Deletion of the RNA-binding proteins *Zfp36l1* and *Zfp36l2* leads to perturbed thymic development and T-lymphoblastic leukaemia

Daniel J. Hodson[1], **Michelle L. Janas**[1], **Alison Galloway**[1], **Sarah E. Bell**[1], **Simon Andrews**[2], **Cheuk M. Li**[1], **Richard Pannell**[3], **Christian W. Siebel**[4], **H. Robson MacDonald**[5], **Kim De Keersmaecker**[6], **Adolfo A. Ferrando**[6,7,8], **Gerald Grutz**[9,10], and **Martin Turner**[1]

[1]Laboratory of Lymphocyte Signalling and Development, The Babraham, Cambridge, CB22 3AT, United Kingdom [2]Bioinformatics Group, The Babraham Institute, Babraham, Cambridge, CB22 3AT, United Kingdom [3]MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 0QH, UK [4]Genentech, Department of Molecular Biology, 1 DNA Way, South San Francisco, CA 94080 USA [5]Ludwig Institute for Cancer Research, Lausanne Branch, University of Lausanne, 1066 Epalinges, Switzerland [6]Institute for Cancer Genetics, Columbia University Medical Centre, New York, USA [7]Department of Pathology, Columbia University Medical Centre, New York, USA [8]Department of Pediatrics, Columbia University Medical Centre, New York, USA [9]Institute of Medical Immunology, Charité-Universitätsmedizin, Campus Mitte, Berlin, Germany [10]Center for Biomaterial Development, Institute of Polymer Research, GKSS, Teltow, Germany

Abstract

ZFP36L1 and ZFP36L2 are RNA-binding proteins (RBPs) which interact with AU-rich elements in the 3'UTR of mRNA, leading to mRNA degradation and translational repression. Mice lacking ZFP36L1 and ZFP36L2 during thymopoiesis develop a Notch1-dependent T cell acute lymphoblastic leukaemia (T-ALL). Prior to the onset of T-ALL, thymic development is perturbed with accumulation of cells which have passed through the β-selection checkpoint without first expressing T cell receptor β (TCR-β). Notch1 expression is increased in non-transformed thymocytes in the absence of ZFP36L1 and ZFP36L2. Both RBPs interact with evolutionarily conserved AU-rich elements within the 3' untranslated region of Notch1 and suppress its expression. These data establish a role for ZFP36L1 and ZFP36L2 during thymocyte development and in the prevention of malignant transformation.

The development of T cells in the thymus proceeds through a series of developmental stages characterised by progressive rearrangement of the T cell receptor (TCR) genes and regulated by a series of developmental checkpoints. This ordered process is orchestrated by transcription factor networks which integrate environmental cues to initiate gene expression programs appropriate to the developmental stage of the thymocyte1¯3. However there is increasing recognition that gene expression during lymphocyte development is also subject to regulation by post-transcriptional mechanisms. These affect the half-life of mRNA though promotion or

---

*

inhibition of mRNA decay. Additional control at the point of mRNA translation also regulates the magnitude of gene expression. These points are exemplified by recent awareness of the regulation of gene expression by microRNAs which act principally through the control of mRNA decay and translation. Post-transcriptional control of gene expression is also mediated by RNA-binding proteins (RBPs) of which over 150 have been found to be expressed in thymus4. However, our knowledge of how post-transcriptional regulation mediated by RNA-binding proteins impacts on thymic development is extremely limited.

ZFP36L1 and ZFP36L2 (also known as TIS11b and TIS11d) belong to a family of CCCH-zinc finger-containing RBPs that includes ZFP36 (tristetraprolin). These regulate gene expression by promoting mRNA decay and might additionally affect translation. A germline *Zfp36* knockout mouse develops a severe inflammatory phenotype attributable to overexpression of TNF[5–6] while germline knockout of *Zfp36l1* is lethal at embryonic day 10.5 due to a failure of chorioallantoic fusion[7–8]. Germline *Zfp36l1* knockout mice die shortly after birth possibly as a consequence of haematopoietic stem cell failure[9]. The tandem zinc fingers are highly conserved between TTP family members and bind to AU-rich elements (ARE) in the 3'-untranslated region (3'UTR) of mRNA, promoting deadenylation and decay. The optimum binding sequence for all family members is UUAUUUAU[10–]11. However sequences as short as UAUUU may be sufficient for binding and genome–wide screens to identify targets have been enriched with transcripts that do not possess the optimal AU-rich binding site12. Thus the criteria for target recognition, and whether this differs between the family members, remain incompletely defined.

There is mounting evidence that escape from post-transcriptional regulation of gene expression is important in the pathogenesis of malignancy. Deletion of the miR15a & miR16-1 cluster in mice leads to development of a disease similar to human chronic lymphocytic leukaemia[13]. Aberrant polyadenylation site usage, leading to a truncated 3'UTR, has been detected in many human malignancies and might allow malignant cells to escape regulation by both microRNA and RBPs14–15. As a physiological mechanism, proliferating T cells preferentially utilise truncated 3'UTRs16. This is consistent with a global reduction in post-transcriptional regulation providing a net proliferative advantage. Circumstantial evidence implicates ZFP36 family members in malignancy. Expression of ZFP36 is suppressed in a variety of human malignancies[17]. ZFP36L2 has been suggested to act downstream of p53 in the induction of apoptosis and ZFP36L1 is implicated in the apoptotic response to rituximab (anti-CD20) in chronic lymphocytic leukaemia[18–19]. A number of oncogenes, including *FOS*, *MYC*, *BCL-2*, and *COX-2* contain AREs in their 3'UTRs and have been proposed as potential targets[20]. However no evidence to date proves an *in vivo*, physiological tumor suppressor role for ZFP36 family members, or indeed any RBP.

To investigate the function of ZFP36L1 and ZFP36L2 in thymic development we generated conditional knockouts of both genes. In anticipation of redundancy between these two closely related family members we inter-crossed the single knockouts to create lymphocyte conditional *Zfp36l1* and *Zfp36l2* double knockout (dKO) mice. Thymic development was normal in the single knockouts, however the dKO mice developed T-lymphoblastic leukaemia T-ALL). Prior to leukaemia the normally ordered process of thymic development was perturbed, with aberrant passage of thymocytes through the β-selection checkpoint. Furthermore the oncogenic transcription factor *Notch1* was identified as a novel target of ZFP36L1 and ZFP36L2. The finding that a transcription factor is itself a target for post-transcriptional regulation, demonstrates how RBPs integrate gene expression at the transcriptional and post-transcriptional level. These findings identify a critical role for ZFP36 family members during lymphocyte development and provide the strongest evidence to date for their function as tumor suppressors.

Results

Double knockout mice develop T-ALL

Zfp36l1 and *Zfp36l2* are expressed throughout thymic development, especially during the early $CD4^{-}CD8^{-}$ double negative stages (Supplementary Fig. 1). To examine their function conditional knockout mice were generated using standard gene targeting techniques (Supplementary Fig. 2). Mice were inter-crossed and bred to homozygosity for the floxed alleles of both *Zfp36l1* and *Zfp36l2*. Transgenic expression of Cre under the control of a *CD2* locus control region was used to effect deletion prior to the double negative 1 (DN1) stage of thymic development[21–22]. Unless otherwise stated control mice were $Zfp36l1^{fl/fl}Zfp36l2^{fl/fl}$. We confirmed that the expression of the *CD2-Cre* transgene alone caused no associated defect in thymic development (data not shown). $Zfp36l1^{fl/fl}Zfp36l2^{fl/fl}$CD2Cre mice, hereafter referred to as double knockout (dKO) for simplicity, were born at expected Mendelian ratios and appeared healthy in early life. However, 90% of dKO mice died or were humanely culled due to ill health by six-months of age (Fig. 1a). All of these mice had developed thymic tumors and many also showed splenomegaly and lymphadenopathy (Supplementary Fig. 3). Tumor development was never observed in $Zfp36l1^{fl/fl}$*CD2-Cre* or $Zfp36l2^{fl/fl}$*CD2-Cre* single mutant mice, or in any intermediate combination of genotypes (e.g. $Zfp36l1^{fl/fl}Zfp36l2^{fl/+}$*CD2-Cre*). Thus a single allele of either *Zfp36l1* or *Zfp36l2* appeared sufficient to prevent tumor development. All thymic tumors showed high CD8 expression, with variable CD4 expression (Fig. 1b). Tumor cells expressed high amounts of heat stable antigen (CD24) but did not express surface T cell receptor beta (sTCR-β). Most, but not all tumors, expressed intracellular TCR-β (icTCR-β). Circulating lymphoblasts were seen upon examination of the peripheral blood (Fig. 1c,d). Flow cytometric analysis of spleen, lymph node and bone marrow frequently demonstrated involvement by tumor cells of identical phenotype to the associated thymic tumor (Fig 1e). Clonality testing by PCR across the TCR-β2 region suggested that thymic tumors were predominantly oligoclonal (Fig. 1f). Taken together, these findings suggest that deletion of *Zfp36l1* and *Zfp36l2* together leads to the development of T-ALL corresponding to the CD8 immature single positive (CD8iSP) and double positive (DP) stages of thymic development.

Perturbed thymopoiesis prior to tumor development

In dKO mice, tumor development was preceded by thymic atrophy. At three and eight weeks of age total thymic cellularity was approximately 50% that of control mice (Fig. 2a,b). This atrophic stage was associated with the gradual expansion of the CD8iSP population ($CD8^{+}CD4^{-}CD24^{hi}$sTCRβ^{-}). By thirteen weeks this population was markedly expanded in

of blasting cells (data not shown). Furthermore, cellular proliferation was elevated, as judged by increased uptake of EdU following pulse administration (Supplementary Fig. 5). Thus it appeared that dKO thymocytes were able to transit the β-selection checkpoint, with associated metabolic activation and proliferation, but without expression of TCR-β. This aberrant passage through β-selection was associated with differentiation of icTCRβ^{-} thymocytes to the CD8iSP and DP developmental stages which lie downstream of β-selection. As expected, in control mice all thymocytes from the CD8iSP and DP stages expressed icTCR-β. However, in dKO thymocytes, icTCR-β was expressed by only 30% of CD8iSP (Fig. 2e & Supplementary Fig. 4). This increased to 90% of DP and 100% at the CD4 and CD8 mature single positive (CD8mSP) stages (Supplementary Fig. 4). Thus although dKO icTCR-β^{-} cells were able to pass β-selection they were unable to progress beyond the DP stage. Despite the accumulation of TCRβ^{-} CD8iSPs, the majority of thymic tumors corresponded to TCRβ^{+} CD8iSP thymocytes suggesting the importance of preTCR signals during leukaemia development.

Elevated Notch1 expression in dKO thymocytes

We hypothesised that the thymic phenotype observed in dKO mice might result from the over-expression of one or more genes normally suppressed by ZFP36L1 or ZFP36L2. We performed microarray analysis upon whole thymus from control and dKO mice, aged five weeks old and nine weeks old, as this was prior to the development of tumor and, at five weeks of age, the relative thymic proportions were normal, as judged by CD4 and CD8 staining (Fig. 1a). At nine weeks of age more than 500 significantly changing genes were elevated more than 1.5 fold. Fewer were elevated at five weeks. When the list was filtered to include only genes elevated in both the five week and the nine week arrays, 17 named genes were identified (Fig. 3a). Of these 17 up-regulated genes, we chose to investigate further the expression of *Notch1* as this gene is critical for thymic development and in the pathogenesis of T-ALL[24]. Elevation of mRNA encoding Notch1 and its target genes, *Hes1*, *Myc* and *Dtx1*, was confirmed by real time PCR (Fig. 3b). We also showed strong expression of Notch1 protein in thymic tumors, both by immunoblot and flow cytometry (Fig. 3c,d). Notch1 expression by flow cytometry was between 5- and 50-fold higher in thymic tumor than .87 0 TTT2 10 3d dKble to eN522 0 Td mfkTf 0 3a)dKble testing the imhe thymated in br thanession of Ndidmalt midelal tflsiocd flnessan of

treatment also led to the disappearance of $CD8^+$

The over-expression of Notch1, observed prior to and after the development of leukaemia, is also intriguing as chimeric mice reconstituted with bone marrow transduced with retrovirus expressing Notch1-intracellular domain, and other mouse models that overexpress Notch1 invariably develop T-ALL[24, 34–36]. The observed phenotype of Notch1-driven T-ALL typically corresponds to the CD8iSP and DP stages of thymocyte development and closely resembles the T-ALL seen in *Zfp36l1-Zfp36l2* dKO mice. The expansion of CD8iSP cells was Notch1-dependent in the dKO mice prior to the onset of T-ALL. The results of luciferase and EMSA experiments suggest that the elevated Notch1 expression is likely to result from alleviation of ZFP36L1 and ZFP36L2-mediated suppression. Regulation of *Notch1* at the post-transcriptional level has been proposed previously in the context of leech development[37] but has not been proposed in the context of thymic development. The degree of elevation of Notch1 in dKO mice prior to onset of leukaemia is modest – about two-fold. However we consider this elevation significant because *Notch1* is normally extremely tightly regulated both at the level of transcription and protein stability. The Notch signaling pathway contains no amplification stage, therefore one ligand-receptor engagement leads to one molecule of Notch intracellular domain complexing with a single promoter and activating transcription from a single target allele. Notch1 is also unusual in that a thymic phenotype has been described in the context of haplo-insufficiency[38]. These factors suggest that the Notch1 signaling pathway has evolved to regulate its expression within very tight limits.

Importantly, overexpression of Notch1 has the capacity to contribute to both the leukaemic phenotype and the aberrant β-selection[24, 31]. This contribution of Notch1 to the leukaemic phenotype is evidenced by the Notch1-dependency of tumor cells both *in vivo* and *in vitro*. However, it is unclear whether the degree of elevation is, by itself, sufficient to initiate leukaemia formation. The oligoclonal nature of the tumors suggests a “second hit”. This might be mutation of the *Notch1* gene itself leading to the co-operative activation of the Notch1 receptor that is already over-expressed in dKO thymocytes. It is also likely that further unidentified ZFP36L1 and ZFP36L2 targets exist that contribute to the leukemic phenotype. We chose to pursue *Notch1* because of its established role in thymic development and the presence of predicted ZFP36L1 and ZFP36L2 binding sites in the 3'UTR. However ZFP36 family members are promiscuous in their binding requirements and targets might exist that do not possess the classicvelopdy-riignn namer12. Fer
be mu

to the changes in gene expression required during thymocyte development. This would provide a mechanism whereby PI3K or MAPK activity could effect rapid changes in expression of transcription factors such as Notch1, thereby integrating extracellular cues with both post-transcriptional and transcriptional control of gene expression.

Our finding that *Notch1* can be regulated at the RNA level is of potentially great significance to human disease. *NOTCH1* is mutated in >50% human T-ALL and co-operating mutations affecting both HD and PEST domains are frequent[46]. Truncation of the 3'UTR as a result of alternative polyadenylation site usage is commonly seen in malignancy and it is plausible that mutation or truncation of the *NOTCH1* 3'UTR might co-operate with coding sequence mutations to further increase NOTCH1 activity. Preliminary experiments involving analysis of array CGH (aCGH) data from 69 human T-ALL samples identified recurrent deletions in chromosome 14q24 including the *ZFP36L1* locus in 3 cases. This finding is intriguing, but of uncertain significance as these were non-focal, heterozygous deletions encompassing several additional genes besides *ZFP36L1*. No genetic alterations involving *ZFP36L2* in chromosomeof arnatiecuky eererun

muta6L2

Gr1, B220, NK1.1, γδTCR). Cells were sorted on a FACSAria (BD): DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+CD98^-$) and DN4 ($CD44^-CD25^-CD98^+$). CD8 populations were sorted following MACS depletion of CD4 then sorted based on: CD8iSP ($CD8^+$sTCR-β^-CD24^{hi}) CD8mSP ($CD8^+$sTCR-β^+CD24^{int}). CD4 and DP populations were sorted by expression of CD4 and CD8.

Antibodies for flow cytometry and immunoblotting

Biotin-Gr1(RB6-8C5), Biotin-NK1.1 (PK136), Biotin-γδ-TCR (GL3), FITC-CD25 (7D4), PerCP-Cy5.5-CD8α (53-6.7), PE-CD98 (RL388) and FITC-CD24 (M1/69) were purchased from BD. PE-Cy7-CD4 (L3T4), PECy5.5-CD44 (IM7), PE-CD71 (R17217), PE-TCR-β (H57-597), Biotin-TER119, Biotin-B220 (RA3-6B2) were purchased from eBioscience. Biotin-CD11b (RM2815) was purchased from Caltag. Notch1 staining was performed using PE-Notch1 (mN1A) from BD and Biotin-Notch1 rat IgG2a (22E5.5)[48]. For immunoblotting BRF1/2(#2119) and Cleaved Notch1 (Val1744) (D3B8) were purchased from Cell Signaling Technology. The EdU staining kit was purchased from Invitrogene and used as per the manufacturer's instructions.

Microarray

Samples were prepared from thymus in Trizol (Invitrogen). RNA was extracted using the RNEasy kit (Qiagen) including the on column DNAse step. Expression profiling was performed using Affymetrix mouse gene ST1.0 array and analysed using Genespring software. Raw data was subjected to RMA normalisation. Probes whose raw intensity fell into the bottom 20% of every replicate across all conditions were removed as being unexpressed. The remaining probes were tested separately for a significant change in expression between control and either 5 or 9 weeks using an unpaired t-test with a cut-off of $p<0.05$ after applying a Benjamini and Hochberg multiple testing correction. The significantly changing genes were further filtered to remove any with an absolute change of <1.5 fold between conditions.

TCR-β2 Clonality PCR

PCR primers were used as previously described[49].

RTPCR

cDNA was synthesised from RNA using Superscript II (Invitrogen). RT-PCR was performed using Platinum QPCR mix (Invitrogen) and primers detailed in Supplementary table 1 and run on a Chromo4 analyser (MJ Research). Results were normalised to the reference genes B2M or to GAPDH as indicated in text. See supplementary table 1 for primer sequences. B2M and GAPDH assay were purchased from ABI.

Plasmid Constructs

pcDNA3.myc.WT *ZFP36L1* expression construct and pFLAG.CMV2-*ZFP36L2* were obtained from Dr Andrew Clark (Kennedy Institute of Rheumatology, London). The *ZFP36L1* tandem zinc finger mutant (TZFM) C135/173R was made by site directed mutagenesis. See Supplementary table 1 for primer sequences. The *ZFP36L1* and *ZFP36L2* coding sequences, generated by PCR from the constructs, above were cloned into the MIGRI retroviral vector using the BglII and EcoRI sites. The mouse *Notch1* 3'UTR reporters were cloned from CHORI BAC clone RP23-306D20 into pSI-Check2 (Promega). See Supplementary table 1 for primer sequences.

Luciferase assays

293T cells were cultured in DMEM, 10% FCS, 2mM Glutamine and 100 U/ml Penicillin/ Streptomycin then transfected with luciferase reporter (20 ng) plus *ZFP36L1* or *ZFP36L2* expression vector (0.3 – 2.5 ng) using the CalPhos kit (Clonotech). Firefly and Renilla luciferase activity was analyzed 24 hours post transfection using the Dual-Glo luciferase reporter assay system (Promega) and analyzed on a TopCount NXT microplate luminescence counter.

EMSA

Complimentary oligonucleotides incorporating a T7 promoter and 61 nucleotides of the Notch1 3'UTR HCR were used for in vitro transcription of ^{32}P labelled RNA probe using the T7 Maxiscript kit (Ambion). 200 fmol of radiolabelled probe was incubated with 20 µg of lysate from transfected 293T cells for 20' at room temperature followed by incubation with 5mg/ml heparin and 50U/ml RNase T1. Protein-RNA complexes were resolved on a 4.5% acrylamide in 0.5× TBE gel then exposed to a Fuji Phosphoimager screen.

Tumor culture

Primary tumor cells were seeded onto OP9-DH31

Acknowledgments

We thank all of our colleagues for their input during the preparation of this manuscript. D.J.H. was funded by a fellowship from Cancer Research UK and by the Addenbrooke's Charitable Trust. A.G. is supported by a MRC CASE studentship. M.T. is a Medical Research Council Senior Non-Clinical Fellow and also received funding for this work from BBSRC grant number BB/C506121/1. This work was supported by the National Institutes of Health grants R01CA120196 and R01CA129382 to A.F and the ECOG tumor bank grant U24 CA114737. A. F. is a Leukemia & Lymphoma Society Scholar. K. D. K. is a postdoctoral researcher funded by the “Fonds voor Wetenschappelijk Onderzoek-Vlaanderen” and recipient of a Belgian American Educational Foundation (BAEF) fellowship.

References

1. Georgescu C, et al. A gene regulatory network armature for T lymphocyte specification. Proc. Natl. Acad. Sci. U. S. A 2008;105:20100–20105. [PubMed: 19104054]
2. Rothenberg EV. Negotiation of the T lineage fate decision by transcription-factor interplay and microenvironmental signals. Immunity 2007;26:690–702. [PubMed: 17582342]
3. Rothenberg EV, Moore JE, Yui MA. Launching the T-cell-lineage developmental programme. Nat Rev Immunol 2008;8:9–21. [PubMed: 18097446]
4. Galante PA, et al. A comprehensive in silico expression analysis of RNA binding proteins in normal and tumor tissue: Identification of potential players in tumor formation. RNA Biol 2009;6:426–433. [PubMed: 19458496]
5. Taylor GA, et al. A pathogenetic role for TNF alpha in the syndrome of cachexia, arthritis, and autoimmunity resulting from tristetraprolin (TTP) deficiency. Immunity 1996;4:445–454. [PubMed: 8630730]
6. Carballo E, Lai WS, Blackshear PJ. Feedback inhibition of macrophage tumor necrosis factor-alpha production by tristetraprolin. Science 1998;281:1001–1005. [PubMed: 9703499]
7. Bell SE, et al. The RNA binding protein Zfp36l1 is required for normal vascularisation and post-transcriptionally regulates VEGF expression. Dev. Dyn 2006;235:3144–3155. [PubMed: 17013884]
8. Stumpo DJ, et al. Chorioallantoic fusion defects and embryonic lethality resulting from disruption of Zfp36L1, a gene encoding a CCCH tandem zinc finger protein of the Tristetraprolin family. Mol. Cell. Biol 2004;24:6445–6455. [PubMed: 15226444]
9. Stumpo DJ, et al. Targeted disruption of Zfp36l2, encoding a CCCH tandem zinc finger RNA-binding protein, results in defective hematopoiesis. Blood 2009;114:2401–2410. [PubMed: 19633199]
10. Blackshear PJ, et al. Characteristics of the interaction of a synthetic human tristetraprolin tandem zinc finger peptide with AU-rich element-containing RNA substrates. J. Biol. Chem 2003;278:19947–19955. [PubMed: 12639954]
11. Hudson BP, Martinez-Yamout MA, Dyson HJ, Wright PE. Recognition of the mRNA AU-rich element by the zinc finger domain of TIS11d. Nat Struct Mol Biol 2004;11:257–264. [PubMed: 14981510]
12. Emmons J, et al. Identification of TTP mRNA targets in human dendritic cells reveals TTP as a critical regulator of dendritic cell maturnals. Irc celF7:89 mRNA 6icats T [Puhis m0esis21]
1
elemen anchrethalyte sthal &

42. Benjamin D, Schmidlin M, Min L, Gross B, Moroni C. BRF1 protein turnover and mRNA decay activity are regulated by protein kinase B at the same phosphorylation sites. Mol. Cell. Biol 2006;26:9497–9507. [PubMed: 17030608]
43. Maitra S, et al. The AU-rich element mRNA decay-promoting activity of BRF1 is regulated by mitogen-activated protein kinase-activated protein kinase 2. RNA 2008;14:950–959. [PubMed: 18326031]
44. Palomero T, et al. Mutational loss of PTEN induces resistance to NOTCH1 inhibition in T-cell leukemia. Nat. Med 2007;13:1203–1210. [PubMed: 17873882]
45. Palomero T, Dominguez M, Ferrando AA. The role of the PTEN/AKT Pathway in NOTCH1-induced leukemia. Cell Cycle 2008;7:965–970. [PubMed: 18414037]
46. Weng AP, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. Science 2004;306:269–271. [PubMed: 15472075]
47. Rodriguez CI, et al. High-efficiency deleter mice show that FLPe is an alternative to CreloxP. Nat. Genet 2000;25:139–140. [PubMed: 10835623]
48. Fiorini E, et al. Dynamic regulation of notch 1 and notch 2 surface expression during T cell development and activation revealed by novel monoclonal antibodies. J. Immunol 2009;183:7212–7222. [PubMed: 19915064]
49. Dumortier A, et al. Notch activation is an early and critical event during T-Cell leukemogenesis in Ikaros-deficient mice. Mol. Cell. Biol 2006;26:209–220. [PubMed: 16354692]

Figure 2. Thymocyte development is perturbed prior to tumor development

A. Flow cytometric plots gated on cells negative for dump channel (B220, Ter119, NK1.1, γδ-TCR, Mac-1, Gr1). B. Total thymic cellularity showing thymic atrophy in dKO mice. Statistical analysis used Mann-Whitney test. C. Flow cytometric plots gated on CD8 single positive population ($CD8^+CD4^-$ as shown in A) showing proportions of immature ($CD24^{hi}sTCR\beta^-$) and mature ($CD24^{int}sTCR\beta^+$) CD8 single positive thymocytes. D. Immature CD8 single positive thymocytes as a percentage of all CD8 SP cells (upper) or absolute number per thymus (lower) at the indicated age of mice. Graphs show the mean and SEM for five mice per genotype. E. Flow cytometric plots from 3-week old mice gated on dump channel negative, $CD4^-CD8^-$ double negative thymocytes. For all flow cytometric plots numbers show the percentage of cells within the indicated gate and represent the mean from one experiment of 4–5 mice per genotype. All plots are representative of at least nine individual mice.

Figure 3. Expression of Notch1 is elevated in *Zfp36l1-Zfp36l2* dKO mice
A. Heatmap summary of microarray performed on cDNA from whole thymus of control and dKO mice at five and nine weeks of age. Criteria for inclusion are genes changing significantly

Figure 4. ZFP36L1 and ZFP36L2 exert suppression via interaction with sequences in the Notch1 3'UTR

A. *Notch1* 3'UTR presented as degree of conservation between human and mouse using the Vista Genome Browser. The highly conserved region (HCR) of interest is underlined in red. **B**. Detailed view showing inter-species sequence conservation for the underlined region in A. Black background indicates 100% conservation between human, mouse, dog, armadillo, opossum and platypus. Predicted ZFP36L1 and ZFP36L2 binding sites are boxed in red. **C**. Luciferase reporters were constructed corresponding to the full length *Notch1* 3'UTR (N1 UTR full length), the proximal HCR underlined in red in A (N1 HCR), or the *Notch1* 3'UTR with the HCR removed (N1 Δ HCR). These reporters were co-transfected into HEK293T cells along with a pCDNA3 empty control vector or pCDNA3 expressing *ZFP36L1*, *ZFP36L2* or a tandem zinc finger mutant of *ZFP36L1* (TZFM). Results are shown as the mean and SEM of five separate transfections and are representative of three experiments. Statistical analysis performed by ANOVA with Tukey post-hoc analysis (* $P < 0.001$) **D**. Titration of transfected *ZFP36L1* or *ZFP36L2*. Mean and SEM of five separate transfections are shown. **E**. Electromobility shift assay (EMSA) following incubation of a radiolabelled *Notch1* probe (corresponding to the 61 nucleotides containing the nonameric AU sequence) with lysates from 293T cells transfected with the indicated expression constructs. pCDNA3 = empty control vector, TZFM = tandem zinc finger mutant.

Figure 5. Inhibition of Notch or re-expression of ZFP36L1 is toxic to tumor growth

A. Primary dKO tumor cells were cultured on OP9-DL1 stromal cells for three days in the presence of increasing concentrations of the gamma-secretase inhibitor Compound E. Graph shows mean and SEM for six tumors presented relative to the number of cells seeded. Statistical analysis was performed by repeated measures ANOVA with Tukey post hoc analysis. (**p<0.01, ***p<0.001) **B**. Notch1 blocking antibody, or saline control, was administered *in vivo* to 11 week old control and dKO mice. After three weeks of treatment thymus was analysed by flow cytometry. Post-treatment thymic cellularity is shown for individual mice. **C**. Representative flow cytometry plots of thymus after treatment with control or Notch1 blocking antibody. CD4–8 plots are gated on thymocytes negative for B220, NK1.1, γδ-TCR, mac-1, Gr1, Ter119. CD24-icTCR-β plots are gated on the CD8 single positive quadrant. **D**. Primary thymic tumors from dKO mice were cultured on OP9-DL1 stromal cells for 24 hours before infection with equivalent titres of retrovirus expressing either *ZFP36L1* or the tandem zinc finger mutant of *ZFP36L1* (TZFM). After three days cells were analysed by flow cytometry. Flow cytometry plots shown are representative of infection of five separate dKO tumors.