Original Research

Mutant U2AF1-induced differential alternative splicing causes an oxidative stress in bone marrow stromal cells

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Impact statement

Alternative splicing (AS) plays a critical role in bone marrow mesenchymal stem cells fate determination by forming distinct isoforms of important regulators. In current study, influence of mutant U2AF1-induced differential AS in bone marrow stromal cells is investigated. We found that mutant U2AF1-induced differential AS causes increased generation of hydrogen peroxide and mitochondria dysfunction in stromal cells. U2AF1 mutation leads to downregulation of genes involved in peroxisome function, which further contribute to oxidative stress in stromal cells. Furthermore, stromal cell-derived oxygen radicals caused by mutant U2AF1 can induce DNA damage in hematopoietic cells. Our data provide evidence that mutant U2AF1-induced oxidative stress in bone marrow microenvironment contributes to DNA damage accumulation in hematopoietic cells.

Abstract

Alternative splicing (AS) is a critical regulatory process of gene expression. In bone marrow microenvironment, AS plays a critical role in mesenchymal stem cells fate determination by forming distinct isoforms of important regulators. As a spliceosome factor, U2AF1 is essential for the catalysis of pre-mRNA splicing, and its mutation can cause differential AS events. In the present study, by forced expression of mutant U2AF1 (U2AF1S34F) in the mouse bone marrow stroma OP9 cells, we determine AS changes in U2AF1S34F transduced OP9 cells and investigate their role in stroma cell biological functions. We find that abundant differential RNA splicing events are induced by U2AF1S34F in OP9 cells. U2AF1S34F causes increased generation of hydrogen peroxide, promotes production of cytokines and chemokines. U2AF1S34F transduced OP9 cells also exhibit dysfunction of mitochondria. RNA-seq data, gene ontology (GO), and gene set enrichment analysis reveal that differentially expressed genes downregulated in response to U2AF1S34F are enriched in peroxisome component and function. U2AF1S34F can also cause release of hydrogen peroxide from OP9 cells. Furthermore, we investigate the influence of U2AF1S34F-induced oxidative stress in stromal cells on hematopoietic cells. When co-culturing mouse bone marrow mononuclear cells with OP9 cells, the U2AF1S34F expressing OP9 cells induce phosphorylation of histone H2AX in hematopoietic cells. Collectively, our results reveal that mutant U2AF1-induced differential AS events cause oxidative stress in bone marrow stromal cells and can further lead to DNA damage and genomic instability in hematopoietic cells.

Keywords: Alternative splicing, bone marrow stromal cells, reactive oxygen species, oxidative stress, hematopoietic cell, DNA damage

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Introduction

Hematopoietic stem cells (HSCs) reside in bone marrow niche and the interplay between HSCs and surrounding stroma is critical in maintaining normal hematopoiesis.[1] Although malignant transformation of HSCs largely results from genetic aberrations within HSCs, emerging evidence indicates that microenvironment dysregulation can also

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cause aberrant hematopoiesis, even initiate leukemogenesis.[2–5] Altered microenvironment regulation can be resulted from mutations in bone marrow stroma cells, and the presence of activating germline mutation in bone marrow mesenchymal stem cells (MSCs) contributes to the development of myelodysplastic syndrome (MDS) and leukemia.[3,6,7]

Alternative splicing (AS) is an essential regulatory step of gene expression through which a pre-mRNA generates multiple mRNA. In MSCs, AS can regulate MSC fate by forming distinct protein isoforms of important regulators.[8] One of the evidences that AS plays a critical role in MSC differentiation come from the study that the shorter RUNX2 protein isoform exhibited attenuated transcriptional activity involved in regulating osteogenic differentiation.[9] By splicing of the key transcriptional regulators, AS plays a pivotal role during differentiation of MSC into osteocyte, adipocyte, and chondrocyte.[8]

AS is regulated by spliceosome factors, which are essential for the catalysis of pre-mRNA splicing. Splicing factor mutations lead to deregulated or abnormal RNA splicing, which has been implicated in various human diseases.[10,11] U2AF1 is one of the splicing factors involved in the initial step of RNA splicing, its mutations have been identified in patients with MDS and hematopoietic malignancies.[12,13] Recent study indicates that U2AF1 downregulation induces a switch in the AS of transcription factors involved in cell-fate determination of pluripotent stem cells.[14]

Considering that mutations in splicing factors can alter the global AS pattern and AS takes a regulation role in MSC fate, we speculate that differential RNA splicing events caused by U2AF1 mutation in MSCs may result in bone marrow MSC dysfunction. Here, to understand how deregulated RNA splicing leads to MSC dysfunction, using OP9 cells, a cell line of bone marrow-derived mouse stromal cells having a MSC phenotype,[15] we generated a mutant U2AF1 (U2AF1S34F) expressing OP9 cells and investigated the role of U2AF1S34F in AS pattern and the biological functions of bone marrow stromal cells.

Materials and methods

Cell line and animals

The mouse bone marrow stromal cell line OP9 was purchased from American Type Culture Collection (ATCC, CRL-2749). The cells were cultured in αMEM supplemented with 20% fetal bovine serum. The MSC phenotype has been confirmed in our cultured OP9 cells.

Four- to six-week-old female C57BL/6 mice were purchased from Beijing HFK Bioscience Co. LTD. All animal experiments were carried out in accordance with standard guidelines and approved by the Institutional Animal Care and Use Committees of State Key Laboratory of Experimental Hematology.

Retroviral vectors construction and transduction

The fragments containing wild-type U2AF1 (U2AF1-WT) and mutant U2AF1 (U2AF1S34F) were amplified by PCR and subcloned into retroviral vector MSCV-IRES-GFP (a gift from Michael H Tomasson, Washington University School of Medicine), respectively. After verified by sequencing, the constructs were co-transfected into 293T cells with Ecopack packaging plasmid using polyethylenimine (Polysciences, USA). The supernatants containing viruses were collected and concentrated. OP9 cells were transduced with prepared retrovirus. GFP-positive cells were sorted by flow cytometry (FACS) on FACS AriaIII (BD Biosciences, San Jose, CA, USA).

RNA-seq analysis

Total RNA was extracted using TRIZOL (Takara, Japan). The sequencing libraries were constructed using the rRNA-depleted RNA by NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina® (NEB, USA) following the manufacturer instructions. The quality of RNA-seq libraries was assessed on the Agilent Bioanalyzer 2100 system. After cluster generation, the libraries were sequenced on an Illumina Hiseq 4000 platform and 150 bp paired-end reads were generated.

Fibroblast colony-forming unit assay

The fibroblast colony-forming unit (CFU-F) assay was performed for evaluating cell proliferation. The 1×10^3 OP9 cells were suspended in 2 mL of MesenCult™ Expansion medium (Cat. No. 05513, STEMCELL Technologies Inc., Vancouver, Canada) and seeded in six-well tissue-culture plate. After culturing for seven days, each well was washed with PBS, and then the remaining attached cells were stained with 0.5% Crystal Violet (Beyotime, Shanghai, China) in methanol for 10 min. All visible colonies were counted.

Measurement of intracellular reactive oxygen species

The cellular reactive oxygen species (ROS) level was determined by FACS analysis using DHE (Cat. No. D11347, Invitrogen, Waltham, MA, USA) and Mitosox (Cat. No. M36008, Invitrogen) labeling. Briefly, 3×10^5 cells were labeled with 1 μmol/L DHE or 5 μmol/L Mitosox. The cellular ROS levels were determined by FACS (LSR Fortessa, BD Biosciences, San Jose, CA, USA) based on the fluorescence intensity.

Cytokine array

The culture supernatants of OP9 cells were collected and analyzed using a cytokine array from R&D Systems (Cat. No. ARY006, Minneapolis, MN, USA) according to the manufacturer's instruction, which was also described previously.[16] The pixel densities of the spots on given blot were quantified by using ImageQuant TL software.

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was determined by using the fluorescent dye tetramethylrhodamine (TMRM). Cells were incubated with TMRM (Cat. No 613404 Invitrogen) at a final concentration of 250 nmol/L for 30 min at 37°C. Then, the fluorescence intensity was

measured by FACS. The mitochondrial membrane potential was assessed by the percentage of $TMRM^+$ cells in GFP^+ cells.

ADP/ATP ratio assay

The ADP/ATP ratio was determined using an ADP/ATP Ratio Assay Kit (Cat. No. MAK135; Sigma-Aldrich, St. Louis., MO, USA). The ATP and ADP levels were measured according to the manufacturer's instructions, which was also described previously.[17] ADP/ATP ratio was calculated.

Detection of hydrogen peroxide released from stromal cells

The hydrogen peroxide (H_2O_2) released from OP9 cells was measured by a Fluorimetric Hydrogen Peroxide Assay Kit (Cat. No. MAK165, Sigma-Aldrich) according to the manufacturer's instructions, which was also described in our previous study.[18] Fifty microliters of cell culture supernatant were plated in 96-well plate and then mixed with 50 μL of the master mix including red peroxidase substrate and peroxidase. The plate was incubated at room temperature in the dark for 15 min. Fluorescence intensity was measure at λex 540 and λem 590 nm using a fluorescence plate reader. The H_2O_2 concentration in the samples was calculated from the standard curve.

Isolation of mouse bone marrow hematopoietic cells and co-culturing of that with OP9 stromal cells

BM mononuclear cells (BM MNCs) were flushed from mouse femurs of two mice. For the co-culture, OP9 cells (3×10^5) were seeded in six-well plate and cultured overnight in αMEM culture medium. BM MNCs (3×10^6) were added directly onto the stromal cells and co-cultured with OP9 cells for 48 h.

Immunofluorescent staining of phosphorylated histone H2AX (γH2AX) for flow cytometry analysis

γH2AX was used for monitoring the cellular response to DNA damage. Cells were fixed in Fixation Buffer (Biolegend, San Diego, CA, USA), permeabilized in Intracellular Staining Perm Wash Buffer (Biolegend) and then stained with anti-H2AX-phosphorylated (Ser139) antibody (Cat. No 613404, Biolegend). γH2AX positive cells were determined by FACS analysis in LSR Fortessa.

Statistical analysis

The data were analyzed using SPSS software. The significance of differences between two groups was determined using the independent-sample *T*-test. The data are presented as the mean ± SD from three independent experiments.

Results

Forced expression of mutant U2AF1 generates a distinct splicing pattern in OP9 cells

To evaluate the potential effects of differential AS in MSCs, we used OP9 cells, a mouse bone marrow stromal cell line which has been characterized as possessing MSC phenotype and differentiation potential,[15] and examine the influence of deregulated RNA splicing on cell functions. We first generated mouse bone marrow stromal OP9 cell lines that stably expressed wild-type U2AF1 (U2AF1-WT) and mutant U2AF1 (U2AF1S34F). The presence of U2AF1S34F in OP9 cells was detected by RT-PCR and confirmed by sequencing (Figure 1(a)). Western-blot analysis indicated that U2AF1 proteins were mildly and stably overexpressed in U2AF1-WT and U2AF1S34F transfected OP9 cells, and U2AF1 proteins were expressed at similar levels in these two transfected cell lines (Figure 1(b)).

We then performed RNA-seq on OP9 cells that stably expressed U2AF1S34F and U2AF1-WT. Using RNA-seq data, the aberrantly spliced genes that resulted from U2AF1S34F were explored. The AS events were analyzed using rMATS and categorized as skipped exon, retained introns, mutually exclusive exons, alternative 3′ splice site usage, or alternative 5′ splice site usage. Relative to U2AF1-WT control cells, U2AF1S34F expressing cells exhibited 2938 differential AS events. Among the main types of AS events, skipped exon accounted for the majority of changed AS events (1625, 55%), followed by mutually exclusive exons (512, 17%) and alternative 3′ splice site (333, 11%). The number of retained introns and alternative 5′ splice site usage events were relatively low, which account for 9% (retained introns, 272) and 7% (alternative 5′ splice site usage, 196) of all changed AS events in U2AF1S34F expressing cells, respectively (Figure 1(c)). This result demonstrates that a distinct splicing pattern can occur in U2AF1S34F transduced cells and the splicing changes have a bias toward exon skipping.

Mutant U2AF1 causes generation of hydrogen peroxide and production of cytokine and chemokine in OP9 cells

We next examined the biological effects induced by mutant U2AF1 in OP9 cells. Cell proliferation was evaluated by CFU-F assay. Compared with U2AF1-WT control cells, U2AF1S34F transduced cells formed slightly smaller colonies; however, there was no significant difference in CFU-F counts between U2AF1S34F and U2AF1-WT cells (Figure 2 (a)). This result indicates that U2AF1S34F has no effect on OP9 cell proliferation. We then assessed the intracellular reactive species using the indicator DHE and Mitosox. As shown in Figure2(b) and Supplemental Figure 1, the percentage of DHE positive cells in U2AF1S34F cells was significantly higher than that in U2AF1-WT cells, indicating that the intracellular ROS production was significantly increased in U2AF1S34F transfected OP9 cells. The increase in ROS production in U2AF1S34F cells was also confirmed by Mitosox analysis, indicating that mutant U2AF1 causes elevated H_2O_2 generation in OP9 cells.

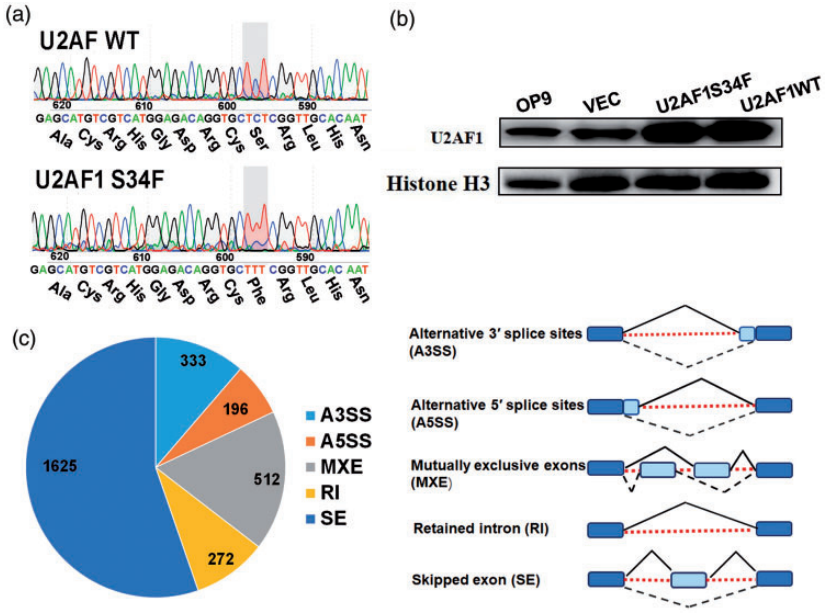

Figure 1. Expression of mutant U2AF1 and distinct splicing pattern in U2AF35S34F transduced OP9 cells. (a) Expression of mutant U2AF1 confirmed by RT-PCR and sequencing in U2AF1S34F transduced OP9 cells. (b) U2AF1 protein levels in U2AF1-WT and U2AF1S34F expressing OP9 cells. (c) Summary of distinct splicing pattern in U2AF1S34F transduced OP9 cells. (A color version of this figure is available in the online journal.)

Impaired oxidative status is often accompanied by increased inflammatory cytokines production. Cytokines and chemokines produced by stromal cells in bone marrow play essential roles in hematopoiesis. We then measured a panel of cytokines and chemokines presented in the culture medium of U2AF1S34F and U2AF1WT cells. As shown in cytokine array analysis, compared with U2AF1WT cells, U2AF1S34F cells produced higher levels of IL-1Ra, CXCL10, CCL-5, CXCL12, and TIMP1 (Figure 2 (c)). This result was supported by our RNA-Seq data. The heatmap in Figure 2(d) revealed that the expression levels of most cytokine and chemokine genes were upregulated in U2AF1S34F cells (Figure 2(d)). Gene set enrichment analysis (GSEA) also showed that up-regulated genes in U2AF1S34F cells were significantly enriched in the gene set that related to inflammatory response. Taken together, our conclusion is that mutant U2AF1 causes an oxidative stress and promotes production of inflammatory cytokine chemokine in OP9 cells.

Mutant U2AF1 induces mitochondria dysfunction in OP9 cells

Mitochondrial dysfunction is associated with increased ROS production, we then measured the mitochondrial membrane potential by TMRM labeling and FACS analysis in U2AF1-WT and U2AF1S34F cells. Figure 3(a) showed that the percentage of TMRM positive cells was significantly decreased in U2AF1S34F cells. Furthermore, to confirm whether mutant U2AF1 causes mitochondria dysfunction, the intracellular ATP and ADP levels were measured and ADP/ATP ratios were calculated. As shown in Figure 3(b), increased ADP/ATP ratio was found in U2AF1S34F cells. Taken together, it indicates that mutant U2AF1 induces mitochondrial membrane potential decrease and mitochondria dysfunction in OP9 cells.

Mutant U2AF1 leads to downregulation of genes involved in peroxisome function in OP9 cells

To better understand the molecular mechanism underlying mutant U2AF1-induced ROS production and mitochondria dysfunction, we compared the expression profile of genes related to the regulation of ROS from the RNA-seq data. It was found that there was no significant difference in the expression levels of genes related to the mitochondria between U2AF1S34F and U2AF1-WT cells. However, it was noticed that most of the genes involved in the peroxisome organization and function were downregulated in U2AF1S34F cells (Figure 4(a)). Gene ontology (GO) enrichment analysis also showed that the downregulated genes in U2AF1S34F cells were significantly enriched in functions related to peroxisome (Figure 4(b)). Furthermore, GSEA was performed to identify biological pathways associated with ROS production in U2AF1S34F cells. Same as the results obtained from GO enrichment analysis, the enrichment of the downregulated genes in U2AF1S34F cells fell into the peroxisome gene set, and especially significant enrichment was observed in the intrinsic component of peroxisomal membrane gene set (Figure 4(c)). No enrichment was found in any mitochondria-related cellular component gene sets. This result indicates that mutant U2AF1 causes peroxisome abnormality. Peroxisomes not only can generate ROS, but also maintain redox balance through removing H_2O_2. It suggests that increased ROS production in U2AF1S34F cells is mainly caused by alteration in peroxisome.

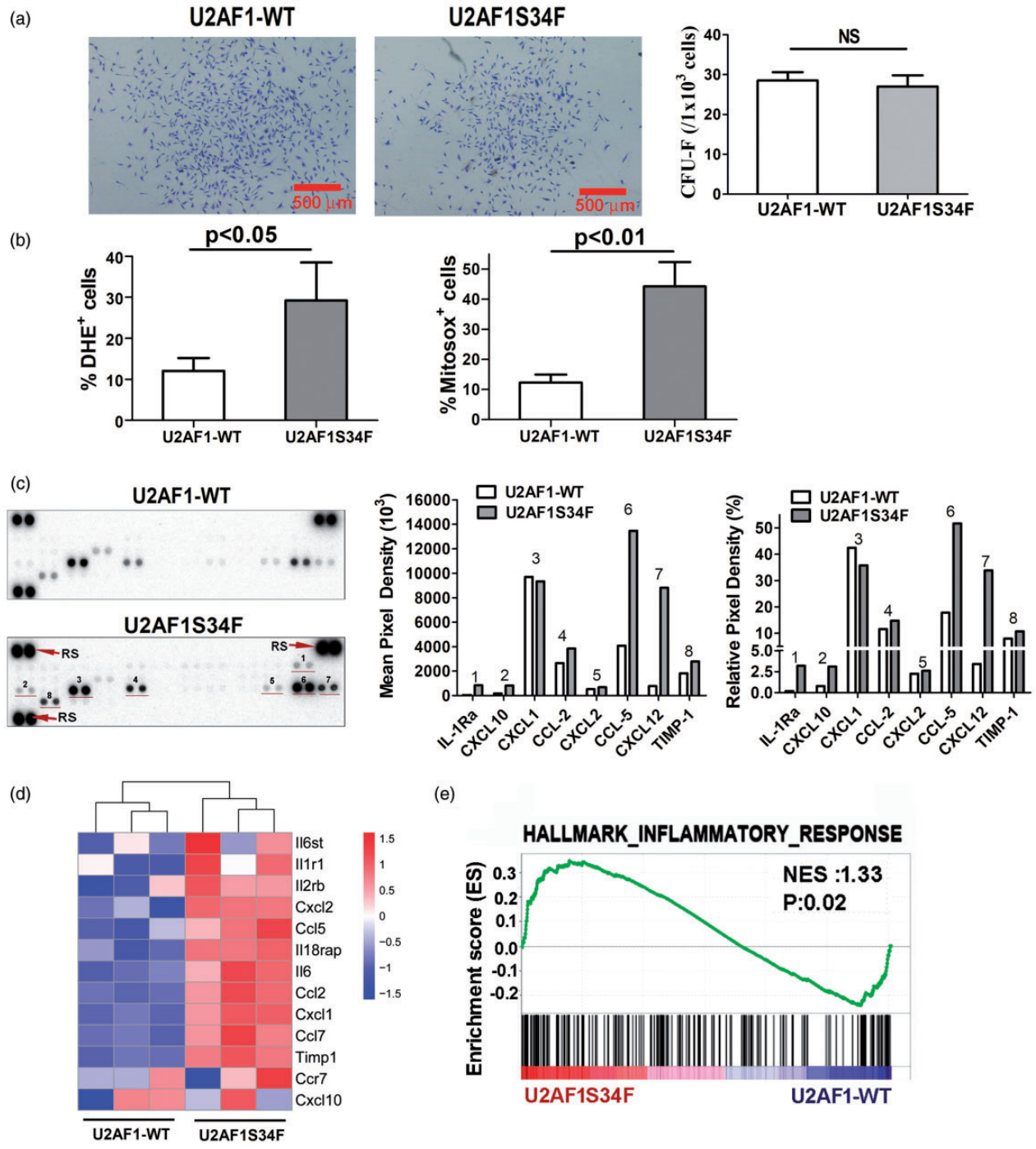

Figure 2. Effects of mutant U2AF1 in ROS generation, cytokine, and chemokine production in OP9 cells. (a) Effect of U2AF1S34F on proliferation of OP9 cells. Cell proliferation was determined by CFU-F assay. In each CFU-F culture, OP9 cells were plated in triplicate. Data are presented as the mean ± SD from three independent experiments. Scale bar: 500 μm. (b) U2AF1S34F induced high intracellular ROS levels in OP9 stromal cells. Intracellular ROS was determined by DHE and Mitosox labeling and FACS analysis. Left panel: DHE labeling. Right panel: Mitosox labeling. Data are presented as the mean ± SD from three independent experiments. (c) Production of cytokines and chemokines in U2AF1S34F and U2AF1-WT expressing OP9 cells. Cytokine array were performed independently twice. One representative data set of cytokine array analysis is presented. RS: reference spot. The pixel density was quantified with ImageQuant TL software. The pixel density value of each cytokine and chemokine was obtained from the dot intensity in duplicate. Relative pixel density = pixel density of cytokine spot/average pixel density of six reference spots on the same membrane (right panel). RS: reference spot. (d) Heatmap analysis of cytokine and chemokine genes. (e) Gene set enrichment analysis. The enrichment of upregulated genes by U2AF1S34F in the gene set related to inflammatory response. (A color version of this figure is available in the online journal.)

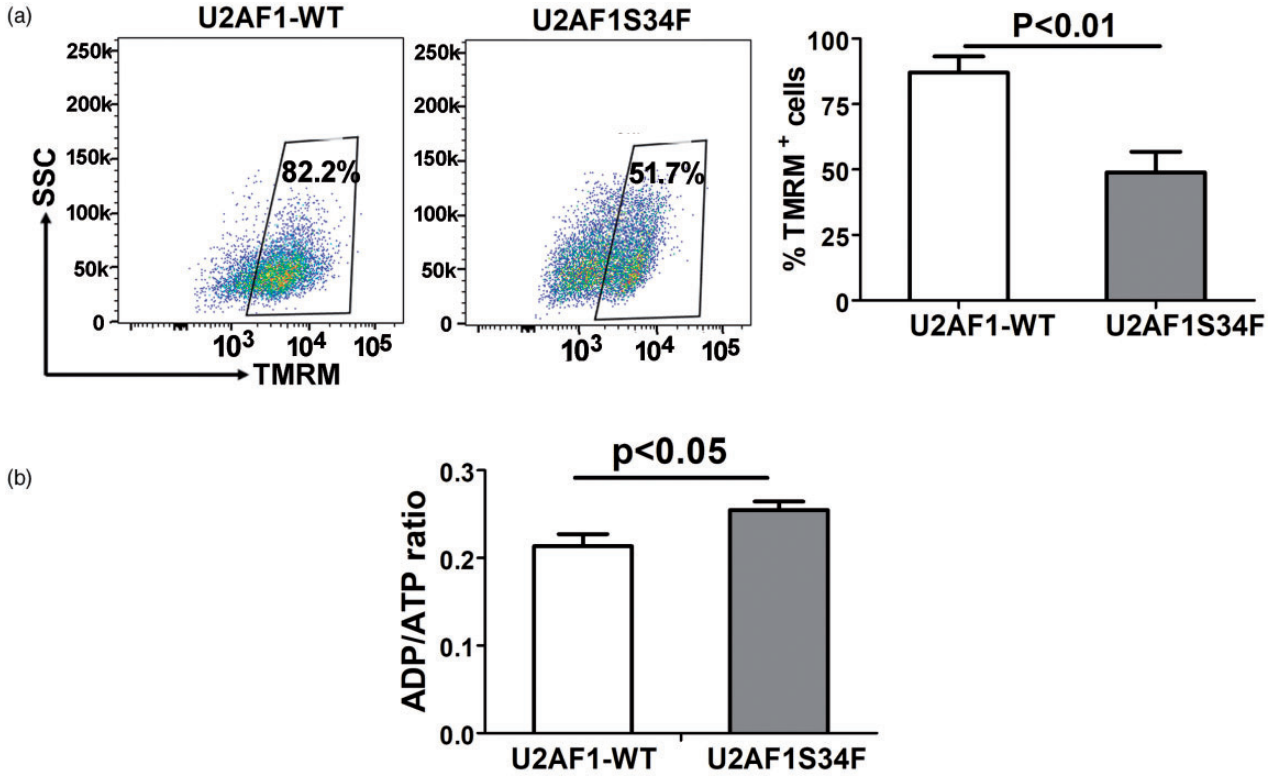

Figure 3. U2AF35S34F-induced mitochondria dysfunction in OP9 cells. (a) Decreased level of mitochondrial membrane potential in U2AF35S34F expressing OP9 cells. Mitochondrial membrane potential was determined by TMRM labeling and FACS analysis. Data are presented as the mean ± SD from three independent experiments. (b) Increase in ADP/ATP ratio in U2AF35S34F expressing OP9 cells. Data are presented as the mean ± SD from six independent experiments. (A color version of this figure is available in the online journal.)

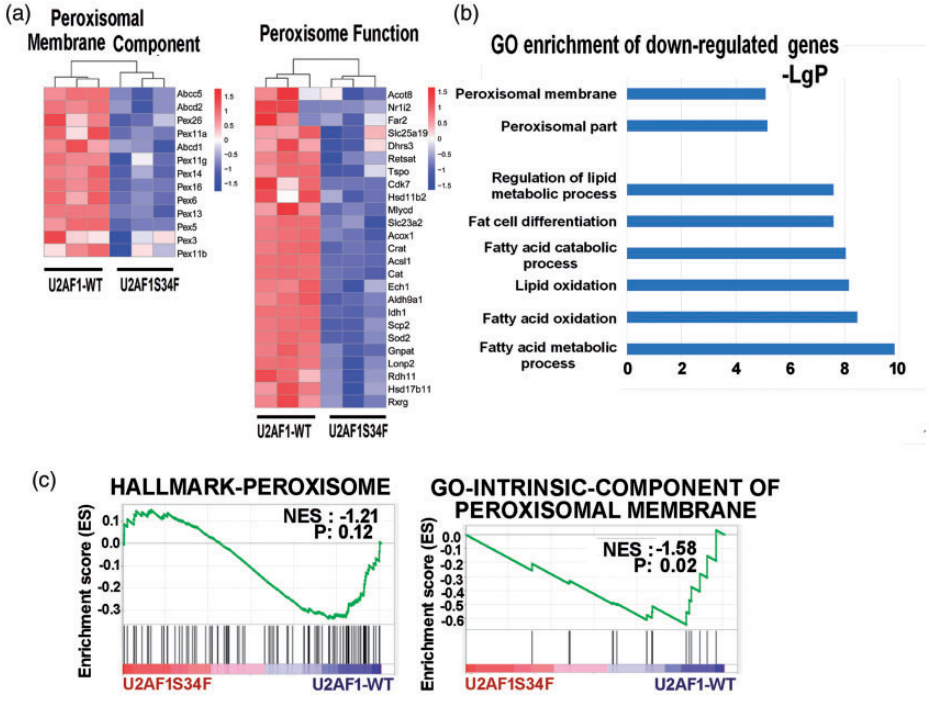

Figure 4. Downregulated genes involved in the peroxisome function in U2AF35S34F transduced OP9 cells. (a) Heatmap analysis of downregulated genes related to peroxisomal membrane component and peroxisome function. (b) GO analysis of significantly downregulated genes. Downregulated genes in U2AF1S34F cells were significantly enriched in functions related to peroxisome. (c) Gene set enrichment analysis. The enrichment of downregulated genes in U2AF1S34F cells fell into the peroxisome and peroxisomal membrane gene sets. (A color version of this figure is available in the online journal.)

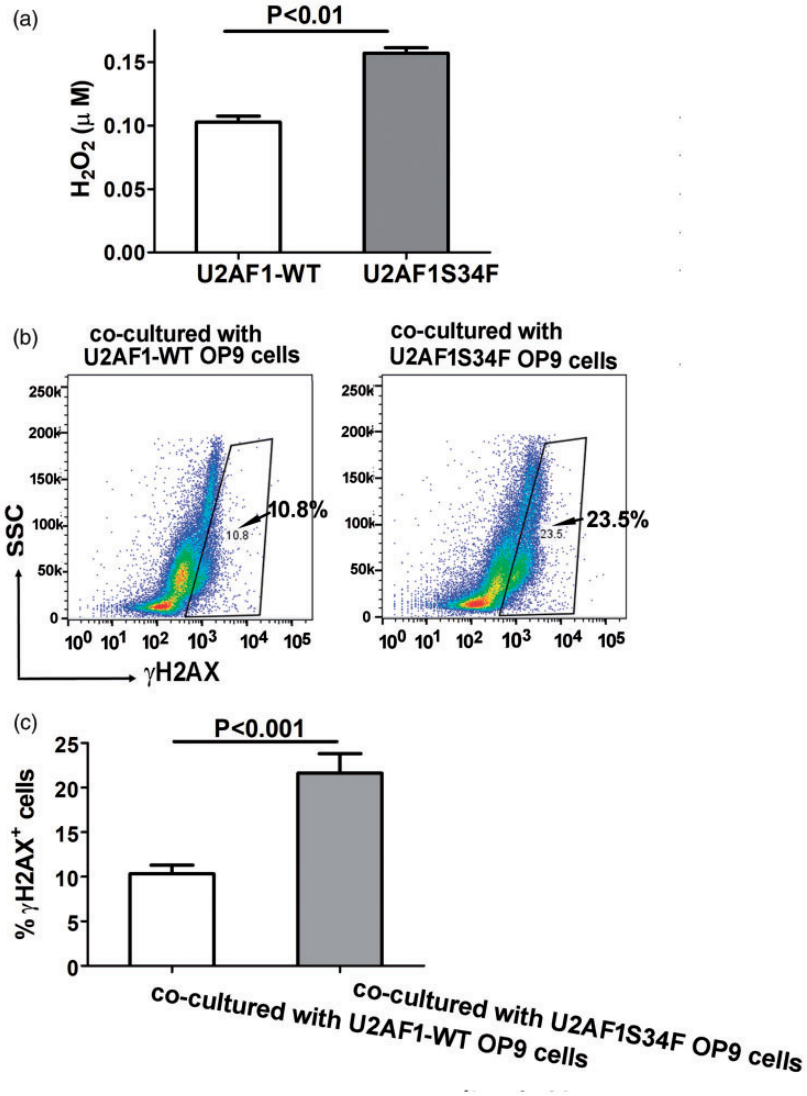

Figure 5. Mutant U2AF1 causes H_2O_2 release from OP9 cells and triggers DNA damage in BM hematopoietic cells. (a) Mutant U2AF1-induced release of H_2O_2 to the culture medium from OP9 cells. All samples were run in triplicate. Data are presented as the mean ± SD from three independent experiments (μM:μmol/L). (b) Representative FACS analysis of γH2AX positive cells in BM MNCs co-cultured with OP9 cells. (c) Percentage of γH2AX positive cells in BM MNCs co-cultured with OP9 cells. Each experiment was performed with two mice and the data are presented as the mean ± SD from three independent experiments. (A color version of this figure is available in the online journal.)

Mutant U2AF1 causes an increase in H_2O_2 release from OP9 cells and leads to an accumulation of DNA damage in co-cultured BM hematopoietic cells

Alterations in peroxisome may affect its function in removing H_2O_2. Furthermore, oxygen radical in the supernatant of cultured U2AF1S34F and U2AF1-WT cells was detected. As expected, the concentration of H_2O_2 was significantly elevated in the culture supernatant of U2AF1S34F cells (Figure 5(a)), indicating that mutant U2AF1 causes more H_2O_2 release from OP9 cells.

U2AF1S34F in stromal cell-induced extra H_2O_2 release from OP9 stromal cells, we speculated that this higher level of oxygen radical may induce DNA damage in hematopoietic cells. We then co-cultured mouse BM MNCs with U2AF1S34F or U2AF1-WT expressing OP9 cells, and determined the oxidative DNA damage in BM MNCs. The percentage of phosphorylated H2AX (γH2AX) positive cells in BM MNCs was measured by flow cytometry. The percentage of $\gamma H2AX^+$ cells in BM MNCs co-cultured with U2AF1S34F OP9 cells was significantly higher than that in BM MNCs co-cultured with U2AF1-WT OP9 cells (Figure 5(b) and (c)). These data suggest that ROS production in OP9 stromal cells caused by mutant U2AF1 can induce DNA damage in hematopoietic cells, and this DNA damage may be induced directly by the oxygen radical released from OP9 stromal cells.

Discussion

As mentioned above, AS plays a critical role in MSC differentiation.[8] Several groups found that differential AS events could be induced by mutation or dysregulation of splicing factors in MSCs, and demonstrated that differential AS controlled MSCs fate determination.[19–22] In our study, we

found that U2AF1S34F induced distinct splicing pattern in OP9 cells, suggesting that mutant U2AF1 may influence the biological functions of stromal cells. Our data indicate that mutant U2AF1 causes generation of intracellular and extracellular ROS, as well as mitochondria dysfunction in OP9 cells, providing evidence that mutation in U2AF1 can induce an oxidative stress in bone marrow.

Mutations in splicing factors including U2AF1 have been identified in malignant hematopoietic cells of patients with MDS and leukemia; however, these mutations in bone marrow MSCs have not yet been reported. Actually, in our investigation on the leukemia-associated mutations in bone marrow MSCs from leukemia patients, we found that in seven MSC samples obtained from leukemia patients, a mutation in splicing factor SF3A1 was observed in one sample. Although U2AF1 mutations were not observed in these seven MSC samples, it could be speculated that differential AS events induced by some other splicing factor maybe exist in bone marrow MSCs of leukemia patients.

Mitochondria and peroxisome are two main cell organelles that maintain cellular ROS homeostasis.[23,24] Abnormal expression of genes related to critical mitochondrial complex and peroxisomal component can lead to dysfunction of these two organelles, and further contribute to oxidative stress.[24–27] Our RNA-seq data showed that there was no significant difference in the expression levels of genes related to the mitochondrial complex between U2AF1S34F and U2AF1-WT cells. However, it was found that most of the genes involved in the peroxisome organization and function were downregulated in U2AF1S34F cells and the enrichment of the downregulated genes in U2AF1S34F cells fell into the peroxisome gene set. Downregulation of genes encoding peroxisomal component may result in peroxisome dysfunction. Peroxisome can not only produce ROS/RNS, but also has a ROS metabolizing capacity by various ROS metabolizing enzymes existed in peroxisome, which contribute to the maintenance of cellular ROS homeostasis.[24] It could be speculated that in U2AF1S34F expressing OP9 cells, downregulated genes of peroxisomal proteins might cause functional disorder in the peroxisomal ROS metabolism, which could lead to persistent high level of ROS in U2AF1S34F cells.

Although significant difference in the expression levels of genes related to the mitochondrial complex was not found between U2AF1S34F and U2AF1-WT cells, mitochondria dysfunction was still observed in U2AF1S34F cells. Mitochondria dysfunction may lead to excess accumulation of ROS, persistent ROS exposure in turn creates oxidative damage to mitochondria and eventually leads to mitochondria dysfunction. [23,25,28] In the present study, a mitochondria damage was observed in U2AF1S34F expressing OP9 cells, which could be induced by persistent high ROS level.

Mutations in the splicing factor U2AF1 have been found in leukemia, myelodysplastic syndrome, and several solid tumors.[29–32] However, reports on the oncogenic activity of mutant U2AF1 are limited. Park *et al*. found that U2AF1S34F could promote Ba/F3 cells proliferation via decreasing autophagy-related factor 7, and they concluded that mutant U2AF1 took a role in the transformation of immortalized hematopoietic cells.[33] Here, in our U2AF1S34F-transfected OP9 stromal cells, no change in cell proliferation was observed. We found that mutant U2AF1 caused production of cytokines and chemokine in OP9 cells. A similar result was reported from Palangat *et al*.'s study, they observed that after radiation, U2AF1S34F caused synthesis of the secreted chemokine interleukin 8 in several cell lines, even at steady state. They conclude that U2AF1 can function as a translational repressor and the U2AF1S34F can cause translational derepression of hundreds of mRNA.[34]

In the hematopoietic system, maintenance of genomic stability is essential for normal hematopoiesis.[35,36] Mounting evidence supports the idea that defects in genome integrity in HSCs limit their self-renewal and hematopoietic function. Accumulation of DNA damage is a main cause of defects in the maintenance of genome stability, which may result in hematopoietic function disorders and even leads to malignant transformation.[37] ROS-induced accumulation of DNA damage has been well documented in human HSCs.[38,39] The studies on the regulation of accumulation of DNA damage in HSCs are mainly focused on the mechanisms of intracellular ROS.[40] Research on the role of extracellular ROS present in microenvironments in accumulation of DNA damage of HSCs are limited. Here, our results suggest that H_2O_2 released from stromal cells can also have an impact on the genome stability in hematopoietic cells. It suggests that ROS presented in microenvironment may influence hematopoietic functions.

In conclusion, in this study the biological functions of BM stromal cell caused by U2AF1 mutation were reported. We show that differential AS induced by U2AF1 mutation causes increased generation of intracellular and extracellular H_2O_2, promotes production of cytokine and chemokine in OP9 stromal cells. U2AF1 mutation-induced ROS production is accompanied by mitochondria damage. Mutant U2AF1 also leads to lower expression of genes involved in the peroxisome organization and function, which may contribute to an imbalance of ROS. Furthermore, stromal cell-derived oxygen radicals can induce DNA damage in hematopoietic cells. Our data provide evidence that differential AS in bone marrow stromal cells causes an oxidative stress in bone marrow microenvironment, which may lead to the genomic instability in hematopoietic cells.

AUTHORS' CONTRIBUTIONS

ZL, QR, and JW conceptualized and designed the study. MZ, YX, and HX developed the method. ZL, XD, YL, ZX, JT, and ZT performed all experiments and analyzed the data. ZC and SQ analyzed the RNA-seq data (e.g. GO, GSEA analysis). ZL and QR wrote and reviewed the manuscript. MW revised the manuscript. MW, QR, and JW supervised the study and finalized the manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

REFERENCES

1. Morrison SJ, Scadden DT. The bone marrow niche for haematopoietic stem cells. *Nature* 2014;**505**:327–34
2. Miller LH, Qu CK, Pauly M. Germline mutations in the bone marrow microenvironment and dysregulated hematopoiesis. *Exp Hematol* 2018;**66**:17–26
3. Kode A, Manavalan JS, Mosialou I, Bhagat G, Rathinam CV, Luo N, Khiabanian H, Lee A, Murty VV, Friedman R, Brum A, Park D, Galili N, Mukherjee S, Teruya-Feldstein J, Raza A, Rabadan R, Berman E, Kousteni S. Leukaemogenesis induced by an activating β-catenin mutation in osteoblasts. *Nature* 2014;**506**:240–4
4. Wiseman DH. Donor cell leukemia: a review. *Biol Blood Marrow Transplant* 2011;**17**:771–89
5. Reichard KK, Zhang QY, Sanchez L, Hozier J, Viswanatha D, Foucar K. Acute myeloid leukemia of donor origin after allogeneic bone marrow transplantation for precursor T-cell acute lymphoblastic leukemia: case report and review of the literature. *Am J Hematol* 2006;**81**:178–85
6. Dong L, Yu WM, Zheng H, Loh ML, Bunting ST, Pauly M, Huang G, Zhou M, Broxmeyer HE, Scadden DT, Qu CK. Leukaemogenic effects of Ptpn11 activating mutations in the stem cell microenvironment. *Nature* 2016;**539**:304–8
7. Zambetti NA, Ping Z, Chen S, Kenswil KJG, Mylona MA, Sanders MA, Hoogenboezem RM, Bindels EMJ, Adisty MN, Van Strien PMH, van der Leije CS, Westers TM, Cremers EMP, Milanese C, Mastroberardino PG, van Leeuwen J, van der Eerden BCJ, Touw IP, Kuijpers TW, Kanaar R, van de Loosdrecht AA, Vogl T, Raaijmakers M. Mesenchymal inflammation drives genotoxic stress in hematopoietic stem cells and predicts disease evolution in human pre-leukemia. *Cell Stem Cell* 2016;**19**:613–27
8. Park JW, Fu S, Huang B, Xu RH. Alternative splicing in mesenchymal stem cell differentiation. *Stem Cells* 2020;**38**:1229–40
9. Makita N, Suzuki M, Asami S, Takahata R, Kohzaki D, Kobayashi S, Hakamazuka T, Hozumi N. Two of four alternatively spliced isoforms of RUNX2 control osteocalcin gene expression in human osteoblast cells. *Gene* 2008;**413**:8–17
10. Wang GS, Cooper TA. Splicing in disease: disruption of the splicing code and the decoding machinery. *Nat Rev Genet* 2007;**8**:749–61
11. David CJ, Manley JL. Alternative pre-mRNA splicing regulation in cancer: pathways and programs unhinged. *Genes Dev* 2010;**24**:2343–64
12. Graubert TA, Shen D, Ding L, Okeyo-Owuor T, Lunn CL, Shao J, Krysiak K, Harris CC, Koboldt DC, Larson DE, McLellan MD, Dooling DJ, Abbott RM, Fulton RS, Schmidt H, Kalicki-Veizer J, O'Laughlin M, Grillot M, Baty J, Heath S, Frater JL, Nasim T, Link DC, Tomasson MH, Westervelt P, DiPersio JF, Mardis ER, Ley TJ, Wilson RK, Walter MJ. Recurrent mutations in the U2AF1 splicing factor in myelodysplastic syndromes. *Nat Genet* 2011;**44**:53–7
13. Visconte V, Makishima H, Maciejewski JP, Tiu RV. Emerging roles of the spliceosomal machinery in myelodysplastic syndromes and other hematological disorders. *Leukemia* 2012;**26**:2447–54
14. Laaref AM, Manchon L, Bareche Y, Lapasset L, Tazi J. The core spliceosomal factor U2AF1 controls cell-fate determination via the modulation of transcriptional networks. *RNA Biol* 2020;**17**:857–71
15. Gao J, Yan XL, Li R, Liu Y, He W, Sun S, Zhang Y, Liu B, Xiong J, Mao N. Characterization of O. P9 as authentic mesenchymal stem cell line. *J Genet Genomics* 2010;**37**:475–82
16. Lo HM, Wang SW, Chen CL, Wu PH, Wu WB. Effects of all-trans retinoic acid, retinol, and β-carotene on murine macrophage activity. *Food Funct* 2014;**5**:140–8
17. Gonçalves SM, Duarte-Oliveira C, Campos CF, Aimanianda V, Ter Horst R, Leite L, Mercier T, Pereira P, Fernández-García M, Antunes D, Rodrigues CS, Barbosa-Matos C, Gaifem J, Mesquita I, Marques A, Osório NS, Torrado E, Rodrigues F, Costa S, Joosten LA, Lagrou K, Maertens J, Lacerda JF, Campos A, Jr., Brown GD, Brakhage AA, Barbas C, Silvestre R, van de Veerdonk FL, Chamilos G, Netea MG, Latgé JP, Cunha C, Carvalho A. Phagosomal removal of fungal melanin reprograms macrophage metabolism to promote antifungal immunity. *Nat Commun* 2020;**11**:2282
18. Li Y, Xue Z, Dong X, Liu Q, Liu Z, Li H, Xing H, Xu Y, Tang K, Tian Z, Wang M, Rao Q, Wang J. Mitochondrial dysfunction and oxidative stress in bone marrow stromal cells induced by daunorubicin leads to DNA damage in hematopoietic cells. *Free Radic Biol Med* 2020;**146**:211–21
19. Peffers MJ, Collins J, Fang Y, Goljanek-Whysall K, Rushton M, Loughlin J, Proctor C, Clegg PD. Age-related changes in mesenchymal stem cells identified using a multi-omics approach. *Eur Cell Mater* 2016;**31**:136–59
20. Mantila Roosa SM, Liu Y, Turner CH. Alternative splicing in bone following mechanical loading. *Bone* 2011;**48**:543–51
21. Baralle FE, Giudice J. Alternative splicing as a regulator of development and tissue identity. *Nat Rev Mol Cell Biol* 2017;**18**:437–51
22. Aprile M, Cataldi S, Ambrosio MR, D'Esposito V, Lim K, Dietrich A, Blüher M, Savage DB, Formisano P, Ciccodicola A, Costa V. PPAR$\gamma\Delta$5, a naturally occurring dominant-negative splice isoform, impairs PPARγ function and adipocyte differentiation. *Cell Rep* 2018;**25**:1577–92 e6
23. Bhat AH, Dar KB, Anees S, Zargar MA, Masood A, Sofi MA, Ganie SA. Oxidative stress, mitochondrial dysfunction and neurodegenerative diseases; a mechanistic insight. *Biomed Pharmacother* 2015;**74**:101–10
24. Fransen M, Nordgren M, Wang B, Apanasets O. Role of peroxisomes in ROS/RNS-metabolism: implications for human disease. *Biochim Biophys Acta* 2012;**1822**:1363–73
25. Castro Mdel R, Suarez E, Kraiselburd E, Isidro A, Paz J, Ferder L, Ayala-Torres S. Aging increases mitochondrial DNA damage and oxidative stress in liver of rhesus monkeys. *Exp Gerontol* 2012;**47**:29–37
26. Islinger M, Voelkl A, Fahimi HD, Schrader M. The peroxisome: an update on mysteries 2.0. *Histochem Cell Biol* 2018;**150**:443–71
27. Fujiki Y. Peroxisome biogenesis and human peroxisome-deficiency disorders. *Proc Jpn Acad Ser B Phys Biol Sci* 2016;**92**:463–77
28. Alexeyev MF. Is there more to aging than mitochondrial DNA and reactive oxygen species? *FEBS J* 2009;**276**:5768–87
29. Esfahani MS, Lee LJ, Jeon YJ, Flynn RA, Stehr H, Hui AB, Ishisoko N, Kildebeck E, Newman AM, Bratman SV, Porteus MH, Chang HY, Alizadeh AA, Diehn M. Functional significance of U2AF1 S34F mutations in lung adenocarcinomas. *Nat Commun* 2019;**10**:5712
30. Ilagan JO, Ramakrishnan A, Hayes B, Murphy ME, Zebari AS, Bradley P, Bradley RK. U2AF1 mutations alter splice site recognition in hematological malignancies. *Genome Res* 2015;**25**:14–26
31. Yoshida K, Sanada M, Shiraishi Y, Nowak D, Nagata Y, Yamamoto R, Sato Y, Sato-Otsubo A, Kon A, Nagasaki M, Chalkidis G, Suzuki Y, Shiosaka M, Kawahata R, Yamaguchi T, Otsu M, Obara N, Sakata-Yanagimoto M, Ishiyama K, Mori H, Nolte F, Hofmann WK, Miyawaki S, Sugano S, Haferlach C, Koeffler HP, Shih LY, Haferlach T, Chiba S, Nakauchi H, Miyano S, Ogawa S. Frequent pathway mutations of splicing machinery in myelodysplasia. *Nature* 2011;**478**:64–9
32. Thol F, Kade S, Schlarmann C, Löffeld P, Morgan M, Krauter J, Wlodarski MW, Kölking B, Wichmann M, Görlich K, Göhring G, Bug G, Ottmann O, Niemeyer CM, Hofmann WK, Schlegelberger B, Ganser A, Heuser M. Frequency and prognostic impact of mutations in SRSF2, U2AF1, and ZRSR2 in patients with myelodysplastic syndromes. *Blood* 2012;**119**:3578–84

33. Park SM, Ou J, Chamberlain L, Simone TM, Yang H, Virbasius CM, Ali AM, Zhu LJ, Mukherjee S, Raza A, Green MR. U2AF35(S34F) promotes transformation by directing aberrant ATG7 pre-mRNA 3′ end formation. *Mol Cell* 2016;**62**:479–90
34. Palangat M, Anastasakis DG, Fei DL, Lindblad KE, Bradley R, Hourigan CS, Hafner M, Larson DR. The splicing factor U2AF1 contributes to cancer progression through a noncanonical role in translation regulation. *Genes Dev* 2019;**33**:482–97
35. Rossi DJ, Bryder D, Seita J, Nussenzweig A, Hoeijmakers J, Weissman IL. Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. *Nature* 2007;**447**:725–9
36. Nijnik A, Woodbine L, Marchetti C, Dawson S, Lambe T, Liu C, Rodrigues NP, Crockford TL, Cabuy E, Vindigni A, Enver T, Bell JI, Slijepcevic P, Goodnow CC, Jeggo PA, Cornall RJ. DNA repair is limiting for haematopoietic stem cells during ageing. *Nature* 2007;**447**:686–90
37. Yahata T, Takanashi T, Muguruma Y, Ibrahim AA, Matsuzawa H, Uno T, Sheng Y, Onizuka M, Ito M, Kato S, Ando K. Accumulation of oxidative DNA damage restricts the self-renewal capacity of human hematopoietic stem cells. *Blood* 2011;**118**:2941–50
38. Zhang X, Sejas DP, Qiu Y, Williams DA, Pang Q. Inflammatory ROS promote and cooperate with the Fanconi anemia mutation for hematopoietic senescence. *J Cell Sci* 2007;**120**:1572–83
39. Miyamoto K, Araki KY, Naka K, Arai F, Takubo K, Yamazaki S, Matsuoka S, Miyamoto T, Ito K, Ohmura M, Chen C, Hosokawa K, Nakauchi H, Nakayama K, Nakayama KI, Harada M, Motoyama N, Suda T, Hirao A. Foxo3a is essential for maintenance of the hematopoietic stem cell pool. *Cell Stem Cell* 2007;**1**:101–12
40. Naka K, Muraguchi T, Hoshii T, Hirao A. Regulation of reactive oxygen species and genomic stability in hematopoietic stem cells. *Antioxid Redox Signal* 2008;**10**:1883–94

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