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SHORT COMMUNICATION

Overexpression of the anti-apoptotic protein AVEN contributes to increased malignancy in hematopoietic neoplasms

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AVEN has been identified as an inhibitor of apoptosis, which binds to the adaptor protein, APAF-1, and thereby prevents apoptosome formation and mitochondrial apoptosis. Recent data have demonstrated high expression levels of *AVEN* messenger RNA in acute leukemias as well as a positive correlation between *AVEN* mRNA overexpression and poor prognosis in childhood acute lymphoblastic leukemia. On the basis of these data, we investigated the potential involvement of AVEN in tumorigenesis. First, we confirmed the overexpression of AVEN in T-cell acute lymphoblastic leukemia/lymphoma (T-ALL) patient samples. We then established a transgenic mouse model with T-cell-specific overexpression of AVEN, with which we demonstrated the oncogenic cooperation of *AVEN* with heterozygous loss of p53. Finally, we used a subcutaneous xenograft mouse model to show that *AVEN* knockdown in the T-ALL cell lines, MOLT-4 and CCRF-CEM, and in the acute myeloblastic leukemia cell line, Kasumi-1, leads to a halt in tumor growth owing to the increased apoptosis and decreased proliferation of tumor cells. Collectively, our data demonstrate that the anti-apoptotic molecule, AVEN, functions as an oncoprotein in hematopoietic neoplasms.

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Keywords: AVEN; apoptosis; T-ALL; acute lymphoblastic leukemia; p53; transgenic mice

INTRODUCTION

The acquired capability of tumor cells to withstand diverse apoptotic stimuli represents a well-recognized characteristic of cancer,[1,2] and in recent years, additional forms of cell death have been identified that have a role in the tumor suppressor function of 'programmed cell death'. Two main apoptotic signaling pathways have been well-characterized: the death receptor (extrinsic) and the mitochondrial (intrinsic) pathways.[3] Mutational interference in both pathways has been identified in many tumors.[4,5] Abrogation of the mitochondrial pathway is a particularly important step in the development of tumor cell resistance to classical chemotherapy and radiation treatment.[6–8] Both the inactivation of pro-apoptotic tumor suppressor molecules and the overexpression of anti-apoptotic oncoproteins, such as BCL-2, have been frequently reported to contribute to the loss of mitochondrial apoptosis sensitivity as well as tumor initiation and development.

The formation of the so-called apoptosome complex is central to the intrinsic apoptotic signal transduction pathway. The apoptotic stimulus promotes the BCL-2 family-regulated release of Cytochrome c (Cytc) from the mitochondria into the cytosol, where it binds to the adaptor protein APAF-1 and subsequently induces APAF-1 oligomerization and Caspase-9 recruitment.[6] The resulting apoptosome complex serves as both the platform for Caspase-9 activation and the molecular trigger for the death-executing caspase cascade.[9]

Several regulators of apoptosome activity have been described, including the anti-apoptotic protein, AVEN.[10] Originally identified in a yeast-two-hybrid screen as a binding partner of BCL-x_L, AVEN has also been shown to interact with APAF-1 and inhibit apoptosome formation.[11] In addition to its inhibitory function in mitochondrial apoptosis, AVEN has also recently been shown to function as an activator of the cell cycle-regulating ATM kinase in the DNA damage response pathway.[12,13]

Evidence supporting the oncogenic involvement of *AVEN* in the inhibition of apoptosis is based on studies describing the high mRNA expression levels observed in acute leukemias. In the first manuscript by Paydas *et al.*, the authors investigated a study group consisting of 37 acute myeloblastic leukemia (AML) and 28 acute lymphoblastic leukemia (ALL) patients.[14] The details regarding the number of ALL patients who were either of the frequent B cell type or had developed T-cell acute lymphoblastic leukemia/lymphoma (T-ALL) were not given. The authors of this study noticed elevated *AVEN* mRNA expression levels of acute leukemias and suggested that AVEN might represent a novel prognostic indicator for acute leukemia. A second paper by Choi *et al.* describes a positive correlation between *AVEN* mRNA overexpression and poor

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prognosis in childhood ALL.[15] Again, no data were presented regarding whether T-ALL samples were included in the study.

The overexpression of AVEN in tumors would not come as a surprise, as it is a cell death-repressing protein; however, no direct experimental proof has been published on the oncogenic potential of *AVEN* until now. Here, we demonstrate strong overexpression of AVEN in acute leukemia/lymphoma, particularly in T-ALLs, thereby confirming the correlation between AVEN expression and the development of acute leukemias. We analyzed a transgenic mouse model with T-cell-specific AVEN overexpression on a heterozygous $p53^{+/-}$ knockout background and found a significant increase in the development of T-cell lymphoma compared with $p53^{+/-}$ heterozygous knockout animals with normal Aven expression levels in thymocytes and T cells. In addition, the results obtained using human leukemic *AVEN* knockdown cell lines in a xenograft mouse model imply that AVEN expression is required for robust tumor formation and progression. Collectively, we obtained significant experimental evidence that the apoptosis inhibitor, *AVEN*, exhibits oncogenic potential during development of hematopoietic neoplasms.

RESULTS AND DISCUSSION

The overexpression of AVEN in both AML and ALL has been reported,[14,15] although the ALL results were not further distinguished between the common B-ALL and rare T-ALL cases. The latter subtypes seem to be quite heterogeneous with regard to their molecular pathogenesis.[16–20] We analyzed tumor material derived from eight patients with T-ALL/T-lymphoblastic lymphoma (WHO classification,[21] ICD-O code 9837/3) by performing immunohistochemistry (IHC) on cryopreserved and on paraffin sections. Detailed clinical information regarding the T-ALL samples is presented in Supplementary Table 1. Additionally, 13 B-ALL NOS samples (WHO classification,[21] ICD-O code 9811/3) were also investigated by IHC. Figure 1 shows representative examples of the T-ALL IHC results (Figures 1a–d: cryosections, Figures 1e–h: paraffin sections). Whereas the cryosection of infiltrate-free bone marrow shown in Figure 1a displays no detectable AVEN expression, the bone marrow samples from T-ALL patients with partial (Figures 1b and c) and complete (Figure 1d) infiltration exhibit elevated AVEN expression levels. In the paraffin section of a normal germinal center displayed in Figure 1e, only single blasts express significant amounts of AVEN protein, while all T-lymphoblastic lymphoma cells from the three patients shown in Figures 1f–h express high amounts of the protein. In total, 7 of the 8 T-ALL/T-lymphoblastic lymphoma samples examined by IHC displayed AVEN (over-) expression, whereas 6 of the 13 B-ALL samples expressed AVEN. To verify that the high AVEN expression levels are not merely associated with the immature state of the T-ALL cells, we quantified the AVEN expression levels in human thymocytes by IHC analysis and did not detect a significant staining for AVEN. We also performed FACS sorting of various mouse thymocyte subpopulations followed by western blot analysis of lysates prepared from double-negative $CD4^{-}CD8^{-}$, double-positive $CD4^{+}CD8^{+}$, and single-positive $CD4^{+}$ and $CD8^{+}$ cells and did not find significant differences in the AVEN expression levels (data not shown). These results confirm a positive correlation between AVEN (over-) expression and ALL leukemogenesis and suggest that *AVEN* fulfills a possible oncogenic function in T-ALLs.

To obtain direct experimental evidence that *AVEN* has an oncogenic role in T-cell leukemia, we established a transgenic mouse model with T-cell-specific expression of the human AVEN protein. For this purpose, *AVEN* cDNA was cloned under the control of the proximal *lck* promoter,[22] which is active in thymocytes and mature T cells (Figure 2a). One founder mouse was identified by Southern blot, and offspring of the transgenic mouse line were assessed by PCR (Figure 2b) and Southern blot

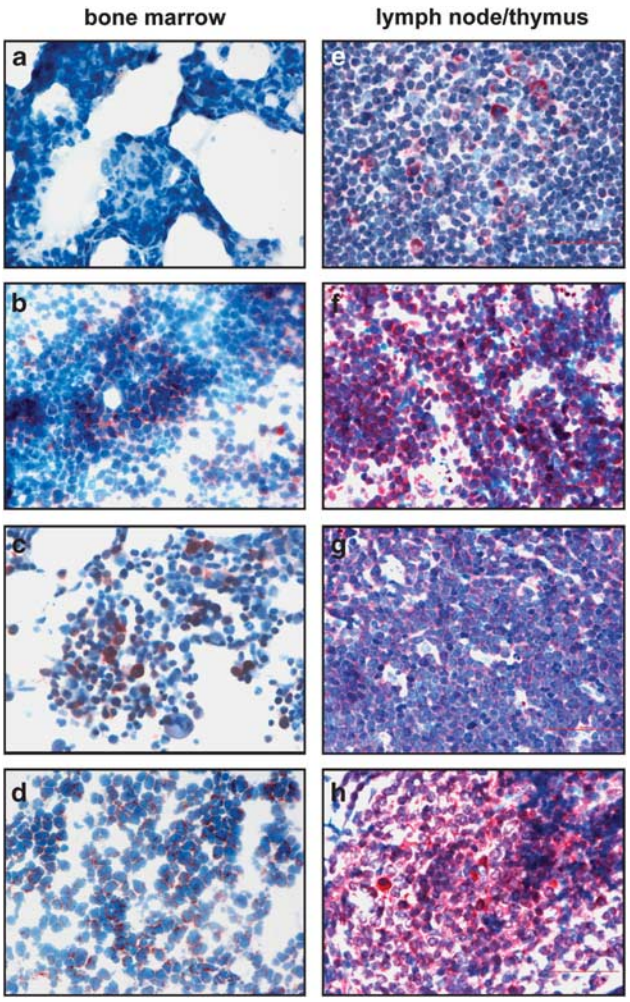

Figure 1. Immunohistochemical (IHC) analysis of human T-lymphoblastic leukemia/lymphoma (ICD-O code 9837/3) reveals the strong expression of AVEN in all tumor cells. (**a**) A cryosection of infiltrate-free bone marrow. Bone marrow samples isolated from T--lymphoblastic leukemia patients with partial (**b**, **c**) and complete (**d**) infiltration. (**e**) A paraffin section of a normal lymph node. (**f**–**h**) Three different cases of T-lymphoblastic lymphoma. IHC analysis was performed according to standard protocols using the polyclonal rabbit antibody against human AVEN (NT, *Pro Sci 2417*) at a 1:1000 dilution. Antibody specificity was validated using MCF-7 cells with and without *AVEN* knockdown. Although cells of the infiltrate-free bone marrow do not show significant AVEN expression (**a**) and only single blasts within a normal germinal center express AVEN (whereas many others do not; **e**), all human T-lymphoblastic leukemia/lymphoma cells display high amounts of AVEN protein (**b**–**d**, **f**–**h**).

(Figure 2c) to genotype wild-type, heterozygous and homozygous *lck AVEN* transgenic animals. Figure 2d demonstrates the strong overexpression of AVEN protein in T cells of the spleen and lymph nodes as well as in thymocytes. AVEN expression levels were greater in homozygous transgenic mice compared with heterozygous animals. As we obtained only one founder mouse line, we determined and analyzed the integration site of the *lck AVEN* transgene to exclude possible insertional mutagenesis of tumor suppressor genes or neighboring oncogenes. mFISH and LAM-PCR analysis revealed the integration of the transgenic DNA in mouse chromosome 6 ~85 kb upstream of the cellular gene *Reg3*γ (see Supplementary Figure 1). The integration of the transgene at this locus, a great distance from any cellular gene, is highly unlikely to result in tumor-promoting insertional mutagenesis.

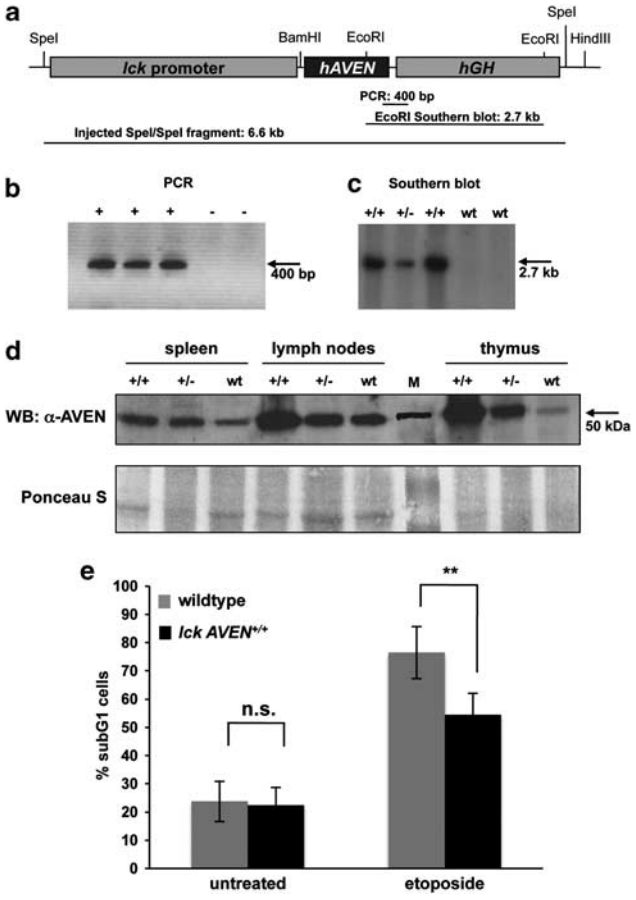

Figure 2. Tissue-specific overexpression of AVEN inhibits etoposide-induced apoptosis in transgenic mouse T cells. (**a**) A schematic presentation of the transgenic construct. Human *AVEN* cDNA is specifically expressed in mouse T cells under the control of the distal *lck* promoter.[22] Genomic *hGH* sequences at the 3′-end enhance transgene expression. The 6.6 kb SpeI/SpeI fragment consisting of the *lck* promoter, *AVEN* cDNA and *hGH* sequences was excised and injected into the pronuclei of FVB/N-derived zygotes, which were then transplanted into CD1 foster female mice. (**b**, **c**) A PCR reaction with *AVEN* and *hGH*-specific primers was performed to identify positive founder animals by amplifying a 400 bp DNA fragment (**b**; '+': positive transgenic mice, '−': wild-type mice), which was confirmed by Southern blot analysis using *Eco*RI-digested genomic tail DNA and a *hGH*-specific ^{32}[P]-labeled probe (**c**; '+/+': homozygous transgenic mice, '+/−': heterozygous transgenic mice, 'wt': wild-type mice). The heterozygous mice were distinguished from the homozygous animals based on the strength of the Southern blot signal, and the genotypes were confirmed by analyzing the offspring derived after mating transgenic mice with wild-type animals (homozygous *lck AVEN*$^{+/+}$ mice yielded 100% heterozygous *lck AVEN*$^{+/-}$ offspring upon mating with wild-type animals). (**d**) Western blot analysis of transgenic AVEN expression in spleen, lymph nodes and thymus. Protein lysates (25 μg) derived from various hematopoietic organs of wild-type, heterozygous and homozygous *lck AVEN* transgenic mice were loaded onto an SDS–PAGE gel. Following electrophoresis and semi-dry transfer, the membrane was incubated with self-raised anti-AVEN antiserum derived against a carboxy-terminal peptide (amino-acid sequence identical for human and mouse AVEN: KNVTEEELEDWLDSMIS). Ponceau S staining of the membrane served as loading control. (**e**) Apoptosis analysis with *lck AVEN* transgenic and wild-type thymocytes. Cell death was measured using the Nicoletti assay[28] 24 h after incubation with or without etoposide (5 μM). Four experiments were performed, and error bars represent s.e. ** indicates $0.01 \geqslant p \geqslant 0.001$ ($P = 0.005$; paired *t*-test).

AVEN has been identified as an inhibitor of apoptosis.[11] Therefore, we aimed to investigate whether transgenic overexpression of *AVEN* influences apoptosis in T cells. We isolated thymocytes from homozygous *lck AVEN* transgenic and wild-type mice, which were then incubated for 24 h with the mitochondrial apoptosis inducer, etoposide (Figure 2e). The AVEN-overexpressing thymocytes were less sensitive towards etoposide treatment, thereby confirming the anti-apoptotic properties of AVEN.

We examined the *lck AVEN* transgenic mice for T-cell lymphomagenesis and observed no tumor development in these animals on a wild-type C57Bl/6 genetic background (data not shown). Next, we investigated whether AVEN overexpression would cooperate as an oncoprotein with loss of the tumor suppressor p53 in T-cell lymphoma.[23,24] For this purpose, we bred the *lck AVEN* mouse line with *p53* knockout animals. We did not observe any further acceleration of lymphoma development in homozygous *p53*$^{-/-}$ knockout mice, following homozygous transgenic *AVEN* overexpression in thymocytes (data not shown). However, any subtle influence of a weak oncogene on tumor development would probably not be detected in the context of the fast T-cell lymphoma occurrence seen in homozygous *p53*$^{-/-}$ knockout mice (all animals died within 5 months after birth); therefore, we decided to analyze the T-cell lymphomagenesis of the *lck AVEN* transgenic mice on a heterozygous *p53*$^{+/-}$ knockout background. While only one of the 23 (4.3%) heterozygous *p53*$^{+/-}$ mice without an *AVEN* transgene developed a T-cell lymphoma within 1 year, 5 of the 20 (25%) *lck AVEN*$^{+/+}$ homozygous animals on a heterozygous *p53*$^{+/-}$ background became terminally ill with lymphomas in the same time frame (Figure 3a). None of the 20 wild-type control mice developed tumors. Our findings provide strong evidence that *AVEN* displays oncogenic potential in a transgenic mouse model. However, as our data were obtained using a single *lck AVEN* transgenic mouse line, we cannot completely rule out the possibility of a distant effect associated with the integration site on the oncogenic phenotype. However, the likelihood of such an influence is rather small, as the integration site is localized a large distance from the neighboring genes.

Figure 3b demonstrates that transgenic overexpression of *AVEN* does not influence the type of thymic lymphoma developing in a p53-deficient background. As in *p53*$^{-/-}$-deficient mice with wild-type Aven expression levels,[23–25] additional homozygous overexpression of AVEN leads to the manifestation of thymic lymphomas with CD4^{+}CD8^{+} double-positive tumor cells resembling an immature thymocyte phenotype.

We were also interested in investigating whether transgenic *AVEN* overexpression would cooperate with the oncoprotein, LMO2, in lymphomagenesis. LMO2 is a nuclear protein that, together with many other proteins (for example, TAL1, E47, GATA-1 and LDB1), forms a functional multi-protein transcriptional complex binding to DNA, which regulates the expression of genes important for differentiation. LMO2 activation has been implicated in the development of human and mouse T-ALL. To investigate the potential oncogene cooperation between *AVEN* and *LMO2*, we performed transplantation experiments. Lineage negative (Lin^{-}) bone marrow cells isolated from homozygous *lck AVEN* and wild-type donor mice were transduced with either a retroviral *LMO2* expression vector or an empty control vector, and then injected intravenously into lethally irradiated recipient *SJL* mice. As expected, *LMO2* overexpression in transplanted hematopoietic stem and progenitor cells led to the development of lymphomas in all injected mice. However, additional over-expression of *AVEN* did not accelerate the development of LMO2-driven hematopoietic malignancies, suggesting that Aven does not cooperate with LMO2 in lymphoma development (Supplementary Figure 2). In contrast, we observed a slight delay in the onset of lymphomagenesis in animals transplanted with LMO2-transduced *lck AVEN* cells compared with mice transplanted

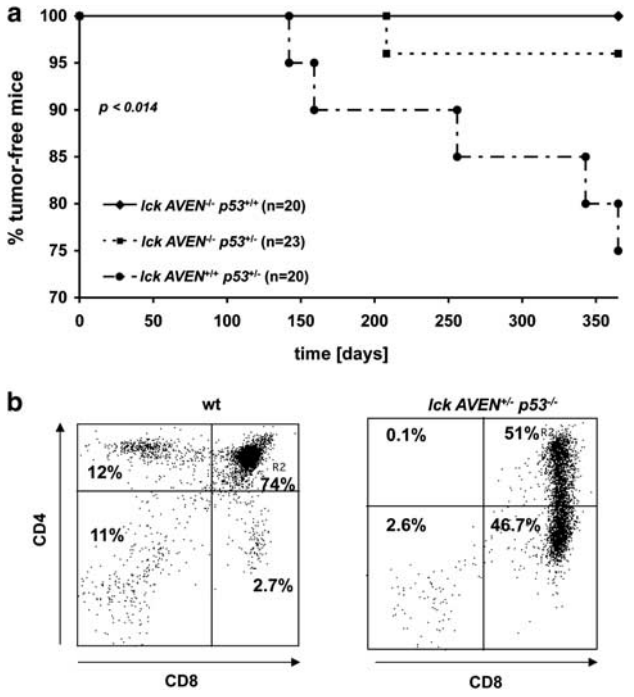

Figure 3. T-cell-specific overexpression of AVEN leads to the acceleration of leukemia development in $p53^{+/-}$ heterozygous knockout mice. (**a**) *lck AVEN* transgenic mice were bred onto a heterozygous $p53^{+/-}$ knockout background and were monitored for leukemia onset over the course of 1 year. Sick animals were sacrificed when moribund. While none of the 20 wild-type control mice (0%) and only one of the 23 non-transgenic $p53^{+/-}$ animals (4.3%) developed T-cell leukemia, 5 of the 20 homozygous *AVEN* transgenic $p53^{+/-}$ mice (25%) died of T-cell leukemia. This represents a significant increase in leukemogenesis (log-rank test, $P<0.014$). In each group, one animal developed a sarcoma; these animals were excluded from the statistical calculations. (**b**) After crossing with *lck AVEN* transgenic animals, heterozygous and homozygous *p53* knockout mice predominantly develop the same immature $CD4^+CD8^+$ double-positive thymic lymphomas as observed in non-*AVEN* transgenic p53-deficient mice.[23] FACS analysis was performed with normal thymocytes isolated from a wild-type C57Bl/6 mouse and T-cell leukemia cells isolated from an *lck* $AVEN^{+/-}$ $p53^{-/-}$ mouse.

with LMO2-transduced wild-type cells. However, the slope of both Kaplan–Meier curves was identical, and we do not believe that AVEN interferes with LMO2-induced lymphoma development.

We proposed to complement our oncogenic *AVEN* overexpression studies with *AVEN* knockdown experiments using human leukemia cell lines. We stably transduced the human T-ALL cell line, MOLT-4 (ATCC no. CRL-1582), with either *AVEN* short hairpin RNA (shRNA) in the lentiviral vector *pLKO.1-tdTomato nucmem* or non-targeting control shRNA in the same vector. In addition, the human T-ALL cell line, CCRF-CEM (ATCC no CCL-119), was transduced with the same shRNA sequences in the *pLKO.1 puro* vector. Efficient *AVEN* knockdown was confirmed via Western blot analysis (Figures 4A and B). Our *in vitro* expansion assays revealed that the number of *AVEN* knockdown MOLT-4 and CCRF-CEM cells increased at a significantly slower rate than the corresponding cells transduced with control shRNA (see Supplementary Figure 3). We then injected either *AVEN* knockdown or control shRNA-transduced MOLT-4 or CCRF-CEM cells into the right flanks of immunocompromised NOD/SCID mice[26] and monitored tumor growth. As shown in Figure 4A, only MOLT-4 cells transduced with control shRNA, which retain normal AVEN protein levels, formed exponentially growing subcutaneous tumors. In contrast, the downregulation of AVEN mediated by lentiviral shRNA strongly diminished the tumorigenicity of MOLT-4 cells in immunodeficient mice. A similar effect of diminished tumor growth in the absence of AVEN was observed in the xenograft tumors established with CCRF-CEM cells (Figure 4B).

After the mice were sacrificed, tumors were explanted and weighed. In both xenograft models, MOLT-4 and CCRF-CEM, tumors established with *AVEN* shRNA-transduced cells weighed significantly less than tumors derived from the control shRNA-transduced cells (Supplementary Figures 4A and B).

An additional xenograft transplantation assay was performed using the human AML cell line, Kasumi-1 (ATCC no. CRL-2724[27]), which was transduced with the pLKO.1*tdTomato nucmem* lentiviral *AVEN* knockdown construct to obtain a significant reduction in the endogenous AVEN protein levels (Supplementary Figure 4C). Again, we injected either *AVEN* knockdown or control shRNA-transduced Kasumi-1 cells into the flanks of NOD/SCID mice and assessed tumor growth. The result of this *in vivo* xenograft transplantation experiment is shown in Supplementary Figure 4C. Whereas the tumors established with AVEN-expressing Kasumi-1 cells grew exponentially, tumor growth was severely diminished in *AVEN* knockdown cells. This result, obtained in a third leukemic AML cell line, substantiates the evidence that the apoptosis inhibitor, AVEN, has an important role in leukemia development and progression.

We performed additional IHC analyses with the xenografted MOLT-4-derived tumors to gain further insight into the consequences of *AVEN* knockdown in tumor cells. As confirmed by positive anti-hCD3 and anti-hCD4 staining of tumor-derived slides, the tumor mass was built up by MOLT-4 cells (Figure 4C: a,b,f,g). Using anti-Ki67 (MIB-1) and anti-phospho-HISTONE H3 (pHH3) antibodies, decreased numbers of proliferating cells were detected in AVEN-deficient MOLT-4-derived tumors compared with MOLT-4 tumors with normal AVEN expression (Figure 4C: c,d,h,i). Of note, HISTONE H3 is not phosphorylated during apoptosis, and therefore, the anti-pHH3 staining allows for the distinction between genuine mitoses and apoptotic nuclei. In addition, we noticed a profound increase in the apoptosis of MOLT-4 tumor cells upon knockdown of *AVEN* when compared with tumors induced by MOLT-4 cells with normal AVEN levels, as revealed by anti-cleaved hCaspase-3 staining (Figure 4C: e,j). Collectively, these IHC results demonstrate that AVEN expression in the T-ALL cell line, MOLT-4, and possibly, in other leukemia cell lines and primary tumor cells, is required for both the proliferation and suppression of apoptosis, two properties that would sufficiently explain the oncogenic potential of *AVEN*.

In 2008, Guo *et al*. have reported that AVEN inhibits the entry into mitosis in *Xenopus* egg extracts and that it is able to function as an ATM activator to inhibit G2/M progression.[13] Immunodepletion of AVEN allowed for mitotic entry even in the presence of damaged DNA, and collectively, the results implicate AVEN in the ATM-dependent DNA damage response pathway. On the basis of these findings, one would expect that, in addition to its oncogenic function as a cell death inhibitor, *AVEN* can also exert a tumor suppressor function by inhibiting mitotic entry, specifically upon DNA damage. However, the findings presented here (that is, AVEN overexpression in human T-ALL samples, oncogenic *AVEN* cooperation in heterozygous $p53^{+/-}$ mice, and reduced tumorigenicity in *AVEN* knockdown T-ALL cell lines measured in a xenograft mouse tumor model) suggest that AVEN functions as an oncoprotein rather than a tumor suppressor, at least in acute lymphomas.

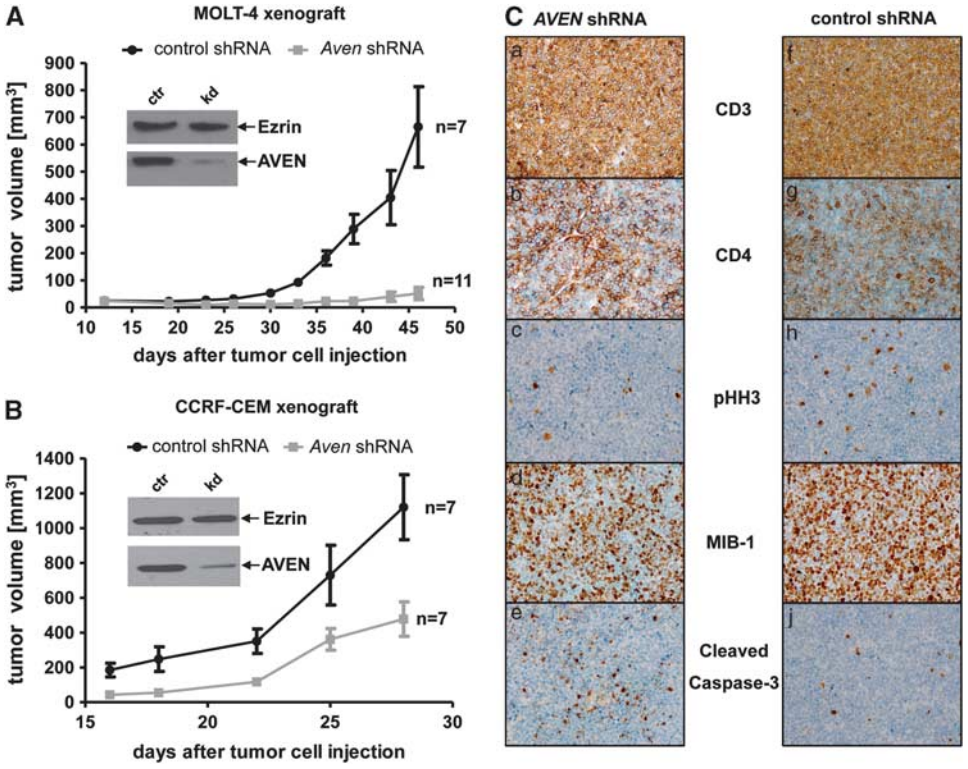

Figure 4. The knockdown of *AVEN* impairs the tumor growth of the T-ALL cell lines MOLT-4 and CCRF-CEM in a subcutaneous murine xenograft tumor model. (**A**) *AVEN* knockdown was performed in MOLT-4 cells using *pLKO.1tdTomato*-csh (control shRNA 5′-CCGGCAACA AGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTT-3′), *pLKO.1tdTomato*-sh*AVEN* (5′-CCGGGAGAATGATGAACAGGGAAATCTCGA GATTTCCCTGTTCATCATTCTCTTTTTTG- 3′) and vsv-g pseudotyped lentiviral transduction (shRNA sequences were derived from Sigma and cloned into the vector *pLKO.1-tdTomato nucmem*). Cells were sorted for positive red fluorescence (tomato) using a BD FACSAria Cell Sorter (BD Biosciences, Heidelberg, Germany), and the downregulation of AVEN was confirmed by western blot analysis (kd: *AVEN* knockdown cells, ctr: control shRNA-transduced cells) with anti-AVEN CT antibody (ProSci; San Diego, CA, USA; 1:2,000 dilution). The anti-Ezrin immunoblot (Clone 3C12, life technologies, Darmstadt, Germany) was used as a loading control. For xenograft experiments, 5×10^6 cells immersed in 30% Matrigel (BD Biosciences, Heidelberg, Germany)/PBS were subcutaneously injected into the right flank of 5- to- 6-week-old NOD/SCID mice, and tumor growth was measured using a caliper gauge. The tumor volume was calculated as follows: tumor volume (mm^3) = length × width^2 × 0.5. Tumor volumes were significantly different with $P = 0.0008$ for day 46 (two-tailed Mann–Whitney- test). (**B**) *AVEN* knockdown and xenograft experiments were performed, as described in **A**, using CCRF-CEM cells with *pLKO.1puro*-csh and *pLKO.1puro*-sh*AVEN* vectors (same shRNA sequences as in *pLKO.1tdTomato*) and vsv-g pseudotyped lentiviral transduction. Cells were selected for several days with 2 μg/ml puromycin (Sigma Aldrich, Munich, Germany) before western blot analysis and xenograft experiments. Tumor volumes were significantly different with $P < 0.0104$ for day 28 (unpaired, two-tailed Student's *t*-test). (**C**) The knockdown of *AVEN* modulates both the proliferative and apoptotic properties of MOLT-4 T-ALL cells *in vivo*. Subcutaneously implanted *AVEN* shRNA (a–e) and control shRNA transduced (f–j) MOLT-4 cells both resulted in tumor formation unequivocally consisting of a T-cell infiltrate characterized by strong CD3 immunopositivity (a and f; anti-hCD3 antibody (Dakocytomation, Glostrup, Denmark)) and moderate CD4 expression (b and g; anti-hCD4 antibody (Ventana, Tucson, AZ, USA)). *AVEN*-deficient MOLT-4 cells (c d) showed reduced mitotic (c) and proliferative (d) activity compared with the corresponding control cells (h and i; anti-pHH3 antibody (BioCare Medical, Concord, CA, USA)) was used to detect mitoses; anti-Ki-67 antibody (MIB-1 (Dakocytomation) was used to stain all proliferative cells exiting the G_0 cell cycle phase). Higher rates of apoptosis were observed in the *AVEN* shRNA- transduced cells (e) compared with the control shRNA-transduced cells (j), as demonstrated by anti-cleaved Caspase-3 immunohistochemistry (Cell Signaling, Danvers, MA, USA). (Original magnification 20× three subcutaneous tumors were analyzed for both *AVEN* shRNA- and control shRNA-transduced MOLT-4 cells; representative images are depicted).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)