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KU HAPLOINSUFFICIENCY CAUSES A LYMPHOPROLIFERATIVE DISORDER OF IMMATURE T-CELL PRECURSORS DUE TO IKAROS MALFUNCTION

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Abstract

Ikaros (IK) malfunction has been implicated in the pathogenesis of acute lymphoblastic leukemia (ALL), the most common form of childhood cancer. Therefore, a stringent regulation of IK activity is very important. Here we provide unique genetic and biochemical evidence that the Ku protein components Ku70 and Ku80 act as positive regulators of IK function via formation of IK-Ku70 and IK-Ku80 heterodimers with augmented sequence-specific DNA binding activity. siRNA-mediated depletion of Ku70 or Ku80 reduced the sequence-specific DNA binding activity of IK in EMSA as well as the RT-PCR measured IK target gene expression levels in human cells. The interaction of Ku components with IK likely contributes to the anti-leukemic effects of IK as a tumor suppressor, because Ku70 as well as Ku80 haploinsufficiency in mice caused development of a lymphoproliferative disorder (LPD) involving CD2⁺CD4⁺CD8⁺CD1⁺IL7R⁺ thymic T-cell precursors with functional IK deficiency.

Introduction

Ikaros (IK) is a zinc finger (ZF)-containing sequence-specific DNA-binding protein that plays an important role in immune homeostasis through transcriptional regulation of the earliest stages of lymphocyte ontogeny by both (a) gene transcriptional activation via efficient transcription initiation and elongation as well as (b) gene repression.^{1,2} Alternatively spliced transcripts of the *IKFZ1* gene with selective inclusion of exons 4–7

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encode at least eight zinc finger proteins with distinct DNA-binding capabilities and specificities (IK isoforms IK1 through IK8) in mice and humans.¹ The formation of homo- and heterodimers among the DNA binding IK isoforms increases their affinity for DNA.¹ Functional IK deficiency has been associated with development of T-cell precursor leukemias in mice and humans.^{2,3} Therefore, a stringent regulation of IK activity is considered of paramount importance.

NHEJ is the predominant DNA-double strand break (DSB) repair pathway in mammalian cells.⁴ The heterodimeric Ku protein (Ku70/Ku80) functions as the versatile DNA-targeting regulatory subunit of the DNA-dependent protein kinase (DNA-PK) holoenzyme, a nuclear serine/threonine kinase that plays a critical role in the NHEJ pathway of DNA-DSB repair.⁵ Here we provide unique genetic and biochemical evidence that the Ku protein components Ku70 and Ku80 act as positive regulators of IK function both *in vitro* and *in vivo*. siRNA-mediated depletion of Ku70 or Ku80 reduced the sequence-specific DNA binding activity of IK in EMSA as well as the RT-PCR measured IK target gene expression levels in human cells. The interaction of Ku components with IK likely contributes to the anti-leukemic effects of IK as a tumor suppressor, because Ku70 as well as Ku80 haploinsufficiency in mice caused development of a lymphoproliferative disorder (LPD) involving immature T-cell precursors with functional IK deficiency. Our study uniquely implicates Ku deficiency for the IK malfunction in T-cell precursors, which is a hallmark of pediatric high-risk T-lineage ALL.

Materials and Methods

Standard Biochemical, Imaging, and Transfection Methods

Confocal Laser Scanning Microscopy, co-immunoprecipitations, Western blot analyses, pull-down assays, and electrophoretic mobility shift assays (EMSA) were performed as per previously described standard procedures. 293T cells were transfected after reaching 70–80% confluence using ON-TARGET^{plus} SMARTpool siRNA and DharmaFECT Transfection Reagent 4 (Catalog No. T-2004) (Thermo Scientific Dharmacon, Lafayette, CO, USA) (supplemental Methods). Reverse transcription (RT) and polymerase chain reaction (PCR) were used to evaluate the expression levels of IK target genes.

Bioinformatics and Statistical Analysis of Gene Expression Profiles

The publically available archived GSE32311 database⁶ was used to compare gene expression changes in CD4⁺CD8⁺ double-positive wild type (N=3; GSM800500, GSM800501, GSM800502) vs. *IK* null mouse thymocytes (N=3, GSM800503, GSM800504, GSM800505) from the same genetic background of (C57BL/6 x 129S4/SvJae), as described.⁷ Probe level RMA signal intensity values were obtained from the mouse 430_2.0 Genome Array. Up-regulated and down-regulated transcripts in *IKZF1/Ikaros* knockout mice were identified by filtering changes greater than 2 fold and T-test P-values less than 0.05 (T-test, Unequal Variances, Excel formula). The GSE32311 database was also used to compare the gene expression profiles of normal thymocytes from 3 wildtype mice (GSM800500, GSM800501, GSM800502) to those of abnormal thymocytes of 8 preleukemic mice of the same genetic background of (C57BL/6 x 129S4/SvJae) with *IKZF1/Ikaros* null mutation (N=3, GSM800503, GSM800504, GSM800505) or expression of dominant negative IK isoforms (N=5, GSM800506, GSM800507, GSM802973, GSM802974, GSM802975).

Characterization of Thymocyte Populations in Ku knockout mice

We examined the surface antigen profiles of thymocytes from Ku knockout mice using flow cytometric immunophenotyping. The IK function of the thymocytes was examined by EMSA (supplemental Methods).

Results

Heterodimerization of Ikaros with Ku70 or Ku80 augments its sequence-specific DNA binding function

In co-immunoprecipitation experiments using Triton X-100 whole cell lysates from EBV-transformed non-leukemic human B-cells, both Ku70 and Ku80 immune complexes contained both Ku70 and Ku80 consistent with a stable physical association between these two components of the Ku70/Ku80 heterodimer (Fig. 1A1). Notably, both Ku70 and Ku80 immune complexes also contained IK indicating that IK constitutively exists in a stable physical association with Ku protein (Fig. A2). This association was further confirmed by demonstrating that IK immune complexes contain Ku both Ku70 and Ku80 (Fig. 1A3). There was more Ku70 in Ku70 immune complexes and more Ku80 in Ku80 immune complexes than in IK immune complexes. Likewise, there was more IK protein in IK immune complexes than in Ku70 or Ku80 immune complexes. These results indicate that not all of the IK protein exists in a complex with Ku components. We next performed “pull-down” experiments with MBP-tagged recombinant IK proteins. DNA-binding isoforms of Ikaros, IK1, IK2, and IK3 each pulled down both Ku70 and Ku80 proteins (Fig. 1B 1&2).

High affinity binding of the most abundant IK isoform IK1 to DNA requires its homo- or hetero-dimerization with other DNA-binding IK isoforms.¹ After demonstrating that native IK can form complexes with both Ku70 as well as Ku80, we next performed electrophoretic mobility shift assays (EMSA) in a cell-free platform devoid of other proteins to evaluate the ability of heterodimers of recombinant IK with recombinant Ku70 or Ku80 to bind the IK-Bs1 oligonucleotide probe containing a high-affinity IK1 binding site. The addition of increasing amounts of Ku70 or Ku80 protein to the MPB-tagged IK1 protein suspension resulted in increased retardation of the IK-specific IK-Bs1 probe in a dose-dependent fashion, which indicates stronger sequence-specific DNA binding by IK1-Ku heterodimers vs. IK1 homodimers (Fig. 1C1). By using anti-IK, anti-Ku70, anti-Ku80 antibodies in EMSA supershift assays, we experimentally confirmed the presence of both Ku70 or Ku80 along with IK1 in the IK-specific DNA binding molecular complexes (Fig. 1C2).

siRNA Mediated Knockdown of Ku Expression Diminishes the Constitutive Transcription Factor Activity of Ikaros in Human Cells

We could not employ RNA interference to evaluate the role of native Ku components for IK function in human lymphoid cells as Ku70- or Ku80-specific siRNA caused apoptosis. In order to experimentally document the significance of Ku components Ku70 and Ku80 to the function of IK, we therefore examined the effects of their depletion by RNA interference on IK-specific DNA binding activity in nuclear extracts from 293T cells using EMSAs with the end-labeled IK-Bs1 oligonucleotide probe containing a high-affinity IK binding site (Fig. 2). The selective depletion of Ku70 and Ku80 by specific siRNA was documented using Western blot analyses (Fig. 2A). We recently published 293T cells as an IK⁺ human cell line that provides the opportunity to study post-translational regulation of native IK.⁷ Nuclear extracts of 293T cells exhibited abundant IK activity in EMSAs performed using the IK-Bs1 oligonucleotide probe containing a high-affinity IK1 binding site (Fig. 2B) and expressed transcripts of validated IK target genes ITGA4, KIF23, TNFAIP8L2, PREP, DNAJC6, and EIF4E3 (Fig. 2C). IK-specific siRNA (but not scrambled control siRNA) abrogated or reduced the expression of each of these 6 IK target genes (Fig. 2C). Notably siRNA-

mediated depletion of Ku70 or Ku80 abolished the DNA binding activity of native IK (Fig. 2B). To formally document the importance of the Ku components Ku70 and Ku80 for the ability of IK to activate the expression of its target genes, we also examined the effects of their depletion by RNA interference on IK target gene expression in human 293T cells using RT-PCR (Fig. 2C). Notably, the expression levels of all IK target genes were reduced by siRNA-mediated depletion of Ku70 or Ku80, whereas treatment with scrambled siRNA (included as a negative control) had no such effect. The striking Ku-dependency of the IK target gene expression levels is consistent with the notion that Ku plays a critical role in regulation of the IK function.

Ku-Knockout Mice Develop a Lymphoproliferative Disorder of Immature T-Cell with Functional Ikaros Deficiency

Complete Ku deficiency is lethal to human cells⁸ as well as C57Bl/6 mice, as the only mouse strain that mimics the human biology in regards to Ku-requirements for viability and survival.⁹ C57Bl/6 mice that are homozygous Ku knockouts are not viable past the early post-natal period.⁹ Therefore, we sought to examine the biologic significance of partial Ku deficiency in C57Bl/6 mice that were heterozygous knockouts for Ku70 or Ku80 genes. These mice developed at 2–3 months of age a lymphoproliferative disorder (LPD) that was characterized by a 9.5-fold above baseline increase of the IL7R-positive immature T-precursor count in the thymus ($29.3 \times 10^6/\text{thymus}$ vs. $3.1 \times 10^6/\text{thymus}$, T-test P-value = 0.016) (Fig. 3A). The composite immunophenotype of the thymocytes from Ku knockout mice was $\text{CD1}^+\text{CD2}^+\text{CD3}^+\text{CD4}^+\text{CD8}^+\text{IL7R}/\text{CD127}^+\text{FLT3}/\text{CD135}^+$ consistent with the surface antigen profile of an immature cortico-thymocyte (Fig. 3B, supplemental Figure 1). CD3 antigen is expressed on both very immature double-positive ($\text{CD4}^+\text{CD8}^+$) T-cell precursors and mature T-cells^{10,11}, whereas CD1 antigen is expressed only on T-cell precursors at a cortico-thymocyte stage.¹²

Notably, IL7R gene is a transcriptional target for IK and expression of dysfunctional dominant negative IK isoforms or *IKZF1* null mutation is associated with increased levels of IL7R gene expression in thymocytes during the preleukemic phase (Fig. 4A). We therefore sought to determine the subcellular localization and DNA binding activity of IK in thymocytes of Ku-knockout mice. In sharp contrast to the nuclear localization of IK in all of the thymocytes from wildtype mice, IK was localized predominantly in the cytoplasm of all of the thymocytes from Ku-knockout mice (Fig. 4B&C). The differences in IK localization between thymocytes from wildtype mice vs. thymocytes from the Ku80- and Ku80-knockout mice were statistically significant (Fisher's Exact Test, 2-tailed, $P < 0.0001$).

Since the nuclear localization of IK is determined by its DNA binding activity, these results uniquely indicated that partial Ku deficiency adversely affects the DNA binding activity of native IK in murine thymocytes. IK has been shown to bind to repetitive sequences within PC-HC that contain consensus IK binding sites, and its localization to the PC-HC in the nucleus is directly related to its ability to bind to these sequences.^{13,14} Therefore, we next performed EMSAs to directly examine the effect of partial Ku deficiency on the binding of native IK to the biotin labeled γ -satellite A probe derived from the centromeric γ -satellite repeat sequences. The γ -satellite A DNA probe contains two consensus IK binding sites in close proximity to each other and is a target for high affinity binding of wildtype IK and the binding affinity to this probe shows an excellent correlation with the homing of IK to PC-HC.^{7,13,14} In agreement with their abnormal subcellular IK localization, thymocytes of Ku knockout mice showed no native IK activity as measured by binding of their nuclear extracts to the γ -satellite A probe (Fig. 4D). The observed development of an LPD involving IL7R positive immature thymocytes with functional IK deficiency in Ku-heterozygous mice

provided unique evidence for haploinsufficiency of Ku70/Ku80 genes and indicated that native Ku is physiologically important for normal IK function in mice.

Discussion

Currently, our knowledge regarding the upstream regulators of IK function is relatively limited. IK function, stability, and subcellular localization are generally thought to be regulated by posttranslational modification and heterodimerization with other members of the IK family of DNA binding proteins.¹ Phosphorylation of IK by casein kinase II (CK2) inhibits its many functions and promotes its degradation via the ubiquitin/proteasome pathway.¹³ Conversely, dephosphorylation of IK by protein phosphatase 1 is critical for its ability to bind to target DNA sequences, localize to PC-HC in the nucleus, and exert its regulatory functions.¹⁴ In a recent study, we identified the spleen tyrosine kinase (SYK) as a posttranslational regulator of IK and determined that SYK-induced activating phosphorylation of IK at unique C-terminal serine phosphorylation sites S358 and S361 is essential for its nuclear localization and optimal transcription factor function.⁷ We now report direct evidence that Ku components Ku70 and Ku80 bind to IK thereby augmenting its nuclear localization and sequence-specific DNA binding activity. The present study provides experimental evidence that Ku colocalizes with IK in human cells and its heterodimerization amplifies the transcription factor function of IK. Ku is the only protein outside the IK family of ZF proteins shown to non-enzymatically improve the function of IK as a sequence-specific DNA binding protein.

In human B-lymphocytes cells, IK was constitutively associated with both Ku70 and Ku80 components of the Ku heterodimer. Recombinant Ku70 and Ku80 formed stable complexes with recombinant IK *in vitro* and enhanced its DNA binding activity. Native IK exhibited a normal multifocal nuclear localization in wildtype thymocytes, but a predominantly cytoplasmic expression in thymocytes from heterozygous Ku knockout mice. These results uniquely indicated that native Ku protein forms a complex with IK, promotes its nuclear localization and enhances its sequence-specific DNA binding activity. siRNA-mediated depletion of Ku70 or Ku80 abolished the sequence-specific DNA binding activity of IK in EMSA and reduced the RT-PCR measured IK target gene expression levels in human cells. We conclude that Ku is critical for the nuclear localization and optimal transcription factor function of IK.

Pull-down experiments using recombinant IK isoforms demonstrated that the Cys₂His₂ zinc finger (ZF) motifs of IK near its N terminus are important for the IK-Ku70/Ku80 interactions. The very similar abilities of recombinant IK1, IK2 and IK3 proteins to pull down Ku70 and Ku80 demonstrate that the protein domains encoded by IK exons 3 (E3) (missing in IK2), or 5 (E5) and 6 (E6) (missing in IK3) are not required for IK-Ku interactions. By comparison, MBP-IK4 and MBP-IK5 pulled down much smaller amounts of Ku70 or Ku80 than the MBP-IK-1, MBP-IK2, and MBP-IK3 fusion proteins. In view of the pull-down efficiency of MBP-IK2 lacking the E3-domain and MBP-IK3 lacking the E5 domain, the diminished Ku-binding of MBP-IK4 cannot be explained by lack of E3 or E5 domains. The poor Ku-binding of MBP-IK5 indicates that E1, E2, E3, and E7 domains are not sufficient for optimal IK-Ku interactions. The most discriminating similarity between MBP-IK1, MBP-IK2, and MBP-IK3 vs. MBP-IK4 and MBP-IK5 is the number of their N-terminal zinc fingers (4 in IK1, 3 in IK2 and IK3 but only 2 in IK4 and 1 in IK5) indicating that the zinc fingers likely participate in the physical contact between IK and Ku proteins. The 3-D structure of IK has not been resolved and the exact roles of the 6 C₂H₂ ZFs of IK in its interactions with DNA and their relative contributions to its DNA binding affinity remain unknown (26). The future elucidation of the structural basis of IK activation by its

interactions with Ku70 or Ku80 will require a 3-D structure determination of IK at atomic resolution using X-ray crystallography or nuclear magnetic resonance (NMR) spectroscopy.

The interaction of Ku components with IK likely contributes to the anti-leukemic effects of IK as a tumor suppressor *in vivo*², because Ku haploinsufficiency caused development of a lymphoproliferative disorder (LPD) in heterozygous Ku70 and Ku80 knockout mice involving IL7R positive immature T-cell precursors with functional IK deficiency. It is noteworthy that comprehensive bioinformatic studies on haploinsufficiency have indicated that the *Ku80/XRCC5* gene is highly likely to be haploinsufficient.¹⁶ As Ku70 and Ku80 are not haploinsufficient for DNA double strand break (DSB) repair and even Ku-null cells have normal DNA repair activity due to hyperactive alternative NHEJ pathway¹⁷, the development of a T-cell precursor hyperplasia in haplodeficient mice cannot be explained by DNA repair deficits and provides compelling evidence that the interaction of Ku components with IK likely contributes to the anti-leukemic effects of IK as a tumor suppressor. Our study uniquely implicates Ku deficiency for the IK malfunction in T-cell precursors, which is a hallmark of pediatric high-risk T-lineage ALL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Ku components Ku70 and Ku80 form heterodimers with Ikaros

[A] immunoprecipitation of Ikaros and Ku. A.1. depicts the results of the Ku70 Western blot analysis of whole cell lysates (WCL) as well as Ku70, Ku80, and IK1 immune complexes immunoprecipitated (IP) from the EBV-transformed human B-cell line BCL1. Ku70 was detected not only in Ku70 immunoprecipitates, Ku80 immunoprecipitates and whole cell lysates that were included as positive controls but also in IK immunoprecipitates. A.2. depicts the results of the Ku80 Western blot analysis of WCL as well as Ku80, Ku70, and IK immune complexes from the same cell lysates. Ku80 was detected not only in Ku70 immunoprecipitates, Ku80 immunoprecipitates and whole cell lysates that were included as positive controls but also in IK immunoprecipitates. A.3. depicts the results of the IK Western blot analysis of IK, Ku70, and Ku80 immune complexes from the same cell lysates which demonstrated the presence of IK1 not only in IK immunoprecipitates and whole cell lysates that were included as positive controls but also in Ku70 and Ku80

immunoprecipitates. **[B] Pull-Down of Ku70 and Ku80 components of Ku with MBP-Ikaros Fusion Proteins.** MBP-IK fusion proteins were used in pull-down (PD) binding assays to examine their ability to interact with native Ku protein in lysates of the BCL-1 cell line. Fusion protein adsorbates and control samples (+CON: BCL-1 WCL, -CON: BCL-1 WCL, PD using amylose beads with no fusion protein added) were resolved by SDS-PAGE, immunoblotted with anti-Ku70 (B.1) or anti-Ku80 (B.2) antibodies using the ECL detection system. MBP pulldown samples were included as additional negative controls. Ku70 and Ku80 were detected not only in whole cell lysates that were included as positive controls but also in protein samples pulled down with MBP-tagged IK1, IK2, and IK3. **[C1] Ku70 and K80 Proteins Enhance Sequence-Specific DNA Binding Ability of Ikaros.** IK-specific DNA binding activity of recombinant MBP-IK1 fusion protein (100 ng/sample) was evaluated by EMSA using the ³²P-labeled double-stranded IK-Bs1 probe (1 ng/sample) in the presence of increasing amounts (50 ng, 100 ng, 150 ng, 200 ng/sample) of recombinant Ku80 (Lanes 2–5), Ku70 (Lanes 6–9), or MBP-IK1 (Lanes 10–13). Lane 1, 1 ng of IK-Bs1 probe alone, Lane 14, 1 ng IK-Bs1 probe mixed with 200 ng recombinant MBP-IK-1 and 100 ng unlabeled cold IK-Bs1. As IK1 binds DNA as a homodimer, the increased binding in the presence of Ku70 or Ku80 indicates that these proteins associate with IK1 to form heterodimers with improved Ikaros-Specific DNA-binding activity. **[C2] Supershift assays.** EMSA's were performed with ³²P-labeled IK-Bs1 oligonucleotide probe containing a high-affinity IK binding site (1 ng/sample, 100,000 cpm) and mixtures of MBP-IK1 + Ku70 (100 ng of each protein), MBP-IK1 + Ku80 (100 ng of each protein). Supershift assays were performed with monoclonal antibodies (2 µg/sample) against IK, Ku70, and Ku80 proteins to confirm the presence of the respective proteins in the retarded DNA-binding protein complexes.

Figure 2. Depletion of Native Ku70 or Ku80 Abrogates Sequence-Specific DNA Binding Activity of Ikaros in situ

[A.1 – A.3] Ku70, Ku80, and Tubulin (TUB) Western blot analysis of whole cell lysates from 293T cells (10×10^6 cells/sample) treated with medium only (CON), Ku80 siRNA, or Ku70 siRNA. Each siRNA was used at a 50 nM concentration. Ku70 siRNA as well as Ku80 siRNA resulted in depletion of Ku70 protein without a decrease in the amount of TUB that was used as a quality control to demonstrate equal protein loading in each lane. The depleting effects of Ku80 siRNA on Ku70 protein levels varied from experiment to experiment which was attributed to the fact that Ku70 levels were not subject to destabilizing low Ku80 protein levels for a sufficient time period as the test was performed at 72 h after treatment with Ku80 siRNA, when depletion of Ku80 is first detected. **[B]** EMSAs were performed on nuclear extracts (E) from untreated control (C) 293T cells as well as 293T cells treated for 72 h with Ku70 siRNA, Ku80 siRNA, or scrambled (scr) siRNA using the Thermo Scientific LightShift Chemiluminescent EMSA Kit and biotin-labeled DNA probes IK-BS1 and IK-BS5 (a mutant probe that was included as a non-IK binding control probe) (supplemental Methods). Each siRNA was used at a 50 nM concentration. IK activity was measured by the electrophoretic mobility shifts of the biotin-labeled IK-Bs1 probe, representing IK-containing nuclear complexes (indicated with arrow heads). The biotin-labeled DNA was detected using streptavidin-horseradish peroxidase conjugate and a chemiluminescent substrate. The membrane was exposed to X-ray film and developed with a film processor. Significant IK activity was detected in control nuclear extracts (CE) from untreated 293T cells as well as 293T cells treated with scr-siRNA, but not in nuclear extracts (E) of 293T cells treated with Ku70 siRNA or Ku80 siRNA. No mobility shifts were observed with the control IK-BS5 probe. **[C]** RT-PCR was used to examine the expression levels of 6 randomly selected IK target genes in 293T cells after 72

h treatment with medium alone (CON), 50 nM scrambled siRNA (sc-siRNA), Ku70 siRNA, or Ku80 siRNA. The expression levels of IK target genes were reduced by siRNA-mediated depletion of Ku70 or Ku80, whereas treatment with sc-siRNA had no such effect. Included as a positive control, IK siRNA also abrogated or reduced the expression of all 6 IK target genes.

Figure 3. Heterozygous Ku-Knockout Mice Develop a PLD of IL7R⁺ Immature Thymic T-Cell Precursors

[A.1 & A.2] The surface antigen profiles of thymocytes from ~3 months old wildtype (Median age: 83 days) and heterozygous Ku knockout mice (Median age: 106 days) were determined by direct immunofluorescence and flow cytometry using a panel of monoclonal antibodies, including those recognizing CD1, CD3, CD127/IL7R, and CD135/FLT3 antigens using standard methods (SI-text). The labeled cells were analyzed using a The LSR II flow cytometer. A.1 compares the percentages for IL7R positive thymocytes in thymocyte populations from wildtype vs. heterozygous Ku-knockout mice. See supplemental Figure 1 for more detailed information about the composite immunophenotypes. A.2 compares the total numbers of IL7R positive thymocytes in thymocyte populations from wildtype vs. heterozygous Ku-knockout mice. The CD3⁺ IL7R⁺ thymocyte numbers were compared between wildtype (WT; N = 8) and heterozygous Ku knockout Mice (Ku/KO; N=8) using T-

tests (2 sample, 2-tailed) for percent of total and $\log_{10}$ transformed values. [B.1 & B.2]
Representative two-color FACS histograms showing differences in the expression levels of the corticothymocyte marker CD1 and IK-deficiency associated IL7R on thymocytes from wildtype vs. heterozygous Ku-knockout mice.

Figure 4. Functional Ikaros Deficiency of Thymocytes in Ku-Knockout Mice

[A1 & A.2] The gene expression profiles (RMA signal intensities) of normal thymocytes from 3 wildtype mice were compared to those of abnormal functionally IK-deficient thymocytes of 8 preleukemic mice with *IK* null mutation (N=3) or expression of dominant negative IK isoforms (N=5). *IL7R* transcript levels were markedly elevated in IK-deficient thymocytes. [B.1] Nuclear localization of IK in normal thymocytes from 2 randomly selected wildtype mice. The thymic sizes in and cell numbers were 0.6 cm/24×10⁶ cells and 0.9 cm/21×10⁶ cells, respectively. The rabbit polyclonal antibody for IK1 (Santa Cruz, CA) and red-fluorescent Alexa Fluor 568 dye-labeled secondary antibody Alexa Fluor 568 F(ab')₂ fragment of goat anti-rabbit IgG (Invitrogen, CA) were used to identify IK localization as red staining in DAPI-stained blue nuclei. MERGE panels depict the merge two-color confocal images showing localization of IK in DAPI-stained nucleus as red immunofluorescent foci. IK showed nuclear localization in 19 of 19 IK-positive thymocytes from the first wildtype mouse as in the representative thymocyte depicted in upper panel and 24 of 24 IK-positive thymocytes from the second wildtype mouse as in the representative thymocyte depicted in lower panel. [B.2 & B.3] Aberrant cytoplasmic localization of IK in thymocytes from a randomly selected heterozygous Ku80-knockout mouse that developed a PLD of IL7R⁺ immature T-cell precursors. The thymic size and cell number were 1.4 cm and 265×10⁶ cells (134.8×10⁶ IL7R⁺ immature thymocytes), respectively. IK showed predominantly cytoplasmic as well as partial nuclear staining in 11 of 12 thymocytes as in

the representative thymocyte depicted in B2 and only cytoplasmic staining in the remaining thymocyte as in the representative thymocyte depicted in B3. The differences in IK localization between thymocytes from wildtype mice in B.1 vs. thymocytes from the Ku80-knockout mouse in B.2 were statistically significant (Fisher's Exact Test, 2-tailed, $P < 0.0001$). **[C]** Aberrant subcellular localization of IK in thymocytes from a randomly selected heterozygous Ku70-knockout mouse that developed a PLD of IL7R positive immature T-cell precursors. The thymic sizes in and cell numbers were $0.5 \text{ cm}/10 \times 10^6$ cells for the wildtype mouse and $0.8 \text{ cm}/40 \times 10^6$ cells for the Ku70-knockout mouse, respectively. There were a total of 32.3×10^6 IL7R⁺ immature thymocytes in the hypercellular thymus. An in-house mouse monoclonal anti-IK antibody (4) and a green-fluorescent Alexa Fluor 488 dye-labeled secondary antibody Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, CA) were used. Depicted are the merge two-color confocal images showing localization of IK in blue-stained nucleus as green immunofluorescent foci. **[C.1]** Normal nuclear IK staining was detected in 8 of 8 thymocytes from the wildtype mouse as in the depicted representative thymocytes. **[C.2&C.3]** IK was localized in the cytoplasm of 26 of 26 IK-positive thymocytes from the Ku70-knockout mouse, as in the depicted representative thymocytes. The differences in IK localization between thymocytes from wildtype mouse in C.1 vs. thymocytes from Ku70-knockout mouse in C.2 were statistically significant (Fisher's Exact Test, 2-tailed, $P < 0.0001$). **[D]** Absent IK DNA binding activity in Ku-deficient thymocytes. EMSAs were performed on nuclear extracts (NE) from wildtype vs. heterozygous Ku-knockout mice using the Thermo Scientific LightShift Chemiluminescent EMSA Kit and biotin-labeled DNA probe γ -satellite A. IK activity was measured by the electrophoretic mobility shifts of the biotin-labeled probe, representing IK-containing nuclear complexes (indicated with arrow head). The biotin-labeled DNA was detected using streptavidin-horseradish peroxidase conjugate and a chemiluminescent substrate. The membrane was exposed to X-ray film and developed with a film processor.