Massone et al., http://www.jcb.org/cgi/content/full/jcb.201011053/DC1

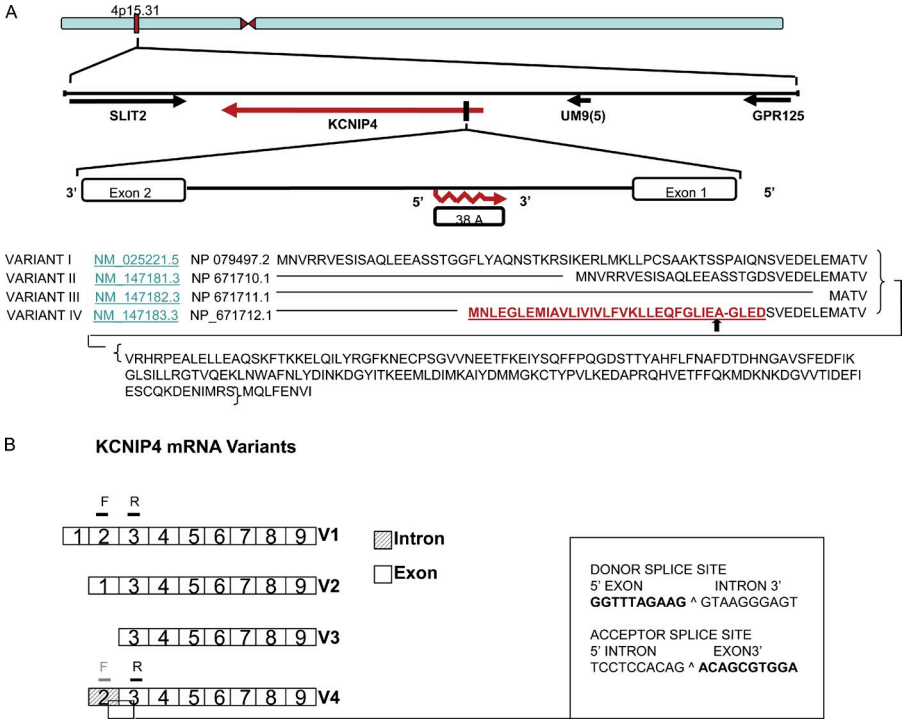

Figure S1. **A schematic view of the KCNIP4 locus.** (A) Protein sequences were analyzed in silico showing that the alternative Var IV (variant b in AceView) harbors a predicted ER localization as a result of a predicted signal peptide (Signal IP), whereas Var I (variant a in Aceview) lacks this structural feature (see Aceview annotations at www.ncbi.nlm.nih.gov/IEB/Research/Acembly). As shown, 38A maps in intron I of the KCNIP4 gene (GC04M020407, 11p15.31) in a region where alternative splicing events give rise to the distinct I and IV protein variants. Together with the other proteins of the same protein family, KCNIP4 regulates the electrophysiological properties of the Kv channel with a specific role of the splice Var IV in determining an altered kinetics of potassium channels (Holmqvist et al., 2002). KCNIP4 is a PS1 and 2 protein interactor (Morohashi et al., 2002). (B) A schematic view of KCNIP4 alternatively spliced mRNAs; the donor site exhibits a very high level of confidence, whereas the acceptor site shows a low level of confidence as a result of three independent programs (NetGene2, GeneSplicer, and the Berkeley Drosophila Genome Project).

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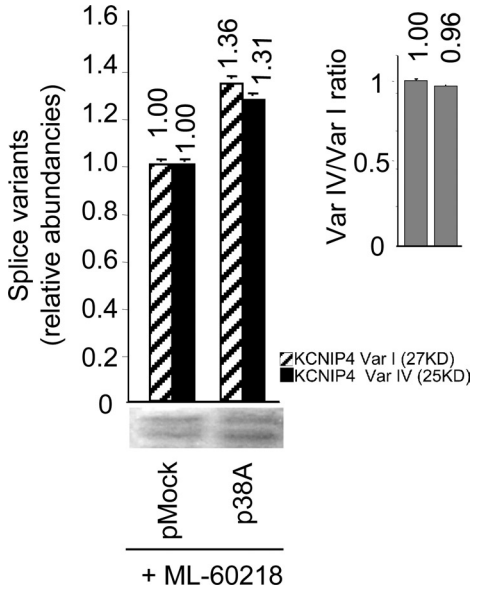

Figure S2. **PolIII dependency of 38A-driven alternative splicing shift.** In this experiment, cells were treated with 20 µM ML-60218, an RNA PolIII–specific inhibitor. Such a condition, by dramatically reducing the expression of 38A (Fig. 5 D), should impair the alternative splicing shift. Indeed, this treatment prevented the splicing shift triggered by 38A, as the KCNIP4 Var IV versus Var I ratio in p38A-transfected cells was similar to that of the pMock-transfected cells. Because at this dosage ML-60218 does not affect directly the transcription performed by PolII, whereas it significantly inhibits PolIII activity (as shown in Fig. 5 D by the unaltered synthesis of c-myc and the concomitant down-regulation of 5s rRNA, 7SK, and 38A synthesis), this result further supports the pivotal role of 38A ncRNA in the determination of KCNIP4 splice variant synthesis, thus bringing to light a novel unexpected role of PolIII-transcribed ncRNAs in gene expression regulation. The histogram shows that no major variations of Var IV versus Var I protein form ratio occurs in p38A-transfected cells as a consequence of the treatment with the PolIII-specific inhibitor ML-60218. Quantitative bars and the correspondent error bars (SD) are referred to the mean of three independent experimental determinations.

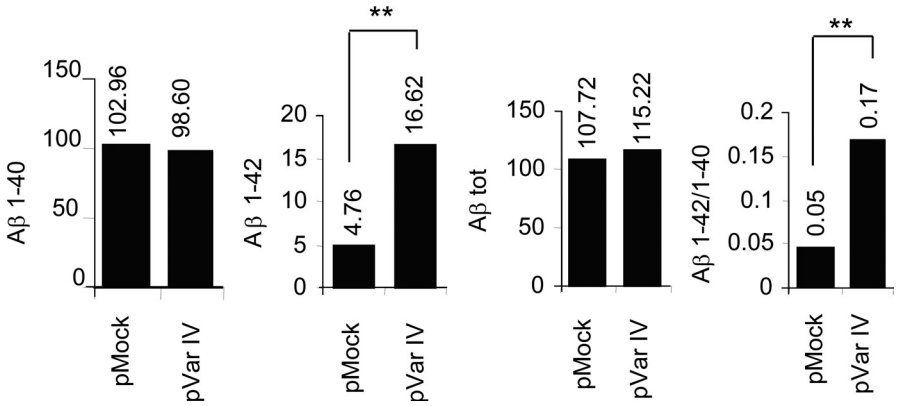

Figure S3. **A demonstration that the alternative splicing of KCNIP4 Var IV plays a key role in the impairment of Aβ processing.** In this experiment, we measured the secretion of Aβ by SKNBE-NDM29 cells (that do not overexpress 38A) in which the transient transfection of a KCNIP4 Var IV–coding cDNA plasmid abolishes the voltage-dependent potassium current (Fig. 3, D and F). The results of three independent determinations showed that the overexpression of the alternative protein form significantly increases the secretion of Aβ x-42, leading to a marked impairment of Aβ x-42/Aβ x-40 ratio demonstrating that the overexpression of recombinant KCNIP4 protein form IV recapitulates the phenotype of 38A-overexpressing cells. **, P = 0.001. The error bars were not relevant enough to be visible in the graphs; additional repetitions of the same experiment gave similar results (not depicted).

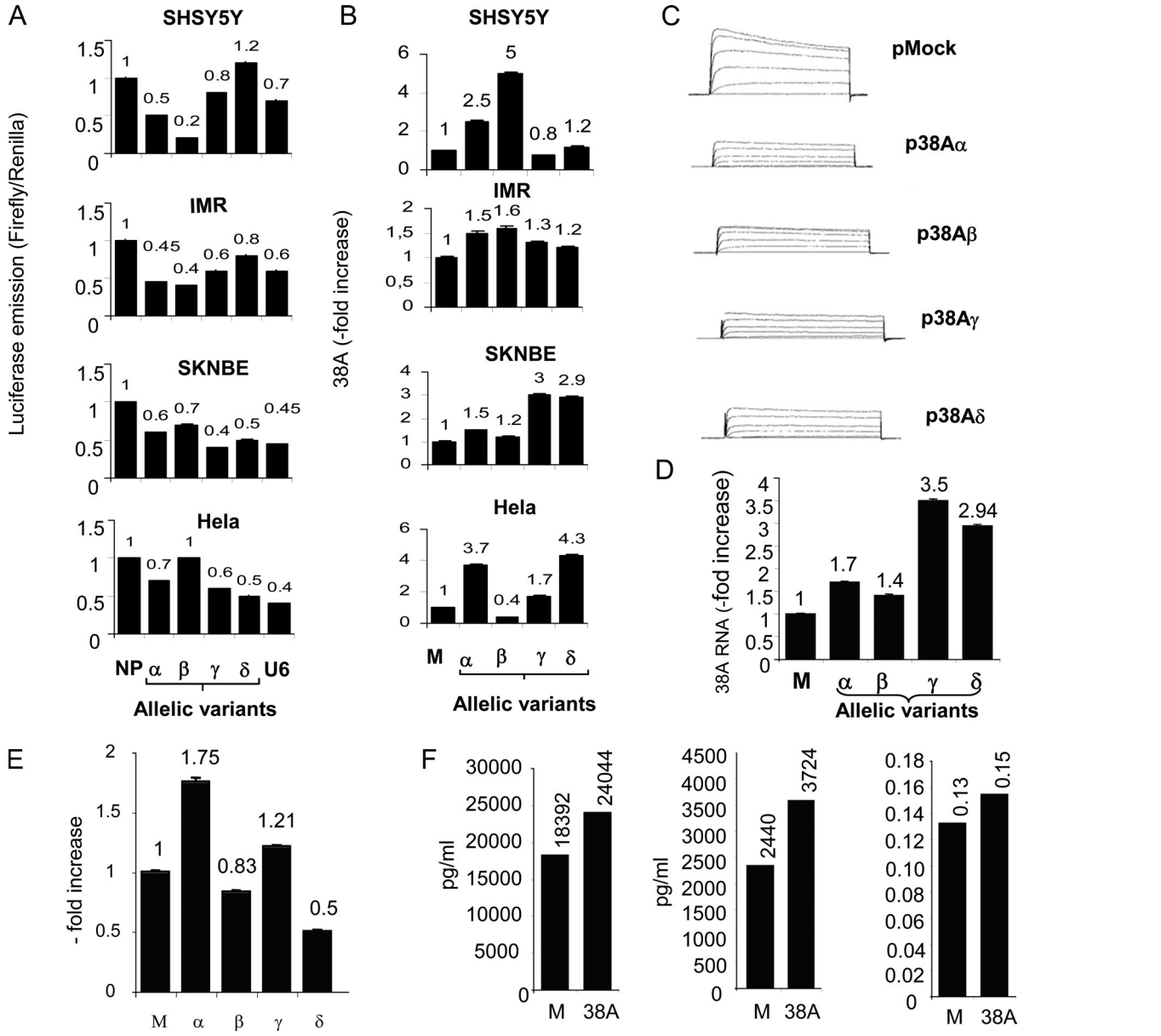

Figure S4. **Transcriptional activity and amyloid secretion in SHSY5Y cells overexpressing 38A under the control of different promoter variants and in HEK293 cells.** (A) An analysis of the transcriptional activity of distinct 38A promoter variants in different cell types by a luciferase-silencing assay. In this experiment, we fused 38A promoters to a luciferase-silencing hairpin that was then cotransfected with a plasmid-expressing luciferase (pGL3) in four different cell lines. The luminescence intensity of luciferase (y axis) silenced by hairpins driven by the different promoter variants is reported. Results normalized to a promoterless hairpin (NP, no promoter) showed that, if compared with the wt promoter α, the allelic variant β is more efficiently transcribed in SHSY5Y and IMR32 cells, whereas its transcription is down-regulated in HeLa and is modest in SKNBE cells. On the contrary, the promoter variant γ is transcriptionally more active than variant α in SKNBE, whereas its transcription is weaker in SHSY5Y and IMR32. Lastly, the promoter variant δ is transcriptionally more active than α in HeLa and SKNBE and is less active in SHSY5Y and IMR32 cell lines. Error bars represent SD of three independent real-time RT-PCR determinations. (B) Direct expression analysis of 38A transcription driven by different promoter variants. The aforementioned results, obtained with an indirect measurement of the promoter's activity, were then corroborated by the real-time RT-PCR analysis of 38A expression in the aforementioned cells transfected with plasmids harboring 38A under the control of the distinct promoter variants that confirmed a high transcription activity of 38A in the same conditions in which the luciferase signal of the aforementioned analysis was inhibited. Thus, complex patterns of altered 38A expression levels, caused by promoter mutations, might contribute to AD. Error bars represent SD of three independent real-time RT-PCR determinations. (C) Perturbation of voltage-dependent potassium channel current by different 38A promoter activities. All of the distinct promoter variants were tested for their possible role in the impairment of the voltage-dependent potassium current. Results showed that all of them are active, indicating that possible differences between diseased and nADc samples are related to 38A promoter activity and to its transcription rate rather than to specific mechanisms associated with its transcribed portions. (D) 38A ncRNA expression driven by the distinct promoter variants in the SKNBE2-NDM29 cells subjected to patch clamp analysis. Error bars represent SD of three independent real-time RT-PCR determinations. (E) Direct expression analysis of 38A transcription driven by different promoter variants in APP-overexpressing HEK293 cells. To assess whether the same phenomenon is detectable in APP-overexpressing cells, we transfected the aforementioned plasmid constructs in HEK293-APP and measured by real-time RT-PCR 38A transcription variations. We found that, also in this cell system, the expression of the ncRNA is dependent on the PolIII promoter that drives its synthesis, as the transcription profile detected in APP-overexpressing HEK293 cells recapitulates the expression pattern previously observed in HeLa cells. As expected, we also found that in APP-overexpressing cells, the enhanced synthesis of 38A ncRNA induces an increased secretion of amyloid and the impairment of its Aβ x-42/Aβ x-40 ratio. (F) Increased β-amyloid secretion and perturbation of Aβ x-42/Aβ x-40 ratio consequent to the increased expresssion of 38A ncRNA in APP-overexpressing HEK293 cells. X axis, transfected plasmids; y axis, quantitative determination of Aβ (picograms/milliliter) secreted in the medium 48 h after medium replacement as determined by sandwich ELISA. M, pMock sample. Error bars represent SD of three independent real-time RT-PCR determinations (E and F).

Table S1. **The principal clinical and demographic findings relative to each patient in terms of postmortem interval, age and sex, severity of the disease, presence of angiopathy, etc.**

Cases	Death	Postmortem delay	Braak stage
	yr	*h*	
AD cases			
A00-102	71	ND	ND
A01-15	ND	ND	ND
A92-404	69	6.5	V
A94-392	81	2.5	ND
A01-171	87	15	IV
A92-274	64	9	ND
A02-11	64	14	III
08-01	78	ND	ND
969	82	3.4	VI
1,142	87	3.4	V
977	80	4.4	V
901	80	4.4	V
124 (fam.)	40	3.3	V
A96-229	60	14	ND
1,024 (fam.)	58	3.3	V
942	88	3.3	V
A97-032	81	2.5	ND
nADc individuals			
A91-432	81	3	
Ctr3	65	10	
Ctr5	69	12	
A95-24	75	24	
A93-300	88	4	
Autopsy 4h	45	4	
984	65	3.3	
591	78	3.3	
543	72	3.4	
963[a]	83	3.3	

After a careful examination of demographical, clinical, and neuropathological data, we are not able to determine with statistic significance whether there is a correlation between 38A increment and clinical or pathological correlates. To date, we can only confirm that there is a significant positive correlation between the expression of 38A and AD and a negative correlation with non-AD cases. Braak stages are as follows: transentorhinal stages, I and II; limbic stages, III and IV; isocortical stages, V and VI.

[a]Case 963 has been enclosed in the control pool of samples, as it is not demented, although it has severe angiopathy and high Braak stage III.

Table S2. **Clinical and demographic findings of patient samples**

Allele	nADc	AD
Allele α (8 nADc, 6 AD)	1169, 787, 432, 945, 95272, 90234, 964 FC, A9525	94282, 801, 901, 969, 1024, A92274
Allele β (7 nADc, 9 AD)	Autopsy 4h, 94207, 93300, 1037, 09527, A92304, A8975	1142, A0115, A92261, A94392, A96104, 0211, 392, 94382, 96100
Allele γ (2 nADc, 5 AD)	CTR5, A97032	960, 942, A00102, A01171, 99219
Allele δ (0 nADc, 2 AD)		977, 124

n = 39 (22 AD and 17 nADc).

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