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Organ specific alteration in caspase expression and STK3 proteolysis during the aging process

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Abstract

Caspases and their substrates are key mediators of apoptosis and strongly implicated in various physiological processes. As the STK family is involved in apoptosis, and STK3 is a recently identified caspase-6 substrate, we assessed the expression and cleavage of STK3 in murine peripheral organs and brain regions during the aging process. We also assessed caspase-3, -6, -7 and -8 expression and activity in order to delineate potential mechanism(s) underlying the generation of the STK3 fragments observed, and their relation to the apoptotic pathway. We demonstrate for the first time the cleavage of STK3 by caspase-7 and show that STK3 protein levels globally increase throughout the organism with age. In contrast, caspase-3, -6, -7 and -8 expression and activity vary significantly amongst the different organs analyzed suggesting differential effects of aging on the apoptotic mechanism and/or non-apoptotic functions of caspases throughout the organism. These results further our understanding of the role of caspases and their substrates in the normal aging process and highlight a potential role for STK3 in neurodegeneration.

1 Introduction

Caspases are cysteinyl peptidases that are activated by proteolysis and dimerization. This leads to cleavage of their substrates and ultimately cell death through the programmed cell death pathway. The caspase family is essential not only for cell death, but also for immunity, neurogenesis, synaptic activity, cell proliferation and differentiation (D'Amelio, Cavallucci, and Cecconi 2010; Kuranaga and Miura 2007; Schwerk and Schulze-Osthoff 2003). Activation of caspases is observed in many physiological and pathological processes including Alzheimer disease, Huntington disease, stroke and aging (Broughton, Reutens, and Sobey 2009; Graham, Ehrnhoefer, and Hayden 2011a; LeBlanc 2013; Lee and Kim 2006; Pattison et al. 2006; Snigdha et al. 2012). The expression levels and activity of caspases during the aging process is organ and tissue specific. For instance, in the cerebral cortex of Wistar rats, caspase-3 (CASP3) protein expression decreases with aging whereas caspase-8 (CASP8) increases from 12 months of age onwards (Shimohama, Tanino, and Fujimoto 2001). In contrast to the cortex, CASP3 expression levels do not change in the skeletal muscle of aged Fischer 344/Brown Norway rats compared to their younger counterparts (Chung and Ng 2006).

The serine/threonine kinase 3 (STK3), also known as STE-20-like kinase MST2 (MST2), is a 56 kDa protein in the germinal center kinase group II of the MAPK (mitogen-activated protein kinase) family and is involved in apoptosis, differentiation, and proliferative signalling (Heallen et al. 2011; Kim et al. 2010; Li et al. 2013; Liu, Wu, et al. 2012; Oh et al. 2009; Qin et al. 2013). This pathway is often referred to as the signalling pathway of its ortholog in *Drosophilia melanogaster*, the Hippo signalling pathway. The activation of STK3 leads to the phosphorylation of Mps Once kinase activator-like 1 (MOB1), Large tumor suppressor 1 and 2 (LATS1/2) and Yes-associated protein (YAP). This results in the cytoplasmic retention and degradation of YAP, inhibiting the latter from promoting the transcription of proliferative genes such as c-myc and cyclin E via the transcription factor Scalloped/TEAD. STK3 also mediates the phosphorylation of the FOXO transcription factor family leading to the transcription of proapoptotic genes such as FasL, Trail and PUMA (Liu, Wong, et al. 2012) **(Fig. 1)**.

The two main post-translational modifications known to regulate STK3 activity are phosphorylation and caspase-mediated cleavage. STK3 can homodimerize or heterodimerize with its homolog STK4 (MST1) allowing its activation by autophosphorylation whereas the protein phosphatase 1 and 2A (PP1 and PP2A) inactivate it (Deng,

Pang, and Wang 2003). STK3 is also regulated by CASP3 cleavage, which results in the production of 34 kDa N-terminal fragments (Deng, Pang, and Wang 2003; Lee et al. 1998). These fragments are resistant to dephosphorylation by protein phosphatases (Deng, Pang, and Wang 2003) and conserve kinase activity. We have recently shown that STK3 is a caspase-6 (CASP6) substrate (Riechers et al. 2016) and a multiple sequence alignment of STK3 demonstrates that the caspase cleavage sites V63EID and D319ELD are conserved down to *C. elegans* and *A. gambia*, respectively, suggesting important roles for the fragments generated. The caspase substrate STK4 is cleaved by CASP3, 6 and 7 (O'Neill, Matallanas, and Kolch 2005).

The majority of the work on STK3 has been made during development. However, the increase of caspase activity and production of reactive oxygen species (ROS) in neurodegenerative diseases and during aging, suggest that the production of STK3 fragments may play a role in neurodegeneration (Merksamer et al. 2013; Patten et al. 2010; Zhang, Zhang, and Herman 2003; Graham, Ehrnhoefer, and Hayden 2011a). We have previously shown that in striatal cells expressing mutant huntingtin the level of STK3 and its fragments are increased compared to controls (Riechers et al. 2016).

In order to gain a better understanding of apoptotic mechanisms and the role of STK3 in aging throughout the organism, we characterized the mRNA, and protein expression of STK3 in the normal aging process in peripheral organs and brain regions of C57BL/6 male mice. Moreover, we assessed the expression and activity levels of CASP3, 6, 7 and 8 in order to further our understanding of the potential mechanism(s) underlying the in vivo generation of STK3 fragments and their relation to the programmed cell death pathway.

2 Material and methods

2.1 Animals

Peripherals organs and brain regions were collected as described previously from C57BL/6 male mice and grouped according to age: 3-4 months, 12 months, 23-28 months and older than 30 months (Lessard-Beaudoin et al. 2015). The animal care and ethics committee of University of Sherbrooke approved the protocols for this study (protocols 303-12 and 133-14B). The mice were anaesthetized (Isoflurane, Abbott) and euthanized by cervical dislocation.

The upper part of the cranium was removed, and the brain was collected. The cortex, cerebellum, striata and hippocampi were then dissected. In parallel, kidney, liver, heart and spleen were harvested.

2.2 Western blot analyses

Peripheral organs and brain regions were homogenized and sonicated in lysis buffer (0.32 mM Sucrose, 20 mM Tris pH 7.2, 1 mM $MgCl_2$, 0.5 mM EDTA pH 7.2) using a cocktail of protease inhibitors (Roche), 4.2 mM PefaBloc SC (Roche) and 10 µM Z-VAD-fmk (Enzo Life Sciences) and clarified by centrifugation at 13,000 rpm. The protein concentration was determined using the BCA protein assay kit (Pierce). Protein lysates (50 µg) were separated on a 7.5% or 10% SDS-PAGE gel and transferred to a PVDF membrane (PerkinElmer). The membranes were probed with anti-STK3 (ab52641, Abcam, 1:1000), anti-CASP3 (9662, Cell signalling, 1:1000), anti-CASP6 (9762, Cell signalling, 1:500), anti-CASP7 (9492, Cell signalling, 1:1000), anti-CASP8 (9746, Cell signalling, 1:1000) or anti-actin (MAB1501, Millipore, 1:10000) antibodies. Peroxidase activity was detected and densitometric values were obtained with the Odyssey Fc imaging system (Mandel) using Luminata Crescendo Western HRP substrate (Millipore). Quantification of β-actin or Coomassie staining was used to standardize the amount of protein in each lane depending on the protein stability in each organ and densitometric values obtained with the Odyssey Fc imaging system (Mandel).

2.3 Real-Time Quantitative RT-PCR

Total RNA was extracted from peripheral organs and brain regions with the RNeasy mini kit (QIAGEN) and cDNAs were prepared using ProtoScript Reverse Transcriptase II (#M0368X, New England BioLabs). Quantification was done using Mx3005P QPCR Systems (Stratagene) with mouse-specific β-actin primers (forward 5'- ACGGCCAGGTCATCACTATTG-3'; reverse 5'-CAAGAAGGAAGGCTGGAAAAGA-3'), STK3 primers (Forward 5'-AGGCCCTATGTCCAACAGTG-3' and Reverse 5'-CCATCATGGGGTCTAGTGCT-3'), CASP3 Primers (Forward 5'-TGTCATCTCGCTCTGGTACG-3' and reverse 5'-TCCCATAAATGACCCCTTCA-3'), CASP6 primers(Forward 5'-TGGCTCCTGGTACATTCAGGAT-3' and reverse 5'-TCCGTGAACTCCAGGGAACT-3') and CASP8 primers(Forward 5'-CCTAGACTGCAACCGAGAGG-3' and reverse 5'-GCAGGCTCAAGTCATCTTCC-3'). Amplification of β-actin was used to standardize the amount of sample RNA in the reaction. Gene-expression levels were measured using MxPro QPCR Software (Stratagene).

2.4 Caspase cleavage assays

Recombinant caspases were expressed, purified and characterized as described elsewhere (Boucher, Duclos, and Denault 2014). Caspases were diluted in a reaction buffer (100 mM Hepes pH 7.4, 200 mM NaCl, 0.2% CHAPS, 2 mM EDTA, 20% glycerol) at a concentration of 2000 nM and serially diluted. Final concentrations used for each caspase in the assay were 0, 7.8, 15.6, 31.3, 62.5, 125, 250, 500, and 1000 nM. The diluted recombinant caspases and 50 µg of brain lysate were pre-heated separately at 37 °C for 30 min, then mixed and incubated for 90 min at 37 °C. Immunoblotting was performed as described above.

2.5 Caspase activity assays

Murine tissues were homogenized as described above without caspases inhibitors and the lysates diluted with reaction buffer (100 mM Hepes pH 7.4, 200 mM NaCl, 0.2% CHAPS, 2 mM EDTA, 20% glycerol and 20% DTT). The activity of endogenous CASP3, 6 and 8 was measured in the lysates using the preferred fluorogenic substrates AcDEVD-AFC, AcVEID-AFC and AcIETD-AFC, respectively (Enzo Life sciences) at 37°C for 60 min. It is noted that AcDEVD-Afc is also the preferred substrate of CASP7, and that no small peptidic substrate is specific to a particular caspase (McStay, Salvesen, and Green 2008). The fluorescence generated was measured by the VICTOR X Multilabel Plate Readers spectrophotometer (PerkinElmer).

2.6 Statistical analysis

Student *t* test, one-way ANOVA and the post hoc Tukey's multiple comparison tests were used for analysis between the age groups.The statistical significance was established at 0.05 (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$). GraphPad Prism 6.0 software was used for all statistical analysis (GraphPad Software, La Jolla, CA).

3 Results

3.1 Differential cleavage of STK3 by CASP3, 6 and 7

As STK4 is differentially cleaved by CASP3, 6 and 7 producing fragments with specific functions, we assessed the cleavage of STK3 by these caspases (Song and Lee 2008). Murine brain lysate was incubated with active-site titrated recombinant CASP3, 6 or 7, and STK3 cleavage was assessed. As expected, CASP3 and CASP6 cleave STK3 (Riechers et al. 2016; Lee et al. 2001). However, we also observed cleavage by CASP7 and noted that the caspases do not cleave STK3 with the same efficacy and do not produce the same fragment sizes. A higher concentration of CASP6 or 7 is required to cleave STK3 and generate the 34 kDa fragment when compared to CASP3. Interestingly, CASP6 is the only caspase that generates the 39 kDa STK3 fragment **(Fig. 2)**.

3.2 Region specific alterations in STK3 expression with aging in peripheral organs

As the STK family is involved in multiple physiological processes, we assessed the mRNA and protein expression of STK3 and its fragments during the aging process. In the heart, a significant decrease in STK3 mRNA expression is observed by >30 months. In contrast, while there is a significant decrease in STK3 mRNA in the kidney at 12 months of age, it then increases after 23-28 months (**Fig. 3**). No significant difference in STK3 mRNA expression is observed with aging in the liver (ANOVA test). However, the mRNA expression decreases significantly (43%) at 23-28 months of age in the liver (*t* test). In contrast to the heart and the kidney, STK3 mRNA expression level increases significantly (47%) in the spleen with aging till 23-28 months of age **(Fig. 3)**.

Overall, no significant change in STK3 protein expression levels is observed in the liver with aging (ANOVA). However, comparing the groups using *t*-test shows that STK3 FL protein levels decrease initially from 3 to 12 months of age then increase at >30 months of age when compared to the 12 months values (**Fig. 4A**). The STK3 39 kDa fragment expression levels in the liver also decrease at 12 months of age whereas the 34 kDa STK3 fragment expression increases by 76% at >30 months of age when compared to 3 and 12 months age-group **(Fig. 4A)**. In the kidney, a 3-fold increase is observed for the STK3 39 kDa fragment expression after the age of 12 months **(Fig. 4B)**. While no significant variation in STK3 protein expression is observed in the heart, a positive correlation is observed in the STK3 34 kDa fragment with age **(Fig. 4C).** No significant variation in STK3 FL protein expression level is observed in the spleen, however a 2 to 3-fold increase is observed in STK3 39 kDa and 34 kDa fragments suggesting an increase in STK3 cleavage occurs with age in the spleen **(Fig. 4D)**.

3.3 Region-specific variations in STK3 expression in the brain with aging

STK3 mRNA expression increases by 68% at 12 months of age in the cortex then decreases after 23 months of age. Hippocampal STK3 mRNA expression increases up to 23-28 months of age then decreases at >30 months of age. In the striatum, the mRNA expression levels of STK3 increase with aging (76%). In the cerebellum, no age dependent effects are observed **(Fig. 5)**.

In the cortex and the cerebellum, both the STK3 FL and the 34 kDa fragment forms increase significantly during the aging process **(Fig. 6A-B)**. In contrast to other brain regions, no significant variation in the STK3 protein expression is observed in the striatum with age. However, a positive correlation is observed for the STK3 34 kDa fragment **(Fig. 6C)**.

3.4 Differential expression and activity of caspases in peripheral organs with aging

In the liver, CASP6 and 8 mRNA expression levels decrease at 23-28 months of age. In contrast, an increase in mRNA expression of CASP3 and 6 is observed in the kidney after the age of 12 months. Similar to the liver, mRNA expression level of all caspases analyzed decreases at >30 months of age in the heart. No significant variation in any caspases is observed in the spleen with age **(Fig. 7)**.

With regards to caspase protein expression levels, cleaved (active) CASP3 and CASP6 proform expression decrease and no significant variation in CASP7 protein level is observed in the liver with the aging **(Fig. 8A, 8B).** In contrast, a ~5 fold increase of all protein forms of casp8 is observed in the liver after 3 months of age **(Fig. 8D)**. In the kidney, the CASP3 zymogen (inactive form) significantly increases (54%) with age **(Fig. 8E).** A global increase in CASP6 and cleaved CASP8 expression levels is also observed in the kidney with aging **(Fig. 8F, 8H).** Interestingly, a 65-fold increase is observed in the CASP8 cleaved p43/41 form and a 26-fold increase is observed in cleaved CASP8 fragment in the kidney **(Fig. 8H)**. Of note, and dissimilar to the other caspases, CASP7 expression level tends to decrease with age in the kidney **(Fig. 8G).** In contrast to the liver and the kidney, no significant variation is observed for CASP3 with aging in the heart and the spleen **(Fig. 8G, 8J).** However, in the heart, the CASP6 cleaved subunits (p20p10) increases by 99% and CASP7 proform decreases at 12 months of age **(Fig. 8J, 8K)**. A 25-30-fold increase is also observed in CASP8 protein expression levels in the heart **(Fig. 8L)**. Similar to the liver, CASP6 protein expression level decreases at 12 months of age in the spleen whereas all protein

forms of CASP8, CASP7 proform and p27 subunit demonstrate increased expression with age in the spleen **(Fig. 8N, 8O, 8P)**.

An increase is observed in CASP3/7 activity in the liver with aging. No significant variation is observed in CASP6 and 8 activities **(Fig. 9A)**. In the kidney, CASP3/7 activity increase at 23-28 months of age while CASP8 activity shows a clear decrease at 12 months of age however than increases at >30 months of age **(Fig. 9B)**. An increase in CASP6 and 8 activities is observed in the heart with aging whereas no significant variation is observed for CASP3/7 activity. Similarly, CASP8 activity increases with aging in the spleen **(Fig. 9C-D)**. No significant variation is observed in CASP3/7 and 6 activities with age in the spleen **(Fig. 9D)**.

3.5 Variation of caspases expression and activities in brain regions with aging

Decreased mRNA expression levels of all caspases analyzed is observed in the cortex with age. In the cerebellum, only a subtle increase in the mRNA expression of casp8 was observed at 23-28 months of age. Striatal mRNA expression levels of CASP6 and 8 increase with aging and hippocampal CASP6 mRNA expression levels increases while CASP3 mRNA decreases at an age of >30 months **(Fig. 10)**.

Striatal expression of the CASP6 zymogen decreases with age whereas the CASP6 cleaved p20p10 form increases suggesting an increase in CASP6 prodomain cleavage upon the aging process **(Fig. 11A)**. Furthermore, a strong increase in all protein forms of CASP8 is observed in the striatum at 23-28 months of age **(**Proform: 85-fold, p43/41: 170-fold, p30: 28-fold, p18: 1684-fold, **Fig. 11D)**. A global increase of caspase protein expression is also detected in the cortex with the exception of CASP7 which does not vary with age in that brain region **(Fig. 11E-H)**. A 28 to 48-fold increase is observed in cortical CASP8 protein expression, a 30-fold increase in CASP6 p20p10 and the CASP3 zymogen increases 43% with age **(Fig. 11D, 11E, 11H)**. In the cerebellum, the CASP6 zymogen and cleaved p20p10 subunits also increase by 43% and 2-fold, respectively, with aging **(Fig. 11J)**. Furthermore, a 15-fold and a 61-fold increase are noted with age in the CASP8 zymogen and the cleaved p43/41 fragments, respectively **(Fig. 11H)**. Thus, similar to the other brain regions, the protein expression level of CASP6 and 8 increase with age in the cerebellum contrary to CASP3, which protein expression decreases in this tissue **(Fig. 11I, 11J, 11L)**.

Despite the increase in CASP6 and 8 mRNA and protein expression levels with aging in the cerebellum, no variation in cerebellar CASP3/7, 6 or 8 activity was observed in our samples **(Fig. 12A)**. However, in the cortex, increasing CASP6 protein levels correlated with an increase in its activity at 23-28 months of age. Surprisingly, although the cortical protein expression of CASP8 increases, a decrease is observed in its activity **(Fig. 12B)**.

4 Discussion

We assessed cleavage of STK3 by CASP3, 6 and 7 and the expression levels of this substrate in several tissues during aging. We also assessed CASP3, 6, 7 and 8 expression and activity in peripheral organs and brain regions in the normal murine aging process (3 to 30 months of age). Overall, the pattern of mRNA and protein expression for CASP3, 6, 7 and 8, and their respective activities, vary strongly amongst the tissues tested and within the same organ, as observed in the different brain regions **(Table 1)**. These results suggest a differential effect of aging on the apoptotic mechanisms and/or non-apoptotic functions of caspases. We demonstrate for the first time cleavage of STK3 by CASP7, which is often seen as redundant to CASP3, but which some studies show unique activities and substrate repertoire (Boucher, Blais, and Denault 2012; Lamkanfi et al. 2008; Slee, Adrain, and Martin 2001). Furthermore, we show the protein expression of STK3 (FL and proteolytic fragments) globally increases throughout the organism upon the aging process.

4.1 Caspase-dependent processing of STK3

STK4, an STK3 homolog, is also a known death substrate of CASP3, 6 and 7 with cleavage resulting in the production of 36 and 40 kDa fragments and STK3 has been previously identified as a CASP3 and 6 substrate (O'Neill, Matallanas, and Kolch 2005; Riechers et al. 2016). In our study, we demonstrate the cleavage of STK3 by all three caspases. However, whereas all caspases generate a 34 kDa STK3 fragment, CASP6 also generates a 39 kDa fragment. The apoptotic functions of the STK4 36 kDa fragment are well described in the literature (Deng, Pang, and Wang 2003; Kilili and Kyriakis 2010; Kim et al. 2010; O'Neill et al. 2004; Qin et al. 2013) and some studies suggest that the 34 kDa STK3 fragment may have a similar role (Deng, Pang, and Wang 2003) . However, to our knowledge, the existence and the production of the STK3 39 kDa fragment by a caspase has not been previously described. It is possible that these two fragments of STK3, as described earlier for two fragments of STK4, may not have the same function *in vivo*. Indeed, the 40 kDa fragment of STK4 produced by CASP7

cleavage has been identified to selectively phosphorylate JNK and p38 while the 36 kDa fragment of STK4, produced by CASP3 cleavage, has been identified to selectively phosphorylate ERK (Song and Lee 2008). Additional studies are required to provide more precision regarding the STK3 caspase cleavage sites and the functions of the STK3 fragments produced by these cleavages events.

4.2 STK3 expression level in peripheral organs

In our studies, STK3 mRNA expression decreases in all peripheral organs except for the spleen where an increase is observed at 23-28 months of age. In contrast to the mRNA pattern, a global increase at the protein level in virtually all forms of STK3 is observed in these organs. Interestingly, the increase in fragment expression levels is not coupled with a decrease in the intact protein expression as may be expected due to cleavage. The increase in mRNA expression (and hence of the protein), in the spleen may compensate for the increased cleavage by caspases thereby stabilizing the FL form of STK3 in this organ. In the other peripheral organs, the stable or increasing level of the intact form may be a result of an increase in the half-life of the protein with age. For instance, the interaction of STK3 with phosphorylated RASSF2 which prevents STK3 degradation may be responsible for this stabilisation of the expression (Cooper et al. 2009).

4.3 Caspases expression and processing of STK3 in the liver

The decrease in CASP6 and 8 mRNA, and the decrease of caspase-3 and 6 protein expression in the liver with age corroborate the low cell death rate in this organ with aging observed in other studies (Suh 2002). The increase in the CASP8 active fragment may be more related to the non-apoptotic functions of the protease in the liver, such as cell proliferation and induction of inflammation (Ben Moshe et al. 2007). Surprisingly, we did not detect a corresponding increase in CASP8 activity in the liver. The absence of any significant variation in activity observed with aging in this organ may be a consequence of the presence of a natural inhibitor of casp8 such as c-flip or PI-9 in the protein lysates (LeBlanc 2003). The increase observed in CASP3/7 activity at >30 months of age in the liver suggests that the increase in the 34 kDa fragment of STK3 observed may be due to CASP3 or CASP7 cleavage. It has been previously demonstrated that STK3 has predominantly anti-proliferative functions within hepatocytes (Jin et al. 2009; Qin et al. 2013). Therefore, the increase in STK3 cleavage in the liver may promote these anti-proliferative functions of STK3 in the aging process rather than cell death *per se*.

4.4 Caspases expression and processing of STK3 in the kidney

The trend increase in the 34 kDa fragment of STK3 present in the kidney may be a consequence of the increased activity of CASP3 observed in that organ. However, the increase in global expression of CASP6 in kidney suggests that it may also be responsible for the production of the 34 kDa fragment and suggests a role for this protease in the generation of the 39 kDa fragment, a fragment only produced by CASP6, and which is significantly increased with age in the kidney. However, no variation in activity is observed using the preferred CASP6 substrate. Therefore, it cannot be ruled out that a protease other than CASP6 cleaves STK3 to generate the 39 kDa STK3 fragments in the kidney. However, in addition to the presence of inhibitors (exogenous or endogenous) that can influence the result, the lack of specificity of the caspase peptidic substrates used in the activity assays that are available further requires caution in interpreting the results (McStay, Salvesen, and Green 2008). The increase in expression of STK3, and of all caspases tested (with exception of CASP7) in the kidney with aging, may reflect the increase in cell death observed previously with aging by TUNEL in the glomerular and corticotubular areas in the kidney (Lim et al. 2012).

4.5 Caspases expression and processing of STK3 in the heart

In the heart, a decrease in CASP3, 6 and 8 mRNA expression levels is observed with aging. Interestingly, these differences were only noted when compared to the >30 months age-group. It is important to note that there are many metabolic changes observed between long lived mice compared to their younger counterparts that could be responsible for these results (Bartke and Westbrook 2012; Milman et al. 2014; Sherlock and Toogood 2007) as in general the normal life span of C57BL/6 mice is ~25 months. Moreover, these mice could be the result of a natural selection of a particular sub-group of individuals which may explain this decrease in caspases mRNA at >30 months of age. In contrast to the mRNA expression levels, CASP6 and 8 protein expression and activity increases with aging, which corroborates the previously observed ~30% loss of cardiomyocytes with age (Sheydina, Riordon, and Boheler 2011). The increase in CASP6 activity observed in the heart may be responsible for the increase in 34 kDa fragment of STK3 production observed in our results. However, the increase in the STK3 fragment is not coupled with a decrease in the FL protein expression. As a decrease in STK3 mRNA level is observed with aging, STK3 may be a target for post-translational modifications that promote an increase in the half-life of the protein or

may interact with some proteins that slow down the degradation rate of STK3, such as RASSF2 (Cooper et al. 2009).

4.6 Caspases expression and processing of STK3 in the spleen

Decreases in caspase-6 protein expression, and an increase in CASP7 and 8 protein expression and CASP8 activity, were observed in the spleen with aging. Of note, *in vivo* CASP6 active fragments are very difficult to detect by Western blotting, possibly due to short protein half-life. The decrease in CASP6 proform levels may be due to the processing and activation of the caspase or a more efficient degradation as we did not detect a decrease in gene expression in the spleen. Similar to our results, an increase in the activity of CASP8 and cell death has previously been observed in old C57BL/6 mice spleenocytes (Itzhaki et al. 2003). However, they also noted an increase in CASP3 activity between 2 and 15 months in old mice which we did not observed in our study. The difference between the ages selected (2 months of age considered in development in contrast to 3 months of age considered an adult), sex or strain of the mice used may be responsible for this difference. A trend increase is detected in all protein forms of STK3 in the spleen with aging. As no variation is observed in CASP3 expression or CASP3/7 activity, the increase in STK3 fragment may be attributable to CASP6 and/or CASP7 as we did detect subtle processing of the zymogens in the spleen.

4.7 Caspases expression and processing of STK3 in the brain

Interestingly, the decrease in mRNA expression of STK3 and all caspases observed in the aging cortex does not reflect the variation at the protein level. All STK3 protein forms increase with age, which may be the result of a less efficient degradation of STK3, an increase of the protein half-life or, more generally, it may be the result of the decrease in proteasome activity previously observed in this brain region with aging (Baraibar and Friguet 2012). An increase in CASP6 and a decrease in CASP8 activities were observed in the cortex with aging while no variation was detected in CASP3/7 activity. Thus, the strong increase in cortical STK3 fragments may be the result of the cleavage of STK3 by CASP6. Interestingly, in contrast to peripheral tissue, no 39 kDa fragment is observed in any of the brain regions assessed which may suggest specific peripheral functions of this particular STK3 fragment.

At the mRNA level, there is no variation observed in aging in any of the genes analyzed in the cerebellum except for CASP8 mRNA that increases. This increase in CASP8 mRNA levels is coupled with a strong increase in CASP8 protein expression. The absence of mRNA variation in CASP3 and 6 is not translated to the protein levels. A decrease in CASP3 and an increase in CASP6 protein expression are observed with aging in the cerebellum which suggest that CASP8, and potentially CASP6, may be a key caspase in neurodegeneration in the cerebellum with aging. A previous study has observed that while there is a stable number of Purkinje cells in the cerebellum with aging, there are some sections of the cerebellum, such as the anterior lobe, in which a loss of these cells is observed with aging (Andersen, Gundersen, and Pakkenberg 2003; Zhang, Zhu, and Hua 2010). As our analysis consisted of the entire cerebellum samples, this region-specific effect might be lost. Surprisingly, the variations at the protein level, even the strong increase in all forms of CASP8 protein levels, are not reflected in the caspase activity assay results. Our data suggest that the increase in the 34 kDa STK3 fragments observed in the cerebellum may be due to CASP6 as both the proform and the fragments increase with age in this brain region. We observe that both the FL and the STK3 34kDa fragments increase in the cerebellum with age supporting a role for apoptosis and caspases in the cerebellum with aging.

The mRNA of CASP6 and 8 increase with age in the striatum. However, this variation is not reflected at the protein level for CASP6 which decreases with age. In contrast, all protein forms of CASP8 strongly increase with aging. Interestingly, between 23-28 months and >30 months of age, the CASP8 proform decreases by 86%, which correlates with the high amount of fragment present. Therefore, the decrease observed in the CASP8 zymogen at >30 months of age may be due to the processing and activation of CASP8. As no variation in CASP3, and a decrease in CASP6 protein expressions is observed in the striatum, the increase observed in 34 kDa STK3 fragment protein in our data may be more attributable to its processing by CASP7, which has a trend increase in expression level with aging, to a less efficient degradation of the protein or an increase of the protein half-life. Of note, no small peptide substrate is available specifically for CASP3, or 7 (McStay, Salvesen, and Green 2008). Therefore, we cannot confirm this hypothesis using activity assays. This increase in STK3 fragment expression correlates with the age-associated increase in apoptosis observed previously in the striatum of Wistar 2BAW rats (Ureshino et al. 2010). Of note, we did not have enough samples to assess caspase activity in the striatum.

Finally, in the hippocampus, a decrease in CASP3 mRNA expression and an increase in the mRNA of CASP6 and STK3 are observed with aging. Of note, experiments in hippocampal primary cultures demonstrate that STK4, an homolog of STK3, is phosphorylated upon oxidative stress by c-abl leading to the stabilization of the protein expression of STK4 and the STK4-FOXO mediated cell death (Xiao et al. 2011). Moreover, CASP6 is activated in neurodegenerative diseases and its activity in the hippocampal CA1 region is sufficient to induce neuronal degeneration, inflammation and age-related memory impairment (LeBlanc et al. 2014; Ramcharitar et al. 2013; Graham, Ehrnhoefer, and Hayden 2011b; Wang et al. 2015). Thus, the increase in mRNA expression of STK3 and CASP6, a protease that cleaves STK3, highlight a possible role for STK3 in the neurodegeneration process in AD and potentially other neurodegenerative diseases. Unfortunately, we did not have enough hippocampal samples to assess protein expression of STK3 and caspases in this brain region.

5 Conclusion

Overall, our results demonstrate that the pattern of mRNA and/or protein expression of CASP3, 6, 7 and 8 and their respective activity vary strongly throughout the organism and even amongst the same organ (as observed in the different brain regions) suggesting differential effects of aging on the apoptotic mechanism and/or non-apoptotic functions of caspases. Moreover, STK3 expression, a newly identified substrate of CASP6 and 7, globally increases throughout the organism with the aging process. To our knowledge, this is the first time that the caspase expression and activity have been investigated as extensively throughout the entire organism during the aging process. This study provides important details regarding the pattern of expression and activity of the caspases and the caspase substrate STK3 throughout the entire organism upon a physiological stress that is the normal aging process. These results further our understanding of the effect of aging on the organism without any pathological condition.

6 Acknowledgements

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Organ specific alteration in caspases expression and STK3 proteolysis during the aging process

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Figures

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Key words: Aging, apoptosis, caspases, serine/threonine kinase 3, peripheral organs, brain region.

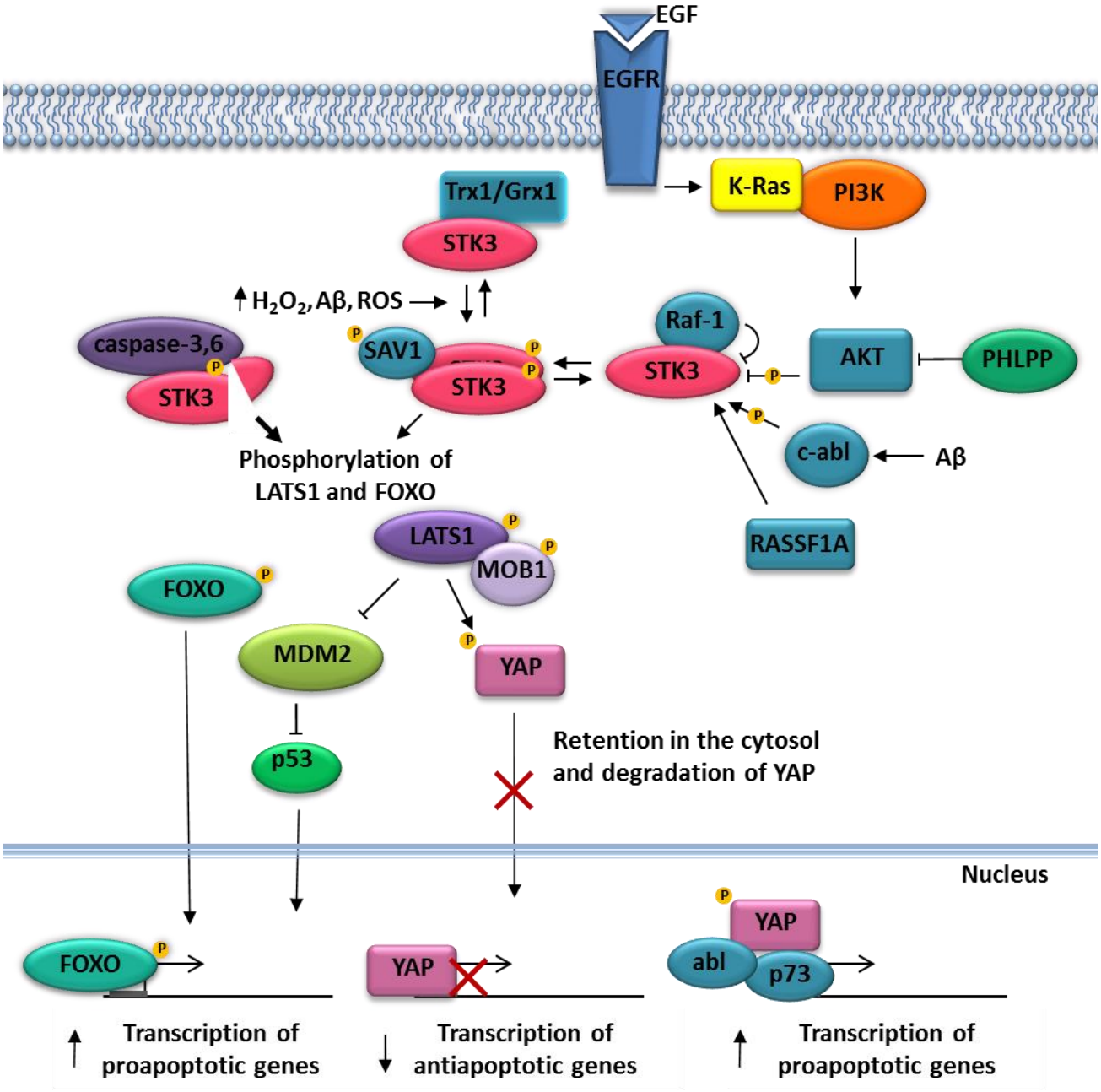

Figure 1. STK3 pathway. In the presence of oxidative stress, glutaredoxine (Grx1) and thioredoxine (Trx1) dissociates from STK3 promoting its activation by autophosphorylation. STK3 is also activated indirectly by the EGFR via PI3K/Akt signaling. The phosphorylation cascade promoted by STK3 activation results in a downregulation of proproliferative genes via the degradation of Yes-associated protein (YAP). The cascade also upregulates of proapoptotic genes by the phosphorylation of YAP and the phosphorylation of FOXO transcription factor. STK3 activity is decreased by the phosphatases (PP1 and PP2A) and Raf-1 and increased by RASSF1A, c-abl and its cleavage by caspase-3 which produces a constitutively active fragment that is resistant to dephosphorylation.

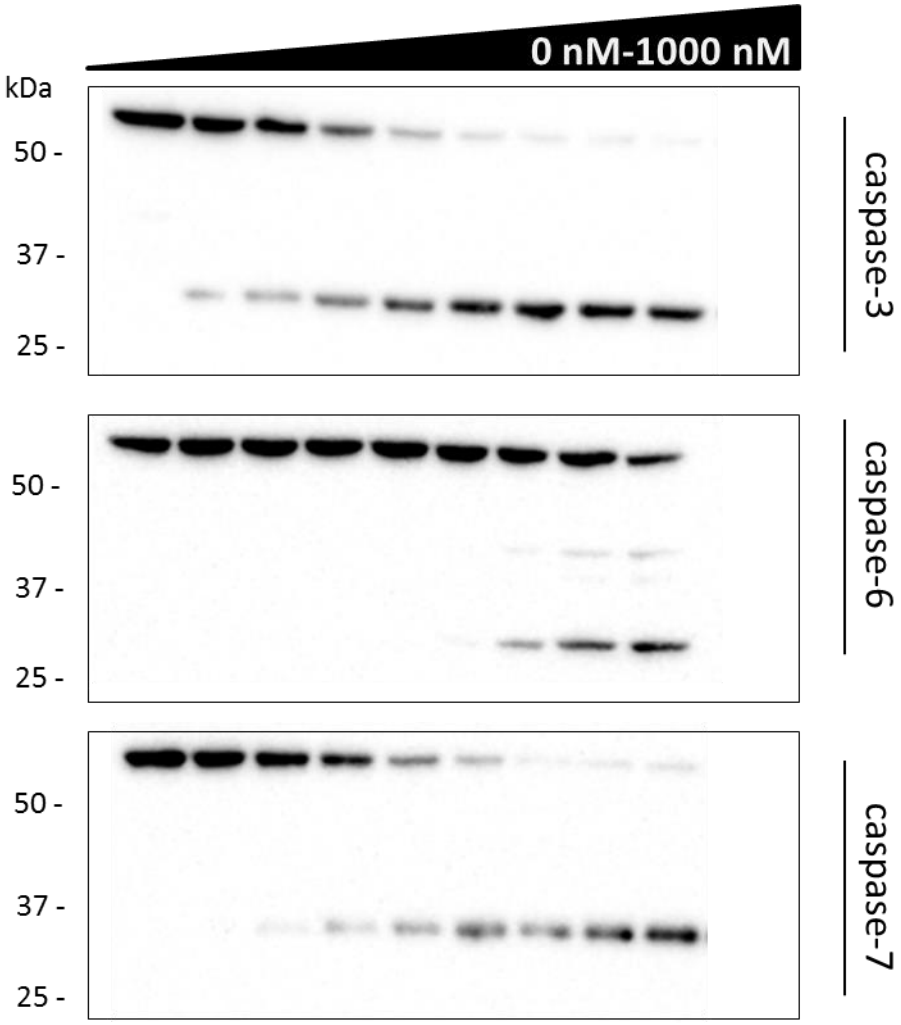

Figure 2. STK3 is cleaved by caspase-3, 6 and 7. Brain lysate was incubated with serial dilutions of recombinant caspase-3, 6 or 7 and analyzed by immonoblotting. The concentration of caspases from lane 1 to 9 are 0, 7.81, 15.63, 31.25, 62.5, 125, 250, 500 and 1000 nM respectively. STK3 is cleaved by the all the executioner caspases albeit with varying efficacies (caspase-3 > caspase-7 > caspase-6).

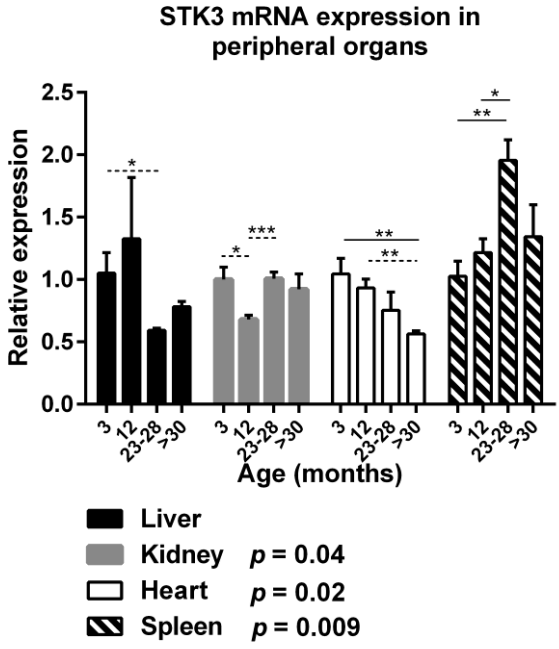

Figure 3. STK3 mRNA expression levels in peripheral organs. STK3 mRNA expression levels tends to decrease at 23-28 months of age in the liver (t test: 3 months *vs.* 23-28 months, p<0.05). In the kidney and the heart, STK3 mRNA decreases with aging (Kidney: ANOVA p=0.04, t test: 3 months vs. 12 months, p<0.05; 12 months *vs.* 23-28 months, p<0.001; Heart: ANOVA p=0.02 *post hoc*: 3 months *vs.* 30 months, p<0.01 ; t test: 12 months *vs.* >30 months, p<0.01). In contrast to the other peripheral organs, an increase of STK3 mRNA expression level is observed with the aging in the spleen(ANOVA p=0.009, *post hoc*: 3 months *vs.* 23-28 months, p<0.01; 12 months *vs.* 23-28 months, p<0.05). n=5 by age-group. Tukey's *post hoc* are presented on the graphs with a continuous line and t test are presented on the graphs with dotted line.

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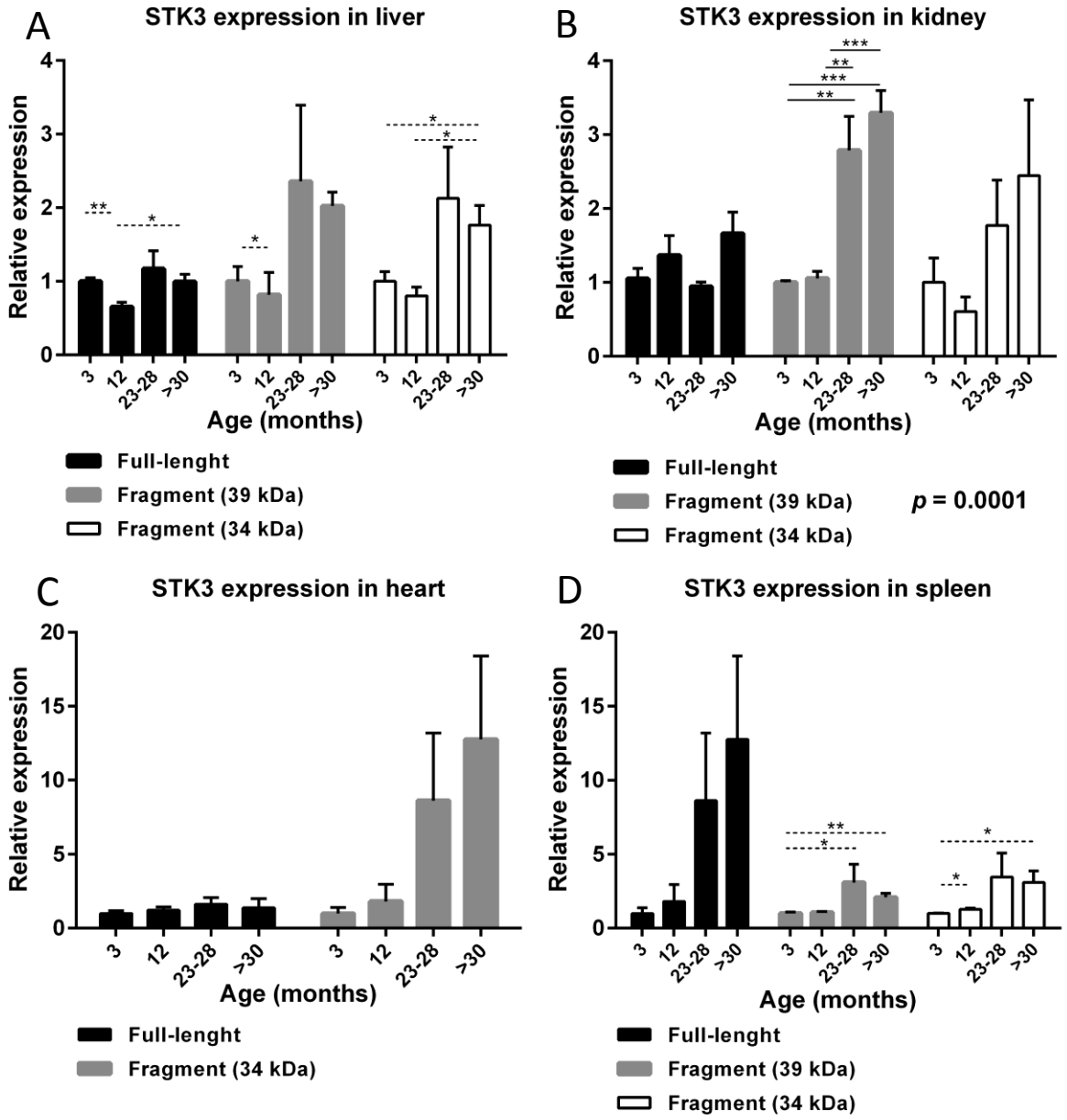

Figure 4. Global increase of STK3 protein expression levels in peripheral tissues. A) The FL form of STK3, and the 34 kDa fragment increase in the liver with age (FL: t test: 3 months vs. 12 months, p<0.01; 12 months *vs.* >30 months, p<0.05; 39 kDa: t test: 3 months *vs.* 12 months, p<0.05; 34 kDa: t test: 3 months *vs.* >30 months, p<0.05; 12 months *vs.* >30 months, p<0.05). **B)** No change in FL STK3 is observed in the kidney. In contrast, the STK3 39 kDa fragment increases in the kidney from 23-28 months of age (ANOVA p=0.0001, *post hoc*: 3 months vs. 23-28 months, p<0.01; 3 months *vs.* >30 months, p<0.001; 12 months *vs.* 23-28 months, p<0.01; 23-28 months *vs.* >30 months, p<0.001). **C)** No significant variation in STK3 protein expression level is observed in the heart. However, an increasing linear trend is observed in the 34 kDa fragment (p=0.0255). **D)** No change in FL STK3 is observed in the spleen with age. However, STK3 39 kDa and 34 kDa fragment expression level increase with aging in the spleen (39 kDa: t test: 3 months *vs.* 23-28 months, p<0.05; 3 months *vs.* >30 months, p<0.01; 34 kDa: t test: 3 months *vs.* 12 months, p<0.05; 3 months *vs.* >30 months, p<0.05). n=4 by age-group. Tukey's *post hoc* are presented on the graphs with a continuous line and t test are presented on the graphs with dotted line.

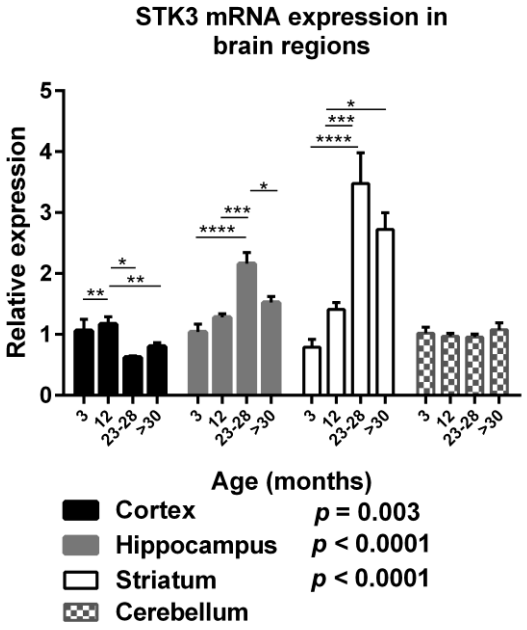

Figure 5. Global increase of STK3 mRNA expression levels in the brain STK3 mRNA expression increases initially then decreases with age in the cortex and the hippocampus (Cortex: ANOVA p=0.003, *post hoc*: 3 months *vs.* 12 months, p<0.01; 12 months *vs.* 23-28 months, p<0.05; 12 months *vs.* >30 months, p<0.01; Hippocampus: ANOVA p<0.0001, *post hoc*: 3 months *vs.* 23-28 months, p<0.0001; 12 months *vs.* 23-28 months, p<0.001; 23-28 months *vs.* >30 months, p<0.05). An increase in striatal STK3 mRNA expression level is observed with age. In contrast, no significant variation are observed in the cerebellum (Striatum: ANOVA p<0.0001, *post hoc*: 3 months vs. 23-28 months, p<0.0001; 12 months *vs.* 23-28 months, p<0.001; 12 months *vs.* >30 months, p<0.05). n=5 by age-group.

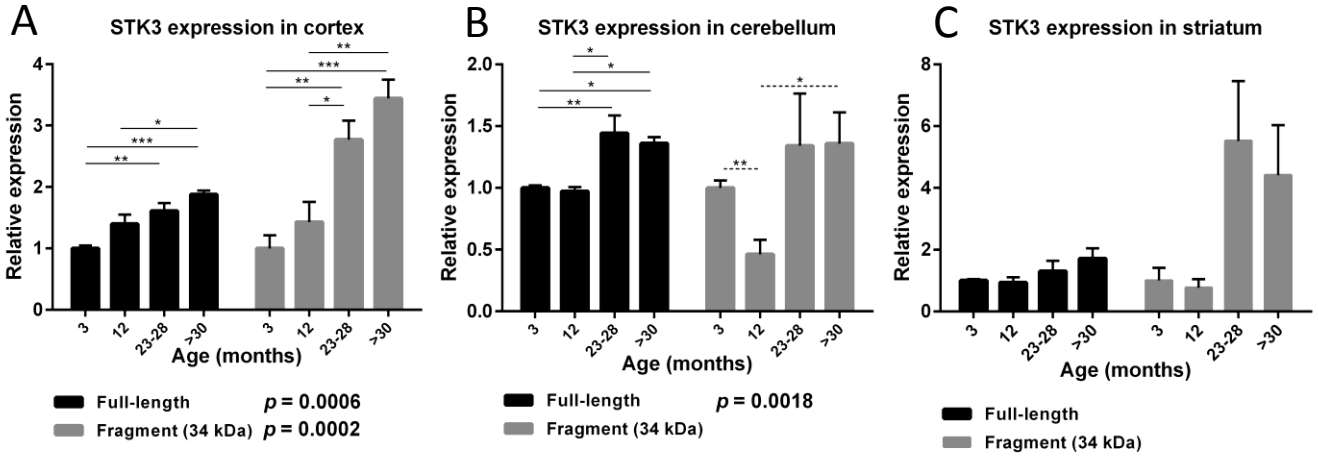

Figure 6. Global increase of STK3 protein expression levels in the brain A,B) STK3 protein expression level increases in the cortex and the cerebellum with age (**A** (FL): ANOVA p=0.0006, *post hoc*: 3 months vs. 23-28 months, p<0.01; 3 months *vs.* >30 months, p<0.001; 12 months *vs.* >30 months, p<0.01; **A** (34 kDa): ANOVA p=0.0002, *post hoc*: 12 months *vs.* 23-28 months, p<0.05; 3 months *vs.* 23-28 months, p<0.01; 3 months *vs.* >30 months, p<0.001; 12 months *vs.* >30 months, p<0.01; **B** (FL): ANOVA p=0.0018, *post hoc*: 3 months *vs.* 23-28 months, p<0.01; 3 months *vs.* >30 months, p<0.05; 12 months *vs.* >30 months, p<0.05; 12 months *vs.* >23-28 months, p<0.05; **B** (34 kDa): t test: 3 months *vs.* 12 months, p<0.01; 12 months *vs.* >30 months, p<0.05). **C)** No significant variation is observed in the FL form of STK3 in the striatum. However, the 34 kDa fragment tends to increase with age (ANOVA p=0.0509, r^2=0.316 p=0.02). n=4 by age-group. Tukey *post hoc* are presented on the graphs. FL= full-length.

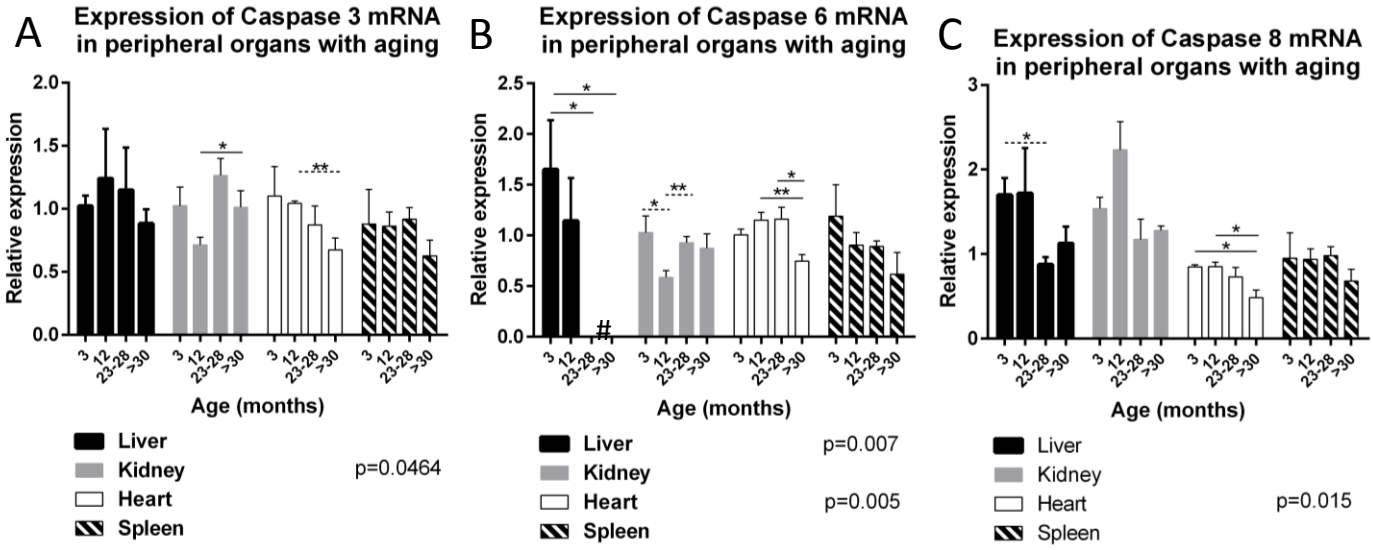

Figure 7. Overall caspase mRNA expression levels decrease in peripheral tissues with aging. A) Caspase 3 mRNA expression increases in the kidney and tends to decrease with aging in the heart (Kidney: ANOVA p=0.04, *post hoc*: 12 months *vs.* >30 months, p<0.05; Heart: t test: 12 months *vs.* >30 months, p<0.01). No significant variation is observed in the liver and the spleen. **B)** Caspase-6 mRNA expression level decreases in the liver and the heart with age (Liver: ANOVA p=0.007, *post hoc*: 3 months *vs.* 23-28 months, p<0.05; 3 months *vs.* >30 months, p<0.05; Heart: ANOVA p=0.005, *post hoc*: 12 months *vs.* >30 months, p<0.01; 23-28 months *vs.* >30 months, p<0.05). In contrast, CASP6 mRNA tends to decrease at 12 months of age then increase at 23-28 months of age in the kidney (t test: 3 months *vs.* 23-28 months, p<0.05; 12 months *vs.* >30 months, p<0.01). **C)** Caspase 8 mRNA expression decreases in the heart and a trend decrease is observed in the liver with age (Liver: 12 months *vs.* >30 months, p<0.05; Heart: ANOVA p=0.015, *post hoc*: 3 months *vs.* >30 months, p<0.05; 12 months *vs.* >30 months, p<0.05). No significant variation in CASP8 mRNA is observed in the kidney and the spleen. n=5 by age-group. Tukey's *post hoc* are presented on the graphs with a continuous line and t test are presented on the graphs with dotted line. *# No Ct is detected for the 23-28 and >30 months group for CASP6.*

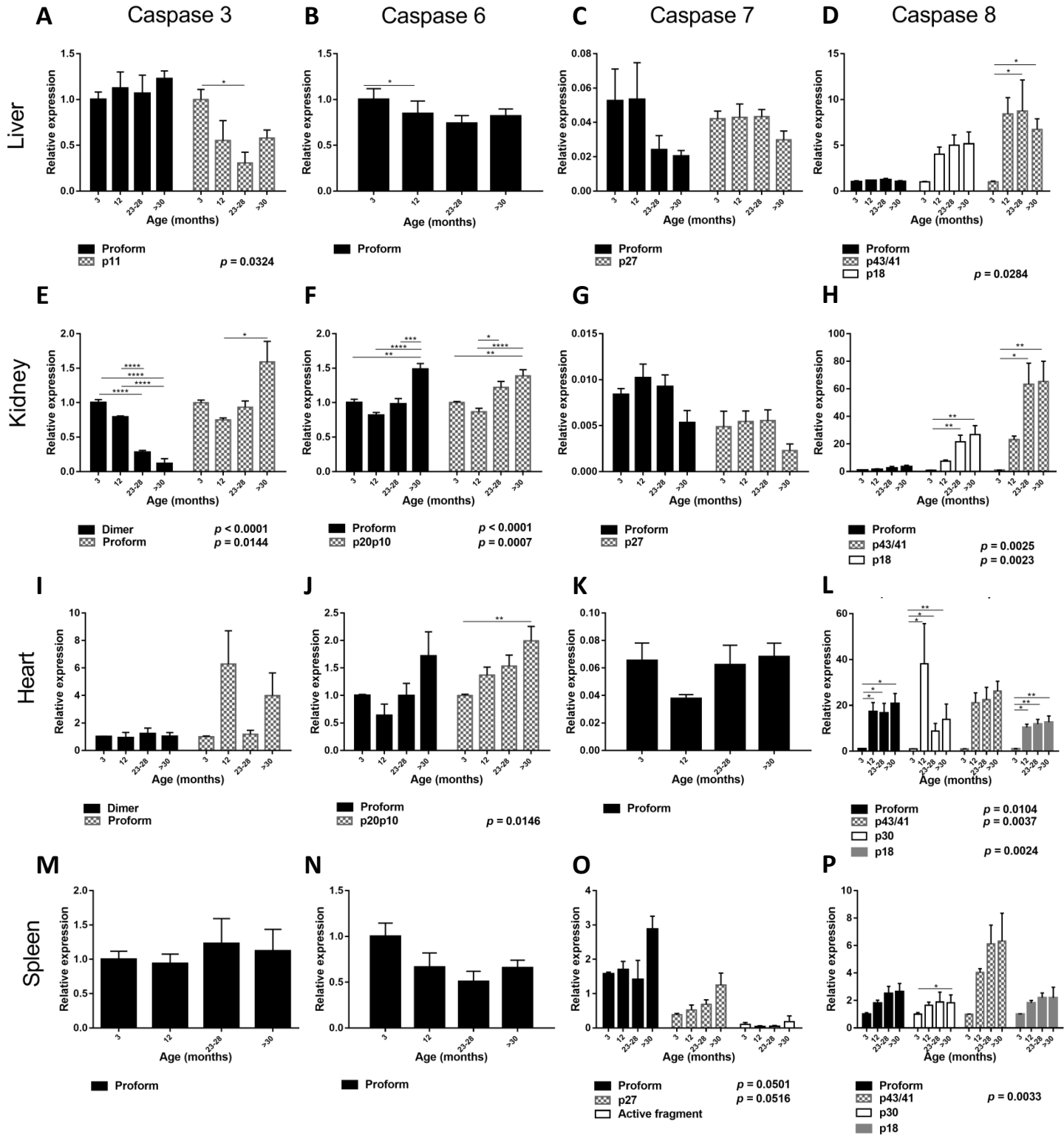
A
Caspase 3
B
Caspase 6
C
Caspase 7
D
Caspase 8
Liver
Relative expression
Age (months)
3
12
23-28
>30
Proform
p11
p = 0.0324
Proform
Proform
p27
Proform
p43/41
p18
p = 0.0284
E
F
G
H
Kidney
Dimer
Proform
p < 0.0001
p = 0.0144
Proform
p20p10
p < 0.0001
p = 0.0007
Proform
p27
Proform
p43/41
p18
p = 0.0025
p = 0.0023
I
J
K
L
Heart
Dimer
Proform
Proform
p20p10
p = 0.0146
Proform
Proform
p43/41
p30
p18
p = 0.0104
p = 0.0037
p = 0.0024
M
N
O
P
Spleen
Proform
Proform
Proform
p27
Active fragment
p = 0.0501
p = 0.0516
Proform
p43/41
p30
p18
p = 0.0033

Figure 8. Caspase protein expression levels vary with aging in peripheral organs. A, B, C, D) The p11 caspase-3 fragment and caspase-6 proform decrease, all forms of caspase-8 increase and no signficant variation is observed with caspase 7 in the liver with aging (A (fragment): ANOVA $p=0.03$; B: t test: 12 months *vs.* 23-28 months, $p<0.05$; D (proform): t test: 3 months *vs.* 23-28 months $p<0.05$; D (p43/41): t test: 3 months *vs.* 12 months $p<0.001$; 3 months *vs.* >30 months, $p<0.01$; D (p18): ANOVA $p=0.03$) **E)** In the kidney, the caspase-3 proform increases with age (ANOVA $p=0.014$) **F, G, H, J, K, L)** A global increase in caspase-6 and caspase-8 protein levels and a trend decrease in caspase-7 protein levels are observed in the kidney and the heart with aging (F (Proform): ANOVA $p<0.0001$; F (p20p10): ANOVA $p=0.0007$; G (Proform): t test: 12 months vs >30 months, $p=0.0502$; 23-28 months vs >30 months, $p=0.07$; G (p27): t test: 12 months vs >30 months, $p=0.0582$; 23-28 months vs >30 months, $p=0.053$; H (proform): t test: 3 months vs. >30 months, $p<0.05$; H (p43/41): ANOVA $p=0.0025$; H (p18): ANOVA $p=0.002$; J (p20p10: ANOVA $p=0.0146$; K (Proform): 3 months *vs.* 12 months, $p<0.05$; L (proform): ANOVA $p=0.0104$; L (p43/41): ANOVA $p=0.0037$; L (p18): $p=0.002$). **I, M)** No significant variation is observed in caspase-3 protein expression levels with aging in the heart and the spleen. **N, O)** Caspase-6 protein expression level decrease in the spleen at 12 months of age (Proform: ANOVA, $p=0.0501$; t test: 3 months *vs.* 12 months, $p<0.05$; t test: 3 months *vs.* >30 months,$p<0.05$; 12 months *vs.* >30 months, $p<0.05$; O (p27) and caspase-7 protein levels increase at >30 months of age (N (Proform): ANOVA, $p=0.0516$; t test: 3 months vs. >30 months, $p<0.05$; 23-28 months *vs.* >30 months, $p<0.05$) **P)** Similar to the other peripheral organs, caspase-8 protein expression level increases with age in the spleen (P (proform): t test: 3 months *vs.* 12 months $p<0.05$; 3 months *vs.* 23-28 months $p<0.05$; 3 months *vs.* >30 months $p<0.05$; P (p43/41): ANOVA $p=0.003$; P (p30): t test: 3 months *vs.* 12 months $p<0.05$; P (p18): t test: 3 months *vs.* 12 months $p<0.01$). n=4 by age-group .Tukey's *post hoc* are presented on the graphs.

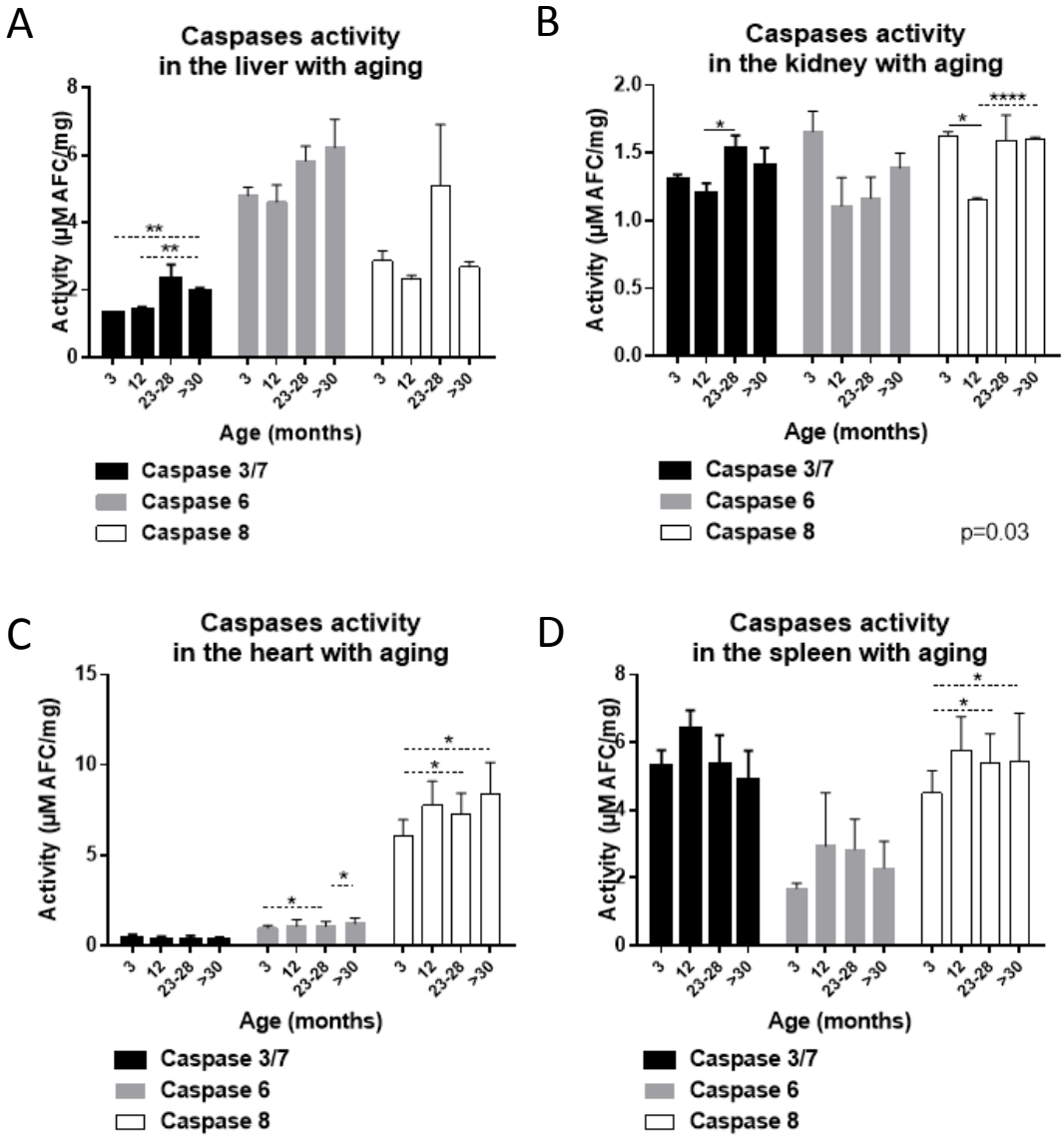

Figure 9. Caspase 6 activity increases in the liver and kidney and Caspase 8 activity increase with aging in the kidney, heart and spleen. A) A trend increase is observed in CASP3/7 activity in the liver (t test: 3 months *vs.* >30 months, p<0.01; 12 months *vs.* >30 months, p<0.01.). No significant variation is observed in CASP6 and 8 activity **B)** In the kidney, CASP3/7 activity increase at 23-28 months of age (t test: 12 months *vs.* 23-28 months , p<0.05). Caspase 8 activity decrease at 12 months and increases at >30 months (ANOVA p=0.03, *post hoc*: 3 months *vs.* 12 months, p<0.05; t test: 12 months *vs.* >30 months, p<0.0001). **C)** An increase is observed in the heart in CASP6 and 8 activity with aging (CASP6: t test: 23-28 months *vs.* >30 months, p<0.05; CASP8: t test: 3 months *vs.* 23-28 months, p<0.05; 3 months vs. >30 months, p<0.05). No significant variation is observed in CASP3/7 with aging in the heart. **D)** Caspase 8 activity increases with aging in the spleen (t test: 3 months vs. 23-28 months, p<0.05; 3 months *vs.* >30 months, p<0.05). No significant variation is observed in the spleen for CASP3/7 and 6 activity with aging. n=4 by age-group. Tukey's *post hoc* are presented on the graphs with lines and t test are presented using dotted lines.

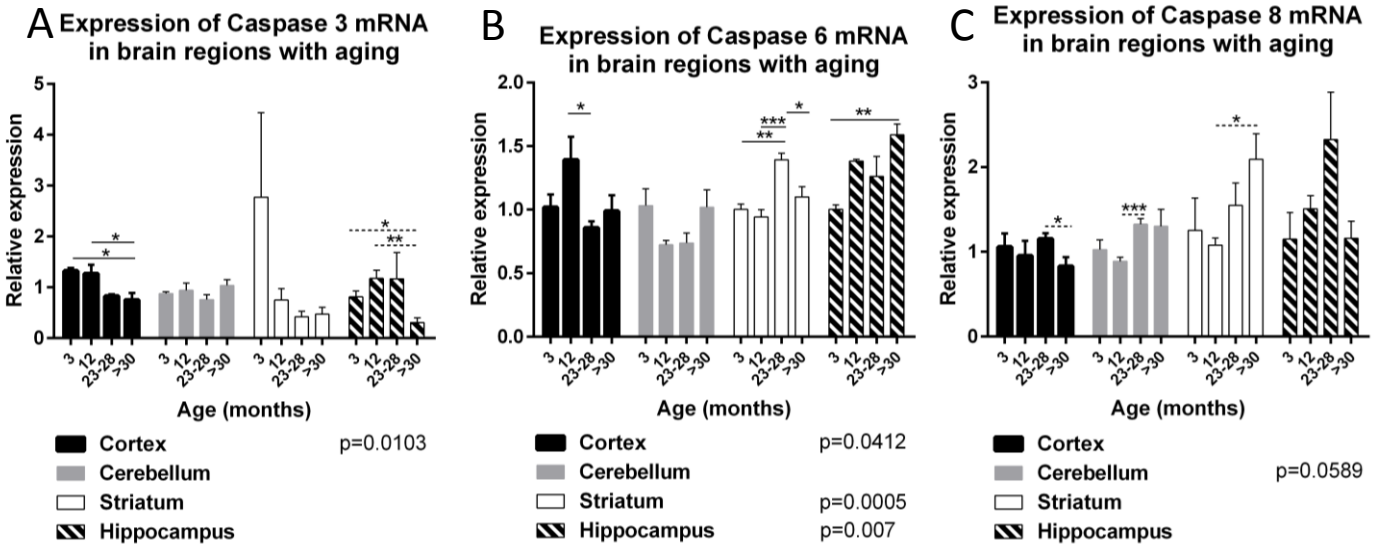

Figure 10. Caspase-6 mRNA expression increases in the striatum and hippocampus with age. A) Caspase 3 mRNA expression decrease in the cortex and the hippocampus with age (Cortex: ANOVA p=0.0103, *post hoc*: 3 months *vs.* >30 months, p<0.05; 12 months *vs.* >30 months, p<0.05; Hippocampus: t test: 3 months *vs.* >30 months, p<0.05; 12 months *vs.* >30 months, p<0.01). No significant variation is observed in CASP3 mRNA expression in the cerebellum and the striatum. **B)** In the cortex, caspase-6 mRNA expression decreases at 23-28 months of age (ANOVA p=0.04, *post hoc*: 12 months *vs.* 23-28 months, p<0.05). In contrast, caspase-6 mRNA expression increases in the striatum and the hippocampus (Striatum: ANOVA p=0.0005, *post hoc*: 3 months vs. 23-28 months, p<0.01; 12 months vs. 23-28 months, p<0.001; 23-28 months *vs.* >30 months, p<0.05; Hippocampus: ANOVA p=0.007, *post hoc*: 3 months *vs.* >30 months, p<0.01) No significant variation is observed in the cerebellum. **C)** Caspase 8 mRNA expression tends to decrease at >30 months of age in the cortex. In contrast, an increase in CASP8 mRNA expression is observed in the cerebellum and the striatum (Cerebellum: ANOVA p=0.0589, t test: 12 months *vs.*. >30 months, p<0.001; Striatum: t test: 12 months *vs.* >30 months, p<0.05). n=5 by age-group . Tukey's *post hoc* are presented on the graphs with a continuous line and t test are presented on the graphs with dotted line.

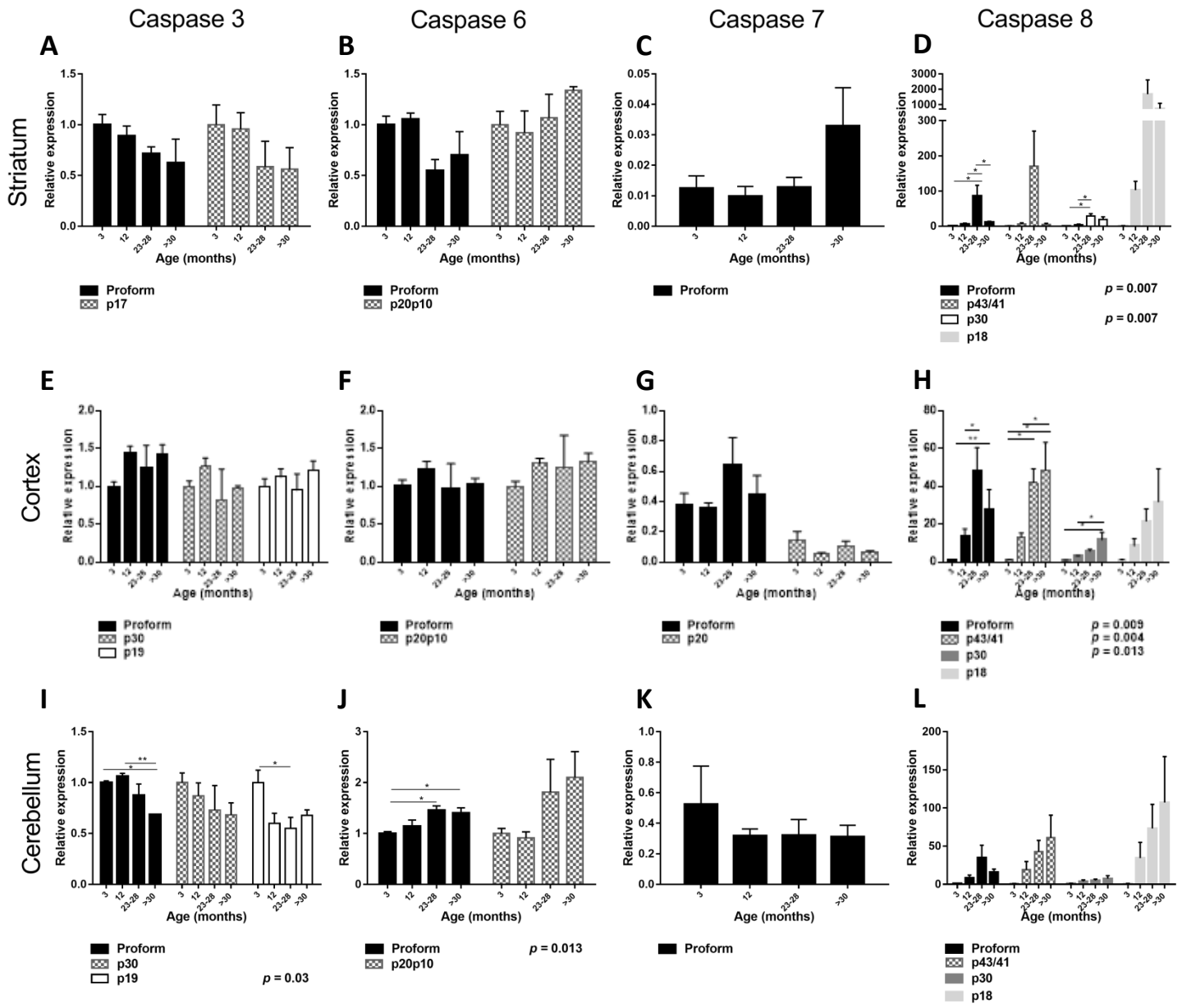

Figure 11. Global increase in caspases protein expression level in the brain with the aging A, B, C, D) Caspase-3 do not vary with age in the striatum whereas caspase-6 tends to decrease and caspase-7 and -8 increase with aging (B (proform) : t test: 3 months *vs.* 23-28 months; C (Proform): r^2=0.29, p=0.04; D (proform): ANOVA p=0,007; D(p30) : ANOVA p=0.007; D (p18) : t test: 3 months *vs.* 12 months, p<0.01). **E ,F ,H)** Caspase-3, 6 and 8 expression increases or tends to increase with age in the cortex (E (proform): t test: 3 months vs. 12 months, p<0.5; 3 months *vs.* >30 months, p<0.5; F (p20p10): t test: 3 months *vs.* 12 months, p<0.5; 3 months *vs.* >30 months, p<0.5; H (proform): ANOVA p=0.009; H (p43/41): ANOVA p=0.004; H (p30) : ANOVA p=0,013; H (p18) : t test 3 months *vs.* 23-28 months p<0,05). **G, K)** No significant variation is observed in caspase-7 protein expression level in the cortex and cerebellum. **I, J, L)** Caspase-6 and 8 expression increases or tends to increase with age in the cerebellum whereas caspase-3 decreases with aging (I (proform): t test: 3 months *vs.* >30 months, p<0.05; 12 months *vs.* >30 months, p<0.05; I (30 kDa): ANOVA p=0.03; J (proform): ANOVA p=0.013; L (proform): t test: 3 months *vs.* >30 months, p<0.05; L (p43/41): t test: 3 months *vs.* >30 months, p<0.05). n=4 by age-group.Tukey's *post hoc* are presented on the graphs.

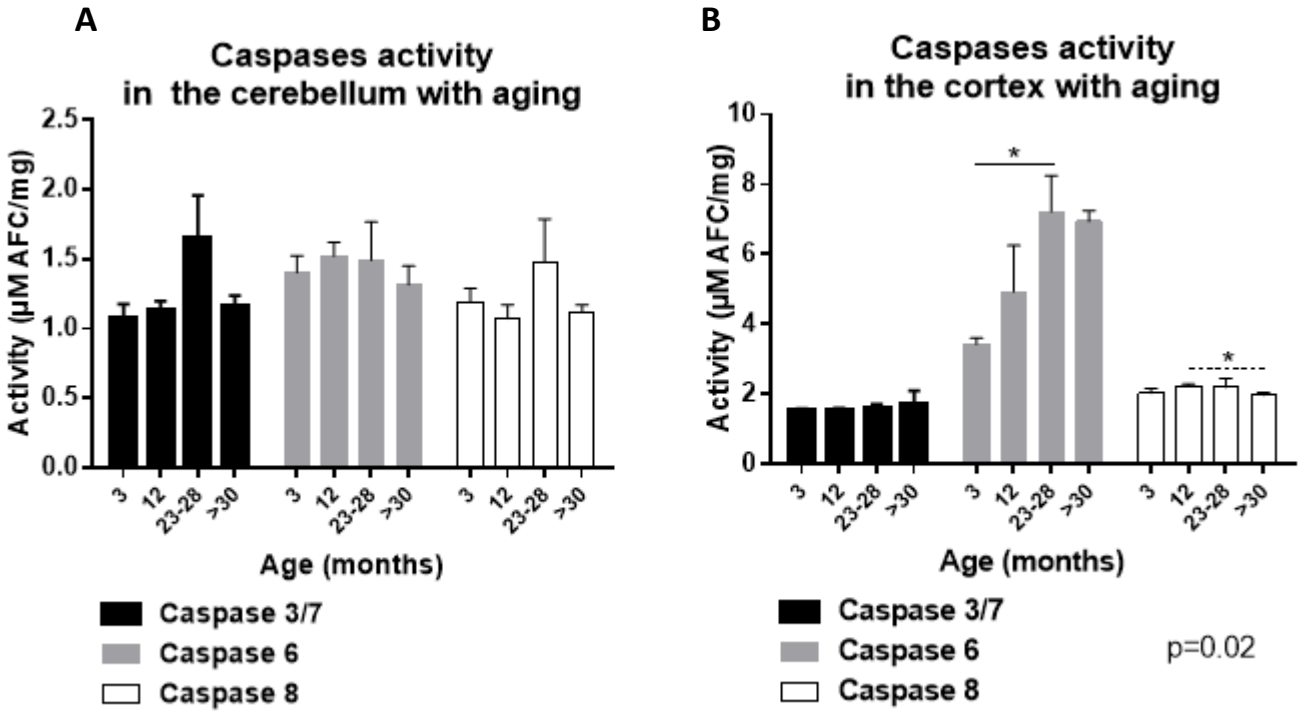

Figure 12. Caspase-6 activity increases in the cortex with aging. A) No significant variation in caspases activty is observed with aging in the cerebellum. **B)** Caspase-6 increase while CASP8 activity tends to decrease in the cortex with aging (Caspase 6: ANOVA p=0.02, *post hoc* : 3 months *vs.* 23-28 months, p<0.05 ; Caspase 8: t test: 12 months *vs.* >30 months) No significant variation is observed in CASP3 activity. n=4 by age-group. Tukey's *post hoc* are presented on the graphs with a continuous line and t test are presented on the graphs with dotted line.

	STK3				Caspase 3					Caspase 6				Caspase7				Caspase 8					
	RNA	FL	39 kDa	34 kDa	RNA	Pro	p30	p19/p17	*Activity	RNA	Pro	p20p10	Activity	Pro	p27	p20	*Activity	RNA	Pro	p43/41	p30	p18	Activity
Liver	↓	↓↑	↓	↑	-	-			↑	↓	↓		-	-	-		↑	↓	↑	↑		↑	-
Kidney	↓	-	↑	-	↑	↑		↓	↑	↓↑	↑	↑	-	↓	↓		↑	-	↑	↑		↑	↓↑
Heart	↓	-		-	↓	-			-	↓	-	↑	↑	↓			-	↓	↑	↑	↑	↑	↑
Spleen	↑	-	↑	↑	-	-			-	-	↓		-	-	↑	-	-	-	↑	↑	↑	↑	↑
Cortex	↑↓	↑		↑	↓	↑	-	-	-	↓	-	↑	↑	-		-	-	↓	↑	↑	↑	↑	↓
Cerebellum	↑	↑		↓↑	-	↓	↓	↓	-	-	↑	-	-	-			-	↑	↑	↑	-	-	-
Striatum	↑	-		-	-	-		-		↑↓	↓	-		↑				↑	↑↓	-	↑	↑	
Hippocampus	↑↓				↓					↑								-					

Table 1. Summary table of the age associated variation in STK3 and caspases expression and activity.
*Analysis of CASP3 and CASP7 activity by the same peptide. Pro = Proform, FL = Full-lenght, p20p10 = CASP6 without the prodomain.

Organ specific alteration in caspases expression and STK3 proteolysis during the aging process

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Supplementary figures

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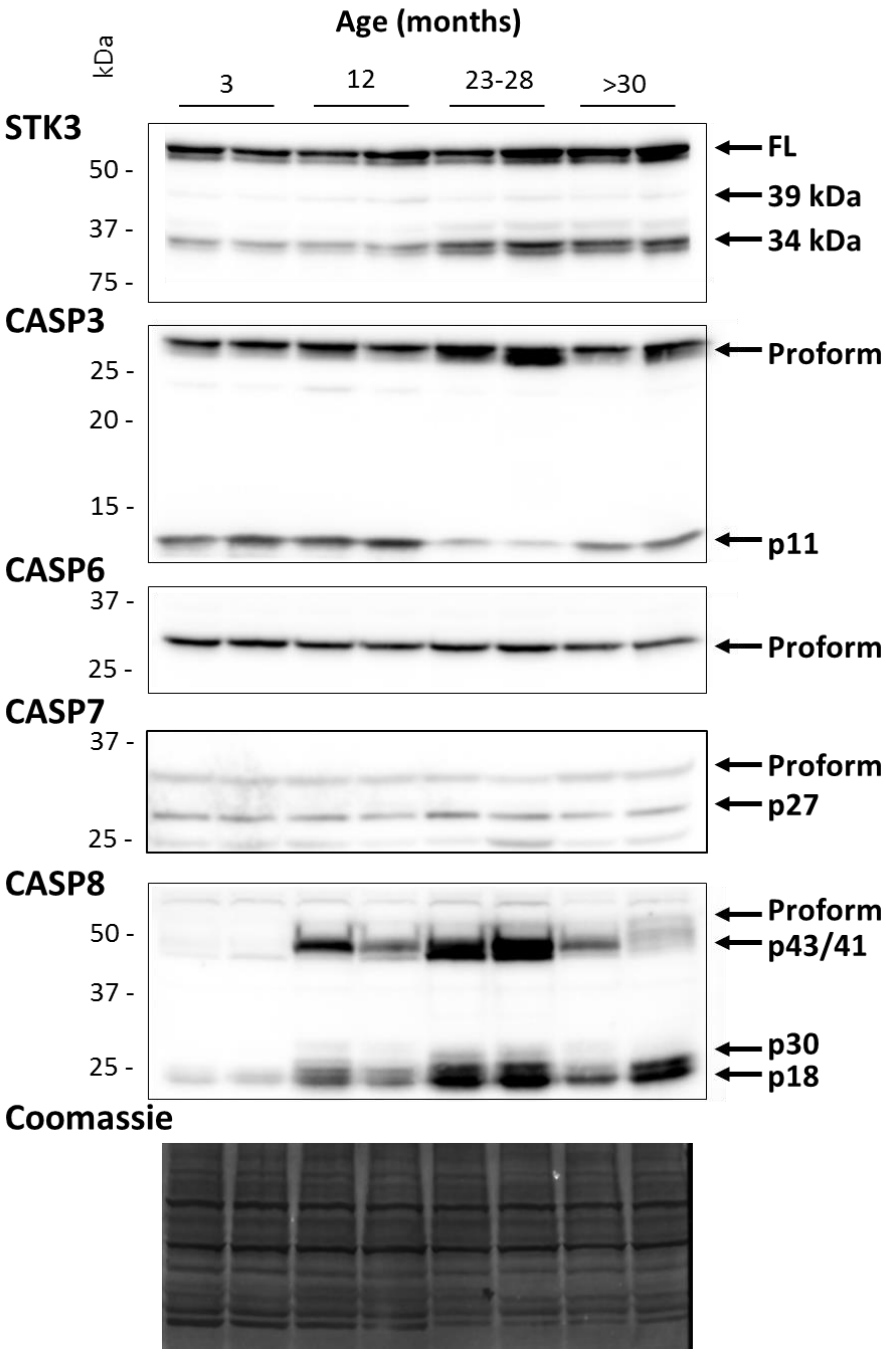
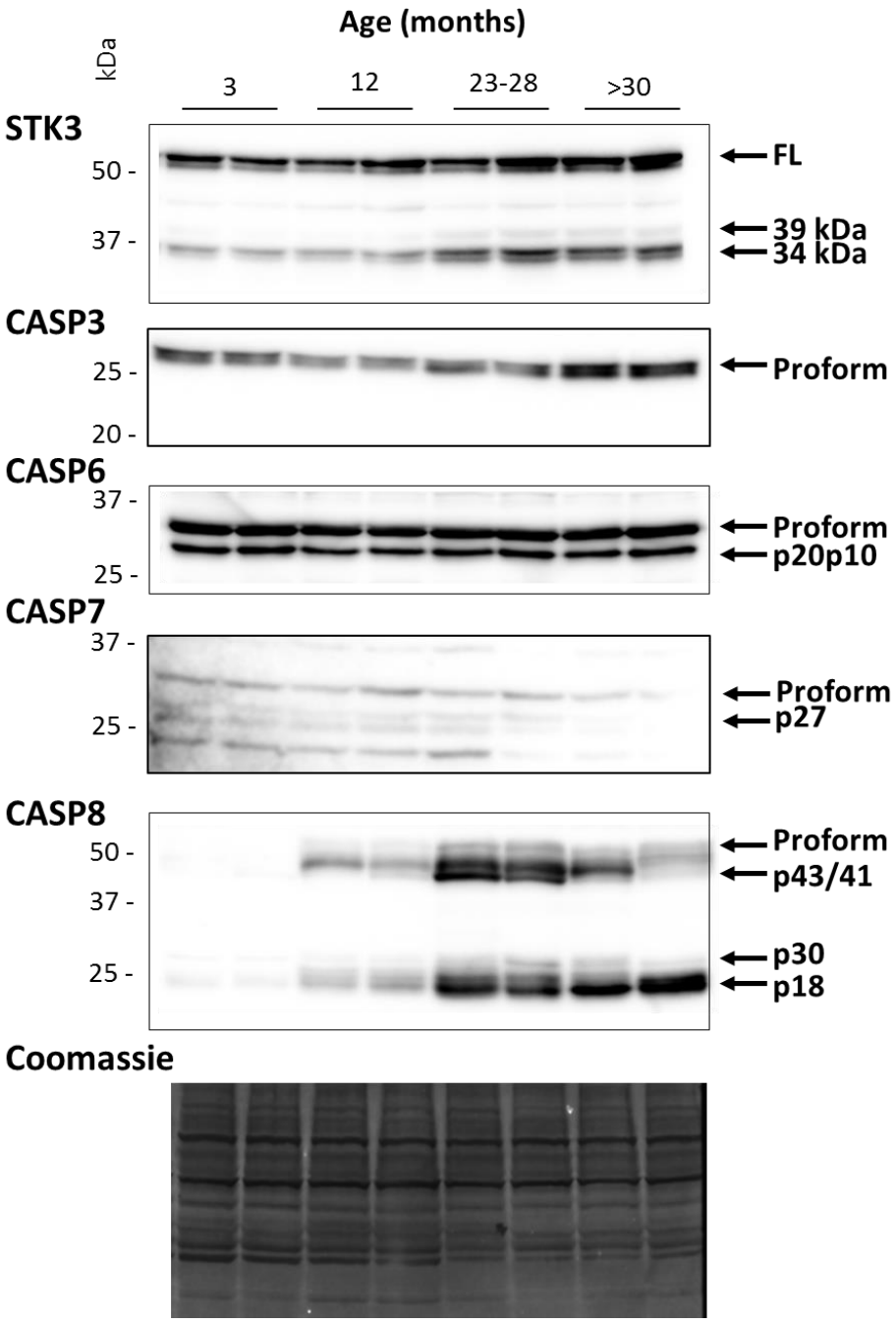
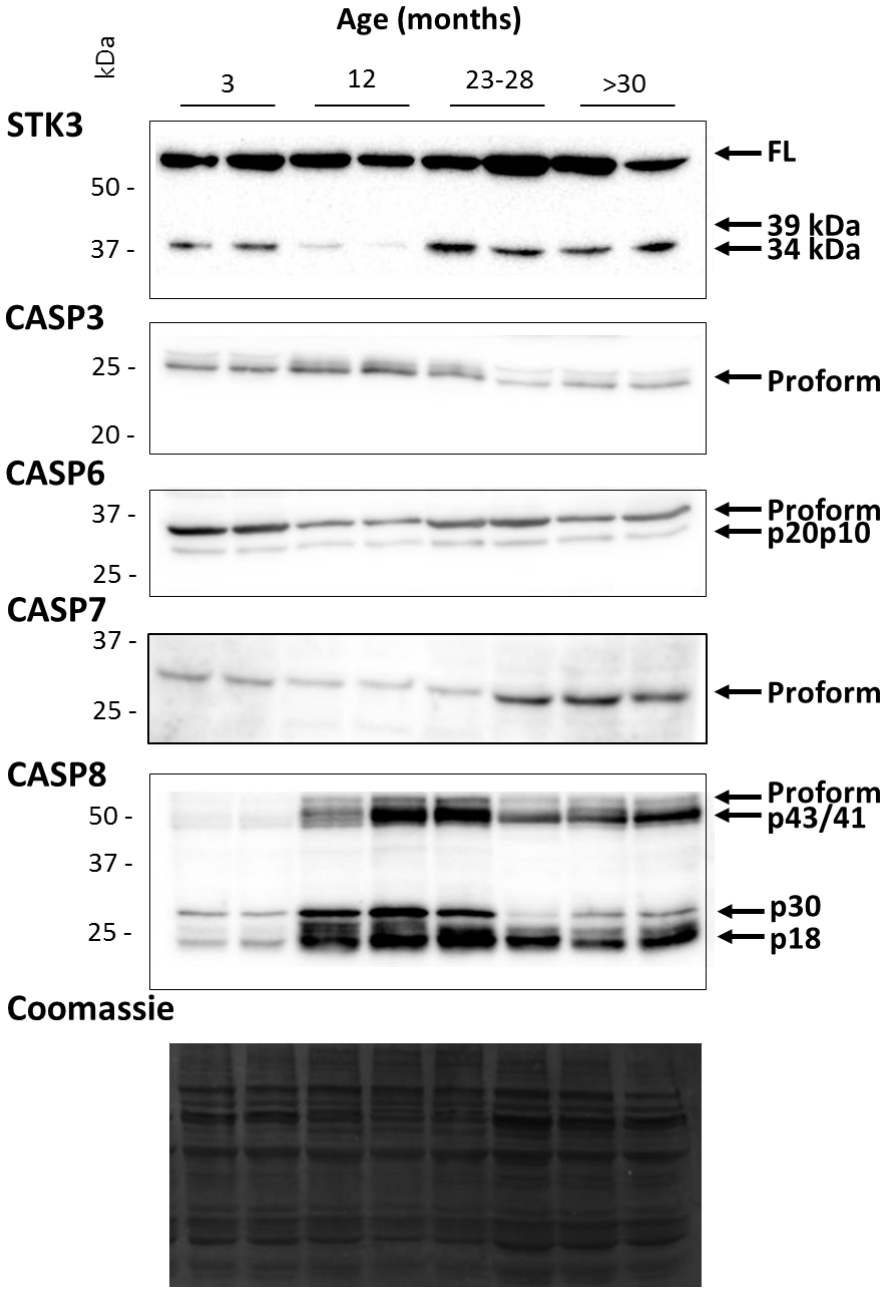
Key words: Aging, apoptosis, caspases, serine/threonine kinase 3, peripheral organs, brain region.



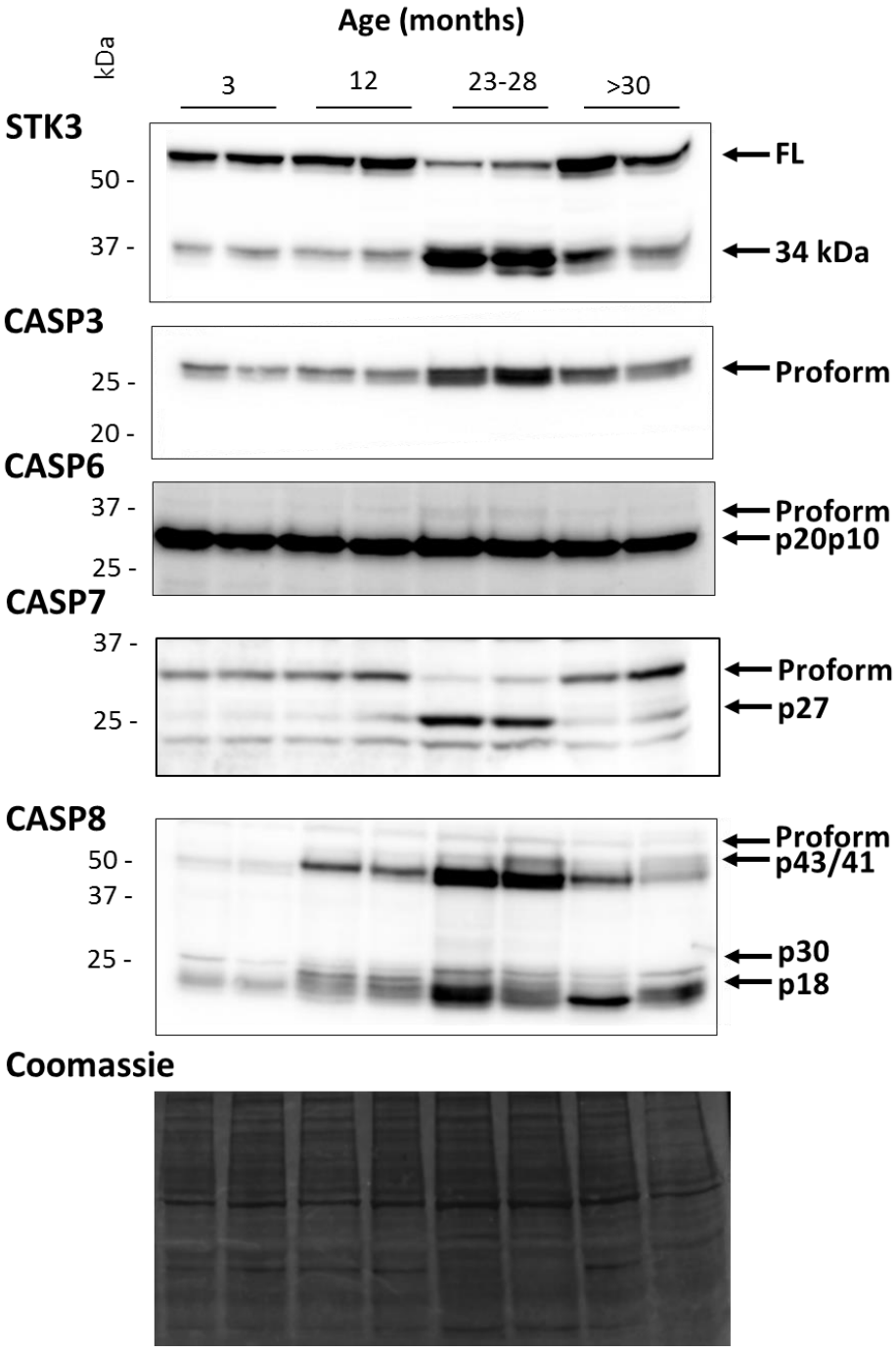
Supplementary figure 1. Western blot of STK3, CASP3, CASP6, CASP7 and CASP8 in the liver. A) An example of STK3, CASP3, CASP6, CASP7 and CASP8 immunoblot is presented of liver samples. The protein loading reference is the Coomassie blue staining for all the peripheral organs.



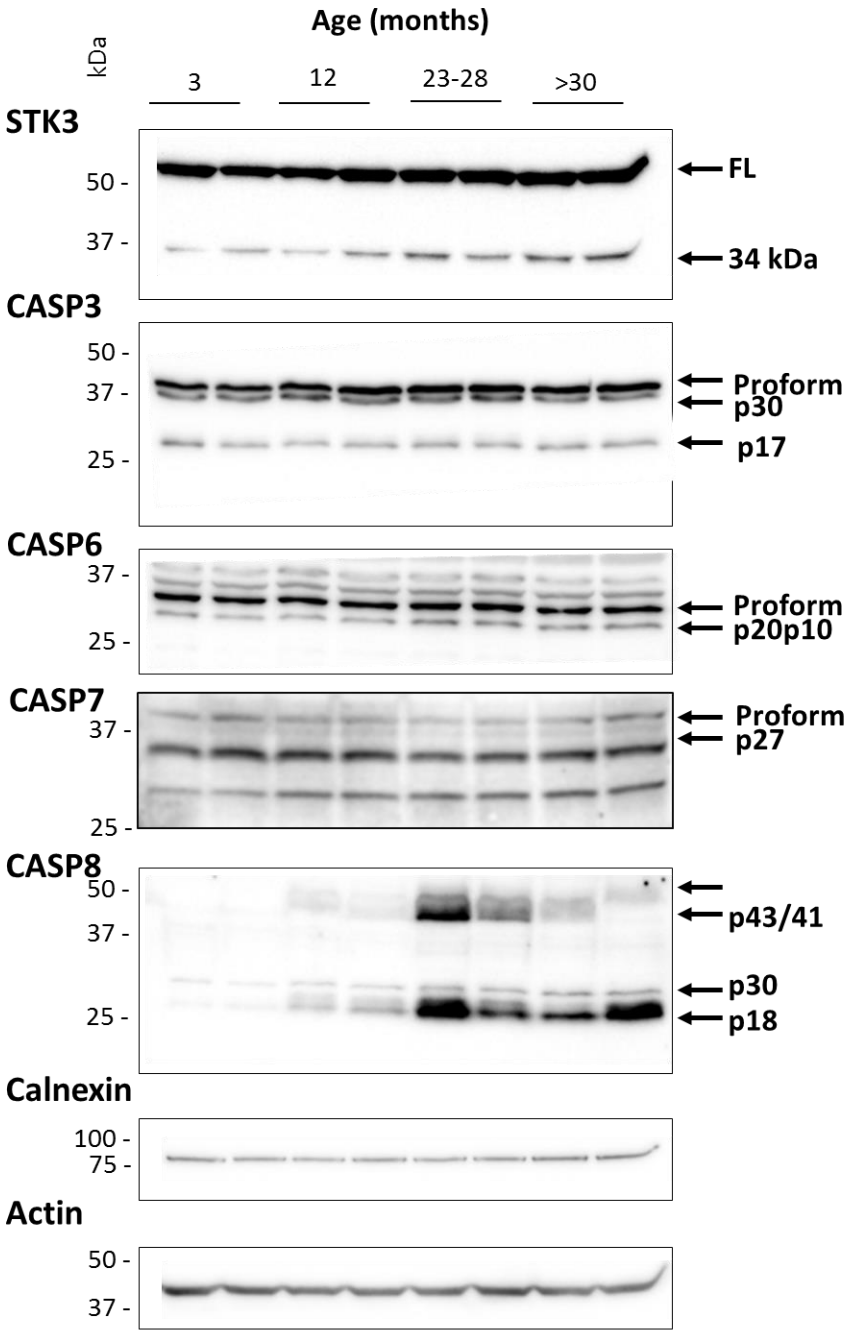
Supplementary figure 2. Western blot of STK3, CASP3, CASP6 , CASP7 and CASP8 in the kidney A) An example of STK3, CASP3, CASP6, CASP7 and CASP8 immunoblot is presented of kidney samples. The protein loading reference is the Coomassie blue staining for all the peripheral organs.



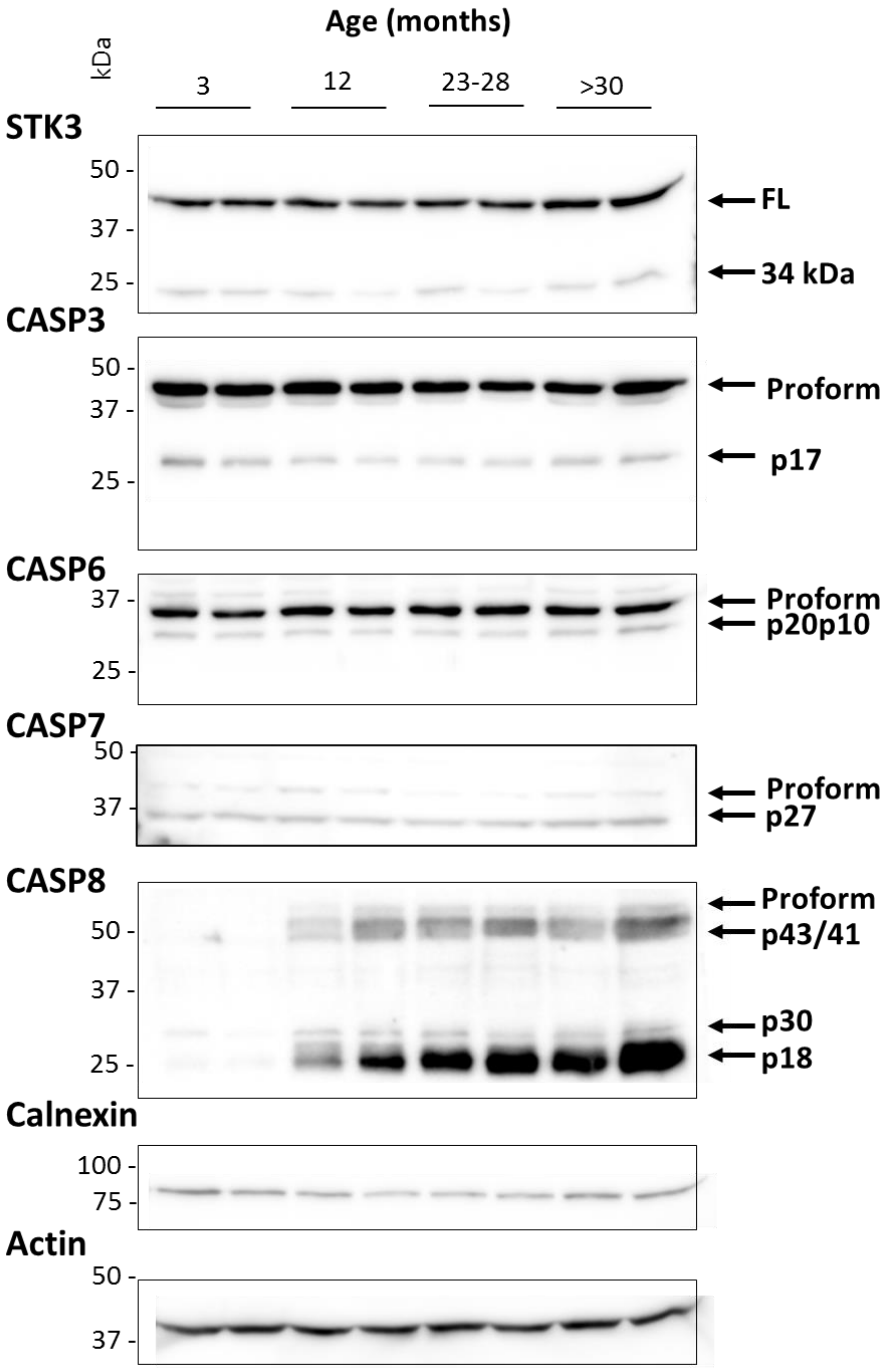
Supplementary figure 3. Western blot of STK3, CASP3, CASP6 , CASP7 and CASP8 in the heart A) An example of STK3, CASP3, CASP6, CASP7 and CASP8 immunoblot is presented of heart samples. The protein loading reference is the Coomassie blue staining for all the peripheral organs.



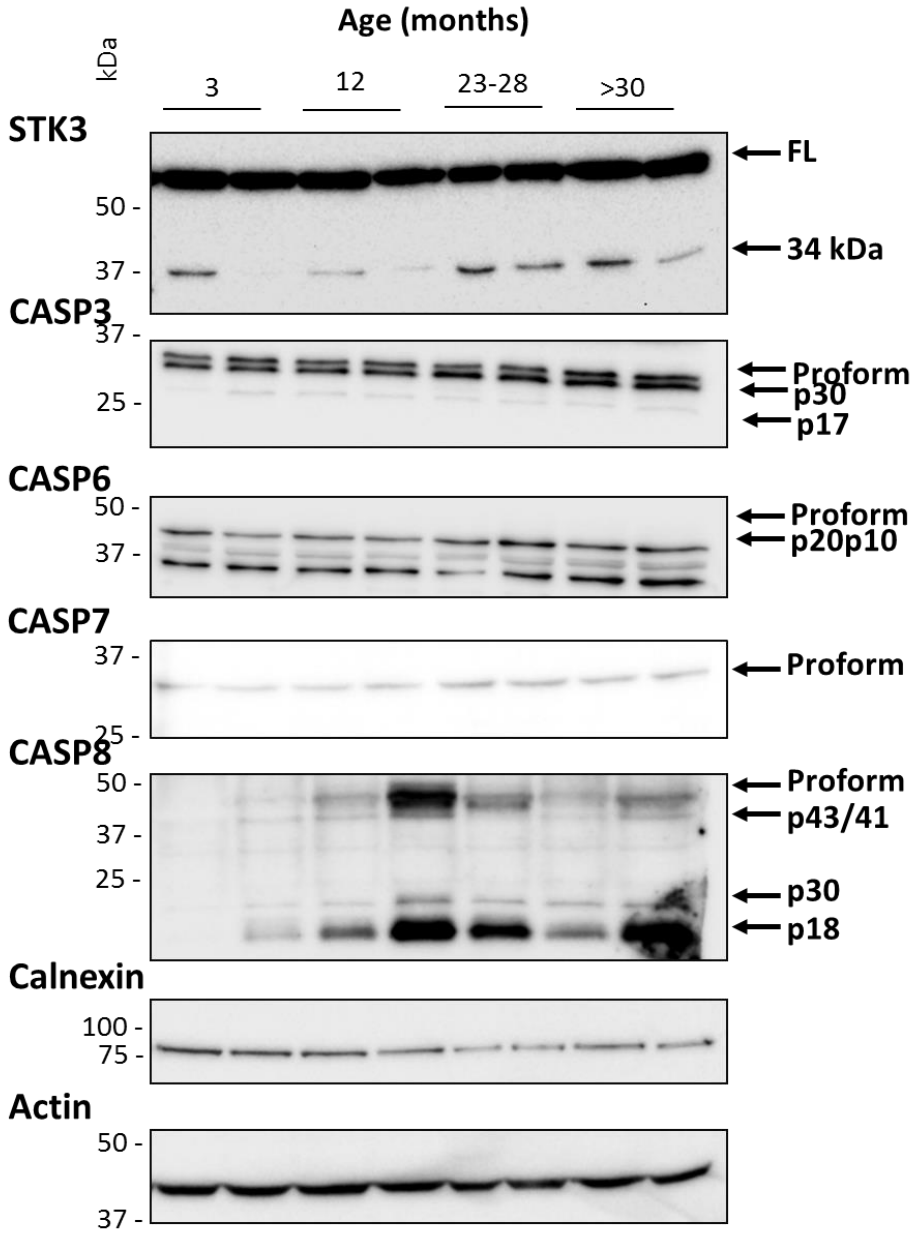
Supplementary figure 4. Western blot of STK3, CASP3, CASP6 , CASP7 and CASP8 in the spleen A) An example of STK3, CASP3, CASP6, CASP7 and CASP8 immunoblot is presented of spleen samples. The protein loading reference is the Coomassie blue staining for all the peripheral organs.



Supplementary figure 5 Western blot of STK3, CASP3, CASP6, CASP7 and CASP8 in the cortex. A) An example of STK3, CASP3, CASP6, CASP7 and CASP8 immunoblot is presented of cortex samples. The protein loading reference calnexin and actin were assessed in all brain regions.



Supplementary figure 6. Western blot of STK3, CASP3, CASP6 , CASP7 and CASP8 in the cerebellum. A) An example of STK3, CASP3, CASP6, CASP7 and CASP8 immunoblot is presented of cerebellum samples. The protein loading reference calnexin and actin were assessed in all brain regions.



Supplementary figure 7. Western blot of STK3, CASP3, CASP6 , CASP7 and CASP8 in the striatum. A) An example of STK3, CASP3, CASP6, CASP7 and CASP8 immunoblot is presented of striatum samples. The protein loading reference calnexin and actin were assessed in all brain regions.