ANALYSIS OF HUNTINGTIN-INTERACTING PROTEIN 1 IN DROSOPHILA MELANOGASTER

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Analysis of Huntingtin-interacting protein 1 in *Drosophila melanogaster*

A thesis presented in partial fulfillment of the requirements for the degree of
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Abstract:

Huntington's disease (HD) is associated with a wide range of effects including selective neuronal death and altered levels of neurogenesis that are ultimately dependent upon altered activities of Huntingtin (Htt) interacting proteins. These effects are similar in nature to those observed with mutations in the Notch signal transduction pathway. Huntingtin interacting protein 1 (Hip1) shows decreased binding to expanded Htt. Hip1 plays a key role in endocytosis and intracellular transport and activation of the Notch signal requires both. Based on this observation links between Hip1 and Notch-dependent neurogenesis were investigated. In Drosophila two *hip1* mRNAs may be naturally produced through the use of alternative splicing of the first exon: full length *hip1* with lipid binding ANTH domain and *hip1ΔANTH* lacking this domain. Directed expression of *hip1* decreases while expression of *hip1ΔANTH* increases microchaetae density in the dorsal notum, a field of sensory bristles on the fruit fly back, suggesting a functional role for Hip1 in neurogenesis. The following studies demonstrate genetic interaction between *hip1* and *deltex*, a key mediator of Notch signaling, with *hip1* enhancing and *hip1ΔANTH* suppressing *deltex* phenotypes. Reduction of bristle microchaetae density associated with $Notch^{MCD}$ alleles is sensitive to *hip1* and *hip1ΔANTH*. This pathway is shown to be independent of classical Notch control through E(spl) and tightly controlled by both GSK3β and achaete levels. Building on this the possibility of an analogous pathway in human neuronal development was investigated using the Ntera-2/D1 neuronal precursor cell line along with siRNA and antibody technologies. These studies demonstrate that the Hip1 functions in a similar deltex-dependent, HES1-independent process of neuronal differentiation. Hip1's novel role in neurogenesis provides a functional link between Notch

signaling and proteins related to HD advancing the understanding of HD neurogenic phenotypes.

During the course of these investigations it was noted by others in our laboratory that high levels of *Gal4* in the developing eye result in elevated apoptosis in the eye imaginal disc. Suppression of apoptosis by expression of the caspase inhibitor *p35* prevented this phenotype. These studies were extended to analyze Gal4 phenotypes associated with microchaetae density using the *pannier-Gal4* transgenic line. The reduction in microchaetae density associated with *pannier-Gal4* is suppressed by co-expression of *p35* but unaffected by expression of *GFP*. These results show that Gal4 has effects outside the Drosophila eye and that similar mechanisms of disruption are at work at least in the eye and dorsal notum.

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Lastly, I'd like to thank my friends and family for their support and patience throughout the course of my degree.

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List of Abbreviations:

AMPA = α- amino-5-hydroxy-3- methyl-4-isoxazole propionic acid
ANOVA = Analysis of variance
ANTH = AP180 N-terminal homology
ap = apterous
ASH1 = Achaete Scute Homolog 1
BAR= bifunctional apoptosis inhibitor
BDNF = Brain derived neurotrophic factor
BDGP = Berkeley Drosophila Genome Project
bHLH = basic helix loop helix
BLAST = Basic local alignment search theorem
CAG = cytidine-adenosine-guanosine trinucleotide repeat
cDNA = complementary DNA
CNS = central nervous system
Ct = threshold cycle
DED = death effector domain
DIG = digoxigenin
DLK1 = Delta like 1
DNA = Deoxyribonucleic acid
dNTP = deoxynucleotide triphosphates
EDTA = ethylenediaminetetraacetic acid
EGFR = Epidermal growth factor receptor
ENTH = Epsin N-terminal homology
E(spl) = Enhancer of split
ExPASy = Expert Protein Analysis System
GABA = Gamma aminobutyric acid
Gal4 = Galactose response transcript 4
GFP = green fluorescent protein
Grb2 = growth factor receptor-bound protein 2
GRIN1 = glutamate receptor, ionotropic, N-methyl D-aspartate 1
GSK3β = Glycogen synthase kinase 3β
HEAT = Huntingtin, Elongation factor 3, protein phosphatase 2A, TOR1
Hepes = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HES = Hairy and Enhancer of split
HD = Huntington's Disease
IP = immunoprecipitation
KCl = Potassium chloride
kDa = kilo Dalton
MCD1 = Microchaetae deficient

List of Abbreviations continued:

mM = mili Molar
mL = mililiters
mRNA = messenger RNA
MuLV = Murine leukaemia virus
N = Notch
NDS = normal donkey serum
NECD = Notch extracellular domain
NeuroD1 = Neuronal differentiation 1
NEXT = Notch Extracellular domain removed
NFκB = Nuclear factor κB
NICD = Notch intracellular domain
NP-40 = Nonidet P40
Nt = nucleotide
NT2-D1 = Ntera2/D1
ORF = open reading frame
PAGE = polyacrylamide gel electrophoresis
PBS = Phosphate buffered saline
PBT = Phosphate buffered saline + Tween 20
PCR = Polymerase chain reaction
Pfam = Protein family
pnr = pannier
Q = Glutamine
RA = retinoic acid
RNA = Ribonucleic acid
RNAi = inhibitory RNA
Rpm = revolutions per minute
RT-PCR = Reverse Transcriptase PCR
SDS = sodium dodecyl sulfate
SEM = Scanning electron microscope
siRNA = short interfering Ribonucleic acid
Su(H) = Suppressor of Hairless
TBS-T=Tris buffered saline + Triton X-100
TOPO = topoisomerase
UAS = Upstream activating sequence
UTR = Untranslated region
YAC128 = Yeast artificial chromosome containing 128 poly glutamine expanded Htt
μL = microliters
μg = microgram
μm = micrometers

Chapter 1: Current understanding of Huntington's disease

1.1 Introduction to Huntington's disease:

Huntington's disease (HD) is one of a growing class of neurodegenerative disorders in which specific regions of the brain and/or peripheral nervous system are lost over time. The neuropathological hallmark of Huntington's disease is the selective elimination of medium-spiny GABAergic neurons of the striatum and disease is accompanied by alterations in cognitive, metabolic, and emotional characteristics (reviewed in (Petersen et al. 1999). A CAG trinucleotide expansion in the gene *Huntingtin* (*Htt*) causes Huntington's disease (HDCRG 1993). This CAG expansion, when translated, leads to a long stretch of the amino acid glutamine (Q) in the ~350 kDa Huntingtin protein (Figure 1.1). This molecular characteristic places Huntington's disease in a subset of degenerative disorders termed Poly-Q or CAG-repeat diseases that include Machado-Joseph disease and some spinocerebellar ataxias, among others (reviewed in (Ross 2002). However, the basic cellular changes that lead to the biological outcome of Huntington's disease have yet to be fully characterized. Investigations of the biological role of Huntingtin and its functional protein partners have led to great advances in the understanding of the biological basis of HD. Nevertheless, preventative therapeutics and/or corrective therapies remain elusive.

This thesis will attempt to outline our present understanding of basis of Huntington's disease, discuss models of HD and explore the foci and controversies of current HD research. This will be followed by a presentation of a series of novel experiments that explore a role for Huntingtin-interacting protein 1 in the process of neurogenesis.

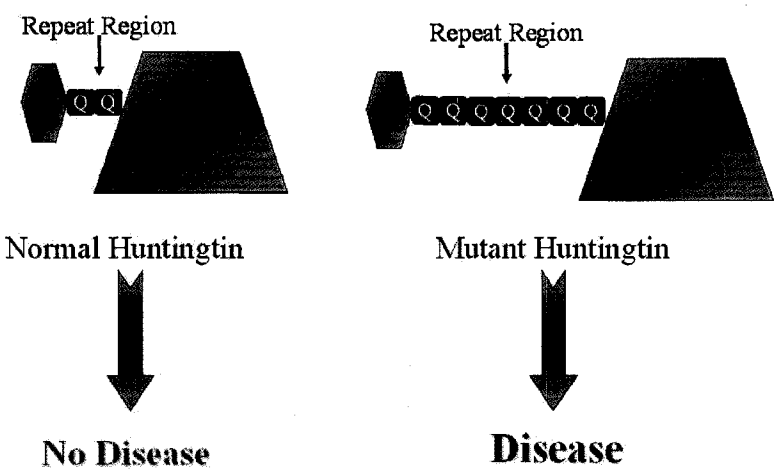

Figure 1.1: Simplified model of the polyglutamine (PolyQ) expansion basis of Huntington's disease. Huntington's disease patients typically have polyQ stretches surpassing 35-36 glutamine repeats with much larger stretches possible. Patients with less than the threshold 35-36 glutamine stretch do not show symptoms of HD while those with 35-36 or more glutamine residues manifest HD. As symptomatic severity and age at onset of HD are negatively correlated with polyQ length, function(s)/interaction(s) affected by expansion may have roles in pathogenic progression and if so may serve to be prime candidates for therapeutic intervention.

1.2 Current understanding of Huntington's disease:

1.2.1 Signs and Symptoms:

Huntington's disease, also referred to as Huntington's chorea, was originally described as an inherited disorder in 1872 by Dr. George Huntington and is characterized by a loss of fine motor control, resulting in a so-called 'dancing disorder' (Huntington 1872). Currently, HD is characterized by complex symptoms including a disruption in the pathways of fine motor control plus additional dysfunctions including difficulties in long term memory formation and declines in reasoning ability and learning (Lemiere et al. 2004). HD patients show a decreased ability to form and recall long term memories. The earliest onset of HD in asymptomatic carriers of the HD gene mutation include changes in attention, working memory, verbal learning, verbal long–term memory and learning of random associations (Lemiere et al. 2004). Subtle dysfunctions that have been shown to be a result of HD also occur in the pathways of neuronal development (Curtis et al. 2003; Tattersfield et al. 2004; Curtis et al. 2005) and in hematopoiesis (Metzler et al. 2000). The brains of HD patients have increased numbers of adult neuronal stem cells in the subependymal layer which suggests that the HD mutation alters the activity of signalling pathways that limit neuronal fate specification. In addition, the targeted deletion of the *Huntingtin* homologue, *Hdh*, in mice has demonstrated that it is required for proper neuronal development (White et al. 1997). Analysis of *Hdh* null cells from mutant mice demonstrate that, under normal conditions, Huntingtin plays at least a partial role in the proper differentiation of hematopoietic progenitor cells (Metzler et al. 2000). In summation, these data suggest alterations to the proper functioning of the Huntingtin gene product through, either poly-glutamine expansion in Huntington's disease, or loss-of-function in *Hdh* -/- mice, lead to changes in the pathways of cellular differentiation. This makes it clear

that understanding the molecular mechanics underlying these changes may be vital to determining the root cause of Huntington's disease.

1.2.2 Molecular functions of Huntingtin:

Since the characterization of the HD mutation (HDCRG 1993), Huntington's disease research has focused upon the molecular function(s) of the Huntingtin (Htt) protein (Figure 1.2). Unfortunately, the large size of the Huntingtin protein, approximately 350 kDa, has hindered both X-ray crystallographic and other three-dimensional modeling techniques. Coupled with this, no clearly defined functional motifs have so far been uncovered in Htt with the exception of numerous repeated Huntingtin, Elongation factor 3, protein phosphatase 2A, and TOR1 (HEAT) motifs, each of which are linked in other systems to protein-protein interaction, suggesting a role for Htt as a 'docking' protein (Takano and Gusella 2002). Despite these limitations several advances into the molecular function of Huntingtin have been uncovered. One critical finding has been the role of Htt in cellular survival systems through its inhibition of caspase activation (Rigamonti et al. 2000; Reiner et al. 2003). Htt is capable of entering the nucleus coincident with the anti-apoptotic NFκB transcription factor dorsal (Takano and Gusella 2002). Huntingtin has also been linked to the transcriptional regulation and transport of the neuronal survival factor, Brain-Derived Neurotrophic Factor (BDNF), required by the striatal neuronal population primarily affected in HD (Reilly 2001; Zuccato et al. 2001; Zuccato et al. 2003; Gauthier et al. 2004; Ross 2004; Cattaneo et al. 2005; Pineda et al. 2005; Zuccato et al. 2005). Proteolytic cleavage of polyQ expanded Htt and, to a lesser extent, wild type Htt by activated caspases leads to the production of nuclear and cytoplasmic inclusions in the brain (Taylor and Ikeda 2000; Sanchez Mejia and Friedlander 2001). These findings strongly suggest a role for Htt in the maintenance of cellular homeostasis, and that alteration

of these functions in HD lead, in turn, to programmed cell death. Additional links to cell survival pathways are still being uncovered as evidenced by findings showing that alterations in the insulin-signalling pro-survival pathway also exist in HD model and patient systems (Humbert et al. 2002; Rangone et al. 2004; Colin et al. 2005; Rangone et al. 2005; Warby et al. 2005; Gines et al. 2006). Htt has also been linked to axonal transport systems implicating alterations in cellular trafficking in the pathogenesis of HD (Block-Galarza et al. 1997; Tukamoto et al. 1997; Feany and La Spada 2003; Gunawardena et al. 2003; Szebenyi et al. 2003; Lee et al. 2004; Trushina et al. 2004). In order to further understand the normal role of Huntingtin in the cell and the changes that must exist under disease conditions, studies have focused on the activities of proteins which interact with Htt under normal and mutant conditions, a summary of these findings will be discussed below. In attempts to uncover the role of Htt directly, several models of Huntington's disease have been established.

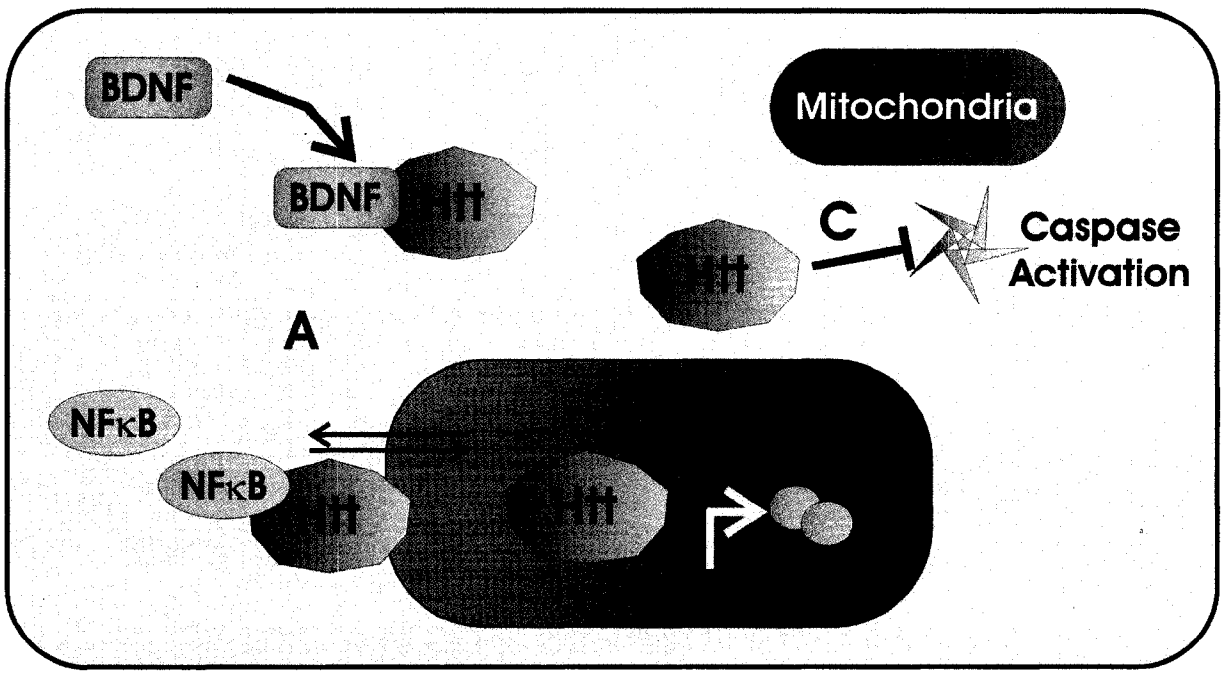

Figure 1.2: Huntingtin is involved in a variety of molecular processes. Huntingtin has been shown to function in: nuclear and cytoplasmic shuttling of several proteins such as brain derived neurotrophic factor (BDNF) and nuclear factor κ B (NFκB) (A), transcriptional co-activation (B) and mechanisms of cell survival, through direct inhibition of caspase activation (C). Adapted from (Ross 2004).

1.2.3 Current Huntington's disease Rodent Models:

Mammalian models of Huntington's disease have been established in an attempt to determine the normal function of the Huntingtin protein and as its interaction partners. Three main classes of mouse lines have been created to investigate various aspects of Huntington's disease: expanded N-terminal Huntingtin transgenics, *Htt* knockout lines, and Poly-Q knock-in/yeast artificial chromosome (YAC) lines. In addition, a commonly used rat model of HD has been established which uses quinolinic acid to induce cell death in the striatum (Beal et al. 1986). Early transgenic mouse models, designated R6/1 and R6/2, over-express the first exon of the *Huntingtin* gene including an expanded poly-glutamine stretch (Mangiarini et al. 1996). These lines have been extensively used to analyze the effects of the truncated polyglutamine-expanded Htt fragments noted to form inclusions in HD. The R6/2 and R6/1 lines have been noted to show neuronal inclusions in the striatum and neurologic phenotypes; however no degenerative phenotype has been noted (Li et al. 2005). Both of these lines show fewer proliferating cells in the hippocampus than age-matched controls (Lazic et al. 2004; Gil et al. 2005; Grote et al. 2005). The YAC128 and Poly-Q knock-in mouse models attempt to recapitulate the genetic basis of HD by adding full-length poly-Q expanded Huntingtin into the mouse genome using different methods, preserving the entire protein context of the HD mutation. Aspects of Huntington's disease have been approximated, and these models display degenerative neurological symptoms in a similar progressive nature to the disease and, in the case of YAC128 mice, the specific loss of striatal neurons (Lin et al. 2001; Slow et al. 2003; Graham et al. 2006). As varied degrees of neuronal phenotypes have been noted with differing protein contexts, these '*in situ*-altered' HD models preserve the nature of polyQ expansion in patients (Yu et al. 2003). Combined, these varied rodent models display many characteristics

of HD and therefore allow, to varying degrees, analyses of the physical changes associated with the disease. However, limitations associated with rodent systems reduce the extent of in depth genetic analysis possible. Invertebrate models can be used, in combination with rodent and *in vitro* systems, to answer difficult questions about HD pathogenesis and development.

1.3 Invertebrate models of Huntington's disease:

Based on their evolutionary similarity to humans, mouse models are often favoured but several drawbacks exist which limit the extent of their usefulness. Mouse models are very difficult and time consuming to establish and in many mammalian systems gene redundancy may mask the effect of genetic alteration (Bernards and Hariharan 2001). In contrast, invertebrate models are very useful in genetic analyses. Numerous *Caenorhabditis elegans* and *Drosophila melanogaster* strains have been engineered to express poly-Q expanded disease proteins in order to take advantage of the well-characterized molecular and genetic systems, and general lack of redundancy found in these models (reviewed in (Bernards and Hariharan 2001; Link 2001; Sipione and Cattaneo 2001; Bonini and Fortini 2003; Marsh et al. 2003; Marsh and Thompson 2004). A number of invertebrate models have been utilized to show that poly-Q expanded Htt fragments cause nuclear aggregation and neurodegeneration (Jackson et al. 1998; Warrick et al. 1998; Faber et al. 1999; Marsh et al. 2000; Morley et al. 2002). In addition several have shown that these phenotypes can be suppressed by expression of certain chaperone proteins and modeled peptides (Warrick et al. 1998; Chan et al. 2000; Kazemi-Esfarjani and Benzer 2000; Higashiyama et al. 2002; Kazantsev et al. 2002). An additional model utilizes RNA interference techniques of gene silencing to show that Huntingtin works in fast axonal transport (Gunawardena et al. 2003; Lee et al. 2004). Importantly, experimental therapeutics are being tested using high throughput *in vitro* screens in parallel with

invertebrate model systems (Bates and Hockly 2003; Shulman et al. 2003; Marsh and Thompson 2004). Parallel studies may prove to be the most effective in therapeutic development with the accurate replication of disease phenotype in mammalian cellular and *in vivo* systems combined with the genetic advantages of invertebrate models.

1.4 Areas of controversy in HD research:

The molecular and cellular nature of the disease and the role of neuronal inclusions are two of the key areas of controversy. Early genetic analysis, prior to the characterization of the *Huntingtin* locus, determined that HD displays dominant gain-of-function inheritance (Wexler et al. 1987; Myers et al. 1989). Early *Hdh* knockout models supported the gain-of-function inheritance as homozygous null animals died during embryogenesis (Duyao et al. 1995). As individuals homozygous for the HD mutation display normal embryogenesis this was inconsistent with the progression of HD, the authors thus determined that the gain-of-function theory was the most likely (Duyao et al. 1995). The earliest examination of an HD homozygous patient and his heterozygous brother also suggested no difference in disease progression (Durr et al. 1999). This suggests that an individual with only one mutant HD allele would be symptomatically indistinguishable from an individual homozygous for the defective CAG-expanded allele, and suggests that the wild type *Htt* gene has no effect on disease outcome. In contrast, recent research suggests that wild type Huntingtin may play a role in disease outcome (Cattaneo et al. 2005; Graham et al. 2006). Decreasing wild type *Hdh* expression enhances the deleterious effects of the YAC128 poly-Q *Htt* chromosome, with earlier age at onset and more severe disease progression (Graham et al. 2006). Indeed, Hdh depletion is seen in several mouse models of neurologic conditions as well as models of HD (Zhang et al. 2003). These findings are further supported by results in an HD knock-in mouse

model in which 150 CAG repeats were inserted into the wild type *Mus musculus Hdh* gene (Reddy et al. 1998; Lin et al. 2001). In this system, homozygotes for the HD mutation have decreased lag times in disease onset (Reddy et al. 1998; Lin et al. 2001). Further evidence supporting a functional role for wildtype *Huntingtin* in disease progression comes from a genetic analysis following 8 homozygous and 75 heterozygous HD patients (Squitieri et al. 2003). Interestingly, this study found that, while age at onset is unaffected by homozygousity, the disease progression and severity were both negatively affected in those individuals homozygous for the HD mutation when compared to heterozygous controls (Squitieri et al. 2003). Several arguments can, however, be identified from these conflicting views. As early analyses were performed using statistical prediction of homozygousity, or on very limited sample numbers, it is difficult to draw firm conclusions from these data. Additionally, variability in the age at onset in relation to CAG length and neurological definition of early Huntington's disease mask subtle changes which may be vitally important. Analyses in animal models, which have clearly defined symptoms and recognizable end points (Reddy et al. 1998; Lin et al. 2001; Graham et al. 2006) along with the more recent analyses using larger numbers of patients, along with molecular determination of homozygousity, would be expected to show more reproducible results (Squitieri et al. 2003). It is apparent that the question of wildtype Htt's role in disease progression remains a topic of heated debate and this debate will remain until enough detailed molecular and neurological data can be compiled to conclusively define age at onset and disease severity in both heterozygous and homozygous HD patients.

A second area of controversy in HD research is the role of protein inclusions in the disease. Theories regarding the function of these protein deposits have ranged from being causative of HD cell death (Li et al. 2001; Lee et al. 2004; Ravikumar et al. 2004; Kim et al. 2006) to

providing a measure of neuroprotection (Chun et al. 2002; Rao et al. 2002; Arrasate et al. 2004; Diaz-Hernandez et al. 2005). This is particularly concerning because a number of potential therapeutic methodologies currently under study focus on the elimination/prevention of these proteinacious inclusions (Kazantsev et al. 2002; Wolfgang et al. 2005). Without a clear understanding of the role of these inclusions, these therapeutics may be detrimental. For example, *in situ* time-course microscopy of cells containing aggregates compared to those lacking aggregates suggests that cells containing aggregates survive better than non-aggregate containing cells (Arrasate et al. 2004). If this protective role is retained in HD patients, therapies directed at the elimination of these aggregates or that induce changes in aggregate structure may be detrimental to those patients. Based on these findings and the aforementioned difficulties in direct functional analysis of Huntingtin (Section 1.2) a great deal of study has been done on proteins that interact with Huntingtin in both the normal and mutant state. Interactions affected by the HD mutation represent promising targets of therapeutic intervention,. Analyzing the molecular function(s) of these interactors could provide an indirect method of understanding the molecular function of Htt and effects of the HD mutation.

1.5 Huntingtin-interacting proteins:

1.5.1 Huntingtin-interacting proteins:

Proteins identified to interact with Huntingtin fit into several functional categories and have varied responses to the HD mutations (as summarized in Table 1.1). Several Htt-binding partners are involved in metabolism, such as glyceraldehyde-3-phosphate dehydrogenase (Burke et al. 1996) and cystathione-β synthase (Boutell et al. 1998). A second group is involved in protein modification, such as Huntingtin-interacting protein 2 (Hip2), a ubiquitin-

conjugating enzyme involved in protein turnover (Kalchman et al. 1996). A third group of Htt-interacting proteins is involved in growth signalling. This group includes Grb2, a growth factor-receptor intermediate (Liu et al. 1997); Akt, a critical cell-survival kinase (Humbert et al. 2002); the proto-oncogenic transcription factor's nuclear factor κB (NFκB) (Takano and Gusella 2002); and the transcription factor p53 (Steffan et al. 2000). The fourth group is involved in the process of intracellular vesicle trafficking. Members of this group include Hip14, a palmitoyl transferase (Singaraja et al. 2002); Huntingtin-associated protein-1 (Hap-1), an adaptor involved in vesicle transport (Li et al. 1995); α-Adaptin, a clathrin adaptor (Faber et al. 1998); and Huntingtin-interacting protein 1 (Hip1), an endocytic adaptor protein linked to numerous roles in the cell (Kalchman et al. 1997; Wanker et al. 1997). Of these, Hip14, Hap1, α-Adaptin, and Hip1, possess altered binding in the presence of the HD mutation. Hip14, α-Adaptin and Hip1 show decreased binding to the mutant forms of Htt, while Hap1 has an increased affinity for poly-Q expanded Htt (Li et al. 1995; Kalchman et al. 1997; Wanker et al. 1997; Singaraja et al. 2002). As the Hip14, Hap1, α-Adaptin and Hip1 proteins bind with differential preference to mutated and non-mutated Htt, they clearly represent strong candidates for preventative or corrective intervention. Although these diverse functional groups of Htt-interactors suggest that an array of biological activities may be affected in HD patients, they are involved in several common processes: all of the described activities depend on intracellular vesicle transport and membrane regulation for proper activity.

Interacting partner	Molecular Function	Affect of Poly-Q on binding activity	Reference
α-Adaptin	Endocytosis	Decrease	(Faber et al. 1998)
Akt	Signalling	No Change	(Humbert et al. 2002)
CBS	Metabolism	No Change	(Boutell et al. 1998)
GAPDH	Metabolism	No Change	(Burke et al. 1996)
Grb2	Signalling	Unknown	(Liu et al. 1997)
Hap1	Trafficking	Increase	(Li et al. 1995)
Hip1	Trafficking	Decrease	(Kalchman et al. 1997; Wanker et al. 1997)
Hip-2	Protein turnover	No Change	(Kalchman et al. 1996)
Hip14	Trafficking	Decrease	(Singaraja et al. 2002)
NFκB	Signalling	Unknown	(Takano and Gusella 2002)
p53	Signalling	No Change	(Steffan et al. 2000)

Table 1.1: Huntingtin interacting proteins, the effect of Huntington's disease polyglutamine (PolyQ) mutation on binding affinity, and current view of molecular function. CBS=Cystathione-β Synthase; GAPDH= Glyceraldehyde Phosphate Dehydrogenase; NFκB= Nuclear factor κ B; Hip = Huntingtin-interacting protein; Hap = Huntingtin associated protein.

1.5.2 Huntingtin-interacting protein 1:

Huntingtin-interacting protein 1 was originally characterized based upon its binding to Huntingtin in the yeast two-hybrid system (Kalchman et al. 1997; Wanker et al. 1997). Importantly, this interaction is reduced in the presence of the HD mutation to suggest that the function of the Hip1/Htt heteromer could have implications in the onset and progression of Huntington's disease (Kalchman et al. 1997; Hackam et al. 2000; Gervais et al. 2002). In support of this theory, Hip1 is capable of inducing apoptotic cell-death in cell-culture through a central coiled-coil domain (Hackam et al. 2000; Gervais et al. 2002). Increased Htt poly-Q length in turn increases cell-death (Hackam et al. 2000; Gervais et al. 2002). A novel Hip1 protein interactor (Hippi) was shown to co-operate with Hip1 to enhance its toxic effects (Gervais et al. 2002). The mechanism of cell death has been undergoing some debate as one study shows activation of the mitochondrial Caspase 9-mediated pathway (Hackam et al. 2000) while another shows activation of the extrinsic Caspase 8-mediated pathway (Gervais et al. 2002). Curiously, Hip1 expression promotes cellular survival and is capable of transforming ordinary cells into malignant masses (Rao et al. 2002; Rao et al. 2003). In addition, Hip1 is highly expressed in prostate and colon cancer and strongly correlates with poor prognosis in prostate cancer (Rao et al. 2002). These apparently counter-intuitive roles for Hip1 have been postulated to stem from Hip1's role in the complex mechanisms of clathrin-mediated endocytosis and cellular targeting.

Hip1 contains domains related to endocytosis and vesicular-trafficking including an AP-180 N-terminal homology domain [ANTH, previously referred to as an ENTH (Epsin N-terminal homology)], a Talin-like/ILWEQ domain, as well as binding sites for actin, adaptor protein 2,

and clathrin (Kalchman et al. 1997; Hackam et al. 2000; Mishra et al. 2001; Rao et al. 2001; Legendre-Guillemin et al. 2002; Metzler et al. 2003; Chen and Brodsky 2005; Legendre-Guillemin et al. 2005; Sun et al. 2005). Studies of the biological role of Hip1 in *Hip1*-null mice and cell culture systems show that these domains are functional in the processes of endocytosis and trafficking. *Hip1*-null mice show defects in neurotransmitter receptor trafficking as well as aspects of cellular differentiation and overall development; in particular, hematopoietic and spermatogenic development are inhibited along with eye and spinal defects (Rao et al. 2001; Metzler et al. 2003; Oravecz-Wilson et al. 2004). Biochemical analyses have shown that these phenotypes are related to the ability of Hip1 to participate in endocytosis (Mishra et al. 2001; Hyun et al. 2004; Chen and Brodsky 2005; Legendre-Guillemin et al. 2005; Sun et al. 2005). Hip1's role in endocytosis and receptor trafficking has been firmly established, however the relation of this function to phenotypes in HD and cancer remain unclear.

Hip1's role in tumourigenesis is believed to be mediated through alterations in epidermal growth factor receptor trafficking leading to enhanced cell-survival signalling (Rao et al. 2003). However, relating the biological functions of Hip1 to HD has proven more difficult. Hip1 null mice do display neurological impairments related to AMPA-receptor transport (Metzler et al. 2003) but these phenotypes have not been linked to HD. These findings, while hinting that Hip1 is involved in HD, leave open for investigation the question of the role of Hip1 in HD.

1.6 Notch and Neurogenesis:

1.6.1 Notch signalling in development:

The Notch signal transduction network (Figure 1.3) was originally described due to its role in lateral inhibition during the process of neurogenesis (reviewed in (Hansson et al. 2004). In the Drosophila embryo null mutations in *Notch* lead to over-production of neurons at the expense of epidermis (Hansson et al. 2004). Since its original description, the roles of Notch signalling in development have expanded with new roles which range from the promotion of cell-cycle progression to the activation of apoptotic cell death (Hansson et al. 2004; Radtke et al. 2005). With this explosion in cell-type specific reactions to Notch-mediated signalling, comes a variety of cross-talk mechanisms and novel Notch regulators. The Delta/Notch signalling pathway is responsible for the classic genetic example of lateral inhibition in the development of cell fates (reviewed in (Hansson et al. 2004). Delta/Serrate/Lag-2, collectively called the DSL ligands, is a family of transmembrane ligands which bind to and lead to the activation of the transmembrane receptor Notch. In the basic Delta/Notch pathway, a group of cells, that express proneural genes, are narrowed down to one cell that will continue to develop into a functional neuron. Initially all cells in this 'proneural cluster' express both Delta and Notch at relatively equal levels to prevent neuronal differentiation. This balance is achieved through inhibition of the neuronal differentiation proteins Achaete/Scute and Neurogenin by Notch signalling within the group of cells. Eventually, one cell in the cluster will express an excess of Delta to lead to strong inhibition of proneural signals in surrounding cells through a corresponding increase in Notch activation. Activation of Notch occurs through a coordinated series of three proteolytic cleavages, termed S1, S2 and S3. The S1 cleavage occurs at the *trans* Golgi network to lead to a functional receptor. The S2 cleavage in the extracellular

domain occurs in response to Delta binding and produces a Notch extracellular domain (NECD) and a membrane-bound activated Notch-extracellular removed 'NEXT' fragment that is subsequently cleaved at the intramembranous S3 site by presenilin. This cleavage event releases the Notch intracellular domain (NICD) into the cytoplasm. NICD then binds to the Su(H) complex thereby leading to transcriptional activation of the *Hairy* and *enhancer of split* (*HES*) complex which down-regulates proneural genes to result in an ectodermal, rather than a neuronal fate. Specialized subsets of Notch signalling have been described which deviate from this canonical pathway mediated through the Notch regulator deltex.

1.6.2 Deltex-dependent Notch-signalling:

Since the discovery of Notch's role in neurogenesis numerous studies have focused on defining modifiers of the standard Notch pathway. One such modifier was described very early using Drosophila genetics, the *deltex* locus, identified based on its phenotypic resemblance to both the *Notch* and *Delta* wing vein phenotypes (Morgan et al. 1931). Molecular and genetic analyses have since defined deltex as an E3 ubiquitin ligase (Cornell et al. 1999) responsible for enhancing Notch's anti-neurogenic effects independent of the classical pathways of lateral inhibition thorough *HES* (Xu and Artavanis-Tsakonas 1990; Matsuno et al. 1998; Yamamoto et al. 2001; Matsuno et al. 2002). This deltex-dependent, HES-independent pathway has since been shown to have clear roles in early neurogenesis (Ramain et al. 2001) and hematopoiesis (Deftos et al. 2000; Suzuki and Chiba 2005). This novel Notch pathway and its connections to Huntington's disease will be described in detail in later chapters with specific focus on the development of the nervous system.

Of particular significance, the deltex-dependent Notch signal has been linked to the development of the sensory bristle field in the *Drosophila melanogaster* dorsal notum, the central segment of the fruit fly back (Ramain et al. 2001). Specifically, within the dorsal notum the development of the small, microchaetae, bristles is affected by the deltex signal while the large, macrochaetae, bristles are unaffected (Ramain et al. 2001). Figure 1.4 illustrates the dorsal notum region highlighting the micro- and macrochaetae. During the development of both micro- and macrochaetae a characteristic pattern of cellular differentiation is carried out, resulting in one sensory neuron, one bristle socket cell, one support cell and one bristle shaft cell (Posakony 1994). This allows for the indirect evaluation of neuronal development through the analysis of bristle formation (Ramain et al. 2001).

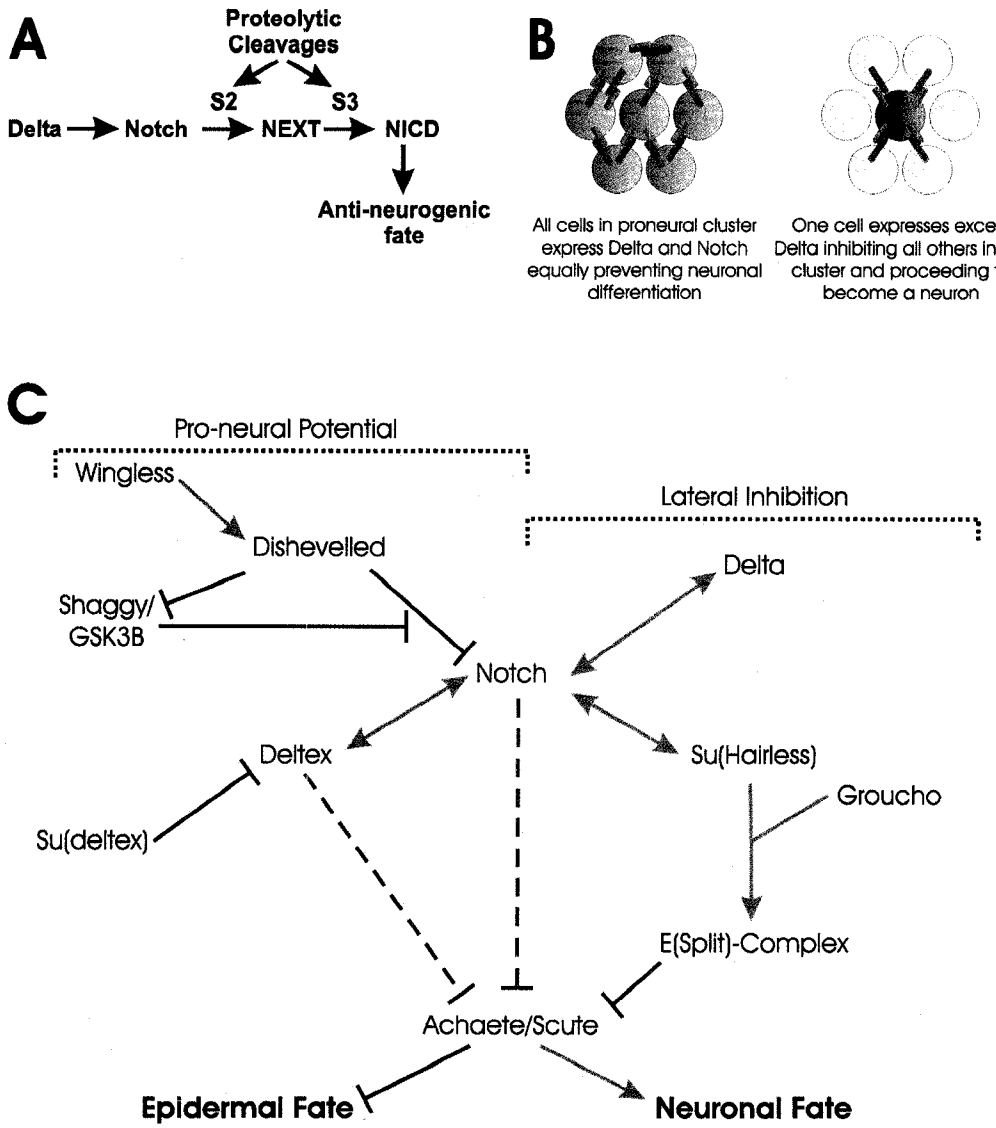

Figure 1.3: The Delta/Notch pathway (A) its role in lateral inhibiton (B) and its regulatory pathways (C). (A) Binding of Delta (Dl) to Notch (N) leads to extracellular S2 cleavage, forming the N extracellular domain (NECD) and N extracellular truncated (NEXT) fragments, followed by intracellular S3 cleavage of the NEXT fragment by presenilins releasing the N intracellular domain (NICD) and anti-neurogenic fate. (B) In the proneural cluster of equipotent cells Dl is expressed at equal amounts by all cells leading to no change in cell fate. Eventually one cell expresses excess Dl leading to inhibition of neural fate for all others through increased N dependent inhibition of neural genes. (C) Genetic and molecular data have gleaned two systems of regulation in the neuronal development through the Notch transmembrane receptor. One regulating pro-neural potential in field of equipotent cells mediated through cross talk between the Notch and Wingless networks and the other controlling the delineation of individual neurons through the classical process of lateral inhibition through the Delta/Notch network (red lines indicate inhibitory processes while green lines indicate activatory processes; double-headed arrows indicate protein-protein binding; dashed lines indicate proteasomal degradation). Adapted from (Hansson et al. 2004).

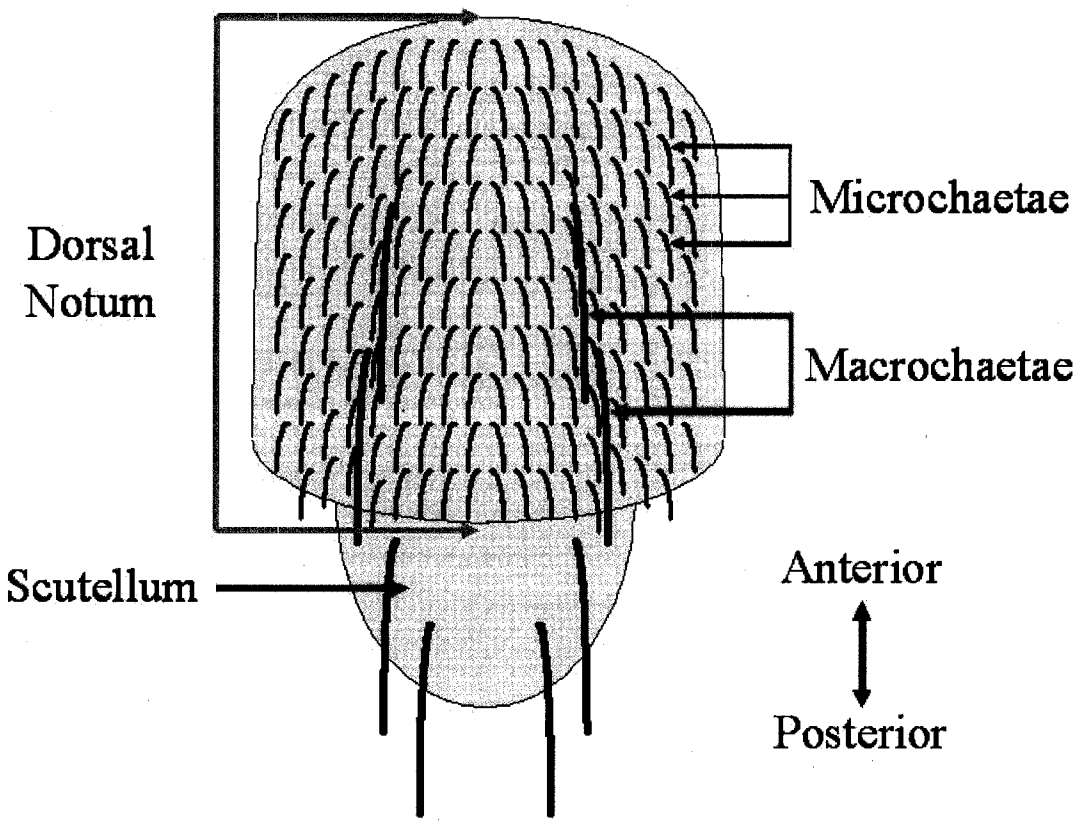

Figure 1.4: The *Drosophila melanogaster* dorsal notum. The dorsal notum contains two separate sensory bristle systems, the densely packed, smaller, microchaetae and the sparse, larger macrochaetae. The scutellum is a shield-shaped dorsal structure posterior to the dorsal notum which contains only macrochaetae.

1.7 Using Drosophila and mammalian cell culture to model human disease:

1.7.1 Drosophila as a model of human disease:

The understanding of the biology of the fruit fly, *Drosophila melanogaster*, exceeds that of most commonly studied organisms owing to over one hundred years of genetic analysis (Bier 2005). This impressive amount of genetic knowledge, coupled with the ease of culture and short life span, have resulted in Drosophila becoming one of the premier systems in which to carry out biological analysis. Recent molecular advances, such as the sequencing of the human and Drosophila genomes, have demonstrated a startling degree of evolutionary conservation (Reiter and Bier 2002; Bier 2005). A large proportion of human disease-related genes and the genes for basic cellular functions, ranging from 65-80 percent depending on comparison methods, have counterparts in the *D. melanogaster* genome (Reiter and Bier 2002; Bier 2005). This includes a well-conserved homologue of the *Htt* gene (Li et al. 1999). The high degree of genetic and functional conservation between the fruit fly and human systems suggests that Drosophila can be used to help determine the function of genes related to Huntington's disease and, in turn, help to determine points for therapeutic intervention. In addition to the great amount of genetic knowledge, the availability of both classical and novel assays of biochemical, behavioural, and physical changes allow for the identification and characterization of very subtle differences in this model system. Virtually all biochemical assays have been translated for use in the fruit fly system. Assays of physical and behavioural parameters allow for the approximation of phenotypes seen in complex human disease. For example, the natural behaviour of negative geotaxis has been used extensively in fly models of Parkinson's disease to demonstrate changes in movement similar to those seen in Parkinson's

patients (Feany and Bender 2000; Haywood and Staveley 2004). Novel assays for learning and memory allow the effective modeling of diseases, such as HD, in which these functions are abrogated (Ge et al. 2004; Presente et al. 2004). *Drosophila melanogaster* therefore represents a fantastic model for the study of complex genetic diseases of the central nervous system.

One great advantage in employing Drosophila over other model organism systems is the availability of both forward and reverse genetic screens. Forward and reverse genetics can quickly determine the existence of additional components of complex genetic systems and can quickly identify the role and location of these components in the given pathway. In addition, the introduction of inducible transgene constructs is a routine activity in Drosophila research that allows directed expression of any gene of interest in virtually any tissue/cell type specific pattern using the bipartite UAS/Gal4 ectopic gene expression system (Brand and Perrimon 1993; Brand et al. 1994). This system allows the evaluation of transgene over-expression in either normal or mutant backgrounds to efficiently evaluate biologically significant genetic interactions. As stated above, Drosophila has already been used in several Huntington's disease studies utilizing transgenics expressing poly-Q expanded Htt, or other disease related poly-Q protein fragments in order to screen for modifiers of the aggregative/ neurodegenerative phenotypes (Jackson et al. 1998; Warrick et al. 1998; Warrick et al. 1999; Chan et al. 2000; Kazemi-Esfarjani and Benzer 2000; Marsh et al. 2000; Higashiyama et al. 2002; Kazantsev et al. 2002). These findings suggest that the molecular basis of polyglutamine toxicity are conserved from flies to humans lending strong support for the use of Drosophila in the genetic dissection of HD.

1.7.2 Use of Human cell culture to investigate conservation of function:

Despite the genetic and logistical advantages afforded in the Drosophila model system, it remains a lower invertebrate system and the possibility exists that some biological divergence in function has occurred. Utilizing mammalian cell culture methods in parallel with Drosophila allows independent confirmation of functionally relevant features from either system in the other. Those mechanisms which are conserved from Drosophila to human cell culture will represent good candidates for directed therapies and, by the nature of this methodology, will also provide multiple parallel screening options for existing therapies. This methodology will allow for the rapid dissection of genetic networks and identification of potential pharmaceutical targets to accelerate drug discovery and design.

1.8 Research Goals:

As no preventative or corrective therapies exist for Huntington's disease despite the fact that the causative mutation has been unequivocally identified since 1993 (HDCRG 1993) I set out to further the understanding of the progression and pathogenesis of this disorder. In order to understand the biological dysfunctions that result in Huntington's disease, we must first understand the function of Huntingtin itself. Due to the limitations in direct study of the Huntingtin protein, one beneficial mechanism to uncover this role is through the functional analysis of Huntingtin protein partners, especially those that display altered function under disease conditions. Based on my review of the Huntingtin interacting partners characterized it was decided that a clear understanding of the biological functions of Huntingtin-interacting protein 1 was desirable. As no analysis of the function of Hip1 in Drosophila had been undertaken and given the unique advantages of the fruit fly system, analysis of the Drosophila

hip1 homologue was selected. Coupling this with mammalian cell culture methods would allow independent confirmation of those functions potentially relevant to patient situations.

The initial goals of this project were to clone and characterize *hip1* in Drosophila homologue and generate transgenic Drosophila which express *hip1* under the control of UAS enhancer element. I analyzed the effects of *hip1* over-expression in numerous tissue/cell-type specific patterns and analyzed several biological parameters for effects. Preliminary results suggested that *hip1* plays a role in the development of the Drosophila nervous system. To evaluate the relevance of any findings to human disease some parallel studies were performed in mammalian cell culture systems. A secondary goal of this project was to initiate the analysis of the Drosophila homologue of *Hippi* based on the pre-defined role of its mammalian counterpart in cell death (Gervais et al. 2002). This consisted of initial cloning and characterization of the gene as well as detailed sequence analysis to determine functional regions of the protein for future study.

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Chapter 2: Huntingtin-interacting protein 1 is a novel regulator of neurogenesis in Drosophila

2.1 Introduction:

Huntington's disease (HD) is a dominantly-inherited progressive neurodegenerative disorder characterized by cognitive, emotional and motor deficits (Petersen et al. 1999; Cattaneo et al. 2005). The manifestation of HD has been linked to the expansion of a CAG trinucleotide within the *Huntingtin (Htt)* gene which, in turn, results in an expanded stretch of glutamine (Q) residues in the Huntingtin protein (HDCRG 1993). Increasing poly-glutamine length above a 36-39 repeat threshold leads to progressively earlier onset and more severe presentation of Huntington's disease symptoms (Rubinsztein et al. 1996). The mechanism underlying this expansion phenomenon has yet to be determined.

Symptoms observed with HD patients arise from the selective death of neurons in specific regions of the brain, primarily the dramatic loss of medium-sized, spiny, GABAergic neurons in the striatum (Petersen et al. 1999). The discovery of the *Htt* gene (HDCRG 1993) has led to advances in unlocking the biological and molecular secrets that underlie HD pathogenesis. As CAG expansion in the *Htt* gene leads to Huntington's disease under normal conditions, the Htt protein must contribute to the prevention of the cellular basis of HD pathogenesis, likely through activities associated with the region of poly-glutamine expansion. The identification of proteins that interact with Huntingtin, including Huntingtin-interacting protein 1 (Hip1), have been particularly enlightening (Kalchman et al. 1997; Wanker et al. 1997). Interestingly, the protein-protein interactions between Htt and Hip1 correspondingly decrease as the poly-glutamine length increases above the pathogenic level to suggest an intimate functional link between the interaction and the cellular mechanism underlying HD (Kalchman et al. 1997; Hackam et al. 2000; Gervais et al. 2002). The identification of a role for Hip1 in intracellular

trafficking and endocytosis suggests that improper protein localization or improper regulation of downstream effects may be involved in HD pathogenesis (Mishra et al. 2001; Rao et al. 2001; Legendre-Guillemin et al. 2002; Rao et al. 2003; Hyun et al. 2004; Chen and Brodsky 2005; Legendre-Guillemin et al. 2005; Sun et al. 2005). Subtle dysfunctions associated with HD involve altered levels of adult stage neurogenesis and reduced numbers of neuronal progenitors as found in both post mortem analysis and isolated tissue culture (White et al. 1997; Metzler et al. 1999; Petersen et al. 1999; Metzler et al. 2000; Curtis et al. 2003; Lazic et al. 2004; Tattersfield et al. 2004; Cattaneo et al. 2005; Curtis et al. 2005; Gil et al. 2005; Grote et al. 2005; Jin et al. 2005). However, no functional links between Hip1 and the HD associated neurogenic phenotype have been established.

Signalling through the Notch transmembrane receptor has been linked to diverse roles in developmental and pathological pathways from the activation of programmed cell death mechanisms to the promotion of cancer progression (reviewed in (Hansson et al. 2004; Radtke et al. 2005). Notch controls lateral inhibition during neurogenesis through interactions with the Suppressor of *Hairless* [Su(H)] protein and subsequent transcriptional regulation of *Hairy and Enhancer of split* (*HES*) basic helix loop helix (bHLH) transcription factors (Hansson et al. 2004; Radtke et al. 2005). Recently, a parallel Notch-mediated signalling mechanism has been described that features the positive regulator of Notch signalling, deltex (Ramain et al. 2001). In fruit flies, this alternative deltex-dependent mechanism regulates the neuronal stem cell field in the dorsal notum, the microchaetae sensory organ precursor population (Ramain et al. 2001). This model of neuronal development allows detailed analysis of novel genetic regulators of Notch-mediated neurogenesis.

Given the parallels between HD related neurogenesis and Notch signalling, potential links between the Htt/Hip1 and Notch/deltex systems were investigated in *Drosophila melanogaster*. We have identified the Drosophila homologue of Hip1, characterized cDNA clones and produced conditional expression transgenic fly lines. Studies utilizing the Drosophila UAS/GAL4 ectopic gene expression system, demonstrated that the Hip1 gene functions in neurogenesis and that this function influences the Notch signalling network. Additionally, data suggest that this function is dependent on Hip1's ability to participate in endocytosis, mediated through its AP180 N-terminal homology (ANTH) domain. Based on review of the current understanding of Hip1 function this novel role in Notch regulation represents the first functional link between Huntington's disease and neurogenesis and may suggest a therapeutic intervention rationale.

2.2 Methods:

2.2.1 Cloning/Sequencing of Drosophila *Hip1* homologues:

Through a tBLASTn search (flybase.net/blast) of the *Drosophila melanogaster* genome utilizing the human *Hip1* homologue (Genbank Accession AAC51257) as reference, a single well-conserved *Hip1* homologue, *CG10971*, was identified. Two clones, containing putative full-length versions of Drosophila *hip1*, LD20514 and LD30041, were obtained from the Berkeley Drosophila Genome Project (BDGP). These cDNAs were subcloned and sequenced using a combination of an ABI 3100 genetic analyzer with BigDye v.3.0 chemistries and a commercial sequencing service (Cortec DNA Laboratory Services, Kingston, Ontario). The longest ORF was determined for each using the Expert Protein Analysis System (ExPASy) translate tool (ca.expasy.org/tools/dna.html). All sequence data will be submitted to appropriate databases preceding publication.

2.2.2 *In vitro* Transcription and Translation:

In vitro transcription and translation was carried out using the Promega *in vitro* T-n-T Coupled reticulocyte lysate system according to manufacturer's instructions. Briefly, 1 μg of LD20514 or LD30041 were added to *in vitro* TnT lysate mixture and transcription and translation were carried out at 30°C for 90 minutes. Radiolabelled 35S-methionine was incorporated into translated products to allow detection. Samples were run onto 4-20% Novex SDS-PAGE gels along with appropriate size standards (Invitrogen, Carlsbad, California). Gel was dried for 1 hour prior to 18 hour exposure to Kodak autoradiographic film and developing.

2.2.3 Drosophila Culture:

The *apterous* and *pannier* Gal4 transcription factor expression lines were obtained from the Bloomington Drosophila Stock Center (BDSC) (University of Indiana, Bloomington) (Calleja et al. 1996). The expression of *pannier-Gal4* radiates laterally from the midline throughout the dorsal notum, the flies back, and *apterous-Gal4* expression is greatest in lateral regions and diminishes at the midline (Calleja et al. 1996). Genetic interactions were investigated using standard genetic techniques and controlled by outcrosses to the appropriate Gal4 driver lines. The *UAS-deltex* line was obtained from Dr. Kenji Matusno (Xu and Artavanis-Tsakonas 1990; Matsuno et al. 1998). The $Notch^{MCD1}$ allele was obtained from Dr. Pascal Heitzler (Ramain et al. 2001). All crosses were performed at 25°C or 19°C as noted and all flies were maintained on standard cornmeal yeast molasses agar media based on recipes available from the BDSC (University of Indiana, Bloomington).

2.2.4 Drosophila Transgenesis:

The *Drosophila melanogaster* cDNAs LD30041 and LD20514 were individually directionally cloned into the pP[UAST] transposable element vector (Brand and Perrimon 1993), transgenics were generated by injection into *white*^{1118} (*w*^{1118}) embryos along with the transposase producing helper plasmid pHSπ and selection for *mini-w*$^{+}$ marker gene expression in progeny. All data are representative of multiple independent transgenic lines for each transgene.

2.2.5 Microchaetae density analysis:

Notum preparations were oriented with dorsal notum facing up on aluminum scanning electron microscope (SEM) studs, desiccated overnight, gold scatter coated and then photographed using a Hitachi 570 SEM. All micrographs were taken at 70X magnification, Polaroid images were scanned and analyzed using ImageJ digital image analysis software (Abramoff 2004). Counts of dorsal microchaetae number in the entire field, as defined by the edges of the dorsal notum (red dotted lines in Figure 2.2), were performed for each image. Total dorsal notum area (μm^2) was calculated with calibration of the software to an internal size standard (red dotted lines in Figure 2.2). Individual microchaetae counts and their respective area measures were used to calculate density values, expressed as number of microchaetae per 100 μm^2, via Microsoft Excel (Microsoft, California). Values for each genotype group were imported into the GraphPad Prism 4 program (Graphpad Software, San Diego California) for display and statistical analysis purposes. Means ± standard error of the mean were plotted and individual groups were subjected to one-way ANOVA analysis along with Neuman-Keuls posthoc-test to determine significance between pairs.

2.3 Results:

2.3.1 Cloning and characterization:

The *Drosophila melanogaster hip1* homologue was identified by a search of the Berkeley Drosophila Genome Project (BDGP) utilizing the tBLASTn search algorithm (www.ncbi.nim.nih.gov/BLAST). The *hip1* gene is located on the left arm of the third chromosome in the polytene chromosome section 69E. Two Drosophila *hip1* cDNA clones, representing alternate mRNA's from the CG10971 locus, clone LD30041 (CG10971-RA) and clone LD20514 (CG10971-RB) were obtained from the Berkeley Drosophila Genome Project and sequenced. As shown in Figure 2.1A, the variants have alternative inclusion of the first exon such that exon 1a (CG10971-RA) begins upstream of exon 1b (CG10971-RB). The cDNAs share a core of six identical exons 2-7. The two cDNAs differ in the site of transcriptional termination within the non-coding region of the eighth exon. CG10971-RA represents a 3881 bp transcript which encodes a predicted peptide of 1124 amino acids. CG10971-RB is a 3938 bp transcript containing two potential initiation codons spaced 18 bp apart. The Kozak sequence (Kozak 1986) for the second potential start codon is a match for the Drosophila Kozak consensus of (C/A)AA(A/C)ATG) (Cavener 1987) while the first possible start codon is less similar. Based on Kozak conformity, a 1026 amino acid protein may be the predominant protein produced from the CG10971-RB transcript.

At the amino acid level, the proteins are highly conserved in structure when compared to the predicted mammalian versions of Hip1 (Figure 2.1B). Protein domain predictions of each variant were performed using the Pfam webtool (www.sanger.ac.uk/cgi-bin/Pfam). Both the 1124 AA and 1026 AA variants contain a complete, well-conserved (Pfam E-value=7.5e-64)

ILWEQ domain at the C-terminus (Red box in Figure 2.1B). In addition, both variants contain well-conserved central pseudo death effector domain structures (Blue box in Figure 2.1B). The protein variants differ at the amino terminus. Importantly, the longer variant encodes a complete AP180 N-terminal homology domain (Orange box in Figure 2.1B, Pfam E-value=2.6e-106), which includes the putative lipid binding pocket (black box in Figure 2.1B). This region is found in all ANTH domains while it is absent in the related Epsin N-terminal homology (ENTH) domain (Sun et al. 2005). Removal of the lipid-binding pocket has been shown to eliminate the function of Hip1 in endocytic regulation of membrane receptors (Rao et al. 2003; Sun et al. 2005). The lipid binding region of Drosophila Hip1 approximates the consensus sequence of (K/G)A(T/I)x_6(P/L/V)KxK(H/Y), with the exception of L20 which is normally a T or I residue based on the consensus (Sun et al. 2005). In contrast, CG10971-RB is incapable of producing a complete ANTH domain containing this lipid-binding region and may be incapable of mediating endocytosis and vesicle trafficking. The putative start methionine residue of CG10971-RB (Arrow head in Figure 2.1B) illustrates that this variant does not encode a fully functional ANTH domain (Pfam E-value=2.4e-25 compared to 2.6e-106 in hip1). The variants are denoted as *hip1*, for CG10971-RA, and *hip1ΔANTH*, for CG10971-RB.

In vitro transcription and translation of both Drosophila transcripts was carried out in order to confirm that both open reading frames could form complete proteins as predicted by bioinformatic analysis (Figure 2.1C, ca.expasy.org/tools/#primary). Translation of *hip1* produced a product of ~125 kDa (predicted = 128 kDa) while *hip1ΔANTH* formed a product of ~113 kDA (predicted = 117 kDa) to conclude that both cDNAs could produce the predicted versions of the Hip1 protein.

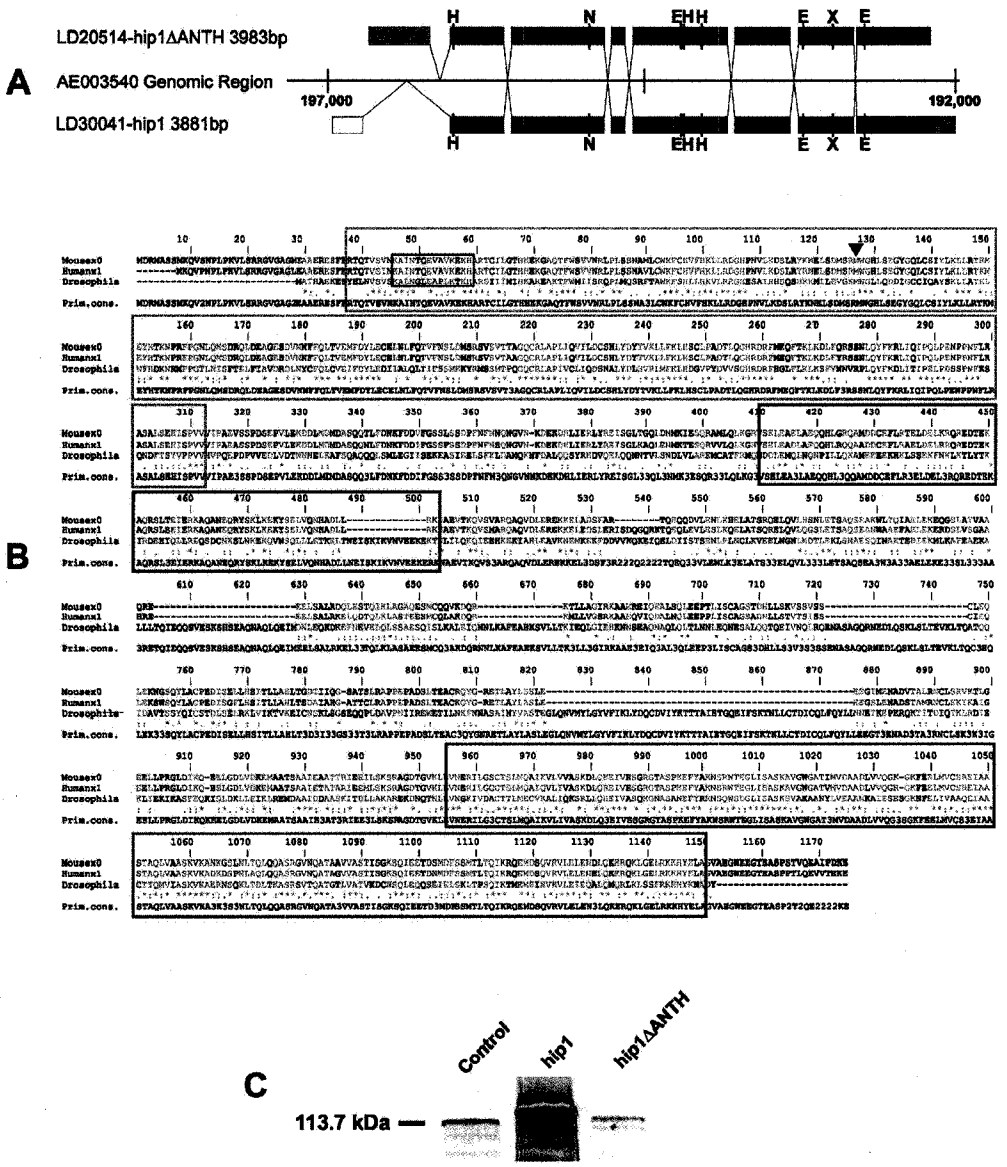

Figure 2.1: The *Drosophila melanogaster* genome contains a single well-conserved *Hip1* homologue. Alternative first exons are coloured as green for *hip1ΔANTH* and yellow for *hip1*, shared exons are shown in red and exons containing alternative termination sites are in blue. Letters represent relevant restriction enzyme sites: E=EcoRI, H=HindIII, X=XhoI, and N=NheI (A). Multiple alignment of the longest ORF in the *hip1* transcript shows that the fruit fly homologue is well conserved including: AP180 N-terminal homology (ANTH) domain (orange boxed region), pseudo death effector domain (blue boxed region), and ILWEQ domain (red boxed region). The consensus lipid-binding pocket within the ANTH domain is located in the amino terminus of longest *hip1* ORF (black box) (B). Characterization of two Drosophila *hip1* cDNAs showed that LD30041 encodes a full length protein of ~128 kDa and LD20514 encoded a truncated product lacking the lipid-binding region of ~117 kDa. *In vitro* transcription and translation showed proteins of approximately the expected sizes (Control =Poly-ADP ribose polymerase C). ► in B indicates putative start methionine for *hip1ΔANTH*.

2.3.2 Novel role for Hip1 in Sensory Bristle formation:

Expression of *hip1* was directed throughout the dorsal notum by *pannier-Gal4* and *apterous-Gal4*, and the bristle development patterns were examined. The gross morphology and development of both microchaetae and macrochaetae, as well as the density of microchaetae per 100 μm^2, of critical class individuals were analyzed. Expression of *hip1* by *pannier-Gal4* led to a decrease in density of the microchaetae bristle type throughout the dorsal notum compared to controls (Figure 2.2A&B). Similar results were obtained with *apterous-Gal4* (Figure 2.2C&D). No differences between experimental and control samples were seen in the number or position of macrochaetae in response to *hip1* expression under either transgene (see Figure 2.2A&C). No differences in gross bristle morphology were seen in response to *hip1* expression as multiple bristle/shaft or empty socket cells were not observed (see Figure 2.2A&C).

2.3.3 Hip1 function in Sensory Bristle formation is altered by the absence of the ANTH Domain:

To clarify the role of the ANTH domain in Hip1 function *hip1ΔANTH* was expressed under the control of *pannier-Gal4* and *apterous-Gal4*. As *hip1* expression decreased microchaetae density, density values were calculated on flies over-expressing *hip1ΔANTH*. Expression of this short variant of *hip1* led to density being significantly greater than controls (Figure 2.2A-D). As with *hip1* expression, no changes in gross bristle morphology or macrochaetae number/position were apparent in response to *hip1ΔANTH* expression. Thus, the lipid-binding ANTH domain is required for *hip1* to regulate microchaetae density in the sensory bristle field of the Drosophila dorsal notum.

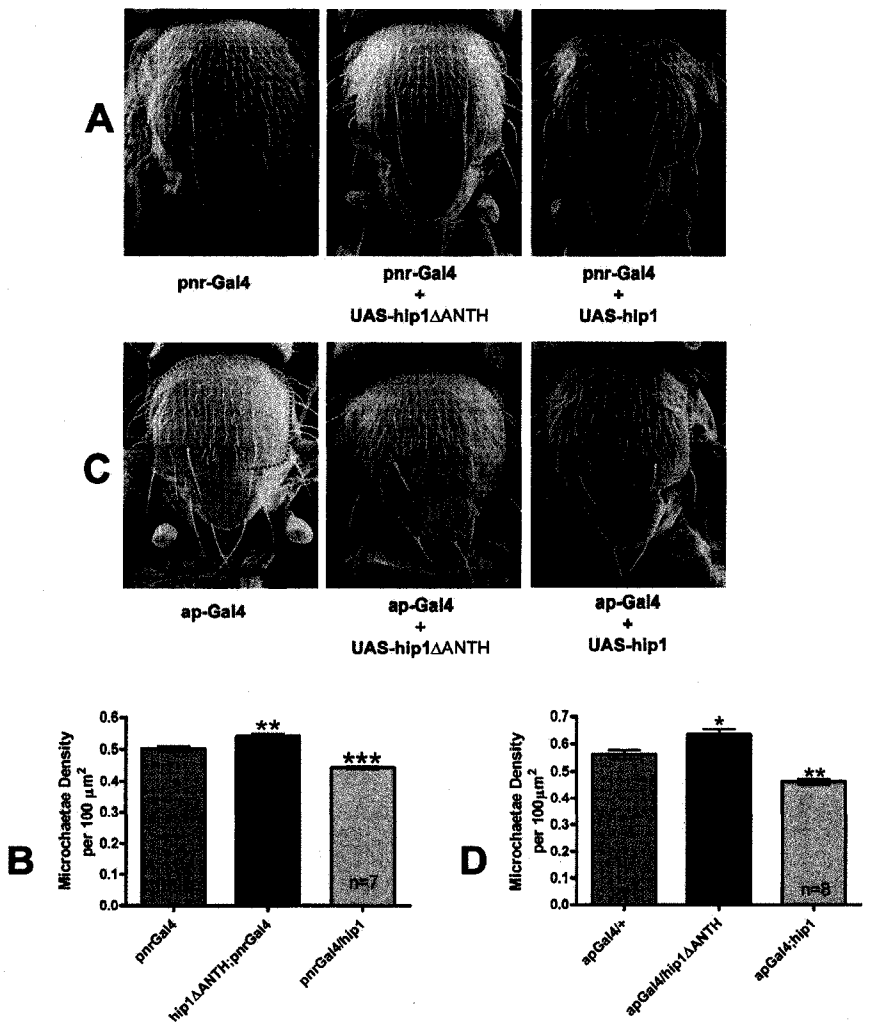

Figure 2.2: Directed expression of *hip1* and *hip1ΔANTH* in the dorsal notum differentially affects microchaetae density. Expression of *hip1* decreases and *hip1ΔANTH* increases microchaetae density under the control of either the *pannier-Gal4* (A and B) or the *apterous-Gal4* (C and D) drivers. Graphic representations of microchaetae density are shown in B and D. Total dorsal notum areas visible in micrographs, represented as red dotted lines in A and C, were included in dorsal microchaetae counts and area measurements (Scale bar in A and C represents 340 µm; Values represent mean ± SEM. *** = P<0.001; ** = P<0.01; *=P<0.05 by Neuman-Keuls posthoc-test).

2.3.4 Hip1 interacts with Deltex:

To determine the relationship of alteration to microchaetae density and deltex-mediated Notch signalling, *deltex* (*dx*) was co-expressed with *hip1* or with *hip1ΔANTH* directed by *pannier-Gal4*. Expression of *UAS-deltex* by *apterous-Gal4* in this pattern led to pupal lethality. At 25°C, *pnr-Gal4/UAS-deltex* expression leads to a near complete loss of microchaetae in the dorsal notum (Ramain et al. 2001). At 19°C, *dx* expression greatly reduces the number of microchaetae in the region of *pannier* expression. Many remaining bristles develop improperly as socket only, double, triple, or groups of five or more bristles, presumably, as excess proneural cluster cells adopt the bristle fate (Figure 2.3A). When *deltex* and *hip1* are co-expressed this phenotype is greatly enhanced to near complete elimination of microchaetae in the region of *pannier* expression (Figure 2.3A&B). Contrary to the effects of *hip1*, co-expression of *hip1ΔANTH* partially suppresses the *pannier/deltex* phenotype, to significantly increase total microchaetae number (Figure 2.3A&B). In addition, the presence of abnormal bristle formations is reduced (Figure 2.3C). This indicates that *hip1* and *deltex* co-operate in the specification of microchaetae in the Drosophila dorsal notum. As *hip1ΔANTH* can counteract the effects of *deltex* expression, both upon bristle morphology and bristle number, these data suggest a dual role for the ANTH domain in microchaetae differentiation.

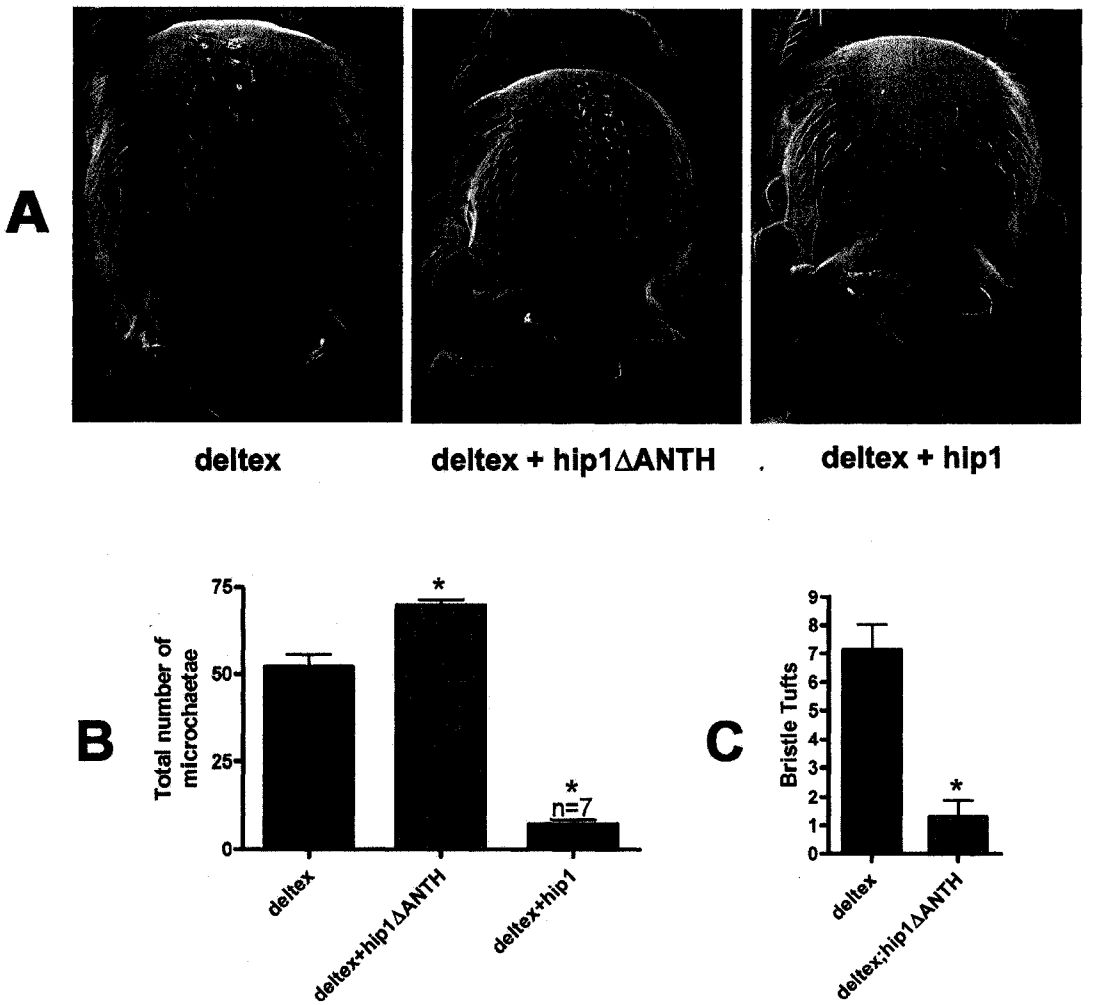

Figure 2.3: Interaction between *hip1* and *deltex*. Both *hip1* and *hip1ΔANTH* were co-expressed with *deltex* in the *pannier-Gal4* expression pattern. (A) *hip1* enhances while *hip1ΔANTH* suppresses the effects of *pannier-Gal4/deltex alone*. (B) Analysis of total single bristle number shows marked rescue of the *deltex* phenotype by *hip1ΔANTH* and enhancement by *hip1*. (C) Analysis of abnormal bristles shows that *hip1ΔANTH* is capable of suppressing the developmental defects of bristle formation associated with *deltex* expression (Scale bar represents 340 μm; Values represent mean ± SEM. * in B =P<0.001 by Neuman-Keuls posthoc-test * in C= P<0.0001 by two-tailed T-test).

2.3.5 Hip1 interacts with microchaetae deficient alleles of Notch:

As *hip1* and *hip1ΔANTH* are capable of modifying the effects of *deltex* expression in the dorsal notum, analysis of expression in a $Notch^{MCD1}$ (N^{MCD1}) genetic background (Ramain et al. 2001) was undertaken to investigate an additional link to the deltex-dependent Notch signalling pathway. The *pannier-Gal4* driver was used to direct *hip1* and *hip1ΔANTH* expression in the N^{MCD1} background at 25°C and analyzed as above. Both *hip1* and *hip1ΔANTH* are capable of modifying the microchaetae densities of the N^{MCD1} phenotype (Figure 2.4A&B). The N^{MCD1} phenotype is enhanced by *hip1* while *hip1ΔANTH* suppresses the microchaetae deficiency.

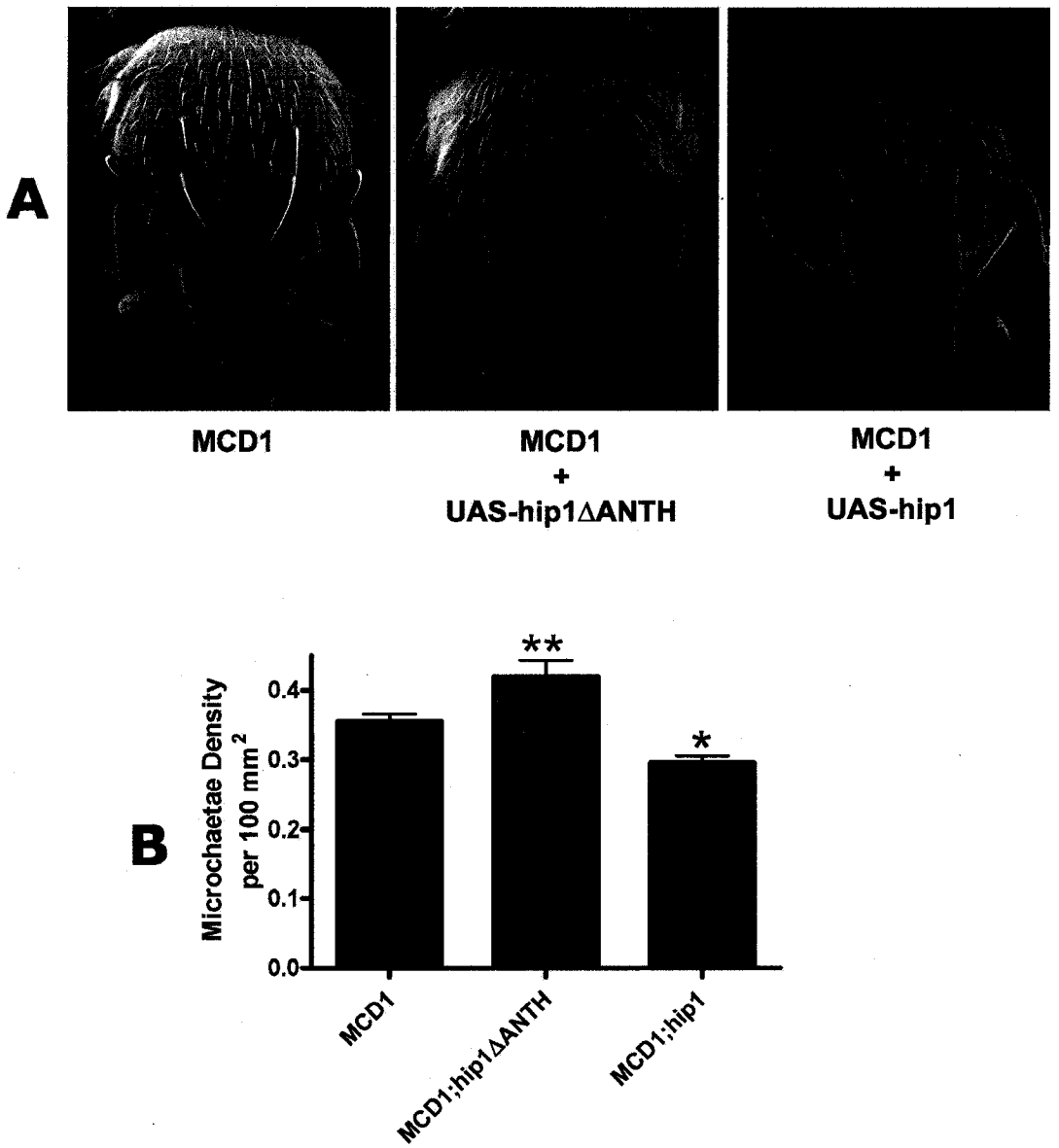

Figure 2.4: *hip1* interacts with *Notch*^{MCD1}. Both *hip1* and *hip1ΔANTH* were expressed in a *Notch*^{MCD1} genetic background in the *pannier-Gal4* expression pattern progeny were compared to *Notch*^{MCD1}; *pannier-Gal4* animals. (A) *hip1* enhances while *hip1ΔANTH* suppresses the *Notch*^{MCD1} phenotype. (B) Analysis of microchaetae density shows suppression of the *Notch*^{MCD1} phenotype by *hip1ΔANTH* and enhancement by *hip1* (Scale bar represents 340 μm; Values represent mean ± SEM. **=P<0.01; *=P<0.05 by Neuman-Keuls posthoc-test).

2.4 Discussion:

Originally, Hip1 was discovered as a binding partner of the Huntingtin protein (Kalchman et al. 1997; Wanker et al. 1997). As this interaction is reduced with polyglutamine expansion, Hip1 is a strong candidate for involvement in HD pathogenesis (Kalchman et al. 1997; Gervais et al. 2002). Since initially discovered, a clear role for Hip1 in clathrin-mediated endocytosis and intracellular trafficking has been well established (Kalchman et al. 1997; Wanker et al. 1997; Gervais et al. 2002; Rao et al. 2002; Metzler et al. 2003; Rao et al. 2003; Hyun et al. 2004). Although Hip1 "knockout" mice have dysfunctions in spermatogenesis, in hematopoietic differentiation and in the regulation of AMPA receptor trafficking, the role of Hip1 in development is not well understood (Rao et al. 2001; Metzler et al. 2003; Oravecz-Wilson et al. 2004). While activation of apoptotic cell death pathways by Hip1 suggests a neuronal cell death mechanism in HD (Hackam et al. 2000; Gervais et al. 2002), other studies indicate that Hip1 over-expression promotes tumourigenesis and is necessary for cellular survival (Rao et al. 2002; Rao et al. 2003). As a result, the biology of Hip1 remains elusive.

The Drosophila notum develops from a neuronal precursor tissue field and through the directed expression of *hip1* throughout the dorsal notum; we have demonstrated that full length *hip1* decreases microchaetae density and that *hip1* lacking a functional ANTH domain leads to an increase in microchaetae density (Figure 2). As the number of individual bristles can be correlated to the number of sensory neurons formed (Jan and Jan 1994; Ramain et al. 2001), these very different changes in bristle density show that *hip1* is capable of a dual-regulatory role in neurogenesis mediated by the presence of a functional ANTH domain. To the best of our knowledge this is the first account of a neurogenic phenotype associated with

Huntingtin-interacting protein-1 and, importantly, the first account of complex neurogenic regulation by any Huntingtin-interacting protein.

As the presence or absence of the lipid-binding ANTH domain changes the role of Hip1 in neurogenesis, this suggests that Hip1 executes its regulation through altered vesicle endocytosis or trafficking. Originally, the amino terminus of Hip1 was suggested to include an Epsin N-terminal homology (ENTH) domain that is structurally related to, but functionally distinct from, the ANTH domain family (Sun et al. 2005). The larger ANTH domain participates in internalization of endocytotic vesicles at PhsIns(4,5)P2 rich regions of the lipid membrane through the highly conserved lipid binding pocket (black box in Fig 1A), absent in all described ENTH domains (Sun et al. 2005). The removal of this domain from Sla2P, the yeast homologue of Hip1, leads to an ablation of Sla2P's endocytic ability (Sun et al. 2005). Early deletion studies removed the lipid-binding pocket to produce a "dominant negative" product similar to our *D. melanogaster hip1ΔANTH* construct (Rao et al. 2002). Multiple splice variants of human Hip1 transcript (Chopra et al. 2000), coupled with two immunoreactive bands detected in Western blots (Chopra et al. 2000; Gervais et al. 2002), suggest the possibility of multiple protein isoforms of human Hip1. This suggests neurogenic control via a binary switch mechanism from the anti-neuronal full length to the pro-neuronal ΔANTH variants.

The deltex-dependent Notch (N/dx) signal maintains the pre-neuronal stem-cell population and modification of this signal leads to alteration of the dorsal microchaetae neuronal field fate. The hip1 and deltex protein products physically interact in whole genome yeast two-hybrid analysis (Giot et al. 2003). Increasing the activity of positive modifiers or decreasing

the activity of negative regulators of the N/dx signal lead to enhancement of the reduced microchaetae density phenotype of the Microchaetae Deficient (MCD) alleles of *Notch*, a phenotype duplicated by *deltex* over-expression (Ramain et al. 2001). Over-expression of *deltex* in the dorsal notum at 25°C led to a near complete loss of microchaetae while loss-of-function alleles of *Notch* or *deltex* result in increased microchaetae density (Ramain et al. 2001; Tattersfield et al. 2004). *hip1* over-expression is reminiscent of Notch activation and *deltex* over-expression (Figure 2.2A&B). Conversly, directed expression of *hip1ΔANTH* leads to an increase in microchaetae density that mirrors the N/dx pathway mutations (Figure 2.2A& B; (Ramain et al. 2001)). When expressed with *deltex*, *hip1* was capable of enhancing the phenotype (compare Figure 2.3A panels 1 and 3). Conversely, *hip1ΔANTH* suppresses the bristle reduction phenotype and *deltex*-dependent bristle deformation (Figure 2.3A-C). Therefore, *hip1* and *deltex* appear to be intimately linked and the nature of *hip1*'s effects depends on the presence or absence of a functional ANTH domain.

Our data support a model of altered neuronal development in HD resulting, to some degree, from a change in the balance of activity of Huntingtin interacting protein 1. Hip1/Htt interactions are decreased when poly-Q levels reach pathologic levels (Kalchman et al. 1997; Wanker et al. 1997). The finding that increased poly-Q levels lead to increased levels of neuronal precursor proliferation in human patients may reflect an imbalance in the activities of the pro- and anti-neurogenic Hip1 isoforms caused by altered Htt binding (Curtis et al. 2003; Tattersfield et al. 2004; Curtis et al. 2005). Disruption of interactions in the disease may be expected to alter the ability of Hip1 to lead to changes in both the number of neuronal precursors and mature neurons formed. The novel role of Huntingtin interacting protein-1 in

neurogenesis may aid in the clarification of the molecular mechanisms underlying Huntington's disease.

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Chapter 3: Hip1 and deltex regulate neurogenesis through Achaetae-Scute

3.1 Introduction:

Notch signalling has been linked to diverse roles in developmental and pathological pathways (reviewed in (Hansson et al. 2004). Primarily, Notch is involved in lateral inhibition in the process of neurogenesis. This occurs through interactions with the suppressor of *Hairless* [Su(H)] protein and subsequent transcriptional regulation through hairy and enhancer of split (HES) basic helix loop helix (bHLH) transcription factors. More recently, a novel Notch-mediated signalling mechanism has been described which functions in parallel with the canonical pathway of transcriptional regulation (Ramain et al. 2001). This pathway involves the positive Notch regulator deltex and the negative Notch regulator dishevelled. Deltex was subsequently shown to be an E3 ubiquitin ligase, capable of altering the transcriptional activity of Achaete-Scute Homologue 1 (ASH1), a pro-neural bHLH transcription factor that is antagonized by Notch activation (Yamamoto et al. 2001; Hori et al. 2004). Negative regulation of ASH1 levels has been linked to transcriptional activity, through the classic Notch pathway, and protein stability and turnover through the ubiquitin proteasome degradation pathway (Sriuranpong et al. 2002). The E3 ubiquitin ligase responsible for this proteasomal regulation of ASH1 has yet to be determined. Chapter 2 of this thesis demonstrates that Huntingtin-interacting protein 1 (Hip1) is a novel regulator of the deltex-dependent Notch signal. Here a potential mechanism for this regulation involving the direct actions of Hip1 and deltex on protein and transcript levels of ASH1 is described. Both Drosophila genetic models and the human pre-neuronal cell line Ntera2-D1 demonstrate that mechanisms involved in the Hip1/deltex/Notch signalling pathway are well conserved between insects and mammals. Short interfering RNA-directed knockdown of *Hip1* and *deltex1* in the Ntera2-D1 cell line prior to neuronal differentiation led to a significant decrease in *ASH1* mRNA levels. In

addition, Hip1 associates with ASH1 at endogenous levels in undifferentiated Ntera2-D1 cells. Genetic studies in the fruit fly using our recently described model of *hip1*-regulated neurogenesis demonstrate that the observed effects of *hip1*, and the inhibitory *hip1ΔANTH*, are sensitive to *acheate* gene dosage.

3.2 Methods

3.2.1 siRNA effects upon Hip1/Deltex in retinoic acid-induced neuronal differentiation:

Custom siRNA duplexes were designed against 3'UTR regions of *hip1* and *deltex1*, and siRNA duplexes against *GL2* luciferase were included as a control (Sigma-Proligo, Boulder, Colorado). siRNA sense and antisense sequences used in this experiment are as follows: *hip1*-sense-5'-CUC AUU GGU GGU AGC CAU CdT dT-3', *hip1*-antisense-5'-GAU GGC UAC CAC CAA UGA GdT dT-3'; *deltex1*-sense-5'-UUG UCU UCG GCC AAC CAG GdT dT-3', *deltex1*-antisense-5'-CCU GGU UGG CCG AAG ACA AdT dT-3'; *GL2* sense-5'-CGU ACG CGG AAU ACU UCG AdT dT-3', *GL2*-antisense-5'-UCG AAG UAU UCC GCG UAC GdT dT-3'. All siRNA duplexes were premixed and diluted to 50 μM in sterile ribonuclease free water.

NT2-D1 cells were plated at ~15-30,000 per well of 24-well Falcon cell culture plates (BD Biosciences, Mississauga, Ontario) in GIBCO GlutaMax™ high glucose media containing sodium pyruvate and antibiotics. Cells were allowed to attach for 24 hours prior to experimental manipulations. siRNA transfection was carried out using Lipofectamine 2000 reagent (Invitrogen Canada Inc, Burlington, Ontario) according to manufacturers instructions for siRNA transfection. At 24 hours post-seeding (~25-30% confluency) cells were transfected with siRNA duplexes (20 pmol) or transfection reagent (1 μl) alone as noted. Cells were

grown for 48-72 hours in the presence of transfection mixture prior to the induction of differentiation to ensure adequate gene silencing. Cells were then treated with 10 μM all-trans retinoic acid (RA) dissolved in dimethyl sulfoxide (DMSO) as described previously (Andrews 1984). Total cellular RNA was collected at 0 h, 24 h, 48 h, and 72 h post RA treatment using QIAShredder and RNeasy mini columns with additional DNase digestion as per manufacturer's instructions (Qiagen, Mississauga, Ontario).

3.2.2 Gene Expression measured by TaqMan Real Time PCR:

Total RNA concentrations and quality for each sample were determined using a NanoDrop spectrophotometer. 100-200 ng total RNA was used as template in RT-PCR reactions using Multiscribe™ reverse transcription reagents as per manufacturer's instructions (PE Applied Biosystems, Foster City, California). Relative mRNA expression for *hip1*, *deltex1*, *ASH1*, *HES1*, *Notch1*, *DLK1*, *Neurogenin*, *NeuroD1*, or *GRIN1* in each sample were determined using Applied Biosystems 7500 Real Time PCR instrument with commercially available probe/primer mixes for each gene and human 18S ribosomal RNA control according to manufacturer's instructions (PE Applied Biosystems, Foster City, California). ΔΔCt was calculated based on 18S ribosomal RNA and untreated control counts and is represented as a fraction of untreated levels. Figures represent means ± standard error of the mean of three replicates per treatment. Statistical significance was determined using one-tailed ANOVA analysis along with Neumann-Keuls posthoc-test for significance between pairs.

3.2.3 Western/Co-IP Protocol:

Co-immunoprecipitation studies were conducted essentially as described (Gervais et al. 2002). Briefly, confluent NT2-D1 cells from two T75 culture flasks were collected and lysed in

buffer containing nonidet P-40 (NP-40) (Gervais et al. 2002). Approximately 500 µg of total protein lysate was brought to a final volume of 1000 µl using ice-cold NP-40 buffer. Antibodies were added as follows to separate lysate aliquots: 10µl of Hip14B10 (Abcam, Cambridge UK), 25µl of MASH1 (Chemicon, Temecula California) (Figure 3.3) and samples were placed at 4°C on an end over end mixing platform over night to allow formation of antibody-protein complexes. EZview™ Protein G/A sepharose beads (Sigma Canada Ltd, Oakville, Ontario) were added as appropriate and immunoprecipitation was carried out for 2-3 hours at 4°C on an end over end mixing platform. Bead complexes were washed three times with ice-cold NP-40 buffer after which samples were split into two equal aliquots. One aliquot of each pair was boiled in 2X sodium dodecyl sulfate (SDS) sample buffer, containing 0.8% SDS, 20mM Tris HCl pH6.8, 4% Glycerol, 0.02% bromophenol blue, supplemented with 0.14 M β-mercaptoethanol while the other was boiled in 2X SDS-buffer without β-mercaptoethanol. Samples were then separated onto 4-20% Novex SDS-PAGE gels (Invitrogen Canada Inc, Burlington, Ontario), Western blotted, and probed with indicated antibodies (1:15,000 dilution of each). Visualization of protein bands was carried out using appropriate HRP-conjugated secondary antibodies, goat anti-mouse-HRP for Hip14B10 and mouse anti-rabbit-HRP for MASH1 (BD Biosciences, San Diego, California) along with pico chemiluminescent reagent (Pierce Biotechnology Inc., Rockford IL) and images were captured using Kodak Biomax autoradiographic film (Kodak, New Haven CT). Mock immunoprecipitation lacking primary antibody, immunoprecipitation using various members of the Notch signalling network, and 50 µg of untreated NT2 lysate were used as specificity controls.

3.2.4 Genetic interaction of Deltex and Hip1 systems with *achaete*, *Enhancer of split*, and *GSK3β*:

To assess the sensitivity of *pannier-Gal4*; *UAS-hip1* and *pannier-Gal4*; *UAS-hip1ΔANTH* phenotypes to dosage of *achaete*, crosses to a hypomorphic allele, *achaete*^{4} (*ac*^{4}), were performed. Briefly, *ac*^{4}/*ac*^{4} homozygous virgin females were crossed to *pannier-Gal4*; *UAS-hip1* and *pannier-Gal4*; *UAS-hip1ΔANTH*. The control arose from similar crosses to *pannier-Gal4* males. Critical class male and female progeny were collected for microchaetae density analysis as described previously (Chapter 2 of this thesis). Similarly, to determine if *UAS-hip1* and *UAS-hip1ΔANTH* are capable of modifying increased density of microchaetae seen in *Enhancer of split* [*E(spl)*] mutants, the transgenes were expressed in *E(spl)*^{1} mutant backgrounds. To determine the effects of *GSK3β* expression on *pannier-Gal4*; *UAS-hip1* and *pannier-Gal4*; *UAS-hip1ΔANTH* phenotypes *UAS-GSK3β* was co-expressed with each and the *pannier-Gal4* driver alone. Both *ac*^{4} and *E(spl)*^{1} alleles were obtained from the Bloomington Drosophila Stock Center. *UAS-GSK3β* was made through standard subcloning and microinjection procedures (BES, unpublished). All crosses were performed at 25°C on standard cornmeal-yeast-molasses-agar medium.

3.2.5 Microchaetae density analysis:

Notum preparations were oriented with dorsal notum facing up on aluminium scanning electron microscope (SEM) studs, desiccated overnight, gold scatter coated and then photographed using a Hitachi 570 SEM. All micrographs were taken at 70X magnification, Polaroid images were scanned and analyzed using ImageJ digital image analysis software (Abramoff 2004). Counts of dorsal microchaetae number in the entire field, as defined by the edges of the dorsal notum (red dotted lines in Figure 3.2), were performed for each image.

Total dorsal notum area (μm^2) was calculated with calibration of the software to an internal size standard (red dotted lines in Figure 3.2). Individual microchaetae counts and their respective area measures were used to calculate density values, expressed as number of microchaetae per 100 μm^2, via Microsoft Excel. Values for each genotype group were imported into the GraphPad Prism 4 program for display and statistical analysis purposes. Means ± standard error of the mean were plotted and statistical significance was determined using one-tailed ANOVA analysis along with Neumann-Keuls post-test for significance between pairs.

3.2.6 Immunocytochemistry in N-tera2/D1 cells:

Cells were plated on Lab-Tek II chambered coverglass (4-well format .5ml total culture volume, Nalge Nunc International, Rochester NY), precoated with Poly-L-Lysine (Sigma, St. Louis, MO) and grown overnight in normal DMEM medium (Invitrogen Canada Inc, Burlington, Ontario). Cells attached to the bottom of chamber were washed twice with PBS then fixed in 3% paraformaldehyde–PBS solution (pH 7.5) for 30 min at room temperature. Cells were washed twice for 10 min in PBS supplemented with 10 mM glycine. Cells were permeabilized by incubation in 1% Triton-PBS solution for 5 min at room temperature followed by two washes in PBS supplemented with 10 mM glycine. Cells were blocked overnight at 4 °C in a humidified chamber with 4% Normal Donkey Serum (NDS) in PBS. Cells were stained for 1 h at 4 °C with 1:100 dilution of Hip14B10 and active Notch1 antibody or control solutions lacking primary antibody followed by two washes in PBS–glycine buffer. Cells were then incubated for another hour in the dark at 4 °C with secondary antibodies, Alexa 594 donkey-anti-rabbit IgG and Alexa 488 donkey-anti-mouse IgG (Molecular Probes, Eugene, OR), at a 1:200 dilution. Cells were given a final round of two washes in

PBS+glycine (in a light protected chamber) before mounting overnight with ProLong Antifade medium (Molecular Probes, Eugene, OR) supplemented with 1.5 μg/ml DAPI nuclear co-stain. Images were scanned with a Zeiss LSM 510 META confocal microscope.

3.3 Results:

3.3.1 siRNA knockdown of *Hip1* or *deltex* reduces *ASH1* expression during NT2 neuronal differentiation:

In order to clarify the molecular mechanics of the co-operation between Hip1 and deltex, NT2 cells were treated with several different siRNA duplexes directed against 3' UTR regions of *Hip1* or *deltex*. SiRNA duplexes directed against *GL2 luciferase* were included as a control and all mRNA levels were standardized to levels calculated for Lipofectamine 2000-treated controls. The *hip1* and *deltex* mRNA levels were reduced by 60-70% by respective siRNA treatment (Figure 3.1A/B). The *ash1* mRNA levels were significantly reduced by treatment of NT2 cells with either *hip1* or *deltex* siRNAs prior to RA induced differentiation (Figure 3.1C). These data show that both Hip1 and deltex are required during the process of RA induced neuronal differentiation of Ntera-2 D1 cells. These findings confirm our previous findings that Hip1 and deltex co-operate in the process of neurogenesis and that this function is conserved between Drosophila and mammals.

3.3.2 siRNA knockdown of *Hip1 or deltex* have no effect on *HES1* expression during NT2 neuronal differentiation:

Upon activation of the canonical Su(H)-dependent Notch mechanism *hairy and Enhancer of split* (*HES*) mRNA levels are increased to prevent neuronal differentiation (Kageyama et al.

1997). Studies of the deltex-dependent Notch signalling network have noted that *HES1* mRNA levels are not increased upon activation of this alternative pathway (Hori et al. 2004). Similarly, levels of *HES1* mRNA, a common transcriptional marker for activation of the canonical Su(H)/Notch anti-neurogenic signal (Kageyama et al. 1997), remained unchanged in response to all siRNA treatments suggesting that the canonical Notch pathway is not influenced by *Hip1* or *deltex* siRNA treatment (Figure 3.1D).

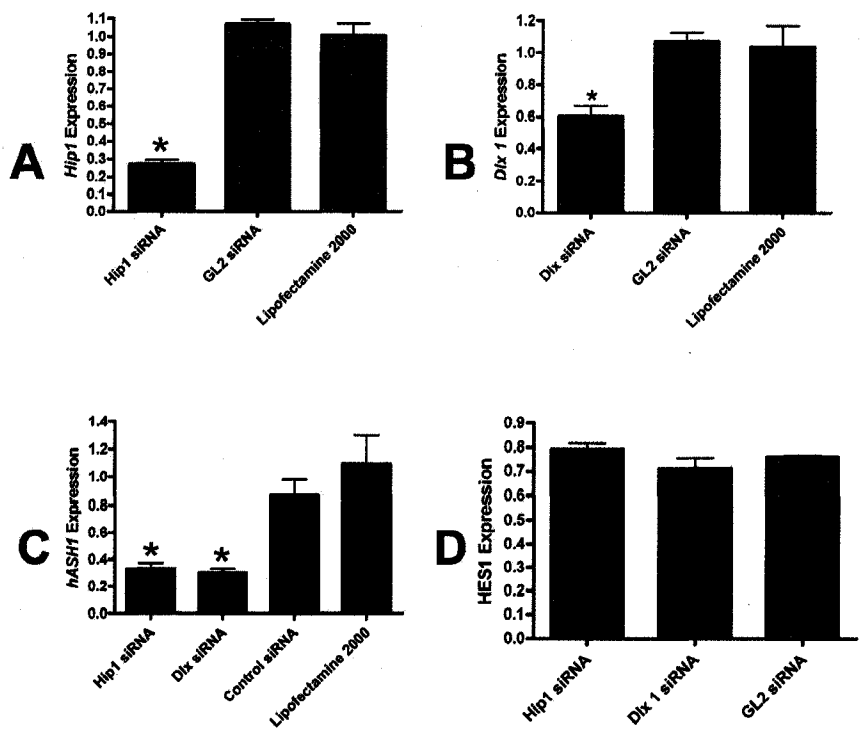

Figure 3.1: Effects of *Hip1* or *deltex (Dlx)* siRNA treatment on their respective mRNAs and the *ASH1* and *HES1* mRNA messages as measured by TaqMan Real Time PCR assay. Treatment of NT2 cells with either *Hip1* or *deltex* siRNA significantly reduces the expression levels of respective mRNAs relative to control siRNA treated cells A and B, respectively. After 72 hours of differentiation induced by retinoic acid, levels of *ASH1* mRNA were significantly reduced relative to controls in response to *Hip1* or *deltex* siRNA treatment (C) while levels of *HES1* mRNA remained unaffected (D) (Values represent mean ± SEM. *=$P<0.001$ in A/C and $P=<0.01$ in B by Neuman-Keuls posthoc-test).

3.3.3 siRNA knockdown of Hip1/Deltex has no effect on *Notch1*, *DLK1*, *Neurogenin*, *NeuroD1*, or *GRIN1* expression during NT2 neuronal differentiation:

***Notch1* and *DLK1*:**

Gene expression of *Notch1* was analyzed to ensure that the observed changes in *ASH1* mRNA levels were not secondary responses to changes in this upstream modifier of the *ASH1* gene (Kageyama et al. 1997). The expression levels of *Notch1* remain unchanged in response to siRNA treatment of NT2 cells (Figure 3.2). This suggests that the observed changes in *ASH1* levels are mediated by the loss of *Hip1* or *deltex1* and not by changes in upstream modifiers. Expression levels of *DLK1* between replicate samples were inconsistent and therefore no conclusions could be drawn (data not shown).

***Neurogenin, NeuroD1,* and *GRIN1*:**

Expression of *Neurogenin*, *NeuroD1*, and *GRIN1* indicate the sequence of gene expression in the progression from stem cell to differentiated neuronal fate in Ntera2-D1 cells (Hartley et al. 1999; Przyborski et al. 2000). Within the 72 hour period of retinoic acid induced differentiation no changes in *Neurogenin*, *NeuroD1*, or *GRIN1* were observed (data not shown). This indicates that within the period of RA treatment naive NT2 cells had not progressed fully to the neuronal fate.

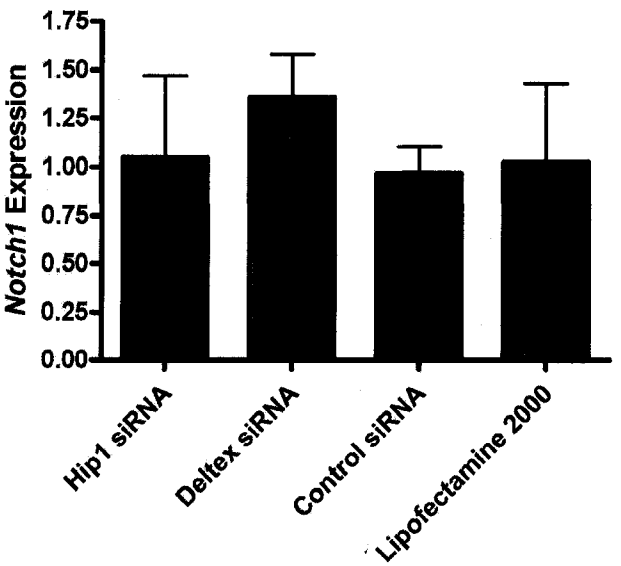

Figure 3.2: ***Hip1* or *deltex* siRNA treatment has no effect on expression levels of *Notch1*.** Treatment of NT2 cells with either *Hip1* or *deltex* siRNA has no effect on the expression levels of *Notch1*, as measured by TaqMan Real Time PCR assay, indicating that no changes occurred in this upstream regulator of *ASH1* expression (Values represent mean ± SEM. Insignificant changes =P>0.05 by Neuman-Keuls posthoc-test).

3.3.4 Hip1 interacts with ASH1 in NT2 neuronal precursors:

Figure 3.3A and B demonstrates that Hip1 antibody is capable of co-precipitating ASH1 while the ASH1 antibody is capable of co-precipitating Hip1 at endogenous levels from untreated NT2-D1 cellular extracts. This suggests that Hip1 and ASH1 interact physically within the cell and lends further evidence to the theory that Hip1 is functional in ASH1 regulation. Taken together the results of *Hip1* and *deltex* siRNA treatment along with co-immunoprecipitation studies demonstrate that Hip1 co-operates with deltex in transmitting a HES1-independent, deltex-dependent Notch signal in human pre-neuronal cells.

3.3.5 ASH1 protein is stabilized in deltex siRNA treated cells:

Given that *ash1* mRNA levels are reduced in response to siRNA treatment, either *ash1* is transcriptionally down-regulated in response to RA treatment (Ichimiya et al. 2001) or ASH1 is spared from proteasomal degradation and leads to its own transcriptional down-regulation. To determine if siRNA knock down of *Hip1* or *deltex* led to ASH1 protein stabilization protein samples were collected at 0 h and 72 h post RA treatment. Western blotting using anti-hASH1 antibody showed that in *deltex* siRNA treated samples that had undergone 72 h RA treatment ASH1 protein was detectable whereas ASH1 was undetectable in control and pre-treatment samples (Figure 3.3C). The finding that ASH1 protein levels are increased when *ASH1* mRNA levels are decreased suggests that protein stabilization and not up-regulation of transcription is responsible for the increase in ASH1 protein presence.

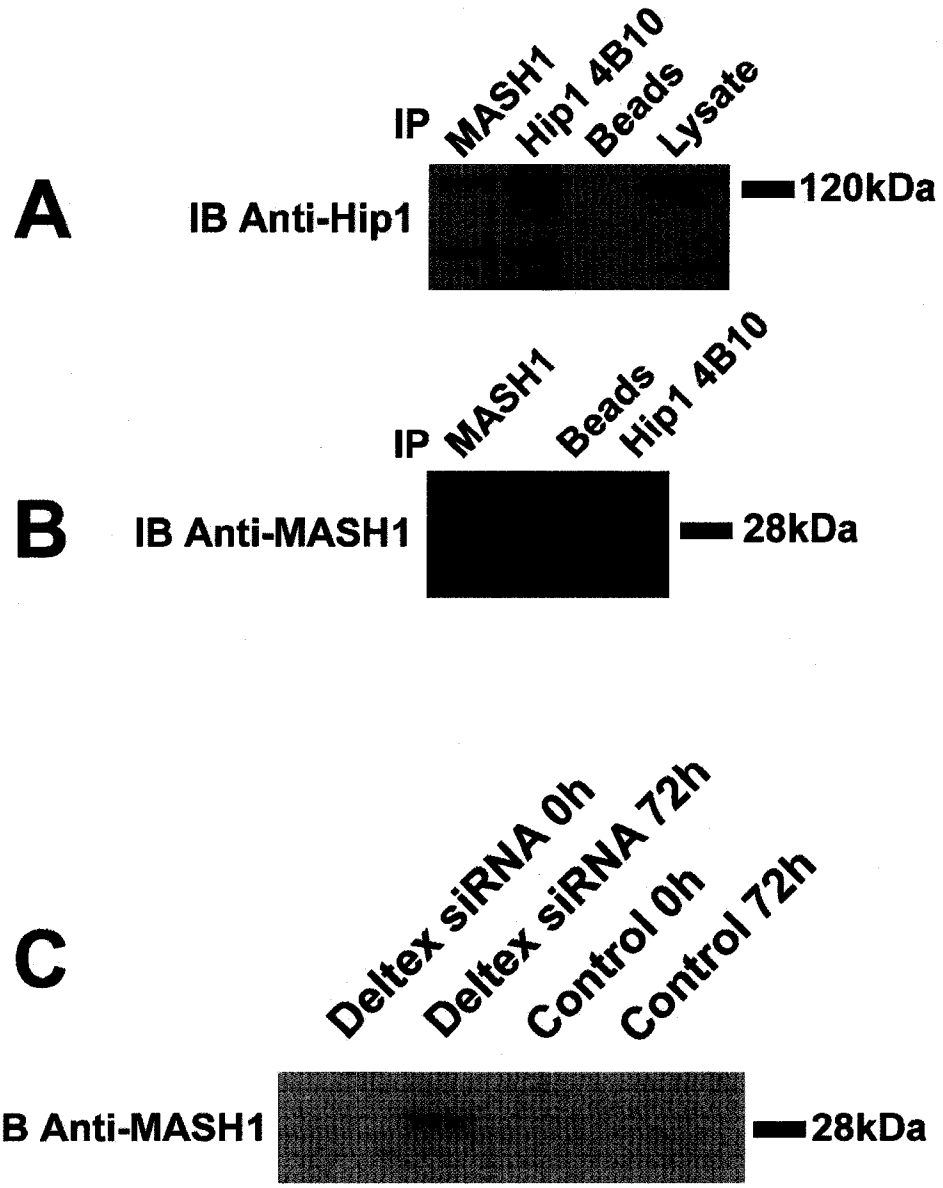

Figure 3.3: Hip1 interacts physically with ASH1 in naive NT2 cells and ASH1 is stabilized following differentiation in *deltex* siRNA treated cells. Immunoprecipitation of NT2 lysates using antibodies directed against mammalian ASH1 (MASH1), Hip1 (Hip14B10), and protein A sepharose beads lacking primary antibody show that Hip1 and ASH1 specifically co-precipitate. Hip1 is co-precipitated using anti-MASH1 (A) while ASH1 is co-precipitated with anti-Hip14B10 (B). This experiment was performed in triplicate with similar results. Western blotting of protein extracts from pre-differentiation (O hours of retinoic acid treatment) or post-differentiation (72 hour retinoic acid treatment) *deltex* siRNA or control treated cells shows that in *deltex* siRNA treated samples ASH1 protein levels are significantly higher than control levels as triplicate blots show similar results (C).

3.3.6 Neurogenic phenotypes in Drosophila:

3.3.6.1 hip1 requires achaete to regulate neurogenesis:

In Drosophila *hip1* plays a dual role in the regulation of deltex-dependent Notch signalling in the dorsal notum microchaetae sensory bristle field (Chapter 2). The directed expression of *hip1* in the developing dorsal notum results in a decreased bristle density while *hip1ΔANTH* leads to increased bristle density consistent with the effects of altered Notch activity. To determine if the effects of *hip1* and *hip1ΔANTH* over-expression are modified in response to the gene dosage of *achaete*, we expressed each transgene in both a heterozygous female and hemizygous male's ac^4 genetic backgrounds. As the *achaete* locus is present on the Drosophila X chromosome, analysis of heterozygous females for ac^4, a severe hypomorphic allele, would be expected to have a reduction in *achaete* expression (Dubinin 1932) and ac^4 hemizygous males should have little or no *acheate* function. *hip1* over-expression in $ac^4/+$ females was capable of reducing microchaetae density (Figure 3.4A). Importantly, expression of the same transgene in the ac^4/Y males did not lead to a reduction in bristle density (Figure 3.4B). *hip1ΔANTH* expression in either the $ac^4/+$ or ac^4/Y backgrounds did not lead to altered bristles densities. Taken together these data show that *hip1* and *hip1ΔANTH* require the presence of functional levels of *achaete* to modulate dorsal notum microchaetae density. In addition, these results demonstrate that the increased microchaetae phenotype seen with *hip1ΔANTH* over-expression is sensitive to changes in *achaete* expression.

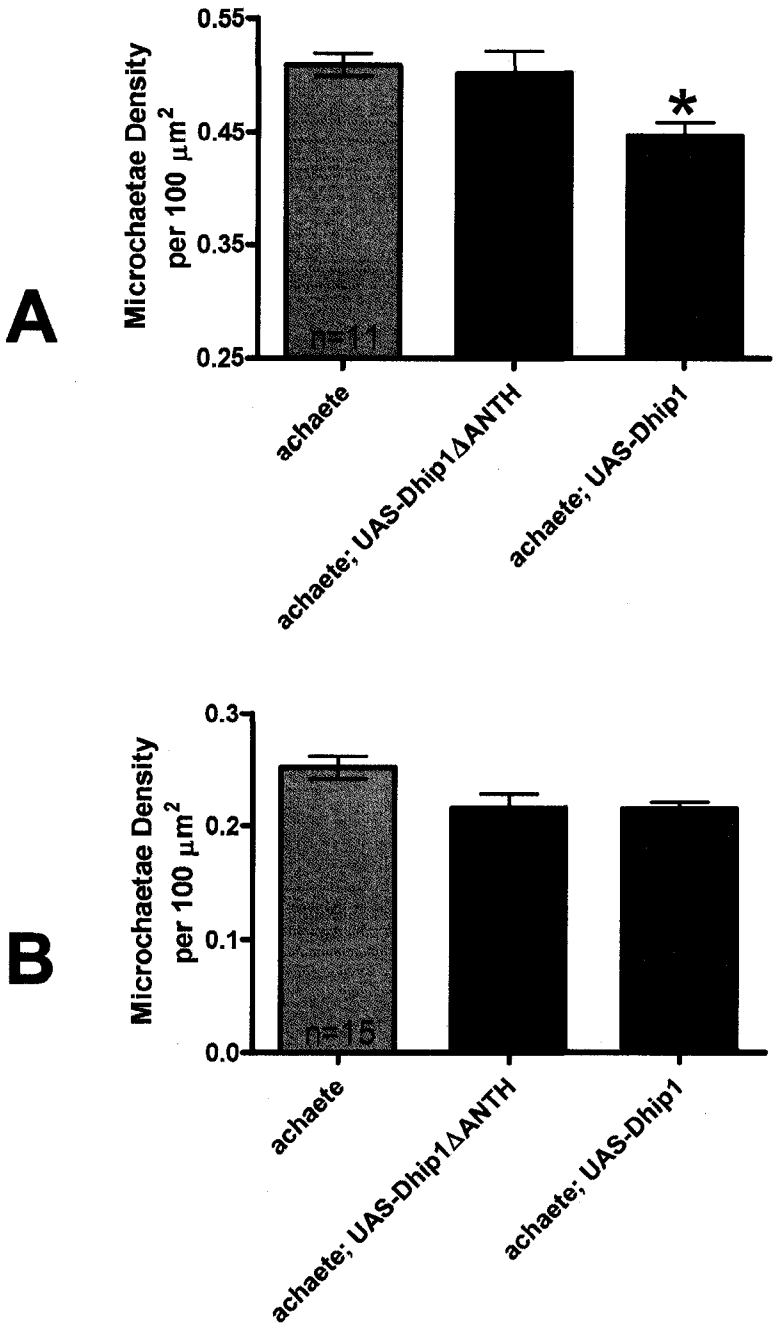

Figure 3.4: *achaete* gene dosage modifies the effects of *hip1* and *hip1ΔANTH* on microchaetae density. When *achaete* gene dosage is reduced in heterozygous females for the *achaete*^{4} allele pro-neurogenic phenotypes associated with *hip1ΔANTH* are blocked while anti-neurogenic *hip1* phenotypes are not (A). In males hemizygous for *ac*^{4}, approximating a null situation, both pro- and anti-neurogenic phenotypes are blocked (B) (values represent mean ± SEM. *=P<0.05 by Neuman-Keuls posthoc-test).

3.3.6.2 hip1 does not alter E(spl)^1-mediated increases in bristle density:

Mutations in *Enhancer of split* lead to increased microchaetae density through the canonical deltex-independent, HES1-dependent subsection of the Notch signalling pathway. As NT2/D1 cells (Figure 3.1) and experiments in Drosophila (Chapter 2) have suggested that Hip1 acts through the deltex-dependent arm of the pathway, sensitivity of *hip1* to the HES1-dependent portion of the neurogenic pathway was examined. When *hip1* and *hip1ΔANTH* are actively expressed in an *E(spl)*^{1} mutant background, no changes to the *E(spl)*^{1}-mediated increase in microchaetae density was observed (Figure 3.5). This suggests that Hip1's function in neurogenesis occurs through the E(spl) -independent pathway.

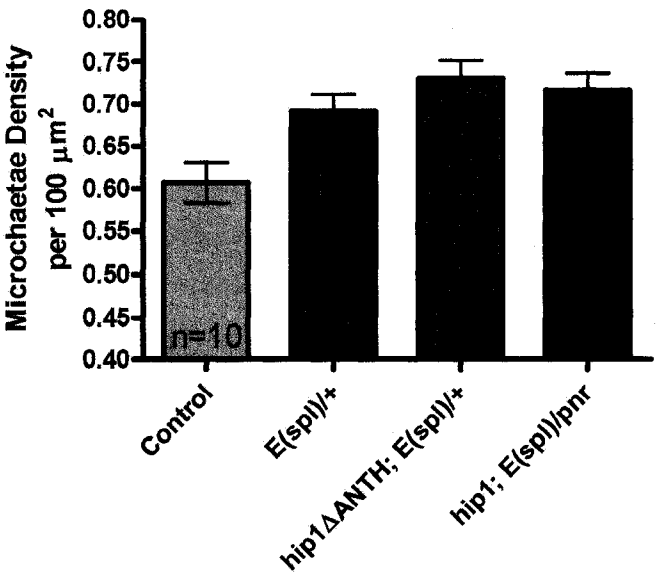

Figure 3.5: *hip1* and *hip1ΔANTH* have no effect on *Enhancer of split* induced increases in microchaetae density. *E(spl)/+* heterozygous females have significantly increased microchaetae density compared to control animals. Expression of *hip1* or *hip1ΔANTH* has no effect on this *E(spl)*-induced increase (values represent mean ± SEM).

3.3.6.3 Co-expression of GSK3β blocks the effects of hip1 and hip1ΔANTH on neurogenesis:

The deltex-dependent, HES1-independent Notch signal has been linked to the activity of *shaggy*, the Drosophila *GSK3β* homologue, with mutations in *shaggy* suppressing the effects of $Notch^{MCD}$ alleles (Ramain et al. 2001). To determine if the anti-neurogenic and neurogenic activities associated with the *hip1* isoforms are altered by GSK3β we co-expressed *hip1* and *hip1ΔANTH* with human *GSK3β* and analyzed microchaetae density. The expression of *GSK3β* alone did not alter microchaetae density from control levels (Figure 3.6). Interestingly, the microchaetae density of animals co-expressing either *hip1* or *hip1ΔANTH* with *GSK3β* also showed no differences from control levels (Figure 3.6). This finding suggests that over-expression of GSK3β blocks the activity of both *hip1* and *hip1ΔANTH*.

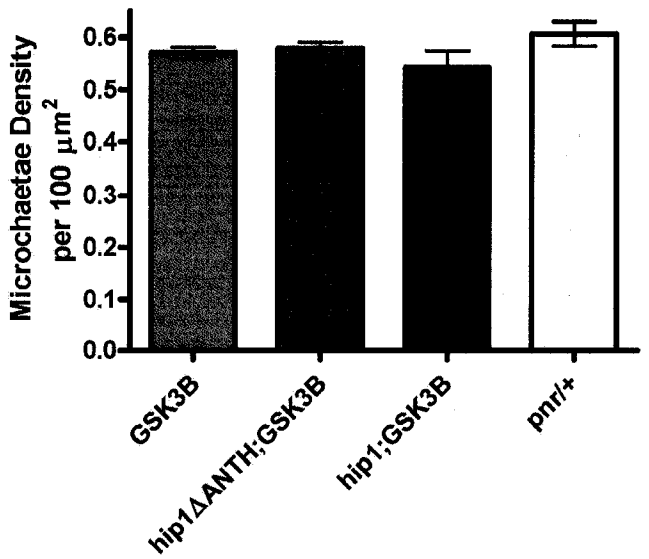

Figure 3.6: *GSK3β* co-expression prevents *hip1* and *hip1ΔANTH*-induced neurogenic phenotypes. Expression of *GSK3β* alone has no effect on microchaetae density as no significant change from the *pnr/+* control is apparent. However, co-expression of *GSK3β* with *hip1* or *hip1ΔANTH* prevent the anti- and pro-neurogenic effects, respectively (values represent mean ± SEM).

3.3.7 Activated-Notch1 co-localizes with Hip1 in NT2 cells:

Confocal analysis of Hip1 (Green) and activated Notch1 (Red) immunostaining in NT2 cells show that the two proteins extensively co-localize throughout the cytoplasm (Supplemental Figure 3.1). This suggests that Hip1 and activated-Notch1 may interact during signal activation. As these findings are preliminary additional experiments will be necessary to confirm the nature of this potential interaction. The Notch receptor is transported from the plasma membrane to various endocytic compartments during activation (Le Borgne 2006) and Hip1 has been shown to alter endocytic transport of membrane bound receptors (Rao et al. 2003). The present findings of co-localization of Hip1 and activated-Notch1 when taken with these findings suggest that Hip1 may be critical for the proper endocytosis, trafficking, and endocytosis of Notch.

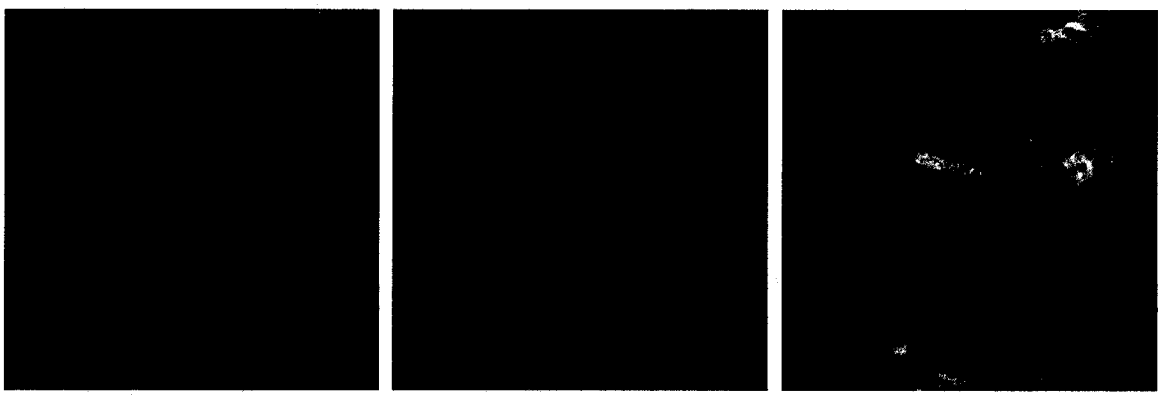

Figure 3.7: Hip1 colocalizes with activated-Notch1 in NT2-D1 cells. Immunostaining for Hip1 (green) and activated Notch1 (red) show extensive co-localization throughout the cytoplasm of NT2-D1 cells (yellow/orange).

3.4 Discussion:

In the standard version of the Notch pathway, ligand binding leads to anti-neuronal fate by up-regulation of transcription by *Hairy and Enhancer of split* (Kageyama et al. 1997). During mammalian neurogenesis, Notch signalling alters protein and transcript levels of the pro-neural ASH1 transcription factor (Sriuranpong et al. 2002). The degradation of ASH1 protein occurs rapidly through a proteasome-dependent pathway while down-regulation of *ash1* transcription occurs over a longer time period following the up-regulation of the HES1 anti-neural transcription factor (Sriuranpong et al. 2002). This activity appears to involve the poly-glutamine, OPA, domain of the Notch receptor as OPA deletion mutants block the proteasomal degradation of ASH1 (Sriuranpong et al. 2002). Analysis of MNS neuronal precursor cells reveal that deltex, like Notch, is capable of reducing activity from an ASH1 responsive transcriptional reporter independent of HES1 activity (Yamamoto et al. 2001). Consistent with findings in Drosophila (Ramain et al. 2001), this suggests the existence of deltex-independent, HES1-dependent and deltex-dependent, HES1-independent functions of Notch in mammalian neurogenesis.

Huntingtin-interacting protein 1 was demonstrated to be a novel regulator of neurogenesis through deltex-dependent Notch signalling in *Drosophila melanogaster* (Chapter 2). To determine if this novel neurogenic role for Hip1 was conserved, we analysed human neuronal precursor, Ntera-2/D1, cells that express Hip1 (Gervais et al. 2002) and additional Notch pathway components (Ichimiya et al. 2001; Walsh and Andrews 2003). Pre-neuronal Ntera-2/D1 cells could be induced to differentiate into mature neurons by retinoic acid treatment (Andrews 1984). The siRNA-mediated knockdown of *Hip1* or *deltex* in Ntera-2 cells led to

down-regulation of *ASH1* mRNA levels after 72 hours of differentiation (Figure 3.1C). As mRNA levels of *HES1* remain unchanged in response to siRNA treatment, the observed changes in *ASH1* do not appear due to the typical Notch/Su(H)/HES1 pathway (Figure 3.1D). As *ASH1* levels are reduced in response to RA-induced differentiation in Ntera-2/D1 cells, *ASH1* down-regulation may be necessary for continued differentiation along the neuronal lineage (Ichimiya et al. 2001). As no expression changes occur in the additional upstream modifiers of ASH1, *Notch1* and *DLK1*, in response to siRNA treatment (Figure 3.2A/B) the changes in *ASH1* are likely due to the direct action of Hip1 and deltex. ASH1 activates downstream determinants of neuronal fate, the transcriptional down-regulation of *ASH1* may therefore represent a feedback mechanism by which ASH1 is removed once it has performed its required activities. Alternatively, the ASH1 protein levels could be quickly decreased by a proteasome-dependent mechanism indicating that protein stabilization may be involved in the mRNA decrease. As it has been shown that Hip1 and deltex interact physically (Giot et al. 2003) and genetically (Chapter 2) the finding that Hip1 complexes with ASH1 at endogenous levels (Figure 3.3A/B) suggests a potential mechanism for the ASH1 proteasomal degradation. As deltex has been shown to be an E3 Ubiquitin ligase, the coincident interaction of Hip1, ASH1, and deltex may promote ASH1 degradation. As ASH1 is stabilized upon differentiation in *deltex* siRNA treated cells supports this theory and suggests that at least under our conditions deltex may be functioning as the E3 Ubiquitin ligase responsible for ASH1 regulation (Figure 3.3C). Notably, this model does not exclude the negative feedback loop of ASH1 on its own transcript. If ASH1 persists at high levels in the cell, it may act to repress its own transcription by an undefined mechanism.

As *achaete* is required for progression along the neuronal fate, reduced levels of the protein in mutant hemizygous males and heterozygous females would be expected to alter the anti- and pro-neural activities previously described for the directed expression of *hip1* and *hip1ΔANTH*, respectively. The finding that the anti-neurogenic function of *hip1* is blocked in the absence of achaete, in *achaete*^{4} hemizygotes, while maintained when *achaete* gene copy number is halved suggests that achaete is the target of *hip1*'s anti-neural activity (compare first and third column of Figure 3.4). If functional levels of achaete remain, as in the female *achaete* heterozygotes, *hip1* can still perform its anti-neural function by blocking the activity of remaining achaete, consistent with results in NT2 cells. In contrast, the pro-neural activity of *hip1ΔANTH* is blocked upon both reduction and absence of functional achaete (Figure 3.4). This finding can also be explained in the context of our proposed model of Hip1/deltex regulation of achaete. As achaete appears to be a key mediator of Hip1/deltex-related neurogenic regulation, it would be predicted that the anti-neural form of Hip1, *hip1ΔANTH*, would also act through achaete regulation. Activation of achaete, in response to *hip1ΔANTH* would be negated upon the reduction of functional levels of achaete in either heterozygous or hemizygous states. Importantly, as *hip1* and *hip1ΔANTH* are both insensitive to mutation in the traditional Notch-signalling pathway, *E(spl)*^{1}, suggests that the deltex-dependent, HES1-independent pathway and not the traditional Notch pathway is involved in Hip1-mediated effects on neurogenesis. As Drosophila *hip1* induced anti- and pro-neurogenic phenotypes are sensitive to *achaete* gene dosage (Figure 3.4) and insensitive to changes in *E(spl)* levels (Figure 3.5), this indicates the evolutionary conservation of the Hip1/deltex/ASH1 pathway in the control of neurogenesis.

Early characterization of the deltex-dependent, HES1-independent Notch signal suggested links between this network and Wingless signalling (Ramain et al. 2001). In particular it was

shown that mutations in *shaggy*, the Drosophila version of GSK3β, suppressed the effects of microchaetae deficient alleles of Notch. Consistent with this both *hip1* and *hip1ΔANTH* actions in neurogenesis are blocked by co-expression of *GSK3β* (Figure 3.6). This suggests a key role for Wingless signalling in maintaining activity of Hip1 with GSK3β correcting perturbations in microchaetae density in either direction in response to increased *hip1* or *hip1ΔANTH*. It will be interesting to determine if the control of Hip1 activity displayed by GSK3β is dependent on its classical role in kinase signalling cascades or an independent function yet to be fully characterized.

Overall these findings clearly show that Hip1's role in neurogenesis is conserved from fruit flies to human neuronal systems. In particular the deltex-dependent, HES1-independent nature of Hip1's role neuronal development in both systems is clearly shown by these data. Further study, especially in the characterization Hip1/Deltex regulation of ASH1 and the GSK3β regulation of Hip1 activity will aid in detailed characterization of this novel neurogenic mechanism. In turn these findings may allow for a better understanding of the neurogenic dysfunctions associated with Huntington's disease and other neurological conditions.

3.5 References:

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Chapter 4: Further characterisation of the relationship between *huntingtin* and *hip1*

4.1 Introduction:

The following studies were undertaken to determine if Drosophila and humans followed similar functional trends with respect to the co-localization of Hip1 and Huntingtin and Hip1 function in neurogenesis.

In mammalian cell culture and mouse immunocytochemical analyses Huntingtin and Hip1 co-localize in both NT2 cells and *in situ* in brain sections (Kalchman et al. 1997; Hackam et al. 2000; Gervais et al. 2002). As these interactions have been characterized in either neuronal cell systems or the mammalian brain, investigations of Hip1 and Huntingtin in Drosophila focussed on the brain. Based on Western blot analyses of immunoprecipitation and protein extract samples, it was determined that a human polyclonal anti-Hip1 antibody [described in (Kalchman et al. 1997)] recognizes a protein in Drosophila resembling Hip1 while an anti-human Huntingtin antibody recognizes a very large molecular weight protein resembling the Drosophila Huntingtin protein.

In addition, studies utilizing novel tools under development and testing for further studies on *hip1* and *hip1ΔANTH* function are presented. An inhibitory RNA method for the down-regulation of endogenous *huntingtin* in Drosophila using a transgenic construct (Gunawardena et al. 2003) was evaluated. Expression of this construct in the dorsal notum does not appear to have an effect on microchaetae density in combination with *hip1* or *hip1ΔANTH* transgenes. Data from potential P-element mutations within the *hip1* locus are also presented here. Homozygous insertional mutants display increased microchaetae density when compared with control animals. As loss-of-function mutations in *deltex*, *Notch*, and *GSK3β* all lead to

increased microchaetae density (Ramain et al. 2001) these results suggest that the insertion may represent a mutation in the *hip1* locus. In particular, based on the dual role of *hip1* and *hip1ΔANTH* in neurogenesis, this insertion may represent a loss-of-function of *hip1* or a gain-of-function of *hip1ΔANTH*.

4.2 Materials and Methods:

4.2.1 Analysis of Drosophila protein extracts using anti-human polyclonal antibodies:

Whole Drosophila protein extracts were obtained as follows: 4 to 8 frozen flies were placed into a 1.5 mL microcentrifuge tube and 150 µL of ice-cold Hepes/EDTA buffer containing phosphatase/protease inhibitor cocktail was added. Hepes/EDTA buffer composition was as follows: 100 mM KCl, 20 mM Hepes, 5% glycerol, 10 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, 1 Complete Mini protease inhibitor tablet (Roche Molecular Biochemicals, Mannheim, Germany), 20 mM β-glycerophosphate, and 100 µM orthovanadate. Flies were homogenized on ice until consistent using a rotary homogenizer. Samples were centrifuged at 14,000 rpm and 4°C for 5 min. Clarified supernatant containing protein was isolated to a new tube. 50-100 µg of total protein extracts were separated on 4-20% SDS-PAGE gels and electroblotted onto nitrocellulose membranes for subsequent Western blotting.

4.2.2 Immunoprecipitation:

Protein for immunoprecipitation experiments was isolated as above. 250-500 µg of total protein extract was immunoprecipitated overnight essentially as described (Gervais et al. 2002) with the exception that Hepes/EDTA buffer was used instead of NP-40 buffer. Anti-Hip1, anti-Hippi, and anti-Huntingtin antibodies were added to aliquots and samples were placed at 4°C on an end over end mixing platform overnight to allow formation of antibody-

protein complexes. Protein A sepharose beads (Sigma, St. Louis, MO) were added and immunoprecipitation was carried out for 2 to 3h at 4°C on an end over end mixing platform. Bead-antibody-protein complexes were washed three times with ice-cold Hepes/EDTA buffer. Aliquot was boiled in 2X SDS-buffer without ß-mercaptoethanol. SDS-buffer lacking ß-mercaptoethanol is incapable of separating antibody light and heavy chains allowing the visualization of immunoprecipitates which may run at levels similar to either the light or heavy chains in SDS-PAGE. Samples were then separated onto 4 to 20% Novex SDS-PAGE gels, Western blotted, and probed with indicated antibodies. Mock immunoprecipitation lacking primary antibody was used as a control for precipitation specificity.

4.2.3 Western Blotting:

Drosophila protein extracts were immunoblotted with a rabbit polyclonal antibody directed against the human Hippi protein (Hackam et al. 2000; Gervais et al. 2002). Immunoprecipitates were also immunoblotted with anti-Hippi. Blots were blocked in 5% non-fat milk in Tris buffered saline-Triton X-100 (TBS-T) prior to incubation with primary antibodies, 1:1000 dilution in TBS-T, for 2 to 4 hours at room temperature on a shaking platform. Blots were washed 3 X 20 minutes in TBS-T prior to incubation with horseradish peroxidase-linked secondary antibodies, 1:10,000 dilutions in TBS-T, for 1 hour. Membranes were washed 3 X 20 minutes in TBS-T to reduce background, exposed to chemiluminescent reagent and documented on autoradiographic film (for suppliers see Methods for Chapter 3).

4.2.4 DIG-labelling of *hip1*:

To determine the mRNA expression pattern of *hip1*, digoxigenin (DIG) labelled DNA probes directed against each transcript were made. DIG labelling was carried according to

manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, ~1000 ng of *hipl* full length cDNA product were denatured by boiling for 10 minutes followed by quick chilling on ice to prevent renaturation. Hexanucleotide random labelling primers, dNTP labelling mix, and Klenow enzyme were added to denatured DNA and labelling was carried out at 37 °C overnight. The reaction was halted by the addition of 2 µl 0.2M EDTA.

4.2.5 *In situ* hybridization to larval brain/discs:

Third instar w^{1118} larvae were dissected in phosphate buffered saline (PBS), fixed in 4% formaldehyde for 15-20 minutes and dehydrated in a methanol and ethanol series. The carcasses were probed with the previously described DIG labeled anti-*hipl* DNA probe. To visualize *hipl* mRNA alkaline phosphatase labeled anti-DIG antibodies were incubated with the carcasses. Following antibody treatment samples were subjected to alkaline phosphate treatment as per the Roche Applied Science DIG application manual. The larval CNS and imaginal discs were dissected out completely and examined under light microscopy.

4.2.6 Immunohistochemical analysis of Hippi in frozen sections of Drosophila:

Staining of the adult CNS was carried out as follows. Whole Drosophila heads were mounted in O.C.T. mounting medium (Tissue Tek, Elkhart, IN) before being sliced in a cryotome at -20°C. Sections were mounted on microscope slides. As these experiments were performed at Merck Frossts centre for therapeutic research access to fresh Drosophila material was limiting thus frozen sections of the Drosophila head were used in these studies. Slides were blocked for one hour in phosphate buffered saline containing 0.3% Triton X-100 and 10% normal donkey serum (10% NDS/PBT). The slides were then exposed to primary antibody at a 1:100 dilution

in 10% NDS/PBT overnight at 4°C. Slides were then rinsed 3 X 5 minutes in PBT and incubated in anti-rabbit Alexa 488 secondary antibody (Molecular Probes, Eugene, OR) at a 1:250 dilution in NDS/PBT in 10% NDS/PBT for one hour. Slides were rinsed an additional 3 X 5 minutes and stored at -20°C prior to image capture on a Ziess Axiovert confocal microscope.

4.2.7 Co-expression of *hip1* or *hip1ΔANTH* with *epidermal growth factor receptor (EGFR)*:

Pannier-Gal4; UAS-hip1 or *pannier-Gal4; UAS-hip1ΔANTH* flies (described in Chapter 2) were crossed to a *UAS-EGFR* line obtained from the BDSC (University of Indiana, Bloomington IN). Effects of *EGFR* Progeny were controlled by crossing to *pannier-Gal4* alone. Critical class progeny were prepared for SEM as described in Chapter 2 and photographed using a Hitachi 570 SEM.

4.2.8 Expression of Drosophila *huntingtin* inhibitory RNA transgenic in the dorsal notum:

UAS-htt RNAi transgenic construct was obtained from Dr. L.S. Goldstein (Gunawardena et al. 2003). *UAS-htt RNAi* was crossed to *pannier-Gal4* and w^{1118} to investigate the effects of *UAS-htt RNAi* on microchaetae density in the dorsal notum. Both *pannier-Gal4* and *UAS-htt RNAi* were crossed to w^{1118} as controls for *UAS-htt RNAi* effects. Critical class progeny were prepared for SEM as described in Chapter 2 and photographed using a Hitachi 570 SEM. Microchaetae density was then calculated and plotted as described in Chapter 2.

4.2.9 P-element 'local hop' mutagenesis of the *hip1* locus:

Bioinformatic analysis of the 69E chromosomal region, containing *hip1*, using the BDGP transposon insertion BLAST tool identified one transposon, *EP3193*, ~17kb from the

transcriptional start of *hip1*. This transposable element was mobilized by crossing the *EP3193* mutant line to a transposase source fly line as previously described (Tower et al. 1993). Sub-lines were isolated which had undergone mobilization and stable re-insertion of the *EP3193* element. These lines were evaluated for phenotypic similarity to *Notch* and *deltex* alleles in both wing vein and microchaetae density defects.

4.3 Results:

4.3.1 Probing immunoprecipitates and Drosophila protein extracts using anti-human Hip1 and anti-human Huntingtin antibodies reveal proteins similar to Hip1 and Huntingtin:

Western blots of both protein extract and immunoprecipitations using the anti-human Hip1 antibody recognized several bands which could represent the Drosophila Hip1 protein (Figure 4.1A and B). The largest of these proteins (~125 kDa) is very similar to the predicted size of hip1 and is recognized in both western blots of whole fly extracts and immunoprecipitations. This strongly suggests that the anti-human Hip1 antibody is capable of recognizing the Drosophila Hip1 protein. Importantly, non-specific bands present in whole fly western blots visualised with this antibody are not present in immunoprecipitation blots, to suggest that these are independent of the anti-human Hip1 antibody. These experiments could be easily expanded using the *hip1* and *hip1ΔANTH* transgenic lines described in Chapter 2. These transgenic lines can over express *hip1* under the control of the Gal4 transcription factor. Performing Western blot, immunoprecipitation, or immunostaining studies on samples from both expressing and non-expressing animals would show, definitively, if the human anti-Hip1 antibody is recognizing the Drosophila protein.

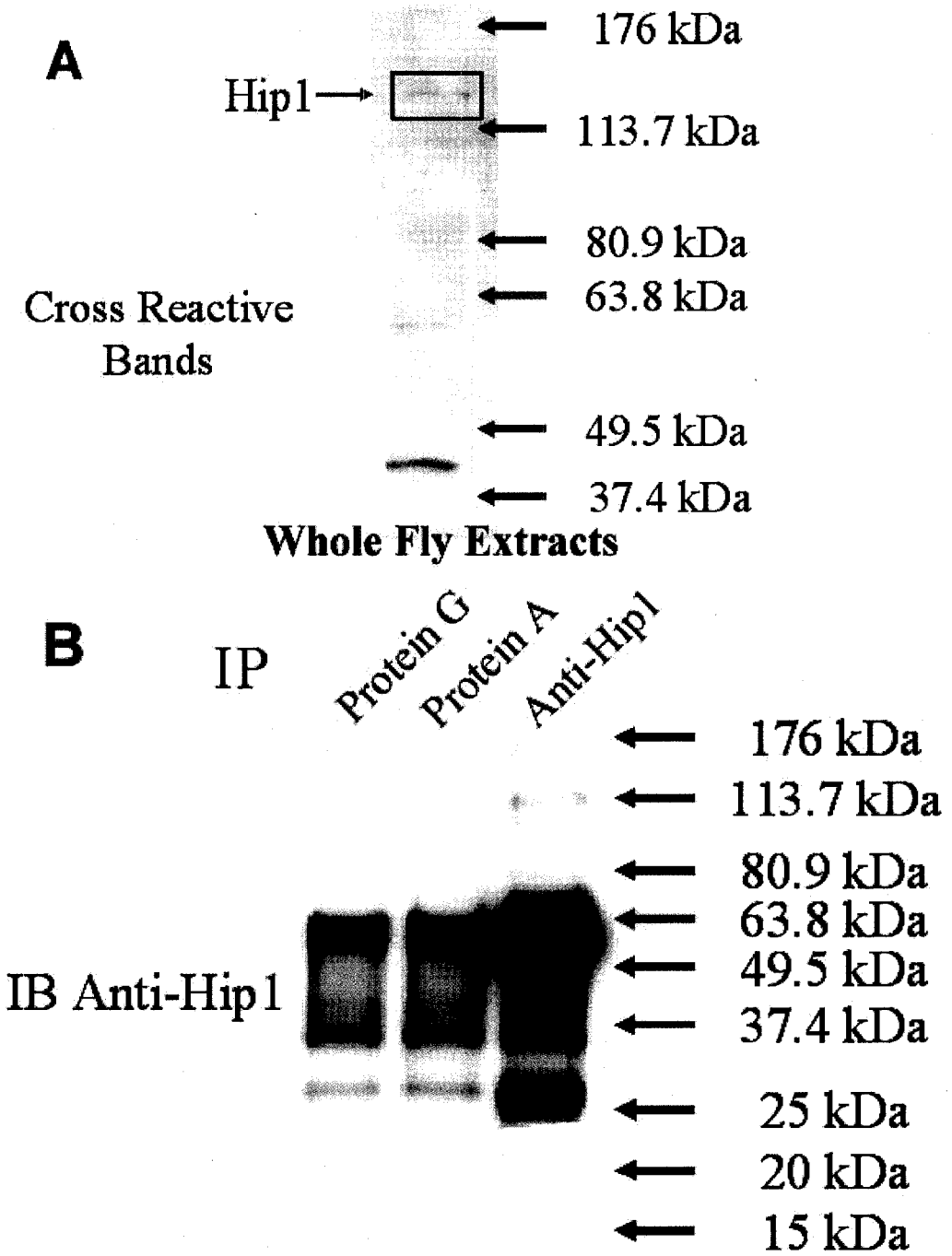

Figure 4.1: Anti-human Hip1 polyclonal antibodies recognize proteins similar in size to Drosophila Hip1 in whole protein extracts and immunoprecipitations. Both whole fly extracts (A) and immunoprecipitation samples (B) show bands of approximately 115 to 125 kDa similar to the predicted size of Hip1 and matching those bands seen in *in vitro* transcription and translation (Figure 2.1C).

4.3.2 Preliminary results of *in situ* hybridization of *hip1*:

Localization of the *hip1* transcript using a DIG labeled probe showed that *hip1* is widely expressed in the Drosophila third instar larvae. Brown regions in Figure 4.2 represent positive staining (Note: imaging problems led to false capture of colouration). Patterned staining was observed in the central brain and optic lobes of the larval central nervous system, in addition bright staining was observed in the larval fat body. Importantly, this staining pattern is identical to that seen for the *hippi* transcript (Figure 5.3) suggesting that *hip1* and *hippi* are expressed in a similar tissue/cell specific pattern. As these are preliminary findings, these experiments must be confirmed for reproducibility of this pattern of expression.

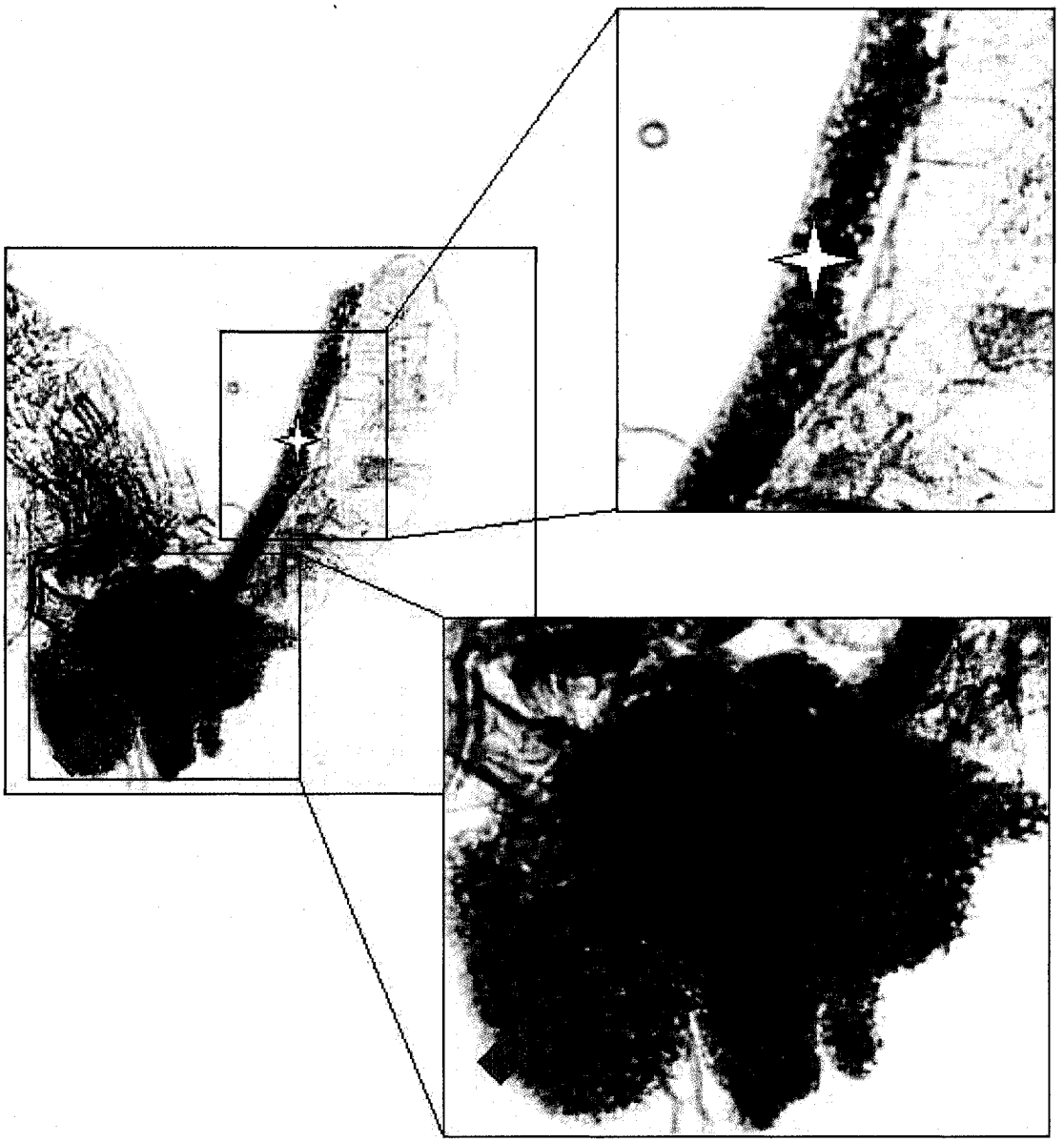

Figure 4.2: DIG-labelled *in situ* hybridization to *hip1* shows mRNA expression pattern in larval central nervous system and fat body. Patterned staining was seen in the central brain (Arrow) and primitive optic lobes (Arrowhead) of the larval CNS as well as the larval fat body (Star).

4.3.3 Immunohistochemical staining of Hip1 and Huntingtin:

Confocal analysis of Hip1 immunostaining in frozen brain sections (Figure 4.3) shows extensive positive staining throughout the optic lobe and central brain regions resembling the staining pattern of Hippi (Figure 5.4). Negative control samples lacking primary antibody show that staining is dependent on the presence of anti-human Hip1 antibody. This suggests that the anti-human Hip1 antibody recognizes a protein in the Drosophila central nervous system. When combined with data from immunoprecipitation showing immunoreactive bands similar in size to the predicted size of Hip1 this CNS expression suggest that the immunostaining is of the Hip1 protein.

Co-localization studies using an anti-human Huntingtin antibody in combination with anti-human Hip1 show areas of co-staining throughout the CNS (Figure 4.4). In particular, strong co-staining is seen in the central complex, with lighter co-staining throughout most regions of the optic lobes and CNS. Combined these results suggest that the Hip1/Huntingtin interaction reported in mammalian systems is conserved in the Drosophila brain.

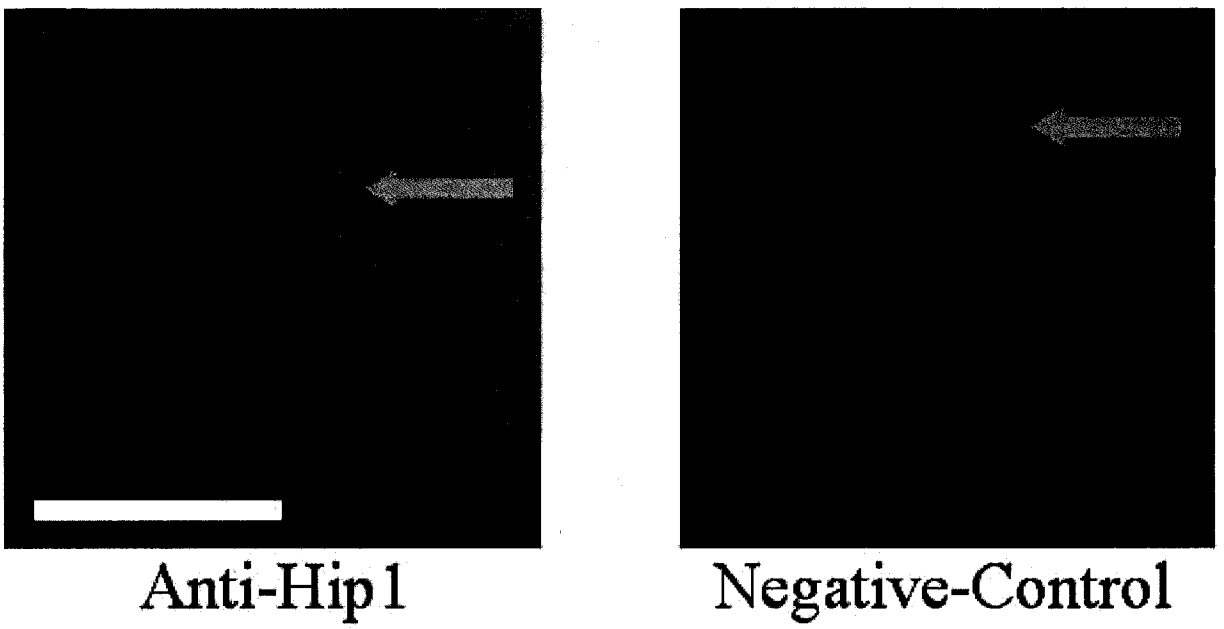

Figure 4.3: Immunohistochemical localization of Hip1 in frozen sections of adult Drosophila CNS. Sections treated with anti-human Hip1 polyclonal antibodies show strong immunoreactive regions throughout the optic lobes/retinal array (red arrowhead), and central brain (orange arrow). Scale bar = 100 μm.

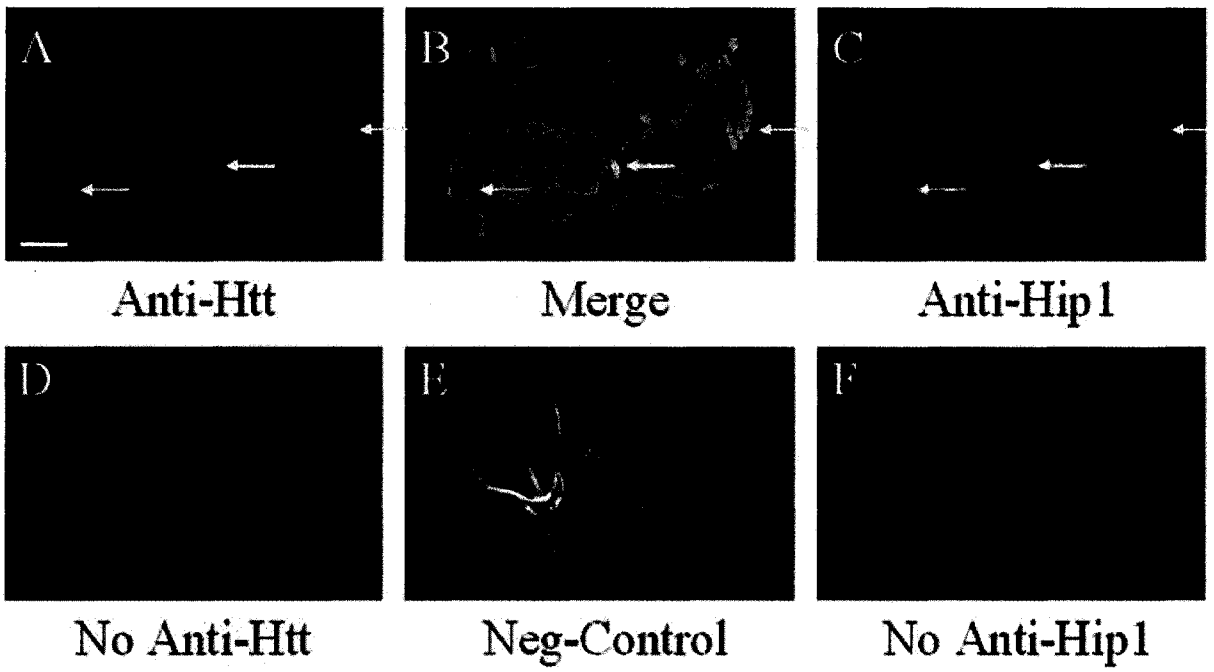

Figure 4.4: Co-localization of Hip1 and Huntingtin immunoreactive regions throughout the Drosophila CNS. Co-immunostaining of Hip1 (C-green) and Huntingtin (A-red) antibodies show areas of co-localization (B-yellow) throughout the central complex and optic lobes. Control samples lacking primary antibodies show light background staining D-F. Arrows indicate regions of strong co-localization. Scale bar in A= 100μm

4.3.4 Co-expression of *hip1* or *hip1ΔANTH* fails to modify the effects of *EGFR* on microchaetae density:

EGFR signalling has been linked to microchaetae development in the dorsal notum (Abdelilah-Seyfried et al. 2000; Culi et al. 2001; Ramain et al. 2001; Escudero et al. 2003). Because of these previously characterized links to microchaetae development *hip1* and *hip1ΔANTH* were individually co-expressed with the *EGFR* using *pannier-Gal4* in hopes of further delineating the pathway of *hip1/hip1ΔANTH* neurogenic signalling. Over-expression of the *EGFR* in the *pannier-Gal4* pattern using a *UAS-EGFR* transgene leads to an extreme over-production of microchaetae bristles and central clefts (Figure 4.5A). Co-expression of *hip1* or *hip1ΔANTH* has no effect on this *pannier/EGFR* phenotype based on qualitative analysis (Figure 4.5B&C). Due to the severity of the *pannier/EGFR* phenotype no analysis could be performed to determine quantitative changes in microchaetae density and in turn subtle effects of *hip1* or *hip1ΔANTH* upon the observed phenotypes.

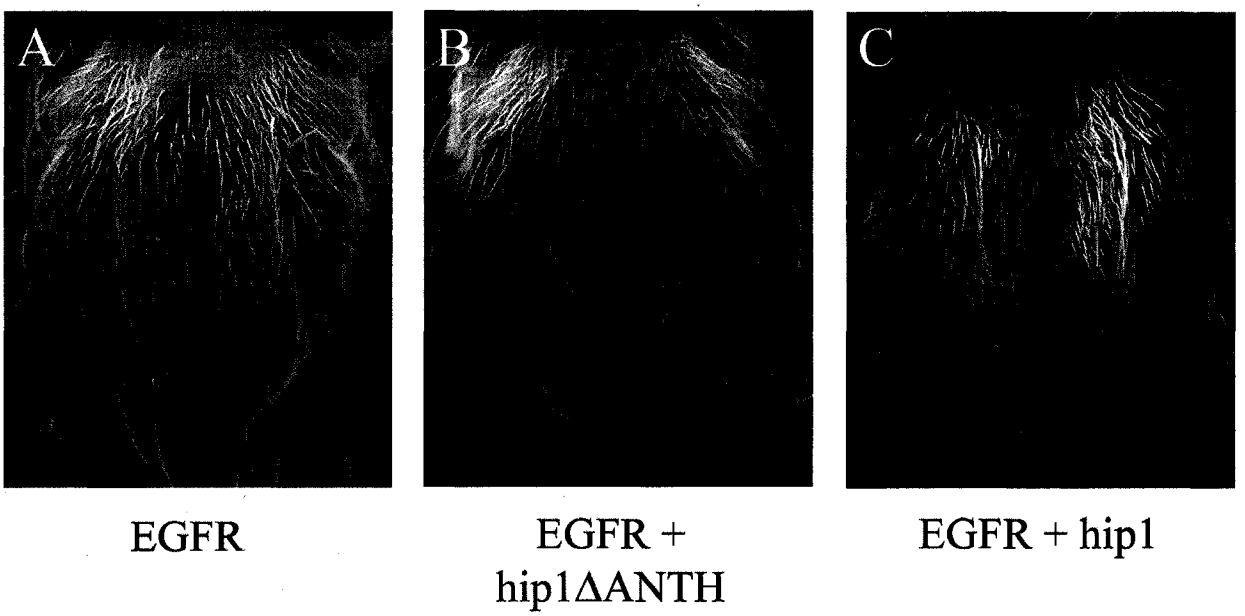

Figure 4.5: *EGFR* dorsal notum phenotypes are not modified by co-expression of *hip1* or *hip1ΔANTH*. Expression of *EGFR* under the *pannier-Gal4* pattern leads to over-production of microchaetae and central clefts (A). These phenotypes are not altered in animals co-expressing *EGFR* and either *hip1ΔANTH* (B) or *hip1* (C). Scale bar = 340 μm.

4.3.5 Reduction of *huntingtin* expression using a double-strand RNAi transgene has no noticable effect on microchaetae density:

Huntingtin expression was reduced using a *UAS-htt RNAi* transgenic construct (Gunawardena et al. 2003). Analysis of microchaetae density in this transgenic line both with and without *pannier-Gal4* show that the *UAS-htt RNAi* construct has no effect on microchaetae density (Figure 4.6).

4.3.6 Potential P-element insertion into *hip1* locus increases microchaetae density:

During the course of this investigation the EP3193 P-element, inserted near *hip1*, was induced to transpose and reinsert and a number of sub lines are being analyzed to uncover mutations in the *hip1* locus. Microchaetae density analysis was performed on of one of these sublines, EP3193-B54A, which was noted to have a wing phenotype reminiscent of members of the Notch pathway (data not shown). Homozygous females for the EP3193-B54A show an increase in microchaetae density when compared to control animals (P-value= 0.0191 by student's T-test n=7 for w^{1118}; n=3 for *B54A* homozygotes, Figure 4.7).

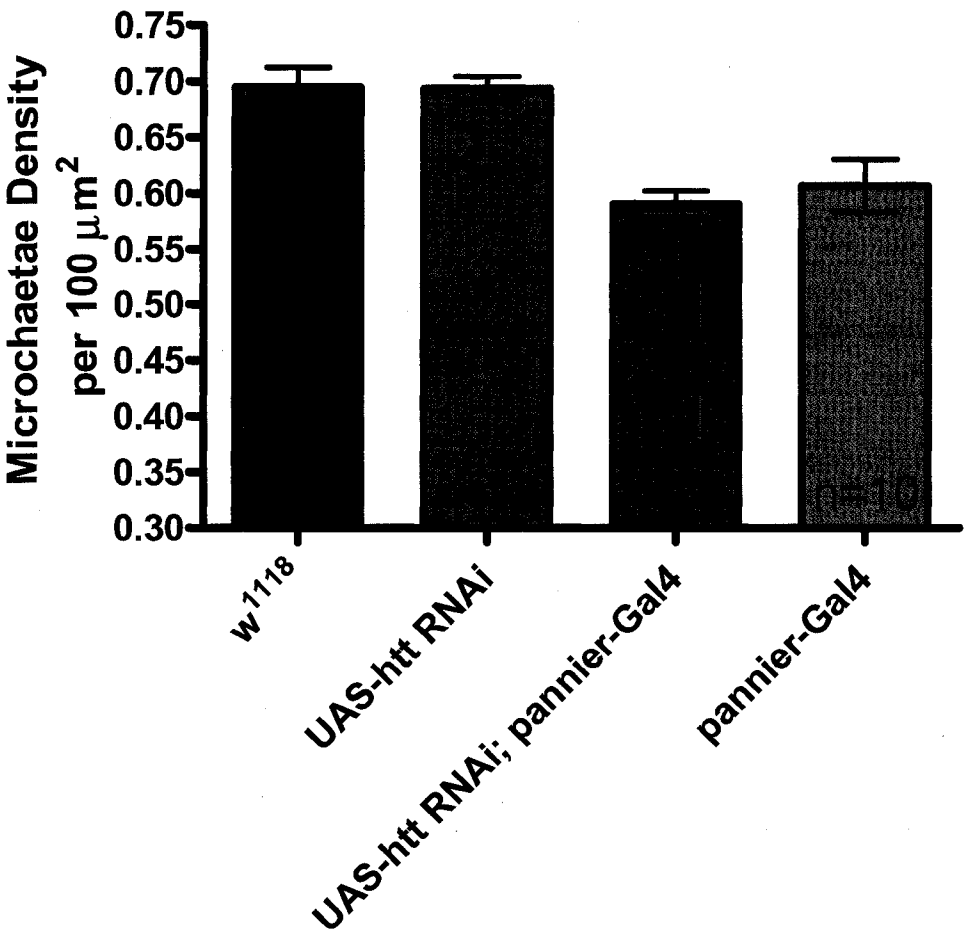

Figure 4.6: Reduction of *huntingtin* expression using a double-strand RNAi transgene has no effect on microchaetae density. Expression of double stranded *huntingtin* RNAi in the dorsal notum had no measurable effect on microchaetae density as compared to control animals expressing *pannier-Gal4* alone. (Genotypes: w^{1118}= no transgene control *UAS-htt RNAi*= non-expressing control, *pannier-Gal4; UAS-htt RNAi* experimental genotype, and *pannier-Gal4*= Gal4 expression control. Values represent mean ± SEM. P>0.05 by Neumann-Keuls posthoc-test for significance between *dshtt/pnr* and *pnr/+* genotypes).

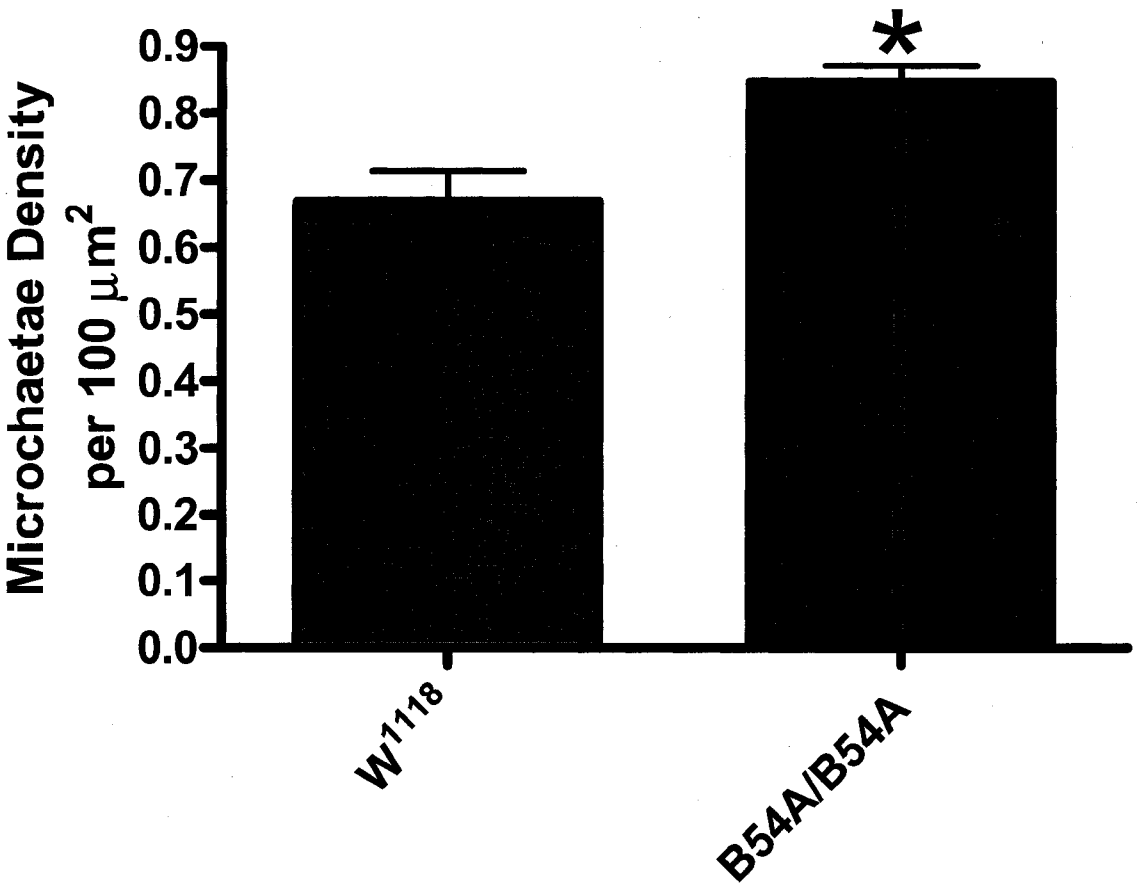

Figure 4.7: Homozygous *EP3193-B54A* P-element transposition mutant near *hip1* locus leads to increased microchaetae density. *B54A/B54A* females show significantly increased microchaetae density when compared with w^{1118} (P=0.0191 by student's T-test).

4.4 Discussion:

To determine details of the conservation of Hip1/Huntingtin interactions and the genetic basis underlying *hip1/hip1ΔANTH*-mediated neurogenic fates, a number of preliminary studies were carried out. As Hip1 and Huntingtin co-localize in the mammalian nervous system (Kalchman et al. 1997; Wanker et al. 1997; Hackam et al. 2000; Gervais et al. 2002), *in situ* hybridization to *hip1* and immunolocalization studies of Hip1 and Huntingtin were carried out in Drosophila. Messenger RNA localization shows that *hip1* is expressed in the larval central nervous system and fat body (Figure 4.2). This may be functionally equivalent to the expression pattern seen in mice where *Hip1* is expressed in all regions of the brain and in high levels in the kidney and liver (Gervais et al. 2002). Both of the latter tissues share some functions with the Drosophila fat body (St Jules et al. 1991). This conserved staining pattern tends to suggest that the Drosophila and human genes may have highly similar tissue and cell type specificity, and possibly conserved functions. This hypothesis is further supported by Western blot and immunoprecipitation experiments performed on whole fly protein extracts. As the antibodies recognize protein bands at the predicted levels for Hip1 (Figure 4.1A&B), this suggests that the Drosophila proteins may be well conserved. Immunocytochemical staining (Figures 4.3 and 4.4) shows that both antibodies recognize antigens in the Drosophila brain and have a degree of co-localization similar to mammals. Additional studies with these reagents and the production of antibodies specific to Drosophila Hip1 and Huntingtin should serve to further clarify these preliminary findings. In particular, production of Hip1 and Hip1ΔANTH specific screening tools should allow for detailed cytological investigations into the cell/tissue specificity and ratios of each isoform. These tools should assist in the

elucidation of the mechanisms controlling Hip1 versus Hip1ΔANTH function and functions mediated by altered localization of these isoforms and/or their interaction partners.

While the above experiments suggest the conservation of Hip1/Huntingtin interactions in flies, we initiated studies into the role of *huntingtin* on the neurogenic role of the Hip1 isoforms. These studies depended upon a *UAS-huntingtin* RNAi transgenic line obtained from Dr. L.S. Goldstein which had been used previously to demonstrate that reduction in *huntingtin* mRNA levels altered fast axonal trafficking (Gunawardena et al. 2003). Using this transgenic under control of the *pannier-Gal4* driver line, reduced levels of *huntingtin* mRNA should be induced in the dorsal notum. However, this method has shown no noticable effects of *UAS-huntingtin RNAi* presence upon microchaetae density (Figure 4.6), therefore further experiments are required. *UAS-htt RNAi* expression fails to alter bristle density from control levels, this finding reflects several possibilities as to the role of *huntingtin* mRNA in microchaetae neurogenesis. Neither the normal expression levels of *huntingtin* nor the degree of *huntingtin* knockdown was measured in the dorsal notum. If the levels of *huntingtin* mRNA are unaltered it will be difficult to determine if *UAS-htt RNAi* transgenics will be useful in unraveling the mechanisms of *hip1/hip1ΔANTH* –neurogenic regulation. For this reason assays must be developed to determine changes in *huntingtin* mRNA, if any, induced by *UAS-htt RNAi* expression.

Several pathways have been shown to have roles in the delineation of the dorsal notum microchaetae (Abdelilah-Seyfried et al. 2000; Culi et al. 2001; Ramain et al. 2001; Escudero et al. 2003). One of these is the EGFR signalling pathway which has been shown to act antagonistically to Notch activity (Culi et al. 2001; Escudero et al. 2003). The role of EGFR on microchaetae development was investigated with respect to *hip1* or *hip1ΔANTH* co-

expression. Consistent with previous studies, *pannier-Gal4* expression of *EGFR* led to greatly increased microchaetae density and large clefts in the dorsal notum (Culi et al. 2001). However, due to the severity of these phenotypes, it was difficult to determine if *hip1* or *hip1ΔANTH* altered EGFR signalling effects upon microchaetae density or clefts of the notum (Figure 4.7). Detailed evaluations of potential links between the *hip1* and *hip1ΔANTH* and *EGFR* must be re-examined under adjusted conditions. Examination of *hip1/hip1ΔANTH* neurogenic signals in *EGFR* mutant animals would be particularly useful in defining potential links between these separate signalling mechanisms. In addition examining changes in the microchaetae phenotype of *deltex/hip1* transgenics in the *EGFR* mutant background would aid in these investigations.

Another key tool that will allow further genetic dissection of the role of *hip1* and *hip1ΔANTH* is the production of null mutations in each transcript as well as the entire *hip1* loci. At the present time several P-element insertional mutations have been created in the genomic regions surrounding *hip1* using P-element local transposition of the EP3193 P-element (Zhang and Spradling 1993; Golic 1994). At this point one derivative line, EP3193-B54A, displays increased microchaetae density (Figure 4.7) and deltex-like wing phenotypes (data not shown) when homozygous similar to mutations in *deltex* and *Notch* (Ramain et al. 2001) to suggest that this mutant may represent the first mutant allele of *hip1*.

Overall these data demonstrate that the Hip1/Huntingtin interaction likely occurs in Drosophila and tools are available to better define these interactions both genetically and molecularly. In parallel, tools are under development to better define links between the confirmed pathways involved in *hip1/ hip1ΔANTH*-mediated events and well studied genetic

pathways. However, additional study is necessary to draw firm conclusions using these new technologies.

4.5 References:

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Chapter 5: Preliminary characterization of *hippi* in Drosophila

5.1 Introduction:

Huntingtin interacting protein 1 (Hip1) regulates neurogenesis through the deltex-dependent/ HES1-independent Notch signalling pathway which is common between flies and humans (Chapters 2 and 3 of this thesis). Parallel with studies on Hip1's role in neurogenesis, investigations into the role of Hip1-protein interactor (Hippi, also called IFT57) were initiated. Hippi was originally characterized based on its modulation of Hip1's pro-apoptotic function (Gervais et al. 2002). Under conditions of polyQ-expanded Htt, free Hip1 binds Hippi and this heterodimer may activate the extrinsic apoptotic cascade through direct binding to caspase 8 (Gervais et al. 2002). This novel cell death pathway suggested a potential polyQ-dependant cell death mechanism in Huntington's disease patients with Hippi being a potential target for therapeutic design (Ferrier 2002; Wanker 2002). To further the hypothesis that Hippi plays a role in cell death, the apoptotic activity of Hippi has been linked to the activity of the bifunctional apoptosis inhibitor (BAR) protein (Roth et al. 2003) and the Apoptin protein (Cheng et al. 2003) by co-immunoprecipitation and co-localization studies. Unexpectedly, the function of Hippi in the BAR and Apoptin systems appears to oppose apoptosis contrary to its role with Hip1. This suggests that the nature of Hippi in cell death may be cell type specific. Drosophila hippi was identified and early investigation and characterization was undertaken.

5.2 Materials and Methods:

5.2.1 Identification of the Drosophila hippi homologue:

The tBLASTn algorithm (flybase.net/blast) was used to search the *Drosophila melanogaster* genome utilizing the human Hippi homologue (Genbank Accession AF245220) as reference (Altschul et al. 1997). The longest ORF for the predicted transcript were determined using the

Expert Protein Analysis System (ExPASy) translate tool (ca.expasy.org/tools/dna.html). Sequence similarity was determined using the BLAST2 program (www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi). The ClustalW webtool (www.npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html) was used to compare the human, mouse, and Drosophila hippi proteins to determine conservation of domain architecture (Thompson et al. 1994).

5.2.2 RT-PCR:

Reverse transcriptase PCR was used to attempt to clone the *hippi* cDNA for further sequence analysis and creation of transgenic Drosophila for additional studies. Briefly, 1 μg of Drosophila brain poly A+ RNA (Clontech Laboratories, Mountain View, CA) was reverse transcribed using a poly T primer and MuLV reverse transcriptase. The cDNA pool was then screened using the following PCR primers and conditions: *hippi* exon1 Forward-1: 5'-ATG CAG CAA GAT GAT GAA CAG GA-3'; *hippi* exon2 Forward-1: 5'-CTG AAT TAG ATG CTC GCA TGA GC-3'; *hippi* exon2 Forward-2: 5'-TCC AGC CCA ATA AGC TGA TTC GT-3'; *hippi* exon2 Reverse: 5'- GCT CAT GCG AGC ATC TAA TTC AG -3'; *hippi* exon3 Reverse: 5'-TGC AGC AAA TCA GGA AAG CGA TC-3'. PCR cycling protocol: 2 min at 95°C for denaturation followed by 30 cycles of 1 min denaturation at 95°C, 1 min of primer annealing at 55°C and 1.5min of extension at 72°C. Cycling was followed by one final round of extension of 5 min at 72°C and soak at 4°C. PCR was carried out using 1 unit of Taq polymerase (Roche Molecular Biochemicals, Mannheim, Germany).

5.2.3 cDNA library creation and screening:

A Marathon cDNA library was then established according to manufacturer's instructions for additional PCR screening and full-length clone isolation (Clontech Laboratories, Mountain View, CA). Briefly, 1 μg of Drosophila brain poly A+ RNA (Clontech Laboratories, Mountain View, CA) was converted into first strand cDNA using the supplied cDNA synthesis primer, AMC reverse transcriptase, and supplied cDNA synthesis buffer and nucleotides for 1 hour at 42°C. Second strand synthesis was carried out using 2nd strand enzyme cocktail at 16°C for 1.5 hours. The complete cDNA was then purified by phenol/chloroform extraction followed by ethanol precipitation. The Marathon adaptor sequence was ligated to an aliquot of the re-dissolved cDNA sample. The cDNA library was then amplified via nested PCR as per manufacturer's instruction using primers directed against the Marathon adaptor sequence and the *hippi* specific primers listed above. Successful PCR products were isolated and cloned using the TOPO T/A system (Invitrogen Corporation, Carlsbad, CA) according to manufacturer's instructions.

5.2.4 Analysis of Drosophila protein extracts for proteins cross-reactive to anti-human Hippi polyclonal antibodies:

Whole Drosophila protein extracts were obtained as follows: 4 to 8 frozen flies were placed into a 1.5 mL microcentrifuge tube and 150 μL of ice cold Hepes/EDTA buffer containing a phosphatase/protease inhibitor cocktail. Hepes/EDTA buffer composition was as follows: 100 mM KCl, 20 mM Hepes, 5% glycerol, 10 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, 1 Complete-Mini protease inhibitor tablet (Roche Molecular Biochemicals, Mannheim, Germany), 20 mM β-glycerophosphate, and 100 μM orthovanadate. Flies were homogenized on ice until consistent using rotary homogenizer. Samples were centrifuged at

14,000 rpm and 4°C for 5 min. Clarified supernatant containing protein was isolated to a new tube. 50 to 100 μg of total protein extracts were separated on 4-20% SDS-PAGE gels and electroblotted onto nitrocellulose membranes for subsequent Western blotting (See Methods section of Chapter 4 for details of Western blotting protocols).

5.2.5 Immunoprecipitation:

Protein for immunoprecipitation experiments was isolated as above. An amount between 250 and 500 μg of total protein extract was immunoprecipitated overnight essentially as described (Gervais et al. 2002) with the exception that Hepes/EDTA buffer was used instead of NP-40 buffer. Anti-Hip1, anti-Hippi, and anti-Huntingtin antibodies were added to aliquots and samples were placed at 4°C on an "end-over-end" mixing platform overnight to allow formation of antibody-protein complexes. Protein A sepharose beads (Sigma, St. Louis, MO) were added and immunoprecipitation was carried out for 2-3 hours at 4°C on an "end-over-end" mixing platform. Bead-antibody-protein complexes were washed three times with ice-cold Hepes/EDTA buffer. Aliquots were boiled in 2X SDS-buffer without ß-mercaptoethanol (See Methods section of Chapter 4 for details of immunoprecipitation and Western blotting protocols). Samples were then separated onto 4-20% Novex SDS-PAGE gels, Western blotted, and probed with indicated antibodies. Mock immunoprecipitation lacking primary antibody was used as a control for specificity.

5.2.6 Western Blotting:

Drosophila protein extracts were challenged with a rabbit polyclonal antibody directed against the human Hippi protein (Hackam et al. 2000; Gervais et al. 2002). Blots were blocked in 5% non-fat milk in Tris-buffered saline-Triton X-100 (TBS-T) prior to incubation with primary

antibodies, 1:1000 dilution in TBS-T, for 2 to 4 hours at room temperature on a shaking platform. Blots were washed 3 X 20 minutes in TBS-T prior to incubation with horseradish peroxidase-linked secondary antibodies, 1:10,000 dilutions in TBS-T, for 1 hour. Membranes were washed 3 X 20 minutes in TBS-T to reduce background, exposed to chemiluminescent reagent and documented on autoradiographic film.

5.2.7 DIG-labelling of *hippi*:

To determine the mRNA expression pattern of *hippi*, digoxigenin (DIG) labelled DNA probes directed against the *hippi* transcript were made. DIG labelling was carried according to manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, ~1000 ng of *hippi* genomic PCR product were denatured by boiling for 10 minutes followed by quick chilling on ice to prevent renaturation. Hexanucleotide random labelling primers, dNTP labelling mix, and Klenow enzyme were added to denatured DNA and labelling was carried out at 37 °C overnight and stopped by addition of 2 µl of 0.2 M EDTA.

5.2.8 *In situ* hybridization to larval brain and imaginal discs:

Third instar w^{1118} larvae were dissected in phosphate buffered saline (PBS), fixed in 4% formaldehyde and dehydrated in methanol and ethanol. The carcases were incubated with the DIG labelled anti-*hippi* DNA probe. To visualize *hippi* mRNA, alkaline phosphatase labelled anti-DIG antibodies were incubated with the carcases and subjected to alkaline phosphate treatment as per the Roche Applied Science DIG application manual. The larval CNS and imaginal discs were dissected and examined under light microscopy.

5.2.9 Immunohistochemical analysis of hippi in frozen sections of Drosophila:

Staining of the adult CNS was carried out by mounting whole Drosophila heads in O.C.T. mounting medium (Tissue Tek, Elkhart, IN) prior to slicing by cryotome at -20°C. Sections were mounted on microscope slides. Slides were blocked for one hour in phosphate buffered saline containing 0.3% Triton X-100 and 10% normal donkey serum (10% NDS/PBT). The slides were exposed to primary antibody in 10% NDS/PBT overnight at 4°C. Slides were then rinsed 3 X 5 minutes in PBT and incubated in anti-rabbit Alexa 488 secondary antibody (Molecular Probes, Eugene, OR) in 10% NDS/PBT for one hour. Slides were rinsed an additional 3 X 5 minutes in PBT and stored at -20°C prior to image capture on a Ziess Axiovert confocal microscope.

5.3 Results:

5.3.1 Hippi is well conserved from *Drosophila melanogaster* to Mammals:

A tBLASTn search of the Drosophila genome with the human Hippi protein sequence as reference, led to the identification of sequence *CG8853* of the Drosophila genome as the sole homologue of *Hippi* in Drosophila. The longest ORF for *CG8853* was determined to be 429 amino acids, or approximately 47 kDa, which is very similar to the 405 amino acid protein of human Hippi. A BLAST2 comparison of the human Hippi amino acid sequence and the predicted sequence of the *CG8853* transcript shows a high degree of conservation, with 36% identical and 57% similar residues and will hereafter be referred to as Drosophila hippi. The central myosin-like domain is highly conserved (Figure 5.1). Interestingly, the carboxy terminal pseudo death effector domain (Gervais et al. 2002) is poorly conserved suggesting that either this region does not exist in Drosophila hippi or the predicted *CG8853* transcript may not be accurate.

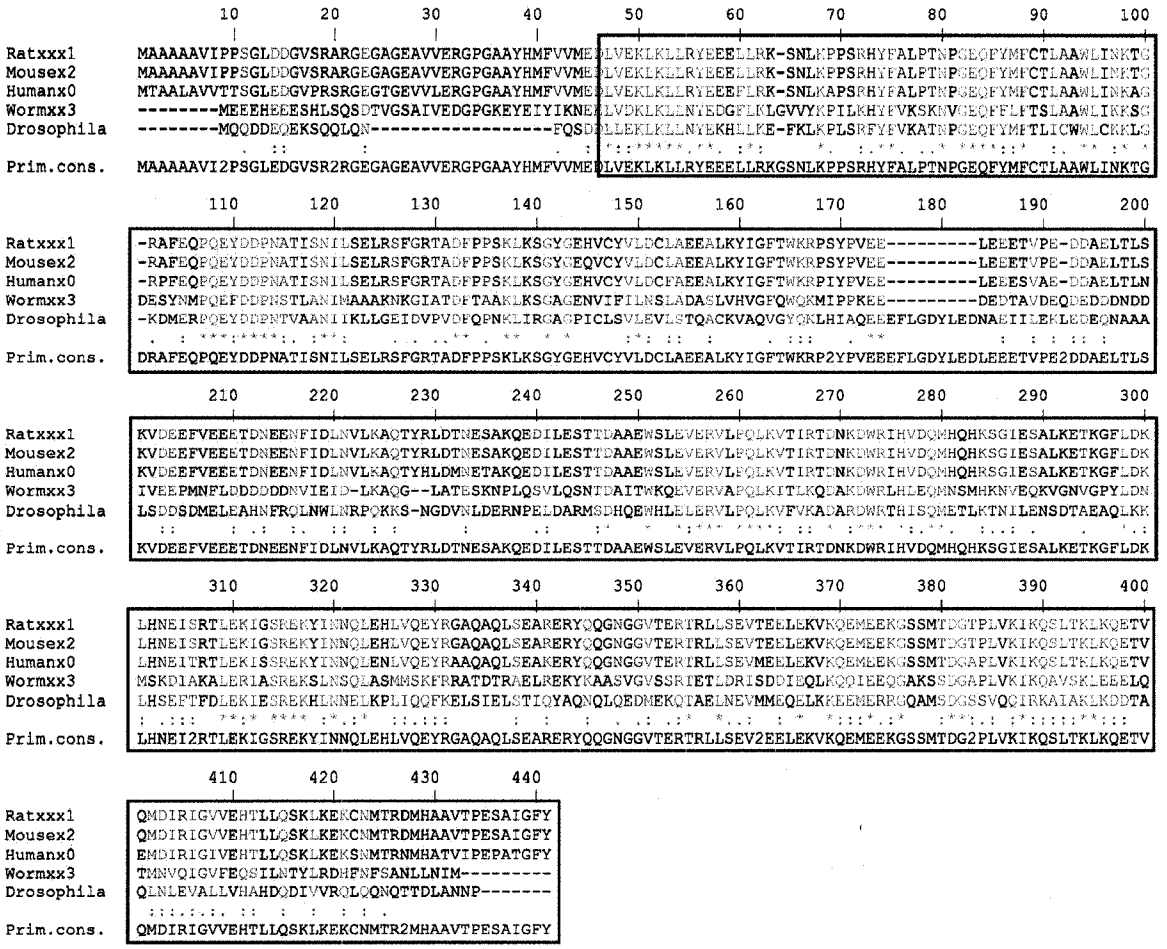

Figure 5.1: The *Drosophila melanogaster* genome contains a single well-conserved *Hip-1 protein interactor* (*Hippi*) homolog. Multiple alignment of the longest ORF in the Drosophila hippi transcript shows that the fruit fly homolog is well conserved including the amino-terminus and central Myosin-like domain (blue box). Notably, little identical conservation is seen in the carboxy-terminal pseudo death effector domain (red box). Red letters indicate identical amino acids, green letters indicated similar amino acids in all sequences, and blue letters indicate partially conserved amino acids.

5.3.2 RT-PCR and Marathon cDNA library screening for *hippi* full length transcript:

RT-PCR of Drosophila brain PolyA+ RNA and subsequent PCR screening revealed that in the Drosophila brain the predicted third exon of *hippi* may not included in the transcript. PCR utilising the *hippi* ex1-F1/ *hippi* ex3-R primer pair failed to produce product while the *hippi* ex1-F1/ *hippi* ex2-R produced a product of the predicted 650 bp (data not shown). Cloning and sequencing of this fragment confirmed that it represents the first 217 amino acid coding sequence of *hippi*. To further characterize and clone the 5' untranslated region upstream of the *hippi* start codon and sequences downstream of base pair 650 from the RT-PCR fragment the Marathon cDNA library was examined using an overlapping PCR cloning strategy. This is based on overlapping gene specific primers and the nested adaptor primers included with the Marathon cDNA system and a touch down PCR approach as per manufacturer's instructions. Clearly defined bands were gel purified and cloned for further sequence analysis. All positive clones were sequenced; however no additional sequence information has yet been obtained. It appears that non-specific cDNA sequences were created during the cDNA production process. Additional screening for 5'and 3'sequence is necessary to fully characterize and clone *hippi.*

5.3.3 Immunoprecipitation using anti-human Hippi antibody reveals Drosophila hippi candidates:

Western blots of crude protein extract and immunoprecipitations using the anti-human Hippi antibody recognized several bands which may represent the Drosophila hippi protein. Figure 5.2A shows that examining extracts with the anti-Hippi antibody reveals two proteins of ~ 115 kDa and 90 kDa, both which are much larger in size than the predicted hippi. Figure 5.2B shows that probing the native state gel of immunoprecipitation samples, which were not exposed to ß-mercaptoethanol, revealed two smaller proteins at ~60 kDa and ~42 kDa, much

nearer the predicted size of hippi at 47 kDa. Additional analysis will be necessary to confirm the identity of these proteins.

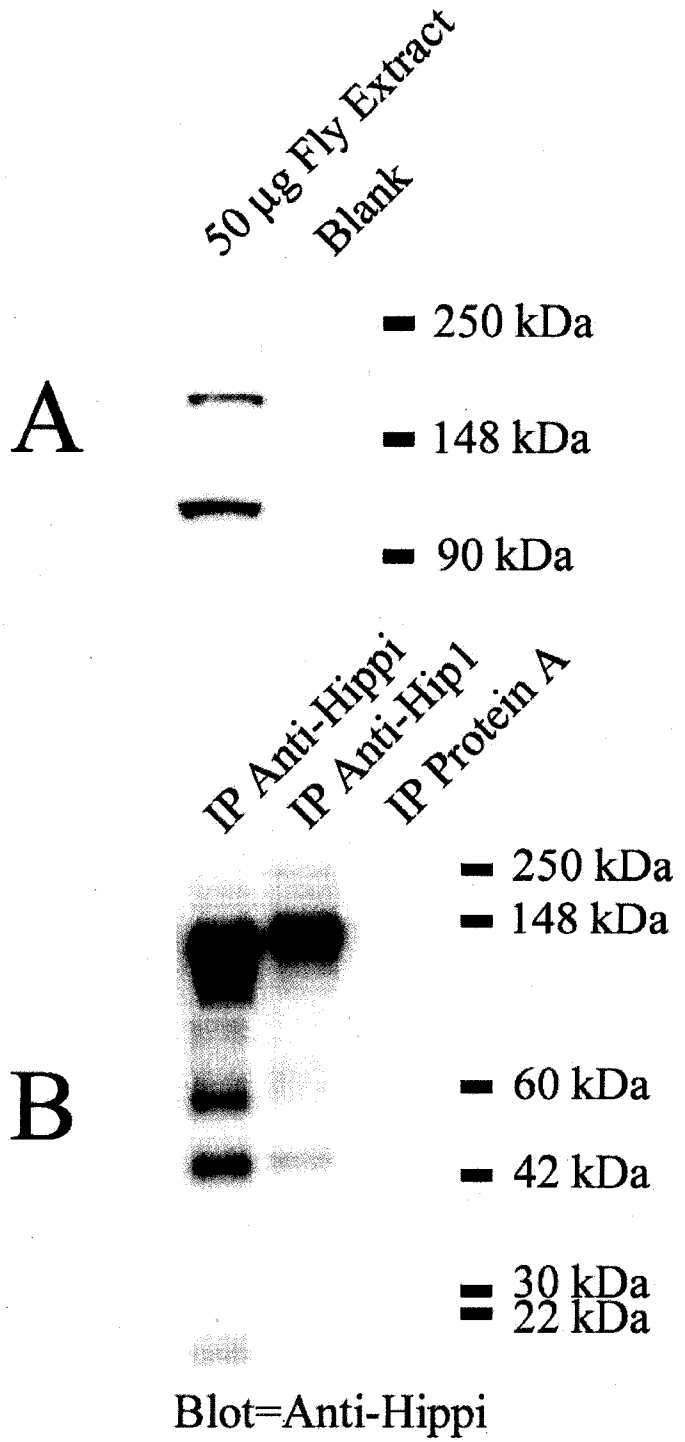

Figure 5.2: Anti-human Hippi polyclonal antibodies recognize proteins similar in size to Drosophila hippi in immunoprecipitates but not in western blots. Whole fly extracts show banding at 215 kDa and 90 kDa much larger than the predicted 47 kDa of *hippi* (A). Immunoprecipitation samples show bands of approximately 60 kDa and 42 kDa much closer in size to that predicted for *hippi* (B).

5.3.4 Preliminary results of *in situ* hybridization of *hippi*:

Localization of the *hippi* transcript using a DIG labelled probe showed that *hippi* is widely expressed in the Drosophila third instar larvae. Brown regions in Figure 5.3 represent positive staining (Note: imaging problems led to false capture of colouration). Patterned staining was observed in the central brain and optic lobes of the larval central nervous system, in addition bright staining was observed in the larval fat body, functionally analogous to the mammalian liver. Importantly, this staining pattern is identical to that seen for the *hip1* transcript (Figure 4.2) suggesting that *hip1* and *hippi* are expressed in similar tissue/cell specific pattern. All experiments need to be confirmed by repetition.

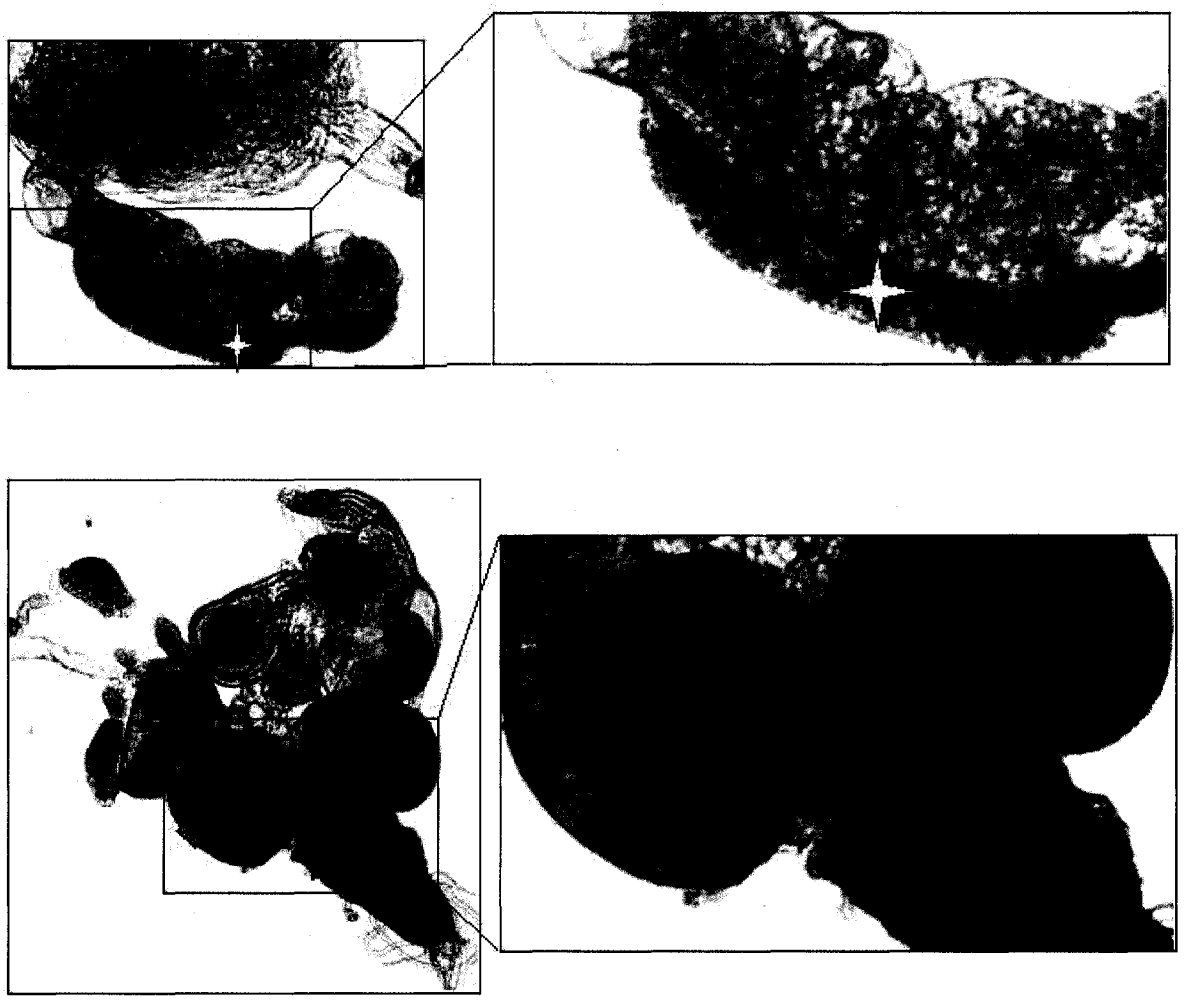

Figure 5.3: DIG-labelled *in situ* hybridization to *hippi* shows mRNA expression pattern in larval central nervous system and fat body. Patterned staining was seen in the central brain (Arrow) and primitive optic lobes (Arrowhead) of the larval CNS as well as the larval fat body (Star). This expression pattern is identical to that seen for *hip1* (Figure 4.2).

5.3.5 Immunohistochemical staining of hippi:

Confocal analysis of anti-human Hippi immunostaining in frozen brain sections (Figure 5.4) showed extensive positive staining throughout the optic lobe and central brain regions resembling the staining pattern of the anti-human Hip1 antibody (Figure 4.3). Negative control samples lacking primary antibody show that staining is dependent on the presence of anti-human Hippi antibody. This suggests that the anti-human Hippi antibody recognizes a protein in the Drosophila central nervous system. Combined with data from immunoprecipitation of whole Drosophila extracts showing immunoreactive bands similar in size to the predicted size of hippi (Figure 4) these data suggest that the protein target may be hippi.

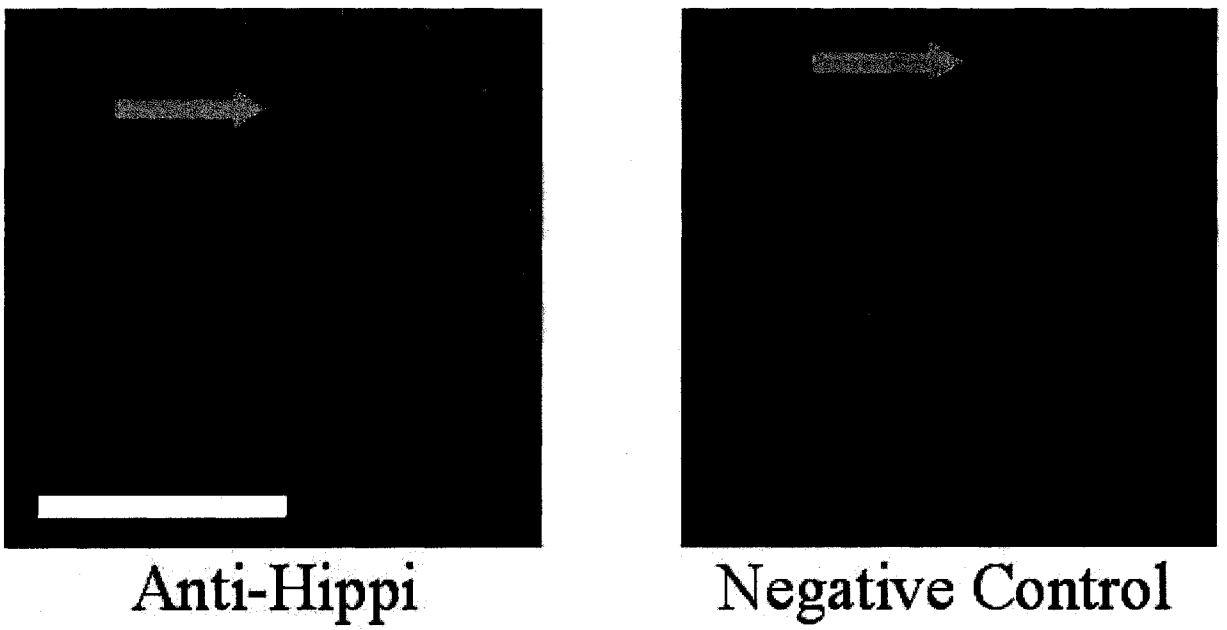

Figure 5.4: Immunohistochemical localization of hippi in frozen sections of adult Drosophila CNS. Sections treated with anti-human Hippi polyclonal antibodies show strong immunoreactive regions throughout the optic lobes/retinal array (red arrowhead) and central brain (orange arrow) identical to staining seen using anti-human Hip1 antibodies (Figure 4.3). Scale bar = 100 μm.

5.4 Discussion:

Hip1 has been described as a mediator of cell-death (Hackam et al. 2000; Gervais et al. 2002), cancer progression (Rao et al. 2002; Rao et al. 2003), and as a novel regulator of neurogenesis (Chapters 2 and 3). Due to the complex roles of the Hip1 protein in such diverse cellular pathways, detailed analysis of modifiers of Hip1, which may act to switch the functions of Hip1 from one end point to another, are critical. One such modifying protein is the Hip1 protein interactor, Hippi, which was originally characterized based on its ability to bind to and enhance the apoptotic activity of Hip1 through shared pseudo death effector domains (pDED) and co-interactions with caspase 8 (Gervais et al. 2002). At the molecular level Hippi has been shown to participate in the assembly of ciliated and flagellated structures through protein interactions with members of the intraflagellar transport system (Baker et al. 2003). The apoptotic role of Hippi has been further linked to the BAR (Roth et al. 2003) and Apoptin systems (Cheng et al. 2003; Rohn and Noteborn 2004) indicating that Hippi functions to counter apoptosis in some situations. As all studies of Hippi function have been undertaken in mammalian cell culture systems, we have initiated studies to characterize hippi in *Drosophila melanogaster*. This system will allow a thorough genetic study on the function(s) of *hippi*.

At present the Drosophila homologue of *hippi* has been identified using bioinformatic tools and the predicted sequence shares significant homology at the amino acid and domain architectural levels with mammalian Hippi proteins (Figure 1). As no clones of the *hippi* gene exist, several methods were used to attempt to clone the full-length transcript. It was shown that the predicted third exon of the *hippi* transcript was incorrect as RT-PCR screens using a third exon specific primer failed to give PCR product. Repeated attempts to determine

additional 5' and 3' sequences to identify a full-length clone of *hippi* failed. Thus far the first 650 bp of the *hippi* transcript have been cloned into the pCR2.1 vector. As a full length clone is necessary to further study *hippi* function *in vivo* this will be a primary goal of future research.

Studies of the localization of hippi transcript and protein have been promising. *in situ* hybridization studies in third instar larval tissues have demonstrated that *hippi* is expressed in the larval CNS and fat body, mirroring the expression pattern of *hip1* (compare Figure 5.4 with Figure 4.3). This similar staining pattern is shared at the protein level as shown by immunohistochemical staining because anti-human Hippi immunoreactive staining is present throughout adult brain frozen sections (Figure 5.4). Native state immunoprecipitation of whole Drosophila extracts using the same anti-human Hippi antibody showed two bands of similar size to the predicted hippi protein sequence (Figure 5.2B). Importantly, anti-human Hippi western blots under denaturing situations show two larger immunoreactive bands suggesting that this antibody does not recognize hippi under denaturing conditions. In particular the cloning of full length *hippi* will allow for correct determination of the full length amino acid sequence. This will allow for delineation of expression patterns of *hippi* and the potential for *hip1/hippi* interactions in Drosophila. This full-length *hippi* transcript will be particularly important for the future determination of mutants in the *hippi* locus by allowing thorough genetic rescue studies.

Based on the preliminary data presented here, study of the function of *hippi* in the fruit fly is promising. Studies of *hippi* localization suggest that the relationship between Hip1 and Hippi seen in mammalian systems (Gervais et al. 2002) may be conserved in the fly. These findings

on Hippi and Hip1 co-localization and the definition of a dual role for *hip1* and *hip1ΔANTH* in neurogenesis leads to the interesting possibility that Hippi's role in Hip1 activity may extend beyond merely enhancing its cell death activity. These findings raise the possibility that Hippi may act in the anti-neurogenic/neurogenic balance between hip1/hip1ΔANTH, respectively, through differential binding to individual isoforms. Such differential binding could explain the pro-apoptotic/anti-apoptotic controversy seen in several studies of Hippi function (Gervais et al. 2002; Baker et al. 2003; Roth et al. 2003; Majumder et al. 2006). In-depth analysis to determine if there are indeed changes in the balance of Hip1 isoforms and in turn Hip1/Hippi heterodimer ratios between the cell types investigated will help answer this question. Future studies will further define the role of Hip1/Hippi interactions both on cell death and neurogenesis and in turn the basis of cellular differentiation processes under both disease and normal conditions.

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Chapter 6: *pannier-Gal4*-mediated decreases in microchaetae density are suppressed by expression of the p35 anti-apoptotic protein.

6.1 Introduction:

The Gal4/upstream activation sequence (UAS) bipartite expression system is perhaps the most widely utilized tool in Drosophila genetics (Duffy 2002). The ectopic expression of Gal4 is believed to be benign due to the lack of complete consensus Gal4 binding sites in the Drosophila genome (Berkeley Drosophila Genome Project). Despite this, recent reports have demonstrated that in some cases expression of Gal4 transgenes alone have detrimental effects (Haywood et al. 2002; Kramer and Staveley 2003). It was shown that these phenotypes were likely a result of programmed cell death as numbers of dying cells stained by acridine orange increased with GMR-Gal4 transgene copy number (Kramer and Staveley 2003) and 'rough eye' phenotypes could be genetically suppressed by the co-expression of p35, an anti-apoptotic protein or the E3 ubiquitin ligase parkin. Increasing the number of Dopa decarboxylase Gal4 transgenes greatly reduced lifespan (Haywood et al. 2002).

As previous co-expression studies were carried out using optimized Gal4 binding sites for transcriptional activation, the UAS promoter region, the possibility remained that the presence of these 'enhanced' binding sites for Gal4 would reduce 'toxic' effects in all circumstances. As these studies demonstrate clear effects of Gal4 on several biological phenotypes it was decided to analyse the effect of ectopic Gal4 expression by the *pannier-Gal4* transgenic line for effects on microchaetae density. It has been noted that the *pannier-Gal4* transgenic line is a hypomorphic allele of the *pannier* locus but that it also displays neomorphic effects including loss of macrochaetae on the head (Heitzler et al. 1996) and also occasionally displays a thoracic cleft and altered microchaetae (Pena-Rangel et al. 2002). The data presented here show that *pannier-Gal4* alone leads to a decrease in microchaetae density from wild-type

control levels. Importantly, the observed phenotype was sensitive to p35 expression but insensitive to green fluorescent protein expression dismissing the general benefit theory and supporting the theory that Gal4 induces apoptotic cell death in a p35 sensitive manner.

6.2 Materials and Methods:

6.2.1 Microchaetae density analysis:

Critical class female progeny were analysed for dorsal notum microchaetae density as described in chapter 2 of this thesis. Briefly, animals were anaesthetized at -70°C, mounted, and photographed by SEM. The following genotypes were analyzed: w^{1118}, *pannier-Gal4*, *UAS-GFP; pannier-Gal4*, *pannier-Gal4/UAS-p35*. Statistical differences were determined by one-tailed ANOVA with Neuman-Keuls posthoc-test for significance between pairs.

6.2.2 Genomic screen for UAS-like regions in the *Drosophila melanogaster* genome:

An optimized nucleotide sequence of the upstream activating sequence (UAS) element, CGG AGT ACT GTC CTC CG (Webster et al. 1988), was used to screen release 4.3 of the *Drosophila melanogaster* genome sequence for similar contiguous sequences using the basic local alignment search tool (BLAST) of the flybase network (www.flybase.net). All hits were analyzed for chromosomal location, associated genes, number of identities, and BLAST E-value.

6.2.3 Genomic screen for siRNA-like homologies between Gal4 DNA sequence in the *Drosophila melanogaster* genome:

The 3271bp *Gal4* mRNA sequence (Genbank Accession: Z73604) was used to screen release 4.3 of the *Drosophila melanogaster* genome sequence for short homologous regions similar to high confidence siRNA complexes, the sequences AA-N_{19}-TT or NA(N_{21}) where N is any

nucleotide were used as high confidence and low confidence siRNAs respectively (Elbashir et al. 2001a). All sequences showing 18 base pairs or more of nucleotide homology were analyzed for potential as siRNAs. The candidates were evaluated based on sequence, length, and intronic/exonic location to determine if they represent potential siRNAs. All candidates were also further categorized for cytologic location, gene, and gene function. In order to be considered siRNA candidates the homologous regions must fit into either the high or low confidence siRNA consensus, match exonic gene sequence, and have no internal mismatches (Elbashir et al. 2001a).

6.3 Results:

6.3.1 Microchaetae density:

The *pannier-Gal4* transgene alone decreases the dorsal notum microchaetae density from that of the w^{1118} genetic background control (Figure 6.1; P-value < 0.01). This *pannier-Gal4* effect is unaltered in the presence of optimized Gal4 binding sites when activating GFP expression by the *UAS-GFP* transgene (P-value > 0.05). The *pannier-Gal4*-dependent decrease in microchaetae density is suppressed back to wild-type levels when *pannier-Gal4* is co-expressed with UAS binding sites coupled to the p35 anti-apoptotic protein (P-value pnr/+ vs. pnr/p35 < 0.001; P-value w1118 vs. pnr/p35 > 0.05).

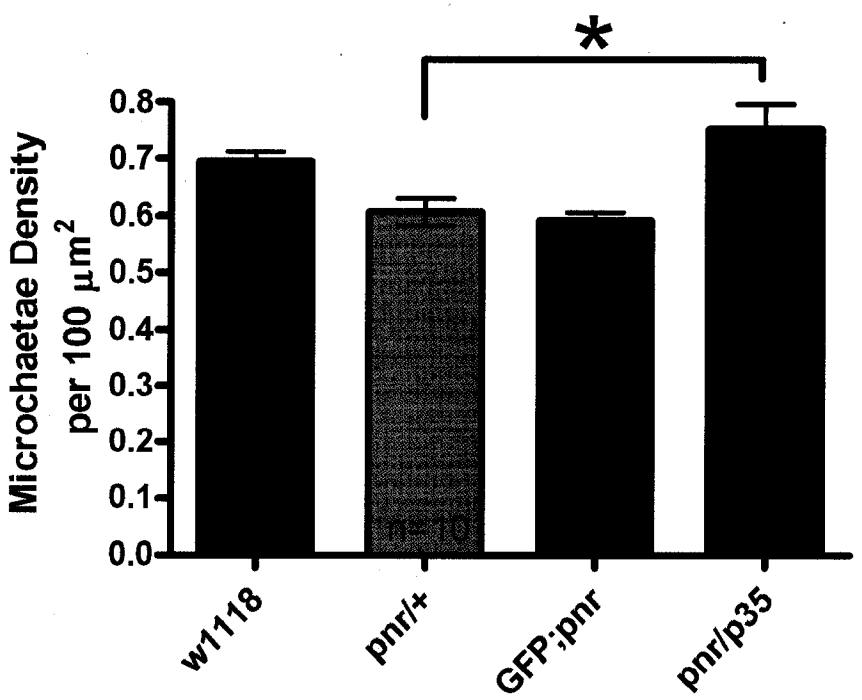

Figure 6.1: *pannier-Gal4* decreases microchaetae density in a *p35* dependent manner. Expression of *Gal4* decreases microchaetae density. Co-expression of the anti-apoptotic p35 protein but not green fluorescent protein suppresses this phenotype back to wild type levels Figure legend: w1118= wild type control, pnr/+= *pannier-Gal4* control, GFP;pnr= *pannier-Gal4* driving expression of green fluorescent protein, and pnr/p35= *pannier-Gal4* driving expression of *p35*(values represent mean ± SEM. * = P<0.001 by Neuman-Keuls posthoc-test).

6.3.2 UAS-like sequences in the Drosophila genome:

BLAST of the optimized sixteen base pair Gal4-binding sequence revealed nine sequences sharing either 13 or 14 contiguous bases with the optimized consensus described in methods. These sequences are summarized in Table 6.1. Of the nine sites identified three were not associated with recognized transcriptional units. Of the remaining six all occur within described transcriptional units with varied molecular functions. Due to the high level of similarity these regions could potentially act as binding sites for the Gal4 transcriptional activator in the absence of complete UAS elements.

Table 6.1: Description of genomic regions resembling optimized Gal4 binding sequence. Characterized genes are denoted by standard gene names. Non-associated = not associated with recognizable transcriptional unit; N/A= not applicable. (data was obtained using Berkely Drosophila Genome Project webtools).

Sequence	Chromosomal location	Identities, E-value	Associated genes	Gene function
1= ggagtactgtcctc	2L; 37A1	14, E=0.83	*Grip71*	γ-tubulin binding
2= cggagtactgtcc	2L; 37A7-B2	13, E=3.3	*burgundy*	purine-biosynthesis
3= agtactgtcctcc	3H	13, E=3.3	Non-associated	N/A
4= agtactgtcctcc	3H	13, E=3.3	Non-associated	N/A
5= agtactgtcctcc	3R; 82E4	13, E=3.3	CG2016	N/A
6= agtactgtcctcc	3R; 84E5	13, E=3.3	CG10445	spliceosome/ transcriptional regulation
7= ggagtactgtcct	3L; 62A8-9	13, E=3.3	CG7955	defence response ; heme transporter
8= cggagtactgtcc	3L; 63A3	13, E=3.3	*always early*	spermatogenesis; transcriptional activation
9= agtactgtcctcc	2R	13, E=3.3	Non-associated	N/A

6.3.3 Regions of the *Gal4* mRNA homologous to *Drosophila melanogaster* genomic sequence which could act as siRNA:

Thirty-one sequences with at least 18 base pairs of identity to regions in the Drosophila genome were characterized. Of those, 27 were between 18-20 nt in length, below the common 21-25 nt limit for efficient RNAi activity (Elbashir et al. 2001a; Elbashir et al. 2001b; Elbashir et al. 2001c). Of the remaining four all contain internal nucleotide differences which would be expected to abolish any siRNA activity (Elbashir et al. 2001a). In addition none of the 31 sequences met the characteristics defined for efficient RNAi activity for one or more of the following reasons: sequence did not match siRNA consensus, homology was not directed to exonic regions, or internal mismatch nucleotides were present (Table 6.2).

Table 6.2: No sections of the Gal4 transcript fit the criteria for efficient inhibitory RNA down regulation of characterized Drosophila transcripts. Specific characteristics are further described in text. N/A= not applicable.

Sequence (at least 18bp)	Chromosomal location	Identities	Gene	Function	Intronic/ Exonic	Matches siRNA Y/N	siRNA candidate Y/N
tgttaatttgttgtaataat	16A5-B1	20/20	*CG8557*	Unknown	intronic	N	N
tatgtaaatactttgaaat	3C9-D1	19/19	*dunce*	cAMP signal	intronic	N	N
ttcattattcatgaagttatcatgaac	9B2	25/27	N/A	N/A	N/A	N	N
aacaattccaggcaaaata	12B4-10	19/19	*CG10990*	apoptosis/ protein synthesis	intronic	N	N
gttaacaatgcttttata	1E1	18/18	*CG3655*	Unknown	exonic	N	N
ttgcagctgttgctgttg	9E1	18/18	*CG32677*	β-Amyloid binding	exonic	N	N
tgcagctgttgctgttgc	10F1	18/18	*CG15740-1*	Unknown	exonic	N	N
tgcagctgttgctgttgc	10F1	18/18	*CG15740-2*	Unknown	exonic	N	N
tatgggtactaggactgc	17B5	18/18	*CG15044*	Unknown	exonic	N	N
cagtctccactgaagccaat	70D7	20/20	N/A	N/A	N/A	N	N
agctcataaaacagaaaaag	78E1	20/20	N/A	N/A	N/A	N	N
tgcagctgttgctgttgc	63E8-F1	18/18	*CG32664*	Unknown	exonic-RB	N	N
agtagagtctttatgggt	64C1-2	18/18	*CG17150-RB*	motor acitivty	exonic-RB	N	N
aatgtatttttcacaagt	65C1	18/18	N/A	N/A	N/A	N	N
ctattcaagcccaatgat	72D1	18/18	*CG13070*	Unknown	exonic	N	N
tgcttcttggtattgtcactgg	75C2	21/22	N/A	N/A	N/A	N	N
tcatttaaaaaattctata	93F14	19/19	*CG6678*	Unkown	intronic	N	N
tcataaaacagaaaaagg	82F6	18/18	N/A	N/A	N/A	N	N
ggtaaaattagtgaaaaa	83D1	18/18	N/A	N/A	N/A	N	N
tgtacaaataatcctgtt	85B2-7	18/18	*polychaetoid*	JNK Cascade	intronic	N	N

Table 6.2 continued: No sections of the Gal4 transcript fit the criteria for efficient inhibitory RNA down regulation of characterized Drosophila transcripts. Specific characteristics are further described in text. N/A= not applicable.

Sequence (at least 18bp)	Chromosomal location	Identities	Gene	Function	intronic/ exonic	Matches siRNA Y/N	siRNA candidate Y/N
gaaggtgtgcttcttggt	86B1	18/18	*Rfx*	RNA Polymerase II	exonic	N	N
gcagctgttgctgttgcc	88A2	18/18	N/A	N/A	N/A	N	N
tccatttgtaaaaacttt	93E9	18/18	N/A	N/A	N/A	N	N
aaatatccatttgtaaaaacttt	35E2	22/23	N/A	N/A	N/A	Y	N
tgaaagaaattgagatggt	35E4	19/19	N/A	N/A	N/A	N	N
attcgattttgagtttga	21E2	18/18	*drongo*	transporter	intronic	N	N
aaaattattgagataactttga	25B6	21/22	N/A	N/A	N/A	Y	N
catttaaaaaattctata	32F3	18/18	N/A	N/A	N/A	N	N
gctcataaaacagaaaaa	III h	18/18	*scare crow*	NK homeobox	intronic	N	N
tgcagctgttgctgttgc	55C2	18/18	N/A	N/A	N/A	N	N
gtgaaaattattgagata	57F2	18/18	N/A	N/A	N/A	N	N

6.4 Discussion:

Due to the widespread use of the UAS/Gal4 ectopic gene expression system throughout Drosophila genetic studies the discovery that numerous Gal4-driver constructs have deleterious effects has the potential for far reaching consequences. Several theories can be proposed to explain the observed Gal4 phenotypes; our findings serve to better define and/or dismiss these in turn. As the Gal4 protein is a transcriptional activator it may alter normal transcriptional activities within associated cells through uncharacterized interactions with the endogenous transcriptional machinery.

Unfortunately, the Gal4 17bp consensus binding sequence, CGG-N_{11}-CCG (Marmorstein et al. 1992), cannot be directly screened using standard genome wide data-mining tools. Direct analysis of Gal4 genomic DNA binding would require a direct analysis of Gal4 DNA binding, such as a chromatin immunoprecipitation assay as has been used in yeast (Ren et al. 2000). This, in turn, inhibits the clear definition of Gal4 binding sites in the fruit fly genome. In addition, many Gal4 regulated transcripts in *Saccharomyces cerevisiae* require multiple binding sites for proper transcription and activity can vary depending on chromosomal state (Liang et al. 1996; Ren et al. 2000). Despite this, from Table 1 we see that at least six sites resembling the optimized Gal4 consensus sequence exist within recognizable transcriptional units. Gal4 binding at sites internal to transcription may disrupt the proper formation of the associated mRNA, or nearby transcriptional units, in a manner similar to transcriptional repressor proteins (Courey and Jia 2001). These findings demonstrate that transcriptional repression is unlikely as a basis for the *pannier-Gal4*-dependent reduction of microchaetae density for several reasons. The microchaetae density phenotype is repressed by the addition

of the anti-apoptotic p35 protein but not by green fluorescent protein under the control of the optimized Gal4 binding UAS sequence (Figure 6.1). If transcriptional dysregulation was primarily responsible for the observed *pannier-Gal4* phenotype then placing optimized UAS sequences into the Gal4 background would be expected to alleviate these effects by reducing the amount of free Gal4 available at dysregulated sites. The independence of UAS presence suggests that the anti-apoptotic activity of the p35 transgene is responsible for the observed suppression. In addition, based on the analysis of the Drosophila genome for optimized Gal4 binding sites none fit the consensus Gal4 binding site as all lack the CGG-N-CCG format (Table 1). As the CGG/CCG sites are the only regions directly bound by the Gal4 protein none of the sites recognized in the BLAST analysis would be expected to allow Gal4 binding.

Aside from transcriptional dysregulation, the transcription of *Gal4* itself, an mRNA foreign to Drosophila has the potential to elicit immunological responses similar to those operating in untargeted RNA interference (Scacheri et al. 2004). If the *Gal4* mRNA induced the activity of the dicer enzyme to form short RNAs, several regions of *Gal4* with homology to Drosophila transcripts could elicit the RNAi response. Table 2 shows that a BLAST of the fruit fly genome searching for candidate siRNA regions within the *Gal4* transcript revealed that no siRNA candidate regions are present. Further to this, it has been shown that a single nucleotide mismatch within an siRNA duplex can abolish effects, further decreasing these candidates (Elbashir et al. 2001c). The data serve to dismiss these theories still further as the anti-apoptotic p35 protein is capable of suppressing the *pannier-Gal4* phenotype while GFP has no effect, suggesting that the apoptotic process and not the RNAi response is elicited in these cells.

Pannier-Gal4 has been suggested to behave as a hypomorphic allele of the *pannier* locus associated with changes in the dorsal notum surface and bristles (Heitzler et al. 1996; Pena-Rangel et al. 2002). Further characterization of this phenotype suggest additional mechanisms may be involved. The data suggest that the *pannier-Gal4* reduced microchaetae phenotype may be related to the expression of Gal4 itself rather than simply hypomorphic activity of pannier as the *pannier-Gal4* phenotype behaves similar to other Gal4 constructs in which defects have been noted. In particular the finding that the *pannier-Gal4* microchaetae phenotype is suppressed by the anti-apoptotic p35 protein, as has been found in the *GMR-Gal4* driver line, suggests that Gal4 is directly or indirectly inducing apoptotic cell death. Other than the presence of Gal4 the drivers are unrelated with *GMR-Gal4* expression primarily in the eye imaginal disc posterior to the morphogenetic furrow (Freeman 1996) while *pannier-Gal4* is expressed primarily in the wing disc in the regions of the primitive dorsal midline and wing (Calleja et al. 1996). Despite unrelated expression patterns both systems share sensitivity to p35. As the sole similarity between the systems is the presence of Gal4, this suggests that the p35 sensitive process is Gal4-induced and not related to the expression system. In further support, the nature of the *pannier-Gal4* and *GMR-Gal4* transgene constructs differs greatly; *pannier-Gal4* was created by the insertion of the pGawB transposable element into the endogenous *pannier* promoter region (Calleja et al. 1996). *GMR-Gal4* (Hay et al. 1994) was created by inserting a P element containing four glass binding sites from the *Rh1* promoter region (Hay et al. 1994) fused to the Gal4 coding region (Brand and Perrimon 1993). Several independent insertions of the *GMR-Gal4* transgene have been noted to display a rough eye phenotype (Helms et al. 1999; Hiesinger et al. 1999; White and Jarman 2000) shown to be associated with increased apoptosis (Kramer and Staveley 2003). This suggests that the

associated phenotype is independent of chromosomal location and dependent only on the presence of Gal4 expression further supporting a direct role of Gal4 in observed effects.

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Chapter 7: General Conclusions and Future Directions

7.1 Huntingtin interacting protein 1 regulates neurogenesis:

Approximately 1 in 10,000 individuals in the Western world are diagnosed with Huntington's disease (HD) (Petersen et al. 1999). This number is compounded when we consider the emotional and physical stress imparted on relatives and caregivers alike. Given these factors, it is critical that we uncover the molecular alterations underlying this debilitating condition. One leap forward in understanding HD came with the discovery of the genetic change underlying the disease, a CAG trinucleotide expansion in the gene *Huntingtin* (HDCRG 1993). Despite 13 years of intensive research since this original discovery, we still lack both preventative and curative therapies for HD. In order to develop these therapies we must first fully understand the multifaceted changes associated with polyglutamine expansion. Polyglutamine expansion of Huntingtin has been linked to the processes of endocytosis/intracellular transport (Tukamoto et al. 1997; Gunawardena et al. 2003; Trushina et al. 2004), intracellular signalling/cellular survival (Liu et al. 1997; Steffan et al. 2000; Reilly 2001; Humbert et al. 2002; Song et al. 2003; Gauthier et al. 2004; Colin et al. 2005; Warby et al. 2005; Zuccato et al. 2005; Gines et al. 2006), and recently neuronal stem cell proliferation (Curtis et al. 2003; Lazic et al. 2004; Tattersfield et al. 2004; Curtis et al. 2005; Gil et al. 2005; Grote et al. 2005; Jin et al. 2005). Analyzing protein interactions and associated activities altered when the *Huntingtin* mutation is present represents one mechanism to understand and, in turn, prevent the cellular changes which result in HD. One interaction that is altered when Huntingtin is expanded involves Huntingtin interacting protein 1 (Hip1).

Hip1 was originally described based on its reduced binding affinity for polyglutamine expanded Huntingtin (Kalchman et al. 1997; Wanker et al. 1997; Hackam et al. 2000; Gervais

et al. 2002). Similar to Huntingtin, Hip1 has been linked to endocytosis/intracellular transport (Mishra et al. 2001; Rao et al. 2001; Legendre-Guillemin et al. 2002; Metzler et al. 2003; Rao et al. 2003; Hyun et al. 2004; Chen and Brodsky 2005; Legendre-Guillemin et al. 2005; Sun et al. 2005) and intracellular signalling/cellular survival processes (Hackam et al. 2000; Gervais et al. 2002; Rao et al. 2002; Rao et al. 2003). This suggests that the function of Hip1 is closely related to that of Huntingtin itself.

It is clear that the proliferation of neuronal stem cell populations is altered in HD patients (Curtis et al. 2003; Tattersfield et al. 2004; Curtis et al. 2005). However, no clear links have been established to classical pathways of stem cell proliferation. As stem cell replacement therapies have been touted as one promising treatment for HD (Lindvall et al. 2004), links between polyglutamine expansion and classical neurodevelopmental pathways may aid in the development of these treatments. Functional similarities between Hip1 and Huntingtin and reduced binding of Hip1 to polyglutamine expanded Huntingtin suggest that Hip1 may play a role in HD pathogenesis or normal Huntingtin function. Based on these similarities potential roles for Hip1 in the process of neurogenesis were investigated. In particular, links between Hip1 and previously described pathways of neurodevelopment were investigated. The present findings have led to the conclusion that Hip1 does indeed function in a specialized pathway of neuronal development.

Studies encompassed in Chapter 2 of this thesis clearly demonstrate that Hip1 plays a regulatory role in the process of neurogenesis in the *Drosophila melanogaster*. Two variants of *hip1* are produced in Drosophila through alternative splicing, *hip1* and *hip1ΔANTH*. These variants differ in that *hip1* includes a complete ANTH domain while *hip1ΔANTH* lacks a

critical lipid binding region of the ANTH domain necessary for endocytotic function. Overexpression studies demonstrated that the isoforms play a dual role in the process of neurogenesis with *hip1* decreasing and *hip1ΔANTH* increasing neurogenesis as measured by changes in microchaetae density. This represents the first description of a role for Huntingtin interacting protein 1 in neurogenesis in any system.

Further characterization of this novel neurogenic function demonstrates that *hip1* works in combination with *deltex*, a positive regulator of the Notch pathway (Chapter 2). Deltex has been shown to act in a specialized Notch signal independent from the classical pathways of lateral inhibition (Ramain et al. 2001). Co-expression studies show *hip1* acts to enhance while *hip1ΔANTH* acts to suppress the anti-neurogenic and developmental phenotypes seen with *deltex* expression (Figure 2.3). In addition, *hip1* enhances and *hip1ΔANTH* suppresses the microchaetae reduction phenotype seen in a $Notch^{MCD}$ mutant background (Figure 2.4). When taken together it can be concluded that *hip1* plays a dual role in neuronal development through a deltex-dependent Notch pathway and that the anti-neurogenic/neurogenic role of *hip1* is dependent on its ability to function in endocytosis, mediated through the lipid binding pocket of the ANTH domain.

The experiments in Chapter 3 demonstrate that *hip1-/hip1ΔANTH* -mediated changes in neurogenesis in Drosophila are independent of Enhancer of (split) but require signalling through GSK3β and achaete (Figure 3.4-3.6). Using siRNA technologies and immunoprecipitation in human cell culture Chapter 3 also shows that this novel role for Hip1 in neurogenesis, including the deltex-dependent, HES-independent nature, is conserved between Drosophila and human development (Figure 3.1). SiRNA-mediated downregulation

of either *hip1* or *deltex1* mRNA levels in pre-neuronal N-tera2/D1 cells led to reductions in the level of *achaete/scute homolog 1* (*ash1*) mRNA with no changes in *Notch1* levels (Figures 3.1 and 3.2). Figure 3.3 shows that Hip1 and ASH1 physically interact and that reduced expression of *deltex,* an E3 ligase, leads to stabilization of ASH1 after the initiation of neuronal differentiation. These findings define a molecular genetic pathway in which *hip1*, *hip1ΔANTH*, and *deltex* co-operate in the regulation of neurogenic and anti-neurogenic signals (Figure 7.1). Hip1 and Hip1ΔANTH act to transmit either anti- or pro-neurogenic signals, respectively, by modifying the effects of deltex and Notch (Figure 7.1A). These anti- or pro-neurogenic signals, while independent of Hairy and Enhancer of split (Figure 7.1B), are intimately linked to GSK3β activity (Figure 7.1C). Finally, Hip1, Hip1ΔANTH, and deltex enact neurogenic regulation through direct binding, stabilization, and transcriptional modification of the bHLH transcription factor ASH1 (Figure 7.1D).

When taken as a whole it can be concluded from these findings that Hip1 is a novel, well-conserved regulator of neurogenesis in both Drosophila and humans. As the Hip1/Huntingtin interaction is reduced under conditions causing HD, these findings suggest that changes in Hip1's activity in neuronal development may result from this reduced interaction. If so, altered levels of neurogenesis noted in HD patient brains (Curtis et al. 2003; Tattersfield et al. 2004; Curtis et al. 2005) could be a result of altered Hip1 activity. These proliferating cells have been suggested as a potential target of therapeutic intervention. By harnessing the regenerative power of these proliferating neuronal stem cell populations it could be possible to replace those cell populations most affected by the HD mutation. If altered Hip1 activity is indeed the root cause of changes in neurogenesis seen in HD then it may also represent a powerful new target to prevent or reverse these changes.

In addition to the potential for these findings in the treatment and prevention of HD our results show that Hip1 is a novel and complex regulator of neuronal development through the deltex-dependent Notch signalling pathway. This pathway is critical in the development of not only neuronal cell populations but also in the proliferation and maturation of cellular systems as diverse as the hematopoietic and myogenic fates (Hansson et al. 2004). Because of this, these data have the potential to link Hip1 to overall mechanisms of cellular development outside the nervous system advancing the basic biological knowledge of cellular development. As well, the Notch signalling network has been implicated in diverse medical conditions including cancer and inflammatory conditions (Hansson et al. 2004). Further study into the function of Hip1 in Notch signalling may garner new insights into the development and progression of these conditions.

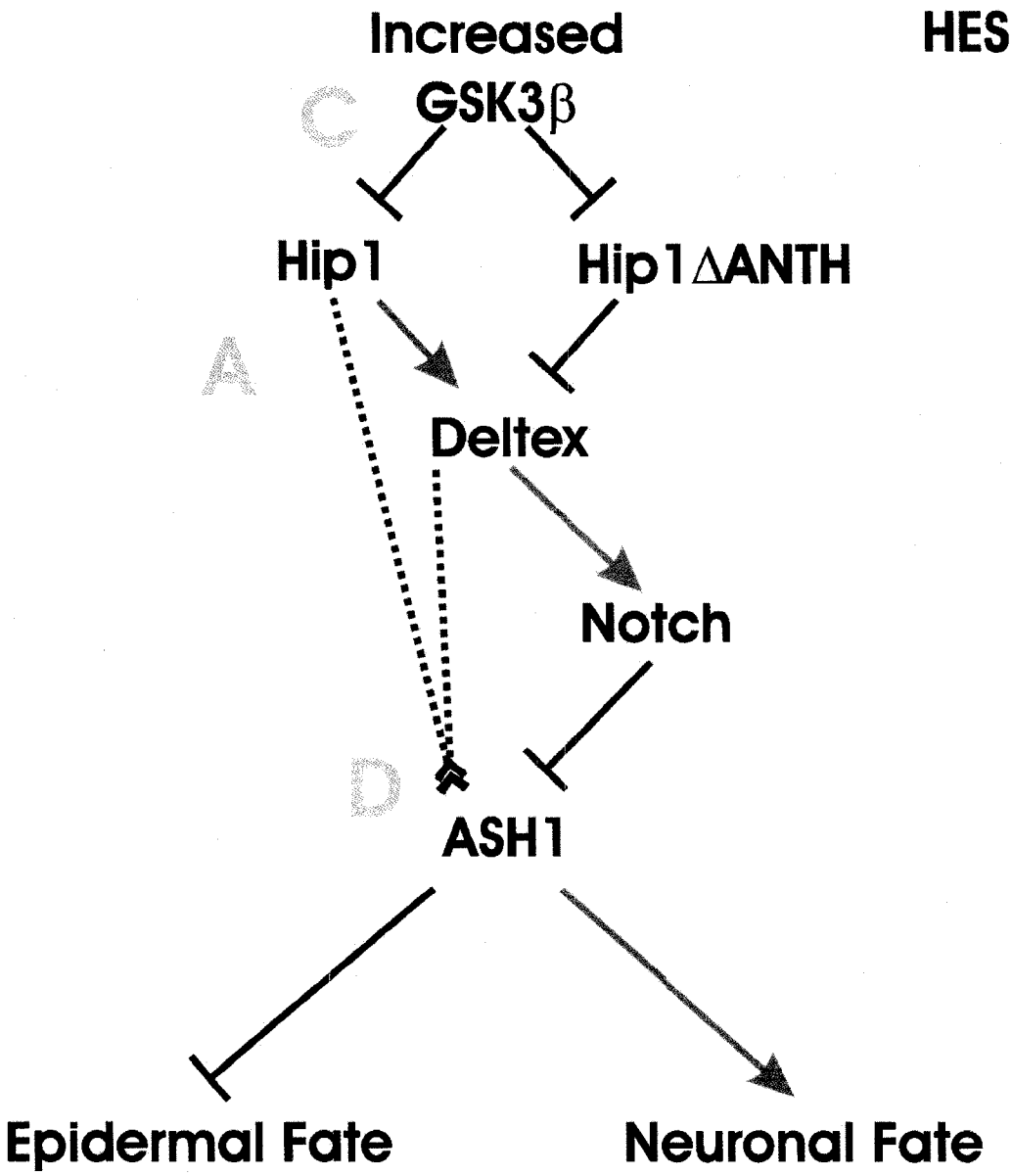

Figure 7.1: Huntingtin interacting protein 1 (Hip1) plays a complex role in the regulation of neuronal fate choices. Hip1 isoforms, Hip1 and Hip1ΔANTH, transmit anti- and pro-neurogenic signals through interactions with the deltex, and in turn, Notch proteins (A). Hip1 neurogenic signalling is independent of classical Notch regulation through the Hairy and Enhancer of split protein, HES1 (B), while inhibited by increased glycogen synthase kinase 3β expression (C). Neurogenic regulation through Hip1 and deltex occurs through physical and transcriptional interactions with the Achaete/Scute homolog (ASH1) transcription factor family (D). Green arrows indicate activatory interactions, red bars indicate inhibitory interactions, blue-dashed lines indicate potential proteasomal regulation.

7.2 *pannier-Gal4* reduces microchaetae density through a p35 sensitive apoptotic mechanism:

Recent findings have raised concerns surrounding the proper use of controls when using the Gal4/UAS ectopic gene expression system (Haywood et al. 2002; Kramer and Staveley 2003). In particular these studies demonstrate that Gal4 expression alone in the absence of UAS target sequences is capable of producing a range of phenotypes including apoptotic cell death (Kramer and Staveley 2003) and reduced life span (Haywood et al. 2002). As the Gal4/UAS system is one of the most widely used tools in Drosophila genetics these findings show that proper experimental controls for any potential Gal4 phenotypes must be incorporated into experimental design. While the apoptotic phenotypes associated with *GMR-Gal4* expression have not been fully defined, it has been shown that the phenotype is sensitive to the expression of the anti-apoptotic p35 protein and the Parkinson's disease related gene *parkin* (Haywood et al. in preparation). Based on these findings the effect of *pannier-Gal4* on microchaetae density was examined and the results of this study are reported in Chapter 6 of this thesis. These studies show that *pannier-Gal4* decreases microchaetae density, and hence *pannier-Gal4/+* animals have been used throughout all studies in this thesis as a control for effects of expression on microchaetae density. In addition to analysis of *pannier-Gal4* effects on microchaetae density, detailed analysis was performed to further define Gal4 phenotypes as a whole.

In particular, it has been suggested that UAS binding sites may exist in the Drosophila genome and that improper transcriptional activity may result in the observed phenotypes. Table 6.1 shows the closest matches to the UAS consensus, CGG AGT ACT GTC CTC CG (Webster et

al. 1988), in the Drosophila genome. As none of these sequences contain the flanking CGG/CCG sequence necessary for Gal4 binding (Marmorstein et al. 1992) transcriptional activation from these sites is not expected. However, this genomic screen could not incontrovertibly demonstrate the lack of Gal4 binding consensus CGG-N11-CCG sites (Marmorstein et al. 1992) due to the lack of complexity and unique features within the Gal4 binding consensus. Due to this the possibility of Gal4 binding sites in the Drosophila genome still exists.

It has also been suggested that in the absence of UAS sequences, fragments of the *Gal4* mRNA itself could activate the immunological RNA inhibitory response. Table 6.2 shows that no fragments of the *Gal4* mRNA share significant sequence homology to efficiently activate the RNAi response based on current understanding of the limitations of RNAi responses (Elbashir et al. 2001a; Elbashir et al. 2001b; Elbashir et al. 2001c). Based on this it can be concluded that the *Gal4* mRNA does not induce RNAi-like responses and that the observed phenotypes are derived though some other mechanism.

In an effort to better define the mechanism underlying observed Gal4 phenotypes co-expression studies using two transgenes, *UAS-p35* and *UAS-GFP*, in the *pannier-Gal4* background were undertaken. Chapter 6 shows that co-expression of *p35* rescued the *pannier-Gal4* microchaetae density phenotype while *GFP* had no effect. Importantly, the UAS constructs used to form each transgene were identical. As the phenotype was shown to be independent of *UAS-GFP,* while sensitive to *UAS-p35,* this argues against transcriptional dysfunction as a cause of the observed phenotype. Both transgenes offered similar opportunities for Gal4 binding, while only the anti-apoptotic *p35* gene was successful in

rescuing the phenotype. It can be concluded that the microchaetae density decrease was caused by a *p35*-sensitive apoptotic mechanism. These results parallel those seen in the otherwise unrelated *GMR-Gal4* system; therefore, it can also be suggested that Gal4 has wide-ranging effects on apoptotic activation and that studies using the Gal4/UAS system should be well controlled, in particular if apoptotic pathways are under investigation.

7.3 Future Directions:

7.3.1 The role of Hip1 in HD-related neurogenesis:

The evidence presented herein strongly suggests that Hip1 functions in Notch-dependent pathways of neurogenic control and its regulation *in vivo* are important in the progression of HD. Detailed analysis in patient samples is required to confirm these relationships. In particular, a detailed analysis of deltex-dependent Notch activation in patient samples would help clarify this role. In addition, detailed expression analyses for Hip1 variants at both the mRNA and protein levels in both the human and Drosophila systems will help to better define the mechanism of *hip1* and *hip1ΔANTH* activity. Reducing the expression levels of all *Hip1* mRNAs in N-tera2/D1 cells (Chapter 3) was successful in eliciting a change in neuronal differentiation. The use of variant specific siRNAs would allow for detailed functional analyses of Hip1 isoforms in this process. At the same time variant specific mutants in Drosophila will help to further characterize the biological functions of these *hip1* variants. Expanding upon the preliminary findings of chapters 4 and 5, the conservation of the Hip1/Huntingtin and Hip1/Hippi interactions, respectively, as well as the development of novel tools to better study these and additional interactions will greatly improve the understanding of this biological system.

Based on the biological conservation demonstrated between the Drosophila and human systems in Hip1 function, the tools created through the course of these studies represent an extraordinary opportunity. Using the combination of genome-wide forward and reverse genetic screens in Drosophila, in parallel with detailed, high-throughput, biochemical analyses available in human NT2 cells will provide a system to rapidly and systematically define the molecular genetic pathways of Hip1 function. This parallel system will allow for both *in vivo* and *in vitro* analysis of therapeutic agents at all stages of research. In turn these tools will help to better define the pathways modified in HD pathogenesis.

7.3.2 Phenotypes associates with pannier-Gal4:

In the case of *pannier-Gal4* microchaetae phenotypes, future research will help to define the exact mechanism of Gal4 effects. Co-expression of the Parkinson's related gene, *parkin*, has been shown to negate the effects of Gal4 in the eye, presumably through its ubiquitin-protein ligase activity (Shimura et al. 2000). Chapter 6 demonstrates that the suppressive effects of p35 expression are conserved from the eye to the notum systems; it will thus be interesting to test for suppression of the *pannier-Gal4* microchaetae phenotype using *parkin* transgenics.

7.4 References:

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