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Nab2 maintains thymus cellularity with aging and stress

K.Taraszka Hastings[a,*], Diana Elizalde[a], Leela Muppana[a], Sarah Levine[a,1], Christy M. Kamel[a,2], Wendy M. Ingram[a,3], Jennifer T. Kirkpatrick[a,4], Chengcheng Hu[b], Matthew P. Rausch[a], Amelia L. Gallitano[a]

[a] Department of Basic Medical Sciences, University of Arizona College of Medicine – Phoenix, 425 N. 5th St., Phoenix, AZ, 85004, USA
[b] Department of Biostatistics, Mel and Enid Zuckerman College of Public Health, University of Arizona, 714 E. Van Buren St., Phoenix, AZ, 85004, USA

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

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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

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.

* Corresponding author.
E-mail addresses: khasting@email.arizona.edu (K.Taraszka Hastings), hucc@email.arizona.edu (C. Hu), amelia@email.arizona.edu (A.L. Gallitano).
[1] University of Arizona College of Medicine – Tucson, Tucson, AZ 85721, USA.
[2] Kansas City University of Medicine and Biosciences, 1750 Independence Ave., Kansas City, MO 64106 USA.
[3] Department of Neuroscience, Geisinger Health System.
[4] Department of Emergency Medicine, University of California, Davis, 2315 Stockton Blvd., Sacramento, CA 95817, USA.

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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 (FcγRIII/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 (9200 × 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 1000 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 coun-

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