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Nab2 maintains thymus cellularity with aging and stress

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Abstract

Thymic cellularity is influenced by a variety of biological and environmental factors, such as age and stress; however, little is known about the molecular genetic mechanisms that regulate this process. Immediate early genes of the Early growth response (Egr) family have critical roles in immune function and response to environmental stress. The transcription factors *Egr1*, *Egr2* and *Egr3*, play roles in the thymus and in peripheral T-cell activation. *Nab2*, which binds Egrs 1, 2, and 3 as a co-regulator of transcription, also regulates peripheral T-cell activation. However, a role for *Nab2* in the thymus has not been reported. Using *Nab2*-deficient (KO) mice we found that male *Nab2*KO mice have reduced thymus size and decreased numbers of thymocytes, compared with age-matched wildtype (WT) mice. Furthermore, the number of thymocytes in *Nab2*KO males decreases more rapidly with age. This effect is sex-dependent as female *Nab2*KO mice show neither reduced thymocyte numbers nor accelerated thymocyte loss with age, compared to female WT littermates. Since stress induces expression of *Nab2* and the *Egr*s, we examined whether loss of *Nab2* alters stress-induced decrease in thymic cellularity. Restraint stress induced a significant decrease in thymic cellularity in *Nab2*KO and WT mice, with significant changes in the thymocyte subset populations only in the *Nab2*KO mice. Stress reduced the percentage of DP cells by half and increased the percentage of CD4SP and CD8SP cells by roughly three-fold in *Nab2*KO mice.

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Conflict of interest

The authors declare no conflict of interest.

These findings indicate a requirement for *Nab2* in maintaining thymocyte number in male mice with age and in response to stress.

Keywords

Immediate early gene; NGFI-A binding protein; early growth response gene; thymus; stress; aging

1. Introduction

The thymus is a lymphoid organ that decreases in size with age and in response to environmental stimuli such as stress. The thymus is the major site of T cell development, producing self-tolerant, mature T cells required for robust immunity. In the thymus, immature T cells, referred to as thymocytes, rearrange TCR genes and gain expression of CD4, CD8 and TCR molecules. The thymus undergoes a precipitous decline in cellularity very early in life, presumably after the thymus has populated the peripheral T cell compartment, and then gradually declines with age (termed thymic involution), as characterized by decreased thymus weight, change in morphology, and loss of thymocyte numbers (Aw, et al. 2010; Li, et al. 2003; Ortman, et al. 2002; Palmer 2013; Sempowski, et al. 2002). Numerous types of stress (e.g. restraint, exercise, and infection) as well as endogenous glucocorticoids, induce transient, acute thymic involution (Ayala, et al. 1995; Compton and Cidlowski 1986; Concordet and Ferry 1993; Gruber, et al. 1994; Gruver and Sempowski 2008; Jondal, et al. 1993; Sun, et al. 1992; Tarcic, et al. 1998). Yet, the molecular pathways involved in control of thymus cellularity are not fully understood.

Immediate early genes of the early growth response gene (Egr) family play roles in the thymus as well as in peripheral T cell function and are activated in response to changes in the environment, including stress. The Egr-family consists of four highly homologous zinc-finger transcription factors: *Egr1* (NGFI-A, zif-268, Krox 24), *Egr2* (Krox 20), *Egr3* (PILOT), and *Egr4* (NGFI-C). *Egr*s 1, 2, and 3 are induced in response to pre-TCR stimulation and are important in thymocyte development (Bettini, et al. 2002; Carleton, et al. 2002; Lauritsen, et al. 2008; Li, et al. 2011; Shao, et al. 1997; Xi and Kersh 2004a; Xi and Kersh 2004b; Xi, et al. 2006). *Egr1* augments, while *Egr2* and *Egr3* inhibit, peripheral T cell function (Collins, et al. 2008; Collins, et al. 2006; Safford, et al. 2005). As immediate early genes, *Egr1* and *Egr3*, and to a lesser extent *Egr2*, are activated in response to numerous types of physical and psychological stressors (Honkaniemi, et al. 2000; Huang and Tunnacliffe 2005; Lyn, et al. 2000; Meaney, et al. 2000; Orsetti, et al. 2008; Senba and Ueyama 1997). In addition, loss of either *Egr1* or *Egr3* alters stress-responsive and anxiety-related behaviors in mice, indicating that the expression of these genes plays a functional role in mediating the stress response (Gallitano-Mendel, et al. 2007; Ko, et al. 2005). These processes may be mediated via glucocorticoids acting either upstream or downstream of *Egr1* and *Egr3* (Gallitano-Mendel, et al. 2007; Leclerc, et al. 2008; Sarrazin, et al. 2009; Weaver, et al. 2007).

At the molecular level, *Egr*s regulate gene expression via interaction with transcriptional co-regulatory proteins NGFI-A binding protein (NAB)1 and NAB2. EGR1, EGR2, and EGR 3,

but not EGR4, proteins contain a conserved NAB-binding (R1) domain through which the NAB proteins enact their co-repressive (Russo, et al. 1995; Svaren, et al. 1996; Svaren, et al. 1998) or co-activating (Collins, et al. 2006; Sevetson, et al. 2000) actions on EGR-mediated transcription. Moreover, EGR1, EGR2, and EGR3 regulate expression of *Nab2*, and the resulting NAB2-EGR complex in turn inhibits expression of *Egr1*, *Egr2*, and *Egr3*, establishing co-regulatory feedback loops among these genes (Kumbrink, et al. 2005; Kumbrink, et al. 2010; Srinivasan, et al. 2007).

Nab2 functions together with *Egr*s 1, 2, and 3 to regulate peripheral T cell activation. However, while *Egr*s 1, 2, and 3 also regulate thymocyte development, little is known about the role of *Nab2* in the thymus. Also, despite the fact that *Nab2* interacts with *Egr1* and *Egr3* in numerous other systems, and both *Egr1* and *Egr3* play critical roles in the stress response (Gallitano-Mendel, et al. 2007; Ko, et al. 2005; Saadane, et al. 2000), a function of *Nab2* in stress has not been identified. To determine the role of *Nab2* in the thymus, we evaluated thymocyte number in *Nab2*KO mice in conditions known to influence thymic size, namely age and stress. In this study, we identified a previously unknown role for the *Nab2* gene in maintaining thymic size and thymocyte number.

2. Materials and methods

2.1. Mice

Previously generated *Nab2*KO mice were back-crossed to the C57BL/6 background strain for greater than 20 generations (Le, et al. 2005). Animals were housed in micro-isolator cages on a 12 h light/dark cycle with ad libitum access to food and water, except where noted for stress experiments. Studies were performed on homozygous progeny derived from breedings of heterozygous males and females. Animals were genotyped, and *Nab2*KO and WT control mice were assigned as matched pairs at the time of weaning. Paired animals underwent all procedures identically. Whenever possible, pairs were assigned from the same litter. Unless otherwise noted, experiments were conducted with adult male mice. Animals were sacrificed by isoflurane anesthesia overdose followed by cervical dislocation. Thymuses were harvested, and thymocytes were liberated by mechanical dissociation and enumerated using a hemocytometer. These studies were conducted in accordance with the guidelines of, and approved by, the Institutional Animal Care and Use Committees of the University of Arizona and Arizona State University.

2.2. Histology

Thymuses were fixed in 10% buffered formalin, washed in 70% ethanol, and paraffin-embedded using a Leica TP1020 automatic tissue processor. Five μm sections were generated from coronal sections of the thymuses and stained with hematoxylin and eosin. Slides were scanned using the Aperio ScanScope GL system and images captured with Aperio ImageScope software. For quantification of thymic cortical thickness, boundaries were drawn around the total thymic section and the corticomedullary junction using ImageJ analysis software. Cross-sectional cortical area was calculated as a percentage of total thymus area.

2.3. Flow cytometry

CD16/CD32 (FcyRIII/II) was blocked using rat anti-mouse CD16/CD32 mAb (Fc block, BD Biosciences, San Jose, CA). Cells were stained in PBS with 1% BSA and 0.05% NaN_3 with anti-CD4-PerCP (clone RM4-5) and anti-CD8-allophycocyanin (clone 53-6.7) mAbs (BD Biosciences). Cell-associated fluorescence was measured using an LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

2.4. Corticosterone assay

Serum samples for corticosterone levels were obtained from adult male mice via mandibular bleed at the circadian nadir, shortly after “lights-on”. To preclude the stress of the procedure affecting the samples, venipuncture was completed on each animal within 2.75 min of touching the cage. Samples were placed on ice until centrifugation (9,200 × g at 4 °C × 5 min), and serum was transferred to fresh tubes and stored at −20 °C. Serum hormone concentrations were determined by radioimmunoassay (MP Biomedicals Diagnostic Division, New York, NY, catalogue # 07-120102) according to the manufacturer’s instructions. Briefly, samples were compared to a standard curve run on standardized samples provided with the kit. For each serum sample, 50 μl of a 1:100 dilution of serum in steroid diluent was mixed with 100 μl of corticosterone ^{125}I reagent and 100 μl of anti-corticosterone reagent in each of two duplicate sample test tubes. Samples were mixed and incubated at room temperature for 120 min. Following addition of 250 μl of precipitating solution, samples were mixed, centrifuged at 1,000 g for 40 min and the supernatant removed by inversion. Precipitated ^{125}I levels were determined by gamma counter. Counts from duplicate samples were averaged and corticosterone concentrations were calculated using the equation from the standard curve of corticosterone concentration versus percent-bound.

2.5. Restraint stress

Mice were restrained using a previously described method (Tarcic, et al. 1998). Briefly, 50 ml polypropylene conical centrifuge tubes were cut to approximately the length of an adult mouse. The conical tip was removed for an anterior ventilation hole approximately 0.5 cm in diameter, and an approximately 1 cm hole in the lid was made to provide space for the tail and posterior ventilation. Four to six ventilation holes were drilled out on the sides to prevent hyperthermia. This device provides sufficient space for chest movements of breathing as well as lateral and anterior-posterior motion, yet is insufficient for the mouse to turn around. For the restraint stress cohort, *Nab2*KO mice and their WT littermates were restrained for two 12 h sessions (during the dark phase) separated by a 12 h recovery interval in the home cage. Animals were sacrificed immediately following completion of the stress. Control *Nab2*KO and WT mice remained in their home cages continuously, and were sacrificed at the same time as their experimental counterparts. For stress recovery experiments, mice were restrained for two 12 h stress periods spaced 12 h apart. After stress, the mice returned to their home cages and allowed to recover for 1, 2 or 3 weeks prior to euthanasia and tissue harvesting.

2.6. Statistical Analyses

Nonparametric tests were used for all comparisons to avoid making the assumption that data were normally distributed. For comparison of outcome measures within matched pairs of *Nab2*KO and WT mice, e.g. thymus weight, thymocyte number, and corticosterone levels, we used the Wilcoxon signed-rank test. For comparison between two independent groups/conditions, such as no stress versus restraint, we used the Wilcoxon rank-sum test. This test can be used to compare a single genotype between two conditions or whether the two genotypes respond differently between two conditions. For comparison across more than two groups/conditions we used the Kruskal-Wallis test, which is an extension of the Wilcoxon rank-sum test to situations with more than two groups/conditions (in Fig. 3D, 3F and 4F). The Kruskal-Wallis test is similar to Analysis of Variance (ANOVA) but does not assume the data are normally distributed and hence is more robust than a standard ANOVA. To check the trend of any outcome measure over time (age) we first used graphical tools like scatter plot and histogram to determine an appropriate transformation of the outcome, if necessary, to remove skewness in the distribution. We found that a log transformation best provided a normal distribution. A linear regression analysis was then performed to test whether there was a trend over time. All tests were two-sided with significance level of 0.05. Due to the small to moderate sample sizes in certain subgroups and the resulting lack of power we reported any test with the p-value between 0.05 and 0.1 as “marginally significant”. Such test results were not conclusive and need to be confirmed by future studies.

3. Results

3.1. Nab2 is required to maintain thymus size and thymocyte number with age in male mice

Our initial evaluation began with gross observation of the thymus in male mice, which revealed that the thymuses of *Nab2*KO male mice appeared much smaller than those of their matched C57BL/6 wildtype (WT) littermates (Fig. 1A). Comparison of the weights of freshly dissected specimens revealed that the thymuses of *Nab2*KO mice weighed significantly less than those of their WT littermates (Fig. 1B; Wilcoxon signed-rank test, $p = 0.004$). Age-related thymic involution is characterized by decreased thymus weight (Aw, et al. 2010; Li, et al. 2003; Ortman, et al. 2002; Sempowski, et al. 2002) and loss of thymic architecture evident as disruption of the corticomedullary junction and thinning of the cortex (Aw, et al. 2008; Li, et al. 2003; Manley, et al. 2012; Pearse 2006) (and reviewed in (Muller and Pawelec 2014; Palmer 2013)). To determine if the smaller thymus size of *Nab2*KO mice was accompanied by histological characteristics seen in normal age-related thymic involution, we examined the histology of hematoxylin and eosin stained sections of *Nab2*KO thymuses. There was no evident difference in cortical thickness or definition of the corticomedullary junction observed between *Nab2*KO and WT thymuses (Fig. 1C). Calculations of the cortical area as a percentage of total thymic area revealed no significant difference between thymuses from *Nab2*KO and WT mice (*Nab2*KO: $72.72 \pm 12.07\%$ (n=6); WT: $74.45 \pm 8.96\%$ (n=4)). To further evaluate the cause of the decreased thymus size and weight in *Nab2*KO mice we counted thymocytes from freshly harvested thymuses from *Nab2*KO and WT littermate adult male mice. Analysis revealed that *Nab2*KO mice display a statistically significant 40% reduction in thymocyte number compared with WT controls

(Fig. 1D; Wilcoxon signed-rank test: $p = 3.6 \times 10^{-5}$). In contrast, analysis of female *Nab2*KO mice revealed no significant difference in thymocyte number from littermate WT controls (Fig. 1E, Wilcoxon signed-rank test: $p = 0.64$).

Since age is one of the factors known to influence thymocyte number in mammals we next evaluated whether the difference we identified in thymocyte number between *Nab2*KO and WT mice varied across ages. Figure 2A shows the thymocyte numbers from male animals presented in Figure 1D graphed with respect to age. Thymocyte number decreased with age in both genotypes, and thymocyte numbers in *Nab2*KO mice are lower than those of WT mice across all ages tested. To determine whether the rate of age-related decline in thymocyte number differed between *Nab2*KO and WT mice we analyzed the data as the ratio of thymocyte number between each *Nab2*KO and its WT pair. The analysis of matched pairs controls for environmental conditions, as matched pairs of *Nab2*KO and WT mice were assigned at the time of weaning and underwent all studies together. For pairs in which the *Nab2*KO mouse has an equal number of thymocytes as the WT, the ratio equals one, and the log of the ratio equals zero, so the data point is on the zero axis. Data points below the zero axis indicate matched pairs in which the *Nab2*KO have fewer thymocytes than their WT matches. If the thymocyte number in both genotypes declined at an equal rate with age, the slope of the line would equal zero. Figure 2B shows that *Nab2*KO mice have fewer thymocytes than their WT matched littermates, indicated by the negative value of the plot line, and that their thymocyte numbers decline more with age, indicated by the downward slope of this line (linear regression: $p = 0.0021$). In contrast, female *Nab2*KO mice do not demonstrate the accelerated age-related loss of thymocytes. Figure 2C shows the data from Figure 1E plotted as the log ratio of thymocyte count (*Nab2*KO/WT) for each matched pair versus age. The slope of this line is not statistically significantly different from zero, indicating no difference in the rate of change in thymocyte number with age between *Nab2*KO and WT females. These results suggest that the age-related loss of thymocytes in *Nab2*KO mice is sex-dependent. All further investigations were performed in male mice.

During T cell development in the thymus, CD4−CD8− double-negative (DN) thymocytes mature into CD4+CD8+ double-positive (DP) thymocytes, which then become either CD4+CD8− or CD4−CD8+ single-positive (SP) cells. To determine whether the accelerated decrease in thymocyte number in aged male *Nab2*KO mice was accompanied by changes in thymocyte development relative to WT mice, we conducted flow cytometric analysis on the thymocytes from these *Nab2*KO and WT mice. Figure 2D shows representative dot plots of CD4 and CD8 staining of thymocytes from matched *Nab2*KO and WT one-year old mice. Using the gating strategy shown, we determined the percentage of DN, DP, CD4SP, and CD8SP thymocytes in each study animal presented in Figure 2A. Figures 2E – 2H show plots of the difference in percentages of CD4SP, DP, DN, and CD8SP thymocytes between *Nab2*KO and WT matched pairs with age. Analysis of the percentages of thymocyte subsets using a linear model revealed no significant differences between *Nab2*KO and WT matched pairs across age. Since the CD4 to CD8 distribution in *Nab2*KO thymocytes is unchanged compared to WT thymocytes, these results indicate that the accelerated age-related loss in *Nab2*KO thymocyte number does not result from a change in maturation from DN to DP to SP. A change in the proportion of DN, DP or SP thymocytes is not usually observed in age-related thymic involution, as the subpopulations decline to the same degree (Aw, et al. 2010;

Li, et al. 2003). Together these data show that male *Nab2*KO mice have decreased size and cellularity of the thymus compared with WT littermates, and an accelerated loss in thymocyte number with age. Consistent with our findings, the Immunological Genome Project (www.immgen.org) (Heng, et al. 2008) revealed high levels of *Nab2* RNA expression in the thymus and T cells, including greater *Nab2* levels in DP and SP thymocytes than in DN thymocytes.

3.2. Loss of Nab2 alters the thymic response to stress

To investigate the possible cause of decreased thymocyte number in *Nab2*KO male mice with age, we evaluated whether another factor known to decrease thymus cellularity may account for this difference. Stress and elevated glucocorticoid levels are factors known to induce acute thymic involution (Ayala, et al. 1995; Compton and Cidlowski 1986; Concordet and Ferry 1993; Gruber, et al. 1994; Gruver and Sempowski 2008; Jondal, et al. 1993; Sun, et al. 1992; Tarcic, et al. 1998). Furthermore, *Egr3*KO mice have elevated corticosterone levels in response to stress (Gallitano-Mendel, et al. 2007). Corticosterone is the major glucocorticoid in rodents, and the rodent equivalent of cortisol. We therefore examined whether *Nab2*KO mice had elevated basal levels of corticosterone, as may be seen with a chronic elevated stress response. The circadian nadir for corticosterone occurs following the transition from dark to light, which is the end of the active period for nocturnal animals. Figure 3A shows that basal corticosterone levels in *Nab2*KO male mice did not display a significant difference from those of matched WT controls (Wilcoxon signed-rank test: $p = 0.67$). To evaluate whether a difference in basal corticosterone levels between *Nab2*KO and WT mice may become evident with age we used a linear regression model to compare the log ratio of corticosterone level (*Nab2*KO/WT) with respect to age (Fig. 3B). The slope of the line is not statistically different than zero; indicating no significant difference between genotypes with age is detected ($p = 0.53$). We also assessed whether corticosterone levels correlated with thymus weight in a cohort of *Nab2*KO and WT mice (n=14 per genotype). Linear regression analysis of plots of thymus weight versus corticosterone level did not reveal a significant correlation in either WT or Nab2−/− mice (not shown). These data suggest that basal corticosterone levels, a measure of chronic stress, do not contribute to the lower thymocyte counts in male *Nab2*KO mice.

We then examined whether thymocyte number in *Nab2*KO mice may be more sensitive to acute stress. To test this we used the paradigm of restraint stress to induce thymic involution, modeled after (Tarcic, et al. 1998). We began by conducting a pilot experiment to determine the level of restraint stress required to induce thymic involution in WT mice. Figure 3C shows a schematic describing the protocol. WT male mice were exposed to either one 12 h episode of restraint, or two 12 h restraint episodes with an intervening stress-free period in their home cages. In addition, separate groups were either immediately sacrificed, or allowed to recover for 12 h post-restraint. Figure 3D shows the results of the pilot study which demonstrated that restraint stress produces a significant overall decrease in thymocyte number (Kruskal-Wallis test across all groups: $p = 0.037$). Comparison of animals that received no restraint to those that received two sequential 12 h restraint exposures, with or without a final 12 h recovery period, demonstrated that two sequential restraint sessions significantly reduced thymocyte number (Wilcoxon rank-sum test: $p = 0.029$).

Because mice undergoing restraint did not have access to food and water during the restraint period, we next conducted a pilot study in WT mice to evaluate whether food and water deprivation may also influence thymic involution. Figure 3E shows a schematic of the protocol. Figure 3F examines the effect of two 12 h periods of either restraint stress or food and water deprivation on thymocyte number in WT mice compared to “no stress” controls. Kruskal-Wallis test across these groups reveals an overall difference in thymocyte number ($p = 0.021$). Subsequent comparison showed a significant decrease in thymocyte number in the restraint group compared to the combined control groups (no food and water, and no stress) (Wilcoxon rank-sum test; $p = 0.004$). These results indicate that two 12 h restraint sessions induce a significant decrease in thymocyte number in WT mice, and that this effect does not appear to be due to lack of access to food and water during the restraint periods.

To evaluate whether male *Nab2*KO mice differed from WT mice in their glucocorticoid response to stress we collected blood at the circadian nadir for corticosterone measurements immediately following the two 12 h restraint stress exposures. Figure 3G shows that the paradigm of two 12 h restraint stresses (with an intervening 12 h period of recovery) induced a significant increase in circadian nadir levels of serum corticosterone in both WT (Wilcoxon rank-sum test: $p = 0.004$) and *Nab2*KO mice ($p = 0.002$), compared to non-stressed controls. To determine if stress increased corticosterone levels differently in *Nab2*KO mice than WT mice, we used the Wilcoxon rank-sum test to compare the corticosterone ratio (*Nab2*KO/WT) between “no stress” and restraint groups. This analysis showed no significant effect of genotype ($p = 0.48$). These results indicate that there is no apparent difference in the corticosterone response to restraint stress in *Nab2*KO compared with WT male mice. Together these data suggest that the reduced thymus size and cellularity in *Nab2*KO mice is not due to either a heightened basal corticosterone level, or an accentuated corticosterone response to acute stress.

Having determined that a 2 × 12 h restraint stress paradigm induced a significant decrease thymocyte number in WT mice (Fig. 3D), we next assessed the effect of stress on thymocyte number in matched pairs of *Nab2*KO and WT mice. Thymocyte numbers from animals sacrificed immediately following 2 × 12 h restraint stress were compared with those from control mice not exposed to stress. Analysis of each genotype independently demonstrated that acute restraint stress significantly reduced thymocyte numbers in both WT and *Nab2*KO groups of mice (Wilcoxon rank-sum test: $p = 0.030$, and $p = 0.017$, respectively; Fig. 4A). Comparison between genotypes in the “no stress” group (Fig. 4A, Wilcoxon signed-rank test: $p = 0.00098$) replicated our initial finding of decreased thymocyte numbers in *Nab2*KO vs WT male mice (Fig. 1D). To determine whether stress affects thymocyte number more severely in *Nab2*KO mice than in WT mice, we used the Wilcoxon rank-sum test to compare the log ratio of thymocyte number (*Nab2*KO/WT) between the “no stress” and restraint groups. This demonstrated a marginally significant result, suggesting that *Nab2*KO mice may have greater sensitivity to stress-induced decrease in thymocyte number, but this is not conclusive ($p = 0.082$; Fig. 4A).

We next evaluated the role of *Nab2* in the response of thymocytes to stress by examining thymocyte subsets (Fig. 4 B–E). Statistical analyses demonstrated that stress resulted in an overall significant increase in the percentage of CD4SP cells (Fig. 4B, Wilcoxon rank-sum

test: p = 0.004), which was due to a significant effect of stress in the *Nab2*KO group (Wilcoxon rank-sum test: p = 0.004), as there was no significant effect of stress in the WT group (Wilcoxon rank-sum test: p = 0.18). Figure 4C shows that restraint produced a marginal effect on the overall percentage of DP thymocytes (Wilcoxon rank-sum test: p = 0.082), showing a significant decrease in the percentage of DP thymocytes in *Nab2*KO mice (Wilcoxon rank-sum test: p = 0.017), but no significant effect on percentage of DP thymocytes in WT mice (Wilcoxon rank-sum test: p = 0.126). Figure 4D shows that there was no significant effect of stress on the percentage of DN thymocytes. Figure 4E shows that restraint stress produced a significant overall increase in the percentage of CD8SP thymocytes (Wilcoxon rank-sum test: p = 0.03), which was due to a significant increase in the percentage of *Nab2*KO cells (Wilcoxon rank-sum test: p = 0.017), as there was no significant increase in percentage of WT thymocytes in response to stress (Wilcoxon rank-sum test: p = 0.13). These results indicate that acute restraint stress induces an immediate suppression of thymocyte number in both *Nab2*KO and matched WT male mice, with significant differences in the response of thymocyte subtypes seen only in the *Nab2*KO mice. Specifically, in *Nab2*KO mice stress resulted in a nearly three-fold increase in the percentage of CD4SP and CD8SP cells, a greater than 50% decrease in the percentage of DP cells, and no change in DN cells, compared to unstressed *Nab2*KO mice.

To test whether the lower thymocyte number in *Nab2*KO mice may be in part due to failure to recover normally from stress-induced loss in thymocyte number, we examined thymocyte number at one-week intervals following restraint stress. Figure 4F shows thymocyte numbers in matched pairs of *Nab2*KO and WT male mice at either 1 week, 2 weeks, or 3 weeks following a 2 × 12 hr restraint paradigm, compared with no stress control animals. To examine the effect of duration of recovery, we performed a Kruskal-Wallis analysis, which showed that there was no significant difference in recovery of thymocyte number following stress between the genotypes (p = 0.20). Analysis of each genotype separately demonstrated a significant effect of stress on the recovery of thymocyte number following restraint stress for both WT and *Nab2*KO mice (Kruskal-Wallis test: p = 0.016 and p = 0.012, respectively). By three weeks following completion of the stress, thymocyte numbers had recovered to the level of baseline, unstressed controls for each genotype. Thus failure to recover from stress does not appear to contribute to the accelerated decrease in thymocyte number with age identified in *Nab2*KO mice.

4. Discussion

Nab2 is a co-regulator of *Egr* transcription factors *Egr1*, *Egr2*, and *Egr3*, and is involved in feedback regulatory relationships between these genes that modulate peripheral T-cell activation. However, while *Egr*s 1, 2, and 3 each also influence thymocyte development, no role for *Nab2* has been previously demonstrated in the thymus. In the present study we identified a requirement for the immediate early gene *Nab2* in maintaining thymus size, weight, and cellularity. In addition, while the thymus normally undergoes involution with age (Aw, et al. 2010; Li, et al. 2003; Muller and Pawelec 2014; Ortman, et al. 2002; Palmer 2013; Sempowski, et al. 2002), we found that the reduction in thymus size and cellularity was greater in *Nab2*KO mice compared to WT mice, and that this difference became larger with age. We did not observe changes in thymocyte subsets with age, which is consistent

with reports of age-related thymic involution (Aspinall 1997; Aw, et al. 2010; Li, et al. 2003). Neither one year old *Nab2*KO or WT mice displayed the histologic changes characteristic of age-related thymic involution. This may be due to their C57BL/6 background strain, as previous studies have shown that WT C57BL/6 mice as old as 15 mo of age do not show a significant difference in the percentages of cortical and medullary areas compared with 3 mo old C57BL/6 mice (Li, et al. 2003). Alternatively, the accelerated loss of thymocyte number in *Nab2*KO mice with age may be due cell intrinsic defects in thymocyte differentiation that become more pronounced with age, such as defects in rearrangement of the TCR β-chain genes (Aspinall 1997).

The role of *Nab2* in maintaining thymocyte number may be related to *Egr1*, *Egr2*, and *Egr3*-mediated transcription, as these are the only known ligands for *Nab2*. *Egr1*, *Egr2*, and *Egr3* regulate multiple steps in thymocyte development. They are expressed in DN and DP thymocytes in response to pre-TCR and TCR signaling, respectively. Prior studies have shown that loss of *Egr1* or *Egr2* results in a defect in positive selection manifested by a partial block in progression from DP to SP stages (Bettini, et al. 2002; Lauritsen, et al. 2008; Lawson, et al. 2010). This survival defect is demonstrated by increased susceptibility to glucocorticoid-induced cell death in *Egr2*-deficient thymocytes in vitro, and is due to *Egr2*-mediated up-regulation of the anti-apoptotic gene Bcl-2 (Lauritsen, et al. 2008; Lawson, et al. 2010). In comparison, *Egr3*KO mice exhibit a block earlier in thymocyte development, within the DN stage (Carter, et al. 2007; Xi and Kersh 2004a). In further support of a role for the *Egr* family within the DN stage, overexpression of *Egr1*, 2 or 3 drives progression from the DN to DP stage and recapitulates the response to pre-TCR signaling (Carleton, et al. 2002; Li, et al. 2011; Miyazaki 1997; Miyazaki and Lemonnier 1998; Xi and Kersh 2004b). While *Egrs* alter thymocyte development, we did not detect a block in thymocyte development in *Nab2*KO mice, as there were no differences in percentages of DN, DP, CD4SP, or CD8SP cells between *Nab2*KO and WT mice under basal conditions.

Prior studies have shown the influence of *Egr*s on thymus cellularity. For example, *Egr1*, *Egr3* double knockout mice, and to a lesser extent individual *Egr1*KO and *Egr3*KO mice, exhibit thymic atrophy (Carter, et al. 2007; Xi and Kersh 2004a). However, the opposite effect, an increase in number of thymocytes, has also been reported in *Egr1*KO mice on a different background strain (Bettini, et al. 2002). Comparison with our results is complicated by the fact that prior studies have examined mice at a much younger age, or have not reported the specific age at which the effect is seen. *Egr1*, *Egr3* double knockout mice demonstrate a 90% loss in thymocyte number that is maximal at two to three weeks of age. Thymus histology in *Egr1*, *Egr3* double knockout mice shows loss of the corticomedullary junction and massive apoptosis (Carter, et al. 2007). This differs from our findings of a much less severe, though still highly significant, decrease in thymocyte number of 40% across all ages of *Nab2*KO male mice (Fig. 1D). This effect is less prominent in young animals, and progresses to a nearly 70% loss, compared with WT mice, by 14 mo of age (Fig. 2A). It is possible that we failed to detect an earlier decrease in thymocyte number in *Nab2*KO mice, since we did not evaluate the thymus in very young animals. However, the fact that we do not see differences in histology in older *Nab2*KO animals suggests that the thymic phenotype of *Nab2*KO mice is, indeed, different than that of *Egr1*, *Egr3* double knockout mice.

An unexpected finding was that the accelerated loss of thymocytes with age was not evident in female *Nab2*KO mice, which maintain a remarkably stable thymocyte number from ages 3 to 15 months (Fig. 2C). Review of the literature did not reveal other examples of sex-dependent genetic determinants of age-related thymic involution. However, numerous studies have identified roles for the sex steroids estrogen, progesterone, and testosterone in both acute and age-related thymic involution ((Greenstein, et al. 1986; Min, et al. 2006; Sutherland, et al. 2005; Tibbetts, et al. 1999; Zubkova, et al. 2005) and reviewed in Chinn (Chinn, et al. 2012)). In addition, glucocorticoids exert sexually dimorphic influences on gene expression that have been hypothesized to influence gender differences in inflammatory diseases (Duma, et al. 2010). Intriguingly, loss of *Egr1* produces sex-specific infertility via its regulation of luteinizing hormone (Lee, et al. 1996), indicating a role for at least one *Nab2* binding partner in sex hormone effects. Future studies comparing the effect of castration, with or without testosterone replacement, will be helpful to decipher the role of sex steroids in the accelerated age-related reduction in thymus size and cellularity of *Nab2*KO male mice.

Acute factors, such as environmental stress and elevated glucocorticoids, can induce thymic involution acutely (Ayala, et al. 1995; Compton and Cidlowski 1986; Concordet and Ferry 1993; Gruber, et al. 1994; Gruver and Sempowski 2008; Jondal, et al. 1993; Sun, et al. 1992; Tarcic, et al. 1998). Numerous kinds of stress, ranging from physical restraint, to bacterial and viral infection, to ionic radiation, induce acute involution of the thymus (reviewed in Gruver and colleagues (Gruver and Sempowski 2008)). Mediators of these processes include lipopolysaccharide, IL-6 family cytokines, and steroid hormones (Greenstein, et al. 1986; Gruver and Sempowski 2008; Hick, et al. 2006; Tibbetts, et al. 1999; Ullewar and Umathe 2015; Wyllie 1980; Zubkova, et al. 2005). In the case of restraint-induced thymic involution, this process is mediated by glucocorticoids, since either adrenalectomy or glucocorticoid antagonism abrogate the effect (Tarcic, et al. 1998). Although *Nab2* has not been shown to play a role in the stress response, *Egr1* and *Egr3* are both activated in response to stressful stimuli and play critical roles in stress-responsive behavior (Honkaniemi, et al. 2000; Huang and Tunnacliffe 2005; Ko, et al. 2005; Lyn, et al. 2000; Meaney, et al. 2000; Orsetti, et al. 2008; Senba and Ueyama 1997). Loss of *Egr3* function in mice results in defects in the response and adaptation to stress including heightened stress-reactivity which is accompanied by elevated corticosterone release (Gallitano-Mendel, et al. 2007). *Egr1* plays a critical role in animal models of early life stress that result in long-lasting changes in hypothalamic-pituitary-adrenal axis regulation and abnormal anxiety-related behaviors later in life (Fish, et al. 2004; Weaver, et al. 2004). This process involves glucocorticoid action both upstream and downstream of *Egr1* activity (Fish, et al. 2004; Revest, et al. 2005; Weaver, et al. 2004; Weaver, et al. 2007).

The roles of *Egr1* and *Egr3* in the stress response to, and regulation of, glucocorticoids suggested a potential mechanism through which *Nab2* may be influencing thymic size and cellularity. Prior studies have shown that thymocytes, in particular DP thymocytes, are exquisitely sensitive to stress-induced, glucocorticoid-mediated apoptosis (Gruver and Sempowski, 2008; Tarcic et al., 1998). If stress plays a role in the decreased thymocyte numbers in *Nab2*KO mice, it could be doing so in either of two ways. Either *Nab2*KO mice have elevated corticosterone levels (at baseline or in response to stress), or thymocytes in

*Nab2*KO mice are more sensitive to the same levels of corticosterone. Our findings that corticosterone levels did not differ between male *Nab2*KO and WT mice at baseline (Fig. 3A) or in response to restraint stress (Fig. 3G) decrease likelihood of the former potential explanation. Instead, our findings support the latter possibility. Figures 4B, 4C and 4E demonstrate that, although restraint stress induces a similar increase in corticosterone in *Nab2*KO mice as in WT mice, it produces a significant decrease in the percentage of DP thymocytes, and increase in the percentage of CD84SP and CD8SP thymocytes, in *Nab2*KO male mice that are not seen WT male mice. This finding suggests that thymocytes in male *Nab2*KO mice are more sensitive to an equivalent level of stress hormone.

Our findings demonstrate a novel requirement for *Nab2* in maintaining thymocyte number in male mice with age. In addition, *Nab2*KO thymocytes appear more sensitive to stress. As an influential gene on age-related and stress-responsive loss of thymus cellularity, *Nab2* may be a protein linking chronic and acute determinants of thymic size.

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Abbreviations

Nab2	NGFI-A binding protein 2
Egr	Early growth response gene
WT	wildtype
SP	single-positive thymocytes
DN	double-negative thymocytes
DP	double-positive thymocytes

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Highlights

- Nab2KO mice have decreased thymus size, weight, and cellularity
- Nab2KO mice show accelerated decrease in thymic cellularity with age
- Nab2KO mice have enhanced thymocyte sensitivity to stress

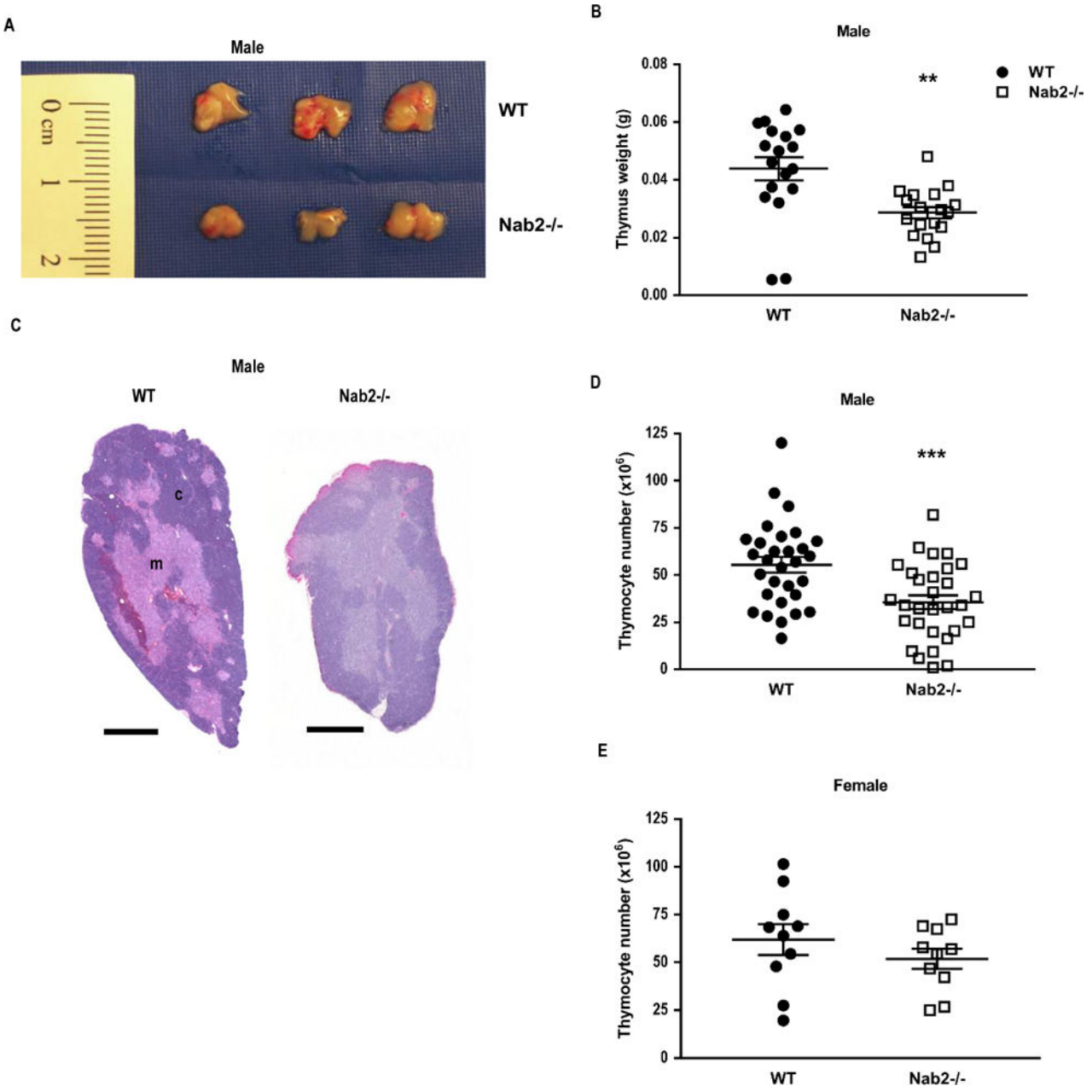

Figure 1. Loss of *Nab2* results in decreased thymus size and cellularity in male mice

A. Representative sample of grossly dissected thymuses from one year old matched pairs shows the reduced thymus size in WT compared to *Nab2*KO male mice. B. Thymus weight in *Nab2*KO males is significantly reduced compared to that of WT littermate controls (n = 18 matched pairs of WT and *Nab2*KO male mice, ages 3 – 14 mo). C. Representative hematoxylin and eosin-stained sections of the thymus from one year old WT and *Nab2*KO male mice. The medulla (m) and cortex (c) are labeled. Scale bars indicate 1 mm. D. The number of thymocytes is significantly lower in *Nab2*KO than WT male mice (n = 30 matched pairs, ages 3 – 14 mo). E. Thymocyte number does not differ between female

*Nab2*KO and WT mice. (n = 10 matched pairs, ages 3 – 15 mo. Points represent values from individual animals, and lines represent the mean ± SEM. **p < 0.01; *** p < 0.001

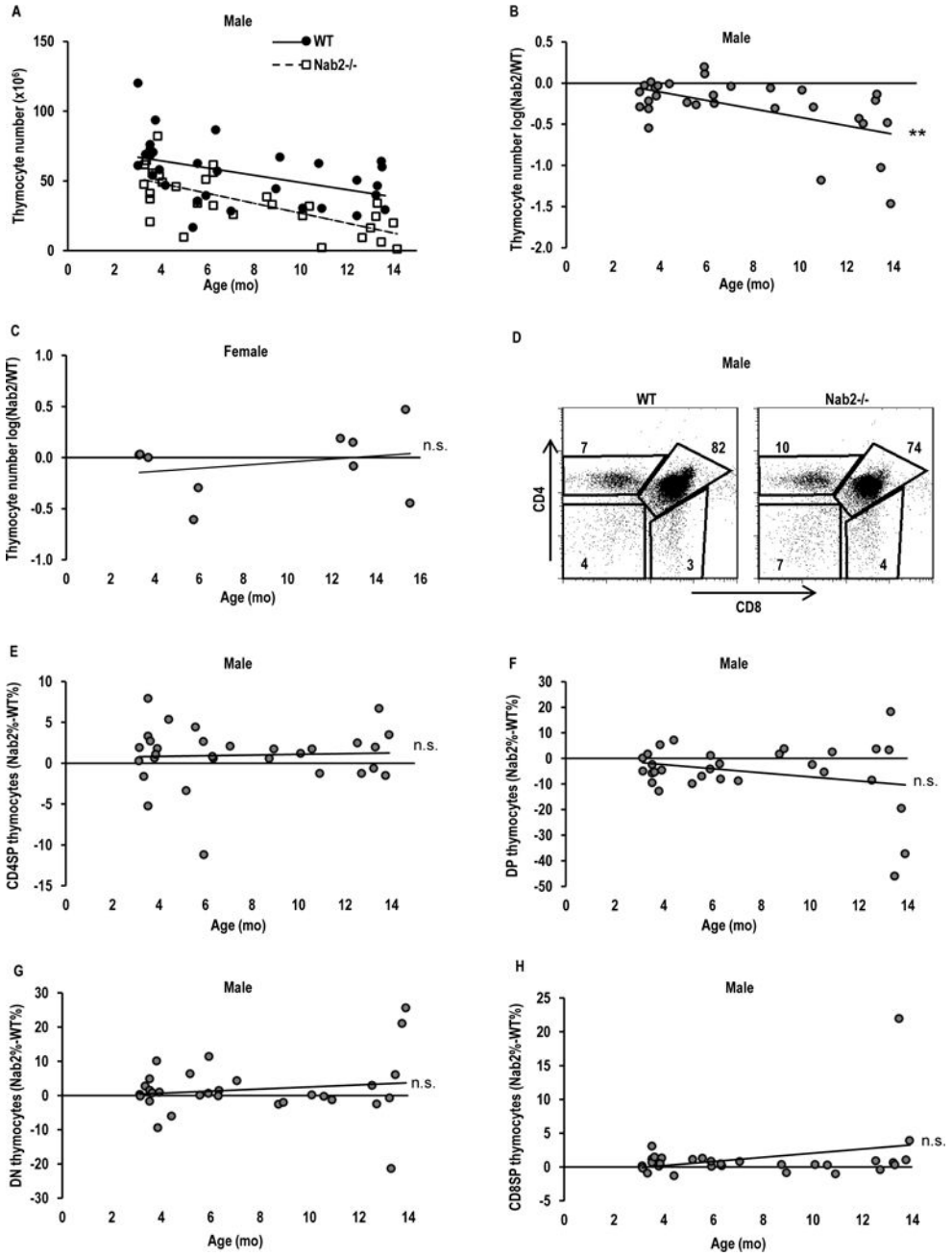

Figure 2. Age-related thymocyte loss is accelerated in *Nab2*KO male mice

A. Scatter plot of total thymocyte number from individual male mice demonstrates loss of thymocyte number with age in both genotypes, and lower thymocyte numbers in *Nab2*KO male mice. B. Linear regression to compare the log ratio of thymocyte number (*Nab2*KO/WT) with respect to age in male mice with age has a negative slope, indicating that age-related thymocyte loss progresses more rapidly in male *Nab2*KO than WT mice ($p < 0.01$; n = 30 matched pairs of WT and *Nab2*KO male mice). C. Similar analysis as in B on female mice indicates that loss of *Nab2* in females does not alter age-related thymocyte loss (slope not significantly different than 0) ($p = 0.50$; n=10 matched pairs of female mice). D.

Dot plots and gating for CD4 and CD8 staining of thymocytes from a representative pair of one year old male *Nab2*KO and WT mice. Numbers represent percentage of total thymocytes in each gate. E – H. Difference in the percentage of thymocyte subsets between WT and *Nab2*KO matched animals across age was not significantly different for: E. CD4 single positive (SP), F. double positive (DP), G. double negative (DN), or H. CD8SP. (n = 30 matched pairs of WT and *Nab2*KO male mice) **$p < 0.01$; n.s. not significant

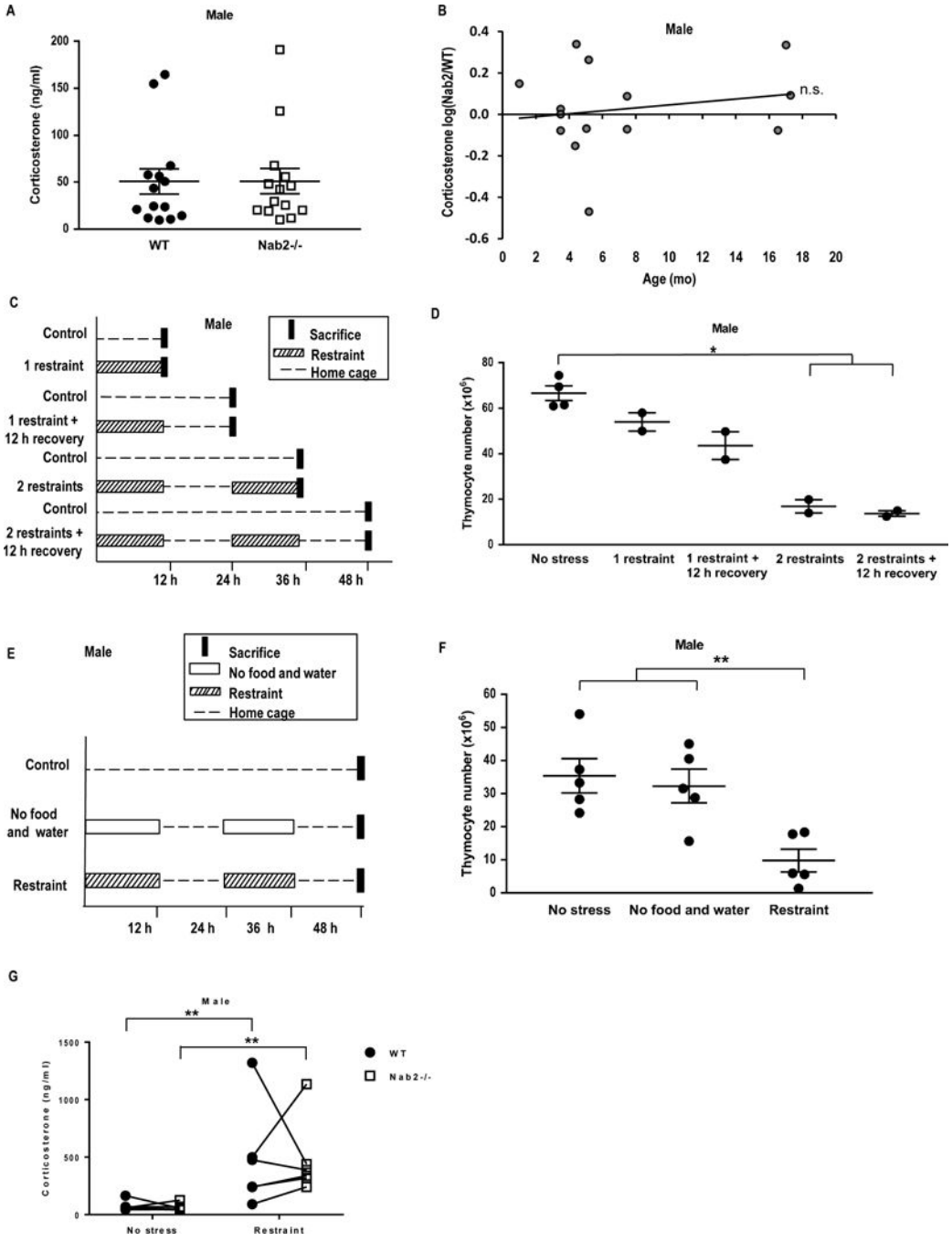

Figure 3. Restraint stress induces thymic involution and elevated corticosterone levels

A. Basal serum corticosterone levels drawn at circadian nadir did not differ between WT and *Nab2*KO male mice. B. Plot of the log ratio of corticosterone levels (*Nab2*KO/WT) with age. (for A and B, n = 14 matched pairs of WT and *Nab2*KO male mice) C. Schematic illustrating restraint stress pilot experiments performed in WT mice to establish the protocol for inducing thymic involution. D. In WT mice, two 12 h restraint periods induced a statistically significant reduction in total thymocyte number compared with non-stressed, age-matched control mice. Data is shown from two pooled experiments; n = 4 “no stress”

control mice and n = 2 mice per stress condition. E. Schematic depicting restraint stress for induction of thymic involution and control conditions. F. Restraint stress reduced thymocyte number compared with “no stress” and “no food and water” controls. Data shown represent pooled results from five experiments with n = 5 male mice per group. G. Comparison of corticosterone levels at baseline (in “no stress” controls) and immediately following 2 × 12 h restraint paradigm showed a significant effect of stress, but no genotype difference. Lines connect WT and *Nab2*KO matched pairs. (n = 12 matched pairs of WT and *Nab2*KO male mice, 6 pairs per condition. Lines in graphs represent the mean ± SEM. *$p < 0.05$; **$p < 0.01$; n.s. not significant

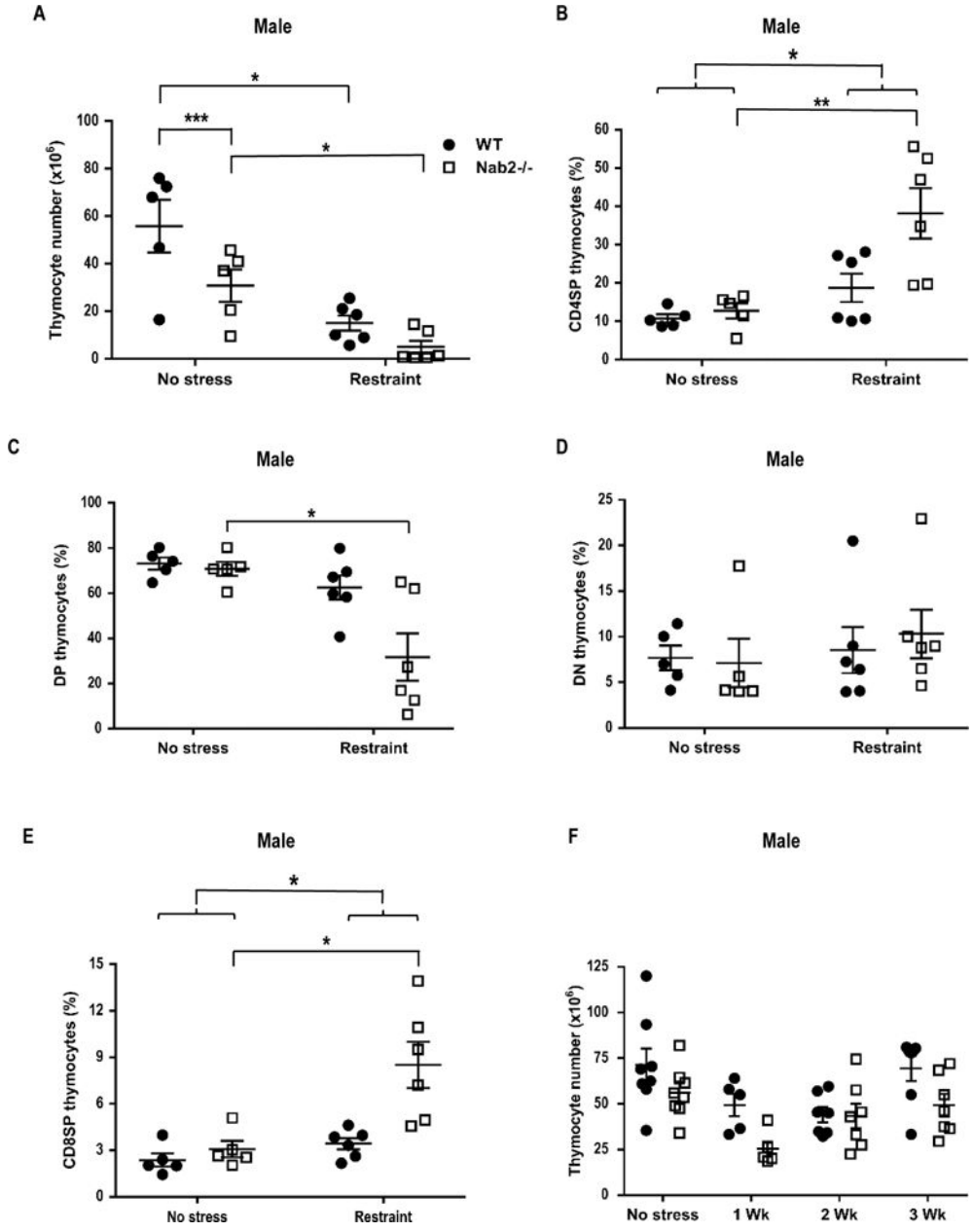

Figure 4. Loss of *Nab2* affects stress-induced thymic involution

Matched pairs of WT and *Nab2*KO mice sacrificed immediately following a 2 × 12 h restraint paradigm were compared with "no stress" control mice. A. Acute restraint reduces total thymocyte number in both WT and *Nab2*KO mice. The effect of stress on the difference between WT and *Nab2*KO thymocyte numbers was marginally significant (i.e. p value between 0.05 – 0.1), suggesting a possible genotype by stress interaction. B – E show percentage of thymocyte subsets: B. CD4 single positive (SP), C. double positive (DP), D. double negative (DN), and E. CD8SP thymocytes. n= 11 matched pairs of WT and *Nab2*KO male mice (n= 5 "no stress" and n= 6 "restraint") ages 2.8–5.4 mo. F. Total thymocyte number in groups of mice exposed to 2 × 12 h restraint stress and allowed to recover for 1, 2, or 3 weeks, compared with "no stress" control mice. (n = matched pairs of WT and *Nab2*KO

male mice, ages 2.8–5.4 mo: n= 8 “no stress” control, n= 5 “1 Wk” recovery, n= 7 “2 Wk” recovery, and n= 7 “3 Wk” recovery. Differences in n between groups resulted from loss of some animals between completion of stress protocol and planned end-date of experiment. Points represent values from individual animals, and lines represent the mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$