FKBP14 in Notch Signalling and *Drosophila* Development

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

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Abstract

Presenilins (PSs) are highly conserved transmembrane proteins that form the catalytic core of the $\gamma$-secretase complex, which cleaves a growing list of transmembrane proteins including Notch and Amyloid Precursor Protein. Autosomal dominant mutations in the genes encoding human PS have been closely linked to cases of early onset familial Alzheimer’s Disease (FAD), and these FAD-linked PS mutations influence the activity of $\gamma$-secretase. The production and maturation of $\gamma$-secretase subunits are highly codependent, yet the mechanisms regulating assembly and activity of the complex are unclear. A screen in *Drosophila* identified *FKBP14*, an FK506-binding proteins (FKBP), as a genetic modifier of *Presenilin (Psn)*, with *FKBP14* mutants exhibiting defects typical of loss of Notch signalling. FKBP1s are highly conserved and involved in a wide array of biochemical processes including protein folding, assembly, and trafficking, yet they are non-essential in yeast and their role in development of multicellular organisms remains unclear.

The work presented in this thesis establishes a role for FKBP14 in multicellular development as a regulator of Notch signalling via Presenilin and the $\gamma$-secretase complex. I show that *FKBP14* mutants genetically interact with components of the Notch pathway, and that Psn-dependent $\gamma$-secretase cleavage, a process required for Notch signalling, is reduced in *FKBP14* mutants. I also
identify a Notch-independent role for FKBP14 in cell viability during *Drosophila* development and present data establishing that both the PPIase and EF hand domains of FKBP14 are required for its function. Altogether, these data demonstrate that FKBP14 plays a critical role in development, one aspect of which includes regulating members of the Notch signalling pathway. Investigation of the role of FKBP14 in Notch signalling via its interaction with *Psn*, and potentially broader roles in development, may provide a greater understanding of FKBP1s in multicellular signalling.
Acknowledgments

I suppose it won’t be a surprise to anyone who has had to write one of these that the acknowledgements was the last section written, and the words were difficult to find. I have so many people to thank that it has been overwhelming figure out where to begin. I’ve had a truly wonderful experience as a graduate student at the University of Toronto and the Hospital for Sick Children. The research community is collaborative, exciting, and inspiring. I hope to live up to the opportunities I have been provided here.

I am grateful to my committee members Sean Egan, Helen McNeill, and Freda Miller, who have provided thoughtful input and valuable advice throughout my Ph.D. The expertise and insight each brought to my project improved the work and challenged me to be a better scientist. I would also like to thank my undergraduate supervisor Elizabeth Meiering, who gave me my first (and third, and fourth) academic research opportunity, and whose excitement for science was infectious. The high standard set by Sean, Helen, Freda, and Liz as both scientists and mentors is one I hope to be able to emulate in my own career.

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scientist for it. She has taught me to be critical but not negative, cautious but hopeful. Gabrielle’s mentorship has been important not only with respect to research; she has also provided invaluable guidance and advice on my career goals, and on finding a fulfilling balance between research and everything else. I know that the training I have received under Gabrielle will allow me to contribute meaningfully to my scientific field and to my research community.

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Martin, my husband and best bud, has been so important to my success I can’t imagine achieving it without him. Science can be pretty unforgiving at times, and fully amazing at others, and Martin shared in all of it with me. He is incredibly encouraging and patient (not to mention handsome and hilarious) and I’m so thankful.
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<tbody>
<tr>
<td>Aβ</td>
<td>Amyloid-beta</td>
</tr>
<tr>
<td>Aβ40</td>
<td>Amyloid-beta 40 amino acid peptide</td>
</tr>
<tr>
<td>Aβ42</td>
<td>Amyloid-beta 42 amino acid peptide</td>
</tr>
<tr>
<td>ADAM</td>
<td>A Disintegrin and Metalloproteinase family</td>
</tr>
<tr>
<td>APH-1</td>
<td>anterior pharynx-defective 1</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid Precursor Protein</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B cell lymphoma 2</td>
</tr>
<tr>
<td>BiP</td>
<td>Binding immunoglobulin protein (GRP-78)</td>
</tr>
<tr>
<td>C99</td>
<td>Amyloid Precursor Protein cleaved by β-secretase, 99 amino acids in length</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CSL</td>
<td>CBF1, Suppressor of Hairless, Lag-1 (transcription factor)</td>
</tr>
<tr>
<td>CTF</td>
<td>C-terminal fragment</td>
</tr>
<tr>
<td>CyO, GFP</td>
<td><em>Drosophila</em> second chromosome balancer bearing a Curly (Cy) mutation and expressing green fluorescent protein (GFP)</td>
</tr>
<tr>
<td>D34</td>
<td>precise excision (wild type) FKBP14 line, used as control in all experiments</td>
</tr>
<tr>
<td>D58</td>
<td>imprecise excision (deletion) FKBP14 line</td>
</tr>
<tr>
<td>da</td>
<td>daughterless</td>
</tr>
<tr>
<td>ΔE9</td>
<td>Presenilin mutant lacking exon 9</td>
</tr>
<tr>
<td>DfN-8</td>
<td>Chromosomal deletion, region deleted includes Notch</td>
</tr>
<tr>
<td>Dl</td>
<td>Delta</td>
</tr>
<tr>
<td>E(spl)</td>
<td>Enhancer of split complex</td>
</tr>
<tr>
<td>EDS</td>
<td>Ehlers-Danlos Syndrome</td>
</tr>
<tr>
<td>EFDM</td>
<td>FKBP14 EF hand double mutant</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>eIF2α</td>
<td>eukaryotic translation initiation factor 2 α</td>
</tr>
<tr>
<td>EP-, P-</td>
<td>Insertion element endoding P transposon, flanked by terminal inverted repeats. EP elements include upstream activation sequences.</td>
</tr>
<tr>
<td>EP2019</td>
<td>EP insertion into FKBP14</td>
</tr>
</tbody>
</table>
ER  endoplasmic reticulum
FAD  familial Alzheimer's Disease
FKBP  FK506 binding protein
FKBP_C  FKBP-type peptidyl prolyl cis/trans isomerase domain
FKBP10  FK506 binding protein 10 (FKBP65)
FKBP11  FK506 binding protein 11 (FKBP19)
FKBP14  FK506 binding protein 14
FKBP15  FK506 binding protein 15 (FKBP133)
FKBP1a  FK506 binding protein 1a (FKBP12)
FKBP2  FK506 binding protein 2 (FKBP13)
FKBP3  FK506 binding protein 3 (FKBP25)
FKBP4  FK506 binding protein 4 (FKBP52)
FKBP5  FK506 binding protein 5 (FKBP51)
FKBP6  FK506 binding protein 6 (FKBP36)
FKBP7  FK506 binding protein 7 (FKBP23)
FKBP8  FK506 binding protein 8 (FKBP38)
FKBP9  FK506 binding protein 9 (FKBP63)
FKDM  FKBP14 FKBP_C domain double mutant
FLP  flippase, yeast site-specific recombinase
FRT  flippase recombination target site
FRT42B, 42D  flippase recombination target site inserted at chromosomal locus 42B or 42D
FSC  follicle stem cell
GFP  green fluorescent protein
GMR  glass multimer reporter
GSC  germline stem cell
HA  human influenza hemagglutinin derived epitope tag
Hsc3  Heat shock 70kDa protein cognate 3
HSP  heat shock protein
imNCSTN  immature (partially glycosylated) nicastrin
IP  immunoprecipitation
Kc cells  Kc 167 Drosophila cells
l(2)cl-R11¹  ethyl methanesulfonate cell lethal recessive mutation
L3  third instar larval stage, approximately 48-96 hours after hatching at 25°C
LN  Lin12-Notch
Mam  mastermind
mNCSTN  mature nicastrin (mammalian)
myc  c-myc protein derived epitope tag
NCSTN  nicastrin (mammalian)
NFT  neurofibrillary tangles
NTF  N-terminal fragment
PEN-2  presenilin enhancer 2
PERK  protein kinase RNA-like endoplasmic reticulum kinase
pnr  pannier
PPIase  peptidyl-prolyl cis/trans isomerase
PS  presenilin (mammalian)
Psn  presenilin (Drosophila)
RyR  Ryanodine receptor
S1  furin mediated cleavage of Notch
S2  ADAM mediated cleavage of Notch
S2 cells  Schneider 2 Drosophila cells
S3  γ-secretase mediated cleavage of Notch
SDS PAGE  sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser  Serrate
SOP  sensory organ precursor
TM  transmembrane
Tm  tunicamycin
TM3, Ser  Drosophila third chromosome balancer bearing a Serrate (Ser) mutation
TPR  tetratricopeptide motif
UAS  GAL4 upstream activating sequence
V5  epitope tag derived from the simian virus 5 (SV5)
wt  wild type
Chapter 1

Introduction
1 Introduction

1.1 Summary

Presenilins (mammalian: PSs, *Drosophila*: Psn) are highly conserved multipass transmembrane proteins, synthesized as 50 kDa precursor proteins that undergo tightly regulated endolytic processing to generate stable PS N- and C-terminal fragments. These fragments form the catalytic core of a complex called γ-secretase, which cleaves a growing list of transmembrane proteins involved in a number of developmental pathways, including Notch signalling. Autosomal dominant mutations in the genes encoding human PS have been closely linked to cases of early onset familial Alzheimer’s Disease (FAD), and these FAD-linked PS mutations influence the activity of γ-secretase. The production and maturation of each of the γ-secretase subunits are highly codependent, yet regulation of assembly and activity of the complex is unclear. FKBP14, a member of the FK506 binding protein (FKBP) family, was identified as a genetic interactor with Psn in *Drosophila*. The FKBP family of proteins is highly conserved, and specificity of recognition between an FKBP and its cognate binding partner can give rise to different complexes and signalling events affecting development and disease. Many members of this family remain largely uncharacterized, and the extent of their contribution to multicellular development is unknown. Investigation of the role of FKBP14 in Notch signalling via its interaction with Psn, and potentially broader roles in development, may provide a greater understanding of FKBPs in multicellular signalling.

1.2 Presenilin and the γ-secretase Complex

PSs were first identified in familial Alzheimer’s Disease (FAD) linkage studies, with mutations in PSs associated with early onset, aggressive forms of FAD (Rogaev, Sherrington et al. 1995, Sherrington, Rogaev et al. 1995). PSs are synthesized as ~50 kDa multi-pass transmembrane (TM) precursor proteins (Vetrivel, Zhang et al. 2006), undergoing highly regulated endolytic processing to generate stable C- and N-terminal fragments (CTF and NTF). The PS fragments associate with each other, nicastrin (NCSTN), anterior pharynx 1 (APH-1) and Presenilin enhancer (PEN-2) proteins to form an intramembrane protease known as the γ-secretase complex (Fig. 1.1 A) (Vetrivel, Zhang et al. 2006).
1.2.1 \(\gamma\)-secretase Components and Assembly

The \(\gamma\)-secretase complex is thought to assemble in a stepwise manner, with several identified subcomplexes. NCSTN and APH-1 associate with each other (LaVoie, Fraering et al. 2003, Shirotani, Edbauer et al. 2004), stabilizing APH-1 independent of PS or PEN-2, and possibly acting as a scaffold for the subsequent addition of PS holoprotein (Fig. 1.1 B, subcomplex \textit{Ia}) (Shirotani, Edbauer et al. 2004). APH-1 has also been shown to interact with the immature forms of NCSTN and PS (Fig. 1.1 B, subcomplex \textit{Ib}), and this interaction requires a conserved GXXXG motif in APH-1 (Lee, Shah et al. 2002, Gu, Chen et al. 2003). Mutations within this motif lead to a “loosely folded” APH-1 protein that can still interact with NCSTN, but disrupts interaction of the subcomplex with PS, and result in rapid degradation of APH-1 (Niimura, Isoo et al. 2005). Loss of APH-1 destabilizes PS (Lee, Shah et al. 2002, Gu, Chen et al. 2003), but in contrast to NCSTN and PEN-2, loss of PS does not significantly affect APH-1 levels (Gu, Chen et al. 2003). PEN-2 is required for coordinated expression of PS and NCSTN (Steiner, Winkler et al. 2002), facilitating endoproteolysis of holo-PS (Fig. 1.1 B, subcomplex \textit{III} to \textit{IV}) (Shiraishi, Sai et al. 2004), and for stabilization of PS N- and C-terminal fragments (Prokop, Shirotani et al. 2004). Conversely, PS is required for maturation and cell surface localization of NCSTN (Leem, Vijayan et al. 2002), and to maintain PEN-2 protein levels (Steiner, Winkler et al. 2002).

Of note, there are two PS (PS1 and PS2) and APH-1 genes in mammals (APH-1a and APH-1b), and alternative splice forms have been identified for APH-1a. These components contribute to unique \(\gamma\)-secretase complexes and appear to direct distinct \(\gamma\)-secretase activities (Lee, Shah et al. 2002, Shirotani, Edbauer et al. 2004, Semeels, Van Biervliet et al. 2009). Together these studies suggest that production, assembly, and maturation of the \(\gamma\)-secretase complex is intricately regulated, requiring multiple levels of feedback.
Figure 1.1: γ-Secretase components and assembly

The γ-secretase complex (A) is assembled in a stepwise manner (B). PS (blue) is the catalytic core (catalytic aspartate residues indicated with red stars), synthesized in the ER as a 50kDa

1.3 Presenilin and the γ-secretase complex in Alzheimer's Disease

γ-secretase cleaves a growing number of transmembrane proteins associated with a wide array of developmental processes, including Notch and Amyloid Precursor Protein (APP). In the case of APP, γ-secretase cleavage within its transmembrane domain generates the APP intracellular domain (AICD, Fig. 1.2), and 39-43 amino acid long peptides, termed β-amyloid peptides (Aβ) (Vetrivel, Zhang et al. 2006). Autosomal dominant mutations in the genes encoding APP and PS proteins have been closely linked to cases of early onset familial Alzheimer’s Disease (FAD) (Vetrivel, Zhang et al. 2006), and are associated with an increase in the γ-secretase mediated cleavage product of APP, amyloid beta peptide 1-42 (Aβ42) (Hashimoto, Rockenstein et al. 2003, Vetrivel, Zhang et al. 2006).

Cases of dementia are projected to nearly double every 20 years as the world’s population ages, with more than 100 million cases predicted by 2050 (Organization 2012). Alzheimer's Disease (AD) is the most common dementia occurring in mid to late life, with up to 40% of individuals over 85 being affected (Gandy and DeKosky 2013). It is a progressive and fatal disease, characterized by the loss of memory and cognitive function associated with the neuropathological appearance of neurofibrillary tangles (NFTs) and extracellular amyloid plaques (Selkoe 2001). NFTs consist of paired helical filaments largely made up of the abnormally phosphorylated, microtubule-associated tau protein. Amyloid plaques are composed primarily of β-amyloid (Aβ) peptide deposits produced as a result of abnormal processing of APP.

As mentioned above, autosomal dominant mutations in genes that encode the PS proteins (Rogaev, Sherrington et al. 1995, Sherrington, Rogaev et al. 1995) and APP (Kang, Lemaire et
al. 1987) have been closely linked to cases of early onset familial AD (FAD) (Vetrivel, Zhang et al. 2006). The γ-secretase complex is capable of cleaving APP within its transmembrane domain into 39-43 amino acid long Aβ peptides (Aβ) (Vetrivel, Zhang et al. 2006). FAD-linked mutations in PS and APP have been shown to influence γ-secretase cleavage of APP by an elusive mechanism, selectively enhancing generation of amyloid beta peptide1-42 (Aβ42), which accumulates in the neuronal endoplasmic reticulum (ER) and extracellularly as toxic plaques (Hashimoto, Rockenstein et al. 2003, Vetrivel, Zhang et al. 2006). Aβ42 forms soluble oligomeric species and insoluble aggregates, both of which have been shown to have toxic properties (Citron 2004). It is unclear whether accumulation of plaques and tangles are causative in AD or simply markers of disease progression, however most models suggest that accumulation of Aβ peptides plays a central role in AD pathology (Tanzi and Bertram 2005).

![Diagram of γ-secretase cleavage of Notch and APP](image)

**Figure 1.2: γ-Secretase cleavage of APP and Notch**

The γ-secretase complex cleaves Notch and APP in their transmembrane domains (black arrows). APP is acted upon by α- or β-secretase (grey arrows), shedding the ectodomain and generating a C-terminal stub (C83 or C99, respectively). Cleavage of C99 by γ-secretase generates the Aβ peptide and the APP intracellular domain (AICD) (Dries and Yu 2008). In the case of Notch, cleavage by γ-secretase follows S1 furin cleavage in the Golgi and S2 cleavage by an ADAM-family metalloprotease (grey arrows), releasing the extracellular domain.
(NEXT) and exposing the γ-secretase cleavage site. Cleavage of the Notch in its transmembrane domain releases the Notch intracellular domain (NICD), allowing it to translocate to the nucleus and upregulate target genes (Selkoe and Kopan 2003, Bray 2006, Fiuza and Arias 2007).

1.4 Presenilin and the γ-secretase complex in Notch Signalling

Though PS was initially identified through FAD linkage studies, it was rapidly shown in multiple models that both PS and γ-secretase are required for Notch developmental signalling (Levitan and Greenwald 1995, De Strooper, Annaert et al. 1999). γ-secretase cleavage of Notch results in release of the intracellular domain (NICD, Fig. 1.2), which translocates to the nucleus to initiate transcription of several target genes involved in a broad array of developmental decisions, including cell-fate specification, progenitor cell maintenance, boundary formation, cell proliferation and apoptosis (Bray 2006).

1.4.1 Overview of the Notch Pathway

Notch signalling is of critical importance in development for vertebrates and invertebrates alike. Though signalling is restricted to communication between adjacent cells, with no secondary messengers, Notch functions in an enormous range of developmental processes (Bray 2006). Defects in Notch signalling have been associated with multiple diseases, including several types of cancer (Jurisch-Yaksi, Sannerud et al. 2013).

Following synthesis, full length Notch protein is processed by a furin-like convertase in the trans-Golgi (S1) during secretion (Selkoe and Kopan 2003). Notch is then presented at the cell surface as a heterodimer consisting of EGF-like and LN repeats linked non-covalently by a heterodimerization region to the transmembrane domain containing the C-terminal fragment (Selkoe and Kopan 2003, Fiuza and Arias 2007). This heterodimer must then interact with one of its ligands, which triggers several cleavage events (Selkoe and Kopan 2003). S2 cleavage is carried out by a member of the ADAM-family metalloproteases, releasing the Notch extracellular domain (Bray 2006) and exposing a third cleavage site within the Notch receptor transmembrane domain (Selkoe and Kopan 2003). Cleavage at this intramembrane site is effected by the γ-secretase complex (S3), resulting in release of NICD, which enters the nucleus and interacts with the DNA-binding protein CSL (CBF1, Su(H) and LAG–1) and its co-activator Mastermind (Mam) to promote transcription of target genes in the signal receiving cell (Fig 1.3) (Selkoe and Kopan 2003, Bray 2006, Fiuza and Arias 2007). Notch activation directs a certain
cell behavior or fate, while signalling a different cell fate for the ligand-bearing (signal sending) cell (Bray 2006).

Figure 1.3: Notch signalling pathway

The Notch receptor trafficks through the ER, is processed by a furin-like convertase in the Golgi (S1), and presented at the cell surface as a heterodimer. The Notch receptor then interacts with one of its ligands on a neighbouring cell (signal sending cell), which results in a second cleavage by an ADAM-family metalloprotease (S2) and shedding of the Notch extracellular domain. Notch is then cleaved in its transmembrane domain by the γ-secretase complex (S3), releasing the Notch intracellular domain (NICD) to translocate to the nucleus and interact with CSL and its co-activator Mastermind (Mam) to upregulate target genes (Selkoe and Kopan 2003, Bray 2006, Fiuza and Arias 2007). Adapted from (Fiuza and Arias 2007).
In some developmental contexts, such as boundary formation, Notch ligands are produced by a distinct population of cells, however under many circumstances, differential ligand expression is not sufficient to explain why certain cells become signal-sending cells instead of signal receiving cells (Bray 2006). Clearly, precise regulation of Notch is of critical importance.

1.4.2 Notch Regulation by Lateral Inhibition in *Drosophila*

In the *Drosophila* peripheral nervous system, Notch signalling controls cell fates by lateral inhibition. From an apparently equipotent group of cells in the third instar larval wing disc known as proneural clusters (Barad, Hornstein et al. 2011, Furman and Bukharina 2011), sensory organ precursors (SOPs) arise and undergo four rounds of asymmetric division, segregating Notch pathway components such as Numb and Sanpodo (Upadhyay, Kandachar et al. 2013), to generate mature cells that comprise a functional mechanosensory organ (Fig. 1.4 A).

Lateral inhibition by Notch at the level of SOP specification functions to restrict the neural cell fate specification in cells that surround the SOP (Barad, Hornstein et al. 2011), where a loss of Notch signalling may result in supernumerary SOPs (Logeat, Bessia et al. 1998, Kidd and Lieber 2002, Mummery-Widmer, Yamazaki et al. 2009). Precise regulation of Notch is also required in both the signal receiving (pIIa) and signal sending (pIIb) cells for correct cell fate specification. Psn and the γ−secretase complex are required in pIIa to perform S3 cleavage of Notch. This has been shown to require the protein Sanpodo, which binds the γ−secretase complex via Psn (Upadhyay, Kandachar et al. 2013). Interestingly, Sanpodo also physically interacts with Numb and Notch (O’Connor-Giles and Skeath 2003), suppressing Notch activation in pIIb by driving Notch internalization (Upadhyay, Kandachar et al. 2013). Thus, a single protein may regulate Notch signalling via distinct mechanisms depending on the cellular context.

1.4.3 Notch Regulation of Boundary Formation in *Drosophila*

Notch signalling is also required in many developmental decisions where cell types are initially distinct, such as across the dorsal-ventral boundary of the *Drosophila* wing margin (Fortini 2009). In the presumptive wing tissue, Notch is widely expressed, but post-translational glycosylation of Notch in the dorsal compartment renders it more sensitive to its ligand Delta, leading to Notch activation in a stripe of dorsal cells along the dorsal-ventral border (Fortini 2009). The Notch receptor in cells just ventral to this stripe lack the same post-translational
modification and show higher responsiveness to its ligand Serrate, which is restricted to the dorsal compartment, resulting in Notch activation in two adjacent rows of cells at the dorsal-ventral boundary to define the presumptive wing margin (Fortini 2009) (Fig. 1.4 B). Similarly, Delta and Serrate are highly expressed in proveins of the developing wing, allowing Notch signalling to refine the boundary between vein and intervein cells (Blair 2007). Loss of Psn or γ-secretase in this context leads to loss of Notch signalling, resulting in a failure to properly define the wing margin or wing veins (Guo, Livne-Bar et al. 1999).

1.4.4 Notch Regulation in *Drosophila* Oogenesis

In a *Drosophila* ovariole (Fig. 1.4 C), the gerarium contains both somatic and germline stem cells. These cells divide asymmetrically to regenerate the stem cell and produce a daughter cell that begins to differentiate. The germline daughter cell undergoes four mitotic divisions with incomplete cytokinesis, forming a 16-cell cyst, one cell of which will differentiate to become the oocyte, while the remaining 15 become polyploid nurse cells (Bastock and St Johnston 2008). Somatic follicle cells surround the cyst as it pinches off from the gerarium, forming an epithelium around individual egg chambers. Each ovariole contains multiple egg chambers which mature as they progress through the ovariole (Bastoock and St Johnston 2008). *Drosophila* oogenesis is divided into 14 stages based on morphological criteria: stage 1 describes the egg chamber budding off of the gerarium, and stage 14 represents the mature egg (Bastoock and St Johnston 2008).

Notch signalling is required in somatic cells to form and maintain the germline stem cell (GSC) niche (Ward, Shcherbata et al. 2006, Song, Call et al. 2007) and to help establish anterior-posterior polarity (Assa-Kunik, Torres et al. 2007). Notch is also required in the somatic follicle cells of developing egg chambers in order to pinch off from the gerarium (Ruohola, Bremer et al. 1991), as well as during stage 5 to 6 to receive the signal from the oocyte for follicle cells to stop mitosis and differentiate (Lopez-Schier and St Johnston 2002). Loss of Notch signalling leads to loss of GSCs (Song, Call et al. 2007), fusion of egg chambers (Ruohola, Bremer et al. 1991), and over-proliferation of follicle cells (Deng, Althauser et al. 2001, Lopez-Schier and St Johnston 2002).
Figure 1.4: Notch signalling in *Drosophila* development (page 11)

Notch signalling in lateral inhibition (A), boundary formation (B), and oogenesis (C).

(A) From an apparently equipotent groups of cells in the third instar larval wing disc known as proneural clusters, sensory organ precursors (SOPs) arise and undergo four rounds of assymetric division, segregating Notch components to generate the mature cells that become a functional mechanosensory organ.

(B) Notch signalling between rows of initially distinct cells. Post-translational modification of Notch in the dorsal compartment renders Notch more sensitive to its ligand Delta. Notch receptor in the cells just ventral to this stripe lack the same post-translational modification and show higher responsiveness to the ligand Serrate, which is restricted to the dorsal compartment, resulting in Notch activation in two adjacent rows of cells at the dorsal-ventral boundary to define the presumptive wing margin.

(C) The *Drosophila* ovariole. The germarium contains both germline (GSCs) and somatic (FSCs) stem cells. The germline daughter cell undergoes four mitotic divisions with incomplete cytokinesis, forming a 16-cell cyst, one cell of which will differentiate to become the oocyte, while the remaining 15 cells become polypliod nurse cells. The cyst is surrounded by somatic follicle cells as it pinches off from the germarium. Each ovariole contains multiple egg chambers, which mature as they progress through the ovariole. Notch signalling is required in the somatic cells to form and maintain the GSC niche and to help establish anterior-posterior polarity. Notch is also known to be required in the somatic follicle cells of developing egg chambers in order for egg chambers to pinch off from the germarium, as well as during stage 5 to 6 to receive the signal from the oocyte for the follicle cells to stop mitosis and differentiate. Adapted from (Fiuza and Arias 2007, Bastock and St Johnston 2008, Fortini 2009).

1.5 **Notch-independent Roles for Presenilin and γ-secretase**

Clearly PS and γ-secretase play critical roles in both disease and development. Indeed, though the Notch pathway was the first developmental role identified for PS and γ-secretase, to date more than 90 γ-secretase substrates have been identified, with diverse structure, localization, and physiological functions (Haapasalo and Kovacs 2011, Jurisch-Yaksi, Sannerud et al. 2013). γ-secretase substrates appear to function largely as signalling proteins, regulating cellular events that range from cell fate determination to axon guidance and formation or maintenance of synapses (Jurisch-Yaksi, Sannerud et al. 2013).

Moreover, cellular functions for PS and other components of the γ-secretase complex independent of γ-secretase cleavage activity have recently been identified. For instance, FAD mutations in PS have been associated with defects in intracellular calcium (Ca\(^{2+}\)) signalling (Michno, Knight et al. 2009, Bezprozvanny 2013). PS mutants also showed defects in lysosomal Ca\(^{2+}\) storage and release, associated with impairment of lysosomal fusion capacity (Coen, Flannagan et al. 2012). It has also been hypothesized that holo-PSs may function as ER Ca\(^{2+}\) leak
channels independent of γ-secretase (Tu, Nelson et al. 2006, Bezprozvanny 2013), though this model has been challenged by other groups (Shilling, Mak et al. 2012). Recent crystallography data on a PS homologue JR1 from *M. marisnigri* revealed a water filled cavity traversing the lipid bilayer, large enough to allow passage of small ions (Li, Dang et al. 2013). Moreover, single cell Ca\(^{2+}\) imaging further showed that knockdown of the mammalian PS2 dramatically reduced ER Ca\(^{2+}\) leak rate, while knockdown of the γ-secretase member PEN-2 had the opposite effect (Bandara, Malmersjo et al. 2013). Though it is still controversial whether PS functions directly as an ER Ca\(^{2+}\) channel, these data indicate that the function of PS in Ca\(^{2+}\) regulation is independent of γ-secretase activity.

PSs are also involved in regulation of apoptotic cell death via an interaction with the immunophilin FKBP38 and the anti-apoptotic protein Bcl-2 (Wang, Nakaya et al. 2005). PSs form a macromolecular complex with FKBP38 and Bcl-2, promoting their degradation and sequestration to the ER/Golgi in a γ-secretase independent manner. This interaction inhibits FKBP38-mediated mitochondrial targeting of Bcl-2, antagonizing it’s anti-apoptotic functions (Wang, Nakaya et al. 2005). FAD mutations in PS enhance this pro-apoptotic activity (Wang, Nakaya et al. 2005), suggesting this function of PS may contribute to FAD pathology.

APH-1 and NCSTN have similarly been shown to have γ-secretase independent functions. In the *Drosophila* ovary, follicle cells mutant for *Psn* or *nct* show defects in the spectrin cytoskeleton not observed in Notch mutants (Lopez-Schier and St Johnston 2002). *Drosophila aph-1* has also been implicated in cell viability in the wing disc (Cooper, Deng et al. 2009), in contrast to both Notch and Psn, indicating that this role is independent of γ-secretase activity. Interestingly, the cell viability defect in aph-1 mutants could be rescued by expressing Psn C- and N-terminal fragments, but not by a version of holo-Psn lacking the endolytic cleavage site (P\(S^{exon9}\)), despite the fact that this version of Psn can reconstitute γ-secretase activity in the absence of wild type Psn (Cooper, Deng et al. 2009). These data indicate that Aph-1 and holo-Psn may both play a role in cell survival, independent of γ-secretase.

Despite the clear importance of PS, the γ-secretase complex, and its components in development and disease, many questions remain regarding their regulation, assembly, and activity.
1.6 **FKBP14 Identified in Presenilin Modifier Screen**

Given the critical role PS plays in both development and disease, identifying novel regulators and co-factors that modify PS dependent processes is of significant interest. A genetic screen carried out in the Boulianne lab sought to identify such modifiers in *Drosophila*. The core components of the γ-secretase complex are functionally conserved in *Drosophila*, as are many of its targets (Guo, Livne-Bar et al. 1999, Ye and Fortini 1999). Moreover, while there are two mammalian PS (PS1 and PS2) and APH-1 genes (APH-1a and APH-1b), *Drosophila* have only one of each (*Psn* and *aph-1*), simplifying the scope of potential interactors in this system.

Taking advantage of two Notch-related phenotypes associated with over-expression of wild type *Psn* in third instar larval wing discs, the Boulianne lab conducted a screen using the GAL4/UAS expression system. Wild type *Psn* expression driven by *pannier* GAL4 (pnr GAL4) in the thoracic region of the wing imaginal disc or *cut* GAL4 along the presumptive wing margin results in supernumerary macrochaetes on the scutellum (Fig. 1.5 B) or a notched wing phenotype (Fig. 1.5 E), respectively (van de Hoef, Hughes et al. 2009), both of which resemble Notch loss-of-function phenotypes (Ye and Fortini 1999, van de Hoef, Hughes et al. 2009). The mechanism leading to these loss-of-Notch phenotypes is not established, but it appears to be attributable to dominant negative effects of over expressed wild type Presenilin (Ye and Fortini 1999). Accumulation of non-functional γ-secretase sub-complexes in the ER or Golgi due to Psn saturation may result in a loss of endogenous γ-secretase activity due to sequestration of γ-secretase components in incomplete complexes, leading to the Notch phenotypes observed.
Figure 1.5: **FKBP14 identified in screen for modifiers of Psn in Drosophila**

Wild type flies have four scutellar bristles (A, arrow), and a well defined wing margin (D, arrowhead). Over-expression of *Psn* results in supernumerary bristles in the thoracic region (B) and wing notching (E). These *Psn*-dependent phenotypes are suppressed by the *FKBP14* mutations, *FKBP14*<sup>EP2019</sup> (C) and *FKBP14*<sup>D58</sup> (F). Adapted from (van de Hoef, Hughes et al. 2009).

A collection of ~1600 P- and EP-element insertions into the *Drosophila* genome were screened against these loss-of-Notch phenotypes, recovering 117 modifiers that enhanced or suppressed the phenotypes (van de Hoef, Hughes et al. 2009), including several known Notch components. Multiple independent insertion alleles identified *FKBP14* as a genetic modifier of *PS*. The insertional mutations *FKBP14*<sup>EP2019</sup> and *FKBP14*<sup>EP2206</sup> both suppressed the notum bristle phenotypes (Fig. 1.5 C), and a deletion mutation in *FKBP14*, *FKBP14*<sup>D58</sup>, suppressed both (Fig. 1.5 F) (van de Hoef 2008, van de Hoef, Hughes et al. 2009). Investigation into the nature and consequences of this observed genetic interaction are discussed in detail in Chapter 2.

1.7 **FKBPs**

*FKBP14* belongs to a superfamily of highly conserved proteins known as immunophilins, originally identified for binding to immunosuppressive drugs such as FK506, rapamycin and cyclosporin A (Barik 2006, Kang, Hong et al. 2008). The FK506-binding protein (FKBP) family is one of the two largest families of immunophilins, many of which bind FK506 as well as possessing peptidyl prolyl cis/trans isomerase (PPlase) activity (Barik 2006, Kang, Hong et al. 2008). The smallest members of this family are composed of a single FK506-like binding
domain (FKBP_C) and little else, while the larger FKBPs are composed of modular domains that are functionally independent (Barik 2006). FKBPs and other PPIases have been identified in several subcellular compartments and organelles, including cytoplasm, ER, nucleus, mitochondria, and polyribosomes (Galat 2013). Functional properties attributed to FKBPs include protein folding, chaperone activity, receptor signalling, protein trafficking, transcription, immunosuppression, and apoptosis (Kang, Hong et al. 2008, Galat 2013).

1.7.1 FKBP Structure and Function Variability

Genomes of eukaryotes encode at least one cytosolic and one ER-anchored FKB with conservation in the PPIase cavity. Humans have 15 FKBPs (Fig. 1.6), as well as several FKBP-like proteins (Galat 2013), whereas 8 FKBPs have been identified in Drosophila (St Pierre, Ponting et al. 2014). The smallest members of the family are monodomain proteins consisting of a single FK506-like binding domain (FKBP_C), where larger multidomain FKBPs consist of one to four FKBP_Cs and diverse motifs such as calmodulin-binding (CaM), EF hand and Ca^{2+} binding, tetracapeptide repeat (TPR), and DNA binding motifs (Fig. 1.6) (Galat 2013). The FKBP_C domain may catalyze cis/trans (PPIase) isomerization of X-Pro epitopes during protein folding and assembly into macromolecular complexes. It may also potentially act as a molecular chaperone, altering the structural or functional attributes of targets (Galat 2013). The physical and chemical properties of each FKB may predispose them to target different interactors in the cell.
Figure 1.6: Schematic of human FKBPs (page 17)

Schematic of the domains and localizations of the human FKBPs repertoire. Known Drosophila homologues are listed. The smallest members of the family (FKBP12s) are monodomain proteins consisting of a single FK506-like binding domain (FKBP_C), where larger multidomain FKBPs consist of one to four FKBP_Cs together with diverse motifs such as EF hand and Ca\(^{2+}\) binding, tetrapeptide repeat (TPR), and DNA binding motifs. Major regions of each FKBP are shown. FKBPs have been identified in several subcellular compartments and organelles, including cytoplasm, ER, nucleus and mitochondria. Specific retention signals are indicated for the ER resident FKBPs. Adapted from (Galat 2013).

1.8 FKBPs in Single Cell Models

The deletion of all members of the two major immunophilin families, cyclophilins and FKBPs, in Saccharomyces cerevisiae is viable. Despite this, both families are highly conserved and distributed across all organelles, suggesting that they interact with unique sets of partners to perform specific functions (Dolinski, Muir et al. 1997). Indeed, a double mutant eliminating the only known cyclophilin and FKBP in another single cell model, Bacillus subtilis, was shown to have strongly impaired growth upon starvation, suggesting that while these proteins may be dispensable under rich growth conditions, they may be required for response to stress (Gothel, Scholz et al. 1998). This is supported by the discovery that S. cerevisiae FKB2 (FKBP13) responds to the accumulation of unfolded proteins in the ER (Partaledis and Berlin 1993), suggesting that a stress response may be one of the conserved functions of this family of proteins.

Though FKBPs are not absolutely required in a single cell context, valuable structural and functional knowledge of the family has been obtained using these models. Structural analysis of the FKBP proteins SlyD and trigger factor from Escherichia coli indicated that while PPIase catalytic activity was carried out by the FKBP_C domain, additional chaperone domains that folded independently conferred a much higher catalysis efficiency (Scholz, Stoller et al. 1997, Zarnt, Tradler et al. 1997, Weininger, Haupt et al. 2009). While FKBP-mediated catalysis, unlike that of cyclophilins, exhibits a high level of target sequence specificity (Harrison and Stein 1990), the presence of such accessory chaperone domains may overcome this inherent high specificity, allowing broader target recognition (Jakob, Zoldak et al. 2009). Analysis of protein complexes in S. cerevisiae, on the other hand, has suggested that FKBPs, including those lacking
accessory domains, interact with distinct sets of proteins (Ho, Gruhler et al. 2002, Krogan, Cagney et al. 2006) and have seemingly discrete functions (Costanzo, Baryshnikova et al. 2010).

Comparative analysis of PPIase repertoires in S. cerevisiae and Schizosaccharomyces pombe with that of Homo sapiens and Drosophila melanogaster revealed that members of other immunophilin families are largely conserved between different organisms and therefore likely evolved to perform conserved functions. However, the FKBP repertoires differ, suggesting that FKBP have evolved to fill changing roles within evolving organisms (Pemberton and Kay 2005).

1.9 FKBP in Multicellular Models

In keeping with the hypothesis that FKBP may have novel roles in higher order organisms (Pemberton and Kay 2005), a number of roles for FKBP in multicellular models have emerged, some of which are conserved with single cell models and others which appear unique to a multicellular context. In multicellular models, critical neurotrophic and neuroprotective roles have been documented for FKBP (Barik 2006, Galat 2008, Kang, Hong et al. 2008), as well as roles in regulation of apoptosis via the interaction of FKBP with their binding partners (Barik 2006, Kang, Hong et al. 2008). The specificity of recognition between an FKBP and its cognate binding partner can give rise to different complexes and signalling events (Barik 2006) affecting multicellular development and disease.

1.9.1 FKBP in Development

In contrast to the collective dispensability of FKBP observed in S. cerevisiae, double and triple mutants for the ER resident FKBP FKB-3, -4, and -5 in Caenorhabditis elegans, which developed normally at permissive temperatures, displayed cold-sensitive larval lethality at low temperatures, establishing that secretory pathway FKBP are collectively essential for development under stress conditions in nematodes (Winter, Eschenlauer et al. 2007). Mutations in the Arabidopsis thaliana FKBP PAS1 (closest human homologue FKBP51/52) lead to deregulated control of cell proliferation resulting in lethality under normal growth conditions (Faure, Vittorioso et al. 1998, Vittorioso, Cowling et al. 1998). Clearly, FKBP have evolved to play important roles in a multicellular context.
One of the most studied members of the FKBP family in a multicellular context is FKBP12, which binds the immunosuppressant FK506 (Goto, Kino et al. 1987) and reduces the immune response through complex formation with FKBP12 and calcineurin in T-lymphocytes (Dumont 2000). Discovery of the link between FK506-mediated immunosuppression and FKBP12 led to a series of revelations about FKBP12 and the role of FKBPs in Ca\(^{2+}\) signalling. Mammalian FKBP12 and its parologue FKBP12.6 bind and modulate the gating properties of two calcium release channels, the inositol 1,4,5-trisphosphate (IP3) and Ryanodine (Ry) receptors (Cameron, Nucifora et al. 1997, Ondrias, Marx et al. 1998, MacMillan 2013). Loss of FKBP12 in mice leads to abnormal cardiac structures, at least partly due to a requirement for FKBP12 as a negative modulator of activated Notch1-mediated signalling within developing endocardial cells (Chen, Zhang et al. 2013). Treatment of FKBP12 deficient embryos with a γ-secretase inhibitor results in partial suppression of these defects (Chen, Zhang et al. 2013). This study, together with our FKBP14 study detailed in Chapter 2 of this thesis, propose a novel role for FKBPs in multicellular development and Notch signalling.

Indeed, a number of important roles for FKBPs have been identified in multicellular models. In Drosophila melanogaster, the FKBP shutdown (homologous to human FKBP6) is essential for the normal function of germline stem cells and in later stages of oogenesis (Munn and Steward 2000, Preall, Czech et al. 2012). The ER resident mammalian FKBP65 regulates lung development and responds to lung injury in mice and humans, in a manner that suggests a distinct set of developmentally regulated protein ligands (Patterson, Abrams et al. 2005). The mitochondrial FKBP38 binds and inhibits calcineurin in human and mouse tissue, and regulates apoptosis via interactions with Bcl2, Bcl\(_{XL}\), and PS proteins (Shirane and Nakayama 2003, Wang, Nakaya et al. 2005, Kang, Hong et al. 2008).

The dynamic nature of FKBP-partner interactions and their regulatory effect on these complexes are one of the principal attributes of this family of proteins (Galat 2008). Indeed, loss or misregulation of FKBPs has been associated with an increasing number of diseases and developmental disorders.

1.9.2 FKBPs in Disease

FKBPs have been associated with a number of disorders and diseases, including cardiovascular disease, cancer, and neurodegeneration. Abberant FKBP12.6-RyR2 receptor interactions are
thought to cause channel dysfunction in acquired and inherited cardiac disease (MacMillan 2013). RyR2 is the channel required for excitation-contraction coupling in the heart, and binding of FKBP12.6 stabilizes and regulates channel structure and function. Defective regulation of RyR2 can result in dissociation of FKBP12.6 from the complex (Marx, Reiken et al. 2000). In failing hearts, dissociation of FKBP12.6 from RyR2 leads to pathological consequences, including potentially fatal cardiac arrhythmia (Marx, Reiken et al. 2000). In support of the critical role of FKBP12.6 in this context, conditional overexpression of FKBP12.6 in mouse cardiac myocytes prevented triggered ventricular tachycardia in normal hearts under stress conditions (Gellen, Fernandez-Velasco et al. 2008).

Several FKBP1s have also been implicated in cancer etiology and chemoresistance, notably the heat shock protein (HSP) 90 co-chaperones FKBP51 and FKBP52. In prostate cancer cell lines, increased levels of FKBP51 and FKBP52 were observed, along with an inhibitory effect of FK506 on androgen-dependent cell growth (Storer, Dickey et al. 2011). FKBP52 expression is also dramatically increased in breast cancer (Ward, Mark et al. 1999, Solassol, Mange et al. 2011), as well as in hepatocellular carcinoma (Liu, Li et al. 2010). FKBP51 was also found to affect the response of cancer cells to chemotherapy (Pei, Li et al. 2009) and radiation (Romano, D'Angelillo et al. 2010).

Roles in neurodegeneration and recovery have been suggested for a number of FKBP1s. FKBP1s are enriched in neuronal tissue, and their expression is elevated after nerve injury (Kang, Hong et al. 2008). Furthermore, FK506 has been suggested to have neuroprotective effects and may induce neuronal regeneration (Galat 2013). Indeed, expression of FKBP12 is increased in the brains of patients with Parkinson’s Disease (PD), Alzheimer’s Disease (AD), and dementia with Lewy bodies, colocalizing with α-synuclein (Avramut and Achim 2002) and neurofibrillary tangles (Sugata, Matsuo et al. 2009). FKBP12 also binds the intracellular domain of amyloid precursor protein (APP) and this interaction can be disrupted by FK506 (Liu, Liu et al. 2006). Moreover, FKBP12 regulates localization and processing of APP (Liu, Liu et al. 2014), potentially contributing to AD pathology. In Drosophila, FKBP52 also binds APP and its overexpression reduces toxicity associated with transgenic expression of Aβ fragments (Sanokawa-Akakura, Cao et al. 2010). FKBP52 also binds specifically to tau in rat brains,
inhibiting production of tau microtubules which further contributes to a neuroprotective effect (Chambraud, Sardin et al. 2010).

Together, these studies place FKBPs at central positions in multiple biological pathways and processes, and suggest critical roles in a number of oncogenic and neurodegenerative pathologies.

1.10 FKBP14

FKBPs have also been associated with complex developmental disorders. Ehlers-Danlos Syndrome (EDS) is a clinically and genetically heterogenous group of heritable connective tissue disorders that affects multiple tissue types (Baumann, Giunta et al. 2012). An autosomal recessive extreme variant of this disorder, which shares features with congenital muscular dystrophy, has been associated with a frameshift in FKBP14, a largely uncharacterized ER resident member of the FKBP family (Baumann, Giunta et al. 2012). The crystal structure of human FKBP14 reveals a classical FKBP_C domain and two EF hand motifs. FKBP14 may form a dimer via its EF hand motifs, with an elongated and primarily hydrophobic cavity potentially available for a ligand (Boudko, Ishikawa et al. 2014).

As detailed above, previous work from the Boulianne lab identified FKBP14 as a genetic interactor with Psn in Drosophila, with mutations in FKBP14 suppressing overexpression of Psn phenotypes (van de Hoef 2008, van de Hoef, Hughes et al. 2009). These data, together with the clinical phenotype of FKBP14 as detailed above, suggest an important role for FKBP14 in multicellular development.

Despite growing evidence for the importance of FKBPs, knowledge of this family of proteins remains vague, with many FKBPs remaining largely uncharacterized. In particular, though many biochemical processes in which FKBPs may play a role have been identified, the extent to which this family of proteins contributes to multicellular development is largely unknown.

1.11 Rationale

Prior to the identification of FKBP14 as a modifier of Psn-dependent Notch phenotypes, no known interaction between FKBPs and Notch signalling had been identified. Moreover, FKBP14 is an almost entirely uncharacterized member of the FKBP protein family, despite its high
conservation. The goal of this thesis, therefore, was to determine the role of FKBP14 in *Drosophila* development, in particular with respect to *Psn*, γ−secretase, and Notch signalling.

In Chapter 2 I describe my experiments analyzing the interaction between *FKBP14*, *Psn*, and Notch in *Drosophila*. Data previously generated by Diana van de Hoef indicated that FKBP14 was an ER resident protein, mutants of which were homozygous lethal, displayed Notch-like phenotypes and showed reductions in Psn protein (van de Hoef, Bonner et al. 2013). I show that FKBP14 endogenously modifies the Notch pathway and that this phenotype is not downstream of ER stress. I further showed that loss of FKBP14 results in defects in γ−secretase cleavage ability, suggesting observed Notch phenotypes are downstream of Psn and γ−secretase. I also show preliminary work suggesting that FKBP14 and Psn may physically interact, suggesting a possible role in γ−secretase assembly/stability.

In Chapter 3 I describe a Notch independent phenotype observed in FKBP14 mutants. Using several variations on the yeast-derived FLP/FRT recombination system (Golic and Lindquist 1989, Chou and Perrimon 1996, Newsome, Asling et al. 2000), I show that *FKBP14* mutant clones are strictly inviable in multiple tissue types and that this inviability cannot be overcome by recombining in the presence of a recessive cell lethal mutation. This is in contrast to both Notch and *Psn* (Lopez-Schier and St Johnston 2002), revealing a novel, Notch-independent role for FKBP14.

In Chapter 4 I describe experiments with a series of deletion and point mutation constructs in conserved residues for both the PPIase and EF hand domains of FKBP14. FKBP14 constructs containing mutations in either the PPIase domain or EF hand domain are unable to rescue the lethality observed in *FKBP14D58* mutants, while robust rescue is observed with wild type FKBP14. These data demonstrate that both the PPIase domain and EF hand domain are required for FKBP14 function in this context.

In Chapter 5 I discuss the data presented in this thesis in the context of a possible model for FKBP14 in Notch signalling and in broader *Drosophila* development, and describe potential experiments to test this model.
FKBP14 is an essential gene that regulates Presenilin protein levels and Notch signalling in *Drosophila*

**Data Attribution:** Julia Maeve Bonner and Diana van de Hoef are co-first authors on a manuscript published in *Development 2013 Feb;140(4):810-9* that includes several figures presented in this chapter. Diana van de Hoef generated the deletion mutant and precise excision lines used for the experiments described, and performed the early characterization of these lines (Fig 2.2). Diana also performed the cell culture co-localization (Fig. 2.3 C-D”), genetic epistasis with DfN-8 (Fig 2.6 I-K), Notch cell surface imaging (Fig 2.8 A-C), and Presenilin Western blots of *FKBP14* mutants (Fig 2.8 D). All other experiments described herein were performed by Julia Maeve Bonner.
2  *FKBP14* is an essential gene that regulates Presenilin and Notch signalling in *Drosophila*

### 2.1 Summary

PSs are highly conserved multipass transmembrane proteins that are required for Notch signalling during development and are implicated in familial Alzheimer’s Disease (Thinakaran, Borchelt et al. 1996, Guo, Livne-Bar et al. 1999, Kimberly, Xia et al. 2000). A screen in *Drosophila* recently identified *FKBP14*, a member of the family of FK506-binding proteins (FKBPs), as a genetic modifier of *Presenilin* with *FKBP14* mutants exhibiting defects typical of a loss of Notch activity. FKBPs are a large, highly conserved family of proteins involved in a wide array of biochemical processes including protein folding, assembly, and trafficking (Barik 2006, Kang, Hong et al. 2008), yet they are non-essential in yeast and their role in the development of multicellular organisms remains unclear (Dolinski, Muir et al. 1997). Here I show that *FKBP14* mutants are null for *FKBP14* transcript and that the observed loss of Notch phenotypes are not associated with upregulation of the ER stress response. I further show that *FKBP14* mutants genetically interact with components of the Notch pathway, indicating that these phenotypes are indeed associated, at least in part, with dysregulation of Notch signalling.

While Notch trafficking to the membrane is unaffected in *FKBP14* mutants, levels of Notch target genes are reduced, suggesting that FKB14 acts downstream of Notch activation at the membrane. Consistent with this model, I show that Psn-dependent γ-secretase cleavage, a process required for Notch signalling, is reduced in *FKBP14* mutants. Finally, I show preliminary data indicating that FKB14 physically interacts with Psn. Altogether, these data demonstrate that FKB14 plays a critical role in development, one aspect of which includes regulating members of the Notch signalling pathway.

### 2.2 Introduction

Presenilins (PSs) are highly conserved multi-pass transmembrane proteins, synthesized as 50 kDa precursor proteins. These precursors are highly unstable and undergo tightly regulated endolytic processing to generate stable PS C-terminal and N terminal fragments that form the catalytic core of the γ−secretase complex (Thinakaran, Borchelt et al. 1996, Guo, Livne-Bar et al. 1999, Kimberly, Xia et al. 2000). γ−secretase cleaves transmembrane proteins associated with a
wide array of developmental processes, including the Notch receptor and Amyloid Precursor Protein (APP). Autosomal dominant mutations in the genes encoding APP and PS have been closely linked to cases of early onset familial Alzheimer’s Disease (FAD) (Vetrivel, Zhang et al. 2006). FAD-linked mutations in PS influence γ–secretase cleavage of APP, enhancing the generation of amyloid beta peptide1-42 (Aβ42), which accumulates in the endoplasmic reticulum of neuronal cells and extracellularly as toxic plaques (Hashimoto, Rockenstein et al. 2003, Vetrivel, Zhang et al. 2006). In the case of Notch, cleavage by γ–secretase releases the Notch intracellular domain, which translocates to the nucleus to initiate transcription of target genes involved in a broad array of developmental decisions (Bray 2006). To gain a greater understanding of how Psn and γ-secretase are regulated, previous members of the Boulianne lab performed a genetic modifier screen and found that loss of function mutations in the immunophilin FKB14 could suppress the phenotype associated with over-expression of Psn (van de Hoef, Hughes et al. 2009).

FKBP14 belongs to a family of highly conserved proteins known as immunophilins that bind to the immunosuppressive drugs FK506, rapamycin and cyclosporin A, and often exhibit peptidyl-prolyl cis-trans isomerase (PPIase) activity (Barik 2006, Kang, Hong et al. 2008). The FK506-binding proteins (FKBPs) are a sub-family of immunophilins, the smallest members of which are composed almost entirely of a single PPIase domain. Larger FKBPs are composed of modular domains that are functionally independent (Barik 2006). FKBPs are found in a broad range of organisms and have been implicated in various biochemical processes including protein folding, receptor signalling, protein trafficking and transcription (Barik 2006, Kang, Hong et al. 2008). FKBP family members also exhibit distinct subcellular localizations and bind specific protein targets (Galat 2008, Kang, Hong et al. 2008).

Despite growing evidence for the importance of FKBPs, there is little data on their roles in development of multicellular organisms. Studies in yeast have shown that all cyclophilins and FKBPs are individually and collectively dispensable for viability (Dolinski, Muir et al. 1997) and few FKBPs have been shown to be essential for viability in multicellular models.

We investigated the function of one member of this family, FKB14, in Drosophila development. To determine the function of Drosophila FKB14, we characterized a lethal P-element insertion, EP(2)2019, located within the first intron of FKB14. The P-element lies 101
bp downstream of the first exon of transcript FKBP14-RA, 818 bp downstream of the first exon of transcript FKBP14-RC, and 1638 bp upstream of the translational start site located in exon 2 of both isoforms (Fig 2.1B). To generate additional *FKBP14* alleles, an excision screen was performed by Diana van de Hoef on *FKBP14*<sup>EP2019</sup> using a previously described procedure (Robertson, Preston et al. 1988). An imprecise excision line with a 2405 bp deletion was isolated, termed *FKBP14*<sup>D58</sup>. The deletion removes residues 105-145 of exon 1 and the entire exon 2, including the translational start site (Fig. 2.1B). A precise excision line, *FKBP14*<sup>D34</sup>, was also isolated and confirmed by DNA sequence analysis. Both *FKBP14*<sup>D58</sup> and *FKBP14*<sup>EP2019</sup> mutants are homozygous lethal and fail to complement an independent P-element insertion, *FKBP14*<sup>D206</sup>. In contrast while *FKBP14*<sup>D34</sup> homozygotes are viable, demonstrating that *FKBP14*<sup>D58</sup> lethality is not due to a second site mutation (van de Hoef, Bonner et al. 2013). *FKBP14*<sup>D34</sup> was used as a genetic control for all subsequent experiments.

![Figure 2.1](image)

**Figure 2.1: Schematic of *Drosophila* FKBP14 domains and genomic locus**

(A) *Drosophila* FKBP14 contains an N-terminal signal peptide (SS; orange), a PPIase domain (FKBP_C; black), an EF-hand calcium binding motif (EFh; blue) and a C-terminal ER retention motif, HDEL (H; green). (B) An *FKBP14* schematic overview (Flybase version FB2009_06, Accession Number AE013599) illustrating the *FKBP14* genomic locus, shows that *FKBP14* is flanked upstream by *CG10496*, and downstream by *Sara*. Excision of *EP*(2)2019 generated *FKBP14*<sup>D58</sup> (imprecise) and *FKBP14*<sup>D34</sup> (precise; not shown) excision alleles.
FKBP14 is an ER-resident protein that is broadly expressed throughout development. Null mutations in *FKBP14* die as larvae and pupae, with rare escapers exhibiting defects in eye, wing and sensory bristle development (van de Hoef, Bonner et al. 2013). The number of microchaetae and macrochaetae are significantly reduced in *FKBP14*<sup>EP2019</sup> (data not shown) and *FKBP14*<sup>DSS</sup> pharate adults (Fig. 2.2 B and B’), compared to control (Fig. 2.2 A and A’; arrows point to single microchaetae, arrowheads mark individual macrochaetae). These phenotypes are highly reminiscent of a defect in Notch signalling, as Notch plays a significant role in sense organ development (Frise, Knoblich et al. 1996, Yaich, Ooi et al. 1998, Lai and Rubin 2001). Indeed, a reduction of the Notch downstream target, E(spl) (Jennings, Preiss et al. 1994, Castro, Barolo et al. 2005), was observed in *FKBP14*<sup>DSS</sup> presumptive nota, particularly in supraalar (Fig. 2.2 D arrow; magnified in D’) and postalar (Fig. 2.2 D arrowhead; magnified in D’’) regions, compared to control. This reduction in E(spl) expression surrounding SOPs suggests a role for FKBP14 in modulating Notch signal transduction during SOP determination. However, in this tissue and presumptive wing tissue (data not shown), a decreased number of SOPs are observed as marked by Senseless (Sens) expression (Nolo, Abbott et al. 2000) in *FKBP14*<sup>DSS</sup> (Fig. 2.2 D’ and D’”) compared to controls. Defects in Notch signalling typically result in supernumerary SOPs (Logeat, Bessia et al. 1998, Kidd and Lieber 2002), indicating FKBP14 may have multiple functions during SOP specification, some of which reflect an effect on Notch signalling and others which appear independent of Notch.
Figure 2.2: FKBP14 is required for bristle formation in adult nota.

(A – B’) Scanning electron microscopy of adult nota. (A; magnified in A’) FKBP14<sup>D34</sup> nota display organized microchaetae (arrows) and macrochaetae (arrowheads). (B; magnified in B’) FKBP14<sup>D58</sup> mutant pharate adults exhibit a severe reduction in the number of bristles on the thorax, compared to control. (C-C’’) E(spl) expression (red) is observed surrounding SOPs that have been marked with Sens (green) in FKBP14<sup>D34</sup> presumptive nota. The supraalar (arrow; magnified in C’) and postalar (arrowhead; magnified in C’’) regions represent normal levels of expression. (D-D’’) In FKBP14<sup>D38</sup> mutants, E(spl) expression (red) is reduced surrounding SOPs (green), and levels of SOPs are reduced, particularly in supraalar (arrow, magnified in D’) and postalar (arrowhead; magnified in D’’) regions. Bars, 20 µM.

** Data provided by Diana van de Hoef, adapted from (van de Hoef, Bonner et al. 2013)

In this chapter I aim to clarify the role of FKBP14 in Notch signalling. I show that FKBP14 mutant phenotypes are associated with a loss of FKBP14 transcript. Further, I show that the phenotypes observed in FKBP14 mutants are not due to ER stress and up-regulation of the unfolded protein response, but are associated, at least in part, with dysregulation of Notch signalling. I show that FKBP14 mutants genetically interact with Delta, a component of the Notch pathway, in a manner similar to the interaction observed for Presenilin. Trafficking of Notch to the membrane appears unaffected in FKBP14 mutants, suggesting that FKBP14 acts downstream of Notch activation at the membrane. Consistent with this model, we find that levels of Presenilin protein, a component of the γ-secretase protease that cleaves Notch (Mahoney, Parks et al. 2006, Stempfle, Kanwar et al. 2010), are reduced in FKBP14 null mutants to variable
degrees, while I show that Presenilin RNA levels are unaffected. I show that the activity of γ-secretase complex, of which Presenilin is the catalytic core, is reduced in FKBP14 mutants. Finally, I show preliminary evidence that FKBP14 may directly bind to Presenilin protein. Altogether, our data demonstrate that FKBP14 plays an essential role in development, and that one of its primary functions is to stabilize Presenilin protein in the endoplasmic reticulum in order to allow generation of the γ-secretase complex, which is required for Notch signal transduction.

2.3 Materials and Methods

2.3.1 Drosophila Genetics

Flies were maintained on standard media. An excision screen was performed by Diana van de Hoef on FKBP14\textsuperscript{EP2019} using a previously described procedure (Robertson, Preston et al. 1988) to generate FKBP14 alleles (van de Hoef, Bonner et al. 2013). An imprecise excision line, FKBP14\textsuperscript{D58}, with a 2405 bp deletion, including residues 105-145 of exon 1 and the entire exon 2, was isolated. Breakpoints were determined by DNA sequence analysis (ACTG). A precise excision line, FKBP14\textsuperscript{D34}, was also generated and confirmed by DNA sequence analysis. This line was used as a genetic control. The balancer CyO-GFP was used to identify homozygotes. Wild type Psn overexpression and P\textsubscript{sn}\textsuperscript{w68p} have been previously described (Guo, Livne-Bar et al. 1999).

The Df(1)N-8/FM7c (729), Dr\textsuperscript{7}/TM2 (485), w\textsuperscript{1118}, hs-flp; Adv\textsuperscript{1}/CyO (6), and FRT(42B) (1956) lines were obtained from the Bloomington Stock Center. FKBP14\textsuperscript{EP2019} (P[EP]Fkbp13\textsuperscript{EP2019}) was obtained from the Szeged Stock Center.

GMR-GAL4 was double balanced with UAS-APP C99\textsuperscript{j6} (Finelli, Kelkar et al. 2004) and males were crossed to virgin FKBP14\textsuperscript{D34}, FKBP14\textsuperscript{EP2019}, FKBP14\textsuperscript{D58}, and P\textsubscript{sn}\textsuperscript{w68p}. Crosses were carried out at 29°C and progeny were raised to 10-14 day old adults prior to ELISA analysis.

2.3.2 Immunohistochemistry

Immunostaining was performed on larval imaginal discs and embryonic cell culture as described (Patel 1994, Yeh, Zhou et al. 2000, Kim, Renihan et al. 2006, Commissio and Boulianne 2007). Briefly, larval imaginal discs were fixed with 4% paraformaldehyde, washed in PBS containing
0.1% Triton (PBT) and blocked in 5% donkey serum diluted in PBT. Tissues were then incubated with primary and secondary antibodies diluted in Block solution, with washes performed using PBT. Embryonic cells (S2, Kc167) were fixed with 4% paraformaldehyde in PBS, washed in PBS, blocked in 1% donkey serum and 3% bovine serum albumin diluted in PBT, and then incubated with primary and secondary antibodies diluted in Block solution. After 3 washes in PBT, cells and larval discs were mounted in Dako (DakoCytomation, Burlington, ON). Embryos were mounted in DABCO mounting media.

Ovaries were dissected in PBS, fixed with 5% paraformaldehyde, washed in PBS followed with PBT (0.3% Triton X-100), and blocked in 5% normal donkey serum and 0.2% bovine serum albumin in PBT. Ovaries were then incubated with primary and secondary antibodies diluted in block, with washes performed using PBT. The ovaries were then stained with DAPI (1:5000 in PBS or PBT), followed by mounting in DABCO or DAKO mounting media.

The primary antibodies used were as follows: Mouse α-Ct (1:100), rat α-ELAV (1:500), mouse α-Notch extracellular domain, EGF repeats #12-20 (C458.2H; 1:100) and mouse α-Wg (1:500) were obtained from the DSHB (University of Iowa, IA). E(spl) antibody mAb323 was a gift of Dr. S. Bray (University of Cambridge; 1:1), while guinea pig α-Sens was a gift of Dr. H. Bellen (Baylor College of Medicine, Houston, TX; 1:1000). Commercial primary antibodies include rabbit α-GFP (Invitrogen, Carlsbad, CA; 1:1000), mouse α-KDEL (MBL; 1:100), and mouse α-p120 (Calbiochem, San Diego, CA; 1:500). Rabbit α-Srp [(Sam, Leise et al. 1996); 1:500], and rabbit α-PsnNTF (Guo, Livne-Bar et al. 1999) were used as previously described. The rabbit α-PsnCTF peptide antibody was generated against amino acids 426-441 (CG18803-PB; NCBI) and was affinity purified (Antibodies Inc., Davis, CA; 1:200). Rat α-FKBP14 (cells - 1:100; ovaries, embryos, larval discs - 1:500) was generated as follows: a Drosophila FKBP14 cDNA (GH08925, amino acids 298-828; BDGP) was cloned into pGEX-4T-1/His 6C (Novagen) to produce an FKBP14 fusion protein for polyclonal antibody production in rats (Antibodies Inc., Davis, CA) and preabsorbed using fixed S2 cells.

A488 and Cy3 secondary antibodies were used (Jackson ImmunoResearch, West Grove, PA; 1:1000). Phalloidin-TRITC (Sigma-Aldrich, St. Louis, MO) was used at 1:100, DAPI was used at 1:5000.
Third instar larval CNS and discs were dissected in PBS, incubated in 0.25 mg/mL acridine orange (Invitrogen) in PBS, rinsed in PBS and mounted immediately in PBS prior to fluorescence microscopy.

2.3.3 Microscopy

Images were acquired at RT using either a Zeiss LSM510 META confocal microscope (Carl Zeiss Canada Ltd., Toronto, ON), using 40x/1.2 and 100x/1.3 objectives and standard fluorescence filters, a Leica (Leica Microsystems Inc., Concord, ON) DMRA2 Fluorescence Microscope equipped with a Hamamatsu Orca-ER digital camera, Improvision Openlab software, and 20x/0.5, 40x/1.25-0.75 or 100x/1.4 objectives, brightfield and standard filters, or a Leica DMLB Fluorescence Microscope, with 5x/0.12 objective, brightfield and CoolSNAP software. Micrographs were analyzed using an FEI (Hillsboro, OR) XL30 Scanning Electron Microscope equipped with XL Docu software. Images were processed in Photoshop CS and Illustrator CS.

2.3.4 Immunoblot Analysis

Fly lysates were prepared using standard procedures and analyzed with mouse α-β-tubulin (DSHB; 1:1000), mouse α-actin (GeneTex Inc., Irvine, CA; 1:1000), mouse α-Dcsp-2 (DSHB; 1:25), mouse α-Fz (SCBT, Santa Cruz, CA; 1:100), guinea-pig α-cad87A (gift from D. Godt, University of Toronto, Toronto, ON; 1:2000), rabbit α-PsnNTF (Guo, Livne-Bar et al. 1999), mouse α-V5 (Invitrogen, 1:1000), rat α-HA (Roche, 1:500) and rat α-FKB14 (1:2000). Fluorescent (Li-Cor) or HRP (Jackson ImmunoResearch) secondary antibodies were used (1:10 000). All blots were performed in triplicate, except where noted.

2.3.5 Quantitative RT PCR

RNA was extracted using Trizol (Invitrogen). RNA was reverse transcribed using the Superscript First-Strand system (Invitrogen). The results were normalized to an internal control, RP49 (three separate experiments, >10 samples each). The primer sequences used were the following: FKBP14: AGCTGATCAACATCGGCAAT (+) and CCAAGAACCCCCATTATTGA (-); CG10496 (TAF1C-like): ATAAAGGGGAAGGAGCTGGA (+) and GGCGGCATATAGCTTTGGTA (-); SARA: CACCGACGATCAGAGTAAGTGAGA (-) and GGCAGCCATATAGCTTTGGTA (-); SARA: CACCGACGATCAGAGTGAGA (+) and
CTCCGCAATCCGTGTATCT (-); Hsc3: GCGAACAAGATACCGATGCT (+), GTTATCGGAGGCAGTGGA (-).

2.3.6 ELISA analysis

The levels of Aβ40 and Aβ42 were determined using commercially available human Aβ specific ELISA kits (Invitrogen) according to the manufacturers instructions. Heads from 10-14 day old adult progeny were lysed in RIPA buffer (50mM Tris, 150mM NaCl, 1% SDS, 1% NP-40, 0.5% deoxycholate, pH 8.0) containing complete protease inhibitor cocktail (Roche). Lysates were diluted 5X or 10X with PBS containing protease inhibitors, followed by a further 1:1 dilution with kit dilution buffer containing protease inhibitors prior to analysis.

2.3.7 Plasmid Construction

The Psn constructs were based on flies generated and described by the Hui-Min Chung lab, with an HA epitope within the large cytoplasmic loop between transmembrane domain (TMD) 6 and 7 (Barakat, Mercer et al. 2009). Both Psn wt HA and Psn ΔE9 HA were amplified from genomic DNA purified from these transgenic lines. GFP HA was generated as a control vector. Insertion of the HA tag was done using Quikchange Lightning Site Directed Mutagenesis Kit (Stratagene). All constructs were cloned into pENTR/D/TOPO vectors and subcloned into cell culture destination vectors using Gateway recombination (Invitrogen).

The primers used were as follows (5’ → 3’):

- **Psn into pENTR**: CACCAAAATGGCTGCTGTCAATCTCC and TTATATAAACACCTGCTTGAGGA T
- **GFP into pENTR**: CACCAAAATGGTGAGCAAGGG

To insert HA at C-terminus of GFP:

TTAGGCGTAATCGGGCACACATCGTAGGCTAGGGTAGGCCTAATCGGGCACATCGTAGGGGTTACTGGCTACAGCTCGTCCATGCC

2.3.8 Coimmunoprecipitation

Transfected or control Kc cells (~1 x 10^7 cells) were harvested in ice cold PBS and pellets were flash frozen on dry ice. Pellets were thawed on ice and lysed for 30 min on ice in 300ml lysis buffer containing 125mM NaCl, 50mM HEPES pH 7.4, 1% chaps (w/v), and Complete protease inhibitor cocktail (Roche). Lysates were pre-cleared with 70% protein G slurry (Sigma-Aldrich) and centrifuged (14k rpm for 15 min). Cleared lysates were incubated overnight at 4°C with
protein G sepharose beads and specific antibodies (mouse anti-V5 or rat anti-FKBP14). Beads were washed with lysis buffer and resuspended in 4X NuPAGE LDS sample buffer (Life Technologies) under reducing conditions and electrophoresed on 4-12% NuPAGE Bis-Tris gels in MOPS running buffer (Life Technologies). Gels were then processed for Western blotting and immunodetection with rat α-HA (Roche, 1:500), rabbit α-V5 (Invitrogen, 1:1000), or rat α-FKBP14 (1:2000). Immunodetection was performed using either Li-Cor fluorescent detection (Li-Cor) or ECL (PerkinElmer).

2.4 Results

2.4.1 **FKBP14** Mutants Display Loss of FKBP14 Transcript and Protein

There are 8 known FKBP12s in *Drosophila* that share homology with the archetypal human FKBP12. Sequence analysis of one of these, *Drosophila* CG9847, hereafter referred to as FKBP14, reveals a signal peptide (SS), peptidyl-prolyl cis-trans isomerase (PPIase) domain (FKBP_C), an EF-hand motif (EFh) and an ER retention signal known as HDEL (Fig. 2.1A). The signal sequence is predicted to be cleaved between positions 23 and 24 (VRA-QD) using neural networks and hidden Markov models trained on eukaryotes (Nielsen and Krogh 1998, Bendtsen, Nielsen et al. 2004). This analysis suggests FKBP14 is an ER resident member of the FKBP family.

Null mutations in **FKBP14** are lethal throughout larval and pupal stages of development with escapers exhibiting defects in eye, wing and sensory bristle development (van de Hoef, Bonner et al. 2013). To ensure that both P-element insertion and deletion of **FKBP14** did not affect expression of neighboring genes, I performed quantitative RT-PCR analysis. The neighboring genes, CG10496 (*TAF1C-like*) and *Sara*, lie 1691 bp upstream of FKBP-RA exon 1 and 58 bp downstream from exon 5, respectively (Fig. 2.1 B). I observed a significant reduction in **FKBP14** expression from **FKBP14**mutant homozygous larvae, compared to controls (*p ≤ 0.001), with no significant changes in expression levels of either CG10496 or *Sara* (Fig. 2.3 A). Similar results were obtained for **FKBP14**mutant (data not shown). Together, these data demonstrate that both the P-element insertion and the deletion significantly affect **FKBP14** transcription but not transcription of neighbouring genes.
Figure 2.3: FKBP14 transcript levels, protein expression, and subcellular localization

(A) qRT-PCR analysis of CG10496, SARA and FKBP14 transcript levels in FKBP14<sup>D58</sup> and FKBP14<sup>EP2019</sup> third instar larval extracts, as compared to FKBP14<sup>D34</sup> control, shown as % FKBP14<sup>D34</sup> control. Error bars represent ± standard deviation. FKBP14 transcript levels are significantly reduced in FKBP14<sup>D58</sup> mutants (**p<0.001; unequal variance t-test), while CG10496 and Sara transcript levels are not significantly reduced, compared with control (n = 3). RP49 was used as an internal control. (B) In pharate adult extracts, FKBP14 expression is absent in FKBP14<sup>D58</sup> and reduced in FKBP14<sup>EP2019</sup>, compared to FKBP14<sup>D34</sup> controls. β-actin was used as a loading control. (C - C") FKBP14 (red) is expressed in a punctate pattern in S2 cells and colocalizes with anti-KDEL (green). Colocalization (yellow) is indicated in the merge. (D - D") FKBP14 (red) does not colocalize with the Golgi marker anti-p120 (green) in S2 cells as indicated by the lack of colocalization (yellow) in the merge. As a control, S2 cells were stained using preimmune sera, and we failed to detect similar localization patterns (data not shown). Cell stains represent single plane images.

** Fig. 2.3 C-D" data provided by Diana van de Hoef, adapted from (van de Hoef, Bonner et al. 2013)
To confirm our transcriptional data, I performed Western analysis using antibodies raised against a *Drosophila* FKBP14 fusion protein (van de Hoef, Bonner et al. 2013) to examine FKBP14 protein expression in wildtype and mutant flies. I observed a dramatic reduction of FKBP14 in animals homozygous for the original insertion line, *FKBP14<sup>EP2019</sup>*, with most samples showing no detectable protein (Fig. 2.3 B). As expected, no protein was detected in the deletion mutant, *FKBP14<sup>D58</sup>*\textsuperscript{1}, confirming that this allele represents an *FKBP14* null mutation (Fig. 2.3 B). These data also demonstrate that the antibody is specific to *Drosophila* FKBP14.

### 2.4.2 FKBP14 is an ER Resident Protein that is Broadly Expressed Throughout Development

To determine the subcellular localization of FKBP14, Diana van de Hoef performed immunocytochemical studies in *Drosophila* Schneider 2 (S2) cells, where FKBP14 is endogenously expressed. She found that *Drosophila* FKBP14 colocalized with anti-KDEL, an ER marker (Fig. 2.3 C-C”), but not with anti-p120, a Golgi marker (Fig 2.3 D-D”). These data are consistent with FKBP14 sequence analysis, which reveals both a signal sequence and an ER retention sequence. FKBP14 is broadly expressed throughout development, including throughout the wing disc, eye disc, CNS optic lobes, and in the ventral nerve cord (van de Hoef, Bonner et al. 2013), suggesting a role for FKBP14 in multiple tissue types during development.

### 2.4.3 *FKBP14* Mutant Phenotypes are Not Due to Global ER Stress

PPIases catalyze the *cis-trans* isomerisation of peptidyl-prolyl amide bonds and are implicated in multiple intracellular processes, including protein folding (Gothel and Marahiel 1999). FKBP14 contains a single PPIase domain, suggesting that reduced FKBP14 expression could result in the accumulation of misfolded proteins, a condition that leads to ER stress and activation of the Unfolded Protein Response (UPR) (Ryoo and Steller 2007). Once activated, the UPR helps to reduce ER stress by attenuating protein synthesis, enhancing degradation of misfolded ER proteins and inducing expression of ER resident chaperones (Ryoo and Steller 2007). The ER chaperone BiP is a well-known component of the UPR and its transcription is upregulated when UPR is active (Ryoo and Steller 2007). To determine if loss of FKBP14 causes a global ER stress response, I examined the transcript levels of a *Drosophila* BiP homolog, *Hsc3*, using quantitative reverse-transcription PCR (qRT-PCR) (Hirota, Kitagaki et al. 2006). As a positive control, *FKBP14<sup>D34</sup>* larvae were placed on food containing Tunicamycin (Tm), which inhibits N-
glycosylation and causes accumulation of unfolded proteins in the ER (Kaufman 1999). This resulted in a significant increase in Hsc3 levels compared to vehicle-treated control (Fig. 2.4 A). However, Hsc3 transcript levels in FKBPI4EP2019 and FKBPI4DS8 third instar larvae were similar to those in control FKBPI4D34 (Fig. 2.4 A). In addition, I examined levels eIF2α phosphorylation in our mutants by Western blot analysis. Upon ER stress, eIF2α is phosphorylated by PERK, resulting in translational attenuation (Ryoo and Steller 2007). I did not observe a significant increase in phosphorylated eIF2α in FKBPI4 mutants compared to controls (Fig. 2.4 B). These data indicate that phenotypes observed in FKBPI4EP2019 and FKBPI4DS8 mutants are not associated with induction of ER stress.

Figure 2.4: Loss of FKBPI4 does not induce the Unfolded Protein Response

(A) qRT-PCR analysis of Hsc3 transcript levels in FKBPI4EP2019 and FKBPI4DS8 in third instar larval extracts, shown as % of FKBPI4D34 control. Tunicamycin (Tm) treated FKBPI4D34 were used as a positive control. While Tm-treated control larvae show a significant increase in Hsc3 transcript (*p=0.01; unequal variance t-test), Hsc3 transcript is at control levels in FKBPI4EP2019 and FKBPI4DS8. (B) Western analysis of phospho-eIF2α levels in FKBPI4EP2019, FKBPI4DS8 and Psnw6rp in third instar larval extracts. Levels of phospho-eIF2α do not appear significantly increased in FKBPI4 or psn mutants relative to control FKBPI4D34, indicating that the UPR is not significantly induced in these animals.

Another possibility is that upon activation of the UPR, FKBPI4 may be among the cohort of chaperones and other components that are upregulated to reduce protein overload in the cell (Ryoo and Steller 2007). Given that FKBPI4 may have protein folding functions, I asked
whether induction of ER stress induces expression of FKBP14. While qRT PCR data were unclear (data not shown), I examined levels of FKBP14 by Western blot in S2 cells under either constant Tm over increasing time (Fig. 2.5 A), or increasing Tm over constant time (Fig. 2.5 B). In both cases I was able to observe increased levels of phospho-eIF2α following Tm treatment while levels of FKBP14 did not change significantly, indicating that FKBP14 levels are not regulated by ER stress (Fig. 2.5). Taken together, these data indicate that loss of FKBP14 does not induce the UPR, nor does UPR induce FKBP14, suggesting that FKBP14 plays a more specific role in the ER.

Figure 2.5: FKBP14 is not induced by ER stress

(A) 10µg Tm was applied to Drosophila S2 cells for 0, 2, 4, or 6hr increments, and levels of phospho-eIF2α and FKBP14 were monitored by Western analysis. Cells treated with carrier only (0µg Tm) were used as a control. While phospho-eIF2α levels increased over time, levels of FKBP14 were unchanged (n=3). (B) Increasing concentrations of Tm (0, 1, 10, 50µg) were applied to Drosophila S2 cells for 6hr, and levels of phospho-eIF2α and FKBP14 were monitored by Western analysis. While phospho-eIF2α levels increased with increasing Tm, FKBP14 levels did not significantly change (n=3).

2.4.4 FKBP14 Mutants Genetically Interact with Notch, Delta, and presenilin

It was previously demonstrated that FKBP14 genetically interacts with Psn, such that mutations in FKBP14 can suppress phenotypes associated with over-expression of Psn (van de Hoef, Hughes et al. 2009). One of the essential developmental roles for Psn is as a core member of the Notch signalling pathway (Bray 2006). Together with the Notch-like phenotypes observed in FKBP14 mutant escapers (Fig. 2.2 and data not shown), these results suggest that FKBP14 may modify the Notch pathway. To determine if FKBP14 interacts with the Notch pathway, I
performed genetic epistasis experiments using \textit{FKBP14} and the Notch ligand \textit{Delta}, while Diana van de Hoef examined the interaction between \textit{Psn} and \textit{FKBP14}, I also examined a mutation in \textit{Psn}, which had previously been shown to modulate both Notch and Delta phenotypes in transheterozygotes (Guo, Livne-Bar et al. 1999). We did not observe any significant defects in wing development in our heterozygous mutant \textit{FKBP14} or \textit{Psn} lines on their own (Fig. 2.6 C and D). I then analyzed whether \textit{FKBP14} mutants genetically interact with a \textit{Delta (Dl)} mutation that causes wing vein defects (Fig. 2.6 E; arrow points to a slight delta between the L4 and L5 wing veins, arrowhead indicates thickening of the L2 wing vein). I found that transheterozygotes of \textit{Dl} and \textit{FKBP14\textsuperscript{DSS}} exhibit enhanced wing vein thickening (arrow) and ectopic deltas (asterisk) (Fig. 2.6 G), compared to controls (Fig. 2.6 C, E, and F). Interestingly, transheterozygotes of \textit{Psn\textsuperscript{w6rp}}, and \textit{Dl} displayed a similar enhancement of wing vein thickening as observed for \textit{FKBP14\textsuperscript{DSS}} (Fig. 2.6 H), relative to controls (Fig. 2.6 D and E).

**Figure 2.6: \textit{FKBP14} mutants genetically interact with Notch and Delta**

Brightfield images of adult wings. WT flies (A), and \textit{FKBP14\textsuperscript{D34}}/\textit{FKBP14\textsuperscript{D34}} control (B) adult wings have a smooth wing margin, similar to \textit{FKBP14\textsuperscript{DSS}}/+ heterozygotes (C), and \textit{psn\textsuperscript{w6rp}}/+ heterozygotes. \textit{Dl\textsuperscript{7}/TM2} flies (E) exhibit mild deltas (arrow) and thickening of L2 wing veins (arrowhead). Distal wing blade notching (arrow) is observed in a Notch deficiency allele, \textit{DfN-8/+} (I). \textit{FKBP14\textsuperscript{D34};Dl\textsuperscript{7}} (F) do not show any enhancement of these phenotypes, while \textit{FKBP14\textsuperscript{DSS};Dl\textsuperscript{7}} transheterozygotes (G) show enhanced Delta phenotypes, including extended L2 vein thickening (arrow) and ectopic deltas (asterisk), similar to that observed for the positive control \textit{Dl\textsuperscript{7}/psn\textsuperscript{w6rp}} transheterozygotes (H). \textit{DfN-8;FKBP14\textsuperscript{D3}} (J) also do not show any enhancement, while notching along the distal blade is enhanced in \textit{DfN-8;FKBP14\textsuperscript{DSS}} transheterozygotes (K). Both phenotypes were fully penetrant.

** Data for DfN-8 crosses provided by Diana van de Hoef
Similar results were observed using a Notch deficiency, Df(1)N-8, that causes notching of the distal wing margin due to haploinsufficiency (Fig. 2.6 I; arrow indicates notching), for which transheterozygotes of FKBPI4\textsuperscript{058} and the Notch deficiency exhibit enhanced wing notching (Fig. 2.6 K), compared to controls (Fig. 2.6 C, I and J). Both phenotypes were fully penetrant.

Together with the previous observation that loss-of-function mutations in FKBPI4 can suppress a dominant phenotype caused by overexpression of Psn (van de Hoef, Hughes et al. 2009), these data demonstrate that FKBPI4 genetically interacts with members of the Notch pathway during wing development and that the phenotypes observed in FKBPI4 mutants may be due, at least in part, to defects in Notch signalling.

To examine whether this interaction with the Notch pathway was detectable for other FKBPs, I performed the same genetic epistasis experiments with Delta, using available mutations in three other Drosophila FKBPs: FKBP59, FK506-bp2 (FKBP12), and CG14715 (FKBP13) (Fig. 2.7 L-N). I used both the deletion and insertion mutant alleles of FKBPI4 and the Psn null mutant as positive controls and observed the same interaction for these lines as described above (Fig. 2.7 A-J). I did not, however, detect a similar level of enhancement of the Dl\textsuperscript{7} wing vein phenotype for the other FKBPs tested (Fig. 2.7 P-R) compared to the Dl\textsuperscript{7} control (Fig. 2.7 O). While a possible interaction between these mutants and the Notch pathway cannot be ruled out by this experiment, these results suggest that FKBPI4 may be affecting the Notch pathway to a greater degree under these circumstances.
Figure 2.7: Mutations in other FKBP's do not appear to genetically interact with Delta to the degree observed for FKBP14

Brightfield images of adult wings. WT flies (A), and FKBP14\textsuperscript{D34}/+ control (B) adult wings have a smooth wing margin, similar to FKBP14\textsuperscript{D58}/+ heterozygotes (C), FKBP1\textsuperscript{EP2019}/+ heterozygotes (D) and psn\textsuperscript{w6rp}/+ heterozygotes (E). Dl\textsuperscript{7}/TM2 flies (F) exhibit mild deltas (arrowhead) and thickening of L2 wing veins (arrow). As previously observed, FKBP14\textsuperscript{D34};Dl\textsuperscript{7} (G) do not show any enhancement of these phenotypes, while FKBP14\textsuperscript{D58};Dl\textsuperscript{7} transheterozygotes (H), and FKBP14\textsuperscript{EP2019};Dl\textsuperscript{7} transheterozygotes (I) show enhanced Delta phenotypes, including extended L2 vein thickening (arrow), enhanced deltas (arrowheads) and ectopic deltas (asterisk), similar to that observed for the positive control Dl\textsuperscript{7}/psn\textsuperscript{w6rp} transheterozygotes (J). However, flies transheterozygous for mutations in Dl\textsuperscript{7} and FKBP59 (P), FK506-bp2 (FKBP12, Q), and CG14715 (FKBP13, R) did not show a significant enhancement of the Delta phenotypes relative to their controls (K – O).

2.4.5 FKBP14 is Not Required for Notch Trafficking to the Plasma Membrane

Given the genetic interaction between FKBP14 mutants and both Notch and Delta mutants, as well as the fact that FKBP14 mutant escapers display Notch-like phenotypes in both the notum and wing, and defects in downstream targets of the Notch pathway in both tissues (Fig. 2.2) (van de Hoef, Bonner et al. 2013), we sought to determine how FKBP14 affects the Notch pathway. In vertebrates, Notch is synthesized in the ER and then processed in the Golgi, leading to formation of a heterodimeric receptor at the plasma membrane, while in Drosophila, the majority of Notch protein at the plasma membrane is uncleaved (Logeat, Bessia et al. 1998, Kidd and Lieber 2002). FKBP14 is an ER-resident protein that may be involved in protein folding; therefore we examined membrane trafficking of the Notch receptor in FKBP14 mutants. We found that Notch is predominantly localized to the plasma membrane in FKBP14\textsuperscript{D58} presumptive nota tissues (Fig. 2.8 B), as in control flies (Fig. 2.8 A). While we cannot rule out subtle defects in Notch trafficking, based on these findings we conclude that trafficking of Notch to the plasma membrane is not grossly affected in FKBP14 mutants. As a control, we examined the surface levels of Notch in a Presenilin null mutant, which interrupts Notch signalling downstream of receptor activation at the plasma membrane. As in our FKBP14 mutants, trafficking of Notch to the cell surface is not grossly affected in the Psn null mutant, w6rp (Fig. 2.8 C).
2.4.6 FKBP14 Acts in the ER to Maintain Psn Protein Levels

While trafficking of Notch to the plasma membrane appears largely unaffected in FKBP14 mutants, Notch target gene expression is reduced suggesting that FKBP14 likely acts downstream of Notch activation at the membrane. The γ-secretase complex plays a key role in Notch activation by cleaving Notch to release the intracellular domain (Selkoe and Kopan 2003). The γ-secretase complex is made up of four essential components: Anterior pharynx defective -1 (Aph-1), nicastrin (Nct), presenilin enhancer 2 (Pen-2), and the catalytic core Psn (Selkoe and Kopan 2003). To assess whether FKBP14 could interact with components of the γ-secretase complex, we examined the subcellular localization of endogenous FKBP14, Psn and transiently transfected Aph-1, Nct, and Pen-2 in Drosophila cells. Colocalization was previously detected between FKBP14 and transiently transfected Aph-1 as well as Psn^{CTF} and Psn^{NTF}, but not Nct or Pen-2 (van de Hoef, Bonner et al. 2013), suggesting that FKBP14 may interact with some members of the γ-secretase within the early secretory pathway.
Figure 2.8: FKBP14 is required to maintain Psn protein levels in the ER.

Notch is detected at the plasma membrane in $FKBP14^{D34}$ (A), $FKBP14^{D58}$ (B) and $psn^{w6rp}$ (C) third instar presumptive wing margins. Bars, 20 µM. (D) Psn$^{NTF}$ protein levels are reduced in $FKBP14^{D58}$, $FKBP14^{EP2019}$ and $psn^{w6rp}$ mutant extracts (top blot). FKBP14 expression is not affected in $Psn^{w6rp}$ mutant extracts (middle blot). β-tubulin shows equal loading (bottom blot). (E) qRT-PCR analysis of Psn transcripts shows reduced Psn expression in $Psn^{w6rp}$ mutant extracts, but no significant changes in $FKBP14^{D58}$ extracts. (F) Loss of FKBP14 does not result in significant loss of other single and multipass transmembrane proteins.

**Fig. 2.8 A-D provided by Diana van de Hoef, adapted from (van de Hoef, Bonner et al. 2013)**
Given that we identified *FKBP14* as a genetic interactor with *Psn* (van de Hoef, Hughes et al. 2009) and observed that *Psn* mutants modify Notch mutant wing phenotypes, similar to *FKBP14* mutants, we tested for loss of *Psn* in *FKBP14* mutant escapers. We examined *Psn* protein levels in *FKBP14* mutants using an anti-*Psn*NTF antibody (Guo, Livne-Bar et al. 1999). It was found that levels of endogenous *Psn* are indeed reduced in *FKBP14* mutants. In the most extreme cases, *Psn* levels are dramatically reduced in *FKBP14* homozygous mutants, as compared to controls (Fig. 2.8 D), however the effect on *Psn* is variable (data not shown), possibly due to variability in maternal loading of *FKBP14* (see Chapter 3). We also tested whether *FKBP14* levels were affected by a loss *Psn* and found no significant change (Fig. 2.8 D), suggesting that *FKBP14* acts upstream of *Psn*. I showed that the effects on *Psn* levels are post-transcriptional, as levels of *Psn* mRNA are not reduced in *FKBP14* mutants compared to controls (Fig. 2.8 E). I also examined levels of several other single and multipass transmembrane proteins in *FKBP14* mutants and found no significant loss (Fig. 2.8 F), suggesting that the loss of *Psn* protein in *FKBP14* mutants may be specific.

### 2.4.7 Loss of *FKBP14* Results in Loss of γ-secretase Cleavage Activity

The reduction in *Psn* protein levels observed in our homozygous mutants suggests that loss of *Psn*-dependent processes, such as γ-secretase cleavage of the Notch receptor, may be at least partially responsible for the Notch-like phenotypes observed in our *FKBP14* pharate adult mutants. To assess whether γ-secretase cleavage is affected in our mutants, I expressed the C99 fragment of human APP (Finelli, Kelkar et al. 2004), a direct γ-secretase target, in wild-type and heterozygous *FKBP14* mutant backgrounds using the GMR-GAL4 driver (Fig. 2.9 A). Specifically, I prepared extracts from heads of 10-14 day old flies and measured the levels of γ-secretase dependent cleavage fragments, Aβ40 or Aβ42, by ELISA. I observed a significant reduction in Aβ42 fragments in flies expressing APP-C99 in a *FKBP14* mutant heterozygous background compared to controls (Fig. 2.9 B). The levels of Aβ40 also trended towards a reduction compared to controls, however these results were not statistically significant. Importantly, the reduction in levels of Aβ40 and Aβ42 observed in *FKBP14* mutants was similar to that observed in heterozygous *Psn* mutants (Fig. 2.9 B). These results indicate that γ-secretase activity is indeed reduced in *FKBP14* mutants relative to controls, perhaps due to the loss of *Psn* protein observed in our homozygous mutants.
Taken together, these data indicate that FKBP14 likely functions directly or indirectly to stabilize Psn in the ER and in the absence of FKBP14, Psn protein levels are reduced resulting in reduced γ-secretase activity and Notch-related developmental defects.

Figure 2.9: γ-secretase cleavage activity is reduced in FKBP14 mutants

(A) Genetic crossing scheme to test endogenous γ-secretase cleavage activity in mutants (FKBP14D58 and FKBP14EP2019, FKBP14EP2019 shown in example final genotype) relative to...
controls (FKBP14ΔD4 and psnΔw6rp) (B) Aβ40 and Aβ42 levels in GMR-GAL4>UAS-APP C99 flies trans-heterozygous for FKBP14 or psn mutations. Measurements are normalized to the levels of Aβ proteins in control flies (precise-excision background), and standard deviation was calculated in cases where at least three experiments of the same genotype were available. Aβ42 levels are significantly reduced in FKBP14 (GMR/2019;UAS-C99/+)) and psn (GMR/+;UAS-C99/w6rp) transheterozygous mutant lines expressing APP C99 compared to control *(p<0.05; unequal variance t-test). Error bars represent ± standard deviation.

2.4.8 FKBP14 May Directly Interact with Psn

The observed loss in γ-secretase activity may occur as a result of a defect in stability of the complex or its components, or assembly of the components into a functional complex. To begin investigating how loss of FKBP14 is affecting γ-secretase activity, I asked whether FKBP14 may directly interact with members of the γ-secretase complex in the ER. I designed and generated cell culture constructs of wild-type FKBP14 and tagged γ-secretase components Psn, Nct, Pen-2, and Aph-1. The Psn constructs were based on flies generated and described by the Hui-Min Chung lab, placing an HA epitope within the large cytoplasmic loop between transmembrane domain (TMD) 6 and 7 (Barakat, Mercer et al. 2009).

Given that FKBP14 and Psn partially colocalize (van de Hoef, Bonner et al. 2013) and the observed effect of FKBP14 mutations on Psn protein levels, I asked whether FKBP14 and Psn could form a stable complex. I carried out immunoprecipitation (IP) experiments based on a method described by Spasic et al., wherein they identified a protein that interacts with γ-secretase in the early secretory pathway (Spasic, Raemaekers et al. 2007). To optimize IP conditions that allows the γ-secretase complex or subcomplexes remain intact, I tested co-IP of known γ-secretase components. Following IP for V5 tagged Nct, I was able to co-IP both wt Psn and a mutant form of Psn missing exon 9 (Psn ΔE9), which removes the endonuclease cleavage site without affecting Psn function (Barakat, Mercer et al. 2009) (Fig. 2.10 B, red arrows indicate IP lanes for Psn wt and ΔE9), compared to the GFP control (Fig. 2.10 B). Importantly, though I was able to detect endogenous FKBP14 in the IP input (Fig. 2.10 A, bottom panel), I did not detect co-IP of FKBP14 with Nct, or with the Nct-Psn complex (Fig. 2.10 B, bottom panel). Thus, under these conditions, FKBP14 either does not associate with Nct or subcomplexes of γ-secretase containing Nct, or perhaps any such interaction may be transient or too low a concentration to detect. This is in keeping with previous data indicating that FKBP14 colocalizes
with both Psn and Aph-1, but not with Nct or Pen-2 in cell culture (van de Hoef, Bonner et al. 2013).

**Figure 2.10: Nct V5 efficiently IPs Psn, but not FKBP14**

(A) IP input showing expression levels of various transfected constructs, as detected by Western analysis for HA (top panel) or V5 (middle panel) tags. FLAG and actin blots were too weak to detect after multiple strips (data not shown). Endogenous levels of FKBP14 are shown (bottom panel), and indicate approximately equal loading for all samples except Pen-2 HA. (B) Psn wt HA and Psn ΔE9 HA co-IP with Nct V5 (top panel, red arrows indicate Psn wt and Psn ΔE9 lanes), while control GFP HA does not co-IP. Note that FKBP14 does not appear to co-IP with either Nct V5 on its own (second lane, bottom panel) or the Nct-Psn complexes (last two lanes, bottom panel) (n=2).
The γ-secretase complex, and components thereof, have been shown to associate with a large range of proteins and targets (Winkler, Hobson et al. 2009, Jurisch-Yaksi, Sannerud et al. 2013), which may obscure a low level or transient interaction with FKBP14. I therefore sought to determine whether an interaction between FKBP14 and Psn may be enriched by IP of endogenous FKBP14. I first established conditions to IP endogenous FKBP14 using the polyclonal FKBP14 antibody (Fig. 2.11 A). I then performed co-IP analysis for cells singly transfected with either Psn wt HA, Psn ΔE9 HA, or a GFP HA control (Fig. 2.11 B). Both Psn wt HA and Psn ΔE9 HA were co-immunoprecipitated with anti-FKBP14 antibody, while control GFP HA was not (Fig. 2.11 B, second panel, red arrows highlight Psn wt and Psn ΔE9 lanes). These data indicate that FKBP14 and Psn physically interact. Of note, while an interaction with full length Psn was seen, there did not appear to be any co-IP with the NTF or CTF. Taken together with the interaction observed between FKBP14 and an uncleavable form of Psn, Psn ΔE9, it is possible that this interaction occurs prior to endolytic cleavage. Indeed, this may explain why the interaction is undetectable by IP of the γ-secretase complex directly.

Taken together, these data indicate that FKBP14 may physically interact with full length Psn in the ER and that such interaction is required for Psn protein stability. Whether FKBP14 binding is required for proper folding of Psn or stabilization until endolytic cleavage and association with other γ-secretase components remains to be seen.
Figure 2.11: Psn co-immunoprecipitates with endogenous FKBP14

(A) Input and IP demonstrating that our polyclonal anti-FKBP14 antibody can successfully IP endogenous FKBP14 from Drosophila Kc cell culture. (B) Input and IP for Kc cells singly transfected with either GFP HA, Psn wt HA, or Psn ΔE9 HA. While GFP-HA is not immunoprecipitated with anti-FKBP14 antibody, both Psn wt and Psn ΔE9 HA are immunoprecipitated (red arrows indicate Psn IP lanes) (n=3).

2.5 Discussion

Presenilin is synthesized within the ER, and rapidly cleaved generating N- and C-terminal fragments that are essential for γ-secretase catalytic activity (Thinakaran, Borchelt et al. 1996, Kimberly, Xia et al. 2000). We identified a novel genetic interactor of Psn, Drosophila FKBP14, which is a previously uncharacterized member of the FKBP family of immunophilins (van de Hoef, Hughes et al. 2009). FKBP14 has a single PPIase domain, a calcium-binding EF hand, as well as an N-terminal signal sequence and C-terminal endoplasmic HDEL motif. Here we have further defined the role of FKBP14 in Notch signalling and begun exploring the broader role of FKBP14 in Drosophila development.
*FKBP14* mutations give rise to developmental defects, a subset of which are similar to those previously identified in Notch loss-of-function mutants, and show a decrease in expression of Notch target genes, such as Ct and E(spl). I have shown that these phenotypes are downstream of a loss of *FKBP14* transcript and protein. Importantly, induction of the UPR does not seem to upregulate *FKBP14* levels, indicating that *FKBP14* does not appear to function as a general ER chaperone in this context. I have further shown that the UPR does not appear to be upregulated in *FKBP14* mutants indicating that the Notch-like phenotypes are not downstream of ER stress.

With respect to Notch signalling, I have shown that *FKBP14* genetically interacts with the *Delta* during wing vein patterning, similar to what was shown for *Psn* and *Delta* transheterozygotes (Guo, Livne-Bar et al. 1999). Further, heterozygous loss of *FKBP14* modifies the *Delta* mutant phenotype in a manner similar to heterozygous loss of *Psn*, suggesting that these mutations may have similar consequences.

Notch signalling can be regulated during a number of steps, including receptor synthesis, transport to the membrane and proteolysis. In *Drosophila*, the majority of Notch protein at the plasma membrane is uncleaved (Kidd and Lieber 2002). Activation of Notch occurs at the cell surface upon interaction with its ligands and subsequent γ-secretase mediated proteolysis (Gupta-Rossi, Six et al. 2004). Loss of *FKBP14* does not affect trafficking of Notch to the membrane, yet it does result in reduced expression of Notch target genes. Though we cannot rule out the possibility that subtle effects on Notch receptor trafficking contribute to the *FKBP14* null phenotype, these results suggest that *FKBP14* likely affects components of the Notch pathway downstream of Notch localization at the plasma membrane. Consistent with this, we find that *Psn* protein but not RNA levels are often reduced in *FKBP14* mutants. Co-IP data indicate that *FKBP14* may physically interact with full length *Psn*. The holoprotein, unlike the N- and C-terminal fragments, is highly unstable and must incorporate into a larger complex for stabilization (Ratovitski, Slunt et al. 1997). A putative role for *FKBP14* may therefore be to properly fold or stabilize *Psn* in the ER allowing for formation of a functional γ-secretase complex that is required for cleavage and activation of Notch. Consistent with this hypothesis, I found that γ-secretase activity is reduced in flies heterozygous for mutations in *FKBP14*, in a manner similar to that observed in flies heterozygous for *Psn* mutations.
Previous studies in yeast have shown that all cyclophilins and FKBPs are individually and collectively dispensable for viability (Dolinski, Muir et al. 1997) and their roles in multicellular organisms have yet to be determined. Of note, a recent study has linked mutations in human FKBP14 to recessive developmental disorders with various congenital symptoms (Baumann, Giunta et al. 2012). These data demonstrate that FKBP14 is required for metazoan development and describes, among other potential roles, a novel requirement for FKBP14 in development, viability, Psn protein stability and Notch signalling.
FKBP14 has Notch-Independent Roles in *Drosophila* Development

Evidence for the clonal inviability phenotype observed in wing discs of *FKBP14* mutants was published in *Development* 2013 Feb;140(4):810-9 (JMB data). Unique data images are shown here (Fig. 3.4).
3  FKBP14 has Notch-Independent Roles in *Drosophila* Development

3.1  Summary

FKBP14 is a member of the highly conserved FK506-binding protein (FKBP) family, members of which are found in a broad range of organisms and have been implicated in various biochemical processes (Barik 2006, Kang, Hong et al. 2008). Despite their high level of conservation, previous studies have shown that all FKBPs are dispensible for viability in yeast (Dolinski, Muir et al. 1997), suggesting that members of this family likely interact with a unique set of partners to perform specific, non-essential functions. However, emerging evidence in multicellular models indicates that FKBPs regulate multiple essential intracellular pathways in highly specific manners, depending on the biological context (Solassol, Mange et al. 2011). We have previously demonstrated that FKBP14 is required for *Drosophila* development, one aspect of which includes regulating Notch signalling via the γ-secretase complex (Chapter 2 and (van de Hoef, Bonner et al. 2013)). Here I show that FKBP14 is required for cell viability in multiple tissue types, in an apparently Notch-independent manner, suggesting that FKBP14 has multiple roles throughout *Drosophila* development.

3.2  Introduction

FKBP14 belongs to the highly conserved FK506-binding protein (FKBP) family, one of the two largest families of immunophilins, with many members sharing the ability to bind FK506, and often exhibiting peptidyl-prolyl cis-trans isomerase (PPIase) activity (Barik 2006, Kang, Hong et al. 2008). FKBPs are found in a broad range of organisms and have been implicated in various biochemical processes, including protein folding, receptor signalling, protein trafficking and transcription (Barik 2006, Kang, Hong et al. 2008). FKBP family members also exhibit distinct subcellular localizations and bind specific protein targets (Galat 2008, Kang, Hong et al. 2008). Several FKBPs and their ligands have been shown to regulate multiple intracellular pathways, depending on the biological context (Solassol, Mange et al. 2011).

We previously identified a requirement for FKBP14 in *Drosophila* development. I have shown that this is at least partly due to a role in regulating Notch signalling via γ-secretase [Chapter 2
and (van de Hoef, Bonner et al. 2013)]. However, *FKBP14* mutants are lethal throughout development and most die during mid-pupal stages. This variation in lethality may be due to perdurance of maternal contribution, which may be concealing early defects associated with loss of FKBP14. Moreover, FKBP14 is expressed over a broad range of tissues and developmental timepoints (van de Hoef, Bonner et al. 2013) and may have multiple related or independent functions throughout development. Indeed, Diana van de Hoef observed a decrease in sense organ precursor (SOP) levels as marked by Senseless (Sens) in *FKBP14*mutants compared to controls (van de Hoef, Bonner et al. 2013), in contrast to typical defects in Notch, which result in supernumerary SOPs (Logeat, Bessia et al. 1998, Kidd and Lieber 2002, Mummery-Widmer, Yamazaki et al. 2009). Moreover, immunoprecipitation/mass spectrometry (IP/MS) using human FKBP14 reveals an interaction with the G2/M checkpoint protein CHEK1, suggesting a potential role in regulating the cell cycle (Appendix A). These data suggest FKBP14 likely has broader roles in development beyond Notch signalling.

In this chapter, I identify a novel requirement for FKBP14 by generating germline and somatic mosaic clones with the *FKBP14*mutants. Using several variations on the yeast-derived flippase/flippase recombination target (FLP/FRT) recombination system (Golic and Lindquist 1989, Chou and Perrimon 1996, Newsome, Asling et al. 2000), I show that *FKBP14*mutant clones are strictly inviable in multiple tissue types. This phenotype may be the result of cell cycle arrest, cell death or a cell competition defect in the *FKBP14*mutant clones. I show that the clonal inviability observed cannot be rescued by eliminating competing cells through the use of a recessive cell lethal mutation, suggesting the defect is not likely to be exclusively due to a defect in cell competition. As it is possible to generate mosaic clones for both Presenilin and Notch (Lopez-Schier and St Johnston 2002), these data reveal a novel, Notch-independent role for FKBP14 in development.

### 3.3 Materials and Methods

#### 3.3.1 *Drosophila* Genetics

The imprecise excision line *FKBP14* was recombined onto an *FRT(42B)* chromosome by Diana van de Hoef and I confirmed presence of the deletion by PCR. Recombination in the ovaries was induced in late L2 to L3 instar larvae heterozygous for hs-flp; *FRT(42B) FKBP14*.
and either FRT(42B) ovoD1 (Chou and Perrimon 1996) or FRT(42B) ubi-GFP by heat shock at 37°C for 1-2 hours for 2-3 days consecutively. Recombination in the imaginal discs was induced in second and early third instar larvae heterozygous for hs::flp; FRT(42B) FKBPI4^{DSS} and FRT(42B) ubi-GFP by heat shock at 37°C for 60 min for 2-3 days consecutively and these clones were analyzed in late third instar larvae imaginal discs (Xu and Rubin 1993).

To generate clones in the eye, I recombined the EP-insertion line FKBPI1P^{EP2019} and the imprecise excision line FKBPI4^{DSS} onto an FRT(42D) chromosome and confirmed the lines by PCR. Virgin females of the stock y^{d2}, w^{1118}, ey::flp; FRT(42D) l(2)cl-R11^{1}/CyO, y+ (Bloomington Drosophila Stock Center #5617) were crossed to male FRT(42D) FKBPI4^{DSS}/CyO, GFP or FRT(42D) FKBPI4^{EP2019}/CyO, GFP at 25°C, and non Cy age-matched adult progeny were assessed.

### 3.3.2 Immunohistochemistry

Ovaries and third instar discs were dissected in cold PBS, fixed with 4% paraformaldehyde, permeabilized in PBS containing 0.3% Triton (PB3T), washed in PBS followed with PBT (0.1% Triton X-100), and blocked in 5% normal donkey serum in PBT, with the addition of 0.2% bovine serum albumin for ovaries. Tissues were then incubated with primary and secondary antibodies diluted in block, with washes performed using PBT. Ovaries stained with phalloidin (1:100 in PBS) were first rinsed with PBS to remove Triton. Tissues were then stained with DAPI (1:5000 in PBS or PBT), followed by mounting in DABCO or DAKO mounting media.

The primary antibodies used were rat anti-FKBPI4 (1:500), and rabbit anti-GFP (Invitrogen, Carlsbad, CA; 1:1000) for ovaries. Natural fluorescence from the ubi-GFP marker chromosome was used for analysis of the somatic mosaic third instar discs.

### 3.3.3 Microscopy

Ovary and larval disc images were acquired at RT using a Zeiss LSM510 META confocal microscope (Carl Zeiss Canada Ltd., Toronto, ON), and fly head images were acquired on a Leica Fluorescence Stereomicroscope (Leica Microsystems Inc., Concord, ON). Image analysis was performed using Volocity (PerkinElmer) software. Images were processed in Photoshop CS and Illustrator CS.
3.4 Results

3.4.1 FKBP14 is Maternally Loaded

By analyzing phenotypes associated with heterozygotes and homozygous mutants, we have identified a role for FKBP14 in the Notch pathway. However, the broad expression pattern of FKBP14 suggests that it may play additional roles in development. Moreover, *FKBP14* mutants are lethal throughout development and most die during mid-pupal stages (van de Hoef, Bonner et al. 2013). We hypothesized that this variation in lethality may be due to perdurance of maternal contribution. I therefore analyzed FKBP14 expression during oogenesis and observed detectable protein at all stages of oogenesis (data not shown). In mid-stage egg chambers, FKBP14 is expressed in nurse cells (arrows) and oocytes (arrowheads) (Fig. 3.1).

![Figure 3.1: FKBP14 is maternally loaded during oogenesis](image)

FKBP14 protein (bottom left panel, false coloured green in merge) is detected in *FKBP14* in nurse cells (arrows) and oocytes (arrowheads) in developing ovarioles. Scale bars = 42μm.
At later stages of oogenesis, FKBP14 expression is maintained in nurse cells and oocytes, with strong expression visible in somatic follicle cells (data not shown). The same pattern was reported for an FKBP14 GFP-trap line (Kelso, Buszczak et al. 2004), indicating that the anti-FKBP14 antibody is specific for immunohistochemistry. Further, while I observed a dramatic reduction of FKBP14 in escaper mutants homozygous for the original insertion line \( FKBP14^{EP2019} \) and no protein in \( FKBP14^{D58} \) escapers (Fig. 2.3 B), maternal protein was often detected in homozygous mutant animals as late as early L3 (data not shown). These data indicate that FKBP14 is maternally loaded and perdurance of maternal protein may contribute to the range of lethality observed in \( FKBP14 \) mutants.

### 3.4.2 Mosaic Clones in the Ovary Reveal a Notch-Independent Function for \( FKBP14 \)

To determine the phenotype associated with a complete loss of FKBP14 and to further define its role during development I attempted to generate maternal-zygotic null animals using the FLP/FRT mosaic clone technique (Perrimon 1998). Using previously generated FRT(42B) recombinant lines wherein \( FKBP14^{D58} \) had been recombined onto a chromosome containing the FRT(42B) insertion, I generated trans-heterozygotes of FRT(42B)\( FKBP14^{D58} \) and FRT(42B)\( ovoD1 \), a dominant female sterile mutation that results in early oogenesis arrest (Chou and Perrimon 1996, Mevel-Ninio, Fouilloux et al. 1996). Under these conditions, only those germline cells which have undergone site-specific recombination at the FRT site, removing the \( ovoD1 \) mutation, should be able to complete oogenesis (Fig. 3.2 A). However, heat-shock mediated FLP/FRT recombination produced apparently sterile females that did not lay eggs, indicating a possible requirement for FKBP14 during oogenesis (data not shown).

As \( ovoD1 \) is a dominant mutation arresting in early oogenesis with a complex phenotype (Chou and Perrimon 1996, Mevel-Ninio, Fouilloux et al. 1996), it was necessary to generate clones using a marker that did not itself interfere with oogenesis when assessing potential oogenesis defects associated with loss of FKBP14. I therefore generated transheterozygotes of FRT(42B)\( FKBP14^{D58} \) and an FRT line containing a transgene ubiquitously expressing GFP, FRT(42B)\( ubi-GFP \). Following FLP-mediated recombination, I dissected and stained ovaries, searching for negatively stained clones neighbouring GFP-positive twin spots (Fig. 3.2 B).
Figure 3.2: Generation of mosaic clones: FLP/FRT schematic

Schematic outlining FLP-recombinase induced site-specific recombination, resulting in homozygous daughter cells from dividing heterozygous cells. FLP recombination target sites (FRT) are indicated in red, FKBP14 (wild-type or mutant) in blue.

(A) FLP/FRT using the dominant female sterile ovoD mutant: Recombination in ovaries results in cells that receive both copies of the ovoD mutant or both copies of the FKBP14^{D58} mutation. Animals heterozygous or homozygous for ovoD do not complete oogenesis.

(B) FLP/FRT using a ubiquitous GFP marker: Recombination in germline or somatic tissue results in cells that receive both copies of the ubi-GFP marker (“GFP marked twin spots”) or both copies of the FKBP14^{D58} mutation (“negatively stained clones”).

While control non-heat shocked heterozygous ovarioles showed no defects in oogenesis (Fig. 3.3 A-A’), the vast majority of recombination events resulted in twin spots only, visible in the germarium (Fig. 3.3 B-B’’, arrowheads indicate twin spots) and occasionally in the somatic follicle cells surrounding the egg chamber (Fig. 3.3 C-C’’, arrowheads). In very rare cases, what appeared to be negatively stained clones were observable in follicle cells (Fig. 3.3 C’, arrow). Egg chambers wherein such clones were visible displayed extreme oogenesis defects, including abnormally distributed nuclei, impaired follicle cell migration, with possible loss of cells as indicated by absence of DAPI nuclear staining (Fig. 3.3 C-C’’).
Figure 3.3: FLP/FRT in ovaries suggests requirement for FKBP14 in cell viability

(A) Control FRT D58 x FRT GFP lines, not heat shocked: Heterozygous lines show normal oogenesis.
(B)-(C) FRT D58 x FRT GFP lines following FRT induced recombination
(B) GFP twin spots (arrowheads) are visible in the gerarium and early egg chambers, seemingly restricted to follicle cells. Negatively stained clones are not observed, and twin spots appear with much less frequency in later stage egg chambers.
(C) Rare follicle cell clones induced by FLP/FRT in the Drosophila ovary: FKBP14 mutant clones are identified by lack of GFP staining (arrows), twin spots are identified by doubly bright GFP (arrowheads). Where observed, clones in follicle cells were associated with severely disordered egg chambers.
Scale bars = 42µm

Importantly, no clones were ever detected in germline tissue, indicating a strict requirement for FKBP14 in oogenesis. This is in contrast to mutations in both Notch and Psn, for which it is possible to generate both maternal-zygotic and somatic null clones (Lopez-Schier and St Johnston 2002, Cooper, Deng et al. 2009). Therefore, FKBP14 has a Notch-independent function required for cell viability in the Drosophila ovary.
3.4.3  **FKBP14** is Required for Viability in Multiple Tissue Types

To determine if the apparent requirement for FKBP14 in oogenesis reflected a broader requirement for FKBP14 in cell viability, I generated clones in somatic tissue using the same ubiquitous GFP marker allele, FRT(42B)ubi-GFP, as described above (Fig. 3.2 B). In control wing discs, I induced recombination in flies bearing the FRT(42B)ubi-GFP and an FRT line with no other mutations, FRT(42B), resulting in clones that lack GFP-positive staining (Fig. 3.4 A-A’, arrows), adjacent to GFP twin spots (Fig. 3.4 A-A’, arrowheads), both of which are wild type for *FKBP14*. In contrast, following recombination between FRT(42B)ubi-GFP and FRT(42B)*FKBP14*ds, I observed GFP twin spots (Fig. 3.4 B-B’, arrowheads), without any associated *FKBP14* null clones. Similar results were observed for all larval imaginal discs wherein recombination had occurred (data not shown). These data indicate that FKBP14 is required for cell viability in multiple tissue types.

![Figure 3.4: FLP/FRT in somatic tissue confirms requirement of FKBP14 for cell viability](image)

(A) Recombination between FRT(42B)ubi-GFP and control FRT(42B) resulted in clones that lack GFP-positive staining (arrows), adjacent to GFP twin spots (arrowheads).

(B) Recombination between FRT(42B)ubi-GFP and FRT(42B)*FKBP14*ds resulted in GFP twin spots only (arrowheads), without any associated *FKBP14* null clones.

Scale bar = 42µm
3.4.4 Recombination Using a Recessive Cell-Lethal Marker Does Not Rescue FKB14 Clones

I have shown that FKB14 clones are not viable, both in the germline and somatic tissue, in contrast to mutations in both Notch and Psn, for which it is possible to generate both maternal-zygotic and somatic null clones (Lopez-Schier and St Johnston 2002, Cooper, Deng et al. 2009). The “cell lethal” phenotype observed in somatic FKB14 clones may be due to cell cycle arrest, apoptosis, or inability of these cells to successfully compete with wild type neighbors. Data from my IP/MS experiments (Appendix A) identified CHEK1 as an interactor with human FKB14, suggesting that cell cycle regulation may be one aspect of FKB14 function. To determine if the phenotype is due to defective cell competition, I performed recombination experiments in a tissue specific manner, using the eyeless-FLP driver and a recessive cell-lethal mutation (Newsome, Asling et al. 2000) to generate mosaic animals in which almost the entire retina should be homozygous mutant for FKB14, while the rest of the animal remained largely heterozygous (Newsome, Asling et al. 2000).

Figure 3.5: eyFLP/FRT recessive lethal schematic

Crossing scheme using eyFLP; FRT-recessive cell lethal to generate mitotic FKB14 null clones. FLP recombination target sites (FRT) are indicated in red, FKB14 (wild-type or mutant) in blue, and a recessive cell lethal mutation (l(2)cl-R111') indicated in black. Following eyFLP induced recombination, the cell receiving both copies of the recessive cell lethal mutation should be inviable (dotted outline), leaving only cells receiving both copies of FKB14 D58.

Expression of the eyFLP driver begins at the 6-23 cell stage within the eye disc primordium and is maintained until the final cell divisions in late L3 larvae, providing high levels of FLP activity throughout the entire proliferative phase of eye development (Newsome, Asling et al. 2000). FLP/FRT recombination then results in a daughter cell homozygous for the desired mutation/control and a daughter cell homozygous for the recessive cell lethal mutation. The population of cells arising from the daughter cell receiving the cell lethal mutation should be
severely impaired, with most cells lost (Fig. 3.5), resulting in a small, highly disordered twin spot, allowing the mutant clone, if viable, to comprise the majority of the eye disc.

To take advantage of the tools available for this system, I first performed recombination crosses to generate fly lines carrying either \textit{FKBP14}^{D58} or \textit{FKBP14}^{EP2019} on a FRT(42D) chromosome. I confirmed by PCR several lines containing both FRT(42D) and either the EP element insertion or deletion in \textit{FKBP14} (data not shown). I then crossed these lines to yw, eyFLP; FRT(42D) \textit{l(2)cl-R11}/CyO,y\textsuperscript{+} and examined non-Cy adult progeny. All parental strains showed normal eye development and uniform eye pigmentation (Fig. 3.6 A-E). Control FRT(42D) and FRT(42D) \textit{ubi-GFP} showed the anticipated outcome, wherein only small disordered patches of eye tissue are composed of dark red tissue arising from the recessive cell lethal twin spot (Fig. 3.6 F and G, arrowheads), while the majority of the adult eye is composed of control clone tissue (Fig. 3.6 F and G, arrows). However, progeny that received the \textit{EP2019} insertion or the \textit{D58} deletion showed only the dark, disordered tissue associated with the recessive cell lethal twin spot (Fig. 3.6 H-J). No associated mutant clone tissue was detected, indicating that following recombination, homozygous mutant tissue is not viable.

Altogether, these data indicate a strict requirement for FKBP14 in cell viability. Since whole-disc recombination using a recessive cell-lethal marker line did not rescue \textit{FKBP14} mutant clones, it is unlikely loss of homozygous mutant cells is due exclusively to a defect in cell competition. It remains unclear whether apoptosis or cell cycle arrest contribute to the observed phenotype.
Figure 3.6: Eye discs following eyFLP/FRT recombination reveal a very strict requirement for FKBP14 in cell viability

**Top panel:** Parental controls showing normal heterozygous eye phenotypes. (A) FRT 42D; ry605. Note that red pigment arises from the w+ background, not present in the recombination progeny. (B) FRT 42D GFP (C) eyFLP; FRT42D cl (D) FRT 42D FKBP14\textsuperscript{D58} (15) (E) FRT 42D FKBP14\textsuperscript{EP2019} (18)

**Bottom panel:** Progeny from mitotic recombination. Control FRT 42D recombination (F) shows eyes made almost entirely of cells not containing the recessive cell lethal mutation (unpigmented), where strong red eye pigment is associated with the recessive cell lethal marker. Similar results are obtained for FRT 42D GFP controls (G), where cells not receiving the recessive cell lethal mutation show pale red pigment. Recombination in the presence of FKBP14\textsuperscript{D58} (H and I) or FKBP14\textsuperscript{EP2019} (J) results in small, disordered eyes made up entirely of tissue arising from cells receiving the recessive cell lethal marker chromosome, indicating a strict requirement for FKBP14 in cell viability.

### 3.5 Discussion

While we observed specific defects in Notch signalling associated with loss of FKBP14, it is likely that FKBP14 also plays a broader role in development. FKBP14 is expressed in many tissues and loss-of-function mutations in FKBP14 are lethal throughout development, likely due to perdurance of maternal transcript or protein. FKBP14 mutants show a range of defects during imaginal disc development, some of which are reminiscent of Notch defects and others that appear independent of Notch (van de Hoef, Bonner et al. 2013). Moreover, IP/MS data from human FKBP14 suggest a potential role in cell cycle regulation (Appendix A). I have shown here that FKBP14 clones are not viable in either germline or somatic tissue, in contrast to mutations in
both Notch and Psn, for which it is possible to generate both maternal-zygotic and somatic null clones (Lopez-Schier and St Johnston 2002, Cooper, Deng et al. 2009).

Specifically, I have shown that FKBP14 mutant clones were never observed in germline tissue, contrary to Notch and Psn. I did, however, observe what appeared to be rare FKBP14 mutant follicle cell clones, which displayed extreme oogenesis defects, including possible loss of cells as indicated by absence of DAPI nuclear staining. (Haack, Bergstralh et al. 2013) Though neither Notch nor Psn are required in the germline for oogenesis progression, Notch signalling is required in follicle cells in order for developing egg chambers to pinch off from the germarium (Ruohola, Bremer et al. 1991), as well as during stage 5 to 6 to transition from a mitotic to endocyclic cell cycle (Lopez-Schier and St Johnston 2002). For the rare FKBP14 mutant follicle cell clones observed, some of the effects may be due to failed Notch signalling. It is possible, however, that these rare clones are a result of damage to the follicular epithelium during dissection, which has previously been reported to result in damage-induced “pseudo-clones” (Haack, Bergstralh et al. 2013). Positively-identifying clones through the use of mosaic analysis with a repressible cell marker (MARCM) should clarify whether these rare follicle cell clones are a true FKBP14 mutant phenotype or an artefact of dissection.

I have also shown a requirement for FKBP14 in cell viability in multiple tissues. While I was able to clearly observe twin spots in imaginal tissue following recombination, confirming that recombination indeed took place, I was not able to observe any associated FKBP14 mutant clones. Interestingly, mutations in one of the γ-secretase components, Aph-1, show a similar defect in cell viability in wing discs and this effect appears independent of its role in regulating γ-secretase activity in at least one context (Cooper, Deng et al. 2009). It is currently thought that Aph-1 and Nct may form a subcomplex that stabilizes Psn early in γ-secretase assembly, prior to Psn endoproteolysis and incorporation of Pen-2 (Hu and Fortini 2003). Of note, partial colocalization of FKBP14 and Aph-1, but not γ-secretase components Nct or Pen-2, was previously observed in Drosophila cell culture (van de Hoef, Bonner et al. 2013). Whether loss of FKBP14 affects Aph-1 function, and whether any such interaction may be responsible for the observed cell viability requirement for FKBP14, is not yet known.

Finally, I have shown that inviability of FKBP14 mutant clones is not rescued by recombination over a recessive cell lethal mutation, indicating that requirement for FKBP14 in cell viability is
strict. These data suggest that the defect is due to apoptosis or a very poor ability to compete against neighbouring cells, even those bearing a cell-lethal mutation. Further analysis using recessive cell-competition defective (Marygold, Roote et al. 2007) marker alleles may determine which of these possibilities is the case. Altogether these data reveal a Notch-independent role for FKBP14 in *Drosophila* development.
Data Attribution: Julia Maeve Bonner performed all experiments described herein with the exception of the S2 transfection/staining of UAS-FKBP14 constructs, which was performed by Tanya Da Sylva and Julia Maeve Bonner (Fig. 4.4 and 4.5). Tanya Da Sylva also provided valuable advice and assistance with balancing and maintaining UAS transgenics and preliminary rescue crosses.
4 FKBP14 Structure/Function Analysis

4.1 Summary

FKBP14 has been shown to have both Notch-dependent and Notch-independent roles in *Drosophila* development. Sequence analysis of FKBP14 reveals a conserved PPIase domain and EF hand domain, including residues predicted to be required for activity. To determine which domains of FKBP14 are required for its various functions, I have generated a number of wild type and mutant alleles, including mutants lacking the entire PPIase domain, the entire EF hand, or with point mutations in each domain designed to reduce or completely abrogate functional activity. In this chapter I show that both the PPIase and EF hand domain are required to rescue lethality associated with loss of FKBP14, indicating that both domains are important for FKBP14 function.

4.2 Introduction

FK506-binding proteins (FKBPs) are a sub-family of immunophilins, many of which bind FK506 and possess PPIase activity (Barik 2006, Kang, Hong et al. 2008). The smallest members of the family are composed of a single PPIase domain, while larger FKBPs contain additional modular domains that can fold and function independently in many cases (Barik 2006). Multi-domain FKBPs may contain single or multiple PPIase domains (FKBP_C) with or without PPIase function, and other diverse sequence motifs such as EF hands, leucine zippers, tetratricopeptide repeats (TPR), and DNA binding motifs (Galat 2013). Co-chaperones FKBP51 and FKBP52, for instance, each contain two FKBP_C domains, only one of which binds FK506 and confers PPIase activity, and a TPR motif that mediates interaction with HSP90, among other roles (Storer, Dickey et al. 2011). FKBP38, shown to physically interact with PS via its TPR motifs, does not bind FK506 or display inherent PPIase activity (Wang, Nakaya et al. 2005), but its calmodulin binding site allows it to respond to intracellular Ca$^{2+}$ via a Ca$^{2+}$/calmodulin/FKBP38 complex that stimulates PPIase activity (Edlich, Weiwad et al. 2005).

Sequence analysis of *Drosophila* FKBP14 reveals a signal peptide (SS), peptidyl-prolyl *cis-trans* isomerase (PPIase) domain (FKBP_C), an EF-hand motif (EFh) and an ER retention signal (van de Hoef, Bonner et al. 2013). Work described in previous chapters outline a critical role for
FKBP14 in Notch signalling via the γ-secretase complex [Chapter 2 and (van de Hoef, Bonner et al. 2013)] and Notch independent roles in Drosophila development, including a requirement in cell viability (Chapter 3). To gain a greater understanding of FKBP14 function, I sought to examine the role of the conserved PPIase and EF hand domains.

In this chapter, I describe experiments to generate and characterize a series of deletion and point mutation constructs in conserved residues for both the PPIase and EF hand domains of FKBP14. I show that mutations in the PPIase domain of FKBP14 appear to affect its intracellular localization in cell culture, as does the addition of a C-terminal tag before the ER retention sequence. FKBP14 constructs containing mutations in either the PPIase domain or EF hand domain do not rescue the lethality observed in FKBP14 D58 mutant, while robust rescue is observed with a wild type FKBP14. These data demonstrate that both the PPIase domain and EF hand domain are required for FKBP14 function.

4.3 Materials and Methods

4.3.1 Plasmid Construction and Mutagenesis

Sequence alignments were performed using ClustalW (Larkin, Blackshields et al. 2007) and Clustal Omega (Sievers, Wilm et al. 2011), and formatted using BoxShade. FKBP_C and EF hand domain boundaries were determined by multiple sequence alignment and those defined in the NCBI National Library of Medicine (NCBI NLM). FKBP14 cDNA was previously amplified from the EST clone GH08925 by Diana van de Hoef and subcloned (van de Hoef 2008). Mutations were generated with and without epitope tags from this template using Quickchange Lightning Site Directed Mutagenesis Kit (Stratagene), cloned into pUASt vectors with Gateway® recombination cloning (Invitrogen), and confirmed by sequencing (TCAG). BAC clones in P[acman] vectors (Venken, He et al. 2006) were obtained from BACPAC Resources (Children’s Hospital Oakland).

The primers used for FKBP14 mutagenesis are as follows (5’ → 3’):

ΔF: GTGATCAGCAGCCGAAACATCGGCAATGCC
D63L: CAGGCCGATGGCAGAAATTCCTGTCGAGCTTCGACCG
Y108A: CCTCCCCAGCTGGGCGCCGGTGACCAGGGTGCC
ΔEF: CCCCGCCCACCACCAATTCGGGACC
E157Q: CAGCTGAGTCGCGAACAGGTCAGCGAG
E207Q: GTTTTCATCTCGCAGCAGTATCGTCTCCTGACCGACCG
To insert V5 before KHDEL:
GATGAGTTCGCGGCCGAGCAGGCTCCCCATCCCCACCCCTGCTGGGGCCTGGATAGC
ACCCACACGACGAGCTG

4.3.2 Drosophila Genetics

Purified pUAST-FKBP14 wild type and mutant constructs were injected into \( w^{1118} \) embryos and transgenic lines obtained (BestGene Inc.) BAC clones in P[acman] vectors were integrated into \( y^{1}, w^{1118} \) flies in the VK31 docking site by PhiC31-integrate mediated site-specific transgenesis (Bischoff, Maeda et al. 2007) to obtain transgenic lines (Genetivision).

For rescue analysis, UAS- and BAC transgenics were double balanced with \( \text{FKBP14}^{D58} \) mutants, then crossed to daGAL4 double balanced with \( \text{FKBP14}^{D58} \). Crosses were raised at 25°C or 29°C, and adult progeny assessed over the course of 5-7 days following the first observed eclosion in each cross. Pupal progeny were assessed by separating GFP positive and negative late L2 and early L3 progeny, and observing the number that formed dark brown pupal cases. Flies were maintained on standard food.

4.3.3 Immunoblot Analysis

S2 cell or whole animal extracts were prepared using standard methods. Briefly, S2 cells harvested 48 h post-transfection, L3 or adult tissue were homogenized in RIPA (50mM Tris, 150mM NaCl, 1% SDS, 1% NP-40, 0.5% deoxycholate, pH 8.0) supplemented with protease inhibitors (Roche) for 15-30 minutes on ice. Following addition of 2X SDS loading buffer supplemented with DTT, samples were boiled for 5 minutes. SDS-polyacrylamide gel electrophoresis and immunoblot analyses were carried out as described (Guo, Livne-Bar et al. 1999).

The primary antibodies used were rat anti-FKBP14 (1:2000), mouse anti-actin (GeneTex Inc., Irvine, CA; 1:1000), and mouse anti-V5 (Invitrogen, 1:1000).

4.3.4 Cell Culture and Immunohistochemistry

Drosophila S2 or Kc167 cells were cultured in Schneider’s media supplemented with 10% fetal bovine serum. Transfections were carried out using Cellfectin (Invitrogen) according to the manufacturer’s specifications. For experiments including pMT-GAL4, 500µm CuSO4 was used to induce protein expression 24 h prior to harvesting cells.
Immunostaining was performed on embryonic cell culture as described (Patel 1994, Yeh, Zhou et al. 2000, Kim, Renihan et al. 2006, Comisso and Boulladonna 2007, Skwarek, Garroni et al. 2007). Briefly, cells were fixed with 4% paraformaldehyde in PBS 48 h post-transfection, washed in PBS, blocked in 1% donkey serum and 3% bovine serum albumin diluted in PBT. Cells were incubated with primary (4°C overnight) and secondary (1 h at room temperature) antibodies diluted in Block solution, followed by mounting in DAKO (DakoCytomation).

Primary antibodies used were rat anti-FKBP14 (1:500), mouse anti-PDI (Abcam, 1:100), rabbit anti-GM130 (Abcam, 1:1000), and mouse anti-V5 (Invitrogen, 1:1000). Alexa555 (Invitrogen, 1:1000), Alexa488 (Invitrogen, 1:1000) and Cy5 (Jackson Immunoresearch, 1:500) secondary antibodies were used. DAPI was used at 1:5000.

4.3.5 Microscopy

Images were acquired at RT using a Zeiss LSM510 META confocal microscope (Carl Zeiss Canada Ltd., Toronto, ON), 63X objective, and standard fluorescence filters equipped with a Hamamatsu C900-13 digital camera. Image analysis was performed using Volocity (PerkinElmer) software. Images were processed in Photoshop CS and Illustrator CS.

4.4 Results

4.4.1 Drosophila FKBP14 PPIase and EF Hand Domains Contain Conserved Amino Acids Required for Activity

Sequence analysis of FKBP14 reveals a signal peptide, conserved PPIase domain, EF hand domain, and KHDEL ER retention sequence. To determine which domains of FKBP14 are required for function, I sought to generate a series of mutants lacking the entire PPIase domain, the entire EF hand, or with point mutations in each domain designed to reduce or completely abrogate functional activity. To determine appropriate boundaries for each domain and whether active residues in each domain are conserved, I performed a number of sequence alignments between Drosophila FKBP14 and other PPIase and EF-hand containing proteins (Fig. 4.1).
A
dFKBP14_PA
Lp_Mip_FKBPA_Cdomain
hFKBP12_FKBPA_Cdomain
hFKBP13_FKBPA_Cdomain
hFKBP14_FKBPA_Cdomain
hFKBP8_FKBPA_Cdomain
consensus
1
1
1
1
1
1

B
dFKBP14_PA
dCam_EFhand1
dCam_EFhand2
hFKBP14
hFKBP9_EFhand
consensus
1
1
1
1
1
1
Figure 4.1: FKBP14 PPIase and EF hand alignments (page 71)

Sequence alignments of FKBP14 PPIase (FKBP_C) and EF hand domains. Conserved residues predicted to abrogate function when mutated are indicated in red. Highly conserved residues (*) and semi-conserved residues (.) are indicated.

(A) Drosophila FKBP14 PA (dFKBP14_PA) sequence aligned with the known FKBP_C domains of L. pneumophila macrophage infectivity potentiator (Lp_Mip_FKBP_Cdomain), Human FKBP12 (hFKBP12_FKBP_Cdomain), FKBP13 (hFKBP13_FKBP_Cdomain), FKBP14 (hFKBP14_FKBP_Cdomain), and FKBP8 (hFKBP8_FKBP_Cdomain).

(B) Drosophila FKBP14 PA (dFKBP14_PA) sequence aligned with the known EF hand domains of Drosophila calmodulin (dCam_EFhand1 and dCam_EFhand2) and Human FKBP9 (hFKBP9_EFhand). Full length Human FKBP14 is also aligned to indicate that though the sequence annotation (NP_060416.1) does not indicate an EF hand, the critical residues are conserved, and it is indeed predicted to have an EF hand motif and to bind Ca$^{2+}$.

Several PPIase mutants have been described in literature, with varying activities. In particular, Tremmel et al generated a site-directed mutation in Neurospora crassa FKBP22 in a conserved aspartate residue at position 60 (FKBP22$_{D60L}$) that reduced PPIase activity without inhibiting dimerization or chaperone activity of the protein (Tremmel and Tropschug 2007). The Legionella pneumophila FKBP Mip was mutated at the same conserved residue (Mip$_{D142L}$), as well as at a conserved tyrosine (Mip$_{Y185A}$). Both mutations dramatically reduced PPIase activity (Wintemeyer, Ludwig et al. 1995). Drosophila FKBP14 contains both of these conserved residues in its predicted PPIase domain (Fig. 4.1A, conserved residues of interest indicated in red). I have thus generated single mutants (D63L and Y108A) and a double mutant (D63L/Y108A, referred to henceforth as FKDM) construct designed to abrogate the putative PPIase activity of FKBP14, ideally without affecting other functions of the protein (Fig. 4.2 B).

Similarly, I performed alignments of FKBP14 with other EF-hand containing proteins, including human FKBP14 (NP_060416.1) and several other FKBPs. EF-hand Ca$^{2+}$ motifs play essential roles in eukaryotic cellular signalling (Gifford, Walsh et al. 2007), thus the predicted EF-hand in FKBP14 may be critical to its role in Drosophila. The EF-hand in FKBP14 appears to be a canonical one, with two putative helix-loop-helix Ca$^{2+}$ binding sites. Mutations in the highly conserved glutamic acid residue at position 12 of each loop have been reported to greatly decrease Ca$^{2+}$ binding affinity (Starovasnik, Su et al. 1992, Buchanan, Ames et al. 2005). These glutamic acid residues are conserved in the predicted EF-hand of FKBP14 (Fig. 4.1 B, residues indicated in red). I therefore generated single (E157Q and E207Q) and double (E157Q/E207Q, referred to henceforth as EFDM) mutant constructs of FKBP14 (Fig. 4.2 C).
Figure 4.2: FKBP14 wild type, mutant, and genomic constructs schematic

Schematic of all FKBP14 wild type and mutant constructs generated and genomic clones obtained. Transgenics were generated for both tagged and untagged versions of domain deletions and double mutants. Tagged and untagged cell culture constructs were generated for all mutants. Single point mutations (greyed out constructs) were generated for cell culture only.

In addition to the point mutations described above, I also generated mutant constructs lacking the entire PPIase (ΔF) or EF-hand (ΔEF) domain, with boundaries defined by sequence alignments (Fig. 4.2 B and C) and the regions predicted by NCBI Entrez Gene (NP_726074.2). Specifically, I
deleted residues 35 to 130 inclusive for the PPIase deletion mutant and residues 141 to 208 inclusive for the EF hand deletion mutant. I have further generated wild type constructs containing the entire coding sequence for FKBP14 (Fig. 4.2 A) and obtained BAC clones containing large overlapping sequences of the *Drosophila* genome that include or surround the FKBP14 locus (Fig. 4.2 C), intended to serve as genomic rescue constructs and controls.

I generated each of these constructs, save the genomic BAC clones, both with and without a V5 tag near the C-terminus of the FKBP14 coding sequence. Due to the presence of an N-terminal signal peptide as well as a C-terminal KHDEL ER retention sequence, I designed these tags within the sequence of the protein so as not to occlude either feature (Snapp 2009). The C-terminal V5 tag has therefore been inserted just upstream of the KHDEL ER retention sequence to allow proper retention of the construct within the ER (Fig. 4.2 A).

### 4.4.2 Cell Culture Localization of Wild Type and Mutant *FKBP14*

Following generation of *pUASt* constructs and confirmation of expression in cell culture (data not shown), I examined subcellular localization of expressed wild type and mutant FKBP14 in *Drosophila* cell culture. To determine if any differences in subcellular localization occurred between mutant proteins, I co-transfected cells with *pUASt* constructs and an inducible *pMT-GAL4* driver construct. Cells were then stained an ER resident protein, PDI, to mark the ER and GM130 to mark the Golgi/cis-Golgi, as well as for FKBP14 using our polyclonal antibody (Fig. 4.3). For those constructs designed with a V5 tag, I also probed with anti-V5 antibody (Fig. 4.4).
Figure 4.3: Localization of untagged wild type and mutant FKBP14 in cell culture (legend page 76)
Figure 4.3: Localization of untagged wild type and mutant FKBP14 in cell culture localization (page 75)

Expression of untagged UAS FKBP14 wild type (wt) and mutant constructs in Kc cells under the control of pMT GAL4, stained with DAPI, rat anti-FKBP14 (yellow), mouse anti-PDI to mark the ER (red), and rabbit anti-GM130 to mark the Golgi and cis-Golgi (green).

(A) Control cells transfected with pMT GAL4 alone. Endogenous FKBP14 is very weakly visible. (B) – (F) Kc cells transfected with pMT GAL4 and the UAS FKBP14 transgene indicated. GAL4 expression was induced with CuSO₄ 24 h prior to fixing and staining.

(B) UAS FKBP14 wild type (wt): FKBP14 is strongly detected throughout the cell, and partial colocalization with PDI observed (arrowhead). (C) UAS FKBP14 ΔF: FKBP14 staining is weak and punctate compared to wild type, and colocalization is not observed for PDI or GM130. (D) UAS FKBP14 FKDM: FKBP14 staining is strongly detected throughout the cell, with partial colocalization with PDI (arrowhead). There did not appear to be colocalization with GM130 (see visibility of green GM130 punctae in merge). (E) UAS FKBP14 ΔEF: FKBP14 staining is weak, partially punctate, and shows partial colocalization with both PDI (arrowhead) and GM130 (arrow). (F) UAS FKBP14 EFDM: FKBP14 staining is weak, partially punctate, and shows partial colocalization with PDI (arrowhead).
Figure 4.4: Localization of V5 tagged wild type and mutant FKBP14 in cell culture localization (legend page 79)
Figure 4.4 (continued): V5 tagged FKBP14 UAS constructs cell culture localization continued (legend page 79)
Figure 4.4: V5 tagged FKBP14 UAS constructs cell culture localization (page 77-78)

Expression of tagged UAS FKBP14 wild type (wt) and mutant constructs in Kc cells under the control of pMT GAL4. The tope panel shows cells stained with DAPI, rat anti-FKBP14 (yellow), mouse anti-PDI to mark the ER (red), and rabbit anti-GM130 to mark the Golgi and cis-Golgi (green), while the bottom panel shows cells stained for DAPI, rat anti-FKBP14 (yellow), rabbit anti-GM130 to mark the Golgi and cis-Golgi (green), and mouse anti-V5 (red).

(A) Control cells transfected with pMT GAL4 alone. Endogenous FKBP14 is very weakly visible. **NOTE: these are the same data as Fig. 4.3 (staining was performed simultaneously).**

(B) – (F) Kc cells transfected with pMT GAL4 and the UAS FKBP14 transgene indicated. GAL4 expression was induced with CuSO$_4$ 24 h prior to fixing and staining.

(B) UAS FKBP14 wild type (wt) V5: strong FKBP14 staining (yellow) and partial colocalization with both the PDI ER marker and the GM130 marker is observed (Top panel: arrowhead and arrow, respectively). Staining for the V5 tag and GM130 did not show obvious colocalization (Bottom panel). (C) UAS FKBP14 ΔF V5: Punctate FKBP14 staining is shown (Top panel: yellow). Partial colocalization with both the PDI ER marker and the GM130 marker is observed (Top panel: arrowhead and arrow, respectively). The less punctate V5 staining also showed partial colocalization with GM130 (Bottom panel: arrow). (D) UAS FKBP14 FKDM V5: Strong FKBP14 staining is shown (Top panel: yellow). Partial colocalization with both the PDI ER marker and the GM130 marker is observed (Top panel: arrowhead and arrow, respectively). V5 staining also showed partial colocalization with GM130 (Bottom panel: arrow). (E) UAS FKBP14 ΔEF V5: Moderate FKBP14 staining is observed, with partial colocalization with PDI (Top panel, arrowheads), and no apparent colocalization with GM130. No colocalization between V5 stain and GM130 (Bottom panel). (F) UAS FKBP14 EFDM V5: Weak FKBP14 staining is observed, with weak partial colocalization with PDI (Top panel, arrowhead). V5 staining is significantly stronger (Bottom panel), with no colocalization observed between V5 and GM130.
FKBP14 wt and FKBP14 FKDM both showed high levels of expression and partial colocalization with the ER marker PDI (Fig. 4.3 B and D, colocalization with PDI indicated with arrowhead), while no apparent colocalization with the Golgi marker GM130 was observed. This is in keeping with previous studies demonstrating that endogenous FKBP14 localizes to the ER in cell culture (van de Hoef, Bonner et al. 2013). Expression of FKBP14 EFDM appeared weaker, though some partial colocalization with PDI was seen (Fig. 4.3 F, arrowhead). FKBP14 ΔF and ΔEF also showed weak expression (Fig. 4.3 C and E). While no obvious colocalization was observed with either PDI or GM130 for ΔF, the ΔEF construct showed partial colocalization with both PDI (Fig. 4.3 E, arrowhead) and GM130 (Fig. 4.3 E, arrow), which is in contrast to that observed for endogenous FKBP14.

Using the polyclonal anti-FKBP14 antibody for visualization, V5-tagged FKBP14 wt showed partial colocalization with the PDI ER marker, but also an apparent partial colocalization with the GM130 Golgi marker (Fig. 4.4 B, top row, arrowheads and arrows, respectively). Visualization of V5-tagged FKBP14 wt using anti-V5, in contrast, did not show obvious colocalization with GM130 (Fig. 4.4 B, bottom row). Of note, the V5 staining looked markedly less punctate than the polyclonal antibody against FKBP14 (Fig. 4.4 B bottom row second panel compared to top row second panel). This may indicate that tagged FKBP14 is not trafficked throughout the cell in the same manner as endogenous protein. Similar colocalization results were observed for ΔF and FKDM when stained with polyclonal anti-FKBP14 (Fig. 4.4 C and D, top rows), though in these cases colocalization with GM130 was also observed with the V5 stain (Fig. 4.4 C and D, bottom rows, arrows). Thus the presence of the V5 tag does not appear to alter subcellular localization of these mutants. The partial colocalization with GM130, however, indicates that the mutation itself may be resulting in some altered subcellular distribution relative to wt (Fig. 4.3 B). The EF hand mutants ΔEF and EFDM appear to partially colocalize with PDI (Fig. 4.4 E and F, top rows) without a partial colocalization with GM130 as observed for the PPIase domain mutants. This may indicate that the EF hand mutations do not affect subcellular localization to the degree observed for the PPIase mutants.

Altogether these data indicate that presence of a V5 tag upstream of the ER retention sequence or mutations in PPIase domain may affect protein subcellular localization relative to wild type FKBP14.
4.4.3 Transgene Expression Levels

I obtained several transgenic lines for each UAS construct. Since the FKBPI4 locus is on the second chromosome, for ease of balancing I selected transgenic insertions on the third chromosome to test for expression. I crossed each third chromosome line received to the ubiquitous driver daGAL4 and tested adult extracts for protein expression by Western blot (Fig. 4.5 A). For those lines showing expression, there did not appear to be a significant difference in levels across lines from the same construct. Of note, one V5 tagged UAS-FKBPI4wt line did not show expression (Fig. 4.5 A, left panel: “wt V5C 1M” lane), and no expression was observed for any of the UAS-FKBPI4ΔF tagged or untagged transgenic lines (Fig. 4.5 A, left panel: “ΔF” lanes, and data not shown). It is possible that FKBPI4ΔF is not recognized by the anti-FKBPI4 polyclonal antibody. However, probing with anti-V5 antibody also failed to detect tagged ΔF protein (data not shown), suggesting that ΔF protein is either not expressed or rapidly degraded.

I further analyzed expression in a subset of lines, comparing them to each other in the context of both daGAL4 and UAS controls (Fig. 4.5 B). I detected strong expression from untagged wt and comparable expression from untagged FKDM (Fig. 4.5 B, left panel: “da x wt” and “da x FKDM”). I observed a lower, but detectable level of expression from untagged EFDM (Fig. 4.5 B, left panel: “da x EFDM”). I also detected strong expression of the V5-tagged wt and V5 tagged-EFDM lines (Fig. 4.5 B, right panel: “da x FKBPI4wt V5” and “da x EFDM V5”) and weaker expression by the V5-tagged ΔEF line (Fig. 4.5 B, right panel: “da x ΔEF V5”).
Expression of UAS FKBP14 transgenes driven by daGAL4. FKBP14 D58 (deletion mutant) and D34 (precise excision control) are compared for control.

(A) Expression of several insertions of single transgene constructs were compared. Where expression is detected with polyclonal anti-FKBP14, levels of expression are similar. One V5 tagged UAS-FKBP14wt line did not show expression (left panel: “wt V5C 1M” lane), one V5 tagged UAS-FKBP14 EFDM line did not show expression (right panel: “EFDM V5C 7M” lane), and no detection was observed for any UAS-FKBP14ΔF tagged or untagged lines (left panel: “ΔF” lanes and data not shown).

(B) Expression of a subset of tagged and untagged insertions to directly compare to each other. Strong expression is detected from untagged wt, and comparable expression from the untagged FKDM UAS line (left panel: “da x wt” and “da x FKDM”). A lower, but detectable level of expression was observed from the untagged EFDM UAS line (left panel: “da x EFDM”). Strong expression was observed for the V5 tagged wt and EFDM lines (right panel: “da x FKBP14wt V5” and “da x EFDM V5”), weaker expression observed for the tagged ΔEF line (right panel: “da x EFDM V5”).
4.4.4 Both the PPIase and EF Hand Domain are Required for FKBP14 Function

Null mutations in *FKBP14* are lethal throughout larval and pupal stages of development with escapers reaching late pupal development (van de Hoef, Bonner et al. 2013). To determine whether the PPIase and EF hand domains are required for FKBP14 function, I sought to perform rescue analysis using the *FKBP14<sup>D58</sup>* lethal mutant. I generated *Drosophila* lines double balanced to carry the *FKBP14<sup>D58</sup>* deletion and either the ubiquitous *daGAL4* driver or a *UAS-FKBP14* transgenic line. Crossing these lines would allow me to determine whether expression of wild type or mutant FKBP14 lines can rescue the lethality observed in *FKBP14<sup>D58</sup>* mutants (Fig. 4.6 A).
Figure 4.6: FKBP14 mutant rescue crossing scheme

Genetic crossing scheme to perform rescue analysis with UAS FKBP14 transgenes. (A) Complete rescue crossing scheme: \( FKBP14^{D58} \), \( daGAL4 \), and insertions onto the third chromosome were double balanced separately. Double balanced \( daGAL4 \) and UAS transgenes were each crossed into the \( FKBP14^{D58} \) background and finally crossed to each other.
Homozygous $FKBP14^{D58}$ progeny were assessed for rescue. Balancers in parentheses indicate that the insertions are homozygous viable. Homozygotes were selected for the final rescue cross. (B) Progeny expected from the final rescue cross of fully double balanced lines: Half of the progeny will be heterozygous for $FKBP14^{D58}$, displaying a Cy wing phenotype. $\frac{1}{4}$ of the progeny will be homozygous for the CyO, GFP balancer which is homozygous lethal (dashed outline indicates lethality). $\frac{1}{4}$ of the progeny should receive both copies of $FKBP14^{D58}$ mutation and the viability of these animals will be assessed (red outline). Discounting the progeny receiving both copies of balancer, the expected ratio of progeny is 33% $D58/D58$, 66% $D58/CyO$, GFP.

Based on cell culture localization and Western expression data, I selected untagged wild type (FKBP14 wt), PPIase, and EF hand double mutant transgenic lines (FKBP14 FKDM and EFDM), as well as V5 tagged wild type transgenic (FKBP14 wt V5) to perform initial rescue analysis. Given that $FKBP14$ mutant escapers reach late pupal stages of development (van de Hoef, Bonner et al. 2013), I first examined the ability of each transgenic line to rescue $FKBP14^{D58}$ lethality to adulthood. Crossing the selected lines at 25°C revealed that only untagged wild type UAS-FKBP14 wt rescued the lethality of $FKBP14^{D58}$ (Fig. 4.7 A, $FKBP14^{D58}/FKBP14^{D58}$ rescued animals indicated in blue, heterozygous animals displaying balancer chromosome markers indicated in grey). Indeed, the rescue was near complete - the expected Mendelian ratio for a full rescue would be 33% (see Fig. 4.6 B), whereas I observed ~28% rescue (Fig. 4.7 A, “wt” column, rescue shown in blue). No viable $FKBP14^{D58}/FKBP14^{D58}$ adults were observed for any other transgenic lines tested (Fig. 4.7 A, “wt V5”, “FKDM” and “EFDM” columns). To ensure robust expression of the UAS transgenes, my analysis was repeated at 29°C (Fig. 4.7 B), with similar results. Compiling data from repeated rounds of rescue analysis at 25°C revealed a strong rescue of the $FKBP14^{D58}$ lethality with UAS-FKBP14 wt, where 21% of $FKBP14^{D58}/FKBP14^{D58}$ mutant adults were rescued to adulthood (Fig. 4.7 C) with no apparent mutant phenotype (data not shown). These data indicate that the lethality observed in our $FKBP14^{D58}$ mutants is indeed due to loss of FKBP14 and can be rescued by expression of wild type FKBP14. However, activity of both the PPIase and EF hand domains are required for viability.
Figure 4.7: FKBP14 mutant rescue reveals requirement for both PPIase and EF hand domains (legend page 87)
Figure 4.7: FKBP14 mutant rescue reveals requirement for both PPIase and EF hand domains (page 86)

Adult rescue results for UAS-FKBP14 transgenes crossed to daGAL4 and pupal rescue results for UAS-FKBP14 transgenes crossed to daGAL4, genomic clone inserts crossed to D58/CyO, GFP. Animals homozygous for FKBP14<sup>ΔSS</sup> are shown in blue. Number of progeny assessed given above each column.

(A) 25°C rescue results: Only untagged UAS-FKBP14 wt rescued the observed lethality of FKBP14<sup>ΔSS</sup> (“wt” lane, rescued animals indicated in blue, 28% of total progeny homozygous for FKBP14<sup>ΔSS</sup>). Neither the V5 tagged wt or double mutants showed any rescue ability (“wt V5”, “FKDM”, “EFDM”). Animals showing Cy phenotype are indicated with dark grey, those showing only a Ser phenotype indicated by light grey.

(B) 29°C rescue results: Only untagged UAS-FKBP14 wt rescued the observed lethality of FKBP14<sup>ΔSS</sup> (“wt” lane, rescued animals indicated in blue). Neither the V5 tagged wt or double mutants tested showed any rescue (“wt V5”, “FKDM”, “EFDM”). Animals showing Cy phenotype are indicated with dark grey, those showing only a Ser phenotype indicated by light grey.

(C) 25°C combined rescue results (2 individual rescue experiments): untagged UAS-FKBP14 wt rescued the observed lethality of FKBP14<sup>ΔSS</sup> (“wt” lane, rescued animals indicated in blue, 21% of total progeny homozygous for FKBP14<sup>ΔSS</sup>).

(D) 25°C pupal rescue results: Only untagged UAS-FKBP14 wt rescued a significant cohort of FKBP14<sup>ΔSS</sup> homozygous progeny to pupal stages (“D58/CyO, GFP;UAS-wt” lane, 41% of total progeny homozygous for FKBP14<sup>ΔSS</sup>), compared to control (“D58/CyO, GFP” lane, 9% of total progeny homozygous for FKBP14<sup>ΔSS</sup>). UAS-FKBP14 FKDM showed a very mild rescue properties compared to FKBP14<sup>ΔSS</sup> controls (“D58/CyO, GFP; UAS-FKDM,” 14% homozygous pupae vs. “D58/CyO, GFP,” 9% homozygous pupae). No rescue was observed for any other lines, including the three BAC genomic clone insertions tested (“D58/CyO, GFP;11402/TM3, Ser”, “D58/CyO, GFP;99104/TM3, Ser”, and “D58/CyO, GFP;187J15/TM3, Ser”).

Of note, in UAS-FKBP14 wt rescue crosses, I observed a number of progeny that appeared to be positive for the TM3, Ser balancer serrated wing phenotype, without obvious Cy wings (Fig. 4.7 A-C, Ser only animals indicated in light grey). The lack of Cy wings seems to indicate that these lines are apparently homozygous for FKBP14<sup>ΔSS</sup> and therefore “rescued” progeny. The serrated wing phenotype may arise from daGAL4 double balanced parental lines that may not have been fully homozygous (i.e. some parents may still have carried a TM3, Ser balancer, see Fig. 4.6 A). If the daGAL4 lines selected for the rescue cross were not in fact fully homozygous, progeny containing the UAS FKBP14 wt transgene alone without daGAL4 may be able to rescue lethality through leaky expression, enhancing a weak serrate phenotype from the daGAL4 double balanced parents. Alternatively, if the CyO balancer is not fully penetrant, these “leaky expression rescue” progeny may in fact be heterozygous for FKBP14<sup>ΔSS</sup> and not rescued by the UAS transgene alone. This conclusion is more in keeping with the expected Mendelian ratio of
progeny. However, non-Cy progeny were not observed in other crosses, suggesting adequate penetrance of the Cy phenotype. It seems therefore probable that the UAS FKBP14 wt transgene exhibits some degree of leaky expression in the absence of GAL4 and this expression is enough to rescue lethality. These animals did not show any obvious bristle or eye phenotypes (data not shown). A wing phenotype could not be determined due to the apparent presence of TM3, Ser.

While only the wild type FKBP14 transgene was able to rescue $FKBP14^{DSS}$ mutants to adulthood, $FKBP14$ mutants are lethal throughout larval and pupal stages of development (van de Hoef, Bonner et al. 2013). I therefore asked whether any of the selected transgenes might partially rescue $FKBP14^{DSS}/FKBP14^{DSS}$ mutants, leading to more escapers at pupal stages of development. After setting up crosses at 25°C, I separated GFP positive (i.e. carrying the CyO, GFP balancer and thus heterozygous for $FKBP14^{DSS}$) late L2 or early L3 larvae from GFP negative (i.e. homozygous for $FKBP14^{DSS}$) and counted how many progressed to form dark brown pupal cases. For these analyses, I included several additional controls, including the $FKBP14^{DSS}$ mutation alone, the precise excision $FKBP14^{D4}$, and BAC genomic transgenics. Taking the lack of GFP fluorescence to indicate homozygous $FKBP14^{DSS}$ progeny (Fig. 4.7 D, blue bars), it appeared again that only UAS-FKBP14 wt rescued a notable number of animals to pupal stages (Fig. 4.7 D, “$D58$/CyO, GFP; UAS-wt”) compared to the $FKBP14^{DSS}/CyO, GFP$ alone control (Fig. 4.7 D, “$D58$/CyO, GFP”). This apparent rescue is greater than expected on the basis of Mendelian genetics, showing 41% of larvae reaching pupal stages, compared to an expected full rescue of 33%. This may be due to a relatively small number of animals examined (n=39), or to leaky rescue in the parental lines. Further analysis is required to confirm rescue in pupae.

No other transgenic lines showed the level of rescue observed for UAS-FKBP14 wt. However, UAS-FKBP14 FKDM (double mutant in the PPIase domain) appeared to show a mild rescue compared to $FKBP14^{DSS}$ controls (Fig. 4.7 C: “$D58$/CyO, GFP; UAS-FKDM,” 14% non GFP pupae vs. “$D58$/CyO, GFP,” 9% non GFP pupae). The number of animals of the correct genotype were too low to be certain that such a small difference is truly representative (n=29 and 45 for FKDM and $D58$/CyO, GFP, respectively). Repeated trials may help determine whether mild rescue of lethality is consistently observed with mutated FKBP14 constructs. Of note, no rescue was observed for any of the BAC genomic clone insertions tested (Fig. 4.7 D, “$D58$/CyO, GFP;114O2/TM3, Ser”, “$D58$/CyO, GFP;99I04/TM3, Ser”, and “$D58$/CyO, GFP;187J15/TM3,
Ser*), including CH322-187J15, which spans the FKBP14 genomic locus (see Fig. 4.2 D). The inability of CH322-187J15 to rescue \textit{FKBP14}\textsuperscript{DSS} lethality to pupae or adulthood (data not shown) suggests that it may be missing one or more important regulatory regions.

Altogether, these data indicate that both the PPIase and EF hand domains are critical for FKBP14 function, as point mutations in conserved residues predicted to abrogate function are not able to rescue the lethality associated with loss of FKBP14.

\section*{4.5 Discussion}

FKBP family members share a PPIase domain (FKBP\textsubscript{C}) which in many cases possess PPIase activity. Many members contain other domains, such as protein-protein interaction TPR motifs or Ca\textsuperscript{2+} binding EF hand domains, which confer multiple functions (Barik 2006, Galat 2013). To determine whether the conserved PPIase and EF hand domains in \textit{Drosophila} FKBP14 are required for its function, I performed a number of sequence alignments between FKBP14 and other PPIase and EF-hand containing proteins (Fig. 4.1). I identified conserved residues in the PPIase domain which are predicted to be required for PPIase activity (Fig. 4.1 A). I next generated single mutants (D68L and Y108A) and a double mutant (D68L/Y108A) construct designed to abrogate putative PPIase activity of FKBP14, hopefully without affecting other functions of FKBP14 (Fig. 4.2 B). Similarly, I identified conserved residues in the EF hand domain, which upon mutation (E157Q and E207Q) should abrogate Ca\textsuperscript{2+} binding ability (Fig. 4.1 B and Fig 4.2 C).

In this chapter I demonstrated that FKBP14 transgenic insertions carrying mutations in either the PPIase domain or EF hand are unable to rescue the lethality observed in \textit{FKBP14} mutants, while a transgenic insertion of full length, untagged wild type FKBP14 does rescue, possibly even in the absence of GAL4. These results show that the lethality observed in \textit{FKBP14} mutants is due to loss of FKBP14 and further indicates that both the PPIase and EF hand domains are required for FKBP14 function.

I also describe the generation of deletion mutations in FKBP14, removing the entire PPIase or EF hand domain (Fig. 4.2 B and C). Cell culture localization analysis and assessment of expression levels by Western analysis indicated that both mutant proteins may partially colocalize with the Golgi compartment, which is in contrast to endogenous FKBP14 (Fig. 4.3
and Fig. 4.5). Similarly, many of the constructs tagged with V5 just upstream of the ER retention sequence, including V5 tagged FKBP14 wt, showed partial colocalization with the Golgi compartment (Fig. 4.4 B - D), indicating that the tag may interrupt proper trafficking of FKBP14, compromising its endogenous restriction to the ER. Importantly, I observed robust rescue of the \( FKBPI4^{D58} \) lethality by untagged FKBP14 wt, no rescue was observed for FKBP14 wt tagged with V5. Despite my efforts to ensure the tag did not occlude either the N-terminal signal sequence or C-terminal ER retention sequence, these data suggest that the tag, or its location within the protein, interrupts FKBP14 function.

The fact that no rescue is observed for the BAC clone spanning the FKBP14 genomic locus, CH322-187J15 (Fig. 4.7 C, “\( D58/CyO, GFP;187J15/TM3, Ser \)”), is of some concern. This clone was chosen with the hope that it included the all or most regulatory regions for \( FKBPI4 \) (Groth, Fish et al. 2004, Venken, He et al. 2006). The inability of CH322-187J15 to rescue \( FKBPI4^{D58} \) lethality to pupae or adulthood indicates that it may be missing one or more important regulatory regions, or lack expression for unknown reasons.

With respect to the apparent rescue by UAS-FKBP14 wt line alone in the absence of GAL4, several possibilities exist. The CyO, GFP balancer may not be fully penetrant, in which case these progeny may in fact be heterozygous for \( FKBPI4^{D58} \). This conclusion is more in keeping with the expected Mendelian ratio of progeny, yet the lack of observed non-Cy progeny in other crosses indicates that this is not likely to be the case. If instead these animals are truly homozygous for \( FKBPI4^{D58} \) but leaky expression of UAS-FKBP14 wt can rescue lethality, it is possible that some of the parents used for the UAS-FKBP14 wt rescue cross may have already been “rescued” and therefore homozygous for \( FKBPI4^{D58} \), interfering with the expected Mendelian ratio of progeny. Western blot analysis of \( FKBPI4^{D58} \) homozygous mutants carrying the wild type transgene without GAL4 must be carried out to distinguish between these possibilities. Finally, while the total lack of apparent rescue in any other lines rules out contamination of the daGAL4 line, it is possible that some contamination of wild type \( FKBPI4 \) may be present in the UAS FKBP14 wt double balanced line used for rescue and that the “rescued” non-Cy progeny may not be homozygous for \( FKBPI4^{D58} \). I have performed PCR on individual “rescued” progeny, both with and without the serrate phenotype, to determine if the deletion is present (data not shown). In almost all animals for both phenotypes, the \( D58 \) deletion
was observed, indicating that they were at least heterozygous for \textit{FKBP14}, and significant contamination is unlikely. However, this analysis cannot differentiate between animals heterozygous or homozygous for the \textit{FKBP14}^{DSS} deletion. To fully rule out contamination, the apparent rescue of the \textit{FKBP14}^{DSS} by UAS \textit{FKBP14} in the absence of GAL4 must be confirmed by sequencing and preferably from animals not containing the TM3, Ser balancer.

Altogether these data indicate that the lethality observed in \textit{FKBP14} mutants is indeed due to loss of \textit{FKBP14} and that both the PPIase and EF hand domains are required for function. This suggests that PPIase activity is necessary for some or all functions of \textit{FKBP14} and that \(\text{Ca}^{2+}\) binding may regulate \textit{FKBP14}. Of relevance, the crystal structure of human \textit{FKBP14}, containing a classical \textit{FKBP} \_C domain and two EF hand motifs, revealed that it may form a dimer via its EF hand motifs, with an elongated and primarily hydrophobic cavity potentially available for ligand binding (Boudko, Ishikawa et al. 2014). Dimerization through the EF hand domain may play an important role in \textit{Drosophila} \textit{FKBP14} function or regulation. Alternatively, it’s possible that the EF hand domain regulates \textit{FKBP14} function in a similar manner as observed for the accessory domains of another member of the FKBP family, \textit{FKBP38}. \textit{FKBP38} does not bind FK506 or display inherent PPIase activity (Wang, Nakaya et al. 2005), but its interaction with calmodulin allows it to respond to intracellular \(\text{Ca}^{2+}\) via a \(\text{Ca}^{2+}/\text{calmodulin/\textit{FKBP38}}\) complex that stimulates PPIase activity (Edlich, Weiwad et al. 2005). Similarly, mouse \textit{FKBP23} was shown to interact with BiP in the ER and this binding is \(\text{Ca}^{2+}\) dependent via its EF hand domain (Zhang, Wang et al. 2004). Thus dimerization, binding of \(\text{Ca}^{2+}\), or a combination of these events may intricately regulate the functions and interactions of \textit{Drosophila} \textit{FKBP14} in a similar manner.

The requirement for conserved residues associated with PPIase function suggests that \textit{FKBP14} likely possesses PPIase activity and that this function contributes to its role in \textit{Drosophila}. Catalytic PPIase activity must be shown by \textit{in vitro} assays, but if confirmed it will be interesting to determine which functions of \textit{FKBP14} are regulated by such activity. For example, another PPIase, Pin1, has been implicated in cell cycle regulation (Stukenberg and Kirschner 2001) and AD progression (Liou, Sun et al. 2003, Gerard, Deleersnijder et al. 2011) via its PPIase activity. If the cell viability defect described in Chapter 3 arises from a requirement for \textit{FKBP14} in cell cycle progression, it is possible that PPIase activity contributes to this aspect of \textit{FKBP14} function. The PPIase activity of \textit{FKBP51} was similarly shown to be critical for its regulation of
tau stability (Jinwal, Koren et al. 2010). The PPIase activity of FKBP14 may affect regulation of the γ-secretase complex at the level of protein folding, stability, or activity.

While unable to rescue viability, further analyses with my mutant constructs could help determine whether, and to what extent, they are able to partially rescue the D58 escaper phenotypes, including the loss of bristles, fused ommatidia and wing margin notching. These constructs may also be used to determine which aspects of FKBP14 function are required within the context of phenotypes and interactions observed with presenilin and the Notch pathway, as well as the Notch-independent clonal inviability. For instance, the first FKBP_C domain of FKBP52 is crucial for receptor potentiation, but its PPIase activity is not required (Storer, Dickey et al. 2011), demonstrating that PPIase activity may not be the exclusive function of the FKBP_C domain. It may be that PPIase activity is required for a subset of FKBP14 functions. Such structure/function analysis of the various deletion and mutation constructs created will provide a deeper understanding of the role that FKBP14 is playing in Drosophila.
Chapter 5

Discussion and Future Directions
5 Discussion and Future Directions

Presenilins are highly conserved multipass transmembrane proteins that are required during development (Levitan and Greenwald 1995, De Strooper, Annaert et al. 1999) and implicated in familial Alzheimer’s Disease (FAD) (Thinakaran, Borchelt et al. 1996, Guo, Livne-Bar et al. 1999, Kimberly, Xia et al. 2000). A screen in Drosophila identified FKB14, a member of the family of FK506-binding proteins (FKBPs), as a genetic modifier of presenilin (van de Hoef, Hughes et al. 2009) with FKB14 mutants exhibiting defects typical of a loss of Notch activity (van de Hoef, Bonner et al. 2013). FKBPs are a large, highly conserved family of proteins involved in a wide array of biochemical processes including protein folding, assembly, and trafficking (Barik 2006, Kang, Hong et al. 2008, Galat 2013), yet they are non-essential in yeast (Dolinski, Muir et al. 1997) and their role in development of multicellular organisms remains unclear. The work presented in this thesis furthers our understanding of FKB14 function with respect to the regulation of Notch signalling via Presenilin and the γ-secretase complex, identifying a Notch-independent role for FKB14 in Drosophila development, and establishing that both the PPIase and EF hand domains are required for FKB14 function. These data establish a novel role for FKB14 in multicellular development.

5.1 Summary of Thesis Results

5.1.1 FKB14 is an Essential Gene that Regulates Presenilin and Notch Signalling in Drosophila Development (Chapter 2)

FKBP14 mutants are lethal and escapers display Notch-like phenotypes (van de Hoef, Bonner et al. 2013). In Chapter 2 I show that these observed phenotypes are not downstream of a general ER stress response. I show that FKB14 endogenously modifies the Notch pathway at the level of γ-secretase activity, where loss of FKB14 results in a decrease in γ-secretase cleavage ability. Loss of Psn protein was observed in FKB14 mutants (van de Hoef, Bonner et al. 2013) and I show that FKB14 and Psn physically interact. These data suggest that FKB14 acts on Psn to effect stability, assembly, or activity of the γ-secretase complex, resulting in the observed effects on Notch signalling. These are among the first data linking FKBPs to Notch signalling and demonstrating a requirement for FKBPs in multicellular development.
5.1.2 FKB14 has Notch-Independent Roles in *Drosophila* Development (Chapter 3)

Despite their high level of conservation, previous studies have shown that all FKBPs are dispensable for viability in yeast (Dolinski, Muir et al. 1997), suggesting that members of this family likely interact with a unique set of partners to perform specific, non-essential functions. However, comparative analysis of the PPIase repertoires in multiple models suggests that FKBPs have evolved to fill changing roles within evolving organisms (Pemberton and Kay 2005). Evidence in multicellular models indicates that FKBPs regulate multiple essential intracellular pathways in highly specific manners, depending on the biological context (Solassol, Mange et al. 2011). I show that FKB14 is required for cell viability in multiple tissue types, in a Notch-independent manner, suggesting that FKB14 has multiple roles throughout *Drosophila* development.

5.1.3 The PPIase and EF hand Domains are Required for FKB14 Function (Chapter 4)

Sequence analysis of FKB14 reveals a conserved PPIase domain and EF hand domain, including residues predicted to be required for activity/function of each. I generated a number of wild type and mutant alleles of FKB14 and show that both PPIase and EF hand domains are required to rescue lethality associated with loss of FKB14, indicating that both domains are critical for FKB14 function.

5.1.4 Human FKB14 Interacts with the Cell Cycle Regulator CHEK1 (Appendix A)

I performed a series of immunoprecipitation and mass spectrometry experiments to identify potential interactors of FKB14. Using human FKB14, I identified CHEK1 as an interactor with FKB14 in cell culture, which I confirmed by Western analysis, suggesting that one aspect of FKB14 function may involve cell cycle regulation. The functional significance of this interaction is as yet unknown, but may provide a new context in which to define the role of FKB14.
5.2 FKBP14 in Notch Signalling

5.2.1 Model for FKBP14 Function in Notch Signalling

The data presented in this thesis and previous work by Diana van de Hoef (van de Hoef, Bonner et al. 2013) indicate a requirement for FKBP14 in Notch signalling. My thesis data suggest that FKBP14 is required in the ER for early γ-secretase assembly, potentially through a role in the stability, folding, or association of members of the γ-secretase. The loss of γ-secretase cleavage activity observed in FKBP14 mutants, together with the observed loss of Psn protein in FKBP14 mutants (van de Hoef, Bonner et al. 2013) and data indicating that FKBP14 physically interacts with holo-Psn (Chapter 2), implies that FKBP14 likely interacts with holo-Psn in the ER (Fig. 5.1, subcomplex F1) and that loss of this interaction results in loss of Psn and downstream γ-secretase activity.

The observation that loss of FKBP14 shows a similar requirement in cell viability as observed for Aph-1 (Cooper, Deng et al. 2009) suggests the possibility that loss of FKBP14 may also be affecting levels of Aph-1. Indeed, partial colocalization of FKBP14 and Aph-1, but not Nct or Pen-2, was previously observed in Drosophila cell culture (van de Hoef, Bonner et al. 2013), suggesting that an interaction between FKBP14 and Aph-1 in the ER may be possible (Fig. 5.1, subcomplex F2). Current models hold that Aph-1 and Nct form a subcomplex that stabilizes Psn early in γ-secretase assembly (Fig. 5.1, subcomplex Ia), prior to Psn endoproteolysis and incorporation of Pen-2 (Hu and Fortini 2003). However, mammalian APH-1 has also been shown to interact with immature forms of PS (Fig. 5.1, subcomplex Ib), and this interaction requires a conserved GXXXG motif in APH-1 (Lee, Shah et al. 2002, Gu, Chen et al. 2003). Mutations to this motif lead to a “loosely folded” APH-1 protein that can still interact with NCSTN, but disrupts interaction of the subcomplex with PS, resulting in rapid degradation of APH-1 (Niimura, Isoo et al. 2005). It is possible that through interaction with Psn, Aph-1, or both, FKBP14 is required for assembly or stability of this subcomplex (Fig. 5.1, subcomplex F3) prior to interaction with Nct or Pen-2. Indeed, disruption of FKBP14 function may lead to destabilization of Aph-1, leading to both the cell viability phenotype and the effects observed on Psn and γ-secretase activity.
Figure 5.1: Model for FKBP14 Function in Notch Signalling

FKBP14 is required in the ER for early γ-secretase assembly, potentially through a role in component stability, folding, or association with other members of the complex. FKBP14 physically interacts with holo-Psn (F1) and potentially other γ-secretase components such as Aph-1 (F2). This interaction is required for folding or stability of these targets, possibly affecting their ability to form γ-secretase subcomplexes (F3) or eventually functional γ-secretase cleavage complexes. FKBP14 is restricted to the ER and does not colocalize with Nct or Pen-2, and is therefore unlikely to associate with more mature γ-secretase subcomplexes (II-V).
Given that endogenous FKBP14 localizes exclusively to the ER, and shows no co-localization with Nct or Pen-2, FKBP14 is not likely to be associated with more mature $\gamma$-secretase complexes. Determining which components and subcomplexes of $\gamma$-secretase with which FKBP14 associates, including at which point FKBP14 leaves the complex (Fig. 5.1, dashed arrows), is critical to understanding precisely how FKBP14 modulates $\gamma$-secretase assembly and activity. This knowledge will provide deeper understanding into the role of FKBP14 in Notch signalling, and potentially provide insight into $\gamma$-secretase dysregulation in Alzheimer’s Disease.

5.2.2 Interaction between FKBP14 and $\gamma$-secretase components

The data I present in Chapter 2 indicate that FKBP14 and Psn physically interact; this is in keeping with previous data indicating that loss of FKBP14 results in loss of Psn (van de Hoef, Bonner et al. 2013), while other transmembrane proteins do not appear similarly affected (Chapter 2). Mapping of this interaction, in conjunction with stability, assembly, and $\gamma$-secretase activity data will allow a more precise understanding of FKBP14 in $\gamma$-secretase biogenesis and function.

Through immunoprecipitation of $\gamma$-secretase components from cell culture we can successfully detect $\gamma$-secretase complexes (e.g. Nct can IP Psn, Chapter 2 Fig. 2.10 B). Using CHAPSO-based IPs, I propose to first confirm the interaction of holo-Psn with endogenous FKBP14 using another tag, followed by experiments to assess whether other $\gamma$-secretase members interact with FKBP14 in a similar manner. This can be done using the tagged constructs of Aph-1, Nct, Pen-2, and Psn variants that I have generated (Chapter 2 and data not shown). I hypothesize that interaction between Aph-1 and FKBP14 will be observed and this interaction may be involved in the cell viability phenotype observed for FKBP14 mutants. I do not anticipate observing any interactions between FKBP14 and Nct or Pen-2 as these components do not colocalize with FKBP14 in cell culture (van de Hoef, Bonner et al. 2013). Co-IPs will be performed using the anti-FKBP14 antibody to IP endogenous protein, and will be confirmed using HA or V5 tagged FKBP14. Reciprocal co-IPs using the tagged $\gamma$-secretase constructs will also be performed.

The tagged Psn and $\gamma$-secretase constructs are based on previously published constructs shown to successfully replicate endogenous activity and incorporate into functional $\gamma$-secretase complexes (Barakat, Mercer et al. 2009, Stempfle, Kanwar et al. 2010). However, new epitopes or epitope
insertion sites must be designed for FKBP14, as those previously designed (discussed in Chapter 4) appear to affect sub-cellular localization. I propose performing secondary structure modeling of FKBP14 to determine a possible site for tag insertion. One possibility is to place the tag immediately downstream of the ER signal peptide, which is predicted to be cleaved between residues 23 and 24 to form the mature protein (Petersen, Brunak et al. 2011).

Using the above methods, it will be possible to determine which members of γ-secretase interact with wild type FKBP14 in cell culture and may identify potential γ-secretase subcomplexes with which FKBP14 associates. Subsequent analysis will include mapping interactions to identify specific regions/functions of FKBP14 using my point mutation and deletion mutant constructs as described in Chapter 4. Reciprocal analyses with γ-secretase members shown to interact with FKBP14 will be performed using small deletions to assess which regions are required for this interaction. With respect to Psn, I hypothesize that FKBP14 interacts with the holo form of the protein, based on co-IP data (Chapter 2) and current models of γ-secretase assembly in which Psn is endolytically cleaved following incorporation of Pen-2 into the γ-secretase complex (Dries and Yu 2008). Initial Psn deletion mutants may thus include those eliminating the endoproteolysis site [DExon9, (Barakat, Mercer et al. 2009) and Chapter2], and those eliminating the entire cytoplasmic loop (Barakat, Mercer et al. 2009), using tagged Psn NTF and CTF constructs as controls. It will be interesting to test whether any regions determined to be required for interaction with FKBP14 include proline residues, which may be acted upon by the PPIase function of FKBP14. One notable region of interest is the conserved Pro-Ala-Leu-Pro (PALP) motif at the C-terminus of Psn. Mutations in the first proline residue of this motif (P433 in PS1) abrogate γ-secretase function and lead to a loss of Notch phenotype, associated with rapid degradation of holo-PS prior to endoproteolysis (Tomita, Watabiki et al. 2001, Wang, Brunkan et al. 2004). If cis/trans isomerization around this proline is effected by FKBP14, loss of FKBP14 may be leading to similar downstream effects as observed for PALP mutations.

5.2.3 How does loss of FKBP14 affect γ-secretase assembly and activity?

Following confirmation of binding between FKBP14 and members of the γ-secretase, and mapping of these regions in both proteins, subsequent analyses will focus on determining the biological relevance of these interactions, including effects on γ-secretase activity.
Using a cell culture short interfering RNA (dsRNA) directed against the 3’UTR of Aph-1, Niimura and colleagues were able to show efficient knockdown of endogenous Aph-1, comparable to coding sequence (CDS) RNAi, and used this technique, along with several cell culture Aph-1 constructs, to discern a role for the GXXXG motif in Aph-1 in early γ-secretase assembly and stability (Niimura, Isoo et al. 2005). Similar experiments with FKBP14 may be used to identify a role for FKBP14 in Psn stability and γ-secretase assembly. Specifically, I propose designing a dsRNA construct against the 3’ UTR of the Drosophila FKBP14 gene, using a scrambled dsRNA construct as a negative control. Levels of knockdown will be assessed by Western blot using the endogenous anti-FKBP14 antibody previously developed in the Boulianne lab (van de Hoef, Bonner et al. 2013). The effects of loss of FKBP14 on Psn and other members of the γ-secretase complex will then be determined by Western blot using antibodies generated by the Merdes Lab (Stempfle, Kanwar et al. 2010). The treated cells will also be co-transfected with SC100, the carboxyl-terminal 99-amino acid fragment of betaAPP fused to a signal peptide of rat preproenkephalin cDNA, to be used as a read out of γ-secretase cleavage activity (similar to the GMR-C99 assay described in Chapter 2), as described in Nimura et al (Niimura, Isoo et al. 2005). Using these tools, we can determine whether knockdown of endogenous FKBP14 results in loss of Psn stability and/or γ-secretase activity. It is anticipated that loss of FKBP14 in cell culture will result in a reduction in Psn protein and γ-secretase activity, as observed in FKBP14 mutants [(van de Hoef, Bonner et al. 2013) and Chapter 2]. If effects are also observed on other γ-secretase members, such as Aph-1, this will provide further insight into the stage of γ-secretase assembly at which FKBP14 acts. We can then assess which domains/residues of FKBP14 are required for this effect by transfecting wild type or mutant FKBP14 cell culture constructs which, having a vector specific UTR, will not be targeted by the dsRNA directed against endogenous FKBP14.

Complementary experiments will be carried out using a 3’UTR dsRNA targeted to endogenous γ-secretase components. Initial experiments will focus on Psn and Psn constructs mutated in regions to which we have mapped the interaction with FKBP14. These experiments will confirm the biological relevance and the mapped location of interaction between FKBP14 and the γ-secretase components with which it associates.
5.2.4 Does FKBP14 regulate Psn conformation?

It has been shown that a conformational change in PS1 can result in a shift in the ratio of Aβ40 to Aβ42 peptides produced (Uemura, Lill et al. 2009, Dolev, Fogel et al. 2013), demonstrating the importance of PS conformation in regulation of γ-secretase activity. Moreover, the initial substrate binding site within γ-secretase is on PS near the active site (Kornilova, Bihel et al. 2005), and FAD mutations in PS1 alter conformation of the γ-secretase active site, affecting its activity and substrate specificity (Chau, Crump et al. 2012), indicating that changes in PS conformation can have pathogenic consequences. Indeed, changes in the conformation of wild type PS1 have been shown to occur in sporadic cases of Alzheimer’s Disease and during normal aging, and this conformational shift is associated with amyloid pathology, preceding Aβ deposition (Wahlster, Arimon et al. 2013).

To determine whether FKBP14 regulates Psn conformation, I propose assessing changes in gross conformation of Psn due to loss or mutation in FKBP14, using a GFP-Psn-RFP construct to monitor Psn conformation (NT-loop proximity) in live cells, based on the construct described in Uemura et al. (Uemura, Lill et al. 2009). Briefly, GFP will be fused to the N-terminus of Drosophila Psn, and RFP will be inserted into the TM6-7 loop domain, resulting in a molecular probe for intramolecular FRET-based analysis. Previous analyses with a mammalian PS1 version of this construct has shown that it traffics appropriately and can incorporate into an active γ-secretase complex (Uemura, Lill et al. 2009). We will use the GFP-Psn-RFP construct to assess Psn NT-loop proximity in the presence or absence of FKBP14, using the UTR dsRNA construct against endogenous FKBP14 described above (Section 5.2.3). We can furthermore assess the effect of various mutations in FKBP14 on Psn NT-loop proximity. In particular, it will be interesting to note whether PPIase activity of FKBP14 is required to maintain correct Psn conformation, which would suggest that FKBP14 is involved in Psn folding. Alternatively, if the EF hand is required, it will be interesting to assess how Ca^{2+} regulates this effect.

If FKBP14 and Aph-1 are shown to interact by the experiments proposed in above sections, similar experiments can be performed to determine if FKBP14 regulates Aph-1 conformation and whether such an effect is relevant to Aph-1 function in cell viability or γ-secretase activity.
5.3 FKBP14 in Cell Viability

I have shown that *FKBP14* is required for cell viability in multiple tissue types, in a Notch-independent manner, suggesting that FKBP14 has multiple roles throughout *Drosophila* development (Chapter 3). This is in keeping with previous data showing that *FKBP14* mutants have reduced SOPs, in contrast to typical loss of Notch mutations (van de Hoef, Bonner et al. 2013). It remains to be determined by what mechanism FKBP14 is required for cell viability. Two interesting possibilities arise based on the previously published *aph-1* cell viability phenotype (Cooper, Deng et al. 2009) and novel data presented in this thesis: FKBP14 may be required for cell viability through interaction with Aph-1, or FKBP14 may be required for cell viability through interaction with the checkpoint protein CHEK1/grp (Appendix A).

Mutations in *aph-1* show a similar defect in cell viability in the wing disc as described for *FKBP14* mutants in Chapter 3, and this effect appears independent of its role in regulating γ-secretase activity (Cooper, Deng et al. 2009). Interestingly, holo-Psn lacking the endolytic cleavage site (PS-\text{exon9}), which was shown to incorporate into functional γ-secretase complexes, could not rescue the *aph-1* mutant cell viability defect. In contrast, cleaved Psn N- and C-terminal fragments were able to rescue cell viability (Cooper, Deng et al. 2009). These data indicate that Aph-1 and holo-Psn may play a role in cell survival, independent of functional γ-secretase. As noted above, partial colocalization of FKBP14 and Aph-1 was previously observed in *Drosophila* cell culture (van de Hoef, Bonner et al. 2013), suggesting that an interaction between these proteins in the ER may be possible (Fig. 5.1, subcomplex F2). Whether loss of FKBP14 affects Aph-1 stability or function and whether any such interaction may be responsible for the observed cell viability requirement for FKBP14, is not yet known. If FKBP14 is shown to interact with Aph-1 through experiments as described above (Sections 5.2.2 and 5.2.3), follow up experiments should be pursued to determine if this interaction plays a part in the cell viability defect observed in *FKBP14* mutants. In particular, if loss of FKBP14 leads to destabilization or loss of Aph-1 protein (Section 5.2.3), the cell viability defect observed in *FKBP14* mosaic clone experiments (Chapter 3) may be downstream of this effect. In this case, it may be possible to rescue the FKBP14 mutant clones by expressing wild type *aph-1*. It will also be important to determine whether *FKBP14* mutant clones can be similarly rescued by Psn fragments but not holo-Psn, as observed for *aph-1* mutant clones (Cooper, Deng et al. 2009). If
FKBP14 mutant clones can indeed be rescued under these circumstances, this data would further support a requirement for FKBP14 in regulating Psn and Aph-1 in the ER.

Alternatively, the cell inviability phenotype observed for FKBP14 mutant clones may be due to a failure of mutant cells to progress through the cell cycle. The IP/MS experiments I performed using human FKBP14 (Appendix A) revealed an interaction between FKBP14 and CHEK1, a highly conserved checkpoint kinase required for G2/M transition (Liu, Guntuku et al. 2000), which I confirmed by Western blot. Interestingly, the Drosophila homologue for CHEK1, grapes (grp), was also identified in the original genetic screen for Psn interactors that identified FKBP14 (van de Hoef, Hughes et al. 2009), suggesting that the interaction observed between FKBP14 and CHEK1 may somehow play into the regulation of Psn by FKBP14. It remains to be seen whether this interaction is conserved in Drosophila, but it suggests that FKBP14 may regulate cell cycle progression.

To define the relevance of an FKBP14/CHEK1 interaction, it must first be confirmed with several control experiments, including reciprocal co-IP of CHEK1, probing for FKBP14. Potential colocalization of FKBP14 and CHEK1 in cell culture must also be determined to rule out the possibility of a false-positive result due to post-lysis interactions. FKBP14 is expected to be exclusively localized to the ER, while CHEK1 is a largely nuclear protein with some cytoplasmic localization during certain cell cycle stages (Wang, Han et al. 2012). It may therefore be necessary to synchronize cells in culture to assess at which point of the cell cycle, if any, FKBP14 and CHEK1 may potentially interact. Further co-IP experiments using the constructs I have generated (Appendix A) are also necessary to determine whether interaction between FKBP14 and CHEK1 is conserved in Drosophila.

If it is shown that FKBP14 and Drosophila CHEK1 (grp) interact in cell culture, this interaction may contribute to the cell viability defect observed in FKBP14 mutants. To determine if grp can modify this phenotype, I propose carrying out mosaic clone analyses with FKBP14 mutants in animals over-expressing or bearing mutations in grp. In addition, I suggest carrying out FKBP14 mutant clonal analyses in animals over-expressing string (stg), a Drosophila Cdc25-type phosphatase that triggers mitosis in G2 arrested cells (Edgar, Lehman et al. 1994, Negre, Ghysen et al. 2003, Shyu, Sun et al. 2009). If loss of FKBP14 is resulting in G2/M arrest through an effect on Grp, the cell viability defect associated with mitotic clones should be modified by loss
or gain of Grp and rescued by stg expression. The resulting FKBP14 mutant clones may then be probed to determine which signalling pathways are being disrupted by loss of FKBP14.

By determining whether an interaction with γ-secretase component Aph-1 or checkpoint kinase Grp contributes to the cell inviability phenotype associated with FKBP14 mutant clones, these analyses should elucidate the broader roles of FKBP14 in *Drosophila* development. Moreover, follow up characterization of the domains and residues of FKBP14 required and the nature of the interaction may be carried out with the tools I have generated and those described in the sections above.

### 5.4 FKBP14 Structure/Function

I have shown that both the PPIase and EF hand domains are required to rescue the lethality associated with FKBP14 mutants (Chapter 4), indicating that both domains are necessary for FKBP14 function. This suggests that PPIase activity may be necessary for some or all functions of FKBP14 and that Ca^{2+} binding may regulate aspects of FKBP14 function.

The crystal structure of human FKBP14, containing a classical FKBP_C domain and two EF hand motifs, revealed that it may form a dimer via its EF hand motifs, with an elongated and primarily hydrophobic cavity potentially available for ligand binding (Boudko, Ishikawa et al. 2014). Dimerization through the EF hand domain may play an important role in *Drosophila* FKBP14 function or regulation (Fig. 5.2 A). It may be that mutations in the EF hand designed to abrogate Ca^{2+} binding also disrupt dimerization, preventing the formation of a ligand cavity (Fig. 5.2 A) and thus preventing FKBP14 from interacting with its target(s). Alternatively, FKBP14 may act as a monomer (Fig. 5.2 B), in which case the loss of function associated with mutations in the EF hand are more likely due to a failure to bind Ca^{2+}. As observed for other FKBPs, such as FKBP38 (Edlich, Weiwad et al. 2005) and FKBP23 (Zhang, Wang et al. 2004), Ca^{2+} binding may regulate FKBP14 complex formation and functionality. Co-IP experiments using differentially tagged wild type and EF hand mutant constructs will clarify whether dimerization occurs in *Drosophila* cells and whether the EF hand is required for this. If so, it will be interesting to ask whether dimerization or Ca^{2+} binding is important for the interaction observed with Psn and Notch phenotypes described in Chapter 2, or the cell viability phenotype described in Chapter 3.
Figure 5.2: FKBP14 Structure/Function Models

Both the PPIase and EF hand domains are required for FKBP14 function (Chapter 4). FKBP14 may act as a dimer via its EF hand, creating a substrate pocket between the PPIase domains (A). This dimerization may be Ca$^{2+}$ dependent.

FKBP14 may also act as a monomer (B), where both domains are required for interaction with or folding of its target protein. Ca$^{2+}$ may in this case regulate the activity of FKBP14 or its target.

The requirement for the FKBP_C domain suggests that PPIase activity comprises an important part of FKBP14 function. It may be that the mutations in the PPIase domain interfere with FKBP14-catalyzed isomerization of targets (Fig. 5.2 B), which may lead to target
misfolding/degradation or the inability of target proteins to recognize and bind other interactors. To determine whether FKBP14 has PPIase activity it will be necessary to purify FKBP14 protein from *E.coli*, and perform a PPIase activity assay as per Wintermeyer *et al* (Wintermeyer, Ludwig et al. 1995). Briefly, PPIase activities of wild type and mutant forms of FKBP14 will be determined by protease coupled assays using the synthetic peptide Suc-Ala-Phe-Pro-Phe-4-nitroanilide as a substrate (Wintermeyer, Ludwig et al. 1995). The enzyme chymotrypsin cleaves nitroanilide from the synthetic peptide when in *trans* conformation only, and this reaction is measured spectrophotometrically at constant wavelength in the presence and absence of a potential PPIase. Following confirmation that FKBP14 indeed possesses PPIase activity, more specific analyses of identified interactors, such as Psn, may be performed using synthetic decapeptides centred on prolines of interest in the Psn backbone, identified by the mapping analyses described in Section 5.2.2. The rate of proline isomerization will be probed by measuring the intensities of exchange crosspeaks correlating *cis* and *trans* specific proline H resonances from NOESY NMR spectra (Monneau, Soufari et al. 2013). It will then be possible to assess how mutations to FKBP14, including mutations in the PPIase domain, affect rates of substrate isomerization and interactions of targets such as Psn with other proteins, such as other members of the γ-secretase complex.

From these analyses we will be able to test for PPIase activity and potentially target sequence specificity of FKBP14. Together with dimerization, conformational and stability experiments, we will gain a greater understanding of the mechanism by which FKBP14 regulates its interactors. In particular this work will clarify the role of FKBP14 in the stability, conformation, assembly and ultimately activity of the γ-secretase complex.

### 5.5 FKBP14 in Disease

The data in this thesis, along with previously published data, establish a critical role for FKBP14 in development, one aspect of which includes regulating the γ-secretase complex, likely through an effect on Psn. PSs have been highly correlated with familial Alzheimer’s Disease (FAD), where mutations in PSs can lead to early onset, aggressive forms of FAD (Rogaev, Sherrington et al. 1995, Sherrington, Rogaev et al. 1995). Cleavage by the γ–secretase complex within the transmembrane domain of another FAD associated protein, Amyloid Precursor Protein (APP), generates the APP intracellular domain (AICD), and 39-43 amino acid long peptides, termed
β-amyloid peptides (Aβ) (Fig. 1.2) (Vetrivel, Zhang et al. 2006). Autosomal dominant mutations in the genes coding for APP and the PS proteins are associated with an increase in γ-secretase mediated cleavage product of APP, amyloid beta peptide 1-42 (Aβ42) (Hashimoto, Rockenstein et al. 2003, Vetrivel, Zhang et al. 2006).

FKBPs are enriched in neuronal tissue and their expression is elevated after nerve injury (Kang, Hong et al. 2008). Several FKBPs have been implicated in neurodegenerative diseases (Avramut and Achim 2002, Liu, Liu et al. 2006, Sugata, Matsuo et al. 2009, Chambraud, Sardin et al. 2010, Sanokawa-Akakura, Cao et al. 2010, Liu, Liu et al. 2014). Indeed, the FKBP ligand FK506 has been shown to have neuroprotective effects and may induce neuronal regeneration (Galat 2013), suggesting significant roles for FKBPs in neural growth, injury, and regeneration.

I have shown that loss of FKBP14 leads to a loss of γ-secretase cleavage activity (Chapter 2), suggesting that FKBP14 may provide an avenue for modulation of γ-secretase function in disease states. Disruption or over-expression of FKBP14 may also potentially modify downstream FAD phenotypes or outcomes. To determine if this is the case, I propose carrying out similar experiments as described by Sanokawa-Akakura et al (Sanokawa-Akakura, Cao et al. 2010), wherein we examine gain or loss of FKBP14 in Drosophila expressing the Aβ toxigenic peptide Aβ42, using the less toxic Aβ40 as a control. If it is found that loss or gain of FKBP14 can modify Aβ phenotypes, follow up experiments may be carried out as per sections 5.2.2 and 5.2.3 examining which domains of FKBP14 are required for this function, or whether interactions with γ-secretase components mediate this function. Further analysis with chemical modifiers, such as FK506, may provide insight into potential Alzheimer’s Disease treatment avenues.

Human FKBP14 has also been associated with Ehlers-Danlos Syndrome (EDS). EDS is a clinically and genetically heterogenous group of heritable connective tissue disorders that affects multiple tissue types (Baumann, Giunta et al. 2012). An autosomal recessive extreme variant of this disorder, which shares features with congenital muscular dystrophy, has been associated with a frameshift in FKB14 (Baumann, Giunta et al. 2012). EDS is associated with defects in collagen production, processing or assembly, and individuals with FKBP14-associated EDS show abnormal distribution and assembly of extracellular matrix components, especially type I and III collagens and fibronectin (Baumann, Giunta et al. 2012). No known link between this
disease and PS or γ-secretase function has been described. However, procollagen III is processed by meprin β (Broder and Becker-Pauly 2013), a metalloprotease that regulates and is itself regulated by ADAM10, the constitutive α-secretase which cleaves APP. Moreover, meprin β also cleaves APP in a manner similar to the β secretase BACE (Bien, Jefferson et al. 2012, Broder and Becker-Pauly 2013), which is then cleaved by γ-secretase to produce Aβ peptides (Vetrivel, Zhang et al. 2006). While no cleavage of procollagen III by γ-secretase has been reported, if such a cleavage were to be required to ensure appropriate maturation of collagen III, the clinical phenotype of FKBP14-associated EDS patients may be related to a defect in FKBP14 regulation of PS. Examination of this possibility would require experiments in mammals to determine if procollagen III is indeed a substrate for γ-secretase, or an interactor with PS or FKBP14. If so, follow up experiments to determine the nature of this interaction may reveal a novel aspect of PS, γ-secretase and/or FKBP14 function in development.

5.6 Conclusions

Despite their high level of conservation and association with multiple regulatory pathways, many FKBPs remain largely uncharacterized and the contribution of FKBPs to multicellular development remains poorly understood. The critical role identified herein for FKBP14 in Notch signalling and cell viability suggests that novel important roles specific to multicellular signalling have emerged for this family of proteins in metazoan evolution. Moreover, these studies provide an additional link between FKBPs and degenerative diseases such as Alzheimer’s. Elucidating the precise mechanism by which FKBP14 regulates Psn, γ-secretase, and cell viability will provide crucial insight into both development and disease, as well as a greater appreciation for the contribution of FKBPs to these cellular processes.
Appendix A: FKB14 Interacts with The Cell Cycle Regulator CHEK1

**Data Attribution:** Julia Maeve Bonner performed all experiments described herein with the following exceptions: A.C. Gingras Lab generously provided the entry vector J.M. Bonner used to generate the tagged hFKBP14 cell culture vectors. Stable cell lines were established from these vectors by the Gingras lab. J.M. Bonner performed the standard IPs with assistance from Marilyn Goudreault. Chaperone IPs and Mass Spec data collection were performed by Zhen-Yuan Lin using samples generated by J.M. Bonner.
A.1 Summary

FKBP14 has been shown to have both Notch-dependent and Notch-independent roles in *Drosophila* development. To gain greater understanding of the broader role of FKBP14, I performed a series of immunoprecipitation and mass spectrometry experiments to identify potential interactors. Using the human FKBP14 homolog, I identified CHEK1 as an interactor with FKBP14 in cell culture, which I confirmed by Western analysis. The functional significance of this interaction is as yet unknown, but may provide a new context in which to understand the role of FKBP14.

A.2 Introduction

Though we have shown that *FKBP14* plays a critical role in development, we still understand very little regarding *FKBP14* targets or regulators that may be important for *FKBP14* to carry out its function. A clearer picture of the breadth of FKBP14 function may be achieved by asking which proteins FKBP14 physically interacts with *in vivo*. Towards this end, I collaborated with the lab of Anne-Claude Gingras, generating stably transfected HEK293 cells expressing the human homologue of *FKBP14* (hFKBP14) to perform a series of unbiased immunoprecipitation experiments followed by Mass Spectrometry (IP/MS). I show that hFKBP14 immunoprecipitates CHEK1 in multiple IP/MS experiments and this interaction is confirmed by Western analysis. I also describe preliminary work exploring whether this interaction is conserved in *Drosophila*.

A.3 Materials and Methods

A.3.1 Plasmid construction

hFKBP14 cell culture vectors were based on the entry clone generously provided by A.C. Gingras lab. A single FLAG tag was inserted upstream of the ER retention sequence (KHDEL) using Quikchange Lightning Site Directed Mutagenesis Kit (Stratagene). Subcloning was performed using Gateway recombination (Invitrogen), and confirmed by sequencing (TCAG, Hospital for Sick Children).
The *grp* cDNA was amplified off of purified genomic DNA from a *grp* transgenic line, cloned into a pENTR/D/TOPO vector and subcloned using Gateway recombination (Invitrogen). A GFP-myc plasmid was generated based on the GFP constructs described in Chapter 2 as a negative control using Gateway recombination (Invitrogen).

The primers used are as follows (5' → 3'):

**grp into pENTR:** CACCAAAATGGCTGCAACGCTGAC

**Insertion of 1XFLAG tag into hFKBP14:**
CTGCCAGAGAATTTACATATGATTACAAGGATGATGATGATAAGAAACACGATGAGTTATAG

A.3.2 Cell culture and immunoprecipitation

Stable cells were generated in Flp-in T-Rex cells (Spitzer, Landthaler et al. 2013) for GFP 1XFLAG and the above described hFKBP14 1XFLAG by the Gingras lab as described (Lambert, Ivosev et al. 2013). Cells were maintained under standard conditions (37°C, 5% CO2) in DMEM high glucose, supplemented with Pen/Strep (100U/ml), Fetal Bovine Serum (5%), Cosmic Calf serum (5%). 2 x 150mm plates of near confluent mammalian cells were used for IP/MS analysis. Expression of stably tranfected genes was induced by treatment with 1 µg/ml tetracycline for 24 hours. Cells were lysed by passive lysis assisted by freeze-thaw. Briefly, to the frozen cell pellet, 1:4 pellet weight:volume ratio of ice-cold lysis buffer was added and the frozen pellet was resuspended by pipetting up and down. Standard lysis buffer was 50 mM Hepes-NaOH pH 8.0, 100 mM KCl, 2 mM EDTA, 0.1% NP40, 10% glycerol, 1 mM PMSF, 1 mM DTT and Sigma protease inhibitor cocktail, P8340, 1:500. Chaperone IP lysis buffer was prepared as above with the addition of 0.01uM Calycin A and 10mM Na2MoSO4. Lysis was assisted by freeze/thaw on dry ice and 37°C water bath. Samples were spun in microfuge at top speed for 20 min at 4°C.

20µl of anti-FLAG magnetic resin was used per pellet from each 2x15cm plate. Beads were washed with lysis buffer, usually 3x1ml, then added to the sample supernatant at 4°C for 2 h. For standard IP, beads were washed with 3x1ml lysis buffer and 2x1ml 20mM Tris-HCl (pH 8.0) + 2mM CaCl₂. For Chaperone IP, beads were washed 1x with each buffer. 500ng trypsin in digestion buffer (20mM Tris-Hcl pH 8.0 in MS grade water) was added to beads, followed by 4 h incubation at 37°C. Supernatant was removed to a clean eppendorf, and another 500ng of trypsin in digestion buffer was added for overnight incubation at 37°C. Formic acid was added to 2 or 5% final concentration. Samples were stored at -40°C until run on MS. Aliquots for Western
analysis were taken of input (spun down, assessed supernatant “whole cell lysate” and pellet separately), IP supernatant (“supernatant”) and IP sample (“IP”).

For *Drosophila* IPs, transfected or control Kc cells (~1 x 10^7 cells) were harvested in ice cold PBS and pellets were flash frozen on dry ice. Pellets were thawed and lysed for 30 min on ice in 300ml lysis buffer containing 125mM NaCl, 50mM HEPES pH 7.4, 1% chaps (w/v) and Complete protease inhibitors (Roche). Lysates were pre-cleared with 70% protein G slurry (Sigma-Aldrich) and centrifuged (14k rpm for 15 min). Cleared lysates were incubated overnight at 4°C with protein G sepharose beads and specific antibodies. Beads were washed with lysis buffer and resuspended in 4X NuPAGE LDS sample buffer (Life Technologies) under reducing conditions.

### A.3.4 Mass spectrometry

Performed as described (Lambert, Ivosev et al. 2013).

### A.3.5 Immunoblot analysis

IP samples from Flp-in T-Rex or transfected *Drosophila* Kc cells were electrophoresed on 10 or 12% polyacrylamide gels. Gels were then processed for Western blotting and immunodetection with mouse anti-FLAG M2 (Sigma-Aldrich, 1:2000), rabbit anti-Chk1 (Millipore, 1:1000), mouse anti-myc (DSHB, 1:200), rabbit anti-myc (Sigma-Aldrich, 1:1000), mouse anti-GFP (Genscript, 1:1000), rabbit anti-V5 (Invitrogen, 1:1000), mouse anti-V5 (Invitrogen, 1:2000), rat anti-FKBP14 (1:2000), and mouse anti-actin (DSHB, 1:200). Immunodetection was performed using either Li-Cor fluorescent detection (Li-Cor) or ECL (PerkinElmer).

### A.4 Results and Discussion

To gain a clearer picture of the breadth of FKBP14 function in *vivo*, I collaborated with the lab of Anne-Claude Gingras, generating stably transfected HEK293 cells expressing the human homologue of *FKBP14* (hFKBP14) (Fig. A.1 A) under the control of the tet operator (Fig. A.1 B-C) to perform a series of unbiased immunoprecipitation experiments followed by Mass Spectrometry (IP/MS). Specifically, I engineered a C-terminal FLAG tag into the human
FKBP14 construct, and used this epitope to perform IP/MS experiments, using a FLAG-tagged GFP stable cell line as a control.

Figure A.1: Human FKBP14 construct design and and stable cell expression schematic

(A) Human FKBP14 FLAG tagged construct generated: The FLAG tag (red) was inserted immediately upstream of the ER retention sequence (KHDEL, purple). The PPIase domain is shown in yellow, and the putative EF hand is shown in blue.

(B) In stably transfected Flp-in T-Rex cells, expression of hFKBP14 is repressed by the tet repressor (tetR).

(C) Following tet addition (black triangles), expression of hFKBP14 is de-repressed. Expression was induced 24 h prior to cell harvest.
The human homologue was selected for these experiments to take advantage of the wealth of previous IP/MS data available from the Gingras lab and others, allowing a more accurate comparison of our data against other co-IPs and complexes they identify, as well as eliminating common “frequent flyer” false positives. As the human and *Drosophila* FKBP14 are highly homologous (44% identity), it is likely that any interactors identified in the human system may be representative of possible interactors in *Drosophila*.

Two initial IPs were performed under standard conditions. In each case, I was able to IP a significant amount of FKBP14 protein, as determined by peptide count and confirmed by Western blot (Fig. A.2 A). Contrary to my expectations, very few proteins co-immunoprecipitated with FKBP14 under standard conditions in two independent experiments as detected by MS (Fig. A.2 A). It is possible that FKBP14 targets may be in the insoluble fraction, including trans-membrane proteins like PS. Alternatively, FKBP14 may interact very transiently with its soluble targets, and these interactions may be susceptible to the lysis buffer or wash steps involved in the IP. Given that FKBP14 contains what appears to be a functional PPIase domain, it may act as a chaperone in the ER. I therefore collaborated with a member of the Gingras lab to perform a further two independent chaperone-specific IP/MS experiments using the FLAG-tagged FKBP14-expressing cells. Under these conditions, we were able to successfully IP CHEK1 (NP_001265), a highly conserved checkpoint protein required for the G2/M transition in the cell cycle (Liu, Guntuku et al. 2000). I had previously observed co-immunoprecipitation of CHEK1 with hFKBP14 in my first IP and observed this interaction a further two times using the chaperone-specific IP, indicating that this is likely a true interaction, possibly stabilized under the chaperone IP conditions (Fig. A.2 A). I was further able to observe this interaction by Western blot (Fig. A.2 B), confirming that full length CHEK1 is indeed interacting with human FKBP14.
Figure A.2: Human FKBP14 IP/MS results

(A) Three (of four total) independent IP/MS identified CHEK1 as an interactor with FKBP14. This interaction appears stabilized under the chaperone specific lysis conditions used for the final two IP/MS experiments.

(B) Western blot confirming Human FKBP14 interacts with CHEK1: FLAG-tagged FKBP14 was able to immunoprecipitate CHEK1 in detectable levels by Western blot, whereas the control FLAG-tagged GFP was not able to do so.

Interestingly, the *Drosophila* homologue for CHEK1, *grapes* (*grp*) (Fig. A.3 A), was also identified in the original genetic screen for *Psn* interactors (van de Hoef, Hughes et al. 2009). To assess whether the interaction with CHEK1/*grp* is conserved in *Drosophila*, I generated cell culture constructs expressing full length *grp* fused to a myc tag, as well as a GFP-myc cell culture control construct (Fig. A.3 B).
Figure A.3: Human CHEK1 and Drosophila Grp alignment and construct expression

(A) Alignment between Human CHEK1 and the Drosophila homologue Grp, revealing high conservation (*) and semi-conservation (.).

(B) Expression of GFP-myc control constructs and grp-myc constructs. Those used in IP experiments are indicated in red.
Using these new tools and my previously generated FKBP14 full length cell culture constructs, I performed a series of preliminary co-IP experiments. From these initial experiments, it appears that the interaction may be conserved in *Drosophila*. However, the grp-myc construct appears to IP with antibody alone under some conditions, obscuring the preliminary results (Fig. A.4 A). Performing the IP with our polyclonal antibody against endogenous FKBP14 showed similar results, with grp-myc immunoprecipitating with antibody alone (Fig. A.4 B). I was therefore unable to determine whether the interaction observed between hFKBP14 and CHEK1 is conserved in *Drosophila*. Further analysis with different tags or IP conditions may resolve this question.

![Figure A.4: Drosophila FKBP14 may IP grp](image)

(A) *Drosophila* cell culture co-IP showing that grp-myc may potentially co-IP with FKBP14 V5, but appears to bind to beads and antibody alone, obscuring results. The control GFP-myc does not IP with FKBP14-V5 or antibody alone. 
(B) *Drosophila* cell culture co-IP with endogenous anti-FKBP14 polyclonal antibody shows similar results as the anti-V5 IP: grp-myc may potentially co-IP with endogenous FKBP14, but this interaction isn’t enhanced by over-expression of FKBP14 V5. GFP myc does not co-IP with endogenous FKBP14.
If the interaction with CHEK1/grp is conserved, the tools I have generated will allow one to assess which domains of FKBP14 are required for this interaction. An understanding of this interaction may reveal some of the roles of *FKBP14*. If conserved, it will be interesting to examine this interaction in the context of the cell viability requirement observed for FKBP14, and whether a failure to progress through the cell cycle may account for the phenotype observed.

Of note, the chaperone IP conditions also identified another potential interactor, RPL13, a cytoplasmic ribosomal protein. RPL13 has been shown to interact with CDC5L (Ajuh, Kuster et al. 2000), another cell cycle regulator important for the G2/M transition. RPL13 is conserved in *Drosophila* and if the interaction with CHEK1 is confirmed, it may be interesting to examine if RPL13 is important for this aspect of FKBP14 function.
References


