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Pharmacological analysis of *Drosophila* γ-secretase with respect to differential proteolysis of Notch and APP

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Running title: Pharmacological analysis of *Drosophila* γ-secretase

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Abbreviations:

APP: Amyloid Precursor Protein, Aβ42: Amyloid β42 peptide, CD44: Cluster of Differentiation 44, ErbB4: Erythroblastosis oncogene B-4, PS1: Presenilin-1, PS2: Presenilin-2, Aph-1: Anterior pharynx defective-1, Pen-2: Presenilin enhancer-2, BACE: β-site of APP cleaving enzyme, ADAM: A disintegrin and metalloproteinase enzyme, NTF: N-terminal fragment, CTF:C-terminal fragment, NICD: Notch intracellular domain, NEXT: Notch extracellular truncation, AICD: Amyloid Precursor Protein intracellular domain, DFK167: Difluoro-ketone peptidomimetic inhibitor-167, DAPT: *N*-[*N*-(3,5-Difluorophenylacetyl)-L-alanyl]-S-phenylglycine *t*-Butyl Ester, CpnE: Compound E, (*S*,*S*)- 2-[2-(3,5-Difluorophenyl)acetylamino]-*N*-(1-methyl-2-oxo-5-phenyl-2,3-dihydro-1H- benzo[e][1,4]diazepin-3-yl)-propionamide, DBZ:

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Dibenzazepine, (*S*,*S*)-2-[2-(3,5-Difluorophenyl)acetylamino]-*N*-(5-methyl-6-oxo-6,7-dihydro-5H-dibenzo[b,d]azepin-7- yl)propionamide , BB94: Batimastat, GM6001: N-[(2R)-2-(Hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide

ABSTRACT

The γ-secretase aspartyl protease is responsible for the cleavage of numerous type I integral membrane proteins, including Amyloid Precursor Protein (APP) and Notch. APP cleavage contributes to the generation of toxic amyloid β peptides in Alzheimer's disease, while cleavage of the Notch receptor is required for normal physiological signaling between differentiating cells. Mutagenesis studies as well as *in vivo* analyses of Notch and APP activity in the presence of pharmacological inhibitors indicate that these substrates can be differentially modulated by inhibition of mammalian γ-secretase, while some biochemical studies instead show nearly identical dose-response inhibitor effects on Notch and APP cleavages. Here we examine the dose-response effects of several inhibitors on Notch and APP in *Drosophila* cells, which possess a homogeneous form of γ-secretase. Four different inhibitors that target different domains of γ-secretase exhibit similar dose-response effects for both substrates, including rank order of inhibitor potencies and effective concentration ranges. For two inhibitors, modest differences in inhibitor dose responses towards Notch and APP were detected, suggesting that inhibitors might be identified that possess some discrimination in their ability to target alternative γ-secretase substrates. These findings also indicate that despite an overall conservation in inhibitor potencies towards different γ-secretase substrates, quantitative differences might exist that could be relevant for the development of therapeutically valuable substrate-specific inhibitors.

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Introduction

The γ-secretase complex is a multi-subunit aspartyl protease that executes the intramembrane proteolysis of certain type I integral membrane proteins, including Amyloid Precursor Protein (APP), the Notch receptor, CD44, ErbB4, and Neuregulin (reviewed in McCarthy et al., 2009; Selkoe and Wolfe, 2007). Cleavage of APP by γ-secretase also contributes to the generation and secretion of amyloid-β peptide, a major constituent of the neurotoxic amyloid plaques found in Alzheimer's disease. In the case of Notch signaling, which regulates a diverse array of developmental processes in many organisms (reviewed in Kopan and Ilagin, 2009; Tien at al., 2009), γ-secretase-mediated cleavage of the Notch receptor is a key step in its activation and signal transduction. Consequently, the development of pharmacological compounds to treat Alzheimer's disease by reducing γ-secretase cleavage of APP is complicated by the need to avoid adverse effects on Notch and other physiologically essential γ-secretase substrates.

Despite the overall mechanistic similarity between APP and Notch cleavage by γ-secretase, subtle differences might exist that could be exploited therapeutically. Several studies involving mutationally altered forms of γ-secretase, including Alzheimer's disease-associated mutant forms, argue that these variants possess differential proteolytic activities towards Notch and APP (Capell et al., 2000; Kulic et al., 2000; Moehlmann et al., 2002; Nakajima et al., 2000; Zhang et al., 2000). Using pharmacological approaches in which Notch and APP intramembrane proteolysis was monitored in parallel to determine their sensitivities to different γ-secretase inhibitors, two studies reported nearly identical dose-dependent inhibitor effects on both substrates (Lewis et al., 2003; Schroeter et al., 2003), whereas one study found that some inhibitors can partially discriminate between the APP and Notch cleavages (Yang et al., 2008).

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Pre-clinical animal studies on these compounds have generally revealed significant toxic side effects attributable to impaired Notch signaling in the mouse gut and immune system (Milano et al., 2004; van Es et al., 2005). Recently, proprietary sulfonamide-based γ-secretase inhibitors have been reported to show significantly higher selectivity towards APP cleavage relative to Notch in cell-based assays and animal models (Barten et al., 2005; Best et al., 2007; Cole et al., 2009; Pu et al., 2009). Overall, the results of previous mutational and pharmacological studies suggest that different assay conditions, drug dosage regimes, or other experimental variations might account for the contradictory outcomes of these analyses.

An additional complication is that mammalian γ-secretase is heterogeneous, with functionally redundant genes encoding two different Presenilins (PS1 and PS2) and two Aph-1 proteins (Aph-1a and Aph-1b), generating several different biologically active complexes (Shirotani et al., 2004). The alternative subunits of mammalian γ-secretase show differential and partially overlapping tissue expression patterns (Hebert et al., 2004), suggesting that different complexes might exhibit distinct biological activities in specific tissues. Indeed, biochemical studies have shown that PS1-containing γ-secretase shows higher activity regarding APP cleavage compared to PS2-containing γ-secretase (Lai et al., 2003), and while Aph-1b-containing complexes contribute significantly to Aβ production in the brain (Serneels et al., 2009), genetic elimination of Aph-1b in mice leads to behavioral deficits associated with schizophrenia (Dejaegere et al., 2008). Thus, results obtained from previous studies of γ-secretase inhibitor effects on APP versus Notch could, in part, reflect the activities of functionally heterogeneous γ-secretase complexes in the various mammalian cell lines and assay systems employed.

To address this issue, we investigated the substrate specificity of several well-characterized γ-secretase inhibitor compounds using *Drosophila* cells, in which γ-secretase has a

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homogeneous composition. The fly genome possesses a single gene for each of the four γ-secretase components Presenilin, Nicastrin, Aph-1, and Pen-2 (Hu and Fortini, 2003). Selecting a panel of γ-secretase inhibitors that target different regions of the complex, we performed dose-response studies on their ability to inhibit the intramembrane cleavage of *Drosophila* Notch and APP in *Drosophila* S2 cells. In general, the effective dose range and rank potencies of the different inhibitors were found to be nearly identical towards both substrates, in agreement with two mammalian studies that performed a similar parallel substrate analysis (Schroeter et al., 2003). However, we also observed that two of the γ-secretase inhibitors (DAPT and DFK167) show a modest, but statistically significant, discrimination between Notch and APP cleavages in *Drosophila*, similar to one mammalian study that reported increased substrate specificity towards APP versus Notch for DAPT (Yang et al., 2008). Our findings also demonstrate that γ-secretase enzyme sensitivity to several inhibitors that were developed to target mammalian γ-secretase is conserved in the invertebrate *Drosophila*, emphasizing the high degree of evolutionary conservation in the regulatory features of this proteolytic mechanism.

Materials and Methods

S2 Cell Culture Studies. The *APPL* gene was obtained by PCR from an embryonic cDNA library. An epitope-tagged form was constructed by inserting a V5-tag at its C-terminus in the vector pMT/V5-His (Invitrogen), co-transfected into S2 cells with pCoHygro using Effectene, and subjected to Hygromycin B antibiotic selection (Invitrogen) to establish the metal-inducible stable cell line S2-MT-APPL. The Notch cell line S2-MT-Notch, S2 cell culture conditions, and preparation of cell extracts for immunoblot analysis were as described (Fehon et al., 1990). Notch lysates were resolved on 3-8% Tris-Acetate gels, while APPL lysates were

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resolved on 12% Bis-Tris or 16.5% Tris-Tricine gels (Bio-Rad). Immunoblots were probed with 1:5000 mouse anti-V5 mAb (Invitrogen), 1:1000 mouse anti-Notch mAb C17.9C6 (Fehon et al., 1990) and 1:2000 mouse anti-β-tubulin mAb (Developmental Hybridoma Bank, University of Iowa). Protein bands were visualized using BioMax XAR film (Kodak) and quantified using Adobe image analysis software.

Pharmacological Inhibition of γ-secretase Activity. Commercially available γ-secretase inhibitors DFK167 (MP Biomedicals), DAPT, DBZ and Compound E (Calbiochem) were used at the concentrations indicated in the text and figure legends. For each inhibitor, pilot experiments were performed with different drug concentrations ranging from 0.1 nM to 150 μM in order to determine the effective linear range and maximal inhibition dose for each compound (cf. Fig. 4 legend). Inhibitors were added at the required concentrations to the S2 cell medium upon induction of Notch or APPL expression, 6 hours prior to protein harvesting. For each sample, the same inhibitor was also included at the corresponding concentration in the lysis buffer for protein extraction and immunoblot analysis.

Statistical Analysis. Data were analyzed using a four-parameter nonlinear logistic regression model to determine IC_{50} values, standard errors, and 95% confidence intervals (DeLean et al., 1978; Pinheiro and Bates, 2002) with S, S-Plus, and R software (Insightful Corp). Inhibitor potencies were calculated using standard nonlinear mixed-effects modeling techniques (52). The regression model used is Y ~ ((A - D)/(1 + (X/C) ^ B)) + D, where Y = the response in relative intensity; X = concentration in nanomoles; A, D = lower and upper asymptotes; C = the concentration giving a response halfway between A and D, referred to as IC_{50}; and B = 'slope' factor corresponding to slope of the logit-log plot, when X is given by natural logarithms;

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specifically, $\boldsymbol{d}Y / \boldsymbol{d} \ln(X) = [(D - A) * B]/4$ when X = C, or $\boldsymbol{d}$ logit $\{(Y - D)/(A - D)\}/\boldsymbol{d} \ln(X) = B$.

Results

***Drosophila* APPL is cleaved by γ-secretase to generate a released intracellular domain.** APP is a member of a multi-protein family with a complex evolutionary history. True APP orthologs are encoded by a single gene in mammals (Coulson et al., 2000) and duplicate genes in the zebrafish (Musa et al., 2001). More distantly related family members include human APLP1 and 2, *Drosophila* APPL and *C. elegans* APL-1 (reviewed in Coulson et al., 2000). In mammals, all three APP/APLP family members undergo similar processing by α-, β- and γ-secretase (Eggert et al., 2004) (Fig. 1A). While *Drosophila* APPL is subject to ectodomain shedding (Luo et al., 1990), it has not been directly demonstrated to be processed via similar proteolytic pathways as mammalian APP, including intramembrane proteolysis by endogenous *Drosophila* γ-secretase.

To examine APPL biochemical processing, we generated a stable inducible *Drosophila* S2 cell line expressing APPL with a V5-tag fused to its C-terminus and analyzed APPL cleavage products on immunoblots following treatments with inhibitors against α- and γ-secretase. APPL holoprotein was readily detected under all conditions as two bands of differing intensities (Fig. 2A, lane 2), which might correspond to immature and glycosylated APPL species (Vingtdeux et al., 2005). Cells treated with the γ-secretase inhibitor Compound E (CpnE) revealed an over-accumulation of two low molecular weight (MW) APPL C-terminal fragment (CTF) bands of 14 and 16 kDa (Fig. 2A, topmost blot, lanes 3 and 5). These APPL CTFs were also found in lysates from untreated cells at longer exposures (Fig. 2B, middle blot, lanes 2 and 4), implying that the

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APPL CTFs are rapidly degraded in S2 cells. Inhibiting γ-secretase activity interferes with the turnover of these fragments, leading to their accumulation in a manner similar to processing of mammalian APP (Xia et al., 1998). To further analyze the APPL CTFs, we treated cells with combinations of α- and γ-secretase inhibitors and resolved the products using 16.5% Tris-Tricine SDS/PAGE, which enhances the separation of low MW species (Fig. 2C, lanes 2-5). With this method, three distinct APPL CTF bands were detected in cells treated with γ-secretase inhibitor for 6 hours, termed APPL CTFa, b and c (Fig. 2C, topmost blot, lane 4). Cells treated with both α- and γ-secretase inhibitors displayed a slight but reproducible accumulation of APPL CTFb and concomitant decrease in CTFc, compared to γ-secretase inhibitor-treated cells alone (Fig. 2C, topmost blot, lanes 4 and 5). Longer treatments for 24 hours with CpnE resulted in a sharp reduction in APPL CTFa and CTFb accompanied by highly elevated levels of CTFc (Fig. 2C, third blot, lane 4), whereas CTFb showed a preferential accumulation compared to CTFc when cells were treated simultaneously with α- and γ-secretase inhibitors (Fig. 2C, third blot, lane 5). The ability of the α-secretase metalloprotease inhibitor GM6001 to reduce levels of the APPL CTFc fragment at both time points indicates that generation of this product involves α-secretase activity in addition to γ-secretase.

Mammalian APP metabolism is also regulated by endocytic trafficking. Interfering with the normal process of endosomal acidification has been shown to cause accumulation of APP CTFs in multivesicular bodies of the endosomal compartment (Vingtdeux et al., 2007). Other studies have implicated the proteasome in regulating the turnover of γ-secretase-generated intracellular domain fragments (Tomita et al., 2006). In an effort to detect directly the *Drosophila* APPL AICD fragment, analogous to the released intracellular AICD fragment from mammalian APP, we treated S2 cells with inhibitors of endosome acidification and proteasomal

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degradation, concanamycin A and epoxomicin, respectively. In cells treated with both compounds, we detected an ~8 kDa APPL CTF fragment with the predicted size of the putative AICD (Fig. 2B, middle blot, lane 4). Production of this fragment was blocked by addition of CpnE to the concanamycin A/epoximicin treatment regime, confirming that this fragment is generated by γ-secretase-mediated proteolysis and thus corresponds to the APPL AICD (Fig. 2B, middle blot, lane 5).

Dose-response analysis of *Drosophila* APPL and Notch intramembrane cleavage with γ-secretase inhibitors. To investigate whether γ-secretase inhibitors can differentially influence APPL and Notch intramembrane proteolysis in *Drosophila*, we performed dose-response studies of these two substrates in S2 cells. Metal-inducible transgenic S2 lines expressing full-length Notch (Fehon et al., 1990) or APPL (this study; see Materials and Methods) were incubated for 6 hours in the presence of different concentrations of γ-secretase inhibitors. Cells were subsequently lysed in EDTA-containing hypotonic buffer for 30 minutes, which promotes ectodomain shedding of γ-secretase substrates and efficiently converts the substrate pool to the membrane-bound CTF forms that are optimal γ-secretase cleavage precursors (Rand et al., 2000). Cell lysates were resolved on SDS-PAGE protein gradient gels and immunoprobed with antibodies recognizing either the Notch intracellular domain or the tagged intracellular domain of expressed APPL.

We have previously used this assay to study the role of γ-secretase core components in *Drosophila* with Notch receptor cleavage as the readout for γ-secretase activity (Hu and Fortini, 2003; Hu et al., 2002). In this assay, the Notch intracellular fragment generated by γ-secretase proteolysis (NICD; Fig. 3A) is sufficiently stable to be detected by immunoblot analysis as a

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distinct fragment whose production is blocked when cells are treated with high doses of γ-secretase inhibitors (Fig. 3B, lanes 2 and 4). Previous characterization of *Drosophila* Notch biochemical processing (Hu et al., 2002) has established that the three co-migrating fragments in the ~ 100 kDa mobility range correspond to the membrane-bound CTFs generated by the extracellular furin-like and ADAM cleavages of Notch (upper bands in Fig. 3B enlarged panel) and the released NICD fragment generated by γ-secretase cleavage (lower band in Fig. 3B enlarged panel). In addition, a faster migrating species that is not derived from the cell-surface pool of Notch and represents a breakdown product is also observed in S2 cells (Hu et al., 2002; this species is indicated by an asterisk in Fig. 3B enlarged panel).

Because the corresponding γ-secretase-generated APPL AICD fragment was found to be highly unstable (Fig. 2B, middle blot, lanes 2 and 4), it could not be used reliably to monitor γ-secretase activity in determining inhibitor dose-response curves. We instead monitored APPL CTF levels, because the APPL CTFs are the direct stoichiometric precursors for the production of AICD. Indeed, mammalian APP CTF levels and AICD production exhibit a direct inverse relationship (Lewis et al., 2003), validating this approach. We therefore quantified the amount of APPL CTFa, the most discrete APPL CTF product detected on immunoblots (Fig. 2C, topmost blot, lanes 2-5), as an indicator of γ-secretase activity towards *Drosophila* APPL.

For the dose-response studies, we selected a panel of four γ-secretase inhibitors that interact directly with the γ-secretase complex and target different functional domains of the complex (Fig. 1B). DFK167 binds to the catalytic site of the Presenilin aspartyl protease subunit (Wolfe et al., 1998), whereas CpnE and DBZ target the N-terminal Presenilin fragment (Fuwa et al., 2007), and DAPT targets the C-terminal Presenilin fragment (Morohashi et al., 2006). Representative immunoblots for the Notch and APPL dose-response studies with the γ-secretase

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inhibitors DBZ, CpnE, DAPT and DFK167 are shown in Figure 4A. Increasing concentrations of these compounds administered to APPL- or Notch-expressing cells leads to the progressive accumulation of APPL CTF fragments and a decrease in NICD production in a strictly dose-dependent manner (Fig. 4A). It is worth noting that the accumulation of APPL CTFs reached a plateau at high inhibitor concentrations, indicating that the maximal inhibitory concentration for each drug was reached. The data obtained were used to plot dose-response curves and derive IC_{50} values for the proteolysis of APPL and Notch (Fig. 4B). Both substrates exhibit the same rank order of potencies for all four inhibitors, with DBZ being most potent, followed by CpnE, then DAPT, and finally DFK167. Importantly, DBZ and CpnE, the two most potent drugs, showed no apparent discrimination in blocking APPL and Notch cleavages, displaying statistically equivalent IC_{50} values for both substrates ($p > 0.05$). In contrast, both DAPT and DFK167 display an approximately four-fold selectivity in their cleavage preferences ($p < 0.05$), with DAPT showing an increased effectiveness in blocking APPL cleavage relative to Notch proteolysis, while DFK167 inhibited Notch cleavage more effectively than APPL proteolysis in this *Drosophila* S2 assay (Fig. 4C).

Discussion

In this study, we demonstrate that APPL, the *Drosophila* ortholog of the Alzheimer's disease-associated protein APP, is rapidly metabolized and processed by α- and γ-secretase activities in *Drosophila* S2 cells. The transmembrane domain and adjacent regions of APPL have not been conserved in evolution, and no sequence homologous to the Aβ region of mammals is present in *Drosophila* APPL (Coulson et al., 2000). Our finding that APPL is nevertheless processed by γ-secretase supports the notion that its three-dimensional structure rather than

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specific residues near the cleavage site(s) renders it accessible to γ-secretase proteolysis (Tanii et al., 2006). Consistent with this idea, overexpression in *Drosophila* of the APPL holoprotein, or the portion of APPL analogous to the Aβ peptide region, produced pathological lesions resembling those in Alzheimer's disease, including age-dependent formation of amyloid deposits, neurotoxic effects, and behavioral deficits (Carmine-Simmen et al., 2009).

The biology of mammalian γ-secretase is complicated by the fact that this multi-subunit enzyme is heterogeneous in composition, with duplicate genes encoding both the Presenilin and Aph-1 subunits (Shirotani et al., 2004). The different complexes have poorly understood and potentially complex expression patterns, with overlapping yet distinct cell- and tissue-specific components (Hebert et al., 2004). Furthermore, the different complexes exhibit distinctive enzymatic activities regarding APP cleavage. Presenilin-2-containing complexes show a reduced ability to process APP compared to Presenilin-1-containing complexes (Lai et al., 2003), and both Presenilin-2- and Aph-1B-containing complexes exhibit a bias towards producing the more amyloidogenic Aβ42 peptide (Placanica et al., 2009; Serneels et al., 2009). To complicate matters further, individual cells might express different γ-secretase complexes that are in equilibrium with one another (Placanica et al., 2009) and subject to strict regulatory control (Thinakaran et al., 1997). The functional heterogeneity in mammalian γ-secretase is a potential confounding factor in studies on the selectivity of various γ-secretase inhibitors with respect to the intramembrane proteolysis of alternative substrates, a critical issue in the search for inhibitors that can therapeutically reduce Aβ peptide generation and amyloid plaque formation while avoiding toxic side effects on Notch and other substrates.

In the current study, we circumvented the problem of γ-secretase heterogeneity by performing dose-response inhibitor studies in parallel on APP and Notch in *Drosophila* cells,

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which possess a homogeneous γ-secretase complex. The fruit fly genome encodes a single variant for each of the four core γ-secretase components Presenilin, Nicastrin, Aph-1, and Pen-2, and functional reconstitution studies have demonstrated that all of these components are required for mature γ-secretase assembly and proteolytic activity in *Drosophila* (Hu and Fortini, 2003; Takasugi et al., 2003). Expressing full-length APPL and Notch proteins in this *Drosophila* assay, we performed dose-response analyses to compare the potencies of four different γ-secretase inhibitors in blocking cleavage of APPL versus Notch. Our results indicate that all of the inhibitors generally have similar effects on APPL and Notch cleavage, including identical rank orders of potency towards each substrate and identical IC_{50} values for two of the inhibitors, namely CpnE and DBZ, which interact with the N-terminal fragment of Presenilin (Fuwa et al., 2007). Despite the overall conservation in inhibitor activity towards APPL and Notch γ-secretase-mediated cleavage in *Drosophila*, two other inhibitors did show modest differences, suggesting that substrate-specific differences in cleavage exist and might be subject to pharmacological targeting. Both DAPT and DFK167, which target the C-terminal fragment of Presenilin and the catalytic site of γ-secretase, respectively (Morohashi et al., 2006; Wolfe et al., 1998), display quantitative differences in their ability to block APPL versus Notch cleavage in *Drosophila*. For DAPT, when APPL cleavage is ~50% inhibited, Notch cleavage is only slightly inhibited, and conversely for DFK167, ~50% inhibition of Notch is accompanied by relatively minor effects on APPL proteolysis. The increased potency of DAPT in blocking APPL cleavage as opposed to Notch proteolysis in *Drosophila* S2 cells is similar to effects of DAPT in human HEK293 cells, as reported in a recent study (Fuwa et al., 2007). While the IC_{50} differences we observed with DAPT and DFK167 regarding APPL versus Notch proteolysis are modest, our results suggest that some aspects of γ-secretase activity might be modulated by the action of

small compounds to yield substrate-specific outcomes. Our findings, together with similar results from some mammalian γ-secretase inhibitor studies, lend credence to the idea that further development of compounds that can partially inhibit human APP cleavage, reducing Aβ load without deleterious toxic effects on Notch signaling, could offer compelling therapeutic benefits in Alzheimer's disease (Selkoe, 2001).

The tertiary structure of γ-secretase is still poorly understood, but recent findings suggest that the C-terminal fragment of the Presenilin aspartyl protease subunit is closely associated with the Aph-1 subunit within the active complex (Steiner et al., 2008). Indeed, these two subunits might be sufficiently tightly linked that mammalian Aph-1 isoforms can differentially regulate γ-secretase through allosteric effects on Presenilin (Serneels et al., 2009). The possibility that Aph-1 might regulate γ-secretase activity makes it an especially attractive potential drug target with the aim of developing drugs having higher discriminatory specificities towards APP rather than Notch proteolysis. In this context, incorporating our *Drosophila* cell-based γ-secretase activity assay in new inhibitor screening and evaluation protocols might prove useful due to the reduced regulatory complexity and uniform subunit composition of the *Drosophila* γ-secretase complex.

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Footnotes

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Figure legends

Figure 1. Proteolytic processing of APP and Notch.

(*A*) Comparison of proteolytic cleavage site locations with respect to the membrane topology of the two γ-secretase substrates Notch and APP. Notch is cleaved extracellularly by a furin-like protease and ADAM metalloprotease, whereas APP is cleaved extracellularly at similar locations by BACE aspartyl proteases and ADAM metalloproteases. Extracellular cleavages are followed by intramembrane proteolysis performed by γ-secretase for both substrates. (*B*) Diagram depicting the human Presenilin domains that are targeted by the four well-characterized γ-secretase inhibitor compounds (DBZ, CpnE, DAPT and DFK167) that we used for dose-response studies of APPL and Notch proteolysis in *Drosophila* S2 cells. The mature PS NTF is depicted in green while the CTF is shown in red.

Figure 2. Processing of *Drosophila* APPL.

Immunoblot analysis of epitope-tagged APPL expressed in *Drosophila* S2 cells treated with different inhibitor compounds that target either γ-secretase, endosome acidification, or metalloprotease activity (see Materials and Methods). Lane numbers, expression constructs and drug combinations are indicated above each immunoblot. (*A*) Full-length APPL is processed into C-terminal fragments (CTFs) that can be detected more readily by preventing their processing by γ-secretase using the inhibitor CpnE. (*B*) APPL also releases a highly unstable γ-secretase-dependent fragment (AICD) which can be detected in longer exposures. (*C*) Extended gel electrophoresis identifies three APPL CTFs (a, b and c) with CTFc generation requiring

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metalloprotease activity. ConA, endosome acidification inhibitor Concanamycin A; Epo, proteasome inhibitor Expoxymicin; GM6001, α-secretase metalloprotease inhibitor. β-tubulin serves as the loading control in all immunoblots.

Figure 3. Relationship of metalloprotease and γ-secretase Notch cleavage in *Drosophila* cells.

(*A*) The Notch receptor is initially synthesized as an ~300 kDa precursor that is processed by furin-like enzyme(s) in the *trans*-Golgi compartment. This cleavage leads to the production of a heterodimeric Notch receptor, which is further processed at the cell surface in a ligand-dependent manner by ADAM and γ-secretase. The ADAM cleavage occurs at an extracellular site and removes the Notch ectodomain, while γ-secretase cleavage (GS) occurs at multiple positions of the Notch transmembrane domain, leading to the release of the intracellular Notch fragment (NICD) that functions as a nuclear transcriptional regulator. (*B*) Immunoblot analysis of Notch biochemical processing following treatment of Notch-expressing S2 cells with pharmacological inhibitors metalloproteases involved in Notch ectodomain shedding (BB94), γ-secretase Notch cleavage (CpnE), and both processes together (BB94 and CpnE). Expression constructs and drug combinations are shown above the immunoblot. The panel at bottom is an enlargement of the fragments detected in the ~100 kDa range, indicating the membrane-bound fragments produced by the extracellular furin-like and ADAM cleavages, including the putative heterodimeric Notch CTF generated by furin-like cleavage in the *trans*-Golgi (put het CTF; upper righthand arrow), the NEXT fragment generated by ADAM-mediated proteolysis (lower righthand arrow), and the NICD fragment produced by γ-secretase cleavage of NEXT (lefthand arrow). The asterisk

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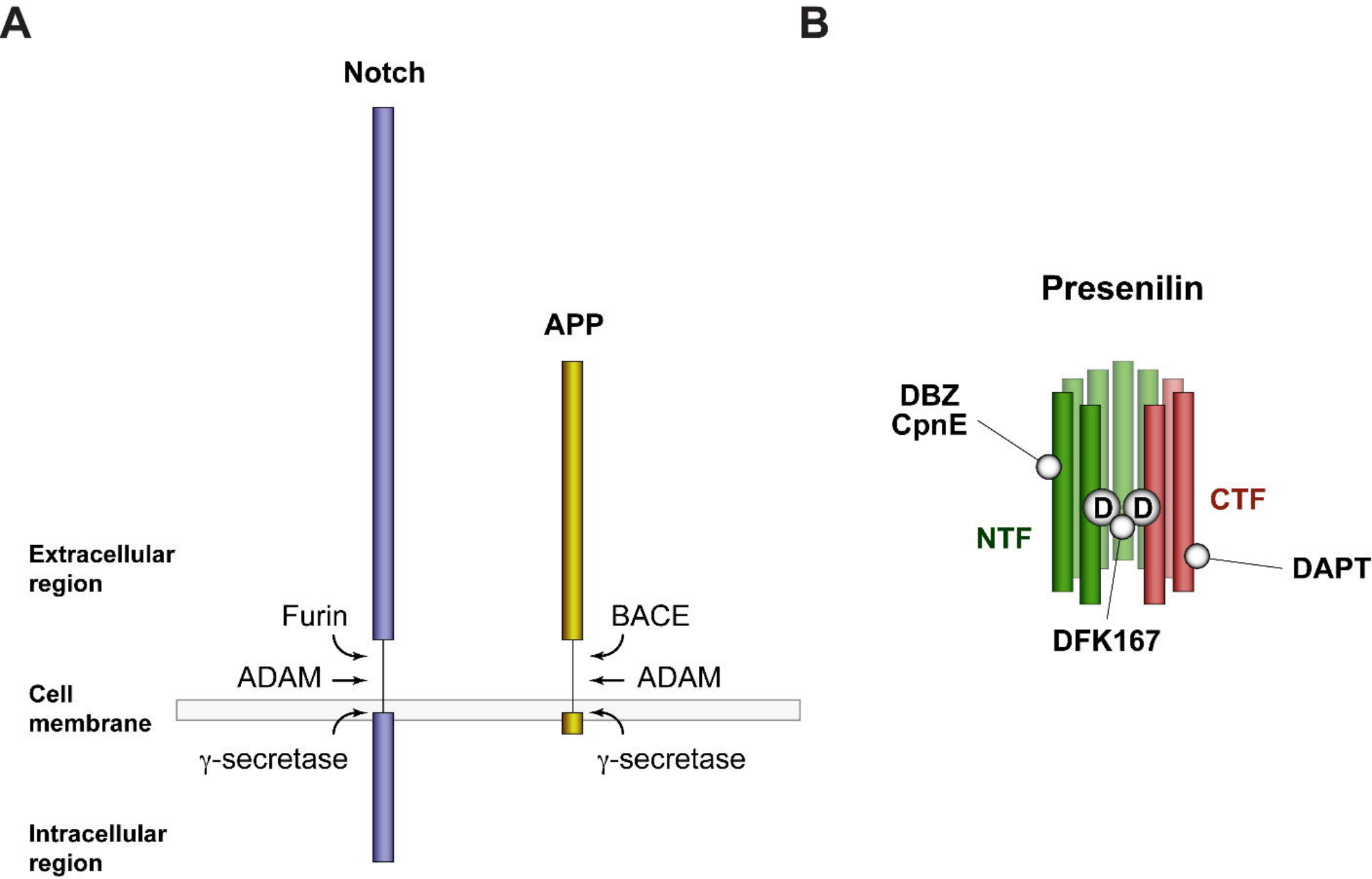
indicates a non-surface-derived Notch breakdown product that is often detected in Notch-expressing S2 cells (Hu et al., 2002). BB94, batimastat; CpnE, Compound E.

Figure 4. Dose-response analysis of Notch and APPL cleavage sensitivity to γ-secretase inhibitors.

(*A*) Representative examples of immunoblots used for dose-response studies of Notch and APPL with four different γ-secretase inhibitors. The substrate under analysis is shown in the black box in the upper left corner of each immunoblot; the inhibitor drug used is shown at upper left above each immunoblot set, with drug concentrations indicated above each lane. Hypotonic lysates from S2 cells were resolved on 3-8% Tris-Acetate or 16.5% Tris-Tricine gels and immuno-probed with antibodies to the C-termini of the expressed constructs (see Materials and Methods). Increasing drug concentrations lead to a progressive accumulation of APPL CTFs and a progressive decrease in Notch NICD levels. Due to difficulties in detecting the highly unstable APPL AICD, immunoblot quantifications to establish the pharmacological potencies of inhibitors are based on CTFa abundance for the APPL studies, while NICD levels were monitored for the Notch dose-response studies. Replicate numbers of dose-response immunoblots performed for each substrate/inhibitor pair were as follows: Notch/DBZ (5), Notch/CpnE (5), Notch/DAPT (7), Notch/DFK167 (7), APPL/DBZ (4), APPL/CpnE (4), APPL/DAPT (4), and APPL/DFK167 (5). (*B*) Dose-response curves for Notch and APPL are shown in black and orange, respectively, for the γ-secretase inhibitors DBZ, CpnE, DAPT, and DFK167 as indicated. The tested concentration range for each compound analyzed was as follows: DBZ (0.1-250 nM); CpnE (1-1000 nM); DAPT (25-2500 nM); DFK167 (1-150 μM). (*C*) The estimated IC_{50} values show that DAPT and DFK167 exhibit modest selectivity in

blocking Notch and APPL intramembrane proteolysis. Notch and APPL IC_{50} estimates for DAPT and DFK167 differ statistically ($p < 0.05$). The two most potent inhibitors, DBZ and CpnE, do not discriminate between the Notch and APPL cleavages, their IC_{50} estimates being statistically equivalent ($p > 0.05$). The IC_{50} parameter estimates are reported along with their standard errors. Replicate numbers of dose-response quantitative datasets used for IC_{50} calculations for each substrate/inhibitor pair were as follows: Notch/DBZ (3), Notch/CpnE (3), Notch/DAPT (2), Notch/DFK167 (3), APPL/DBZ (3), APPL/CpnE (2), APPL/DAPT (2), and APPL/DFK167 (3).

Fig. 1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Extracellular region
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ADAM
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NTF
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D
CTF
DAPT
DFK167

Fig. 2

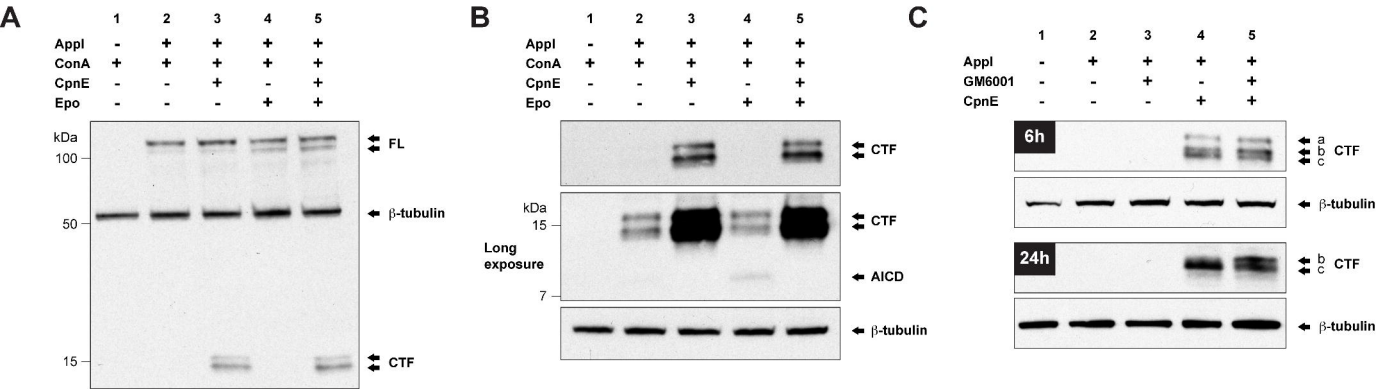
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Fig. 3

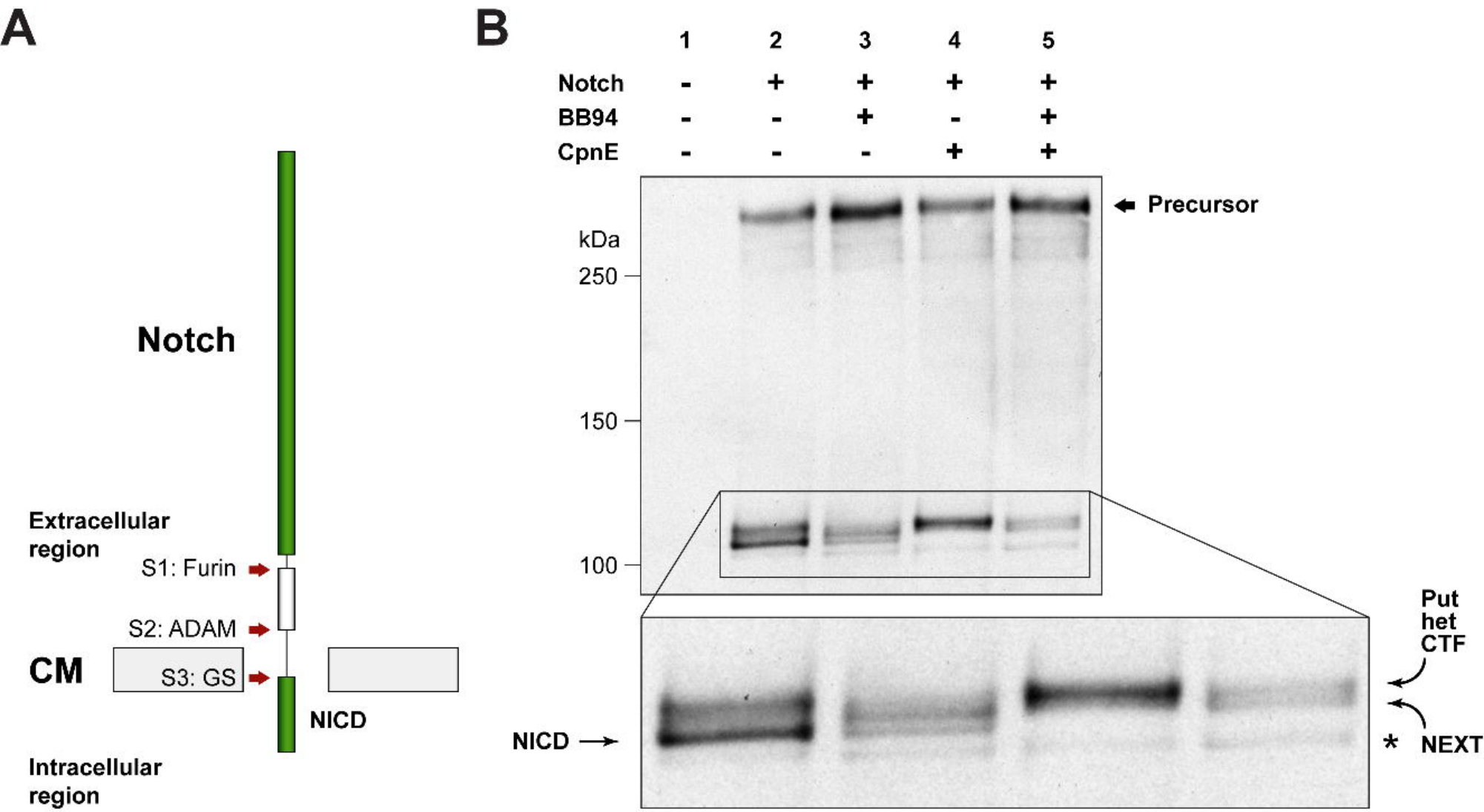
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Fig. 4

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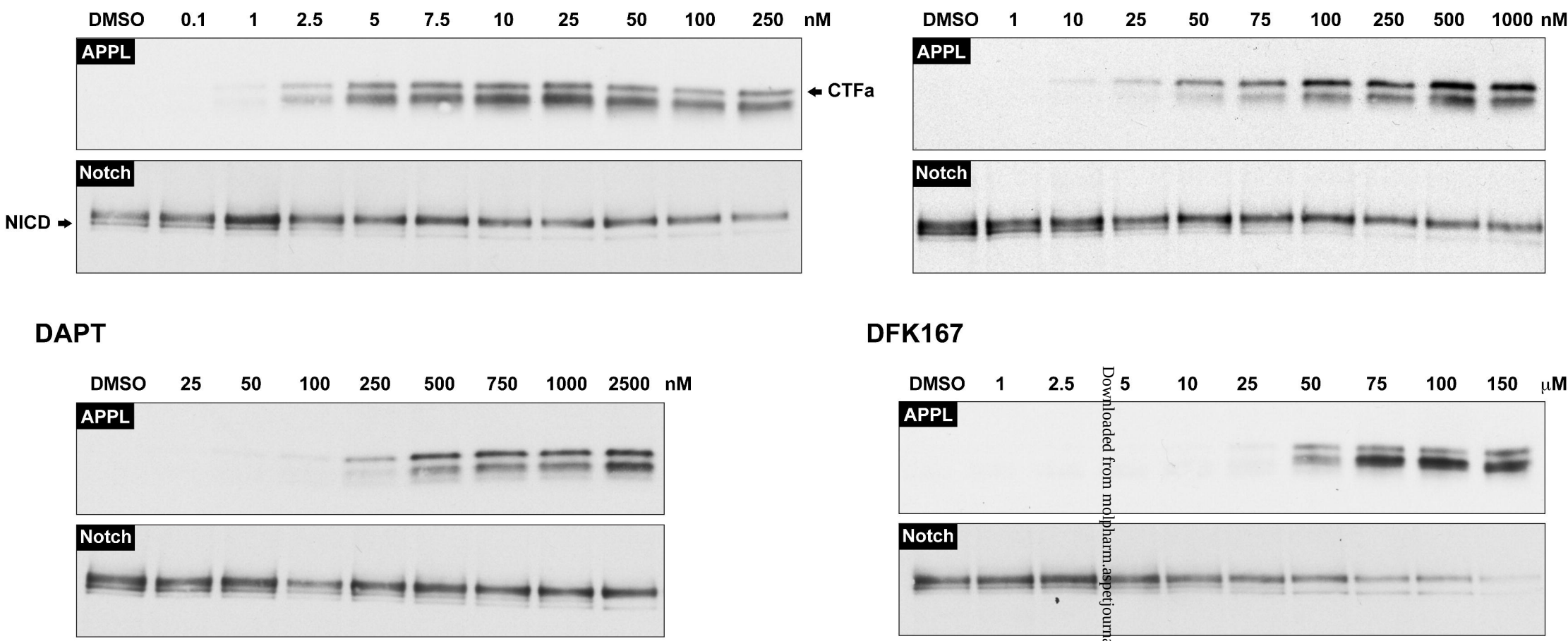
A



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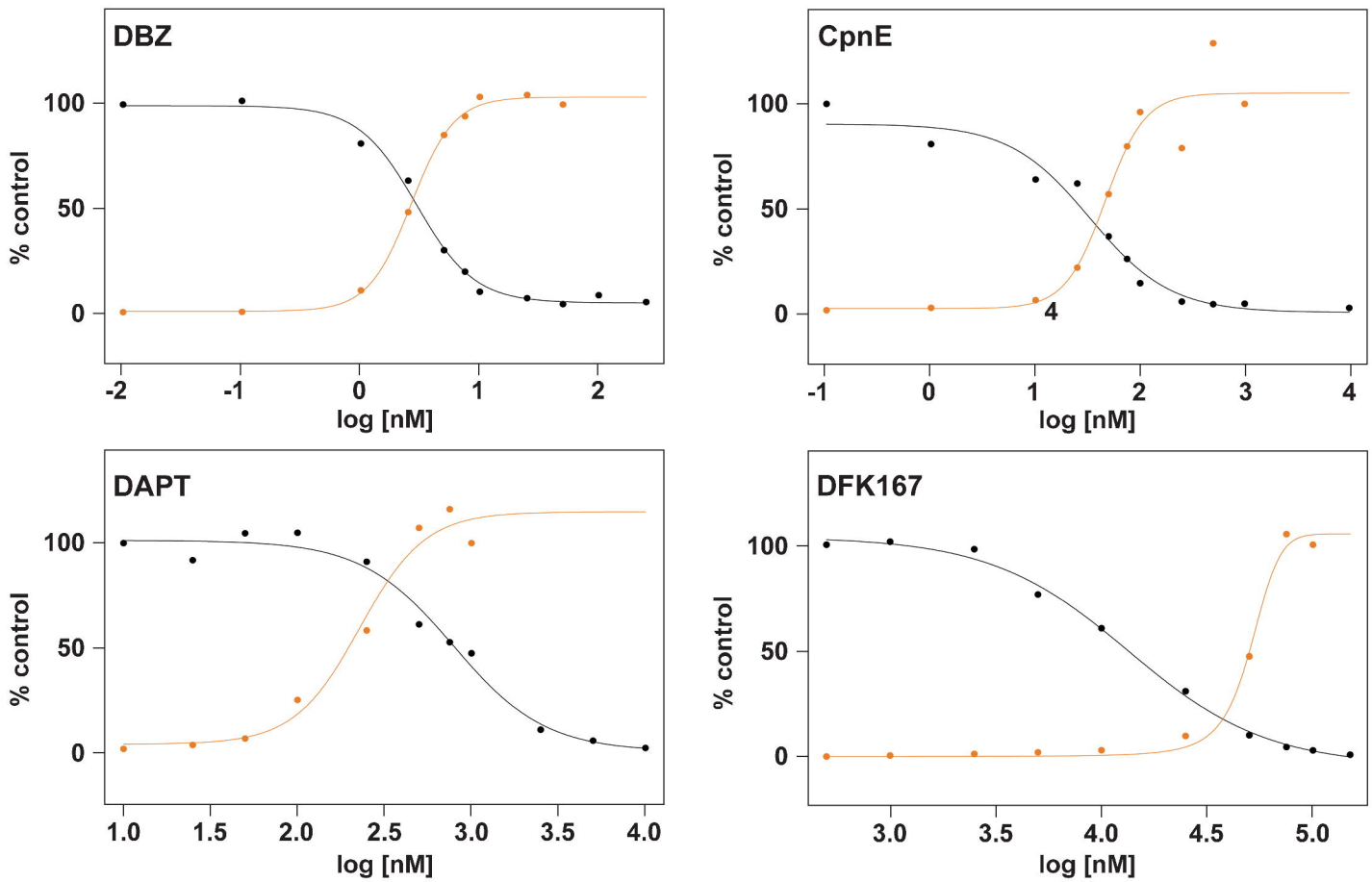

C

	APPL	Notch
DBZ	2.64 ± 0.30	2.92 ± 0.22
CpnE	46.26 ± 8.57	33.10 ± 6.02
DAPT	227.46 ± 50.75	788 ± 113.14
DFK167	50939.90 ± 1860.39	13452.10 ± 2046.82