Protein signaling and regulation of gene transcription in leukemia: role of the Casein Kinase II-Ikaros axis

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ABSTRACT

Protein signaling and regulation of gene expression are the two major mechanisms that regulate cellular proliferation in leukemia. Discerning the function of these processes is essential for understanding the pathogenesis of leukemia and for developing the targeted therapies. Here, we provide an overview of one of the mechanisms that regulates gene transcription in leukemia. This mechanism involves the direct interaction between Casein Kinase II (CK2) and the *Ikaros* transcription factor. Ikaros (*IKZF1*) functions as a master regulator of hematopoiesis and a tumor suppressor in acute lymphoblastic leukemia (ALL). Impaired Ikaros function results in the development of high-risk leukemia. Ikaros binds to the upstream regulatory elements of its target genes and regulates their transcription via chromatin remodeling. In vivo, Ikaros is a target for CK2, a pro-oncogenic kinase. CK2 directly phosphorylates Ikaros at multiple amino acids. Functional experiments showed that CK2-mediated phosphorylation of Ikaros, regulates Ikaros' DNA binding affinity, subcellular localization and protein stability. Recent studies revealed that phosphorylation of Ikaros by CK2 regulates Ikaros binding and repression of the terminal deoxytransferase (TdT) gene in normal thymocytes and in T-cell ALL. Available data suggest that the oncogenic activity of CK2 in leukemia involves functional inactivation of Ikaros and provide a rationale for CK2 inhibitors as a potential treatment for ALL.

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common cancer of childhood. Recent studies used a large number of leukemia samples from children and next generation sequencing to identify alterations in multiple genes.[1] Among altered genes, *IKZF1* deletion or deactivation has proven to be the most important independent negative prognostic marker.[2 3] Since *IKZF1* (Ikaros) was first identified in 1992,[4] extensive studies have been focused on understanding the role of Ikaros in normal hematopoiesis and leukemogenesis. Studies to elucidation the signal transduction pathways that affect Ikaros's regulatory function have been performed.[5] Several Ikaros target genes involved in important biological pathways have been identified. Recent studies have focused on identifying pathways that can be targeted to restore and enhance Ikaros function as a tumor suppressor. There is promising data to support the use of this strategy in developing potential treatments for leukemia.

STRUCTURE AND BIOLOGIC FUNCTION OF IKAROS

The *IKZF1* gene encodes several Ikaros isoforms via alternate splicing.[6] Ikaros protein contains two separate zinc finger domains. There are four zinc fingers in the amino half of the protein that take part in sequence-specific DNA binding. The two zinc fingers at the C-terminus of the protein are responsible for protein–protein interaction. This enables Ikaros proteins to form dimers or multimers with different Ikaros isoforms, as well as with other members of Ikaros family genes that share the same protein-interaction domain.[7] Small Ikaros isoforms lack DNA-binding zinc fingers resulting in a functionally inactive complex when associated with full-length Ikaros.[8]

Ikaros binds to the upstream regulatory element (URE) of its target genes and aids in their recruitment to pericentromeric heterochromatin (PC-HC), resulting in repression or activation of transcription of those genes via chromatin remodeling.[9] The ability of Ikaros to localize to PC-HC is essential for Ikaros' function as an activator or repressor of transcription.[10 11] Several gene disruption experiments have shown that Ikaros is a master regulator of lymphoid development and suggest its involvement in lymphocyte activation and tumor suppression.[12]

ROLE OF IKAROS AS A TUMOR SUPPRESSOR IN LEUKEMIA

The role of Ikaros in tumor suppression was first described in Ikaros knockout mice. The absence of Ikaros has a severe impact on normal hematopoiesis with the absence of B, natural killer cell (NK) and dendritic cells, along with reduced T cells. However, Ikaros haplo knockout mice that lack one copy of *IKZF1* develop an early T cell leukemia with 100% penetrance.[12 13] Reintroduction of Ikaros into T-cell leukemia with Ikaros deficiency resulted in cellular arrest with partial T-cell differentiation.[13]

Altered Ikaros expression has been associated with the development of malignancies

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including childhood ALL, infant T-cell ALL, adult B cell ALL, myelodysplastic syndrome, AML and adult and juvenile CML.[2 14] *IKZF1* defects leading to a loss of Ikaros activity have been detected in 30% of pediatric B-cell ALL, in >80% of BCR-ABL1 ALL and BCR-ABL-like ALL, and approximately 5% of T-cell ALL.[14] Overall, 90% of the Ikaros gene defects involve *IKZF1* gene deletion and the rest are nonsense or functionally inactivating mutations of a single allele.[15] Thus, Ikaros haploinsufficiency is sufficient to result in malignant transformation and the development of ALL. Deletion of *IKZF1* confers poor prognosis and is known to be an independent negative prognostic marker in childhood ALL.[3 16]

PHOSPHORYLATION OF IKAROS BY CK2 CONTROLS IKAROS ACTIVITY

Ikaros protein is abundant during the most stages of hematopoiesis. Although it has been shown that Ikaros transcription is controlled by a complex regulatory network.[17] post-translational modifications are hypothesized to play an important role in the regulation of Ikaros function in hematopoietic cells. In vivo phosphopeptide mapping of Ikaros has shown that Ikaros is phosphorylated at multiple sites.[18] The first evidence that phosphorylation is one of the major mechanisms regulating Ikaros function came with the discovery that Ikaros DNA binding to target genes is controlled in a cell cycle-dependent manner, during mitosis. This control occurs via direct phosphorylation at a linker sequence that connects the DNA binding zinc finger units.[19] Studies from the Georgopoulos group identified several additional phosphorylated amino acids in Ikaros protein that are directly phosphorylated by CK2. These studies suggested that CK2-mediated phosphorylation of Ikaros can regulate its ability to control G1-to-S cell cycle progression in mice.[20] Subsequent studies in human leukemia cells revealed that phosphorylation by CK2 regulates Ikaros DNA-binding in human leukemia cells in S phase of the cell cycle.[20 21]

Gurel *et al*,[22] identified four novel sites in the N terminus of Ikaros that are phosphorylated by CK2 in vivo. Functional analysis of Ikaros phosphomimetic mutants (where phosphosites are mutated to aspartate to mimic phosphorylation) and phosphoresistant mutants (where phosphosites are mutated to alanine to mimic the dephosphorylated state) were used to study effects of CK2-mediated phosphorylation on Ikaros function. These analyses demonstrated that phosphorylation of Ikaros at specific amino acids by CK2 regulates two important Ikaros functions:

A. *Pericentromeric localization*: In the nucleus, Ikaros exhibits a punctate staining pattern in normal hematopoietic cells and leukemia cells. Confocal microcopy showed that this punctate staining pattern is due to the localization of Ikaros to PC-HC. Ikaros ability to localize to PC-HC is thought to be important for its function in chromatin remodeling and transcriptional regulation of its target genes. Ikaros phosphomimetic mutants at amino acids in the N-terminal region show severely reduced DNA binding affinity for probes that are derived from PC-HC. In contrast, phopshoresistant mutants were able to bind probes derived from PC-HC at a rate similar to wild-type Ikaros. These data suggested that Ikaros phosphorylation by CK2 at specific sites regulates DNA binding of Ikaros to PC-HC.[22] Confocal microscopy analysis showed that Ikaros protein with phosphomimetic mutations at CK2 phospho acceptor sites loses its ability to localize to PC-HC, resulting in a diffuse nuclear distribution of Ikaros mutant protein. These results demonstrated that CK2-mediated phosphorylation regulates the ability of Ikaros to bind DNA at PC-HC, but also its subcellular localization and function in chromatin remodeling.[22]
B. *DNA binding ability to Ikaros target genes*: Ikaros binds to the upstream regulatory sequence of the terminal deoxytransferase (TdT) (*dntt*) gene and negatively regulates expression of TdT.[23] However, molecular mechanisms that results in increased binding of Ikaros to the TdT D' regulatory sequence during thymocyte differentiation were unknown. The aforementioned study by Gurel *et al*, showed that reversible phosphorylation of Ikaros at specific amino acids regulates the expression of TdT during thymocyte differentiation. Experiments using phosphoresistant mutants at CK2 phospho acceptor sites (amino acids #13 or #294) showed increased Ikaros' DNA-binding affinity toward the TdT D' regulatory sequence, while phosphomimetic mutants (at amino acid #294) had decreased Ikaros' DNA binding by threefold as compared to wild type, suggesting that, phosphorylation of Ikaros controls its ability to bind to the regulatory sequence of the TdT gene. Furthermore, phosphopeptide mapping of endogenous Ikaros, in unstimulated thymocytes and in thymocytes induced to differentiate, showed that Ikaros undergoes dephosphorylation at amino acids #13 and #294 following the induction of thymocyte differentiation. These data suggest that the DNA-binding affinity of Ikaros for the TdT D' regulatory element during thymocyte differentiation is controlled by phosphorylation at specific amino acids.

Treating cells in the above studies with a specific CK2 inhibitor, resulted in the loss of phosphorylation at several amino acid sites including #13 and #294, suggesting that high CK2 kinase activity is necessary for in vivo phosphorylation of these amino acids. Overall, this study provided an in-depth analysis of the molecular mechanism through which phosphorylation by CK2 controls the function of Ikaros in regulating gene transcription and chromatin remodeling.

DEPHOSPHORYLATION OF IKAROS BY PROTEIN PHOSPHATASE 1

The above studies demonstrated the strong connection between the CK2 signal transduction pathway and transcriptional regulation by the Ikaros tumor suppressor. The discovery that Ikaros is a substrate for protein phosphatase 1 (PP1) provided new insights on the role of phosphorylation in regulating Ikaros function. Ikaros contains an evolutionarily-conserved PP1 recognition motif R/K-X_{0-1}-[V/I]-X-[F/W] at amino acids located at the C-terminal end of the protein.[24 25] Interaction between Ikaros and PP1 was studied in detail by Popescu *et al*,[26] using an Ikaros mutant that is unable to interact with PP1 (IK-A465/7, mutation of valine and alanine at amino acids 465 and 467). These studies determined that Ikaros

dephosphorylation by PP1 regulates both Ikaros binding to pericentromeric DNA, as well as its subcellular localization. Importantly, the introduction of phosphoresistant mutations at CK2-phosphorylated residues restored DNA-binding affinity and PC-HC localization of this mutant. These results show that PP1 is important in preventing hyperphosphorylation of Ikaros by CK2 kinase, and the loss of Ikaros DNA binding activity. Hence, CK2-mediated phosphorylation is one of the major mechanisms regulating Ikaros function in DNA binding and chromatin remodeling and dephosphorylation of Ikaros is essential for preserving its function in hematopoietic cells.[26]

PHOSPHORYLATION REGULATES STABILITY OF IKAROS PROTEIN

An unexpected finding was that the loss of interaction with PP1 results in a severely shortened Ikaros half-life. The aforementioned study[26] showed that, protein levels of the PP1-nonbinding Ikaros mutant (Ikaros A465/7) was more than fivefold decreased compared to wild type Ikaros, despite the presence of similar levels of Ikaros mRNA. The introduction of phosphoresistant mutations at CK2 phosphoacceptor sites stabilized the Ikaros A465/7 mutant protein and extended its half-life by fivefold. This demonstrated that phosphorylation of Ikaros by CK2 kinase at specific amino acids promotes its degradation while dephosphorylation by PP1 stabilises the Ikaros protein and extends its half-life.[26]

CK2 AND PP1 REGULATE IKAROS-MEDIATED REPRESSION OF TDT AND CELLULAR PROLIFERATION IN LEUKEMIA

The above data showed that Ikaros activity can be regulated in an opposing manner by the CK2 and PP1 signaling pathways. Subsequent studies extended the analysis of CK2 and PP1 pathways in regulation of Ikaros function as regulator of TdT transcription.

CK2 and PP1 regulate Ikaros binding to URE of TdT

To study the role of CK2 and PP1 in regulating Ikaros function, the CK2 phosphomimetic and phopshoresistant Ikaros mutants were used, together with the PP1-non-binding Ikaros mutant (IK-A465/7) and PP1-non-binding, CK2-phosphoresistant Ikaros mutant (IKA11+A465/7). In addition, thymocytes were treated with PP1 or CK2 specific inhibitors. Results showed that PP1 activity and/or PP1-mediated dephosphorylation are essential for Ikaros binding to the TdT URE. However, inhibition of CK2 by specific inhibitor or the introduction of phosphoresistant mutations at CK2 phospho acceptor sites restored Ikaros binding to the TdT URE despite the absence of PP1 activity. These results provide evidence that CK2 and PP1 signaling have critical and opposing roles in the regulation of Ikaros binding to the TdT URE in primary thymocytes and identified the physiological relevance of CK2 and PP1 in normal thymocyte differentiation.

CK2 and PP1 signaling regulate Ikaros-mediated transcriptional repression in primary thymocytes and in T cell leukemia

Impaired differentiation of lymphoid cells is often associated with malignant transformation and the development of leukemia. TdT is an enzyme that is involved in lymphoid differentiation. During thymocyte differentiation, Ikaros represses TdT, and Ikaros-mediated repression of TdT is an important part of normal T cell development. The role of CK2 and PP1 signaling in regulating Ikaros-mediated repression of TdT transcription was studied in normal thymocytes and in T cell leukemia using the above-described Ikaros mutants and specific CK2 and PP1 inhibitors. Results showed that dephosphorylation of Ikaros by PP1, is essential for transcriptional repression of TdT. Inhibition of PP1 activity abolishes the Ikaros-mediated repression of TdT. Loss of CK2 activity or the presence of phosphoresistant Ikaros mutations restores transcriptional repression of TdT. The opposing roles of CK1 and PP1 on Ikaros-mediated transcriptional repression of TdT were demonstrated in primary thymocytes and in T cell leukemia, which established the significance of these signaling pathways in normal lymphopoiesis and in leukemia.

TARGETING CK2-IKAROS PATHWAY IN B CELL LEUKEMIA

Our recently published data showed that Ikaros controls cellular proliferation by repressing genes that regulate cell cycle progression, PI3K pathway and histone methylation. CK2-mediated phosphorylation of Ikaros impairs its ability to regulate its target genes in B-cell ALL (B-ALL).[27–30] CK2 inhibition restores Ikaros tumor suppressor function in B-ALL, as well as in high-risk B-ALL with IKZF1 deletion and showed strong therapeutic effect in vivo .These data provided validation and the rationale for targeting CK2 as a novel therapeutic approach for high-risk B-cell ALL.

CONCLUSION

The described studies showed a strong correlation between CK2 and PP1 activity and the regulation of Ikaros function in normal thymocytes and in T cell leukemia.[31] Evidence suggests that phosphorylation of Ikaros by CK2 impairs its function as a tumor suppressor by, (1) decreasing its DNA binding ability to the URE of its target genes (2) decreasing its localization to PC-HC and (3) impairing Ikaros function as transcriptional regulator of its target genes. This was demonstrated in primary thymocytes and in T cell leukemia. PP1 has an opposing effect on all the above Ikaros functions (figure 1).[22 26 32] The use of CK2 inhibitors reversed these processes and restored or enhanced of Ikaros function in thymocytes and/or in T cell leukemia.

In summary, the available data provide evidence that the phosphorylation of Ikaros protein lies at the intersection of two signaling pathways—CK2 and PP1. While the CK2 pathway has been known to promote oncogenesis, PP1 has previously been shown to act as a tumor suppressor. Data from the studies described above revealed the opposing effects of two signaling pathways on cellular transformation that occur via their regulation of Ikaros tumor suppressor function. The above data demonstrate the functional

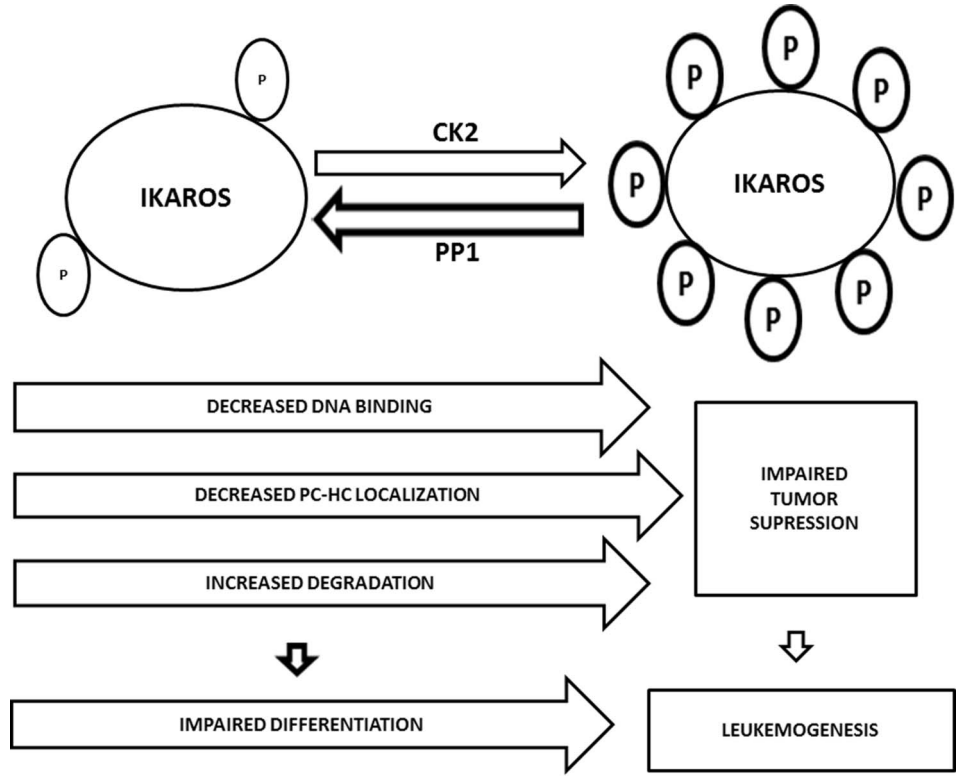

Figure 1 Schematic showing regulation of Ikaros by CK2 and PP1. PP1, protein phosphatase 1.

integration of signal transduction and transcriptional regulation in the control of gene expression.[33 34]

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REFERENCES

1 Mullighan CG. Genomic profiling of B-progenitor acute lymphoblastic leukemia. *Best Pract Res Clin Haematol* 2011;24:489–503.
2 Mullighan CG, Goorha S, Radtke I, *et al*. Genome-wide analysis of genetic alterations in acute lymphoblastic leukaemia. *Nature* 2007;446:758–64.
3 Mullighan CG, Su X, Zhang J, *et al*, Children's Oncology Group. Deletion of IKZF1 and prognosis in acute lymphoblastic leukemia. *N Engl J Med* 2009;360:470–80.
4 Georgopoulos K, Moore DD, Derfler B. Ikaros, an early lymphoid-specific transcription factor and a putative mediator for T cell commitment. *Science* 1992;258:808–12.
5 Georgopoulos K, Winandy S, Avitahl N. The role of the Ikaros gene in lymphocyte development and homeostasis. *Annu Rev Immunol* 1997;15:155–76.
6 Li Z, Perez-Casellas LA, Savic A, *et al*. Ikaros isoforms: the saga continues. *World J Biol Chem* 2011;2:140–5.
7 Molnar A, Georgopoulos K. The Ikaros gene encodes a family of functionally diverse zinc finger DNA-binding proteins. *Mol Cell Biol* 1994;14:8292–303.
8 Sun L, Liu A, Georgopoulos K. Zinc finger-mediated protein interactions modulate Ikaros activity, a molecular control of lymphocyte development. *EMBO J* 1996;15:5358–69.
9 Dovat S. Ikaros in hematopoiesis and leukemia. *World J Biol Chem* 2011;2:105–7.
10 Brown KE, Guest SS, Smale ST, *et al*. Association of transcriptionally silent genes with Ikaros complexes at centromeric heterochromatin. *Cell* 1997;91:845–54.
11 Liberg D, Smale ST, Merkenschlager M. Upstream of Ikaros. *Trends Immunol* 2003;24:567–70.
12 Winandy S, Wu P, Georgopoulos K. A dominant mutation in the Ikaros gene leads to rapid development of leukemia and lymphoma. *Cell* 1995;83:289–99.
13 Dovat S, Payne KJ. Tumor suppression in T cell leukemia--the role of Ikaros. *Leuk Res* 2010;34:416–17.
14 Mullighan CG, Miller CB, Radtke I, *et al*. BCR-ABL1 lymphoblastic leukaemia is characterized by the deletion of Ikaros. *Nature* 2008;453:110–14.
15 Mullighan C, Downing J. Ikaros and acute leukemia. *Leuk Lymphoma* 2008;49:847–9.
16 Volejnikova J, Mejstrikova E, Dörge P, *et al*. Ikaros (IKZF1) alterations and minimal residual disease at day 15 assessed by flow cytometry predict prognosis of childhood BCR/ABL-negative acute lymphoblastic leukemia. *Pediatr Blood Cancer* 2013;60:420–7.
17 Rebollo A, Schmitt C. Ikaros, Aiolos and Helios: Transcription regulators and lymphoid malignancies. *Immunol Cell Biol* 2003;81:171–5.
18 Dovat S, Ronni T, Russell D, *et al*. A common mechanism for mitotic inactivation of C2H2 zinc finger DNA-binding domains. *Genes Dev* 2002;16:2985–90.
19 Georgopoulos K. Haematopoietic cell-fate decisions, chromatin regulation and ikaros. *Nature Rev Immunol* 2002;2:162–74.
20 Gómez-Del Arco P, Maki K, Georgopoulos K. Phosphorylation controls Ikaros's ability to negatively regulate the G(1)-S transition. *Mol Cell Biol* 2004;24:2797–807.
21 Li Z, Song C, Ouyang H, *et al*. Cell cycle-specific function of Ikaros in human leukemia. *Pediatr Blood Cancer* 2012;59:69–76.
22 Gurel Z, Ronni T, Ho S, *et al*. Recruitment of ikaros to pericentromeric heterochromatin is regulated by phosphorylation. *J Biol Chem* 2008;283:8291–300.
23 Trinh LA, Ferrini R, Cobb BS, *et al*. Down-regulation of TDT transcription in CD4(+)CD8(+) thymocytes by Ikaros proteins in direct competition with an Ets activator. *Genes Dev* 2001;15:1817–32.

24 Wakula P, Beullens M, Ceulemans H, *et al*. Degeneracy and function of the ubiquitous RVXF motif that mediates binding to protein phosphatase-1. *J Biol Chem* 2003;278:18817–23.
25 Zhao S, Lee EY. A protein phosphatase-1-binding motif identified by the panning of a random peptide display library. *J Biol Chem* 1997;272:28368–72.
26 Popescu M, Gurel Z, Ronni T, *et al*. Ikaros stability and pericentromeric localization are regulated by protein phosphatase 1. *J Biol Chem* 2009;284:13869–80.
27 Song C, Gowda C, Pan X, *et al*. Targeting casein kinase II restores Ikaros tumor suppressor activity and demonstrates therapeutic efficacy in high-risk leukemia. *Blood* 2015;126:1813–22.
28 Song C, Pan X, Ge Z, *et al*. Epigenetic regulation of gene expression by Ikaros, HDAC1 and Casein Kinase II in leukemia. *Leukemia* 2015. doi:10.1038/leu.2015.331
29 Wang H, Song C, Ding Y, *et al*. Transcriptional Regulation of JARID1B/KDM5B Histone Demethylase by Ikaros, Histone Deacetylase 1 (HDAC1), and Casein Kinase 2 (CK2) in B Cell Acute Lymphoblastic Leukemia. *J Biol Chem* 2015. pii: jbc.M115.679332.
30 Ge Z, Guo X, Li J, *et al*. Clinical significance of high c-MYC and low MYCBP2 expression and their association with Ikaros dysfunction in adult acute lymphoblastic leukemia. *Oncotarget* 2015;6:42300–11.
31 Dovat S, Song C, Payne KJ, *et al*. Ikaros, CK2 kinase, and the road to leukemia. *Mol Cell Biochem* 2011;356:201–7.
32 Wang H, Song C, Gurel Z, *et al*. Protein phosphatase 1 (PP1) and Casein Kinase II (CK2) regulate Ikaros-mediated repression of TdT in thymocytes and T-cell leukemia. *Pediatr Blood Cancer* 2014;61:2230–5.
33 Gowda C, Dovat S. Genetic targets in pediatric acute lymphoblastic leukemia. *Adv Exp Med Biol* 2013;779:327–40.
34 Buontempo F, Orsini E, Martins LR, *et al*. Cytotoxic activity of the casein kinase 2 inhibitor CX-4945 against T-cell acute lymphoblastic leukemia: targeting the unfolded protein response signaling. *Leukemia* 2014;28:543–53.