## SUPPLEMENTAL APPENDIX I

### METHODS

### Antibodies

Specific antibodies against the IK-H protein were generated (by ProSci, Poway, CA) by immunizing rabbits with the KLH-conjugated peptide TYGADDFRDFHAIIIPKSF (made by Biosynthesis Inc., Lewisville, TX) as previously described [9]. This sequence is encoded by exon 3B that is present in IK-H, but not in IK-1 isoforms (Suppl. Fig. 1). The resulting rabbit antiserum was used at 1/1000 dilution. Antibodies used to detect the C-terminus (IK-CTS) of murine and human Ikaros have been described previously [35]. The structure of the two largest Ikaros isoforms, IK-1 and IK-H, are outlined in Suppl. Fig 1. The segments of Ikaros protein that are recognized by IK-H and IK-CTS antibodies are indicated. Thus, IK-H antibody detects only IK-H isoform, but not IK-1, while IK-CTS antibodies detect both Ikaros isoforms. Anti-Ikaros antibodies were visualized using FITC-goat anti-rabbit IgG antibodies (Jackson Immuno Research, West Grove, PA). The HA-specific mouse monoclonal antibody HA.11 (Covance Research Products, Harrisburg, PA), visualized with Texas Red-goat anti-mouse IgG (Jackson Immuno Research, West Grove, PA), was used to detect HA-tagged Ikaros isoforms.

### Cells, Plasmids, Transfection, and Retroviral Transduction

MOLT-4, CCRF-CEM (CEM) and Nalm6 human cell lines have no deletion of the *IKAROS* gene, and express all of the Ikaros isoforms found in actively dividing human lymphocytes. The T-ALL cell lines produce DNA-binding results for Ikaros proteins that are similar to those obtained from activated peripheral human T cells in electromobility shift assays (EMSA) with the CENP-B, $\gamma$ satellite 8, $\alpha$ satellite, VPAC-1, Granzyme B and *IkCa1* probes [9] used here. For retrovirus generation, IK-H or HA-tagged IK-1 (HA tag in N terminus) were amplified by PCR and cloned between BglII and EcoRI sites of the MSCV IRES GFP (MIG) vector as described previously [9].

293T cells were transfected *via* the calcium phosphate method. CEM cells were infected with amphotropic retrovirus as previously described [19] for 24 hours. This retroviral vector has been used to study the subcellular localization of murine and human Ikaros isoforms in the past [19, 20, 47] because it expresses Ikaros protein at the level physiologically seen in hematopoietic cells [47,48]. Although the introduction of the extra copy of Ikaros *via* retroviral infection into cells might result in altered subcellular localization, this was not the case in the previous studies [9,47], and it is an acceptable way to study changes in Ikaros subcellular localization.

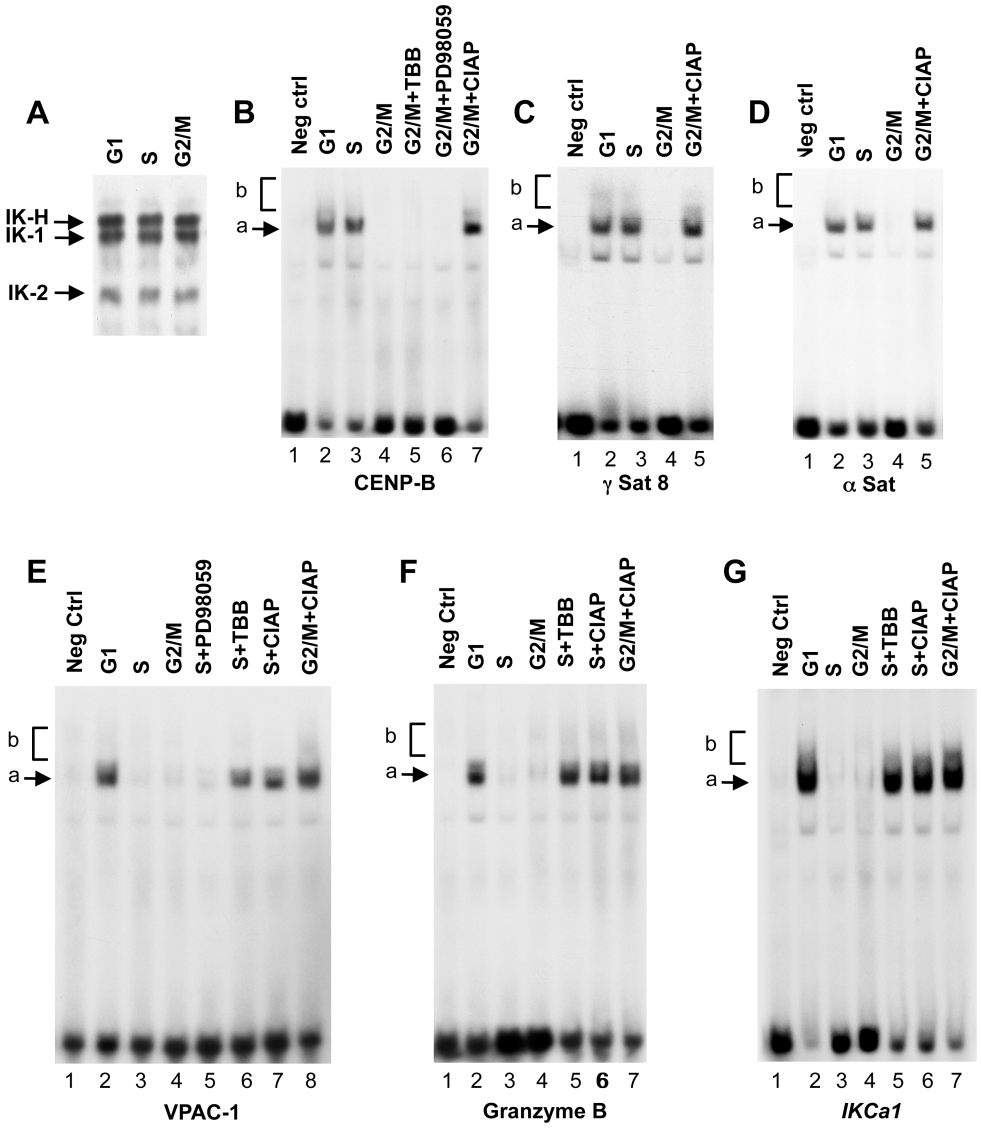
**Confocal Microscopy**

CEM cells were infected with amphotropic retrovirus and analyzed by confocal microscopy as described previously [19]. Images were acquired at room temperature by a Leica TCS-SP MP Confocal and Multiphoton Microscope with a Leica DM-LFS body (upright fixed-stage microscope) using a 100X Leica HX PLAPO (Planapo) oil immersion lens with numerical aperture of 1.4 (Heidelberg, Germany).

**Biochemical Experiments**

PC-HC gel shift probes CENP-B, $\gamma$ sat 8, and $\alpha$ Sat are derived from repetitive sequences locted near human PC-HC and have been described previously [9]. The $\gamma$ sat 8 probe contains two Ikaros consensus binding sites, and thus represents a high-affinity Ikaros binding probe, while CENP-B, and $\alpha$ Sat, probes contain a single Ikaros consensus binding site, and represent lower Ikaros affinity binding probes [9]. Gel shift probes from derived from the URE of Ikaros target genes (VPAC-1, Granzyme B, and *IKCa1)* have been described previously [9]. It has been demonstrated that Ikaros binds these sites [9] and represses *VPAC-1* and activates *Granzyme B*, and *IKCa1* genes [37,38]. The phosphopeptide mapping of IK-1 and IK-H shown for MOLT-4 cells in Fig. 3 is the same as previously observed for these isoforms in activated peripheral T cells and thus, is not the result of mimosine treatment or of malignant transformation.

**Phosphorylation Regulates the Cell Cycle-Specific DNA-Binding Ability of Human Ikaros in B-ALL**




**Supplemental Figure 1: DNA binding of Ikaros during cell cycle.** (A) Nalm6 cells were arrested in G1 (with mimosine), in S (with hydroxyurea, followed by 3 hours release) or in G2/M phase (with vinblastine) of the cell cycle. Nuclear extracts were obtained from cells at each phase, run on western blot, visualized with anti-CTS antibody, and normalized for Ikaros content. (B-G) DNA binding of nuclear extracts from cells at indicated stages of cell cycle with probes derived from PC-HC (B-D) or the URE of indicated genes (E-G). Ikaros DNA-binding complexes are indicated as a) arrows indicating dimers that contain Ikaros isoforms and b) brackets indicating tetramers/higher order complexes of Ikaros isoforms.

To determine whether Ikaros function during the cell cycle in B-ALL is regulated similarly to T-ALL, DNA-binding experiments were performed using the human Nalm6 B-ALL cell line, and the same probes as shown in Fig. 2 for the T-ALL cell lines (Suppl. Fig. 1). Cells were arrested in G1, S, or G2/M phase, and nuclear extracts from each stage of the cell cycle were normalized for the amount of expressed Ikaros protein by Western blot (Suppl. Fig. 1A). The DNA-binding ability of Ikaros in cells from each stage of the cell cycle was studied by EMSA using probes derived from human PC-HC repeats (CENP-B, $\gamma$ satellite 8, and $\alpha$ satellite) (Suppl. Fig. 1B-D), and probes derived from the upstream regulatory elements (URE) of Ikaros target genes (VPAC-1, Granzyme B and *IkCa1*) (Suppl. Fig. 1E-G). The results show that Ikaros' DNA-binding is strongest during G1 phase of the cell cycle (Suppl. Fig 1B-G). The DNA-binding ability of Ikaros was almost completely abolished during mitosis (G2/M) (Suppl. Fig. 1B-G).

During S phase, the DNA-binding ability of Ikaros varied depending on the DNA probe in a manner similar to that seen for T-ALL cells. With probes derived from human PC-HC, Ikaros was able to bind equally well in G1 and S phases (Suppl. Fig. 1B-D lane 3 compared to lane 2). However, with DNA probes that are derived from the URE of Ikaros target genes (VPAC-1, Granzyme B, and *IKCa1*), Ikaros binding was diminished in S phase compared to G1 phase of the cell cycle (Suppl. Fig. 1 E-G lane 3 compared to lane 2). These results demonstrated that the ability of human Ikaros to bind DNA is cell cycle-regulated and that it can vary based on the target sequence.

Taken together these data suggest that during the S phase of the cell cycle, Ikaros loses its ability to bind the URE of its target genes, while retaining its DNA binding activity toward PC-HC in human B-ALL.

The role of phosphorylation in the regulation of Ikaros DNA-binding activity toward the URE of its target genes during S phase of the cell cycle was tested. To dephosphorylate Ikaros proteins, nuclear extracts from Nalm6 cells in G1 and S phase were treated with calf intestinal alkaline phosphatase (CIAP) followed by EMSA. Dephosphorylation of Ikaros proteins in S

phase by CIAP, restored the DNA-binding ability of Ikaros to the DNA probes derived from URE of Ikaros target genes (Suppl. Fig. 1E lane 7 and Suppl. Fig. 1F-G lanes 6). These data provide evidence that the phosphorylation of Ikaros is responsible for the cell cycle-specific loss of Ikaros binding to the URE of its target genes in the S phase of the cell cycle in human B-ALL.

CK2 kinase phosphorylates Ikaros *in vivo* [19]. We tested whether CK2-mediated phosphorylation of Ikaros regulates its S phase-specific DNA-binding. Inhibition of CK2 kinase by a specific inhibitor (TBB) in cells in S phase resulted in the restoration of Ikaros DNA-binding (Suppl. Fig. 1E lane 6, and Suppl. Fig. 1G-F lanes 5). Treatment with the inhibitor of ERK2 kinase, PD98059, did not restore Ikaros binding activity (Suppl. Fig. 1E lane 6), confirming that CK2-specific inhibition is required to restore Ikaros binding activity during the S phase of the cell cycle. These results suggest that the phosphorylation of Ikaros by CK2 causes the decreased Ikaros binding to the UREs of Ikaros target genes observed during the S phase of the cell cycle in B-ALL.

Dephosphorylation of Ikaros following CIAP-treatment restored the ability of Ikaros to bind both PC-HC and URE probes in nuclear extracts from cells in G2/M phase (Suppl. Fig. 1B, lane 7 and Suppl. Fig 1C-D lanes 5; Suppl. Fig. 1E, lane 8; Suppl. Fig. 1F-G lane 7). This confirms that phosphorylation is responsible for the lack of DNA-binding activity of Ikaros during the G2/M phase of the cell cycle. The treatment of the cells with TBB or PD598059 failed to restore Ikaros DNA-binding activity during the G2/M phase providing evidence that a kinase other than CK2 or ERK2 is responsible for the absence of Ikaros binding during the G2/M phase in B-ALL.

In summary, the DNA-binding activity of Ikaros during the cell cycle in Nalm6 B-ALL cells (presented in Suppl. Figure 1) were highly similar to those obtained in MOLT-4 cells (Figure 2), suggesting that the cell cycle-specific DNA-binding activity of Ikaros and its regulation by CK2 kinase during S phase is preserved in both B- and T-cell ALL.