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FKBP14 is an essential gene that regulates Presenilin protein levels and Notch signaling in *Drosophila*

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SUMMARY

Presenilins were identified as causative factors in familial Alzheimer's disease and also play an essential role in Notch signaling during development. We previously identified FKBP14, a member of the family of FK506-binding proteins (FKBPs), as a modifier of *Presenilin* in *Drosophila*. FKBPs are highly conserved peptidyl-prolyl *cis-trans* isomerases that play integral roles in protein folding, assembly and trafficking. Although FKBPs have been implicated in a broad range of biological processes, they are non-essential in yeast and their role in the development of multicellular organisms remains unclear. We show that *FKBP14* is an essential gene in *Drosophila* and that loss of FKBP14 gives rise to specific defects in eye, bristle and wing development. *FKBP14* mutants genetically interact with components of the Notch pathway, indicating that these phenotypes are associated, at least in part, with dysregulation of Notch signaling. We show that whereas Notch trafficking to the membrane is unaffected in *FKBP14* mutants, levels of Notch target genes are reduced, suggesting that FKBP14 acts downstream of Notch activation at the membrane. Consistent with this model, we find that Presenilin protein levels and γ-secretase activity are reduced in *FKBP14* null mutants. Altogether, our data demonstrate that FKBP14 plays an essential role in development, one aspect of which includes regulating members of the Notch signaling pathway.

KEY WORDS: *Drosophila*, FKBP, Notch, Presenilin

INTRODUCTION

Presenilins (PSNs) are highly conserved multi-pass transmembrane proteins that are synthesized as highly unstable 50 kDa precursor proteins that undergo tightly regulated endolytic processing to generate stable PSN C-terminal and N-terminal fragments that form the catalytic core of the γ-secretase complex (Thinakaran et al., 1996; Guo et al., 1999; Kimberly et al., 2000). γ-secretase cleaves a number of type I transmembrane proteins associated with a wide array of developmental processes, including the Notch receptor and Amyloid precursor protein (APP). In the case of APP, γ-secretase cleavage results in the generation of small amyloid β peptides, including Aβ40 and Aβ42. Mutations associated with Alzheimer's Disease (AD) result in the selective enhancement of Aβ42, which accumulates in the endoplasmic reticulum (ER) of neuronal cells and extracellularly as toxic plaques and is thought to initiate the AD pathogenic cascade (Hashimoto et al., 2003; Vetrivel et al., 2006). In the case of Notch, cleavage by γ-secretase results in release of the intracellular domain, 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). Despite the clear importance of the γ-secretase complex and, in particular, the role of PSN in development and disease, little is known about how PSN itself is regulated.

Each of the identified components of the γ-secretase complex, including PSN, are functionally conserved in *Drosophila* (Greeve et al., 2004). Further, many of the known γ-secretase targets and pathways are also conserved, making *Drosophila* a useful model with which to identify novel PSN interactors and regulatory mechanisms. We previously carried out a screen for modifiers of *Drosophila Presenilin* (*Psn*) and APP (van de Hoef et al., 2009) and found that mutations in *FKBP14* could suppress the phenotype associated with overexpression of *Psn* (van de Hoef 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 et al., 2008). The FK506-binding proteins (FKBPs) are a subfamily of these immunophilins, the smallest members of which are composed almost entirely of a single PPIase domain, whereas 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 signaling, protein trafficking and transcription (Barik, 2006; Kang et al., 2008). FKBP family members also exhibit distinct subcellular localizations and bind specific protein targets (Galat, 2008; Kang et al., 2008). Despite the growing evidence for the important roles of various FKBPs, knowledge of their specific functions remains vague. In particular, there is little information on the roles that FKBPs play in the development of multicellular organisms. Studies in yeast have demonstrated that all cyclophilins and FKBPs are individually and collectively dispensable for viability (Dolinski et al., 1997) and no FKBPs have been shown to be essential for viability in any multicellular model.

We have investigated the function of one member of this family, FKBP14, in *Drosophila* development. We show that FKBP14 is an ER resident protein that is broadly expressed throughout development. Null mutations in *FKBP14* are lethal throughout the larval and pupal stages of development, with escapers exhibiting defects in eye, wing and sensory bristle development. We show that *FKBP14* mutants do not appear to induce the unfolded protein response, indicating that phenotypes are not likely to be due to ER

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stress, but are instead associated, at least in part, with the dysregulation of Notch signaling. *FKBP14* mutants genetically interact with components of the Notch pathway, including *Notch*, *Delta* and *Psn*. We also find that although trafficking of Notch to the membrane appears unaffected in *FKBP14* mutants, the levels of several Notch targets, including Cut, Wingless and Enhancer of split, are significantly reduced, suggesting that FKBP14 acts downstream of Notch activation at the membrane. Consistent with this model, we find that Psn protein levels and γ-secretase activity are reduced in *FKBP14* null mutants. Altogether, our data demonstrate that FKBP14 plays an essential role in development, and we propose that one of its primary functions is to stabilize Psn protein in the ER, which is essential for formation of the γ-secretase complex that is required for effective Notch signal transduction.

MATERIALS AND METHODS

Fly genetics

Flies were maintained on standard media. An excision screen was performed on $FKBP14^{EP2019}$ to generate *FKBP14* alleles as described (Robertson et al., 1988). The P-element lies 101 bp downstream of the first exon and 1639 bp upstream of the translational start site within exon 2. *Sara* lies 58 bp downstream of exon 5 and *CG10496* is 1691 bp upstream of the first exon of *FKBP14-RA*, as described in FlyBase (Wilson et al., 2008). An imprecise excision line, $FKBP14^{D58}$, has a 2405 bp deletion that includes residues 105-145 of exon 1 and the entire exon 2. Breakpoints were determined by DNA sequence analysis (ACTG). A precise excision line, $FKBP14^{D34}$, was also generated and confirmed by DNA sequence analysis. This line is used as a genetic control. The balancer *CyO-GFP* was used to identify homozygotes. Wild-type Psn overexpression and Psn^{w6rp} have been described previously (Guo et al., 1999).

To achieve RNAi-mediated inactivation of Psn, $UAS\text{-}Psn\text{-}RNAi^{43082}$ transgenes were expressed at 29°C during larval and pupal stages of development using *daughterless-GAL4* (*da-GAL4*) or from late embryogenesis until adulthood using *pannier-GAL4* (*pnr-GAL4*) drivers.

To generate clones, the imprecise excision line $FKBP14^{D58}$ was recombined onto an *FRT(42B)* chromosome and confirmed by PCR. Recombination was induced in second and early third instar larvae heterozygous for *hs-flp; FRT(42B) FKBP14*^{D58} and *FRT(42B) ubi-GFP* by heat shock at 37°C for 60 minutes for 2-3 days consecutively and were analyzed in late third instar larvae imaginal discs (Xu and Rubin, 1993).

The *da-GAL4* (5460), *Df(1)N-8/FM7c* (729), $Dl^{7}/TM2$ (485), w^{1118},*hs-flp;* Adv^{1}/CyO (6), *FRT(42B)* (1956) and *pnr-GAL4* (3039) lines were obtained from the Bloomington Stock Center. $FKBP14^{EP2019}$ ($P[EP]Fkbp13^{EP2019}$) is available from the Szeged Stock Center. The $UAS\text{-}Psn\text{-}RNAi^{43082}$ (43082) line is available from the Vienna Drosophila RNAi Center (VDRC) (Dietzl et al., 2007).

GMR-GAL4 was double balanced with $UAS\text{-}APP\ C99^{J6}$ (Finelli et al., 2004) and males were crossed to virgin $FKBP14^{D34}$, $FKBP14^{EP2019}$, $FKBP14^{D58}$ and Psn^{w6rp}. Crosses were carried out at 29°C and progeny were raised to 10- to 14-day-old adults prior to ELISA.

Immunohistochemistry

Immunostaining was performed on embryos, larval imaginal discs and embryonic cell culture as described (Patel, 1994; Yeh et al., 2000; Kim et al., 2006; Commisso and Boulianne, 2007). The primary antibodies used were mouse anti-Ct (1:100), rat anti-Elav (1:500), mouse anti-Notch extracellular domain, EGF repeats #12-20 (C458.2H; 1:100) and mouse anti-Wg (1:500) [all from Developmental Studies Hybridoma Bank (DSHB), University of Iowa, IA, USA], mAb323 [E(spl); gift of Dr S. Bray, University of Cambridge, UK; 1:1], rabbit anti-GFP (Invitrogen; 1:1000), mouse anti-KDEL (MBL; 1:100), mouse anti-p120 (Calbiochem; 1:500), guinea-pig anti-Sens (gift of Dr H. Bellen, Baylor College of Medicine, Houston, TX, USA; 1:1000), rabbit anti-Srp (Sam et al., 1996) (1:500), rabbit anti-Psn^{NTF} (Guo et al., 1999) and a rabbit anti-Psn^{CTF} peptide antibody corresponding to amino acids 426-441 (CG18803-PB; NCBI), which was affinity purified (Antibodies Incorporated, Davis, CA, USA; 1:200). Rat anti-FKBP14 (cells, 1:100; ovaries, embryos, larval discs, 1:500) was generated as follows: a *Drosophila FKBP14* cDNA [GH08925, amino acids 298-828; Berkeley Drosophila Genome Project (BDGP)] was cloned into *pGEX-4T-1/His 6C* (Novagen) to produce an FKBP14 fusion protein for polyclonal antibody production in rats (Antibodies Incorporated) and preabsorbed using fixed S2 cells. A488 and Cy3 secondary antibodies were used (Jackson ImmunoResearch; 1:1000). Phalloidin-TRITC (Sigma-Aldrich) was used at 1:100 and DAPI (Invitrogen) 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.

Microscopy

Images were acquired at room temperature using either an LSM510 META confocal microscope (Carl Zeiss), 40×/1.2 and 100×/1.3 objectives and standard fluorescence filters, or a DMRA2 fluorescence microscope (Leica Microsystems) equipped with a Hamamatsu Orca-ER digital camera and Improvision Openlab software, 20×/0.5, 40×/1.25-0.75 and 100×/1.4 objectives, brightfield and standard filters, or a DMLB fluorescence microscope (Leica Microsystems), 5×/0.12 objective, brightfield and CoolSNAP software. Micrographs were analyzed using an XL30 scanning electron microscope (FEI, Hillsboro, OR, USA) equipped with XL Docu software. Images were processed in Photoshop CS and Illustrator CS (Adobe).

Immunoblot analysis

Fly lysates were prepared using standard procedures and analyzed with mouse anti-β-tubulin (DSHB; 1:1000), mouse anti-actin (GeneTex, Irvine, CA, USA; 1:1000), mouse anti-Csp-2 (DSHB; 1:25), mouse anti-Fz (Santa Cruz Biotechnology; 1:100), guinea-pig anti-Cad87A (gift from D. Godt, University of Toronto, ON, Canada; 1:2000), rabbit anti-Psn^{NTF} (1:1000) and rat anti-FKBP14 (1:2000). HRP secondary antibodies were used (Jackson ImmunoResearch; 1:10,000). All blots were performed in triplicate. Quantitation was performed using a Fluorchem 8000 Gel Documentation System and Alpha Innotech software (Alpha Innotech, San Leandro, CA, USA).

RT-PCR and qRT-PCR

RNA was extracted using Trizol (Invitrogen). For reverse transcription (RT)-PCR, RNA was reverse transcribed using the Superscript First-Strand System (Invitrogen). For quantitative (q) RT-PCR, the results were normalized to an internal control, *Rp49* (*RpL32* – FlyBase) (three separate experiments, at least ten animals per sample). Primer sequences (5′-3′) were: *FKBP14*, AGCTGATCAACATCGGCAAT (+) and CCAAGAACCCCCATTATTGA (–); *CG10496*, ATAAAGGGGAAGGAGCTGGA (+) and GGCGCCATATAGCTTTGGTA (–); *Sara*, CACCGACGATCAGAGTGAGA (+) and CTCCGCAATCCGTGTTATCT (–); *Hsc3*, GCGAACAAGATACCGATGCT (+) and GTTATCGGAGGCAGTGGAGA (–); and *Rp49*, AGTGCGTCGCCGCTTCAAGG (+) and AGAACGCAGGCGACCGTTGG (–).

ELISA

The levels of Aβ40 and Aβ42 were determined using commercially available human Aβ-specific ELISA kits (Invitrogen) according to the manufacturer's instructions. Heads from 10- to 14-day-old adult progeny were lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 1% SDS, 1% NP40, 0.5% sodium deoxycholate, pH 8.0) containing Complete Protease Inhibitor Cocktail (Roche). Lysates were diluted 1:5 or 1:10 with PBS containing protease inhibitors, followed by a further 1:1 dilution with the kit dilution buffer containing protease inhibitors prior to analysis.

RESULTS

Drosophila FKBP14 is an ER resident protein that is broadly expressed throughout development

There are eight known FKBPs in *Drosophila* that share homology with the archetypal human FKBP12 (FKBP1A – Human Gene Nomenclature Committee). Sequence analysis of one of these, *Drosophila* CG9847, reveals a signal peptide (SS), peptidyl-prolyl

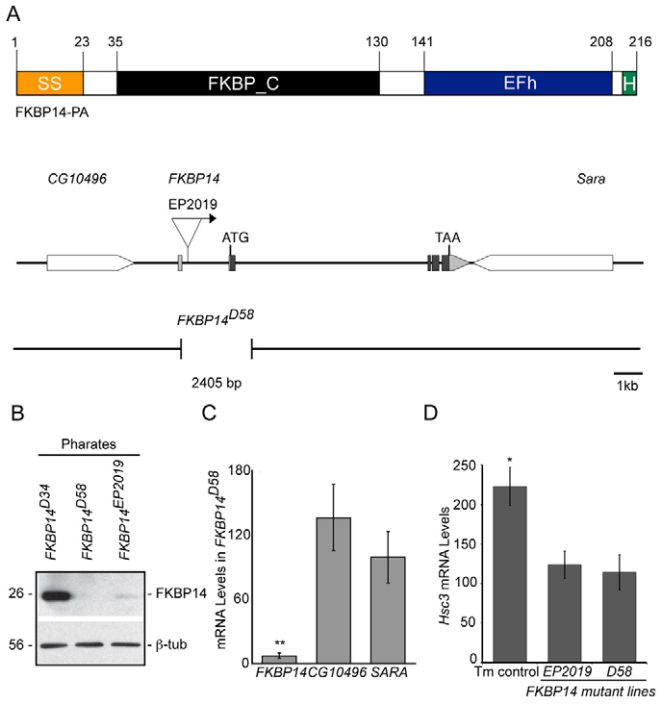

Fig. 1. *Drosophila* FKBP14 is an essential ER protein; mutations in FKBP14 do not result in ER stress. (**A**) *Drosophila* FKBP14 contains an N-terminal signal peptide (SS), a PPIase domain (FKBP_C), an EF-hand calcium-binding motif (EFh) and a C-terminal ER retention motif, HDEL (H). Beneath is shown the *FKBP14* genomic locus (FlyBase version FB2009_06, accession number AE013599) illustrating that *FKBP14* is flanked downstream by *Sara* and upstream by *CG10496*. Excision of *EP(2)2019* generated $FKBP14^{D58}$ (imprecise) and $FKBP14^{D34}$ (precise; not shown) excision alleles. (**B**) Western blot showing that in pharate adult extracts, FKBP14 expression is absent in $FKBP14^{D58}$ and reduced in $FKBP14^{EP2019}$ as compared with $FKBP14^{D34}$ controls. β-tubulin was used as a loading control. (**C**) qRT-PCR analysis of *Sara*, *CG10496* and *FKBP14* transcript levels in $FKBP14^{D58}$ third instar larval extracts shown as a percentage of the $FKBP14^{D34}$ control. Error bars represent ± s.d. *FKBP14* transcript levels are significantly reduced in $FKBP14^{D58}$ mutants (**P<0.001, unequal variance t-test), whereas *Sara* and *CG10496* transcript levels are not significantly reduced, compared with control (n=3). *Rp49* was used as an internal control. (**D**) qRT-PCR analysis of *Hsc3* transcript levels in $FKBP14^{EP2019}$ and $FKBP14^{D58}$ third instar larval extracts shown as a percentage of the $FKBP14^{D34}$ control. Tunicamycin (Tm)-treated $FKBP14^{D34}$ were used as a positive control. Whereas 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 $FKBP14^{EP2019}$ and $FKBP14^{D58}$.

cis-trans isomerase (PPIase) domain (FKBP_C), an EF-hand motif (EFh) and an ER retention signal known as HDEL (Fig. 1A; supplementary material Fig. S1A). Although it was originally identified as Fkbp13 (Spradling et al., 1999), its closest mammalian ortholog is FKBP14, with 37% overall sequence identity (supplementary material Fig. S1B). By contrast, mammalian FKBP13 shares 26% sequence identity with *Drosophila* CG9847 and 57% sequence identity with another *Drosophila* FKBP, CG14715 (supplementary material Fig. S1B). This indicates that *Drosophila CG9847* encodes an ortholog of mammalian FKBP14. Hereafter, we will refer to the protein encoded by *CG9847* as *Drosophila* FKBP14.

To determine its subcellular localization, we raised antibodies against a *Drosophila* FKBP14 fusion protein. Immune serum detected a 26 kDa band in western blots of fly protein lysates, which was not observed with pre-immune serum (data not shown). The specificity of the primary antibody was confirmed using hypomorphic and null *FKBP14* mutants (Fig. 1B). Using this anti-FKBP14 antibody, we examined the subcellular localization of FKBP14 in *Drosophila* Schneider 2 (S2) cells, in which it is endogenously expressed. *Drosophila* FKBP14 colocalized with KDEL, an ER marker (supplementary material Fig. S2A-A″), but not with p120 (p120ctn – FlyBase), a Golgi marker (supplementary material Fig. S2B-B″).

We then examined FKBP14 expression in *Drosophila* embryos and larval imaginal tissues. We found that FKBP14 is broadly expressed throughout development. In mid- and late-stage embryos, FKBP14 colocalizes with a number of pro-hemocyte and plasmatocyte markers, including the GATA transcription factor Serpent (Tepass et al., 1994; Sam et al., 1996), which colocalized with FKBP14 in pro-hemocytes that circulate from cephalic mesoderm (supplementary material Fig. S3B-B″) and partially colocalized with FKBP14 in differentiated pro-hemocytes in the dorsal region of 16- to 18-hour embryos (supplementary material Fig. S3C-C″). FKBP14 also colocalized in plasmatocytes with the collagen type IV factor Viking (Yasothornsrikul et al., 1997) at late stages of embryogenesis (supplementary material Fig. S3D-D″). In third instar larvae FKBP14 was broadly expressed in the wing disc (supplementary material Fig. S3E), eye disc (supplementary material Fig. S3F), CNS optic lobes (supplementary material Fig. S3G) and in the ventral nerve cord (supplementary material Fig. S3H). Altogether, these data suggest a role for FKBP14 in multiple tissue types during development.

FKBP14 is an essential gene that is required for viability

To determine the function of *Drosophila* FKBP14, we characterized a lethal P-element insertion, *EP(2)2019*, located within the first intron of *FKBP14* (Fig. 1A). We also identified an independent P-element insertion in *FKBP14*, $FKBP14^{2206}$, which failed to complement $FKBP14^{EP2019}$. We also generated additional alleles of *FKBP14* through imprecise excision of the *EP(2)2019* insertion, including $FKBP14^{D58}$, which removes part of the first exon and completely removes the second exon, including the translation start site (Fig. 1A). $FKBP14^{D58}$ failed to complement both $FKBP14^{EP2019}$ and $FKBP14^{2206}$. $FKBP14^{D58}$ and $FKBP14^{EP2019}$ mutants are homozygous lethal, with escapers reaching late pupal development as pharate adults. In addition to the deletion allele, we obtained a revertant line, $FKBP14^{D34}$, which precisely removed the original P-element insertion. $FKBP14^{D34}$ homozygotes are viable, demonstrating that $FKBP14^{D58}$ lethality is not due to a second site mutation. $FKBP14^{D34}$ was used as a genetic control for all subsequent experiments.

To further characterize the *FKBP14* alleles, we examined FKBP14 protein expression in wild-type and mutant flies by western blot. We observed up to 80% reduction of FKBP14 in pharate adults from the original insertion line, $FKBP14^{EP2019}$, demonstrating that this is a strong hypomorphic allele (Fig. 1B). No protein was detected in pharate adults from the deletion mutant $FKBP14^{D58}$, confirming that this allele represents a null mutation (Fig. 1B). These data also demonstrate that our antibody is specific to *Drosophila* FKBP14.

To ensure that neither P-element insertion nor deletion of *FKBP14* affected the expression of neighboring genes, we performed qRT-PCR analysis. The neighboring genes *Sara* and *CG10496* lie 58 bp downstream from exon 5 and 1691 bp upstream of *FKBP14-RA* exon 1, respectively (Fig. 1A). We observed a

significant reduction in *FKBP14* expression from *FKBP14* homozygous mutant larvae, as compared with controls ($P \leq 0.001$), and no significant changes in the expression levels of *CG10496* or *Sara* (Fig. 1C). Together, these data demonstrate that both the P-element insertion and the deletion significantly affect *FKBP14* transcription alone.

FKBP14 mutant phenotypes are not due to global ER stress

PPIases catalyze the *cis-trans* isomerization of peptidyl-prolyl amide bonds and are implicated in multiple intracellular processes, including protein folding (Göthel 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 member of the UPR, and its transcription is upregulated when UPR is active (Ryoo and Steller, 2007). To determine whether loss of FKBP14 causes a global ER stress response, we examined the transcript levels of a *Drosophila* BiP homolog, *Hsc3* (*Hsc70-3* – FlyBase), by qRT-PCR (Hirota et al., 2006). As a positive control, $FKBP14^{D34}$ 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 with the vehicle-treated control (Fig. 1D). However, we found similar *Hsc3* transcript levels in $FKBP14^{EP2019}$ and $FKBP14^{D58}$ third instar larvae as compared with control $FKBP14^{D34}$ (Fig. 1D). Taken together with the mutant qRT-PCR results, these data indicate that the phenotypes observed in 2019 and D58 mutants are due to loss of FKBP14 and are likely independent of ER stress.

FKBP14 mutants genetically interact with *Notch*, *Delta* and *Psn*

We previously demonstrated that *FKBP14* genetically interacts with *Psn*, such that mutations in *FKBP14* can suppress phenotypes associated with overexpression of *Psn* (van de Hoef et al., 2009). One of the essential developmental roles for *Psn* is as a core member of the Notch signaling pathway (Bray, 2006). To determine whether *FKBP14* interacts with the Notch pathway, we performed genetic epistasis experiments using *FKBP14*, *Notch* and the Notch ligand *Delta*. We also examined a mutation in *Psn* that we have previously shown to modulate both Notch and Delta phenotypes in transheterozygotes (Guo et al., 1999). We did not observe any significant defects in wing development in either heterozygous mutant *FKBP14* or *Psn* lines on their own (Fig. 2C,D).

We then analyzed whether *FKBP14* mutants genetically interact with a *Delta* (*Dl*) mutation that causes wing vein defects (Fig. 2E) and a *Notch* deficiency that causes notching of the distal wing margin due to haploinsufficiency (Fig. 2I). We found that transheterozygotes of Dl^{7} and $FKBP14^{D58}$ exhibit enhanced wing vein thickening and ectopic deltas (Fig. 2G), compared with controls (Fig. 2C,E,F). Similarly, transheterozygotes of $FKBP14^{D58}$ and the *Notch* deficiency *Df(1)N-8* exhibit enhanced wing notching (Fig. 2K), compared with controls (Fig. 2C,I,J). Interestingly, transheterozygotes of Psn^{w6rp} and Dl^{7} displayed a similar enhancement of wing vein thickening as observed for $FKBP14^{D58}$ and Dl^{7} transheterozygotes (Fig. 2H), relative to controls (Fig. 2D,E). Both phenotypes were fully penetrant.

These data demonstrate that *FKBP14* genetically interacts with members of the Notch pathway during wing development and that the phenotypes observed in *FKBP14* mutants, including the ability of loss-of-function mutants in *FKBP14* to suppress the dominant phenotype caused by overexpression of *Psn* (van de Hoef et al., 2009), may be due, at least in part, to defects in Notch signaling.

Loss of FKBP14 impairs Notch signaling in proneural clusters and at the wing margin

Although *FKBP14* mutants are homozygous lethal during a broad range of larval development, we also observed 'escapers' that survived to become pharate adults, probably owing to perdurance of a maternal contribution. In pharate adults, loss of FKBP14 function during wing development causes mild wing margin notching (Fig. 3B), loss of bristles (Fig. 3B′) and bristle doublets

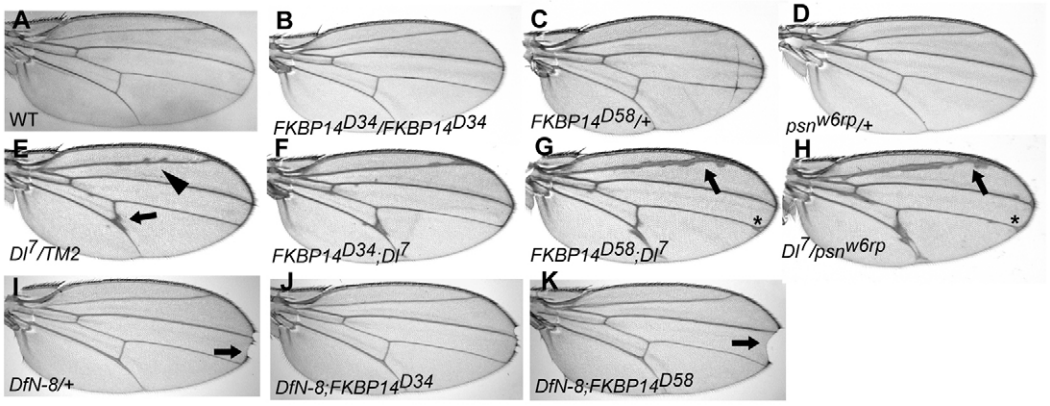

Fig. 2. *FKBP14* mutants genetically interact with *Notch* and *Delta*. Brightfield images of adult wings. Wild-type (WT) flies (**A**) and $FKBP14^{D34}/FKBP14^{D34}$ control (**B**) adult wings have a smooth wing margin, similar to $FKBP14^{D58}/+$ heterozygotes (**C**) and $Psn^{w6rp}/+$ heterozygotes (**D**). $Dl^{7}/TM2$ flies (**E**) exhibit mild deltas (arrow indicates a slight delta between the L4 and L5 wing veins) and thickening of L2 wing veins (arrowhead). Distal wing blade notching (arrow) is observed in a *Notch* deficiency allele, *DfN-8*/+ (**I**). $FKBP14^{D34};Dl^{7}$ wings (**F**) do not show any enhancement of these phenotypes, whereas $FKBP14^{D58}/Dl^{7}$ transheterozygotes (**G**) show enhanced *Delta* phenotypes, including extended L2 vein thickening (arrow) and ectopic deltas (asterisk), similar to that observed for the positive control Dl^{7}/Psn^{w6rp} transheterozygotes (**H**). *DfN-8;*$FKBP14^{D34}$ wings (**J**) also do not show any enhancement, whereas notching along the distal blade is enhanced in *DfN-8;*$FKBP14^{D58}$ transheterozygotes (**K**). Both phenotypes were fully penetrant.

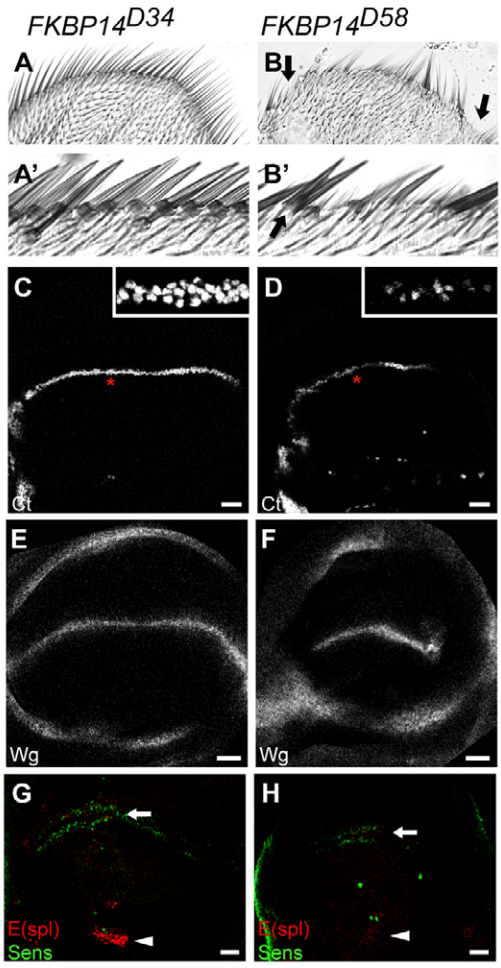

Fig. 3. FKBP14 mediates Notch target gene expression during presumptive wing margin specification. (**A**) Pharate adult bristle patterning at the wing tip in *FKBP14*^{D34} flies. (**B**) Defects in bristle patterning and mild wing notching (arrows) are observed in *FKBP14*^{D58} wings. (**A′**) *FKBP14*^{D34} anterior wing margins exhibit rows of mechanosensory bristles and chemosensory bristles, consistent with wild type (Lai and Rubin, 2001). (**B′**) *FKBP14*^{D58} anterior wing margins exhibit reduced numbers of mechanosensory bristles and show socket-to-shaft transformations (arrow points to a double-shafted bristle), consistent with some Notch loss-of-function phenotypes (Yaich et al., 1998; Lai and Rubin, 2001). (C-H) Third instar larval wing discs, anterior left, ventral up. (**C**) Ct expression is observed in rows of cells at the presumptive wing margin in *FKBP14*^{D34} discs, consistent with wild type (de Celis and Bray, 2000). The region marked by a red asterisk is magnified in the inset (100×) to show about three rows of cells that express Ct. (**D**) In *FKBP14*^{D58} mutants, Ct staining is disrupted across the margin. Cells marked by a red asterisk show significantly reduced Ct expression (inset; 100×). (**E**) Wg expression is observed in cells at the presumptive wing margin and in regions of the wing pouch in *FKBP14*^{D34} discs, consistent with wild type (Klein, 2001). (**F**) In *FKBP14*^{D58} mutants, Wg expression is mildly reduced in cells at the presumptive wing margin, whereas wing pouch expression appears normal. (**G**) Sens (green) expression is detected in cells at the presumptive wing margin (arrow) and in sense organ precursors (SOPs) of the pouch and hinge (arrowhead) regions of *FKBP14*^{D34} wing discs. E(spl) (red) expression is detected in cells surrounding SOPs. (**H**) Sens (green) localization is reduced at the presumptive wing margin (arrow) and in SOPs of the pouch and hinge (arrowhead) regions of *FKBP14*^{D58} wing discs. Similarly, E(spl) expression (red) is significantly reduced in cells that surround SOPs. Scale bars: 20 μm.

(Fig. 3B′), compared with the control (Fig. 3A,A′). These defects are phenotypically consistent with some Notch loss-of-function mutants (de Celis et al., 1996; Guo et al., 1999) and with a role for *FKBP14* in Notch regulation during wing development, as seen in our genetic epistasis experiments (Fig. 2). Patterning of the adult wing begins at the larval stage and requires activity of the transcription factors Cut (Ct) and Wingless (Wg) to define the presumptive wing margin (de Celis and Bray, 1997; Micchelli et al., 1997). Notch signaling maintains Ct expression at the margin and Ct may be required to maintain *wg* transcription (Micchelli et al., 1997). To examine the effects of FKBP14 on Notch signaling in third instar wing disc development we analyzed the levels of Ct and Wg expression and found that both were reduced in *FKBP14*^{D58} mutants (Fig. 3D,F), compared with the control (Fig. 3C,E). The reduction in Ct expression is more extreme than that in Wg, similar to what has been shown in *Notch* loss-of-function alleles (Micchelli et al., 1997). These data indicate that loss of FKBP14 may affect Notch-dependent wing margin specification.

Notch signaling in presumptive wing tissues activates the expression of proteins encoded by the E(spl) complex in cells targeted for an epidermal cell fate that surround sense organ precursors (SOPs) (de Celis et al., 1996). To further define the potential role of FKBP14 in mediating a Notch signal, we analyzed the expression of E(spl) in *FKBP14*^{D58} mutant wing discs. We found that E(spl) levels are significantly reduced in cells at the presumptive wing margin and hinge region in *FKBP14* mutant wing discs (Fig. 3H), compared with control (Fig. 3G). These data, together with the mild wing margin notches observed in *FKBP14* null mutants, suggest that FKBP14 function is required to maintain Notch signaling in third instar presumptive wing tissues. However, we also observed reduced numbers of cells marked by the nuclear protein Senseless (Sens) (Nolo et al., 2000) at the presumptive wing margin in *FKBP14* mutants, indicating a loss of SOPs (Fig. 3H). This is in contrast to typical defects in Notch, which result in supernumerary SOPs (Logeat et al., 1998; Kidd and Lieber, 2002).

In *FKBP14* mutant pharate adult nota, the numbers of microchaetae and macrochaetae are significantly reduced in *FKBP14*^{EP2019} (data not shown) and *FKBP14*^{D58} (Fig. 4B,B′) mutants, compared with control (Fig. 4A,A′). Once again, these phenotypes are highly reminiscent of a defect in Notch signaling (Frise et al., 1996; Yaich et al., 1998; Lai and Rubin, 2001). We therefore examined whether FKBP14 was modulating Notch signaling during sense organ patterning by analyzing the effects of FKBP14 loss on the expression of the Notch downstream target E(spl) in third instar presumptive notum tissue. As in the presumptive wing tissue, Notch signaling in the presumptive notum activates the expression of proteins that are encoded by the E(spl) complex in cells targeted for an epidermal cell fate, which surround SOPs (Jennings et al., 1994; Castro et al., 2005). In control larvae, SOPs marked by Sens are surrounded by cells expressing E(spl) proteins, particularly in supraalar (Fig. 4C,C′) and postalar (Fig. 4C,C″) regions. We observed reduced E(spl) expression in *FKBP14*^{D58} presumptive nota, particularly in supraalar (Fig. 4D,D′) and postalar (Fig. 4D,D″) regions, compared with the control. This reduction in E(spl) expression surrounding SOPs suggests a role for FKBP14 in modulating Notch signal transduction during SOP determination. However, we again observed a decrease in SOP levels as marked by Sens in *FKBP14*^{D58} larval presumptive nota (Fig. 4D′,D″; quantified in supplementary material Fig. S4), similar to that seen for

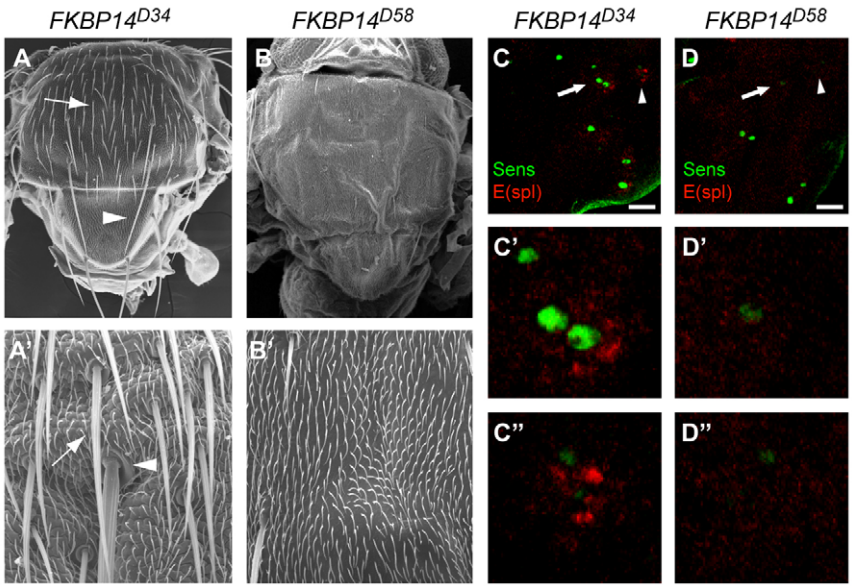

Fig. 4. FKBP14 is required for bristle formation in adult nota. (**A-B′**) Scanning electron microscopy of adult nota. (A; magnified in A′) *FKBP14*^{D34} nota display organized microchaetae (arrows) and macrochaetae (arrowheads). (B; magnified in B′) *FKBP14*^{D58} mutant pharate adults exhibit a severe reduction in the number of bristles on the thorax, as compared with the control. (**C-C″**) E(spl) expression (red) is observed surrounding SOPs that have been marked with Sens (green) in *FKBP14*^{D34} presumptive nota. The supraalar (arrow; magnified in C′) and postalar (arrowhead; magnified in C″) regions exhibit normal levels of expression. (**D-D″**) In *FKBP14*^{D58} 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. Scale bars: 20 μm.

FKBP14^{D58} presumptive wing tissue. This indicates that FKBP14 may have multiple functions during SOP specification, some of which reflect an effect on Notch signaling, whereas others appear to be independent of Notch.

FKBP14 is not required for Notch trafficking to the plasma membrane

Since our *FKBP14* mutants display Notch-like phenotypes in both the notum and wing, and we observed defects in downstream targets of the Notch pathway in both tissues, 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 the formation of a heterodimeric receptor at the plasma membrane, whereas in *Drosophila* the majority of Notch protein at the plasma membrane is uncleaved (Logeat et al., 1998; Kidd and Lieber, 2002). FKBP14 is an ER resident protein that might be involved in protein folding; therefore, we examined membrane trafficking of the Notch receptor in *FKBP14* mutants. We found that the Notch receptor appears predominantly localized at the plasma membrane in *FKBP14*^{D58} presumptive nota tissues (Fig. 5B), similar to the control (Fig. 5A). Moreover, Notch expression at the membrane is intact in cells that border third instar larval presumptive wing margins in control and *FKBP14*^{D58} mutant tissues (supplementary material Fig. S5A,B).

Although we cannot rule out subtle defects in Notch trafficking, based on these findings we conclude that trafficking of the Notch receptor to the plasma membrane is not grossly affected in our *FKBP14* mutants. As a control, we examined the surface levels of Notch ligand in *Psn* null mutants, which interrupt Notch signaling downstream of the Notch receptor reaching the cell surface. As in our *FKBP14* mutants, trafficking of the Notch ligand to the cell surface was not grossly affected in the *Psn* null mutant *w6rp* (Fig. 5C).

FKBP14 acts in the ER to maintain Psn protein levels

Although trafficking of Notch to the membrane appears largely unaffected in *FKBP14* mutants, the levels of Notch target genes are reduced suggesting that FKBP14 is likely to act downstream of Notch activation at the membrane. The γ-secretase complex plays a key role in Notch activation by cleaving Notch, leading to translocation of the intracellular domain to the nucleus where it activates downstream genes (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 interacts with members 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. We detected colocalization of FKBP14 with transiently transfected Aph-1 (supplementary material Fig. S6A″) but not Nct or Pen-2 (supplementary material Fig. S6B″-C″). We also found that although Psn is broadly distributed throughout the cytoplasm, it colocalizes with endogenous FKBP14 within ER-localized puncta (supplementary material Fig. S6D″,E″), suggesting that these proteins might interact within the early secretory pathway.

Given that we identified *FKBP14* as a *Psn* modifier (van de Hoef et al., 2009), and observed that *Psn* mutants modified *Notch* mutant wing phenotypes, similar to *FKBP14* mutants, we examined whether loss of Psn might be partially responsible for the Notch-like traits that we observe in our mutant escapers. To this end, we utilized RNAi-mediated knockdown of Psn during larval and pupal development (*UAS-Psn-RNAi*^{43082} from VDRC) (Dietzl et al., 2007) driven either ubiquitously (*da*-GAL4) or in the medial presumptive notum (*pnr*-GAL4). In Psn knockdown flies, we observed a reduction in bristle sense organs, similar to what was observed in *FKBP14* mutants (supplementary material Fig. S7A-C). This was not due to a loss of FKBP14 function because FKBP14 levels were unaffected by Psn knockdown (supplementary material Fig. S7E).

To determine how the loss of FKBP14 affects Psn, we examined Psn protein levels in *FKBP14* mutants using an anti-Psn^{NTF} antibody. We found that levels of endogenous Psn are reduced in our *FKBP14* mutants. In the most extreme cases, Psn appears reduced by up to 90% in *FKBP14* mutants as compared with controls (Fig. 5D); however, the effect on Psn is highly variable. The effects on Psn levels are post-transcriptional, as levels of *Psn* mRNA are not reduced in *FKBP14* mutants compared with controls (Fig. 5E). We also examined whether FKBP14 levels were

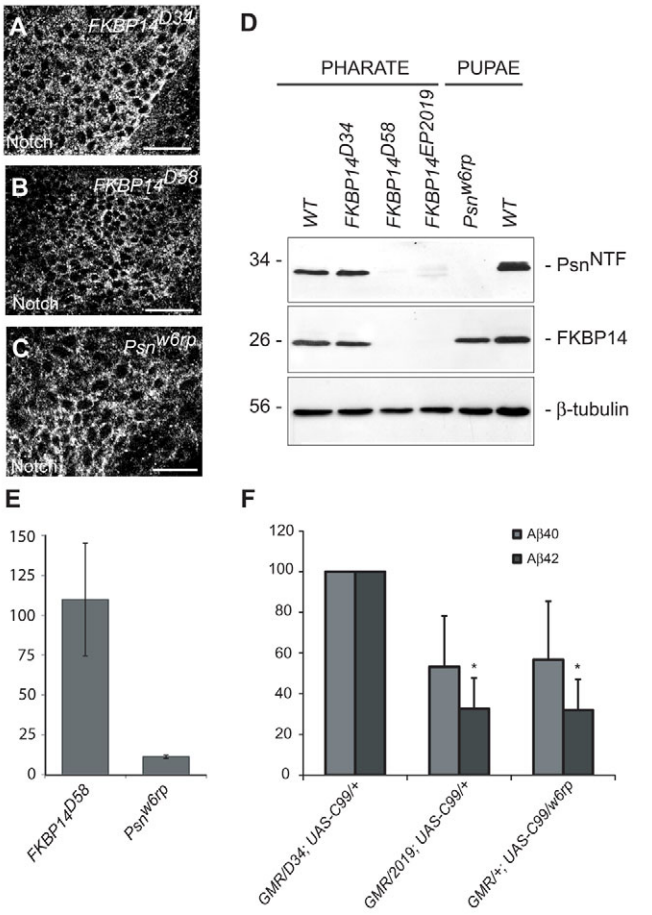

Fig. 5. FKBP14 is required to maintain Psn protein levels in the ER. (**A-C**) Notch is detected at the plasma membrane in *FKBP14*^{D34} (A), *FKBP14*^{D58} (B) and *Psn*^{w6rp} (C) third instar presumptive wing margins. Scale bars: 20 μm. (**D**) Psn^{NTF} protein levels are reduced in *FKBP14*^{D58}, *FKBP14*^{EP2019} and *Psn*^{w6rp} mutant extracts (top). FKBP14 expression is unaffected in *Psn*^{w6rp} mutant extracts (middle). β-tubulin shows equal loading (bottom). (**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**) Aβ40 and Aβ42 levels in *GMR-GAL4>UAS-APP C99* flies transheterozygous for *FKBP14*^{EP2019} or *Psn*^{w6rp} mutations. Measurements are normalized to the levels of Aβ proteins in control flies (precise excision background). Aβ42 levels are significantly reduced in *FKBP14*^{EP2019} and *Psn*^{w6rp} transheterozygous lines expressing APP C99 as compared with control (*P<0.05, unequal variance t-test). Error bars represent ± s.d.

affected by Psn loss and found no significant changes (Fig. 5D), suggesting that FKBP14 acts upstream of Psn. The effect of loss of FKBP14 on Psn protein levels appears relatively specific, as we also examined the levels of several other single and multipass transmembrane proteins in *FKBP14* mutants and found no gross differences in protein levels to controls (supplementary material Fig. S7D).

To assess whether Psn-dependent cleavage by γ-secretase is affected in our mutants, we expressed the C99 fragment of human APP (Finelli et al., 2004), a direct γ-secretase target, in wild-type and heterozygous *FKBP14* mutant backgrounds using the GMR-GAL4 driver. Specifically, extracts were prepared from heads of 10- to 14-day-old flies and the levels of the γ-secretase-dependent cleavage fragments Aβ40 and Aβ42 were measured by ELISA. A significant reduction in the Aβ42 fragment was observed in flies expressing APP-C99 in an *FKBP14* heterozygous mutant background compared with controls (Fig. 5F). The levels of Aβ40 were also reduced compared with controls, although these results were not statistically significant. Importantly, the reduction in the levels of Aβ40 and Aβ42 observed in *FKBP14* mutants was similar to that observed in heterozygous *Psn* mutants (Fig. 5F). These results indicate that γ-secretase activity is indeed reduced in *FKBP14* mutants relative to controls, probably owing to the loss of Psn protein observed in our mutants.

Taken together, these data indicate that FKBP14 is likely to function, directly or indirectly, to stabilize Psn in the ER and that, in the absence of FKBP14, Psn protein levels are reduced resulting in reduced γ-secretase activity and Notch-related developmental defects.

FKBP14 is required for cell viability

By analyzing the phenotypes of FKBP14 escapers we have identified a role for FKBP14 in the Notch pathway. However, the broad expression pattern of FKBP14 suggests that it might play additional roles in development. Moreover, *FKBP14* mutants are lethal throughout development, and most die during mid-pupal stages. Defects include ventral nerve cord retraction defects, small optic lobes and misshapen imaginal discs (supplementary material Fig. S8B,D,F), compared with controls (supplementary material Fig. S8A,C,E); however, the predominant mutant phenotype is a loss of imaginal discs, with 80% of *FKBP14*^{D58} homozygotes lacking imaginal discs (supplementary material Fig. S8G; n=250). Since we hypothesized that this variation in lethality might be due to perdurance of maternal contribution, we examined FKBP14 expression during oogenesis and detected FKBP14 expression in unfertilized eggs of control *FKBP14*^{D34} flies (Fig. 6A). In mid-stage egg chambers, FKBP14 is expressed in nurse cells and oocytes (Fig. 6B). At later stages of oogenesis, FKBP14 protein is maintained in nurse cells, oocytes and somatic follicle cells (data not shown), and in syncytial blastoderm embryos FKBP14 expression is detected throughout the cytoplasm (supplementary material Fig. S3A-A″).

To determine the phenotype associated with a complete loss of FKBP14 and to further define its role during development we first attempted to generate maternal-zygotic null animals using the FLP/FRT mosaic clone technique (Perrimon, 1998). Initially, we generated transheterozygotes of FRT(42B)*FKBP14*^{D58} and FRT(42B)*ovoD*, a dominant female-sterile mutation (Chou and Perrimon, 1996). 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). To further determine the effect of a complete loss of FKBP14 we generated clones in somatic tissues. Using the ubiquitous *GFP* marker allele FRT(42B)*ubi-GFP*, we induced recombination and assessed clones in imaginal discs. In control wing discs, we induced recombination between FRT(42B)*ubi-GFP* and FRT(42B), resulting in clones that lack GFP staining (Fig. 6D) adjacent to GFP twin spots (Fig. 6D). By contrast, following recombination between FRT(42B)*ubi-GFP* and FRT(42B)*FKBP14*^{D58}, we observed GFP twin spots (Fig. 6E) without any associated *FKBP14* null clones. These data indicate that FKBP14 is required for cell viability. This is in contrast to mutations in *Notch* and *Psn*, for which it is possible to generate both maternal-zygotic and somatic null clones, but is similar to what has been observed for *aph-1* (López-Schier and St Johnston, 2002; Cooper et al., 2009). Whether this result is due to cell death or to a defect in cell competition has yet to be determined.

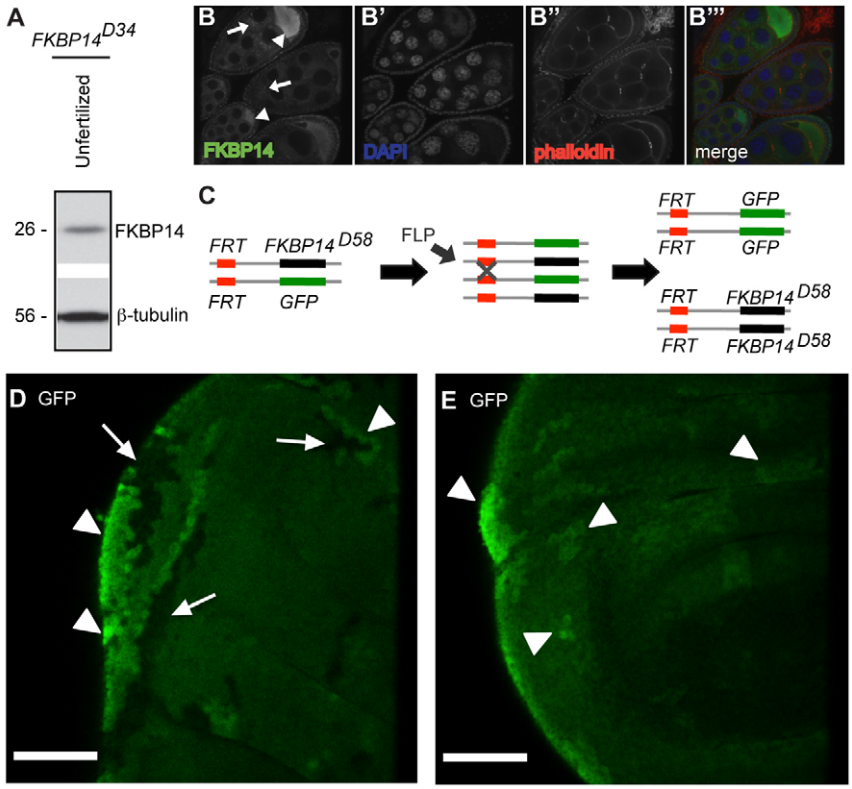

Fig. 6. FKBP14 is required for cell viability. (**A-B'''**) FKBP14 protein is detected in *FKBP14*^{D34} unfertilized embryos (A, 26 kDa) and in nurse cells (arrows) and oocytes (arrowheads) in developing ovarioles (B-B''', green). (**C**) Schematic outlining FLP recombinase-induced site-specific recombination, resulting in homozygous daughter cells from dividing heterozygous cells. FLP recombination target sites (FRTs) are indicated in red, *FKBP14*^{D58} in black and the *GFP* marker allele in green. Control recombination crosses are not shown. (**D**) Recombination between FRT(42B)*ubi-GFP* and FRT(42B) resulted in clones that lack GFP staining (arrows) adjacent to GFP twin spots (arrowheads). (**E**) Recombination between FRT(42B)*ubi-GFP* and FRT(42B)*FKBP14*^{D58} resulted in GFP twin spots only (arrowheads), without any associated *FKBP14* null clones. Scale bars: 35 μm.

DISCUSSION

Psn is synthesized within the ER and is rapidly cleaved, generating N- and C-terminal fragments that are essential for γ-secretase catalytic activity (Thinakaran et al., 1996; Kimberly et al., 2000). We identified a novel genetic interactor of *Psn*, *Drosophila* FKBP14, which is a member of the large FKBP family of immunophilins (van de Hoef et al., 2009). FKBP14 has a single PPIase domain, a calcium-binding EF hand, an N-terminal signal sequence and C-terminal endoplasmic HDEL motif. Here we have further defined a potential role of FKBP14 in Notch signaling and begun exploring the broader role of FKBP14 in *Drosophila* development.

We have shown that *FKBP14* genetically interacts with *Notch* and *Delta* during wing patterning, similar to what has been shown in *Psn* and *Notch* or *Delta* transheterozygotes (Guo et al., 1999). Our data show that *FKBP14* mutations give rise to developmental defects, a subset of which are similar to those previously identified in Notch loss-of-function mutants. In particular, *FKBP14* escaper pharate adults exhibit bristle phenotypes in the notum and wing tissues that suggest defects in Notch signaling. In keeping with a role for FKBP14 in Notch signaling, we observe a decrease in the expression of Notch target genes, such as *ct* and *E(spl)*, in third instar wing disc tissues.

Notch signaling can be regulated at a number of steps, including Notch 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 proteolysis of Notch within the endocytic pathway leads to Notch signaling events (Gupta-Rossi et al., 2004). We found that loss of FKBP14 does not affect trafficking of Notch to the membrane, yet it does result in reduced expression of Notch target genes. Although we cannot rule out the possibility that subtle effects on Notch receptor trafficking contribute to our *FKBP14* null phenotype, these results suggest that FKBP14 is likely to affect components of the Notch pathway downstream of Notch localization at the plasma membrane. Consistent with this model, we find that Psn protein but not RNA levels are often reduced in *FKBP14* mutants. Further, we found that FKBP14 and Psn colocalize in the ER, where the majority of Psn protein is uncleaved, suggesting that FKBP14 might interact with Psn prior to endoproteolytic cleavage. The holoprotein, unlike the N- and C-terminal fragments, is highly unstable and must incorporate into a larger complex for stabilization (Ratovitski et al., 1997). A putative role for FKBP14 might therefore be to properly fold or stabilize Psn in the ER, allowing for the formation of a functional γ-secretase complex that is required for cleavage and activation of Notch. Consistent with this hypothesis, we found that γ-secretase activity is reduced in flies heterozygous for mutations in *FKBP14*, in a manner similar to those heterozygous for *Psn* mutations.

Although we observed specific defects in Notch signaling associated with the loss of FKBP14, it is likely that FKBP14 also plays a broader role in development. FKBP14 is broadly expressed in many tissues and loss-of-function mutations in *FKBP14* are lethal throughout development. Perdurance of maternal contribution likely leads to the broad time span of lethality observed, depending on the amount of maternal protein and transcript each animal received. Moreover, *FKBP14* mutants show a range of defects during imaginal disc development, some of which are reminiscent of Notch defects whereas others appear to be independent of Notch. *FKBP14* mutants, for instance, show fewer SOPs than controls. Defective Notch signaling at this stage of SOP specification results in supernumerary SOPs (Mummery-Widmer et al., 2009). We also found that *FKBP14* clones are not viable, neither in the germline nor in somatic tissue, in contrast to

mutations in both *Notch* and *Psn* (Lopez-Schier and St Johnston, 2002; Cooper et al., 2009). Interestingly, mutations in one of the γ-secretase components, Aph-1, show a similar defect in cell viability, and this effect appears to be independent of its role in regulating γ-secretase activity (Cooper et al., 2009). Indeed, we observe partial colocalization of FKBP14 with Aph-1, but not with the other γ-secretase components Nct and Pen-2, in *Drosophila* cells. It is currently thought that Aph-1 and Nct might form a subcomplex that stabilizes Psn early in γ-secretase assembly, prior to Psn endoproteolysis and incorporation of Pen-2 (Hu and Fortini, 2003). Whether loss of FKBP14 affects Aph-1 function, however, is not yet known. It is also unclear whether the cell lethality phenotype observed in somatic *FKBP14* clones is due to apoptosis or the inability of these cells to successfully compete with wild-type neighbors. Further analysis using recessive cell-lethal (Menut et al., 2007) and cell competition-defective (Marygold et al., 2007) marker alleles will enable us to distinguish between these possibilities.

Previous studies in yeast have demonstrated that all cyclophilins and FKBPs are individually and collectively dispensable for viability (Dolinski 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 et al., 2012). Our study demonstrates that FKBP14 is required for metazoan development and describes, among other potential roles, a novel requirement for FKBP14 in development, cell viability, Psn protein stability and Notch signaling.

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Competing interests statement
The authors declare no competing financial interests.

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