	*MGC4728*	NM_198542	1.36
Chr.19	*ZIK1*	NM_001010879	1.99
Chr.19	*ZNF211*	NM_006385	1.76
Chr.19	*ZNF671*	NM_024833	1.65
Chr.19	*ZNF256*	NM_005773	1.66
Chr.19	*C19orf18*	NM_152474	1.58
Chr.19	*ZNF606*	NM_025027	1.41
Chr.6	***SIRT5***	**NM_012241**	**0.72**
Chr.6	***PRSS16***	**NM_005865**	**0.67**

6.3.8 The expression patterns of SOX11 regulated genes in dentate gyrus of the hippocampus

The expression pattern of Sox11 in the adult mouse hippocampus, the site of neurogenesis that continues into adulthood, was investigated using immunofluorescence microscopy. Sections from frozen mouse brain were cut using a thermo cryostat. The following antibody solutions and dilutions were used: 1/500 Sox11 rabbit anti mouse, 1/400 Gpc2 goat anti mouse (Santa Cruz Biotechnology). The sections were mounted on slides with Prolong anti-fade reagent with 4'6-diamidino-2-phenylindole (DAPI). Sox11 was found to be expressed in many brain regions including the whole dentate gyrus, in accordance with the literature. The distribution of several Sox11-regulated genes was examined. Gpc2, a gene up-regulated by SOX11 upregulated gene was localised to the inner sub-granular zone of dentate gyrus, where neurogenesis occurs, and had much lower expression in other regions of dentate gyrus (Figure 6.5).

As SOX11 is down regulated by NPAS3 in the microarray data set, colocalization between these two proteins in hippocampus were looked for. However, no overlap of expression was found in these experiments. (NPAS3 expression pattern in mouse hippocampus result is shown Figure 3.1)
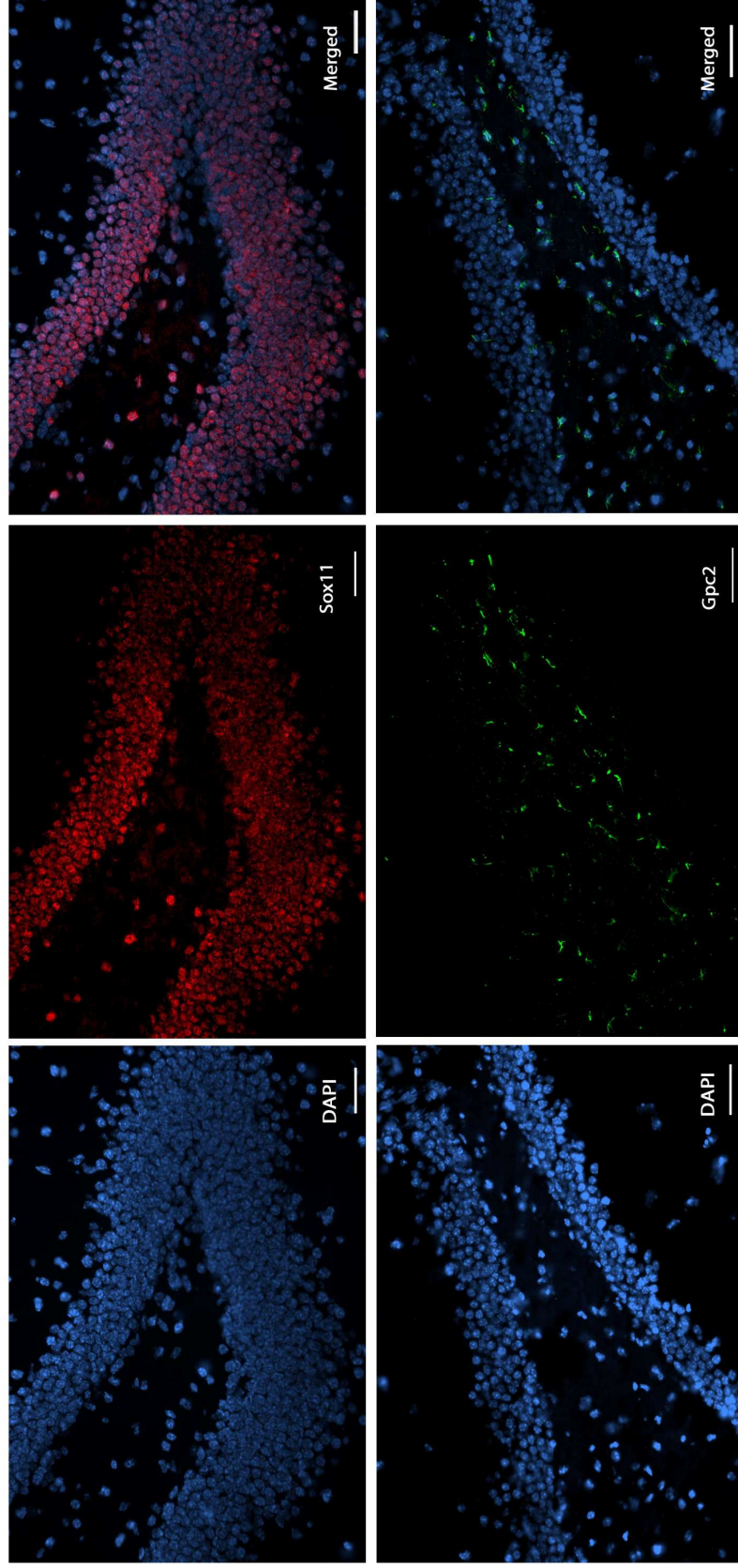
Figure 6.5 Sox11 and Gpc2 expression patterns in the dentate gyrus region of mouse hippocampus

Sox11 and Gpc2 protein expression was examined using immunofluorescence on frozen mouse brain sections. Sox11 shows a nuclear distribution in all dentate gyrus granule cells. Gpc2 expression is highly enriched in the subgranular zone where neurogenesis occurs. Localization is often seen in short axonal projections towards the CA3 region. Scale bar: 80µm

6.4   Discussion

A cell line-based over-expression study coupled with microarray analysis was carried out to characterise the set of genes regulated by SOX11. Evidence suggests that a number of SOX11 targets may play a role in the maturation and differentiation of neurons as predicted by previous studies.

Sox11 is expressed in the subventricular zone of lateral ventricles and the subgranular zone of the dentate gyrus, But Sox11 is strictly localized in Dcx-expressing neuronal precursors and immature neurons, but not Sox2-expressing cells. (Haslinger et al., 2009), *Doublecortin (Dcx)* is a neurogenesis marker which is expressed in newly formed neurons between the timing of their birth and final maturation (Brown et al., 2003). This suggests that Sox11 is involved in the transcriptional regulation of the specific stage of converting immature neurons into final maturation in adult neurogenesis. Although Sox11 does not show colocalization with Npas3 in the adult mouse hippocampus, they are both expressed in Dcx-positive cells. This suggests that they might have the same temporal and spatial roles in the process of adult neurogenesis.

In the analysis of relative fold change in gene expression for different time points, several genes responsible for adult and developmental neurogenesis and psychiatric disease are differentially expressed by more than 2 fold after over-expression of SOX11. The fold changes of several genes, like *CYP39A1, TUBB3, and FILIP*, are increased before 24h, but significantly decrease after 48h. These results are consistent with the possibility that SOX11 might be a part of a special transcriptional

regulatory network. Other factors, which depress gene expression, may also be activated by the overexpression of SOX1 and together with SOX11 might function as a network to control the expression of pivotal genes in different stage of neurogenesis.

SOX11 may be regulated by several different mechanisms. These include binding to the promoter region directly or through the alteration of local chromatin states (Pevny and Lovell-Badge, 1997). These hypotheses are consistent with the finding that clusters of genes are regulated by SOX11. At least two clusters of SOX11 targets exist, located on chromosomes 6 and 19. And it might be the case that gene duplication events in these clusters have produced multiple SOX11-regulated genes. This could explain the activation of that SOX11 target genes at different time points.

The presence of non-histone/zinc finger genes in Table 6.3 suggests that SOX11 exerts its control over the region through indirect means. It has been previously suggested that SOX genes can act by altering chromatin conformation (Wilson and Koopman, 2002). This may be compatible with a regulatory model whereby all genes within a given 'loop' of chromosomal DNA are affected by the local chromatin state and its control. The zinc finger genes are often transcription factors themselves while the histone genes regulate chromatin state; together these targets may be responsible for a co-ordinated programme of genome regulation promoting neuronal fate commitment and neuronal gene expression.

Recent genome-wide association studies in schizophrenia have identified the strongest susceptibility signal over the chromosome 6 cluster (Purcell et al., 2009; Shi et al., 2009; Stefansson et al., 2009). The genes on chromosome 6 involved in schizophrenia are not yet identified and it is a possibility that the association signal is due to the involvement of SOX11.

CHAPTER SEVEN

7 Discussion

In this thesis I have described experiments designed to study the biological functions of NPAS3 and other transcription factors in the hope that the results would help our understanding of its role in mental illness.

In order to pinpoint the location of NPAS3 activity in the brain and identify the biological processes NPAS3 regulates relevant to mental illness, I carried out Immunofluorescence on mouse brain sections and microarrays of NPAS3 over-expressing cell lines.

Strong Npas3 expression was seen in the hippocampal subgranular zone - the site of adult neurogenesis. It is found to be colocalised with Dcx, not other neurogenesis makers, in the dentate gyrus. NPAS3 and the SOX family of transcription factors shared many target genes suggesting overlapping neuro-developmental roles. Glycolytic and hypoxia-response genes were significantly enriched in the set of NPAS3 down-regulated genes. Additionally, in the circadian condition, NPAS3 activity was shown to be sensitive to circadian time-point and protein truncation.

Here, in the discussion chapter, I want to talk about the methods used, the implication of the findings and limitation of this work and future plan.

7.1 Why a HEK293 cell line was used in my microarray studies

The use of an over-expression model in the human epithelial kidney HEK293 cell line may not fully represent a transcription factor's role in a neuronal context but I believe that, in addition to the convergence of transcriptional and metabolic findings discussed below, three observations suggest the data are biologically relevant. Firstly, there is a consistency of target gene sets between different transcription factors, which suggests technical quality and biological relevance. Our microarray data matched the evolutionary distance between the SOX genes. For example, closely related SOX D members (SOX5 and SOX6) showed more internal overlap that with SOX E members (SOX9 and SOX10). If the data in HEK293 cells were just artefacts, these would not be expected. Secondly, the neuronal-exclusivity, or enrichment in comparison to kidney, of many up-regulated NPAS3/SOX targets such as *VGF*, *NEFH*, *CHGA*, *GALR2*, *GADD45B*, and *VASN* suggests that the HEK293 cellular phenotype does not preclude the expression of CNS genes. Indeed, there is some speculation (http://www.fda.gov/ohrms/dockets/ac/01/transcripts/3750t1_01.pdf) from the laboratory that originally derived the cell line in 1973 that HEK293 cells might actually be of neuronal origin because of the expression of many neuronal markers (including NEFH mentioned above) and because neuronal cells are readily transformed by the adenovirus 5 used. Thirdly, as our experiments involve transfecting a gene of interest and then analyzing the expression profile, the behaviour of the cell itself is not of interest. It is well known that HEK293 cell is a particularly good model for this kind of experiment (Malagon et al., 2009; Tominaga, 2010).

7.2 Why not use a stem cell model to look at the neurodevelopmental role of NPAS3?

Human adult neuronal stem cells originated from hippocampus might be a good cell model to clarify the change in adult hippocampal neurogenesis in response to NPAS3 or SOX11. However, the progeny of stem cell division that normally undergo a strictly limited number of replication cycles *in vitro* and the efficiency of neurosphere transfection are very low (Doetsch et al., 2002). The small number of cells makes the synthesis of microarray probe very difficult. Therefore, it is very difficult to obtain a set of labelled cRNA with high RIN value from neural stem cell.

Furthermore, the gene expression environment provided by human neuronal stem cells is still quite different from *in vivo,* as neural stem cells change properties in culture. The neurosphere-derived cells do not behave as stem cells when transplanted back into the brain (Marshall et al., 2006). Based on all these reasons, HEK293 cells were chosen to be used in our studies. The approach offers a simple, high-throughput and reproducible analysis of transcriptional activity

7.3 NPAS3 interacts with several SOX members

Doublecortin (Dcx) is a neurogenesis marker which is expressed in newly formed neurons between the timing of their birth and final maturation (Brown et al., 2003). Sox11 is strictly expressed in Dcx-expressing, non-committed precursors and immature cells in the neurogenic area of adult brain, but not with Sox2-expressiong cells (Haslinger et al., 2009). In this study, Npas3 was also found to be colocalised with Dcx in cells with a differentiating morphology in the adult mouse hippocampus. Expression patterns of NPAS3 and SOX11 in the Dcx-expressing cells suggest that these two factors may exert their function during the same stage of neurogenesis.

SOX D and SOX E members are involved in the regulation of the later stage of adult neuogenesis. SOX5 is involved in the regulation of neurogenesis by controlling cell cycle progression in neural progenitors (Martinez-Morales et al., 2010). Another SOX gene studied, SOX9, has been shown to be important for differentiation of SVZ stem cell into neurons through miR-124 mediation (Cheng et al., 2009). In addition, mRNA of SOX10, a highly similar transcriptional factor to SOX9, was reduced in the hippocampus and anterior cingulated cortex of schizophrenia patients (Dracheva et al., 2006).

Based on this information, I hypothesise that SOXD, SOXE, SOX11 and NPAS3 exert their functions at similar timing of neurogenesis, probably after the birth of newly formed neurons and during their final maturation. As transcriptional regulators, whether there are any interactions between these genes. Thus, microarray studies were carried out to investigate the overlaps of the regulatory profile of these

transcription factors. We demonstrated that NPAS3 participates in a neuro-developmental regulatory network overlapping and directly interacting with the SOX transcription factor family. The identification of multiple targets of these transcription factors should clarify their regulatory roles in embryonic and adult neurogenesis - processes that seem to correlate with the risk of depression and psychosis (Kempermann et al., 2008). We propose that the overlap between NPAS3 and SOX5/6 target genes may reflect their activities downstream of the proliferative transcriptional stage, perhaps at the differentiation and maturation stages.

7.4 Altered metabolite levels in brain tissue from Npas3 knockout mice

We collaborated with Lynsey MacIntyre and Dr. David Watson at Strathclyde University and Dr. Steven Clapcote at Leeds University to investigate metabolic changes in the brain of *Npas3* (-/-) mice using high-resolution mass spectrometry-based metabonomic analysis. This work is not directly part of my thesis – it is included in a collaborative paper submitted for publication on which I am joint first author (Sha, submitted) - but produced data that are highly relevant to my microarray analysis which I will discuss now.

Four half-brains from homozygote knockout (KO) and four half-brains from wild-type littermates were homogenised and extracted using solvents and then separated and identified using a Finnigan LTQ-Orbitrap fitted with a Surveyor HPLC pump. Many cellular metabolites showed changed levels in the knockout brain tissue. Decreased levels of dihydroxyacetone phosphate/glycerone phosphate and octulose-1,8-bisphosphate in the KO (6% and 5% of wild-type levels, respectively) and increased levels of NAD^+, cystathione and dTDP-glucose/galactose (12.4-, 3.7- and 3.4-fold increases, respectively) were found in this study (Appendix table S6 a-d).

It is very interesting that there is a large (over 12-fold) increase in the oxidised form of the coenzyme nicotinamide adenine dinucleotide (NAD^+). This may indicate a slow-down in glycolysis rate (which produces the reduced form, NADH) or abnormally oxidative conditions in the cell. UDP-glucose/galactose, were increased

in knockout brain (TDP-glucose/-galactose level changes might be similarly explained), which indicates that ineffective glycolysis is possibly resulting in glucose build-up and storage as glycogen. In the same way, the altered concentrations of Sedoheptulose and Octulose-1,8-bisphosphate may indicate that ineffective glycolysis is impacting on the pentose phosphate pathway which commences with Glucose-6-phosphate. The decreases in Fructose 1,6-bisphosphate and Dihydroxyacetone Phosphate (DHAP) directly suggest glycolysis abnormalities. Further speculation indicated that there may be a direct link between DHAP decrease and the observed increase in another metabolite, Glycerol-3-Phosphate (G-3-P). To recycle NADH to NAD^+ while simultaneously transferring the reducing power to the oxidative phosphorylation process within the interior of the mitochondrion, the glycerol-3-phosphate shuttle is employed at the outer mitochondrial membrane. This converts DHAP and NADH into G-3-P and NAD^+ , catalysed by cytosolic G-3-P Dehydrogenase (GPD1); with the reverse process catalysed by mitochondrial GPD2 generating reduced FADH within the mitochondrial matrix for subsequent energy production. A decrease in the efficiency of the reverse reaction (perhaps due to mitochondrial dysfunction) might contribute to shuttle failure and the altered levels of DHAP, G3P and NAD^+ observed in knockout brain. Also correlated with low DHAP were reduced levels of lactoyl glutathione which can be formed from DHAP via methylglyoxal.

The transcriptional inhibition of argininosuccinate synthase (*ASS1*) expression by NPAS3 over-expression was mirrored by significant changes in its substrates L-citrulline (increased) and aspartate (decreased) in *Npas3* knockout mice. *ASS1*

deficiency causes classic citrullinemia (MIM #215700) which can present with manic episodes and psychosis in adult patients (Ikeda et al., 2001).

There is a high consistency between the metabolic findings in the NPAS3 overexpression cells (microarray analysis) and knockout mouse brain (mass spectrometry analysis). Genes in the glycolysis pathway have been switched off in the NPAS3 overexpressed cells. This implicates that in the Npas3 (-/-) mice brain; the glycolysis pathway might be switched on (activated). In the *Npas3* (-/-) mice brain, concentrations of upstream compounds of glucose-6-p are higher than normal condition. But as the genes which control the downstream of glucose-6-p in the glycolysis pathway are activated due the loss of Npas3, the downstream compounds, fructose-1,6-bisP and dihydroxyacetone-p, in the glycolysis pathway are metabolized much more quickly than normal condition.

Pyruvate produced by glycolysis is transported across the inner mitochondrial membrane into the matrix and formed CO_2 and acetyl-CoA and NADH (Voet, 2006). The acetyl-CoA is the substrate for the TCA cycle (also called Krebs cycle). The enzymes of the TCA cycle are located in the mitochondrial matrix, except the succinate dehydrogenase, which is a part of ComplexII of the inner mitochondria membrane (King et al., 2006). In the *Npas3* (-/-) mice brain, concentrations of α-Ketoglutarate and Succinate, both are the compounds of TCA cycle, are higher. This implies that the TCA cycle in the mitochondria of brain might be accelerated due to the loss of Npas3. Whether the abnormal energy production from the TCA could affect other aspects of mitochondrial function is not yet determined. Is the abnormal

energy metabolism in the mitochondria is associated with the lack of neurogenesis in the Npas3 (-/-) mice?

However, the metabolic study by the mass spectrometry analysis is based on the whole brain (not just subgranular zone). In a whole organism, the effects of loss of Npas3 might be modified by homeostasis, energy control by other factors etc. Thus, study in the NPAS3 effects in a simple model (autonomous cells) is very necessary.

Glycogen

UDP-Glucose ——— UDP-Galactose

Glucose-1-P　Glucose

PGM5 (1.95)　HK2 (2.13)

Glucose-6-P ——→ 'Pentose Phosphate Pathway'

Fructose-6-P —— PFKFB4 (1.65) —— Fructose-2,6-bisP

PFKP (1.82)

Fructose-1,6-bisP　'Glycolysis'

ALDOC (2.90)
ALDOA (1.59)

Dihydroxyacetone-P —— Glyceroaldehyde-3-P

FADH2

GPD1　GPD2　GAPDH (1.62)　NADH

NAD+

Glycerol-3-P

'G-3-P shuttle'

Bisphosphoglycerate

PGK1 (1.85)

3-Phosphoglycerate

2-Phosphoglycerate

ENO2 (1.94)

Acetate

ALDH2 (1.5)

Acetaldehyde ——— Phosphoenolpyruvate

PCK2 (2.15)

Ethanol

Pyruvate

pyruvate dehydrogenase complex　NADH

PDK4 (1.53)

Acetyl-CoA

Glutamine

Carbamoyl-P

Citrulline

Aspartate

ASS1 (2.03)

Ornithine

Urea

Arginine

Arginine Succinate

'Urea cycle'

GENE (fold decrease)

metabolic change

'TCA Cycle'

α-Ketoglutarate

NADH　Isocitrate　Succinyl-CoA　NADH

Citrate　Succinate

Oxaloacetate　Fumarate

NADH　Malate

Figure 7.1 Three metabolic pathways with coincident findings from the transcriptomic and metabonomic analyses.

These were glycolysis, the tricarboxylic acid (Citric acid/Krebb's) cycle and the urea cycle. In each case the enzyme or regulatory protein is listed in italics with transcript fold down-regulation upon NPAS3 over-expression shown in brackets. Heavy black arrows indicate the direction by which metabolite abundance is changed in the knock-out mouse brain. The Glycerol-3-phosphate shuttle uses the interconversion of DHAP and G-3-P to transfer reducing power from cytosolic NADH to mitochondrial FADH$_2$. One interpretation of the metabolic changes in knockout brain invokes a reduction in mitochondrial GPD2 function resulting in reduced shuttle efficiency and the observed directional concentration changes of DHAP, G-3-P and NAD$^+$. Emboldened –P indicates Phosphate.

7.5   Comparing my NPAS3 findings with newly published data.

Recently Pieper et al. (Pieper et al., 2010) identified P7C3, an aminopropyl carbazole, which has proneurogenic activity by protecting newborn neurons from apoptosis. Their results are summarised in Figure 7.4 A. Prolonged administration of P7C3 increased Dcx+ neurons in mouse hippocampus. In collaboration with Wang et al., they showed that P7C3 protected mitochondrial membrane integrity, thus preventing apoptosis. An intrinsic pathway leading to apoptosis emanates from mitochondria (Liu et al., 1996; Yang et al., 1997). NPAS3 (-/-) mice were reported with devoid of hippocampal neurogenesis and display malformation and electrophysiological dysfunction of the dentate gyrus (Erbel-Sieler et al., 2004; Pieper et al., 2005) (Figure 7.2 A). Prolonged administration of P7C3 to Npas3 (-/-) mice corrected these deficits by normalising levels of apoptosis of newborn hippocampal neurons (Pieper et al., 2010)

In this PhD project, Thirty-six of the 282 up-regulated genes (corrected hypergeometric p-value = 2.07 X 10^{-8}) were either transcription factors or DNA-binding proteins with regulatory function (Figure 7.2 B). Combined with Pieper's finding and our finding of the colocalization with Dcx in the dentate gyrus, we hypothesis that NPAS3 is involved in the regulation of neurogenesis at the stage of maturation and survival.

Glycolysis pathways were disturbed by altering NPAS3 activity. The increased levels of 2-Oxoglutarate/α-Ketoglutaric acid and Succinate in Kreb's cycle (TCA cycle), which is within mitochondria, were found in *Npas* (-/-) mouse brains. Linking with

the finding of Pieper et al. that P7C3 acts on deficient mitochondrial function and corrects the lack of hippocampus neurogenesis in *Npas* (-/-) mouse, I suppose that in the *Npas3* (-/-) mice, there might be some connection between neurogenesis and intervention in metabolic pathways, including glycolysis and TCA (Figure 7.2 B).
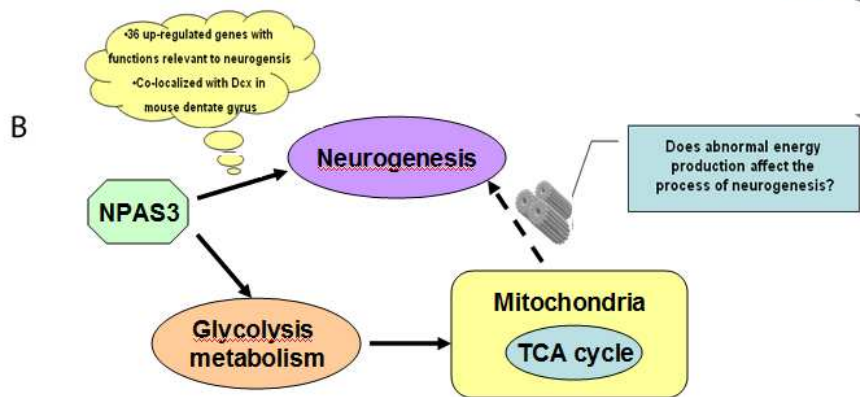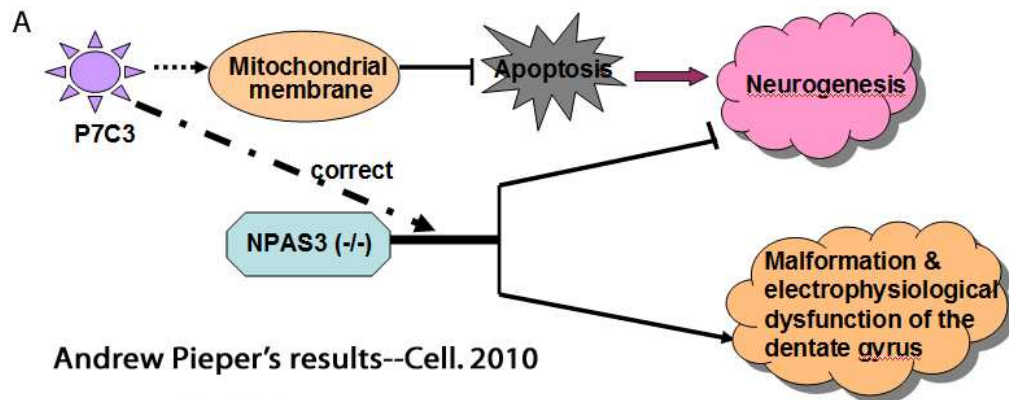
Figure 7.2 Comparison of my NPAS3 findings with Andrew Pieper's recent result. A: P7C3 increase neurogenesis by protecting new born neurons from apoptosis. P7C3 also can correct the malformation and electrophysiological dysfunction of the dentate gyrus in Npas3 (-/-) mice. B: Several transcription factors and genes involved in neurogenesis were upregulated in NPAS3 overexpression cells. Many genes in glycolysis pathways were also down regulated in NPAS3 overexpressing cells. Compounds involved in glycolysis, TCA cycle (within mitochondria), 'G-3-P shuttle' and Urea cycle are increased in the brain of Npas3 (-/-) mice.

7.6   A link between metabolism and circadian rhythms.

I think it is important to investigate the linkage of metabolic aspect of NPAS3 and effect of circadian rhythms on the regulatory prolife of NPAS3. There is ample experimental evidence suggesting that the circadian clock controls metabolic activity and metabolism feeds back to impinge upon the rhythm (Roenneberg and Merrow, 1999). There is much evidence for a functional link between metabolism and circadian rhythms in the brain. First, certain genes encoding metabolic enzymes and transport systems for energy metabolites are under circadian control, including glycogen phosphorylase (Harley and Rusak, 1993; Harley et al., 2001), cytochrome oxidase, lactate dehydrogenase (Rutter et al., 2001) and the monocarboxylate transporter 2 (MCT2). Secondly, glucose uptake in the SCN and other brain areas fluctuates in a circadian rhythm (Newman and Hospod, 1986; Schwartz and Gainer, 1977; Shibata and Moore, 1988). Finally, the concentration of ATP exhibits marked circadian fluctuation (Yamazaki et al., 1994).

Astrocytes can regulate the balance between neuronal activity and energy substrate uptake and utilization at the cellular level (Bergles and Jahr, 1998). Magistretti and Pellerin developed a model to explain how neural activity induces glucose uptake and glycolysis in astrocytes (Figure 7.3). Glutamate, which is from neuronal activity, can be absorbed by astrocytes via glutamate transporters and with concomitant Na+ transmission. Increased Na+ concentrations in the cell activates the astrocytic Na+/K+-ATPase, which can further activate glucose uptake and glycolysis (Pellerin and Magistretti, 1994; Pellerin and Magistretti, 1997). Lactate, the glycolysis product, is shuttled into neurons through two monocarboxylate transporters MCT1

and MCT2 to provide energy for these active neurons (Pellerin et al., 1998).

Strikingly, it was reported that the expression of a fly MCT-like gene is in a

circadian rhythm in the brain (Claridge-Chang et al., 2001)
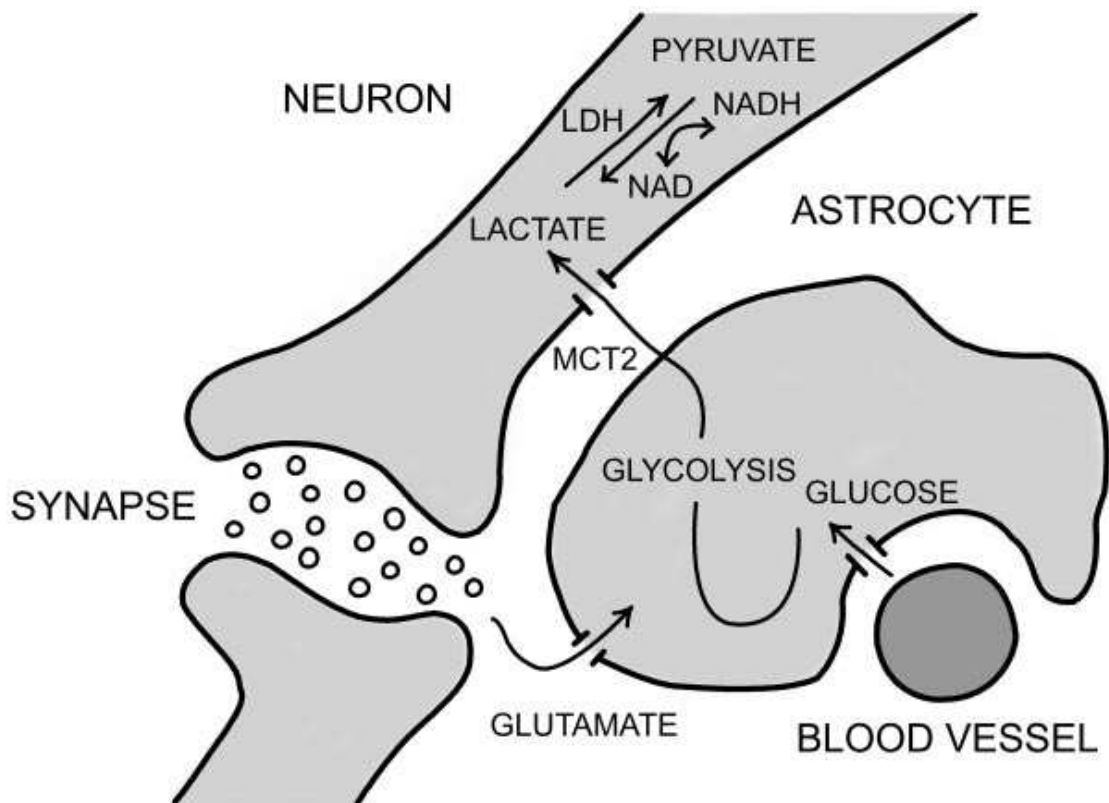
Figure 7.3 Model for electrical activity-induced change in neuronal redox status. An
increase in synaptic glutamate stimulates glycolytic flux and lactate export in
astrocytes. The monocarboxylate transporter, MCT2, facilitates neuronal uptake of
lactate, which increases the NADH:NAD ratio via the lactate dehydrogenase (LDH)
reaction. Adapted from (Magistretti and Pellerin, 1999).

The energy metabolites induced by neuronal activity possibly feed back on to the circadian rhythm. This was supported by two observations: first, Rats injected with 2-DG (2-deoxyglucose), which inhibits glucose utilization, have a blocked light-induced phase shift (Challet et al., 2000; Challet et al., 1999). Second, caloric restriction can entrain the rhythm of peripheral oscillators (Challet et al., 2000; Damiola et al., 2000; Stokkan et al., 2001).

This is related to what we see in our microarray results that genes involved in glycolysis were negatively regulated in cells over-expressing NPAS3. Also disrupted metabolic control, including glycolysis, was discovered in the brains of Npas3 (-/-) mice by mass spectrometry (Sha et al. submitted). The ternary plot results (Figure 5.4 b) also show that FLNPAS3 mainly exerts an inhibitory regulation on the circadian genes at +12 hours after circadian rhythm induction but not after +24 hours. Based on the model developed by Magistretti and Pellerin (Pellerin and Magistretti, 2004),we support the hypothesis that NPAS3 might inhibit the utilization of glucose which then blocks the normal circadian rhythm in the cell. There might be another possibility behind the phenomenon of the altered circadian in the HEK293 cells. Many genes controlling the circadian rhythm are members of the bHLH transcription family and they might have the properties of binding to NPAS3 or NPAS3's partner (such as BMAL)(Nievergelt et al., 2006). Therefore, there is an opportunity for these factors to interact with each other or to sequester common binding partners. These findings not only link psychiatric illness into the cycle composed of neuronal activity, energy metabolism and circadian rhythm, but also suggest the underlying mechanism at the molecular level.

7.7   Limitations of my work and Future directions worthy of study

This study of disease gene function in cell and animal models provides valuable clues to the understanding of the pathology of psychiatric illness. However, confirmation of these findings in patients will be necessary. NPAS3-mediated changes in metabolic activity may have a direct impact on brain imaging techniques such as functional magnetic resonance imaging or positron emission tomography that are sensitive to glucose and oxygen consumption. In the context of psychiatric patient studies, such imaging data may be a reflection of primary metabolic failures rather than the intended indirect measurement of regional neuronal activity. In my PhD project, this kind of experiments can not be carried out due to the limited time and funding.

Our study suggests that many NPAS3 regulated genes are involved in adult neurogenesis. Disturbance in the metabolic pathways can also affect the cell growth. Investigation of the roles of NPAS3 and SOX members in adult neural stem cells is an indispensable stage to understand and characterise how these genes contribute adult neurogenesis. Although we tried to isolate primary mouse hippocampus progenitor cells and purchased human hippocampus stem cell from European Collection of Cell Cultures (ECACC), functional studies on these models were not successful due to limited time.

7.8 Relating our observations to the known biology of psychiatric disorders

Although metabolic disturbance, including weight gain, insulin sensitivity and increased risk of diabetes (Obesity, 2004), is often found in psychiatric patients, this metabolic syndrome has often been attributed to side effects of the medical treatments (such as typical and atypical antipsychotic drugs) rather than inherent deficits. Several genes involved in glycolysis do appear to be involved in the response to drug therapies for psychiatric disorders. A gene expression study of lithium-regulated transcriptional changes on mice brain reveals that ALDOC was one of the top up-regulated genes (McQuillin et al., 2007). Olanzapine, one of the antipsychotic drugs, has been shown to increase ALDOA, ENO1, ENO2, PGAM1 and GAPDH protein expression (Ma et al., 2009a).

However, inherent vulnerability also contributes to metabolic disturbance in psychiatric diagnosis (van Winkel et al., 2008). Accumulating evidence suggests that glycolytic dysfunction (Martins-de-Souza et al.), mitochondrial failure, and oxidative stress (Do et al., 2009) contribute to disturbed metabolism in schizophrenia and bipolar disorder. Significant changes in multiple glycolytic enzyme levels were identified in peripheral blood monocytes of schizophrenia patients who were receiving no treatment in a recent proteomic analysis (Herberth et al., ; Herberth et al., 2010). My results show that metabolic pathways, including glycolysis, are disturbed by NPAS3. These NPAS3-mediated metabolic changes support the view that inherent deficits may also contribute to metabolic dysregulation.

It is still not clear whether risk of psychiatric illness contributed by NPAS3 is through deficits in the process of neurogenesis or metabolic regulation, or both are required for full expression of illness. We suggest that NPAS3 metabolism defects might have a particularly detrimental effect on labile new neurons produced in the fluctuating perfusion conditions present in the vascularised neurogenic niche (Nikolova et al., 2007; Palmer et al., 2000; Pereira et al., 2007). Hence, the therapeutic control of metabolism in mental illness may be a promising route for future exploration.

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APPENDICES

Appendix table S1: Circadian (control samples) +12hrs to +24hrs top differentially expressed genes.

Unique id	Description	Gene symbol	FoldDiff
ILMN_1751607	FBJ murine osteosarcoma viral oncogene homolog B (FOSB), mRNA.	FOSB	4.61
ILMN_1762899	early growth response 1 (EGR1), mRNA.	EGR1	4.27
ILMN_1743199	early growth response 2 (Krox-20 homolog, Drosophila) (EGR2), mRNA.	EGR2	3.44
ILMN_1669523	v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS), mRNA.	FOS	2.52
ILMN_1790317	RAB26, member RAS oncogene family (RAB26), mRNA.	RAB26	2.21
ILMN_1813386	coronin 6 (CORO6), mRNA.	CORO6	2.20
ILMN_1809931	N-myc downstream regulated gene 1 (NDRG1), mRNA.	NDRG1	2.14
ILMN_1700268	quinolinate phosphoribosyltransferase (nicotinate-nucleotide pyrophosphorylase (carboxylating)) (QPRT), mRNA.	QPRT	2.03
ILMN_1755974	aldolase C, fructose-bisphosphate (ALDOC), mRNA.	ALDOC	2.01
ILMN_2192694	eukaryotic translation initiation factor 3, subunit M (EIF3M), mRNA.	EIF3M	-2.67
ILMN_2205350	chromosome 6 open reading frame 66 (C6orf66), mRNA.	C6orf66	-2.67
ILMN_1781039	vacuolar protein sorting 26 (yeast) (VPS26), mRNA.	VPS26	-2.68
ILMN_1775829	PERP, TP53 apoptosis effector (PERP), mRNA.	PERP	-2.68
ILMN_2343332	TAF9 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 32kDa (TAF9), transcript variant 2, mRNA.	TAF9	-2.68
ILMN_2181540	YY1 transcription factor (YY1), mRNA.	YY1	-2.68
ILMN_2362368	U2 small nuclear RNA auxiliary factor 1 (U2AF1), transcript variant b, mRNA.	U2AF1	-2.69
ILMN_2121555	F-box protein 5 (FBXO5), mRNA.	FBXO5	-2.70
ILMN_2149400	SPC25, NDC80 kinetochore complex component, homolog (S. cerevisiae) (SPC25), mRNA.	SPC25	-2.71
ILMN_2145250	nascent-polypeptide-associated complex alpha polypeptide pseudogene 1 (NACAP1) on chromosome 8.	NACAP1	-2.71
ILMN_1721868	karyopherin alpha 2 (RAG cohort 1, importin alpha 1) (KPNA2), mRNA. XM_001133262 XM_001133265 XM_001133267 XM_001133271	KPNA2	-2.72
ILMN_1742813	transmembrane protein 167 (TMEM167), mRNA.	TMEM167	-2.74
ILMN_2379788	hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor) (HIF1A), transcript variant 2, mRNA.	HIF1A	-2.74
ILMN_1689400	CDC-like kinase 1 (CLK1), transcript variant 2, mRNA.	CLK1	-2.75
ILMN_2298511	bolA homolog 2 (E. coli) (BOLA2), mRNA.	BOLA2	-2.75

ILMN ID	Description	Gene	Value
ILMN_1719870	gastric cancer up-regulated-2 (GCUD2), mRNA.	GCUD2	-2.76
ILMN_2062524	retinoblastoma binding protein 4 (RBBP4), mRNA.	RBBP4	-2.76
ILMN_2221006	RAD21 homolog (S. pombe) (RAD21), mRNA.	RAD21	-2.78
ILMN_1734176	glycoprotein hormones, alpha polypeptide (CGA), mRNA.	CGA	-2.80
ILMN_2183885	chromosome 7 open reading frame 11 (C7orf11), mRNA.	C7orf11	-2.85
ILMN_1687036	mitochondrial ribosomal protein L47 (MRPL47), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA.	MRPL47	-2.85
ILMN_1695588	heterogeneous nuclear ribonucleoprotein C (C1/C2) (HNRPC), transcript variant 1, mRNA.	HNRPC	-2.87
ILMN_1710428	cell division cycle 2, G1 to S and G2 to M (CDC2), transcript variant 1, mRNA.	CDC2	-2.93
ILMN_2116556	LSM5 homolog, U6 small nuclear RNA associated (S. cerevisiae) (LSM5), mRNA.	LSM5	-2.95
ILMN_2196347	cyclin-dependent kinase inhibitor 1B (p27, Kip1) (CDKN1B), mRNA.	CDKN1B	-2.98
ILMN_1778617	TAF9 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 32kDa (TAF9), transcript variant 3, mRNA.	TAF9	-2.99
ILMN_2299072	cisplatin resistance-associated overexpressed protein (CROP), transcript variant 2, mRNA.	CROP	-3.02
ILMN_1657153	ARP3 actin-related protein 3 homolog (yeast) (ACTR3), mRNA.	ACTR3	-3.05
ILMN_2195914	gamma-glutamyl hydrolase (conjugase, folylpolygammaglutamyl hydrolase) (GGH), mRNA.	GGH	-3.10
ILMN_1679045	Shwachman-Bodian-Diamond syndrome (SBDS), mRNA.	SBDS	-3.10
ILMN_1738150	SMT3 suppressor of mif two 3 homolog 2 (S. cerevisiae) (SUMO2), transcript variant 2, mRNA.	SUMO2	-3.12
ILMN_1719749	prostaglandin E synthase 3 (cytosolic) (PTGES3), mRNA.	PTGES3	-3.16
ILMN_2351548	Fas apoptotic inhibitory molecule (FAIM), transcript variant 3, mRNA.	FAIM	-3.20
ILMN_1751816	malignant T cell amplified sequence 1 (MCTS1), mRNA.	MCTS1	-3.26
ILMN_2219712	high-mobility group box 2 (HMGB2), mRNA.	HMGB2	-3.31
ILMN_2041101	annexin A2 pseudogene 1 (ANXA2P1) on chromosome 4.	ANXA2P1	-3.34
ILMN_2181241	ribosomal protein L23a pseudogene (LOC649946) on chromosome 11.	LOC649946	-3.40
ILMN_2371590	DEAD (Asp-Glu-Ala-Asp) box polypeptide 17 (DDX17), transcript variant 2, mRNA.	DDX17	-3.42
ILMN_1759954	prothymosin, alpha (PTMA), transcript variant 1, mRNA.	PTMA	-3.45
ILMN_1657790	splicing factor, arginine/serine-rich 11 (SFRS11), mRNA.	SFRS11	-3.64
ILMN_1789074	heat shock 70kDa protein 1A (HSPA1A), mRNA.	HSPA1A	-3.70
ILMN_2092536	heat shock 10kDa protein 1 (chaperonin 10) (HSPE1), mRNA.	HSPE1	-3.92
ILMN_1708160	karyopherin alpha 2 (RAG cohort 1, importin alpha 1) (KPNA2), mRNA. XM_001133262 XM_001133265 XM_001133267 XM_001133271	KPNA2	-4.01
ILMN_2344455	GTPase activating protein (SH3 domain) binding protein 1 (G3BP1), transcript variant 1, mRNA.	G3BP1	-4.10

Appendix table S2: FLNPAS3 vs. ΔNPAS3 +12hrs top differentially regulated genes. Those genes showing increased (positive) or reduced (negative) expression changes in FLNPAS3 over-expressing cells compared to ΔNPAS3 over-expressing cells at the +12 hr time-point after circadian induction. Exogenous FLNPAS3 is the apparent top gene as exogenous ΔNPAS3 is not detectable on the microarray.

Unique id	Gene symbol	Description	FoldDiff
ILMN_1752550	NPAS3	neuronal PAS domain protein 3 (NPAS3), transcript variant 1, mRNA.	46.32
ILMN_1759954	PTMA	prothymosin, alpha (PTMA), transcript variant 1, mRNA.	3.53
ILMN_1657790	SFRS11	splicing factor, arginine/serine-rich 11 (SFRS11), mRNA.	3.13
ILMN_2299072	CROP	cisplatin resistance-associated overexpressed protein (CROP), transcript variant 2, mRNA.	2.97
ILMN_2344455	G3BP1	GTPase activating protein (SH3 domain) binding protein 1 (G3BP1), transcript variant 1, mRNA.	2.92
ILMN_1708160	KPNA2	karyopherin alpha 2 (RAG cohort 1, importin alpha 1) (KPNA2), mRNA. XM_001133262 XM_001133265 XM_001133267 XM_001133271	2.87
ILMN_2219712	HMGB2	high-mobility group box 2 (HMGB2), mRNA.	2.72
ILMN_1657153	ACTR3	ARP3 actin-related protein 3 homolog (yeast) (ACTR3), mRNA.	2.72
ILMN_2371590	DDX17	DEAD (Asp-Glu-Ala-Asp) box polypeptide 17 (DDX17), transcript variant 2, mRNA.	2.69
ILMN_2221006	RAD21	RAD21 homolog (S. pombe) (RAD21), mRNA.	2.56
ILMN_2379788	HIF1A	hypoxia-inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor) (HIF1A), transcript variant 2, mRNA.	2.55
ILMN_1738150	SUMO2	SMT3 suppressor of mif two 3 homolog 2 (S. cerevisiae) (SUMO2), transcript variant 2, mRNA.	2.53
ILMN_2402936	LOC440926	H3 histone, family 3A pseudogene (LOC440926) on chromosome 2.	2.46
ILMN_2181540	YY1	YY1 transcription factor (YY1), mRNA.	2.41
ILMN_1690894	TRA1P2	PREDICTED: tumor rejection antigen (gp96) 1 pseudogene 2 (TRA1P2), misc RNA.	2.41
ILMN_2211672	TSNAX	translin-associated factor X (TSNAX), mRNA.	2.39
ILMN_1789074	HSPA1A	heat shock 70kDa protein 1A (HSPA1A), mRNA.	2.37
ILMN_2195914	GGH	gamma-glutamyl hydrolase (conjugase, folylpolygammaglutamyl hydrolase) (GGH), mRNA.	2.37
ILMN_2183885	C7orf11	chromosome 7 open reading frame 11 (C7orf11), mRNA.	2.37
ILMN_2116556	LSM5	LSM5 homolog, U6 small nuclear RNA associated (S. cerevisiae) (LSM5), mRNA.	2.36
ILMN_1751816	MCTS1	malignant T cell amplified sequence 1 (MCTS1), mRNA.	2.35
ILMN_2059452	SLC12A2	solute carrier family 12 (sodium/potassium/chloride transporters), member 2 (SLC12A2), mRNA.	2.34
ILMN_1782488	RNASEH2B	ribonuclease H2, subunit B (RNASEH2B), mRNA.	2.32
ILMN_1755419	EIF1AX	eukaryotic translation initiation factor 1A, X-linked (EIF1AX), mRNA.	2.31
ILMN_2041101	ANXA2P1	annexin A2 pseudogene 1 (ANXA2P1) on chromosome 4.	2.29
ILMN_2182348	SMC3	structural maintenance of chromosomes 3 (SMC3), mRNA.	2.28

ILMN_1768582	PPP2CB	protein phosphatase 2 (formerly 2A), catalytic subunit, beta isoform (PPP2CB), transcript variant 1, mRNA.	2.27
ILMN_1679045	SBDS	Shwachman-Bodian-Diamond syndrome (SBDS), mRNA.	2.25
ILMN_2351548	FAIM	Fas apoptotic inhibitory molecule (FAIM), transcript variant 3, mRNA.	2.25
ILMN_2181241	LOC649946	ribosomal protein L23a pseudogene (LOC649946) on chromosome 11.	2.24
ILMN_1719749	PTGES3	prostaglandin E synthase 3 (cytosolic) (PTGES3), mRNA.	2.23
ILMN_1664294	LEPRE1	leucine proline-enriched proteoglycan (leprecan) 1 (LEPRE1), mRNA.	-1.60
ILMN_1723978	LGALS1	lectin, galactoside-binding, soluble, 1 (galectin 1) (LGALS1), mRNA.	-1.61
ILMN_1693014	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta (CEBPB), mRNA.	-1.61
ILMN_1729208	NGFRAP1	nerve growth factor receptor (TNFRSF16) associated protein 1 (NGFRAP1), transcript variant 1, mRNA.	-1.61
ILMN_1779182	TMEM98	transmembrane protein 98 (TMEM98), transcript variant 2, mRNA.	-1.62
ILMN_1738347	RNPEP	arginyl aminopeptidase (aminopeptidase B) (RNPEP), mRNA.	-1.62
ILMN_1694504	C1orf164	chromosome 1 open reading frame 164 (C1orf164), mRNA.	-1.62
ILMN_1812031	PALM	paralemmin (PALM), transcript variant 1, mRNA.	-1.62
ILMN_1701413	PIGQ	phosphatidylinositol glycan anchor biosynthesis, class Q (PIGQ), transcript variant 2, mRNA.	-1.62
ILMN_1673305	RHOC	ras homolog gene family, member C (RHOC), transcript variant 2, mRNA.	-1.62
ILMN_2049303	DCI	dodecenoyl-Coenzyme A delta isomerase (3,2 trans-enoyl-Coenzyme A isomerase) (DCI), nuclear gene encoding mitochondrial protein, mRNA.	-1.63
ILMN_2186061	PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), mRNA.	-1.63
ILMN_1660793	PAQR4	progestin and adipoQ receptor family member IV (PAQR4), mRNA.	-1.64
ILMN_1700541	FBLN1	fibulin 1 (FBLN1), transcript variant C, mRNA.	-1.64
ILMN_1726981	VEGFB	vascular endothelial growth factor B (VEGFB), mRNA.	-1.64
ILMN_1696046	SIVA	CD27-binding (Siva) protein (SIVA), transcript variant 2, mRNA.	-1.65
ILMN_1739241	CHAC1	ChaC, cation transport regulator homolog 1 (E. coli) (CHAC1), mRNA.	-1.65
ILMN_1737157	GRAMD1A	GRAM domain containing 1A (GRAMD1A), mRNA.	-1.67
ILMN_1706521	CSNK1G2	casein kinase 1, gamma 2 (CSNK1G2), mRNA.	-1.67
ILMN_1733110	RASSF7	Ras association (RalGDS/AF-6) domain family (N-terminal) member 7 (RASSF7), mRNA.	-1.68
ILMN_2209671	DCDC5	doublecortin domain containing 5 (DCDC5), mRNA.	-1.68
ILMN_1720373	SLC7A5	solute carrier family 7 (cationic amino acid transporter, y+ system), member 5 (SLC7A5), mRNA.	-1.70
ILMN_1677200	CYFIP2	cytoplasmic FMR1 interacting protein 2 (CYFIP2), transcript variant 3, mRNA.	-1.71
ILMN_1679809	GSTP1	glutathione S-transferase pi (GSTP1), mRNA.	-1.73
ILMN_1711566	TIMP1	TIMP metallopeptidase inhibitor 1 (TIMP1), mRNA.	-1.73
ILMN_1702933	ADM2	adrenomedullin 2 (ADM2), mRNA.	-1.73

ILMN_1658289	WDR54	WD repeat domain 54 (WDR54), mRNA.	-1.75
ILMN_1724658	BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3 (BNIP3), nuclear gene encoding mitochondrial protein, mRNA.	-1.75
ILMN_1651237	CDT1	chromatin licensing and DNA replication factor 1 (CDT1), mRNA.	-1.79
ILMN_1787885	NUDT18	nudix (nucleoside diphosphate linked moiety X)-type motif 18 (NUDT18), mRNA.	-1.80
ILMN_1661599	DDIT4	DNA-damage-inducible transcript 4 (DDIT4), mRNA.	-1.83
ILMN_1756469	GAMT	guanidinoacetate N-methyltransferase (GAMT), transcript variant 1, mRNA.	-1.85
ILMN_1756417	ANKRD37	ankyrin repeat domain 37 (ANKRD37), mRNA.	-1.92
ILMN_1659027	SLC2A1	solute carrier family 2 (facilitated glucose transporter), member 1 (SLC2A1), mRNA.	-1.93
ILMN_1809931	NDRG1	N-myc downstream regulated gene 1 (NDRG1), mRNA.	-2.01
ILMN_1755974	ALDOC	aldolase C, fructose-bisphosphate (ALDOC), mRNA.	-2.06
ILMN_1653292	PFKFB4	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (PFKFB4), mRNA.	-2.08

Appendix table S3: FLNPAS3 vs. Control +12hrs top up-/down-regulated genes. The majority of genes showing altered expression in the presence of FLNPAS3 at +12 hrs were down-regulated.

Unique id	Gene symbol	Description	FoldDiff
ILMN_1752550	NPAS3	neuronal PAS domain protein 3 (NPAS3), transcript variant 1, mRNA.	45.76
ILMN_1810418	LBR	lamin B receptor (LBR), transcript variant 1, mRNA.	1.77
ILMN_2184184	ANXA1	annexin A1 (ANXA1), mRNA.	1.64
ILMN_1659086	NEFL	neurofilament, light polypeptide 68kDa (NEFL), mRNA.	-1.71
ILMN_1745075	RPLP0	ribosomal protein, large, P0 (RPLP0), transcript variant 2, mRNA.	-1.72
ILMN_1667716	TMEM101	transmembrane protein 101 (TMEM101), mRNA.	-1.73
ILMN_1668863	LYPD1	LY6/PLAUR domain containing 1 (LYPD1), transcript variant 1, mRNA.	-1.74
ILMN_1653055	SOX3	SRY (sex determining region Y)-box 3 (SOX3), mRNA.	-1.74
ILMN_1704418	FOXD1	forkhead box D1 (FOXD1), mRNA.	-1.75
ILMN_1701875	ZYX	zyxin (ZYX), transcript variant 1, mRNA.	-1.76
ILMN_2181241	LOC649946	ribosomal protein L23a pseudogene (LOC649946) on chromosome 11.	-1.77
ILMN_1758623	HIST1H2BD	histone cluster 1, H2bd (HIST1H2BD), transcript variant 2, mRNA.	-1.77
ILMN_1659936	PPP1R15A	protein phosphatase 1, regulatory (inhibitor) subunit 15A (PPP1R15A), mRNA.	-1.78
ILMN_2391765	C6orf48	chromosome 6 open reading frame 48 (C6orf48), transcript variant 1, mRNA.	-1.78
ILMN_1726981	VEGFB	vascular endothelial growth factor B (VEGFB), mRNA.	-1.79
ILMN_2395451	ASS1	argininosuccinate synthetase 1 (ASS1), transcript variant 2, mRNA.	-1.79
ILMN_2329914	SPRY1	sprouty homolog 1, antagonist of FGF signaling (Drosophila) (SPRY1), transcript variant 1, mRNA.	-1.79
ILMN_1752394	CCNB1IP1	cyclin B1 interacting protein 1 (CCNB1IP1), transcript variant 2, mRNA.	-1.79
ILMN_2130180	LOC283345	RPL13-2 pseudogene (LOC283345) on chromosome 12.	-1.79
ILMN_1710284	HES1	hairy and enhancer of split 1, (Drosophila) (HES1), mRNA.	-1.81
ILMN_1708728	H2AFJ	H2A histone family, member J (H2AFJ), mRNA.	-1.81
ILMN_1775743	BTG1	B-cell translocation gene 1, anti-proliferative (BTG1), mRNA.	-1.81
ILMN_1658289	WDR54	WD repeat domain 54 (WDR54), mRNA.	-1.82
ILMN_1671731	AVPI1	arginine vasopressin-induced 1 (AVPI1), mRNA.	-1.82
ILMN_1708778	ASS1	argininosuccinate synthetase 1 (ASS1), transcript variant 1, mRNA.	-1.83
ILMN_1674243	TFRC	transferrin receptor (p90, CD71) (TFRC), mRNA.	-1.83
ILMN_2410924	PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2), transcript variant 2, mRNA.	-1.85
ILMN_1792356	DPYSL4	dihydropyrimidinase-like 4 (DPYSL4), mRNA.	-1.88

ILMN_2115340	HIST2H4A	histone cluster 2, H4a (HIST2H4A), mRNA.	-1.88
ILMN_1735014	KLF6	Kruppel-like factor 6 (KLF6), transcript variant 2, mRNA.	-1.89
ILMN_2347349	CCNB1IP1	cyclin B1 interacting protein 1 (CCNB1IP1), transcript variant 3, mRNA.	-1.89
ILMN_1691884	STC2	stanniocalcin 2 (STC2), mRNA.	-1.89
ILMN_1737406	KLF6	Kruppel-like factor 6 (KLF6), transcript variant 1, mRNA.	-1.94
ILMN_2298818	RPS29	ribosomal protein S29 (RPS29), transcript variant 2, mRNA.	-1.94
ILMN_1802205	RHOB	ras homolog gene family, member B (RHOB), mRNA.	-1.95
ILMN_1682717	IER3	immediate early response 3 (IER3), mRNA.	-1.96
ILMN_1693334	P4HA1	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide I (P4HA1), transcript variant 1, mRNA.	-1.96
ILMN_1757406	HIST1H1C	histone cluster 1, H1c (HIST1H1C), mRNA.	-2.00
ILMN_1806023	JUN	jun oncogene (JUN), mRNA.	-2.01
ILMN_1764986	HIST3H2BB	histone cluster 3, H2bb (HIST3H2BB), mRNA.	-2.01
ILMN_1736015	PHF17	PHD finger protein 17 (PHF17), transcript variant S, mRNA.	-2.02
ILMN_1663092	CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2 (CITED2), mRNA.	-2.04
ILMN_2064655	CXorf40A	chromosome X open reading frame 40A (CXorf40A), mRNA.	-2.07
ILMN_2276952	TSC22D3	TSC22 domain family, member 3 (TSC22D3), transcript variant 2, mRNA.	-2.07
ILMN_2074860	RN7SK	RNA, 7SK small nuclear (RN7SK) on chromosome 6.	-2.07
ILMN_1787885	NUDT18	nudix (nucleoside diphosphate linked moiety X)-type motif 18 (NUDT18), mRNA.	-2.07
ILMN_1653292	PFKFB4	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 (PFKFB4), mRNA.	-2.07
ILMN_1689378	CCRN4L	CCR4 carbon catabolite repression 4-like (S. cerevisiae) (CCRN4L), mRNA.	-2.08
ILMN_1724658	BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3 (BNIP3), nuclear gene encoding mitochondrial protein, mRNA.	-2.11
ILMN_1809931	NDRG1	N-myc downstream regulated gene 1 (NDRG1), mRNA.	-2.14
ILMN_1733559	LOC100008589	28S ribosomal RNA (LOC100008589).	-2.14
ILMN_1708934	ADM	adrenomedullin (ADM), mRNA.	-2.15
ILMN_1731349	HOXA13	homeobox A13 (HOXA13), mRNA.	-2.17
ILMN_1755974	ALDOC	aldolase C, fructose-bisphosphate (ALDOC), mRNA.	-2.17
ILMN_2150112	NRN1	neuritin 1 (NRN1), mRNA.	-2.18
ILMN_2374865	ATF3	activating transcription factor 3 (ATF3), transcript variant 4, mRNA.	-2.19
ILMN_1758164	STC1	stanniocalcin 1 (STC1), mRNA.	-2.24
ILMN_1664706	LOC653604	PREDICTED: Homo sapiens similar to H3 histone, family 2 isoform 2 (LOC653604), mRNA.	-2.25

ILMN_2186061	PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), mRNA.	-2.29
ILMN_1768534	BHLHB2	basic helix-loop-helix domain containing, class B, 2 (BHLHB2), mRNA.	-2.29
ILMN_1652287	NOG	noggin (NOG), mRNA.	-2.33
ILMN_2320888	CXCR4	chemokine (C-X-C motif) receptor 4 (CXCR4), transcript variant 1, mRNA.	-2.35
ILMN_1748124	TSC22D3	TSC22 domain family, member 3 (TSC22D3), transcript variant 1, mRNA.	-2.36
ILMN_2376403	TSC22D3	TSC22 domain family, member 3 (TSC22D3), transcript variant 2, mRNA.	-2.39
ILMN_1659027	SLC2A1	solute carrier family 2 (facilitated glucose transporter), member 1 (SLC2A1), mRNA.	-2.42
ILMN_1801584	CXCR4	chemokine (C-X-C motif) receptor 4 (CXCR4), transcript variant 2, mRNA.	-2.46
ILMN_1703123	AXUD1	AXIN1 up-regulated 1 (AXUD1), mRNA.	-2.49
ILMN_1775708	SLC2A3	solute carrier family 2 (facilitated glucose transporter), member 3 (SLC2A3), mRNA.	-2.54
ILMN_2156172	HK2	hexokinase 2 (HK2), mRNA.	-2.62
ILMN_1695880	LOX	lysyl oxidase (LOX), mRNA.	-2.76
ILMN_1736670	PPP1R3C	protein phosphatase 1, regulatory (inhibitor) subunit 3C (PPP1R3C), mRNA.	-2.87
ILMN_1768973	HIST2H2AC	histone cluster 2, H2ac (HIST2H2AC), mRNA.	-2.90
ILMN_1669523	FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS), mRNA.	-2.94
ILMN_1756417	ANKRD37	ankyrin repeat domain 37 (ANKRD37), mRNA.	-3.06
ILMN_1661599	DDIT4	DNA-damage-inducible transcript 4 (DDIT4), mRNA.	-3.20
ILMN_1779648	HIST3H2A	histone cluster 3, H2a (HIST3H2A), mRNA.	-3.26
ILMN_1781285	DUSP1	dual specificity phosphatase 1 (DUSP1), mRNA.	-3.44
ILMN_1659990	HIG2	hypoxia-inducible protein 2 (HIG2), transcript variant 1, mRNA.	-4.03
ILMN_2144426	HIST2H2AA3	histone cluster 2, H2aa3 (HIST2H2AA3), mRNA.	-4.15
ILMN_1732071	HIST2H2BE	histone cluster 2, H2be (HIST2H2BE), mRNA.	-4.98

Appendix table S4: FLNPAS3 vs. Control +24hrs top up-/down-regulated genes

Unique id	Gene symbol	Description	FoldDiff
ILMN_1752550	NPAS3	neuronal PAS domain protein 3 (NPAS3), transcript variant 1, mRNA.	68.10
ILMN_1732296	ID3	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein (ID3), mRNA.	2.41
ILMN_2321064	BAX	BCL2-associated X protein (BAX), transcript variant sigma, mRNA.	2.17
ILMN_1661516	POLR2J3	RPB11b2 protein (POLR2J3), mRNA.	1.97
ILMN_2305112	CTH	cystathionase (cystathionine gamma-lyase) (CTH), transcript variant 2, mRNA.	1.90
ILMN_1733847	GALR2	galanin receptor 2 (GALR2), mRNA.	1.81
ILMN_2086095	ID2	inhibitor of DNA binding 2, dominant negative helix-loop-helix protein (ID2), mRNA.	1.80
ILMN_1777060	CTH	cystathionase (cystathionine gamma-lyase) (CTH), transcript variant 1, mRNA.	1.80
ILMN_1729281	SPHK2	sphingosine kinase 2 (SPHK2), mRNA.	1.78
ILMN_2252160	UBC	ubiquitin C (UBC), mRNA.	1.77
ILMN_1723480	BST2	bone marrow stromal cell antigen 2 (BST2), mRNA.	1.76
ILMN_2224486	C3orf14	chromosome 3 open reading frame 14 (C3orf14), mRNA.	1.76
ILMN_2266005	C21orf51	chromosome 21 open reading frame 51 (C21orf51), transcript variant 2, mRNA.	1.76
ILMN_1790962	FLJ45909	FLJ45909 protein (FLJ45909), mRNA.	1.70
ILMN_1771051	RPL29	ribosomal protein L29 (RPL29), mRNA.	1.69
ILMN_1714445	SLC6A9	member 9 (SLC6A9), transcript variant 3, mRNA.	1.69
ILMN_1717934	SYT11	synaptotagmin XI (SYT11), mRNA.	1.68
ILMN_1740938	APOE	apolipoprotein E (APOE), mRNA.	1.63
ILMN_1687384	IFI6	interferon, alpha-inducible protein 6 (IFI6), transcript variant 3, mRNA.	1.62
ILMN_1710543	SLC39A3	solute carrier family 39 (zinc transporter), member 3 (SLC39A3), transcript variant 1, mRNA.	1.61
ILMN_2090802	TMEM79	transmembrane protein 79 (TMEM79), mRNA.	1.61
ILMN_1739885	SLC41A3	solute carrier family 41, member 3 (SLC41A3), transcript variant 1, mRNA.	1.59
ILMN_1809467	VAMP5	vesicle-associated membrane protein 5 (myobrevin) (VAMP5), mRNA.	1.59
ILMN_2112811	RPL36A	ribosomal protein L36a (RPL36A), mRNA.	1.59
ILMN_1710170	PPAP2C	phosphatidic acid phosphatase type 2C (PPAP2C), transcript variant 2, mRNA.	1.58
ILMN_1760649	PCK2	phosphoenolpyruvate carboxykinase 2 (mitochondrial) (PCK2), nuclear gene encoding mitochondrial protein, transcript variant 2, mRNA.	1.57
ILMN_2110281	UFC1	ubiquitin-fold modifier conjugating enzyme 1 (UFC1), mRNA.	1.56
ILMN_2375651	SCNM1	sodium channel modifier 1 (SCNM1), mRNA.	1.56

ILMN_2060413	CD24	CD24 molecule (CD24), mRNA.	1.56
ILMN_1784602	CDKN1A	cyclin-dependent kinase inhibitor 1A (p21, Cip1) (CDKN1A), transcript variant 1, mRNA.	1.55
ILMN_1764096	CCBL1	cysteine conjugate-beta lyase, cytoplasmic (CCBL1), transcript variant 1, mRNA.	1.55
ILMN_1747204	HTRA2	HtrA serine peptidase 2 (HTRA2), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA.	1.55
ILMN_1730351	FLJ35767	FLJ35767 protein (FLJ35767), mRNA.	1.55
ILMN_1669286	YWHAZ	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ), transcript variant 1, mRNA.	1.54
ILMN_1796417	ASNS	asparagine synthetase (ASNS), transcript variant 1, mRNA.	1.54
ILMN_1747067	NPAS1	neuronal PAS domain protein 1 (NPAS1), mRNA.	1.53
ILMN_1702933	ADM2	adrenomedullin 2 (ADM2), mRNA.	1.53
ILMN_1692938	PSAT1	phosphoserine aminotransferase 1 (PSAT1), transcript variant 2, mRNA.	1.53
ILMN_1662470	C10orf35	chromosome 10 open reading frame 35 (C10orf35), mRNA.	1.53
ILMN_1671791	PCK2	phosphoenolpyruvate carboxykinase 2 (mitochondrial) (PCK2), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA	1.52
ILMN_2234970	SLC39A3	solute carrier family 39 (zinc transporter), member 3 (SLC39A3), transcript variant 1, mRNA.	1.52
ILMN_1791726	TUBB3	tubulin, beta 3 (TUBB3), mRNA.	1.52
ILMN_1664861	ID1	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein (ID1), transcript variant 2, mRNA.	1.52
ILMN_1660021	M6PRBP1	mannose-6-phosphate receptor binding protein 1 (M6PRBP1), mRNA.	1.52
ILMN_1675156	CDC42	cell division cycle 42 (GTP binding protein, 25kDa) (CDC42), transcript variant 3, mRNA.	1.52
ILMN_1756071	MFGE8	milk fat globule-EGF factor 8 protein (MFGE8), mRNA.	1.51
ILMN_2275248	ECE2	endothelin converting enzyme 2 (ECE2), transcript variant 1, mRNA.	1.51
ILMN_2191822	ALG14	asparagine-linked glycosylation 14 homolog (S. cerevisiae) (ALG14), mRNA.	1.51
ILMN_1701483	SYP	synaptophysin (SYP), mRNA.	1.50
ILMN_1663220	MRPL22	mitochondrial ribosomal protein L22 (MRPL22), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA.	1.50
ILMN_1796245	DNASE2	deoxyribonuclease II, lysosomal (DNASE2), mRNA.	1.50
ILMN_2217630	CDKL3	cyclin-dependent kinase-like 3 (CDKL3), mRNA.	1.50
ILMN_1702065	MFSD5	major facilitator superfamily domain containing 5 (MFSD5), mRNA.	1.50
ILMN_1711208	CELSR2	Homo sapiens cadherin, EGF LAG seven-pass G-type receptor 2 (flamingo homolog, Drosophila) (CELSR2), mRNA.	-1.66
ILMN_2410924	PLOD2	Homo sapiens procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2), transcript variant 2, mRNA.	-1.67
ILMN_1651557	KDELC2	KDEL (Lys-Asp-Glu-Leu) containing 2 (KDELC2), mRNA.	-1.67

ILMN ID	Symbol	Description	Value
ILMN_1773117	BCOR	BCL6 co-repressor (BCOR), transcript variant 1, mRNA.	-1.69
ILMN_1696991	DYRK1B	dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1B (DYRK1B), transcript variant c, mRNA.	-1.70
ILMN_1771599	PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2), transcript variant 2, mRNA.	-1.70
ILMN_2366634	PKM2	pyruvate kinase, muscle (PKM2), transcript variant 3, mRNA.	-1.70
ILMN_1772876	ZNF395	zinc finger protein 395 (ZNF395), mRNA.	-1.71
ILMN_1756573	NDUFA4L2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2 (NDUFA4L2), mRNA.	-1.71
ILMN_1724658	BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3 (BNIP3), nuclear gene encoding mitochondrial protein, mRNA.	-1.72
ILMN_1762899	EGR1	early growth response 1 (EGR1), mRNA.	-1.73
ILMN_1686664	MT2A	metallothionein 2A (MT2A), mRNA.	-1.73
ILMN_2117904	ZNF22	zinc finger protein 22 (KOX 15) (ZNF22), mRNA.	-1.73
ILMN_1731349	HOXA13	homeobox A13 (HOXA13), mRNA.	-1.73
ILMN_1658411	CHD4	chromodomain helicase DNA binding protein 4 (CHD4), mRNA.	-1.74
ILMN_1701918	KLHDC9	kelch domain containing 9 (KLHDC9), transcript variant 3, mRNA.	-1.74
ILMN_1741214	NXPH4	PREDICTED: Homo sapiens neurexophilin 4 (NXPH4), mRNA.	-1.75
ILMN_2070052	LOC613037	nuclear pore complex interacting protein pseudogene (LOC613037) on chromosome 16.	-1.75
ILMN_1744665	EP300	E1A binding protein p300 (EP300), mRNA.	-1.75
ILMN_1696434	LAMA1	laminin, alpha 1 (LAMA1), mRNA.	-1.75
ILMN_2216852	PGK1	phosphoglycerate kinase 1 (PGK1), mRNA.	-1.75
ILMN_1674662	C15orf42	chromosome 15 open reading frame 42 (C15orf42), mRNA.	-1.76
ILMN_1722532	JMJD1A	jumonji domain containing 1A (JMJD1A), mRNA.	-1.78
ILMN_1789909	TBC1D9B	TBC1 domain family, member 9B (with GRAM domain) (TBC1D9B), transcript variant 2, mRNA.	-1.79
ILMN_1736670	PPP1R3C	protein phosphatase 1, regulatory (inhibitor) subunit 3C (PPP1R3C), mRNA.	-1.79
ILMN_1768534	BHLHB2	basic helix-loop-helix domain containing, class B, 2 (BHLHB2), mRNA.	-1.83
ILMN_1782385	POLR2A	polymerase (RNA) II (DNA directed) polypeptide A, 220kDa (POLR2A), mRNA.	-1.83
ILMN_1760563	BAT2	HLA-B associated transcript 2 (BAT2), mRNA.	-1.84
ILMN_1672650	PKM2	pyruvate kinase, muscle (PKM2), transcript variant 1, mRNA.	-1.86
ILMN_1758164	STC1	stanniocalcin 1 (STC1), mRNA.	-1.88
ILMN_1674243	TFRC	transferrin receptor (p90, CD71) (TFRC), mRNA.	-1.90
ILMN_1775327	PKM2	pyruvate kinase, muscle (PKM2), transcript variant 2, mRNA.	-1.91
ILMN_1762835	HELZ	helicase with zinc finger (HELZ), mRNA.	-1.93
ILMN_1693334	P4HA1	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide I (P4HA1),	-1.94

		transcript variant 1, mRNA.	
ILMN_2095840	MYST3	MYST histone acetyltransferase (monocytic leukemia) 3 (MYST3), mRNA.	-1.94
ILMN_1756417	ANKRD37	ankyrin repeat domain 37 (ANKRD37), mRNA.	-2.01
ILMN_2186061	PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), mRNA.	-2.16
ILMN_1659990	HIG2	hypoxia-inducible protein 2 (HIG2), transcript variant 1, mRNA.	-2.18
ILMN_1775708	SLC2A3	solute carrier family 2 (facilitated glucose transporter), member 3 (SLC2A3), mRNA.	-2.23
ILMN_1669523	FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS), mRNA.	-2.32
ILMN_1775170	MT1X	metallothionein 1X (MT1X), mRNA.	-2.54
ILMN_1695880	LOX	lysyl oxidase (LOX), mRNA.	-2.72

Appendix table S5: genes significant by SAM - Significance analysis of Microarray.

The estimated false discovery rate among the 932 significant genes is 0.00931. The delta value used to identify the significant genes is 1.79503. The fudge factor for standard deviation is computed as 0.03882.

Gene symbol	GB acc	Unique id	Description	Fold-change
FILIP1	NM_015687	ILMN_17533	Homo sapiens filamin A interacting protein 1 (FILIP1), mRNA.	6.25
HIST1H4H	NM_003543	ILMN_30163	Homo sapiens histone 1, H4h (HIST1H4H), mRNA.	5.26
GPC2	NM_152742	ILMN_6771	Homo sapiens glypican 2 (cerebroglycan) (GPC2), mRNA.	4.17
NDP	NM_000266	ILMN_22946	Homo sapiens Norrie disease (pseudoglioma) (NDP), mRNA.	4
CD24	NM_013230	ILMN_28723	Homo sapiens CD24 antigen (small cell lung carcinoma cluster 4 antigen) (CD24), mRNA.	4
HIST1H2BD	NM_138720	ILMN_17622	Homo sapiens histone 1, H2bd (HIST1H2BD), transcript variant 2, mRNA.	3.7
KIAA1913	NM_052913	ILMN_19059	Homo sapiens KIAA1913 (KIAA1913), mRNA.	2.94
P2RY5	NM_005767	ILMN_1003	Homo sapiens purinergic receptor P2Y, G-protein coupled, 5 (P2RY5), mRNA.	2.94
HIST2H4	NM_003548	ILMN_22069	Homo sapiens histone 2, H4 (HIST2H4), mRNA.	2.86
CYP39A1	NM_016593	ILMN_24012	Homo sapiens cytochrome P450, family 39, subfamily A, polypeptide 1 (CYP39A1), mRNA.	2.86
LOC400566	NM_001013672	ILMN_7731	Homo sapiens hypothetical gene supported by AK128660 (LOC400566), mRNA.	2.86
LRRC17	NM_001031692	ILMN_5522	Homo sapiens leucine rich repeat containing 17 (LRRC17), transcript variant 1, mRNA.	2.7
LRP5L	NM_182492	ILMN_650	Homo sapiens low density lipoprotein receptor-related protein 5-like (LRP5L), mRNA.	2.7
TUBB3	NM_006086	ILMN_16399	Homo sapiens tubulin, beta 3 (TUBB3), mRNA.	2.7
DOC1	NM_182909	ILMN_18421	Homo sapiens downregulated in ovarian cancer 1 (DOC1), transcript variant 1, mRNA.	2.63
DOC1	NM_014890	ILMN_28977	Homo sapiens downregulated in ovarian cancer 1 (DOC1), transcript variant 2, mRNA.	2.56
C1orf162	NM_174896	ILMN_19455	Homo sapiens chromosome 1 open reading frame 162 (C1orf162), mRNA.	2.5
HIST1H4E	NM_003545	ILMN_692	Homo sapiens histone 1, H4e (HIST1H4E), mRNA.	2.5
CLK1	NM_004071	ILMN_27652	Homo sapiens CDC-like kinase 1 (CLK1), transcript variant 1, mRNA.	2.5
ZNF435	NM_025231	ILMN_12847	Homo sapiens zinc finger protein 435 (ZNF435), mRNA.	2.44
FGD6	NM_018351	ILMN_4727	Homo sapiens FYVE, RhoGEF and PH domain containing 6 (FGD6), mRNA.	2.33
LOC400566	NM_001013672	ILMN_7731	Homo sapiens hypothetical gene supported by AK128660 (LOC400566), mRNA.	2.33
B3GALNT	NM_152490	ILMN_22440	Homo sapiens UDP-GalNAc:betaGlcNAc beta 1,3-galactosaminyltransferase, polypeptide 2	2.33

Gene	Accession	ILMN	Description	Value
2			(B3GALNT2), mRNA.	
ZNF193	NM_006299	ILMN_10151	Homo sapiens zinc finger protein 193 (ZNF193), mRNA.	2.33
FSBP	NM_006550	ILMN_588	Homo sapiens fibrinogen silencer binding protein (FSBP), mRNA.	2.33
C10orf82	NM_144661	ILMN_24579	Homo sapiens chromosome 10 open reading frame 82 (C10orf82), mRNA.	2.27
HIST1H1C	NM_005319	ILMN_18282	Homo sapiens histone 1, H1c (HIST1H1C), mRNA.	2.27
RBM4	NM_002896	ILMN_11057	Homo sapiens RNA binding motif protein 4 (RBM4), mRNA.	2.22
ST7	NM_021908	ILMN_15870	Homo sapiens suppression of tumorigenicity 7 (ST7), transcript variant b, mRNA.	2.22
GABPB2	NM_181427	ILMN_17949	Homo sapiens GA binding protein transcription factor, beta subunit 2 (GABPB2), transcript variant gamma-3, mRNA.	2.22
HIST1H2BG	NM_003518	ILMN_21089	Homo sapiens histone 1, H2bg (HIST1H2BG), mRNA.	2.22
ZNF184	NM_007149	ILMN_22535	Homo sapiens zinc finger protein 184 (Kruppel-like) (ZNF184), mRNA.	2.22
SEMA3B	NM_001005914	ILMN_25666	Homo sapiens sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B (SEMA3B), transcript variant 2, mRNA.	2.17
IFI44	NM_006417	ILMN_28905	Homo sapiens interferon-induced protein 44 (IFI44), mRNA.	2.17
ARRDC3	NM_020801	ILMN_22538	Homo sapiens arrestin domain containing 3 (ARRDC3), mRNA.	2.17
UBE1DC1	NM_198329	ILMN_19687	Homo sapiens ubiquitin-activating enzyme E1-domain containing 1 (UBE1DC1), transcript variant 2, mRNA.	2.17
FLJ39575	NM_182597	ILMN_11266	Homo sapiens hypothetical protein FLJ39575 (FLJ39575), mRNA.	2.17
ANTXR1	NM_018153	ILMN_16129	Homo sapiens anthrax toxin receptor 1 (ANTXR1), transcript variant 3, mRNA.	2.17
WDR47	NM_014969	ILMN_25145	Homo sapiens WD repeat domain 47 (WDR47), mRNA.	2.17
SLC6A16	NM_014037	ILMN_10688	Homo sapiens solute carrier family 6, member 16 (SLC6A16), mRNA.	2.13
PTPRR	NM_002849	ILMN_1395	Homo sapiens protein tyrosine phosphatase, receptor type, R (PTPRR), transcript variant 1, mRNA.	2.13
FBXL20	NM_032875	ILMN_11563	Homo sapiens F-box and leucine-rich repeat protein 20 (FBXL20), mRNA.	2.13
SCG2	NM_003469	ILMN_17827	Homo sapiens secretogranin II (chromogranin C) (SCG2), mRNA.	2.13
CBLB	NM_170662	ILMN_18286	Homo sapiens Cas-Br-M (murine) ecotropic retroviral transforming sequence b (CBLB), mRNA.	2.13
RFX3	NM_134428	ILMN_21620	Homo sapiens regulatory factor X, 3 (influences HLA class II expression) (RFX3), transcript variant 2, mRNA.	2.13
SERPINI1	NM_005025	ILMN_7089	Homo sapiens serpin peptidase inhibitor, clade I (neuroserpin), member 1 (SERPINI1), mRNA.	2.08
EIF2C3	NM_177422	ILMN_8765	Homo sapiens eukaryotic translation initiation factor 2C, 3 (EIF2C3), transcript variant 2, mRNA.	2.08

Gene	Accession	ILMN	Description	Value
TFDP2	NM_006286	ILMN_29976	Homo sapiens transcription factor Dp-2 (E2F dimerization partner 2) (TFDP2), mRNA.	2.08
RFX3	NM_134428	ILMN_21620	Homo sapiens regulatory factor X, 3 (influences HLA class II expression) (RFX3), transcript variant 2, mRNA.	2.08
TTC25	NM_031421	ILMN_14828	Homo sapiens tetratricopeptide repeat domain 25 (TTC25), mRNA.	2.08
OVGP1	NM_002557	ILMN_7829	Homo sapiens oviductal glycoprotein 1, 120kDa (mucin 9, oviductin) (OVGP1), mRNA.	2.08
GSTA2	NM_000846	ILMN_15139	Homo sapiens glutathione S-transferase A2 (GSTA2), mRNA.	2.04
ZNF548	NM_152909	ILMN_16141	Homo sapiens zinc finger protein 548 (ZNF548), mRNA.	2.04
ARL9	NM_206919	ILMN_25508	Homo sapiens ADP-ribosylation factor-like 9 (ARL9), mRNA.	2.04
KLHL7	NM_018846	ILMN_21425	Homo sapiens kelch-like 7 (Drosophila) (KLHL7), transcript variant 2, mRNA.	2
MRPL21	NM_181512	ILMN_2878	Homo sapiens mitochondrial ribosomal protein L21 (MRPL21), nuclear gene encoding mitochondrial protein, transcript variant 2, mRNA.	2
WDR78	NM_207014	ILMN_746	Homo sapiens WD repeat domain 78 (WDR78), transcript variant 2, mRNA.	2
GSTA1	NM_145740	ILMN_30031	Homo sapiens glutathione S-transferase A1 (GSTA1), mRNA.	2
CHN1	NM_001822	ILMN_6252	Homo sapiens chimerin (chimaerin) 1 (CHN1), transcript variant 1, mRNA.	2
PLA2G4C	NM_003706	ILMN_29328	Homo sapiens phospholipase A2, group IVC (cytosolic, calcium-independent) (PLA2G4C), mRNA.	2
PAIP2	NM_001033112	ILMN_14455	Homo sapiens poly(A) binding protein interacting protein 2 (PAIP2), transcript variant 1, mRNA.	2
CLK1	NM_001024646	ILMN_27286	Homo sapiens CDC-like kinase 1 (CLK1), transcript variant 2, mRNA.	2
ZIK1	NM_001010879	ILMN_137064	Homo sapiens zinc finger protein interacting with K protein 1 (ZIK1), mRNA.	2
FKBP7	NM_016105	ILMN_4459	Homo sapiens FK506 binding protein 7 (FKBP7), transcript variant 1, mRNA.	1.96
MAP2	NM_031845	ILMN_137325	Homo sapiens microtubule-associated protein 2 (MAP2), transcript variant 2, mRNA.	1.96
LOC132321	NM_173487	ILMN_4988	Homo sapiens hypothetical protein LOC132321 (LOC132321), mRNA.	1.96
IFIT2	NM_001547	ILMN_28123	Homo sapiens interferon-induced protein with tetratricopeptide repeats 2 (IFIT2), mRNA.	1.96
FOXP1	NM_001012505	ILMN_21389	Homo sapiens forkhead box P1 (FOXP1), transcript variant 2, mRNA.	1.92
NME1	NM_198175	ILMN_22180	Homo sapiens non-metastatic cells 1, protein (NM23A) expressed in (NME1), transcript variant 1, mRNA.	1.92
PLXNB1	NM_002673	ILMN_22628	Homo sapiens plexin B1 (PLXNB1), mRNA.	1.92
ARL4	NM_005738	ILMN_137281	Homo sapiens ADP-ribosylation factor-like 4 (ARL4), transcript variant 1, mRNA.	1.92
RP9	NM_203288	ILMN_23874	Homo sapiens retinitis pigmentosa 9 (autosomal dominant) (RP9), mRNA.	1.92
UACA	NM_001008224	ILMN_29424	Homo sapiens uveal autoantigen with coiled-coil domains and ankyrin repeats (UACA), transcript variant 2, mRNA.	1.92
ZMYM5	NM_014242	ILMN_25227	Homo sapiens zinc finger, MYM-type 5 (ZMYM5), mRNA.	1.89

Gene	Accession	ILMN ID	Description	Value
AKR1C3	NM_003739	ILMN_11871	Homo sapiens aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II) (AKR1C3), mRNA.	1.89
TIA1	NM_022173	ILMN_29910	Homo sapiens TIA1 cytotoxic granule-associated RNA binding protein (TIA1), transcript variant 2, mRNA.	1.89
RWDD1	NM_016104	ILMN_29174	Homo sapiens RWD domain containing 1 (RWDD1), transcript variant 2, mRNA.	1.89
IVNS1ABP	NM_016389	ILMN_26908	Homo sapiens influenza virus NS1A binding protein (IVNS1ABP), transcript variant 2, mRNA.	1.89
FLJ21062	NM_024788	ILMN_21254	Homo sapiens hypothetical protein FLJ21062 (FLJ21062), mRNA.	1.85
ZNF610	NM_173530	ILMN_8884	Homo sapiens zinc finger protein 610 (ZNF610), mRNA.	1.85
IL8	NM_000584	ILMN_2247	Homo sapiens interleukin 8 (IL8), mRNA.	1.85
PTBP2	NM_021190	ILMN_556	Homo sapiens polypyrimidine tract binding protein 2 (PTBP2), mRNA.	1.85
DLG3	NM_021120	ILMN_8485	Homo sapiens discs, large homolog 3 (neuroendocrine-dlg, Drosophila) (DLG3), mRNA.	1.85
ZSCAN2	NM_181877	ILMN_10070	Homo sapiens zinc finger and SCAN domain containing 2 (ZSCAN2), transcript variant 1, mRNA.	1.85
DNAH17	NM_003727	ILMN_16763	Homo sapiens dynein, axonemal, heavy polypeptide 17 (DNAH17), mRNA.	1.85
ZNF93	NM_031218	ILMN_24222	Homo sapiens zinc finger protein 93 (HTF34) (ZNF93), transcript variant 1, mRNA.	1.85
PPP1R15A	NM_014330	ILMN_1024	Homo sapiens protein phosphatase 1, regulatory (inhibitor) subunit 15A (PPP1R15A), mRNA.	1.85
HIST1H2AC	NM_003512	ILMN_26493	Homo sapiens histone 1, H2ac (HIST1H2AC), mRNA.	1.85
SPAG9	NM_172345	ILMN_21524	Homo sapiens sperm associated antigen 9 (SPAG9), transcript variant 2, mRNA.	1.85
RKHD3	NM_032246	ILMN_29516	Homo sapiens ring finger and KH domain containing 3 (RKHD3), mRNA.	1.85
FAM80B	NM_020734	ILMN_19439	Homo sapiens family with sequence similarity 80, member B (FAM80B), mRNA.	1.82
C1orf50	NM_024097	ILMN_12960	Homo sapiens chromosome 1 open reading frame 50 (C1orf50), mRNA.	1.82
NEDD9	NM_182966	ILMN_137978	Homo sapiens neural precursor cell expressed, developmentally down-regulated 9 (NEDD9), transcript variant 2, mRNA.	1.82
FLJ41603	NM_001001669	ILMN_139156	Homo sapiens FLJ41603 protein (FLJ41603), mRNA.	1.82
ZBTB40	NM_014870	ILMN_6671	Homo sapiens zinc finger and BTB domain containing 40 (ZBTB40), mRNA.	1.82
TOR1AIP2	NM_145034	ILMN_12227	Homo sapiens torsin A interacting protein 2 (TOR1AIP2), mRNA.	1.82
MSI2	NM_170721	ILMN_25750	Homo sapiens musashi homolog 2 (Drosophila) (MSI2), transcript variant 2, mRNA.	1.82
C13orf24	NM_006346	ILMN_23420	Homo sapiens chromosome 13 open reading frame 24 (C13orf24), mRNA.	1.82
TGIF	NM_170695	ILMN_2941	Homo sapiens TGFB-induced factor (TALE family homeobox) (TGIF), transcript variant 1, mRNA.	1.82
KIAA0409	NM_015324	ILMN_1461	Homo sapiens KIAA0409 (KIAA0409), mRNA.	1.82

Gene	Accession	ILMN	Description	Value
PELI1	NM_020651	ILMN_11771	Homo sapiens pellino homolog 1 (Drosophila) (PELI1), mRNA.	1.82
ZNF35	NM_003420	ILMN_15315	Homo sapiens zinc finger protein 35 (clone HF.10) (ZNF35), mRNA.	1.82
ST7	NM_018412	ILMN_16575	Homo sapiens suppression of tumorigenicity 7 (ST7), transcript variant a, mRNA.	1.82
UBE2H	NM_003344	ILMN_23401	Homo sapiens ubiquitin-conjugating enzyme E2H (UBC8 homolog, yeast) (UBE2H), transcript variant 1, mRNA.	1.82
PCDHB12	NM_018932	ILMN_7611	Homo sapiens protocadherin beta 12 (PCDHB12), mRNA.	1.79
DMRTA1	NM_022160	ILMN_10416	Homo sapiens DMRT-like family A1 (DMRTA1), mRNA.	1.79
C6orf188	NM_153711	ILMN_138017	Homo sapiens chromosome 6 open reading frame 188 (C6orf188), mRNA.	1.79
EIF5	NM_001969	ILMN_16924	Homo sapiens eukaryotic translation initiation factor 5 (EIF5), transcript variant 1, mRNA.	1.79
TEX9	NM_198524	ILMN_6690	Homo sapiens testis expressed sequence 9 (TEX9), mRNA.	1.79
FLAD1	NM_201398	ILMN_17093	Homo sapiens Fad1, flavin adenine dinucleotide synthetase, homolog (yeast) (FLAD1), transcript variant 2, mRNA.	1.79
BLOC1S2	NM_001001342	ILMN_8691	Homo sapiens biogenesis of lysosome-related organelles complex-1, subunit 2 (BLOC1S2), transcript variant 2, mRNA.	1.75
C21orf66	NM_013329	ILMN_15558	Homo sapiens chromosome 21 open reading frame 66 (C21orf66), transcript variant 2, mRNA.	1.75
SBSN	NM_198538	ILMN_1498	Homo sapiens suprabasin (SBSN), mRNA.	1.75
CDKN2C	NM_078626	ILMN_138785	Homo sapiens cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4) (CDKN2C), transcript variant 2, mRNA.	1.75
HIST1H2AE	NM_021052	ILMN_1822	Homo sapiens histone 1, H2ae (HIST1H2AE), mRNA.	1.75
CCDC66	NM_001012506	ILMN_20485	Homo sapiens coiled-coil domain containing 66 (CCDC66), mRNA.	1.75
ZNF570	NM_144694	ILMN_11817	Homo sapiens zinc finger protein 570 (ZNF570), mRNA.	1.75
ZNF211	NM_006385	ILMN_8413	Homo sapiens zinc finger protein 211 (ZNF211), transcript variant 1, mRNA.	1.75
FLJ11021	NM_023012	ILMN_17793	Homo sapiens similar to splicing factor, arginine/serine-rich 4 (FLJ11021), transcript variant 1, mRNA.	1.75
MGC11335	NM_030819	ILMN_16965	Homo sapiens hypothetical protein MGC11335 (MGC11335), mRNA.	1.75
LOC221955	NM_139179	ILMN_1764	Homo sapiens KCCR13L (LOC221955), mRNA.	1.75
PAMCI	NM_005447	ILMN_26625	Homo sapiens peptidylglycine alpha-amidating monooxygenase COOH-terminal interactor (PAMCI), mRNA.	1.72
C14orf45	NM_025057	ILMN_24020	Homo sapiens chromosome 14 open reading frame 45 (C14orf45), mRNA.	1.72
MAK	NM_005906	ILMN_24831	Homo sapiens male germ cell-associated kinase (MAK), mRNA.	1.72
RUFY3	NM_014961	ILMN_22291	Homo sapiens RUN and FYVE domain containing 3 (RUFY3), transcript variant 2, mRNA.	1.72

Gene	ILMN ID	Accession	Description	Value
C10orf78	ILMN_1251	NM_145247	Homo sapiens chromosome 10 open reading frame 78 (C10orf78), transcript variant 2, mRNA.	1.72
CCNJ	ILMN_3931	NM_019084	Homo sapiens cyclin J (CCNJ), mRNA.	1.72
ING4	ILMN_3900	NM_198287	Homo sapiens inhibitor of growth family, member 4 (ING4), transcript variant 2, mRNA.	1.72
SEC22L3	ILMN_28430	NM_032970	Homo sapiens SEC22 vesicle trafficking protein-like 3 (S. cerevisiae) (SEC22L3), transcript variant 1, mRNA.	1.72
C6orf141	ILMN_3806	NM_153344	Homo sapiens chromosome 6 open reading frame 141 (C6orf141), mRNA.	1.72
TAIP-2	ILMN_20070	NM_024969	Homo sapiens TGF-beta induced apotosis protein 2 (TAIP-2), mRNA.	1.72
MAT2B	ILMN_19777	NM_182796	Homo sapiens methionine adenosyltransferase II, beta (MAT2B), transcript variant 2, mRNA.	1.72
NSUN6	ILMN_30073	NM_182543	Homo sapiens NOL1/NOP2/Sun domain family, member 6 (NSUN6), mRNA.	1.72
ZNF3	ILMN_25682	NM_032924	Homo sapiens zinc finger protein 3 (A8-51) (ZNF3), transcript variant 2, mRNA.	1.72
ZNF34	ILMN_7366	NM_030580	Homo sapiens zinc finger protein 34 (KOX 32) (ZNF34), mRNA.	1.72
ST7	ILMN_16575	NM_018412	Homo sapiens suppression of tumorigenicity 7 (ST7), transcript variant a, mRNA.	1.72
ALS2CR4	ILMN_23219	NM_152388	Homo sapiens amyotrophic lateral sclerosis 2 (juvenile) chromosome region, candidate 4 (ALS2CR4), mRNA.	1.72
FYTTD1	ILMN_5513	NM_001011537	Homo sapiens forty-two-three domain containing 1 (FYTTD1), transcript variant 2, mRNA.	1.72
GALR2	ILMN_4188	NM_003857	Homo sapiens galanin receptor 2 (GALR2), mRNA.	1.72
TNRC4	ILMN_8091	NM_007185	Homo sapiens trinucleotide repeat containing 4 (TNRC4), mRNA.	1.69
TP53AP1	ILMN_684	NM_007233	Homo sapiens TP53 activated protein 1 (TP53AP1), mRNA.	1.69
ST8SIA4	ILMN_7432	NM_175052	Homo sapiens ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 4 (ST8SIA4), transcript variant 2, mRNA.	1.69
CARD9	ILMN_22365	NM_052813	Homo sapiens caspase recruitment domain family, member 9 (CARD9), mRNA.	1.69
PLEKHO1	ILMN_23081	NM_016274	Homo sapiens pleckstrin homology domain containing, family O member 1 (PLEKHO1), mRNA.	1.69
TRIT1	ILMN_7478	NM_017646	Homo sapiens tRNA isopentenyltransferase 1 (TRIT1), mRNA.	1.69
DNAJA1	ILMN_5819	NM_001539	Homo sapiens DnaJ (Hsp40) homolog, subfamily A, member 1 (DNAJA1), mRNA.	1.69
METTL2A	ILMN_7564	NM_0010005372	Homo sapiens methyltransferase like 2A (METTL2A), mRNA.	1.69
TRAPPC2	ILMN_19285	NM_001011658	Homo sapiens trafficking protein particle complex 2 (TRAPPC2), transcript variant 1, mRNA.	1.69
PHF21B	ILMN_22331	NM_138415	Homo sapiens PHD finger protein 21B (PHF21B), mRNA.	1.69
RKHD1	ILMN_27274	NM_203304	Homo sapiens ring finger and KH domain containing 1 (RKHD1), mRNA.	1.69
AKAP8L	ILMN_12359	NM_014371	Homo sapiens A kinase (PRKA) anchor protein 8-like (AKAP8L), mRNA.	1.69
MCL1	ILMN_18397	NM_021960	Homo sapiens myeloid cell leukemia sequence 1 (BCL2-related) (MCL1), transcript variant 1, mRNA.	1.69

Gene	Accession	Probe	Description	Value
FLJ40113	NM_198079	ILMN_27191	Homo sapiens golgi autoantigen, golgin subfamily a-like (FLJ40113), mRNA.	1.69
IL1A	NM_000575	ILMN_25320	Homo sapiens interleukin 1, alpha (IL1A), mRNA.	1.67
UFD1L	NM_005659	ILMN_138971	Homo sapiens ubiquitin fusion degradation 1-like (UFD1L), mRNA.	1.67
GMIP	NM_016573	ILMN_26995	Homo sapiens GEM interacting protein (GMIP), mRNA.	1.67
LRRTM2	NM_015564	ILMN_4242	Homo sapiens leucine rich repeat transmembrane neuronal 2 (LRRTM2), mRNA.	1.67
DYNC1I1	NM_004411	ILMN_7312	Homo sapiens dynein, cytoplasmic 1, intermediate chain 1 (DYNC1I1), mRNA.	1.67
HIST2H2AA	NM_003516	ILMN_26733	Homo sapiens histone 2, H2aa (HIST2H2AA), mRNA.	1.67
ZNF256	NM_005773	ILMN_29477	Homo sapiens zinc finger protein 256 (ZNF256), mRNA.	1.67
LUC7L	NM_018032	ILMN_27491	Homo sapiens LUC7-like (S. cerevisiae) (LUC7L), transcript variant 1, mRNA.	1.67
ZNF79	NM_007135	ILMN_23438	Homo sapiens zinc finger protein 79 (pT7) (ZNF79), mRNA.	1.64
MAPK10	NM_138980	ILMN_7405	Homo sapiens mitogen-activated protein kinase 10 (MAPK10), transcript variant 3, mRNA.	1.64
LPXN	NM_004811	ILMN_25554	Homo sapiens leupaxin (LPXN), mRNA.	1.64
MGC17330	NM_052880	ILMN_15026	Homo sapiens HGFL gene (MGC17330), mRNA.	1.64
BAG3	NM_004281	ILMN_9420	Homo sapiens BCL2-associated athanogene 3 (BAG3), mRNA.	1.64
ZNF671	NM_024833	ILMN_24381	Homo sapiens zinc finger protein 671 (ZNF671), mRNA.	1.64
LOC88523	NM_033111	ILMN_6087	Homo sapiens CG016 (LOC88523), mRNA.	1.64
ZNF585B	NM_152279	ILMN_19168	Homo sapiens zinc finger protein 585B (ZNF585B), mRNA.	1.64
C14orf104	NM_018139	ILMN_2733	Homo sapiens chromosome 14 open reading frame 104 (C14orf104), mRNA.	1.64
LOC125893	NM_001031665	ILMN_15160	Homo sapiens hypothetical protein LOC125893 (LOC125893), mRNA.	1.64
KLHL7	NM_018846	ILMN_21425	Homo sapiens kelch-like 7 (Drosophila) (KLHL7), transcript variant 2, mRNA.	1.64
KIAA1370	NM_019600	ILMN_2444	Homo sapiens KIAA1370 (KIAA1370), mRNA.	1.64
DLGAP4	NM_014902	ILMN_11104	Homo sapiens discs, large (Drosophila) homolog-associated protein 4 (DLGAP4), transcript variant 1, mRNA.	1.64
MRRF	NM_199176	ILMN_4576	Homo sapiens mitochondrial ribosome recycling factor (MRRF), nuclear gene encoding mitochondrial protein, transcript variant 3, mRNA.	1.61
SCG3	NM_013243	ILMN_8523	Homo sapiens secretogranin III (SCG3), mRNA.	1.61
HIST1H2BN	NM_003520	ILMN_27335	Homo sapiens histone 1, H2bn (HIST1H2BN), mRNA.	1.61
PHF6	NM_032335	ILMN_21948	Homo sapiens PHD finger protein 6 (PHF6), transcript variant 3, mRNA.	1.61
SPAG9	NM_003971	ILMN_19153	Homo sapiens sperm associated antigen 9 (SPAG9), transcript variant 1, mRNA.	1.61
PDZD6	NM_015693	ILMN_13017	Homo sapiens PDZ domain containing 6 (PDZD6), mRNA.	1.61
TBPL1	NM_004865	ILMN_3787	Homo sapiens TBP-like 1 (TBPL1), mRNA.	1.61

Gene	Accession	ILMN	Description	Value
HIST1H2BD	NM_138720	ILMN_17622	Homo sapiens histone 1, H2bd (HIST1H2BD), transcript variant 2, mRNA.	1.61
PBX1	NM_002585	ILMN_27588	Homo sapiens pre-B-cell leukemia transcription factor 1 (PBX1), mRNA.	1.61
C8orf70	NM_016010	ILMN_13979	Homo sapiens chromosome 8 open reading frame 70 (C8orf70), mRNA.	1.61
ZNF187	NM_001023560	ILMN_4390	Homo sapiens zinc finger protein 187 (ZNF187), transcript variant 2, mRNA.	1.61
SNRP70	NM_001009820	ILMN_22033	Homo sapiens small nuclear ribonucleoprotein 70kDa polypeptide (RNP antigen) (SNRP70), transcript variant 2, mRNA.	1.61
SCYL2	NM_017988	ILMN_25841	Homo sapiens SCY1-like 2 (S. cerevisiae) (SCYL2), mRNA.	1.61
CARF	NM_017632	ILMN_1978	Homo sapiens collaborates/cooperates with ARF (alternate reading frame) protein (CARF), mRNA.	1.61
SRPK2	NM_182691	ILMN_29914	Homo sapiens SFRS protein kinase 2 (SRPK2), transcript variant 2, mRNA.	1.61
SKIL	NM_005414	ILMN_15145	Homo sapiens SKI-like (SKIL), mRNA.	1.59
RBM9	NM_014309	ILMN_13392	Homo sapiens RNA binding motif protein 9 (RBM9), transcript variant 2, mRNA.	1.59
CELSR3	NM_001407	ILMN_12021	Homo sapiens cadherin, EGF LAG seven-pass G-type receptor 3 (flamingo homolog, Drosophila) (CELSR3), mRNA.	1.59
ST8SIA4	NM_005668	ILMN_13523	Homo sapiens ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 4 (ST8SIA4), transcript variant 1, mRNA.	1.59
LOC55565	NM_017530	ILMN_22894	Homo sapiens hypothetical protein LOC55565 (LOC55565), mRNA.	1.59
HSPA4L	NM_014278	ILMN_22150	Homo sapiens heat shock 70kDa protein 4-like (HSPA4L), mRNA.	1.59
C19orf18	NM_152474	ILMN_23331	Homo sapiens chromosome 19 open reading frame 18 (C19orf18), mRNA.	1.59
CPEB3	NM_014912	ILMN_11977	Homo sapiens cytoplasmic polyadenylation element binding protein 3 (CPEB3), mRNA.	1.59
PRDM8	NM_020226	ILMN_22013	Homo sapiens PR domain containing 8 (PRDM8), mRNA.	1.59
CLK3	NM_003992	ILMN_1044	Homo sapiens CDC-like kinase 3 (CLK3), transcript variant phclk3, mRNA.	1.59
ZNF17	NM_006959	ILMN_13772	Homo sapiens zinc finger protein 17 (HPF3, KOX 10) (ZNF17), mRNA.	1.59
LOC63920	NM_022090	ILMN_10601	Homo sapiens transposon-derived Buster3 transposase-like (LOC63920), mRNA.	1.59
CTTNBP2NL	NM_018704	ILMN_19791	Homo sapiens CTTNBP2 N-terminal like (CTTNBP2NL), mRNA.	1.59
ETNK1	NM_018638	ILMN_137282	Homo sapiens ethanolamine kinase 1 (ETNK1), mRNA.	1.59
ZP3	NM_007155	ILMN_17555	Homo sapiens zona pellucida glycoprotein 3 (sperm receptor) (ZP3), mRNA.	1.59
ANTXR1	NM_053034	ILMN_24001	Homo sapiens anthrax toxin receptor 1 (ANTXR1), transcript variant 2, mRNA.	1.56
PEX13	NM_002618	ILMN_27585	Homo sapiens peroxisome biogenesis factor 13 (PEX13), mRNA.	1.56
MSI2	NM_138962	ILMN_525	Homo sapiens musashi homolog 2 (Drosophila) (MSI2), transcript variant 1, mRNA.	1.56
USP36	NM_025090	ILMN_23872	Homo sapiens ubiquitin specific peptidase 36 (USP36), mRNA.	1.56

Symbol	RefSeq	ILMN	Description	Value
HNRPDL	NM_005463	ILMN_29342	Homo sapiens heterogeneous nuclear ribonucleoprotein D-like (HNRPDL), transcript variant 1, mRNA.	1.56
COX11	NM_004375	ILMN_24495	Homo sapiens COX11 homolog, cytochrome c oxidase assembly protein (yeast) (COX11), nuclear gene encoding mitochondrial protein, mRNA.	1.56
COL17A1	NM_130778	ILMN_5067	Homo sapiens collagen, type XVII, alpha 1 (COL17A1), transcript variant short, mRNA.	1.56
CLEC4A	NM_194448	ILMN_8463	Homo sapiens C-type lectin domain family 4, member A (CLEC4A), transcript variant 4, mRNA.	1.54
YAF2	NM_001012424	ILMN_19509	Homo sapiens YY1 associated factor 2 (YAF2), transcript variant 2, mRNA.	1.54
C1orf187	NM_198545	ILMN_138946	Homo sapiens chromosome 1 open reading frame 187 (C1orf187), mRNA.	1.54
CCDC11	NM_145020	ILMN_14245	Homo sapiens coiled-coil domain containing 11 (CCDC11), mRNA.	1.54
COMMD5	NM_014066	ILMN_16200	Homo sapiens COMM domain containing 5 (COMMD5), mRNA.	1.54
ZNF585A	NM_199126	ILMN_9003	Homo sapiens zinc finger protein 585A (ZNF585A), transcript variant 2, mRNA.	1.54
TEAD2	NM_003598	ILMN_4452	Homo sapiens TEA domain family member 2 (TEAD2), mRNA.	1.54
LOC91614	NM_139160	ILMN_17145	Homo sapiens novel 58.3 KDA protein (LOC91614), mRNA.	1.54
HIST1H4K	NM_003541	ILMN_29808	Homo sapiens histone 1, H4k (HIST1H4K), mRNA.	1.54
DYNLRB2	NM_130897	ILMN_9895	Homo sapiens dynein, light chain, roadblock-type 2 (DYNLRB2), mRNA.	1.54
DLNB14	NM_198489	ILMN_6803	Homo sapiens similar to DLNB14 (DLNB14), mRNA.	1.54
THUMPD2	NM_025264	ILMN_25512	Homo sapiens THUMP domain containing 2 (THUMPD2), mRNA.	1.54
MLLT11	NM_006818	ILMN_21397	Homo sapiens myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 11 (MLLT11), mRNA.	1.54
ST7L	NM_138729	ILMN_24639	Homo sapiens suppression of tumorigenicity 7 like (ST7L), transcript variant 4, mRNA.	1.54
C7orf36	NM_020192	ILMN_6410	Homo sapiens chromosome 7 open reading frame 36 (C7orf36), mRNA.	1.54
C9orf152	NM_001012993	ILMN_753	Homo sapiens chromosome 9 open reading frame 152 (C9orf152), mRNA.	1.54
PTPN21	NM_007039	ILMN_10404	Homo sapiens protein tyrosine phosphatase, non-receptor type 21 (PTPN21), mRNA.	1.54
ZNF323	NM_030899	ILMN_13350	Homo sapiens zinc finger protein 323 (ZNF323), transcript variant 1, mRNA.	1.54
DPF1	NM_004647	ILMN_13188	Homo sapiens D4, zinc and double PHD fingers family 1 (DPF1), mRNA.	1.54
IHPK2	NM_001005911	ILMN_4437	Homo sapiens inositol hexaphosphate kinase 2 (IHPK2), transcript variant 4, mRNA.	1.54
ZNF431	NM_133473	ILMN_7008	Homo sapiens zinc finger protein 431 (ZNF431), mRNA.	1.54
POMZP3	NM_012230	ILMN_9563	Homo sapiens POM (POM121 homolog, rat) and ZP3 fusion (POMZP3), transcript variant 1, mRNA.	1.54
ZBTB17	NM_003443	ILMN_23973	Homo sapiens zinc finger and BTB domain containing 17 (ZBTB17), mRNA.	1.54
POLB	NM_002690	ILMN_15404	Homo sapiens polymerase (DNA directed), beta (POLB), mRNA.	1.54
RNF7	NM_183063	ILMN_12234	Homo sapiens ring finger protein 7 (RNF7), transcript variant 2, mRNA.	1.52

Symbol	Accession	Probe	Description	Value
FLJ10260	NM_018042	ILMN_454	Homo sapiens likely ortholog of mouse schlafen 3 (FLJ10260), mRNA.	1.52
MMP10	NM_002425	ILMN_4442	Homo sapiens matrix metallopeptidase 10 (stromelysin 2) (MMP10), mRNA.	1.52
MRPS12	NM_021107	ILMN_28352	Homo sapiens mitochondrial ribosomal protein S12 (MRPS12), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA.	1.52
MUM1L1	NM_152423	ILMN_2521	Homo sapiens melanoma associated antigen (mutated) 1-like 1 (MUM1L1), mRNA.	1.52
ZSCAN2	NM_017894	ILMN_10192	Homo sapiens zinc finger and SCAN domain containing 2 (ZSCAN2), transcript variant 2, mRNA.	1.52
RIT1	NM_006912	ILMN_8608	Homo sapiens Ras-like without CAAX 1 (RIT1), mRNA.	1.52
FLJ37266	NM_175892	ILMN_14294	Homo sapiens hypothetical protein LOC283225 (FLJ37266), mRNA.	1.52
PPP2R2A	NM_002717	ILMN_24841	Homo sapiens protein phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), alpha isoform (PPP2R2A), mRNA.	1.52
BTN2A2	NM_181531	ILMN_13569	Homo sapiens butyrophilin, subfamily 2, member A2 (BTN2A2), transcript variant 2, mRNA.	1.52
C6orf113	NM_145062	ILMN_24644	Homo sapiens chromosome 6 open reading frame 113 (C6orf113), mRNA.	1.52
LOC285989	NM_213603	ILMN_12699	Homo sapiens hypothetical protein LOC285989 (LOC285989), transcript variant 1, mRNA.	1.52
SYT4	NM_020783	ILMN_21875	Homo sapiens synaptotagmin IV (SYT4), mRNA.	1.52
PARP6	NM_020213	ILMN_5118	Homo sapiens poly (ADP-ribose) polymerase family, member 6 (PARP6), transcript variant 1, mRNA.	1.52
CDKN2B	NM_078487	ILMN_20121	Homo sapiens cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4) (CDKN2B), transcript variant 2, mRNA.	1.52
PTRH2	NM_001015509	ILMN_6658	Homo sapiens peptidyl-tRNA hydrolase 2 (PTRH2), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA.	1.52
MGC33839	NM_152353	ILMN_15940	Homo sapiens hypothetical protein MGC33839 (MGC33839), mRNA.	-1.5
GALNT12	NM_024642	ILMN_15198	Homo sapiens UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 12 (GalNAc-T12) (GALNT12), mRNA.	-1.5
OR51E2	NM_030774	ILMN_1888	Homo sapiens olfactory receptor, family 51, subfamily E, member 2 (OR51E2), mRNA.	-1.5
CD151	NM_004357	ILMN_139293	Homo sapiens CD151 antigen (CD151), transcript variant 4, mRNA.	-1.5
C9orf123	NM_033428	ILMN_1979	Homo sapiens chromosome 9 open reading frame 123 (C9orf123), mRNA.	-1.5
ZNF277	NM_021994	ILMN_2173	Homo sapiens zinc finger protein 277 (ZNF277), mRNA.	-1.5
C15orf15	NM_016304	ILMN_20374	Homo sapiens chromosome 15 open reading frame 15 (C15orf15), mRNA.	-1.5
DOCK1	NM_001380	ILMN_24487	Homo sapiens dedicator of cytokinesis 1 (DOCK1), mRNA.	-1.5
LAMA3	NM_198129	ILMN_6835	Homo sapiens laminin, alpha 3 (LAMA3), transcript variant 1, mRNA.	-1.5
MGST3	NM_004528	ILMN_8534	Homo sapiens microsomal glutathione S-transferase 3 (MGST3), mRNA.	-1.5
HLCS	NM_000411	ILMN_2816	Homo sapiens holocarboxylase synthetase (biotin-(proprionyl-Coenzyme A-carboxylase	-1.5

(ATP-hydrolysing)) ligase) (HLCS), mRNA.

Gene	Accession	Probe	Description	Value
PFKL	NM_002626	ILMN_25945	Homo sapiens phosphofructokinase, liver (PFKL), transcript variant 2, mRNA.	-1.5
AUH	NM_001698	ILMN_16053	Homo sapiens AU RNA binding protein/enoyl-Coenzyme A hydratase (AUH), nuclear gene encoding mitochondrial protein, mRNA.	-1.5
LIPA	NM_000235	ILMN_17379	Homo sapiens lipase A, lysosomal acid, cholesterol esterase (Wolman disease) (LIPA), mRNA.	-1.5
DHPS	NM_001930	ILMN_3527	Homo sapiens deoxyhypusine synthase (DHPS), transcript variant 1, mRNA.	-1.5
MPP1	NM_002436	ILMN_19796	Homo sapiens membrane protein, palmitoylated 1, 55kDa (MPP1), mRNA.	-1.5
CTNNBIP1	NM_001012329	ILMN_22311	Homo sapiens catenin, beta interacting protein 1 (CTNNBIP1), transcript variant 2, mRNA.	-1.5
COQ10A	NM_144576	ILMN_29873	Homo sapiens coenzyme Q10 homolog A (yeast) (COQ10A), mRNA.	-1.5
C6orf108	NM_199184	ILMN_5240	Homo sapiens chromosome 6 open reading frame 108 (C6orf108), transcript variant 2, mRNA.	-1.5
C2orf30	NM_015701	ILMN_22881	Homo sapiens chromosome 2 open reading frame 30 (C2orf30), mRNA.	-1.5
LOC57228	NM_001031628	ILMN_22527	Homo sapiens small trans-membrane and glycosylated protein (LOC57228), transcript variant 1, mRNA.	-1.5
C16orf9	NM_032039	ILMN_12959	Homo sapiens chromosome 16 open reading frame 9 (C16orf9), mRNA.	-1.5
METTL4	NM_022840	ILMN_16027	Homo sapiens methyltransferase like 4 (METTL4), mRNA.	-1.5
LAMA4	NM_002290	ILMN_4021	Homo sapiens laminin, alpha 4 (LAMA4), mRNA.	-1.5
F8A1	NM_012151	ILMN_23293	Homo sapiens coagulation factor VIII-associated (intronic transcript) 1 (F8A1), mRNA.	-1.5
RPL22	NM_000983	ILMN_10289	Homo sapiens ribosomal protein L22 (RPL22), mRNA.	-1.5
PPIAL4	NM_178230	ILMN_1203	Homo sapiens peptidylprolyl isomerase A (cyclophilin A)-like 4 (PPIAL4), mRNA.	-1.5
MOSPD2	NM_152581	ILMN_28307	Homo sapiens motile sperm domain containing 2 (MOSPD2), mRNA.	-1.5
MORC4	NM_024657	ILMN_5233	Homo sapiens MORC family CW-type zinc finger 4 (MORC4), mRNA.	-1.5
SYTL1	NM_032872	ILMN_7184	Homo sapiens synaptotagmin-like 1 (SYTL1), mRNA.	-1.5
GBL	NM_022372	ILMN_137375	Homo sapiens G protein beta subunit-like (GBL), mRNA.	-1.5
ZNF291	NM_020843	ILMN_22657	Homo sapiens zinc finger protein 291 (ZNF291), mRNA.	-1.5
GPNMB	NM_001005340	ILMN_20483	Homo sapiens glycoprotein (transmembrane) nmb (GPNMB), transcript variant 1, mRNA.	-1.5
PTPN13	NM_080685	ILMN_9656	Homo sapiens protein tyrosine phosphatase, non-receptor type 13 (APO-1/CD95 (Fas)-associated phosphatase) (PTPN13), transcript variant 4, mRNA.	-1.5
DKFZP686A01247	NM_014988	ILMN_3090	Homo sapiens hypothetical protein (DKFZP686A01247), mRNA.	-1.5
FYN	NM_002037	ILMN_5919	Homo sapiens FYN oncogene related to SRC, FGR, YES (FYN), transcript variant 1, mRNA.	-1.5
GAMT	NM_000156	ILMN_20028	Homo sapiens guanidinoacetate N-methyltransferase (GAMT), transcript variant 1, mRNA.	-1.5

Symbol	Accession	ILMN ID	Description	Value
PRSS16	NM_005865	ILMN_10360	Homo sapiens protease, serine, 16 (thymus) (PRSS16), mRNA.	-1.5
IGSF4	NM_014333	ILMN_19233	Homo sapiens immunoglobulin superfamily, member 4 (IGSF4), mRNA.	-1.5
C6orf57	NM_145267	ILMN_23605	Homo sapiens chromosome 6 open reading frame 57 (C6orf57), mRNA.	-1.5
KIAA0141	NM_014773	ILMN_8708	Homo sapiens KIAA0141 (KIAA0141), mRNA.	-1.5
MAN1C1	NM_020379	ILMN_12633	Homo sapiens mannosidase, alpha, class 1C, member 1 (MAN1C1), mRNA.	-1.5
APOC1	NM_001645	ILMN_14337	Homo sapiens apolipoprotein C-I (APOC1), mRNA.	-1.5
FLJ14503	NM_152780	ILMN_6575	Homo sapiens hypothetical protein FLJ14503 (FLJ14503), mRNA.	-1.5
MRE11A	NM_005590	ILMN_6718	Homo sapiens MRE11 meiotic recombination 11 homolog A (S. cerevisiae) (MRE11A), transcript variant 2, mRNA.	-1.5
BCL2	NM_000633	ILMN_3868	Homo sapiens B-cell CLL/lymphoma 2 (BCL2), nuclear gene encoding mitochondrial protein, transcript variant alpha, mRNA.	-1.5
EXTL2	NM_001439	ILMN_28046	Homo sapiens exostoses (multiple)-like 2 (EXTL2), transcript variant 1, mRNA.	-1.5
CREG1	NM_003851	ILMN_11645	Homo sapiens cellular repressor of E1A-stimulated genes 1 (CREG1), mRNA.	-1.5
MGC3265	NM_024028	ILMN_26079	Homo sapiens hypothetical protein MGC3265 (MGC3265), mRNA.	-1.5
PDCD4	NM_145341	ILMN_12916	Homo sapiens programmed cell death 4 (neoplastic transformation inhibitor) (PDCD4), transcript variant 2, mRNA.	-1.5
RHOT1	NM_018307	ILMN_6709	Homo sapiens ras homolog gene family, member T1 (RHOT1), transcript variant 3, mRNA.	-1.5
LMBR1	NM_022458	ILMN_1755	Homo sapiens limb region 1 homolog (mouse) (LMBR1), mRNA.	-1.5
ARPC2	NM_005731	ILMN_1434	Homo sapiens actin related protein 2/3 complex, subunit 2, 34kDa (ARPC2), transcript variant 2, mRNA.	-1.5
ITPR1	NM_002222	ILMN_12245	Homo sapiens inositol 1,4,5-triphosphate receptor, type 1 (ITPR1), mRNA.	-1.5
YPEL3	NM_031477	ILMN_17925	Homo sapiens yippee-like 3 (Drosophila) (YPEL3), mRNA.	-1.5
BNIP3	NM_004052	ILMN_3871	Homo sapiens BCL2/adenovirus E1B 19kDa interacting protein 3 (BNIP3), nuclear gene encoding mitochondrial protein, mRNA.	-1.5
PCK2	NM_001018073	ILMN_18787	Homo sapiens phosphoenolpyruvate carboxykinase 2 (mitochondrial) (PCK2), nuclear gene encoding mitochondrial protein, transcript variant 2, mRNA.	-1.5
RNF170	NM_030954	ILMN_24753	Homo sapiens ring finger protein 170 (RNF170), mRNA.	-1.5
MGC24665	NM_152308	ILMN_5047	Homo sapiens hypothetical protein MGC24665 (MGC24665), mRNA.	-1.5
RHOQ	NM_012249	ILMN_2265	Homo sapiens ras homolog gene family, member Q (RHOQ), mRNA.	-1.5
DDX59	NM_001031725	ILMN_10085	Homo sapiens DEAD (Asp-Glu-Ala-Asp) box polypeptide 59 (DDX59), transcript variant 1, mRNA.	-1.5
UNC84A	NM_025154	ILMN_12719	Homo sapiens unc-84 homolog A (C. elegans) (UNC84A), mRNA.	-1.5
LRRC8D	NM_018103	ILMN_16484	Homo sapiens leucine rich repeat containing 8 family, member D (LRRC8D), mRNA.	-1.5

Gene	Accession	Probe	Description	Value
CDCA7L	NM_018719	ILMN_14646	Homo sapiens cell division cycle associated 7-like (CDCA7L), mRNA.	-1.5
EPHA4	NM_004438	ILMN_21869	Homo sapiens EPH receptor A4 (EPHA4), mRNA.	-1.5
MT1F	NM_005949	ILMN_21370	Homo sapiens metallothionein 1F (functional) (MT1F), mRNA.	-1.5
HEBP1	NM_015987	ILMN_4128	Homo sapiens heme binding protein 1 (HEBP1), mRNA.	-1.5
ZNF533	NM_152520	ILMN_16766	Homo sapiens zinc finger protein 533 (ZNF533), mRNA.	-1.5
B4GALT1	NM_001497	ILMN_20711	Homo sapiens UDP-Gal:betaGlcNAc beta 1,4- galactosyltransferase, polypeptide 1 (B4GALT1), mRNA.	-1.5
COL4A6	NM_001847	ILMN_1137	Homo sapiens collagen, type IV, alpha 6 (COL4A6), transcript variant A, mRNA.	-1.5
MARS	NM_004990	ILMN_3087	Homo sapiens methionine-tRNA synthetase (MARS), mRNA.	-1.5
NPY	NM_000905	ILMN_11990	Homo sapiens neuropeptide Y (NPY), mRNA.	-1.5
HSDL2	NM_032303	ILMN_18734	Homo sapiens hydroxysteroid dehydrogenase like 2 (HSDL2), mRNA.	-1.5
SLC25A1	NM_005984	ILMN_28181	Homo sapiens solute carrier family 25 (mitochondrial carrier; citrate transporter), member 1 (SLC25A1), mRNA.	-1.5
DDX59	NM_001031725	ILMN_10085	Homo sapiens DEAD (Asp-Glu-Ala-Asp) box polypeptide 59 (DDX59), transcript variant 1, mRNA.	-1.5
SHMT2	NM_005412	ILMN_19915	Homo sapiens serine hydroxymethyltransferase 2 (mitochondrial) (SHMT2), mRNA.	-1.5
C9orf91	NM_153045	ILMN_21211	Homo sapiens chromosome 9 open reading frame 91 (C9orf91), mRNA.	-1.5
VRK3	NM_001025778	ILMN_12794	Homo sapiens vaccinia related kinase 3 (VRK3), transcript variant 2, mRNA.	-1.5
ENOSF1	NM_017512	ILMN_12398	Homo sapiens enolase superfamily member 1 (ENOSF1), mRNA.	-1.5
EPS15	NM_001981	ILMN_8810	Homo sapiens epidermal growth factor receptor pathway substrate 15 (EPS15), mRNA.	-1.5
PIGV	NM_017837	ILMN_3048	Homo sapiens phosphatidylinositol glycan, class V (PIGV), mRNA.	-1.5
SREBF1	NM_004176	ILMN_21143	Homo sapiens sterol regulatory element binding transcription factor 1 (SREBF1), transcript variant 2, mRNA.	-1.5
MT1A	NM_005946	ILMN_1797	Homo sapiens metallothionein 1A (functional) (MT1A), mRNA.	-1.5
P4HA2	NM_001017974	ILMN_9080	Homo sapiens procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide II (P4HA2), transcript variant 3, mRNA.	-1.5
MT1X	NM_005952	ILMN_16629	Homo sapiens metallothionein 1X (MT1X), mRNA.	-1.5
GPR37	NM_005302	ILMN_6027	Homo sapiens G protein-coupled receptor 37 (endothelin receptor type B-like) (GPR37), mRNA.	-1.5
PLAT	NM_000930	ILMN_1289	Homo sapiens plasminogen activator, tissue (PLAT), transcript variant 1, mRNA.	-1.5
CCPG1	NM_004748	ILMN_6585	Homo sapiens cell cycle progression 1 (CCPG1), transcript variant 1, mRNA.	-1.5
MOCOS	NM_017947	ILMN_29346	Homo sapiens molybdenum cofactor sulfurase (MOCOS), mRNA.	-1.5
TSPAN7	NM_004615	ILMN_20684	Homo sapiens tetraspanin 7 (TSPAN7), mRNA.	-1.5

Gene	Accession	Probe	Description	Fold
GRPEL2	NM_152407	ILMN_138454	Homo sapiens GrpE-like 2, mitochondrial (E. coli) (GRPEL2), mRNA.	-1.5
ATP5D	NM_001001975	ILMN_6639	Homo sapiens ATP synthase, H+ transporting, mitochondrial F1 complex, delta subunit (ATP5D), nuclear gene encoding mitochondrial protein, transcript variant 2, mRNA.	-1.5
LACTB2	NM_016027	ILMN_18082	Homo sapiens lactamase, beta 2 (LACTB2), mRNA.	-1.5
SLC43A1	NM_003627	ILMN_20441	Homo sapiens solute carrier family 43, member 1 (SLC43A1), mRNA.	-1.5
XPOT	NM_007235	ILMN_22427	Homo sapiens exportin, tRNA (nuclear export receptor for tRNAs) (XPOT), mRNA.	-1.5
FLJ12788	NM_022492	ILMN_2918	Homo sapiens hypothetical protein FLJ12788 (FLJ12788), mRNA.	-1.5
CRYL1	NM_015974	ILMN_17181	Homo sapiens crystallin, lambda 1 (CRYL1), mRNA.	-1.5
SH3KBP1	NM_031892	ILMN_18782	Homo sapiens SH3-domain kinase binding protein 1 (SH3KBP1), transcript variant 1, mRNA.	-1.5
CTDSP1	NM_021198	ILMN_12001	Homo sapiens CTD (carboxy-terminal domain, RNA polymerase II, polypeptide A) small phosphatase 1 (CTDSP1), transcript variant 1, mRNA.	-1.5
SULF1	NM_015170	ILMN_28982	Homo sapiens sulfatase 1 (SULF1), mRNA.	-1.5
BBS2	NM_031885	ILMN_12583	Homo sapiens Bardet-Biedl syndrome 2 (BBS2), mRNA.	-1.5
GPR64	NM_005756	ILMN_26937	Homo sapiens G protein-coupled receptor 64 (GPR64), mRNA.	-1.5
SIAE	NM_170601	ILMN_10502	Homo sapiens sialic acid acetylesterase (SIAE), mRNA.	-1.6
PAQR8	NM_133367	ILMN_25860	Homo sapiens progestin and adipoQ receptor family member VIII (PAQR8), mRNA.	-1.6
PYCR1	NM_153824	ILMN_8761	Homo sapiens pyrroline-5-carboxylate reductase 1 (PYCR1), transcript variant 2, mRNA.	-1.6
NUP210	NM_024923	ILMN_22933	Homo sapiens nucleoporin 210kDa (NUP210), mRNA.	-1.6
EIF2S2	NM_003908	ILMN_23053	Homo sapiens eukaryotic translation initiation factor 2, subunit 2 beta, 38kDa (EIF2S2), mRNA.	-1.6
GRB10	NM_005311	ILMN_2830	Homo sapiens growth factor receptor-bound protein 10 (GRB10), transcript variant 1, mRNA.	-1.6
RAB26	NM_014353	ILMN_22043	Homo sapiens RAB26, member RAS oncogene family (RAB26), mRNA.	-1.6
PDGFD	NM_025208	ILMN_138561	Homo sapiens platelet derived growth factor D (PDGFD), transcript variant 1, mRNA.	-1.6
LRP16	NM_014067	ILMN_13854	Homo sapiens LRP16 protein (LRP16), mRNA.	-1.6
ARHGEF6	NM_004840	ILMN_26689	Homo sapiens Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6 (ARHGEF6), mRNA.	-1.6
FCGRT	NM_004107	ILMN_17135	Homo sapiens Fc fragment of IgG, receptor, transporter, alpha (FCGRT), mRNA.	-1.6
ALDH2	NM_000690	ILMN_17598	Homo sapiens aldehyde dehydrogenase 2 family (mitochondrial) (ALDH2), nuclear gene encoding mitochondrial protein, mRNA.	-1.6
CDCA1	NM_031423	ILMN_16808	Homo sapiens cell division cycle associated 1 (CDCA1), transcript variant 2, mRNA.	-1.6
ANKRD37	NM_181726	ILMN_2423	Homo sapiens ankyrin repeat domain 37 (ANKRD37), mRNA.	-1.6
ATF5	NM_012068	ILMN_6490	Homo sapiens activating transcription factor 5 (ATF5), mRNA.	-1.6
CCND2	NM_001759	ILMN_1503	Homo sapiens cyclin D2 (CCND2), mRNA.	-1.6
HINT2	NM_032593	ILMN_20073	Homo sapiens histidine triad nucleotide binding protein 2 (HINT2), mRNA.	-1.6

Gene	Accession	Probe	Description	Value
SH3KBP1	NM_001024666	ILMN_22300	Homo sapiens SH3-domain kinase binding protein 1 (SH3KBP1), transcript variant 2, mRNA.	-1.6
DOCK11	NM_144658	ILMN_4260	Homo sapiens dedicator of cytokinesis 11 (DOCK11), mRNA.	-1.6
IGFBP5	NM_000599	ILMN_3066	Homo sapiens insulin-like growth factor binding protein 5 (IGFBP5), mRNA.	-1.6
CLDN1	NM_021101	ILMN_24855	Homo sapiens claudin 1 (CLDN1), mRNA.	-1.6
PDCD6IP	NM_013374	ILMN_29002	Homo sapiens programmed cell death 6 interacting protein (PDCD6IP), mRNA.	-1.6
DEPDC1	NM_017779	ILMN_25582	Homo sapiens DEP domain containing 1 (DEPDC1), mRNA.	-1.6
SRPX	NM_006307	ILMN_26705	Homo sapiens sushi-repeat-containing protein, X-linked (SRPX), mRNA.	-1.6
CRTAP	NM_006371	ILMN_2952	Homo sapiens cartilage associated protein (CRTAP), mRNA.	-1.6
FLJ10922	NM_018273	ILMN_8453	Homo sapiens hypothetical protein FLJ10922 (FLJ10922), mRNA.	-1.6
NXPH4	NM_007224	ILMN_17963	Homo sapiens neurexophilin 4 (NXPH4), mRNA.	-1.6
SNAP91	NM_014841	ILMN_15248	Homo sapiens synaptosomal-associated protein, 91kDa homolog (mouse) (SNAP91), mRNA.	-1.6
MTHFD1L	NM_015440	ILMN_20988	Homo sapiens methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 1-like (MTHFD1L), mRNA.	-1.6
CBX4	NM_003655	ILMN_20703	Homo sapiens chromobox homolog 4 (Pc class homolog, Drosophila) (CBX4), mRNA.	-1.6
HTRA1	NM_002775	ILMN_10981	Homo sapiens HtrA serine peptidase 1 (HTRA1), mRNA.	-1.6
TBL1X	NM_005647	ILMN_24329	Homo sapiens transducin (beta)-like 1X-linked (TBL1X), mRNA.	-1.6
SUMF2	NM_015411	ILMN_9757	Homo sapiens sulfatase modifying factor 2 (SUMF2), mRNA.	-1.6
DEPDC6	NM_022783	ILMN_24376	Homo sapiens DEP domain containing 6 (DEPDC6), mRNA.	-1.6
GPR126	NM_020455	ILMN_5536	Homo sapiens G protein-coupled receptor 126 (GPR126), transcript variant a1, mRNA.	-1.6
C1orf24	NM_052966	ILMN_8647	Homo sapiens chromosome 1 open reading frame 24 (C1orf24), transcript variant 2, mRNA.	-1.6
CDH11	NM_001797	ILMN_11789	Homo sapiens cadherin 11, type 2, OB-cadherin (osteoblast) (CDH11), mRNA.	-1.6
NT5DC1	NM_152729	ILMN_22046	Homo sapiens 5'-nucleotidase domain containing 1 (NT5DC1), mRNA.	-1.6
C1orf24	NM_022083	ILMN_6530	Homo sapiens chromosome 1 open reading frame 24 (C1orf24), transcript variant 1, mRNA.	-1.6
SELENBP1	NM_003944	ILMN_17578	Homo sapiens selenium binding protein 1 (SELENBP1), mRNA.	-1.6
ARRDC4	NM_183376	ILMN_11014	Homo sapiens arrestin domain containing 4 (ARRDC4), mRNA.	-1.6
PLCB1	NM_182734	ILMN_3945	Homo sapiens phospholipase C, beta 1 (phosphoinositide-specific) (PLCB1), transcript variant 2, mRNA.	-1.6
GPC4	NM_001448	ILMN_11696	Homo sapiens glypican 4 (GPC4), mRNA.	-1.6
ALCAM	NM_001627	ILMN_21054	Homo sapiens activated leukocyte cell adhesion molecule (ALCAM), mRNA.	-1.6
GPC3	NM_004484	ILMN_24093	Homo sapiens glypican 3 (GPC3), mRNA.	-1.6
TMEM80	NM_174940	ILMN_10875	Homo sapiens transmembrane protein 80 (TMEM80), mRNA.	-1.6
RPS29	NM_001030001	ILMN_15031	Homo sapiens ribosomal protein S29 (RPS29), transcript variant 2, mRNA.	-1.7

Symbol	RefSeq	Probe ID	Description	Fold
GFRA2	NM_001495	ILMN_24176	Homo sapiens GDNF family receptor alpha 2 (GFRA2), mRNA.	-1.7
CEBPG	NM_001806	ILMN_4860	Homo sapiens CCAAT/enhancer binding protein (C/EBP), gamma (CEBPG), mRNA.	-1.7
LOC389432	NM_001030060	ILMN_18130	Homo sapiens SAM domain containing 1 (LOC389432), mRNA.	-1.7
RGS16	NM_002928	ILMN_16445	Homo sapiens regulator of G-protein signalling 16 (RGS16), mRNA.	-1.7
FLJ21657	NM_022483	ILMN_5037	Homo sapiens hypothetical protein FLJ21657 (FLJ21657), mRNA.	-1.7
TMEM18	NM_152834	ILMN_8053	Homo sapiens transmembrane protein 18 (TMEM18), mRNA.	-1.7
IGFBP7	NM_001553	ILMN_894	Homo sapiens insulin-like growth factor binding protein 7 (IGFBP7), mRNA.	-1.7
HSD17B8	NM_014234	ILMN_9238	Homo sapiens hydroxysteroid (17-beta) dehydrogenase 8 (HSD17B8), mRNA.	-1.7
APCDD1	NM_153000	ILMN_21788	Homo sapiens adenomatosis polyposis coli down-regulated 1 (APCDD1), mRNA.	-1.7
KIAA0367	NM_015225	ILMN_23214	Homo sapiens KIAA0367 (KIAA0367), mRNA.	-1.7
KDELC2	NM_153705	ILMN_23677	Homo sapiens KDEL (Lys-Asp-Glu-Leu) containing 2 (KDELC2), mRNA.	-1.7
KRTAP19-1	NM_181607	ILMN_24171	Homo sapiens keratin associated protein 19-1 (KRTAP19-1), mRNA.	-1.7
GAJ	NM_032117	ILMN_28552	Homo sapiens GAJ protein (GAJ), mRNA.	-1.7
PPM1M	NM_144641	ILMN_4690	Homo sapiens protein phosphatase 1M (PP2C domain containing) (PPM1M), mRNA.	-1.7
C18orf56	NM_001012716	ILMN_21911	Homo sapiens chromosome 18 open reading frame 56 (C18orf56), mRNA.	-1.7
PNMA2	NM_007257	ILMN_10930	Homo sapiens paraneoplastic antigen MA2 (PNMA2), mRNA.	-1.7
KARCA1	NM_152366	ILMN_13490	Homo sapiens kelch/ankyrin repeat containing cyclin A1 interacting protein (KARCA1), transcript variant 1, mRNA.	-1.7
FAM43A	NM_153690	ILMN_21212	Homo sapiens family with sequence similarity 43, member A (FAM43A), mRNA.	-1.7
PSIP1	NM_033222	ILMN_5095	Homo sapiens PC4 and SFRS1 interacting protein 1 (PSIP1), transcript variant 2, mRNA.	-1.7
KLF9	NM_001206	ILMN_2670	Homo sapiens Kruppel-like factor 9 (KLF9), mRNA.	-1.7
PSPH	NM_004577	ILMN_14445	Homo sapiens phosphoserine phosphatase (PSPH), mRNA.	-1.7
MXD3	NM_031300	ILMN_21984	Homo sapiens MAX dimerization protein 3 (MXD3), mRNA.	-1.7
FZD4	NM_012193	ILMN_13859	Homo sapiens frizzled homolog 4 (Drosophila) (FZD4), mRNA.	-1.7
SLC1A4	NM_003038	ILMN_12585	Homo sapiens solute carrier family 1 (glutamate/neutral amino acid transporter), member 4 (SLC1A4), mRNA.	-1.7
PCDH20	NM_022843	ILMN_4787	Homo sapiens protocadherin 20 (PCDH20), mRNA.	-1.7
DNASE2	NM_001375	ILMN_27899	Homo sapiens deoxyribonuclease II, lysosomal (DNASE2), mRNA.	-1.7
STC1	NM_003155	ILMN_16225	Homo sapiens stanniocalcin 1 (STC1), mRNA.	-1.8
RPS6KA2	NM_021135	ILMN_11318	Homo sapiens ribosomal protein S6 kinase, 90kDa, polypeptide 2 (RPS6KA2), transcript variant 1, mRNA.	-1.8
LNK	NM_005475	ILMN_5130	Homo sapiens lymphocyte adaptor protein (LNK), mRNA.	-1.8

Gene	Accession	Probe	Description	Value
DDR2	NM_006182	ILMN_20698	Homo sapiens discoidin domain receptor family, member 2 (DDR2), transcript variant 2, mRNA.	-1.8
SLC7A1	NM_003045	ILMN_22619	Homo sapiens solute carrier family 7 (cationic amino acid transporter, y+ system), member 1 (SLC7A1), mRNA.	-1.8
EIF4EBP1	NM_004095	ILMN_6626	Homo sapiens eukaryotic translation initiation factor 4E binding protein 1 (EIF4EBP1), mRNA.	-1.8
ASNS	NM_133436	ILMN_14195	Homo sapiens asparagine synthetase (ASNS), transcript variant 1, mRNA.	-1.8
SLC38A2	NM_018976	ILMN_10001	Homo sapiens solute carrier family 38, member 2 (SLC38A2), mRNA.	-1.8
BHLHB2	NM_003670	ILMN_24095	Homo sapiens basic helix-loop-helix domain containing, class B, 2 (BHLHB2), mRNA.	-1.8
PLCXD1	NM_018390	ILMN_8273	Homo sapiens phosphatidylinositol-specific phospholipase C, X domain containing 1 (PLCXD1), mRNA.	-1.8
EGR1	NM_001964	ILMN_20932	Homo sapiens early growth response 1 (EGR1), mRNA.	-1.8
SLCO2A1	NM_005630	ILMN_14229	Homo sapiens solute carrier organic anion transporter family, member 2A1 (SLCO2A1), mRNA.	-1.9
DDIT4	NM_019058	ILMN_13176	Homo sapiens DNA-damage-inducible transcript 4 (DDIT4), mRNA.	-1.9
ADM2	NM_024866	ILMN_19761	Homo sapiens adrenomedullin 2 (ADM2), mRNA.	-1.9
PSAT1	NM_021154	ILMN_17460	Homo sapiens phosphoserine aminotransferase 1 (PSAT1), transcript variant 2, mRNA.	-2
RAPGEF3	NM_006105	ILMN_24486	Homo sapiens Rap guanine nucleotide exchange factor (GEF) 3 (RAPGEF3), mRNA.	-2
SLC7A5	NM_003486	ILMN_25446	Homo sapiens solute carrier family 7 (cationic amino acid transporter, y+ system), member 5 (SLC7A5), mRNA.	-2
FOS	NM_005252	ILMN_27030	Homo sapiens v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS), mRNA.	-2
WNT5A	NM_003392	ILMN_14624	Homo sapiens wingless-type MMTV integration site family, member 5A (WNT5A), mRNA.	-2
ASS	NM_000050	ILMN_18789	Homo sapiens argininosuccinate synthetase (ASS), transcript variant 1, mRNA.	-2
ULBP1	NM_025218	ILMN_1673	Homo sapiens UL16 binding protein 1 (ULBP1), mRNA.	-2
CBS	NM_000071	ILMN_5029	Homo sapiens cystathionine-beta-synthase (CBS), mRNA.	-2
HERPUD1	NM_001010990	ILMN_19884	Homo sapiens homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1 (HERPUD1), transcript variant 3, mRNA.	-2.1
MTHFD2	NM_006636	ILMN_23782	Homo sapiens methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase (MTHFD2), nuclear gene encoding mitochondrial protein, mRNA.	-2.1
SESN2	NM_031459	ILMN_7290	Homo sapiens sestrin 2 (SESN2), mRNA.	-2.1
TSC22D3	NM_004089	ILMN_9893	Homo sapiens TSC22 domain family, member 3 (TSC22D3), transcript variant 2, mRNA.	-2.1
PCK2	NM_001018073	ILMN_18787	Homo sapiens phosphoenolpyruvate carboxykinase 2 (mitochondrial) (PCK2), nuclear gene encoding mitochondrial protein, transcript variant 2, mRNA.	-2.2

Gene	Accession	Probe	Fold	Description
TRIB3	NM_021158	ILMN_21257	-2.2	Homo sapiens tribbles homolog 3 (Drosophila) (TRIB3), mRNA.
STC2	NM_003714	ILMN_28725	-2.2	Homo sapiens stanniocalcin 2 (STC2), mRNA.
CEBPB	NM_005194	ILMN_4674	-2.4	Homo sapiens CCAAT/enhancer binding protein (C/EBP), beta (CEBPB), mRNA.
SLC6A9	NM_006934	ILMN_8399	-2.9	Homo sapiens solute carrier family 6 (neurotransmitter transporter, glycine), member 9 (SLC6A9), transcript variant 1, mRNA.
PCK2	NM_004563	ILMN_2603	-2.9	Homo sapiens phosphoenolpyruvate carboxykinase 2 (mitochondrial) (PCK2), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA.
CHAC1	NM_024111	ILMN_2489	-3.2	Homo sapiens ChaC, cation transport regulator-like 1 (E. coli) (CHAC1), mRNA.

Appendix table **S6**:Results of high-resolution mass spectrometric-based metabonomic analysis of *Npas3* wild-type and knockout brain tissue showing molecular formula of metabolites, tentative assignment of metabolites, KEGG pathways, m/z ratios & retention times. Sieve was used to identify metabolites altered in the KO animals by calculating a p-value and ratio based on the average intensities of individual peaks corresponding to different metabolites. A significant difference in the level of each metabolite between genotypes was set at p-value <0.05 and/or ratio less than 0.5 for down-regulated metabolites and greater than 2 for up-regulated metabolites. For metabolite identification, masses were searched against the exact masses of 6,000 biomolecules using an in-house developed macro which also contained the retention times of metabolites obtained from analysis of standards (Excel, Microsoft). Decreased (a,c) and increased (b,d) metabolites are shown from positive (a,b) and negative (c,d) ionisation modes. Asterisked compounds in (d) highlight ambiguous identities.

a

Formula	Compounds	Pathway(s)	M/Z	Time	P-Value	Ratio
$C_3H_7O_6P$	Glycerone phosphate (DHAP)	Nicotinamide_Glyco_Fructo_Galacto_GPL_IP_Lipid_Pent-phosph_Pyruv	171.0053	13.7	2.62×10^{-5}	0.061
$C_{13}H_{21}N_3O_8S$	(R)-S-Lactoylglutathione	Pyruv	380.1124	12.5	1.77×10^{-5}	0.259
$C_7H_{16}N_2O_2$	N(6)-Methyllysine	None	161.1284	32.5	2.28×10^{-4}	0.328
$C_8H_{17}NO_2$	Propionyl Acetylcholine	None	160.1332	12.8	3.49×10^{-4}	0.36
$C_6H_{12}O_4$	(R)-Pantoate	Panto_ CoA	149.0808	15.3	6.17×10^{-4}	0.515
$C_5H_8N_2O_2$	5,6-Dihydrothymine	Pyrimidine	129.0658	14.4	8.07×10^{-4}	0.555
$C_5H_{10}N_2O_3$	Glutamine	Glu_Purine_Pyrimidine	147.0765	15.3	1.06×10^{-3}	0.596
$C_5H_{11}N_3O_2$	Guanidino butyric acid	Urea	146.0925	13.0	4.60×10^{-5}	0.605
$C_{10}H_{17}N_3O_6$	Gamma glutamyl glutamine	None	276.119	15.0	4.25×10^{-4}	0.633
$C_5H_9NO_2S$	Thiomorpholine 3-carboxylate	None	148.0427	10.577	7.96×10^{-3}	0.641
$C_{10}H_{16}N_4O_3$	Homo-carnosine	Urea	241.1296	26.3	1.33×10^{-2}	0.688
$C_5H_7NO_3$	Pyrroline-4-hydroxy-2-carboxylate	Arg/Pro	130.0498	15.3	1.28×10^{-3}	0.722
$C_6H_9N_3O_2$	L-Histidine	His_btAla	156.0769	26.3	3.39×10^{-2}	0.761
$C_4H_7NO_4$	L-Aspartate	His_ Arg/Pro_Urea_Ser/Gly/Thr_Nicotinamide_Lys_Ala/Asp_Pantt	134.0448	15.0	1.03×10^{-3}	0.766
$C_4H_5NO_3$	Maleamate	Nicotinamide	116.0342	15.0	1.08×10^{-3}	0.766
CH_5O_4P	Hydroxymethylphosphonate	AminoPhos	112.9998	13.3	2.20×10^{-2}	0.786
$C_6H_6N_2O$	Nicotinamide	Nicotinamide	123.0553	8.2	2.97×10^{-3}	0.824
$C_6H_{11}NO_4$	O-Acetyl-L-homoserine	Met_Sul	162.0761	11.8	4.34×10^{-2}	0.824

b

Formula	Compounds	Pathway(s)	M/Z	Time	P-Value	Ratio
$C_{21}H_{28}N_7O_{14}P_2^+$	NAD$^+$	Nicotinamide_Glu_OxidPhos	664.1162	16.9	2.69×10^{-8}	12.36
$C_7H_{14}N_2O_4S$	L-Cystathionine	Ser/Gly/Thr_Met_Sul	223.0748	19.2	8.05×10^{-6}	3.741
$C_4H_{10}N_3O_5P$	Phospho-creatine	Arg/Pro	212.0431	13.682	1.39×10^{-5}	2.042
$C_{11}H_{20}N_2O_3$	L-leucyl-L-proline	None	229.155	13.0	1.63×10^{-5}	1.859
$C_6H_{14}N_2O_2$	L-Lysine	Lys(Syn)_Lys(Deg)_Biot	147.1129	24.5	4.24×10^{-11}	1.845
C_3H_7NO	Aminoacetone	Ser/Gly/Thr	74.06009	14.82	1.82×10^{-10}	1.704
$C_9H_{15}N_3O_2S$	Ergothioneine	None	230.0959	15.8	5.76×10^{-5}	1.688
$C_3H_9O_6P$	Glycerol 3-phosphate	Ala_GPL_Lipid	173.021	13.8	7.66×10^{-6}	1.635
$C_{12}H_{20}N_2O_3$	Slaframine	Bio-alkaloid	241.1548	12.745	4.31×10^{-5}	1.414
$C_{10}H_{19}NO_4$	Propionyl carnitine	None	218.139	10.7	5.37×10^{-3}	1.402
$C_5H_4N_4O_2$	Xanthine	Purine	153.0408	8.2	1.97×10^{-4}	1.389
$C_6H_{13}N_3O_3$	L-Citrulline	Arg/Pro_Urea	176.103	15.821	2.62×10^{-4}	1.372
$C_9H_{17}NO_4$	O-Acetylcarnitine	Ala/Asp	204.123	11.7	5.39×10^{-4}	1.361
$C_5H_{15}NO_4P$	Phosphoryl-choline	GPL	184.0734	19.3	1.78×10^{-4}	1.314
$C_9H_{13}N_3O_5$	Cytidine	Pyrimidine	244.0929	16.4	1.12×10^{-3}	1.247
$C_6H_{14}N_4O_2$	L-Arginine	Arg/Pro_Urea	175.1189	24.1	7.27×10^{-5}	1.244
$C_5H_9NO_2$	L-Proline	Arg/Pro	116.0707	13.6	4.15×10^{-4}	1.234
H_3PO_4	Phosphoric acid	Oxidphos_Photo_Peptiglyc_ABC_Parki	98.98418	14.9	1.39×10^{-5}	1.223
$C_4H_5N_3O$	Cytosine	Pyrimidine	112.0506	16.4	2.04×10^{-3}	1.197
$C_2H_7NO_3S$	Taurine	Taurine	126.0219	14.3	5.63×10^{-6}	1.184

c

Formula	Compounds	Pathway(s)	M/Z	Time	P VALUE	RATIO
$C_8H_{18}O_{14}P_2$	Octulose-1,8-bisphosphate	Pent-phosph	399.01035	13.7	1.99×10^{-3}	0.052
$C_{15}H_{12}O_5$	Naringenin	None	271.06073	14.5	2.76×10^{-2}	0.431
$C_6H_{14}O_{12}P_2$	Fructose 1,6-bisphosphate	Pent-phosph, Glyco, Fructo	338.98907	19.3	5.61×10^{-2}	0.443

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d

Formula	Compounds	Pathway(s)	M/Z	Time	P VALUE	RATIO
$C_{16}H_{26}N_2O_{16}P_2$	*dTDP-glucose, dTDP-galactose,	Galacto- Nucleo	563.06958	16.6	2.73×10^{-5}	3.446
$C_7H_{14}O_7$	Sedoheptulose	Pent-phosph	209.06688	10.6	4.59×10^{-5}	2.653
$C_{14}H_{25}N_3O_6S_2$	Aspartylmethionylmethionine	None	394.11221	15.1	4.41×10^{-6}	2.566
$C_4H_6O_4$	Succinate	Glu_Tyr/Phe_Ala/Asp_Benzt_Butan_CO2fix_Glyoxy_Propn	117.01945	5.9	1.28×10^{-4}	2.05
$C_{15}H_{24}N_2O_{17}P_2$	*UDP-D-galactose; UDP-glucose	Galacto_Nucleo	565.04822	16.8	3.49×10^{-4}	1.968
$C_3H_7O_5P$	Propanoyl phosphate	Propn	152.996	12.5	1.88×10^{-4}	1.943
$C_{20}H_{31}N_4O_{16}P$	CMP-N-acetyl-neuraminate	AmnoSug	613.14081	15.1	7.91×10^{-4}	1.939
$C_5H_6O_5$	α-ketoglutaric acid	Glu_His_Lys(syn)_Ala/Asp_VB6_Ascob_Butan_CO2fix_Glyoxy	145.0144	8.4	6.07×10^{-4}	1.793
$C_3H_4O_4$	*Hydroxypyruvate; Malonate; 2-Hydroxy-3-oxopropanoate	Ser/Gly/Thr ; Pyrimidine	103.00379	7.9	2.74×10^{-3}	1.595
$C_5H_4N_4O_3$	Uric Acid (Urate)	Purine	167.02121	10.6	1.30×10^{-2}	1.506
$C_7H_{14}O_8$	Glucoheptonic acid	None	225.06163	15.1	1.67×10^{-2}	1.481
$C_2H_6O_4S$	2-Hydroxyethanesulfonate	Taurine	124.99155	10.4	1.71×10^{-2}	1.469
$C_5H_{13}O_7P$	2-C-Methyl-D-erythritol 4-phosphate	Sterd	215.033	15.1	2.91×10^{-2}	1.4
$C_6H_8O_6$	Ascorbate	GSH_Ascob	175.02481	10.3	4.11×10^{-2}	1.369