THE HIP1 FAMILY OF CYTOSKELETAL-ASSOCIATED PROTEINS

by

Vikramjit Chopra

B.Sc., McGill University, 1992
M.Sc., McGill University, 1995

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

In

THE FACULTY OF GRADUATE STUDIES
Department of Medical Genetics

We accept this thesis as conforming
to the required standards

THE UNIVERSITY OF BRITISH COLUMBIA
August 25, 2003

Abstract

Huntingtin-interacting protein 1 (HIP1) is a membrane associated protein which interacts with huntingtin, the protein altered in Huntington Disease (HD). It was determined that the HIP1 gene consists of 32 exons spanning approximately 215 kb of genomic DNA and gives rise to two alternate splice forms termed HIP1-1 and HIP1- 2. Additionally, a novel homolog of HIP1, termed HIP12, was identified. HIP1 and HIP12 share significant amino acid identity and both proteins contain an epsin N-terminal homology (ENTH), coiled coil and talin-like domains. While overexpression of HIP1 is toxic in cell culture, HIP12 did not confer toxicity in the same assay systems. Interestingly, HIP12 did not interact with huntingtin but was able to interact with HIP1 suggesting a potential interaction *in vivo* which may influence the function of each respective protein.

Tissue distribution studies indicated that HIP1 was expressed predominantly within the brain whereas HIP12 expression was more ubiquitous. Within cells, HIP1 and HIP12 displayed a similar punctate distribution at the plasma membrane and underlying cortical actin cytoskeleton. HIP1 was shown to interact with the endocytic proteins clathrin heavy chain and AP2. However, HIP12 displayed weak binding to clathrin heavy chain and no interaction with AP2. HIP1 and HIP12 were also shown to interact with the clathrin light chain. In addition, it was also revealed that HIP1 and HIP12 were capable of inducing the assembly of the clathrin coat *in vitro*. Finally, it was determined that HIP12 interacts with F-actin suggesting that HIP12 may interact with the cortical actin

cytoskeleton underlying clathrin-coated pits and vesicles. HIP1 may be indirectly associated with the actin cytoskeleton by forming heterodimers with HIP12. Thus, HIP1 and HIP12 may act as a molecular "link" between the endocytic machinery and the cortical actin cytoskeleton. Taken together, these results demonstrate that HIP1 and HIP12 are components of the endocytic machinery that participate in the recruitment, assembly and stabilization of the clathrin lattice structure.

The observation that huntingtin associates with HIP1 suggests that it may function in endocytosis and transport. Since mutant huntingtin displays an altered interaction with HIP1, it raises the possibility that HD may be the result of defects in endocytosis and transport. The determination of the cellular functions of the HIP1 family and huntingtin in endocytosis and transport may provide valuable insights into the pathogenic mechanism(s) underlying HD.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Adapter protein 1 (AP2)

Adaptor protein 180 (AP180)

α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)

Analysis of variance (ANOVA)

Base pair (bp)

Brain-derived neurotrophic factor (BDNF)

Calmodulin (CAM)

Cdc42-interacting protein 4 (CIP4)

Clathrin assembly lymphoid myeloid leukemia protein (CALM)

Clathrin-coated vesicle (CCV)

Clathrin heavy chain (CHC)

Clathrinlight chain (CLC)

Clathrin-mediated endocytosis (CME)

Chronic myelomonocytic leukemia (CMML)

Death effector domain (DED)

Dentatorubropalliduysian atrphy (DRPLA)

Epsin N-terminal Homology (ENTH)

γ-amino butyric acid (GABA)

Glutathione S-transferase (GST)

Hemagglutinin (HA)

Huntingtin associated protein 1 (HAP1)

Huntingtin interacting protein 1 (HIP1)

Huntingtin interacting protein 12 (HIP12)

Huntingtin interacting protein 14 (HIP14)

HIP1 protein interactor (HIPPI)

Huntington Disease (HD)

Huntington Disease Collaborative Research Group (HDCRG)

Huntingtin, elongation factor 3, protein phosphatase 2A subunit 65, and the lipid kinase TOR (HEAT)

type 1 inositol (1,4,5)-triphosphate receptor ($InsP_3R1$)

Kilo-base pair (kb)

Neuronal intranuclear inclusion (NIIs)

N-methyl-D-aspartate (NMDA)

Phosphatidylinositol-4,5-bisphosphate [$PtdIns(4,5)P_2$]

Platelet-derived growth factor β receptor (PDGFβR)

Postsynaptic density 95 (PSD95)

Spinal and Bulbar muscular atrophy (SBMA)

Spinocerebellar ataxia type 1 (SCA1)

Spinocerebellar ataxia type 2 (SCA2)

Spinocerebellar ataxia type 3 (SCA3)

Spinocerebellar ataxia type 6 (SCA6)

Spinocerebellar ataxia type 1 (SCA7)

Src homology 2-containing inositol 5-phosphatase (SHIP1)

Synthetic Lethal with Actin binding protein-1 #2 (Sla2)

Transgenic mouse model for prostate cancer (TRAMP)

Ubiquitin-conjugating enzyme (hE2-25K)

Wiskott-Aldrich syndrome protein (WASP)

Yeast artificial chromosome (YAC)

Dedication

To my wife, mother, father, and brother for their love, patience, and support.

Acknowledegements

I would very much like to thank Dr. M. R. Hayden for giving me the opportunity to work in his laboratory and providing me with guidance throughout my graduate training. I would also like to thank members of my thesis committee, Dr. F. Jirik, Dr. D. Mager, and Dr. R. McMaster for keeping me on the right track through their thoughtful comments throughout the years. I am also very grateful to all the past and present members of Dr. Hayden's laboratory and my fellow colleagues at the Centre for Molecular Medicine who generously answered my many questions and provided me valuable advice. I would especially like to thank Roshni, Brett, Susie, Keith, Ryan, Simon, Ed and Rebecca. I would like to thank my collaborators Dr. M. Metzler, members of Dr. P. McPherson's Lab, and members of Dr. D. Nicholson's team at Merck Frosst Canada. I would like to give thanks and love to my mom and dad for their incredible support and love. All my achievements are because of you two. The mistakes are mine. Finally, I would like to give love and thanks to my wife, Manjit for her love, patience and faith in me. I cannot thank you enough for everything that you have given me. But I will try. This thesis was supported in part by scholarships from the Huntington Disease Society of Canada, the University of British Columbia Graduate Fellowship, and the British Columbia Children and Women's Research Institute.

Chapter 1:

Introduction

1.1 Clinical Presentation, Epidemiology and Natural History

Huntington Disease (HD) is a severe autosomal dominant neurodegenerative disorder that occurs with equal frequency in both sexes and has no known cure (Hayden, 1981). The incidence of HD in North American and European populations is ~1/10000 with other populations showing lower frequencies. HD is characterized by chorea, dementia and psychiatric disturbances (Hayden, 1981). The initial symptoms can include behavioral disturbances and minor motor disturbances. As the disease progresses, the classical hallmark of HD, chorea, becomes apparent (Martin and Gusella, 1986). During the later stages of the disease the motor disturbances worsen resulting in rigidity, dystonia, dysphagia and dysarthria. HD patients often develop marked weight loss. Furthermore there is significant cognitive deficits including intellectual decline, memory loss and dementia.

Although the age of onset in HD is variable, in most instances symptoms first appear at approximately 40 years of age. The mean life expectancy after first presentation of the symptoms is 18 years. HD displays the phenomenon of genetic anticipation where, in this case, earlier age of onset and more rapid progression of the disorder correlate with inheritance of the mutant allele from the father. Individuals with onset under 20 years of age are defined as juvenile HD and comprise 5 to 10% of total cases (Hayden, 1981). Juvenile HD displays somewhat different clinical features including rigidity, spasticity, and severe intellectual decline. Furthermore, the progression of the disorder is much more rapid in juvenile patients (Hayden, 1981).

1.2 Neuropathology

Neuropathological findings in HD brains indicate that there is a selective loss of neurons in the neostriatum (caudate nucleus and putamen) and neurons within the deeper layers of the cerebral cortex (Vonsattel et al., 1985). Medium spiny neurons of the striatum produce the neurotransmitters γ-amino butyric acid (GABA) which upon release exerts inhibitory effects (via the direct and indirect pathways) on neurons within the globus pallidus. In HD patients the loss of the GABAergic medium spiny neurons results in the loss of inhibition of neurons in the globus pallidus, which in turn results in choreic movements (Albin et al., 1992; Kremer et al., 1992; Vonsattel and DiFiglia, 1998). Loss of regulation of neurons in the internal segments of the globus pallidus also results in the rigid state seen in the advance stages of the disease (Kremer et al., 1992). Glutamate receptor hyperactivity, referred to as excitotoxic cell death, may also play a role in the selective neuronal loss seen in HD patients and mouse models of HD (Albin et al., 1992; Hodgson et al., 1999; Zeron et al., 2002). Overactivation of glutamate receptors results in high calcium influx that activates calcium–dependent degradative proteases and endonucleases and creation of reactive oxygen species that can lead to cellular dysfunction and death (Albin et al., 1992; Zeron et al., 2002). In addition to the loss of medium spiny neurons, the striatum also exhibits signs of inflammation such as astrogliosis, microgliosis and complement activation (Singrao et al., 1999). Examination of the degenerating neurons has demonstrated that these cells contain swollen mitochondria, nuclear and cytoplasmic inclusions, condensed cytoplasm, and condensed and marginalized heterochromatin (Difiglia et 1997).

1.3 HD Gene

The HD gene was first identified by the HD Collaborative Research Group in 1993 (HDCRG, 1993). The HD gene is located on the short arm of chromosome 4 (4p16.3), spans ~210kb and consists of 67 exons. The HD gene encodes 2 differentially polyadenylated transcripts that are 10.3 and 13.6kb in size that are ubiquitously expressed (Lin et al., 1994). Furthermore, no differences are observed between normal and affected individuals, with respect to expression levels or patterns (Li et al, 1993). These transcripts encode a 348kDa protein (called huntingtin) which, like the mRNA, is expressed in all tissues of normal and affected individuals (Sharp et al., 1995; Trottier et al., 1995). Within exon 1 of the HD gene is a tract of multiple [CAG]n, which code for glutamine residues. The mutation underlying HD stems from an expansion of this CAG tract beyond 35 repeats. The nature of the mutations has placed HD into a growing list of neurodegenerative disorders associated with repeat expansions within the coding portion of their respective genes (table 1.1).

1.4 CAG Repeat Length is Inversely Correlated with the Onset of HD

The normal range of CAG repeats in huntingtin is 9-35 with a mean of ~18. The range in HD patients is 36-150, with a mean of 46 (Duyao et al., 1993; Snell et al., 1993; Andrew et al., 1993; Read et al., 1993; Nance et al., 1999). There is a significant inverse correlation between the age of onset and the repeat size length (Trottier et al., 1994; Brinkman et al., 1997). For example, the range of repeats observed in juvenile HD is 44-121 (Telenius et al., 1993). Although the sex incidence of juvenile HD is equal, ~80% of

Table 1.1 The CAG trinucleotide repeat diseases

DISEASE	CLINICAL PRESENTATION	SITE OF PATHOLOGY	INHERITANCE	AFFECTED CAG	PROTEIN	GENOME LOCATION	REFERENCE
Huntington Disease	1) Chorea 2) Dementia 3) Psychiatric disturbances 4) Juvenile rigidity	1) Caudate nucleus 2) Putamen 3) Cerebral cortex	Autosomal dominant	36-150	Huntingtin	4q16.3	HDCRG, 1993
Spinocerebellar ataxia type 1	1) Ataxia 2) Dysphagia 3) Amyotrophy	1) Cerebellum (Purkinje cells) 2) Inferior olive	Autosomal dominant	39-81	Ataxin-1	6p23	Orr et al., 1993
Spinocerebellar ataxia type 2	1) Ataxia 2) Sensorimotor neuropathy	1) Cerebellum (Purkinje cells) 2) Inferior olive 3) Substantia nigra	Autosomal dominant	34-64	Ataxin-2	12q24	Imbert et al., 1996; Pulst et al., 1996; Sanpei et al., 1996
Spinocerebellar ataxia type 3	1) Ataxia 2) Cranial nerve deficits	1) Dentate nucleus 2) Pontine nucleus 3) Anterior horn cells 4) Spinocerebellar tracts 5) Subthalamic tracts	Autosomal dominant	61-84	Ataxin-3	14q24.3	Kawaguchi et al., 1994
Spinocerebellar ataxia type 6	1) Ataxia 2) Defects in proprioception	1) Cerebellum (Purkinje cells) 2) Inferior olive	Autosomal dominant	21-27	α-1A-voltage-dependent calcium channel	19q13	Zhuchenko et al. 1997
Spinocerebellar ataxia type 7	Ataxia	Degenerative retinal blindness	Autosomal dominant	38-130	Unknown	3p12	Del-Favero et al., 1998; David et al 1997
Dentatorubral-pallidoluysian atrophy	1) Ataxia 2) Dementia 3) Myoclonic epilepsy	1) Dentate nucleus 2) Globus pallidus	Autosomal dominant	52-88	Atrophin	12p13.31	Koide et al., 1994
Spinal and bulbar muscular atrophy	1) Gynecomastia 2) Motor deficits 3) Amyotrophy 4) Sensory deficits	1) Anterior horn 2) Cranial motor nuclei	X-linked	38-75	Androgen receptor	Xq11	La Spada et al., 1991

juvenile HD patients inherit the defective gene from the father (Hayden, 1981). Moreover, Ridley et al. (1988) demonstrated that the average age of onset is earlier in offspring of affected males. However, the mean age of onset in the offspring of affected females does not vary significantly from that of their mothers (Ridley et al., 1988). Taken together, these findings show that the CAG repeat is more unstable upon transmission through the male germline. It has been suggested that the higher instability of CAG tracts in the paternal germline and the inverse correlation between the age of onset and the repeat length represent the molecular basis of genetic anticipation in HD.

1.5 Do CAG Expansions lead to a Gain of Function Mutation?

The development of HD knockout mice has indicated that the polyglutamine expansion in the huntingtin protein results in a gain of function mutation. Nasir et al. (1995), Duyao et al. (1995) and Zeitlin et al. (1995) generated targeted disruption in exon 5, in exon 4 and the promoter of the HD gene, respectively. Mice heterozygous for the disruption did not develop a phenotype reminiscent of HD. Instead, mice homozygous for the disruption died before embryonic day 8.5 demonstrating that the HD gene plays a critical role in development. Examination of the embryos indicated that they were able to initiate gastrulation, but do not proceed to form somites or undergo organogenesis. For example, using embryonic stem cells lacking any HD gene expression Metzler et al. (2000) demonstrated that huntingtin is also required for the normal generation and expansion of hematopoietic cells.

The development of conditional HD gene knockout mice, however, has provided evidence that the disease may in part be due to a loss of huntingtin function. Dragatsis et

al. (2000) selectively inactivated wild-type huntingtin in the brains and testes of adult mice. These mice developed progressive neurodegeneration within the striatum and cortex and displayed abnormal limb-clasping behavior, slight tremor and hypoactivity (Dragatsis et al., 2000).

1.6 Inclusions and Aggregates in HD

The hypothesis that polyglutamine expansion results in a gain of function mutation may explain the development of insoluble aggregates or inclusions within the cytoplasm and nucleus of neurons in HD mouse models as well as affected individuals. These aggregates contain ubiquinated amino-terminal fragments of huntingtin (Davies et al.1997; DiFiglia et al., 1997). In addition, the aggregates contain a number of other proteins including chaperones (Jana et al., 2000), and transcription factors (Boutell et al, 1999; Steffan et al., 2000; Suhr et al., 2001; Nucifora et al., 2001).

The aggregates were initially observed in transgenic mice that over-expressed exon 1 of the HD gene containing an expanded CAG repeat length (Davies et al., 1997). The mice contained neuronal intranuclear inclusions (NIIs) in several regions of their brains including the striatum, cortex, cerebellum and spinal cord. NIIs have been observed in post mortem brain material from HD patients (DiFiglia et al., 1997; Becher et al., 1997). In patients affected with HD, these aggregates are found solely in the dystrophic neurites and neuropil but never in glial cells (DiFiglia et al., 1997). In addition, larger numbers of aggregates have been found in the striatum and cortex in the more severe juvenile-onset HD (DifFiglia et al., 1997). Interestingly, aggregate accumulation seems to be common feature in a number of different trinucleotide repeat

disorders including SCA-1 (Skinner et al., 1997), SCA-3 (Paulson et al., 1997), SCA-7 (Holmberg et al., 1998), SBMA (Li et al., 1998) and DRPLA (Becher et al., 1997).

It has been demonstrated that the accumulation of aggregates in neurons does not necessarily play a causal role in cell death. Saudou et al. (1998) demonstrated that nuclear import of mutant huntingtin is sufficient to induce cell death and does not require the formation of NIIs. Furthermore, neurodegeneration without the appearance of aggregates has been observed in a YAC transgenic mouse model for HD (Hodgson et al., 1999; Slow et al., 2003). It has been hypothesized that aggregates are not the cause of cell death but are rather a marker for cellular dysfunction.

At this time it is unclear what are the underlying mechanisms underlying the formation of aggregates. Recently, it has been demonstrated that the phosphorylation occurs of mutant ataxin-1 at discrete sites (Emamian et al., 2003). The phosphorylated ataxin-1 interacts with the regulatory protein 14-3-3, which in turn, leads to the formation of aggregates (Chen et al., 2003). Two additional biochemical mechanisms have also been proposed to explain why mutant huntingtin tends to form aggregates. One possibility is that the expanded polyglutamine tract in huntingtin can form stable hairpin structures consisting of anti-parallel β-sheets (Perutz, 1999, Thakur et al, 2002). Another possibility is that huntingtin and other polyglutamine containing proteins may be the target of transglutaminases. Transglutamination would result in the formation of protein complexes that are cross-linked via isopeptide bonds (Green, 1993). Interestingly, transglutaminase activity has been shown to be increased in HD patients (Karpuj et al., 1999).

1.7 Proteolysis of Huntingtin

The presence of amino terminal fragments of huntingtin has been demonstrated in brain material from HD patients (DiFiglia et al., 1997; Becher et al., 1997). The generation of these fragments may in part be due to cleavage of huntingtin by proteases. *In vitro* studies have shown that huntingtin is a target for proteolysis by caspase-3 and 6 in a polyglutamine length-independent manner (Wellington et al., 2000; Wellington et al., 1998; Goldberg et al., 1996). Caspases are a family of cysteine aspartic acid proteases involved in the execution phase of apoptosis (Thornberry et al., 1997). The presence of activated caspase 3 and 6 has been confirmed in the brains of a YAC mouse model of HD, as well as post-mortem brain material from HD patients (Wellington et al., 2002; Kim et al., 2001). In addition, huntingtin cleavage can occur in the absence of an apoptotic stimulus suggesting that caspase cleavage of this protein may be a normal physiological mechanism to regulate huntingtin levels within the cell (Wellington et al., 2000; Wellington et al., 2002).

It has been hypothesized that caspase cleavage of huntingtin having an expanded polyglutamine tract may lead to amino terminal fragments which may be toxic to the cell, and also lead to further activation of caspases (and other proteases) that ultimately lead to cell death. Indeed, it has been established that amino terminal fragments of expanded huntingtin can induce neuronal cell death (Hackam et al, 1998; Martindale et al., 1998; Saudou et al., 1998, Cooper et al, 1998). Moreover, inhibition of cleavage of expanded huntingtin by caspases inhibitors has been shown to greatly reduce both cellular toxicity and the accumulation of cytoplasmic and nuclear inclusions (Kim et al., 1999; Wellington

et al., 2000). These studies suggest that cleavage of mutant huntingtin may play an important role in the pathogenesis of HD.

Huntingtin can also undergo proteolysis by calpains, a family of Ca^{2+}-dependent cysteine proteases (Kim et al., 2001; Gafni and Ellerby, 2002). Calpains cleave huntingtin in a polyglutamine–length dependent manner, where larger tracts confer greater cleavage (Gafni and Ellerby, 2002). These studies may be linked to previous findings indicating that intracellular Ca^{2+} levels are altered in both *in vitro* and *in vivo* models for HD (Li et al., 2000, Hodgson et al., 1999; Chen et al., 1999).

Recently, Lunkes et al (2002) have demonstrated that an undefined aspartic endopeptidase can cleave huntingtin to generate two amino-terminal fragments termed cp-A and cp-B. These two fragments appear to play an important role in the biogenesis of inclusion within the nucleus and cytoplasm. The cp-A fragments are found exclusively in nuclear aggregates whereas the cp-B is found in the cytoplasmic inclusion in association with cytosolic proteins (Lunkes et al., 2002).

1.8 Cellular Functions of Huntington

Although the exact functions of huntingtin are unknown, a growing body of literature has indicated that this protein may play an important role in a number of different cellular processes including cellular survival, transcriptional regulation, intracellular signaling, and cellular transport and endocytosis.

1.8.1 Cellular Survival

Several groups have demonstrated that neuronal cell death in HD may involve apoptosis (Nasir et al., 1995; Thomas et al., 1995; Portera-Cailliau et al., 1995; Dragunow

et al., 1995). Similarly, Zeitlin et al. (1995) reported homozygote HD knockout mice that displayed higher levels of apoptosis. They postulated that wild-type huntingtin is involved in processes to counterbalance the apoptotic pathway. *In vivo* studies have shown that wild-type huntingtin can also mitigate the toxic effects of mutant huntingtin in a YAC mouse model of HD (Leavitt et al., 2001). *In vitro* studies suggest that expression of wild-type huntingtin can protect both neural and non-neuronal cells from toxic stressors such as serum deprivation, 3-nitropropionic acid (a mitochondrial toxin), and the expression of either full-length or amino-terminal fragments of mutant huntingtin (Rigamonti et al., 2000; Ho et al., 2001; Cattaneo et al., 2001). Huntingtin is thought to mediate its protective effect by down-regulating apoptotic signals upstream of caspase-3 activation and downstream of pro-apoptotic Bcl-2 family members, Bik and Bak (Rigamonti et al., 2000). Interestingly, Zuccato et al. (2001) have demonstrated that normal huntingtin can upregulate transcription of brain-derived neurotrophic factor (BDNF) whereas mutant huntingtin decreases BDNF expression. The loss of BDNF in cells expressing mutant huntingtin may ultimately lead to cell death (Zuccato et al., 2001; Zuccato et al., 2003). Finally, wild-type huntingtin can interact with the pro-apoptotic protein huntingtin interacting protein 1 (HIP-1) and thereby prevent it from inducing apoptosis (Hackam et al., 2000). HIP-1 will be discussed in greater detail in later sections.

1.8.2 Transcriptional Regulation

It has been hypothesized that huntingtin may play a role in transcriptional regulation (Perutz, 1999). This theory is based, in part, on the observation that glutamine-rich regions are present in a number of transcription factors including the TATA binding protein (Kao et al., 1990) and Sp1 protein (Courey and Tjian, 1988). The glutamine

repeats within the HD protein may allow it to interact with proteins in the transcriptional machinery and the DNA itself (Perutz 1999; Perutz, 1994; Stot et al., 1995). The glutamine-rich region in the androgen receptor, which is expanded in SBMA, is believed to be important for interaction with other transcription factors. In addition, huntingtin can be found within the nucleus where it may interact with a number of different transcription factors and influence their activity. For a complete list of huntingtin-interacting transcription factors and their known functions please refer to table 1.2. Finally, examination of mouse models of HD expressing either full-length or an amino terminal fragment of mutant huntingtin exhibit marked alterations in the expression of various genes (Luthi-Carter et al, 2002; Chan et al., 2002).

1.8.3 Intracellular Signaling

Huntingtin has been shown to be play a role of intracellular signaling based on its interaction with a number of different signaling proteins such as epidermal growth factor receptor signaling complex (Liu et al., 1997) and the JNK activator, mixed lineage kinase 2 (MLK2; Liu et al., 2000). For a complete list of huntingtin-interacting signaling proteins and their known functions please refer to table 1.2.

Recently, huntingtin has also been shown to be phosphorylated by the serine / threonine kinase, Akt (Humbert et al, 2002). Activation of Akt has been shown to have a neuroprotective effect in models of neurodegenerative diseases (For reviews see Dore et al., 2000; Brunet et al., 2001). Interestingly, *in vitro* studies indicated that the phosphorylation abrogates the ability of mutant huntingtin to induce apoptosis (Humbert et al., 2002).

1.8.4 Cellular Transport and Endocytosis

Immunohistochemical studies indicate that huntingtin is localized to the intracellular membranes and the cytoskeleton. Huntingtin is present in the axon and nerve terminals where it appears to be associated with microtubules and synaptic vesicles (DiFiglia et al., 1995; Gutekunst et al., 1995; Tukamoto et al., 1997). In addition, Velier et al. (1998) have demonstrated that huntingtin may play a role in vesicle transport in the endocytic pathways through associations with clathrin-coated vesicles. Consistent with these findings is the observation that huntingtin contains HEAT repeat domains (Andrade and Bork, 1995; for a review see Andrade et al., 2001). The HEAT domains mediate protein-protein interactions and are present in proteins that perform multiple cellular functions. Of particular interest is importin, a HEAT repeat containing protein, which is involved in the transport of proteins from the cytoplasm to the nucleus (Andrade and Bork, 1995). By analogy, huntingtin may also play a role in transport. Huntingtin has also been shown to interact with a number of proteins that are proposed to have a role in transport and endocytosis (see table 1.2). These interacting proteins will be discussed in greater detail below.

Table 1.2 Huntingtin interacting proteins and their functions

Huntingtin Interacting Protein	Interaction	Function	Reference
Cystathione β-Synthase	No CAG modulation	Synthesis of cystathione	Boutell et al., 1998
growth factor receptor-bound protein 2 (Grb2)	No CAG modulation	Activation of the Ras protein kinase pathway	Liu et al., 1998
Ras GTPase-activating protein (RasGAP)	No CAG modulation	Inactivation of the Ras protein kinase pathway	Liu et al., 1998
Mixed-Lineage Kinase 2 (MLK2)	Does not interact with mutant huntingtin	Activation of the JNK-mediated apoptosis	Liu et al., 2000
Calmodulin	Wild-type huntingtin requires calcium for binding whereas mutant huntingtin does not.	Regulation of various calcium-dependent enzymes	Bao et al., 1996
Heat Shock Proteins 70 and 40	Increased binding with larger CAG lengths	Molecular chaperones	Jana et al., 2000
Postsynaptic Density 95	Does not interact with mutant huntingtin	Regulation of glutamate receptor activity	Sun et al., 2001
GAPDH	Increased binding with larger CAG lengths	Glycolytic enzyme	Burke et al., 1996
Ubiquitin-conjugating enzyme	No CAG modulation	Links ubiquitin residues to glycine residues on target proteins	Kalchman et al., 1996
Nuclear Receptor Co-Repressor	Increased binding with larger CAG lengths	Transcriptional repressor	Boutell et al.; 1998
mSin3a	Increased binding with larger CAG lengths	Transcriptional co-repressor	Steffan et al., 2000
Transcriptional Co-Repressor C-Terminal Binding Protein (CtBP)	Decreased binding with larger CAG lengths	Transcriptional co-repressor	Kegel et al.; 2002
CREB binding protein	Increased binding with larger CAG lengths	Acetylation of Histone H3 and H4	Kegel et al.; 2002

p300/CBP-associated factor	No CAG modulation	Acetylation of Histone H3 and H4	Steffan et al, 2000; Steffan et al., 2001; Nucifora et al. 2001
p53	No CAG modulation	1)Transcriptional regulator 2) Tumor suppressor 3) Activator of apoptosis	Steffan et al., 2000
Forminin binding protein 11 (HYPA)	Unknown	Involved in pre-mRNA splicing	Farber et al., 1998
HYPB	Unknown	Transcriptional regulator	Farber et al., 1998
HYPC	Unknown	Unknown	Farber et al., 1998
CA150	No CAG modulation	Transcriptional regulator	Holbert et al., 2001
Cdc42-interacting protein 4	Increased binding with larger CAG lengths	1) Regulation of cortical actin cytoskeletal dynamics 2) Recycling of the Glucose transporter Glut4	Holbert et al., 2003
Huntingtin Associated Protein 1	Increased binding with larger CAG lengths	Intracellular transport	Li et al., 1995; Englender et al, 1997; Li et al., 1998
α-adaptin C subunit (HYPJ)	Unknown	1) Subunit of adapter protein 2 (AP2). 2) AP2 is essential for clathrin coat formation and interacts with various members of the receptor mediated endocytosis machinery	Faber et al., 1998
Endophillin A3 (SH3GL3)	Increased binding with larger CAG lengths	Component of the endocytic machinery	Sittler et al., 1998
Pacsin 1	Increased binding with larger CAG lengths	Component of the endocytic machinery	Modregger et al., 2002
Huntingtin Interacting Protein 14	Decreased binding with larger CAG lengths	1) Component of the endocytic machinery 2) Intracellular transport	Singaraja et al., 2002
Huntingtin Interacting Protein 1	Decreased binding with larger CAG lengths	1) Component of the endocytic machinery 2) Regulation of cortical actin cytoskeletal dynamics 3) Cellular survival 4) Activation of apoptosis	Kalchman et al., 1997; Wanker et al., 1997; Metzler et al., 2001; Hackam et al., 2000; Gervais et al., 2002

1.9 Interacting Proteins involved in Endocytosis and Cellular trafficking

The HD gene product is constitutively expressed in both the brain and peripheral tissues. Since HD results in the selective loss of neurons, it has been suggested that the mutant HD protein mediates its effects by interacting with other brain-specific proteins. Several studies have determined that huntingtin interacts with components of the endocytic machinery and cellular transport. These proteins include HAP1, (Li et al., 1995), HIP1 (Wanker et al., 1997; Kalchman et al., 1997); SH3GL3 (Sittler et al, 1998), α-adaptin-C (Faber et al., 1998), HIP14 (Singaraja et al., 2002) and Cdc42-interacting protein 4 (Holbert et al., 2003). Each of these proteins have been implicated in the process of clathrin-mediated endocytosis (CME) and vesicle transport. CME will be discussed in greater detail in section 1.10. Please refer to figure 1.2 for a diagram of the different stages of clathrin mediated endocytosis. The nature of these interactions and their relevance to the pathogenesis of HD are discussed below.

1.9.1 Huntingtin Associated Protein 1

The first known huntingtin-interacting protein HAP1 (Huntingtin associated protein) was identified using the yeast 2-hybrid system (Li et al., 1995). The interaction between HAP1 and huntingtin has been shown to be enhanced by an expanded polyglutamine tract (Li et al., 1995). HAP1 is highly expressed in the neurons of the cortex and striatum, regions involved in HD pathology. However, it is also expressed in the cerebellum and brainstem, regions unaffected in HD (Gutekunst et al., 1998). Interestingly, the expression of HAP1 in the striatum was more widespread than huntingtin suggesting that the interaction between these two proteins only occurs in a

subset of the striatal neurons (Gutekunst et al., 1998). HAP1 shows no similarity with other known proteins that could provide clues as to its normal cellular function.

Within the neuron, HAP1 and huntingtin displayed a similar cellular distribution, including several different membranous organelles such as mitochondria, endoplasmic reticulum, lysosomal, endocytic and synaptic vesicles (Gutekunst et al., 1998; Martin et al., 1999). In addition, huntingtin and HAP1 were also associated with microtubules (Gutekunst et al., 1998). Taken together these findings suggest that HAP1 and huntingtin may play a role in vesicle trafficking and organelle transport. Consistent with this idea is the observation that HAP1 interacts with $p150^{glued}$, a subunit of the microtubule-based transporter complex dynein (Englender et al, 1997, Li et al., 1998). In addition, HAP1 has been shown to interact with duo, a guanine nucleotide exchange factor that is a regulator of actin cytoskeletal dynamics (Colomer et al., 1997). HAP1 has also been shown to interact with hepatocyte growth factor-regulated tyrosine kinase (Hrs), a protein involved in the regulation of vesicle transport from the early endosomes to the late endocytic compartments (Li et al., 2002). At this time it is unclear how the enhanced binding of HAP1 to mutant huntingtin may lead to some of the pathological changes seen in HD. It may be possible that the increased interaction may somehow perturb HAP1's ability to transport vesicles which may ultimately lead to the cellular dysfunction or death seen in HD.

Recently, it has been demonstrated that HAP1 interacts with type 1 inositol (1,4,5)-triphosphate receptor, $InsP_3R1$ (Tang et al., 2003). $InsP_3R1$ is an intracellular calcium channel which functions in calcium signaling within neurons. In addition, Tang et al. (2003) have identified a ternary complex consisting of $InsP_3R1$, HAP1 and

huntingtin. Interestingly, activation of $InsP_3R1$ by its ligand, $InsP_3$, is sensitized by the presence of mutant huntingtin, resulting in an increase in intracellular calcium. It is postulated that the increased calcium levels may activate calcium–dependent degradative enzymes and generate reactive oxygen radicals, both of which result in cellular dysfunction and death (Albin et al., 1992). Indeed, several studies have reported that intracellular calcium levels are elevated in HD patients and mouse models of HD (Albin and Greenamyre et al, 1992, Hodgson et al., 1999; Zeron et al., 2002).

Chan et al. (2002) have generated mice with a targeted disruption in the HAP1 gene (*HAP1*$^{-/-}$). The *HAP1*$^{-/-}$ mice displayed a depressed feeding behavior which resulted in postnatal death. The role of HAP1 in normal feeding activity is unclear.

1.9.2 α-Adaptin-C

A yeast two hybrid screen of a human fetal brain library demonstrated that huntingtin with an expanded polyglutamine tract (58 and 62 repeats) interacted with the endocytic machinery component α-adaptin-C (Faber et al., 1998). At this time it has not been tested whether α-Adaptin-C also interacts with wild-type huntingtin. α-adaptin-C is a subunit of the adapter protein 2 complex (AP2). AP2 consists of four subunits: α-adaptin, β2-adaptin, μ2-adaptin and σ-adaptin (Slepnev and DeCamilli, 2000). This heterotetrameric complex has been shown to interact with membrane lipids and various components of the endocytic machinery (Slepnev and DeCamilli, 2000). Indeed, the AP2 complex has been shown to play a role in the cellular process referred to as clathrin mediated endocytosis (CME) at the cytoplasmic membrane (Brodsky et al., 2001). During CME, AP2 is implicated in the recruitment and polymerization of clathrin

subunits which ultimately form clathrin-coated vesicles (Brodsky et al., 2001). Furthermore, AP2 is also thought to help synchronize the process of endocytosis with intracellular signaling pathways (Slepnev and DeCamilli, 2000; Brodsky et al., 2001). The process of CME will be discussed in greater detail in section 1.12. Since it is unknown whether wild-type and mutant huntingtin differ in their binding affinity to α-adaptin-C, it is difficult to speculate as to role these interactions play in the pathogenesis of HD.

1.9.3 Endophillin A3

Sittler et al. (1998) have demonstrated that huntingtin can interact with the endocytic protein endophillin A3 (also known as SH3GL3). Endophillin A3 contains a SH3 domain which has been shown to mediate its interaction with the polyproline-rich domain in huntingtin. Moreover, an increased strength of interaction was shown to correspond to larger lengths in the polyglutamine tract of huntingtin (Sittler et al., 1998). Although the exact function of endophillin A3 is unclear, it has been postulated that it plays a role in the induction of membrane curvature during clathrin-dependent endocytosis (Slepnev and De Camilli, 2000). Similar to huntingtin, the highest levels of endophillin A3 expression are seen in the brain and testes (Sittler et al., 1998). *In vitro* studies using cultured cells have indicated that endophillin A3 co-localizes with mutant huntingtin in both nuclear and cytoplasmic inclusions (Sittler et al., 1998). In addition, over-expression of endophillin A3 was shown to enhance the accumulation of aggregates in cultured cells expressing mutant huntingtin (Sittler et al., 1998). These findings suggest that endophillin A3 plays a role in aggregate formation that is seen in the brains

of HD patients. Furthermore, endophillin A3's functions in endocytosis may be compromised due to sequestration within aggregates. Thus, disruption of clathrin-mediated endocytosis might play a role in the pathogenesis of HD.

1.9.4 Pacsin 1

Recently, huntingtin has been shown to interact with the neurospecific protein Pacsin 1 (also known as syndapin; Modregger et al., 2002). The interaction was mediated by the SH3 domain of Pacsin 1 and the polyproline region of huntingtin. In addition, the strength of this interaction was enhanced by increased length of the polyglutamine tract in huntingtin (Modregger et al., 2002). Pacsin 1 is thought to play an important role in the recruitment and stimulation of the actin polymerization machinery at the site of newly formed endocytic vesicles (Modregger et al., 2002; Qualmann and Kessels, 2002). Within the neuron, Pacsin 1 is normally located throughout the cytoplasm and along neurites and presynaptic boutons (Modregger et al., 2002). Immunohistochemical studies by Modregger et al. (2002) have demonstrated that huntingtin shows partial co-localization with Pacsin 1 in the cytoplasm of neurons. However, examination of brain material from early-stage HD patients indicated a conspicuous loss of Pacsin 1 in the neuronal processes (Modregger et al., 2002). The altered distribution of Pacsin 1 may be due to its enhanced interaction with mutant huntingtin. Taken together, the loss of Pacsin 1 at the synaptic boutons may cause defects in endocytosis which may play a role in the pathogenesis of HD.

1.9.5 Huntingtin Interacting Protein 14

Singaraja et al. (2002) have recently demonstrated that huntingtin interacts with a novel trafficking and endocytosis protein termed huntingtin interacting protein 14 (HIP14). The strength of the interaction was found to be inversely correlated with the length of the polyglutamine tract in huntingtin (Singaraja et al., 2002). HIP14 was found to be predominately expressed in neurons within the brain. Interestingly, HIP14 was found to co-localize with huntingtin in the striatal medium spiny neurons; the cells that are selectively lost in HD (Singaraja et al., 2002). Within these neurons, HIP14 was localized to the Golgi apparatus and cytoplasmic vesicles. Similarly, previous studies by Velier et al. (1998) have indicated that huntingtin is also enriched on Golgi membranes and cytoplasmic vesicles.

HIP14 has been shown to have 41% amino acid conservation to the *Saccharomyces cerevisiae* endocytic protein Akr1p. Akr1p has been shown to be essential for the endocytosis and transport of the yeast pheromone receptor Ste3p to degradatory vesicles (Kao et al., 1996). Thus, by analogy, HIP14 may also play a role in endocytosis and vesicle trafficking within neurons. Consistent with this hypothesis is the observation that HIP14 can partially restore endocytosis of Ste3p when expressed in *S. cerevisiae* cells lacking Akr1p (Singaraja et al., 2002). Thus, it may be postulated that the decreased interaction between HIP14 and mutant huntingtin may disturb endocytosis and intracellular transport and that these dysfunctions may play a role in the pathogenesis of HD.

1.9.6 Cdc42-Interacting Protein 4

Using the yeast two-hybrid system to screen a *Caenorhabtidis elegans* cDNA library, Holbert et al. (2003) demonstrated that huntingtin can interact with Cdc42-interacting protein 4 (CIP4). The two-hybrid and biochemical analyses indicated that CIP4 exhibits greater affinity for mutant huntingtin than wild-type. Furthermore, Holbert et al. (2003) showed that the SH3 domain present in CIP4 mediates binding to the proline-rich domain in huntingtin. Presumably, the enlarged polyglutamine tract in mutant huntingtin causes a conformational change in the protein that enhances CIP4 interaction. Immunohistochemical examination of the brain material from HD patients and age-matched controls indicated that CIP4 protein levels positively correlated with the severity of neuropathology in the striatum of HD patients (Holbert et al., 2003). Within the neurons CIP4 was found to partially colocalize with ubiquitin-positive aggregates (Holbert et al., 2003). Interestingly, overexpression of CIP4 in cultured rat striatal neurons resulted in apoptotic cell death (Holbert et al., 2003).

As the name implies, CIP4 interacts with the activated form of the small GTPase, Cdc42 (Aspenstrom et al., 1997). In addition, CIP4 also independently interact with Wiskott-Aldrich syndrome protein (WASP; Tian et al., 2000). Both Cdc42 and WASP have been shown to be important regulators of cytoskeletal dynamics (Ramesh et al., 1999). In addition, CIP4 interaction with WASP has been shown to play an essential role in the transport and recycling of the glucose transporter 4 (Glut4; Jiang et al., 2002). Taken together, these findings suggest that CIP4 is involved in several cellular functions including endocytosis, transport and regulation of actin cytoskeleton. It is possible that

the enhanced interaction of mutant huntingtin with CIP4 may result in disruptions of these cellular functions.

1.9.7 Huntingtin Interacting Protein 1

Two research groups independently isolated the huntingtin interacting protein, HIP1, using the yeast 2-hybrid system (Kalchman et al., 1997; Wanker et al., 1997). HIP1 has 2 features that are suggestive of its role in HD: 1) HIP1 is highly expressed in the brain, although the mRNA can be found throughout the body (Kalchman et al., 1997). 2) Increased length of the huntingtin polyglutamine tract is associated with decreased interaction with HIP1 (Kalchman et al., 1997). The HIP1 gene has been shown to map to human chromosome 7q11.2 and produce a 120kDa protein.

Subcellular localization of HIP1 in the brain revealed that the protein is associated with the intracellular membranes in a manner similar to many cytoskeletal and cytoskeletal-associated membrane proteins including actin (Kalchman et al., 1997). As mentioned previously, huntingtin is membrane-associated, co-localizes with microtubules and may be involved in vesicle trafficking (Difiglia et al., 1995; Gutekunst et al., 1995). The fact that HIP1 is seen exclusively bound to the cytoskeleton, a site where huntingtin can also be found, suggests that the huntingtin-HIP1 interaction occurs at this site (Kalchman et al., 1997). Interestingly, HIP1 also shares significant sequence similarity and biochemical properties with the known cortical cytoskeletal protein Sla2p (Synthetic Lethal with Actin binding protein-1 #2) in *Saccharomyces cerevisiae* and CeHIP1 (or ZK370.3) in *Caenorhabtidis elegans* (Kalchman et al, 1997; Wanker et al., 1997). Furthermore, HIP1 has been shown to have a homolog termed HIP12 (or HIP1r) which

will be discussed in depth throughout this thesis (Chopra et al., 2000, Engqvist-Goldstein et al., 1999; Seki et al., 1999). Taken together, these findings suggest that huntingtin-HIP1 interaction may play an important role in the normal membrane dynamics and vesicular trafficking and that the loss of normal interaction may play a role in HD.

Recently, Metzler et al. (2003) have generated mice with a targeted disruption in the HIP1 gene (*HIP1*$^{-/-}$) to determine the cellular function of HIP1 *in vivo*. The *HIP1*$^{-/-}$ mice exhibited gait ataxia, tremor and the development of a rigid thoracolumbar kyphosis which was the result of neurological dysfunction. Furthermore, these mice exhibited a profound dose-dependent defect in the internalization of glutamate subtype, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor. These findings suggest that HIP1 regulates AMPA receptor internalization in the CNS. It may be postulated that by association huntingtin may also play a role in AMPA receptor trafficking.

HIP1 expression has been shown to correlate with neoplastic changes resulting in uncontrolled cellular growth (Ross et al., 1998; Ross and Gilliland, 1999; Saint-Dic et al., 2001; Rao et al., 2002, Rao et al., 2003). Paradoxically, it has also been suggested that HIP1 is a pro-apoptotic protein in light of evidence demonstrating that it can induce both the intrinsic and extrinsic cellular death pathways (Hackam et al., 2000; Gervais et al., 2002). In order to gain greater understanding of the role of HIP1 in HD pathogenesis, the following sections will review evidence implicating HIP1 in apoptosis, neoplasia, cytoskeletal dynamics, and endocytosis.

1.9.7.1 The Pro-Apoptotic Function of HIP1

As mentioned previously, several lines of evidence suggest that activation of cell death pathways may play a role in the pathogenesis of HD. Interestingly, Hackam et al.

(2000) have demonstrated that overexpression of HIP1 in NT2 cell lines results in the morphological changes consistent with apoptosis including retraction of cytoplasmic extensions, cell rounding, blebbing and fragmentation of nuclear DNA. In addition, overexpression of HIP1 *in vitro* induced caspase 3 activation in a caspase 8 and usurpin-independent manner. These findings suggest that HIP1 can activate the intrinsic cell death pathway. The intrinsic pathway is normally activated by cytochrome c release from the mitochondria. This release leads to the activation of caspase 9 that in turn, activates caspase 3 (for review see Ashkenazi and Dixit, 1999).

Sequence analyses have demonstrated that HIP1 contains an 82 amino acid death effector domain (DED) starting at amino acid position 376 (Hackam et al., 2000). Furthermore, site-directed mutagenesis of a phenylalanine residue at amino acid residue 398 completely inhibited the ability of HIP1 to induce apoptosis. Several activators of apoptosis such as caspases 8 and 10, also contain DEDs that are thought to mediate protein-protein interactions that play a role in the induction of apoptosis (for review see Rasper et al., 1998). Since HIP1 binds mutant huntingtin with reduced affinity, it is possible that the unbound HIP1 may induce cell death via the intrinsic pathway. Thus, the "free" HIP1 may play a role in the apoptosis seen in brain tissue of individuals affected with HD.

More recently, *in vitro* studies have reported that HIP1 can also induce the activation of the extrinsic cell-death pathway (Gervais et al., 2002). The extrinsic pathway is usually initiated by the activation of either the fas or the tumor necrosis factor receptor. The stimulated receptor interacts with DED-containing proteins that recruit pro-caspase 8 to the plasma membrane and activate it. The activated caspase 8 induces

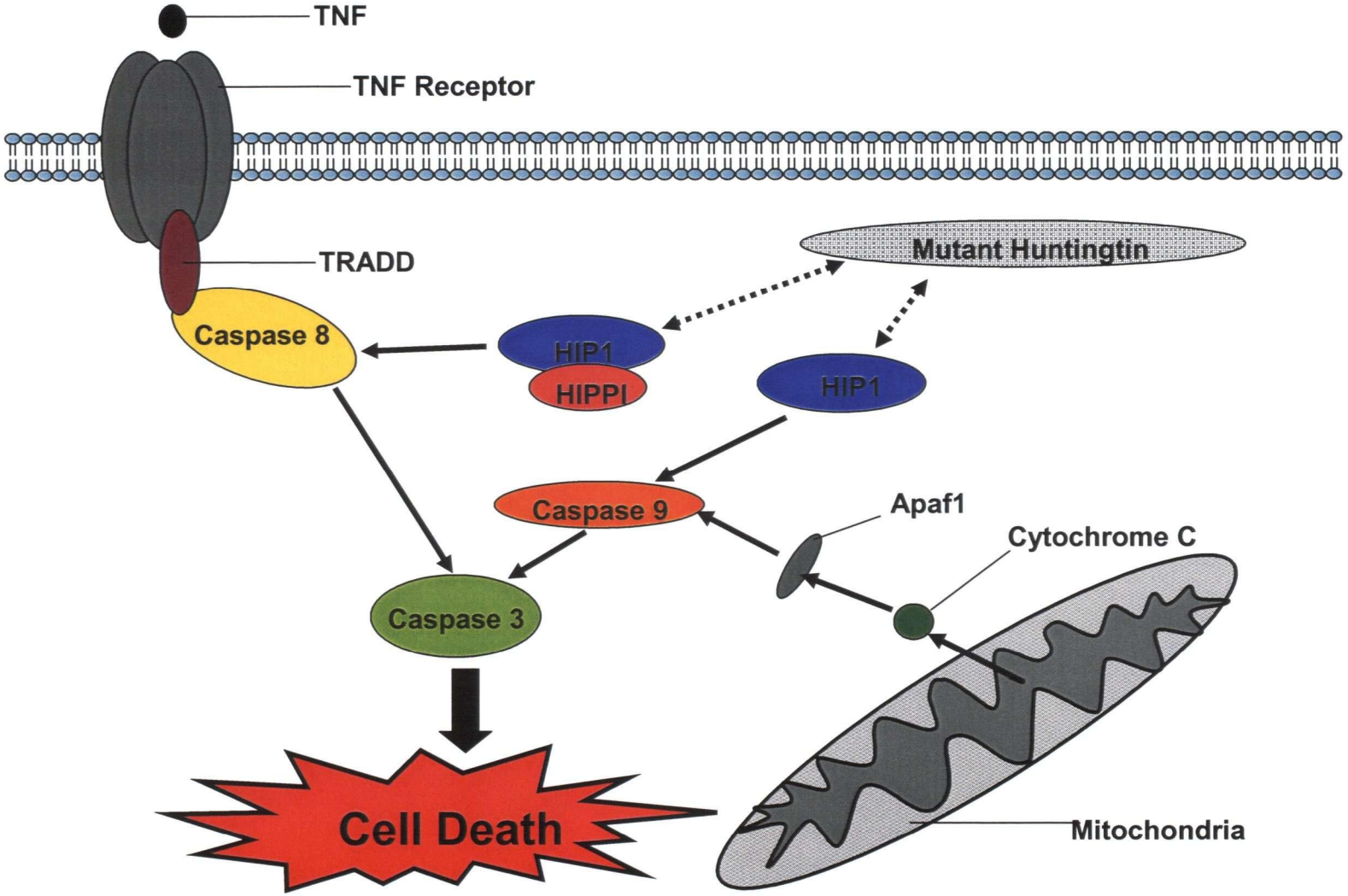
caspase 3 activity which results in the initiation of apoptosis (for review see Ashkenazi and Dixit, 1999). Gervais et al. (2002) established that HIP1 interacts with a novel DED-containing protein termed HIPPI (HIP1 protein interactor). Immunohistochemical studies indicated that HIP1 and HIPPI proteins have similar patterns of expression in the brain and peripheral tissues (Gervais et al., 2002). Interestingly, the HIP1- HIPPI heterodimers were shown to interact with and activate caspase 8 (Gervais et al., 2002). The HIP1-HIPPI mediated activation of caspase 8 may represent the signal inducing apoptosis. In HD, mutant huntingtin would be expected to bind HIP1 with decreased efficiency. The unbound HIP1 may then interact with HIPP1 and induce activation of the extrinsic cell death pathway. HIP1 activation of the intrinsic and extrinsic cell death pathways are illustrated in figure 1.1.

1.9.7.2 The Role of HIP1 in Cellular Transformation

There is accumulating evidence to suggest that HIP1 may play a role in the development of neoplastic changes in cells. Ross et al (1998) identified a t(5;7)(q33;q11.2) chromosomal translocation in a patient with chronic myelomonocytic leukemia (CMML). The translocation resulted in the generation of a chimeric protein containing amino acids 1 to 950 of HIP1 fused with the transmembrane and tyrosine kinase domain of the platelet-derived growth factor β receptor (PDGFβR). Ross et al. (1998) demonstrated that expression of the HIP1- PDGFβR chimeric protein (HIP1/ PDGFβR) can induce cellular transformation of the murine hematopoietic cell line, Ba/F3. Within the transformed Ba/F3 cells, HIP1/ PDGFβR was shown to form oligomers and contain a constitutively active PDGFβR tyrosine kinase domain (Ross and

Gilliland, 1999). It has been demonstrated that HIP1/ PDGFβR expression correlates with tyrosine phosphorylation of cell signaling proteins STAT5 and Src homology 2-containing inositol 5-phosphatase (SHIP1) (Ross and Gilliland, 1999; Saint-Dic et al., 2001). The tyrosine phosphorylated form of STAT5 has been shown to induce DNA synthesis and cell division (deGroot et al., 1998; Saint-Dic et al., 2001). However, phosphorylation of SHIP1 has been shown to inhibit its ability to down-regulate hemapoietic proliferation (Saint-Dic et al., 2001). Thus, activation of STAT5 and inhibition of SHIP1 may result in unregulated cell growth resulting in transformation. Primary tissue microarray studies have indicated that HIP1 protein is highly expressed in aggressive forms of prostate and colon cancer (Rao et al., 2002). Rao et al. (2002) examined cancer tissue microarrays using samples generated from small biopsies from patients with various types of cancer including prostate, colon, central nervous system, lung, ovary breast, kidney, and various leukemia and melanomas. These studies found that HIP1 was overexpressed in prostate and colon cancers relative to normal epithelium of prostate and colon. Furthermore, analysis of tissue microarrays from patients with various stages of prostate or colon cancer progression indicated that the level of HIP1 protein was directly correlated with the severity of these cancers. Immunohistochemical examination in the transgenic mouse model for prostate cancer (TRAMP) demonstrated that HIP1 was highly expressed in the prostate tumors as compared to adjacent benign tissue (Rao et al., 2002). Interestingly, HIP1 was not highly expressed in all cells of the TRAMP mouse prostate tumors suggesting that HIP1 overexpression is not a prerequisite to tumor formation.

Figure 1.1. Two potential mechanisms how HIP1 induces apoptotic cell death. HIP1 displays reduced interaction to mutant huntingtin. The "unbound" HIP1 may then stimulate apoptosis through a pathway that involves activation of caspase-9, which then activates the downstream effector caspase-3. Caspase-9 is normally a component of the intrinsic cell death pathway that is activated by cytochrome c release from the mitochondria. HIP1, when combined with the HIP1 interactor HIPPI, has also been shown to elicit cell death via activation of caspase-8, which then activates caspase-3. Caspase-8 is part of the "extrinsic" cell death pathway that is normally stimulated by activation of cell-surface death receptors such as tumor necrosis factor.


TNF
TNF Receptor
TRADD
Mutant Huntingtin
Caspase 8
HIP1
HIPPI
HIP1
Caspase 9
Apaf1
Cytochrome C
Caspase 3
Cell Death
Mitochondria

Recently, Rao et al., (2003) have reported that overexpression of HIP1 *in vitro* promotes transformation of NIH/3T3 fibroblasts and is also associated with alterations in the trafficking of several receptors including epidermal growth factor receptor (EGFR). Interestingly, overexpression of HIP1 within the brains of HIP1 transgenic mice does not appear to result in tumor formation (Dr. Metzler, personal communication). Thus, it is possible that the *in vitro* studies by Rao et al (2003) do not necessarily reflect physiological events underlying tumor formation *in vivo.* Alternatively, HIP1 may simply perform different cellular functions in the brain as compared to peripheral tissues.

1.9.7.3 The HIP1 Family of Proteins

HIP1 is a member of a growing family of membrane cytoskeletal-associated proteins. HIP1 has been shown to share significant sequence identity and biochemical characteristics with the known SLA2p in *S. Cerevisiae* and CeHIP1 in *C. elegans* and the hypothetical protein CG10971-PA in *D. melanogaster*. Furthermore, humans and mice express a HIP1 homolog termed HIP12 (Chopra et al., 2000; Engqvist-Goldstein et al., 1999; Seki et al., 1999). All of the HIP1 family of proteins are similar in molecular weight and contain an amino-terminal ENTH (Epsin N-terminal Homology), a central coiled coil and a carboxy terminal talin-like domain.

The ENTH domain is postulated to induce clathrin-mediated endocytosis through binding to phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2]-containing membranes (Ford et al., 2001; Itoh et al., 2001; DeCamilli et al., 2002). Furthermore, ENTH domains are also found in several other endocytic proteins including epsin 1 and 2, adaptor protein 180 (AP180) and its homologue, clathrin assembly lymphoid myeloid leukemia protein

(CALM; Ford et al, 2002; Ford et al., 2001; Itoh et al., 2001). Interestingly, each of these proteins also binds to clathrin. The ENTH domains of epsin 1 and 2, contain a type-I clathrin-binding sequence, referred to as a "clathrin box" which interacts with the amino-terminal domain of the clathrin heavy chain (Ford et al, 2002; Ford et al., 2001; Itoh et al., 2001). Proteins that bind both PtdIns(4,5)P_2 and clathrin are thought to promote the accumulation and assembly of clathrin coat proteins to sites of receptor-mediated endocytosis (DeCamilli et al., 2002 Kay et al., 1999; Rosenthal et al., 1999).

The central region of HIP1, HIP12, Sla2p, CeHIP1 and CG10971 contains a predicted coiled coil domain that is postulated to mediate protein-protein interactions (Engqvist-Goldstein et al., 1999; Kalchman et al., 1997; Lupas, 1996; Parker, 2001; Wanker et al., 1997). In the case of Sla2p and HIP12, the coiled coil domains are critical for the localization of these proteins to the plasma membrane (Engqvist-Goldstein et al., 1999; Wesp et al., 1997; Yang et al., 1999). It may be speculated that the coiled coil domains may tether Sla2p (or HIP12) to proteins that are present at the plasma membrane (Yang et al., 1999; Barr, 2000).

The carboxy-terminal domains of the HIP1 family of proteins share significant homology with the mammalian membrane cytoskeletal-associated protein, talin (Kalchman et al., 1997). Talin is found at sites where the cytoskeletal element actin is linked to the extracellular membrane via integral membrane proteins (Critchley, 2000; Moulder et al., 1996). The talin-like domain present in the HIP1 family members consists of four sub-domains collectively referred to as the I/LWEQ module. The I/LWEQ modules of Sla2p and HIP12 have been shown to interact with F-actin (McCann and Craig, 1997; Engqvist-Goldstein et al., 1999). Thus, it is likely that the talin-like domain

in the HIP1 family of proteins plays an essential role in targeting these proteins to the cortical cytoskeleton.

Taken together, the ENTH, coiled coil and talin-like domains suggest that the HIP1 family of proteins may be involved in the functioning of the actin cytoskeleton and endocytosis. The following sections will discuss what is known about the cellular functions of Sla2p and CeHIP1 in order to suggest possible functions for HIP1.

1.9.7.4 *Saccharomyces cerevisiae* Sla2p

The yeast actin-binding protein Sla2p has been shown to play an essential role in the assembly and function of the actin cytoskeleton in *Saccharomyces cerevisiae* (Holtzman et al., 1993). In addition, Sla2p has also been implicated in endocytosis, vesicle transport and cAMP signaling (Mulholland et la., 1997; Wesp et al., 1997; Blader et al., 1999). Sla2p was originally characterized as a null mutation ($Sla2^{-/-}$) causing temperature sensitive growth defects related to a general disorganization of cell surface and actin cytoskeleton (Holtzman et al., 1993). For example, the $Sla2^{-/-}$ cells possess abnormally thick cell walls and display a spherical shape as opposed to the characteristic ellipsoid appearance seen in wild-type cells (Holtzman et al., 1993). Moreover, during cellular division the $Sla2^{-/-}$ cells do not undergo polarized cell surface growth. These cells have actin patches distributed evenly across the surface of the mother and bud, whereas in wild-type cells the cortical actin patches are spatially restricted to growing domains within the cell cortex (Holtzman et al, 1993; Yang et al, 1999). Interestingly, these defects in bipolar budding are also been reported in yeast with mutations in various components of the cortical actin cytoskeleton (Holtzman et al, 1993; Raths et al 1993, Na et al, 1995).

The two other known alleles at the SLA2 gene locus are referred to as *End4* and *Mop2* (Na et al 1995, Raths et al 1993). The mutants described as end4 were originally described as having temperature-sensitive growth phenotype and defects in receptor-mediated endocytosis (Raths et al., 1993). The mutants referred to as mop2 were shown to have temperature-sensitive growth defects that resulted an inability to regulate the levels of the yeast H^+-ATPase (Pma1p) in the plasma membrane (Na et al., 1995). Taken together these findings suggest that Sla2p is required for endocytosis and the maintenance and distribution of integral membrane proteins.

The generation of yeast cells with various SLA2 deletion mutants has helped to delineate the role of the talin-like, ENTH, and coiled-coil domains in Sla2p function. Biochemical studies by McCann and Craig (1997) have demonstrated that Sla2p can bind to F-actin through its talin-like domain. In addition, it has been shown that the ENTH domain contains a localization sequence that allows Sla2p to be associated with the cortical actin cytoskeleton. Thus, it would seem that the ENTH and talin-like domain both play a role in the localization of Sla2p to the cortical actin cytoskeleton (Wesp et al., 1997; Ayscough et al., 1997; Yang et al., 1999). At this time it is unclear what subtle differences in distribution can be attributed to these two domains. The central coiled-coil domain has been shown to play an essential role for the cellular activity of Sla2p. Yeast cells expressing Sla2p that lack the central coiled-coil domain display normal cellular distribution, however, they also contain defects in endocytosis, cellular growth and cytoskeletal organization (Yang et al., 1999). It has been postulated that the coiled-coil domain mediates its effects by interacting with other proteins involved in cytoskeletal dynamics and endocytosis (Lupas et al, 1991; Yang et al, 1999).

1.9.7.5 The *Caenorhabtidis elegans* HIP1 (CeHIP1)

The CeHIP1 gene spans 4808 bp, contains 10 exons and codes for a 2798 bp message. The CeHIP1 is 927aa in length and has a molecular weight of 104kDa. Within the adult hermaphrodites, CeHIP1 is present in the pharynx, spermatheca, vulvar muscles and the gonads. In the adult male *C. elegans*, CeHIP1 was found in the pharynx, vas deferens and cloacae (Parker, 2001). Through chemical mutagenesis (using trimethylpsoralen and ultraviolet light) and RNA interference (RNAi) studies it was determined that the CeHip1 protein was found to be essential for normal development since no animals with a homozygous or heterozygous deletion of CeHIP1 could be detected. Transgenic *C. elegans* containing an inducible CeHIP1 construct were also developed to determine the effect of CeHIP1 over-expression in these worms. Upon induction of the transgene the worms failed to thrive and developed intracellular vacuoles suggesting that excess CeHIP1 may in some be toxic to the worm (Parker, 2001).

RNAi studies were performed to silence the CeHIP1 gene in adult *C. elegans* (Fire 1998; Parker 2001). The silencing of CeHIP1 expression resulted in altered morphology and functioning of the pharynx as well as reduced production of fertilized oocytes (Parker, 2001). The exact function of CeHIP1 in pharynx and gonads is unclear at this time. It has been postulated that since CeHIP1 contains a talin domain, the protein most likely interacts with the actin cytoskeleton and thereby helps to maintain the structural integrity of the cells within the pharynx and the gonads (Parker, 2001). Furthermore, since CeHIP1 contains an ENTH and coiled coil domains, it may also perform a critical role in the endocytic and transport pathways within the pharynx and gonads of *C. elegans* (For a review see Riddle et al 1997; Parker, 2001).

1.9.7.6 Huntingtin Interacting Protein 12 (HIP12)

The role of the HIP1 in membrane dynamics and vesicle transport is supported by the observation that it is structurally similar to its homolog HIP12 (Chopra et al., 2000; Engqvist-Goldstein et al., 1999). The murine homolog of HIP12, mHIP12 (or mHIP1r) has been shown to play an essential role in receptor-mediated endocytosis and vesicular transport (Engqvist-Goldstein et al., 1999). mHIP12 was initially isolated by our laboratory by screening a mouse brain cDNA library with mouse expressed sequence tags (ESTs) showing significant nucleotide identity (60%) with human HIP1 (Engqvist-Goldstein et al., 1999). The mHIP12 protein is 1068 amino acids in length and has a molecular mass of approximately 120 kDa. Like HIP1, Sla2p, and CeHIP1, the mHIP12 protein contains an amino-terminal ENTH domain, a central coiled-coil domain and a carboxy-terminal talin domain. Furthermore, mHIP12 is expressed in mouse embryos 7 days post-fertilization suggesting that this protein may play an important role during development. In the adult mouse, mHIP12 is expressed in both the central nervous system and peripheral tissues including kidney, liver, lung, spleen, testes (Engqvist-Goldstein et al., 1999). Within the cell, mHIP12 was shown to exhibit a distinctive punctate staining throughout the cytoplasm but not in the nucleus.

Several lines of experimental evidence suggest that mHIP12 is involved in clathrin mediated endocytosis and actin cytoskeleton dynamics. *In vitro* binding assays indicated that the mHIP12 talin-like domain interacts with F-actin (Engqvist-Goldstein et al., 1999). Similarly, immunohistochemical studies demonstrated that mHIP12 colocalized with F-actin at the cortical cytoskeleton and in the perinuclear region of the cultured cell lines (Engqvist-Goldstein et al., 1999). At the plasma membrane, mHIP12

was found to colocalize with components of the receptor-mediated endocytic machinery. It was determined that mHIP12 co-localizes with the coat protein, clathrin, which is essential for vesicle formation at the plasma membrane and the *trans*-Golgi network (Engqvist-Goldstein et al., 1999; Brodsky et al., 2001). Moreover, mHIP12 was also found to colocalize with the adapter protein, AP2, which is known to play a role in the recruitment of clathrin at sites undergoing receptor-mediated endocytosis (Brodsky et al., 2001). In addition, mHIP12 was also found to colocalize with newly endocytosed vesicles containing labeled transferrin. However, as the transferrin-containing vesicles traveled further into the cytoplasm, their co-localization with mHIP1r decreased (Engqvist-Goldstein et al., 1999). Taken together, these findings suggest that mHIP1r may play an important role in the initial stages of receptor-mediated endocytosis.

The generation and expression of various mHIP12 deletions constructs in culture have indicated that the ENTH, coiled-coil and talin-like domains play an essential role in cellular localization and receptor mediated endocytosis. Engqvist-Goldstein et al. (1999) demonstrated that both the ENTH and coiled-coil domains are required for normal cellular distribution and association with the endocytic machinery (Engqvist-Goldstein et al., 1999). The mHIP12 talin-like domain has been shown to be necessary for localization to F-actin. These findings suggest that mHIP12 may act a link between the endocytic machinery and the underlying actin cytoskeleton.

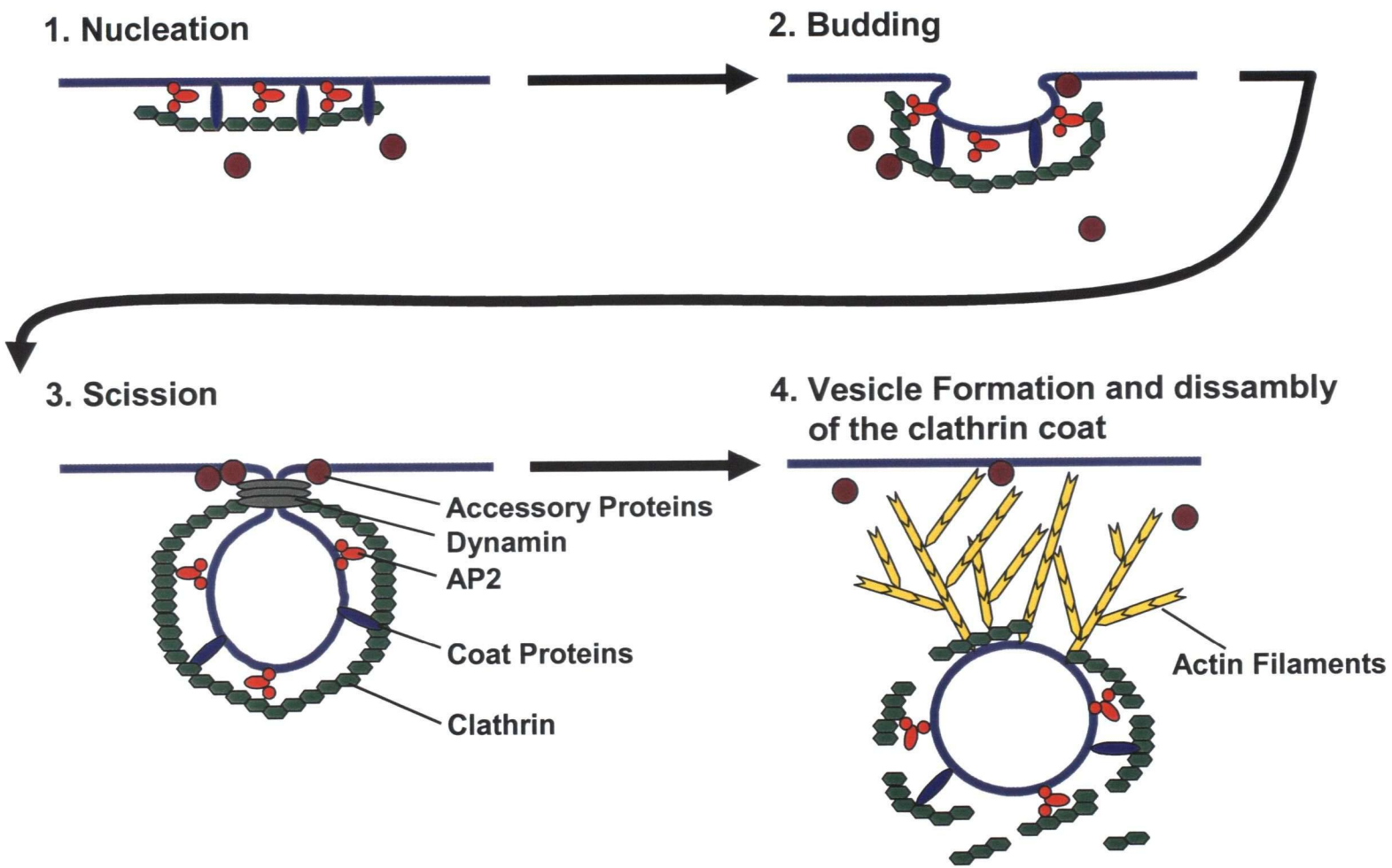
1.10 Clathrin-Mediated Endocytosis and the Cortical Actin Cytoskeleton

As previously mentioned, HIP12 (and potentially HIP1) have been shown to play a role in clathrin-mediated endocytosis (CME; Engqvist-Goldstein et al., 2000; Waelter et

al., 2001). CME is the major mechanism for the internalization and transport of proteins and lipids from the plasma membrane to the cytoplasm (Hirst and Robinson, 1998). For example, at the neuronal synapse, CME has been shown to be critical in the recycling of synaptic vesicles following neurotransmitter release (Slepnev and DeCamilli, 2000).

CME in neurons can be divided into 4 different stages: 1) nucleation, 2) budding, 3) scission and 4) uncoating of the clathrin-coated vesicle (please refer to figure 1.2). During nucleation, the adapter proteins, AP2 and AP180, are recruited to discrete sites on the plasma membrane. The adapter proteins aid in the recruitment of the clathrin units to the cell membrane. The clathrin units are composed of three heavy and light chains that form a three-legged triskelion. These clathrin triskelia self-assemble into the curved polygonal lattice structure which is referred to as the clathrin-coated pit (Brodsky et al., 2001). During the budding phase, the clathrin-coated pit develops a pronounced curvature until a narrow neck forms at the plasma membrane. The highly complex budding process is regulated by the coordinated activity of several proteins that are associated with the clathrin-coat. These proteins include the adapter proteins amphiphysin, intersectin, EPS15, and epsin. In addition, the phospholipid metabolism enzymes endophillin and synaptojanin have also been shown to regulate the budding process (Brodsky et al., 2001; Slepnev and DeCamilli, 2000). During fission, ring-like structures form around the neck of the clathrin-coated pits and "pinch" off clathrin coated vesicles. The ring-like structure is composed of the GTPase, dynamin, in association with amphiphysin and endophillin (Slepnev and DeCamilli, 2000). Finally, the clathrin-coated vesicle is rapidly uncoated through the activity of the chaperone HSP70 and its neuron-specific cofactor auxilin (Slepnev and DeCamilli, 2000).

Figure 1.2. The different stages of clathrin mediated endocytosis (CME; see section 1.10). The light blue line represents the cytoplasmic membrane. The green hexagons represent the clathrin subunits. The red "Y" structure represent AP2. The dark blue ovals represent other coat proteins such as AP180 (and potentially HIP1 and HIP12). The plum circles represent other accessory proteins including amphiphysin, endophillin and intersectin. The grey ovals represent dynamin. Finally, the yellow rods represent actin filaments. The huntingtin interacting proteins HAP1, AP2, endophillin, pacsin, HIP1, HIP14, and CIP4 are all thought to play important during four stages of CME.


1. Nucleation
2. Budding
3. Scission
4. Vesicle Formation and dissambly of the clathrin coat
Accessory Proteins
Dynamin
AP2
Coat Proteins
Clathrin
Actin Filaments

The cortical actin cytoskeleton has been speculated to perform a number of different functions associated with CME. The actin cytoskeleton may help to anchor the endocytic machinery to specific sites on the plasma membrane by either binding to the endocytic components or restraining their mobility (Qualmann et al., 2000). Consistent with this idea are the observations that exposure of cultured cells to the actin depolymerizing drug latrunculin B, causes a diffuse localization of the endocytic machinery across the cell surface and an inability to perform CME (Santini et al., 2002). The cortical actin cytoskeleton is also hypothesized to undergo deformation or invagination at the site of endocytosis, and thereby assist in the generation of the clathrin-coated pits (Qualmann et al., 2000). Alternatively, there may be a selective depolymerization of the actin cytoskeleton at the site of endocytosis to allow the expansion and growth of the clathrin-coated pit (Fujimoto et al., 2000; Qualmann et al., 2000). Finally, rapid polymerization of actin at the site of endocytosis may generate the force necessary to release the clathrin-coated vesicle from the plasma membrane and transport it further into the cytoplasm (Qualmann et al., 2000; Merrifield et al., 1999). The propulsive force of the actin cytoskeleton has been visualized as a "comet tail" where long chains of actin extend from the plasma membrane to the newly formed clathrin-coated vesicle (Merrifield et al., 1999).These findings suggest that clathrin-mediated endocytosis requires the complex interplay between endocytic machinery and the underlying cortical actin cytoskeleton. Interestingly, HIP1 and HIP12 contain structural domains that allow these proteins to interact with both the endocytic machinery and the

cortical actin cytoskeleton. Thus, HIP1 and HIP12 may represent a molecular link between the endocytic machinery and the underlying cortical actin cytoskeleton.

1.11 Hypotheses

The following hypotheses will be addressed in this thesis

1) HIP1 is a member of a family of cytoskeletal-associated proteins that exhibit differences in their ability to both interact with huntingtin and to induce apoptosis.

2) The HIP1 family of proteins are components of the clathrin-mediated endocytic machinery and the actin cytoskeleton.

1.12 Thesis Objectives

To address the above hypotheses, these are specific objectives for this thesis:

1) To identify members of the huntingtin interacting protein 1 family by using ESTs to screen both mouse and human cDNA libraries.

2) To sequence and assess the similarities or identities of the isolated HIP1 family members with HIP1, Sla2p and CeHIP1.

3) To identify the chromosomal location of the isolated HIP1 family members.

4) To characterize the mRNA and protein expression pattern of the isolated HIP1 family members.

5) To assess whether the novel HIP1 family members interact with huntingtin or HIP1 using the yeast two-hybrid system.

6) To address whether HIP1 family members activate apoptosis.

7) To determine whether the human HIP1 family members are associated with components of clathrin-mediated endocytic machinery.

8) To determine whether the human HIP1 family members interact with actin filaments.

9) To identify domains present in the human HIP1 family members that are required for normal function and localization.

Chapter 2:

Materials and Methods

2.1 Materials and Methods for Chapter 3

2.1.1 Isolation of HIP12

Human HIP12 was initially identified as a result of our efforts to obtain a murine homolog of HIP1. The published nucleotide sequence of human *HIP1* cDNA (accession # U79734) was used to screen the GenBank EST database (dbEST) using BLASTN. We obtained 2 different murine ESTs which showed homology to *HIP1*: aa110840 (clone 520282) and w82687 (404331). Please see figure 2.1. A 1.3kb *Eco* RI & *Nco* I fragment of clone 404331 was used to screen a mouse brain lambda ZAPII cDNA library (Stratagene) using manufacturer's suggested protocols. We isolated a 3.9 Kb cDNA clone, which we termed *mHIP12*, that showed nucleotide and amino acid identity with HIP1. The entire *mHIP12* cDNA sequence was used to screen the non-redundant database of GenBank EST Division. We identified a human EST, T08283 (clone HIBBB80), which showed homology to *mHIP12* but not *HIP1*. A 1.6Kb *Hind* IIII & *Not* I fragment of clone 404331 (containing EST T08283) was used to screen a human frontal cortex lambda ZAPII cDNA library (Gift from M. Montal, University of California, San Diego). We isolated a 3.2 Kb cDNA clone, termed *HIP12*, that showed nucleotide and amino acid identity with mHIP12 and HIP1.

The remaining 5′ portion of the *HIP12* cDNA was isolated using the 5′ Rapid Amplification of cDNA Ends (RACE) Kit (Canadian Life Technologies). The first strand cDNA was synthesised using the oligo HIP12-950 (5′ ACC GCC

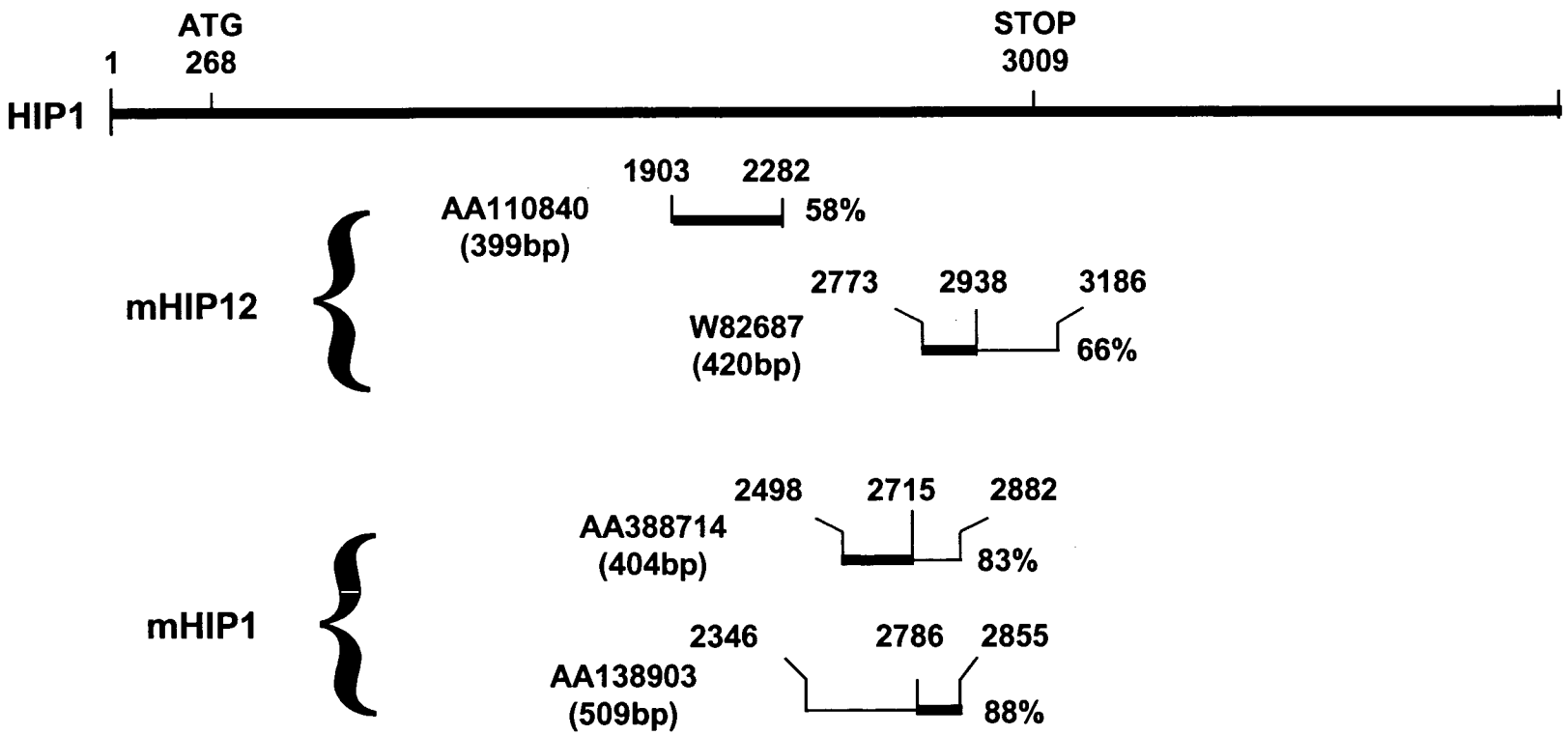


Figure 2.1 Mouse expressed sequence tags (ESTs) that were identified by BLAST search of the National Centre of Biological Information EST database using human HIP1 nucleotide sequence.

ATT GAT GAT GGT AT). After dCTP tailing the first strand cDNA with terminal deoxynucleotide transferase, 2 rounds of 30 cycles (94 °C for 1 min; 58 °C for 1 min; 72 °C for 2 min) of PCR using HIP12300 (5′ CAC ATG GAC GAG CTC ACT GT) and the anchor primer (5′$(CAU)_4$ GGC CAC GCG TCG ACT AGT ACG GGI IGG GII GGG IIG) (Canadian Life Technologies) were performed. The subsequent 1.5Kb PCR product was cloned using the TA cloning system (Invitrogen) and sequenced.

2.1.2 DNA and Amino Acid Sequence Analyses

The DNA sequences were analysed using the BLAST server at NIH (http://www.ncbi.nlm.nih.gov) to search for homology with nucleotide (BLASTN) and protein (BLASTP) sequences. Amino acid alignments were performed using CLUSTALW, converted to MSF format and transferred to GeneDoc. Coiled-coil prediction was performed using COIL at BCM search launcher (http://kiwi.imgen.bcm.tmc.edu:8088/search-launcher /launcher. html).

2.1.3 Determination of Additional 5′ HIP1 sequences

A theoretical translation of the published *HIP1* 5′ UTR showed significant sequence similarity to the amino terminal sequences of Sla2p, ZK370.3 and HIP12. 5′ RACE and RT-PCR analyses were performed to determine whether *HIP1* contained additional 5′ coding sequence.

5′ RACE Analysis: 0.5 μg of Human Brain Marathon-ready cDNA library (Clontech) was used to isolate the 5′ portion of HIP1 by Rapid Amplification of

cDNA ends (RACE), following the manufacturer's recommendations. The primers used in the first round of PCR were a gene-specific primer-1 (5′ CTT TCA ACT TTG TTA ACT GCT CC) and the supplied adaptor primer 1. The primers used for the second round of PCR were a nested gene-specific primer-2 (5′ GGT TGC TGG AGC GGT AGA ACA G) and a supplied nested adaptor primer 2. The resulting PCR product was subcloned using the TA cloning system (Invitrogen) and sequenced.

RT-PCR Analysis: 5 μg of total human brain RNA (Clontech) was used for first strand cDNA synthesis using the SUPERSCRIPT™ preamplification system (Canadian Life Technologies). The synthesized cDNA was diluted 1:10 and 1 μl of cDNA was used for PCR amplification (30 cycles: 92°C for 10 seconds; 60°C for 30 seconds and 72°C for 1 minute). The following primers were used: an exon 1-specific primer (P1) encompassing the upstream translation initiation site (5′ GGA TGG CCA GCT CCA TGA AG), an exon 2-specific primer (P2; 5′ GAG AGC TTC GAG CGG ACT CAG) and two exon 3-specific primers (P3 and P4) encompassing the downstream translation initiation site (5′ GCC TCG GTC ATG GAT GTG AGC or 5′ GGT CAT GGA TGT GAG CAA GA, respectively) were used in combination with an exon 10-specific primer (5′ GAC ATG TCC AGG GAG TTG AA). The PCR products were separated on a 1% agarose gel and visualized following ethidium bromide staining.

2.1.4 Genome mapping of HIP12: FISH detection system and image analysis

The full-length HIP12 cDNA obtained by screening the human brain cDNA library and 5′ RACE amplification was mapped by fluorescent *in situ* hybridization (FISH) to normal human lymphocyte chromosomes counter-stained with propidium iodide and DAPI. Biotinylated probe was detected with avidin-fluorescein isothiocyanate (FITC). Images of metaphase preparations were captured by a thermoelectrically cooled charge coupled camera (Photometrics). Separate images of DAPI banded chromosomes and FITC targeted chromosomes were obtained. Hybridization signals were acquired and merged using image analysis software and pseudo coloured blue (DAPI) and yellow (FITC) as described and overlaid electronically.

2.1.5 Northern Blot Analyses

A commercially prepared blot with mRNA from various human tissues and different brain regions (Clontech) was hybridized with a 1.2Kb HIP1 fragment labelled using random-priming and incorporation of [α^{32} P]-dCTP. After washing the blot according to manufacturer's recommendations, the filter was exposed to X-ray film (Hyperfilm, Amersham). The filters were then stripped using 0.5% sodium dodecyl sulfate, reprobed using a [α^{32} P]-dCTP-labelled 675bp *Pvu* II *HIP12* fragment and washed as before. The blots were then hybridized with randomly [α^{32} P]-dCTP-labelled β-actin as an internal control to ensure that the mRNA on the blots was intact and to ensure equal loading.

2.1.6 Generation of Antibodies

The HIP1 and HIP12 antibodies were generated using GST-tagged HIP1 and HIP12. The HIP1-GST expression vector was constructed by subcloning a 1.2 Kb *HIP1 Sal* I fragment into pGEX-5X-1 (Pharmacia). The HIP12-GST expression vector was generated by subcloning a 675bp HIP12 *Pvu* II fragment into pGEX-4T-2 (Pharmacia). Each of the vectors was transformed into *E. coli* DH5-alpha (Canadian Life Technologies). The HIP1-GST and HIP12-GST proteins were isolated using methods previously described (Wanker et al., 1997). New Zealand white rabbits were injected with either HIP1-GST or HIP12-GST protein in Freund's adjuvant (Pierce, Rockford, Illinois). Antibodies against HIP1 fusion protein (HIP1FP)or HIP12 fusion protein (HIP12FP) were purified from rabbit sera using affinity column with low pH elution. The affinity columns were made by incubation of either HIP1-GST or HIP12-GST with activated thiol-sepharose (Pharmacia).

2.1.7 Protein Preparation and Western Blot Analyses

Frozen human tissues were homogenized using a Polytron homogenizer in a buffer containing 0.25 M sucrose, 20 mM Tris-HCl (pH 7.5), 10 mM EGTA, 2 mM EDTA supplemented with a protease-inhibitor cocktail (Roche Diagnostics). The homogenate was centrifuged at 4000 rpm for 10 min at 4 °C to remove cellular debris. 100 μg / lane of protein was separated on a SDS-PAGE (7% acrylamide) gel and transferred to Immobilon-P membranes (Millipore) using standard protocols (Towbin et al., 1979). Western blot analyses were performed using

purified antibodies HIP1FP (1:100), HIP12FP (1:100) or a control monoclonal antibody for glyceraldehyde phosphodehydrogenase (1:500; Chemicon) and then detected using enhanced chemiluminescence (Amersham).

2.1.8 Construction of Yeast Two-Hybrid Vectors

Huntingtin cDNA constructs with either 15 or 128 CAG repeats were generated encompassing amino acids 1-548 of the published huntingtin sequence. The yeast expression vectors pGBT9-1955-15 and pGBT9-1955-128 were constructed by subcloning a *EcoR*1-*Sal*1 fragment from pCI1955-15 or pCI1955-128 into pGBT9 (Clontech; Kalchman et al., 1997). Similarly, the *EcoR*1-*Sal* 1 fragment from pCI1955-15 or pCI1955-128 into pGBT9 (Clontech)were fused in-frame adjacent to the GAL4–activating domain (AD) of the yeast two-hybrid vector pGAD (Clontech) generating pGAD-1955-15 and pGAD-1955-128. A 1513bp PCR product comprising nucleotide 642 to 2155 of *HIP12* was subcloned into the pCR2.9 TA cloning vector (HIP12pCR2.9). The fragment was sequenced to ensure that it was intact, excised from the TA vector using *EcoR*1, and blunt-end ligated into the *Sma* I site of both pGAD424 (HIP12pGAD) and pGBT9 (HIP12pGBT9). Yeast transformations were performed using a modified lithium acetate transformation protocol and grown at 30 °C using the appropriate synthetic complete (SC) dropout media (Geitz et al., 1996). β-galactosidase chromogenic assays were performed using procedures previously described (Kalchman et al., 1997).

2.1.9 Immunocytochemical Analyses of Neuronal NT2 cells and ES Cell Derived Neurons

The neuronal precursor cell line NT2 (gift from Dr. L. Ellerby, Buck Institute, San Francisco, California) were washed three times with PBS and fixed with 4% (w/v) paraformaldehyde at room temperature (RT) for 15 minutes. Cells were permeabilized in PBS containing 1% paraformaldehyde and 0.3% Triton X-100 for 5 minutes, washed with PBS three times, and incubated with either HIP1FP or HIP12FP antibodies in PBS, 2% normal goat serum overnight at 4°C. After extensive washing in PBS, slides were incubated with goat anti rabbit Alexa 488 (Molecular Probes, Eugene, Oregon) at a 1:800 dilution in PBS containing 2% normal goat serum for 2 hours at RT. Cells were washed 3 times with PBS, counterstained with TO-PRO (Molecular Probes) mounted in Mowiol (Aldrich) and analyzed by confocal laserscanning microscopy (BioRad Radiance Plus Confocal Microscope). Images were analyzed using NIH Image software.

Double immunofluorescence was performed on embryonic stem (ES) cell-derived neurons 12 days after plating as previously described (Metzler et al., 1999). After fixation and permeabilization, the cells were incubated with either the HIP1FP or HIP12FP antibodies overnight at 4°C. Subsequently, cells were washed with PBS and incubated with a mouse monoclonal antibody directed against GM130 (Transduction Laboratories, Lexington, Kentucky) in PBS containing 2% normal goat serum. After extensive washing in PBS, slides were incubated with goat anti-rabbit Alexa 488 and goat anti-mouse Alexa 594 antibodies (Molecular Probes) at a 1:800 dilution in PBS containing 2% normal goat serum for 2 hours at

RT. Cells were washed and mounted in Mowiol (Aldrich). Immunofluorescence was detected using an Axioscope microscope (Carl Zeiss Inc.) Images were captured digitally with a CCD camera (Princeton Instruments Inc.).

2.1.10 Cell Viability Assays

The entire coding sequence of HIP1-2 and HIP12 were each fused into the mammalian expression vector pCI (Promega). Human embryonic kidney 293T cells were plated in 96 and 6 well dishes (Becton-Dickinson) for the MTT and Caspase-3 activation assays, respectively in Dulbecco's modified eagle medium, 10% fetal bovine serum, 50 units/ml penicillin-streptomycin, and 2 mM L-glutamine (Canadian Life Technologies) and cultured at 37°C in 6% CO_2. Cells were transfected with either the HIP1-2 or HIP12 constructs using the $CaPO_4$ method (Jordan et al., 1996), and the media was changed 24 hours post transfection. Transfection efficiency was 80-95% as measured by β-galactosidase staining. The HIP1 and HIP12 constructs produced similar levels of protein as determined by immunoblot assays. These transfected cells were used for either MTT or Caspase 3 activation assays.

MTT Assays: At 48 hours post transfection, 10 µl of the cell proliferation reagent WST-1 (Roche Diagnostics) was added to each well, and the plates were incubated for an additional hour. The assay was quantified using a test filter of 450 nm and a reference filter of 630 nm. Statistical analyses were performed using one way ANOVA with a Neuman-Keuls post-test.

2.1.11 Caspase 3 Activation Assays

At 24 hours post transfection, 2 ml of media, containing 35 μM tamoxifen (Sigma) was added to each well for 4 hours. Cells were scraped on ice, washed with cold PBS, and DEVD-ase assays were performed as specified using the ApoAlert fluorometric kit (Clontech). The fluorescent substrate N-acetyl-Asp-Glu-Val-Asp-AFC (7-amino-4-trifluoromethyl coumarin) (BIOMOL) was used at a final concentration of 50μM instead of the Clontech substrate. The plate was incubated in a Labsystems Fluoroscan Ascent fluorescent plate reader at 37^0C for 75 minutes, with a reading obtained every 5 minutes, using excitiation at 390nm and emission at 510nm and Ascent Research edition 2.24 software. The DEVD-ase activity was normalized to protein concentrations. Statistical analyses were performed using one way ANOVA with a Neuman-Keuls post-test.

2.2 Materials and Methods for Chapter 4

2.2.1 Antibodies

The mouse monoclonal anti-HIP1 antibody (mAb HIP1#9) was generated by immunization of Balb/C mice with a GST-fusion protein encoding the huntingtin-interacting domain of human HIP1 (1). Hybridomas were generated and cloned and HIP1 mAbs were selected by ELISA with purified HIP1. Positive hybridomas were further characterized by Western blot and mAb HIP1#9 was found to specifically interact with human and mouse HIP1. Mouse mAbs recognizing α-adaptin, CHC or Rab5 were from Transduction Laboratories; mouse mAb directed against CHC was from Affinity Bioreagents Inc.; rabbit polyclonal anti α-adaptin was from Santa Cruz. Mouse mAbs directed against the Flag- and tetra-His epitope were from Sigma and Qiagen, respectively.

2.2.2 DNA constructs

HIP1 mammalian expression constructs were generated with HIP1 splice variant 2 (4) that encodes the 1003 amino acid isoform of HIP1. HIP1 cDNA sequences were cloned into the mammalian expression vector pCI (Promega) and tagged at their respective C-termini with the following cDNA sequence 5'-GGAGGTGGAGACTACAAGGACGACGATGACAAGTAG. This cDNA encodes a linker of 3 glycines, the Flag-tag, and a stop codon. The following HIP1-GST-fusion proteins were created by PCR amplification from full-length HIP1 cDNA with subsequent cloning into the appropriate pGEX vectors (Pharmacia Biotech): GST-HIP1-219-616, GST-HIP1-276-335. For the His_6-tagged terminal domain (TD) of clathrin, a

GST-construct encoding the 579 amino acids of the CHC in pGEX-2T (generous gift of James Keen, Thomas Jefferson University, Philadelphia) was digested with *Bam*HI and *Eco*RI and the resulting insert was subcloned into the pTrcHisA (Invitrogen) at the same sites. All expression constructs were verified by DNA sequencing.

2.2.3 Immunofluorescence

Cells were washed in phosphate-buffered saline (PBS) (20 mM NaH_2PO_4, 0.9% NaCl, pH7.4), fixed in 4% paraformaldehyde in PBS, and permeabilized in 0.3% Triton X-100, 1% paraformaldehyde. Non-specific binding sites were blocked by incubation with 3% normal goat serum (NGS) in PBS. For double immunofluorescence, HIP1 polyclonal antibody was incubated in 2% NGS in PBS at 4°C overnight followed by incubation with mAbs directed against endocytic markers for 2 h at room temperature. Cells were extensively washed in PBS between each incubation. Cells were then incubated with secondary antibodies, washed, mounted and observed by confocal microscopy.

2.2.4 Transferrin uptake assays

COS-7 cells plated on poly-L-lysine-coated coverslips were transfected, cultured 24 h and serum-starved overnight. The cells were then incubated with Cy3-conjugated human transferrin (25 μg/ml) for 20 min at 37°C, washed three times with PBS, and fixed with 3.5% paraformaldehyde in PBS. Following fixation, coverslips were processed for immunostaining with a monoclonal anti-Flag antibody as above.

2.2.5 Purification of CCVs

CCVs from rat brain were purified as described (Maycox et al., 1992). A short version of the purification was adapted for transfected cells. Specifically, cells were homogenized in buffer A [0.1 M 2-(N-morpholino)ethanesulfonic acid pH 6.5, 1 mM EGTA, 0.5 mM $MgCl_2$, 0.83 mM benzamidine, 0.23 mM PMSF, 0.5 μg/ml aprotinin and 0.5 μg/ml leupeptin] using a glass-Teflon homogenizer (10 strokes, 1500 rpm). The homogenate (H) was centrifuged at 17,800 X g_{max} for 20 min and the supernatant (S1) was collected and centrifuged at 56,100 X g_{max} for 1 h. The pellet (P2) was resuspended in buffer A using a glass-Teflon homogenizer (3 strokes, 1500 rpm) followed by dispersion through a 25-gauge needle. The resuspended pellet was loaded on top of a solution containing 8% sucrose in buffer A made in D_2O and centrifuged for 2 h at 115,800 g_{max}. The pellet containing the CCVs was collected.

2.2.6 Pull-down and binding assays

Adult rat brains were homogenized in buffer B (10 mM HEPES pH 7.4, 0.83 mM benzamidine, 0.23 mM PMSF, 0.5 μg/ml aprotinin and 0.5 μg/ml leupeptin) and a cytosolic fraction was generated by centrifugation at 205,000 g_{max} for 30 min at 4°C. Triton X-100 was added to the cytosolic fraction (2 mg) to a 1% final concentration and the samples were incubated overnight at 4°C with GST-fusion proteins pre-coupled to glutathione-Sepharose beads. After incubation, samples were washed three times in buffer B containing 1% Triton X-100. In other cases, a His_6-tagged fusion protein encoding the clathrin terminal domain (TD; 25 μg) was incubated overnight at 4°C in

buffer B with the various GST-fusion proteins pre-coupled to glutathione-Sepharose beads. After incubation, samples were washed three times in buffer B. For all assays, specifically bound proteins were eluted in SDS-PAGE sample buffer and processed for Western blot analysis.

2.2.7 Immunoprecipitation analyses

Forty-eight hours following transfection, 293T cells were washed in PBS, scraped in buffer B, sonicated and Triton X-100 was added to a 1% final concentration. The lysates were rocked for 30 min at 4°C and then centrifuged at 245,000g_{max} for 15 min. Supernatants were incubated overnight at 4°C with anti-Flag antibody pre-coupled to protein G-Sepharose. After incubation, samples were washed three times in buffer B containing 1% Triton X-100. Bound proteins were eluted in SDS-PAGE sample buffer and processed for Western blot analysis.

2.3 Materials and Methods for Chapter 5

2.3.1 Antibodies

Rabbit polyclonal antibodies against HIP1 (HIP1FP) and HIP12 (HIP12FP) and a mouse monoclonal antibody against HIP1 (mAb HIP no. 9) were previously described (section 2.1.5 and 2.2.1). Monoclonal antibodies against the clathrin heavy chain and α-adaptin were purchased from Transduction Laboratories. Monoclonal antibody against the clathrin light chain was purchased from Santa Cruz. Monoclonal antibodies against the FLAG, HA, and His_6 epitopes were purchased from Sigma, Roche Molecular Biochemicals, and Qiagen, respectively.

2.3.2 DNA Constructs and Recombinant Proteins

Mammalian expression constructs encoding full-length HIP1 and HIP12 and the talin homology domain of HIP1, each with a FLAG epitope tag at the C terminus, were previously described (Metzler et al., 2001). A construct encoding human full-length HIP12 with a C-terminal HA epitope tag was generated by insertion of a cDNA sequence, 5'-GGAGGTGGATATCCCTATGATGTCCCCGATTATGCC, encoding a linker of 3 glycines followed by the HA tag. The integrity of the HA-tagged HIP12 construct was verified by DNA sequencing. Bacterial fusion proteins encoding His_6-tagged terminal domain of the clathrin heavy chain and the amino acids 276-335 of HIP1 fused to GST (GST-HIP1-(276-335)) were previously described (Metzler et al., 2001). A His_6-tagged construct encoding full-length clathrin light chain b was generated by subcloning the GFP-clathrin light chain b cDNA (kindly provided by Dr. Juan Bonifacino) into the

pTrcHisB bacterial expression vector (Qiagen). The following HIP1 and HIP12 GST fusion proteins were created by PCR amplification from either full-length HIP1 or HIP12 cDNAs with subsequent cloning into pGEX-2T or pGEX-4T vectors (Amersham Biosciences): GST-HIP12-(302-348), GST-HIP1-(336-610), GST-HIP12-(349-644), GST-HIP1-(731-1003) (GST-HIP1-talin), and GST-HIP12-(765-1068) (GST-HIP12-talin).

2.3.3 Pull-down Assays

Rat brains were homogenized in buffer A (10 mM HEPES-OH, pH 7.4, 0.83 mM benzamidine, 0.23 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml aprotinin, and 0.5 μg/ml leupeptin) and the homogenates were centrifuged at 205,000 × g_{max} for 30 min at 4 °C. The supernatant (cytosolic extract) was collected and Triton X-100 was added to 1% final concentration. Aliquots of the extract (2 mg) were incubated overnight at 4 °C with GST fusion proteins pre-coupled to glutathione-Sepharose beads. After incubation, samples were washed three times in buffer A containing 1% Triton X-100. For binding experiments, purified His_6-tagged clathrin terminal domain or clathrin light chain b fusion proteins (15 μg) in buffer A were incubated overnight at 4 °C with various GST fusion proteins pre-coupled to glutathione-Sepharose beads. After incubation, samples were washed three times in buffer A. In all cases, proteins specifically bound to the beads were eluted with gel sample buffer and analyzed by SDS-PAGE and Western blot.

For pull-downs from transfected cells, HeLa cells were grown on 10-cm^2 dishes and transfected with 6 μg of HIP1 or HIP12 cDNA using FuGENE (Roche Molecular Biochemicals). Twenty-four h post-transfection, cells were washed twice with ice-cold

phosphate-buffered saline (PBS) and lysed in buffer A (containing 1% Triton X-100 and 10 μM Pefabloc (asulfonyl-type serine protease inhibitor; Roche Molecular Biochemicals). Samples were incubated overnight at 4 °C with GST fusion proteins pre-coupled to glutathione-Sepharose beads. After incubation, samples were washed three times in buffer A containing 1% Triton X-100 and 10 μM Pefabloc. Bound proteins were analyzed by SDS-PAGE and transferred to nitrocellulose for Western blot analysis.

2.3.4 Immunoprecipitation Assays

HEK-293T cells were grown on 10-cm^2 plates and transfected with 4 μg of HIP12 cDNA by $CaPO_4^-$ precipitation. Transfected and nontransfected cells were washed 24 h post-transfection with ice-cold PBS and lysed in ABL buffer (20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100) supplemented with a 1:100 dilution of aprotinin (Sigma), 20 ng/ml leupeptin (Roche Molecular Biochemicals), and 10 μM Pefabloc. The cell lysates were sonicated and centrifuged at 245,000 × g_{max} for 15 min. The supernatants were then incubated for 6 h at 4 °C with mouse monoclonal antibody HIP1 no. 9 pre-bound to protein G-Sepharose (Amersham Biosciences). Subsequently, samples were washed 6 times in ABL buffer and specifically bound proteins were analyzed by SDS-PAGE and Western blot.

2.3.5 Immunofluorescence

Cells were plated onto gelatinized glass slides in 6-well tissue culture plates and transfected with 1 μg of HIP1 or HIP12 cDNA. Twenty-four h post-transfection, the cells were washed in PBS, fixed in PBS containing 4% paraformaldehyde for 15 min at room

temperature, and permeabilized in PBS containing 0.3% Triton X-100 and 1% paraformaldehyde for 5 min. The cells were then incubated in PBS containing 3% normal goat serum for 30 min to block nonspecific binding. Depending on the experiment, the cells were then incubated with monoclonal antibodies against the FLAG or HA epitopes for 1 h at room temperature or overnight at 4 °C with a polyclonal antibody against HIP1, followed by a 1-h incubation at room temperature with appropriate secondary antibodies in PBS containing 2% normal goat serum. Staining of the actin cytoskeleton was achieved by incubation with Texas Red-phalloidin. Cells were then extensively washed in PBS, mounted, and observed on a BioRad Radiance Plus confocal microscope (BioRad, Hercules, CA) using Laser Sharp software (BioRad).

2.3.6 Actin-binding Assay

Purified human non-muscle monomeric actin (Cytoskeleton Inc.) was polymerized as described previously (Engqvist-Goldstein et al., 1999) and incubated with purified GST-HIP1-talin and GST-HIP12-talin (20 μg each) for 1 h at room temperature in a final volume of 50 μl. The samples were then centrifuged for 30 min at 362,000 × g_{max}. Protein components of the pellets and supernatants were analyzed by SDS-PAGE followed by Coomassie Blue staining. Gels were scanned on a ScanJet 6300 (Hewlett Packard) and protein amounts were determined using Quantity One (Bio-Rad) software. Statistical analysis was done by the Student's *t* test.

2.3.7 Clathrin Purification and Clathrin Assembly Assay

CCVs were purified from adult rat brains as described (Maycox et al., 1992). To purify clathrin, coats were stripped from the vesicles by incubation in buffer B (0.5 M Tris, pH 7.0, 2 mM EDTA, 0.83 mM benzamidine, 0.23 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml aprotinin, and 0.5 μg/ml leupeptin) for 15 min on ice. The samples were then centrifuged for 15 min at 245,000 × g_{max} with the purified coat fraction remaining in the supernatant. The stripping and centrifugation steps were repeated a second time and the supernatants were pooled. The pooled supernatants were loaded onto a continuous 5-20% sucrose gradient made in buffer B and then centrifuged for 3.5 h at 195,000 × g_{max} in a Sorvall Step Saver vertical rotor. Fractions of 2 ml were collected and analyzed by SDS-PAGE and Coomassie Blue staining. Peak clathrin fractions were pooled and dialyzed overnight in clathrin assembly buffer (10 mM Tris-Cl, pH 8.5) and subsequently used for clathrin assembly assays.

Clathrin assembly assays were performed with 0.5 μM purified clathrin and different concentrations (0.12-2 μM) of fusion proteins in a final volume of 90 μl of clathrin assembly buffer. Assembly was initiated at 4 °C by addition of 10 μl of 1 M 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.7. The mixture was kept on ice for 45 min and then centrifuged at 400,000 × g_{max} for 6 min. The supernatant (80 μl) was loaded on a SDS-PAGE and the clathrin assembly was quantified by Coomassie Blue staining.

Chapter 3:

HIP12 is a non-proapoptotic member of a gene family including HIP1, an interacting protein with huntingtin

The data presented in this chapter contributed to the manuscript:

Chopra, V. S., Metzler, M., Rasper, D. M., Engqvist-Goldstein, A. E., Singaraja, R., Gan, L., Fichter, K. M., McCutcheon, K., Drubin, D., Nicholson, D. W., and Hayden, M. R. (2000) HIP12 is a non-proapoptotic member of a gene family including HIP1, an interacting protein with huntingtin. *Mamm. Genome* 11, 1006-1015.

Unless otherwise stated the data presented in this chapter was obtained by myself. All contributions by other individuals will be discussed in the results section 3.2.

3.1 Introduction

Huntingtin-interacting protein 1 (HIP1) is a 120 kDa membrane associated protein that was first identified through its interaction with huntingtin, a polyglutamine-containing protein associated with Huntington Disease (HD) (Kalchman et al., 1997; Wanker et al., 1997). The interaction between HIP1 and huntingtin is polyglutamine-length dependent; the longer the polyglutamine repeat the less the interaction with huntingtin. Although huntingtin is expressed in all cell types, HD results from a selective loss of medium spiny neurons in the neostriatum and neurons within the deeper layers of the cerebral cortex (Hayden, 1981; Vonsattel et al., 1985; Sharp et al., 1995; for a review see Wellington et al., 1997). This selectivity of neuronal degeneration may be influenced by altered interactions with proteins such as HIP1, which is highly expressed in the brain.

HIP1 shows homology to Sla2p in *Saccharomyces cerevisiae* and to ZK370.3 in *Caenorhabditis elegans* (Wanker et al., 1997; Kalchman et al., 1997). Sla2p is essential for endocytosis and the assembly and function of the cytoskeleton (Holtzman et al., 1993, Raths et al., 1993 and Na et al., 1995) and for vesicle transport from the Golgi apparatus in yeast (Mulholland et al, 1997; Blader et al, 1999). A potential role in vesicle transport has also been suggested for huntingtin which is a cytosolic protein and often found associated with intracellular membranes, microtubules, synaptic and clathrin-coated vesicles (DiFiglia et al. 1995; Gutekunst et al., 1995; Velier et al., 1998). These observations suggest that the interaction between HIP1 and huntingtin is necessary for vesicle trafficking and maintenance of the membrane cytoskeleton. As a consequence, the

altered interaction of HIP1 with huntingtin could result in a disruption of cellular transport leading to neuronal cell death.

Interestingly, huntingtin has been shown to be a substrate for the proapoptotic protease caspase 3 (Goldberg et al, 1996; Wellington et al., 1998). Furthermore, the caspase 3 cleavage sites present in huntingtin are adjacent to the domain which interacts with HIP1 (Wellington et al., 2000). Thus, it is possible that the reduced interaction between HIP1 and huntingtin containing an expanded polyglutamine tract may render huntingtin more susceptible to cleavage by caspases.

In this chapter we determined the genomic organization of *HIP1* and demonstrated that alternative splicing occurs at the *HIP1* locus. We also identified a novel protein termed HIP12 that shows profound sequence and biochemical similarities to HIP1 and also shows high sequence similarity to Sla2p and ZK370.3. However, HIP1 and HIP12 differed in their abilities both to interact with huntingtin and to activate proapoptotic pathways.

3.2 Results

3.2.1 Identification of HIP12, a paralog of *HIP1*

In an attempt to identify the murine homolog of *HIP1*, a mouse brain lambda ZAPII cDNA library was screened with the mouse EST w82687 (clone 404331). EST w82687 was 420bp in length and showed 66% nucleotide identity with *HIP1* cDNA. The EST was initially isolated from the non-redundant database of GenBank EST Division by performing a BLASTN search using a fragment of the human *HIP1* cDNA as the query. Subsequently a cDNA clone was isolated, termed *mHIP12*, that exhibited 47% nucleotide identity and 65% amino acid conservation with *HIP1*. The characterization of mHIP12 is published elsewhere (Engqvist-Goldstein et al., 1999).

The entire *mHIP12* cDNA sequence was used to screen the non-redundant database of GenBank EST Division. We identified a human EST, T08283, which showed 87% nucleotide identity with *mHIP12* cDNA but not HIP1, indicating that HIP1 is a member of a gene family. TO8283 was used to isolate a 3.2 Kb cDNA clone from a human frontal cortex cDNA library. Subsequently, 5′ RACE was used in order to isolate 1.2 Kb of additional 5′sequence resulting in a 4.4 Kb cDNA sequence which contained an open reading frame of 3204 nucleotides encoding a protein of 1068 amino acids in length, which was termed *hHIP12*. Flourescence *in situ* hybridization demonstrated that *hHIP12* was localized to chromosome 12q24.32 (figure 3.2.1). Recently, Seki et al. (1998) have also identified an 888 amino acid protein which is identical to HIP12 but lacks the first 180 amino acid residues.

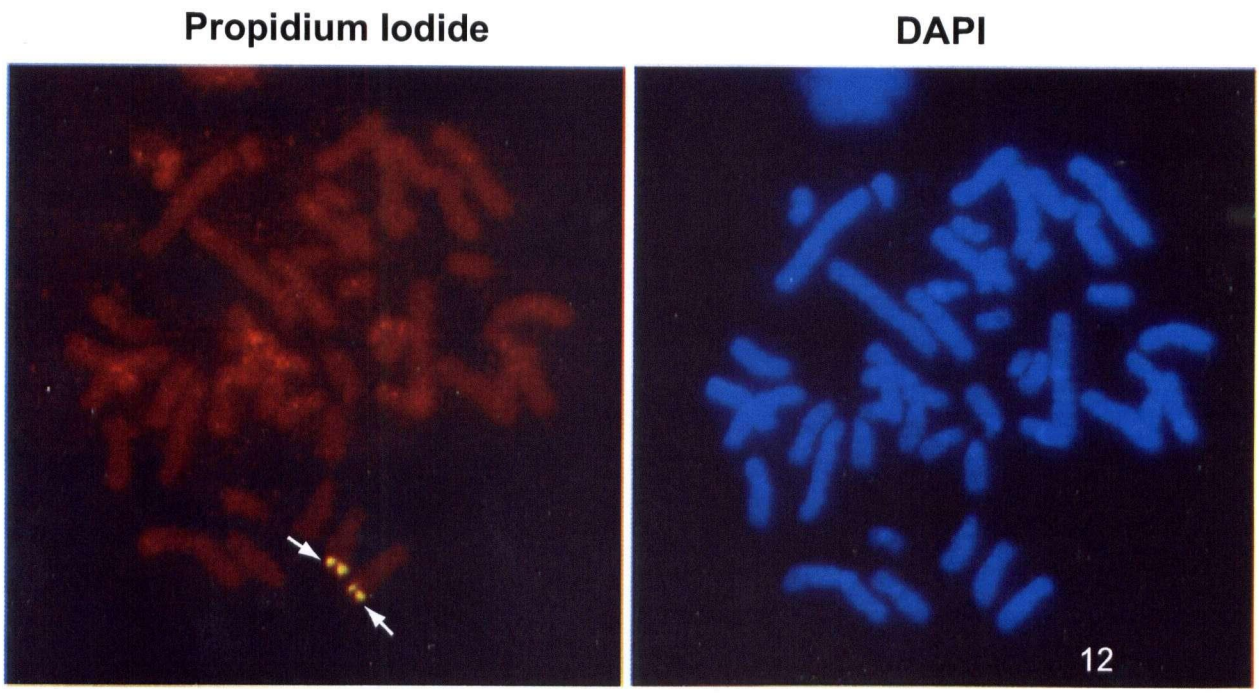


Figure 3.2.1. Genomic mapping of the HIP12 locus. A single genomic locus for HIP12 was identified at 12q24.32 using the original cDNA probe isolated from the two-hybrid screen as the probe.

3.2.2 Alternative splicing at the human *HIP1* locus

Protein sequence comparisons between HIP12, HIP1, ZK370.3 and Sla2p suggested that the published sequence of HIP1 (Genbank, accession number U79734) may be missing additional amino terminal sequences. Translation of the published *HIP1* 5′ UTR showed significant sequence similarity to the amino terminal sequences of Sla2p and ZK370.3. Moreover, antibodies generated against a HIP1 fusion protein detected an approximately 120 kDa protein which is larger than the expected molecular weight of approximately 100 kDa predicted from the previously published HIP1 encoded by 914 amino acids. Subsequent analysis of human *HIP1* genomic sequences deposited in GenBank (accession number AC004491), and RT-PCR and 5′ RACE analyses performed on total human brain RNA confirmed that human HIP1 contained additional 5′ sequences. Moreover, these studies led to the identification of 2 alternative splice variants, HIP1-1 and HIP1-2, which differ in their 5′ sequences (figure 3.2.2A and B). The identification of the HIP1 isoforms was a collaborative effort by Dr. M. Metzler, D. Rasper and myself. Both HIP1 variants contain in frame translation initiation sites with a strong Kozak consensus sequence present in HIP1-2. HIP1-1 contains 2 in frame translation initiation sites that are 12 nucleotides apart. The more upstream ATG is surrounded by sequences which conform better to the Kozak consensus sequence (figure 3.2.2A). Therefore, this site was chosen as the translation initiation site and produced a protein of 1034 amino acids. The HIP1-2 open reading frame is 3009 nucleotides long and encodes a smaller protein isoform of 1003 amino acids. Both splice forms share a high degree of homology with Sla2p and ZK370.3 among their respective N-termini.

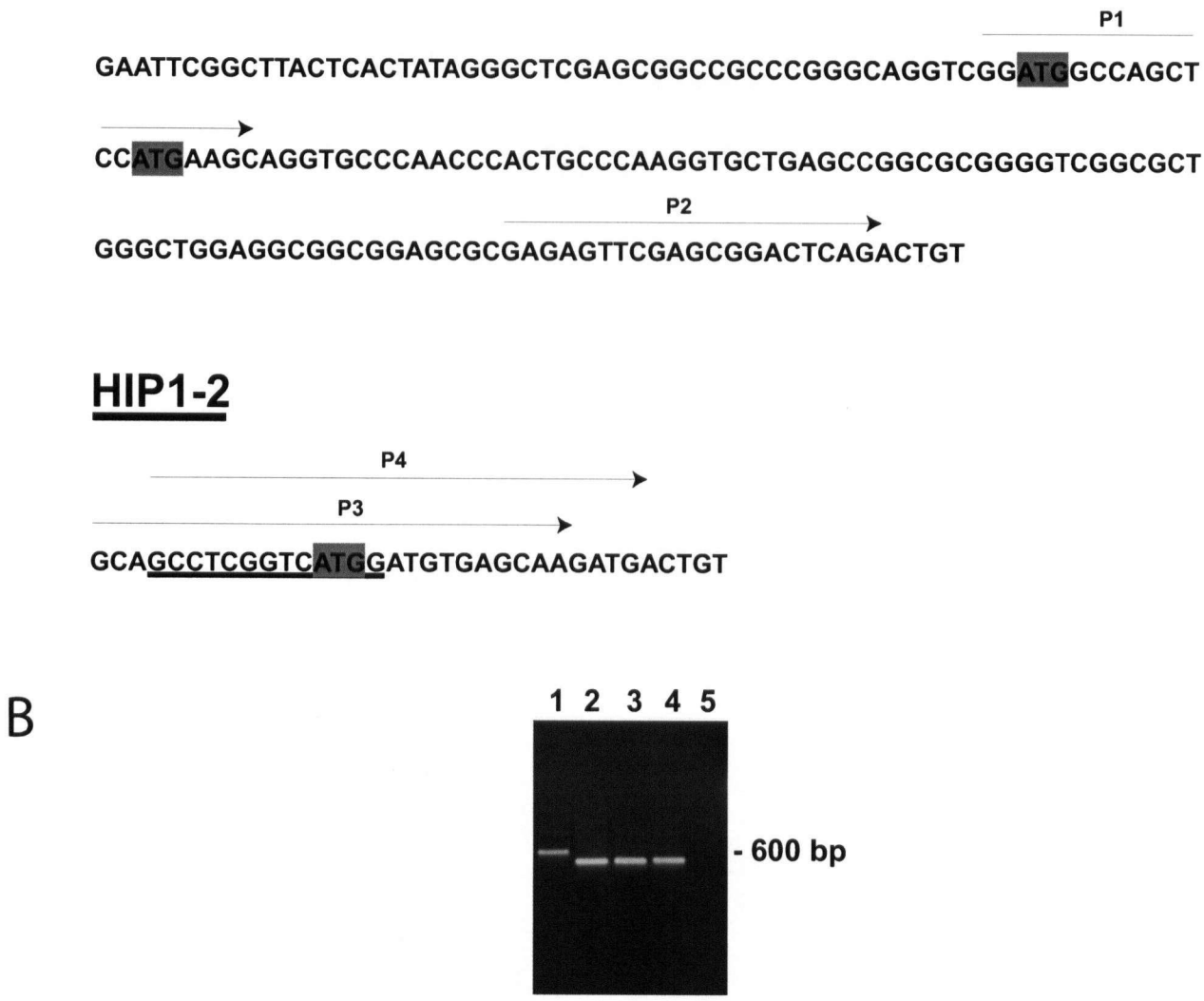


Figure 3.2.2. Alternative splicing at the HIP1 locus. A: Sequences of splice variant 1 (HIP1-1) and 2 (HIP1-2) are shown. The translation initiation sites are shaded in grey and the Kozak consensus sequence preceeding the translation initiation site in HIP1-2 is shown as the underlined sequence. Arrows P1 and P2 represent forward primers that recognize 5′ sequences in HIP1-1 whereas arrows P3 and P4 denote primers that recognize 5′ sequences sequences in HIP1-2. B: RT-PCR amplification of alternative transcripts at the HIP1 locus using total RNA isolated from human brain. The forward primers P1, P2, P3 and P4 were used in combination with the same reverse primer (lanes 1, 2, 3, and 4 respectively). This results in the amplification of a 619 bp fragment in lane 1, a 527 bp fragment in lane 2, a 536 bp fragment in lane 3 and a 531 bp fragment in lane 4. In lane 5 no RNA was present for reverse transcription. Lanes 1 and 2 confirm the presence of HIP1-1 sequences whereas lanes 3 and 4 confirm the presence of HIP1-2 sequences.

Moreover, RT-PCR analyses indicate that both splice variants are widely expressed since they are present in all tissues analyzed (data not shown). Thus far, 5′ and 3′ RACE analyses of HIP12 sequences have lead to the identification of a single transcript indicating that no alternative splicing occurs at the HIP12 locus (data not shown).

3.2.3 Genomic organization of human *HIP1*

The genomic organization of human *HIP1* was determined using *HIP1* cDNA specific primers followed by PCR amplification across intronic sequences. In addition, exon-intron boundaries were identified comparing *HIP1* genomic sequences with *HIP1* cDNA sequences deposited in GenBank (accession # AC004491). The *HIP1* gene consists of 32 exons spanning approximately 215 Kb of genomic sequence (figure 3.2.3 and table 3.2.3). Moreover, translation initiation sites are present in exon 1 and exon 3 and alternative splicing occurs either from exon 2 to exon 4 or from exon 3 to exon 4. At present it is unclear whether additional untranslated 5′ exons exist at the *HIP1* locus. Interestingly, exons 1, 2, 4, 5 did not conform to the GT-AG rule for splice-donor and splice-acceptor sites.

3.2.4 Sequence comparisons of HIP12 with HIP1, Sla2p and ZK370.3

Protein sequence alignments of HIP12, HIP1, ZK370.3 and Sla2p displayed significant sequence similarities. HIP12 showed 47% identity and 64% amino acid conservation with HIP1. Moreover, HIP12 and HIP1 showed virtually identical levels of amino acid identity and amino acid conservation with ZK370.3 and Sla2p (Table 3.2.4).

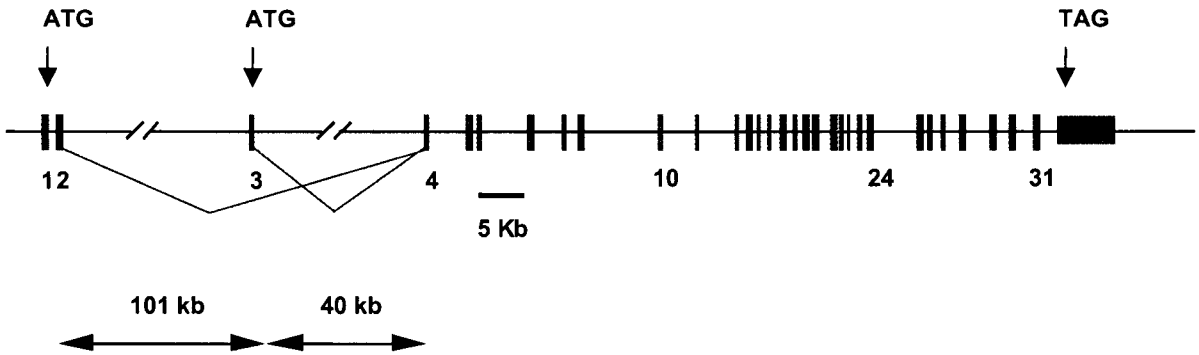


Figure 3.2.3. Genomic organization of HIP1. The relative distance between exons is shown by horizontal bars; exons are shown by vertical grey bars. Alternative splicing occurs from exon 2 or exon 3 to exon 4. Exon 32 contains the stop codon and the 3′ UTR is shown as a grey box.

Table 3.2.3. Splice-acceptor and splice -donor sequences at the human *HIP1* gene. Exonic sequences are indicated by capital letters. Intronic sequences are shown by small letters.

Exon	5' Splice junction	cDNA position	3' splice junction	Exon size (bp)	Intron size (Kb)
1*	ATGGCCAGCT	1-62	GGCGCGGGGTtcctgctaagcatcacac	62**	1
2	tggggatcccggggcagccCGGCGCTGGG	63-111	GCGGACTCAGgtgaggacgggggaccc	49	101
3*	ATGGATGTGA	112-129	GAGCAAGATGgtaagtgtgagcaggggc	18**	40
4	ccccccttcttgtttttcagACTGTCAGCATCA	130-193	CACGCCAGAAatatccttttggatgttgctt	64	5.6
5	ttttccataacccccctcacCGTGCATACT	194-337	ACACCCGAACgtgagttcctggggctatgggg	144	0.35
6	gtgttcttttgcccctgcagGTCCTGAAGGAC	338-395	AGCAGGATGTgtgtgagtttggagatgtact	58	5.2
7	ttcccctcctcttcctaaaagGGCCACCTGA	396-476	CCACACCAAAgtgagtctctgcggacagtt	81	4.6
8	ttcctctgcctcccttccagAATCCCAGGT	477-553	TGAACAACTTgtaagtggctcctgccctgag	77	0.8
9	ctctttcctcttgggtccagTTTCCAGTTA	554-615	TTCCAAACAGgtgagtctcttccctcccgt	62	10
10	ccctcacttttatttcctagTATTCAACTC	616-756	CTCCACTCCTgtgagtaccgcgggccagat	141	5.8
11	ctctttccaatttcttccagGCCTCCCAGC	757-814	AGTTTACAAAgtaagtggttcaagtaacag	58	5.5
12	aggtccttctccacccctagGTTGAAAGAT	815-890	GCTGCCTGAGgtaagcatgcccaaccaca	76	0.1
13	ggatgtgtctccgtcttcagACCCACCCAA	891-1031	CTCTCAGCAGgtgaggaccacttgggagaga	141	1.5
14	tcactccttttttggtcaaccacagAATTTATTT	1032-1129	AGGATGAGAAgtgagtccaagctgggttcaa	98	1.1
15	cattccccttctctttcccagGGACCACTTAAT	1130-1202	GAAGACTGAGgtataactttggatctgctctgcc	73	1.6
16	tgaccggagtccccccacagAGCCAGCGGG	1203-1386	GAGATAGAAAgtgagcggtgggtgggggcg	184	1.4
17	cttggtcctttacaatacagGGAAAGCTCAAG	1387-1475	GCTGCGGAAGgtaagaccctcagcccctgtca	89	0.8
18	acactgttcttcttttgcagAATGCAGAGGT	1476-1592	GGCCAGCGGAAGgtgagtgggacgaggagc	117	1
19	ctgtttttcttctctttcagACTCAAGAACA	1593-1694	CTTCTGCCCAGgtaaatacctcctttttttt	102	1
20	ccttgtggtcacctttgcagTCAGAAGCAAA	1695-1839	AGCACAGAGGCAGgtcacggacatggac	145	0.8
21	acctccctggaccatttttagAATCTATGTG	1840-1974	GGGTCTGCAGgtacacttgcaattgcccagct	135	1.4
22	ccctgtgttccctctcacagATCACCTCCT	1975-2061	TGCCCAGAAGgtaagaatggccaaggacagt	87	0.3
23	cttctctttccaatcctggcagACATCAGTGG	2062-2168	CCTGCCGACTgtgagtactggggcatgagg	107	0.5
24	ctattgccttgtatctccagCACTTGACCGA	2169-2305	CATCGGCGAGgtacttggagtagtatcattg	137	5
25	cctttgtgatgtgttcacagGAGCTCCTGCCC	2306-2416	CCAGAATAGAGgtaggaggttcctgcaggatc	111	1.7
26	accatgcttctttttcacagGAGATGCTCAG	2417-2475	TGAATGAAAGgtcggtctgagcggcatggtgg	59	1.3
27	tcgacttgtttgtttggcagGATCCTTGGTTG	2476-2569	GAGCGGCAGGgtgagcgtgggtgtgggccct	94	2.2
28	actatttttttatttccctagGGTACAGCATCCC	2570-2670	CCACTGTCATGgtaagtatctattggtaccaa	101	3.8
29	cccaggggacacagggcaagGTGGATGCAG	2671-2776	TGCATCCAAGgtaggacctggctggacctcc	106	1.8
30	aatctgctttgtcctggtagGTGAAAGCTGAA	2777-2899	GAAGAGACAGgtagcctttccaaagggaccct	123	3.6
31	ttgtttccatccttgcagACAACATGGAC	2900-3070	TGGGAAGAAGgtaagctgactcaaaggat	171	1.4
32	aacataaattatcattgtcttttagGAACAGAGG	3071-3123	3' UTR	53***	

* First 10 nucleotides within respective exon starting at the translation initiation site.

** Length of nucleotide sequence in between translation initiation site and 3' end of exonic sequences.

*** Length of nucleotide sequence in between 5' end of exon 32 and translation stop codon.

Table 3.2.4. Pairwise comparisons between the nucleotide and amino acid sequences of HIP1-1, HIP1-2, HIP12, Sla2p and ZK370.3.

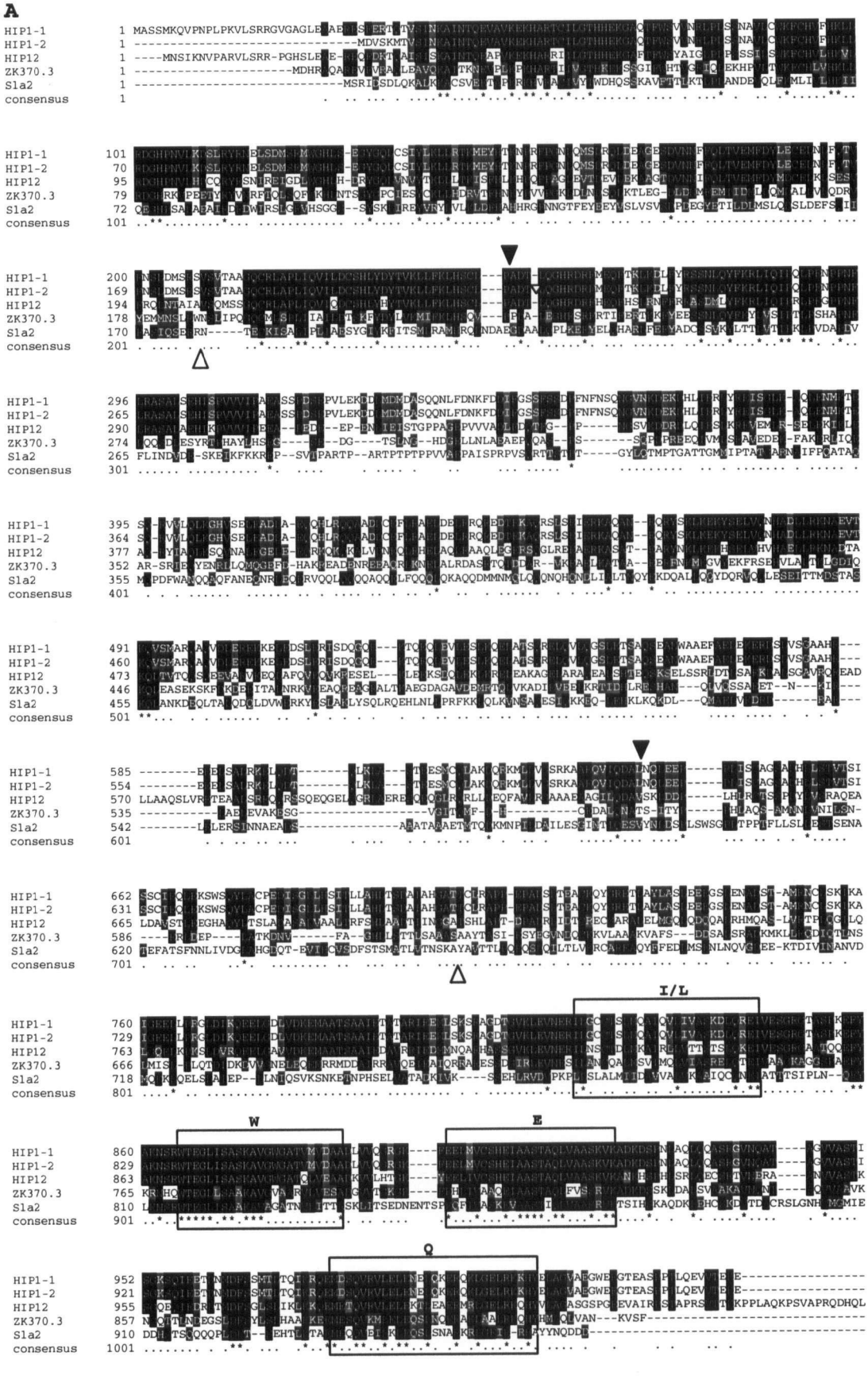
Alignment	Percentage Nucleotide Identity	Percentage Amino Acid Identity	Percentage Amino Acid Conservation
HIP1-1 vs HIP1-2	98	96	96
HIP1-1 vs HIP12	64	47	65
HIP1-1 vs ZK370.3	34	30	49
HIP1-1 vs Sla2p	31	17	39
HIP1-2 vs HIP12	63	46	64
HIP1-2 vs Zk370.3	34	30	50
HIP1-2 vs Sla2p	32	18	40
HIP12 vs ZK370.3	33	30	47
HIP12 vs Sla2p	32	18	36

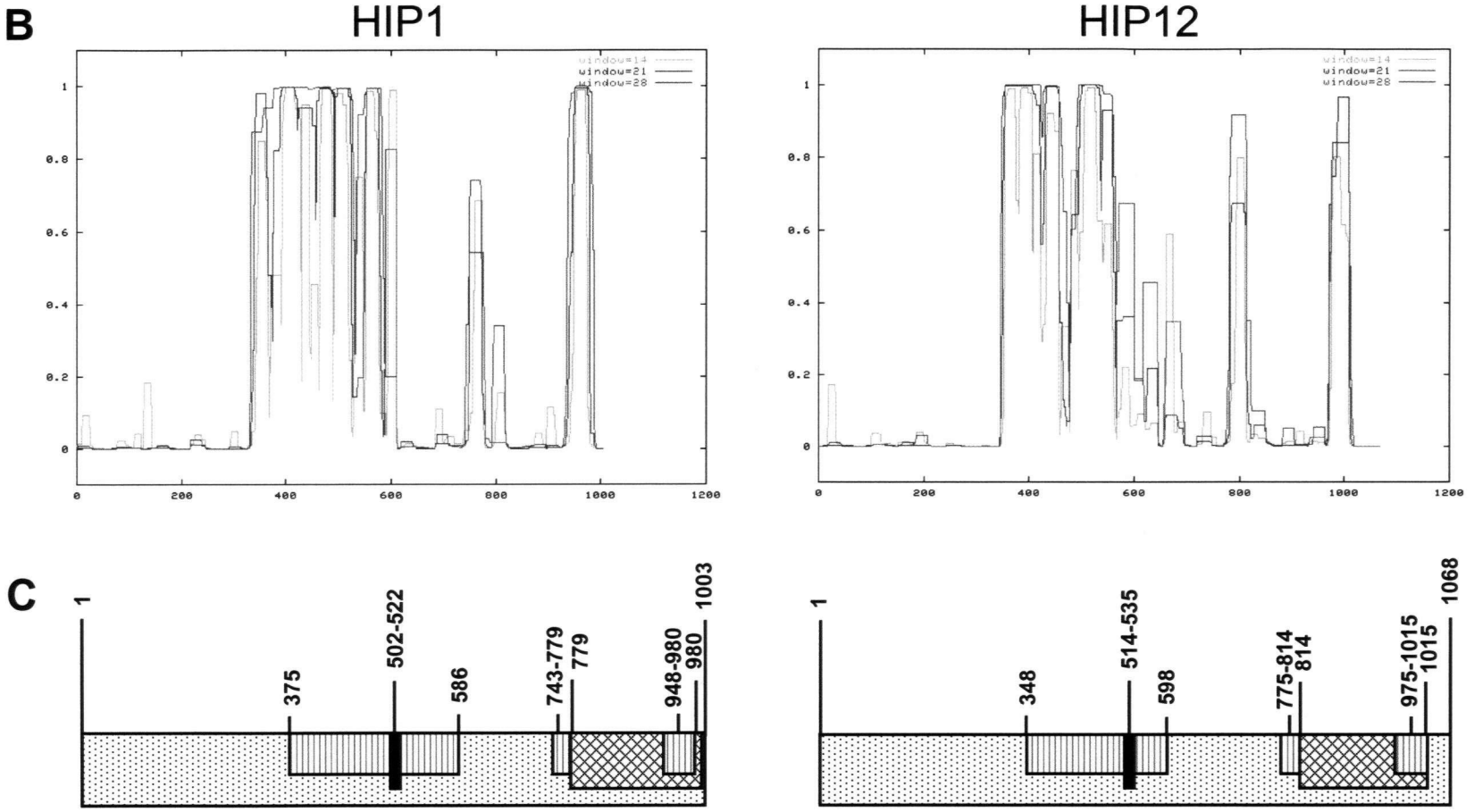
HIP12 shows high nucleotide and amino acid identity with HIP1. HIP1 and HIP12 show similar levels of nucleotide and amino acid identity with ZK370.3 and Sla2p.

The carboxy-terminal region of HIP12, like Sla2p, ZK370.3, and HIP1 shows considerable homology to the cytoskeletal protein talin. In Sla2p, the talin domain has been shown to contain 4 sub-domains referred to as the I/LWEQ module (McCann and Craig, 1997) that play a critical role in the binding of Sla2p to F-actin (figure 3.2.4). Amino acid residues in subdomain Q in HIP1 and HIP12 are likely to be essential in mediating actin binding for these proteins.

Using the COILs program, HIP12 similar to HIP1, Sla2p and ZK370.3 contains predicted coiled-coil domains (figure 3.2.4B and C). The coiled-coil domain in Sla2p has been shown to play an important role in actin polarization and protein dimerization in yeast (Wesp et al., 1997; Yang et al., 1999; Turunen et al., 1998). These observations suggest that HIP1 and/or HIP12 may play a role in actin polarization in mammalian cells. Further analyses of the HIP12 protein sequence revealed that, similar to HIP1 and Sla2p, HIP12 contains a leucine zipper at amino acid residues 514 to 535 (LEKLKRELEAKAGELARAQEAL). Leucine zippers are known to mediate protein-protein interactions observed among cytoskeletal proteins and transcriptional activators (Luscher and Larsson, 1999).

Figure 3.2.4. A: Amino acid alignment of HIP1-1, HIP1-2, HIP12, Sla2p and ZK370.3. The alignment between the 5 proteins is shown with the dark shading representing identical or conserved amino acids between all 5 proteins. The lighter shading representing identical or conserved amino acids in 3 or 4 of the proteins. ▼ Refers to the amino and carboxy terminal sequences present in the HIP1 yeast 2-hybrid vectors. △ Refers to amino and carboxy terminal sequences present in the HIP12 yeast 2-hybrid vectors. Boxed sequences represent the I/LWEQ sub-domains. **B**) The COILS program was used to predict the presence of coiled-coil domains in HIP1-2 and HIP12. The probability of the coiled-coil domain is plotted as a function of the amino acid position in the protein. **C**) Schematic representation of the various domains within HIP1-2 and HIP12, where the striped regions represent predicted coiled coil domains, solid portions represent the leucine zipper and hatched regions represent the talin domain.


A
HIP1-1
HIP1-2
HIP12
ZK370.3
Sla2
consensus
I/L
W
E
Q


B
HIP1
HIP12
window=14
window=21
window=28
0
0.2
0.4
0.6
0.8
1
0
200
400
600
800
1000
1200
C
1
375
502-522
586
743-779
779
948-980
980
1003
348
514-535
598
775-814
814
975-1015
1015
1068

3.2.5 mRNA Expression of Human *HIP1* and *HIP12*

In order to determine the expression pattern of *HIP1* and *HIP12*, Northern blot analyses were performed on various human tissues. Using a 1.2Kb fragment of the *HIP1* cDNA as a probe, we observed a 9.0 Kb transcript, which was present in all tissues with significant levels of expression found in the brain (figure 3.2.5A). Within the brain, *HIP1* mRNA expression was observed in all brain regions with highest expression levels present in the frontal cortex and putamen (figure 3.2.5B). RT-PCR analyses indicated that both splice variants, *HIP1-1* and *HIP1-2*, are expressed in various human brain regions and peripheral tissues (data not shown) however, the difference in transcript size is too small to be detectable by Northern blot analysis.

Northern blot analyses of *HIP12* indicated a different expression pattern in comparison with *HIP1*. Using a 675 bp cDNA of *HIP12*, a 5.0 Kb transcript was present in the brain, heart, kidney, pancreas, and liver but not in the lung or placenta (figure 3.2.5C). In addition, a 7.0 Kb transcript was also present in the heart and skeletal muscle. Furthermore, a 3.5 Kb transcript was observed in the skeletal muscle and occipital lobe. RT-PCR was performed in order to determine the source of the 7.0 and 3.5 KB transcripts. Primer pairs within the coding region of *HIP12* amplified identical size products in all tissues examined which indicated that the source of the larger transcript is not due to alternative splicing within the coding region (data not shown). We performed 3′ RACE to determine whether these transcripts arose due to differential polyadenylation and did not detect any differences in RT-PCR products that could account for the 3.5 and 7.0 Kb bands (data not shown). Therefore, it is likely that the 3.5 and 7.0 kb transcripts result from cross reactivity of the HIP12-specific cDNA probe.

3.2.6 Protein Expression of Human HIP1 and HIP12

Western blots were performed using novel polyclonal antibodies generated against either a HIP1 or HIP12 fusion protein. The HIP1 and HIP12 fusion protein antibodies recognized 120 and 130 kDa proteins, respectively. The specificity of each anti-fusion protein antibody was confirmed by competition studies using either excess HIP1 or HIP12 fusion proteins (data not shown). Within the brain, both HIP1 and HIP12 were found at similar levels in all brain regions examined (figure 3.2.6B and D). In various tissues examined, HIP1 was found to be expressed at highest levels in the frontal cortex and the spleen. Significant levels of HIP1 were also observed in the liver, and kidney (figure 3.2.6A). Similarly, HIP12 was expressed predominantly in the brain, liver, and to a lesser extent in the kidney (figure 3.2.6C). HIP1 Western blots exhibited faint lower molecular weight proteins in the spleen, kidney, cerebellum, caudate and putamen. And, in the case of HIP12, faint lower molecular weight proteins were detected in the heart, liver, lung and cerebellum. These bands most likely represent proteolytic degradation products.

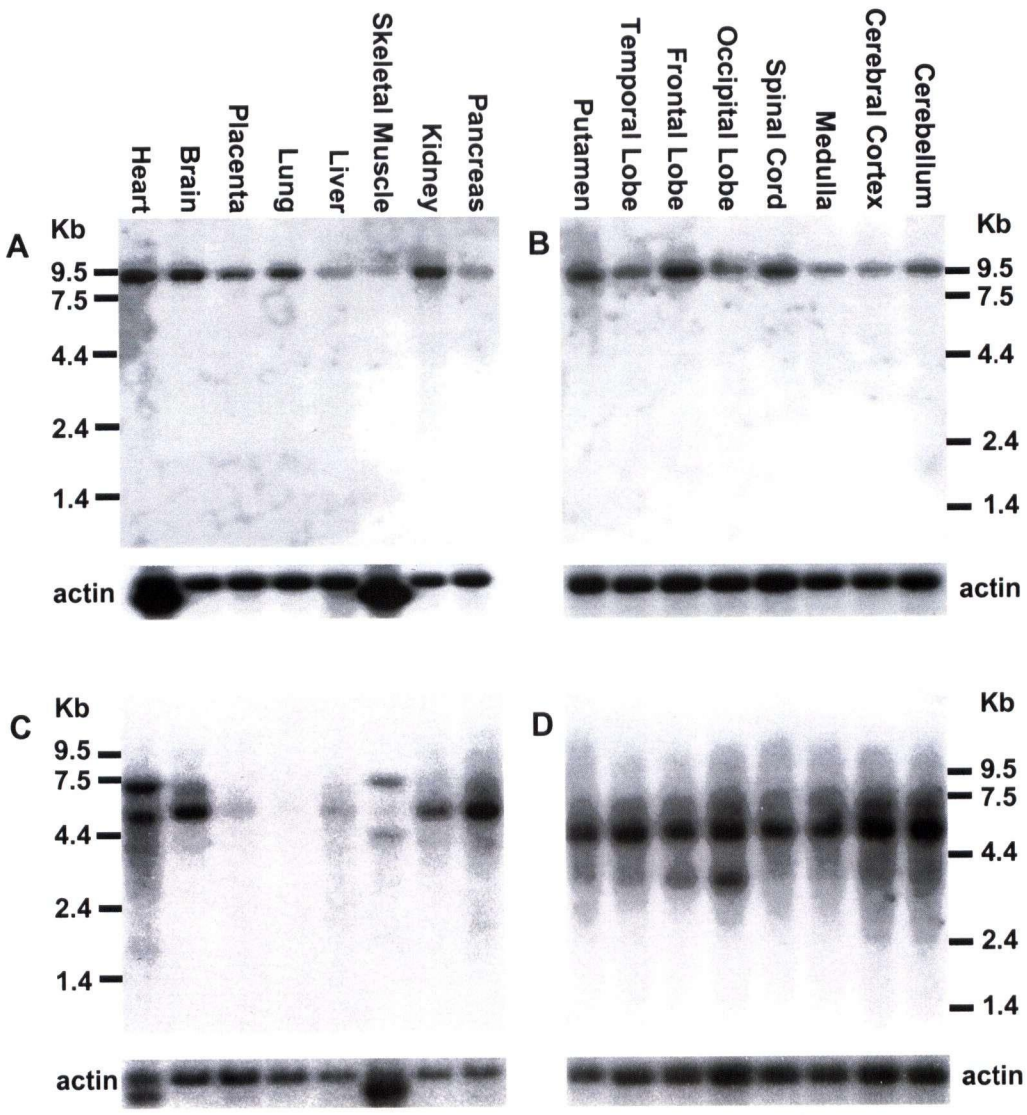


Figure 3.2.5. Northern blot analysis of the human HIP1 and HIP12. Northern blots containing 2μg of poly A+ mRNA from various adult human tissues were hybridized with either a 1.2 Kb fragment of HIP1 (A and B) or a 675 bp fragment of HIP12 (C and D). The lower panel represents the same blot hybridized with the human actin cDNA to confirm RNA quality and relative loading (C and D). RNA size markers are shown on the right and left.

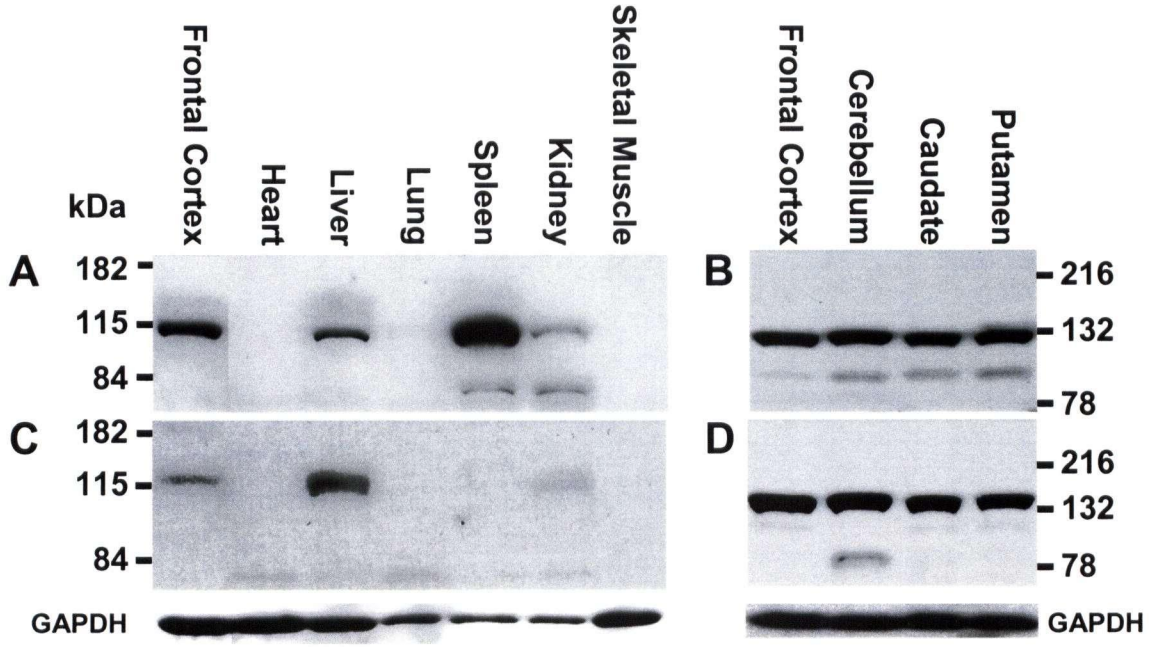


Figure 3.2.6. HIP1 and HIP12 protein expression in normal human CNS and peripheral tissues. HIP1 protein expression in normal human CNS and peripheral tissues (A). HIP1 protein expression in normal human brain regions (B). HIP12 protein expression in normal human CNS and peripheral tissues (C). HIP12 protein expression in normal human brain regions (D). The lower panels represent the same blots reprobed with the antibody against human glyceraldehyde phosphodehydrogenase to confirm protein quality and relative loading.

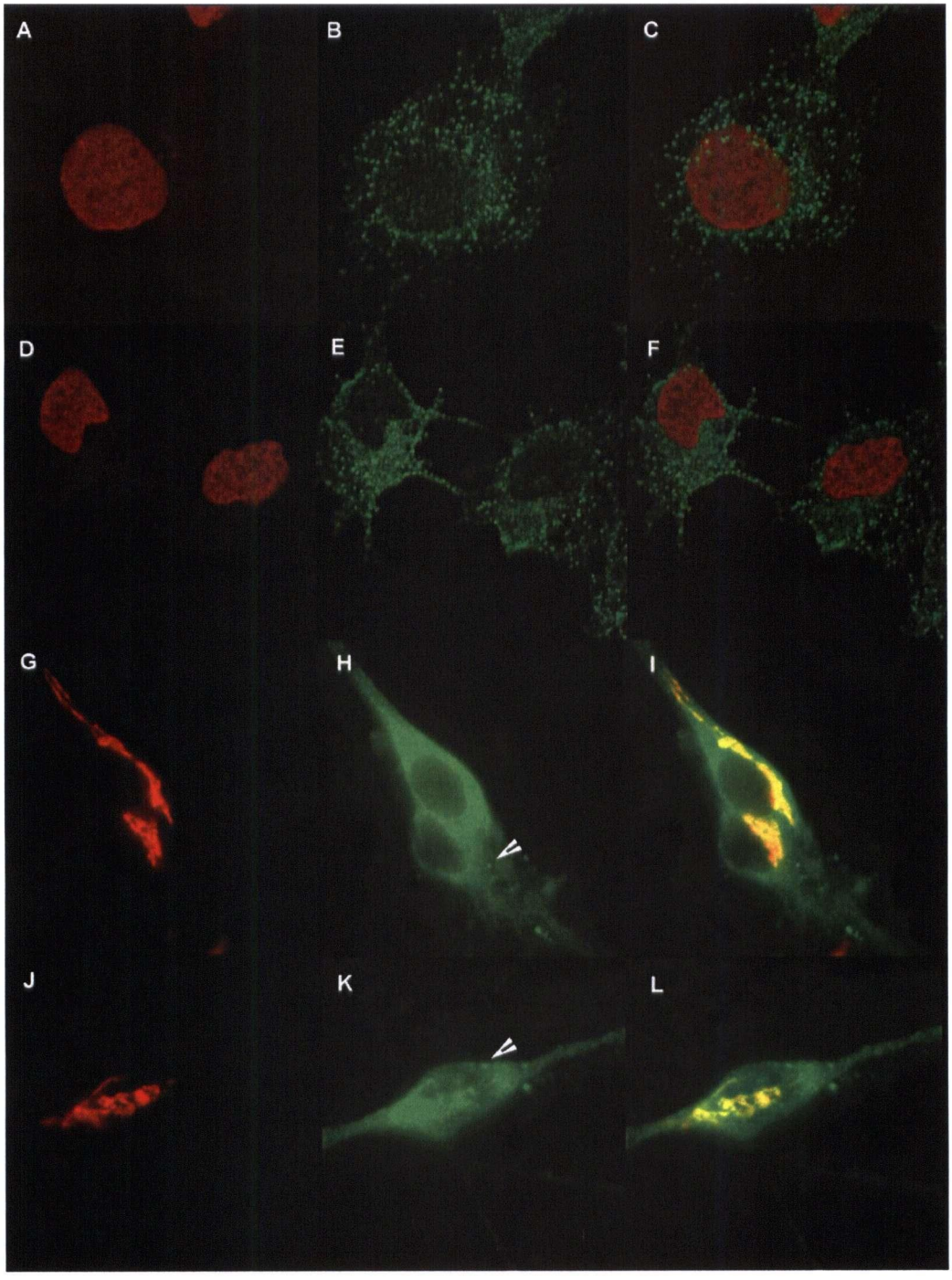
3.2.7 Immunofluorescence in NT2 cells and ES Cell-Derived Neurons.

In order to determine the subcellular localization of HIP1 and HIP12, immunofluorescence studies were undertaken in the neuronal precursor cell line NT2 and functional ES cell-derived neurons (Metzler et al, 1999; figure 3.2.7). These studies were performed in collaboration with Dr. M. Metzler and myself and revealed that HIP1 and HIP12 display a very similar intracellular distribution. In NT2 cells, HIP1 and HIP12 exhibited a non-uniform punctate staining pattern throughout the cell body except the nucleus (figure 3.2.7A-F). Omission of the primary antibodies resulted in no detectable staining within the cells (data not shown). In ES cell-derived neurons, HIP1 and HIP12 were highly both expressed and distributed throughout the cytoplasm and cell processes with enrichment within the *cis*-Golgi (figure 3.2.7 G and L). However, only a few strong punctae were visible (indicated by arrows) at high levels within these cells (figure 3.2.7 H and J).

3.2.8 Yeast 2-Hybrid Interactions of HIP1, HIP12 and Huntingtin

In order to determine if HIP12, like HIP1, can interact with huntingtin, we performed yeast 2-hybrid assays. A 1.5 Kb fragment of HIP12, corresponding to amino acids 514 to 738 was fused in frame, adjacent to both the GAL4-DNA binding domain (BD) of the yeast two-hybrid vector pGBT9 and to the GAL4–activating domain (AD) of the yeast two-hybrid vector pGAD. This 1.5 Kb fragment of HIP12 corresponds to the region in HIP1 that interacts with huntingtin. We determined that unlike HIP1, HIP12 does not interact with huntingtin containing 15 polyglutamine repeats. Furthermore,

Figure 3.2.7. Immunolocalization of HIP1 and HIP12 in NT2 (**A-F**) and ES cell-derived neurons (**G-L**). Expression of HIP1 (**E, green**) and HIP12 (**B, green**) in NT2 cells was visualized by confocal immunofluorescence microscopy using the DNA stain TO-PRO to visualize the nuclei (**A, D, red**). In ES cell-derived neurons HIP1 (**H, green**) and HIP12 (**K, green**) expression was visualized by conventional immunofluorescence microscopy and compared with the cis-golgi marker GM130 (**G, J, red**). Images in the left and middle panel were overlaid and merged electronically and are shown in the right panel (**C, F, I, L**).


A
B
C
D
E
F
G
H
I
J
K
L

HIP12 did not show any interaction with huntingtin containing 128 polyglutamine repeats (data not shown). However, HIP12 can interact with HIP1 and may therefore compete with huntingtin in the binding to HIP1. Moreover, HIP1 can interact with itself indicating that HIP1 may form dimers *in vivo* (figure 3.2.8).

3.2.9 Overexpression of HIP12 in Human Embryonic Kidney (293T) Cells

Several studies have demonstrated that overexpression of HIP1 in cultured cells decreases cell viability (Hackam et al., 1998; Hackam et al, 2000). HIP12 was transiently transfected into HEK 293T cells by R. Singaraja and me to test if overexpression of HIP12 is also toxic (figure 3.2.9A-B). Interestingly, 48 hours post-transfection, cells expressing HIP1 showed a significant decrease in cell viability when compared to cells expressing LacZ whereas overexpression of HIP12 did not influence cell viability ($p > 0.05$, $n=6$). Cells expressing the first 540 amino acids of huntingtin and containing 128 CAG repeats (1955-128) was used as a positive control and displayed reduced cell viability relative to cells expressing lacZ ($p<0.001$, $n=6$).

Caspase-3 activation assays were performed by R. Singaraja and me in order to determine whether the decreased viability of HEK 293T cells overexpressing HIP1 or 1955-128 may be due to the activation of the pro-apoptotic enzyme caspase 3. At 24 hours post-transfection, cells expressing either HIP1 or 1955-128 showed significantly elevated levels of caspase 3 activity when compared to cells expressing LacZ ($p<0.001$, $n=6$) whereas overexpression of HIP12 did not influence caspase 3 activity ($p > 0.05$, $n=6$).

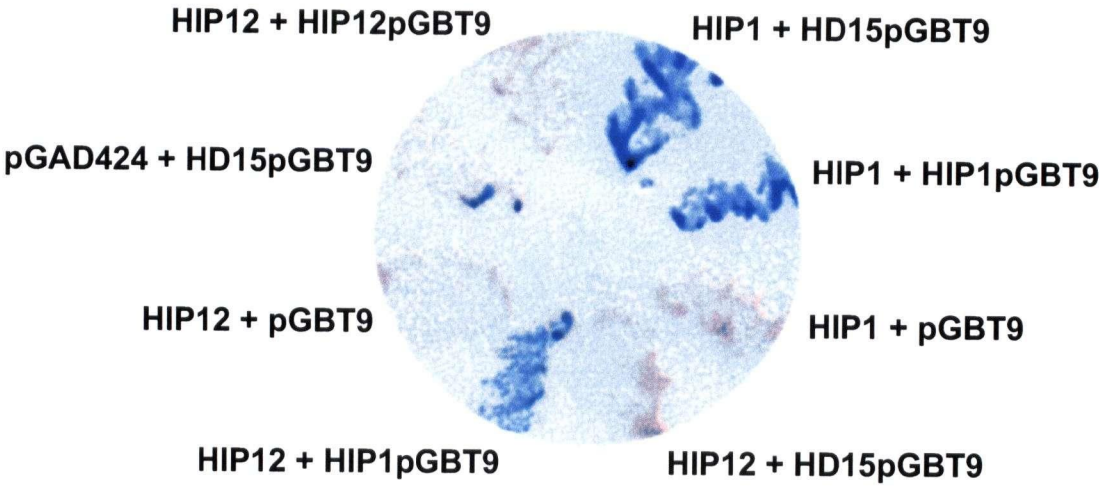


Figure 3.2.8. Yeast 2-Hybrid interactions of HIP1 and HIP12 with various GAL4 DNA-binding domain constructs. Chromogenic β -galactosidase filter assay showing the interaction between HIP1 with the first 540 amino acids of huntingtin with 16 CAG repeats and with HIP1. HIP1 did not interact with GAL4-BD vector alone. The pGBT9-AD vector alone did not interact HIP1 pGAL-BD vector. HIP12 interacted with HIP1 but did not interact with a GAL4-BD vector containing HIP12. HIP12 did not show any interaction with GAL4-BD vector alone. pGBT9-AD vector alone did not interact HIP12 pGAL-BD vector.

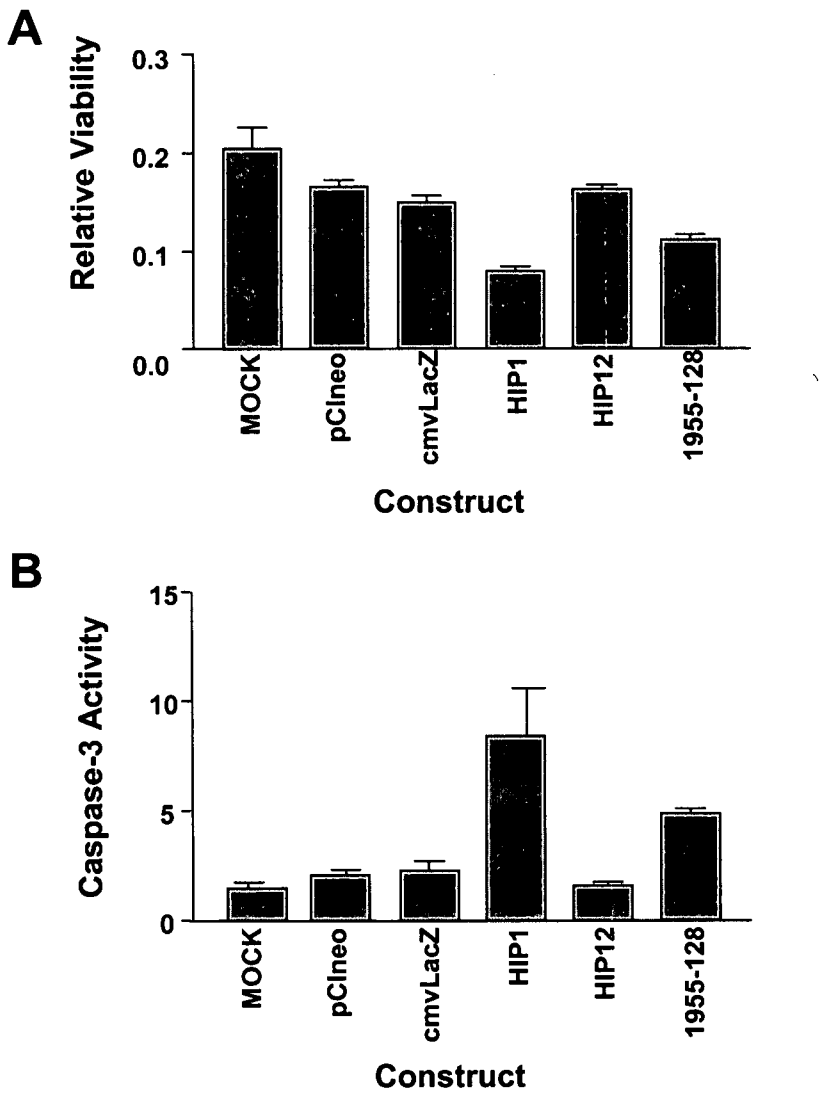


Figure 3.2.9. MTT assays as a measure of cell viability. (A) Transfection and tamoxifen treatment of vector expressing β-galactosidase (LacZ; n=6) served as controls. Expression of huntingtin and HIP1 but not HIP12 constructs results in increased cell death relative to control vector LacZ (n=6). Absorbance values (y-axis) reflect the proportion of viable cells. Increased absorbance is equivalent to increased viability. **Caspase-3 activation assays as a measure of cellular Apoptosis.** (B) Transfection and tamoxifen treatment of vector expressing β-galactosidase (LacZ; n=6) served as controls. Expression of huntingtin and HIP1 but not HIP12 constructs results in increased levels of activated caspase-3 relative to control vector LacZ (n=6). Absorbance values (y-axis) reflect the level of caspase 3 activation. Increased absorbance is equivalent to increased level of apoptosis.

3.3 Discussion

In this study we have demonstrated that HIP1 is a member of a family of cytoskeletal-associated proteins through the isolation of a novel protein termed HIP12. HIP12 is 1068 amino acids in length and shows structural similarity with not only the two HIP1 splice variants, HIP1-1 and HIP1-2, but also Sla2p and ZK370.3. HIP12 shows an overall 47% identity and 64% amino acid conservation with HIP1. The highest degree of sequence similarity between both proteins occurs at the carboxy-terminal region, which shows considerable homology to talin, an actin filament binding protein which is found at focal cell adhesions (Moulder et al, 1996).

HIP1 has been shown to interact with huntingtin in a CAG length-dependent manner, the larger repeat conferring weaker interaction. Thus, it could be expected that in cells expressing mutant huntingtin, there would be more unbound HIP1. This free HIP1 may then initiate biochemical events which may play a role in the pathogenesis of HD. Overexpression of HIP1 in HEK 293T cells results in a significant decrease in cell viability as well as a significant increase in caspase 3 activity. We have shown that transfection of HIP12 into HEK 293T cells does not cause any alteration in cell viability or caspase 3 activity. These findings indicate that HIP12, in contrast to HIP1, is not a proapoptotic protein. Using the yeast 2-hybrid system we demonstrated that HIP1 can interact with huntingtin whereas HIP12 shows no detectable interaction with huntingtin. Interestingly, HIP1 interacts with itself and can interact with HIP12 in the yeast 2-hybrid system. Western blot analyses indicate that HIP1 and HIP12 are both highly expressed in the brain. Furthermore, immunolocalization studies of both proteins in NT2 cells and ES cell-derived neurons demonstrate a similar intracellular distribution indicating that both

proteins may co-localize in specific cellular compartments. Taken together these findings suggest that such interactions may also occur *in vivo* and that HIP12 may be able to modulate the toxic effects of HIP1.

The yeast ortholog of HIP1 and HIP12, Sla2p, was originally characterised as a null mutation causing temperature-sensitive growth defects related to a general disorganization of the membrane cytoskeleton (Holtzman et al., 1993). Two other alleles of Sla2p have been identified based on their mutant phenotype which have defects in endocytosis and regulation of cell surface H+ ATPase (Raths et al., 1993; Na et al., 1995). More recently, Sla2p mutants have been shown to be defective in their ability to transport secretory vesicles from the Golgi apparatus to the plasma membrane (Mulholland et al., 1997; Blader et al., 1999; Bowman and Kahn, 1995). Thus, Sla2p and, by analogy, HIP1 and possibly HIP12 may play a role in the regulation of membrane events through interactions with the underlying cytoskeleton and vesicle trafficking. Recently, Engqvist-Goldstein et al. (1999) have demonstrated that the murine homolog of HIP12 is a component of clathrin-coated pits and vesicles and that it may link the endocytic machinery with the actin cytoskeleton.

Interestingly, other huntingtin-interacting proteins may be involved in vesicle transport and the regulation of membrane events. HAP1 interacts with Duo (Colomer et al., 1997) and the $p150^{Glued}$ subunit of dynactin (Engelender et al., 1997 ; Lippincott-Schwartz, 1998), proteins known to play a role in vesicle trafficking. The huntingtin-interacting protein, SH3GL3, is a member of a novel SH3-containing family of proteins (Sittler et al., 1998). In the central nervous system, certain SH3 containing proteins are involved in vesicle trafficking, signalling at the cell surface and organization of the

cytoskeletal matrices (McPherson et al, 1994; Ringstad et al., 1997).

Huntingtin is membrane associated, co-localizes with microtubules and may also be involved in vesicle trafficking (DiFiglia et al., 1995; Gutekunst et al., 1995). In addition, huntingtin has also been shown to be localized with clathrin in membranes of the *trans*-Golgi network and in clathrin-coated and non-coated endosomal vesicles in the cytoplasm and plasma membrane (Velier et al., 1998). Together, these observations suggest a role for HIP1 and huntingtin as two intimately associated proteins essential for vesicle transport. Thus, the loss of interaction between HIP1 and huntingtin may perturb the organization of the cytoskeleton and vesicle transport, which may ultimately play an important role in the pathogenesis of Huntington disease.

Chapter 4:

HIP1 Interacts with Clathrin and AP2

The data presented in this chapter contributed to the manuscript:

Metzler M, Legendre-Guillemin V, Gan L, Chopra V, Kwok A, McPherson PS, Hayden MR. HIP1 functions in clathrin-mediated endocytosis through binding to clathrin and adaptor protein 2. *J Biol Chem* (2001) 276:39271-39276

My contribution towards obtaining the data within the manuscript shall be discussed in the results section 4.2.

4.1 Introduction

In humans, HIP1 belongs to a family of proteins consisting of HIP1 and HIP12. The HIP1 and HIP12 proteins have a similar molecular weight and contain an amino-terminal ENTH (Epsin N-terminal Homology), a central coiled coil and a carboxy terminal talin-like domain (Chapter 3). The ENTH domain is proposed to induce clathrin-mediated endocytosis through binding to phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2]-containing membranes (Ford et al., 2001; Itoh et al., 2001). Furthermore, ENTH domains are also present in several other endocytic proteins including epsin 1 and 2, AP180 and its homolog, CALM (Ford et al, 2002; Ford et al., 2001; Itoh et al., 2001). The carboxy-terminal domains of the HIP1 and HIP12 proteins share significant sequence similarity to the mammalian membrane cytoskeletal-associated protein, talin (Kalchman et al., 1997). Proteins that bind both PtdIns(4,5)P_2 and clathrin are thought to promote the linkage of clathrin triskelia to the lipid bilayer (Hao et al., 1999). Consistent with this idea is the observation that HIP12 is involved in clathrin mediated endocytosis (CME) and actin cytoskeleton dynamics (Engqvist-Goldstein et al., 1999). For example, HIP12 has been shown to colocalize with markers of receptor endocytosis in mammalian cells including AP2 and clathrin. In addition, HIP12 has also been revealed to be associated with clathrin coated vesicles (CCVs; Engqvist-Goldstein et al., 1999). Finally, HIP12 was also found to colocalize with newly endocytosed vesicles containing labeled transferrin. However, as the transferrin-containing vesicles traveled further into the cytoplasm, their co-localization with HIP12 decreased (Engqvist-Goldstein et al., 1999). Since HIP1 shows significant sequence and biochemical similarities, we hypothesize that HIP1 may also be

involved in CME and actin cytoskeleton dynamics. To address this possibility, we tested whether HIP1 colocalizes with markers of clathrin-mediated endocytosis in neuronal cells and assessed whether HIP1 is enriched on CCVs purified from brain homogenates. In addition, we examined whether HIP1 and HIP12 interact directly with components of receptor mediated endocytosis. The findings in this chapter indicate that HIP1 is a component of the endocytic machinery.

4.2 Results

4.2.1 Co-localization of HIP1 with components of the endocytic machinery.

In order to address whether HIP1 may play a role in clathrin-mediated endocytosis, Dr. M. Metzler and I performed immunofluoresence studies in the neuronal precursor cell line NT2. The NT2 cell line was chosen because HIP1 is highly expressed in the brain (Kalchman et al., 1997; Wanker et al., 1997). Within the NT2 cells, HIP1 displayed a characteristic punctate staining pattern throughout the cytoplasm (figure 4.2.1A). Furthermore, HIP1 co-localized with the clathrin heavy chain (CHC) and the adapter protein AP2 (figure 4.2.1A). In addition, HIP1 partially co-localized with the endocytic vesicle trafficking protein, Rab5 (figure 4.2.1A). Rab5 is a guanosine triphosphatase that has been shown to regulate the formation, transport and fusion of clathrin-coated vesicles with target organelles (Zerial and McBride, 2001). These finding suggest that HIP1 may play an important role at the early stages of clathrin-mediated endocytosis.

To determine the subcellular localization of HIP1, tissue from rat brain was fractionated by differential centrifugation (figure 4.21B). The different cytosolic fractions consisted of the homogenate (H), pellet fraction #1 (P1) containing cell debris and nuclei, pellet fraction #2 (P2) containing the mitochondria and some synaptosomes, supernatant fraction #2 (S2) containing clathrin-coated vesicles and synaptosomes, the supernatant after sucrose gradient centrifugation of the S2 fraction (SGs) containing synaptosomes and membranous debris, and the pellet after sucrose gradient centrifugation of S2 fraction (SGp) containing the purified clathrin-coated vesicles. The clathrin coat was separated

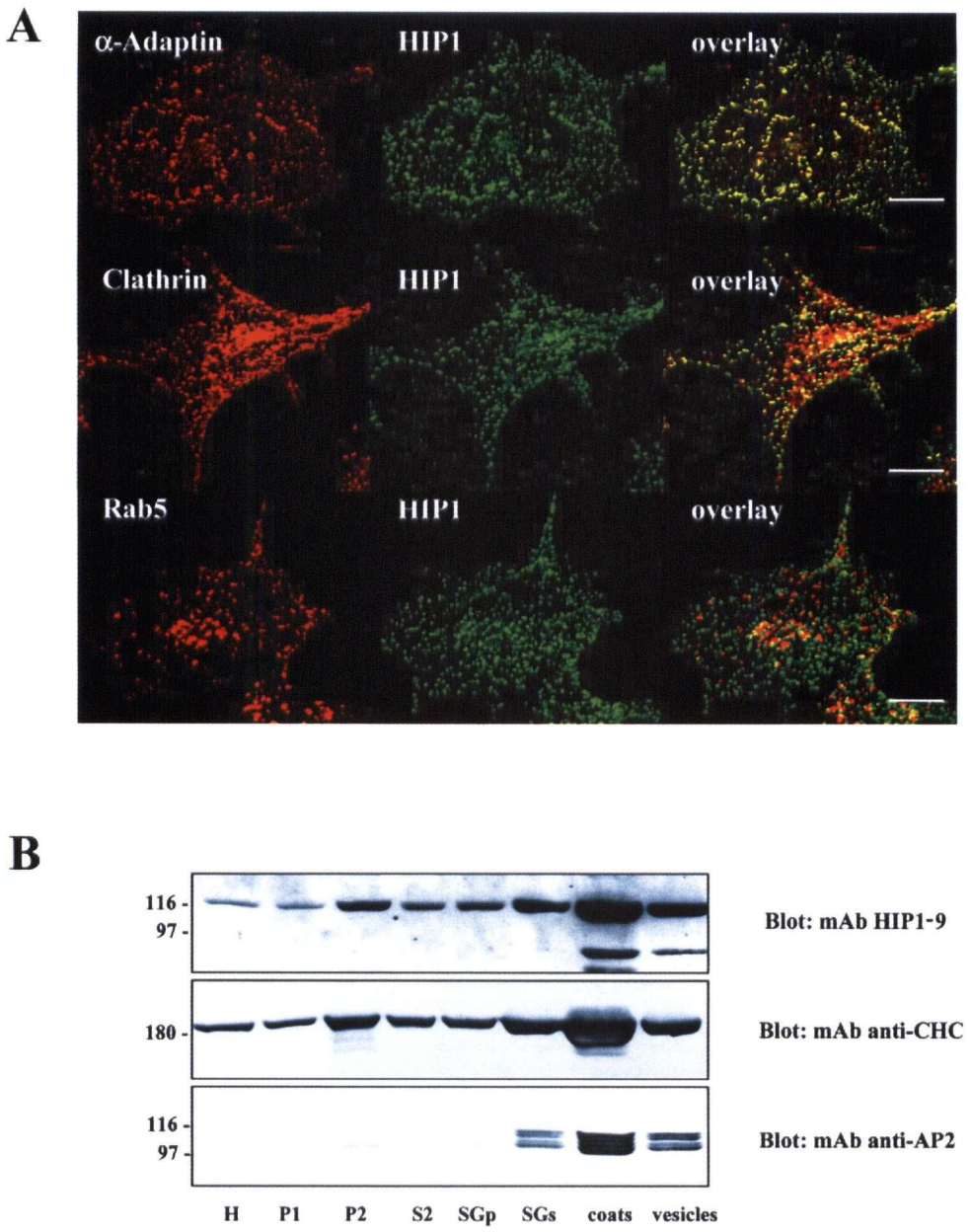


Figure 4.2.1. HIP1 colocalizes with markers of endocytosis and is enriched in CCVs. (A) Localization of HIP1 (green) with α-adaptin, clathrin, and Rab5 (red) was analyzedin neuronal NT2 cells. Overlays of the images are shown in the right panels. Bars, 10 mm. (B) Purification of CCVs was performed by subcellular fractionation of E18 rat brain homogenates. Aliquots of 100 mg of fraction H (homogenate), P1 (pellet 1), P2 (pellet 2), S2 (supernatant 2), SGp (pellet obtainedafter sucrose gradient centrifugation), SGs (supernatant obtained after sucrose gradient centrifugation), coats and vesicles were analyzed on SDS-PAGE and immunoblotted with anti-HIP1, anti-CHC and anti-α-adaptin.

from the vesicle using 0.5M Tris pH 9.0. The proteins in the different cytosolic fractions were separated by SDS-PAGE, transferred to a nylon membrane, and probed with antibodies against HIP1, CHC and the AP2 subunit, α-adaptin. Similar to CHC and α-adaptin, HIP1 was enriched in the SGp (coat and vesicles fraction). These findings indicate that HIP1 is a component of CCVs within the brain.

4.2.2 Identification of HIP1 domains necessary for endocytic targeting.

Five different HIP1 expression constructs were generated by L. Gan and me in order to determine the HIP1 domains that are required for localization to the CCV (figure 4.2.2A). The constructs were: 1) full-length HIP1 construct which expressed the HIP1-2 splice variant (see chapter 3) 2) HIP1-Δcc which expressed a HIP1-2 fragment lacking the coiled coil domain (corresponding to amino acids 336-610) 3) HIP1-ΔN-term which expressed a HIP1-2 fragment lacking the amino-terminal sequences including the ENTH domain (corresponding to amino acids 1-335) 4) HIP1-155-610 which expressed a HIP1-2 fragment lacking the ENTH domain and carboxy terminal sequences including the talin-like domain (corresponding to amino acids 1-155 and 611-1003) 5) HIP1-talin which expressed the talin-like domain of HIP1-2 (corresponding to amino acids 732-1003). Each of the constructs contained a Flag-tag at their respective carboxy-termini. The protein expression from the constructs was verified by Western blot analyses using transfected human embryonic kidney 293T (HEK293T) cell lines (figure 4.2.2B).

To determine which HIP1 constructs interacts with the CHC, the CCVs were isolated from transfected HEK293T cells, transferred to membrane by Western blot and probed with antibodies against HIP1, Flag and CHC. The cells overexpressing full length

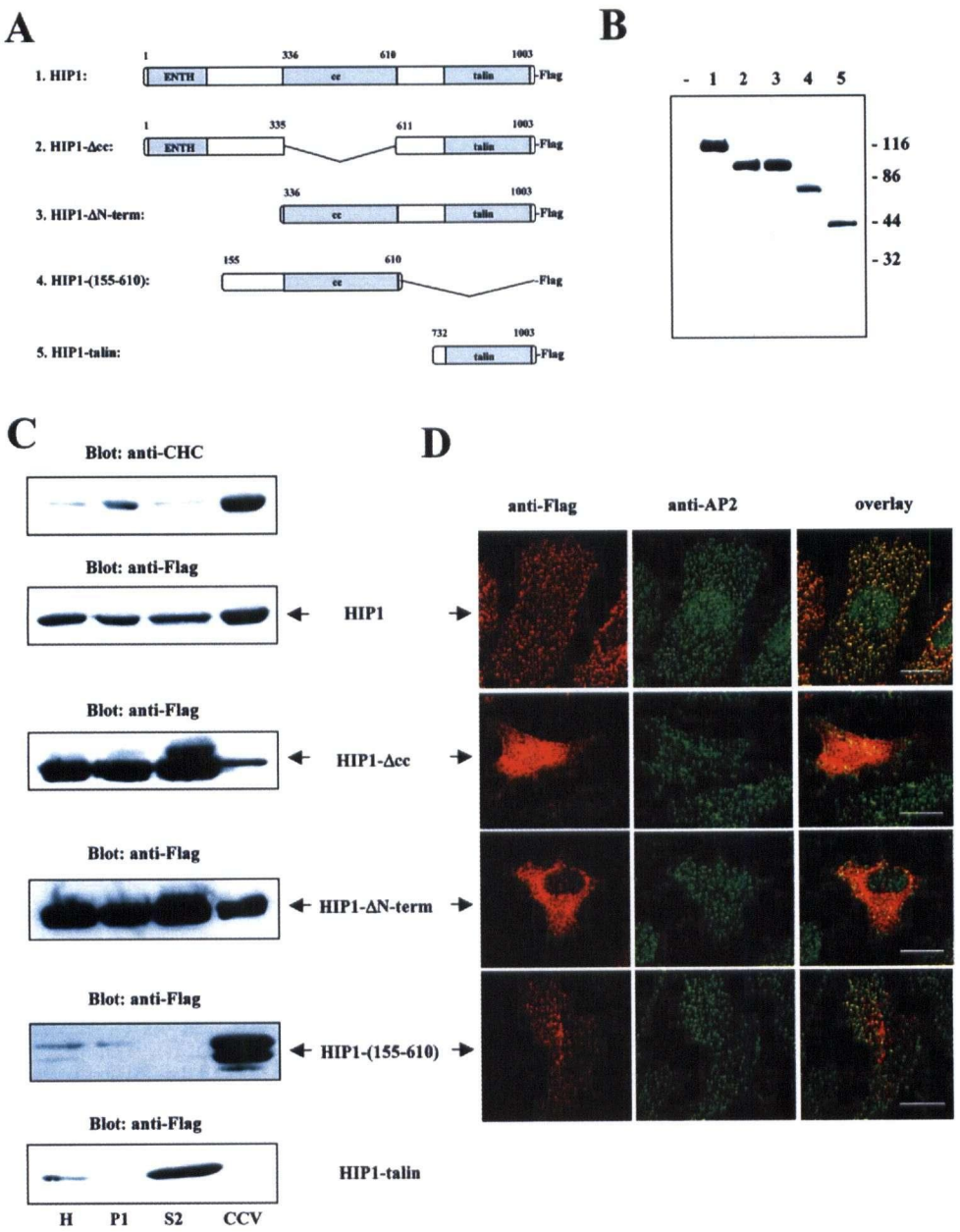


Figure 4.2.2. HIP1 targeting to CCVs. (A) Domain model of full-length HIP1 and HIP1 mutants (amino acid numbersare indicated above the constructs). (B) Expression of each construct (1-5) was analyzed by Western blot and compared to non-transfected cells (-) using an anti-Flag antibody. (C) 293T cells were transfected with various constructs of HIP1 and CCVs were purified as described in Experimental Procedures. Aliquots of various fractions from the purification (100 mg) were analyzed by Western blot with anti-CHC antibody or with anti-Flag antibody. (D) Expression of transfected HIP1 and various HIP1 mutants (red) was visualized using an anti-Flag antibody following transfection in HeLa cells. Expression of AP2 is shown in green. Overlays of the images are shown on the right panels. Bars, 10 μm.

HIP1 did not exhibit as large an enrichment in the CCV as the cell expressing endogenous HIP1 (figure 4.2.2C). Cells overexpressing HIP1 may contain excess amounts of the protein that is incapable of interacting with the CCV and are therefore found in other cellular fractions. The protein expressed by the HIP1-155-610 construct was found to be enriched in the CCV. However, the protein products of the HIP1-Δcc, HIP1-ΔN-term and HIP1-talin constructs were not found to be enriched in the CCV (figure 4.2.2C). These studies demonstrate that sequences between the ENTH and coiled-coil domain are necessary for targeting HIP1 to CCVs.

To assess whether the HIP1 constructs also localized with AP2, transfected HEK 293T cells were examined by immunofluoresence (by Dr. M. Metzler) using antibodies against AP2 and the Flag sequence. The transfected full-length HIP1 displayed extensive colocalization with AP2 (figure 4.2.2D, top panel). However, the proteins expressed by HIP1-Δcc, HIP1-ΔN-term constructs did not colocalize with AP2 (figure 4.2.2D, second and third panel). The protein produced by the HIP1-155-610 construct was exhibit significant co-localization with AP2. Thus, the sequence contained between amino acids 155-610 are required for the colocalization of HIP1 with AP2.

4.2.3 HIP1 interaction with AP2 and clathrin terminal domain.

Sequence analyses demonstrated that HIP1-2 contains the consensus sequence for a clathrin box sequence, LMDMD, at amino acid residues 298-302 (figure 4.2.3A). The clathrin box has been shown to mediate binding to the clathrin terminal domain (Ramjaun and McPherson, 1998; Drake and Traub, 2001). In addition, HIP1 also contained a DPF motif, at amino acid residues 324-326, which is known to interact with AP2 (figure

4.2.3A; Ramjaun and McPherson, 1998; Drake and Traub, 2001). In order to assess whether the clathrin box and DPF motifs in HIP1 can interact with clathrin and AP2, two GST-fusion proteins constructs containing these motifs were generated. The first construct, GST-HIP1-(219-616) encompassed HIP1 sequence containing the clathrin box, DPF motif and the coiled coil domain (figure 4.2.3B, middle panel). The second construct, GST-HIP1-(276-335), encompassed HIP1 sequence containing the clathrin box and DPF motif (figure 4.2.3B, bottom panel). Affinity purification studies demonstrated that the two fusion proteins were both able to bind clathrin and AP2 from rat brain extracts. Interestingly, interaction with clathrin was significantly greater to the protein product of GST-HIP1-(219-616) than to GST-HIP1-(276-335) (figure 4.2.3C, top panel). The stronger interaction of GST-HIP1-(219-616) to clathrin suggests that the coiled coil domain may contain additional site(s) that interact with clathrin. AP2 binding was significantly higher to GST-HIP1-(276-335) than to GST-HIP1-(219-616) (figure 4.2.3C, bottom panel). The different binding affinities suggest that AP2 interaction occurs independently of clathrin binding. Immunoprecipitation studies also showed that both of the fusion proteins were able to bind His_6-tagged clathrin terminal domain (figure 4.2.3C, middle panel). These findings suggest that the interaction between clathrin and HIP1 is direct. Finally, AP2 was immunoprecipitated from HEK 293T overexpressing full-length HIP1 (figure 4.2.3D).

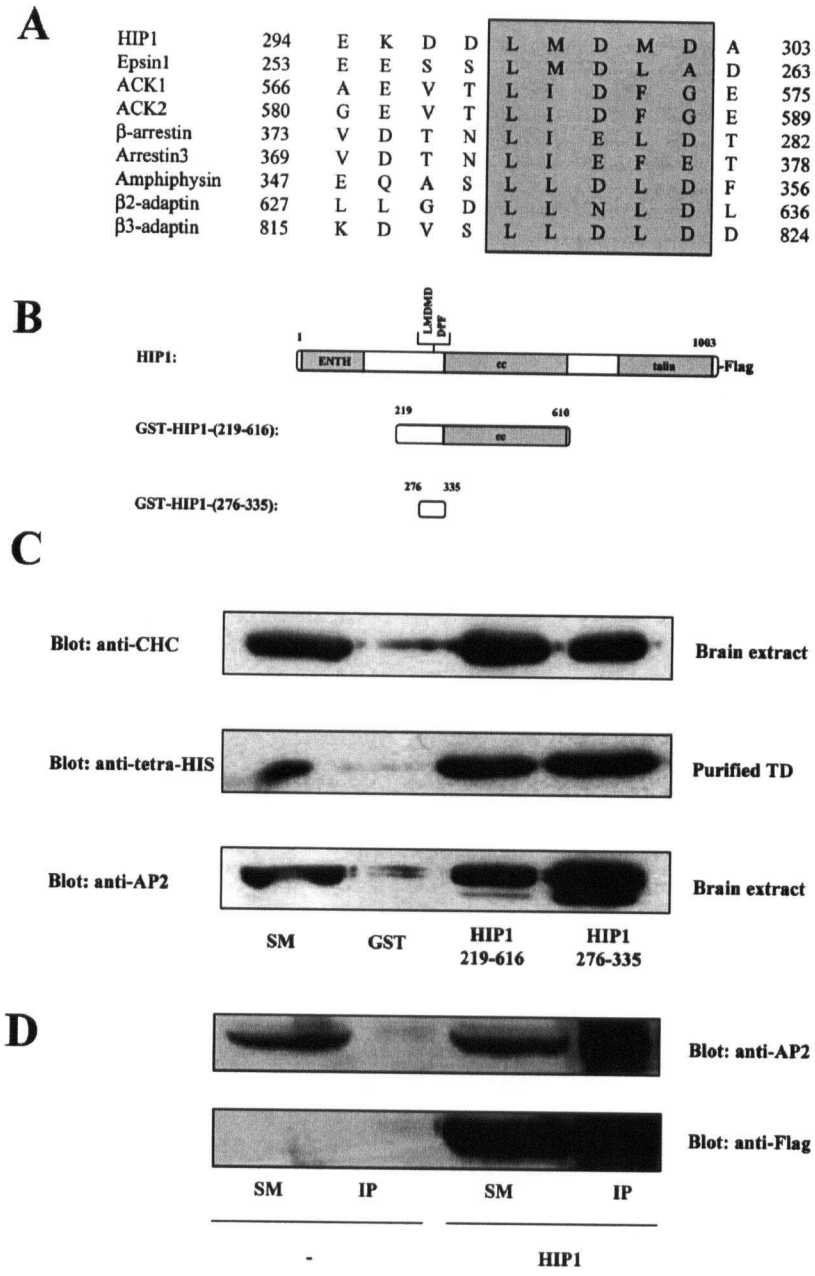


Figure 4.2.3. HIP1 binds clathrin and AP2. (A) Sequence alignment of HIP1 with various clathrin box-containing proteins. (B) Schematic presentation of Flag-tagged full-length HIP1 and sequences present in different HIP1-GST-fusion proteins. (C) Soluble proteins from brain extracts or purified His6-tagged clathrin TD fusion protein were affinity-purified with equal amounts of GST fusion proteins encoding domains of HIP1 bound to glutathione-Sepharose beads. Proteins specifically bound to the beads were analyzed by Western blotting with anti-CHC, anti-tetra-His, and anti-α-adaptin antibodies as indicated. (D) 293T cells were transfected with Flag-tagged full-length HIP1 and processed in parallel with non transfected cells (-). Proteins were extracted and immunoprecipitated with anti-Flag antibody. Immunoprecipitated proteins were analyzed by Western blotting with anti α-adaptin and anti-Flag antibodies. SM refers to starting material.

4.2.4 HIP1 plays a role in clathrin-mediated endocytosis.

In order to determine if HIP1 play a role in endocytosis, transferrin uptake assays were performed by Dr. V Legendre-Guillemin. Four different HIP1 constructs were transfected into COS7 cells in order to assess their effect on transferrin uptake (figure 4.2.4A). The ability of the transfected cells to endocytose transferrin was determined by immunofluorescent microscopy. COS7 cells transfected with constructs expressing either full length HIP1 or the HIP1 coiled coil domain (HIP1-cc) exhibited efficient transferrin endocytosis (figure 4.2.4B, panels *i, ii, v* and *vi*). However, cells transfected with constructs expressing either HIP1-155-610 or HIP1-155-335 displayed a markedly reduced transferrin uptake (figure 4.2.4B, panels *iii, iv, vii* and *viii*). It is possible that the clathrin box and DFP motif present in the HIP1-155-610 and HIP1-155-335 constructs act as inhibitors of transferrin uptake. Figure 4.2.4C represents a graph of the percentage transferrin uptake for the different constructs in six separate experiments. These findings are consistent with previous findings indicating that the HIP1 homolog, HIP12, colocalizes with newly endocytosed vesicles containing labeled transferring (Engqvist-Goldstein et al., 1999).

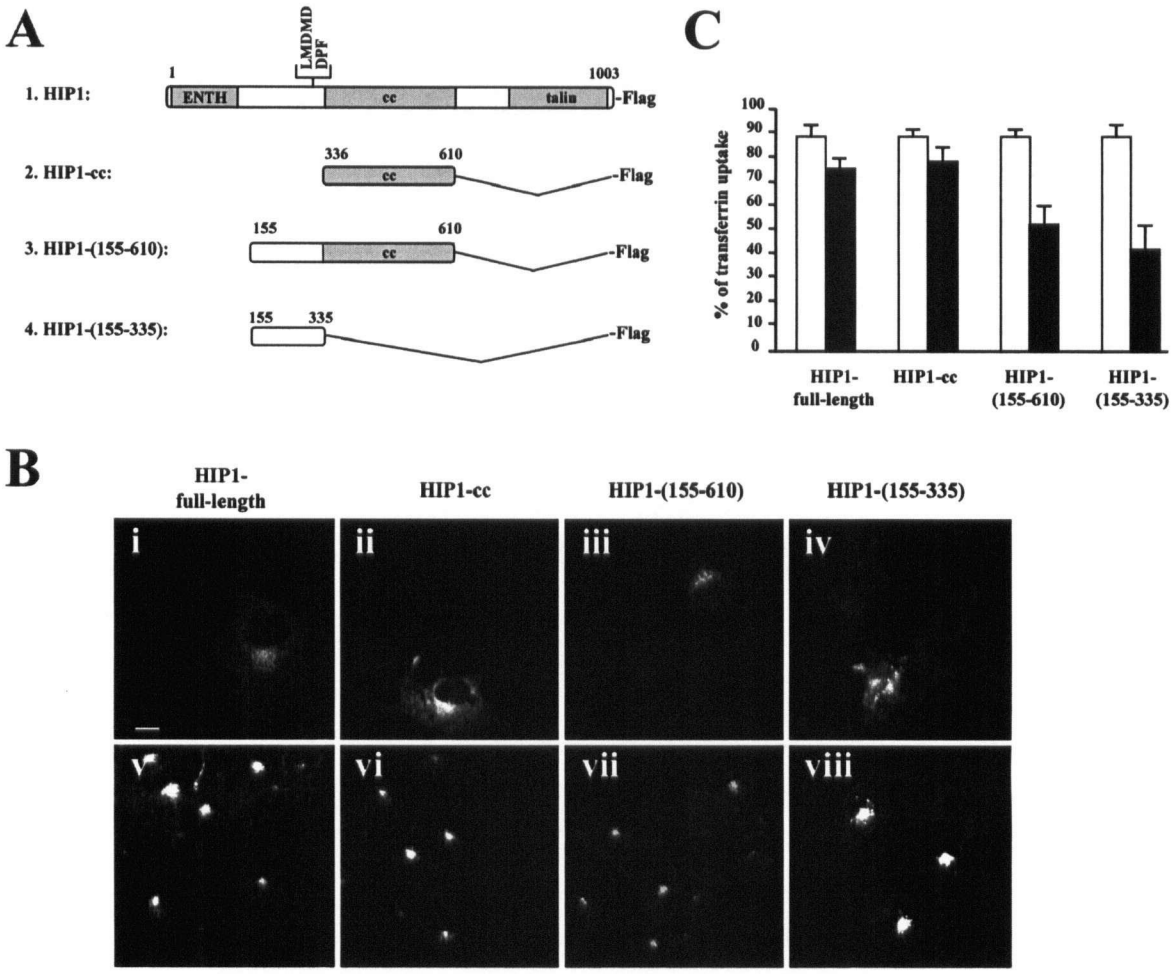


Figure 4.2.4. Transferrin uptake in COS7 cells. (A) Schematic of full-length HIP1 and HIP1 mutants. (B) The uptake of Cy3-labelled transferrin into COS7 cells (v-viii) was analyzed in the presence of various constructs of HIP1. Transfected cells were revealed by immunofluorescence with an anti-Flag antibody (i-iv). Bars, 10 mm. (C) The graph represents the mean ± SEM from 6 separate experiments of the percentage of transferrin uptake for the different constructs. The white and black bars represent non-transfected and transfected cells, respectively.

4.3 Discussion

In this study we have demonstrated that HIP1 is a major component of the clathrin-mediated endocytic machinery. HIP1 was found to interact with CHC via its type 1 clathrin box sequence LMDMD at amino acid position 318 to 322. These findings have been confirmed by site-directed mutagenesis studies performed by Mishra et al. (2001). They demonstrated that the conversion of the HIP1 LMDMD sequence to AAAMD completely abolished CHC binding. The consensus sequence for the type 1 clathrin box is L(L/I)(D/E/N)(L/F)(D/E) (Ramjaun et al., 1998; Drake et al., 2001). Variations of this sequence has been identified in a several endocytic machinery proteins including AP180, amphiphysin, epsins, and β-arrestin (Ford et al., 2001; Slepnev et al., 2000; Drake et al., 2000; Laporte et al., 1999).

In addition, we have shown that HIP1 interacts with AP2 in part through its consensus AP2 binding sequence DPF at amino acid position 324 to 326. Variations of the AP2 binding sequence are present in the endocytic proteins epsin (DPW) and eps15 (DPF) (Mishra et al., 2001). Our findings have been confirmed by binding studies showing that the HIP1 DFP sequence interacts directly with the α-adaptin subunit of AP2 (Mishra et al., 2001; Waelter et al., 2001). Interestingly, Mishra et al (2001) also identified additional AP2 binding motifs FDNKP, FDDIF, FGSSF at amino acids 309 to 321. The consensus sequence FXDXF (where X represents any amino acid) is also present in the endocytic proteins amphiphysin 1 and AP180 (Slepnev et al., 2000; Hao et al.,1999). Examination of our HIP1 expression constructs that bound to AP2 contained the DPF and the FDNKP, FDDIF, FGSSF sequences. Thus, it is possible that both these motifs interact with AP2. The tandem arrangement of the clathrin box and AP2-binding

motifs present in HIP1 has also been previously observed in epsin (Drake et al., 2000). The arrangement of these two binding sites has been postulated to help stabilize the relatively weak interaction between AP2 and clathrin.

We have demonstrated that, similar to the adapter proteins AP2 and AP180, HIP1 was also highly enriched in clathrin-coated vesicles (CCVs) isolated rat brain homogenate. AP2 has been shown to play an important role in the recruitment and polymerization of clathrin subunits which ultimately form clathrin-coated vesicles (Brodsky et al., 200). The brain-specific protein AP180 has been shown to interact with AP2 and clathrin heavy and is postulated to assist in the formation of CCVs (Kay et al., 1999; Ford et al., 2001; Itoh et al., 2001). Furthermore, the carboxy-terminal region of AP180 contains several copies of the amino acid sequence, DLL, which is thought to function in clathrin coat assembly (Slepnev and DeCamilli, 2000). Interestingly, the HIP1-2 central coiled coil domain contains a single DLL motif at amino acid position 450-452. At this time it is unclear whether the DLL motif present in HIP1 plays a direct role in clathrin assembly.

HIP1 was initially identified on the basis of its interaction with huntingtin (Kalchman et al., 1997; Wanker et al., 1997). The strength of the interaction has been shown to be inversely correlated with the length of the polyglutamine tract present in huntingtin. HIP1 and huntingtin have been shown to be highly enriched within the brain and display a similar subcellular distribution (Kalchman et al., 1997; Wanker et al., 1997; Chopra et al., 2000). The present study indicates that HIP1 is a component of the clathrin-mediated endocytic machinery. Similarly, huntingtin has been shown to interact with the endocytic proteins α-adaptin and endophilin A3 (Faber et al., 1998; Sittler et al., 1998). In

addition, huntingtin is also associated with clathrin-coated vesicles and thought to function in vesicle trafficking in the secretory and endocytic pathways (Velier et al., 1998). Taken together, these findings suggest that interaction of huntingtin with HIP1 may play a role in endocytosis. Within the neurons of the brain it may be postulated that these proteins may be involved in synaptic vesicle endocytosis.

Chapter 5:

Differential Binding of HIP1 and HIP12 to Components of the Endocytic Machinery

The data presented in this chapter contributed to the manuscript:

Legendre-Guillemin V, Metzler M, Charbonneau M, Gan L, Chopra V, Philie J, Hayden MR, McPherson PS. HIP1 and HIP12 display differential binding to F-actin, AP2, and clathrin. Identification of a novel interaction with clathrin light chain. *J Biol Chem.* (2002) 277:19897-19904.

My contribution towards obtaining the data within the manuscript shall be discussed in the results section 5.2.

5.1 Introduction

Accumulating evidence suggests that HIP1 and HIP12 both play an important role in endocytosis and regulation of the cortical actin cytoskeleton. As mentioned previously, both these proteins contain an ENTH (epsin N-terminal homology) domain which can bind to phosphatidylinositol 4,5-bisphosphate-containing membranes and thought to induce clathrin-mediated endocytosis (Kay et al., 1999; Ford et al., 2001; Itoh et al., 2001; DeCamilli et al., 2002). The central portion of these proteins contains a coiled-coil domain which is thought to mediate protein-protein interactions. Finally, these proteins contain a C-terminal talin-homology domain that has been shown to bind F-actin (Engqvist-Goldstein et al., 1999; McCann and Craig, 1997). These findings suggest that HIP1 and HIP12 may play a role in linking membrane attachment and clathrin-coated vesicle (CCVs) formation with actin dynamics (Engqvist-Goldstein et al., 1999; Bennet et al., 2001).

Recent studies have shown that both HIP1 and HIP12 are enriched at discrete cytoplasmic membrane sites that are undergoing clathrin-mediated endocytosis. Furthermore, HIP1 and HIP12 co-localize with markers of endocytosis including clathrin, AP2 and Rab5 (Engqvist-Goldstein et al 1999, Metzler et al., 2001; Mishra et al., 2001; Waelter et al., 2001). The HIP1 protein contains a type 1 clathrin box motif (LMDMD) which is thought to mediate the direct interaction of HIP1 with clathrin heavy chain (CHC; Chapter 4, section 4.2.3; Metzler et al., 2001; Mishra et al., 2001; Waelter et al., 2001). In addition, HIP1 also possesses the consensus AP2 binding sequences FDNKP, FDDIF, FGSSF and DPF which have been shown to interact with the AP2 subunits α-

adaptin A and C (Chapter 4, section 4.2.3; Metzler et al., 2001; Mishra et al., 2001; Waelter et al., 2001). The previous studies mentioned have not confirmed whether HIP12 can also interact directly with clathrin or AP2. However, sequence analyses have demonstrated HIP12 also contains the clathrin box motif (LIEIS) but does not contain any AP2 binding sites (Mishra et al.; 2001).

In this chapter the protein interaction and functional properties of HIP1 and HIP12 were analyzed in order to elucidate their roles in clathrin-mediated endocytosis. We demonstrated that HIP1 strongly interacts with clathrin and AP2 but does not interact with F-actin. However, relative to HIP1, HIP12 displayed weaker affinity for clathrin and AP2 but did interact with F-actin. In addition, HIP1 and HIP12 were both shown to bind directly to the clathrin light chain (CLC) through their central helical domain. These results suggest related but distinct functions for HIP1 and HIP12 in clathrin-mediated endocytosis.

5.2 Results

5.2.1 Comparison of HIP1 and HIP12 protein expression

5.2.1.1 HIP1 and HIP12 protein expression in adult and embryonic rat brain

To further characterize the functions of HIP1 and HIP12, their developmental expression profiles were assessed in adult and embryonic rat brain. These studies were performed in collaboration with Dr V. Legendre-Guillemin. Western blot analyses indicated that HIP1 was highly expressed at embryonic day 13 (E13) and E16 (figure 5.2.1A, top panel). However, HIP12 protein levels were highest in post-natal day 2 and adult rat brain but relatively low in all the embryonic days examined (figure 5.2.1A, bottom panel). Thus, HIP1 is expressed earlier in rat brain development than HIP12.

5.2.1.1 Subcellular fractionation studies.

To compare the subcellular localization of HIP1 and HIP12, tissue from rat brain was fractionated by differential centrifugation (figure 5.2.2B). The different cytosolic fractions consisted of the homogenate (H), pellet fraction #1 (P1) which contained cell debris and nuclei, pellet fraction #2 (P2) containing the mitochondria and some synaptosomes, supernatant fraction #2 (S2) containing clathrin-coated vesicles and synaptosomes, the supernatant after sucrose gradient centrifugation of the S2 fraction (SGs) containing synaptosomes and membranous debris, and the pellet after sucrose gradient centrifugation of S2 fraction (SGp) containing the purified clathrin-coated vesicles. The clathrin coat was separated from the vesicle using 0.5M Tris pH9.0. The proteins in the different cytosolic fractions were separated by SDS-PAGE, transferred to a nylon membrane, and probed with antibodies against HIP1, HIP12, CHC and the AP2

subunit, α-adaptin. HIP1 and HIP12 were both found to be enriched in the clathrin-coated vesicle fractions (CCV; figure 5.2.1B, top two panels). As expected , CHC and the clathrin associated protein subunit, α-adaptin, were also present in the CCV fractions (figure 5.2.1B, bottom 2 panels).

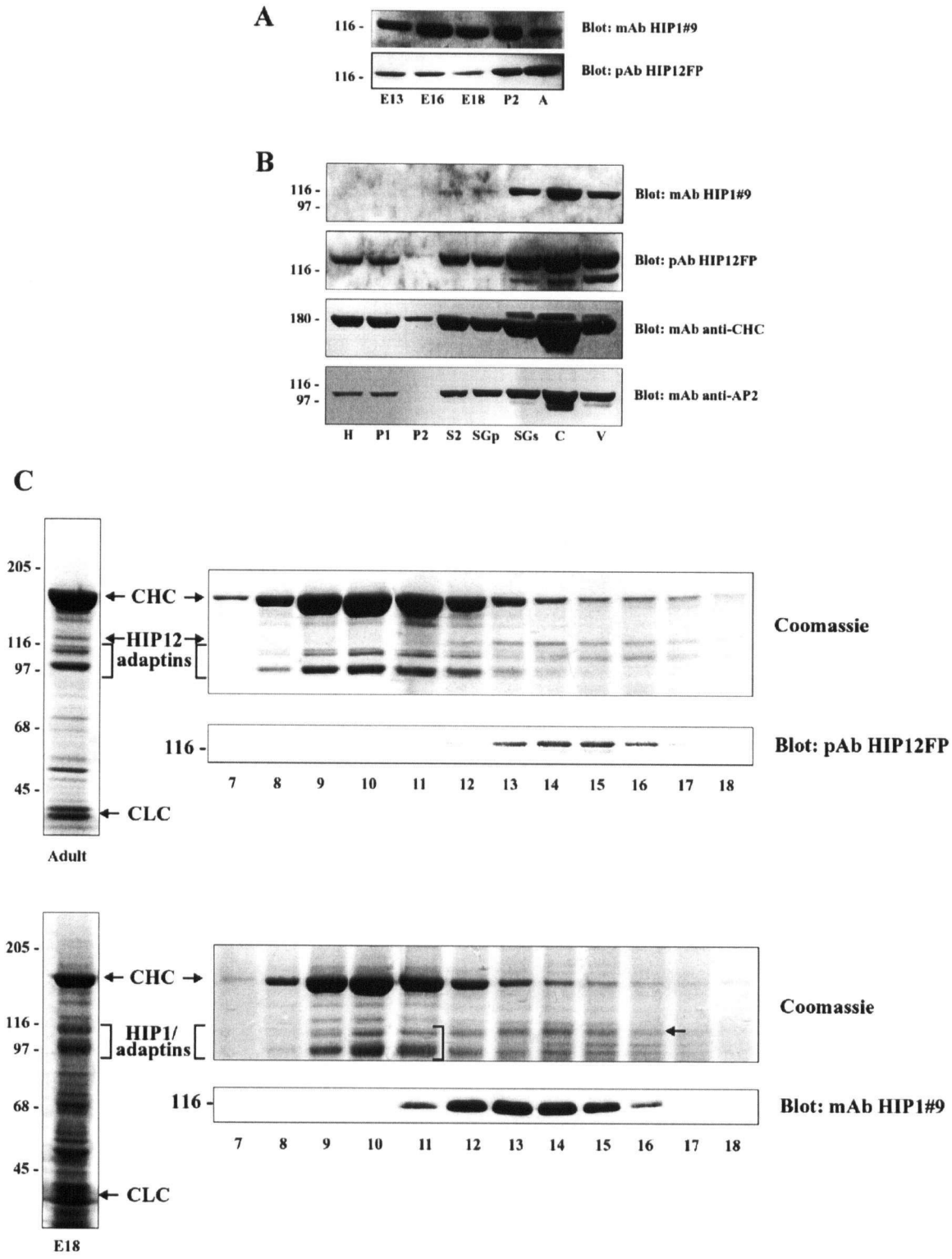
The clathrin coat proteins were separated, from the embryonic day 18 tissue (E18) and adult rat brain CCVs using 0.5M Tris pH 9.0 and fractionated by linear 5-20% sucrose gradients (figure 5.2.1C, bottom 2 panels). The proteins in the different cytosolic fractions were separated by SDS-PAGE, and either stained using Coomassie Blue or transferred to a nylon membrane, and probed with antibodies against HIP1 or HIP12. Coomassie Blue staining indicated that HIP1 and HIP12 were present on both E18 and adult rat brain CCVs.

5.2.2 Interaction of HIP1 with HIP12

5.2.2.1 Co-immunoprecipitation

As mentioned previously, yeast two-hybrid studies have demonstrated that fragments of HIP1 and HIP12 can interact and form heterodimers. Furthermore, the fragments of HIP1 and HIP12 which mediate interaction both contain their central coiled-coil domains (Chapter 3, Chopra et al., 2000). To confirm that the full-length HIP1 and HIP12 proteins interact, co-immunoprecipitation studies were performed in HEK 293T cells using the monoclonal HIP1 antibody, HIP1 #9. The immunoprecipitation of endogenous HIP1 led to co-immunoprecipitation of HIP12 (figure 5.2.2A). Furthermore, transfection of HEK 293T cells with HIP12 cDNA resulted in a greater amounts of HIP12 co-immnopreciptated by the endogenous HIP1 (figure 5.2.2A). Negative controls

Figure 5.2.1. HIP1 and HIP12 are coat proteins of CCVs and display distinct developmental expression profiles. *A,* post-nuclear supernatants (100 μg) from brain extracts of various rat developmental stages were analyzed by Western blot with anti-HIP1 or anti-HIP12 antibodies as indicated. *E*, embryonic day; *P*, post-natal day; *A*, adult. *B*, purified CCVs were obtained by subcellular fractionation of adult rat brain homogenates. Aliquots (100 μg) of the various fractions were analyzed by Western blot with antibodies against clathrin heavy chain (*CHC*), the α-adaptin subunit of AP2 (AP2), HIP1 and HIP12, as indicated. *H*, homogenate; *P*, pellet; *S*, supernatant; *SGp*, sucrose gradient pellet; *SGs*, sucrose gradient supernatant; *C*, coats; *V*, vesicles. The coats and vesicles correspond to the supernatant and pellet fractions, respectively, following incubation of the purified CCVs with 0.5 M Tris, pH 9.0. *C*, purified CCVs from adult and E18 brains were stripped of their coats in 0.5 M Tris, pH 7.0, 2 mM EDTA. Aliquots of the coat fractions were analyzed on SDS-PAGE and Coomassie Blue staining (*panels at left*). The coat proteins were then fractionated on continuous 5-20% sucrose gradients. The gradients were collected in 20 equal fractions and aliquots from fractions 7-18 were analyzed by SDS-PAGE with Coomassie Blue staining (*Coomassie*). The migration of HIP1 and HIP12 was determined by Western blot as indicated. The *bracket* on the E18 gradient demonstrates the migratory positions of the large subunits of the adaptins, whereas the *arrow* indicates the migratory position of HIP1.


A
116 -
116 -
Blot: mAb HIP1#9
Blot: pAb HIP12FP
E13 E16 E18 P2 A
B
116 -
97 -
116 -
180 -
116 -
97 -
Blot: mAb HIP1#9
Blot: pAb HIP12FP
Blot: mAb anti-CHC
Blot: mAb anti-AP2
H P1 P2 S2 SGp SGs C V
C
205 -
116 -
97 -
68 -
45 -
CHC
HIP12
adaptins
CLC
Adult
Coomassie
116 -
Blot: pAb HIP12FP
7 8 9 10 11 12 13 14 15 16 17 18
205 -
116 -
97 -
68 -
45 -
CHC
HIP1/
adaptins
CLC
E18
Coomassie
116 -
Blot: mAb HIP1#9
7 8 9 10 11 12 13 14 15 16 17 18

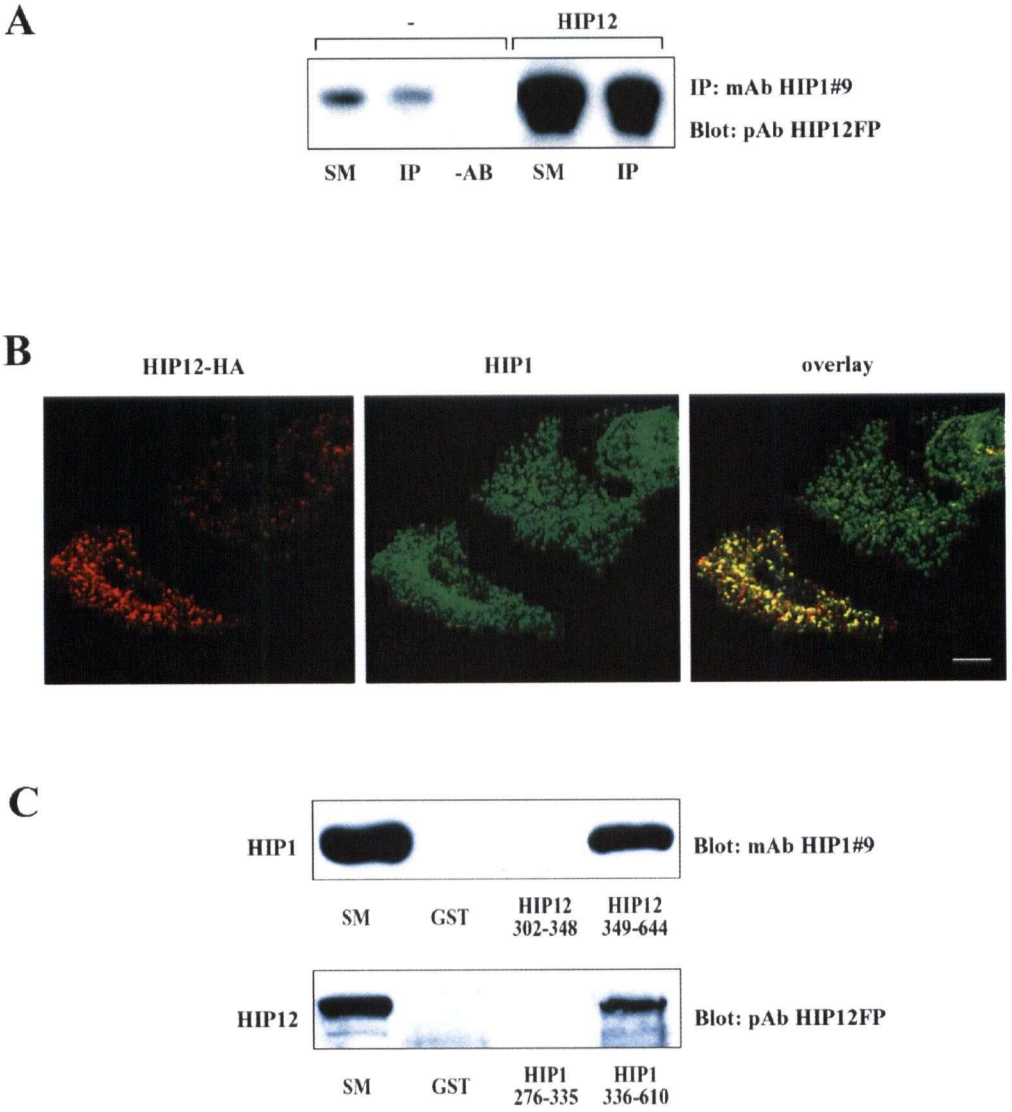


Fig. 5.2.2. HIP1 and HIP12 interact via their helical domains. A, HEK 293T cells were transfected with full-length HIP12 cDNA (HIP12) or were left untransfected ( ). Cell lysates were prepared and immunoprecipitations were carried out in the presence or absence (AB) of mouse mAb HIP1#9. Cell lysate (SM) and immunoprecipitated protein (IP) were analyzed by SDS-PAGE and Western blot with anti-HIP12FP antibody. B, co-localization of transfected HIP12-HA (red) and endogenous HIP1 (green) was analyzed in neuronal NT2 cells. Overlays of the images are shown in the panel on the right (overlay). Scale bar = 10 μM. C, HeLa cells were transfected with either HIP1 or HIP12 full-length cDNA as indicated. Cell extracts were prepared and tested for binding to GST alone or various HIP12 and HIP1.

in which no HIP1 #9 was added resulted in a lack of immunoprecipiptation of HIP12 (figure 5.2.2A).

5.2.2.2 Confocal microscopy

To assess the intracellular locations where HIP1 and HIP12 may interact, confocal microscopy studies were performed in collaboration with Dr M. Metzler. An hemagglutinin-tagged HIP12 (HIP12-HA) expression construct was generated and transfected into the neuronal cell line NT2. The intracellular localization of HIP12-HA and endogenous HIP1 was determined using a monoclonal antibody against hemaagglutinin and a polyclonal antibody against HIP1. Within the NT2 cells, HIP12-HA exhibited punctate staining throughout the cell and displayed significant co-localization with HIP1 (figure 5.2.3B). The HIP1 and HIP12 staining pattern was similar to the distribution of clathrin-coated pits and vesicles

5.2.2.3 Affinity purifications using deletion constructs

To confirm whether the coiled-coil domains of HIP1 and HIP12 are required for their interaction, pull-down assays were performed using HIP1 and HIP12 deletion constructs. Four different deletion constructs were generated by L. Gan and I and pull-down assays were performed by Dr V. Legendre-Guillemin. The constructs were: 1) HIP1 336-610 which expressed an HIP1 fragment containing the central coiled coil domain (corresponding to amino acids 336-610) 2) HIP1 276-335 which expressed an HIP1 fragment expressing the sequences between the coiled coil and ENTH domains (corresponding to amino acids 276-335) 3) HIP12 349-644 which expressed an HIP12

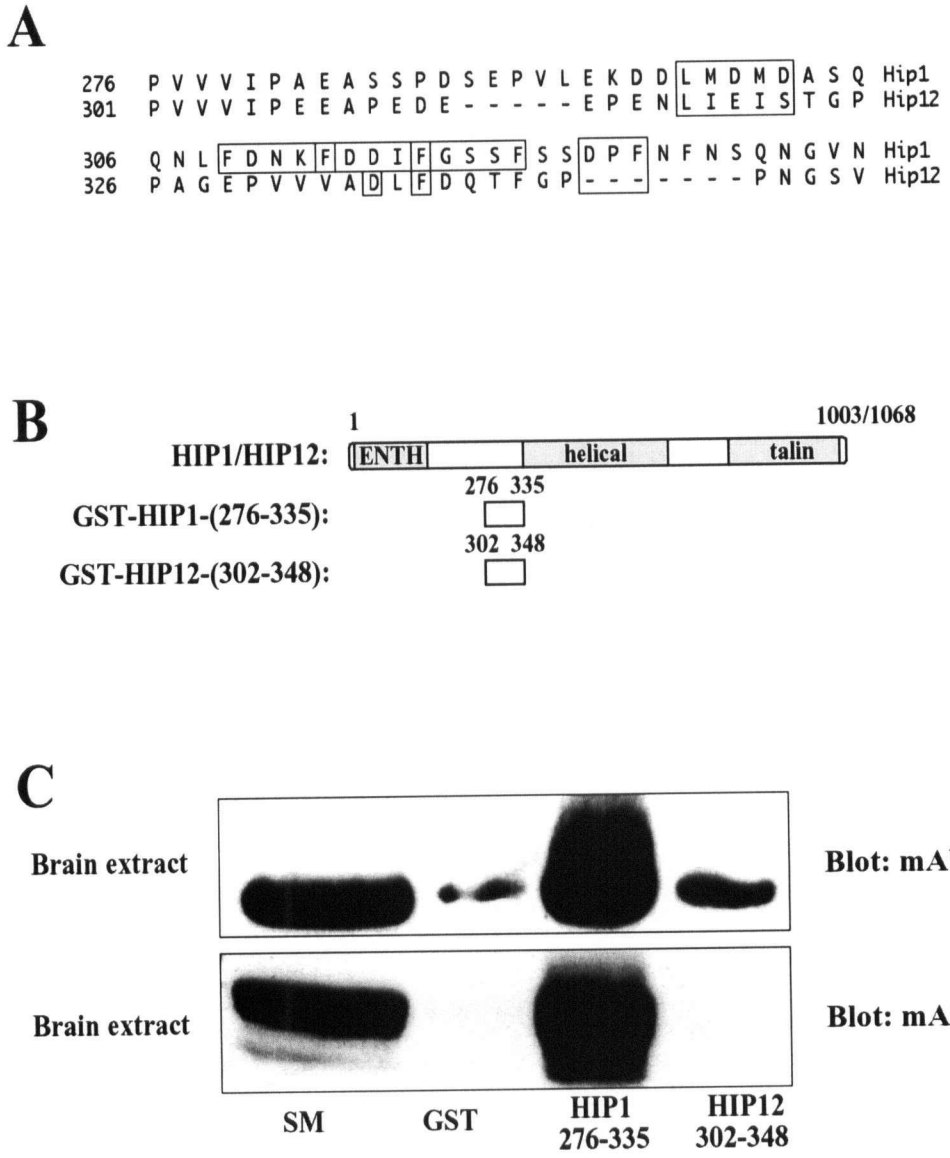


Fig. 5.2.3. HIP1 and HIP12 display differential binding to clathrin and AP2. A, sequence alignment of a region of HIP1 and HIP12 demonstrating consensus clathrin and AP2-binding sites. B, schematic representation of various GST fusion proteins used for the binding assays. C, soluble proteins from brain extracts were affinity purified with equal amounts of HIP1 or HIP12-GST fusion proteins bound to glutathione-Sepharose beads. Proteins specifically bound to the beads were analyzed by Western blot with antibodies against CHC or the α-adaptin subunit of AP2 (AP2) as indicated.

fragment containing the central coiled coil domain (corresponding to amino acids 349-644) 4) HIP12 302-348 which expressed an HIP12 fragment expressing the sequences between the coiled coil and ENTH domains (corresponding to amino acids 302-348) Each of the proteins contained a glutathione s transferase-tag (GST-tag) at their respective carboxy-termini. It was determined that HIP1 336-610 and HIP12 349-644 were able to specifically "pull-down" HIP12 and HIP1 respectively (figure 5.2.3C). Thus, the coiled-coil domains are necessary for HIP1-HIP12 interaction.

5.2.3 Differential binding of HIP1 and HIP12 to Clathrin

HIP1 has previously been shown to contain the consensus sequence for a clathrin box sequence, LMDMD, at amino acid residues 298-302 (Metzler et al., 2001; Chapter 4, section 4.2.3). In addition, HIP1 also possesses the consensus AP2 binding sequences FDNKP, FDDIF, FGSSF (at amino acids 309 to 321) and DPF (at amino acids 324 to 326) (Mishra et al., 2001; Metzler et al., 2001; Chapter 4, section 4.2.3). Sequence analyses by Mishra et al. (2001) have shown that HIP12 also contains the clathrin box sequence LIEIS (at amino acids 318-322) (figure 5.2.3A). The HIP12 protein sequence did not contain adjacent AP2 binding motifs. To determine whether the clathrin box and AP2 binding motifs present in HIP1 and HIP12 can interact with clathrin and AP2, two deletion constructs were generated by me. The deletion constructs were: 1) HIP1 276-335 which contained the HIP1 clathrin box and its adjacent AP2 binding sequences (corresponding to amino acids 276-335) 2) HIP12 302-348, which contains the HIP12 clathrin box sequence. These studies were performed in collaboration with Dr. V.

Legendre-Guillemin. The HIP1 276-335 and HIP12 302-348 fusion proteins were bound to glutathione-sepharose, and incubated with rat brain material and washed several times. The bound proteins were eluted from the columns, separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and probed with antibodies against clathrin and AP2. The HIP1 276-335 fusion protein was found to bind strongly to both CHC and AP2 (figure 5.2.3C, top panel). However, HIP12 302-348 protein displayed relatively weak clathrin binding and no AP2 binding (figure 5.2.5C, bottom panel).

5.2.4 Differential binding of HIP1 and HIP12 to F-actin

5.2.4.1 Co-sedimentation studies

As mentioned previously, the talin-like domain in HIP12 has been shown to interact with F-actin (Chapter 1; Engqvist-Goldstein et al., 1999). Since the HIP1 talin-like domain displayed high sequence identity to that of HIP12, co-sedimentation studies were performed in order to determine whether the HIP1 talin-like domain also interacts with F-actin. To perform these studies two different constructs were generated by L. Gan and me. The constructs were: 1) GST-HIP1-talin which expressed the HIP1-2 talin-like domain (corresponding to amino acids 731-1003) 2) GST-HIP12-talin which expressed an HIP12 fragment expressing its talin-like domain (corresponding to amino acids 765-1068). Both of these proteins contained a GST-tag fused to their respective carboxy-termini. As previously reported, a substantial proportion of GST-HIP12-talin, 87.6% $\pm$6.0 (mean $\pm$S.E., n=3), bound to F-actin (figure 5.2.4A). Unexpectedly, a significantly reduced amount of GST-HIP1-talin bound to the F-action 19.3% $\pm$3.7 (mean $\pm$S.E., n=3)

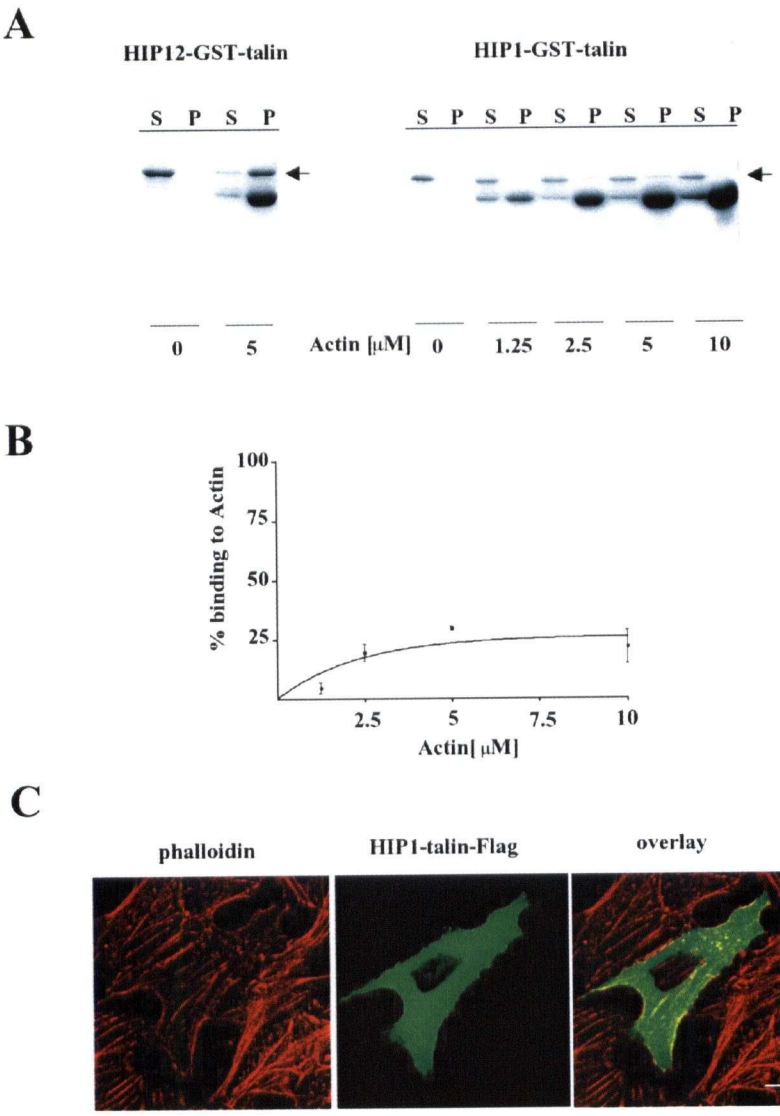


Fig. 5.2.4. HIP1 does not bind actin in contrast to HIP12. A, binding of the HIP1-talin homology domain expressed as a GST fusion protein (HIP1-talin) to filamentous actin was analyzed by cosedimentation in the presence of various concentrations of assembled actin (0-10 μM). Binding of the HIP12-talin homology domain GST fusion protein (HIP12-talin) was used as positive control. The arrows indicate HIP1 or HIP12 fusion proteins in the supernatant (S) and pellet (P) fractions. B, the percentage of GST-HIP1-talin bound to actin is shown as mean ± S.E. of three independent experiments. C, HeLa cells expressing a FLAG-tagged construct encoding the HIP1-talin homology domain (HIP1-talin-Flag) were immunostained with an anti-FLAG antibody shown in green and with Texas Red-phalloidin shown in red to reveal F-actin. Overlays of the confocal images are shown in the right. Scale bar = 10 μM.

(figure 5.2.4B). These findings were consistent when tested under different parameters including pH and ion concentration (data not shown).

5.2.4.2 Confocal microscopy

To confirm that HIP1 does not interact with F-actin, GST-HIP1-talin was expressed in HELA cells and examined using confocal microscopy. Using the F-actin stain, phalloidin, and an antibody against GST, it was determined that GST-HIP1-talin does not co-localize with the cortical actin cytoskeleton (figure 5.2.4C). These findings are in constrast to previous findings clearly demonstrating that the talin-like domain in HIP12 can interact with F-actin (Chapter 1; Engqvist-Goldstein et al., 1999).

5.2.5 The Coiled-Coil Domains of HIP1 and HIP12 Bind to Clathrin

It has previously been demonstrated that the HIP1 clathrin box can interact with clathrin heavy chain (CHC; Metzler et al., 2001; Chapter 4 section 4.2.3). Furthermore, the adjacent coiled coil domain seems to enhance HIP interaction with CHC (CHC; Metzler et al., 2001; Chapter 4 section 4.2.3). To determine whether HIP1 (and potentially HIP12) contain clathrin binding domains within their respective coiled coil domains, affinity purification studies were performed using HIP1 and HIP12 expression constructs. The constructs used were: 1) GST-HIP1-(336-610) which expressed the HIP1-2 coiled coil domain (corresponding to amino acids 336-610) 2) GST-HIP12-(348-644) which expressed an HIP12 fragment expressing its talin-like domain (corresponding to amino acids 348-644). Both of these proteins contained a GST-tag fused to their respective carboxy-termini. The HIP1-(336-610) and HIP12-(348-644) fusion proteins

were bound to glutathione-sepharose, and incubated with rat brain material and washed several times. The specifically bound proteins were eluted from the columns, separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and probed with antibodies against clathrin and AP2. The HIP1 and HIP12 fusion proteins were both capable of interacting with clathrin but not to AP2 (figure 5.2.5A-B). Thus, the coiled coil domains present in both HIP1 and HIP12 contain sequence which mediate interaction with CHC.

5.2.6 Clathrin Coat Assembly using the Coiled-Coil Domains of HIP1 and HIP12

Sequence analyses have demonstrated the presence of DLL motifs within the coiled coil domains of both HIP1 and HIP12. The DLL motifs are present on a several adapter proteins (including AP2 and AP180) and are required for clathrin cage formation (Morgan et al., 2000). Therefore, it is possible that HIP1 and/or HIP12 may promote clathrin coat assembly. This suggestion is supported by studies which demonstrated that full-length HIP12 can induce the formation of clathrin lattice structures (Engqvist et al., 2001). The fusion protein produced by the constructs GST-HIP1-(336-610) and GST-HIP12-(348-644) were used to determine whether the coiled coil domain of HIP1 and HIP12 could stimulate clathrin assembly *in vitro*. These studies were performed in collaboration with Dr V. Legendre-Guillemin. As expected, the negative control, GST protein, was unable to induce clathrin lattice formation *in vitro*. The GST-HIP1-(336-610) and GST-HIP12-(348-644) protein products were both capable of stimulating clathrin assembly in a protein concentration-dependent manner (figure 5.2.6).

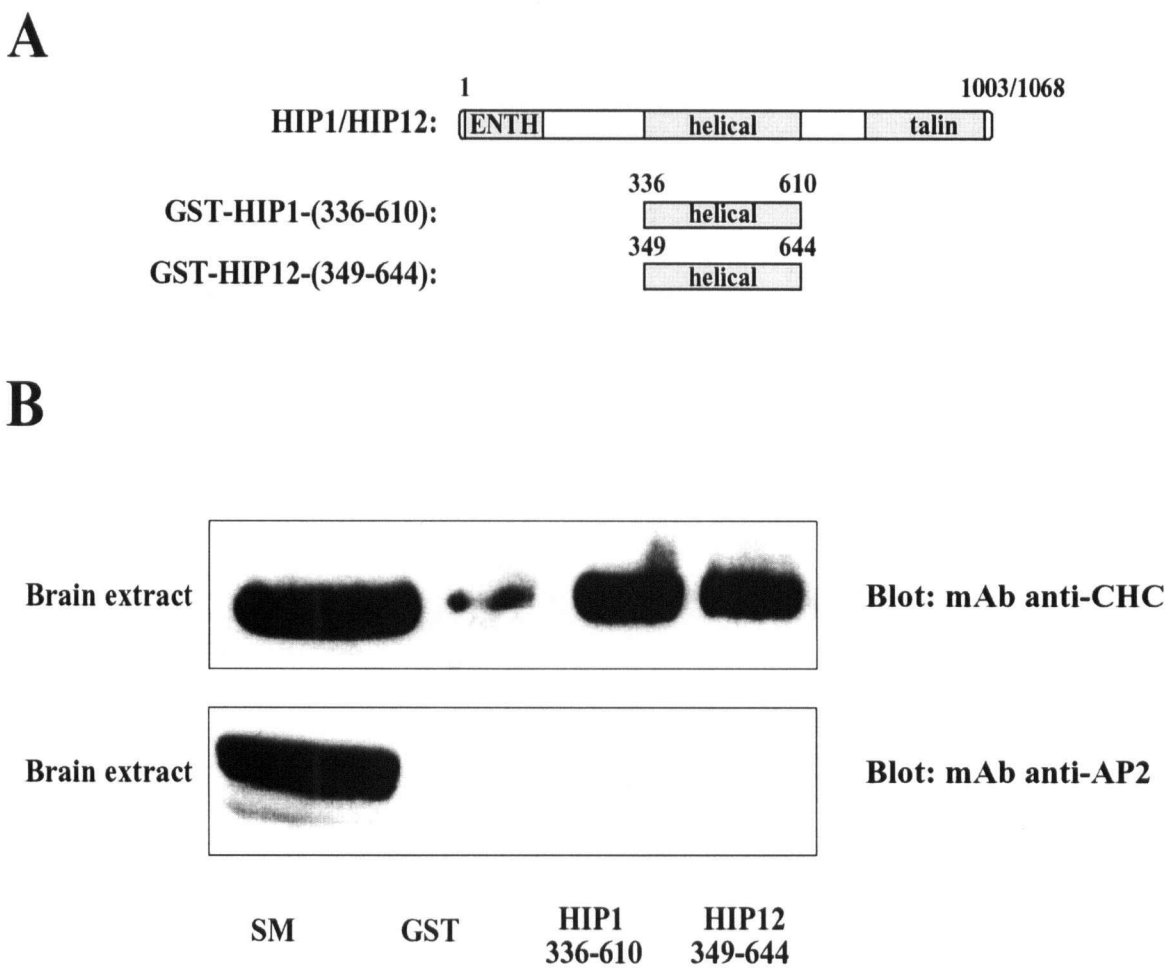


Fig. 5.2.5. The helical domains of HIP1 and HIP12 bind to clathrin but not AP2. A, schematic representation of various GST fusion proteins used for the binding assays. B, soluble proteins from brain extracts were affinity purified with equal amounts of HIP1 or HIP12 GST fusion proteins bound to glutathione-Sepharose beads. Proteins specifically bound to the beads were analyzed by Western blot with antibodies against CHC or the α-adaptin subunit of AP2 (AP2) as indicated.

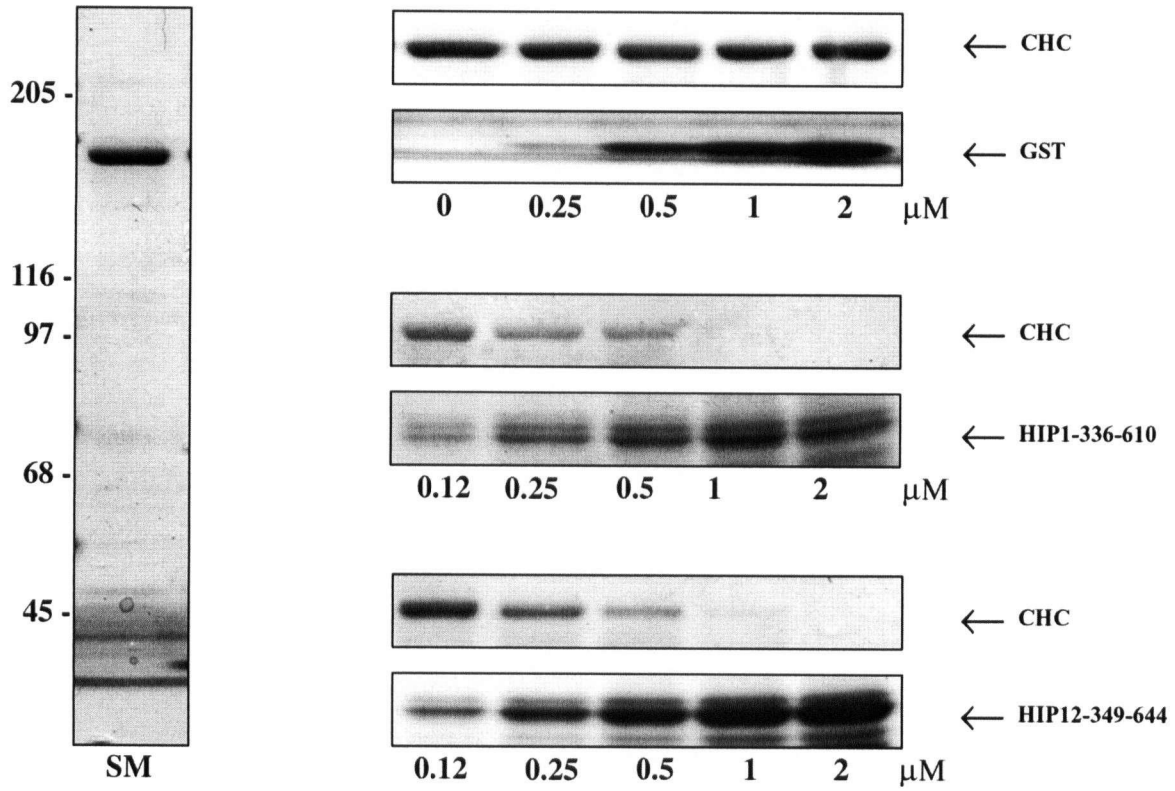


Fig. 5.2.6. HIP1 and HIP12 promote clathrin assembly through their helical domains. An aliquot of the purified clathrin used as a starting material (SM) for the clathrin assembly assays was resolved on SDS-PAGE and stained with Coomassie Blue. Clathrin assembly assays were performed with increasing amounts of fusion proteins GST, GST-HIP1-(336-610), and GST-HIP12-(348-644) as indicated. The GST fusion proteins and the clathrin remaining in the supernatant after high-speed centrifugation following initiation of clathrin assembly were analyzed by SDS-PAGE and Coomassie Blue staining.

5.2.7 HIP1 and HIP12 Binding to the Clathrin Light Chain

Although the coiled coil domains of HIP1 and HIP12 can interact with clathrin from rat brain; sequences examinations have not identified any clathrin box motifs within these domains. These findings suggest that either the coiled coil domains contain a novel clathrin binding motif or, these domains bind indirectly with the CHC. In order to confirm the interaction of the coiled coil domains with CHC, affinity purification studies were performed using the GST-HIP1-(336-610) and GST-HIP12-(348-644) fusion proteins. As expected, the fusion proteins were able to bind to CHC from rat brain extracts HIP1-(336-610) and GST-HIP12-(348-644) (figure 5.2.7A, top panel). However, GST-HIP1-(336-610) and GST-HIP12-(348-644) were unable to interact with purified His_6-tagged CHC terminal domain (figure 5.2.7A, bottom panel). These findings suggest that either the coiled coil domains of HIP1 and HIP12 do not interact directly with the CHC or, these domains interact with other segments of the CHC. Since clathrin triskelia are composed of three CHC interacting with three CLCs, it may be possible that the HIP1 and HIP12 coiled coil domain interacts with CLC. Affinity purification studies were performed using HIP1-(336-610) and GST-HIP12-(348-644) fusion proteins to determine whether HIP1 and HIP12 could interact with *in vitro* synthesized CLC b. Both HIP1-(336-610) and GST-HIP12-(348-644) were found to interact with CLC b (figure 5.2.8B, bottom panel). These findings are consistent with previous findings indicating that CLC is required for HIP12 interaction with the CHC (Engqvist et al., 2001).

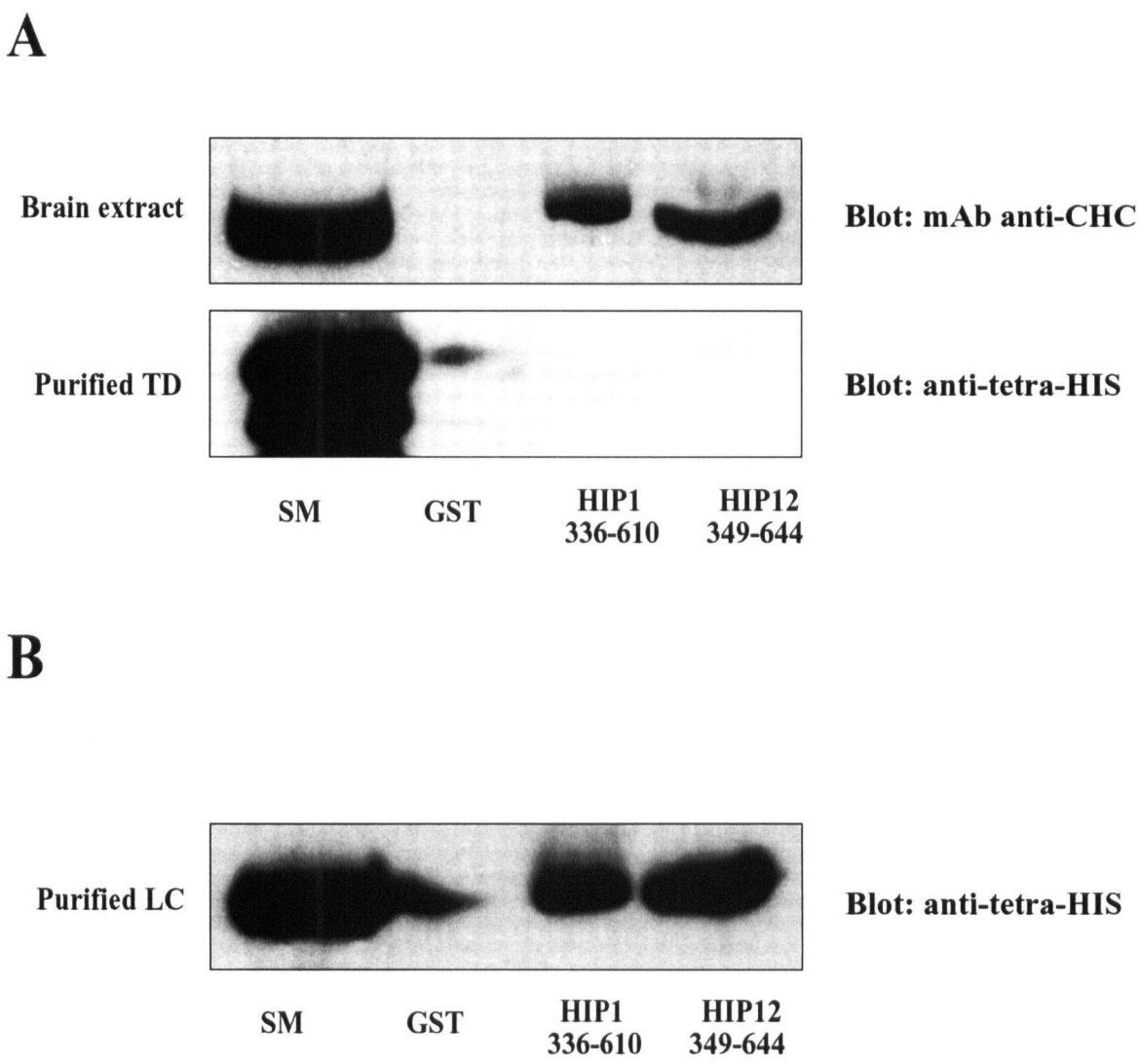


Fig. 5.2.7. HIP1 and HIP12 bind directly to the clathrin light chain through their helical domains. A, soluble proteins from brain extracts or purified His6-tagged clathrin terminal domain (TD) fusion protein were affinity purified with equal amounts of HIP1 or HIP12 GST fusion proteins bound to glutathione-Sepharose beads. Proteins specifically bound to the beads were analyzed by Western blots with antibodies against CHC or the His epitope (tetra-HIS) as indicated. B, purified His6-tagged clathrin light chain b (LCb) fusion protein was affinity purified with equal amounts of HIP1 or HIP12 GST fusion proteins bound to glutathione-Sepharose beads. Proteins specifically bound to the beads were analyzed by Western blot with the anti-His epitope antibody (tetra-HIS).

5.3 Discussion

In this study we have demonstrated that HIP1 and HIP12 are components of the endocytic machinery. HIP1 and HIP12 were found to be enriched in purified clathrin-coated vesicles (CCVs) at similar levels to clathrin and the adapter proteins α-adaptin and AP180. Furthermore, like the AP2 subunit, α-adaptin, HIP1 and HIP12 was stripped from the coated vesicles using Tris pH 9.0. The buffer Tris pH 9.0 has previously been shown to detach coat components from CCVs (Keen et al, 1979).

The association of HIP1 and HIP12 with the CCVs, indicate that these proteins are clathrin coat components and may play a role in the recruitment and assembly of the clathrin lattice structure. Indeed, HIP1 was found to interact directly with clathrin and AP2. HIP1 was found to contain a type 1 clathrin box sequence LMDMD. The consensus sequence for the type 1 clathrin box is L(L/I)(D/E/N)(L/F)(D/E) (Ramjaun et al., 1998; Drake et al., 2001). The endocytic machinery proteins AP180, amphiphysin, epsins, and β-arrestin have previously been shown to contain a type 1 clathrin box (Ford et al., 2001; Slepnev et al., 2000; Drake et al., 2000; Laporte et al., 1999). HIP1 also contains the AP2 binding motifs DPF and FDNKP, FDDIF, FGSSF. The consensus AP2 binding motifs DPF/W and FXDXF (where X represents any amino acid) are also present in the endocytic proteins epsin, amphiphysin 1 and AP180 (Mishra et al, 2001; Slepnev et al., 2000; Hao et al.,1999).

Unlike HIP1, HIP12 exhibited weak binding to clathrin and no interaction with AP2. Sequence analyses indicated that HIP12 contained the clathrin box sequence but did not contain any known AP2 binding motifs. The HIP12 clathrin box was similar to the endocytic protein β-arrestin and to the CDC42-interacting proteins ACK1 and ACK2

(Mishra et al.; 2001; Teo et al., 2001; Yang et al., 2001). As mentioned previously, Cdc42 is known to play an important role in the regulation actin cytoskeletal dynamics, endocytosis and cellular transport (Chapter 1, section 1.9.6; Ramesh et al., 1999).

These studies have also demonstrated that HIP1 and HIP12 are not only components of CCVs but also play a role in the assembly of the clathrin coat. Specifically, the coiled coil domains present in both HIP1 and HIP12 were capable of inducing the assembly of the clathrin lattice *in vitro*. Furthermore, these coiled coil domains did not interact with CHC but rather bound to CLC. Thus, the interaction of HIP1 and/or HIP12 with the CLC may play a role in clathrin assembly. Indeed, CLC has been shown to negatively regulate clathrin assembly (Ybe et al., 1998). The generation of the polyhedral clathrin lattice structure requires the interaction of clathrin triskelions units via salt bridges and electrostatic interactions. Ybe et al. (1998) have demonstrated that CLC can inhibit clathrin assembly by preventing the CHCs from forming salt bridges. More recently, Wilde et al. (1999) have demonstrated that Src-mediated phosphorylation of CHC, at a tyrosine residue located within the site of CHC-CLC interaction, results in both decreased CLC binding and induction of clathrin assembly. Taken together, it is possible that the coiled coil domains of HIP1 and HIP12 stimulate clathrin assembly by interacting with CLC and thereby prevent its inhibitory effects.

The carboxy-terminal sequences of HIP1 and HIP12 contain domains which have significant sequence identity with the cytoskeletal-associated protein, talin (Chapter 3, section 3.2.4; Chopra et al., 2000; Engqvist-Goldstein et al., 1999; Kalchman et al., 1997). The talin-like domain present in Sla2p, the yeast ortholog of HIP1/HIP12, has previously been shown to interact with F-actin (McCann and Craig, 1997). Similarly, we

have confirmed previous findings that HIP12 can also interact with F-actin (Engqvist-Goldstein et al., 1999). The talin-like domain in Sla2p and HIP12 function in the localization of these proteins to the cortical actin cytoskeleton (Wesp et al 1997; Engqvist-Goldstein et al., 1999). Unexpectedly, the HIP1 talin-like domain did not exhibit any interaction with F-actin. In this study we have confirmed previous yeast two-hybrid data showing that HIP1 can interact with HIP12 (Chapter 3, section 3.2.8; Chopra et al., 2000). It is possible that the formation of this heterodimers may play a role in localization of HIP1 to the cortical actin cytoskeleton.

Several studies have shown that HIP1, HIP12 and Sla2p are localized to the cytoplasmic membrane and, in particular, sites undergoing clathrin mediated endocytosis (CME; Holtzman et al, 1993; Raths et al., 1993; Engqvist-Goldstein et al., 1999; Metzler et al., 2001; Chapter 4, section 4.2.2). The amino terminal sequences of HIP1, HIP12 and Sla2p contain ENTH domains which has been shown to play a role in the localization of these proteins to the plasma membrane (Wesp et al, 1997; Engqvist-Goldstein et al., 1999; Metzler et al., 2001; Chapter 4, section 4.2.2). ENTH domains are proposed to stimulate clathrin-mediated endocytosis by binding to phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2]-containing membranes (Ford et al., 2001; Itoh et al., 2001). ENTH domains are also present in several other endocytic proteins including epsin 1 and 2 and AP180 (Ford et al, 2002; Ford et al., 2001; Itoh et al., 2001). Within neurons, PtdIns(4,5)P_2 levels have been found to be increased on the plasma membrane at sites of CME and on newly endocytosed synaptic vesicles (Micheva et al., 2001). It is possible that the localization of HIP1 and HIP12 to the endocytic machinery may be mediated by their ENTH domains.

The present studies suggest that HIP1 and HIP12 are components of the endocytic machinery that can link the plasma membrane to the underlying cortical actin cytoskeleton. HIP1 was originally identified as an interactor of huntingtin, the protein altered in HD (Kalchman et al., 1997; Wanker et al., 1997). The strength of the interaction is decreased with increased lengths of the polyglutamine tract present in huntingtin. HIP1 and huntingtin have been shown to be highly enriched within the brain and display a similar subcellular distribution (Kalchman et al., 1997; Wanker et al., 1997; Chopra et al., 2000). Huntingtin has also been shown to interact with the endocytic proteins α-adaptin and endophilin A3 (Faber et al., 1998; Sittler et al., 1998). In addition, huntingtin is also associated with clathrin-coated vesicles and thought to function in vesicle trafficking in the secretory and endocytic pathways (Velier et al., 1998). Take together, the altered interaction between mutant huntingtin and HIP1 may result in alterations in vesicle internalization within the neurons of the brain that ultimately lead to the neuropathological changes observed in HD.

Chapter 6:

General Discussion

6.1 Summary

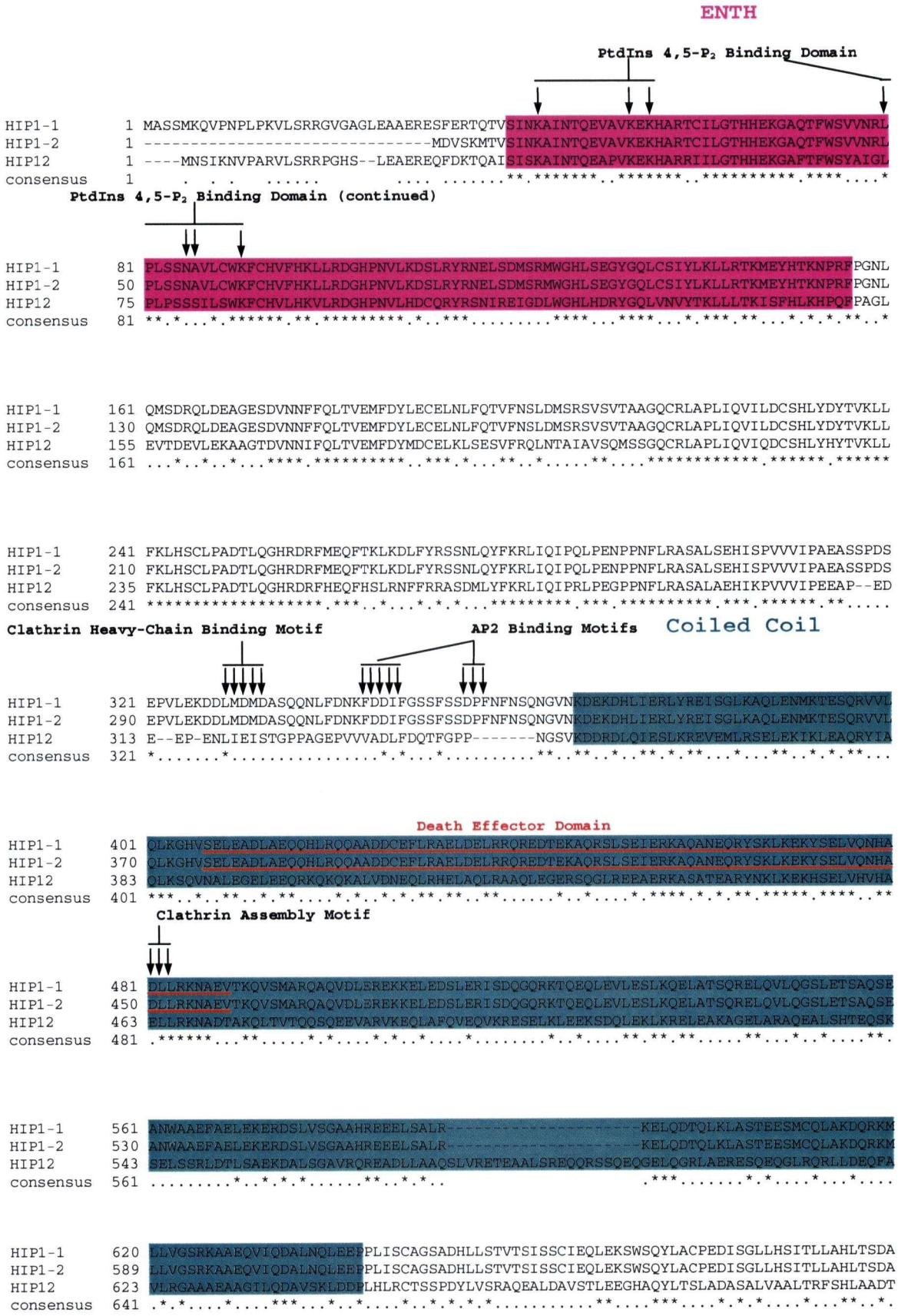
The objective of this thesis was to identify and characterize members of the HIP1 family of cytoskeletal-associated proteins. HIP1 was initially identified as an interactor of huntingtin (Kalchman et al., 1997; Wanker et al., 1997). Increased lengths of the huntingtin polyglutamine tract were associated with decreased interaction with HIP1 (Kalchman et al., 1997). By gaining greater insights into the cellular functions of the HIP1 family, it may be possible to determine their role in the pathogenesis of HD.

In this thesis we have determined that HIP1 is a member of a family of cytoskeletal-associated proteins that function in clathrin-mediated endocytosis (CME). In addition, *in vitro* studies indicated that HIP1 (but not HIP12) can induce programmed cell death by activating the pro-apoptotic enzyme caspase-3. The following sections will discuss the cellular functions of the HIP1 family and their significance in the pathogenesis of Huntington Disease.

6.2 HIP1 and HIP12 are components of the endocytic machinery

In this thesis I have identified a homolog of HIP1 termed HIP12. HIP1 and HIP12 shared 47% amino acid identity and both proteins contained ENTH, coiled coil and talin-like domains. However, HIP12 did not interact with huntingtin but could bind to HIP1 and form heterodimers. Please refer to figure 6.1 for the location of the different binding sites of HIP1 and HIP12. Tissue distribution studies indicated that HIP1 was expressed predominantly within the brain whereas HIP12 expression was more ubiquitous. Within cells, HIP1 and HIP12 displayed a similar punctate distribution at the plasma membrane and underlying cortical actin cytoskeleton. Specifically, HIP1 and HIP12 were both

Figure 6.1 HIP1 and HIP12 Domains. The ENTH , coiled coil, and talin-like domains are represented by red, blue and green shading respectively. The red line represents the death effector domain present in HIP1-1 and HIP1-2. The location of the clathrin box sequences and binding sites for phosphatidylinositol-4,5-bisphosphate, AP2, and clathrin light chain are depicted by arrows.


ENTH
PtdIns 4,5-P2 Binding Domain
HIP1-1 1 MASSMKQVPNPLPKVLSRRGVGAGLEAAERESFERTQTVSINKAINTQEVAVKEKHARTCILGTHHEKGAQTFWSVVNRL
HIP1-2 1 ----------MDVSKMTVSINKAINTQEVAVKEKHARTCILGTHHEKGAQTFWSVVNRL
HIP12 1 ----MNSIKNVPARVLSRRPGHS--LEAEREQFDKTQAISISKAINTQEAPVKEKHARRIILGTHHEKGAFTFWSYAIGL
consensus 1
PtdIns 4,5-P2 Binding Domain (continued)
HIP1-1 81 PLSSNAVLCWKFCHVFHKLLRDGHPNVLKDSLRYRNELSDMSRMWGHLSEGYGQLCSIYLKLLRTKMEYHTKNPRFPGNL
HIP1-2 50 PLSSNAVLCWKFCHVFHKLLRDGHPNVLKDSLRYRNELSDMSRMWGHLSEGYGQLCSIYLKLLRTKMEYHTKNPRFPGNL
HIP12 75 PLPSSSILSWKFCHVLHKVLRDGHPNVLHDCQRYRSNIREIGDLWGHLHDRYGQLVNVYTKLLLTKISFHLKHPQFPAGL
consensus 81
HIP1-1 161 QMSDRQLDEAGESDVNNFFQLTVEMFDYLECELNLFQTVFNSLDMSRSVSVTAAGQCRLAPLIQVILDCSHLYDYTVKLL
HIP1-2 130 QMSDRQLDEAGESDVNNFFQLTVEMFDYLECELNLFQTVFNSLDMSRSVSVTAAGQCRLAPLIQVILDCSHLYDYTVKLL
HIP12 155 EVTDEVLEKAAGTDVNNIFQLTVEMFDYMDCELKLSESVFRQLNTAIAVSQMSSGQCRLAPLIQVIQDCSHLYHYTVKLL
consensus 161
HIP1-1 241 FKLHSCLPADTLQGHRDRFMEQFTKLKDLFYRSSNLQYFKRLIQIPQLPENPPNFLRASALSEHISPVVVIPAEASSPDS
HIP1-2 210 FKLHSCLPADTLQGHRDRFMEQFTKLKDLFYRSSNLQYFKRLIQIPQLPENPPNFLRASALSEHISPVVVIPAEASSPDS
HIP12 235 FKLHSCLPADTLQGHRDRFHEQFHSLRNFFRRASDMLYFKRLIQIPRLPEGPPNFLRASALAEHIKPVVVIPEEAP--ED
consensus 241
Clathrin Heavy-Chain Binding Motif
AP2 Binding Motifs
Coiled Coil
HIP1-1 321 EPVLEKDDLMDMDASQQNLFDNKFDDIFGSSFSSDPFNFNSQNGVNKDEKDHLIERLYREISGLKAQLENMKTESQRVVL
HIP1-2 290 EPVLEKDDLMDMDASQQNLFDNKFDDIFGSSFSSDPFNFNSQNGVNKDEKDHLIERLYREISGLKAQLENMKTESQRVVL
HIP12 313 E--EP-ENLIEISTGPPAGEPVVVADLFDQTFGPP-------NGSVKDDRDLQIESLKREVEMLRSELEKIKLEAQRYIA
consensus 321
Death Effector Domain
HIP1-1 401 QLKGHVSELEADLAEQQHLRQQAADDCEFLRAELDELRRQREDTEKAQRSLSEIERKAQANEQRYSKLKEKYSELVQNHA
HIP1-2 370 QLKGHVSELEADLAEQQHLRQQAADDCEFLRAELDELRRQREDTEKAQRSLSEIERKAQANEQRYSKLKEKYSELVQNHA
HIP12 383 QLKSQVNALEGELEEQRKQKQKALVDNEQLRHELAQLRAAQLEGERSQGLREEAERKASATEARYNKLKEKHSELVHVHA
consensus 401
Clathrin Assembly Motif
HIP1-1 481 DLLRKNAEVTKQVSMARQAQVDLEREKKELEDSLERISDQGQRKTQEQLEVLESLKQELATSQRELQVLQGSLETSAQSE
HIP1-2 450 DLLRKNAEVTKQVSMARQAQVDLEREKKELEDSLERISDQGQRKTQEQLEVLESLKQELATSQRELQVLQGSLETSAQSE
HIP12 463 ELLRKNADTAKQLTVTQQSQEEVARVKEQLAFQVEQVKRESELKLEEKSDQLEKLKRELEAKAGELARAQEALSHTEQSK
consensus 481
HIP1-1 561 ANWAAEFAELEKERDSLVSGAAHREEELSALR----------KELQDTQLKLASTEESMCQLAKDQRKM
HIP1-2 530 ANWAAEFAELEKERDSLVSGAAHREEELSALR----------KELQDTQLKLASTEESMCQLAKDQRKM
HIP12 543 SELSSRLDTLSAEKDALSGAVRQREADLLAAQSLVRETEAALSREQQRSSQEQGELQGRLAERESQEQGLRQRLLDEQFA
consensus 561
HIP1-1 620 LLVGSRKAAEQVIQDALNQLEEPPLISCAGSADHLLSTVTSISSCIEQLEKSWSQYLACPEDISGLLHSITLLAHLTSDA
HIP1-2 589 LLVGSRKAAEQVIQDALNQLEEPPLISCAGSADHLLSTVTSISSCIEQLEKSWSQYLACPEDISGLLHSITLLAHLTSDA
HIP12 623 VLRGAAAEAAGILQDAVSKLDDPLHLRCTSSPDYLVSRAQEALDAVSTLEEGHAQYLTSLADASALVAALTRFSHLAADT
consensus 641

```
HIP1-1      700 IAHGATTCLRAPPEPADSLTEACKQYGRETLAYLASLEEEGSLENADSTAMRNCLSKIKAIGEELLPRGLDIKQEELGDL
HIP1-2      669 IAHGATTCLRAPPEPADSLTEACKQYGRETLAYLASLEEEGSLENADSTAMRNCLSKIKAIGEELLPRGLDIKQEELGDL
HIP12       703 IINGGATSHLAPTDPADRLIDTCRECGARALELMGQLQDQQALRHMQASLVRTPLQGILQLGQELKPKSLDVRQEELGAV
consensus   721 *..*..*...**..***.*...*...*...*.....*.....*........*..*..*...*.**.*..**..*****..
```

Talin-Like

```
                                                                  I/L
HIP1-1      780 VDKEMAATSAAIETATARIEEMLSKSRAGDTGVKLEVNERILGCCTSLMQAIQVLIVASKDLQRETVESGRGTASPKEFY
HIP1-2      749 VDKEMAATSAAIETATARIEEMLSKSRAGDTGVKLEVNERILGCCTSLMQAIQVLIVASKDLQRETVESGRGTASPKEFY
HIP12       783 VDKEMAATSAAIEDAVRRIEDMMNQARHASSGVKLEVNERILNSCTDLMKAIRLLVTTSTSLQKETVESGRGAATQQEFY
consensus   801 *************.*..***.*....*....**********..**.**.**..*...*..**.********.*...***
```

```
                          W                                   E
HIP1-1      860 AKNSRVTEGLISASKAVGWGATVMVDAADLVVQGRGKFEELMVCSHEIAASTAQLVAASKVKADKDSPNLAQLQQASRGV
HIP1-2      829 AKNSRVTEGLISASKAVGWGATVMVDAADLVVQGRGKFEELMVCSHEIAASTAQLVAASKVKADKDSPNLAQLQQASRGV
HIP12       863 AKNSRVTEGLISASKAVGWGATQLVEAADKVVLHTGKYEELIVCSHEIAASTAQLVAASKVKANKHSPHLSRLQECSRTV
consensus   881 **********************..*.***.**...**.***.**********************.*.**.*..**..**.*
```

```
                                                          Q
HIP1-1      940 NQATAGVVASTISGKSQIEETDNMDFSSMTLTQIKRQEMDSQVRVLELENELQKERQKLGELRKKHYELAGVAEGWEEGT
HIP1-2      909 NQATAGVVASTISGKSQIEETDNMDFSSMTLTQIKRQEMDSQVRVLELENELQKERQKLGELRKKHYELAGVAEGWEEGT
HIP12       943 NERAANVVASTKSGQEQIEDRDTMDFSGLSLIKLKKQEMETQVRVLELEKTLEAERMRLGELRKQHYVLAGASGSPGEEV
consensus   961 *...*.*****.**..***..*.****...*...*.***..********..*..**..******.**.***......*..
```

```
HIP1-1     1020 EASPPTLQEVVTEKE--------------------------------
HIP1-2      989 EASPPTLQEVVTEKE--------------------------------
HIP12      1023 AIRPSTAPRSVTTKKPPLAQKPSVAPRQDHQLDKKDGIYPAQLVNY
consensus  1041 ...*.*....**.*.
```

found to be localized to plasma membrane sites undergoing CME and associated with clathrin-coated vesicles.

The findings in this thesis also indicated that HIP1 and HIP12 differ in their associations with components of the endocytic machinery. Please refer to figure 6.2 for the HIP1 and HIP12 domains and their respective interacting proteins. HIP1 was shown to interact with the clathrin heavy chain (via its clathrin box sequence) and AP2 (via its DPF and FXDXF motifs). The tandem arrangement of the clathrin box and DPF present in HIP1 has been suggested to help stabilize the relatively weak interaction between AP2 and clathrin. Relative to HIP1, HIP12 displayed weaker binding to clathrin heavy chain (through its clathrin box sequence) and no interaction with AP2. In this thesis it was also shown that the coiled coil domains present in both HIP1 and HIP12 were capable of inducing the assembly of the clathrin coat *in vitro*. The HIP1 and HIP12 coiled coil domains were also shown to interact with the clathrin light chain. The clathrin light chain has been shown to inhibit the assembly the clathrin lattice structure (Ybe et al., 1998; Wilde et al., 1999). It may be possible that HIP1 and HIP12 stimulate with clathrin coat assembly by binding to clathrin light chain and abrogating its inhibitory activity. Taken together, these results demonstrate that HIP1 and HIP1 are components of the endocytic machinery that participate in the recruitment, assembly and stabilization of the clathrin lattice structure.

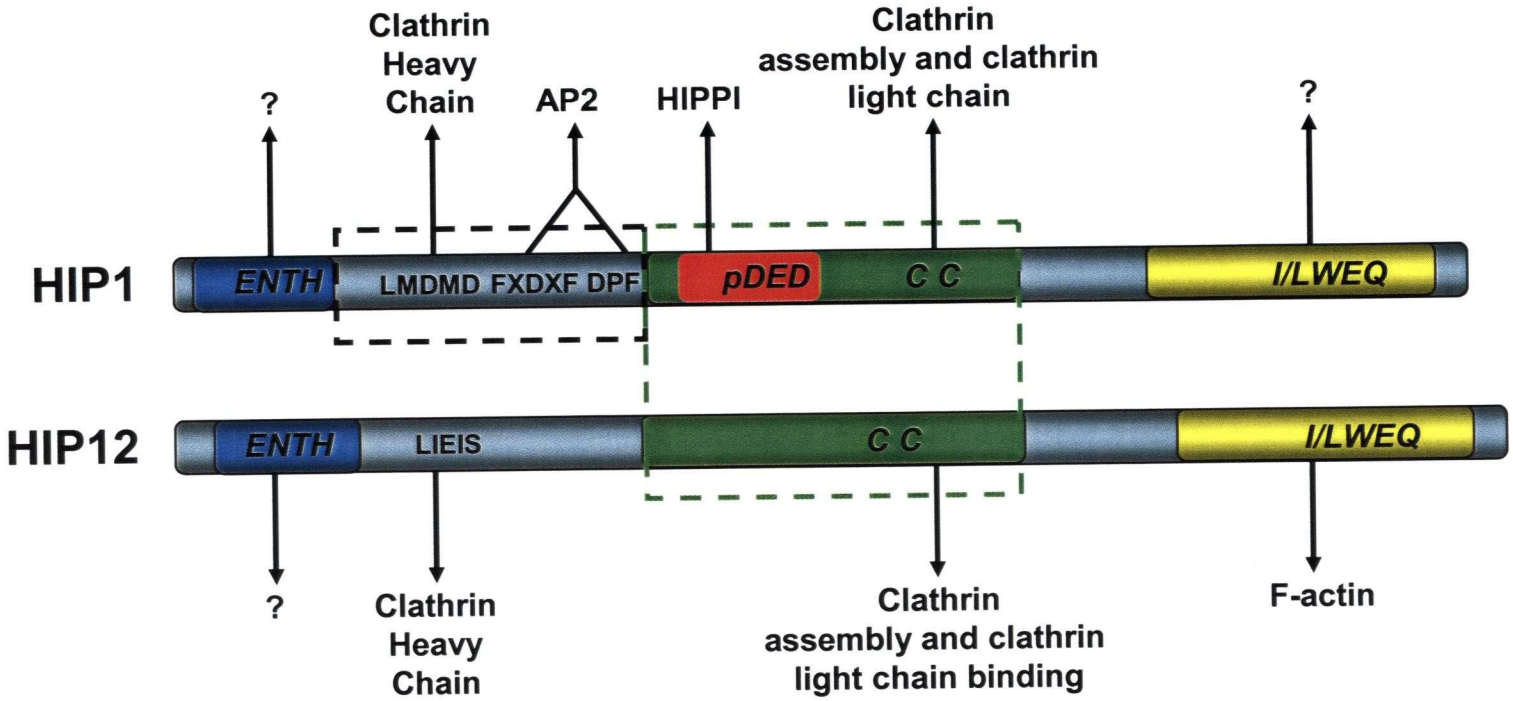


Figure 6.2 Domain structures of HIP1 and HIP12. The arrows point to interacting proteins and specific function associated with the domain or sequence motif. The black dashed box refers to the domain required for interaction with huntingtin. The dashed green box refers to the domains necessary for interaction of HIP1 with HIP12.

6.3 HIP1 and HIP12 Interact with the Cortical Actin Cytoskeleton

The observation that HIP12 interacts with F-actin suggests that HIP12 may interact with the cortical actin cytoskeleton underlying clathrin-coated pits and vesicles. Furthermore, HIP1 may be indirectly associated with the actin cytoskeleton by forming heterodimers with HIP12. Thus, HIP1 and HIP12 may act as a molecular "link" between the endocytic machinery and the cortical actin cytoskeleton. Consistent with this suggestion is the observation that a number of proteins involved in CME also influence cortical actin cytoskeletal dynamics. For example, dynamin has been shown to interact with several actin-binding proteins including profilin, Abp1, and cortactin (Kessels et al., 2001). Cortactin is known to activate actin assembly at sites adjacent to clathrin-coated pits (Schafer, 2002; Uruno et al, 2001). The endocytic protein, intersectin, has also been shown to induce the polymerization of actin filaments at site of CME (Qualmann et al., 2000). In addition, the huntingtin-interacting proteins, pacsin 1 and CIP4 are involved in the stimulation of the actin polymerization machinery at the site of newly formed endocytic vesicles (Modregger et al., 2002; Holbert et al., 2003). The cortical actin cytoskeleton is thought to perform a number of different functions during CME. The actin cytoskeleton may be involved in localizing the endocytic machinery to specific sites on the cytoplasmic membrane by physically interacting with components of endocytic machinery and/ or acting as a barrier that prevents their lateral movement (Qualmann et al., 2000).

6.4 In vivo Evidence of HIP1 Function in Endocytosis

The data presented in this thesis presents immunohistochemical and biochemical evidence that the HIP1 family of proteins are involved in endocytosis. Recently, Metzler et al. (2003) have generated mice with a targeted disruption in the HIP1 gene ($HIP1^{-/-}$) to determine the cellular function of HIP1 *in vivo*. The $HIP1^{-/-}$ mice exhibited gait ataxia, tremor and developed a distinctive "hunchback" phenotype due to the development of a rigid thoracolumbar kyphosis. These changes were not due to muscular or skeletal defects but rather they were the result of neurological dysfunction. Examination of the $HIP1^{-/-}$ mouse neurons indicated that these cells had a reduced ability to form the endocytic protein complexes. Furthermore, these mice exhibited a profound dose-dependent defect in clathrin-mediated internalization of glutamate subtype, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor. These findings suggest that HIP1 regulates AMPA receptor trafficking in the CNS through its function in clathrin-mediated endocytosis. It may be postulated that by association huntingtin may also play in AMPA receptor trafficking.

Interestingly, huntingtin also interact with the glutamate receptor-binding protein postsynaptic density 95 (PSD95; Sun et al., 2001). Furthermore, the expanded polyglutamine tract in mutant huntingtin reduces its interaction with PSD95 (Sun et al., 2001). Sun et al. (2001) demonstrated that the lack of PSD95 interaction with mutant huntingtin results in the sensitization and activation of NMDA receptors. PSD95 is a membrane-associated scaffolding protein which binds directly to N-methyl-D-aspartate (NMDA) receptor and indirectly to (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor and has also been shown to bind to the cytoskeleton and cell-

adhesion molecules (El-Husseini and Bredt, 2002). Through these multiple interactions PSD95 induces clustering of NMDA and AMPA receptors in the postsynaptic membrane and regulates neuronal activities such as long-term potentiation and long-term depression (Sun et al., 2001; El-Husseini and Bredt, 2002). Taken together, huntingtin interaction with PSD95 may play an essential role in the regulation of glutamate receptor activity. Moreover, the loss of interaction between mutant huntingtin and PSD95 may result in a dysregulation of glutamate receptor activity which results in the glutamate-induced cell death seen in HD.

Recently, Wang et al. (2003) have demonstrated that excessive activation of NMDA receptors results the activation of apoptosis and the internalization of AMPA receptors via CME. Using inhibitors of CME, Wang et al. (2003) established that inhibition of AMPA endocytosis selectively inhibits NMDA-induced apoptosis. Thus, clathrin-dependent endocytosis of AMPA is required for NMDA receptor-mediated endocytosis. It may be postulated that clathrin-coated AMPA receptor may form a pro-apoptotic complex that results in the activation of cell death signals. Since HIP1 and huntingtin and HIP1 are both components of the endocytic machinery and can induce apoptosis, it may be possible that these proteins play an important role in NMDA-induced apoptosis.

6.5 The Role of Huntingtin in Endocytosis and Transport

Several lines of evidence suggest that huntingtin may play a role in endocytosis and transport. Within neurons of the brain, huntingtin has been shown to be present in the axon and nerve terminals where it seems to be associated with microtubules and synaptic

vesicles (DiFiglia et al., 1995; Gutekunst et al., 1995; Tukamoto et al., 1997). Furthermore, huntingtin has been shown to be associated with clathrin-coated and non-coated endosomal vesicles in the cytoplasm and along plasma membranes (Velier et al., 1998). In this thesis it has been confirmed that huntingtin interacts with HIP1, a component in the endocytic and transport machinery of the cell. Thus, by association huntingtin may also play a role in endocytosis and transport. Please refer to figure 6.2 illustrating the known functions of several huntingtin-interacting proteins in endocytosis and transport.

6.5.1 Endocytic and Transport Proteins that interact with Huntingtin

In addition to HIP1, huntingtin has been shown to interact with other proteins that play a role in endocytosis and transport. Huntingtin interacts with the brain-enriched protein HAP1which is thought to play a role in vesicle transport and actin dynamics. HAP1 interacts with $p150^{glued}$, a subunit of the microtubule-based transporter complex dynein (Engelender et al, 1997, Li et al., 1998). HAP1 binds to hepatocyte growth factor-regulated tyrosine kinase (Hrs), a protein involved in the regulation of vesicle transport from the early endosomes to the late endocytic compartments (Li et al., 2002). Finally, HAP1 interacts with duo, a guanine nucleotide exchange factor that is a regulator of actin cytoskeletal dynamics (Colomer et al., 1997). Huntingtin also interacts with α-adaptin-C which is a subunit of the adapter protein 2 complex (AP2). AP2 is involved in the recruitment and polymerization of clathrin subunits into clathrin-coated vesicles (Brodsky et al., 2001). AP2 is also thought to coordinate the process of endocytosis with intracellular signaling pathways (Slepnev and DeCamilli, 2000, Brodsky et al., 2001).

Huntingtin can also interact with endophillin A3, a protein hypothesized to play a role in the induction of membrane curvature during clathrin-dependent endocytosis (Sittler et al., 1998; Slepnev and De Camilli, 2000). Huntingtin has been shown to interact with the neurospecific protein Pacsin 1. Pacsin 1 is implicated in the recruitment and stimulation of the actin polymerization machinery at the site of newly formed endocytic vesicles (Modregger et al., 2002; Qualmann and Kessels, 2002). Huntingtin also binds to the brain-enriched protein huntingtin interacting protein 14 (HIP14). Within neurons, HIP14 has been suggested to play a role in clathrin-coated vesicle formation and vesicle trafficking at the cytoplasmic and Golgi apparatus membranes (Singaraja et al., 2002). Finally, huntingtin can interact with Cdc42-interacting protein 4 (CIP4). CIP4 is involved in several cellular functions including endocytosis, transport and regulation of actin cytoskeleton (Holbert et al., 2003; Aspenstrom et al., 1997; Ramesh et al., 1999; Tian et. al, 2000; Jiang et al., 2002).

6.5.2 Huntingtin-Interacting Proteins that Influence Endocytosis and Transport

Interestingly, Huntingtin has been shown to interact with a number of different proteins which may be "loosely" categorized as cellular signaling proteins. Several of these proteins may, in part, be involved in endocytosis and cellular transport. These huntingtin interacting proteins include HSP70, ubiquitin-conjugating enzyme, and calmodulin.

The chaperone HSP70 has been shown to interact with huntingtin in a polyglutamine-length dependent manner; the larger the repeat the greater the interaction (Jana et al., 2000). Within the brains of HD patients HSP70 have been shown to co-

localize to inclusions in the nucleus but not in the cytoplasm (Jana et al. 2000). The cellular function of HSP70 is to prevent aggregation of denatured proteins and aid in their renaturation (Ohtsuka and Suzuki, 2000). It has been suggested that in HD, the induction HSP70 may be a cellular attempt to "clear" the nuclear aggregates containing mutant huntingtin. With respect to endocytosis and cellular transport, HSP70 and its neuron-specific cofactor auxilin are involved in the rapid disassembly of the clathrin lattice structure from newly formed endocytic vesicles (Slepnev and DeCamilli, 2000).

Huntingtin can interact with the ubiquitin-conjugating enzyme hE2-25K (Kalchman et al., 1996). The interaction of hE2-25K with huntingtin is CAG length independent. This enzyme belongs to a family of proteins that participate in the linking of C-terminal glycine residues of ubiquitin to specific lysine residues on target proteins including huntingtin and ubiquitin (Kalchman et al., 1996; Hicke, 2001). Recent evidence suggests that the addition of a single ubiquitin residue (monoubiquitylation) to a target protein may result its transport to various intracellular compartments including late endosomes and lysosomal vacuoles (Shih et al., 2002; for a review see Hicke, 2001). Furthermore, monoubiquitylation has been shown to down-regulate activated receptors on the plasma membrane by inducing their endocytosis (Shih et al., 2000). It has been postulated that ubiquitin regulates protein transport at discreet site on the plasma membrane by serving as a sorting signal on protein cargo and by controlling the activity of trafficking machinery (Hicke, 2001). Interestingly, target proteins can also be conjugated with several ubiquitin "units" and form large multi-ubiquitin chains (Hicke, 2001). These multi-ubiquitin-tagged proteins are then directed to the multi-protein complex known as the proteosome where they are degraded (Hicke, 1999). Thus, ubiquitination also plays a

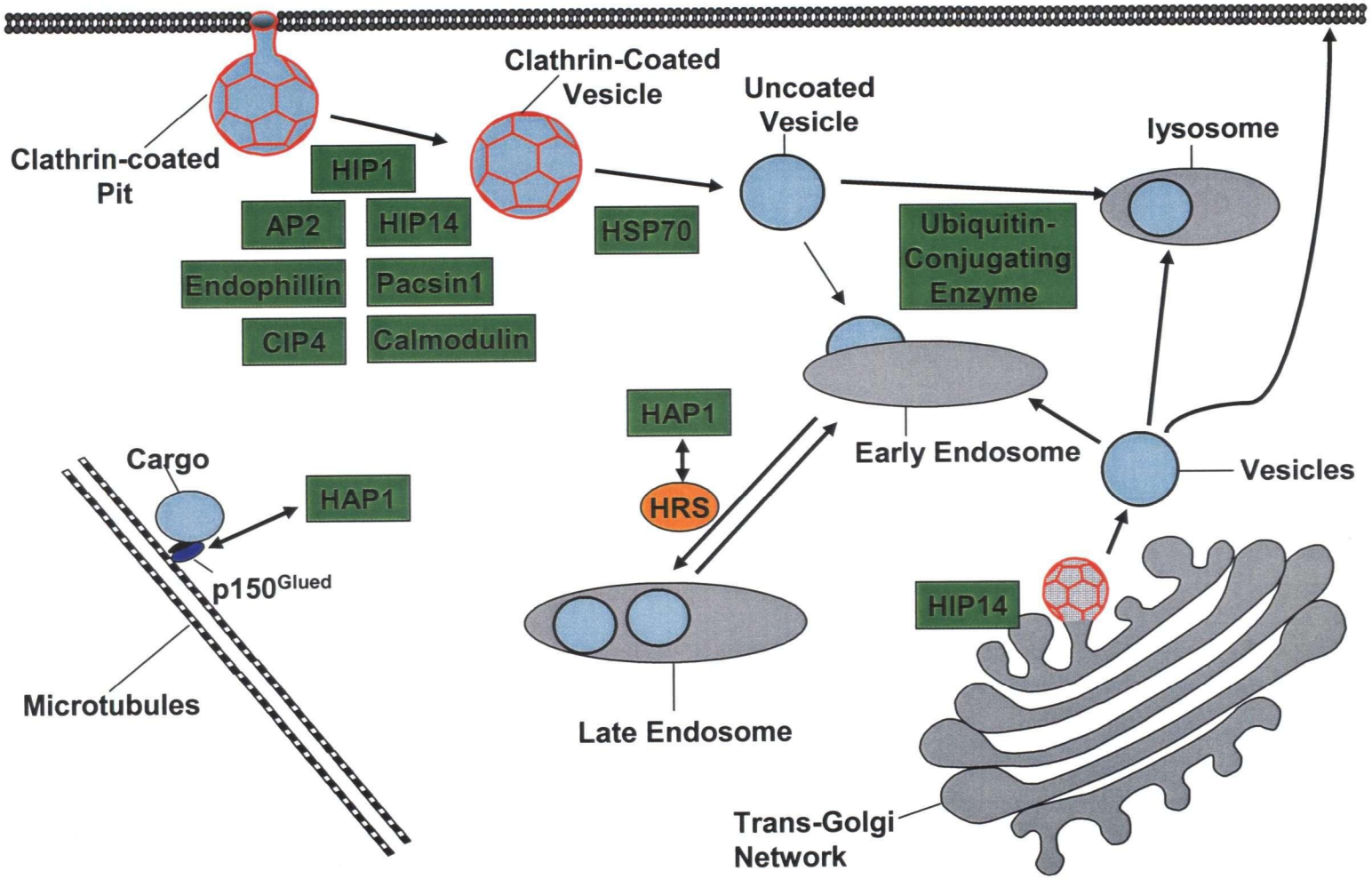
major role in the regulated catabolism of various proteins including huntingtin (Hicke, 1999; Wyttenbach et al., 2000).

The Ca^{2+}-binding protein calmodulin (CAM) has also been shown to interact with huntingtin in the presence of calcium (Bao et al., 1996; Tukamoto et al., 1997). In addition, the mutant form of huntingtin interacts with CAM in the absence of calcium and with greater affinity than with wild-type huntingtin. A number of different functions have been ascribed to CAM including smooth muscle contraction, transport, endocytosis, proliferation, protein catabolism, and cellular adhesion (Deckel, 2001). Furthermore, CAM has been shown to regulate the activity of several protein kinases and phosphatases, ion channels and nitric oxide synthase (Deckel, 2001). Of particular interest is CAM's ability to activate the calcium-binding phosphatase, calcineurin. During endocytosis calcineurin is required for the dephosphorylation of several components of the endocytic machinery including dynamin, amphiphysin, synaptojanin, epsin ESP15, AP2 and AP180 which are collectively referred to as dephosphin (Cousin et al., 2001; Cousin and Robinson, 2001). The prevention of dephosphorylation of the dephosphins results in an inhibition of endocytosis (Cousin et al., 2001). Since huntingtin interacts with CAM, it is conceivable that huntingtin also indirectly influences endocytosis.

Taken together, these findings strongly suggest that huntingtin may play a role in endocytosis and transport. Please refer to figure 6.2 illustrating the known functions of several huntingtin-interacting proteins in endocytosis and transport. Recently, several studies have demonstrated that huntingtin can interact with transcription factors. At this time these interactions have not been shown to have direct consequences on endocytosis and transport. It may be speculated that huntingtin may instead play a role in the transport

of the transcription factors to the nucleus. Consistent with this idea is the observation that huntingtin is found in the nucleus (DiFiglia et al., 1997). Furthermore, the huntingtin protein contains HEAT repeats domains which are also nuclear transport protein importin (Andrade and Bork, 1995). Thus, by analogy huntingtin may also play a role in transport.

Figure 6.3 Potential roles for huntingtin interacting proteins in endocytosis. HIP1, AP2, HIP14, endophillin, pacsin, Cdc42-interacting protein 4 (CIP4) and calmodulin play important in the formation of clathrin-coated pits and release of clathrin-coated vesicles during endocytosis. HIP14 may also be involved in formation of clathrin-coated pits and vesicles at the trans-Golgi network. HSP70 plays an important in the "shedding" of the clathrin coat. Ubiquitin-conjugating enzyme may play a role in targeting endocytosed vesicles to intracellular organelles including lysosomes and early endosomes. HAP1 in conjunction with HRS is involved in the transport of vesicles from the early to late endosomes. HAP1 also interacts with $p150^{Glued}$ complex and may help transport cargo along microtubules chains.


Clathrin-coated Pit
Clathrin-Coated Vesicle
Uncoated Vesicle
lysosome
HIP1
AP2
HIP14
Endophillin
Pacsin1
CIP4
Calmodulin
HSP70
Ubiquitin-Conjugating Enzyme
HAP1
Early Endosome
Vesicles
Cargo
HAP1
HRS
p150Glued
HIP14
Microtubules
Late Endosome
Trans-Golgi Network

6.6 The Potential Role of Endocytosis Defects in the Pathogenesis of HD

In this thesis it has been demonstrated that HIP1 is one of several huntingtin-interacting that are involved in endocytosis and transport. It may be hypothesized that altered interactions between these proteins and mutant huntingtin that potentially result in defects in endocytosis. These defects in endocytosis and transport may play a role in the pathogenesis of HD. Even if this is the case, it is still difficult explain how defects in endocytosis and transport could account for the selective loss of medium spiny neurons (MSNs) within the striatum. One possibility is that the survival of MSNs requires the endocytosis of trophic factors. For example, brain-derived neurotrophic factor (BDNF) is not produced by cells in the striatum. Instead, medium spiny neurons obtain BDNF from cortical connections (Katoh-Semba et al., 1998). Thus, if the MSNs are unable to endocytose BDNF, they may eventually die. Therefore, if HD is a result of defects in endocytosis, it would be expected that the MSNs in affected individuals would have difficulty in obtaining the trophic support of BDNF. Interestingly, BDNF has also been shown to protect cultured striatal neurons from excitotoxic cell death (Nakao et al., 1995). Several studies have reported that excititoxicity may play an important role in the selective neuronal loss seen in HD patients and mouse models of HD (Albin and Greenamyre, 1992; Hodgson et al., 1999; Zeron et al., 2002). It may be hypothesized that if BDNF cannot be taken up by the MSNs, this may result in an inability to respond to excitotoxic insults which could in turn produce changes reminiscent of HD.

6.7 Future Directions

The isolation and characterization of the HIP1 family has helped to elucidate its role in clathrin-mediated endocytosis and suggest its potential role in the pathogenesis of HD. However, a great deal of further investigations is required to extend the findings within this thesis. The following are suggestion for further investigation of the function HIP1 family and huntingtin.

1) In this thesis I have determined that the *HIP1* gene comprises 32 exons spanning approximately 215 Kb of genomic DNA and gives rise to two alternate splice forms designated as HIP1-1 and HIP1-2. At this time the physiological relevance of these two splice forms is unclear. Using reverse transcriptase (RT) PCR it was determined that both these proteins are expressed at similar levels throughout the body. However, the use of quantitative RT-PCR may help to determine if the mRNA expression levels vary between HIP1-1 and HIP1-2. Alternatively, it may be necessary to generate antibodies that can distinguish between these isoforms. Using these reagents it may be possible to determine whether HIP1-1 and HIP1-2 have differences in their spatial and temporal expression patterns. These findings (if any) may suggest that these 2 isoforms perform exclusive functions within the cell.

2) In this thesis it was demonstrated that overexpression of HIP1 in HEK 293T cells causes both a significant decrease in cell viability and an increase in caspase 3 activity. However, overexpression of HIP12 into HEK 293T cells does not cause any alteration in cell viability or caspase 3 activity. These findings indicate that HIP12 in contrast to HIP1 is not a pro-apoptotic protein. Since HIP12 interacts with HIP1 it may be possible that

HIP12 modulates the pro-apoptotic activity of HIP1. To address this possibility, it would be useful to perform co-expression studies in HEK 293T cells to determine whether HIP12 influences HIP1's ability to reduce cell viability and increase caspase 3 activation.

3) The development of a transgenic mouse overexpressing HIP1 would serve as a useful reagent to further address the pro-apoptotic function of HIP1. These mice may help to determine whether certain cell regions are more vulnerable to HIP1 overexpression.

4) The recent development of a HIP1 knockout mice has demonstrated that HIP1 plays a critical role in plasma receptor internalization. Future studies may require the development of a HIP12 knockout in order to determine its functions *in vivo*. The phenotypes of the HIP1 and HIP12 knockouts mice could then be compared in order to gain insights in the function of HIP1 and HIP12.

5) Huntingtin binds to HIP1 at location overlapping the binding sites for AP2, clathrin light and heavy chains. At this time it is unclear whether huntingtin may in fact impede the binding of these endocytic proteins to HIP1. It would be useful to develop deletion constructs for HIP1 in order to determine the exact location where huntingtin interacts. In addition, HIP1-huntingtin co-immunoprecipitation assays could be performed in the presence or absence of the clathrin subunits to determine whether these proteins compete for binding to HIP1. These studies will help to address whether huntingtin can modulate the interaction HIP1 with the endocytic proteins or if huntingtin is a component of the endocytic machinery.

6) This thesis presented further evidence suggesting that huntingtin plays a role in endocytosis. It would be useful to perform endocytosis assays (such as transferrin uptake assays) in cell culture to address whether wildtype and mutant huntingtin differ in their ability to perform clathrin-mediated endocytosis.

7) Members of the HIP1 family contain a putative amino-terminal ENTH domain. At this time it has not been confirmed that the HIP1 and HIP12 ENTHs can interact with phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P_2]-containing membranes. Future studies examining the binding of HIP1 and HIP12 to purified [PtdIns(4,5)P_2]-containing membranes will confirm the function of their ENTH domains.

8) It is unclear whether all of the HIP1 and HIP12 interacting proteins have been identified. It may be useful to perform yeast 2-hybrid assays to screen for any additional interactor(s) that would help to further delineate the functions of the HIP1 family of proteins.

6.8 Conclusion

The findings in this thesis demonstrate that the HIP1 family of proteins plays an important role in clathrin-mediated endocytosis. The interaction of huntingtin with other interacting proteins suggests that it may also function in endocytosis and transport. Since mutant huntingtin displays altered interactions with HIP1 and other endocytic proteins; it may be speculated that the pathogenesis of HD is due to defects in endocytosis and transport. A greater understanding of the role the HIP1 family and huntingtin in endocytosis and transport could aid in unraveling the mechanism underlying HD and potentially point to novel therapeutic strategies.

Chapter 7:

References

References

1. Ahle, S., Mann, A., Eichelsbacher, U., and Ungewickell, E. (1988) Structural relationships between clathrin assembly proteins from the Golgi and the plasma membrane. *EMBO J.* 7, 919-929.

2. Albin, R.L., Reiner, A., Anderson, K.D., Dure, L.S., Handelin, B., Balfour, R., Whetsell, W.O., Jr., Penney, J.B., and Young, A.B. (1992). Preferential loss of striato-external pallidal projection neurons in presymptomatic Huntington's disease. *Ann. Neurol.* 31, 425-430.

3. Andrade, M.A. and Bork, P. (1995). HEAT repeats in the Huntington's disease protein. *Nat. Genet.* 11, 115-116.

4. Andrade, M.A., Petosa, C., O'Donoghue, S.I., Muller, C.W., and Bork, P. (2001). Comparison of ARM and HEAT protein repeats. *J. Mol. Biol.* 309, 1-18.

5. Andrew, S.E., Goldberg, Y.P., Kremer, B., Telenius, H., Theilmann, J., Adam, S., Starr, E., Squitieri, F., Lin, B., Kalchman, M.A., and . (1993). The relationship between trinucleotide (CAG) repeat length and clinical features of Huntington's disease. *Nat. Genet.* 4, 398-403.

6. Ashkenazi, A. and Dixit, V.M. (1999). Apoptosis control by death and decoy receptors. *Curr. Opin. Cell Biol.* 11, 255-260.

7. Aspenstrom, P. (1997). A Cdc42 target protein with homology to the non-kinase domain of FER has a potential role in regulating the actin cytoskeleton. *Curr. Biol.* 7, 479-487.

8. Ayscough, K.R., Stryker, J., Pokala, N., Sanders, M., Crews, P., and Drubin, D.G. (1997). High rates of actin filament turnover in budding yeast and roles for actin in establishment and maintenance of cell polarity revealed using the actin inhibitor latrunculin-A. *J. Cell Biol.* 137, 399-416.

9. Bao, J., Sharp, A.H., Wagster, M.V., Becher, M., Schilling, G., Ross, C.A., Dawson, V.L., and Dawson, T.M. (1996). Expansion of polyglutamine repeat in huntingtin leads to abnormal protein interactions involving calmodulin. *Proc. Natl. Acad. Sci. U. S. A* 93, 5037-5042.

10. Barr, F. (2000). Vesicular transport. *Essays Biochem.* 36, 37-46.

11. Beal, M.F. (1996). Mitochondria, free radicals, and neurodegeneration. *Curr. Opin. Neurobiol.* 6, 661-666.

12. Becher, M.W., Rubinsztein, D.C., Leggo, J., Wagster, M.V., Stine, O.C., Ranen, N.G., Franz, M.L., Abbott, M.H., Sherr, M., MacMillan, J.C., Barron, L., Porteous, M., Harper, P.S., and Ross, C.A. (1997). Dentatorubral and pallidoluysian atrophy (DRPLA). Clinical and neuropathological findings in genetically confirmed North American and European pedigrees. *Mov Disord.* 12, 519-530.

13. Bennett, E. M., Chen, C. Y., Engqvist-Goldstein, A. E., Drubin, D., and Brodsky, F. M. (2001) Clathrin hub expression dissociates the actin-binding protein Hip1R from coated pits and disrupts their alignment with the actin cytoskeleton. *Traffic* 2, 851-858.

14. Blader, I.J., Cope, M.J., Jackson, T.R., Profit, A.A., Greenwood, A.F., Drubin, D.G., Prestwich, G.D., and Theibert, A.B. (1999). GCS1, an Arf guanosine triphosphatase-activating protein in Saccharomyces cerevisiae, is required for normal actin cytoskeletal organization in vivo and stimulates actin polymerization in vitro. *Mol. Biol. Cell* 10, 581-596.

15. Block-Galaraza, J., Chase, K.O., Sapp, E., Vaughn, K.T., Vallee, R.B., DiFiglia, M., Aronin, N. (1997) Fast transport and retrograde movement of huntingtin and HAP1 in axons. *NeuroReport* 8, 2247-2251.

16. Brodsky, F.M., Chen, C.Y., Knuehl, C., Towler, M.C., Wakeham, D.E. (2001) Biological basket weaving: formation and function of clathrin-coated vesicles. *Annu Rev Cell Dev Biol.* 17, 517-68.

17. Boutell, J.M., Thomas, P., Neal, J.W., Weston, V.J., Duce, J., Harper, P.S., and Jones, A.L. (1999). Aberrant interactions of transcriptional repressor proteins with the Huntington's disease gene product, huntingtin. *Hum. Mol. Genet.* 8, 1647-1655.

18. Boutell, J.M., Wood, J.D., Harper, P.S., and Jones, A.L. (1998). Huntingtin interacts with cystathionine beta-synthase. *Hum. Mol. Genet.* 7, 371-378.

19. Bowman, A.L. and Kahn, R.A. (1995) ARF proteins, the membrane traffic police? *Trends Biochem. Sci.* 20, 147-150.

20. Brinkman, R.R., Mezei, M.M., Theilmann, J., Almqvist, E., and Hayden, M.R. (1997). The likelihood of being affected with Huntington disease by a particular age, for a specific CAG size. *Am. J. Hum. Genet.* 60, 1202-1210.

21. Brodsky, F.M., Chen, C.Y., Knuehl, C., Towler, M.C., and Wakeham, D.E. (2001). Biological basket weaving: formation and function of clathrin-coated vesicles. *Annu. Rev. Cell Dev. Biol.* 17, 517-568.

22. Brunet, A., Datta, S.R., and Greenberg, M.E. (2001). Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. *Curr. Opin. Neurobiol.* 11, 297-305.

23. Burke, J.R., Enghild, J.J., Martin, M.E., Jou, Y.S., Myers, R.M., Roses, A.D., Vance, J.M., and Strittmatter, W.J. (1996). Huntingtin and DRPLA proteins selectively interact with the enzyme GAPDH. *Nat. Med.* 2, 347-350.

24. Cattaneo, E., Rigamonti, D., Goffredo, D., Zuccato, C., Squitieri, F., and Sipione, S. (2001). Loss of normal huntingtin function: new developments in Huntington's disease research. *Trends Neurosci.* 24, 182-188.

25. Chan, E.Y., Luthi-Carter, R., Strand, A., Solano, S.M., Hanson, S.A., DeJohn, M.M., Kooperberg, C., Chase, K.O., DiFiglia, M., Young, A.B., Leavitt, B.R., Cha, J.H., Aronin, N., Hayden, M.R., and Olson, J.M. (2002). Increased huntingtin protein length reduces the number of polyglutamine-induced gene expression changes in mouse models of Huntington's disease. *Hum. Mol. Genet.* 11, 1939-1951.

26. Chen, H.K., Fernandez-Funez, P., Acevedo, S.F., Lam, Y.C., Kaytor. M.D., Fernandez, M.H., Aitken, A., Skoulakis, E.M., Orr, H.T., Botas, J., Zoghbi, H.Y. (2003). Interaction of akt-phosphorylated ataxin-1 with 14-3-3 mediates neurodegeneration in spinocerebellar ataxia type 1.*Cell.* 113, 457-468.

27. Chen, N., Luo, T., Wellington, C., Metzler, M., McCutcheon, K., Hayden, M.R., and Raymond, L.A. (1999). Subtype-specific enhancement of NMDA receptor currents by mutant huntingtin. *J. Neurochem.* 72, 1890-1898.

28.Chen, H., Fre, S., Slepnev, V.I., Capua, M.R., Takei, K., Butler, M.H., Di Fiore, P.P. and De Camilli, P. (1998) Epsin is an EH-domain-binding protein implicated in clathrin-mediated endocytosis. *Nature* 394, 793-797.

29. Chopra, V.S., Metzler, M., Rasper, D.M., Engqvist-Goldstein, A.E., Singaraja, R., Gan, L., Fichter, K.M., McCutcheon, K., Drubin, D., Nicholson, D.W., and Hayden, M.R. (2000). HIP12 is a non-proapoptotic member of a gene family including HIP1, an interacting protein with huntingtin. *Mamm. Genome* 11, 1006-1015.

30. Colomer, V., Engelender, S., Sharp, A.H., Duan, K., Cooper, J.K., Lanahan, A., Lyford, G., Worley, P., and Ross, C.A. (1997). Huntingtin-associated protein 1 (HAP1) binds to a Trio-like polypeptide, with a rac1 guanine nucleotide exchange factor domain. *Hum. Mol. Genet.* 6, 1519-1525.

31. Cooper, J.K., Schilling, G., Peters, M.F., Herring, W.J., Sharp, A.H., Kaminsky, Z., Masone, J., Khan, F.A., Delanoy, M., Borchelt, D.R., Dawson, V.L., Dawson, T.M., and Ross, C.A. (1998). Truncated N-terminal fragments of huntingtin with expanded glutamine repeats form nuclear and cytoplasmic aggregates in cell culture. *Hum. Mol. Genet.* 7, 783-790.

32. Courey, A.J. and Tjian, R. (1988). Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. *Cell* 55, 887-898.

33. Cousin, M.A., Robinson, P.J. (2001) The dephosphins: dephosphorylation by calcineurin triggers synaptic vesicle endocytosis. *Trends Neurosci.* 24, 659-665.

34. Cousin, M.A., Tan, T.C., Robinson, P.J. (2001) Protein phosphorylation is required for endocytosis in nerve terminals: potential role for the dephosphins dynamin I and synaptojanin, but not AP180 or amphiphysin. *J Neurochem.* 76, 105-16.

35. Critchley, D.R. (2000). Focal adhesions - the cytoskeletal connection. *Curr. Opin. Cell Biol.* 12, 133-139.

36. David, G., Abbas, N., Stevanin, G., Durr, A., Yvert, G., Cancel, G., Weber, C., Imbert, G., Saudou, F., Antoniou, E., Drabkin, H., Gemmill, R., Giunti, P., Benomar, A., Wood, N., Ruberg, M., Agid, Y., Mandel. J.L., and Brice, A. (1997). Cloning of the SCA7 gene reveals a highly unstable CAG repeat expansion. *Nat. Genet.* 17, 65-70.

37. Davies, S.W., Turmaine, M., Cozens, B.A., DiFiglia, M., Sharp, A.H., Ross, C.A., Scherzinger, E., Wanker, E.E., Mangiarini, L., and Bates, G.P. (1997). Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation. *Cell* 90, 537-548.

38. De Camilli, P., Chen, H., Hyman, J., Panepucci, E., Bateman, A., and Brunger, A.T. (2002). The ENTH domain. *FEBS Lett.* 513, 11-18.

39. Deckel, A.W. (2001) Nitric oxide and nitric oxide synthase in Huntington's disease. *J Neurosci Res.* 64, 99-107.

40. de Groot, R.P., Coffer, P.J., and Koenderman, L. (1998). Regulation of proliferation, differentiation and survival by the IL-3/IL-5/GM-CSF receptor family. *Cell Signal.* 10, 619-628.

41. Del Favero, J., Krols, L., Michalik, A., Theuns, J., Lofgren, A., Goossens, D., Wehnert, A., Van den Bossche, D., Van Zand, K., Backhovens, H., van Regenmorter, N., Martin, J.J., and Van Broeckhoven, C. (1998). Molecular genetic analysis of autosomal dominant cerebellar ataxia with retinal degeneration (ADCA type II) caused by CAG triplet repeat expansion. *Hum. Mol. Genet.* 7, 177-186.

42. DiFiglia, M., Sapp, E., Chase, K.O., Davies, S.W., Bates, G.P., Vonsattel, J.P., and Aronin, N. (1997). Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. *Science* 277, 1990-1993.

43. DiFiglia, M., Sapp, E., Chase, K., Schwarz, C., Meloni, A., Young, C., Martin, E., Vonsattel, J.P., Carraway, R., Reeves, S.A., and . (1995). Huntingtin is a cytoplasmic protein associated with vesicles in human and rat brain neurons. *Neuron* 14, 1075-1081.

44. Dore, S., Kar, S., Zheng, W.H., and Quirion, R. (2000). Rediscovering good old friend IGF-I in the new millenium: possible usefulness in Alzheimer's disease and stroke. *Pharm. Acta Helv.* 74, 273-280.

45. Dragatsis, I., Levine, M.S., and Zeitlin, S. (2000). Inactivation of Hdh in the brain and testis results in progressive neurodegeneration and sterility in mice. *Nat. Genet.* 26, 300-306.

46. Dragunow, M., Faull, R.L., Lawlor, P., Beilharz, E.J., Singleton, K., Walker, E.B., and Mee, E. (1995). In situ evidence for DNA fragmentation in Huntington's disease striatum and Alzheimer's disease temporal lobes. *Neuroreport* 6, 1053-1057.

47. Drake, M.T., and Traub, L.M. (2001) Interaction of two structurally distinct sequence types with the clathrin terminal domain beta-propeller. *J. Biol. Chem.* 276, 28700-28709.

48. Drake, M.T., Downs, M.A., and Traub, L.M. (2000) Epsin binds to clathrin by associating directly with the clathrin-terminal domain. Evidence for cooperative binding through two discrete sites. *J. Biol. Chem.* 275, 6479-6489.

49. Duyao, M., Ambrose, C., Myers, R., Novelletto, A., Persichetti, F., Frontali, M., Folstein, S., Ross, C., Franz, M., Abbott, M., and . (1993). Trinucleotide repeat length instability and age of onset in Huntington's disease. *Nat. Genet.* 4, 387-392.

50. Duyao, M.P., Auerbach, A.B., Ryan, A., Persichetti, F., Barnes, G.T., McNeil, S.M., Ge, P., Vonsattel, J.P., Gusella, J.F., Joyner, A.L., and . (1995). Inactivation of the mouse Huntington's disease gene homolog Hdh. *Science* 269, 407-410.

51. El-Husseini, Ael-D. and Bredt, D.S. (2002) Protein palmitoylation: a regulator of neuronal development and function. *Nat Rev Neurosci.* 3, 791-802.

52. Engelender, S., Sharp, A.H., Colomer, V., Tokito, M.K., Lanahan, A., Worley, P., Holzbaur, E.L., and Ross, C.A. (1997). Huntingtin-associated protein 1 (HAP1) interacts with the p150Glued subunit of dynactin. *Hum. Mol. Genet.* 6, 2205-2212.

53. Engqvist-Goldstein, A.E., Warren, R.A., Kessels, M.M., Keen, J.H., Heuser, J., and Drubin, D.G. (2001). The actin-binding protein Hip1R associates with clathrin during early stages of endocytosis and promotes clathrin assembly in vitro. *J. Cell Biol.* 154, 1209-1223.

54. Engqvist-Goldstein, A.E., Kessels, M.M., Chopra, V.S., Hayden, M.R., and Drubin, D.G. (1999). An actin-binding protein of the Sla2/Huntingtin interacting protein 1 family is a novel component of clathrin-coated pits and vesicles. *J. Cell Biol.* 147, 1503-1518.

55. Emamian ES, Kaytor MD, Duvick LA, Zu T, Tousey SK, Zoghbi HY, Clark HB, Orr HT. (2003) Serine 776 of ataxin-1 is critical for polyglutamine-induced disease in SCA1 transgenic mice. *Neuron.* 38, 375-387.

56. Faber, P.W., Barnes, G.T., Srinidhi, J., Chen, J., Gusella, J.F., and MacDonald, M.E. (1998). Huntingtin interacts with a family of WW domain proteins. *Hum. Mol. Genet.* 7, 1463-1474.

57. Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* 391, 806-811.

58. Ford, M.G., Mills, I.G., Peter, B.J., Vallis, Y., Praefcke, G.J., Evans, P.R., and McMahon, H.T. (2002). Curvature of clathrin-coated pits driven by epsin. *Nature* 419, 361-366.

59. Ford, M.G., Pearse, B.M., Higgins, M.K., Vallis, Y., Owen, D.J., Gibson, A., Hopkins, C.R., Evans, P.R., and McMahon, H.T. (2001). Simultaneous binding of PtdIns(4, 5)P2 and clathrin by AP180 in the nucleation of clathrin lattices on membranes. *Science* 291, 1051-1055.

60. Fujimoto, L.M., Roth, R., Heuser, J.E., and Schmid, S.L. (2000). Actin assembly plays a variable, but not obligatory role in receptor-mediated endocytosis in mammalian cells. *Traffic.* 1, 161-171.

61. Gafni, J. and Ellerby, L.M. (2002). Calpain activation in Huntington's disease. *J. Neurosci.* 22, 4842-4849.

62. Gaidarov, I., Santini, F., Warren, R. A., and Keen, J. H. (1999) Spatial control of coated-pit dynamics in living cells. *Nat. Cell. Biol.* 1, 1-7.

63. Geitz, R.D., Woods, R.A., Manivasakam, P., Scheistl, R.H. Yeast growth and yeast transformation. in Cell Biology:Alaboratory Manual (eds Spector, D., Goldman, R., and Leinwand, L.) (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1996).

64. Gervais, F.G., Singaraja, R., Xanthoudakis, S., Gutekunst, C.A., Leavitt, B.R., Metzler, M., Hackam, A.S., Tam, J., Vaillancourt, J.P., Houtzager, V., Rasper, D.M., Roy, S., Hayden, M.R., and Nicholson, D.W. (2002). Recruitment and activation of caspase-8 by the Huntingtin-interacting protein Hip-1 and a novel partner Hippi. *Nat. Cell Biol.* 4, 95-105.

65. Goldberg, Y.P., Nicholson, D.W., Rasper, D.M., Kalchman, M.A., Koide, H.B., Graham, R.K., Bromm, M., Kazemi-Esfarjani, P., Thornberry, N.A., Vaillancourt, J.P., and Hayden, M.R. (1996). Cleavage of huntingtin by apopain, a proapoptotic cysteine protease, is modulated by the polyglutamine tract. *Nat. Genet.* 13, 442-449.

66. Green, H. (1993). Human genetic diseases due to codon reiteration: relationship to an evolutionary mechanism. *Cell* 74, 955-956.

67. Greenamyre JT, Higgins DS, Eller RV. (1992) Quantitative autoradiography of dihydrorotenone binding to complex I of the electron transport chain. *J Neurochem.* 59, 746-749.

68. Gutekunst, C.A., Levey, A.I., Heilman, C.J., Whaley, W.L., Yi, H., Nash, N.R., Rees, H.D., Madden, J.J., and Hersch, S.M. (1995). Identification and localization of huntingtin in brain and human lymphoblastoid cell lines with anti-fusion protein antibodies. *Proc. Natl. Acad. Sci. U. S. A* 92, 8710-8714.

69. Gutekunst, C.A., Li, S.H., Yi, H., Ferrante, R.J., Li, X.J., and Hersch, S.M. (1998). The cellular and subcellular localization of huntingtin-associated protein 1 (HAP1): comparison with huntingtin in rat and human. *J. Neurosci.* 18, 7674-7686.

70. Hackam, A.S., Singaraja, R., Wellington, C.L., Metzler, M., McCutcheon, K., Zhang, T., Kalchman, M., and Hayden, M.R. (1998). The influence of huntingtin protein size on nuclear localization and cellular toxicity. J. Cell Biol. 141, 1097-1105.

71. Hackam, A.S., Singaraja, R., Wellington, C.W., Rasper, D., Roy, S., Nicholson, D. Huntingtin interacting protein-1 (HIP1) accelerates cell death and intracellular aggregate formation in the presence of mutant huntingtin. (1998. Abstract # 1894 The American Society of Human Genetics 49th Annual Meeting October 27-31. Denver, Colorado).

72. Hackam, A.S., Yassa, A.S., Singaraja, R., Metzler, M., Gutekunst, C.A., Gan, L., Warby, S., Wellington, C.L., Vaillancourt, J., Chen, N., Gervais, F.G., Raymond, L., Nicholson, D.W., and Hayden, M.R. (2000). Huntingtin interacting protein 1 induces apoptosis via a novel caspase-dependent death effector domain. *J. Biol. Chem.* 275, 41299-41308.

73. Hao, W., Luo, Z., Zheng, L., Prasad, K., and Lafer, E.M.. (1999) AP180 and AP-2 interact directly in a complex that cooperatively assembles clathrin. *J. Biol. Chem.* 274, 22785-22794.

74. Hicke, L. (2001) Protein regulation by monoubiquitin. *Nat Rev Mol Cell Biol.* 2, 195-201.

75. Hicke, L. (1999) Gettin' down with ubiquitin: turning off cell-surface receptors, transporters and channels. *Trends Cell Biol.* 9, 107-112.

76. Hayden, M.R. (1981). Huntington's Chorea. (Berlin and New York: Springer-Verlag).

77. Hirst, J. and Robinson, M.S. (1998). Clathrin and adaptors. *Biochim. Biophys. Acta* 1404, 173-193.

78. Ho, L.W., Brown, R., Maxwell, M., Wyttenbach, A., and Rubinsztein, D.C. (2001). Wild type Huntingtin reduces the cellular toxicity of mutant Huntingtin in mammalian cell models of Huntington's disease. *J. Med. Genet.* 38, 450-452.

79. Hodgson, J.G., Agopyan, N., Gutekunst, C.A., Leavitt, B.R., LePiane, F., Singaraja, R., Smith, D.J., Bissada, N., McCutcheon, K., Nasir, J., Jamot, L., Li, X.J., Stevens, M.E., Rosemond, E., Roder, J.C., Phillips, A.G., Rubin, E.M., Hersch, S.M., and Hayden, M.R. (1999). A YAC mouse model for Huntington's disease with full-length mutant huntingtin, cytoplasmic toxicity, and selective striatal neurodegeneration. *Neuron* 23, 181-192.

80. Holbert S, Dedeoglu A, Humbert S, Saudou F, Ferrante RJ, Neri C. (2003) Cdc42-interacting protein 4 binds to huntingtin: neuropathologic and biological evidence for a role in Huntington's disease. *Proc Natl Acad Sci U S A.* 100, 2712-2717.

81. Holbert, S., Denghien, I., Kiechle, T., Rosenblatt, A., Wellington, C., Hayden, M.R., Margolis, R.L., Ross, C.A., Dausset, J., Ferrante, R.J., and Neri, C. (2001). The Gln-Ala repeat transcriptional activator CA150 interacts with huntingtin: neuropathologic and genetic evidence for a role in Huntington's disease pathogenesis. *Proc. Natl. Acad. Sci. U. S. A* 98, 1811-1816.

82. Holbert, S., Dedeoglu, A., Humbert, S., Saudou, F., Ferrante, R.J., and Neri, C. (2003). Cdc42-interacting protein 4 binds to huntingtin: Neuropathologic and biological evidence for a role in Huntington's disease. *Proc. Natl. Acad. Sci. U. S. A* 100, 2712-2717.

83. Holmberg, M., Duyckaerts, C., Durr, A., Cancel, G., Gourfinkel-An, I., Damier, P., Faucheux, B., Trottier, Y., Hirsch, E.C., Agid, Y., and Brice, A. (1998). Spinocerebellar ataxia type 7 (SCA7): a neurodegenerative disorder with neuronal intranuclear inclusions. *Hum. Mol. Genet.* 7, 913-918.

84. Holtzman, D.A., Yang, S., and Drubin, D.G. (1993). Synthetic-lethal interactions identify two novel genes, SLA1 and SLA2, that control membrane cytoskeleton assembly in Saccharomyces cerevisiae. *J. Cell Biol.* 122, 635-644.

85. Humbert, S., Bryson, E.A., Cordelieres, F.P., Connors, N.C., Datta, S.R., Finkbeiner, S., Greenberg, M.E., and Saudou, F. (2002). The IGF-1/Akt pathway is neuroprotective in Huntington's disease and involves Huntingtin phosphorylation by Akt. *Dev. Cell* 2, 831-837.

86. Hussain, N.K., Yamabhai, M., Ramjaun, A.R., Guy, A.M., Baranes, D., O'Bryan, J.P., Der , C.J., Kay, B.K., and McPherson, P.S. (1999) Splice variants of intersectin are components of the endocytic machinery in neurons and nonneuronal cells. *J. Biol. Chem.* 274, 15671-15677.

87. Imbert, G., Saudou, F., Yvert, G., Devys, D., Trottier, Y., Garnier, J.M., Weber, C., Mandel, J.L., Cancel, G., Abbas, N., Durr, A., Didierjean, O., Stevanin, G., Agid, Y., and Brice, A. (1996). Cloning of the gene for spinocerebellar ataxia 2 reveals a locus with high sensitivity to expanded CAG/glutamine repeats. *Nat. Genet.* 14, 285-291.

88. Ishikawa, K., Nagase, T., Suyama, M., Miyajama, N., Tanaka, A., Kotani, H., Nomura, N., Ohara, O. (1998) Prediction of the coding sequences of unidentified human genes. X. The complete sequences of 100 new cDNA clones from brain which can code for large proteins *in vitro*. *DNA Res.* 5, 169-76.

89. Itoh, T., Koshiba, S., Kigawa, T., Kikuchi, A., Yokoyama, S., and Takenawa, T. (2001). Role of the ENTH domain in phosphatidylinositol-4, 5-bisphosphate binding and endocytosis. *Science* 291, 1047-1051.

90. Jana, N.R., Tanaka, M., Wang, G., and Nukina, N. (2000). Polyglutamine length-dependent interaction of Hsp40 and Hsp70 family chaperones with truncated N-terminal huntingtin: their role in suppression of aggregation and cellular toxicity. *Hum. Mol. Genet.* 9, 2009-2018.

91. Jiang, Z.Y., Chawla, A., Bose, A., Way, M., and Czech, M.P. (2002). A phosphatidylinositol 3-kinase-independent insulin signaling pathway to N-WASP/Arp2/3/F-actin required for GLUT4 glucose transporter recycling. *J. Biol. Chem.* 277, 509-515.

92. Jordan, M., Schallhorn, A., Wurm, F.M. (1996) Transfecting mammalian cells:optimization of critical parameters affecting calcium-phosphate precipitate formation. *Nucleic Acids Res.* 24, 596-601.

93. Kalchman, M.A., Koide, H.B., McCutcheon, K., Graham, R.K., Nichol, K., Nishiyama, K., Kazemi-Esfarjani, P., Lynn, F.C., Wellington, C., Metzler, M., Goldberg, Y.P., Kanazawa, I., Gietz, R.D., and Hayden, M.R. (1997). HIP1, a human homologue of S. cerevisiae Sla2p, interacts with membrane-associated huntingtin in the brain. *Nat. Genet.* 16, 44-53.

94. Kalchman, M.A., Graham, R.K., Xia, G., Koide, H.B., Hodgson, J.G., Graham, K.C., Goldberg, Y.P., Gietz, R.D., Pickart, C.M., and Hayden, M.R. (1996). Huntingtin is ubiquitinated and interacts with a specific ubiquitin-conjugating enzyme. *J. Biol. Chem.* 271, 19385-19394.

95. Kao, C.C., Lieberman, P.M., Schmidt, M.C., Zhou, Q., Pei, R., and Berk, A.J. (1990). Cloning of a transcriptionally active human TATA binding factor. *Science* 248, 1646-1650.

96. Karpuj, M.V., Garren, H., Slunt, H., Price, D.L., Gusella, J., Becher, M.W., and Steinman, L. (1999). Transglutaminase aggregates huntingtin into nonamyloidogenic polymers, and its enzymatic activity increases in Huntington's disease brain nuclei. *Proc. Natl. Acad. Sci. U. S. A* 96, 7388-7393.

97. Katoh-Semba R, Semba R, Takeuchi IK, Kato K. (1998) Age-related changes in levels of brain-derived neurotrophic factor in selected brain regions of rats, normal mice and senescence-accelerated mice: a comparison to those of nerve growth factor and neurotrophin-3. *Neurosci Res.* 31, 227-234.

98. Kawaguchi, Y., Okamoto, T., Taniwaki, M., Aizawa, M., Inoue, M., Katayama, S., Kawakami, H., Nakamura, S., Nishimura, M., Akiguchi, I. (1994). CAG expansions in a novel gene for Machado-Joseph disease at chromosome 14q32.1. *Nat. Genet.* 8, 221-228.

99. Kay, B.K., Yamabhai, M., Wendland, B., and Emr, S.D. (1999). Identification of a novel domain shared by putative components of the endocytic and cytoskeletal machinery. *Protein Sci.* 8, 435-438.

100. Keen, J. H., Willingham, M. C., and Pastan, I. H. (1979) Clathrin-coated vesicles: isolation, dissociation and factor-dependent reassociation of clathrin baskets. *Cell* 16, 303-312.

101. Kegel, K.B., Meloni, A.R., Yi, Y., Kim, Y.J., Doyle, E., Cuiffo, B.G., Sapp, E., Wang, Y., Qin, Z.H., Chen, J.D., Nevins, J.R., Aronin, N., and DiFiglia, M. (2002). Huntingtin is present in the nucleus, interacts with the transcriptional corepressor C-terminal binding protein, and represses transcription. *J. Biol. Chem.* 277, 7466-7476.

102. Kegel, K. B., Kim, M., Sapp, E., McIntyre, C., Castano, J. G., Aronin, N., and DiFiglia, M. (2000) Huntingtin expression stimulates endosomal-lysosomal activity, endosome tubulation, and autophagy. *J. Neurosci.* 20, 7268-7278.

103. Kessels, M.M., Engqvist-Goldstein, A.E., Drubin, D.G., and Qualmann, B. (2001). Mammalian Abp1, a signal-responsive F-actin-binding protein, links the actin cytoskeleton to endocytosis via the GTPase dynamin. *J. Cell Biol.* 153, 351-366.

104. Kim, J.A. and Kim, H.L. (2001). Cleavage of purified neuronal clathrin assembly protein (CALM) by caspase 3 and calpain. *Exp. Mol. Med.* 33, 245-250.

105. Kim, M., Lee, H.S., Laforet, G., McIntyre, C., Martin, E.J., Chang, P., Kim, T.W., Williams, M., Reddy, P.H., Tagle, D., Boyce, F.M., Won, L., Heller, A., Aronin, N., and DiFiglia, M. (1999). Mutant huntingtin expression in clonal striatal cells: dissociation of inclusion formation and neuronal survival by caspase inhibition. *J. Neurosci.* 19, 964-973.

106. Kim, Y.J., Yi, Y., Sapp, E., Wang, Y., Cuiffo, B., Kegel, K.B., Qin, Z.H., Aronin, N., and DiFiglia, M. (2001). Caspase 3-cleaved N-terminal fragments of wild-type and mutant huntingtin are present in normal and Huntington's disease brains, associate with membranes, and undergo calpain-dependent proteolysis. *Proc. Natl. Acad. Sci. U. S. A* 98, 12784-12789.

107. Koide R, Ikeuchi T, Onodera O, Tanaka H, Igarashi S, Endo K, Takahashi H, Kondo R, Ishikawa A, Hayashi T, and et al. (1994). Unstable expansion of CAG repeat in hereditary dentatorubral-pallidoluysian atrophy (DRPLA). *Nat. Genet.* 6, 9-13.

108. Kremer, B., Weber, B., and Hayden, M.R. (1992). New insights into the clinical features, pathogenesis and molecular genetics of Huntington disease. *Brain Pathol.* 2, 321-335.

109. Laporte, S. A., Oakley, R. H., Zhang, J., Holt, J. A., Ferguson, S. S., Caron, M. G., and Barak, L. S. (1999) The beta2-adrenergic receptor/betaarrestin complex recruits the clathrin adaptor AP-2 during endocytosis. *Proc. Natl. Acad. Sci. U. S. A.* 96, 3712-3717.

110. LaSpada, Wilson EM, Lubahn DB, Harding AE, and Fischbeck KH (1991). Androgen receptor gene mutations in X-linked spinal and bulbar muscular atrophy. *Nature* 352, 77-79.

111. Leavitt, B.R., Guttman, J.A., Hodgson, J.G., Kimel, G.H., Singaraja, R., Vogl, A.W., and Hayden, M.R. (2001). Wild-type huntingtin reduces the cellular toxicity of mutant huntingtin in vivo. *Am. J. Hum. Genet.* 68, 313-324.

112. Li, S.H., Lam, S., Cheng, A.L., and Li, X.J. (2000). Intranuclear huntingtin increases the expression of caspase-1 and induces apoptosis. *Hum. Mol. Genet.* 9, 2859-2867.

113. Li, S.H., Gutekunst, C.A., Hersch, S.M., and Li, X.J. (1998b). Interaction of huntingtin-associated protein with dynactin P150Glued. *J. Neurosci.* 18, 1261-1269.

114. Li, S.H., Schilling, G., Young, W.S., III, Li, X.J., Margolis, R.L., Stine, O.C., Wagster, M.V., Abbott, M.H., Franz, M.L., Ranen, N.G., and . (1993). Huntington's disease gene (IT15) is widely expressed in human and rat tissues. *Neuron* 11, 985-993.

115. Li, M., Miwa, S., Kobayashi, Y., Merry, D.E., Yamamoto, M., Tanaka, F., Doyu, M., Hashizume, Y., Fischbeck, K.H., and Sobue, G. (1998a). Nuclear inclusions of the androgen receptor protein in spinal and bulbar muscular atrophy. *Ann. Neurol.* 44, 249-254.

116. Li, X.J., Li, S.H., Sharp, A.H., Nucifora, F.C., Jr., Schilling, G., Lanahan, A., Worley, P., Snyder, S.H., and Ross, C.A. (1995). A huntingtin-associated protein enriched in brain with implications for pathology. *Nature* 378, 398-402.

117. Li, Y., Chin, L.S., Levey, A.I., and Li, L. (2002). Huntingtin-associated protein 1 interacts with hepatocyte growth factor-regulated tyrosine kinase substrate and functions in endosomal trafficking. *J. Biol. Chem.* 277, 28212-28221.

118. Lila, T. and Drubin, D.G. (1997). Evidence for physical and functional interactions among two Saccharomyces cerevisiae SH3 domain proteins, an adenylyl cyclase-associated protein and the actin cytoskeleton. *Mol. Biol. Cell* 8, 367-385.

119. Lin, B., Nasir, J., MacDonald, H., Hutchinson, G., Graham, R.K., Rommens, J.M., and Hayden, M.R. (1994). Sequence of the murine Huntington disease gene: evidence for conservation, alternate splicing and polymorphism in a triplet (CCG) repeat [corrected]. *Hum. Mol. Genet.* 3, 85-92.

120. Lippincott-Schwartz, J. (1998) Cytoskeletal proteins and Golgi dynamics. *Curr. Opin. Cell Biol.* 10, 52-59.

121. Liu, Y.F. (1998). Expression of polyglutamine-expanded Huntingtin activates the SEK1-JNK pathway and induces apoptosis in a hippocampal neuronal cell line. *J. Biol. Chem.* 273, 28873-28877.

122. Liu, Y.F., Deth, R.C., and Devys, D. (1997). SH3 domain-dependent association of huntingtin with epidermal growth factor receptor signaling complexes. *J. Biol. Chem.* 272, 8121-8124.

123. Liu, Y.F., Dorow, D., and Marshall, J. (2000). Activation of MLK2-mediated signaling cascades by polyglutamine-expanded huntingtin. *J. Biol. Chem.* 275, 19035-19040.

124. Lunkes, A., Lindenberg, K.S., Ben Haiem, L., Weber, C., Devys, D., Landwehrmeyer, G.B., Mandel, J.L., and Trottier, Y. (2002). Proteases acting on mutant huntingtin generate cleaved products that differentially build up cytoplasmic and nuclear inclusions. Mol. Cell 10, 259-269.

125. Lupas, A. (1996). Coiled coils: new structures and new functions. *Trends Biochem. Sci.* 21, 375-382.

126. Luthi-Carter, R., Strand, A.D., Hanson, S.A., Kooperberg, C., Schilling, G., La Spada, A.R., Merry, D.E., Young, A.B., Ross, C.A., Borchelt, D.R., and Olson, J.M. (2002). Polyglutamine and transcription: gene expression changes shared by DRPLA and Huntington's disease mouse models reveal context-independent effects. *Hum. Mol. Genet.* 11, 1927-1937.

127. Mangiarini, L., Sathasivam, K., Seller, M., Cozens, B., Harper, A., Hetherington, C., Lawton, M., Trottier, Y., Lehrach, H., Davies, S.W., and Bates, G.P. (1996). Exon 1 of the HD gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. *Cell* 87, 493-506.

128. Marsh, M., and McMahon, H. T. (1999) The structural era of endocytosis. *Science* 285, 215-220.

129. Martin, E.J., Kim, M., Velier, J., Sapp, E., Lee, H.S., Laforet, G., Won, L., Chase, K., Bhide, P.G., Heller, A., Aronin, N., and DiFiglia, M. (1999). Analysis of Huntingtin-associated protein 1 in mouse brain and immortalized striatal neurons. *J. Comp Neurol.* 403, 421-430.

130. Martin, J.B. and Gusella, J.F. (1986). Huntington's disease. Pathogenesis and management. *N. Engl. J. Med.* 315, 1267-1276.

131. Martindale, D., Hackam, A., Wieczorek, A., Ellerby, L., Wellington, C., McCutcheon, K., Singaraja, R., Kazemi-Esfarjani, P., Devon, R., Kim, S.U., Bredesen, D.E., Tufaro, F., and Hayden, M.R. (1998). Length of huntingtin and its polyglutamine tract influences localization and frequency of intracellular aggregates. *Nat. Genet.* 18, 150-154.

132. Maycox, P. R., Link, E., Reetz, A., Morris, S. A., and Jahn, R. (1992) Clathrin-coated vesicles in nervous tissue are involved primarily in synaptic vesicle recycling. *J. Cell Biol.* 118, 1379-1380.

133. McCann, R.O. and Craig, S.W. (1997). The I/LWEQ module: a conserved sequence that signifies F-actin binding in functionally diverse proteins from yeast to mammals. Proc. Natl. Acad. Sci. U. S. A *94*, 5679-5684.

134. McPherson, P. S. (1999) Regulatory role of SH3 domain-mediated protein-protein interactions in synaptic vesicle endocytosis. *Cell. Signal.* 11, 229-238.

135. McPherson, P.S., Czernik, Chilcote, T.J., Onfori, F., Benfati, F, Greengard, P., Schlessinger, J., De Camilli, P. (1994) Interaction of Grb2 via its Src homology 3 domains with synaptic proteins including synapsin 1. *Proc. Natl. Acad. Sci. USA* 91, 6486-6490.

136. Merrifield, C.J., Moss, S.E., Ballestrem, C., Imhof, B.A., Giese, G., Wunderlich, I., and Almers, W. (1999). Endocytic vesicles move at the tips of actin tails in cultured mast cells. *Nat. Cell Biol.* 1, 72-74.

137. Metzler M, Li B, Gan L, Georgiou J , Gutekunst CA, Torre E, Devon RS, Oh R, Legendre-Guillemin V, Rich M, Alvarez C, Gertsenstein M, McPherson PS, Nagy A, Roder JC, Raymond LA, Hayden MR. (2003) Targeted Disruption of the Endocytic Protein HIP1 Results in Major Neurological Deficits Associated with Defects in AMPA Receptor Trafficking. *EMBO 22,* 3254-326

138. Metzler, M., Legendre-Guillemin, V., Gan, L., Chopra, V., Kwok, A., McPherson, P.S., and Hayden, M.R. (2001). HIP1 functions in clathrin-mediated endocytosis through binding to clathrin and adaptor protein 2. *J. Biol. Chem.* 276, 39271-39276.

139. Metzler, M., Helgason, C.D., Dragatsis, I., Zhang, T., Gan, L., Pineault, N., Zeitlin, S.O., Humphries, R.K., and Hayden, M.R. (2000). Huntingtin is required for normal hematopoiesis. *Hum. Mol. Genet.* 9, 387-394.

140. Metzler, M., Chen N., Helgason, C.D., Graham, R.K., Nichol, K., McCutcheon, K., Nasir, J., Humphries, R.K., Raymond, L.A., Hayden, MR. (1999) Life without huntingtin: normal differentiation into functional neurons. *J. Neurochem.* 72, 1009-18.

141. Micheva, K.D., Holz, R.W., and Smith, S.J. (2001) Regulation of presynaptic phosphatidylinositol 4, 5-biphosphate by neuronal activity. *J. Cell Biol* 154, 355-368.

142. Mishra SK, Agostinelli NR, Brett TJ, Mizukami I, Ross TS, Traub LM. (2001) Clathrin- and AP-2-binding sites in HIP1 uncover a general assembly role for endocytic accessory proteins. *J Biol Chem.* 276, 46230-46236.

143. Modregger, J., DiProspero, N.A., Charles, V., Tagle, D.A., and Plomann, M. (2002). PACSIN 1 interacts with huntingtin and is absent from synaptic varicosities in presymptomatic Huntington's disease brains. *Hum. Mol. Genet.* 11, 2547-2558.

144. Moore, R.H., Sadovnikoff, N., Hoffenberg, S., Liu, S., Woodford, P., Angelides, K., Trial, J.A., Carsrud, N.D., Dickey, B.F., and Knoll, B.J. (1995) Ligand-stimulated beta 2-adrenergic receptor internalization via the constitutive endocytic pathway into rab5-containing endosomes. *J. Cell Sci.* 108, 2983-2991.

145. Morgan, J.R., Prasad, K, Hao, W., Augustine, G.J., and Lafer, E.M. (2000) A conserved clathrin assembly motif essential for synaptic vesicle endocytosis. *J. Neurosci.* 20, 8667-8676.

146. Morgan, J. R., Zhao, X., Womack, M., Prasad, K., Augustine, G. J., and Lafer, E. M. (1999) A role for the clathrin assembly domain of AP180 in synaptic vesicle endocytosis. *J. Neurosci.* 19, 10201-10212.

147. Moulder, G.L., Huang, M.M., Waterston, R.H., and Barstead, R.J. (1996). Talin requires beta-integrin, but not vinculin, for its assembly into focal adhesion-like structures in the nematode Caenorhabditis elegans. *Mol. Biol. Cell* 7, 1181-1193.

148. Mulholland, J., Wesp, A., Riezman, H., and Botstein, D. (1997). Yeast actin cytoskeleton mutants accumulate a new class of Golgi-derived secretary vesicle. *Mol. Biol. Cell* 8, 1481-1499.

149. Na, S., Hincapie, M., McCusker, J.H., and Haber, J.E. (1995). MOP2 (SLA2) affects the abundance of the plasma membrane H(+)-ATPase of Saccharomyces cerevisiae. *J. Biol. Chem.* 270, 6815-6823.

150. Nakao N, Brundin P, Funa K, Lindvall O, Odin P. (1995) Trophic and protective actions of brain-derived neurotrophic factor on striatal DARPP-32-containing neurons in vitro. *Brain Res Dev Brain Res.* 90, 92-101.

151. Nance, M.A., Mathias-Hagen, V., Breningstall, G., Wick, M.J., and McGlennen, R.C. (1999). Analysis of a very large trinucleotide repeat in a patient with juvenile Huntington's disease. *Neurology* 52, 392-394.

152. Nasir, J., Floresco, S.B., O'Kusky, J.R., Diewert, V.M., Richman, J.M., Zeisler, J., Borowski, A., Marth, J.D., Phillips, A.G., and Hayden, M.R. (1995). Targeted disruption of the Huntington's disease gene results in embryonic lethality and behavioral and morphological changes in heterozygotes. *Cell* 81, 811-823.

153. Nucifora, F.C., Jr., Sasaki, M., Peters, M.F., Huang, H., Cooper, J.K., Yamada, M., Takahashi, H., Tsuji, S., Troncoso, J., Dawson, V.L., Dawson, T.M., and Ross, C.A. (2001). Interference by huntingtin and atrophin-1 with cbp-mediated transcription leading to cellular toxicity. *Science* 291, 2423-2428.

154. Orr, H.T., Chung, M.Y., Banfi, S., Kwiatkowski, T.J., Jr., Servadio, A., Beaudet, A.L., McCall, A.E., Duvick, L.A., Ranum, L.P., and Zoghbi, H.Y. (1993). Expansion of an unstable trinucleotide CAG repeat in spinocerebellar ataxia type 1. *Nat. Genet.* 4, 221-226.

155. Ohtsuka, K., Suzuki, T. (2000) Roles of molecular chaperones in the nervous system. *Brain Res Bull.* 53, 141-146.

156. Owen, D. J., Vallis, Y., Noble, M. E., Hunter, J. B., Dafforn, T. R., Evans, P. R., and McMahon, H. T. (1999) A structural explanation for the binding of multiple ligands by the alpha-adaptin appendage domain. *Cell* 97, 805-815.

157. Parker, J.A., Connolly, J.B., Wellington, C., Hayden, M., Dausset, J., and Neri, C. (2001). Expanded polyglutamines in Caenorhabditis elegans cause axonal abnormalities and severe dysfunction of PLM mechanosensory neurons without cell death. *Proc. Natl. Acad. Sci. U. S. A* 98, 13318-13323.

158. Parker, JA. The Caenorhabtidis Elegans Homologue of Huntingtin Interacting Protein 1 has multiple roles in development. 2001. The University of British Columbia. Ref Type: Thesis/Dissertation

159. Paulson, H.L., Perez, M.K., Trottier, Y., Trojanowski, J.Q., Subramony, S.H., Das, S.S., Vig, P., Mandel, J.L., Fischbeck, K.H., and Pittman, R.N. (1997). Intranuclear inclusions of expanded polyglutamine protein in spinocerebellar ataxia type 3. *Neuron* 19, 333-344.

160. Perutz, M.F. (1999). Glutamine repeats and neurodegenerative diseases. *Brain Res. Bull.* 50, 467.

161. Perutz, M.F., Johnson, T., Suzuki, M., and Finch, J.T. (1994). Glutamine repeats as polar zippers: their possible role in inherited neurodegenerative diseases. *Proc. Natl. Acad. Sci. U. S. A* 91, 5355-5358.

162. Portera-Cailliau, C., Hedreen, J.C., Price, D.L., and Koliatsos, V.E. (1995). Evidence for apoptotic cell death in Huntington disease and excitotoxic animal models. *J. Neurosci.* 15, 3775-3787.

163. Pulst, S.M., Nechiporuk, A., Nechiporuk, T., Gispert, S., Chen, X.N., Lopes-Cendes, I., Pearlman, S., Starkman, S., Orozco-Diaz, G., Lunkes, A., DeJong, P., Rouleau, G.A., Auburger, G., Korenberg, J.R., Figueroa, C., and Sahba, S. (1996). Moderate expansion of a normally biallelic trinucleotide repeat in spinocerebellar ataxia type 2. *Nat. Genet.* 14, 269-276.

164. Qualmann, B. and Kessels, M.M. (2002). Endocytosis and the cytoskeleton. *Int. Rev. Cytol.* 220, 93-144.

165. Qualmann, B., Kessels, M.M., and Kelly, R.B. (2000). Molecular links between endocytosis and the actin cytoskeleton. *J. Cell Biol.* 150, F111-F116.

166. Ramesh, N., Anton, I.M., Martinez-Quiles, N., and Geha, R.S. (1999). Waltzing with WASP. *Trends Cell Biol.* 9, 15-19.

167. Ramjaun, A. R., and McPherson, P. S. (1998) Multiple amphiphysin II splice variants display differential clathrin binding: identification of two distinct clathrin-binding sites. *J. Neurochem.* 70, 2369-2376.

168. Ramjaun, A. R., Micheva, K. D., Bouchelet, I., and McPherson, P. S. (1997) Identification and characterization of a nerve terminal-enriched amphiphysin isoform. *J. Biol. Chem. 272*, 16700-16706.

169. Rao DS, Bradley SV, Kumar PD, Hyun TS, Saint-Dic D, Oravecz-Wilson K, Kleer CG, Ross TS. (2003). Altered receptor trafficking in Huntingtin Interacting Protein 1-transformed cells. *Cancer Cell.* 3, 471-482.

170. Rao, D.S., Hyun, T.S., Kumar, P.D., Mizukami, I.F., Rubin, M.A., Lucas, P.C., Sanda, M.G., and Ross, T.S. (2002). Huntingtin-interacting protein 1 is overexpressed in prostate and colon cancer and is critical for cellular survival. *J. Clin. Invest* 110, 351-360.

171. Rasper, D.M., Vaillancourt, J.P., Hadano, S., Houtzager, V.M., Seiden, I., Keen, S.L., Tawa, P., Xanthoudakis, S., Nasir, J., Martindale, D., Koop, B.F., Peterson, E.P., Thornberry, N.A., Huang, J., MacPherson, D.P., Black, S.C., Hornung, F., Lenardo, M.J., Hayden, M.R., Roy, S., and Nicholson, D.W. (1998). Cell death attenuation by 'Usurpin', a mammalian DED-caspase homologue that precludes caspase-8 recruitment and activation by the CD-95 (Fas, APO-1) receptor complex. *Cell Death. Differ. 5*, 271-288.

172. Raths, S., Rohrer, J., Crausaz, F., and Riezman, H. (1993). end3 and end4: two mutants defective in receptor-mediated and fluid-phase endocytosis in Saccharomyces cerevisiae. *J. Cell Biol.* 120, 55-65.

173. Read, A.P. (1993). Huntington's disease: testing the test. *Nat. Genet.* 4, 329-330.

174. Reddy, P.H., Williams, M., Charles, V., Garrett, L., Pike-Buchanan, L., Whetsell, W.O., Jr., Miller, G., and Tagle, D.A. (1998). Behavioural abnormalities and selective neuronal loss in HD transgenic mice expressing mutated full-length HD cDNA. *Nat. Genet.* 20, 198-202.

175. Riddle, D.L., Blumenthal, R., Meyer, B.J., and Preiss, J.R. (1997). C. elegans II. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

176. Ridley, R.M., Frith, C.D., Crow, T.J., and Conneally, P.M. (1988). Anticipation in Huntington's disease is inherited through the male line but may originate in the female. *J. Med. Genet.* 25, 589-595.

177. Rigamonti, D., Bauer, J.H., De Fraja, C., Conti, L., Sipione, S., Sciorati, C., Clementi, E., Hackam, A., Hayden, M.R., Li, Y., Cooper, J.K., Ross, C.A., Govoni, S., Vincenz, C., and Cattaneo, E. (2000). Wild-type huntingtin protects from apoptosis upstream of caspase-3. *J. Neurosci.* 20, 3705-3713.

178. Ringstad, N., Nemoto, Y, De Camilli, P. (1997) The SH3p4 / SH3p8 / SH3p13 protein family: binding partners for synaptojanin and dynamin via a Grb-2 like Src homology domain. *Proc. Natl. Acad. Sci. USA* 94, 8569-8574.

179. Rosenthal, J.A., Chen, H., Slepnev, V.I., Pellegrini, L., Salcini, A.E., Di Fiore, P.P., and De Camilli, P. (1999). The epsins define a family of proteins that interact with components of the clathrin coat and contain a new protein module. *J. Biol. Chem.* 274, 33959-33965.

180. Ross, T.S., Bernard, O.A., Berger, R., and Gilliland, D.G. (1998). Fusion of Huntingtin interacting protein 1 to platelet-derived growth factor beta receptor (PDGFbetaR) in chronic myelomonocytic leukemia with t(5;7)(q33;q11.2). *Blood* 91, 4419-4426.

181. Ross, T.S. and Gilliland, D.G. (1999). Transforming properties of the Huntingtin interacting protein 1/ platelet-derived growth factor beta receptor fusion protein. *J. Biol. Chem.* 274, 22328-22336.

182. Saint-Dic, D., Chang, S.C., Taylor, G.S., Provot, M.M., and Ross, T.S. (2001). Regulation of the Src homology 2-containing inositol 5-phosphatase SHIP1 in HIP1/PDGFbeta R-transformed cells. *J. Biol. Chem.* 276, 21192-21198.

183. Sanpei, K., Takano, H., Igarashi, S., Sato, T., Oyake, M., Sasaki, H., Wakisaka, A., Tashiro, K., Ishida, Y., Ikeuchi, T., Koide, R., Saito, M., Sato, A., Tanaka, T., Hanyu, S., Takiyama, Y., Nishizawa, M., Shimizu, N., Nomura, Y., Segawa, M., Iwabuchi, K., Eguchi, I., Tanaka, H., Takahashi, H., and Tsuji, S. (1996). Identification of the spinocerebellar ataxia type 2 gene using a direct identification of repeat expansion and cloning technique, DIRECT. *Nat. Genet.* 14, 277-284.

184. Santini, F., Gaidarov, I., and Keen, J.H. (2002). G protein-coupled receptor/arrestin3 modulation of the endocytic machinery. *J. Cell Biol.* 156, 665-676.

185. Saudou, F., Finkbeiner, S., Devys, D., and Greenberg, M.E. (1998). Huntingtin acts in the nucleus to induce apoptosis but death does not correlate with the formation of intranuclear inclusions. *Cell* 95, 55-66.

186. Schafer, D.A. (2002). Coupling actin dynamics and membrane dynamics during endocytosis. *Curr. Opin. Cell Biol.* 14, 76-81.

187. Schmid, S. L. (1997) Clathrin-coated vesicle formation and protein sorting: an integrated process. *Annu. Rev. Biochem.* 66, 511-548.

188. Seki, N., Muramatsu, M., Sugano, S., Suzuki, Y., Nakagawara, A., Ohhira, M., Hayashi, A., Hori, T., and Saito, T. (1998). Cloning, expression analysis, and chromosomal localization of HIP1R, an isolog of huntingtin interacting protein (HIP1). *J. Hum. Genet.* 43, 268-271.

189. Sharp, A.H., Loev, S.J., Schilling, G., Li, S.H., Li, X.J., Bao, J., Wagster, M.V., Kotzuk, J.A., Steiner, J.P., Lo, A., and . (1995). Widespread expression of Huntington's disease gene (IT15) protein product. *Neuron* 14, 1065-1074.

190. Shih, S.C., Katzmann, D.J., Schnell J.D., Sutanto M., Emr S.D., Hicke L. (2002) Epsins and Vps27p/Hrs contain ubiquitin-binding domains that function in receptor endocytosis. *Nat Cell Biol.* 4, 389-393.

191. Shih SC, Sloper-Mould KE, Hicke L. (2000) Monoubiquitin carries a novel internalization signal that is appended to activated receptors. *EMBO J.* 19, 187-198.

192. Singaraja, R.R., Hadano, S., Metzler, M., Givan, S., Wellington, C.L., Warby, S., Yanai, A., Gutekunst, C.A., Leavitt, B.R., Yi, H., Fichter, K., Gan, L., McCutcheon, K., Chopra, V., Michel, J., Hersch, S.M., Ikeda, J.E., and Hayden, M.R. (2002). HIP14, a novel ankyrin domain-containing protein, links huntingtin to intracellular trafficking and endocytosis. *Hum. Mol. Genet.* 11, 2815-2828.

193. Singhrao, S.K., Neal, J.W., Morgan, B.P., and Gasque, P. (1999). Increased complement biosynthesis by microglia and complement activation on neurons in Huntington's disease. *Exp. Neurol.* 159, 362-376.

194. Sittler, A., Walter, S., Wedemeyer, N., Hasenbank, R., Scherzinger, E., Eickhoff, H., Bates, G.P., Lehrach, H., and Wanker, E.E. (1998). SH3GL3 associates with the Huntingtin exon 1 protein and promotes the formation of polygln-containing protein aggregates. Mol. Cell 2, 427-436.

195. Skinner, P.J., Koshy, B.T., Cummings, C.J., Klement, I.A., Helin, K., Servadio, A., Zoghbi, H.Y., and Orr, H.T. (1997). Ataxin-1 with an expanded glutamine tract alters nuclear matrix-associated structures. *Nature* 389, 971-974.

196. Slepnev, V.I. and De Camilli, P. (2000a). Accessory factors in clathrin-dependent synaptic vesicle endocytosis. *Nat. Rev. Neurosci.* 1, 161-172.

197. Slepnev, V. I., Ochoa, G. C., Butler, M. H., and De Camilli, P. (2000b) Tandem arrangement of the clathrin and AP-2 binding domains in amphiphysin 1 and disruption of clathrin coat function by amphiphysin fragments comprising these sites. *J. Biol. Chem.* 275, 17583-17589.

198. Slow EJ, Van Raamsdonk J, Rogers D, Coleman SH, Graham RK, Deng Y, Oh R, Bissada N, Hossain SM, Yang YZ, Li XJ, Simpson EM, Gutekunst CA, Leavitt BR, Hayden MR. (2003). Selective striatal neuronal loss in a YAC128 mouse model of Huntington disease. *Hum Mol Genet.* 12, 1555-1567.

199. Snell, R.G., MacMillan, J.C., Cheadle, J.P., Fenton, I., Lazarou, L.P., Davies, P., MacDonald, M.E., Gusella, J.F., Harper, P.S., and Shaw, D.J. (1993). Relationship between trinucleotide repeat expansion and phenotypic variation in Huntington's disease. Nat. Genet. 4, 393-397.

200. Steffan, J.S., Bodai, L., Pallos, J., Poelman, M., McCampbell, A., Apostol, B.L., Kazantsev, A., Schmidt, E., Zhu, Y.Z., Greenwald, M., Kurokawa, R., Housman, D.E., Jackson, G.R., Marsh, J.L., and Thompson, L.M. (2001). Histone deacetylase inhibitors arrest polyglutamine-dependent neurodegeneration in Drosophila. *Nature* 413, 739-743.

201. Steffan, J.S., Kazantsev, A., Spasic-Boskovic, O., Greenwald, M., Zhu, Y.Z., Gohler, H., Wanker, E.E., Bates, G.P., Housman, D.E., and Thompson, L.M. (2000). The Huntington's disease protein interacts with p53 and CREB-binding protein and represses transcription. *Proc. Natl. Acad. Sci. U. S. A* 97, 6763-6768.

202. Sun, Y., Savanenin, A., Reddy, P.H., and Liu, Y.F. (2001). Polyglutamine-expanded huntingtin promotes sensitization of N-methyl-D-aspartate receptors via post-synaptic density 95. *J. Biol. Chem.* 276, 24713-24718.

203. Tebar, F., Bohlander, S.K., and Sorkin, A. (1999) Clathrin assembly lymphoid myeloid leukemia (CALM) protein: localization in endocytic-coated pits, interactions with clathrin, and the impact of overexpression on clathrin-mediated traffic. *Mol. Biol. Cell* 10, 2687-2702.

204. Telenius, H., Kremer, H.P., Theilmann, J., Andrew, S.E., Almqvist, E., Anvret, M., Greenberg, C., Greenberg, J., Lucotte, G., Squitieri, F., and . (1993). Molecular analysis of juvenile Huntington disease: the major influence on (CAG)n repeat length is the sex of the affected parent. *Hum. Mol. Genet.* 2, 1535-1540.

205. Teo, M., Tan, L., Lim, L., and Manser, E. (2001) The tyrosine kinase ACK1 associates with clathrin-coated vesicles through a binding motif shared by arrestin and other adaptors. *J. Biol. Chem.* 276, 18392-18398.

206. ter Haar, E., Harrison, S.C., and Kirchhausen, T. (2000) Peptide-in-groove interactions link target proteins to the beta-propeller of clathrin.*Proc. Natl. Acad. Sci. USA.* 97, 1096-1100.

207. Thakur AK, Wetzel R. (2002) Mutational analysis of the structural organization of polyglutamine aggregates.*Proc Natl Acad Sci U S A.* 99, 17014-17019.

208. Thomas, L.B., Gates, D.J., Richfield, E.K., O'Brien, T.F., Schweitzer, J.B., and Steindler, D.A. (1995). DNA end labeling (TUNEL) in Huntington's disease and other neuropathological conditions. *Exp. Neurol.* 133, 265-272.

209. Thornberry, N.A., Rano, T.A., Peterson, E.P., Rasper, D.M., Timkey, T., Garcia-Calvo, M., Houtzager, V.M., Nordstrom, P.A., Roy, S., Vaillancourt, J.P., Chapman, K.T., and Nicholson, D.W. (1997). A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. *J. Biol. Chem.* 272, 17907-17911.

210. Tian, L., Nelson, D.L., and Stewart, D.M. (2000). Cdc42-interacting protein 4 mediates binding of the Wiskott-Aldrich syndrome protein to microtubules. *J. Biol. Chem.* 275, 7854-7861.

211. Towbin, H., Staehelin, T., Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some Applications. *Proc. Natl. Acad. Sci. USA* 76, 4350-4354.

212. Traub, L. M., Downs, M. A., Westrich, J. L., and Fremont, D. H. (1999) Crystal structure of the alpha appendage of AP-2 reveals a recruitment platform for clathrin-coat assembly. *Proc. Natl. Acad. Sci. U. S. A.* 96, 8907-8912.

213. Trottier, Y., Biancalana, V., and Mandel, J.L. (1994). Instability of CAG repeats in Huntington's disease: relation to parental transmission and age of onset. *J. Med. Genet.* 31, 377-382.

214. Trottier, Y., Devys, D., Imbert, G., Saudou, F., An, I., Lutz, Y., Weber, C., Agid, Y., Hirsch, E.C., and Mandel, J.L. (1995). Cellular localization of the Huntington's disease protein and discrimination of the normal and mutated form. *Nat. Genet.* 10, 104-110.

215. Tukamoto, T., Nukina, N., Ide, K., and Kanazawa, I. (1997). Huntington's disease gene product, huntingtin, associates with microtubules in vitro. *Brain Res. Mol. Brain Res.* 51, 8-14.

216. Turunen O, Sainio M, Jaaskelainen J, Carpen O, Vaheri A (1998) Structure-function relationships in the ezrin family and the effect of tumorassociated point mutations in neurofibromatosis 2 protein. *Biochim Biophys Acta* 1387, 1-16.

217. Uruno T, Liu J, Zhang P, Fan Yx, Egile C, Li R, Mueller SC, Zhan X. (2001) Activation of Arp2/3 complex-mediated actin polymerization by cortactin. *Nat Cell Biol.* 3, 259-266.

218. Velier, J., Kim, M., Schwarz, C., Kim, T.W., Sapp, E., Chase, K., Aronin, N., and DiFiglia, M. (1998). Wild-type and mutant huntingtins function in vesicle trafficking in the secretory and endocytic pathways. *Exp. Neurol.* 152, 34-40.

219. Vonsattel, J.P. and DiFiglia, M. (1998). Huntington disease. *J. Neuropathol. Exp. Neurol.* 57, 369-384.

220. Vonsattel, J.P., Myers, R.H., Stevens, T.J., Ferrante, R.J., Bird, E.D., and Richardson, E.P., Jr. (1985). Neuropathological classification of Huntington's disease. *J. Neuropathol. Exp. Neurol.* 44, 559-577.

221. Waelter, S., Scherzinger, E., Hasenbank, R., Nordhoff, E., Lurz, R., Goehler, H., Gauss, C., Sathasivam, K., Bates, G.P., Lehrach, H., and Wanker, E.E. (2001). The huntingtin interacting protein HIP1 is a clathrin and alpha-adaptin-binding protein involved in receptor-mediated endocytosis. *Hum. Mol. Genet.* 10, 1807-1817.

222. Wang, Y., Ju, W., Fam, S., D'Souza, S., Taghibiglou, C., Salter, Wang, Y.T. (2003) AMPA receptor is essential for NMDA-induced neuronal apoptosis. *Submitted.*

223. Wanker, E.E. (2002). Hip1 and Hippi participate in a novel cell death-signaling pathway. *Dev. Cell* 2, 126-128.

224. Wanker, E.E., Rovira, C., Scherzinger, E., Hasenbank, R., Walter, S., Tait, D., Colicelli, J., and Lehrach, H. (1997). HIP-I: a huntingtin interacting protein isolated by the yeast two-hybrid system. *Hum. Mol. Genet.* 6, 487-495.

225. Wellington, C.L., Ellerby, L.M., Gutekunst, C.A., Rogers, D., Warby, S., Graham, R.K., Loubser, O., van Raamsdonk, J., Singaraja, R., Yang, Y.Z., Gafni, J., Bredesen, D., Hersch, S.M., Leavitt, B.R., Roy, S., Nicholson, D.W., and Hayden, M.R. (2002). Caspase cleavage of mutant huntingtin precedes neurodegeneration in Huntington's disease. *J. Neurosci.* 22, 7862-7872.

226. Wellington, C.L., Singaraja, R., Ellerby, L., Savill, J., Roy, S., Leavitt, B., Cattaneo, E., Hackam, A., Sharp, A., Thornberry, N., Nicholson, D.W., Bredesen, D.E., and Hayden, M.R. (2000). Inhibiting caspase cleavage of huntingtin reduces toxicity and aggregate formation in neuronal and nonneuronal cells. *J. Biol. Chem.* 275, 19831-19838.

227. Wellington, C.L., Ellerby, L.M., Hackam, A.S., Margolis, R.L., Trifiro, M.A., Singaraja, R., McCutcheon, K., Salvesen, G.S., Propp, S.S., Bromm, M., Rowland, K.J., Zhang, T., Rasper, D., Roy, S., Thornberry, N., Pinsky, L., Kakizuka, A., Ross, C.A., Nicholson, D.W., Bredesen, D.E., and Hayden, M.R. (1998). Caspase cleavage of gene products associated with triplet expansion disorders generates truncated fragments containing the polyglutamine tract. *J. Biol. Chem.* 273, 9158-9167.

228. Wellington, C.L., Brinkman, R.R., 0'Kusky, J.R., Hayden, M.R. (1997) Toward understanding the molecular pathology of Huntington's disease. *Brain Pathol.* 7, 979-1002.

229. Wesp, A., Hicke, L., Palecek, J., Lombardi, R., Aust, T., Munn, A.L., and Riezman, H. (1997). End4p/Sla2p interacts with actin-associated proteins for endocytosis in Saccharomyces cerevisiae. *Mol. Biol. Cell* 8, 2291-2306.

230. Wilde, A., Beattie, E. C., Lem, L., Riethof, D. A., Liu, S. H., Mobley, W. C., Soriano, P., and Brodsky, F. M. (1999) EGF receptor signaling stimulates SRC kinase phosphorylation of clathrin, influencing clathrin redistribution and EGF uptake. *Cell* 96, 677-687.

231. Wyttenbach A, Carmichael J, Swartz J, Furlong RA, Narain Y, Rankin J, Rubinsztein DC. (2000) Effects of heat shock, heat shock protein 40 (HDJ-2), and proteasome inhibition on protein aggregation in cellular models of Huntington's disease. *Proc Natl Acad Sci U S A.* 97, 2898-2903.

232. Yang, W., Lo, C. G., Dispenza, T., and Cerione, R. A. (2001) The Cdc42 target ACK2 directly interacts with clathrin and influences clathrin assembly. *J. Biol. Chem.* 276, 17468-17473.

233. Yang, S., Cope, M.J., and Drubin, D.G. (1999). Sla2p is associated with the yeast cortical actin cytoskeleton via redundant localization signals. *Mol. Biol. Cell* 10, 2265-2283.

234. Ybe, J. A., Greene, B., Liu, S. H., Pley, U., Parham, P., and Brodsky, F. M. (1998) Clathrin self-assembly is regulated by three light-chain residues controlling the formation of critical salt bridges. *EMBO J.* 17, 1297-1303.

235. Ye, W. and.Lafer, E.M. (1995) Clathrin binding and assembly activities of expressed domains of the synapse-specific clathrin assembly protein AP-3. *J. Biol. Chem.* 270, 10933-10939.

236. Zeron M.M., Hansson O., Chen N., Wellington C.L., Leavitt B.R., Brundin P., Hayden M.R., Raymond L.A. (2002) Increased sensitivity to N-methyl-D-aspartate receptor-mediated excitotoxicity in a mouse model of Huntington's disease.*Neuron.* 33, 849-860.

237. Zeitlin, S., Liu, J.P., Chapman, D.L., Papaioannou, V.E., and Efstratiadis, A. (1995). Increased apoptosis and early embryonic lethality in mice nullizygous for the Huntington's disease gene homologue. *Nat. Genet.* 11, 155-163.

238. Zhuchenko, O., Bailey, J., Bonnen, P., Ashizawa, T., Stockton, D.W., Amos, C., Dobyns, W.B., Subramony, S.H., Zoghbi, H.Y., and Lee, C.C. (1997). Autosomal dominant cerebellar ataxia (SCA6) associated with small polyglutamine expansions in the alpha 1A-voltage-dependent calcium channel. *Nat. Genet.* 15, 62-69.

239. Zuccato, C., Ciammola, A., Rigamonti, D., Leavitt, B.R., Goffredo, D., Conti, L., MacDonald, M.E., Friedlander, R.M., Silani, V., Hayden, M.R., Timmusk, T., Sipione, S., and Cattaneo, E. (2001). Loss of huntingtin-mediated BDNF gene transcription in Huntington's disease. *Science* 293, 493-498.

240. Zuccato, C., Tartari, M., Crotti, A., Goffredo, D., Valenza, M., Conti, L., Cataudella, T., Leavitt, B.R., Hayden, M.R., Timmusk., T., Rigamonti., D., and Cattaneo, E. (2003). Huntingtin interacts with REST/NRSF to modulate the transcription of NRSE-controlled neuronal genes. *Nat. Genet. 35, 76-83.*