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Austin David Cape

Huntingtin-Associated Protein 1 (Hap1) is Involved in Insulin Secretion From Pancreatic Beta Cells

By

Austin David Cape
Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences
Genetics and Molecular Biology

_____
Xiao-Jiang Li, M.D., Ph.D.
Advisor

_____
Guy M. Benian, M.D.
Committee Member

_____
Anthony W.S. Chan, DVM, Ph.D.
Committee Member

_____
Joseph Cubells, M.D., Ph.D.
Committee Member

_____
Victor Faundez, M.D., Ph.D.
Committee Member

Accepted:

_____
Lisa A. Tedesco, Ph.D.
Dean of the James T. Laney School of Graduate Studies

_____
Date

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Austin David Cape
B.S., Georgia State University, 2006

Advisor: Xiao-Jiang Li, M.D., Ph.D.

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Abstract

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Hap1 was identified as the first binding partner with huntingtin, the Huntington disease (HD) protein. Studies have revealed Hap1 participates in intracellular trafficking in neuronal cells, which is adversely affected by the mutant version of huntingtin (mHtt). Recently, Hap1 was found in pancreatic beta (β) cells and other endocrine cells that secrete hormones via similar exocytic mechanisms. However, the role of Hap1 in these endocrine cells is unknown. We generated a mouse model selectively depleted of Hap1 in pancreatic β cells using the Cre-loxP genetic manipulation system. These Hap1 knockout (KO) mice displayed impairment of glucose tolerance without affecting insulin sensitivity. We validated that decreased Hap1, via its shRNA, reduced glucose-stimulated insulin release in Min6 cells, a well-studied pancreatic β cell line. In addition, ELISA measurement of insulin levels in blood confirmed Hap1 KO mice are defective for insulin release in response to glucose treatment. Electron microscopy revealed decreased numbers of insulin-containing vesicles at the docking sites in β cell membranes of Hap1 KO mice. Glucose-stimulated insulin release also reduced the phosphorylation of pHap1A in both cell and mouse models. Moreover, this glucose-induced dephosphorylation caused more Hap1 to associate with kinesin light chain and dynactin p150, both of which are involved in microtubule-dependent trafficking. These results suggest Hap1-associated intracellular trafficking is important for vesicular release of insulin from pancreatic β cells, providing a potential target for treating the impairment of β cell insulin release seen in HD and other metabolic disorders.

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Chapter 1

General Introduction

1.1 Huntingtin Associated Protein 1 (Hap1)

Hap1 was initially described as a neuronal protein and the first binding partner to huntingtin (Htt), the protein responsible for Huntington's disease (HD) (Li et al., 1995). HD is a severe neurodegenerative disease caused by a mutation in Htt that results in an expansion of the poly-glutamine (pCAG) tract in exon 1. The expanded CAG repeats in mutant Htt (mHtt) confer a gain-of-function causing aberrant protein interactions (Li SH 1998). As a result, Hap1 binds more strongly to mHtt than to normal Htt in a repeat length dependent manner (Albin and Tagle 1995; Li SH 1998). Several studies have demonstrated a role for Htt and Hap1 in intracellular trafficking, which can be adversely affected by the aberrant mHtt interactions (Li et al., 2003; Tang et al., 2003; Tang et al., 2004; Twelvetrees et al., 2010). As such, molecular trafficking defects are a pathological characteristic of HD, therefore investigating Htt binding proteins like Hap1 is a central issue in understanding HD in general, and molecular transport in particular.

Taking a closer look at Hap1, we find it is a brain-enriched protein highly expressed in the olfactory bulbs, spinal cord and brain stem with less expression in the striatum, hippocampus and cortex (Gutekunst et al., 1998; Li et al., 1995; Li et al., 1996; Page et al., 1998). Htt, on the other hand, is differentially expressed throughout the human body, but HD neurodegeneration is highly selective to striatum of neuronal cells. This selective degradation is the paradox currently driving basic and translational research in HD. Hap1 intracellular localization significantly overlaps Htt; both associate with microtubules and membrane-bound organelles (Gutekunst et al., 1998). Taken together, selective expression of Hap1 in specific brain regions and the colocalization with Htt suggest that

Hap1 dysfunction contributes to HD pathogenesis. Understanding Hap1's function may be a key to solving the complicated puzzle of selective neurodegeneration. Moreover, the importance of Hap1 is demonstrated in Hap1 null mice that die within days postnatal due to a feeding defect (Chan et al., 2002).

Hap1 seems to have conserved homology across several species. Two Hap1 isoforms have been described in rat and mouse, differing only at their C-terminal ends (amino acids 579-599 in Hap1-A; 579-629 in Hap1-B) ((Nasir et al., 1998; Nasir et al., 1999). In humans, only a single Hap1 form has been described, which is more similar to murine Hap1-A (Li SH 1998). Hap1 has also been studied in other model organisms including *Drosophila melanogaster*, the common fruit fly, and *Caenorhabditis elegans*, a nematode worm. In flies, a Hap1 homolog called Milton was found in a mosaic screen for mutants of axonal and synaptic function (Aldridge et al., 2007). Milton functions to link mitochondria to kinesin for transport in neurons (Aldridge et al., 2007). A follow up study confirmed this finding using human Milton transfected in Cos7 cells, and also showed that Milton binds kinesin heavy chain and mitochondria at distinct sites (Koutsopoulos et al., 2010). Similarly, worms express a protein known as T27A3.1 that shares significant homology with Hap1 (Mercer et al., 2009), however, functional studies in worms have yet to be reported. These data are nice clues that overlap in explaining a role for Hap1 in molecular trafficking along microtubules.

All Hap1 isoforms contain conserved coiled-coil domains that promote homodimerization and support binding to other proteins, including Htt in mice and rat (Li

SH 1998). Hap1 contains no transmembrane domains nor nuclear localization signals, consistent with the evidence that Hap1 is a cytoplasmic protein (Li et al., 1998). The different C-terminal ends confer unique functions to Hap1 isoforms. For example, Hap1-A seems to promote cytoplasmic inclusions compared to aggregate inhibition by Hap1-B (Li et al., 1998).

1.2 Hap1 Function In Molecular Trafficking

The majority of proteins known to bind Hap1 are associated with vesicular trafficking and endocytosis, although a few are strictly molecular signaling or gene transcription players (**Table 1-1**). The proteins that bind Hap1 are potentially important in the disease mechanisms of HD and in microtubule based vesicle trafficking. Given that Hap1 binds more strongly to mHtt compared to normal, or wild type (WT), in a polyQ length dependent manner (Li SH 1998), understanding Hap1's role is essential in elucidating the toxicity of mHtt and its impact on molecular transport.

Hap1 binds dynactin p150Glued (p150) (Engelender et al., 1997; Li et al., 1998), a subunit of the dynactin complex that activates microtubule-based motor complexes kinesin and dynein (Berezuk and Schroer 2007). The p150 protein is critical for dynein powered retrograde axonal transport, membrane trafficking, nuclear migration and mitotic spindle positioning (Holzbaur and Vallee 1994; Kardon and Vale 2009). Together, p150, Htt and Hap1 form a complex that enhances vesicular transport of brain-derived neurotrophic factor (BDNF), supporting neuronal health and survival (Gauthier et al., 2004).

Hap1 also associates with kinesin light chain (KLC) (McGuire et al., 2006). Kinesins are ATP-dependent molecular motors that transport cellular cargo along microtubule roadways (Hirokawa et al., 2009). Conventional kinesins are formed as dimers of kinesin heavy chain (KHC) (Hirokawa 1997). A motor, or head, domain binds microtubules and processes energy from ATP, while a tail domain binds cargo, adaptors or scaffold proteins (Hirokawa 1997; Hirokawa et al., 2009). Attached at the C-terminal ends of KHCs, KLCs interact with cargo, and also function to regulate KHC activity (Hirokawa et al., 2009; McGuire et al., 2006). Reducing Hap1 protein by siRNA has been shown to disrupt kinesin-dependent neurite outgrowth and transport of amyloid precursor protein vesicles (McGuire et al., 2006).

It is important to mention here that microtubule transport is dynamic, with molecular motor complexes moving in a forward (anterograde) and reverse (retrograde) direction en route to a final destination. High magnification studies have shown vesicular movement on microtubules is characterized broken starts and stops, mixed with sidesteps and turn-arounds (Welte 2004). Despite excellent descriptions of bi-directional transport, it remains unknown how microtubule dependent bi-directional transport is regulated. Precedence suggests Hap1 may participate in this regulation. For example, the association of Hap1-A with kinesin light chain (KLC) and dynactin p150Glued (p150) is regulated by phosphorylation of Hap1-A at the C-terminus (Rong et al., 2006). Modulation of Hap1 binding to microtubule-associated proteins by phosphorylation may act as a molecular switch to promote or inhibit vesicular trafficking.

Hap1A's phosphorylation appears to be related to its association with 14-3-3. The family of 14-3-3 proteins has widespread functionality and binds many protein classes including those of signal transduction and trafficking (Aitken et al., 1992; Rong et al., 2007). The binding of 14-3-3 to Hap1 is specific to Hap1A, which is enhanced by the phosphorylation of Hap1A (Rong et al., 2007). This interaction comes at the expense of Hap1 binding to KLC, decreasing Hap1A transport to neurite tips (Rong et al., 2007). Hap1 dependent trafficking seems to be characterized by the antagonistic relationship with 14-3-3 and KLC, where Hap1 binding is regulated by phosphorylation of Hap1A.

In addition to exocytosis, the association of Hap1 with microtubule motor complexes is likely to modulate endocytosis of membrane receptors. As an example, Hap1 interacts with hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) (Li et al., 2002), an endosome-associated protein that functions in regulating vesicular trafficking of endocytosed membrane receptors destined to either recycle back to the membrane or for lysosomal degradation (Komada and Kitamura 2005). Down regulation of Hrs leads to inhibition of intracellular degradation, but not internalization, of platelet derived growth factor and epidermal growth factor (EGF), suggesting Hrs is a regulator of endocytosis (Komada and Kitamura 2001; Li et al., 2002). Similarly, Hap1 over-expression promotes endosomal stabilization and inhibits the degradation of internalized EGF (Li et al., 2002), supporting a role for Hap1 in early endocytic trafficking pathways.

Further support for Hap1 having a function in endocytosis comes from γ-aminobutyric acid type A receptors ($GABA_A$Rs), the major receptors for the inhibitory neurotransmitter GABA (Olsen and Tobin 1990). GABA is the primary regulator of fast synaptic inhibition in the brain (Moss and Smart 2001). Proper control of neuron excitation and inhibition is a fine balancing act mediated by dynamic plasma membrane receptors at the neuronal synapse. Synaptic membrane receptors are endocytosed in clathrin-coated vesicles and sorted for either membrane recycling or lysosomal degradation. Regulation of membrane receptor concentration is a major cellular technique used to control neuronal communication. Hap1 was shown to bind $GABA_A$R β subunit and regulate receptor recycling by preventing $GABA_A$R lysosomal degradation via endocytic trafficking, similar to the stabilizing role Hap1 provides for EGF (Kittler et al., 2004).

Hap1 is also found to be involved in stabilizing endocytic neurotrophin receptors. Tropomyosin-related kinase receptor tyrosine kinases (Trk) are receptors that bind neurotrophin growth factors supporting cell survival and differentiation (Klesse and Parada 1999). The Trk family consists of three types: TrkA, TrkB and TrkC, preferentially activated by nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3), respectively (Klesse and Parada 1999). In the synapse, endocytosis and trafficking of neurotrophin receptors are critical for proper function and health of neurons. This trafficking is microtubule-dependent and involves Hap1 participation. In cell culture and primary neuronal experiments, reducing Hap1 levels decreased intracellular TrkA amounts and diminished neurite outgrowth (Rong et al., 2006). In addition, lower Hap1 levels resulted in suppressed TrkB internalization and

reduced signaling (Sheng et al., 2008). Again, Hap1 is functioning in a positive role supporting membrane receptors through stabilization.

Hap1 also associates with the inositol 1,4,5-triphosphate receptor (InsP3R), which functions as an intracellular Ca^{2+} release channel critical for signaling in neuronal and endocrine cells (Tang et al., 2004). The endoplasmic reticulum (ER) contains InsP3 receptors that signal calcium release from the ER into the cytoplasm during signaling events. Disrupted calcium signaling is known to trigger cell death through apoptosis (Cheek and Barry 1993). Hap1A, not Hap1B, has been shown to bind InsP3R and Htt, forming a tertiary complex required for proper calcium signaling (Tang et al., 2003). This complex, and therefore calcium signaling, is disrupted by mHtt, which over sensitizes the InsP3 receptor and leads to over secretion of calcium from the ER. These signaling events are also critical for β cell insulin secretion. In fact, some reports have hinted that ER calcium secretion defects in β cells are involved in the diabetes like symptoms reported in HD patients (Burcelin et al., 2008; Yamamoto et al., 2003; Cheek et al., 1993).

As mentioned previously, further support for Hap1 in molecular trafficking comes from functional analysis in fly and worm systems. Depletion of the Hap1 fly homolog Milton, which binds KHC and promotes mitochondrial transport via binding of Milton's coiled coil region to microtubules (Koutsopoulos et al., 2010), disrupted mitochondrial trafficking and resulted in defective spermatogenesis in male flies (Aldridge et al., 2007).

1.3 Neuroendocrine System

Endocrine cells secrete hormones. They are classified according to the type of hormone secreted, as either amino acid-derived hormones or steroid-derived hormones. Tissue types include pituitary, adrenal, thyroid and pancreas. The term neuroendocrine is inclusive for all cells that secrete hormones that link the nervous system to the rest of the body (Langley 1994). For example, the β cells of the pancreas secrete insulin, which is a powerful hormone responsible for physiological actions like up taking glucose from blood after eating a meal, and is also important for neuronal function in signaling events connected to the autonomic nervous system (Woods et al., 1985). Insulin also participates in cognition tasks such as learning and memory (Ashcroft et al., 2005).

Most of Hap1 functional investigations have focused on neuronal environments due to its relationship with Htt and HD. However, Hap1 is also found in ovaries, testes, and in endocrine cells of pituitary, adrenal, thyroid and pancreas (Dragatsis et al., 2000; Liao et al., 2005). Functional similarities in exocytosis between these endocrine tissues and neuronal cells suggest Hap1 is important for endocrine exocytosis and endocytosis function. Analogous characteristics include expression of neuropeptides, presence of dense core granules and regulated exocytosis in response to specific stimuli (Barakat et al., 2004; Day and Salzet 2002). Case in point, regulated secretion of acrosomes in sperm, and cortical granules in egg, follows a common Ca^{2+} dependent pathway (Ducibella and Matson 2007) also required for β cell insulin release. The acrosome is located in the anterior half of spermatozoa head, where Hap1 expression has been shown

(Dragatsis et al., 2000). The presence of Hap1 in these cells supports our hypothesis that Hap1 is a key regulator of endocrine exocytosis.

1.4 The Pancreas

Pancreatic tissue is a heterogeneous mixture of cell types that function in food digestion, metabolic regulation and hormonal signaling for both exocrine and endocrine systems. Often referred to as a "bag of juices", the pancreas secretes digestive enzymes including trypsin, chymotrypsin and lipase from the exocrine acinar cells (Haist et al., 1971(Lin and Haist 1971)). The exocrine portion of the pancreas constitutes about 98% of the total mass. The remaining 2% is composed of the endocrine cells known as the islets of Langerhans, an island shaped structure described initially in 1869 by Paul Langerhans (Namazi et al., 2008).

The islets are composed of several cell types; all are hormone secreting endocrine cells **Fig. 1-2)**. First, the alpha (α) cells produce glucagon, a hormone that functions to increase blood glucose levels when they drop too low by stimulating the liver to convert stored glycogen into glucose for release (Burcelin et al., 2008). Glucagon's actions are extremely important in maintaining steady levels of blood glucose. Second, β cells produce insulin that acts opposite to glucagon by stimulating glucose uptake in peripheral tissues and liver when blood sugar levels rise (Woods et al., 1985). In addition to signaling peripheral tissues to take up glucose from blood, insulin also promotes DNA replication in supporting protein synthesis, decreases autophagy and stimulates arterial muscle tone for increased blood flow (Haist 1971) Third, delta (δ) cells produce

somatostatin (SST), a multifunctional hormone involved in endocrine regulation. Both alpha and beta cells contain SST receptors that respond to extra-islet SST secreted from neuroendocrine hypothalamic cells (Hauge-Evans et al., 2009). Delta cell secreted SST is less understood, with a few recent publications suggesting SST functions to block glucagon release during starvation states (Hauge-Evans et al., 2009; Strowski et al., 2000). Fourth, PP cells (also known as F cells) produce pancreatic polypeptide, which is less well understood. Some evidence suggests a link between low blood plasma levels and obesity, however these results were obtained from unhealthy individuals (Chaudhri et al., 2008). Finally, epsilon cells (ε) produce ghrelin, the hunger hormone. Ghrelin stimulates appetite and acts counter to leptin, which is produced by adipose tissues that signals satiation (Inui et al., 2004).

Hap1 expression has been documented in the islets of Langerhans (Liao et al., 2005). This initial study captured a novel view of Hap1 protein expression throughout the endocrine system, but more detailed analysis was needed to determine if Hap1 is present in all pancreatic islet cells, or unique to specific cell types. A follow up to this original publication demonstrated by immunofluorescent staining that Hap1 is strictly expressed in the β cells of the islets of Langerhans (Liao et al., 2010). Curiously, all of the islet cell types secrete hormones in a similar manner, yet only β cells seem to express Hap1, unless non-β cell Hap1 protein was not detected by IHC in the aforementioned study. If this was the case, the antibodies used may be of no value given that Hap1 is a member of a group of proteins that include T27A3.1d, Milton, amyotrophic lateral sclerosis 2 chromosomal

region candidate gene protein 3 (hALS2CR3), and O-GlcNAc transferase interacting protein of 106 kDa (hOGT-IP) (Mercer et al., 2009).

The phenomenon of multiple isoforms regulating a cellular event is a universally common strategy used by cells throughout the body. A paragon example is the protein synaptotagmin. Synaptotagmins are membrane associated proteins that control calcium-dependent trafficking and come in various isoforms that are expressed differentially depending on which cargo they assist, serving as a key of sorts to unlock full capacity of vesicle transport in the appropriate cell type (Adolfsen et al., 2004; Yoshihara and Montana 2004). Hap1, and other family members, may serve a similar function to assist microtubule motor complexes in recognizing specific cargos, and guiding directional transport.

Pancreatic β cells are the body's sole source of the hormone insulin. As the master regulator of metabolic functions, insulin's most critical job is signaling the body to take up glucose from the blood into cells such as fat, muscle and liver. Glucose is the key carbohydrate source of cellular energy, and also serves as building material for proteins and lipids (Mertz et al., 1996). This simple sugar is derived from more complex carbohydrates in the diet and is absorbed by the intestines into the circulating blood stream. Blood glucose levels are tightly regulated within a narrow range by insulin. In β cells, insulin production, packaging, transport and secretion are constantly adjusted, regulated by a highly sensitive blood sugar-feedback loop. The importance of insulin in metabolism is underscored by the fact that insulin disruption, either by misregulation of

gene transcription or through interrupted secretion, can severely affect the health of individuals (Stolar et al., 2008; Urakami et al., 2005). These effects manifest as unstable blood glucose levels, often leading to diabetes mellitus (DM).

DM consists of two major forms. Type I DM is characterized by a lack of insulin due to destruction of the β cells. Pancreatic β cells are targeted by one's own immune system, known as autoimmunity. Patients suffering from type I DM are insulin dependent and must carefully monitor blood glucose levels and administer insulin appropriately (Salsali and Nathan 2006). Type II DM is far more common, accounting for nearly 95% of all diabetes cases. For this population the medical issue is generally insulin resistance, the reduced ability of insulin to effectively trigger glucose uptake from blood. Thus, insulin production and secretion are functioning properly; dysfunction occurs at target tissues like muscle and adipose cells that receive insulin signal input via membrane receptors and then cue uptake of glucose from blood. Reduced insulin-mediated glucose transport leads to overproduction of insulin from β cells working to compensate for elevated blood glucose levels, or hyperglycemia (Edelman 1998). Both type I and type II are defined by hyperglycemia. Chronically elevated glucose levels significantly increase the chances of heart disease, stroke, amputation and kidney failure due to changes in microvascular structure, atherosclerosis, compromised immune response to infection and albuminuria, respectively (Edelman 1998; Salsali and Nathan 2006)

β cells sit at the center of the delicate blood sugar homeostatic control. Proper secretion of insulin vesicles from β-cells is kinesin and dynein dependent (Meng et al., 1997,

Varadi et al., 2002). Reducing kinesin I protein by siRNA, or introducing a dominant negative kinesin I mutant shuts down insulin vesicle movement in Min6 pancreatic cell culture model (Varadi et al., 2003). Without dynein, insulin vesicle recovery at the β cell membrane is reduced (Varadi et al., 2003). An understanding of how microtubule motor complexes function during insulin exocytosis remains to be uncovered. Thus, Hap1's relationship with microtubule motor complexes is an interesting investigative direction that warrants more attention.

1.5 β Cell Dysfunction in HD

The role of Hap1 in pancreatic β-cells is particularly interesting in light of several reports suggesting a link between HD and impaired insulin secretion (Andreassen et al., 2002; Bjorkqvist et al., 2005; Farrer 1985; Hurlbert et al., 1999; Podolsky S 1972; Smith et al., 2009). These studies report a higher prevalence of diabetes in HD patients and in HD mouse models. For example, mHtt is found to form aggregates in β-cells and interferes with insulin exocytosis (Andreassen et al., 2002; Bjorkqvist et al., 2005; Smith et al., 2009). In addition, insulin trafficking relies on the microtubule component β-tubulin, which mHtt aberrantly binds, disrupting insulin vesicle movement (Smith et al., 2009). However, a full description of the diabetic disease condition in HD has not been offered.

1.6 Dissertation Goals

A survey of the HD field shows a high density of research in topics such as mHtt proteolytic fragments, impaired gene transcription, selective neurodegeneration and disrupted molecular transport. However, there seemed to be little attention paid to issues

regarding HD pathology outside of the brain, despite published data connecting HD to cardiac problems and diabetes dating back 25 years. Interestingly, nearly one third of all HD patients die from cardiac problems (Mihm et al., 2007), and all HD patients are four times more likely to develop diabetes compared to healthy individuals (Hurlbert et al., 1999). Research has neglected these issues, overlooking secondary pathologies in favor of focusing on the devastating neurological consequences of HD.

Given the findings that Hap1 is expressed in neuronal and endocrine tissues that rely on similar mechanisms for vesicle transport and secretion, we hypothesized that Hap1 is involved in exocytosis of insulin from β cells. Our hypothesis is based on previously published results showing that Hap1 is an integral trafficking protein in neuronal environments. Our proposed model is represented in **figure 1-2**. The specific aims of this dissertation were as follows:

1. Create a mouse model conditional for Hap1 depletion in pancreatic β cells. Chapter 2 describes that breeding strategy used for mice, explains the genotype of the novel Hap1 β KO mice, and offers insight into initial characterization of these mice regarding phenotype.
2. Characterize this mouse model regarding metabolic phenotype. Chapter 3 builds upon chapter 2 data to offer a comprehensive description of the negative effects Hap1 depletion has on insulin secretion. Investigations were carried out in a cell culture model, Min6, and confirmed in our mouse model.

3. Describe the molecular events involved in Hap1-dependent trafficking of insulin vesicles. Finally, a mechanism for Hap1-dependent microtubule trafficking is offered in chapter 4. Using a variety of molecular and cellular biology approaches Hap1's role in insulin vesicle transport is described.

This dissertation offers a comprehensive scientific study of the function of Hap1 protein during insulin secretion from pancreatic β cells. I offer *in vitro* and *in vivo* data from endocrine cells that confirms previously reported evidence from neuronal tissues, cementing the hypothesis that Hap1 plays a general role in molecular trafficking in multiple cell types. In addition to constructing a useful mouse model, a sound mechanism of action explains how post-translational modification of Hap1 is connected with microtubule motor proteins p150 and KLC. Lastly, preliminary results in a HD mouse model strengthens the notion that mHtt interferes with molecular transport and puts Hap1 at the center of attention in the correlation between HD and diabetes.

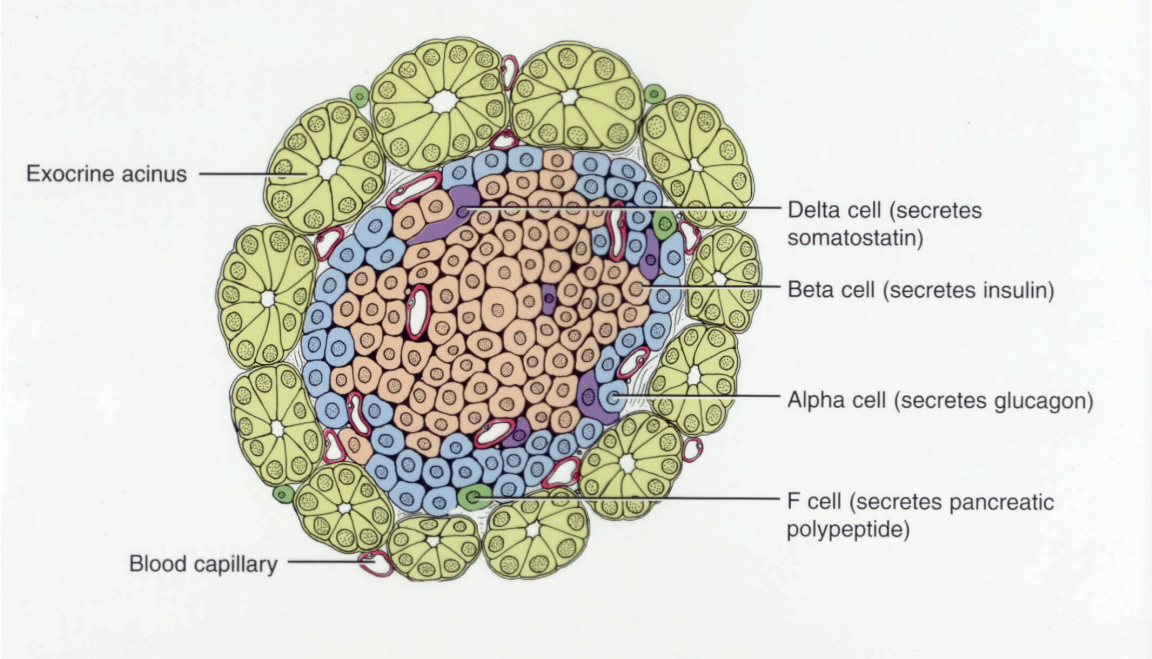

Figure 1-1. Diagram of Islets of Langerhans.

The diagram shows a typical islet surrounded by exocrine acinar cells that secrete digestive enzymes. Within the islet are delta, beta, alpha and epsilon endocrine cells. Blood capillaries innervate both exocrine and endocrine pancreas tissue.

Name	Function
Huntingtin	Scaffold protein
P150 Glued	Trafficking protein
KLC	Vesicular trafficking
Hrs	Vesicular trafficking
$GABA_A$ receptors	Membrane receptor
$InsP_3R1$	Membrane receptor
TrkA	Nerve growth factor receptor
14-3-3	Multifunctional regulatory protein
Ahi	Multifunctional protein
EGFR NeuroD	Neuronal transcription factor
Duo	GDP-GTP exchange factor
AR	Androgen receptor
TBP	Transcription factor

Wu et al., Cell Adh & Mig 2009

Table 1-1. Hap1 Interacting Proteins.

The table lists currently known proteins that associate with Hap1.

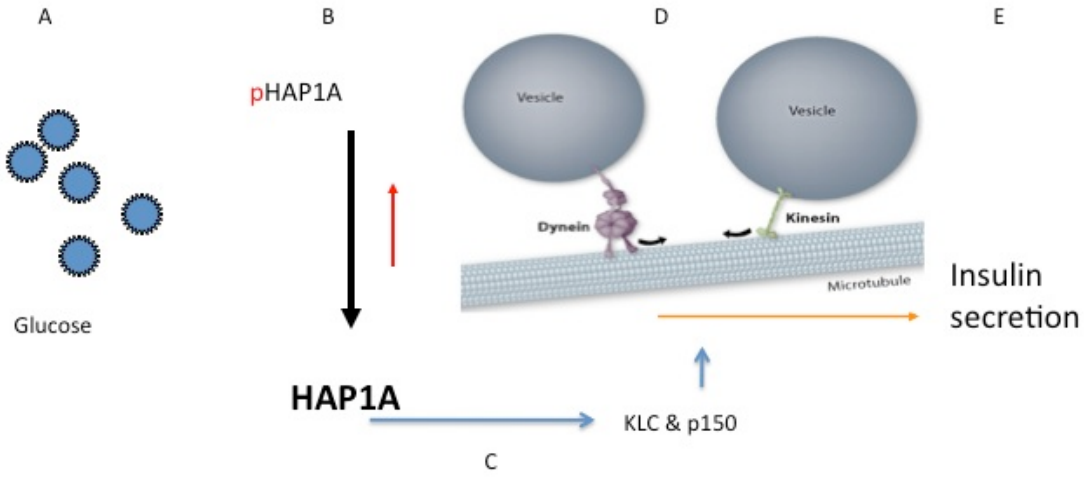

Figure 1-2. Model of Hap1-Dependent Trafficking in β Cells.

Graphical interpretation of the function of Hap1 during glucose stimulated insulin secretion. An increase in blood sugar levels, by glucose stimulation (A), phosphorylated Hap1A is dephosphorylated quickly (B), increasing the concentration of Hap1 species. (C) The increased binding affinity of Hap1A to trafficking proteins KLC and p150 supports vesicular transport of insulin cargo along microtubules (D), leading to insulin secretion (E).

Chapter 2

Creation of Hap1 Conditional β-Cell Knockout Mice

Introduction

The classical mantra in genetic research is “create a mutant”. If one wants to understand the function of a particular protein or event, a direct approach is to remove that factor from the animal. As a graduate student studying genetics and molecular biology, a central theme to my dissertation is using the power of genetic research tools for investigating biological phenomena. In my case, the Hap1 protein is under investigation. The power of genetic engineering offers sophisticated tools designed for tinkering with genes.

Although Hap1 was originally found to be a neuronal protein that is enriched in the brain, recent studies have revealed its presence in endocrine cells including pituitary, thyroid, adrenal and pancreas (Dragatsis et al., 2000; Liao et al., 2005). Genetic deletion of Hap1 expression is a powerful approach to investigate the function of Hap1 in cells. Our lab and others have produced Hap1 null mice via germ line depletion of the Hap1 gene. However, Hap1 null mice die within 2-3 days after birth (Chan et al., 2002), and proper *in vivo* analysis of Hap1 function in specific cell types could not be investigated using this model. Therefore, I chose to create a Hap1 conditional KO mouse model deficient for Hap1 protein in β cells of the pancreas. This new system would become a useful platform to investigate Hap1’s function during insulin secretion.

I used a Cre-loxP knockout strategy to selectively delete Hap1 in pancreatic β cells. This sophisticated genetic method, developed by Brian Sauer at DuPont in the 1980’s (Sauer 1987; Sauer and Henderson 1988) is a great tool to alter DNA at specific sites. For site-specific recombination, the cyclic recombinase (Cre) enzyme functions as molecular

scissors that cut only at special sites in DNA called locus of X-over P1 (*loxP*). The advantage of this system lies in the specificity of Cre expression in time or tissue, providing precise control over gene manipulation. My mouse breeding strategy began with a male mouse that expressed Cre, under the control of the pre-pro insulin (*Ins2*) gene promoter, in only β cells of pancreas. When crossed with a female mouse containing *loxP* sites flanking exon 1 of the Hap1 gene, Cre recombinase selectively deletes this portion of Hap1 in the β cells. As a result, the truncated Hap1 gene in β cells is unable to express Hap1 protein.

I confirmed Hap1 depletion in β cells using PCR on genomic DNA and immunohistochemical staining in the pancreatic tissue of conditional Hap1 knockout mice. In addition, Hap1 β cell KO mice were evaluated for phenotypic changes in glucose tolerance, insulin sensitivity, and gross morphological alterations to insulin granule pools. Finally, metabolic measurements of body weight, and intake of water and food were analyzed. These analyses revealed that depletion of Hap1 in β cells in mice can affect glucose tolerance, which turns out to be a solid clue that gave me confidence in my hypothesis that Hap1 is required for insulin secretion from pancreatic β cells.

Results

Conditional Hap1 knockout in β cells of mouse pancreas.

To investigate the role of Hap1 in endocrine cells, I first generated gene-targeting mice by inserting the loxP sites flanking exon1 of the mouse Hap1 gene. I then selectively deleted exon1 of the *Hap1* gene in mouse pancreatic β cells by crossing floxed Hap1 mice (C57BL/6/SV129) carrying homozygous *loxP*-flanked *Hap1* exon1 ($Hap1^{Flox/Flox}$) to *Ins2-Cre* C57BL/6 mice (**Fig. 2-1A**), which express Cre in pancreatic β cells under the control of the rat insulin II promoter (Postic, et al. JBC 1999). Mice heterozygous for Loxp-Hap1 with Cre were mated to obtain homozygous and heterozygous floxed Hap1 mice that also carry the Cre transgene (**Fig. 2-1B**). Because the deletion of the Hap1 gene selectively occurs in a few β cells in pancreas, we analyzed the expression of Hap1 via immunohistochemistry. I did not find any significant difference in Hap1 expression in β cells between wild type and heterozygous floxed Hap1 ($Ins2\text{-}Hap1^{+/-)}$ mice that also express Cre (**Fig. 2-2**). However, Hap1 in β cells of homozygous floxed mice with Cre ($Ins2\text{-}Hap1^{-/-}$) is apparently reduced when compared with the heterozygous $Ins2\text{-}Hap1^{+/-}$ mice (**Fig. 2-2**). Since heterozygous $Ins2\text{-}Hap1^{+/-}$ also carry the Cre transgene, a genetic background similar to homozygous $Ins2\text{-}Hap1^{-/-}$ mice, we used $Ins2\text{-}Hap1^{+/-}$ (referred to as control) mice as a control for comparison with $Ins2\text{-}Hap1^{-/-}$ (referred to as KO) mice to define the specific effects caused by Hap1 deficiency in β cells.

Reduced glucose tolerance in Hap1 KO mice.

Since β cells in the pancreas secrete insulin to regulate blood glucose levels, I performed glucose tolerance experiments on KO mice and their controls. Following i.p. injection of glucose at 2.0 g/kg body weight, there was a marked increase of plasma glucose at 15-30 min, which then declined within 120 min. KO mice showed a significantly higher level of plasma glucose compared with WT or control mice in both males (**Fig. 2-3A**) and females (**Fig. 2-3B**), suggesting a defect in insulin release.

Insulin tolerance is unaffected in Hap1 KO mice.

To verify that defective glucose tolerance is not due to impairment of insulin receptor function in peripheral tissues, I performed an insulin tolerance test. I injected insulin (1.0 unit/kg body weight) i.p. into KO mice and their controls. There was no significant difference in plasma glucose levels between KO and control mice after insulin injection (**Fig. 2-4**), indicating insulin signaling was unaffected in KO mice.

Other metabolic measures.

Since β cells in the pancreas are important for the body's metabolism, I wondered whether selective elimination of Hap1 in β cells would cause any alteration of body weight. I monitored the body weight of KO mice along with control mice and found a significant difference in both males and females at one month of age (**Fig. 2-5**). However, KO mice overcame the body weight difference within two months and grew comparably with controls over their lifespan. Male KO mice maintained a body weight slightly less than controls over time without significant differences (**Fig. 2-5A**).

Disrupted insulin and glucose levels can have a dramatic impact on thirst and hunger. Therefore, water and food consumption were measured in Hap1 KO mice and their controls. In both male and female, no significant change in water or food intake was found from 3 months to 12 months of age (**Fig. 2-6**).

Gross morphology of insulin vesicle pools.

To analyze the effect of Hap1 depletion on insulin vesicle transport, I visualized the insulin pools of KO and control mice using immunohistochemistry. Pancreas tissues from postnatal day 2 mice were isolated and prepared for fluorescent staining of insulin vesicles. I found that Hap1 KO β cells displayed a higher staining concentration of insulin compared to controls (**Fig. 2-7**). Visually, Hap1 KO β cells appear to either contain more insulin vesicles or have larger volume of insulin per vesicle. Although this difference is more qualitative than quantitative, it does suggest Hap1 depletion interferes with insulin vesicle dynamics.

Taken together, selective depletion of Hap1 in the mouse pancreatic β cells impairs glucose tolerance without affecting insulin signaling in peripheral tissues, and no significant changes in the body weight or nutrient consumption were found.

Discussion

In order to study the function of Hap1 in β cells, I have created a conditional knockout mouse model in which Hap1 is specifically depleted in β cells using a Cre/LoxP strategy. I was confident our KO mice were properly created based on genotyping data and immunohistochemistry of pancreas tissue, both of which are standard confirmation tools used in similar conditional KO strategies for other β cell proteins {Maximov, 2008 #852;Gorogawa, 2004 #853;(Ellingsgaard et al., 2008; Gorogawa et al., 2004; Maximov et al., 2008; Zhang et al., 2009).

As a direct consequence of Hap1 depletion, KO mice had impaired glucose tolerance compared to controls. This reduced ability to metabolize a physiologically relevant dose of sugar suggested insulin secretion was disrupted in KO mice. A rise in blood glucose levels should trigger β cells to release insulin, signaling the target peripheral tissues muscle, liver and fat cells to take up extra glucose from blood. A second possibility for this defect could reside in the insulin signaling in peripheral tissue responsible for taking up excess glucose from blood. However, challenging mice with a dose of insulin to directly measure the effect of insulin on the blood glucose levels in peripheral tissue confirmed that insulin function was not affected in KO mice. Thus, I concluded the glucose intolerance resulted from a lack of insulin secreted from the pancreatic β cells rather than malfunction of insulin at target peripheral tissue. **Figure 2-8** shows these two possibilities that explain glucose intolerance.

Food and water intake measurements showed no difference between Hap1 KO and their control littermates. This data suggests the glucose intolerance is not related to size of the animals, nor is it dependent upon appetite and thirst patterns. Thus, generation of conditional Hap1 knockout mice allowed me to obtain important *in vivo* evidence for the participation of Hap1 in insulin secretion.

Materials and Methods

Animals. Generation of conditional Hap1 knockout mice, in which exon1 of the mouse *Hap1* two loxP sites to allow the Cre-mediated deletion of exon1 flanked gene, was described in our recent study (Lin et al., 2010). β cell conditional Hap1 knockout mice were generated by crossing INS2-Cre transgenic mice (B6.Cg-Tg(Ins2-cre)25Mgn/J, The Jackson Laboratory) with $Hap1^{flox/flox}$ mice. All mice were generated on C57BL/6J background. The resulting heterozygous mice were used to generate homozygous conditional knockout (*Ins2-*Cre^{+}*/Hap1*$^{loxp/loxp}$, referred to as *Ins2-Hap1*$^{-/-}$*, or simply KO throughout this dissertation*) or heterozygous (*Ins2-*Cre^{+}*/Hap1*$^{+/loxp}$, referred to as *Ins2-Hap1*$^{+/-}$*, or controls*) mice. Genotyping of these mice was performed with genomic DNA extracted from the tails via PCR to amplify the mouse Hap1 DNA fragment (from 4929 nt to 5003 nt) using the forward (5'- TTT TTC TGG GGA GCA TAC GTC-3') and reverse (5'- ATC CGT TAT CCC AGG GTC TGA-3') primers. Primers (forward: 5'-GCG GTC TGG CAG TAA AAA CTA TC-3' and reverse: 5'-TGT TTC ACT ATC CAG GTT ACG G-3') that amplify Cre recombinase were also used to determine the presence of the Cre.

PCR conditions:

Both Cre and LoxP primer sets were run together in same reaction.

1. 96^{o} C for 3 min
2. 96^{o} C for 45 sec
3. 60^{o} C for 45 sec
4. 72^{o} for 1 min

5. Repeat steps 2-4 for 34 cycles
6. 72^{o} C for 5 min
7. Soak at 18^{o} C

Expression analysis of Hap1 and behavioral tests of heterozygous *Ins2-Hap1*$^{+/-}$ (control) and wild-type mice revealed no differences between these two groups. Because homozygous (*Ins2-Hap1*$^{-/-}$) and heterozygous (*Ins2-Hap1*$^{+/-}$) mice share the same mixed genetic background, mice of these two genotypes were used mainly to reveal differences related to Hap1 deficiency in β cells. Mice were housed in a light [12-h dark (19:00-7:00) and 12-h light (7:00-19:00) cycle] and temperature (22°C)-controlled chamber. Animals were fed *ad libitum* (Lab Diet 5001) and maintained in the animal facility at Emory University in accordance with institutional animal care and use guidelines.

Immunohistochemistry. For pancreatic tissue, mice were sacrificed by cervical dislocation and the pancreas was removed. Pancreas tissues were submerged in Bouin's fixative (saturated picric acid, formaldehyde, glacial acetic acid) overnight. Tissue was washed in H_20 until yellow color faded then placed in neutral buffered formalin for storage until processed by the WCI Pathology Core Lab at Emory University. The paraffin embedded slides were rehydrated in xylene, EtOH then water. Antigens were unmasked in sodium citrate buffer for 3 min in microwave at low heat. Slides were blocked in 2% normal goat serum, 0.5% Triton X-100, 1% BSA in PBS for 30 min. After block, slides were incubated with primary antibodies to insulin, Hap1 (EM 78), and nuclei (Hoechst) overnight at 4°C. Light micrographs were taken on a Zeiss microscope

(Axiovert 200 M) with a digital camera (Hamamatsu ORCA-100) and processed with Openlab software (Improvision, Inc).

Glucose Tolerance Test. After an overnight fast, mice were given an i.p. injection of glucose at 2.0 g/kg body weight. Blood glucose levels were measured with Lifescan SureStep glucose monitor. Each time point reading was performed twice for each mouse.

Insulin Tolerance Test. Fasted mice were i.p. injected with 1.0 unit/kg body weight human insulin (Sigma). Blood glucose levels were measured over a time course using Lifescan SureStep glucose monitor.

Metabolic Measurements. Body weights of mice were measured every month beginning at weaning date, 21 days, and continued through life span. For water and food intake, mice were housed in static cages with consumption monitored manually every 2 days. Weight of food and water consumption was calculated as grams per mouse per day. Mice were housed in groups of 2-3 each genotype for 1 month of analysis.

Statistical Analysis. Student's t-test was used to determine significance for single time point measurements. ANOVA was used to calculate significance for values in timed series for GTT.

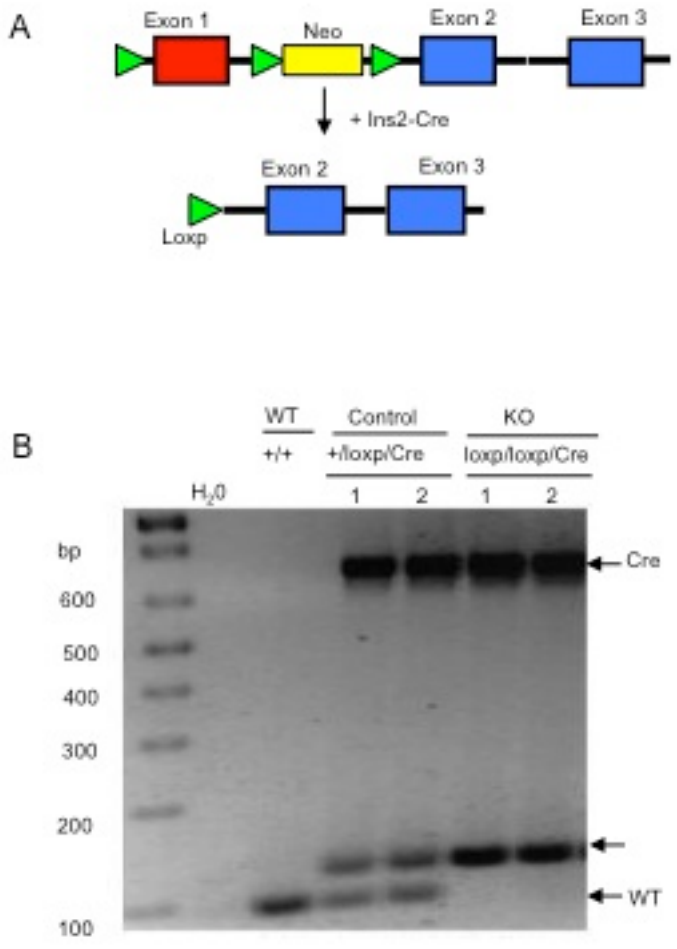

Figure 2-1. Generation of Hap1 Conditional Knockout Mice.

(**A**) LoxP sites flank exon1 of the mouse Hap gene in targeted allele. Mice carrying this targeted gene were mated with transgenic mice that express Cre in pancreatic beta cells driven by the preproinsulin 2 (Ins2) promoter, resulting in offspring mice that have deleted exon1 of the Hap1 gene. (**B**) PCR genotyping of crossed mice showing the presence of the Cre and loxP alleles in homozygous (loxp/loxp/Cre), heterozygous (+/loxp/Cre), but not in wild type (+/+) mice. For simplicity, homozygous loxp/loxp/Cre mice are referred to as Hap1 KO and heterozygous (+/loxp/Cre) as control. All mice were bred on C57/BL6 background.

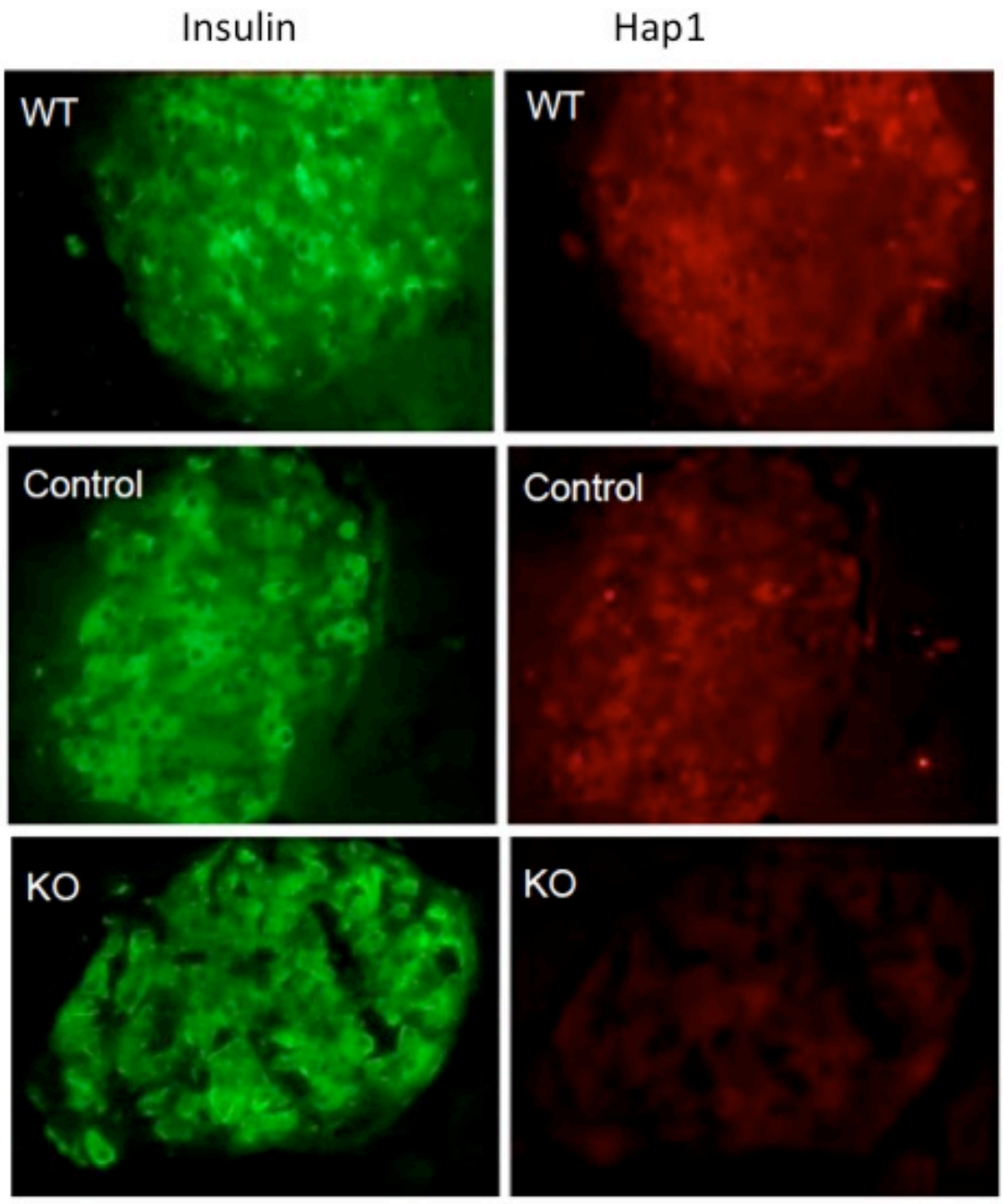

Figure 2-2. Confirmation of Hap1 Depletion in Pancreatic β Cells.

Double immunofluorescence staining of insulin (green) and Hap1 (red) showing Hap1 depletion in insulin positive beta cells in Hap1 KO male mice 3 months of age.

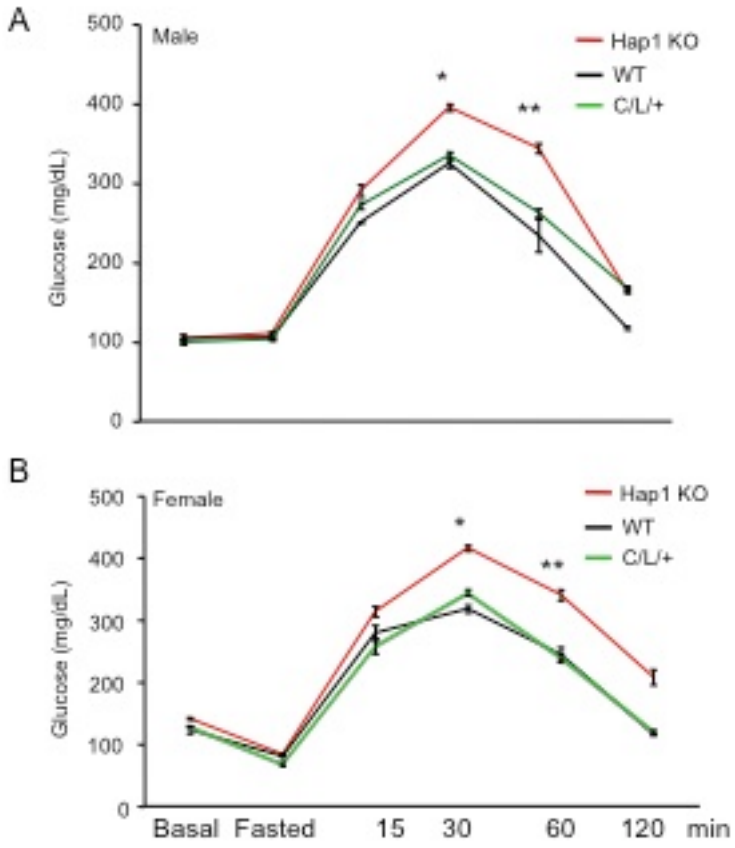

Figure 2-3. Glucose Tolerance is Impaired in Hap1 KO Mice.

A glucose tolerance test was performed on overnight-fasted **(A)** male mice (n= 10 each group) and **(B)** female mice (n=5 each group) at the age of 3 months. Hap1 KO mice displayed glucose clearing impairment measured by a blood glucose monitor following i.p. of glucose (2 g/kg bodyweight). *$p<0.05$, ** $p<0.01$ compared to control.

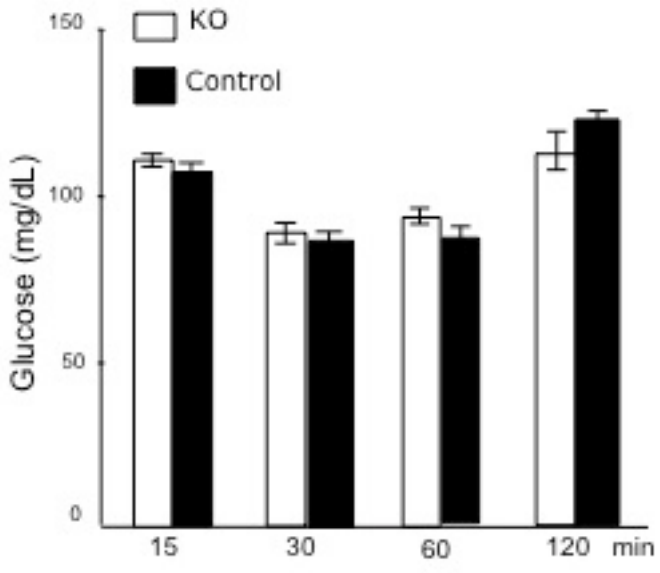

Figure 2-4. Insulin Sensitivity is Unaffected in Hap1 KO Mice.

An insulin tolerance test on 3-month-old male mice (n=4 each group) fasted overnight showed no significant difference in KO compared to control mice in insulin sensitivity. Blood glucose levels were measured after i.p. injection of insulin (1 unit /kg body weight). $p>0.05$; p values calculated using student's t-test.

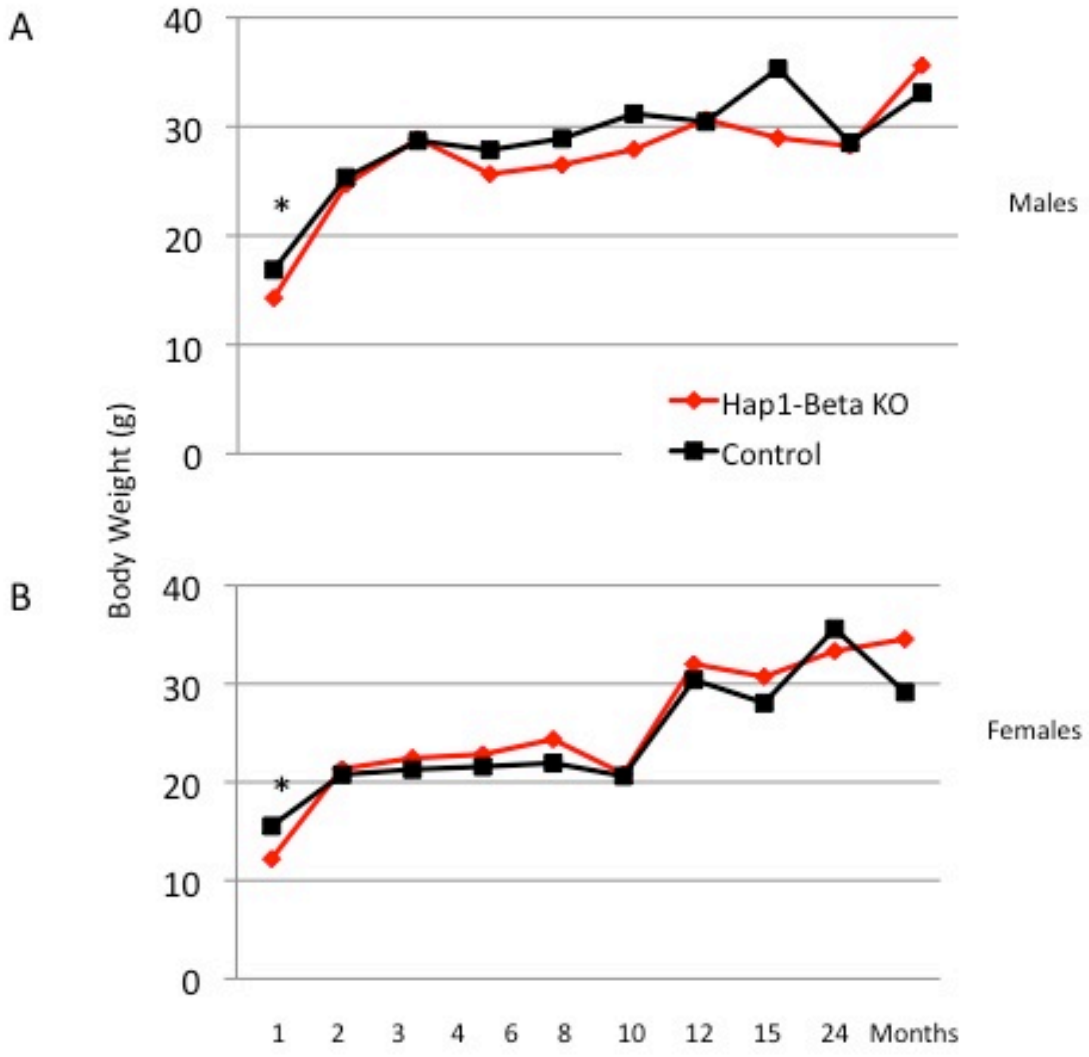

Figure 2-5. Body Weight Measurements.

Body weight in male **(A)** and female **(B)** Hap1 KO mice compared with the littermate control mice under the normal feeding conditions over time. N=10 per group. *$p<0.05$ according to ANOVA analysis.

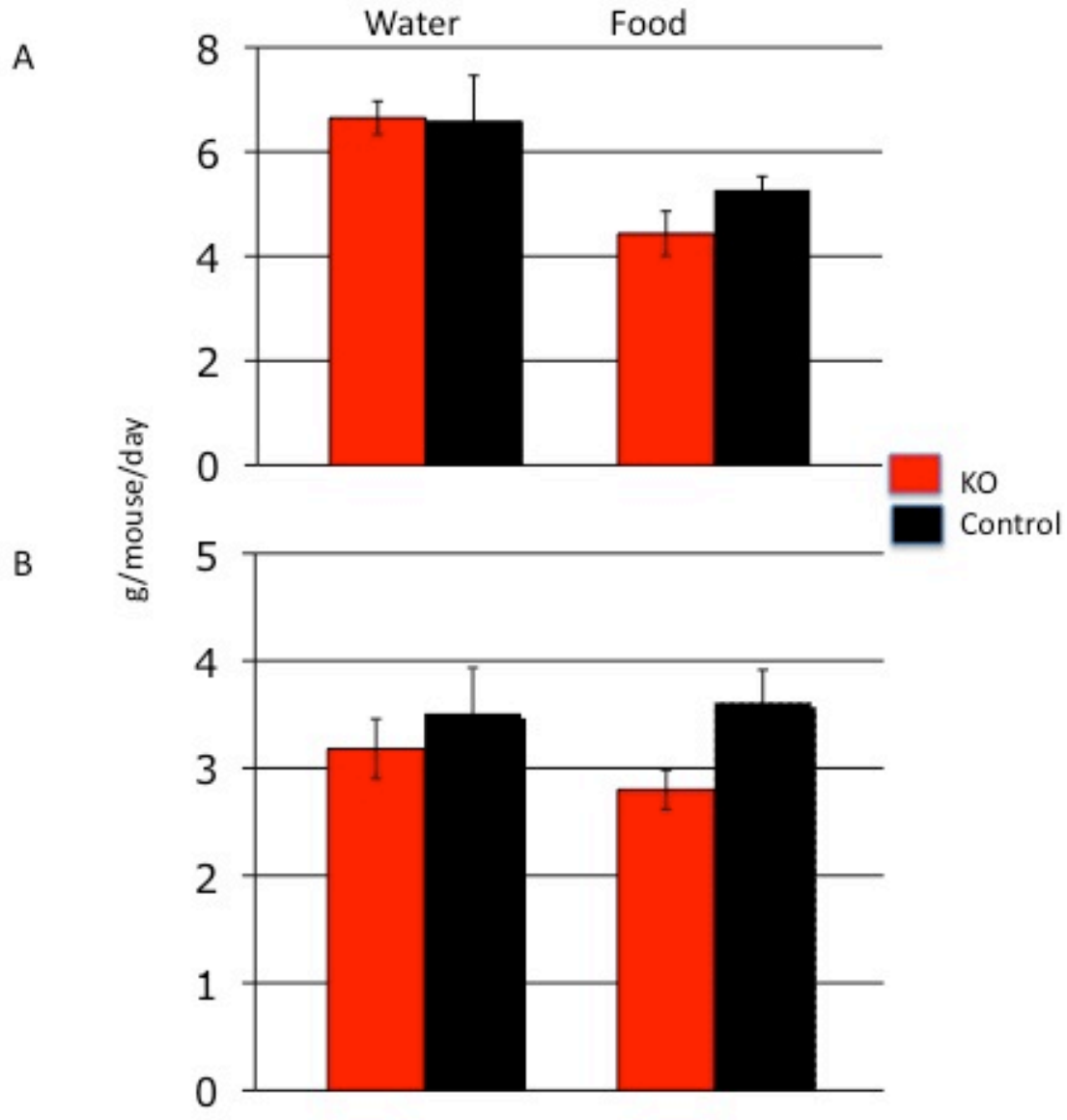

Figure 2-6. Water and Food Consumption.

Water and food intake of mice aged 3-12 months for male **(A)** and female **(B)**. N=6 per group. Intake was averaged over 1 month of measurement as grams/mouse/day. All p values >0.05 using student's t-test.

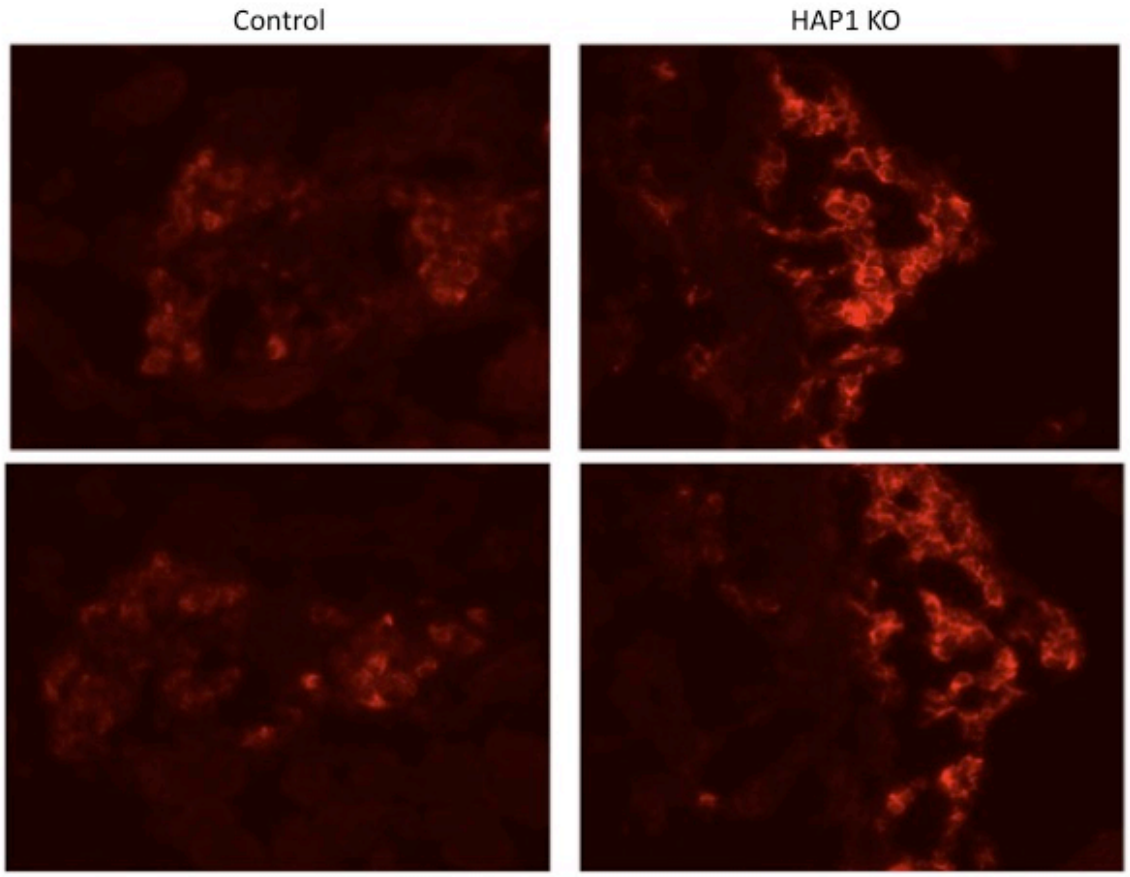

Figure 2-7. More Intense Insulin Staining in Hap1 KO Islets.

Immunofluorescent staining of insulin in WT and Hap1 KO postnatal day 2 mice. Staining indicates a more intense concentration of insulin in KO mice. Images are of islets in pancreatic tissue slice (15 μm) representative of three mice from each genotype.

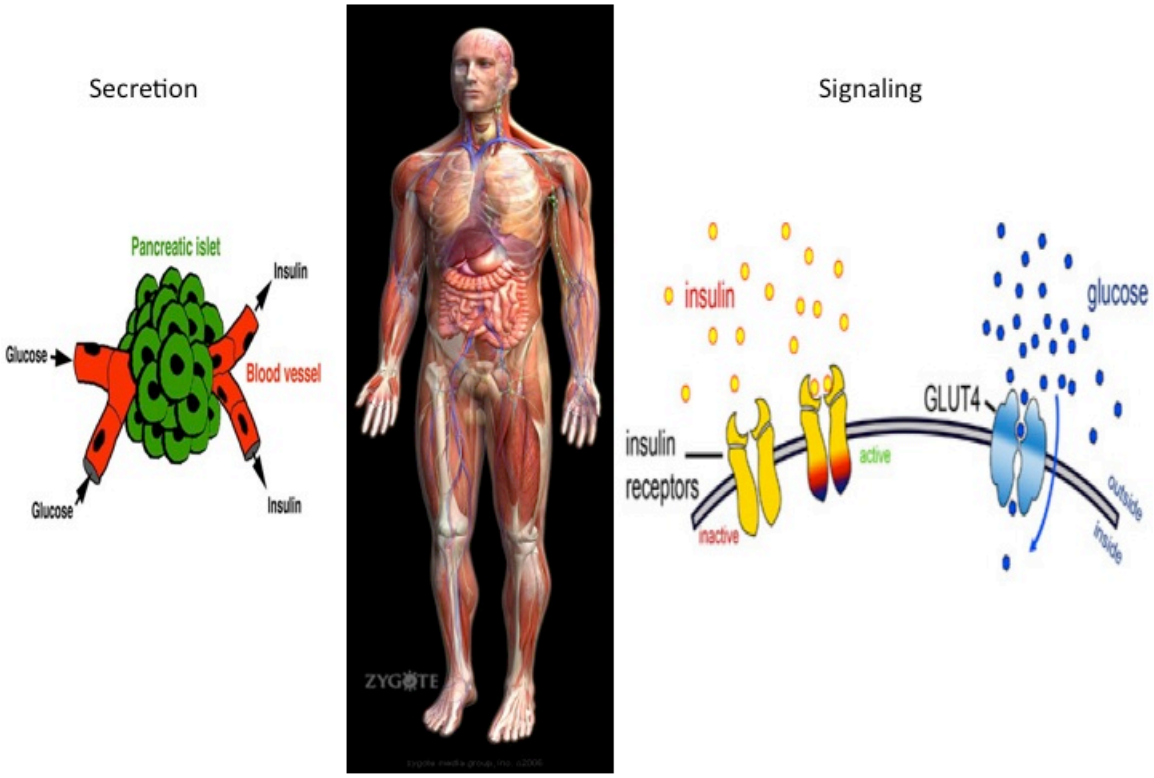

Figure 2-8. **Simple schematic of glucose stimulation response in the body**.

The upstream component of blood sugar regulation is direct sensing of glucose by β cells on the left that secreted insulin in response. Downstream events take place after insulin is released into the blood and travels to target tissues like muscle to signal glucose membrane transporters GLUT4 to take up excess glucose from blood into the cells.

Chapter 3

Hap1 Depletion Reduces Insulin Secretion From Pancreatic β Cells

Introduction

Insulin secretion from pancreatic β cells is a well characterized phenomenon (**Fig. 3-1**). Increased glucose blood levels trigger uptake of the sugar into pancreatic β cells through glucose transporters called GLUT2. Glycolysis quickly metabolizes glucose and produces ATP energy molecules. Increased intracellular ATP in β cells triggers closure of potassium (K^+) channels leading to membrane depolarization. Voltage controlled calcium (Ca^{2+}) channels open to allow diffusion of Ca^{2+} into the cytoplasm. An increase in calcium concentration activates phospholipase C that cleaves membrane phospholipid phosphatidyl inositol 4,5-bisphosphate into phosphatidyl inositol 1,4,5-triphosphate (IP3) and diacylglycerol. IP3 binds endoplasmic reticulum (ER) receptors that further facilitate calcium release from ER to cytoplasm. High cytoplasmic calcium concentrations signals mature insulin vesicles to fuse with the plasma membrane dumping insulin contents directly into pancreatic capillaries (Burgoyne and Morgan 2003; Cheek and Barry 1993; Varadi et al., 2002).

Glucose stimulated insulin secretion is the physiologically significant mechanism responsible for nearly all insulin exocytosis. As regulators, β cells are structured to sense and respond to slight changes in blood sugar, which is of course glucose (**Fig. 3-2**). Other molecules are capable of triggering insulin release, however (Liang and Matschinsky 1994). These secretagogues are often used in experimental biology for various reasons, but are not worth mentioning here given that their combined contribution to insulin release in mouse or human is either negligible or artificial. I sought to recreate a

condition of induced insulin secretion as close to an *in vivo* natural setting as possible using the sugar glucose at a physiologically relevant dose and delivery method.

Much attention has been paid to insulin transcription, vesicle formation in the Golgi body, signaling pathways that signal exocytosis and fusion of mature vesicles at the plasma membrane. However, less is known regarding the trafficking of insulin cargo along microtubules and the molecular motor complexes that transport it (Liang, 1994; Wang, 2009). While nuclear and membrane related molecular events are littered with extensive research projects, the microtubule networks in endocrine cells have far less description. As a consequence, important questions remain regarding how molecular motor complexes form to correctly hook up with specific cargo, what signals guide directionality of the vesicles along microtubules, and mechanisms of trafficking disruptions in disease conditions such as HD. I hypothesize that understanding Hap1's role during β cell insulin secretion will help understand vesicular trafficking in more detail.

According to my hypothesis, Hap1 is required for proper insulin exocytosis. If this is the case, Hap1 deletion should interfere with insulin secretion, which can be directly measured as a concentration in blood samples from mice, or in media from beta cell culture. During the lengthy time required for breeding the Hap1 KO mice, I also studied the β cell tissue culture model Min6 for the effect of Hap1 expression on insulin release.

Min6 cells were created from insulinomas obtained by targeted expression of the simian virus 40 T antigen gene in transgenic mice (Miyazaki et al., 1990). These cells have been well characterized and respond to insulin secretagogues in a similar manner as isolated mouse β cells and *in vivo* measurements (Miyazaki et al., 1990; Nakashima et al., 2009; Oliver-Krasinski and Stoffers 2008). As a model system, this cell line has produced significant data in the fields of diabetes and pancreatic β cell development. They have been used to demonstrate a role for synatxin-4 and actin in insulin exocytosis (Jewell et al., 2008), 3-D imaging of individual insulin vesicle release events (Rutter et al., 2006), and introduce a role for Pdx-1 during β cell maturation (Szabat et al., 2009) as well as many other findings.

I confirmed that both isoforms of Hap1 are expressed in Min6 cells by western blot. Immunocytochemistry showed Hap1A is localized in the cytoplasm, where insulin vesicle trafficking occurs. In addition, fluorescent staining demonstrated Hap1 colocalizes with insulin vesicles. Finally, I show Hap1 depletion in Min6 cells and in KO mice inhibits insulin secretion. All of these findings support the idea that Hap1 deficiency in pancreatic β cells reduces insulin release in response to glucose challenge.

Results

Hap1 isoforms are expressed in β cells and colocalize with insulin vesicles.

Building on the evidence provided by Liao *et al.* showing Hap1 presence in pancreas islets, I took a closer look at Hap1 using Min6 β cells. Using tissue culture I confirmed by western blot Hap1 isoforms A and B are expressed at similar molecular weights in Min6 cells compared with PC12 control (**Fig. 3-3**). Because insulin vesicle trafficking occurs in the cytoplasm, I performed fluorescent staining in Min6 cells to confirm Hap1 expression localization. Based on the previous reported function of Hap1A as a modifiable regulator of molecular trafficking, I looked at the distribution in Min6 cells. As expected from neuronal data, Hap1A is found strictly in the cytoplasm (**Fig. 3-4**). In addition, immunofluorescent staining of Min6 cells shows Hap1 colocalizes with both insulin and phogrin, a membrane protein marker in insulin vesicles (**Fig. 3-5**). These data suggest that Hap1 protein is closely associated with insulin vesicles in β cells and may participate in insulin secretion.

Hap1 knockdown by RNAi reduces insulin secretion in Min6 β cells .

To provide direct evidence for my hypothesis, I cultured Min6 cells to measure insulin released in the medium via an enzyme-linked immunosorbent assay (ELISA). Min6 cells were infected with adenoviral Hap1 shRNA (Sheng 2006) to reduce Hap1 levels. I confirmed the shRNA was specific to Hap1 protein using western blot (**Fig. 3-6A**). The amount of insulin released into the medium was significantly reduced only in Hap1 shRNA treated cells, but not in untreated or scramble siRNA treated cells (**Fig. 3-6B**). This suggests that Hap1 deficiency negatively affects insulin release from β cells.

Insulin secretion is reduced in Hap1 β cell KO mice.

To examine the levels of insulin secreted from β cells of mice, I measured plasma insulin levels by an enzyme linked immunosorbent assay (ELISA) following i.p. injection of glucose at 2.0 g/kg body weight. I observed a significant decrease in plasma insulin concentration in KO mice compared to control mice (**Fig. 3-7**). These findings verified that loss of Hap1 in β cells can affect release of insulin into blood in response to elevated blood glucose.

Discussion

Evidence has shown insulin secretion is microtubule dependent. Molecular motor proteins such as kinesin and dynein are critical for proper vesicle transport along the microtubule network. Nevertheless, evidence is weak regarding which motor proteins are important and how vesicle cargos are recognized by trafficking complexes (Welte 2004).

Although Hap1 expression has been shown in pancreatic islets, no published information was available regarding expression levels of its isoforms. One recent publication demonstrated Hap1 expression is limited to the β cells of the islets (Liao et al., 2010). This evidence supports my hypothesis that Hap1 plays a role in insulin secretion from β cells. Immunohistochemistry is particularly useful to assess the selective expression of the Hap1 protein given that β cells comprise such a small population of pancreatic cells. My data demonstrates Hap1 colocalizes with insulin vesicles, also supporting the role of Hap1 in function of insulin release from β cells.

The strongest evidence for the important function of Hap1 in β cells lies in its effect on the secretion of insulin from pancreatic β cells. I found Hap1 depletion results in reduced insulin secretion in both Min6 cell culture and KO mice challenged with glucose. The level of reduced insulin secretion in siRNA treated Min6 cells is dependent on the inhibition of Hap1 expression, which may not be as effective as of the elimination of Hap1 *in vivo* via the Cre-Loxp system. However, these two complementary findings are consistent and clearly show Hap1 depletion reduces insulin secretion.

These data support the findings that Hap1 KO mice suffer from glucose intolerance. As glucose levels rise, an effective β cells response is a robust insulin release to signal peripheral tissues to up-take excess glucose, thereby maintaining a balanced blood sugar level. The failure of Hap1 KO mice to effectively deal with increased glucose load and the fact that insulin sensitivity is not affected suggests the metabolic defect is insulin secretion.

In humans, chronic glucose intolerance combined with defective insulin secretion are fundamental factors leading to DM. Patients suffering from DM suffer from multiple health conditions including higher risk of cardiovascular disease, obesity, stroke, and shortened lifespan (Edelman 1998). In Hap1 β cell KO mice, however, my investigations did not uncover similar health defects. The most likely explanation comes from the drastic difference in the daily diets of humans versus lab mice. Although genetic risk factors exist for DM, nutritional intake is the most powerful contributor to disease onset (Salsali and Nathan 2006). Poor dietary habits including high fat, high sodium, and excess calories are highly correlated with DM in humans. Many studies have clearly shown individuals diagnosed with DM are able to resolve blood glucose abnormalities with nutritional intervention (Stolar et al., 2008). Compared to the typical human diet, lab mice survive on an unchanged balanced diet free from excessive caloric swings. Therefore, phenotypes like glucose intolerance, which is dependent upon metabolic deficiencies, are muted in the lab setting. One interesting experiment yet to be conducted is to observe the long term effect of a human type diet on my Hap1 KO mice. I suspect Hap1 deficient mice would acquire changes to their body mass index, body weight, and

possibly activity levels, as suggested by reports in humans showing DM, increased body fat, and reduced physical activity are strongly correlated (Salsali and Nathan 2006).

Materials and Methods

Cell culture and Hap1 shRNA treatment. Adenoviral Hap1 shRNA was generated in our previous studies (McGuire et al., 2006). Min6 cells were grown in standard media (DMEM, Sigma D5671) with 15% FCS Sigma F7524), 71 μM 2-mercaptoethanol (Sigma M7522), 2 mM glutamine (Sigma G6392), 100 U/ml penicillin/ 100 μg/ml streptomycin (Sigma P0781) in 12-well plates to 60% confluency. Cells were treated with adenoviral Hap1 shRNA or scramble shRNA for 12 hours in standard culture media. Cells were cultured for additional 48 h before measuring glucose induced insulin release.

Insulin secretion. Insulin secretion was measured using an ELISA Kit (Crystal Chemicon). For *in vivo* measurements, mice were fasted overnight and then i.p. injected with 2.0 g/kg glucose. Plasma insulin levels were measured using tail blood collected at different times. For *in vitro* measurements, MIN6 cells were plated in 12-well plates and maintained in DMEM (Sigma D5671) with 15% FCS (Invitrogen), 71uM 2-mercaptoethanol (Sigma M7522), 2mM glutamine (Sigma G6392), 100 U/ml penicillin/ 100 μg/ml streptomycin (Sigma P0781). Cells were kept in a 37°C tissue culture incubator with 5% CO_2 and plated in 12-well plates for 48 hours. Culture media was removed and replaced with glucose free Krebs buffer for 1 hour. Cells were then treated with Krebs buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 4.2 mM NaHCO3, 2 mM CaCl2, 200 mM sulphinpyrazone and 10 mM Hepes, pH 7.4) containing 20 mM glucose. Insulin content was measured from collected media via ELISA assay in triplicate.

Western Blotting. PC12 cells were cultured in DMEM with 5% fetal bovine serum (FBS), 10% horse serum, 100 μg/ml penicillin, 100 μg/ml streptomycin. Min6 cells were grown in DMEM with 25mM glucose, 15% FCS, 71 μM 2-mercaptoethanol, 2mM glutamine, 100 μg/ml penicillin, 100 μg/ml streptomycin. Cells were harvested by scraping 10 cm plate with cell scraper in NP40 lysis buffer (protease inhibitor, PMSF). Lysed samples were sonicated 10 seconds, SDS running buffer added, then boiled 10 minutes for SDS-Page on tris-glycine gel. Western blots were carried out using primary antibodies to Hap1 (EM 78) and tubulin control. Blots were visualized with Kodak x-ray developer with ECL Plus HRP-conjugated enzyme signal reagent.

Immunofluorescent Assays. Immunocytochemistry was performed on cultured cells grown 48 hours at 37^0 C in 5% CO_2 chamber. Cells were fixed in 4% paraformaldehyde for 10 minutes, then blocked in PBS (0.5% Triton X-100, 3% BSA) for 30 min. After blocking, cells were incubated with primary antibodies to insulin, Hap1 (EM 78), phogrin ("cyrus" gift from Dr. Sutton), and nuclei (Hoechst) overnight at 4°C. Secondary fluorescent antibodies were added in same block buffer for 30 minutes at 40C. Light micrographs were taken on a Zeiss microscope (Axiovert 200 M) with a digital camera (Hamamatsu ORCA-100) and processed with Openlab software (Improvision, Inc).

Statistical Analysis. Student's t-test was used to calculate significant differences in all time points as single events.

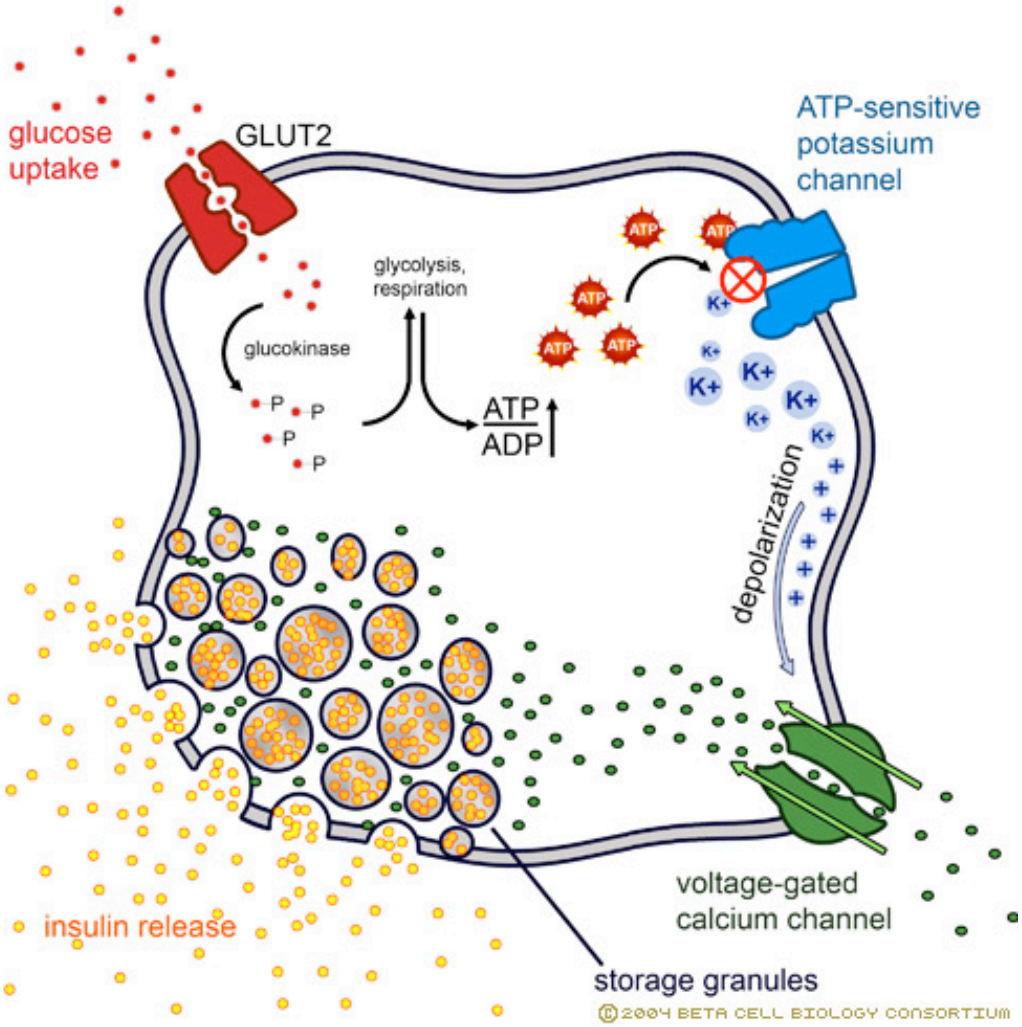

Figure 3-1. Overview of Glucose Stimulated Insulin Secretion from β Cells.

From top left, membrane receptor GLUT 2 senses increased glucose concentration in blood and open in response, flooding β cells with glucose. Metabolism of glucose sugar produces ATP that triggers ATP-sensitive potassium channels to close, preventing K^+ from exiting the cell. This increased positive charge depolarizes the membrane and signals voltage-gated calcium channels to import Ca^{2+} that serves as the cue for insulin vesicles to fuse with the membrane and release insulin into blood.

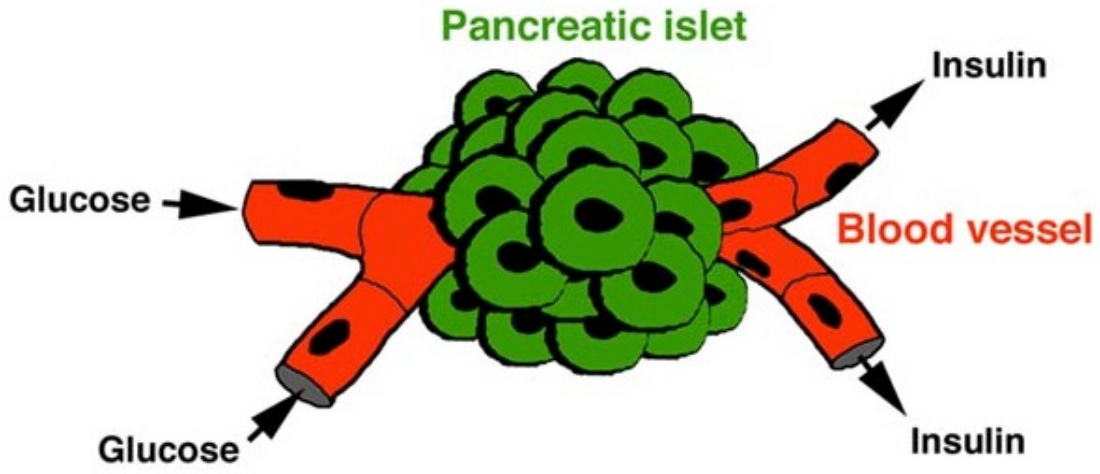

Figure 3-2. Pancreatic Islets are Control Centers for Sensing Glucose and Releasing Insulin. Blood vessels innervating the pancreas carry glucose in and send insulin out to tightly regulate blood sugar levels.

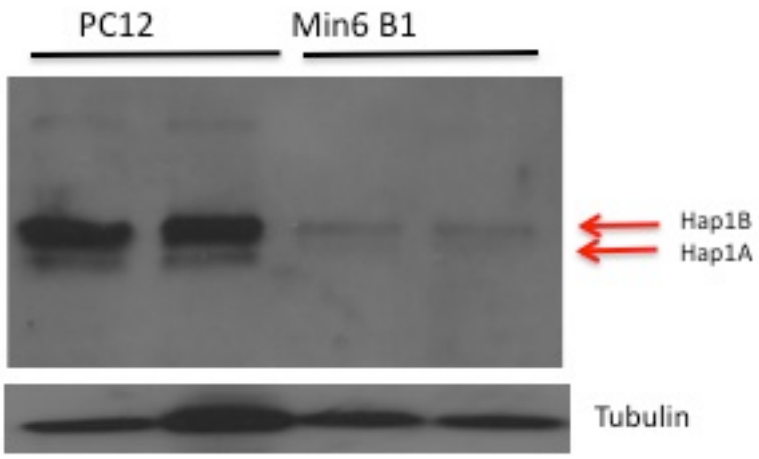

Figure 3-3. Hap1 Isoforms are Expressed in β Cells.

Western blot showing Hap1 expression in PC12 neuronal cells and Min6 β cells. Hap1A and Hap1B are found in a similar ratio and molecular weight in both cell types. Total Hap1 expression in β cells is significantly less than PC12 neuronal cells. Western blot image is representative of multiple experiments. Hap1 antibody used was EM78.

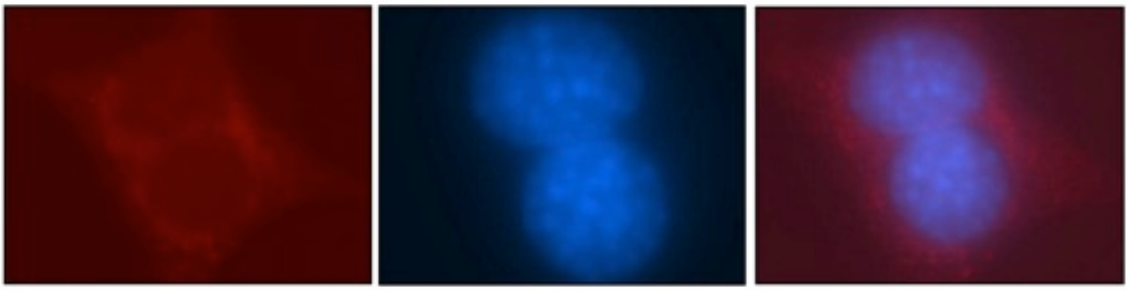

Figure 3-4. Hap1A is a Cytoplasmic Protein.

Immunocytochemistry image of Min6 cells stained for Hap1A in red and nucleus in blue. Image shows that Hap1A is localized to the cytoplasm where insulin vesicle trafficking occurs. Data supports Hap1A cytoplasmic localization in other cell models previously reported.

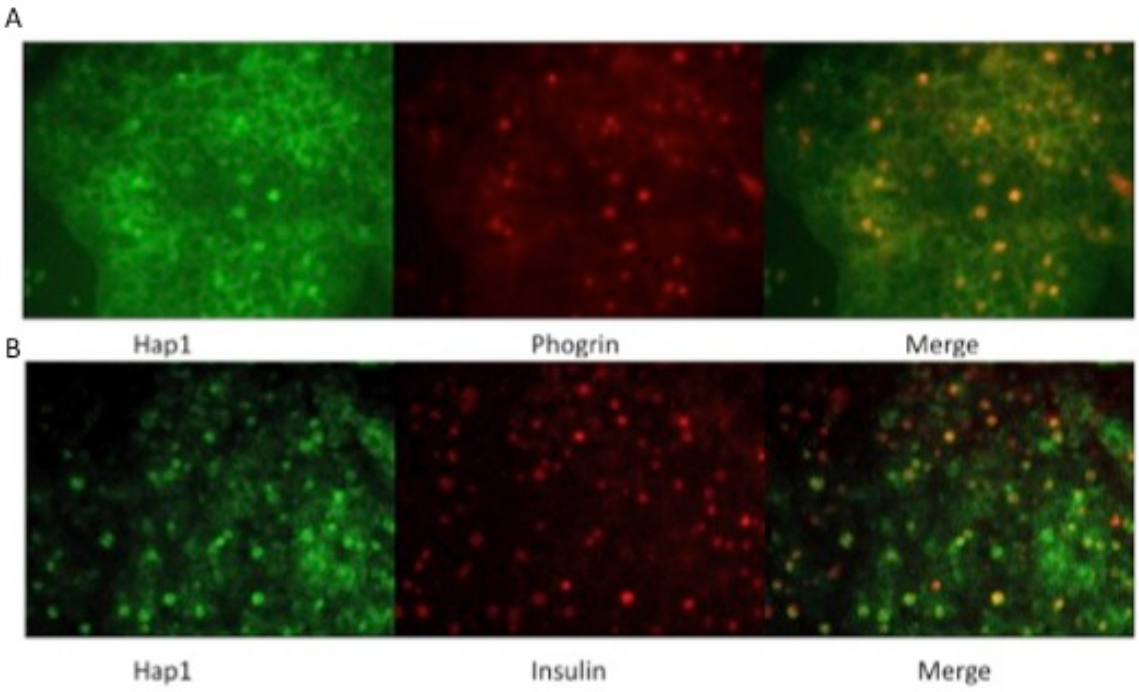

Figure 3-5. Hap1 Colocalizes with Insulin Granules.

Immunocytochemistry of Min6 cells in culture stained for Hap1 and insulin vesicles using insulin antibody (A) and phogrin antibody (B) as a membrane marker for insulin granules. Images are of multiple cells. Note overlap of Hap1 and insulin granules in the merged image.

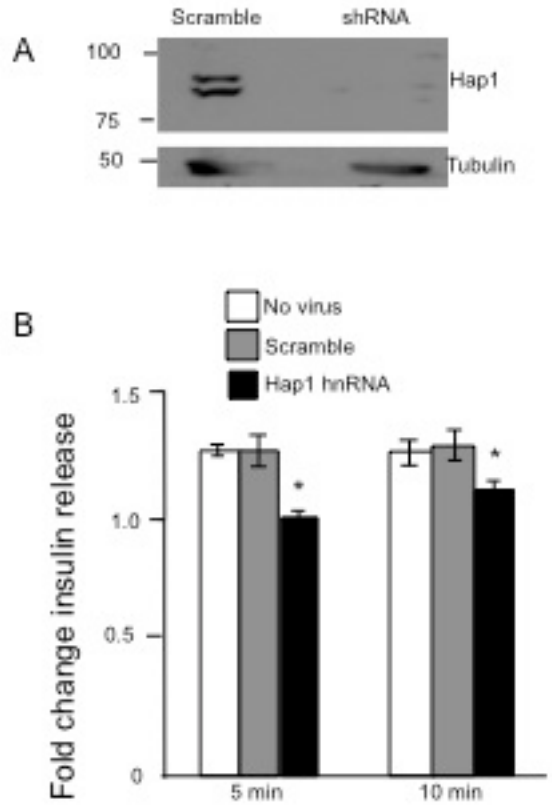

Figure 3-6. Hap1 Deficiency Reduced Insulin Secretion in Min6 β Cells.

(A) Western blot shows Hap1 level was reduced by adenoviral Hap1 shRNA treatment, but not by scramble shRNA control. (B) ELISA assay showing cells treated with Hap1 shRNA displayed a significant reduction in insulin release after glucose stimulation. n=3, * $p<0.05$ compared with no virus or scramble shRNA virus control.

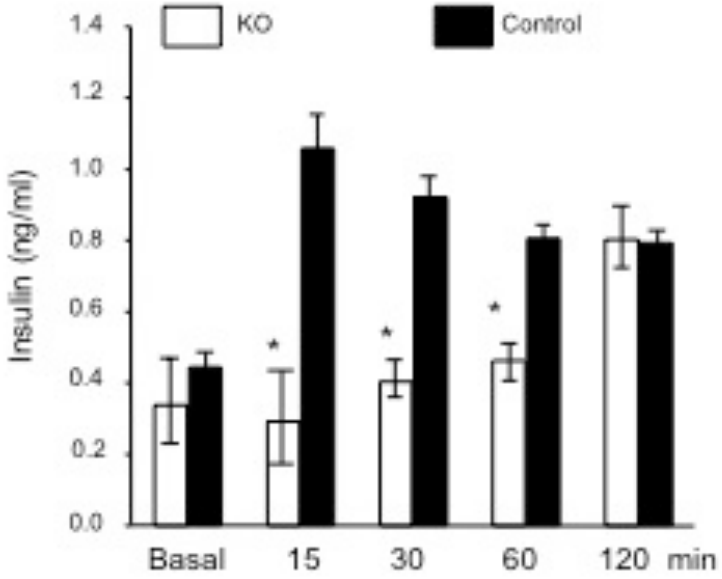

Figure 3-7. Insulin Secretion is Reduced in Hap1 KO Mice.

ELISA results showing that insulin secretion is reduced in Hap1 KO mice. Blood plasma from male mice aged 3 months fasted overnight was collected at various time points after i.p. injection of glucose (2 g/kg body weight). n=8 each group, * $p<0.05$ compared to control.

Chapter 4

Biochemical and Mechanistic Study of Hap1 During Insulin Secretion

Introduction

Insulin release is composed of two distinct waves, or phases of secretion. The fast, first wave of release activates those granules primed for membrane fusion via molecular events at the actin network coating the interior cell membrane. Readily releasable pools of insulin granules in close proximity to β cell plasma membranes do not rely on microtubule movement for release. Effective insulin secretion during the second wave from pancreatic β cells is entirely dependent upon microtubule transport (Varadi et al., 2002; Burgoyne et al., 2003; Yamamoto et al., 2003). Proper insulin secretion after the pools are empty is unsustainable without microtubule transport of fresh insulin cargo reinforcements (Rutter et al., 2006; Jewell et al., 2008).

Insulin release is a dynamic process that shares many characteristics with other endocrine tissues and neuronal cells. All of these cells that secrete cargo from dense core granules do so through exocytosis. This process has been thoroughly described in many cell types, with chromaffin cells as the most well-studied example (Oliver-Krasinski et al., 2008; Langley et al., 1994; Bader et al., 2002).

Earlier studies from our lab connected Hap1 to motor associated proteins KLC and p150 (McGuire et al., 2006; Rong et al., 2006). The results showed that modification of Hap1A by phosphorylation at T598 reduced the association of Hap1A to both KLC and p150. The inhibition of Hap1 expression decreases microtubule transport of several cargos including APP and inhibits neurite tip outgrowth.

Similar mechanisms of exocytosis in neuronal and endocrine cells suggest conserved functions in trafficking proteins involved in microtubule-based transport. Based on this background, I studied Hap1 function in β cells using the presumptive model that Hap1 post-translational modification modulates its function. Here I show Hap1 phosphorylation is modulated as a physiologically relevant consequence to glucose stimulation. I demonstrate that reduced phosphorylation of Hap1A correlates with the timing of both waves of insulin release. Further, I confirm previously published data from neuronal experiments demonstrating Hap1A de-phosphorylation increases association with trafficking proteins p150 and KLC in pancreatic β cells. Finally, I show reduced numbers of readily releasable insulin granules in Hap1 KO mice compared to controls. These data support the hypothesis that insulin vesicle trafficking is Hap1 dependent.

Results

Glucose stimulation reduces phosphorylation of Hap1A.

Hap1 consists of two isoforms, Hap1A and Hap1B, which are alternative splice forms with different C-terminal sequences and can form heterodimers (Li et al., 1998). Our early studies identified a unique phosphorylation site (T598) at the C-terminal region of Hap1A and that this phosphorylation can regulate the association of Hap1A with trafficking proteins (Rong et al., 2006). I thus examined the phosphorylation of Hap1A in Min6 cells that had been stimulated with 20 mM glucose. A marked decrease of phosphorylated Hap1A was observed at 5 and 10 min after glucose stimulation despite the expected increase of phosphorylation in positive controls (Erk and Akt) (**Fig. 4-1A**). To explore whether this change also occurs *in vivo*, I treated wild type mice with i.p. injection of glucose and then isolated their pancreatic tissues for analysis. Western blotting also demonstrated a decrease in Hap1A phosphorylation in pancreatic tissues at 5-10 min following glucose challenge (**Fig. 4-1B**). Immunocytochemical staining verified a decreased labeling of phosphorylated Hap1A (pHap1A) in insulin-containing β cells after glucose stimulation (**Fig. 4-2**). The timing of pHap1A dephosphorylation within minutes correlates with both the first and second wave of insulin release, suggesting the recruitment of granules to the fusion sites is dependent upon Hap1A modulating trafficking complexes.

Glucose stimulation does not affect total Hap1 protein levels.

To determine if decreased phosphorylated Hap1A levels is due to protein modification rather than protein degradation, a western blot was performed using Min6 cell lysates after a

glucose stimulation time course. Total Hap1 protein levels are constant through time points measured (**Fig. 4-3**). Hap1 levels do appear to increase at the 40 minute time point, however by that point insulin secretion has leveled off to basal levels.

Dephosphorylation of pHap1A increases association with trafficking proteins.

In order to connect the physiological glucose induced Hap1A phosphorylation change with insulin secretion output, I investigated whether Hap1A binding characteristics are altered upon glucose stimulation. Therefore, I performed immunoprecipitation to assess the association of Hap1 with KLC and dynactin p150 in the pancreas of mice. I found increased precipitation of KLC with Hap1A at 5 and 10 min after i.p. injection of glucose (**Fig. 4-4A**). Quantitative analysis of the ratios of precipitated proteins to the input also showed that more KLC and dynactin p150 were associated with Hap1A in pancreatic tissues after glucose stimulation than that without glucose treatment (**Fig. 4-4B**). Tubulin protein was also probed as an internal control. Taken together, these findings suggest that glucose stimulation can alter the phosphorylation of Hap1A and increase its association with trafficking proteins, which may promote the movement and docking of insulin-containing vesicles to release insulin from β cells.

Docked insulin granules are decreased in Hap1-deficient β cells.

Insulin secretion involves the trafficking of insulin-containing vesicles and their docking to the plasma membrane of β cells. I performed electron microscopic examination of the pancreas of *Ins2-Hap1*$^{-/-}$ and control mice after they had been stimulated by i.p. glucose injection. Insulin vesicles travel to the β cell membrane adjacent to capillary walls lined

by epithelial cells (**Fig. 4-5A**). I observed a decreased number of vesicles lining the plasma membrane of mutant β-cells compared with controls (**Fig. 4-5B**). Vesicles in Hap1 mutant β- cells were largely scattered in the region proximate to the plasma membrane, with a few docked vesicles at the plasma membrane (**Fig. 4-5B**). In contrast, more vesicles were docked or fused to the plasma membrane in control β cells. To quantitatively analyze the docked vesicles, I used the imaging software ImageJ (NIH) to count the relative numbers of vesicles docked at the capillary plasma membrane per μm. This quantification also verified the decreased number of docked vesicles in Hap1 mutant β cells (3.4 ±0.08, n=10, $P<0.05$) versus control cells (4.64 ±0.16) (**Fig. 4-6A**).

If fewer insulin vesicles are present near the capillary membrane in KO β cells, is this due to a reduction in total vesicle numbers or a transport issue in cytoplasmic vesicles? Using ImageJ software, electron micrographs were analyzed for insulin vesicle density by counting total vesicles using a grid system overlaying images of 5,000 magnification. This magnification was chosen because a total cell count of approximately 100 vesicles in 5 μm^2 offered precision measurements. I observed significantly more insulin-containing vesicles in Hap1 KO compared with control β cells (**Fig. 4-6B**). High-magnification micrographs showed no structural differences in insulin-containing vesicles between control and Hap1-deficient β cells (**Fig. 4-7**). Thus, these studies suggest that lack of Hap1 functionally affects the movement, docking, and fusion of insulin-containing vesicles to the plasma membrane, which can lead to a decrease in the release of insulin.

Discussion

Glucose is the primary trigger for insulin release. Although other secretagogues such as potassium are capable of triggering insulin release, they all merge at the step of Ca^{2+} influx. The advantage of using glucose over other secretagogues to stimulate insulin release is that canonical signaling pathways, rather than secondary or supplementary mechanisms, are activated in the β cells. This is significant given my hypothesis arguing phosphorylation of Hap1A modulates glucose stimulated insulin secretion. Thus, I studied Hap1 in a relevant environment mirroring endogenous molecular events.

The dephosphorylation of Hap1A in response to glucose stimulation is an important clue to elucidating the mechanistic function of Hap1. As shown previously, glucose stimulation triggers a biphasic insulin release from β cells within minutes (**Fig. 4-8**). The first phase of insulin release occurs around the 2 minute point and peaks at 5 minutes. This initial wave fades with a drop in secretion, followed by a second prolonged wave of lesser intensity over 30-60 minutes. My studies clearly show that dephosphorylation of Hap1 bridges both phases of insulin release with respect to timing of secretion. Whether this protein modification is important for the first or second insulin release wave is unknown. The data from glucose tolerance testing suggests the first wave is robust enough in Hap1 KO mice, although no time measurement less than 15 minutes was measured.

I confirmed that dephosphorylation of pHap1A was not due to protein degradation, as glucose stimulation did not affect Hap1 protein levels up to the 40 minute time point. At

this mark, an increase in Hap1 levels was shown. While this finding may be interesting to pursue, we chose to focus on earlier time points relevant to first and second insulin secretion waves.

The fact that Hap1 depletion does not affect Erk or Akt signaling and is involved in both phases of insulin secretion suggests Hap1 is a general trafficking protein downstream of signaling events. However, this evidence does not preclude Hap1 from participating as a signaling molecule itself. As previously noted, Hap1 binds the inositol 1,4,5-triphosphate receptor (InsP3R1) that functions at the ER membrane to pump out calcium into the cytoplasm during insulin secretion signaling (Tang et al., 2003; Ashcroft et al., 2005). It is possible that Hap1 functions in InsP3 receptor stability, similar to Hap1's stabilizing effect on GABAA receptors in neurons. If this is the case, we would expect that calcium release from the ER is reduced, delaying or stunting the signal for insulin secretion. One of the functional consequences of defective calcium signal at the β cell membrane would be a higher number of docked insulin vesicles postponed from fusing to the membrane. However, our EM data contradicts this possibility, suggesting that Hap1 deficiency is more likely to affect the transport and docking of insulin-containing vesicles rather than upstream signaling events.

Our findings from EM show Hap1 KO β cells have fewer readily releasable insulin vesicles compared to control. This concept supports the idea that Hap1 depletion reduces insulin trafficking to the membrane. One anticipated outcome of inhibited vesicle transport is an increase in total vesicles in Hap1 KO β cells slowly moving through

cytoplasm. Consistently, we found the total vesicle density was significantly higher in KO compared with control β cells. This finding further suggests insulin granule production is unaffected, although I did not directly measure total insulin mRNA levels.

Materials and Methods

Electron Microscopy. After mice were perfused with 4% paraformaldehdye, pancreas tissue was removed from animal and further dissected into 1-1.5 mm3 pieces. Fixation was continued in 4% paraformaldehdye at 4 °C overnight. After washing with 0.1 M phosphate buffered (PB, pH 7.2) twice, the tissue blocks were dehydrated in ethanol series to 100%, and then infiltrated overnight with 50% L.R. White resin (Electron Microscopy Sciences, Hatfield, PA) in 100% ethanol followed by pure L.R. White resin overnight. Embedding of the tissue blocks was carried by placing pieces in capped gelatin capsule and letting resin polymerizing in 50 °C oven for 24 hours. Ultrathin sections were cut at 70-80 nm and placed on Formvar coated nickel grids for immunogold labeling.

For immunogold labeling, ultrathin sections on nickel grids were treated with 1% hydrogen peroxidase for 5 min and washed with double distilled water before a 30 min incubation in phosphate buffered saline (PBS) containing 5% normal goat serum, 5% BSA, and 0.1% gelatin to block potential non-specific binding sites in the sections. After blocking, sections were incubated at 4°C overnight with mouse anti-insulin (Fitzgerald Industries International, Inc, Concord, MA) primary antibody diluted 1:100 in PBS containing 0.1 % acetylated BSA (Aurion, the Netherlands), washed, incubated with 10 nm gold particles conjugated goat anti-mouse secondary antibodies (Aurion, The Netherlands) at 1:20 dilution in the same solution with primary antibody. After washing off excess antibody, sections were fixed with 2.5% glutaraldehyde in 0.1 M PB, and then counter-stained with 4% aqueous uranyl acetate and 2% lead citrate. Insulin immunogold

labeled sections were examined using a Hitachi H-7500 transmission electron microscope (Hitachi High Technologies of America, Inc., Pleasanton, CA) equipped with a Gatan BioScan CCD camera. Pancreas tissue was labeled with anti-insulin antibody (Fitzgerald). All electron microscopy performed by Hong Li.

Insulin granule counting. Images were analyzed using ImageJ software (NIH). Images of endothelial cells of capillaries were used to count the number of readily releasable insulin granules per 1μm distance along capillary membrane periphery. Three β cell images each for control and KO were used, ten different distances along capillary membranes were compared and averaged to display results as vesicles per μm distance. Total insulin vesicle density was calculated by counting number of vesicles inside random squares of grid place on top of multiple β cells images.

Immunofluorescent staining. Mice were sacrificed by cervical dislocation and the pancreas was removed. Pancreas tissues were submerged in Bouin's fixative (saturated picric acid, formaldehyde, glacial acetic acid) overnight. Tissue was washed in H_20 until yellow color faded then placed in neutral buffered formalin for storage until processed by the WCI Pathology Core Lab at Emory University. The paraffin embedded slides were rehydrated in xylene, EtOH then water. Antigens were unmasked in sodium citrate buffer for 3 min in microwave at low heat. Slides were blocked in 2% normal goat serum, 0.5% Triton X-100, 1% BSA in PBS for 30 min. After block, slides were incubated with primary antibodies to insulin, pHap1A (EM 41A), and nuclei (Hoechst) overnight at 4°C. Light micrographs were taken on a Zeiss microscope (Axiovert 200 M) with a digital

camera (Hamamatsu ORCA-100) and processed with Openlab software (Improvision, Inc).

Western blotting. Cells were harvested by scraping 10 cm plate with cell scraper in NP40 lysis buffer (protease inhibitor, PMSF). Pancreatic samples were collected from anesthetized mice, quickly placed in buffer on ice. Lysed samples were kept in NP40 buffer with phosphatase inhibitors (50mM sodium fluoride, 2mM sodium vanadate) and 10mM sodium phosphate. Samples were sonicated 10 seconds, SDS running buffer added, then boiled 10 minutes for SDS-Page on tris-glycine gel. Western blots were carried out using primary antibodies to Hap1 (EM 78) and tubulin control. Blots were visualized with Kodak x-ray developer with ECL Plus HRP-conjugated enzyme signal reagent.

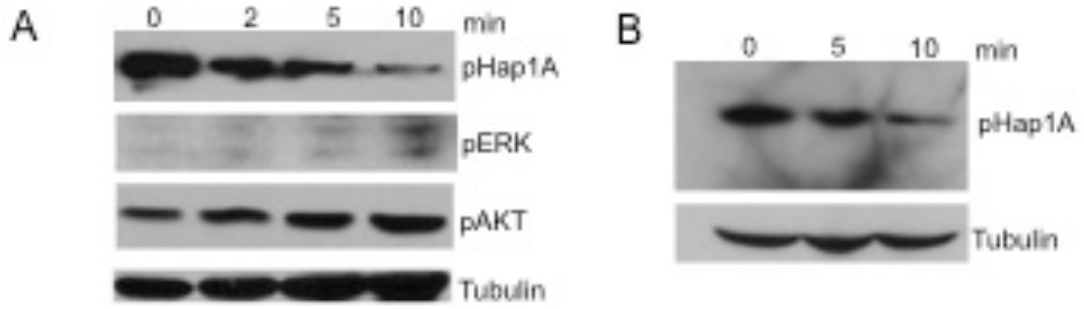

Figure 4-1. Glucose Stimulation Reduces Phosphorylated Hap1A Levels.

(**A**) Western blot data from Min6 cells showing reduction of phosphorylated Hap1A (pHap1A) after treatment with 20 mM glucose for different times (min). The blots were probed with antibodies to phosphorylated ERK (pERK) or AKT (pAKT), which are positive controls known to be up-regulated after glucose stimulation. (**B**) Western blot of phosphorylated Hap1A (pHap1A) levels in the pancreas from wild type mice stimulated with i.p. injection of glucose (2g/kg) for 5 or 10 minutes.

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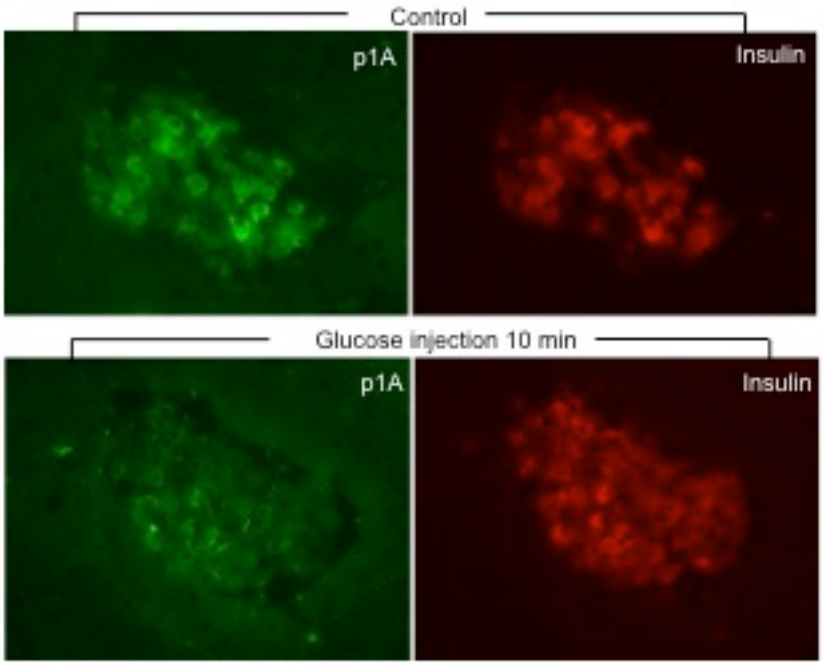

Figure 4-2. Glucose Stimulation Dephosphorylates pHap1A.

Double immunofluorescence of pancreatic tissues collected from WT mice showing that phosphorylated Hap1A (p1A, green) was reduced after glucose challenge for 10 min in wild type mice compared with control mice without glucose treatment. Image is of an islet, representative of N=3 mice.

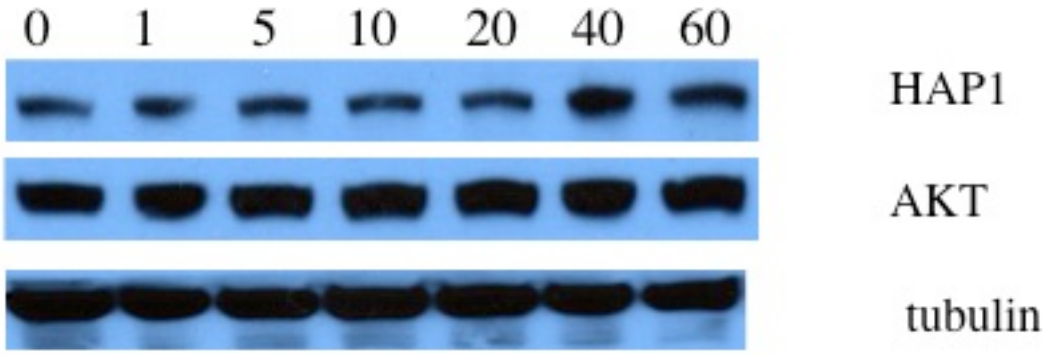

Figure 4-3. Glucose Stimulation Does Not Decrease Hap1A Protein Levels.

Western blot of Min6 cells stimulated with glucose over time (minutes) showing Hap1A protein level is stable up to 40 minutes when a marked increase becomes apparent. Total Akt level is unaffected over time course. N=3.

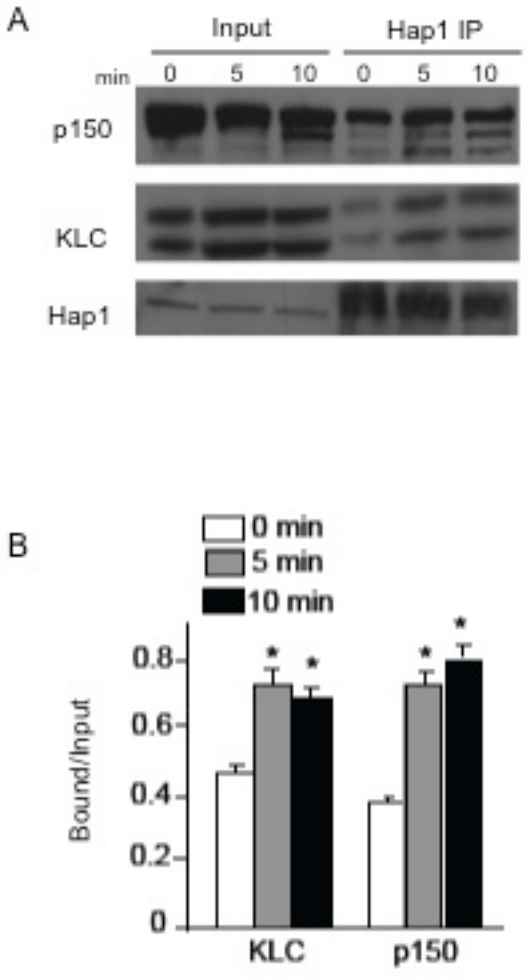

Figure 4-4 Dephosphorylation of pHap1A Increases Association with Trafficking Proteins. (**A**) Immunoprecipitation of Hap1A from pancreatic tissues of mice i.p. injected with glucose (2g/kg body weight) for 5 or 10 min. The precipitates were probed with antibodies to dynactin p150, kinesin light chain (KLC), and Hap1A. (**B**) Quantification of the relative precipitated proteins (ratio of precipitated to input) showing a significant increase of KLC and p150 associated with Hap1A in mouse pancreatic tissues after glucose stimulation. Fold changes (mean±SE, n=3) are shown as bound protein relative to input. *p<0.05 compared to unstimulated samples.

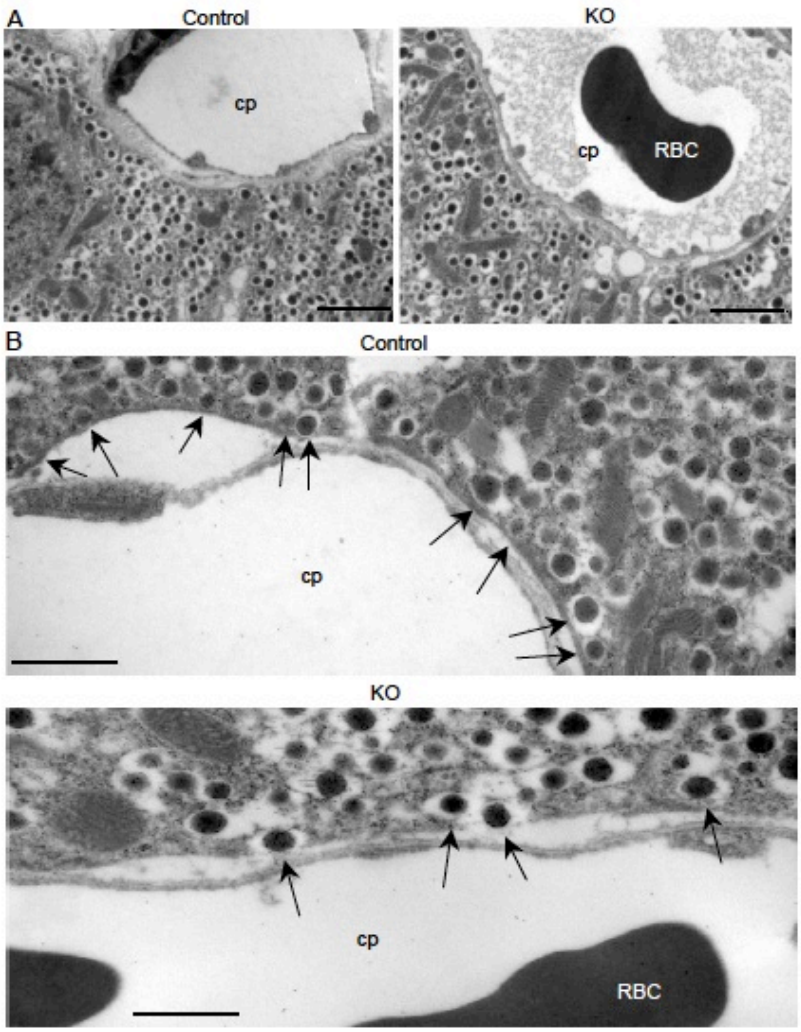

Figure 4-5. Reduced Number of Insulin Vesicles at the Plasma Membrane in Hap1 KO β Cells. Electron microscopic images of gold-labeled insulin vesicles (arrows to dark structures with dots) in β cells of KO control mice i.p. injected with glucose (2g/kg) for 10 min. (**A**) Micrographs at 5000X magnification showing fewer vesicles lining the plasma membrane in KO β cells than control cells. Scale bars: 2.5 μm. (**B**) High magnification (15000X) micrographs showing the reduced number of insulin vesicles that are docked on the plasma membrane in the KO pancreatic tissue. Arrows indicate docking insulin vesicles. cp, capillary; RBC: red blood cell. Scale bars: 1.0 μm.

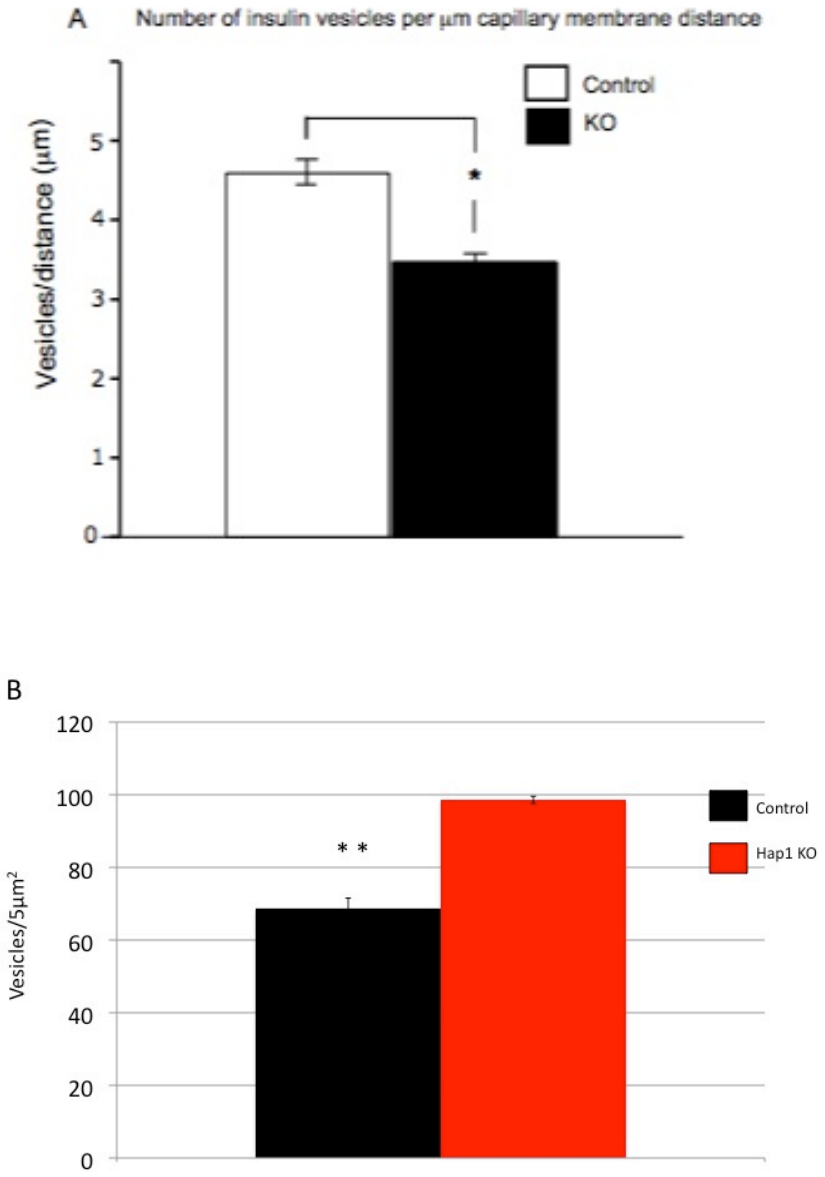

Figure 4-6. Quantification of Electron Microscopy Data.

Quantification of the number of insulin vesicles per μm capillary membrane distance (A) and the relative density of insulin vesicles per 5μm^2 (B) in β cells of control and KO mice. n=10. * P<0.05, ** P<0.01.

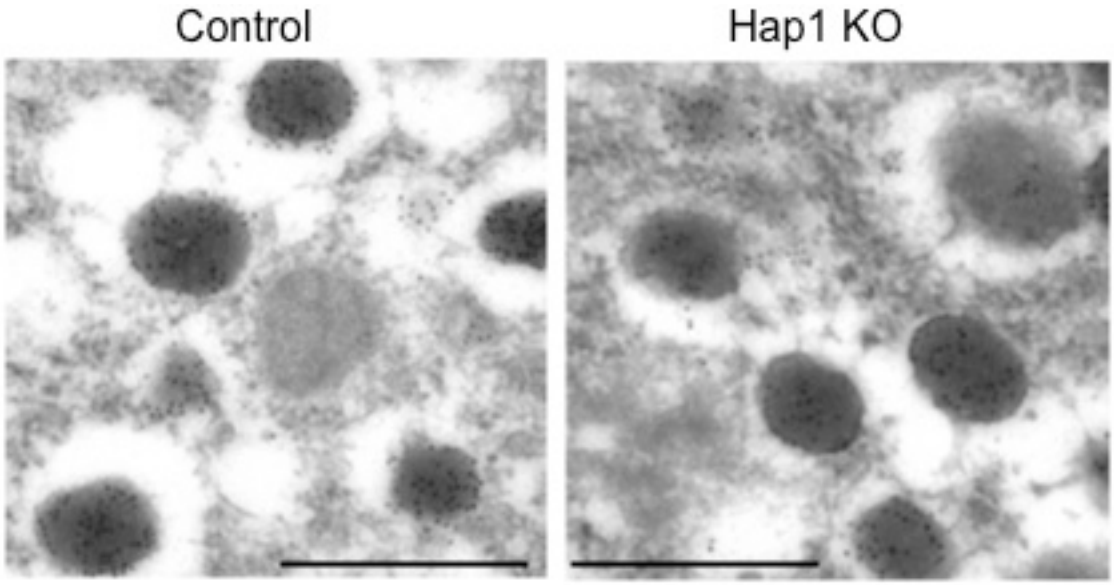

Figure 4-7. Insulin Vesicle Morphology is Unchanged in Hap1 KO Mice.

Insulin immunogold labeling (40000X) showing no difference in the morphology of insulin-containing vesicles between control and KO β cells. Scale bars: 0.5 μm

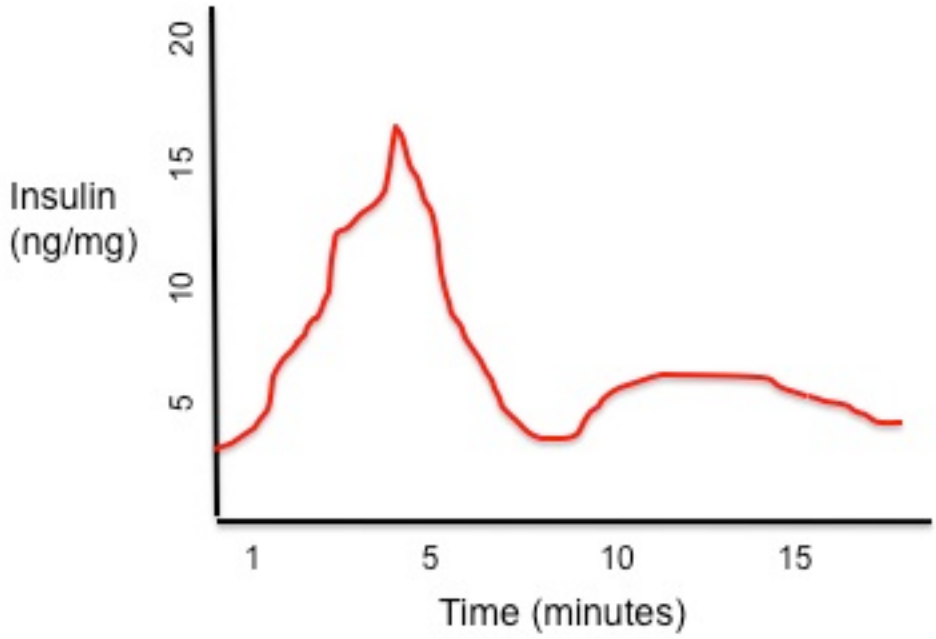

Figure 4-8. Biphasic Insulin Release.

Graphical representation of insulin release in mice taken from Shigeto et al., J Pharmacol Sci 2006. Graph is consistent with insulin release from both Min6 cells and isolated islets. Initial wave and peak is followed by dip and a second blunted sustained wave.

Chapter 5

Summary and Discussion

Summary

HD has a complex disease pathology that includes disruptions in gene transcription, protein degradation, membrane receptor signaling, synaptic toxicity and microtubule transport. As the first protein found to bind Htt, Hap1 has been suspected in many of the molecular crimes that mHtt commits. As a coconspirator with mHtt, Hap1's role in microtubule dependent trafficking is well documented in neuronal studies.

Although Hap1 is enriched in the brain, it is also present in endocrine cells (Liao et al., 2010);(Dragatsis et al., 2000; Liao et al., 2005). Nevertheless, the *in vivo* role of Hap1 in endocrine cells remained unknown until my research. By selectively eliminating Hap1 expression in β cells in the mouse pancreas, here I provide evidence that Hap1 deficiency in β cells impairs insulin release and glucose tolerance. I further demonstrate that glucose stimulation alters Hap1A's phosphorylation and increases its association with microtubule-dependent trafficking proteins. These findings not only support the role of Hap1 in intracellular trafficking, but also reveal its critical function for vesicular release of hormones from endocrine cells. Given the vital roles hormones play in a variety of the body's functions, our findings have broader implications for the pathogenesis of metabolic conditions that result from impaired vesicular trafficking or exocytosis in endocrine cells, offering new therapeutic targets for the treatment of such conditions. The confirmation of a role for Hap1 in vesicular trafficking in endocrine cells unifies the previously hypothesized function in neuronal cells.

Future studies

Hap1 is an important protein for microtubule-based intracellular trafficking that is required for endocytosis and exocytosis. We know that Hap1's function can be disrupted through the aberrant binding of mutant Htt. In addition, the abundant expression of Hap1 in neurons and in the hormone secreting cells of endocrine tissues strongly argues for a unifying role in neuroendocrine secretion. Although plenty of other proteins have been shown to be important in neuroendocrine secretion, Hap1 stands out as unique given its role in HD and selective expression in β cells. Therefore, I propose the following two lines of investigation, independent in strategy and approach, connected in the general sense of understanding not only the function of Hap1, but more importantly, in elucidating regulation of hormone release in endocrine cells.

1. Study what biochemical events engage Hap1.

Previously, we showed Hap1A is phosphorylated by protein kinase A (PKA) at T598 (Rong et al., 2006). However, evidence is scarce regarding other potential kinases and phosphatases acting on Hap1A. Even fewer details are known regarding Hap1's role in signaling pathways. It would be interesting to identify why glucose stimulation reduces Hap1A phosphorylation and what phosphatase is involved in this regulation. Identifying such targets would be useful for the development of druggable targets to treat the diseases associated with insulin release defect.

One piece of evidence comes from studies showing the Wnt signaling pathway participates in glucose stimulated insulin secretion. The Wnt pathway is generally associated with development and differentiation, but is also been described in endocrine cell function (Heller et al., 2002). In fact, several publications have demonstrated defects in Wnt signaling are associated with diabetes (Kanazawa et al., 2004). In addition, experiments with isolated mouse islets treated with Wnt ligands caused potentiation of glucose-dependents insulin exocytosis (Fujino et al., 2003).

Of course, what would a study of signaling be without extreme complexity and crosstalk? Indeed, Wnt signaling in the β cells appears to overlap with PPARχ, a pathway supporting adipose tissue differentiation (Schinner et al., 2008). Interestingly, PPARχ signaling promotes glucose uptake into fat cells from blood (Lemberger et al., 1996).

Even more intriguing is the molecule protein kinase A (PKA). In addition to phosphorylating Hap1A, PKA also mediates the signaling of Wnt, which also requires Akt phosphorylation (Welters and Kulkarni 2008). Recall that Akt phosphorylation is a required event for glucose stimulated insulin secretion. It would be interesting to investigate this further to tease out what Wnt isoforms are important in insulin secretion, for example. Also, looking for other Hap1 binding partners may help narrow the focus on regions of the cell such as the ER that support signaling events culminating in calcium spikes that drive insulin release.

2. Explore the role of Hap1 deficiency in diabetic symptoms and hormone secretion abnormalities in HD.

As a binding partner to Htt, Hap1's role in HD pathology presents a critical piece in understanding both diseases fully. First, HD patients have higher prevalence of diabetes (Farrer 1985; Podolsky S 1972). mHtt interferes with gene transcription factors PDX-1, E2A, CBP and p300 (Andreassen et al., 2002). These experiments in R6/2 mice highlight reduced insulin mRNA and reduced glucagon and somatostatin levels (Andreassen et al., 2002). Mechanistically, one group has also used the R6/2 model and INS-1 cells showing mHtt slows insulin vesicle speed along microtubules by aberrantly binding β-tubulin, the building blocks of microtubules (Smith et al., 2009). This study shows no change in insulin content in β cells of 832/13 INS-1 cells after transfection with mHtt, and no alteration in vesicle biogenesis. They argue mHtt does not interfere with microtubule dynamics, but binds aberrantly and forms road bumps. This study does agree with previous reports showing less insulin in HD R6/2 mice (Hakvag et al., 2008; Hurlbert et al., 1999; Josefsen et al., 2008).

Given the correlation of HD with disrupted insulin secretion and the fact that mHtt binds aberrantly to Hap1, it seems logical to hypothesize that mHtt would interfere with Hap's role in endocrine exocytosis. Early evidence from this dissertation using Min6 cells shows Htt and Hap1 colocalize (**Fig. 5-1**). In addition, an HD knock-in mouse model with full length Htt and 140 CAG repeats has provided evidence that mHtt interferes with glucose tolerance (**Fig. 5-2**). Our HD KI 140Q mouse model can be used to experimentally test whether there is an insulin secretion defect compared to WT mice.

The binding characteristics of Hap1 to p150 and KLC can be measured by immunoprecipitation (IP) in the pancreatic tissues from HD KI 140Q mice. I anticipate that IP data will show mHtt in HD mouse pancreatic tissue samples decreases Hap1 binding to p150 and KLC. This data would strongly argue in favor of our model.

Finally, it has been argued that mHtt also affects the secretion of several other hormones from endocrine cells (Li et al., 2003; Saleh et al., 2009). It would be interesting if these adverse effects occur via abnormal Htt-Hap1 interactions as well. Thus, the findings in my study showing that Hap1 is involved in insulin secretion have implications for investigating the pathogenesis of HD as well.

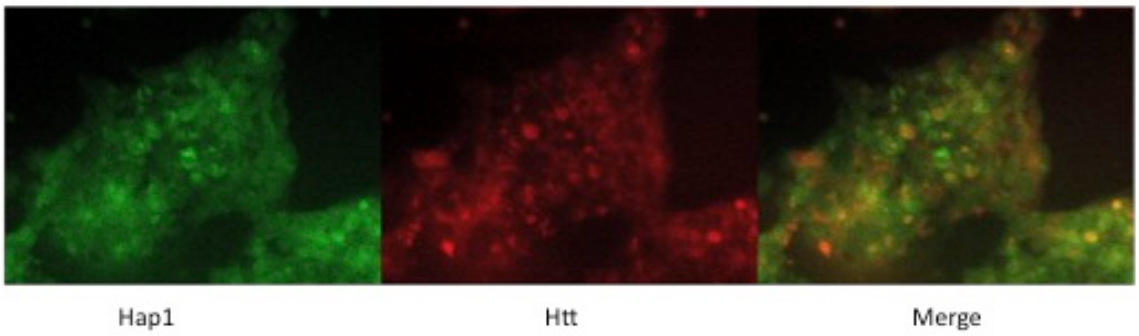

Figure 5-1. Hap1 and Huntingtin Colocalize in β Cells.

Immunocytochemical staining of Min6 cells shows Hap1 (green) colocalizes with Htt (red). Representative image is of multiple β cells in culture.

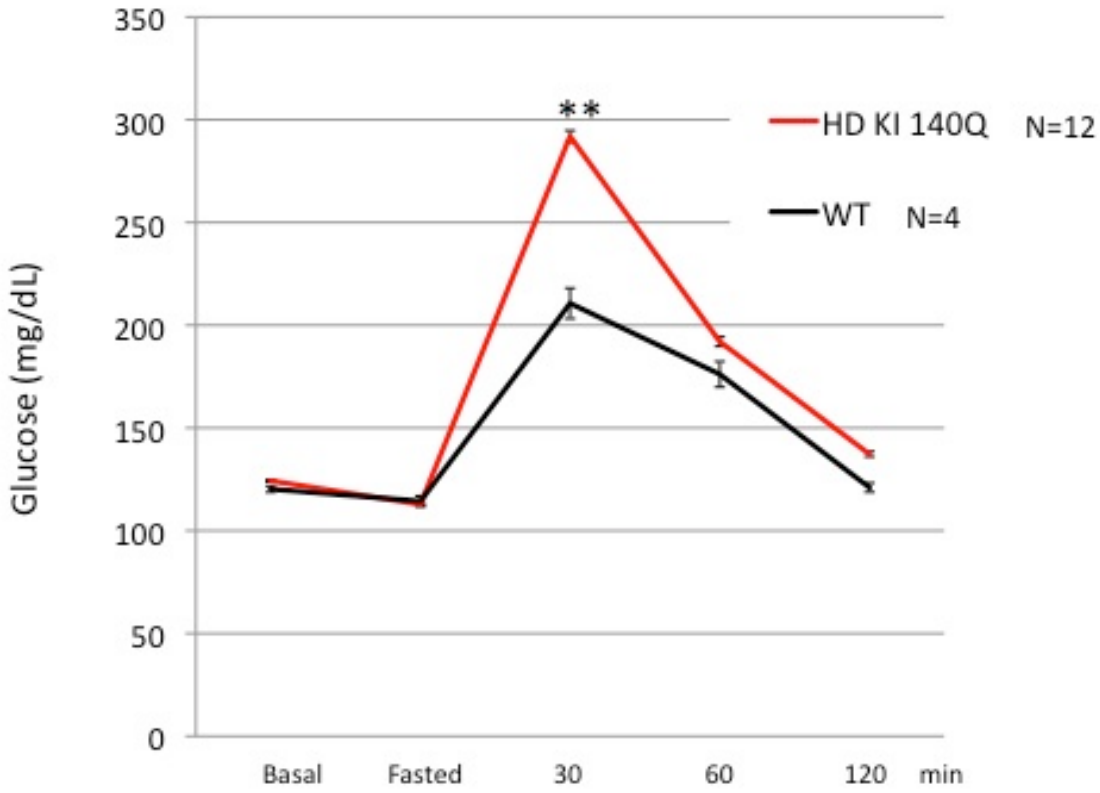

Figure 5-2. Glucose Intolerance in HD Mouse Model.

A glucose tolerance test was performed on HD KI mice containing full length Htt with 140 CAG repeats. Mice were tested after overnight fasting. Tail blood glucose concentration was measured in male (KI, N=6; WT, N=3, 24 months of age) and female (KI, N=6; WT N=2, 16 months of age) challenged with 20 mM i.p. glucose injection. **p<0.01.

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