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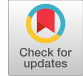
Research paper

Alternative splicing of the tumor suppressor ASPP2 results in a stress-inducible, oncogenic isoform prevalent in acute leukemia



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ABSTRACT

Background: Apoptosis-stimulating Protein of TP53-2 (*ASPP2*) is a tumor suppressor enhancing TP53-mediated apoptosis via binding to the TP53 core domain. *TP53* mutations found in cancers disrupt ASPP2 binding, arguing for an important role of ASPP2 in TP53-mediated tumor suppression. We now identify an oncogenic splicing variant, *ASPP2κ*, with high prevalence in acute leukemia.
Methods: An mRNA screen to detect ASPP2 splicing variants was performed and *ASPP2κ* was validated using isoform-specific PCR approaches. Translation into a genuine protein isoform was evaluated after establishing epitope-specific antibodies. For functional studies cell models with forced expression of *ASPP2κ* or isoform-specific *ASPP2κ*-interference were created to evaluate proliferative, apoptotic and oncogenic characteristics of *ASPP2κ*.
Findings: Exon skipping generates a premature stop codon, leading to a truncated C-terminus, omitting the TP53-binding sites. *ASPP2κ* translates into a dominant-negative protein variant impairing TP53-dependent induction of apoptosis. *ASPP2κ* is expressed in CD34+ leukemic progenitor cells and functional studies argue for a role in early oncogenesis, resulting in perturbed proliferation and impaired induction of apoptosis, mitotic failure and chromosomal instability (CIN) – similar to *TP53* mutations.
Importantly, as expression of *ASPP2κ* is stress-inducible it defines a novel class of dynamic oncogenes not represented by genomic mutations.
Interpretation: Our data demonstrates that *ASPP2κ* plays a distinctive role as an antiapoptotic regulator of the *TP53* checkpoint, rendering cells to a more aggressive phenotype as evidenced by proliferation and apoptosis rates – and ASPP2κ expression results in acquisition of genomic mutations, a first initiating step in leukemogenesis. We provide proof-of-concept to establish ASPP2κ as a clinically relevant biomarker and a target for molecule-defined therapy.
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1. Introduction

The current understanding of leukemogenesis is based on an evolutionary process where stepwise acquisition of somatic mutations drive hematopoietic progenitor/stem cells to malignant transformation, impairing hematopoietic differentiation and control of cellular proliferation and viability [1–3]. The TP53 pathway is well known as a central player in cellular stress response and tumor suppression.

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Research in context

Before this study, it had been shown that ASPP2 is an independent haploinsufficient tumor suppressor, which initiates induction of apoptosis after cellular damage in a *TP53* dependent manner. Importantly, most *TP53* mutations result in impaired ASPP2 binding, which again results in abrogated induction of apoptosis.

Added value of this study

Studying the underlying mechanisms of deregulated ASPP2 function, we uncovered an oncogenic stress-inducible dominant-negative isoform, which is C-terminally truncated. Intriguingly, this results in loss of the TP53 binding sites – which in turn results in impaired induction of apoptosis.

Implication of all the available evidence

These observations have far reaching implications for the prediction of response to (chemo)therapy. Further, the discovered isoform defines a novel class of *dynamic stress-inducible oncogenes*, which facilitate the acquisition of gene/chromosomal abberations further driving oncogenesis.

Not surprisingly, inactivation of the TP53 pathway is a universal event in human cancers (reviewed by Vousden and Prives [4]) and *TP53* is one of the most highly mutated genes in human cancers with >50% of human malignancies harboring inactivating *TP53* mutations [5].

Interestingly, in most cases of de novo acute leukemias inactivating mutations of *TP53* or chromosomal aberrations of the long arm of chromosome 17 (locus of *TP53*) are uncommon [6,7]. Therefore, acute leukemias must inactivate the TP53 pathway by other means besides mutation. However, the molecular mechanisms that inactivate the TP53 pathway in acute leukemia remain unclear. We have evidence, that dysregulation of a family of TP53-binding proteins (Apoptosis Stimulating Proteins of TP53; ASPPs) may be involved in leukemogenesis:

Three members of the ASPP family exist so far: ASPP1 and ASPP2 (TP53BP2 or 53BP2L) bind to the TP*53* core domain and enhance DNA binding and transactivation function of TP53 on the promoters of selected pro-apoptotic target genes in vivo [8,9]. Inhibitory-(i)ASPP acts as an inhibitor of apoptosis by binding to an adjacent region of the core domain and the proline rich region of TP53 [10].

Co-crystallization of the C-terminus of ASPP2 with the TP53 core domain has revealed that many of the *TP53* hotspot mutations found in cancers disrupt the ASPP2 and TP53 interaction [11]. This suggests that ASPP2 may play an important role in tumor suppression and that attenuation of ASPP2 function might ultimately result in tumorigenesis. Consequently, targeting of ASPP2 in mouse models demonstrates that ASPP2 functions as a tumor suppressor [8,12].

Studies in different human tumor types have indeed suggested that attenuation/loss of ASPP2 expression is a common occurrence: Attenuated ASPP2 function was found in different tumors such as *TP53* wildtype breast cancer [13], lymphoma subtypes [14] and acute leukemia [9], where low *ASPP2* expression levels associated with biologically more aggressive disease, therapy failure and poor clinical outcome. These observations not only argue for a role in tumorigenesis – but for a role in therapy response via mediating induction of apoptosis. In this context, we have previously demonstrated that ASPP2 is damage-inducible by anthracyclines, and failure to do so leads to impaired induction of apoptosis in vitro [9,15].

Altered expression and function of ASPP2 in physiologic and tumor tissues is incompletely understood and involves complex mechanisms, such as promoter methylation [15–21].

Tantalizingly, additional mechanisms may also serve to alter ASPP2 function. *ASPP2* is subject to alternative splicing to generate a 1005 amino-acid isoform (BBP aka 53BP2S) that is less efficient in stimulating apoptosis [13,22] – implying that ASPP2 function may be controlled by the generation of different gene products from the *ASPP2* locus. Recently, an N-terminal truncated ASPP2 isoform (ΔN-ASPP2), generated by an alternative transcription start site, was found to be overexpressed in breast cancer, promote cellular proliferation, and inhibit apoptosis [23]. Indeed, the complex structure of the *ASPP2* allele suggests that there are likely additional gene products important for its function.

In an attempt to identify novel *ASPP2* gene products, we performed a systematic screen of the entire *ASPP2* transcript – and herein describe a novel stress-inducible splicing variant, named *ASPP2κ*, with high prevalence in acute leukemia. We will show, that ASPP2κ displays dominant-negative functions, implying a functional role in oncogenesis and therapy response.

2. Material and methods

2.1. Cell lines

The IL3-dependent mouse hematopoietic pro-B cell line Ba/F3 and the acute myelogenous leukemia cell line MOLM14 were generous gifts from Dr. Heinrich, OHSU, OR. The K562 cell line, derived from a patient with chronic myeloid leukemia in blast crisis, was obtained from the Leipniz Institute DSMZ, Germany. The human HMC-1.2 mastocytosis cell line was kindly provided by Dr. Akin (Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD). Cell lines were cultured in RPMI media containing 10% FBS.

The HCT116 metastatic human colon adenocarcinoma cell strains, containing wildtype TP53 (+/+) or a TP53-deleted derivative (−/−), were gifts from Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). Cell lines were cultured in DMEM media containing 10% FBS.

2.2. Ficoll hypaque extraction of physiologic or leukemic mononuclear cells

Bone marrow aspirate and peripheral blood samples from patients and healthy donors were collected in 5000 U heparin after informed consent and approval of the ethics committee of the University of Tübingen. Mononuclear cells were isolated by Ficoll Hypaque density gradient fractionation. For functional experiments, native ex vivo blasts were cultured in DMEM media containing 20% FBS [24].

2.3. Polymerase chain reaction (PCR)

mRNA was isolated and reversely transcribed (RT) using standard techniques following the RNeasy® RNA purification kit (Qiagen, Hilden, Germany). ASPP2 cDNA (exon 1-18) was sequencially amplified using the following primer pairs:

fragment 1 (amplicon bp1-143) 5′-ATGCGGTTCGGGTCCAAGATG ATGCCG-3′ (sense primer), 5′- CTTGGTCCACTCACAATGTCCC -3′ (antisense primer),

fragment 2 (amplicon bp313-856) 5′- CCAGTTGCGGATAATGAGCG -3′ (sense primer), 5′- CTACTTTTGACACAGCCAGGACG -3′ (antisense primer),

fragment 3 (amplicon bp1201-1752) 5′- ATCCAGTCGTCTACTATGC CTC-3′ (sense primer), 5′- AAGCCTGAATGACCAAGGAACC -3′ (antisense primer),

fragment 4 (amplicon bp750-1291) 5′- GGAACAGAAGAGACTAAGC AATGGG -3′ (sense primer), 5′- TGACAACTGCTGAGAACTTCCGTC -3′ (antisense primer),

fragment 5 (amplicon bp1599-2132) 5′- TATCTTGCGGGATGCT CAGG -3′ (sense primer), 5′- TCTGGGTGCTGCTGTTGATTC -3′ (antisense primer),

fragment 6 (amplicon bp1915-2465) 5′- CCTTCCAAAGACACCTTAC TTCCAC -3′ (sense primer), 5′- GATGGGTATGATGGGACAGAGATG -3′ (antisense primer),

fragment 7 (amplicon bp2364-2943) 5′- TACAGAGCCAGAGGGTCCT AATGG -3′ (sense primer), 5′- TAGCAGTAAAGCAAGGGGGTTG -3′ (antisense primer),

fragment 8 (amplicon bp2864-3436) 5′- GGACAAACTTGCGTAAAAC TGGC -3′ (sense primer), 5′- CGATTTCATCTTCGTCTTCCCTG -3′ (antisense primer),

fragment 9 (amplicon bp3347-3537) 5′- GGGATTATGAACCTCAGAA TG -3′ (sense primer), 5′- TCAGGCCAAGCTCCTTTGTCT -3′ (antisense primer),

fragment 8 (WT)-nested (including exon 17) 5′- TGAAATTCAACCCC CTTGC – 3′ (sense primer), 5′ ACTCCACCAAAAACTTACACAC -3′,

fragment 8 (kappa)-nested (excluding exon 17) 5′- AAGGCATCACG GCTCTTCAC -3′ (sense primer), 5′ CTTCTTTCATGGGCAGCTCATC -3′.

PCR amplification was performed using 500 ng of cDNA [25], or 1 μl of amplicon product for nested PCR.

2.4. Sequencing

Bidirectional Sanger sequencing was performed after amplicon purification and sequences were analyzed using Chromas software.

Templates of the wildtype sequence of ASPP2 gDNA, cDNA, peptide sequences, reading frames and restriction sites were generated using NCBI gene data banking and pDraw software.

Original Sanger sequencing data of *ASPP2κ* can be found online at https://ncbi.nlm.nih.gov/genbank/

2.5. qRT-PCR

Quantitative RT-PCR methodology was used according to manufacturer protocols. Isoform-specific primers detecting the transcript fusion site of the specific splice variant have been established in our lab. To further increase specificity, restriction digest may be applied prior to PCR amplification using restriction enzymes targeting motifs specifically occuring in exon 17 - which is spliced out in the newly described splice variant - to digest the wildtype isoform but leaving the ASPP2κ isoform unaffected. ASPP2 mRNA expression levels, relative to GAPDH as the housekeeping gene, were determined by qRT-PCR Roche® LightCycler Technology (Roche, Basel, Switzerland) [15].

2.6. Cell sort

CD34+ positive leukemia blasts were isolated using standard protocols on a FACSAria assembly.

2.7. Restriction digest

pDraw32 software was used to compute for potential isoform-specific restriction motifs to specifically digest the wildtype cDNA isoform. Two motifs targeting *Hpy*CH4IV or *Tsp*509I restriction enzymes were identified. Restriction digest was set up prior to RT-PCR for 3 h at 37°C and stopped at 65°C for 20 min.

2.8. Development of isoform-specific antibodies

Immunization of rabbits with isoform-specific peptides targeting the fusion epitope was custom made by a commercial supplier (Biogenes, Berlin, Germany). Specificity of the unique fusion epitope was confirmed by BlastN search.

2.9. Immunoprecipitation and immunoblotting

Protein cell lysates were used for immunoprecipitation experiments using ASPP2κ-specific antibodies incubated over night. After microbead capturing immunoblots to target ASPP2 WT was performed as described before [24,26].

2.10. Immunohistochemistry

In Paraffin-embedded EDTA-decalcified bone marrow biopsies, the immunohistochemistry was performed in a dilution of 1:500 using an automated immunostainer (Ventana BenchMark ULTRA IHC/ISH staining module, Ventana Medical Systems, Tuscon, USA) with the U OptiView DAB IHC v5 procedure according to the manufacturer's protocols. Slides were evaluated on a Zeiss Axioscope 40.

2.11. Flow cytometry based (intracellular) protein expression

Cells were fixed and permeabilisized using the Fix & Perm® Fixation and Permeabilisation kit (Invitrogen). The unlabeled primary antibody was added in a 1:1000 dilution to the cell suspension and incubated for 1 h at room temperature followed by rinsing and resuspension of cells. Fluorescent dye-conjugated (AlexaFluor®) secondary antibody was added in a 1:10000 dilution and cells were incubated for 30 min at room temperature. After rinsing and resuspension, protein expression levels were assayed using a FACScalibur® flow cytometer loaded with CellQuest® analysis software (BD, Heidelberg, Germany).

2.12. Induction of apoptosis

Determination of cellular integrity after successful transfection of ASPP2κ was performed by analysis of induction of apoptosis, which was measured in an Annexin V-based assay (Immunotech, Marseilles, France) [26,27] targeting phosphatidylserine. Translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane accounts for an early indicator of apoptosis.

2.13. Cytogenetic analysis

Cytogenetic analysis was performed using standard protocols: In short, cells were cultured and treated with KaryoMAX colcemid (Life Technologies), hypotonic KCl-solution and harvested in fresh fixative containing 25% acetic acid and 75% methanol. Karyotyping was performed on G-banded metaphase chromosomes.

2.14. Irradiation

γ-irradiation as a cellular stressor was performed at 5Gy (Kampa et al., PNAS 2009). These experiments were performed in cooperation with the physics section of the Department of Radiooncology (University of Tübingen).

2.15. Isoform-specific knock-down

Specific ASPP2κ siRNA constructs were developed to be used with standard lipofection transfection techniques following the manufacturer protocols.

The constructs or a scrambled siRNA control were lipofected into leukemia cell lines or freshly harvested native leukemia cells using a lipofectamine 2000®-based protocol (Invitrogen, CA). Briefly, 5×10^5 cells/well were plated in a 24-well plate and cultured in medium containing serum without antibiotics. Constructs are diluted in serum-free medium and mixed with a lipofectamine. The mix is then added to the cell suspension followed by incubation at 37 °C. Transfection efficacy was independently validated by siGLO transfection indicator (Dharmacon).

2.16. Cloning and transfection/transduction of specific ASPP isoforms in defined cell models

ASPP2κ cDNA was custom made (Eurofins, D) encoding for the peptide sequence of the *ASPP2κ* splicing variant and cloned into a HisMax-based plasmid vector to be transfected into the target cell model (Ba/F3 cells) using lipofection according to manufacturer protocols. After successful transfection as determined by Xpress epitope detection and definite inhibition of the specific ASPP2 isoform in an immunoblot, IL3 weaning was used to evaluate whether the transfected gene of interest is capable to uphold viability pathways after withdrawal of an obligate growth stimulus – arguing for oncogenic potential.

2.17. Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

3. Results

3.1. Identification of a highly prevalent, exon-skipping splicing variant of ASPP2, ASPP2κ, in native acute leukemia samples

To identify novel ASPP2 mutations or transcripts that could be clinically important for regulating ASPP2 function, we set up a screen using mononuclear cell isolates derived from 30 patient samples with confirmed diagnosis of acute myeloid ($n = 25$) or lymphoid ($n = 5$) leukemia.

cDNA, spanning exon 1-18, was analyzed by Sanger sequencing using primer pairs amplifying 9 overlapping fragments (ref. *Material and methods, 2.3*).

No novel missense pointmutations were identified in any of the amplified fragments. However, amplification of fragment 8 (spanning exon 14-18) revealed a C-terminal deletion mutation, in ten patients, which fused mRNA transcripts at base pairs (bp) 3289 (corresponding to bp53554 in gDNA) with bp3457 (bp61659, gDNA).

This site marks a fusion of end of exon 16 with start of exon 18, therefore highly suggesting an exon-skipping splicing variant, which we named *ASPP2κ* (Fig. 1a-c).

To exclude genomic deletion, quantitative (q)PCR was set up to measure relative gDNA content generated from amplicons 5 prime (exon 15 and following intron), and 3 prime (exon 17 and following intron), from the fusion site. Both amplicons displayed similar relative expression levels in several patient samples tested (Fig. 1d) confirming aberrant splicing rather than a gDNA deletion.

Since *ASPP2κ* would be predicted to have aberrant function, we wished to further test its prevalence in human cancers. We therefore performed an extended screen of 100 blood samples derived from patients with hematologic neoplasms or healthy blood or bone marrow donors (mostly AML (bm 31, pb 42), but also ALL/AUL (bm 5, pb 3),

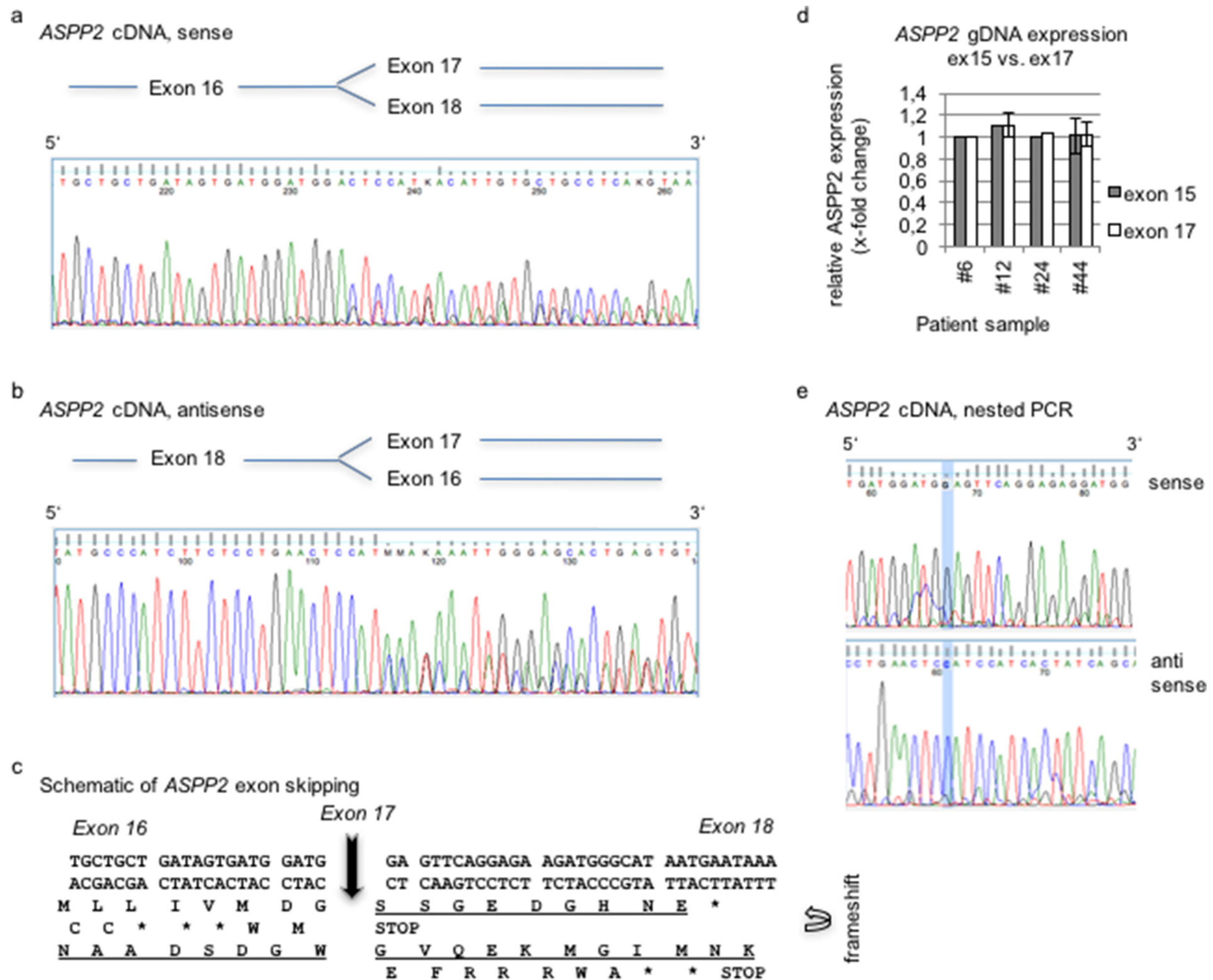

Fig. 1. Identification of a C-terminally truncated exon-skipping splicing variant of *ASPP2.* (a, b) A systematic mRNA screen reveals a novel mutation fusing exon 16 with exon 18. Bi-directional Sanger sequencing is shown. (c) Schematic of exon skipping indicating a reading frame shift followed by an early STOP if translated. (d) Relative quantification of gDNA expression to compare exon 15 with exon 17 levels excludes genomic deletion (decrease of exon 17) rather than splicing variance via exon skipping (no change of exon 17 expression on the genomic level). SDC4 served as housekeeping gene. Patient samples #6, #24 and #44 harbor the wildtype isoforms of ASPP2, patient sample #12 harbors the truncated splicing variant (as validated by bi-directional Sanger sequencing). Data are mean ± SEM. (e) Nested RT-PCR approach on CD34+ isolated leukemia blasts verifies expression of *ASPP2κ* in leukemia.

CML/CMML [6], lymphoma and B-CLL (6, including the DOHH-2 cell line) and healthy blood [3] or bone marrow [4] donors). We identified *ASPP2κ* in 55 of 90 (61%) evaluable patients (10 samples with poor quality material were excluded from the analysis).

Specifically, we found *ASPP2κ* in: AML-bm 57,7% (15/26, 5 samples w/o result), AML-pb 70% (28/40, 2 samples w/o result), ALL-bm 40% (2/5, 1 sample w/o result), ALL-pB 33,3% (1/3), CML/CMML 75% (3/4, 2 samples w/o result), B-CLL/T-NHL 83,3% (5/6). In contrast, we did not detect *ASPP2κ* in blood or bone marrow from healthy donors. These data suggest a high prevalence of the *ASPP2κ* splicing variant in acute leukemias as well as in other hematologic malignancies.

Notably, no other aberrant splicing variants, which were predicted or reported in previous screens in various tissues, were detected in our patient cohort – with the exception of the identification of two additional C-terminally truncating splicing variants of ASPP2 in two leukemia patients fusing exon 15 with exon 18 (*ASPP2μ*), respectively fusing exon 16 with mid of exon 18 (*ASPP2λ*). Both variants again result in a frame-shift resulting in a premature stop codon – similar to *ASPP2κ* (not shown). In this report, we concentrate on the prevalent splicing variant *ASPP2κ*, since the functional consequences for the rare splicing events are predicted to be similar.

3.2. Validation of ASPP2κ expression in CD34+ leukemia stem cells

To validate the presence of the *ASPP2κ* splicing variant in early human CD34+ leukemia blasts, we performed a cell sort to detect *ASPP2κ*-expression in fresh human leukemia blasts. Using a nested RT-PCR approach spanning the fragment 8 amplicon (ref. *Material and methods, 2.3*) and bi-directional Sanger sequencing, we confirmed presence of *ASPP2κ* along with the ASPP2 wildtype isoform (supplemental fig. S1). Interestingly, in one patient, only the exon-skipping *ASPP2κ* splicing variant was found whereas no ASPP2 WT was detected in the CD34+ isolated leukemia subpopulation (Fig. 1e, compare with schematic provided with Fig. 1c). These findings may point to a functional role of the truncated *ASPP2κ* splicing variant in leukemogenesis.

3.3. Validation of the fusion product using an isoform-specific PCR approach

To further confirm alternative splicing via exon skipping, we designed an isoform-specific RT-PCR assay to distinguish wildtype (WT) *ASPP2* from *ASPP2κ* isoforms:

WT-specific primer pairs, located within exon 17 (missing in *ASPP2κ*), were designed to detect WT *ASPP2* amplicon (Fig. 2a). A non-specific nested RT-PCR using an antisense exon 18 primer is shown in Fig. 2b. Additionally, we designed an *ASPP2κ*-isoform specific RT-PCR assay uniquely recognizing the fusion site (antisense primer in exon 18 and the sense primer located in exon 15) (Fig. 2c/d).

We further validated the *ASPP2κ* qRT-PCR assay, using restriction enzymes to specifically digest the *ASPP2* WT isoform. pDraw32 software identified isoform-specific restriction motifs not present in *ASPP2* cDNA (Fig. 3a).

After reverse transcription and digestion with either *Hpy*CH4IV or *Tsp*509I prior to PCR amplification (fragment 8, compare *Material and methods 2.3*), HpyCH4IV but not Tsp509I digestion cleaved *ASPP2κ* as

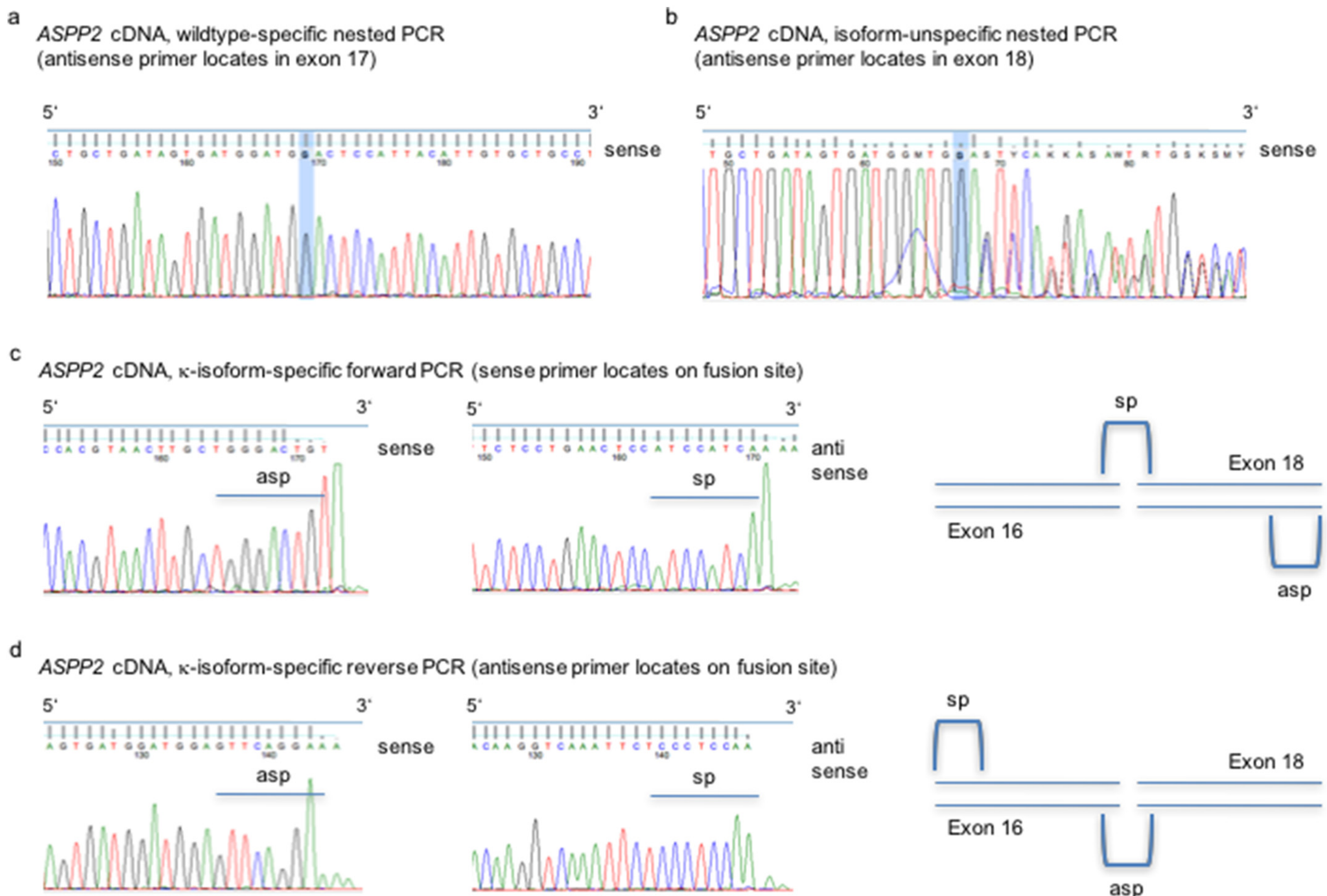

Fig. 2. Isoform-specific RT-PCR. (a, b) Isoform-specific nested RT-PCR segregates *ASPP2* WT (a) compared to isoform-unspecific nested RT-PCR (b) overlapping the wildtype sequence with the exon-skipping splice variant. Sanger sequences in sense direction are provided; compare schematic provided with Fig. 1c. (c, d) *ASPP2κ* isoform-specifc RT-PCR approach uniquely detecting the exon16/18 fusion site in sense (c) or antisense (d) direction. Bi-directional sequencing of the primer sites is provided. Left panels: sense direction, middle panels: antisense direction. Right panels provide a schematic of the primer design. Sp, sense primer; asp, antisense primer.

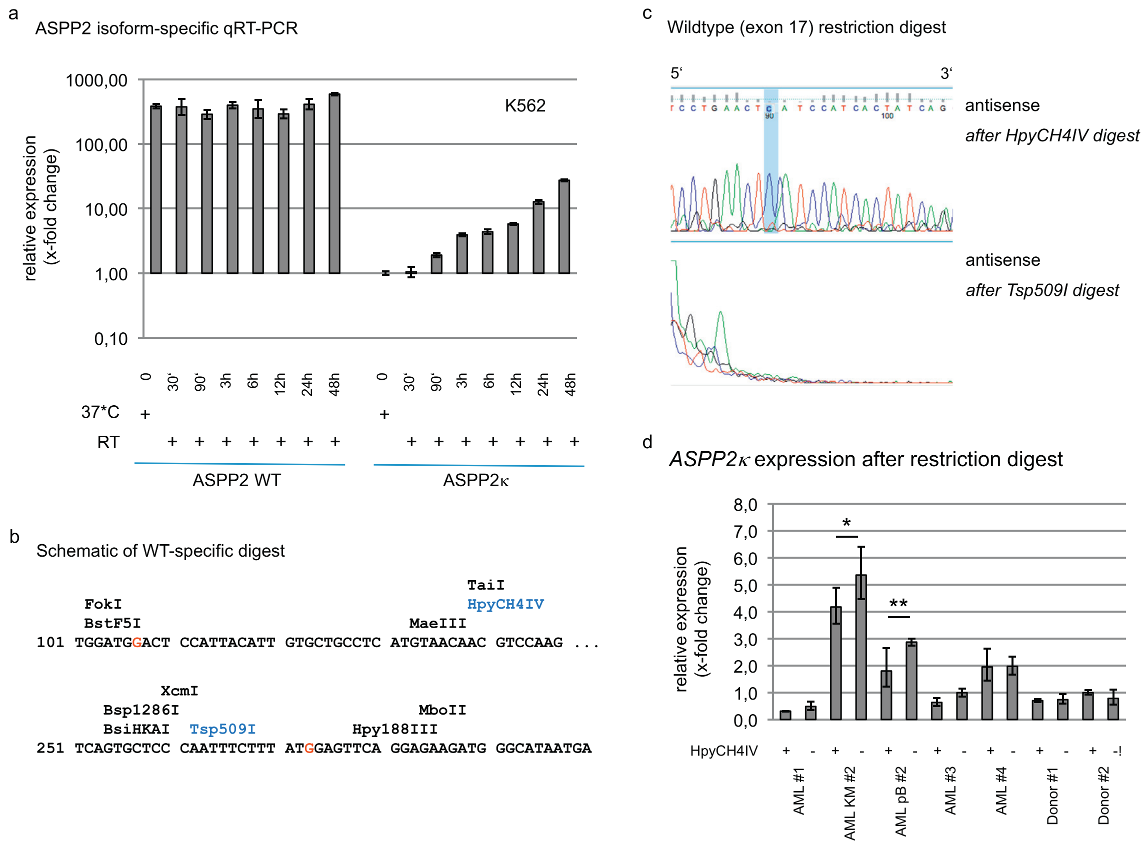

Fig. 3. Isoform-specific quantitative (q)RT-PCR. (a) Successful design of isoform-specific primer pairs to use in a qRT-PCR approach clearly distinguishes the *ASPP2* WT from the *ASPP2κ* splice variant. In contrast to the WT isoform, *ASPP2κ* is stress-inducible by changing culture conditions (RT, cultured at room temperature). Data are mean ± SEM. (b) Schematic of WT (exon 17)-specific digest sites. Red letters mark start/end of exon 17. Exon 17-specific restriction enzymes used for analysis are marked in blue. (c) An isoform-unspecific RT-PCR using fragment 8 primers was set up after restriction digest with either *Hpy*CH4IV or *Tsp*509I. Only HpyCH4IV enzyme digest proved to be effective – leaving the ASPP2κ isoform unaffected as demonstrated by Sanger sequencing (upper panel). Ineffective digest using Tsp509I (lower panel). (d) Optimized *ASPP2κ*-specific qRT-PCR approach including restriction digest of exon 17 (WT isoform) demonstrates high specificity of the qRT-PCR primer set – with only moderate improvement after pre-digest of the WT isoform. (R) restriction digest sample. Bm, bone marrow; pb, peripheral blood; donor, bone marrow donor sample. Data are mean ± SEM. *,** not statistically significant ($p > 0.05$, Student's *t*-test).

predicted (Fig. 3b). To exclude non-specific binding of the *ASPP2κ*-specific qRT-PCR primer set, qRT-PCR with or without prior wildtype-specific HpyCH4IV restriction site digest was performed on native cell samples derived from patients with AML or healthy bone marrow donors. As shown in Fig. 3c, this confirmed (a) preferential expression of the *ASPP2κ* isoforms in leukemia blasts compared to physiologic mononuclear cells, (b) high specificity of the qRT-PCR assay to detect the *ASPP2κ* splicing variant.

To further evaluate the robustness of our optimized qRT-PCR assay, we confirmed presence of the *ASPP2κ* splicing variant in native leukemia cells and various tumor cell lines, including those derived from solid tumor neoplasms (supplemental fig. S2a), which points to a potential role of *ASPP2κ* beyond leukemia in hematologic as well as solid tumors.

Since expression of *ASPP2* WT is inducible upon cellular stressors such as DNA damaging agents or radiation [9,15,28], we wished to quantify if *ASPP2κ* expression changed in response to cellular stress. We therefore performed quantitative (q)RT-PCR specific for *ASPP2κ* in two leukemia cell line models (K562, MOLM14) before and after treatment with either gamma irradiation or cytarabine (Fig. 3d).

To assess if *ASPP2κ* expression after cell stress was physiological and occurred in vivo in patients, we quantified *ASPP2κ*-expression using our qRT-PCR assay in two patients with AML undergoing induction chemotherapy. Samples at diagnosis and after 1st induction chemotherapy were analyzed. Intriguingly, we found persistent elevated *ASPP2κ* levels in AML blasts from a patient refractory to induction chemotherapy; in contrast, *ASPP2κ* levels declined in a patient who had a complete response to induction chemotherapy. This observation suggests that *ASPP2κ* expression is specific in leukemia blasts (supplemental fig. S2b).

3.4. ASPP2κ translates into a genuine truncated protein isoform

Since *ASPP2κ* mRNA expression is physiologic, we next wished to confirm ASPP2κ protein expression. The sequence of *ASPP2κ* with a premature stop codon (Fig. 1c), predicts the generation of a C-terminally truncated protein isoform. This is of interest as the C-terminus of ASPP2 WT harbors the TP53 contact sites (Fig. 4a); thus ASPP2κ protein would be predicted to be attenuated in promoting TP53 binding and TP53-mediated apoptosis compared to ASPP2 WT [11]).

To specifically detect ASPP2κ protein, we generated isoform-specific rabbit antibodies, targeting the putative unique fusion-site of the ASPP2κ-specific epitope (as confirmed by BlastN search), (compare Fig. 1c).

We performed immunoprecipitation using ASPP2κ-specific antibodies on lysates from three patients with AML. Western blotting was then performed with an N-terminal ASPP2 antibody (Fig. 4b). We found an ASPP2 immunoreactive band at 111 KDa, which is the predicted size of ASPP2κ (Fig. 4c). In contrast, we did not detect this band in healthy bone marrow donors. Of note, it is well described for many commercially available N-terminal ASPP2 antibodies, that – besides ASPP2 – a "non-specific" band is shown at −110 KDa. Our data suggest, that this band may represent the ASPP2κ isoform (supplemental fig. S3).

We additionaly tested our ASPP2κ specific antibiodies on immunohistochemistry of a paraffin-embedded bone marrow trepanates from

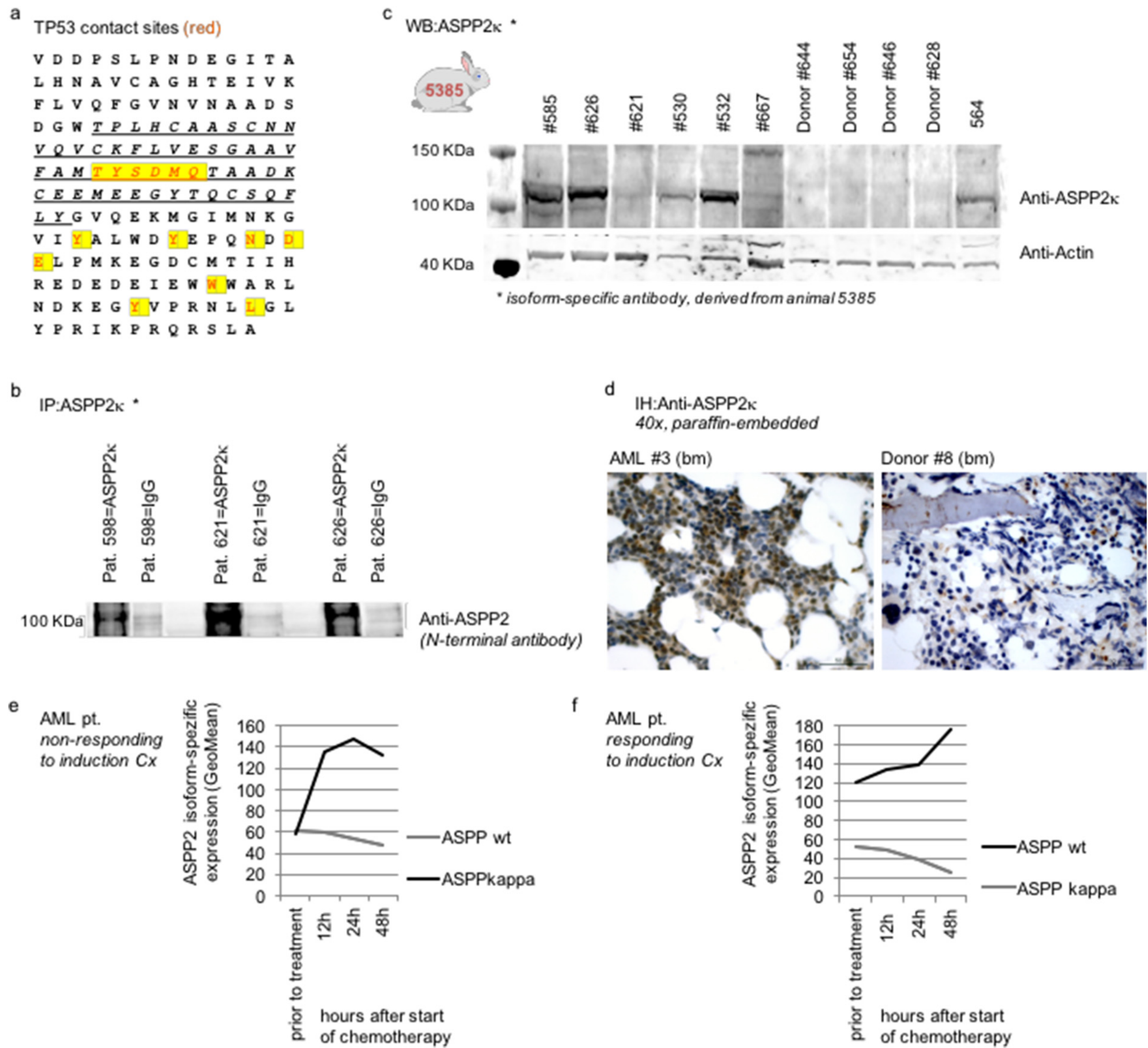

Fig. 4. Identification of a C-truncated protein splicing variant of ASPP2. (a) Schematic of the wildtype ASPP2 amino acid sequence (corresponding to exon 16, exon 17 and exon 18/19) and the TP53 binding sites (in red) – which are lost in the truncated splice variant. (b) Immunoprecipitation (IP) using ASPP2κ-specific antibodies in lysates derived from three acute leukemia samples and probed with N-terminal ASPP2 antibodies confirm genuine translation of an ASPP2κ-protein isoform at ~110 KDa (anticipated size 111 KDa). IP using unspecific polyclonal IgG antibodies served as negative controls. (c) ASPP2κ is specifically detected in acute leukemia samples compared to mononuclear cells extracted from healthy bone marrow donors. A representative western immunoblot (of 5 separate experiments) using equally loaded whole cell lysates of patient samples and donors is shown. Actin served as loading control. (d) Immunohistochemistry analysis using anti-ASPP2κ specific antibodies confirms cytosolic expression of ASPP2κ in acute leukemia bone marrow biopsies. A representative sample is shown (left panel). Bone marrow of a healthy donor serves as negative control (right panel). (e, f) Dynamic monitoring of ASPP2 isoform expression levels to predict therapy response: ASPP2 wildtype and ASPP2κ isoform protein expression levels were analyzed in newly diagnosed patients with acute myeloid leukemia undergoing induction chemotherapy. A monoclonal C-terminal antibody (A480, Sigma) detecting full-length (wildtype) ASPP2 and an ASPP2κ isoform-specific antibody were used. (e) non-responsive patient (i.e. blast persistence) after one cycle of induction chemotherapy; (f) therapy responsive patient (i.e. complete remission after one cycle of induction chemotherapy).

patient samples with AML – further confirming genuine in vivo expression of ASPP2κ (Fig. 4d).

Since we found that *ASPP2κ* mRNA expression is inducible upon response to cellular stressors such as gamma irradiation or chemotherapy (compare Fig. 3d), we next determined whether ASPP2κ protein expression is stress-inducible in vivo: ASPP2κ-specific or C-terminal ASPP2 WT antibodies were used to monitor ASPP2κ and ASPP2 WT protein expression levels, as quantified by FACS flow cytometry, in two patients with AML undergoing induction chemotherapy.

Interestingly, we found upregulation of ASPP2κ protein within the first hours of therapy in a patient with refractory disease (Fig. 4e) – whereas a patient achieving complete remission showed decreasing levels of ASPP2κ and increasing levels of the proapoptotic WT isoform at the same time points (Fig. 4f).

These findings are noteworthy as monitoring dynamic expression levels of ASSP2 isoforms may provide a novel early marker to predict response towards chemotherapy.

3.5. ASPP2κ has dominant-negative apoptotic functions

To shed light into the functions of the ASPP2κ isoform, we have cloned the cDNA into a pcDNA4/HisMax/LacZ vector in order to express ASPP2κ in the hematopoietic IL3-dependent murine pro B-cell line Ba/F3. The Ba/F3 cell model is broadly used to evaluate the tumorigenic potential of expressed genes to render cells IL3-factor independent – a sign of autonomous, oncogenic cellular proliferation [25]. Succesful transfection and expression was confirmed using the Xpress-epitope tagged-β-galactosidase (LacZ) and ASPP2κ specific antibodies, as demonstrated in Fig. 5a/b.

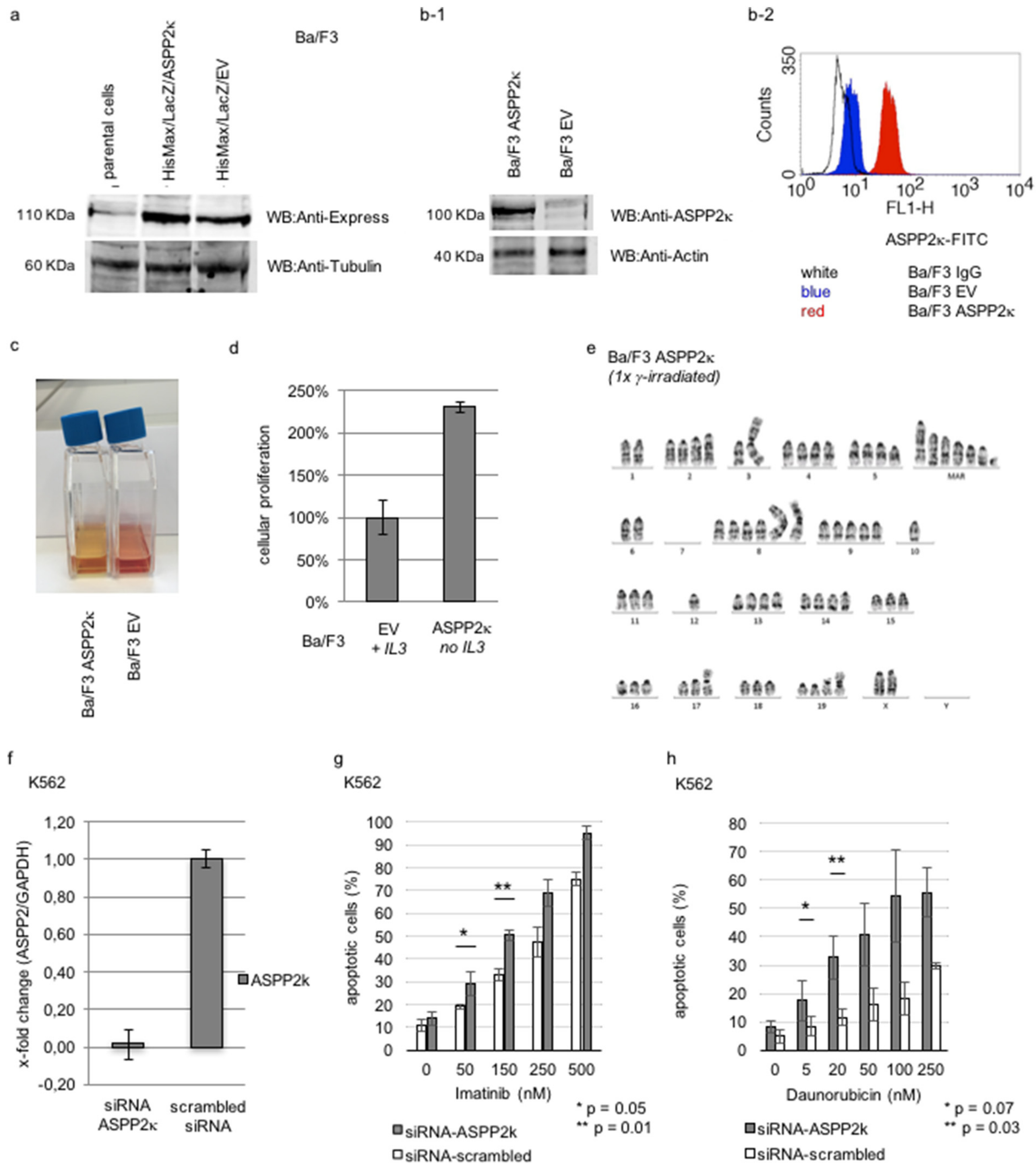

Fig. 5. Forced expression of ASPP2κ perturbs oncogenic transformation. (a)*Transfection control*: Successful transfection of a pcDNA4/HisMax/LacZ vector (+/− encoding the *ASPP2κ* isoform) into the hematopoietic pro-B Ba/F3 cell line as verified by Xpress-epitope tagged to β-galactosidase (LacZ) using specific antibodies. (b) *Expression control:* Western immunoblot of whole cell lysates *(b-1)* or intracellular flow cytometry stain *(b-2)* of Zeocin-selected Ba/F3 transfectants with ASPP2κ-specific antibodies confirm selective upregulation of ASPP2κ. (c) Change of color of cell culture medium from red to yellow is highly suggestive for outgrowth of a growth-factor independent clone after IL3 weaning. The empty vector controls did not survive IL3 weaning (microscopic validation). (d) Engineered overexpression of ASPP2κ in Ba/F3 cells leads to increase of proliferation rates compared to cells transfected with an empty vector control. Data are mean ± SEM of three independent experiments. (e) Karyogram of IL3-weaned and 1 ×g-irradiated cells is shown demonstrating poyploidity, loss of chromosomes and addition of marker chromosomes. (f-h) ASPP2κ has a negative dominant activity abrogating the proapoptotic effect of the wildtype isoforms. Successful establishment of *ASPP2κ*-specific siRNA knock down (f). ASPP2κ-interference increases proapoptotic activity in BCR-ABL1 positive K562 cells treated with imatinib (g), resp. daunorubicin (h). Data are mean ± SEM. Statistical significance at $p < 0.05$ (Student's t-test).

After withdrawal of IL3, the parental Ba/F3 negative controls did not survive. In contrast, Ba/F3 cells with forced expression of ASPP2κ lost IL3-dependency and survived without growth factor supplementation (Fig. 5c). We also found increased cellular proliferation rates of IL3-independent Ba/F3 strains expressing the ASPP2κ isoform compared to Ba/F3 parental cells cultured in IL3-supplemented media (Fig. 5d). Given our findings that ASPP2κ is overexpressed in native leukemia blasts, these findings stongly suggest that ASPP2κ promotes malignant transformation.

As ASPP2κ lacks the TP53-binding site, this may result in impaired TP53-mediated apoptosis and attenuated cellular repair mechanisms. To shed insight into this possibility, ASPP2κ-transfected Ba/F3 cells were IL3-weaned and γ-irradiated (1 × 5 Gy) and monitored for IL3-independent growth. IL3-dependent parental Ba/F3 cells (with and

without γ-irradiation) served as negative controls. Cytogenetic analyses were performed on IL3-independently proliferating clones after radiation.

Parental as well as empty vector control cell strains did not survive IL3-weaning. In contrast, Ba/F3-ASPP2κ cell strains survived IL3-weaning as well as γ-irradiation – arguing for ASPP2κ promoted malignant transformation. In line, cytogenetics demonstrated chromosomal instability (CIN) with loss of chromosomes and addition of marker chromosomes (Fig. 5e and supplemental table S1 for all experiments). Notably, lentiviral knock-down of total-*ASPP2* reveals similar effects compared to the ASPP2κ cell strains – arguing fo a dominant-negative function of ASPP2κ.

Since enforced ASPP2κ expression promoted proliferation and inhibited damage-induced cell death, we reasoned that attenuation of ASPP2κ expression would inhibit cell viability. We therefore developed a specific siRNA-based lipofection assay to selectively target the novel fusion site of the *ASPP2κ* isoform (compare Fig. 1c). Using the *BCR-ABL1* positive K562 leukemia model, for which we have shown stress-dependent induction of *ASPP2κ* (compare Fig. 3d), cells were lipofected with specific *ASPP2κ*-siRNA or a scrambled negative control and then treated either with the *BCR-ABL1*-directed tyrosine kinase inhibitor imatinib mesylate or a DNA-damaging anthracycline. Successful knock down of *ASPP2κ* transcription is provided with Fig. 5f. Consequently, ASPP2κ-specific knock-down leads to restoration of proapoptotic capacities: K562 cells treated with imatinib in a dose-dilution assay were more prone to induction of apoptosis when *ASPP2κ* was silenced – compared to cell strains treated with a scrambled siRNA control. Similarly, dose-escalating treatment of K562 cells with daunorubicin showed even more pronounced rescue of proapoptotic efficacies in *ASPP2κ*-interferenced cell strains compared to the control strains (Fig. 5g/h).

Together, these data highly support findings that *ASPP2κ* has converse function of full-length tumor-suppressing proapoptoic ASPP2 [12].

3.6. Validation of functional data and demonstration of TP53-dependence in an independent ASPP2κ solid tumor model

As we pointed out earlier, aberrant splicing of ASPP2 may be more prevalent in human cancers and also affect solid tumors (compare supplemental fig. S2a).

To challenge our functional data obtained in the leukemia setting, we obtained an isogenic *TP53*+/+ vs. *TP53*−/− colorectal cancer tumor cell line model. Besides evaluation of proapoptotic efficacy of cytotoxic agents in dependence *ASPP2κ* expression, this tumor model further allows to evaluate whether functional activity of *ASPP2κ* depends on the presence of *TP53.*

Lipofection experiments were performed as described above to either induce enforced *ASPP2κ* expression – or to knock-down basal *ASPP2κ* (successful transfection provided with Fig. 6a).

Cells were exposed to DNA damaging agents (oxaliplatin or irinotecan) and induction of apoptosis was followed for the transfected strains compared to untreated controls. Fig. 6b-e demonstrates that forced expression of *ASPP2κ* attenuates induction of apoptosis in *TP*53 +/+ cells – but apoptotic efficacy remains mainly unaffected in a *TP53*−/− background.

Consistent with this, *ASPP2κ*-knockdown restores induction of apoptosis in *TP53*+/+ cells, whereas *TP53*−/− strains remain largely unaffected (Fig. 6f-i).

Together, these data suggest that ASPP2κ functions at least in part through a TP53-dependent mechanism. Further, this model argues for a universal role of *ASPP2κ* beyond hematologic malignancies, which deserves further expolaration in future studies.

4. Discussion

Although ASPP2 functions as a tumor suppressor (as reviewed by Kampa, Sullivan, Li and colleagues [8,12,16,29] – and references within), precisely how it does so and how it is regulated remains unclear. The role of ASPP2 in human acute leukemia has important prognostic and therapeutic implications that are not well understood. Our discovery of *ASPP2κ* now provides significant new insight into understanding the complex regulation and function of ASPP2 in human leukemia and malignancies in general.

We demonstrate that the *ASPP2κ* isoform is generated by alternative splicing that truncates the C-terminal TP53 binding domain. Moreover, *ASPP2κ* is frequently expressed in acute leukemia blasts – but is also detected in other hematologic and solid tumors, implicating a common role in human cancer. Our methods to specifically detect the *ASPP2κ* isoform separately from WT ASPP2 are important for the design of future studies: Prior studies exploring *ASPP2* mRNA expression in human cancer did not segregate *ASPP2* WT and *ASPP2κ* isoforms, which may have confounded robust interpretation of these clinical datasets.

Our data demonstrate that ASPP2κ promotes cellular proliferation and inhibits damage-induced apoptosis – as confirmed by enforced expression as well as siRNA knockdown of *ASPP2κ.* Since *ASPP2κ* is specifically expressed in early leukemic progenitor blasts – and higher expression correlates with in vivo resistance to chemotherapy induction, our findings suggest, that *ASPP2κ* exerts a role as a pro-leukemogenic isoform. Importantly, we functionally confirmed that ASPP2κ expression inhibits therapy-induced apoptosis in vitro as well as promotes growth factor independent cell growth and proliferation.

Since *ASPP2κ* is missing the C-terminal TP53 binding domain, which has been described to be fundamental for ASPP2 WT pro-apoptotic function by Ahn et al. [30] or as reviewed by Rotem and colleagues [31], it seems likely that the anti-apotoic and pro-proliferative functions of ASPP2κ may be the consequence of disrupted binding to TP53. It implies further, that high levels of ASPP2κ may serve to antagonize the ASPP2-TP53 axis that normally promotes TP53-mediated apoptosis and growth arrest. This notion is supported by our data demonstrating that enforced ASPP2κ expression inhibits chemotherapy-induced apoptosis more profoundly in *TP53*+/+ cells then in isogenic *TP53*−/− cells.

Consistent with these findings, *ASPP2κ* specific knock down demonstrates increased sensitivity to chemotherapy-induced apoptosis in *TP53*+/+ but not *TP53*−/− isogenic cells lines. Given that human leukemias harbor a low frequency of *TP53* mutations, this suggests that the *ASPP2κ* isoform may be an important mechanism for suppressing the TP53 pathway and promoting a more aggressive disease with resistance to induction chemotherapy and resultant poor clinical outcome. The mechanisms of how exactly *ASPP2κ* inhibits the TP53 pathway remain unknown and are subject to ongoing research. One possibilitiy is that ASPP2κ may bind ASPP2 – therefore inhibiting ASPP2 interaction with TP53 to promote apoptosis. This potential ASPP2κ-ASPP2 interaction is supported by structural studies, predicting ASPP2-ASPP2 associations and seems feasible since ASPP2κ maintains the putative interaction domains [32,33]. It may also be possible that ASPP2κ has unique interactions with other proteins that could inhibit TP53 function independently. In this context, it is noteworthy that anti-apoptotic BCL-2 and BCL-XL are known direct interaction partners of the wildtype isoform of ASPP2 [34] – but binding sites are lost in ASPP2κ. In the rise of clinical importance of BCL-2/XL inhibitors, the ASPP-BCL interactions may be of special interest with regard to definition of biomarkers and trial design – and are under active investigation.

Interestingly, we found that *ASPP2κ* is stress-inducible. Moreover, *ASPP2κ* induction was observed in AML patients not responding to induction chemotherapy. Since we have demonstrated that increased ASPP2κ expression inhibits apoptosis, this suggests that alternative splicing of *ASPP2* is a potential mechanism for promoting resistance to therapy.

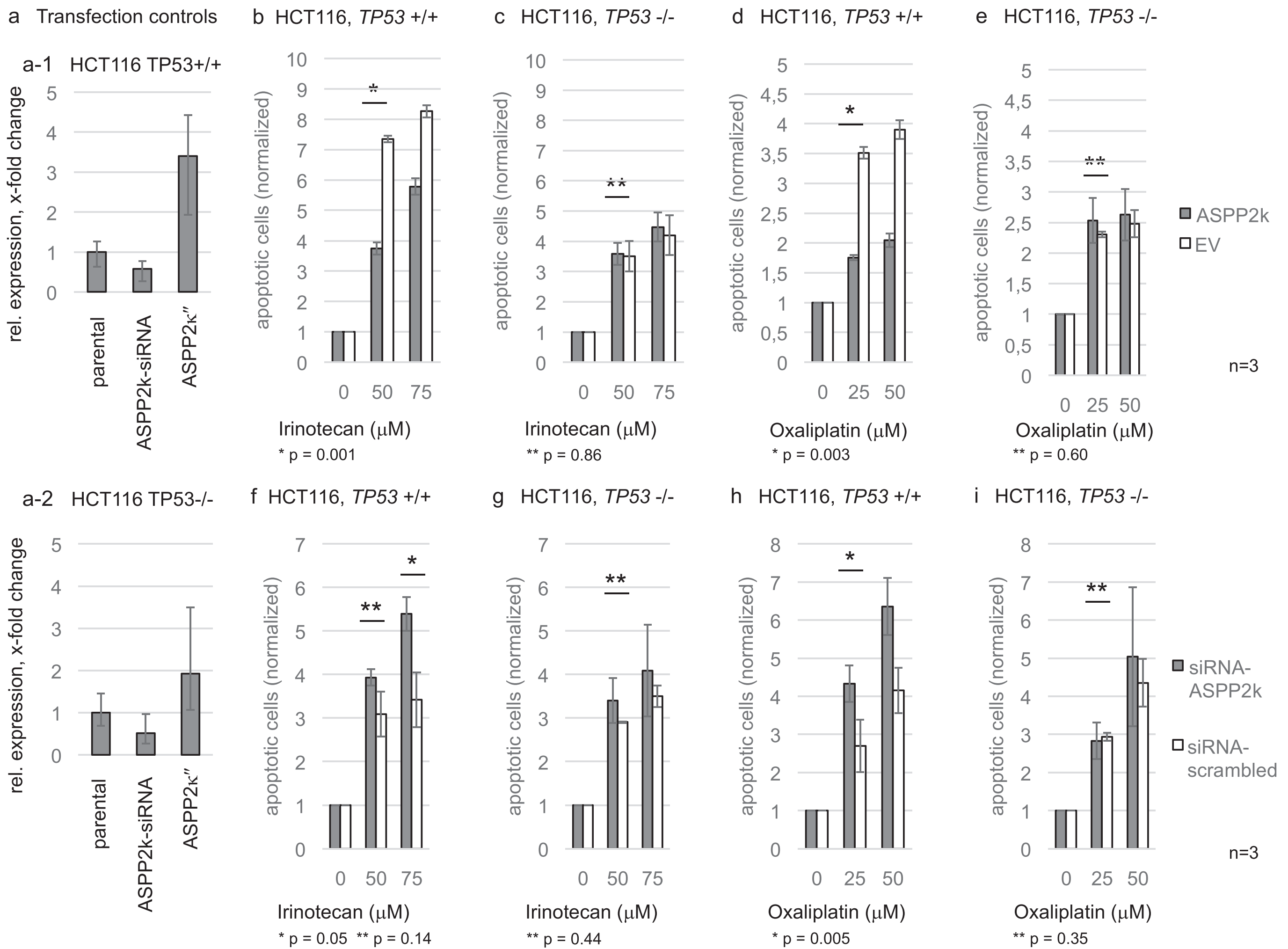

Fig. 6. *ASPP2κ* displays TP53-dependent functions. (a) *Transfection controls*: Successful transfection of a pcDNA4/HisMax/LacZ vector (encoding the ASPP2κ isoform) or ASPP2κ-specific siRNA knock down in HCT116 TP53+/+ *(a-1)*, resp. TP53−/− *(a-2)* cell strains is provided (qRT-PCR, GAPD house keeping gene). Induction of apoptosis in response to oxaliplatin or irinotecan in dependence of TP53 status and forced *ASPP2κ* (b-e), respectively *ASPP2κ*-interferenced (f-i) expression. Data are mean ± SEM. ** not statistically significant ($p > 0.05$, Student's *t*-test)

In addition, ASPP2κ promotes perturbed cellular proliferation, genomic alterations and chromosomal instability with acquisition of marker chromosomes, indicating oncogenic transformation. As ASPP2κ is stress-inducible, our findings may have far reaching consequences, arguing for alternative splicing of *ASPP2* as a dynamic mechanism in inactivating the TP53 pathway – independently of genomic mutations.

Noteworthy, it was shown that marker chromosomes are found in high-risk AML, associating with poor prognosis and therapy failure [35].

Prior studies demonstrate that *ASPP2* expression is suppressed in cancer [36–38]. While informative, these studies did not distinguish between full-length *ASPP2* and *BBP*. The recent description of another *ASPP2* isoform, *ΔN-ASPP2* [23], that is missing the N-terminus and promotes cellular survival and inhibits apoptosis, underscores the importance of identifying *ASPP2* isoforms that have functional and clinical relevance. Hence, our description of the novel *ASPP2κ* isoform provides significant new insight into the mechanisms of regulating *ASPP2* function – and emphasizes the need to interpret *ASPP2* expression data in human tumors in the context of all isoforms.

Due to the higher prevalence in leukemias compared to healthy individuals, *ASPP2κ* may serve as a clinically important prognostic marker.

Monitoring of dynamic *ASPP2κ* expression levels while under therapy may also be a useful tool to predict therapy response – thereby allowing treatment modifications such as dose escalations or addition of drugs in case of imminent therapy failure. The data presented herein may help to develop prospective clinical trials for *ASPP2κ*-guided therapies. A response guided therapy strategy has been proposed for pediatric leukemia patients [39]. Dynamic molecular markers predicting suboptimal response while under therapy would further improve this concept. The presented proof-of-principle experiments, employing stress-inducible pro- and antiapoptotic ASPP2 isoforms may provide such a tool and should further be developed in order to monitor and steer therapy early in the course by intensifying or adjusting treatment as necessary.

In summary, we describe a dynamic dominant-negative splicing variant of *ASPP2* with oncogenic potential and demonstrate a role of *ASPP2κ* in early leukemogenesis and therapy resistance. We further demonstrate prevalence of *ASPP2κ* in other malignancies, including solid tumors – which argues for a much more universal role of *ASPP2κ* in human cancers. These observations blaze the trail for future studies in this field and are currently under active investigation.

Author's contribution.

M.S. designed the research study, analyzed experimental data and wrote the paper, B.W. and V.T. performed functional experiments and contributed with data analysis and writing of the paper, B.F. performed histology analysis and contributed with data analysis and writing of the paper, M.BS. performed qRT-PCR experiments and contributed with

data analysis, B.I. and F.A. performed expression and functional experiments and contributed with data analysis, U.MH. performed cytogenetic experiments and contributed with data analysis and writing of the paper, F.F. performed histology experiments and contributed with data analysis, C.D.L. contributed to research design, data analysis and wrote the paper, K.KS. designed the research study, performed expression and functional experiments, analyzed the data and wrote the paper.

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Competing financial interests

Dr. Schittenhelm reports grants from IZKF Program of the Medical Faculty Tübingen, grants from Wilhelm Sander Foundation for Cancer Research, during the conduct of the study; personal fees from Pfizer, personal fees from Astellas, outside the submitted work; In addition, Dr. Schittenhelm has a patent U.S. 13/753,354 issued, a patent PCT/EP 2011/063283 issued, and a patent GER 102010033575.4-41 issued. V. Tsintari has nothing to disclose. B. Walter has nothing to disclose. Dr. Federmann has nothing to disclose. M. Bajrami Saipi has nothing to disclose. Dr. Illing has nothing to disclose. F. Akmut has nothing to disclose. Dr. Mau-Holzmann has nothing to disclose. Dr. Fend has nothing to disclose. Dr. Lopez has nothing to disclose. Dr. Kampa-Schittenhelm reports grants from Brigitte Schlieben-Lange Program Baden-Württemberg, grants from Margarete von Wrangell Program Baden-Wuerttemberg, grants from Athene Program of the University of Tübingen, grants from Wilhelm Sander Foundation for Cancer Research, during the conduct of the study; In addition, Dr. Kampa-Schittenhelm has a patent U.S. 13/753,354 issued, a patent PCT/EP 2011/063283 issued, and a patent GER 102010033575.4-41 issued.

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Data availability

The corresponding author had access to all data and had final responsibility to submit for publication.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.03.028.

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