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Mammalian ISWI gene knockdown modulates growth and differentiation properties of neural progenitor cells

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Mammalian ISWI gene knockdown modulates growth and differentiation properties of neural progenitor cells

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THESIS
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Abstract

Epigenetic modifications during cellular differentiation are critical for establishing the expression of tissue specific genes characterizing particular cell types. There are two groups of chromatin modifying enzymes that regulate these processes, the ATP-dependent complexes, and the histone modifying complexes. In ATP-dependent remodelling complexes, the imitation switch (ISWI) group has been studied extensively in the past. ISWI proteins were initially discovered in *Drosophila* and mammals contain two orthologs called *SNF2H* and *SNF2L*. Past experiments demonstrated that these remodelers have distinct roles in cellular differentiation and proliferation. *SNF2H* expression is greatest in proliferating neuroprogenitor populations, whereas *SNF2L* predominates in mature neurons. Additionally, *SNF2H* null mice are periimplantation lethal due to defective cellular proliferation. Other studies showed that neuronal cell cultures ectopically expressing increased levels of *SNF2L* display a dramatic increase in neurite extension and spontaneous differentiation. These findings point to requirements for *SNF2L* during neuronal differentiation but the distinct manner in which ISWI proteins oversee this process remains unknown. To elucidate the role of ISWI proteins in the regulation of neuronal proliferation and differentiation we used shRNA transfection to stably knockdown the genes of interest in established neuronal cell lines. Concurrently, we examined the growth and differentiation properties of primary neuronal cultures derived from mice functionally ablated for *Snf2l*. Results in neuroblastoma cultures show that upon *Snf2l* knockdown proliferation is maintained in 30% of the population under differentiation conditions at 4 days of differentiation. In contrast, Snf2H knockdown cells proliferate normally but undergo extensive apoptosis when induced to differentiate. Aberrant cell replication was also observed in E14.5 and E12.5 neurosphere cultures and resulted in a 2-fold decrease in the number of neurons generated and a 4-fold reduction in astrocyte production at 4 days of differentiation. Moreover, both astrocytes and neurons expressing differentiation markers had reduced neurite extensions and branching patterns suggesting a distinct developmental delay. Taken together these studies define distinct roles for ISWI in growth and differentiation of CNS cells.

Acknowledgements

Initially in my life I was raised to keep an open mind and be curious about the world around me by the two people who were most important in my life at that time, my parents. Their teaching and quiet support knew no bounds and I was able to grow and develop at my own pace under their guidance. Today their staunch support has not lapsed in any way. My father grew up in a working class family but through hard work and determination succeeded in many areas early on and then returned to complete his Master's degree from the University of Waterloo. His determination and unflagging desire to make his life and that of his family better is the exact model that I hold myself up to. My mother grew up in a rural setting and while post-secondary education was not in the cards for her she dedicated a great portion of her working life to the Board of Education in a supportive role. There is no doubt in my mind that this support allowed many children to gain the utmost from their time in the schools where she worked. The greatest portion of this thesis is dedicated to my parents because without their direction and aid I would not be in a position to accomplish what I desire.

I am also indebted to my supervisor Dr. David Picketts who took an inexperienced student and gave him the opportunity to further his education in an ideal environment for scientific learning. This would also not have been possible without the other members of the Picketts laboratory who had to put up with my constant questions and mistakes. And finally this thesis is mine, it allowed me to experience an aspect of the world that I have long been curious about. Through this experience I have learned that analytical thinking is critical in all aspects of life but also that over analysis is not always required.

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List of Abbreviations

ACF	ATP-utilizing chromatin assembly and remodeling factor
ADP	adenosine diphosphate
ATP	adenosine triphosphate
ATRX	α-thalessemia X-linked mental retardation gene
BAF	Brg/Brm-associated factor
BDNF	brain derived neurotrophic factor
BLAST	basic local alignment and search tool
BMP4	bone morphogenetic protein 4
BPTF	bromodomain PHD finger transcription factor
BRAF35	Brca2-associated factor 35
Brca1	breast cancer 1 gene
Brca2	breast cancer 2 gene
BrdU	5-bromo-2-deoxyuridine
BRG1	brahma-related gene 1
BRM	Brahma
Camk2a	Ca^{2+}/calmodulin kinase II inhibitor α gene
cAMP	cyclic adenosine monophosphate
Casp3	caspase 3 gene
Ccnb2	cyclin B2 gene
cDNA	complimentary DNA
CECR2	cat eye syndrome chromosome region candidate 2
CERF	CECR2-containing remodeling factor
CHD	chromodomain helicase DNA-binding family
ChIP	chromatin immunoprecipitation
CHRAC	chromatin accessibility complex
CLS	Coffin-Lowry syndrome
CNS	central nervous system
CREB	Cyclic AMP Response Element-binding Protein
CSB	Cockayne syndrome group B family
DAPI	4',6-diamidino-2-phenylindole
DDM1	Deficient in DNA Methylation 1 family
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Dnajc2	DnaJ (Hsp40) homolog, subfamily C, member 2
dNTP	Deoxyribonucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EtOH	ethanol
EV	empty vector
E2F3	E2F transcription factor 3
Fabpi	Fatty acid-binding protein, intestinal
FBS	fetal bovine serum

FITC	Fluorescein isothiocyanate
FRAXA	fragile X syndrome
FMR2	fragile X mental retardation-2 gene
GDI1	guanosine diphosphate dissociation inhibitor 1
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
hEGF	human epithelial growth factor
HAT	histone acetyltransferase
HDAC	histone deacetylase
HoxB9	homeo box B9 gene
HP1	heterochromatin protein 1
HPLC	high performance liquid chromatography
Hus1	Hus1 homolog
IL1RAPL	X-linked interleukin-1 receptor accessory protein-like 1 precursor
INK4	inhibitor of cyclin-dependent kinase 4
IQ	intelligence quotient
ISWI	imitation switch
Isw1	insertion switch 1
Isw2	insertion switch 2
KCl	potassium chloride
LICAM	ligand-induced cell adhesion molecule
LTP	long-term potentiation
MeCP2	methyl CpG-binding protein 2
MeOH	methanol
MID-1	Midline 1 RING finger protein
Msh2	mutant S homolog
NaCl	sodium chloride
NCAM	neural cell adhesion molecule
NoRC	nucleolar remodeling complex
NURD	nucleosome remodeling and histone deacetylase complex
NURF	nucleosome remodeling factor
OPHN1	oligophrenin-1
PAK3	p21-activating protein kinase 3
Pax6	paired box gene 6
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PHD	plant homeodomain
PMSF	phenylmethylsulfonyl fluoride
qPCR	quantitative polymerase chain reaction
RbAP48/46	retinoblastoma-associated protein 48 and 46
Rb	retinoblastoma gene
Rbl1	Retinoblastoma-like 1 (p107)
RNA	ribonucleic acid
RSF	remodeling and spacing factor
RSC	remodelling the structure of chromatin complex
Rsk-2	ribosomal S6 protein kinase 2

RTS	Rubinstein-Taybi syndrome
SANT	SWI3 ADA2 N-CoR TFIIIB domain
Sesn2	sestrin 2 gene
Shh	sonic hedgehog gene
shRNA	short hairpin ribonucleic acid
SH2	Src homology 2 domain
SLIDE	SANT-like ISWI domain
Snf2H	sucrose nonfermenting 2 homolog
Snf2L	sucrose nonfermenting 2 like
Sry	sex-determining Region Y gene
SWI/SNF	Switch / Sucrose nonfermenting family
Tip5	termination factor TTFI-interacting protein 5
Tris	trishydroxymethylaminomethane
Tuj1	Neuronal Class III β-Tubulin
TUNEL	Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling
VXC-A	variably charged X chromosome mRNA on CRI-S232A
WAL/BAZ/BAF	wstf-acf-like/ bromo-adjacent zinc finger/ bromo-adjacent homology
WCRF	Williams syndrome transcription factor-related chromatin remodeling
WICH	WSTF–ISWI chromatin remodeling complex factor
WICH	WSTF–ISWI chromatin remodeling complex
WSTF	Williams syndrome transcription factor
XLMR	x-linked mental retardation
Xmeis	Xenopus homolog of Meis1 (Myeloid Ecotropic viral Integration Site 1)
Wt	wild type

1.0 Introduction

1.1 Genetic causes of X-linked mental retardation

1.1.1 Overview

Mental retardation is the most commonly occurring genetic disorder causing people to seek genetic counseling in North America (1). These conditions are typified by an intelligence quotient (IQ) score of <70 and will affect between two and three percent of the population worldwide (2). Severe retardation conditions result in IQ scores <50 and are represented in a smaller but still significant 0.3 percent of the population. Current scientific study has determined that there is a definitive genetic component to more than half of these clinical cases. Unfortunately the root cause of many of these conditions remains unknown. As research techniques advance the list of genetic mutations causing developmental delay is rapidly growing. There are many different types of mental retardation and when the Online Mendelian Inheritance in Man website is queried it results in 790 autosomal recessive, 451 autosomal dominant, 395 X-linked and approximately 121 that have yet to be classified.

Mental retardation conditions may be divided into two general categories; those where the intellectual restriction is only a secondary feature of structural brain malformation resulting in symptomatic features (syndromal mental retardation), and those where this intellectual restriction is the only symptom to occur (non-specific mental retardation) (1-4). The X chromosome has been a key region studied for both forms of mental retardation due its relative ease of genetic mapping and to the haploid state of the encoded genes. As a result when conditions are found to be due to mutation events to the X chromosome they are termed X-linked mental retardation conditions (XLMR) (1, 2). These conditions are

represented by various mutation events in a wide variety of genes and occur much more significantly in males than in females.

Currently there have been more genetic mutations found on the X chromosome resulting in mental retardation than any other though this may be a result of the hemizygous state of this chromosome making for easier gene identification as compared to other autosomal regions (3). There have so far been 61 different genes detected on the X chromosome resulting in either syndromal or non-specific XLMR, (Figure 1). One of the most significant genes causing XLMR to date is called *FMR1* which when mutated causes the fragile X syndrome (FRAXA), and accounts for 15-20 percent of diagnosed XLMR conditions. Other genes such as *Rsk-2, MID-1* and *LICAM* affect various essential factors like growth factor-induced kinase, transcriptional zinc-finger proteins and cellular adhesion molecules respectively and indicate the wide range of essential cellular processes that may be affected. No matter the gene that is examined they all play key roles in the developmental stages for the brain and may also have regulatory functions for other essential genes.

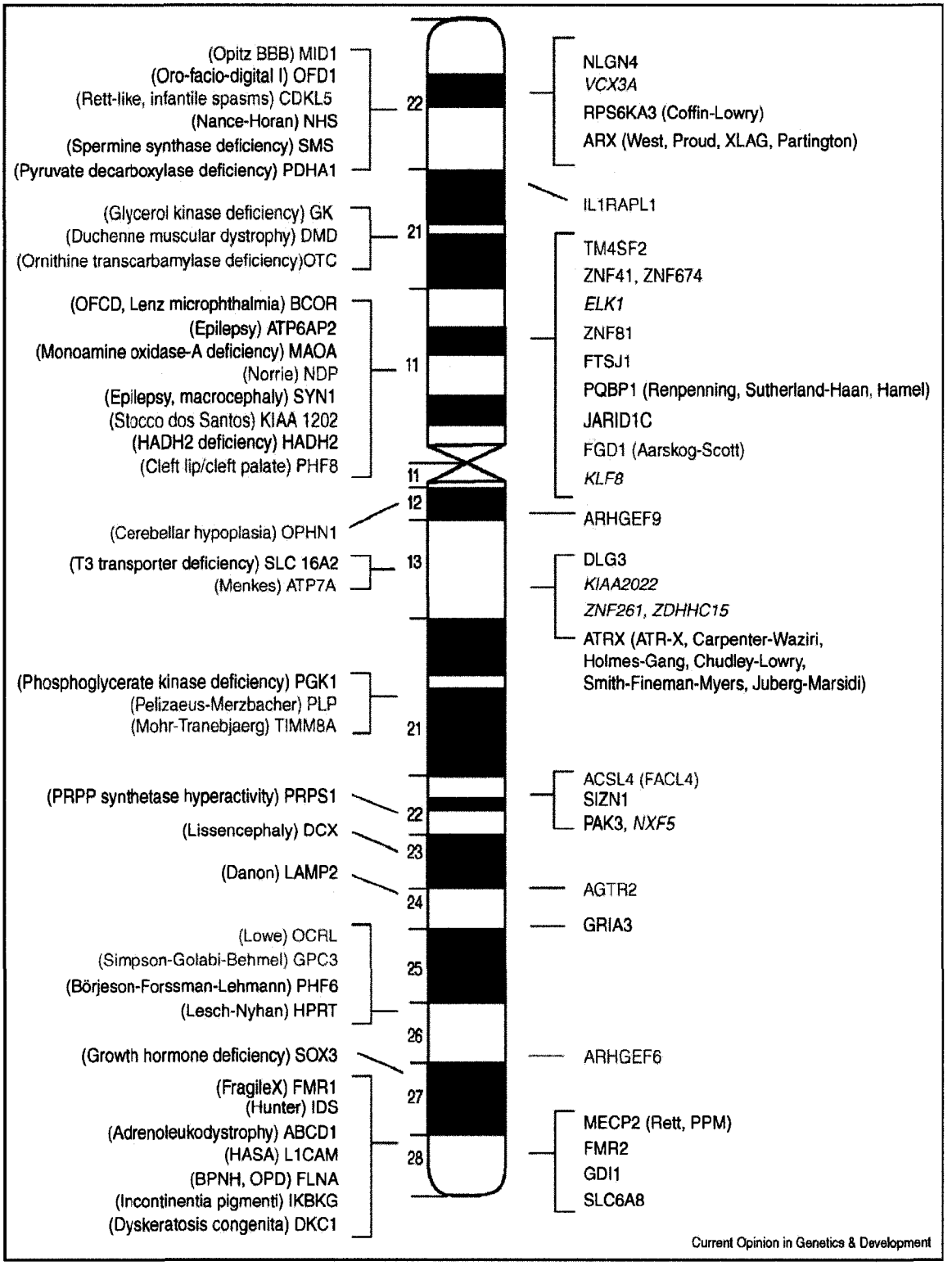
Non-specific mental retardation genes are much fewer in number with approximately 10-12 having been identified. The initial gene discovered for this condition was fragile X mental retardation gene 2 (*FMR2*) (5). The mutational event in *FMR2* that results in non-specific retardation is an unstable CGG repeat of over 200 copies that silences gene expression. Other non-specific XLMR genes that have been identified include oligophrenin-1 (*OPHN1*), p21-activating protein kinase 3 (*PAK3*) and Rab GDP-dissociation inhibitor 1 (*GDI1*), interleukin-1 receptor accessory protein-like (*IL1RAPL*), variably charged X chromosome mRNA on CRI-S232A (*VXC-A*) to name a few (5). The complete list is shown in Figure 1.

Many of the genes involved in XLMR conditions can be classified into two categories, those involved in the regulation of signaling pathways and those involved in chromatin regulation. For example genes such as *OPHN1* and *PAK3* function in RhoGTPase signaling pathways along the cytoskeleton from the nucleus to the cell surface membrane and are essential for correct neuronal development and synaptogenesis (6). Here we focus on those genes involved in the regulation of chromatin and chromatin remodeling such as *ATRX, MeCP2, RSK2* and *ISWI.*

1.1.2 Chromatin remodeling and XLMR

One group of genes that is rapidly coming to the forefront for XLMR conditions is the chromatin remodeling group. These genes will be discussed in further detail below but are essential transcriptional regulatory units capable of both transcriptional activation and inhibition. Various members of the chromatin remodeling genes are known to be essential for brain development and as may be expected when mutation occurs there are a variety of conditions that will develop. One of the key founding genes for the importance of chromatin remodeling complexes in brain development is called *ATRX*, α-thalessemia X-linked mental retardation gene (7, 8). This gene contains a plant homeodomain-type (PHD-type) zinc finger motif and a sucrose nonfermenting 2 family (SNF2)/ ATPase domain. It is these two regions of the gene that are most

FIGURE 1: X CHROMOSOME MENTAL RETARDATION GENES. Map of the X chromosome detailing the genes identified resulting in both syndromic (on left) and non-specific (on right) mental retardation, red indicates genes detected from patient screening. Those shown in italics represent genes whose status has not yet been proven. Figure adapted from Ropers 2006.


(Opitz BBB) MID1
(Oro-facio-digital I) OFD1
(Rett-like, infantile spasms) CDKL5
(Nance-Horan) NHS
(Spermine synthase deficiency) SMS
(Pyruvate decarboxylase deficiency) PDHA1
(Glycerol kinase deficiency) GK
(Duchenne muscular dystrophy) DMD
(Ornithine transcarbamylase deficiency)OTC
(OFCD, Lenz microphthalmia) BCOR
(Epilepsy) ATP6AP2
(Monoamine oxidase-A deficiency) MAOA
(Norrie) NDP
(Epilepsy, macrocephaly) SYN1
(Stocco dos Santos) KIAA 1202
(HADH2 deficiency) HADH2
(Cleft lip/cleft palate) PHF8
(Cerebellar hypoplasia) OPHN1
(T3 transporter deficiency) SLC 16A2
(Menkes) ATP7A
(Phosphoglycerate kinase deficiency) PGK1
(Pelizaeus-Merzbacher) PLP
(Mohr-Tranebjaerg) TIMM8A
(PRPP synthetase hyperactivity) PRPS1
(Lissencephaly) DCX
(Danon) LAMP2
(Lowe) OCRL
(Simpson-Golabi-Behmel) GPC3
(Börjeson-Forssman-Lehmann) PHF6
(Lesch-Nyhan) HPRT
(Growth hormone deficiency) SOX3
(FragileX) FMR1
(Hunter) IDS
(Adrenoleukodystrophy) ABCD1
(HASA) L1CAM
(BPNH, OPD) FLNA
(Incontinentia pigmenti) IKBKG
(Dyskeratosis congenita) DKC1
22
21
11
11
12
13
21
22
23
24
25
26
27
28
NLGN4
VCX3A
RPS6KA3 (Coffin-Lowry)
ARX (West, Proud, XLAG, Partington)
IL1RAPL1
TM4SF2
ZNF41, ZNF674
ELK1
ZNF81
FTSJ1
PQBP1 (Renpenning, Sutherland-Haan, Hamel)
JARID1C
FGD1 (Aarskog-Scott)
KLF8
ARHGEF9
DLG3
KIAA2022
ZNF261, ZDHHC15
ATRX (ATR-X, Carpenter-Waziri,
Holmes-Gang, Chudley-Lowry,
Smith-Fineman-Myers, Juberg-Marsidi)
ACSL4 (FACL4)
SIZN1
PAK3, NXF5
AGTR2
GRIA3
ARHGEF6
MECP2 (Rett, PPM)
FMR2
GDI1
SLC6A8
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often mutated in various XLMR syndromes. Under normal conditions the ATRX protein plays roles in chromatin accessibility through ATPase activity, associates with HP1 at heterochromatin, is recruited to promyelotic nuclear bodies through association with other proteins and functions in methylation states of various regions of DNA (9, 10). ATRX patients are subjected to symptoms such as facial/genital/skeletal abnormalities, α-thalassemia, optic atrophy, and severe cognitive delay.

In studies conducted on mice it was found that complete loss of the *Atrx* gene results in embryonic death during early developmental stages (10). Regionally specific knockout studies in the murine forebrain demonstrated that there was a reduced size in knockout animals. When this was examined in detail it was found that this was due to a vastly reduced number of neurons in the upper cortical layers without any loss of the proliferative capacity of the neuroprogenitor cells (7). This loss of neurons came about as a result of increased apoptosis specifically in the differentiating population. Recent discovery has demonstrated links between ATRX and another chromatin remodeling associated protein called methyl CpG-binding protein 2 (MeCP2) (11).

The MeCP2 protein functions as a gene silencer due to its ability to recruit various complexes, such as histone deacetylases (HDACs) and SWI/SNF complexes (12). It contains two functional domains the first being a methyl-CpG binding site and the second being a transcriptional repression domain. When mutation occurs to this gene a condition called Rett syndrome develops. This syndrome is typified by the postnatal loss of certain motor skills, language capability, and autism (13). Clinically, Rett syndrome affects more females than males and is one of the most common severe mental retardation conditions for females, though this is likely due to more frequent neonatal death in affected males (14).

Molecular investigation of Rett syndrome using mouse models demonstrated that there are distinct transient delays in post mitotic neuronal development characteristic of the developmental abnormalities observed in Rett patients (13). These results are most evident in cortical development with pyramidal neurons being smaller and having less dendritic and axonal extensions in cortical layers 2 and 3. Knockout mice also show decreased spatial learning and memory along with altered 3-methoxy-4-hydroxyphenylethylene glycol levels in the cerebrospinal fluid.

Recently, a study was conducted to find proteins that interact with MeCP2 in order to further clarify the neuronal abnormalities found in Rett syndrome patients. This study culminated in the discovery that *MeCP2* is capable of direct association with *ATRX* during in vitro studies (11). When mutations such as p.A140V or p.R133C occur in the *MeCP2* gene it is no longer capable of recruiting the ATRX protein to its binding site and therefore there is altered repression of the target genes. Previously it was shown that under normal conditions the N-terminal region of ATRX is responsible for its localization to pericentromeric heterochromatin. However upon the loss of MeCP2 the ATRX localization is diffuse throughout the nucleus suggesting that in the brain the interaction of these two proteins is critical for correct ATRX localization.

The gene brain derived neurotropic factor (*BDNF*) has been shown to be a direct target of *MeCP2* (15). In *MeCP2* knockout mice models there is a distinct down regulation of *BDNF* compared to wild type levels which was surprising given that *MeCP2* acts as a repressor of transcription (12). *BDNF* is a member of the neurotrophin family of secretory proteins and is directly involved in neuronal survival, differentiation and also acts as a chemoattractant for extending neurites in the developing brain (16). Therefore the loss of *BDNF* in Rett syndrome is a likely cause for the developmental abnormalities that are found.

The final gene that will be discussed here is the ribosomal S6 protein kinase gene 2 (*RSK2*). This gene codes for a serine threonine protein kinase growth factor on the X chromosome that acts in the RAS signaling cascade (17, 18). When mutation occurs to this gene a rare X-linked syndromic form of mental retardation develops called Coffin-Lowry syndrome (CLS). CLS patients are affected by morphological defects to the head, face, and hands accompanied by shortened stature, hearing loss, and severe mental disruption in males with often less severe effects in females. To date there are 75 specific genetic mutations that will result in CLS. Humans have four RSK family members and structurally they are made up of two functional catalytic kinase regions attached to each other by a regulatory linker region. The N-terminal region will always contain a region related to the AGC kinase family while the C-terminal region contains calcium/calmodulin dependent kinase relatives. Transcripts of these genes are detected throughout the body and have direct functional roles in cellular proliferation/differentiation, stress response and apoptosis. These proteins have both cytosolic and nuclear substrates and will regulate genes such as glycogen synthase kinase 3, pro-apoptotic BCL2 antagonist of cell death, cAMP responsive element binding protein and many others.

The *ATRX, MeCP2* and *RSK2* genes represent only a few of the chromatin remodeling genes that are essential for proper brain development and neurological function. In the following sections the processes of chromatin remodeling will be examined in detail with a focus on the imitation switch (ISWI) family. Recent discovery has indicated that this family will play key roles in brain development and is therefore a likely candidate underlying XLMR.

1.2 Chromatin Remodeling

1.2.1 Chromatin: the basics

The term chromatin, meaning "coloured material", was first coined by Walther Flemming in 1882 and was created based on his assumption that there was some sort of nuclear scaffolding contained within the nucleus. This estimation was based on the observation that when the nucleus was stained, using various techniques, there was a definitive substance that reliably took up the dye (19). Since these times intensive study has discovered that Flemming's early estimation was correct and we now know that chromatin is the building block of chromosomes in the eukaryotic nucleus.

In the modern era intensive study has resulted in a focused understanding of the structure and function of chromatin. Overall the eukaryotic nucleus has two forms of chromatin; euchromatin and heterochromatin (19). Heterochromatin refers to the highly condensed, typically transcriptionally inactive, chromatin and may be broken down into two groupings; constitutive and facultative heterochromatin. The term constitutive heterochromatin refers to the highly repetitive DNA regions contained within the brightly stained areas after nuclear stains such as 4', 6-diamidino-2-phenylindole (DAPI) have been used. DNA in areas such as these is tightly compressed and therefore transcriptionally inactive. Facultative heterochromatin encompasses DNA regions that have previously been transcriptionally permissive but which have since been transcriptionally silenced. In many cases these regions are often difficult to visualize using standard techniques. An exception to this rule is the formation of the Barr body, or inactivated X chromosome, in mammalian female cells. From a transcriptional standpoint euchromatin is opposite to heterochromatin. Euchromatic regions contain the genes that are actively transcribed, or have the potential to be, and are therefore much more loosely packaged than heterochromatin.

While all of the cells contained within an organism contain an exact DNA sequence compliment not all cells are identical. This is a result of variation in gene expression profiles due to epigenetic modifications. The term epigenetics was first coined by Conrad Waddington and today is used to describe all of the changes occurring to cells that do not involve direct changes in the underlying DNA sequence. Many of these epigenetic modifications are heritable and are passed down from an individual to its offspring while others are more dynamic and may change as a cell ages. Processes such as histone acetylation, DNA methylation, ADP-ribosylation, ubiquitylation, chromatin remodeling and RNA interference have reshaped the way that scientists view the processes of cellular differentiation.

1.2.2 Chromatin architecture

The human genome is made up of approximately $3.0x10^9$ basepairs that encode all of the genes that result in a human being (20, 21). As may be expected, if the DNA double helical string were stretched out its length would greatly exceed the storage capacity of any eukaryotic nucleus. As a result DNA is reorganized into a highly structured architecture allowing it to be condensed into the nucleus. When this compression is discussed in scientific terms it is referred to as the packaging ratio which refers to the length of the DNA divided by the length that it is compressed into, in most cases the nucleus. In humans the shortest chromosome is approximately $4.6x10^7$ basepairs in length which will equal out to 14,000 μm when the DNA string is unfolded. But when the strand is at its most condensed, during mitosis, it is contained in a length of only 2 μm giving a packaging ratio of 7000 times.

This condensation is highly structured and follows three basic processes the first one being the formation of nucleosomes. The nucleosome is the most basic packaging unit in eukaryotic cells and occurs when 146-147 basepairs of DNA wrap two and one half times around an octamer set of two copies each of the core histones H2A, H2B, H3 and H4. When this structure forms it increases the packaging ratio by six times and during initial microscopic studies was termed a beads-on-a-string formation since the nucleosomes will occur either in ordered arrays or intermittently along the DNA string. This arrangement may be broken down into three separate domains; the core DNA wrapped directly around the histones, the histone core, and the linker DNA found between each histone bead.

Each histone protein contains a C-terminal globular domain and an N-terminal tail domain (22). The globular domains are the areas of each histone molecule that form the protein core of the nucleosome particles and the tail domains are the regions that will interact with the adjacent DNA and associated proteins. These N-terminal tails are all highly conserved throughout evolution in part due to intense posttranslational modifications which allow for specific epigenetic modifications to occur. These posttranslational modifications to the histone tails are termed the "histone code" and are heritable and conserved from one generation to the next.

The second architectural arrangement is the 30 nm fiber which appears in interphase chromatin and during mitosis. Thirty nm fibers result in another 40x increase to the packaging ratio and makes for a solenoid structure that contains approximately six nucleosomes per turn. This means that for every 1 μm of 30 nm fiber there is 40 μm of the original DNA string. In order for these fibers to form, histone H1 binds to the linker DNA and facilitates helical packaging of the chromatin fibers. Finally these fibers will coil and loop together to form the chromosome. This process is extremely complicated but will result

in the formation of the 700 nm metaphase chromosome and can impart up to a 10,000x increase to the packaging ratio. Please refer to Figure 2 for graphical representations of these packaging processes.

One of the key aspects for all of these condensation activities is that when they form they present a barrier to the binding and subsequent activity of the transcriptional machinery contained within the cell. This repression of gene transcription is one of the major factors that will contribute to the ability of two cells to develop into two different phenotypes while containing an identical genetic compliment. In order for these modifications to occur each cell must contain enzymatic complexes capable of their own reorganization activity.

1.2.3 Chromatin Dynamics

Chromatin remodeling refers to various dynamic structural alterations that occur to the chromatin architecture either locally or globally during cell cycle progression, development or differentiation of specific cell types (23). These epigenetic changes will result through two different manners; either covalently or directly. Covalent modifications are carried out on the terminal ends of the core histone tails and include such actions as acetylation, methylation, ubiquitination, glycosylation, ADP-ribosylation, carbonylation, sumoylation and phosphorylation (23). Complexes that carry out these modifications are called histone modifying enzymes. Direct structural changes will result in nucleosome translocations, histone removal or DNA shifts and bulges. The complexes that achieve this end are all dependent upon the energy produced through ATP hydrolysis and are therefore called ATP-dependent chromatin remodelers. No matter the specific identity, proteins that carry out these functions work as multiprotein complexes and have influences on a wide

variety of cellular processes such as transcriptional activation or repression, DNA replication, DNA repair, cellular differentiation/proliferation, and cell cycle progression.

Chromatin conformational changes in regard to histone modifications have been studied intensely over the past few years. The most understood process is lysine acetylation which involves the use of two different enzyme groups; the histone acetyltransferases (HATs) and histone deacetylases (HDACs) (24-26). HATs impart their action on the N-terminal lysine residues and effectively reduce the interaction of the negatively charged phosphate DNA backbone and the positively charged histone tail. This process loosens the binding of the histone tails and allows association between the transcriptional machinery and the genes previously tightly bound in the nucleosome structure. HDACs have the exact opposite functionality as interactions here create a more tightly bound inaccessible chromatin architecture and a suppressive transcriptional setting. There have been eleven HDACs isolated in humans to date and are divided into three different classes. Class 1, HDACs 1-3 and 8, are widely expressed while class 2, HDACs 4-7 and 9-10, are much more restricted in their expression and finally the class 3 which are all related to the transcriptional repressor Sir2 from yeast. The class 2 members have their highest expression levels in both the striated muscle and brain.

Another process coming to the forefront of study is phosphorylation and is exemplified by activity at serine 10 (Ser10) of histone H3 which has been shown to be important during processes such as transcriptional activation and chromatin condensation (27, 28). The underlying method for this activity still remains elusive though theories suggest that phosphorylation here disrupts the electrostatic interaction of the N-terminal histone H3 tail with the negatively charged DNA backbone therefore allowing for accessibility (28). Other theories suggest that this phosphorylation acts as a recognition site for specific

transcriptional complexes contained within the nucleus. This theory is represented by the discovery of the SH2 domains of receptor tyrosine kinase mediated transduction pathways. This domain recognizes the 4-10 amino acid residues around the phosphorylated Ser10 and is typically located downstream of protein-tyrosine-kinase stimulated signal transduction activation (29).

Methylation of histones typically results in the inactivation of genes coded by the underlying DNA (30). This process is carried out by enzymes called histone methyltransferases (HMTs) which will catalyze the transfer of methyl groups to specific arginine or lysine residues on histone tails. Specifically histone H3 lysines 9 and 27 (H3K9 and H3K27) are typically associated with inactivation events. Methylation of H3K9 results in the binding of heterochromatin protein 1 (HP1) via its chromodomain and results in regional chromatin condensation and therefore effective inactivation (31). However, methylation of histone H3K4 is a mark of active chromatin.

The final process that will be described here is that of ubiquitination. This process involves the post-translational attachment of the ubiquitin protein to other proteins via covalent bonding for targeting towards protein degradation (32). For DNA ubiquitination activity on histones provokes a myriad of responses with regard to DNA damage. In bacteria the cell cycle arrest associated with these DNA breaks is dependent upon ubiquitination of lysine 123 of histone H2B by the complex Rad6-Brel (33, 34). Upon loss of Rad6 activity there is a loss of Rad53 phosphorylation resulting in a variety of extra double strand DNA break formations. Rad6 is an ubiquitin conjugating enzyme which plays direct roles in postreplication repair systems, which is a critical step in double strand break repair in bacteria.

All of these covalent activities indicate the wide degree of functionality of histone modifying enzymes in a variety of essential cellular processes. The following section will focus on the remaining group of chromatin modifiers, the ATP-dependent chromatin remodeling complexes.

1.2.4 ATP-dependent chromatin remodling complexes:

All ATP-dependent chromatin remodeling complexes contain an ATPase subunit from the SNF2 superfamily of proteins. Based upon the identity of this subunit the complexes may be separated into seven different groupings: SWI/SNF, ISWI, CHD, INO80, CSB, RAD54 and DDM1 (35). The three most commonly studied groups are: ISWI, CHD and SWI/SNF. The most extensively studied grouping out of these three is the SWI/SNF group. This group is made up of two complexes from yeast, ySWI/SNF and yRSC, one from *Drosophila*, Brahma complex, and two from humans, hBRG1 and hBRM. These members are all closely related not only in their ATPase subunits but they also contain a C-terminal bromodomain and are similar through uncharacterized regions called domains 1 and 2. The yeast SWI/SNF complex was the first to be characterized and the nomenclature comes from the isolation of genetic mutants that showed that the complex was required for the regulated expression of the *HO* endonuclease gene, which is essential for mating type switching (SWI), and the *Suc2* gene, which is essential for growth on sucrose and is therefore a sucrose nonfermenter (SNF). Later testing demonstrated that this complex had a wide range of effects on the yeast genome and that it had direct relationships with the chromatin backbone where it was capable of nucleosome mobilization through ATP hydrolysis.

The CHD group is made up of protein complexes that contain both chromatin remodeling and deacetylase functions and is identified by the presence of a

chromodomain/PHD zinc finger (35). The most studied complex here is called the nucleosome remodeling and histone deacetylase complex (NURD) and contains the ATPase subunit CHD4 which is a Swi2/Snf2 homologue, also called Mi-2β. The deacetylation activity of this complex has been attributed to the presence of HDAC 1 and 2 in the complex and the remodeling activity is a result of the Mi2/CHD proteins (36, 37).

ISWI complexes were first isolated in *Drosophila* and represented three different complexes. These complexes are ATP-utilizing chromatin assembly and remodeling factor (ACF), nucleosome remodeling factor (NURF) and chromatin accessibility complex (CHRAC). This group will be discussed in greater detail in following sections.

1.3 ISWI chromatin remodeling complexes

1.3.1 Overview

While other enzymatic complexes alter chromatin structure by methods such as methylation, acetylation, or phosphorylation, those that contain ISWI alter the actual structure of the nucleosomes in sliding or translocation processes (38). The *Drosophila melanogaster* complexes NURF, CHRAC and ACF have been studied extensively. NURF has potent remodeling activity at the hsp70 locus in distinct GAGA dependent manners. ACF, on the other hand, has been found to arrange nucleosomes into consistent smooth arrangements from disorganized unevenly spaced structures (39). CHRAC activity can modulate the sliding of complexes on DNA and is only changed from ACF in two of the subunits contained within the complex (40).

Since these initial discoveries from insects, ISWI has been isolated from mammals and all other organisms that have been studied. In mammals there are two orthologs to the Drosophila ISWI protein called Snf2H and Snf2L. To date there have been eight different

complexes isolated from humans, six of which contain the Snf2H protein and only two of which contain the Snf2L protein. Four complexes have been detected in *Xenopus* and yeasts and one in *Arabidopsis*. Please refer to Figure 3 for graphical representation of these complexes.

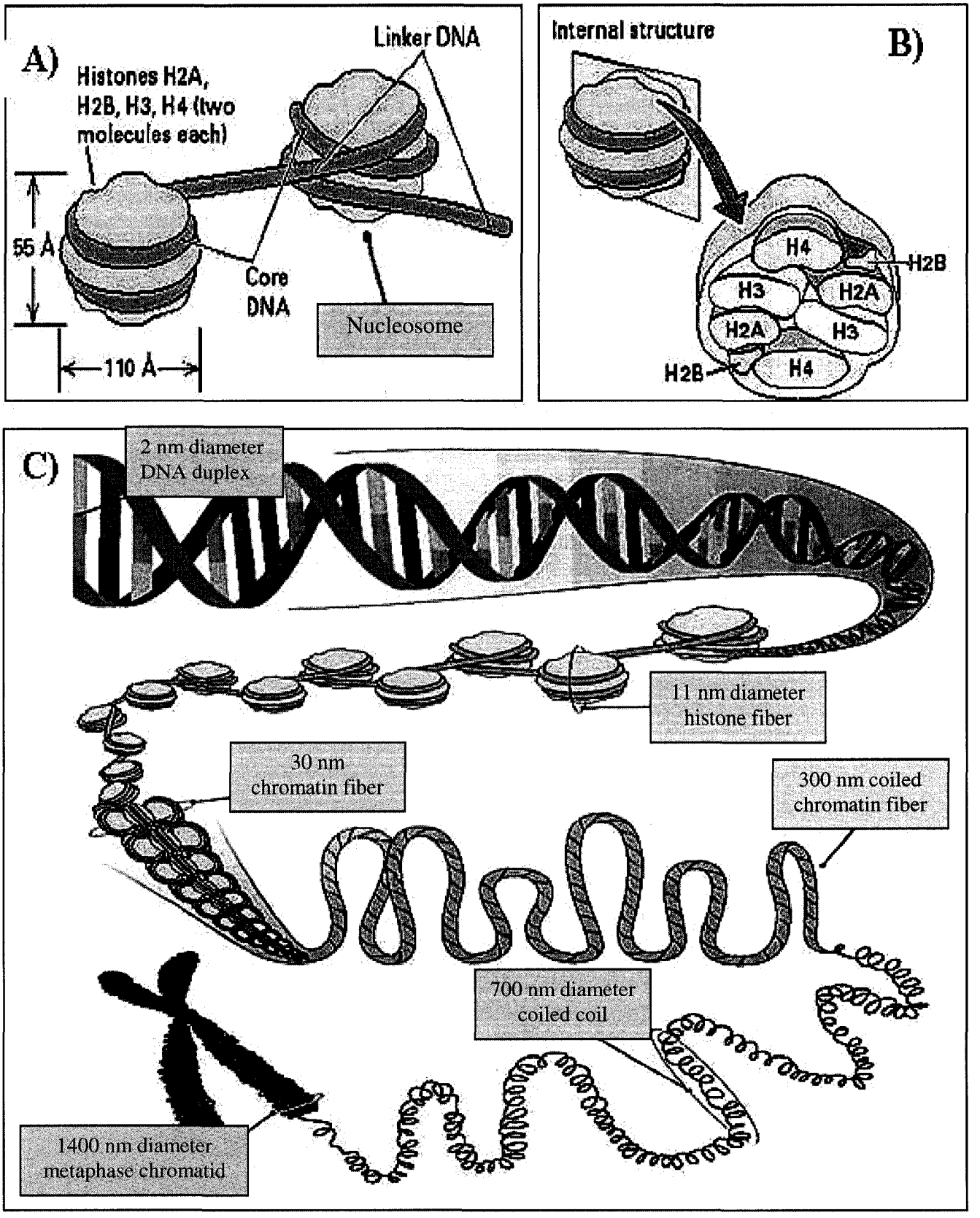
As mentioned briefly above, the imitation switch (ISWI) complexes were first discovered in *Drosophila* using in vitro assays for nucleosome remodeling activity from embryo extracts (41). Since then they have been detected in all species examined to date which portrays the conserved nature of these essential cellular constituents. The ISWI proteins differ from the other SNF2 family members in that they only function maximally in the presence of nucleosomal DNA, while free DNA and histones only elicit modest activity (42). ISWI ATPases have been shown to function in two general roles with regards to chromatin remodeling activity; they may either work to arrange the nucleosome particles into an ordered evenly spaced array or create a situation whereby specific DNA sequences are unpackaged from their nucleosome particles via sliding mechanisms. These processes will be examined in detail in the following sections.

1.3.2 The ISWI Protein Family

As mentioned briefly above, the imitation switch (ISWI) complexes were first discovered in *Drosophila* using in vitro assays for nucleosome remodeling activity from embryo extracts (41). Since then they have been detected in all species examined to date which portrays the conserved nature of these essential cellular constituents. The ISWI proteins differ from the other SNF2 family members in that they only function maximally in the presence of nucleosomal DNA, while free DNA and histones only elicit modest activity (42). ISWI ATPases have been shown to function in two general roles with regards to

chromatin remodeling activity; they may either work to arrange the nucleosome particles into an ordered evenly spaced array or create a situation whereby specific DNA sequences are unpackaged from their nucleosome particles via sliding mechanisms. These processes will be examined in detail in the following sections.

FIGURE 2: DNA PACKAGING IN THE MAMMALIAN CELL. A) Schematic representation of the DNA strand wrapped around the core histone particles also revealing the particle sizes and DNA regional sections. B) Histone core particles are represented here in the inner histone core. C) This figure details the various packaging processes as DNA is compressed into the classical chromosome particle. Figure adapted from Connexions webpage at www.cnx.org/content/m11413/latest .


A)
Histones H2A, H2B, H3, H4 (two molecules each)
Linker DNA
55 Å
110 Å
Core DNA
Nucleosome
Internal structure
B)
H4
H2B
H3
H2A
H2A
H3
H2B
H4
C)
2 nm diameter DNA duplex
11 nm diameter histone fiber
30 nm chromatin fiber
300 nm coiled chromatin fiber
700 nm diameter coiled coil
1400 nm diameter metaphase chromatid

1.3.3 ISWI structure and remodeling activity

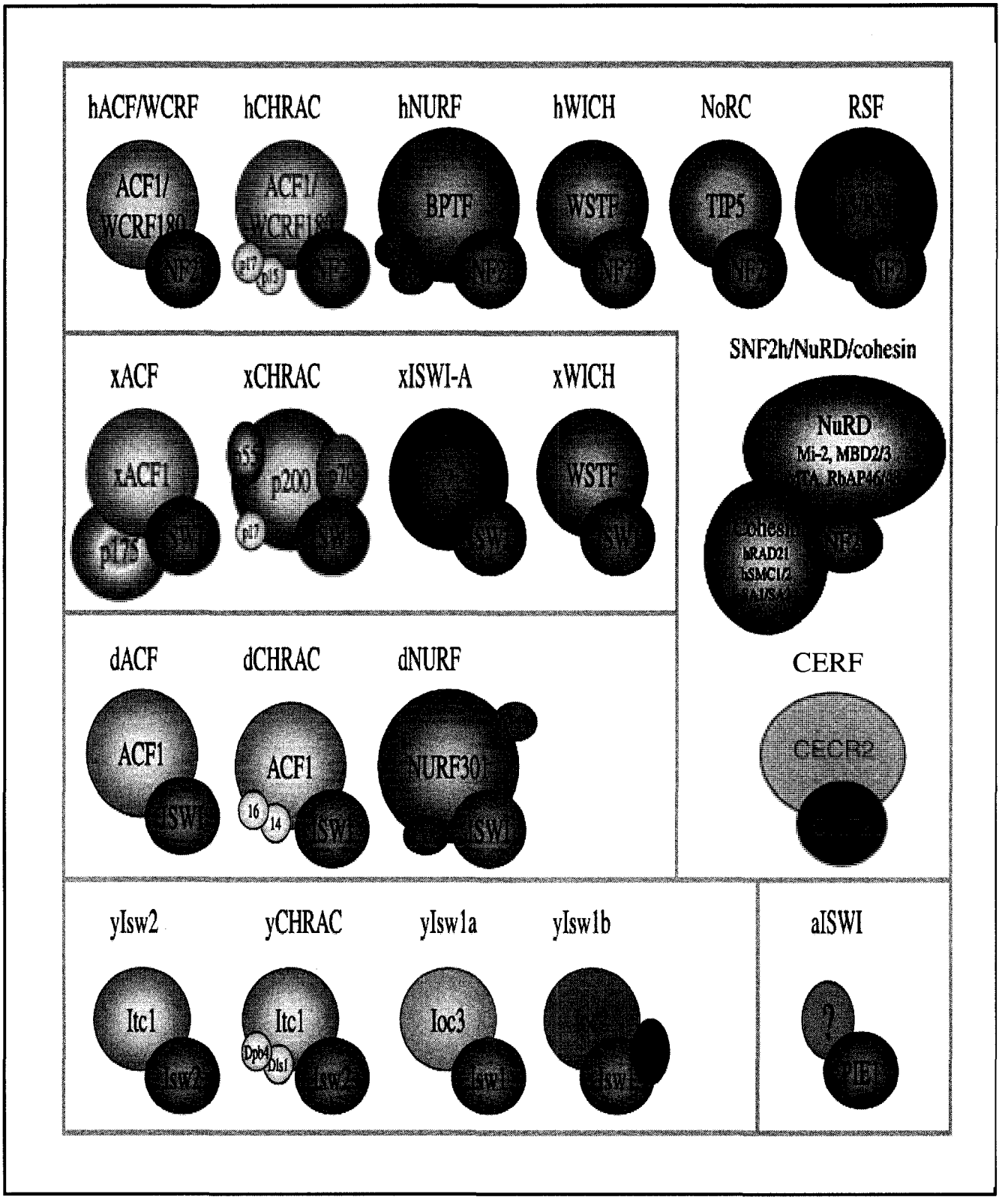
The mammalian ISWI homologs Snf2h and Snf2l are highly conserved to the ISWI proteins from yeasts and *Drosophila* and have over an 80% identity at the protein level in mouse extracts (please refer to Figure 4 for cross species percent homology chart) (43). The protein domain composition is also highly homologous with both proteins containing a SNF2 ATPase domain in their N-terminal ends and a SANT domain in their C-terminal ends (see Figure 4 for the amino acid percent homology) (42, 44, 45). The murine Snf2h gene contains 24 exons and codes for a protein 134 kDa in size. The murine Snf2l gene is also encoded by 24 exons but codes for a slightly larger protein that is 140 kDa in size. Interestingly, the expression patterns for Snf2l in mice are restricted to the brain and gonad tissues but in humans it is expressed ubiquitously. This is a result of humans containing a novel splice variant called Snf2l+13 (46-48). This variant is capable of forming the same complexes as its wild type relative but is functionally inert. During development there is a strong selection for the wild type isoform in the brain and gonads therefore from a functional standpoint the expression of active Snf2l is conserved in mice and humans. Snf2h expression is ubiquitous in both mice and humans (43, 46).

The ISWI proteins contain a conserved ATPase domain and two other domains called SANT (SWI3 ADA2 N-CoR TFIIIB) and SLIDE (SANT-like ISWI domain) motifs (42). Even though these regions are conserved and highly related they are not able to be interchanged while retaining the same remodeling activity (49). The N-terminal region is the section where the ATPase domain resides while the C-terminal area contains the SANT and SLIDE motifs (42). Currently the functionality of these regions has only been examined in *Drosophila* extracts and to a limited extent in yeast. The SLIDE domain of ISWI functions as a DNA binding and sequence specificity module. This region of the protein will recognize appropriate DNA regions by a helix-turn-helix motif where it binds to the major

DNA groove. Experimental evidence has demonstrated that the SANT domain is nonessential for protein function. In order to have proper clamping of the ISWI complex onto the DNA the SLIDE domain must be intact. This region will interact with the major DNA groove directly adjacent to the histone H4 tail. Therefore the histone tail of H4 is essential for the ISWI interaction. In yeast the SANT domain has been associated with binding to histone H3 from various complexes such as SWI/SNF, GCN5, and RSC (for review here see Boyer et al 2000).

Currently there are two different hypotheses for the method of ISWI induced sliding of nucleosomes along the DNA backbone. The first is that the DNA will be twisted over the surface of the histone particles or that it may be translationally altered by looping or bulging of the DNA. The twisting model is dependent upon the buildup of torsional strain in the region of the DNA between the remodeling complex and the nucleosome (50). This strain will build up to the point where the histone particles in the nucleosome core are then shifted to new positions along the DNA strand. Problems occur with this theory in that it is known that nucleosomal DNA is very flexible and would likely inherently adjust and absorb any strain sufficient enough to shift the nucleosome particles. Another significant point is that the linker DNA between nucleosome particles is much more open and would therefore allow for more mobility to resist torsional strain events.

FIGURE 3: SCHEMATIC REPRESENTATION OF THE KNOWN ISWI COMPLEXES ISOLATED TO DATE FROM HUMANS, XENOPUS, DROSOPHILA, YEASTS AND ARABIDOPSIS. The colour of the bubbles indicate protein homology. Figure adapted from Dirscherl and Krebs 2004


hACF/WCRF
hCHRAC
hNURF
hWICH
NoRC
RSF
ACF1/
WCRF180
BPTF
WSTF
TIP5
xACF
xCHRAC
xISWI-A
xWICH
xACF1
p200
WSTF
SNF2h/NuRD/cohesin
NuRD
Mi-2, MBD2/3
cohesin
hRAD21
hSMC1/2
dACF
dCHRAC
dNURF
CERF
ACF1
ACF1
NURF301
CECR2
yIsw2
yCHRAC
yIsw1a
yIsw1b
aISWI
Itc1
Itc1
Ioc3
?

The bulging model is beginning to come to the forefront as the favored process for ISWI induced sliding events. In this model the remodeling complexes will clamp the DNA at two points, one in the linker region and one around the nucleosome. This initiates a conformational change where a loop is formed due to dissociation of the histone tails with the DNA backbone. This bulge is then shifted through the core particle until an end point is reached. The end point is theorized to be a result of the overall geometry of the ISWI complex whereby adjacent nucleosomes and DNA strands will create barriers to further movement (50).

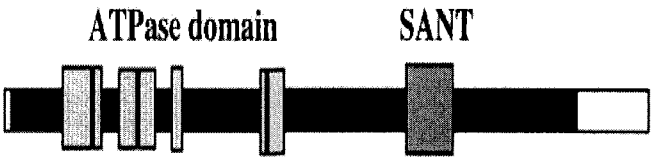
As stated earlier there is a direct requirement for the histone tail regions of the core particles in order for maximal ISWI function. These tail regions extend from the tightly packaged core particle and interact with linker DNA, adjacent chromatin or nonhistone regulatory areas (49). The requirement for these tail sections is a unique aspect of ISWI remodeling activity and was one of the key identifications used in initial screening. The tails emerging from each histone particle are not monotonous in their function and each has distinct roles with regards to varying global DNA functionality. As a result of this, experiments have shown that ISWI remodeling is directly dependent upon the tail extending from histone H4. The H4 histone tail will generally extend out from the nucleosome particle and assume an α-helical structure (49, 51). This extension allows it to interact with other chromatin fibers or histone particles and create a stabilized structure. Currently the actual significance of the H4 histone tail and ISWI interaction is not known but experimental evidence has shown that when these tails are removed from nucleosomal arrays there is no detectable level of ISWI function.

FIGURE 4: PERCENT HOMOLOGY OF ISWI PROTEINS. A) Protein sequence alignment demonstrating percent homology from yeast, fly, human and mouse extracts. B) Alignment of proteins indicating percent of identity between ATPase and SANT domains. Figure adapted from Lazzaro and Picketts 2001.

A)

hSNF2L2	100						
mSNF2L	93	100					
hSNF2H	81	83	100				
mSNF2H	84	83	97	100			
Drosophila ISWI	73	73	74	75	100		
Yeast ISW1	49	48	50	50	49	100	
Yeast ISW2	49	48	50	50	49	53	100
	hSNF2L2	mSNF2L	hSNF2H	mSNF2H	*D.* ISWI	Yeast ISW1	Yeast ISW2

B)



Snf2l	95% (88%)	100% (93%)
SNF2H	87% (100%)	87% (100%)
Snf2h	85% (99%)	92% (100%)
***Drosophila* ISWI**	78% (80%)	87% (85%)
Yeast ISW1	65% (67%)	48% (49%)
Yeast ISW2	65% (67%)	55% (59%)

1.3.4 Function And Composition Of ISWI Complexes

The ISWI group of complexes contains many structurally highly related protein subunits. Of these subunits many are from the WAL/BAZ/BAF (wstf-acf-like/bromo-adjacent zinc finger/bromo-adjacent homology) family (52-54). These subunits are ACF1, Itc1, WSTF, Tip5 and NURF301 and while they do not share a high degree of homology at an amino acid sequence level they are all highly related in their protein domains and structural morphology. Please refer to Figure 5 for graphical representation.

The ISWI complexes all appear to have unique functions and the diversity of these functions is as numerous as the complexes themselves and the list continues to grow as new complexes are isolated. From a general standpoint these complexes may be broken up into those that function as chromatin assembly and replication factors and those that play roles in transcription (55). Those that function in chromatin assembly and replication are ACF, CHRAC, RSF and WICH. ACF and CHRAC are both closely related and work to reassemble nucleosomes into regular, evenly spaced arrays when histone fold chaperones are present such as the subunits 16 and 14 in dCHRAC. RSF action results in similar nucleosome reorganization but differs from ACF and CHRAC in that its p325/RSF-1 subunit has intrinsic histone chaperone activity therefore separate chaperone subunits are not required. The role of these complexes in chromatin assembly was determined using RNA interference and genetic knockouts for ACF and CHRAC.

These studies demonstrated that when ACF and CHRAC are inactivated there is impairment to the replication of pericentromeric heterochromatin through inability of the replication fork to travel through dense heterochromatin (56). There is also a loss of transcriptional silencing in these same areas in Acf1 deficient flies (57). The WICH complex has been found to associate directly with mitotic chromosomes and to function in both replication and transcription though the specific function has not yet been determined.

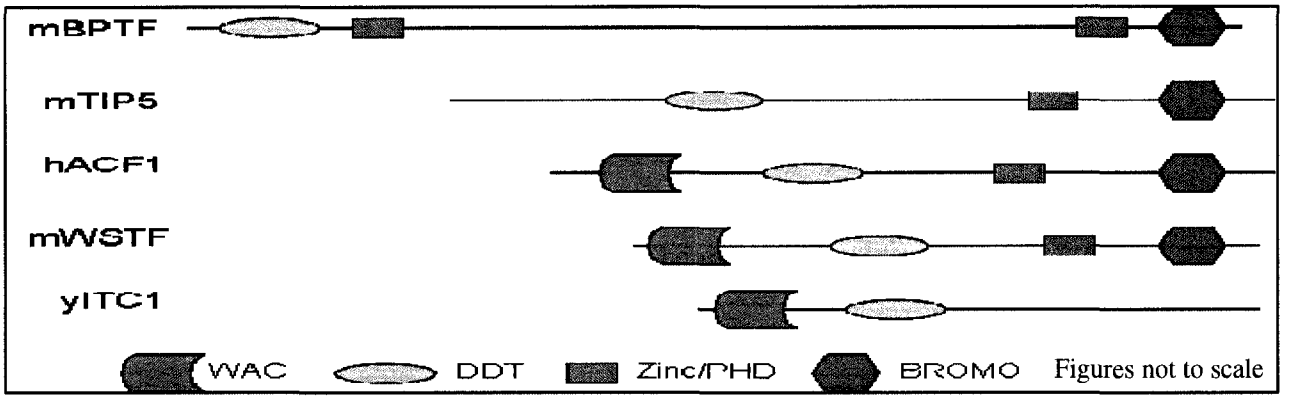
From a regulatory transcriptional standpoint the ISWI complexes that are involved are Isw1, Isw2, NURF and NoRC (58). The initial studies done to show that ISWI complexes played roles during transcription came from investigations conducted on *Drosophila* NURF. This complex is made up of three subunits: BPTF, a 350 kDa protein that contains a bromodomain and a PHD finger, the Snf2L protein, and the smallest subunit is 50 kDa and is the retinoblastoma-associated protein 48 and 46 (RbAP48/46) (47). BPTF and RbAP48/46 are the mammalian orthologs to the subunits found in *Drosophila melanogaster*, NURF301 and NURF55 respectively. From this identification, and the fact that the complex contained Snf2L, it was expected that this complex would act in a chromatin remodeling manner. In previous experiments on other ISWI containing complexes, such as WCRF/hACF and CHRAC, there is an intrinsic ATPase activity when the complexes are exposed to naked DNA and that this activity may be increased by the addition of nucleosomal DNA. The *Drosophila* NURF activity in this regard has been shown to be almost exclusively dependent upon the presence of nucleosomes. This trend was also found to be true in hNURF as when quantified the ATPase activity of hNURF was found to be ~100 fold stronger on nucleosomal DNA then unbound DNA. When there is deletion or mutation to the nurf301 or ISWI subunits of *Drosophila* NURF there is impaired transcription to the heat shock genes hsp70 and hsp26 as well as two homeotic genes ubx and engrailed (55).

The nucleolar remodeling complex (NoRC) has not been found to have activation roles like its counterpart NURF but demonstrates distinct repressive actions (59, 60). This remodeling complex will recruit other complexes that contain HDAC subunits such as the SIN3/HDAC1 corepressor complex to ribosomal genes (55, 61). Isw1 and Isw2 have both been shown to play repressive roles in yeast gene transcription. These two complexes will repress a wide range of genes from stress responsive genes to early meiotic ones. Given that

ISWI proteins are highly expressed in the brain many studies are beginning to examine the role of these complexes in neural development.

FIGURE 5: PROTEIN DOMAINS AND SEQUENCE ALIGNMENT. A) Representative schematic demonstrating the sequence motifs of Tip5, hACF1 and WSTF and their placement within the protein structure, created using ProDom website at www.ebi.ac.uk. B) Chart indicating the specific amino acids making up the given domains. C) Amino acid sequence alignments of the given domains, asterisks indicate matching residues, created using ClustalW from www.ebi.ac.uk/tools/clustalw.

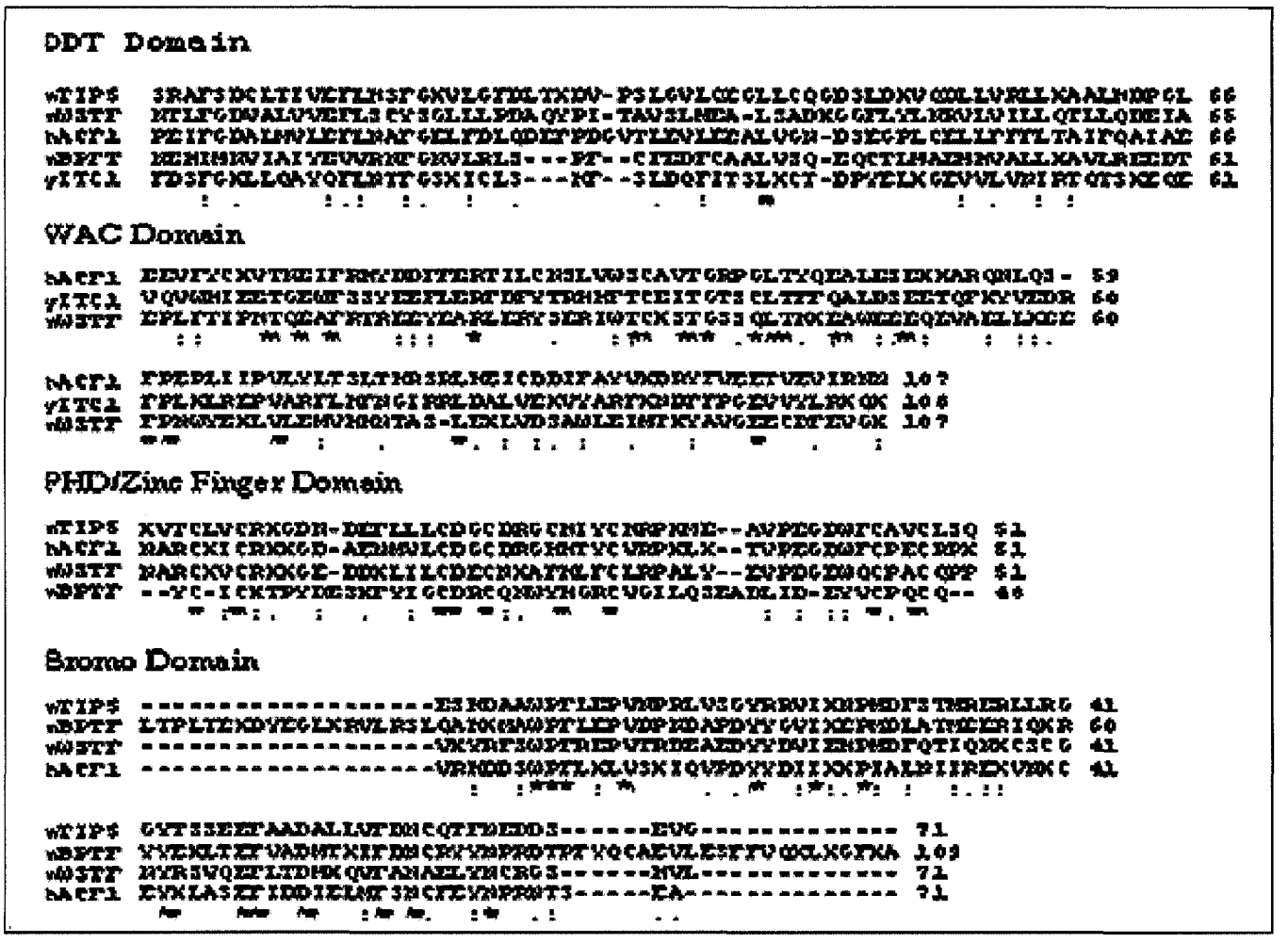
A)



B)

Protein Identity	Total Length (amino acids)	Domain Location			
		WAC	DDT	PHD/Zinc Finger	Broma
mBPTF	2921		252-312	402-449 2742-2793	2819-2889
mTIP5	1660		600-665	1623-1673	1755-1825
hACF1	1556	22 - 126	422-487	1146-1196	1446-1516
mWSTF	1479	20 - 126	605-669	1164-1234	1352-1422
yITC1	1264	23 - 130	423-483		

C)



1.4 Neural development and the role of chromatin remodeling complexes

1.4.1 Cortical development

There are three main sections to the mammalian brain; the forebrain, midbrain, and hindbrain (62). The forebrain may be further divided into the cerebrum, thalamus and hypothalamus. By far the largest part of the brain is the cerebrum, also called the cortex, and it is associated with functions of higher and more evolved species such as thought, memory and action. This cortex is separated into four lobes, the frontal, parietal, occipital and temporal lobes each having its own specific roles in various cognitive processes. Contained within all of these lobes is the neocortex which is unique to mammals and is relatively new in evolutionary terms.

The fundamental principles of cortical development have been understood for some time but the underlying molecular mechanisms still remain poorly understood. The developing neocortex is the most highly organized and complicated structure in the brain (62, 63). When the neocortex is fully developed it consists of six distinct layers that are composed of different and distinct types of cells. Each layer retains its own morphological characteristics and architecture that give unique traits depending upon the subpopulation that is being examined. This structure develops in an inside out pattern where new progenitor cells are produced in the innermost layer, the ventricular zone, and move outwards/upwards as they differentiate. These outward movements have been termed migration and there are two overall migratory streams that exist, these are radial and tangential migration (62).

Radial migration is the process that governs the vast majority of the positioning of cells in the neocortex and occurs between E11 and E18-19 (62). During the early stages of cortical formation (E11) there is a mass of rapidly proliferating pluripotent stem cells present

in the ventricular zone. The ventricular zone is the deepest layer of the cortex and will always contain cells that are capable of proliferation both natally and, to lesser extents, into adulthood. Cells born at this timepoint will migrate upwards, once exiting the cell cycle, and form a developmental layer called the preplate. After two more days of development, E13 approximately, there is another burst of activity where an additional front of postmitotic cells leave the ventricular zone and create a new layer called the cortical plate. Additionally at this time as the second migration is occurring it breaks the preplate into the superficial marginal zone and a lower layer called the subplate. Development continues in this way from E14 to E18 whereby each subsequent wave of postmitotic neurons migrates past the previous layers to establish a new layer and thus all of the different levels of the cortex are established. After E18 the subplate will recede until only six cortical layers remain (Figure 6).

1.4.2 The role of SWI/SNF complexes

The development of the mammalian brain proceeds in a highly complex and structured manner but may be broken down into three basic stages with regards to postnatal and subsequent adult neurogenesis (63). Firstly is the stage where the neural precursor cells self regenerate and then move into a lineage defined specification. The second stage contains all of the cellular extension targeting, migration and synapse formation with other adjacent cells and finally the third stage where the neural connectivity of the existing cells will reorganize under the experience of sensory perception. Currently the actions of epigenetic mechanisms overseeing the progression and completion of neural development are an intense area of study. While this is the case it is well clarified that mutation or

disruption of specific chromatin remodeling complexes, which are key during epigenetic modification, results in moderate to severe mental retardation conditions.

During normal early stage brain development neuroepithelial neuroprogenitor cells rapidly divide to build up population numbers and this division occurs in a symmetrical style which later changes into asymmetrical division (64, 65) . During later stages when glial cells are on the rise the division switches back to symmetrical division and gives rise to the astrocyte and oligodendrocyte stocks. No matter the timepoint examined the processes are all highly regulated and there must be an ordered array of epigenetic transcriptional regulation in place to direct the transcriptional activity.

The chromatin remodeling complexes of the SWI/SNF and ISWI family are prime candidates to oversee part of this regulation control. All of these complexes interact in varying ways with the previously mentioned HATs and HDACs to either allow or repress transcriptional activity. Kondo and Raff in 1994 demonstrated that the switch early on in oligodendrocyte cells from a precursor cell back into a neural stem-like cell is partially dependent upon the recruitment of Brahma (*Brm*) and Breast-ovarian cancer susceptibility gene 1 (*Brca1*), these are the catalytic subunits of several SWI/SNF complexes, to Sox2 a key promoter for neural stem-like cells.

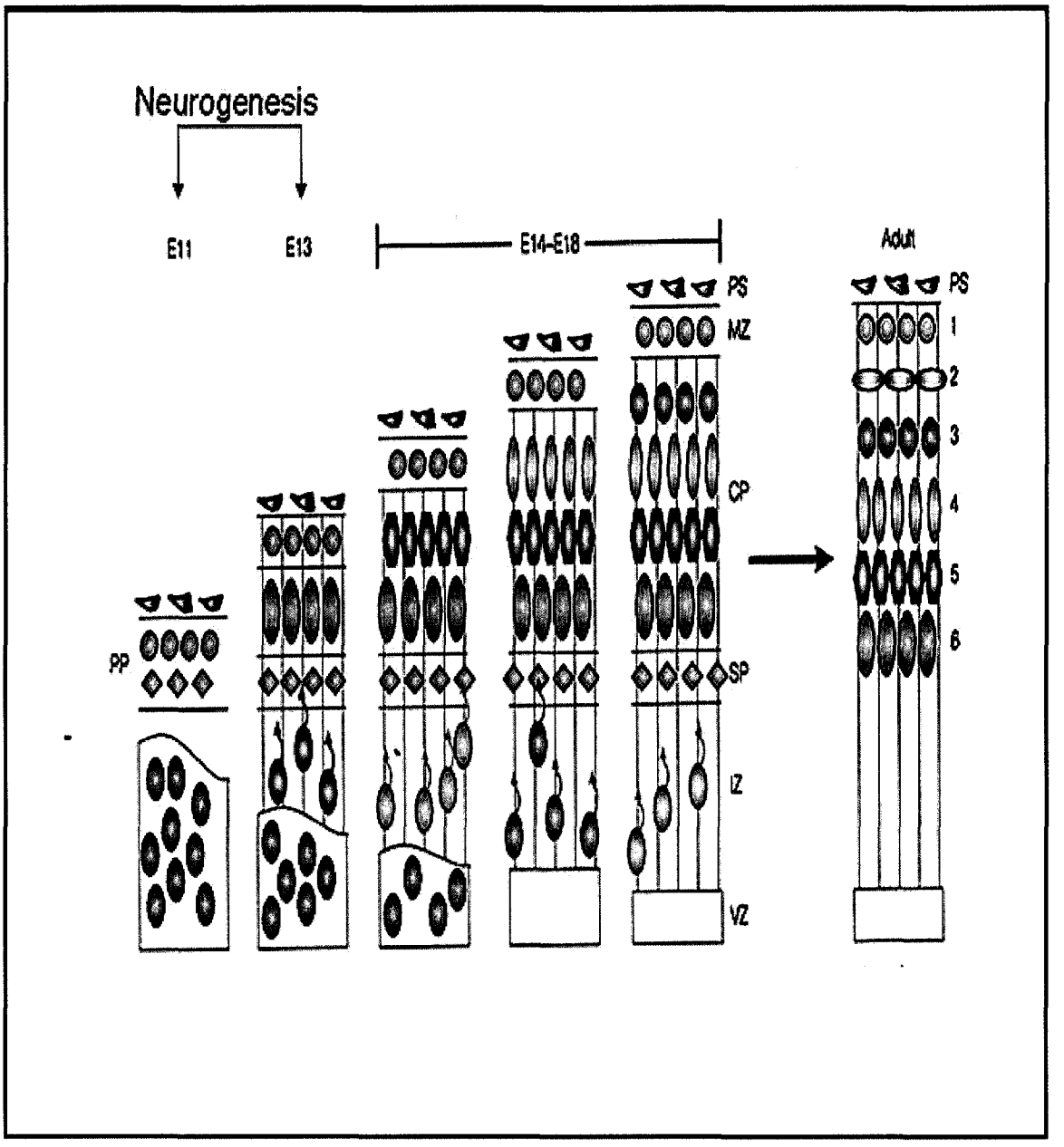
Epigenetic control through chromatin remodeling complexes has also been found to be essential for memory formation and neural plasticity specifically in areas such as polyADP-ribosylation and DNA methylation (25). For example in murine studies of Rubinstein-Taybi syndrome (RTS) when the histone acetylation activity carried out by the HAT CREB-binding protein is removed there is a definitive disruption to long term memory as examined by fear conditioning and object recognition studies. These lapses were reversed when the

HDAC inhibitor Trichostatin A was utilized suggesting possible pharmacological approaches to RTS (65).

Recent study has discovered a novel Snf2l containing complex called CERF (CECR2-containing remodeling factor) (66). This complex contains the subunit CECR2 or cat eye syndrome chromosome region candidate 2 a factor that when mutated is involved in the human cat eye syndrome disorder. This condition is typified by a wide degree of defects to the eye, brain, kidney, skeleton, and heart. During development individuals afflicted with this condition will have neural tube defects with improper or malformed closure.

The initial identification of the Cecr2 protein found that it contained three domain motifs that are typical of ISWI chromatin remodeling complexes. These three are DDT, BAZ, and a bromodomain. Also between 9.5 and 13.5 days post coitus, during rapid neurogenesis, the expression profile of Cecr2 is identical to that for Snf2L. At these timepoints there is a neural enrichment in the rostral portion of the brain and in individuals with excencephaly there is a strong upregulation around the edges of the neural fold that have failed to close. During identification of the novel Cecr2 and Snf2L containing complex ATPase studies were conducted and it was found that the potent ATP hydrolyzing activity of the CERF complex is a result of its association with Snf2L. It was also found that when the Cecr2 gene is mutated in murine models extensive and severe neural tube defects occur. Neural tube defects are the second most commonly occurring mutation to develop in humans and present themselves at approximately one in one thousand births. Since there is a high rate of penetrance of neural tube defects in the homozygous Cecr2 mutant mice developed for this study this leads to

FIGURE 6: CORTICAL DEVELOPMENT. This figure details the cortical layering process and indicated the progression from E11 into adulthood. The approximate time for neurogenesis is also indicated whereby most cells from the ventricular zone will give rise to neurons. (PP) preplate, (VZ) ventricular zone, (IZ) intermediate zone, (SP) subplate, (CP) cortical plate, (MZ) marginal zone, (PS) parietal zone. This figure has been adapted from Gupta et al 2002.


Neurogenesis
E11
E13
E14-E18
Adult
PS
MZ
CP
SP
IZ
VZ
PP
PS
1
2
3
4
5
6

strong implications that CERF partially regulates neurulation which in turn implicates Snf2l as well.

The regional mapping of transcripts for both BPTF and SNF2L are found throughout the cerebellum, cortex and hippocampus (67). Upon closer magnification the transcripts were located in the granule and Purkinje layers of the cerebellum and in the pyramidal neurons of the hippocampus. These results strongly point to distinct requirements for this ISWI complex in the regulation of both hippocampal and cerebellar development.

At this point the question remained; what are some of the downstream consequences of ISWI mutation? Previous studies in flies demonstrated that the cerebellar polarity genes *engrailed-1* and *engrailed-2* are severely disrupted in flies with mutations to ISWI or the NURF component NURF301 (47). These genes are vital for the patterning and proper regional development of the brain and are conserved from flies to mammals. Upon examination in murine models it was found that the hNURF complex localized directly to the promoters for both en1 and en2. In a paper by Barak et al (2003) this question was examined through the use of siRNAs directed towards *Snf2L*. Their results show a two fold decrease in the levels of *en-1* and *en-2* when *Snf2L* levels are reduced.

The engrailed homeodomain protein is known to promote the outgrowth of neuronal process extensions such as neurites and axons (47, 63, 68). Therefore logic would dictate that upon down regulation of the engrailed products that neuronal process extension would be inhibited with Snf2l knockdown. This situation has been examined in mouse N1E115 neuroblastoma cells as by simple serum starvation they develop into fully mature neurons with the associated cellular extensions. When cells were transfected with a *Snf2l*-WT expressing plasmid there is a significant increase in cellular extension length and number. These transfected cells also resulted in a spontaneous switch to differentiation in cell

populations maintained under proliferation conditions (47). This result was found to be directly related to the upregulation of Snf2L as when similar cells were transfected with an ATPase dead Snf2L mutant there was no corresponding increase. It was also found to be specific to only Snf2L as in Snf2H transfected cells there was again no increased extension activity.

hNURF, the human equivalent of dNURF, has been found to contain only two of the three subunits that dNURF contains, BPTF and RbAP46/48 but not the smaller inorganic pyrophosphatase protein NURF38. This difference aside the complex shows potent nucleosome dependent chromatin remodeling activity in ATPase assays. This activity is also required by neurons for the steps leading to neurite process extension. The human NURF complex was shown here to regulate both *en1* and *en2* gene expression. Both of these genes are essential for the regulation of development in the mid- and hindbrain.

ISWI function has also been examined in *Xenopus laevis* where two forms of ISWI exist which are ISWI 1 and ISWI 2 (69). ISWI transcripts are expressed throughout the neural tissue during development and into adulthood. Levels are especially high in the cranial crest, brachial arches, neural folds and in the otic vesicle. When techniques such as anti-ISWI morpholino were used to knockout ISWI, distinct neural developmental disorders were found. These were represented by widespread gastrulation disorders and failure of the neural tube to close. In an attempt to explain these severe deformities Dirscherl et al 2005 examined the expression levels of several neural genes in ISWI knockout samples. The genes that were examined were *BMP*4, bone morphogenic protein 4 a neural tissue inhibitor, *Xmeis*, a neural crest marker, *Shh*, sonic hedgehog an early notochord marker and cell proliferation gene, *Pax6*, a developmental gene critical for eye formation, *NCAM*, neural cell adhesion molecule a pan-neural tissue marker, *Sox9*, a neural crest formation progenitor,

Slug, a lateral plate and neural crest marker, and finally *HoxB9*, a central nervous system marker. Interestingly all of these genes except BMP4 were strongly downregulated with ISWI knockout. *BMP4* on the other hand was increased two fold suggesting an essential role for *ISWI* function at the *BMP4* promoter region. Under normal conditions as development continues there is an associated down regulation of *BMP4* that allows neural tissues to develop. The authors here have proposed that *ISWI* is required for the repression of BMP4 and in subsequent chromatin immunoprecipitation studies it was found that *ISWI* binds directly to the *BMP4* gene. This also suggests that ISWI regulates the other genes as they may be activated by *ISWI* while *BMP4* is normally repressed. Associated with these neural defects are distinct eye-specific deformities such as cataract formation.

Evidence for the essential functions of chromatin remodeling complexes has recently been discovered for the replication of the multipotent neuroepithelial cells establishing the central nervous system (CNS) (70). It has been known for some time that the switch of these cells to either undergo self-replication or shift to lineage specific differentiation is controlled by various nuclear receptors developmentally regulated transcription factors such as basic helix-loop-helix (bHLH) and Signal Transducers and Activators of Transcription/Sma Mothers Against Decapentaplegic (STAT/Smad) (70, 71). The ISWI related proteins from the SWI/SNF group Brg and Brahma (Brm) are known to modulate the accessibility of DNA to transcriptional machinery among other roles (72, 73). There is strong evidence that these ATPase proteins have direct roles in neuronal development in the CNS as studies in *Xenopus* have demonstrated that they are required for the modulation of Neurogenin and NeuroD (65). Both Brg and Brahma are subunits of the chromatin remodeling complex called BAF (Brg/Brm-associated factor). This complex is made up of ten different subunits the composition of which is the same for all tissues except in post mitotic neurons where the

standard subunit BAF53a is substituted for BAF53b Arp-4 like protein (71). Lessard et al have demonstrated that in order to maintain a proliferative state in the neuroepithelial ventricular zone cells a specialized npBAF complex is required. This complex will contain the previously mentioned BAF53a as well as a unique subunit BAF45a. When these cells are directed towards differentiation, either into neuronal cell fates or glial, there is a switch in the BAF subunit composition where the BAF53a and BAF45a are exchanged for either BAF45b or a complex of BAF45c/BAF53b. Taken together these studies highlighted the importance of chromatin remodeling complexes in neurodevelopment.

One intriguing possibility is that ISWI mutation can cause mental retardation. In this regard, the mammalian Snf2L gene is mapped on the X chromosome at Xq25-26 where a large number of X-linked mental retardation conditions have been linked making it a prime candidate for these conditions (43). For a graphical representation of the Snf2L gene location and known linkage intervals from XLMR families refer to Figure 7.

1.4.3 Neural expression profiles of ISWI

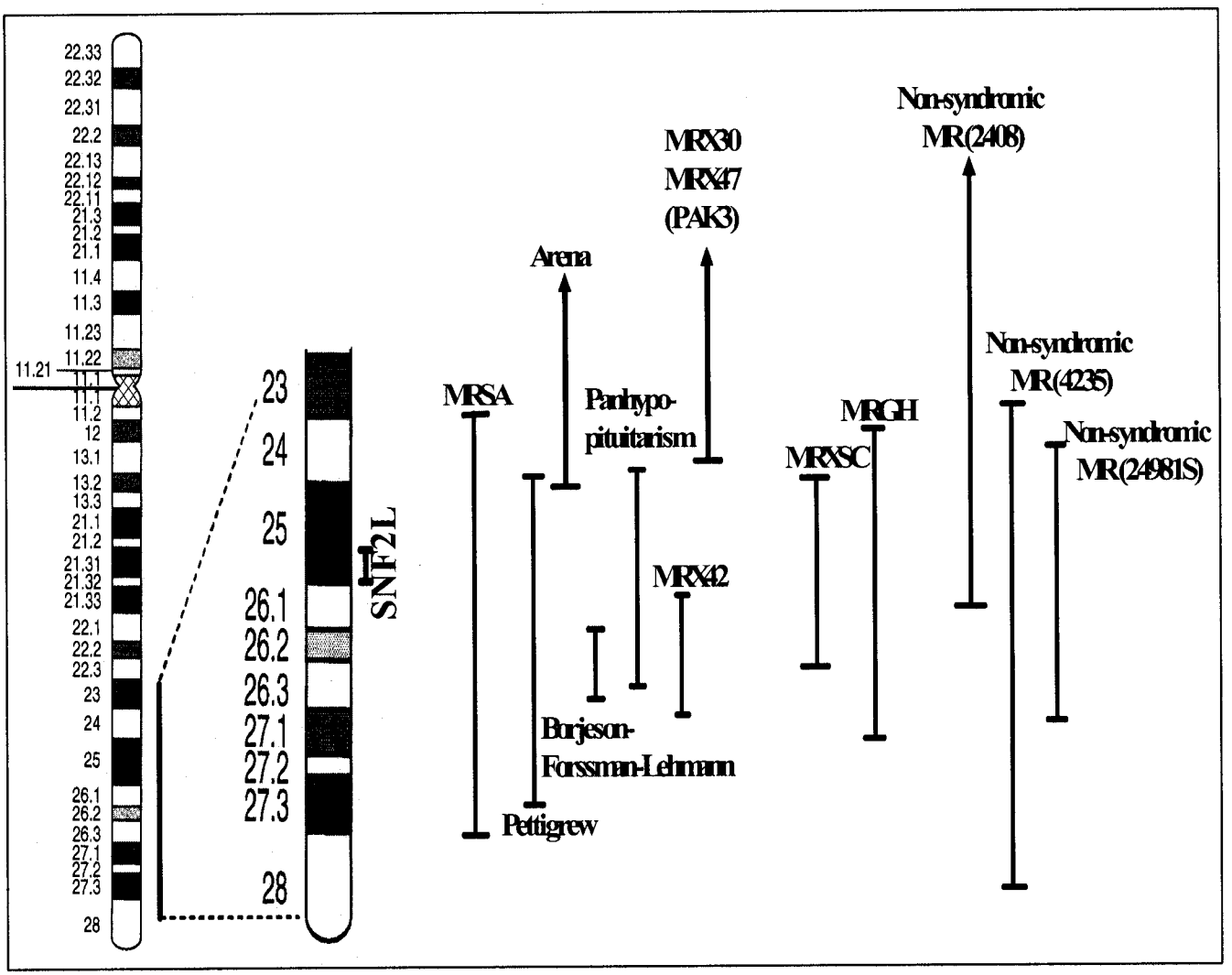
The expression profiles for Snf2h and Snf2l differ for each protein and strongly indicate a distinct difference in functions during developmental progression (43). During spatial and temporal studies using mouse cDNAs it was found that Snf2h was expressed in the brain at high levels mainly in rapidly proliferating cellular populations. Snf2l on the other hand is upregulated in cellular populations that are exiting the cell cycle and are post mitotic, such as differentiating neurons.

The expression for Snf2h and Snf2l has been examined in the murine brain using RNA *in situ* hybridization techniques at several timepoints during development (43). Specifically at E9.5 to E15.5 of development both proteins are expressed ubiquitously

throughout the embryo. Snf2l, at these times, is not localized to any specific cell type in the brain and levels stay low until later, postnatal, stages. Snf2h transcripts are somewhat elevated when compared to those of Snf2l in the developing neocortex and cerebellum, and outside the brain in areas such as the kidneys, gut, lungs and olfactory epithelium.

Postnatally there is a progressive switch where Snf2l transcript expression comes to the forefront while Snf2h levels decrease in regionally specific manners (43, 44). At postnatal day 1 the expression levels remain constant with Snf2l being lowly expressed in all areas and cell types. However this changes during the initial two weeks post birth. At these times there is a rapid elevation in Snf2l abundance in all neurons throughout the brain. The opposite trend is true for Snf2h levels as there is a marked decrease in mRNA amounts. During this period there are two areas where these trends are most prevalent which are the cerebellum and the hippocampus. The cerebellum has been examined in further detail and shows that the Purkinje cells and the internal granule cell layer represent the most upregulated areas. While these two regions show distinct high Snf2l expression there is concurrent elevation in many other areas such as the thalamic nuclei, both superior and inferior colliculi, the pontine nuclei and all layers of the cortex and this continues into adulthood. Interestingly the presence of Snf2h in proliferating cellular populations and Snf2l in postmitotic populations remains

FIGURE 7: SCHEMATIC REPRESENTATION OF THE MAMMALIAN X CHROMOSOME. The location of the Snf2L gene locus along with an assortment of linkage intervals from several XLMR families are shown.


22.33
22.32
22.31
22.2
22.13
22.12
22.11
21.3
21.2
21.1
11.4
11.3
11.23
11.22
11.21
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12
13.1
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13.3
21.1
21.2
21.31
21.32
21.33
22.1
22.2
22.3
23
24
25
26.1
26.2
26.3
27.1
27.2
27.3
28
23
24
25
26.1
26.2
26.3
27.1
27.2
27.3
28
SNF2L
MRSA
Pettigrew
Arena
Parhypo-
pituitarism
Borjeson-
Forssman-Lehmann
MRX30
MRX47
(PAK3)
MRX42
MRXSC
MRGH
Non-syndromic
MR(2408)
Non-syndromic
MR(4235)
Non-syndromic
MR(24981S)

constant for all regions of ISWI expression in the reproductive organs as well which further indicates specific roles for both of these ATPases.

1.5 Rationale and Specific Aims

Epigenetic modification allows for the development of different cellular phenotypes while containing an exact genetic compliment and is carried out in part by various chromatin remodeling enzyme complexes. It has been shown that during brain development if mutations occur in genes encoding the chromatin remodeling complexes severe mental retardation conditions can develop. As scientific experimentation advances new genes are rapidly being discovered that underlie such developmental disorders. Obvious areas of focus for new candidate genes are those that oversee the establishment of neuronal differentiation and proliferation. Past experimentation has clearly demonstrated that the *ISWI* genes are prime candidates for these processes but to date their exact roles in the establishment of distinct mature neuronal or glial phenotypes remains unknown. As a result, we hypothesize that the levels of Snf2h and Snf2l are important for the proper regulation of the switch from proliferation to differentiation in neural progenitor cells. Specifically, we propose that *Snf2H* is required for proliferation in neural progenitors while *Snf2L* is necessary to promote cell cycle exit and differentiation. To address this hypothesis we will utilize existing mouse lines in our laboratory that have conditional *Snf2l* knockout via cre recombinase along with N1E115 and SH-SY5Ycell lines in which we have used shRNA-mediated knockdown of the ISWI proteins. The specific aims addressed in this thesis are:

- AIM 1 – to design shRNA constructs geared towards human and mouse *SNF2H* and *SNF2L* and transfect them into established neuroblastoma cell lines

- AIM 2 – to examine these knockdown cell lines for their proliferative potential and ability to differentiate
- AIM 3 – to examine the growth and differentiation properties of neural progenitors isolated from *Snf2L* functional knockout mice

It is our hope that these studies will allow for a greater understanding of the role that the *ISWI* genes play in the development of the murine brain. Furthermore this will allow for insight into how these genes will regulate the development of the human brain and allow for greater understanding of mental retardation conditions.

2.0 Materials and Methods

2.1 Materials

2.1.1 General Materials and Reagent Sources

All chemicals, such as Tris, NaCl, and EDTA, for the preparation of buffers were purchased from Fisher Scientific, Toronto Ontario unless otherwise stated. Agarose for DNA and RNA electrophoresis was purchased from Life Technologies, Carlsbad California. Bradford assay dye reagent was purchased from Bio-Rad Laboratories, Hercules CA and was used for protein concentration analysis. Precast Western gels, NuPage 3-8% Tris-Acetate 1.0 mm x 10 well, were obtained from Invitrogen, Burlington ON, and were run in accordance with their instructions. Pierce CL-X Posure x-ray film for western development was purchased from Fisher Scientific. ECL Plus Western Blotting Detection System reagents came from GE Healthcare, Buckinghamshire UK. All restriction enzymes and their specific buffers were purchased from New England BioLabs (Ipswich, MA).

The primary antibodies for neural cell characterization, TUJ1 (mouse IgG), GFAP (rabbit polyclonal), and Nestin (mouse IgG) were all purchased from StemCell Technologies, Vancouver BC. Secondary antibodies for fluorescent detection of primary antibodies used were donkey anti-mouse Alexa 594 or 488, goat anti-mouse FITC and goat anti-rabbit FITC and they were purchased from Sigma Aldrich, Oakville ON. Rabbit anti-BrdU-20 primary antibody was purchased from the Developmental Studies Hybridoma Bank at the University of Iowa, and the goat anti-rabbit FITC antibody was purchased from Amersham, Buckinghamshire UK. DAPI counterstain was purchased from VWR, Westchester PA.

Antibodies used for western detection of both Snf2h and Snf2l were created specifically for our laboratory by Affinity Biologicals Incorporated, Hamilton ON. Snf2h primary antibody was produced in sheep and is a polyclonal N-terminal directed affinity purified antibody. The Snf2l antibody is also a sheep polyclonal N-terminal directed antibody. Other primary antibodies used for western blotting were mouse anti-β-actin and rabbit anti-γ-tubulin, both purchased from Santa Cruz Biotechnology Incorporated, Santa Cruz CA. All horse radish peroxidase conjugated secondary antibodies were also purchased from Santa Cruz.

Plasticware such as Falcon tubes, Petri dishes, T75 culture flasks and 6-well culture plates were purchased from VWR unless stated otherwise. 8-well Chamber Slide System glass bottom slides were obtained from Nalge Nunc International, Rochester NY.

2.1.2 Cell Culture Reagent Sources

All neuroblastoma cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM) containing high glucose 1x purchased from Invitrogen in Carlsbad CA. This media was supplemented with 10% heat inactivated fetal bovine serum purchased from Sigma Aldrich. Intermittently culture media was supplemented with 1% Antibiotic-Antimycotic from Invitrogen. All other neuroblastoma cell culture reagents such as trypsin, dimethyl sulfoxide (DMSO), lipofectamine, poly-D-lysine, retinoic acid, 5-Bromo-2'-deoxyuridine and laminin etc. were purchased from Sigma Aldrich.

Neurosphere cultures were grown in NeuroCult NSC Basal Medium supplemented with NeuroCult NSC Proliferation Supplements from StemCell Technologies. Added to this was human epithelial growth factor (hEGF) purchased from Sigma Aldrich. For the

purposes of differentiation the NeuroCult Differentiation Medium was used and was purchased from StemCell Technologies.

2.1.3 Oligonucleotides

Unless otherwise stated all oligonucleotides utilized in these experimental procedures were purchased from Cortec DNA Service Laboratories Incorporated, Kingston ON. The experimental procedure the oligonucleotides were designed for and the specific sequences chosen are listed below.

shRNA target sequences
Snf2L - 5' AAAACCATTTTGTTACGCCG 3'

Snf2H 1- 5' AAGATTATGGCTCAGATTGAA 3'

2- 5' AAATGCTTATTAAAGAGAAGT 3'

Genotyping primers
mSnf2l – 1) Rev 5' GTATGGACAAGTGTGTGAAGCC 3'

2) Rev 5' CCATGTGGGGTCCAGGAATG 3'

3) For 5' CCTGGGCTGGAACCATGATC 3'

Primers here were used to detect the deletion of exon 6 in the murine Snf2l gene, in knockout animals, and the normal wild type allele. Primers 1 and 3 are located in sections of the gene that are common for all animal types while primer 2 is located in exon 6. As a result of this design wild type animals will amplify a PCR product of 508 basepairs while knockout animals will generate a band of 438 basepairs and heterozygous animals will have both bands. (PLEASE see Figure 14 for representative genotyping results)

hSnf2l – For 5' CTGGTCCACCTTATACCAC 3'

Rev 5' GTGGCTCCATGCCGTATC 3'

These primers recognize human SNF2L and amplify a PCR product 504 basepairs in size.

mSnf2h – For 5' GATCAAAAGCTAGTTGAGAGGC 3'

Rev 5' GATGATTGCAGCATTTCCTCAAC 3'

These primers recognize mouse Snf2h and amplify a PCR product 669 basepairs in size.

hSNF2H – For 5' GTGCTGAGATGAAACTCAG 3'

Rev 5' CCTTTGCATTTTGCTGAGG 3'

SNF2H primers amplify a PCR product of 350 basepairs from human samples

βactin – For 5' CTGAACCCTAAGGCCAACCGT 3'

Rev 5' CCGTCAGGCAGCTCATAGCTCT 3'

βactin primers amplify a PCR product that is 407 basepairs from murine or human samples for both PCR and RT-PCR reactions.

Sry - For 5' TTGTCTAGAGAGCATGGAGGGCCATGTCAA 3'

Rev 5' CCACTCCTCTGTGACACTTTAGCCCTCCGA 3'

This is a male specific gene allowing for sex determination and amplifies a PCR product that is 300 basepairs in size from murine samples.

Fabpi – For 5' TGGACAGGACTGGACCTCTGCTTTCCTAGA 3'

Rev 5' TAGAGCTTTGCCACATCACAGGTCATTCAG 3'

This gene is used as a reaction control and is expressed in every murine cell, the PCR product size is 200 basepairs from murine samples.

Cre Recombinase - For 5' ATGCTTCTGTCCGTTTGCCG 3'

Rev 5' CCTGTTTTGCACGTTCACCG 3'

These primers detect the presence of Cre recombinase and amplify a PCR product 200 basepairs in size from murine samples.

RT-PCR primers

mSnf2l – For 5' CCTCTCAACATGAAACTGGC 3'

Rev 5' TCACGCAAACATCCCACTCT 3'

These primers recognize mouse Snf2l transcripts and amplify a PCR product 504 basepairs in size.

hSNF2L – For 5' GTGGCTCCATGCCGTATC 3'

Rev 5' CTGGTCCACCTTATACCAC 3'

These primers recognize human SNF2L and amplify a PCR product 609 basepairs in size.

mSnf2h – For 5' GCGAGGATTGAATTGGCTC 3'

Rev 5' CCTGTGAGCTTCATCTATAAC 3'

These primers recognize mouse Snf2h and amplify a PCR product 381 basepairs in size.

hSNF2H – For 5' TCCAATTTGTCACACAGAAGAA 3'

Rev 5' AATCCCTCCTGCCACTTCTC 3'

These primers detect the murine Snf2h gene and amplify a PCR product 354 basepairs in size.

2.2 Methods

2.2.1 shRNA Oligonucleotide Sequence Selection

All oligonucleotides chosen for shRNA knockdown purposes were designed to target both human and murine sequences. These target sequences were selected using the online siRNA Target Finder found on the Ambion website (www.ambion.com/techlib/misc/siRNA_finder.html). In the design sequences were selected to end with TT and to contain less than 40% GC content and finally to avoid sequences containing five or more T's in a row which is a stop codon for the vector's pol III promoter. In order to form the hairpin structure of the shRNA inner chain a noncomplementary sequence was chosen, specifically 5' CCACC 3'. All sequences were also constructed to contain *Bbs1* restriction enzyme sites on their 5' ends for ligation purposes.

The mRNA sequences for human SNF2H and SNF2L may be found on the NCBI website, (www.ncbi.nlm.nlh.gov), and have accession numbers NM003601 and NM003069 respectively. Mouse mRNA Snf2h and Snf2l sequences were also found on the NCBI website and have accession numbers NM053124 and NM053123 respectively. After selection the shRNA target sequences were run through nucleotide BLAST to ensure no cross reaction would occur with other gene sequences.

2.2.2 Plasmid Selection, Annealing, Ligation and Bacterial Transformation

The plasmid chosen for stable shRNA expression was psiRNA-hH1 neo G2 generated by InvivoGen, San Deigo and is shown in Figure 8. Initially, shRNA oligonucleotides were resuspended to a concentration of 500 μM using sterile water. Then following the guidelines described by Sigma Genosys, a division of Sigma-Aldrich Oakville, shRNA oligonucleotides were annealed together. Briefly, annealing was conducted at room temperature using 300 ng of both sense and antisense shRNA oligonucleotides in TENS buffer (10mM Tris pH 8, 50mM NaCl, 1mM EDTA). Ligations were then preformed using the Rapid DNA Ligation Kit (Roche Diagnostics, Laval QC) following their guidelines. In brief, 10 ng of digested vector and 20ng of the annealed oligonucleotide insert were incubated at room temperature for 5 minutes with 10 μl T4 DNA ligation buffer and 1 μl T4 DNA ligase. Prior to this step the vector was digested using the *Bbs*1 restriction enzyme for two hours at 37°C and the resulting linear DNA was separated in a 1% agarose gel. The DNA was then extracted and purified from the gel using Qiagen's QIAquick Gel Extraction Kit. Then 10 ng of the ligation product was transformed into Stbl2 Competent Cells (Invitrogen) and LB broth (10 g tryptone, 10 g NaCl 5 g yeast extract and 1 L distilled water) for amplification purposes using standard protocols where the culture was incubated on ice for 20 minutes followed by 45 seconds at 42°C and then another 2 minutes on ice. To this culture 1mL of SOC broth (10g Bacto Tryptone, 2.5 g yeast extract, 5 mL 1M NaCl, 1.25 mL 1 M KCl, 1 mL 2 M Mg^{2+}, 1 mL 2 M glucose and 488 mL distilled water) was added and the solutions were left on the bench top for one hour to aid in their recovery from heat shock. From here 150 μL of the culture was spread onto LB agar plates containing 30 μg/mL kanamycin for selection purposes. These plates were incubated at 37°C for 24 hours. The resulting bacterial colonies were removed from the LB plates and inoculated into 5 mL LB

kanamycin media and grown for another 24 hours to produce sufficient stock for Qiagen Plasmid Mini Kit preparation.

Isolated plasmid DNA was then digested using the restriction enzyme *Ase1* for 2 hours at 37°C. Positive clones were identified as linearized DNA after *Ase*1 digestion whereas negative clones contained two bands upon 1% agarose gel examination (please see Figure 8 for representative figure). In order to confirm the integrity of the shRNA inserts they were sequenced by StemCore Laboratories (Ottawa, ON). Results were compared to the normal sequence using the program DS Gene 1.5 from Accelyrs Software Incorporated (San Diego, CA). Once positive clones were obtained they were grown for a larger DNA preparation using Qiagen Plasmid Maxi Kits.

2.2.3 Neuroblastoma Cell Line Selection, Culturing and Transfection

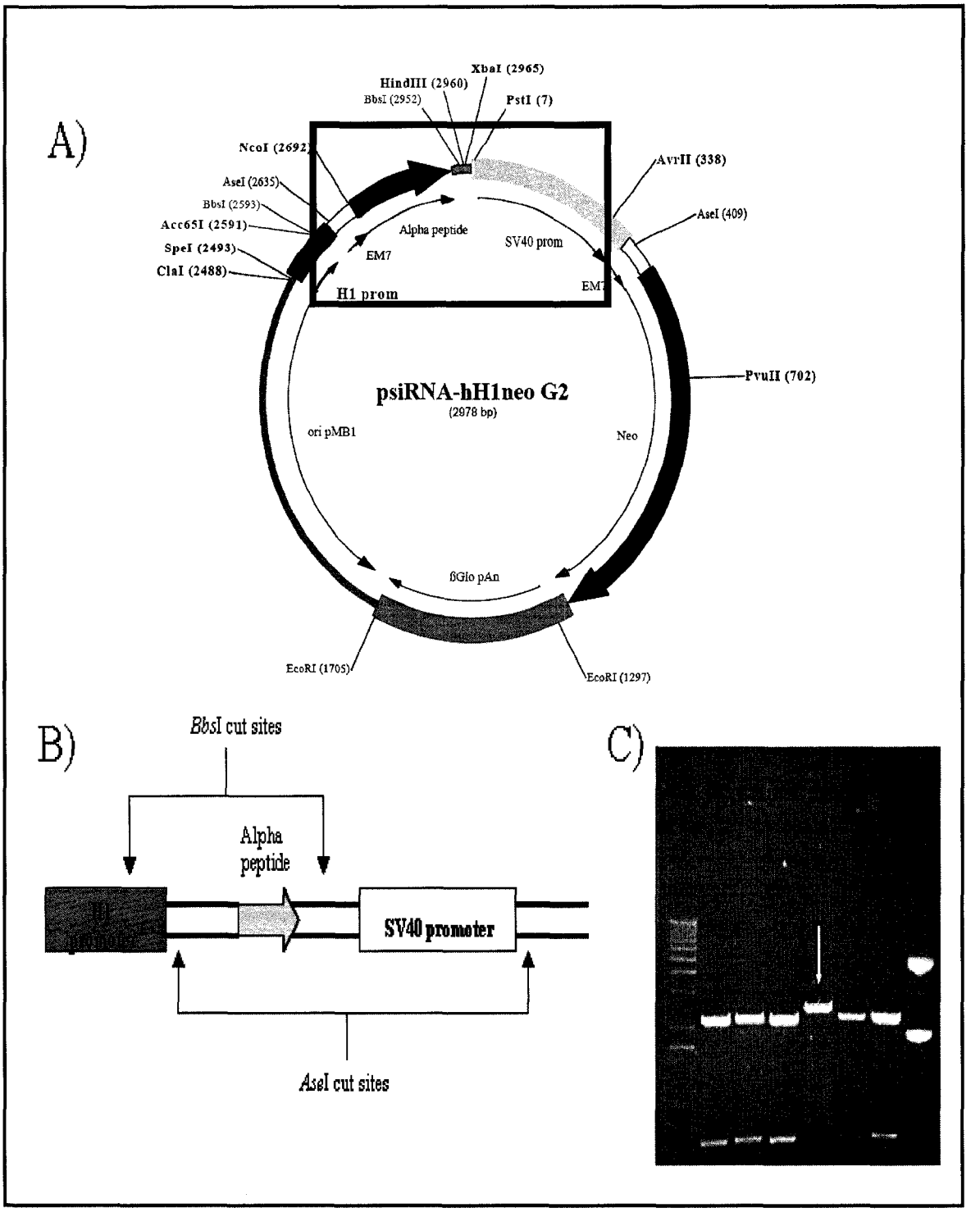
The mouse neuroblastoma cell line N1E115 (CRL-2263) and the human neuroblastoma cell line SH-SY5Y (CRL-2266) were purchased from American Type Culture Collection (ATCC), (Rockville, Ma). Both cell lines were maintained in T75 culture flasks containing DMEM supplemented with 10% heat inactivated FBS for proliferation purposes and were grown at 37°C and 5% CO_2 (Sanyo CO_2 Incubators). These cultures were passaged approximately every 3 days, or as they achieved 80% confluency. Cells were initially washed with sterile PBS and then trypsinized with 1 mL trypsin-EDTA for 5 minutes at 37 °C. Cells were then harvested in 4mL of proliferation media and 1 mL was transferred to each of 4 T75 flasks containing 9mL of proliferation media. For cryopreservation a similar process was followed except that after resuspension in new proliferation media 900 µL of the sample was transferred to a 1.5 mL cryovial and 100 µL of DMSO was added. These cryovials were then immediately placed in a Forma Scientific -86 °C freezer.

In order to induce differentiation of the N1E115 cells the media was changed to DMEM supplemented with 1% heat inactivated FBS and 1% DMSO. SH-SY5Y cells were differentiated in DMEM supplemented with 1% FBS and 0.001% retinoic acid (stock 100mM).

Transfection of both cell types followed the protocol developed by Life Technologies Incorporated of Gaithersburg with several changes. Briefly, 35 mm culture plates were seeded with $3x10^5$ cells in 2 mL of proliferation media and allowed to incubate at 37°C and 5% CO_2 for 24 hours, or until reaching 80% confluency. A solution of 12 μL Lipofectamine and 100 μL serum free DMEM was made and allowed to incubate at room temperature for 25 minutes. To this solution 1.5 μg of DNA and another 100 μL serum free DMEM was added and allowed to incubate for 30 minutes at room temperature. This solution was then overlaid onto the growing cells and they were returned to the incubator for 6 hours. After this time the medium was replaced with growth media supplemented with 20% FBS for 24 hours after which standard growth media was used.

Transfection efficiency was monitored by co-transfection with the pExpress-GFP plasmid from ExpressOn Biosystems of Roslin, UK. Both human and murine neuroblastoma cells were grown in 35mm culture dishes and 1.5 μg of DNA and 12μL of lipofectamine were utilized. For future experimental processes the amounts of reagents used was scaled up or down accordingly.

FIGURE 8: TRANSFORMATION OF PSIRNA-HH1NEO G2 VECTOR. A) Graphical representation of the vector, green box indicates the focus area for B, schematic adapted from supplier website at www.invivogen.com. B) closeup of the restriction enzyme sites of interest for plasmid transformation and screening of recombinant clones. C) Example of positive clone, yellow arrow, along with the uncut vector, red arrow, and a negative clone (uncut by *Ase*1, green arrow.


A)
XbaI (2965)
HindIII (2960)
BbsI (2952)
PstI (7)
NcoI (2692)
AvrII (338)
AseI (2635)
AseI (409)
BbsI (2593)
Acc65I (2591)
Alpha peptide
SV40 prom
SpeI (2493)
EM7
ClaI (2488)
H1 prom
EM7
PvuII (702)
psiRNA-hH1neo G2
(2978 bp)
ori pMB1
Neo
ßGlo pAn
EcoRI (1705)
EcoRI (1297)
B)
BbsI cut sites
Alpha peptide
SV40 promoter
AseI cut sites
C)

2.2.4 Nuclear Protein Extraction and Western Blotting

Initially, N1E115 or SH-SY5Y cells used for Western Blot analysis were grown to approximately 80% confluency in T75 culture flasks and pelleted. The cell pellet was washed then resuspended in 400 µL of cold Buffer A (10mM Tris-HCl at pH 8, 1.5 mM $MgCl_2$, 5 mM KCl and 0.5% NP-40, 0.5 mM *threo*-1,4-Dimercapto-2,3-butanediol (DTT) and 0.2 mM phenylmethylsulfonyl fluoride (PMSF)) and then gently transferred to a 1.5mL eppendorf tube. This cellular resuspension was allowed to incubate at 25 °C for 15 minutes after which it was centrifuged at 1000 rpm for 5 minutes using an Eppendorf 5417R centrifuge set at 4 °C. From here on all samples are kept in ice to prevent protein degradation. After centrifugation the supernatant was discarded and another 400 µL of Buffer A was added along with 4 µL of DNase 1 (100 U/mL). This was allowed to incubate on ice for 15 minutes and was then centrifuged for another 5 minutes at 1000 rpm and 4 °C. The supernatant was then discarded and the pellet was resuspended in 80 µL of cold Buffer B (20 mM Tris-HCl pH 8, 25% glycerol, 1.5 mM $MgCl_2$, 400 mM KCl and 0.2 mM EDTA, 0.5 mM DTT and 0.2 mM PMSF). This mixture was allowed to incubate on ice for 20 minutes after which it was centrifuged at 13,000 rpm for 5 minutes at 4 °C. The supernatant at this stage contains the desired nuclear protein content for further processing and was either used immediately for Western Blotting or was aliquoted and stored at -20 °C.

For Western Blotting the nuclear protein extracts were quantified using a Bradford Assay as described in reference 74 (74). Once quantified 30 µg of protein was loaded into the 3-8% NuPage precast gels and were electrophoresed at 80 volts for 2.5 hours, then were transferred to a Millipore Immobilion-P membrane using a BioRad Trans-Blot SD Semidry Transfer Cell at 40 mA for 35 minutes in a transfer buffer made up of 25 mM Tris, 190 mM glycine, 0.05 mM SDS and 20% MeOH. After the transfer, the membrane was wetted in

MeOH and then transferred into a Ponceau S solution made up of 0.5% Ponceau Red and 1% acetic acid. The membrane was incubated for 5 minutes then gently rinsed in tap water until the protein transfer became visible. To encourage complete protein binding the membranes were allowed to dry on the desktop for 1 hour. Membranes were blocked overnight at 4 °C in a solution of TBST (100 mM Tris pH 8, 1.5 M NaCl, and 0.5% Tween 20) with 5% fat free milk. This blocking solution was then replaced with the primary antibody solution containing the antibody and TBST with 5% fat free milk. For Snf2L detection the antibody was used at a concentration of 1:1700, Snf2H at 1:2000, γ-tubulin at 1:2000, and β-actin at 1:2500 (antibody: milk solution). Primary antibodies were incubated overnight at 4 °C and in the morning were rinsed 3 times for 15 minutes each time in 15 mL TBST. Once the rinses were complete the secondary antibodies were added at a concentration of 1:5000 for all. Secondary antibodies were allowed to incubate for 1 hour at 4 °C after which they were rinsed 3 times for 15 minutes each time in 15 mL TBST. Blots were then exposed to ECL Western Blotting Detection System (Pierce, Rockford, IL) following the manufacturer's guidelines. The resulting protein expression was detected on CL-X Posure™ Film. Films were processed in a Kodak M35A-X-Omat Processor. For reprobing blots were stripped for 30 minutes at 56 °C in a buffer containing 100mM β-mercaptoethanol, 2% SDS and 62.5mM Tris pH 6.7. The membrane was then washed 3 times for 10 minutes each time in 15 mL TBST and then the detection process began again at the initial blocking stage. The extraction process is identical for neurosphere cultures except that they were grown to confluency prior to extraction. Protein extraction has been adapted from the protocol followed by Aoki et al (2006) and westerns from Berube et al (2000) (75, 76).

2.2.5 RNA Isolation, RT-PCR Reactions and Electrophoresis Detection

RNA from neurosphere and neuroblastoma cultures was isolated using the RNeasy Plus Mini Kit (Qiagen) and quantified using a Beckman DU640 spectrophotometer. In order to confirm the integrity of the RNA 1 μg was run on a 1% agarose gel made with 1x MOPS (3-[N-morpholino]propanesulfonic acid) buffer (200 mM MOPS, 50 mM NaAc and 1 mM EDTA and 2.2 M formaldehyde). First strand cDNA synthesis was preformed using 4μg of RNA and 300ng of random primers. This mixture was heated to 70°C for 5 minutes then rapidly cooled on ice before adding 8μL of Invitrogen's First Strand Synthesis Buffer, 4 μL DTT, 2 μL dNTP, 0.8 μL of SuperScript II Reverse Transcriptase (SSRI) and 15.6 μL of diethyl pyrocarbonate (DEPC) water. The first strand cDNA was amplified in a thermal cycler (PTC 200 Peltier Thermo Cycler, Watertown, MA) using the following cycles; 42°C for 5 minutes, 50°C for 50 minutes, 70°C for 15 minutes and finally 4°C for 1 hour. Tubes without the SSRI enzyme were used as control.

PCR reactions for the investigation of shRNA construct activity on Snf2h or Snf2l and for βactin were conducted in the following manner. Each reaction contained 3 μL cDNA product, 1 μL of each primer from 25 μM stock solution, 5 μL of Invitrogen's 10x PCR buffer, 2 μL dNTPs, $MgCl_2$ to a final concentration of 15 mM, 2 units of Invitrogen's TAQ polymerase and up to 50 μL of HPLC water (Sigma-Aldrich). These reactions were placed into a PTC-200 Peltier Thermo Cycler PCR machine and run at 95°C for 2 minutes followed by 35 cycles at 94°C for 30 seconds, 60°C for 33 seconds, 72°C for 45 seconds and a final step at 72°C for 10 minutes. All reaction products were then electrophoresed in 1.5% agarose gels containing 1 μg/mL ethidium bromide and 1x TAE buffer (40 mM Tris-Acetate and 1 mM EDTA) using a BioRad Power-Pac 200 power supply. DNA loading buffer contained trace bromophenol blue, trace xylene cyanol and 200 mg/mL Ficoll 400. To

identify the band sizes a 1 kb plus DNA ladder from Invitrogen was run in the first lane of the gels.

2.2.6 DNA Genotyping and Electrophoresis Detection

DNA from cell cultures or murine tissue samples was extracted using the HotShot DNA extraction protocol developed by Truett et al (2000) and was used for PCR amplification with the following conditions: 1 μL of DNA, 5 μL of Invitrogen's PCR Buffer, 1 μL of each primer from 25 μM stock solution, 1 unit of TAQ polymerase, 2 μL dNTPs, 1.5 mM $MgCl_2$, and 38 μL HPLC water. The reaction mixtures were PCR amplified by heating at 95°C for 2 minutes, 94°C for 30 seconds, 60°C for 40 seconds, 72°C for 40 seconds for 35 cycles followed by one 72°C 10 minute cycle extension hold. Products were analyzed by electrophoresis as outlined in section 2.2.5.

2.2.7 Q-PCR Analysis of Cell Cycle Genes

To detect gene changes in Snf2l knockout neurosphere cultures a qPCR array specific for cell cycle genes was used. The array that was chosen was the Cell Cycle RT^2 Profiler PCR Array from SuperArray, Frederick MD. All processes for these experiments strictly followed the manufacturer's guidelines outlined for a Stratagene Mx3000p machine. These experiments were conducted in triplicate for two control samples, Mar24 D and Mar27 I, and two experimental samples, Mar24 B and Mar24 C all of which were time matched at P4. The results from these experiments were analyzed using the ΔΔCT method contained in the template found at (www.superarray.com/manual.php?target=pcrarrayQCanalysisV2.xls).

2.2.8 BrdU Incorporation Assays and Fluorescent Antibody Detection

To determine the percentage of the cell culture population that was in S-phase both SH-SY5Y and neurosphere populations were exposed to BrdU (77). Briefly, for SH-SY5Y cells a haemocytometer was used to count cells and thus transfer ~150,000 cells to 22x22 mm glass micro covers (VWR) coated with 1x poly-D-lysine (SH-SY5Y and N1E115 cells). For neurosphere cultures cells were seeded into 8-well Chamber Slides treated with 1x poly-D-lysine and 15 μg/mL laminin (neurospheres) for 5 hours at room temperature and then allowed to air dry. Neurosphere cultures were first dissociated using a Neurocult Chemical Dissociation Kit (StemCell Technologies). Cultures were added to their dishes in their respective proliferation medias and allowed to acclimatize for 12 hours after which the media was changed to proliferation media, supplemented with 10 mM BrdU for SH-SY5Y and N1E115 cells or 100 mM for neurospheres, in all 0 hour plates and were left for a 3 hour pulse for SH-SY5Y cells and an 8 hour pulse for neurosphere cultures. The media in all remaining cultures was changed to its respective differentiation media. Neurosphere cultures were then allowed to differentiate up to 4 hour before the fixation time. At this time the media was replaced with differentiation media containing the appropriate amount of BrdU. This was left for the 8 hour pulse and then cells were fixed. SH-SY5Y cells followed the same process except that they were allowed to differentiate up to 3 hours pre-fixation at which time BrdU supplemented media was added for 3 hour BrdU pulse and then they were fixed. For fixation of SH-SY5Y and N1E115 cells cold (-20°C) 3:1 EtOH/MeOH was added to the cells for 10 minutes at room temperature while for neurospheres 4% paraformaldehyde (PFA) (10 mL 20%PFA, 20.25 mL 0.2 M Na_2HPO_4 4.75 mL 0.2 M NaH_2PO_4 and 15 mL of dH_2O) was used for 30 minutes at room temperature. After fixation cells were permeabilized with 2 N HCl for 20 minutes at room temperature. HCl was then aspirated and the plates

were washed using 0.1 M Tris buffer at pH 8.4 for 10 minutes. Followed by three 5 minute washes with sterile PBS supplemented with 0.1% Tween 20. Plates were then blocked for 30 minutes at 37°C in sterile PBS containing 5% non-fat milk prior to the addition of the primary antibody. The primary BrdU20a antibody was used at a 1:20 concentration in sterile PBS containing 5% non-fat milk. These were allowed to incubate for 4 hours at 37°C after which the cover slips were rinsed in sterile PBS 3x for 10 minutes each time. The secondary antibody used was goat α mouse 488 nm antibody from Sigma Aldrich at 1:200 in sterile PBS containing 5% non-fat milk and was incubated for 1 hour at 37°C. These were then washed for 3x 10 minutes each time in sterile PBS and were then counterstained using 1μg/mL DAPI for 5 minutes. The plates were then washed 3x with PBS for 5 minutes each time and were mounted on VWR Colorfrost slides using one drop of Vector Vectashield and sealed with clear nail polish. Cells were visualized using a Zeiss Axiovert 200 M microscope, Toronto ON, with an X-Cite 120 Fluorescence Illumination System (EXFO, Orlando, FL).

For quantification purposes three pictures were taken for each slide that was prepared and each experiment was conducted in triplicate. All the cells in the field of view at 20x magnification were counted using the Axiovision software for the 488 nm fluorescent BrdU antibody and were compared against the total population detected with the DAPI counterstain.

2.2.9 Isolation and Growth of Primary Neurospheres

Frontal cortex neurosphere cultures were isolated from embryos extracted at either embryonic day 12.5 or 14.5 (E12.5 or E14.5) from pregnant female mice ($Snf2L^{f/f}$;$Gata1Cre^{-/-}$) time-mated with male mice ($Snf2L^{+/+}$;$Gata1Cre^{+/-}$). Pregnant females were allowed to develop for the required length of time and were then sacrificed using CO_2 asphyxiation followed by cervical dislocation. The embryos were then removed from the uterine sac and each decidua was separated and placed into warm (37°C) sterile Hank's Balanced Salt Solution (Sigma Aldrich) to wash away excess blood. Individual embryos were then processed using #5 Dumoxel tweezers, Switzerland. A thin longitudinal line was inscribed into the embryo head and the overlying meninges were removed. Gentle pressure was then applied to the temporal sides of the head causing the frontal cortex to project out of the inscribed line. This was excised and transferred into sterile eppendorf tubes and placed on ice. Samples of the remaining embryo were placed into another eppendorf tube for genotyping purposes. The frontal cortex samples were then titurated into single cell suspensions using fire polished glass Pasteur pipettes and approximately 1.5 mL of neurosphere proliferation media (450 mL NeuroCult Basal Media supplemented with 50 mL NeuroCult Neural Stem Cell Proliferation Supplement, Stem Cell Technologies Inc., and 20 ng/mL human Epithelial Growth Factor, Sigma Aldrich). These suspensions were then transferred into T75 culture flasks and an additional 25 mL of proliferation media was added. These cultures were allowed to develop for 10 days, or until robust spheres had formed.

2.2.10 TUNEL Staining

Terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was carried out on SH-SY5Y clones expressing shRNA targeting Snf2h. The kit that was used was the In Situ Cell Death Detection Kit, TMR Red from Roche Diagnostics (Mississauga, ON). Initially 35 mm culture dishes and glass micro covers were treated as per the processes described in section 2.2.8. After the adherence chemicals had air dried 200,000 cells were seeded into the dishes in proliferation media and allowed to adhere to the substrate for 24 hours. At this time point the proliferation dishes were fixed using cold (-20°C) 3:1 EtOH:MeOH for 10 minutes. All of the media in the remaining dishes was changed to differentiation media and at 24 hour intervals the remaining cultures were fixed up to 4 days of differentiation. All cultures were kept at 4°C until the TUNEL assay was conducted. Processes for the TUNEL assay were conducted following the guidelines laid out by the manufacturer. In order to quantify the percentage of the population that was TUNEL positive a DAPI counter stain was used. These studies were all completed in triplicate for each sample. The staining was visualized and counted using the previously mentioned Axiovert 200M microscope and three different pictures were taken for each culture sample.

2.2.11 Cellular Proliferation Assay

SH-SY5Y populations from SNF2L knockdown clones and controls were seeded at an initial density of 80,000 cells into T25 culture dishes as described above. These cultures were then subjected to the differentiation protocols described previously and at 24 hour intervals were then trypsinized and counted using a haemocytometer. This process was repeated three times for each culture sample in the study with the study being completed 6 times and the average number from these counts was taken as the population number.

Coulter counts were conducted on neurosphere populations after using Neurocult chemical dissociation treatment described above. Initially 500,000 cells were seeded into T25 culture flasks using the VWR haemocytometer. For the duration of this study all populations were maintained in proliferation media. At each 24 hour interval from proliferation up to 3 days cells were collected, dissociated and counted using a 22^{TM} Coulter Counter Cell and Particle Counter, Beckman Coulter Mississauga ON. All samples were run in triplicate and three counts were taken for each replicate.

2.2.12 Neurosphere Cell Specific Labelling and Cell Population Quantification

In order to determine the specific cell types contained in the wild type and experimental neurosphere populations the previously mentioned StemCell fluorescent antibodies were used. These antibodies were specific to neuronal class III β-tubulin (TUJ1), glial fibrillary acidic protein (GFAP), and nestin. Cultures were grown in 8-well Lab-Tek II chamber slides treated with adherence chemicals as per the process described in section 2.2.8. Cells were allowed to adhere for 24 hours then media was changed to differentiation media and cultures were fixed at 24 hour intervals up to 7 days of differentiation. Cells were fixed using 4%PFA as described earlier and were then permeabilized using sterile PBS containing 0.3% Triton-X for 5 minutes at room temperature. This was then aspirated and washed 3x for 5 minutes each time in sterile PBS. Cultures were then blocked for 1 hour in sterile PBS supplemented with 10% horse serum (Sigma Aldrich). After blocking, the primary antibody was mixed in fresh blocking solution in the following concentrations: TUJ1 1:600, GFAP1:100, nestin 1:50. Primary antibodies were allowed to incubate for 4 hours at 37°C after which the primary antibody solution was aspirated and the plate was washed 3x for 10 minutes each time using sterile PBS. Secondary antibodies were exposed

for 1 hour at 37°C and then washed 3x for 10 minutes each time using sterile PBS. The secondary antibodies used were; donkey α mouse Alexa 594nm or 488nm for TUJ1, goat α mouse FITC or 594nm for nestin and goat α rabbit FITC for GFAP. Cultures were then counterstained using 1μg/mL DAPI in PBS for 5 minutes after which they were washed 3x for 10 minutes each time. Once these processes were completed the plates were mounted as per the process outlined in section 2.2.8. The resulting data was visualized on the previously mentioned Axiovert 200M microscope and population counts were quantified in similar manners. These processes are adapted from the protocols described by StemCell Technologies.

3.0 Results

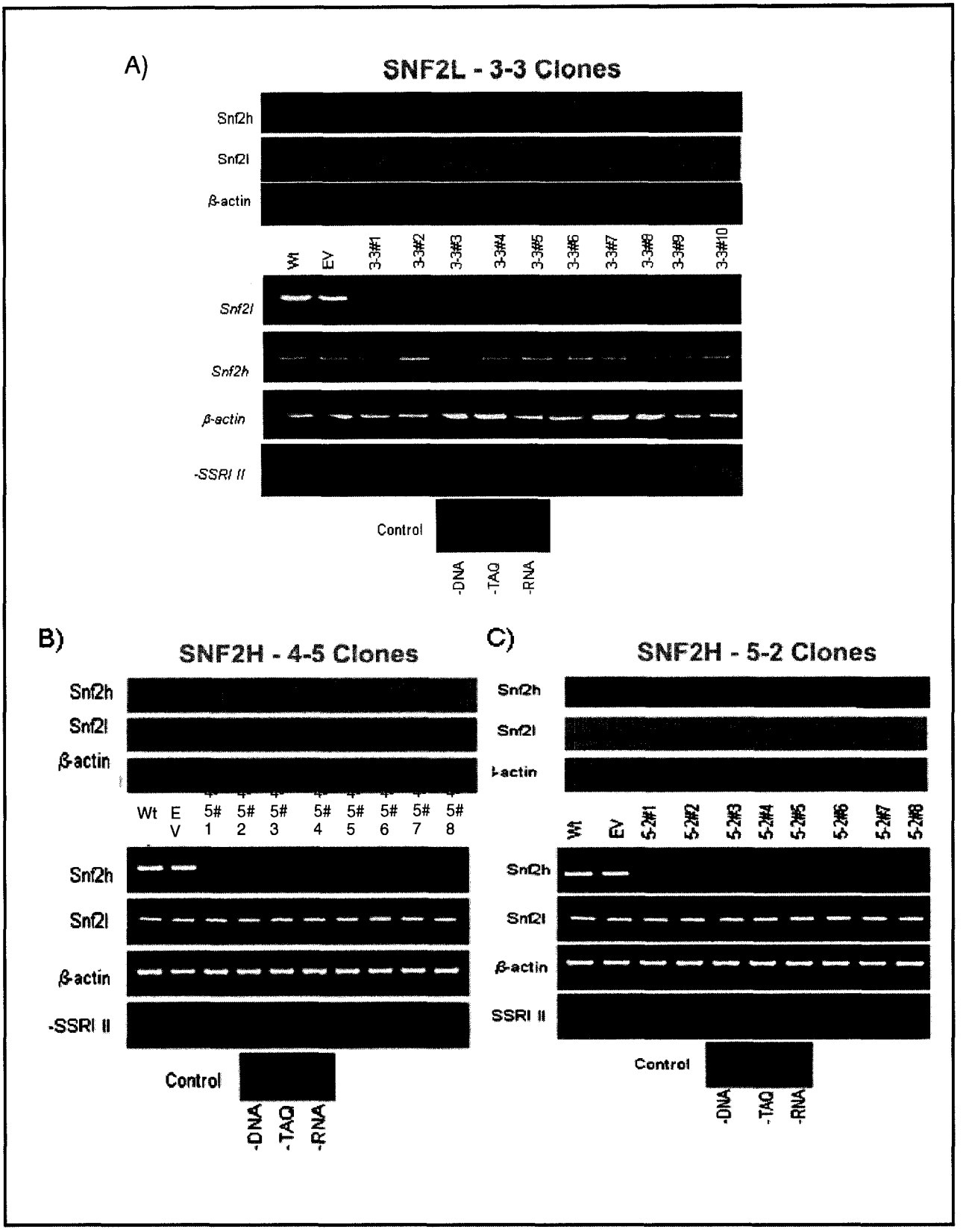
3.1 Initial characterization and selection of ISWI knockdown clones

In order to determine the manner in which the ISWI genes control development and differentiation in mammalian neurons the human neuroblastoma cell line SH-SY5Y was transfected with plasmid derived shRNA constructs for stable gene knockdown of Snf2L and Snf2H (see section 2.2.1). Following transfection and drug selection ten clones transfected with shRNA construct against Snf2L (3-3) and eight clones transfected with each of two different shRNA constructs targeting Snf2H (4-5 and 5-2) were selected for analysis (Figure 9 B and C). The clones were examined for expression of both the targeted ISWI gene and the remaining highly homologous ISWI gene using both RT-PCR and Western blot analysis. The Snf2L knockdown clones, panel A Figure 9, resulted in a complete knockdown of the Snf2L gene when examined by RT-PCR analysis and a 91.3% reduction as analyzed by

Western Blot as quantified by densitometry via the online program Image J. For all other experiments we utilized clones 3-3#1, 2 and 3. The Snf2H knockdown clones, panel B and C Figure 9, were also examined for successful gene knockdown via RT-PCR and Western blot analysis. This resulted in a complete knockdown of the Snf2H gene upon RT-PCR examination and a 95.4% decrease in protein level by Western blot analysis. Further studies utilized clones 1 and 2 for the 4-5 construct and clones 2 and 4 for the 5-2 construct.

The controls used for these experiments were a wild type SH-SY5Y cell population that was not transfected and SH-SY5Y cells transfected with the vector alone, termed "empty vector" (EV). The EV control allowed for the determination of possible

FIGURE 9: RT-PCR (LOWER 5 PANELS) AND WESTERN (UPPER 3 PANELS) ANALYSIS OF SH-SY5Y CELLS EXPRESSING SHRNA CONSTRUCTS. A) Wild type (Wt) and Empty Vector (EV) controls with ten clones containing the Snf2L shRNA construct 3-3. B,C) Western and Rt-PCR results for shRNA constructs 4-5 and 5-2 targeting Snf2H with eight clones each. –DNA, no DNA control; -TAQ, no reverse-transcriptase control; -RNA, no RNA control.


A)
SNF2L - 3-3 Clones
Snf2h
Snf2l
β-actin
Wt
EV
3-3#1
3-3#2
3-3#3
3-3#4
3-3#5
3-3#6
3-3#7
3-3#8
3-3#9
3-3#10
Snf2l
Snf2h
β-actin
-SSRI II
Control
-DNA
-TAQ
-RNA
B)
SNF2H - 4-5 Clones
Snf2h
Snf2l
β-actin
Wt
E V
5# 1
5# 2
5# 3
5# 4
5# 5
5# 6
5# 7
5# 8
Snf2h
Snf2l
β-actin
-SSRI II
Control
-DNA
-TAQ
-RNA
C)
SNF2H - 5-2 Clones
Snf2h
Snf2l
β-actin
Wt
EV
5-2#1
5-2#2
5-2#3
5-2#4
5-2#5
5-2#6
5-2#7
5-2#8
Snf2h
Snf2l
β-actin
SSRI II
Control
-DNA
-TAQ
-RNA

outside effects due to plasmid transfection and/or drug selection. The antibiotic used for selection was geneticin and it is the most commonly used G-418 chemical for the selection of neo cassette expressing plasmid transfected eukaryotic cells currently available (78). We have found that when SH-SY5Y cells were grown in the presence of geneticin there is no disruption to our genes of interest but that there is a moderate affect to the morphology of the cell soma (Supplemental Figures 24 and Figure 9).

3.2 Examination of ISWI knockout in neuroblastoma cells

3.2.1 The effect of SNF2L knockdown on proliferation

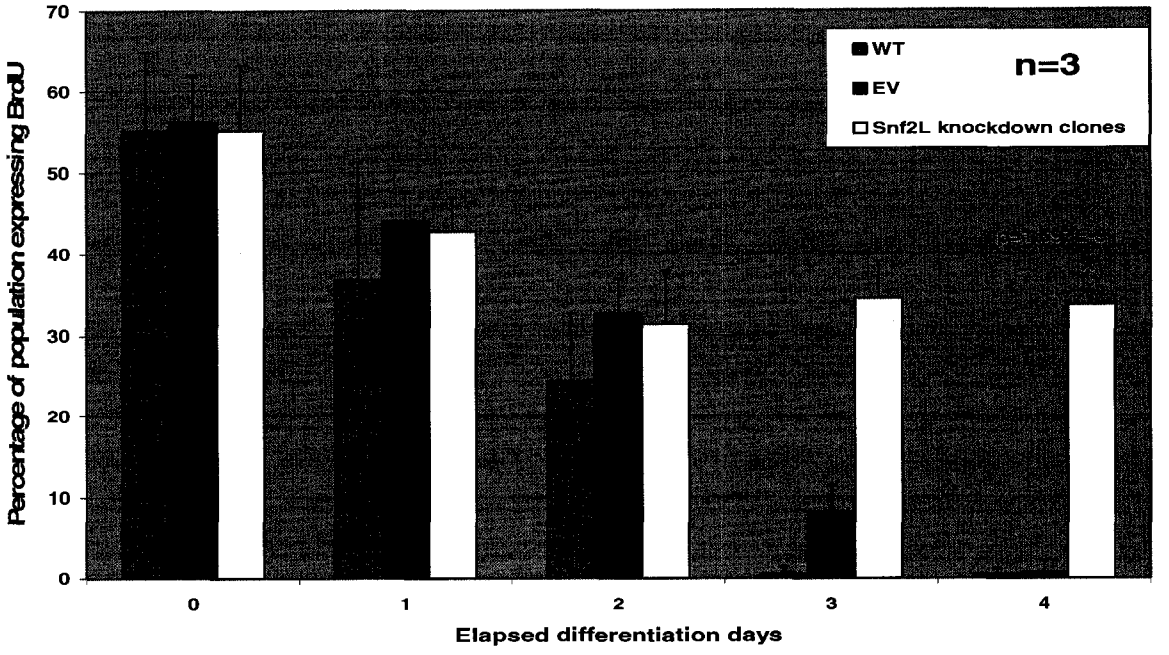
Since previous results have demonstrated that the SNF2L gene is strongly upregulated in differentiated cell populations and that overexpression creates spontaneous neuronal differentiation, Supplemental Figures 25 and 26, we decided to first examine whether there are any effects on the ability of SNF2L knockout cells to differentiate. We hypothesized that the knockdown of the SNF2L gene will result in neuronal populations that are unable to differentiate properly as we believe that this gene is required for correct neuronal development. This experiment was done by exposing 3-3 clones 1, 2 and 3 to a BrdU incorporation assay to determine the population undergoing S-phase. Initially cell stocks were grown to an approximate 80% confluence in proliferation media then were supplemented with 10 mM BrdU and left for a 3 hour pulse after which the cells were fixed. All remaining cultures were switched to differentiation media and 3 hours prior to the time of harvest (1, 2, 3 and 4 days) were pulsed with BrdU. Cells were fixed and counterstained using DAPI and then analyzed for BrdU expression. BrdU positive cells were quantified as a percentage of the total number of DAPI positive cells. We observed that SNF2L knockdown and control cultures had a similar level of BrdU incorporation at time 0 (~55%) and Day 1

(~40%) but were beginning to show differences by Day 2. By Day 4 it was found that the SNF2L knockdown cultures resulted in an average of 33.8% of the cells still incorporating BrdU while wild type and empty vector controls are at 0.6% and 0.8% respectively (Figure 10 A). When the results were compiled and analyzed using a Student's T-test the p value was 1.697×10^{-9} indicating that this is a statistically significant result. Examples of the fluorescent antibody labeling and DAPI counter stain microscopy can be seen in Figure 10 B. We observed no BrdU positive cells by Day 4 in the controls but that there are many positive cells in the SNF2L knockdown clones 3-3#1 and 3. These results indicate that the removal of the SNF2L gene does indeed affect the differentiation capacity of the neuronal cell population in culture conditions.

In order to further examine this result we conducted a cellular proliferation assay under differentiation conditions where at the beginning of the study 80,000 cells from wild type, empty vector and clones 3-3#1 and 3-3#3 were seeded into their own culture flasks. These cells were put through the same differentiation time course as for the BrdU experiment described above but at each 24 hour interval they were trypsinized and the population was counted using a haemocytometer. In order to achieve statistical significance each population was counted three times and the study was completed six times in total. The results for these experiments are displayed in Figure 11 and show continued proliferation, similar to the initial BrdU study. The wild type and empty vector controls had an average population count of 200,000 and 141,667

FIGURE 10: BRDU LABELLING ASSAY OF SNF2L SHRNA CELL LINES. Three hour pulse labeling study using 10mM BrdU and SH-SY5Y human neuroblastoma cells during differentiation time course. Wild type (WT) and empty vector (EV2) cell lines were used as controls along with three clones, 3-3#1,#2, and #3 expressing shRNA sequences against the SNF2L gene. Cells were grown in DMEM containing 10%FBS and 1% penn/strep (SNF2L-/- clones also contain 400ug/mL kanamycin). For differentiation the media was changed to DMEM with 1%FBS, 10uM retinoic acid and 1%penn/strep. Three hours pre-fixation time differentiation media was supplemented with 10mM BrdU. A) Graph showing cumulative data from 3 replicates. The three SNF2L shRNA clones 3-3#1, 2 &3 were averaged together and are represented by the yellow bars. Asterisks indicate statistical significance analyzed by Student's T-test. B) DAPI stained (blue) and BrdU labelled (green) cells demonstrating that many of the SNF2L knockdown cells continue to incorporate BrdU after 4 days of differentiation. Scale bar is 56μm.

A)


70
60
50
40
30
20
10
0
Percentage of population expressing BrdU
WT
EV
Snf2L knockdown clones
n=3
0
1
2
3
4
Elapsed differentiation days

B)

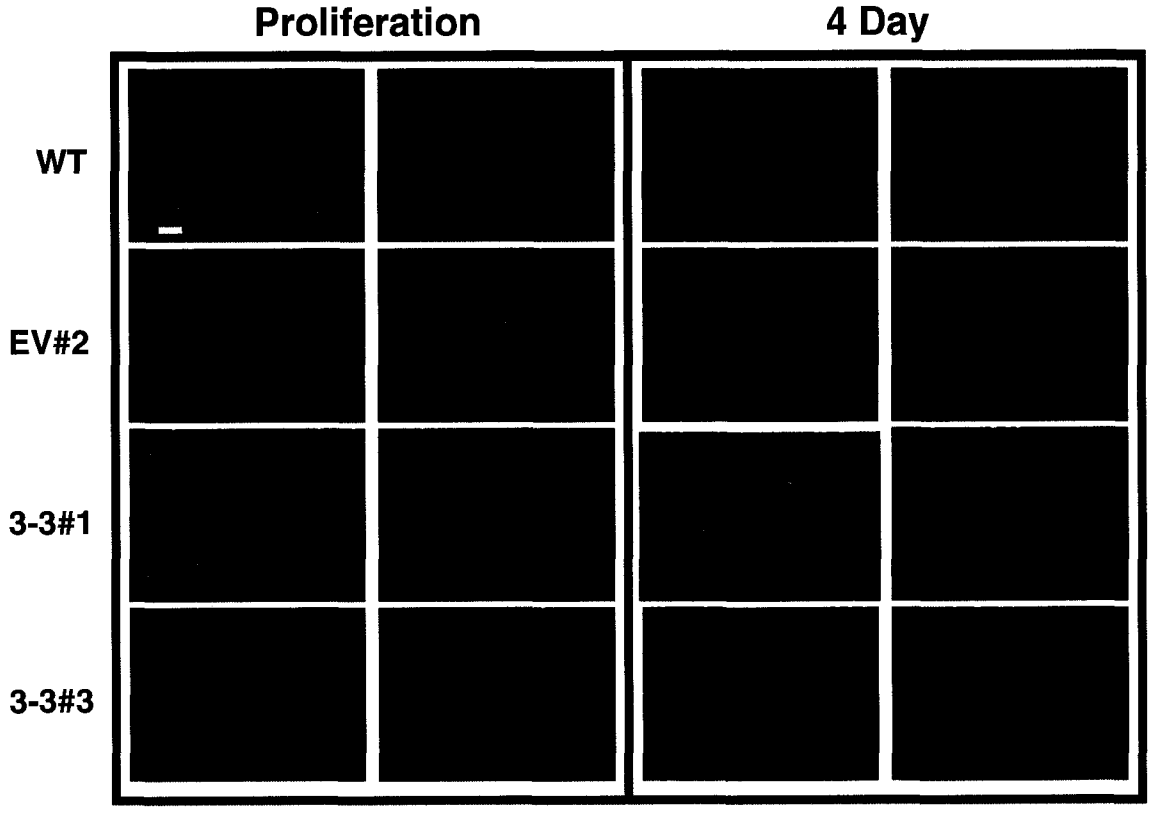
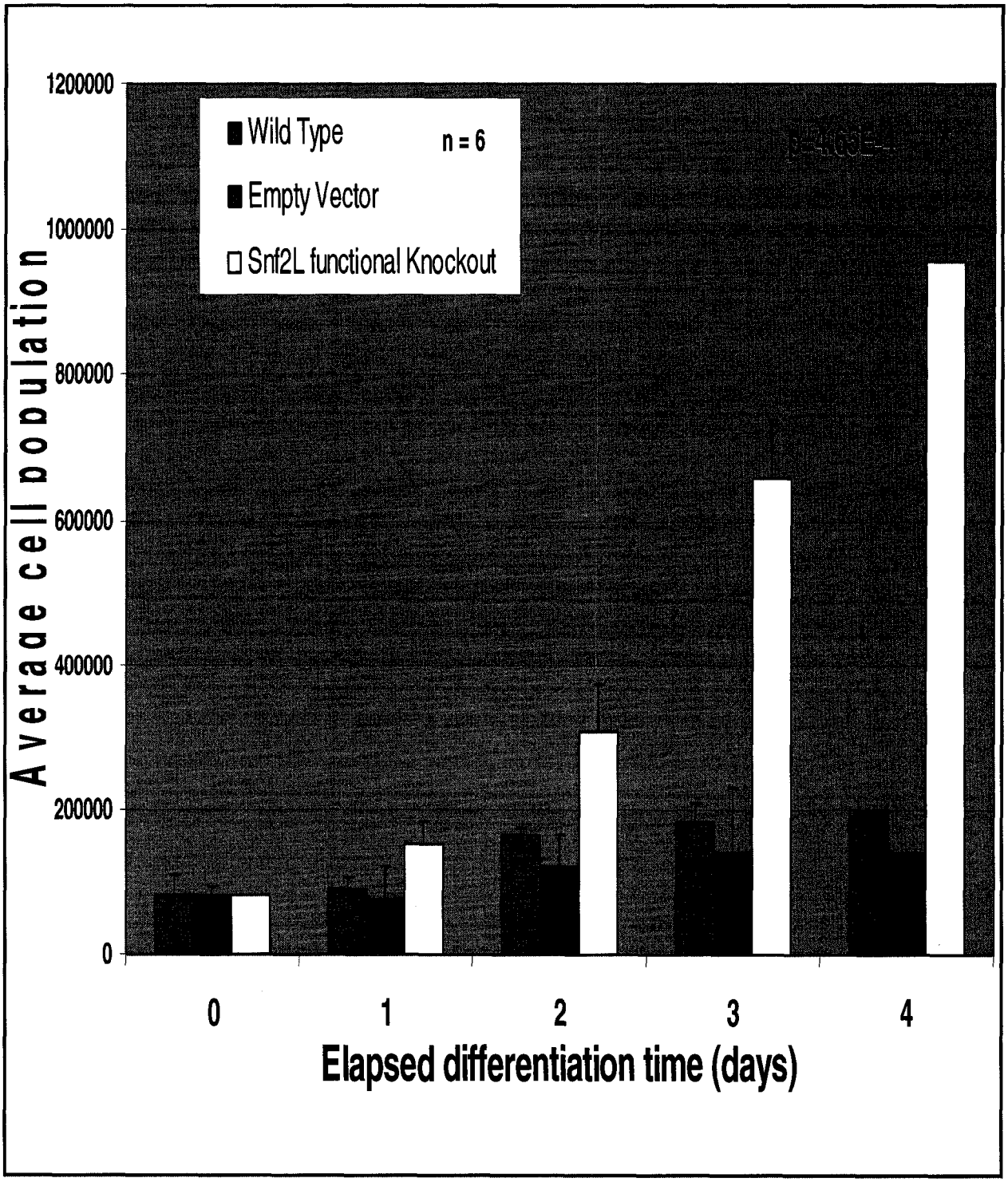

Proliferation
4 Day
WT
EV#2
3-3#1
3-3#3

FIGURE 11: INCREASED CELL COUNTS IN SNF2L KNOCKDOWN CELL LINES DURING FORCED DIFFERENTIATION. Cellular proliferation after induction of differentiation for SH-SY5Y cells containing Snf2L targeting shRNA constructs with wild type (Wt, blue bars) and empty vector (EV, red bars) controls. At the start of the study 80,000 cells were plated into T25 culture flasks containing differentiation media made up of DMEM, 0.5%FBS, and 10uM retinoic acid. At 24 hour intervals cells were trypsinized and counted using a haemocytometer, three counts for each cell strain were conducted at each time increment. The yellow bars represent data pooled and averaged from 3 SNF2L knockdown clones. Note the increasing cell number during differentiation of the knockdown clones. Asterisk indicates statistical significance analyzed by Student's T-test.


Wild Type
n = 6
Empty Vector
Snf2L functional Knockout
Average cell population
1200000
1000000
800000
600000
400000
200000
0
0
1
2
3
4
Elapsed differentiation time (days)

at 4 days of differentiation indicating that there is a small percentage of cells that continue to proliferate in control populations. Indeed this is expected as cells will continue through the cell cycle one final time upon serum withdrawal (79). The SNF2L clones resulted in an average total population of 956,250 at Day 4 of differentiation with a Student's T-test value of $4.65x10^{-4}$ indicating statistical significance. When these results are examined as a population fold increase, we found that the wild type and empty vector controls averaged an increase of only 1.29 and 1.18 fold increase in a 24 hour interval respectively, while the Snf2L clones averaged at an increase of 1.83 fold (Table 1). The knockout clones are also still significantly higher at the final differentiation day examined, day 4, but appear to be declining which likely indicates the continued proliferation is only a temporary condition (Table 1). Control populations have also leveled out after approximately 2 days of forced differentiation.

Table 1: Fold increase in total population

	Elapsed Differentiation Days			
	1	2	3	4
Wild Type	1.1458	1.8181	1.1	1.0909
Empty Vector	0.9375	1.6111	1.1724	1
Snf2L 3-3 clones	1.901	1.9829	2.0813	1.3643

Taken together these results indicate that the removal of the SNF2L gene causes *in vitro* neuronal populations to continue to proliferate under forced differentiation conditions. As mentioned in previous sections the Snf2L gene is known to be important in established, differentiated cellular populations and also causes spontaneous differentiation when overexpressed in proliferating N1E115 murine neuroblastoma populations.

3.2.2 The effect of Snf2H knockdown on proliferation

In contrast to the upregulation of the Snf2L gene during neuronal differentiation the Snf2H gene has previously been demonstrated to be essential for cellular proliferation. Stopka and Skoultchi clearly demonstrated that the ablation of Snf2h in a mouse model results in embryonic lethality during periimplantation due to insufficient cellular proliferation (80). As a result of these findings we decided to conduct a similar BrdU incorporation assay on the SNF2H knockdown clones 4-5 and 5-2 as was completed for those knocked down for Snf2L. We hypothesized that the knockdown of the SNf2H gene will result in rapid cellular death during proliferation. These studies were conducted in the same manner as those mentioned in section 3.2.1 but were done using 4-5 clones 1 and 2 along with 5-2 clones 2 and 4 (Figure 9). During the proliferation time point of this experiment the control populations, Wt and EV, incorporated BrdU to very similar rates as those found for our SNF2L BrdU incorporation assay. As differentiation continued this BrdU incorporation decreased steadily until by Day 4 there was no detectable level in the EV population and only a widely variably 3.7% incorporation in the WT population. Analysis of the 4 Day time point demonstrated that there is no significant difference between the control populations and the experimental Snf2h knockdown populations. However, during proliferation there was a 36.24% lower BrdU incorporation rate in the experimental cells (Figure 12). These data suggest that while these cells are able to proliferate there is a definitive decrease in their replication time course as fewer cells incorporated the label. It was also found that there was a constant wide degree of variability in the population counts in the experimental populations that increases throughout the differentiation time course. This is likely the result of a high level of cellular death when the cells are changed into differentiation media as seen during microscopic investigation (data not shown).

To further examine the effect of SNF2H removal in our SH-SY5Y clones further study will be required. It would be beneficial to conduct proliferation assays in proliferation media as were discussed in section 3.2.1 for SNF2L knockdown. This will allow for closer examination into the differences that were found at time 0 and will determine if there is an associated disruption to the rate of proliferation in these SNF2H knockdown clones.

3.2.3 Snf2H knockdown causes increased apoptosis during differentiation

Due to the fluctuating results found during the BrdU incorporation assay and the fact that an apparent increase in cell death was found on microscopic examinations we decided to conduct a TUNEL assay to determine the extent of the apoptotic changes. We hypothesized that because of these findings there would be a strong increase in cell death associated with SNF2H knockdown. For this study both wild type and empty vector controls were grown alongside two clones each for the 4-5 and 5-2 inserts (4-5 #1,2 and 5-2 #2,4). After reaching an approximate 80% confluence the proliferation time point was fixed and the media in the remaining cultures was changed to standard differentiation media. These cells were allowed to differentiate for 1-4 days then they were fixed, and processed for TUNEL. Figure 13 A shows some representative pictures from the TUNEL labeling process. As may be seen very few TUNEL positive cells could be detected in the control samples and when quantified was less than 1%. In contrast, at Day 2 and 4 of differentiation our experimental SNF2H knockdown cultures contained close to 100% TUNEL positive cells. This result is somewhat surprising as initially we believed that all cells knocked down for Snf2H would undergo apoptosis during early proliferation stages.

Figure 13 B documents the percentage of the total population, through DAPI counter stain, that is TUNEL positive. It was found that in the Snf2H knockdown clones

there was a significant increase in TUNEL staining in 4-5 and 5-2 clones with 38.56% and 51.66% death respectively after only 1 day of differentiation. This compares to a complete absence in TUNEL staining for both control populations. By the last day of differentiation, day 4, that was examined all experimental populations had between 97-100% of the cells positive for TUNEL staining clearly demonstrating that these populations are unable to survive in the absence of Snf2H when induced to differentiate.

3.3 Generation of Snf2L knockout neurosphere cultures

To determine if we could recapitulate these results in primary cultures we isolated neurospheres from WT and Snf2l knockout mice. These cultures were generated from existing mouse lines in our laboratory. These experimental mice have been functionally ablated for the Snf2l gene by cloning *loxP* sites around exon 6 of the ATPase domain of the Snf2l gene. Upon the introduction of Cre recombinase exon 6 is excised creating a protein that is unable to perform any ATP hydrolyzing activity yet still producing a protein product that is capable of incorporation into its multiprotein complexes (81). Female 129SV mice from background Snf2Lf/f;Gata1Cre$^{-/-}$ were time mated to male C57B6 mice from Snf2L$^{+/y}$;Gata1Cre$^{+/-}$ background. After either 12.5 or 14.5 days of development pregnant mares were sacrificed and the embryos were removed. The frontal cortex was excised as described in section 2.2.9 and allowed to grow in culture for approximately 10 days. During

FIGURE 12: SNF2H KNOCKDOWN CLONES DO NOT SHOW ENHANCED BRDU INCORPORATION DURING DIFFERENTIATION. Three hour pulse labeling with 10mM BrdU on SH-SY5Y cells containing Snf2H shRNA constructs with wild type (Wt) and empty vector (EV) controls. Two clones were selected for both 4-5 (yellow bars) and 5-2 (light blue bars) shRNA constructs. BrdU incorporation decreases during differentiation in SNF2H clones similar to Wt and EV controls.

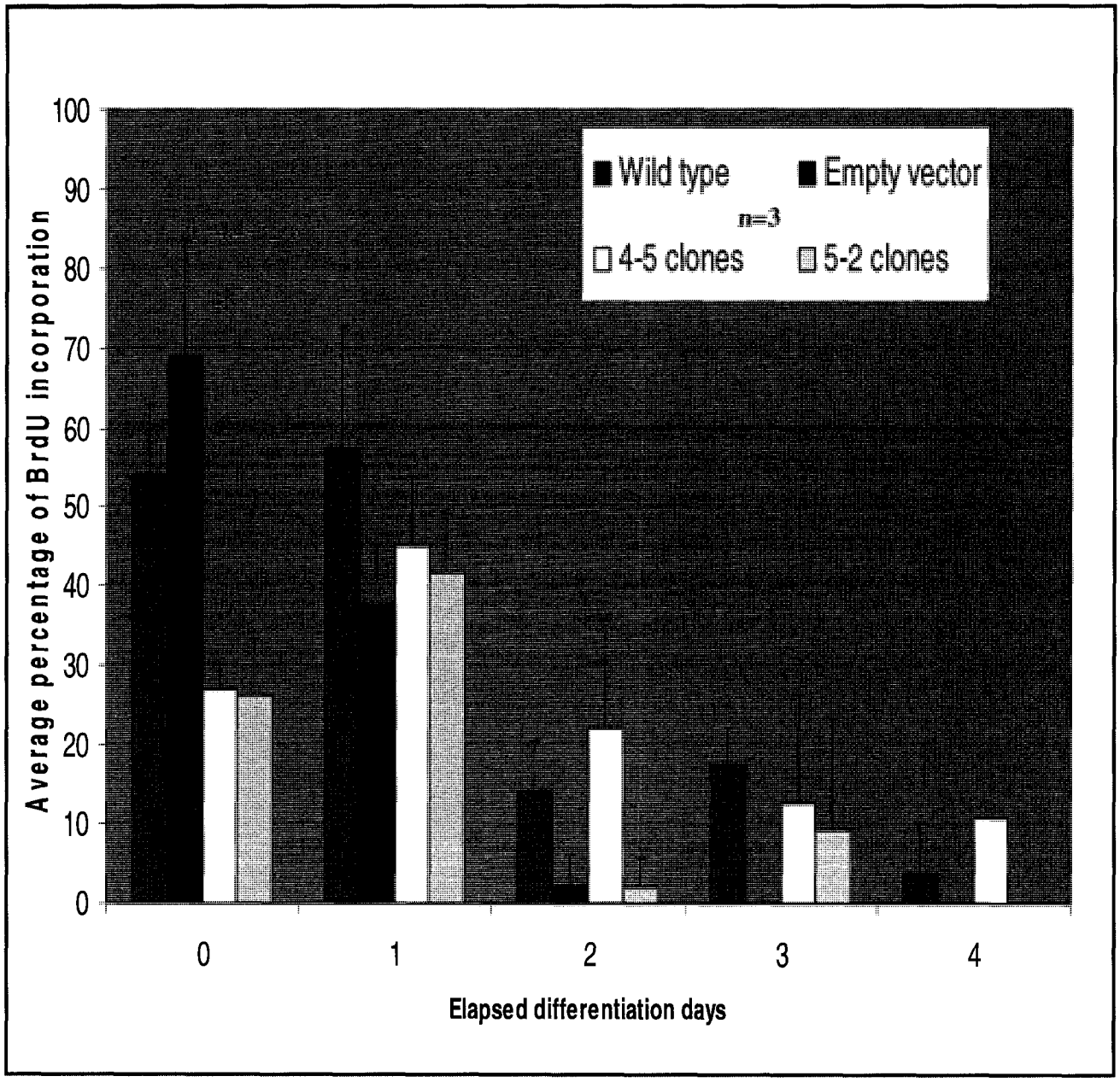
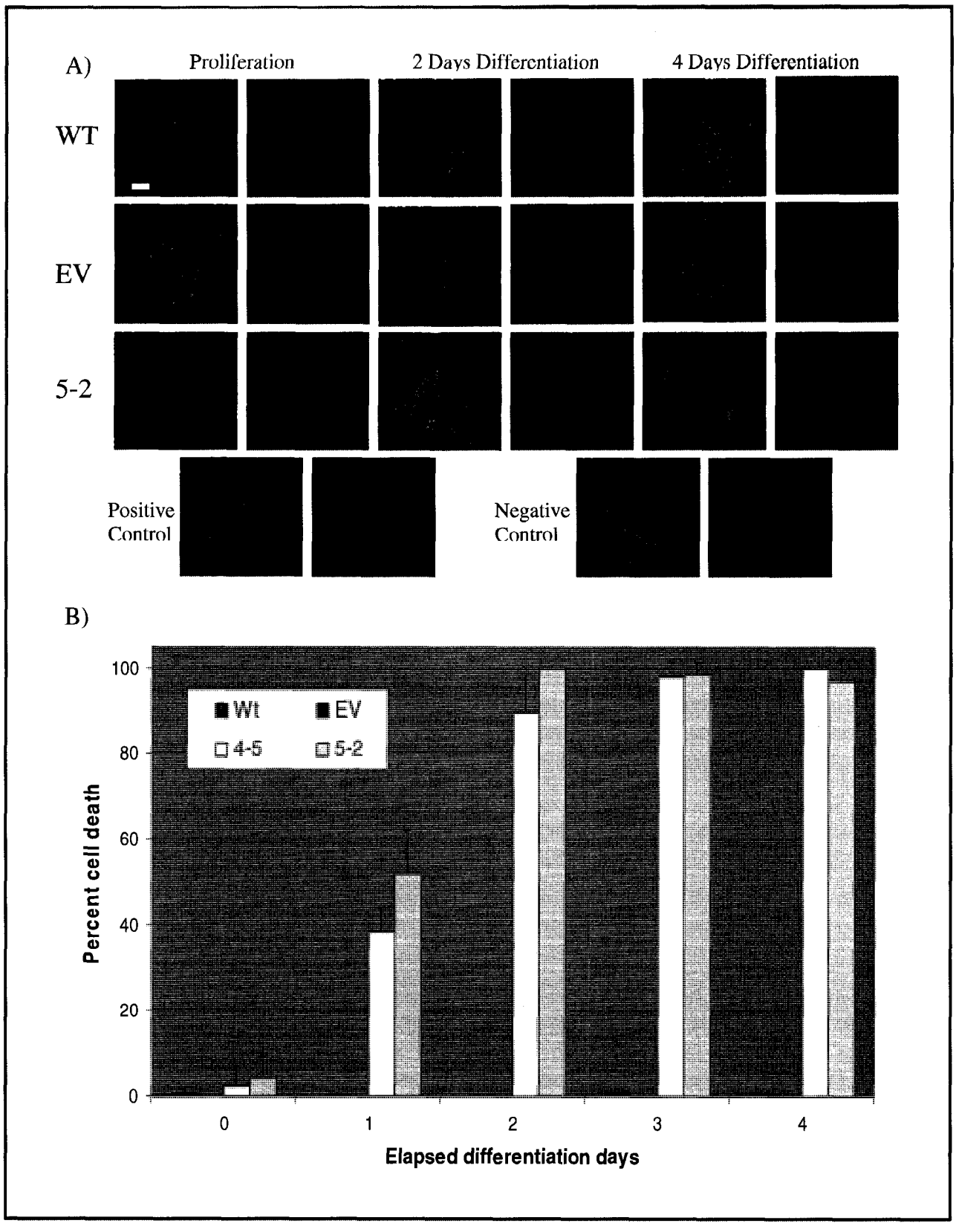

Wild type
Empty vector
n=3
4-5 clones
5-2 clones
Average percentage of BrdU incorporation
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
Elapsed differentiation days

FIGURE 13: TUNEL ASSAY ON SH-SY5Y CELLS EXPRESSING SNF2H DIRECTED SHRNA CONSTRUCTS WITH WILD TYPE (WT) AND EMPTY VECTOR (EV) CONTROLS. A) Representative pictures of cells seeded onto glass slides and fixed at 24 hour intervals and then examined for apoptosis. Cells were stained with DAPI (blue) and TUNEL (red) dyes, positive controls for TUNEL staining were treated with DNase1 for 30 minutes at room temperature. B) Apoptotic cells were counted and compared against DAPI counterstain and the average population of apoptotic cells was quantified. Approximately 100% of the SNF2H knockdown clones are TUNEL positive after 2 days of differentiation. Scale bar is 56μm.


A)
Proliferation
2 Days Differentiation
4 Days Differentiation
WT
EV
5-2
Positive Control
Negative Control
B)
Wt
EV
4-5
5-2
Percent cell death
100
80
60
40
20
0
0
1
2
3
4
Elapsed differentiation days

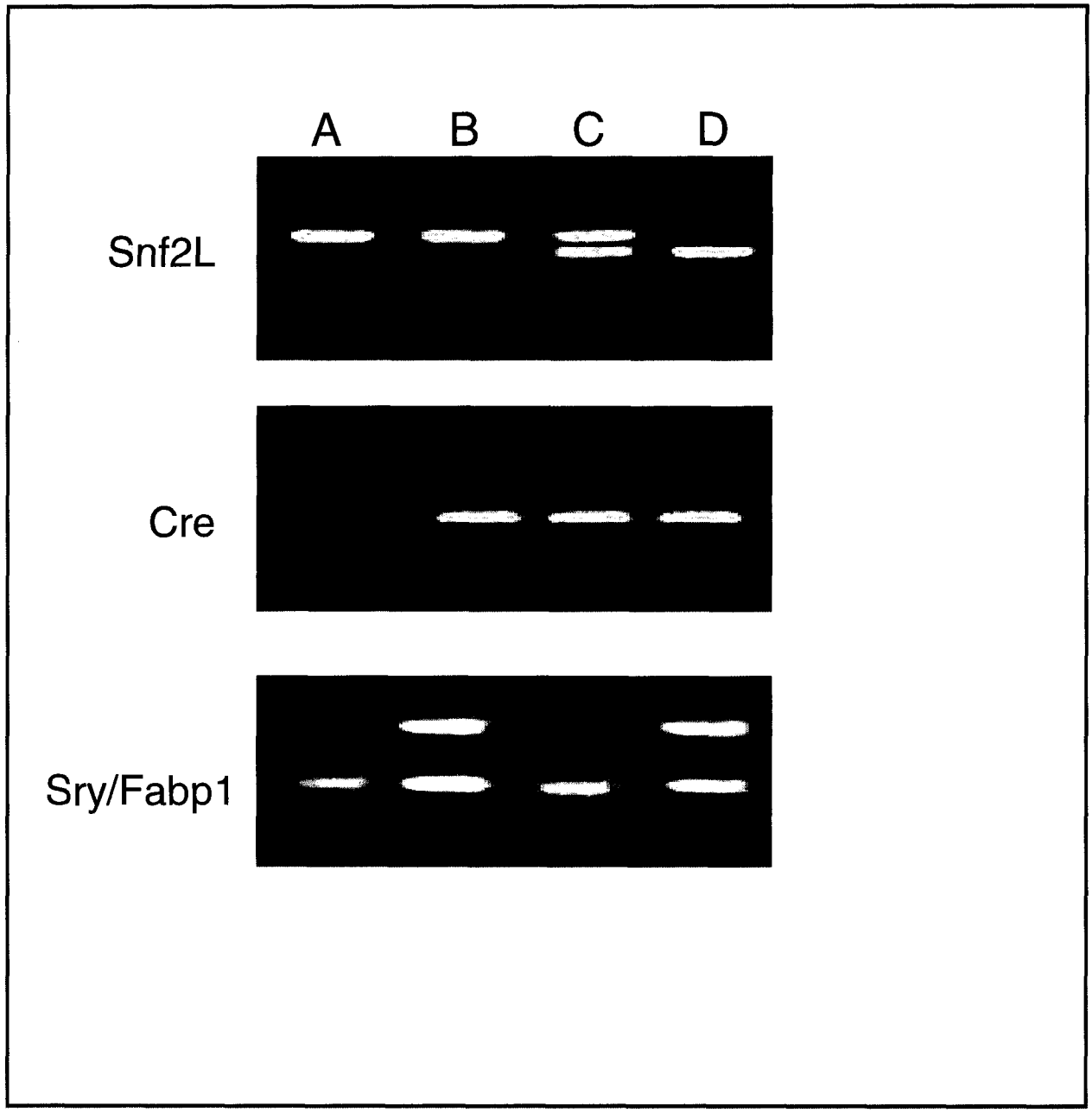
this time PCR genotyping was conducted on the remaining embryo carcass. Our PCR primers detect the WT Snf2l allele, 508 basepairs, and the exon 6 deleted Snf2l functional knockout allele, 438 basepairs. For sex determination and an appropriate housekeeping gene we have utilized a two gene multi-primer mixture for Sry, male specific, and Fabpi respectively. The products from these primers are 300 basepairs for Sry and 200 basepairs for Fabpi. These processes resulted in the production of 9 neurosphere cultures that were utilized for experimental procedures. We obtained 6 cultures for embryonic day 12.5 (E12.5); 2 wild type, Mar24D (♀ cre+) and Mar27I (♂ cre-), 1 heterozygote, Mar1H (♀ cre-), and three knockouts, Mar24B/C and Mar27J (all ♂ cre+). For E14.5 we generated three cultures; 1 wild type, Nov4C (♂ cre+), 1 heterozygote, Nov4B (♂ cre-), and 1 knockout, Nov4D (♂ cre+) (Figure 14 for representative results).

3.4 Characterization of growth and differentiation properties of Snf2l knockout neurosphere cultures

3.4.1 Snf2l knockout in neurosphere culture mimics results in SH-SY5Y cells

The first study we decided to conduct on our neurosphere cultures was a BrdU incorporation assay similar to the study completed with our neuroblastoma cell lines. Initially, we allowed the cultures to proliferate in standard neurosphere proliferation media. Once a sufficient population density was reached cultures were seeded into 8-well culture slides and allowed to acclimatize for 12 hours. After this time a differentiation time course was set up with an 8 hour BrdU pulse before harvesting cells at 1, 2, 3, and 4 differentiation days. The media was then switched to differentiation media supplemented with 100mM BrdU and was left for an 8 hour pulse after which they were fixed and processes as described

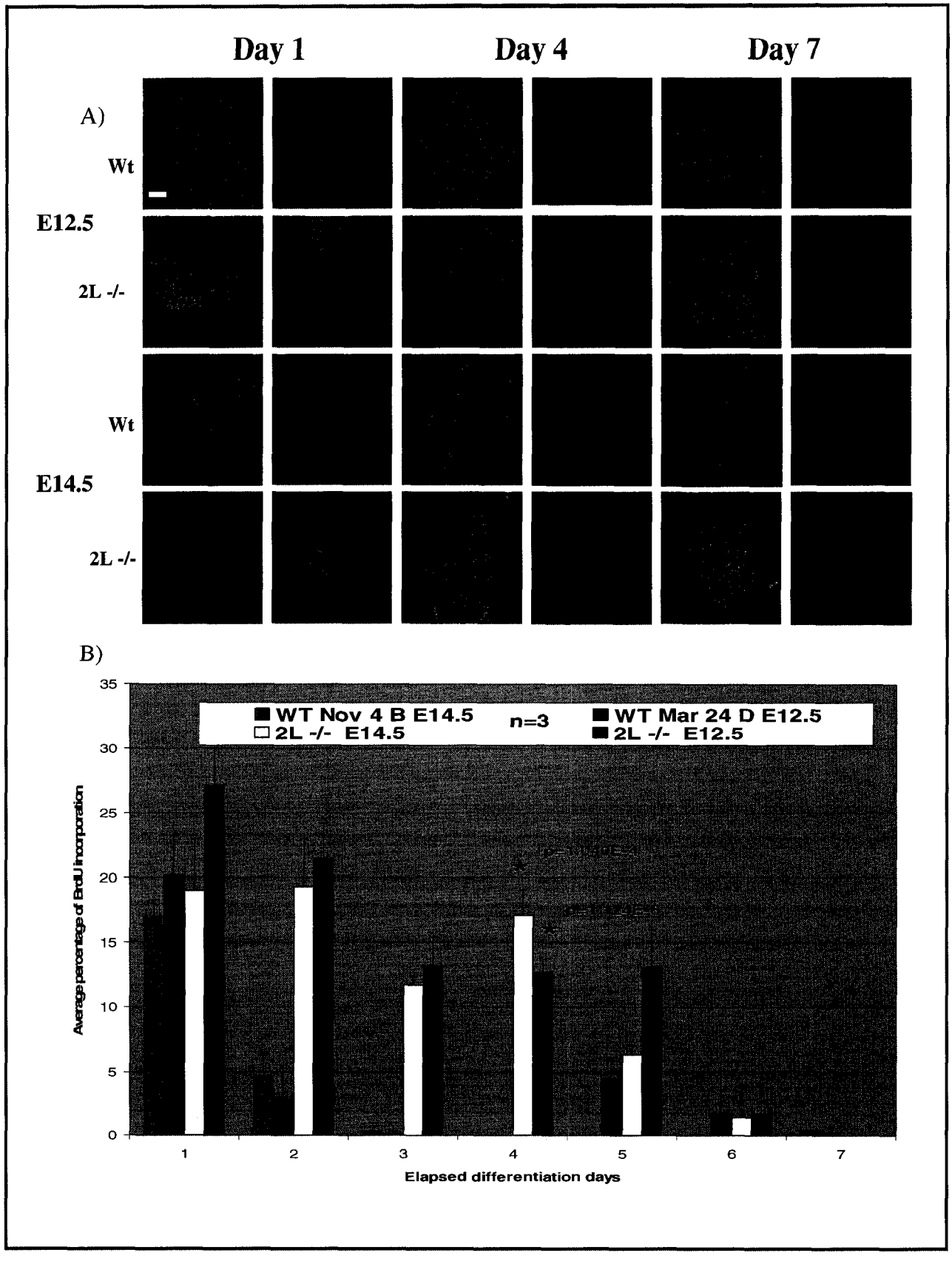
FIGURE 14: REPRESENTATIVE GENOTYPING ANALYSIS OF PRIMARY NEUROSPHERE CULTURES. Neurosphere genotyping for embryo cultures derived from pregnant mares of Snf2Lf/f;Gata1Cre-/- background crossed with males of Snf2L+/y;Gata1Cre+/- background. Snf2l primers recognize the wild tyoe allele, 508 basepairs, and the exon 6 deleted allele, 438 basepairs. The Sry primers amplify a portion of the SRY gene of 300 basepairs in size and is used to identify males whereas the Fabpi primers amplify a portion of the Fabpi gene 200 basepairs in size and are used as a positive reaction control due to expression in every murine cell. Cre primers amplify a portion of the Cre recombinase gene 200bp in size. A is Wt female, B is Wt cre+ male, C is Het female cre+ and D is knockout male cre+.


A
B
C
D
Snf2L
Cre
Sry/Fabp1

in section 2.2.8. Results here demonstrate continued proliferation under forced differentiation conditions, similar to what we found in our neuroblastoma cultures (Figure 10). Figure 15 A are pictures from the fluorescent antibody staining and show that a large number of BrdU positive cells were present after 4 days of differentiation. Since we know that Snf2l knockout in these mice is non lethal and that during our neuroblastoma cellular proliferation assay the fold increase began to drop back to control levels after 4 days, it was our estimation that this continued proliferation would only be transient in nature. As a result of this we decided to allow our cultures to differentiate up to 7 days. This resulted in a reduction in the BrdU incorporation returning to control levels.

Figure 15 B displays the quantification of the total BrdU incorporation as a percentage of DAPI stained cells. At 4 days of differentiation there was no BrdU incorporation observed in the control cultures but in E12.5 and E14.5 Snf2l knockouts it was found that 12.6% ($p=1.1x10^{-6}$) and 17.1% ($p=1.0x10^{-4}$) of cells continued to incorporate BrdU respectively. These results indicate that in neurosphere cultures the removal of Snf2l results in continued proliferation at 4 days of differentiation but that this condition is indeed only transient and resolves itself by day 7 of differentiation. As a result of this it is possible that during *in vivo* situations there will be at least 1 or possible 2 additional rounds of cellular proliferation in the developing cortex that may produce an increase in brain size as documented in Rb-/- in the telencephalon (82). It is important to realize that neurosphere cultures are a heterogeneous population of neurons, astrocytes and all other cells that make up the developing neocortex. This means that further study will be required to determine if the changes found here are specific to only a certain population or are a broad spectrum

FIGURE 15: CONTINUED PROLIFERATION IN SNF2L KNOCKOUT CELL POPULATIONS EXTENDS INTO NEUROSPHERE CULTURES. For E12.5 and E14.5 cultures one wild type (Wt) control was used along with 1 knockout for E14.5 and 3 for E12.5. 100mM BrdU pulse labeling time is 8 hours. A) Microscope pictures at 20x magnification showing DAPI counterstain (blue) and BrdU labeled cells after 1, 4 and 7 differentiation days. B) Quantification of the percentage of BrdU positive cells (green) at 24 hour intervals up to 7 differentiation days. Scale bar is 56μm.


Day 1
Day 4
Day 7
A)
Wt
E12.5
2L -/-
Wt
E14.5
2L -/-
B)
WT Nov 4 B E14.5
2L -/- E14.5
n=3
WT Mar 24 D E12.5
2L -/- E12.5
Average percentage of BrdU incorporation
0
5
10
15
20
25
30
35
1
2
3
4
5
6
7
Elapsed differentiation days

result. We have now determined that there is an increase in the period of proliferation during forced differentiation but have not examined the proliferation rate of the cultures as yet.

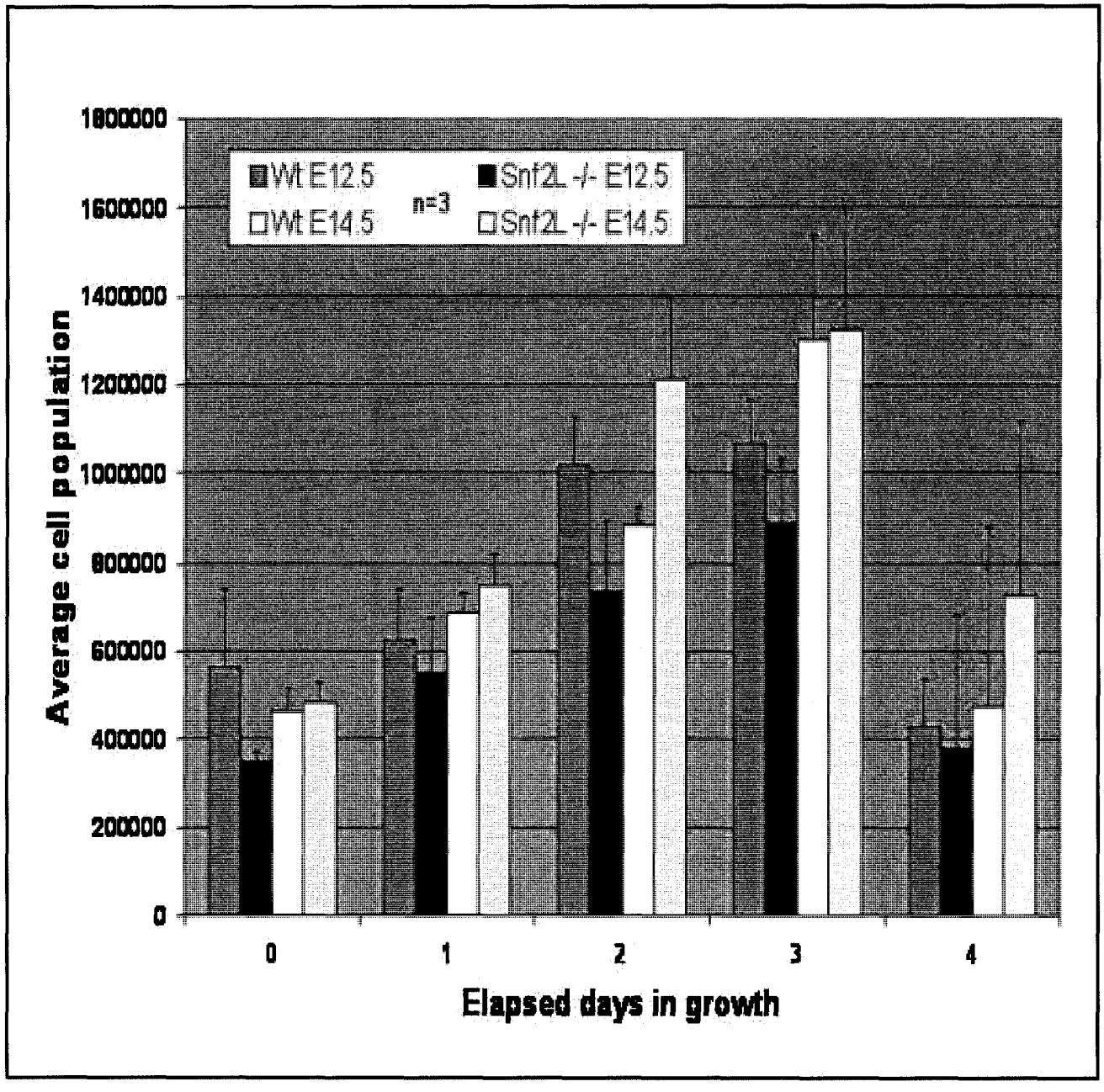
3.4.2 Proliferation rate remains unaffected in Snf2l knockout neurospheres

We have established that neurospheres knocked out for Snf2l continue to proliferate and delay proliferation, but the question remains whether the rate of proliferation of the cultures is altered. In order to examine the proliferation rate of the neurosphere cultures we conducted a proliferation assay as described in section 2.2.11. These results demonstrated that there were no significant difference between control cultures and experimental populations (Figure 16). Since all cultures were maintained in proliferation media at all times, this indicates that there is no difference in the rate that the cells are replicating. This result suggests that the cell cycle has not been shortened in our experimental cells but that there is an inability of these cells to exit the cell cycle efficiently. This experiment resulted in a wide degree of variability which is likely a result of the use of different neurosphere cultures obtained from different animals.

3.4.3 Fewer neurons are generated in Snf2l knockout neurosphere cultures

To determine if there are any quantifiable differences in the generation of neurons when neurosphere cultures are induced to differentiate we determined the fraction of neurons within the culture by staining with a Tuj1 antibody. At 24 hour intervals for 7 days cells were fixed and processed for Tuj1 antibody labeling as described in section 2.2.12. The neuronal population, Tuj1 positive, was plotted as a percentage of the total number of cells within the sphere as determined by DAPI counter staining. After 1 day of differentiation of

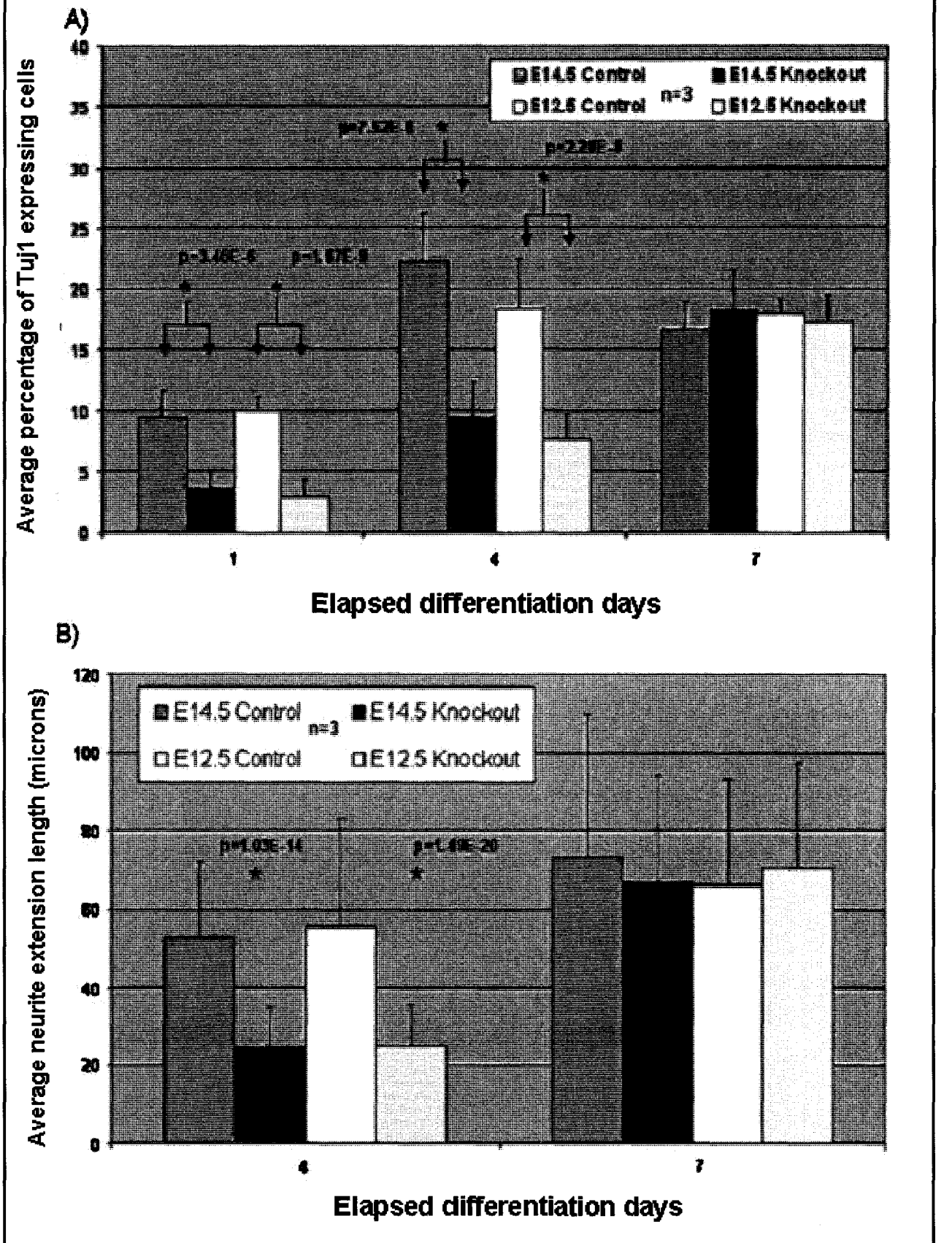
FIGURE 16: SNF2L KNOCKOUT NEUROSPHERE CULTURES PROLIFERATE AT SIMILAR RATE TO CONTROLS. Cellular proliferation under growth conditions remains unchanged in Snf2L knockout neurosphere cultures (red bar E12.5, light blue bars E14.5) as compared to control populations (navy blue E12.5, yellow E14.5). Cells were seeded into culture flasks and at 24 hour intervals were dissociated and analyzed using a Coulter Counter.


1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
Average cell population
Wt E12.5
Wt E14.5
n=3
Snf2L -/- E12.5
Snf2L -/- E14.5
0
1
2
3
4
Elapsed days in growth

the E14.5 neurospheres there is a distinct decrease in the number of Tuj1 positive cells compared to control samples, 3.6% versus 9.4% respectively, creating an average loss of 5.8% (Figure 17 A). In E12.5 knockout neurosphere cultures at this time interval there is also fewer Tuj1 positive cells (a drop of 7.135%) between control and knockout populations (10% versus 2.8% respectively). By Day 4 of differentiation the differences become even more evident as there are 12.9% and 10.7% fewer Tuj1 positive cells in E14.5 and E12.5 proliferation media at all times, this indicates that there is no difference in the rate that the cells are replicating. This result suggests that the cell cycle has not been shortened in our experimental cells but that there is an inability of these cells to exit the cell cycle efficiently. knockout populations respectively. Student's T-tests were calculated for all of these results and all have proven statistically significant (Figure 17 A). These results demonstrate that the continued proliferation found during all BrdU incorporation experiments has delayed the development of the total neuronal population. Our previous results have indicated that this delay to exit the cell cycle is a transient condition and was resolved by 7 days of differentiation. As a result, these cultures were allowed to differentiate up to the 7 day time point. This resulted in the neuronal populations between controls and knockouts reaching equivalent levels.

To further quantify the delay in neuronal differentiation we examined whether neurite extension was affected. The longest neurite extension was measured in 50 different neurons from control and Snf21 knockout cultures. The average extension length as a total population at 4 and 7 days of differentiation are displayed in Figure 17 B. For E14.5 control neurons after 4 days of differentiation there is an average extension length of 52.9μm while knockout

FIGURE 17: FEWER NEURONS ARE GENERATED AND CONTAIN SHORTENED NEURITE EXTENSIONS FROM SNF2L KNOCKOUT NEUROSPHERE CULTURES. Snf2L knockdown (red bars E14.5, light blue bars E12.5) results in reduced neuronal population with delayed neurite extension formation at 4 days of differentiation but recovers by 7 days when compared to control populations (navy blue bars E14.5, yellow bars E12.5). A) Quantification of immunocytochemistry where Tuj1 population was compared against DAPI counterstaining. E12.5 knockout results are averages taken from 3 distinct cultures and compared against 2 distinct control cultures. E14.5 results are represented by 1 culture each for control and knockout. B) Quantification and measurement of neuronal extensions in the total population. Results here utilized the same cultures as for graph A, notice the reduction by approximately half in neurite extension length at 4 differentiation days with tighter standard deviation bars in Snf2l knockout cultures.

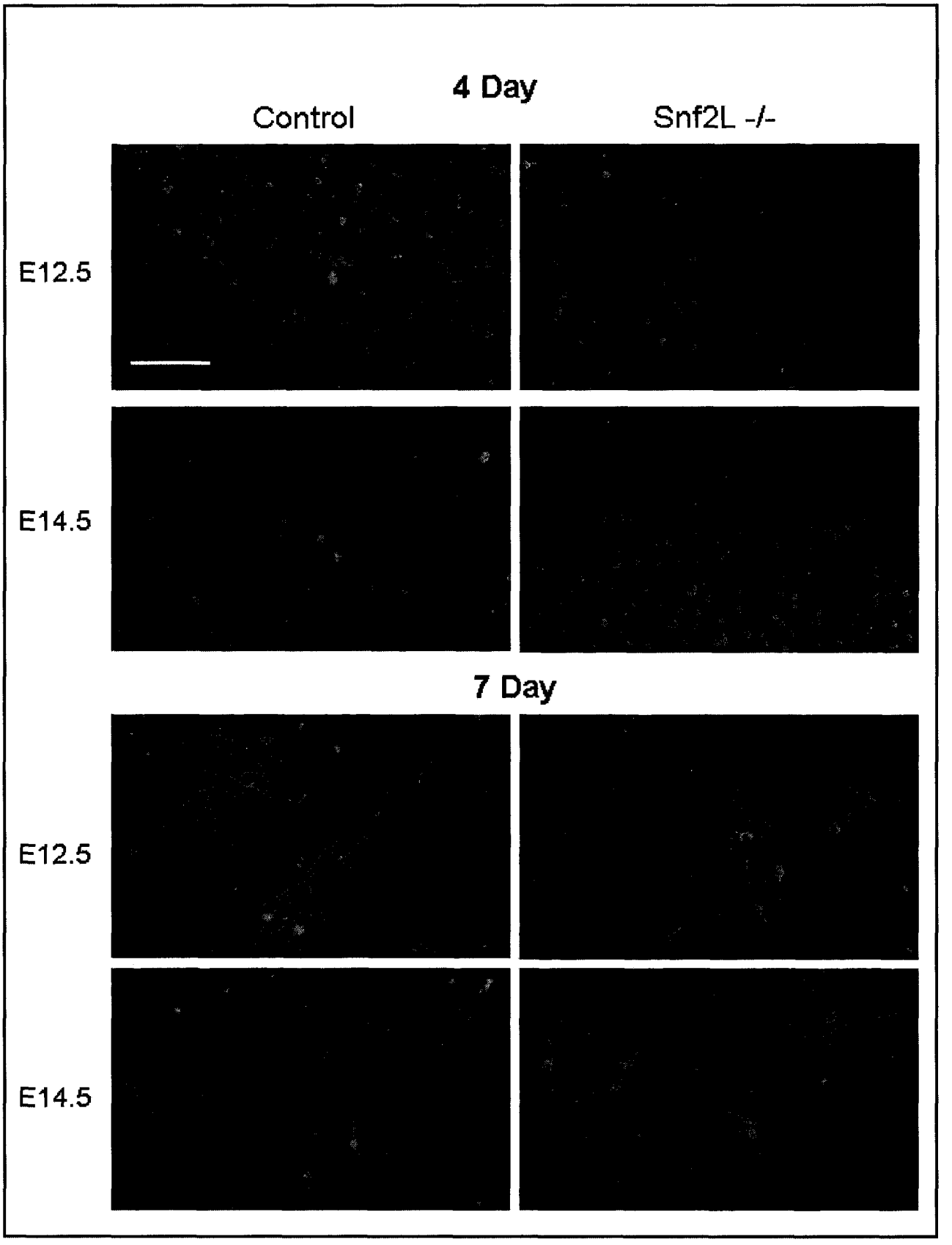

A)
Average percentage of Tuj1 expressing cells
E14.5 Control
E14.5 Knockout
E12.5 Control
n=3
E12.5 Knockout
p=3.48E-5
p=1.67E-5
p=2.28E-8
1
4
7
Elapsed differentiation days
B)
Average neurite extension length (microns)
E14.5 Control
n=3
E14.5 Knockout
E12.5 Control
E12.5 Knockout
p=1.03E-14
p=1.48E-20
4
7
Elapsed differentiation days

cells only have an average of 24.8 μm. In E12.5 control cells there is an average length of 55.4 μm while in knockout neurons the average is 25.3 μm. When a Student's T-test was used to analyze these results it was found that the p values for both the E14.5 and E12.5 cultures were statistically significant with $1.03x10^{-14}$ and $1.49x10^{-20}$ respectively.

These cultures were allowed to continue differentiating and were re-examined at 7 days whereby the neurons in the knockout populations had equivalent neurite extension length compared to wild type neurons. Pictures of our immunocytochemical microscopic investigations may be found in Figure 18. The morphology of the Tuj1 positive neurons in the knockout cultures is identical to those from the control populations and both have long, multi-branching processes. This indicated that while there is a developmental delay in their production there does not appear to be any overt morphological effect once the Snf2l knockout neurons have reached maturity.

More in depth examination of this delay in neurite extension is shown in Figure 19, for E12.5, and Figure 20, for E14.5. There is a much higher population of neurons having neurite extensions shorter than 30 μm for E12.5 and less than 40 μm for E14.5 knockout cultures after 4 days of differentiation. Control populations at this time point have a much broader spectrum of neurite extension lengths with no definitive population being more dominant than the rest. This further illustrates that neurons derived from the Snf2l knockout animals are delayed in the formation of neurite extensions during this transient cell cycle disruption. By 7 days of differentiation the distribution of both the E12.5 and E14.5 neurons from Snf2l knockout animals has achieved a comparable state as the control neurons.

FIGURE 18: NEURITE EXTENSIONS ARE DELAYED IN SNF2L KNOCKOUT NEUROSPHERE CULTURES AFTER 4 DAYS OF DIFFERENTIATION BUT RECOVER BY 7 DAYS. Cells were fluorescently tagged using a Tuj1 antibody which targets β-III-tubulin (red and green) and total population numbers were counted against DAPI (blue) counterstain. Detailed counts were taken and demonstrated that the total population is also reduced at 4 days and shows the same ability to recover by 7 days. These micrographs are representative of all study processes and detail the distinctly shortened and disorganized neurite extensions in Snf2l knockouts after 4 differentiation days in both E12.5 and E14.5 cultures compared to the long highly branched control counterparts. After 7 days of differentiation Snf2l knockout neurons have neuronal processes which are much more representative in both total number and extension length as their control counterparts. Scale bar is 56 μm in length.

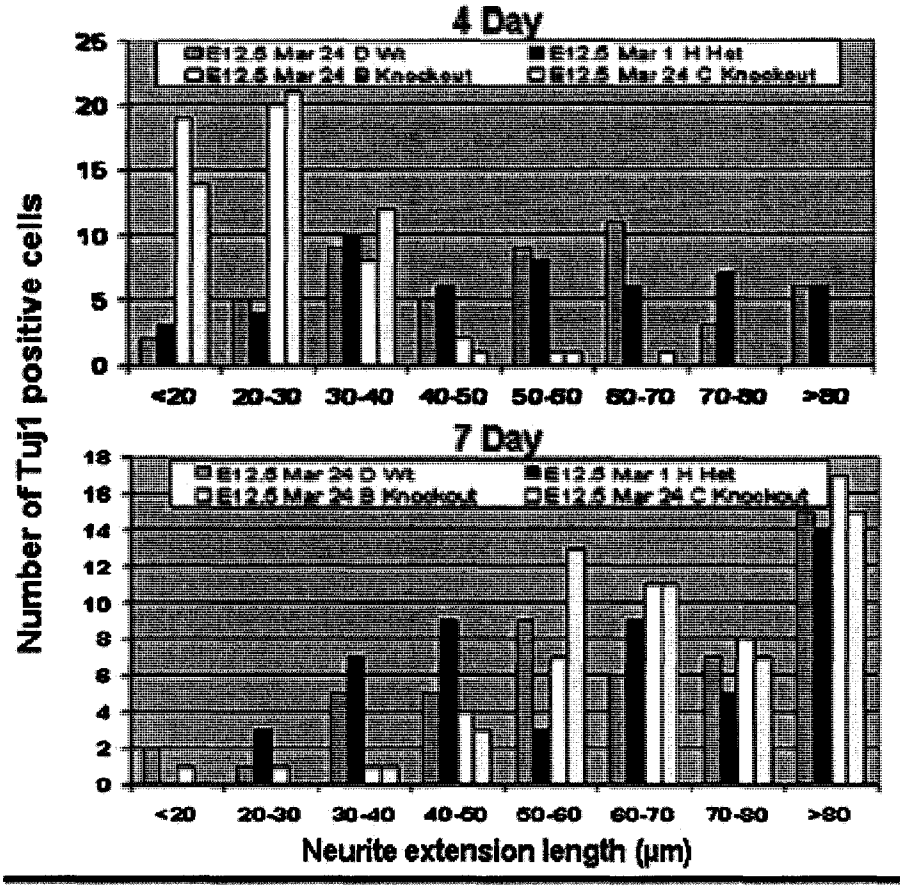

4 Day
Control
Snf2L -/-
E12.5
E14.5
7 Day
E12.5
E14.5

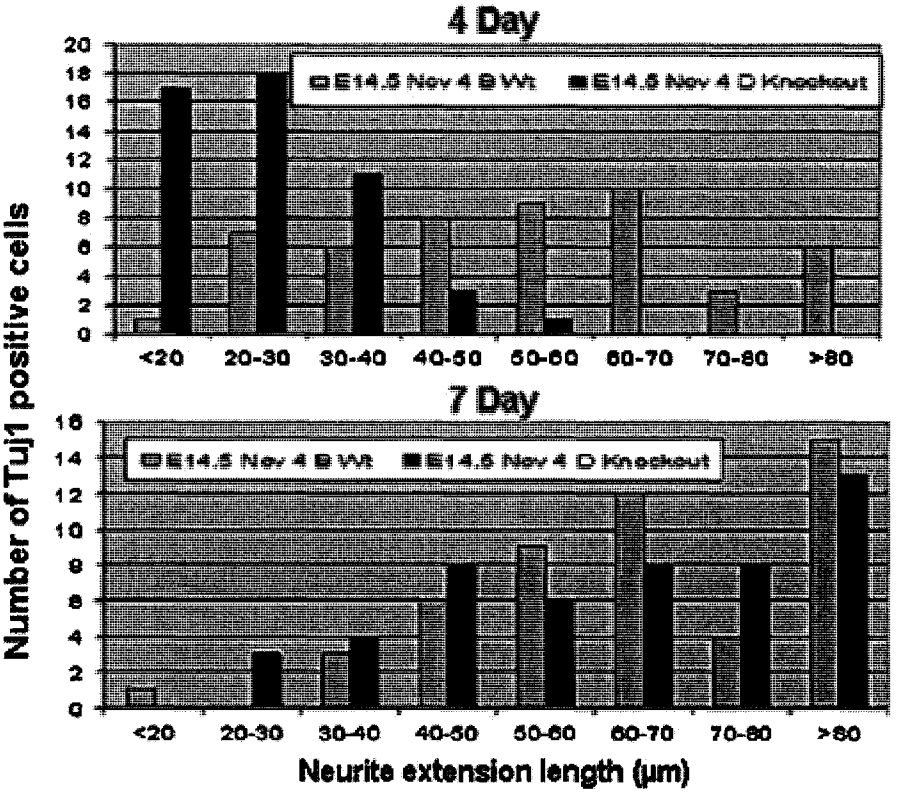
3.4.4 Snf2l knockout neurosphere cultures display reduced astrocyte numbers and delayed differentiation ability

As mentioned briefly earlier primary cortical neurosphere cultures contain a mixed population of all the cells making up the neocortex. In order to determine if the altered proliferation and development of neurons will extend into any other cortical cell type we decided to first investigate the astrocyte population as they account for approximately 80% of the cellular mass in the cortex (83, 84). We utilized a similar approach to our neuronal analysis with antibodies against the astrocytic marker GFAP. Since we generated our E14.5 cultures first we began our analysis with these cultures (Figure 21 B). The total astrocyte population was quantified against a DAPI counter stain and during early stages of culture life, P3, resulted in an average reduction of 58.8% in the astrocyte population of Snf2l knockout cultures compared to controls at 4 days of differentiation. This trend mimics that found for the neuronal population and we therefore examined the morphological characteristics of these astrocytes to see if the delayed development was also found. We concluded that the developmental inhibition had been extended into the astrocyte population as examined by fluorescent microscopy (data not shown). We then examined E12.5 neurosphere cultures for GFAP positive cells and found that they resulted in a population reduction of 34.1% (Figure 21 C). Figure 21A demonstrates that the astrocytes generated from Snf2l knockout neurosphere populations at 4 days of differentiation resemble control cells at 1 day of differentiation. When all Snf2l knockout cultures were allowed to differentiate to the 7 day time point the astrocytes were found to have a similar morphological appearance and total GFAP positive cell number to the controls.

FIGURE 19: DISTRIBUTION OF NEURITE EXTENSION LENGTHS IN DIFFERENTIATING CONTROL AND SNF2L KNOCKOUT NEUROSPHERE CULTURES FROM E12.5. Neurite extensions from 50 E12.5 Snf2L knockout neurons (yellow and light blue bars) were measured and have shortened length at 4 days of differentiation (upper panel) but recover by 7 days (lower panel) when compared to their control counterparts (navy blue and red bars). Notice the knockout extension lengths are shifted towards the left (shorter) end of the spectrum at 4 days which returns to control levels by 7 days.

FIGURE 20: DISTRIBUTION OF NEURITE EXTENSION LENGTHS IN DIFFERENTIATING CONTROL AND SNF2L KNOCKOUT NEUROSPHERE CULTURES FROM E14.5. Neurite extensions from 50 E14.5 Snf2L knockout neurons (red bars) were measured and have shortened length at 4 days of differentiation (upper panel) but recover by 7 days (lower panel) when compared to their control counterparts (navy blue bars). Notice the knockout extension lengths are shifted towards the left (shorter) end of the spectrum at 4 days which returns to control levels by 7 days.


4 Day
E12.5 Mar 24 D Wt
E12.5 Mar 1 H Het
E12.5 Mar 24 B Knockout
E12.5 Mar 24 C Knockout
Number of Tuj1 positive cells
<20
20-30
30-40
40-50
50-60
60-70
70-80
>80
7 Day
Neurite extension length (µm)


4 Day
E14.5 Nov 4 B Wt
E14.5 Nov 4 D Knockout
Number of Tuj1 positive cells
<20
20-30
30-40
40-50
50-60
60-70
70-80
>80
7 Day
Neurite extension length (µm)

3.4.5 Snf2l knockout neurosphere cultures maintain nestin expression

To determine if the cells that are developmentally delayed remain as progenitors we stained the neurospheres for nestin, an intermediate filament protein restricted to progenitor cells. When the total population expressing nestin was quantified against the DAPI counter stain after 1 day of differentiation the populations were virtually the same (Figure 22 B). When the differentiation time course was extended to 4 days a definitive difference was detected. The E14.5 cultures expressed an 11.1% increase in the number of nestin positive cells while the E12.5 cultures had a 27.1% increase in nestin staining. When Student's T-tests were calculated they showed that both E14.5 and E12.5 results were significantly different with p values of $5.1x10^{-4}$ and $9.3x10^{-5}$ respectively. Representative pictures of these results are displayed in Figure 22 A. When these cultures are differentiated up to the 7 day time point the nestin expression returns to control levels.

3.5 Snf2l knockout results in specific gene changes in cortical neurospheres

All of the results discussed above for our Snf2l knockout neurospheres and neuroblastoma cells have indicated that there are likely disruptions to the genes controlling cell cycle exit. As a result, we decided to examine the RNA expression profiles of cell cycle genes in a qPCR array. The array that was chosen was the Cell Cycle RT^2 Profiler PCR Array from SuperArray. This array contains 96 primers that are all designed for uniform amplification and all of the appropriate controls. At the start of this study two knockout cultures, Mar24 B and C, were grown in standard proliferation media along with two control cultures, Mar24 D and Mar27 I. Once these populations had reached approximately 80% confluence the media was changed to differentiation media and they were allowed to

FIGURE 21: ASTROCYTES IN SNF2L KNOCKOUT NEUROSPHERE CULTURES HAVE DELAYED DIFFERENTIATION AS FOUND IN NEURONS. A) Immunocytochemical micrographs of GFAP positive cells. Differences in morphological characteristics are found in both E12.5 and E14.5 cultures (these pictures are from P9 cultures). 1 day pictures for controls have been included for comparison with 4 day knockout cultures B) Early neurosphere cultures show large difference in total astrocyte population as compared to controls. C) Cultures from later in culture life no longer result in decreased astrocyte population in E14.5 cultures but do in E12.5 ones. Scale bar is 18 μm.

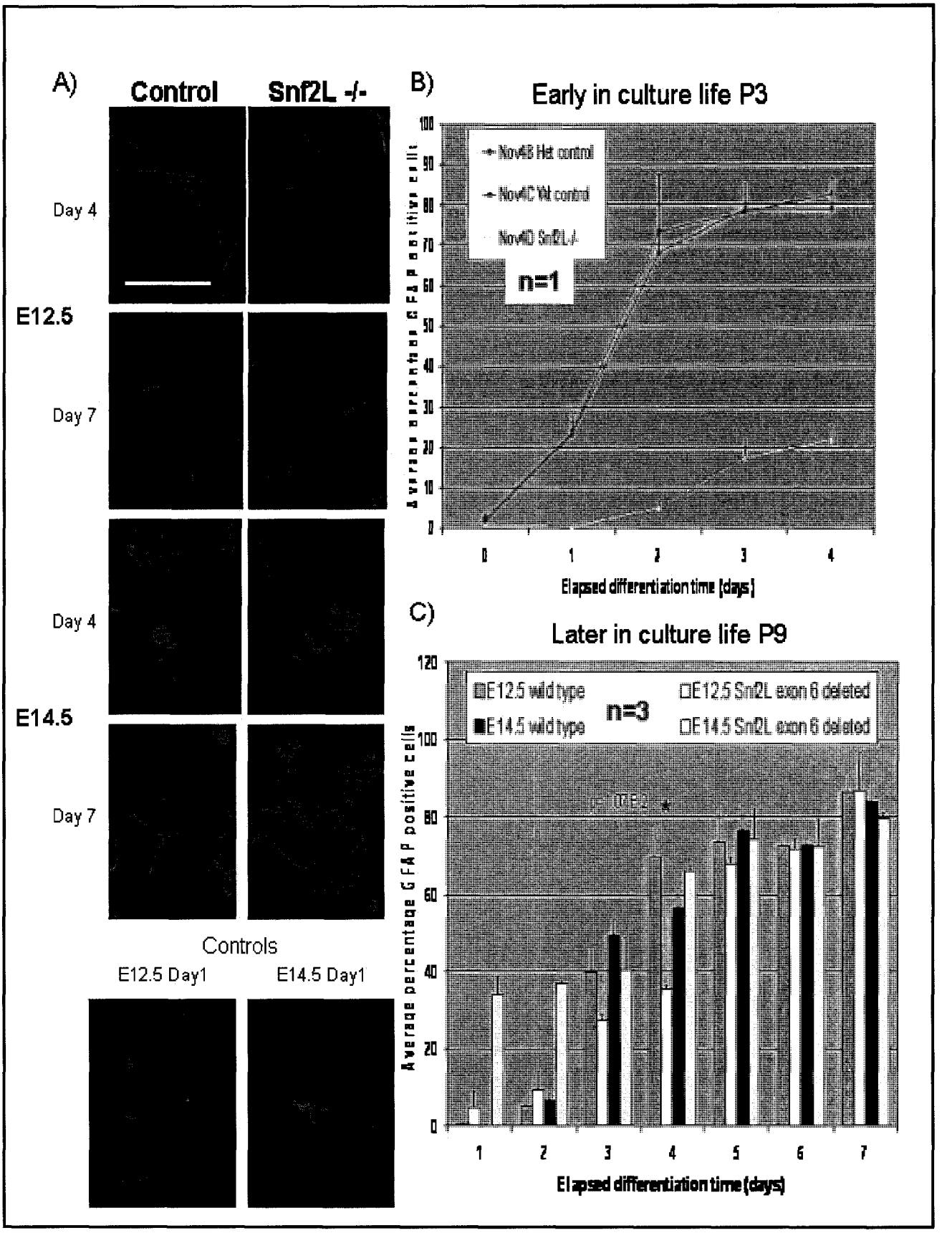
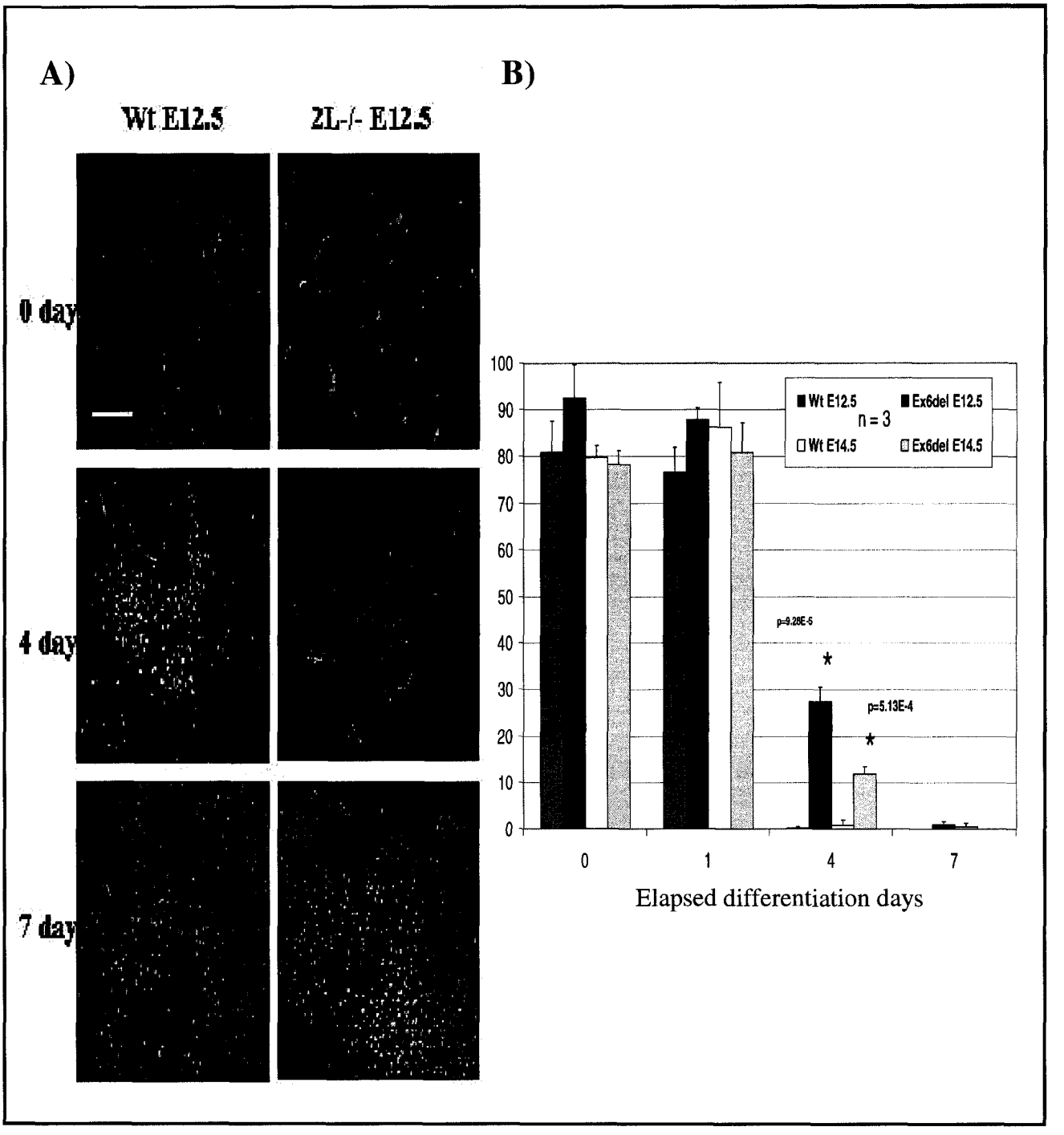

A)
Control
Snf2L -/-
Day 4
E12.5
Day 7
Day 4
E14.5
Day 7
Controls
E12.5 Day1
E14.5 Day1
B)
Early in culture life P3
n=1
Elapsed differentiation time (days)
C)
Later in culture life P9
E12.5 wild type
E14.5 wild type
n=3
E12.5 Snf2L exon 6 deleted
E14.5 Snf2L exon 6 deleted
Elapsed differentiation time (days)

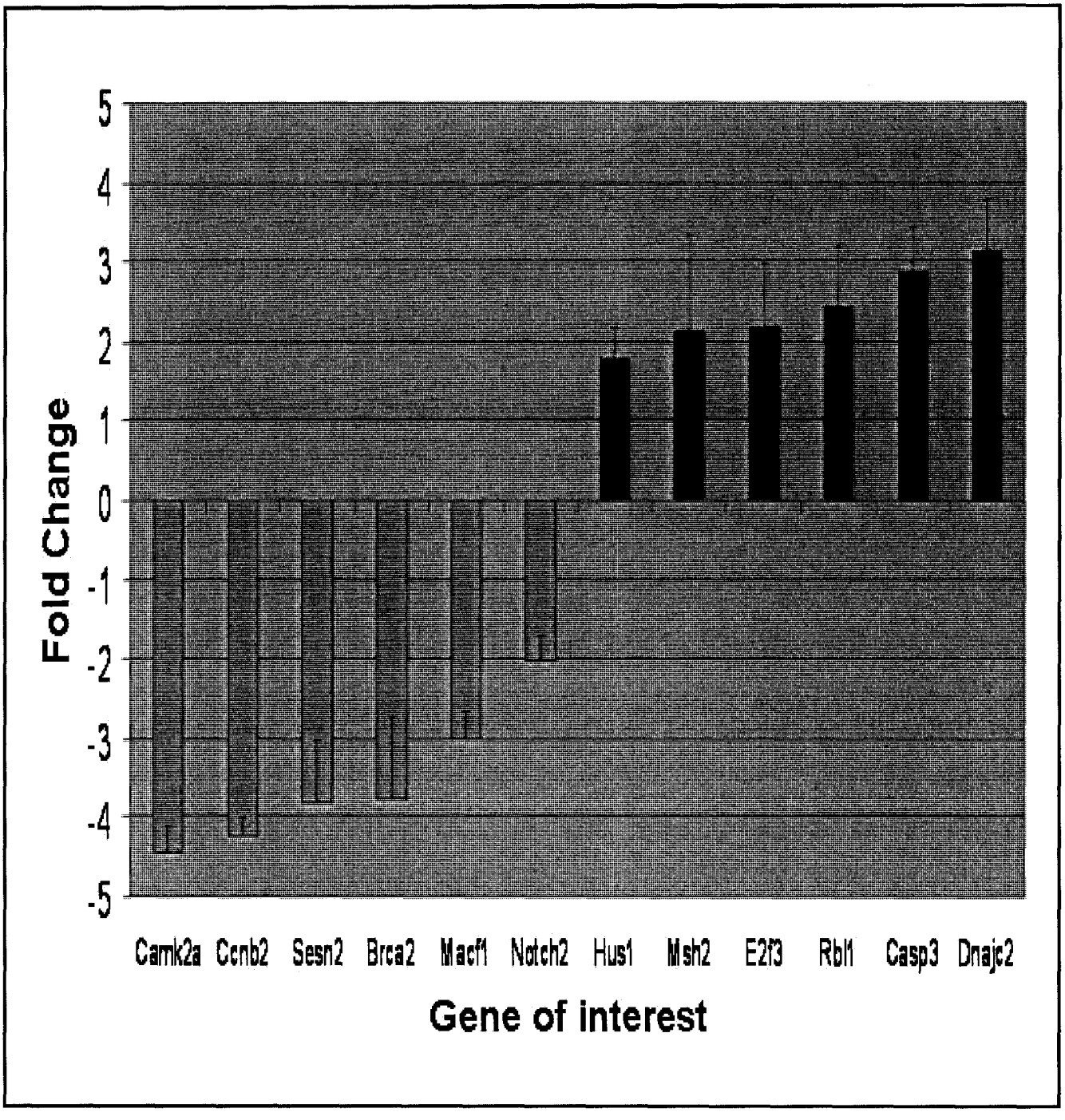
FIGURE 22: NESTIN LABELLING REMAINS IN SNF2L KNOCKOUT NEUROSPHERE CULTURES AFTER 4 DAYS OF FORCED DIFFERENTIATION BUT DISAPPEARS BY 7 DAYS. A) Neurosphere cultures were seeded onto glass slides and forced to differentiate and fixed at 24 hour intervals up to 7 days after which they were laballed using a primary nestin antibody with a 594 secondary (red). Scale bar is 56μm in length. B) The total number of nestin positive cells was quantified against DAPI counterstain (blue) in order to determine the nestin positive population percentage.


A)
Wt E12.5
ZL-/- E12.5
0 day
4 day
7 day
B)
Wt E12.5
Ex6del E12.5
n = 3
Wt E14.5
Ex6del E14.5
p=9.28E-5
p=5.13E-4
100
90
80
70
60
50
40
30
20
10
0
0
1
4
7
Elapsed differentiation days

differentiate for 4 days, as this is the time point where the greatest changes had been observed. RNA was isolated in triplicate and used for quantitative RT-PCR as described in section 2.2.7. Once the results were obtained they were run through the ΔΔCT method in the online template file. It was found that there were 6 genes down regulated and 6 genes with increased expression with statistical significance, >2 fold change and p-value <0.05 (Figure 23). Genes down regulated included: Camk2a, Ccnb2, Sesn2, Brca2, Macf1, and Notch2.

We also found that 6 genes were significantly up regulated which were Hus1, Msh2, E2F3, Rbl1, Casp3, and Dnajc2. Further examination of these genes will need to be conducted in order to determine if the up or down regulation found here results in a change in the protein expression level.

FIGURE 23: QUANTITATIVE RT-PCR EVIDENCE DEMONSTRATES DISTINCT CHANGES IN 12 DIFFERENT CELL-CYCLE RELATED GENES. Results here are the average fold change of 3 separate trials. We found that 12 of the 84 cell cycle genes in the qPCR array were either downregulated (navy blue bars) or upregulated (red bars) by 2 fold with p-values of less than 0.05 indicating statistical


Fold Change
5
4
3
2
1
0
-1
-2
-3
-4
-5
Camk2a
Ccnb2
Sesn2
Brca2
Macf1
Notch2
Hus1
Msh2
E2f3
Rbl1
Casp3
Dnajc2
Gene of interest

4.0 Discussion

From their initial discovery in *Drosophila* extracts it was recognized that the ISWI proteins functioned as distinct chromatin remodeling ATPases contained in multi-subunit complexes. The involvement of chromatin remodeling complexes in the development of X-linked mental retardation is rapidly gaining focus in primary research today. Various mutations to chromatin remodeling genes such as *ATRX*, *MeCP2* and *RSK2* result in severe developmental retardation conditions (5, 18). With approximately three percent of the global population affected to varying degrees by mental retardation there is a growing strain on federal agencies for both primary care and the development of effective treatment options. As a result of this we initially decided to examine the role that the *ISWI* genes played during the differentiation of the human SH-SY5Y neuroblastoma cell line. The primary goal of this research was to determine the contribution of the *ISWI* genes during mammalian neuronal differentiation with the ultimate goal of gaining insight into whether *ISWI* would be a candidate gene for human brain developmental disorders. While there is a growing amount of information on the mechanism underlying the remodeling activity for the *ISWI* genes their exact contribution to the development of a neuron remains lacking.

4.1 Snf2l knockout results in delayed neural cell differentiation

In our examination of the proliferation of human neuroblastoma cells knocked out for the chromatin remodeling gene *SNF2L* we found they had a delay in cell cycle exit. This was examined using BrdU incorporation assays and differentiation experiments. In the BrdU incorporation experiments we demonstrated that the *SNF2L* knockout cultures had a similar number of cells in S-phase during proliferation and out to 2 days of differentiation but that by 3 and 4 differentiation days there was a significantly higher percentage of cells in S-

phase. This finding was confirmed by using forced differentiation studies and directly counting the cell population using a haemocytometer. Results here showed that there was indeed an increased population in *SNF2L* knockouts with a 2.08 fold increase at 3 days of differentiation while controls had only a 1.1 fold increase. By day 4 of differentiation the fold increase had dropped but was still significantly higher at 1.36 fold increase while controls showed no enlargement in population. When we changed our model system to primary neurospheres from conditional *Snf2l* knockout mice we discovered a similar delay in cell cycle exit. The relevance of neurosphere cultures for developmental neurobiology was first recognized over ten years ago and has since been utilized in many different neurological applications with three main goals in mind; firstly it is useful as a model of the neural stem cell population, secondly as a model of regional brain development, and thirdly as a potential process for cell proliferation for use in transplantation (85, 86). Our BrdU examinations here demonstrated that there was indeed a significantly higher S-phase population in our *Snf2l* knockout cultures by day 2 of differentiation. It was found that the increased S-phase percentage continued until approximately day 5 of differentiation but by days 6 and 7 there were no significant differences. This suggests that the condition is only transient in nature as there are no differences found in our day 7 time point. This fits well with the phenotype of the knockout *Snf2l* mice as they have larger brains with more cells but do not suffer from exencephaly as would be expected if the cells failed to exit the cell cycle completely. We next examined the proliferation rate when cells were maintained in proliferation media and discovered that there is no significant difference between controls and experimental populations with regards to the speed of cell cycle progression.

This delay in cell cycle exit also resulted in neuronal cells that had much shortened neurite extensions and decreased their population percentage by approximately half at day 4

of differentiation. Interestingly this reduced population and delayed development was also found in the astrocytes of Snf2l knockout neurospheres indicating that Snf2l activity is important in glial cell populations as well as neuronal. Astrocytes differentiated for 4 days more closely resembled those at day 1 of differentiation from littermate control cultures. In all cases for neuronal or glial cells when the cultures were allowed to continue differentiating for 7 days the morphology and population percentage returned to control levels again indicating this condition is only transient in nature. This suggests that Snf2l is only required in a limited window during the development of these neural cell types and also indicates that there may be a compensatory mechanism that is capable of carrying out Snf2l's function in its absence. Unpublished data in our laboratory demonstrates that in functionally ablated Snf2l mice both of these cell phenotypes function normally as these mice do not show any overt behavioral phenotypes as a result of Snf2l loss.

We next examined the expression of an intermediate filament protein that is used for the identification of neural progenitor populations and is expressed in all neural precursor cells which is nestin (87). Nestin is expressed ubiquitously throughout embryogenesis and as progression proceeds towards adulthood it becomes restricted to regenerating cellular populations (88). A recent study has also proved links between the ability of neural cells to differentiate and early nestin expression. Typically in neurosphere cultures nestin expression will gradually be reduced as differentiation proceeds until it is no longer detectable. Our experimental results correlated well with these previous findings and nestin was not detectable in control populations by 4 days of forced differentiation. Our *Snf2l* knockout populations on the other hand resulted in a significantly higher percentage of cells continuing to express nestin after 4 differentiation days in both E14.5 and E12.5 populations, ~12% and ~27% respectively. This strongly suggests that the removal of *Snf2l* has caused these cells to

be held in a precursor state for an extended period. All of these results show that there are distinct disruptions to the ability of the cells to exit the cell cycle and as such there will likely be changes in regulatory gene expression because Snf2l cannot repress or activate them properly. The ability of these cells to recover is similar to that seen in experimental procedures on microtubule-associated protein 1B (MAP1B). MAP1B is part of the neuronal cytoskeleton and initially was believed to be essential for proper development as disruption caused death at E8 in mouse models (89). Recent gene targeting studies demonstrated that knockout actually results in a reduced brain weight and underdeveloped brain that is able to recover with progressing development and this ability to recover is similar to our Snf2l knockout situation after 7 days of differentiation (90).

Transcripts of Snf2l are expressed in a regionally specific manner in the developing brain in cellular populations that are differentiating (43). In the cerebellum increased Snf2l levels are found in the Purkinje neurons especially during the first 15 days of postnatal development. This time period corresponds to the time when cells are exiting the cell cycle and there is a drastic increase in neurite formation and branching (62). In the brain there are three Snf2l transcripts of 3.7, 4 and 5 kb in size with the 3.7kb transcript being the most abundantly expressed in the cortex and cerebellum. All of these splice variants are expressed as early as E9.5 at low levels but at E11.5 there is an upregulation of the 4kb transcript in the brain. During later postnatal stages, P15-P18, there is again a switch where the 3.7kb variant becomes the dominant species. Therefore the brain specific 3.7kb species has been shown to be strongly upregulated during the times when neuronal cell populations are exiting the cell cycle and progressing towards terminal differentiation (43). All of the experimentation discussed so far demonstrates that when the Snf2l gene is removed there is a delay in the cell's ability to differentiate. Interestingly the capability of the cell to differentiate is not

abolished which indicates that the activity of Snf2l is important but not essential in the developing frontal cortex for the processes of differentiation.

4.2 Regulation of brain size

In order for the brain to develop there is a requirement for regionally specific cues that will control the progression and exit of cells towards a predetermined fate. Chromatin remodeling is one of the critical processes overseeing the expression of various genes that are required for this developmental regulation (63, 65). Previous research has demonstrated that the remodeling activity of the *ISWI* genes on the promoters of *En1* and *En2* are essential for their expression. Other studies have shown that the engrailed genes are critical for cerebellar and midbrain development. It is also possible that both the *Snf2l* and *Snf2h* genes are required for the transcription of many cell cycle regulation genes as is suggested by their temporal and regional expression during brain development. The cell cycle is a complicated process during which regulatory genes are turned on in a cascade of events whereby cells develop in a concerted effort for organ development. There are several genes that are potential candidates for regulation by Snf2l. These are *p27^{kip1}*, *αE-Catenin, E2F3* isoform *a* and *b*, *p107, Shh*, and various Wnt proteins.

p27^{kip1} is one member of the cyclin-dependent kinase inhibitor family of proteins. This family is divided into the Cip/Kip family, which includes p21^{Cip1}, p27^{kip1}, and p57^{Kip2}, and the INK4 family which includes p15 ^{Ink4b}, p16^{Ink4c} and p19^{Ink4d9} (91). The cyclin-dependent kinase inhibitors work to promote cell cycle exit at the G1 stage during cell cycle progression. This is achieved via a relationship with the cyclin-dependent kinases where the inhibitors will block the kinases ability to bind to ATP therefore halting their catalytic activity. p27^{kip1} is the most abundantly expressed cyclin-dependent kinase inhibitor in the

cerebral cortex and when it is knocked out in mouse experiments it results in an increase in proliferation and therefore an enlarged brain develops (91, 92). The cortex from these mice is enlarged in the upper layers as a result of a decrease in early born neurons and a subsequent drastic increase in later born neuron production. There is also evidence suggesting that $p27^{kip1}$ plays roles in the determination of cell fate. It is not likely that this protein pathway is involved in the phenotype that we have found in our experimental cultures as we detected no misregulation in the RNA in our qPCR examination.

Another set of genes that are critical for the regulation of organ size, including brain, are the cell-cell adhesion molecules such as cadherins, β-catenins and α-catenins (93, 94). Of chief importance for progenitor cell regulation is αE-catenin which is a critical cellular adhesion molecule (94). During conditional knockout experiments in mice for this gene it was found that there was no significant disruption at E12.5 but by E13.5 there was a 40% increase in the total number of cells throughout the brain. These mice also had enlarged heads and shrunken body stature and died by 2 to 3 weeks after birth. Along with these mutations was a distinct dysplasia in the ventricular zone neuronal precursor cell morphology. These cells are typically bipolar with one projection extending down towards the ventricle and the other projecting up into the higher layers of the cortex. In the αE-catenin -/- animals these cells were nonpolarized with a rounded morphology that was loosely connected to adjacent cells (95, 96). While we have not yet examined the morphological characteristics of the ventricular precursor neurons in our *Snf2l* knockout mice there are morphological abnormalities in neurons from our neurosphere cultures suggesting that further work is needed to determine whether these genes are altered and whether *Snf2l* plays a role in their expression.

The E2F family of transcription factors are recognized as being some of the most critical components regulating many aspects of cellular proliferation and differentiation. Currently there are six family members that have been identified so far (97). While E2F's 2-5 are all strongly expressed in the embryonic brain E2F3 is likely the most possible target for ISWI mediated activity due to its direct association with the retinoblastoma proteins. The loss of E2F3 is associated with absolving the ectopic proliferation in the central nervous system of Rb-/- mouse models (Rb will be discussed in further sections). There are two different isoforms of the E2f3 protein; isoform a and b (82). Isoform a is directly involved in the enhancement of self renewal in the stem cell pool but has no effects on the progenitor population. Isoform b on the other hand is expressed at similar levels in both proliferating and quiescent populations and is implicated in the control of progenitor cell proliferation.

p107, or Rbl1, is one of the members of the Rb family of proteins which is made up of three members, Rb, p107 and p130 (98). p107 is typically only expressed in proliferating cells located around the ventricles (99). Each of these proteins has strong interactions with various members of the E2F protein family and for p107 its partner is mainly, though not exclusively, E2F4. Previous studies indicated that when null mutations are engineered into mice for p107 that p130 is able to compensate for its loss and these mice develop normally. Recent analysis has demonstrated that in these *p107*$^{-/-}$ mice there is an increase in the number of neurosphere producing cells and an enhanced ability for stem cell self renewal (100). These *p107* null mice also had an upregulation in *Notch-1* and when ChIP studies were conducted they demonstrated that p107 is a potential regulator of *Notch*-1 expression. We found both *p107* and *E2F3* were upregulated in our *Snf2l* knockout neurosphere cultures suggesting that *Snf2l* may negatively regulate stem cell self renewal or proliferation thereby promoting differentiation via this pathway.

Another gene that is critical for the growth of the brain is sonic hedgehog (*Shh*). Shh is a member of the Hedgehog family of secreted glycoproteins and is critical for the growth of many different organs and cell populations (101). This glycoprotein achieves its activity through the Patched 1 – Smoothened receptor complex and cells that respond to Shh have an increased level of the zinc-finger transcription factor Gli1. Shh production has been found to be essential for virtually all areas of brain development from the midbrain to the hindbrain and forebrain. Currently it remains unclear whether Shh is produced directly by the ventricular progenitor cells themselves in the developing neocortex or if they are responding to secreted Shh produced higher up in the cortical layers (layer V) by differentiated cells. Experimentation has determined that overexpressed Shh will increase the production of nestin expressing neocortical progenitors and reduced levels are associated with drastic reductions in progenitor populations (102). This fits nicely with our data where Snf2l removal resulted in an extended expression of nestin in neurosphere cultures. Other research has documented direct links between *Xenopus* ISWI and Shh expression (77, 103). In these studies when an anti-ISWI morpholino was used to inhibit ISWI production there was a drastic reduction in the level of Shh that was expressed resulting in a failure of gastrulation and neural tube closure. In *Xenopus* ISWI is expressed in four complexes three of which are homologous to those found in other species which are xACF, xCHRAC and xWICH all of which contain the Snf2h protein in mammals. Further studies will be required in order to determine if the Shh pathway is involved in our Snf2l knockout phenotype.

The final gene family of note is the Wnt family. The Wnts are a large and diverse family of cellular morphogens that are related to the *Drosophila* wingless protein (104). These proteins are known to regulate various aspects of cell to cell communication throughout embryonic development. Their activity is carried out through binding to the

Frizzled receptor family which in turn will trigger a canonical pathway of events inside the cell that results in the transport of β-catenin into the nucleus to activate the transcription of the Wnt target genes. In studies conducted on *Wnt1* it was found that inactivation of this gene resulted in mouse embryos that had no midbrain or hippocampus. Further study demonstrated that overexpression of Wnt1 or Wnt3a resulted in a dramatic increase in neuronal cell proliferation while the cell identity remained unaffected (105). These results are similar to our findings that when the Snf2l gene is removed we see an extended proliferation time period suggesting that the Wnt pathway may be regulated to some extent by Snf2l.

The genes discussed above represent a small portion of those that are required for the proper progression and removal from the cell cycle in the growth of the mammalian brain. The results from our qPCR experimentation will be discussed in further sections.

4.3 SNF2H knockout causes death upon forced differentiation

Previous testing has demonstrated that the Snf2h gene transcripts are directly involved in cellular proliferation (43). When null mutations are engineered into the murine Snf2h gene it causes lethality at the periimplantation stage of development due to an inability of the trophectoderm and inner cell mass to proliferate (80). Snf2h is widely expressed in mice all throughout early neonatal development in areas such as the brain, testis, ovaries and kidneys. In the brain elevated levels are detected in all proliferating progenitor cell populations and in the cortex are found most highly in the ventricular zone. Interestingly during further development these transcripts are maintained at similar levels in all areas of expression. As a result of this we expected that our human neuroblastoma cells knocked down for *SNF2H* would rapidly die during proliferation. Interestingly this is not the result

that we found. When these cultures were grown in standard proliferation media they reproduced themselves with only a slight increase in TUNEL staining, 4.49% for 5-2 clones and 2.56% for 4-5 clones, while control populations maintained 0% TUNEL positive staining. After only one day of differentiation the experimental *SNF2H* knockdown cells had an average of 45.11% TUNEL positive result. This indicates that there is a strong requirement for the *SNF2H* gene during these times and may also indicate that the stress of forced differentiation resulted in an inability of the cells to survive. The ability of our *SNF2H* knockout cells to survive during proliferation is most likely due to the SH-SY5Y cell line being a metastatic cancer cell line that has compromised growth control pathways such as p53 (106, 107).

The finding that our *SNF2H* knockdown cells die upon forced differentiation is very similar to that found in primary myoblasts derived from mice knocked out for *Rb* (108). In culture primary myoblasts from these mice are able to proliferate but upon the stimulation of differentiation they undergo rapid apoptosis due to a severe defect in the ability of the cells to control cell cycle exit mediated by *MyoD*. Similar results aside, *Rb* functions primarily as a cell cycle regulator in the brain (109). Additionally *Rb* loss in the telencephalon causes ectopic proliferation of neural precursor cells resulting in increased brain size and is associated with the vast majority of human carcinomas (82, 110). Links have also been made between *C. elegans* isw-1 and members of the Rb pathway where a reduction in isw-1 mediated the loss of *lin-35* Rb in carcinogenic cells (110). In this regard, the loss of a specific interaction between SNF2H and Rb may result in a similar death phenotype as loss of Rb alone.

The development of an organism is a complicated process whereby defined populations must receive cues that trigger the regulation of the genes required for proper

phenotypic expression. In the murine brain part of this regulation is carried out by the Rb protein and its associated binding partners E2F1 and E2F3 (82) Previous study has demonstrated that the Rb protein plays key roles in various critical aspects of neuronal proliferation, cell-cycle exit, cellular survival and neuronal migration. Therefore all of our results so far suggest that there may be disruption in the expression of this protein in both our *SNF2H* and *SNF2L* knockout clones.

4.4 Snf2l knockout neurosphere cultures contain mutations to several cell cycle genes

Our results, and those discussed previously, have shown that indeed the *Snf2l* gene is required for the correct differentiation of neuronal and specific glial cells. Therefore with the removal of a chromatin remodeling gene believed to be essential for differentiation it is logical to find that cells will be maintained in a proliferative state.

As discussed briefly in our results we found 6 genes that were down regulated in *Snf2l* knockout neurospheres after 4 days of forced differentiation. These genes are *Camk2a, Ccnb2, Sesn2, Brca2, Macf1*, and *Notch2*. In the brain Ca^{2+}/calmodulin kinase II inhibitor α (*Camk2a*) is associated with a wide variety of phosphorylation events to various neural substrates (111, 112). This family of proteins is made up of 4 members α,β,γ,δ of which the α form is predominantly expressed in the brain where it can make up to 2% of the total protein in certain brain regions. It has been strongly implicated in areas such as spatial learning, memory and neural plasticity. Loss of this protein has resulted in distinct disruptions in the spatial encoding of hippocampal place cells and mutations in long term potentiation (LTP). Surprisingly, engineered upregulations in *Camk2a* is also associated with defects to LTP (113). The genes *cyclin B2* (*Ccnb2*) and *breast cancer 2* (*Brca2*) are

both involved in neural apoptotic pathways. *Ccnb2* is upregulated in the apoptosis of retinal ganglion cells (RGCs) and is directly involved in the transition from G2 phase to mitosis via the activation of CDK1/CDC2 (114). In RGCs under apoptotic stimulation by nerve growth factor (NGF) there is a concurrent increase in Ccnb2 levels which results in a re-entry of the cell cycle leading to apoptotic cell death. Brca2 is a key regulator of the homologous recombination repair machinery in neural populations (115). The loss of Brca2 drastically decreases neurogenesis especially during embryonic development (116). This occurs through interaction with BRAF35 and Smad3 which are key S-phase regulators. Further examination will need to be conducted to determine if these downstream genes are misregulated in our Snf2l knockout cells. In experimental cell populations decreased Brca2 levels result in a drastic upregulation of meduloblastoma formation in p53 deficient populations and an associated reduction in the ability of neurons to differentiate. The reduction in neuronal populations and the continued proliferation in our Snf2l knockout neurosphere cultures indicate that both Brca2 and Ccnb2 pathways may be involved in the cell cycle exit problems.

Sestrin 2 (Sesn2) is a member of the sestrin family of proteins that are involved in the decomposition of hydrogen peroxide produced in response to receptor signalling (117). During shRNA mediated removal of *Sesn2* there is a rapid increase in reactive oxygen species and also chromosome breaks in cell populations undergoing reproduction (118). The contribution of this gene towards the phenotype we found in our *Snf2l* knockout neurospheres will need further examination. Microtubule actin cross linking factor 1 (*Macf1*) is a housekeeping gene expressed throughout neural cells and acts in the transport of inner cellular communication (119, 120). Murine models containing reduced *Macf1* levels present with distinct reductions in the ability of neuronal cells to produce axonal outgrowth

and dendritic sprouting. These findings fit very well with the phenotype we have discovered in our Snf2l knockout neurosphere cultures where there is a delay the ability of both neurons and astrocytes to produce cellular extensions.

The final gene that we found to be reduced was *Notch2*. The Notch family of proteins is highly conserved across the species barrier and all members are known to act as lateral inhibitors whereby these transcripts will be secreted and act to inhibit neighbouring cells from turning into neurons (121, 122). The upregulation of Notch2 is directly associated with a reduction in glial cell differentiation. Our results appear to be contradictory to these previous studies and further examination will need to be conducted to determine the exact mechanism that Notch2 plays in our phenotype. It is possible that this is a compensatory reaction by the cells whereby the reduction of a gene that inhibits differentiation is reduced when Snf2L function is lost.

We have also found that there are 6 cell cycle regulation genes that were upregulated which are: *Hus1, Msh2, E2F3, Rbl1, Casp3, and Dnajc2.* Hus1 is one of the members of the 911 (Rad9-Rad1-Hus1) DNA damage checkpoint proteins (123, 124). Under DNA damage this complex binds to chromatin and promotes the phosphorylation of Atr, a phosphatidylinositol kinase-like protein kinase, substrates such as Brca1/2 and Chk which in turn will cause misregulation in S-phase checkpoints. We have found that Brca2 expression is reduced in our knockout neurospheres which may be a result of increased phosphorylation resulting in protein degradation via the upregulation found in *Hus1*. Mutant S homologue 2 (*Msh2*) is a housekeeping gene that is typically mutated in gliomas (125, 126). It is also a chief member of the mismatch repair system during initial nucleotide mismatch recognition and is directly involved with p53 during tumorigenesis. In these glioma cells an increase in Msh2 results in an associated increase in proliferation (127). This suggests that the extended

proliferation of our experimental neurosphere populations may be a result of disruption to processes governed by *Msh2*.

As mentioned previously *E2F3* is a critical transcription factor that carries out roles in a wide range of early developmental stages in the brain and contains two different isoforms, a and b (82). Unfortunately the qPCR array used in these studies is not able to distinguish between these two isoforms and further study will be required to determine if one or both of the isoforms are increased.

p107, or Rb-like 1 (Rbl1), is a potent tumour suppressor that plays key roles during entry into the cell cycle (128). Interestingly, increased levels of this transcript are associated with decreases in proliferative potential indicating that upon gross examination our results may conflict with previous studies and further investigation will be required in order to both confirm and investigate how *p107* misregulation is affecting both up and downstream pathway members. While this result appears to conflict with other investigations it is likely that this is a compensation event by the cells in response to the loss of functional Snf2L. Caspase 3 (*Casp3*) is a pro-apoptotic member of the interleukin-1β-converting enzyme family (129). This protein is proteolytically activated and cleaved into two subunits, a 10kDA and a 20kDa species, both of which will act to cleave various substrates such as members of the cytoskeleton, proteases and various DNA repair enzymes in the nucleus (130). During past experimentation it was believed that an upregulation of this protein was only associated with increased apoptosis subsequent to brain trauma. However more recent studies have implicated caspase 3 in processes such as proliferation, cell cycle regulation and also embryonic differentiation. These findings strongly implicate that this gene may be regulated either directly or indirectly in both our *SNF2H* knockout neuroblastoma cells and *Snf2l* knockout neurospheres.

The final gene that will be discussed is Dnajc2 a member of the heat-shock protein 40 family (131). Currently little information is known of the over 20 Dnaj homologs that have been discovered, and the total number still remains unknown. These proteins are known to assist in protein translation and are also implicated in many neurodegenerative diseases. With the limited amount of information on this gene further study will be required in order to determine how it is influencing cell cycle progression in our experimental cell cultures.

4.5 Future directions

Throughout our experimental procedures here we have discovered that there are twelve candidate genes that are prime candidates to explain the extended proliferation of our Snf2l knockout cultures. For all of these potential candidates further examination is going to be required for confirmation purposes. So far it remains unknown whether these genes are either direct targets or downstream effectors of Snf2l regulation. To determine this ChIP studies could be conducted which would determine if Snf2l is actively remodelling the given promoter regions. We will also need to conduct Western blot analysis for each of the candidate genes in order to determine if the altered RNA expression has caused any modification to the amount of protein that is produced.

One shortcoming of this study is that our qPCR examination has been conducted on a mixed population. The neurospheres that were examined for this section of the enquiry are made up of a combination of neurons, astrocytes and oligodendrocytes. This means that each gene change that we have detected may only be isolated to a certain cellular population. It would also be valuable to generate more neurosphere cultures from new Snf2l knockout embryos in order to increase the biological replicates, especially for the E14.5 time point. Currently we are in the process of designing our own RT-PCR primers and extracting RNA

from new neurosphere cultures isolated at day 4 of differentiation in order to confirm the gene changes found during this qPCR array.

Another area that will need further examination is the oligodendrocyte population. These cells are the third type making up the community found in neurosphere cultures. We are currently in the process of examining whether Snf2l knockout will result in the same delayed differentiation and extended proliferation in these cells as was found in the neuronal and astrocyte cells. It is our belief that there will be a disruption to them as a paper by Collarini et al 1991 it was found that neurons are capable of releasing different factors that influence myelination and therefore the development of oligodendrocytes. As a result of this even if the Snf2l knockout does not directly cause disruption to the cultures of oligodendrocytes it is likely that there will be secondary downstream developmental problems created.

In all of the experimental procedures conducted in these studies no focus has been given to the specific Snf2l complexes that are involved in the chromatin remodelling processes. The Snf2l inactivation utilized here was knocked down ubiquitously and as a result of this Snf2l function will be eliminated in every complex that it is present in. It would be valuable to examine if the genes we have identified in this study are regulated by either NURF or CERF complexes. It would also be beneficial to conduct some rescue experiments whereby *Snf2l* is reintroduced into our knockout cultures to determine if the phenotype created by knockout will be rescued.

4.6 Conclusions

Chromatin and chromatin remodelling ATPases are rapidly coming to the forefront for various developmental regulation processes especially when X-linked mental retardation is examined. The overall goal of our investigation here was to examine how the ISWI genes aided in the development of a fully differentiated neuronal cell phenotype. Initially we began this study using established neuroblastoma cells transfected with shRNA constructs. From here we produced neurosphere cultures functionally ablated for Snf2l. These experiments allowed us to determine that Snf2h removal in neuroblastoma cells causes a rapid increase in cell death when the cells are induced to differentiate. In cells knocked down for Snf2l there was an extension to the proliferation time course as examined by BrdU incorporation that suggested there were disruptions to the ability of these cells to exit the cell cycle. Our functionally ablated Snf2l neurosphere cultures allowed us to examine these results in a more complex system. This resulted in the same sort of cell cycle exit problems during forced differentiation and also resulted in a delayed ability of these experimental Snf2l cultures to produce both neuronal and astrocytic cell populations. This indicated that the problems caused by Snf2l knockout were not specific to the neuronal population but affected glial lineages as well. This situation was found to be only transient in nature as when these experimental cell populations were allowed to differentiate further the Snf2l knockout cells returned to the control morphology and total population count.

In the experiments detailed herein we have clearly demonstrated that the ISWI genes are key regulators for neuronal development and survival during differentiation. We have also shown that this regulation extends into the astrocyte cells as well. In conclusion, while these results did not indicate that the ISWI genes are directly involved in X-linked mental retardation they have demonstrated that there is a definitive requirement for both neuronal

and astrocyte cells to correctly exit the cell cycle which in turn implicates the ISWI genes in proper brain development.

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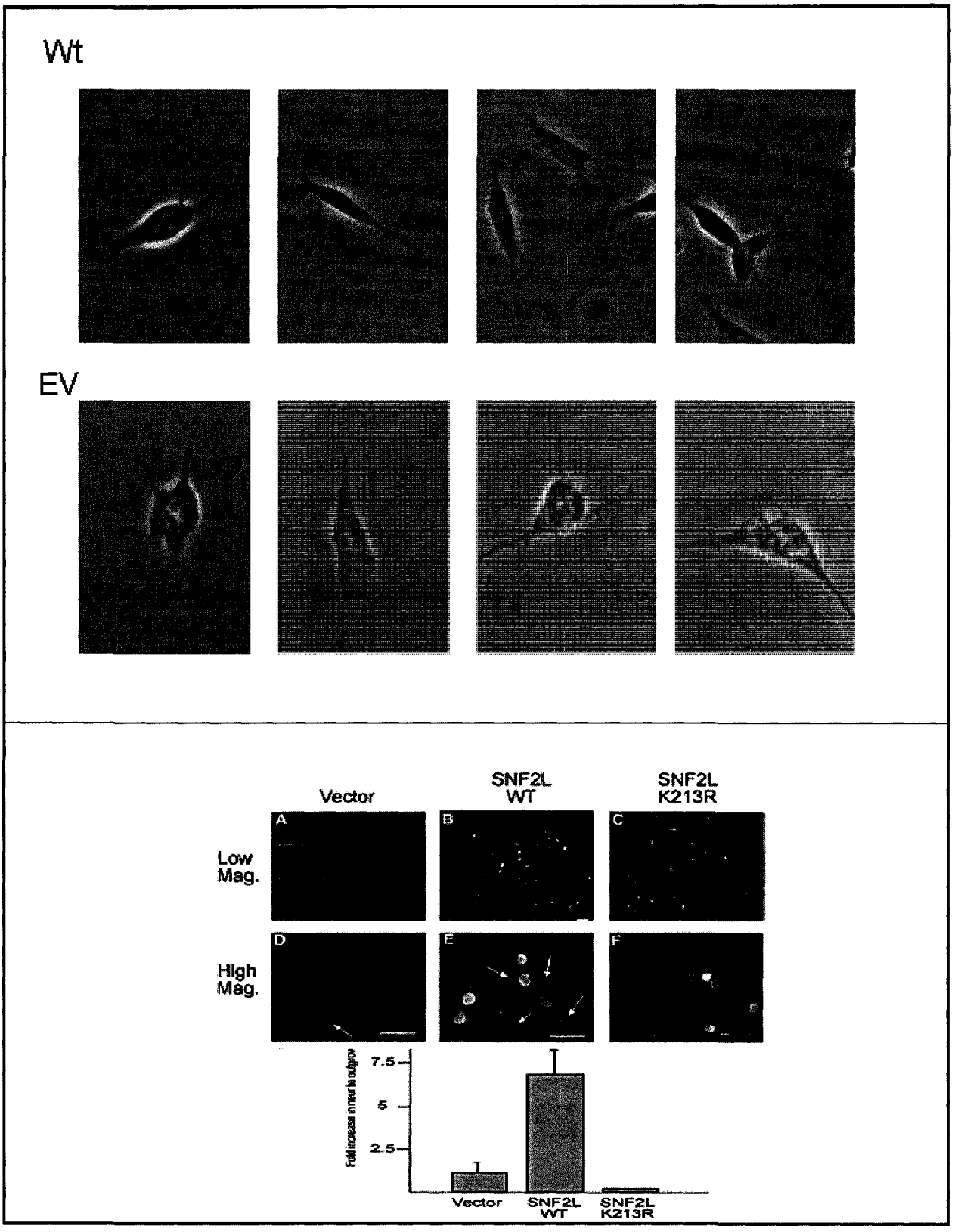
Supplemental Figures

Figure 1: Selection chemicals cause morphological changes in SH-SY5Y cells. Proliferating SH-SY5Y wild type (Wt) and empty vector (EV) demonstrate the morphological differences between cells grown in the presence of geneticin (EV) and those not (Wt).

Figure 2: Over expression of Snf2L results in increased neurite outgrowth under proliferation conditions. A) Pictures A and D demonstrate N1E115 cells transfected with control vector. Pictures B and E show cells transfected with vector containing wild type Snf2L. Finally pictures C and F show cells transfected with a vector containing an ATPase null Snf2L mutant. B) Graphical quantification of the resulting neurte outgrowths from the three N1E115 cell populations. Figure taken from Barak et al 2003.


Wt
EV
Vector
SNF2L WT
SNF2L K213R
Low Mag.
High Mag.
A
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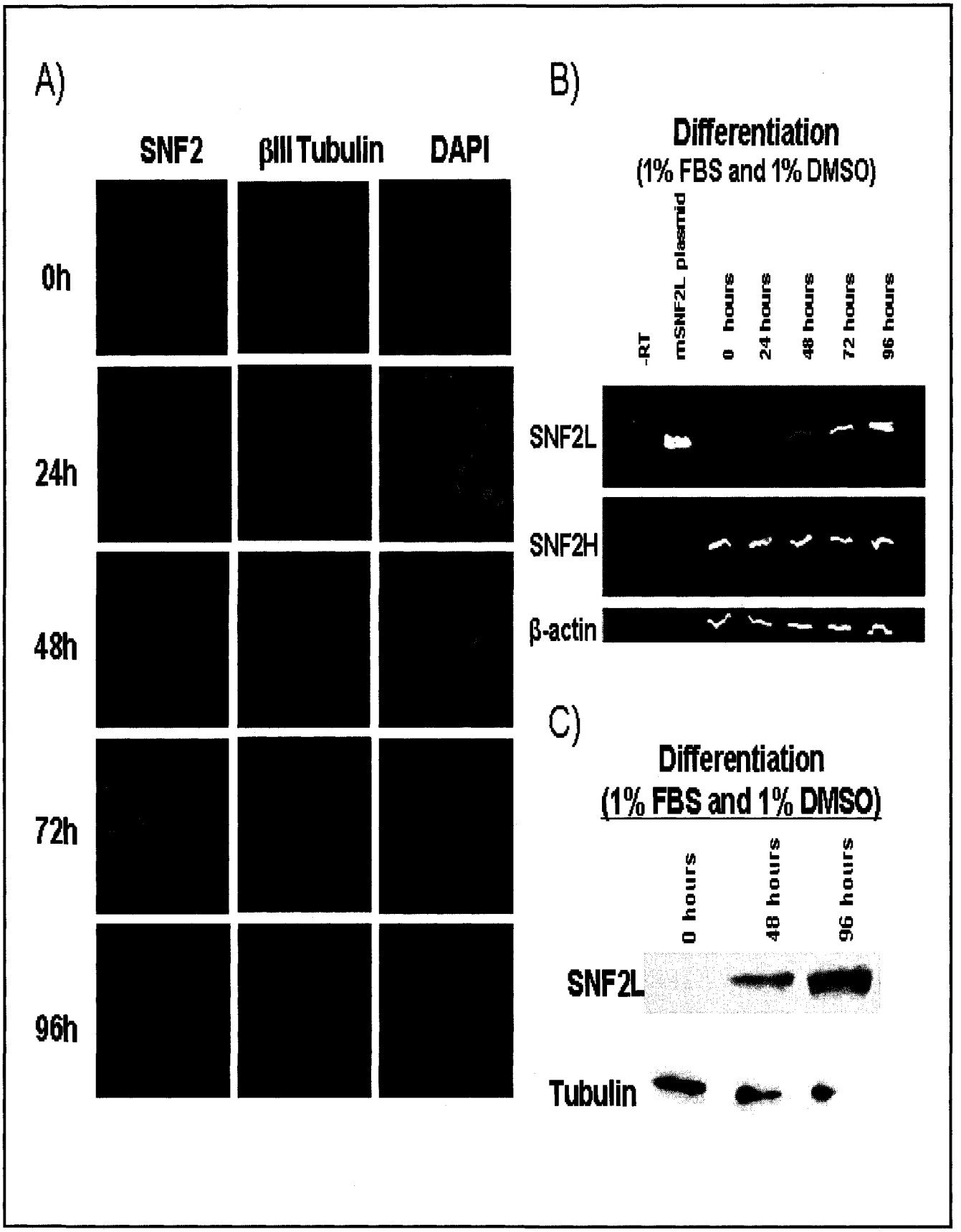
Fold increase in neurite outgrow
7.5
5
2.5
Vector
SNF2L WT
SNF2L K213R

Figure 3: Snf2l expression increases with differentiation. A) Immunocytochemical investigation of N1E115 cells demonstrates increased nuclear Snf2l (green) expression during forced differentiation along with Tuj1 (red) and DAPI (blue). B) RT-PCR analysis of N1E115 extracts confirms the Snf2l upregulation during differentiation while Snf2h remains constant. C) Western blot analysis of Snf2l protein expression shows Snf2l is also upregulated.


A)
SNF2
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B)
Differentiation
(1% FBS and 1% DMSO)
-RT
mSNF2L plasmid
0 hours
24 hours
48 hours
72 hours
96 hours
SNF2L
SNF2H
β-actin
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Differentiation
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