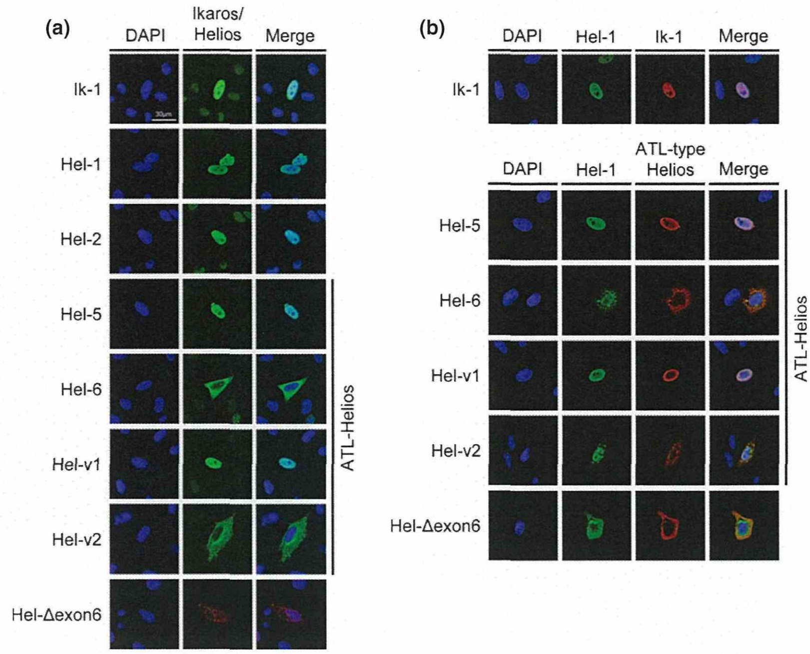

Fig. 4. Subcellular localization of adult T-cell leukemia (ATL)-type Helios isoforms. Immunostaining analyses of Helios and Ikaros proteins. HeLa cells were transfected with each individual expression vector (a) or the indicated combination of expression vectors (b). Each protein was visualized with anti-FLAG (green) or anti-HA antibodies (red). Nuclei were detected by DAPI staining (blue). Colocalization between Ik-1 and ATL-type Helios was shown in Fig. S2. Hel-v1, Hel-variant 1; Hel-v2, Hel-variant 2.

(Fig. 6d). To examine whether the cellular effect of Hel-5 was due to its dominant-negative function against Hel-1 and Ik-1, we carried out further knockdown analyses with specific shRNAs (Fig. 6e). The results showed that knockdown of wild-type Helios or Ikaros led to enhanced cell growth (Fig. 6f), which was consistent with the results of enforced Hel-5 expression. These results collectively suggested that counteraction of Ikaros or Helios by dominant-negative isoforms contributed to T cell growth.

Helios deficiency causes expression of various genes in T cells. We globally searched mRNA expression changes using microarray analysis of Jurkat cells expressing Hel-5 and those of knocked-down Helios or Ikaros (Fig. 7a,b). The results clearly showed differentially expressed gene sets between the transformants and control cells (Fig. 7c). Furthermore, pathway analysis[37] of each upregulated gene set identified activation of several signaling cascades. In particular, we focused on six common pathways identified in both Hel-5 transduced and Helios or Ikaros knocked-down Jurkat cells (Fig. 7d). These pathways are important for various T cell regulations, for example, cell growth, apoptosis resistance, and migration activity. Among these pathways, it has not been reported that the shingosine-1-phosphate (S1P) pathway is regulated by the Ikaros family. We confirmed overexpressed *S1PR1* and *S1PR3*, which are critical receptors for the activation of the S1P pathway, in manipulated Jurkat samples (Fig. 7e).

Discussion

In the present study, on the basis of the integrated analysis of ATL cells using our biomaterial bank in Japan, we revealed a novel molecular characteristic of ATL cells, which is a profound abnormality in the expression of Helios. The abnormal alternative splicing and, in some cases, loss of Helios expression appear to be a part of the basis for advantageous cell growth and survival in ATL cells. We also showed the tumor-suppressive function and target genes, as well as pathways of Helios, in mature human T cells.

Characterization of Ikaros family members revealed profound abnormalities in Helios expression in ATL cells: (i) biased and increased expression of alternatively spliced variants; (ii) suppression of Hel-1 expression; (iii) lack of Helios expression in some cases; and (iv) frequent genomic defects of the *Helios* locus. Our results also revealed that alternatively spliced Helios variants are expressed in PBMCs of HTLV-1 carriers, suggesting that the abnormal splicing of Helios may occur in HTLV-1-infected cells at the carrier state until progression to leukemia development. However, the genomic deletions appear to be one of the important genetic events during the latter stages of leukemia development, as they were observed only in aggressive subtypes of ATL.

The structural characteristics of the ATL-type Helios variants involve a selective lack of one or more zinc fingers in the N-terminal domain. The results of this study indicated that these variant proteins lost DNA binding activity, whereas the capacity of dimerization was preserved. Therefore, these variant proteins hindered transcriptional activities of Ikaros family proteins, showing dominant-negative effects. In addition, a part of ATL-type Helios isoform, which lacks exon 6, is linked to abnormal localization of wild-type Helios and I-karos. We confirmed that Helios isoforms lacking exon 6 were overexpressed in primary ATL cells (Fig. S5). Interestingly, Hel-2 has reduced transcriptional suppressive activity compared with Hel-1, although it can bind to the target sequence as well as Hel-1. This is similar to a previous report,[36] which noted that the activity of mouse Ik-2 protein for the reporter gene was remarkably lower than that of Ik-1, whereas the binding affinities of Ik-1 and Ik-2 were similar. The exon 3 skip occurred more frequently in ATL cells, compared to PBMCs from normal volunteers (Fig. S6). These results collectively indicate that all abnormalities of Helios expression, including loss of or decreased Hel-1 expression and upregulated Hel-2 and ATL-type Helios, result in abrogation of Ikaros family functions in ATL cells.

We also confirmed that *Hes1*, a target gene of the Notch pathway, is one of the targets of Helios as well as Ikaros.[34,35] A recent study reported that activated Notch signaling may be important to ATL pathogenesis and that Hes1 is upregulated in ATL cells.[38] Thus, we examined expression levels of Hes1 mRNA by quantitative RT-PCR and confirmed the

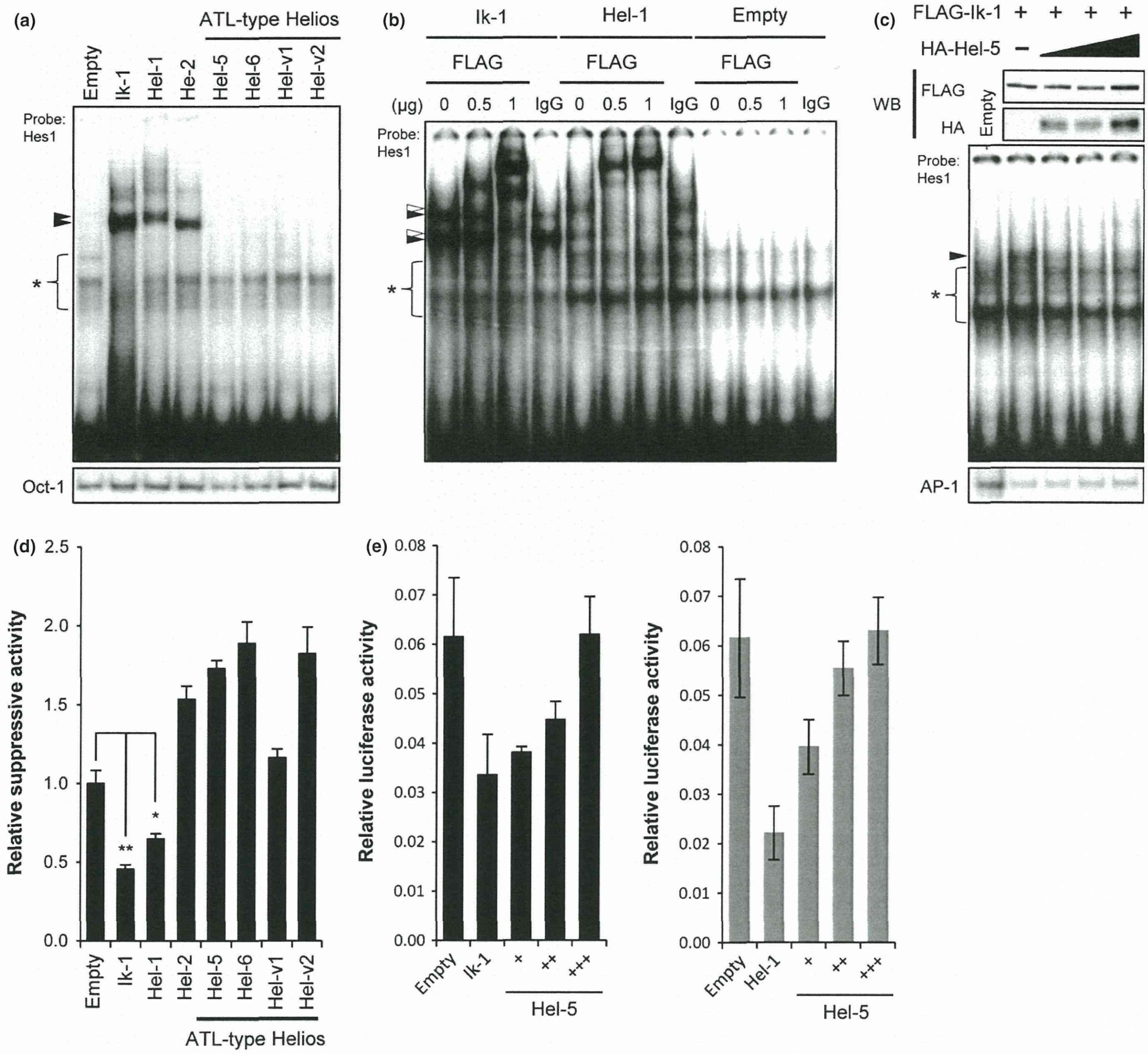

Fig. 5. Dominant-negative function of adult T-cell leukemia (ATL)-type Helios isoforms. (a) DNA-binding activities of wild-type Helios or Ikaros and ATL-type Helios proteins. Each FLAG-tagged Helios or Ikaros isoforms were ectopically expressed in 293T cells and their nuclear extracts were subjected to EMSA with a [γ-^{32}P]-labeled *Hes1* promoter probe. Oct-1 probe was used as an internal control. Arrowheads indicate Helios or Ikaros complexes. *Non-specific bands. Hel-v1, Hel-variant 1; Hel-v2, Hel-variant 2. (b) Results of supershift assays. Anti-FLAG (0, 0.5, 1 μg) or control IgG (1 μg) antibodies were added to each nuclear extract prior to electrophoresis. The black and white arrowheads indicate the supershifted bands of Ik-1 and Hel-1, respectively. (c) Antagonistic effects of Hel-5 on DNA-binding of Ik-1 tested by EMSA. The molar ratios of Ik-1 to Hel-5 plasmids are 1:1, 1:4, and 1:8. Expression levels of FLAG-Ik-1 and HA-Hel-5 were assessed by immunoblotting. The arrowheads indicate the Ik-1 specific band. AP-1 probe was used as an internal control. WB, western blot. (d) Transcriptional suppression activities of various Helios or Ikaros isoforms tested by *Hes1* promoter-luciferase reporter systems (n = 3, mean ± SD). Basal *Hes1* promoter activity was defined as firefly/renilla ratio, and suppression activities of Helios or Ikaros are relatively presented. Statistical significance was evaluated by unpaired Student's t-test (*$P < 0.05$; **$P < 0.01$). (e) Inhibitory function of Hel-5 against Ik-1 and Hel-1 tested by *Hes1* promoter assay (n = 3, mean ± SD). The molar ratios of Ik-1 or Hel-1 to Hel-5 plasmids are 1:1, 1:2, and 1:3. Relative luciferase activities were defined as firefly/renilla ratio.

upregulation in our ATL samples (Fig. S7). Hes1 has been reported to directly promote cell proliferation through the transcriptional repression of p27kip1.[39] Taken together, our results suggest a possibility that abnormalities in Helios expression are one of the causes of Hes1 activation, which may be one of the genetic events involved in ATL leukemogenesis.

Our results show that the Hel-5 variant may have an oncogenic role, whereas the wild-type Helios, Hel-1, shows tumor suppressor-like activity. These findings are consistent with previous findings in mice.[15] Furthermore, our description of expression profiles of stable cells followed by pathway analyses showed activation of several important pathways in lymphocytes for the regulation of proliferation, survival, and others. In particular, we discovered novel molecular cross-talk between the Ikaros family and the S1P pathway. The S1P–S1PR1 axis is known to play important

doi: 10.1111/cas.12181

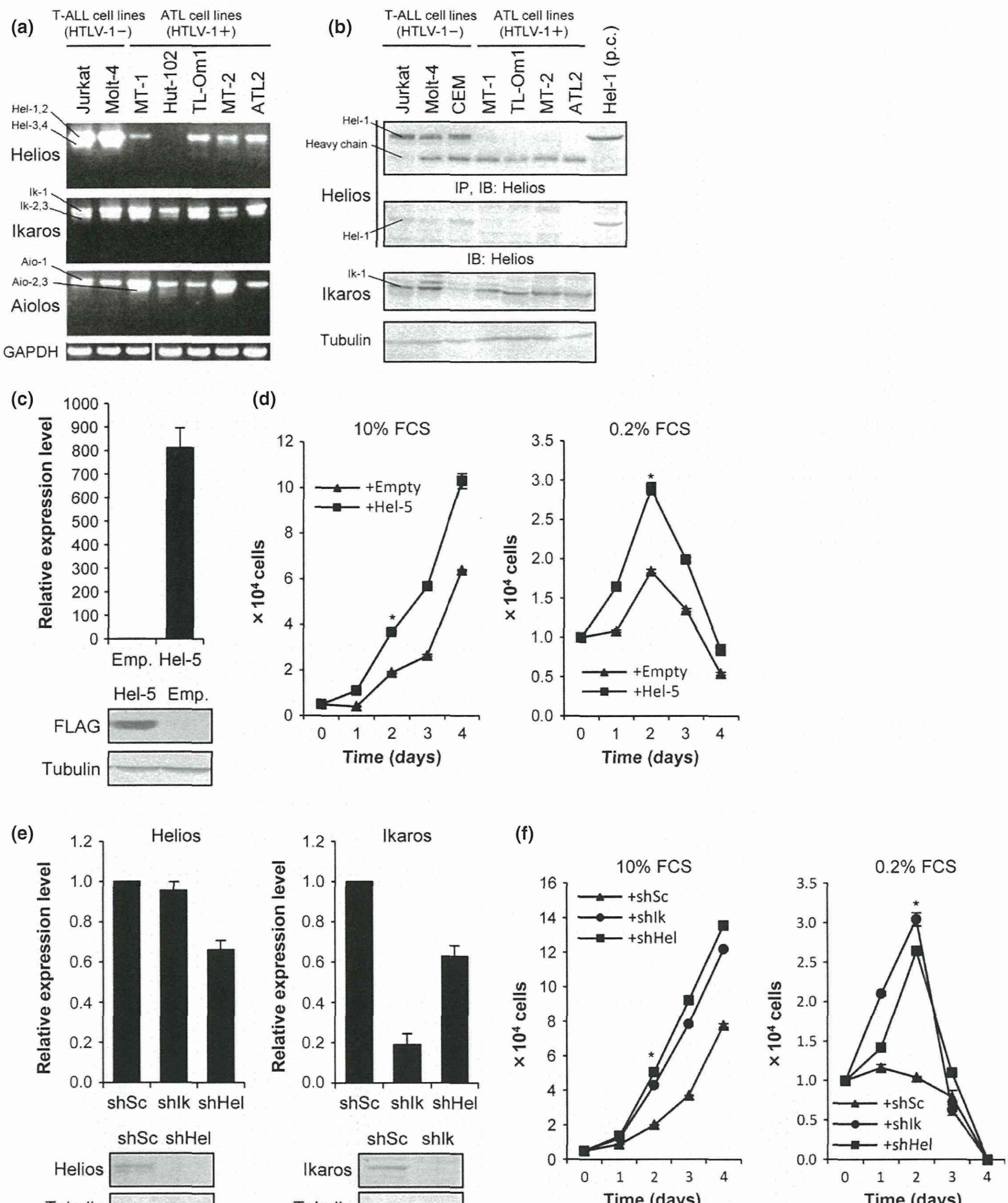

Fig. 6. Hel-5 functions in T cell growth and survival. (a) Expression patterns and levels of Ikaros family genes in various cell lines examined by RT-PCR. ATL, adult T-cell leukemia; T-ALL, acute T lymphoblastic leukemia. (b) Results of immunoblotting analyses of the immunoprecipitants (top panel) and cell lysates (lower panels). Positive control (p.c.), Hel-1 transfectant. IB, immunoblot; IP, immunoprecipitant. (c) Establishment of Jurkat cells stably expressing Hel-5. The Hel-5 level was quantified by quantitative RT-PCR (top, $n = 3$, mean ± SD) and immunoblotting (bottom). (d) Cell proliferation analysis of control cells (▲) and Hel-5-expressing Jurkat cells (■) under two FCS conditions ($n = 3$, mean ± SD). Statistical significance was observed (*P <0.01, Student's t-test). (e) Knockdown analyses of Helios or Ikaros in Jurkat cells. The Helios and Ikaros levels were evaluated by quantitative RT-PCR (top, $n = 3$, mean ± SD) and immunoblotting (bottom), respectively. (f) Cell proliferation curves of scrambled shRNA (shSc) cells (▲), shIkaros (shIk) cells (•), and shHelios (shHel) cells (■) were examined in two FBS conditions ($n = 3$, mean ± SD; *$P < 0.01$).

roles in regulation of the immune system, apoptosis, cell cycle, and migration of lymphocytes.[40–42] Recently, activation of the S1P pathway in various diseases, including leukemia, has been reported, and the therapeutic potential of S1PR1 inhibitors was suggested.[42] Studies of functional roles of S1P pathway activation in ATL cells are now underway in our laboratory.

In conclusion, our present study revealed a novel aspect of molecular abnormalities in ATL cells: a profound deregulation in Helios expression, which appears to play an important role in T-cell proliferation. Our experimental approaches also imply that, in addition to genetic and epigenetic abnormalities, ATL shows abnormal splicing, which has been observed in various human diseases including cancers.[43–45]

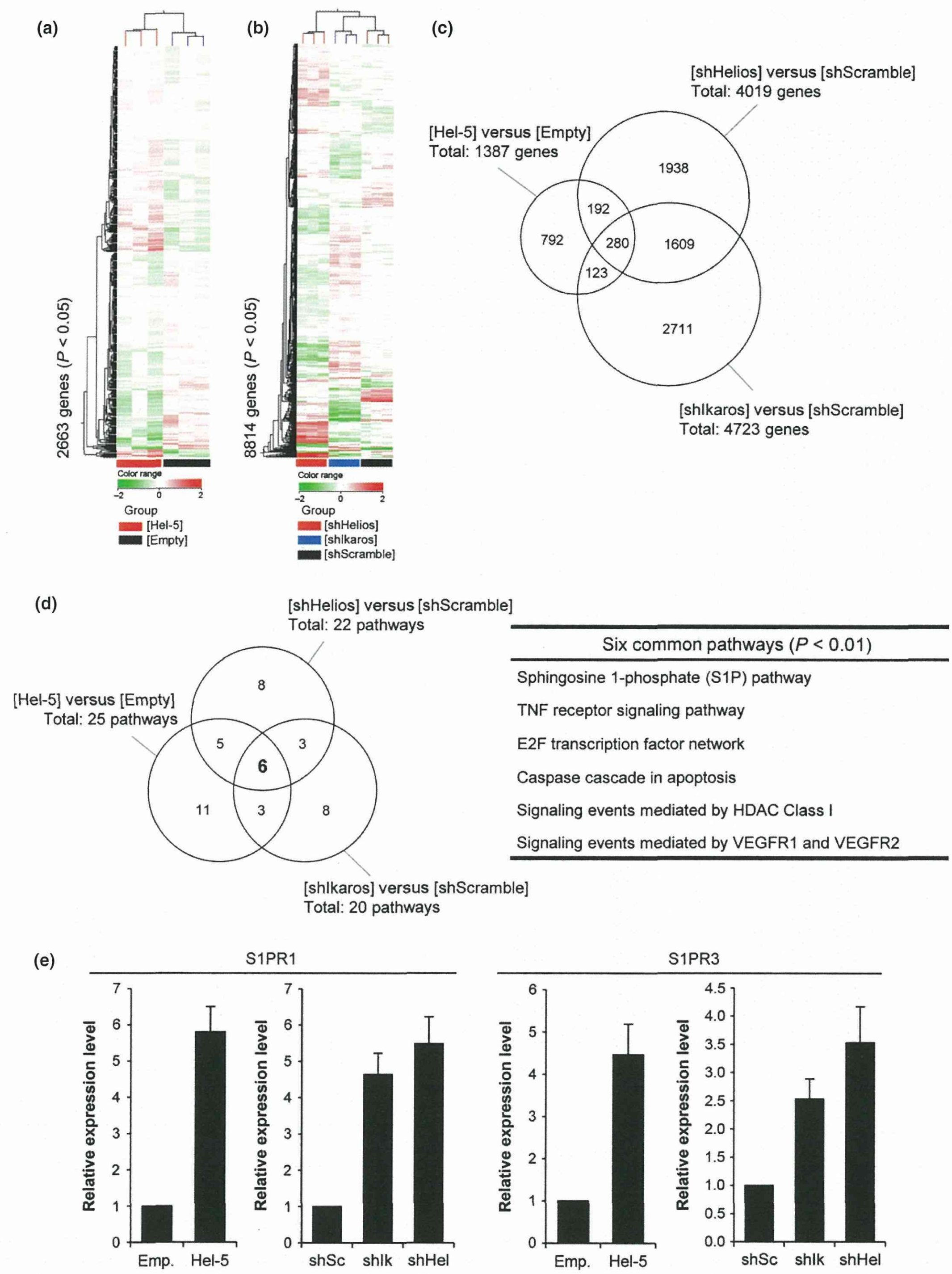

Fig. 7. Comprehensive search for Helios target genes by microarray analysis. (a,b) Gene expression analysis of Jurkat stable cells. The gene expression patterns of Jurkat cells expressing Hel-5 ($n = 3$), shIkaros ($n = 3$), and shHelios ($n = 3$) were comprehensively analyzed by microarray technique. The obtained 2D hierarchical clusters and Pearson's correlation between the cells expressing Hel-5 or not (a) and the cells introducing shHel, shIk, or shSc (b). (c) Venn diagram of differential gene expression pattern in the Jurkat sublines. The each differential expression gene set (5-fold changes, $P < 1 \times 10^{-5}$) was compared. (d) Venn diagram depicting the overlap between the outputs of pathway analysis in Jurkat sublines. The analysis was based on the NCI-Nature Pathway Interaction Database.[37] Each differential pathway set (t-test, $P < 0.01$) was compared and the common pathways listed. (e) Results of quantitative RT-PCR of shingosine-1-phosphate receptor 1 (S1PR1) and receptor 3 (S1PR3) in Jurkat sublines ($n = 3$, mean $\pm$ SD). HDAC, histone deacetylase; VEGFR, vascular endothelial growth factor receptor.

Acknowledgments

We thank Mr. M. Nakashima and Ms. T. Akashi for support and maintenance of the Joint Study on Prognostic Factors of ATL Development. This work is supported by JSPS KAKENHI Grant Numbers 24790436 (M.Y.), 23390250 (T.W.), 23659484 (T.W.), 23 6291 (S.A.), NEXT KAKENHI Grant Number 221S0001 (T.W.), and a Grant-in-Aid from the Ministry of Health, Labor and Welfare of Japan H24-G-004 (M.Y. and T.W.).

doi: 10.1111/cas.12181

Disclosure Statement

The authors have no conflict of interest.

References

1 Yamaguchi K, Watanabe T. Human T lymphotropic virus type-I and adult T-cell leukemia in Japan. *Int J Hematol* 2002; **76**: 240–45.

2 Iwanaga M, Watanabe T, Utsunomiya A *et al.* Human T-cell leukemia virus type I (HTLV-1) proviral load and disease progression in asymptomatic HTLV-1 carriers: a nationwide prospective study in Japan. *Blood* 2010; **116**: 1211–19.

3 Yamagishi M, Nakano K, Miyake A *et al.* Polycomb-mediated loss of miR-31 activates NIK-dependent NF-κB pathway in adult T cell leukemia and other cancers. *Cancer Cell* 2012; **21**: 121–35.

4 Yamagishi M, Watanabe T. Molecular hallmarks of adult T cell leukemia. *Front Microbiol* 2012; **3**: 334.

5 Lo K, Landau NR, Smale ST. LyF-1, a transcriptional regulator that interacts with a novel class of promoters for lymphocyte-specific genes. *Mol Cell Biol* 1991; **11**: 5229–43.

6 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.

7 Hahm K, Ernst P, Lo K, Kim GS, Turck C, Smale ST. The lymphoid transcription factor LyF-1 is encoded by specific, alternatively spliced mRNAs derived from the Ikaros gene. *Mol Cell Biol* 1994; **14**: 7111–23.

8 Sun L, Liu A. Zinc finger-mediated protein interactions modulate Ikaros activity, a molecular control of lymphocyte development. *EMBO J* 1996; **15**: 5358–69.

9 Morgan B, Sun L, Avitahl N *et al.* Aiolos, a lymphoid restricted transcription factor that interacts with Ikaros to regulate lymphocyte differentiation. *EMBO J* 1997; **16**: 2004–13.

10 Kelley CM, Ikeda T, Koipally J *et al.* Helios, a novel dimerization partner of Ikaros expressed in the earliest hematopoietic progenitors. *Curr Biol* 1998; **8**: 508–15.

11 Cobb BS, McCarty AS, Brown KE *et al.* Helios, a T cell-restricted Ikaros family member that quantitatively associates with Ikaros at centromeric heterochromatin. *Genes Dev* 1998; **12**: 782–96.

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 Wang JH, Nichogiannopoulou A, Wu L *et al.* Selective defects in the development of the fetal and adult lymphoid system in mice with an Ikaros null mutation. *Immunity* 1996; **5**: 537–49.

14 Wang JH, Avitahl N, Cariappa A *et al.* Aiolos regulates B cell activation and maturation to effector state. *Immunity* 1998; **9**: 543–53.

15 Zhang Z, Swindle CS, Bates JT, Ko R, Cotta CV, Klug CA. Expression of a non-DNA-binding isoform of Helios induces T-cell lymphoma in mice. *Blood* 2007; **109**: 2190–7.

16 Sun L, Crotty ML, Sensel M *et al.* Expression of dominant-negative Ikaros isoforms in T-cell acute lymphoblastic leukemia. *Clin Cancer Res* 1999; **5**: 2112–20.

17 Nakase K, Ishimaru F, Avitahl N *et al.* Dominant negative isoform of the Ikaros gene in patients with adult B-cell acute lymphoblastic leukemia. *Cancer Res* 2000; **60**: 062–4065.

18 Takanashi M, Yagi T, Imamura T *et al.* Expression of the Ikaros gene family in childhood acute lymphoblastic leukaemia. *Br J Haematol* 2002; **117**: 525–30.

19 Nishii K, Katayama N, Miwa H. Non-DNA-binding Ikaros isoform gene expressed in adult B-precursor acute lymphoblastic leukemia. *Leukemia* 2002; **16**: 1285–92.

20 Tonnelle C, Imbert M-C, Sainty D, Granjeaud S, N'Guyen C, Chabannon C. Overexpression of dominant-negative Ikaros 6 protein is restricted to a subset of B common adult acute lymphoblastic leukemias that express high levels of the CD34 antigen. *Hematol J* 2003; **4**: 104–9.

21 Klein F, Feldhahn N, Herzog S *et al.* BCR-ABL1 induces aberrant splicing of IKAROS and lineage infidelity in pre-B lymphoblastic leukemia cells. *Oncogene* 2006; **25**: 1118–24.

22 Zhou F, Mei H, Jin R, Li X, Chen X. Expression of ikaros isoform 6 in chinese children with acute lymphoblastic leukemia. *J Pediatr Hematol Oncol* 2011; **33**: 429–32.

23 Mullighan CG, Miller CB, Radtke I *et al.* BCR-ABL1 lymphoblastic leukaemia is characterized by the deletion of Ikaros. *Nature* 2008; **453**: 110–14.

24 Kano G, Morimoto A, Takanashi M *et al.* Ikaros dominant negative isoform (Ik6) induces IL-3-independent survival of murine pro-B lymphocytes by activating JAK-STAT and up-regulating Bcl-xl levels. *Leuk Lymphoma* 2008; **49**: 965–73.

25 Iacobucci I, Lonetti A, Messa F *et al.* Expression of spliced oncogenic Ikaros isoforms in Philadelphia-positive acute lymphoblastic leukemia patients treated with tyrosine kinase inhibitors: implications for a new mechanism of resistance. *Blood* 2008; **112**: 3847–55.

26 Mullighan CG, Su X, Zhang J *et al.* Deletion of IKZF1 and prognosis in acute lymphoblastic leukemia. *N Engl J Med* 2009; **360**: 470–80.

27 Kuiper RP, Waanders E, van der Velden VHJ *et al.* IKZF1 deletions predict relapse in uniformly treated pediatric precursor B-ALL. *Leukemia* 2010; **24**: 1258–64.

28 Nakase K, Ishimaru F, Fujii K *et al.* Overexpression of novel short isoforms of Helios in a patient with T-cell acute lymphoblastic leukemia. *Exp Hematol* 2002; **30**: 313–17.

29 Fujii K, Ishimaru F, Tabayashi T *et al.* Over-expression of short isoforms of Helios in patients with adult T-cell leukaemia/lymphoma. *Br J Haematol* 2003; **120**: 986–9.

30 Fujiwara SI, Yamashita Y, Nakamura N *et al.* High-resolution analysis of chromosome copy number alterations in angioimmunoblastic T-cell lymphoma and peripheral T-cell lymphoma, unspecified, with single nucleotide polymorphism-typing microarrays. *Leukemia* 2008; **22**: 1891–8.

31 Fujimoto R, Ozawa T, Itoyama T, Sadamori N, Kurosawa N, Isobe M. HELIOS-BCL11B fusion gene involvement in a t(2;14)(q34;q32) in an adult T-cell leukemia patient. *Cancer Genet* 2012; **205**: 356–64.

32 Shimoyama M. Diagnostic criteria and classification of clinical subtypes of adult T-cell leukaemia-lymphoma. A report from the Lymphoma Study Group (1984–87). *Br J Haematol* 1991; **79**: 428–37.

33 Tabayashi T, Ishimaru F, Takata M *et al.* Characterization of the short isoform of Helios overexpressed in patients with T-cell malignancies. *Cancer Sci* 2007; **98**: 182–8.

34 Kathrein KL, Chari S, Winandy S. Ikaros directly represses the notch target gene Hes1 in a leukemia T cell line: implications for CD4 regulation. *J Biol Chem* 2008; **283**: 10476–84.

35 Kleinmann E, Geimer Le Lay AS, Sellars M, Kastner P, Chan S. Ikaros represses the transcriptional response to Notch signaling in T-cell development. *Mol Cell Biol* 2008; **28**: 7465–75.

36 Molnár A, Georgopoulos K. The Ikaros gene encodes a family of functionally diverse zinc finger DNA-binding proteins. *Mol Cell Biol* 1994; **14**: 8292–303.

37 Schaefer CF, Anthony K, Krupa S *et al.* PID: the pathway interaction database. *Nucleic Acids Res* 2009; **37**: D674–9.

38 Pancewicz J, Taylor JM, Datta A. Notch signaling contributes to proliferation and tumor formation of human T-cell leukemia virus type 1-associated adult T-cell leukemia. *Proc Natl Acad Sci USA* 2010; **107**: 16619–24.

39 Murata K, Hattori M, Hirai N *et al.* Hes1 directly controls cell proliferation through the transcriptional repression of p27Kip1. *Mol Cell Biol* 2005; **25**: 4262–71.

40 Maeda Y, Seki N, Sato N, Sugahara K, Chiba K. Sphingosine 1-phosphate receptor type 1 regulates egress of mature T cells from mouse bone marrow. *Int Immunol* 2010; **22**: 515–25.

41 Spiegel S, Milstien S. The outs and the ins of sphingosine-1-phosphate in immunity. *Nat Rev Immunol* 2011; **11**: 403–15.

42 Maceyka M, Harikumar KB, Milstien S, Spiegel S. Sphingosine-1-phosphate signaling and its role in disease. *Trends Cell Biol* 2012; **22**: 50–60.

43 Ghigna C, Valacca C, Biamonti G. Alternative splicing and tumor progression. *Curr Genomics* 2008; **9**: 556–70.

44 David CJ, Manley JL. Alternative pre-mRNA splicing regulation in cancer: pathways and programs unhinged. *Genes Dev* 2010; **24**: 2343–64.

45 Blair CA, Zi X. Potential molecular targeting of splice variants for cancer treatment. *Indian J Exp Biol* 2011; **49**: 836–9.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Deregulated expression of Ikaros family genes in primary adult T-cell leukemia cells.

Fig. S2. Colocalization of wild-type Ikaros and adult T-cell leukemia-type Helios.

Fig. S3. Dominant-negative inhibition of Hel-6, Hel-v1, and Hel-v2 in the suppressive activities of wild-type Helios and Ikaros.

Fig. S4. Downregulation of the expression of Helios mRNA in HTLV-1-positive T cell lines.

Fig. S5. Overexpression of abnormal Helios isoforms lacking exon 6 in adult T-cell leukemia samples.

Fig. S6. Relative value of Helios transcripts skipping exon 3 to all is upregulated in primary adult T-cell leukemia cells.

Fig. S7. Upregulated expression of Hes1 in primary adult T-cell leukemia cells.

Table S1. Clinical characteristics of adult T-cell leukemia patients and HTLV-1 carriers.

Table S2. Primer list and probe sequences.

doi: 10.1111/cas.12181