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THE AGE-RELATED DECREASE IN E47 DNA-BINDING DOES NOT DEPEND ON INCREASED ID INHIBITORY PROTEINS IN BONE MARROW-DERIVED B CELL PRECURSORS

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1. ABSTRACT

Our previous results showed that decreased numbers of pre-B cells in aged mice were associated with decreases in surrogate light chain, λ5, and transcription factors E47/E12. In the present paper, we have analyzed IL-7-expanded populations of pro-B/early pre-B cells to evaluate whether the age-related reduction in E47 DNA-binding could be attributed to reduced E47 expression and/or increased Id expression. According to the percentage of pre-B cells present in their bone marrow, old mice were classified as severely depleted (>80% loss in pre-B cells, 50% loss in pro-B cells), or moderately depleted (20-80% loss in pre-B cells). Results herein show that IL-7-stimulated pro-B/pre-B cells from both severely depleted and moderately depleted old mice exhibit a reduced E47 DNA-binding capacity compared to young mice, and this defect in severely depleted old mice is more dramatic than that in moderately depleted old mice. Id proteins, which are inhibitors of E47, are not increased in nuclear extracts of IL-7-expanded pro-B/early pre-B cells from severely depleted or moderately depleted old mice. We therefore conclude that the reduced expression of E47 protein alone in severely depleted and to a lesser extent in moderately depleted old mice explains the reduced amount of E47-DNA binding.

2. INTRODUCTION

The E2A-encoded transcription factors E12 and E47 are class I basic helix-loop-helix (bHLH) proteins, which are involved in the B cell differentiation processes and in the regulation of mature B cell functions. E47 and E12 bind to the palindromic DNA sequence CANNTG, referred to as an E box site (1), found in the promoter and enhancer regions of many B lineage-specific genes, including the enhancers in the immunoglobulin (Ig) loci and the promoters of mb-1, λ5 and RAG-1 (2-6). They regulate the expression of the surrogate light chain (λ5, VpreB), which promotes cell survival of early pre-B cells, initiating Ig rearrangements and promoting class switching. Disruption of the E2A gene results in a complete block in B lymphocyte development because D_H-J_H gene rearrangements of Ig are impaired (7). E47 is also involved in the ability of mature B cells to undergo class switch recombination (8). E47 binds DNA as a homo- or heterodimer. DNA-binding in bone marrow-derived B cell precursors is due to E47/E12 complexes, whereas in mature splenic B cells it is due to E47/E47 complexes (9-12). In non-B cells, E47 binds DNA as a heterodimer together with tissue-restricted bHLH proteins, such as MyoD or NeuroD (13, 14). The formation and the function of the homodimer or heterodimer depend on the balance between E47 and Id proteins, which lack the DNA-binding domain and function as inhibitors of E proteins (15, 16). For this reason, these proteins were named as Id (Inhibitors of DNA-binding). Mammals have four *Id* genes, namely *Id1*, *Id2*, *Id3* and *Id4* (17). In general, the expression level of Id proteins is high in proliferating cells and low in differentiating cells and in quiescent cells. Recent studies have provided evidence that Id proteins can interact not only with bHLH proteins but also with cell cycle regulatory proteins, such as pRB and Cdk2 (18, 19), thus activating cell cycle progression and blocking cell differentiation. The Id name was also related

to this potential role of Id proteins as inhibitors of differentiation.

Our laboratory has previously shown that numbers of pre-B cells as well surrogate light (SL) chain, composed of the VpreB and λ5 peptides, are reduced in old mice (20-22). The SL chain, together with the μ heavy chain and the associated signal-transducing molecules Igα and Igβ, forms the pre-B cell receptor (pre-BCR), which is critical for Ig variable heavy (V_H) chain selection, and for cellular proliferation and survival at the pre-B cell stage (23-26). The transcription of the SL chain is, at least in part, regulated by E47 (4), and both λ5 and E47 have been found by us to be significantly decreased by aging in IL-7-expanded pro-B/pre-B cells (12, 22). As Id proteins are expressed during B-cell development and function as negative regulators of B lymphopoiesis, we investigated whether the age-related reduction in E47 DNA-binding and expression in IL-7-expanded pro-B/pre-B cells could be attributed to increased levels of the Id inhibitory proteins. Results herein suggest that the reduced expression of the E47 protein itself in nuclear extracts of IL-7-expanded pro-B/early pre-B cells from old mice, rather than increased expression of Id proteins, accounts for the reduced DNA-binding of E47.

3. MATERIALS AND METHODS

3.1. Mice

Young male and female BALB/c mice 3-4 months of age and old BALB/c mice 22-29 months of age were purchased from the National Institute of Aging colony. Mice bearing visible tumors in the thoracic or abdominal cavities or splenomegaly were eliminated from this study.

3.2. Flow cytometry

Single-cell suspensions from young and old bone marrow were prepared by flushing cells from femur and tibia pairs with PBS. Red blood cells were lysed with a hypotonic ammonium chloride buffer (ACK). Cells ($3x10^5$) were stained for surface expression of CD43, B220 and μ chain. Briefly, cells were incubated with PE- conjugated anti-CD43 antibodies (1:40 diluted, BD Pharmingen 553271), CY-conjugated anti-B220 antibodies (1:40 diluted, BD Pharmingen 553091), and FITC- conjugated anti-μ antibodies (1:100 diluted, Jackson ImmunoResearch) in FACS buffer (HBSS, buffered in 0.02% sodium azide and 0.1% BSA) for 30 min on ice, using the protocol previously described (20). After staining, cells were fixed with 0.25% paraformaldehyde. After *in vitro* expansion in the presence of IL-7, cultures were harvested, cells counted and $3x10^5$ cells were stained for surface expression of CD43, B220 and for intracytoplasmic μ chain. Briefly, cells were incubated with PE-labeled anti-CD43 antibodies and CY-conjugated anti-B220 antibodies as described above and fixed with 0.25% paraformaldehyde. The following day, cells were permeabilized with PBS-TWEEN (1X PBS containing 0.5% Tween-20) for 30 min at room temperature and then stained with FITC-labeled anti-μ antibodies for 30 min on ice, fixed and analyzed. Samples of 10^4 cells were analyzed on a LSR flow cytometer (BD) using logarithmic amplification.

3.3. *In vitro* expansion of IL-7-dependent pro-B/pre-B cells

Bone marrow cells from young and old mice were cultured in 6-well culture plates at the concentration of 10^6/ml in complete medium (RPMI 1640, supplemented with 10% fetal calf serum, 10 μg/ml penicillin/streptomycin, $3x10^{-5}$ M 2-mercaptoethanol and 2 mM L-glutamine). Recombinant murine IL-7 (Biosource Int., CA) was used at the concentration of 5 ng/ml. Seven days later, non-adherent cells were harvested, stained, lysed and nuclear and cytoplasmic extracts prepared. Typically, IL-7-stimulated bone marrow results in about a 10X expansion of precursor cells which gives pro-B cells (~80%) and early pre-B cells (~20%) (22). The expansion of pro-B/early pre-B cells to IL-7 was decreased significantly in the bone marrow from old as compared to young mice beginning at day 3 after culture initiation and continuing through day 7. At day 7 we observed the maximum difference in expansion between young and old mice (22). After day 7, a great proportion of dead cells are found in both young and old mice.

3.4. Preparation of nuclear extracts

Nuclear extracts were prepared from cultured bone marrow cells essentially as previously published (27); briefly, cells were harvested and centrifuged in a 5415C Eppendorf microfuge (2,000 rpm, 5 min.). The pellet was resuspended in 30 μl of solution A containing Hepes 10 mM pH 7.9, KCl 10 mM, EDTA 1.0 mM DTT 1 mM, $MgCl_2$ 1.5 mM, PMSF 1 mM, 1 tablet of protease inhibitor cocktail (Boeringer Manheim, Germany), and Nonidet P-40 (0.1%), briefly vortexed and centrifuged (8,000 rpm, 5 min., 4°C). The supernatant containing the cytoplasmic extract was removed and the pellet containing the nuclei was resuspended in 30 μl of solution B containing Hepes 20 mM pH 7.9, EDTA 0.1 mM, DTT 1 mM, $MgCl_2$ 1.5 mM, PMSF 2 mM, 1 tablet of protease inhibitor cocktail, and glycerol 10%). The lysate was incubated on ice for 20 min, protein sonicated for a few seconds and centrifuged (14,000 rpm, 15 min., 4°C). Aliquots of the nuclear extract were stored at -80°C. Protein content was determined by Bradford assay.

3.5. Preparation of the DNA probe

The μE5 probe, whose sequence is 5'CCC-GGC-GCG-GGG-GCG-ATT-TCG-AGT-CA-3', present in the IgH intronic enhancer (28) was prepared as follows: one hundred μl of each single strand (26 bp), at the concentration of 100 ng/μl, were annealed at the following temperatures: 85°C (2 min), 65°C (15 min), 37°C (15 min), 25°C (15 min) and on ice (15 min) and then end labeled for 40 min at 37°C, using T4 DNA polynucleotide kinase in the presence of 1 μl of γ-^{32}P ATP. The probe was then purified over a G-25-50 sepharose column.

3.6. Electrophoretic Mobility Shift Assay (EMSA)

A gel mobility shift assay to determine DNA-binding of E47 was performed as follows. The radiolabeled DNA probe was incubated with 10 μg of nuclear extract in

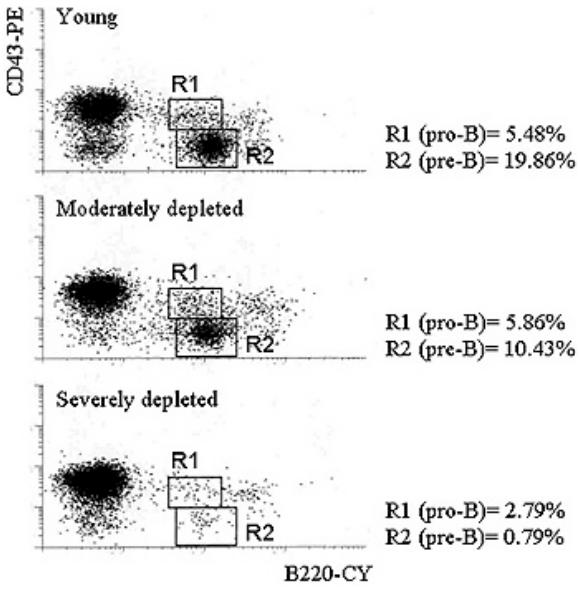

Figure 1. Flow staining of bone marrow cells from young and severely depleted or moderately depleted old mice. Bone marrow cells from young and severely depleted or moderately depleted old mice were stained for surface expression of CD43, B220 and μ chain. Severely depleted and moderately depleted mice have been defined in the text. Results obtained are representative of 10 pairs of mice. Means +/- S.E. of pro-B ($IgM^-CD43^+B220^{low}$) and pre-B ($IgM^-CD43^-B220^{low}$) percentages from these 10 young, 10 severely and 10 moderately depleted old mice were: 4.40 +/- 0.12 and 19.40 +/- 1.28; 4.41 +/- 0.20 and 10.37 +/- 1.18; 2.2 +/- 0.25 and 2.72 +/- 0.59, respectively.

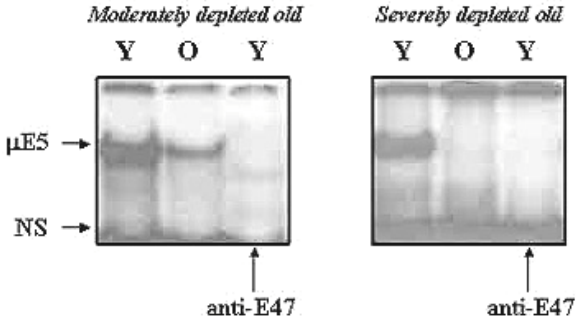

Figure 2. Aging down-regulates DNA-binding of E47 in IL-7-expanded pro-B/early pre-B cells. Nuclear extracts from the same numbers (10^7) of IL-7-expanded pro-B/pre-B cells from young and severely depleted or moderately depleted old mice were run in EMSA (10 μg/lane). In this particular example, severely depleted old mice showed 1% pre-B cells *versus* 21% in young mice, whereas moderately depleted old mice showed 10% pre-B cells *versus* 20% in young mice. Results obtained are representative of 10 pairs of mice. NS: non specific bands. Anti-E47: supershift (inhibition), described in Materials and Methods.

the presence of 0.5 μg poly-dIdC as non-specific competitor for μE5. The reaction was performed at room temperature in 15 μl of binding buffer (Tris-HCl 10 mM pH 7.5, NaCl 75 mM, DTT 1 mM, PMSF 1 mM, glycerol 6%). To supershift (and/or inhibit DNA-binding of) E47, the nuclear extracts were preincubated with rabbit polyclonal anti-mouse E47 (2 μl, sc-763 Santa Cruz), before the addition of the radiolabeled probes. This antibody was the best supershifting antibody, as compared to others commercially available, based on the evidence that it completely prevented the binding of the μE5 complexes in IL-7-expanded pro-B/pre-B cells (12). The samples were electrophoresed in a 5% polyacrylamide gel at 175 V for 3 hours at room temperature. The gels were dried on Whatman 3M and exposed to Kodak X-ray films at -80°C overnight.

3.7 Western Blotting

For the evaluation of the amounts of E47 in the nucleus of IL-7-expanded pro-B/pre-B cells, nuclear extracts at equal protein concentration were denatured by boiling for 4 min in Laemli sample buffer and subjected to SDS-PAGE using a 4-12% polyacrylamide gel under reducing conditions. Proteins were then electrotransferred onto nitrocellulose filters (Biorad). Non-specific sites were blocked by incubation of the membranes with PBS-TWEEN (0.05%) containing 5% milk, for 1 hr at room temperature (blocking solution). Filters were incubated with the following antibodies: mouse monoclonal anti-E47 (1:400 diluted, BD Pharmingen 554077), rabbit polyclonal anti-Id1 (1:400 diluted, Santa Cruz sc-488), and rabbit polyclonal anti-Id3 (1:400 diluted, Santa Cruz sc-490). Mouse monoclonal anti-histone H-1 (1:200 diluted, sc-8030 Santa Cruz) was used to detect histone H-1, as loading controls. Following overnight incubation with the primary antibody, immunoblots were incubated with the appropriate horseradish peroxidase-labeled secondary antibodies (610-1319 Rockland, 1:16000 diluted for mouse primary antibodies; Santa Cruz sc-2313, 1:5000 diluted, for rabbit primary antibodies) for 3 hours at 4°C, developed by enzyme chemiluminescence (1 min incubation for E47, Ikaros and histone H-1 blots and 10-20 min incubation for Id1 and Id3 blots) and exposed to CL-XPosure Film (Pierce).

4. RESULTS

4.1. Effects of aging on the percentage of pre-B cells from *ex vivo* stained bone marrow

Bone marrow cells from young and old mice were analyzed for surface CD43, B220, and μ to identify pro-B and pre-B cell subsets. IgM-CD43+$B220^{low}$ are designated as pro-B cells, whereas IgM-CD43-$B220^{low}$ are pre-B cells. As shown in Figure 1, old mice can be classified as severely depleted (>80% loss in pre-B cells, 50% loss in pro-B cells, compared to young), or moderately depleted (20-80% loss in pre-B cells). Percentages of pro-B and pre-B cells are shown in R1 and R2 squares, respectively. Results show that aging significantly affects pro-B cell numbers in severely depleted old mice and pre-B cell numbers in both severely and moderately depleted old mice. Reduced pre-B cell numbers have been previously

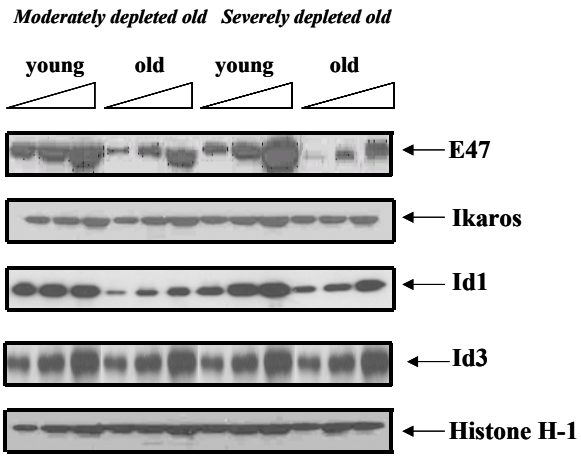

Figure 3. Expression of E47, Id1-3, and Ikaros in nuclear extracts of IL-7-expanded pro-B/early pre-B cells. Nuclear extracts from same numbers (10^7) of IL-7-expanded pro-B/pre-B cells from young and severely depleted or moderately depleted old mice were run in Western blot experiments. The titration is 4, 8 and 16 μg of cell lysate/lane. E47, Ikaros, Id1, Id3 and Histone H-1 were detected with specific antibodies. Results are representative of 10 (E47), 8 (Id 1) and 6 (Id3, Ikaros) pairs of mice

shown to correlate with decreased expression of E2A by Western blot analyses in IL-7-expanded pro-B/pre-B cells from old mice (12, 22). We next examined DNA-binding of the E2A-encoded transcription factor E47 in nuclear extracts of these IL-7 blasts from young and severely depleted and moderately depleted old mice.

4.2. Effects of aging on DNA-binding of E47 in IL-7-expanded pro-B/early pre-B cells

IL-7-stimulated pro-B/pre-B cells from both severely depleted and moderately depleted old mice exhibit a reduced E47 DNA-binding as compared to young mice, the defect in severely depleted old mice being more dramatic than that in moderately depleted old mice (Figure 2). Anti-E47 antibodies effectively prevent DNA-binding, confirming previous results showing that E47/E12 complexes bind DNA in these IL-7-expanded pro-B/pre-B cells (12).

4.3. Effects of aging on the expression of E47 and Id1-3 in IL-7-expanded pro-B/early pre-B cells

Since DNA-binding of E47 is decreased in IL-7-expanded pro-B/early pre-B cells from both severely depleted and moderately depleted old mice, we asked whether this could be dependent on reduced levels of E47 and/or increased levels of inhibitory Id1-3 proteins in the nuclear extracts from IL-7-expanded pro-B/early pre-B cells. We have previously shown that the expression of E2A (22) and E47 (12) by Western blot in whole cell lysates of IL-7-expanded pro-B/pre-B cells is decreased by aging. Results in Figure 3 show that E47 expression is decreased in nuclear extracts of pro-B/pre-B from both severely depleted and, to a lesser extent, from moderately depleted old mice. Thus, differences in DNA-binding between young and severely/moderately depleted old mice are likely to reflect differences in E47 expression in these IL-7-expanded pro-B/early pre-B cells. Id1 was also found to be decreased in IL-7-expanded pro-B/early pre-B cells from old as compared to young mice, whereas Id 3 was found not to be modified by aging. Id2 was undetectable in either young or old mice (data not shown). Ikaros, a zinc-finger transcription factor exerting a critical role during the early stages of B cell development (29), was unaffected by aging in these cells, demonstrating that not all proteins nor transcription factors are down-regulated during aging. In our experiments, Id1 was found to be decreased in IL-7 blasts of all old mice tested. In 4 independent experiments with young, moderately and severely depleted old mice, moreover, Id1 was found to be slightly higher in severely depleted as compared to moderately depleted old mice. Results from the densitometric analyses of the bands obtained in Western analyses indicate that severely depleted old mice have 3 times more Id1 than moderately depleted mice (means of the densitometric estimates +/- SE were: 10 in young mice, 6.00 +/- 0.41 in severely depleted and 1.98 +/- 0.72 in moderately depleted old mice). This result might indeed account for the higher reduction in E47 amount in severely depleted as compared to moderately depleted old mice, but not for the age-related difference in E47 between young and old mice, because Id1 was not increased in old as compared to young mice. Thus, from these results obtained so far we can conclude that the reduced expression of E47 in nuclear extracts of IL-7-expanded pro-B/early pre-B cells from both severely depleted and moderately depleted old mice accounts for the age-related reduction in DNA-binding of E47. Id proteins, which are inhibitors of E47, are not increased during aging and therefore do not contribute to decreased E47 DNA-binding activity seen in aged mice. However, the higher levels of Id1 in severely depleted as compared to moderately depleted old mice could account for the different DNA-binding of E47 in these mice.

5. DISCUSSION

The E2A-encoded transcription factor E47 is a key regulator of B cell differentiation processes. The percentages of pre-B cells in the bone marrow are positively correlated with the levels of E2A proteins during aging (22), and E2A protein activities can be modulated by Id proteins (18, 28, 30, 31). It is generally accepted that the role of Id proteins is to negatively regulate E2A during B cell development, because they efficiently heterodimerize with E proteins and act as dominant-negative HLH proteins. As a result of inhibiting E protein DNA-binding, Id proteins also inhibit E-box-dependent activation of E protein target genes. This loss of E protein activity can result in the loss of tissue-specific gene expression and of differentiation programs in different cell types (1, 32-34).

Id proteins are expressed in the fetal liver, bone marrow, spleen and thymus (35). Studies on B cell lines have demonstrated that the expression of Id proteins is high in pro-B cells and decreases with maturation (36). In mature B cells, expression of these proteins has been shown to repress transcriptional activity of the IgH and *k* intronic

enhancers (36). Id3 transcripts are rapidly induced after BCR engagement, suggesting a role of Id3 in promoting cell cycle entry (18, 37). Although several published data strongly suggest that Id proteins negatively regulate E proteins, unequivocal evidence of the direct interaction between Id and E proteins *in vivo* has come from genetic analysis of $Id^{-/-}$ mice and from studies on ectopic expression of these proteins. Id1 expression was shown to be dispensable for normal murine development (38), as $Id1^{-/-}$ mice do not show impaired B cell development when tested either alone or in combination with E2A deficiency (37, 38). Conversely, $Id2^{-/-}$ mice display profound defects in peripheral lymphoid organ formation and development of NK cells (39, 40). $Id3^{-/-}$ mice show a severely compromized humoral immunity, although they do not display overt abnormalities in tissue and embryo development and have normal numbers of B and T lymphocytes: B cells respond poorly to BCR cross-linking signals, alone or delivered together with cytokines such as IL-4, and are unable to switch to IgG2a and IgG3 isotypes (8, 37). Analysis of $Id3^{-/-}/E2A^{-/-}$ mice failed to show any effect of *Id3* deficiency on B cell development (37): thus, $E2A^{-/-}$ mice cannot be rescued by the deletion in *Id3*. This suggests that *Id3* does not play a major role in early B cell development.

Ectopic expression of Id1 inhibits development and differentiation of myoblasts and of myeloid, erythroid and mammary epithelial cell lines (41-44), whereas ectopic expression of Id3 in primary splenic B lymphocytes blocks class switch recombination but not Ig constant region germline transcription, suggesting that E2A plays a role in isotype switch downstream of germline transcription and may be required for the regulation of genes involved in the class switch process (8) or in accessibility/transcription of the upstream (V(D)JEμ) sequence involved in class switch.

Little is known about *in vivo* expression of Id proteins in B cell precursors. In the present paper, we have analyzed IL-7-expanded populations of pro-B/early pre-B cells to evaluate whether the reduced age-related E47 DNA-binding could be attributed to reduced age-related E47 expression and/or increased age-related Id expression. Results herein suggest that Id proteins do not play a major role in B cell development at this stage. This may be due either to their level of expression, which may be insufficient in IL-7-expanded pro-B/early pre-B cells, due to their rapid turnover, or to the fact that these proteins could be inactive in these cells. Id3 is indeed phosphorylated by cyclin-dependent kinase 2 in late G1 (18) and the phosphorylation may alter its activity and interaction with other proteins. Moreover, Id3 undergoes rapid turnover during the cell cycle and its levels are regulated by the ubiquitin-proteasome degradation pathway (45). Thus, Id family proteins are small and transiently expressed proteins, whose detection is difficult not only *in vivo* but even after ectopic expression. Because of their instability inside the cell, their levels are tightly regulated by degradation processes in order to avoid deleterious consequences. Interestingly, the half-life of Id3 is increased by co-expression with E47 (46), suggesting that interaction with its binding partner protects Id3 protein from degradation. Recent studies have reported that also some Id interacting proteins are degraded by the ubiquitin–proteasome pathway. The HLH proteins E12 and MyoD are ubiquitinated, and turned over rapidly (47, 48) and treatment with proteasome inhibitors increases accumulation of these HLH proteins in transfected cells. In the experiments presented in this paper, Id1 was found decreased in old as compared to young mice. Thus, we do not think that it is involved in the reduction of DNA-binding observed in IL-7 blasts from old mice. The fact that Id1 is slightly increased in IL-7 blasts from severely depleted as compared to moderately depleted old mice could indeed account for the higher reduction in E47 amount in severely depleted as compared to moderately depleted old mice. However, further experiments on stability of the Id proteins, in particular Id1, within the cell are required in order to provide additional explanation of the mechanisms that control the balance between E and Id proteins, allowing the cell to fine-tune the regulatory activities of these transcription factors.

6. ACKNOWLEDGMENTS

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7. REFERENCES

1. Quong, MW, WJ Romanow & C Murre: E protein function in lymphocyte development. *Annu Rev Immunol* 20, 301-322 (2002)

2. Schlissel M, A Voronova & D Baltimore: Helix-loop-helix transcription factor E47 activates germ-line immunoglobulin heavy-chain gene transcription and rearrangement in a pre-T-cell line. *Genes Dev* 5, 1367-1376 (1991)

3. Bain G, EC Robanus Maandag, HP te Riele, AJ Feeney, A Sheehy, M Schlissel, SA Shinton, RR Hardy & C Murre: Both E12 and E47 allow commitment to the B cell lineage. *Immunity* 6, 145-154 (1997)

4. Sigvardsson, M, M O'Riordan & R Grosschedl: EBF and E47 collaborate to induce expression of the endogenous immunoglobulin surrogate light chain genes. *Immunity* 7, 25-36 (1997)

5. Kee, BL & C Murre: Induction of early B cell factor (EBF) and multiple B lineage genes by the basic helix-loop-helix transcription factor E12. *J Exp Med* 188, 699-713 (1998)

6. Kee, BL, G Bain & C Murre: IL-7Ralpha and E47: independent pathways required for development of multipotent lymphoid progenitors. *EMBO J* 21, 103-113 (2002)

7. Bain, G., EC Robanus Maandag, D Izon, D Amsen, AM Kruisbeek, BC Weintraub, I Krop, MS Schlissel, AJ Feeney, M van Roon, M van der Valk, HPJ te Riele, A Berns & C Murre: E2A proteins are required for proper B

cell development and initiation of immunoglobulin gene rearrangements. *Cell* 79, 885-892 (1994)

8. Quong, MW, DP Harris, SL Swain & C Murre: E2A activity is induced during B-cell activation to promote Immunoglobulin class switch recombination. *EMBO J* 18, 6307-6318 (1999)
9. Murre, C., A. Voronova & D Baltimore: B-cell- and myocyte-specific E2-box-binding factors contain E12/E47-like subunits. *Mol Cell Biol* 11: 1156-1160 (1991)

10. Sloan, SR, CP Shen, R McCarrick-Walmsley & T Kadesh: Phosphorylation of E47 as a potential determinant of B-cell-specific activity. *Mol Cell Biol* 16, 6900-6908 (1996)

11. Chu, C & DS Kohtz: Identification of the E2A gene products as regulatory targets of the G1 cyclin-dependent kinases. *J Biol Chem* 276, 8524-8534 (2001)

12. Frasca, D, D Nguyen, RL Riley & BB Blomberg: Decreased E12 and/or E47 transcription factor activity in the bone marrow as well as in the spleen of aged mice. *J Immunol* 170, 719-726 (2003)

13. Lassar, AB, RL Davis, WE Wright, T Kadesch, C Murre, A Voronova, D Baltimore & H Weintraub: Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins *in vivo*. *Cell* 66, 305-315 (1991)

14. Lee, JE, SM Hollenberg, L Snider, DL Turner, N Lipnick & H Weintraub: Conversion of Xenopus ectoderm into neurons by NeuroD, a basic helix-loop-helix protein. *Science* 268, 836-844 (1995)

15. Herblot, S, PD Aplan & T Hoang: Gradient of E2A activity in B-cell development. *Mol Cell Biol* 22, 886-900 (2002)

16. Rivera R & C Murre: The regulation and function of the Id proteins in lymphocyte development. *Oncogene* 20, 8308-8316 (2001)

17. Riechmann, V, I van Cruchten & F Sablitzky: The expression pattern of Id4, a novel dominant negative helix-loop-helix protein, is distinct from Id1, Id2 and Id3. *Nucleic Acids Res* 22, 749-755 (1994)

18. Deed, RW, E Hara, GT Atherton, G Peters & JD Norton: Regulation of Id3 cell cycle function by Cdk-2-dependent phosphorylation. *Mol Cell Biol* 12, 6815-6821 (1997)

19. Zebedee, Z & E Hara: Id proteins in cell cycle control and cellular senescence. *Oncogene* 20, 8317-8325 (2001)

20. Riley, RL, MG Kruger & J Elia: B cell precursors are decreased in senescent Balb/c mice, but retain normal mitotic activity *in vivo* and *in vitro*. *Clin Immunol Immunopathol* 59, 301-313 (1991)

21. Sherwood, EM, BB Blomberg, W Xu, CA Warner & RL Riley: Senescent Balb/c mice exhibit decreased expression of λ5 surrogate light chains and reduced development within the pre-B cell compartment. *J Immunol* 161, 4472-4475 (1998)

22. Sherwood, EM, W Xu, AM King, BB Blomberg & RL Riley: The reduced expression of surrogate light chains in B cell precursors from senescent Balb/c mice is associated with decreased E2A proteins. *Mech Ageing Dev* 118, 45-59 (2000)

23. Kitamura, D, J Roes, R Kuhn & K Rajewsky: A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin mu chain gene. *Nature* 350, 423-426 (1991)

24. Kitamura, D, A Kudo, S Schaal, W Muller, F Melchers & K Rajewsky: A critical role of lambda 5 protein in B cell development. *Cell* 69, 823-831 (1992)

25. Karasuyama, H, A Rolink, Y Shinkai, F Young, FW Alt & F Melchers: The expression of Vpre-B/lambda 5 surrogate light chain in early bone marrow precursor B cells of normal and B cell-deficient mutant mice. *Cell* 77, 133-143 (1994)

26. Teh, YM & MS Neuberger: The immunoglobulin Igα and Igβ cytoplasmic domains are independently sufficient to signal B cell maturation and activation in transgenic mice. *J Exp Med* 185, 1753-1758 (1997)

27. Jacobs, Y, C Vierra & C Nelson: E2A expression, nuclear localization, and *in vivo* formation of DNA- and non-DNA-binding species during B-cell development. *Mol Cell Biol* 13, 7321-7333 (1993)

28. Bain, G, CL Cravatt, C Loomans, J Alberola-Ila, SM Hedrick & C Murre: Regulation of the helix-loop-helix proteins, E2A and Id3, by the Ras-ERK MAPK cascade. *Nat Immunol* 2, 165-170 (2001)

29. Kirstetter, P, M Thomas, A Dierich, P Kastner & S Chan: Ikaros is critical for B cell differentiation and function. *Eur J Immunol* 32, 720-730 (2002)

30. Norton, DD: Id helix-loop-helix proteins in cell growth, differentiation and tumorigenesis. *J Cell Sci* 113, 3897-3905 (2000)

31. Norton, JD, RW Deed, G Craggs & F Sablitzky: Id helix-loop-helix proteins in cell growth and differentiation. *Trends Cell Biol* 8, 58-65 (1998)

32. Engel, I & C Murre: The function of E- and Id proteins in lymphocyte development. *Nat Rev Immunol* 1, 193-199 (2001)

33. Chen, B, BH Han, XH Sun & RW Lim: Inhibition of muscle-specific gene expression by Id3: requirement of the C-terminal region of the protein for stable expression and function. *Nucleic Acids Res* 25, 423-430 (1997)

34. Moldes, M, F Lasnier, B Feve, J Pairault & P Djian: Id3 prevents differentiation of preadipose cells. *Mol Cell Biol* 17, 1796-1804 (1997)

35. Riechmann, V, I van Cruchten & F Sablitzky: The expression pattern of Id4, a novel dominant negative helix-loop-helix protein, is distinct from Id1, Id2 and Id3. *Nucleic Acids Res* 22, 749-755 (1994)

36. Wilson, RB, M Kiledjian, CP Shen, R Benezra, P Zwollo, SM Dymecki, SV Desiderio & T Kadesch: Repression of immunoglobulin enhancers by the helix-loop-helix protein Id: implications for B-lymphoid-cell development. *Mol Cell Biol* 11, 6185-6191 (1991)

37. Pan, L, S Sato, JP Frederick, XH Sun & Y Zhuang: Impaired immune responses and B-cell proliferation in mice lacking the Id3 gene. *Mol Cell Biol* 19, 5969-5980 (1999)

38. Yan, W, AZ Young, VC Soares, R Kelley, R Benezra & Y Zhuang: High incidence of T-cell tumors in E2A-null mice and E2A/Id1 double-knockout mice. *Mol Cell Biol* 17, 7317-7327 (1997)

39. Yokota, Y, A Mansouri, S Mori, S Sugawara, S Adachi, S Nishikawa & P Gruss: Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. *Nature* 397, 702-706 (1999)

40. Becker-Herman, S, F Lantner & I Shachar: Id2 negatively regulates B cell differentiation in the spleen. *J Immunol* 168, 5507-5513 (2002)

41. Jen, Y, H Weintraub & R Benezra: Overexpression of Id protein inhibits the muscle differentiation program: *in vivo* association of Id with E2A proteins. *Genes Dev* 6, 1466-1479 (1992)

42. Kreider, BL, R Benezra, G Rovera & T Kadesch: Inhibition of myeloid differentiation by the helix-loop-helix protein Id. *Science* 255, 1700-1702 (1992)

43. Shoji, W, T Yamamoto & M Obinata: The helix-loop-helix protein Id inhibits differentiation of murine erythroleukemia cells. *J Biol Chem* 269, 5078-5084 (1994)

44. Desprez, PY, E Hara, MJ Bissell & J Campisi: Suppression of mammary epithelial cell differentiation by the helix-loop-helix protein Id-1. *Mol Cell Biol* 15, 3398-3404 (1995)

45. Bounpheng, MA, JJ Dimas, SG Dodds & BA Christy: Degradation of Id proteins by the ubiquitin-proteasome pathway. *FASEB J* 13, 2257-2264 (1999)

46. Deed, RW, S Armitage & JD Norton: Nuclear localization and regulation of Id protein through an E protein-mediated chaperone mechanism. *J Biol Chem* 271, 23603-23606 (1996)

47. Kho, C, GS Huggins, WO Endege , C-M Hsieh , M-E Lee & E Haber: Degradation of E2A proteins through a ubiquitin-conjugating enzyme, UbcE2A. *J Biol Chem* 272, 3845-3851 (1997)

48. Breitschopf, K, E Bengal, T Ziv, A Admon & A Ciechanover: A novel site for ubiquitination: the N-terminal residue, and not internal lysines of MyoD, is essential for conjugation and degradation of the protein. *EMBO J* 17, 5964-5973 (1998)

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