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► To cite this version:

Alessia Nisco, Maria Tolomeo, Mariafrancesca Scalise, Katia Zanier, Maria Barile. Exploring the impact of flavin homeostasis on cancer cell metabolism. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, 2024, 1879 (5), pp.189149. <10.1016/j.bbcan.2024.189149>. <hal-04776914>

HAL Id: hal-04776914

<https://hal.science/hal-04776914v1>

Submitted on 12 Nov 2024

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Exploring the impact of flavin homeostasis on cancer cell metabolism

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Abstract

Flavins and their associated proteins have recently emerged as compelling players in the landscape of cancer biology. Flavins, encompassing flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), serve as coenzymes in a multitude of cellular processes, such as metabolism, apoptosis, and cell proliferation. Their involvement in oxidative phosphorylation, redox homeostasis, and enzymatic reactions has long been recognized. However, recent research has unveiled an extended role for flavins in the context of cancer. In parallel, riboflavin transporters (RFVTs), FAD synthase (FADS), and riboflavin kinase (RFK) have gained prominence in cancer research. These proteins, responsible for riboflavin uptake, FAD biosynthesis, and FMN generation, are integral components of the cellular machinery that governs flavin homeostasis. Dysregulation in the expression/function of these proteins has been associated with various cancers, underscoring their potential as diagnostic markers, therapeutic targets, and key determinants of cancer cell behavior. This review embarks on a comprehensive exploration of the multifaceted role of flavins and of the flavoproteins involved in nucleus-mitochondria crosstalk in cancer. We journey through the influence of flavins on cancer cell energetics, the modulation of RFVTs in malignant transformation, the diagnostic and prognostic significance of FADS, and the implications of RFK in drug resistance and apoptosis. This review also underscores the potential of these molecules and processes as targets for novel diagnostic and therapeutic strategies, offering new avenues for the battle against this relentless disease.

Keywords: cancer, flavins, flavoenzymes, metabolism

Abbreviations

ABCG2 ATP-binding cassette G2
ACADM acyl-CoA dehydrogenase medium chain
ACADL acyl-CoA dehydrogenase long chain
AF AlphaFold
AIF apoptosis inducing factor
ARE antioxidant response element
BAX BCL2 Associated X, Apoptosis Regulator
Bcl-2 B-cell lymphoma 2
BCR biochemical recurrence
BCRP breast cancer resistance protein
BSO buthionine sulfoximine
ChIP chromatin immunoprecipitation
CIN cervical intraepithelial neoplasia
CoREST corepressor of REST
CRC colorectal cancer
CSB Chicago sky blue
CSCC cervical squamous cell carcinoma
EMSA electrophoretic mobility shift assay
ESCC esophageal squamous cell carcinoma
FAD flavin adenine dinucleotide
FADS FAD synthase
FAO fatty acid β -oxidation
FDA Food and Drug Administration
FH fumarate hydratase
FMN flavin mononucleotide
FP first progression
GBM glioblastoma
GC gastric cancer
GEO Gene Expression Omnibus
GLUTs glucose transporters
GR glutathione reductase
GSC GBM cancer stem cells
GSH reduced glutathione
GST π glutathione S-transferase- π

HDM histone demethylase
HPV human papillomavirus
HTS High Throughputs Screening
JmjC Jumonji C
JMJD JmjC domain demethylases
KD knockdown
KO knockout
LC-MS Liquid Chromatography coupled Mass Spectrometry
LSD1 lysine demethylase 1
NADPH Nicotinamide Adenine Dinucleotide Phosphate
NRF2 Nuclear factor, erythroid 2-like 2
NQO1 NAD(P)H Quinone Dehydrogenase 1
NuRD nucleosome remodeling and deacetylase
OAT3 organic acid transporter 3
OGT O-linked N-acetylglucosamine (GlcNAc) transferase
OS overall survival
OSCC oral squamous cell carcinoma
PDAC pancreatic ductal adenocarcinoma
PGC1- α peroxisome proliferator-activated receptor gamma coactivator 1 alpha
PPS post-progression survival
pRb retinoblastoma protein
Rf riboflavin
RFVT riboflavin transporter
RFK riboflavin kinase
RNS reactive nitrogen species
ROS reactive oxygen species
SCC squamous cell carcinoma
SDHA succinate dehydrogenase A
SLC solute carrier
SNHG3 Small Nucleolar RNA Host Gene 3
TCA tricarboxylic acid cycle
TCGA The Cancer Genome Atlas
THF tetrahydrofolate
THUMPD3-AS1 THUMPD3 Antisense RNA 1

1. Introduction

1.1 Preface

The metabolic alterations observed in the malignant transformation of cells are now recognized as a distinct hallmark of carcinogenesis [1]. The “Warburg effect” was first noticed by Otto Warburg, who observed that cancer cells use glycolysis to generate energy, even in the presence of an adequate oxygen supply. This metabolic shift in cancer cells towards aerobic glycolysis involves increased glucose uptake facilitated by glucose transporters (GLUTs) and the conversion of pyruvate to lactate. It has been proposed that the increased glycolysis of the Warburg effect stimulates tumour growth by allowing glycolytic intermediates to enter anabolic side pathways and facilitating the *de novo* synthesis of nucleotides, lipids, and amino acids, all of which are essential for cell proliferation [2].

The concept of the tumour-specific metabolic phenotype, initially proposed by Otto Warburg, has been revisited [3]. In fact, in addition to the Warburg effect, elevated levels of fatty acid synthesis and glutaminolysis are also well-known hallmarks of cancer. Glutaminolysis plays a crucial role in supplying nitrogen and carbon skeletons to fuel the tricarboxylic acid cycle (TCA), thus supporting the production of nucleotides and amino acids [4]. However, the rapid division of cancer cells necessitates increased *de novo* synthesis of fatty acids from acetyl-CoA and reducing power (NADPH) to facilitate membrane biogenesis [5].

Differently from the lipogenic phenotype, the precise function of mitochondrial fatty acid β -oxidation (FAO) in cancer remains unclear. More recent studies demonstrate that FAO can furnish ATP as fast as needed to support cancer proliferation in SiHa cells and mouse models [6, 7]. Moreover, the Acetyl-CoA produced in the mitochondria may contribute to the synthesis of cytosolic NADPH [8, 9], the reducing power needed to sustain biosynthesis and offset oxidative stress. FAO plays a crucial role in various aspects of cancer cell biology, including growth, survival, maintenance of stemness, resistance to drugs, and metastasis. Inhibition of FAO has been linked to reduced viability or the induction of apoptosis in cell lines associated with myeloid leukemia, glioma, and hepatocellular carcinoma [10, 11]. The disruption of NADPH homeostasis, the generation of reactive oxidative species (ROS), oxidative stress, and/or mitochondrial damage are most likely the causes of the anti-survival effect of FAO inhibition [12]. Furthermore, over the last ten years, the TCA cycle has also emerged as a pivotal anabolic hub that supports the growth of tumours in cancer patients as well as in mouse models [13, 14]. It has been demonstrated that pyruvate carboxylase, which converts pyruvate into oxaloacetate, is essential for the formation of primary and metastatic tumours [15]. In contrast, the production of aspartate from oxaloacetate and its derivative, asparagine, may have a limited effect on tumour growth [16]. For all these reasons, mitochondria are increasingly recognized as crucial organelles that mediate important steps in various interconnected intracellular pathways [4, 17]. Importantly, these pathways may operate concurrently, underscoring the significance of targeting them to address the metabolic adaptability of cancer cells [18].

Riboflavin (Rf) has been recognized as an important player in the economy of mitochondrial energy metabolism since 1975 [19]. Rf (also known as vitamin B2) is a water-soluble vitamin belonging to the B-group, and it represents the precursor of the flavin cofactors flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which enable the enzymatic activation of the cellular flavoproteome [20]. Rf status can be altered by malabsorption resulting from various disorders, including alcoholism, cancer, and celiac disease [21]. Rf nutritional status and Rf availability in intestinal cells have been demonstrated to be important for normal cellular functions, such as growth, survival and proliferation [22]. Additionally, since Rf plays a role in folate metabolism, a diet low in Rf could enhance the harmful effects of a low-folate diet [23].

The development of colorectal cancer (CRC) has been correlated to Rf deficiency in [24, 25], even before the genes involved in Rf homeostasis were identified (Figure 1). Further confirmation that Rf deficiency is linked to the development of CRC was obtained from studies on patients suffering from esophageal squamous cell carcinoma (ESCC) and later gastric cardia-adenocarcinoma [26, 27]. Moreover, cervical dysplasia, a condition predisposing to invasive cervical cancer, may also be associated with Rf deficiency. A case-control study showed an increased risk of occurrence of cervical dysplasia for Rf intake less than 1.2 mg per day [28]. More recently, the association between serum Rf and FMN and pancreatic cancer has been demonstrated [29].

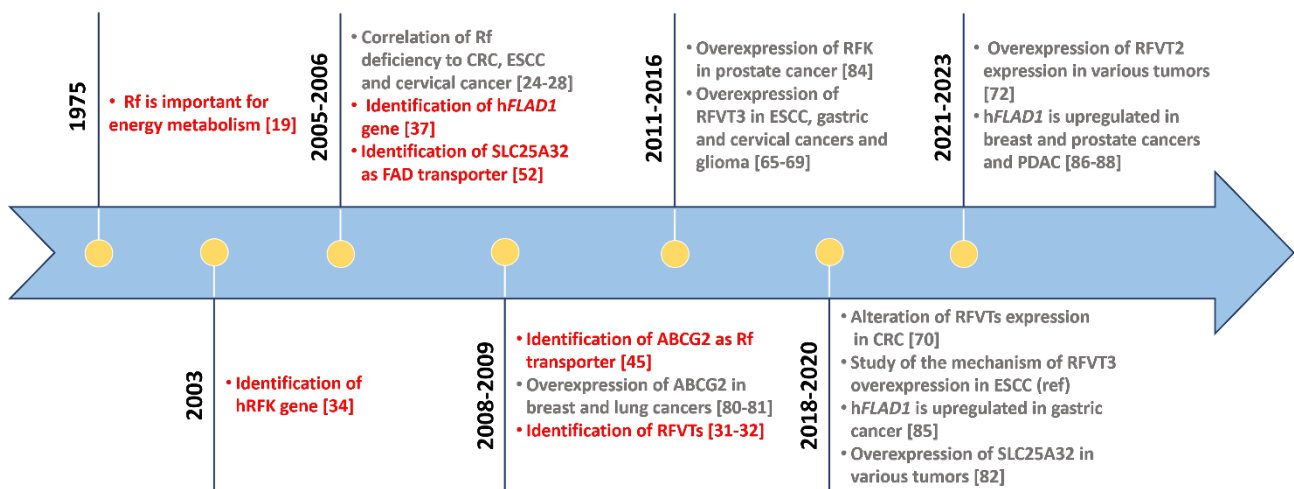


Figure 1. Landmarks timeline of the characterization of the human genes involved in flavin homeostasis and their alteration in cancer.

1.2 The molecular components of cellular Rf homeostasis

Rf intestinal absorption [30] is facilitated by recently discovered carrier-mediated pathways that are aided by three members of the solute carrier family 52 (SLC52), namely Rf transporter 1 (RFVT1), RFVT2 and RFVT3, encoded by the three genes *SLC52A1-3*, respectively. These transporters are associated with different functional and kinetic properties [31] and specific localisation in the polarized cells of the intestinal

epithelium [32], with RFVT3 mainly expressed on the luminal side and RFVT1/2 on the basolateral membrane. Recently, three-dimensional structural models for all three human RFVTs were obtained by state-of-the-art artificial intelligence-based methods (including AlphaFold (AF) and the newly released RoseTTaFold.29). These models were subsequently improved upon using molecular dynamics simulations [33].

Within the cells, two enzymes are required for Rf conversion into flavin cofactors: riboflavin kinase (RFK, ATP:riboflavin 5'phosphotransferase, EC 2.7.1.26), encoded by *RFK* gene. RFK transfers a phosphoryl group from ATP to Rf, resulting in the formation of FMN [34]. The other enzyme involved is FAD synthase, alternatively known as ATP:FMN adenylyltransferase (FADS or FMNAT, EC 2.7.7.2), encoded by *FLAD1*. FADS catalyzes the adenylation of FMN to produce FAD. Following the identification of the human *FLAD1* by our research group, two protein isoforms were reported: hFADS1 located in the mitochondria [35] and hFADS2 in the cytosol [36, 37]. Additionally, the nuclear localisation of FADS has been demonstrated [38], suggesting a potential role in regulating redox epigenetic events alongside FAD and its derivatives [39]. Although FAD hydrolysis has also been reported in the nucleus, it remains unclear whether this process is due to one of the bi-functional isoforms produced by *FLAD1* or if it occurs *via* independent hydrolases [38]. The specific FADS isoform responsible for nuclear activity has yet to be determined.

The “recycling” of flavin cofactors takes place in the plasma, where CD73, or ecto-5' nucleotidase, has the ability to hydrolyse FAD to FMN. Then, alkaline phosphatase hydrolyses FMN to Rf, which is efficiently transported into cells [40]. Rf can also be produced inside the cells by the recycling of flavin cofactors due to the sequential activities of diphosphatases and phosphomonoesterases, which are still poorly characterised [41]. Quite interestingly, in humans, FADS can switch from a FAD synthase to a FAD diphosphatase activity, leading to the production of FMN and AMP. The physiological significance of this switch is still poorly clarified [42].

Rf can enter or be extruded from cells either by simple diffusion *via* RFVTs [43, 44] or by other less specific transporters, among which we can find the ATP-binding cassette G2 (ABCG2) transporter, which belongs to the family of ABC translocators. ABCG2, also known as the breast cancer resistance protein (BCRP), was initially identified in a multidrug-resistant breast cancer cell line due to its role in drug resistance. Besides its involvement in drug resistance, ABCG2 is responsible for secreting Rf into milk and other extracellular fluids [45]. Its distribution in specific tissues is closely linked to various physiological functions, such as limiting the absorption of substances in the intestine, facilitating distribution across important barriers like the blood-brain and blood-placental barriers, and aiding in the elimination and excretion of a wide range of drugs, carcinogens, and food toxins [46].

The “efflux” organic acid transporter 3 (OAT3- SLC22A8) is another less selective transporter that efficiently transports Rf, as shown in the kidney and choroid plexus [47].

A fascinating biochemical puzzle that remains unresolved is how Rf and flavin cofactors are distributed among cellular organelles. This question is closely correlated with the issue of sub-cellular

compartmentalisation of enzymes involved in FAD-forming/destroying. The transport of Rf into mitochondria was proposed at the functional level many years ago [41], but the sole mitochondrial Rf transporter currently identified is SLC22A14, which is responsible for the supply of FAD into spermatozoa [48]. SLC25A17 serves as human peroxisomal transporter for several free cofactors, including FAD, essential for peroxisomal β -oxidation processes while exporting FMN as a countersubstrate [49]. SLC25A32, located in the inner mitochondrial membrane, is closely related to human diseases. Initially recognised as a folate transporter [50], it was later identified as the human orthologue of the yeast mitochondrial FAD transporter FLX1 [51, 52]. SLC25A32 facilitates the transport of FAD synthesized in the cytosol into mitochondria, as well as the movement of FAD produced inside mitochondria to the intermembrane space, regulating flavoproteome biogenesis [53].

1.3 Flavin cofactors and the cellular flavoproteome

The fundamental role of the flavin cofactors, which are essential molecular components of around one hundred of dehydrogenases, reductases, and oxidases, is to transfer reduction equivalents, resulting in semi-reduced or fully reduced forms [54, 55]. While flavoenzymes exhibit specificity for either FMN or FAD, they may employ either cofactor. A comprehensive examination of FMN and FAD utilization revealed that 25% of flavoenzymes utilize FMN [56], suggesting a preference for FAD-dependent enzymes within the human flavoproteome [57].

Flavoenzymes are mostly localised in mitochondria [20, 58], where they are involved in the terminal energetic metabolism of fatty acids, carbohydrates, amino acids, and choline. Additionally, metabolic conversions of folates and pyridoxine involve flavin-dependent enzymes situated outside of mitochondria. Within the endoplasmic reticulum and nucleus, FAD-dependent enzymes play roles in both generating and scavenging ROS and reactive nitrogen species (RNS) [59]. For instance, the FAD-dependent nuclear amine oxidase, lysine demethylase 1 (LSD1), targets mono- and dimethyl groups on lysine 4 (H3K4) and lysine 9 (H3K9) of histone H3, facilitating transcriptional repression and activation, respectively. This function of LSD1 serves as a pivotal "molecular switch" in gene transcription [60].

As an example of the importance of vitamin B2 and derived cofactors on flavoproteome activation, we reported experiments performed in murine melanoma cells [61]. Upon restriction of the supply of Rf, a substantial part of the flavoproteome was inactivated due to the loss of protein stability, similar to what occurs under the suppression of the chaperone HSP90. In support of this analogy, mass spectrometry analysis of the FAD-free NQO1 aggregates revealed an increased association between flavin-depleted proteins and multiple components of the proteostasis network. Furthermore, the mevalonate pathway of cholesterol synthesis was shown to be profoundly inactivated in a proteome-wide investigation of Rf-starved cells. This finding highlights several cellular vulnerabilities caused by flavoproteome inactivation [62].

Since their discovery, changes to Rf homeostasis components have been associated with neuromuscular illness. This association makes sense given the role that vitamin B2 plays in flavoenzymes, which are essential for oxidative metabolism and, consequently, for muscular and nervous system economy [36, 63, 64]. In contrast, the relationship between Rf and the metabolic switch related to cancer transformation is indeed poorly understood. Given its important biochemical roles, Rf could be an important player in the development and progression of different types of cancer [21].

2. Aim of the study

In this review, we aim at unravelling the intricate network of flavin transporters, RFK and FADS, in the frame of the flavin-dependent mitochondria-nucleus crosstalk and to shed light on their roles in cancer-related pathways.

3. Flavin transporters and cancer

3.1 RFVTs

Immediately after the discovery of the human RFVTs [65-67], in 2012, RFVT3 expression and blood Rf concentrations were investigated in gastric carcinoma (GC), using blood samples and both tumour and normal tissue specimens from 60 GC patients. The findings revealed a significant reduction in RFVT3 expression levels in GC samples when compared to those in the normal mucous membrane. This decrease in RFVT3 expression was strongly linked to the tumour stage, histological grade, and blood Rf levels. Importantly, these observations were negatively associated with the development of GC. Furthermore, in GC patients, the defective expression of the RFVT3 protein was correlated with elevated plasma Rf levels [68].

Human papillomavirus (HPV) infections are etiological agents of cervical squamous cell carcinoma (CSCC) and of its precancerous cervical intraepithelial neoplasia (CIN) lesions [69]. Individuals with CIN or CSCC exhibited lower plasma Rf levels compared to healthy controls. Moreover, in CSCC patients, levels of Rf were significantly lower in tumour tissue compared to normal cervical epithelium. Consistently, Rf levels have been found to be significantly lower in tissue samples positive for the 'high-risk' HPV16 and HPV18 viruses as compared to HPV16/18-negative tissue [70]. On the other hand, surprisingly, RFVT3 expression (both at the mRNA and protein level) was higher in CSCC tissue samples compared to a matched control group and was found to correlate with tumour stages [70], but the other way around with respect to GC [68].

RFVT3 was also found to be frequently overexpressed in patients with ESCC, as assessed by comparing tumour samples to normal adjacent tissue. Notably, when RFVT3 was knocked down in ESCC cells, several significant effects were observed. These included reductions in intracellular flavin levels, mitochondrial membrane potential, and cellular ATP levels. Knockdown (KD) of RFVT3 reduced cell proliferation, colony formation, and the ability of cells to grow independently of anchorage. Additionally,

RFVT3 KD resulted in increased p21 and p27 protein levels and a decrease in the levels of their downstream targets, cyclin E1 and Cdk2. This caused hypophosphorylation of retinoblastoma protein (pRb), leading to cell cycle arrest at the G1-G1/S phase. Furthermore, the downregulation of RFVT3 led to a reduction in anti-apoptotic proteins such as B-cell lymphoma 2 (Bcl-2), Bcl-xl, resulting in the activation of caspase-3 and the induction of apoptosis. Conversely, when RFVT3 was ectopically overexpressed in ESCC cells, it promoted cell proliferation under restricted conditions (such as soft agar), conferred resistance to cisplatin, and enhanced tumorigenicity in nude mice. These findings collectively suggest that RFVT3 plays a significant role in the tumorigenesis of ESCC and may represent a potential therapeutic target for this condition [71].

The role of RFVT3 in glioma development was also investigated. RFVT3 is overexpressed in glioma samples when compared to normal brain tissue. Like in ESCC, silencing of RFVT3 in glioma cells inhibited cell proliferation by inducing cell cycle arrest and apoptosis, which was associated with changes in the expression of proteins known to regulate the cell cycle (such as p21 and p27) and apoptosis (including Bcl-2 and BCL2 Associated X, Apoptosis Regulator, BAX). Furthermore, RFVT3 silencing in glioma cells had a notable impact on migration and invasiveness. Importantly, the reduction in RFVT3 levels was found to decrease tumour growth in *in vivo* experiments [72].

As reported in Figure 1, in 2018, our group studied the alterations of the expression levels of the three RFVTs in human CRC samples, providing the first evidence that changes not only in RFVT3, but also in the other RFVTs, occur in human CRC. When examining tumour tissues from CRC patients, we found that RFVT1 protein and mRNA levels were reduced compared to normal mucosa. We also found decreased protein content in RFVT2 and RFVT3, whereas their mRNA levels increased, suggesting an alteration of the transcriptional/translational responses of RFVTs to tumorigenesis. To gain further insight into this point, we used as models different CRC cell lines with different degrees of differentiation. The findings revealed an increase in the gene and protein expression levels of RFVT3 and RFVT2 in less differentiated cells, while the RFVT1 protein level was significantly lower. These differences in transporter expression were believed to contribute to the higher total flavin content [73]. These results inspired the view that cancer cells and tissues become greedy for flavins and shed light on the intricate regulation of RFVTs in CRC and their impact on flavin content in cancer cells and tissues. Yet, a unique mechanistic model and a correlation with metabolic changes are missing.

The potential of Rf and its derivatives as binding targets to facilitate the internalization of drugs *via* RFVT1-3 has garnered interest due to their association with enhanced Rf uptake in various cancers. To investigate this further, the expression patterns of RFVTs were examined in different types of cancer samples, as well as in healthy tissues. In the latter, low constitutive expression levels of the three transporters were observed. However, distinct patterns of overexpression were identified for the different types of cancer. For example, melanoma exhibited significant overexpression of RFVT2 and RFVT3, luminal A breast cancer displayed elevated levels of RFVT1 and RFVT3, and squamous cell carcinoma (SCC) showed increased

expression of all three transporters. As part of this investigation, the SCC cell line A431 was found to be highly positive for all RFVTs. This makes A431 a suitable *in vitro* model for studying the potential targeting and drug internalization *via* these Rf transporters [74].

More recently, the significance of RFVT2 has been extensively studied across various human tumours. The authors systematically explored the association of RFVT2 with 33 different types of tumours, utilizing publicly available databases like The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO). They found RFVT2 expression in specific cancers, including hepatocellular carcinoma, gastric cancer, colon cancer, and rectal cancer, through immunohistochemistry. Results from immunohistochemical analyses confirmed RFVT2 overexpression. Importantly, RFVT2 expression was linked to several clinical parameters, among them overall survival, disease-specific survival, tumour mutational weight, microsatellite instability, common immune checkpoint genes, and immune cell infiltration. In the case of hepatocellular carcinoma, RFVT2 expression was identified as an independent prognostic factor. Furthermore, the study identified potential regulatory pathways involving SNHG3 (Small Nucleolar RNA Host Gene 3) and THUMP3-AS1 (THUMP3 Antisense RNA 1) la-miR-139-5p-SLC52A2 axes in hepatocellular carcinoma [75].

Little is known about the regulation of RFVT3 gene expression in cancer. One study focused on the hypermethylation of the RFVT3 gene in biopsies from resected tissues from Uighur CSCC patients [76]. The methylation sequences in the RFVT3 promoter region were mapped using bisulfite-sequencing PCR in the SiHa cell line. Results indicated that two CpG sites (CpG 2 and CpG 3) out of 12 were consistently methylated. Subsequently, quantitative detection of methylated DNA was performed in 30 pairs of Uighur CSCC and adjacent normal tissues using the MassARRAY technique. Results revealed significant differences in the methylation levels between CpG 2 and CpG 3. Interestingly, there was a tendency for the RFVT3 protein to translocate from the cytoplasm/membrane to the nucleus in CSCC. This shift was associated with an increase in methylation at CpG 2 and CpG 3 in the RFVT3 gene promoter regions and was therefore proposed to play a role in the development of CSCC [76].

Another regulating mechanism was also illustrated in [77], where, by decreasing the intracellular Rf levels by 20%, an upregulation of Rf transporter genes, particularly SLC52A3, was observed. To investigate a plausible molecular explanation, chromatin immunoprecipitation (ChIP) analysis and histone H3 tail modifications at promoter regions were performed. The results showed that H3K4me3 and H3K9me3 were present at low levels, suggesting that the induction in SLC52A3 promoter activity observed may involve epigenetic mechanisms.

In later studies involving ESCC cells, two transcript variants of the *SLC52A3* gene coding for RFVT3 were identified, which differ in their transcriptional start sites and consequently encode different proteins: SLC52A3a and SLC52A3b. It's worth noting that SLC52A3a, but not SLC52A3b, exhibited a strong ability to promote the proliferation and colony formation of ESCC cells. Moreover, the 5'-flanking regions of the *SLC52A3* gene were found to contain binding sites for NF- κ B p65/Rel-B, which are crucial for RFVT3

expression in ESCC cells. Results from CHIP and electrophoretic mobility shift assay (EMSA) experiments demonstrated that p65/Rel-B proteins indeed bind to these 5'-flanking regions of SLC52A3. Consistently, the NF- κ B signaling pathway was found to upregulate the transcription of SLC52A3 in response to TNF α stimulation [78].

Differently from *SLC52A3*, the regulatory elements in the promoters of *SLC52A1* and *SLC52A2*, encoding for RFVT1 and RFVT2 respectively, remain poorly characterized. Recently, upstream of the transcriptional start site of *SLC52A2* was found an antioxidant response element (ARE), a known binding site of Nuclear factor, erythroid 2-like 2 (Nfe2l2/NRF2) [79]. This could be relevant since the NRF2 pathway has been established as a driver of cancer progression, metastasis, and resistance to therapy [80]. On the other hand, *SLC52A1* appears to be instead regulated by p53 during senescence [81]. Further investigations on the impact of this event on cancer are needed.

3.2 ABCG2

ABCG2 transporter is not only responsible for secreting Rf into the milk in lactating mammary glands, but it has been established that the overexpression of ABCG2 in the membranes of extracellular vesicles plays a role in conferring resistance to the chemotherapy drug mitoxantrone in breast cancer. These vesicles are specifically localized in cell-cell attachment regions. Importantly, it was noticed that ABCG2 overexpression resulted in the accumulation of an unidentified fluorescent green compound within these vesicles [82], which, later on, turned out to be Rf. Furthermore, the intravesicular concentration of Rf in ABCG2-overexpressing breast and lung cancer cells strongly correlates with the extent of ABCG2 overexpression. This accumulation of Rf occurs within the vesicular membrane and not the plasma membrane, which is surrounded by growth medium. Based on these findings, it was proposed that the ABCG2-dependent concentration of Rf within these intercellular compartments can serve as a novel, sensitive, and non-cytotoxic functional marker to quantify the levels of multidrug resistance in various malignant cells [83]. Later, an inherent autofluorescent phenotype in cancer stem cells (CSCs) of different epithelial cancers was identified and characterised. The autofluorescence resulted from Rf accumulation in membrane-bound cytoplasmic structures containing the ABCG2 transporter [84].

In addition, in 2021, it was discovered that the intracellular accumulation of Rf mediated by ABCG2 occurs in a subset of autofluorescent (Fluo⁺) glioblastoma (GBM) cells. These cells exhibit all the functional traits of GBM cancer stem cells (GSCs), including a heightened capacity to grow as neurospheres, long-term self-renewal ability, increased expression of stem cell markers, and enhanced tumorigenicity *in vivo* [85].

3.3 SLC25A32

As outlined in the introduction, SLC25A32 plays a crucial role in transporting tetrahydrofolate (THF) and FAD into mitochondria, thereby regulating mitochondrial one-carbon metabolism and redox balance [52]. In a study conducted in 2020, it was demonstrated that the SLC25A32 gene is amplified in various types of tumours, and this amplification is associated with increased mRNA expression and reduced patient survival. Using siRNA-mediated KD and CRISPR-mediated knockout (KO) techniques and cancer cells from different origins, cell lines sensitive or resistant to SLC25A32 inhibition were identified. Interestingly, SLC25A32 KD did not appear to affect the mitochondrial/cytosolic folate flux as determined by Liquid Chromatography coupled Mass Spectrometry (LC-MS) experiments. Instead, it led to dysfunction in the respiratory chain at the FAD-dependent complex II enzyme, induction of ROS, and depletion of reduced glutathione (GSH). These effects ultimately impair cancer cell proliferation. Moreover, treatment with buthionine sulfoximine (BSO) further sensitized cells to ROS-mediated inhibition of cell proliferation upon SLC25A32 KD. However, treatment with Rf and GSH rescued cancer cell proliferation following SLC25A32 down-regulation. These findings shed light on the critical role of SLC25A32 in mitochondrial function, redox balance, and cancer cell survival, and they suggest potential therapeutic strategies for targeting this transporter in cancer therapy [86].

Recently, the mechanisms and functions of SLC25A32 in the progression of GBM were thoroughly examined. SLC25A32 exhibited elevated expression in GBM, with higher expression levels being associated with a higher glioma grade and a poorer prognosis. These results were further validated through immunohistochemistry using anti-SLC25A32 antibodies on samples from an independent cohort of patients. Moreover, the study demonstrated that SLC25A32 KD in GBM cells inhibited their proliferation and invasion, while overexpression of SLC25A32 significantly promoted cell growth and invasion. These effects were primarily attributed to the activation of the PI3K-AKT-mTOR signalling pathway, shedding light on the molecular mechanisms underlying the role of SLC25A32 in the progression of glioblastoma [87].

4. RfK and cancer

RfK expression in human tumours is indeed poorly investigated. RfK overexpression was found to confer resistance to cisplatin and to hydrogen peroxide (H₂O₂) and diamide, which are agents that induce oxidative stress, in human prostate cancer cells PC3 and P/CDP6. Conversely, RfK KD induced apoptosis in the cells, sensitizing them to cisplatin. Furthermore, RfK overexpression increased FAD, FMN, and total glutathione levels, as well as the expression of the FAD-dependent glutathione reductase (GR) and of the glutathione S-transferase- π (GST π). Interestingly, RfK expression levels correlated well with the Gleason score, which is a known indicator of patient prognosis in prostate cancer. In summary, this study suggests that RfK plays a role not only in cellular protection against oxidative stress but also in the malignant progression of prostate cancer.

Its overexpression appears to confer resistance to various stressors and chemotherapeutic agents, while its downregulation sensitizes cells to cisplatin treatment [88].

5. FADS and cancer

Differently from what was found for the RFVTs, the relationship between FADS and cancer was poorly investigated. The first evidence was demonstrated in 2008, before the gene coding for this enzyme was discovered. In fact, a mass spectrometry analysis performed in the serum of nasopharyngeal carcinoma patients revealed a downregulation of the FADS protein.

More recently, in 2020, evidence on the role of *FLAD1* in cancer was demonstrated in a study on *FLAD1* expression in GC and normal tissues. Initially, the expression of *FLAD1* at the mRNA level was analysed in GC tumour tissues and normal tissues using the OncoPrint database. Results were validated by RT-qPCR experiments on 10 pairs of GC and their adjacent normal tissues, which confirmed the overexpression of *FLAD1* mRNA in GC tissues. The study further examined *FLAD1* protein expression by immunohistochemistry revealing that *FLAD1* was overexpressed in 59.4% of the patients. The prognostic value of *FLAD1* mRNA expression was assessed using the Kaplan-Meier plotter (www.kmplot.com), which pointed to an association between *FLAD1* overexpression and GC. In addition, *FLAD1* overexpression was found to be associated with a higher TNM stage, indicating a more advanced stage of the disease. Multivariate analysis demonstrated that *FLAD1* was an independent prognostic factor for GC, underscoring its significance in predicting patient outcomes. *FLAD1* mRNA overexpression was not only associated with unfavourable overall survival (OS) but also with first progression (FP) and post-progression survival (PPS) in GC patients. Moreover, *FLAD1* was identified as an independent prognostic factor with a strong correlation to patient survival outcomes, making it a potential target for further investigations in the context of gastric cancer [89].

The diagnostic and prognostic value of *FLAD1* expression was extensively investigated across various cancer types in a pan-cancer analysis, utilizing data from multiple online databases. [90]. *FLAD1* was found to be overexpressed in a variety of cancer types, with a particular emphasis on breast cancer. Higher *FLAD1* expression levels were associated with specific subgroups of breast cancer, including p53 mutant cancers. These studies revealed that *FLAD1* expression may have clinical relevance in characterizing breast cancer subtypes. This comprehensive analysis underscores the significance of *FLAD1* in various cancer types and suggests that it has the potential to serve as a valuable biomarker for cancer diagnosis and prognosis.

In [91], a research identified the metabolic genes associated with the progression of non-metastatic prostate cancer. This study utilized genetic information (335 patients) from TCGA and validated the prognostic significance of these genes by assessing patients' immunohistochemical prostatectomy specimens. A number of metabolic candidate genes were examined that showed strong correlations with the development of cancer, its biochemical recurrence (BCR), and mortality. The authors identified 20 metabolic genes using their own software, TarGA, and found that the median expression levels of these genes consistently increased with

cancer progression to BCR and deaths. Five metabolic genes, among them *FLAD1*, were significantly associated with overall survival in the TCGA dataset. Together with clinicopathological data, the immunohistochemical validation of these genes in prostatectomy specimens showed that OGT (O-linked N-acetylglucosamine (GlcNAc) transferase) and *FLAD1* remained significant factors for BCR and cancer-specific survival.

In our laboratory, the prognostic significance of *FLAD1* was investigated in pancreatic ductal adenocarcinoma (PDAC). Our work focused on the study of the FAD synthesis process in CSCs. For this purpose, we used two sets of PDAC cell lines carrying two distinct mutations in the p53 tumour suppressor gene, namely: i) PANC-1 and PANC-1-derived CSCs cell lines expressing p53 R273H; ii) MiaPaca2 and MiaPaca2-derived CSCs cell lines with p53 R248W. HPDE cells expressing wild type p53 were used as a control for comparison. FADS expression and activity increased with malignancy, and this effect was even more pronounced in the context of cancer stemness. Furthermore, the study investigated the inhibitory effect of the molecule Chicago Sky Blue (CSB) on the enzymatic activity of FADS, which was found to be effective on the enzymatic activity of the recombinant 6His-hFADS2 and extracts from PANC-1-derived CSCs with an IC_{50} in the μM range. Furthermore, CSB demonstrated effectiveness in inhibiting the growth of PANC-1 and its derived CSC line, suggesting its potential as a chemotherapy [92].

Given the significance of FAD availability for mitochondrial and nuclear redox epigenetics [93], we suggested that the increased FAD synthesis rate in cancer cell lines may be related to the higher expression levels of the FAD-dependent LSD1 protein in PDAC cell lines and the flavoprotein subunit of complex II of the mitochondrial respiratory chain, succinate dehydrogenase (SDHA). When stemness is induced, this enzyme is expected to parallel the cellular metabolism [92]. Together, the results support the view that FAD plays a crucial role in various cellular processes associated with cancer.

6. Flavoproteins of nuclear-mitochondrial crosstalk and cancer

There are around 100 human flavoproteins, and because of their central metabolic roles, a huge body of experimental information has been accumulated over many decades regarding their involvement in diverse aspects of tumour biology. Here, we have reported just a few examples. For instance, Acyl-CoA dehydrogenase family member 11 (ACAD11) contributes to p53-dependent cell survival and OXPHOS upon glucose starvation [94]. Moreover, the acyl-CoA dehydrogenase medium chain (ACADM) has been linked to the advancement of neuroblastoma, pancreatic cancer, and hepatocellular carcinoma [95-97]. The acyl-CoA dehydrogenase long chain (ACADL) has also been linked to ESCC, lung adenocarcinoma, and hepatocellular carcinoma [98-101]. Additionally, the role of dihydrolipoamide dehydrogenase (DLD) has been explored in several cancers [102].

Among the mitochondrial flavoproteins, one of the most relevant is complex I (CI) of the electron transport chain. Changes in CI are strongly linked to carcinogenesis and the development of cancer, since it is

a critical component of OXPHOS and plays a vital role in the synthesis of ATP, the maintenance of the cellular NAD⁺/NADH ratio, and ROS production. There is growing evidence that different types of tumours have overexpressed CI [103].

In this section, we will discuss the role of nuclear and mitochondrial flavoproteins that are potentially involved in the nucleus-mitochondria crosstalk in cancer. Indeed, the latter has gained importance in the cancer field since it controls several cellular functions, such as stress adaptation and differentiation. Mitochondria produce metabolites for epigenetic changes and other nuclear-associated processes, and, on the other hand, some mitochondrial proteins have been found in the nucleus [104].

The apoptosis-inducing factor (AIF) is a flavoprotein that resides in the nucleus but begins its journey at the mitochondrial inner membrane. AIF translocates to the nucleus in response to oxidative stress or other proapoptotic triggers, ultimately contributing to DNA degradation. Beyond its role in apoptosis, AIF contributes to the biogenesis of respiratory chain complexes through its interaction with the flavoprotein CHCHD4, the human homolog of yeast Mia40, a regulator of the intermembrane space import machinery [105]. The relocalization hints at the possibility that AIF may function as a mediator, conveying vital metabolic information from the mitochondria to the nucleus, thereby integrating metabolic signalling with nuclear responses [106, 107].

AIF overexpression in untreated tumours suggests that AIF may provide cancer cells with pro-survival activities associated with energy and ROS production and regulation of the cell cycle [108]. Apart from this evidence, there are no other indications for the AIF-tumor connection. Actually, it is not surprising since no oncogenic phenotype was reported in either Harlequin mice, who are naturally AIF deficient [109], or in a transgenic deficiency model [110].

As outlined in the Preface, it is now well recognised that many cancer cells depend on both glycolysis and mitochondrial energy metabolism to efficiently balance energy production and glucose-derived macromolecule biosynthesis, allowing aggressive growth and survival [111]. AIF's significance in cancer is underscored by its role in maintaining effective mitochondrial electron transport and oxidative phosphorylation. As cancer cells develop into more aggressive phenotypes and become more dependent on mitochondrial activity, this function becomes particularly important [112]. Recent studies have shed light on the role played by oxidative phosphorylation in enhancing the invasiveness of cancer cells, [113, 114].

Lysine methylation plays a pivotal role in regulating gene expression, and its effects can be dual, either promoting transcriptional activation or repression, depending on the specific lysine residue and the degree of methylation involved. Notably, methylation marks on histone H3 lysine residues like H3K4, H3K48, H3K36, or H3K79 are typically associated with transcriptional activation, while methylation on H3K9, H3K27, and H4K20 is correlated with transcriptional silencing [115]. These modifications are orchestrated by histone methyltransferases, which add methyl groups, and histone demethylases (HDMs), which remove them. Lysine demethylases (KDMs) are an important category in the landscape of HDMs. They are classified into

two main families: Lys-specific demethylases (LSD) and Jumonji C (JmjC) domain demethylases (JMJD). LSDs are essentially amine oxidases composed of two domains: a substrate-binding domain and a FAD-binding domain [116, 117]. This versatile enzyme can be recruited by several multiprotein complexes, including NuRD (nucleosome remodeling and deacetylase) or CoREST (corepressor of REST), involved in the demethylation of H3K4me2 and H3K4me1 marks [118]. Additionally, LSD1 can form complexes with nuclear receptors like androgen or estrogen receptors, contributing to the demethylation of H3K9 [117]. Interestingly, several studies have shed light on the crucial role of LSD1 in the regulation of mitochondrial gene expression, particularly within adipose tissue, although the interpretation of this activity remains somewhat contentious [119]. Additionally, it has been observed that in human hepatocellular carcinoma cells, inhibiting LSD1 can decrease glycolysis and enhance mitochondrial respiration [120]. Moreover, it has been determined that the loss of LSD1 function is a major modulator of regulators, including PPAR γ coactivator-1 α (PGC1- α) that has been involved in energy expenditure and mitochondrial metabolism. The intricate relationship between LSD1 and cellular energy balance is highlighted by the fact that its loss causes the activation of mitochondrial respiration. In contrast, a paper suggests that LSD1 diminishes mitochondrial respiration and energy expenditure [121]. Besides its role in histone demethylation, LSD1 also participates in the demethylation of non-histone proteins, including p53, Stat3, and Dnmt1. This broad range of substrates suggests that LSD1 contributes to selective biological processes in cancer cells [121]. The progress on the study of LSD1 functions in cancer and immune regulation and on LSD1 inhibitors in clinical trials is reviewed in [122-126].

Mitochondrial dysfunction can induce histone hypermethylation *via* various mechanisms. Elevated levels of ROS, succinate, or fumarate, for instance, inhibit histone demethylases [127]. This link is particularly significant for various cancers that originates from mutations in fumarate hydratase (FH) and in the SDH complex components, leading to the accumulation of succinate and fumarate, thereby inhibiting the function of histone demethylases [128]. According to *in vitro* experiments, overexpressing JMJD3 histone demethylase (KDM6B) can counteract the increased H3 methylation that results from SDH inhibition [129].

The relationship between flavins and epigenetic regulation extends further through the actions of SDHA, the FAD-dependent subunit of SDH. Within mitochondria, FAD serves as a vital cofactor for the SDH complex in the TCA cycle and for acyl-CoA dehydrogenase in β -oxidation, where it undergoes reduction to FADH₂. This reduced form carries two high-energy electrons through the electron transport chain (ETC), ultimately contributing to ATP production via OXPHOS while reverting FAD back to its oxidized form.

Moreover, flavination of SDHA is associated with the formation of a complex with SDHB, essential for function [130]. Immunohistochemical staining consistently shows normal SDHA but decreased expression of SDHB in gastrointestinal tumours associated with SDHA [131]. By using SDHB levels as an indicator to compare adjacent normal tissues to the tumour ones in the same tissue sections of multiple patient samples, it was possible to demonstrate decreased functional or flavinated SDHA in tumour tissues. This finding suggests that altered cellular metabolism is linked to the loss of tissue autofluorescence, which is mediated by

mitochondrial flavoproteins. This idea is corroborated by a study conducted in oral squamous cell carcinoma (OSCC), which showed a decrease in the autofluorescence caused by a reduction in SDHA flavination rather than a decrease in FAD levels. This was followed by an increase in OXPHOS, which may be a necessary condition for cancer progression [132]. Succinate accumulation has the potential to stimulate NF- κ B signalling and stabilize HIF-1 α expression [133, 134]. While HIF-1 α is a key transcriptional driver of glycolysis, OXPHOS can be sustained by NF- κ B signaling [135, 136], providing an explanation for the simultaneous increase in OXPHOS and glycolysis observed in OSCC.

7. Conclusions

In conclusion, the exploration of flavins and some related proteins in the context of cancer unveils a multifaceted landscape where metabolic and epigenetic processes intricately intersect. The involvement of flavin transporters, FADS, Rf, and certain flavoproteins, extends beyond traditional metabolic pathways, encompassing critical roles in cellular functions with direct implications for cancer biology (Figure 2). Flavins, known for their participation in redox reactions, emerge as central players not only in energy metabolism but also in epigenetic regulation. The identification of various flavin-dependent enzymes, including LSD1, as key contributors to cancer progression underscores the intricate connection between metabolic and epigenetic pathways. The unique role of LSD1 as a nuclear flavoprotein adds a layer of complexity, bridging the gap between nuclear and mitochondrial processes in the regulation of cellular energy balance. The emerging role of AIF in connecting the mitochondria and the nucleus further underscores the tricky crosstalk governing cellular functions. As tumour cells adapt to stress and exhibit altered energy metabolism, the reciprocal communication between these cellular compartments could become a focal point for understanding cancer cell behaviour.

Moreover, the identification of specific metabolic genes, including *FLAD1* and *SLC25A32*, as prognostic indicators in various cancers highlights the potential clinical relevance of targeting flavin-related pathways. A thorough knowledge of these mechanisms in the context of cancer is crucial, given the complex interplay among these metabolic genes, histone modifications, and mitochondrial function.

A crucial, still little considered aspect when thinking about flavin homeostasis and cancer is the need for improved drug design; indeed, developing pharmacological modulators of key players in flavin homeostasis represents an open field with interesting perspectives in the near future. As an example, research for new, more specific drugs for FADS, beyond CSB [92], is ongoing, particularly with the recent resolution of the crystal structures of the full-length protein and the C-terminal domain [137]. The availability of 3D structure is, indeed, a fundamental pre-requisite for *in silico* testing to reduce and drive the number of experimental tests.

Considering the forefront localization of membrane transporters, targeting Rf and FAD transporter(s) may be considered the first line of therapeutic interventions for influencing flavin homeostasis in human diseases

characterized by its alteration, including, but not limited to, cancer. Indeed, efforts are being made to discover novel therapeutic inhibitors for RfVTs through both *in silico* studies, utilizing newly described molecular structures [33], and *in vitro* studies with reconstituted proteins [44]. This approach could be significant for simulating Rf deficiency and causing destabilization of the flavoproteome, as previously discussed by Vabulas and colleagues [61, 62]. It must be stressed that membrane transporters, besides being targets, may also be involved in the direct transport of pharmacological compounds, as already proposed by Bartmann and colleagues for RfVTs [74], particularly in the case of molecules with substrate-like structures. In this respect, the International Transporter Consortium periodically updates the list of membrane transporters to be considered when designing novel drugs [138]. Furthermore, in recent years, the race for rapid identification of drugs with high efficacy and low side effects drove research towards drug repurposing, *i.e.*, the use of old and Food and Drug Administration (FDA)-approved drugs in applications other than those for which the drugs were originally designed and tested [139]. Technologies like High Throughputs Screening (HTS), lab automation, and AI are evolving to create platforms useful for drug discovery.

In summary, the integration of flavins into the broader framework of cancer biology provides a rich tapestry of interconnected pathways. Unravelling the complexities of these interactions holds promise for the development of novel therapeutic strategies targeting specific nodes in these networks, offering new avenues for intervention in cancer progression.

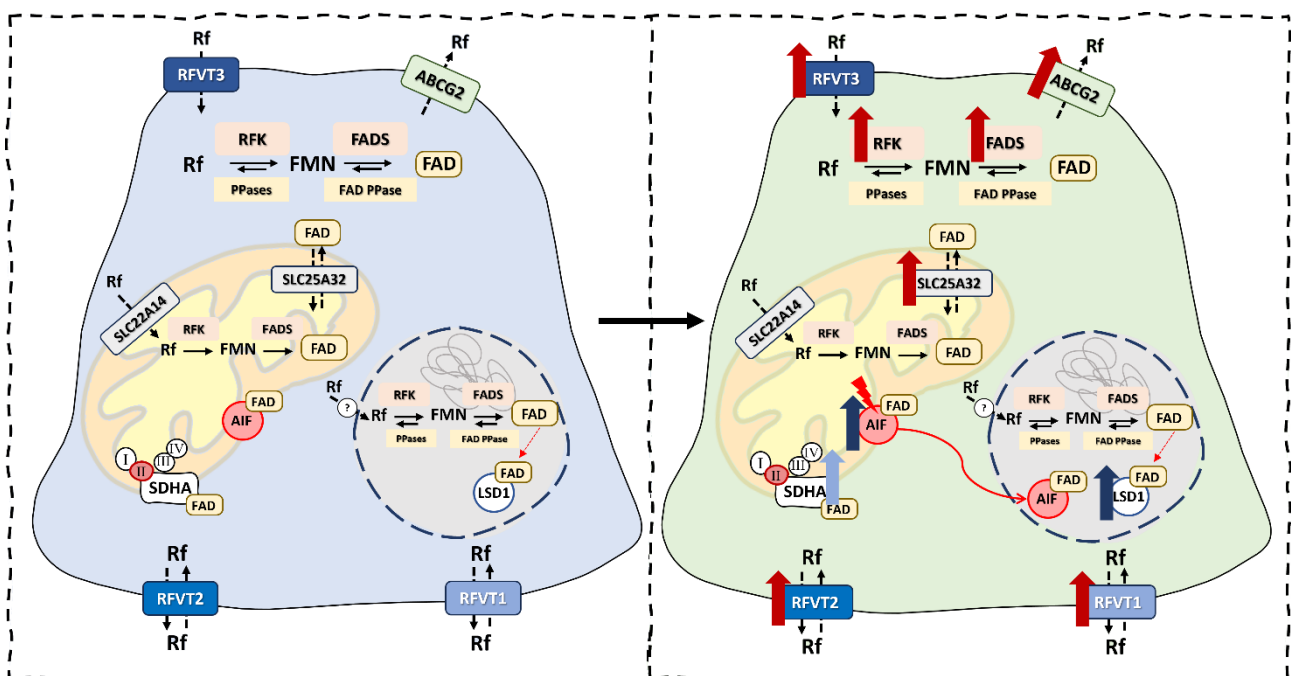


Figure 2: Sketch representation depicting the main events of flavin homeostasis and how they are affected during tumour cell transformation. Light blue: non-cancerous cell; green: cancer cell. All main events of flavin homeostasis were reported to be upregulated in different types of cancer (dark red arrows), except for RFVT3 in gastric cancer [65]. Concomitant overexpression of nuclear and mitochondrial flavoproteins is indicated by blue arrows. SDHA overexpression in PDAC CSCs is indicated by a faint blue arrow [88]. Noteworthy is the translocation of AIF to the nucleus in response to proapoptotic triggers (red flash).

Author contributions

Conceptualization: AN; Writing - Original Draft: AN; Writing - Review & Editing: AN, KZ, MT, MS and MB;
Funding acquisition: MB

Funding

This work was supported by a grant from Association d'Aide à la Recherche sur les Thérapies Innovantes "Role of flavins in therapy" (2018–20) to MB.

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