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Klotho is a substrate for α -, β - and γ -secretase

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Abstract

Klotho is an anti-aging protein with different functions of the full-length membrane protein and the secreted hormone-like form. Using overexpression and knock-down approaches as well as embryonic fibroblasts of knock-out mice we present evidence that Klotho is shedded by the α -secretases ADAM10 and 17 as well as by the β -secretase BACE1. The remaining membrane-bound fragment is a substrate for regulated intramembrane proteolysis by γ -secretase. Our data suggest that therapeutic approaches targeting these proteases should be carefully analyzed for potential side effects on Klotho-mediated physiological processes.

Keywords

Klotho; gamma-secretase; ADAM protease; BACE

1. Introduction

Klotho (Kl) is a protein directly linked to aging. Transgenic mice with an insertion in the Kl locus were found to develop drastic symptoms of premature aging starting after 3-4 weeks [1]. Symptoms included shortened life span, arteriosclerosis, osteoporosis, skin atrophy and emphysema and resemble human aging. On the contrary, transgenic mice overexpressing Kl have an extended life span, suggesting that it has anti-aging properties [2]. Kl is a 120-135 kDa type-I transmembrane protein [for review see 3]. Interestingly, Kl is shedded and the extracellular part is released into CSF and serum [4]. Kl is predominantly expressed in the kidney in distal convoluted tubules and in brain in the choroid plexus, with weaker expression in pituitary, hippocampus, parathyroid and others [1].

Kl seems to be involved in Vitamin D dependent phosphate and calcium homeostasis [1,5]. It was shown that the transmembrane form of Kl is a co-receptor for FGF-receptor 1, which converts that receptor into a specific receptor for FGF23 [6,7].

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The anti-aging properties of KI might be caused by inhibition of insulin and IGF-1 signaling, since mice lacking KI are hypoglycemic and hypersensitive to insulin [8]. This would be in line with numerous findings showing the involvement of insulin/IGF-1 signaling pathways in longevity [for review see 3]. KI overexpressing mice have a higher resistance to oxidative stress [9]. This resistance might be induced via KI-mediated downregulation of insulin/IGF-1 signaling, which is involved in the inactivation of FOXO transcription factors. Target genes of FOXO transcription factors are, among others, antioxidant enzymes [for review see 3].

Type-I transmembrane proteins often are shedded by metalloproteases of the ADAM (a disintegrin and metalloprotease) family. Release of ectodomains by ADAMs can have important physiological functions, for example in the case of TNF α or Notch signaling or the release of soluble Amyloid Precursor Protein (APP) by ADAM10 and ADAM17 [for review see 10,11]. Shedding can also be mediated by β -secretase, also called BACE1 (β -APP cleaving enzyme 1), a key enzyme in the pathology of Alzheimer's Disease [for review see 12]. The remaining membrane-bound fragments "stubs" are in many instances substrate for a process called regulated intramembrane proteolysis. This process is mediated by an enzymatic complex called γ -secretase [for review see 13]. BACE1 and γ -secretase are potential drug targets for the treatment of Alzheimer's Disease and the latter also for a number of Notch-driven cancer types. It is therefore important to carefully study potential substrates of these enzymes and to be aware of potential side effects caused by interfering with their function.

We here show that KI is processed by ADAM10 and 17 and by BACE1. The remaining stubs are further processed by γ -secretase.

2. Material and Methods

Antibodies and cell lines

The following antibodies were used. Anti-flag was purchased from Sigma-Aldrich; monoclonal anti-GFP from Invitrogen; monoclonal anti-KI (KM2119 from Kyowa HAKKO Kogyo Co [14]); polyclonal anti-ADAM10 and anti-BACE1 from Abcam. HEK293 cells stably expressing PS1 and PS1(D385N), respectively, were described before [15]. MEF devoid of PS1/2 [16], ADAM10, ADAM17, ADAM10/17 and BACE1 [17,18] were described before. 293KI (HEK293 cells stably expressing flag-tagged KI) were described [6]. Cells were grown in DMEM supplemented with 10% FCS and Pen/Strep.

cDNA-constructs and transfections

Flag-tagged KI was described before [6]. Human cDNA clones containing the ADAM10 or BACE1 coding region (OpenBiosystems) were cloned into pcDNA3.1(+) expression vector (Invitrogen). 293KI and MEF cells were transfected using Lipofectamine 2000 (Invitrogen) and TurboFect (Fermentas), respectively.

Immunoprecipitation, immunoblotting and shedding assay

For shedding assays cells were plated in 6-well plates and grown to 70-80% confluency. 24h after transfection with KI-flag (in case of MEF cells) or 48h after transfection of siRNA or 16h after drug treatment (in case of HEK293KI), cells were washed in PBS and incubated in DMEM without serum for 3-6h. The cleared conditioned media were subjected to immunoblot analysis for Klotho protein directly or after trichloric acid precipitation. Cells were lysed in STEN-lysis buffer (50 mM Tris pH 7.6, 150 mM NaCl, 2 mM EDTA, 1% NP40 and protease inhibitor mix). Proteins were separated on 6% SDS-PAGE (for shedding experiments) or 10-20% Tris-Tricine gels (Invitrogen, for γ -secretase processing), and transferred to PVDF membranes. Membranes were cut at appropriate positions and blotted with antibodies as indicated. For

quantitation, a Fuji LAS4000 Luminescent Image Analyzer was used. The ratio of cellular vs secreted KI indicated shedding efficiency.

Drug treatments

To inhibit γ -secretase, cells were incubated overnight with 1 μ M DAPT (Axxora) or 5 μ M L385,345 (Sigma-Aldrich), respectively. To stimulate or inhibit ADAM mediated shedding, Phorbol-12-myristate-13-acetate (PMA) was used at 1 μ M for 16h and TAPI-1 at 25 μ M for 16h, respectively. To inhibit BACE1, C3 (Calbiochem) dissolved in DMSO was used at 2 μ M for 16 h.

siRNA-mediated knock-down

Specific siRNAs were transfected using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol and cells and media collected 48h after transfection. For qPCR RNA was isolated with Trizol reagent (Sigma) 48h post-transfection and the PCR was performed with the SYBR Green Master Mix (Applied Biosystems, Darmstadt, Germany).

3. Results

KI is processed by ADAM10, 17 and BACE1

KI is shedded into extracellular fluids similar to APP, suggesting that a surface protease of the ADAM family or BACE1 might be involved. To get an initial idea if these proteases could cleave KI, HEK293 cells stably expressing KI (HEK293KI) were first subjected to ADAM10 analysis (Fig. 1A). Activation of ADAMs by PMA increased shedding of KI by 50%, whereas the metalloprotease inhibitor TAPI-1 decreased shedding of KI by 90%. More specifically, overexpression of ADAM10 increased shedding of KI by 60% whereas knockdown by two different siRNAs decreased shedding by 25 and 30%, respectively, suggesting that ADAM10 is involved in shedding of KI (Fig. 1A). Efficient knock-down of ADAM10 but not control siRNAs was verified on mRNA level (Fig. 1B). Next we analyzed if BACE1 can mediate shedding of KI. Overexpression of BACE1 cDNA but not expression vector alone induced shedding of KI (Fig. 1C). Two different BACE1 siRNAs but not control siRNA decreased BACE1-induced shedding of KI by 70%. In addition, using a BACE1 inhibitor, C3, reduced the BACE1-induced shedding of KI by 50% (Fig. 1B). Taken together, these data suggest that ADAM10 and BACE1 can shed KI. Overexpression of an enzyme can only give hints to its potential role, and knock-down with siRNAs might cause off-target effects. We therefore made use of mouse embryonic fibroblasts (MEF) devoid of ADAM10 and BACE1, respectively. In addition, we tested MEF cells deficient in ADAM17 and deficient in both ADAM10 and 17. Transient transfection of KI in these MEF cells showed a strong reduction in shedding in all KO cells, with some variability between different lines (Fig. 2A, quantitation in 2B). A very strong reduction in shedding was observed in ADAM10/17 double KO MEFs, again supporting the idea that these two proteases are involved in shedding of KI. Taken together, these data indicate that the processing of KI is similar to APP in that both α - and β -secretases are able to cleave KI.

KI is processed by γ -secretase

The shedding of KI is reminiscent of APP, Notch and many other type I transmembrane proteins. In all these cases the remaining, membrane-bound stub is substrate for γ -secretase. To test if γ -secretase would cleave the KI stub, we incubated HEK293KI cells with or without two different γ -secretase inhibitors, L-685,458 and DAPT (Fig. 3A). In the presence of both inhibitors a small 5 kDa fragment was observed, which was hardly seen in the absence of the inhibitor. To further test if γ -secretase is mediating the processing of the KI-stub, we transfected KI-flag in HEK293 cell overexpressing functional PS1(wt) or non-functional PS1(D385N)

[15]. Only in PS1(D385N) cells without functional γ -secretase the 5 kDa fragment was visible, suggesting that it is a substrate of γ -secretase which is processed in PS1(wt) cells (Fig. 3B). Further evidence for γ -secretase processing of the KI-stub was provided by transiently transfecting KI-flag in mouse embryonic fibroblasts (MEF) derived from PS1^{-/-}/PS2^{-/-} double KO mice and from wild type mice, respectively. Fig. 3C demonstrated that the 5 kDa KI-stub could be detected only in transfected MEF PS1^{-/-}/PS2^{-/-}, but not in transfected wild type MEF cells, strongly suggesting that the KI-stub is substrate for γ -secretase (Fig. 3C). The specificity of the immunoprecipitation was demonstrated in Fig. 3D. Taken together, these data show that after shedding of the extracellular domain KI is a substrate for intramembrane proteolysis by γ -secretase.

4. Discussion

Many of the physiological functions of KI can be attributed to its membrane bound full-length form, for example its role as coreceptor for FGF23 [6,7]. Its limited expression mainly in the kidney and choroid plexus together with the occurrence of the secreted form of KI in serum and CSF as well as the plethora of affected tissues in KI deficient mice suggest a hormone like role of the secreted KI [for review see 3]. One function of the secreted KI is to antagonize wnt signaling [19], another the activation of the calcium channel TRPV5 [20]. It is therefore important to dissect in molecular detail KI processing and its regulation that would influence the ratio of full length versus secreted form. Here we show that KI processing is similar to APP, Notch and other cell surface proteins. KI can be shedded by ADAM10 and 17. Our data confirm earlier findings by Chen et al [21] and extend their findings by showing that also BACE1 is able to shed KI. The findings presented here suggest future experiments to study the role of BACE1 in KI processing *in vivo*, since BACE1 inhibitors developed to reduce amyloid-beta load in Alzheimer's Disease patients might interfere with KI-mediated physiological processes. BACE KO mice do not have obvious age-related phenotypes, but such phenotypes might have been overlooked because the potential connection to KI was not known. Using inhibitors, dominant-negative PS1 mutations as well as PS1^{-/-}/PS2^{-/-} cells we demonstrate that the KI-stub remaining after shedding is substrate for γ -secretase. γ -secretase cleavage might serve to remove the stub from the membrane, as has been suggested for other substrates [22]. It remains to be shown whether the intracellular domain, which would be very small, has a physiological function. Efforts to visualize and purify this peptide were unsuccessful so far, probably because of its very low half-life (DR and CK, unpublished). The work presented here provides the basis for future analysis of the role of shedding in the physiology of KI. It will be interesting to investigate if the ratio full-length/secreted form is changed during aging or under pathophysiological conditions and what the underlying mechanisms are.

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List of abbreviations

ADAM	a disintegrin and metalloprotease
APP	amyloid precursor protein
BACE1	β-APP cleaving enzyme 1

KI Klotho
MEF mouse embryonic fibroblast
PS1 Presenilin

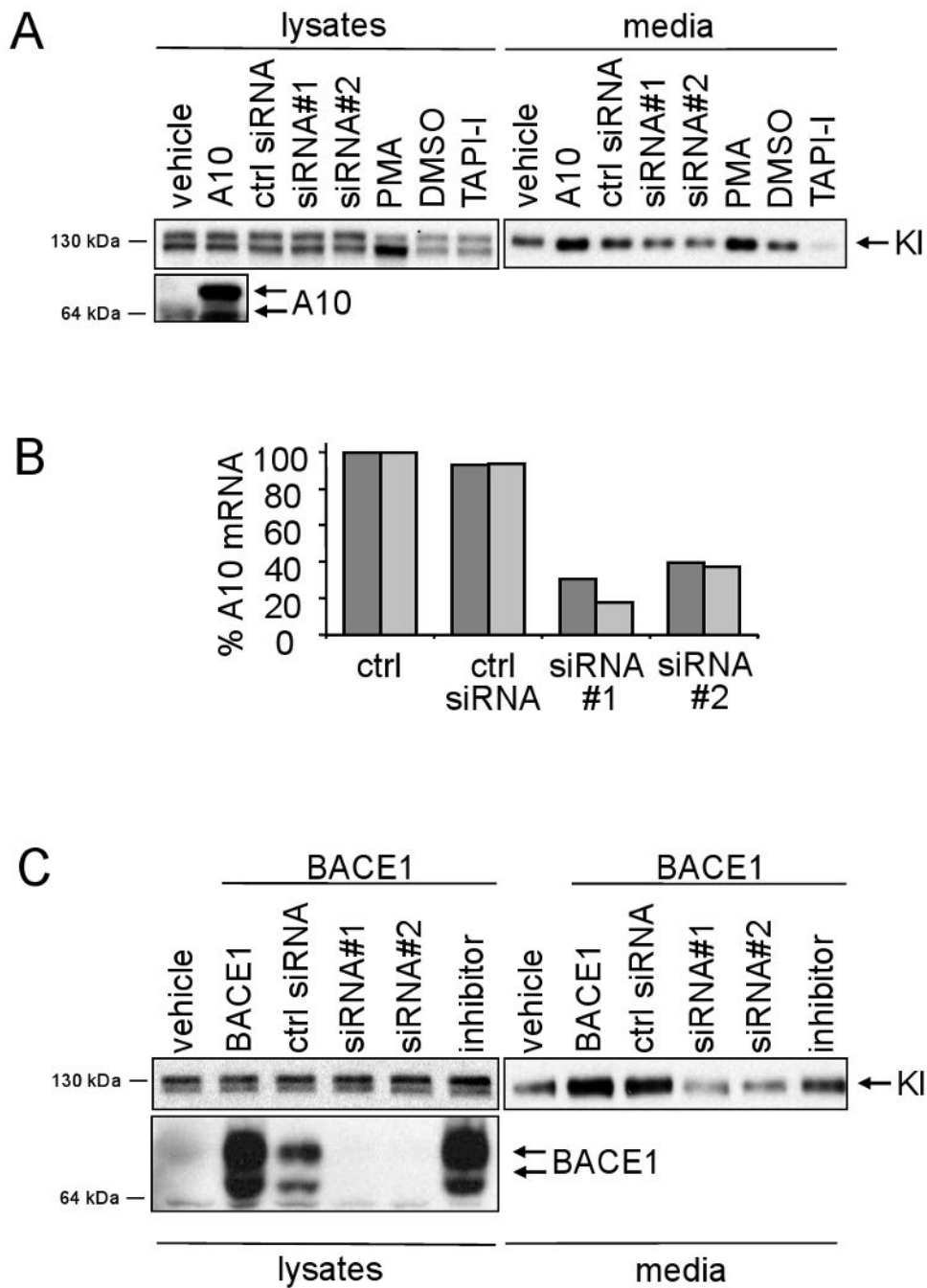


Fig. 1. KI is shedded by ADAM10 and BACE1. A) 293KI cells were transiently transfected with ADAM10 (A10) or empty vector (vehicle) or with siRNAs as indicated or treated with PMA or TAPI-1 for 16h and shedding of KI was assayed after 4h. After Western blotting, lysates and media were probed with KI antibodies and relevant lysates with ADAM10 antibodies. B) mRNA from cells transfected with transfection reagent only (ctrl) or control siRNAs or two different siRNAs against ADAM10 was isolated and qPCR using primers specific for ADAM10 was performed. mRNA level in ctrl cells was set to 100% and levels in siRNA treated cells related to that. Results from two independent experiments are shown. C) 293KI cells were transiently transfected with empty vector (vehicle) or with BACE1 cDNA or with BACE1

cDNA and in addition with siRNAs as indicated or treated with BACE inhibitor. Thereafter shedding of KI was assayed as in A). Lysates in addition were probed with BACE1 antibodies.

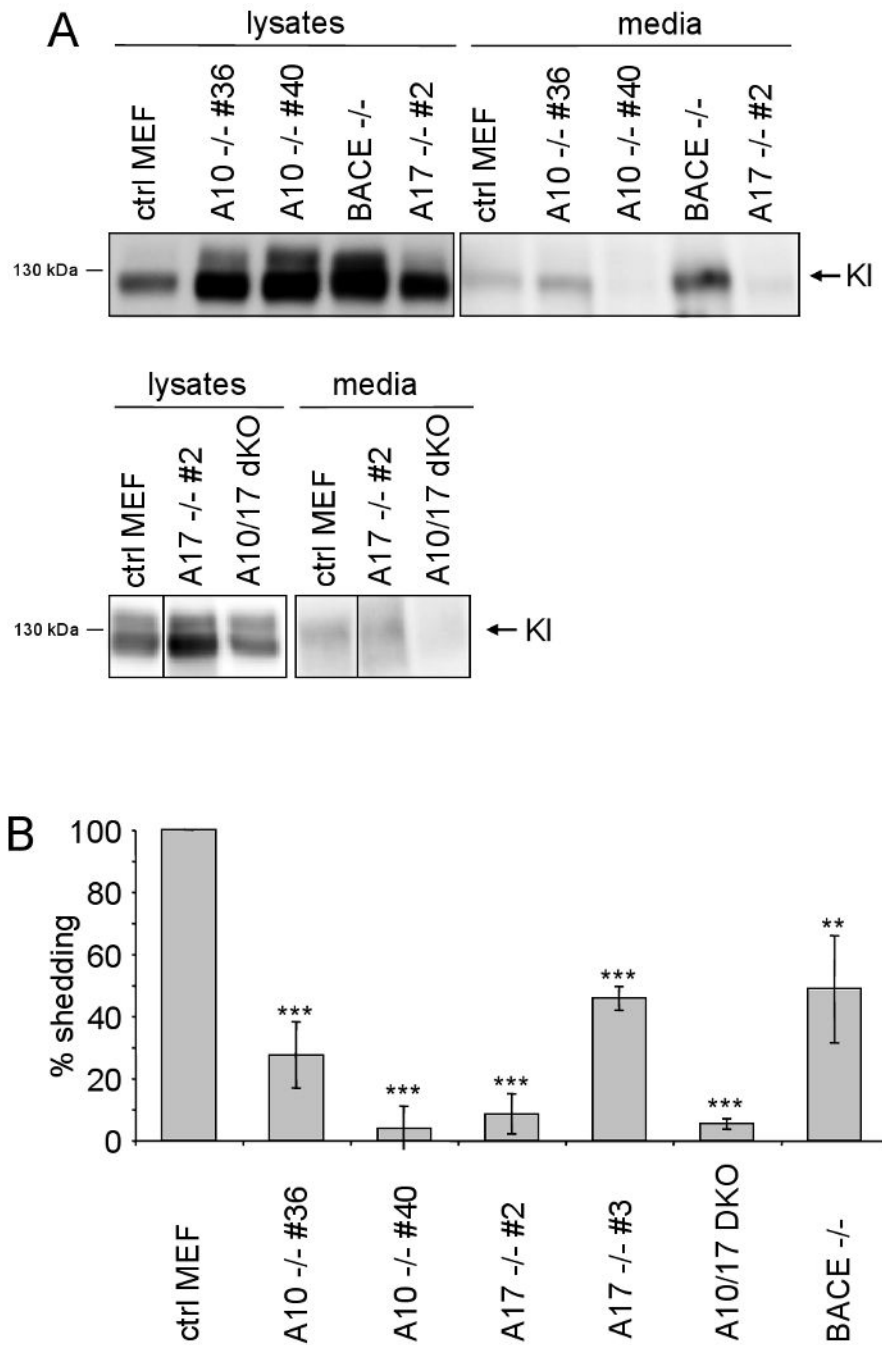


Fig. 2. KI is shedded by ADAM10, 17 and BACE1. A) MEFs cells derived from KO mice as indicated were transiently transfected with KI-flag and shedding was assayed after 24h. After Western blotting, lysates and media were probed with KI antibody. Representative gels are shown. B) Quantitation of shedding. The ratio of shedded KI in the medium versus KI in cell lysates was determined in all MEF lines. In ctrl MEF cells this ratio was set to 100% and the ratios from the other cell lines related to that. Error bars depict standard deviation from 4-7 independent experiments. ***, $p < 0.001$; **, $p < 0.01$.

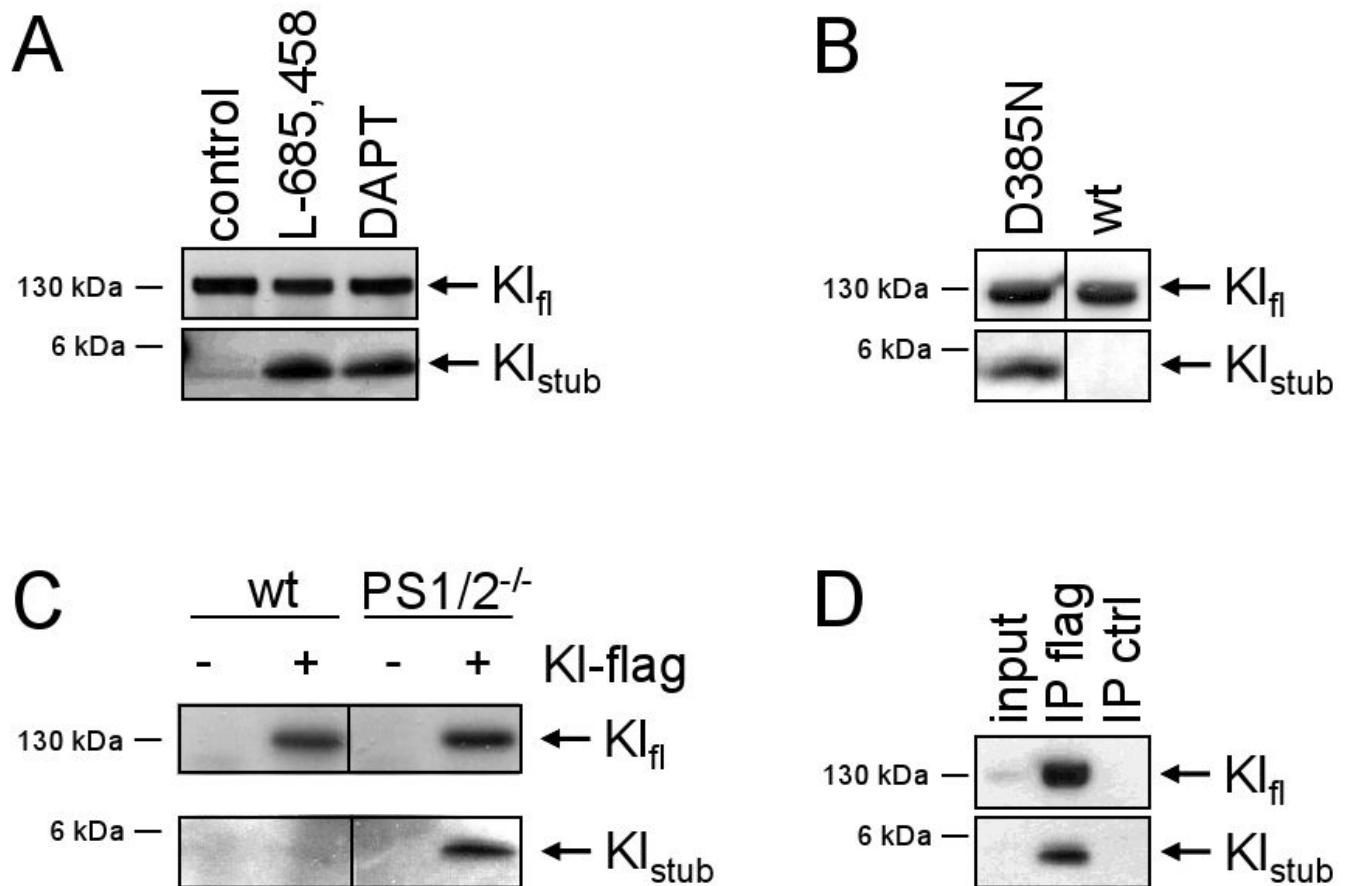


Fig. 3.

KI is processed by γ -secretase. A) 293KI cells were incubated with or without two different γ -secretase inhibitors for 24h as indicated, lysed and immunoprecipitated with flag-antibody, separated on 10-20% Tris-Tricine gels, blotted and probed with flag-antibody. B) KI-flag cDNA was transiently transfected in HEK293 cells stably expressing PS1^{wt} (wt) or dominant negative PS1(D385N). Lysates were immunoprecipitated and processed as in A). C) MEFs derived from wild type mice (wt) or PS1/2 double knock-out mice were transiently transfected with KI-flag and processed as in A). D) To demonstrate specificity of the antibody lysates from HEK293KI cells were subjected to immunoprecipitation with flag antibody (IP flag) and with beads only (IP ctrl) and processed as in A). In the input lane 1/25th of the lysate used for IP was loaded.