Mitochondrial Fus1/Tusc2 and cellular Ca\(^{2+}\) homeostasis: tumor suppressor, anti-inflammatory and anti-aging implications

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**INTRODUCTION**

Execution of cellular programs including proliferation, differentiation, apoptosis, senescence is based on fine-tuned signal transduction cascades. Growth receptor signaling (such as receptor tyrosine kinases or RTK) should be tightly controlled at the spatial and temporal levels to execute appropriate cell decisions. Uncontrolled RTK signaling (via EGFR, HER2/neu, or VEGFR) could be oncogenic and conducive to a variety of other pathologies [1, 2]. Indeed, tumor suppressors integrate various signaling networks pivotal for tissue homeostasis [1, 2]. They control DNA repair (e.g., BRCA1, MSH2), cell cycle progression (RB1, CDKN2A, TGFβ1), angiogenesis (VEGF, ANGPTL4), apoptosis (TP53, BCL2), and cell adhesion (CADM1, FAT, CDH) [1-8]. A separate class of tumor suppressors are miRNAs, small hairpin RNAs regulating gene expression (miR-7, miR-29, miR-145) [5, 6, 9] and https://bioinfo.uth.edu/TSGene/. Loss of TSG functions in malignant cells occurs at the DNA, transcriptional and post-transcriptional levels and may be achieved via different mechanisms. According to the Knudson’s two-hit paradigm (stemming from clinical observations on Rb deficiency), first hit comes from the loss of heterozygosity (LOH) due to deletion or loss-of-function mutation in one of two TSG alleles that can be inherited as a recessive mutation. Second hit causes a biallelic mutation eliminating the function of the remaining TSG allele. This transforms the cell towards malignancy. The effect of haploinsufficiency, when mutation of a single gene copy is sufficient for malignant transformation, has been described for some TSGs (CDKN1B, TP53, NF1, PTEN) [7, 8]. Later, importance of epigenetic silencing via promoter hypermethylation or histone modification was also recognized [7, 8]. At the protein level, TSG products can be functionally inactivated via proteasomal degradation (MDM2/p53 axis) or through altered cellular compartmentalization (mislocalization of SMAD4 in cytosol vs. nucleus) [5]. Transcriptional silencing of TSGs in tumors has also been reported (TWIST/CDH1 repression axis) [5].

In this review, tracing the history of the tumor suppressor Fus1/Tusc2 studies “from bench to bedside”, we discuss what is known of its role in tumor development, inflammation, and aging. Initially characterized as one of several genes belonging to potential 3p21.3 TSG cluster [10-12], recently Fus1/Tusc2 gene enters clinical trials as a gene-based therapy in the form of plasmid DNA encapsulated in cation lipid nanoparticles (REQORSA) in combination with other drugs for patients with non-small cell lung cancer (NSCLC) [13].

Reported ability of Fus1/Tusc2 to inhibit receptor (EGFR, PDGFR, c-Kit) and non-receptor (c-Abl, Akt) tyrosine kinases as well as promote apoptosis [14] makes Fus1/Tusc2 a promising adjuvant for successful anti-tumor chemotherapy (https://clinicaltrials.gov/ct2/show/NCT01455389, https://clinicaltrials.gov/ct2/show/NCT04486833). By affecting the listed above upstream pathways, Fus1/Tusc2 can potentially prevent cancer cell evolution towards drug-resistant variants. During the last years, ongoing revolution in immunotherapy including series of Abs targeting immune checkpoint (IC) molecules altered therapeutic landscape in tumor treatment and significantly improved therapeutic outcomes [15-17]. However, broad spectrum of immune evasion

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mechanisms let cancer cells escape from immune surveillance stimulated by using IC inhibitors in clinics (e.g., expression of alternative IC molecules such as TIM-3 after anti-PD-1 therapy) \cite{18, 19}. This issue was suggested to be solved by using REQORSA as Fus1/Tusc2-based genetic therapy in mouse model of lung cancer, reduced expression of array of IC molecules (TIM-3, CTLA-4, PD-1) concomitant with anti-tumor response \cite{20}.

Noteworthy, studies on mice with the Fus1 loss extended its biological activity beyond tumorigenesis to aging, inflammation, infections, and geriatric conditions \cite{21–23}. Fus1 was demonstrated to be the novel regulator of mitochondrial Ca\(^{2+}\) (miCa\(^{2+}\)) transport \cite{24, 25, 23}. Fine-tuning of Ca\(^{2+}\) signaling by Fus1/Tusc2 may control cellular senescence and its disturbance may contribute to various pathologies.

\textbf{FUS1/TUSC2 GENE DISCOVERY AND FUNCTION}

The pioneering concept describing the importance of loss of chromosomal segments responsible for tumor suppression was put forward by Theodor Boveri in 1914 \cite{26}. In the 1980s, chromosomal abnormalities identified in small cell lung carcinoma (SCLC) demonstrated that SCLC cell lines and tumor biopsies shared the deletion of 3p14-23 region \cite{27, 28}. Further, this chromosomal segment was narrowed down to 3p \cite{21–23, 29} and 3p21 \cite{30} as two most common overlapping deletions in SCLC and other lung cancers. In parallel, it was established that the 3p deletions followed the LOH pattern and matched the Knudson’s two-hit paradigm \cite{31, 32}. The localization of TSGs in the 3p21 region was reinforced by detection of deletions in this area in other solid (e.g., breast, cervix, and renal carcinoma) and hematopoietic (chronic myeloid leukemia) tumors \cite{33–41}. Few research groups used a microcell fusion technique to transfer 3d chromosome into tumor cells. Such hybrid tumor cells displayed signs of senescence, growth arrest, and decreased tumorigenicity in athymic nude mice. This demonstrated a tumor suppressor potential for the entire chromosome 3 \cite{42} and regions 3p21 \cite{43} and 3p21.3 \cite{44}.

To perform detailed mapping of the 3p21 region, initial analysis of growth suppression of human-mouse tumor hybrid cells was done and resulted in the isolation of a subclone HA \cite{3} B99F carrying a 2-Mb fragment of human 3p21-22 chromosome with tumor suppressor function \cite{43}. Further, this region was narrowed down to the 3p21.2-21.3 area using specific chromosomal markers \cite{45}. A long-range physical map spanning about 1.8-Mb DNA over the deleted region helped to construct a 700-kb clone. This library (23 cosmids and one PI phage) included a genetically defined 370-kb segment containing lung cancer TSGs from the 3p21.3 fragment \cite{40}. This led to the identification of a 220-kb segment deleted in primary breast cancer. By overlapping chromosomal fragments deleted in SCLC cells, the TSGs area was narrowed down to the minimal 120 kb deletion nested within three small homologous deletions in the 3p21.3 segment (3p21.3 C or LUCA, 3p21.3 or CER1, and 3p21.3 T or AP20). Within this 120 kb deletion were identified eight genes: HYAL2, Fus1/TUSC2, RASSF1, BLU/ ZMYND10, NPR2L1, 101F6, P6, and CACA2D2 \cite{11}. The original name of TUSC2, Fus1, was introduced based on its position at the junction (FUSion) of cosmids LUCA12 and LUCA13. Fus1 remains to be commonly used even though it is sometimes confused with an unrelated gene, Fus \cite{10}.

Shortly after gene identification, it was demonstrated that overexpression of the Fus1/TUSC2 transgene in Fus1/TUSC2-deficient lung cancer cells suppressed proliferation, blocked G1/S or G2/M transition, and increased doubling time implying a tumor suppressor role for Fus1/TUSC2 \cite{46, 47}. Likewise, intratumoral adenoviral delivery of the Fus1/Tusc2 transgene suppressed tumor growth and lung metastases in mice \cite{46}.

For further insights into the biological role of Fus1, Fus1/Tusc2 \textit{−/−} mice were developed. These animals showed increased frequencies of lupus-like autoimmune conditions (vasculitis, glomerulonephritis, anemia, circulating autoantibodies) and spontaneous vascular tumors \cite{22}, confirming tumor suppressor properties of Fus1. Moreover, these mice exhibited increased susceptibility to irradiation \cite{48, 49}, enhanced response to \textit{A. baumannii} infection \cite{21}, premature aging \cite{23}, hearing loss \cite{50}, and olfactory and spatial memory impairments \cite{51}. These pleiotropic effects of Fus1/Tusc2 loss expanded its role beyond tumor suppression activities. Also, these data suggested that Fus1/Tusc2-dependent therapeutic approaches could alleviate/treat different diseases associated with mitochondrial dysfunction, which is linked to inflammation, infection, metabolic imbalance, and aging.

\textbf{FUS1/TUSC2 LOCALIZATION, MUTATIONS, AND EXPRESSION IN NORMAL AND TUMOR TISSUES}

The Fus1/Tusc2 gene is located in the centromeric segment of 3p21.3 (chromosome 9 in mice) encoding a small (110 aa) protein without recognizable domains \cite{10, 12}. The TUSC2/Fus1 gene is 3.3 kb long and contains three exons, which encode a 1691 bp mRNA with 5'UTR (untranslated region) spanning positions 1–147 and 3'UTR spanning positions 481–169 \cite{10} (Fig. 1A).

At the beginning of 3p21.3 cluster analysis, it was expected that TSGs from this region would possess cancer-associated spectrum mutations. Surprisingly, extensive studies found little or no mutations in the genes from 3p21.3 cluster, and in the Fus1/Tusc2 gene, except for a few lung cancer cell lines with gene deletions and stop mutations. Overall, the mutational rate in this area did not exceed 5% \cite{10}. The identified stop mutation (28 bp deletion at the 3' terminus of the Fus1/Tusc2 exon 2) resulted in the expression of nonfunctional C-terminal truncation at position 82 \cite{47}.

In normal tissues, Fus1/Tusc2 is ubiquitously expressed with the highest levels in all brain regions, followed by blood vessels, stomach, esophagus, colon, adrenal, pituitary, and thyroid glands, skeletal muscle, kidney, spleen, lung, testis, and fallopian tubes (https://gtexportal.org/home/gene/TUSC2).

Fus1/Tusc2 mRNA was detected in lung and sarcoma cancer cell lines and sarcoma tissues, but no protein expression was detected in SCLC or non-small cell lung carcinoma (NSCLC) cells \cite{2, 47, 52, 53}. Low or no protein expression was explained by its reduced half-life in tumor cells due to the loss of myristoylation, a post-translational modification that prevents Fus1/Tusc2 from proteasome-mediated degradation \cite{53}. Detailed information on systemic, cellular and molecular manifestations of Fus1 decrease/loss/increase in normal and tumor tissues is presented in Table 1.

\textbf{FUS1/TUSC2 GENE REGULATION IN NORMAL AND TUMOR TISSUES}

Besides the post-translational modification affecting Fus1/Tusc2 expression due to the loss of myristoylation, its expression can also be regulated at the translational level. At the 5'UTR, the Fus1/Tusc2 mRNA contains two alternative highly conserved open reading frames (uORF1 and uORF2) and a secondary structure, which can suppress ribosomal scanning during translation. The 3'UTR also displays a negative regulatory activity mediated via miRNAs and regulation of mRNA stability \cite{54}. Thus, mir-93, mir-98, and mir-197 target 3'UTR and down-regulate expression of Fus1/Tusc2 mRNA (Fig. 1A, Table 2). Elevated mir-93 and mir-197 expression correlated with reduced Fus1/Tusc2 expression in NSCLC tumors \cite{55}. Other miRNAs suppressing Fus1/Tusc2 mRNA expression include mir-663 in ovarian cancer \cite{56}, mir-19a in lung cancer \cite{57}, mir-378 in mesenchymal stem cells \cite{58}, and mir-584 in thyroid cancer \cite{59}. In triple-negative breast cancer cells, mir-138 binds to the 5'-UTR site on Fus1/Tusc2 mRNA containing translation initiation region and interferes with its translation \cite{60}. An additional layer regulating Fus1/Tusc2 mRNA expression includes two Fus1/Tusc2 pseudogenes (TUSC2P) on
The FUS1/TUSC2 gene was reported in head-and-neck [67] as well as in complementary to several miRNAs: miR-17, miR-93, miR-299-3p, acetylation leads to the opposite effect [64]. As for predominance of H3 acetylation (H3K9ac) over methylation their origin. The expression is reported to be downregulated by [65, 66, 47, 10]. However, partial promoter methylation of the hypermethylation in the neighboring gene nasopharyngeal, and breast cancers, even though promoter CpG methylation in the promoter was demonstrated in lung, 20% of NSCLC cancers [68]. As for histone modifications, predominance of H3 acetylation (H3K9ac) over methylation (H3K9me3) was reported [66].

Physiological stimuli affecting FUS1/TUSC2 mRNA levels vary in their origin. The expression is reported to be downregulated by reactive oxygen species (ROS) [69, 70], and upregulated by hypoxia [58] and the factors stimulating differentiation [71]. Summary of mechanisms regulating Fus1 levels is presented in Fig. 2. However, a fuller understanding of how FUS1/TUSC2 expression is regulated by physiological and pathological stimuli is still lacking.

**PROTEIN MOTIFS AND POSTTRANSLATIONAL MODIFICATIONS OF FUS1/TUSC2**

Human FUS1/TUSC2 is a small (110 aa) protein with an estimated MW of 12 kD. The protein is basic and predicted to have a pI (isoelectric point) of 9.69 [10]. According to a computer-based modeling, FUS1/TUSC2 lacks transmembrane domains, is highly hydrophobic and contains helix-coil domain secondary structures [53]. A 3D model for FUS1/TUSC2 protein is shown in Fig. 1B. Tertiary FUS1/TUSC2 structure is still debatable. Below are the functional motifs identified in the protein sequence.

1. At the N-terminus, FUS1/TUSC2 contains a myristoylation signal (Met-Gly-X-X-Ser/Thr), and experiments confirmed that FUS1 is myristoylated [53] (Fig. 1C).

2. Motif-based profile scanning found a protein kinase A interaction site, an A-kinase anchoring protein interaction site, and a PDZ class II domain [53].

3. *In silico* analysis revealed that protein fragment 54–65 aa is highly homologous to the EF-hand Ca2+-binding domains found in calmodulin as well as in mitochondrial proteins MICU1 and LETM1 [24, 25] (Fig. 1C).
Table 1. Systemic, cellular and molecular manifestations of Fus1 decrease/loss/increase in normal and tumor tissues.

| Type of tumor or normal tissue | Systemic and molecular effects of Fus1 loss/overexpression | Reference |
|-------------------------------|-------------------------------------------------------|-----------|
| 3p21.3-deficient lung cancer cells H1299 and A549. (Fus1 is lost as the part of 3p21.3 deletion) | Overexpression of FUS1/TUSC2 transgene suppresses proliferation, blocks G1/S or G2/M transition, and increases doubling time Intratumoral adenoviral delivery of the FUS1/ TUSC2 transgene suppressed growth of tumor xenografts and inhibited experimental lung metastases in nu/nu mice | [45, 46] |
| Human white blood cells, human keratinocyte cell line HaCaT, human bronchial epithelial cell line BEAS-2B, human breast cancer cell lines MDA-MB231, MB468 and MT-1, a human glioblastoma cell line U87, and a mouse breast cancer cell line 4T1. All cancer cells have lower (TUSC2 expression is lower in all cancer cells than in all normal cells in the study) | Increase of Fus1/Tusc2 mRNA expression after sequestration of miRNAs by TUSC2P inhibits cell proliferation, survival, migration, invasion, colony formation and stimulates tumor cell death | [61] |
| Human NSCLC cell lines A549, H1299, H358, H226, H322, H460, normal human lung fibroblast cell line WI-38. (TUSC2 expression is lower in all cancer cells than in a normal cell line) | Methylstabilization-deficient Fus1/TUSC2 loses its abilities to induce apoptosis and suppress tumor cell proliferation in vitro and promotes tumor growth and metastases in vivo | [53] |
| Murine osteoclasts from bone marrow. Normal Tusc2 levels. | Overexpression of Tusc2 positively regulates osteoclast differentiation induced by RANKL. Tusc2 induces activation of Ca2+-dependent RANKL-mediated NF-kB and CaMKIV/CREB signaling cascades. | [71] |
| Human NSCLC cell lines A549 and H1299, normal human lung fibroblast 32D P210 cells. (TUSC2 expression is lower in all cancer cells than in a normal cell line) | Deletion of B3-110 aa at the Fus1/TUSC2 C-terminus leads to the loss of its ability to inhibit tyrosine kinase c-Abl | [98] |
| Human NSCLC cell lines H1299, H460, A549, H322 and normal human bronchial epithelial cells HBEC. (TUSC2 expression is lower in all cancer cells than in a normal cell line) | Co-expression of FUS1/TUSC2 and p53 synergistically increased apoptosis associated with down-regulation of MDM2 and activation of Apaf/caspase-3 | [113] |
| Anaplastic thyroid cancer cell line B505 C and papillary thyroid cancer cell line TPC-1 (Fus1 level is decreased) | Overexpression of FUS1/TUSC2 increases levels of Smac/ Diablo, suppressor of IAPs blocking caspase- and cytochrome c-mediated apoptosis | [115] |
| Cell-free (Protein Chip array and SELDI-TOF mass spectrometry) | Direct interaction between PDZ domains of FUS1/TUSC2 and Apaf | [116] |
| Fus1 KO mouse model (Fus1 is deleted in all tissues) | Increased frequencies of lupus-like autoimmune conditions (vasculitis, glomerulonephritis, anemia, circulating autoantibodies) and spontaneous vascular tumors, defective NK cell maturation in Fus1 KO mice completely rescued by in vivo injections of IL-15 expressing plasmid. Increased susceptibility to irradiation, enhanced response to A. baumanii infection, premature aging, hearing loss, and olfactory and spatial memory impairments. | [21–23, 48–50] |
| Gastrointestinal epithelial cells from irradiated Fus1 KO mice (Fus1 is deleted) | After in vivo irradiation, epithelial cells demonstrated accelerated cell cycle arrest, aberrant mitosis, lack of proper DNA repair (mitotic catastrophe), early activation of p53, inadequate cellular antioxidant defenses, defective redox homeostasis and death of gastrointestinal crypt cells. IR sensitivity in Fus1 KO cells could be alleviated by antioxidant treatment with Pyridoxamine. | [48, 49] |
| Activated mouse CD4+ T cells (Fus1 is deleted), human tumor cells (Fus1 is silenced) | Deletion or silencing of Fus1/Tusc2 accelerates cell proliferation | [24, 129] |
| Fus1 KO CD4+ T cells, mouse embryonic fibroblasts, kidney epithelial cells (Fus1 is deleted) | Loss of Fus1 altered Ca2+ signaling including mitochondrial Ca2+ accumulation during cytosolic Ca2+ rises, which led to hyperactivation of basal NFAT/NFkB and decreased NFAT/ NFkB activation during Ca2+ elevations induced by cell stimulation | [24, 25, 23] |
| Lung tissues and BALF cells from Fus1 KO mice infected with A. Baumanii (Fus1 is deleted) | Early recruitment of lymphocytes to infection site, early activation of anti-bacterial pathways, (PI3K/Akt/mTOR pathways activation, PTEN downregulation), increased mitochondrial membrane potential and UCP2 (UnCoupled Protein 2) expression | [21] |
| Peritoneal granulocytes from Fus1/Tusc2 KO mice (Fus1 is deleted) | After intraperitoneal injection of asbestos, infiltrating cells demonstrate signatures of enhanced genotoxic stress (elevated γH2AX, DNA damage response molecule, and phosphorylated pro-inflammatory NFkB and ERK1/2) | [70] |
| Gastrointestinal epithelial cells from Fus1/Tusc2 KO mice (Fus1 is deleted) | After in vivo irradiation, epithelial cells demonstrated accelerated cell cycle arrest, aberrant mitosis, lack of proper | [49] |
During translation, Fus1/Tusc2 sequesters miRNAs and prevents their binding to TUSC2 mRNA. mRNA for TUSC2 pseudogene (TUSC2P) is degraded at a faster rate via proteasome machinery. Normal cells; in the absence of Myr tail (in tumor cells), protein is myristoylation (Myr) which is necessary for protein stability in tumor cells. After effective translation, Fus1/Tusc2 undergoes stoppage of ribosomes along TUSC2 mRNA observed in NSCLC.

Table 1. A list of miRNA molecules regulating Fus1/Tusc2 levels in various cancers.

| Name          | UTR on mRNA | Expression, tissue                                                                 | Reference |
|--------------|-------------|-----------------------------------------------------------------------------------|-----------|
| miR-93       | 3’          | Expressed at higher levels in SCLC lines compared to NSCLC lines and immortalized human bronchial epithelial cells (HBECs); miR-93 binds to TUSC2P mRNA (see below) | [55, 61]  |
| miR-663      | 3’          | Ovarian cancer                                                                     | [56]      |
| miR-197      | 3’          | Expressed at higher levels in both SCLC and NSCLC compared to HBECs                 | [55]      |
| miR-19a      | 3’          | Lung cancer                                                                        | [57]      |
| miR-378      | 3’          | Mesenchymal stem cells                                                             | [58]      |
| miR-584      | 3’          | Thyroid cancer                                                                     | [59]      |
| miR-138      | 5’          | Triple-negative breast cancer                                                       | [60]      |
| miR-17       | miR-93      | Binds to TUSC2P mRNA expressed at high levels in normal cells (human white blood cells, human keratinocyte cell line HaCaT, and human bronchial epithelial cell line BEAS-2B), TUSC2P mRNA expressed at low levels in human breast cancer cell lines (MDA-MB231, MB468 and MT-1), a human glioblastoma cell line U87, and a mouse breast cancer cell line 4T1 | [61]      |

Table 2. A list of miRNA molecules regulating Fus1/Tusc2 levels in various cancers.

| Name          | UTR on mRNA | Expression, tissue                                                                 | Reference |
|--------------|-------------|-----------------------------------------------------------------------------------|-----------|
| miR-93       | 3’          | Expressed at higher levels in SCLC lines compared to NSCLC lines and immortalized human bronchial epithelial cells (HBECs); miR-93 binds to TUSC2P mRNA (see below) | [55, 61]  |
| miR-663      | 3’          | Ovarian cancer                                                                     | [56]      |
| miR-197      | 3’          | Expressed at higher levels in both SCLC and NSCLC compared to HBECs                 | [55]      |
| miR-19a      | 3’          | Lung cancer                                                                        | [57]      |
| miR-378      | 3’          | Mesenchymal stem cells                                                             | [58]      |
| miR-584      | 3’          | Thyroid cancer                                                                     | [59]      |
| miR-138      | 5’          | Triple-negative breast cancer                                                       | [60]      |
| miR-17       | miR-93      | Binds to TUSC2P mRNA expressed at high levels in normal cells (human white blood cells, human keratinocyte cell line HaCaT, and human bronchial epithelial cell line BEAS-2B), TUSC2P mRNA expressed at low levels in human breast cancer cell lines (MDA-MB231, MB468 and MT-1), a human glioblastoma cell line U87, and a mouse breast cancer cell line 4T1 | [61]      |

4. The N-terminal fragment of FUS1/TUSC2 (positions 45–110) is 53% homologous to myristoyl-binding domain of recoverin, a typical Ca\(^+\)/myristoyl switch protein. Remarkably, 9 of 11 key amino acids that form myristoyl-binding hydrophobic pocket of recoverin share similarity with the FUS1 protein [24, 25] (Fig. 1C).

5. Fus1/Tusc2 of C. elegans carries a bipartite NLS (nuclear localization signal) (residues 84–101) and possesses weak similarity to DNA-directed RNA polymerase subunit A [10].

6. The N-terminus of Fus1/Tusc2 displays 40% similarity to the DNA-binding domain of IRF7 [69].

7. The Fus1/Tusc2 with pos. 57–65 is identical to the 9 aa transactivation domains of transcription factors (TFs) p53, NFAT, and Nfkb as predicted by 9aa TAD tool [72].

Myristoylation is extremely important for cell localization and functioning of the FUS1/TUSC2 protein [53]. N-myristoylation is a post-translational modification consisting of the removal of the N-terminal methionine from a protein and ligation of the released NH2 group to the residue of myristic acid, a 14-carbon saturated fatty acid (Fig. 1D) [73]. The resulting lipid tail participates in protein folding and anchoring myristoylated proteins to different membrane compartments, where they execute their functions (Fig. 1E) [74, 73, 75]. A mutant form of FUS1/TUSC2 missing the myristoyl tail has a shorter half-life (6 h vs 12 h) due to improper protein folding and increased proteasome degradation [53]. Also, myristoylation-deficient FUS1/TUSC2 loses its characteristic mitochondria/ER localization and its abilities to induce apoptosis and suppress tumor cell proliferation in vitro. Importantly, it also acquires the abilities to

Fig. 2 Transcriptional, post-transcriptional, and post-translational regulation of Fus1/Tusc2. Fus1/Tusc2 gene is regulated on transcriptional level by different physiological and pathological factors such as ROS, hypoxia, and differentiation molecules (e.g., RANKL). In some tumors (e.g., NSCLC), DNA methylation may lead to down-regulation of TUSC2 gene transcription. Binding of microRNAs (miRs) to 3’ and 5’ untranslated regions (UTR) of TUSC2 mRNA suppresses its translation. mRNA for TUSC2 pseudogene (TUSC2P) sequesters miRNAs and prevents their binding to TUSC2 mRNA. During translation, 5’ UTR can adopt conformation preferring stoppage of ribosomes along TUSC2 mRNA observed in NSCLC tumor cells. After effective translation, Fus1/Tusc2 undergoes myristoylation (Myr) which is necessary for a protein stability in normal cells; in the absence of Myr tail (in tumor cells), protein is degraded at a faster rate via proteasome machinery.
promote tumor growth and metastases in vivo [53]. Therefore, loss of myristoylation may be considered a key event that leads to insufficiency of Fus1 function, when compared to mutations and hypermethylation [14, 53].

The predicted Ca\(^{2+}\) EF hand binding motif and hydrophobic binding pocket of Fus1/Tusc2 prompted it to be classified as a novel calcium/myristoyl switch protein (Fig. 1D) [24]. Its involvement in Ca\(^{2+}\)-dependent signaling have been reported for different cell types including CD4\(^+\) T lymphocytes, kidney epithelial cells, osteoclasts, and mouse embryonic fibroblasts [71, 23, 24, 23, 25]. The presence of myristoylation and Ca\(^{2+}\) binding motifs in one protein is pivotal for regulation of cellular processes. In has been established that Ca\(^{2+}\)/myristoyl switch proteins, in response to Ca\(^{2+}\) binding to EF-hand motifs, release their lipid myristoyl tail from a protein hydrophobic pocket and anchor it to membranes during Ca\(^{2+}\) elevations (Fig. 1E) [74, 75].

The Fus1/Tusc2 protein can undergo other posttranslational modifications such as arginine mono-methylation (R9) [76], phosphorylation (SS0, site for PKA and RSK) [77], and acetylation (K93) [78]. Moreover, NetPhos 3.1 software (http://www.cbs.dtu.dk/services/NetPhos/) predicts phosphorylation at S4 (site for Ser/Thr kinases) (Fig. 1C). Thus, several functional protein motifs and a spectrum of potential posttranslational modifications facilitate Fus1/Tusc2 involvement in sensing and execution of various cellular programs.

**FUS1/TUSC2 IN MITOCHONDRIAL Ca\(^{2+}\) REGULATION**

**Mitochondrial Ca\(^{2+}\) transport: main players**

Mitoco\(^{2+}\) transport is balanced by Ca\(^{2+}\) import and export. The main route for mito\(^{2+}\) entry is mitochondrial Ca\(^{2+}\) uniporter holocomplex (MCUcx), consisting of membrane channel protein MCU (mitochondrial Ca\(^{2+}\) uniporter) and MCU regulatory proteins MICU1, MICUB, MCRY1, EMRE [79–81]. MCU is an inner mitochondrial membrane protein that forms a pentameric channel with a selectivity filter for Ca\(^{2+}\) ions (a DIME motif) (Fig. 3A) [82, 83]. MCU becomes permeable for Ca\(^{2+}\) when ion concentration reaches 1-10 \(\mu\)M, which is only possible at the contact with the endoplasmic reticulum (ER) where Ca\(^{2+}\) is released via inositol-1,4,5-triphosphate receptors (IP3Rs). The 1-10 \(\mu\)M Ca\(^{2+}\) threshold is set by the Ca\(^{2+}\)-binding EF-hand motif of the mitochondrial protein MICU1 (mitochondrial Ca\(^{2+}\) uptakeI) (Fig. 3A) [84, 85]. In the absence of MICU1, MCU transports Ca\(^{2+}\) at much lower cytoplasmic concentrations (hundreds of nM range).
accompanied by mitoCa2+ overload [80]. However, other reports suggested that the lack of MICU1 inactivates MCU in a Ca2+-dependent manner at a faster rate via autophosphorylation. Accordingly, MICU1 plays a gatekeeper role for MCU and prevents its premature inactivation [86]. MICU1 heterodimerizes with its analogs, MICU2 and MICU3. Thus, MICU1/MICU2 heterodimer fine-tunes Ca2+ currents: MICU1 stimulates Ca2+ uptake at high cytosolic Ca2+ level while MICU2 inhibits MCU at low cytosolic Ca2+ content [87].

The importance of MCU in tumor growth is based on its involvement in cell death. Malignant cells benefit from reduction of mitoCa2+ uptake. This is especially important as tumor cells experience increased ROS production. Interestingly, MCU contains ROS sensing cysteines in its N-terminus. Their oxidation promotes MCU/α subcomplex, persistent channel activity, and mitoCa2+ overload following by opening the permeability transition pore, which triggers cell death (Fig. 3B) [80]. However, for some breast cancers it was reported that activation of MCU promotes cancer cells motility, invasion, and growth [79]. So, it is not surprising that mitoCa2+ accumulation undergoes regulation by tumor suppressors. Thus, p53 directly interacts with the Ca2+/Ca2+ ATPase in ER, promotes its negative oxidative modification, and stimulates enhanced Ca2+ release from ER with further Ca2+ transfer to mitochondria via the MCU protein (Fig. 3A) [88]. The lipid/protein phosphatase PTEN binds IP3R and, thus, counteracts PKB/Akt phosphorlyating and inhibiting IP3R; this results in a higher mitochondrial Ca2+ accumulation and apoptosis (Fig. 3A) [89]. On the other hand, down-regulation of the MCU mRNA by miR-25 is accompanied by increased cancer cell survival and apoptosis resistance (Fig. 3A) [90].

Extrusion of Ca2+ from mitochondria into cytosol is mediated by NCLX (Na+/Ca2+/Li+), a mitochondrial form of Na+/Ca2+ exchanger (mNCX), and Ca2+-binding EF-hand motif containing LETM1, a 2H+/Ca2+ exchanger (Fig. 3A) [79, 91]. Diminished NCLX activity leads to mitoCa2+ overload and elevated ROS production due to the activation of Krebs cycle [92]. In a few tested non-tumor cell models, these events led to cell death. In cancer cells, however, although increased ROS blocked cell proliferation, they also induced metastasis via the ROS/HIF1α signaling axis [93]. Therefore, Ca2+ transients in mitochondria play a decisive role in the normal and tumor cell biology.

Potential mechanism of mitochondrial Ca2+ regulation by Fus1/Tusc2 protein

Localization of Fus1/Tusc2 in mitochondria and the discovery of a Ca2+-binding domain in its structure [22, 24] suggested that Fus1/Tusc2 could regulate mitoCa2+ accumulation. Indeed, Fus1 loss reduced mitoCa2+ accumulation resulting in retention of Ca2+ in cytosol [24, 23]. Like MICU1, Fus1 showed a dual effect on mitoCa2+: Fus1-deficient cells had increased steady-state mitoCa2+ levels, while decreased cytoCa2+ levels. Moreover, during Ca2+ response, Fus1—mitochondria accumulated Ca2+ at a faster initial rate than WT mitochondria and reached higher Ca2+ values at the peak of response. However, levels of mitoCa2+ declined faster during the recovery phase of Ca2+ response in Fus1-deficient cells [24, 23, 25]. This effect of Fus1/Tusc2 deficiency on mitoCa2+ was partially alleviated by inhibition of mNCX via arrhythmia sodium channel [24, 25]. Accordingly, Fus1/Tusc2 may set a threshold for Ca2+ uptake similar to MICU1 and prevent mitoCa2+ accumulation at steady-state or rapid Ca2+ accumulation after initial rise in cytoCa2+ (Fig. 3C, left). However, when cytoCa2+ concentration reaches high values, we propose that Fus1/Tusc2 binds Ca2+ ions, and releases its lipid tail that anchors protein to the mitochondrial membrane, thus maintaining mtoCa2+ uptake. Finally, Fus1/Tusc2 inhibits mNCX and promotes maximal Ca2+ accumulation in mitochondria (Fig. 3C, right) [24, 23, 25]. When levels of cytoCa2+ start recovering, drop in Ca2+ initiates reversed events: Ca2+ import via MCU declines and mNCX accelerates Ca2+ export out of mitochondria. Although regulation of cytoCa2+ by mitochondria is complex, usually deficiency in MCU translates into cytoCa2+ retention (Fig. 3D) [94]. Sustained cytoCa2+ results in prominent activation of Ca2+-dependent proteins, i.e., NADPH oxidase [95], CAMKII [94] or Miro1 [80].

Initially, it was proposed that MICU1 regulates MCU-mediated Ca2+ currents via Ca2+/myristoyl switch mechanism [96]. However, the MICU1 and MCU interaction is mediated via direct protein binding. At the MICU1 C-terminus, KQRLMRGL peptide represents an MCU-binding domain interacting with with DIME motif via salt bridges [97]. Structural analysis of the MCU N-terminal domain (NTD) surprisingly revealed unidentified lipid molecule bound to a hydrophobic protein surface formed by residues in the L1 loop, two helices (a2 and a3) and C-terminal tail. This lipid was described as a linear lipid-like structure consisting of 13–16 carbon atoms that is similar to a tetra-ethylene glycol molecule [67]. It is noteworthy that myristic acid (a substrate for N-terminal myristoylation) is a saturated linear long-chain lipid with a 14-carbon backbone complying with the features of an unidentified lipid. Therefore, Fus1/Tusc2 could regulate the activity of MCU via a novel mechanism of inter-protein lipid tail exchange. We suggest that the myristic acid residue of Fus1/Tusc2 protein released from the hydrophilic pocket after Ca2+ binding to EF-hands could interact with the hydrophobic surface of MCU and maintain its Ca2+ transport function (Fig. 4A). This is likely in view of the fact that Fus1/Tusc2 has the amino acid sequence KARGLWPF resembling MCU-binding domain of MICU1–3 proteins (Figs.1, 4B and C). Moreover, Fus1/Tusc2 can potentially interact with MCU via its inter-protein interaction site at the C-terminus (positions 81–96) (Figs. 3 and 4C). This mechanism needs further elucidation by functional and structural studies.

Putative Fus1 protein-protein interaction via myristoyl tail exchange

An intriguing opportunity for protein-protein interactions was demonstrated by mutational analysis of Fus1/TUSC2 binding to a pro-tumorigenic tyrosine kinase c-Abl [98, 99]. Initiated by discovery of a truncated form of Fus1/TUSC2 (deletion of C-terminal 83-110 aa) in tumor cells, Lin et al. showed that the Fus1/TUSC2 81-96 aa C-terminal peptide linked with stearate inhibits c-Abl activity and facilitates its degradation. On the contrary, the Fus1/TUSC2 N-terminal peptide 1-80 aa, although capable of binding to c-Abl, was unable to inhibit its kinase activity [98]. Like Fus1/TUSC2, c-Abl is a myristoylated protein. It is activated by releasing the myristoyl tail from the hydrophobic pocket and conforming to an active open state, which allows c-Abl to bind its substrates via the SH2-domain [100]. Fus1/TUSC2 mutational analysis showed that while the N-portion of Fus1/TUSC2 binds to myristoyl-binding pocket of c-Abl, the C-terminal portion of Fus1/TUSC2 (containing c-Abl inhibitory peptide 81-96 aa) interacts with the ATP-binding site in the N-lobe of c-Abl. Alternatively, Fus1/TUSC2 could interact with an activation loop in the C-lobe of c-Abl [99].

Initially, it was thought that myristoylation is exclusive for targeting proteins to certain membrane compartments but, nowadays, this posttranslational modification is appreciated to play roles in protein folding and prevention from premature degradation [100, 101, 53]. A synthetic ligand for myristoyl binding pocket named GNF-5 maintains closed inactive conformation of c-Abl apo form or convert open conformation to a closed one [102]. Therefore, one could speculate that the myristoyl tail of Fus1/TUSC2 inserted into the hydrophobic pocket of c-Abl inhibits its tyrosine kinase activity. This tail-pocket interaction can be an important feedback loop in the network of interactions initiated by RTKs. Thus, platelet-derived growth factor receptor (PDGFR) stimulates signaling molecules such as PLCγ1, c-Src, and c-Abl. In turn, PLCγ1 generates IP3 and promotes Ca2+ release from the ER [103].
We suggest that activation of FUS1/TUSC2 by Ca\(^{2+}\) leading to the release of its myristoyl tail could suppress c-Abl activity. Noteworthy, c-Abl-deficient B cells exhibited reduced Ca\(^{2+}\) flux in response to antigen receptor or CD19 stimulation [104] reinforcing the concept of mutual regulatory loops between FUS1/TUSC2 and c-Abl.

**Ca\(^{2+}\) signaling fine-tuned via Fus1/Tusc2 impacts cell fate**

Maintaining mitoCa\(^{2+}\) at moderate levels is important due to the effect of Ca\(^{2+}\) on ROS production and cell death enhancing effect of Ca\(^{2+}\) overload [53, 77]. The MICU1-3 proteins fine-tune MCU activity by setting a threshold to filter out mitoCa\(^{2+}\) elevations at low cytotoxic Ca\(^{2+}\) levels (<350 nM) and cooperatively increasing MCU currents at high Ca\(^{2+}\) elevations (Fig. 3A) [89, 97]. Similarly, Fus1/TUSC2 may control basal mitoCa\(^{2+}\) by inactivating MCUcx, which prevents rapid initial Ca\(^{2+}\) accumulation and promotes adequate mitoCa\(^{2+}\) elevation (Fig. 3B) [23, 24, 25, 26]. This translates into appropriate activation of Krebs cycle and sufficient formation of antioxidants (NADH, NADPH) maintaining ROS at low levels. Accordingly, loss of antioxidant defense in Fus1-deficient cells [24, 25, 49, 69], would reduce cell proliferation, tissue repair, and promote cell death. Indeed, MICU1 deficiency accompanies compromised liver regeneration after partial hepatectomy due to inflammation, overloaded mitoCa\(^{2+}\), blocked proliferation, and increased necrosis of hepatocytes [105].

Compromised capability of Fus1\(^{-/-}\) adult stem cells to repopulate tissues was reported in aged Fus1\(^{-/-}\) animals (e.g., hair follicles, thymus) [23] or younger mice after exposure to radiation (e.g., GI crypt epithelial cells, melanocyte stem cells) [49]. In vitro data suggest that Fus1 is involved in bone remodeling shaped by bone deposition (osteoblasts) and resorption (osteoclasts) [106]. Silencing of Fus1/Tusc2 gene in bone marrow precursor cells in vitro diminished RANKL-induced osteoclast differentiation without affecting osteoblast formation [71]. Thus, it is possible that Fus1/Tusc2 loss in vivo could also affect bone remodeling, shifting it towards bone deposition that would reduce tissue repair potential [106] corroborating with accelerated aging and development of age-related disorders in Fus1\(^{-/-}\) mice [23].

Thresholds in the mitogenic signal transduction demand tight control of activation of proteins involved in cell proliferation. Malfunction of key tumor suppressors (e.g., E3 ligase PML) may switch cell fate from senescence to malignant transformation [50, 107]. The ability of Fus1/Tusc2 to calibrate Ca\(^{2+}\) responses would translate into adequate cell responses based on the characteristics of input signals such as signal strength. In this regard, Fus1/Tusc2 reminds other tumor suppressors (e.g., PTEN, Spry), which prevent cells from over-stimulation by mitogenic signals maintaining their survival and responsiveness to proliferation signals [108–112].

**REGULATION OF APOPTOSIS BY FUS1/TUSC2**

Numerous studies demonstrated that Fus1/Tusc2 overexpression in cancer cells that lack 3p21.3 or Fus1/Tusc2 gene expression induces cell death [2, 46, 47, 53, 113, 114]. Co-expression of Fus1/Tusc2 and p53 synergistically increased apoptosis in NSCLC cells.

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**Fig. 4 Hypothetical mechanisms of Fus1/Tusc2 and MCU (mitochondrial Ca\(^{2+}\) uniporter) interaction. A** A proposed mechanistic model of inter-protein interaction between Fus1/Tusc2 and MCU. At low Ca\(^{2+}\) levels, Fus1/Tusc2 maintains a closed conformation with its myristoyl tail hidden in a hydrophobic pocket that promotes protein folding, stabilizes inactive state, and prevents premature protein degradation. At high Ca\(^{2+}\) levels, myristoyl tail is released triggered by Ca\(^{2+}\) binding to EF-hand of Fus1/Tusc2. Two possible scenarios could be proposed after the tail release: [1] the lipid tail anchors Fus1/Tusc2 to the membrane followed by the Fus1/MCU interaction in the mitochondrial intermembrane space (IMS) or matrix side of the inner membrane (IMM), or [2] the lipid tail of Fus1/Tusc2 binds the hydrophobic surface of the MCU N-terminal domain (NTD) and affects activity/kinetics of the mitochondrial channel. **B** The Fus1/Tusc2 protein motif, which may interact with the MCU DIME motif (Ca\(^{2+}\) selectivity filter). The 7-15 aa fragment from Fus1/Tusc2 was compared with motifs of the MICU1-3 proteins responsible for binding to MCU in the IMS. Critical amino acids responsible for formation of salt bridges with the DIME motif in MCU are shown in red. Blue/green color highlights identical residues in similar positions in Fus1/Tusc2 as compared to MCU1-3 proteins. **C** Docking simulation of predicted interaction between the MCU/EMRE complex and Fus1/Tusc2 protein. In the configuration 1, Fus1/Tusc2 interacts with MCU close to its DIME sequence (Ca\(^{2+}\) selectivity filter) in the mouth of the channel. Lys (K) and Arg (R) from the Fus1/Tusc2 motif homologous to MCU-binding motif of MICU1-3 (KxRxRgx) (see Fig. 4B) are positioned against the DIME motif. Critical Ser and Asp residues in the DIME motif required for formation of salt bridges as well as potential Fus1/Tusc2-binding sequence (including K and R) are marked with licorice sticks. (see 3B and text). In the configuration 2, Fus1/Tusc2 interacts with the MCU/EMRE complex via the 81-96 aa fragment (highlighted in blue) that was experimentally shown to be involved in the inter-protein interactions. Docking simulation has been performed using the ClusPro server [143] and visualized using PyMOL software (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC; https://pymol.org/2/#page-top). The MCU sequence was retrieved from the Protein Data Bank (acc # 6O5B).
This synergistic effect was associated with the ability of Fus1/Tusc2 to down-regulate expression of MDM2, an E3 ubiquitin ligase that suppresses p53, and thereby stabilizes p53 levels. Importantly, combined effect of Fus1 and p53 co-expression required Apaf-1 that mediates mitochondria- and caspase-dependent apoptosis [113]. Additionally, in thyroid cancer cell lines, overexpression of FUS1/TUSC2 increased levels of Smac/Diablo that blocks caspase regulatory inhibitors of apoptosis proteins and cytochrome c [115]. Protein Chip array and SELDI-TOF mass spectrometry revealed direct interaction between PDZ domains of FUS1/TUSC2 and Apaf [116] corroborating the link of FUS1 with the Apaf-1-mediated apoptosis.

**FUS1/TUSC2 IN CELLULAR SENOENCE**

**Cellular senescence: main players**

Cellular senescence refers to cellular aging characterized by a highly stable cell cycle arrest accompanied by biochemical and morphological alterations [117, 118]. The phenomenon of a limited number of cell divisions discovered by Leonard Hayflick in 1961 became known as the Hayflick limit [119]. A role of p53 in senescence is evident from its effect on cell cycle arrest usually mediated by up-regulation of p21, an inhibitor of cyclin-dependent kinases CDK1, CDK2, and CDK4/6 required for the G1/S transition [120, 121]. Senescence can be induced by oncogene overactivation, oxidative stress, genotoxic drugs, radiation, CDK suppression, demethylating and acetylating agents, etc. [121]. Cell cycle arrest accumulates unphosphorylated form of Rb protein, upregulating the lysosomal compartment and subsequently stimulating mTOR signaling (Fig. 5) [122], mTOR signaling, in turn, upregulates mitochondrial biogenesis via TF PGC1α. Increased mass and dysfunctional state of mitochondria enhance intracellular ROS and DNA damage. Downstream of this cascade, DNA damage kinases (i.e., ATM) further activate the AKT/mTOR signaling axis stimulating PGC1α and creating a positive feedback loop stabilizing senescent state (Fig. 5) [117, 118].

DNA damage is responsible for activation of the NFκB pathway regulating senescence-associated secretory phenotype (SASP); senescent cells secrete proinflammatory cytokines (IL-1β, IL-8, IL-6), chemokines (MCP-1, CCL3, CXCL1), matrix metalloproteinases (MMP3, MMP9), etc. (Fig. 5) [123, 117, 118]. Moreover, SASP reinforces senescence via autocrine pathway and induces it in neighboring cells. Secreted cytokines and chemokines attract immune cells necessary for clearance of senescent cells (Fig. 5). However, accumulation of senescent cells results in the development of chronic inflammatory disorders [124, 125, 121]. Initially considered an in vitro phenomenon, senescence was recently confirmed in vivo; transplantation of senescent cells from ear cartilage into knee joint caused an osteoarthritis-like phenotype in mice [126]. Also, senescent fibroblasts can promote growth and proliferation of tumor cells via secretion of SASP intermediates (e.g., fibroblast growth factors 10 and 19, IL-1β), epithelial-mesenchymal transition (IL6, MMP2-3), and immune evasion mechanisms (i.e., IL-6 drives accumulation of suppressive myeloid cells and their activity) [124]. Therefore, senescence and SASP-mediated chronic inflammation could underlie the development of aging-related disorders including tumors.

**Ca²⁺ signaling and senescence**

Fine-tuned control of cellular Ca²⁺ signaling by tumor suppressors and oncogenes [127] established that increased cellular Ca²⁺ (cytosolic and/or mitochondrial) results in senescence or apoptosis, whereas moderate levels of Ca²⁺ favor cell proliferation. Ca²⁺ regulates SASP via activation of calpain that converts pro-IL-1α into functional IL-1α [128]. Another Ca²⁺ effector is NFAT, a TF regulated by calmodulin/calciurein complex that has both pro-proliferative and pro-senescent effects. In particular, NFAT induces p53 by suppressing expression of ATF3, a p53 negative regulator. NFAT acts as a pro-senescent factor by activating expression of IP3R2 that mobilizes Ca²⁺ from ER elevating cytosolic and mitoCa²⁺ levels [128].

IP3Rs are instrumental in cancer cells, where the number of oncogenes and tumor suppressors cross their paths [127]. Pro-survival/pro-tumorigenic PKB/Akt phosphorylates and inactivates IP3R3. Tumor suppressor protein PML binds to IP3R3 and recruits protein phosphatase PP2A to dephosphorylate IP3R3 leading to IP3R3-marked opening (Fig. 3A). Consequently, Ca²⁺ accumulation in mitochondria would increase ROS production leading to a senescent state (Fig. 3B). It is noteworthy that abrogation of MCU helps cells avoid senescence [128] and apoptosis, and promotes uncontrolled proliferation, migration, and metastases [80].

**Fus1/Tusc2-dependent processes mediating cellular senescence**

**Proliferation.** The role of Fus1/Tusc2 in the control of senescence appears to be a part of its role as a TSG. FUS1/TUSC2 overexpression in tumor cells is associated with cell cycle arrest at G1/S [47] and G2/M [115] transition checkpoints. Deletion or silencing of Fus1/Tusc2 accelerates proliferation of activated mouse CD4⁺ T cells [24] and human tumor cells [129]. Upregulation of miR-197 miRNA that increases FUS1/TUSC2 levels and, thus, suppresses tumor metastasis was attributed to the ability of Fus1 to regulate proliferation of human glioblastoma cells [129].

**Genotoxic stress.** In several models of cell injury, Fus1/−/− cells showed increased levels of genotoxic stress and senescence markers compared to wild-type counterparts. For example, intraperitoneal injection of asbestos, infiltrating cells from Fus1/−/− mice had higher levels of γH2AX, DNA damage response molecule, and phosphorylated pro-inflammatory NFκB and ERK1/2. This was associated with increased number of macrophages and accelerated accumulation of granulocytes in the peritoneal cavity. Among others, IL-1β, a signature cytokine of SASP, was up-regulated in peritoneal Fus1/−/− cells.
The increased sensitivity to irradiation in Fus1−/− mice was accompanied by accelerated cell cycle arrest, aberrant mitosis, lack of proper DNA repair (mitotic catastrophe), early activation of p53, and death of gastrointestinal crypt cells, which are especially susceptible to ionizing radiation [49]. Thereby, Fus1-deficient mice demonstrated enhanced cell damage upon challenging stimuli like other Tsg deficiency models (i.e., p27) [8].

Clearance of senescent cells. The most striking alteration in the chemokines/cytokines profile of Fus1-deficient mice was down-regulation of RANTES/CCL5, a chemokine for T and NK cells [70]. Since T and NK cells are necessary for clearance of senescent cells [130], lack of CCL5 production in Fus1−/− mice could lead to accumulation of senescent cells during the course of lifetime and result in chronic inflammation, aging-related diseases, and malignant transformation.

CONSEQUENCES OF FUS1/TUSC2 DEFICIENCY AT ORGANISMAL LEVEL

Fus1/Tusc2-associated systemic pathologies

Several Fus1-dependent pathologies developing in mice have been reported following the targeted Fus1 inactivation. These could be divided into two groups:

Spontaneous pathologies developed in young or middle-aged mice and progressing with time.

a. Chronic systemic inflammation [23].

b. Progressive development of SLE-like autoimmune disease in some mice (incomplete penetration) (vasculitis, glomerulonephritis, anemia, circulating autoantibodies) [22].

c. Increased frequency of spontaneous vascular tumors [22].

d. Preponderance of aging signs that include lordokyphosis, absence of vigor, diminished hair regrowth, reduced sperm count and motility, enlarged seminal vesicles, and compromised stem cells self-renewal [22, 23].

e. Early development of aging-associated diseases:

1. Premature progressive hearing loss (higher threshold for sound intensity, longer latency to respond to sound, and smaller amplitude in auditory brainstem responses (ABR) waves, compared to wild-type mice [50]. The hearing loss corresponded with impaired PTEN/Akt/mTOR pathways in cochlear cells and was ameliorated by administration of N-acetyl cysteine, an antioxidant agent [50].

2. Impairments in olfactory and spatial memory at a relatively young age (4-5 months old), as indicated by habituation test, hidden cookie test, and Morris water-maze test [51].

Injury-induced pathologies in young mice.

a. Higher sensitivity to γ-irradiation [48, 49].

b. Higher sensitivity to peritoneal asbestos injury [70].

c. Resistance to A. baumanii lung infection [21].

Most likely, defects in common Fus1-dependent mechanism(s)/pathways that are discussed below underlie these pathologies.

The mTOR pathway activation

Fus1−/− mice showed prominent mTOR signaling activation and oxidative stress, signature hallmarks of senescence and early aging. In young Fus1−/− mice, cochlear cells are distinguished with reduction of antioxidant enzymes (mitochondrial SOD2, PRDX1) and concomitant activation of the Akt/mTOR pathway (decrease in PTEN levels, up-regulation of phospho-Akt and S6). This molecular pattern was associated with low-grade chronic inflammation observed in bone marrow cells in the temporal bone surrounding the cochlea [50]. During senescence, activation of Akt/mTOR (for example, via DNA damage/ATM pathway) stimulates PGC1α followed by up-regulation in mitochondrial biogenesis. Mitochondria-derived ROS damage DNA and, thereby, maintain mTOR activation. Additionally, mTOR regulates translation of mRNA related to SASP via MAP kinase-activated protein kinase 2 phosphorlyating protein ZFP36L1 responsible for mRNA degradation [117]. Therefore, it is not surprising that chronic inflammation accompanies senescent phenotype of cochlear Fus1−/− cells [50].

Oxidative stress

Increased ROS stabilize p21, a p53 activator and key suppressor of cell proliferation in senescence, and inhibit autophagy 23. Increased ROS production was evident in Fus1/+/Tusc2-deficient head-and-neck cancer cells JHU012 [70] and in mouse splenocytes [24]. Additionally, cochlear cells from Fus1−/− mice demonstrated up-regulation of antioxidant defense proteins (Prdx1) in steady-state epithelial cells as well as gradual decrease in the expression of Sod2 and Prdx1 [50]. Further, Fus1−/− primary mouse embryonic fibroblasts and immortalized kidney epithelial cells showed defects in respiration such as significantly decreased maximal mitochondrial respiration and respiratory reserve capacity, likely due to down-regulation of mitochondrial respiratory proteins [127]. However, upon challenging conditions (i.e., irradiation) Fus1−/− deficient cells displayed delayed up-regulation of Sod2, a mitochondrial form of antioxidant superoxide dismutase [49]. Treatment with antioxidants (Tempol, pyroxidamine) restored Sod2 expression and significantly improved survival of whole-body irradiated Fus1-deficient mice [48]. Another antioxidant, N-acetylcysteine, rescued expression of Prdx1 and respiratory chain compounds, restored normal mitochondrial morphology, and prevented progression of death in Fus1−/− animals [50].

Disruption of mitochondrial Ca2+ homeostasis

Increased ROS production in Fus1+/+/Tusc2-deficient tissues may derive from Fus1 ability to regulate mitoCa2+ transport [23-25]. Indeed, inhibition of MICU1 via Akt/PKB phosphorylation increased ROS production and downstream Akt/PKB activation [85, 90]. The deficiency in mitoCa2+ leads to elevated cytosolic Ca2+ and activation of Ca2+-dependent proteins in the cytosol. For example, Ca2+-dependent stimulation of Miro1 results in the remodeling of long filamentous mitochondria into globe-shaped mitochondria, step prerequisite for autophagosomal degradation [80]. Indeed, presence of globule-shaped giant mitochondria in Fus1-deficient epithelial and cochlear cells point to preferred mitochondrial fission in these cells [24, 51]. This process should activate mitophagy, but in Fus1−/− mice it rather leads to accumulation of nonfunctional mitochondria. Fus1−/− cochlear cells displayed a major down-regulation in the expression of PTEN-induced kinase-1 (PINK1) [51], a sensor of mitochondrial quality control, which directs dysfunctional mitochondria towards autophagy/mitophagy [131]. Importantly, PINK1 protects cells from oxidative stress and premature senescence [132]. Therefore, Fus1−/− deficient cells accumulate dysfunctional mitochondria leading to further increase in ROS production and senescence [50].

Inflammation

Transcriptomic analysis of Fus1+/−/−CD4+ T cells revealed that at the basal level T lymphocytes significantly up-regulate gene expression of secretory pro-inflammatory markers such as Mmp8-9, S100a8-9, Lcn2, Ltf, Retnlg [24]. Some of these markers (S100a8-9, Mmp8-9) are signatures for SASP [133-135] and associate with chronic inflammation observed in Fus1−/− mice [23, 50, 70]. Persistent oxidative stress leads to chronic inflammation, whereas senescence limits stem cell turnover culminating in early aging. Therefore, Fus1+/Tusc2 loss links systemic aging to early
senescence, geriatric diseases as well as to tumor escape from immunosurveillance [136].

CONCLUSIONS AND CLINICAL RELEVANCE

The Fus1-mediated cellular homeostasis is at the crux of its tumor suppressor, anti-inflammatory, and anti-aging activities. Developing Fus1-based genetic therapies for cancer patients became an apparent step after revealing its TSG properties.

In preclinical studies, intra-tumoral injection of cationic liposome nanoparticles complexed with plasmid DNA encoding Fus1/Tusc2 gene (REQORSA or quаратасугенез озеплазмид formerly known as Oncoprex) significantly inhibited growth of human NSCLC cells H1299 and A549 in subcutaneously inoculated mice. Administered intravenously, REQORSA suppressed metastases and extended survival of tumor-bearing mice [114]. Phase-I clinical trial reported the effective dosage and safety of REQORSA [13].

Conventional therapy for metastatic NSCLC currently uses epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors such as erlotinib, gefitinib, and osimertinib [137]. Beside toxicity, this type of therapy often leads to treatment-resistant cancer as a result of selecting tumor cell clones carrying mutations. Thus, continuous treatment with erlotinib prescribed to NSCLC patients with overexpression or mutations in EGFR (deletion in 19th exon or L858R point mutation), leads to accumulation of cell clones with T790M mutation in EGFR or up-regulation in other growth receptor signaling pathways (e.g., HGF, RB33/Pi3K) leading to drug resistance. Osimertinib allows to overcome this issue as it can block EGFR mutated at T790M site; however, resistance to this treatment can also develop after losing tumor cells with T790M [137]. Therefore, combination of EGFR inhibitors with other treatments affecting upstream signaling pathways involved into drug resistance is highly desirable. Ability of Fus1/Tusc2 to suppress receptor and non-receptor kinases as well as sensitize NSCLS cells to chemotherapy [14, 98, 138, 139] makes this tumor suppressor a promising therapeutic candidate for combinatory therapy. Currently, two clinical trials based on combination of Fus1-gene based drug REQORSA and EGFR inhibitors erlotinib (https://clinicaltrials.gov/ct2/show/NCT01455389) and osimertinib (Acclaim-1, https://clinicaltrials.gov/ct2/show/NCT04486833) are underway for treatment of NSCLC patients.

Ongoing revolution in immunotherapy started with a clinical introduction of checkpoint inhibitors significantly improved therapeutic outcomes in patients with different types of cancer (melanoma, breast cancer, colon cancer, Hodgkin lymphoma, etc.) [15–17]. However, effectiveness of immunotherapeutics is limited due to evasion of tumor cells from immunosurveillance mechanisms (e.g., up-regulation of TIM-3 immune suppressive molecules on cancer cells after PD-1 blocking therapy) eventually leading to tumor resistance similar to chemotherapy [18, 19]. Thus, treatments with drugs targeting pathways upstream of drug resistance would be beneficial.

Overexpression of Fus1/Tusc2 down-regulates mTOR signaling, which stimulates PD-L1, an immunosuppressive ligand up-regulated in many types of tumors including NSCLC. Fus1/Tusc2-induced decrease of PD-L1 expression in response to its main inducer, IFN gamma, modifies tumor microenvironment, unleashes T and NK cells from inhibition, and allows effective use of PD-1 blockers [24, 140]. REQORSA and anti-PD-1 combined therapy demonstrated significantly stronger immune response than individual therapies. This resulted in the development of favored anti-tumor response (Th1 differentiation, NK and CD8+ CTL recruitment) and down-regulation of immune suppression signatures (PD-1, CTLA-4, TIM-3) [20]. Recently, a clinical trial Acclaim-2 using REQORSA (also called GPX-001) combined with PD-1 blocking Abs (Pembrolizumab) in treated non-small lung cancer patients has been launched (https://clinicaltrials.gov/ct2/show/NCT05062980).

Thus, studies initiated about 40 years ago to dissect chromosomal aberrations in lung cancer cells culminated in a new promising gene therapy aimed to battle most aggressive lung cancer stages. In addition, Fus1/Tusc2-mediated anti-inflammatory and anti-aging activities provide avenues for development of new approaches to fight conditions of chronic inflammation, infections, premature aging and geriatric diseases.

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AUTHOR CONTRIBUTIONS

RU conceived and prepared the first draft. AVI, SSC, AkS, SVI, and AnS reviewed and edited the manuscript. RU designed figures. All authors read and approved the final manuscript for publication.

CONFLICT OF INTEREST

The authors declare no competing interests.

ADDITIONAL INFORMATION

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