Coordination in the unfolded protein response during aging in outbred deer mice

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Short title: UPR regulation during aging in deer mice

Summary

Endoplasmic reticulum (ER) stress has been linked to various metabolic pathologies, neurodegeneration and aging. Although various mechanistic aspects of the resulting unfolded protein response (UPR) have been elucidated, its regulation in genetically diverse populations remains elusive. In the present study we evaluated the expression of chaperones BiP/GRP78, GRP94 and calnexin (CANX) in the lungs, liver and brain of 7 months old and 2-3 years old outbred deer mice *P. maniculatus* and *P. leucopus*. Chaperones' expression was highly variable between species, tissues and ages suggesting that levels of expression of individual chaperones do not change consistently during aging. Despite this variation, a high degree of coordination was maintained between chaperones' expression indicating the tight regulation of the UPR which is consistent with its adaptive activity to maintain homeostasis. In the brain though of older *P. maniculatus*, at which neurodegenerative changes were detected, loss of coordination was revealed, especially between BiP and either of GRP94 or calnexin which indicates that de-coordination rather than aberrant expression is linked to deregulation of the UPR in aging. These findings underscore the involvement of UPR in the onset of aging-related pathologies and suggest that beyond levels of expression, concerted activation may be of significance to attain homeostasis. These findings emphasize the value of genetically diverse models and suggest that beyond levels of expression of individual targets the coordination of transcriptional networks should be considered when links to pathology are explored.

Introduction

It is well known that cells cope with changes in their environment or respond to specific stimuli by altering extensively their transcriptional profile and adapt accordingly to attain homeostasis (1,2). This transcriptional reprogramming can be highly variable for different transcripts and among individuals, yet a high degree of coordination is maintained for genes that belong to the same transcriptional networks (3-5). This is particularly true for stress response pathways at which transcription must be altered in a highly concerted manner. The unfolded protein response (UPR) is a component of the integrated stress response that is triggered at conditions of endoplasmic reticulum (ER) stress and aims to attain cellular and tissue homeostasis (6,7). Various pathologies have been linked to the deregulation of ER stress-associated signaling and include metabolic diseases, neurodegeneration and aging (8,9). The overexpression or downregulation of various UPR-associated genes has been demonstrated for various pathologies and their degree of deregulation is considered informative for the impact of the corresponding genes in disease development. During aging the progressive deregulation of proteostasis has been demonstrated, and a loss of chaperones in the brains of older animals has been described, which is indicative for their reduced ability to resolve stress (10-12).

Despite the detailed mechanistic insights we have obtained for the regulation of the UPR, how this is coordinated in genetically diverse individuals and how it changes during aging remains elusive. By analyzing primary fibroblasts from outbred deer mice (*Peromyscus maniculatus*) we recently showed that the UPR is highly variable among individuals but despite its variation a high degree of coordination in the levels of

expression between different UPR-associated genes was maintained (13). Furthermore, this profile of the UPR *in vitro* predicted the onset of diet-induced metabolic disease later in life (13). Noteworthy, depending on the intensity of the UPR, animals could be classified as high and low UPR responders which were differentially distributed between high or low altitude-derived populations of deer mice, indicating evolutionary relevance (13). The power of this coordination analysis in gene expression in deer mice was further demonstrated by RNA sequencing studies in cultured fibroblasts and showed that the hierarchical clustering of genes according to the strength of coordination with specific targets - as opposed to their clustering according to the magnitude of overexpression or downregulation – accurately predicted the particular functions of specific UPR branches (14). In example, the apoptotic response was readily linked to ATF4 and the ER stress associated degradation to GRP94 (14).

In the present study we sought to explore how the UPR is regulated in different tissues from outbred *Peromyscus*. For our studies we used 2 closely related species of deer mice that reportedly differ in their lifespans: *P. leucopus* that lives for up to 8 years and *P. maniculatus* that exhibits a lifespan of about 4 years (15,16). These animals reach reproductive maturity when they are about 2 months old and at 2.5 years they are still active but show reduced reproductive activity. In the present study we focused on how UPR is regulated and our analysis extended at 2 different levels: First, we evaluated collectively the overall levels of UPR chaperones as a group, in different tissues, species, and ages. Second, we calculated the degree of coordination in the levels of expression between the different chaperones within the same individuals. For our studies we focused on BiP/GRP78, GRP94 and calnexin that according to our earlier studies exhibit high

coordination with each other under physiological condition and during ER-stress associated challenge (13). BiP is considered the master regulator of the UPR that upon dissociation of the intracellular receptors PERK, IRE1 and ATF6 elicits the UPR (9). GRP94 is a glycoprotein that participates in protein folding, assists in the targeting of misfolded proteins for degradation and also interacts with other components of the ER protein folding machinery (17). Calnexin is an ER lectin chaperones that facilitates quality control in the ER of glycoproteins (18,19). Our results suggest that chaperone levels are highly variable at different ages among tissues, individuals and species, yet a high degree of coordination in expression is maintained that is being progressively abolished during aging when the latter was associated with degenerative changes in histology.

Material and Methods

Animals: Deer mice, *P. maniculatus bairdii* (BW stock) was closed colony bred in captivity since 1948. Descended from 40 ancestors wild-caught near Ann Arbor, Michigan and the *P. leucopus* (LL stock) was derived from 38 wild ancestors captured between 1982 and 1985 near Linville. We obtained animals from the Genetic Stock Center (PGSC), University of South Carolina (USC), Columbia, SC (RRID:SCR_002769). We picked twenty outbred females of *P. maniculatus* and twenty-two outbred females of P*. leucopus*. The animals were divided into two groups of young (7 months old for both species) and old (25–29 months old for *P. maniculatus* and 19-33 months old for *P. leucopus*) animals in each species at the time we sacrificed them. The exact age at sacrifice, and the ID number of the animals are shown in Table 1. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and the Department of Health and

Human Services, Office of Laboratory Animal Welfare, University of South Carolina (Approval No. 2349-101211-041917).

RNA extraction, cDNA synthesis and qPCR: RNA was extracted from three different tissues (brain, liver and lung) using the RNeasy Mini Kit (Qiagen). RNA was extracted based on the kit's supplied protocol with some modifications. The extracted RNA was eluted using 30 µl of nuclease-free water. Due to high fatty nature of the brain tissues and in lack of any specific fat degradation kit we used 80 mg of brain tissue instead of 30-50 mg which was mentioned in the kit. RNA yield was evaluated by spectrophotometric absorbance (NanoDrop™ 2000/2000c Spectrophotometers Thermo Scientific™) at 260 nm (A260) and the purity was evaluated at 260/280 nm (A260/280). RNA extractions were stored in -80 ^{o}C. Complementary DNA (cDNA) synthesis was performed using iScript cDNA synthesis kits (Bio-Rad) according to the supplied protocol on a T100 thermal cycler (Bio-Rad). Quantitative PCR (qPCR) analyses were performed on a Bio-Rad Real Time PCR detection system using iTaq Universal SYBR Green Supermix (Bio-Rad). We used designed and optimized primers (Table 2). Oligonucleotide sequences used for qPCR amplification were designed using Primer3 and PrimerBLAST.

Histology. Twenty *P. maniculatus* and twenty-two *P. leucopus* were divided into two groups of older (about 2.5 -year old) and young (7-month old) animals. The animals were sacrificed at the ages indicated in Table 1. The liver, lung and brain were harvested from each animal and divided in two parts. On part of the tissue was snap-frozen on dry ice and stored at -80 ^{o}C, for RNA and protein extraction. The second part of the tissue was

fixed in 4% neutral buffered formalin and subsequently embedded in paraffin. Sections of the tissues were stained with Hematoxylin and Eosin (H&E) as previously described and Congo Red (Sigma-Aldrich Lot#MKCF4499) Histopathological analysis was performed by a certified pathologist (I.C.) blinded to the experimental conditions.

Statistical analysis. Results analyzed using 2-tailed t-test and Pearson correlation, $P<0.05$ considered as significant, by GraphPad Prism 8.

Results

Tissue and species-specific changes in UPR genes expression during aging. 7 months old and 19-33 months old female deer mice (*P. maniculatus and P. leucopus*) were analyzed for the expression of the major chaperones BiP, calnexin and GRP94, in the brain, liver and lung, by qPCR. As expected, RNA levels were highly variable for all tissues tested which is not surprising considering that they are genetically diverse individuals (Figure 1 and Figure 2).

With regards to age, different trends were revealed in the 2 species and between the tissues tested: In *P. maniculatus*, in the lungs reduced expression of chaperones was detected in the group of the aged animals ($P<0.05$) while in the brain, GRP94 levels were moderately elevated ($P<0.05$).

In the long-living *P. leucopus* expression profiles were different. In the brain and the liver, expression of chaperones was consistently enhanced in the older animals but statistical significance was attained only in the brain for GRP94 ($P<0.05$). In the lungs, a

strong suppression of BiP was detected in the older animals ($P<0.05$). This probably reflects the fact that with aging, the accumulation of reactive oxygen species and of other stressors initiates an adaptive UPR that in order to resolve ER stress requires elevated expression of chaperones in the brain and liver (20,21). In the lungs of the older *P. leucopus* both calnexin and GRP94 expression remained at levels similar to those of the younger animals. These results should be interpreted with caution since GAPDH levels may differ between experimental groups during aging.

Tissue histology in aged animals. In order to test if the modulated expression of UPR-associated genes is reflected in the morphology of the affected tissues we examined the histology of the lung, liver and brain of these animals. Degenerative changes were presented with consistency only in the brain specimens of older *P. maniculatus*. As shown in Figure 3a, in sections from the brain, neurons with condense eosinophilic cytoplasm and dark nuclei were noticed. These alterations were absent in the brain of the younger *P. maniculatus* as well as the younger or older *P. leucopus*. Congo red staining was also performed in the same areas of the brain and demonstrated the deposition of amyloid (Figure 3b). In the lungs, no morphological (architectural or cellular) changes were seen in any of the animals from both species and age groups (Figure 4a). In the liver of older *P. maniculatus* we also noticed small scant areas with macrovesicular steatosis without any signs of inflammation or cellular degeneration (Figure 4b).

Coordination of the UPR-associated genes. Since the only tissue at which degenerative changes consistent with aging were seen was the brain of older *P. maniculatus*, yet high variation in chaperones expression was detected among individuals

of the same group of animals and between the animals of different groups, which suggests that chaperones abundance is not directly associated with aging. Furthermore, this observation also shows that chaperones levels can adjust accordingly to attain homeostasis in a manner that highly depends on the individual requirements and tissue context. However, their levels are not indicative for tissue damage and degeneration, especially in genetically diverse individuals. This notion was also supported by the variation of chaperones' levels in *P. leucopus* at which no degenerative changes were detected, especially in the brain. The significant reduction in the expression of BiP in the lungs of *P. leucopus*, did not appear sufficient to trigger pathology since this tissue retained its normal morphology in the older animals.

In view of our earlier findings showing that in cultured fibroblasts from genetically diverse individuals UPR-associated genes are expressed in a concerted manner and are highly coordinated, we sought to investigate if this profile of UPR expression also occurs in tissues and whether this co-regulation is altered during aging. Thus, we performed pairwise comparisons in chaperones' levels in the individual specimens in all groups. As shown in Figure 5 and Suppl. Figure 1, all tissues tested maintained high degree of correlation between the expression of BiP, GRP94 and calnexin in all pairwise comparisons in younger *P. maniculatus*. In the aged group, and despite the differential regulation in expression levels, coordination was maintained in both the liver and the lungs between BiP, GRP94 and calnexin, at levels comparable to those of the younger animals. In the brain though of the aged *P. maniculatus* at which degenerative changes were seen, the correlation was lost between BiP and either of GRP94 and calnexin (Figures 5 and Suppl. Figure 1).

Contrary to *P. maniculatus*, in *P. leucopus* and for both age groups and all 3 tissues tested, coordination was maintained (Figure 5 and Suppl. Figure 2). Despite their variation in expression, that in certain cases was significantly enhanced or inhibited, such as for GRP94 in the brain and BiP in the lungs respectively, chaperones' levels during aging were adjusted accordingly to maintain a balanced and well-orchestrated activity. Thus, we conclude that the levels of expression may vary considerably between different individuals, tissues, species and age groups, but this variation is not indicative for the onset of pathological changes in tissue histology. What appears to characterize the onset of aging-related pathologies, such as neurodegeneration is the loss of coordination between different chaperones during the execution of the UPR.

Discussion

Aging has been linked to several progressive molecular alterations such as telomere shortening (22), inefficient DNA repair (23,24), differential methylation (25,26) and others, yet the regulation of stress response signaling pathways that are responsible for maintaining cellular and tissue homeostasis has been overlooked. In order to maintain homeostasis, cells respond to environmental or intrinsically inflicted changes, by adapting their transcriptional program and attain physiological function and performance. Such changes in transcription operate in concert, affecting multiple genes, yet they occur in a highly regulated and well orchestrating manner.

Considering that the progressive degenerative changes that are associated with aging constitute deviation from homeostasis, we hypothesized that they should be reflected in the transcriptional profile of the cells, influencing the robustness by which

coordination in gene expression operates. The UPR represents a signaling network that is appropriate to study these notions during aging since it is activated during chronic or acute stress in a manner that is concerted in genetically diverse specimens. To that end, although different stimulation is triggered in diverse specimens, within the same individuals the expression of different chaperones is highly correlated. In the context of aging, the role of UPR has been established, yet how the profile of UPR changes over time and whether such genes when they occur alter the coordination in gene expression remains elusive.

By using 2 closely related rodent species of *Peromyscus* as a model, and by focusing on the expression profile of chaperones BiP, GRP94 and calnexin, we show that during aging, what appears to mark pathology is the reduction in the degree of coordination. While tissues such as the brain, lung and liver exhibited distinct and frequently diverged UPR profiles as compared to those of the younger individuals, always a high degree of coordination between these chaperones, in all pairwise comparisons, was maintained. This profile of UPR expression was not identical between the two species tested implying that species-specific regulation may operate during aging. However, these changes were not associated with aging, with the exception of the coordination profile in older *P. maniculatus* which indicated that correlation in the expression of chaperones was abolished, and this was associated with the onset of neurodegenerative lesions.

Contrary to *P. maniculatus*, in long-living *P. leucopus* coordination was maintained and this was related to an overall induction in the expression of chaperones in the brain, while in *P. maniculatus* expression of BiP, but not of GRP94 and calnexin, was drastically

reduced. This moderate reduction of BiP expression as such, does not appear to be the cause for the loss of coordination because an even more pronounced suppression of BiP was recorded in the lungs of *P. leucopus* in the older animals, however, coordination in chaperones' expression was maintained.

In parallel with the loss of UPR coordination in the brain, in *P. maniculatus*, some aging-associated changes were also seen in their livers, exemplified by the accumulation of yellowish material resembling lipids. This however was not associated with inflammation and apparently does not compromise liver homeostasis and the execution of a canonical UPR.

The molecular phenotyping of histopathological lesions by using transcriptional data remains a challenge in modern biology. Conventionally, the differential expression of specific genes is being linked to particular lesions and states due to their significant overexpression or under-expression at given states. By using outbred rodents from 2 different species as a model this study shows that under physiological conditions the UPR exhibits patterns that are highly specific for each individual and are also specific for the tissue, the species, and the age at which they have been evaluated. Despite this variation, a high degree of coordination is maintained under physiological conditions among the major chaperones BiP, GRP94 and calnexin. During aging though, this coordination is abolished by a manner that appears to be lesion-specific and not just age-specific. Between the two *Peromyscus* species tested, the one that exhibits the longer lifespan maintains coordination in the brain over time, and does not develop neurodegenerative lesions, as opposed to the reportedly shorter-living *Peromyscus* that abolishes UPR coordination and develops neurodegenerative lesions. Since the loss of coordination in

the brain of aged P. maniculatus could be unveiled and indeed affected, collectively all animals analyzed as a group, outliers escaping coordination could not be identified to test if these exhibited exaggerated pathology. Probably, inclusion of progressively aging groups of animals could have permitted the identification of such animals that lost individually coordination while the majority of the animals in the same group retained it.

A limitation of the study is that GAPDH was used as internal control. It is plausible though that GAPDH expression changes during aging and between tissues. Thus, no comparison between different tissues was made. Furthermore, while changes in different age groups might have influenced our results in evaluating expression levels, provided that such changes would have been consistent between the different age groups, even if it had occurred it would not have influenced the results pertinent to the coordination analysis.

How this selective, age-specific loss of coordination of the UPR integrates with established aging-associated alterations such as methylation or DNA repair efficiency remains to be seen. It is plausible that expansion of this approach to other UPR genes and signaling networks may provide valuable insights regarding the degenerative changes underlining aging and potentially other pathologies. Furthermore, this study underscores the power of outbred animal models in illuminating aspects of the transcriptional changes occurring during aging that conventional models cannot.

Competing interests

None

Authors' contributions

ES, EF, YZ, AN and VK designed and performed experiments, interpreted results, edited and approved MS; IC, designed experiments, performed histopathology, interpreted results, edited and approved MS, HK designed experiments, interpreted results, drafted and approved MS.

Acknowledgments

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Data Availability Statement

Data available on request from the authors

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Table 1. Age of animals at sacrifice.

P. leucopus		*P. maniculatus*	
Animal ID	Age (months)	Animal ID	Age (months)
22188	7	35992	7
22186	7	35993	7
22177	7	36017	7
22174	7	36021	7
22189	7	36023	7
22182	7	35997	7
22183	7	36172	7
22500	7	36184	7
22547	7	36183	7
22488	7	36175	7
22507	7	35279	28
20980	24	35274	28
20956	24	35261	29
21907	19	35266	29
21022	31	35505	26
21195	28	35506	26
21069	32	35529	25
20960	33	35534	26
21989	24	35263	29
21001	33	35260	29
20967	33		
21211	29		

Table 2. Oligonucleotide used for the qPCR analysis.

Peromyscus maniculatus	*Peromyscus leucopus*
BIP	BIP
5’-cgccaagcaaccaaagatg-3’	5’- ctcggtggggaagactttga-3’
5’-cccaggtcaaacacaaggatg-3’	5’- agaagacagggctcgcttag-3’
PRODUCT SIZE 137	PRODUCT SIZE 147
GRP94	GRP94
5’-gtctccctgtgctcttgtgg-3’	5’-acacaacaatgacacccagc-3’
5’-gtctttgcccgtctggtatg-3’	5’-cctagtgtgtttcctctgggg-3’
PRODUCT SIZE 91	PRODUCT SIZE 84
Calnexin	Calnexin
5’-ccttgccagtgttccttgtg-3’	5’-tgtgttggctactggtcctt-3’
5’-cttccttcccttcttcctcctt-3’	5’-tgaaggagtgctggtatctga-3’
PRODUCT SIZE 120	PRODUCT SIZE 146
GAPDH	GAPDH
5’-ggagccgagtatgttgtggag-3’	5’-cttcctgcaccaccaact-3’
5’-ggagatgatgacccgtttgg-3’	5’-ggatgcagggatgatgttct-3’
PRODUCT SIZE 94	PRODUCT SIZE. 182

Figure legends

Figure 1. Expression of BiP, GRP94, and calnexin in the brain, lung and liver of *P. maniculatus* at different ages. Expression was evaluated by qPCR and was expressed in arbitrary units following normalization with GAPDH. Mean ± 95% CI is shown. *, P<0.05 student's t-test.

Figure 2. Expression of BiP, GRP94, and calnexin in the brain of *P. leucopus* at different ages. Expression was evaluated by qPCR and was expressed in arbitrary units following normalization with GAPDH. Mean ± 95%CI is shown. *, P<0.05 student's t-test; **, P<0.01 student's t-test

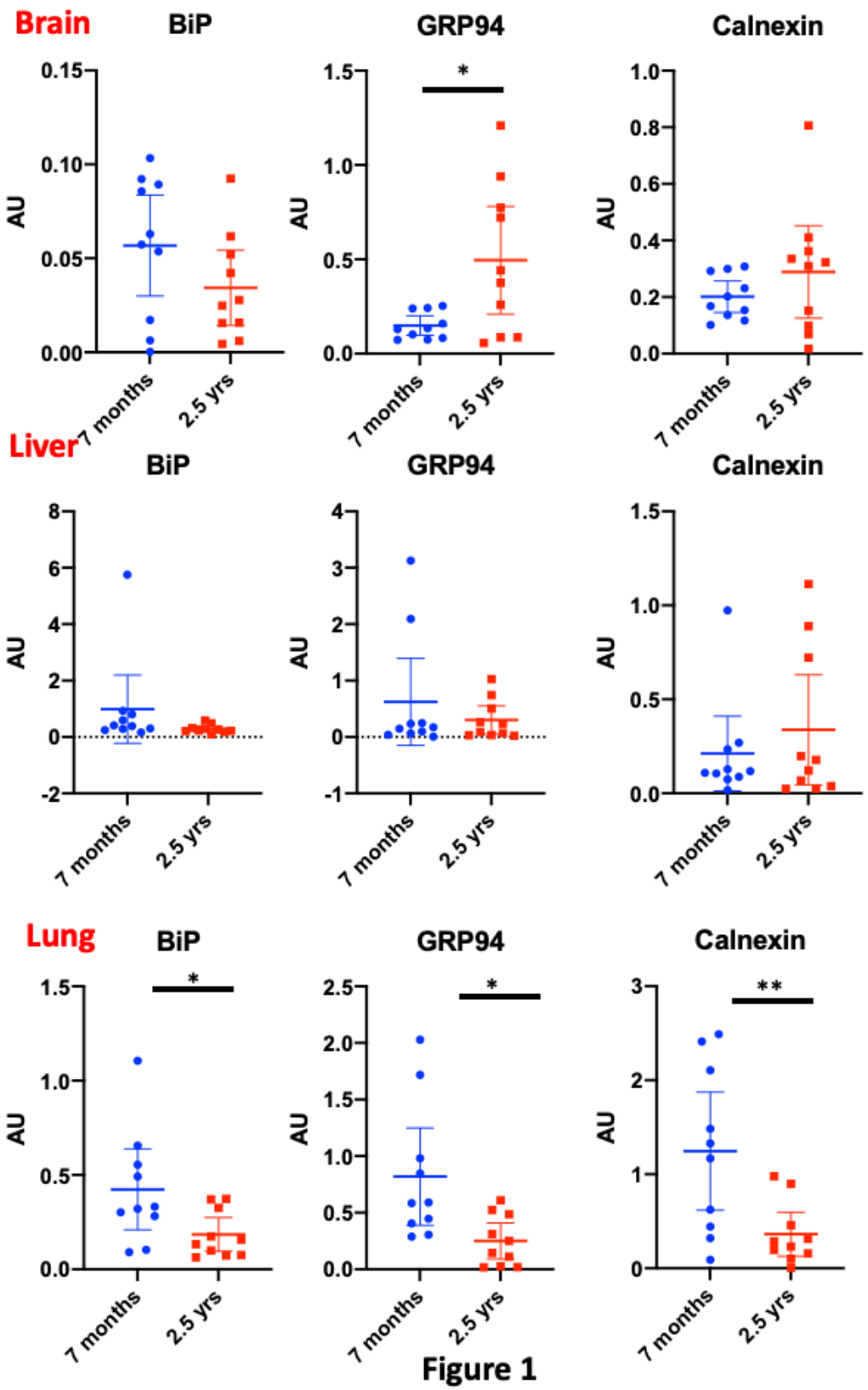
Figure 3. Brain histology in *P. leucopus* (LL stock) and *P. maniculatus* (BW stock) at 7 months and about 2.5 years. **a.** H&E staining. Neurodegenerative changes including neuronal esosinophilia of the cytoplasm and darkening of nuclei are pointed by yellow arrows. **b.** Congo red staining for amyloid deposits. Positivity was only detected in older BW animals. Representative images (10x) are shown.

Figure 4. Liver **(a)** and lung **(b)** histology in *P. leucopus* (LL stock) and *P. maniculatus* (BW stock) at 7 months and 2.5 years. In the liver of older *P. maniculatus* (BW stock), sparse areas with macrovesicular steatosis were observed (inset). Representative images (10x) are shown.

Figure 5. Heat maps indicating the correlation (Pearson's) between different combinations of chaperones in *P. maniculatus* (left) and *P. leucopus* (right panel) at different ages and different tissues. R values (Pearson's) are shown in white. P values

are indicated as asterisks as follows: *, P<or =0.05; **, P<0.01, ***P<0.001

BW


Brain
BiP
GRP94
Calnexin
AU
0.15
0.10
0.05
0.00
1.5
1.0
0.5
0.0
*
1.0
0.8
0.6
0.4
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0.0
7 months
2.5 yrs
Liver
BiP
GRP94
Calnexin
8
6
4
2
0
-2
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3
2
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-1
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0.5
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Lung
BiP
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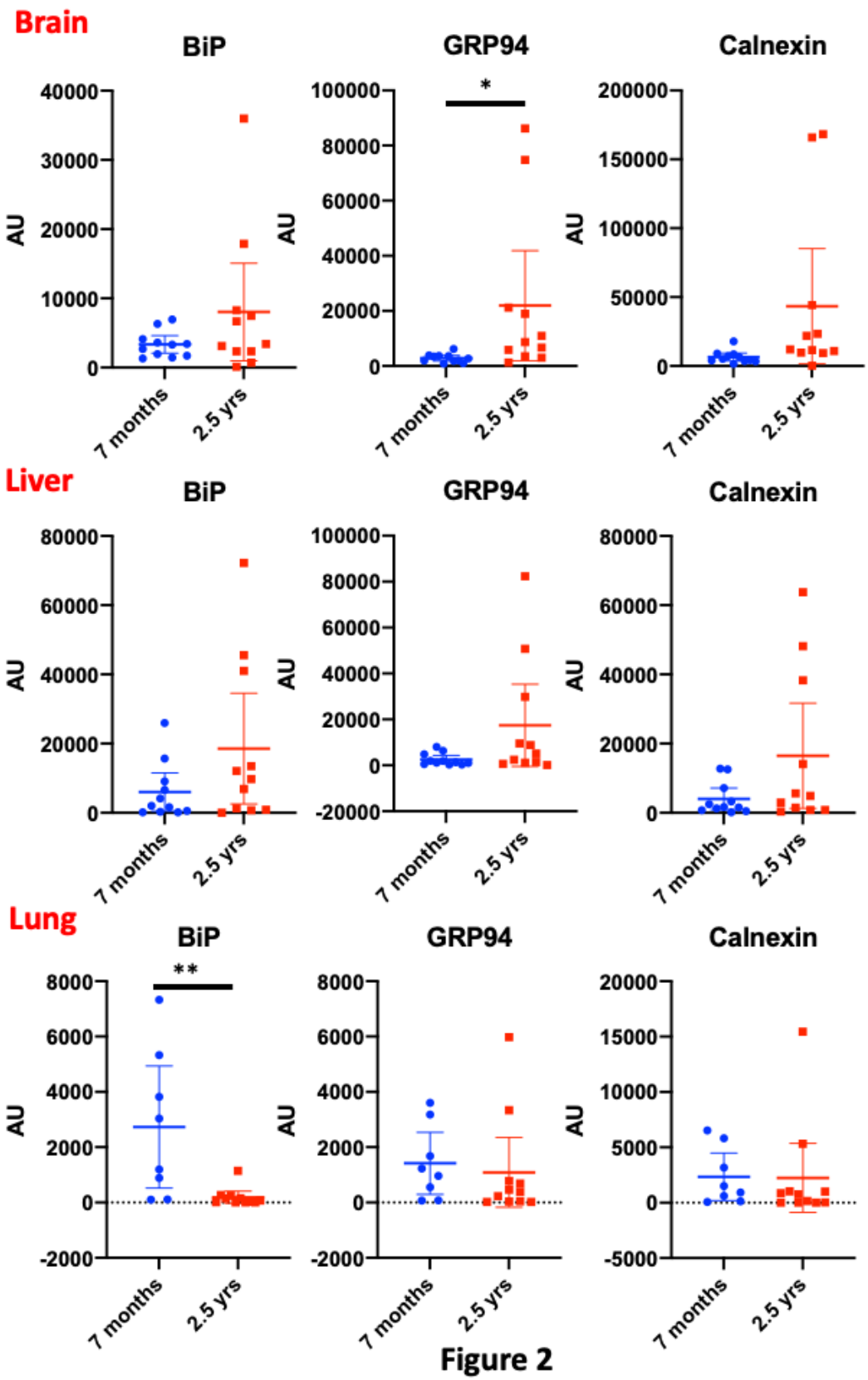
Figure 1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-5000

Figure 2

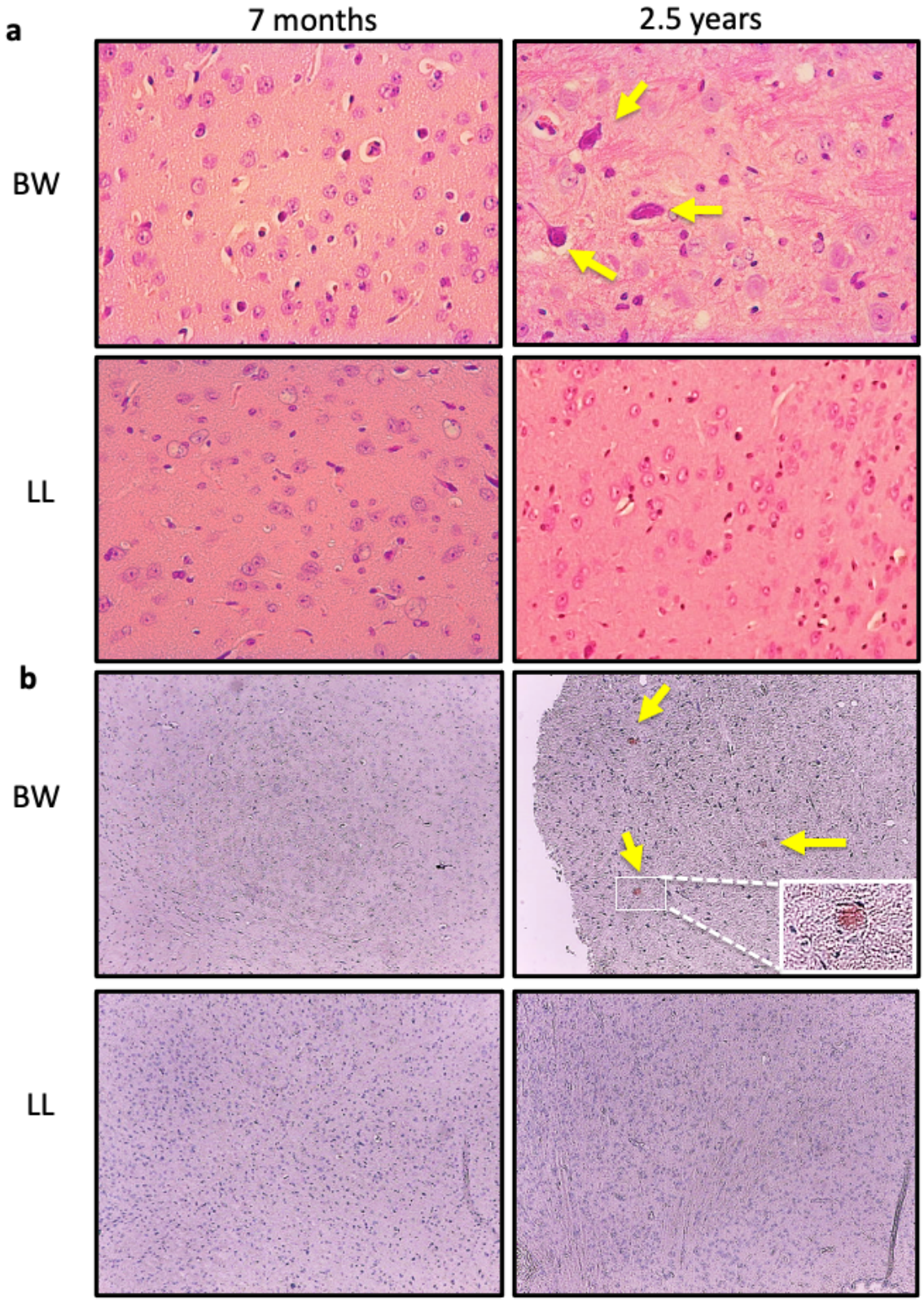

Figure 3

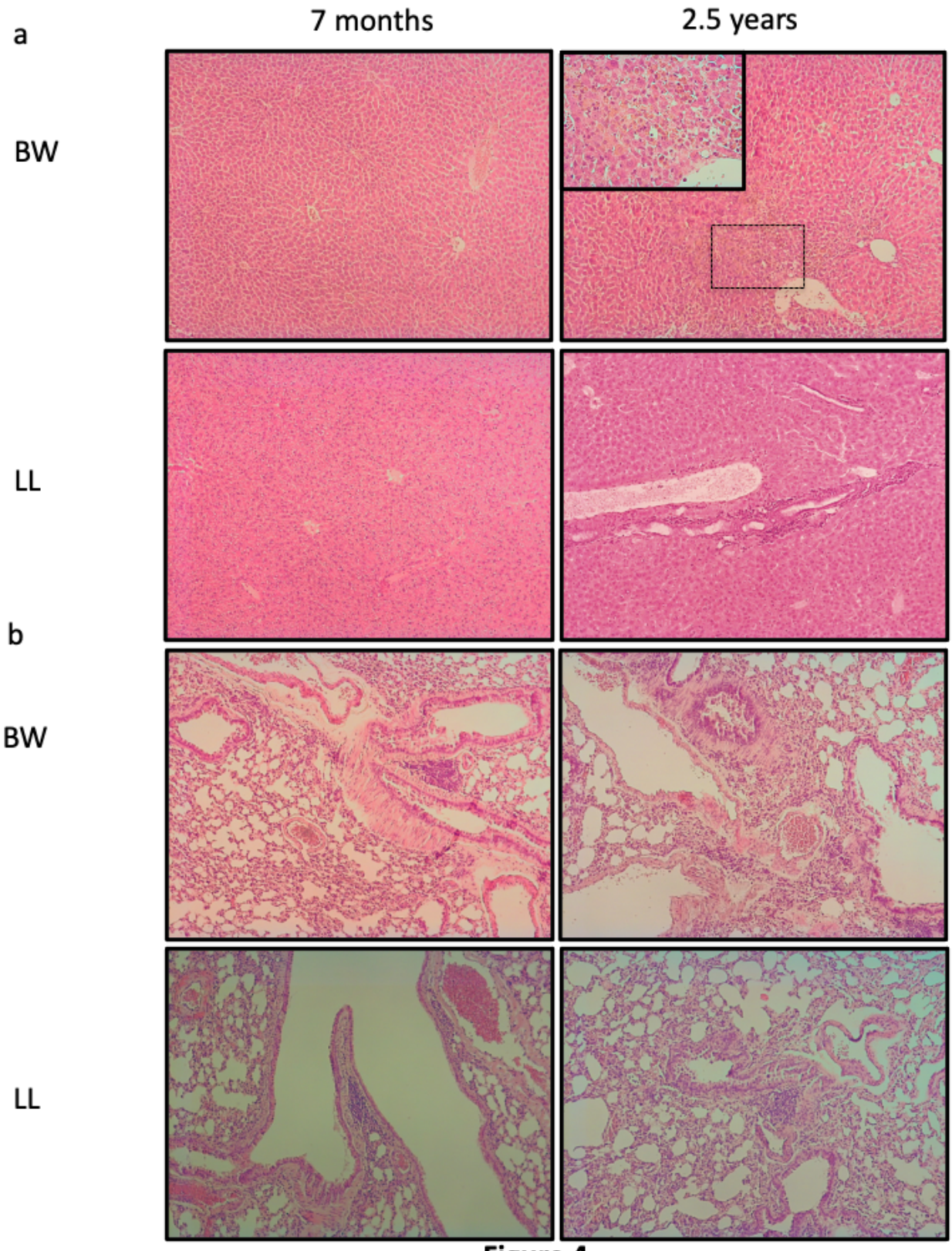

Figure 4

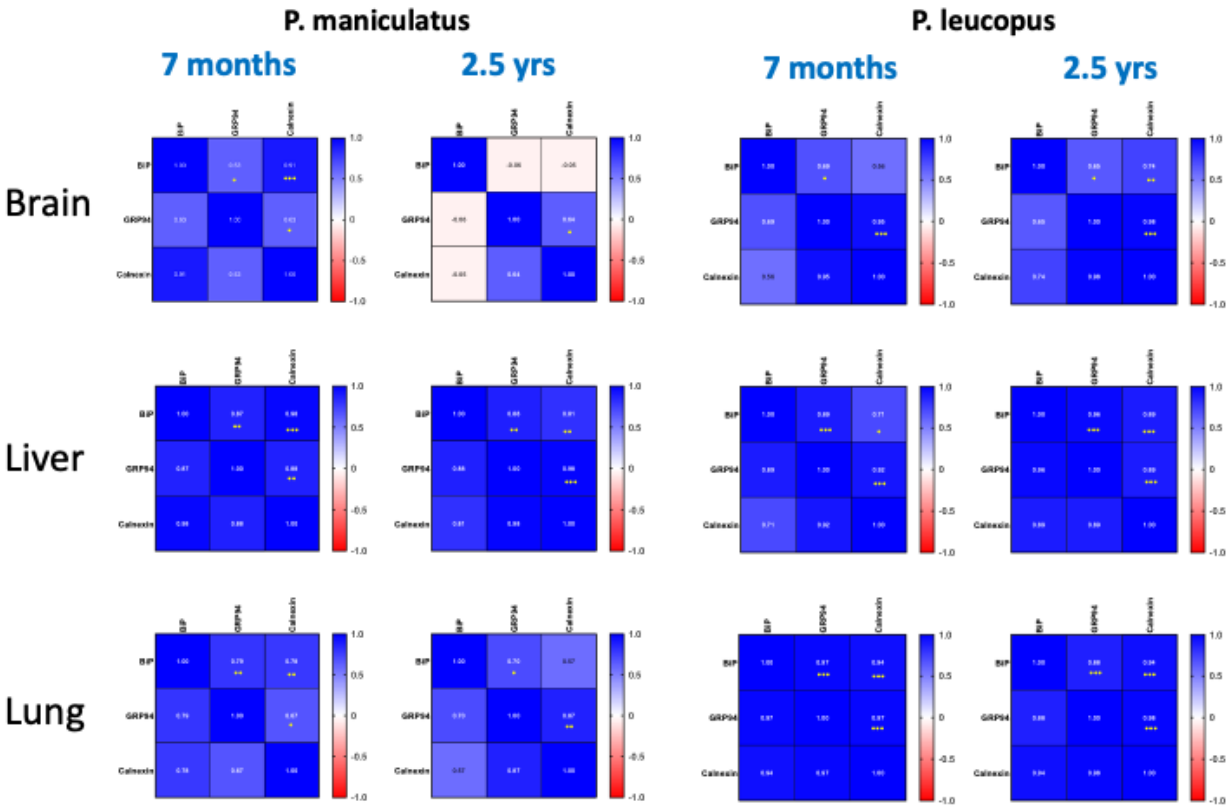
P. maniculatus
P. leucopus
7 months
2.5 yrs
7 months
2.5 yrs
Brain
Liver
Lung
BiP
GRP94
Calnexin
1.0
0.5
0
-0.5
-1.0

Figure 5