Regulation of Mint1-dependent APP trafficking by N1-Src

A thesis submitted to the

Department of Biology

For the degree of

Master of Science by Research

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December 2012

Abstract

Alzheimer's disease (AD) is a neurodegenerative disease characterised by the accumulation of Amyloid-beta (Aβ) plaques and neurofibrillary tangles. Aβ plaques form as a result of improper trafficking and processing of the Amyloid precursor protein (APP). APP trafficking is highly spatially and temporally regulated through a complex network of protein-protein interactions. Mint1/X11α is one of four neuronal trafficking adaptor proteins that bind to the YENPTY motif in the APP C-terminus. Whilst these adaptor proteins are known to regulate APP trafficking and processing, it is as yet unclear how these interactions with APP are regulated. Previous data from the laboratory shows that Mint1 is phosphorylated on Y202 and that this phosphorylation regulates APP trafficking. Here I have further investigated the role of Mint1-Y202 phosphorylation by Src in the regulation of APP trafficking and processing. I have utilized a stable, inducible APP expressing HeLa cell line to show that disruption of Mint1-Y202 phosphorylation disrupts APP trafficking following internalisation of the protein. In addition to this, data suggests that the neuronal isoform of the kinase, N1-Src, has a higher affinity for Mint1 than its ubiquitously expressed isoform C-Src. Previous studies have shown that Mint1 overexpression results in accumulation of APP in the trans-Golgi network (TGN). Interestingly, I also see this effect and moreover, observe the recruitment of N1-Src, along with APP to the perinuclear region in a Mint1-dependent manner. Together, these data suggest Mint1 and N1-Src are recruited to regulate correct APP trafficking and processing.

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Acknowledgements

I would like to thank Dr Gareth Evans for his guidance and support throughout the study. I would also like to thank the other members of the Evans laboratory, Sarah Keenan, Philip Lewis and Clare Dennison. In addition I wish to thanks my parents and family for their continued support.

Declaration

I hereby declare that this thesis is my own work and effort and that it has not been submitted anywhere for any award. Where other sources of information have been used, they have been acknowledged.

1: Introduction

1.1: Alzheimer's disease

Alzheimer's disease (AD) is the most common form of dementia. According to the Alzheimer's society, around 800,000 people in the UK have some form of dementia. As well as the emotional burden of the disease, it costs the UK economy approximately £20 billion per year (http://www.alzheimers.org.uk/site/scripts/documents_info.php?documentID=3 41, accessed 6/3/13). Despite its prevalence, relatively little is known about the cause and molecular background of the disease. The two main microscopic pathologies of AD are the presence of amyloid-β (Aβ) plaques and neurofibrillary tangles, found in the brains of patients. Neurofibrillary tangles mainly consist of filaments of hyperphosphorylated tau protein, a microtubule associated protein, while Aβ plaques are formed by alternative cleavage of the amyloid precursor protein (APP), producing the aggregation prone peptide, Aβ-42. Macroscopically, AD is characterised by atrophy of the hippocampal formation and cerebral cortex (Perl 2010). AD symptoms can vary between patients, adding to the difficulty faced when diagnosing the disease. Generally, the disease presents as a gradual decline in memory, with an inability to retain new information being the initial sign, leading to severe memory loss. Other symptoms include changes in mood, declines in abstract reasoning and language. In the later stages of the disease, a number of neurological symptoms may present, such as seizures, hypertonia, myoclonus and mutism (Bekris *et al.* 2010).

In 1984, a set of criteria defining Alzheimer's disease was outlined, including aspects of medical history, clinical examination, neuropsychological testing and laboratory testing (McKhann *et al.* 1984). They were out together to allow clinicians and researchers to maintain consistency when dealing with the disease. Following improvements in research and diagnostic techniques these criteria were recently revised (McKhann *et al.* 2011). Following these revisions

it was found that the incidence of the disease symptoms were more widespread than previously thought, for example some symptoms, such as neuropathological changes, can present years before the onset of the classically recognised dementia. With these clinical criteria a number of risk factors for the disease were out lined. For example, it is widely known that the prevalence rates for AD rise exponentially with age, it was found there is around a 15-fold increase in the prevalence of AD between the ages of 60 and 85 years old (Evans *et al.* 1989). Other risk factors for the disease include cerebrovascular disease (Blennow *et al.* 2006; Wen *et al.* 2008; Schneider and Bennett 2010), diabetes (Farris *et al.* 2003; Yu and Ginsberg 2005), hypertension (Morris *et al.* 2001; Skoog and Gustafson 2006), smoking (Whitehouse *et al.* 1988; Perry *et al.* 2002) and obesity (Profenno *et al.* 2010).

Recent advances in genetic research have revealed a number of genetic mutations associated with AD. These generally fall into two classes – i) familial, Mendelian inherited mutations associated with early onset AD (40-50 years of age) ii) sporadic, non-inherited mutations associated with late onset AD (at >60 years of age).

1.2: Genetics of AD

Familial cases of AD account for less than 1 % of cases, however investigations into the mutations found in these cases has proved informative in identifying a number of proteins involved in the progression of both familial and sporadic forms of the disease.

Over 32 mutant forms of APP itself have been reported in around 85 families. The majority of these are found at the sites where the secretases cleave the protein. Of these the missense 'Swedish' (APP$_{sw}$, *APP*K670N, and M671L) and 'London' (APP$_{lon}$, *APP*V717I) mutations are perhaps the most studied (Goate *et al.* 1991; Mullan 1992). Mice expressing APP$_{sw}$ and APP$_{lon}$ mutations are often used to study the mechanisms behind AD progression. Mice carrying these mutations exhibit different APP expression levels and neurological abnormalities. For example the APP$_{sw}$ mutant mouse, Tg2576

exhibits high APP and Aβ levels that get progressively worse and begin from around 6 months of age (Irizarry *et al.* 1997; Westerman *et al.* 2002).

Presenilins are a major component of the APP γ-secretase complex, mutations found in Presenilin 1 (PSEN1) are the most common cause of early onset AD accounting for 18-50 % of cases (Theuns *et al.* 2000). PSEN1 mutations cause an increase in the ratio of Aβ42 to Aβ40 (Citron *et al.* 1997), and are responsible for some of the most severe cases of AD with onset seen as early as 30 years of age. There have been 176 PSEN1 mutations reported, the majority of which are missense mutations present throughout the protein. PSEN1 null mice are not viable (Shen *et al.* 1997), however, mice with conditional knockout of PSEN1 in the postnatal forebrain show mild cognitive impairment in long term memory retention, coupled with the accumulation of C-terminal APP fragments and a reduction in Aβ production (Yu *et al.* 2001).

Another important component of the γ-secretase complex is Presenilin 2 (PSEN2). Mutations in this protein are rare and patients carrying the mutations show different disease progression to those with PSEN 1 mutations. The age of onset is often later, and this may vary widely between members of the same family (Sherrington *et al.* 1996). Mutations within PSEN 2 are generally of lower penetrance that those in PSEN 1, they therefore may be subject to modifications by other genes or environmental factors (Tandon and Fraser 2002).

Whilst it is apparent a number of mechanisms may lead to cases of sporadic AD, a number of mutations associated with late onset AD have also been identified. Of these, the most common mutations are those found in the Apolipoprotein E (ApoE) gene (Götz *et al.* 2012). ApoE plays a role in receptor-mediated endocytosis of lipoproteins. Under certain conditions, ApoE is expressed at low levels in neurons in the brain (Xu *et al.* 2006), however the protein is mainly expressed in non-neuronal cells such as astrocytes and microglia (Grehan *et al.* 2001). Although mutations in the protein differ by only

a couple of amino acid, the structure and function of the protein are significantly changed. Although the effects of ApoE mutations are clearly present in AD patients, it is unclear by what mode of action this occurs, although a number of mechanisms have been proposed including those involving neurite outgrowth, cytoskeletal changes in addition to direct effects on Aβ (Mahley *et al.* 2006).

1.3: Amyloid precursor protein

There are 3 members of the APP gene family in humans; APP and APP-like proteins (APLP) 1 and 2 (reviewed in (Muller and Zheng 2012). They are all type one membrane bound proteins with large extracellular N-termini and a shorter, very highly conserved intracellular C-terminal domain. The extracellular domain of APLP2 and some APP isoforms contain a Kunitz protease inhibitor (KPI) domain, which is lacking in APLP1 and APP695 (the main neuronally expressed isoform). The KPI domain is thought to play a role in APP dimerisation in addition to affecting trafficking of the protein (reviewed in (Khalifa *et al.* 2012). The variation between APP gene family proteins lies in the transmembrane and extracellular juxtamembrane regions. Importantly only APP, not APLP1 or APLP2, contains the sequence encoding Aβ peptides.

Figure 1.1 illustrates the alternative mechanisms via which APP is processed. The integral membrane protein can be cleaved by either α, β or γ secretases (reviewed in Thinakaran & Koo 2008). α secretase cleaves the protein near the extracellular side of the cell membrane, when further cleaved by γ secretase within the plasma membrane, the 40 amino acid p3 peptide is produced. However, initial cleavage by β secretase followed by γ secretase produces the longer Aβ-42 peptide (reviewed in King & Scott Turner, 2004, Tang, 2009). It is not fully understood why this differential cleavage occurs, or why Aβ curiously only appears to aggregate in brain cells, although it is most likely this is due to the highly specialised nature of neuronal cells including the specialised trafficking processes APP undergoes due to the distance the

protein travels along the axon. A number of functions of APP have been proposed, including regulation of cell signaling mechanisms, calcium regulation and cell adhesion (reviewed in Brunholz *et al.*, 2011). The importance of APP in the onset of AD is highlighted by the increased incidence of the disease in individuals with Down syndrome. Down syndrome occurs as a result of chromosome 21 being present in triplicate, which coincides with the location of the APP gene (Nistor *et al.*, 2007).
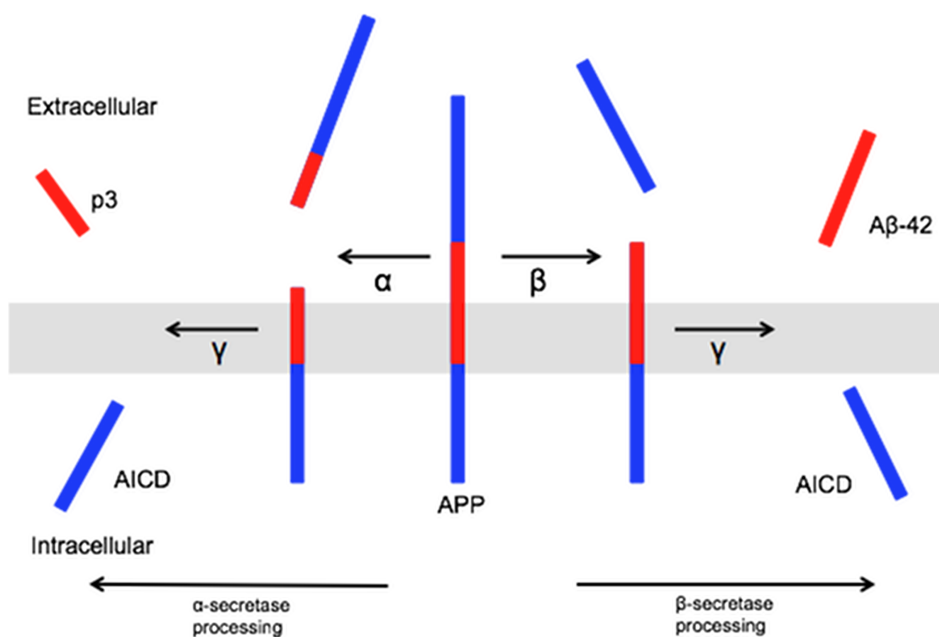
Figure 1.1 – The alternative cleavage pathways of APP. The membrane protein APP is processed by secretase enzymes through one of two pathways. The α-secretase cleaving pathway (left) is a non-pathogenic pathway in which α-secretase cleaves the protein extracellularly, before γ-secretase cleaves the protein within the membrane to release the soluble peptide, p3, and the C-terminal APP intracellular domain (AICD). However, the protein can also be processed through the pathogenic β-secretase pathway (right). Here the β-secretase enzyme cleaves the protein before γ-secretase cleaves the protein in the membrane again, this time producing the aggregation prone peptide Aβ-42, as well as the AICD.

1.4: The secretases

1.4.1: α-secretase

Multiple transmembrane metalloproteases have been proposed to act as α-secretases. Members of the A Disintegrin And Metalloprotease (ADAM) family have been proposed as α-secretases– including ADAM 9, 10 and 17 (Asai *et al.* 2003). Although all display α-secretase activity the expression levels of the proteases vary in the brain, with ADAM10 being expressed at high levels whereas ADAM 9 and 17 are found at lower concentrations (Weskamp *et al.* 1996; Black *et al.* 1997). Coupled with varied expression levels, experimental evidence suggests ADAM10 is the major α-site protease that cleaves APP in neurons (Kuhn *et al.* 2010). It has been shown ADAM10 is synthesised in the Endoplasmic Reticulum (ER) and is present as a proenzyme mainly in the Golgi (Lammich *et al.* 1999), whereas the majority of mature ADAM10 is found at the cell surface in neurons (Parvathy *et al.* 1999). It has been reported that mutations in the ADAM10 prodomain could cause late-onset AD (Kim *et al.* 2009), perhaps because this domain is important for preventing the protease from being degraded along the secretory pathway (Roghani *et al.* 1999). The presence of active ADAM10 in neurons has been shown to be important in the processing of APP and AD, for example ADAM10 KO mice were shown to have almost no soluble alpha-secretase cleaved APP (sAPPα) secretion (Jorissen *et al.* 2010). It has been suggested cholesterol plays a role in regulating α-secretase activity (Kojro *et al.* 2001). It has recently been shown that cholesterol-lowering drugs, such as statins, can upregulate α-secretase activity via their cholesterol lowering properties (Kojro *et al.* 2010).

1.4.2: β-secretase

The APP β-secretase has been identified as the enzyme BACE1 (β-site APP cleaving enzyme), a transmembrane aspartic protease (Vassar *et al.* 1999). After being identified as having β-secretase activity and shown to cleave APP, inhibition of BACE1 in the brain was proven to lower levels of Aβ (Li *et al.*

2006). Studies utilizing Förster resonance energy transfer techniques and microscopy have shown that BACE1 mainly interacts with APP in endosomes (Kinoshita *et al.* 2003). The activity of BACE1 is regulated by the low pH in endosomes, a favourable environment for the enzyme (Knops *et al.* 1995). It was recently shown that sAPPα directly modulates BACE1, decreasing Aβ production in mice (Obregon *et al.* 2012). In addition to this it was suggested Aβ can upregulate BACE1 and APP transcription by direct interaction with an Aβ interacting domain in the APP gene promoter, thus facilitating its own production (Bailey *et al.* 2011).

1.4.3: γ-secretase

Following cleavage by α- or β-secretase APP is further cleaved by γ-secretase in a process known as Regulated Intermembrane Proteolysis (RIP) (reviewed by (Prox *et al.* 2012). γ-secretase is a complex consisting of a 1:1:1:1 ratio (Renzi *et al.* 2011) of Nicastrin (Nct), Anterior pharynx defective one (APH1), Presenelin enhancer 2 (PEN2) and Presenilin 1 and/or 2 (PSEN1/2) (Steiner *et al.* 2008). The individual roles of the proteins that make up this complex are not yet fully understood; it is thought PSEN1/2 is the active catalytic subunit (Li *et al.* 2000), whilst Nct is responsible for anchoring the substrate (Shah *et al.* 2005), however a role for APH1 has not yet been elucidated. Mutations in the PSEN component are responsible for the majority of familial cases of AD (Bertram *et al.* 2010). One of the proteins that interacts with and is involved in regulation of γ-secretase is Arc (Dickey *et al.* 2004). Arc is a postsynaptic protein that regulates trafficking of a number of proteins, and interacts with PSEN1. APP and BACE1 have been found to be trafficked in Arc-endosomes and deletion of Arc causes a decrease in Aβ in a mouse model of AD (Wu *et al.* 2011).

1.5: APP in neurons

Much of the research into APP metabolism and processing thus far has been carried out in non-neuronal cell systems. Historically it has not been clear how

similar APP processing in these systems is to APP processing in the brain, largely due to the difficulty of culturing and manipulating neurons as well as producing sufficient materials for protein biochemistry compared to other cell systems. However, more information about how APP behaves in neurons is beginning to emerge and is critical to our understanding of how the protein contributes to the progression of AD, in addition to being informative in studies using non-neuronal cell systems.

There have been a number of discrete functional *in vivo* roles suggested for APP metabolites in the brain. It had been previously reported that APP levels in the brain increase following traumatic brain injury, and this was traditionally thought to be detrimental to the patients and lead to an increase in β-secretase cleavage of APP and Aβ production (Chen *et al.* 2004). However, it has more recently been reported that sAPPα has neuroprotective properties. Mice administered with a post-traumatic treatment of sAPPα showed a decrease in the number of apoptotic neurons and reduction in axonal injury (Thornton *et al.* 2006). Additionally, exogenous sAPPα was shown to enhance both short- and long-term memory in mice (Meziane *et al.* 1998). This effect was attributed to increased synaptic density and enhanced NMDA receptor mediated currents. Mounting evidence that presynaptic terminals are a major site for sAPPα secretion (Nitsch *et al.* 1992), coupled with more recent observations that sAPPα secretion correlates with enhanced synaptic activity (Nitsch *et al.* 1993; Hoey *et al.* 2009) suggest sAPPα may act as a ligand for post-synaptic neurotransmitter receptors (reviewed in (Brunholz *et al.* 2011).

Much research has also been carried out on the physiological role of Aβ peptides, however conclusions from experimental data have varied substantially (reviewed in (Lahiri and Maloney 2010). Several studies have suggested lower concentrations of Aβ may positively regulate long-term potentiation and memory (Puzzo *et al.* 2008), as well as regulating cholesterol transport and homeostasis (Grimm *et al.* 2005). Alternatively, it has been reported Aβ has inhibitory effects on synaptic transmission (Moreno *et al.*

2009) and axonal transport (Pigino *et al.* 2009). A number of studies have reported that APP is localised in both axons and dendrites in neurons (Schubert *et al.* 1991; Ferreira *et al.* 1993; Back *et al.* 2007), these observations coincide with data showing BACE1 is also localised in axons and dendrites (Tienari *et al.* 1996). Tienari and colleagues also examined a number of sorting signals on APP itself, finding the Aβ region to be essential for axonal trafficking (Tienari *et al.* 1996). Interestingly it has been suggested that APP is differentially processed at pre- and post-synaptic sites, and that the majority of aggregated Aβ results from APP that has been transported along axons rather than dendrites (Lazarov *et al.* 2002).

Recent research into APP processing in neurons suggests that there are some neuron-specific features of APP trafficking due to the complex and specialised subcellular organisation of the cell. Although the majority of APP in both neuronal and non-neuronal cells is processed by α-secretase (Sisodia *et al.* 1990), it has been shown that in neurons a larger fraction of APP is processed by β-secretase (Simons *et al.* 1996). Due to their size and complexity neurons have developed a specialised mechanism for trafficking proteins, known as fast axonal transport. APP interacts with a number of different proteins in neurons that are involved in fast axonal transport (reviewed in (Morfini *et al.* 2002). For example, APP has been shown to interact, via a YKFFE sequence in its cytosolic tail, with a subunit of the adaptor protein 4 (AP4) complex to facilitate the sorting of APP into specific post-Golgi transport vesicles (Burgos *et al.* 2010). Consistent with the proposed function of APP in neurons APP has been shown to interact with a number of pre-synaptic proteins in these transport vesicles (Groemer *et al.* 2011). Due to the observation that aggregation prone Aβ-42 is generated from APP that has been transported along axons, a number of groups investigated whether APP was co-transported with the secretases responsible for its processing. Kamal *et al.* suggested that APP, BACE1 and γ-secretase are co-transported in the same discrete transport vesicles, and that APP undergoes processing as the components are trafficked along the axon and that fast axonal transport of this compartment is mediated by APP and kinesin-I

(Kamal *et al.* 2001). However, more recent studies failed to detect Aβ within axonal compartments (Goldsbury *et al.* 2006). This study also used live cell imaging to show APP and BACE1 in separate vesicles along the axon (Goldsbury *et al.* 2006). Furthermore, studies with all secretase components found only active ADAM10 was detected in APP-containing vesicles (Szodorai *et al.* 2009). Together, these data suggest APP and its secretases are trafficked separately and that pathogenic cleavage of the protein occurs in specific areas within the neuron, after it has been transported.

1.6: APP trafficking

A number of groups have investigated the path though which APP is trafficked around the cell, in numerous cell lines and begun to clarify which proteins are involved at each stage. It is generally accepted that APP is trafficked through the endoplasmic reticulum (ER), Golgi and trans-Golgi network (TGN) to the plasma membrane, where it can either be processed and secreted or recycled through clathrin coated endocytic vesicles (reviewed in (Thinakaran and Koo 2008; Claeysen *et al.* 2012). It is believed the majority of β-secretase activity occurs as APP is endocytosed and rapid, unperturbed transport to the plasma membrane favours non-amyloidogenic cleavage (Claeysen *et al.* 2012). A recent study by Khalifa *et al.* showed high levels of APP are found in the ERGIC, the compartment between the ER and Golgi (Khalifa *et al.* 2012). They also highlighted the importance of the KPI domain, found in the APP795 isoform but not in the neuronally expressed APP695 isoform, in allowing transport of APP out to the plasma membrane. This may be one explanation to account for high levels of Aβ in neurons when compared to other cells.

When in the Golgi and TGN, APP is heavily post-translationally modified. The protein is glycosylated, phosphorylated and tyrosine sulphated. It has been reported there is some ADAM 10 activity in the TGN, however this is a very small proportion when compared to α-secretase activity at the plasma membrane (Skovronsky *et al.* 2012). One study into α-secretase activity also reported ADAM 10 activity in trafficking vesicles moving towards the

membrane (Szodorai *et al.* 2009). This observation serves to back up the widely believed theory that APP trafficking along the secretory pathway leads to non-amyloidogenic cleavage of the protein.

In contrast to this experimental data suggests that Aβ-42 production occurs mainly as APP is recycled through the endocytic sorting pathway. The C-terminally located YENPTY motif of APP, to which a number of neuronal trafficking adaptors including Mint1, bind is essential for this internalisation (Perez *et al.* 1999). High levels of β secretase activity have been reported in endocytic compartments (Grbovic *et al.* 2003). The relevance of APP endocytosis in Aβ-42 production is also highlighted when the process is disrupted. It was recently observed that overexpressing a component of the E3 ubiquitin ligase complex, FBL2 (F-box and leucine rich repeat protein 2), inhibited APP endocytosis by promoting its ubiquitination (Watanabe *et al.* 2012). Increased ubiquitination was shown to have an effect on both cell surface APP, inhibiting endocytosis and reducing Aβ production, and on intracellular APP, by promoting its ubiquitin dependent degradation. Mutations in APP have also been shown to affect the protein's degradation in the lysosome. A recent study by Lorenzen *et al.* demonstrated that APP can be rapidly and directly transported to the lysosome for degredation, however the presence of either London or Swedish mutations of the protein inhibited this direct transport (Lorenzen *et al.* 2010).

In contrast to reports of high β and γ secretase activity in endosomes, Burgos *et al.* proposed high levels of the enzymes were actually found in the Golgi (Burgos *et al.* 2010). This study demonstrated APP interacts with adaptor protein 4 (AP-4), which is involved in sorting of proteins in post-Golgi compartments, to move out of the TGN to endosomes. They reported disruption of this interaction resulted in an increase in γ secretase activity on APP and an increase in Aβ-42 production.

1.7: APP adaptor proteins

Processing and trafficking of the APP protein is by the interaction of a number of adaptor proteins with the conserved YENPTY sequence in the C-terminal domain of the protein. Interaction with these adaptor proteins has been shown to alter the levels of Aβ in the cell, as discussed later. These interactions are therefore important for studying the progression of AD and might be exploitable as therapeutic targets for the disease. There are four known neuronal trafficking adaptors of APP that bind to this motif via conserved phosphotyrosine binding (PTB) domains; Fe65, Mint, JIP and DAB.

1.7.1: Fe65

There are three members of the Fe65 family; Fe65, Fe65-like 1 (Fe65L1) and Fe65-like 2 (Fe65L2). Their name is derived from the initials of the first author (FE), plus the clone number (65) and they were found when characterising brain specific cDNA fragments (Esposito *et al.* 1990). They were subsequently identified as APP adaptor proteins using the yeast two-hybrid system (Fiore *et al.* 1995). They bind to the YENPTY motif of APP in a phosphotyrosine independent manner through their PTB domain (Zambrano *et al.* 1997). The proteins all contain two PTB domains, as well as a WW domain, which binds to particular proline rich motifs. This WW domain links the proteins to actin via Mama (mammalian active) (Ermekova *et al.* 1997), thus indicating the family plays a role in cellular trafficking.

There are contrasting reports as to what role Fe65 plays in regulating APP processing. It was reported that APP and Fe65 colocalise in the ER and Golgi and that Fe65 increases translocation of APP to the cell surface resulting in an increase of sAPPα and Aβ in a non-neuronal cell system (Sabo *et al.* 1999). One study also showed Fe65 null primary neuronal cultures from transgenic mice displayed a reduction in Aβ secretion. However, expression of human Fe65 has been reported to decrease Aβ production in APP

transgenic mice known to show Aβ plaque loads by 6 months (Santiard-Baron *et al.* 2005).

The mechanism by which γ-secretase cleavage of APP occurs is homologous to the process of cleaving the Notch receptor. The Notch signaling pathway is involved in cell proliferation, cell fate and cell death in development. One mechanism involved in this pathway is intramembranous γ-cleavage of the Notch receptor, with the translocation of the intracellular domain to the nucleus in order to regulate the transcription of related genes (Bray 2006). Much support has been put forward for APP to act in this manner. It has been reported that when Fe65 is bound to the intracellular domain of APP (AICD) the complex can stimulate transcription of the APP and BACE1 (which cleaves APP at the β-secretase site) (von Rotz *et al.* 2004). Fe65 interacts with a number of nuclear proteins (reviewed by (McLoughlin and Miller 2008). It has been reported by numerous groups that Fe65 forms a transcriptionally active complex with the AICD in heterologous gene reporter systems (Scheinfeld *et al.* 2003; Araki *et al.* 2004; Perkinton *et al.* 2004; Cao and Südhof 2006). Although it is as yet unclear precisely how this mechanism works, it has been proposed the interaction between Fe65 and the AICD could be regulated by phosphorylation. Phosphorylation or mutation of Thr668 in the AICD region of APP blocks Fe65 binding (Ando *et al.* 2001; Chang *et al.* 2006). Similarly phosphorylation of the residue tyrosine 547 in the second PTB domain of Fe65 stimulated transcription (Perkinton *et al.* 2004).

1.7.2: Dab1

Disabled 1 (Dab1) is active in embryogenesis and has a role in regulating the position of neurons during development. Dab1 has been shown to bind to the YENPTY motif of APP in a phosphtyrosine-dependant manner (Howell *et al.* 1999). The effects of Dab1 on APP trafficking and processing are largely unclear. Dab1 regulates neuronal positioning via the Reelin signaling pathway (Bar *et al.* 2000). Reelin is a secreted glycoprotein that is involved in the cell-cell interactions that are vital for neuronal migration and development (Rice *et*

al. 1998). Treatment of cells with the extracellular Reelin protein has been seen to increase co-immunoprecipitation of Dab1 and APP; and Reelin itself can increase cleavage of APP (Hoe *et al.* 2006).

It has been shown that Dab1 can be phosphorylated and regulated by the Src family kinase Fyn, which, in turn, can be regulated by reelin (Hoe *et al.* 2008). Fyn has been shown to localise to detergent resistant membranes (DRM) in cells (Kramer *et al.* 1999), interestingly high amounts of β- and γ-secretases are also found to localise to DRMs (Parkin *et al.* 1999). It was found that treatment of cells with reelin increased the localisation of Dab1 and APP to DRMs, however the treatment also increased phosphorylation of Dab1 therefore decreasing its interaction with APP. This effect was not seen in Fyn knockout mutants, suggesting reelin promotes recruitment of APP and Dab1 to DRMs and regulates the activity of Fyn, which regulates the Dab1-APP interaction (Minami *et al.* 2011).

A number of studies have shown that Dab1 can compete for binding to APP with other neuronal adaptors. One study has also shown that Mint1 expression can counteract the effect of Dab1 on APP levels (Parisiadou and Efthimiopoulos 2007). They found that increasing levels of Mint1 reduced the levels of Dab1 bound to APP, whereas the opposite effect was not observed. Surprisingly, it was found that the unphosphorylated C-terminus of Dab1 can bind to Fe65, and decreases the effect of Fe65 on APP processing (Kwon *et al.* 2010). This competition for binding to the C-terminal YENPTY motif of APP could provide a further level of regulation for APP processing and trafficking.

1.7.3: JIP

JIPs are a family of c-Jun N-terminal kinases (JNK) interacting proteins; they act as scaffold proteins for JNK and MAP kinases. There are 4 members of the JIP family; JIP1 and 2 have an SH3 domain, a PTB domain and a JNK interacting domain, whereas JIP3 and 4 have a coiled-coil domain, a transmembrane domain and the JNK interacting domain. JIP1 and 3 have

been shown to be important in axonal transport via interaction with a number of proteins (Koushika 2008). APP has been identified as one of the cargoes to which JIP1 can bind (Matsuda *et al.* 2001) however the relevance of this interaction is still under debate. It has been shown that JIP1 can stabilise immature APP and decrease the production of Aβ, suggesting the adaptor directly regulates APP processing (Taru *et al.* 2002). It is possible JIP1 acts as a scaffold protein for JNK and APP as the presence of JIP1 enhanced *in vitro* phosphorylation of APP at threonine668 (Inomata *et al.* 2003).

Coprecipitation experiments suggest JIP proteins, pre-loaded with cargo proteins, interact with kinesin light chain (KLC) acting as scaffolds for motor-protein trafficking (Verhey *et al.* 2001). It has been suggested that this interaction may mediate APP interaction with kinesin light-chain 1 in a mechanism regulating APP trafficking (Matsuda *et al.* 2003). However, others suggest that APP and KLC interact directly (Kamal *et al.* 2000). It has also been illustrated JIP1 can interact with the AICD to promote transcription of APP, however, unlike Fe65, the study suggests JIP1 and AICD interact in the cytosol rather than in the nucleus (Scheinfeld *et al.* 2003).

1.7.4: Mints

There are 3 isoforms of Mint (Mint1/X11α, Mint2/X11β, Mint3/X11) all of which bind to the YENPTY motif in the intracellular domain of APP (Figure 1.2). Mints1 and 2 are specifically expressed in the brain, whereas Mint 3 is expressed ubiquitously (Okamoto and Sudhof 1997). The isoforms have conserved C-terminal domains, differing in their N-terminus. The domain structure of Mint1 is shown in figure 1.2. They are all highly conserved and homologues have been found in a number of organisms, such as the *C. Elegans* homologue lin-10. The proteins were named Mint (Munc18 interacting proteins) when they were found to be adaptors for the synaptic protein Munc18 (Okamoto and Sudhof 1997).

Mint proteins have been implicated in neuronal trafficking. Mint 1 has been shown to bind, via its PDZ domain, to the motor protein KIF17 (Setou *et al.*

2000), whilst Mint 2 can be immunoprecipitated with kinesin-heavy chain (Araki *et al.* 2003). In neurons, APP is processed whilst being trafficked to the neuronal terminals (Wilquet and Strooper 2004). It is thought this process is associated with the kinesin light-chain subunit of kinesin-1 (Kamal *et al.* 2000). These factors indicate Mint-APP interactions are of physiological importance for studying AD pathology.
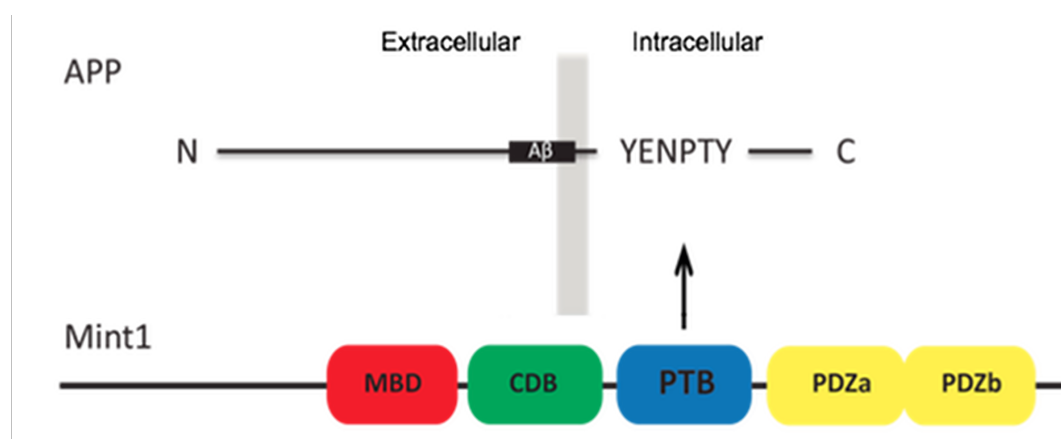
Figure 1.2 – Domain structure of APP adaptor protein Mint1. Showing the Munc18 binding domain (MBD), CASK binding domain (CBD), phosphotyrosine binding domain (PTB) and the two PDZ domains (post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1) domain). The PTB domain of the protein binds to the YENPTY sequence of APP situated in the intracellular section of the protein and has been shown to play a role in regulating APP trafficking. The grey line represents the plasma membrane.

A number of laboratories have shown that altering expression levels of Mint 1 or 2 has an effect on APP processing. For example, over expression of Mint 1 and 2 have been shown to stabilise APP and therefore reduce the levels of Aβ produced (Saito *et al.* 2008), (Borg *et al.* 1998).

Mice with Mint mutations were crossed with Alzheimer model mice (Tg2576) predisposed to the early accumulation of Aβ plaques. Deletion of all three Mint proteins was shown to reduce the level of both Aβ40 and Aβ42, as well as the number of Aβ plaques in the Tg2576 mice (Ho et al. 2008). However, the mechanism by which this occurs is unknown. It has been suggested that

Mints could interfere with Fe65-APP interactions. Mints 1 and 2 may block the upregulation of BACE1 and APP genes, which is thought to be regulated by Fe65 (Lau *et al.* 2000). Alternatively, more recent studies have suggested Mints 1 and 2 may interfere with γ-secretase cleavage of APP. In order to function γ-secretase requires four components; PSEN1, PSEN2, APH1 and nicastrin. Mint may interfere directly with this complex in a number of ways, either at the level of transcription or by interfering with binding (reviewed in (Miller *et al.* 2006). It was reported that Mint1 impairs γ but not β cleavage of APP, potentially by preventing the APP from being trafficked to sites of active γ-secretase (King *et al.* 2004). Mint 2 and the Mint 2 binding protein, alcadein, have also been shown to inhibit Presenelin-1 APP interactions (Araki *et al.* 2003).

The Mint proteins have recently been shown to play a role in APP trafficking. Mint knock-out neurons were shown to have reduced internalisation of APP from the plasma membrane (Chaufty *et al.* 2012).

1.8: Regulating the Mint-APP interaction

There is still little known about the mechanisms by which the Mint-APP interaction is modulated. Earlier studies indicated the N- and C- termini of Mint1 play an important role in the regulation of the protein. It was shown that overexpression of Mint1 N-terminal domains were sufficient for promoting APP and Aβ secretion, whereas overexpression of the C-terminus alone reduced APP metabolism (Mueller *et al.* 2000). The C-terminus, including the two PDZ domains, has since been implicated in regulating Mint1 activity via conformational changes, resulting in autoinhibition. One study found the C-terminal tail of Mint1 displayed intramolecular interactions with the first PDZ domain of the protein, thus forming an autoinhibited conformation (Long *et al.* 2005). More recently, it has been shown a similar mechanism; again involving the C terminal domain of Mint1 can inhibit binding between the Mint1 PTB domain and target proteins (Matos *et al.* 2012). This study showed a C-terminal linker region of the protein is structurally located adjacent to the PTB domain and the short alpha helix can sterically inhibit APP binding.

Furthermore, the study suggests tyrosine phosphorylation could play a part in this regulatory mechanism. Mutating the residue Tyr633 was shown to disrupt this inhibitory interaction and increase APP binding and Aβ production. Similar structural regulatory mechanisms have been suggested for the Mint2-APP interaction. One recent study demonstrated the protein undergoes a conformational change between an 'open' and 'closed' structure in which the region between the PDZ and PTB domains blocks the peptide binding region of the PTB domain in a ligand free structure (Xie *et al.* 2012). Despite early studies suggesting the Mint N-terminal played an important role in regulating Mint activity, little work has since been done to elucidate the specific mechanisms by which this occurs.

1.9: Regulation of APP trafficking by protein phosphorylation

The mechanisms for how the interactions of APP with its trafficking adaptors and secretases are regulated, both temporally and spatially, are poorly understood. However, phosphorylation, and in particular tyrosine phosphorylation, has been shown to add a layer of regulation to APP function. Reports from several laboratories have shown that Src family kinases (SFKs) can modulate APP adaptors via tyrosine phosphorylation. For example, Dab1 has been shown to interact with the SFK Fyn (Howell *et al.*, 1997). Fyn has been shown to phosphorylate both Dab1 and APP to affect the level of APP processing (Hoe *et al.*, 2008). It was found that, whilst Fyn alone could alter the processing of APP, this effect was increased in the presence of Dab1, suggesting the regulation is a collaborative process. In addition to this Abl, another tyrosine kinase related to Src, has been shown to interact with APP and the Fe65 adaptor protein. It has been shown Abl can stimulate Fe65/APP-mediated transcription (Perkinton *et al.*, 2004). It is unclear how this phosphorylation affects their transcriptional activity. The study by Perkinton *et al.* suggested it didn't do this through inhibiting the Fe65-APP interaction, as was initially thought. A previous study revealed that phosphorylation of two serine residues (Ser 236 and 238), which are located N-terminal to the PTB domain, enhances the association between Mint2 and

APP (Sakuma *et al.*, 2009). This phosphorylation was seen in Mints extracted from the brain, but not in the ubiquitously expressed Mint3, indicating it is an important feature of APP regulation. Another recent study has identified 3 tyrosine residues in Mint2 which are phosphorylated by the tyrosine kinase c-Src (Chaufty *et al.* 2012). Moreover the study found the phosphorylation state of Mint2 had an effect on the sub-cellular localisation of the protein. They found that a phospho-mimetic mutant of Mint2 heavily colocalised with the autophagosome marker LC3, whereas a phospho-resistant mutant form of the protein colocalised with a Golgi marker (Chaufty *et al.* 2012). Importantly the study found the phosphorylation state of Mint2 had no effect on the proteins binding affinity for APP, nor the expression of the protein.

1.10: Src family kinases

Tyrosine phosphorylation has been implicated in the regulation of a number of cellular processes, including cell motility, proliferation and differentiation. There are a number of different protein tyrosine kinase families, one of which is the Src family kinases (SFKs). SFKs were first identified in their oncogenic from, v-Src, in the Rous sarcoma virus (Brugge and Erikson 1977). v-Src is a mutated version of the ubiquitously expressed c-Src. Src was found to possess tyrosine kinase activity in 1978 (Collett and Erikson 1978), and has since been implicated in a number of cellular functions. Src is one of 9 non-receptor tyrosine kinases (nRTKs) that make up the SFKs. The proteins share six common, functional domains- a myristylation domain that associates the kinase with cellular membranes; a unique domain; Src homology (SH) domains SH2 and SH3 that interact with target proteins; the tyrosine kinase domain; an autophosphorylation site which is involved in autoregulation; and a C-terminal regulatory domain.

1.11: Src activation and regulation

Src activity itself is regulated by tyrosine phosphorylation. It was noted that tyrosine-416 of c-Src was phosphorylated in transforming, oncogenic forms of Src, but was not phosphorylated in inactive wild-type c-Src (Cooper *et al.* 1986; Parsons and Weber 1989). It has since been found there are two sites on which Src in phosphorylated that cause conformational changes in the protein (Figure 1.3).

When inactive, Src is phosphorylated on Y527 in the regulatory C-terminal domain, this interacts with the SH2 domain (Roussel *et al.* 1991) causing the linker region between the kinase and SH2 domains to interact leaving the protein in a folded,

Figure 1.3 – The domains and regulatory mechanism of Src kinases. **A)** Src kinase activity is regulated by phosphorylation causing a conformational shift between 'closed' inactive conformations and the 'open' active structure. Y527 can be phosphorylated by Csk or Chk. The phosphotyrosine then forms intramolecular interactions with the SH2 domain causing it to be in the 'closed' conformation. Alternatively the protein is active and 'open' following dephosphorylation of Y527, by any of a number of phosphatases, and intermolecular autophosphorylation of Y416 promoting kinase activity. **B)** The domain structure of Src kinases. Shown is the myristolation domain, the SH3 (Src homology 3) and SH2 domains, and the tyrosine kinase domain. The SH3 domain recognizes a PXXP motif in target proteins. Sh2 domains typically bind to phosphotyrosines. The neuron specific Src isoforms N1 and N2-Src have short amino acid inserts in their SH3 domains of 6 and 18 residues respectively.

'closed' conformation, at basal levels 90-95 % of c-Src is in this state (Zheng *et al.* 2000). Src is phosphorylated at Y527 by either C-terminal Src kinase

(Csk) (Okada and Nakagawa 1989) or Csk homology kinase (Chk) (Zrihan-Licht *et al.* 1997). Dephosphorylation of Y527 and phosphorylation of Y416 disrupts the intramolecular interactions resulting in the protein being in an 'open' active conformation. Src undergoes an intermolecular autophosphorylation at Y416 (Roskoski 2004). There are numerous candidate phosphatases which have been implicated in Y527 dephosphorylation, these include PTP1B, Shp1 (Src homology 2 domain-containing tyrosine phosphatase 1), Shp2, PTPα, PTPγ and PTPε (Levin 2004). The importance of this regulatory mechanism is highlighted in the consequences of a mutation in the c-terminus of c-Src which causes a frame shift and disrupts it's interaction with the SH2 domain, this mutation causes the protein to be constitutively active and giving rise to the oncogenic v-Src form of the protein (Takeya and Hanafusa 1983).

In an additional regulatory mechanism, it has been found that the activity state of Src is also dependent on its cellular localisation. Trafficking studies carried out using a temperature sensitive mutant of v-Src found that the protein was present in its inactive state localised to the perinuclear region, whereas the active form of the protein was found at the periphery of the cell (Welham and Wyke 1988). The same activity-localisation pattern was noticed with c-Src, where the inactive form of the protein was associated with the perinuclear region and colocalised with endosomal markers (Kaplan *et al.* 1992). Despite these early observations, until recently, very little was known about the spatial regulation of SFK activity and whether the association with endosomal and membrane compartments was significant (Kaplan *et al.* 1992). Recent studies, using a phospho-antibody specific to active c-Src, showed that c-Src was held, in its inactive form, in the perinuclear region. However, stimulation of the protein with growth factors resulted in translocation of c-Src to the plasma membrane, where it is activated and localises in cytoplasmic endosomes (Sandilands *et al.* 2004).

1.12: Src in neurons

It is well documented that one of the main functions of Src in neurons is to regulate the ion channels involved in synaptic plasticity. Src has been found to regulate the NMDA receptor (Wang and Salter 1994), potassium channels (Fadool *et al.* 1997), calcium channels (Cataldi *et al.* 1996), γ-aminobutyric acid type A (GABA$_A$) (Moss *et al.* 1995) and nicotinic acetylcholine receptors (Wang *et al.* 2004). It has been shown for example, that Src mediates NMDA receptor activity by balancing receptor phosphorylation and dephosphorylation, one study showed that inhibiting phosphotyrosine phosphatase (PTP) activity and increasing exogenous Src expression in cells enhances NMDA mediated currents (Wang and Salter 1994). This process highlights the flexibility and reversible nature of regulating protein function by phosphorylation. NMDA receptors are involved in numerous aspects of neuronal plasticity, including long-term potentiation (LTP) in learning and memory. Importantly NMDA receptor upregulation by Src is required for the induction of LTP at synapses in the hippocampus (Lu *et al.* 1998; Huang *et al.* 2001). Src expression in significantly upregulated in neuronal development (Cartwright *et al.* 1988), however it is also expressed at high levels in differentiated, post mitotic neurons (Cotton and Brugge 1983; Sudol and Hanafusa 1986). Src null mice show no significant neuronal phenotypes however do present with bone formation defects and osteopetrosis (Soriano *et al.* 1991). This is most likely due to redundancy in function between SFKs in neurons. Another SFK that is highly expressed in neurons is Fyn. Fyn knockout mice, in contrast to Src deficient ones, do show abnormal neuronal phenotypes. These mice show impaired LTP and decreased short and long term contextual fear memory (Grant *et al.* 1992). Impairments in ion channels and the hippocampal region of the brain have been linked to a number of neurological diseases including AD (Santos *et al.* 2010), especially in the early stages of the disease(Gotz *et al.* 2011). In fact the NMDA receptor inhibitor memantine is used as an AD treatment (Schmitt *et al.* 2007), thus

highlighting the importance of SFK mediated regulation of neuronal processes.

Following the identification of the ubiquitously expressed c-Src it was found that neurons expressed a higher level of a more active form of Src than seen in cells from other tissues (Brugge *et al.* 1985). This neuronal Src was shown to be one of two neuronal splice variants of c-Src, both posses short amino acid inserts in their SH3 domain (Brugge *et al.* 1985; Pyper and Bolen 1990).

Since the identification of the two variants, N1 and N2-Src, little progress has been made in elucidating whether the kinases have any specific targets or functions that differ from c-Src. However, research into the roles of SH2 and SH3 domains does suggest that, due to their important role in regulating the interactions of kinases to their target proteins, splice variations in these domains would effect the affinity of the kinases for certain targets. A recent study by Groveman *et al.* (2011) examined the differential effects of the SH2 and SH3 domains of neuronal Src on phosphorylation. They found that dysfunction of the SH2 domain has a greater detrimental effect on Src kinase activity than that of the SH3 domain. Previous studies have also shown that conformational changes in Src kinases, shifting the position of the SH2 and SH3 domains, can alter its kinase activity (Nagar *et al.*, 2006, Filippakopoulos *et al.*, 2008).

1.13: Project aims

Mint1 has previously been identified as a C-Src substrate in the laboratory (Figure 1.4; Dunning *et al.*, manuscript in preparation). When screening the Mint1 amino acid sequence for functional domains and motifs, it was noted there was a YEEI motif, the canonical motif recognised by Src kinases, in the N-terminus. *In vitro* kinase assays using Mint1 N-terminus (1-431) showed that this motif showed tyrosine phosphorylation by C-Src. In addition to this it was noted, as the N-terminus of Mint proteins differ significantly, no phosphorylation was detected on the equivalent regions of Mint 2 and 3 (Figure 1.4A). Subsequent *in vitro* phosphorylation of recombinant Mint1 and

analysis of the phosphorylated protein following 2D-gel electrophoresis and LC-MS/MS (liquid chromatography-tandem mass spectrometry) found the phosphorylated peptide (the sequence of which is shown in figure 1.4) was present in its mono-, di- and tri-phosphorylated forms. All detected peptides were phosphorylated on Y202, with subsequent phosphorylation at Y191 and Y187. Significantly neither phosphorylation of Y191/Y187 alone nor phosphorylation of Y202 and Y187 was observed. More over, mutation of Y202 to a phospho-resistant phenylalanine resulted in abolition of all phosphorylation. It was therefore concluded that Mint1 is processively phosphorylated by Src kinase at the three residues (Dunning *et al.,* manuscript in preparation, Figure 1.4).

In addition to this it was noted that overexpression of Mint1-WT disrupted the regular appearance of APP distribution in COS7 cells, and moreover that this disruption was not observed in cells overexpressing Mint1-Y202F (Dunning *et al.*, manuscript in preparation). These findings suggest phosphorylation of the N-terminal region of Mint1 may play an important role in regulation of APP trafficking, and that this regulation is specific for Mint1. I therefore aim to study the effects of Mint1-WT or -Y202F and Src kinases on the APP subcellular trafficking cycle. In order to do this I used a combination of *in vivo* studies of the APP trafficking cycle in a HeLa cell system, along with *in vitro* assays.
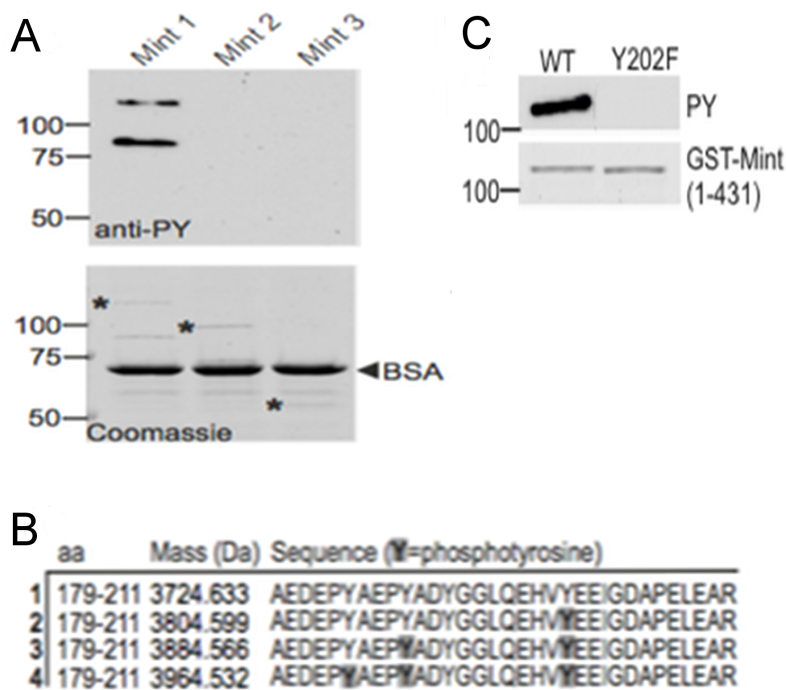
Figure 1.4- Preliminary data from the laboratory (adapted from Dunning *et al.*, manuscript in preparation. **A)** Mint1 (1-431) is seen to be phosphorylated by Src, whereas Mints 2 (1-341) and 3 (1-184) show no phosphorylation. A kinase assay with C-Src was carried out with GST-fusions of N-terminal Mint regions. Phosphorylation was detected by immunoblotting (top panel). Coomassie loading control (* show Mint1-3 loading) is shown in the bottom panel. **B)** Sequences and masses of the phosphopeptides detected by mass spectrometry. Highlighted are the tyrosine residues that are phosphorylated by Src. **C)** Mutation of Mint1-Y202 to phenylalanine was shown to stop all Mint1 N-terminal phosphorylation by C-Src in a recombinant Mint1 kinase assay when probed with an anti-phosphotyrosine antibody (top panel). Coomassie staining for protein loading is shown in the bottom panel.

2: Materials and methods

2.1: Materials

Rabbit, monoclonal anti-Myc antibody and rabbit, polyclonal anti-FLAG were purchased from Cell Signalling Technologies, Hitchin, UK. Mouse anti-phosphotyrosine (PY20) was purchased from BD Biosciences, Oxford, UK. The anti-GFP antibody (used to detect CFP and YFP) was a generous gift from Paul Pryor's laboratory (University of York). Mouse anti-β-actin antibody was obtained from Proteintech, Manchester, UK. Mouse, monoclonal anti-FLAG was purchased from Sigma Aldrich Company Ltd, Dorset, United Kingdom. Anti-mouse and anti-rabbit, Horseraddish peroxidase-linked secondary antibodies produced in goat were obtained from Sigma Aldrich Company Ltd, Dorset, UK. Alexa-fluor conjugated secondary antibodies used in immunocytochemistry were as follows; Anti-mouse Alexa-594 and -568, Anti-rabbit Alexa-488, and were purchased from Invitrogen, Paisley, UK.

Miniprep purification of plasmids was carried out using the QIAprep Spin MiniPrep Kit according to the manufacturers instructions. Gel extraction of DNA was carried out using QIAquick Gel Extraction Kit according to the manufacturer's instructions (QIAGEN, Sussex, UK). Proteins were produced using XL10 Gold Ultracompetent *E. Coli* purchased from Agilent technologies, Berkshire, United Kingdom. For culturing of cell lines Dulbecco's modified eagle medium, Penicillin-streptomycin and FBS were all obtained from Invitrogen Ltd, Paisley, United Kingdom. For transfections, Ecotransfect was purchased from Oz biosciences, Nottingham, UK.

Table 2.1: Plasmids used in cell expression experiments

Gene of interest	Species of gene	Tag (Terminal)	Vector
Mint1WT **1-842** **(full length)**	Mouse	CFP (N) MYC (N)	pmCer pMH (Roche)
Mint1-Y202F **1-842** **(full length**	Mouse	CFP (N) MYC (N)	pmCer pMH (Roche)
APP(695)	Human	FLAG (C) YFP (C)	pcDNA5/FRT/TO pFLAG-N1(YFP introduced to replace FLAG)
N1-Src	Rat	FLAG (C)	pcDNA5/FRT/TO
C-Src	Rat	FLAG (C)	pcDNA5/FRT/TO

Table 2.2: Constructs used in *in vitro* assays

Protein	Region	Tag	Plasmid
Mint1	**1-341** **(N-terminus)**	**GST**	pGEX-6P1
	183-208 **(Phospho-box)**	**GST**	
N1-Src		**His**	pGEX4T-1 (PTP1B fusion and 3C protease site)
C-Src		**His**	pGEX4T-1 (PTP1B fusion and a 3C protease site)

2.2: Expression, production and purification of His-tagged Src kinases

Transformed, competent XL10 Gold were grown to an appropriate cell density (showing an absorbance at 600 nm of around 0.6-1) in 1 L autoclave sterilised supermedia containing 5 g/l NaCl, 15 g/l tryptone, 25 g/l yeast extract and the appropriate antibiotic (Ampicillin at 100 µg/ml, kanamycin at 50 µg/ml). Protein production was induced by the addition of isopropyl-β-D-1 thiogalactopyranoside (IPTG) at a concentration of 1 mM and incubated overnight with aggitation at 18 °C. The culture was then spun at 5000 rpm for 10 minutes. Pellets were resuspended in PBS containing 1 mM of the serine protease inhibitor phenylmethanesulfonylfluoride (PMSF) and incubated on ice for 30 minutes. The suspensions were then sonicated at 10 kHz to lyse bacteria cells on a 30 second on 30 second off cycle 6X on ice. The sonicated suspension was then centrifuged at 15000 rpm for 30 minutes at 4 °C. The protein, now present in the supernatant, was bound to 2 mL of glutathione-sepharose beads in a 50 % slurry in PBS by incubating the two for 2-3 hours at 4 °C with agitation. Following the incubation the beads were washed in PBS and centrifuged at 860 rpm, for 3 minutes at 4 °C 5X, once in 1.2 M NaCl in PBS, and a final wash in PBS. Excess PBS was removed to leave beads in a 50 % slurry and beads were transferred to a 1.5 mL eppendorf. The protein was cleaved by overnight incubation at 4 °C with 3 units of 3C PreScission protease. The presence and effective cleavage of the proteins was tested using SDS-PAGE. Samples were separated on a 10 % SDS-PAGE gel at 160 V for around one hour. Gels were then stained with Coomassie protein stain (50 % Methanol, 9 % Acetic acid, 0.3 % Brilliant blue R) for 30 minutes and destained using 14% acetic acid, 7% methanol in distilled H_2O for 30 minutes. Proteins were stored in kinase storage buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.05 mM EDTA, 1 mM DTT, 10 % glycerol, 1 mg/mL BSA (prevents aggregation of kinase and adsorption to tube wall) and 0.05 % NP-40) at -80 °C.

2.3: SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a Bio Rad Mini-PROTEAN Tetra cell gel apparatus (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK) according to the manufacturer's protocol. Gels were prepared with either 7.5 % or 10 % resolving gel (375 mM Tris.HCl, pH 8.8, 0.1 % SDS, 10 or 7.5 % acrylamide, 0.05 % Ammonium persulfate and 0.01 % TEMED) with 4 % stacking gel (125 mM Tris.HCl, pH 6.8, 0.1 % SDS, 4 % acrylamide, 0.05 % Ammonium persulfate and 0.01 % TEMED). Gels were run in SDS running buffer (25 mM Tris, 0.192 M glycine and 0.1 % SDS in distilled H_2O). Gels were run at 160 V for around 60 minutes, or until the dye front reached the bottom of the gel. Gels were then either stained using Coomassie blue, or transferred for Western blot analysis.

2.4: Western blotting

Proteins were transferred from SDS-PAGE gels using Bio-Rad Mini Trans-Blot transfer apparatus (Bio-Rad Laboratories Ltd, Hemel Hempstead, Hertfordshire, HP2 7DX). Proteins were transferred onto Polyvinylidene fluoride (PVDF) membranes. PVDF membranes were used for their increased protein retention, and increased resilience allowing reprobing of membranes when compared to the alternative nitrocellulose membranes. PVDF membranes were first activated in 100 % methanol for around 30 seconds, before being equilibrated along with SDS-PAGE gels in transfer buffer (25 mM Tris, 190 mM Glycine and 20 % Methanol). Transfer apparatus was assembled in cassettes containing the gel and PVDF membrane surrounded by 3 MM filter paper and pads, taking care to ensure there was no air bubbles between the gel and membrane. The transfer was carried out at either 66 V for 1 hour or 20 V overnight, with an ice block in the transfer tank to avoid overheating. Transfer efficiency was tested by staining the membrane with Pancau-Red (6.5 mM ponceau S and 1 % acetic acid), before de-staining in dH_2O. Membranes were then incubated in PBS + 3 % milk powder (or 3 % BSA if probing for phospho-tyrosines) for one hour, all incubations were

carried out on a rocking platform. Primary antibodies were then added at concentrations as recommended by manufacturers, in PBS with or without 0.1% Tween-20 and 0.5 % BSA, for either 2 hours at room temperature or overnight at 4 °C. Membranes were washed in PBS with 0.5% Tween-20 in between primary and secondary antibodies. Horse-raddish peroxidase (HRP)-linked secondary antibodies raised against the desired species were added at 1:5000-1:2000 dilution in PBS plus 0.5 % Tween-20 for around an hour at room temperature. Membranes were washed again in PBS plus 0.5% Tween-20. Proteins were detected by incubation with ECL (Equal volumes of solution A – 2.5 µM luminol, 1% DMSO, 0.1M Tris/HCl, pH 8.5, and solution B – 0.06 % hydrogen peroxide, 0.1M Tris/HCl, pH 8.5, plus 'enhancer' 90 µM p-coumaric acid in DMSO at 2 µL/mL of A+B) for 1 minute before being exposed to X-Ray film for between 30 seconds – 5 minutes, and developed. Membranes were probed with an anti β-actin antibody as a loading control. For reprobing membranes the previous antibodies were stripped in a buffer containing 136mM NaCl, 20mM glycine, pH 2.5.

2.5: *In vitro* kinase assay

All kinase assays were carried out at in a final volume of 25 µL, at 30°C for 90 minutes. Reactions contained 100 mM Tris, pH 7.5, 10 mM $MgCl_2$, 0.5 mM ATP, 5 nM kinase, and up to 25 µM substrate. Reagents were mixed on ice, minus ATP, and pre-warmed for around 5 minutes. ATP was added to start the reaction. The assays were stopped using an equal amount of 2X Laemmli buffer (final concentrations 2 % SDS, 10 % glycerol, 5 % beta-mercaptoethanol, 0.002 % bromophenol blue, 62.5 mM Tris.HCl pH 6.8) to the reaction mixture. The assays were then analysed using SDS-PAGE and Western blotting.

2.6: Cell culture

Cells were cultured in Dulbecco's modified eagle medium (DMEM) plus filter sterilized 1 % penicillin/steptromycin and 10 % FBS, in 25 cm culture flasks.

Cells were kept at 37 °C, in a humidified atmosphere of 5 % CO_2 and 95 % air.

2.7: Transfection

Cells were transfected using Ecotransfect according to the manufacturers protocol. In summary, cells were seeded in either 24 or 6 well plates, 24 hours later (or when cells reached 75-80 % confluency) cells were transfected with 2 µl Ecotransfect plus 1 µg total DNA in 100 µl DMEM for 24 well plates, or 6 µl Ecotransfect plus 3 µg total DNA in 200 µl DMEM for 6 wells plates. Cells were cultured in transfection media for 24 hours.

2.8: Generating a stable, inducible APP expressing HeLa cell line

APP-HeLas were produced using the Flp-In system (Invitrogen). Flp-In HeLas were received as a generous gift from Dani Ungar's laboratory (University of York). The Flp-In HeLas contain a Flp Recombination target (FRT) site, the presence of which had been previously selected for using Zeomycin resistance. Zeomycin resistant clones are then screened to select for clones that contain only one FRT site per cell. The Flp-In HeLas received were co-transfected with plasmids containing the gene of interest (GOI) (pcDNA5/FRT/TO-APP-FLAG) and Flp-recombinase (pOG44). Approximately 1.5 x 10^5 cells were plated in 6 well plates and co-transfected using the Ecotransfect protocol mentioned previously, 0.5 µg of each of the plasmids was co-transfected using 6 µl of EcoTransfect per well. Expression of the Flp recombinase facilitates a recombination event between FRT sites in the genome and that included in the GOI plasmid. The resulting site has an SV40 promoter; ATG initiation codon in frame with the GOI and a hygromycin resistance gene, as a result of this recombination event the Zeomycin resistance gene is interrupted. Clones that have been successfully transfected were then selected for using 200 µg/mL hygromycin. 24 hours after transfection, cells were transferred to 10 cm culture dishes to select for successfully transfected clone. Cells were removed from 6 well plates by incubating with 500 µl trypsin at 37 °C for around 3-5 minutes, trypsinised

cells were the dislodged and 1.5 mL of media was added. Cells were plated at various concentrations and cultured until colonies of hygromycin resistant cells formed, 200 µg/mL hygromycin containing culture medium was changed every 2 days until colonies formed. Individual colonies were then selected, firstly the medium was removed and cells were carefully washed 3X with sterile PBS. Colonies were isolated using autoclave sterilized wide ends of 1 mL pipette tips attached to the plate using autoclaved silicon grease. Selected colonies were then incubated with 100 µL trypsin for around one minute before being suspended by trituration with the trypsin. Colonies were expanded, and APP expression was characterised using Western blotting of cell lysates and immunocytochemistry. A total of 5 APP-HeLa clones were selected, one of which showed slightly increased APP expression above the others and was used in all future experiments. APP-HeLas were cultured as with other cells, with the inclusion of 200 µg/mL hygromycin in the culture medium.

N1-Src and C-Src HeLa cell lines were also generated, following the same protocol, by Philip Lewis, Evans Laboratory, University of York. Once transfected HeLa cells were treated with 1 µg/mL doxycycline to induce expression of either APP of N1-Src. Cells were treated with doxycycline for up to 48 hours.

2.9: Cell lysis

Around 1.5×10^5 cells were plated in 6 well plates. Following transfection and treatments, cells were first washed 3X in PBS before being lysed by scraping in 200 µl Laemmli buffer. Whole cell lysates were analysed by SDS-PAGE and Western blotting. Between 10-20 µl of cell lysates in 2X Laemmli buffer were loaded on the gel, along with Precision plus protein standards from Bio Rad.

2.10: Immunocytochemistry

For immunocyctochemical analysis, 1.5×10^4 cells were plated on 13 mm coverslips in 24 well plates. Following transfections and treatments, cells were fixed. Cells were first washed 3X in PBS, before being fixed in 4% PFA for 20 minutes. Cells were stained using commercially available antibodies, all incubations were carried out at room temperature. They were first permeabilised and blocked in PBS containing 0.1% Triton-X100 and 1% BSA for 30 minutes. Primary antibodies were applied at 1/500 concentrations in PBS containing 1% BSA for 2 hours. Cells were then washed 3X in PBS before secondary antibodies, linked to the desired fluorophore, were applied, again at 1:500 concentration in PBS + 1% BSA, for 1 hour in the dark. Following subsequent washes in PBS (3X) and distilled H_2O (1X) coverslips were air-dried and mounted on slides using Mowiol (13 % Mowiol, 30 % glycerol, 0.2 M Tris, pH 8.5) plus 1μg/mL DAPI. Slides were stored at 4 °C prior to image analysis.

2.11: Imaging and statistics

Slides were imaged on confocal microscopes in the Technology Facility, Department of Biology, University of York. Confocal microscopes used were Zeiss LSM 710 on an Axio Observer.Z1 invert and Zeiss LSM 510 meta on an Axioplan 2M, both running ZEN software (Zeiss microscopy, Cambridge, UK). All image analysis was carried out using Image J software. Analysis of APP particles was carried out using threshold, particle analysis. The threshold of each image was adjusted to a point where the maximum number of individual APP containing particles could be counted, this was carried out for each cell analysed. Binary images were then analysed using the 'Analyse particles' function in imageJ. One-way ANOVAS were used to calculate statistical significance, where appropriate, with post-hoc Tukey test. Adjusted P values were also calculated; values have been adjusted to compensate for multiple comparisons. Chi squared analysis was used to examine the statistical significance of phenotyping data. Bonferroni correction was used to correct for

multiple comparisons. The following equation was used to calculate the adjusted α value; with the normal α value set at 0.05 -

$$\alpha_{adjusted} = \alpha/\text{number of comparisons}$$

All statistics and graphs were carried out using Graphpad Prism 6.0 software.

3: Results

3.1: Generating and characterising a stable, inducible APP expressing HeLa cell line

Mint1 is a neuronally expressed adaptor protein that binds to and facilitates trafficking of APP. Previous data from the Evans laboratory has shown that Mint1 is N-terminally phosphorylated by the tyrosine kinase C-Src. It was also shown that this phosphorylation is dependent on a canonical YEEI motif in Src and mutation of the tyrosine residue Y202 to a phospho-resistant phenylalanine (Y202F) abolishes all phosphorylation (Dunning *et al.,* manuscript in preparation). In light of these observations this study aims to evaluate the mechanisms by which Mint1 and Src regulate APP trafficking.

In order to thoroughly examine this process, a stable, inducible, APP expressing HeLa cell line was generated. To generate this cell line HeLa cells containing an FRT site, a site that is recognised by the enzyme Flp recombinase, were co-transfected with a plasmid encoding Flp recombinase and one encoding the gene of interest (GOI). The GOI is then incorporated into the genome at the FRT site. Importantly this system ensures only one copy of the GOI is inserted into each cell. Cells therefore display similar levels of the GOI thus improving analysis and quantification of images from the cells. Exploiting a stable cell line reduces the need for multiple co-transfections of plasmids, and ensures higher expression efficiency as each cell is derived from a single clone, and is continuously cultured in media containing 200 µg/mL hygromycin to select for cells which keep the GOI incorporated. The inducible nature of this cell line allows the localisation and expression of APP to be examined as early as possible after translation. The expression of the gene of interest is under the control of a CMV promoter, containing tetracycline operon sequences allowing doxycycline inducible expression, which is known to give high levels of expression in mammalian cells and so the system will provide sufficient levels of protein to be visualized using biochemical techniques.

Figure 3.1 shows APP expression in a selected, single clone of FLAG tagged APP-HeLa cells. Figure 3.1A shows immunocytochemical images of cells cultured in the presence (bottom) or absence (top) of doxycycline. APP was visualized using an anti-FLAG antibody 48 hours after doxycycline induction. Figure 3.1B shows Western blot analysis of APP-HeLa cell lysates, probed with an α-FLAG antibody. Two bands representing APP at around 120 and 130 kDa can be seen, the presence of more than one band may represent APP that has been differentially post-translationally modified, as seen in previous studies (Tomita *et al.* 1998; Jacobsen and Iverfeldt 2011). Figure 3.1B also shows an increase in the amount of APP detected between 24 and 48 hours of doxycycline treatment, for this reason 48 hours was used as a standard doxycycline induction period throughout the study. Importantly the lack of FLAG staining in cells or lysates that have not been treated with doxycycline, demonstrates the system doesn't exhibit leaky expression of the GOI. The FLAG staining of the protein is representative of endogenous APP localisation, with the majority being perinuclear, as well as some in punctate trafficking vesicles (Seen clearer in Figure 3.2A) (von Rotz *et al.* 2004).
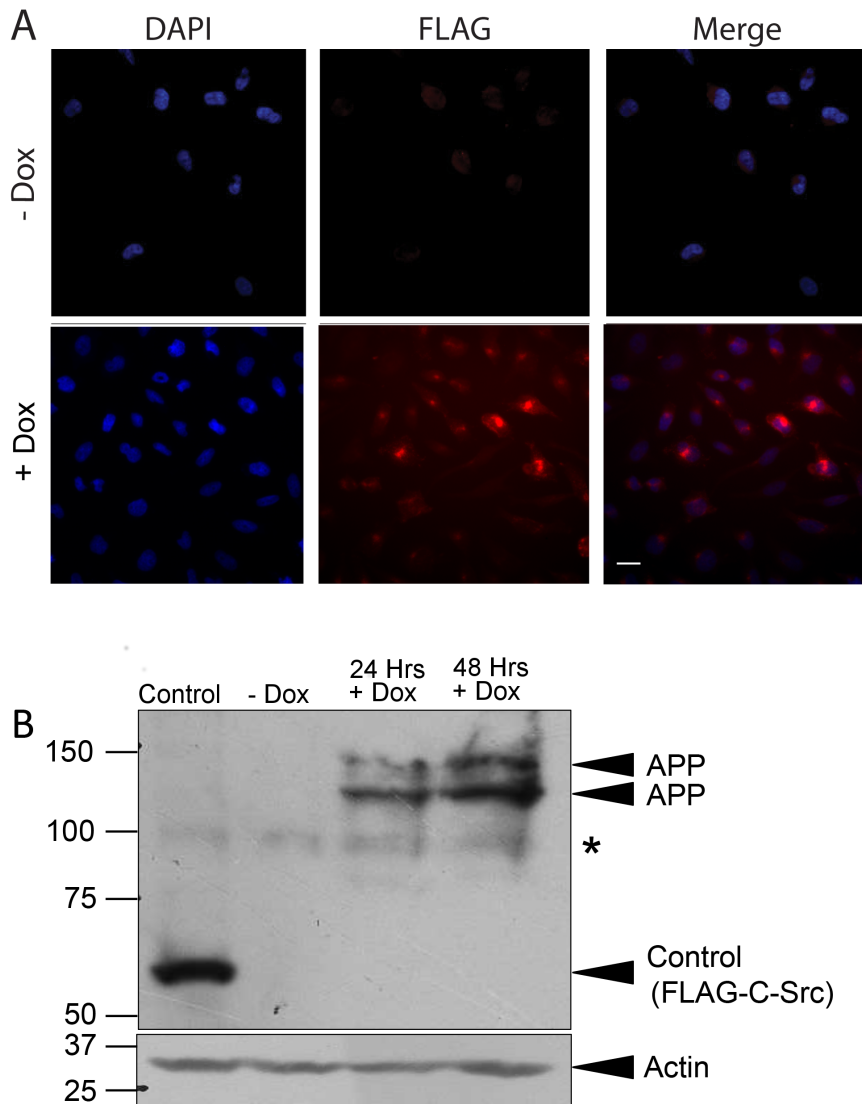
Figure 3.1 – Characterisation of a stable, inducible APP HeLa cell line. **A)** Representative images of APP HeLa cells with or without doxycycline treatment. APP (red) can be seen in doxycycline treated cells but is not present in non-treated cells. Cells were cultured in 1 µg/mL doxycycline for 48 hours. Scale bar = 20 µm. **B)** Western blot analysis of APP expression in HeLa cell lysates cultured in 1 µg/mL doxycycline for 24 or 48 hours. Expression is seen to increase with prolonged exposure to doxycycline. Lane 1 shows a positive FLAG control of whole cell lysates expressing FLAG-tagged C-Src. Lane 2 shows lysates in which APP expression has not been induced. * denotes non-specific binding of the antibody.

The Evans laboratory has previously obtained data that demonstrates the phosphorylation state of Mint1 disrupts APP trafficking in COS7 cells (Dunning *et al.,* manuscript in preparation). The data shows APP has a perinuclear distribution when Mint1 is overexpressed in heterologous cells (Figure 1.4). In order to characterise whether the same effect is observed in APP-HeLa cells, the cells were transfected with CFP-fusions of mutant, wild type Mint1 or a CFP control plasmid; 24 hours later APP expression was induced by incubating cells with 1 µg/mL doxycycline for 48 hours. Figure 3.2 shows immunocytochemical analysis of APP-HeLa cells expressing CFP-Mint1-WT or -Y202F. APP is seen in puncta throughout the cell, most likely in trafficking vesicles, upon transfection of Mint1–WT the distribution of these punctae changes significantly. Overexpression of Mint-WT results in a 70 % reduction in the number of APP containing particles per cell compared to those over-expressing APP alone (Mean number of particles/cell 360.32 and 1186.06 respectively, adjusted P value <0.0001). Additionally, overexpression of Mint1–WT resulted in a 45 % decrease in APP containing particles when compared to cells expressing Mint1–Y202F or an empty CFP control plasmid (Mean particle number/cell CFP = 727, Mint1-Y202F = 808, adjusted P values 0.0057 and <0.0001 respectively when compared to Mint1-WT). Whilst transfection alone is responsible for around a 25 % reduction in APP particle number (Fig 3.2B) the 45 % reduction of APP particles in cells expressing Mint1–WT compared to phospho-resistant Mint1–Y202F suggests the change in APP distribution, and therefore particle number, is a result of Mint1 phosphorylation at Y202F.
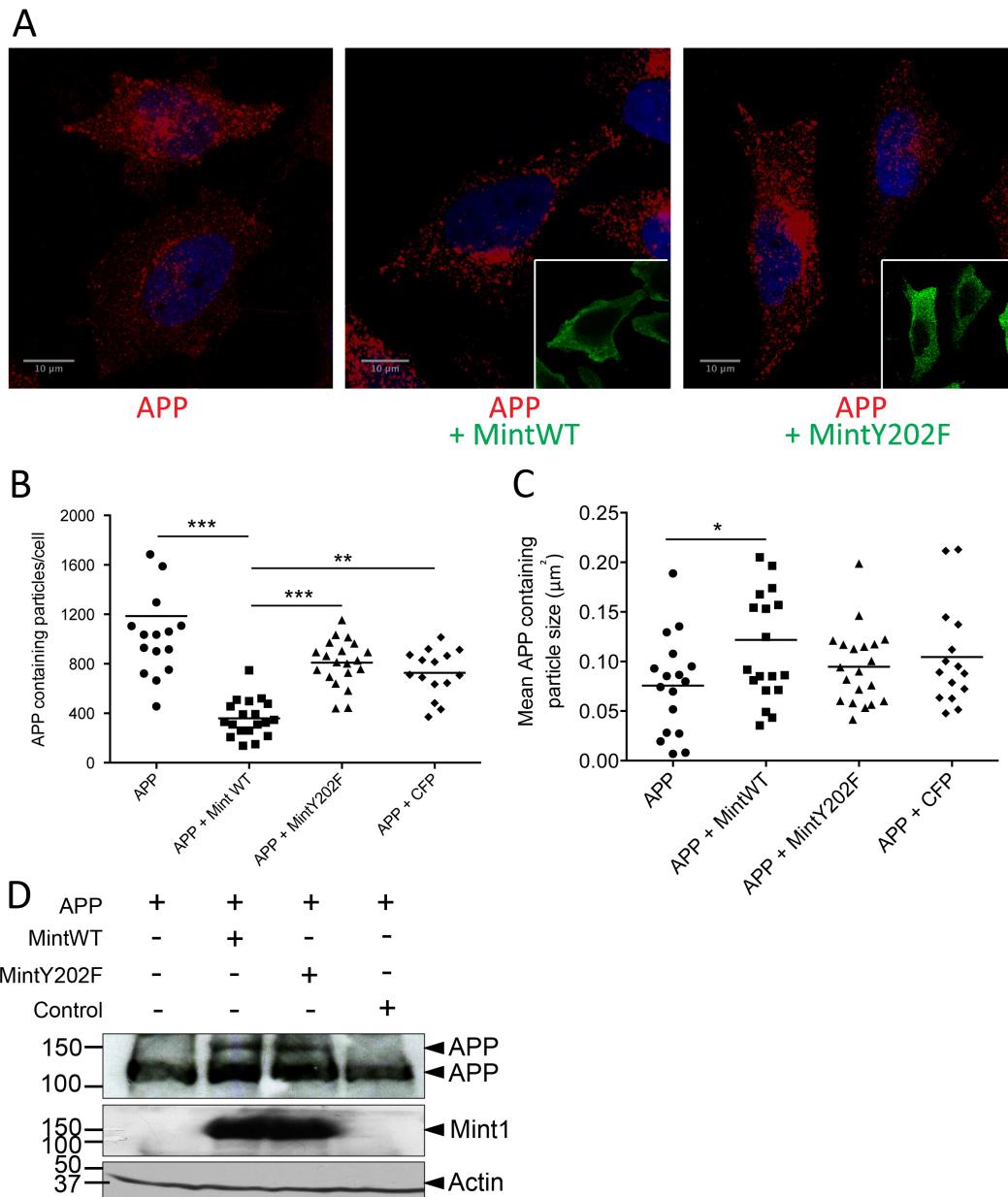
Figure 3.2 – Mutation of the phosphorylation site Y202 of Mint1 alters APP localisation. Cells were transfected with either Mint-WT or Y202F and 24 hours later cultured in 1 µg/mL doxycycline for 48 hours. Empty CFP plasmid is used as a control for the effect of transfection. **A)** Representative images showing altered localisation of induced APP (red) following overexpression of Mint1 constructs (green). Images show a change in the distribution and number of APP containing particles in the cell when Mint-WT is overexpressed, the same change is not observed when overexpressing the Mint1 phospho-resistant mutant Y202F. B) Graphs show quantification of APP containing particle number **B)** and size **C)**. Between 15 and 20 cells were analysed for each condition from three separate coverslips. *s denote statistical difference between means, data was subjected to one-way ANOVA with post-hoc tukey test. **D)** Western blot analysis of APP-HeLa lysates ± Mint1 constructs. The presence of Mint1 results in a higher molecular weight FLAG-APP band, suggesting Mint1 alters APP processing in a non-phosphodependent manner.

In addition to noting the change in APP particle number the size of the APP containing trafficking vesicles appeared to change with Mint1 transfection (Fig 3.2A). In an attempt to quantify this change the mean size of APP containing particles per cell was measured (Fig 3.2C). HeLa cells overexpressing Mint-WT showed a 38 % increase in mean APP particle size per cell (Mean particle size μm^2 Mint1-WT = 0.122 and APP alone = 0.0757, adjusted P value 0.032). However, no significant difference was observed between cells overexpressing Mint-WT and Mint-Y202F (Mean particle size μm^2 0.0947), or CFP (Mean particle size μm^2 0.104). Although Mint-Y202F and CFP transfected HeLa cells also showed no significant difference to cells expressing APP alone. The lack of difference in particle size between cells expressing Mint-WT and Mint-Y202F is due to the wide variation in particle sizes seen per cell. Figure 3.2C shows mean particle size for each cell plotted, it is clear the cells exhibit a wide range of particles sizes due to the different localisation of APP in the cell, for example a cell that is overexpressing Mint-Y202F may have many smaller APP containing particles than that seen in a cell overexpressing Mint-WT however the presence of a collection of perinuclear APP skews the mean particle size increasing it to around that seen in Mint-WT cells. In order to address this issue, it could be possible to quantify the particle size, discounting the perinuclear collection, perhaps by taking a z-stack image of cells and quantifying each layer to compare particle size in a layer of the cell that doesn't include the perinuclear region. Additionally it could be possible to quantify the intensity of fluorescence across the cell, therefore assessing the distribution of APP in the cell, in a similar manner to the phenotyping seen later in this study.

Previous reports, as well as observations in our laboratory, have suggested overexpression of Mint1 stabilises full length APP (Borg *et al.* 1998; Biederer *et al.* 2002). Figure 3.2D shows Western blot analysis of APP-HeLa cells that have been transfected with CFP-fusion Mint1 constructs. The top panel shows APP levels seen in the cells (visualised using an anti-FLAG antibody). This shows the intensity of the levels of APP detected does not significantly change between each condition, in contrast to previous reports. This may be due to the cell system used, or the inducible nature of APP production.

Although the levels of APP do not appear to change, the appearance of a higher molecular weight APP band, at around 140 kDa, is seen when APP is co-expressed with both wild type and phospho-resistant Mint1. As APP is highly post-translationally modified it is possible this heavier APP form is due to increased post-translational modification; such as increased glycosylation. Notably, and curiously this change doesn't appear to occur in a phospho-dependent manner.

Although Mint-WT has a clear effect on APP particle number in the cell line (Figure 3.2B), the lack of difference in particle size between cells expressing Mint-WT and Mint-Y202F led me to further characterise APP distribution in the cell line. As the data for mean particle size exhibited a wide range of values, it was thought the more accurate way to quantify APP localisation and assess whether APP is accumulating in the perinuclear region as hypothesised, was to quantify the phenotype of APP localisation in the APP HeLa cells. Cells were catagorised into 3 phenotypes; cytoplasmic, perinuclear or cytoplasmic and perinuclear. Figure 3.3A shows examples of each of the three phenotypes. Cytoplasmic APP was characterised as being visualised in punctate vesicles throughout the cell, with no distinct pool in the cell. Whereas APP classed as perinuclear showed a large, clear accumulation of APP around the nucleus, with little or no APP containing particles though the cytoplasm of the cell. Cytoplasmic & perinuclear was classed as a combination of the two. Analysis revealed there was a basal level of around 40 % of cells with an APP distribution of cytoplasmic & perinuclear (Figure 3.3B) in cells treated expressing APP alone, APP + CFP and APP + Mint1-Y202F. This proportion increased to 51 % in APP + Mint1-WT cells. However, the principal change in APP localisation following expression of the different Mint1 constructs was shown in the large increase in perinuclear localised APP. Cells expressing APP, APP + CFP or APP + Mint1-Y202F show little or no exclusively perinuclear APP (APP – 0 %, APP + CFP – 1.5 % and APP + Mint1-Y202F – 0 %). Whereas 31 % of cells overexpressing Mint1-WT exhibit a perinuclear phenotype for APP distribution (Figure 3.3B). Chi squared statistical analysis of the data, with Bonferroni correction, showed that the phenotypes observed in cells expressing APP and Mint1 were highly

significantly different to all other conditions (p value <0.0001 when compared to all other conditions, adjusted α value was 0.008).

This observation coupled to the quantification of the number and size of APP containing particles per cell (Figure 3.2) suggests Mint1 recruits APP to the perinuclear region in a phosphorylation dependent manner.

It is known that APP is trafficked through the Golgi, TGN and to the plasma membrane before being recycled through the endocytic pathway (Claeysen *et al.* 2012). With this in mind, it is unclear, from the data in this study thus far, at which stage of the APP trafficking pathway Mint1 acts. From the literature we know that APP interacts with the YENPTY motif in the C-terminus of APP and that this motif is essential for APP endocytosis (Perez *et al.* 1999). With this in mind I hypothesise the effect of Mint1 on APP trafficking seen in figures 3.2 and 3.3 occurs after APP is trafficked to the plasma membrane.
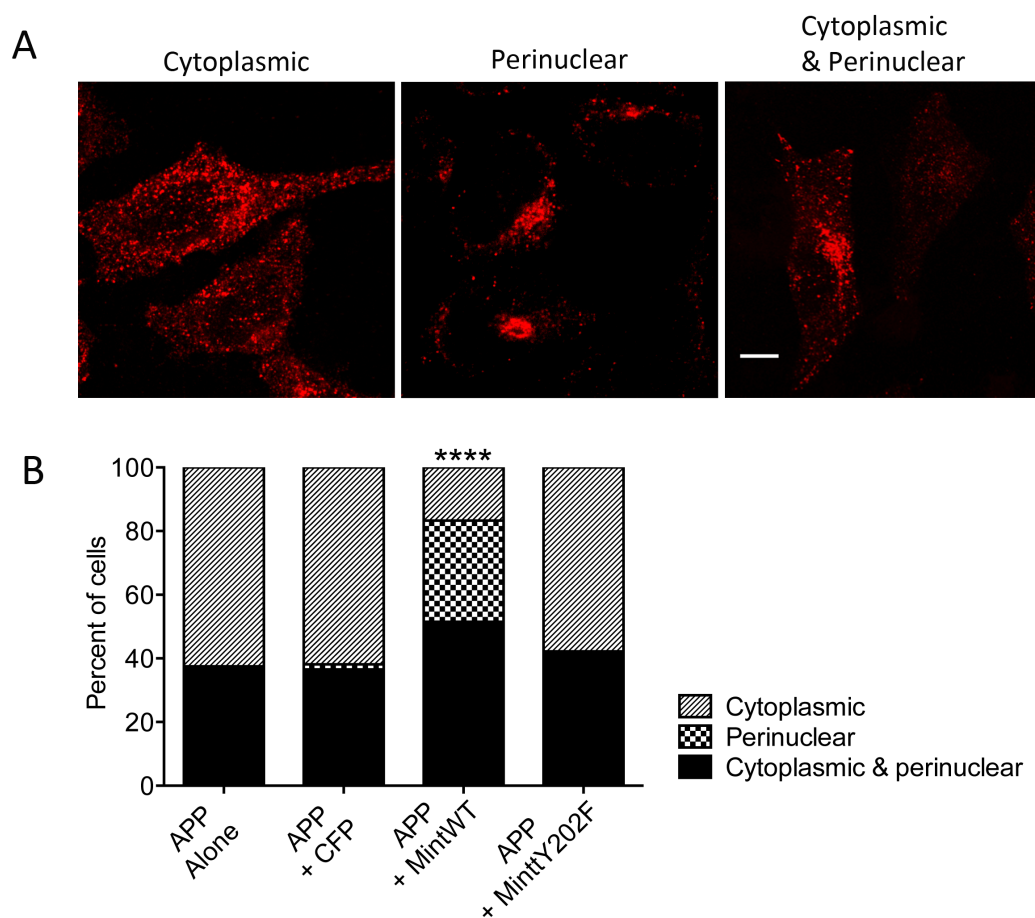
Figure 3.3 – Mint-WT increases the proportion of perinuclear localised APP, in a phospho-dependent manner. Phenotypic analysis was carried out on APP-HeLa cells that had been transiently transfected with Mint1 constructs and APP expression induced for 48 hours. **A)** Representative images of the three phenotypes of APP localisation analysed. Scale bar represents 10 μm. **B)** Quantification of the representation of each phenotype, normalised to the percentage of cells analysed. Between 45-60 cells were analysed for each treatment across three separate coverslips. Chi squared statistical analysis was carried out, * indicate APP + MintWT was highly significantly different to other coditions.

3.2: Mint1 recruits APP to the perinuclear region after APP has been trafficked through the cytoplasm.

Thus far this study has shown that overexpression of Mint-WT in a stable, APP expressing HeLa cell line, recruits APP to the perinuclear region in a phospho-dependent manner (Figures 3.2 and 3.3). However, it is unclear at which stage in the trafficking cycle APP is arrested. In order to address this, a time course experiment was carried out. It was first established, by Western blot analysis of APP-HeLa cell lysates, how early APP could be detected following doxycycline induction of the APP-HeLa cell line. APP-HeLa cells were cultured in media containing 1 µg/mL doxycycline and lysed 0, 3, 6, 12 and 24 hours after the addition of the doxycycline. Figure 3.4A shows APP can begin to be detected by Western blotting between 3 and 6 hours after doxycycline induction. The blot also shows that the APP doublet band seen on previous Western blots (Figure 3.1B) is only detected after 12 hours, this indicates that post-translational modifications don't occur immediately after APP expression.

Using the same time points, I examined whether the cellular localization of APP changed over time when over expressing Mint1. Cells were first transfected with Myc-tagged Mints and APP expression induced after 24 hours using 1 µg/mL doxycycline. Figure 3.4B shows representative images of APP and Mint expression in APP-HeLa cells. The images from 3, 6 and 12 hours show a fairly even distribution and amount of APP across all conditions. However, at 24 hours the distribution of APP in cells over-expressing Mint-WT looks considerably different to that seen in APP and APP + MintY202F cells. The number of APP containing particles is quantified in Figure 3.4C. APP alone and APP + Mint-WT show particle numbers of no significant difference to each other (all Adjusted P values >0.999), however, at 24 hours the number of APP containing particles in APP alone cells is around 2 fold higher than the number of APP containing particles in APP + Mint-WT cells (Mean number of APP containing particles 281 and 142 respectively, Adjusted P value <0.001). Although following the same trend as the number of particles in APP alone cells, cells overexpressing Mint-Y202F show particle numbers

considerably higher at all time points, with the most significant being at 24 hours, where the number of APP containing particles in APP + Mint-Y202F cells is 1.5 fold higher that the number seen in APP alone cells, and 2.8 fold higher than that seen in APP + Mint-WT cells (Mean number of APP containing particles in APP + Mint-Y202F 407) (Figure 3.4C).
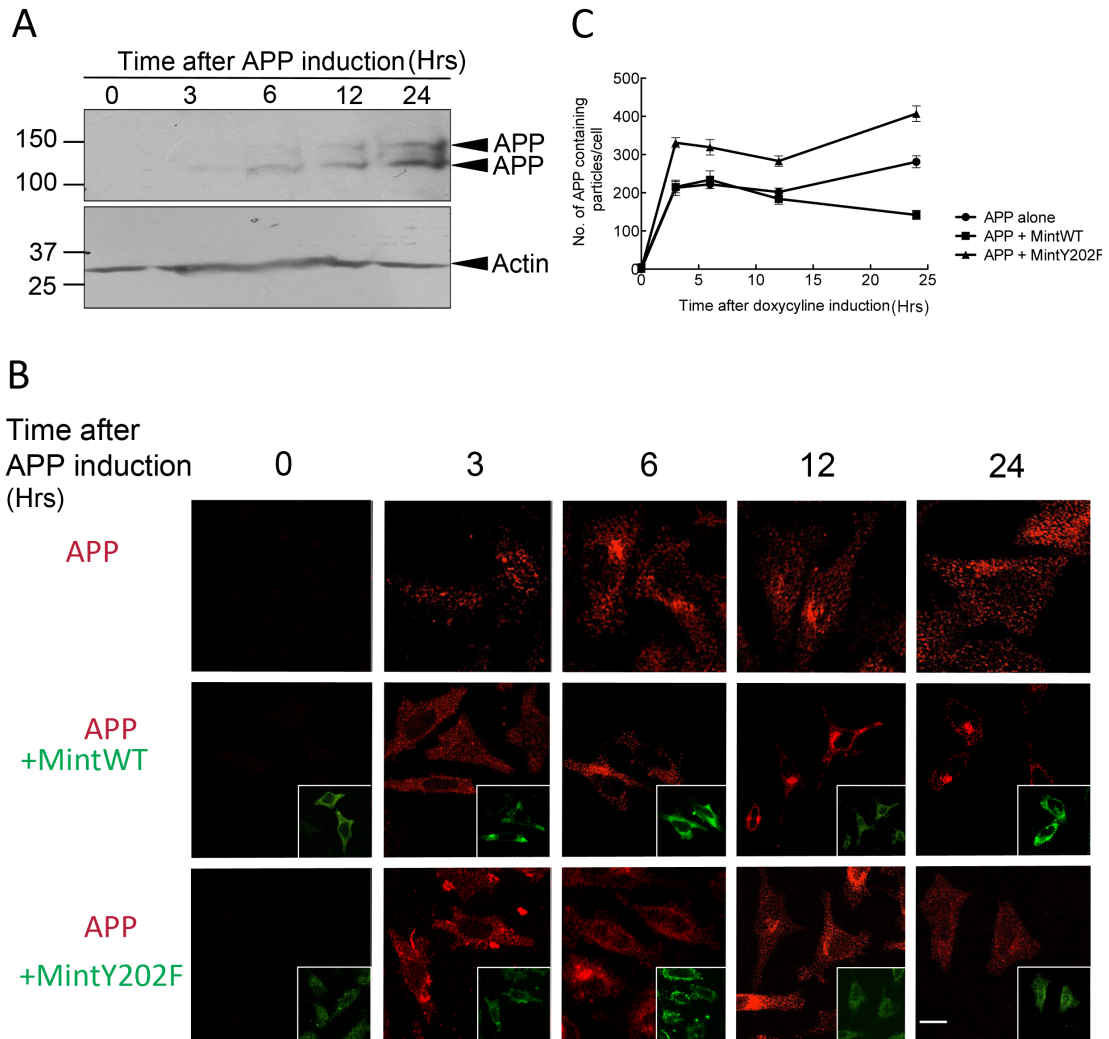
Figure 3.4 – Mint1 recruits APP to the perinuclear region once the protein has been trafficked through the cytoplasm. 24 hours after transfection with Mint1 constructs in APP-HeLa cell lines APP expression was induced. Cells were fixed or lysed at fixed time points following induction. **A)** Representative images showing the localisation of APP 0, 3, 6, 12 and 24 hours after APP induction. Cells transfected with Mint-WT show APP is internalized after 6 hours and held, mainly in the perinuclear region, whereas cells expressing APP alone or Mint-Y202F show APP moving towards the perinuclear region before being recycled back towards the plasma membrane. **B)** Western blot analysis showing APP can be detected around 6 hours following induction. Actin is used as a loading control. **C)** Quantification of the number of APP containing particles per cell. The number of particles in cells expressing Mint-WT decreases significantly after 12 hours when compared to cells expressing Mint-Y202F or APP alone. Between 15 and 40 cells from three separate coverslips were analysed for each condition and time point across three different cover slips. Error bars show the standard error of the mean.

Further quantification of the distribution of APP over time was carried out using phenotypic analysis as previously described for Figure 3.3. Figure 3.5A shows representative images for each phenotype. There was no statistical difference in APP localisation between conditions at 3, 6 and 12 hours (All P values >0.1, α value=0.0166). Under all conditions APP was mainly characterized as being cytoplasmic at 3 hours, whereas at 6 hours APP localisation is evenly divided between cytoplasmic and cytoplasmic & perinuclear. However, after 12 hours APP distribution in cells expressing Mint1-WT becomes increasingly perinuclear, whereas in those expressing Mint1-Y202F and APP alone the distribution of APP returns to being mostly cytoplasmic. 24 hours after APP induction, cells expressing Mint1-Y202F and APP alone show phenotypes similar to the distribution of APP at 3 hours, with 90 % and 78 % of APP respectively being cytoplasmic (Figure 3.5B). In contrast to this, 26 % of cells expressing Mint1-WT are characterised as having a perinuclear phenotype with respect to APP distribution (At 24 hours P=<0.0001 when APP + MintWT was compared with other conditions). Data from figures 3.4 and 3.5 indicate that the phosphorylation state of Mint1 affects APP localisation after internalisation of the protein.
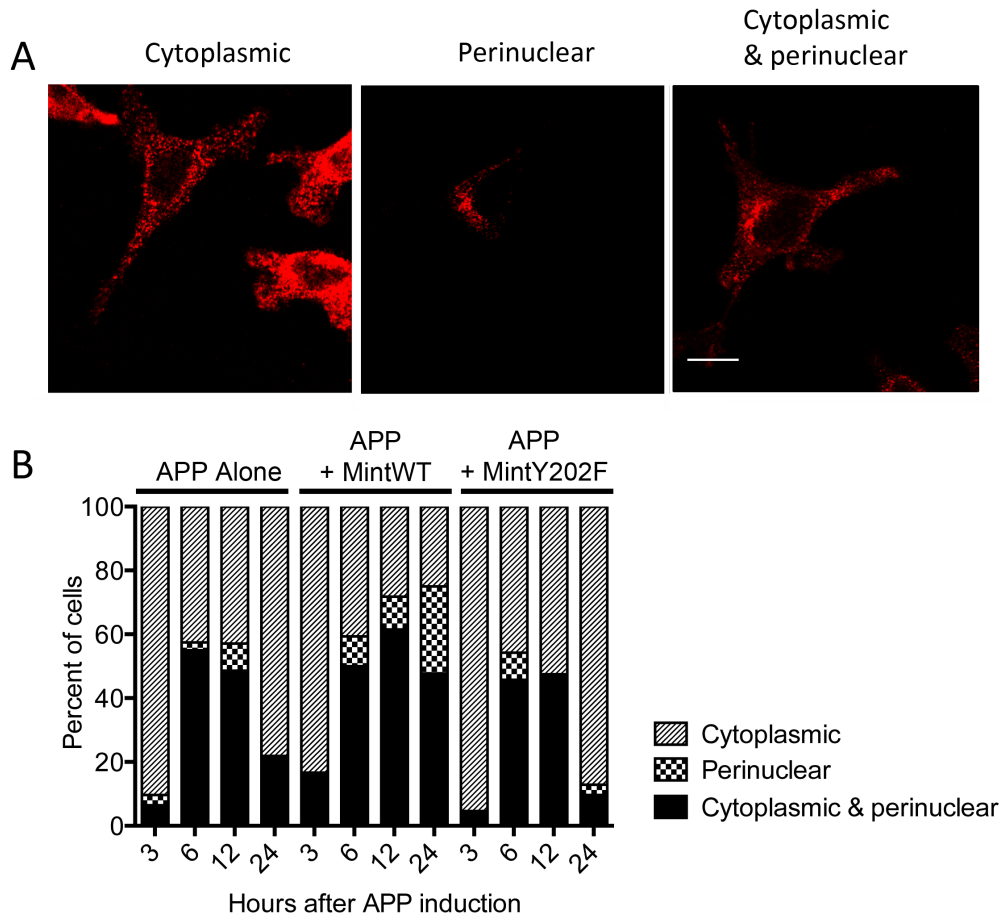
Figure 3.5 – The phosphorylation state of Mint1 alters the subcellular distribution of APP after 12 hours. APP expression was induced in HeLa cells transiently expressing Mint1 constructs, cells were fixed at intervals and APP distribution scored according to three phenotypes. **A)** Representative images of phenotypes. **B)** Quantification of the proportion of cells for each of the three phenotypes characterised. The phenotypes are similarly represented up to 6 hours for each treatment. However, after 6 hours post-APP induction, the proportion of perinuclear and cytoplasmic & perinuclear APP rises in cells overexpressing Mint1-WT, whereas the proportion of cells with cytoplasmic APP increases in cells with no Mint1 or Mint1-Y202F. Between 30 and 60 cells were analysed for each condition from three separate coverslips. Pair wise chi squared statistical analysis, with Bonferroni correction, was carried out for each condition.

All images were collected and figures compiled using the same magnification and objective. Figure 3.4A shows cells expressing APP alone appear to be larger than those expressing Mint1. Initially it was thought this size difference observed between cells not expressing Mints and those which do was a significant effect brought on by Mint1. However, from analysing the area of cells that had been transfected with Mint1-WT, Mint1-Y202 or CFP before APP expression was induced, no significant difference was observed (data not shown). The apparent size difference seen between APP alone cells and those expressing Mint1 was therefore attributed to the effect of transfection alone as APP only cells have not been transfected, this observation highlights the need for an empty plasmid control.

3.3: N1-Src phosphorylates N-terminal regions of Mint1

Previous data from the laboratory has shown that Mint1 is phosphorylated by C-Src (Figure 1.4). Mint1 is a neuronal specific protein, and pathogenic processing of APP only occurs in neurons, therefore the neuronal splice variant N1-Src was examined for it's ability to phosphorylate Mint1 in order to establish whether the neuronal kinase is more likely to be the neuronal Mint1 kinase isoform. Figure 3.6 shows a Western blot an *in* vitro kinase assay comparing the phosphorylation of varying concentrations of the Mint1 N-terminal peptide with both N1- and C-Src. Also shown, is the sequence structure of the phospho-box region of Mint1 used in kinase assays (Figure 3.6A). Highlighted are the three tyrosine residues that can be phosphorylated processively. Evidence from the lab shows that phosphorylation is dependent on the canonical YEEI SH2 binding motif. From initial experiments, using the Mint1 1-314, it was assumed there was an additional PXXP motif (a known SH3 binding motif) in the N terminal of Mint1. However, the fact that the short phospho-box peptide, which contains no PXXP motif, shows a similar discrepancy between C- and N1-Src phosphorylation suggests there are additional factors in the preferential binding by N1-Src. In order to draw firm conclusions from the assay the experiment would need to be repeated numerous times in order to establish the kinetics of the reaction. It is also

notable that clear loading controls to demonstrate the equal concentrations of Src kinases would be needed in future experiments.

A   183 EDEP$\underline{Y}$AEPYAD$\underline{Y}$GGLQEHV$\underline{Y}$EEIGDA 208
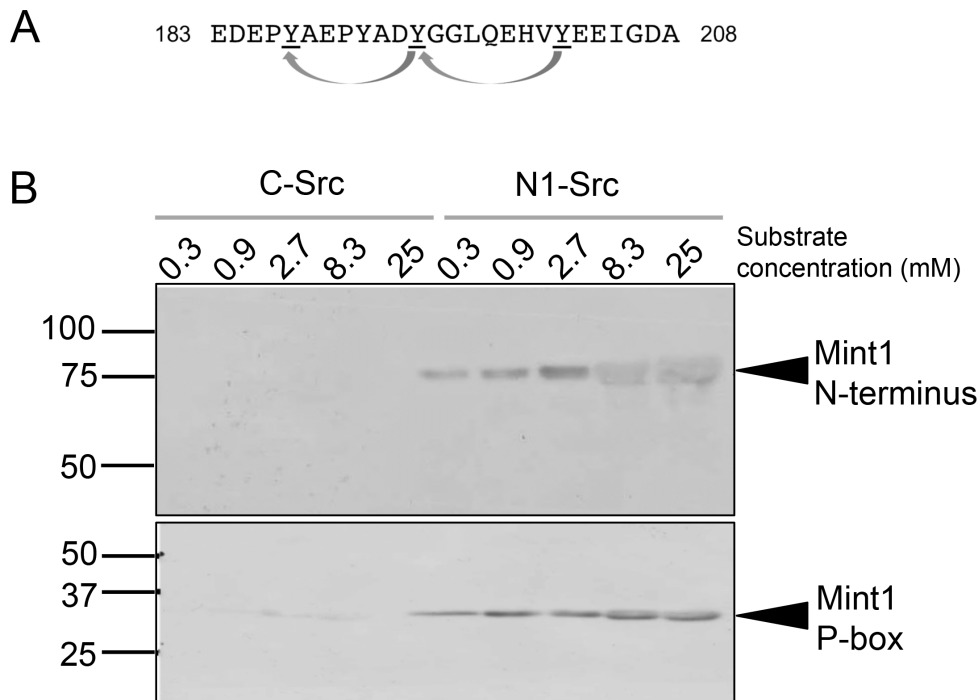
Figure 3.6 – N1-Src phosphorylates Mint1 N-terminal regions. **A)** The 'phospho-box' region of Mint1, amino acids 183-208. Underlined are the three tyrosine residues on which Mint1 is phosphorylated. These residues are processively phosphorylated in the order indicated by the arrows. **B)** *In vitro* kinase assays of Mint1 N-terminus (1-341, top panel) and phospho-box (183-208, bottom panel). Varying concentrations of Mint1 N-terminal regions were incubated with C- or N1-Src in the presence of ATP for 90 minutes. Phosphorylation of the peptides was visualised using an anti-phospho tyrosine (PY20) antibody. Kinase concentrations were all at 5 nM. Smeared bands seen with high concentrations of N1-Src are likely due to photo bleaching from high signal. Figure is missing positive controls.

3.4: N1-Src increases the accumulation of perinuclear APP when in the presence of Mint1, independent of tyrosine phosphorylation at Y202.

In vitro data (Figure 3.6) suggests N1-Src may have a higher affinity for Mint1 than C-Src. I therefore went on to examine the effect of overexpression of N1-Src on the process of Mint1 mediated APP trafficking. I hypothesised the overexpression of N1-Src in cells expressing APP and Mint1-WT will increase the interference of APP trafficking seen in figures 3.2-5. To test this I utilized a stable, inducible Flp-In N1-Src-HeLa cell line, which had been produced in the laboratory by Phillip Lewis. N1-HeLas were transfected with YFP-fusions of APP plus CFP-fusions of Mint1 proteins and N1-Src expression induced, where required, 24 hours later. After 48 hours of N1-Src induction cells were fixed and imaged.

Figure 3.7A shows illustrative images of N1-HeLa cells. The effect of N1-Src on APP localisation has been quantified in Figure 3.7B. The number of APP particles was analysed for each condition, from the quantification of the number of APP containing particles its appears that when APP and Mint1 are expressed along with N1-Src the accumulation of APP in the perinuclear region increases when compared to APP and Mint1 alone. In cells lacking N1-Src expression, Mint1-WT expressing cells had 47 % fewer APP containing particles than those expressing Mint1-Y202F (Mean particle number/cell 229 and 430 respectively, Adjusted P value 0.0266). Similarly, in cells expressing N1-Src, Mint1-WT cells exhibited 56 % fewer APP vesicles than Mint1-Y202F cells (Mean particle number/cell 113 and 263 respectively, Adjusted P value 0.0044)   (Figure 3.7B). This demonstrates that inhibiting Mint1-Y202 phosphorylation has the same effect in cells over-expressing N1-Src as seen previously in APP-HeLa cells. Importantly however, cells expressing Mint1-WT + N1-Src had 50 % fewer APP containing particles than cells expressing Mint1-WT without N1-Src (Adjusted P value 0.0251). On a similar note, Mint1-Y202F + N1-Src expressing cells had 40 % less APP containing particles than cells expressing Mint1-Y202F with no N1-Src (229 and 430 respectively, this difference was found to be not significant) (Figure 3.7B). Importantly, this effect is not seen to be dependent on phosphorylation at Y202. The

expression of N1-Src is seen to cause a similar decrease in cells expressing both Mint1-WT and the phospho-resistant mutant Mint1-Y202F. This suggests an additional level of regulation of APP trafficking and processing.
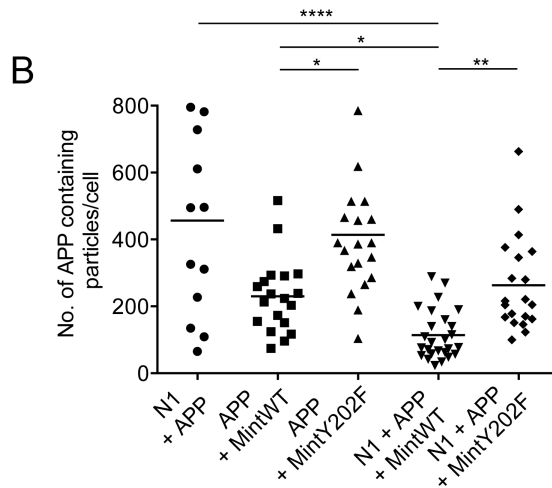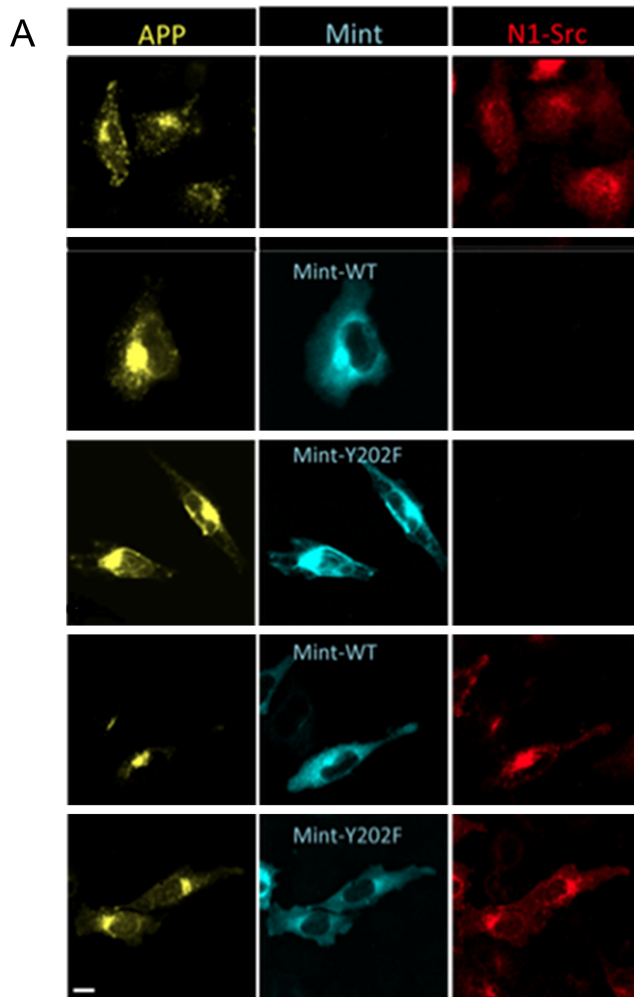
Figure 3.7 – N1-Src increases the accumulation of perinuclear APP when in the presence of Mint1, in a Y202 phospho-independent manner. N1-Src-HeLa cells were co-transfected with APP ± Mint1 constructs, prior to N1-Src induction for 48 hours. **A)** Representative images showing YFP-APP (yellow), CFP-Mint1 (blue) and FLAG-N1-Src (red) expression and localisation in HeLa cells. **B)** Quantification of APP particle number in N1-Src-HeLa cells. 12 – 26 cells from three different coverslips were analysed for each condition. Statistical significance was calculated using one-way ANOVA with a post-hoc Tukey test, stars denote statistically significant means. Scale bar= 10μm

3.5: Mint1 recruits N1-Src to the perinuclear region, in a Y202 phospho-independent manner in HeLa cells

Data from figure 3.7 suggests Mint1 and N1-Src work to recruit APP to the perinuclear region through a mechanism independent of Mint1 phosphorylation at Y202. In order to further study this mechanism, the cellular localisation of N1-Src in the HeLa cells was examined. Srcs are membrane-associated kinases usually expressed throughout the cell and have seen to be associated with endosomes and the TGN (Kaplan *et al.* 1992). Phenotypic analysis was carried out in order to quantify the change in N1-Src localisation (Figure 3.8). It was found around half of N1-Src under all conditions is distributed in both the perinuclear region and the cytoplasm. However co-expression of N1-Src and APP with either Mint1-WT or Mint1-Y202F significantly increases the proportion of cell in which N1-Src is localised specifically perinuclearly to 42 % and 35 % respectively compared to 5.3 % in cells lacking Mint1 (Figure 3.8B). Notably, this effect does not appear to be dependent on APP or Y202 phosphorylation. Cells expressing N1-Src and either of the Mint1 constructs, but not APP, also showed that N1-Src was localised to the perinuclear region in 25 % of cells (Figure 3.8B). Statistical analysis of the data, using chi squared tests with Bonferroni correction, shows that N1-Src localisation differs greatly between N1 alone or N1 + APP cells and those expressing N1-Src, APP and Mint1-WT or -Y202F (P=<0.0001 for each pairwise comparison, adjusted α= 0.0033). Similarly, cells expressing N1-Src ± APP show significant difference to those expressing N1-Src + Mint1-WT or –Y202F (P=0.003 and 0.002 respectively) but to a lesser extent. Although the change in N1-Src localisation is not as pronounced in cells expressing Mint1 in the absence of APP, there is still a noticable increase in the proportion of perinuclear N1-Src.
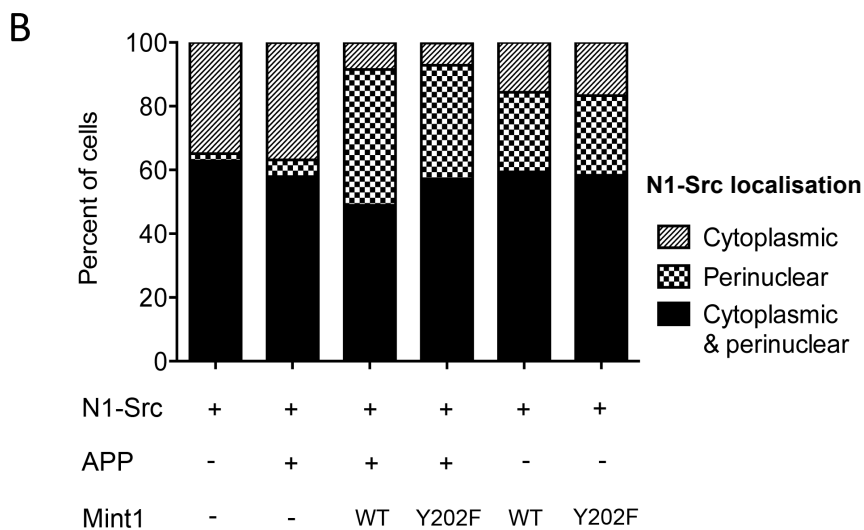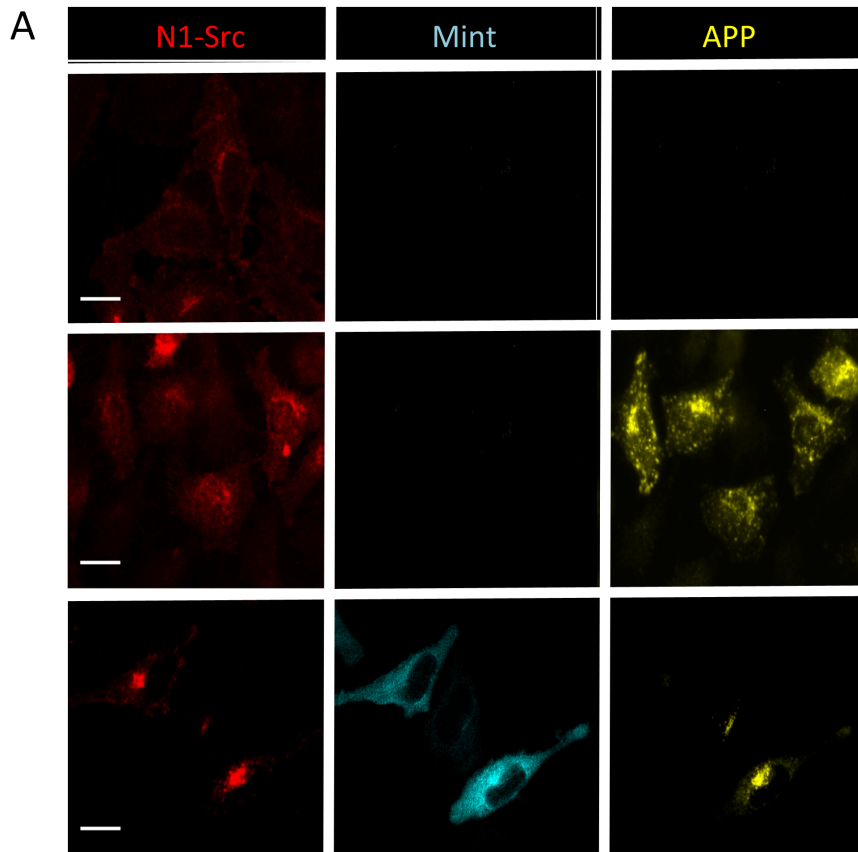
Figure 3.8 – Mint1 recruits N1-Src to the perinuclear region in a Y202 phospho-independent manner. N1-HeLa cells we co-transfected with CFP-Mint1 and YFP-APP constructs before N1-Src expression was induced. Cells were fixed and stained after 48 h of N1-Src induction. **A)** Representative images of N1-HeLa cells expressing combinations of Mint1, APP and N1-Src. Images show examples of each of the three phenotypes of N1-Src localisation; cytoplasmic (top), cytoplasmic and perinuclear (middle) and perinuclear (bottom). Scale bars= 10 μm **B)** Quantification of the change in N1-Src phenotypes observed. Data showed as the % of cells analysed displaying each phenotype. Between 25-50 cells were analysed for each condition from three different coverslips.

3.6: Co-expression of Mint1 and N1-Src stabilises APP and leads to a change in YFP-APP fragment size

Following the observations that N1-Src increases accumulation of APP in the perinucler region, and that Mint1 recruit N1-Src to the perinuclear region in an N1-HeLa cell line, I then investigated the effect of overexpression of Src on APP expression levels in the cell line. A C-Src HeLa cell line was also available in the laboratory (also prepared by Philip Lewis) and so I compared expression levels of APP, Mint1 and Src in both N1- and C-Src HeLa cell lines (Figure 3.9). Src-HeLa cell lines were transfected with YFP-APP and myc-Mint1 constructs, before Src expression was induced by incubation with 1 µg/mL doxycycline for 48 hours. Analysis by Western blot shows APP levels increase with the co-expression of N1-Src and Mint1, irrespective of Mint1 phosphorylation state. Cells co-expressing Mint1-Wt or Mint1-Y202F and N1-Src showed a significant increase in YFP-APP signal at between 110-150 kDa. The presence of the heavier APP band at 140 kDa seen in cells co-expressing N1-Src and Mint1 isn't present in samples not expressing the kinase (Figure 3.9A). This suggests the presence of N1-Src may result in increased post-translational modifications of APP. The increase of APP expression seen in N1 expressing cells is not observed in those co-expressing C-Src and Mint1. Co-expression of Mint1 and APP in C-Src HeLas results in the presence of an YFP-APP band just above the original at 130 kDa. This suggests that expression of Mint1 increases some posttranslational modification of APP, and co-expression of Mint1 (Figure 2.9B), APP and N1-Src further increases this causing the protein to run at 140 kDa. In addition to affecting full length APP, the expression of Mint1 and N1-Src also causes a change in molecular weight of lower mass YFP-APP fragments from 30 kDa to 25 kDa (Figure 2.9A, second panel). This shift is not observed when APP + Mint1 are expressed in the presence of C-Src (Figure 2.9B, second panel). Curiously, although the effect seen differs between the ubiquitous C-Src and neuronal N1-Src the effect does not appear to be Y202 phosphorylation dependent as overexpression of both Mint1-WT and Mint1-Y202F alter APP expression levels and size of APP fragments detected in the presence of N1-Src. These observations, coupled with previous the previous observations of

N1-Src and APP localisation (Figures 2.7 and 2.8) suggest the accumulation of APP and N1-Src, most likely in the TGN, causes a change in APP modification, stabilisation and processing.
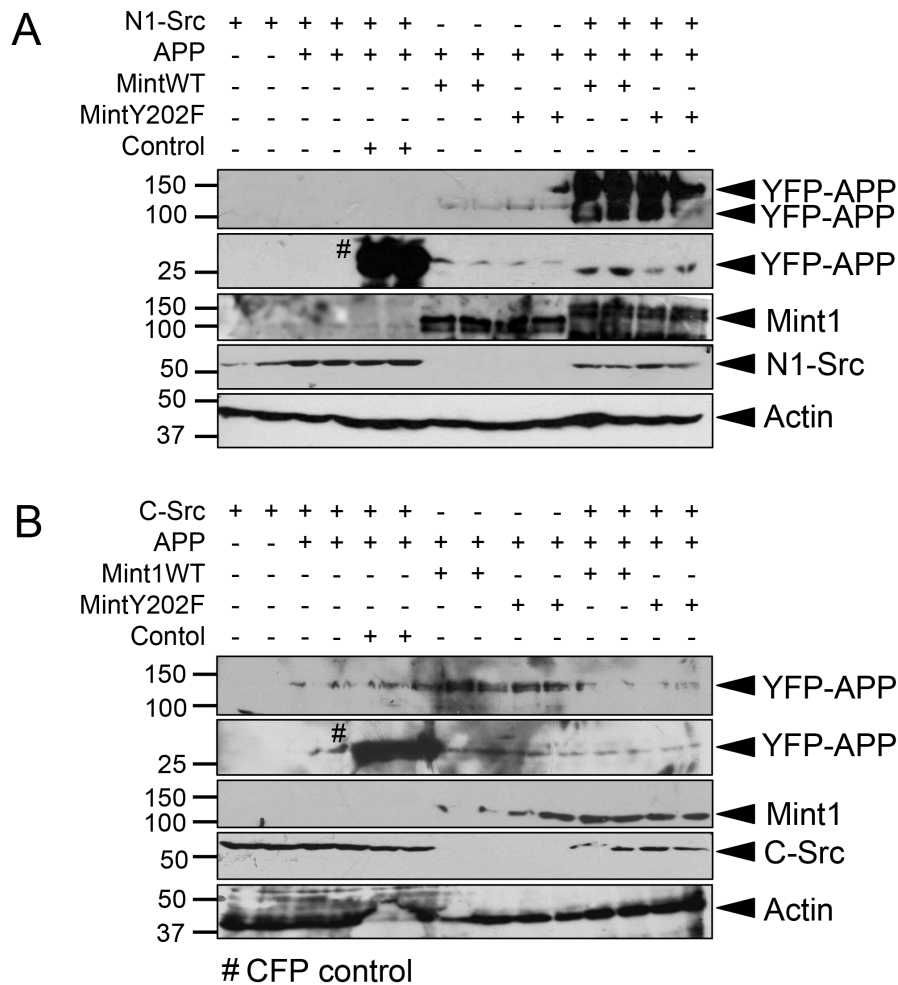
Figure 3.9 – N1-Src stabilises full length APP and results in a change in the mass of YFP-APP fragments detected. N1- or C-Src HeLa cell lines were transfected with Myc-Mint1, YFP-APP and Src kinase expression induced for 48 hours before lysing. Samples were analysed by Western blotting and probed with α-Myc (Mint1), α-GFP (YFP-APP +CFP-control), α-FLAG (Src) and α-actin. **A)** Western blot analysis of N1-HeLa cell lysates. YFP-APP (top panel) levels are seen to increase when Mint1 and N1-Src are co-expressed (lanes 11-14). A lower molecular weight YFP-APP fragment is also seen when co-expressed with Mint1 and N1-Src. This fragment is most-likely the C-terminal end of APP fused with the YFP fluorophore (Second panel lanes 7-14). **B)** C-Src HeLa cells were transfected with Mint1 and APP as above. Unlike when co-expressed with N1-Src and Mint1 YFP-APP levels are not seen to increase. The presence of the APP doublet seen in previous experiments was detected with co-expression of Mint1 and APP, however the intensity of the YFP-APP band does not change upon expression of C-Src. Similarly, no change is seen in the molecular weight of smaller YFP-APP fragments. All conditions are shown in duplicate. Actin was used as a loading control, whilst CFP was used as a transfection control replacing CFP-fusion Mint1 constructs (represented by second panel in A and B lanes 5 and 6).

4: Discussion

It is well documented that the neuronal adaptor protein Mint1 binds to and alters the trafficking and processing of APP (Borg *et al.* 1998; King *et al.* 2003; Miller *et al.* 2006). Although the mechanisms behind this regulation were previously unclear, recent studies have highlighted a number of regulatory mechanisms in both the C- and N- terminal ends of Mints 1 and 2 (Chaufty *et al.* 2012; Matos *et al.* 2012; Xie *et al.* 2012). In addition, recent observations in the laboratory have identified a further regulatory mechanism in the Mint1 N-terminus via Src mediated tyrosine phosphorylation (Dunning *et al.,* manuscript in preparation). In this study I further examined the regulation of APP trafficking by Mint1 and N1-Src. I have shown that abolition of Mint1 phosphorylation by Src at Y202 disrupts the APP trafficking cycle, most likely after internalisation, in a stable, inducible APP-HeLa cell model (Figures 3.1-3.5). In addition to this it has been observed that APP and N1-Src are recruited to the perinuclear regions of cells in the presence of Mint1 in an apparent phospho-independent mechanism suggesting that N1-Src is the preferential isoform of the kinase rather than c-Src (Figures 3.6-3.9).

4.1: Src phosphorylation of Mint1-Y202 regulates APP trafficking, most likely after internalistation

By utilizing a stable, inducible APP expressing HeLa cell line I examined the effect of overexpressing both Mint1-WT and the phospho-resistant mutant Mint1-Y202F. It was seen that after 48 hours of Mint1 and APP expression, a large proportion of APP was recruited to the perinuclear region. However, when overexpressing Mint1-Y202F the majority of APP was seen to be cytoplasmic (Figures 3.2 and 3.3). As specific cellular localisation markers weren't used in this study I cannot categorically identify the compartments in which APP is localised. From studying the literature it has been shown that APP and Mint colocalise in the TGN (Borg *et al.* 1996; Sastre *et al.* 1998; Biederer *et al.* 2002) I therefore hypothesize that the accumulation of APP observed in this study would also show APP mainly localizing to the TGN

when in the presence of Mint1-WT. However, it would be interesting and informative to examine whether this is the case and also to monitor APP localisation in endosomes, and more importantly which endosomes using specific subcellular markers.

In addition to this time course experiments, exploiting the inducible nature of the Flp-In HeLa cell system, demonstrated that mutation of Y202 disrupted APP trafficking between 6 and 12 hours after doxycycline induction of the protein (Figures 3.4 and 3.5). These results suggest that the phosphorylation of Mint1 at tyrosine residues 202, 191 and 187 by Src kinase is an important regulatory step in APP trafficking, and therefore processing. This regulatory mechanism is similar to that seen to regulate the Mint2-APP interaction in a study by Chaufty *et al.* Whilst this study also observed an increase in APP internalisation with Mint2 phosphorylation they observed very different subcellular localisation of the proteins involved.

It was also recently found that Mint proteins may play a role in the insertion of APP into the plasma membrane (Chaufty *et al.* 2012). This study showed that Mint knock-out neurons showed a significant decrease in the amount of APP which had been newly inserted into the membrane. Data from time course experiments in this study has shown that the APP trafficking cycle is disrupted following internalisation of APP (Figure 3.4). APP in cells overexpressing the Mint1-WT is seen to remain localised to the perinuclear region whereas over-expression of Mint1-Y202F allows APP to be trafficked back to the plasma membrane. It is possible that phosphorylation of Mint1 at these residues either increases endocytosis of APP or may decrease the reinsertion of APP into the membrane following endocytosis. Data from this and previous studies suggest it may regulate both processes. Previous studies carried out in the lab found that Mint1-WT causes APP to stabilise in COS7 cells, although this result is not seen in HeLa cells in this study unless also expressed in the presence of N1-Src, this may be due to increased endocytosis and decreased insertion of APP into the membrane causing it to accumulate in the cell.

4.2: The phosphorylation state of Mint1-Y202 alters APP and N1-Src localisation.

Here I have shown that APP accumulates in the perinuclear region when Mint1-WT is expressed and this phenotype isn't seen in cells expressing Mint1-Y202F. Chaufty *et al.* report that both APP and Mint2 localise predominantly in autophagic vesicles when phosphorylated whereas expression of phospho-resistant Mint2 results in APP and Mint2 localising to the perinuclear region (Chaufty *et al.* 2012). Interestingly, in many studies examining the effect of Mint proteins on APP processing and trafficking, the two isoforms are used interchangeably and previous differences in results have been attributed to varying expression levels (Ho *et al.* 2008). Although the proteins appear to have the same affect on the overall levels of APP and processing of APP, comparisons between this study and the one carried out by Chaufty *et al.* suggest that while the end products may not differ, the mechanisms by which Mint1 and 2 regulate APP and the trafficking pathways involved differ significantly. This suggests the two proteins have distinct roles in regulating trafficking of the protein and moreover that these roles are regulated by the N-terminus, as this is the region in which the proteins differ (Rogelj 2006).

In addition to the change in APP localisation observed in this study, a change in N1-Src localisation was observed when the kinase was co-expressed with Mint1. Curiously, the accumulation of N1-Src in the perinuclear region appears to be phospho-independent (Figure 3.8). Previous work had been carried out in the laboratory to assess whether this effect was seen with C-Src with no success (data not shown). These observations, coupled with the *in vitro* kinase assay and HeLa cell lysate data from this study (Figures 3.6 and 3.7), strongly suggest that N1-Src is the preferential Src kinase isoform for Mint1 phosphorylation at this site and that the kinase has specific effects on APP trafficking and processing. There have been numerous theories as to why Aβ plaques only form in the brain and not in other tissues as APP is expressed ubiquitously (Gotz *et al.* 2011). One of the factors thought to

influence this is the specialised trafficking pathway APP goes through in neuronal cells. The observations shown in this study indicate that the specificity of neuronal isoforms of proteins, Mint1 and N1-Src, add to neuron specific regulatory pathways and might explain why APP is only pathologically processed in the brain.

4.3: The potential affect of Mint1 phosphorylation on Aβ accumulation

Previous studies have shown that the majority of Aβ-40 is produced at the plasma membrane (Skovronsky *et al.* 2012), with an increase in APP internalisation correlating with an increase in toxic Aβ-42 production (Claeysen *et al.* 2012). It is therefore possible to hypothesize that disruption of Mint1-Y202 phosphorylation by Src would also correlate with a decrease in Aβ-42 production, this effect would need to be assessed in further studies. However, it would also be important to quantify the amount of APP in the cells. The distribution of APP in cells overexpressing Mint1-Y202F is mainly cytoplasmic so it is possible that it is present in endosomal vesicles that have been described as the compartment where the majority of Aβ-42 cleavage occurs (Ferreira *et al.* 1993; Vassar *et al.* 1999). On the other hand overexpression of Mint1-WT, which can be phosphorylated, results in the accumulation of APP in the perinuclear region. Previous studies, using both cell and animal models, have suggested that intracellular Aβ accumulation is more cytotoxic than extracellular plaque deposits (Koistinaho *et al.* 2001; Yu *et al.* 2004; LaFerla *et al.* 2007). Therefore the accumulation of APP in the perinuclear regions may result in a build up of highly toxic intracellular Aβ. For this reason a potential treatment for AD may be to disrupt the Mint1-APP interaction by targeting Y202 phosphorylation and therefore allowing normal trafficking of APP from the cell. This coupled with previous conflicting evidence as to what specific effect Mint1 has on APP suggests that levels of Mint1 must be precise and any alterations in expression levels can affect APP processing.

4.4: The Mint1-N1-Src-APP interaction may alter C-terminal APP processing

Whilst it is known that APP is cleaved by α-, β- and γ- secretases, and these cleavage events and products have been widely studied, other APP cleaving sites have been identified. Lu and colleagues identified a short C-terminal peptide fragment of APP, named C31 (Lu *et al.* 2000). They found this fragment was produced as a result of caspases or a caspase-like protease at aspartic acid residue 664 (D664), and that the presence of this fragment has cytotoxic effects. Figure 3.9 shows the effect of APP levels and processing in cells overexpressing N1- or C-Src kinases. In cells co-expressing N1-Src, Mint1 and APP a change in the mass of the C-terminal YFP-tagged peptide is seen. The mass of the peptide changes from around 28 kDa to 25 kDa. It is possible this change is due to a shift in APP cleavage from the expected γ-secretase cleavage site, which would result in a YFP-C57 peptide having a mass of around 28 kDa, to the potential caspase cleavage site producing the shorter YFP-C31 fragment having a mass of around 25 kDa. Figure 4.1 shows a schematic of the two C-terminal products and their masses. The 3 kDa difference between the two peptides is representative of the shift seen in Figure 3.9, thus making it feasible that the presence of N1-Src and Mint1 results in significant changes in APP processing.
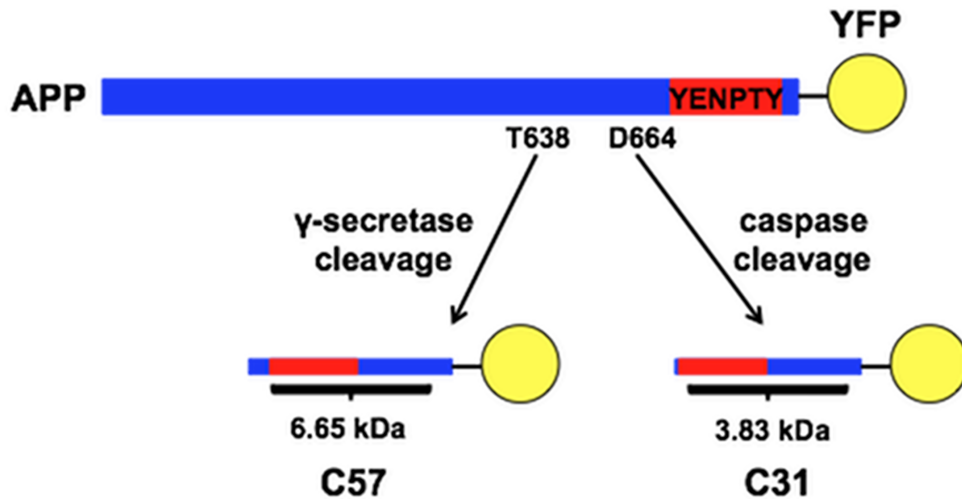
Figure 4.1 – Diagram showing the alternative cleavage sites and products of the APP C-terminus. APP can be cleaved either at threonine 638 by γ-secretase to produce the longer C57 fragment. Alternatively caspase can cleave APP at aspartic acid residue 664 to produce the shorter C31 fragment. The presence of these differential cleavage sites may explain the change in mass of YFP-tagged C-terminal APP seen in Fig 3.9 upon cotransfection of APP with Mint1 and N1-Src.

One study noted that production of the C31 fragment curiously appeared to rely on the presence of full length APP. They proposed that dimerisation of full length APP triggered this alternative cleaving mechanism, resulting in cytotoxic C31 production (Park *et al.* 2009). Taking these studies in to consideration, one possible explanation for the shift in C-terminal APP processing is that the presence of N1-Src and Mint1 causes APP to dimerise, thus stabilising the protein and explaining the increased levels of full length APP seen in the N1-HeLas but not in the APP-HeLas and, in turn, causing a change in the C-terminal cleavage site. Significantly, neither the increase in full length APP or the shift in C-terminal APP fragment size is seen in C-Src HeLa cells, further implying that N1-Src plays a more important role in APP trafficking and processing.

Notably this effect does not appear to be dependent on Y202 phosphorylation as it is seen in cells expressing both Mint1-WT and Mint1-Y202F. Whilst this observation indicates that the change observed does not rely on phosphorylation of Mint1 at Y202, it is possible that N1-Src kinase

phosphorylates Mint1 or APP at a different location in the protein and that this site of phosphorylation is specific to N1-Src, most likely as a result of the amino acid inset in the SH3 domain. As multiple phosphorylation sites on both APP and Mint1 have been identified (Borg *et al.* 1996; Chang *et al.* 2006; Sakuma *et al.* 2009; Chaufty *et al.* 2012), it is reasonable to suspect that N1-Src may phosphorylate a number of residues on either protein. This again complicates the mechanisms by which APP trafficking and processing is regulated by adding yet another layer of transient post-translational modification in order to influence the fate of the protein. Importantly the change in APP cleavage products is only seen in cells co-expressing N1-Src, APP and Mint1 and is not seen in those cells expressing only N1-Src and APP. This suggests that the three proteins are interacting, possibly forming a complex to regulate APP processing and that the effects seen in figure 3.9 are both Mint1 and N1-Src dependent.

4.5: Regulation of APP adaptor proteins by tyrosine phosphorylation

Despite the volume of studies which have been carried out investigating how neuronal PTB adaptor proteins regulate APP, and notable recent advances in our understanding of these mechanisms, the overall picture of how the proteins regulate APP trafficking and processing and specifically how they work together to do this is generally unknown. However, it is apparent that phosphorylation is an important process in this regulation. Not only can APP be phosphorylated on multiple residues, so can the adaptor proteins. Mint1, for example, can be both N- and C-terminally phosphorylated (Sakuma *et al.* 2009; Chaufty *et al.* 2012; Matos *et al.* 2012). In addition to this it has been shown that the other PTB adaptor proteins can be regulated by tyrosine phosphorylation; Fe65 by Abl (Perkinton *et al.* 2004; Vazquez *et al.* 2009), Dab1 by Src and Fyn (Hoe *et al.* 2008; Minami *et al.* 2011) and JIP1 by JNK (Nihalani *et al.* 2007). As the proteins all bind to the same YENPTY motif of APP to regulate trafficking and processing of the protein, understanding the mechanisms behind regulating which adaptor protein binding is favoured under certain conditions may be a key step in understanding the triggers behind the development of AD. A study by Tamayev and colleagues in 2009

sought to investigate the role of phosphorylation in regulating the proteins that interact with APP (Tamayev *et al.* 2009). They investigated the effect of phosphorylating Y682, in general the study found that phosphorylation of this residue increased binding of SH2 domain containing proteins, but had no real effect on proteins containing a PTB binding domain. The observation that these PTB domain adaptor proteins bind to APP in a phospho-APP independent manner highlights the importance of third party regulation, likely by tyrosine phosphorylation, on regulating their interaction with APP. With this in mind it is likely that the adaptor proteins compete for binding with APP, with tyrosine phosphorylation, potentially along with other interactions, regulating which adaptor is bound.

4.6: Conclusions and future work

Earlier investigations into the effects of Mint proteins on APP in mice models have been largely conflicting. Studies investigating the effect of Mint knockdowns (Sano *et al.* 2006; Saito *et al.* 2008) and overexpression (Borg *et al.* 1998; Mueller *et al.* 2000; King *et al.* 2003; Parisiadou and Efthimiopoulos 2007) studies have reported both increases and decreases in Aβ production. The conflicts in these studies may be attributable to the different mice strains used, extent of knockdown or methods of overexpression and knockdown. It is likely that it is necessary to have the right levels of Mint proteins in order for them to correctly regulate their interaction with APP. It is important to remember there is a whole catalogue of proteins with which APP interacts, and specifically with the YENPTY motif (Tamayev *et al.* 2009) suggesting the protein has many functions and that disrupting it's interaction with one may affect the interaction of the protein with the others. It is therefore important to take data from both *In vitro*, cell and whole organism studies when attempting to elucidate the complex mechanisms of regulation involved.

Despite the conflicting results it is apparent that Mint proteins do play a significant role in regulating APP trafficking and processing. Evidence of altered Aβ accumulation in mice models suggests that the Mint1-APP could

be an important therapeutic target for treating AD. Whilst it is feasible that knockdown of Mint expression would reduce Aβ levels evidence from this study suggests that by targeting the Mint1-APP interaction specifically by disrupting Mint1 Y202 phosphorylation may be a more specific target therefore having fewer side effects on other Mint1 functions. Previous experiments in the laboratory have suggested that the effects of mutating Y202 is specific to the Mint1-APP interaction as trafficking of the NR2B subunit of the NMDA receptor, another Mint1 interacting protein (Setou *et al.* 2000), was not disrupted (Dunning *et al.*, manuscript in preparation). This could potentially be done via small molecule inhibitors or gene therapies.

All together, the number of mechanisms by which the Mint-APP interaction is regulated, and the fact that they are regulated by phosphorylation (a reversible process) suggests that, under physiological conditions, these regulatory mechanisms are transient and highly spatially and temporally regulated. The complexity produced my numerous regulatory systems, coupled with the difficulty of working with neuronal cells and the use of heterologous cell systems, may go some way to explain the contradictory results seen from previous studies looking at the Mint-APP interaction. Future studies should incorporate the findings of this and other studies together to elucidate the precise mechanisms behind the regulation of the Mint-APP interaction and to assess how the various forms of regulation work together in order to find potential treatment targets for the prevention of Aβ plaque accumulation in the brains of patients with AD.

This study has shed some light on the possible mechanisms behind the regulation of the Mint1 APP interaction. However, much future work would need to be done to fully elucidate the importance of Mint1-Y202 phosphorylation by Src. Initially it would be important to identify the cellular compartments in which APP is located when coexpressed with either Mint1-WT and Mint1-Y202F. Detailed knowledge of the cellular compartments in which APP and Mint1 are located would help to understand the effect on APP processing as the different APP cleaving enzymes are known to localise in

different compartments within the cell. Another key feature would be to assess the effect of Y202 phosphorylation on the production of both intra- and extracellular Aβ. This could be done using enzyme-linked immunosorbent assays (ELISA) as used in a number of previous studies (King *et al.* 2003; Sano *et al.* 2006; Ho *et al.* 2008). The effect of both N1-Src and Mint1 on APP trafficking would also need to be further assessed. Further co-localisation and Src knock out and recovery experiments would confirm whether the effects on N1-Src and APP trafficking seen in this study (Figures 3.6-3.9) were specific to these interactions. Given that the effects seen in N1-Src HeLa cells do not appear to be dependent on the phosphorylation of Y202 it would also be informative to screen the protein for further Src phosphorylation sites, most likely in the N-terminus of the protein. A number of further *In vitro* kinase and binding assays would allow the kinetics of N1- and C-Src phosphorylation of Mint1 to be elucidated as well as assessing whether the three proteins act to form a complex and in what ratio this may occur. As data from figure 3.9 suggests that the presence of N1-Src may alter C-terminal cleavage of APP in the presence of Mint1 it may be that N1-Src causes a change in the complex formed leading to possible dimerisation of APP resulting in a switch from γ-secretase cleavage to caspase cleavage of the protein.

Finally, it would be important to validate the results seen in the HeLa cell lines by assessing whether the same effects are seen in neuronal cell lines. As AD is a neuronal disease these experiments are critical before firm conclusions can be made about the potential of the Mint1-APP interaction as a target for AD treatment.

Definitions and abbreviations

°C	Degrees Celsius
α	alpha
$A\beta$	Amyloid beta peptide
AD	Alzheimer's disease
APP	Amyloid precursor protein
APS	Amonium persulphate
ATP	Adenosine 5'-triphosphate
β	beta
BSA	Bovine serum albumin
C-terminal	Carboxy terminal
CFP	Cerulium fluorescent protein
CMV promoter	Cytomegalovirus promoter
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
E. coli	Escherichia coli
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
γ	gama
g	Gram
GFP	Green fluorescent protein
GST	Glutathione S transferase
HCl	Hydrochloric acid
HEPES	2-[4-(2-Hydroxyethyl)-1-piperazine] ethanesulphonic acid
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IPTG	Isopropyl-β-D-thiogalactopyranoside
k	kilo (prefix)
L	Litre

μ	micro
m	milli (prefix)
M	molar
Mint	Munc18 interacting protein
NMDA	*N*-methyl-D-aspartate
N-terminal	Amino terminal
OD	Optical density
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulfonyl fluoride
RNA	Ribonucleic acid
rpm	Rotations per minute
SH	Src homology domain
TEMED	N, N, N', N'-tetramethylenediamine
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
Tween-20	Polyoxyethylene sorbitan monolaurate
YFP	Yellow fluorescent protein

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