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## Transcriptome analysis of age-, gender- and diet-associated changes in murine thymus <sup>★</sup>

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### Abstract

The loss of thymic function with age may be due to diminished numbers of T-cell progenitors and the loss of critical mediators within the thymic microenvironment. To assess the molecular changes associated with this loss, we examined transcriptomes of progressively aging mouse thymi, of different sexes and on caloric-restricted (CR) vs. *ad libitum* (AL) diets. Genes involved in various biological and molecular processes including transcriptional regulators, stress response, inflammation and immune function significantly changed during thymic aging. These differences depended on variables such as sex and diet. Interestingly, many changes associated with thymic aging are either muted or almost completely reversed in mice on caloric-restricted diets. These studies provide valuable insight into the molecular mechanisms associated with thymic aging and emphasize the need to account for biological variables such as sex and diet when elucidating the genomic correlates that influence the molecular pathways responsible for thymic involution.

### Keywords

Thymus; Involution; Aging; Microarray; AGEMAP; Thymocytes; Caloric restriction

## 1. Introduction

Alterations in immune function with age in both humans and animals are important to the health of aging individuals. Older humans are much more susceptible to microbial infections of the soft tissues, skin, the abdomen and both the urinary and respiratory tracts [1,2]. These older subjects also demonstrate an increased incidence of infectious endocarditis, tuberculosis, meningitis and herpes zoster, and the mortality rates for these diseases in older patients are often two to three times higher than in younger people with the same disease [3-6]. In addition, an increased prevalence of specific cancers and certain autoimmune diseases have been observed with advancing age [6-9]. The increased prevalence of these conditions and the higher morbidity and mortality from infections strongly suggest functional defects in a deteriorating immune system with advancing age [10-16]. Many studies have centered on possible loss or alterations in the number of circulating T lymphocytes and T-cell subsets [17,18]. This focus on T lymphocytes seems reasonable given that the thymus is known to atrophy with progressive

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aging and correlates with a significant loss in its capability to generate de novo T cells for export into the peripheral T-cell pool [19]. Interestingly, this loss in thymic output with age does not result in any significant change in the total number of peripheral T cells as the maintenance of peripheral T-cell numbers appears to be regulated via a thymus-independent homeostatic process involving expansion of mature peripheral T cells. This peripheral homeostatic expansion of mature T cells results in a much more limited T-cell receptor (TCR) repertoire with age [19,20] and is believed to contribute to the diminished capacity of older T cells to proliferate upon mitogenic and other stimulation when compared with their younger counterparts [20-23]. However, given that the loss in thymic function is one of the earliest and most consistent steps in the progression to immune dysfunction, the involuting thymus seems to be a promising target to which to direct therapy with the specific goal of reversing thymic atrophy and restoring thymopoiesis and T-cell export [19,24-26].

For a number of years in gerontological research, efforts have been made to identify new and unique biomarkers that will define the biological, physiological and chronological changes that occur with age [27]. Numerous studies have focused on the comparison of calorically restricted (CR) primates and rodents to their *ad libitum* (AL)-fed animal controls, where significant differences in a variety of physiological processes have been observed as well as a prolongation in the animals' lifespan [28,29]. This delay of aging by CR appears to depend upon a delay, or sometimes ablation, of a broad spectrum of age-associated pathophysiological changes and a 30–50% increase in maximum life span. These studies are quite complicated as CR induces a plethora of biological changes including decreases in oxidative stress [30], glycation or glycoxydation [31,32], body temperature and circulating thyroid hormone levels associated with a hypometabolic state [33,34] as well as alterations in gene expression and protein degradation [29,32] and a number of neuroendocrine and inflammatory changes [35-37]. Despite the identification of many notable physiological differences between such dietary-restricted animals, no specific biomarkers have ever been reproducibly identified that could accurately identify the age of the organism or predict lifespan.

Microarray analysis has been an efficient way to profile the changes associated with disease onset, especially in the arena of both cancer and infectious disease [27]. By identifying the global gene expression profiles between disease and non-disease states, researchers are often able to isolate individual genes that may be used either as markers for disease, or as molecular targets as well as whole pathways that may be involved in the studied disease. Similar studies have now been initiated to examine age-related changes in a number of tissues and cellular subsets by microarray analysis in an effort to identify genes or patterns of gene expression associated with physiological aging, lifespan, and age-related disease states [27,38-42]. Only a few studies to date have focused on immunologic aging with the majority of studies focusing on an examination of bulk T-cell populations [43]. Limited replication of analysis, small sample sizes and poor array validation have restricted the interpretation of such data with regard to age-related immunological changes. To date, no studies have focused on gene expression changes within the aging thymus using microarray analysis with an additional focus on additional biological variables such as gender and dietary restrictions.

In an effort to understand the potential forces driving age-associated thymic involution and the cellular pathways involved in these phenomena, we have performed microarray analyses on thymi derived from young and old mice to identify differences in gene expression patterns which may be attributed to aging. We have included mice of both sexes in this study to determine sex-specific differences in aging, as well as mice that have been placed on various dietary regimens. Our results demonstrate that many of the putative thymic aging-responsive genes are in fact dependent upon variables like sex and diet, some of which are independent of age. Only a small fraction of the total gene products examined demonstrated thymic aging-dependent gene expression changes that were also independent of sex or diet. Complex

biological interactions are therefore responsible for the breadth of alterations in gene expression generally observed in the expression analyses of progressively aging thymi.

## 2. Materials and methods

### 2.1. Mice

Specific pathogen-free C57BL/6 mice were purchased through the Office of Biological Resources and Resource Development of the National Institute on Aging (Bethesda, MD). All mice were maintained in an AAALAC-certified barrier facility and were acclimated for 2 weeks before use. All mice were fed autoclaved food. Water was ingested *ad libitum*. All mice with evidence of disease (e.g., enlarged spleen, gross tumors) were excluded from these studies. The distribution and characteristics of the animals utilized in this study are described in Table 1. In this study, mice were fed either diets consisting of 100% regular feed (*ad libitum*) or caloric-restricted (CR) mice as follows: Up to 13 weeks of age, 100% regular feed, followed by 90% fortified feed for 1 week, 75% for 1 week, then 60% fortified feed after that until the age at which the mice were sacrificed. For the verification studies, flash-frozen thymi for RNA and protein analyses from both C57BL/6 and Balb/C mice were obtained from the NIA aged rodent tissue bank (Bethesda, MD).

### 2.2. RNA extraction and microarray analysis

All data from microarray experiments have been deposited into the GEO Accession Database (number in progress). For each array sample, the RNA was prepared using the entire thymus. The tissue was processed using a Bead Beater (Bio-Spec, Bartlesville, OK) followed by RNA purification using the RNEasy Mini Kit (Qiagen, Valencia, CA). The RNA was examined for quantity and quality using a Bioanalyzer (Agilent Technologies, Palo Alto, CA). Five micrograms of each RNA sample was used in a PCR reaction with [<sup>33</sup>P]dCTP (Valeant, Costa Mesa, CA). Radiolabelled cDNA was allowed to hybridize overnight at 43 °C to the mouse 17K DNA filters developed by the Laboratory of Genetics, NIA [44]. The hybridized filters were washed and placed under imaging screens for 3 days to allow for sufficient exposure. The images were developed and scanned in a Storm Phosphoimager (Molecular Dynamics, Piscataway, NJ) and the data was extracted using ArrayPro Software (Media Cybernetics, San Diego, CA).

### 2.3. Statistical data analysis

All data were processed in Excel spreadsheets using a Z score statistical analysis method developed at NIA [45]. In order to be selected for the final gene list, the expression value of a particular gene had to be at least 1.5 times different from the Z score of the control. Differences were considered statistically significant only if they had a *p* value less than 0.01.

### 2.4. Gene expression profiles

The selected gene lists were uploaded along with their Z scores to the Microarray Data Analysis website of the National Human Genome Research Institute (<http://arrayanalysis.nih.gov/>). Using distance-based gene selection, gene expression profiles were created in order to visualize differences between age, gender and diet.

### 2.5. Thymocyte isolation

Freshly extracted thymi from mice of various ages were dissociated in RPMI using a syringe and forceps. Cell clumps were broken up with repeated pipetting and then pouring through 70 µm nylon mesh cell strainers (BD Falcon, Bedford, MA). The cells were washed once after which the red blood cells were lysed with ammonium chloride buffer. The thymocytes were then washed twice in RPMI followed by PBS, counted, and pelleted. Cell pellets were either

used directly in the Qiagen RNeasy mini kits for RNA preparation or lysed in RIPA buffer containing protease and phosphatase inhibitors (Sigma, St. Louis, MO) to use in Western blot analysis.

## 2.6. Real-time PCR

Array results were verified by semi-quantitative RT-PCR. One microgram of RNA from each array sample or from thymus or thymocyte RNA samples was used to make cDNA with the TaqMan Reverse Transcription kit (Applied Biosystems, Branchburg, NJ) or the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). One microliter of each cDNA sample was then used to measure quantity using the SYBR Green PCR master mix (Applied Biosystems) and reactions were run on the ABI Prism 7700 Sequence Detector (Applied Biosystems). The results were normalized to GAPDH or 18S using the QuantumRNA universal 18S (Ambion, Austin, TX) and were also used to determine relative quantities. The primers used were:

CCT3: forward (F)—GCCATTCTTCGAGAGATTCAA, reverse (R)—TGCATCTGCTGCTCTAGGAA; CTSL: F—AGAAGGGGAGGCTTTTTTCAG, R—TGCCGGTCTTAAGGAACATC; DOCK7: F—TTGATGGAAACCTGGCTACTG, R—TGCAGATAGACTGCACTTTGG; EIF3S10: F—CAAAGGAGCGAGAGAAGGAA, R—CCAGTTGTTCAACCTGTTTGG; EIF4G2: F—TGCTTCTCGTTTCAGTGCTT, R—TTCATTTGCGGAGTTGTTTGG; ETFB: F—AAAGTGGACCTTTTGTTCCTG, R—TCAATTTCCCCTTCCAC TTT; HSPA8: F—TTGCTTTCACGGACACAGAG, fR—GCATCATTCACCACCATGAA; HSPCA: F—CA AGACCAACCAATGGAGGA, R—TCAGGCTCTCGTAACGGATT; HSPCB: F—TCGGCCTATCTAGTTGCAGA, R—TGAGGTGAAGGATCACTTTGG; IGH6: F—ATGGAATGGACCTGGGTCTT, R—ATGTCCAG GCCTCTGCTTTA; IGHG: F—TGCAGTCTGGACTCTACACTATGA, R—GGGCATTTGTGACTCCTT; NR1I3: F—TTGCATATCTCACTCAACACTACG, R—TCCTGGAGATGCAGTCCTTT; NR3C1: F—TTCCTTGGGGGCTATGAACT, R—AAGCTTCATCGGAGCACA; SMARCD2: F—ATTCCGAAAAC GCCTGCT, R—GGATTCGCTGAGGTAGAACCCTT; VIL2: F—GG ATTCCTACCTGGCTGAA, R—TT GACTTGCAGGA AGAAGAGC.

## 2.7. Western blot analysis

Equal amounts of protein from total thymus or thymocyte pellets were run in a 10% Tris-glycine gel and transferred to nitrocellulose on the Novex gel-blot system (Invitrogen, Carlsbad, CA). The nitrocellulose filters were then probed using specific antibodies to the glucocorticoid receptor (GR), the immunoglobulin heavy chain M (IgM), IgG and GAPDH obtained from Abcam (Cambridge, MA). In addition, the antibody to eIF4G was also utilized (Santa Cruz Biotechnology Inc, Santa Cruz, CA). The HRP-conjugated secondary antibodies were from Amersham (Piscataway, NJ). Bands were visualized using the ECL Plus western blotting detection reagents (Amersham) and CL-X Posure film from Pierce (Rockford, IL).

## 2.8. Immunohistochemistry (IHC)

Paraffin sections of thymus from young (3.5 months) and old (24 months) C57/BL6 and Balb/C mice were sliced at 5  $\mu$ m of thickness and mounted onto positively charged slides by Paragon (Baltimore, MD). The slides were then steamed for 30 min in the presence of antigen unmasking solution (Vector Laboratories, Burlingame, CA). These slides were subsequently washed and then blocked with rodent block (Vector Laboratories) for 1 h, followed by an overnight at 4 °C incubation with anti-GR antibody (Abcam, Cambridge, MA). The cells were washed again with PBS for 30 min and then probed with a biotin-conjugated secondary antibody for 1 h (Vector Laboratories). Staining was visualized using a Lab Vision ABC (streptavidin/biotin) detection system (Fremont, CA). After probing, the slides were then washed, mounted in

permount and examined on a Zeiss Axiovert 200 microscope using Axio-Vision software (Zeiss, Thornwood, NY).

### 3. Results

#### 3.1. Microarray analysis reveals distinct transcriptional changes with thymic aging

To examine the molecular events associated with aging in mammals, we utilized cDNA-based microarray analysis to elucidate the transcriptional response to the aging process in the murine thymus. In the current study, a total of 67 mice were utilized and divided into seven separate categories. Four age groups of mice, namely 1-, 6-, 16-, and 24-month-old animals, were placed on *ad libitum* diets. The three additional groups also included mice at the ages of 6, 16 and 24 months of age fed a 60% caloric-restricted diet beginning at 4 months of age. These conditions are summarized in Table 1. The thymi of these various animals were obtained and utilized in microarray analysis to assess any differences with age. Out of the 17,000 genes on the cDNA array, 1066 genes significantly changed with age in these various groups. A complete list of all genes that changed with age can be accessed at (GEO Accession in progress). Many replicate genes were spotted at several different locations on the filters to verify reproducibility of the results. The robust quality of the array is reflected in the fact that the statistical analysis of the data identifies the repeated spots (data not shown).

Using the in-house Microarray Data Analysis website of the National Human Genome Research Institute (<http://arrayanalysis.nih.gov/>), a distance-based gene selection analysis was performed on this data set and an expression profile was generated (Fig. 1a). Dramatic changes in gene expression were observed between mice aged 1 month and 6 months (240 genes changed; 130 up and 110 down) and those aged 16 months (123 genes changed; 93 up and 30 down) or 24 months (1066 genes changed; 587 up and 479 down) (Fig. 1b). The early changes in expression may possibly reflect the known peak of thymic decline between the initiation of puberty and the midlife period in mice [46]. The results in Table 2 list some of the genes demonstrating the greatest increases or decreases when comparing the oldest mice to the youngest mice on the *ad libitum* diet. A complete list of these genes can be accessed at (GEO Accession in progress). Most of the proteins encoded by these genes are fairly equally divided between the nucleus and the cytoplasm as well as a large number of genes associated with the plasma membrane. The most common genes that changed with age include transcription regulators such as various ETS, forkhead P and the SMARC transcription factors. There were also a number of genes coding for enzymes, such as ATP synthases, MAP kinases and ubiquitin-conjugating enzymes. These proteins perform a variety of functions within the cells and are most commonly associated with cellular growth and proliferation, cell death, cancer and protein synthesis. A striking observation was that of the four most significantly changed genes involved in DNA repair and recombination, every one was down-regulated. The relevance of these gene changes to thymic aging remains to be determined.

Given that the thymus undergoes significant involution with progressive aging, one would expect to see changes in genes that are associated with T-cell development and thymic function (Table 3). Examples of such genes include thymosin beta 10 (Tmsb10), whose expression decreased with age (-2.72-fold) but was restored in older mice on a caloric-restricted diet (+2.90-fold). Tmsb10 is a member of a family of proteins originally isolated from calf thymus and it is critical for proper maintenance of the actin cytoskeleton within cells [47]. Tmsb4, which is in the same family, has previously been shown to decline with age [48]. Several other immune-related genes were also downregulated with aging including cathepsin L (which ranged in value from -2.53-fold at 6 months to -5.70-fold at 24 months), a protease that has been shown to be involved in MHC class II processing in cortical thymic epithelial cells [49]. Interestingly, expression of  $\beta$ -actin RNA also decreased (-4.58-fold) with age in the array analysis and this may correspond to reports demonstrating that actin polymerization is impaired



in aged mouse T lymphocytes [50]. All of these above changes were consistent with the literature regarding age-associated changes to the thymus, lending credence to our observations. Whether these changes in expression are due to the global changes in the thymic structure and make-up or due simply to the loss of thymocyte numbers needs to be addressed. For example, the data in Fig. 2a confirms our microarray results demonstrating an age-associated increase in IgM and IgG heavy chain levels using real time RT-PCR on mRNA derived from total thymus and purified thymocytes. This increase is also evident in the protein expression of these immunoglobulin genes in aged thymocytes Fig. 2b. These data suggest that the changes in the expression of certain genes such as immunoglobulins may be due to changes in the cellular make-up of the thymocyte subpopulations rather than global changes in the thymic structure or thymic cellularity.

### 3.2. Caloric restriction prevents gene expression changes observed within aging murine thymi

Laboratory animals placed on a caloric-restricted diet have exhibited an extended lifespan directly correlated with the degree of restriction [51,52]. Caloric restriction also delays the onset and reduces the incidence of age-associated diseases and complications [52-54]. In light of these observations, it was of interest to examine how calorie restriction affected the gene expression profiles of aging mouse thymi (Table 4). The data in Fig. 3a show the gene expression profile for thymi derived from mice on a CR diet compared to mice on an AL diet. Surprisingly, in all groups, changes associated with the aging profile are prevented by a CR diet by 24 months of age, reflecting the profile observed in the youngest AL mice. There were 472 genes expressed in the thymus that demonstrated significantly different levels of expression when the 24-month-old mice on a CR diet were compared to their age-matched controls on an AL diet. Dramatic changes in gene expression were observed between mice 1-month-old AL mice versus 6-month-old AL (8 genes changed; 5 up and 3 down) and CR mice (476 genes changed; 341 up and 135 down) and those of the AL (0 genes changed) and CR (165 genes changed; 97 up and 68 down) of 12 months of age or 24-month-old AL (278 genes changed; 153 up and 125 down) and CR (472 genes changed; 222 up and 250 down) (Fig. 3b). Many of the proteins encoded by these genes are fairly equally divided between the nucleus and the cytoplasm, with fewer gene products associated with the plasma membrane (data not shown). The most common genes affected by CR included transcription regulators such as BCL2-associated transcription factor 1 (+2.1, data not shown) and histone deacetylase 1 (+1.66, data not shown) and enzymes such as fatty acid desaturase 1 (+2.32) and thioredoxin (+1.68, data not shown). Moreover, genes encoding transporter proteins (including transportin 1 [-1.5], not shown) and kinases (including Pak2 [+3.61] and PTK2 [+2.19]) also demonstrated significant changes with age and diet. These proteins perform a wide variety of functions within the cells but are most commonly associated with cell death, gene expression, cancer growth and development and cell cycle.

The most significantly affected genes that changed with age and were also affected by CR in each of the top gene categories, appear to be transcription regulators, enzymes, kinases and transporters, while additional diet-associated gene expression differences are observed in several smaller categories. Aging seemed to affect a greater proportion of peptidases, transmembrane receptors, ion-channel genes and cytokines, while a CR diet influences a greater proportion of ligand-dependent nuclear receptors and growth factors. These data are summarized in Fig. 4a. Fig. 4b shows that many of the genes affected by aging tended to also be involved in cellular growth and proliferation and protein synthesis, while a greater percentage of the genes affected by a CR diet are involved in development and survival. Focusing on the genes in these latter categories may provide some insight into the mechanism by which a CR diet exerts its beneficial effects.

The results in Table 4 demonstrate that several of the genes which increase or decrease by the greatest margins were age-associated when comparing the oldest CR mice to the oldest AL mice (Table 4). Immediately evident in this list are several heat-shock proteins that are more highly expressed in aged CR mice compared to age-matched AL-fed mice. These genes were found to decrease in expression within the thymus with age; however, mice placed on CR diets demonstrated heat shock protein levels similar to those seen in the youngest AL mice. Similar reversals were observed in quite a number of genes including PTPNS1 (Table 4), cathepsin L and the glucocorticoid receptor.

### 3.3. Sex-specific transcriptional changes were also noted within the thymus and over progressive aging

Statistically significant changes in thymic gene expression were observed between male and female mice at specific ages with 158 genes (38 up and 120 down) demonstrating a change at 6 months, 159 genes (113 up and 46 down) demonstrating a change at 16 months and 215 genes (151 up and 64 down) demonstrating a change at 24 months. Fig. 5a shows the expression profile of statistically significant gene expression differences between males and females at 24 months of age. Values for other age groups in this figure are not statistically significant, but aging trends are quite evident. The gene list can be separated into three groups, namely genes that increase with age, decrease with age or genes that decrease with age in males but are highly expressed throughout the females' lifetime. In addition, in the first two groups, the thymic expression pattern between males and females is quite similar; however, the degree of such expression changes is more subtle in the females. Interestingly, many of these gender-associated changes in gene expression were found to be related to cancer development and growth.

In addition, the differences observed between male and female mice in the context of thymic aging revealed approximately 532 genes significantly different between the oldest males and females. Although trends are evident in Fig. 5a, only four statistically significant changes in gene expression were observed between 1- and 6-month-old male vs. female mice (1 up, 3 down), many with only subtle changes in expression (+1.07- to -1.34-fold). The same is true for 1- and 16-month-old male vs. female mice (5 genes changed; 3 up and 2 down), although the differences are much larger (+3.87 to -3.33). However, very pronounced differences begin to develop when comparing the 1- and 24-month-old male vs. female mice (58 genes changed; 35 up and 23 down) (Fig. 5b). The majority of these genes code for proteins that function within the nucleus, while several others code for cytoplasmic proteins. The most common differences in gene expression encode transcription regulators and enzymes as well as genes involved in a variety of functions within the cells, including cell cycle, DNA replication, recombination and repair and cancer development. The genes that demonstrate the largest differences between males and females are listed in Table 5.

Some genes were expected to change, lending validation to the array data, such as the Xist gene, which is involved in the silencing of the second X chromosome [55], and was higher in females than males. Other genes such as Tmsb10, which decreased in expression overall with age as mentioned before (Table 2), decreased more acutely in males than females. However, on a caloric restricted diet, the oldest mice exhibited Tmsb10 levels equivalent to those seen in young mice on an unrestricted diet, regardless of gender. Interestingly, thymosin beta 10 upregulation has been shown to be associated with carcinogenesis [56]. Another carcinogenesis-related gene on this list was checkpoint kinase 1, which is regulated by tumor suppressor protein p53 [57] and may also be involved in carcinogenesis. In fact, of the top 100 identifiable genes showing differential expression levels between the oldest males and females, 53 are tumor-associated (Table 6).

### 3.4. Age-associated changes in gene expression levels reflect changes in thymic cellularity and thymocytes

Given that the thymus involutes with age and a significant loss in thymocyte numbers occurs, it is important to determine whether the observed changes in the array samples were due to the relative decrease in thymocyte number or due to intrinsic changes within the thymocytes and/or thymic tissue. Table 7 shows the array results that were confirmed by quantitative real-time PCR using the total thymus samples used for the array. These same genes were then examined using purified age-matched thymocytes to determine whether these confirmed changes could be attributed simply to a diminished thymic cellularity or changes within the thymocytes themselves. Several of these genes demonstrated changes with age in the purified thymocytes, reflecting the changes observed in the thymic arrays. In contrast to the thymic array data, other genes including several heat-shock proteins failed to demonstrate any changes in expression in thymocytes at various ages. Clearly, both thymocyte-specific and architectural changes appear to be important contributors to thymic involution. The involution process could be influenced by increases in gene expression attributed to the infiltration of fat cells in the thymus and/or decreases in gene expression due to changes in the thymocyte number and profile.

### 3.5. Control of protein translation in aging thymocytes by elongation initiation factors

Another group of genes which changed expression with age and whose age-associated expression levels were reversed by a restricted diet were the elongation initiation factors (eIFs). The eIFs are a group of proteins that control translation within the cell, and are important in protein synthesis and cell proliferation [58]. Both the eIF3 and the eIF4 families act as scaffolding proteins during ribosomal complex assembly and translation initiation [59,60], and have been associated with sarcopenia and muscle wasting during aging [61]. Importantly, a recent proteomics study showed that eIF is decreased in aging brain tissue, and that its expression can be reversed by caloric restriction [62]. Our data validated these observations in thymic tissue, where several members of the IF family, specifically, eIF3S2, eIF3S9, eIF3S10, eIF4A1 and eIF4G2 (Fig. 3 and Table 7), all decreased within the thymus with age were restored in caloric-restricted old mice. Age-associated changes in eIF3S10 and eIF4G2 were confirmed by real-time RT-PCR on independent total thymus samples, and changes in levels of eIF4G2 were further confirmed by real-time RT-PCR on purified thymocytes (Table 7), thus indicating that these changes are intrinsic to the thymocytes and may play a role in involution. Further, Western analysis of protein lysates from young and old thymocytes using antibody to eIF4G demonstrates that there is a clear decrease in the level of eIF4G protein in the old samples when compared to the young (Fig. 6).

### 3.6. Involvement of the glucocorticoid receptor in the aging of thymocytes

After validating the observed array changes using real-time PCR, pathway analysis was utilized to examine if multiple genes associated with specific signaling pathways may be altered within the aging thymus. Genes that changed were submitted to the Ingenuity Pathways Analysis program available from Ingenuity Systems (<http://www.ingenuity.com>), and a representative pathway is shown in Fig. 7a. This particular pathway was of great interest because it represented regulation of the glucocorticoid pathway, and glucocorticoids are produced by thymic epithelial cells and it has been postulated that GR is necessary for thymocyte development [63]. Furthermore, as mentioned in the earlier section referring to CR prevention of age-associated changes, we had also observed that the glucocorticoid receptor and HSPs display similar relationships but exhibit expression levels opposite from those seen in aging mice on an AL diet. Thus it was of great interest to us that this particular pathway was identified by gene ontology analysis of our data. At the center of the network is the nuclear receptor NR3C1, also called the glucocorticoid receptor (GR), which decreased in expression with age in both total thymus and purified thymocytes. Fig. 7b shows a network derived using the list of genes



that were affected by CR. This was in concordance with the array results, where a CR diet restored the expression levels of this gene to the levels seen in the thymi of young AL mice. To confirm these results and also confirm that this was not due to decreases in thymocyte numbers, we performed Western analysis using purified thymocytes. Fig. 7c shows that the glucocorticoid receptor levels also decrease with age specifically within the thymocytes themselves. To ascertain the region in which this loss of GR was occurring, we then performed immunohistochemistry of various thymic sections and revealed that the decrease of glucocorticoid receptor protein in the thymus with age appears to be within both the cortex and medulla of the thymus (Fig. 7). Loss of GR expression in the cortex appears to be in the cytoplasm of all the cells, as well as a complete loss within the nucleus of many cells (Fig. 7d). In the thymic medulla, age-associated loss of GR appears to be cytoplasmic (Fig. 7e). Taken together, these data support the loss of the GR with thymic aging and this loss may have potential implications for the thymic involution process.

#### 4. Discussion

The specific aim of the current study was to create an atlas of changes in thymus gene expression with age and assess those affected by a CR diet. There have been numerous array papers published that reveal profiles of age-associated gene expression in various tissues. However, there are no data on global changes in gene expression associated specifically with thymic aging and involution. Many genes decreased in age in the thymus, and were restored to their original levels by a CR diet, indicating that these may play a role in thymocyte death and involution. These included housekeeping genes such as actin and tubulin, heat- and stress-related genes such as HSP90 and HSC70, and the glucocorticoid receptor, as well as elongation initiation factors. Decreased production of these types of proteins in the aging thymus could adversely affect cell function and contribute to involution within the thymus. Indeed, eIF4G2, for example, has been specifically associated with apoptosis and is referred to as a death associated protein (DAP) [64,65]. Decreased production of eIF4G2 specifically has been associated with inhibition of cell proliferation, and has been proposed to be involved in the switching of the cell from generalized translation to translation related to stress response [66], implicating other proteins previously mentioned such as the HSPs. Further scrutiny of these pathways is required to examine their roles in thymic involution. An important note is that, although our initial analyses were restricted to C57/B1 mice, we have expanded and validated these observations in Balb/C mice as well.

Regardless of whether the comparison was by age, diet or sex, the most common types of genes that changed expression levels were transcription regulators, enzymes, transporters and kinases. These genes code for proteins that perform a variety of functions within cells. Strikingly, genes involved in DNA repair and recombination were significantly downregulated, suggesting that aging thymocytes have a decreased ability to repair DNA damage, a common feature of both aging and cancer cells. The largest functional group of genes that changed with age is involved in cellular growth and proliferation, while the largest functional group of genes affected by a caloric-restricted diet is involved in cellular death. With regard to immune function, CR has been shown to reduce inflammatory cytokine expression in young and aged subjects and mediates an enhancement of immune cell proliferation and activation in response to mitogens and antigen [21-23,67,68]. However, the literature on the effects of CR on immune and thymic function is both sparse and contradictory. Several researchers have reported that CR induces a significant decrease in splenic cellularity and an elevation in the percentage of splenic CD4<sup>+</sup> T cells compared with CD8<sup>+</sup> T cells [69], while others have not observed any subset changes in CR mice vs. AL controls [70]. The studies by Chen and co-workers suggest that CR may preserve cortical thymocytes and may directly influence thymopoiesis and thymocyte survival. It would be interesting to speculate that these changes may relate to the possible effects of a caloric-restricted diet on thymocyte survival and delay of the aging process

within the cells. Also, given the dramatic changes in gene expression in aged CR thymi compared to their AL counterparts and the similarities to young AL thymic gene expression, studies are underway attempting to further elucidate the effects of CR on thymocytes and the involution process. Of specific interest were genes known to be associated with T cell function and aging. Cathepsin L, which decreased with age in our arrays, is critical for MHC II processing and thymocyte survival [49]. Age-specific decreases in this gene may thus be responsible for the lowered maturation and survival of thymocytes with age. Other processes known to be altered in aging T cells also included actin polymerization and altered MHC processing, and genes known to be involved in these processes are indeed altered in our current array data (Table 2).

Using published data, the ingenuity pathways program designed possible networks of interactions between genes in our lists. The networks shown in Fig. 6 highlight the interaction between the glucocorticoid receptor, androstane receptor and heat shock proteins, which change with age and are reversed by a CR diet. Western blot and immunohistochemical analysis demonstrate that significant alterations in GR protein expression can be observed with age and supports our gene expression analysis. Glucocorticoids, acting through the glucocorticoid receptor (GR), potently modulate immune function and are a mainstay of therapy for treatment of inflammatory conditions, autoimmune diseases, leukemias and lymphomas [71-74]. Removal of systemic glucocorticoids, by adrenalectomy in animal models or adrenal insufficiency in humans, has shown that endogenous glucocorticoid production is required for regulation of physiologic immune responses [75,76]. These effects have been attributed to suppression of cytokines, although the crucial cellular and molecular targets remain unknown. In addition, considerable controversy remains as to whether glucocorticoids are required for thymocyte development [76,77] GR knockout mice are lethal perinatally but mice with a T-cell-specific disruption of the GR gene show no impairment in thymocyte development or T-cell populations [78]. In a recent paper by Brewer and co-workers [78], they demonstrate in T-cell-specific GR KO mice that T-cells are a critical cellular target of glucocorticoid receptor signaling, as immune activation in these mice resulted in significant mortality. This lethal activation is rescued by the presence of cyclooxygenase-2 (COX-2) inhibition but not glucocorticoid administration or cytokine neutralization. Thus, it would appear that glucocorticoid receptor suppression of COX-2 is crucial for curtailing lethal immune activation, and suggests new therapeutic approaches for regulation of T-cell-mediated inflammatory diseases. Enhanced expression of the GR increases the cells' resistance to stress [79]. Also, mice with an increased sensitivity to glucocorticoids exhibit delayed involution of the thymus [80]. Perhaps GR may not be necessary for thymocyte development, but its presence may make it easier for the cells to survive under stress conditions associated with aging. Thus, decreased levels and activity of the GR could contribute to thymocyte death and thymic involution. Our data demonstrate that the GR practically disappears in thymus and thymocytes derived from old mice and that mice maintained under a CR regimen demonstrate restored GR levels to those of young AL-fed mice. This age-associated decrease in GR expression occurs within the thymocytes themselves. This implies that there could be a critical balance between GR and the HSPs required for thymocyte survival. Further study of the role of GR in thymocyte survival and thymic involution is planned.

Overall, this study was performed in combination with a larger study being conducted at the Gerontology Research Center of the National Institute on Aging called the "AGEMAP Project". This project aims to generate a database of gene expression patterns associated with aging and caloric restriction in many of the mammalian organ systems, which may provide a valuable source of information about genetic influences involved in aging including those unique to specific organ systems and others that may be universally associated with aging. Additional studies are currently underway examining the spleens and bone marrow cells of these mice. In our current report, the data have demonstrated that microarray analysis can

robustly identify significant differences in the profiles of aging thymi, and also isolate sex-specific and dietary effects upon this process. It will be interesting to attempt to define roles and manipulate pathways indicated by the array data that are involved in thymic aging. Many more genes from this array will need to be identified and tested for their contributions to the cellular aging process as well as their ability to be rescued by a CR diet. Further studies are under way in order to create a clearer picture of the dynamics involved in thymic involution and aging within the immune system.

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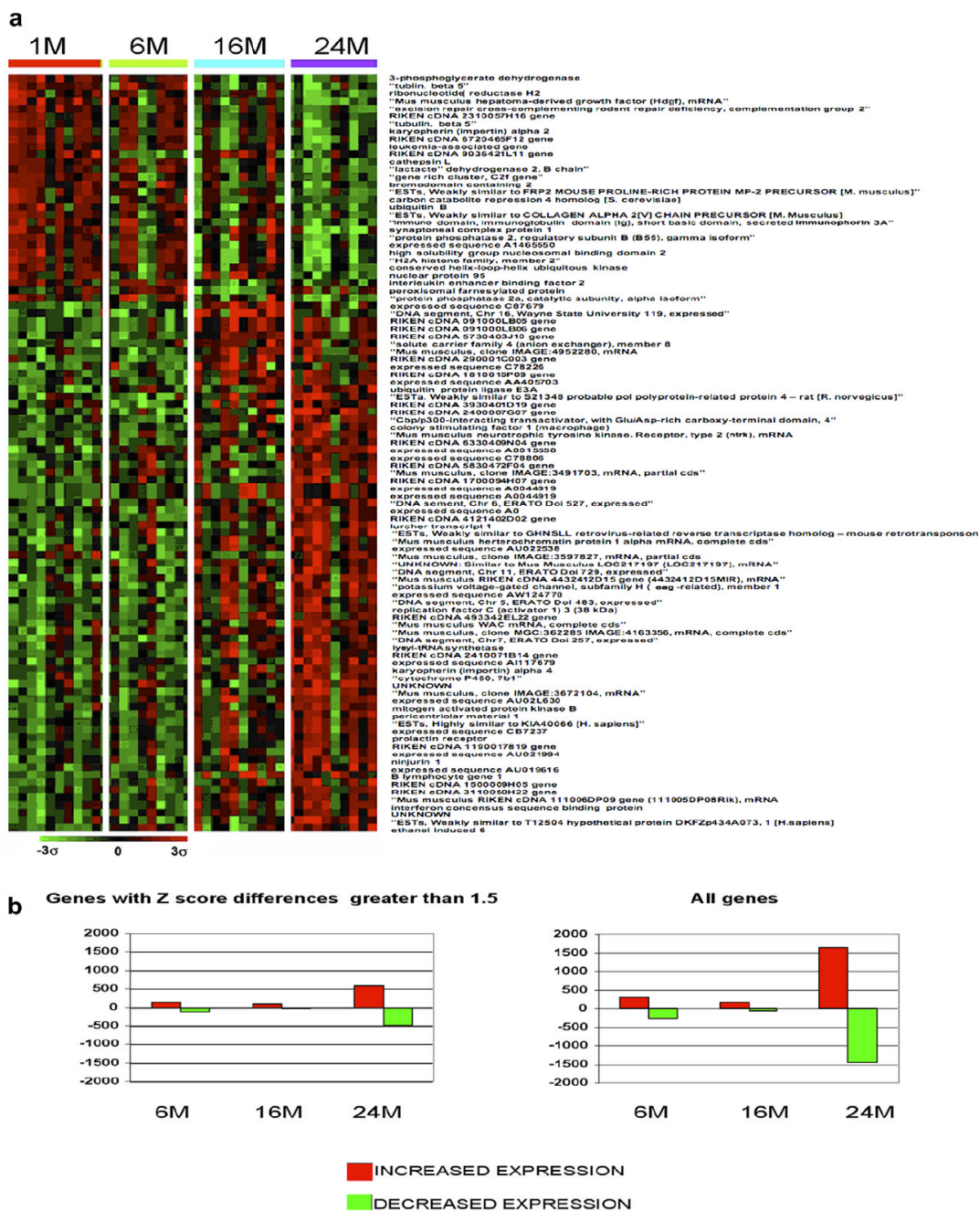
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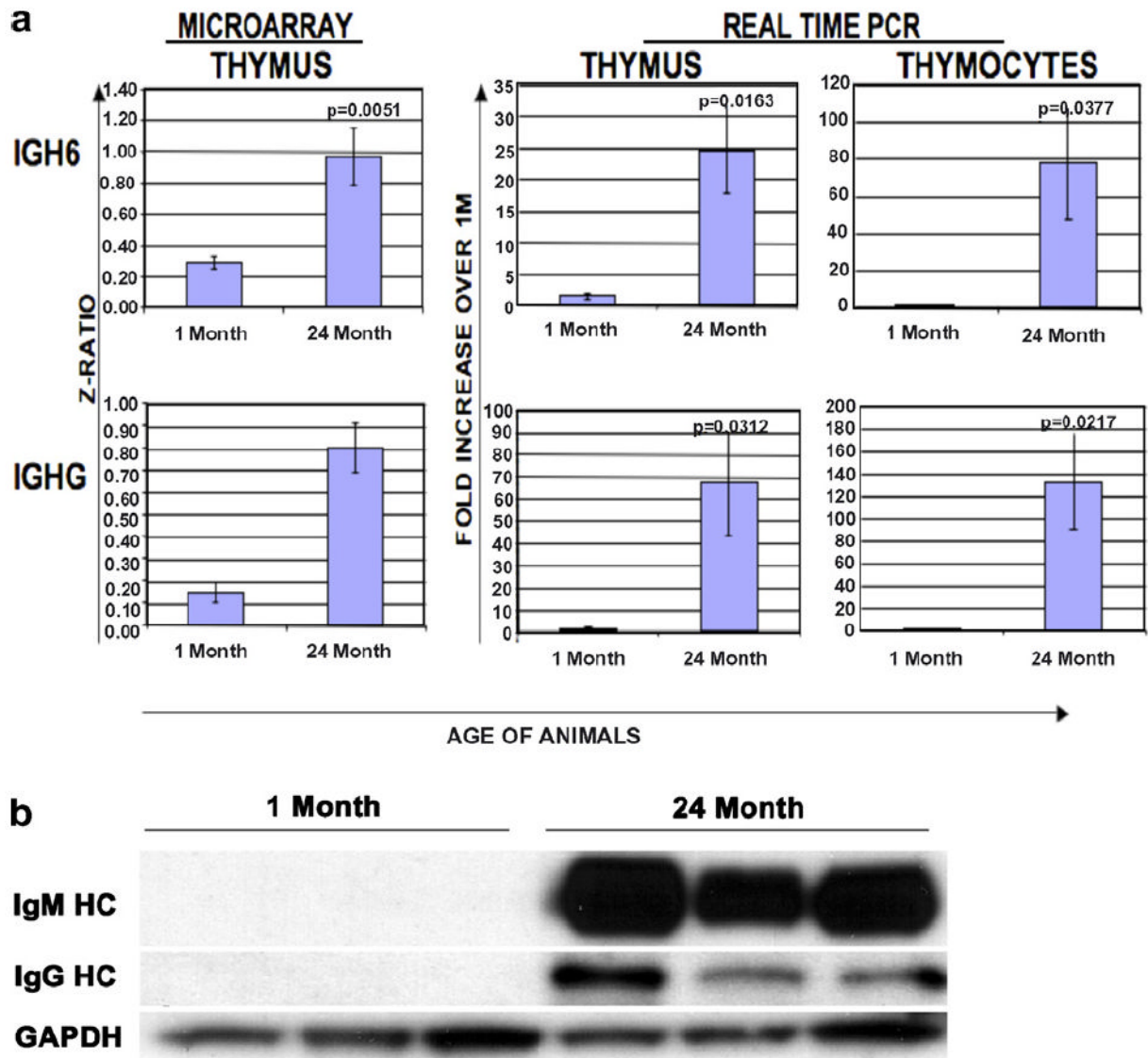


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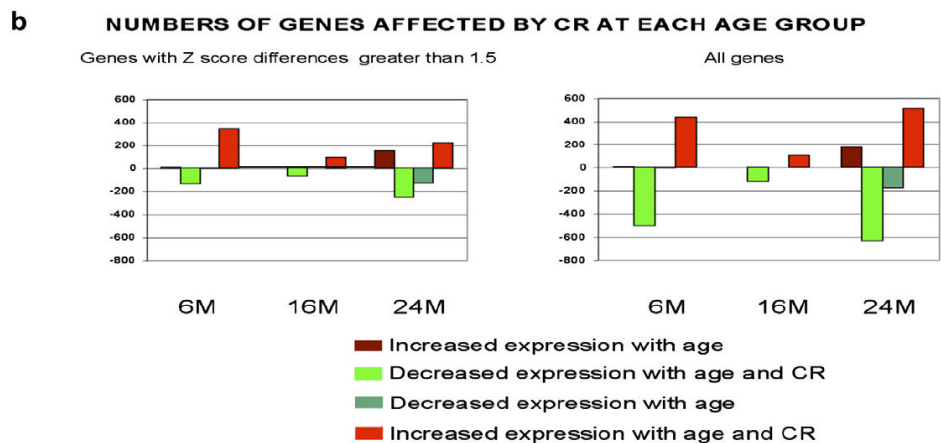
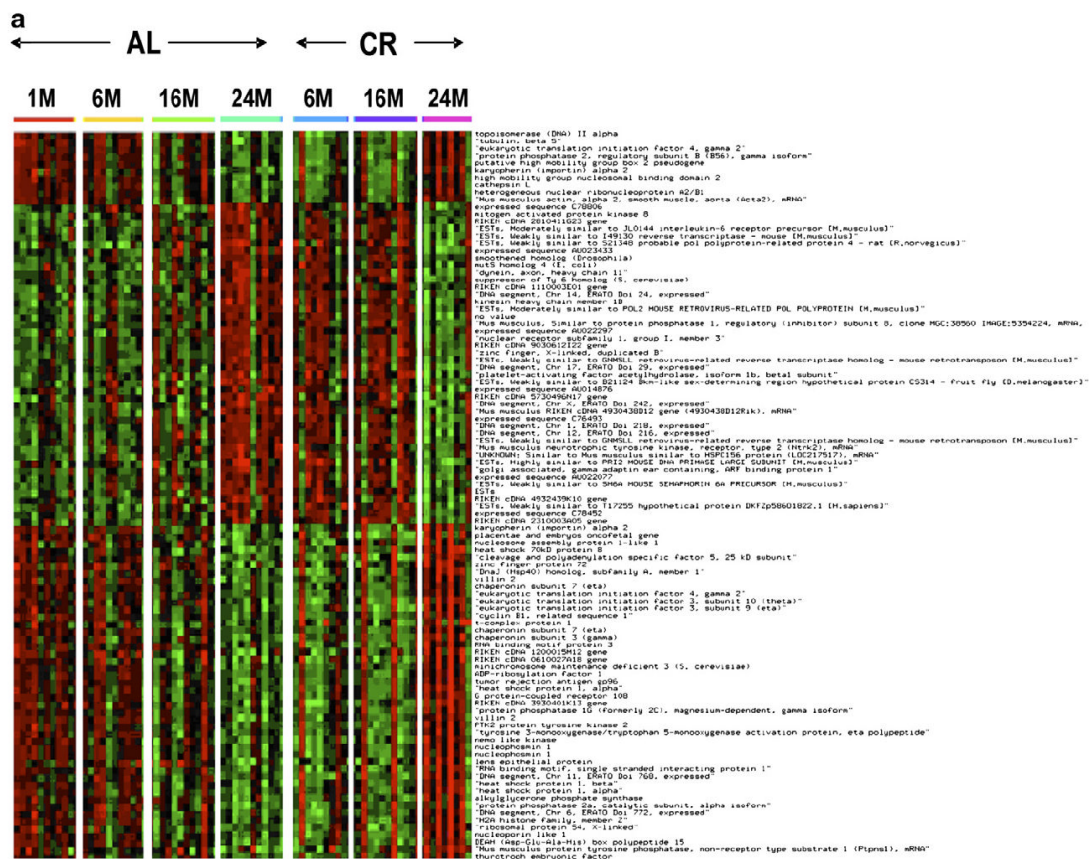


**Fig. 1.** Gene expression profiles of genes which change with age. (a) This profile was created using the Ingenuity program for the genes that exhibited the greatest degree of change when comparing the oldest thymic group to the youngest. The brighter the red, the higher the expression level above the mean for all genes, and the brighter the green the lower the expression level. Expression levels are statistically significant in the 24-month age group. The other age groups are included to exhibit expression trends and may or may not be statistically significant. (b) Comparison of the total numbers of genes affected at each age group.



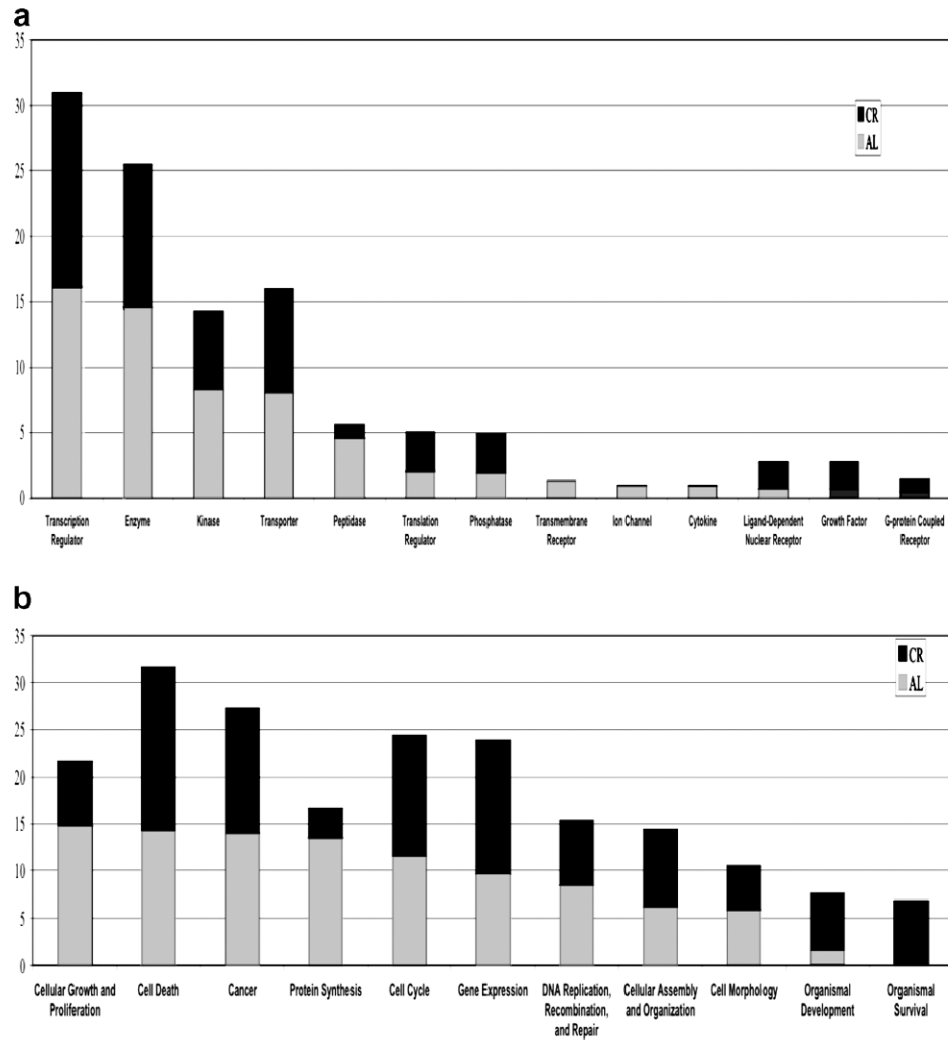
**Fig. 2.** Confirmation of age-associated changes in gene expression data in thymic tissue. (a) Side-by-side comparison of the array results for the genes for the heavy chains, IgM and IgG and the results when comparing young and old thymocyte and thymus samples using real-time PCR. (b) Age-associated differences in immunoglobulin protein expression are evident when comparing protein lysates derived from the total thymocytes of young (1 month) and old (24 months) mice.



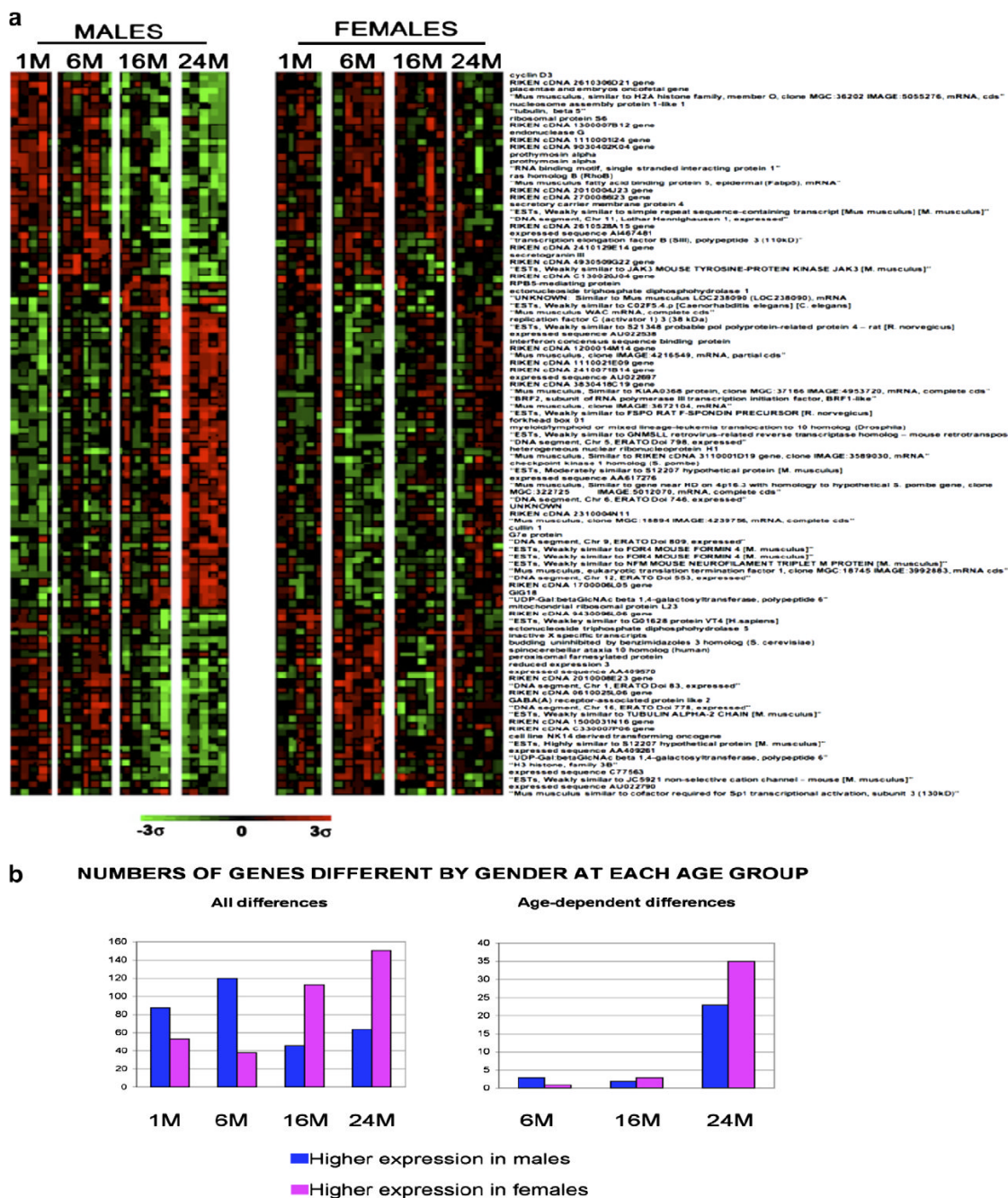


**Fig. 3.** Expression profile of genes exhibiting the greatest differences when comparing the oldest CR mice to the oldest AL mice. (a) This profile was created using the Ingenuity program for the genes that exhibited the greatest degree of change when comparing the oldest (24 months) CR group to the oldest AL group. The brighter the red, the higher the expression level above the mean for all genes, and the brighter the green the lower the expression level. Expression levels are statistically significant at the 24-month age group. The other age groups are included to exhibit expression trends and may or may not be statistically significant. (b) Comparison of the total numbers of genes affected at each age group.

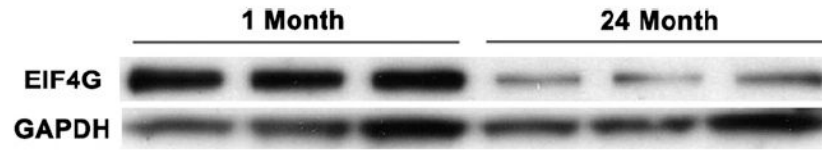




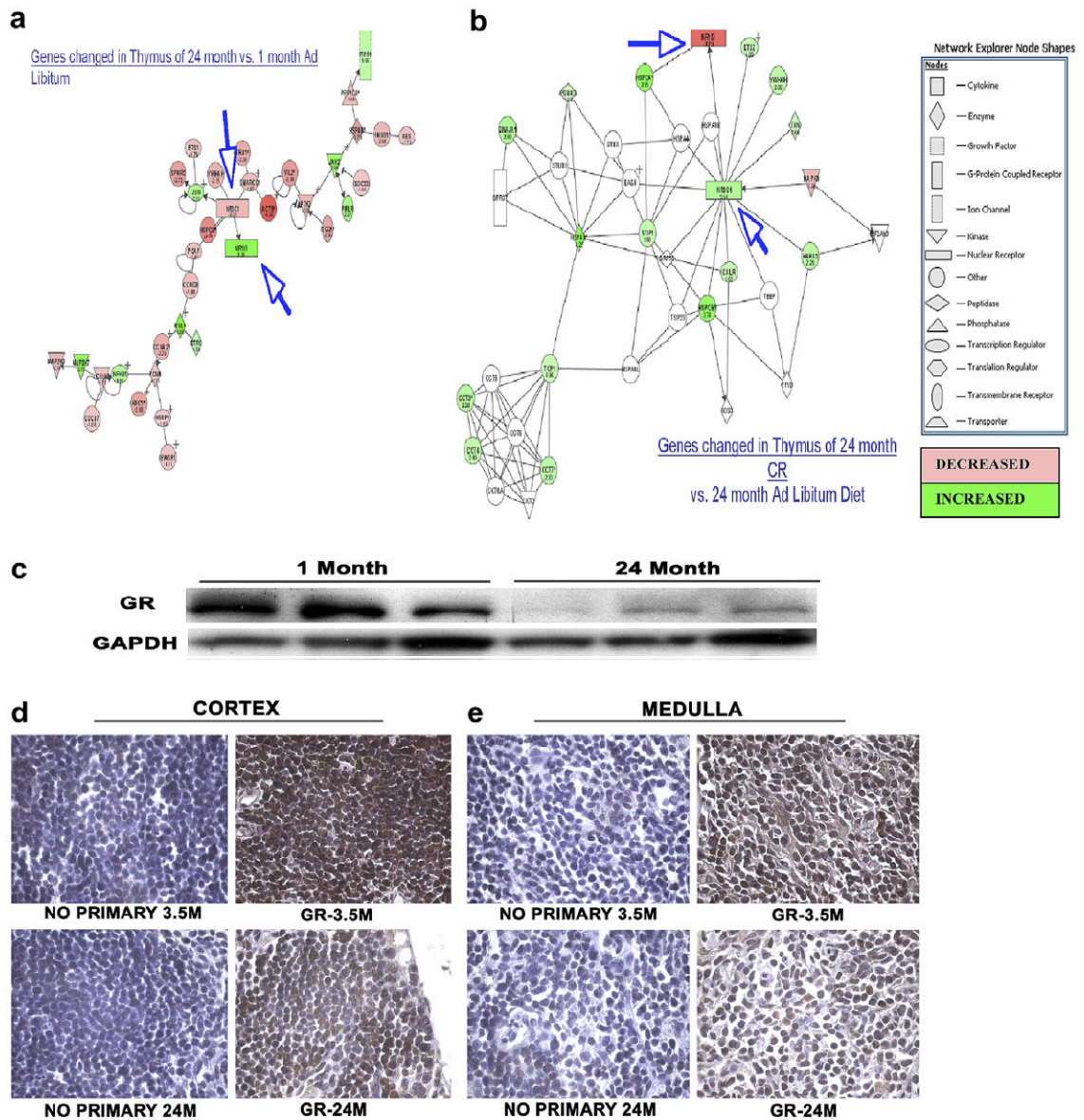
**Fig. 4.** Relative percentages of genes that changed with age and were affected by CR in each of the top categories of gene type (a) and gene function (b). In each graph, the percentage of age and CR-affected genes are shown together for a direct comparison.



**Fig. 5.** Expression profiles of genes that demonstrate expression differences with gender. (a) This profile was created using the Ingenuity program for the genes that exhibited the greatest degree of change when comparing the oldest female *ad libitum* group to the oldest male *ad libitum* group. The brighter the red, the higher the expression level above the means for all genes, and the brighter the green the lower the expression level. Expression levels are statistically significant for the 24-month age group. The other age groups are included to display expression trends and may or may not be statistically significant. (b) Comparison of the total numbers of genes affected in each gender in the context of age.



**Fig. 6.** Western analysis of protein lysates from young and old thymocytes using antibody to eIF4G. In 24-month-old mice, levels of eIF4G protein are significantly decreased as compared to young mice.



**Fig. 7.** Possible pathways for cellular senescence (a) and CR rescue (b) involving the glucocorticoid receptor, androstane receptor and heat-shock proteins. These pathways were designed by the Ingenuity program by compiling published associations between genes in the lists whose expression levels changed with age and were then reversed by a CR diet. (c) Western blot analysis of protein lysates derived from thymocytes of C57BL/6 mice of designated ages confirm that GR is changed in aging thymocytes. (d,e) Whole thymus was also analyzed for GR expression by immunohistochemistry. Sections were stained with a nuclear DAPI stain (blue) and a mouse anti-GR following a standard protocol for DAB staining of paraffin sections. The images (100× magnification) clearly show the GR staining in the young thymus is much darker than that in the old thymus in both the cortex (d) and medulla (e). Data are representative of three experiments, each with one sample from each age.

Table 1

Mouse age, gender and diet distribution for cDNA array experiments

Mice at age of sacrifice		6 months				16 months				24 months			
<i>Ad libitum</i>		<i>Ad libitum</i>		Restricted		<i>Ad libitum</i>		Restricted		<i>Ad libitum</i>		Restricted	
M	F	M	F	M	F	M	F	M	F	M	F	M	F
5	5	5	5	4	5	5	5	5	5	5	5	3	5
Total = 67													

The mice were sacrificed at 1, 6, 16 or 24 months of age. Each age group except the youngest included mice placed on either an *ad libitum* (AL) or a caloric-restricted (CR) diet. These groups were further divided to include both male and female mice in the numbers indicated along the bottom row of the above table.



**Table 2**

Genes with the greatest differences in expression when comparing the oldest age group to the youngest age group

Gene function	No. of genes	Examples	Entrez ID	Fold change
Cellular growth and proliferation	110	Exportin 4	57258	<b>+3.9</b>
		Tnfrsf12a (tumor necrosis factor receptor superfamily)	27279	<b>+3.19</b>
Cell death Cancer	106	Dock7	67299	<b>+3.57</b>
	104	RioK1 (rio kinase)	71340	<b>+3.43</b>
Protein synthesis/ maintenance	100	Gnb2-rs1 (guanine nucleotide binding protein)	14694	<i>-4.05</i>
		ZKscan1 (Zinc finger with KRAB and scan domains)	74570	<b>+3.53</b>
		Zfp294 (Zinc finger)	78913	<b>+3.33</b>
		Eef1a1 (elongation factor)	13627	<i>-4.00</i>
Cell cycle	86	PPIA (peptidyl isomerase)	19034	<i>-4.14</i>
		Cullin 1	26965	<b>+4</b>
		Chek1 (checkpoint kinase 1)	12649	<b>+3.63</b>
Gene expression and transcription	72	Cyclin H	66671	<i>-3.84</i>
		TCF7 (T-cell-specific transcription factor)	21414	<i>-3.58</i>
		Preb (prolactin regulatory element)	50907	<b>+3.46</b>
		Ribosomal S9	76846	<i>-3.53</i>
DNA replication, recombination, and repair	63	ARbp (ribosomal phosphoprotein)	11837	<i>-3.69</i>
		NR1i3 (nuclear receptor subfamily)	12355	<b>+3.36</b>
		HAT1 (histone aminotransferase)	107435	<b>+3.26</b>
		Hmgb2p (high-mobility group, pseudogene)	15352	<i>-3.66</i>
Cellular assembly and organization	46	Hmgn2 (high-mobility group, binding)	15331	<i>-4.95</i>
		H2A, z (histone 2A)	51788	<i>-3.86</i>
		Hnrpa2b1 (heteronuclear riboprotein)	53379	<i>-3.54</i>
Cell morphology Post-translational modification	44	Tubulin A1	22142	<i>-4.32</i>
	41	Actin, $\beta$	11461	<i>-4.58</i>
Other genes of highly significant weight		Ubiquitin B	22187	<i>-3.61</i>
		Cathepsin L	13039	<i>-5.70</i>
		SLC16A2 (solute carrier)	20502	<b>+3.40</b>
		SLC25 (solute carrier)	11740	<i>-3.57</i>
		IgH6 (immunoglobulin)	16019	<b>+3.70</b>
IgHg	380794	<b>+3.60</b>		
LDH2 (lactate dehydrogenase)	16832	<i>-3.55</i>		

This table includes the grouping of gene products into their functional classes as determined by the Ingenuity gene ontology program. The examples shown here encompass the 16 most significantly upregulated (bolded) and 16 most significantly down regulated genes (italicized) in the data set. All data presented is statistically significant at the 24-month age group.

Table 3

Changes in immune-related genes with age

Gene	Gene name	Entrez Gene ID	Change at 24 months	Function
Actb	Actin, $\beta$ , cytoplasmic	11461	<i>-4.58</i>	Impaired in older T cells
Actr3	ARP3 actin-related protein 3 homolog (yeast)	74117	<i>-2.01</i>	Involved in actin polymerization for migration of leukocytes
Arf1	ADP-ribosylation factor 1	11840	<i>-1.75</i>	Cytoskeletal remodeling of lymphoid cells
Biklk	Bcl2-interacting killer-like	12124	<i>-1.61</i>	B cell-specific tyrosine kinase
Calm1	Calmodulin 1	12313	<i>-2.38</i>	Upregulated in activated T cell lines
Capns1	Calpain, small subunit 1	12336	<i>-2.88</i>	Plays important role in CD4+ T-cell selection in the thymus
Ctsl	Cathespin L	13039	<i>-5.70</i>	CD4+ T-cell selection in the thymus
Cullin1	Cullin 1	26965	<b>4.00</b>	T cell production
Diablo	Diablo homolog ( <i>Drosophila</i> )	66593	<i>-2.51</i>	T cell apoptosis
Ets2	E26 avian leukemia oncogene 2, 3' domain	23872	<i>-2.03</i>	Proliferation and survival of thymocytes
Hmgn2	High mobility group nucleosomal binding domain 2	15331	<i>-2.29</i>	Associated with immune diseases
Hspa8	Heat-shock 70 kDa protein 8	15481	<i>-2.79</i>	Induce CTL; carries antigenic peptides
Hspca	Heat-shock protein 1, $\alpha$	15519	<i>-2.82</i>	High levels in rabbit thymus
Hspcb	Heat-shock protein 1, $\beta$	15516	<i>-3.28</i>	High levels in rabbit thymus
Lsm2	LSM2 homolog, U6 small nuclear RNA associated ( <i>Saccharomyces cerevisiae</i> )	27756	<i>-1.97</i>	Variant predominantly expressed in thymus
Mapk8	Mitogen-activated protein kinase 8	26419	<b>2.10</b>	B cell survival
Mcm8	Minichromosome maintenance deficient 3 ( <i>Saccharomyces cerevisiae</i> )	17215	<i>-1.74</i>	B cell response to T-dependent antigens
Ncl	Nucleolin	17975	<i>-2.92</i>	Thymocyte apoptosis and differentiation
Neu1	Neuraminidase 1	18010	<i>-1.57</i>	IL-4 synthesis in T cells
Nr3c1	Nuclear receptor subfamily 3, group C, member 1	14815	<i>-2.01</i>	Necessary for thymocyte survival
Ntrk2	Mus musculus neurotrophic tyrosine kinase, receptor, type 2 (Ntrk2)	18212	<b>2.93</b>	Thymocyte differentiation
Pena	Proliferating cell nuclear antigen	18538	<i>-1.75</i>	Decreased thymocyte proliferation
Ptk2	PTK2 protein tyrosine kinase 2	14083	<i>-2.09</i>	Chemokine response
Rpl7	Ribosomal protein L7	19989	<i>-2.98</i>	High levels of anti-RPL7 IgG in SLE
Socs3	Suppressor of cytokine signaling 3	12702	<i>-1.65</i>	Immune regulation
Tia1	Cytotoxic granule-associated RNA binding protein 1	21841	<i>-1.51</i>	Expressed mainly in T cells and NK cells
Tmsb10	Thymosin, $\beta$ 10	19240	<i>-2.72</i>	Thymosin, $\beta$ 10
Tra1	Tumor rejection antigen gp96	22027	<i>-2.38</i>	Antitumor immunity; CD8+ T-cell responses
Tubb5	Tubulin, $\beta$ 5	22154	<i>-2.40</i>	Abundance in spleen, thymus, and brain
Ywhah	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, $\eta$ polypeptide	22629	<i>-2.15</i>	Control of T cell apoptosis
Ywhaz	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, $\zeta$ polypeptide	22631	<i>-2.18</i>	Control of T cell apoptosis

In the list of genes with the greatest differences in expression levels when comparing oldest to youngest groups, there were many genes with published associations with the immune system. The vast majority are downregulated (italicized). Changes 1.5-fold or greater are in bold and changes  $-1.5$ -fold or less are in italics.

**Table 4**  
Age-associated differences in expression when comparing the oldest CR mice to the oldest AL group

Gene	ACC	Name	6 months AL	16 months AL	24 months AL	6 months CR	16 months CR	24 months CR
(A)								
EST	AW537151	<i>Mus musculus</i> cDNA clone G0112H12	-1.22	-3.62	-2.82	-0.85	1.77	4.46
Hspa8	BG087426	Heat-shock 70 kDa protein 8	-0.68	-2.95	-2.79	-0.49	2.43	4.22
Hsp86-1	BG064773	Heat-shock protein, 86 kDa 1	-0.89	-1.56	-2.76	-0.68	-1.14	3.95
Ctsl	BG078496	Cathepsin L	-2.53	-4.44	-5.70	-2.85	-1.70	3.85
Pkn2	BG078640	Protein Kinase N2	-0.93	-2.93	-3.13	-2.14	-1.71	3.79
Hsp84-1	BG079631	Heat-shock protein, 84 kDa 1	-1.78	-3.53	-3.28	-1.81	-0.80	3.74
EST	AU023376	<i>Mus musculus</i> cDNA clone J0431A10	-1.39	-2.88	-2.77	-1.31	-0.05	3.71
Slc25a5	BG078462	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5	-1.46	-2.93	-3.57	-2.12	-1.03	3.64
Pak2	BG065345	p21 (CDKN1A)-activated kinase 2	-0.50	-3.33	-2.90	-2.18	-0.68	3.61
Hmgn2	BG078806	High mobility group nucleosomal binding domain 2	-2.29	-4.45	-4.95	-4.43	-1.91	3.50
EST	BG071906	<i>Mus musculus</i> cDNA clone H3104E04	0.46	1.22	2.88	2.85	3.76	-3.21
AA209988	AW555450	<i>Mus musculus</i> cDNA clone L0253F11	0.22	1.20	3.36	2.49	3.03	-3.23
EST	BG068086	ESTs, Weakly similar to GNM5LL retrovirus-related reverse transcriptase homolog DNA segment, Chr 1, ERATO Doi 218, expressed	0.00	1.40	2.86	2.27	1.68	-3.25
DIERTD218e	BG066358	Insulin-like growth factor binding protein 7	-0.29	1.18	2.63	2.61	1.02	-3.25
Igfbp7	BG068536	Insulin-like growth factor binding protein 7	0.48	1.65	2.62	2.48	1.32	-3.28
EST	BQ551169	<i>Mus musculus</i> cDNA clone H4007G03	1.00	1.68	4.17	2.97	2.71	-3.33
EST	BG070998	<i>Mus musculus</i> cDNA clone H3093E11	-0.59	1.04	2.47	2.92	1.70	-3.35
EST	BG067761	<i>Mus musculus</i> cDNA clone H3057H05	-0.26	3.47	3.98	3.45	1.44	-3.41
EST	BQ551211	<i>Mus musculus</i> cDNA clone H4008A02	1.00	1.67	4.06	2.55	3.24	-3.90
EST	BG070193	<i>Mus musculus</i> cDNA clone H3084F12	0.15	1.38	4.27	2.57	5.44	-4.02
(B)								
Pipns1	BQ551724	<i>Mus musculus</i> protein tyrosine phosphatase, non-receptor type substrate 1 (Pipns1)	-1.39	-0.24	-2.86	-1.67	-0.50	5.20
EST	BQ551457	<i>Mus musculus</i> cDNA clone H4009D09	-4.38	1.73	-2.56	2.47	-2.23	2.94
EST	BG082760	<i>Mus musculus</i> cDNA clone H3079H08	-0.47	-1.30	-1.37	-1.25	-0.34	2.52
Meap2	BG086961	Methionine aminopeptidase 2	-0.22	0.61	-2.18	-0.54	-0.78	2.42
Fads1	BG078755	RIKEN cDNA 0710001O03 gene	-0.25	1.19	-1.26	-1.32	-3.42	2.32
EST	BG086658	<i>Mus musculus</i> cDNA clone H3129A03	0.64	-0.55	-1.95	-1.21	-0.22	2.32
Zfp87	NA	Zinc finger protein 87	-0.21	-0.25	-2.25	-0.53	-0.59	2.30
AU021304	BG069921	Expressed sequence AU021304	-0.96	2.22	-1.36	-0.66	-4.43	2.28
Hspa5	BG078795	Heat-shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa)	-0.51	-2.16	-1.08	-0.52	1.64	2.26

Gene	ACC	Name	6 months AL	16 months AL	24 months AL	6 months CR	16 months CR	24 months CR
EST	NA	ESTs, Weakly similar to SCP3 mouse synaptonemal complex protein 3	-0.56	-0.14	-1.10	-0.39	-1.44	2.19
C78452	BG066208	Expressed sequence C78452	-1.74	<b>1.50</b>	<b>2.41</b>	<b>3.89</b>	<b>2.71</b>	-2.96
EST	BG069906	<i>Mus musculus</i> cDNA clone H3081D07	-4.00	-5.77	-1.52	-4.17	-4.16	-3.10
EST	BQ551932	<i>Mus musculus</i> cDNA clone H4012D07	-1.20	-0.54	0.71	0.60	0.03	-3.20
EST	AU015698	<i>Mus musculus</i> cDNA clone J0715C06	-1.10	1.39	<b>2.51</b>	<b>2.98</b>	<b>1.69</b>	-3.22
EST	BG066049	ESTs, Weakly similar to I49130 reverse transcriptase—mouse [ <i>Mus musculus</i> ]	-1.02	<b>1.98</b>	<b>2.63</b>	<b>3.21</b>	1.27	-3.26
EST	BQ552641	<i>Mus musculus</i> cDNA clone H4017B10	-1.19	<b>1.57</b>	<b>2.60</b>	<b>4.11</b>	<b>2.24</b>	-3.29
EST	BG068246	<i>Mus musculus</i> cDNA clone H3063C12	0.79	<b>1.75</b>	<b>2.96</b>	<b>2.56</b>	<b>2.81</b>	-3.53
Vps41	NA	Vacuolar protein sorting 41 (yeast)	-0.48	1.22	<b>2.27</b>	0.89	<b>1.55</b>	-3.65
EST	BG068614	<i>Mus musculus</i> cDNA clone H3067D11	0.62	<b>2.77</b>	<b>3.10</b>	<b>2.87</b>	<b>1.91</b>	-3.65
Cenpa	BG082881	Centromere autoantigen A	0.04	<b>2.76</b>	1.47	-0.10	-3.55	-4.33

(A) The data presented are statistically significant at the 24-month age group comparing animals under different dietary regimens. (B) The data presented are statistically significant differences in gene expression affected by a CR diet independent of age. Values for other age groups are shown for complete information but may not necessarily be substantial or statistically significant values. Changes 1.5-fold or greater are in bold and changes -1.5-fold or less are in italics.

Table 5

Gender- and age-associated differences in gene expression

Gene symbol	Gene name	Entrez ID	Overall				Female vs male						
			6 months	16 months	24 months	6 months	1 month	6 months	16 months	24 months			
(A)													
Xist	Inactive X specific transcripts	22438	2.48	1.71	1.49	6.52	3.55	3.22	3.66				
Abilm1	Actin-binding LIM protein 1	107346	0.41	-2.38	-2.17	-0.13	-1.07	2.16	3.40				
Igfbp5	Insulin-like growth factor binding protein 5	98292	1.46	-0.16	-0.43	-0.02	-0.66	-1.48	3.04				
H3f3b	H3 histone, family 3B	15081	-0.26	-2.10	-1.92	1.36	-0.55	2.17	2.19				
Rnpc2	RNA-binding region (RNPI, RRM) containing 2	170791	0.17	-1.07	-1.02	0.20	-1.65	0.89	2.06				
In	Leadon	16920	0.63	0.31	0.76	0.27	-0.09	-0.53	1.93				
Wasl	Wiskott-Aldrich syndrome-like (human)	73178	0.21	0.57	-0.35	-0.37	-1.40	-0.71	1.91				
Adrm1	Adhesion regulating molecule 1	56436	-0.33	-0.96	-1.09	1.21	0.34	0.85	1.86				
Shyc	Selective hybridizing clone	20430	0.06	-0.59	-0.47	1.08	-0.74	0.46	1.85				
Beas3	Breast carcinoma amplified sequence 3	104502	0.56	-0.21	-0.10	-0.18	-0.31	-0.22	1.84				
D5Erd798e	DNA segment, Chr 5, ERATO Doi 798, expressed	52409	-0.45	0.57	2.56	0.67	2.48	-0.03	-3.67				
Plazg7	Phospholipase A2 group VII	27226	-0.71	1.67	1.34	1.19	0.27	-1.40	-3.70				
Brf2	BRF2, subunit of RNA polymerase III transcription initiation factor	66653	-0.85	2.72	3.07	-1.65	2.90	-1.76	-3.80				
Hat1	Histone aminotransferase 1	67376	-0.58	3.17	3.26	-0.93	1.69	-2.42	-4.15				
Oog3	Oogenesis 3	100012	-0.50	2.59	2.63	-1.01	1.89	-0.91	-4.34				
Chek1	Checkpoint kinase 1 homolog ( <i>Schizosaccharomyces pombe</i> )	12649	-0.33	3.40	3.63	0.39	3.01	-1.94	-4.56				
1200014M14Rik	RIKEN cDNA 1200014M14 gene	67463	-0.21	1.13	3.51	-1.26	2.94	-0.64	-4.57				
No value	<i>Mus musculus</i> , clone IMAGE: 4216549, mRNA, partial cds	No value	-0.80	3.74	3.98	-1.20	4.21	-1.73	-5.07				
Cul1	Cullin 1	26965	-0.18	1.12	4.00	0.75	3.42	-0.37	-5.27				
No value	<i>Mus musculus</i> , clone IMAGE: 3672104, mRNA	No value	-0.02	2.00	4.50	-0.98	3.24	-0.96	-5.33				
(B)													
Adrm1	Adhesion regulating molecule 1	56436	-0.33	-0.96	-1.09	1.21	0.34	0.85	1.86				
Arhb	Ras homolog B (RhoB)	11852	0.17	-0.97	-0.94	0.09	-0.94	0.80	1.81				
No value	ESTs, Weakly similar to G01628 protein VT4 ( <i>Homo sapiens</i> )	No value	-0.80	-1.28	-1.19	-0.52	0.81	0.15	1.79				
No value	<i>Mus musculus</i> , clone IMAGE: 4504748, mRNA	No value	-0.43	-1.86	-1.21	1.26	-0.60	2.29	1.77				
Fabp5	<i>Mus musculus</i> fatty acid binding protein 5, epidermal (Fabp5)	16592	-0.05	-1.09	-1.11	0.66	-0.64	1.64	1.70				
Bzw2	Basic leucine zipper and W2 domains 2	66912	-0.55	-1.58	-1.42	0.04	-1.21	0.05	1.65				
Tubb5	Tubulin, $\beta$ 5	22154	-0.23	-1.18	-1.40	0.69	-0.83	0.37	1.63				
Mki67ip	Mki67 (FHA domain) interacting nuclear phosphoprotein	67949	-0.27	-0.10	-1.10	-0.26	0.20	-0.61	1.62				
Nap111	Nucleosome assembly protein 1-like 1	53605	-0.89	-0.79	-1.27	0.33	-1.33	0.22	1.57				
Rfc3	Replication factor C (activator 1) 3 (38 kDa)	69263	-0.53	2.58	2.56	-0.39	2.17	-1.48	-3.47				
D5Erd798e	DNA segment, Chr 5, ERATO Doi 798, expressed	52409	-0.45	0.57	2.56	0.67	2.48	-0.03	-3.67				



Gene symbol	Gene name	Entrez ID	Overall			Female vs male			
			6 months	16 months	24 months	1 month	6 months	16 months	24 months
Brf2	BRF2, subunit of RNA polymerase III transcription initiation factor	66653	-0.85	2.72	3.07	2.90	-1.65	-1.76	-3.80
Hat1	Histone aminotransferase 1	67376	-0.58	3.17	3.26	1.69	-0.93	-2.42	-4.15
Ovg3	Oogenesis 3	100012	-0.50	2.59	2.63	1.89	-1.01	-0.91	-4.34
Chek1	Checkpoint kinase 1 homolog ( <i>Schizosaccharomyces pombe</i> )	12649	-0.33	3.40	3.63	3.01	0.39	-1.94	-4.56
1200014M14Rik	RIKEN cDNA 1200014M14 gene	67463	-0.21	1.13	3.51	2.94	-1.26	-0.64	-4.57
No value	<i>Mus musculus</i> , clone IMAGE:4216549, mRNA, partial cds	No value	-0.80	3.74	3.98	4.21	-1.20	-1.73	-5.07
Cul1	Cullin 1	26965	-0.18	1.12	4.00	3.42	0.75	-0.37	-5.27
No value	<i>Mus musculus</i> , clone IMAGE:3672104, mRNA	No value	-0.02	2.00	4.50	3.24	-0.98	-0.96	-5.33

(A) This table shows the genes with the greatest age-associated differences in expression when comparing the oldest female *ad libitum* age group to the oldest male *ad libitum* age group. All data presented is statistically significant at the 24-month age group. (B) Age-associated differences between males and females on an AL diet. These genes not only showed differences in expression levels between males and females but also had statistically significant differences when comparing the oldest to the youngest age group as a whole. Values for other age groups are shown for complete information but may not necessarily be substantial or statistically significant values. Changes 1.5-fold or greater are in bold and changes -1.5-fold or less are in italics.

Tumor-associated genes which showed the greatest differences in expression levels at age 24 months when female values were compared to male values

Table 6

Gene symbol	Gene name	1 month	6 months	16 months	24 months
Xist	Inactive X specific transcripts	<b>3.55</b>	<b>6.52</b>	<b>3.22</b>	<b>3.66</b>
Ablim1	Actin-binding LIM protein 1	-1.07	-0.13	<b>2.16</b>	<b>3.40</b>
Igfbp5	Insulin-like growth factor binding protein 5	-0.66	-0.02	-1.48	<b>3.04</b>
Tmsb10	Thymosin, $\beta$ 10	-0.46	<b>3.31</b>	<b>3.26</b>	<b>2.44</b>
Pima	prothymosin $\alpha$	-1.23	0.77	<b>4.00</b>	<b>2.11</b>
Wasl	Wiskott-Aldrich syndrome-like (human)	-1.40	-0.37	-0.71	<b>1.91</b>
Beas3	Breast carcinoma amplified sequence 3	-0.31	-0.18	-0.22	<b>1.84</b>
Rhob	Ras homolog gene family, member B	-0.94	0.09	0.80	<b>1.81</b>
Col2a1	Procollagen, type II, $\alpha$ 1	-0.64	-0.24	0.87	<b>1.79</b>
Fabp5	<i>Mus musculus</i> fatty acid binding protein 5, epidermal (Fabp5), mRNA	-0.28	0.66	<b>1.64</b>	<b>1.70</b>
Pem	Placenta and embryos oncofetal gene	-0.56	-0.81	0.96	<b>1.65</b>
Bzw2	Basic leucine zipper and W2 domains 2	-1.21	0.04	0.05	<b>1.65</b>
Ncor1	Nuclear receptor co-repressor 1	-0.52	1.12	1.26	<b>1.64</b>
Sp3	Trans-acting transcription factor 3	0.52	-0.17	0.36	<b>1.57</b>
Nap1l1	Nucleosome assembly protein 1-like 1	-1.33	0.33	0.22	<b>1.57</b>
Got2	Glutamate oxaloacetate transaminase 2, mitochondrial	-1.98	0.17	-0.41	<b>1.56</b>
Fanc1	Fanconi anemia, complementation group L	-0.59	-1.22	0.42	<b>1.56</b>
Hif1a	Hypoxia inducible factor 1, alpha subunit	0.07	0.65	1.00	<b>1.51</b>
Clic4	Chloride intracellular channel 4 (mitochondrial)	-0.70	1.01	0.41	<b>1.50</b>
Edf1	Endothelial differentiation-related factor 1	-0.41	<b>2.65</b>	<b>2.97</b>	1.46
Sdcbp	Syndecan binding protein	-0.58	-0.11	-0.07	1.46
Ldb1	LIM domain binding 1	0.57	-0.21	-0.77	1.45
Rab8a	RAB8A, member RAS oncogene family	-0.50	0.95	-0.13	1.44
Aip2a2	ATPase, Ca <sup>2+</sup> transporting, cardiac muscle, slow twitch 2	-0.59	-3.52	-0.29	1.40
Entpd1	Ectonucleoside triphosphate diphosphohydrolase 1	0.00	-0.13	-0.93	1.38
Rps16	Ribosomal protein S16	-0.75	0.58	<b>2.55</b>	1.37
Cdc25a	Cell division cycle 25 homolog A ( <i>Saccharomyces cerevisiae</i> )	-0.57	-0.38	-0.78	1.37
Bcl9	B-cell CLL/lymphoma 9	<b>1.51</b>	-2.02	-0.50	-1.03
Mad1l1	Mitotic arrest-deficient 1-like 1	0.92	-1.15	1.24	-1.06
Ptprg	Protein tyrosine phosphatase, receptor type, G	-1.40	0.29	1.05	-1.09
Bcas1	Breast carcinoma amplified sequence 1	0.31	-0.40	0.51	-1.11
Jak2	Janus kinase 2	0.92	0.79	-1.06	-1.26
Foxo1	Forkhead box O1	1.06	-0.40	-1.62	-1.38
Rgs2	Regulator of G-protein signaling 2	<b>2.09</b>	0.48	-1.69	-1.43
Lanc2	LanC (bacterial lantibiotic synthetase component C)-like 2	<b>1.88</b>	-0.69	0.13	-1.46
Tde2	Tumor differentially expressed 2	-0.79	0.54	<b>2.21</b>	-1.74
Eps8	Epidermal growth factor receptor pathway substrate 8	-0.01	-1.59	-0.82	-1.75
Icsp8	Interferon consensus sequence binding protein	<b>2.31</b>	-1.07	-0.95	-1.77
Lgals8	Lectin, galactose binding, soluble 8	0.11	1.11	-0.59	-1.80
Llgh	Lethal giant larvae homolog	-0.38	0.80	0.50	-1.82
Mdm4	Transformed mouse 3T3 cell double minute 4	0.71	<b>3.74</b>	-0.96	-1.90
G3bp2	Ras-GTPase-activating protein (GAP<120>) SH3-domain binding protein 2	1.02	-0.98	-0.23	-2.10
Cend1	Cyclin D1	-0.35	-1.79	-1.69	-2.23
Greb1	Gene regulated by estrogen in breast cancer protein	<b>2.87</b>	-0.56	-1.04	-2.29
Mllt10	Myeloid/lymphoid or mixed lineage-leukemia translocation to 10 homolog ( <i>Drosophila</i> )	<b>1.64</b>	-1.13	-0.81	-2.32
Ube2h	Ubiquitin-conjugating enzyme E2N	1.31	-0.88	-0.55	-2.56
Rassf5	Ras association (RalGDS/AF-6) domain family 5	<b>1.87</b>	-0.37	0.27	-2.73
Cbl	Casitas B-lineage lymphoma	<b>1.88</b>	-1.23	-0.91	-3.44
Plat2g7	Phospholipase A2 group VII (platelet-activating factor acetylhydrolase, plasma)	0.27	1.19	-1.40	-3.70
Brf2	BRF2, subunit of RNA polymerase III transcription initiation factor, BRF1-like	<b>2.90</b>	-1.65	-1.76	-3.80

Gene symbol	Gene name	1 month	6 months	16 months	24 months
Hat1	Histone aminotransferase 1	<b>1.69</b>	-0.93	-2.42	-4.15
Chk1	Checkpoint kinase 1 homolog ( <i>Schizosaccharomyces pombe</i> )	<b>3.01</b>	0.39	-1.94	-4.56
Cul1	Cullin 1	<b>3.42</b>	0.75	-0.37	-5.27

All data presented are statistically significant in at least one age group. Changes 1.5-fold or greater are in bold and changes  $-1.5$ -fold or less are in italics.

Summary of real-time PCR confirmation of array results and comparison to thymocyte gene expression

Table 7

Symbol	Gene name	Entrez ID	Changes with age in:		
			Array	Thymus	Thymocytes
CCT3	Chaperonin subunit 3 ( $\gamma$ )	12462	D	D*	D
CTS1	Cathepsin L	13039	D	D	D*
Dock7	Dedicator of cytokinesis 7	67299	U	U	U
EIF3S10	Eukaryotic translation initiation factor 3, subunit 10 (H)	13669	D	D	NC
EIF4C2	Eukaryotic translation initiation factor 4, $\gamma$ 2	13690	D	D*	D*
ETFB	Electron transferring flavoprotein, $\beta$ polypeptide	110826	U	U*	NC
HSPA8	Heat-shock 70 kDa protein 8	15481	D	D	NC
HSPCA	Heat-shock protein 1, $\alpha$	15519	D	D	D*
HSPCB	Heat-shock protein 1, $\beta$	15516	D	D	NC
IGH6	Immunoglobulin heavy chain 6 (heavy chain of IgM)	16019	U	U*	U*
IGHG	Immunoglobulin heavy chain ( $\gamma$ polypeptide)	380794	U	U*	U*
NR1B	Nuclear receptor subfamily 1, group 1, member 3	12355	U	U	NC
NR3C1	Nuclear receptor subfamily 3, group C, member 1	14815	D	D*	D
SMARCD2	SWI/SNF-related, matrix associated, actin-dependent regulation of chromatin, subfamily d, 2	83796	D	D	D*
VIL2	Villin 2	22350	D	D*	D

Data represent comparison of results using six samples from each of the 1- and 24-month age groups. D, downregulated; U, upregulated; NC, no change. Asterisk indicates data with a  $p$  value  $\leq 0.05$ .