INVESTIGATING *DROSOPHILA* MIDGUT COPPER HOMEOSTASIS AND NOVEL REGULATION MECHANISMS

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ABSTRACT

All animals require the essential biometal copper as an enzymatic cofactor for processes as diverse as energy production, free radical detoxification and pigmentation. The human body has an absolute need for copper but also must provide protection against toxicity. Therefore each cell produces a set of copper homeostasis proteins which are required for copper to enter the cell, be utilized and then be removed. In mammalian cells, hCtr1 and the Cu-ATPases have been identified as the primary transport proteins for import and export of copper respectively. Whilst the basic role of each copper transporter has been identified, many questions remain in regard to their regulation, localization and tissue specific function. In recent years *Drosophila melanogaster* (referred to as *Drosophila*) has emerged as an alternative model used to further investigate these questions due to the high conservation of the key copper transporters. As the major source of copper exposure to *Drosophila* (and humans) is ingestion of the nutrient, the first aim of this project was to explore the mechanisms of midgut copper absorption and its importance to distribution of the nutrient throughout the organism. The second and third aims explored regulation of copper homeostasis by investigating protein localization and the role of novel interacting proteins.

Ctr1A and Ctr1B, orthologues of the human Ctr1, are the primary *Drosophila* copper import proteins and were suspected to be crucial for midgut absorption. However, midgut specific suppression of both in combination does not impact upon viability implying that reduced copper entry may be sufficient for survival, *Drosophila* as a whole can compensate for low intestinal copper import or that there are other sources of uptake. Ectopic expression of *Ctr1B* causes lethality suggesting that this protein is the more efficient importer and results in toxicity of both the midgut and lymph. Localization of both Ctr1A/B was predominantly at the apical membrane yet some was at the basolateral membrane implying that copper import back from the lymph was also possible. DmATP7, the sole orthologue of the mammalian Cu-ATPases, localizes predominantly to the basolateral membrane of enterocytes, with some protein likely at the *trans*-Golgi network. Midgut specific suppression of DmATP7 does not impact upon viability yet

increases enterocyte copper content and is detrimental when copper import is increased. It appears likely that loss of *DmATP7* expression results in midgut copper toxicity but the effect of limited copper delivery to the lymph appears negligible. Ectopic expression of *DmATP7* results in lethality likely caused by copper toxicosis and in combination with suppression data further implies that *Drosophila* are more capable surviving decreased enterocyte copper import rather than increased.

Using the midgut model (and other *Drosophila* tissues such as the eye and midline) this project was also able to demonstrate putative roles for dRab5 and HipK in copper homeostasis. This project shows that copper levels may influence dRab5 stability, whilst at the same time *dRab5* expression is crucial in determining the localization of DmATP7. As dRab5 is a key component in the maturation of the early endosome, this suggests a link between endocytosis and the regulation of copper transporters. HipK is a homeodomain serine/threonine kinase that inhibits the activity of Slimb, an E3 ubiquitin ligase and therefore can stabilize targets proteins by blocking degradation. This project demonstrates that increased expression of *HipK* exacerbates *Drosophila* midline and midgut phenotypes caused by ectopic expression of *DmATP7* implying that degradation of import protein is inhibited. At the same time, midline phenotypes caused by ectopic expression of *HipK* may be partially as a result of copper deficiency as the phenotypes are rescued by increased cellular copper levels. Work on both novel interacting proteins clearly demonstrated their regulation of copper homeostasis and will stimulate further use of *Drosophila* to explore this area of research.

DECLARATION

This is to certify that that:

(i) the thesis comprises of my original work except where indicated

(ii) due acknowledgement has been made in text to all other material used

(iii) the thesis is less than 100,000 words in length, exclusive of tables, figures and bibliographies

Tim Binks

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ABBREVIATIONS

AAS	Atomic absorption spectrophotometry
A-Domain	Energy transduction (A) domain
Atox1	Antioxidant-1
ATP	Adenosine triphosphate
BCS	Bathocuproine disulfonate
Caco2	Human epithelial colorectal adenocarcinoma
CCS	Copper Chaperone for Superoxide dismutase
COMMD1	Copper Metabolism MURR1 Domain 1
CHO	Chinese Hamster Ovary
Ctr	Copper transporter
Cu^{+}	Cuprous Ion
Cu^{2+}	Cupric Ion
Cu-ATPases	Copper-transporting ATPases
DβM	Dopamine-β-monoxygenase
DMT1	Divalent metal transporter 1
DHMN	Distal Hereditary Motor Neuropathy
DNA	Deoxyribonucleic Acid
HUVEC	Human primary umbilical vein endothelial cell
ICP-MS	Inductively coupled plasma mass spectrometry
IM	Inner mitochondrial membrane
OH	Hydroxyl radicals
MAPK	Mitogen-activated protein kinase
MBD	Membrane binding domain
MDCK	Madin-Darby Canine Kidney
MRE	Metal responsive element
MT	Metallothionein
MTF-1	Metal responsive transcription factor-1
N-Domain	Nucleotide binding domain
OHS	Occipital Horn Syndrome

P-Domain	Autocatalytic phosphorylation domain
PAM	Peptidylglycine α-amidated monoxygenase
PDZ	Post synaptic density protein, *Drosophila* disc large tumor suppressor and Zonula occludens-1 protein
SOD1	Copper-zinc-superoxide dismutase
TGN	*trans*-Golgi network
TM	Transmembrane spanning domain

CHAPTER ONE: INTRODUCTION

Synopsis

The human body requires copper as an essential enzymatic cofactor, yet must also provide protection against its toxicity when in excess. Therefore each cell produces a set of copper homeostasis proteins which are required for copper to enter the cell, be utilized and then be removed. This chapter introduces the basic mechanisms of cellular copper transport, how these processes are regulated and how disruption to copper homeostasis can lead to disease. This chapter also introduces *Drosophila* as an alternative model for exploring copper homeostasis and how this study aims to expand on current knowledge.

1.1 The fine balance of copper homeostasis

Copper is a micronutrient with significant implications in human physiology. Humans require a tight homeostatic regulation of copper to avoid either a copper deficiency or toxicity situation. The major source of copper exposure to humans is via the consumption of food and water (Harrison and Dameron, 1999). Copper is found in a wide variety of foods including meat, shellfish, nuts and chocolate (Lonnerdal, 1996). The copper content of natural drinking water is quite low but can be marginally increased by copper piping or storage containers (Lonnerdal, 1996). Other minor sources of copper exposure can include air inhalation (from industrial processes or the environment) and in rare occasions skin contact with soil or jewelry (Barceloux, 1999).

1.1.1 Copper acts as an important co-factor for several key enzymes

Copper was first discovered as an essential micronutrient in 1928 when laboratory animals fed on a low copper diet were shown to have a lower than average survival rate (Hart et al., 1928). Copper deficiency in humans leads to growth defects, neurological impairment and ultimately early childhood death if severe enough (Danks, 1988). These symptoms are relieved by dietary copper supplementation confirming they are caused by a copper deficiency (Karpel and Peden, 1972; Danks, 1988).

As a transition metal, copper can exist in either the oxidized Cu^{2+} (cupric ion) or the reduced Cu^{+} (cuprous ion) state. Copper has the ability to readily donate or accept an electron, allowing switching between the cuprous and cupric states (Linder and Hazegh-Azam, 1996). This transition between states facilitates the role of copper as a co-factor for enzymes in key pathways such as cellular respiration, anti-oxidant defense, pigment formation and neural development (Linder and Hazegh-Azam, 1996; Itoh, 2006; Miao and St Clair, 2009; Olivares and Solano, 2009) (Table 1.1).

Table 1.1: The main mammalian cuproenzymes

Cuproenzyme	Enzymatic role	How does copper act as a co-factor?
Cytochrome *c* oxidase	Crucial for cellular respiration. The enzyme obtains electrons to reduce oxygen and then translocate protons across the inner mitochondrial membrane (Linder and Hazegh-Azam, 1996).	Cytochrome *c* oxidase forms a subunit with two copper atoms and two heme moieties (Linder and Hazegh-Azam, 1996).
Copper-zinc-superoxide dismutase (SOD1)	An enzyme vital for antioxidant defence (Miao and St Clair, 2009).	SOD1 requires bound copper to convert reactive oxygen species into oxygen and hydrogen peroxide (Linder and Hazegh-Azam, 1996).
Tyrosinase	A key pigment regulatory enzyme required for the synthesis of melanin (Olivares and Solano, 2009).	Active tyrosinase requires binding of two copper ions (Olivares and Solano, 2009).
Dopamine-*β*-monoxygenase (D*β*M)	Converts dopamine to norepinephrine (a neurotransmitter of the central nervous system) (Itoh, 2006).	Active D*β*M requires one copper ion bound to its mononuclear site (Itoh, 2006).
Ceruloplasmin	Ferroxidase associated with the oxidation of iron (Healy and Tipton, 2007)	Copper delivered from the secretory pathway is incorporated into ceruloplasmin (Healy and Tipton, 2007)
Peptidylglycine α-amidated monoxygenase (PAM)	A key enzyme required for the synthesis of all amidated neuropeptides (Bousquet-Moore *et al.*, 2010).	Active PAM requires one copper ion bound to its mononuclear site (Itoh, 2006).

A list of mammalian cuproenzymes including their subsequent role and how copper is required to act as a cofactor.

1.1.2 Copper toxicity

Despite being an important dietary heavy metal, an excess of copper is toxic to cells. In elevated copper situations, the redox activity generated by the transition between the cupric and cuprous states leads to toxicity. After the reduction of cupric ions, free cuprous ions can react with hydrogen peroxide causing the production of hydroxyl radicals (OH) via the Fenton reaction (Moriwaki *et al.*, 2008; Jomova and Valko, 2011). Copper-generated hydroxyl radicals are highly reactive and cause oxidative damage resulting in a wide range of biological effects such as DNA damage, peroxidation of lipids, incorrect activation of Mitogen-activated protein kinase (MAPK) signaling pathways and formation of lipid radicals (Mattie and Freedman, 2004; Mattie *et al.*, 2008; Moriwaki *et al.*, 2008). When copper levels are moderate there is no oxidative damage but as levels increase, copper accumulates, causing repetitive free radical formation (Powell, 2000).

1.2 Cellular copper homeostasis

1.2.1 Copper uptake

The human body has an absolute need for copper but also must provide protection from its toxicity. Therefore each cell produces a set of copper homeostasis proteins which are required for copper to enter the cell, be utilized and then be removed (Figure 1.1). In eukaryotes copper uptake is predominantly controlled by the copper transporter (Ctr) protein family (Kaplan and Lutsenko, 2009). The Ctr1 family was first linked to copper homeostasis in 1994 via the identification of the *Saccharomyces cerevisiae Ctr1p* gene as a high affinity copper transporter (Dancis *et al.*, 1994). Mutations in *Ctr1p* were found to cause decreased copper uptake, whilst the gene transcript was shown to be heavily regulated by copper availability (Dancis *et al.*, 1994).

In 1997 hCtr1 was identified as the probable human copper uptake protein (Zhou and Gitschier, 1997). *hCtr1* was found to complement the mutation in yeast *Ctr1p* which was

disabling copper uptake (Zhou and Gitschier, 1997). hCtr1 was then confirmed as a copper pump in mice embryonic cells. Measurements of copper uptake were achieved by tracking ^{64}Cu uptake into these cells (Eisses and Kaplan, 2002; Lee *et al.*, 2002).

1.2.1.1 The structure, expression and localization of hCtr1

hCtr1 is 190 amino acids long and contains three membrane spanning domains, a amino(N)-terminus located outside the cell, a large cytoplasmic loop and a short carboxy-terminal tail (Eisses and Kaplan, 2002) (Figure 1.2). The extracellular N-terminus of hCtr1 contains two metal binding methionine motifs. The first of these motifs is M*x*M*xx*M, whilst the second motif is the more methionine-rich, MMMM*x*M (Eisses and Kaplan, 2002). Mutational analysis of both motifs revealed that only the second methionine motif of the N-terminus ($Met_{40} - Met_{45}$) is required for copper uptake (Puig *et al.*, 2002; Eisses and Kaplan, 2005). However, as this mutational analysis has been completed *in vitro*, larger portions of the N-terminus may be important for *in vivo* functions such as binding to potential copper donor proteins (Eisses and Kaplan, 2005; Kaplan and Lutsenko, 2009). The second transmembrane domain also contains a methionine motif ($Met_{150} + Met_{154}$) which when mutated reduces copper uptake by 70% (Eisses and Kaplan, 2005).

hCtr1 is expressed ubiquitously and deletion of the mouse homologue *mCtr1* (92% identical to *hCtr1*) results in mortality in early mouse embryogenesis (Lee *et al.*, 2000; Kuo *et al.*, 2001). The lack of human diseases associated with the hCtr1 protein suggests that loss of the copper transporter also produces a lethal phenotype in humans (Kuo *et al.*, 2001). hCtr1 can cycle between an outer plasma membrane localization and intracellular organelles located in the perinuclear region (Klomp *et al.*, 2002). The recycling of hCtr1 away from the outer plasma membrane can be stimulated by increased extracellular copper and can also be dependent on cell type (Petris *et al.*, 2003; van den Berghe and Klomp, 2009). For example, in intestinal enterocytes (to be discussed later), the majority of hCtr1 is localized to the outer plasma membrane or intracellular regions adjacent to the membrane (Zimnicka *et al.*, 2007; Nose *et al.*, 2010). It is not well

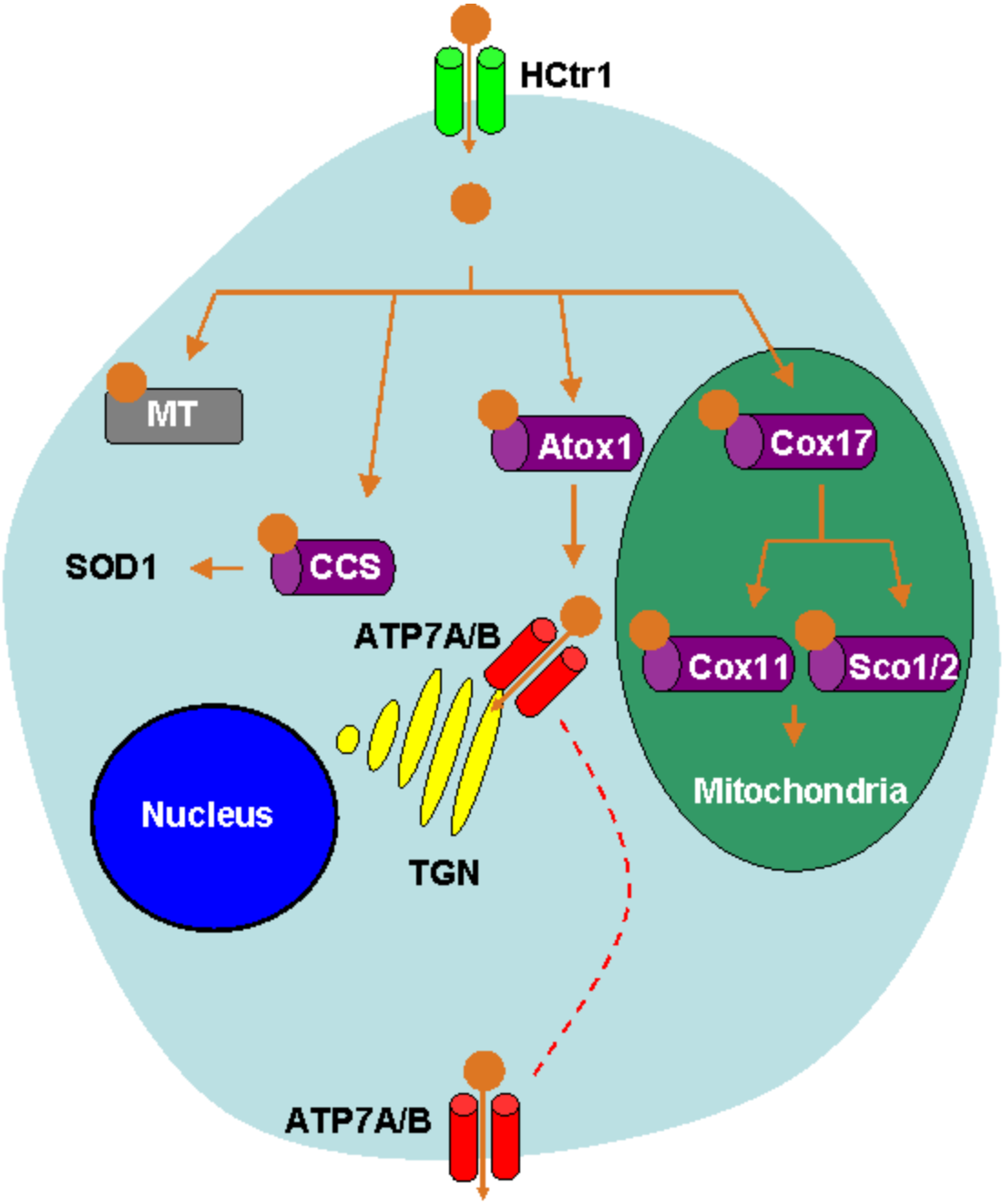

HCtr1
MT
Atox1
Cox17
SOD1
CCS
ATP7A/B
Cox11
Sco1/2
Nucleus
Mitochondria
TGN
ATP7A/B

Figure 1.1: Mammalian cellular copper homeostasis

Copper enters the cell via HCtr1 and is then distributed by the chaperones CCS, Cox17, Cox11, Sco1/2 and Atox1. CCS delivers copper to the antioxidant enzyme SOD1, the Cox-network of chaperones control the passage of copper in the mitochondria whereby it acts as a co-factor for cytochrome c oxidase and Atox1 delivers copper to ATP7A/B at the TGN. ATP7A/B can traffic from the TGN to the outer plasma membrane to facilitate copper efflux out of the cell. Excess copper is sequestered via metallothioneins (MT).

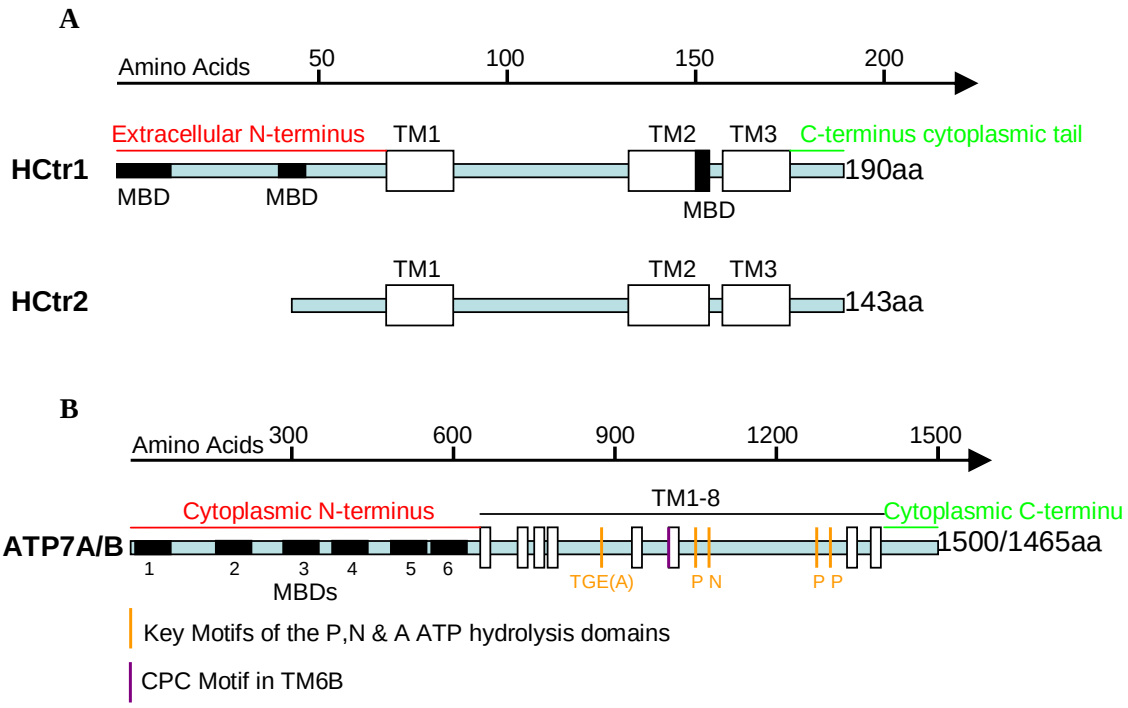

A
Amino Acids
50
100
150
200
Extracellular N-terminus
TM1
TM2
TM3
C-terminus cytoplasmic tail
HCtr1
190aa
MBD
MBD
MBD
TM1
TM2
TM3
HCtr2
143aa
B
Amino Acids
300
600
900
1200
1500
TM1-8
Cytoplasmic N-terminus
Cytoplasmic C-terminu
ATP7A/B
1500/1465aa
1
2
3
4
5
6
MBDs
TGE(A)
P N
P P
Key Motifs of the P,N & A ATP hydrolysis domains
CPC Motif in TM6B

Figure 1.2: Protein domain structure of transporters for copper import and export

HCtr1 (190 amino acids long) and HCtr2 (143aa) each have three transmembrane domains (TMs), whilst only HCtr1 has MBDs in the N-terminus region. ATP7A/B (1500/1465aa) contains 6 MBDs and 8 TMs. Also highlighted are the locations of key motifs of the ATP hydrolysis domains (P,N & A).

understood whether hCtr1 has a function at intracellular organelles or whether this is simply a mobile pool of the protein awaiting recycling back to the outer plasma membrane.

1.2.1.2 How does hCtr1 function?

How hCtr1 binds and transports copper into the cell is not completely understood yet mutations in the N-terminus suggest that binding of copper to at least one of the methionine motifs is required for uptake (Puig *et al.*, 2002; Eisses and Kaplan, 2005). This suggests that copper is initially bound or sensed via the N-terminus, initiating uptake. As mutations in the methionine motif of the second transmembrane domain do not block copper uptake it is suggested that a tight interaction between copper and binding sites in the trans-membrane domains is not required for hCtr1-facilitated uptake (Eisses and Kaplan, 2005). Therefore rather than membrane transport being reliant on tight binding to the transmembrane domains, it is believed that hCtr1 can transport copper across the membrane via a permeation model. This hypothesis is based upon electron crystallography data which shows that hCtr1 forms a symmetric trimer which creates a channel-like pore in-between the three connected subunits (De Feo *et al.*, 2009). Each monomer of the trimer contains the three membrane spanning domains which together create the central pore (De Feo *et al.*, 2009).

It was earlier suggested that the binding site in the second transmembrane domain of hCtr1 is required for efficient copper uptake. Mutations in this binding site may cause interruptions to the opening and closing of the central pore (Eisses and Kaplan, 2005). It is proposed that as copper binds to the binding sites in the second transmembrane domain there are changes to the cytosolic loop resulting in the formation of an opening at the other end of the pore allowing for copper to exit the hCtr1 channel (Eisses and Kaplan, 2005). Whilst a permeation model for hCtr1 copper uptake has strong support, further research is required as questions remain with regards to how copper may cause conformational changes in the trimer and whether there are protein-specific interactions with intracellular chaperones.

1.2.1.3 Could hCtr2 also be involved in copper uptake?

Although hCtr1 is essential for embryonic development, there may be other mechanisms that can contribute to total copper uptake. *hCtr1*-deficient mouse embryonic cells still have some ^{64}Cu transport activity, indicating the potential for hCtr1 independent mechanisms of uptake (Lee *et al.*, 2002). One potential candidate for hCtr1-independent copper uptake is hCtr2, the 143 amino acid long homologue of hCtr1 (Figure 1.2) (Zhou and Gitschier, 1997). Homology is strong in the transmembrane domains of each protein, but hCtr2 lacks the two metal binding methionine motifs at the N-terminus (Zhou and Gitschier, 1997). *hCtr2* is expressed ubiquitously, but at lower levels than *hCtr1* (Zhou and Gitschier, 1997).

hCtr1 can localize to the outer plasma membrane, supporting a possible copper uptake function, however the cellular localization of hCtr2 is predominantly intracellular. In human embryonic kidney cell lines, hCtr2 was not localized to the plasma membrane, but to late endosomes and lysosomes (van den Berghe *et al.*, 2007). Contradictory research has shown that in the monkey kidney COS-7 cell line, endogenous hCtr2 was able to localize at the outer plasma membrane as well as to late endosomes and lysosomes (Bertinato *et al.*, 2008). Further research is required to confirm whether hCtr2 can localize to the outer plasma membrane *in vivo*.

The function of hCtr2 in copper homeostasis is still not completely understood but there is some evidence suggesting the protein could mediate an alternative copper uptake pathway. Exogenous expression of *hCtr2 in vitro* can increase cytosolic copper bioavailability (van den Berghe *et al.*, 2007). Furthermore, siRNA interference (RNAi) suppression of hCtr2 in monkey kidney COS-7 cells resulted in cytosolic copper deficiency (Bertinato *et al.*, 2008). As hCtr2 is predominantly localized to intracellular organelles, these functional results could suggest that the protein is releasing trapped copper from lysosomes or other vesicles (van den Berghe *et al.*, 2007). Further research on the effects of loss of *hCtr2* expression could give further support to a role as an hCtr1-independent copper uptake pathway.

1.2.1.4 Divalent metal transporter 1 (DMT1)

Another potential candidate for hCtr1 independent copper uptake is the divalent metal transporter 1 (DMT1). DMT1 is a 561-amino acid protein, containing 12 transmembrane domains and expressed ubiquitously in mammals with higher levels in the intestine (Gunshin *et al.*, 1997). DMT1 also localizes to the outer apical plasma membrane of polarized cells, suggesting a possible role in metal uptake (Yanatori *et al.*, 2010). DMT1 is largely known as an iron transporter but has a broad substrate range that includes other metals (copper, zinc, nickel and cobalt) and uses energy gained from exchanging protons to actively transport these metals across membranes (Gunshin *et al.*, 1997).

Inactivation of DMT1 in Belgrade rats demonstrated only an iron deficiency phenotype (Knopfel *et al.*, 2005). However in *Drosophila*, a mutation in *Malvolio* (*Drosophila* homologue of DMT1) resulted in flies sensitive to both copper excess and limitation. It was suggested that *Malvolio* mutants were sensitive to copper limitation due to impaired midgut uptake and sensitive to copper excess due to reduced transport to detoxification tissues (Southon *et al.*, 2008). These results give rise to the suggestion that DMT1 may play an important role in copper homeostasis.

In vitro analysis has also provided more evidence supporting a role for DMT1 in copper homeostasis as partial knockdown of the protein in human epithelial colorectal adenocarcinoma (Caco2) cells resulted in reduced copper uptake, whilst over-expression of *Malvolio* in *Drosophila* S2 cells resulted in copper accumulation (Arredondo *et al.*, 2003; Southon *et al.*, 2008). Although not conclusive, this research implies that DMT1 may be involved in an hCtr1 independent copper uptake pathway.

1.2.1.5 Could anion exchangers also transport copper?

Recent research has also raised the possibility of intestinal copper uptake via a member of the SLC26 family of human anion exchangers (Zimnicka *et al.*, 2010) (to be discussed further in Section 1.3.1.1.2). These proteins transport anions (such as chloride, iodide,

bicarbonate and oxalate) across membranes (Mount and Romero, 2004), typically have between 10 and 14 transmembrane domains and have varied expression including the intestine (Mount and Romero, 2004).

In human red blood cells it has been shown that anion exchanger channels are the major source of copper uptake (Alda and Garay, 1990). Copper uptake in these cells is inhibited by the addition of stilbene disulfonic acid (DIDS), which is a common anion exchange inhibitor (Alda and Garay, 1990). It was proposed that copper could be transported into the red blood cells in copper-chloride complexes suggesting that anion exchange may be an hCtr1-independent copper uptake pathway.

1.2.2 The role of copper p-type ATPases

A crucial aspect of copper homeostasis is the controlled delivery of the metal ion from the cytosol into the secretory pathway (Kaplan and Lutsenko, 2009). At the secretory pathway copper acts as a cofactor for enzymes in key pathways such as pigment formation and neural development (Linder and Hazegh-Azam, 1996; Itoh, 2006; Miao and St Clair, 2009; Olivares and Solano, 2009). The delivery of copper to the secretory pathway is controlled by copper-transporting ATPases (Cu-ATPases) (Kaplan and Lutsenko, 2009). Cu-ATPases use the energy of ATP hydrolysis to translocate copper ions from the cytosol across membranes (Kaplan and Lutsenko, 2009). Humans have two Cu-ATPases, ATP7A (Menkes disease protein) and ATP7B (Wilson's disease protein), characterized by the diseases caused by their loss of function (Kaplan and Lutsenko, 2009).

1.2.2.1 Cu-ATPase structure and function

ATP7A and *ATP7B* are both relatively large genes with coding regions spanning approximately 4.5 kb and the translated proteins share ~60% amino acid identity (Lutsenko *et al.*, 2007). Both Cu-ATPases are part of the P_{1B} – subfamily of the P-type

ATPases which translocate metal ions across a membrane using the energy of ATP hydrolysis (Barry *et al.*, 2010). The general protein structure of P-type ATPases contains specific regions for metal binding, ATP hydrolysis and transport through the transmembrane domain (Figure 1.2). Additionally, P_{1B} –ATPases (Cu-ATPases) have some copper-specific structures which facilitate chaperone recognition, copper binding and regulation (Barry *et al.*, 2010).

Human Cu-ATPases have six metal binding domains (MBD1-6) found on the cytoplasmic N-terminal tail which each contain a CxxC (double cysteine binding site) motif (Barry *et al.*, 2010; Veldhuis *et al.*, 2011). Each MBD is approximately 72 amino acids long and they are separated by linker regions of varying length (Veldhuis *et al.*, 2011). X-ray crystallography and absorption spectrometry of MBD1-6 revealed that copper binds to the CxxC motif which is located on an exposed loop of the protein and each MBD forms a ferredoxin-like fold (Jones *et al.*, 2003; Walker *et al.*, 2004; Banci *et al.*, 2008).

Copper is not free in the cytosol, therefore is transferred to the MBDs of ATP7A/B by an intracellular copper chaperone protein known as Antioxidant-1 (Atox1) (discussed in detail in 1.2.3.1) (Barry *et al.*, 2010). Atox1 contains homologous MBDs and binds copper at a similar rate to the Cu-ATPases (Yatsunyk and Rosenzweig, 2007). In *Escherichia coli* cells, Atox1 was shown to transfer copper to each of the human ATP7B MBDs, suggesting no MBD specificity for Atox1 (Banci *et al.*, 2009). However MBD2 has been proposed as the initial site of copper loading via Atox1 as this was the only MBD protected from cysteine labeling when one copper ion was transferred from Atox1 (Walker *et al.*, 2004). Atox1 may preferably transfer copper first to MBD2 before it is then bound to other MBDs (Barry *et al.*, 2010). Interestingly, deletion studies of the N-terminal domain in ATP7A/B have shown that only MBD5 and 6 are required for copper transport (Cater *et al.*, 2004). Both domains are essential but may not be the first site of Atox1 copper transfer as it is believed that interdomain transfer of copper between MDBs is possible (Achila *et al.*, 2006; Bunce *et al.*, 2006). It is likely however that copper must

have to pass through MBD5 and 6 before being transferred to the transmembrane domains.

Cu-ATPases facilitate ATP hydrolysis via three domains; the autocatalytic phosphorylation (P) domain, the nucleotide binding (N) domain and the energy transduction (A) domain (Barry *et al.*, 2010). These three domains are crucial for generating the energy for copper transport (Figure 1.3). ATP binds to the N-domain which triggers donation of γ-phosphate to a motif containing an aspartic acid in the P-domain (Kuhlbrandt, 2004). The aspartic acid is phosphorylated during hydrolysis, and then later dephosphorylated after copper transport to start the cycle again (Kuhlbrandt, 2004). The A-domain contains the conserved TGE or SGE motif which is required for dephosphorylation (Barry *et al.*, 2010). Dephosphorylation is associated with the release of copper from the Cu-ATPase into the extracellular side (Barry *et al.*, 2010).

Cu-ATPase-mediated copper transport across the membrane is controlled by the eight transmembrane spanning domains (TM1-TM8) (Lutsenko *et al.*, 2007). Motifs in TM6-8 of ATP7A/B are believed to be crucial for copper binding and transport (Lutsenko *et al.*, 2007). Mutational analysis of the bacterial Cu-ATPase, *CopA*, has elucidated two possible sites for copper binding in the transmembrane domains (Gonzalez-Guerrero *et al.*, 2008). Both sites are thought to bind copper with trigonal coordination between copper and transmembrane domain sequences (Gonzalez-Guerrero *et al.*, 2008). One site is formed with the CPC motif (TM6) and a Tyr residue (TM7), whilst the second site is formed with an Asn (TM7) and Met and Ser (TM8) (Gonzalez-Guerrero *et al.*, 2008). These high affinity copper binding regions are conserved in human Cu-ATPases (Barry *et al.*, 2010) and may be involved in the translocation of copper through the membrane and subsequent delivery to the extracellular side (Barry *et al.*, 2010).

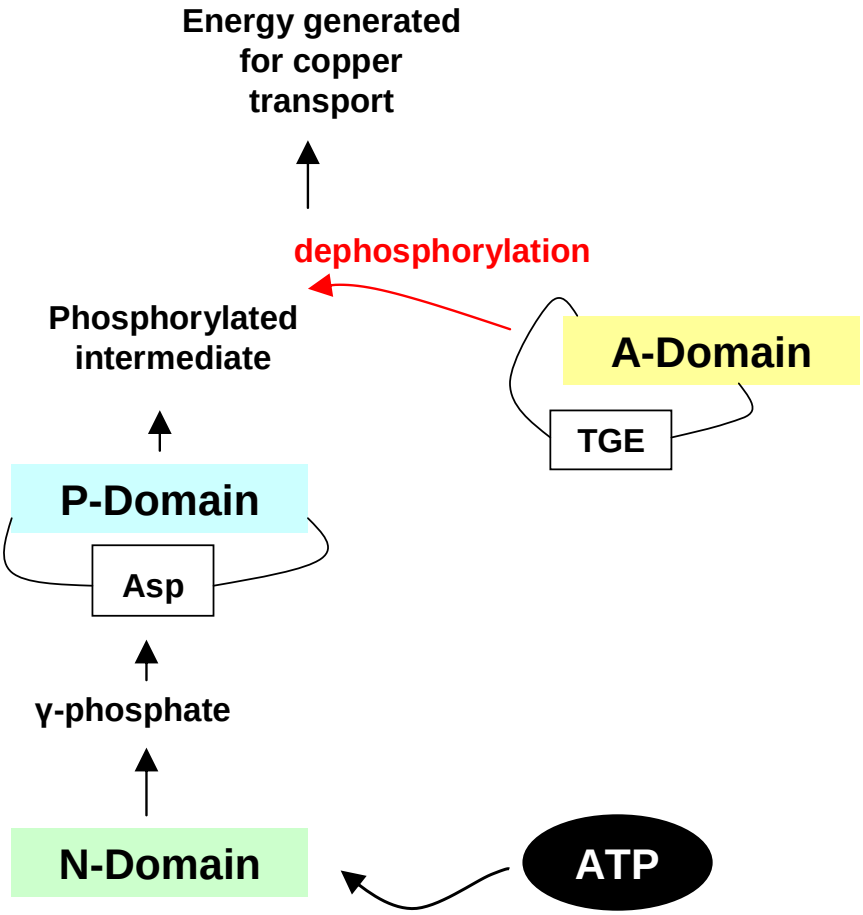

Energy generated
for copper
transport
dephosphorylation
Phosphorylated
intermediate
A-Domain
TGE
P-Domain
Asp
γ-phosphate
N-Domain
ATP

Figure 1.3: The ATP hydrolysis cycle

Cu-ATPases use ATP hydrolysis to generate the energy required for copper transport. ATP hydrolysis is facilitated by protein domains; the autocatalytic phosphorylation (P) domain, the nucleotide binding (N) domain and the energy transduction (A) domain. ATP binds to the N-domain triggering the donation of γ-phosphate to an aspartic acid residue in the P-domain. The aspartic acid becomes phosphorylated producing a intermediate product which is then dephosphorylated (coordinated by the TGE motif of the A-domain) generating the energy for copper transfer.

1.2.2.2 Expression of ATP7A and ATP7B

Although both Cu-ATPases share a similar protein structure, differences in gene expression patterns can help explain the contradictory phenotypes of Menkes and Wilson's diseases (Lutsenko *et al.*, 2007). *ATP7A* is expressed in the intestine, choroid plexus, vascular smooth muscle cells, vascular endothelial cells and most non-hepatic cell types (Monty *et al.*, 2005; Niciu *et al.*, 2006; Lutsenko *et al.*, 2007). This correlates with the required functions of ATP7A to export copper out of the intestine and the ensuing delivery to the brain (Lutsenko *et al.*, 2007). Consequently, Menkes disease is caused by a systemic copper deficiency due to defective ATP7A activity.

Conversely, *ATP7B* is expressed predominantly in the liver correlating with the function of exporting excess copper from the liver and with the copper accumulation phenotype associated with Wilson's disease (Lutsenko *et al.*, 2007). There is also expression of *ATP7B* in the midbrain and basal ganglia but it is not well understood how the protein functions in these regions (Saatci *et al.*, 1997). The lung, kidney, placenta and mammary gland all show co-expression of both *ATP7A* and *ATP7B* and roles for each protein in these cells types are still being elucidated (Lutsenko *et al.*, 2007).

1.2.2.3 Cu-ATPase localization can determine its cellular role

The localization of Cu-ATPases is also crucial as it determines the cellular site of copper transport. At basal cellular copper levels both ATP7A and ATP7B localize to the *trans*-Golgi network (TGN) (Petris *et al.*, 1996; Mercer *et al.*, 2003). At the TGN, copper is delivered to the secretory pathway to act as cofactor for key cuproenzymes. However the localization of both Cu-ATPases can change in response to different stimuli such as elevated copper levels, post-translational modification and hormone signaling (Petris *et al.*, 1996; Mercer *et al.*, 2003; Veldhuis *et al.*, 2009). Polarized cell culture studies have revealed that in response to elevated copper levels, Cu-ATPases will relocalize to endocytic vesicles at the basolateral membrane (ATP7A) or the apical membrane (ATP7B) (Guo *et al*, 2005, Monty *et al*, 2005) This suggests that under elevated copper

conditions ATP7A relocalizes to the basolateral membrane of cells in the intestine or choroid plexus to export excess copper. Conversely, ATP7B relocalizes to vesicles in the canalicular membrane to export excess copper into the bile for excretion. The trafficking of the Cu-ATPases is reversible and is crucial to regulating intracellular copper levels (trafficking mechanisms are further discussed in regards to regulation of copper transport).

1.2.2.4 ATP7A-related disease

Symptoms of Menkes disease were first observed by Dr John Menkes in 1962 as he attended a family of sufferers who had five members die in early childhood (Kaler, 2011). His early description of symptoms included brittle hair, low weight and small brain size (Menkes *et al.*, 1962). Positional cloning was later able to link Menkes disease to *ATP7A* on the X chromosome (Chelly and Monaco, 1993; Mercer *et al.*, 1993; Vulpe *et al.*, 1993). As discussed, Menkes disease is caused by a block in copper absorption in the small intestine which results in low copper levels in the brain, liver and serum (DiDonato and Sarkar, 1997; Kaler, 2011). The lack of copper delivery to key cuproenzymes such as tyrosinase, Peptidylglycine α-Amidating Monooygenase and Dopa *β*-Monooygenase, cause growth defects, neurological impairment, loss of pigment and ultimately early childhood death (Danks, 1988; Kaler, 1998; Lutsenko *et al.*, 2007). Parenteral administration of copper-histidine can treat some of the milder symptoms of Menkes disease, however a successful treatment for neurological symptoms remains to be discovered (Kodama *et al.*, 2011).

Two other milder allelic variants have also been associated with *ATP7A* mutations, Occipital Horn Syndrome (OHS) and Distal Hereditary Motor Neuropathy (DHMN) (Lazoff *et al.*, 1975; Tsukahara *et al.*, 1994; Kennerson *et al.*, 2010). OHS is a milder form of Menkes diseases where ATP7A function is partially lost (Kaler *et al.*, 1994). Neurological symptoms are less severe however sufferers form calcifications (horns) on the upper back (Kaler, 2011). Phenotypes caused by DHMN are not similar to those caused by Menkes or OHS as sufferers display muscle weakness and poor movement of

the hands and feet (Kennerson *et al.*, 2010). The disease does not alter serum copper levels but may result in the failure of ATP7A to return to the TGN from the outer plasma membrane (Kaler, 2011; Yi *et al.*, 2012). Mutations causing the diseases are located in the carboxy region of *ATP7A* (aa1386 P-S & aa994 T-I) , but not in any known functional domains (Kennerson *et al.*, 2010).

1.2.2.5 ATP7B-related disease

Wilson's disease was first described by Kinnear Wilson early in the twentieth century (Wilson, 1912). The disease was linked to a copper overload and determined to be inherited in an autosomal recessive manner (Cumings, 1948; Bearn, 1953). Positional cloning was later able to link Wilson's disease to *ATP7B* on chromosome 13. The disease is caused by a block in copper export from hepatocytes leading to toxicity of the liver, the release of free copper ions into the bloodstream and build up of copper in the major organs including the brain and kidneys (DiDonato and Sarkar, 1997; Roberts and Cox, 1998; Huster, 2010). Therefore Wilson's disease sufferers have a range of both hepatic and neuronal symptoms including chronic liver necrosis, behavioral disturbances, movement disorders and dementia (DiDonato and Sarkar, 1997; Huster, 2010). One recognisable symptom is the formation of granular copper depositions in the cornea creating what is known as a Kayser-Fleischer ring (Ferenci, 2004). Wilson's disease patients range in age from childhood to early fifties and treatment involves the delivery of copper chelating agents such as pencillimate, trientine or zinc (DiDonato and Sarkar, 1997; Kodama *et al.*, 2011). These treatments are only partially effective as in some cases neurological symptoms of Wilson's disease actually worsened (Kodama *et al.*, 2011). Another copper chelating agent, known as tetrathiomolybdate, is currently being trialed as a more effective treatment method (Kodama *et al.*, 2011).

1.2.3 Cellular copper distribution

After entering the cell copper is not free in the cytosol but rather bound to proteins known as metallochaperones (Figure 1.1). The role of metallochaperones is to bind copper and deliver the metal ion to specific partner proteins which fuel cellular copper requirements (Robinson and Winge, 2011). The major metallochaperones found in the human copper homeostasis system are Atox1, Copper Chaperone for Superoxide dismutase (CCS) and the network of metallochaperones for cytochrome *c* oxidase (Robinson and Winge, 2011).

1.2.3.1 Antioxidant-1 (Atox1) is the metallochaperone for the Cu-ATPases

A specific copper chaperone for Cu-ATPases was first identified in both yeast and bacteria (Robinson and Winge, 2011). CopZ (bacteria) and Atx1p (yeast) were both identified as chaperones capable of transporting copper (Lin and Culotta, 1995; Odermatt and Solioz, 1995). Atox1 (originally known as HAH1) was then characterised as the human homologue of Atx1p (Klomp *et al.*, 1997).

Atox1 is 68 amino acids long and contains one copy of the CxxC MBD motif associated with a ferredoxin-like structural loop (Muller and Klomp, 2009; Robinson and Winge, 2011). Mouse mutants lacking *Atox1* suffered copper deficiency symptoms such as growth retardation, hypopigmentation and congenital eye defects (Hamza *et al.*, 2001). A loss of human *Atox1* results in copper accumulating in the cell as shown in null *Atox1* fibroblast cells (Hamza *et al.*, 2003). These cells also had impaired copper-mediated trafficking of ATP7A leading to decreased copper export (Hamza *et al.*, 2003).

It is proposed that Atox1 accepts copper entering the cell from a direct interaction with hCtr1. This is supported by the electron crystallography of hCtr1 which reveals a potential Atox1 docking site (De Feo *et al.*, 2009). Copper initially binds to the sole Atox1 MBD forming a copper dependent homodimer (Wernimont *et al.*, 2000;

Anastassopoulou *et al.*, 2004). The interaction between Atox1 and ATP7A or ATP7B involves the direct transfer of copper between respective MBDs (Wernimont *et al.*, 2000; van Dongen *et al.*, 2004). Research has demonstrated a direct copper transfer from Atox1's MBD to MBD2 of ATP7B, suggesting this domain is the initial Atox1 loading site (Walker *et al.*, 2004).

1.2.3.2 Copper chaperone for superoxide dismutase (CCS)

Copper is distributed to the antioxidant defense enzyme SOD1 by CCS which is 274 amino acids long and contains three major domains (Lamb *et al.*, 2001; Kawamata and Manfredi, 2010). Domains I and III both contain one copy of the CxxC MBD motif for copper binding (Lamb *et al.*, 2001). Domain II directly interacts with SOD1, whilst domain III contains a key residue involved in formation of a intermolecular disulfide bond with SOD1 (Lamb *et al.*, 2001).

SOD1 is predominantly localized in the cytosol, however it has been recently found in the mitochondrial intermembrane space (IMS) as well. Loss of *CCS* causes a decrease in SOD1 activity and increased sensitivity to reactive oxygen species (Wong *et al.*, 2000). The role of SOD1 is to provide a buffer against reactive species in the cytosol and mitochondrial intermembrane space (Robinson and Winge, 2011). CCS delivers copper to SOD1, triggering the formation an intermolecular disulfide bond and protein dimerisation (Kawamata and Manfredi, 2010). It has been recently shown that SOD1 can also be activated via a CCS-independent pathway. Human, mice and *Drosophila* SOD1 can be partially activated independently of CCS, however the nature of this independent mechanism is unclear (Subramaniam *et al.*, 2002; Kirby *et al.*, 2008; Robinson and Winge, 2011).

1.2.3.3 Metallochaperones for cytochrome c oxidase

Copper plays a key role in cellular respiration by acting as a co-factor for the mitochondrial enzyme cytochrome *c* oxidase which is crucial for cellular respiration (Cobine *et al.*, 2006). Cytochrome *c* oxidase obtains electrons to reduce oxygen and then translocate protons across the inner mitochondrial membrane (IM) (Cobine *et al.*, 2006). The enzyme consists of subunits that are derived from both the mitochondrial and nuclear genomes (Robinson and Winge, 2011). Key core components, Cox1-3, are encoded by the mitochondrial genome, whilst surrounding components are encoded by the nuclear genome (Yoshikawa *et al.*, 1998). The enzyme structure revolves around the insertion of two copper ions and two heme α moieties triggering electron transfer and binding of oxygen (Yoshikawa *et al.*, 1998; Cobine *et al.*, 2006). The enzyme itself is fixed into the IM with separate portions protruding into the mitochondrial inner membrane space (IMS) and mitochondrial matrix (Beinert, 1995).

Subunits Cox1 and Cox2 contain the copper binding motifs Cu_B and Cu_A respectively (Cobine *et al.*, 2006). Both these motifs require the action of a system of metallochaperones to deliver copper (Cobine *et al.*, 2006) (Figure 1.4). Localized in the IMS and associated with the IM, Cox11 delivers copper to the Cu_B site of Cox1 (Cobine *et al.*, 2006). Also localized in the IMS and associated with the IM, Sco1 and Sco2 deliver copper to the Cu_A site of Cox2 (Cobine *et al.*, 2006). Both copper-loaded core units, Cox1 and Cox2, are inserted into the IM to form cytochrome c oxidase with other accessory components (Robinson and Winge, 2011).

Cox11, Sco1 and Sco2 also require copper to be donated via a separate metallochaperone Cox17. Cox17 was originally identified as the protein responsible for transferring copper from cellular uptake proteins to the mitochondria (Beers *et al.*, 1997). It was thought that as Cox17 was localized to both the cytosol and mitochondria, it could shuttle from the outer plasma membrane to deliver copper to the mitochondria (Beers *et al.*, 1997). This was supported by the early embryonic death and decreased cytochrome *c* oxidase activity in mice lacking Cox17 (Takahashi *et al.*, 2002). However when Cox17 was

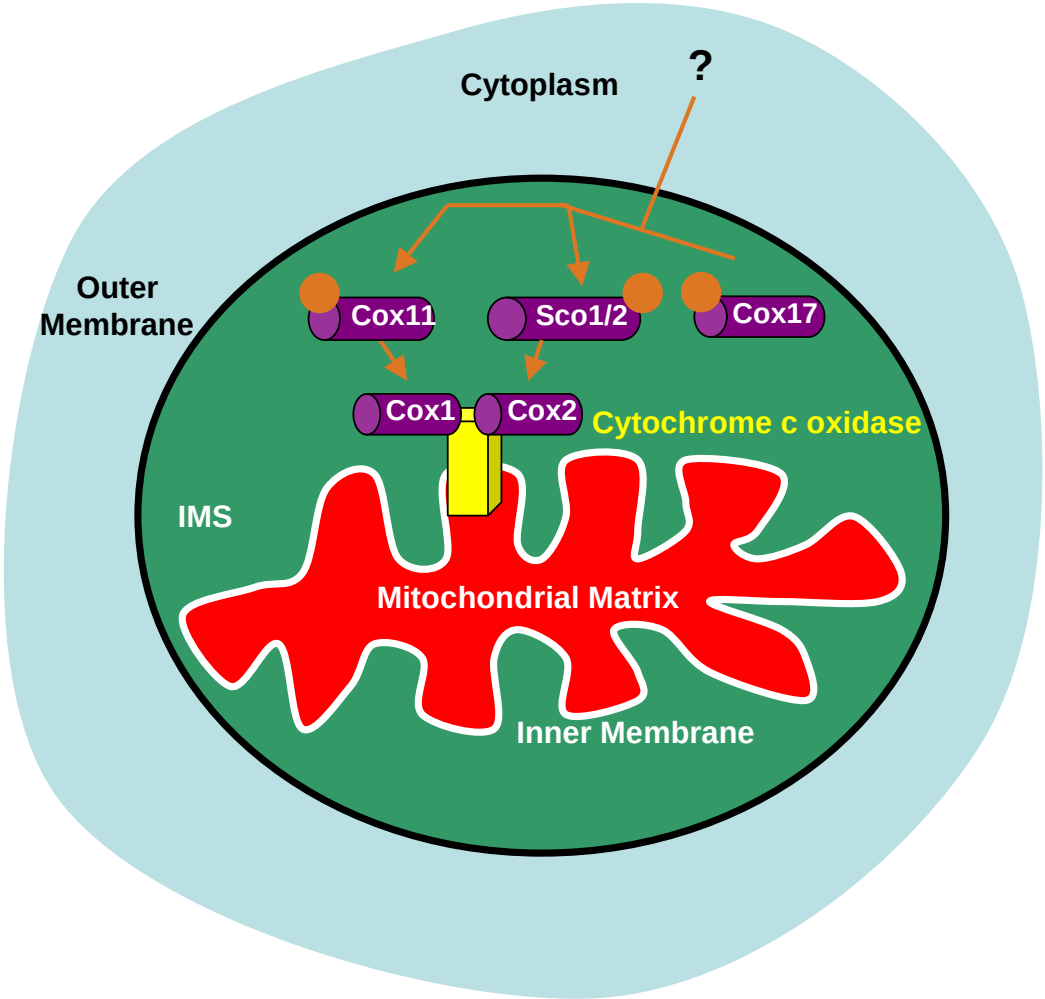

Cytoplasm
?
Outer
Membrane
Cox11
Sco1/2
Cox17
Cox1
Cox2
Cytochrome c oxidase
IMS
Mitochondrial Matrix
Inner Membrane

Figure 1.4: The network of metallochaperones for cytochrome *c* oxidase

The mitochondrial enzyme cytochrome *c* oxidase requires copper as a cofactor to drive cellular respiration. After entering the cytoplasm, it remains unclear how copper travels across the outer mitochondrial membrane and into the IMS. Once inside the IMS Cox17 delivers copper to both Cox11 and Sco1/2 which in turn load the respective copper binding sites of Cox1 and Cox2. The copper-loaded chaperones then insert into the inner mitochondrial membrane and form cytochrome *c* oxidase with other accessory components (not shown in this diagram).

tethered to the mitochondrial IM there was no reduction in cytochrome c oxidase activity (Maxfield *et al.*, 2004). This suggests that Cox17 functions only at the mitochondria to transfer copper to Cox11, Sco1 and Sco2 (Horng *et al.*, 2004). It has been shown that the mitochondrial matrix contains a store of copper, suggesting Cox17 may accept copper from the matrix via an unknown mechanism (Cobine *et al.*, 2004). What remains unclear is how the mitochondria receive copper from the cytosol after uptake.

1.2.4 Cellular copper sequestration by metallothionein

When copper is not bound by metallochaperones, the cytoplasm can be buffered from the metal's toxic effect by metallothioneins. Human metallothionein proteins are separated into four classes, MT-I, MT-II, MT-III and MT-IV (Balamurugan and Schaffner, 2006). All classes of metallothioneins are approximately 60 amino acids long and contain a cysteine content of around 30% (Balamurugan and Schaffner, 2006). Copper binds to the cysteine-rich region of metallothionein whereby it is sequestered from the cytoplasm. Loss of metallothionein function, as shown by gene knockout in *Drosophila*, results in sensitivity to high copper environments (Egli *et al.*, 2006).

MT-I and MT-III are expressed almost ubiquitously at all stages of mammalian development, MT-II is restricted to neurons and MT-V is found only in differentiated squamous epithelia (Quaife *et al.*, 1994; Aschner *et al.*, 1997; Andrews, 2000). With the exception of MT-III, metallothionein expression is triggered by increases in intracellular copper levels (Balamurugan and Schaffner, 2006). Expression of MT-I, II and IV is transcriptionally activated by MTF-1 (metal responsive transcription factor-1) (Heuchel *et al.*, 1994). MTF-1 can bind to MREs (metal responsive elements) contained in the promoter regions of metallothionein genes. MTF-1 is normally localized to the cytoplasm but under heavy metal load shifts to the nucleus to drive target gene transcription (Saydam *et al.*, 2001). MTF-1 contains six zinc-finger binding domains and is activated directly *in vitro* by high zinc levels (Zhang *et al.*, 2003). Copper activates MTF-1 indirectly by displacing metallothionein-bound zinc into the cytoplasm (Zhang *et*

al., 2003) (the MTF-1 activation of Metallothionein transcription will be further discussed in *Drosophila* copper homeostasis).

1.3 Copper distribution throughout different tissues

Copper distribution involves nutrient absorption, regulation at the liver and subsequent delivery to the rest of the body (Lutsenko *et al.*, 2007) (Figure 1.5). The major source of copper exposure to humans is via ingestion and absorption of the nutrient through the small intestine after which copper is transported into the hepatic portal system (also known as the portal venous system) which controls direct blood flow from the small intestine to the liver (Linder and Hazegh-Azam, 1996; Harrison and Dameron, 1999; van den Berghe and Klomp, 2009). The liver regulates copper levels by delivering the nutrient to the heart and the rest of the body or by removing excess via the bilary network (van den Berghe and Klomp, 2009).

1.3.1 Intestinal copper homeostasis

An emerging area of copper research is trying to understand the mechanics of absorption at the small intestine. Copper is absorbed by the small intestine at a daily rate of approximately 0.6 to 1.6 mg/day (Linder and Hazegh-Azam, 1996). Copper absorption is the first site of distribution and homeostasis in action, yet the intestinal specific roles of copper transport proteins in the absorption process are unclear. The small intestine mucosa is made up of a set of simple, microvilli-coated epithelial cells known as enterocytes (Ballard *et al.*, 1995). Enterocytes are polarized, differentiated cells which stack to form an epithelial barrier between the lumen and the bloodstream (Ballard *et al.*, 1995). The epithelial barrier is maintained by tight junctions which join the enterocytes side by side, allowing each cell to have separate apical (luminal) and basolateral (bloodstream) outer plasma membranes (Ballard *et al.*, 1995). Dietary absorption involves uptake at the enterocyte apical membrane, passage through the cell and export across the basolateral membrane. Additionally, enterocytes must fuel their own cell-

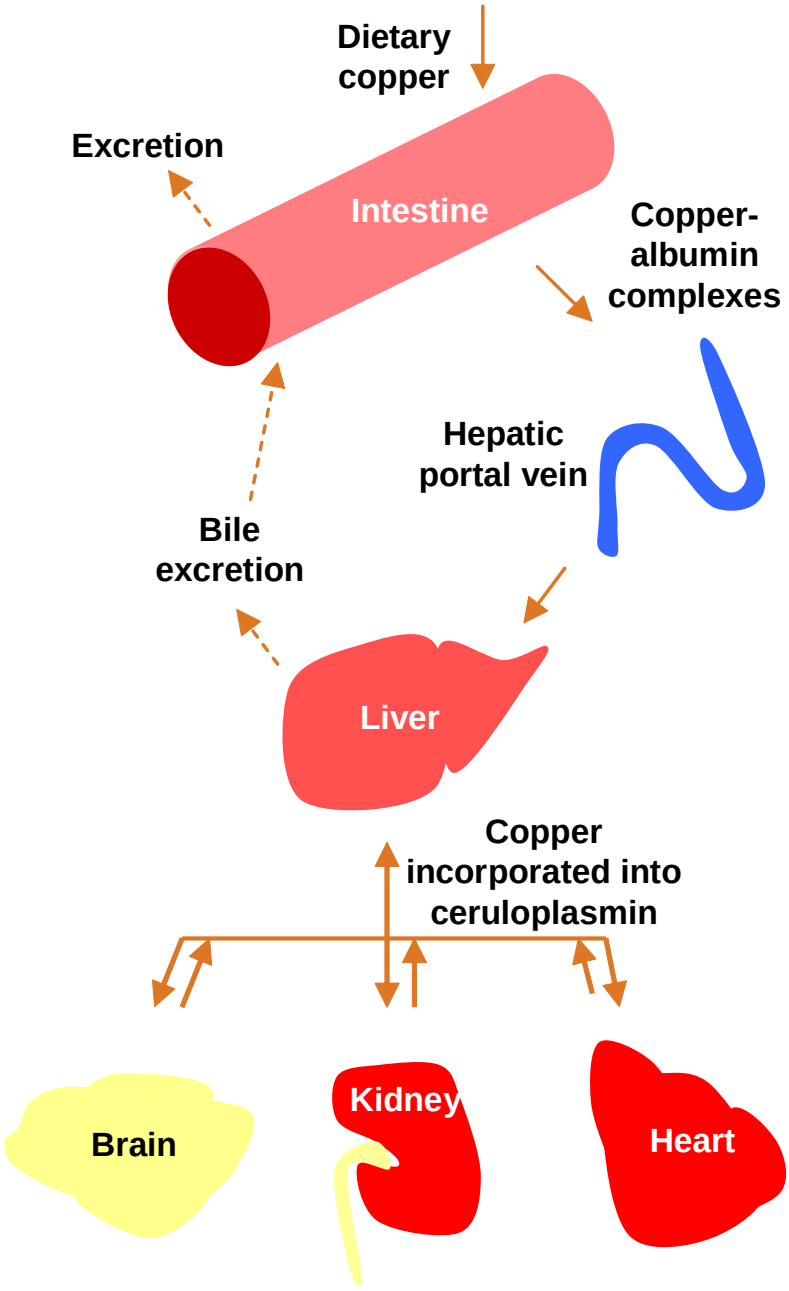

Dietary copper
Excretion
Intestine
Copper-albumin complexes
Hepatic portal vein
Bile excretion
Liver
Copper incorporated into ceruloplasmin
Brain
Kidney
Heart

Figure 1.5: Distribution of copper around the human body

The major source of copper exposure to humans is via dietary absorption through the small intestine after which copper is transported into the hepatic portal vein bound to albumin. Copper-albumin complexes enter hepatocytes whereby the liver regulates body copper levels. Copper is delivered via ceruloplasmin to the rest of the body including the heart, kidney and brain. Excess copper can be removed from the liver as bile and excreted from the intestine.

specific copper requirements and prevent against toxicity (van den Berghe and Klomp, 2009). Although the exact mechanisms of enterocyte copper transport are unclear there is a strong indication that key homeostasis proteins such as hCtr1 and ATP7A are involved (Figure 1.6).

1.3.1.1 Intestinal copper uptake

The first step of intestinal copper absorption is the uptake of the nutrient at the apical membrane of enterocytes. Research into the mechanism controlling copper uptake into enterocytes is conflicting but the one constant is an essential role for hCtr1. Intestinal specific *hCtr1* knockout mice suffer copper deficient phenotypes such as growth defects, loss of cardiac function, hypopigmentation and a heavily shortened lifespan (Nose *et al.*, 2006). A post-intestinal copper deficiency was proven by the fact that accumulation was significantly decreased in peripheral tissues (Nose *et al.*, 2006). The deficiency was a result of lost intestinal absorption as rescue was achieved via intraperitoneal injection, bypassing intestinal absorption (Nose *et al.*, 2006). This research concluded that hCtr1 was the obvious candidate for uptake of copper at the apical membrane of enterocytes.

Research into the candidate for intestinal copper uptake becomes controversial when considering the localization of hCtr1 in enterocytes. One line of thinking suggests that hCtr1 is predominantly localized to the apical membrane of enterocytes (Nose *et al.*, 2010). Immunohistochemical and biotinylation analysis on mouse, rat and pig small intestinal sections demonstrated predominant apical membrane localization, with low levels of intracellular protein (Nose *et al.*, 2010). This supported earlier work which also showed apical or internal-compartment localization of hCtr1 in intestinal epithelial cells (Kuo *et al.*, 2006; Nose *et al.*, 2006). If localized to the apical membrane of intestinal epithelial cells, hCtr1 could facilitate copper uptake from the lumen into the cytosol of enterocytes.

In contrast, it has also been proposed that hCtr1 is localized to intracellular organelles and the basolateral membrane of intestinal enterocytes (Zimnicka *et al.*, 2007).

Immunohistochemical and biotinylation analysis on Caco-2 cells (an established model of intestinal enterocytes) demonstrated that hCtr1 was localized predominantly at the basolateral membrane (Zimnicka *et al.*, 2007). In addition silver ions inhibited copper uptake only when added at the basolateral membrane (Zimnicka *et al.*, 2007). This model suggests that hCtr1 is localized at the basolateral membrane controlling uptake of copper from the bloodstream back into the intestine (Zimnicka *et al.*, 2007). As discussed earlier, intestinal enterocytes must fuel their own requirements for copper hence basolateral-localized hCtr1 could import copper from the bloodstream for this function. It must be noted that doubts have been raised about the basolateral hCtr1 enterocyte localization due to variability in Caco2 cells and antibody differences (Nose *et al.*, 2010).

The basolateral hCtr1 localization model proposes that enterocytes would have separate systems for copper delivered from the lumen (dietary copper required to fuel the whole body) and from the bloodstream (enterocyte specific requirements). This model is supported by the intriguing result that copper accumulates in the enterocytes of the intestinal specific *hCtr1* knockout mice therefore implying there is a separate mechanism importing copper (Nose *et al.*, 2006; Zimnicka *et al.*, 2010). Cuproenzyme activity remained low in these cells suggesting that the copper accumulated was not available to the cell (Nose *et al.*, 2006). In theory the secondary mechanism may import dietary copper from the lumen and into intracellular pools but as hCtr1 is still required to prevent a post-intestinal copper deficiency it may still be needed to release copper from intracellular bio-unavailable pools (Zimnicka *et al.*, 2007). This could give hCtr1 an essential role at both basolateral (copper uptake from the bloodstream for enterocyte specific requirements) and intracellular locations (release of dietary copper pools). So regardless of whether hCtr1 is also localized at the apical membrane or not, research suggests there is potential for other uptake mechanisms (Zimnicka *et al.*, 2010).

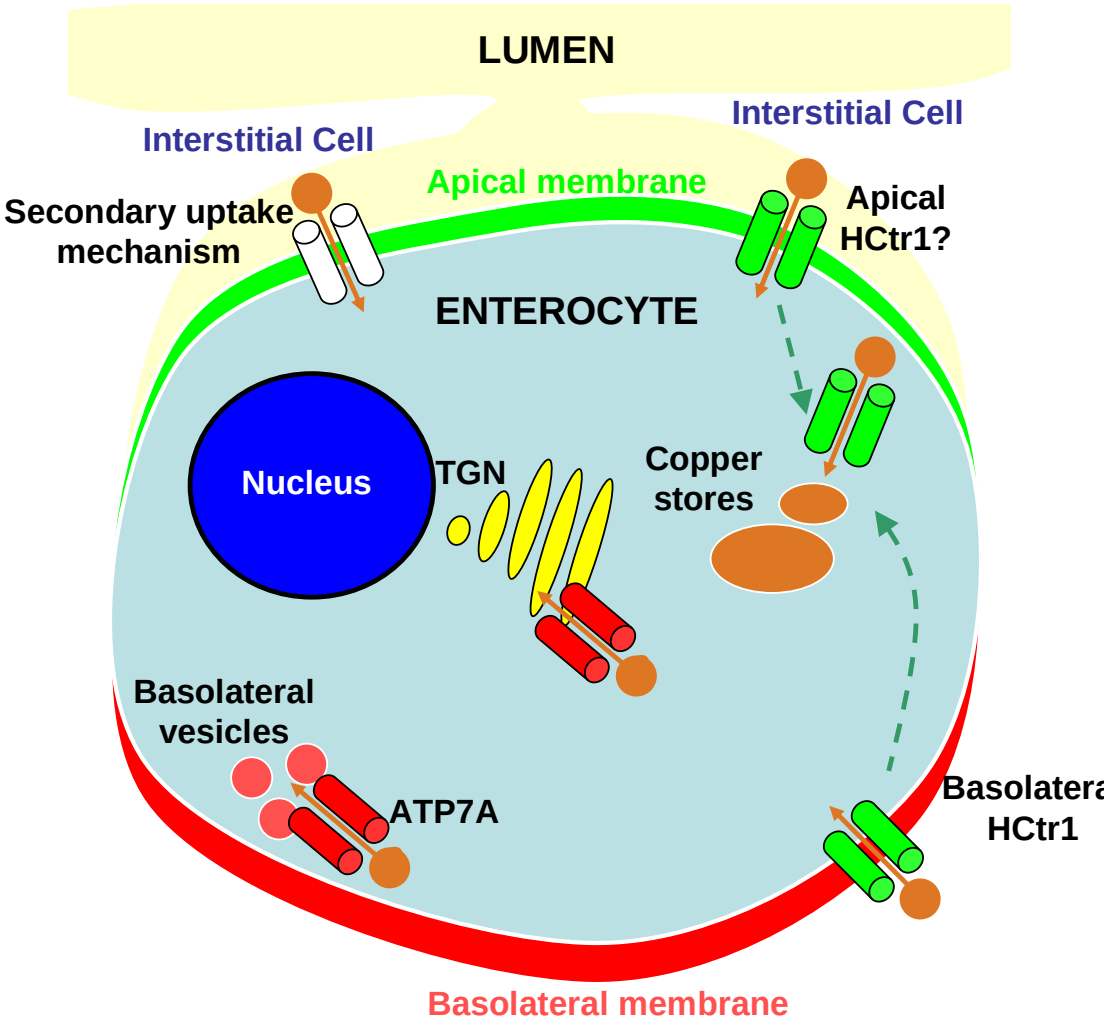

LUMEN
Interstitial Cell
Interstitial Cell
Apical membrane
Secondary uptake mechanism
Apical HCtr1?
ENTEROCYTE
Nucleus
TGN
Copper stores
Basolateral vesicles
ATP7A
Basolatera
HCtr1
Basolateral membrane

Figure 1.6: Mammalian intestinal copper transport

Polarised cells known as enterocytes form the epithelial barrier between the intestinal lumen and the bloodstream and are the site of nutrient absorption in the small intestine. Dietary copper is imported from the lumen across the apical membrane into the cell before export across the basolateral membrane into the bloodstream. HCtr1 is the main candidate for copper uptake at the apical membrane, however it has also been shown to localize to both intracellular organelles and the basolateral membrane. Other candidates such as DMT1 or anion exchangers have been proposed as alternative apical copper transporters. ATP7A delivers copper to the secretory pathway and also translocates to vesicles adjacent to the basolateral membrane to facilitate export into the bloodstream.

1.3.1.1.1 Are there candidates for hCtr1-independent apical uptake?

DMT1 is a potential candidate involved in the apical uptake of copper in the intestinal enterocytes. As discussed earlier, *in vitro* analysis has shown that the partial knockdown of *DMT1* in Caco2 cells resulted in reduced copper uptake. However it has recently been suggested that DMT1 may play a minor role in intestinal copper absorption. When iron or manganese, preferred substrates of DMT1, are added to the cellular medium of Caco2 cells there is no alteration to copper uptake levels (Zimnicka *et al.*, 2010).

Another likely candidate for apical uptake of copper in intestinal enterocytes is one of the anion exchanger channels. Like in red blood cells, it has been suggested that copper may be transported into enterocytes in copper-chloride complexes. In Caco2 cells, the removal of chloride ions from the extracellular medium resulted in decreased copper uptake (Zimnicka *et al.*, 2010). Copper uptake also decreased when anion exchange was inhibited by the addition of DIDS (Zimnicka *et al.*, 2010). The most likely anion exchanger channel candidate is the family of SLC26 exchangers as they are expressed apically in the intestine (Zimnicka *et al.*, 2010).

1.3.1.2 Intestinal copper export

Once copper is absorbed into enterocytes, it is then transported across the basolateral membrane into the hepatic portal bloodstream. Due to its high expression levels and the fact that Menkes disease patients suffer from systemic copper deficiency, ATP7A is the likely candidate for basolateral copper export in enterocytes (Nyasae et al., 2007). During copper limiting conditions, intestinal ATP7A localizes to the TGN in transgenic mice and polarized Caco2 cells (Bauerly *et al.*, 2004; Monty *et al.*, 2005; Nyasae *et al.*, 2007). The proposed model of intestinal copper absorption suggests that ATP7A would translocate to the basolateral membrane during increased intracellular copper levels (Petris *et al.*, 1996). However under increased copper load ATP7A relocalizes predominantly to organelles adjacent to the basolateral membrane, with only a small portion shifting to the actual surface of the membrane (Monty *et al.*, 2005; Nyasae *et al.*,

2007). The role of ATP7A at these intracellular organelles is still unclear. ATP7A may be exporting copper into vesicles which undergo exocytosis, releasing the nutrient into the bloodstream. Alternatively, ATP7A may directly export copper at the basolateral membrane before being recycled back to adjacent (holding) organelles.

There is also evidence that there may be some apical localization of intestinal ATP7A (Ravia *et al.*, 2005). In rat duodenal enterocytes, ATP7A protein was detected at the apical membrane in control rats and then shifted to a basolateral localization in iron-deprived rats (Ravia *et al.*, 2005). It was proposed that intestinal copper absorption increases during times of iron deficiency and apical ATP7A may be required to export excess copper back into the lumen (Ravia *et al.*, 2005; van den Berghe and Klomp, 2009). Despite some apical ATP7A being detected, it is widely accepted that ATP7A is required chiefly for the export of copper from enterocytes into the hepatic portal bloodstream.

1.3.2 Hepatic copper regulation

The liver regulates copper homeostasis by distributing adequate amounts of the nutrient to peripheral organs and by excreting excess to prevent toxicity (Figure 1.5). To manage both of these processes, hepatic cells have a unique cell-specific copper transport system. Copper exported from the intestinal epithelium into the hepatic portal bloodstream becomes bound to the plasma transport protein serum albumin. The endothelial cells of the liver allow copper-albumin complexes to pass through into the perisinusoidal space where entry into hepatocytes can take place.

1.3.2.1 The hepatic role of hCtr1

hCtr1 is expressed highly in the liver and the protein controls the majority of copper uptake into hepatocytes (Zhou and Gitschier, 1997; Kim *et al.*, 2009). Mice with a liver-specific deletion of *hCtr1* had decreased hepatic copper content and cuproenzyme

activity (Kim *et al.*, 2009). As the liver-specific deletion caused only a mild copper deficiency (considering there was only ~10% residual hCtr1 remaining), hepatic cells were somehow compensating for the loss of *hCtr1*, possibly by reducing copper export or activating a compensatory uptake mechanism.

1.3.2.2 The hepatic Cu-ATPase: ATP7B

ATP7B is a fundamental component of hepatic copper homeostasis as its gene is expressed almost exclusively in the liver and mutations cause Wilson's Disease (Lutsenko *et al.*, 2007; Huster, 2010). Under basal conditions, ATP7B is localized at the TGN driving the transfer of copper into the secretory pathway to be incorporated into ceruloplasmin, a copper dependent ferroxidase associated with the oxidation of iron (Healy and Tipton, 2007; Lutsenko *et al.*, 2007). Copper incorporated into ceruloplasmin is secreted into the bloodstream for delivery to the heart and more peripheral organs (Lutsenko *et al.*, 2007). Aceruloplasminemia, the disease caused by loss of ceruloplasmin, is characterized by iron accumulation (Jeong and David, 2003). Mice suffering Aceruloplasminemia have a lack of serum ferroxidase activity and iron accumulation but surprisingly normal copper homeostasis (Meyer *et al.*, 2001). It has been shown that copper accumulates in the liver during Aceruloplasminemia but a systemic effect on copper homeostasis is yet to be discovered (Meyer *et al.*, 2001).

Under elevated copper conditions, ATP7B relocalizes to export excess copper out of hepatic cells into vesicles near the canalicular membrane of hepatocytes (Schaefer *et al.*, 1999; Roelofsen *et al.*, 2000, Guo *et al*, 2005, Cater *et al*, 2006). Vesicles can then bind to the membrane, releasing copper out of the cell into the bile (Bartee and Lutsenko, 2007). In rats, bilary-copper excretion has been detected after gastrointestinal injection of copper (Bissig *et al.*, 2005). Most copper regulated by the liver is excreted via bile, with only a small amount incorporated into ceruloplasmin (Madsen and Gitlin, 2007). Deletion of *ATP7B* in mice results in a copper overload of the liver and production of inactive ceruloplasmin. These phenotypes highlight the dual role of ATP7B at the

secretory pathway and canalicular membrane and reflect the symptoms suffered by Wilson's disease patients.

1.3.3 Systemic copper distribution

As the key site of copper homeostasis regulation, the liver distributes an adequate amount of the nutrient for peripheral organs, whilst controlling excretion to prevent toxicity. Copper-bound ceruloplasmin is exported from the liver into the bloodstream to supply several organs including the brain, nervous systems, heart, kidneys and mammary gland (Lutsenko *et al.*, 2007) (Figure 1.5). Each system has specific copper needs and therefore the delivery of copper must be regulated. To maintain homeostatic control of copper distribution it has been recently suggested that organs, such as the heart, may be able to communicate with the liver and also the intestine (Kim *et al.*, 2010).

Mice with copper deficient hearts were investigated by generating a cardiac-specific knockout of *hCtr1* (Kim *et al.*, 2010). These mice suffered from impaired cardiac function suggesting the heart has a high requirement for copper (Kim *et al.*, 2010). Interestingly, mice deficient in cardiac *hCtr1* also displayed decreased copper content in the liver and a simultaneous rise in serum copper (Kim *et al.*, 2010). The increased transfer of copper from the liver into the bloodstream was proposed to be a response to a cardiac copper deficiency. In support of this theory , *ATP7A* was found to be expressed at high levels in the livers of cardiac copper deficient mice (Kim *et al.*, 2010). As discussed earlier, *ATP7A* is not normally expressed in the liver and hepatic copper export is controlled by ATP7B. Higher expression of *ATP7A* could lead to the increased export of copper from the liver into the bloodstream. The liver had previously not been known as a copper storage organ or to have an ATP7A export mechanism. Interestingly *ATP7A* expression was also much higher than normal in the intestine of cardiac copper deficient mice suggesting a second feedback mechanism for increasing serum copper content (Kim *et al.*, 2010). In addition, serum from cardiac copper deficient mice induced *ATP7A* expression in human primary umbilical vein endothelial cells (HUVECs) (Kim *et al.*, 2010). This demonstrates that the serum itself may contain a signaling molecule which is

inducing the expression of *ATP7A* and is the first evidence of systemic regulation of copper homeostasis.

1.4 The regulation of cellular copper homeostasis

Copper homeostasis is strictly regulated to maintain the tight balance between requirement and toxicity. Regulation of copper homeostasis can be either by transcriptional changes, post-translational changes or protein-protein interactions.

1.4.1 Copper-induced transcriptional regulation

Whilst post-translational modification of copper transporters has been demonstrated successfully, identifying transcriptional regulators has remained relatively elusive. Apart from the previously introduced MTF-1, whole genome cDNA microarrays have not been successful in discovering copper dependent transcription factors (Huster *et al.*, 2007; Muller *et al.*, 2007). However, the copper chaperone Atox1 has recently been also classified as a copper-dependent transcription factor (Itoh *et al.*, 2008). This section will discuss the roles of both MTF-1 and Atox1 in regulating copper homeostasis.

1.4.1.1 MTF-1 transcriptional regulation

First discovered in yeast, the major transcriptional regulator of heavy metal load is MTF-1. Mammalian MTF-1 consists of six zinc-finger domains and can be directly activated by zinc (Zhang *et al.*, 2003). Other metals such as copper and cadmium indirectly activate MTF-1 by displacing metallothionein-bound zinc into the cytoplasm (Zhang *et al.*, 2003). MTF-1 is normally localized in the cytoplasm but under heavy metal load shifts to the nucleus to drive transcription of MT-I, II and IV (Saydam *et al.*, 2001). Copper-induced activation of MTF-1 may be due to phosphorylation as inhibitors of protein kinase C can block induction (Saydam *et al.*, 2002). *Drosophila* MTF-1 differs from the mammalian version, as it can be activated by both copper excess and deficiency. When copper is in excess *Drosophila* MTF-1 drives expression of the *metallothionein*

genes, but low copper levels trigger expression of the secondary uptake gene, *Ctr1B* (Selvaraj et al., 2005).

1.4.1.2 Atox1 may also be a copper-dependent transcription factor

Atox1 is known as a copper chaperone required for the transfer of copper to Cu-ATPases for export, but recently a novel function for the protein has been established. Atox1 has been demonstrated as a copper-dependent transcription factor driving expression of the cell proliferation gene *Cyclin D1* (*Ccnd1)* (Itoh et al., 2008). Copper has been shown to stimulate cell proliferation, but in *Atox*$^{-/-}$ mouse fibroblast cells proliferation was not increased and *Ccnd1* expression dropped (Itoh *et al.*, 2008). Mobility shift and chromatin immunoprecipitation (ChIP) assays were used to establish that Atox1 binds and activates the promoter of *Ccnd1* in the presence of copper (Itoh *et al.*, 2008). These results imply that copper stimulates Atox1 to drive transcription of *Ccnd1* and subsequently increase cell proliferation. Whether Atox1 acts as a chaperone or transcription factor may be determined by its cellular localization and/or the protein forming a homodimer (Muller and Klomp, 2009).

1.4.2 Post translational regulation of the trafficking of copper transporters

1.4.2.1 Post translational copper dependent trafficking of Cu-ATPases

The majority of copper homeostasis regulation mechanisms discovered in the mammalian system are posttranslational (van den Berghe and Klomp, 2009). Key copper transporters hCtr1, ATP7A and APT7B can be regulated by posttranslational mechanisms such as direct protein interactions or phosphorylation (van den Berghe and Klomp, 2009). As discussed earlier, the cellular localization of ATP7A and ATP7B is crucial for determining function. At basal cellular copper levels both proteins localize to the TGN yet under elevated copper conditions, ATP7A traffics to the outer plasma (basolateral) membrane and ATP7B traffics to vesicles adjacent to the canalicular (apical) membrane (Guo *et al*, 2005, Monty *et al*, 2005). When copper concentrations are restored to basal levels, both Cu-ATPases return to the TGN. Localization of the transporters is not static

and the proteins are constantly cycled between the TGN, endocytic or transport vesicles and apical/basolateral membranes (Lutsenko *et al.*, 2007).

A key component in understanding the copper stimulated trafficking of the Cu-ATPases was determining whether copper is triggering the release of the proteins from the TGN or preventing retrograde transport from the membrane. Experiments using a myc-tagged ATP7A suggest that copper stimulates trafficking away from the TGN (Petris and Mercer, 1999). The myc-tag was in an extracellular loop of ATP7A and could only bind antibody when at the plasma membrane. Under increased copper conditions some myc-ATP7A was still found at the TGN suggesting that transport back from the outer plasma membrane was not impaired (Petris and Mercer, 1999). Despite understanding the basic concepts of copper stimulated trafficking of Cu-ATPases, the mechanisms involved are not completely clear. What is known is that the process is complex and may involve several factors including copper binding, catalytic activity, targeting motifs, protein interactions and phosphorylation.

1.4.2.1.1 Copper binding and catalytic activity can influence localization

Copper induced trafficking of Cu-ATPases can be influenced by both copper binding and the ATP hydrolysis cycle. As discussed earlier, copper initially binds to MBDs found at the N-terminus of ATP7A/B and deletion studies determined that MBD5/6 in particular are required for copper transport (Cater *et al.*, 2004; Yatsunyk and Rosenzweig, 2007). Interestingly inactivation of both domains also resulted in inhibition of copper-induced trafficking, whereas truncation of MBD1-4 did not have the same effect (Cater *et al.*, 2004; Guo *et al.*, 2005). In a similar vein, mutating the CPC motif, the key copper binding motif in the transmembrane domain, also results in inhibition of trafficking (Petris *et al.*, 2002; Mercer *et al.*, 2003). Therefore copper binding in both the MBDs and CPC motif strongly correlates with copper-induced trafficking.

After binding copper, Cu-ATPases require the catalytic activity of ATP hydrolysis to transfer the metal into the extracellular space (Kuhlbrandt, 2004). ATP hydrolysis is

controlled by P, N and A domains (Kuhlbrandt, 2004). ATP binds to the N-domain triggering the donation of the γ-phosphate to an aspartic acid in the P-domain. This is followed by transient acyl-phosphorylation of the aspartic acid, then subsequent dephosphorylation and copper transport (Kuhlbrandt, 2004; Veldhuis *et al.*, 2009). The A-domain contains the conserved TGE motif required for dephosphorylation (Barry *et al.*, 2010) and interestingly mutations in this motif restore trafficking to the outer plasma membrane in MBD and CPC mutants (Cater *et al.*, 2004; Barry *et al.*, 2010). TGE motif mutations prevent dephosphorylation, maintaining the Cu-ATPase in the transient acyl-phosphorylation state leading to a loss of copper transport function but restoring trafficking (Petris *et al.*, 2002; Cater *et al.*, 2004; Lutsenko *et al.*, 2007). This restored trafficking is independent of copper binding but can be used to help describe the mechanism of copper-induced trafficking (Petris *et al.*, 2002; Cater *et al.*, 2004; Lutsenko *et al.*, 2007). Increased intracellular copper levels and subsequent increase in copper binding to the Cu-ATPases may maintain the protein in the transient acyl-phosphorylation state (Veldhuis *et al.*, 2009). This may result in trafficking to the outer plasma membrane, copper export and then dephosphorylation. Cu-ATPases maintained in the transient acyl-phosphorylation state may be more susceptible to trafficking mechanisms.

1.4.2.1.2 Targeting motifs of Cu-ATPases

In the Cu-ATPases, targeting motifs have been identified which are important for trafficking to / retention at the TGN and outer plasma membrane (Figure 1.2). Under increased copper conditions ATP7A/B traffic to opposing membranes and as such each has different targeting motifs. ATP7B contains a specific nine amino acid sequence in the N-terminus that is required for apical targeting (Braiterman *et al.*, 2009). The sequence is required to retain the protein at the TGN of polarized hepatic cells in basal conditions and for apical targeting in increased copper conditions (Braiterman *et al.*, 2009). How this apical signal targets ATP7B in increased copper conditions remains to be identified but it has been hypothesised to bind to a unknown trafficking protein (Braiterman *et al.*, 2009). ATP7A does not contain the apical targeting motif, but rather a

basolateral targeting signal in the C-terminus (Greenough *et al.*, 2004). In polarized Madin-Darby Canine Kidney (MDCK) cells, removal of the targeting signal (a di-leucine) results in apical localization rather than basolateral (Greenough *et al.*, 2004). In addition, ATP7A also interacts with PDZ domain trafficking proteins (named after the first proteins identified with the domain; Post synaptic density protein, Drosophila disc large tumor suppressor and Zonula occludens-1 protein) (Stephenson *et al.*, 2005). PDZ domain proteins are typically required for the membrane retention of proteins in various sub cellular locations. The PDZ domain protein AIPP1 has been identified to interact with the ATP7A C-terminus PDZ targeting signal (Stephenson *et al.*, 2005).

Cu-ATPases also have targeting motifs responsible for the return of the proteins back to the TGN as basal copper levels are restored. ATP7B contains a leucine residue near the boundary of TM8 and the C-terminal tail that is required for retention at the TGN (Braiterman *et al.*, 2011). Mutations in this residue (leucine to proline or arginine) result in the protein leaving the TGN and becoming degraded (Braiterman *et al.*, 2011). At the opposite end of the C-terminus, a tri-leucine motif is required for retrograde transport back to the TGN after copper-induced trafficking (Braiterman *et al.*, 2011). Interestingly in ATP7A the same di-leucine basolateral membrane signal, is responsible for retrograde transport to the TGN (Petris *et al.*, 1998). Mutations in the di-leucine motif inhibit the endocytic return of the protein from the plasma membrane to the TGN (Petris *et al.*, 1998; Petris and Mercer, 1999). This suggests that the signal read from the di-leucine motif is dependent on ATP7A sub-cellular localization. There is evidence to suggest that di-leucine motifs in other membrane proteins, such as LIMPII and GLUT4, could also be differentially read dependent on protein sub-cellular localization and structural changes (Sandoval *et al.*, 2000; Greenough *et al.*, 2004).

1.4.2.1.3 Retrograde transport of APT7A involving endocytosis

How ATP7A returns from the basolateral membrane to the TGN when basal copper levels are restored is yet to be discovered. It has been suggested that the process may involve endocytic removal of ATP7A from the basolateral membrane. The endocytic

pathway consists of membrane compartments that can internalize molecules and cycle them back and forth between outer plasma membrane and TGN. The process could involve several regulator proteins such as clathrin and the Rab proteins.

ATP7A retrograde transport is dependent on a C-terminus di-leucine motif indicating the involvement of clathrin-mediated endocytosis (Lane *et al.*, 2004). Clathrin is a coating protein involved in vesicle trafficking and di-leucine motifs can bind to clathrin-adaptor complexes at both the TGN and basolateral membrane (Dietrich *et al.*, 1997). Contradicting this assumption, when clathrin-mediated endocytosis was blocked in HeLa cells, ATP7A was still able to transport back from the basolateral membrane (Lane *et al.*, 2004). Treating these cells with hypertonic sucrose, which blocks both clathrin-mediated and fluid-phase endocytosis, was able to block ATP7A retrieval from the basolateral membrane (Lane *et al.*, 2004). This lead to the suggestion that both clathrin dependent and independent endocytosis may be involved in the retrograde transport of ATP7A (Lane *et al.*, 2004).

Rab proteins have also been suggested as candidates to be involved in endocytic trafficking of ATP7A. The Rab proteins are members of the Ras-like GTPase family that regulate vesicle trafficking (Zhang et al., 2007). Many of the Rab proteins play important roles in different stages of the endocytic pathway (such as Rab5 with early endosomes and Rab7 in late endosomes). Immunostaining experiments have shown that at high copper conditions, Rab7 co-localizes with ATP7A in Chinese Hamster Ovary (CHO) cells (Pascale *et al.*, 2003). At basal copper levels, APT7A co-localizes with Rab5 at early endosomes (Pascale *et al.*, 2003). Whether or not this implies the Rab proteins are involved in endocytic removal of APT7A from the basolateral membrane remains to be elucidated.

1.4.2.1.4 Copper-induced trafficking can also be linked to kinase-mediated phosphorylation

Kinase-mediated phosphorylation can regulate protein function, stability and localization. Phosphorylation of the Cu-ATPases has been shown *in-vitro* and increases under elevated copper levels (Vanderwerf *et al.*, 2001; Voskoboinik *et al.*, 2003). As elevated copper levels also stimulate post TGN trafficking of the Cu-ATPases, the trafficking process may be regulated by phosphorylation. Phosphopeptide mapping studies on ATP7A discovered 28 sites for phosphorylation across both the cytosolic N and C-termini (Veldhuis *et al.*, 2009). The C-terminus contained 8 sites regulated by copper and in close proximity to previously discussed trafficking signals (Veldhuis *et al.*, 2009). Mutagenesis of one of these copper regulated phosphorylation sites resulted in mis-localization of ATP7A in high copper medium (Veldhuis *et al.*, 2009). The kinase/s involved in phosphorylation of APT7A or ATP7B are yet to be identified but the process appears to be a key regulating factor in copper-induced trafficking.

1.4.2.2 Copper dependent recycling of hCtr1

hCtr1 can shift between an outer plasma membrane localization and intracellular organelles located in the perinuclear region (Klomp *et al.*, 2002). Using cell surface biotinylation the majority of hCtr1 was found to localized at the outer plasma membrane and a small portion internalized (Molloy and Kaplan, 2009). hCtr1 was increasingly internalized as extracellular copper levels rose (Molloy and Kaplan, 2009). The mechanism of copper dependent recycling of hCtr1 remains elusive but the process is seen as a physiologically relevant as it can regulate copper import from the extracellular space.

1.4.3 Protein-protein interactions regulating degradation of the Cu-ATPases

Recently research into the regulation of ATP7A and ATP7B has focused on interactions between the Cu-ATPases and other proteins. Discovering what protein partners interact with the Cu-ATPases has lead to an increased understanding of copper binding, protein trafficking and protein degradation. Understanding these interactions can help unravel to pathology of ATP7A/B-related disease. This section will elaborate on two partner proteins linked to Cu-ATPase degradation, COMMD1 and Clusterin.

1.4.3.1 COMMD1 regulates proteolysis of ATP7B

Reports of copper accumulation in the livers of Bedlington terriers (dogs) lead to the discovery of a protein capable of regulating copper homeostasis. The condition was mapped to the loss of a gene encoding Copper Metabolism MURR1 Domain 1 (COMMD1) (van De Sluis *et al.*, 2002). Cultured hepatic cells (HEK293T cells) suppressing *COMMD1* accumulated copper and co-immunoprecipitation analysis identified a direct interaction between COMMD1 and the amino terminus of ATP7B. This implied that a COMMD1/ATP7B interaction was required for copper export out of hepatic cells (Tao *et al.*, 2003; Burstein *et al.*, 2004; Spee *et al.*, 2007).

Interestingly, Wilson's disease-causing mutations increased the amount of ATP7B interacting with COMMD1 (de Bie *et al.*, 2007). These mutations also lead to the mis-localization of ATP7B to the endoplasmic reticulum, often a destination for degraded proteins (Meusser *et al.*, 2005). Together these results indicated that increased COMMD1 binding lead to enhanced ATP7B proteasomal degradation and development of Wilson's disease. Therefore endogenous COMMD1 would be required to degrade poorly synthesized ATP7B and prevent that mis-functioning protein causing copper accumulation. This theory was further supported by the fact that over-expression of COMMD1 resulted in increased proteolysis of newly synthesized ATP7B (de Bie *et al.*, 2007).

COMMD1 has been shown to have a similar role as a negative regulator of protein stability in the HIF-1 and NF-κB pathways and interacts with many subunits of the E3-ubiquitin ligase complex implying a role in ubiquitin related degradation (van de Sluis *et al.*, 2007; van de Sluis *et al.*, 2009). COMMD1 also interacts with ATP7A and in contrast to the ATP7B interaction has recently been shown to improve ATP7A stability (de Bie *et al.*, 2007; Materia et al, 2011; Vonk *et al.*, 2011)

1.4.3.2 Clusterin regulates lysosomal degradation of ATP7B

A yeast two-hybrid screen identified that the chaperone protein, Clusterin, interacts with ATP7B (Lim *et al.*, 2006). Clusterin was further demonstrated via co-immunoprecipitation to bind to both ATP7A and ATP7B (Materia *et al.*, 2011). Similar to COMMD1, these interactions increased when the Cu-ATPases were unstable and misfolded (Materia *et al.*, 2011). Studies using the same ATP7B mutants as in the COMMD1 investigation demonstrated that Clusterin targeted degradation of the protein was via the lysosomal rather than the proteasomal pathway (Materia *et al.*, 2011). This suggested that ATP7B could be degraded by either the proteasomal (COMMD1) or lysosomal (Clusterin) pathway. Interestingly, Clusterin can also promote ubiquitination of COMMD1 leading to proteasomal degradation (Zoubeidi *et al.*, 2010). This shows that Clusterin is capable of targeting degradation via either pathway but also highlights the potential complexity of ATP7B degradation. Nonetheless, both Clusterin and COMMD1 have demonstrated that Cu-ATPase function can be regulated by partner proteins that target degradation.

1.5 *Drosophila* as a model for copper homeostasis research

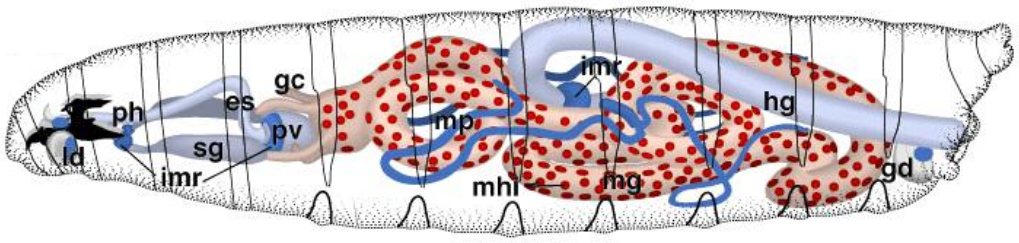
Most research into the mechanisms of copper homeostasis makes use of human cell culture or mammalian model experimental systems. Both systems are very useful for studying copper transport but have disadvantages and limitations. Human cell culture

experimental systems, such as Caco2 or MDCK, use cells that are derived from cancers with substantial changes in gene expression in comparison to normal cells and cannot be judged as a true *in vivo* system. Mammalian model systems are expensive with animals having long life cycles and maintenance costs. An exciting alternative *in vivo* model for studying copper transport is *Drosophila melanogaster* (referred to herein as *Drosophila*).

Drosophila possesses many key qualities that have lead researchers around the world to choose it as a model organism. Advantages can include a very short life cycle (approximately two weeks), ease of growth and maintenance, a large community of researchers and a full sequenced genome (Ryder and Russell, 2003). *Drosophila* genetics includes useful techniques such as the generation of mutants via *P-element* excision, gene trapping, the *GAL4-UAS* system and RNAi suppression (Ryder and Russell, 2003). Despite the disadvantage of being evolutionarily distant, *Drosophila* can still be regarded as useful model for studying human genetics (Ryder and Russell, 2003).

1.5.1 Investigating tissue specific function in *Drosophila*

One of the most useful techniques associated with research in *Drosophila* is the *GAL4-UAS* system. This two-part system allows for investigation into tissue specific copper homeostasis mechanisms. This work will focus on exploring the *Drosophila* midgut, whilst also investigating the eye and thorax. *Drosophila* contains a midgut region which is visible in the developing larvae and easily accessible via dissection (Figure 1.7). Despite the simplicity of the *Drosophil*a gut, there are still a number of specialized cell types which can be correlated to mammalian counterparts. In *Drosophila* larvae, food enters through the mouthparts before passing into the pharynx and esophagus. The passage continues into the foregut region, beginning at the gastric caeca, and then onto the midgut. The midgut region is the major site of nutrient absorption and contains the copper cells which are the major site of copper accumulation, as shown by the induction of orange fluorescence after copper feeding (Dubreuil, 2004). Acting as the *Drosophila* equivalent of the kidney, excretion is carried out by two pairs of closed tubules known as the malpighian tubules (Wessing and Eichelberg, 1978; Jung *et al.*, 2005). Solutes



Foregut / Midgut Hindgut Malpighian tubules

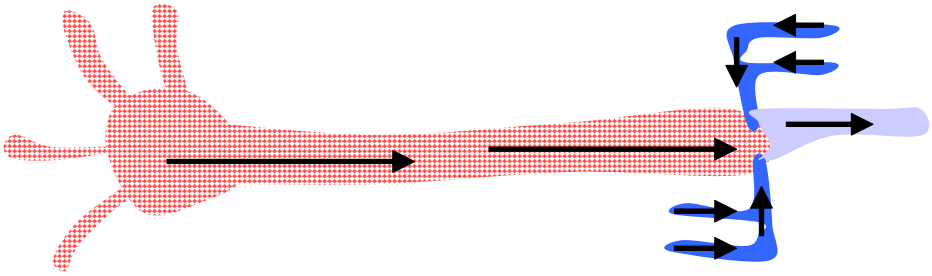


Figure 1.7: The Drosophila midgut region

The *Drosophila* midgut region is visible in developing third instar larvae and is easily dissectible. Larvae feed using mouthparts and the passage of food enters the gut region via the proventriculus. Nutrient absorption occurs in the midgut region and excretion via the hindgut. The malpighian tubules are the *Drosophila* equivalent of the kidney and excrete solutes into the hindgut.

The image is modified from the Atlas of *Drosophila* development.

collect in the distal regions of the tubules and drain towards the renal discharge duct in the hindgut (Wessing and Eichelberg, 1978; Jung *et al.*, 2005). Sheets of monolayers known as the fat bodies have been suggested to play a similar function to that of the liver.

1.5.2 *Drosophila* copper homeostasis

Research into copper transport in *Drosophila* is aided by strong conservation of the key homeostasis mechanisms across eukaryotes (Table 1.2). *Drosophila* has one sole efflux protein DmATP7, which was identified by conservation of key motifs from ATP7A and ATP7B (Southon *et al.*, 2004; Norgate *et al.*, 2006). Homology studies discovered that *Drosophila* have three members of the Ctr1 family, known as *Ctr1A*, *Ctr1B* and *Ctr1C* (Zhou et al., 2003). Recent studies on *Malvolio*, the homologue of *DMT1*, has initiated research into other modes of uptake (Southon et al., 2008). Putative orthologues for key members of the chaperone network have also been identified, including *Atox1*, *CCS*, *Sco1* and *Sco2* (Southon et al., 2004).

1.5.2.1 Ctr mediated copper uptake in Drosophila

In *Drosophila* there are three proteins (Ctr1A, Ctr1B and Ctr1C) which belong to the Ctr family associated with copper uptake. The *Drosophila* Ctr proteins were identified on the basis of key structural characteristics such as three transmembrane spanning domains and conserved methionine residues in both the N-terminus and second transmembrane domain (Zhou *et al.*, 2003). The strong conservation of copper homeostasis mechanisms between mammals and *Drosophila* has been demonstrated by the ability of hCtr1 to compensate for the loss of *Ctr1A* in flies (Hua *et al.*, 2009). The multiple Ctr proteins of *Drosophila* were confirmed to transport copper by complementing a yeast mutant deficient in high affinity copper uptake (Zhou *et al.*, 2003). Ctr1A and Ctr1B, but not Ctr1C, can also stimulate copper uptake in cultured *Drosophila* S2 cells
(Zhou *et al.*, 2003; Southon *et al.*, 2004).

Table 1.2: The conservation of Human and *Drosophila* copper homeostasis genes

Protein Function	Human	*Drosophila*
Copper Import	*hCtr1*	*Ctr1A* (CG3977) *Ctr1B* (CG7459) *Ctr1C* (CG15551)
Copper Export	*ATP7A* *ATP7B*	*DmATP7* (CG1886)
Chaperone for the Cu-ATPases	*Atox1*	*dAtox1* (CG32444)
Chaperone for SOD1	*CCS*	*dCCS* (CG17753)
Chaperones for cytochrome *c* oxidase	*Cox17* *Sco1* *Sco2*	*Cox17* (CG9065) *Sco1 (Scox)* (CG8885)
Copper sequestration	*Mtn* (10 members)	*MtnA* (CG9470) *MtnB* (CG4312) *MtnC* (CG5097) *MtnD* (CG3743)

There is a high level of conservation of copper homeostasis genes between humans and *Drosophila melanogaster (Drosophila).*

1.5.2.1.1 Ctr1A is the primary source of copper uptake

Expression of the two subtle splice variants of *Ctr1A* (207 and 217 amino acids long respectively) is ubiquitous throughout embryogenesis (Zhou *et al.*, 2003). Loss of *Ctr1A* expression is lethal with mutant larvae dying in the first or second instar of larval development (Turski and Thiele, 2007). *Ctr1A* null mutants also have reduced mouthpart pigmentation, reduced cuproenzyme activity, heart beat defects and reduced total copper content (Turski and Thiele, 2007). *Ctr1A* null mutants were rescued to adult viability by increases in dietary copper yet rescued mutants still showed signs of copper deficiency such as hypopigmentation (Turski and Thiele, 2007). Furthermore, tissue specific loss of *Ctr1A* in the eye and thorax/abdomen via RNAi suppression results in copper deficient phenotypes (Binks *et al.*, 2010). Specific loss of *Ctr1A* in the eye results in a flat, sunken eye phenotype (Binks *et al.*, 2010). Specific loss of *Ct1A* in the thorax/abdomen results in copper-deficient hypopigmentation (Binks *et al.*, 2010). Ctr1A localizes predominantly to the apical membrane in cultured *Drosophila* cells, embryos and third instar larval tissues (salivary glands and epithelial cells) (Turski and Thiele, 2007). Loss of function and localization analysis suggest that Ctr1A is the primary copper uptake protein of *Drosophila*

1.5.2.1.2 Ctr1B is required when copper is scarce and in excess

Ctr1B is 174 amino acids long and is expressed mainly in the intestine of *Drosophila. Ctr1B* is only highly expressed during late embryonic stages or larval development and its expression is activated during times of copper scarcity and down-regulated in times of overload (Zhou *et al.*, 2003). Transcriptional regulation of *Ctr1B* is controlled by the MTF-1 transcription factor (to be further discussed in a later section). $Ctr1B^{-/-}$ mutants are viable in normal conditions but die when copper is either scarce or in excess (Zhou *et al.*, 2003). Lethality when copper is scarce demonstrates that Ctr1B is required for uptake when copper levels are low. However lethality when copper is in excess implies that Ctr1B is required as a detoxification mechanism (Zhou *et al.*, 2003). This role of the Ctr1B is yet to be elucidated but may involve a second intracellular function (Zhou *et al.*,

2003). Heterozygous *Ctr1B* mutants are viable in all conditions but when copper is scarce show pigment defects associated with copper deficiency (Zhou *et al.*, 2003). There are no phenotypic changes associated with tissue specific loss of *Ctr1B* in the eye and suppression in the thorax /abdomen only exacerbates the *Ctr1A* suppression phenotype (Binks *et al.*, 2010). Notably, ectopic expression of *Ctr1B* in the eye results in a mild copper toxicity rough eye phenotype (worsened by increased dietary copper) (Balamurugan *et al.*, 2007; Binks *et al.*, 2010). In agreement with results from functional experiments, Ctr1B localizes to the apical membrane of larval gut cells and does not internalize under increased copper conditions (Balamurugan *et al.*, 2007). Ctr1B appears to be a more efficient, secondary source of copper uptake that is only required for larval development in high or low copper environments.

1.5.2.1.3 Ctr1C has a role in male fertility

The remaining member of the family, Ctr1C, is 270 amino acids long and is expressed exclusively in the male germline (specifically maturing spermatocytes and sperm) (Zhou *et al.*, 2003; Steiger *et al.*, 2010). Ctr1C is a copper transporter, as ubiquitous over-expression of the protein can rescue *Ctr1A* null mutants, contradicting earlier work which showed that Ctr1C could not stimulate copper uptake in cultured *Drosophila* S2 cells (Zhou *et al.*, 2003; Southon *et al.*, 2004). Ctr1C null mutants are viable, fertile and show no sensitivity to changing copper levels (Zhou *et al.*, 2003). When reared in a $Ctr1B^{-/-}$ background, *Ctr1C* null mutant males are infertile indicating that when there is a systemic copper scarcity, Ctr1C is required for import into maturing spermatocytes and that copper has an important role in male fertility.

1.5.2.2 Other sources of copper uptake in *Drosophila*

Drosophila is also a useful system to investigate Ctr1-independent copper uptake pathways. A potential candidate is *Malvolio*, the *Drosophila* homologue of DMT1 which has been suggested as an alternative copper uptake mechanism in mammals (Arredondo

et al., 2003; Southon *et al.*, 2008). *Malvolio* is expressed only in the larval malpighian tubules, testis, brain and alimentary canal indicating a tissue specific role (Folwell *et al.*, 2006). As discussed earlier, knockout of *Malvolio* resulted in flies sensitive to both copper excess and limitation. It was proposed that *Malvolio* mutants were sensitive to copper limitation due to impaired uptake and sensitive to copper excess due to reduced transport to detoxification tissues (Southon *et al.*, 2008). Malvolio localizes to the cytoplasm, the outer plasma membrane and punctate vesicles suggesting a role in uptake and detoxification may be possible (Folwell *et al.*, 2006). Despite additional evidence demonstrating that Malvolio can transport copper into *Drosophila* cultured S2 cells, a specific role in copper uptake remains to be shown (Southon *et al.*, 2008).

1.5.2.3 DmATP7 is the *Drosophila* Cu-ATPase

Drosophila, like other lower multicellular organisms, has only one copper efflux protein which is known as DmATP7 and is the sole orthologue of the human *ATP7A* and *ATP7B* (Southon et al., 2004). DmATP7 shares 54% sequence identity with the human Cu-ATPases, sharing key motifs including the N-terminus MBDs (only four compared to six in ATP7A/B), eight transmembrane spanning domains and ATP hydrolysis domains (Southon *et al.*, 2004). DmATP7 function was first linked to that of its mammalian orthologue using suppression analysis in *Drosophila* cultured S2 cells which resulted in cellular copper accumulation and implied an export role (Southon *et al.*, 2004).

1.5.2.3.1 Expression of *DmATP7*

DmATP7 is strongly expressed in embryonic, larval and adult life stages of *Drosophila* (Norgate *et al.*, 2006; Burke *et al.*, 2008). Embryonic expression of *DmATP7* was investigated using an ATP7A-antibody which cross reacts with the *Drosophila* homologue (Norgate *et al.*, 2006). During early embryogenesis *DmATP7* is expressed at the plasma membrane of all cells and becomes more cytoplasmic as the embryo ages

(Norgate *et al.*, 2006). When embryos are aged at around four hours, *DmATP7* expression is seen in the developing tracheal dorsal trunk (Norgate *et al.*, 2006).

Larval and adult expression has been investigated by creating a reporter-*GAL4* construct using the *DmATP7* promoter region (Burke *et al.*, 2008). During larval development *DmATP7* is expressed in the larval brain, peripheral nervous system, salivary glands, male gonads, malpighian tubules and the gut region (Burke *et al.*, 2008). Interestingly larval midgut and malpighian tubule expression of *DmATP7* is up-regulated under increased copper conditions (Burke *et al.*, 2008). This copper-induced change in expression requires the known copper transcription factor, MTF-1 (Burke *et al.*, 2008). A role for MTF-1 in mammalian Cu-ATPase transcriptional regulation has yet to be identified; however there has been evidence of copper induced transcription in the gut region (Bauerly *et al.*, 2005). The adult midgut also showed *DmATP7* expression suggesting a constant copper efflux role throughout development (Burke *et al.*, 2008). In addition, adult neuronal cells of the head, wings and legs also have strong *DmATP7* expression (Burke *et al.*, 2008). The role for DmATP7 in the nervous system of *Drosophila* is still under investigation.

1.5.2.3.2 Loss of *DmATP7* prevents function at both the TGN and outer plasma membrane

In vitro analysis of *DmATP7* suppression in cultured *Drosophila* S2 cells by RNAi interference resulted in an increase in copper accumulation (Southon *et al.*, 2004). Generation of a $DmATP7^{-/}Y$ mutants resulted in larvae that are more lethargic, have smaller and less pigmented mouthparts and eventually die before reaching the second instar of development (Norgate *et al.*, 2006). Viability could not be restored by increasing or decreasing copper levels (Norgate *et al.*, 2006). The transcript levels of copper responsive genes in the mutant larvae suggested copper was accumulating in intestinal cells before death (Norgate *et al.*, 2006). This suggests that DmATP7, like ATP7A, is required for the efflux of dietary copper from the intestine. In order to further investigate the mutant phenotype, mosaics were created with patches of cells

homozygous for the loss of *DmATP7* (Norgate et al., 2006). These cells were morphologically normal except for a severe loss of pigment implying that DmATP7, like both human Cu-ATPases, functions at the TGN to provide copper for cuproenzymes such as tyrosinase (Norgate *et al.*, 2006).

Tissue specific loss of *DmATP7* gives further evidence for its role in copper export at the TGN and outer plasma membrane. Targeted suppression of *DmATP7* in the thorax/abdomen using RNAi results in copper specific hypopigmentation (Binks *et al.*, 2010). This loss of pigment cannot be rescued by increased uptake, suggesting a loss of copper export to the secretory pathway (Binks *et al.*, 2010). Suppression of *DmATP7* in the eye produces no phenotype alone suggesting minimal requirement for the protein in eye development (Binks *et al.*, 2010). DmATP7 may, however, be required in the eye in times of elevated copper uptake as its suppression can exacerbate the rough eye caused by ectopic expression of *Ctr1B* (Binks *et al.*, 2010). Suppression of *DmATP7* in the midgut using a *Sym-PUAST-DmATP7* RNAi silencing construct resulted in a reduction in viability on low copper media and decreased copper content in the brain (Bahadorani *et al.*, 2010). This further implies a role for DmATP7 in intestinal copper export.

1.5.2.3.3 Cellular copper deficiency as a result of ectopic expression of *DmATP7*

Ectopic expression of *DmATP7* specifically in the thorax/abdomen results in hypopigmentation, bristle loss, reduction of the scutellum and a small thoracic cleft (Norgate *et al.*, 2006). These phenotypes can be rescued by increasing intracellular copper levels by ectopically expressing uptake genes for thorax/abdomen expression (Norgate *et al.*, 2006). This indicates that ectopic expression of *DmATP7* is causing a cellular copper deficiency leading to reduced delivery to the secretory pathway (Norgate *et al.*, 2006). Endogenous DmATP7 would still be active at the TGN and ectopic DmATP7 may be forced to the outer plasma membrane causing increased efflux leading to a cellular copper deficiency (Norgate *et al.*, 2006). Additional phenotypes observed in the thorax/abdomen such as bristle loss and reduced scuttellum may be a result of low copper delivery to other cuproenzymes caused by the cellular copper deficiency. Ectopic

expression of *DmATP7* in the eye has no harmful effect on eye development but can rescue the rough eye caused by *Ctr1B* ectopic expression (Binks *et al.*, 2010). This demonstrates that DmATP7 can also cause copper export in the eye even if this is not normally required.

1.5.2.3.4 Localization of DmATP7

As DmATP7 shares functional similarity with mammalian ATP7A/B, the *Drosophila* orthologue was also expected to localize to the TGN and outer plasma membrane. In cultured *Drosophila* S2 cells, DmATP7 predominantly localized to the TGN with some protein found at the outer plasma membrane (Southon *et al.*, 2010). This localization was consistent under both basal and elevated copper conditions (Southon *et al.*, 2010). Although not observed, it was assumed DmATP7 could shift between the TGN and the outer plasma membrane locations. Interestingly, when incorporated with the mammalian trafficking machinery, DmATP7 could undergo copper induced translocation to the outer plasma membrane. DmATP7 transfected into *ATP7A*$^{-/-}$ Me32a human fibroblast cells was found to traffic to the outer plasma membrane when excess copper was added (Southon *et al.*, 2010).

DmATP7 localization and its ability to traffic were further explored *in vivo*. using an anti-ATP7A antibody in early *Drosophila* embryogenesis, , DmATP7 was found to localize to the plasma membrane of all cells (Norgate *et al.*, 2006). As the antibody was no longer sufficiently specific in later life stages, an EGFP (Enhanced Green Fluorescent Protein)-tagged UAS-expression construct for DmATP7 was used to investigate localization in third instar larvae (Burke *et al.*, 2008). When driven in its endogenous expression domain using *DmATP7-GAL4*, the fusion protein localized to the basolateral membrane of midgut cells (Burke *et al.*, 2008). This localization could not be shifted by growing the larvae on copper supplemented food. DmATP7 localization is consistent with that of ATP7A/B and although the protein is capable of trafficking in mammalian cells there is not yet any conclusive evidence of this occurring in the fly.

1.5.2.4 Copper chaperones proteins in *Drosophila*

Despite the lack of research into *Drosophila* copper chaperones, fly orthologues of mammalian Atox1, CCS and members of the Cox network of proteins (Sco1 & Cox17) have been identified based on sequence similarity. In cultured *Drosophila* S2 cells no transcriptional responses to increased copper were observed from any of the putative chaperone orthologues (Southon *et al.*, 2004). However *Drosophila* with chromosomal deletions containing these genes have been shown to be sensitive to supplemented copper media (Norgate *et al.*, 2007).

dAtox1 and dSco1 (Scox) have been further investigated by mutant analysis and tissue specific suppression. *dAtox*$^{-/-}$ mutants die at the third instar larval stage when raised on low copper media (Hua *et al.*, 2011). Analysis of these mutant third instar larvae determined that there was no increase in *Ctr1B* expression on low copper media but rather an increase in *MetalliothioneinB* expression in the midgut (Hua *et al.*, 2011). These results implied that loss of Atox1 resulted in less copper export from the midgut likely caused by inadequate delivery to DmATP7. *Scox* $^{-/-}$ mutants are larval lethal and site-specific mutants in the promoter region had phenotypes associated with motor dysfunction and female sterility indicating that the Cox network is not functioning properly but not necessarily as a consequence of impaired copper transport (Porcelli *et al.*, 2010). Suppression of *Scox* in a tissue-specific manner in the thorax and abdomen resulted in mild thoracic cleft, reduction in scutellum and thin bristles (Binks *et al.*, 2010). This phenotype is similar to *DmATP7* ectopic expression (an intracellular copper deficiency phenotype) but without the hypopigmentation phenotype normally seen. This suggests that the phenotypic similarities are likely to be caused by loss of copper transport specifically to the mitochondria. This thesis also investigates CutC, a putative copper chaperone previously implicated in *E.coli* copper homeostasis (Gupta *et al.*, 1995). Mis-expression of *Drosophila* CutC has been shown to influence loss of *MTF-1* viability phenotypes (unpublished data from the Burke laboratory). The protein localizes to the nucleus and where it is speculated to interact with MTF-1.

1.5.2.5 Investigating the regulation of copper homeostasis in *Drosophila*

Regulation of copper homeostasis in *Drosophila* is an exciting new area for research as the fly is a unique system to explore both transcriptional and post-translational mechanisms. *Drosophila* MTF-1-mediated transcriptional regulation is well understood but whether post translational modification is also important remains unclear. Two interesting areas of research into the regulation of mammalian Cu-ATPases are intracellular trafficking and protein stability / degradation. The role of both processes in the regulation of DmATP7 remains to be elucidated.

1.5.2.5.1 The *Drosophila* endocytic pathway and dRab5

The importance of localization of Cu-ATPases to their function is highlighted by research into ATP7A/B trafficking in mammals. The trafficking process is complex and involves factors including copper binding, targeting motifs, protein-protein interactions, phosphorylation and the endocytic pathway. DmATP7 trafficking is yet to be established in vivo however functional analyses suggest roles for the protein at both the TGN and outer plasma membrane. Endocytosis is a process which can internalize membrane compartments, nutrients or membrane associated proteins (Huotari and Helenius, 2011). The process can be clathrin dependent or independent and is commonly a route for proteins between the outer plasma and TGN membrane. Endocytic transport begins with membrane invaginations forming from the plasma membrane and these vesicles maturing into early and then late endosomes (Huotari and Helenius, 2011). Vesicles can then either continue to the TGN, to the lysosomes for degradation or they can recycle back to the outer plasma membrane (Huotari and Helenius, 2011).

Many of the endocytic processes are regulated by proteins such as clathrin or the Rabs. The Rab proteins are members of the Ras-like GTPase family that regulate vesicle trafficking (Zhang et al., 2007). Rab proteins localize to distinct membrane-bound sub compartments and are involved in vesicle formation, motility, tethering and fusion to

other membranes (Corbeel and Freson, 2008). Rab proteins are active when bound to Guanosine-5'-triphosphate (GTP) and not active when bound to Guanosine diphosphate (GDP) (Corbeel and Freson, 2008). Before activation, Rab-GDP undergoes prenylation which allows the protein to bind to a lipid bilayer and form a transport vesicle (Corbeel and Freson, 2008). This vesicle may contain nutrients or membrane associated proteins that are being transported. After activation, the Rab-GTP associated transport vesicle binds to an effector protein which enables fusion with other membrane associated vesicles (Corbeel and Freson, 2008).

The Rab proteins are essential for endocytosis and are involved in vesicle maturation. Different Rabs have specialized roles throughout the process, such as Rab5 in early endosomes. Rab5 and its effector VPS34/p150 produce the phosphoinositide PtdIns(3)P, which leads to maturation of the early endosome (Behnia and Munro, 2005). Rab5 is constantly associated with the endocytic membrane during the early endosome stage and is important for their maturation into the late endosomes (Huotari and Helenius, 2011). As previously discussed, Rab5 has been proposed as a candidate to be involved in endocytic trafficking of ATP7A. At basal copper levels, APT7A was shown to co-localize with Rab5 in early endosomes (Pascale *et al.*, 2003). *Drosophila* Rab (dRab) proteins are beginning to be characterized and most can be clearly related to their mammalian orthologues (Zhang *et al.*, 2007). This thesis will explore dRab5-mediated endocytosis as a source of DmATP7 translocation.

1.5.2.5.2 Protein-protein interactions causing degradation in *Drosophila*

The involvement of protein partners in the regulation of *Drosophila* copper homeostasis is not well understood. As with the discovery that COMMD1 and Clusterin regulate Cu-ATPases degradation in mammals, it is expected that there are still novel proteins for regulating copper transport to be found in *Drosophila*. A screen of over 500 UAS-containing *P-element (UAS)* insertions identified genes that weakened or exacerbated the *UAS-DmATP7* thorax/abdomen phenotype (unpublished data from the Burke Laboratory). One of the candidates identified to have a lethal interaction with *UAS-*

DmATP7 was the *Drosophila* Homeodomain interacting protein kinase (HipK). HipK is the sole *Drosophila* member of a family of serine/threonine kinases (Kim *et al.*, 1998). Mammalian members of this family are known as transcription factors regulating various genes (Rinaldo *et al.*, 2007).

Drosophila *HipK*$^{-/-}$ mutants are embryonic lethal and hypomorpic mutations cause small and rough eyes due a loss of photoreceptor cells in homozygous animals (Lee *et al.*, 2009). Ectopic expression of *HipK* in the eye resulted in large overgrowths in the eye imaginal disc and adult eye (Lee *et al.*, 2009). HipK was found to be a positive regulator of the Notch signaling pathway, which is involved in eye development. HipK phosphorylates and inactivates Groucho, the key repressor of Notch signaling, resulting in increased tissue growth (Lee *et al.*, 2009).

Research into other *Drosophila* signaling pathways, such as Wnt/Wingless (Wg) and Hedgehog, demonstrated that HipK may gave a conserved role in preventing protein degradation (Lee *et al.*, 2009; Swarup and Verheyen, 2011). The Wnt/Wg pathway is initiated by the Wnt/Wg ligand binding to a Frizzled receptor leading to the prevention of degradation of the transcriptional effector Armadillo (Cadigan and Peifer, 2009). This then allows Armadillo to begin driving transcription of Wg target genes. Over-expression of *HipK* in the *Drosophila* wing increased activation of the Wg pathway by inhibiting the degradation of Armadillo (Swarup and Verheyen, 2011). HipK binds to and phosphorylates Slimb, a component of the E3 ubiquitin ligase responsible for Armadillo degradation (Swarup and Verheyen, 2011), inhibiting its ubiquitination of Armadillo. The HipK-Slimb interaction was also responsible for regulating the Hedgehog pathway (Swarup and Verheyen, 2011).

These results suggest that HipK has a general role in ubiquitination pathways and that just as COMMD1 and Clusterin are mammalian regulators of Cu-ATPase degradation, HipK could play a role in the degradation of DmATP7. This role may explain the lethal interaction observed between *UAS-DmATP7* and the *P-element (UAS)* insertion mutant of *HipK*.

1.6 This study

All animals require the essential biometal copper as an enzymatic cofactor for processes as diverse as energy production, free radical detoxification and pigmentation. The human body has an absolute need for copper but also must provide protection against toxicity. Therefore each cell produces a set of copper homeostasis proteins which are required for copper to enter the cell, be utilized and then be removed. Whilst the basic role of each copper transporter has been identified, many questions remain in regard to their regulation, localization and tissue specific function.

Drosophila has been established as an alternative model for research on copper transport by several groups. This study uses *Drosophila* as a model to further investigate those unidentified questions in copper homeostasis. The particular aims of this study are to:

- Explore the mechanisms of midgut copper absorption and its importance to distribution of the nutrient throughout the organism.
- Investigate the localization of copper homeostasis proteins in the midgut, salivary glands and malpighian tubules.
- Determine functional roles for dRab5 and HipK in *Drosophila* copper homeostasis.

CHAPTER TWO: MATERIALS AND METHODS

Synopsis

This chapter describes the materials and methods used throughout this thesis.

2.1 *Drosophila* maintenance, stocks and procedures

2.1.1 Fly maintenance and medium

All *Drosophila* stocks were maintained at 22°C on Cordonbleu food in 20mm wide vials with adults removed and progeny turned into new vials on alternative weeks. Cordonbleu food was made freshly each week based upon the following recipe (for 10 litres of food). 96g of potassium tartrate, 6g of calcium chloride, 64g of agar, 144g of yeast, 640g of glucose, 320g of raw sugar was brought to the boil in 9.44 litres of water. 800g of semolina was pre-mixed in 2.4 litres of water, then added to the mixture and again brought to boiling point. After the mixture cooled, 42 milliliters of propionic acid and 85 milliliters of nipagin (methyl-p-hydroxybenzoate in 100% ethanol) was added before being dispensed into plastic vials. $CuSO_4$ (25mg/ml) was added to supplement the food with various levels of copper (ranging from 5µM – 1mM CU), whilst BCS (bathocuproinedisulfonic acid) was added to chelate copper in the food.

2.1.2 Fly stocks

The following *Drosophila* stocks were used:

Table 2.1: Summary of *Drosophila* stocks

Stock	Genotype	Stock origin
General Stocks		
w1118	+/+; +/+	Obtained from Bloomington Stock centre
Double Balancer	*IF/Cyo;TM6B/MKRS*	Obtained from Bloomington Stock centre
Gal4 drivers		
mex-GAL4	*mex-GAL4/Cyo; +/+*	Obtained from Bloomington Stock centre
pannier-GAL4	*+/+; pnr-GAL4/TM6B*	Obtained from Bloomington Stock centre
C42-GAL4	*+/+; C42-GAL4/TM6B*	Julian Dow Laboratory
actin-GAL4	*actin-GAL4/Cyo;+/+*	Obtained from Bloomington Stock centre
Mutants		
$HipK^{11361}$	*+/+;+/+*	Obtained from Bloomington

		Stock centre
UAS-DmATP7^{DOMNEG}	*UAS-DmATP7^{DOMNEG} /TM3*	Generated in the Richard Burke laboratory
UAS-*cDNA*		
UAS-DmATP7	*+/+; UAS-DmATP7/TM6B or UAS-DmATP7/Cyo; +/+*	Generated in the Richard Burke laboratory
UAS-Ctr1A	*+/+; UAS-Ctr1A/TM6B or UAS- Ctr1A /Cyo; +/+*	Generated in the Richard Burke laboratory
UAS-Ctr1B	*+/+; UAS-Ctr1B/TM6B or UAS-Ctr1B/Cyo; +/+*	Generated in the Richard Burke laboratory
UAS-ScoX	*+/+; UAS-ScoX/TM6B*	Generated in the Richard Burke laboratory
UAS-dAtox1	*+/+; UAS-dAtox1/TM6B*	Generated in the Richard Burke laboratory
UAS-dCCS	*+/+; UAS-dCCS/TM6B*	Generated in the Richard Burke laboratory
UAS-CutC	*+/+; UAS-CutC/TM6B*	Generated in the Richard Burke laboratory
UAS-HipK	*+/+; UAS-HipK/TM6B*	Generated in the Richard Burke laboratory
***UAS*-RNAi**		
DmATP7 (8315)	*+/+; 8315/8315*	Obtained from VDRC
DmATP7 (108159)	*108159/108159; +/+*	Obtained from VDRC
Ctr1A (46757)	*+/+; 46757/TM6B*	Obtained from VDRC
Ctr1A (46758)	*46758/46758; +/+*	Obtained from VDRC
Ctr1B (5804)	*+/+; 5804/5804*	Obtained from VDRC
ScoX (7861)	*+/+; 7861/7861*	Obtained from VDRC
dAtox1 (23057)	*+/+; 23057/23057*	Obtained from VDRC
dCCS (20536)	*20536/20536; +/+*	Obtained from VDRC
CutC (22238)	*+/+; 22238/22238*	Obtained from VDRC
HipK (32854)	*+/+;32854/32854*	Obtained from VDRC
dRab5 (34096)	*+/+;34096/34096*	Obtained from VDRC
Enterocyte copper reporter lines		
pCtr1B-EGFP	*pCtr1B-EGFP/ pCtr1B-EGFP; +/+*	Walter Shaffner laboratory
pMtnB-EGFP	*+/+; pMtnB-EGFP/ pMtnB-EGFP*	Walter Shaffner laboratory
Fluorescence tagged fusion proteins		
UAS-mCherry-DmATP7	*+/+; UAS-mCherry-DmATP7/TM6B or UAS-mCherry-DmATP7/Cyo; +/+*	Generated in the Richard Burke laboratory
UAS-Ctr1A-GFP	*+/+; UAS-Ctr1A-GFP/TM6B or UAS-Ctr1A-GFP/Cyo; +/+*	Generated in the Richard Burke laboratory

UAS-Ctr1B-GFP	*+/+; UAS-Ctr1B-GFP/TM6B or UAS-Ctr1B-GFP/Cyo; +/+*	Generated in the Richard Burke laboratory
UAS-Apical-GFP	*UAS-Apical-GFP/Cyo; +/+*	Obtained from Bloomington Stock centre
UAS-GalNAc-GFP	*UAS-GalNAc-GFP/Cyo; +/+*	Obtained from Bloomington Stock centre
UAS-KDEL-GFP	*+/+;KDEL-GFP/TM6B*	Obtained from Bloomington Stock centre
UAS-dRab5-WT-EYFP	*9775/9775; +/+*	Obtained from Bloomington Stock centre
UAS-dRab5-DN-EYFP	*+/+; 9772/TM3*	Obtained from Bloomington Stock centre
UAS-dRab5-CA-EYFP	*+/+; 9773/9773*	Obtained from Bloomington Stock centre

2.1.3 Basic *Drosophila* crosses

All basic fly interactions were investigated using crosses that consisted of between 1-5 males and 5-10 virgin females. All crosses were made at constant humidity and at 25°C unless otherwise stated. Adults were removed from crosses after 6-9 days and progeny were collected, anesthetized with CO_2 and then viewed under Nikon/Leica MZ6 microscopes. Adult emergence was used as a measure to determine viability, whilst midline or eye phenotypes were assessed on 2-day old adults.

2.1.4 *Drosophila* caged crosses

Drosophila 'caged crosses' were used to grow 2nd instar larvae to be picked to different food types. A 'caged cross' consisted of fifty virgin females and between 10 and 20 males crossed inside a mesh cage which sits above a normal food medium plate. After 15 hours at 25°C, adults were removed from the cage and fifty 2nd instar larvae were picked to 20mm wide vials containing each food type. Four repetitions of each vial were reared at 25°C and larval survival into adulthood was measured. Progeny were scored as either non-Cyo (containing *mex-GAL4*) or Cyo (the no GAL4 control) (Cyo is the *Drosophila* phenotypic marker for 'curled wings'). Genotype scores were then converted to a

percentage of the expected progeny number (25). The mean survival and standard error for each genotype was determined via statistical analysis (Chapter 2.6).

2.1.5 Dissections of third instar *Drosophila* larvae

Progeny for dissection were grown on normal media using a *Drosophila* caged cross. Selected third instar larvae were place into cold PBS and viewed under the Leica Dissecting stereoscope. Dissection consisted of pulling from the larval mouthparts, removing the salivary glands, brain and imaginal discs. The remaining larvae were then inverted and the midgut and malpighian tubules removed before the lymph was searched for tumours. The investigated tissue (gut region, malpighian tubules, wing imaginal disc or tumours) were placed onto microscope slides in mounting media. Slides were viewed under the Leica Dissecting stereoscope to check orientation of the tissues and a cover slip was placed on top of the sample (See Chapter 2.3 for microscopy methods)

PBS (10x)
80gNaCl, 2g KCl, 14.4g Na_2HPO, 2.4 g KH_2PO_4, pH to 7.4 with NaOH and made up to IL with dH_2O

2.2 DNA cloning methods

2.2.1 Generation of UAS-cDNA constructs

Ectopic expression was achieved when the transcript downstream of a UAS-construct contained *cDNA* from the targeted copper transport gene (a procedure previously used to investigate copper homeostasis (Norgate *et al.*, 2006; Binks *et al.*, 2010; Norgate *et al.*, 2010). *UAS-cDNA* constructs for Ctr1A, Ctr1B, DmATP7, ScoX, dAtox1, dCCS and CutC were previously created by Richard Burke. The remaining *UAS-cDNA* constructs and *UAS-cDNA-fluorescent tag* constructs were created during this project.

2.2.1.1 cDNA extraction, PCR and primer design

For all constructs wild type *cDNA* was originally extracted from either *w1118* larvae or S2 cells. In each case, the targeted gene was PCR amplified using gene-specific primers and the stop codon was omitted if a FLAG epitope or fluorescent tag was required to be attached at the C-terminus (Table 2.2). Standard PCR reactions were performed on the thermal cycler. For fragments less than 1kb, the reaction started with a denaturation step at 94°C for 2 minutes. The next 35 cycles consisted of a denaturing step at 94°C for 30 seconds, a primer annealing step at 54°C for 30 seconds and an extension step at 72°C for 1 minute. Finally there is a longer extension step at 72°C for 10 minutes. For larger fragments the extension steps were lengthen and for higher stringency the annealing temperature was increased. Primer design was completed using Vector NTI and the relevant *cDNA* sequences. Primers were obtained from Invitrogen and resuspended in dH_2O before being stored at -20°C.

Standard PCR reaction

5µl 10X Buffer, 1.5µl 25mM $MgCl_2$, 1.0µl of each 20µM primer, 2.5µl 2mM dNTPs, 2.5µl DNA template, 0.15µl Taq Polymerase and 11.32 H_2O.

Table 2.2: PCR primers

Gene	Forward Primer	Reverse Primer
DmATP7	GAGGTACCATGTCCACGGTGCCGCCTGCC	GCTCTAGACAGCTTTTGCAGTTCGGTACT
Ctr1A	GAGGTACCATGGACCACGCCCATCACAG	GCTCTAGAGTGACAGTGCTCGGTTACG
Ctr1B	GAGGTACCATGGATCACGGCTCGATGA	GCTCTAGAAGGACAGCACTCGCCTCG
ScoX	GAGGTACCATGTCCCGCTCCCTGCAAC	GCTCTAGAGCTGAACCATCCCTTTTTGTT
dAtox1	GAGGTACCATGACAGTGCAACGAATTCAAG	GCTCTAGATTTCTTCACCCCGACGTAAG
dCCS	GCGGTACCATGAGCTCCATTAAGATCGAAAT	GCTCTAGACAGCTTTTGTGAGCGGTCCT
CutC		
HipK	CGGAATTCATGAAAACGTCCTACCCC	GCTCTAGACTACTCAGCCCCATACCA

2.2.1.2 Digestion, gel electrophoresis, purification and ligation

cDNA from each gene was inserted into the *pUAST* Drosophila expression vector R256 for storage (ligation method discussed in 2.2.1.3). The vector was digested using

appropriate Promega enzymes and buffers. Enzyme buffers were selected on the basis of the Promega restriction enzyme buffer technical appendix. Incubations were made in 37°C heating baths for 1.5-2.5 hours. Loading dye was added to the digested product before being loaded into agarose gels. Gels were run at 100V for 50 minutes and then bands cut out under a UV-transilluminator. Gel slices were purified following the Promega Wizard gel purification kit. Purified fragments were always checked using another digest (in these cases only 10 µl of the purified product was loaded into the gel). The purified product was ligated into the appropriate vector, pUAST or pUAST-attb, which had also been digested and purified. Standard ligation mixtures were incubated for 3 hours at room temperature.

Standard restriction digest (purifying fragment)

5µl of DNA, 3µl of enzyme buffer, 1µl of each restriction enzyme and 5µl of H_2O.

Agarose gels

Prepared by dissolving 0.8-3% agarose (w/v) in TBE buffer (1X). Once the mixture cooled ethidium bromide was added and the mixture was poured into small gel trays to set.

Standard ligation mixture

4µl of the enzyme restricted insert, 1µl of the enzyme restricted vector, 2µl of ligase buffer (Promega), 1µl of DNA ligase (Promega) and 2µl H_2O.

2.2.1.3 Transformation and mini-prep

Competent *E.coli* cells were stored at -70°C and standard transformation was achieved following a heat shock method. The competent cells and ligation mixture were incubated on ice for 5 minutes. The mixture was then heat shocked for 3 minutes at 37°C, before being placed back on ice for a further 5 minutes. Cells were then streaked out on LB solid medium plates containing ampicillin (100mg/ml) and incubated for 16-20 hours at 37°C. Single colonies, with resistance to ampicillin, were picked with a sterile pipette tip

and placed in 10ml McCartney bottles with 2ml LB (with penicillin). Colonies were incubated, shaking at 230 RPM for 16-20 hours at 37°C. Mini prep of the colonies was achieved by either the Camakaris method or via the Wizard mini prep kit. Mini-prep DNA was always tested via an analytical restriction enzyme digest and before sequencing. A standard sequencing reaction underwent a program in the thermal cycler which started with an initial step at 96°C for 1 minute. The next 30 cycles consisted of steps at 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. After amplification, the mixture entered sequence clean-up before being submitted to the Micromon for sequencing.

Competent cell preparation

A single *E.coli* colony was grown, with moderate shaking, overnight in 100ml LB medium with antibiotic. 2ml of culture was then used to inoculate 100ml LB and left until OD_{590} reached 0.375. 50ml of that culture was used to inoculate 1000ml and again left until OD_{590} reached 0.375. The solution was then spun at 3000 RPM, resuspended in competence solution and left on ice for 40 minutes. After another spin, the solution was resuspended in competence solution again before being allocated to aliquots kept at -70°C.

Camakaris mini-prep method

The overnight bacterial culture was spun down and resuspended in Solution I. After 5 minutes at room temperature, fresh Solution II was added. The culture was then placed on ice for 10 minutes before Solution III was added. After a further 10 minutes on ice the culture was spun down for 7 minutes. The supernatant was then mixed with isopropanol and kept at -20°C for 30 minutes. The solution was then spun down for 10 minutes and the supernatant removed. 70% EtOH was added before being spun for 3 minutes. The ethanol was aspirated, the pellet dried and then resuspended in TE + RNAase.

Standard Sequencing Reaction

1µl of fluorescent labeled oligonucleotides (Big Dye Premix), 3.5µl of 5X Buffer, 1µl of either 3.2uM forward or reverse primer, 1.5µl – 5µl of template DNA (dependent on concentration) and 9.5 – 13µl of H_2O.

Sequencing clean-up

80µl of Isoproponal was added to the sequencing reaction and then left at room temperature for 15 minutes. After a 20 min spin, the supernatant was discarded and 250µl of Isoproponal added. The mixture was then spun for 5 minutes and the supernatant was discarded. There was a final spin to remove all isopropanol and the pellet dried.

2.2.1.4 Microinjection and identification of transgenic flies

pUAST vectors with the correct inserts were microinjected into *w1118 Drosophila* eggs following standard microinjecting protocol (Venken and Bellen, 2007). pUAST-attb vector with the correct insert were microinjected into PhiC31 attP 51C and 96E strains of *Drosophila.* Injected females were crossed to Double Balancer (DB) males. The progeny of this cross were selected for on the basis of non-white eye colour. This was due to the fact that the vectors contained the *white*$^{+}$ marker gene.

2.3 Microscopy

2.3.1 Imaging adult *Drosophila*

For imaging the adult *Drosophila* thorax and abdomen, two-day old flies had their legs and wings removed before imaging. For imaging the *Drosophila* eye, two-day old flies had their heads removed and the heads were placed with the anterior end facing to the right of the observed image. Flies were observed using the Leica MZ6 dissecting microscope, imaged using the Leica DFC295 camera and analysed using Leica IM50 software.

2.3.2 Imaging whole *Drosophila* guts

Dissected midguts from third instar larvae were mounted as described earlier and viewed under GFP light using a Leica dissecting stereoscope. Images were recorded with a Leica DC300 camera using Leica Application Suite software

2.3.3 Confocal imaging of the *Drosophila* midgut, salivary glands, malpighian tubules and imaginal wing discs

Dissected tissues from third instar larvae were mounted as described earlier without being fixed and viewed at 40X using the Nikon C1 Upright Confocal Microscope**.** Images were analysed using NIS-Elements and ImageJ software.

2.4 Assays

2.4.1 SOD1 activity assay

SOD1 activity was assayed using a SOD1 determination kit from Sigma which measured the ability of SOD1 to inhibit superoxide anion. The assay measures the absorbance of a water-soluble tetrazolium salt that produces a formazan dye upon reduction with superoxide anion. Increased SOD1 activity results in inhibition of superoxide anion and a reduction in absorbance at 450nm determined on a 96-well plate reader. Correctly genotyped Drosophila were grown on normal media using a *Drosophila* caged cross. Five larvae were homogenized in a centrifuge tube with squishing buffer containing Proteinase K. The larvae ground using a mortar and the resultant mixture was centrifuged for 15 minutes. The supernatant underwent a 2-fold dilution before being used as the sample for the assay. Each detection mixture contained 20µl of sample, 200µl of WST working solution and 20µl of enzyme working solution. Absorbance at 450nm was calculated for each sample as well as three controls, Blank 1 (no sample), Blank 2 (no superoxide anion enzyme) and Blank 3 (no sample or superoxide anion enzyme. SOD1 activity of each sample was calculated as a percentage of colour-loss compared to the no sample control using the following formula: {[(Ab-Blank 1 – Ab-Blank 3) - Ab-Sample – Ab-Blank 2)]/ (Ab-Blank 1 – Ab-Blank 3)} x 100. Each assay was repeated four times to establish the mean and standard error for SOD activity of each genotype.

2.4.2 Measuring tumour rate

The tumour rate of third instar larvae was measured by scoring larvae as containing tumours or not and six repetitions were completed. The average and standard error of the percentage of larvae containing tumours was calculated.

2.5 Synchrotron analysis

Imaginal discs were dissected from third instar larvae as described earlier but in this case were fixed in 4% Paraformaldehyde in PBS for 30 minutes. Fixed tissues were washed in PBS then rinsed briefly in H_20 prior to two final 2-minute washes in 0.1 M ammonium acetate. Discs were then transported to a silicon nitride window (Silson Ltd: SiRN-7.5-200-3.0-500) with a small drop of ammonium acetate. Windows were then left to dry overnight. Samples were then scanned at vertical and horizontal increments using the XFM beamline at the Australian Synchrotron to generate elemental maps. The beamline uses an undulator source, a pair of Si(111) crystals, and a Kirkpatrick-Baez mirror pair to form a focus of monochromatic x-rays with spot size around 2×2 µm. Elemental maps are generated by using X-ray energy to excite K-shell fluorescence emission from first row transition metals (Lye *et al.*, 2011). Elemental maps were analysed using GeoPIXE software, which uses Dynamic Analysis to subtract background and resolve overlapping peaks allowing for the generation of semi-quantitative values for each metal investigated. Distribution of the metal within the tissue could then be summarised using a heat map.

2.6 Statistical analysis

Statistical analysis was completed using Microsoft Excel. In all cases genotype numbers were converted to a proportion (%) of the expected progeny. Means and standard errors were determined from four repetitions of each scenario. The sample means and standard error were compared to the internal control using a simple paired t-test. Statistical analyses where the p value <0.05 were deemed to be statistically significant.

CHAPTER THREE: THE FUNCTIONAL ANALYSIS OF COPPER TRANSPORT IN THE *DROSOPHILA* MIDGUT

Synopsis

This chapter explores the mechanisms responsible for midgut copper absorption and its importance to distribution of the nutrient throughout the organism. Midgut-specific copper transport is explored by determining the outcomes of targeted suppression and ectopic expression of key copper transporters. The chapter demonstrates that the genetic manipulation of copper homeostasis proteins can alter fly viability as well as enterocyte and lymph copper content.

3.1 Introduction

3.1.1 Investigating gene function in the *Drosophila* midgut

The major source of copper exposure to *Drosophila*, like humans, is via ingestion and absorption of the nutrient. This chapter explores the mechanisms of midgut copper absorption and its importance to distribution of the nutrient throughout the organism. Copper absorption involves copper import from the intestinal lumen into the enterocytes, intracellular transport within the enterocytes and subsequent export into the bloodstream (lymph in the case of *Drosophila*). Here, intestinal absorption of copper is investigated by determining the role of copper import proteins (Ctr1A and Ctr1B), chaperones (Scox, dAtox1, dCCS and CutC) and the sole copper exporter (DmATP7).

Analysis of *in vivo* gene function in *Drosophila* is facilitated using the GAL4//UAS system. The GAL4/UAS system can be used to express a gene or RNAi construct in a cell/tissue specific manner (Brand and Perrimon, 1993; Kennerdell and Carthew, 2000). This is a two-part system that consists of a cell or tissue specific GAL4 transcriptional activator which can bind to a responsive Upstream Activating Sequence (UAS) triggering expression of the transcript following the UAS-construct. This two-part system is useful as the GAL4 drivers and UAS-constructs can be maintained in different *Drosophila* lines preventing interaction. *mex-GAL4*, created using the promoter region of the gene *Midgut expression (Mex1)*, was used to investigation gene function in the midgut. This gene is expressed in the middle midgut sections of developing *Drosophila* larvae. The expression pattern of *mex-GAL4* was confirmed by driving *UAS-GFP* (Figure 3.1).

3.1.2 Suppression and ectopic expression of copper transporters

The GAL4/UAS system can be used to investigate both ectopic (over) expression and suppression of targeted genes. Ectopic expression was achieved when the transcript downstream of the UAS-construct contained *cDNA* from the targeted copper transport gene (constructs created by Richard Burke) (Norgate *et al.*, 2006; Binks *et al.*, 2010; Norgate *et al.*, 2010). For each gene full length *cDNA* was PCR amplified from w^{1118}

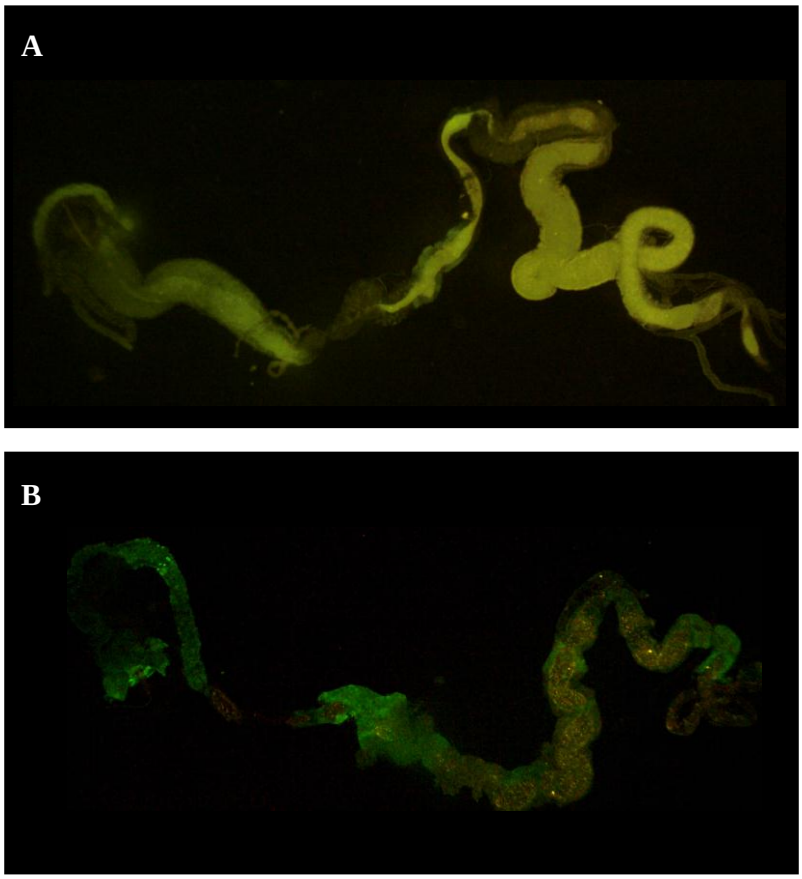

A
B

Figure 3.1: The expression pattern of *mex-GAL4* in the *Drosophila* midgut

mex-GAL4 is expressed in the subsections of the middle midgut of *Drosophila*. *UAS-GFP* driven under the control of *mex-GAL4* (A) is shown to highlight the midgut expression pattern in comparison with w^{1118} (B). Dissected midguts from third instar larvae were mounted and viewed under GFP light using a Leica dissecting stereoscope. Images were recorded with a Leica DC300 camera using Leica Application Suite software

Drosophila, cloned into a pUAST vector and the stop codon was omitted to allow the attachment of an in-frame FLAG epitope tag at the C-terminus. Vectors with the correct insert were confirmed by restriction digest and sequencing. The pUAST constructs were microinjected into w^{1118} *Drosophila* embryos and transgenic flies were identified by the presence of the $white^{+}$ marker gene, contained in the pUAST vector, which results in orange/red coloured eyes. Stable lines were then generated containing the inserted *UAS-cDNA* constructs. Constructs for *Ctr1A*, *Ctr1B*, *DmATP7* and *Scox* were shown to be functional as determined by phenotypes generated when driven by *pannier-GAL4* (thorax/abdomen) and *GMR-GAL4* (eye) (Binks *et al.*, 2010; Norgate *et al.*, 2010). *pannier/GMR-GAL4* driven expression of constructs for *dAtox1*, *CutC* or *dCCS* does not result in any phenotypic differences compared to wild type.

Suppression was achieved via RNAi using lines designed specifically for each copper transporter. RNAi is a process where the transcript downstream of the UAS-construct contains an inverted repeat sequence of the targeted gene (Kennerdell and Carthew, 2000). Transcription of the repeated sequence leads to the production of dsRNA which then mediates suppression via RNA interference (RNAi). Stable *Drosophila* lines containing specific suppression transgenes were obtained from the Vienna *Drosophila* RNAi Centre (VDRC). RNAi constructs for *Ctr1A*, *Ctr1B*, *DmATP7*, *Scox* where shown to be functional as determined by thorax/abdomen and eye phenotypes (Binks *et al.*, 2010; Norgate *et al.*, 2010), whereas expression of RNAi constructs for *dAtox1*, *CutC* or *dCCS* resulted in a wild type thorax/abdomen or eye.

3.2 Results

3.2.1 *Drosophil*a midgut-specific suppression of key copper uptake and efflux transporters

Driving RNAi suppression of key copper transporters for import and export using *mex-GAL4* was used to advance our understanding of the specific roles Ctr1A, Ctr1B and DmATP7 play in midgut. Endogenous expression patterns of *Ctr1A* (ubiquitous), *Ctr1B* (induced in the midgut by low dietary copper levels) and *DmATP7* (midgut amongst

other areas) imply that each transporter is functionally important in enterocytes (Zhou *et al.*, 2003; Burke *et al.*, 2008). To test the importance on endogenous copper transport genes in midgut copper absorption, stable compound *mex-GAL4*/Cyo; *UAS-RNAi/UAS-RNAi* lines were crossed to *+/+;UAS-RNAi/UAS-RNAi* lines in population cages to generate progeny with one copy of the *mex-GAL4* driver and two copies of the RNAi line. Progeny were picked as first instar larvae, transferred to various test media and flies that survived to adulthood were scored as either non-Cyo (containing *mex-GAL4*) or Cyo (the no GAL4 control). Genotype scores were then converted to a percentage of the expected progeny number. The mean survival and standard error for each genotype was determined over four independent experiments and compared using a t-test for paired samples.

3.2.1.1 Suppression of *Ctr1A* and *Ctr1B* driven by mex-GAL4 did not alter *Drosophila* viability

The phenotypes of flies mutant for *Ctr1A* or *Ctr1B* imply that both genes have a functional role in the midgut. Ctr1A is proposed to import dietary copper into developing larvae, because first instar $Ctr1A^{-/-}$ larvae mutants are copper deficient as determined by Inductively Coupled Plasma - Mass Spectrometry (ICP-MS) and subsequently die by early second instar (Turski and Thiele, 2007). $Ctr1B^{-/-}$ mutants are viable on normal food yet die when dietary copper levels decrease (Zhou *et al.*, 2003). *Ctr1B* expression is transcriptionally activated during times of copper scarcity and down-regulated in times of overload (Zhou *et al.*, 2003). These results indicate that Ctr1B is likely to be a secondary uptake source whose expression is triggered in the midgut in times of copper need.

Suppression of *Ctr1A* in the midgut resulted in no change to viability on normal or decreased dietary copper food (Figure 3.2), although there was a significant drop in viability on food supplemented with 1mM Cu (Figure 3.2). Suppression of *Ctr1B* resulted in no change to viability on normal, increased or decreased dietary copper food (Figure 3.2). Midgut co-suppression analysis was therefore carried out to explore whether Ctr1A or Ctr1B could act to compensate for the loss of the other. For this

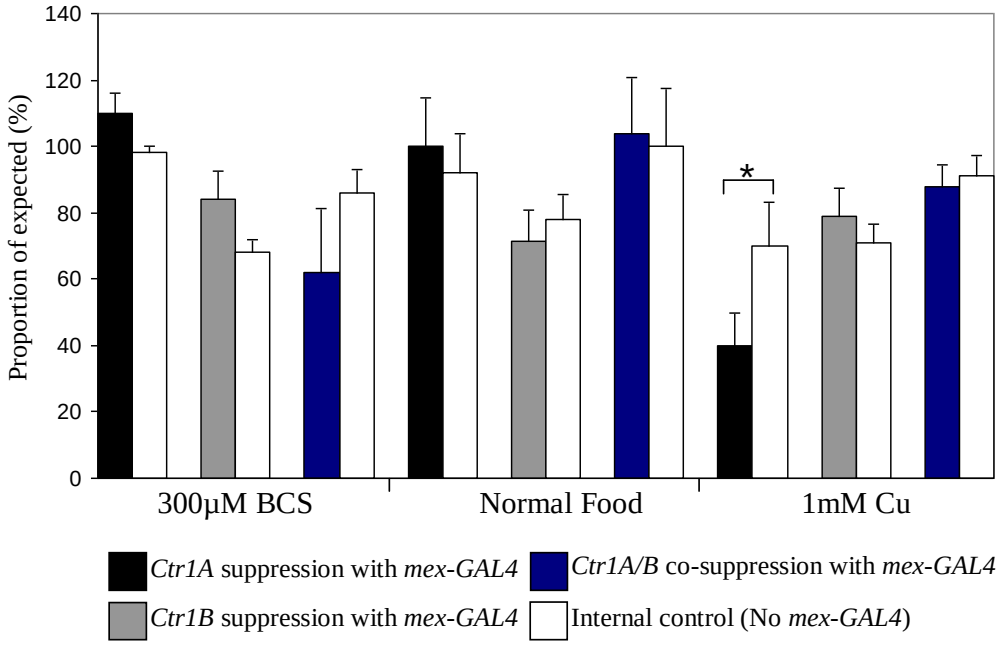

140
120
100
80
60
40
20
0
Proportion of expected (%)
*
300µM BCS
Normal Food
1mM Cu
Ctr1A suppression with mex-GAL4
Ctr1A/B co-suppression with mex-GAL4
Ctr1B suppression with mex-GAL4
Internal control (No mex-GAL4)

Figure 3.2: Larvae to adulthood survival of *mex-GAL4;Ctr1A/B* RNAi *Drosophila*

In each case *mex-GAL4/Cyo; RNAi/RNAi* males were crossed to *+/+; RNAi/RNAi* female in population cages to generate progeny with one copy of the *mex-GAL4* driver and either two copies of the one RNAi line or one copy of each. Progeny were transferred as first instar larvae to normal food, 1mM copper and 300µM BCS (Bathocuproine disulfonate) supplemented food. Surviving adults were scored as either non-Cyo (containing *mex-GAL4*) or Cyo (the no GAL4 control). The genotype numbers were converted to a proportion (%) of the expected progeny. Means and standard errors were determined from four repetitions of each scenario. *mex-GAL4* was compared to the internal control using a simple paired t-test. Suppression of *Ctr1A* or *Ctr1B* resulted in no change to viability on normal or 300µM BCS food but there was significant drop in viability on increased dietary copper for *Ctr1A* RNAi (p value < 0.05). Co-suppression of *Ctr1A* and *Ctr1B* resulted in no change to viability on all media types.

experiment *mex-GAL4/Cyo; Ctr1A-RNAi/Ctr1A-RNAi* lines were crossed to *+/+; Ctr1B-RNAi/Ctr1B-RNAi* lines generating progeny with one copy of the *mex-GAL4* and one copy of each RNAi construct. Co-suppression of *Ctr1A* and *Ctr1B* resulted in viable *Drosophila* on all media types (Figure 3.2). The results in this section suggest that midgut suppression of the two copper importers, alone or in combination, is not detrimental to the organism as a whole.

3.2.1.2 *mex-GAL4* driven suppression of the sole export protein *DmATP7* did not alter *Drosophila* viability

DmATP7 is the sole known efflux protein of *Drosophila* and hypothesized to control copper efflux in the midgut. *DmATP7*$^{-/-}$ mutant larvae are small, lethargic, have less pigmented mouthparts and eventually die before reaching the second instar of development (Norgate *et al.*, 2006). Viability could not be restored by increasing copper levels and the nutrient was shown to accumulate in enterocytes implying that the overall mutant phenotype is at least partially caused by impairment of the midgut function of DmATP7 (Norgate *et al.*, 2006). Bahadorani *et al* has investigated suppression of *DmATP7* in the midgut driving the *Sym-PUAST-DmATP7* RNAi silencing construct with *2020-GAL4* which resulted in a reduction in viability on low copper media and decreased copper content in the brain (Bahadorani *et al.*, 2010). In this study however, *mex-GAL4* driven suppression of *DmATP7* resulted in no change to viability on normal, increased or decreased dietary copper food (Figure 3.3).

3.2.1.3 Co-suppression of copper uptake and efflux with *mex-GAL4* does not alter *Drosophila* viability

As suppression of import or export mechanisms alone was not detrimental to *Drosophila* adult viability, *DmATP7* was co-suppressed with both *Ctr1A* and *Ctr1B* under *mex-GAL4* control. Midgut-specific co-suppression of *DmATP7* with either *Ctr1A* or *Ctr1B* did not alter viability on normal, increased or decreased dietary copper (Figure 3.4). This

indicates that suppressing candidates for both copper import and export at the same time is not detrimental to *Drosophila*.

3.2.2 *Drosophila* midgut-specific ectopic expression of key copper transporters for uptake and efflux

Targeted ectopic expression of key copper transporters in the midgut is an alternative method of examining any intestine-specific roles of Ctr1A, Ctr1B and DmATP7. As *Ctr1A*, *Ctr1B* and *DmATP7* are all endogenously expressed in the midgut it was hypothesized that increasing the expression of these genes could alter copper transport in a cell-type specific manner. Ectopic expression was achieved when the transcript downstream of the UAS-construct contained *cDNA* from *Ctr1A*, *Ctr1B* or *DmATP7*. Stable *Drosophila* lines containing specific *UAS-cDNA* constructs on the third chromosome and *mex-GAL4* on the second chromosome were generated. As with the suppression analysis, the consequence of ectopic expression was determined by measuring larval-to-adult survival using the earlier described population caged crosses.

3.2.2.1 Midgut-specific ectopic-expression of *Ctr1B* and *DmATP7* is lethal on food supplemented with copper

Elevating the expression of copper import and export genes above endogenous levels in the *Drosophila* midgut was hypothesized to alter absorption of the nutrient, consequently impacting on viability. Ectopic expression of either uptake protein, *Ctr1A* or *Ctr1B*, in the eye and thorax resulted in phenotypes indicative of increased intracellular copper levels (Binks *et al.*, 2010). In intestinal cells it was hypothesized that ectopic expression of *Ctr1A* or *B* would increase copper content of the midgut leading to higher absorption rates and potential changes in larval development. Midgut-specific ectopic expression of *Ctr1A* resulted in no alteration to viability on normal, increased or decreased dietary copper food (Figure 3.5). However *Ctr1B* ectopic expression resulted in a significant drop in viability on food supplemented with 1mM Cu (Figure 3.5).

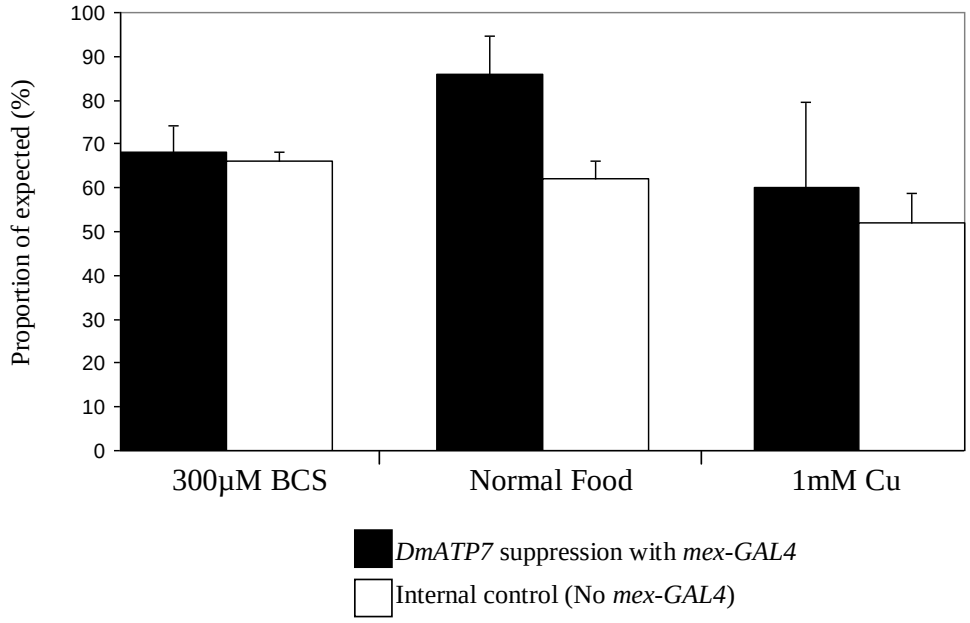

Proportion of expected (%)
100
90
80
70
60
50
40
30
20
10
0
300µM BCS
Normal Food
1mM Cu
DmATP7 suppression with mex-GAL4
Internal control (No mex-GAL4)

Figure 3.3: Larvae to adulthood survival of *mex-Gal4;DmATP7* RNAi *Drosophila*

mex-GAL4/Cyo; DmATP7-RNAi/ DmATP7-RNAi males were crossed to +/+; *DmATP7*-RNAi/ *DmATP7*-RNAi females in population cages to generate progeny with one copy of the *mex-GAL4* driver and two copies of the *DmATP7* RNAi construct. Progeny were transferred as first instar larvae to normal food, 1mM copper and 300µM BCS supplemented food. Surviving adults were scored as either non-Cyo (containing *mex-GAL4*) or Cyo (the no GAL4 control). The genotype numbers were converted to a proportion (%) of the expected progeny. Means and standard errors were determined from four repetitions of each scenario. *mex-GAL4* was compared to the internal control using a simple paired t-test. Suppression of *DmATP7* resulted in no change to viability on all media types.

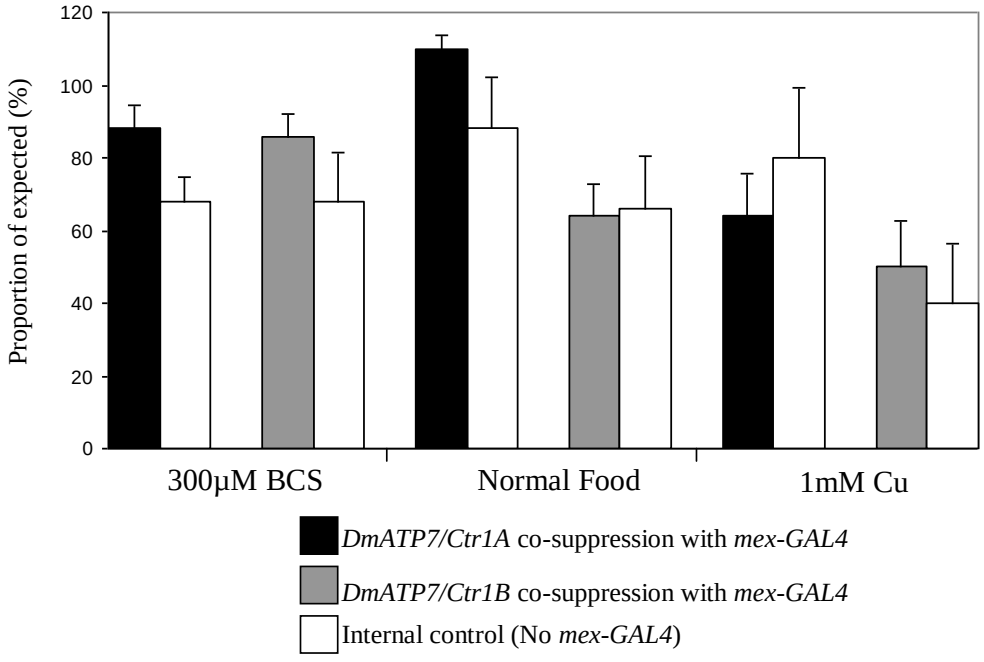
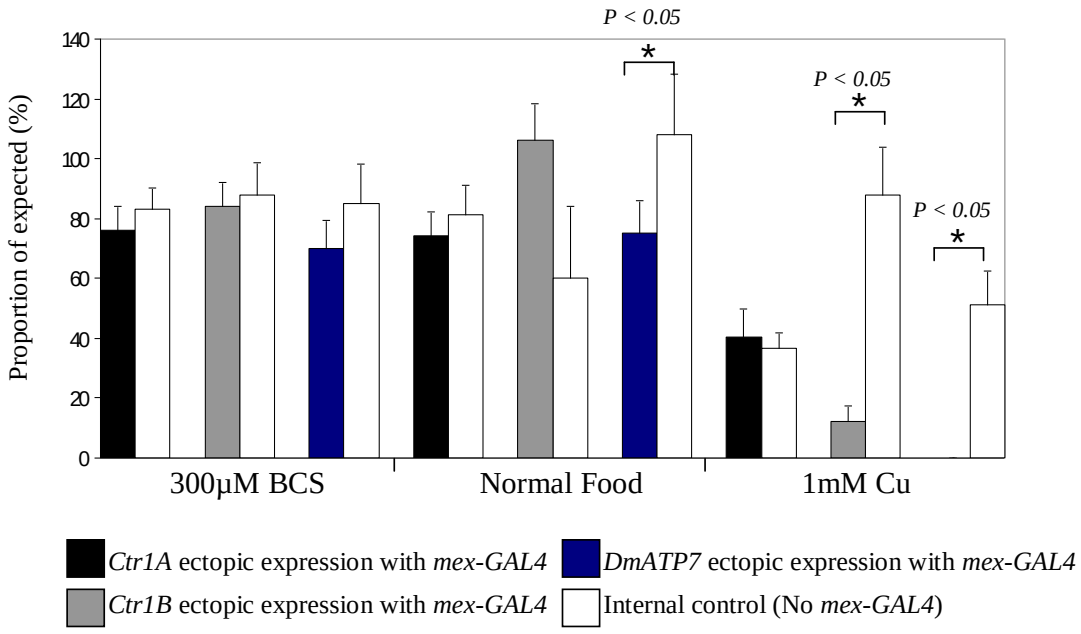

120
100
80
60
40
20
0
Proportion of expected (%)
300µM BCS
Normal Food
1mM Cu
DmATP7/Ctr1A co-suppression with mex-GAL4
DmATP7/Ctr1B co-suppression with mex-GAL4
Internal control (No mex-GAL4)

Figure 3.4: Larvae to adulthood survival of *mex-GAL4;DmATP7/Ctr1A or Ctr1B* RNAi *Drosophila*

mex-GAL4/Cyo; DmATP7-RNAi/ DmATP7-RNAi males were crossed to *+/+; Ctr1A/-RNAi/ Ctr1A-RNAi* or *+/+; Ctr1B/-RNAi/ Ctr1B-RNAi* females to generate progeny with one copy of the *mex-GAL4* and one copy of each RNAi construct. Progeny were transferred as first instar larvae to normal food, 1mM copper and 300µM BCS supplemented food. Surviving adults were scored as either non-Cyo (containing *mex-GAL4*) or Cyo (the no GAL4 control). The genotype numbers were converted to a proportion (%) of the expected progeny. Means and standard errors were determined from four repetitions of each scenario. *mex-GAL4* was compared to the internal control using a simple paired t-test. Suppression of *DmATP7* in combination with either *Ctr1A* or *B* RNAi resulted in no alteration to viability.


P < 0.05
P < 0.05
P < 0.05
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Proportion of expected (%)
140
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60
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20
0
300µM BCS
Normal Food
1mM Cu
Ctr1A ectopic expression with mex-GAL4
DmATP7 ectopic expression with mex-GAL4
Ctr1B ectopic expression with mex-GAL4
Internal control (No mex-GAL4)

Figure 3.5: Larvae to adulthood survival of *mex-Gal4; UAS-Ctr1A, Ctr1B* and *DmATP7*

For each experiment, *mex-GAL4/Cyo;UAS-cDNA/UAS-cDNA* males were crossed to *+/+; UAS-cDNA/UAS-cDNA* females to generate progeny with one copy of the *mex-GAL4* and two copies of each UAS construct. Progeny were transferred as first instar larvae to normal food, 1mM copper and 300µM BCS supplemented food. Surviving adults were scored as either non-Cyo (containing Mex-Gal4) or Cyo (the no Gal4 control). The genotype numbers were converted to a proportion (%) of the expected progeny. Means and standard errors were determined from four repetitions of each scenario. *mex-GAL4* was compared to the internal control using a simple paired t-test. Midgut-specific ectopic expression of *Ctr1B* resulted in a significant drop in viability on 1mM Cu (p value < 0.05). Ectopic expression of *DmATP7* resulted in a significant drop in viability of normal food and complete lethality on 1mM Cu (p value < 0.05).. Ectopic expression of *Ctr1A* did not alter viability on any media type.

UAS-DmATP7 expression driven ubiquitously or in the thorax resulted in phenotypes reflective of increased export leading to copper deficiency (Norgate *et al.*, 2007). Elevating midgut expression *DmATP7* above endogenous levels was expected to lead to increased export out of enterocytes. This would result in increased copper absorption whilst also creating a copper deficiency in the midgut itself. Both outcomes could conceivably alter the viability of the organism. It was found that midgut-specific ectopic expression resulted in 100% lethality on increased copper media (Figure 3.5).

To further explore both the *Ctr1B* and *DmATP7* copper toxicity phenotypes, the survival to adulthood of first instar larvae was scored on smaller variations in dietary copper levels. *UAS-Ctr1B* expression resulted in a significant drop in viability by 500µM Cu and complete lethality on 1.5mM Cu supplemented media (Figure 3.6). *UAS-DmATP7* was more sensitive to increased copper levels as there was a significant drop in viability on normal food and complete lethality by 50µM Cu (Figure 3.7). These results imply that increased midgut import (Ctr1B) or export in combination with increased dietary copper levels can be detrimental to *Drosophila.*

3.2.3 *Drosophila* midgut copper homeostasis is further explored by investigating ectopic expression and suppression in combination

The midgut-specific role of copper transporters for uptake and efflux has been examined by analysis of changes to *Drosophila* viability as a consequence of suppression or ectopic expression alone. This has revealed that there is little effect associated with *mex-GAL4* suppression but ectopic expression of *Ctr1B* and *DmATP7* is lethal on increased copper media. This section now investigates the consequences of combining suppression and ectopic expression of *Ctr1A, Ctr1B* and *DmATP7*. Stable compound *mex-GAL4; UAS-RNAi* lines were crossed to *mex-GAL4; UAS-cDNA* lines on various test media and progeny were scored for survival to adulthood (Table 3.1).

Combining *Ctr1A* or *Ctr1B* RNAi with *UAS-DmATP7* resulted in no change to viability on normal food, implying that if suppression of the two uptake candidates causes less

copper to enter the enterocyte, the addition of increased efflux does not make that more detrimental to the organism.

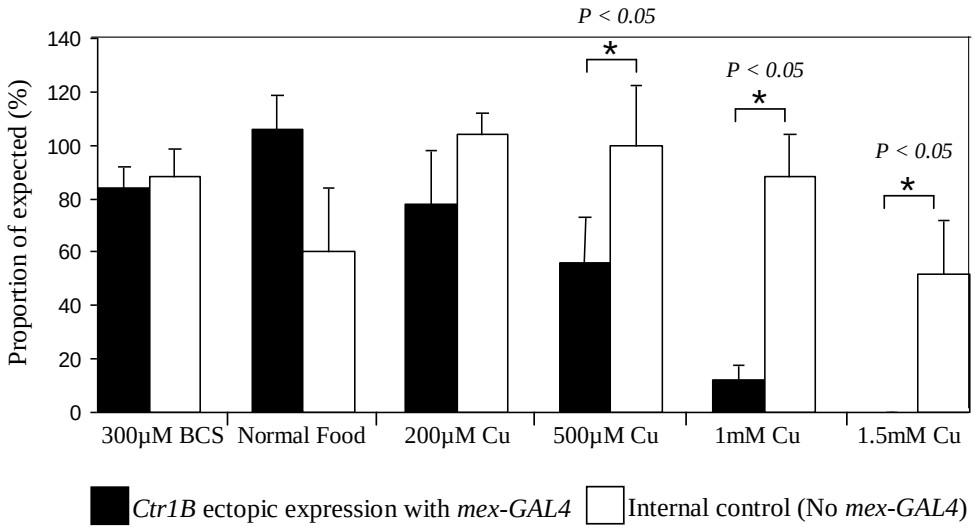
Viability was unchanged when suppressing *DmATP7* in combination with expression of either *UAS-Ctr1A* or *UAS-Ctr1B* alone. However the combination of *DmATP7* suppression with expression of both *UAS-Ctr1A* and *UAS-Ctr1B* caused lethality on all media types. This result indicates increasing import and blocking export can lead the entrapment of copper in the midgut resulting in toxicosis (further investigated in 3.2.6).

mex-GAL4;UAS-Ctr1B flies (lethal on 1.5mM Cu) were rescued by suppressing either *Ctr1A* or *B* (Table 3.1). Suppression of *Ctr1B* was expected to rescue from lethality by counteracting the original over-expression, however the *Ctr1A* suppression result suggests that limiting copper uptake in general can reverse the effect of *Ctr1B* ectopic expression. This further implies a similarity in the midgut-specific roles of Ctr1A and Ctr1B.

Neither *DmATP7* suppression nor ectopic expression could rescue the *mex-GAL4;UAS-Ctr1B* lethality, although *UAS-DmATP7* alone causes lethality at 50µM Cu. Interestingly, the *UAS-DmATP7* + Cu lethality is only reverseed by *DmATP7* suppression, indicating that *Drosophila* are highly sensitive to increased efflux of copper out of the midgut. All of these results have been summarized in Table 3.1.

3.2.4 Enterocyte copper levels during midgut-specific suppression and ectopic expression of *Ctr1A*, *Ctr1B* and *DmATP7*

The transcription factor MTF-1 is responsive to changes in intracellular copper levels, activating midgut expression of either *Ctr1B* or *MetallothioneinB* (*MtnB*). When copper is scarce, *Ctr1B* is activated to increase uptake, whilst when copper levels increase beyond requirement, expression of *MtnB* is triggered to sequester excess copper. This


P < 0.05
P < 0.05
P < 0.05
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140
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80
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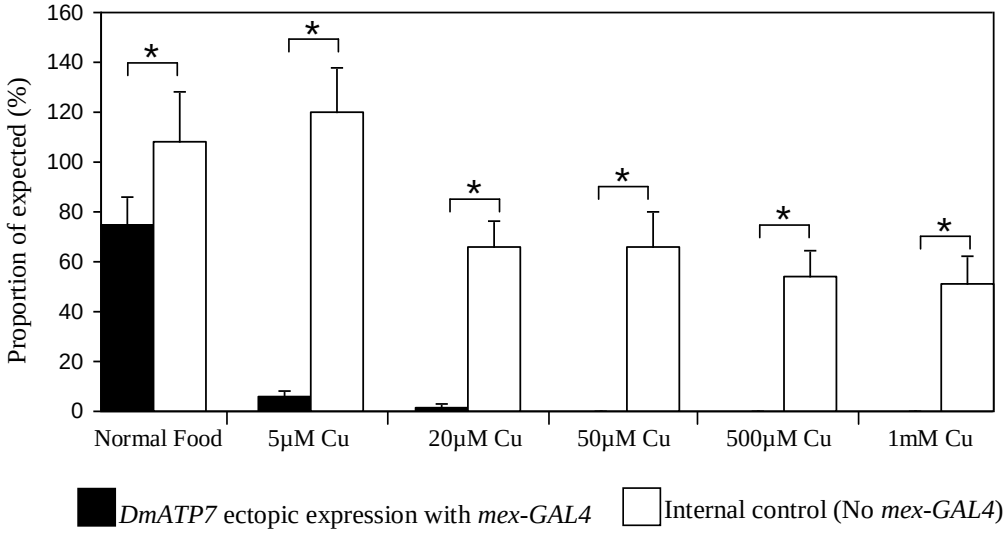
20
0
Proportion of expected (%)
300µM BCS
Normal Food
200µM Cu
500µM Cu
1mM Cu
1.5mM Cu
Ctr1B ectopic expression with mex-GAL4
Internal control (No mex-GAL4)

Figure 3.6: Larvae to adulthood survival of *mex-GAL4*; *Ctr1B* on varying copper levels

mex-GAL4/Cyo; UAS-Ctr1B / TM6B males were crossed to a *+/+; UAS-Ctr1B /UAS-Ctr1B* females. Progeny were transferred at first instar to different variations of copper or chelator supplemented media (300µM BCS, Normal food, 200µM Cu, 500µM Cu, 1mM Cu and 1.5mM Cu). Surviving adults were scored as either non-Cyo (containing *mex-GAL4*) or Cyo (the no Gal4 control). The genotype numbers were converted to a proportion (%) of the expected progeny. Means and standard errors were determined from four repetitions of each scenario. *mex-GAL4* was compared to the internal control using a simple paired t-test. Midgut-specific ectopic expression of *Ctr1B* resulted in a significant drop in viability by 500µM Cu and complete lethality at 1.5mM Cu (p value < 0.05).


160
140
120
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80
60
40
20
0
Proportion of expected (%)
*
*
*
*
*
*
Normal Food
5µM Cu
20µM Cu
50µM Cu
500µM Cu
1mM Cu
DmATP7 ectopic expression with mex-GAL4
Internal control (No mex-GAL4)

Figure 3.7: Larvae to adulthood survival of *mex-GAL4; DmATP7* on varying copper levels

mex-GAL4/Cyo; UAS-DmATP7 / TM6B males were crossed to a *+/+; UAS-DmATP7 /UAS-DmATP7* females. Progeny were transferred at first instar to different variations of copper supplemented media (Normal food, 5µM Cu, 20µM Cu, 50µM Cu, 500µM Cu and 1mM Cu). Surviving adults were scored as either non-Cyo (containing *mex-GAL4*) or Cyo (the no GAL4 control). The genotype numbers were converted to a proportion (%) of the expected progeny. Means and standard errors were determined from four repetitions of each scenario. *mex-GAL4* was compared to the internal control using a simple paired t-test. Midgut-specific ectopic expression of *DmATP7* results in complete lethality at 50µM Cu (p value < 0.05).

Table 3.1: Summary of *mex-GAL4* driven ectopic expression in combination with suppression of copper importers and exporters

	Ctr1A RNAi	***Ctr1B RNAi***	***DmATP7 RNAi***	***UAS-Ctr1A***	***UAS-Ctr1B***	***UAS-DmATP7***
Alone	Viable	Viable	Viable	Viable	Lethal on increased copper	Lethal on increased copper
Ctr1A RNAi		Viable	Viable	Viable	Viable	Lethal on increased copper
Ctr1B RNAi	Viable		Viable	Viable	Viable	Lethal on increased copper
Ctr1A RNAi & Ctr1B RNAi			Viable	Viable	Viable	Lethal on increased copper
DmATP7 RNAi	Viable	Viable		Viable	Lethal on increased copper	Viable
UAS-Ctr1A	Viable	Viable	Viable		Lethal on increased copper	Lethal on increased copper
UAS-Ctr1B	Viable	Viable	Lethal on increased copper	Lethal on increased copper		Lethal on increased copper
UAS-Ctr1A & UAS-Ctr1B	Viable	Viable	Lethal on Normal Food			Lethal on increased copper
UAS-DmATP7	Lethal on increased copper	Lethal on increased copper	Viable	Lethal on increased copper	Lethal on increased copper	

transcriptional mechanism is useful in maintaining homeostasis both in the enterocytes and also systemically.

MTF-1 activity can be used to gauge enterocyte copper content by examining the expression patterns of its target genes, *Ctr1B* and *MtnB.* *Drosophila* lines containing *Ctr1B* and *MtnB* expression reporter constructs were used for this experiment (Transgenic lines were created by Professor Walter Schaffner's group). The constructs were created so the endogenous promoter for either *Ctr1B* or *MtnB* drives expression of EYFP (Enhanced Yellow Fluorescent Protein), and consequently fluorescence acts as a proxy measure of gene expression. Dissected midguts from wild type *Drosophila* expressing *pCtr1B-EYFP* show no fluorescence on normal food, but EYFP expression is enhanced in the middle midgut, foregut and hindgut when larvae are raised on food supplemented with the copper chelator BCS (Figure 3.8). Conversely, *pMtnB-EYFP* expression in the gut is very low on low copper food, but increases substantially when copper levels increase (Figure 3.10). These measures were used to determine enterocyte copper levels during genetic manipulation of copper uptake and efflux transporters in the midgut.

3.2.4.1 *Ctr1A* suppression or ectopic expression of *DmATP7* causes enterocyte copper deficiency

Lines containing *pCtr1B-EYFP* were crossed to compound stocks containing *mex-GAL4*; RNAi or *cDNA* for *Ctr1A*, *Ctr1B* and *DmATP7*. Progeny were reared on either normal food or food supplemented with BCS and dissected to remove the entire midgut region, which was viewed under EYFP (GFP) fluorescence. Midgut suppression of *Ctr1A* resulted in an increase in *pCtr1B-EYFP* expression on normal food (Figure 3.8). This change was not observed with suppression of *Ctr1B* or *DmATP7* (Figure 3.8). This implies that Ctr1A is required for copper entry into the *Drosophila* midgut.

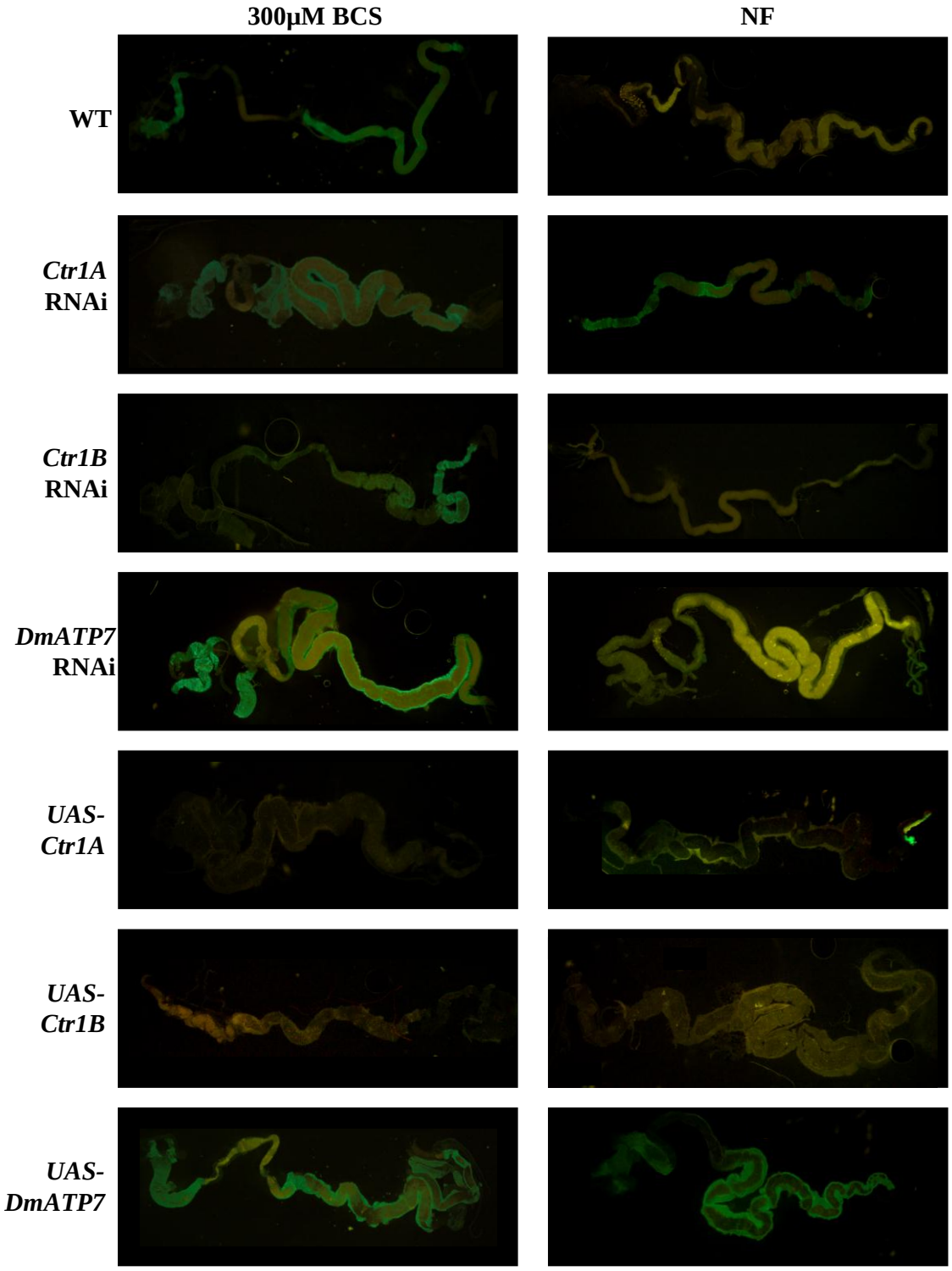

300µM BCS
NF
WT
Ctr1A RNAi
Ctr1B RNAi
DmATP7 RNAi
UAS-Ctr1A
UAS-Ctr1B
UAS-DmATP7

Figure 3.8: Expression of *pCtr1B-EYFP* in the *Drosophila* midgut during manipulation of copper uptake and efflux transporters

Expression of the reporter construct *pCtr1B-EYFP* was used to measure enterocyte copper content in the midgut of developing third instar *Drosophila* larvae. Larvae were dissected to remove the entire gut region, which was mounted and viewed under EYFP fluorescence using the Leica Stereoscope. Midguts from wild type flies containing *pCtr1B-EYFP* show no yellow/green fluorescence when raised on normal food, yet EYFP expression is enhanced in the middle midgut, foregut and hindgut when raised on food supplemented with the copper chelator, BCS. Suppression of *Ctr1A* by RNAi results in increased EYFP expression on normal food indicating a copper deficient midgut. *Ctr1B* or *DmATP7* suppression does not alter *pCtr1B-EYFP* expression. *UAS-DmATP7* results in enhanced EYFP expression on normal food, whilst *UAS-Ctr1A/B* partially decreases EYFP observed on BCS supplemented food. These results demonstrate that increasing *DmATP7* expression generates a midgut copper deficiency, whilst increasing *Ctr1A/B* expression leads to higher midgut copper levels than wild type. Images were recorded with a Leica DC300 camera using Leica Application Suite software.

(Transgenic *Drosophila* containing the *pCtr1B-EYFP* construct were obtained from Walter Shaffner)

Similarly, ectopic expression of *DmATP7* resulted in an increase in *pCtr1B-EYFP* expression on normal food (Figure 3.8). This supports a role for DmATP7 in export of copper from enterocytes into the lymph. Ectopic expression of *Ctr1A* or *Ctr1B* resulted in no *pCtr1B-EYFP* expression on normal food and partially reduced fluorescence on low copper food (Figure 3.8). These results demonstrate that increasing expression of *Ctr1A/B* generates higher enterocyte copper content as evidenced by reduced expression of *pCtr1B-EYFP*.

These scenarios were further explored by determining *pCtr1B-EYFP* expression when *mex-GAL4*; *Ctr1A* RNAi was combined with *DmATP7* suppression or *Ctr1B* ectopic expression. In both experiments the midgut copper deficiency caused by *Ctr1A* suppression was rescued (Figure 3.9). Decreasing export (*DmATP7* RNAi) and increasing import (UAS-*Ctr1B*) restored midgut copper levels as demonstrated by the reduced *pCtr1B-EYFP* expression.

3.2.4.2 *DmATP7* suppression or ectopic expression of *Ctr1A/B* cause enterocyte copper accumulation

Suppression of *DmATP7* resulted in an increase in *pMtnB-EYFP* expression in the midgut of *Drosophila* reared on low copper media (Figure 3.10). This increase indicates that suppressing *DmATP7* results in a loss of copper export and consequently high enterocyte copper levels. In contrast, *Ctr1A* and *Ctr1B* suppression induced no change to *pMtnB-EYFP* expression (Figure 3.10). Ectopic expression of either *Ctr1A* or *Ctr1B*, but not *DmATP7*, resulted in enhanced *pMtnB-EYFP* expression in the midgut when larvae are raised on food supplemented with BCS (Figure 3.10). These results imply that Ctr1A/B can transport copper into the midgut and increased uptake can cause copper accumulation. The enhanced *pMtnB-EYFP* expression generated by *DmATP7* suppression was rescued by *Ctr1A* (partially) and *Ctr1B* suppression as decreased uptake prevented copper accumulation (Figure 3.11).

3.2.5 Modification of midgut copper transport causes changes to peripheral copper levels in *Drosophila*

Observing changes in viability is only one measure to determine the systemic effect on *Drosophila* caused by midgut-specific suppression and ectopic expression of *Ctr1A*, *Ctr1B* and *DmATP7*. Copper accumulation in the entire organism can be measured to help determine the role of each protein in midgut absorption. Previous work investigating the general roles of copper transporters has used various methods to measure copper content in *Drosophila* larvae or adults. *Ctr1A*$^{-/-}$ mutant larvae have a 30% decrease in accumulated copper as determined by ICP-MS and a 3-fold reduction in the activity of the copper dependent enzyme cytochrome *c* oxidase (Turski and Thiele, 2007). *Ctr1B*$^{-/-}$ mutants were similarly affected with a 40% reduction in total copper as established by ICP-MS and low tyrosinase activity (Zhou *et al.*, 2003). Work using the *Sym-pUAST-DmATP7* suppression construct driven by *2020-GAL4* showed a drop in copper content in the adult body and brain using atomic absorption spectrophotometry (AAS) (Bahadorani *et al.*, 2010). Each of these results established that loss of copper uptake or efflux resulted in a significant change to copper levels of whole *Drosophila.* It was presumed that manipulating expression of these genes in the midgut alone would also impact total copper levels.

For this investigation, SOD1 activity was used to determine total copper content of *Drosophila* third instar larvae. SOD1 is a cuproenzyme vital for antioxidant defence which when bound to copper can convert superoxide radicals into oxygen and hydrogen peroxide (Linder and Hazegh-Azam, 1996; Miao and St Clair, 2009). Increased SOD1 activity is an indication of copper accumulation and was assayed using a SOD1 determination kit from Sigma which measured the enzyme's ability to inhibit superoxide anion. Variations of this assay have been used recently to determine SOD1 activity in *Drosophila* (Gupta *et al.*, 2005; Fujiwara *et al.*, 2011). The assay measures the absorbance of a water-soluble tetrazolium salt that produces a formazan dye upon reduction with superoxide anion. Increased SOD1 activity results in inhibition of superoxide anion and a reduction in absorbance. The SOD1 activity of *Drosophila* third instar larvae containing *mex-GAL4* plus *RNAi* or *cDNA* constructs for *Ctr1A*, *Ctr1B* and

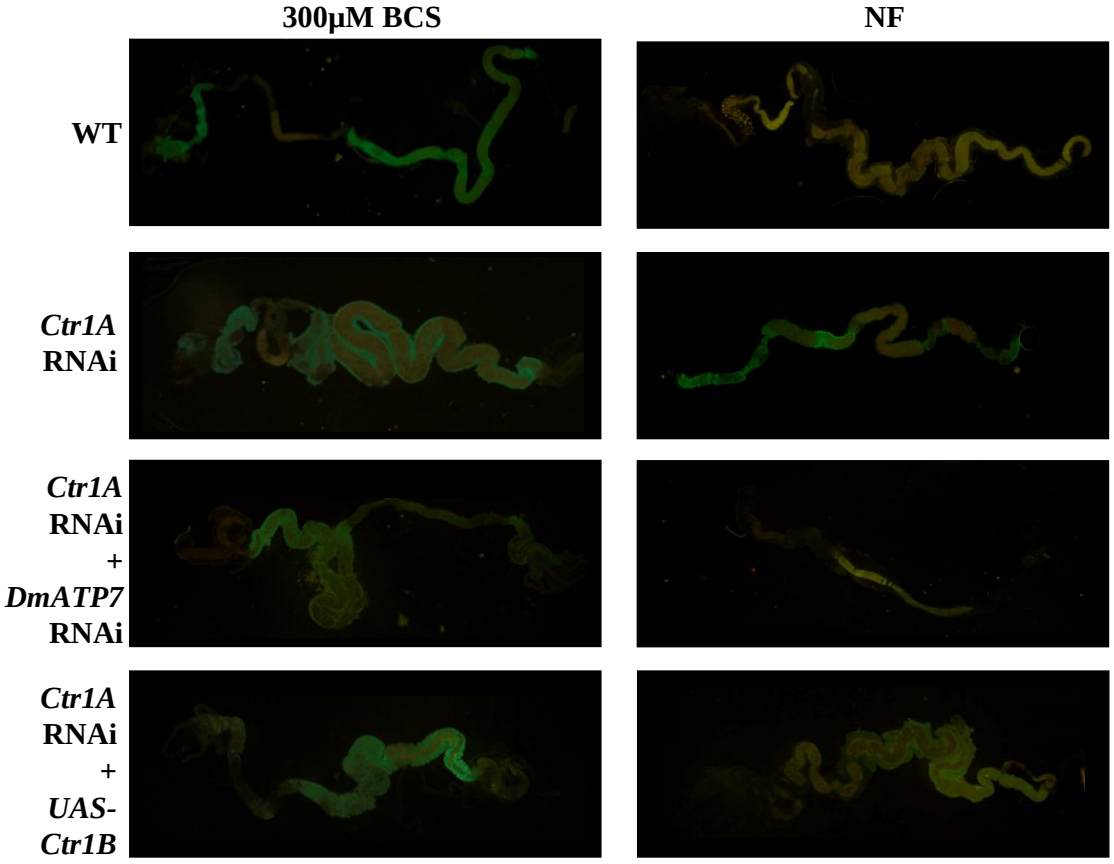

300µM BCS
NF
WT
Ctr1A RNAi
Ctr1A RNAi + DmATP7 RNAi
Ctr1A RNAi + UAS-Ctr1B

Figure 3.9: Expression of *pCtr1B-EYFP* in the *Drosophila* midgut when *mex-GAL4;Ctr1A* RNAi is combined with *DmATP7* RNAi and UAS-*Ctr1B*

Suppression of *Ctr1A* by RNAi results in increased expression of the reporter construct *pCtr1B-EYFP* when larvae are reared on normal food in comparison with the wild type control demonstrating a copper deficient *Drosophila* midgut. The combination of either *DmATP7* suppression or ectopic expression of *Ctr1B* rescues the copper deficiency as *pCtr1B-EYFP* expression returns to basal levels on normal food. Larvae were dissected to remove the entire gut region, which was mounted and viewed under EYFP fluorescence using the Leica Stereoscope. Images were recorded with a Leica DC300 camera using Leica Application Suite software.

(Transgenic *Drosophila* containing the *pCtr1B-EYFP* construct were obtained from Walter Shaffner)

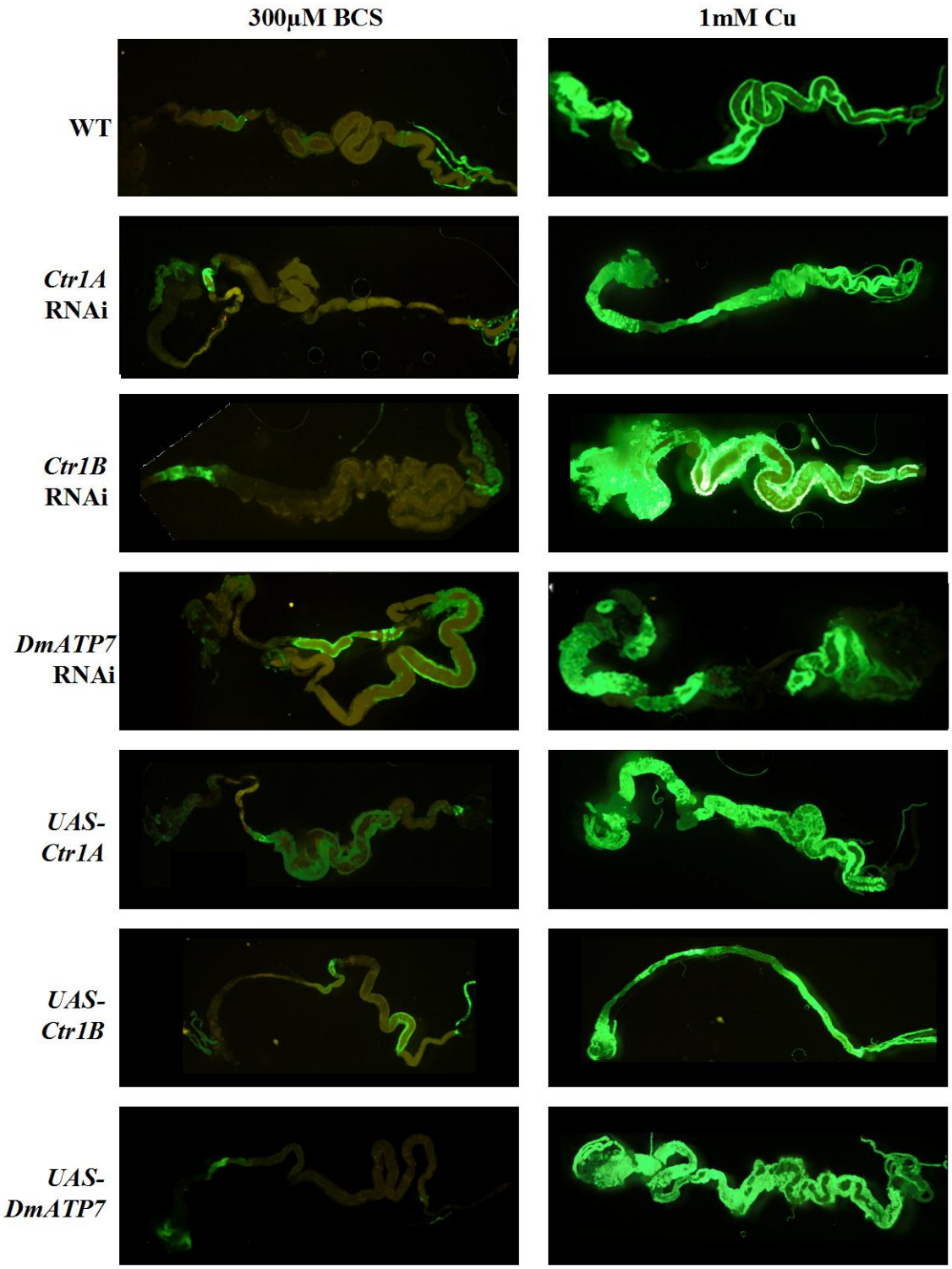

300µM BCS
1mM Cu
WT
Ctr1A RNAi
Ctr1B RNAi
DmATP7 RNAi
UAS-Ctr1A
UAS-Ctr1B
UAS-DmATP7

Figure 3.10: Expression of *pMtnB-EYFP* in the *Drosophila* midgut during manipulation of copper uptake and efflux transporters

Expression of the reporter construct *pMtnB-EYFP* was used to measure enterocyte copper content in the midgut of developing third instar *Drosophila* larvae. Larvae were dissected to remove the entire gut region, which was mounted and viewed under EYFP fluorescence using the Leica Dissecting Stereoscope. Midguts from wild type flies containing *pMtnB-EYFP* show little yellow/green fluorescence when raised on food supplemented with the copper chelator BCS, yet EYFP expression increases throughout the whole gut region when raised on 1mM Cu supplemented food. Suppression of *DmATP7* by RNAi results in increased EYFP expression on 300µM BCS food indicating copper accumulation in the midgut. *Ctr1A* or *Ctr1B* suppression does not alter *pCtr1B-EYFP* expression. *UAS-Ctr1A* and *UAS-Ctr1B* also caused increased *pMtnB-EYFP* expression on 300µM BCS food demonstrating a role for these transporters in copper uptake. *UAS-DmATP7* did not alter the wild type expression of *pMtnB-EYFP*.. Images were recorded with a Leica DC300 camera using Leica Application Suite software.

(Transgenic *Drosophila* containing the *pMtnB-EYFP* construct were obtained from Walter Shaffner)

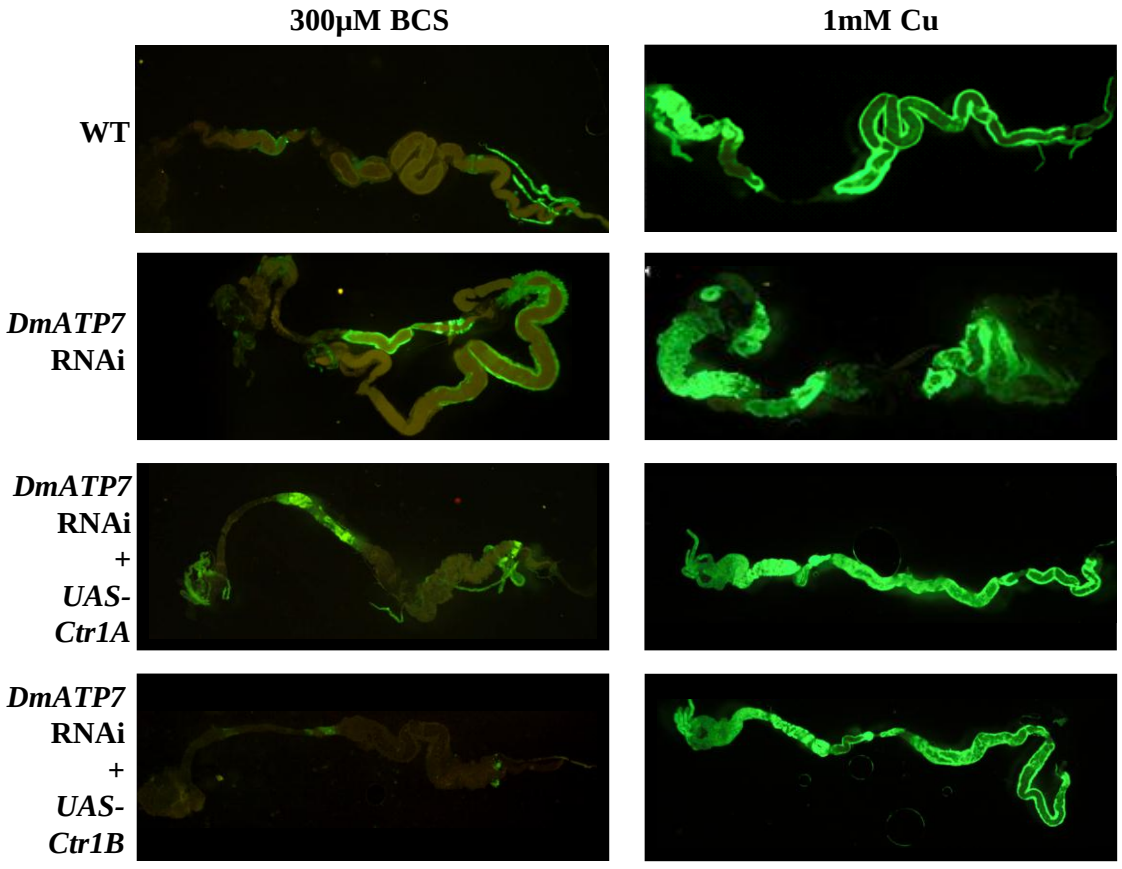

300µM BCS
1mM Cu
WT
DmATP7 RNAi
DmATP7 RNAi + UAS-Ctr1A
DmATP7 RNAi + UAS-Ctr1B

Figure 3.11: Expression of *pMtnB-EYFP* in the *Drosophila* midgut when *mex-GAL4;DmATP7* RNAi is combined with *Ctr1A/B* RNAi

Suppression of *DmATP7* by RNAi results in increased expression of the reporter construct *pMtnB-EYFP* when larvae are reared on food supplemented with 300µM BCS, in comparison with the wild type control demonstrating copper accumulation in the *Drosophila* midgut. The combination of wither *Ctr1A* or *Ctr1B* suppression with *mex-GAL4;DmATP7* RNAi partially rescues the copper accumulation phenotype as *pMtnB-EYFP* expression returns to normal levels on 300µM BCS food. *Ctr1B* RNAi generates a stronger rescue that *Ctr1A* RNAi. Larvae were dissected to remove the entire gut region, which was mounted and viewed under EYFP fluorescence using the Leica Stereoscope. Images were recorded with a Leica DC300 camera using Leica Application Suite software.

(Transgenic *Drosophila* containing the *pMtnB-EYFP* construct were obtained from Walter Shaffner)

DmATP7 was compared to a wild type control. In each case, five third instar larvae were homogenized in a grinding buffer, centrifuged and the supernatant diluted 2-fold before being used as the sample for the assay. Each assay was repeated four times to establish the mean and standard error for SOD activity of each genotype.

3.2.5.1 *Drosophila* suppressing or ectopically expressing *Ctr1A*, *Ctr1B* or *DmATP7* in the midgut resulted in little change to larval SOD1 activity

Wild type (*w1118*) *Drosophila* larvae were found to have a mean superoxide anion inhibition activity of 79.11% which equated to a SOD1 concentration of approximately 5 units/per ml (Figure 3.12). Midgut specific suppression of *Ctr1A*, *Ctr1B*, *DmATP7* or *Ctr1A* and *Ctr1B* in combination resulted in no significant change to the SOD1 activity observed in the wild type (Figure 3.12). Whilst not significant, there was a small increase observed with *Ctr1A* RNAi and a small decrease observed with *DmATP7* RNAi. Furthermore, midgut specific ectopic expression of *Ctr1A*, *Ctr1B*, *DmATP7* or *Ctr1A* and *Ctr1B* in combination also resulted in no significant change to wild type SOD1 activity (Figure 3.13). *UAS-Ctr1B* did slightly increase the mean inhibition activity, whilst all other ectopic expression assays resulted in a small decrease. The SOD1 assay has shown that genetic manipulation of copper transporters for import and export in the midgut may not notably alter systemic copper levels.

3.2.6 Copper accumulation in enterocytes can be detrimental to *Drosophila*

Suppression of *DmATP7* caused increased expression of *pMtn-EYFP* in the midgut implying copper accumulation in enterocyte cells. In addition, the *mex-GAL4* combination of *DmATP7* suppression with ectopic expression of both *Ctr1A* and *Ctr1B* resulted in complete lethality on all media types. Together these phenotypes suggest that copper export by DmATP7 out of the midgut is required to prevent intestinal copper toxicity. This section describes an experiment used to support this theory.

3.2.6.1 Loss of oxidative stress response increases the susceptibility of the *Drosophila* midgut to copper toxicity

Copper accumulation leads to the formation of dangerous free radicals causing oxidative damage. To protect against toxicity, copper can be distributed to the antioxidant defence enzyme SOD1 by the chaperone protein CCS. Loss of *CCS* causes a decrease in SOD1 activity and increased sensitivity to oxidative damage (Wong *et al.*, 2000). This section investigates whether the increased sensitivity to oxidative damage generated by the loss of *CCS* could exacerbate the midgut copper accumulation phenotype caused by *DmATP7* suppression. It has already been shown that *mex-GAL4*-driven *DmATP7* RNAi in combination with *UAS-Ctr1A+B* results in complete lethality, highlighting that the phenotype can be exacerbated by increasing copper levels. *mex-GAL4* driving *CCS* RNAi alone has no effect on viability (data not shown). The effect of the combination of *DmATP7* and *CCS* RNAi on *Drosophila* was assessed on the viability of progeny generated. In comparison with the internal control there was a significant drop in survival observed on normal, 300 µM BCS and 1mM Cu food indicating that *CCS* suppression was worsening the effect of the copper accumulation caused by *DmATP7* suppression (Figure 3.14).

3.2.7 Copper distribution in enterocytes

To further examine copper transport in enterocytes, the role of copper chaperones was explored by investigating the outcome of midgut specific suppression and ectopic expression. *dAtox1*, *Scox* and *dCCS* are homologues of known mammalian copper chaperones and have been implicated in *Drosophila* copper homeostasis. *dAtox1*$^{-/-}$ mutants are lethal on low copper food and likely cause inadequate delivery of copper to *DmATP7* in the midgut (Hua *et al.*, 2011). *Scox*$^{-/-}$ mutant or RNAi suppression phenotypes reflect a role in the Cox network of chaperones required for copper delivery to Cytochrome *c* oxidase (Binks *et al.*, 2010; Porcelli *et al.*, 2010). The role *dCCS* has not been characterized but it is likely the protein is involved in the delivery of copper to SOD1. Also explored here is the putative *Drosophila* chaperone *CutC*, a homologue of

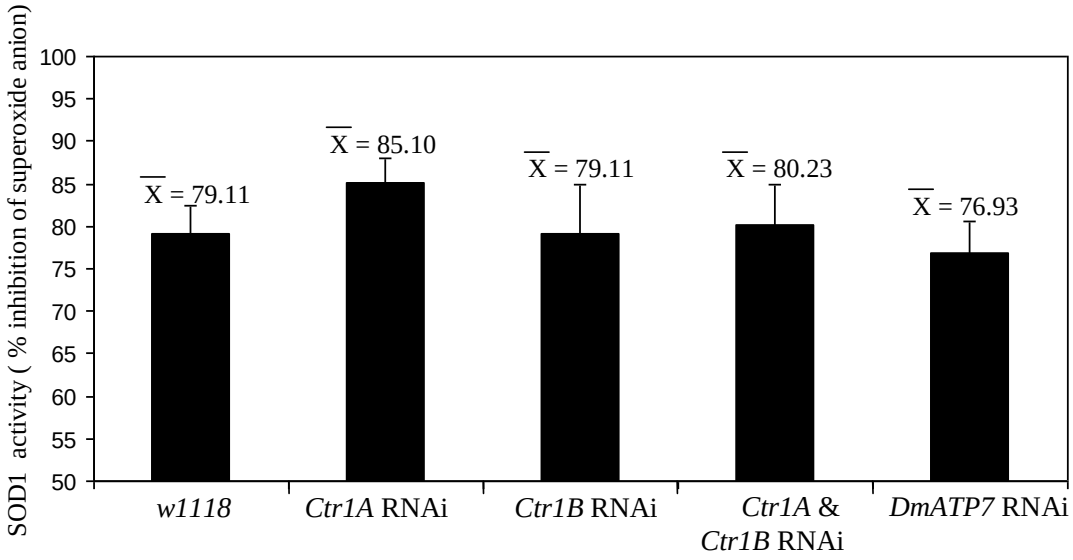

SOD1 activity (% inhibition of superoxide anion)
100
95
90
85
80
75
70
65
60
55
50
X = 79.11
X = 85.10
X = 79.11
X = 80.23
X = 76.93
w1118
Ctr1A RNAi
Ctr1B RNAi
Ctr1A & Ctr1B RNAi
DmATP7 RNAi

Figure 3.12: SOD1 activity of *Drosophila* larvae during midgut suppression of *Ctr1A*, *Ctr1B* or *DmATP7*

SOD1 activity was assayed using a SOD1 determination kit from Sigma which measured the ability of SOD1 to inhibit superoxide anion. Increased SOD1 activity results in inhibition of superoxide anion and a reduction in absorbance at 450nm determined on a 96-well plate reader. Absorbance at 450nm was calculated for each sample as well as three controls, Blank 1 (no sample), Blank 2 (no superoxide anion enzyme) and Blank 3 (no sample or superoxide anion enzyme. SOD1 activity of each sample was calculated as a the percentage of colour-loss compared to the no sample control using the following formula: {[(Ab-Blank 1 – Ab-Blank 3) - Ab-Sample – Ab-Blank 2)]/ (Ab-Blank 1 – Ab-Blank 3)} x 100. Assay samples were created by homogenizing five third instar larvae in grinding buffer before being centrifuged and undergoing a 2-fold dilution. Three absorbance values were calculated for each sample assay and a mean calculated. Each assay was repeated four times to establish the mean and standard error for SOD levels of each genotype. Wild type *Drosophila* (*w1118*) were calculated to have a mean superoxide inhibition activity of 79.11% which equates to a SOD1 concentration of approximately 5 SOD1 units per ml. The SOD1 activity of *Drosophila* third instar larvae containing *mex-GAL4-RNAi* constructs for *Ctr1A*, *Ctr1B* and *DmATP7* was compared to wild-type. There was no significant change associated with suppression of any of *Ctr1A*, *Ctr1B* or *DmATP7*.

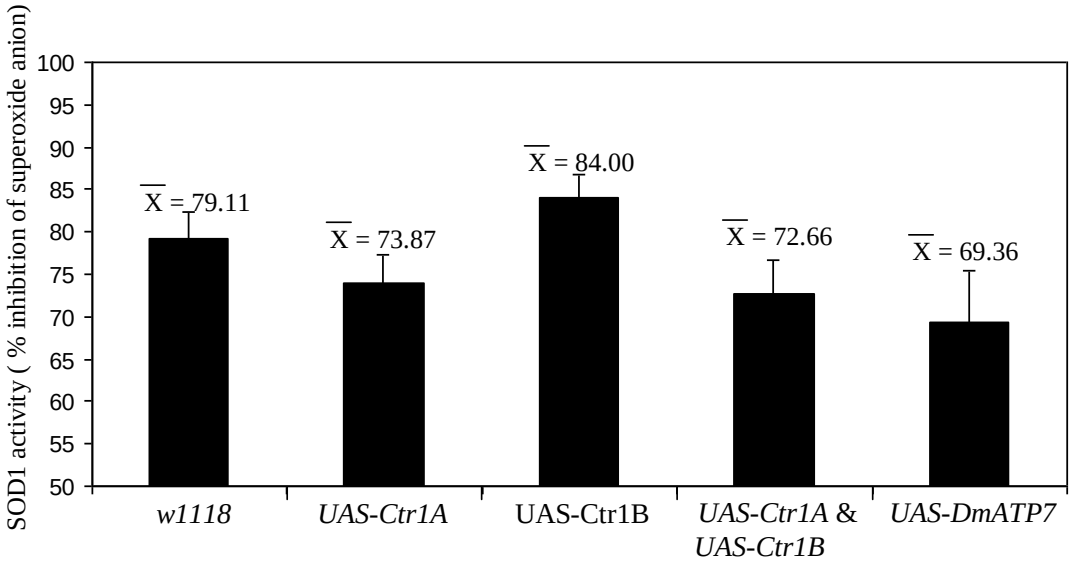

SOD1 activity (% inhibition of superoxide anion)
100
95
90
85
80
75
70
65
60
55
50
X = 79.11
X = 73.87
X = 84.00
X = 72.66
X = 69.36
w1118
UAS-Ctr1A
UAS-Ctr1B
UAS-Ctr1A & UAS-Ctr1B
UAS-DmATP7

Figure 3.13: SOD1 activity of *Drosophila* larvae during midgut ectopic expression of *Ctr1A*, *Ctr1B* or *DmATP7*

SOD1 activity was assayed using a SOD1 determination kit from Sigma which measured the ability of SOD1 to inhibit superoxide anion. Increased SOD1 activity results in inhibition of superoxide anion and a reduction in absorbance at 450nm determined on a 96-well plate reader. Absorbance at 450nm was calculated for each sample as well as three controls, Blank 1 (no sample), Blank 2 (no superoxide anion enzyme) and Blank 3 (no sample or superoxide anion enzyme. SOD1 activity of each sample was calculated as a the percentage of colour-loss compared to the no sample control using the following formula: {[(Ab-Blank 1 – Ab-Blank 3) - Ab-Sample – Ab-Blank 2)]/ (Ab-Blank 1 – Ab-Blank 3)} x 100. Assay samples were created by homogenizing five third instar larvae in grinding buffer before being centrifuged and undergoing a 2-fold dilution. Three absorbance values were calculated for each sample assay and a mean calculated. Each assay was repeated four times to establish the mean and standard error for SOD levels of each genotype. Wild type *Drosophila* (*w1118*) were calculated to have a mean superoxide inhibition activity of 79.11% which equates to a SOD1 concentration of approximately 5 SOD1 units per ml. The SOD1 activity of *Drosophila* third instar larvae containing *mex-GAL4-UAS* constructs for *Ctr1A*, *Ctr1B* and *DmATP7* was compared to wild-type. There was no significant change associated with ectopic expression of any of *Ctr1A*, *Ctr1B* or *DmATP7*. Whilst not significant there was a slight decrease in SOD1 activity during *Ctr1A* and *DmATP7* ectopic expression, whilst a slight increase with *Ctr1B* ectopic expression.

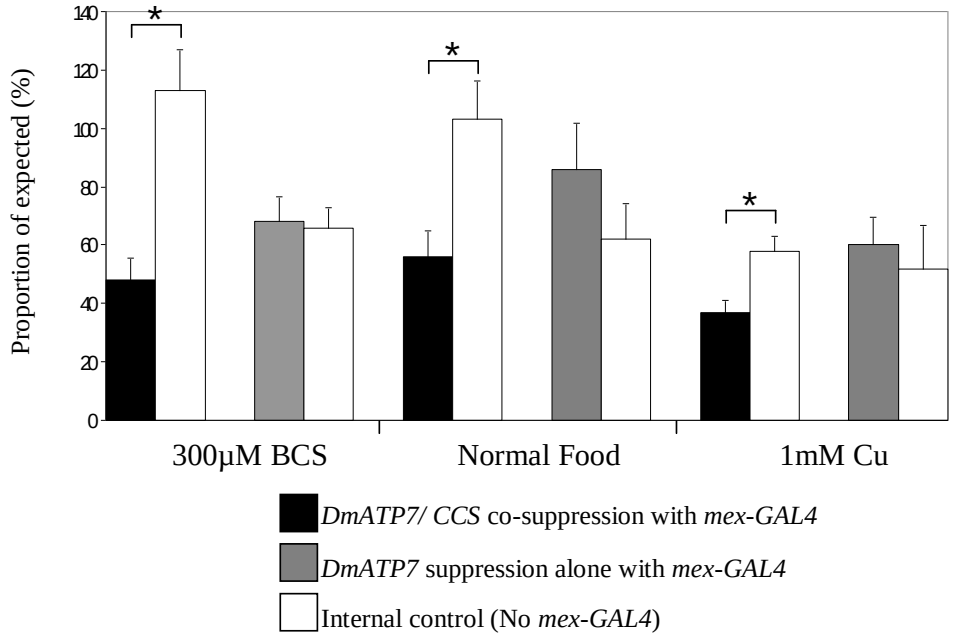

Proportion of expected (%)
140
120
100
80
60
40
20
0
*
*
*
300µM BCS
Normal Food
1mM Cu
DmATP7/ CCS co-suppression with mex-GAL4
DmATP7 suppression alone with mex-GAL4
Internal control (No mex-GAL4)

Figure 3.14: Larvae to adulthood survival of *mex-GAL4;DmATP7* RNAi *Drosophila* in combination with *CCS* RNAi.

Mex-GAL4/Cyo; DmATP7 RNAi lines were crossed to *CCS* RNAi lines in population cages to generate progeny with one copy each of the *mex-GAL4* driver and either RNAi line. Progeny were transferred at the first instar to normal food, 1mM copper and 300µM BCS supplemented food. Surviving adults were scored as either non-Cyo (containing *mex-GAL4*) or Cyo (the no GAL4 control). The genotype numbers were converted to a proportion (%) of the expected progeny as determined by Mendelian genetics. Means and standard errors were determined from four repetitions of each scenario. *mex-GAL4* was compared to the internal control using a simple paired t-test. The *DmATP7* RNAi/ *CCS* RNAi combination was also compared to *DmATP7* suppression alone. The combination of suppressing both *DmATP7* and *CCS* resulted in a significant drop in comparison with the control on all food types (p value < 0.05). There was also a small decrease in survival of the *DmATP7* RNAi/ *CCS* RNAi combination in comparison to *DmATP7* suppression alone on all food types.

the *CutC* implicated in *E.coli* copper homeostasis, which may interact with the transcription factor MTF-1 (Gupta *et al.*, 1995). The effect of the midgut genetic manipulation of these chaperones on *Drosophila* viability and enterocyte copper content was assessed.

3.2.7.1 Suppression of *Scox* and *Ctr1B* in combination results in a drop in viability on low copper food

For suppression and ectopic expression of each copper chaperone, stable compound *mex-GAL4/Cyo; construct/construct* lines were crossed to *+/+; construct/construct* lines in population cages to generate progeny with one copy of the *mex-GAL4* driver and two copies of either the RNAi or *cDNA* construct. Progeny were picked, scored and analysed in the same way as with the import/export experiments and it was found that suppression or ectopic expression of *dAtox1*, *Scox*, *dCCS* or *CutC* resulted in no change to viability on any food type (summarized in Table 3.2). Genetic manipulation of each chaperone was then explored in combination with the suppression and ectopic expression of *Ctr1A*, *Ctr1B* and *DmATP7*. In these experiments, *+/+; Chaperone RNAi* or *cDNA* lines were crossed to *mex-GAL4/Cyo; RNAi / cDNA* lines for *Ctr1A*, *Ctr1B* and *DmATP7*. The combination of *Scox* and *Ctr1B* suppression resulted in a drop in viability on food supplemented with BCS, indicating that reduced dietary copper levels and inhibited import creates a midgut scenario more susceptible to *Scox* suppression (Table 3.2). As already discussed in section 3.2.6.1, the combination of *dCCS* and *DmATP7* suppression resulted in a drop in viability on all food types (Table 3.2).

3.2.7.2 Ectopic expression of *CutC* results in an increased enterocyte copper content

pCtr1B-EYFP and *pMtnB-EYFP* expression was used to determine enterocyte copper content in these chaperone investigations in the same way as in the Ctr1A, Ctr1B and DmATP7 experiments. *pCtr1B-EYFP* expression is triggered when larvae are raised on food supplemented with BCS, whilst *pMtnB-EYFP* expression is elevated on increased

copper food. Both reporter lines were crossed to lines containing *mex-GAL4; RNAi* or *cDNA* constructs for *dAtox1*, *Scox*, *dCCS* and *CutC*. *pCtr1B-EYFP* expression did not change from the wild type during genetic manipulation of the copper chaperones in the midgut (not shown). However, *pMtnB-EYFP* expression was triggered on food supplemented with BCS when *UAS-CutC* was driven by *mex-GAL4*, demonstrating an increase in enterocyte copper content despite the presence of chelator in the food (Figure 3.15).

3.3 Discussion & Conclusion

Drosophila is a useful model organism for investigating copper homeostasis due to the high conservation of copper transport mechanisms and the techniques available in fly genetics (Ryder and Russell, 2003; Southon *et al.*, 2004). Copper import is controlled by Ctr1A and Ctr1B (homologues of hCtr1), whilst DmATP7 (homologue of ATP7A/B) is the export protein (Zhou *et al.*, 2003; Southon *et al.*, 2004). Known or putative copper chaperones such as Atox1, Sco1, CCS and CutC also have equivalent *Drosophila* homologues (Southon *et al.*, 2004). Inactivation of many of these genes results in copper deficient phenotypes such as pigment loss, decreased cuproenzyme activity and lethargy, which mimic phenotypes observed in mammalian and human models (Zhou *et al.*, 2003; Norgate *et al.*, 2006; Turski and Thiele, 2007). Research into the roles of *Drosophila* copper transporters benefits from the GAL4/UAS system which is used to suppress or enhance gene expression in specific cell types. The results presented in this chapter explored the role of copper homeostasis proteins in the midgut and the importance of that role to the organism as a whole.

3.3.1 Midgut suppression of copper import results in enterocyte copper deficiency but no alteration to viability

Ctr1A and Ctr1B have been identified as being the principal sources of cellular copper uptake in *Drosophila* and are therefore the major candidates for copper entry into enterocytes from the lumen. Midgut suppression of *Ctr1A* or *Ctr1B* alone resulted in

Table 3.2: Summary of Mex-Gal4 driven suppression and ectopic expression of copper chaperones in combination with genetic manipulation of *Ctr1A*, *Ctr1B* and *DmATP7*.

	***Atox* RNAi**	***ScoX* RNAi**	***dCCS* RNAi**	***Cut C* RNAi**
Alone	Viable	Viable	Viable	Viable
***Ctr1A* RNAi**	Viable	Viable	Viable	Viable
***Ctr1B* RNAi**	Viable	**Drop in viability on BCS**	Viable	Viable
***DmATP7* RNAi**	Viable	Viable	**Drop in viability**	Viable
UAS-Ctr1A	Viable	Viable	Viable	Viable
UAS-Ctr1B	Lethal on Cu	Lethal on Cu	Lethal on Cu	Lethal on Cu
UAS-DmATP7	Lethal on Cu	Lethal on Cu	Lethal on Cu	Lethal on Cu
	UAS-dAtox1	***UAS-ScoX***	***UAS-dCCS***	***UAS-CutC***
Alone	Viable	Viable	Viable	Viable
***Ctr1A* RNAi**	Viable	Viable	Viable	Viable
***Ctr1B* RNAi**	Viable	Viable	Viable	Viable
***DmATP7* RNAi**	Viable	Viable	Viable	Viable
UAS-Ctr1A	Viable	Viable	Viable	Viable
UAS-Ctr1B	Lethal on Cu	Lethal on Cu	Lethal on Cu	Lethal on Cu
UAS-DmATP7	Lethal on Cu	Lethal on Cu	Lethal on Cu	Lethal on Cu

Table 3.2: Summary of *mex-GAL4* driven suppression and ectopic expression of copper chaperones in combination with genetic manipulation of *Ctr1A, Ctr1B* and *DmATP7*.

Summary of the outcome of midgut-specific suppression and ectopic expression of copper chaperones *dAtox1, ScoX, dCCS* and *CutC* on *Drosophila* viability. Stable compound *mex-GAL4/Cyo; construct/construct* lines were crossed to *+/+; construct/construct* lines in population cages to generate progeny with one copy of the *mex-GAL4* driver and two copies of either the RNAi or *cDNA* construct. Progeny were picked as first instar and transferred to normal food, 1mM copper and 300µM BCS supplemented food. Surviving adults were scored as either non-Cyo (containing mex-GAL4) or Cyo (the no GAL4 control). The genotype numbers were converted to a proportion (%) of the expected progeny. Means and standard errors were determined from four repetitions of each scenario. *mex-GAL4* was compared to the internal control using a simple paired t-test and all results were then summarised. Suppression and ectopic expression alone of *dAtox1, ScoX, dCCS* or *CutC* resulted in no change to viability on any food type. Interactions were also explored between the midgut specific suppression and ectopic expression of copper chaperones with genetic manipulation of *Ctr1A, Ctr1B* and *DmATP7*. In each experiment, *Drosophila* containing suppression and ectopic expression constructs for *dAtox1, ScoX, dCCS* and *CutC* were crossed to *mex-GAL4/Cyo; UAS* or *RNAi* constructs for the transporters for uptake and efflux. The combination of *ScoX* and *Ctr1B* suppression resulted in a drop in viability on BCS supplemented food. The combination of *dCCS* and *DmATP7* suppression resulted in a drop in viability of all food types.

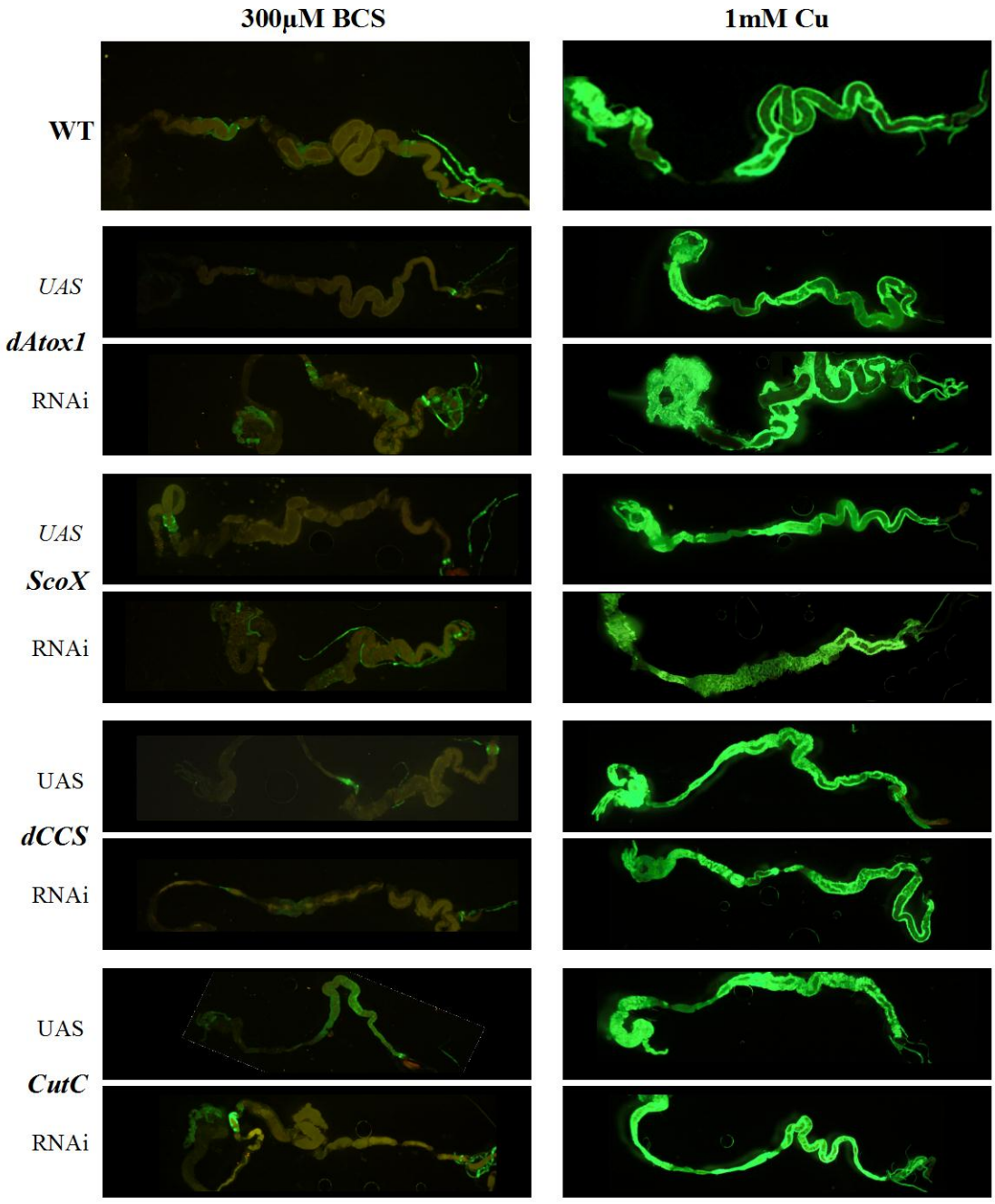

300µM BCS
1mM Cu
WT
UAS
dAtox1
RNAi
UAS
ScoX
RNAi
UAS
dCCS
RNAi
UAS
CutC
RNAi

Figure 3.15: Expression of *pMtnB-EYFP* in the *Drosophila* midgut during manipulation of copper chaperones.

Expression of the reporter construct *pMtnB-EYFP* was used to measure enterocyte copper content in the midgut of developing third instar *Drosophila* larvae. Larvae were dissected to remove the entire gut region, which was mounted and viewed under EYFP fluorescence using the Leica Dissecting Stereoscope. Midguts from wild type flies containing *pMtnB-EYFP* show little yellow/green fluorescence when raised on food supplemented with the copper chelator BCS, yet EYFP expression increases throughout the whole gut region when raised on 1mM Cu supplemented food (A). The effect of either ectopic expression or suppression of *dAtox1* (B), *ScoX* (C), *dCCS* (D) or *CutC* (E) was investigated. *UAS-CutC* resulted in increased expression of *pMtnB-EYFP* expression on 300µM BCS food (E). All other genetic manipulation of the copper chaperones resulted in no change to the wild type. Larvae were dissected to remove the entire gut region, which was mounted and viewed under EYFP fluorescence using the Leica Stereoscope. Images were recorded with a Leica DC300 camera using Leica Application Suite software.

(Transgenic *Drosophila* containing the *pMtnB-EYFP* construct were obtained from Walter Shaffner)

wild type viability on all food types, except for a significant drop caused by *Ctr1A* suppression on 1mM Cu supplemented food. As viability does not change, it might be expected that Ctr1A and Ctr1B can complement the loss of each other. On high copper medium, *Ctr1B* expression is reduced, explaining the drop of viability generated from *Ctr1A* suppression. However, the combination of *Ctr1A* and *Ctr1B* suppression (albeit one copy of each suppression construct) also resulted in no change to viability on all food types. These results infer that suppressing the principal sources of cellular copper import in midgut enterocytes is not detrimental to *Drosophila* when measured by viability. An explanation for this may be the fact that the relative total copper levels (SOD1 activity) of third instar larvae did not alter during *Ctr1A* and/or *B* suppression. Interestingly *Ctr1A* suppression resulted in decreased enterocyte copper content which could then be rescued by either *DmATP7* suppression or *Ctr1B* ectopic expression. When efflux was reduced or a secondary uptake source enhanced, the copper deficiency was rescued. These results suggest that although not impacting the organism as a whole, *Ctr1A* suppression still resulted in less copper import into enterocytes. During *Ctr1B* suppression, enterocyte copper levels remained normal suggesting copper could still enter the cell via Ctr1A.

As copper import is reduced yet viability and total larval copper content remains unchanged, two linked scenarios are proposed: 1) reduced copper entry, as a result of the inhibition of Ctr1A and Ctr1B, is still sufficient for survival through to adulthood and/or; 2) the organism as a whole compensates for low copper import from the midgut lumen. If apical copper entry during *Ctr1A/B* suppression is sufficient for survival, then the question remains how is copper still imported into the enterocyte? It may be possible that not all intestinal *Ctr1A* or *Ctr1B* is knocked down during suppression, yet the RNAi constructs used have previously generated strong phenotypes when driven ubiquitously or in the thorax, abdomen or eye . Homozygous *Ctr1A* null mutants are non-viable yet can be partially rescued via increased dietary copper, inferring that other sources of uptake (presumed to be Ctr1B) can replenish copper needs in many cell types, whilst the homozygous *Ctr1B* null mutant indicates that the Ctr1B protein is only required at times of copper scarcity or toxicity (Zhou *et al.*, 2003; Turski and Thiele, 2007). Both mutant phenotypes suggest that Ctr1A and Ctr1B are the major sources of midgut copper uptake

yet also do not disprove the concept that other avenues of uptake could further supplement enterocyte copper levels in times of need.

The results demonstrated in this chapter add weight to the concept that other avenues of copper import may mitigate the effect of midgut-specific loss of *Ctr1A* and *Ctr1B*. This is consistent with the mammalian system where although hCtr1 is the likely importer of copper into the small intestine, other avenues of import have been postulated (Nose *et al.*, 2006; Zimnicka *et al.*, 2007; Zimnicka *et al.*, 2010). There has been some evidence in mammalian research suggesting that the iron transporter DMT1 and/or anion exchanger channels could be involved in the apical uptake of copper from the lumen of the small intestine (Arredondo *et al.*, 2003; Zimnicka *et al.*, 2010). The *Drosophila* homologue of DMT1, Malvolio, can transport copper *in vitro* and mutants are sensitive to copper excess and limitation (Southon *et al.*, 2008). *Drosophila* homologues of anion exchanger genes are relatively uncharacterized and remain to be tested as possible copper transporters. Both DMT1 and fly anion exchangers are ideal candidates to be further investigated as alternative avenues for apical copper uptake in the *Drosophila* midgut.

Another possible scenario is that the organism as a whole can sense the drop in enterocyte copper levels and adjust accordingly, preventing a total body deficiency. This could involve more efficient use of the limited imported copper by preventing storage in proposed regulatory organs such as the fat bodies or preventing copper excretion. In theory, transcription of *DmATP7* in fat bodies may increase when enterocyte copper levels drop, triggering copper release from the storage organs. Alternatively *DmATP7* expression could decrease in excretion organs such as the malpighian tubules. Both situations might prevent an overall copper deficiency and help maintain viability. This could explain why SOD1 activity levels remained normal during *Ctr1A/B* suppression. There is some evidence that *Drosophila* larvae import extra copper for storage during times of exposure to high dietary levels (Balamurugan *et al.*, 2007). A short exposure to high copper food was able to hasten the development of wild type *Drosophila* larvae otherwise reared on food supplemented with BCS (Balamurugan *et al.*, 2007). *Ctr1B* mutant larvae undergoing the same experiment were unable to take advantage of the

copper pulse (Balamurugan *et al.*, 2007). It was proposed that in wild type larvae, copper could be imported by Ctr1B during the short exposure and stored to be used when the fly returns to the chelator environment. Results in this chapter may demonstrate a reversed situation, whereby when import is limited, copper may be better utilized by preventing storage or limiting excretion.

Although highly speculative there has been some preliminary evidence of serum signals which could communicate between the heart, liver and intestine in mice (Kim *et al.*, 2010). In this case, serum from mice deficient in heart *hCtr1* could induce *ATP7A* expression in the intestine and liver, leading to increased copper in the bloodstream (Kim *et al.*, 2010). Further investigation could determine the expression levels of *ATP7A*, *Ctr1A* and *Ctr1B* in other *Drosophila* organs during midgut suppression of *Ctr1A/B* to test if expression levels change in response to changes to intestinal copper transport.

3.3.2 Ectopic expression of *Ctr1B* in the midgut causes lethality on food supplemented with copper

Ectopic expression of *Ctr1B* in the *Drosophila* midgut resulted in increased enterocyte copper levels and animal lethality when larvae were reared on food supplemented with 1.5mM Cu. It also leads to an increase, although not significant, in SOD1 activity in developing third instar larvae reared on normal food. These results imply that enhancing *Ctr1B* expression leads to both increased enterocyte copper content and increased copper absorption into the lymph. In a wild type midgut, *Ctr1B* expression is activated when dietary copper levels are decreased (Zhou *et al.*, 2003). In times of copper scarcity it is proposed that *Ctr1B* expression is enhanced in the midgut to supplement Ctr1A mediated copper import (Zhou *et al.*, 2003). Therefore it is reasonable to expect that increased *Ctr1B* expression on a high copper load would be detrimental to viability.

Drosophila are likely to be highly susceptible to the effects of ectopic Ctr1B as the protein is thought to almost be exclusively regulated at the transcriptional level and localisation does not shift from the plasma membrane during copper shock treatment

(Balamurugan *et al.*, 2007). On normal food ectopic Ctr1B results in higher enterocyte and whole fly copper content, but as dietary copper levels rise the effects become detrimental to viability. It can be proposed that as more copper is imported there is increased susceptibility to oxidative damage in the midgut and throughout the organism.

Suppression of *Ctr1A* rescues Ctr1B-mediated lethality, implying that ectopic Ctr1B is only detrimental to the organism when in addition to the normal import machinery. The fact that midgut *Ctr1A* ectopic expression does not result in the same phenotype may be due to the fact that Ctr1B is the more efficient copper importer possibly due to increased protein stability (Balamurugan *et al.*, 2007). Ctr1B was found to induce metalliothionein expression in the midgut more rapidly then other uptake sources and therefore is a more proficient importer (Balamurugan *et al.*, 2007). The results in this chapter have demonstrated that increased dietary copper is lethal in combination with rapid import of this copper into the midgut.

3.3.3 Suppression of *DmATP7* causes enterocyte copper toxicity

Midgut-specific suppression of the sole *Drosophila* copper efflux gene, *DmATP7*, resulted in no changes to viability on any food types. This result contrasts with research by Bahadorani *et al* which demonstrated that RNAi silencing of *DmATP7* in the midgut could alter viability. Instead of using *mex-GAL4* and *DmATP7* RNAi obtained from VDRC, they achieved suppression by using the *2020-GAL4* midgut driver to drive expression of the *Sym-pUAST-DmATP7* RNAi silencing construct. As viability was only partially lost in their experiment, it can be proposed that changes in their method resulted in slightly more suppression of *DmATP7*. As the majority of flies suppressing *DmATP7* in the midgut still developed to adulthood in both experiments, other means of enterocyte copper absorption cannot be ruled out. This concept has been explored in mammalian systems where removing ATP7A from intestinal cells in mice using the Cre/Loxp system only resulted in partial lethality (Petris, 2010). Although the mice were unhealthy due to a severe copper deficiency, the survival of some suggests that a secondary low affinity copper exporter could exist in the intestinal epithelium (Petris, 2010).

Bahadorani *et al* also showed that suppression of *DmATP7* in the midgut resulted in lowering the total copper content of adult bodies as measured by AAS. Results in this chapter demonstrated that loss of *DmATP7* in the midgut lead to a slight though not significant drop in SOD1 activity of developing third instar larvae. The fact that the drop in SOD1 activity was not as substantial as the Bahadorani *et al* result is likely due to differences in the assay used. It is likely that atomic absorption spectrometry, as a direct measure of copper, may detect larger differences than the SOD1 assay used here. Future investigations could use AAS or ICP-MS to determine copper content of *mex-GAL4; DmATP7 RNAi* adults and larvae. Despite subtle differences, both assays demonstrate that midgut *DmATP7* suppression results in less copper entering the lymph and rest of the organism. This correlates well with phenotypes associated with *DmATP7* mutants and with the symptoms of Menkes Disease in humans (Mercer, 1998; Norgate *et al.*, 2006).

Midgut suppression of *DmATP7* also resulted in increased copper content of enterocytes, further supporting the notion that the protein is required to prevent copper accumulation in the midgut (Norgate *et al.*, 2006). Both *mex-GAL4; DmATP7 RNAi* and *DmATP7* mutant flies showed increased metallothionein induction in enterocytes indicating copper accumulation (Norgate *et al.*, 2006). It is proposed that blocking the major avenue of copper export leads to less systemic copper delivery as the nutrient becomes trapped in the midgut. Decreasing copper import by co-suppressing either *Ctr1A* or *Ctr1B* with *DmATP7* returns enterocyte copper levels to normal. Correspondingly, increasing import by ectopically expressing both *Ctr1A* and *Ctr1B* exacerbates copper accumulation, ultimately causing lethality. This lethality is likely due to copper accumulating in the midgut to toxic levels causing oxidative damage. This theory is supported by the fact that co-suppression of *CCS* with *DmATP7* results in a significant drop in viability on all food types. Removal of CCS leads to less delivery of copper to SOD1, the key defensive enzyme against oxidative damage. Oxidative damage has been shown to increase the transcription of genes leading to autophagy and ultimately cell death in the *Drosophila* midgut (Wu *et al.*, 2009). Copper toxicity in the midgut of *Drosophila* is likely to form a component of the overall phenotype associated with the *DmATP7* null mutant. This may

also be relevant to the human loss of ATP7A as copper can accumulate in the intestinal epithelia of Menkes sufferers (Mercer, 1998).

3.3.4 Ectopic expression of *DmATP7* in the midgut causes lethality on food supplemented with copper

Midgut ectopic expression of *DmATP7* resulted in lethality when larvae were reared on food supplemented with 50µM Cu. *DmATP7* is normally expressed in a variety of cell types in developing larvae, but is up-regulated specifically in the midgut under increased copper conditions (Burke *et al.*, 2008). This up-regulation is likely due to increased enterocyte copper triggering enhanced export into the lymph. Results in this chapter have shown that further expressing *DmATP7* during increased copper conditions is detrimental to development into adulthood. Possible causes of this lethality are either an enterocyte copper deficiency or copper toxicosis of the lymph and the organs situated in it.

Drosophila midgut-specific ectopic expression of *DmATP7* corresponds with phenotypes observed in the thorax and eye, whereby copper export is increased and the cell becomes deficient in the nutrient (Binks *et al.*, 2010). *pCtr1B-EYFP* results illustrated that increased *DmATP7* expression lead to a copper deficient enterocyte. Although consistent with other cell types, this phenotype is not likely to cause the observed lethality alone as the viable *Ctr1A* suppression flies also demonstrated an enterocyte copper deficiency. It is also proposed that if an enterocyte copper deficiency alone was responsible then there would also have been a lethal phenotype observed when *mex-GAL4;UAS-DmATP7* flies were reared on normal or copper chelated food.

Enterocytes differ from other cell types as the cell has the dual roles of supplying its own intracellular copper needs and also controlling the absorption of dietary copper from the intestinal lumen into the lymph. The role of DmATP7 to export copper out of enterocytes is crucial in providing adequate copper to the lymph and other organs. Lethality caused by enhancing enterocyte *DmATP7* expression on high copper load may be due to increased copper delivery to the lymph resulting in toxicosis. Copper toxicosis

is major component of Wilson's disease in humans where the copper levels increase in the liver before the release of free ions into the bloodstream, brain and other major organs (DiDonato and Sarkar, 1997; Huster, 2010). The phenotype observed in *mex-GAL4; UAS-DmATP7* flies on high copper load may be similar to that suffered by Wilson's disease patients. To assess this similarity, future investigations could use neurological assays, such as climbing tests, to determine if enhanced copper export from enterocytes can lead to increased copper levels in the brain and CNS. One contradiction to this is the fact that there was no significant alteration to the SOD1 activity in Mex; UAS-*DmATP7* larvae. It could be that the level of SOD1 is rate limiting and that illustrating a copper toxic situation may be difficult. This also needs to be further investigated when larvae are reared on high copper medium as it is likely that on normal food the effect would not be as severe. The results discussed in this section illustrate that *Drosophila* struggle to cope with increased copper absorption, possibly because wild type flies are generally reared in environments where copper scarcity is more of a threat than excess.

3.3.5 Some preliminary evidence of the midgut role of Scox and CutC

Results in this chapter also sought determine the role of the copper chaperone network in the *Drosophila* midgut. Decreased copper import (via suppression of *Ctr1B)* and low dietary copper levels created an enterocyte more susceptible to *Scox* suppression. *Scox* is part of system of proteins required to provide copper to the cuproenzyme *Cytochrome c oxidase.* Suppression of *Scox* in the thorax/abdomen resulted in a phenotype suggestive of reduced copper delivery to *Cytochrome c oxidase* and the result presented here supports a similar role for Scox in the midgut (Binks *et al.,* 2010). Ectopic expression of *CutC* resulted in increased enterocyte copper content and preliminary unpublished data has indicated that CutC is localized in the nucleus, where it may interact with the transcription factor MTF-1. Although highly speculative this result may suggest that increased CutC can induce MTF-1 to stimulate transcription of Ctr1B, resulting in increased copper import.

3.3.6 Conclusion

This chapter has demonstrated that the enterocytes represent a unique cell type in *Drosophila*, fuelling their own cellular copper needs whilst also containing the mechanisms required for dietary copper to enter the fly. Copper import into the cell is likely controlled by Ctr1A and Ctr1B, yet other mechanisms may be able to supplement the cell in times of copper need. Reducing this import can cause enterocyte copper deficiency but is not detrimental to the organism as whole. Increasing export leads to copper toxicity in both the enterocyte and throughout the organism. DmATP7 is responsible for the majority of copper export out of the cell into the lymph, providing copper throughout the fly, but also for preventing toxic copper levels in the midgut. It was found that enhancing export on a high copper load is detrimental to the organism, revealing that the lymph and major organs are sensitive to high copper content.

Midgut copper absorption as a whole is vitally important to survival of the organism but appears to be a flexible process that can be supplemented by many sources. Mutant phenotypes generated by the loss of function of Ctr1A, Ctr1B and DmATP7 were more severe than those observed when suppressed in the midgut alone. This implies that the loss of each protein in other tissues such as the brain, CNS and fat bodies also contributes to the loss of function phenotype

Results from this chapter also lead to the hypothesis that *Drosophila* are capable of surviving scenarios where midgut copper absorption is limited, but are susceptible when absorption is enhanced. This may be due to the fact that wild type *Drosophila* are generally reared in environments where copper is scarce rather than in excess. This concept provides the foundation for future investigation into the mechanics of copper absorption and also raises many questions as to the transportation of copper once it has entered the lymph.

CHAPTER FOUR: LOCALIZATION OF COPPER TRANSPORTERS IN THE *DROSOPHILA* MIDGUT

Synopsis

This chapter explores the localization of Ctr1A, Ctr1B and DmATP7 in *Drosophila* enterocytes supporting functional analyses and helping determine the midgut-specific role of each protein. Ctr protein localization is shown to be predominantly at the apical membrane however there is some protein at the basolateral membrane. DmATP7 localizes to either the basolateral membrane or intracellular locations which include the TGN. This chapter also determines localization of these proteins in the secretory cells of salivary glands and the principal cells of malpighian tubules.

4.1 Introduction

4.1.1 Determining the midgut localization of copper transporters is important for understanding midgut-specific function

An important component to understanding the mechanisms which control absorption of copper by the *Drosophila* midgut is determining the localization of key transporters for uptake and efflux. This chapter investigates the localization of Ctr1A, Ctr1B and DmATP7 in enterocytes, supporting the functional analyses presented in Chapter Three to establish a model for midgut copper transport. In humans, identifying the intracellular localization of copper transporters hCtr1, ATP7A and ATP7B has been a crucial aspect in determining the cell-specific function of each protein (Petris *et al.*, 1996; Klomp *et al.*, 2002; Mercer *et al.*, 2003). Intracellular localization can reveal where a protein is functioning and the role it is playing in cell-specific homeostasis. Determining the localization of copper transport proteins in the polarized enterocyte cells lining the epithelial barrier has lead to a greater understanding of intestinal transport (Nyasae *et al.*, 2007; Zimnicka *et al.*, 2007; Nose *et al.*, 2010).

hCtr1 cycles between the outer plasma membrane and intracellular organelles located in the perinuclear region, consistent with a role in cellular copper import (Klomp *et al.*, 2002). Typically in polarized cells hCtr1 is localized at the apical membrane, however, in intestinal enterocytes the protein has separately been shown to localize to both the apical and basolateral membranes (Zimnicka *et al.*, 2007; Nose *et al.*, 2010). Contrasting localizations lead to different assumptions about the intestine-specific function of hCtr1. Apical localization suggests a role in copper absorption from the lumen, whilst basolateral localization suggests hCtr1 could import copper from the bloodstream. This highlights the importance of determining the midgut localization for Ctr1A and Ctr1B in *Drosophila*.

ATP7A/B localize to the TGN at basal copper levels, shifting to the outer plasma membrane following stimulation by copper or post-translational modification (Petris *et al.*, 1996; Mercer *et al.*, 2003). ATP7A can relocalize to endocytic vesicles at the

basolateral membrane of cells, whilst ATP7B can shift to the canaicular (apical) membrane (Guo *et al*, 2005, Monty *et al*, 2005). This remains consistent in intestinal cells where ATP7A localizes to the TGN but under copper stress shifts to vesicles adjacent to the basolateral membrane, with a small portion at the actual membrane surface (Nyasae *et al.*, 2007). Intestinal localization of ATP7A indicates a dual role, fueling cell-specific requirements at the TGN and exporting copper out of enterocytes into the bloodstream. This chapter establishes the *Drosophila* midgut-specific localization of Ctr1A, Ctr1B and DmATP7, whilst also investigating other *Drosophila* polarized cell types such as the salivary glands and malpighian tubules.

4.1.2 Creation of fluorescent-tagged fusion proteins

Determination of localization was facilitated by the GAL4/UAS system (described in detail in Chapter Three), whereby fluorescent-tagged *UAS*-expression constructs (fusion proteins) are driven in a tissue specific manner. Fusion proteins were created by initially PCR amplifying the targeted gene from w^{1118} *Drosophila cDNA* but cloning and microinjection was completed using a different system to that previously described. Here, each PCR product was cloned in-frame with either an N or C terminal fluorescent-tag into the pUAST-attb vector. Vectors with the correct insert were confirmed by restriction digest and sequencing, before being microinjected into PhiC31 attP 51C and 96E strains of *Drosophila.* Transgenic flies were identified by the presence of the $white^{+}$ marker gene, contained on the pUAST-attB vector, which results in yellow/orange/red coloured eyes. Stable lines were then generated containing the inserted *UAS-cDNA* constructs. For Ctr1A and Ctr1B, an eGFP tag was attached at the C-terminus, whilst for DmATP7 a mCherry (red) tag was attached at the N-terminus.

4.2 Results

4.2.1 Fusion protein constructs show copper transport activity

As creation of fusion proteins differed from the previously used ectopic expression constructs (the addition of a fluorescent tag), their function was investigated. As the insertion of a fluorescent tag can alter the function of the targeted protein, Ctr1A, Ctr1B and DmATP7 fusion proteins had to be confirmed as functional before being used to investigate localisation.

pannier-GAL4 driven expression was used to assess the function of the fluorescent-tagged fusion proteins. Ectopic expression of *DmATP7* in adult thorax/abdomen using *pannier-GAL4* results in a copper deficiency phenotype which includes hypopigmentation, bristle loss, reduction of scutellum and a small thoracic cleft (Norgate *et al.*, 2007) (Figure 4.1). This phenotype can be rescued by increasing copper uptake with co-expression of *Ctr1A* or *Ctr1B* (Figure 4.1 B) (Binks *et al.*, 2010). The midline ectopic *DmATP7* phenotype and subsequent rescue by increased uptake form the basis for determining if the fluorescent-tagged fusion proteins are functional.

Expression of *mCherry-DmATP7* driven by *pannier-GAL4* replicates the ectopic *DmATP7* phenotype as the flies have a strong band of hypopigmentation down the thorax/abdomen, some bristle loss, reduction in scutellum and a small thoracic cleft (Figure 4.1). Co-expression of *UAS-Ctr1A* with *mCherry-DmATP7* using *pannier-GAL4* results in a complete rescue of the phenotype (Figure 4.1). Both results confirm that the mCherry-DmATP7 fusion protein functions the same way as DmATP7-FLAG, supporting the validity of localizations observed using this construct.

Expression of either *Ctr1A-GFP* or *Ctr1B-GFP* driven by *pannier-GAL4* has no phenotype alone (not shown), similar to the FLAG-tagged versions of these genes. However both fusion proteins are able to rescue the copper deficiency phenotype caused by ectopic *DmATP7* under *pannier-GAL4* control (Figure 4.1). This verifies that both fusion proteins function in the same way as Ctr1A-FLAG and Ctr1B-FLAG.

4.2.2 The Ctr proteins are primarily localized to the apical membrane of intestinal enterocytes, but some Ctr1A is located at the basolateral membrane

The *Drosophila* midgut is made up of enterocytes, polarized cells which together with adjacent interstitial cells form an epithelial barrier between the lumen and lymph (Figure 4.2). Ingested food travels through the lumen and into enterocyte invaginations whereby nutrients can be absorbed across the apical membrane into the cell and then across the basolateral membrane into the lymph.

To investigate localization in the *Drosophila* midgut, fusion proteins were expressed in cell-specific manner using *mex-GAL4*. *mex-GAL4*; *Ctr1A/B-GFP* larvae were raised on normal, 1mM Cu and 300µM BCS food then dissected to remove the entire midgut region, which was mounted and viewed using the Nikon C1 Upright Confocal Microscope. Ctr1A-GFP and Ctr1B-GFP both localize predominantly to the apical membrane of intestinal enterocytes when compared to the nuclear Dapi staining (Figure 4.2 and 4.3). There is also some signal of both proteins adjacent to or at the basolateral membrane (Figure 4.2 and 4.3). Predominant apical localization shows that both uptake proteins are capable of importing copper from the lumen into enterocytes. Basolateral Ctr implies that copper may also be transported from the lymph back into enterocytes. When larvae were raised on copper-supplemented or chelated food there was no change to localisation.

4.2.3 DmATP7 localizes predominantly to the basolateral membrane in intestinal enterocytes

To determine midgut-specific localization of DmATP7, *mex-GAL4; mCherry-DmATP7* larvae were raised on normal, 1mM Cu and 300µM BCS food and dissected as above. mCherry-DmATP7 localizes predominantly to the basolateral membrane of intestinal enterocytes consistent with a role in copper export into the lymph (Figure 4.4). There is

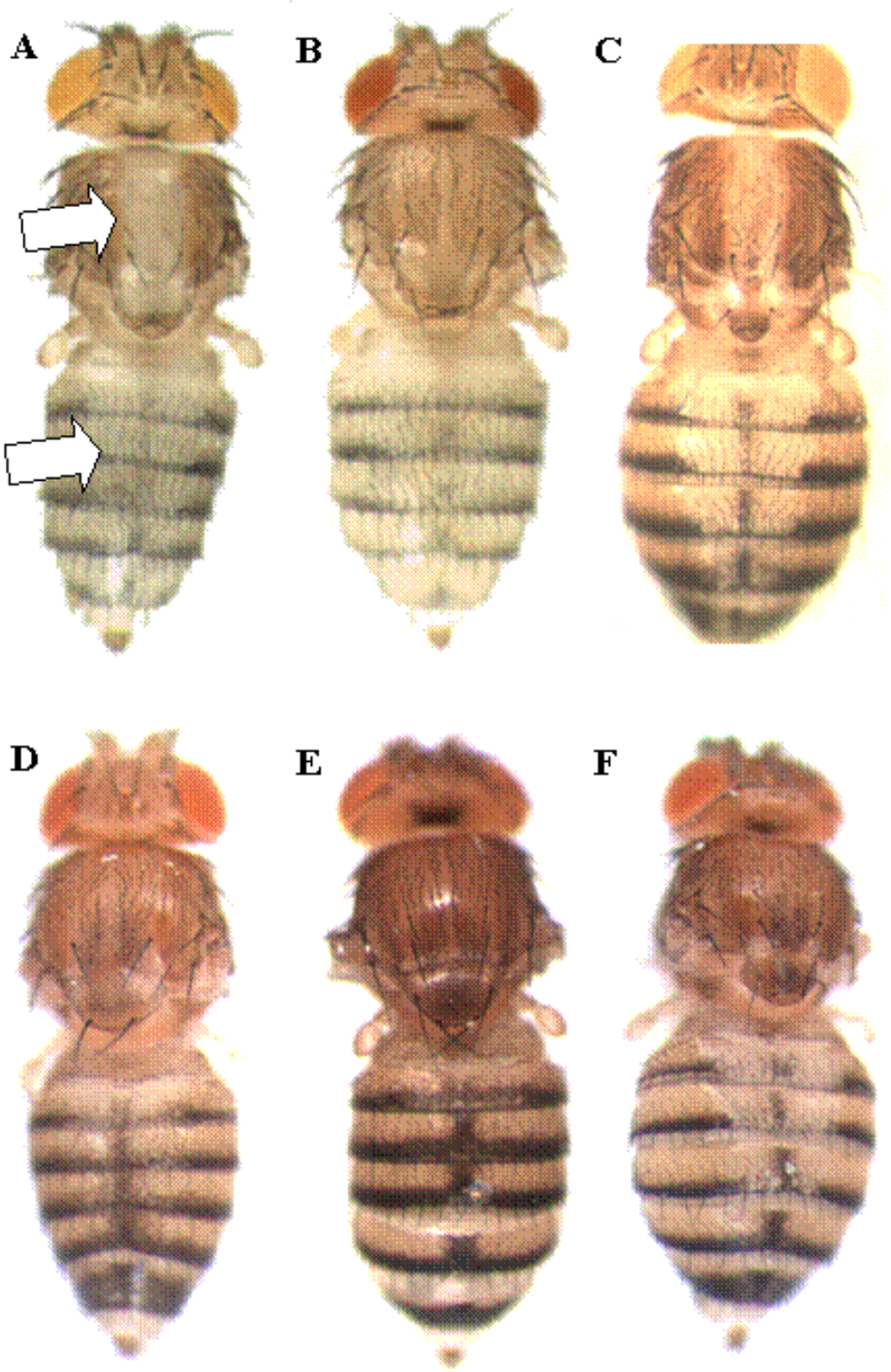


Figure 4.1: Fusion proteins for Ctr1A, Ctr1B and DmATP7 are functional

Figure 4.1: Fusion proteins for Ctr1A, Ctr1B and DmATP7 are functional

To explore whether the *mCherry-DmATP7* fusion protein was functional the construct was crossed to *pannier-GAL4* and the thorax/abdomen was compared to the observed *UAS-DmATP7* phenotype. *pannier-GAL4* driven expression of *UAS-DmATP7* results in a copper deficiency phenotype that includes hypopigmentation, bristle loss, reduction of scutellum and a small thoracic cleft (A – as shown by the arrows). Expression of *mCherry-DmATP7* resulted in a similar phenotype implying shared function between both constructs (C). The thorax/abdomen copper deficiency phenotype can be rescued increasing uptake by also ectopically expressing *Ctr1A* (B). This phenotype rescue was also observed when *pannier-GAL4*; *mCherry-DmATP7* was crossed to *UAS-Ctr1A* (D). The Ctr1A-GFP and Ctr1B-GFP fusion proteins were also deemed functional as both could rescue the *pannier-GAL4*; *UAS-DmATP7* phenotype (E + F). For all photos, two-day old *Drosophila* adults had their legs and wings removed before imaging. Flies were observed using the Leica MZ6 dissecting microscope, imaged using the Leica DFC295 camera and analysed using Leica IM50 software.

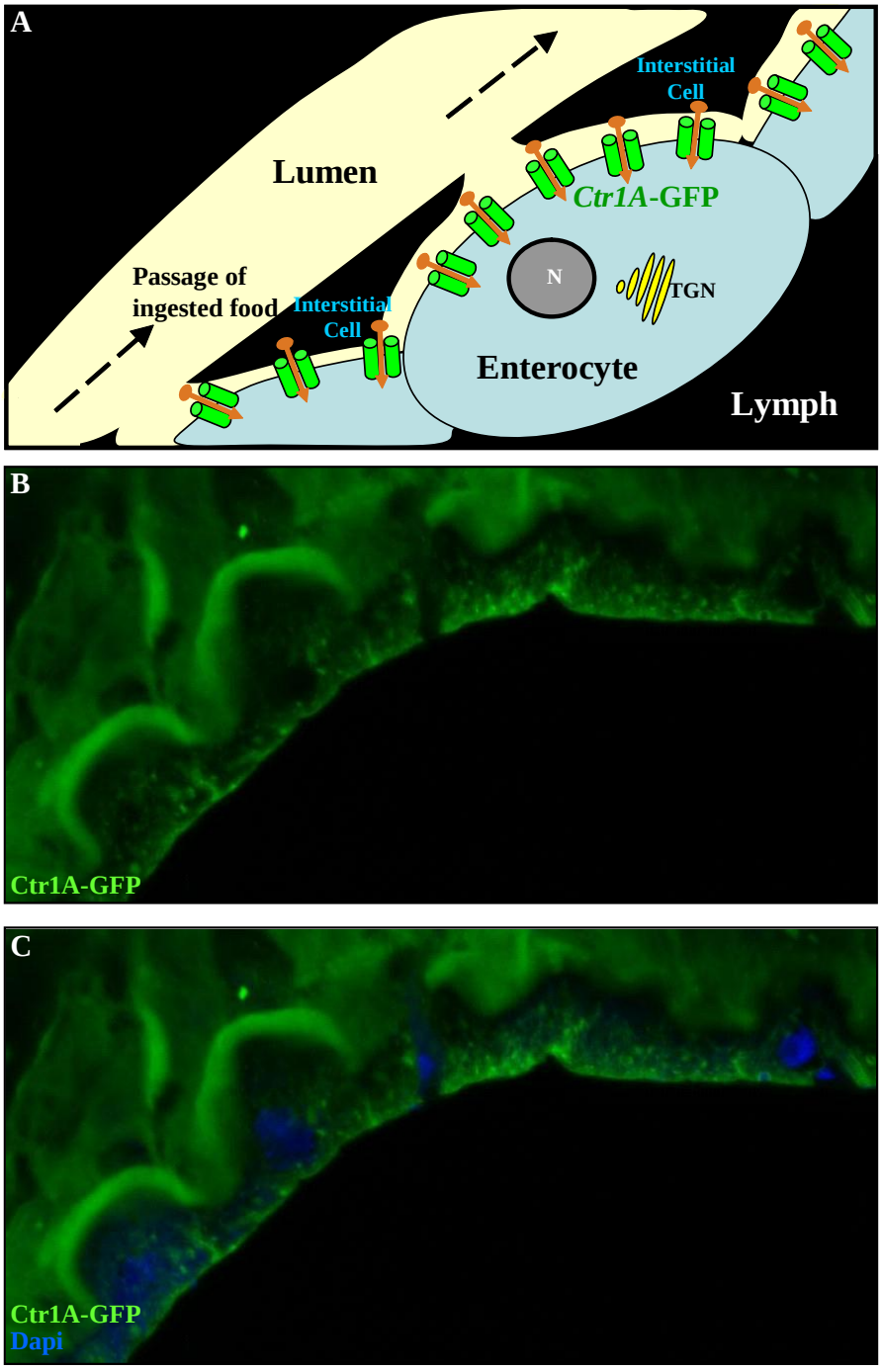

A
Interstitial Cell
Lumen
Ctr1A-GFP
N
TGN
Passage of ingested food
Interstitial Cell
Enterocyte
Lymph
B
Ctr1A-GFP
C
Ctr1A-GFP
Dapi

Figure 4.2: Both apical and basolateral localization of Ctr1A in *Drosophila* midgut enterocytes

Ctr1A-GFP expressed under the control of *mex-GAL4* in *Drosophila* midgut enterocytes is shown in green (B+C) and Dapi (nuclear) staining is shown in blue (C). Ctr1A localized to the apical membrane of midgut enterocytes as described in a schematic (A) and observed (B+C). There is also some Ctr1A-GFP signal adjacent to or at the basolateral membrane (C). Dissected midguts from third instar larvae were unfixed and viewed at 40X using the Nikon C1 Upright Confocal Microscope (B+C). Images were analysed using NIS-Elements and ImageJ software.

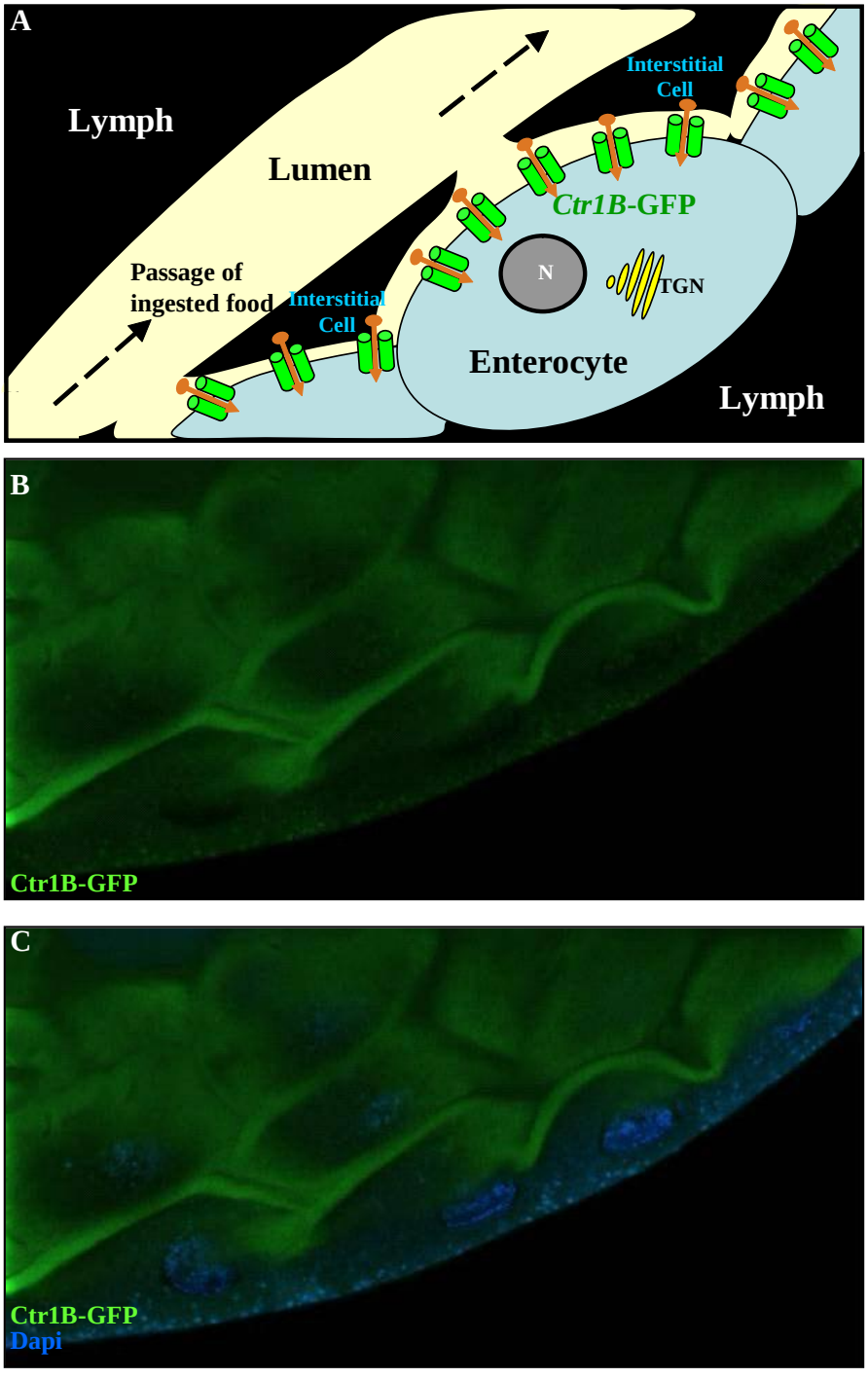
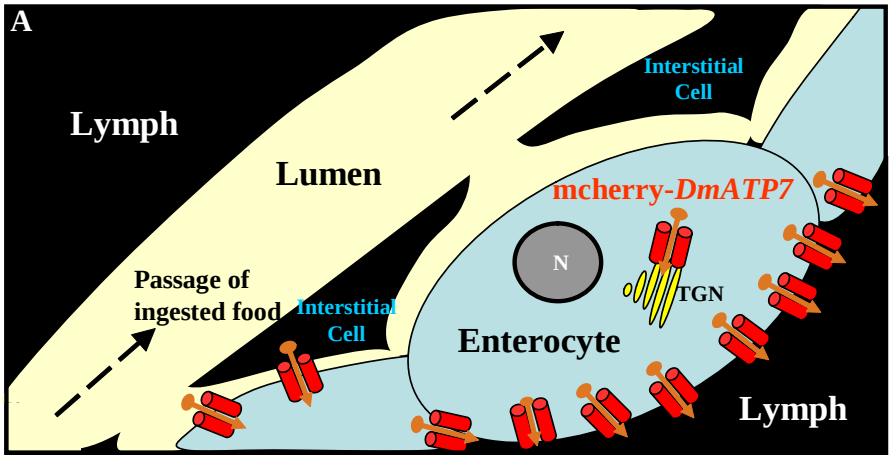
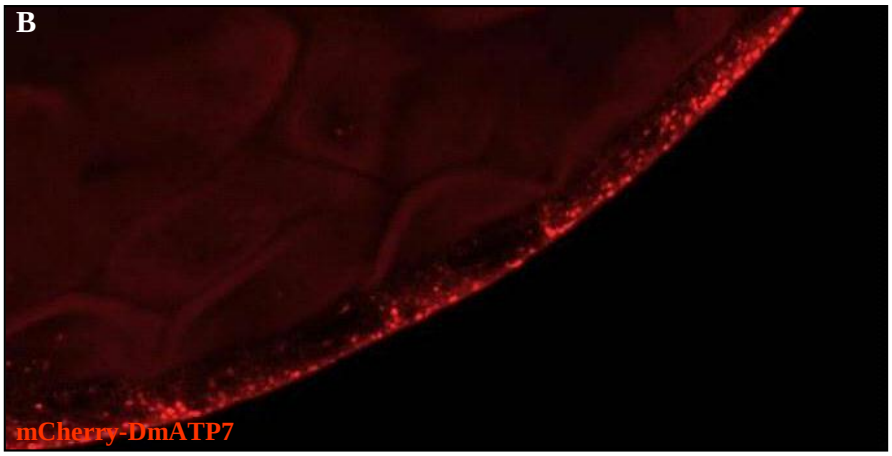

A
Lymph
Lumen
Interstitial Cell
Ctr1B-GFP
N
TGN
Passage of ingested food
Interstitial Cell
Enterocyte
Lymph
B
Ctr1B-GFP
C
Ctr1B-GFP
Dapi

Figure 4.3: Apical localization of Ctr1B in *Drosophila* midgut enterocytes

Ctr1B-GFP expressed under the control of *mex-GAL4* in *Drosophila* midgut enterocytes is shown in green (B+C) and Dapi (nuclear) staining is shown in blue (C). Ctr1B localized to the apical membrane of midgut enterocytes as described in a schematic (A) and observed (B+C). There is also some Ctr1B-GFP signal adjacent to or at the basolateral membrane (C). Dissected midguts from third instar larvae were unfixed and viewed at 40X using the Nikon C1 Upright Confocal Microscope (B+C). Images were analysed using NIS-Elements and ImageJ software.


A
Lymph
Lumen
Interstitial Cell
mcherry-DmATP7
N
TGN
Passage of ingested food
Interstitial Cell
Enterocyte
Lymph


B
mCherry-DmATP7

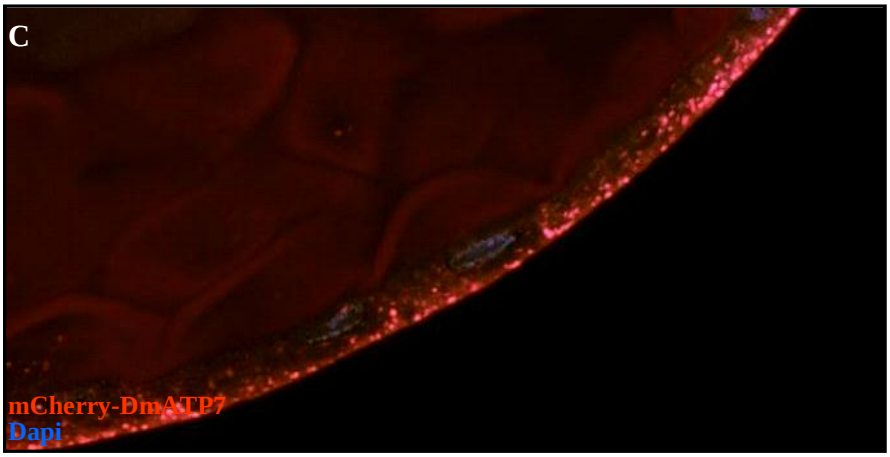

C
mCherry-DmATP7
Dapi

Figure 4.4: Basolateral localization of DmATP7 in *Drosophila* midgut enterocytes

mCherry-DmATP7 expressed under the control of *mex-GAL4* in *Drosophila* midgut enterocytes is shown in red (B+C) and Dapi (nuclear) staining is shown in blue (C). DmATP7 predominantly localized to the basolateral membrane of midgut enterocytes as described in the schematic (A) and observed (B+C). There is also some intracellular localization of DmATP7 (A-C) (investigated later in this chapter). Dissected midguts from third instar *Drosophila* larvae were viewed at 40X using confocal microscopy (B+C). Images were analysed using NIS-Elements and ImageJ software.

some mCherry-DmATP7 localized to internal regions of the intestinal enterocytes and this localization is investigated in further detail in section 4.2.5. When larvae were raised on copper-supplemented or chelated food there was no change to localisation. To demonstrate the distinct apical and basolateral membrane of enterocytes, *mex-GAL4; mCherry-DmATP7* was co-expressed with *Ctr1B-GFP* (Figure 4.5). mCherry-DmATP7 was predominantly localized at the basolateral membrane, with Ctr1B-GFP seen at the apical membrane. TGN, basolateral and apical signals did not overlap, implying location-specific targeting of each protein. This also demonstrated that the basolateral and apical membranes are distinctly separated in polarized enterocytes.

4.2.4 Localization of copper transporters is consistent in other polarized cell types

The localization of Ctr1A, Ctr1B and DmATP7 in enterocytes reflected the hypothesized function of each protein in the midgut. In order to assess whether this would be consistent in other *Drosophila* polarized cell types, localization was explored in the salivary glands and malpighian tubules. The salivary glands are the key secretory organ in *Drosophila* larvae and consist of two polarized cell types, duct and secretory cells (Andrew, 2000). Duct cells form a connective tube during early development which joins the mouthparts to the secretory cells. Secretory cells are large, columnar cells which surround the tubular duct, secreting proteins into the lymph (Andrew, 2000). *Ctr1A*, *Ctr1B* and *DmATP7* are all expressed, albeit not at high levels, in the larval salivary gland implying localization is functionally relevant (Chintapalli *et al.*, 2007)

To determine the salivary gland-specific localization of Ctr1A, Ctr1B and DmATP7, *pannier-GAL4* driven expression of each fusion protein was investigated and larvae were reared on normal, 1mM Cu and 300µM BCS food. Third instar larvae were dissected to remove the salivary glands, which were mounted and viewed using the Nikon C1 Upright Confocal Microscope. Both Ctr1A-GFP and Ctr1B-GFP located to the apical side (adjacent to the duct) of secretory cells, implying copper is imported into secretory cells from the lumen/duct of the salivary glands (Figure 4.6). Ctr1B-GFP also show some

intracellular signal suggestive of a secondary role or internalisation of the protein (Figure 4.6). Localization of mCherry-DmATP7 is predominantly at the basolateral membrane of secretory cells, whilst there is some located in intracellular regions (Figure 4.6). Basolateral location suggests salivary gland DmATP7, like in enterocytes, exports copper into the lymph, whilst the intracellular protein may be functioning at the TGN (further investigated in section 4.2.5). Localization of any of Ctr1A, Ctr1B or DmATP7 did not shift under increased or decreased copper conditions. Co-localisation analysis was able to clearly define an apical membrane and basolateral membrane of the secretory cells by demonstrating that the GFP and mCherry signals are separate and do not overlap.

Drosophila malpighian tubules excrete toxic substances from the midgut and maintain ionic balance (Wessing, 1999). The major functions of the tubules are facilitated by the principal cells, which are separated by smaller stellate cells (Wessing, 1999). There is high expression of *Ctr1A* and *DmATP7* in larval malpighian tubules, whilst *Ctr1B* expression is also seen at lower levels (Chintapalli *et al.*, 2007). In this experiment, *C42-GAL4* was used to drive expression of each fusion protein in the principal cells of malpighian tubules. Ctr1A-GFP and Ctr1B-GFP are both largely localized to the apical membrane of principal cells, corresponding with the apical-GFP marker (Figure 4.7).
There is much more internal Ctr-GFP signal than the apical-GFP marker (Figure 4.7).
Predominant Apical localization indicates a role in importing copper into principal cells from the inner duct which is attached to the hindgut. mCherry-DmATP7 mostly localized to the basolateral membrane of principal cells but was also observed in a peri-nuclear region. DmATP7 location reflects an intracellular role as well as exporting copper into the lymph (Figure 4.7). Ctr1A, Ctr1B or DmATP7 localization did not alter under increased or decreased copper conditions. Co-localization analysis demonstrated separate apical and basolateral signals, but more overlap was observed than in the midgut or salivary glands (Figure 4.7).


A
Ctr1B-GFP
B
mCherry-DmATP7
C
mCherry-DmATP7
Ctr1B-GFP
Dapi

Figure 4.5: Comparing the localization of Ctr1B (apical) and DmATP7 (basolateral) in *Drosophila* midgut enterocytes

Ctr1B-GFP and *mCherry-DmATP7* both expressed under the control of *mex-GAL4* in *Drosophila* midgut enterocytes is shown in green and red respectively (A-C). Dapi (nuclear) staining is shown in blue (C). Dissected midguts from third Instar *Drosophila* larvae were viewed at 40X using confocal microscopy (A-C). In midgut enterocytes, Ctr1B localized to the apical membrane and DmATP7 predominantly to the basolateral membrane. There is little co-localization observed between the proteins and clear distinction between the apical and basolateral membranes. Images were analysed using NIS-Elements and ImageJ software.

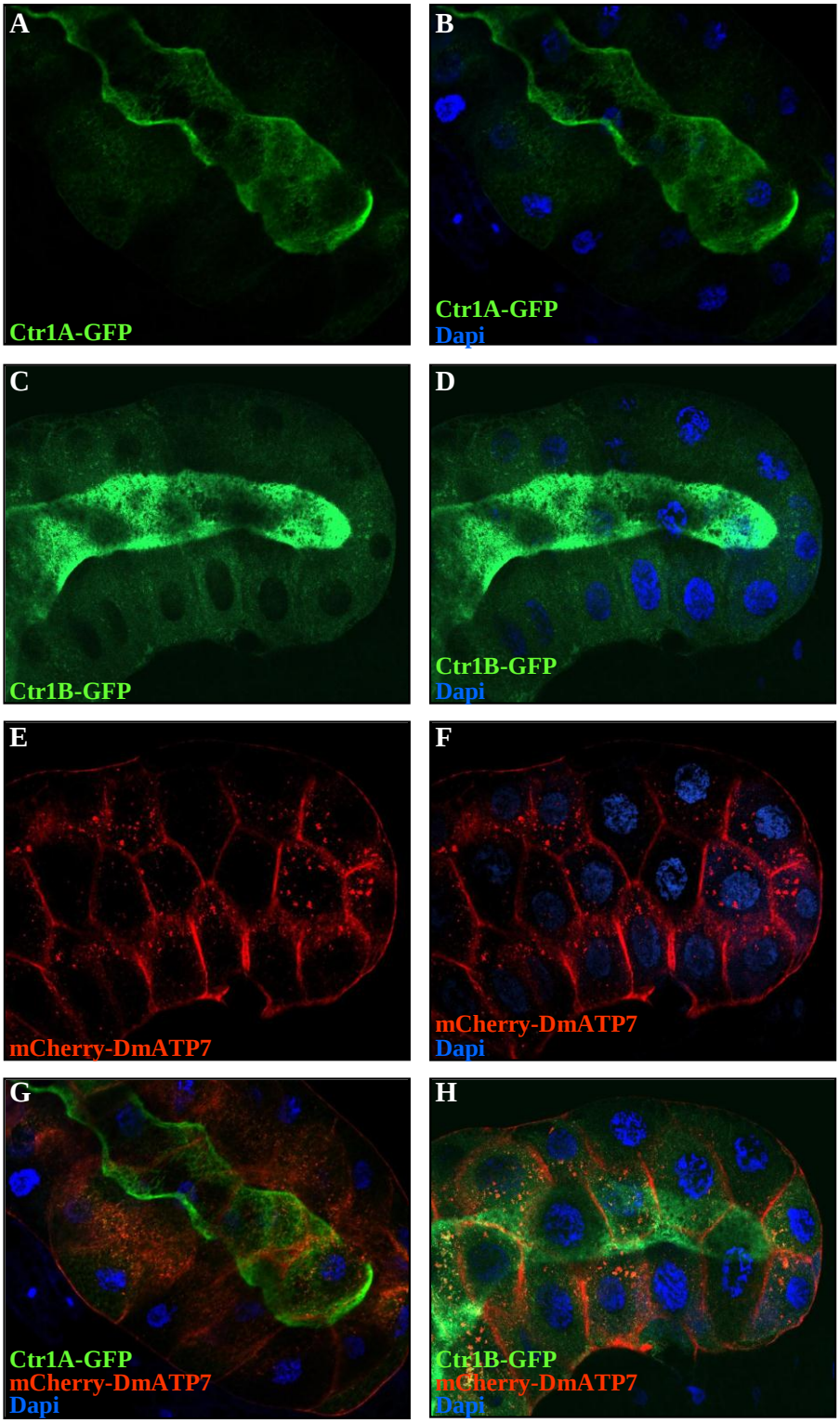

A
Ctr1A-GFP
B
Ctr1A-GFP
Dapi
C
Ctr1B-GFP
D
Ctr1B-GFP
Dapi
E
mCherry-DmATP7
F
mCherry-DmATP7
Dapi
G
Ctr1A-GFP
mCherry-DmATP7
Dapi
H
Ctr1B-GFP
mCherry-DmATP7
Dapi

Figure 4.6: The localization of Ctr1A, Ctr1B and DmATP7 in secretory cells of the salivary glands

Ctr1A-GFP, *Ctr1B-GFP* and *mCherry-DmATP7* were expressed under the control of *pannier-GAL4* in the secretory cells of third instar larval salivary glands. Dissected salivary glands were viewed at 40X using confocal microscopy and images were analysed using NIS-Elements and ImageJ software. Ctr1A and Ctr1B were located at the apical side of secretory cells, adjacent to the inner duct, when observed in contrast to the Dapi nucleus stain (blue) (A-D). There is also some intracellular Ctr1B (C+D). DmATP7 is located at the basolateral membrane of the secretory cells (E+F). Co-localization analysis confirmed that the uptake and efflux proteins localized distinctly at separate membranes in *Drosophila* salivary glands (G+H).

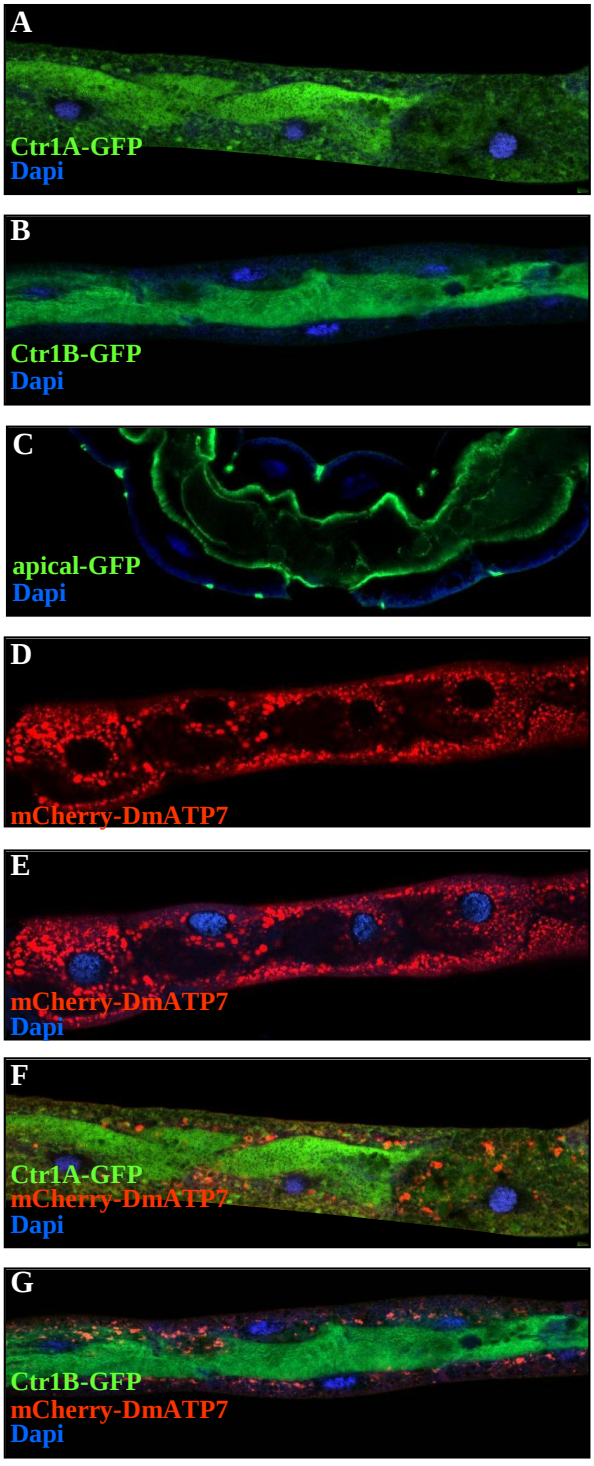

A
Ctr1A-GFP
Dapi
B
Ctr1B-GFP
Dapi
C
apical-GFP
Dapi
D
mCherry-DmATP7
E
mCherry-DmATP7
Dapi
F
Ctr1A-GFP
mCherry-DmATP7
Dapi
G
Ctr1B-GFP
mCherry-DmATP7
Dapi

Figure 4.7: The localization of Ctr1A, Ctr1B and DmATP7 in stellate cells of the *Drosophila* malpighian tubules

Ctr1A-GFP, *Ctr1B-GFP* and *mCherry-DmATP7* were expressed using *C42-GAL4* in the stellate cells of the *Drosophila* malpighian tubules. Dissected malpighian tubules from third instar larvae were viewed at 40X using confocal microscopy and images were analysed using NIS-Elements and ImageJ software. Ctr1A and Ctr1B predominantly localized to the inner apical membrane of the malpighian tubule cells in comparison with Dapi nuclear marker (blue) (A+B). Although predominantly matching the localization pattern of the Apical-GFP marker, there was more intracellular signal observed with both Ctr proteins (A-C). DmATP7 localized to both the basolateral membrane and in the peri-nuclear region of malpighian tubule cells (D+E). Co-localization analysis partially demonstrated that the uptake and efflux proteins localized to separate membranes in *Drosophila* malpighian tubules (F+G). There was still regions in the cells where there was overlap observed.

4.2.5 Investigating intracellular DmATP7

DmATP7, like mammalian Cu-ATPases, is proposed to play two roles, delivering copper to the secretory pathway at the TGN and exporting copper from the cell at the outer plasma membrane. In the polarized cells investigated in this chapter DmATP7 has been found to be located primarily at the basolateral membrane but also at intracellular regions, hypothesized to be the TGN. To help identify whether there was TGN-localized DmATP7, two intracellular markers were expressed: *GalNAc-GFP* and *KDEL-GFP*. Co-localization was explored in salivary glands where the secretory cells are large and protein location is easily detectable. Each of the marker GFPs were driven in combination with *mCherry-DmATP7* by *pannier-GAL4*.

In the *Drosophila* TGN the enzyme N-acetylgalactosaminyltransferase is required for the donation of the sugar GalNAc to serine and threonine residues initiating the formation of mucin-type *O*-glycans (Kelly, 2003). The addition of a GFP tag enables ectopic expression of the enzyme to act as TGN-localized reporter. GalNAc-GFP generates a web-like localization spreading from a peri-nuclear region up to, but not including, the outer plasma membrane. At joining points of the web pattern, GalNAc-GFP is observed as punctate protein (Figure 4.8). In some areas intracellular mCherry-DmATP7 co-localizes with this punctate protein (yellow signal), although the spread of the GalNAc-GFP far exceeds mCherry (Figure 4.8). There is also mCherry-DmATP7 found at regions that do not overlap with the GFP signal. This partial co-localization suggests that some but not all intracellular DmATP7 is located at the TGN.

The second intracellular marker, KDEL, is a transport protein located predominantly at the endoplasmic reticulum with some seen at the TGN and intermediate vesicles between these organelles such as transitional elements or vesicular tubule clusters (Martinez-Menarguez, 1999). *KDEL*-GFP signal is observed throughout the cytoplasm except in spotted regions of no expression and there is also some irregular punctate signal (Figure 4.9). mCherry-DmATP7 does not co-localize with KDEL but rather occasionally locates

to regions just adjacent to the punctate KDEL accumulations (Figure 4.9). The lack of co-localisation suggests DmATP7 is not localized to the endoplasmic reticulum.

4.3 Discussion & Conclusion

Determining sub-cellular localization is crucial in investigating the cell-specific role of each of the mammalian copper homeostasis transporters. Determining which membranes (apical outer, basolateral outer, vesicle or organelle) the efflux or uptake transporters are located in can help in generating functional models of copper transport (Petris *et al.*, 1996; Klomp *et al.*, 2002). In *Drosophila*, there have been preliminary *in vivo* and *in vitro* investigations demonstrating that Ctr1A and Ctr1B localize to the apical membrane of polarized cells, whilst DmATP7 is basolateral (Norgate *et al.*, 2006; Balamurugan *et al.*, 2007; Turski and Thiele, 2007; Burke *et al.*, 2008; Southon *et al.*, 2010). This chapter further explored these localization models using the polarized cells of the midgut, salivary glands and malpighian tubules.

4.3.1 Although both Ctr proteins are primarily apical, there is some protein observed at the basolateral membrane of enterocytes

Ctr1A and Ctr1B have been identified as the principal sources of cellular copper uptake in *Drosophila* and have both been shown to have apical localisation. Ctr1A-specific antibody staining is primarily detected at the outer plasma membrane in cultured S2 cells, whole embryos and third instar salivary glands (Turski and Thiele, 2007). Similarly, Ctr1B-EGFP fusion protein was observed at the apical plasma membrane of larval gut epithelial cells (Balamurugan *et al.*, 2007). GFP-tagged fusion proteins used in the experiments presented here demonstrated copper transport activity implying that localizations observed were a valid representation of the location of the endogenous protein.

In midgut enterocytes, Ctr1A and Ctr1B predominantly localized to the apical membrane implying as expected that the proteins have a role in copper import from the lumen.

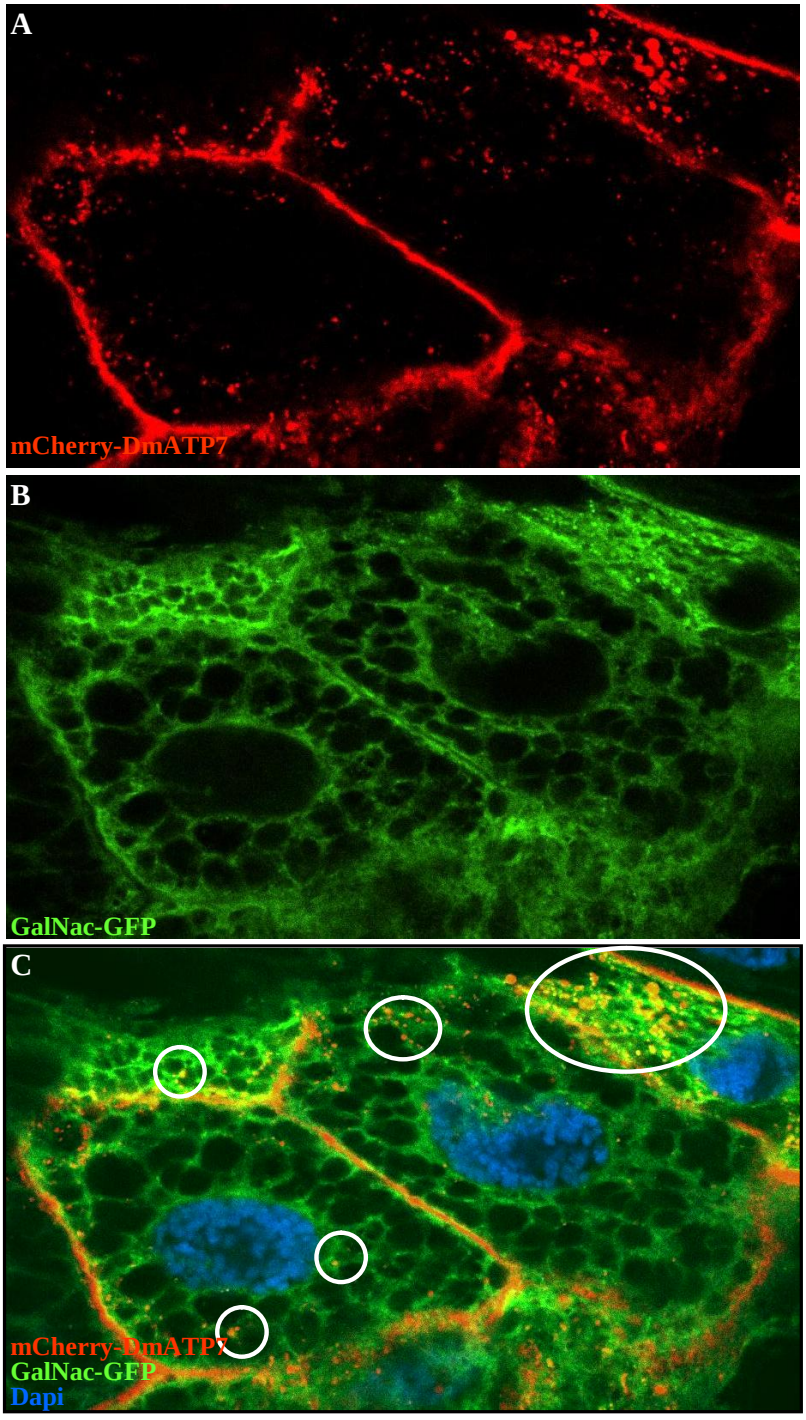

A
mCherry-DmATP7
B
GalNac-GFP
C
mCherry-DmATP7
GalNac-GFP
Dapi

Figure 4.8: Co-localization of mCherry-DmATP7 and GalNAc-GFP in salivary gland secretory cells

TGN marker *GalNAc-GFP* and *mCherry-DmATP7* were expressed in combination under the control of *pannier-GAL4*. Dissected salivary glands from third instar *Drosophila* larvae were viewed at 40X using confocal microscopy and images were analysed using NIS-Elements and ImageJ software. mCherry-DmATP7 was located predominantly at the outer plasma membrane although partially at intracellular regions (A). GalNAc-GFP has a web-like localization starting in a peri-nuclear region and spreading to, but not including, the basolateral membrane (B) There were also punctate protein observed generally at joins of the web pattern. mCherry-DmATP7 co-localized with punctate GalNAc-GFP with yellow signal observed (identified in the white circles) (C). The spread of GalNAc signal is much more extensive than intracellular mCherry-DmATP7 and only co-localizes in some regions.

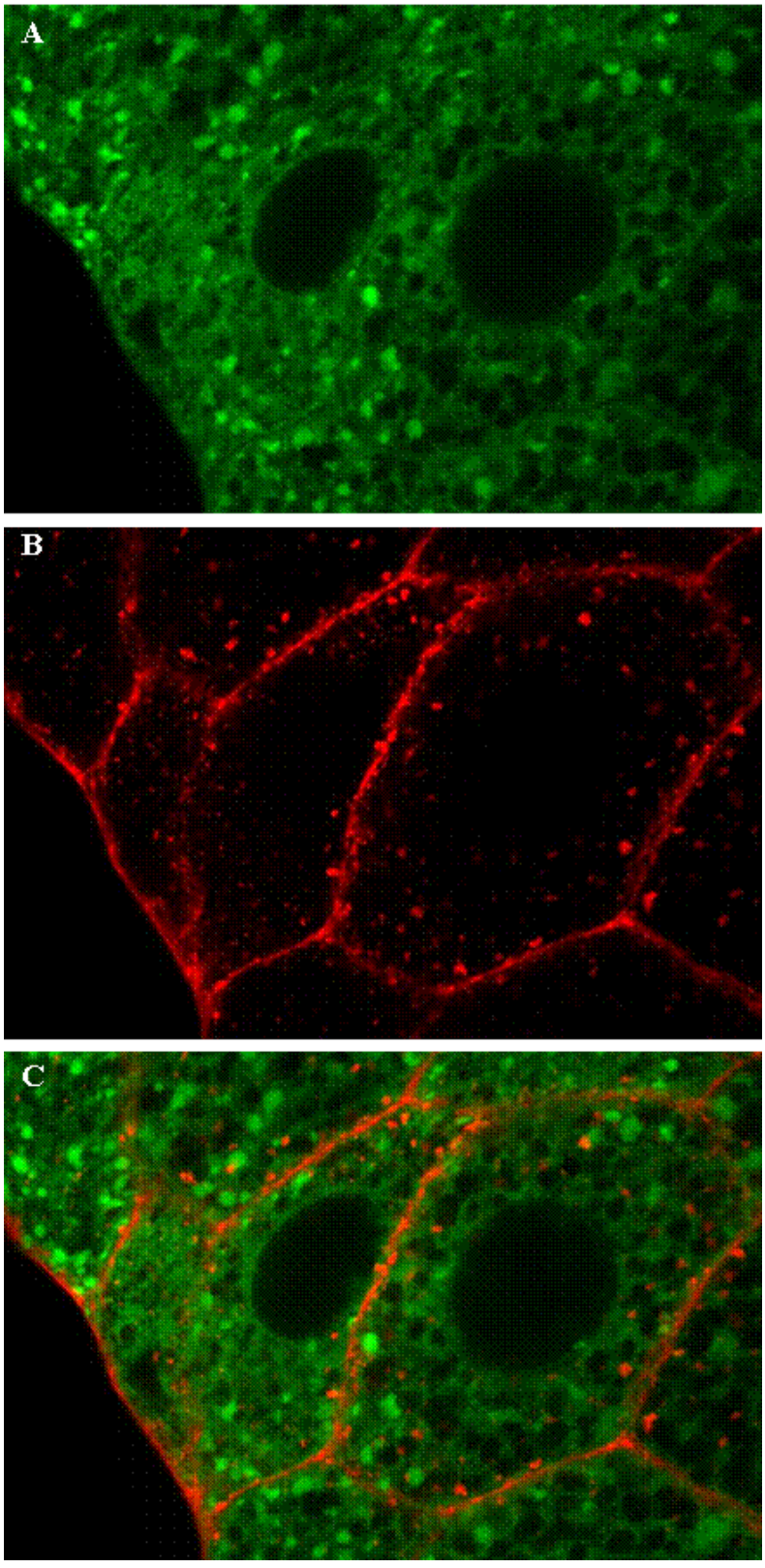

A
B
C

Figure 4.9: There is no co-localization of mCherry-DmATP7 with KDEL-GFP in salivary gland secretory cells

Endoplasmic reticulum marker *KDEL-GFP* and *mCherry-DmATP7* were expressed in combination under the control of *pannier-GAL4* in *Drosophila* salivary glands. Dissected salivary glands from third instar *Drosophila* larvae were viewed at 40X using confocal microscopy and images were analysed using NIS-Elements and ImageJ software. KDEL-GFP is located throughout the cytoplasm but not at the outer plasma membrane (A) Weak signal is observed except for a spotted pattern which contains no GFP. There is also distinct punctate GFP observed irregularly across the cytoplasm. mCherry-DmATP7 was located predominantly at the outer plasma membrane although partially at intracellular regions (B). mCherry-DmATP7 does not co-localize with KDEL-GFP but is occasionally adjacent to the edges of punctate GFP (C).

However, some signal was also observed adjacent to or at the basolateral membrane. Basolateral Ctr1A or Ctr1B would have interesting implications in regards to the model of dietary copper absorption in *Drosophila* and the results are also similar to what has been observed in mammalian epithelia. Conflicting research has shown that hCtr1 localizes to the apical membrane in mouse, rat and pig intestinal sections but is found adjacent to the basolateral membrane in Caco-2 cells (Zimnicka *et al.*, 2007; Nose *et al.*, 2010). Basolateral hCtr1 would suggest that the protein is required for copper import back from the bloodstream into enterocytes and consequently other sources of uptake may be responsible for dietary copper absorption from the lumen (Zimnicka *et al.*, 2007; Zimnicka *et al.*, 2010). Whilst the question remains as to which hCtr1 location is accurate (or whether they both are possible), *Drosophila* localization data clearly demonstrates the majority of the Ctr proteins would be required for apical copper import but some may have a role to play at the basolateral membrane. Enterocytes may require a source of copper from the lymph that could be exclusively used for cellular copper requirements and which is separately regulated from imported dietary copper. This would be an enterocyte-specific role for the Ctr proteins, highlighted by the fact that protein is apically localized in both salivary glands and malpighian tubules. There is also the possibility that basolateral Ctr could serve as a mechanism to protect against copper toxicity in the lymph.

Whilst the predominant locations of Ctr1A and Ctr1B are indicative of apical copper import, the possibility of other sources of import at this membrane cannot be ruled out. Alternative mechanisms may not be principal sources of enterocyte copper import, but work alongside or as secondary mechanisms to the Ctr1 proteins. The fact that increased copper levels do not cause the internalisation of either Ctr1A/B in the midgut is supported by similar results in previous studies (Balamurugan *et al.*, 2007; Turski and Thiele, 2007).

The salivary glands in *Drosophila* form by a complex process involving cell specification, the ceasing of mitotic division, secretory cell invagination, elongation and migration and the concomitant formation of a tube (Kerman, 2006). In this process the

secretory cells become polarized with a distinct apical membrane facing the inner duct and an outer basolateral membrane (Kerman, 2006). Both Ctr proteins are expressed in the salivary glands so apical localization of the Ctr proteins implies that copper is being transported from the duct/lumen of the gland into the secretory cells. The need for copper in these cells remains to be confirmed but a possible requirement is suggested later in this discussion. These results differ from investigations using a Ctr1A-specfic antibody which showed outer plasma membrane localization but not specially on the apical side (Turski and Thiele, 2007). Differences may have occurred as confocal imaging of the salivary glands may better discriminate between the apical and basolateral membranes. There was also some intracellular Ctr1B observed implying that the protein has an unidentified intracellular role. A more likely scenario however is that Ctr1B regulation involves internalization of the protein. Ectopic Ctr1B is likely to increase copper import into the secretory cells and internalization of the protein could prevent cellular toxicity. hCtr1 has been shown to be internalized under increased extracellular copper levels but the mechanism remains elusive (Molloy and Kaplan, 2009).

The malpighian tubules, the kidney-like organ of insects, typically import solutes/ions from the lymph into its inner luminal tube (Beyenbach, 2003). Water then follows by osmosis, causing the solutes/ions to be pushed down the tubules towards the gut for excretion (Beyenbach, 2003). Like the kidneys however, solutes/ions can be re-absorbed back into the lymph to maintain ionic balance (Beyenbach, 2003). GFP-signal from both fusion proteins was observed at the apical membrane of the principal cells implying either could import copper. However endogenous Ctr1A is more likely to be required for this role as gene expression is high in the malpighian tubules and *Ctr1B* expression is typically low. Increased expression of *Ctr1B* is not induced in the malpighian tubules when copper levels are scarce as observed in the midgut (see Chapter Three experiments using pCtr1B-EYFP). Endogenous Ctr1A may be required for the re-absorption process, importing copper from the luminal tube into principal cells but further research is required into the regulation of copper excretion by the malpighian tubules.

4.3.2 DmATP7 predominantly localizes to the basolateral membrane of midgut enterocytes

DmATP7 has the dual role of being responsible for copper efflux out of the cell and for delivering copper to the secretory pathway at the TGN. An antibody for the mammalian ATP7A, which also reacts specifically with DmATP7 in *Drosophila,* is detected at both the TGN and the outer plasma membrane in *Drosophila* S2 cells, but only at the plasma membrane in developing embryos (Southon *et al.*, 2004; Norgate *et al.*, 2006; Southon *et al.*, 2010). Further work on *Drosophila* larvae used a *GFP-DmATP7* construct driven under the control of the endogenous *DmATP7-GAL4*, which localized to the basolateral membrane of midgut enterocytes (Norgate *et al.*, 2006; Burke *et al.*, 2008). This localization was consistent during all conditions and no intracellular protein was observed (Burke *et al.*, 2008). Experiments in this chapter used a similar approach but drove *mCherry-DmATP7* in the entire midgut under *mex-GAL4* control. In addition to the predominant basolateral signal, mCherry-DmATP7 was also observed in intracellular regions. The fact that some intracellular protein was observed in this case is likely due to improved detection methods, as these midguts were observed using confocal microscopy. It is highly likely that some of the intracellular DmATP7 is located at the TGN as studies in mammals and yeast have demonstrated a requirement for copper delivery to the secretory pathway in intestinal cells (Yuan *et al.*, 1995; Nyasae *et al.*, 2007). Cu-ATPases in both species are TGN-localized to incorporate copper into multi-copper oxidase enzymes (Hephaestin in mammals and Fet3p in yeast) required for dietary iron absorption and it is likely that DmATP7 is necessary for this role in *Drosophila* enterocytes (Yuan *et al.*, 1995; Nyasae *et al.*, 2007).

In the mammalian model of intestinal ATP7A localisation, the protein is located at the TGN before undergoing a copper-dependent translocation to the organelles adjacent to the basolateral membrane to facilitate copper efflux (Monty *et al.*, 2005; Nyasae *et al.*, 2007). Midgut DmATP7 differed from the mammalian ATP7A, as basolateral localization was observed when dietary copper levels were normal and the protein did not undergo copper induced trafficking. The possibility that the mCherry-tag influenced localization was ruled out, as the fusion protein acted the same as the FLAG tagged

version in a functional assay. One possible reason for the apparent difference between ATP7A and DmATP7 may be due to expression levels as ectopic *mCherry-DmATP7*, expressed in addition to endogenous *DmATP7* may be forced mostly to the basolateral membrane as the TGN is occupied. This may make observing a copper-induced increase in basolateral protein difficult to detect. Nevertheless, the possibility that *DmATP7* may not be able to translocate in the midgut or other cell types must also be considered. It has been shown in cultured *Drosophila* S2 cells that DmATP7 failed to translocate from the TGN to the outer plasma membrane when copper concentration increased (Southon *et al.*, 2010). The same study did show however that DmATP7 was capable of translocation when exposed to mammalian copper trafficking mechanisms in Me32a human fibroblast cells (Southon *et al.*, 2010).

Another possibility is that midgut enterocytes may be a specialized cell type whereby DmATP7 is predominantly localized at the basolateral membrane and does not require translocation. *Drosophila* may have adapted to low copper environments where the need for intestinal efflux meant that more DmATP7 was required to be permanently at the basolateral membrane instead of TGN. DmATP7 may also be constantly recycling between both locations, but be predominantly located at the basolateral membrane. Further research could explore midgut DmATP7 translocation by using a specific antibody and staggered time-point microscopy methods.

These experiments were also able to observe DmATP7 and Ctr localization at the same time in enterocytes. The results demonstrated that TGN, basolateral and apical signals did not overlap, implying location-specific targeting of each protein. As the apical and basolateral membranes are clearly separate, this research has also been able to make further assumptions about enterocyte copper transport. As apical fluorescent protein signal does not come in to contact with basolateral fluorescent protein signal, there must be enterocyte-to-enterocyte membrane that copper is not actively transported through. This would mean that each separate enterocyte must import copper from the lumen and export it into the lymph.

4.3.3 DmATP7 localization is both basolateral and intracellular in the salivary glands and malpighian tubules

Endogenous *DmATP7* is expressed in larval salivary glands and both basolateral and intracellular localization of the fusion protein was observed. Some intracellular DmATP7 is likely to be located at the TGN fuelling any copper requirements for the secretory cells. Despite a lack of research into the copper requirements of secretory cells, the cuproenzyme lysyl oxidase has been detected in adult *Drosophila* salivary glands and it may function in larvae (Molnar *et al.*, 2005). Lysyl oxidase is generally required for the production of cross-links in collagens, but also for cellular processes such as motility and differentiation (Molnar *et al.*, 2005). Further research would be required to determine if this enzyme is expressed in larval salivary glands and whether DmATP7 is required to deliver copper to the enzyme at the TGN. Basolateral DmATP7 in the secretory cells suggested that copper is also exported out of the glands into the lymph which may be to prevent against toxicity when too much copper is imported from the salivary gland lumen.

Intracellular and basolateral DmATP7 is also found in principal cells of the malpighian tubules implying copper delivery to the secretory pathway and cellular export. DmATP7 is highly expressed in the malpighian tubules and further up-regulated when reared on food supplemented with copper (Burke *et al.*, 2008). The function for DmATP7 in malpighian tubules remains to be discovered but some roles can be proposed. A family of malpighian tubule specific multi-copper oxidases have been identified in Anopheles gambiae and it is likely that TGN-DmATP7 could deliver copper to these enzymes in *Drosophila* (Gorman *et al.*, 2008). Basolateral DmATP7 is likely to export copper from principal cells into the lymph and the fact that Ctr proteins are located apically suggests copper transport occurs from the inner lumen to the lymph. Although highly speculative this may suggest that copper is re-absorbed as solutes travel down the tubules towards the hindgut. While these results can be used to generate a hypothetical model for copper homeostasis in the malpighian tubules, further functional analysis of the role of each copper transporter is required.

4.3.4 Intracellular DmATP7 is partially located at the TGN

The results of suppression and ectopic expression analysis in the *Drosophila* thorax/abdomen gave strong evidence for a TGN role for the protein (Norgate *et al.*, 2006). TGN-located DmATP7 was demonstrated in cultured S2 cells but has not yet been demonstrated in *Drosophila* tissues (Southon *et al.*, 2010). Results in this chapter established that some DmATP7 was localized in intracellular regions in three separate polarized cell types. Using the large secretory cells of the salivary gland, this intracellular DmATP7 was determined to partially co-localize with TGN reporter, GalNAc-GFP, and not at all with endoplasmic reticulum marker, KDEL-GFP.

For the most part GalNAc-GFP signal did not co-localize with mCherry-DmATP7 but where there was yellow signal it can be presumed to be DmATP7 at the TGN. This suggests, not surprisingly, that not all TGN locations are occupied by DmATP7. At the same time intracellular mCherry-DmATP7 was also found elsewhere in the cell away from the TGN. The endoplasmic reticulum can be ruled out as another possible location as there was no co-localization with KDEL-GFP. One possibility is that DmATP7 is pumping copper into specialized vesicles which would then undergo exocytosis to remove copper from the cell. This has been observed in mammalian intestinal cells but in that case vesicles were adjacent to the membrane whereas here intracellular DmATP7 is throughout the secretory cell (Nyasae *et al.*, 2007). One likely scenario may be that DmATP7 is observed in the process of translocating from the outer plasma membrane to the TGN or in the other direction. This would raise the possibility that the protein is located in endocytic or other transport vesicles as part of the trafficking process. DmATP7 trafficking and the possible mechanisms involved have not been demonstrated in *Drosophila* but remain a priority for future investigations, such as the experiments detailed in Chapter Five.

4.3.5 Conclusion

This work primarily added further understanding to the model of intestinal copper absorption in *Drosophila*. Ctr1A and Ctr1B are both capable of being the chief copper importers at the apical membrane, but in addition are also partially located at the basolateral membrane. In both the salivary glands and malpighian tubules both Ctr proteins were predominantly located at the apical membrane. This is the first time in *Drosophila* that a possible role for the Ctr proteins in importing copper from the lymph back into enterocytes has been observed. Midgut DmATP7 appears to be primarily located at the outer membrane in enterocytes implying that the protein's most important function in these cells is export into the lymph. Predominant basolateral localization was also observed in the salivary glands and malpighian tubules.

DmATP7 was partially located in intracellular regions in all polarized cell types investigated. Co-localization analysis implied that some intracellular DmATP7 was located at the TGN but the majority was at unknown locations. It has been proposed that DmATP7 may have been observed trafficking between the TGN and outer plasma membrane. There was no copper induced translocation observed but it remains likely that the protein does exchange between pools at the TGN and basolateral membrane. The process may not be as pronounced as in mammals but remains a key component in future research into the localization of DmATP7 in *Drosophila*. This chapter has generated useful localization data which helps in determining the cell-specific functions of Ctr1A, Ct1B and DmATP7.

CHAPTER FIVE: INVESTIGATION OF PUTATIVE REGULATORS OF COPPER TRANSPORT PROTEINS IN *DROSOPHILA*

Synopsis

This chapter explores putative regulators of copper homeostasis in *Drosophila.* The role of dRab5 and HipK in regulating DmATP7 is explored by functional assays in the *Drosophila* midgut, midline and eye. Both proteins are then demonstrated to significantly alter DmATP7 localization in the secretory cells of salivary glands.

5.1 Introduction

5.1.1 Investigating the regulation of copper transport in *Drosophila*

This chapter explores the regulation of copper homeostasis in *Drosophila* by investigating the role of two candidate interacting proteins, dRab5 and HipK. Both candidates are involved in intracellular processes that have been implicated in the post-translational regulation of the mammalian Cu-ATPases. Rab5 is a vesicle transport protein that is required for early endosome maturation during endocytosis, a process suspected to be involved in the retrieval of Cu-ATPases from the outer plasma membrane (Pascale *et al.*, 2003). HipK on the other hand is a protein shown to regulate a ubiquitin ligase implicated in proteasomal degradation, which together with lysosomal degradation has been implicated in the regulation of ATP7A and ATP7B (de Bie *et al.*, 2007; Materia *et al.*, 2011; Swarup and Verheyen, 2011). This chapter explores dRab5 and HipK function in *Drosophila* and determines a role for both in the regulation of DmATP7.

5.1.2 Exploring the role of dRab5-mediated endocytosis in DmATP7 transport

Endocytosis is one of the key intracellular processes implicated in the trafficking of ATP7A. After copper-induced trafficking to the basolateral membrane, the return of ATP7A to the TGN when basal copper levels are restored is thought to involve endocytic retrieval (Mercer *et al.*, 2003; Lutsenko *et al.*, 2007). Endocytosis can be regulated by several proteins including the family of Ras-like GTPases collectively known as the Rabs. Active Rabs are essential for maturation of endocytic vesicles and are useful tools to investigate the role of endocytosis in protein trafficking. In fact it has been suggested that endocytosis is required for transport of copper homeostasis proteins as Rab5 was shown to co-localize with ATP7A at basal copper levels (Pascale *et al.*, 2003). This gave rise to the theory that ATP7A may require Rab5-mediated endocytosis to return to the TGN after copper-induced translocation to the basolateral membrane.

Drosophila Rab5 is one of thirty-three dRab proteins and is required for the endosomal trafficking of various proteins including members of the Wnt signaling pathway and Toll receptors (Daulat *et al.*, 2011; Tanaka and Nakamura, 2011). *dRab5* is expressed ubiquitously and the protein localizes to punctate vesicles in photoreceptor cells (Zhang *et al.*, 2007). This expression pattern and localisation is consistent with the mammalian version of *Rab5*. This research will explore dRab5 localisation, function and potential involvement in the trafficking of DmATP7. The work will utilise a set of fluorescent *dRab* transgenic fly lines from Zhang *et al*, which were created by cloning *cDNA* into a pUAST vector with a YFP tag at the N-terminus. This set of lines included Wild type (WT), Dominant-Negative (DN) and Constitutively-Active (CA) versions for each *dRab*. *dRab5-DN* and *dRab5-CA* lines were created by generating separate mutations in the GTP-binding domain (T/S to N change for *dRab5-DN* and Q to L change for *dRab5-CA*) (Zhang *et al.*, 2007). Suppression of *Rab5* was also achieved using RNAi lines designed and obtained from VDRC.

5.1.3 Investigating a role for HipK in copper homeostasis

The involvement of protein partners in the regulation of *Drosophila* copper homeostasis is a promising area of research. Previously unpublished research from the Richard Burke laboratory screened over 500 UAS-containing *P-element (UAS)* insertions. The screen determined if ectopic expression of any of the investigated genes could weaken or exacerbate the midline phenotype caused by *pannier-GAL4*; *UAS-DmATP7* expression. This screen discovered that the combination of a line containing a *P-element (UAS)* insertion in *HipK* and *pannier-GAL4*; *UAS-DmATP7* was lethal (R. Burke, pers comm.). This interaction suggested that increased HipK activity was exacerbating the *DmATP7* over expression phenotype.

HipK is the sole *Drosophila* member of the homeodomain family of serine/threonine kinases (Kim *et al.*, 1998). Mammalian members of this family are known transcription factors which regulate various genes (Rinaldo *et al.*, 2007). Recent research has discovered that HipK binds to Slimb, an E3 ubiquitin ligase and is thus involved in

inhibiting the degradation of members of several pathways including Wnt/Wg, Notch and Hedgehog (Lee *et al.*, 2009; Swarup and Verheyen, 2011). Ubiquitination is the process of adding ubiquitin molecules to a protein so that it is targeted for degradation via the 26S proteasome (Pickart, 2001). The role of E3 ubiquitin ligase in this process is to transfer ubiquitin onto the targeted substrate (Pickart, 2001). Ubiquitination has been identified to regulate mammalian Cu-ATPases as demonstrated by research into COMMD1 and Clusterin. COMMD1 can bind to mis-functioning mutants of both ATP7A and ATP7B, inducing proteolysis via an interaction with components of E3-ubiquitin ligase complex (Meusser *et al.*, 2005; de Bie *et al.*, 2007; Vonk *et al.*, 2011). Clusterin can interact with both ATP7A and ATP7B and targets their degradation by the lysosomal pathway (Materia *et al.*, 2011). It remains unlikely that HipK would bind directly to DmATP7 but nevertheless the mammalian protein-protein interactions highlight that the function of Cu-ATPases can be regulated by changes in protein stability. This research will investigate *HipK* by using the GAL4/UAS system to suppress and ectopically express the gene in various cell types. A DmATP7/HipK interaction is also explored by functional analysis and localisation studies.

5.2 Results

5.2.1 *dRab5* is an essential *Drosophila* gene

Ectopic expression (WT, DN and CA versions) and RNAi suppression of *dRab5* in a cell-specific manner was used to determine the role of the gene in *Drosophila*. dRab5 is a key component of both non clathrin-mediated and clathrin-mediated endocytosis and is likely involved in various systems requiring vesicle transport. Homozygous *dRab5* null mutants, created via p-element excision, are paralyzed and embryonic lethal, probably due to the failure of embryos to form early endosomes in the embryonic nervous system (Wucherpfennig *et al.*, 2003). Further research into $dRab5^{-/+}$ heterozygous flies has highlighted roles for the protein in germline endocytosis, JAK-STAT signaling, Toll receptor signaling and the control of cell polarity/proliferation preventing neoplastic tumours (Lu and Bilder, 2005; Devergne *et al.*, 2007; Compagnon *et al.*, 2009; Lund and

Delotto, 2011). The protein's function in *Drosophila* appears similar to that of Rab5 in mammals, which was shown to be important for early endosome maturation (Bucci *et al.*, 1992). A study in hamster kidney cells ectopically expressing *Rab5* resulted in an increase in endocytosis and the generation of large early endosomes (Bucci *et al.*, 1992). Conversely, expression of a *Rab5* mutant deficient in GTP binding was detrimental to endocytosis as early endosomes decreased in size and small vesicles formed adjacent to the outer membrane (Bucci *et al.*, 1992).

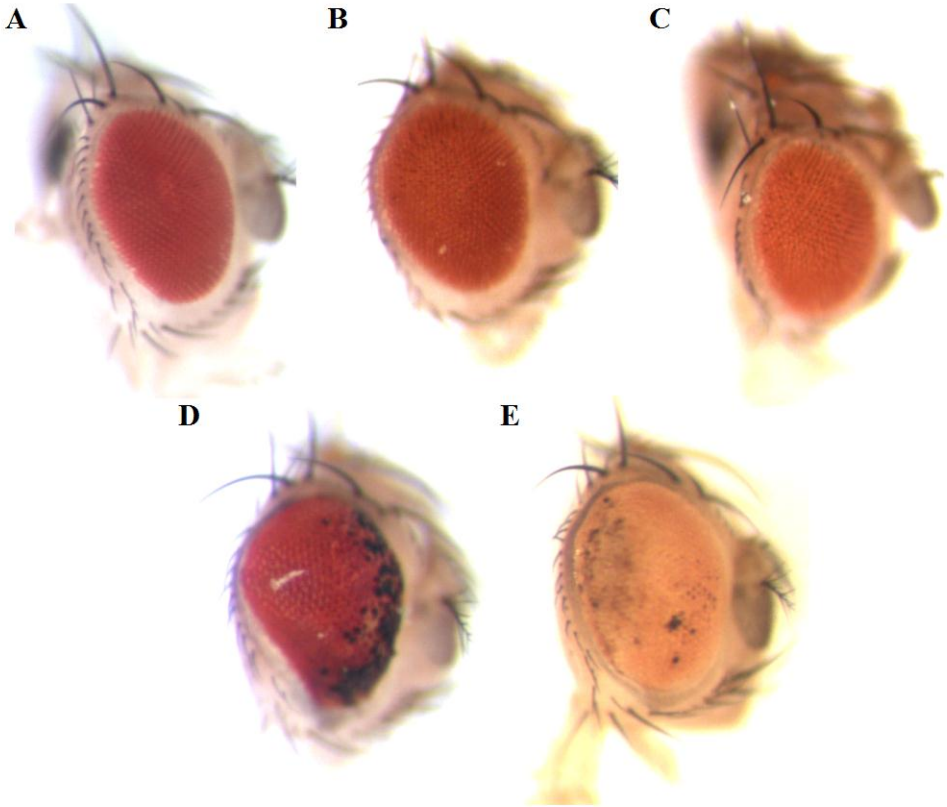
This investigation will further explore the importance of *dRab5* to *Drosophila* using the GAL4/UAS system. The importance of *dRab5* to *Drosophila* viability was assessed when lines containing each of the constructs were crossed to *actin-GAL4* (ubiquitous expression), *GMR-GAL4*, *pannier-GAL4* and *mex-GAL4*. There was no change in viability observed when either *dRab5-WT* or *dRab5-CA* were driven by any of the tested drivers (Table 5.1). Conversely, *dRab5-DN* and *dRab5* RNAi caused lethality with all drivers except *GMR-GAL4*, indicating that the consequence of losing dRab5-mediated endocytosis is more severe than increasing it (Table 5.1).

5.2.1.1 *dRab5-DN* and *dRab5* RNAi are both detrimental to the *Drosophila* eye

The viable adults generated from *GMR-GAL4* expression of either *dRab5-DN* or *dRab5* RNAi displayed severe alterations to the *Drosophila* eye in comparison with the GAL4 control (Figure 5.1). *GMR-GAL4* drives expression in cells posterior of and including the morphogenetic furrow in the developing eye disc (Freeman *et al.*, 1996). This GAL4-driver has been reported to cause a phenotype on its own when homozygous and/or when flies are reared at 29°C (Kramer and Staveley, 2003). Therefore these experiments were carried out at 25°C with flies heterozygous for *GMR-GAL4*. Expression of *dRab5-DN* resulted in the formation of black spots on the anterior side of the eye (closet to the front of the head) (Figure 5.1), whereas expression of *dRab5* RNAi resulted in smaller and less severe black spots forming mostly on the posterior side of the eye. *GMR-GAL4; dRab5* RNAi eyes were also pale orange in colour when compared to the strong red colour of the

Table 5.1: Summary of phenotypes caused by the expression of *dRab5-WT*, *dRab5-CA*, *dRab5-DN* and *dRab5* RNAi constructs when driven by various GAL4-drivers.

	dRab5-WT	***dRab5-CA***	***dRab5-DN***	***dRab5* RNAi**
actin-GAL4	Viable (No phenotype)	Viable (No phenotype)	Lethal (Late larvae)	Lethal (Late larvae)
GMR-GAL4	Viable (No phenotype)	Viable (No phenotype)	Rough eye with anterior darkening	Glazed rough eye
pannier-GAL4	Viable (No phenotype)	Viable (No phenotype)	Lethal (Late larvae) (Larvae have increased wing discs and large tumours)	Lethal (Late larvae) (Larvae have increased wing discs and large tumours)
mex-GAL4	Viable (No phenotype)	Viable (No phenotype)	Lethal (Late larvae)	Lethal (Late larvae)


A
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E

Figure 5.1: *Drosophila* eye phenotypes observed during *GMR-GAL4* driven expression of *dRab5-Wild type (WT), dRab5- Constitutively active (CA), dRab5-Dominant negative (DN)* and *dRab5* RNAi.

To explore *dRab5* function in the *Drosophila* eye *UAS-dRab5* constructs were crossed to *GMR-GAL4* and adult phenotypes observed. Expression of *UAS-dRab5-WT* (B) or *UAS-dRab5CA* (C) did not alter from the *GMR-GAL4* alone control (A). However, expression of *UAS-dRab5-DN* (D) or *UAS-dRab5* RNAi (E) resulted in significant alterations to the normal eye. *UAS-dRab5-DN* caused black necrosis spots to form in the anterior margin of the eye (D), whilst *UAS-dRab5* RNAi caused less severe black necrosis spots mostly on the posterior margin. *GMR-GAL4; dRab5 RNAi* eyes also appeared pale orange in colour as compared to the red eyes observed in the GAL4 control. For all photos, two-day old *Drosophila* adults had their heads removed and placed with the anterior end facing to the right of the observed image. Flies were observed using the Leica MZ6 dissecting microscope, imaged using the Leica DFC295 camera and analysed using Leica IM50 software.

GAL4 controls (Figure 5.1). These results demonstrated that expression of *dRab5-DN* or *dRab5* RNAi suppression disrupts normal eye development.

5.2.1.2 *dRab5-DN* or *dRab5* RNAi expression driven by *pannier-GAL4* causes larval abnormalities

Previous research has illustrated that the homozygous *dRab5* null mutant has disrupted cellular polarity, increased cell proliferation and neoplastic tumours (Lu and Bilder, 2005). These phenotypes are consistent with mutants of several genes involved in the endocytic pathway inferring that they may be caused by inhibition of endocytosis (Lu and Bilder, 2005). This investigation aimed to explore this phenomenon in distinct tissues using the adult midline specific *pannier-GAL4* driver. However, as *pannier-GAL4* driven expression of either *dRab5-DN* or *dRab5* RNAi is adult lethal, third instar larvae were investigated. The wing imaginal disc is the larval precursor to the adult wing and thorax and *pannier-GAL4* is expressed at the dorsal tip of the larval organ (Lye *et al.*, 2011). Notably, the GAL4-driver also expresses in the larval salivary glands and hemocytes (*Drosophila* blood cells).

Imaginal wings discs from *pannier-GAL4; dRab5DN* or *dRab5* RNAi were larger than wild type controls demonstrating over-proliferation (Figure 5.2). By monitoring YFP expression in *pannier-GAL4; dRab5DN* larvae, disc overgrowth was determined to be both cell autonomous (same cell where the construct is expressed) and non-cell autonomous to the *pannier-GAL4* expression zone (Figure 5.2). In addition to the wing disc phenotypes, both *dRab5-DN* and *dRab5* RNAi caused the majority of third instar larvae to display large melanotic tumours throughout the lymph (Figure 5.2). Tumour size varied from larvae to larvae but they were all found to be autonomous to the *pannier-GAL4* expression zone as seen by co-expression of *UAS-GFP*. Both larval phenotypes verified that the cell-specific loss of *dRab5* caused phenotypes reminiscent of the endocytosis inhibition phenotypes displayed by the *dRab5* null mutant.

5.2.2 *dRab5-DN* or *dRab5* RNAi eye phenotypes are modified when combined with mis-expression of copper transporters

Cell-specific loss of *dRab5* by expression of either the DN-construct or RNAi suppression resulted in dramatic eye phenotypes as a consequence of inhibition of endocytosis. This chapter aimed to use these phenotypes to investigate whether the loss of endocytosis could alter copper transport or vice versa. There is some understanding of the mechanisms of copper homeostasis in the *Drosophila* eye with roles proposed for Ctr1A, Ctr1B and DmATP7 (Binks *et al.*, 2010). Eye-specific suppression of *Ctr1A* results in a sunken eye phenotype with loss of ommatidia and a folding of the eye surface, implying that copper uptake is required for eye formation (Binks *et al.*, 2010). Ectopic expression of *Ctr1A-FLAG* resulted in no alteration to the eye, but expression of fully functional, untagged *Ctr1A* resulted in no viable progeny when reared on normal food and minimal survival with a severely distorted eye when reared on food supplemented with BCS (see figure 5.10). Suppression of *Ctr1B* caused no variation from the wild-type eye, but ectopic expression of the gene resulted in a rough eye phenotype when the flies were reared on copper supplemented food (Binks *et al.*, 2010). Neither suppression nor ectopic expression of *DmATP7* resulted in any phenotype alone. However the *UAS-Ctr1B* phenotype could be exacerbated by suppression of *DmATP7* and rescued by ectopic expression of *DmATP7* (Binks *et al.*, 2010). These experiments were now repeated but in the genetic background of *dRab5-DN* expression or *dRab5* RNAi suppression.

The *dRab5-DN* / RNAi eye phenotypes are not typical of those caused by copper deficiency or accumulation and rearing these flies on food at different dietary copper levels did not alter either phenotype. However, loss of *dRab5* in combination with *UAS-DmATP7* or *DmATP7* RNAi resulted in a dramatic worsening of the eye phenotypes observed with *dRab5-DN* or *dRab5* RNAi alone (Figure 5.3 and 5.4). *dRab5-DN* + *UAS-DmATP7* eyes were rough and reduced in size, whilst *Rab5* RNAi + *UAS-DmATP7* eyes had increased black necrosis throughout the eye. *UAS-DmATP7* causes cellular copper deficiency so this phenotype may be due to low copper levels impacting on the function of dRab5. Alternatively, the combination of the loss of *dRab5* and increased *DmATP7*

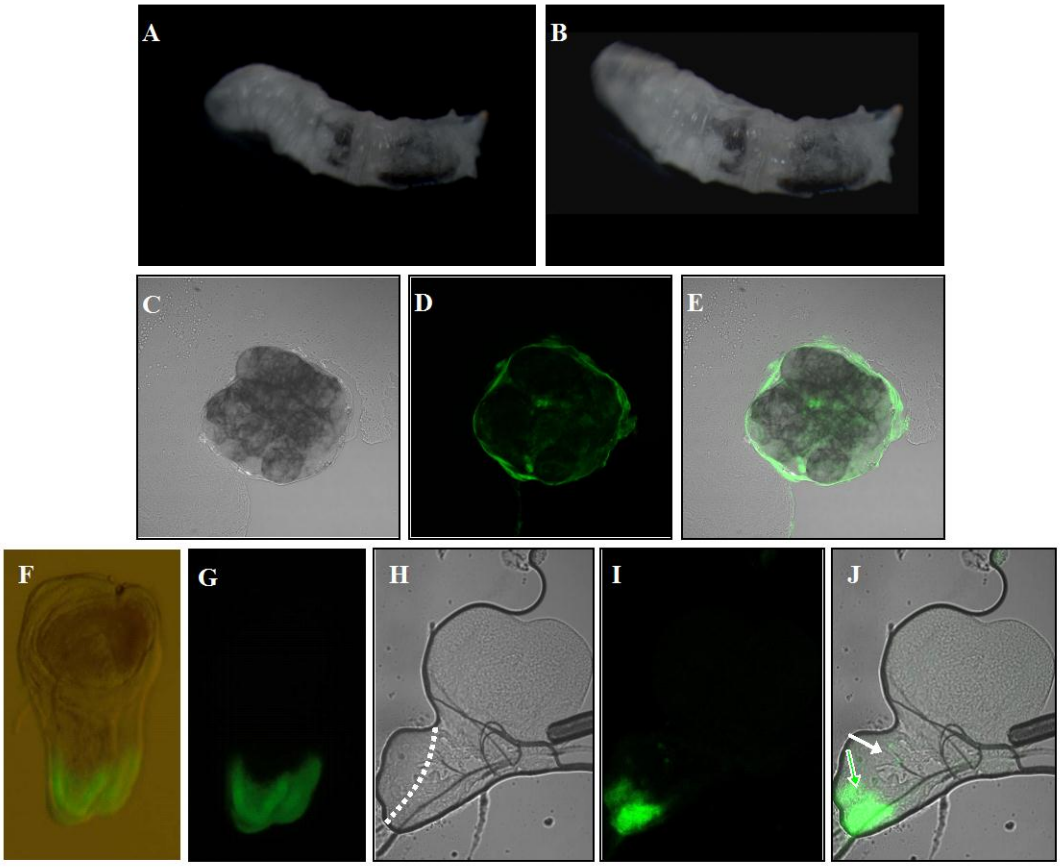
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Figure 5.2: *Drosophila* larval phenotypes observed during *pannier-GAL4* driven expression of *dRab5-Dominant negative (DN)* and *dRab5* RNAi.

Expression of either *UAS-dRab5-DN* or *UAS-dRab5* RNAi with *pannier-GAL4* resulted in lethality at the late larval stage of *Drosophila* development. Third instar larvae of both interactions were investigated to determine if there were any signs of impeding lethality. Both *UAS-dRab5-DN* (A) and *UAS-dRab5* RNAi (B) larvae showed the formation of large, black tumours in the lymph. The tumours exhibited GFP signal implying that there are in fact cell autonomous (C-E). In addition, when compared to the wild type (F+G)*UAS-dRab5-DN* and *UAS-dRab5* RNAi third instar larval imaginal wing discs demonstrated over-proliferation (over-growth outside the normal imaginal wing disc shown by the white dotted line) (H – only *UAS-dRab5-DN* shown). The *UAS-dRab5-DN* construct is GFP-tagged which allows this experiment to investigate whether the observed phenotypes are autonomous to the *pannier-GAL4* expression zone. Imaginal wing disc overgrowth was both cell autonomous and non-cell autonomous (I+J) (indicated by arrows). Wild-type discs were imaged by Richard Burke

	Phenotype Alone with *GMR-GAL4*	**Interaction with *GMR-GAL4* ; *dRab5-DN***
UAS-DmATP7	No Phenotype	Worsening of *dRab5-DN* phenotype
***DmATP7* RNAi**	No Phenotype	Lethal (Pupae eye severely distorted)
***Ctr1B* RNAi**	No Phenotype	Worsening of *dRab5-DN* phenotype
UAS-Ctr1B	Rough eye (only on Cu)	Partial rescue of *dRab5-DN* phenotype
UAS-Ctr1A	Lethal on NF	Lethal on all food types
***Ctr1A* RNAi**	Sunken eye phenotype	Lethal

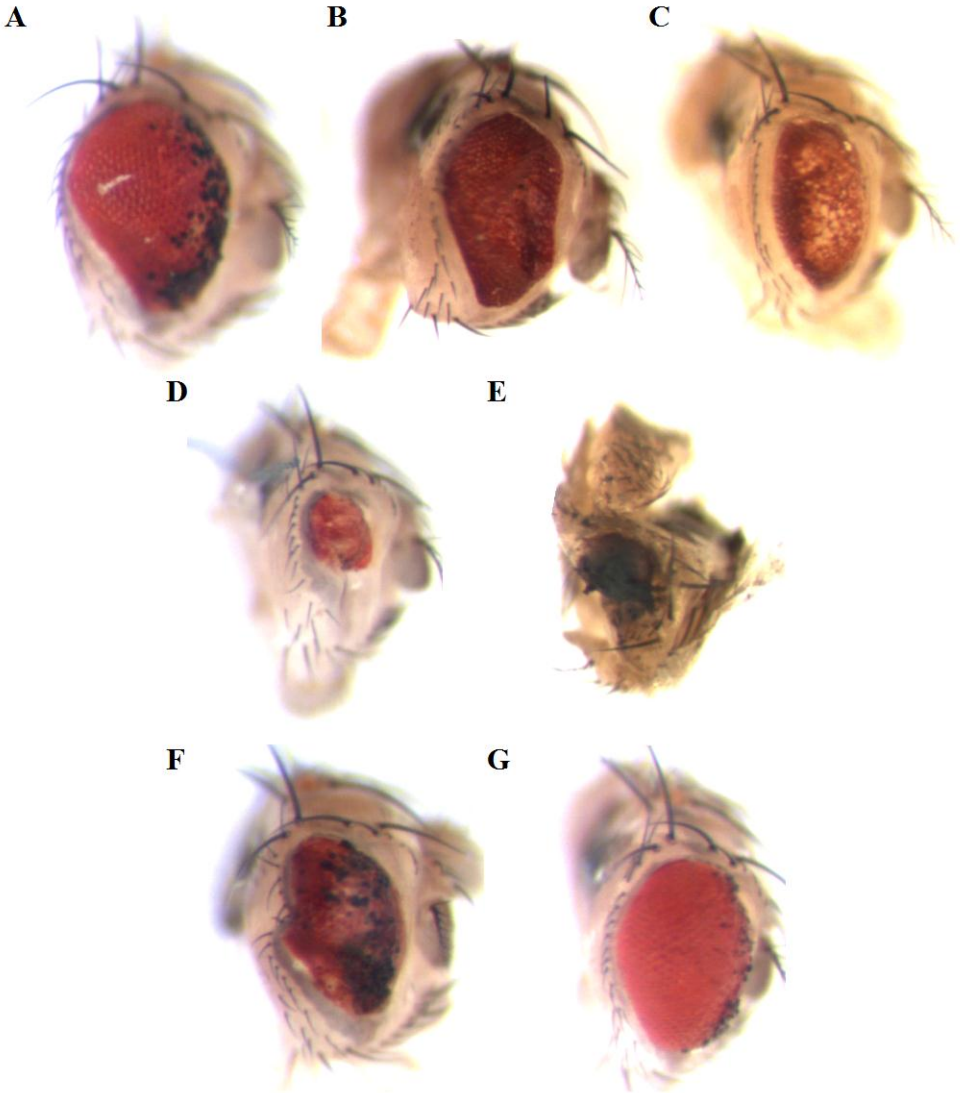

Figure 5.3: Interactions observed between manipulation of *Ctr1A, Ctr1B* and *DmATP7* expression with the *GMR-GAL4; dRab5-DN* phenotype

GMR-GAL4;UAS-dRab5-DN flies (A), which have black necrosis spots on the anterior of the eye were crossed to lines containing the *Ctr1A*, *Ctr1B* and *DmATP7* ectopic expression and suppression constructs. Suppression or ectopic expression of *DmATP7* in the eye results in no phenotype alone. *UAS-Ctr1A* when driven by *GMR-GAL4* is lethal, whilst *Ctr1A* RNAi results in copper deficient sunken eye phenotype (B). *UAS-Ctr1B* when driven by *GMR-GAL4* only causes a rough eye when reared on copper supplemented food (C), whilst *Ctr1B* RNAi has no phenotype. Expression of either manipulation of *DmATP7* with *dRab5-DN* resulted in a severe worsening of the phenotype; a small, distorted eye with *UAS-DmATP7* (D) and lethality with *DmATP7* RNAi. Flies crossed with *DmATP7* RNAi were dissected out of the pupae case and revealing a severely damaged eye (E). Crossing *GMR-GAL4;dRab5-DN* to *UAS-Ctr1B* resulted in a reduction in the black anterior necrosis observed in the *dRab5-DN* phenotype (F). The same cross of 1mM Cu resulted in a similar phenotype. Crossing *GMR-GAL4; dRab5-DN* to *Ctr1B* RNAi resulted in a worsening of the *dRab5-DN* phenotype with increased dark spots throughout the eye (G). Crossing *GMR-GAL4;UAS-dRab5-DN* to either of these constructs results in no viable progeny. For all photos, two-day old *Drosophila* adults had their heads removed and placed with the anterior end facing to the right of the observed image. Flies were observed using the Leica MZ6 dissecting microscope, imaged using the Leica DFC295 camera and analysed using Leica IM50 software.

	Phenotype Alone with *GMR-GAL4*	**Interaction with *GMR-GAL4*; *dRab5* RNAi**
UAS-DmATP7	No Phenotype	Worsening of *dRab5* RNAi phenotype
***DmATP7* RNAi**	No Phenotype	Worsening of *dRab5* RNAi phenotype
***Ctr1B* RNAi**	No Phenotype	Little Change to *dRab5* RNAi phenotype
UAS-Ctr1B	Rough eye (only on Cu)	Little Change to d*Rab5* RNAi phenotype
UAS-Ctr1A	Lethal on NF	Lethal on all food types
***Ctr1A* RNAi**	Sunken eye phenotype	Lethal

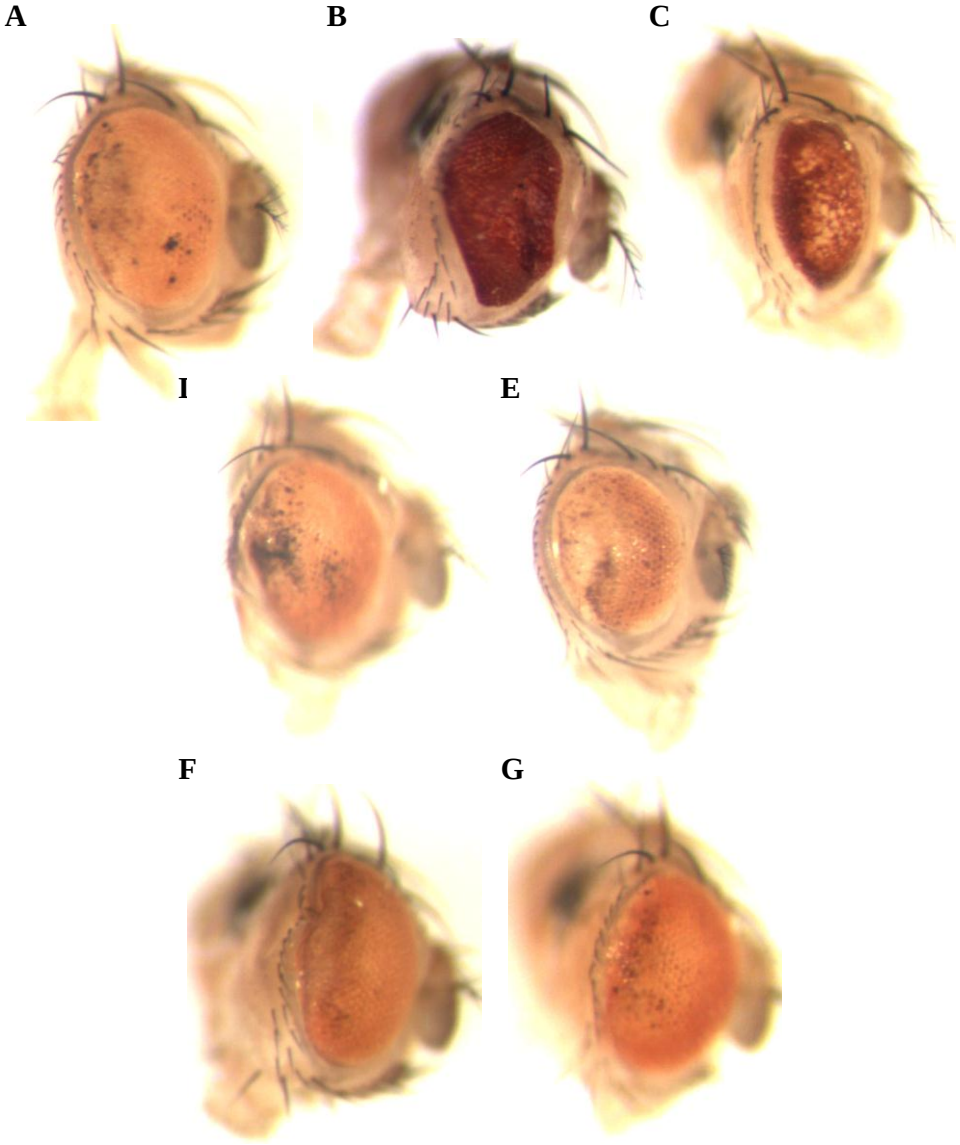

Figure 5.4: Interactions observed between manipulation of *Ctr1A, Ctr1B* and *DmATP7* expression with the *GMR-GAL4; dRab5* RNAi phenotype

GMR-GAL4;UAS-*dRab5* RNAi flies (A), which have black spots mostly on the posterior margin of the eye, were crossed to lines containing the *Ctr1A*, *Ctr1B* and *DmATP7* ectopic expression and suppression constructs. Suppression or ectopic expression of *DmATP7* in the eye results in no phenotype alone. UAS-*Ctr1A* when driven by *GMR-GAL4* is lethal, whilst *Ctr1A* RNAi results in copper deficient sunken eye phenotype (B). *UAS-Ctr1B* when driven by *GMR-GAL4* only causes a rough eye when reared on copper supplemented food (C), whilst *Ctr1B* RNAi has no phenotype. Expression of either DmATP7 construct with *Rab5* RNAi resulted in a subtle worsening of the phenotype in both cases (D – *UAS-DmATP7* + E – *DmATP7* RNAi). Crossing *GMR-Gal4;dRab5* RNAi to either *UAS-Ctr1B* (F) or *Ctr1B* RNAi (G) resulted in little change to the *Rab5* RNAi phenotype. Crossing *GMR-GAL4;dRab5* RNAi to either of Ctr1A constructs results in no viable progeny. For all photos, two-day old *Drosophila* adults had their heads removed and placed with the anterior end facing to the right of the observed image. Flies were observed using the Leica MZ6 dissecting microscope, imaged using the Leica DFC295 camera and analysed using Leica IM50 software.

expression may generate an extreme copper deficiency phenotype which has not been identified in previous manipulations of copper transporters in the *Drosophila* eye. Loss of *dRab5* in combination with *DmATP7* RNAi also caused much more severe phenotypes; adult lethality with *Rab5-DN* and increased black necrosis throughout the eye with *Rab5* RNAi (Figure 5.3 and Figure 5.4). Dissections of dying *GMR-GAL4*; *dRab5-DN*/*DmATP7* RNAi pupae demonstrated that eye development had been severely distorted (Figure 5.3). Suppression of *DmATP7* presumably results in less copper delivery to the secretory pathway but total cellular copper levels should increase due to reduced efflux. Therefore the loss of *dRab5* phenotypes appear to be exacerbated specifically by the reduction of copper delivery to the TGN.

The combination of *Ctr1B* RNAi with *Rab5-DN* resulted in black necrosis starting at the anterior margin but spread throughout the eye, which was a much more severe phenotype then observed with *Rab5-DN* alone (Figure 5.3). Suppression of *Ctr1B* is anticipated to result in reduced copper import into the eye, therefore this is further evidence of low copper levels impacting on the function of dRab5. *UAS-Ctr1B* (one copy) with *Rab5-DN* resulted in a phenotype less severe than observed with *Rab5-DN* alone as the necrosis was limited to a thin margin in the anterior eye. Flies of the same genotype reared on food supplemented with copper generated the same phenotype (Figure 5.3). In this situation it appears that high cellular copper levels may result in improved function of dRab5 and slight rescue of the necrosis phenotype. In contrast *UAS-Ctr1B* and *Ctr1B* RNAi had no impact on the *Rab5* RNAi phenotype (Figure 5.4). Loss of *dRab5* in combination with either *Ctr1A* RNAi or *UAS-Ctr1A* resulted in lethality in all cases, likely due to the additive combination of each dramatic phenotype (Figure 5.3 and 5.4)

5.2.3 The impact of *Rab5-DN* and *Rab5* RNAi on cellular copper levels

Results in the *Drosophila* eye demonstrated an interaction between copper transporters and dRab5 which may imply that loss of the endocytic protein may alter cellular copper content. Measuring cellular copper content directly in *Drosophila* remains elusive although indirect measures such as the previously discussed *pCtr1B-EGFP* and *pMtnB-*

EGFP reporters are useful. First, this chapter explores the use of reporter constructs to determine the enterocyte copper content when *dRab5* is lost in developing third instar larvae. Second, cellular copper levels were determined by synchrotron technology using X-ray fluorescence microscopy (XFM) to generate X-ray fluorescence (XRF) maps detailing the metal content (including copper, zinc and iron) of biological tissue (McRae *et al.*, 2010; Lye *et al.*, 2011).

5.2.3.1 *dRab5* suppression does not alter enterocyte copper levels

As previously introduced in Chapter Three, enterocyte copper content can be examined by determining the expression of target genes of the MTF-1 transcription factor, *Ctr1B* and *MtnB*. Compound lines containing *pCtr1B-EYFP* or *pMtnB-EYFP* constructs with *mex-GAL4* were crossed to *dRab5* RNAi lines. *dRab5-DN* was not used as the construct contained a YFP tag and therefore would be indistinguishable from EYFP expression from the reporter lines. Progeny were reared on the appropriate food type and dissected to remove the entire gut region, which was viewed under EYFP (GFP) fluorescence. Wild-type *Drosophila* have enhanced expression of *pCtr1B-EYFP* in the middle midgut, foregut and hindgut when larvae are raised on food supplemented with BCS, whilst *pMtnB-EYFP* expression increases when copper levels rise (Figure 5.5). *dRab5* suppression in the midgut did not cause any deviation from the wild type suggesting that enterocyte copper content remained normal.

5.2.3.2 The copper content of third instar imaginal wing discs during expression of *Rab5-DN*

XFM was used to explore the copper content of third instar imaginal wing discs as the tissue is only one cell thick and, as discussed earlier, *pannier-GAL4* only drives in the dorsal tip of the disc allowing for comparison between genetically manipulated and control sections (as illustrated in Figure 5.2) (Lye *et al.*, 2011). Expression of *dRab5-DN* in the wing imaginal disc causes cell autonomous over-proliferation; these sections can also be compared to the controls or regions of non-cell autonomous overgrowth.

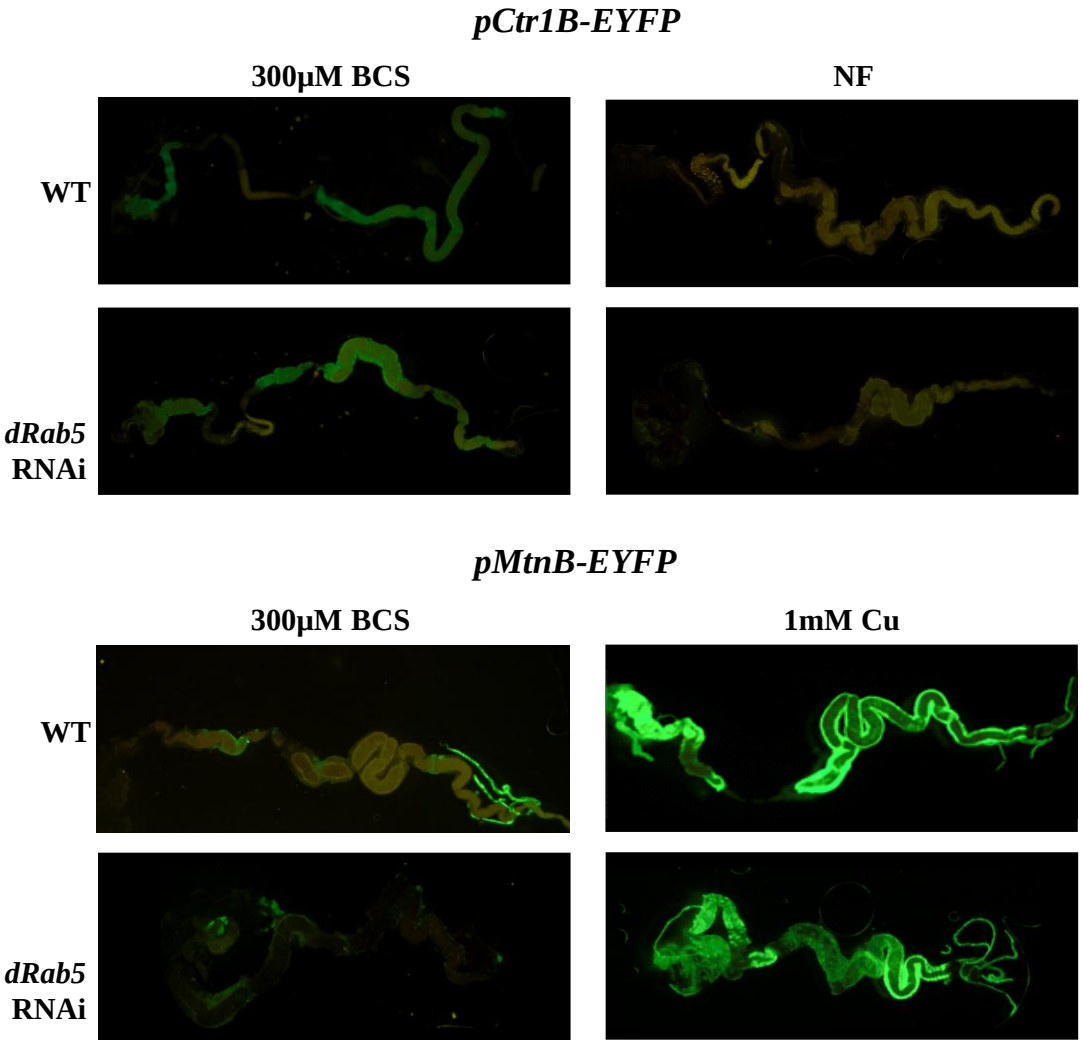
pCtr1B-EYFP
300µM BCS
NF
WT
dRab5 RNAi
pMtnB-EYFP
300µM BCS
1mM Cu
WT
dRab5 RNAi

Figure 5.5: Expression of *pCtr1B-EYFP* and *pMtnB-EYFP* in the *Drosophila* midgut when *dRab5* RNAi is driven by *mex-GAL4*.

Expression of both reporter constructs *pCtr1B-EYFP* and *pMtnB-EYFP* was used to determine the enterocyte copper content during *dRab5* suppression. Lines containing each reporter construct and *mex-GAL4* were crossed to lines containing the *dRab5* RNAi construct. Larvae were dissected to remove the entire gut region, which was mounted and viewed under EYFP fluorescence using the Leica Stereoscope. Images were recorded with a Leica DC300 camera using Leica Application Suite software. Wild-type larvae have little *pCtr1B-EYFP* expression on normal food but enhanced when reared on BCS food. *dRab5* RNAi larvae *pCtr1B-EYFP* expression levels did not differ from the wild type. *pMtnB-EYFP* expression is largely not observed when wild-type larvae are reared on low copper food, but increases substantially as copper levels rise. This remained consistent in larvae where *dRab5* was suppressed.

Imaginal discs were dissected from third instar larvae before being fixed, washed and mounted on silicon nitride windows. Samples were scanned at vertical and horizontal increments using the XFM beamline at the Australian Synchrotron, generating elemental maps. Elemental maps were generated by using X-ray energy to excite K-shell fluorescence emission from first row transition metals (Lye *et al.*, 2011). Elemental maps were analysed using GeoPIXE software allowing for the generation of semi-quantitative values for each metal investigated. Distribution of the metal within the tissue could then be summarized using a heat map (Figure 5.6).

Wild-type wing imaginal discs showed consistent distribution of the three metals investigated here, copper, zinc and iron (Figure 5.6). Copper levels increased at the ventral margin of the disc, zinc levels were high throughout the disc and iron levels were higher in the dorsal half (Figure 5.6). Elemental heat maps from *pannier-GAL4; Rab5-DN* wing discs differed significantly from the wild-type. In the dorsal tip of the discs where *dRab5-DN* is expressed tissue outgrowth was observed. Zinc and iron levels were elevated in these outgrowths in comparison with the remainder of the disc (Figure 5.6) whereas copper levels remained constant, with higher levels at the margin of the out-growths (Figure 5.6). In the out-growths the metal content was predicted to increase as the over-proliferated tissue was thicker than the remaining parts of the disc and therefore no change in copper levels may actually indicate a deficiency. Non-autonomous out-growths showed the same pattern, with similar, albeit less, increases in zinc and iron, whilst copper levels were again elevated only at the margins (Figure 5.6).

5.2.4 Genetic manipulation of the *Ctr1A* and *DmATP7* does not alter the formation of tumours in *pannier-GAL4; dRab5-DN* larvae

One of the results of *dRab5-DN* expression under the control of *pannier-GAL4* was autonomous tumours throughout the lymph of developing larvae. These tumours were found in 86.7% of *pannier-GAL4; dRab5-DN* genotyped larvae (Figure 5.7). This investigation aimed to determine if the manipulation of copper transporters *Ctr1A* or *DmATP7* and resultant changes in cellular copper levels could alter tumour phenotype.

Results from *dRab5* suppression via RNAi are not shown, as in all cases the tumour rate was 100%. For each cross, larvae were simply scored as containing tumours or not and six repetitions were completed. The average and standard error of the percentage of larvae containing tumours was calculated. It was found that neither *Ctr1A* RNAi, *UAS-Ctr1A*, *DmATP7* RNAi nor *UAS-DmATP7* expression made any significant impact to the tumours observed, implying that the loss of *dRab5* tumour phenotype is not copper dependent (Figure 5.7).

5.2.5 Both dRab5-WT and dRab5-DN partially colocalize with DmATP7

The genetic interactions described above suggest that dRab5-mediated endocytosis may have implications on copper homeostasis and vice versa. A likely role for endocytosis may be in the regulation of DmATP7 localization. Co-localisation analysis of dRab5-WT and dRab5-DN with DmATP7 was investigated to further explore this theory. As discussed in Chapter Four, the secretory cells of salivary glands are a useful cell-type due to their size and the ease in detecting protein localisation. In the secretory cells of the salivary glands mCherry-DmATP7 is located predominantly at the basolateral membrane, with some intracellular protein at the TGN (Figure 5.8). dRab5-WT-EYFP was located throughout the cell, with strong GFP signal observed at large vesicular structures, most likely early endosomes (Figure 5.8). Co-expression of *dRab5-WT* with *DmATP7* resulted in increased levels of intracellular DmATP7 with almost no basolateral membrane protein observed (Figure 5.8 compare D with B). The increased intracellular DmATP7 was shown to co-localize with dRab5-WT-EYFP particularly at the early endosomes (yellow), implying that the shift in DmATP7 location may have been as a result of enhanced endocytic retrieval from the basolateral membrane (Figure 5.8). dRab5-DN is located throughout the cytoplasm, with a small number of punctate signals observed throughout the cell (Figure 5.9). This localization is similar to the less punctate dRab5-DN-EYFP pattern observed in photoreceptor cells (Zhang *et al.*, 2007). Co-expression of *dRab5-DN* with *DmATP7* also resulted in increased intracellular DmATP7 (Figure 5.9 compare D with B). There was partial co-localisation between DmATP7 and dRab5DN (yellow), yet the majority of DmATP7 was not co-localized (Figure 5.9).

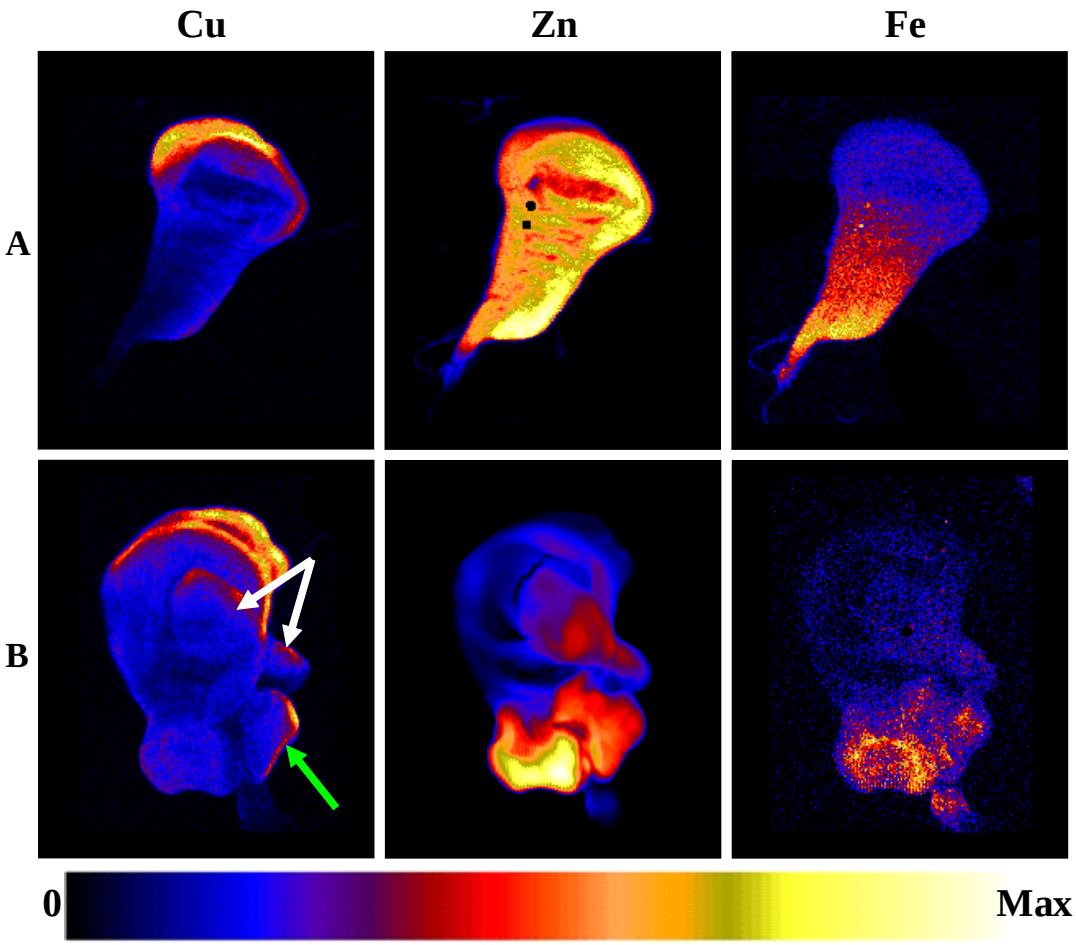
Cu
Zn
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Figure 5.6: Elemental maps of *pannier-GAL4-Rab5DN* third instar larvae imaginal discs generated by X-ray Fluorescence Microscopy (XFM) at the Australian Syncatron.

XFM was used to explore the copper content of third instar imaginal wing discs of larvae from a cross between *pannier-GAL4* and *UAS-Rab5-DN*. Imaginal discs were dissected from third instar larvae before undergoing wash, fixation and transport to silicon nitride windows. Samples were scanned at vertical and horizontal increments using the XFM beamline at the Australian Synchrotron generating elemental maps. Elemental maps are generated by using X-ray energy to excite K-shell fluorescence emission from first row transition metals (Lye *et al.*, 2011). Elemental maps were analysed using GeoPIXE software allowing for the generation of semi-quantitative values for each metal investigated. Distribution of the metal within the tissue could then be summarised using a heat map (shown at the bottom) The distribution of the investigated metals, copper (left), zinc (middle) and iron (right) is shown. Wild-type imaginal wing discs (A) demonstrate that copper levels increase at the ventral margin of the disc, zinc levels are high throughout the disc and iron levels are higher in the dorsal half. *pannier-GAL4-Rab5-DN* imaginal wing discs (B) show increased levels of zinc and iron, but not copper, in the dorsal tip of *pannier* expression, autonomous over-growths (green arrow) and non-autonomous over-growths (white arrow).

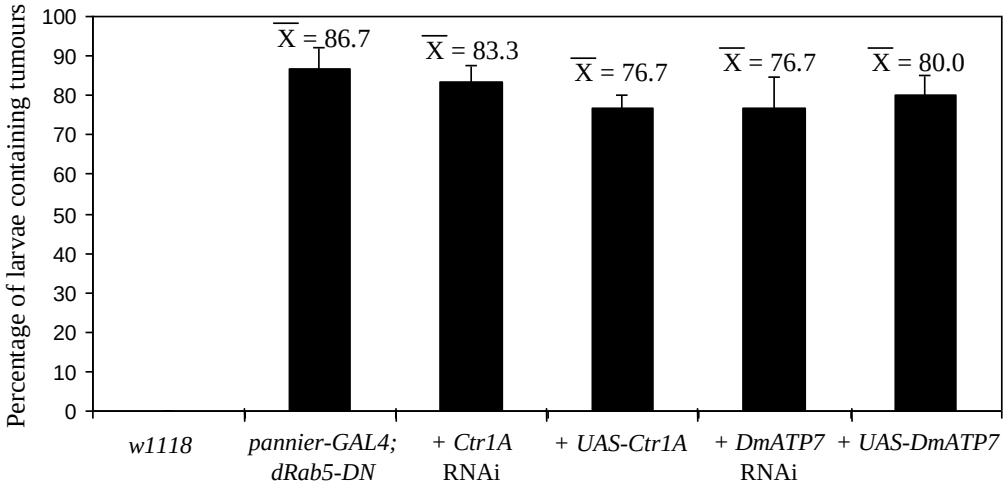
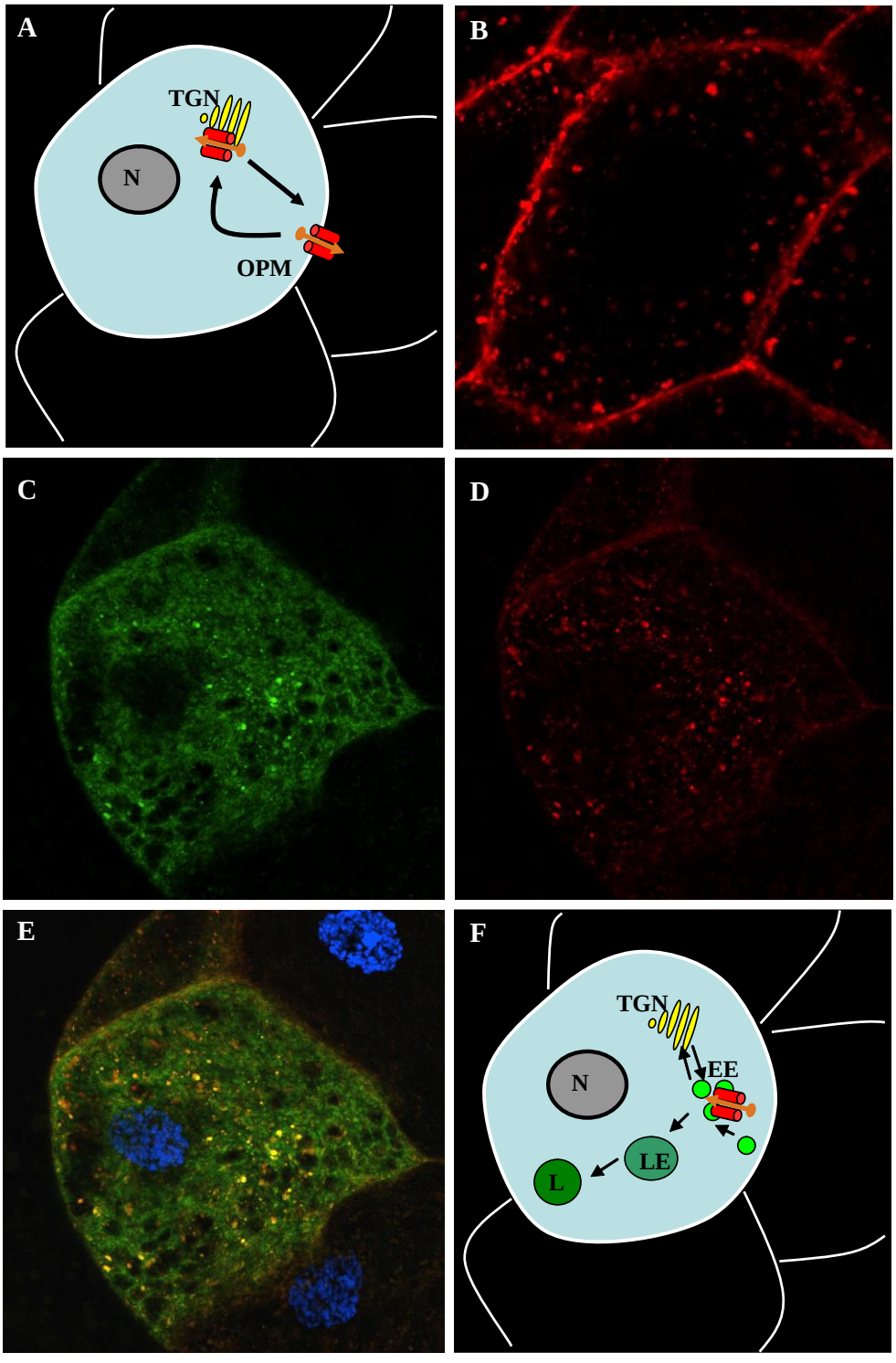

Percentage of larvae containing tumours
100
90
80
70
60
50
40
30
20
10
0
X = 86.7
X = 83.3
X = 76.7
X = 76.7
X = 80.0
w1118
pannier-GAL4; dRab5-DN
+ Ctr1A RNAi
+ UAS-Ctr1A
+ DmATP7 RNAi
+ UAS-DmATP7

Figure 5.7: Percentage tumour count of *pannier-GAL4; dRab5-DN* larvae during genetic manipulation of *Ctr1A* and *DmATP7*

dRab5-DN expression under the control of *pannier-GAL4* generated autonomous tumours throughout the lymph of 86.7% of developing larvae. To investigate if the manipulation of copper transporters *Ctr1A* or *DmATP7* and resultant changes in cellular copper levels could alter the phenotype, compound stocks of each construct with *pannier-GAL4* was crossed to *UAS-dRab5-DN*. For each cross larvae were simply scored as containing tumours or not and six repetitions were completed. The average and standard error of the percentage of larvae containing tumours was calculated. It was found that *Ctr1A* RNAi, *UAS-Ctr1A*, *DmATP7* RNAi or *UAS-DmATP7* made no significant impact to the tumours observed.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TGN
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N
LE
L

Figure 5.8: UAS-dRab5-WT-EYFP colocalises with and causes a shift in mcherry-DmATP7 localisation in the *Drosophila* salivary glands

mCherry-DmATP7 expressed under the control of *pannier-GAL4* in the secretory cells of *Drosophila* salivary glands localises predominantly to the basolateral membrane with some located at the TGN (A+B). *pannier-GAL4;mCherry-DmATP7* was crossed *UAS-dRab5-WT-EYFP* and dissected salivary glands from third instar progeny were viewed at 40X using confocal microscopy before images were analysed using NIS-Elements and ImageJ software. dRab5-WT-GFP is throughout the cell, with strong YFP signal indicating the larger early endosomes (C). Expression of the *dRab5-WT-EYFP* resulted in increased intracellular DmATP7 and almost no basolateral membrane protein (D). Intracellular DmATP7 was shown to co-localise with dRab5-WT-EYFP, likely at the early endosomes (E). This implies that ectopic expression of *dRab5* results in increased DmATP7 being recruited to the early endosomes (modeled in F).

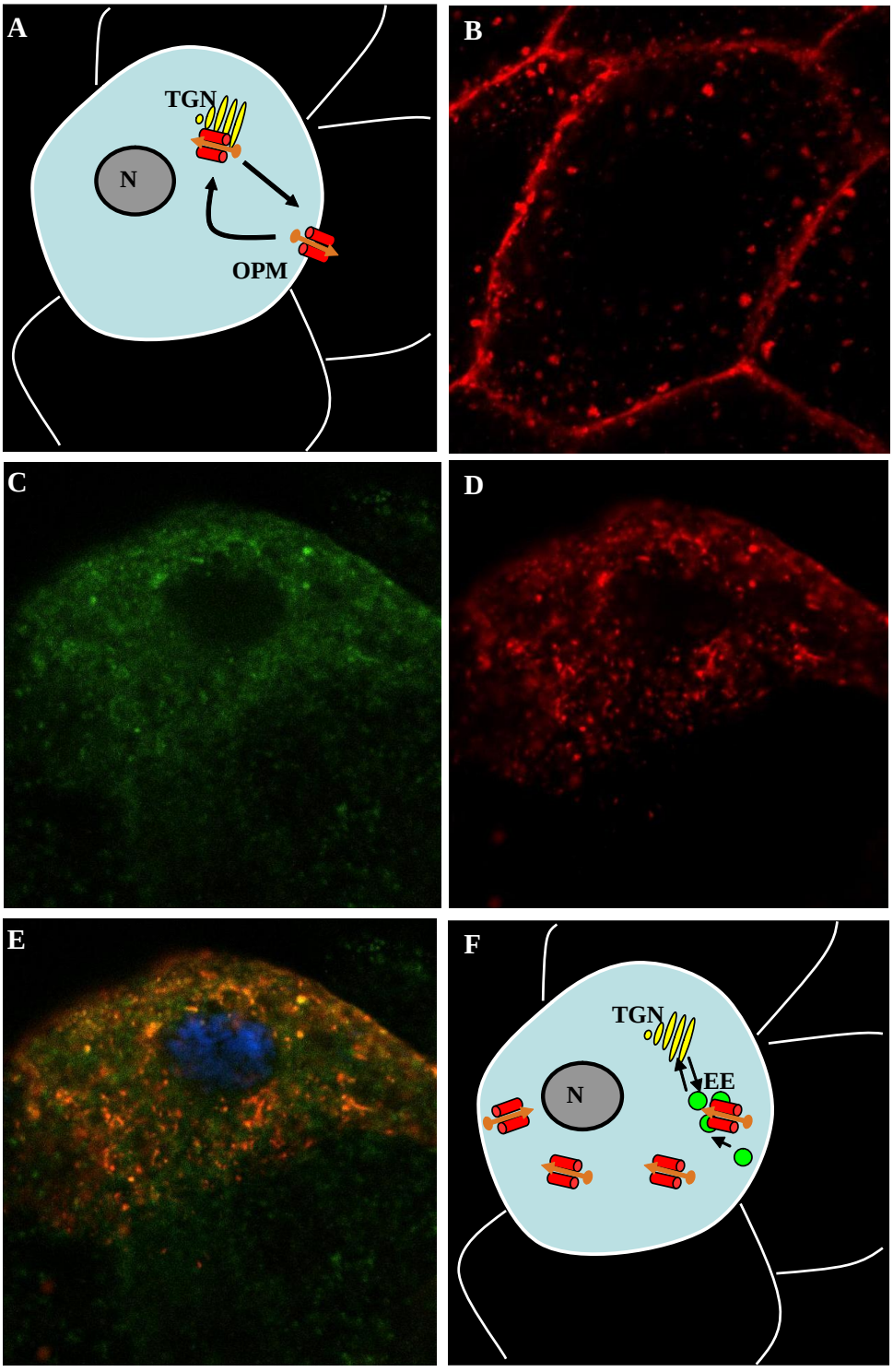
A
TGN
N
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Figure 5.9: UAS-dRab5-DN-EYFP colocalises with and causes a shift in mcherry-DmATP7 localisation in the *Drosophila* salivary glands

mCherry-DmATP7 expressed under the control of *pannier-GAL4* in the secretory cells of *Drosophila* salivary glands localises predominantly to the basolateral membrane with some located at the TGN (A+B). *pannier-GAL4; mCherry-DmATP7* was crossed to *UAS-dRab5-DN-EYFP* and dissected salivary glands from third instar progeny were viewed at 40X using confocal microscopy before images were analysed using NIS-Elements and ImageJ software. dRab5-DN-EYFP is throughout the cell, with a small number of punctate signals (C). Expression of the *dRab5-DN-EYFP* also resulted in increased intracellular DmATP7 (D). Some intracellular DmATP7 was shown to co-localise with dRab5-DN-EYFP, but the majority of DmATP7 was not co-localised (E) (modeled in F).

5.2.6 *HipK* is an essential *Drosophila* gene and cell-specific ectopic expression leads to dramatic phenotypes of the eye and thorax

The second putative copper homeostasis regulator investigated was the sole *Drosophila* homeodomain serine/threonine kinase, HipK. Null mutants of *HipK*, a gene shown to be a positive regulator of the Notch, Wnt/Wingless and Hedgehog signaling pathways, are embryonic lethal and hypomorphic mutants display a loss of photoreceptor cells (Lee et al., 2009; Lee et al., 2009; Swarup and Verheyen, 2011). *HipK* expression has been shown in various organs throughout developing *Drosophila* larvae including the brain, midgut, salivary glands, malpighian tubules and fat body cells (Chintapalli *et al.*, 2007). Given our laboratory's preliminary results suggesting an interaction between HipK and DmATP7, this work aimed to use a GAL4/UAS system approach to mis-express *HipK* in the *Drosophila* eye, midline and midgut before investigating the possible role of the kinase in DmATP7 regulation. *HipK* RNAi was designed and obtained from the VDRC, whilst the UAS-*HipK* construct was created following the same methods as described for the generation of *UAS-cDNA* constructs for the copper transporters.

Driving *HipK* RNAi with *pannier-GAL4* resulted in viable flies with a normal midline, however ectopic expression of the *UAS-HipK* construct lead to a dramatic cleft of the thorax, loss of scutellum and a band of pigment loss down the entire abdominal midline (Figure 5.10). Pigment loss is a phenotypic trait of copper deficiency and hence gives initial evidence, albeit indirect, linking HipK to copper homeostasis. In addition, there was a partial rescue of the scutellum, less thorax cleft and restored abdominal pigment when these flies were reared on food supplemented with copper (Figure 5.10). *GMR-GAL4* driven suppression of *HipK* resulted in no alteration from the wild type eye, but *UAS-HipK* caused a mild rough eye (Figure 5.10). This phenotype was similar, albeit less severe, than the phenotype observed when expression was driven with *eyeless-GAL4* (Lee *et al.*, 2009). Expression of either construct with the midgut-specific *mex-GAL4* resulted in no change to viability. These cell-specific results demonstrated that increased *HipK* expression has dramatic consequences likely to be caused by the enhanced positive regulation of several cellular processes, one of which may lead to a copper deficiency.

5.2.7 Characterisation of the interaction between the *HipK* P-element (UAS) mutant and *pannier-GAL4; UAS-DmATP7*

As introduced earlier, HipK was first implicated as a potential partner protein in copper transport during a screen of *P-element (UAS)* insertions identifying genes interacting with DmATP7. The $HipK^{11361}$ allele was found to produce no viable progeny when crossed to *pannier-GAL4*; *UAS-DmATP7* (Table 5.2). The mutant allele was homozygous lethal, but when crossed with *pannier-GAL4* alone also generated the thorax cleft phenotype (confirming the *P-element* insertion was ectopically expressing *HipK*) (Table 5.2). It was also found that combining $HipK^{11361}$ with *pannier-GAL4; UAS-Ctr1A* resulted in a partial rescue of the thorax phenotype (Table 5.2). These results lead to speculation that HipK could be enhancing DmATP7 activity leading to an increase in cellular copper export.

5.2.8 *HipK* ectopic-expression supports an interaction with *DmATP7*

5.2.8.1 *UAS-HipK* enhances *DmATP7* in the *Drosophila* midline

Results demonstrating that the $HipK^{11361}$ allele could influence and be influenced by shifts in copper transport prompted further research using the *UAS-HipK* construct. For this investigation crosses were setup between *UAS-HipK* flies and lines containing both *pannier-GAL4* and either *UAS-DmATP7*, *UAS-DmATP7 RNAi*, $UAS\text{-}DmATP7^{DOMNEG}$, *UAS-Ctr1A* or *UAS-dRab5*. Ectopic expression of *DmATP7* in the midline generates a cellular copper deficiency resulting in hypopigmentation, loss of scutellum and a small thoracic cleft (Norgate *et al.*, 2006) (Figure 5.11). Expressing both *UAS-HipK* and *UAS-DmATP7* together with *pannier-GAL4* generates no viable progeny, implying the combination of phenotypic alterations is detrimental to the organism or that there is an interaction between the ectopically-expressed proteins (Figure 5.11).

Suppression of *DmATP7* alone results in midline pigment loss due to the loss of copper transport function at the TGN and when combined with ectopic expression of *HipK* displays an additive phenotype reflective of both *DmATP7* RNAi and *UAS-HipK* (Figure 5.11). Expression of $UAS\text{-}DmATP7^{DOMNEG}$, a catalytically inactive mutant which cannot

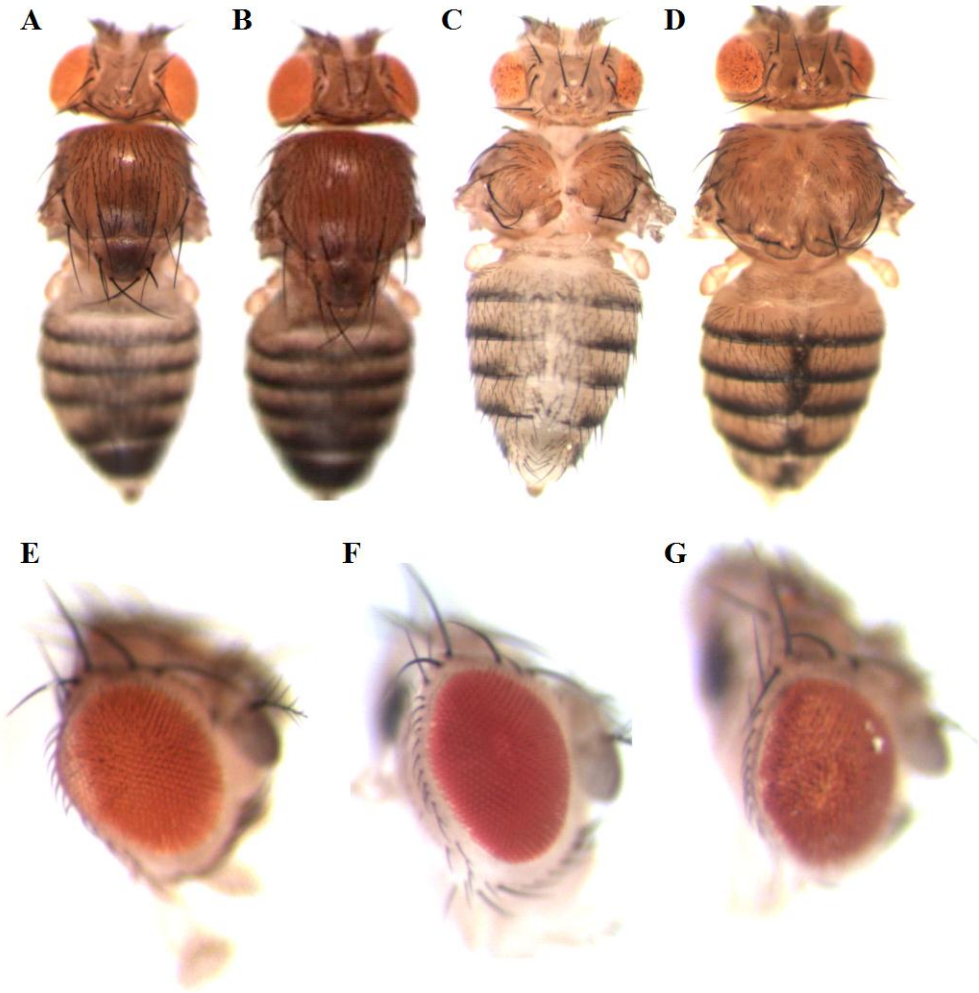
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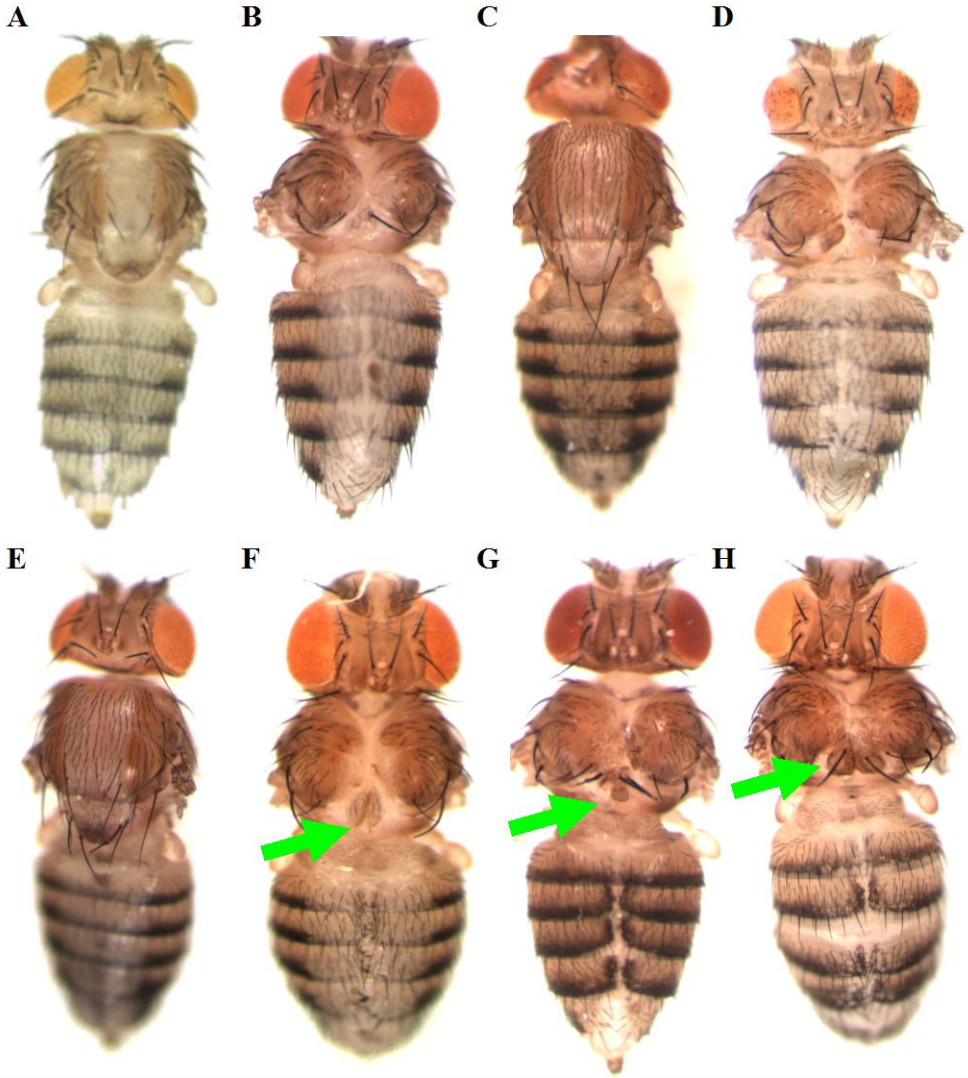
Figure 5.10: Phenotypes associated with *pannier-GAL4* and *GMR-GAL4* expression of *HipK* RNAi and *UAS-Hipk*

To investigate HipK function in cell-specific manner in *Drosophila*, *HipK* RNAi and *UAS-HipK* constructs were driven by *pannier-GAL4* and *GMR-GAL4*. Lines containing the constructs or GAL4 drivers were crossed, reared on various levels of dietary copper and the progeny observed for phenotypic alterations in the thorax/abdomen and eye respectively. *HipK* RNAi driven by *pannier-GAL4* (A), resulted in no alteration to the wild-type (B), whilst *UAS-HipK* caused a dramatic cleft of the thorax, loss of scutellum and a band of pigment loss down the entire abdominal midline (C). This phenotype was much less severe when the flies were reared on 1mM Cu supplement food (D). *GMR-GAL4 HipK* RNAi (E) resulted in no alteration from the wild type eye (F), whilst *UAS-HipK* caused a mild rough eye (G). For all photos, two day old *Drosophila* adults were observed using the Leica MZ6 dissecting microscope, imaged using the Leica DFC295 camera and analysed using Leica IM50 software. For eye photos, the flies had their heads removes and placed with the anterior end facing to the right of the observed image, whilst for midline photos the wings and legs were removed.



	UAS-HipK
pannier-GAL4	Thorax cleft, reduced scutellum, midline hypopigmentation
pannier-GAL4-UAS-DmATP7	Lethal
***pannier-GAL4-DmATP7* RNAi**	Increasing hypopigmentation, No change to thorax
pannier-GAL4-UAS-DmATP7^{MUT}	Scutellum rescue
pannier-GAL4-UAS-Ctr1A	Partial scutellum rescue
pannier-GAL4-UAS-Rab5	Scutellum rescue

Figure 5.11: Interactions observed between *UAS-HipK* and copper transporters in the *Drosophila* thorax/abdomen

The *pannier-GAL4-UAS-HipK* phenotype was used to investigate the potential interaction between HipK and DmATP7. *pannier-GAL4;UAS-DmATP7* flies have a copper deficiency midline phenotype which includes hypopigmentation down the midline, a small thoracic cleft and bristle loss (A). Expression of *UAS-HipK* alone using *pannier-GAL4* results in a thorax cleft, lost scutellum and band of pigment loss down the midline (B). The combination *UAS-HipK* with *pannier-GAL4-UAS-DmATP7* generates no viable progeny. The combination with *pannier-GAL4*-UAS-*DmATP7* RNAi, which alone results in a band of pigment loss down to the midline (C), results in no significant alteration to the thorax but increased hypopigmentation (D). Combination with *pannier-GAL4-DmATP7*^{DOMNEG}, which alone also results in hypopigmentation down the midline (E), results in a partial rescue of the scutellum (inidicated by the green arrow) (F). The same phenotype is also observed when combined with *pannier-GAL4-UAS-Ctr1A* (G) or *Rab5* (H). For all photos, two day old *Drosophila* adults had their wings and legs removed before being observed using the Leica MZ6 dissecting microscope, imaged using the Leica DFC295 camera and analysed using Leica IM50 software.

Table 5.2: Summary of phenotypes observed with the *HipK^{11361}* allele

	HipK^{11361}
Basis of *P-element*	A stable *P-element* in the regulatory region of *HipK* which contains a *UAS*-construct
Likely effect to HipK expression	Increased expression
Viability of the mutant	Third instar larvae homozygous lethality
Adult heterozygote phenotype with *pannier-GAL4*	Thorax cleft and reduced scutellum
pannier-GAL4-UAS-DmATP7	Lethal
pannier-GAL4-UAS-Ctr1A	Partial rescue

transport copper, results in hypopigmentation but intact bristle formation, thorax and scutellum (similar to *DmATP7* RNAi) (Norgate *et al.*, 2006) (Figure 5.11). This phenotype is thought to be caused by the inactive protein replacing the endogenous DmATP7 at the TGN but not being able to transport copper (Norgate *et al.*, 2006). In this case when inactive *DmATP7* is expressed with *UAS-HipK* there is a partial rescue of the HipK scutellum defect (Figure 5.11).

Intriguingly, progeny from the remaining crosses also demonstrated a partial or full rescue of the scutellum (Figure 5.9). Expression of *UAS-Ctr1A*, likely to lead to increased copper import, can also rescue the scutellum in *pannier-GAL4; UAS-HipK* flies (Figure 5.11). And, *UAS-dRab5*, likely to trap DmATP7 in large early endosomes, can also rescue scutellum loss when co-expressed with *UAS-HipK*. Interestingly, in the case of both *UAS-Rab5* and *UAS-Ctr1A* cellular copper levels are likely to increase.

5.2.8.2 There was little interaction between HipK and DmATP7 in the eye

Due to the dramatic interactions observed in the midline, *UAS-HipK* was further investigated in the eye by combining it with constructs mis-expressing *Ctr1A* and *DmATP7*. *GMR-GAL4; UAS-HipK* flies, which have a subtle rough eye, were crossed to lines containing *UAS-DmATP7*, *DmATP7* RNAi, *UAS-Ctr1A* and *Ctr1A* RNAi. There was no observable interaction observed with either suppression or ectopic expression of *DmATP7* implying that the *UAS-HipK* rough eye may not be copper related (Figure 5.11). Similarly, there was also no interaction observed with *Ctr1A* suppression or ectopic expression (Figure 5.12)

5.2.9 *HipK* suppression driven by *pannier-GAL4* supports an interaction with DmATP7

Suppression of *HipK* driven by *pannier-GAL4* results in no change from the wild-type, but when crossed to *pannier-GAL4; UAS-DmATP7* partially rescues the midline hypopigmentation caused by ectopic expression of *DmATP7* (Figure 5.11). This

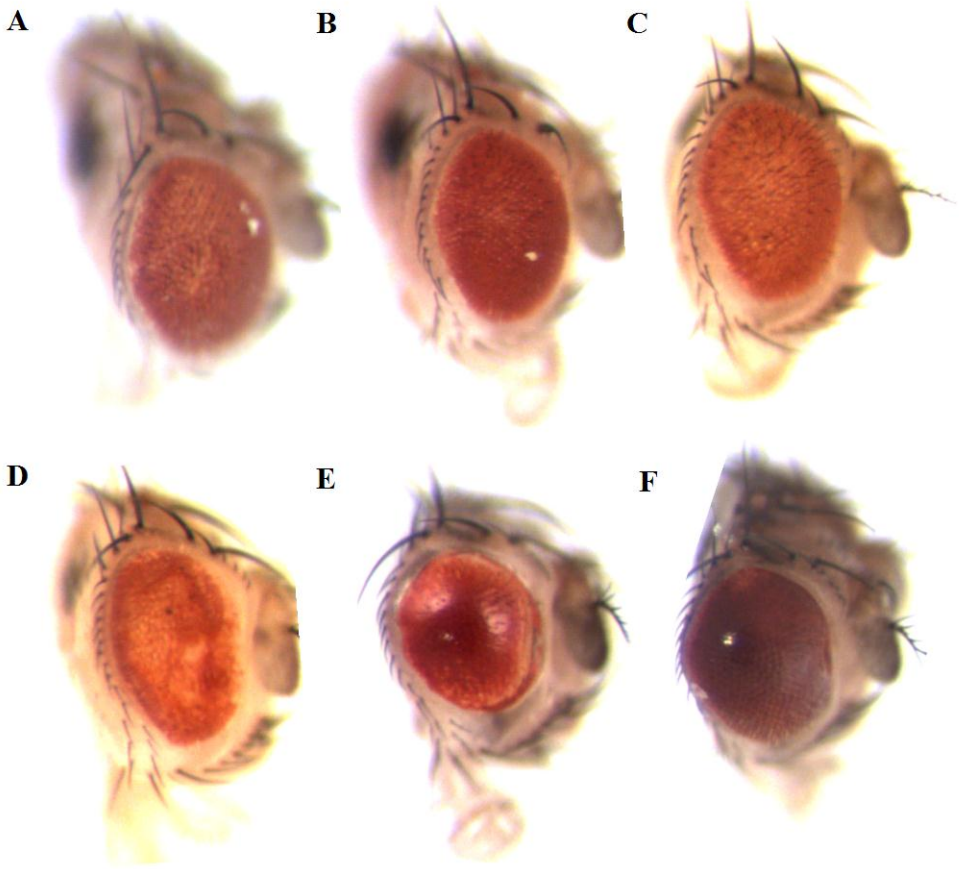

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Figure 5.12: Interactions observed between *UAS-HipK* and copper transport in the *Drosophila* eye

The *GMR-GAL4-UAS-HipK* phenotype was used to investigate the potential interaction between HipK and DmATP7. Expression of *UAS-HipK* alone using *GMR-GAL4* results in a mild rough eye phenotype (A). Combinations with either *UAS-DmATP7* or DmATP7 RNAi do not alter this phenotype (B,C). Co-expression with *Ctr1A* RNAi, which causes a sunken rough eye alone, results in a phenotype combining both original phenotypes (D). Expression of *UAS-Ctr1A* with *GMR-GAL4* causes lethality on normal food but some flies survive with a severe rough eye when reared on food supplemented with BCS (E). *UAS-HipK* and *UAS-Ctr1A* driven with *GMR-GAL4* resulted in a combination of both phenotypes (F). For all photos, two day old *Drosophila* adults had their heads removed and placed with the anterior side facing the right of the image before being observed using the Leica MZ6 dissecting microscope, imaged using the Leica DFC295 camera and analysed using Leica IM50 software.

phenotype may indicate that the loss of *HipK* expression leads to an increase in cellular copper levels due to reduced export by ectopic DmATP7. Interestingly *HipK* RNAi had no impact on the hypopigmentation observed with *pannier-GAL4* driven suppression of *DmATP7* or expression of *UAS-DmATP7*^{MUT}, both of which inhibit copper delivery to the secretory pathway of the TGN (Figure 5.13).

5.2.10 *UAS-HipK* alters the *mex-GAL4; UAS-DmATP7* phenotype

It has been shown in Chapter Three that *mex-GAL4* driven ectopic expression of *DmATP7* is viable on normal food but causes lethality when larvae are reared on 5µM copper-supplemented food, presumably due to increased midgut copper export. This section aimed to determine if altering the expression levels of midgut *HipK* could influence the *mex-GAL4; UAS-DmATP7* phenotype. Repeated interaction crosses of *UAS-HipK* to *mex-GAL4; UAS-DmATP7* flies generated no viable progeny when reared on normal food whilst expression of *HipK* RNAi did not alter the *mex-GAL4; UAS-DmATP7* phenotype. These results provide further evidence that ectopic HipK could be preventing degradation of DmATP7, heightening its cellular copper export function which results in larval lethality as excess copper is absorbed through the midgut enterocytes.

5.2.11 *UAS-HipK* alters DmATP7 localisation in the salivary glands

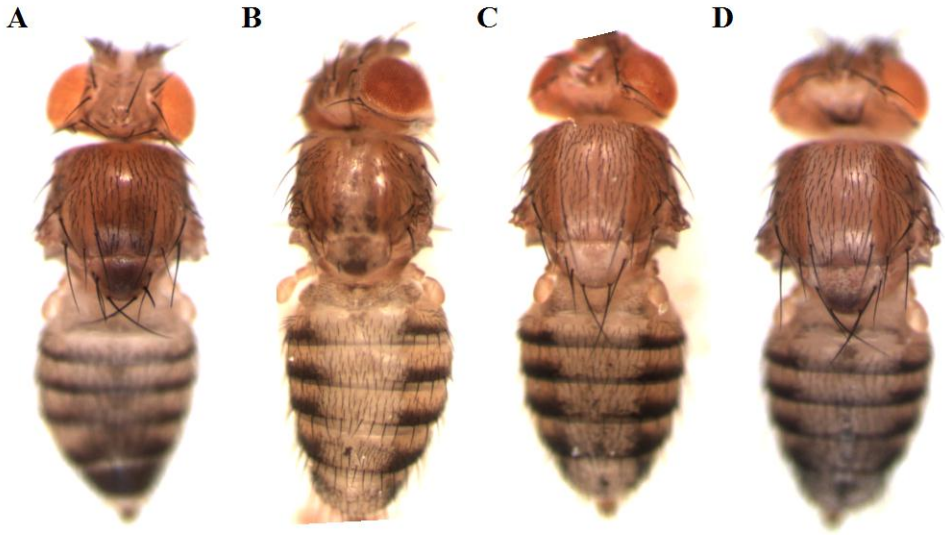
This chapter has demonstrated through functional analysis that HipK may prevent degradation of DmATP7 leading to a cellular copper deficiency via increased efflux. The investigation then aimed to determine if DmATP7 localisation would be altered when *HipK* was ectopically expressed or suppressed. *pannier-GAL4* was used to again investigate localisation in the secretory cells of salivary glands where DmATP7 is predominantly basolateral with some intracellular protein at the TGN (Figure 5.14). Expression of *UAS-HipK* resulted in increased and mis-localized DmATP7 throughout the cell (Figure 5.14). Unlike wild type cells, DmATP7 did not localize predominantly to

the basolateral membrane with significant amounts of the proteins at unknown intracellular locations (Figure 5.14). If this shift was to cause a cellular copper deficiency, DmATP7 may be exporting copper from the cytoplasm into a vesicular compartment where it is no longer available. Expression of *HipK* RNAi had no impact on the localisation of DmATP7 supporting the lack of midline phenotype caused by suppression of *HipK* alone (Figure 5.14). This result also implies that the *HipK* RNAi rescue of the hypopigmentation caused by *pannier-GAL4-UAS-DmATP7* was only partial.

5.3 Discussion & Conclusions

Despite a good understanding of the basic mechanisms involved in copper homeostasis, many questions remain unanswered with regards to the mode of action of import and export transporters, their regulation and the level of involvement of other proteins. For example, knowledge of the post-translational regulation of the chief copper export mechanisms, the Cu-ATPases, remains relatively scant (Lutsenko *et al.*, 2007). Key questions remain: How does the protein return to the TGN from outer plasma membrane after copper-induced trafficking? If the Cu-ATPases are degraded, what are the mechanisms involved in lysosomal or proteasomal degradation? Does the regulation of Cu-ATPase localisation or stability use known or novel cellular signaling/transport systems?

Drosophila has become a very useful tool in attempting to answer these and many more questions in copper homeostasis. Fly research takes advantage of useful tools such as the GAL4/UAS system, RNAi suppression and the generation of mutants via P-element insertion and excision (Ryder and Russell, 2003). As shown in previous research and earlier chapters in this investigation, the *Drosophila* copper homeostasis machinery is highly conserved with that of mammals, (Norgate *et al.*, 2006; Balamurugan *et al.*, 2007; Turski and Thiele, 2007; Burke *et al.*, 2008). Recent fly research has also been able to identify new protein interactions involved in copper transport (Norgate *et al.*, 2007; Southon *et al.*, 2008; Norgate *et al.*, 2010; Southon *et al.*, 2010). This investigation has



	***HipK* RNAi**
pannier-GAL4	Wild-type thorax/abdomen
pannier-GAL4-UAS-DmATP7	Partial rescue of hypopigmentation
***pannier-GAL4-DmATP7* RNAi**	Midline hypopigmentation
pannier-GAL4-UAS-DmATP7^{MUT}	Midline hypopigmentation

Figure 5.13: Interactions observed between HipK RNAi and copper transporters in the Drosophila thorax/abdomen

HipK RNAi was crossed to various *DmATP7* expression lines under the control of *pannier-GAL4* to further investigate a interaction between the two proteins. *HipK* RNAi alone under *pannier-GAL4* control does not generate a midline phenotype (A). When *HipK* RNAi is crossed to *pannier-GAL4-UAS-DmATP7* there is a partial rescue of the hypopigmentation caused by ectopic *DmATP7* implying copper export has been altered (B). Expression of *HipK* RNAi does not alter the phenotypes observed with *pannier-GAL4*-UAS-*DmATP7* RNAi (C) and *pannier-GAL4-DmATP7*^{DOMNEG} (D). For all photos, two day old *Drosophila* adults had their wings and legs removed before being observed using the Leica MZ6 dissecting microscope, imaged using the Leica DFC295 camera and analysed using Leica IM50 software.

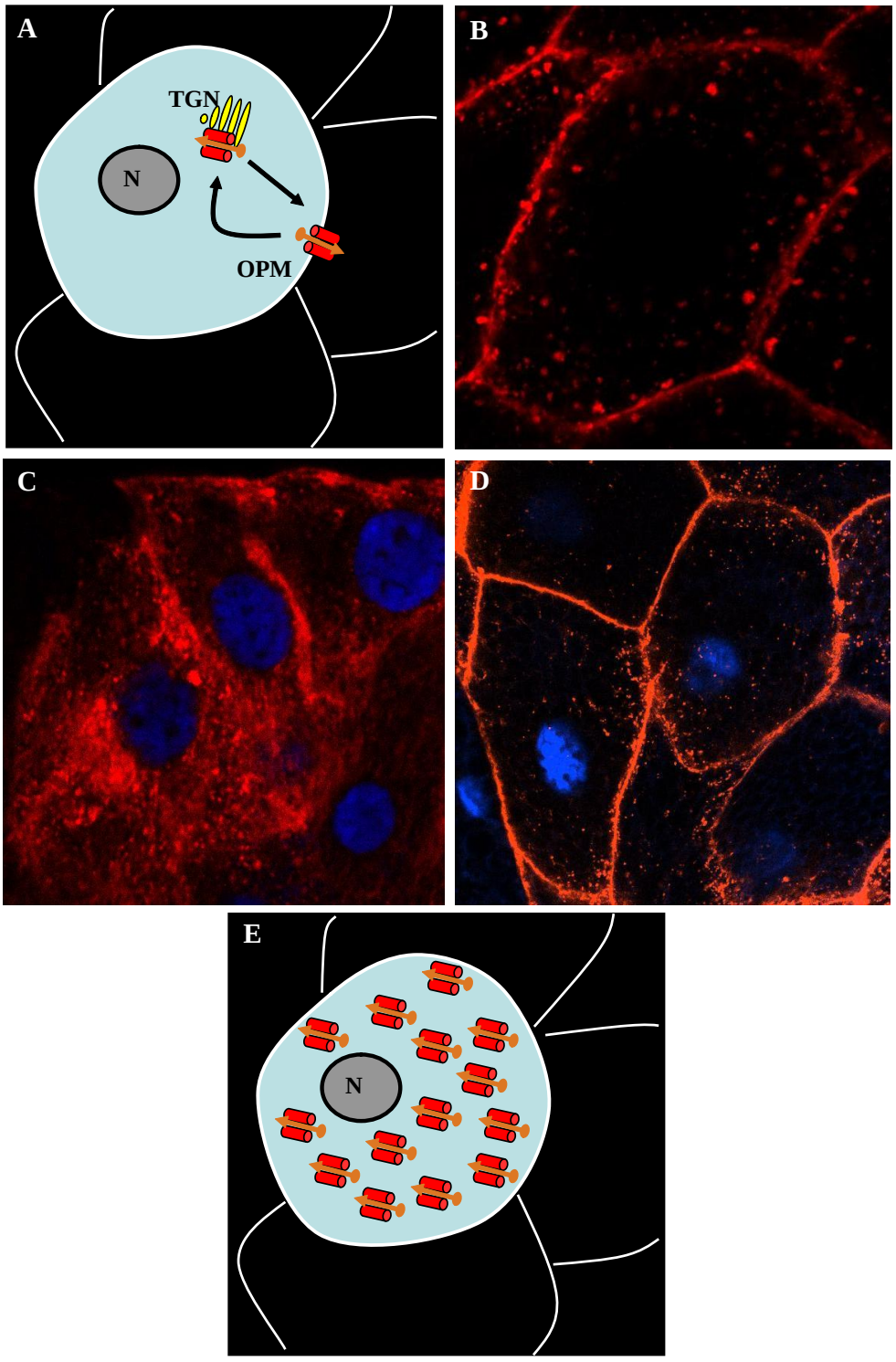

A
TGN
N
OPM
B
C
D
E
N

Figure 5.14: *UAS-HipK* causes increased and mis-localised DmATP7 in the *Drosophila* salivary glands

mCherry-DmATP7 expressed under the control of *pannier-GAL4* in the secretory cells of *Drosophila* salivary glands localises predominantly to the basolateral membrane with some located at the TGN (A+B). *mCherry-DmATP7* was crossed either *UAS-HipK* or *HipK* RNAi and dissected salivary glands from third instar progeny were viewed at 40X using confocal microscopy before images were analysed using NIS-Elements and ImageJ software. Expression of *UAS-HipK* resulted in increased and mis-localised DmATP7 throughout the cell, with less observed at the basolateral membrane (C). Expression of *HipK* RNAi did not alter DmATP7 localisation (D). Both results implied that HipK may be involved in the regulation of DmATP7 (modeled in E).

used the *Drosophila* model to define likely roles for dRab5 and HipK in the regulation of DmATP7.

5.3.1 Loss of *dRab5* in a cell-specific manner has severe consequences

Mammalian Rab5 is a crucial component of endocytosis, a process whereby vesicles bud off from membrane invaginations then mature into early and then late endosomes (Huotari and Helenius, 2011). Endocytosis is important to a cell as it can transport proteins, receptors or other molecules required for various different cellular processes. Transport can be to the TGN, to lysosomes and also back from early endosomes to the outer plasma membrane via recycling endosomes (Huotari and Helenius, 2011). Mammalian *Rab5* is ubiquitously expressed and both *in vivo* and *in vitro* studies have confirmed it is required for early endosome maturation (Gorvel *et al.*, 1991; Bucci *et al.*, 1992). *dRab5* is highly conserved with its mammalian counterpart, is also expressed ubiquitously and homozygous mutants are embryonic lethal (Wucherpfennig *et al.*, 2003; Zhang *et al.*, 2007).

This investigation used ectopic expression (WT, DN and CA versions) and RNAi suppression of *dRab5* in a cell-specific manner to expand on previous functional studies. Ubiquitous expression of either *dRab5-DN* or *dRab5* RNAi was lethal, as expected from the *dRab5* null mutant phenotype. Expression of *dRab5-WT* or *dRab5-CA* did not generate any phenotype in the tissues explored implying that ectopic expression of the vesicle transporter is less detrimental than loss of expression. It was highlighted in mammals that increased *Rab5* expression leads to the generation of large early endosomes but there is little evidence of a impact in *Drosophila* except for a increase in Toll signaling (Bucci *et al.*, 1992; Lund and Delotto, 2011).

5.3.1.1 Loss of *dRab5* inhibits endocytosis triggering cell over-proliferation

Due to the mutant being homozygous lethal, previous analysis of the effects of losing *dRab5* expression had been restricted to the heterozygous mutant phenotype (Wucherpfennig *et al.*, 2003). It was shown that $dRab5^{+/-}$ mutant larvae had reduced cellular polarity and increased cell proliferation leading to the formation of neoplastic tumour tissue in the larvae eye imaginal disc (Lu and Bilder, 2005). Similarly, results from this chapter demonstrated that *pannier-GAL4* expression of either *dRab5-DN* or *dRab5* RNAi resulted in over-proliferation of the wing disc. The over-proliferation phenotype was not unique to *dRab5* mutants but rather shared strong similarity to mutants of several other endocytic genes such as *avalanche*, *vps25* and *erupted tumour susceptibility gene* (Gilbert et al., 2009). Inhibition of endocytosis appears to prevent intracellular transport of important cargo required to maintain cell polarity and prevent over-growth. As these phenotypes are general traits of cancer progression, proteins such as dRab5 have been implicated as tumour suppressors (Vaccari and Bilder, 2009).

In the eye imaginal disc it was shown that endocytosis would normally regulate apical membrane proteins such as cellular polarity determinants (Crumbs) and growth factor regulators such as the Notch signaling receptor by transporting them via the early endosomes to lysosomes (Lu and Bilder, 2005). Loss of *dRab5* or other crucial genes in endosome maturation results in these proteins being trapped at the apical membrane causing inhibited cellular polarity and increased cell proliferation (Lu and Bilder, 2005). Further research into the *erupted tumour susceptibility gene* demonstrated that the mutant displayed reduced levels of the tumour suppressor orthologue Rbf1, which regulates progression through the G1-S phase of the cell cycle (Gilbert *et al.*, 2009). This coincided with mislocalisation of Notch signaling receptor and it was postulated that the Notch signaling pathway may be involved in Rbf1 regulation and subsequent cell over-proliferation (Gilbert *et al.*, 2009).

Although Notch signaling is a likely candidate for wing disc over-proliferation, it is also possible that inhibition of endocytosis would lead to mis-localisation of wing disc

specific polarity determinants. Wing disc polarity is controlled by the established morphogen gradients of the Wg (dorsal to ventral) and Decapentaplegic (Dpp) (anterior to posterior) pathways (Erickson, 2011). Morphogen concentration is highest in producing cells and slowly decreases towards each margin of the wing disc (Erickson, 2011). Producing cells excrete morphogen into the extracellular space before it binds to receptors of the adjacent cell (Erickson, 2011). It was thought that differing diffusion rates were responsible for maintaining the morphogen gradient but recent research has implicated a role for endocytosis. It is postulated that endocytosis may be required to internalize the morphogen then either re-secrete to the next cell or degrade via the lysosomes depending on where the cell lies in the gradient (Erickson, 2011). Loss of *dRab5* or other genes required for endocytosis has been shown to result in the failure of both Dpp and Wg to internalize (Gonzalez-Gaitan, 2003; Seto and Bellen, 2006). This then leads to a reduction in the range of the gradient of both morphogens, implying that more distant cells would lose polarity (Gonzalez-Gaitan, 2003; Rives *et al.*, 2006). This loss of polarity may help explain both autonomous and non-autonomous over-proliferation observed during *dRab5-DN* expression and *dRab5* suppression. Although not in the *pannier-GAL4* expression zone, non-autonomous cells would likely still be altered by changes to the morphogen gradients.

Another explanation for non-autonomous over-proliferation may be due to the activation of JAK/STAT signaling in non-autonomous cells. JAK/STAT signaling is involved in variety of processes including inducing cell growth and differentiation (Arbouzova, 2006). *dRab5* loss may result in ectopic expression of the JAK/STAT ligand, Unpaired 1 (Upd1), due to inhibited internalisation of Upd1 caused by the block in endocytosis (Devergne, 2007). Ectopic Upd1 can be secreted into the extracellular matrix and then bind to the Dome receptor of neighboring cells triggering JAK/STAT signaling and over proliferation in cells adjacent to the original *dRab5* mutant cells (Harrison, 1998; Devergne, 2007).

Third instar larval tumours generated from expression of *dRab-DN* or *dRab5* RNAi with *pannier-GAL4* are also likely to be caused by over-proliferation. *pannier-GAL4*

expresses in larval hemocytes (blood cells) which have previously been studied as a model of cell over-proliferation (Asha *et al.*, 2003). It was shown that ectopic expression of the fellow GTP-binding Ras proteins resulted in increased hemocyte numbers due to cellular over-proliferation and inhibition of endocytosis is likely to have a similar outcome (Asha *et al.*, 2003). These tumours were shown to occur at a consistent rate regardless of changes in the expression levels of *Ctr1A* and *DmATP7* demonstrating that the phenotype is not copper related.

5.3.1.2 Loss of *dRab5* in the eye may impact upon photoreceptor development

The dramatic phenotypes generated by *dRab5-DN* and *dRab5* RNAi expression with *GMR-GAL4* were similar to those previously identified by Houalla *et al*. Expression of *dRab5-DN* in the eye resulted in the failure of photoreceptor nuclei to maintain apical localisation (Houalla *et al.*, 2010). As discussed earlier in terms of eye disc over-proliferation, it could be postulated that the inhibition of endocytosis causes the deregulation of proteins required to maintain apical localisation. The over-proliferation of the eye disc itself may also impact upon the photoreceptor cells. It remains unclear why the dRab5-DN and dRab5 RNAi phenotypes differ in terms of the location (anterior versus posterior) of the black necrotic spots but it may be due to there still being partial *Rab5* function during RNAi suppression. *dRab5* RNAi suppression in the eye also results in a loss of pigment. This may be due to failed internalisation of the Notch receptor as over activation of the Notch signaling pathway has also been shown to be responsible for pigment cell death (Peralta S, 2009). It is unclear why expression of *UAS-dRab5-DN* did not result in the same pigment phenotype but it may suggest that the DN-mutation in the GTP-binding domain does not inhibit *dRab5* from influencing eye pigment.

5.3.2 dRab5 may be influenced by intracellular copper levels

Whilst initially clarifying the function of *dRab5*, the main objective of this work was to determine if there is any interaction between dRab5-mediated endocytosis and copper

homeostasis. Mammalian studies have suggested that ATP7A may co-localize with Rab5 but there has been no further research on any interaction (Pascale *et al.*, 2003). Work in this chapter has investigated a relationship between dRab5 and copper, by explorations in the *Drosophila* eye, imaginal wing disc, hemocytes and salivary glands.

As discussed earlier, the mechanisms causing the necrotic eye phenotypes observed in *Rab5-DN* or *Rab5* RNAi flies are likely caused by impeded development of photoreceptor cells. Investigations into the requirement for copper in the eye have discovered metal homeostasis is also needed to maintain normal eye development (Binks *et al.*, 2010). However, targeted manipulation of copper transport genes typically caused rough or sunken eyes and not the necrotic growths observed with the loss of *dRab5* (Binks et al., 2010). Therefore a change in copper transport is not likely to contribute to the loss of *dRab5* phenotypes. It is still clear however that there were phenotypic differences observed when copper transporters were manipulated in *GMR-GAL4*; *dRab5-DN* or *dRab5* RNAi flies.

Three of the interactions observed may be explained by changes in copper levels impacting upon the activity of *dRab5*. Suppression of *Ctr1B* or *DmATP7* results in the reduction in copper delivery to the TGN and resulted in an exacerbation of the *dRab5-DN* necrotic eye phenotype. The additional fact that lethality was observed with *GMR-Gal4; dRab5-DN/DmATP7* RNAi flies is likely due to the fact that the *GMR-Gal4* driver also expresses in a component of the ring gland known as the corpus cardiacum. The eye itself is actually dispensable for adult viability whereas production of hormones from the ring gland is essential for correct progression through pupariation. Conversely, ectopic expression of *Ctr1B* results in increased copper import, subsequent delivery of copper to the TGN and a slight rescue of the necrotic phenotype. Although highly speculative, these results suggest that copper levels may induce endogenous *dRab5* activity. This may be a mechanism use to internalize copper import proteins and prevent against cellular toxicity. The concept of internalization is not foreign to the Ctr-protein family as hCtr1 shifts between the outer plasma membrane and perinuclear organelles in cervical carcinoma and HEK293 cells (Klomp *et al.*, 2002; Molloy and Kaplan, 2009). Internal

localization was found to be dependent on increased extracellular copper levels but a mechanism of translocation remains elusive (Molloy and Kaplan, 2009). Previous work and this thesis have only shown both *Drosophila* Ctr proteins localizing to the outer plasma membrane and not internally (Balamurugan *et al.*, 2007; Turski and Thiele, 2007). However, these speculative results with *dRab5* give rise to the idea that the *Drosophila* Ctr proteins could be internalized by endocytosis. Further research is required to identify shifts in Ctr localization during the manipulation of endocytic genes such as dRab5. Techniques such as membrane surface biotinylation could be used to further quantify membrane Ctr versus internal.

5.3.3 dRab5-mediated endocytosis is required for DmATP7 localisation

The small, rough necrotic eye generated from the combination of *dRab5-DN* with *UAS-DMATP7* may also be considered a low copper situation exacerbating the *dRab5-DN* necrotic phenotype. However, the severity of the phenotype and changes in eye size lead to speculation that it may be caused by an interaction between increased DmATP7 and the inhibition of endocytosis. This is further supported by the localization data which indicated that *dRab5-DN* expression caused increased intracellular DmATP7. This is in contrast with discussed research where inhibition of endocytosis lead to the entrapment of proteins such as the Notch receptor or Crumbs at the outer plasma membrane (Lu and Bilder, 2005). In this case however, inhibited endocytosis may lead to incorrect localisation of DmATP7 at intracellular locations. Some DmATP7 co-localized with dRab5-DN-GFP, likely to be in either immature early endosomes or pre-endocytic vesicles adjacent to the membrane, but the majority was at unidentified intracellular localisations (Bucci *et al.*, 1992). Increased intracellular DmATP7 may be able to cause copper transport abnormalities that would result in the observed small, necrotic eye phenotype. There may be increased copper export into unknown organelles such as storage vesicles or pre-endocytic vesicles. Could this alteration to cellular copper homeostasis cause the reduction in eye size? Copper has been shown to be essential for cellular proliferation due to an unanticipated role in the mitogen-activated (MAP)_kinase pathway (Turski *et al.*, 2012). In this study, inhibition of copper uptake resulted in a

reduction in the ability of the MAP kinase Mek1 to phosphorylate the MAP kinase Erk (Turski *et al.*, 2012). This demonstrated that copper was required to activate the cellular proliferation pathway.

Co-localisation of dRab5-WT-GFP with DmATP7 also provides further evidence of an interaction between the proteins. Punctate dRab5-WT-GFP signal, likely to represent enlarged early endosomes caused by increased *dRab5* expression, co-localized with the majority of DmATP7 and in turn reduced basolateral localization of the copper importer (Bucci *et al.*, 1992). This suggested that increased *dRab5* expression could also internalize DmATP7 but in this case predominantly to early endosomes. It must be noted that *pannier-GAL4* expression of *UAS-dRab5-WT* did not rescue the *UAS-DmATP7* phenotype (results not shown). In this case it may be that some ectopic DmATP7 remains present at the outer plasma membrane and continues to cause a cellular copper deficiency. Both localization results add weight to the argument that endogenous DmATP7 would use the endocytic pathway for transport from the outer plasma membrane to the TGN.

Emerging synchrotron technology also identified that expression of *dRab5-DN* in the dorsal pannier domain of the wing imaginal disc resulted in both autonomous and non-autonomous over growth and that these hyper proliferating tissues were high in zinc and iron but not copper. Measuring the trace metal content using XFM is an exciting option for directly measuring copper levels and has been demonstrated to be effective in various tissues (McRae *et al.*, 2010; Lye *et al.*, 2011; Qin *et al.*, 2011). As the overgrowth was a thicker tissue than the remaining disc it was anticipated that metal content would rise, so the copper result is intriguing as it suggests that copper levels are decreased in these cells. This copper deficiency could be due to increased export of copper vesicles that may later leave the cell via exocytosis. This interpretation of the synchrotron data remains preliminary and it must be considered that the non-autonomous overgrowths also shared a similar metal content profile. These cells are clearly also compromised and factors leading to overgrowth may also impact upon metal content. More detailed analysis of

metal content in the eye imaginal disc and other tissues is required to confirm the observation that loss of *dRab5* causes a change to copper levels.

5.3.4 Conclusions regarding the role of dRab5-mediated endocytosis in DmATP7 transport

Results from this chapter support the notion that endocytosis is an important cellular process that is required for the internalisation of many different proteins, receptors or other molecules. Clear evidence has also been presented suggesting that endocytosis may be an important process for the regulation of copper homeostasis. Results from this chapter led to the postulation that copper levels may influence *dRab5* activity, whilst at the same time *dRab5* expression is crucial in determining the localization of the key copper export protein DmATP7.

These results will trigger further research into the role of endocytosis in the regulation of copper homeostasis. Endocytosis is a constant network of vesicles moving in both directions and many questions remain. How would DmATP7 be transported from early endosomes to the TGN? Can recycling endosomes be involved in transport back to the membrane? Using *Drosophila* as a model organism to study Cu-ATPase regulation by endocytosis may provide the future answers to these questions. Determining the exact effect of the loss of dRab5 on DmATP7 localization remains the first key question to be answered. Quantifying co-localisation with intracellular markers and membrane surface biotinylation could be used to measure the changes in DmATP7 localization. Future studies should explore the $Rab5^{-/+}$ mutants to determine if changed copper levels can influence the mutant phenotypes and additionally identify if cellular copper homeostasis is altered. Other endocytic genes such as the effector protein of Rab5 *VPS34/p150*, *avalanche*, *vps25* and *erupted tumour susceptibility gene* could also be explored to further confirm the observed interactions.

5.3.5 HipK may regulate copper homeostasis in *Drosophila*

The second aspect of copper homeostasis regulation investigated in this chapter was the potential involvement of HipK in DmATP7 degradation. HipK is a homeodomain serine/threonine kinase that inhibits the activity of Slimb, a E3 ubiquitin ligase. This inhibition of a key proteasomal degradation pathway has been shown to help regulate the stability of members of several cellular pathways including Wnt/Wg, Notch and Hedgehog (Lee *et al.*, 2009; Swarup and Verheyen, 2011). Similar processes have been implicated in regulation of the mammalian Cu-ATPases when targeted by partner proteins COMMD1 and Clusterin (Meusser *et al.*, 2005; de Bie *et al.*, 2007).

HipK is an essential *Drosophila* gene but results from this chapter demonstrated that targeted RNAi suppression of the gene in the eye, midgut or midline did not alter the wild-type phenotype. Some residual HipK expression is likely as the null mutant caused a rough eye phenotype (Lee et al., 2009). In contrast, cell-specific ectopic expression of *HipK* resulted in strong phenotypes in the midline and eyes. The midline phenotype consisted of three components, a dramatic cleft of the thorax, complete loss of scutellum and a band of pigment loss down the entire abdominal midline. These phenotypes are likely the result of the culmination of many cellular processes that *UAS-HipK* could positively regulate by inhibiting the proteasomal degradation pathway. The dramatic cleft in the thorax is likely caused by the inability of the two hemithoraxes to come together at the dorsal midline for thoracic closure, possibly due changes in either the JNK or Dpp signaling pathways (Pena-Rangel *et al.*, 2002). The scutellum forms as a result of the both Hedgehog and Dpp signaling cascades, one of which (Hedgehog) has been shown to be regulated by HipK (Pena-Rangel *et al.*, 2002; Swarup and Verheyen, 2011). Midline pigmentation is a complex process that involves the production of pigment components dopa melanin, dopamine melanin and NBAD sclerotin (Wright, 1987). Although *UAS-HipK* is likely to disrupt several of the above signaling networks during development of the thorax, scutellum and pigmentation, the midline phenotypes are also indicative of a copper deficiency (Norgate *et al.*, 2006; Binks *et al.*, 2010). Interestingly all three phenotypes were partially rescued when *UAS-HipK* flies were reared on food

supplemented with copper. This could imply that the midline phenotype, although largely caused by the disruption of major developmental pathways, is partially caused by a copper deficiency or alternatively that HipK itself is down regulated by increased copper levels.

Ectopic expression of *HipK* in the *Drosophila* eye caused a mild rough eye phenotype which was less severe than observed in previous research (Lee et al., 2009). In that case *UAS-HipK* was driven with *eyeless-GAL4*, a stronger driver than *GMR-GAL4*. The rough eye phenotype is not likely to be copper related as it was not altered by the genetic manipulation of copper transporters or by changing dietary copper levels. Ectopic HipK expression resulted in no phenotype in the midgut and also did not affect enterocyte copper levels as determined by copper-responsive reporter genes (data not shown in results).

5.3.6 HipK inhibits the degradation of DmATP7

Lethality caused by the co-expression of *UAS-HipK* and *UAS-DmATP7* may be caused by an additive amalgamation of two unrelated severe midline phenotypes or it could indicate a genetic interaction. Interestingly, *HipK* suppression partially rescues the midline phenotype caused by *UAS-DmATP7* implying there is in fact a genetic interaction. *UAS-DmATP7* increases copper export leading to cellular deficiency and therefore the addition of *UAS-HipK* may cause lethality by exacerbation of this export. This theory is supported in the *Drosophila* midgut where ectopic *DmATP7* expression combined with a high copper diet results in lethality, presumably due to increased export of copper into the lymph. As discussed in Chapter Three, there was no lethality observed on normal food implying that although copper deficient, the enterocyte cells were viable. The addition of *UAS-HipK* expression may increase the copper deficiency or increase flux of copper through to the lymph causing lethality on normal food. In both midline and midgut situations, inhibited degradation of DmATP7 could lead to increased copper export from the cytoplasm and a resultant deficiency. This would lead to the assumption that *UAS-HipK* expression alone would stabilize endogenous DmATP7, leading to a copper deficiency and partially explaining the *UAS-HipK* midline phenotype.

Supporting evidence that DmATP7 could be degraded was found in the other interactions with HipK in the midline. Increasing *Ctr1A* or *Rab5* expression partially rescued the scutellum of *UAS-HipK* mutants. Increasing *Ctr1A* will lead to elevated copper import, compensating for the hypothesized cellular copper deficiency. Increasing *Rab5*, as shown earlier in this chapter, can result in the mis-localisation of DmATP7 to the early endosomes. This would prevent copper export and also reverse a cellular copper deficiency. Alternatively as suggested earlier, the increased cellular copper levels could down regulate HipK. It is interesting that only the scutellum aspect of the phenotype was rescued in these cases when compared to the rescue observed by increasing dietary copper levels. Scutellum loss is a trait of cellular copper deficiency rather than inhibited delivery of the metal to the TGN (Binks *et al.*, 2010). It may be in this case that rescue of scutellum requires a more subtle increase in copper levels than thoracic formation or pigmentation.

Expression of the $DmATP7^{DOMNEG}$ mutant with *UAS-HipK* resulted in rescue of the scutellum implying it could also rescue cellular copper deficiency. Initially this appears to be in disagreement with the proposed model of the $DmATP7^{DOMNEG}$ mutant which suggests the inactive form of DmATP7 replaces endogenous at the TGN, inhibiting copper transport to the secretory pathway and causing hypopigmentation (Norgate *et al.*, 2006). It may be that when DmATP7 degradation is inhibited, the inactive mutant can also replace the endogenous DmATP7 at other intracellular locations, preventing a copper deficiency. This may also happen in the absence *UAS-HipK*, but it is not observed as the resultant increase in cellular copper does not generate a phenotype. It remains unclear why expression of *DmATP7* RNAi did not result in scutellum rescue as it would be expected to suppress the ectopic DmATP7. One possible scenario is that the increased levels of endogenous protein may swamp the RNAi. Expression of $DmATP7^{DOMNEG}$ may also be more effective than RNAi suppression as HipK may also stabilize the mutant form of DmATP7.

Functional studies suggested that *HipK* ectopic expression leads to the increased stability of DmATP7 and a resultant cellular copper deficiency, possibly via the inhibition of an E3 ubiquitin ligase. While it would seem likely that increased DmATP7 would localize to the outer plasma membrane for copper export, surprisingly this theory was not supported by localisation data. When *UAS-HipK* was expressed in the secretory cells of the salivary gland, DmATP7 was dramatically increased but not at the outer plasma membrane. Instead, the increased amounts of DmATP7 were observed throughout the cytoplasm. This raises the possibility that DmATP7 is exporting copper from the cytoplasm into bio-unavailable stores or into vesicles which are then exported from the cell. There is some evidence of ATP7A localising to vesicles adjacent to the basolateral membrane to export copper but the localisation shown is not consistently adjacent to a membrane (Nyasae *et al.*, 2007). This phenotype may be caused by stabilized DmATP7 not being susceptible to endogenous trafficking mechanisms, resulting in mis-localisation, in a similar effect to that caused by loss of *dRab5* as discussed earlier. This theory requires further investigation into the localization shift of DmATP7. As discussed with the dRab5 investigation, membrane surface biotinylation could be used to quantify the localization shift. Co-localization with a variety of intracellular and endocytic markers could also be investigated to determine just where stabilized DmATP7 is located.

5.3.7 Conclusions as to the role of HipK in DmATP7 regulation

HipK is a homeodomain serine/threonine kinase that inhibits the activity of Slimb, an E3 ubiquitin ligase and therefore can stabilize target proteins by blocking degradation. Results in this chapter provided strong evidence that post-translational regulation of DmATP7 may involve HipK-inhibited degradation. Increased *HipK* exacerbates *Drosophila* midline and midgut phenotypes caused by ectopic expression of *DmATP7* implying that degradation of the export protein is inhibited. Midline phenotypes generated by *UAS-HipK* appear to be partially caused by copper deficiency as the phenotypes are rescued by increased cellular copper levels. It appears that degradation of DmATP7 may be required as a form of regulation to prevent against copper deficiency

Post-translational modification of DmATP7 has not been demonstrated to date but both mammalian Cu-ATPases undergo degradation, targeted by Clusterin and COMMD1 and it would appear likely that the *Drosophila* copper importer would also undergo this form of regulation. Despite identifying the protein interaction, future research is required to confirm that DmATP7 is degraded and the process is regulated by HipK. The likely experiment to confirm this interaction would be *in vitro* ubiquitination assays with HipK, Slimb and DmATP7. This would be able to confirm that Slimb can bind DmATP7 and cause degradation. Ectopic expression and suppression of Slimb can also be investigated to determine if there are any changes to DmATP7 function and localization. Another approach would be to inhibit Slimb by using proteasome degradation inhibitors.

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CHAPTER SIX: FINAL DISCUSSION AND CONCLUSIONS

Synopsis

Drosophila has emerged as a useful model organism to provide insight into human copper homeostasis mechanisms. This thesis explored midgut-specific copper homeostasis and investigated novel regulatory systems. This chapter discusses results from midgut-specific suppression and ectopic expression of key copper transporters. The sub-cellular localization of these key transporters in the midgut, salivary glands and malpighian tubules is also discussed. Investigations into the role of dRab5 and HipK in regulating DmATP7 are also further discussed. Furthering this research in the *Drosophila* model will expand upon mammalian copper homeostasis research and increase understanding of copper related disease.

6.1 Introduction

Research into the mechanisms of copper transport began when early twentieth century experiments identified that the metal was an essential micronutrient (Hart *et al*., 1928). Two human conditions, Menkes and Wilson's disease, were later determined to be the consequence of systemic copper deficiency and overload respectively (DiDonato and Sarkar, 1997; Kaler, 2011). Subsequently, mechanisms of importing, distributing, sequestering and exporting copper were identified in humans (Chelly and Monaco, 1993; Mercer *et al.*, 1993; Klomp *et al.*, 1997; Zhou and Gitschier, 1997; Lamb *et al.*, 2001; Robinson and Winge, 2011). Homologous copper transport systems in model organisms such as mice, *Drosophila* and fish have been identified leading to further research in each species. *Drosophila*, in particular, has emerged as an ideal model organism for genetic research due to its very short life cycle, ease and growth of maintenance and the sophisticated genetic techniques available (Ryder and Russell, 2003). The key components of the *Drosophila* copper homeostasis machinery have been revealed over the past decade with the identification of the Ctr and Cu-ATPase homologues (Zhou *et al.*, 2003; Southon *et al.*, 2004; Norgate *et al.*, 2006; Turski and Thiele, 2007). This thesis has expanded on this knowledge by focusing specifically on the function of these genes in the midgut as well as exploring novel regulatory mechanisms identified through genetic screen, thus thereby providing important new insights into the complex genetic interactions required to maintain copper homeostasis in animals.

6.2 Ctr1A and Ctr1B are the major copper importers in *Drosophila*

Ctr1A and Ctr1B, homologues of hCtr1, have been previously identified as the principal sources of cellular copper uptake in *Drosophila*. Homozygous $Ctr1A^{-/-}$ mutants are larval lethal and tissue-specific loss of *Ctr1A* in the midline or eye results in phenotypes reflective of a copper deficiency (Turski and Thiele, 2007; Binks *et al.*, 2010). *Ctr1B* expression is up-regulated in times of copper scarcity and null mutants of this gene are lethal when dietary copper levels are altered (Zhou *et al.*, 2003). Taken together these results indicate that Ctr1A is the primary source of copper import, whilst Ctr1B is

required to boost import when dietary copper levels are low. Both proteins were previously shown to localize to the apical membrane of polarized cells and are major candidates to control copper entry into enterocytes from the midgut lumen (Balamurugan *et al.*, 2007; Turski and Thiele, 2007).

6.2.1 The Ctr proteins localize to both the apical and basolateral membrane of intestinal enterocytes

Results from this thesis demonstrated that Ctr1A-GFP and Ctr1B-GFP both localize predominantly to the apical membrane of intestinal enterocytes, but there is also some signal of both proteins from adjacent to or at the basolateral membrane (Chapter 4.2.2). It appears that copper import may be more specialized in enterocytes than in other cell-types in *Drosophila*. Predominant localization of both Ctr proteins at the apical membrane of enterocytes implies that both proteins are capable of importing copper from the lumen of the midgut into the midgut cells. However the fact that there was also some basolateral localization of both Ctr proteins was unexpected and implies that they can import copper at both membranes.

This concept is not novel to *Drosophila*, as the location of hCtr1 in human enterocytes remains a point of contention with both apical and basolateral localization demonstrated in separate studies (Zimnicka *et al.*, 2007; Nose *et al.*, 2010; Zimnicka *et al.*, 2010). Immunohistochemical and biotinylation studies in Caco-2 cells identified basolateral hCtr1, whilst mouse, rat and pig small intestinal sections demonstrated apical hCtr1 (Zimnicka *et al.*, 2007; Nose *et al.*, 2010; Zimnicka *et al.*, 2010). Enterocytes must facilitate dietary copper absorption whilst also having a cell-specific requirement for copper, incorporating the nutrient into multi-copper oxidase enzymes such as Hephaestin in mammals which is required for dietary iron absorption (Yuan *et al.*, 1995; Nyasae *et al.*, 2007). Could these results from the *Drosophila* midgut support the suggestion that separate sources of enterocyte copper are required for basolateral export and delivery to the TGN? Is Ctr-mediated copper import back from the bloodstream / lymph across the basolateral membrane required because dietary copper absorbed from the lumen is not

available to enterocytes for cell-specific use? This remains a difficult hypothesis to test and would require determination of bioavailable versus not available copper in enterocytes.

6.2.2 Could there be alternative sources of midgut apical copper uptake in *Drosophila*?

Research which demonstrated basolateral hCtr1 in enterocytes suggested that alternative sources of copper import may be required at the apical membrane with candidates including DMT1 or anion exchanger channels. Whilst it appears highly likely that *Drosophila* Ctr1A/B do have a major role in apical import due to their predominant apical localization, non-Ctr mediated import cannot be ruled out. This is due to the fact that midgut-specific suppression of both *Ctr* genes had no impact on the larval to adulthood survival nor did it cause reduced larval copper content as determined by monitoring expression of *Ctr1B* and *MtnB* expression reporter constructs (Chapter 3.2.1 and 3.2.5.1). The *Ctr* null mutant phenotypes did suggest that Ctr1A and Ctr1B were the major sources of midgut copper uptake yet also do not disprove the concept that other avenues of uptake could further supplement enterocyte copper levels in times of need (Zhou *et al.*, 2003; Turski and Thiele, 2007). In fact, the individual Ctr1A and Ctr1B null mutant phenotypes were both partially rescued by copper supplementation. This may have been due to increased import by the other Ctr protein but may also be due to a non-Ctr alternative source of uptake. To further assess this concept a double *Ctr1A/Ctr1B* knockout needs to be investigated to determine if copper supplementation can still partially rescue the lethal phenotype.

Alternative sources of apical copper import may not be the principal source but rather a mechanism which could substitute for the loss of *Ctr* expression by providing sufficient dietary copper import for survival through to adulthood. Mammalian research has identified likely candidates for this role which could be further investigated in *Drosophila*. Iron transporter DMT1 has been shown to localize to the apical membrane of polarized cells and partial knockdown results in reduced copper uptake (Arredondo *et al.*, 2003; Yanatori *et al.*, 2010). The *Drosophila* homologue of DMT1, Malvolio, can

transport copper *in vitro* and null mutants are sensitive to copper excess and limitation (Southon *et al.*, 2008). Malvolio is a likely candidate for additional copper import in *Drosophila* enterocytes and future experiments should investigate enterocyte copper content in mutants and co-suppress the gene in combination with *Ctr1A* and *Ctr1B*. Recent research has also raised the possibility of intestinal copper import by anion exchangers, as inhibition of the ion channels reduced copper uptake in cultured xxx cells (Zimnicka *et al.*, 2010). The SLC26 anion exchangers may play this role as they are apically expressed in the mammalian intestine (Zimnicka *et al.*, 2010). The SLC26 anion exchangers are relatively uncharacterized in *Drosophila* but future research could also determine if mutation of any of these genes results in reduced copper uptake *in vitro* or in enterocytes in vivo.

If other avenues of uptake can mask some of the affect of *Ctr* suppression, it remains likely that *Drosophila* may be able to survive with minimal midgut copper import. Could the minimal copper import from alternative sources be supplemented by better utilization of the nutrient? Previous research has shown that *Drosophila* that undergo short pulses of growth on high copper food can then develop more efficiently than controls when transferred to low copper environments (Balamurugan *et al.*, 2007). It was suggested that when dietary copper levels are in excess the nutrient could be stored, likely by metallothioneins, before being released to cope with periods of copper scarcity (Balamurugan *et al.*, 2007). It can be hypothesized that in normal conditions some copper would also be stored, albeit not enough to improve development if larvae are then transferred to a low copper environment. Could limiting this storage mechanism, creating more available copper, be a means for better utilization of the nutrient? Could the minimal copper import from a non-Ctr transporter be supplemented by inactivation of a storage mechanism? This theory remains highly speculative but could be tested by over-expressing metallothionein in combination with *Ctr1A/B* suppression and determining if the further loss of available copper could cause lethality.

6.2.3 Ectopic expression of *Ctr1A* and *Ctr1B* in the *Drosophila* midgut

Ectopic *Ctr1A* or *Ctr1B* causes increased enterocyte copper content as determined by monitoring expression the *MtnB* expression reporter construct (Chapter 3.2.4.2). In the case of *Ctr1B* ectopic expression alone this resulted in lethality on increased copper load (Chapter 3.2.2.1). The dual localization of Ctr1A and B discussed above implies that increased Ctr protein, predominantly apical, enhances copper import, possibly triggering oxidative damage in the midgut leading to lethality. *Drosophila* would be very susceptible to the enhanced import caused by ectopic *Ctr1B* as the gene is thought to be almost exclusively regulated at the transcriptional level and localisation of the protein does not shift from the plasma membrane during copper shock treatment (Balamurugan *et al.*, 2007). The lower impact of Ctr1A in the midgut compared to Ctr1B suggests that the effect of ectopic *Ctr1A* may be blunted by some form of post-translational inhibition. Interestingly *Ctr1A* expression has been shown to be more deleterious that *Ctr1B* in the fly eye (Balamurugan *et al*, 2007) indicating that this inhibition is enterocyte specific. As the majority of the Ctr1B was apically located it must also be considered that lethality may be caused by increased copper content of the lymph. This is supported by the partial increase in SOD activity, although not significant, demonstrated in developing third instar *UAS-Ctr1B* larvae reared on normal food.

6.2.4 The localizations of Ctr1A and Ctr1B reflect a role in apical import in the *Drosophila* salivary glands and malpighian tubules

Localization of the Ctr proteins was also explored in other polarized cell types; the secretory cells of salivary glands and the principal cells of malpighian tubules. The salivary glands are the key secretory organ in *Drosophila* larvae and are made up of a connective tube joining the mouthparts to secretory cells (Andrew, 2000). *Ctr1A* and *Ctr1B* are both expressed in the salivary glands suggesting a role for both proteins (Chintapalli *et al.*, 2007). Results from this thesis demonstrated that both Ctr1A and Ctr1B predominantly localized to the apical side of secretory cells of salivary glands, consistent with a role in import from the inner duct (Chapter 4.2.4). Copper would be

required to be transported across the apical membrane into secretory cells to act as a cofactor for the cuproenzyme Lysyl oxidase which is required for the production of cross-links in collagens, cellular motility and/or cellular differentiation (Molnar *et al.*, 2005). Future studies could confirm this role by assaying Lysyl oxidase activity in the salivary glands when either/both *Ctr1A* or *Ctr1B* are specifically ectopically expressed or suppressed.

The role of the malpighian tubules is to import ions from the lymph into the principal cells of the tubules before excretion down a inner luminal tube towards the gut for removal (Beyenbach, 2003). *Ctr1A* is strongly expressed in malpighian tubules, whilst Ctr1B expression is low (Chintapalli *et al.*, 2007). Both Ctr1A and Ctr1B localized predominantly to the apical membrane of principal cells implying that copper can be transported against the excretion gradient from the luminal tube into principal cells (Chapter 4.2.4). Like in the mammalian kidneys, copper ions may be re-absorbed back into the lymph to maintain ionic balance (Beyenbach, 2003). Further research could use the malpighian tubule specific *C42-GAL4* to suppress or ectopically express both *Ctr1A* and *Ctr1B* to determine if the hypothesized functions are required for survival.

6.2.5 Regulation of Ctr1A and Ctr1B localization

HCtr1 shifts between the outer plasma membrane and perinuclear organelles in cervical carcinoma and HEK293 cells (Klomp *et al.*, 2002; Molloy and Kaplan, 2009). Internal localization was found to be dependent on increased extracellular copper levels but a mechanism for this translocation remains elusive (Molloy and Kaplan, 2009). Previous work has only shown both *Drosophila* Ctr proteins localizing to the outer plasma membrane and not internally (Balamurugan *et al.*, 2007; Turski and Thiele, 2007). This thesis demonstrated that some Ctr1B in salivary gland secretory cells was located in intracellular regions away from the apical membrane (Chapter 4.2.4). My genetic interaction studies in the fly eye have indicated that dRab5-mediated endocytosis may be involved in internalisation of the Ctr proteins.

Expression of *dRab5-DN* in the eye caused the formation of black spots on the anterior side of the eye (closet to the front of the head) (Chapter 5.2.1.1). Suppression of *Ctr1B* or *DmATP7* both result in reduced copper delivery to the TGN and both resulted in an exacerbation of the *dRab5-DN* necrotic eye phenotype (5.2.2). Conversely, ectopic expression of *Ctr1B* results in increased copper import, subsequently increased copper delivery to the TGN and a slight rescue of the necrotic phenotype (5.2.2). Could these results suggest that copper levels induce endogenous *dRab5* activity? A potential reason for increased dRab5-mediated endocytosis could be to regulate Ctr function by internalizing the protein to prevent further copper import. To further investigate this theory membrane surface biotinylation could be used to further quantify membrane versus internal Ctr levels. The same comparison could be made when *dRab5* and other endocytic genes are over-expressed or suppressed.

6.3 Copper distribution and export

After being imported into the cell, copper is not free in the cytosol but rather bound to proteins known as metallochaperones. Copper entering mammalian enterocytes is likely to be distributed via metallochaperones such as Atox1, CCS and the Cox network of chaperones yet there is little specific evidence for this. There is some data showing that Atox1 is required for dietary copper absorption but further research is required to clarify intestinal specific roles for each chaperone (Hamza *et al.*, 2003). This study examined the effect of manipulation of *Drosophila* homologues of mammalian metallochaperones on animal viability and on the copper content of the enterocytes (Chapter 3.2.7). Reduced copper uptake, in combination with reduced *ScoX* activity resulted in a drop in animal viability presumably due to less delivery of copper to Cytochrome *c* oxidase in midgut cells. Midgut-specific ectopic expression of the putative *Drosophila* copper chaperone *CutC* increased enterocyte copper content. Unpublished data from the Burke laboratory has indicated CutC is localized in the nucleus and may interact with the transcription factor MTF-1. Although highly speculative this result may suggest that increased CutC can induce MTF-1 to stimulate transcription of Ctr1B, resulting in increased copper import.

Both results give preliminary evidence that copper distribution is important in *Drosophila* enterocytes and localization data may assist in identifying cell-specific roles for each chaperone.

6.3.1 Midgut DmATP7 predominantly exports copper at the basolateral membrane

The dual localisation of DmATP7 at both the Golgi and basolateral plasma membrane in larval enterocytes leads to the likely hypothesis that the protein is involved in both copper export from the cell and copper delivery to the secretory pathway at the TGN (Chapter 4.2.3). Supporting the localization analysis was the fact that midgut-specific suppression of *DmATP7* resulted in increased metallothionein induction, implying enterocyte copper accumulation (Chapter 3.2.4.2). In fact, simultaneous increasing of midgut copper import with suppression of *DmATP7* caused lethality which was likely due to enterocyte copper toxicity (Chapter 3.2.3). These results were supported by previous copper accumulation phenotypes observed during *DmATP7* suppression in S2 cells and in the intestinal cells of *DmATP7*$^{-/-}$ mutant larvae prior to death (Southon *et al.*, 2004; Norgate *et al.*, 2006). Copper accumulating in the midgut is likely to cause oxidative damage which increases the transcription of genes leading to autophagy and ultimately cell death in the *Drosophila* midgut (Wu *et al.*, 2009). Supporting this, co-suppression of *CCS* with *DmATP7* results in a significant drop in viability on all food types (Chapter 3.2.6.1). Removal of CCS leads to less delivery of copper to SOD1 (the key defensive enzyme against oxidative damage) which would compromise the cells' ability to withstand oxidative damage caused by excess copper accumulation.

A role for DmATP7 in exporting copper from midgut cells into the lymph/bloodstream is analogous with that of ATP7A in mammals. There may be subtle differences between the mammalian/*Drosophila* models as ATP7A is located at vesicles adjacent to the basolateral membrane rather than at the actual membrane as observed with DmATP7. These differences may suggest a simpler model of copper export in *Drosophila*. In mammalian enterocytes, copper is removed either by ATP7A cycling between adjacent

organelles and the basolateral membrane or by exporting copper into organelles before exocytosis (Nyasae *et al.*, 2007). As a consequence, copper accumulation in intestinal epithelia of mammals with inhibited *ATP7A* expression is a symptom of Menkes Disease (Mercer, 1998). The current model in *Drosophila* suggests that copper is directly exported at the basolateral membrane by DmATP7. Inhibited *DmATP7* expression also causes midgut copper accumulation which forms a component of the overall phenotype associated with the *DmATP7* null mutant.

The death of Menkes Disease patients as a result of systemic copper deficiency is evidence that the other likely consequence of inhibiting enterocyte copper export is less copper delivery to the bloodstream (Mercer, 1998). However, results from this thesis (and previous work) demonstrate that in *Drosophila,* midgut-specific suppression of *DmATP7* does not cause complete lethality (Chapter 3.2.1.2) (Bahadorani *et al.*, 2010). It remains likely that these flies are copper deficient but still have a means of survival via alternative sources of midgut copper export. Interestingly, one third of mice lacking ATP7A specifically in the intestinal epithelium were able to survive, albeit with a severe copper deficiency. Their survival suggests that a secondary low affinity mechanism of copper export could exist in the intestinal epithelium (Petris, 2010). Could the absence of intestinal Cu-ATPase result in copper being directly transported across the basolateral membrane via exocytosis? This remains highly speculative but alternative mechanisms of cellular copper export may be required in intestinal cells to prevent toxic levels of accumulation. The obvious way to further explore this in *Drosophila* is by blocking exocytosis during different stages of development, but this is likely to be detrimental to several cellular processes. It would be more useful to identify a gene involved in exocytosis, which when mutated is still adult viable. Midgut-specific suppression of *DmATP7* could then be investigated in these flies.

Increasing midgut DmATP7 expression further highlighted its role at the basolateral membrane as increased export lead to lethality on food supplemented with copper (Chapter 3.2.2.1). This phenotype is likely to be reflective of copper toxicosis of *Drosophila* lymph or even the brain and CNS. *Drosophila* brain sections have previously

shown that that copper depletion leads to a smaller brain with less stainable material (Bahadorani *et al.*, 2010). Could copper toxicity also have a similar deleterious effect? To investigate this, future studies should section the brains of *mex-GAL4; UAS-DmATP7* flies grown on normal food and pupae grown on copper food. The effect of copper toxicity on the CNS can be assessed using neurological assays on flies reared on normal food, such as negative geotaxis climbing tests.

In addition to these midgut investigations, localization of DmATP7 in the secretory cells of the salivary glands and principal cells of the malpighian tubules was also predominantly basolateral (Chapter 4.2.4). Basolateral DmATP7 in the salivary glands suggested that copper is exported out of the glands into the lymph which may be to prevent against toxicity when too much copper is imported from the salivary gland lumen. Malpighian tubule DmATP7 at the basolateral membrane may export copper from principal cells into the lymph. As Ctr proteins are located at the apical membrane, the general direction of copper transport appears to be from the inner lumen to the lymph. Although highly speculative this may suggest that copper is re-absorbed as solutes travel down the tubules towards the hindgut.

6.3.2 Intracellular DmATP7: TGN and the endocytic pathway

DmATP7 was also observed to localize to intracellular regions in the midgut, salivary glands and malpighian tubules (Chapter 4.2.3 & 4.2.4). In the principal cells of the salivary glands, intracellular DmATP7 was shown to partially colocalize with TGN reporter, GalNAc-GFP, and not at all with endoplasmic reticulum marker, KDEL-GFP (Chapter 4.2.5). It is likely that the intracellular DmATP7 observed in *Drosophila* enterocytes is also partially located at the TGN as ATP7A is required at the TGN of mammalian enterocytes. ATP7A fuels copper delivery to the secretory pathway where copper is required as a cofactor for cuproenzymes, one of which is the multi-copper oxidase enzyme Hephaestin. Hephaestin is one of the key enzymes involved in dietary iron absorption (Nyasae *et al.*, 2007). An intracellular role for DmATP7 in the *Drosophila* midgut does not appear to be vital for survival as *DmATP7* suppression in the

enterocytes did not alter viability. Considering the TGN-requirement of APTP7A in iron absorption, the intracellular role of DmATP7 in enterocytes could be further investigated by demonstrating that intestinal iron absorption is altered. Midgut-specific suppression of DmATP7 could be investigated on iron-depleted food to determine if this is the case.

Unlike ATP7A, DmATP7 localisation was predominantly basolateral on all food types rather than shifting from the TGN to the basolateral membrane after exposure to copper. Two scenarios could explain this difference; either the localisation of ectopic DmATP7 does not truly reflect that of the endogenous protein or the *Drosophila* intestinal Cu-ATPase is required predominantly at the basolateral membrane. Ectopic *mcherry-DmATP7* may not reflect the endogenous protein as expression levels of ectopic *mCherry-DmATP7*, expressed in addition to endogenous *DmATP7* may be forced mostly to the basolateral membrane as the TGN is occupied. However, it can not be ruled out that *Drosophila* may have adapted to low copper environments and therefore require predominantly basolateral DmATP7 to maximize copper absorption potential. Relative quantification of golgi versus basolateral DmATP7 prior and post exposure to increased copper levels would be required to detect any shift in distribution of the protein.

Copper stimulated trafficking was also not observed in the secretory cells of salivary glands or in the principal cells of malpighian tubules (Chapter 4.2.4). Whilst DmATP7 was predominantly basolateral, some TGN-localized protein in both cell types implies that the Cu-ATPase has a role to play in delivering copper to the secretory pathway of these cells. As discussed earlier, copper will act as a cofactor for the cuproenzyme Lysyl oxidase in salivary glands, whilst in malpighian tubules DmATP7 may be required to deliver copper to cell-specific multi-copper oxidases (Gorman *et al.*, 2008).

Although copper-stimulated trafficking was not observed in any of the polarized cell types, some protein was observed at locations other than the TGN or the basolateral membrane. Could it be possible that DmATP7 is being observed in transport vesicles in the process of translocating from the basolateral membrane to the TGN or in the other

direction? The likelihood of this proposal was supported by the results of Chapter Five which demonstrated an interaction between dRab5-mediated endocytosis and DmATP7.

Rab5 is a crucial component of endocytosis and is specifically required for the maturation of the early endosome (Huotari and Helenius, 2011). *dRab5* is expressed ubiquitously, homozygous mutants are embryonic lethal, and results from this thesis demonstrated the protein is required for roles in eye development and cellular over-proliferation (Wucherpfennig *et al.*, 2003; Zhang *et al.*, 2007). Expression of either *dRab5-DN* or *dRab5* RNAi was shown to disrupt cellular polarity causing cellular proliferation in the *Drosophila* wing disc, hemocytes and eye (Chapter 5.2.1). Loss of *dRab5* expression causes a halt in endocytosis likely resulting in mis-localization of signaling ligands such as Notch receptor, Crumbs, Wg, Dpp or Upd1.

Interestingly, expression of *dRab5* was also shown to be crucial in determining the sub-cellular localization of DmATP7 (Chapter 5.2.5). Expression of a dominant negative form of *dRab5* internalizes DmATP7 with partial co-localization of the proteins at pre-endocytic vesicles, with most DmATP7 at unidentified cellular locations. This result contrasts with the previous mis-localization effects of the loss of *dRab5* expression where proteins such as the Notch receptor or Crumbs were trapped at the outer plasma membrane (Lu and Bilder, 2005). DmATP7 mis-localization at unidentified intracellular localisations may cause copper transport abnormalities. Evidence of a copper transport abnormality may be demonstrated by the small, rough necrotic eye generated from the combination of *dRab5-DN* with *UAS-DmATP7* (Chapter 5.2.2). Whilst this observation could be considered another low copper situation exacerbating the *dRab5-DN* necrotic phenotype (discussed earlier with the *dRab5-DN* interactions with *Ctr1B* and *DmATP7* RNAi), the severity of the phenotype suggests it may be caused by an interaction between increased DmATP7 and the inhibition of endocytosis. Could mis-localized DmATP7 cause increased copper export from the cytoplasm into unknown organelles such as storage vesicles or pre-endocytic vesicles resulting in the reduction in eye size? Previous work has demonstrated that copper is essential for cellular proliferation due to an unanticipated role in the mitogen-activated (MAP)_kinase pathway (Turski *et al.*, 2012).

Inhibition of copper uptake resulted in a reduction in the ability of the MAP kinase Mek1 to phosphorylate the MAP kinase Erk (Turski *et al.*, 2012). This demonstrated that copper was required to activate the cellular proliferation pathway and therefore in the case of *dRab5-DN* flies, low cellular copper levels could cause eye reduction.

Increased *dRab5* expression also resulted in the internalization of DmATP7, causing co-localization of the two proteins in the early endosomes of salivary gland secretory cells. Interpreted together with the *dRab5-DN* result, it appears that dRab5-mediated endocytosis may be required to transport DmATP7 from the basolateral membrane to the TGN. Over-expression of *dRab5-WT* or *dRab5-DN* disrupts endocytosis and disrupts localization of DmATP7. This is supported by mammalian research which has shown that Rab5 co-localizes with ATP7A at basal copper levels (Pascale *et al.*, 2003). This gave rise to the theory that ATP7A may require Rab5-mediated endocytosis to return to the TGN after copper-induced translocation to the basolateral membrane.

Further research is needed to determine the exact effect of the loss/gain of *dRab5* on DmATP7 localization. Quantifying co-localisation with intracellular markers and membrane surface biotinylation could be used to measure the changes in DmATP7 localization. To confirm the phenotypic interactions observed, $Rab5^{-/+}$ heterozygotes? could be explored to determine if changed copper levels can influence the known mutant phenotypes and additionally identify if cellular copper homeostasis is altered in the mutants. Interactions could also be confirmed by exploring the role of other known endocytic genes, such as the effector protein of Rab5 *VPS34/p150*, *avalanche*, *vps25* and *erupted tumour susceptibility gene* on the localization and activity of DmATP7.

6.3.3 Degradation may be another form of DmATP7 regulation

Whilst endocytosis may regulate the intracellular location of DmATP7, evidence from this thesis also lead to the idea that DmATP7 is post-translationally regulated by protein degradation. In previous research, the serine/threonine kinase HipK has been demonstrated to bind to Slimb, an E3 ubiquitin ligase and inhibit the degradation of

members of several pathways including Wnt/Wg, Notch and Hedgehog (Lee *et al.*, 2009; Swarup and Verheyen, 2011). Slimb transfers ubiquitin onto a targeted substrate initiating the process of ubiquitination via the 26S proteasome (Pickart, 2001). Loss of *HipK* results in increased Slimb-induced degradation and is an essential *Drosophila* gene (Lee *et al.*, 2009). Ectopic expression of HipK causes dramatic eye phenotypes likely to be a result of increased HipK positively regulating the key signaling pathways discussed (Wright, 1987; Pena-Rangel *et al.*, 2002; Swarup and Verheyen, 2011).

Results from Chapter Five showed that tissue-specific suppression of *HipK* by RNAi in the *Drosophila* eye, midline or midgut caused no alteration to wild type. Interestingly ectopic expression of *HipK* resulted in strong phenotypes in the midline and eyes. The midline phenotype consisted of three components, a dramatic cleft of the thorax, complete loss of scutellum and a band of pigment loss down the entire abdominal midline (Chapter 5.2.6). Each aspect of the phenotype could be explained by increased *HipK* positively regulating key signaling pathways such as JNK, Dpp and Hedgehog, and production of pigments components such as dopa melanin, dopamine melanin and NBAD sclerotin (Wright, 1987; Pena-Rangel *et al.*, 2002; Swarup and Verheyen, 2011). As the *UAS-HipK* midline phenotype was partially rescued by increased copper supplementation it can also be speculated that the phenotype is partially caused by a copper deficiency or alternatively that HipK itself is down regulated by increased copper levels (Chapter 5.2.6). Increased *HipK* in eye caused a mild rough eye phenotype, albeit less severe than observed in previous research (Lee et al., 2009).

Results from this\is thesis demonstrated that increased HipK may also inhibit the degradation of DmATP7, leading to increased amounts of intracellular protein. Salivary gland-specific expression of *UAS-HipK* resulted in increased levels of mcherry-DmATP7 throughout the cytoplasm (Figure 5.2.11). Under the assumption that increased HipK inhibits degradation of DmATP7, it appears that stabilized DmATP7 is no longer susceptible to endogenous trafficking mechanisms, resulting in mis-localisation. This phenotype is similar to the effect caused by the loss of *dRab5* as discussed earlier. To further investigative this theory, membrane surface biotinylation could be used to

quantify the localization shift. Co-localization with a variety of intracellular and endocytic markers could also be investigated to determine just where stabilized DmATP7 is located.

Functional studies in the *Drosophila* midline and midgut supported the hypothesis that HipK may indirectly inhibit DmATP7 degradation. The combination of *UAS-HipK* and *UAS-DmATP7* was lethal on normal food when co-expressed in either the midline or midgut (Chapter 5.2.8 and Chapter 5.2.10). The midline phenotype could be an additive amalgamation of two unrelated severe midline phenotypes, but I argue it is likely to be a genetic interaction as *HipK* suppression partially rescues the midline phenotype caused by *UAS-DmATP7* (Chapter 5.2.9). Considering that midgut-specific expression of *UAS-DmATP7* alone is only lethal on increased copper load, the midgut interaction with *UAS-HipK* again implies exacerbation of the phenotype. Both the midline and midgut scenarios appear to be explained by an interaction causing inhibited degradation of DmATP7. This may result in stabilized DmATP7 throughout the cytoplasm causing increased copper export from the cytoplasm into unavailable vesicles and a cellular copper deficiency.

Other interactions in the midline also supported an interaction between HipK and DmATP7. Expression of *UAS-Ctr1A* or *UAS-Rab5* partially rescued the scutellum defect caused by *UAS-HipK* over expression (Chapter 5.2.8). Increasing *Ctr1A* will lead to elevated copper import, whilst increasing *Rab5* can result in the mis-localisation of DmATP7 to the early endosomes preventing copper export. As both cause increased cellular copper levels, each result could indicate a partial copper deficiency being reverseed or that increased cellular copper levels can down regulate HipK. These results are similar to the earlier discussed partial rescue of *pannier-GAL4;UAS-HipK* flies on copper supplemented food.

Further exploration of the role of HipK in regulation of DmATP7 will likely involve *in vitro* ubiquitination assays with HipK, Slimb and DmATP7. Could DmATP7 be ubiquitinated in the presence of Slimb? And would this still occur in the presence of

HipK? This experiment could confirm protein-protein interactions between Slimb and DmATP7. Other future experiments could involve investigating the ectopic expression and suppression of Slimb to determine if there are any changes to DmATP7 function and localization.

The regulation of Cu-ATPase degradation was also observed in mammalian research with COMMD1 and Clusterin. COMMD1 is capable of binding to mis-functioning mutants of either ATP7A or ATP7B and then inducing proteolysis via interaction with the E3-ubiquitin ligase complex (Meusser *et al.*, 2005; de Bie *et al.*, 2007; Vonk *et al.*, 2011). Clusterin can interact with both ATP7A and ATP7B but in this case target degradation by the lysosomal pathway (Materia *et al.*, 2011). In these mammalian examples the regulator protein binds directly to the Cu-ATPase and then induces degradation. In the case of HipK in *Drosophila*, it is hypothesized that degradation is induced indirectly by activation of the E3 ubiquitin ligase rather than direct binding of HipK to DmATP7. Nevertheless, both mammalian and *Drosophila* examples highlight that Cu-ATPase stability must be regulated to prevent copper deficiency or toxicity. These mechanisms have likely evolved to counteract low intracellular copper levels caused by the environment or malfunctioning import proteins.

6.4 Final Conclusions and future directions

The results presented in this thesis have expanded on the current knowledge of copper homeostasis in *Drosophila* and in doing so have illuminated novel research areas to be pursued in parallel in mammalian systems. Copper absorption through the *Drosophila* midgut was shown to be a complex process that is not as simple as 'copper in and copper out'. Whilst DmATP7, Ctr1A and Ctr1B predominantly play expected roles in enterocytes, the function of each protein appears to be flexible. The Ctr proteins are the primary mechanisms for apical copper import but there was strong evidence to suggest other import avenues not only exist but can complement Ctr1A and Ctr1B. Ctr1 proteins also appear to have a role at the basolateral membrane highlighting that apically imported copper may not be available for the cell-specific requirements of enterocytes. The

described midgut-specific roles of the *Drosophila* Ctr proteins appear strikingly similar to hCtr1 and provide impetus for further mammalian research. How a cell might differentiate between copper imported from two different membranes is a fascinating question. Could it be that different chaperones transport the imported copper to different cellular destinations? Or is copper coming from the bloodstream / lymph in a different form to that coming from the intestinal lumen?

Like in most cell types, midgut DmATP7 has dual copper transport roles to play at the TGN and at the basolateral membrane. DmATP7 localization in *Drosophila* enterocytes implies that its primary role is to transport copper across the basolateral membrane rather than at the TGN. This localisation would reflect the premium placed on copper export into the lymph which may have evolved due to *Drosophila* being reared in low copper environments. Regulation of *DmATP7* midgut expression levels is crucial to preventing copper accumulation, maintaining copper delivery to the lymph and preventing systemic toxicity.

Results from the midgut have highlighted that *Drosophila*, and likely mammals, can adapt well to targeted mis-expression of copper transport proteins. There are regulatory systems in play, some likely known and others yet to be discovered, which work to mask the effect of the system-specific mis-expression. This thesis illustrated that the breakdown in regulation of localization and stability can significantly alter copper homeostasis. The involvement of dRab5 in determining DmATP7 localization demonstrated that general cellular processes like endocytosis are likely to be involved in regulation of copper transport. This leads to speculation that Ctr proteins and copper chaperones may also rely on endocytic transport for proper localization. The role of HipK and subsequently the E3 ubiquitin ligase in stabilizing DmATP7 demonstrates that cells can trigger degradation of copper transporters. This implies that cells can sense intracellular copper levels and then regulate the stability of high affinity copper transporters such as DmATP7.

This thesis has developed a working model of midgut copper transport, identified two methods of posttranslational regulation and consequently generated intrigue about other systems of *Drosophila*. How is copper in the lymph transported to other organs? Is there systemic regulation and what mechanisms are involved? Are there copper storage organs in *Drosophila* and what can trigger release*?* Understanding the organization and dynamics of organ-organ communication will become a key component of the future of copper research in *Drosophila.* Answering these questions in *Drosophila* will broaden the knowledge of mammalian copper homeostasis resulting in further understanding of copper related disease.

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